# Synthesis and Characterization of Oligonucleotides Containing a Nitrogen Mustard Formamidopyrimidine Monoadduct of Deoxyguanosine

Plamen P. Christov, Kyu-Jun Son, and Carmelo J. Rizzo*

Departments of Chemistry and Biochemistry, Center in Molecular Toxicology, and Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, Tennessee 37235, United States

S *Supporting Information*

ABSTRACT: N^5-Substituted formamidopyrimidine adducts have been observed from the reaction of dGuo or DNA with aziridine containing electrophiles, including nitrogen mustards. However, the role of substituted Fapy-dGuo adducts in the biological response to nitrogen mustards and related species has not been extensively explored. We have developed chemistry for the site-specific synthesis of oligonucleotides containing an N^5-nitrogen mustard Fapy-dGuo using the phosphoramidite approach. The lesion was found to be a good substrate for *Escherichia coli* endonuclease IV and formamidopyrimidine glycosylase.
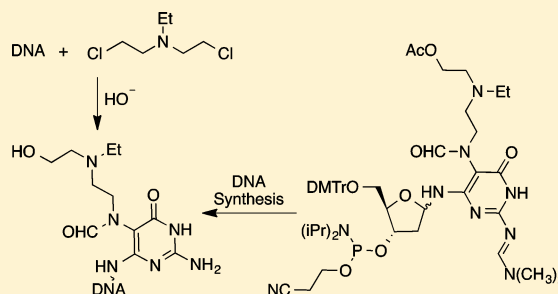
INTRODUCTION

Nitrogen mustards (bis(2-chloroethyl)amine derivatives; Figure 1) are a family of bifunctional DNA alkylating agents that were first introduced into a clinical setting in 1942 for the treatment of non-Hodgkin lymphoma.[1,2] Nitrogen mustards (NM) are still used in combination with other agents for the treatment of a variety of cancers.[2] The predominant site of reaction of nitrogen mustards with DNA is at N7-dGuo. Cationic N7-dGuo adducts are generally considered to be benign,[3,4] but, like most DNA alkylating agents, an array of minor products are also formed.[5−7] The cytotoxicity of simple monofunctional alkylating agents has been ascribed to minor O^6-dGuo, O^4-dThd, and N3-dAdo adducts.[7−9]

The mechanism of action of nitrogen mustards involves an initial intramolecular S_N2 reaction to form an aziridinium ion, which is the DNA modifying agent.[10,11] The nucleophilicity of the N atom can be attenuated by the third substituent, designated as R in Figure 1. Although the N7-dGuo adduct is generally the major adduct (N7-NM-dGuo, Figure 1), with the second chloride (X = Cl) partially or fully hydrolyzed to the corresponding alcohol (X = OH),[12] the bifunctional nature of nitrogen mustards allows them to react with a second nuclophilic site in DNA to afford intra- and interstrand cross-links as well as with nucleophilic sites of proteins to form DNA−protein cross-links.[13−15] This second adduction reaction presumably involves aziridine formation of the initial N7-adduct. Cross-links between the N7 positions of dGuo (N7:N7-NM-dGuo), between the N3-positions of dAdo, and between N7-dGuo and N3-dAdo have been identified, among others.[16−19] Many crucial cellular processes (i.e., replication and repair) require the transient separation of the DNA strands. DNA interstrand cross-links prevent this strand separation and are therefore predicted to be highly cytotoxic to cells.[20] Indeed, although DNA interstrand cross-links typically account for only 1−5% to the total adduct burden, they are believed to be the key lesion in the mechanism of action of nitrogen mustards and related agents. Interestingly, nitrogen mustards prefer to form interstrand cross-links in a 5′-GNC-3′ rather than in a 5′-GC-3′ sequence context.[21−24]

The products from the reaction of nitrogen mustards with Guo, dGuo, and DNA have been well-studied. Chetsanga[25] and Hemminki[26−28] have independently characterized the imidazole ring-opening of the N7-dGuo adduct of a nitrogen mustard to afford the corresponding N^5-substituted formamidopyrimidine (Fapy) lesion (Figure 1). N^5-(2-Aminoethyl)-Fapy-dGuo (AE-Fapy-dGuo) has been observed to be the major product from the reaction of aziridine with dGuo and DNA.[29,30] A Fapy-dGuo adduct has also been characterized from the reaction of DNA with acid-activated mitomycin, an aziridine-containing natural product.[31] However, little attention has been paid to nitrogen mustard Fapy-dGuo adducts (NM-Fapy-dGuo). Previous studies have shown that Fapy-dGuo adducts have mutagenic potential[32−34] and are substrates for the base excision repair pathway.[35−40] Oligonucleotides containing site-specific NM-Fapy-dGuo lesions will allow for future replication and repair studies both *in vitro* and in cells. We have reported the synthesis of oligonucleotides containing the N^5-methyl-Fapy-dGuo lesion (MeFapy-dGuo) by solid-phase methods.[41] The extension of this chemistry to the synthesis of oligonucleotides containing the NM-Fapy-dGuo lesion (R = Et) is described herein. We find that the NM-Fapy-dGuo lesion is a substrate for *Escherichia coli* endonuclease IV (Endo IV) and formamidopyrimidine glycosylase (FPG) *in vitro*.
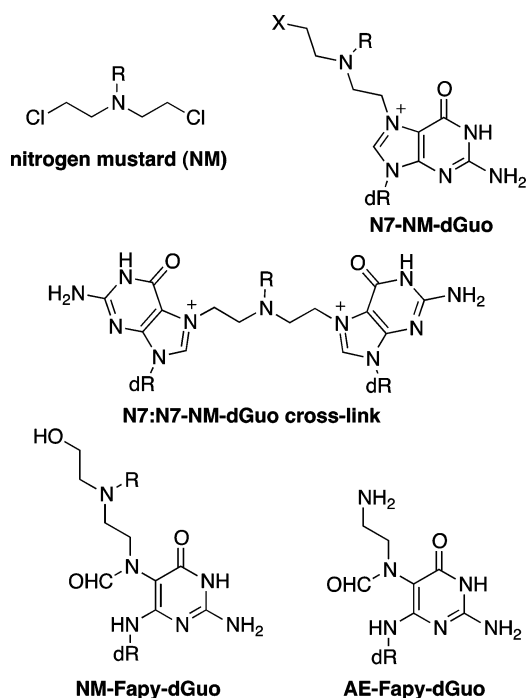
Figure 1. General structure of a nitrogen mustard (NM) and some of their dGuo adducts.

■ EXPERIMENTAL PROCEDURES

N^2-[(Dimethylamino)methylene]-O^6-[2-(trimethylsilyl)-ethyl]-2′-deoxyguanosine (2). A solution of 1[42] (426 mg, 1.16 mmol) and N,N-dimethylformamide dimethyl acetal (1.22 mL, 9.3 mmol) in dry methanol (20 mL) was heated at 60 °C for 6 h. The methanol was then removed *in vacuo* on a rotary evaporator. Purification of the resulting solid by flash chromatography on silica afforded 2 (441 mg, 90%). The eluent was initially 96:4 CH$_2$Cl$_2$/CH$_3$OH, and the CH$_3$OH content was increased by 1% every column volume (~200 mL) to a final ratio of 92:8. ^{1}H NMR (DMSO-d_6): δ 8.57 (s, 1H, N=CH), 8.25 (s, 1H, H-8), 6.30 (t, 1H, J = 6.0 Hz, H-1′), 5.29 (s, 1H, 3′-OH), 5.14 (s, 1H, OH-5′), 4.59 (t, 2H, J = 6.0 Hz, OCH$_2$CH$_2$Si), 4.39 (m, 1H, H-3′), 3.85 (m, 1H, H-4′), 3.62−3.58 (m, 2H, H-5′), 3.17 (s, 3H, CH$_3$-N-CH=), 3.08 (s, 3H, CH$_3$-N-CH=), 2.65 (m, 1H, H-2′), 2.27 (m, 1H, H-2′), 1.20 (t, 2H, J = 6.0 Hz, OCH$_2$CH$_2$Si), 0.15 (s, 9H, Me$_3$-Si). HRMS (FAB+) m/z calcd for C$_{18}$H$_{31}$N$_6$O$_4$Si [M + H]$^+$, 423.2180; found, 423.2171.

