VOLUME 116, NUMBER 20
OCTOBER 5, 1994

JOURNAL
OF THE
AMERICAN
CHEMICAL
SOCIETY

Adenine Adduct of Aflatoxin B_1 Epoxide

Rajkumar S. Iyer, Markus W. Voehler, and Thomas M. Harris*

Contribution from the Department of Chemistry and Center in Molecular Toxicology, Vanderbilt University, Nashville, Tennessee 37235

Received May 31, 1994®

Abstract: The reaction of aflatoxin B_1 *exo*-8,9-epoxide with calf thymus DNA gives small quantities of the previously unknown adenine N7 adduct in addition to the well-characterized deoxyguanosine N7 derivative. The adenine derivative can also be prepared by the reaction of epoxide with an oligodeoxynucleotide and with poly(dAdT). The deoxyadenosine adduct is more labile than the deoxyguanosine adduct, undergoing loss of deoxyribose at ambient temperature. The adenine adduct was independently synthesized by reaction of the epoxide with the 3′,5′-bis(*O*-tert-butyldimethylsilyl) derivative of 2′-deoxyadenosine. The linkage to adenine was shown to be at N7 by NMR spectroscopy employing C–H heteronuclear correlations, $^3J_{C-H}$ coupling constants, and an NOE between aflatoxin H9 and adenine H8. NMR spectroscopic studies were also carried out with 1-, 3-, 7-, and 9-methyladenines to establish guidelines for making assignments on the aflatoxin adduct. Aflatoxin adduction carried out in D_2O gave deuterium substitution at the adenine 8 position in the product.

Introduction

Aflatoxins are potently carcinogenic metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, fungi that frequently infest peanuts, corn, and other seed crops.[1] Health hazards of the aflatoxins are of world-wide concern, and even minute traces of these compounds in agricultural products are cause for alarm. Their genotoxic effects are ascribed primarily to aflatoxin B_1 (AFB_1, **1**, Scheme 1), a key structural feature of which is the furofuran ring system. AFB_1 undergoes enzymatic epoxidation to form AFB *exo*-epoxide **2**[2] plus small amounts of *endo*-epoxide **3**.[3] The *exo*-epoxide reacts avidly with DNA to give high adduction yields, but the *endo*-epoxide fails to react.[4]

Epoxide **2** has long eluded isolation from and direct spectroscopic detection in natural systems, but its existence was deduced from the structure of guanine adduct **5** obtained from DNA by a thermal depurination of deoxyguanosine adduct **4** (see Scheme 2.)[5] For many years the synthesis of epoxide **2** was also elusive due not to low reactivity of the dihydrofuran double bond, which is in fact electron-rich, but to the unusually high reactivity of the epoxide.[6] Speculation that **2** was too unstable to ever be isolated[6c] was laid to rest in 1988, when this laboratory reported a synthesis using dimethyldioxirane as the oxidant.[4a] The epoxide proved to

* Abstract published in *Advance ACS Abstracts*, September 1, 1994.

(1) For a review, see: Busby, W. F., Jr.; Wogan, G. N. In *Chemical Carcinogens*, 2nd ed.; Searle, C., Ed.; American Chemical Society; Washington, DC, 1984; pp 945–1136.

(2) Garner, R. C.; Miller, E. C.; Miller, J. A.; Hansen, R. S. *Biochem. Biophys. Res. Commun.* **1971**, *45*, 774–780.

(3) (a) Baertschi, S. W.; Raney, K.; Shimada, T.; Harris, T. M.; Guengerich, F. P. *Chem. Res. Toxicol.* **1989**, *2*, 114–122. (b) Raney, K. D.; Coles, B.; Guengerich, F. P.; Harris, T. M. *Chem. Res. Toxicol.* **1992**, *5*, 333–335.

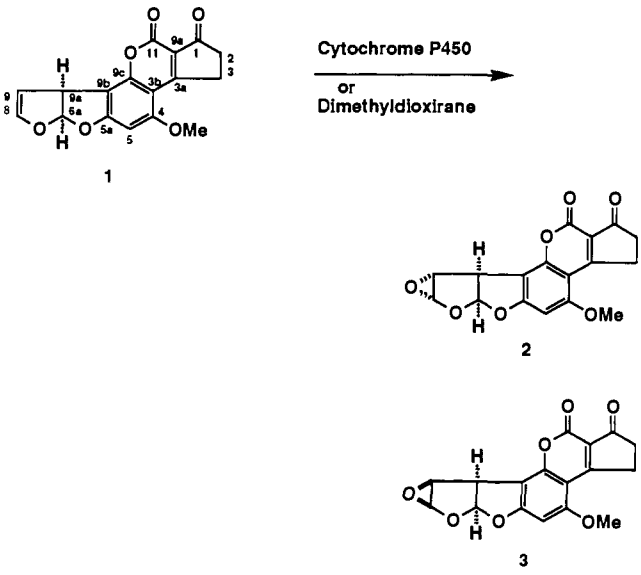
(4) (a) Baertschi, S. W.; Raney, K. D.; Stone, M. P.; Harris, T. M. *J. Am. Chem. Soc.* **1988**, *110*, 7929–7931. (b) Iyer, R. S.; Coles, B. F.; Raney, K. D.; Thier, R.; Guengerich, F. P.; Harris, T. M. *J. Am. Chem. Soc.* **1994**, *116*, 1603–1609.

(5) (a) Essigmann, J. M.; Croy, R. G.; Nadzan, A. M.; Busby, W. F., Jr.; Reinhold, V. N.; Büchi, G.; Wogan, G. N. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1870–1874. (b) Lin, J. K.; Miller, J. A.; Miller, E. C. *Cancer Res.* **1977**, *37*, 4430–4438. (c) Croy, R. G.; Essigmann, J. M.; Reinhold, V. M.; Wogan, G. N. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 1745–1749. (d) Martin, C. N.; Garner, R. C. *Nature (London)* **1977**, *267*, 863–865. (e) Büchi, G.; Fowler, K. W.; Nadzan, A. M. *J. Am. Chem. Soc.* **1982**, *104*, 544–547. (f) Coles, B. F.; Welch, A. M.; Hertzog, P. J.; Lindsay Smith, J. R.; Garner, R. C. *Carcinogenesis* **1980**, *1*, 79–90. (g) Swenson, D. H.; Miller, J. A.; Miller, E. C. *Biochem. Biophys. Res. Commun.* **1973**, *53*, 1260–1267.

(6) (a) