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Chemical Biology of N^5-Substituted Formamidopyrimidine DNA Adducts

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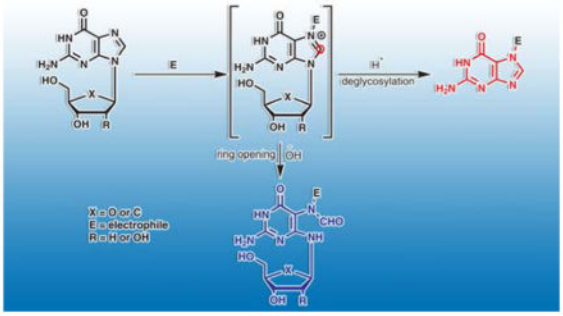
Abstract

DNA nucleobases are the prime targets for chemical modifications by endogenous and exogenous electrophiles. Alkylation of the N7 position of guanine and adenine in DNA triggers base-catalyzed imidazole ring opening and the formation of N^5-substituted formamidopyrimidine (N^5-R-FAPy) lesions. Me-FAPy-dG adducts induced by exposure to methylating agents and AFB-FAPy-dG lesions formed by aflatoxin B_1 have been shown to persist in cells and to contribute to toxicity and mutagenicity. In contrast, the biological outcomes of other N^5-substituted FAPy lesions have not been fully elucidated. To enable their structural and biological evaluation, N^5-R-FAPy adducts must be site-specifically incorporated into synthetic DNA strands using phosphoramidite building blocks, which can be complicated by their unusual structural complexity. N^5-R-FAPy exist as a mixture of rotamers and can undergo isomerization between α, β anomers and furanose-pyranose forms. In this Perspective, we will discuss the main types of N^5-R-FAPy adducts and summarize the strategies for their synthesis and structural elucidation. We will also summarize the chemical biology studies conducted with N^5-R-FAPy-containing DNA to elucidate their effects on DNA replication and to identify the mechanisms of N^5-R-FAPy repair.

Graphical Abstract



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1. Introduction

N^5-Alkyl-formamidopyrimidines (N^5-R-FAPy) are ring open DNA adducts that form upon imidazole ring opening of the corresponding N7-alkylpurine lesions.[1–8] N7 positions of guanine and adenine in DNA are susceptible to electrophilic attack by a variety of alkylating agents. The resulting N7-substituted purines are destabilized due to the presence of positive charge at the N7 position[9] and can undergo two competing reactions: depurination to form apurinic sites and imidazole ring opening to give N^5-R-FAPy.[1, 2, 10–13] While depurination is accelerated at low pH, N^5-R-FAPy formation is preferred under basic conditions (Scheme 1). Although under physiological conditions, N^5-R-FAPy adducts are formed in much lower yields than the corresponding depurinated adducts, they may have a significant biological impact because of their persistence in cells and their ability to induce mutations.

Many simple alkylating agents including epoxides, nitrogen mustards, and alkyl halides preferentially alkylate the nucleophilic N7 position of guanine in DNA.[14–21] However, not all of the resulting N7-dG adducts form the corresponding FAPy adducts under physiological conditions. Imidazole ring opening of N7-alkyl-dG is favored by electron withdrawing groups on the N7 substituent, which makes the C7-C8 bond more susceptible towards attack by hydroxyl anions.[22, 23] Interestingly, imidazole ring opening of N7-alkyl-G adducts in RNA is 2–3 times faster than of their DNA counterparts, presumably due to the electron withdrawing effect of the 2′-hydroxyl group.[24] Aflatoxin B1 epoxide,[25–27] *N*-methylnitrosamines,[28–32] dimethyl sulfate,[33, 34] tobacco carcinogen 4-(methylnitrosamino)-1-(pyridyl)-1-butanone (NNK),[35] N-methylnitrosourea,[36] 1, 2-dimethylhydrazine, *N*, *N*-dimethylnitrosamine,[28–32, 37] cyclophosphamide,[38, 39] mitomycin C,[40, 41] and ethyleneimine[42] are some examples of alkylating agents that give rise to N^5 substituted FAPy adducts. N^5-substituted FAPy adducts are also induced by leinamycin,[20] pluramycins,[43] azinomycin,[44, 45] and *S*-(2-haloethyl)glutathione.[46, 47] Structurally related unsubstituted FAPy adducts can be formed by a radical mechanism upon exposure to reactive oxygen species (ROS)[48–50] but are beyond the scope of this review.

Imidazole ring opening drastically changes the molecular shape and the hydrogen bonding characteristics of the parent purine nucleobase. As a result, N^5-substituted FAPy lesions are likely to induce DNA polymerase stalling, toxicity, and mutations. For example, N^5-AFB1-FAPy adducts induced by Aflatoxin B1 are thought to play a major role in its hepatocarcinogenicity.[51, 52] However, our understanding of the cellular formation and biological outcomes of N^5-R-FAPy adducts induced by other DNA modifying agents is incomplete. Chemical synthesis of N^5-R-FAPy nucleosides and N^5-R-FAPy-containing DNA strands represents a special challenge due to the structural complexity of these unusual ring open lesions and their propensity to undergo isomerization. The goal of this Perspective is to summarize the current understanding of the mechanisms of formation, synthesis, isomerism, and biological consequences of N^5-R-FAPy adducts. For additional information on N7-guanine alkylation and the chemistry of unsubstituted FAPy adducts induced by ROS, readers are referred to several recent reviews.[7, 48–50, 53,54]

2. Structural Identification and Synthesis of N^5-Substituted FAPy Lesions (N^5-R-FAPy)

Since their discovery in the early 1960s, N^5-substituted FAPy adducts have been a subject of intense investigation. In addition to simple DNA alkylating agents, a number of antitumor drugs and natural products have been shown to induce N^5-R-FAPy adducts under physiological conditions, generating significant interest in these ring open structures. In this section, we will describe the discovery, mechanisms of formation, and synthesis of the major types of N^5-R-FAPy adducts.

2.1. Methyl-FAPy-dG

DNA can be methylated upon exposure to exogenous agents such as dimethyl sulfate,[17, 33, 34] N-methylnitrosamines,[28–32] and tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).[35] DNA methylation can also occur from the reactions with endogenous enzyme cofactor, S-adenosyl-L-methionine (SAM).[55] The most reactive site in DNA towards methylating agents is the N7 position of guanine.[9, 16] Haines *et al.* described imidazole ring opening of N7-methylguanosine in the presence of ammonia or sodium hydroxide at room temperature.[2] The ring open adduct was cleaved with acid, and the resulting aglycone was assigned the structure of 2,4-diamino-6-hydroxy-5N-methylformamidine (**1** in Scheme 2) based on its spectroscopic properties.[2]

The biological significance of this finding remained unclear until 1983, when Beranek *et al.* isolated a novel nucleobase adduct from liver DNA of rats treated with the methylating agents, *N, N*,-dimethylnitrosoamine and 1,2-dimethyl hydrazine.[37] The same adducts were subsequently found in bladder epithelial DNA of rats treated with *N*-methylnitrosourea.[36] The unknown lesions were chromatographically identical to the ring opened derivative of 7-methylguanine prepared by alkaline hydrolysis of N7-methyl-Guo, followed by cleavage of the glycosidic bond with acid (Scheme 2).[36] Two isomers of the adduct were isolated by HPLC. Further experiments revealed that following isolation as two separate peaks, these two isomers interconverted with each other to give a 1:1 mixture.[36, 37] Thermal desorption mass spectra of the two isomers were identical, giving a molecular ion peak *m/z* 183 and major fragments at *m/z* 155 and 140, corresponding to the loss of CO and CO+CH_3, respectively.[37] ^{1}H-NMR spectra of the two products were also identical, both exhibiting two distinct sets of resonances (Figure 1).[36] NMR spectra of these isomers were consistent with *cis* and *trans* isomers around the C_5-N_5 amide bond (Figure 2). NMR spectra revealed two sets of resonances, each corresponding to two different forms of N^5-methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine, which interconverted with each other.[36] The relative abundances of the two rotamers were 1:9, 1:4, and 1:2 when spectra were taken in dimethylsulfoxide-d_6, methanol-d_4, and dimethylsulfoxide-d_6/D_2O, respectively (Figure 1).[36] These results indicated that ring-open N^5-methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine adducts are found *in vivo* (and thus may not require strongly basic conditions to be formed) and exist as at least two interconverting forms (**1** and **2** in Figure 2).

The structural identities of the interconverting isomers of N^5-methyl-FAPy adducts were further examined by proton NMR by Boiteux *et al.*[10] Two methyl group signals were

observed at 2.7 and 2.81 ppm, with the relative intensities of 88% and 12%, while the corresponding formamido signals were observed at 7.61 ppm (88%) and 7.88 ppm (12%) (Figure 3). Nuclear Overhauser Enhancement (NOE) was observed on the formamido proton at 7.61 ppm by irradiation of the methyl signal at 2.7 ppm, whereas no NOE effect was observed for formamido proton when methyl signal at 2.81 ppm was irradiated. This indicated that the protons observed as resonances at 2.7 ppm and 7.61 ppm are in a close proximity to each other and therefore belong to the *Z* conformer of the N-methyl-formamido bond, while the other isomers giving rise to resonances at 2.8 and 7.88 ppm are the *E* rotamer (**3** and **4** in Figure 2).

In summary, these early studies have revealed that, ring open N7-methyl-G adducts can form under physiological conditions. These adducts can exist as a mixture of four rotational isomers due to a hindered rotation about the C5-N5 and N-methyl-formamido bonds (**1–4** in Figure 2). In the free nucleoside form these conformers rapidly interconvert with an estimated half-life of 8 min at 37 °C.[10] Subsequently, these results were unambiguously supported by NMR studies of ^{15}N-labeled adducts.[56] As discussed below, the distribution of conformational isomers of N^5-methyl-FAPy and other FAPy lesions is altered in double stranded DNA due to steric factors and hydrogen bonding interactions.

The first synthesis of Me-FAPy-dG nucleoside was reported by Christov *et. al.* in 2008.[57] In their approach, 2′-deoxyguanosine was protected at the exocyclic amine with *N,N*-dimethylformamide dimethylacetal and at the 5′-OH with DMT to give the doubly protected nucleoside **5** (Scheme 3). N7-methylation of **5** was carried out using CH_3I in DMSO to give the N7-methyl-dG intermediate **6**, which was not isolated (Scheme 3). Subsequent treatment of **6** with 1M NaOH and immediate neutralization yielded protected Me-FAPy-dG **7**, which was characterized by NMR spectroscopy and high resolution mass spectrometry.[57] The corresponding Me-FAPy-G ribonucleoside was synthesized using a similar route.[58] N7-methylation of guanosine was achieved by reaction of unprotected guanosine with diazomethane, which was obtained from nitrosomethylurea as reported by Farmer *et. al.*[59] Next, N7-methylguanosine was incubated with 0.15M ammonia for 30 min at 25 °C, yielding the Me-FAPy-G in 60% yield. The availability of Me-FAPy-dG and Me-FAPy-Guo has made it possible to incorporate these structures in nucleic acids chains using the phosphoramidite approach (Section 3 below).

2.2. Ethyl FAPy Adducts

N7-ethylguanine is the main DNA lesion formed upon exposure to ethylating agents, and it can be converted to the corresponding Et-FAPy adducts.[19, 58] van Delft *et al.* reported the synthesis of Ethyl-FAPy-dG (**9** in Scheme 4) from N7-ethyl-dG **8** by basic treatment in 0.5 M ammonia for 70 min at 25 °C, followed by cooling to −80 °C and lyophilization (Scheme 4).[58] The conversion yield was reported as 95%.[58] The corresponding ribonucleoside (Et-FAPy-Guo) was prepared analogously by treating N7-Et-Guo with 0.1 N KOH at ambient temperature.[19] A characteristic change in UV spectra was observed when N7-Et-Guo (λ_{max}, 243 and 272 nm) was converted to Et-FAPy-Guo (λ_{max} 265 nm in acid and 247 nm under basic conditions (pH 13, Figure 4).[19]

2.3. 2-Hydroxyethyl FAPy Adducts

N7-(2-hydroxyethyl)guanine (N7-heG, structure **10a** in Scheme 5) represents the major adduct produced upon exposure of DNA to ethylene oxide, which is commonly used as an intermediate in chemical industry. Ethylene oxide can also be formed endogenously through P450-mediated metabolism of ethylene.[60, 61] As a result, N7-heG adducts are ubiquitously present in tissues of mice exposed to ethylene oxide and are among the most abundant endogenous DNA lesions measured, with > 30,000 N7-heG lesions per cell.[53, 62]

Roe *et. al.*[63] reported the first synthesis of he-FAPy adducts. Guanosine was treated with ethylene oxide in glacial acetic acid at 50–55 °C to give N7-he-Guo (**10b** in Scheme 5).[58] This intermediate was incubated with 0.5 M ammonia for 40 min at 25 °C, followed by lyophilization to give N7-(2-hydroxyethyl)-FAPy-Guo (compound **11b**).[63] The 2′-deoxy counterparts **10a** and **11a** were prepared in a similar fashion.[63]

2.4. 2-Oxoethyl-FAPy

Vinyl chloride is an important industrial chemical classified as a known carcinogen.[64–67] It is epoxidized by cytochrome P450 2E1 to chlorooxirane, which reacts extensively with DNA.[68] Acetoxyoxirane, an acetylated analogue of chlorooxirane, was used in many studies of DNA alkylation because it is more chemically stable than chlorooxirane, but generates the same types of DNA adducts.[69] Acetoxyoxirane can be readily prepared from vinyl acetate and dimethyldioxirane.[69]

Similar to other simple epoxides, chlorooxirane preferentially alkylates N7G in DNA, and the resulting adducts can undergo imidazole ring opening.[69] Christov *et. al.* reported the first synthesis of oxoethyl-FAPy-dG (Scheme 6).[69] In brief, dG was reacted with acetoxyoxirane in acetic acid at room temperature for 3 h to give N7-oxyethyl-dG **12**. At pH 7.8, compound **12** was found to undergo two competing reactions: deglycosylation to give an abasic site (major pathway) and imidazole ring opening to give compound **13** in 10 % yield (Scheme 6, top). Compound **13**, which was accounted for 10% of total reaction mixture, was highly unstable and underwent spontaneous cyclization to give a ring closed compound (cyclized product, Scheme 6). In a similar way the allyl-FAPy-dG was obtained by the alkylation of dG with allyl bromide to give compound **14** which was further treated with 1M NaOH to give the corresponding FAPy adduct **15** (Scheme 6, bottom).

2.5. Ethylamine-FAPy

Ethyleneimine (aziridine) is an industrial chemical widely used in the production of polymers, coatings, adhesives, drugs, dyes, cosmetics, and antineoplastic agents.[70, 71] Carboxylic acid derivatives of aziridine have been reported as immunomodulators.[72] Ethyleneimine is an extremely reactive alkylating agent which targets the N7-purine positions in DNA. In 1984, Hemminki *et. al.*[42] reported that ethyleneimine reactions with guanosine and 2′-deoxyguanosine at pH 6.5 in 0.2 M ammonium formate for 6 h gave rise to N7-dG adducts **16a** or **16b** (Scheme 7).[42] The corresponding ring opened adducts **17a** and **17b** were formed in 80% yield. Unlike N7 adducts of simple alkylating agents, the conversion of aziridine adducts to the corresponding FAPy structures took place under mild conditions, probably due to protonation of the amino side chain, which facilitates hydroxyl

ion attack at C-8 position of N7-alkylated nucleoside (Scheme 7). As described below, a similar mechanism accelerates FAPy adduct formation from antitumor nitrogen mustards.

2.6. FAPy Adducts of Nitrogen Mustards

Nitrogen mustards (NM) are *bis*-electrophiles capable of cross-linking DNA to form toxic interstrand and intrastrand cross-links (Figure 5). Nitrogen mustard drugs such as cyclophosphamide, chlorambucil, and mechlorethamine (**18–21**) are widely used in the treatment of immune diseases, lymphoma, leukemia, multiple myeloma, and ovarian carcinoma (Figure 5).[73–76] The antitumor activity of NMs has been attributed to their ability to cross-link the twin strands of DNA.[77] The resulting bifunctional lesions, if not repaired, can inhibit DNA replication and transcription, eventually leading to cell cycle arrest, apoptosis, and the inhibition of tumor growth. In 1982, Chetsanga *et. al.* first reported imidazole ring opening of N7-guanine adducts generated by phosphoramide mustard in DNA.[78] Alkylated DNA was treated with 0.2 N NaOH for 30 min at 37 °C to obtain the corresponding FAPy adducts. The reaction mixture contained five isomers of phosphoramide mustard-imidazole ring-opened dG complexes.[78] Recently, the Turesky group for the first time detected NM-FAPy adduct in mustard-treated DNA and in human cell culture.[79]

Christov *et al.* recently reported the first synthesis of NM-FAPy adducts, which were subsequently incorporated into DNA strands by phosphoramidite chemistry (Section 3 below).[80] The synthesis began with N^2 – formamidine protected compound **22**, which was further protected at 5′OH treated with DMT-Cl to give DMT protected dG (**23**, Scheme 8). Compound **23** was reacted with ethyl nitrogen mustard in trifluoroethanol to give N7-dG intermediate **24**, which was not isolated. Further imidazole ring opening of **24** was performed in the presence of 1M NaOH to give NM-FAPy-dG **25** (85% yield).[80]

2.7. Aflatoxin B1 (AFB 1)-FAPy-dG

Aflatoxins are carcinogenic mycotoxins produced by certain molds that can contaminate agricultural products such as peanuts and corn.[81, 82] Specifically, Aflatoxin B1 is produced by *Aspergillus flavus* and *A. parasiticus* and has been implicated in liver cancer in populations consuming contaminated grains.[83] Aflatoxin B1 is metabolically activated to epoxide **26** (Figure 6), which is capable of alkylating guanine nucleobases of DNA to give N7-guanine adducts **27**, which spontaneously depurinate to give abasic sites.[84]

Aflatoxin exposure induces high levels of G→T transversions.[85–88] AFB-N7-dG adducts were initially hypothesized to be responsible for these genetic changes.[83, 89, 90] However, site-specific mutagenesis experiments have revealed that N7-dG adducts **27** are only weakly mutagenic in *E. coli.* (4%).[91] It was then proposed that apurinic (AP) sites arising from depurination of *N7*-guanine adducts may be the source for AFB genotoxicity.[14, 92] However, Essigmann and coworkers have shown that AFB 1 epoxide treated cells exhibit a unique mutagenic signature distinct from that of AP sites. It was subsequently shown that N7-AFB-dG undergoes imidazole ring opening at physiological pH to give the corresponding AFB-FAPy-dG adduct **28**.[89, 90] AFB-FAPy-dG formation occurs readily at physiological pH,[83] and the resulting lesions are highly mutagenic[84, 90, 93–95] and persist *in vivo*.[84, 93, 94] It is now generally accepted that ring open adducts AFB-FAPy-dG (**28** in Figure 6) are largely

responsible for the key G to T mutations that lead to hepatocarcinoma development following exposure to Aflatoxin B1.[96]

The first synthesis of AFB-FAPy-dG adducts was reported by Harris and coworkers (Scheme 9).[82] 5′-DMT-protected-2′-deoxyguanosine was treated with AFB epoxide **26** in THF to give the corresponding *N7*-dG adduct **29**. Protected AFB-FAPy-dG **30** was obtained by incubating compound **29** at pH 9.5 overnight (15 mM Na_2CO_3/30 mM $NaHCO_3$ buffer at ambient temperature). Subsequent detritylation of compound **30** in 0.1 M HCl for 15 min gave AFB-FAPy-dG nucleoside **31**.[82] The use of 5′-protected dG was critical to prevent anomerization of the sugar (see below Section 3).[82]

The presence of multiple isomeric forms of AFB-FAPy-dG has made it challenging to deduce the exact chemical structure of AFB-FAPy-dG. Initial NMR studies by Miller and Garner groups have revealed that AFB-FAPy-dG existed as a mixture of at least four isomers.[89, 90] These four isomers were isolated by HPLC as two separate peaks, with each fraction containing a pair of inseparable species. Miller and Garner initially hypothesized that the structures of the AFB-FAPy isomers involved ring closed forms **32B** and **33B**, along with the corresponding ring open structures **32A** and **33A** (Figure 7).[89, 90] Subsequent structural studies by Hertzog *et. al.*[90] have reassigned the structures of AFB-FAPy isomers as two pairs of geometrical isomers **34** and **35**, which were in equilibrium with the rotamers **36** and **37** (Figure 8). UV absorption spectra of **34** and **35** were reported to have maxima at 265, 340 and 364 nm, with pH having only a minor effect on the spectra.[71] However, the presence of structural isomers **34** and **35,** with different position of the formyl group (N^5 vs N^6), due to different direction of imidazole ring opening following hydroxyl ion attack, was in contrast with previous studies of FAPy adducts generated by methylating agents, sterigmatocystin, and mitomycin, in which the formyl group was always placed at the N^5 atom as in **34** and **36**.[10, 36, 41, 97]

Most recently, detailed NMR experiments were conducted by the Harris group[82] to elucidate the correct structure of AFB-FAPy adducts (Figure 9). Two sets of stereoisomers are possible for AFB-FAPy adducts; (a) geometrical isomers around the formamide group and (b) atropisomers at the pyrimidine C5–N^5 bond (**38**–**41** in Figure 9). The first pair of isomers (**38**/**40** and **39**/**41**) forms as a result of rotation about the formamide bond (highlighted in blue, Figure 9). Since aflatoxin is a chiral molecule, this leads to a pair of diastereomers separable by HPLC. Additional isomers (**38**/**39** and **40**/**41**) are produced due to hindered rotation about the C5–N^5 bond (highlighted in red). The four isomers (**38**–**41**) in Figure 9 are separable by HPLC, but interconvert with each other.[82] The rotational barrier for this interconversion is relatively high due to the steric bulk of the AFB substituent at the N^5.[82] ^{1}H NMR spectra of nucleobases **38** and **39** are very similar. The pair of formyl signals are observed at 8.29 and 7.59 ppm (compound **38**) and 8.22 and 7.52 ppm (compound **39**). These four formyl signals are split into doublets with coupling constants ~17 Hz, confirming the attachment of the formyl group to the N5 position of pyrimidine ring. Similar observations were made for the isomers of FAPy nucleoside **31** (AFB-FAPy-dG). The complete NMR studies of AFB-FAPy-dG included COSY, TOCSY, HMQC, HMBC, NOESY, and ROESY.[82]

Structurally related FAPy adducts of sterigmatocystin, a mycotoxin that can form in moldy grain, green coffee, and cheese, have been reported by Baertschi *et. al.*[98] Sterigmatocystin is produced by some strains of *Aspergillus, Penicillium*, and *Bipolaris* sp.[99, 100] Sterigmatocystin 1,2 epoxide **42** (Scheme 10) was prepared from sterigmatocystin and incubated with DNA for 7 days at 5 °C to form the corresponding N7-alkylated adduct **43**. Imidazole ring opening of this adduct was performed by incubating compound 43 at pH 9.8 at 25 C for 2 hr to obtain sterigmatocystin-FAPy **44** (Scheme 10).[97, 99, 100]

2.8. Mitomycin C-FAPy Adducts

Mitomycin C (MMC, in Scheme 11) is an antitumor antibiotic widely used in cancer chemotherapy.[101] It is a bifunctional alkylating agent capable of cross-linking DNA, leading to cell cycle arrest and apoptosis. MMC alkylates N7-G position in DNA to give the corresponding guanine adduct.[40] In 1987, Tomasz *et. al.* reported that the mitomycin C forms FAPy-dG lesions under basic conditions.[41] d(GpC) dinucleotide was treated with MMC at pH 3.5–4 to give the corresponding N7-alkylguanine adduct **45**. Subsequent imidazole ring opening under basic condition yielded MMC-FAPy-dG **46** (Scheme 11).

Five isomers of MMC-FAPy-dG (**47–50**) were identified, including α and β anomers and furanose/ pyranose nucleosides (Figure 10).[41] The α anomer **53** may form through imino intermediate **52** (Scheme 12), a rearrangement that can take place in double stranded DNA, but is much faster for free nucleosides. The pyranose isomers of FAPy-dG adducts (**54** in Scheme 12) may form by intramolecular attack of C5′-hydroxyl on the imine functionality of intermediate **52**.[41, 102] It should be noted that the rearrangement to pyranose form is not possible in the absence of a free C5′-hydroxyl group (as in DNA strands).[7, 103] Therefore, during nucleoside and phosphoramidite synthesis of FAPy adducts, the formation of pyranose isomers can be minimized by protecting the 5′position of the sugar, typically using the DMT group.[57]

3. Synthesis of DNA Oligodeoxynucleotides Containing N^5-R-FAPy Adducts

In order to establish the role of N^5-substituted FAPy adducts in mutagenicity and to uncover their possible contributions to the therapeutic effects of DNA modifying agents, it is necessary to establish the chemical structures, stability, and mispairing characteristics of N^5-R-FAPy adducts. This requires chemical synthesis of DNA molecules containing structurally defined, site-specific N^5-R-FAPy adducts. Such a synthesis can be a challenging task due to the unusual structural complexity of this class of adducts and their ability to undergo isomerization.[82] Standard solid phase synthesis coupling conditions can result in a mixture of DNA strands containing α and β anomers, as well as both furanose and pyranose forms, and special precautions must be taken to minimize this structural complexity.

Previous attempts to prepare N^5-R-FAPy containing DNA strands can be broadly divided into three general approaches; (i) direct treatment of DNA with alkylating agents and base to introduce FAPy, (ii) chemical synthesis of alkylated-FAPy-phosphoramidite building blocks and their incorporation into DNA *via* solid phase synthesis (SPS), and (iii) solid phase synthesis employing carbocyclic nucleoside analogues.

3.1. Direct Alkylation of DNA to Generate N^5-R-FAPy

In the most straightforward approach, DNA strands containing a FAPy adduct can be prepared by treating oligodeoxynucleotides containing a single guanine base with alkylating agents, followed by basic treatment to introduce N^5-R-FAPy (Scheme 13). This methodology was employed by Brown *et al.* to generate DNA containing AFB-FAPy-dG.[82] Synthetic DNA 13-mer containing a single dG residue was treated with AFB-epoxide in 100 μL phosphate buffer (10 mM sodium phosphate, 100 mM NaCl, pH 7.0) for 30 min (Scheme 14). Further, the alkylated DNA was dissolved in sodium carbonate solution (pH 10) to open the imidazole ring, and the resulting AFB-FAPy-dG containing oligodeoxynucleotide was purified by HPLC. The main limitations of this approach are that it is limited to DNA sequences containing a single guanine and that a mixture of isomers can be generated.

Tudek *et al.*[104] employed a similar direct alkylation approach to study the mutagenic specificity of Me-FAPy-purines in M13mp18 phage DNA. Single stranded M13 phage DNA was incubated with dimethylsulfate (DMS) to obtain DNA containing N7-methyl-dG (83%), and N7-methyl-dA (2.2%) (Scheme 15). This DNA was further incubated in 0.2M NaOH for 15 min at 37 °C to obtain DNA containing the corresponding FAPy adducts.[104] It should be noted that this approach induced Methyl-FAPy-dG and Methyl-FAPy-dA adducts at random sites within the plasmid.

Chetsanga *et. al.* treated DNA containing [^{3}H]-dG with phosphoramide mustard. To obtain labelled DNA, a guanine requiring *Bacilius Subtilis* strain was grown in cell culture supplemented with deoxy[8-^{3}H]guanosine, and cells were lysed by lysosome treatment.[78] The purified DNA consisting [^{3}H]guanosine was treated with phosphoramide mustard to obtain alkylated DNA and with 0.2N NaOH to produce PM-FAPy containing DNA (Scheme 16). As in the paper by Tudek *et al.,* this approach does not produce site-specific adducts.[104]

3.2. Incorporation of Alkylated-FAPy Adducts into DNA via Phosphoramidite Chemistry

To enable the preparation of DNA strands containing site specific, structurally defined N^5-R-FAPy adducts, solid phase synthesis starting with nucleoside phosphoramidites can be employed. In 2008, the Rizzo group reported the synthesis of Me-FAPy-dG phosphoramidite (Scheme 17). Compound **55** (generated as shown in Scheme 3 above) was treated with phosphoramidite reagent in the presence of tetrazole in anhydrous dichloromethane for 2 h at room temperature to obtain Me-FAPy-dG phosphoramidite **56** in 78% yield (Scheme 17).[57] This phosphoramidite building block was employed in solid phase synthesis (SPS) experiments in order to incorporate Me-FAPy-dG into short DNA sequence 5′-d(TT-Me-FAPy-dG-TTC)-3′.

