

## Synthesis, DNA Polymerase Incorporation, and Enzymatic Phosphate Hydrolysis of Formamidopyrimidine Nucleoside Triphosphates

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Abstract: The nucleoside triphosphates of N6-(2-deoxy- $\alpha,\beta$ -D-erythro-pentofuranosyl)-2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-dGTP) and its C-nucleoside analogue ( $\beta$ -C-Fapy-dGTP) were synthesized. The lability of the formamide group required that nucleoside triphosphate formation be carried out using an umpolung strategy in which pyrophosphate was activated toward nucleophilic attack. The Klenow fragment of DNA polymerase I from *Escherichia coli* accepted Fapy+dGTP and  $\beta$ -C-Fapy+dGTP as substrates much less efficiently than it did dGTP. Subsequent extension of a primer containing either modified nucleotide was less affected compared to when the native nucleotide is present at the 3'-terminus. The specificity constants are sufficiently large that nucleoside triphosphate incorporation could account for the level of Fapv-dG observed in cells if 1% of the dGTP pool is converted to Fapv-dGTP. Similarly, polymerasemediated introduction of  $\beta$ -C-Fapv-dG could be useful for incorporating useful amounts of this nonhydrolyzable analogue for use as an inhibitor of base excision repair. The kinetic viability of these processes is enhanced by inefficient hydrolysis of Fapy-dGTP and  $\beta$ -C-Fapy-dGTP by MutT, the *E. coli* enzyme that releases pyrophosphate and the corresponding nucleoside monophosphate upon reaction with structurally related nucleoside triphosphates.

DNA lesions are produced when nucleic acids are exposed to oxidative stress and are associated with aging and a variety of diseases.<sup>1-3</sup> When present in DNA, damaged nucleotides can be mutagenic and/or cytotoxic.4-7 A lesion can also be incorporated in DNA following damage to the nucleoside triphosphate pool provided the respective triphosphate is a substrate for DNA polymerase(s). Organisms have developed elaborate, redundant repair systems that excise DNA lesions before they affect replication or transcription.<sup>8,9</sup> Enzymes that guard against mutations by cleansing the nucleoside triphosphate pool also exist.<sup>10</sup> Described below are the results of investigations on two nucleoside triphosphates that are related to the formamidopyrimidine derived from 2'-deoxyguanosine (N6-(2deoxy- $\alpha$ , $\beta$ -D-*erythro*-pentofuranosyl)-2,6-diamino-4-hydroxy-5formamidopyrimidine, Fapy•dG). We examined the ability of a model Escherichia coli polymerase (Klenow) to incorporate Fapy•dGTP and its C-nucleotide analogue ( $\beta$ -C-Fapy•dGTP)



into duplex DNA. Hydrolysis of the nucleoside triphosphates by the hydrolase from E. coli (MutT) that acts on the related 8-oxopurine lesion OxodGTP was also examined. The results of these experiments raise interesting possibilities regarding DNA mutagenesis and repair. The efficiency with which Klenow incorporates the modified nucleosides in DNA and the reluctance of MutT to hydrolyze the triphosphates indicate that Fapy•dGTP could be a source of Fapy•dG in DNA and that  $\beta$ -C-Fapy•dGTP could be useful as a DNA repair inhibitor.

Fapy•dG is believed to result from a radical intermediate (Scheme 1) that serves as a common precursor to 7,8-dihydro-8-hydroxy-2'-deoxyguanosine (OxodG).<sup>11</sup> The lesions are detected in comparable quantities, particularly when DNA is subjected to oxidative stress under O<sub>2</sub>-deficient conditions.<sup>12,13</sup> Fapy•dG and OxodG affect polymerases and repair enzymes

