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Novel post-synthetic generation, isomeric resolution, and characterization of Fapy-dG within oligodeoxynucleotides: differential anomeric impacts on DNA duplex properties

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ABSTRACT

Accumulation of damaged guanine nucleobases within genomic DNA, including the imidazole ring opened N^6-(2-Deoxy-α,β-D-*erythro*-pentafuranosyl)-2,6-diamino-4-hydroxy-5-formylamidopyrimidine (Fapy-dG), is associated with progression of age-related diseases and cancer. To evaluate the impact of this mutagenic lesion on DNA structure and energetics, we have developed a novel synthetic strategy to incorporate cognate Fapy-dG site-specifically within any oligodeoxynucleotide sequence. The scheme involves the synthesis of an oligonucleotide precursor containing a 5-nitropyrimidine moiety at the desired lesion site via standard solid-phase procedures. Following deprotection and isolation, the Fapy-dG lesion is generated by catalytic hydrogenation and subsequent formylation. NMR assignment of the Fapy-dG lesion (X) embedded within a TXT trimer reveals the presence of rotameric and anomeric species. The latter have been characterized by synthesizing the tridecamer oligodeoxynucleotide d(GCGTACXC ATGCG) harboring Fapy-dG as the central residue and developing a protocol to resolve the isomeric components. Hybridization of the chromatographically isolated fractions with their complementary d(CGCATGCGTACGC) counterpart yields two Fapy-dG·C duplexes that are differentially destabilized relative to the canonical G·C parent. The resultant duplexes exhibit distinct thermal and thermodynamic profiles that are characteristic of α- and β-anomers, the former more destabilizing than the latter. These anomer-specific impacts are discussed in terms of differential repair enzyme recognition, processing and translesion synthesis.

INTRODUCTION

Hydroxyl radicals arising from exposure to environmental oxidants or produced by cell metabolism react with DNA causing thousands of oxidatively damaged nucleotides per diem (1). The reaction of hydroxyl radicals with DNA can generate different base and sugar radicals, including the C8-OH guanine adduct. As illustrated in Scheme 1, oxidation of the C8-OH radical forms 8-oxo-7,8-dihydroxy-2′-deoxyguanosine (8-oxodG), while the competing one-electron reduction that predominates under anoxic conditions generates N^6-(2-Deoxy-α,β-D-*erythro*-pentafuranosyl)-2,6-diamino-4-hydroxy-5-formylamidopyrimidine (Fapy-dG) via imidazole ring opening (2). Formamidopyrimidine (Fapy) lesions are abundant under a variety of experimental conditions. Cell exposure to γ-radiation or treatment with reactive oxygen species generates 8-oxodG and Fapy-dG, the latter forming even in the presence of oxygen (3–6). Mammalian cells possess background levels of both lesions, which rise rapidly following oxidative stress (7–9) and are elevated in a number of circumstances including chronic inflammatory processes

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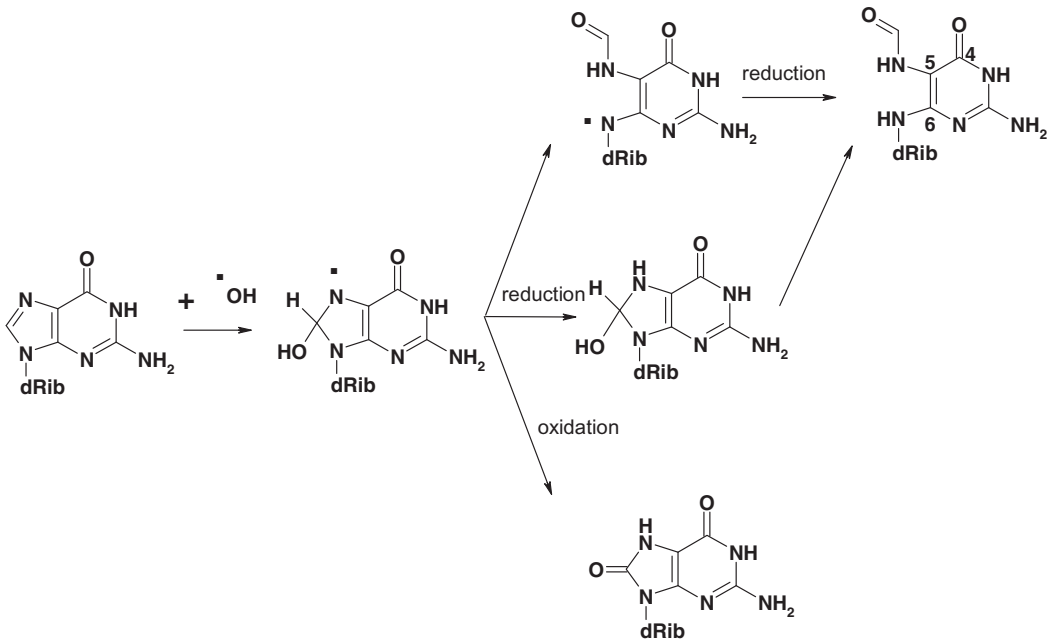


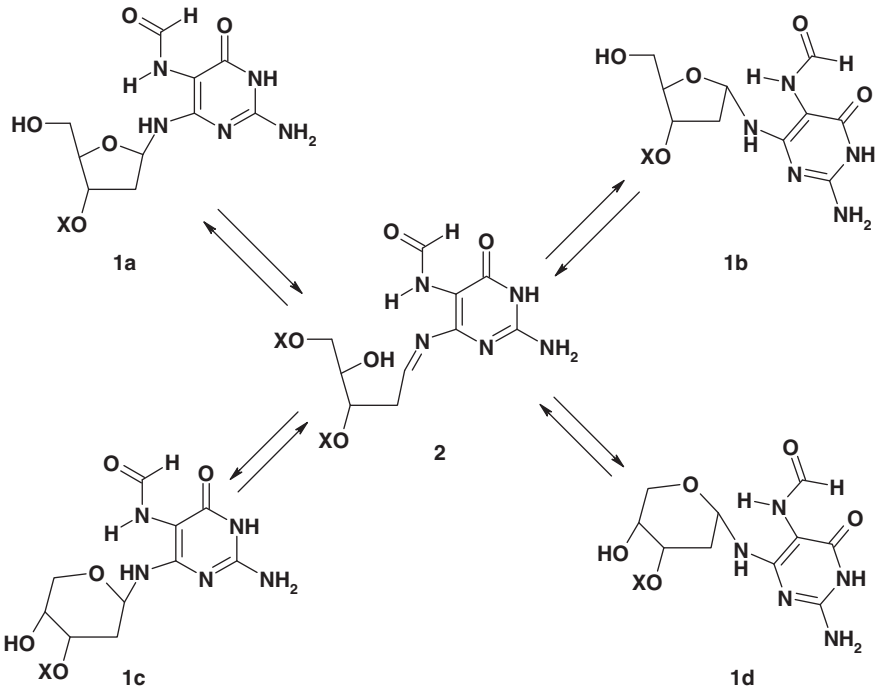
Scheme 1. Oxidation pathways from a common precursor that yields 8-oxodG and Fapy-dG.

and pathological conditions such as Alzheimer's disease and cancer (10–14).