The critical step in the solid phase synthesis of Me-FAPy-dG containing ODNs is the deprotection of the 5′-OH group of Me-FAPy-dG, since the unprotected nucleoside undergoes rapid rearrangement to the pyranose form (Scheme 12 and discussion above).[57] During solid phase synthesis, a “short” deprotection step was employed rather than the traditional deprotection step.[57] Upon HPLC analysis of enzymatic digests, five Me-FAPy-dG peaks were observed (Figure 11), of which one peak was the pyranose form (Peak 1)

whereas other four peaks corresponded to furanose nucleosides in an α and β anomeric forms.[57]

A similar strategy was employed to prepare NM-FAPy-dG building block (**59** in Scheme 18).[80] The protected NM-FAPy-dG (compound **25**) was prepared as shown in Scheme 8. Compound **25** was then treated with cesium acetate in 18-crown-6 ether to replace chloro group of NM with an acetyl group (**57**). The 6-oxo group was deprotected with TBAF in THF to give compound **58**, which upon phosphitylation in dichloromethane in the presence of tetrazole gave phosphoramidite building block **59** in 50% yield. This nucleoside was incorporated into 12 and 24-mer oligodeoxynucleotides by solid phase synthesis.[80]

During automated solid-phase synthesis, a short deprotection cycle was employed in order to minimize the rearrangement of NM-FAPy-dG to the pyranose form of the nucleoside. However, HPLC analysis of the resulting DNA strands revealed two peaks, both having the expected molecular weight and representing furanose and pyranose forms of the adduct. When the standard DNA synthesis protocol (normal deprotection time) was employed, a 1:1 ratio of furanose to pyranose ring containing product formation was observed. The thermal melting profile of the NM-FAPy-containing 12 mers gave inconsistent results due to the presence of α and β anomers (1:1).[80]

3.3. Carbocyclic Nucleoside Analogues of FAPy

As described above, a major obstacle in solid phase synthesis of FAPy-dG containing DNA strands is that they readily undergo rapid isomerization to give α anomers and pyranose forms under standard DNA synthesis conditions.[105, 106] Due to this rapid anomerization, it has only been possible to incorporate α/β anomeric mixtures of FAPy adducts into DNA. Hydrolysis of glycosidic bond of FAPy-dG at elevated temperatures further complicates their synthesis.

To circumvent these problems, Carell *et al.* have developed the nonhydrolizable and non-epimerizable β analogues of the FAPy-dG lesions.[105–107] In this approach, the oxygen atom of the deoxyribose moiety was replaced with a methylene group to give the corresponding carbocyclic nucleoside. This replacement had a minor effect on base pairing.[59]

The synthesis of Bz-cFAPy-dG (Scheme 19)[105–107] started with enantiomerically pure cyclopentylamine **60,** which was synthesized as described by Cullis and Dominguez.[108] Coupling of compound **60** with protected 2-amino-6-chloro-5-nitro-4-oxopyrimidine (**61**) furnished the nitro pyrimidine derivative **62** (86%), which was subsequently subjected to reduction to give the corresponding aminopyrimidine compound **63** (58%). Further, the primary amine of compound **63** was coupled with benzaldehyde and subjected to reduction with sodium cyanoborohydride to give the N5-benzylated compound **64**. The formyl group was introduced at the C-5 position by reaction of compound **64** with acetic formic anhydride to give Bz-cFAPy-dG **65** (89%) (Scheme 19).

To prepare the nucleoside phosphoramidite of **65**, compound **65** was protected at 5′-OH by DMTCl in pyridine at room temperature to give the DMT derivative **66** in 55% yield (Scheme 19). Phosphitylation of **66** was carried out under argon in acetonitrile in the

presence of tetrazole and DIPEA to give phosphoramidite **67** in 54% yield, which was subsequently incorporated into DNA. These authors reported that since the standard capping procedure during DNA synthesis was not compatible with Bz-cFAPy-dG, a solution of 2,6-lutidine and pivaloylic anhydride in THF (v/v/v 1:1:8) was used in place of phenoxyacetyl anhydride for capping, and 4,5-dicyanoimidazole (0.25 M) was used as the coupling reagent. The coupling time for the Bz-cFAPy-dG phosphoramidite was much longer compared to standard phosphoramidite methods (10 min vs 144 s).[106]

DNA strands containing Bz-cFAPy-dG were purified by HPLC and analyzed by MALDI-TOF. LC-MS of enzymatic digests showed no structural alteration of Bz-cFAPy-dG during ODN synthesis and purification. Thermodynamic studies of c-FAPy (no substitution on the formamide group) oligodeoxynucleotides revealed that c-FAPy conferred significant duplex destabilization.[105, 106] Interestingly, the base excision repair enzyme Fpg recognized the unnatural N7-benzyl-cFAPy-dG lesion via an unproductive binding mode, leading to enzyme inhibition.[107]

4. Effects of N^5-R-FAPy Adducts on DNA Replication

4.1. Methyl-FAPy-dG

Structurally, ring open N^5-R-FAPy adducts are substituted pyrimidines, and are expected to mispair with purines during DNA replication. However, initial studies with bacterial DNA polymerases (e.g. Klenow fragment of *E. coli*. DNA polymerase I) have shown that Methyl-FAPy-dG blocked bacterial DNA replication *in vitro*, but did not induce any mutations.[11, 53, 109, 110] Similarly, O'Connor and others reported that *E. coli*. DNA polymerase I exo and T4 DNA polymerases were inhibited one nucleotide before Me-FAPy-G.[11] Inhibition of DNA synthesis by Me-FAPy-G is stronger than ROS-induced lesion 8-oxo-dG, but is weaker than that of apurinic sites and FAPy-A (FAPy-Ade ≈AP site>FAPy-7Me-G>8-oxoG).[54, 111]

Rizzo *et al*. examined *in vitro* bypass of Me-FAPy-dG in the presence of eukaryotic DNA polymerases α, β, and hPol δ/PCNA.[112] Me-Fapy-dG blocked high fidelity polymerases at either the insertion or the extension step. Translesion synthesis was observed for hPols η, κ, and hRev1/Pol ζ. These polymerases primarily inserted the correct base (C) opposite the lesion, however hPols η and κ also catalyzed the misinsertion of Thy, Gua, and Ade opposite Me-Fapy-dG, and generated a single nucleotide deletion product. These authors concluded that although the amounts of Me-FAPy-dG lesions in cells are relatively low, their miscoding potential could contribute to genomic instability.[112]

Tudek *et. al*.[111, 113] investigated the biological properties of Me-Fapy-dG and Me-Fapy-dA in M13mp18 phage DNA. Lesions containing plasmids were generated as described above in section 3.1 (Scheme 15), and were transfected into *E. coli*. The presence of Me-FAPy adducts led to a significant decrease in transfection efficiency and increased mutational frequency in the lacZ gene following SOS induction.[111, 113] However, sequencing analyses have revealed primarily A →G transitions, while mutations at GC base pairs were only slightly elevated. These results suggest that Me-FAPy-G is primarily a lethal lesion in *E. coli*. In contrast, the corresponding Me-FAPy-A adducts are a more miscoding, causing

A→G transitions.[54, 104] Me-FAPy-A (Figure 12) was two orders of magnitude more mutagenic than Me-FAPy-G.[54, 111]

4.2. AFB-FAPy-G

The biological outcomes of AFB1-FAPy-dG adducts (Figure 13) have been examined in detail due to their proposed roles in aflatoxin-mediated liver cancer. In contrast to N7-AFB-G, AFB1-FAPy-G is highly persistent in rat liver DNA, reaching maximum adduct amounts 2 weeks after exposure.[89] One of the AFB1-FAPy rotamers (**68A** in Figure 13) has been shown to be a potent block to DNA synthesis, even when DNA polymerase of lowered replication fidelity was used (MucAB).[96] Both AFB1-FAPy-G and N7-AFB1-G caused G→T transversions, which is consistent with the observed G→ T mutations in codon 249 of the *p53* tumor suppressor gene in 50% of hepatocellular carcinomas and in AFB1-treated human hepatocytes cultures.[51, 52] In addition, AFB1-induced G→ T mutations in the *ras* oncogene appear to be important for liver tumor progression.[86, 87] Taken together, these results indicate that AFB1-FAPy adducts may be the ultimate lesions responsible for mutagenesis and genotoxicity of aflatoxin.[96, 114]

5. Cellular Repair of N^5-R-FAPy Adducts

Many of the previous studies of FAPy adduct repair have been limited to unsubstituted FAPy induced by ROS.[49, 50, 53] Repair studies of N^5-substituted FAPy-adducts are less extensive, and several examples are given below.

FPG glycosylase: Formamidopyrimidine DNA glycosylase (Fpg) was first identified in 1978–1979 as a DNA glycosylase that removes Me-FAPy-G from DNA.[13] Along with Me-FAPy-G, this glycosylase also excises ROS-induced unsubstituted FAPy-G, unsubstituted FAPy-A, as well as damaged pyrimidines and 8-oxo-dG.[13,115–119] Substituent size on the N^5 position of -the adduct has been shown to influence enzyme activity. For example, Tudek *et. al*. have shown that Me-FAPy-G was excised by Fpg 7-times faster than Et-FAPy-G.[120] It is not known whether other N^5-R-FAPY adducts are also substrates for this repair pathway.

yOgg 1: 8-oxo-G DNA glycosylase (yOgg 1) excises Me-FAPy-G, but does not remove Me-FAPy-A.[121, 122] Furthermore, Fpg and its eukaryotic homolog Ogg1 have been reported to recognize unsubstituted FAPy-dG and the carbocyclic analog of Bz-FAPy-dG (Bz-cFAPy-dG, Figure 14) with high affinity.

hNEIL and mNEIL 1: Both hNEIL 1 (human NEIL 1) and mNEIL 1 (mouse NEIL 1) excised Me-FAPy-G, along with a number of pyrimidine-derived nucleobase lesions. However, hNEIL is the only human enzyme that excises FAPy-Ade (unsubstituted).[123–127]

E. coli. Endonuclease IV: *E. coli*. Endonucleoase IV (Endo IV) is an AP endonuclease specific for double stranded DNA . It also removes the 3′-blocking phosphate groups[128–130] and possess the 3′ →5′ endonuclease activity.[131, 132] ODNs containing α-dA, α-dT, α-dC, α-FAPy-dG, α-FAPy-dA lesions are substrates to Endo IV.[133–137] Asagoshi *et. al*. reported that oligodeoxynucleotides containing α-

Me-FAPy-dG are not substrates for Endo IV.[110, 138] However, in 2000 Christov and others showed that α-Me-FAPy-dG is indeed a substrate for Endo IV. [139] The Me-FAPy-dG lesion is a substrate for Fpg/Nei family of glycosylases as well. It is possible that Endo IV and Fpg glycosylases play specialized roles in FAPy adduct repair, with Endo IV recognizing only the α-anomer of N5-alkyl-FAPy lesions and Fpg glycosylase recognizing the β-anomer form of the adduct.

Nucleotide excision repair Alekseyev and Essigmann reported that Aflatoxin B1 formamidopyrimidine adducts (AFB1-FAPy-dG, Figure 13) are preferentially repaired by the nucleotide excision repair pathway *in vivo.*[140] These authors transfected plasmids containing site-specific AFB1-FAPy-dG lesions into *E.coli* cells and employed the host cell reactivation assay to monitor lesion repair in wild type cells and their repair deficient clones. Cells deficient in nucleotide excision repair (*uvrA*) were unable to remove the damage, while BER mutants (*mutM)* were affected to a lesser extent.[140] This was confirmed by in vitro experiments with site-specifically modified oligodeoxynucleotides and purified MutM protein, which revealed excision products characteristic of NER.[140]

6. Future Prospects and Outlook

Although the chemistry and biology of the N^5-substituted FAPy lesions have been a subject of interest for several decades, the majority of the published studies have focused on two types of FAPy lesions (Me-FAPy and AFB-FAPy). The chemical biology of other N^5-R-FAPy adducts is incompletely understood, and their roles in chemical carcinogenesis remain unknown. Based on the published studies of Me-FAPy and AFB-FAPy, such lesions may be extremely important for the biological mechanisms of many DNA damaging agents, if formed *in vivo*. However, with a few exceptions, it is not known whether significant amounts of N^5-R-FAPy adducts form in cells treated with DNA alkylating drugs and environmental agents. Future mass spectrometry based studies are urgently needed to establish the concentrations of these secondary adducts in cells and tissues. Chemical synthesis of DNA strands containing structurally defined N^5-R-FAPy represents a significant challenge due to their propensity to undergo isomerization. Furthermore, FAPy lesions exist as a mixture of rotamers which present a range of possibilities for base pairing due to changes in hydrogen bond donor acceptor patterns. Future studies employing novel solid phase synthesis methodologies are needed in order to establish the relationship between N^5-R-FAPy adduct structures and their biological outcomes, as well as to elucidate their effects on DNA structure.

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Abbreviations

FAPy	formamidopyridines
dG	2′-deoxyguanosine
dA	2′-deoxyadenosine
DNA	deoxynucleic acid
N7-heG	N7-(2-hydroxy)ethyl guanine
PM	phosphoramide
NM	nitrogen mustard
MMC	mitomycin C
AFB	aflatoxin
THF	tetrahydofuran
DCM	dichloromethane
CH_3I	methyliodide
HCl	hydrochloric acid
DMSO	dimethylsulfoxide
NaOH	sodium hydroxide
AcOH	acetic acid
EI	ethyleneimine
DMTCl	dimethoxytrityl chloride
HPLC	high performance liquid chromatography
UV	ultraviolet
NMR	nuclear magnetic resonance
ppm	parts per million
Me	methyl
ROS	reactive oxygen species
ODN	oligodeoxynucleotides
DMS	dimethylsulfate
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium bicarbonate

AFB-FAPy	aflatoxin formamidopyrimidine
Me-FAPy	methyl formamidopyrimidine
Et-FAPy	ethyl formamidopyrimidine
MMC-FAPy	mitomycin C formamidopyridine
NM-FAPy	nitrogen mustard formamidopyridine
PM-FAPy	phosphoramide formamidopyridine
Bz-cFAPy	benzyl carbocyclic formamidopyridine

