AFLATOXIN B_1 ADDUCTS WITH $d(ATCGAT)_2$ AND $d(ATGCAT)_2$: DO METHYLATION AND HYDROXYMETHYLATION INFLUENCE REACTION STOICHIOMETRY?

By

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CHAPTER I

INTRODUCTION

The relationship between fungi and humans has a long and complex history. While certain varieties of mushrooms and yeast have served as food sources for millennia, many fungi produce harmful secondary metabolites known as mycotoxins. The earliest accounts of mycotoxicosis involve the infamous syndrome "ergotism", caused by ergot alkaloids in crops contaminated with *Claviceps purpurea* mold[1]. While the ergot alkaloids and most other mycotoxins are not carcinogenic, several carcinogenic mycotoxins have recently been identified. Among these are aflatoxin, luteoskyrin[2], and sterigmatocystin[3].

Aflatoxin was discovered in the 1960's when the widespread hepatotoxicity observed in poultry in the United Kingdom was traced back to ground-nut meal contaminated with *Aspergillus flavus* mold[1]. Soon after this finding, *A. flavus* mold was implicated in an outbreak of liver cancer among hatchery-reared rainbow trout[4]. Subsequent laboratory experiments showed that rats fed the contaminated meal developed liver cancer, providing the first evidence for the existence of carcinogenic mycotoxins[1].

Hepatotoxicity has also been documented in humans following aflatoxin exposure. Consumption of 2-6 mg aflatoxin daily for a month or longer leads to acute aflatoxicosis, characterized by jaundice, portal hypertension, and ascites[5]. Far more common, however, is long-term exposure to lower levels of aflatoxin, which is implicated in the high rates of hepatocellular carcinoma seen in some developing countries[6]. Accordingly, aflatoxin has been classified as a Group 1 carcinogen in humans[7]. There is a synergistic relationship between aflatoxin exposure and hepatitis B infection, both of which are prevalent in many developing countries. The risk of liver cancer is doubled by chronic aflatoxin exposure and quintupled by hepatitis B infection. However, the combination of chronic aflatoxin exposure and hepatitis B infection leads to an alarming sixtyfold increase in the rate of liver cancer[8].

Four toxic compounds have been isolated from *A. flavus* (Figure 1). All share a common difuranocoumarin moiety. Named according to their chromatographic properties, aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) fluoresce blue, while aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) fluoresce green[1]. Of these, AFB_1 is of the greatest concern to human health. AFB_1 is not only highly genotoxic, but it is also the most commonly found aflatoxin in food.

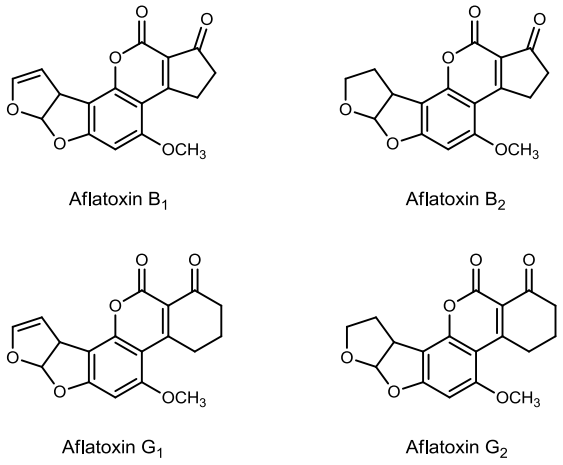


Figure 1. The structures of aflatoxins B_1, B_2, G_1, and G_2.

Early experiments demonstrated that the metabolism of AFB_1 is integral to its mutagenicity. While treatment of nucleic acid with AFB_1 results in a reversible nucleic acid-carcinogen complex[9], coincubation of monooxygenases with nucleic acid and AFB_1 leads to the covalent DNA adduct[1]. Incubation of *Salmonella typhimurium* with AFB_1 and a rat liver preparation leads to bacterial killing, but this killing effect could be blocked by the addition of exogenous nucleic acid or CYP450 inhibitors to the assay. The nucleic acids in this assay were found to be covalently bound to the AFB_1 metabolite[1].

The structure of AFB_1 led researchers to envision the 8,9-epoxide (Figure 2) as the ultimate carcinogen[10]. While the epoxide has never been directly observed in biological systems, likely due to its half-life of less than ten seconds in aqueous solution[11], its existence has been confirmed by indirect means. For example, acid hydrolysis of AFB_1-DNA adducts yields AFB_1-8,9-dihydrodiol, a product consistent with the proposed epoxide[12].

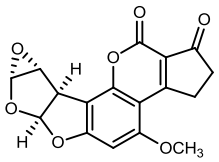


Figure 2. Structure of AFB_1-*exo*-8,9-epoxide.

Early attempts to prepare the epoxide *in vitro* were unsuccessful. Synthetic attempts have included oxidation by m-chloroperbenzoic acid[13], sensitized photooxidation[14], and solvolysis of 8-(acyloxy)-9-hydroxy AFB_1 derivatives[15]. These methods failed largely because of acidic or nucleophilic components of the reaction mixture causing cleavage of the epoxide[16]. The ultimate carcinogen, AFB_1-*exo*-8,9-epoxide, was finally prepared by DMDO oxidation[11] (Figure 3).

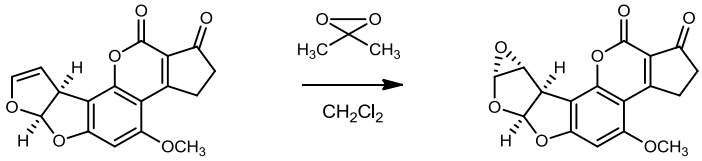


Figure 3. Preparation of AFB_1-*exo*-8,9-epoxide by DMDO oxidation of AFB_1 in dichloromethane.

AFB_1 epoxide is highly genotoxic. Over half of hepatocellular carcinoma cases resulting from aflatoxin exposure are associated with a G to T mutation at the third position of codon 249 in the p53 gene[17]. Similar G to T mutations in the first and second positions of codon 12 in the Ha-*ras* protooncogene are also likely to be involved in tumorogenicity[18]. Binding occurs preferentially at CpG islands[19]. Site-specific mutagenesis assays reveal AFB_1-induced G→T transversions at a level of 5% in E. coli[20]. The major adduct *in vitro* is *trans*-8,9-dihydro-(*N*7-guanyl)-9-hydroxy AFB_1 (Figure 3)[12]. DNA is thermodynamically stabilized by the adduct[21].

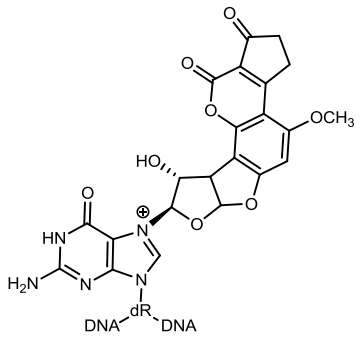


Figure 4. Structure of *trans*-8,9-dihydro-(*N*7-guanyl)-9-hydroxy AFB_1.

The AFB_1-*N*7-dG adduct may either depurinate to yield an abasic site, or hydrolyze to the formamidopyrimidine (FAPY) adduct[22]. The FAPY derivative results from opening of the imidazole ring of the initial cationic adduct[12]. While the β anomer is favored in duplex DNA, there is a 2:1 α:β mixture in single-stranded DNA[23]. The α anomer blocks replication and the β anomer is mutagenic[23]. In fact, the AFB_1-β-FAPY adduct causes G→T transversions at levels up to 36%, which is six times as mutagenic as the cationic adduct[24]. The overall reaction pathway for AFB_1 is summarized below (Figure 5).

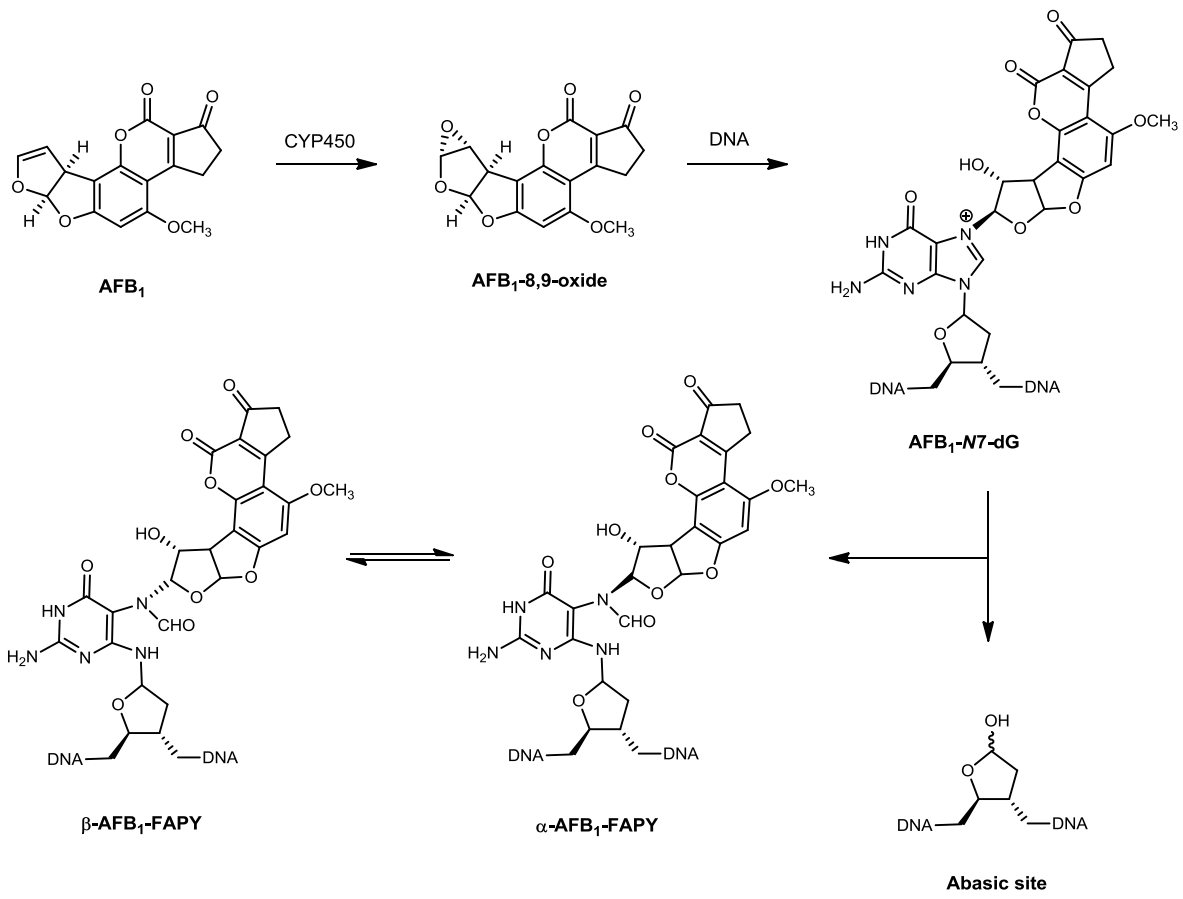


Figure 5. Summary of AFB_1 metabolism.

The stereospecificity of the reaction suggests that AFB_1 epoxide is held in a reaction-promoting orientation upon its association with DNA. An intercalated transition state with the epoxide positioned above the 5' face of dG would facilitate nucleophilic attack by *N*7[21]. The planar difuranocoumarin structure of AFB_1 alone is suggestive of intercalation. In agreement with this hypothesis, molecular modeling studies have demonstrated intercalation of AFB_1 in DNA[25]. Furthermore, binding of AFB_1 to DNA causes increased proton shielding[21]. Later studies confirmed intercalation of the mutagen above the 5' face of dG by NMR. Interestingly, the sterigmatocystin adduct adopts a similar intercalated structure[26].

Intercalation is responsible for the differential stoichiometry of the reactions of AFB_1 with certain oligonucleotide sequence isomers. Only one equivalent of epoxide can react with d(ATCGAT), while two equivalents of epoxide can react with d(ATGCAT). This difference in stoichiometry is due to intercalation of the epoxide above the 5' face of dG, which precludes binding of another AFB_1 equivalent to the complementary strand in d(ATCGAT) but leaves open a second binding site in d(ATGCAT)[21].

Due to the fact that AFB_1 epoxide binds preferentially at CpG islands, which are frequently methylated[27], it is important to know the chemistry of the reaction between AFB_1 epoxide and methylated DNA sequences. Therefore, the current work aims to describe the reaction between AFB_1 epoxide and the methylated sequence isomers d[AT(5-methyl-dC)GAT]$_2$ and d[ATG(5-methyl-dC)AT]$_2$, as well as the hydroxymethylated sequence isomers d[AT(5-hydroxymethyl-dC)GAT]$_2$ and d[ATG(5-hydroxymethyl-dC)AT]$_2$. Each sequence was reacted with a molar excess of AFB_1 epoxide. The limiting stoichiometry was determined for each sequence by high performance liquid chromatography (HPLC).

The reactions of AFB_1 with d[AT(5-methyl-dC)GAT]$_2$ and d[AT(5-hydroxymethyl-dC)GAT]$_2$ reached equilibrium after only half of the strands had reacted, as predicted by the 1:1 stoichiometry of the unmodified sequence. In contrast, the reactions of AFB_1 with d[ATG(5-methyl-dC)AT]$_2$ and d[ATG(5-hydroxymethyl-dC)AT]$_2$ reached equilibrium after all of the strands had reacted, as predicted by the 2:1 stoichiometry of the unmodified sequence. These findings suggest that methylation and hydroxymethylation do not influence the equilibrium conditions for the reaction between AFB_1 epoxide and these sequence isomers.

CHAPTER II

METHODS

Synthesis of Dimethyldioxirane (DMDO)

DMDO was prepared by fractional distillation as a 0.025 M solution in acetone[28]. A fractional distillation apparatus was assembled and equipped with separate attachments for adding solid and liquid. $NaHCO_3$ (16 g, 0.20 mol) was added to a 250 mL distilling flask. $KHSO_5$ (30 g, 0.20 mol) was added to the solid addition attachment and 50 mL of a 1:1 mixture of water and acetone was added to the liquid addition attachment. The entire apparatus was placed under a nitrogen atmosphere and cooled to -78°C in a dry ice/acetone bath. The $KHSO_5$ was added slowly over a period of 15 minutes, then the reaction was allowed to proceed for another 20 minutes. The liquid in the receiving flask was removed and stored over anhydrous $MgSO_4$ at -4°C. Formation of DMDO was confirmed by NMR spectroscopy by placing ten µL of the solution combined with 600 µL of dry deuterated chloroform in a 5mm NMR tube. A ^{1}H NMR spectrum was obtained on a 500 MHz Bruker NMR spectrometer. The concentration of DMDO was determined by comparing the height of the methyl peak to the height of the ^{13}C satellite peak of acetone.

Synthesis of Aflatoxin B_1 Epoxide (AFB_1)

Aflatoxin B_1 is extremely hazardous due to its carcinogenicity and should be handled using appropriate safety procedures. All reactions were conducted in a well-ventilated hood using disposable latex gloves. Afterwards, the work area was bleached with NaOCl solution.

DMDO (10.5 µmol) was added to AFB_1 (10 mg, 7.0 µmol) dissolved in 0.5 mL dichloromethane. After 20 minutes, excess DMDO solution was removed by evaporation under a stream of nitrogen gas. One mg of the product was dissolved in 600 µL of dry deuterated chloroform and added to a 5mm NMR tube. A ^1H NMR spectrum was obtained on a 500 MHz Bruker NMR spectrometer. The remaining epoxide was stored at -4°C.

Preparation of AFB_1 DNA Adducts

The oligonucleotides 5'-ATCGAT-3', 5'-ATGCAT-3', 5'-AT(5-methyl-dC)GAT-3', 5'-ATG(5-methyl-dC)AT-3', 5'-AT(5-hydroxymethyl-dC)GAT-3', and 5'-ATG(5-hydroxymethyl-dC)AT-3' were synthesized by Midland Certified Reagent Company. The purity of the samples was confirmed using HPLC and the mass of the oligonucleotides was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

AFB_1 epoxide (1 mg, 3 μmol) was dissolved in 0.2 mL dichloromethane and added to the oligonucleotide (0.36 μmol) dissolved in 0.2 mL buffer (10 mM sodium phosphate dibasic, 100 mM sodium chloride, 10 mM magnesium chloride, 0.05 mM ethylenediaminetetraacetic acid). The mixture was stirred at 5°C for 30 min. The aqueous layer was collected and analyzed by HPLC.

High-Performance Liquid Chromatography

The sample was dissolved in water and a glass syringe was used to inject 200 nmol of the sample onto a 5 micron Phenomenex 250 x 10 mm column attached to a Beckman Coulter HPLC. All samples were run in acetonitrile and 0.1 M ammonium formate buffer, adjusted to pH 7.2 by titration with 5 M NaOH. The HPLC gradient was as follows: acetonitrile remained at 5% from 0 to 5 min, slowly raised to 16% from 5 to 60 min, slowly dropped back down to 5% from 60 to 65 min, then kept constant at 5% from 65 to 70 min. Absorbance was read at 254 nm and 360 nm. The area under the peaks was used to determine the ratio of unreacted oligonucleotide (absorbs at 254 nm) to the adduct (absorbs at both 254 nm and 360 nm).

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Ten μL of 3-hydroxypicolinic acid (0.5 M in 50/50 acetonitrile/water), 5 μL of ammonium citrate (0.1 M in 50/50 acetonitrile/water), and 3 nmol of DNA in 1 μL water were added to a vial. One μL of the mixture was added to a 96 well MALDI plate and allowed to dry. The MALDI spectrum was obtained in negative ion mode on a Voyager-DE STR MALDI mass spectrometer.

CHAPTER III

RESULTS

Dimethyldioxirane

The ^{1}H NMR spectrum of DMDO showed a diagnostic methyl peak at 1.65 ppm (Figure 6). The concentration of DMDO was calculated by comparing the height of the methyl peak to the height of the ^{13}C satellite peak of acetone at 1.95 ppm.

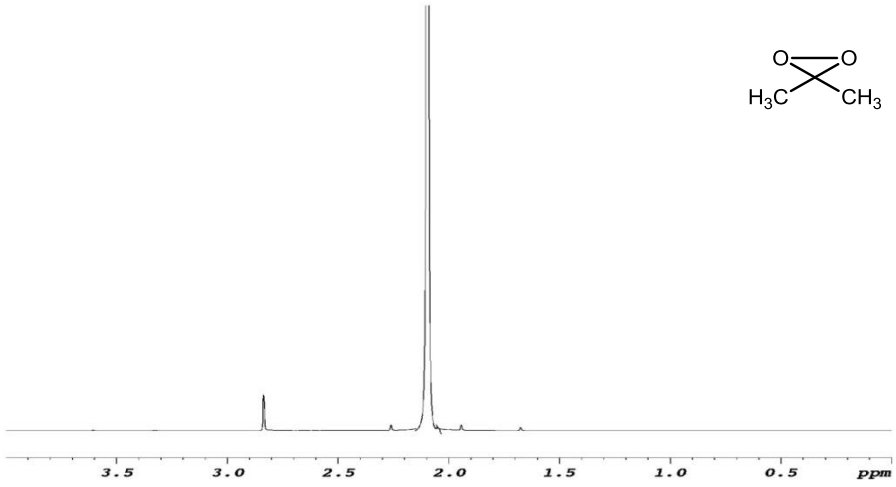


Figure 6. ^{1}H NMR spectrum of DMDO.

AFB_1 Epoxide

The ^{1}H NMR spectrum of aflatoxin B_1 epoxide showed diagnostic epoxide peaks at 3.5 and 5.4 ppm (Figure 7).

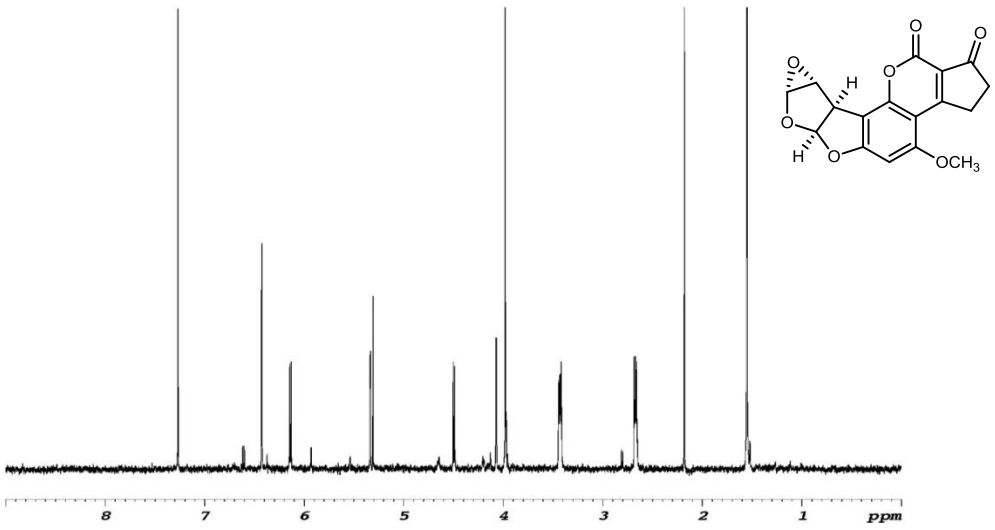


Figure 7. ^{1}H NMR spectrum of aflatoxin B_1 epoxide.

ATCGAT-AFB$_1$

ATCGAT was analyzed before and after reaction with AFB_1 epoxide (Figure 8). Pure ATCGAT gave a single large peak at 34 min. After the reaction, two peaks were observed at 34 min (35% intensity) and 53 min (37% intensity). This finding indicates a 1:1 AFB_1:d$(ATCGAT)_2$ limiting stoichiometry in the formation of the d$(ATC^{AFB}GAT)$·d(ATCGAT) adduct.

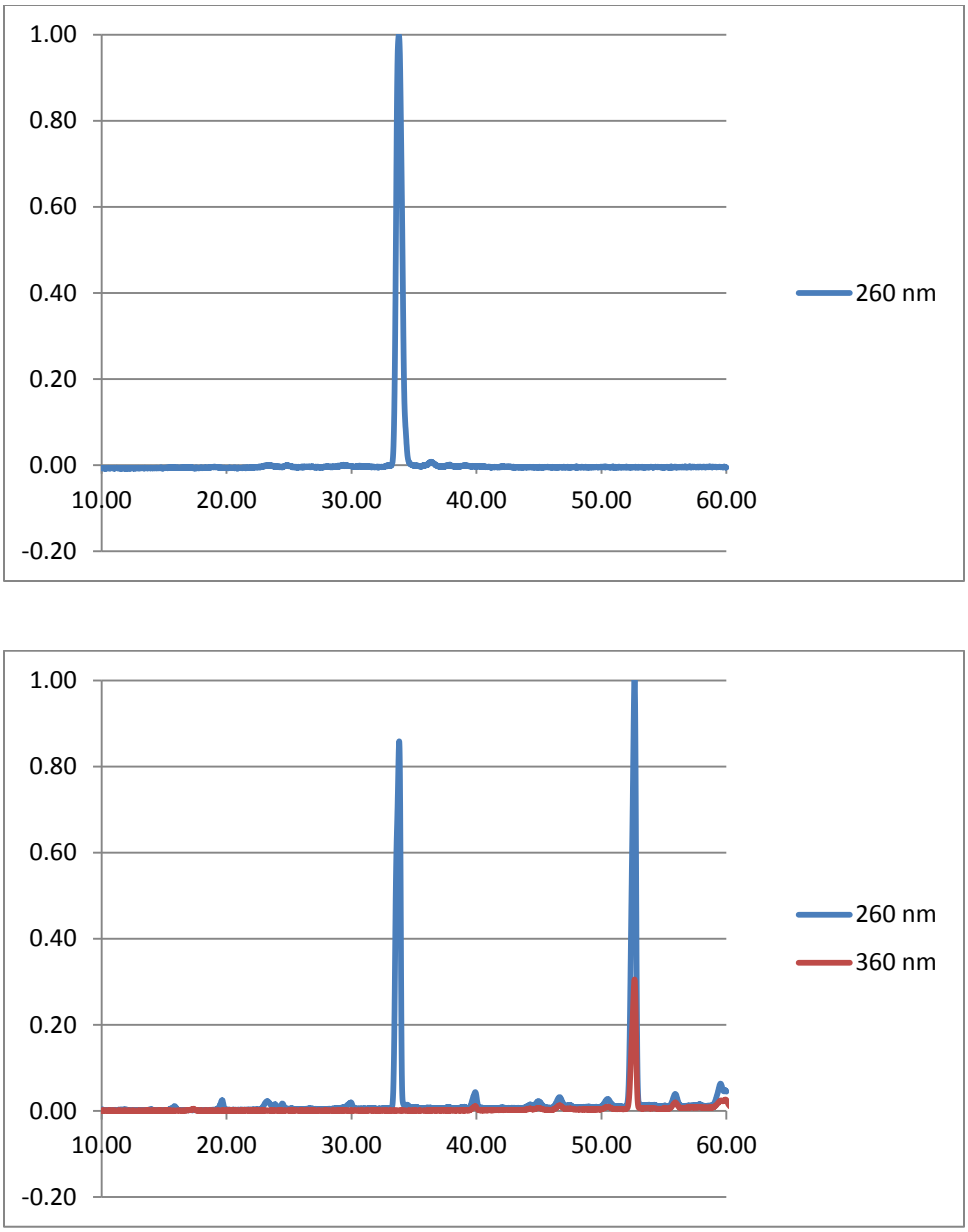
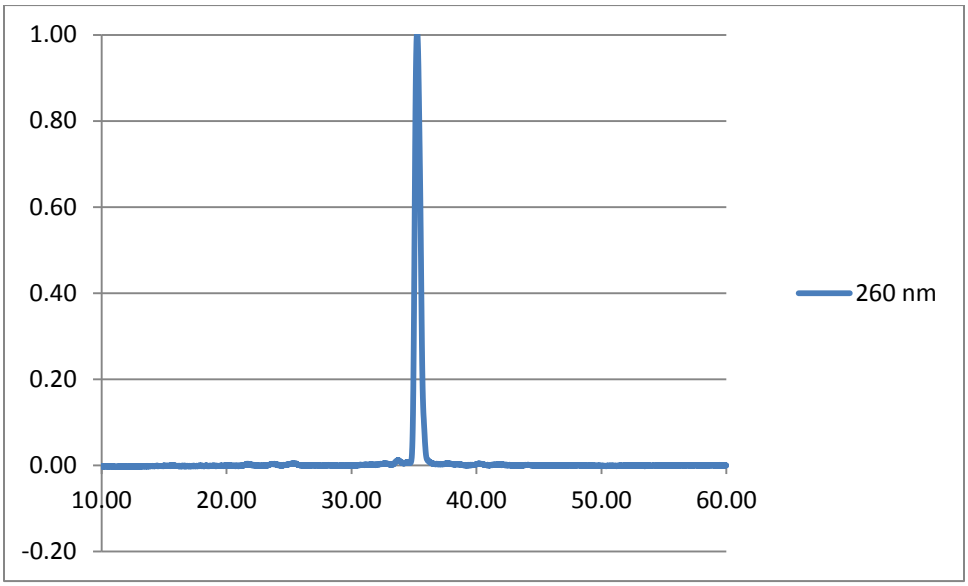


Figure 8. ATCGAT before (above) and after (below) reaction with AFB_1 epoxide.

ATGCAT-AFB$_1$

ATGCAT was analyzed before and after reaction with AFB_1 epoxide (Figure 9). Pure ATGCAT gave a single large peak at 35 min. After the reaction, two peaks were observed at 35 min (7% intensity) and 54 min (69% intensity). This finding indicates a 2:1 AFB_1:d$(ATGCAT)_2$ limiting stoichiometry in the formation of the d$(AT^{AFB}GCAT)_2$ adduct.



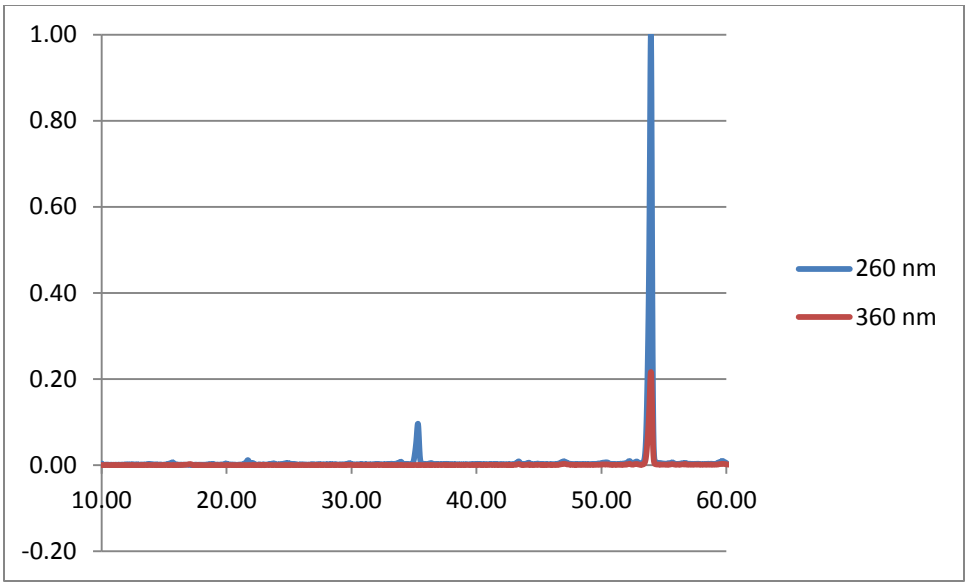


Figure 9. ATGCAT before (above) and after (below) reaction with AFB_1 epoxide.

AT(5-methyl-dC)GAT-AFB_1

AT(5-methyl-dC)GAT was analyzed before and after reaction with AFB_1 epoxide (Figure 10). Pure AT(5-methyl-dC)GAT gave a single peak at 36 min. After the reaction, two peaks were observed at 35 min (23% intensity) and 54 min (20% intensity). This finding indicates a 1:1 AFB_1:d[AT(5-methyl-dC)GAT]$_2$ limiting stoichiometry in the formation of the d[AT(5-methyl-dC)^{AFB}GAT)·d[AT(5-methyl-dC)GAT] adduct.