The base excision repair (BER) pathway efficiently removes Fapy-dG and 8-oxodG lesions from DNA. Pioneering studies using irradiated or photosensitized DNA harboring multiple oxidative lesions have demonstrated that the bacterial Fapy-DNA glycosylase (Fpg, MutM) removes a broad range of oxidative damage, including 8-oxodG, Fapy-dG, methyl-Fapy-dG and Fapy-dA (15–17). These findings have been confirmed in recent investigations of Fapy lesions incorporated site-specifically within DNA constructs to explore the impact of counter-base effects on enzymatic activity (18,19). The specificity constant reported for Fpg excision of Fapy-dG is ~17-fold higher when it pairs with dC rather than dA. Eukaryotic cells possess several enzymes that can remove Fapy lesions from DNA duplexes including Ogg1, the functional homologue of Fpg. Ogg1 excises both Fapy-dG and 8-oxodG, yet is largely inactive in the presence of Fapy-dA adducts (20–24). Mammalian Nei-like enzymes efficiently remove Fapy-dG and Fapy-dA lesions from damaged duplexes, but do not process 8-oxodG containing substrates, in contrast to Ogg1 and Fpg (25–28).

Despite the impressive number of studies that specifically address the impact of Fapy-dG lesions and their consequence on the host cell, there is a noticeable dearth of parallel synthetic efforts to obtain suitable DNA constructs containing the cognate lesion. The high reactivity of Fapy lesions in standard oligonucleotide synthesis schemes coupled with the challenge of developing efficient synthetic strategies has hampered biochemical and biophysical studies of Fapy-dG embedded within defined DNA duplexes. The existing synthetic approach for preparation of Fapy-containing oligomers requires the synthesis of a phosphoramidite dimer with a standard nucleotide at the lesion 5′-side and its subsequent incorporation into DNA following a modified solid-phase protocol (29,30). Despite these important advances, the dimeric synthesis approach imposes a significant limitation in terms of readily exploring the influence of sequence context on Fapy properties. Specifically, the 5′-neighboring base is generally a thymine as other bases are not easily incorporated within the synthetic scheme to facilitate sequence-dependent studies. This serious deficiency may only be alleviated by a massive synthetic effort that entails preparing all four possible dimeric precursors.

The synthesis and isolation of homogeneous Fapy-dG 2′-oligodeoxynucleotides represent formidable tasks, given that the cognate lesion is chemically labile and exists as a mixture of α- and β-anomers (Scheme 2; **1a** and **1b**) at the nucleoside level, which re-arrange to the pyranosides **1c** and **1d**. The impact of lesion anomerization on biological outcomes has only been assessed indirectly via synthesis of chemically stable analogues that do not epimerize. Although proven useful in a number of applications including translesion synthesis assays, such non-hydrolyzable analogues preclude kinetic and thermodynamic characterization of the forces driving Fapy-dG excision/repair. Recent crystallographic studies of Fpg complexes with damaged DNA duplexes employing the carbocyclic analogue of Fapy-dG (i.e. carba-Fapy-dG) (31) have yielded intriguing results in terms of conformational preferences that differ markedly from those reported for 8-oxodG (32). Whereas these differences may be attributed to sequence variations within Fpg (i.e. $S^{77}E$ in *Lactococcus lactis*), there are legitimate concerns regarding whether non-hydrolyzable analogues (e.g. carba-Fapy-dG) truly mimic their cognate counterparts in lesion recognition processes (33–35). Specifically, analogue binding in the absence of turnover might actually reflect fortuitous interactions that are not necessarily biologically relevant. These findings underscore the



Scheme 2. Proposed mechanism of Fapy isomerization via an open intermediate.

need to develop synthetic protocols that yield stable cognate lesions at isomeric resolution for direct assessment and characterization of their biophysical properties and biological outcomes.

In the present study, we report a novel post-synthetic strategy for the site-specific incorporation of Fapy-dG residues within any oligodeoxynucleotide sequence context. This methodology overcomes inherent limitations of previous approaches by establishing the Fapy moiety as the 5-formamido group in the pyrimidino xenonucleotide (Scheme 3). We discuss the application of this strategy to the preparation of isotopically labeled Fapy-dG incorporated within a single-strand DNA trimer for NMR assignment of the anomeric species. In order to evaluate the impact of Fapy-dG on DNA duplex energetics, we synthesized a tridecameric oligodeoxynucleotide with the lesion embedded as the central residue. Subsequent isolation and hybridization with the corresponding complementary strand generates Fapy-dG•C duplexes exhibiting distinct thermal and thermodynamic properties that are consistent with α- and β-anomers. The combination of structural and thermodynamic analysis provides insights into the properties of Fapy-dG•C duplexes, which sets the stage for a detailed characterization of the respective anomers in terms of lesion formation, translesion replication and BER.

MATERIALS AND METHODS

Reagents and equipment

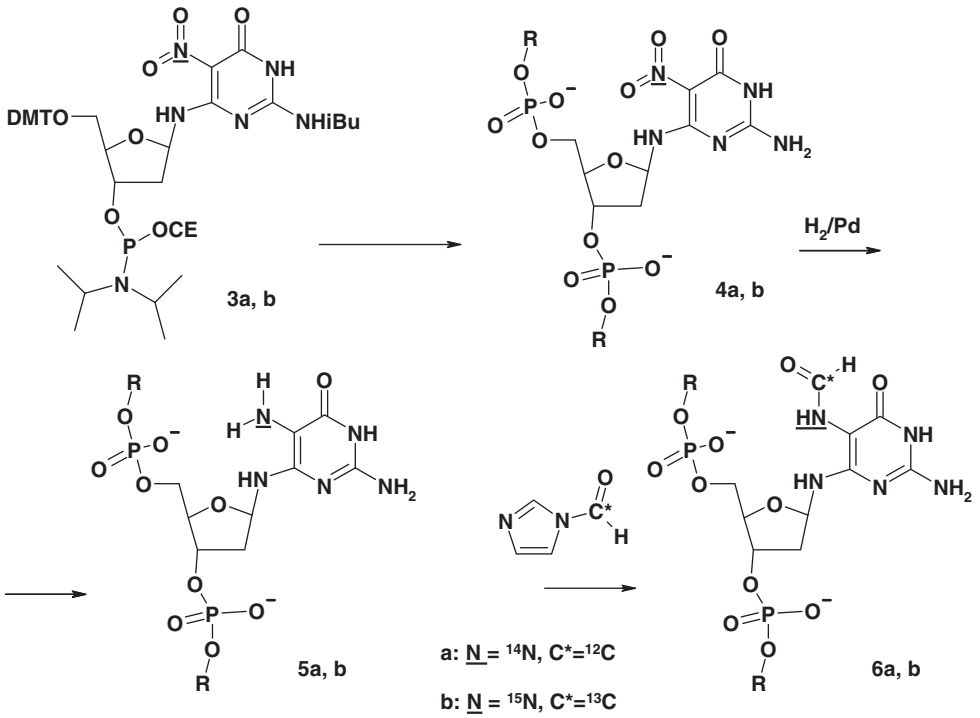
Chemicals and solvents were purchased from Fluka-Sigma-Aldrich and DNA syntheses were performed on an Applied Biosystems instrument using reagents from Glenn Research. Isotopically labeled compounds were purchased from Cambridge Isotope Laboratories. HPLC analysis and purification were performed on a Gilson system equipped with a Zorbax C_{18} semi-preparative column (7.5×300 mm). Further purification was achieved using a high resolution Mono Q^{TM} HR 5/5 (4.6×100 mm) pre-packed anion exchange column (Amersham Pharmacia, Piscataway, NJ, USA). NMR spectra were recorded on Varian Inova instruments operating at 400, 500 and 600 MHz, or on a Bruker Avance spectrometer at 700 MHz. Time domain data were processed using Vnmr (Varian, CA, USA), Felix or NMRPipe (36) software. Two dimensional spectra were visualized using Sparky (37). Oligodeoxynucleotides were analyzed by nano-ESI on a Micromass Platform time-of-flight mass spectrometer operated in negative mode. Molecular modeling was performed using HyperChem (Hypercube, Inc.).