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Biographies

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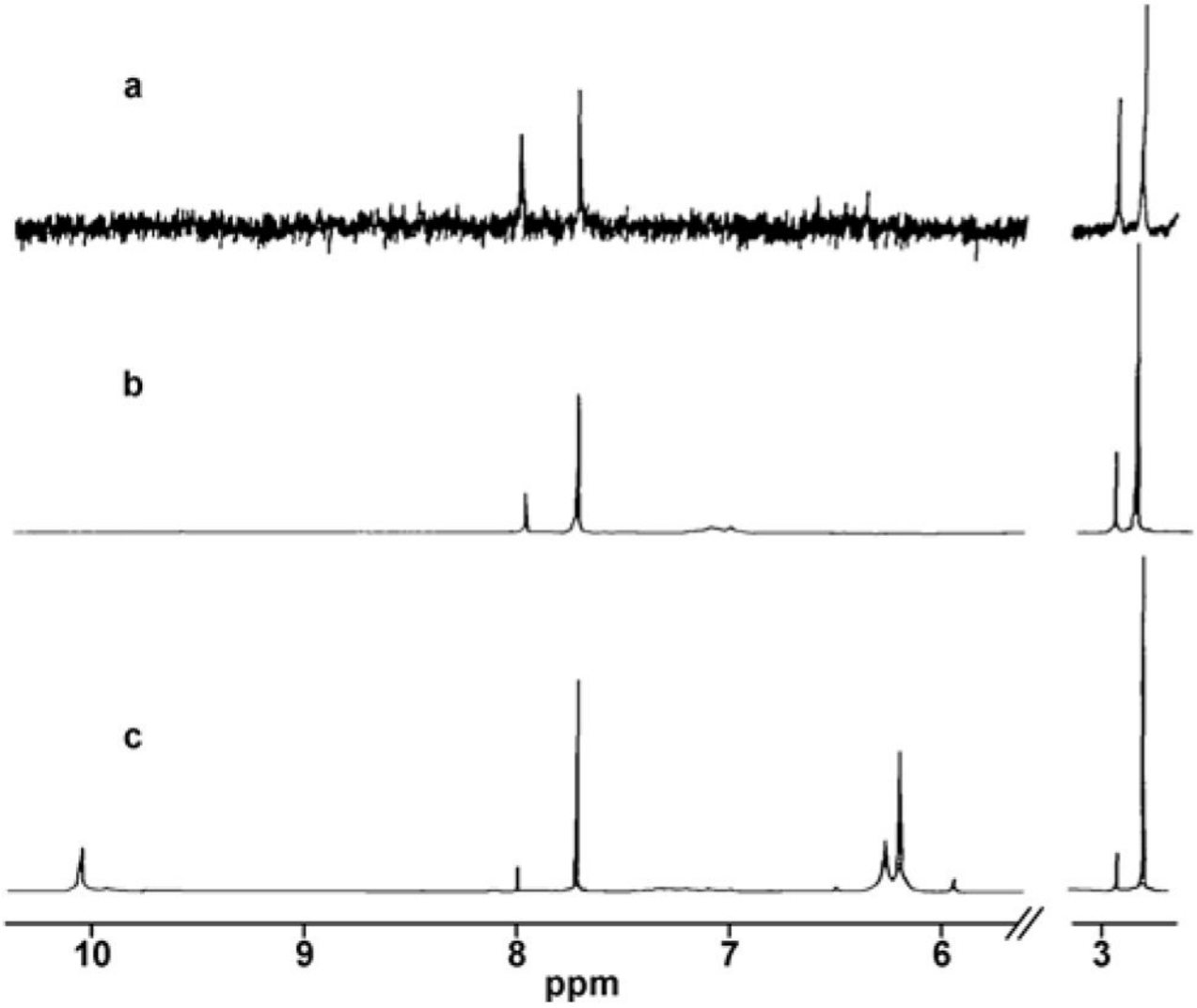


Figure 1.

500 MHz NMR spectra of N^5-methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine. Spectra were obtained in DMSO-d_6/D_2O (1:1) (a), in MeOH-d_4 (b); and in DMSO-d_6 (c).[36] Used by permission of Oxford University Press.

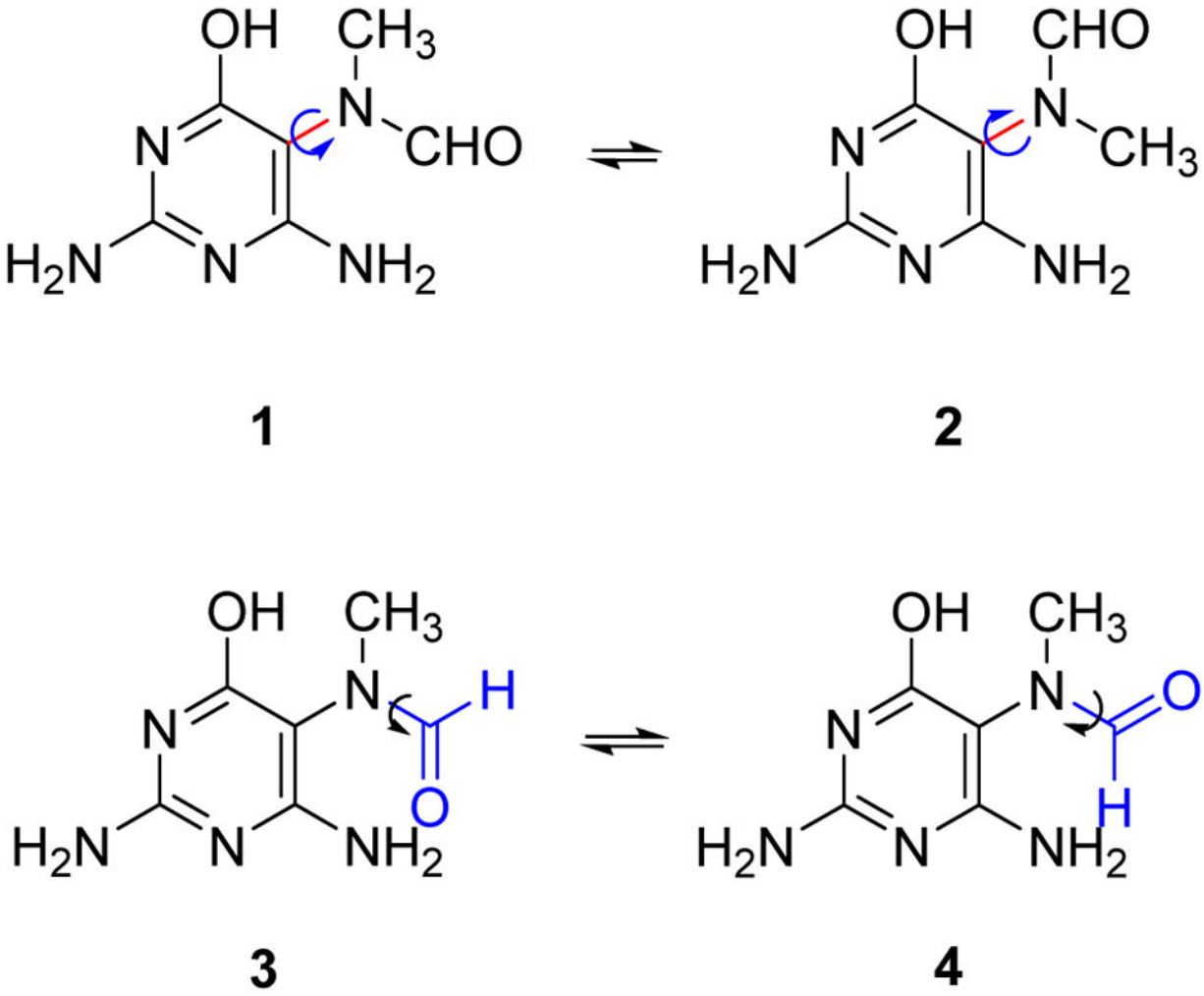


Figure 2.
Rotational isomers of Methyl-FAPy-Gua adducts identified by Beranek *et al.*[36, 37]

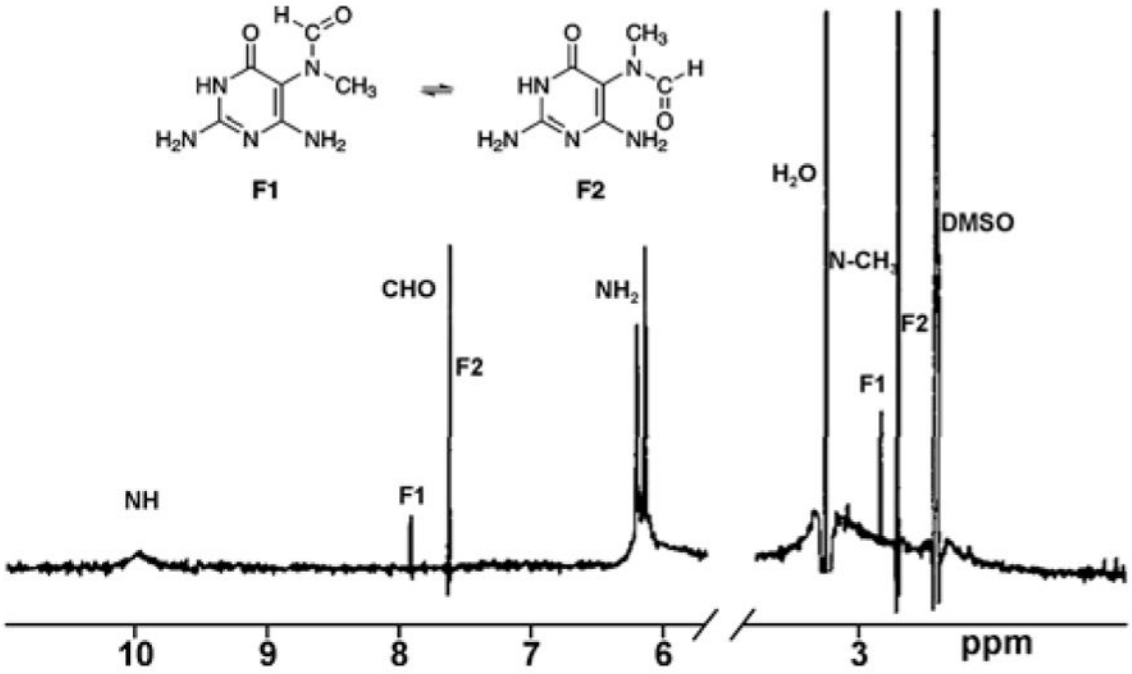


Figure 3.

Proton NMR spectra showing formamido signals with methylene protons of Methyl-Fapy isomers. Spectra were taken in DMSO-d_6.[10] Used by permission of Oxford University Press.

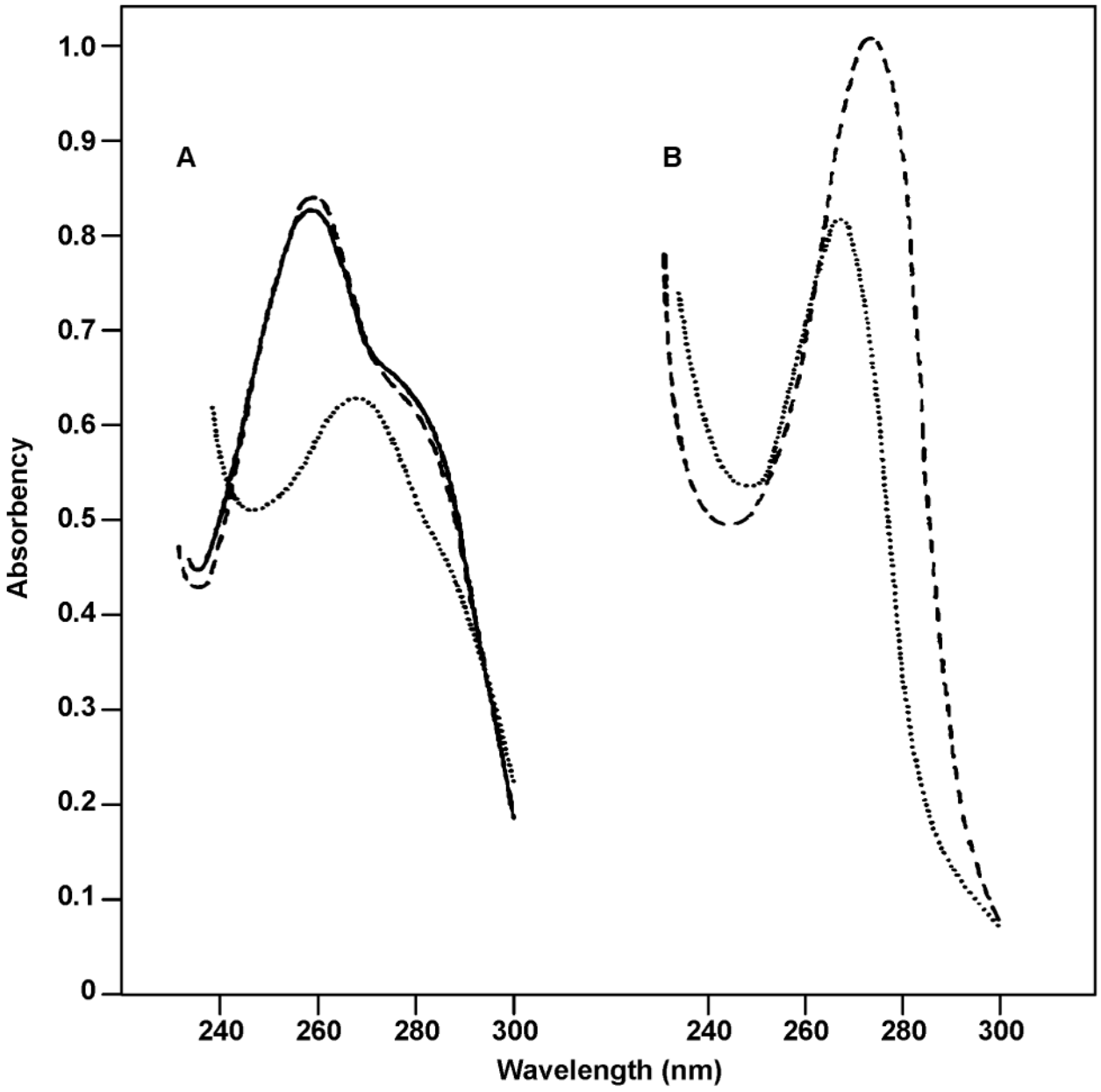


Figure 4.

UV absorption spectra of (A) 7-Et-Guo in water (—), 0.1 N HCl (--) and 0.1N KOH (….). Spectrum shown for 0.1N KOH is for Et-FAPy-G. (B) Ethyl-FAPy-Guo in 0.1 N KOH (….), 0.1N HCl (--).[19]

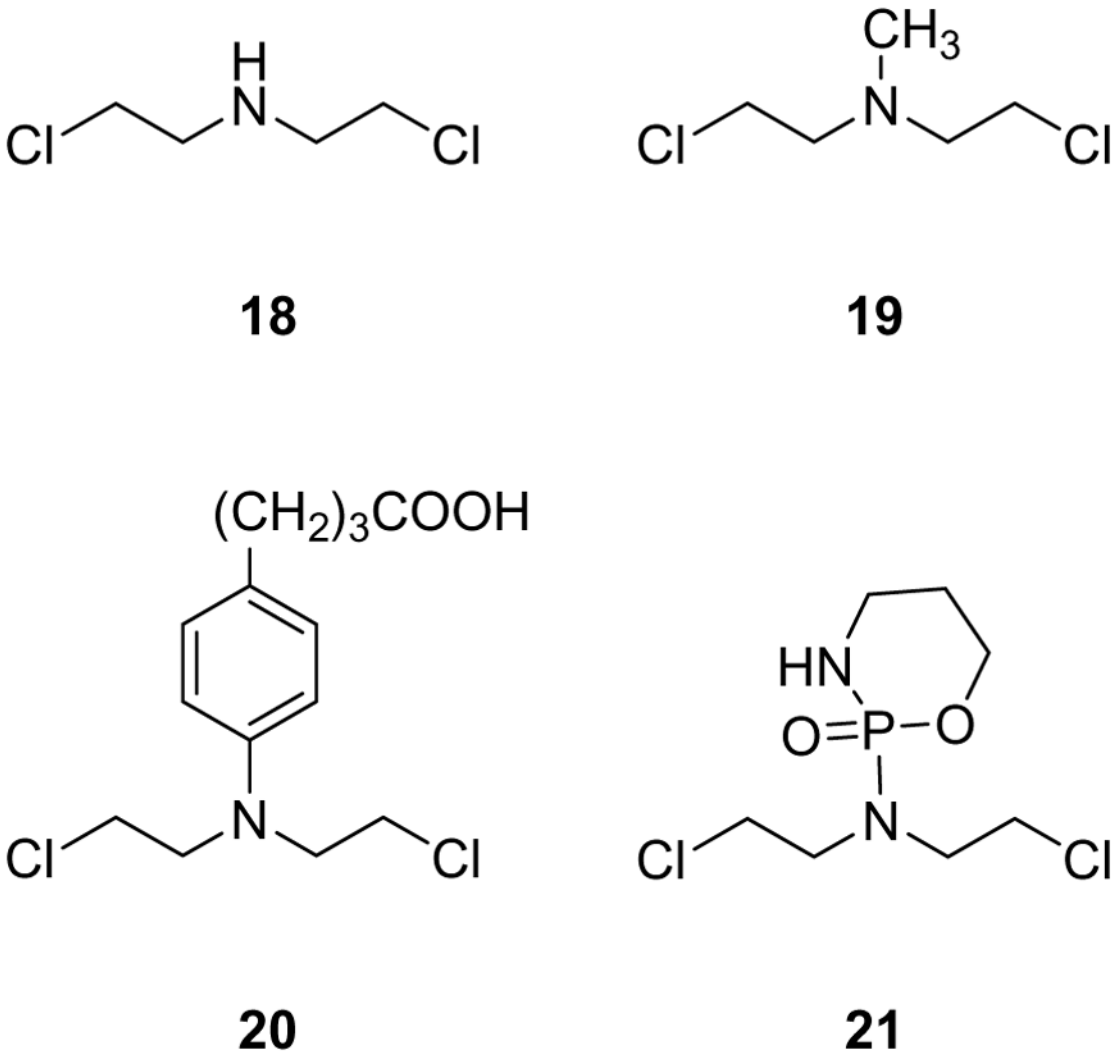


Figure 5.
Structures of nitrogen mustards.

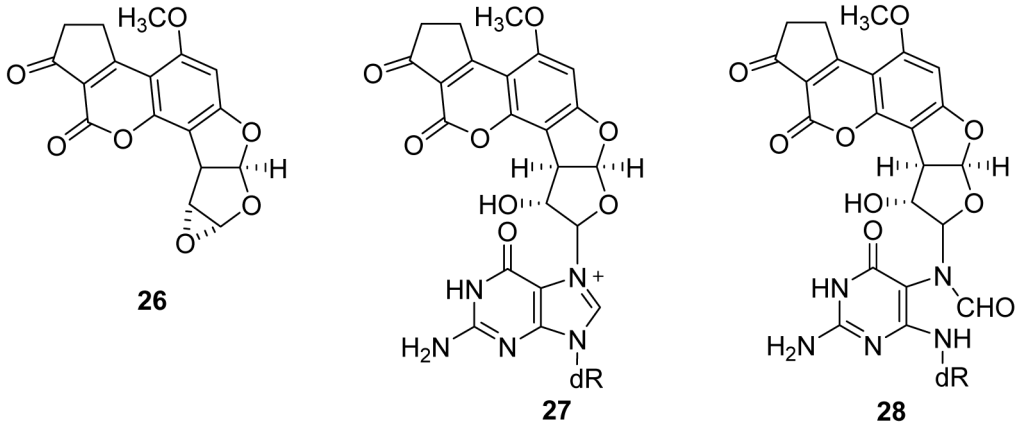


Figure 6.
Structures of aflatoxin B1 epoxide **26**, its cationic *N7*-dG adduct **27**, and AFB-FAPy-dG **28**.

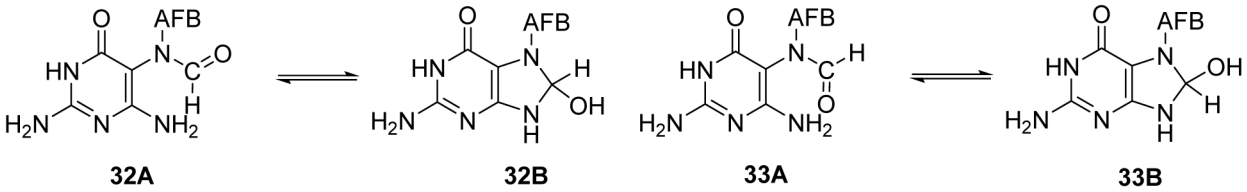


Figure 7.
Proposed structures of AFB-FAPy-dG isomers put forward by Miller and others.[89]

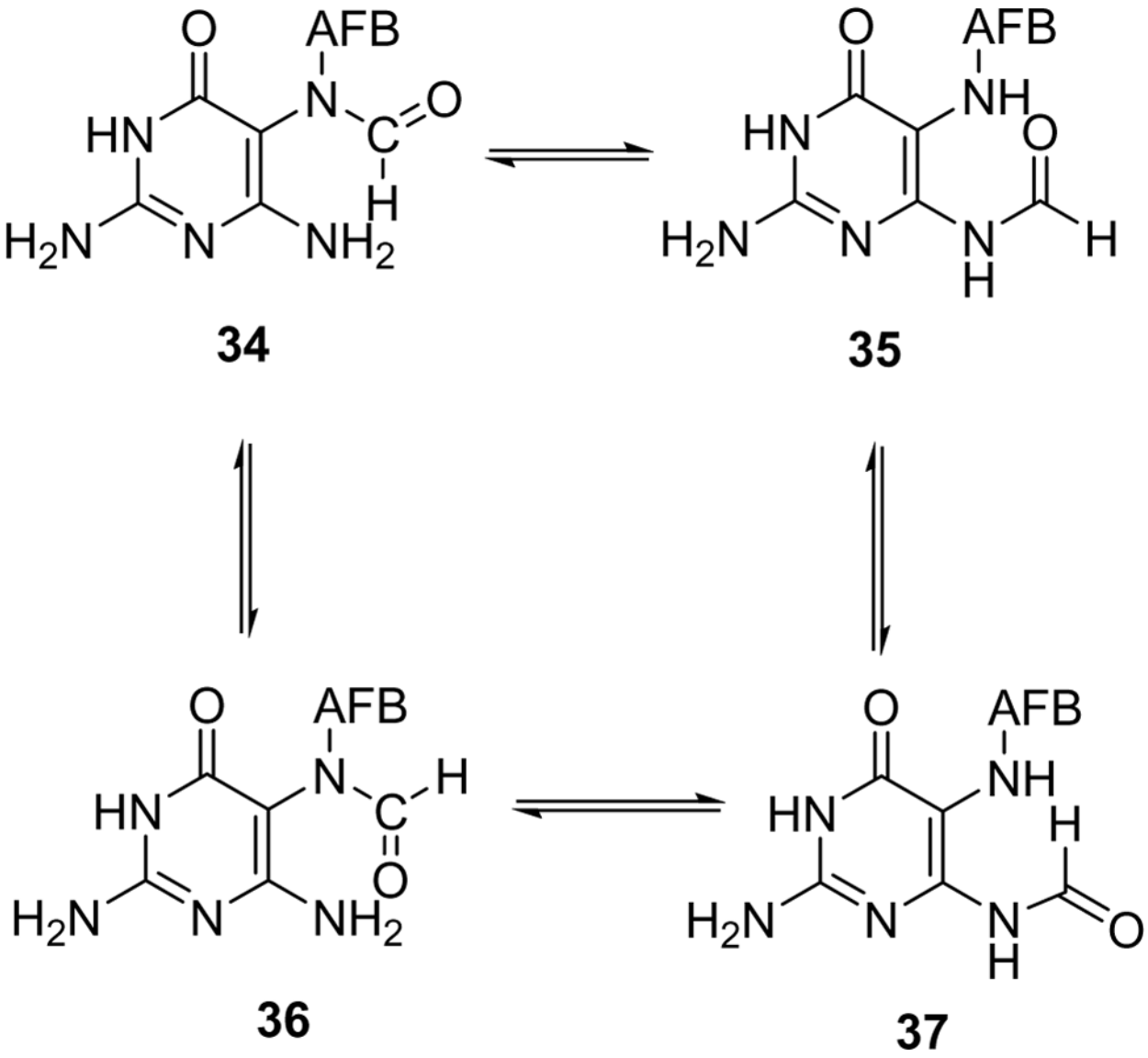


Figure 8.
Structures of AFB-FAPy isomers proposed by Hertzog *et. al.*[90]

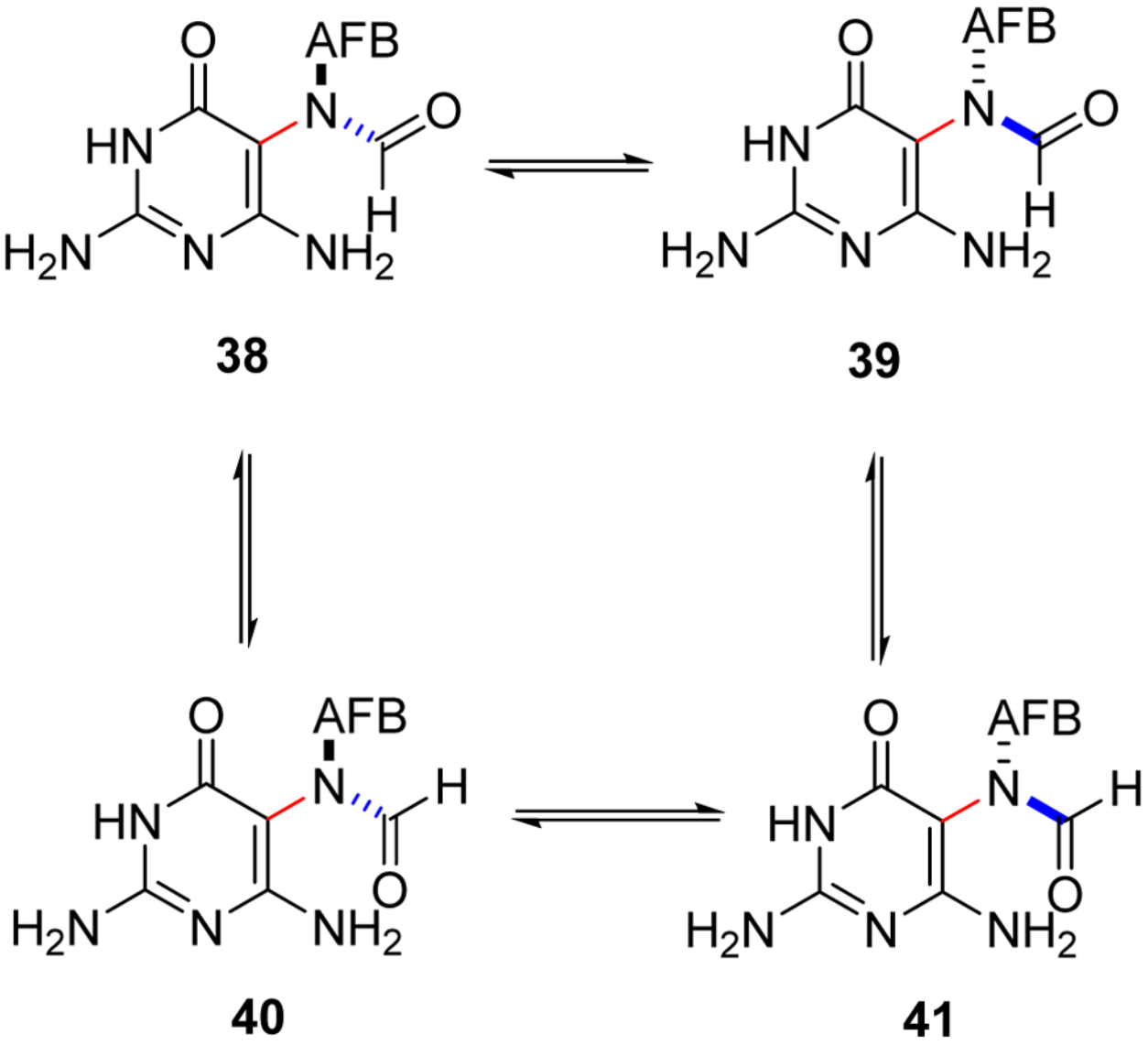


Figure 9.
Structures of AFB-FAPy isomers elucidated by Harris *et al.*[82]

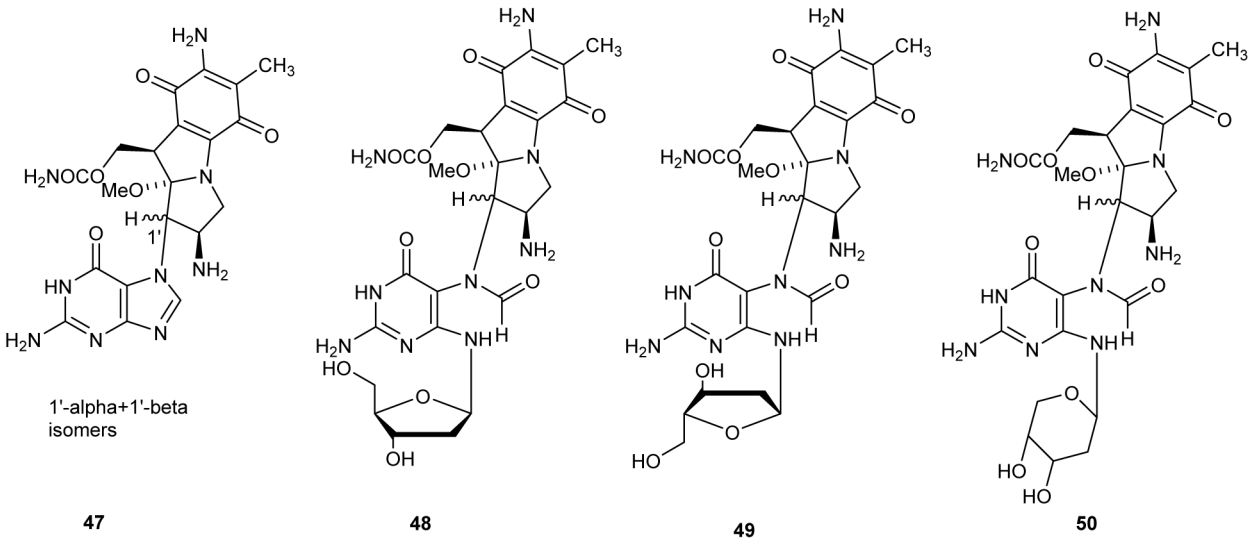


Figure 10.
Structural isomers of MMC-FAPy-dG.

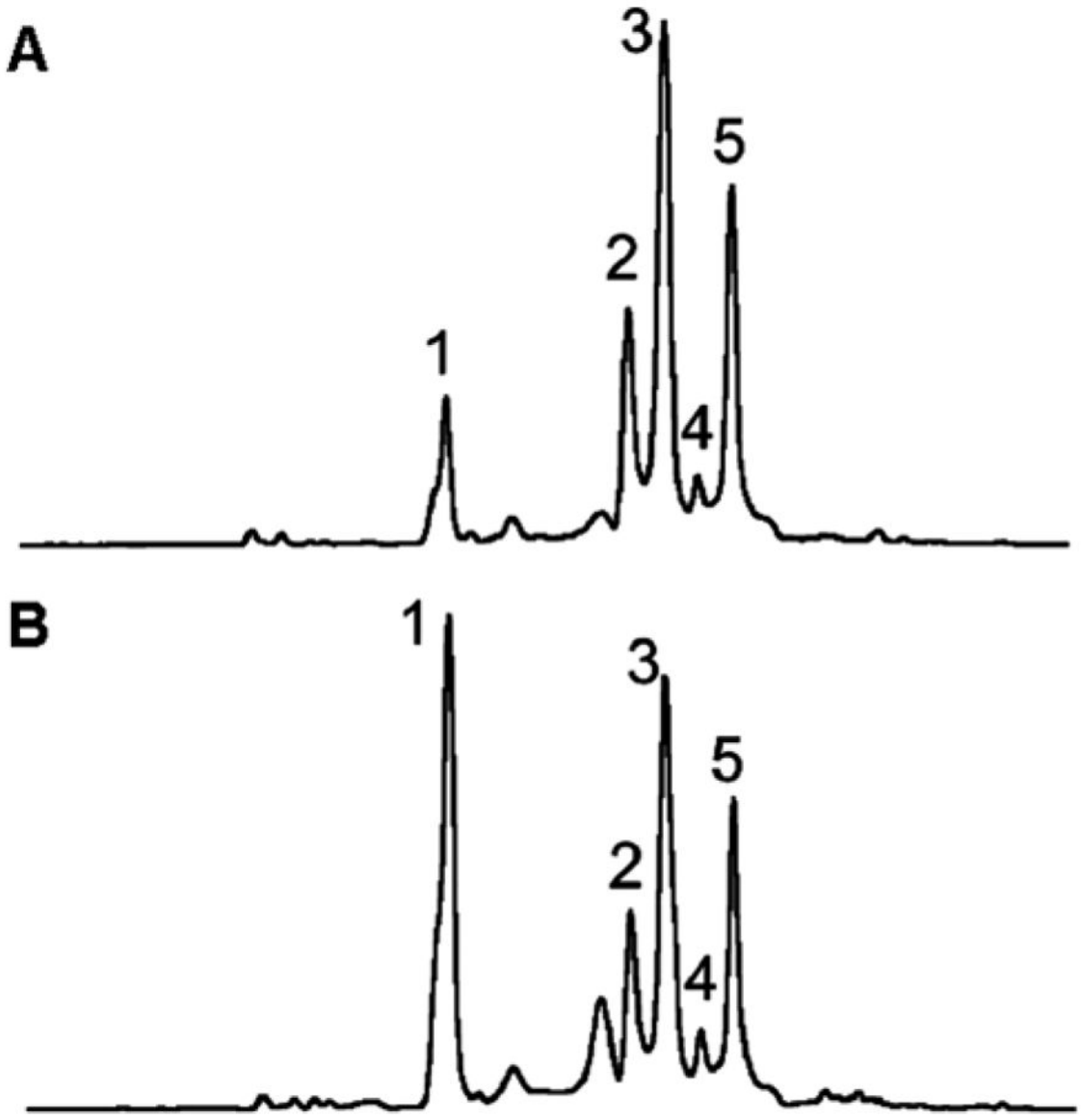


Figure 11.

HPLC analysis of ODN 5′-d(TT-Me-FAPy-dG)-TTC-3′. (A) Analysis of ODN synthesis with a short deprotection cycle. (B) Analysis of ODN synthesis with a long deprotection cycle. In both figures, peak 1 represents the formation of pyranose adduct, whereas other peaks are mixture of α and β isomers.[57]

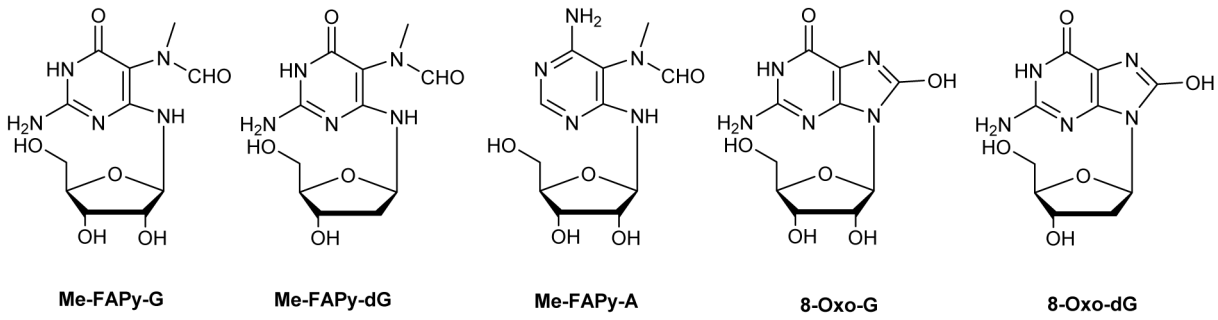


Figure 12.
Structures of N^5-R-FAPy lesions investigated in biological studies.

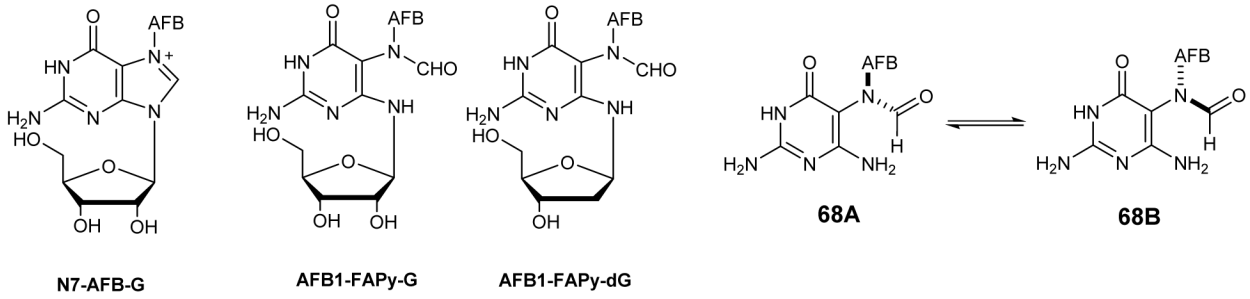


Figure 13.
Structures of N7-AFB1-G and AFB1-FAPy lesions investigated in biological studies.[82]

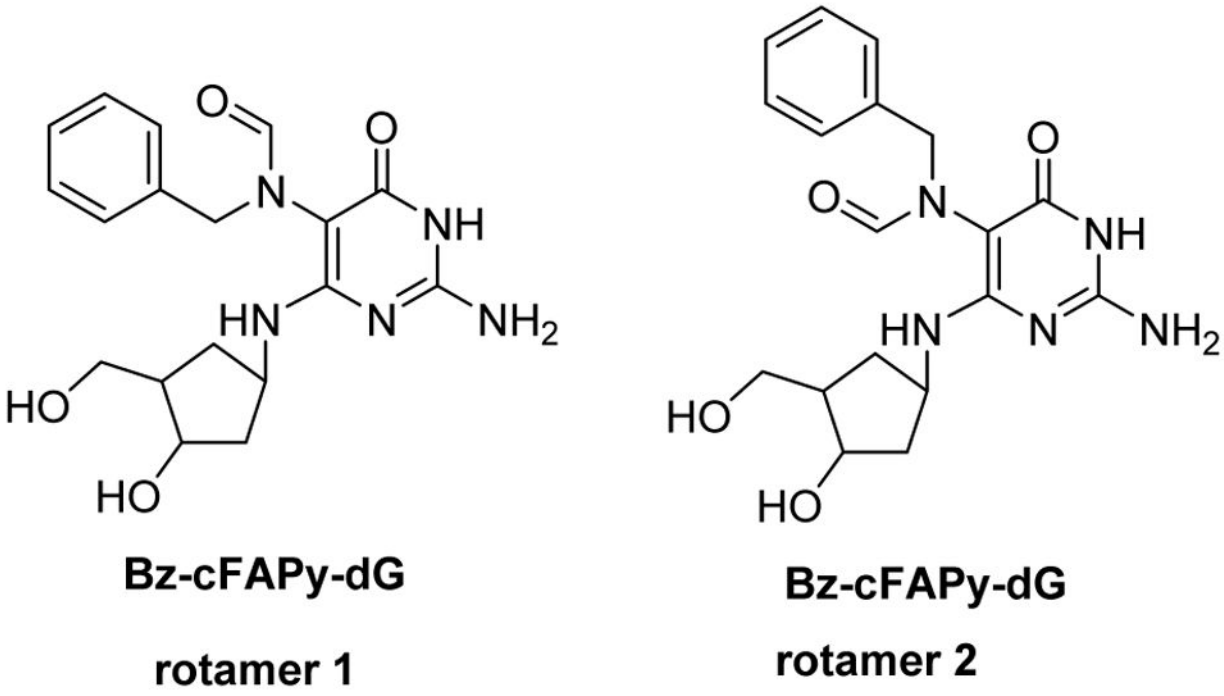
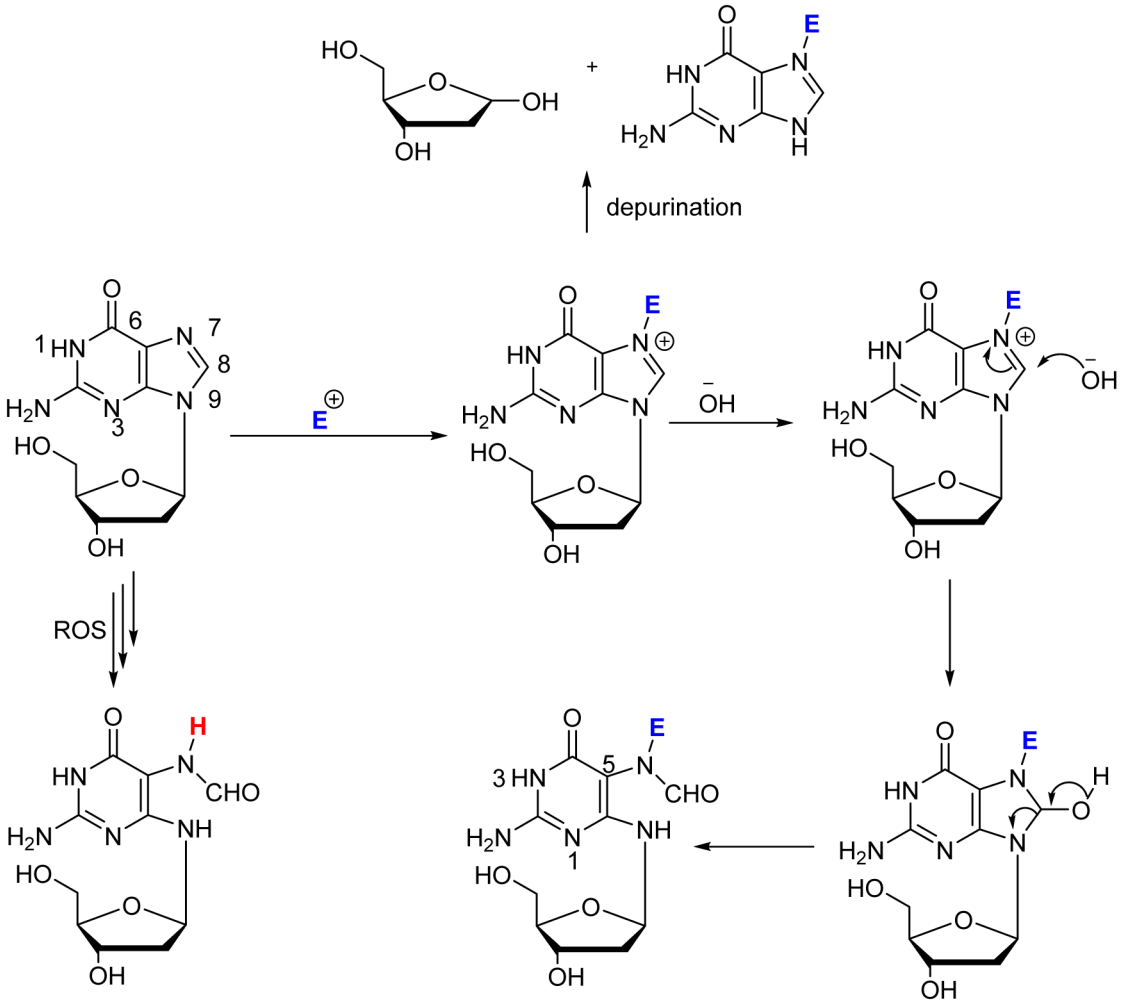
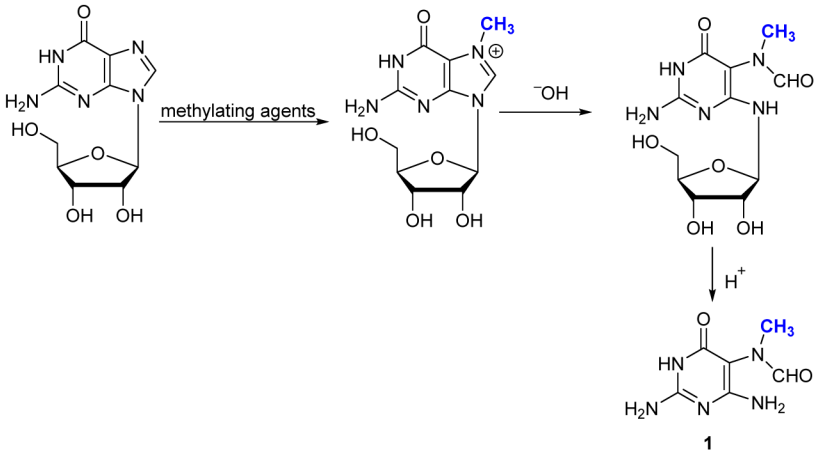


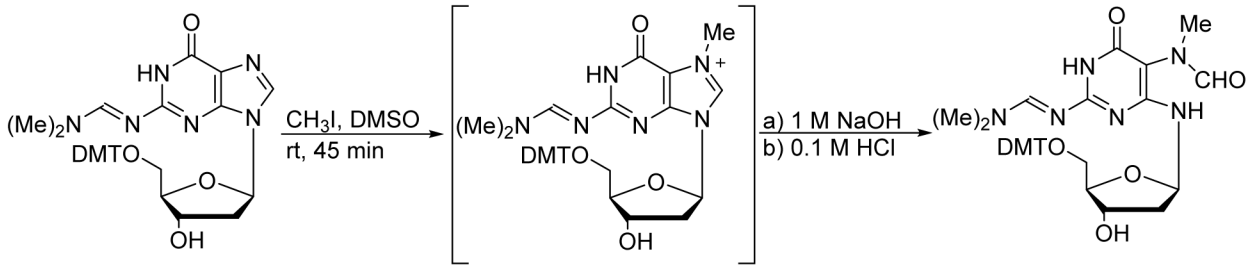
Figure 14.
Rotamers of Bz-cFAPy-dG.[107]



Scheme 1.
Mechanisms leading to the formation of FAPy adducts in DNA.

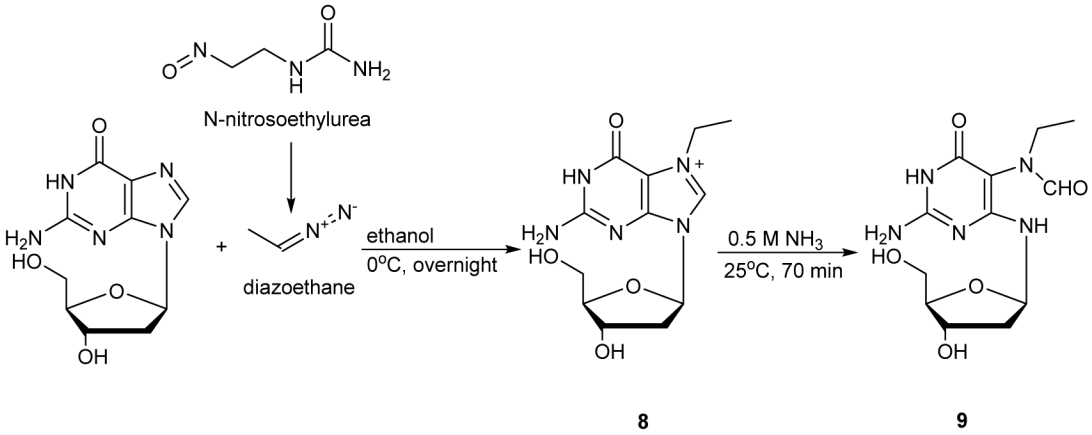


Scheme 2.
Formation of methyl-FAPy from dG.

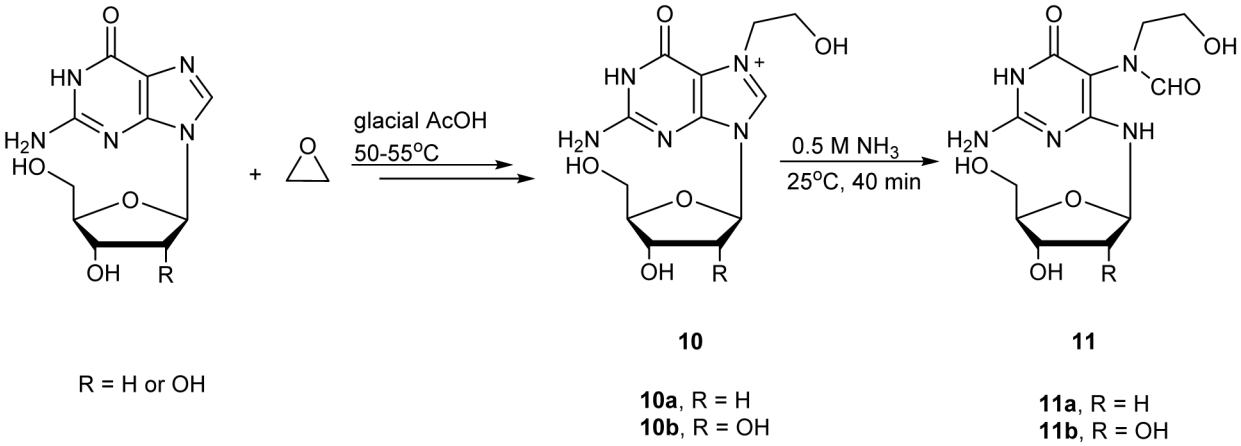


5 **6** **7**

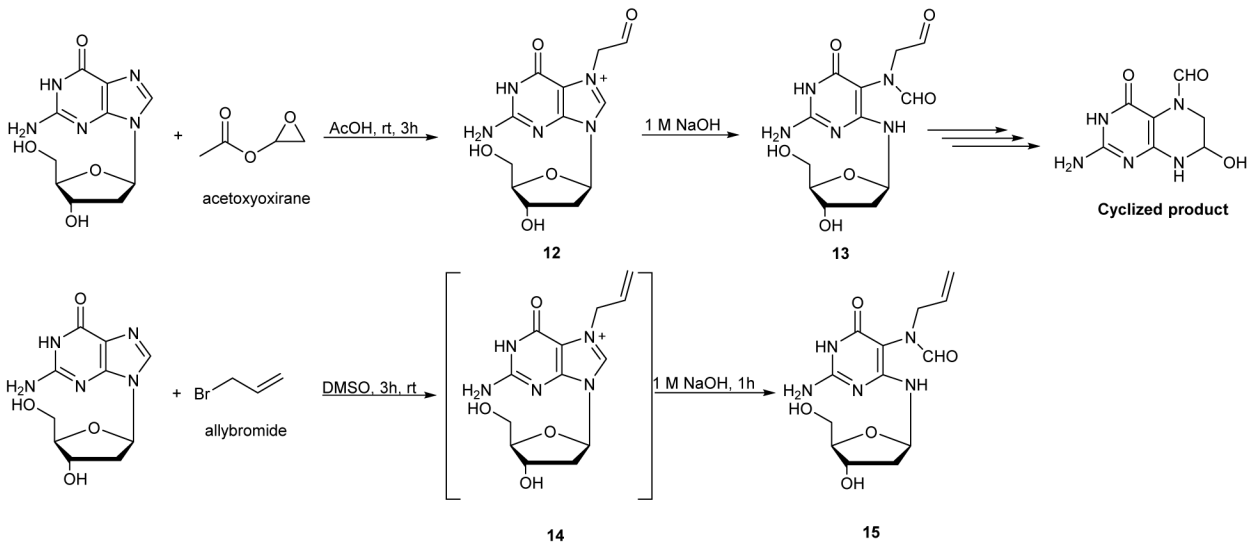
Scheme 3.
Synthesis of Me-FAPy-dG from N^2, 5′-OH -protected dG.[57]



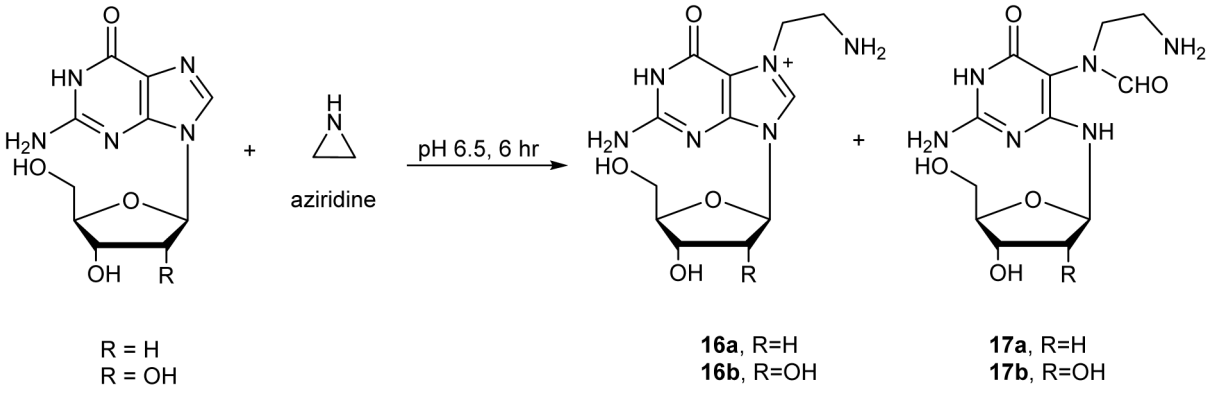
Scheme 4.
Synthetic scheme for Ethyl-FAPy-dG.[58]



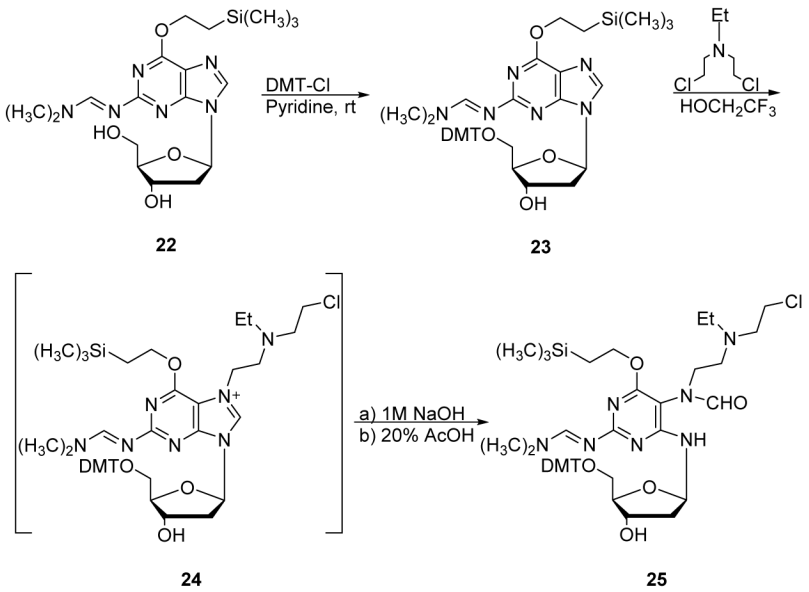
Scheme 5.
Synthesis of N7-(2-hydroxyethyl)-FAPy-dG.[63]



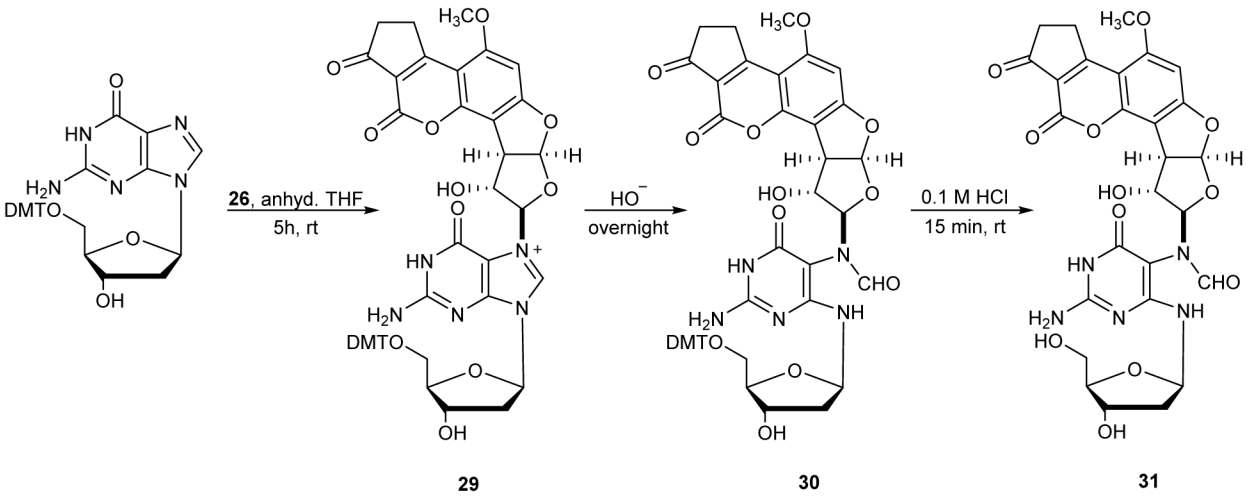
Scheme 6.
Synthesis of 2-oxyethyl-FAPy-dG.[69]



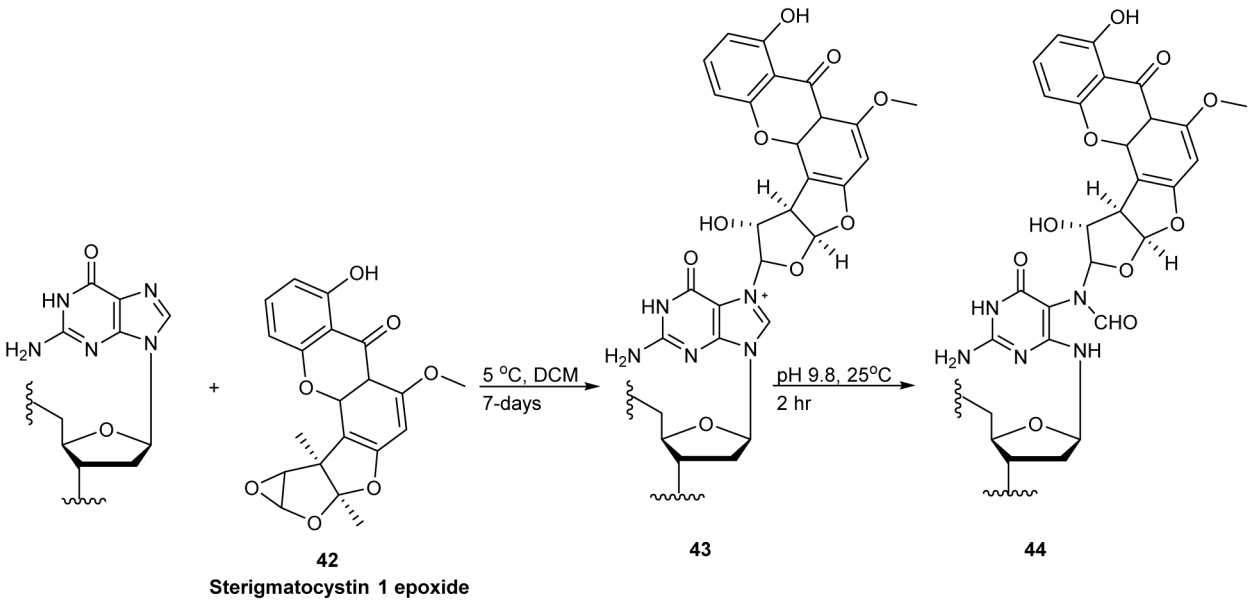
Scheme 7.
Synthesis of Ethylamine-FAPy-dG.[42]