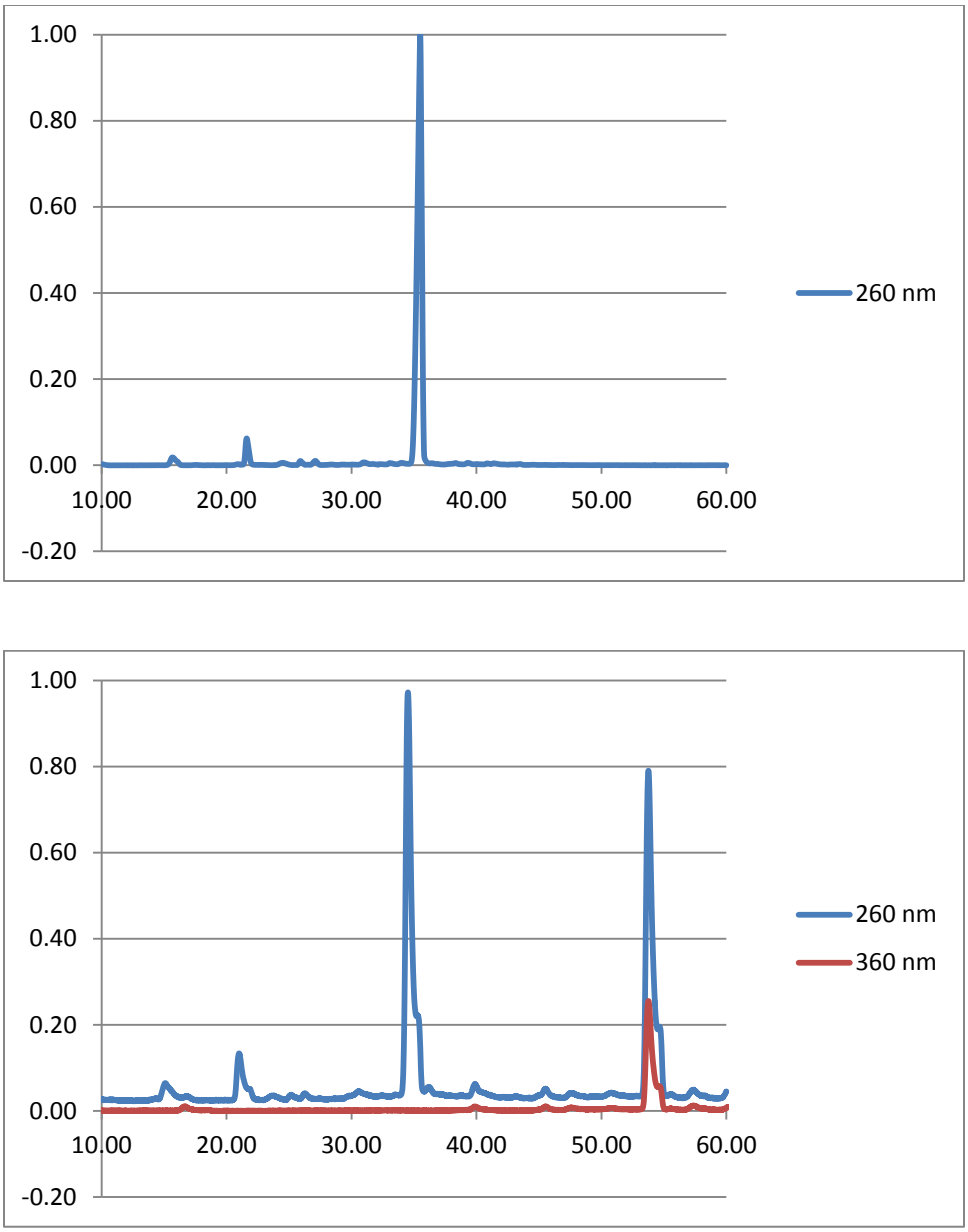
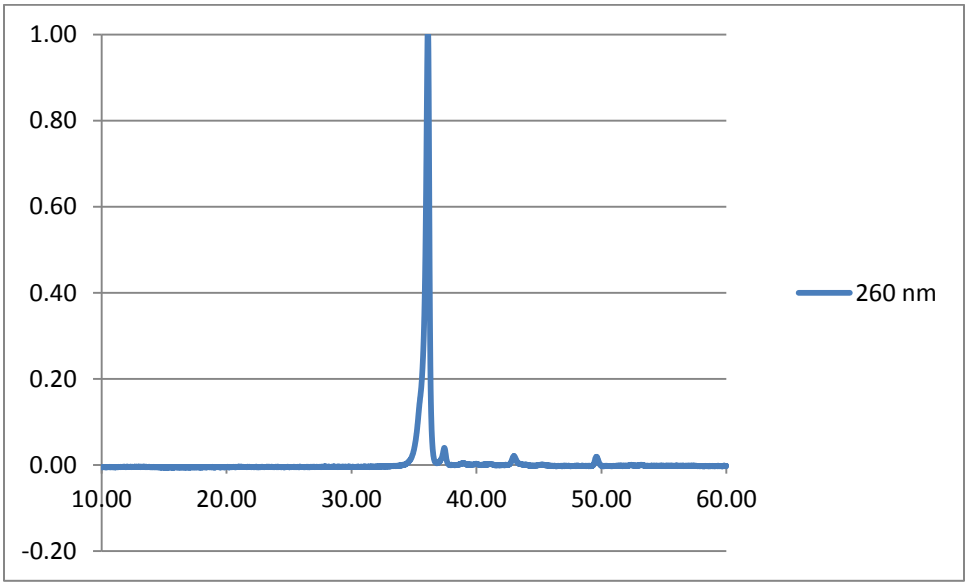


Figure 10. AT(5-methyl-dC)GAT before (above) and after (below) reaction with AFB_1 epoxide.

ATG(5-methyl-dC)AT-AFB$_1$

ATG(5-methyl-dC)AT was analyzed before and after reaction with AFB$_1$ epoxide (Figure 11). Pure ATG(5-methyl-dC)AT gave a single peak at 36 min. After the reaction, two peaks were observed at 35 min (2% intensity) and 53 min (58% intensity). This finding indicates a 2:1 AFB$_1$:d[ATG(5-methyl-dC)AT]$_2$ limiting stoichiometry in the formation of the d[ATG(5-methyl-dC)^{AFB}AT]$_2$ adduct.