N^2-[(Dimethylamino)methylene]-[5′-O-(bis(4-methoxyphenyl)phenylmethyl)-O^6-(trimethylsilylethy)-2′-deoxy-guanosine (3). Compound 2 (420 mg, 1.00 mmol) was dissolved in anhydrous pyridine, evaporated on a rotary evaporator, dried overnight under high vacuum, and then dissolved in dry pyridine (15 mL). Dimethoxytrityl chloride (372 mg, 1.1 mmol) was added in three equal portions, and the mixture stirred at room temperature overnight. The pyridine was removed *in vacuo* on a rotary evaporator. Purification of the residue by flash chromatography on silica afforded 3 (677 mg, 85%). The eluent was initially 94:5:1 CH$_2$Cl$_2$/CH$_3$OH/pyridine, and the methanol content was increased by 1% every column volume (~200 mL) to a final ratio of 91:7:1. ^{1}H NMR (DMSO-d_6): δ 9.02 (s, 1H, N=CH), 8.42 (s, 1H, H-8), 7.30−7.13 (m, 9H, ArH), 6.80−6.70 (m, 4H, ArH), 6.43 (t, 1H, J = 6.0 Hz, H-1′), 5.54 (s, 1H, 3′-OH), 4.74 (t, 2H, J = 6.0 Hz, OCH$_2$CH$_2$Si), 4.45 (m, 1H, H-3′), 3.96 (m, 1H, H-4′), 3.69 (s, 6H, 2CH$_3$O), 3.33 (s, 3H, CH$_3$-N-CH=), 3.20 (s, 3H, CH$_3$-N-CH=), 3.15−3.12 (m, 2H, H-5′), 2.54−2.52 (m, 1H, H-2′), 2.48−2.46 (m, 1H, H-2′), 1.19 (t, 2H, J = 6.0 Hz, OCH$_2$CH$_2$Si), 0.10 (s, 9H, Si). HRMS (FAB+) m/z calcd for C$_{39}$H$_{49}$N$_6$O$_6$Si [M + H]$^+$, 725.3477; found, 725.3484

N^6-[4-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-2-deoxy-D-*erythro*-pentofuranosyl]amino]-2-[[(dimethylamino)-methylene]amino]-O^4-[2-(trimethylsilyl)ethyl]-5-N-[2-(2-

chloroethyl)ethylamino]ethylformamidopyrimidine (5). Tri-ethylamine (460 µL, 3.3 mmol) and N,N-bis(2-chloroethyl)ethylamine hydrochloride (566 mg, 2.75 mmol) were sequentially added to a stirred solution of 3 (400 mg, 0.55 mmol) in dry trifluoroethanol (10 mL). The reaction mixture was stirred at ambient temperature for 4 h, after which time it was concentrated to ~1 mL *in vacuo* on a rotary evaporator. Methanolic sodium hydroxide (0.5 M in 1:3 water/methanol, 10 mL) was added, and the mixture was stirred for 5 min followed by neutralization by the careful addition of 20% acetic acid. Water (15 mL) was added, and the mixture extracted with CH$_2$Cl$_2$ (3 × 20 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated. Purification of the gummy residue by flash chromatography on silica afforded 5 as a mixture of isomers (410 mg, 85%). The eluent was initially 98:1:1 CH$_2$Cl$_2$/CH$_3$OH/pyridine; the CH$_3$OH content was increase by 1% every column volume (~200 mL) to a final ratio of 93:6:1. ^{1}H NMR (DMSO-d_6) mixture of isomers: δ 8.56 (s, 0.5H, N=CH), 8.52 (s, 0.5H, N=CH), 7.70 (s, 0.5H, CHO), 7.38 (s, 0.5H, CHO), 7.29−7.18 (m, 9H, ArH), 7.05 (d, 0.5H, J = 12.0 Hz, NH), 6.95 (d, 0.5H, J = 12.0 Hz, NH), 6.83−6.79 (m, 4H, ArH), 6.05 (m, 1H, H-1′), 5.12 (d, 1H, J = 6.0 Hz, 3′-OH), 4.36−4.31 (m, 2H, OCH$_2$CH$_2$Si), 4.07 (m, 1H, H-3′), 3.76 (m, 1H, H-4′), 3.70 (s, 6H, 2CH$_3$O), 3.60−3.55 (m, 2H, ClCH$_2$CH$_2$N), 3.50−3.35 (m, 2H, EtNCH$_2$CH$_2$NCHO), 3.07 (s, 3H, CH$_3$-N-CH=), 2.90 (s, 3H, CH$_3$-N-CH=), 3.00−2.98 (m, 2H, H-5′), 2.81−2.60 (m, 4H, ClCH$_2$CH$_2$N, EtNCH$_2$CH$_2$NCHO), 2.55−2.52 (m, 2H, CH$_2$CH$_3$), 1.95−1.87 (m, 2H, H-2′), 0.98 (t, 2H, J = 6.0 Hz, OCH$_2$CH$_2$Si), 0.84 (t, 1.5H, J = 6.0 Hz, CH$_2$CH$_3$), 0.70 (t, 1.5H, J = 6.0 Hz, CH$_2$CH$_3$), 0.07 (s, 9H, Me$_3$-Si). HRMS (FAB+) m/z calcd for C$_{45}$H$_{63}$ClN$_7$O$_7$Si [M + H]$^+$, 876.4252; found, 876.4241.

N^6-[4-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-2-deoxy-D-*erythro*-pentofuranosyl]amino]-2-[[(dimethylamino)-methylene]amino]-O^4-[2-(trimethylsilyl)ethyl]-5-N-[2-(2-acetoxyethyl)ethylamino]ethylformamidopyrimidine (6). 18-Crown-6 (422 mg, 1.6 mmol) and cesium acetate (306 mg, 1.6 mmol) were sequentially added to a stirred solution of 5 (350 mg, 0.4 mmol) in dry toluene (20 mL). The mixture was heated at 70 °C for 2 h. The toluene was removed *in vacuo* on a rotary evaporator, and the gummy residue was purified by flash chromatography on silica to afford 6 as a mixture of isomers (277 mg, 77%). The eluent was initially 98:1:1 CH$_2$Cl$_2$/CH$_3$OH/pyridine; the CH$_3$OH content was increased by 1% every column volume (~200 mL) to a final ratio of 95:4:1. ^{1}H NMR (DMSO-d_6) mixture of isomers: δ 8.42 (s, 0.5H, N=CH), 8.40 (s, 0.5H, N=CH), 7.81 (s, 0.5H, CHO), 7.78 (s, 0.5H, CHO), 7.32−7.21 (m, 9H, ArH), 7.16 (d, 0.5H, J = 12.0 Hz, NH), 6.83−6.79 (m, 4.5H, ArH, NH), 6.21−6.19 (m, 1H, H-1′), 5.37−5.35 (m, 1H, 3′−OH), 4.45 (m, 2H, OCH$_2$CH$_2$Si), 4.33 (m, 1H, H-3′), 4.10−4.00 (m, 2H, AcOCH$_2$CH$_2$N), 3.90 (m, 1H, H-4′), 3.75 (s, 6H, 2CH$_3$O), 3.50−3.35 (m, 2H, EtNCH$_2$CH$_2$NCHO), 3.10−3.00 (m, 2H, H-5′), 2.86−2.84 (multiple s, total 6H, CH$_3$-N-CH=), 2.71−2.60 (m, 6H, CH$_2$CH$_3$, AcOCH$_2$CH$_2$N, EtNCH$_2$CH$_2$NCHO), 2.31−2.29 (m, 1H, H-2′), 1.95−1.87 (m, 4H, H-2′, AcO), 0.98 (t, 2H, J = 6.0 Hz, OCH$_2$CH$_2$Si), 0.98 (t, 1.5H, J = 6.0 Hz, CH$_2$CH$_3$), 0.91 (t, 1.5H, J = 6.0 Hz, CH$_2$CH$_3$), 0.08 (s, 9H, Me$_3$-Si). HRMS (FAB+) m/z calcd for C$_{47}$H$_{66}$N$_7$O$_9$Si [M + H]$^+$, 900.4686; found, 900.4692

N^6-[4-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-2-deoxy-D-*erythro*-pentofuranosyl]amino]-2-[[(dimethylamino)-methylene]amino]-1,4-dihydro-4-oxo-5-N-[2-(2-hydroxyethyl)-ethylamino]ethylformamidopyrimidine (7). Tetrabutylammonium flouride (1 M in THF, 420 µL, 0.42 mmol) was added to a stirred solution of 6 (250 mg, 0.28 mmol) in dry methylene chloride (15 mL). The mixture was stirred at room temperature for 4 h. The solvent was removed, and the residue was purified by flash chromatography on silica to afford 7 as a mixture of isomers (177 mg, 80%). The eluent was initially 98:1:1 CH$_2$Cl$_2$/CH$_3$OH/pyridine; the CH$_3$OH content was increased by 1% every column volume (~200 mL) to a final ratio of 95:4:1. ^{1}H NMR (DMSO-d_6) mixture of isomers: δ 11.09 (s, 1H, NH), 8.56 and 8.53 (two s, total 0.5H, N=CH), 8.32 and 8.29 (two s, total 0.5H, N=CH), 7.81 and 7.79 (two s, total 0.5H, CHO), 7.66 and 7.50 (two s, total 0.5H, CHO), 7.40−7.20 (m, 9.5H, ArH, NH), 6.99 (d, 0.5H, J = 12.0 Hz, NH), 6.90−6.85 (m,