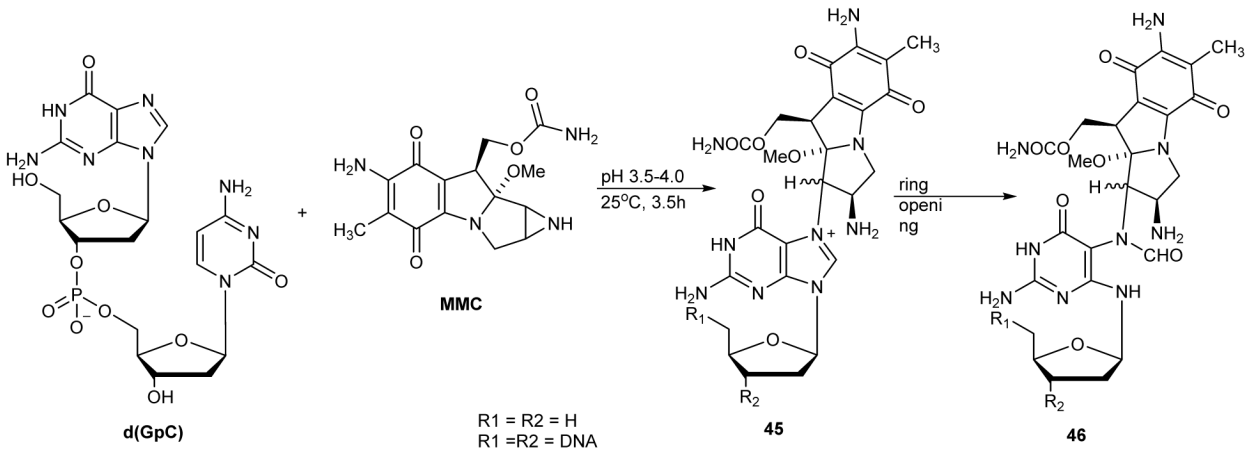
Scheme 8.
Synthesis of NM-FAPy-dG by Christov *et. al.*[80]



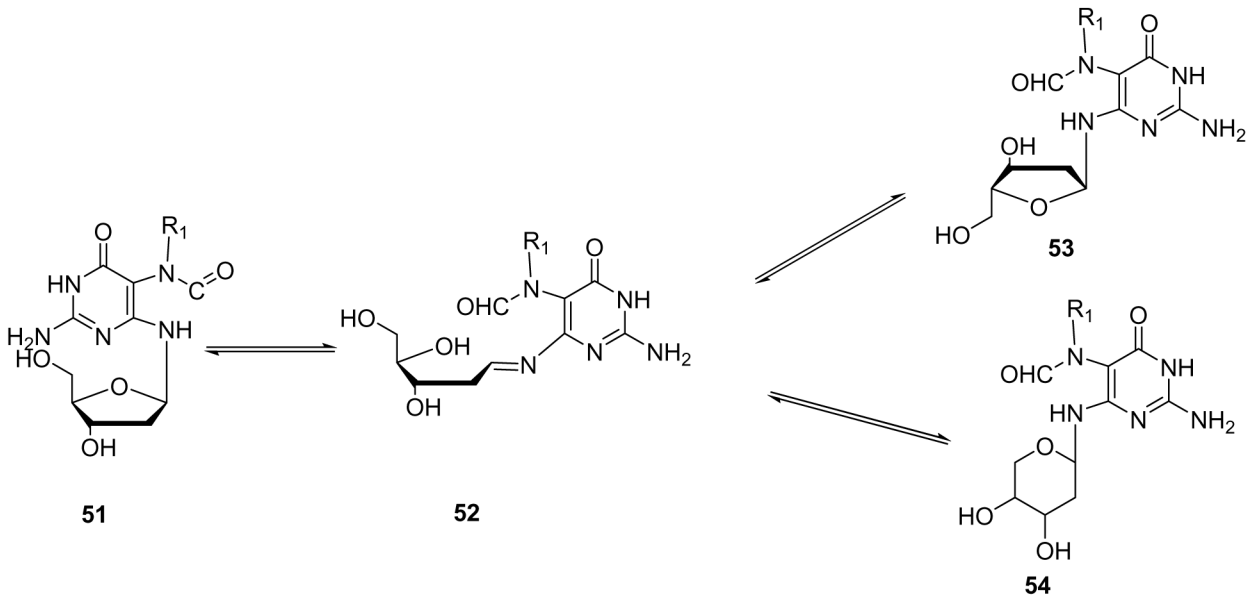
Scheme 9.
Synthesis of AFB-FAPy-dG.[82]



Scheme 10.
FAPy formation of sterigmatocystin 1 epoxide.[97]

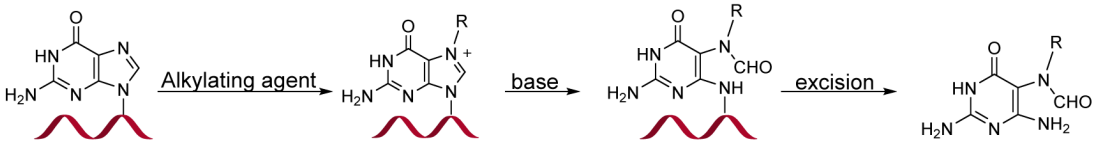


Scheme 11.
Synthesis of MMC-FAPy-dG.[41]



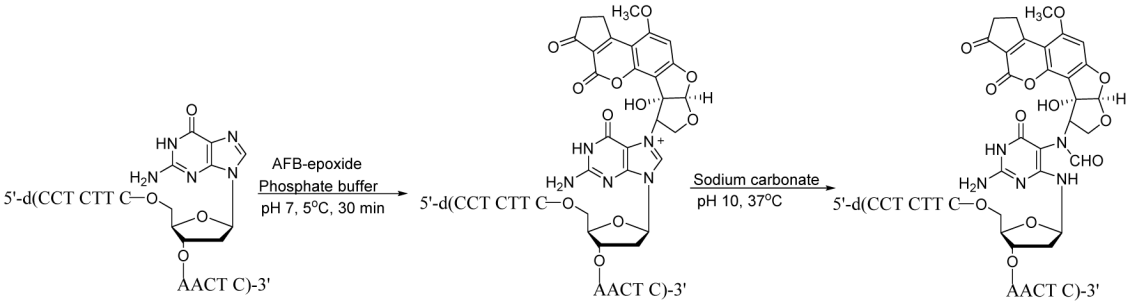
Scheme 12.

Isomerization of N5-R-FAPy adducts to α, β anomers **51, 53** and pyranose derivatives **54** via an imine intermediate.

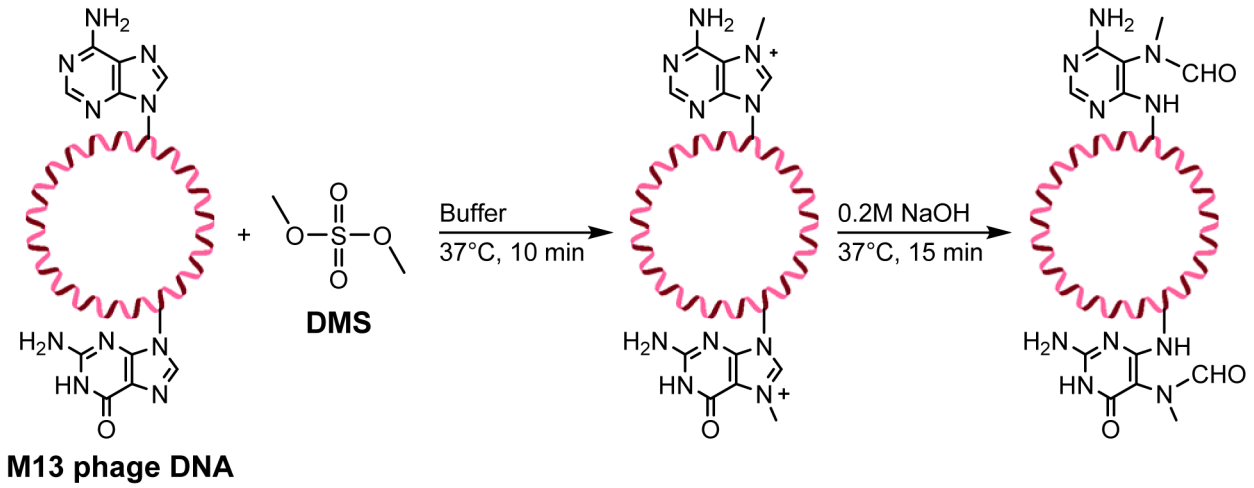


Scheme 13.

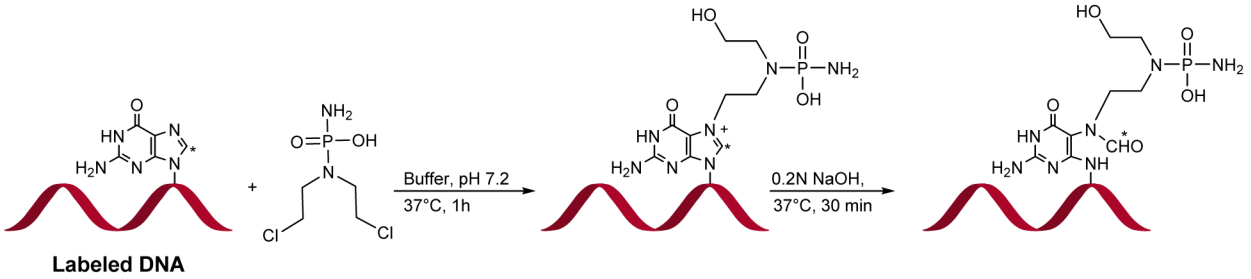
Direct treatment of DNA containing a single dG residue to introduce N^5-R-FAPy-residue.



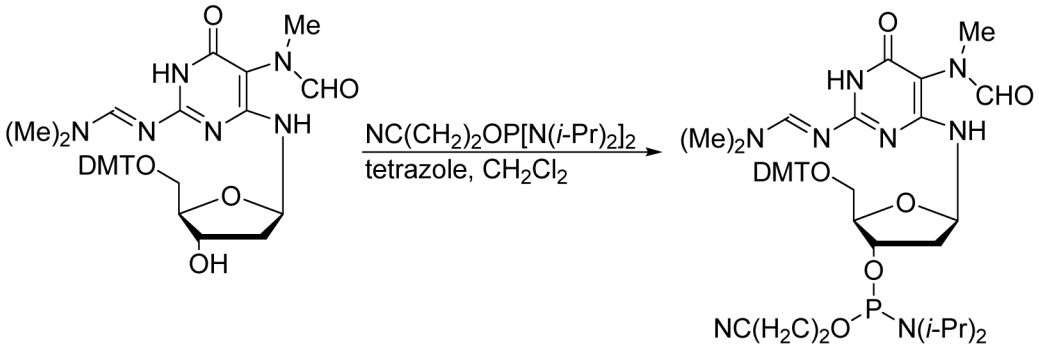
Scheme 14.
Synthesis of AFB-FAPy-dG oligonucleotide.[82]



Scheme 15.
Direct methylation of M13 phage DNA to form Me-FAPy adducts.[111]

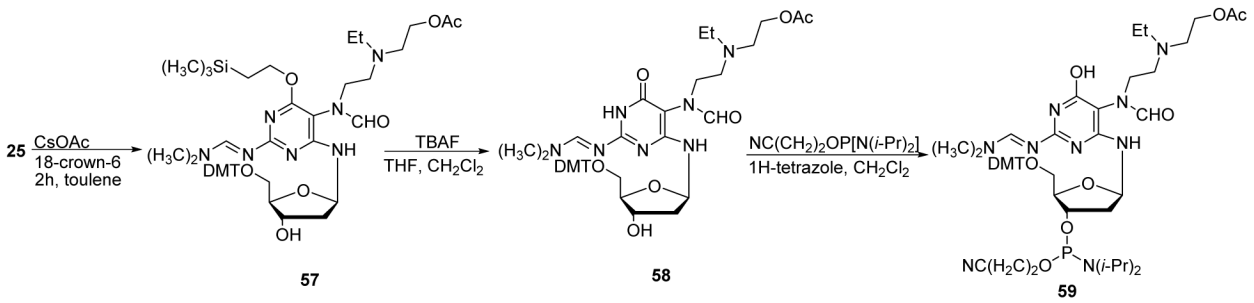


Scheme 16.
Preparation of DNA containing radilabaled phosphoramide mustard-FAPy adducts.[104]



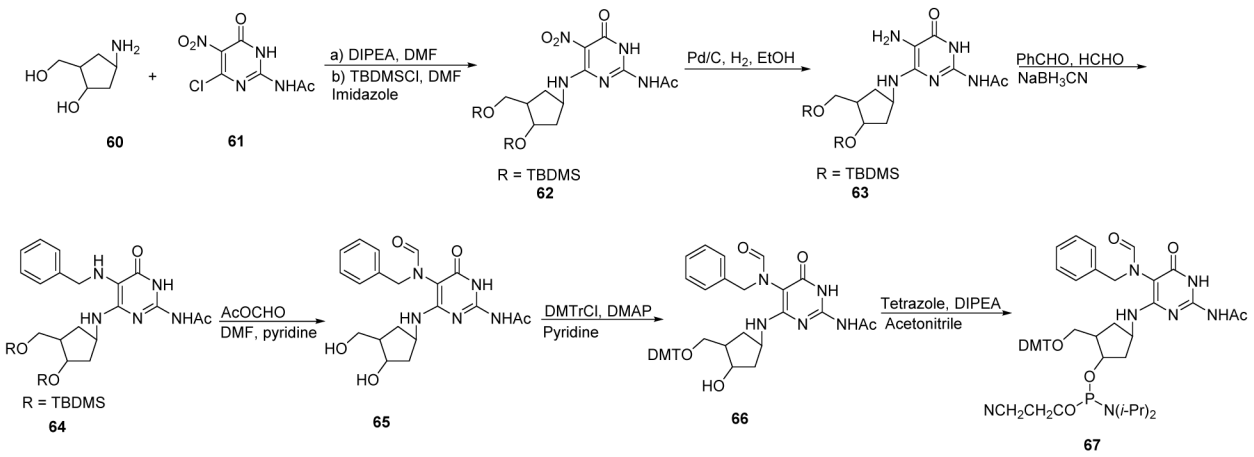
55 **56**

Scheme 17.
Synthesis of Me-FAPy-dG phosphoramidite.[57]



Scheme 18.

Preparation of NM-FAPy-dG phosphoramidite building block for solid phase synthesis.[80]



Scheme 19.

Synthesis of Bz-cFAPy-dG phosphoramidite.[105–107]