Preparation of phosphoramidites for solid-phase DNA synthesis

Details of the synthesis are presented as Supplementary Data.

Synthesis of oligodeoxynucleotides

The NPym-dG phosphoramidite **3** was prepared using the standard protocol (38) (Supplementary Scheme S1). Oligodeoxynucleotide synthesis followed a standard solid-phase protocol using ultra-mild phosphoramidites. Saturated ammonia treatment for 2 h at 50°C removed all protecting groups and the oligomers were purified using a standard solid-phase protocol.



Scheme 3. Post-synthetic generation of Fapy-dG in DNA.

Standard procedure for post-synthetic reduction and formylation of 5-nitropyrimidine oligodeoxynucleotides

Approximately 300 OD_{260} of oligodeoxynucleotide was dissolved in 1 ml of water, followed by the addition of 500 µl of methanol and 50 µl of triethylamine to maintain a basic medium. A total of 2 mg of Pd black was added to the mixture and shaken under hydrogen (3 atm pressure) for 3–6 h. The reaction was monitored by mass-spectroscopy. After the starting material disappeared completely, 50 ml of mercaptoethanol was added to the mixture followed by 1 ml of a freshly prepared THF solution of formylimidazole (38). The addition was conducted portion-wise via a syringe with 200 µl of solution injected at 30 min time intervals over 2 h. When MS revealed that the aminopyrimidine oligodeoxynucleotide had completely disappeared, the reaction mixture was centrifuged, evaporated to remove organic solvents and the remaining water solution diluted with 0.1 M triethylammonium acetate buffer and purified using ion-pair reverse phase HPLC with a linear gradient of acetonitrile, 0–40%, 30 min in 0.1 M TEAA.

Analytical and preparative ion exchange chromatographic protocol to isolate and identify Fapy-dG oligodeoxynucleotide species

Purification of Fapy-dG fractions was performed via anion-exchange chromatography employing previously published procedures (39) with specific modifications conducive for the isolation of anomeric Fapy-dG species. The anion-exchange column was equilibrated with TA buffer composed of 50 mM Tris–HCl and 15% acetonitrile (pH 8.0) at a flow rate of 1.5 ml min^{-1}. The reverse-phase purified and lyophilized Fapy-dG samples were suspended in de-ionized water (1.5–2.0 ml), injected into the column and the fractions eluted with a linear gradient (typically 20–80%) of TA buffer containing 1.0 M NaCl over a period of 20 min with the outflow monitored at 260 nm. The procedure was repeated by reapplying the enriched isolated fractions following a 3-fold dilution in TA buffer. In the final step, each of the primary components (i.e. Fractions I and II) eluted at 11 and 11.5 min, respectively. The fractions were collected conservatively to avoid peak overlap and immediately combined with an excess of complementary strand in TA buffer. The resultant disproportionate single-strand mixtures were then reapplied into the column to separate the duplex from the excess of single-strand species. The duplex peaks that elute typically at ~14 min (i.e. ~0.7 M NaCl) were collected and immediately diluted in cold absolute ethanol and equilibrated at −20°C for a minimum of 30 min. The ethanol precipitated material was collected in a Model 5417R refrigerated centrifuge (Brinkmann Instruments, Westbury, NY, USA) operating at 16 000 rpm for 20 min at 5.0°C. The resultant pellet was dried in a SpeedVac Concentrator (Savant, Hicksville, NY, USA) under vacuum and suspended in cold deionized water followed by freezing in liquid nitrogen. The procedure was repeated and the final pellet suspended in a buffer comprising 10 mM sodium phosphate and 150 mM NaCl (pH 7.0).

Fapy-dG·C duplex concentration determination and standard preparation

An aliquot of the duplex stock in 10 mM sodium phosphate and 150 mM NaCl (pH 7.0) was diluted in the

identical buffer system to prepare a Fapy-dG•C standard for concentration determination. The absorbance was measured spectrophotometrically by monitoring the temperature-dependent hyperchromicity at 260 nm via conventional methods. Linear extrapolation of the post-transition baseline to 25°C yields A_{260} of the dissociated oligodeoxynucleotide that represents the sum of the single-strand extinction coefficients measured via traditional enzyme digestion methods (40,41). Alternatively, an aliquot of the duplex was digested using an excess of nucleases and the resultant absorbance divided by the sum of the extinction coefficients calculated for the nucleotides. The extinction coefficient for the Fapy-dG single strand was corroborated via the method of continuous fractions by hybridizing defined ratios of the complementary strand with known extinction coefficient in isothermal mixing experiments and casting the data in the form of Job plots (42).

Thermodynamic analysis

Temperature-dependent UV melting experiments were performed on an Aviv Model 14 UV/Vis spectrophotometer (Aviv Biomedical, Inc., Lakewood, NJ, USA). Samples in quartz cuvettes of 1.0 cm path length were heated in the thermostatted sample compartment over the temperature range of 0–90.0°C. The absorbance at 260 nm was recorded at 0.5°C increments following integration for 10 s to monitor the hyperchromicity change as a function of temperature. The duplex dissociation temperatures and van't Hoff thermodynamic parameters have been determined by shape analysis of the temperature-dependent hyperchromicity profiles for a bimolecular two-state dissociation process assuming a zero heat capacity in accordance with traditional protocols (43).

RESULTS AND DISCUSSION

Rationale for characterizing cognate Fapy-dG at anomeric resolution

Fapy lesions have been the subject of considerable interest and inquiry since their discovery over three decades ago as some of the most ubiquitous lesions implicated in DNA mutagenesis. Despite progress in elucidating its promutagenic properties and *in vivo* processing by the BER pathway, there are no structural or thermodynamic characterizations of DNA duplexes harboring a cognate Fapy-dG. One of the primary obstacles impeding systematic investigations is the fact that a canonical undamaged DNA base in a conventional β-configuration may potentially yield a mixture of α- and β-anomeric species upon conversion to Fapy. Moreover, the equilibrium ratio and sequence context-dependence of the isomerization process within the DNA duplex is not adequately understood. Since epimerization rates are conceivably faster than competing deglycosylation reactions, there is supporting evidence to conclude that isomeric species are present in the cell during biological processes (44), thereby rendering characterization of BER processing specificities a formidable task. A recent study on Fapy-dG promutagenic potential in *Escherichia coli* suggests that the lesion is 3- to 5-fold less mutagenic than 8-oxodG embedded within an identical sequence context (45). Conversely, Fapy-dG may promote G to T transversions in mammalian (COS) cells at a greater magnitude than 8-oxodG, given that the latter occurs in a highly sequence-context dependent manner (46). The structural and energetic determinants associated with Fapy recognition and the possible stabilization of its mutagenic intermediates remains a matter of inquiry. Since most of these studies employ either synthetic strategies that produce anomeric mixtures or utilize stable bio-isosteric analogues, systematic investigations of the isolated cognate Fapy-dG conformers are warranted to elucidate unequivocally the anomer-dependent replication and/or repair outcomes.