Scheme 1. Synthesis of the NM-Fapy-dGuo Phosphoramidite
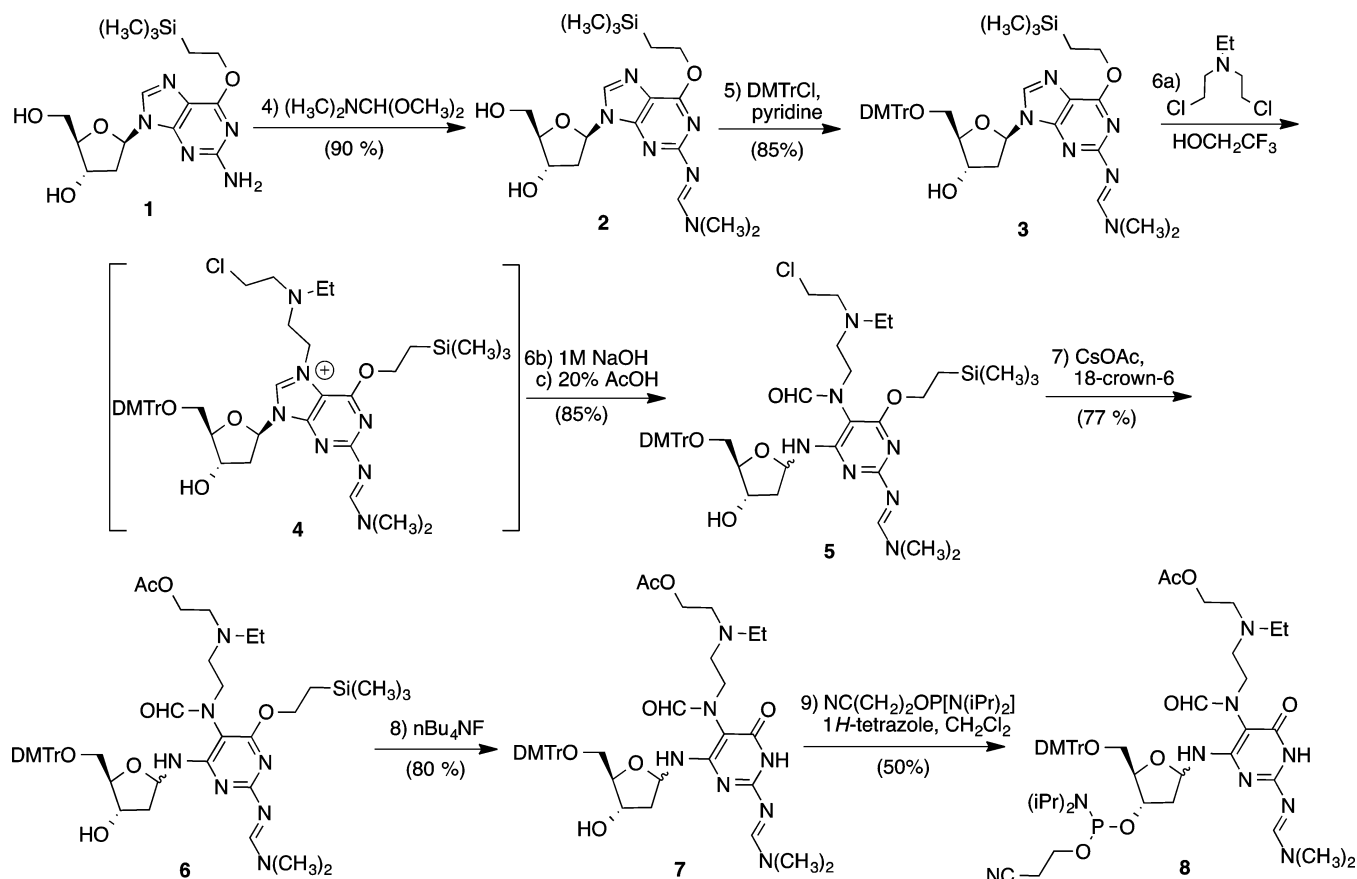
4H, ArH), 6.27 and 6.23 (two m, 1H, H-1′), 5.31 and 5.12 (s, total 1H, 3′-OH), 4.28 (m, 1H, H-3′), 4.10−4.00 (m, 2H, AcO$\underline{CH_2}$CH$_2$N), 4.23 (m, 1H, H-4′), 3.72 (s, 6H, 2CH$_3$O), 3.50−3.35 (m, 2H, EtN$\underline{CH_2}$CH$_2$NCHO), 3.22−3.17 (multiple s, total 6H, 2CH$_3$-N-CH═), 2.98−2.90 (m, 2H, H-5′), 2.60−2.52 (m, 6H, $\underline{CH_2}$CH$_3$, AcOCH$_2$$\underline{CH_2}$N, EtNCH$_2$$\underline{CH_2}$NCHO), 1.98−1.85 (m, 5H, H-2′, AcO), 1.33−1.22 (multiple t, total 3H, CH$_2$$\underline{CH_3}$). HRMS (FAB+) m/z calcd for C$_{42}$H$_{54}$N$_7$O$_9$ [M + H]$^+$, 800.3978; found, 800.3985.

N^6-[4-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-3-O-[(N,N-diisopropylamino)(2-cyanoethoxy)phosphino]-2-deoxy-D-*erythro*-pentofuranosyl]amino]-2-[[(dimethylamino)methylene]-amino]-1,4-dihydro-4-oxo-5-N-[2-(2-hydroxyethyl)-ethylamino]ethylformamidopyrimidine (8). Compound 7 (100 mg, 0.13 mmol) was dissolved in anhydrous pyridine, evaporated on a rotary evaporator, and dried overnight under high vacuum. The gummy residue was dissolved in dry CH$_2$Cl$_2$ (20 mL), and a solution of anhydrous 1H-tetrazole (12.6 mg, 0.18 mmol) was added followed by 2-cyanoethyl-N,N,N',N'-tetraisopropyl-phosphorodiamidite (54.2 mg, 0.18 mmol). The solution was stirred at room temperature for 3 h. The solvent was removed *in vacuo* on a rotary evaporator, and the residue was purified by flash chromatography on silica to afford **8** as a mixture of isomers (63.0 mg, 50%). The eluent was initially 97:2:1 CH$_2$Cl$_2$/CH$_3$OH/pyridine; the CH$_3$OH content was increased by 1% every column volume (∼200 mL) to a final ratio of 95:4:1. ^{1}H NMR (acetone-d_6) mixture of isomers: δ 8.34 (s, 0.5H, N═CH), 8.23 (s, 0.5H, N═CH), 7.92 (s, 0.5H, CHO), 7.89 (s, 0.5H, CHO), 7.48−7.30 (m, 9H, ArH), 6.90−6.85 (m, 4H, ArH), 6.61 (m, 1H, H-1′), 4.84 (m, 1H, H-3′), 4.35−4.17 (m, 3H, H-4′, AcO$\underline{CH_2}$CH$_2$N), 3.78 (s, 6H, 2-CH$_3$O), 3.70−3.63 (m, 2H, POCH$_2$), 3.63−3.43 (m, 4H, isopropyl CH, EtN$\underline{CH_2}$CH$_2$NCHO), 3.10−3.03 (m, 2H, H-5′), 2.91−2.68 (multiple s and m, total 12H, 2CH$_3$-N-CH═, OCH$_2$-CN, AcOCH$_2$$\underline{CH_2}$N, EtNCH$_2$$\underline{CH_2}$NCHO), 2.55−2.52 (m, 2H, $\underline{CH_2}$CH$_3$), 2.03−1.90 (m, 5H, H-2′, AcO), 1.33−1.21 (multiple t, total 3H, CH$_2$$\underline{CH_3}$), 1.22−1.01 (m, 12H, isopropyl CH$_3$). ^{31}P NMR (acetone-d_6, 125 MHz) δ 149.56, 149.25, 149.12, 148.98. HRMS (FAB +) m/z calcd for C$_{51}$H$_{71}$N$_9$O$_{10}$P [M + H]$^+$, 1000.5056; found, 1000.5079.

Oligonucleotide Synthesis. The oligodeoxynucleotides were synthesized on a Perspective Biosystems Model 8909 DNA synthesizer on a 1 μmol scale using Expedite reagents (Glen Research) with the standard synthetic protocols for the coupling of unmodified bases. The coupling of the NM-Fapy-dGuo phosphoramidite was performed off-line for 30 min as previously described.[41,43]

Enzymatic Digestion and Analysis of Oligonucleotides. The enzymatic digestion of oligonucleotides was carried out in a single step as follows: the oligonucleotide (0.5 A$_{260}$ units) was dissolved in 70 μL of buffer (pH 7, 10 mM Tris-HCl, 10 mM MgCl$_2$). DNase I (5 units), alkaline phosphatase (1.7 units), and snake venom phosphodiesterase I, type II (0.02 units), were added, and the solution was incubated at 37 °C for 1.5 h. HPLC analysis was performed using solvent gradient 1. Conditions for the HPLC and UPLC-MS analyses are provided in the Supporting Information.

5′-GCT AGC-(NM-Fapy-dGuo)-AG TCC-3′ (9). Purified by reversed-phase HPLC using gradient 2 (see Supporting Information for conditions). MALDI-TOF MS (HPA) m/z calcd for [M − H]$^-$, 3775.7; found, 3777.2

5′-ACC ACG CTA GC-(NM-Fapy-dGuo)-AGT CCT AAC AAC-3′ (10). Purified by reversed-phase HPLC using gradient 3 (see Supporting Information for conditions). MALDI-TOF MS (HPA) m/z calcd for [M − H]$^-$, 7406.9; found, 7407.7

5′-ACC ACG CTA GC-(8-oxo-dGuo)-AGT CCT AAC AAC-3′. This oligonucleotide was synthesized using commercially available 8-oxo-dGuo phosphoramidite and purified by reversed-phase HPLC using gradient 4 (see Supporting Information for conditions). MALDI-TOF MS (HPA) m/z calcd for [M − H]$^-$, 7289.8; found, 7287.0.