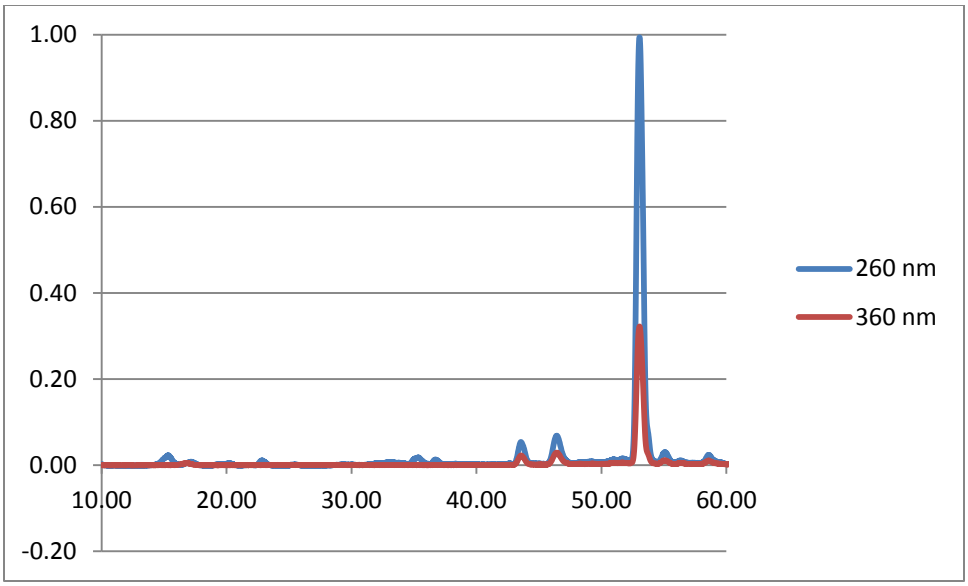


Figure 11. ATG(5-methyl-dC)AT before (above) and after (below) reaction with AFB_1 epoxide.

AT(5-hydroxymethyl-dC)GAT-AFB_1

AT(5-hydroxymethyl-dC)GAT was analyzed before and after reaction with AFB_1 epoxide (Figure 12). Pure AT(5-hydroxymethyl-dC)GAT gave a single peak at 34 min. After the reaction, two peaks were observed at 34 min (22% intensity) and 52 min (19% intensity). This finding indicates a 1:1 AFB_1:d[AT(5-methyl-dC)GAT]$_2$ limiting stoichiometry in the formation of the d[AT(5-hydroxymethyl-dC)^{AFB}GAT)·d[AT(5-hydroxymethyl-dC)GAT] adduct. However, there were several peaks in the HPLC trace that have not been accounted for.

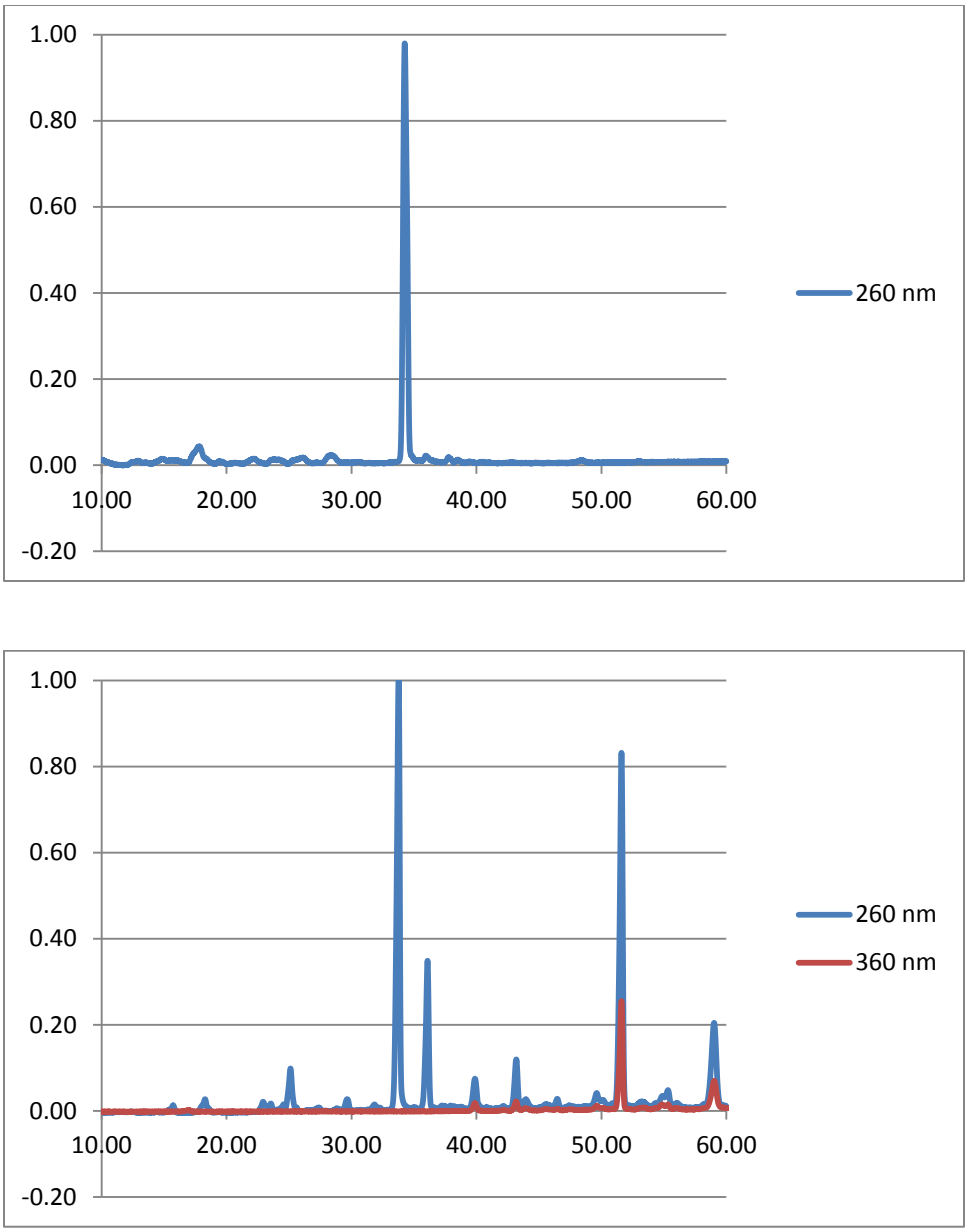
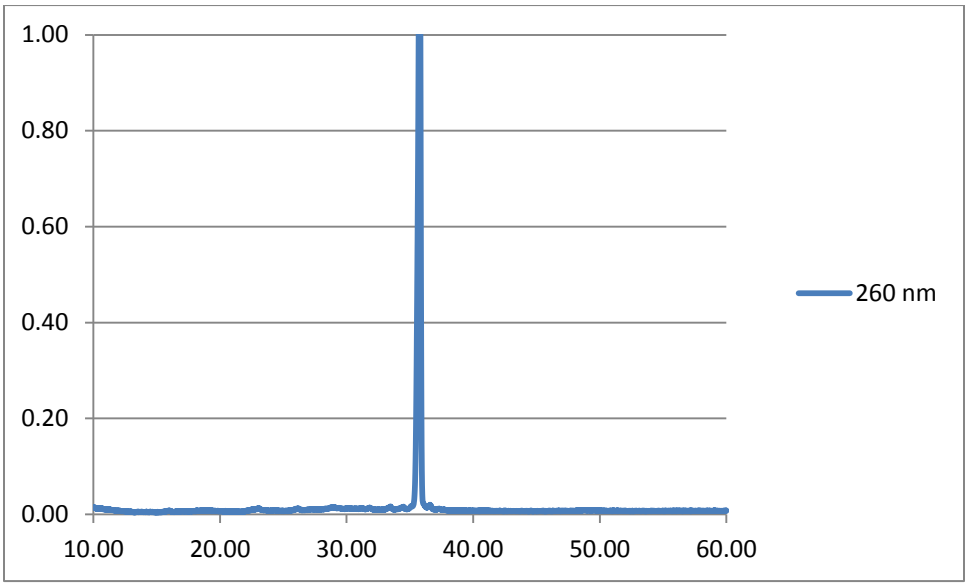


