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

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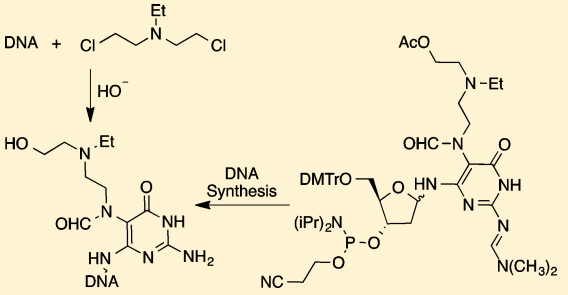
Supporting Information

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*.

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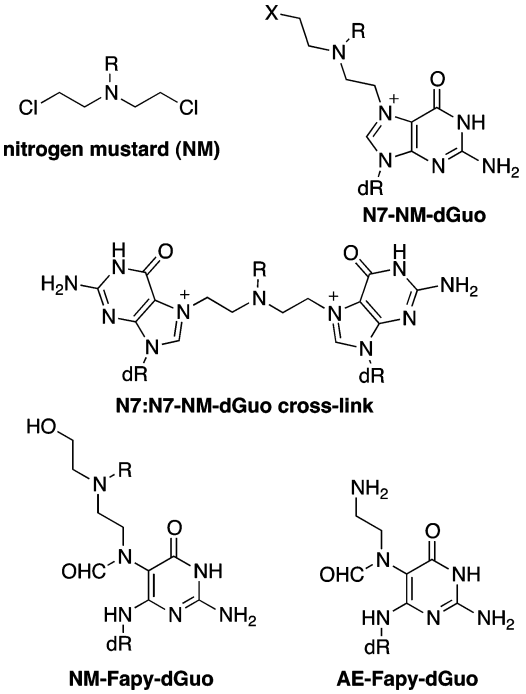

Figure 1. General structure of a nitrogen mustard (NM) and some of their dGuo adducts.

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_2Cl_2/CH_3OH, and the CH_3OH 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, O$\underline{CH_2}CH_2Si$), 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, $OCH_2\underline{CH_2}Si$), 0.15 (s, 9H, Me_3-Si). HRMS (FAB+) m/z calcd for $C_{18}H_{31}N_6O_4Si$ $[M + H]^+$, 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_2Cl_2/CH_3OH/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, O$\underline{CH_2}CH_2Si$), 4.45 (m, 1H, H-3′), 3.96 (m, 1H, H-4′), 3.69 (s, 6H, $2CH_3O$), 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, $OCH_2\underline{CH_2}Si$), 0.10 (s, 9H, Si). HRMS (FAB+) m/z calcd for $C_{39}H_{49}N_6O_6Si$ $[M + H]^+$, 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).** Triethylamine (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_2Cl_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_2Cl_2/CH_3OH/pyridine; the CH_3OH 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, O$\underline{CH_2}CH_2Si$), 4.07 (m, 1H, H-3′), 3.76 (m, 1H, H-4′), 3.70 (s, 6H, $2CH_3O$), 3.60−3.55 (m, 2H, Cl$\underline{CH_2}CH_2N$), 3.50−3.35 (m, 2H, EtN$\underline{CH_2}CH_2NCHO$), 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, $ClCH_2\underline{CH_2}N$, $EtNCH_2\underline{CH_2}NCHO$), 2.55−2.52 (m, 2H, $\underline{CH_2}CH_3$), 1.95−1.87 (m, 2H, H-2′), 0.98 (t, 2H, J = 6.0 Hz, $OCH_2\underline{CH_2}Si$), 0.84 (t, 1.5H, J = 6.0 Hz, $CH_2\underline{CH_3}$), 0.70 (t, 1.5H, J = 6.0 Hz, $CH_2\underline{CH_3}$), 0.07 (s, 9H, Me_3-Si). HRMS (FAB+) m/z calcd for $C_{45}H_{63}ClN_7O_7Si$ $[M + H]^+$, 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_2Cl_2/CH_3OH/pyridine; the CH_3OH 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, O$\underline{CH_2}CH_2Si$), 4.33 (m, 1H, H-3′), 4.10−4.00 (m, 2H, $AcO\underline{CH_2}CH_2N$), 3.90 (m, 1H, H-4′), 3.75 (s, 6H, $2CH_3O$), 3.50−3.35 (m, 2H, EtN$\underline{CH_2}CH_2NCHO$), 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, $\underline{CH_2}CH_3$, $AcOCH_2\underline{CH_2}N$, $EtNCH_2\underline{CH_2}NCHO$), 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, $OCH_2\underline{CH_2}Si$), 0.98 (t, 1.5H, J = 6.0 Hz, $CH_2\underline{CH_3}$), 0.91 (t, 1.5H, J = 6.0 Hz, $CH_2\underline{CH_3}$), 0.08 (s, 9H, Me_3-Si). HRMS (FAB+) m/z calcd for $C_{47}H_{66}N_7O_9Si$ $[M + H]^+$, 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_2Cl_2/CH_3OH/pyridine; the CH_3OH 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,