5′-ACC ACG CTA GC-(MeFapy-dGuo)-AGT CCT AAC AAC-3′ and 5′-ACC ACG CTA GC-(α-dG)-AGT CCT AAC AAC-3′ were prepared previously.[44]

Oligonucleotide Labeling and Annealing. The oligonucleotides were labeled using T4 polynucleotide kinase and γ-^{32}P-ATP as previously described.[44]

Time Course of the Endo IV Incision of the NM-Fapy-dGuo Containing Duplexes. These experiments were performed as previously described.[44]

Kinetics for the Endo IV Incision of the NM-Fapy-dGuo Containing Duplex. These experiments were performed as previously described.[44]

Time Course Incision of the NM-Fapy-dGuo Containing Duplex by Endo IV at pH 7.0, 7.5, and 8.0. These experiments were performed as previously described.[44]

Incision of the NM-Fapy-dGuo Containing Duplex after Denaturation and Reannealing. These experiments were performed as previously described.[44]

Excision of MeFapy-dGuo, NM-Fapy-dGuo, and 8-Oxo-dGuo Containing Duplexes by E. coli Formamido-pyrimidine Glycosylase. The 5'-^{32}P-labeled modified oligonucleotide (200 nM) was annealed to an equal volume of its complementary strand (600 nM) in Tris buffer (50 mM). Oligonucleotide duplex (100 nM, 4 μL) was added to the formamidopyrimidine glycosylase (FPG) reaction buffer (1×: 10 mM Bis-Tris Propane-HCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, 74 μL), followed by addition of FPG (10 nM). Reactions were incubated at 37 °C. Aliquots (5 μL) were removed at the appropriate times, added to 10 μL of 95% formamide loading buffer containing xylene cyanol and bromophenol blue dyes, and heated for 1 min at 90 °C. Aliquots (6 μL) were separated by electrophoresis on a denaturing gel.

Kinetics for the Excision of MeFapy-dGuo, NM-Fapy-dGuo, and 8-Oxo-dGuo Containing Duplexes by E. coli Formamido-pyrimidine Glycosylase. DNA duplex was formed by heating ^{32}P-labeled oligonucleotide (100 nM in 50 mM Tris) and its complementary strand (1.5 equiv) at 95 °C for 5 min and then slowly cooling to ambient temperature over 1 h. FPG (0.1 nM) was added to varying concentrations of DNA duplex (5–100 nM for MeFapy-dGuo, 10–180 nM for NM-Fapy-dGuo, and 1–40 nM for 8-oxo-dGuo in FPG reaction buffer (1×)) to a final volume 50 μL. Reactions were run at 37 °C for 10 min. Aliquots (5 μL) were taken every 1 min, added to loading buffer (10 μL), and heated at 90 °C for 1 min. Separation was achieved by PAGE. The kinetics parameters were calculated using KaleidaGraph (v. 4.5, Synergy Software). Reactions were carried out in duplicate.

■ RESULTS AND DISCUSSION

Phosphoramidite Synthesis. The synthesis of the NM-Fapy-dGuo phosphoramidite reagent is shown in Scheme 1. O^6-(2-Trimethylsilyethyl)-dGuo (**1**) was prepared in three steps according to a literature procedure[42] and was further protected at the 5'-O- and N^2-positions to afford **3** in 43% overall yield from dGuo. Reaction of **3** with bis(2-chloroethyl)ethylamine in trifluoroethanol presumably gives the cationic N7-adduct (**4**),[18,26,28,45] which was not isolated. Rather, the reaction mixture was briefly treated with methanolic NaOH followed by careful neutralization with 20% acetic acid to afford protected NM-Fapy-dGuo intermediate **5**. Competing alkylation at O^6 was observed if this position was not protected. The second chloride, which surprisingly survived the hydroxide treatment, was displaced by cesium acetate, and the O^6-protecting group was removed by fluoride ion. Phosphitylation of the 3'-hydroxyl group provided the desired phosphoramidite reagent (**8**) as a mixture of isomers. The synthesis required nine steps from dGuo and proceeded in ~11% overall yield.

Oligonucleotide Synthesis. Phosphoramidite **8** was used to synthesize 12- and 24-mer oligonucleotides containing the NM-Fapy-dGuo lesion shown in Table 1. An off-line, manual coupling protocol was used to incorporate the modified nucleotide.[41,43] Fapy-dGuo lesions with a free 5' hydroxyl group can rearrange to the pyranose form.[31,46] Therefore, the

Table 1. Sequences and Mass Spectrometric Characterization of the Oligonucleotides Containing the NM-Fapy-dGuo Lesion

		m/z	
	oligonucleotide	found	calcd
9	5'-GCTAGC-(NM-Fapy-dGuo)-AGTCC-3'	3777.2	3775.7
10	5'-ACCACGCTAGC-(NM-Fapy-dGuo)-AGTCCTAACAAC-3'	7407.7	7406.9

critical step of the oligonucleotide synthesis was the detritylation of the NM-Fapy-dGuo nucleotide. We previously observed that standard DNA synthesis protocols resulted in significant rearrangement to the pyranose form of the MeFapy-dGuo adducts. However, shortening the deprotection cycle minimized the furanose to pyranose rearrangement.[41] Oligonucleotides containing the NM-Fapy-dGuo adduct were synthesized using a "short" deprotection cycle. Two products were identified as having the correct mass in a ~1:2.6 ratio (see Figure S10 of the Supporting Information). The major product (later eluting) was assumed to be the furanose form based on our previous work with the MeFapy-dGuo adduct in the same sequence. The two products were observed in a ~1:1 ratio under a long deprotection cycle, which is consistent with the assignment because the long deprotection should favor the isomerization to the pyranose form.

The NM-Fapy-dGuo containing 12-mer oligonucleotide was enzymatically digested to the individual nucleosides. The unmodified nucleosides were readily observed by HPLC analysis in the expected ratio (Figure 2A),[47] but NM-Fapy-dGuo was not detected. Substituted Fapy-dGuo nucleosides exist as multiple, slowly interconverting species, which may include the furanose and pyranose forms of the deoxyribose, α- and β-anomers, cis and trans geometric isomers of the formamide, and possibly atropisomers.[31,46,48−53] The slow interconversion of the Fapy-isomers often results in poor HPLC chromatographic behavior and diminished detection.[54] Therefore, the enzymatic digestion reaction was examined by UPLC-MS^n. The extracted ion chromatograms are shown in Figure 2B, in which the neutral loss of the deoxyribose (−116 Da) was monitored. A mass consistent with NM-Fapy-dGuo (400 → 285) was observed along with the unmodified nucleosides. As anticipated, NM-Fapy-dGuo was observed as multiple broad peaks, reflecting its conformational heterogeneity. The enzymatic digestion reaction was also subjected to acid hydrolysis and UPLC-MS analysis (Figure 2C). A broad peak with a mass consistent with the NM-Fapy-Gua base (m/z 285.2) was observed. The broad nature of this peak likely reflects geometric isomers of the formamide group; the geometric isomers of the MeFapy-Gua base could be resolved into two separate peaks by HPLC.

Duplexes containing the MeFapy-dGuo and N^5-aflatoxin B$_1$-Fapy-dGuo (AFB$_1$-Fapy-dGuo) lesions showed biphasic thermal melting profiles.[41,55] It was hypothesized that the two phases represented the α- and β-anomers of the Fapy-dGuo adduct. Attempts to characterize the thermal melting profile of the NM-Fapy-dGuo containing 12-mer gave inconsistent results in our hands. We cannot offer an explanation for this observation.

Incision of the NM-Fapy-dGuo Duplex with E. coli Endonuclease IV (Endo IV). An unusual property of Fapy lesions is that they can isomerize to the unnatural α-anomer. Endo IV incises the 5'-phosphodiester bond of an abasic site in
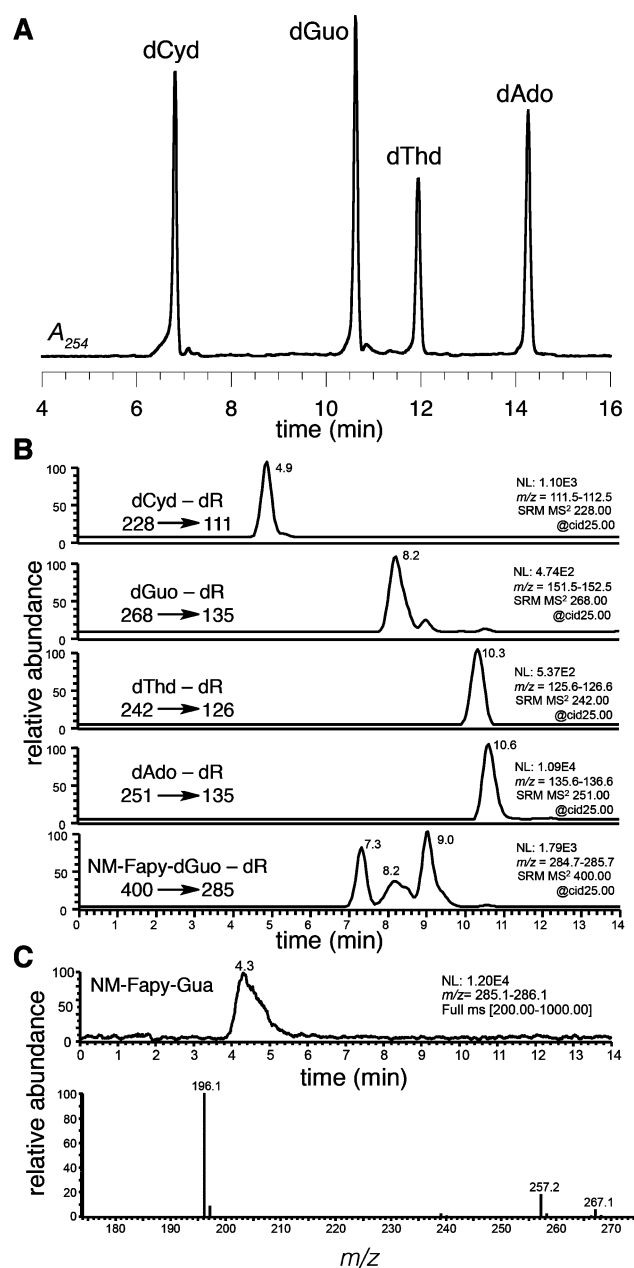
Figure 2. (A) HPLC (A_{254}) and (B) UPLC-SRM-MS^2 analysis of the enzymatic digestion of NM-Fapy-dGuo containing 12-mer oligonucleotide (**9**). (C) UPLC-MS analysis of the NM-Fapy-Gua after acid hydrolysis of the enzymatic digestion reaction of oligonucleotide (**9**). The reconstructed ion chromatogram of NM-Fapy-Gua ([M + H]$^+$ m/z 285.2) is shown at the top of this panel, and the CID fragmentation of the parent ion is shown at the bottom. See the Supporting Information for the conditions for each analysis.