Synthesis of nitropyrimidine oligodeoxynucleotides

The low chemical stability of the Fapy moiety coupled with the ability of the Fapy-dG nucleoside to isomerize represents a major challenge for solid-phase DNA synthesis. Previous studies have demonstrated that the Fapy nucleosides readily isomerize to yield a mixture of anomeric 2′-deoxyribofuranosides (Scheme 2; **1a** and **1b**) (47). Moreover, deprotection of the 5′-hydroxyl in Fapy furanosides leads by rearrangement to an anomeric mixture of corresponding pyranosides (i.e. **1c** and **1d**). The latter renders a standard solid-phase protocol unsatisfactory for the synthesis of Fapy-containing DNA. A solution to this problem has been provided by the Greenberg group, who initially reported the synthesis of Fapy-dG containing DNA using a modified 5′- to 3′-solid-phase protocol (29), and subsequently prepared the dinucleotide phosphoramidite with protected Fapy-dG at its 3′-end (48). Since these approaches require substantial modification of the standard solid-phase protocol using intermediates that are not trivial to synthesize, we decided to develop a universal post-synthetic method for the generation of Fapy lesions in DNA. Our strategy is based on reduction of a nitropyrimidine residue present in oligomeric DNA followed by formylation of the resulting amino group in aqueous media. Initially, we surmised that the problem of furanoside isomerization to the pyranoside with the 5′-hydroxyl group unprotected might be due to an increase in the acid-sensitivity of the system given the basic nature of the associated pyrimidine. We hypothesized that this might be solved by retaining the 5-nitro group, the progenitor of the formamido group, during oligomer synthesis. This reasoning is based on the idea that the strongly electron-withdrawing nitro group would deplete the electrons on the 6-amino group, thus reducing its ability to facilitate acid-catalyzed opening of the ribosyl ring. This concept subsequently proved to be correct. Following reduction of the nitro group, formylation of the 5-amino group in the precursor Fapy lesion is expected to be highly selective since all other amino and hydroxy functions in the deprotected oligomers are much less basic in character. To verify the feasibility of this synthesis strategy, we prepared a model TpNPympT trimer **7** (Figure 1) using NPym-dG amidite **3** as a building block. For preparation of the starting nucleoside, we

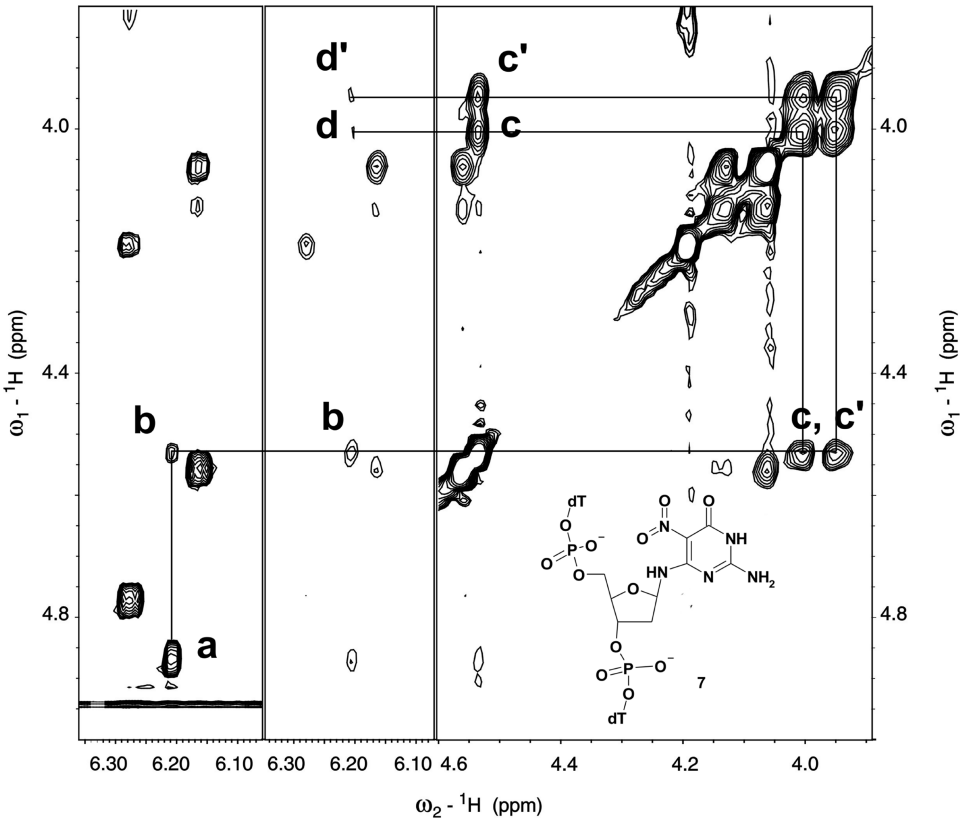


Figure 1. Expanded region of ^{1}H-TOCSY (left panel) and NOESY (central and right panels) spectra of the major isomer of the TpNPypT trimer 7. Cross-peaks between the sugar protons of the nitropyrimidine nucleotide are assigned as follows: a: 1′3′, b: 1′–4′, c, c′: 4′–5′, d, d′: 1′–5′.

utilized a scheme similar to that reported by the Greenberg group (48) implementing specific modifications that allowed us to obtain the amidite as a pure β-anomer (Supplementary Scheme S1).

Generation of the TpNPympT trimer

We employed a standard solid-phase protocol to synthesize the TpNPympT trimer with the slight modification of increasing the coupling time of NPym-dG amidite to 5 min, which resulted in a 97% coupling efficiency approximating that of standard amidites. Deprotection in concentrated ammonia followed by trityl-on HPLC purification yields trimer **7**, which is detritylated via standard treatment with acetic acid. Subsequent HPLC analysis of the resulting trimer reveals only two components, one of which is the predominant species reflective of a non-equilibrium mixture. Examination of ^{1}H-NMR spectra confirms that the major fraction is a single compound, and comparison with the crude trimer reveals that the second component is also a single compound (Supplementary Figure S5). 2D-NOESY and TOCSY spectra of the major component (Figure 2) corroborated the furanoside structure of trimer **7**, indicating that isomerization of NPym-dG to pyranosides does not occur during the monomer synthesis deprotection and coupling steps. Such isomerization requires significant time for completion and all four isomers would be present in the mixture during the reaction course (49). The spectral evidence allows us to conclude that the minor component is the α-furanoside isomer, which arises during deprotection and chromatographic separation, thus eliminating the possibility of pyranoside isomer formation. Our results are therefore consistent with previous studies, indicating that pyranoside rearrangement is unattainable when Fapy-dG is successfully incorporated into oligodeoxynucleotides, whereas anomerization can potentially occur within the DNA strand (29,49,50). As reported in subsequent sections, we have utilized the identical synthetic scheme to prepare the tridecameric d(GCGTACXCATGCG) oligodeoxynucleotides, where X denotes a nitropyrimidine residue. In order to minimize possible degradation of the nitropyrimidine residue, we employed ultra-mild protective groups on the dC, dA and dG phosphoramidites used in the solid-phase synthesis, followed by standard deprotection and purification protocols.