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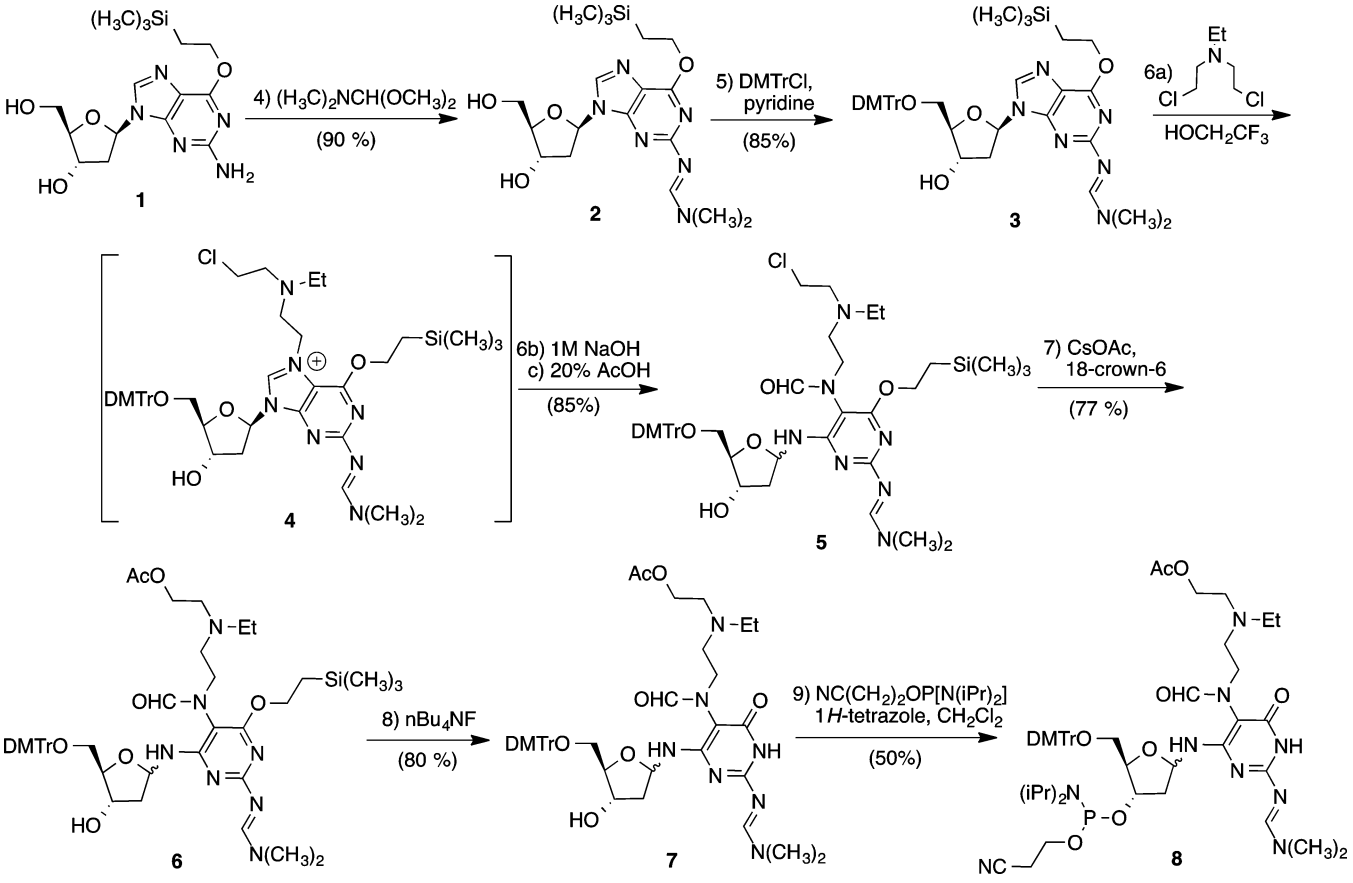
Scheme 1. Synthesis of the NM-Fapy-dGuo Phosphoramidite