duplex DNA[56−58] and has been shown to incise α-nucleotides. Endo IV has been used previously to approximate the α/β ratio of Fapy-dGuo, Fapy-dAdo, and MeFapy-dGuo in duplex DNA by the selective incision of the α-anomer.[44,59−61] We applied this assay to the NM-Fapy-dGuo containing 24-mer to determine the α/β ratio of the lesion. The modified 24-mer (**10**) was 5′-^{32}P-labeled, annealed to its complement, and incubated with Endo IV (Figure 3). Approximately 50% of the duplex was incised after a 40 min incubation period at 37 °C, affording a product consistent with phosphodiester hydrolysis at the 5′-side of NM-Fapy-dGuo. The reaction mixture,
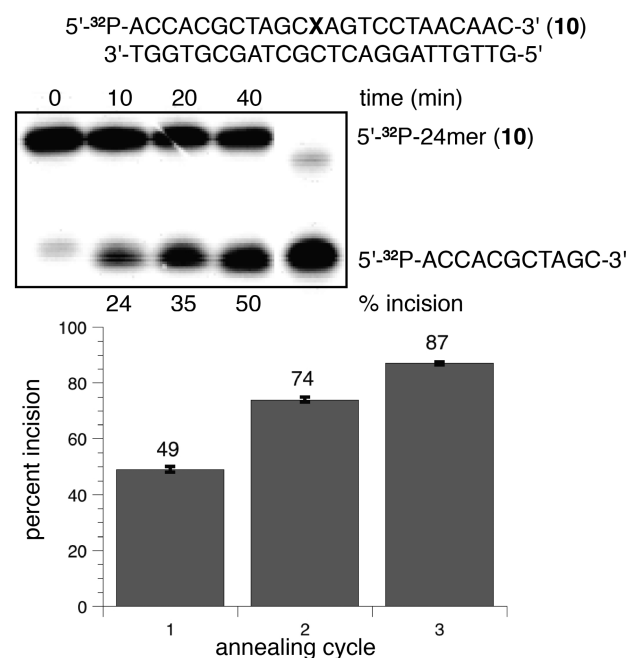
Figure 3. (Top) Gel electrophoretic analysis of the incision of the NM-Fapy-dGuo containing 24-mer (**10**) duplex with *E. coli* Endo IV. The right lane contains a standard of the 5′-^{32}P-ACCACGCTAGC-3′ incision product. (Bottom) Percentage of incision product after denaturation−reannealing and additional Endo IV (average of two experiments).

containing the remaining 50% of the duplex as the β-anomer, was denatured by heating at 90 °C and slowly cooling to reanneal the duplex. This process should re-equilibrate the NM-Fapy-dGuo lesion to its original mixture of anomers.[44,61] Additional Endo IV was added, and after 40 min, gel analysis indicated that the total level of incision was ∼75%; the 25% increase represents 50% of the duplex remaining after the first incision reaction. The reaction mixture was denatured and reannealed again, and a third portion of Endo IV was added. The third cycle resulted in an additional ∼12% of the incised product (∼87% total), which represents approximately half of the duplex remaining after the second Endo IV treatment (25%). The results are consistent with a 1:1 mixture of α- and β-anomers after initial annealing of the duplex.

We previously observed that the initial α/β-anomeric ratio of the MeFapy-lesion slowly equilibrated over time.[44] The labeled NM-Fapy-dGuo containing 24-mer duplex was incubated at 37 °C and pH 7.5. Aliquots were removed after 0.5 h and then approximately every 24 h and were subjected to Endo IV treatment. The level of incision steadily decreased over time to about 14% after 5 days (Figure 4). The anomerization was also examined at pH 7.0 and 8.0 and was surprisingly insensitive to
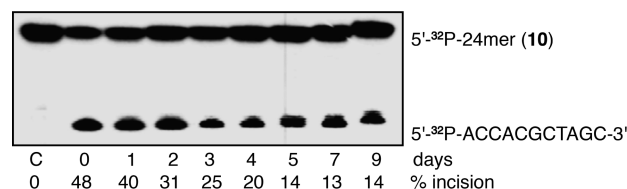
Figure 4. Time course for Endo IV incision of the NM-Fapy-dGuo containing 24-mer (**10**). Endo IV was omitted in the control lane (C). The day 0 time point was after an incubation time of 30 min.

pH over this range; this is in contrast to that of the MeFapy-dGuo lesion, which anomerized more slowly at pH 7.5 and not at all at pH 8.0.

The catalytic efficiency (k_{cat}/K_m, Table 2) for the Endo IV incision of the NM-Fapy-dGuo containing 24-mer was 0.16 nM^{-1} min^{-1} (k_{cat} = 5.4 ± 0.5 min^{-1}, K_m= 33 ± 6.8 nM). The incision efficiency was approximately half of that of the MeFapy-dGuo lesion and ~4.3-fold lower than α-dGuo in the same sequence context (Table 2).[44] The k_{cat} values for the three substrates were similar, and differences in their incision efficiency were largely due to K_m. An active site pocket of Endo IV is hypothesized to accommodate α-nucleotides.[62] The affinity for our three substrates decreases with increasing steric bulk, perhaps reflecting the ability of the active site pocket to accommodate the substrate. We previously reported that the very bulky AFB$_1$-Fapy-dGuo adduct is a poor substrate for Endo IV.

Excision of the NM-Fapy-dGuo Lesion of *E. coli* Formamidopyrimidine Glycosylase (FPG). Fapy lesions are substrates for the base excision repair pathway and are excised by FPG in bacteria and by OGG1, NEIL1, and NTH1 in eukaryotes.[35−40] Less is known about the excision of N^5-substituted Fapy-dGuo lesions. The MeFapy-dGuo, ethyl-Fapy-dGuo, (2-hydroxyethyl)-Fapy-dGuo, (pyridyloxobutyl)-Fapy-dGuo, and Fapy-dGuo lesions derived from a phosphoramide and sulfur mustard have been reported to be substrates for FPG,[25,52,63−66] whereas AFB$_1$-Fapy-dGuo is not a substrate.[66] A ring-opened oxidation product of dThd is also a substrate for FPG.[67] MeFapy-dGuo is a substrate for human[68] and yeast[69] OGG1, hNEIL1,[70] and yeast,[71] mouse,[72] and human[70,73] NTH1, but it is a poor substrate for hNEIL2,[70] mouse NEIL3,[74] and *E. coli* Endo III and Endo VIII[70,72,75] when paired with dCyd. Excision by NEIL3 improved markedly in single-stranded DNA.[74] There is indirect evidence that FPG and hOGG1 will excise the AE-Fapy-dGuo lesion. The over-expression of FPG or hOGG1 was up to 100-fold protective of cells treated with thioTEPA or aziridine, suggesting a role for the AE-Fapy-dGuo lesion in the cytotoxic mechanism of these agents.[76−79] FPG expression also provided a 10- and 2-fold protective effect against 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and the nitrogen mustard mafosfamide,[76,80] respectively, suggesting that the corresponding N^5-substituted Fapy-dGuo lesions are part of the mechanism of action of these agents as well. The lower protective effect against BCNU and mafosfamide is probably reflective of the greater role of interstrand cross-links in their cytotoxicity.

The excision of MeFapy-dGuo and NM-Fapy-dGuo opposite dCyd from duplex DNA by *E. coli* FPG was examined and compared to that of 8-oxo-dGuo; all three lesions were incorporated into the same 24-mer sequence. Asagoshi et al. previously reported the excision of MeFapy-dGuo and 8-oxo-dGuo from a 25-mer duplex in a 5′-T$\underline{X}$G-3′ sequence by FPG

and hOGG1.[68] Both glycosylases had nearly identical catalytic efficiencies for the two substrates opposite dCyd, although the activity for FPG was significantly higher than that of hOGG1. We found that the MeFapy-dGuo and NM-Fapy-dGuo lesions were good substrates for *E. coli* FPG when paired with dCyd (Figure 5). Our k_{cat} and K_m values for the MeFapy-dGuo and 8-oxo-dGuo substrates are in reasonable agreement with those previously reported (Table 3);[68] however, we found 8-oxo-dGuo to be a better substrate for FPG by ~2-fold. The excision of NM-Fapy-dGuo was 2.5 and 4.6 times less efficient than that for MeFapy-dGuo and 8-oxo-dGuo, respectively. The excision efficiencies are largely reflective of differences in the K_m values, which roughly correlate with the steric demands of the modification. Consistent with our observation, the K_m for the excision of N^5-ethyl-Fapy-dG was reported to be 7-fold higher than that for MeFapy-dGuo.[52] However, the apparent K_D's of nonhydrolyzable cyclopentane analogues of Fapy-dGuo and N^5-benzyl-Fapy-dGuo with FPG were nearly identical.[81]

CONCLUSIONS

MeFapy-dGuo and AFB$_1$-Fapy-dGuo have been reported to be persistent lesions from exposure to methylating agents and aflatoxin B$_1$, respectively.[82,83] This observation suggests that N^5-substituted Fapy-dGuo lesions play a role in the carcinogenicity of DNA alkylating agents and secondary tumor development from chemotherapeutic agents such as temozolomide, thio-TEPA, BCNU, and nitrogen mustards.

We have site-specifically incorporated an N^5-nitrogen mustard Fapy-dGuo lesion into oligonucleotides using the phosphoramidite approach. A DNA duplex containing the NM-Fapy-dGuo lesion is a good substrate for *E. coli* Endo IV and FPG when paired with dCyd. The Endo IV incision indicates that NM-Fapy-dGuo exists as a ~50:50 α/β-anomeric ratio after annealing, but it will slowly equilibrate to a ~14:86 ratio. The NM-Fapy-dGuo lesion will be initially formed as the natural β-anomer in cellular DNA and will slowly equilibrate to a mixture of anomers over time. Therefore, the α-anomer will be a relevant lesion if NM-Fapy-dGuo persists. The efficient excision of NM-Fapy-dGuo from duplex DNA suggests that FPG could be used as part of an enrichment protocol for the mass spectrometric detection of NM-Fapy-Gua from treated cells, animals, or clinical samples. Oligonucleotides containing NM-Fapy-dGuo will also be useful in repair and replication studies *in vitro* and in cells. Our work compliments previously reported work in which a nitrogen mustard interstrand cross-link model was engineered into oligonucleotides.[84] It should also be noted that the parent Fapy-dGuo lesion derived from

Table 2. Steady Kinetic Parameter for the Incision of the NM-Fapy-dGuo, MeFapy-dGuo, and α-dGuo Containing 24-mer Duplexes by *E. coli* Endo IV

	NM-Fapy-dGuo	MeFapy-dGuo[a]	α-dGuo[a]
k_{cat} (min^{-1})	5.4 ± 0.5	5.5 ± 0.4	4.9 ± 0.5
K_m (nM)	33 ± 6.8	18 ± 3.8	7.1 ± 1.0
k_{cat}/K_m (nM^{-1} min^{-1})	0.16	0.31	0.69

[a]This data is reprinted from ref.[44]
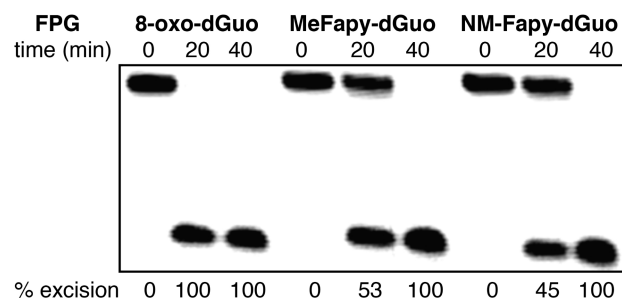
Figure 5. Gel analysis of the excision of 8-oxo-dGuo, MeFapy-dGuo, and NM-Fapy-dGuo containing duplexes by *E. coli* formamidopyrimidine glycosylase (FPG).

FPG	8-oxo-dGuo			MeFapy-dGuo			NM-Fapy-dGuo		
time (min)	0	20	40	0	20	40	0	20	40
% excision	0	100	100	0	53	100	0	45	100

Table 3. Steady Kinetic Parameter for the Excision of the NM-Fapy-dGuo, MeFapy-dGuo, and 8-Oxo-dGuo Containing Duplexes by *E. coli* FPG

	NM-Fapy-dGuo	MeFapy-dGuo	8-oxo-dGuo
k_{cat} (min^{-1})	7.6 ± 0.9	9.5 ± 0.4	4.6 ± 0.3
K_m (nM)	60 ± 18	29 ± 3.8	7.7 ± 1.4
k_{cat}/K_m (nM^{-1} min^{-1})	0.13	0.33	0.60

oxidative damage has previously been incorporated into oligonucleotides.[85−87]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR spectra of the phosphoramidite and synthetic intermediates, HPLC traces, MS analysis, and kinetic plots. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: 615-322-6100. Fax: 615-343-1234. E-mail: c.j.rizzo@vanderbilt.edu.

Notes

The authors declare no competing financial interest.

■ DEDICATION

This article is dedicated to Professor Thomas M. Harris on the occasion of his 80th birthday.

■ ABBREVIATIONS

NM, bis(2-chloroethyl)ethylamine; Fapy, formamidopyrimidine; MeFapy-dGuo, N^6-(2-deoxy-D-*erythro*-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-*N*-methylformamidopyrimidine; NM-Fapy-dGuo, N^6-(2-deoxy-D-*erythro*-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-*N*-[2-(2-hydroxylethyl)-ethylamino)-ethyl]-formamidopyrimidine; NM-dGuo, N7-[2-[(2-hydroxyethyl)ethylamino]ethyl]-2′-deoxyguanosine; 8-oxo-dGuo, 7,8-dihydro-8-oxo-2′-deoxyguanosine; Endo IV, *E. coli* endonuclease IV; FPG, *E. coli* formamidopyrimidine glycosylase; AFB$_1$-Fapy-dGuo, 8,9-dihydro-8(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B$_1$; AE-Fapy-dGuo, N^6-(2-deoxy-D-*erythro*-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-*N*-(2-aminoethyl)-formamidopyrimidine; thioTEPA, 1,1′,1″-phosphorothioyltriaziridine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; HPA, 3-hydroxypicolinic acid

■ REFERENCES

(1) Chabner, B. A., and Roberts, T. G. (2005) Timeline: chemotherapy and the war on cancer. *Nat. Rev. Cancer 5*, 65−72.

(2) Emadi, A., Jones, R. J., and Brodsky, R. A. (2009) Cyclophosphamide and cancer: golden anniversary. *Nat. Rev. Clin. Oncol. 6*, 638−647.

(3) Boysen, G., Pachkowski, B. F., Nakamura, J., and Swenberg, J. A. (2009) The formation and biological significance of N7-guanine adducts. *Mutat. Res. 678*, 76−94.

(4) Gates, K. S., Nooner, T., and Dutta, S. (2004) Biologically relevant chemical reactions of N7-alkylguanine residues in DNA. *Chem. Res. Toxicol. 17*, 839−856.

(5) Beranek, D. T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res. 231*, 11−30.

(6) Sedgwick, B. (2004) Repairing DNA-methylation damage. *Nat. Rev. Mol. Cell. Biol. 5*, 148−157.

(7) Shrivastav, N., Li, D., and Essigmann, J. M. (2010) Chemical biology of mutagenesis and DNA repair: Cellular responses to DNA alkylation. *Carcinogenesis 31*, 59−70.

(8) Bignami, M., O'Driscoll, M., Aquilina, G., and Karran, P. (2000) Unmasking a killer: DNA O^6-methylguanine and the cytotoxicity of methylating agents. *Mutat. Res. 462*, 71−82.

(9) Fronza, G., and Gold, B. (2004) The biological effects of N3-methyladenine. *J. Cell. Biochem. 91*, 250−257.

(10) Rajski, S. R., and Williams, D. M. (1998) DNA cross-linking agents as antitumor drugs. *Chem. Rev. 98*, 2723−2796.

(11) Povirk, L. F., and Shuker, D. E. (1994) DNA damage and mutagenesis induced by nitrogen mustards. *Mutat. Res. 318*, 205−226.

(12) Brookes, P., and Lawley, P. D. (1961) The reaction of mono- and di-functional alkylating agents with nucleic acids. *Biochem. J. 80*, 496−503.

(13) Baker, J. M., Parish, J. H., and Curtis, J. P. E. (1984) DNA−DNA and DNA−protein crosslinking and repair in *Neurospora crassa* following exposure to nitrogen mustard. *Mutat. Res. 132*, 171−179.

(14) Ewig, R. A., and Kohn, K. W. (1978) DNA−protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells. *Cancer Res. 38*, 3197−3203.

(15) Michaelson-Richie, E. D., Ming, X., Codreanu, S. G., Loeber, R. L., Liebler, D. C., Campbell, C., and Tretyakova, N. Y. (2011) Mechlorethamine-induced DNA−protein cross-linking in human fibrosarcoma (HT1080) cells. *J. Proteome Res. 10*, 2785−2796.

(16) Osborne, M. R., Wilman, D. E., and Lawley, P. D. (1995) Alkylation of DNA by the nitrogen mustard bis(2-chloroethyl)-methylamine. *Chem. Res. Toxicol. 8*, 316−320.

(17) Osborne, M. R., and Lawley, P. D. (1993) Alkylation of DNA by melphalan with special reference to adenine derivatives and adenine-guanine cross-linking. *Chem.—Biol. Interact. 89*, 49−60.

(18) Balcome, S., Park, S., Quirk Dorr, D., Hafner, L., Phillips, L., and Tretyakova, N. Y. (2004) Adenine-containing DNA−DNA cross-links of antitumor nitrogen mustards. *Chem. Res. Toxicol. 17*, 950−962.

(19) Rojsitthisak, P., Jongaroonngamsang, N., Romero, R. M., and Haworth, I. S. (2011) HPLC-UV, MALDI-TOF-MS and ESI-MS/MS analysis of the mechlorethamine DNA crosslink at a cytosine-cytosine mismatch pair. *PLoS One 6*, e20745.

(20) Schärer, O. D. (2005) DNA interstrand crosslinks: natural and drug-induced DNA adducts that induce unique cellular responses. *ChemBioChem 6*, 27−32.

(21) Ojwang, J. O., Grueneberg, D. A., and Loechler, E. L. (1989) Synthesis of a duplex oligonucleotide containing a nitrogen mustard interstrand DNA−DNA cross-link. *Cancer Res. 49*, 6529−6537.