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Scheme 1. Formation of Fapy-dG and OxodG via a Common Intermediate



in similar ways when they are present in DNA. Both lesions induce DNA polymerases to misinsert dA when they are present in duplex DNA.<sup>14–16</sup> G  $\rightarrow$  T transversions result when this misreplication event occurs in cells.<sup>17-20</sup> Base excision repair enzymes, such as formamidopyrimidine-DNA glycosylase (Fpg) also excise Fapy•dG and OxodG, but discriminate with respect to the opposing nucleotide to prevent transforming a promutagenic base pair (e.g., Fapy•dG:dA) into a mutation.<sup>21-23</sup> Introduction of OxodG into DNA via its nucleoside triphosphate (OxodGTP) has also been investigated. OxodGTP is a substrate for several DNA polymerases.<sup>24,25</sup> The efficiency with which the modified nucleotide is incorporated into DNA relative to dG via its respective triphosphate varies. For instance, the Klenow fragment of DNA polymerase I from E. coli that lacks proofreading capability (Klenow exo<sup>-</sup>) accepts OxodGTP more than 150000-fold less efficiently than dGTP. However, incorporation of OxodG by the  $\alpha$ -subunit of the major replicative polymerase in E. coli occurs at almost 3% the rate of that of dG, suggesting that OxodGTP could be a significant source of the lesion in DNA.<sup>25</sup> This hypothesis is corroborated by the determination that OxodGTP is hydrolyzed by the E. coli nucleoside-triphosphate pyrophosphohydrolase, MutT, as much as 200-fold more efficiently than dGTP.<sup>10,25,26</sup> These data have given rise to a widely accepted proposal that a biological role for MutT is to sanitize the nucleoside triphosphate pool by removing OxodGTP. Consequently, MutT is often referred to as a member of the GO (guanine oxidation) family of repair enzymes. Fpg and the mismatch repair enzyme MutY are the other members of this repair enzyme family. However, this model was recently questioned when OxodGTP was not detected in cell extracts of E. coli mutT mutants that were subjected to oxidative stress.<sup>27</sup> An unknown compound, presumably a different nucleoside triphosphate, was detected. It is conceivable that this molecule is Fapy dGTP or one of several oxidation products of OxodGTP that have recently been characterized. 4,20,28-30

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We sought to determine whether Fapy•dGTP could be a source of Fapy•dG in DNA by synthesizing this molecule and characterizing its interactions with MutT and Klenow. In addition, we carried out the analogous synthesis and study of  $\beta$ -C-Fapy•dGTP. We were motivated to carry out this exercise



due to the considerable interest in analogues of DNA lesions as mechanistic probes and as base excision repair inhibitors.<sup>21,22,31-33</sup> Duplex DNA containing hydrolytically stable  $\beta$ -C-Fapy•dG was previously shown to strongly bind the enzyme that cleaves the glycosidic bond in Fapy·dG.<sup>21</sup> Synthesizing  $\beta$ -C-Fapy·dGTP enabled us to determine whether this molecule was a viable base excision repair inhibitor.

Scheme 2. Umpolung Preparation of Fapy-dGTP<sup>a</sup>



<sup>a</sup> Conditions and reagents: (a) pyrophosphate; (b) 1,1-carbonyldiimidazole; (c) Fap•dGMP; (d) hv (350 nm), MeOH.



<sup>a</sup> Conditions and reagents: (a) (i) bis(2-cyanoethyl)-N,N-diisopropylaminophosphine, tetrazole, CH<sub>3</sub>CN, 25 °C; (ii) t-BuOOH, 25 °C; (b) (i) H<sub>2</sub>, Pd/C, DIPEA, THF; (ii) pyridine, formic acetic anhydride, 0 °C; (c) (i) DBU, BSA, pyridine, 25 °C; (ii) AcOH, TBAF·3H<sub>2</sub>O, MeOH; (iii) K<sub>2</sub>CO<sub>3</sub>, MeOH, 25 °C.

## **Results and Discussion**

Synthesis of Fapy·dGTP and  $\beta$ -C-Fapy·dGTP. Nucleoside triphosphate synthesis is typically achieved from the respective nucleoside via the monophosphate, which is activated for nucleophilic attack by pyrophosphate.<sup>34</sup> Isolated yields of the triphosphates are often low, but pure products are obtained following anion exchange or reversed-phase chromatography in sufficient quantities for enzymology experiments. These issues notwithstanding, it was not possible to synthesize Fapy+dGTP or  $\beta$ -C-Fapy•dGTP by this method. For instance, attempted activation of the corresponding monophosphates with 1,1carbonyldiimidazole resulted in deformylation. Consequently, we developed an umpolung-type approach for synthesizing Fapy•dGTP and  $\beta$ -C-Fapy•dGTP from the respective nucleoside monophosphates (Scheme 2). The o-nitroveratrole ether of pyrophosphate (1) was activated by 1,1-carbonyldiimidazole.35 After excess electrophile was guenched with methanol, the photolabile pyrophosphate was coupled to Fapy•dGMP and  $\beta$ -C-Fapy•dGMP. The photolabile, protected nucleoside triphosphates were partially purified by anion exchange chromatography and photolyzed (350 nm) to yield Fapy•dGTP and  $\beta$ -C-Fapy•dGTP, which were purified using a mono-Q column on an FPLC instrument.