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, AcOCH$_2$CH$_2$N), 4.23 (m, 1H, H-4′), 3.72 (s, 6H, 2CH$_3$O), 3.50−3.35 (m, 2H, EtNCH$_2$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, CH$_2$CH$_3$, AcOCH$_2$CH$_2$N, EtNCH$_2$CH$_2$NCHO), 1.98−1.85 (m, 5H, H-2′, AcO), 1.33−1.22 (multiple t, total 3H, CH$_2$CH$_3$). HRMS (FAB+) m/z calcd for $C_{42}H_{54}N_7O_9$ [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_2Cl_2 (20 mL), and a solution of anhydrous 1*H*-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_2Cl_2/CH_3OH/pyridine; the CH_3OH 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′, AcOCH$_2$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, EtNCH$_2$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$_2$CH$_2$N, EtNCH$_2$CH$_2$NCHO), 2.55−2.52 (m, 2H, CH$_2$CH$_3$), 2.03−1.90 (m, 5H, H-2′, AcO), 1.33−1.21 (multiple t, total 3H, CH$_2$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 $C_{51}H_{71}N_9O_{10}P$ [M + H]$^+$, 1000.5056; found, 1000.5079.

Oligonucleotide Synthesis. The oligodeoxynucleotides were synthesized on a Perseptive 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]

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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 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.

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

	oligonucleotide	*m/z* found	*m/z* 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