(22) Grueneberg, D. A., Ojwang, J. O., Benasutti, M., Hartman, S., and Loechler, E. L. (1991) Construction of a human shuttle vector containing a single nitrogen mustard interstrand, DNA−DNA cross-link at a unique plasmid location. *Cancer Res. 51*, 2268−2272.

(23) Millard, J. T., Raucher, S., and Hopkins, P. B. (1990) Mechlorethamine cross-links deoxyguanosine residues at 5′-GNC sequences in duplex DNA fragments. *J. Am. Chem. Soc. 112*, 2459−2460.

(24) Rink, S. M., and Hopkins, P. B. (1995) A mechlorethamine-induced DNA interstrand cross-link bends duplex DNA. *Biochemistry 34*, 1439−1445.

(25) Chetsanga, C. J., Polidori, G., and Mainwaring, M. (1982) Analysis and excision of ring-opened phosphoramide mustard−deoxyguanine adducts in DNA. *Cancer Res. 42*, 2616−2621.

(26) Hemminki, K. (1987) DNA-binding products of nornitrogen mustard, a metabolite of cyclophosphamide. *Chem.—Biol. Interact. 61*, 75−88.

(27) Kallama, S., and Hemminki, K. (1984) Alkylation of guanosine by phosphoramide mustard, chlormethine hydrochloride and chlorambucil. *Acta Pharmacol. Toxicol. 54*, 214−220.

(28) Kallama, S., and Hemminki, K. (1986) Stabilities of 7-alkylguanosines and 7-deoxyguanosines formed by phosphoramide mustard and nitrogen mustard. *Chem.−Biol. Interact. 57*, 85−96.

(29) Hemminki, K. (1984) Reactions of ethyleneimine with guanosine and deoxyguanosine. *Chem.−Biol. Interact. 48*, 249−260.

(30) Hemminki, K., Peltonen, K., and Vodicka, P. (1989) Depurination from DNA of 7-methylguanine, 7-(2-aminoethyl)-guanine and ring-opened 7-methylguanines. *Chem.−Biol. Interact. 70*, 289−303.

(31) Tomasz, M., Lipman, R., Lee, M. S., Verdine, G. L., and Nakanishi, K. (1987) Reaction of acid-activated mitomycin C with calf thymus DNA and model guanines: elucidation of the base-catalyzed degradation of N7-alkylguanine nucleosides. *Biochemistry 26*, 2010−2027.

(32) Kalam, M. A., Haraguchi, K., Chandani, S., Loechler, E. L., Moriya, M., Greenberg, M. M., and Basu, A. K. (2006) Genetic effects of oxidative DNA damages: comparative mutagenesis of the imidazole ring-opened formamidopyrimidines (Fapy lesions) and 8-oxo-purines in simian kidney cells. *Nucleic Acids Res. 34*, 2305−2315.

(33) Earley, L. F., Minko, I. G., Christov, P. P., Rizzo, C. J., and Lloyd, R. S. (2013) Mutagenic spectra arising from replication bypass of the 2,6-diamino-4-hydroxy-N^5-methyl formamidopyrimidine adduct in primate cells. *Chem. Res. Toxicol. 26*, 1108−1114.

(34) Lin, Y.-C., Li, L., Makarova, A. V., Burgers, P. M., Stone, M. P., and Lloyd, R. S. (2014) Molecular basis of aflatoxin-induced mutagenesis-role of the aflatoxin B1−formamidopyrimidine adduct. *Carcinogenesis 35*, 1461−1468.

(35) Dizdaroglu, M., Kirkal, G., and Jaruga, P. (2008) Formamidopyrimidines in DNA: mechanisms of formation, repair, and biological effects. *Free Radical Biol. Med. 45*, 1610−1621.

(36) Greenberg, M. M. (2012) The formamidopyrimidines: purine lesions formed in competition with 8-oxopurines from oxidative stress. *Acc. Chem. Res. 45*, 588−597.

(37) David, S. S., and Williams, S. D. (1998) Chemistry of glycosylases and endonucleases Involved in base-excision repair. *Chem. Rev. 98*, 1221−1262.

(38) Prakash, A., Doublié, S., and Wallace, S. S. (2012) The Fpg/Nei family of DNA glycosylases: substrates, structures, and search for damage. *Prog. Mol. Biol. Transl. Sci. 110*, 71−91.

(39) Hu, J., de Souza-Pinto, N. C., Haraguchi, K., Hogue, B. A., Jaruga, P., Greenberg, M. M., Dizdaroglu, M., and Bohr, V. A. (2005) Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes. *J. Biol. Chem. 280*, 40544−40551.

(40) Krishnamurthy, N., Haraguchi, K., Greenberg, M. M., and David, S. S. (2008) Efficient removal of formamidopyrimidines by 8-oxoguanine glycosylases. *Biochemistry 47*, 1043−1050.

(41) Christov, P. P., Brown, K. L., Kozekov, I. D., Stone, M. P., Harris, T. M., and Rizzo, C. J. (2008) Site-specific synthesis and characterization of oligonucleotides containing an N^6-(2-deoxy-D-erythro-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-N-methylformamidopyrimidine lesion, the ring-opened product from N7-methylation of deoxyguanosine. *Chem. Res. Toxicol. 21*, 2324−2333.

(42) Harris, T., and Harris, C. (2000) Synthesis of N2-substituted deoxygunaosine nucleosides from 2-fluoro-6-O-(trimethylsilylethyl)-2′-deoxyinosine. *Curr. Protoc. Nucleic Acid Chem.*, 1.3.1−1.3.19.

(43) Elmquist, C. E., Stover, J. S., Wang, Z., and Rizzo, C. J. (2004) Site-specific synthesis and properties of oligonucleotides containing C8-deoxyguanosine adducts of the diatery mutagen IQ. *J. Am. Chem. Soc. 126*, 11189−11201.

(44) Christov, P. P., Banerjee, S., Stone, M. P., and Rizzo, C. J. (2010) Selective incision of the α-N^5-methyl formamidopyrimidine anomer by *Escherichia coli* endonuclease IV. *J. Nucleic Acids*, 1−10.

(45) Hemminki, K. (1985) Binding of metabolites of cyclophosphamide to DNA in a rat liver microsomal system and *in vivo* in mice. *Cancer Res. 45*, 4237−4243.

(46) Berger, M., and Cadet, J. (1985) Isolation and characterization of the radiation-induced degradation products of 2′-deoxyguanosine in oxygen-free aqueous solutions. *Z. Naturforsch. 40B*, 1519−1531.

(47) Cavaluzzi, M. J., and Borer, P. N. (2004) Revised UV extinction coefficients for nucleoside-5′-monophosphates and unpaired DNA and RNA. *Nucleic Acids Res. 32*, e13.

(48) Greenberg, M. M., Hantosi, Z., Wiederholt, C. J., and Rithner, C. D. (2001) Studies on N4-(2-deoxy-D-pentofuranosyl)-4,6-diamino-5-formamidopyrimidine (Fapy·dA) and N6-(2-deoxy-D-pentofuranosyl)-6-diamino-5-formamido-4-hydroxypyrimidine (Fapy·dG). *Biochemistry 40*, 15856−15861.

(49) Burgdorf, L. T., and Carell, T. (2002) Synthesis, stability, and conformation of the formamidopyrimidine G DNA lesion. *Chem.—Eur. J. 8*, 293−301.

(50) Humphreys, W., and Guengerich, F. P. (1991) Structure of formamidopyrimidine adducts as determined by NMR using specifically ^{15}N-labeled guanosine. *Chem. Res. Toxicol. 4*, 632−636.

(51) Boiteux, S., Belleney, J., Roques, B. P., and Laval, J. (1984) Two rotameric forms of open ring 7-methylguanine are present in alkylated polynucleotides. *Nucleic Acids Res. 12*, 5429−5439.

(52) Tudek, B., Van Zeeland, A., Kusmierek, J., and Laval, J. (1998) Activity of *Escherichia coli* DNA-glycosylases on DNA damaged by methylating and ethylating agents and influence of 3-substituted adenine derivatives. *Mutat. Res. 407*, 169−176.

(53) Brown, K. L., Deng, J. Z., Iyer, R. S., Iyer, L. G., Voehler, M. W., Stone, M. P., Harris, C. M., and Harris, T. M. (2006) Unraveling the aflatoxin-FAPY conundrum: structural basis for differential replicative processing of isomeric forms of the formamidopyrimidine-type DNA adduct of aflatoxin B$_1$. *J. Am. Chem. Soc. 128*, 15188−15199.

(54) Christov, P. P., Kozekov, I. D., Rizzo, C. J., and Harris, T. M. (2008) The formamidopyrimidine derivative of 7-(2-oxoethyl)-2′-deoxyguanosine. *Chem. Res. Toxicol. 21*, 1777−1786.

(55) Mao, H., Deng, Z., Wang, F., Harris, T. M., and Stone, M. P. (1998) An intercalated and thermally stable FAPY adduct of aflatoxin B$_1$ in a DNA duplex: structural refinement from ^{1}H NMR. *Biochemistry 37*, 4374−4387.

(56) Ramotar, D. (1997) The apurinic−apyrimidinic endonuclease IV family of DNA repair enzymes. *Biochem. Cell Biol. 75*, 327−336.

(57) Garcin, E. D., Hosfield, D. J., Desai, S. A., Haas, B. J., Björas, M., Cunningham, R. P., and Tainer, J. A. (2008) DNA apurinic-apyrimidinic site binding and excision by endonuclease IV. *Nat. Struct. Mol. Biol. 15*, 515−522.