Fapy dGMP was synthesized using an approach (and intermediate) employed during the preparation of the corresponding phosphoramidite (3, Scheme 3).<sup>36-38</sup> These previous studies

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Scheme 4. Synthesis of  $\beta$ -C-Fapy·dGMP<sup>a</sup>



<sup>a</sup> Conditions and reagents: (a) (i) bis(2-cyanoethoxy)-N,N-diisopropylaminophosphine, pyridine hydrochloride, pyridine, -20 °C; (ii) t-BuOOH, 25 °C; (b) (i) DBU, BSA, pyridine, 25 °C; (ii) K<sub>2</sub>CI<sub>3</sub>, MeOH, 25 °C.



Figure 1. Determination of stereochemistry in  $\beta$ -C-Fapy•dGMP via NOE.

determined that it was necessary to introduce the phosphate group prior to transformation of the nitro group into a formamide to prevent rearrangement to the pyranose isomer. Consequently, Fapy•dGMP was prepared as a mixture of  $\alpha$ - and  $\beta$ -anomers from previously reported 3 (Scheme 3).<sup>38</sup> The anomers of 5 were separated by chromatography, but it was unnecessary to do so for practical preparative purposes because the diastereomers readily equilibrated. After treatment of 5 with excess N,Obis(trimethylsilyl)acetamide and DBU, the amine and alcohol groups of the crude monophosphate were sequentially deprotected.<sup>39</sup> The anomeric mixture of Fapy•dGMP, which was purified by anion exchange chromatography, was coupled as described above (Scheme 2) to form the desired nucleoside triphosphate as a 1:1 mixture of epimers. The coupling yield and subsequent photochemical deprotection proceeded in 59% yield.

 $\beta$ -C-Fapy•dGMP was prepared from **6** (Scheme 4) using the same strategy employed above for the synthesis of Fapy-

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Table 1. Specificity Constants for Hydrolysis of Nucleoside Triphosphates by MutT

dNTP	$k_{\rm cat}/K_{\rm m}{}^a({\rm M}^{-1}{\rm s}^{-1})$	dNTP	$k_{\rm cat}/K_{\rm m}{}^a({\rm M}^{-1}{\rm s}^{-1})$
dGTP	$(4.3 \pm 0.9) \times 10^4$	$\beta$ -C-Fapy•dGTP	$\begin{array}{c} (2.2\pm 0.5)\times 10^2 \\ (5.4\pm 0.7)\times 10^6 \end{array}$
Fapy•dGTP	$(3.8 \pm 1.3) \times 10^2$	8-OxodGTP <sup>b</sup>	

<sup>a</sup> Results are the average  $\pm$  standard deviation of at least three experiments. <sup>b</sup> Taken from ref 26.

dGMP.<sup>40</sup> The anomers of  $\beta$ -C-Fapy•dGMP were separated by anion exchange chromatography. The stereochemistry of the  $\beta$ -anomer was determined via an NOE experiment in which a 2.8% enhancement of the pseudoanomeric proton was observed when the respective 4'-proton was irradiated (Figure 1). Following synthesis from the monophosphate using the abovedescribed umpolung approach,  $\beta$ -C-Fapy•dGTP was purified in 22% yield and characterized.