Post-synthetic treatment and structural characterization of the TpNPympT trimer

We dissolved the TpNPympT trimer in 30% methanol and reduced the nitro group by hydrogenation over Pd black in the presence of triethylamine, employing ESI-MS to monitor reaction completion (i.e. 3–6 h). After reduction, the formylation reaction is performed directly without removal of the catalyst or purification of the aminopyrimidine to minimize possible oxidation of the reduced

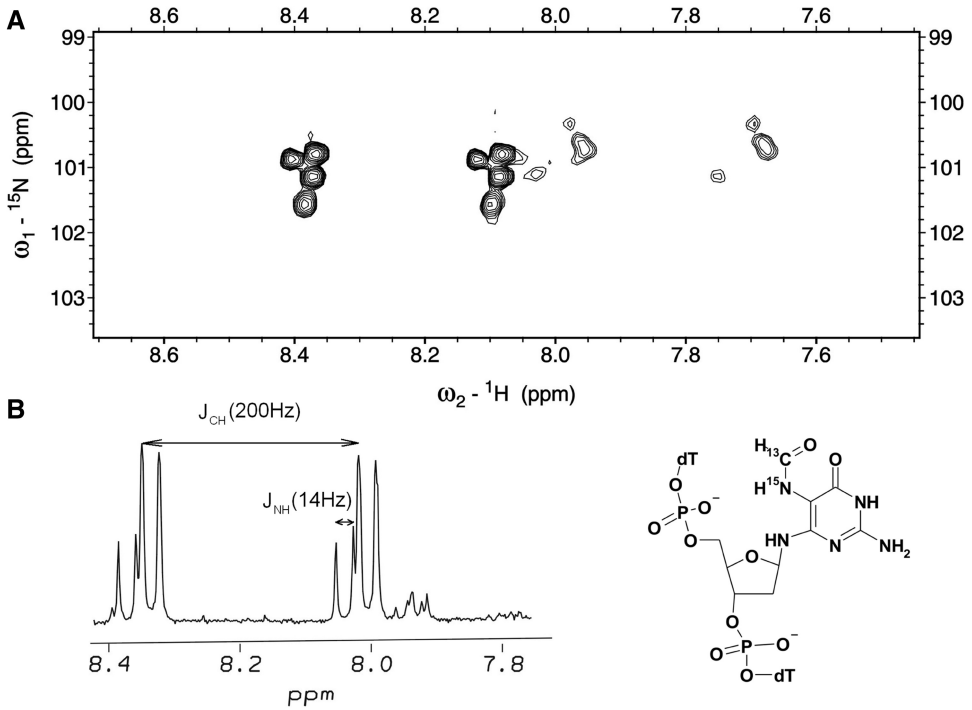


Figure 2. (**A**) Expanded region of a [^{13}C-coupled] ^{1}H-^{15}N-HSQC spectrum (700 MHz) of the doubly labeled Fapy-dG containing trimer. (**B**) Expanded region of a [^{13}C, ^{15}N-coupled] ^{1}H-NMR spectrum (600 MHz) illustrating the formyl proton resonances.

oligonucleotide. We selected *N*-formylimidazole as the formylating agent since it does not decompose immediately in the presence of water. This mild formylating agent is prepared from *N,N′*-carbonyldiimidazole and formic acid as described in (51) and used as a THF solution. The reaction normally requires 2–6 h for completion and is monitored by ESI-MS. The Fapy-containing trimer is purified by reverse-phase HPLC with an approximate yield of 60% based on the nitropyrimidine precursor.

Structural characterization of the trimer confirms the presence of a single formyl susbtitution attached exclusively to the N5 amino group. Specifically, we prepared the TpNPympT trimer with the nitrogroup ^{15}N labeled, and formylated the reduction product with ^{13}C-formyl imidazole, obtaining a trimer containing a double labeled (^{13}C,^{15}N) Fapy moiety (Figure 2). The ^{1}H-NMR spectrum of this trimer (Figure 2) reveals the formyl proton with one-bond ^{13}C–H (~200 Hz) and two-bond ^{15}N–H (15 Hz) couplings, indicating that the formamido group is situated at N5 as opposed to alternate positions (i.e. N2 or N6). Since both post-synthetic steps (i.e. catalytic hydrogenation and treatment with formyl imidazole in aqueous media) do not affect natural nucleotides, and incorporation of the nitropyrimidylamino residue into DNA follows standard solid-phase protocols, the proposed synthetic scheme represents the first universal method for the preparation of DNA containing a Fapy lesion in any sequence context. The versatility of the synthetic strategy can therefore be exploited to incorporate either Fapy-dG or Fapy-dA within defined oligodeoxynucleotide sequences.

Conformational properties and isomerism of Fapy-dG in single-strand DNA

One of the problematic issues in characterizing the biophysical properties of Fapy-dG lesions and their impact on nucleic acid structure and energetics is the feasibility of accurately determining the overall degree of isomerism within single strand and duplex DNA. Concurrently, one must understand the factors that play an integral role in modulating Fapy-dG equilibrium anomerization following incorporation of the cognate lesion within the DNA chain. The presence of a doubly labeled ^{13}C,^{15}N Fapy-oligodeoxynucleotide within the TXT model system provides us with a valuable tool for studying the conformational behavior of the formamido group and Fapy-dG isomerism. The [^{13}C-coupled] ^{1}H-^{15}N-HSQC spectrum of the TpFapyGpT trimer exhibits two groups of resonances with one-bond ^{1}H-^{13}C-splitting as evidenced in Figure 2. This observation is in full accordance with earlier reports of Cadet and co-workers (49) who determined that Fapy-dG adopts two primary conformations corresponding to the *cis*- and *trans*-isomers around the formamido bond. The *cis*-isomer is the predominant species and is characterized by a CHO resonance that is shifted downfield. Since the formyl chemical shifts in both isomers and their populations correspond approximately to those observed for the nucleoside in prior investigations (49,52), we conclude that the formamido group of Fapy-dG exists predominantly as the *cis*-isomer in single-stranded oligodeoxynucleotide.

Inspection of the [^{13}C-coupled] ^{1}H-^{15}N-HSQC spectrum for the TXT trimer (Figure 2) reveals that in addition to *cis–trans* isomerism of the amido group, the Fapy-dG

moiety exists in four different states. NMR spectroscopy provides partial insight regarding the identity of these conformers. Specifically, NOESY and TOCSY spectra reveal that the 1′-proton of one Fapy-dG isomer is closer to its 4′- than the 3′-sugar proton, whereas the opposite situation is encountered for the second isomer (Figure 3). Based on such characteristics, we assign the anomeric configuration of these isomers as β for the former and α for the latter. Due to the absence of spin–spin correlation and NOE interactions between the formyl and sugar protons, the NMR spectra do not furnish additional information regarding the nature of the second set of resonances. Nevertheless, the observed double set of signals might conceivably arise from the non-planar Fapy aglycone (52) that can exist in two conformations with no mirror symmetry (Figure 4). Molecular modeling reveals that the transition between these two conformations is restricted by the steric clash between H-N6 and H-N5, and the pyrimidine O-4 and formyl oxygen. Consequently, the resultant conformers exhibit traits of atropisomerism. Whereas the presence of the atropisomers is not detected in the 1D NMR spectra of the Fapy nucleoside (52), the highly unsymmetrical environment of an oligodeoxynucleotide reveals the magnetic non-equivalence of the formyl proton and 5-nitrogen. The latter are clearly visible in the ^{1}H-^{15}N-HSQC spectrum, primarily along the nitrogen axis (Figure 2).

