Synthesis of Oligonucleotides Containing Fapy·dG (*N6*- (2-Deoxy-α,*β*-D-*erythro*-pentofuranosyl)-2,6-diamino-4-hydroxy-5-formamidopyrimidine)

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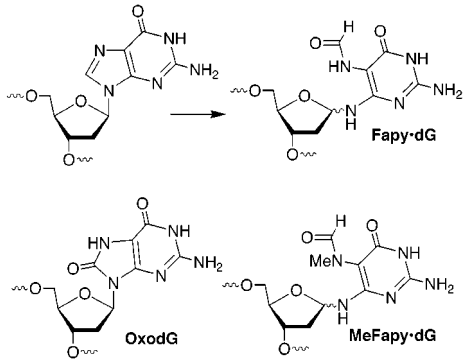
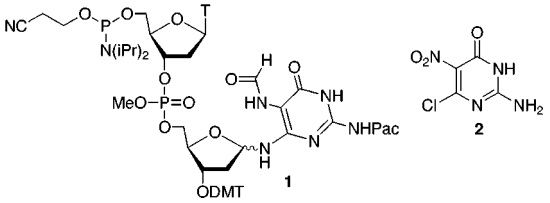
Exposure of DNA to various forms of oxidative stress results in its structural alteration. The effects of the lesions produced on the structure of DNA and their interaction with repair and polymerase enzymes have important consequences in aging and in the etiology of diseases, including cancer and neurodegenerative diseases such as Cockayne syndrome and xeroderma pigmentosum.[1] The formamidopyrimidine lesions (e.g. Fapy·dG) are produced from the purine nucleotides in DNA due to the effects of ionizing irradiation and agents that produce reactive oxygen species.[2,3] Under O_2 limiting conditions the yield of Fapy·dG formed via γ-radiolysis is greater than that of the well-studied lesion, OxodG.[2a] An indication that Fapy·dG formation is biologically significant is its excision by the base excision repair enzyme that bears its name, formamidopyrimidine glycosylase (fpg, mutM).[4] Furthermore, there is evidence that related molecules MeFapy·dG and Fapy·dA adversely affect DNA polymerase activity.[5,6] However, examination of the effects of Fapy·dG on DNA structure and function is limited by the inability to prepare nucleic acids containing this lesion at a defined site. We wish to report the first synthesis of oligonucleotides containing Fapy·dG.



DNA containing *N*-methylformamidopyrimidines (e.g. MeFapy·dG) has been prepared via random methylation by dimethyl sulfate, followed by alkaline hydrolysis.[5a] Recently, a chemoenzymatic method for preparing DNA containing MeFapy·dG at a defined site was reported.[7] These methods are not applicable to the synthesis of DNA containing the parent Fapy·dG. Consequently, studies on Fapy·dG are limited to DNA in which it and other lesions are produced via photolysis, γ-radiolysis, or radiomimetic methods.[2−6]

Solid-phase oligonucleotide synthesis is extremely useful for providing oligonucleotides containing DNA lesions that are useful in physicochemical and biological studies.[8−11] However, the chemical properties of Fapy·dG presented unique challenges for oligonucleotide synthesis. The greatest anticipated hurdle for the successful synthesis of oligonucleotides containing Fapy·dG was the facile epimerization of *N*-(2-deoxy-α,*β*-D-*erythro*-pentofuranosyl)formamidopyrimidine nucleosides and their rearrangement to pyranose isomers, a process that cannot occur when the lesion is produced within a biopolymer.[12] Facile epimerization of formamidopyrimidines obviated the need for stereoselective synthesis of oligonucleotides containing α- or *β*-Fapy·dG. Consequently, we adopted a synthetic strategy that was unconcerned with stereochemistry at the anomeric center, but avoided exposing the primary hydroxyl group of the sugar in the presence of the formamidopyrimidine. For oligonucleotide synthesis this required incorporating Fapy·dG as part of a dinucleotide phosphoramidite (**1**).[13]



The nitro group also served to accelerate nucleophilic aromatic substitution by the requisite 2-deoxyribosylamine, which was prepared from the triacetate (**3**) and used crude (Scheme 1).[15] The dimethoxytrityl group was used to protect the C3-hydroxyl because we wanted to unmask the primary hydroxyl under nonacidic conditions in order to reduce the likelihood that **6** rearranges and minimize protecting group manipulations later in the synthesis. This required that we synthesize the oligonucleotides in the 5′→3′ direction using reverse phosphoramidites.[16] Since Fapy nucleosides epimerize in water, it was not necessary to separate anomers following glycosylation.[12] But the anomers of **6** were separated following protection of the *N*2-amino group and desilylation, to facilitate characterization. Neither anomer of **6** rearranged to the pyranose isomer, indicating that the electron

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(13) DMT = dimethoxytrityl; Pac = phenoxyacetyl

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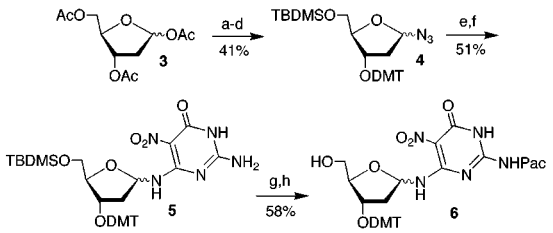
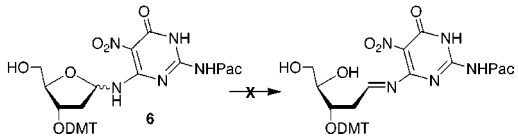
Scheme 1[a]

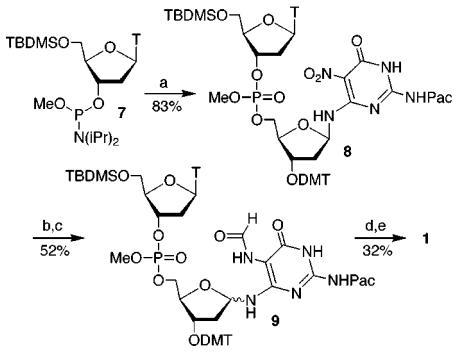


[a] Key: (a) $TMSN_3$, TMSOTf, CH_2Cl_2. (b) NaOMe, MeOH. (c) TBDMSCl, pyridine. (d) DMTCl, pyridine. (e) H_2, $Pd/CaCO_3$, EtOH. (f) **2**, diisopropylethylamine, EtOH. (g) $PhOCH_2CO_2H$, PyBOP, CH_2Cl_2. (h) $Bu_4N^+ F^-$, AcOH, THF.