Fapy dGTP and  $\beta$ -C-Fapy dGTP Are Poor Substrates for **MutT.** If Fapy•dGTP and/or  $\beta$ -C-Fapy•dGTP are to serve as sources of the respective nucleotides in DNA, they must escape hydrolysis by nucleotide hydrolases. MutT was chosen as a potential hydrolase because of the biochemical similarity between Fapy•dG and OxodG. The latter is an excellent substrate for this enzyme.14,21,41 Preliminary studies indicated that the  $K_{\rm m}$  for Fapy•dGTP and  $\beta$ -C-Fapy•dGTP hydrolysis by MutT was very high. Saturation was not achieved at triphosphate concentrations >400  $\mu$ M. To spare valuable triphosphate substrates, we utilized a steady-state kinetic method that provides the specificity constant by measuring the amount of product as a function of time at a single concentration of substrate. This simplification of Michaelis-Menten kinetics assumes that the amount of enzyme-bound substrate is much smaller than the total amount of enzyme in solution.<sup>42</sup> This approximation is valid when the substrate concentration is much lower than the  $K_{\rm m}$ . Consequently, we measured the hydrolysis of Fapy+dGTP and  $\beta$ -C-Fapy•dGTP at 20  $\mu$ M (Table 1).<sup>43</sup> Furthermore, to validate the method, we determined the specificity constant for MutT hydrolysis of dGTP (10  $\mu$ M). The value of  $k_{cat}/K_m$  determined for dGTP hydrolysis (Table 1) is within error of that reported in the literature.<sup>26</sup> MutT hydrolyzes Fapy•dGTP and  $\beta$ -C-Fapy• dGTP 100-200 times less efficiently than even dGTP. These data indicate that MutT will not protect the nucleoside triphosphate pool against Fapy•dGTP. Inefficient hydrolysis of  $\beta$ -C-Fapy•dGTP is also potentially significant. DNA containing  $\beta$ -C-Fapy•dG strongly binds the glycosylase that excises Fapy• dG.<sup>21</sup> MutT's reluctance to act on the C-nucleotide suggests that  $\beta$ -C-Fapy•dGTP will be useful as a base excision repair enzyme inhibitor if the triphosphate is accepted as a substrate by DNA polymerase(s) and an alternative nucleotide hydrolase does not act on the molecule.<sup>10</sup>

An additional observation worth noting is that although the starting material is a 1:1 mixture of  $\alpha$ - and  $\beta$ -anomers, the hydrolysis of Fapy•dGTP, which proceeds to ~75% conversion in 45 min, is fit to a single exponential. The reactions are completed in too short a period of time for equilibration of the anomers and preferential hydrolysis of one isomer to explain



Figure 2. Qualitative incorporation of  $\beta$ -C-Fapy•dG (A) and Fapy•dG (B) at multiple positions in 8. Pause sites were identified using an independently synthesized oligonucleotide ladder.

the kinetics.<sup>44,45</sup> The kinetic behavior can be explained in one of two ways. Either epimerization of the anomers is accelerated in the presence of MutT or the two anomers are comparable substrates for the enzyme. In the absence of evidence supporting the former, mechanistic economy favors the latter.

Effect of Fapy  $\cdot$  dGTP and  $\beta$ -C-Fapy  $\cdot$  dGTP on Replication by the Klenow Fragment of DNA Polymerase I. The ability of Klenow to incorporate the formamidopyrimidine triphosphates and extend the primer following modified nucleotide incorporation was qualitatively examined. Extension of the radiolabeled primer in 8 in the presence of Fapy dGTP (200

5'-d(ACC ATG GGA CGT GCT GA 3'-d(TGG TAC CCT GCA CGA CTC TGA GAT CTG ATA TCT GGA) 8

 $\mu$ M) or  $\beta$ -C-Fapy•dGTP (200  $\mu$ M) and dATP, dCTP, and dTTP  $(10 \,\mu\text{M})$  was followed as a function of time (Figure 2). Greater than 85% of the primer was extended to full-length product in the presence of either nonnative nucleoside triphosphate. No full-length product was observed in the absence of Fapy•dGTP or  $\beta$ -C-Fapy•dGTP (data not shown). The template contained 3 2'-deoxycytidines in the single-stranded region, indicating that Klenow was able to incorporate 3 of the modified nucleotides in a short sequence (19 nucleotides). In addition, pause sites were only observed at the position prior to incorporation of  $\beta$ -C-Fapy•dG. None were detected for Fapy•dG incorporation under these conditions. If extension of the primer following modified nucleotide incorporation was strongly inhibited, additional pause sites opposite dC would have been observed. Although fulllength extension of 8 required a higher concentration of FapydGTP or  $\beta$ -C-Fapy•dGTP than the native nucleoside triphosphate (dGTP,  $10 \,\mu$ M, data not shown), the qualitative experiments indicated that neither modified nucleotide blocked replication by Klenow. The ability to tolerate multiple incorporations of Fapy•dG and  $\beta$ -C-Fapy•dG in a growing primer distinguishes these molecules from other nonnative nucleoside triphosphates, in which extension of primers containing them at their 3'-termini