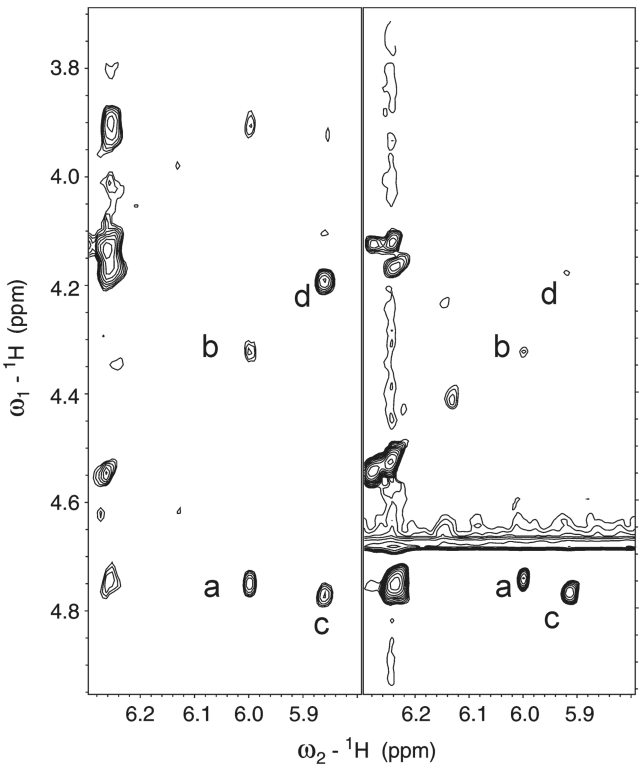


Figure 3. Expanded region of a NOESY (1 s mixing time, 277 K) (left) and a TOCSY (70 ms isotropic mixing, 298 K) (right) spectra of the Fapy-dG trimer. Interactions between the sugar protons of the Fapy-dG residue identify the anomeric forms of the lesion. Labeled peaks are assigned as follows: a: 1′–3′ (α), b: 1′–4′ (α), c: 1′–3′ (β)-5′, d: 1′–4′ (β).

Our detailed structural analysis of the Fapy trimer including possible conformations of the lesion within the TXT context sets the stage for characterization of longer oligodeoxynucleotide sequences harboring Fapy-dG. Such studies enable further investigation of the biophysical and biological properties of the corresponding DNA duplexes and modulation of lesion properties due to Fapy isomerization.

Characterization of Fapy-dG incorporated within a tridecameric oligodeoxynucleotide

Our primary objective is to elucidate the energetic and structural signatures of homogeneous Fapy-dG species. Given the degree of isomerism evident in NMR spectra of the TXT trimer, the resultant equilibrium mixture may effectively preclude isolation and characterization of the respective Fapy anomers. We utilized our novel synthetic strategy to embed Fapy-dG (represented as X) as the central residue within the d(GCGTACXCATGCG) oligodeoxynucleotide and employed a standard purification protocol. Mass spectroscopic analysis of the reverse phase purified Fapy-dG strand reveals an apparently homogeneous species with an expected mass of 3992.7 Da. Following hybridization with its complementary counterpart d(CGCATGCGTACGC) to form the tridecameric Fapy-dG oligodeoxynucleotide duplex, thermal dissociation profiles exhibit non-characteristic biphasic melting behavior (data not shown). Analysis of the transitions suggests the presence of two Fapy-dG species with distinct thermal stabilities. Previous studies have reported similar thermal dissociation profiles for an N^5-alkylated Fapy adduct in which the corresponding Me-Fapy-dG duplexes display biphasic melting behavior (53). These findings have been attributed to the presence of an α/β-anomeric mixture with differential thermal stabilities. The bulky aflatoxin B Fapy-dG lesion exhibits comparable behavior (54) with the notable exception that its adduct intercalating properties shift the α/β-anomeric

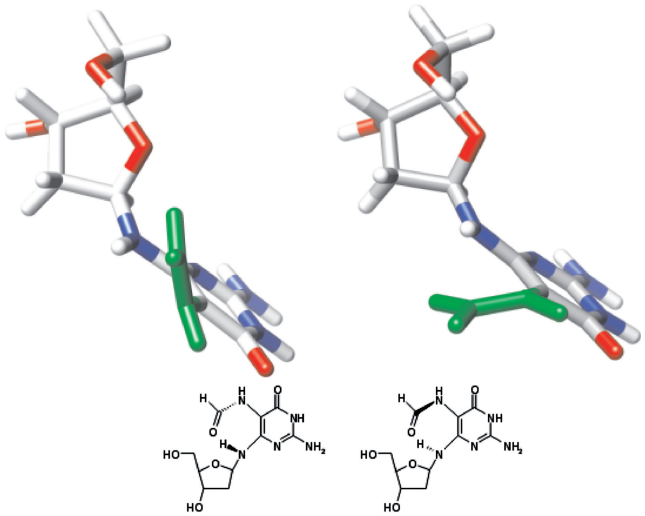


Figure 4. Atropisomeric states of Fapy-dG residue. These forms were minimized with HyperChem using the Amber force field. The formyl group is depicted in green.

equilibrium in favor of the β-conformation. Remarkably, favorable stacking interactions actually stabilize the β-anomeric-intercalated conformer relative to its parent duplex (54). In order to separate and characterize the respective components within our Fapy-dG isomeric mixture, we have introduced a series of chromatographic fractionation steps into the standard purification protocol. In the following sections, we describe the isolation, purification and characterization of the two predominant isomers of natural Fapy-dG and provide compelling evidence that these isomers represent the α- and β-anomers.

Chromatographic separation of the isomeric Fapy-dG·C oligodeoxynucleotides

Conventional reverse-phase chromatography of oligodeoxynucleotides harboring cognate Fapy-dG yields a pseudo-homogeneous deprotected species as reflected in the equilibrium mixture of isomers with distinct thermal stabilities (data not shown). Isolation of the natural Fapy anomers necessitates incorporation of a multi-step ion exchange chromatographic protocol to fractionate the heterogeneous mixture. Although ion exchange chromatography is normally employed for separations based on size and/or charge, application of this technique allows resolution of two major fractions eluting at lower (Fraction I) and higher (Fraction II) ionic strengths as depicted in Figure 5A. Successive re-chromatographic isolation of the respective fractions (Figure 5B and C) yields separable species exhibiting indistinguishable mass spectra that are characterized by identical molecular weights (i.e. Fraction I = 3992.71 ± 0.85 Da; Fraction II = 3993.01 ± 0.89 Da) consistent with the predicted value of 3992.7 Da for the Fapy-dG oligodeoxynucleotides (Supplementary Figures S7 and S8). Although the origins for this unique chromatographic behavior warrants further exploration, our results suggest that subtle local changes at the lesion site and/or within neighboring bases provide alternate interactions with the separating chromatographic medium that may result in the distinct elution times/volumes. In fact, recent studies have reported that oligodeoxynucleotides of identical base composition can be discriminated on the basis of their relative nucleotide sequence (55), suggesting that the differential retention ability of the ion exchange resin may resolve sequence context interactions and/or local conformational changes under specific solution conditions. Following exchange on a G-25 sepharose gel filtration column to triethylammonium acetate buffer (pH 6.0), each of the fractions interconvert rapidly yielding a mixture of Fractions I and II. The finding that each oligodeoxynucleotide fraction isomerizes to the starting mixture as a function of time and solution conditions provides additional corroboration of their anomeric nature. The exchanged fractions maintain their original mass and may be re-chromatographed to isolate the Fapy-dG anomers. Each of the fractions comprising the single-strand sequence d(GCGTAC XCATGCG) harboring Fapy-dG as the central residue is rapidly hybridized with its complementary counterpart d(CGCATGCGTACGC) to circumvent the unavoidable re-equilibration of isomeric species. The isolated Fapy-dG·C duplexes are subsequently re-chromatographed and buffer exchanged to ensure preparation of homogeneous solutions for temperature-dependent studies.