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

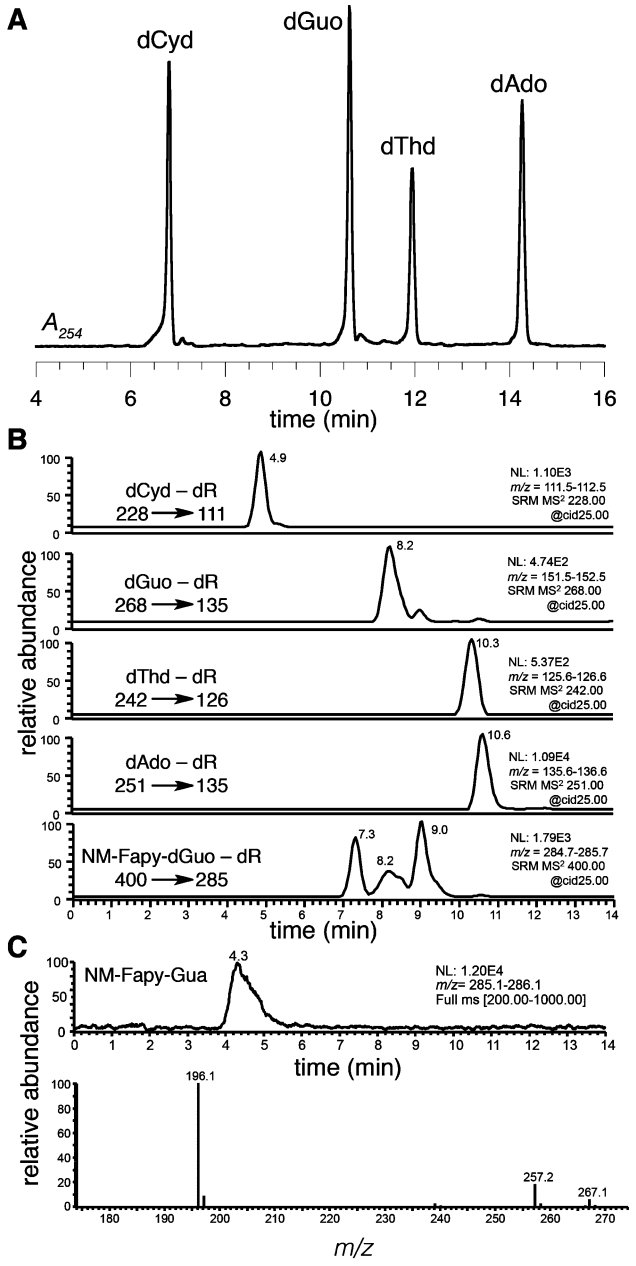

Figure 2. (A) HPLC (A_{254}) and (B) UPLC-SRM-MS^2 analysis of the enzymatic digestion of NM-Fapy-dGuo containing 12-mer oligonucleotide (**9**). (C) UPLC-MS analysis of the NM-Fapy-Gua after acid hydrolysis of the enzymatic digestion reaction of oligonucleotide (**9**). The reconstructed ion chromatogram 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,

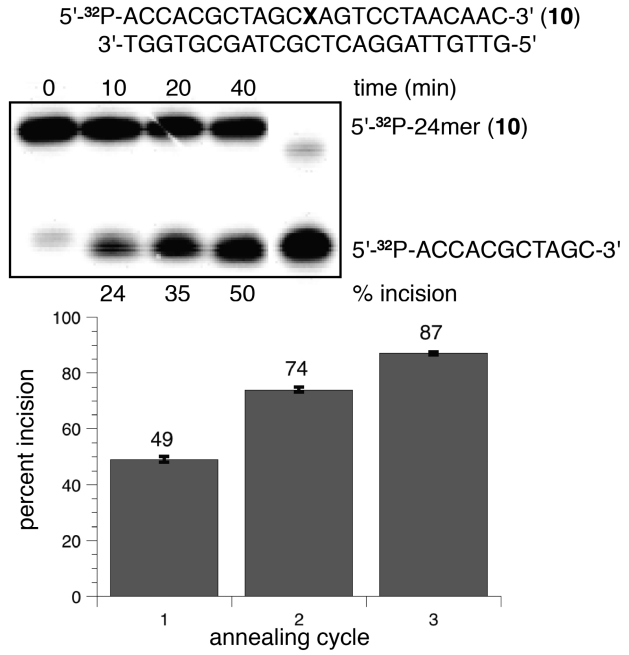

Figure 3. (Top) Gel electrophoretic analysis of the incision of the NM-Fapy-dGuo containing 24-mer (**10**) duplex with *E. coli* Endo IV. The right lane contains a standard of the 5′-^{32}P-ACCACGCTAGC-3′ incision product. (Bottom) Percentage of incision product after denaturation−reannealing and additional 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

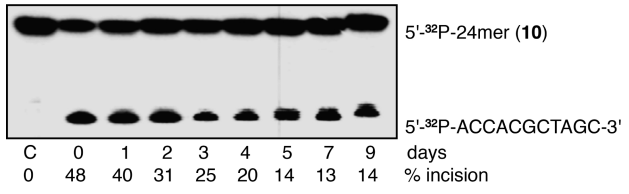

Figure 4. Time course for Endo IV incision of the NM-Fapy-dGuo containing 24-mer (**10**). Endo IV was omitted in the control lane (C). The day 0 time point was after an incubation time of 30 min.

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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 overexpression 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′-TXG-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]

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]

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

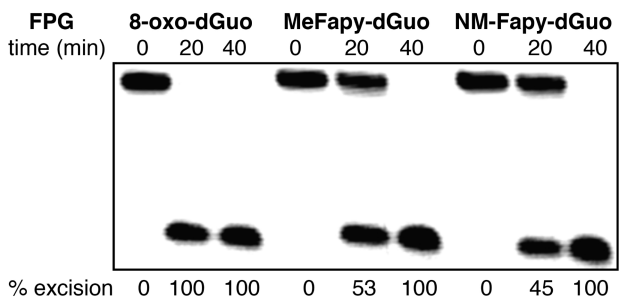

Figure 5. Gel analysis of the excision of 8-oxo-dGuo, MeFapy-dGuo, and NM-Fapy-dGuo containing duplexes by *E. coli* formamidopyrimidine glycosylase (FPG).

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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.

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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

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