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 Table 2.
 Specificity Constants for Nucleoside Triphosphate

 Incorporation by Klenow
 Vector

5'-d(ACC ATG GGA CGT GCT GA 3'-d(TGG TAC CCT GCA CGA CT**X** TGA CGT GCA ACT TGC GGA)

## 9 Klenow dNTP

5'-d(ACC ATG GGA CGT GCT GA**N** 3'-d(TGG TAC CCT GCA CGA CT**X** TGA CGT GCA ACT TGC GGA)

dNTP	Х	$k_{\rm cat}/K_{\rm m}{}^a$ (M <sup>-1</sup> min <sup>-1</sup> )	dNTP	Х	$k_{\rm cat}/K_{\rm m}{}^a$ (M <sup>-1</sup> min <sup>-1</sup> )
	dC dC dC dA	$\begin{array}{c} (2.0\pm0.3)\times10^7\\ (1.2\pm0.5)\times10^4\\ (1.5\pm0.4)\times10^3\\ (4.3\pm0.7)\times10^4 \end{array}$	Fapy•dGTP β-C-Fapy•dGTP dTTP	dA dA dA	$(4.8 \pm 1.6) \times 10^{2}$ $26^{b}$ $(1.2 \pm 0.1) \times 10^{7}$

<sup>*a*</sup> Results are the average  $\pm$  standard deviation of at least two experiments containing three replicates. <sup>*b*</sup> The result is from a single experiment (three replicates).

by Klenow can be inefficient, and rarely does one observe multiple incorporation sites in such a short region. $^{46-50}$ 

Klenow incorporation of Fapy•dG and  $\beta$ -C-Fapy•dG and subsequent extension were quantitatively evaluated under steadystate conditions. Incorporation of the modified nucleotides in 9 was examined using a standing start process (Table 2).<sup>51</sup> Preliminary experiments again established that the  $K_{\rm m}$  values for the triphosphate substrates were very high. Consequently, specificity constants were extracted from plots of product formation as a function of time using a single concentration of triphosphate.<sup>42,43</sup> Experiments with Fapy•dGTP were carried out using 5  $\mu$ M opposite dC and 100  $\mu$ M when dA was the opposing template nucleotide. The specificity constant for incorporation of  $\beta$ -C-Fapy•dG opposite dC was determined using three different concentrations of the triphosphate (50–200  $\mu$ M). The small variation of the specificity constant indicates that the experiments were carried out considerably below the  $K_m$  and justifies using the kinetic method.42 Insertion product grew in linearly as a function of time, because even at the low concentrations of triphosphates used the substrate was in large excess relative to DNA. Hence, the substrate concentration did not vary. Fapy•dG and  $\beta$ -C-Fapy•dG were incorporated ~1000 and 10000 times less efficiently, respectively, than dG opposite dC in the template. Klenow also incorporated the modified nucleotides opposite dA considerably less efficiently than it did dG. Moreover, Fapy•dG and  $\beta$ -C-Fapy•dG were incorporated more than 40000-fold less efficiently opposite dA than was dTTP. Incorporation of Fapy dG opposite dT or dG was not observed under these conditions (data not shown). These data indicate that if Fapy•dG or  $\beta$ -C-Fapy•dG are incorporated into DNA via their respective nucleoside triphosphates, it will very likely be opposite dC.

The qualitative observation that primer extension following modified nucleotide incorporation was not strongly inhibited (Figure 2) was confirmed using a running start kinetic experi*Scheme 5.* Determination of Relative Velocities for Incorporation of a Modified Nucleotide and Extension

5'-d(ACC ATG GGA CGT GCT GA

3'-d(TGG TAC CCT GCA CGA CTC TGA CGT GCA ACT TGC GGA)