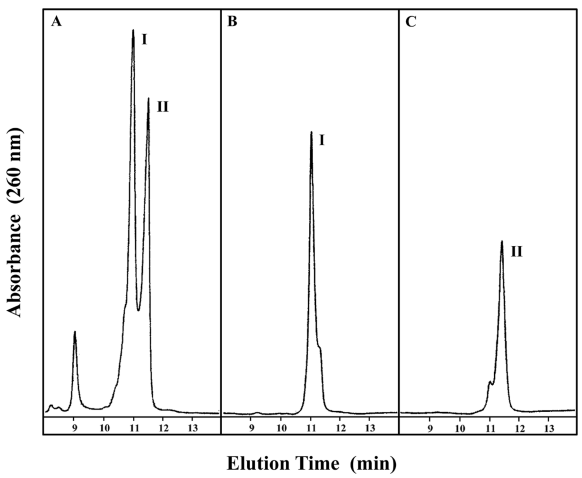


Figure 5. Anion exchange chromatographic profiles depicting fractionation of reverse-phase-purified Fapy-dG-containing deoxyoligonucleotide. Panel A illustrates the resolution of two major species eluting at lower (Fraction I) and higher (Fraction II) ionic strength. Successive re-chromatographic isolation of each fraction (as visualized in panels B and C) yields homogeneous species that retain their respective elution times and exhibit indistinguishable mass spectra (Supplementary Figures S7 and S8).

Impact of Fapy-dG isomerization on DNA duplex energetics

The thermal and thermodynamic stability of the isolated Fapy-dG·C duplexes has been evaluated by temperature-dependent UV spectroscopy. Thermal dissociation profiles of the Fraction I- and Fraction II-derived Fapy-dG·C duplexes and the canonical G·C parent are presented in Figure 6. Inspection of the profiles reveals that the duplexes harboring each of the chromatographically

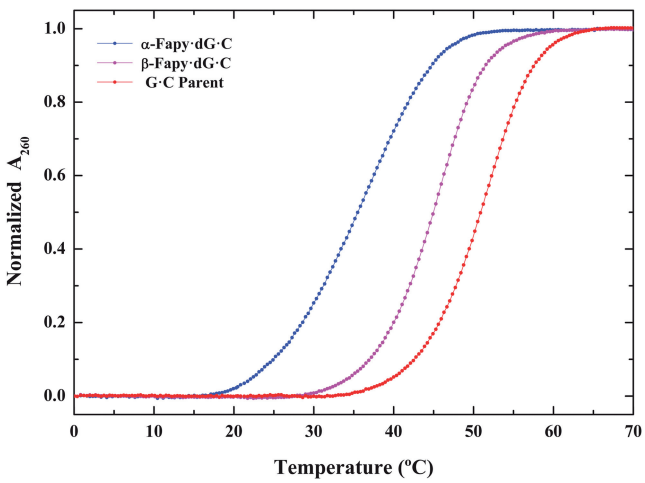


Figure 6. Thermally-induced dissociation profiles of α-Fapy-dG·C (blue), β-Fapy-dG·C (magenta) and canonical G·C (red) duplexes expressed as normalized absorbance at 260 nm.

resolvable Fapy-dG isomers exhibit monophasic transitions characteristic of a single anomeric species. The transition temperatures of the differentially destabilized Fapy-dG•C duplexes are 35.1 and 44.7°C. Evaluation of the duplex energetics via van't Hoff analysis of the denaturation profiles reveals that both Fapy-dG•C duplexes are destabilized relative to the parent G•C tridecamer. The energetic profiles summarized in Table 1 delineate the magnitudes of the observed thermal ($\Delta T_m \sim -15.4$ and -6.0°C) and thermodynamic ($\Delta\Delta G_{vH} \sim -5.5$ and -1.5 kcal•mol^{-1}) destabilizations for Fapy-dG•C duplexes I and II, respectively. Significantly, the transition temperatures of the isolated Fapy-dG•C duplexes are in qualitative agreement with the midpoints of a biphasic dissociation profile previously reported for Me-Fapy-dG•C (53). Moreover, one observes comparable differential thermal destabilization of Fapy-dG•C (i.e. $\Delta T_m \sim -15.4$ and -6.0°C) and Me-Fapy-dG•C (i.e. $\Delta T_m \sim -20$ and -8°C) relative to the corresponding parent duplex. Considering the diversity of solution conditions and sequence contexts employed, as well as the distinct nature of the lesions studied, the similarities observed between Fapy-dG•C and Me-Fapy-dG•C are remarkable. In subsequent sections, we provide evidence for our assignment of the isolated oligomeric Fapy-dG•C isomers.

Isolated fractions I and II correspond to the Fapy-dG α- and β-anomers

Synthetic approaches designed to incorporate the Fapy-dG lesion within DNA duplexes have historically utilized isomeric mixtures comprised of varying relative ratios. The resultant single-strand species are hybridized with their corresponding complementary strands to form a heterogeneous mixture of Fapy-dG duplexes (29,30,48,50). Consequently, data accumulated on the cognate lesion to date does not specifically resolve the unique properties of each isomer populated within the DNA host duplex. The dearth of investigations aimed at resolving isomeric species may be attributed to the practical notion that once incorporated within DNA, sequence environment plays a significant role on the overall anomeric distribution (50) regardless of initial isomeric ratios in the free adduct. In the present study, we find that under specific solution and chromatographic conditions, single-strand Fapy-dG oligonucleotides may be resolved into two primary species that exhibit unique thermal dissociation profiles upon duplex formation. Our findings infer that the Fapy-dG conformation within each of the isolated isomeric single strands may be preserved upon association to form the DNA host duplex, provided the isolation and hybridization processes are conducted promptly and under optimized solution conditions. Our data therefore challenges the conventional wisdom that anomeric properties can only be evaluated via synthesis of stable bio-isosteric analogues. Nevertheless, the wealth of data accumulated on such analogues has proven insightful in terms of assessing the biological and biophysical impacts of Fapy-dG and their modulation by epimerization. In the following section, we discuss our data in view of recent advances employing stable bio-isosteric analogues to characterize lesion anomerization properties.

Table 1. Thermal and thermodynamic dissociation parameters of α- and β-Fapy-dG•C duplexes relative to the parent G•C tridecamer

Duplex acronym	T_m (°C)	ΔT_m (°C)	ΔG_{vH} (kcal mol^{-1})	$\Delta\Delta G_{vH}$ (kcal mol^{-1})
Parent G•C	50.8		15.9	
α-Fapy-dG•C	35.4	−15.4	10.6	−5.3
β-Fapy-dG•C	44.7	−6.0	14.4	−1.5

Conformationally stable analogues for assessment of Fapy-dG α- and β-anomer properties