Figure 12. AT(5-hydroxymethyl-dC)GAT before (above) and after (below) reaction with AFB_1 epoxide.

ATG(5-hydroxymethyl-dC)AT-AFB$_1$

ATG(5-hydroxymethyl-dC)AT was analyzed before and after reaction with AFB$_1$ epoxide (Figure 12). Pure ATG(5-hydroxymethyl-dC)AT gave a single peak at 36 min. After the reaction, two peaks were observed at 36 min (2% intensity) and 55 min (56% intensity). This finding indicates a 2:1 AFB$_1$:d[ATG(5-hydroxymethyl-dC)AT]$_2$ limiting stoichiometry in the formation of the d[ATG(5-hydroxymethyl-dC)^{AFB}AT]$_2$ adduct.



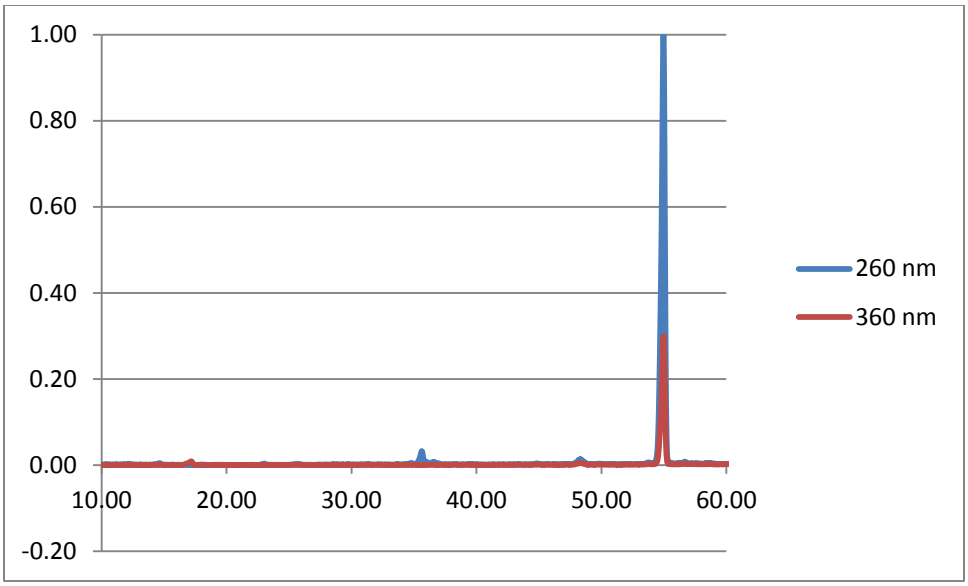


Figure 13. ATG(5-hydroxymethyl-dC)AT before (above) and after (below) reaction with AFB_1 epoxide.

CHAPTER IV

DISCUSSION

The sequence isomers ATCGAT and ATGCAT were selected due to their utility in previous studies of AFB_1 epoxide reactivity[21]. Since the sequence is self-complementary, the task of synthesizing and annealing two different but complementary strands was avoided. Furthermore, palindromic sequences are not susceptible to regioselectivity issues that could arise if the two guanines in the duplex were not identical. The placement of cytosine and guanine beside one another was driven by the finding that 5'-CpG-3' is the preferred binding site for the epoxide[19].

In agreement with previous work by Gopalakrishnan et al.[29], the limiting stoichiometry for the reaction between AFB_1 epoxide and the oligonucleotide duplex was found to be 1:1 for ATCGAT and 2:1 for ATGCAT. The epoxide intercalates above the 5' face of dG, preventing another equivalent of epoxide from binding to the complementary strand of ATCGAT, but not ATGCAT. Interestingly, methylation and hydroxymethylation do not cause the reaction to deviate from this limiting stoichiometry.

While the results approximated the predicted stoichiometry, they did not go to completion. One possible explanation is the formation of hairpin loops, which is not uncommon for palindromic sequences such as the ones utilized in this study. The reaction could be optimized by adjusting variables such as pH and salt concentration in order to minimize the formation of hairpin loops. In this reaction, DNA and water compete for reaction with AFB_1 epoxide. Even though the reaction with DNA occurs rapidly, it is known that the epoxide is hydrolyzed to AFB_1-8,9-dihydrodiol in aqueous solution. It is possible that a small portion of the epoxide used in the reaction is hydrolyzed to the diol, which could intercalate the oligonucleotide and effectively block the epoxide binding site. This problem could be addressed by extracting the reaction mixture in dichloromethane to remove hydrolyzed epoxide and adding another excess of fresh epoxide. The protocol could be further optimized by taking aliquots at different times during the reaction to identify the exact point at which equilibrium is reached.

While the current work addresses the stoichiometry of the reaction, it does not answer the important question of the relative stability of the adducts. Previous studies have shown that AFB_1 has a stabilizing effect on DNA[21]. However, the magnitude of this effect is unknown for the sequences in this study. Future work could utilize melting experiments to determine the effect of AFB_1 on the thermodynamic stability of the oligonucleotides.

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