Scheme 2



Scheme 3[a]



[a] Key: (a) β-**6**, tetrazole, then t-BuOOH. (b) H_2, Pd/C, MeOH. (c) HC(O)OAc, pyridine, THF. (d) $Bu_4N^+ F^-$, AcOH, THF. (e) 2-cyanoethyl phosphoramidic chloride, diisopropylethylamine, CH_2Cl_2.

withdrawing nitro group inhibited the ability of the N6 nitrogen to stabilize the acyclic intermediate necessary for rearrangement and epimerization (Scheme 2).

Dinucleotide couplings were carried out by using single diastereomers of **6** for characterization purposes (Scheme 3). However, for large-scale synthesis anomeric mixtures of **6** were employed. The *O*-methyl phosphoramidite (**7**) was employed because the alternative β-cyanoethyl group was unstable to subsequent transformations. Formylation of the *N*5-amino group obtained upon reduction of the nitro group (**8**) produced **9** as a mixture of diastereomers and formamide rotamers. Diastereomeric products differing at the anomeric center of Fapy·dG (β:α = 2:1) were separated from one another.[17] Desilylation of the β-anomer of **9** yielded a mixture of dinucleotides epimeric at the Fapy·dG anomeric center (β:α = 1.7:1).[17] The major isomer assigned as the β-Fapy·dG dinucleotide was carried on to **1** by using standard phosphitylation methods.[11]

Oligonucleotides (**10**, **11**) were prepared by using **1** and reverse β-cyanoethyl phosphoramidites containing "fast-deprotecting" groups on the exocyclic amines of dA, dC, and dG.[11] Native nucleotide phosphoramidites (0.05 M) were coupled by using automated synthesis cycles comparable to those previously reported for reverse phosphoramidites,[16] but **1** (0.05 M) required double-coupling for extended times (30 min/coupling) to achieve a 70% yield. Unreacted hydroxyl groups on the growing oligonucleotide were capped by using trimethylacetic anhydride/lutidine instead of acetic anhydride to guard against transamidation of deoxyguanosine and deformylation of Fapy·dG.[18] Deprotection was carried out in two steps. Demethylation of the phosphate

5′-d(TGC AGT **X**AC AGC)
10
5′-d(TGC AGT **X**TC AGC)
11
5′-d(AGG CGT TCA ACG TGC AGT **X**AC AGC ACG TCC CAT GGT)
12
5′-d(ACG TCC CAT GGT)
13
5′-d(AGG CGTTCA ACG)
14
5′-d(TCA GAC ACC ATG GGA CGT GCT GTC ACT GCA CGT TGA ACG CCT)
15
X = Fapy·dG

triester introduced along with **1** was achieved with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (0.2 M, 20 min).[19] The remainder of the protecting groups were removed and the oligonucleotide cleaved from the support by using anhydrous K_2CO_3 (0.05 M)/MeOH (4 h). Denaturing polyacrylamide gel electrophoresis (PAGE) purified oligonucleotides (**10**, **11**) exhibited the expected molecular weight by ESI-MS and showed no evidence for deformylation, dehydration, or deglycosylation of Fapy·dG.[20] Due to the modest coupling yield of **1**, a 36mer (**12**) was prepared enzymatically from **10** on a 5 nmol scale.[21] Oligonucleotides **10** and **13** were 5′-phosphorylated by using polynucleotide T4 kinase and then hybridized along with **14** to a DNA template (**15**). Following incubation with T4 DNA ligase, **12** was purified by PAGE and analyzed by MALDI-TOF MS.[20] The 36mer was characterized further by reacting 5′-^{32}P-**12** hybridized to its complement with fpg protein (200 nM). Quantitative cleavage within 5 min provided additional evidence that none of the Fapy·dG underwent dehydration to form dG during any of the synthesis or purification procedures.[20]

In summary, we have developed chemical and enzymatic methods for synthesizing oligonucleotides containing Fapy·dG at a defined site. These biopolymers will facilitate elucidation of the physical, chemical, and biological effects of this lesion.

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Supporting Information Available: Procedures for the synthesis of **1**, synthesis/purification of oligonucleotides **10**−**12**, and the cleavage of 5′-^{32}P-**12** by fpg protein; mass spectra of **10**−**12**; and phosphor image showing the cleavage of 5′-^{32}P-**12** by fpg protein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Stereochemical assignments of anomers in **9** and the desilylated product were based upon the chemical shift of the formyl protons. Shielding by the phenyl rings of the dimethoxytrityl protecting group results in an upfield shift of this proton in the respective α-isomers (**9**, 0.52 ppm; desilylated product, 0.42 ppm).

(18) Control experiments on protected monomers revealed that acetic anhydride/lutidine/*N*-methyl imidazole capping conditions resulted in acylation of Fapy·dG. For the effects of capping conditions using "fast-deprotecting" phosphoramidites see: Zhu, Q.; Delaney, M. O.; Greenberg, M. M. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1105.

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