5'-d(ACC ATG GGA CGT GCT GAN AC 3'-d(TGG TAC CCT GCA CGA CTC TGA CGT GCA ACT TGC GGA)

dNTP = Fapy•dGTP or  $\beta$ -C-Fapy•dGTP



*Table 3.* Relative Kinetic Parameters for Nucleoside Triphosphate Extension by Klenow<sup>a</sup>

dNTP	rel V <sub>max</sub>	<i>K</i> <sub>m</sub> (μΜ)	(rel V <sub>max</sub> )/K <sub>m</sub> (µM <sup>-1</sup> )
Fapy•dGTP β-C-Fapy•dGTP	$6.4 \pm 0.1$ $10.6 \pm 3.2$	$\begin{array}{c} 1.7\pm0.5\\ 1.9\pm0.8 \end{array}$	$4.6 \pm 0.3 \\ 5.7 \pm 0.6$

 $^a$  Results are the average  $\pm$  standard deviation of at least two experiments (three replicates).

ment. The ratio  $(V_{rel})$  of the velocity for extending the primer after incorporation of the modified nucleotide relative to the velocity for its incorporation was obtained by measuring the amounts of products  $(I_{t-1}, I_t, I_{t+1})$  as a function of dATP concentration (Scheme 5, Table 3).43,52 The radiolabeled primer in 10 was extended in the presence of dCTP (10  $\mu$ M) to prevent removal of product by proofreading ("rescue nucleotide") and either Fapy•dGTP (70  $\mu$ M) or  $\beta$ -C-Fapy•dGTP (200  $\mu$ M). The amount of product extended past Fapy•dGTP or  $\beta$ -C-Fapy•dGTP depended on the concentration of dATP (1–10  $\mu$ M). Primer  $(I_{t-2})$  extension was not observed in the absence of Fapy•dGTP or  $\beta$ -C-Fapy•dGTP. The relative velocities ( $V_{rel}$ ) were plotted versus [dATP] and fitted via nonlinear regression analysis.<sup>51</sup> These data indicate that primer extension is faster following modified nucleotide incorporation than for incorporation of the modification (rel  $V_{\text{max}} > 1$ ).<sup>52</sup> Moreover, this implies that Klenow does not treat the presence of Fapy dG or  $\beta$ -C-Fapy dG like it does a mismatch (rel  $V_{\text{max}} < 1$ ), suggesting that these modified nucleotides do not significantly perturb the structure of the nascent duplex.

## Conclusions

The kinetic data raise the possibility that if Fapy•dGTP is produced in the nucleoside triphosphate pool as a result of oxidative stress, it could be a source of Fapy•dG in duplex DNA. The triphosphate is a kinetically competent source of Fapy•dG in DNA because it is a poor substrate for MutT, but is incorporated opposite dC by Klenow, a model DNA polymerase. Fapy•dGTP is incorporated opposite dC ~1000 times less efficiently than is dGTP. If Fapy•dGTP is present at even 1% of the concentration level of dGTP, our kinetic data lead us to estimate that the nucleotide would be incorporated opposite 1 every 100000 dC's  $(10^{-3} \times 10^{-2} = 10^{-5})$  as a substitute for dG. This would yield several Fapy•dG lesions per million base pairs of DNA and is of sufficient efficiency to achieve the levels

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of Fapy•dG detected in cellular DNA.<sup>12,13</sup> Admittedly, this estimate is based upon data obtained using a bacterial enzyme. However, the Klenow fragment of DNA polymerase I from *E. coli* is often used to study DNA lesions. In view of the fact that it is less certain that 8-OxodGTP is the source of dG  $\rightarrow$  dT transversions, these data suggest that one should consider Fapy• dGTP as the source of these mutations.<sup>27</sup>

The interaction of  $\beta$ -C-Fapy•dGTP with MutT and the DNA polymerase suggests that the triphosphate could be useful as an inhibitor of base excision repair. It is known that when the nonhydrolyzable analogue  $\beta$ -C-Fapy•dG is in DNA, it strongly binds the repair enzyme (Fpg) responsible for removing many purine lesions.<sup>21</sup> Provided  $\beta$ -C-Fapy•dGTP could be delivered or produced in cells (i.e., via a prodrug), this molecule would

have advantages over other base excision repair inhibitors because it is a substrate for a DNA polymerase.<sup>31</sup>

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Supporting Information Available: Experimental procedures for the syntheses of Fapy•dGTP and  $\beta$ -C-Fapy•dGTP and enzyme experiments, NMR spectra of previously unreported compounds, and sample kinetic plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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