(58) Ivanov, I., Tainer, J. A., and McCammon, J. A. (2007) Unraveling the three-metal-ion catalytic mechanism of the DNA repair enzyme endonuclease IV. *Proc. Nat. Acad. Sci. U.S.A. 104*, 1465−1470.

(59) Ide, H., Tedzuka, K., Shimzu, H., Kimura, Y., Purmal, A. A., Wallace, S. S., and Kow, Y. W. (1994) α-Deoxyadenosine, a major anoxic radiolysis product of adenine in DNA, is a substrate for *Escherichia coli* endonuclease IV. *Biochemistry 33*, 7842−7847.

(60) Ishchenko, A. A., Ide, H., Ramotar, D., Nevinsky, G., and Saparbaev, M. (2004) α-Anomeric deoxynucleotides, anoxic products of ionizing radiation, are substrates for the endonuclease IV-type AP endonucleases. *Biochemistry 43*, 15210−15216.

(61) Patro, J. N., Haraguchi, K., Delaney, M. O., and Greenberg, M. M. (2004) Probing the configurations of formamidopyrimidine lesions Fapy·dA and Fapy·dG in DNA using endonuclease IV. *Biochemistry 43*, 13397−13403.

(62) Hosfield, D. J., Guan, Y., Haas, B. J., Cunningham, R. P., and Tainer, J. A. (1999) Structure of the DNA repair enzyme endonuclease IV and its DNA complex: double-nucleotide flipping at abasic sites and three-metal-ion catalysis. *Cell 98*, 397−408.

(63) Laval, J., Lopès, F., Madelmont, J. C., Godenèche, D., Meyniel, G., Habraken, Y., O'Connor, T. R., and Boiteux, S. (1991) Excision of imidazole ring-opened N7-hydroxyethylguanine from chloroethylnitrosourea-treated DNA by *Escherichia coli* formamidopyrimidine-DNA glycosylase. *IARC Sci. Publ.*, 412−416.

(64) Lacoste, S., Castonguay, A., and Drouin, R. (2006) Formamidopyrimidine adducts are detected using the comet assay in

human cells treated with reactive metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Mutat. Res. 600*, 138−149.

(65) Li, Q., Laval, J., and Ludlum, D. B. (1997) Fpg protein releases a ring-opened N-7 guanine adduct from DNA that has been modified by sulfur mustard. *Carcinogenesis 18*, 1035−1038.

(66) Alekseyev, Y. O., Hamm, M. L., and Essigmann, J. M. (2004) Aflatoxin B$_1$ formamidopyrimidine adducts are preferentially repaired by the nucleotide excision repair pathway *in vivo*. *Carcinogenesis 25*, 1045−1051.

(67) Jurado, J., Saparbaev, M., Matray, T. J., Greenberg, M. M., and Laval, J. (1998) The ring fragmentation product of thymidine C5-hydrate when present in DNA is repaired by the *Escherichia coli* Fpg and Nth proteins. *Biochemistry 37*, 7757−7763.

(68) Asagoshi, K., Yamada, T., Terato, H., Ohyama, Y., Monden, Y., Arai, T., Nishimura, S., Aburatani, H., Lindahl, T., and Ide, H. (2000) Distinct repair activities of human 7,8-dihydro-8-oxoguanine DNA glycosylase and formamidopyrimidine DNA glycosylase for formamidopyrimidine and 7,8-dihydro-8-oxoguanine. *J. Biol. Chem. 275*, 4956−4964.

(69) Karahalil, B., Girard, P. M., Boiteux, S., and Dizdaroglu, M. (1998) Substrate specificity of the Ogg1 protein of *Saccharomyces cerevisiae*: excision of guanine lesions produced in DNA by ionizing radiation- or hydrogen peroxide/metal ion-generated free radicals. *Nucleic Acids Res. 26*, 1228−1233.

(70) Katafuchi, A., Nakano, T., Masaoka, A., Terato, H., Iwai, S., Hanaoka, F., and Ide, H. (2004) Differential specificity of human and *Escherichia coli* endonuclease III and VIII homologues for oxidative base lesions. *J. Biol. Chem. 279*, 14464−14471.

(71) Eide, L., Bjørås, M., Pirovano, M., Alseth, I., Berdal, K. G., and Seeberg, E. (1996) Base excision of oxidative purine and pyrimidine DNA damage in *Saccharomyces cerevisiae* by a DNA glycosylase with sequence similarity to endonuclease III from *Escherichia coli*. *Proc. Nat. Acad. Sci. U.S.A. 93*, 10735−10740.

(72) Asagoshi, K., Yamada, T., Okada, Y., Terato, H., Ohyama, Y., Seki, S., and Ide, H. (2000) Recognition of formamidopyrimidine by *Escherichia coli* and mammalian thymine glycol glycosylases. Distinctive paired base effects and biological and mechanistic implications. *J. Biol. Chem. 275*, 24781−24786.

(73) Luna, L., Bjørås, M., Hoff, E., Rognes, T., and Seeberg, E. (2000) Cell-cycle regulation, intracellular sorting and induced overexpression of the human NTH1 DNA glycosylase involved in removal of formamidopyrimidine residues from DNA. *Mutat. Res. 460*, 95−104.

(74) Liu, M., Bandaru, V., Bond, J. P., Jaruga, P., Zhao, X., Christov, P. P., Burrows, C. J., Rizzo, C. J., Dizdaroglu, M., and Wallace, S. S. (2010) The mouse ortholog of NEIL3 is a functional DNA glycosylase in vitro and in vivo. *Proc. Nat. Acad. Sci. U.S.A. 107*, 4925−4930.

(75) Wiederholt, C., Patro, J., Jiang, Y., Haraguchi, K., and Greenberg, M. (2005) Excision of formamidopyrimidine lesions by endonucleases III and VIII is not a major DNA repair pathway in *Escherichia coli*. *Nucleic Acids Res. 33*, 3331−3338.

(76) Xu, Y., Hansen, W. K., Rosenquist, T. A., Williams, D. A., Limp-Foster, M., and Kelley, M. R. (2001) Protection of mammalian cells against chemotherapeutic agents thiotepa, 1,3-*N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea, and mafosfamide using the DNA base excision repair genes Fpg and α-hOgg1: Implications for protective gene therapy applications. *J. Pharmacol. Exp. Ther. 296*, 825−831.

(77) Cussac, C., and Laval, F. (1996) Reduction of the toxicity and mutagenicity of aziridine in mammalian cells harboring the *Escherichia coli fpg* gene. *Nucleic Acids Res. 24*, 1742−1746.

(78) Gill, R., Cussac, C., Souhami, R., and Laval, F. (1996) Increased resistance to *N*,*N*′,*N*″-triethylenethiophosphoramide (thiotepa) in cells expressing the *Escherichia coli* formamidopyrimidine-DNA glycosylase. *Cancer Res. 56*, 3721−3724.

(79) Kobune, M., Xu, Y., Baum, C., Kelley, M. R., and Williams, D. A. (2001) Retrovirus-mediated expression of the base excision repair proteins, formamidopyrimidine DNA glycosylase or human oxoguanine DNA glycosylase, protects hematopoietic cells from *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA)-induced toxicity *in vitro* and *in vivo*. *Cancer Res. 61*, 5116−5125.

(80) He, Y.-H., Xu, Y., Kobune, M., Wu, M., Kelley, M. R., and Martin, W. J. (2002) *Escherichia coli* FPG and human OGG1 reduce DNA damage and cytotoxicity by BCNU in human lung cells. *Am. J. Physiol.: Lung Cell. Mol. Physiol. 282*, L50−L55.

(81) 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 N^7-substituted-FapydG lesion via unproductive binding mode. *Chem. Biol. 15*, 706−717.

(82) Kadlubar, F. F., Beranek, D. T., Weis, C. C., Evans, F. E., Cox, R., and Irving, C. C. (1984) Characterization of the purine ring-opened 7-methylguanine and its persistence in rat bladder epithelial DNA after treatment with the carcinogen N-methylnitrosourea. *Carcinogenesis 5*, 587−592.

(83) Essigmann, J. M., Croy, R. G., Bennett, R. A., and Wogan, G. N. (1982) Metabolic activation of aflatoxin B$_1$: patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. *Drug Metab. Rev. 13*, 581−602.

(84) Angelov, T., Guainazzi, A., and Schärer, O. D. (2009) Generation of DNA interstrand cross-links by post-synthetic reductive amination. *Org. Lett. 11*, 661−664.

(85) Haraguchi, K., and Greenberg, M. M. (2001) Synthesis of oligonucleotides containing Fapy·dG ($N6$-(2-deoxy-α,β-D-*erythro*-pentofuranosyl)-2,6-diamino-4-hydroxy-5-formamidopyrimidine). *J. Am. Chem. Soc. 123*, 8636−8637.

(86) Jiang, Y. L., Wiederholt, C. J., Patro, J. N., Haraguchi, K., and Greenberg, M. M. (2005) Synthesis of oligonucleotides containing Fapy·dG (N^6-(2-deoxy-α,β-D-erythropentofuranosyl)-2,6-diamino-4-hydroxy-5-formamidopyrimidine) using a 5′-dimethoxytrityl dinucleotide phosphoramidite. *J. Org. Chem. 70*, 141−149.

(87) Lukin, M., Minetti, C. A., Remeta, D. P., Attaluri, S., Johnson, F., Breslauer, K. J., and de Los Santos, C. (2011) Novel post-synthetic generation, isomeric resolution, and characterization of Fapy-dG within oligodeoxynucleotides: differential anomeric impacts on DNA duplex properties. *Nucleic Acids Res. 39*, 5776−5789.