In order to alleviate the need to resolve potential anomer-specific properties of Fapy lesions, a number of studies have reported the synthesis of stable bio-isosteric analogues mimicking each of the Fapy-dG anomers (30,56–58). The latter do not undergo anomerization or processing by the cellular repair machinery and may be incorporated into template-primer duplexes for *in vitro* and/or *in vivo* mutagenesis studies. A specific example is the conformationally stable β-anomer of Fapy-dG (i.e. β-C-Fapy-dG) in which N6 is replaced by a methylene group (56). The latter has been synthesized and its biochemical and biophysical properties characterized extensively (18,19,30,59). Carbocyclic derivatives designed to resemble cognate damage in the absence of turnover have been employed to study a number of lesions (33,60–63), as these analogues mimic the structural and biochemical properties of the cognate lesion (61). Conformationally stable Fapy-dG isomers have been prepared as *carba*-analogues to mimic both the β- (57,64) and α- (58) anomeric conformations. NMR analysis of the α-carba-Fapy•dG reveals that in spite of local perturbations at the lesion site, the overall duplex structure is maintained (62). Comparison of these carba-analogues within identical sequence contexts reveals that the Fapy-dG α-anomer is thermally destabilized to a greater extent than its β-anomeric counterpart (58). The finding that the Fapy-dG α-anomer is more destabilizing than the β-anomer is not unprecedented and is generally supported by studies comparing the β-conformation of canonical nucleobases with their α-counterparts. The latter are relatively rare yet readily repaired in vivo by specific endonucleases. One such example is α-adenosine (α-dA) that thermally destabilizes the parent duplex and is processed by endonuclease IV (65,66). Recent evidence reveals that endo IV processes α-anomers of Fapy lesions including those of Fapy-dG (50) and Me-Fapy-dG (67). Collectively, the existing database of Fapy-dG analogues conforms to the consensus regarding the relative energetic impacts of α- and β-isomers. Our observations on the cognate Fapy-dG anomeric species provide further corroboration of such anomer-specific impacts that may assume a critical role in modulating lesion-mediated cytotoxicity and/or mutagenicity.

Relevance of our findings in terms of Fapy-dG mutagenic potential

Lesion outcomes *in vivo* are likely to be dependent on cell type, polymerase properties, replication/repair proficiencies and sequence-context effects. Parallel *in vitro* energetic characterizations yield insights regarding the propensity of a particular lesion to exhibit mutagenic or replication blocking properties. As an empirical probe of mutagenicity/cytotoxicity, we already have demonstrated that energy data can assist in predicting a lesion outcome during polymerase-mediated replication (68; C.A.S.A. Minetti., D.P. Remeta, C.R. Iden, F. Johnson and K.J. Breslauer, submitted for publication). The present study provides compelling evidence that β-anomeric species are significantly more stable than their α-counterparts, a finding that is generally consistent with previous observations employing synthetic analogues. The use of stable carbocyclic analogues in template-primer extension analysis reveals unique anomer-dependent consequences on polymerase-mediated replication. Such damage-induced biological outcomes may be correlated with differential energetic impacts as we have proposed for a number of oxidative lesions and exocyclic adducts (68; C.A.S.A. Minetti., D.P. Remeta, C.R. Iden, F. Johnson and K.J. Breslauer, submitted for publication).

Our finding that cognate α-Fapy-dG significantly destabilizes the host duplex is consistent with studies demonstrating that α-*carba*-Fapy-dG blocks polymerase-mediated DNA synthesis. Conversely, the lower degree of destabilization observed for the cognate β-Fapy-dG corroborates previous assertions that the β-anomer is solely responsible for the mutagenic potential of this lesion. The latter is confirmed by the observation that polymerases may bypass β-Fapy-dG analogues in an error-free or error-prone manner depending on cell type and sequence context (58). Ongoing studies to characterize the replication and repair of cognate Fapy-dG duplexes should provide significant insight regarding the modulatory impact of anomerization on biological processes at the cellular level. Our novel large-scale post-synthetic strategy and anomeric separation protocol will be exploited to furnish a complete structural and thermodynamic characterization of duplexes containing the Fapy-dG α- and β-anomers within varying sequence contexts and as a function of the counterbase.

Lesion analogues and their cognate counterparts are not necessarily isoenergetic

Despite the absence of a detailed structure for the cognate Fapy-dG lesion, a number of reports suggest that conformationally stable Fapy-dG analogues may fulfill this void. Preliminary studies on the solution conformation of β-*carba*-Fapy-dG reveals that the lesion adopts conventional Watson–Crick interactions yet significantly disrupts the duplex structure to an extent that exceeds the promutagenic 8-oxodG lesion (62). While bio-isosteric analogues are designed to mimic nascent damage, there is evidence that Fapy analogues might not always resemble the cognate lesion given the greater thermal/thermodynamic destabilization observed for the latter in specific cases. Representative examples include the β-C-Fapy-dG analogue in which N6 is replaced by the methylene group (18,56), and the carbocyclic β-*carba*-Fapy-dG analogue in which O4′ is replaced by a CH_2 moiety (57,58). Interestingly, a study employing β-*carba*-Fapy-dG reveals *aberrant* temperature-dependent profiles that are not typical of a homogeneous species (64). Under the premise that bio-isosteric analogues are resistant to anomerization, the anomalous biphasic behavior reported for β-carba-Fapy-dG is intriguing and may actually arise from the choice of sequence context and solution conditions. Our energy data on the cognate isomeric species assigned as β-Fapy•dG reveals that lesion-induced thermal and thermodynamic destabilizations are generally less dramatic than those reported for conformationally stable β-analogues. Inasmuch as studies of Fapy-analogues have furnished significant insight on lesion-induced perturbations, caution must be exercised when attempting to extrapolate these findings to elucidate energetic/structural properties of the cognate lesion within genomic DNA and underlying biological consequences at the molecular level.

Although valuable information has been gleaned from studies employing surrogate Fapy analogues, there are reports suggesting that specific interactions with glycosylases might differ from those of the cognate lesion. The basis for such concerns are the distinct lesion-residue interactions observed when comparing crystal structures of the Fpg glycosylase complexed with the *carba*-Fapy-dG analogue (31) versus a mutant form bound to the cognate 8-oxodG lesion (32). Recent molecular mechanics studies reveal that lesion-substrate interactions with Fpg occur via a *syn*-conformation (34), yet crystal structure analysis suggests that the *carba*-Fapy-dG analogue adopts a non-planar anti-conformation in its complex with Fpg (31). These disparities have been attributed to a number of factors, including the participation of a non-conserved amino acid residue in Fpg:*carba*-Fapy-dG interactions (i.e. $S^{77}E$ in *L. lactis* Fpg). Considering that the *carba*-analogue interacts with a residue that is *not* conserved within the Fpg-family of glycosylases, it has been hypothesized that the crystallographic structure of the Fpg:*carba*-Fapy-dG complex might not accurately reflect biologically relevant Fpg interactions that mediate cognate lesion recognition and processing. Future studies employing Fpg mutants that lack catalytic activity yet retain wild-type binding ability are required to probe cognate Fapy-dG lesion interactions, the energy landscape of BER recognition, and the impact of lesion anomerization on the overall process.

CONCLUSION

The experimental strategy outlined herein provides a facile and robust synthetic scheme for the large-scale production of Fapy-dG embedded within any oligodeoxynucleotide sequence. The overall utility of the methodology has been enhanced by optimizing a multistep purification protocol that permits chromatographic isolation of tridecameric deoxynucleotides containing cognate

Fapy-dG at anomeric resolution. Hybridization of the isomeric species with their complementary counterpart yields two Fapy-dG•C duplexes that exhibit unique thermal and thermodynamic properties. The resultant duplexes are differentially destabilized relative to the canonical G•C parent and thereby reflect two primary conformations of the cognate Fapy-dG lesion that are consistent with α- and β-anomers, respectively. Comparison of the respective energetic profiles reveals that α-Fapy-dG•C is more destabilizing than β-Fapy-dG•C. Our findings underscore the importance of characterizing anomer-specific impacts in terms of biological outcomes, particularly given the fact that anomerization is likely to occur within genomic DNA and pose significant challenges to the cellular replication/repair machinery.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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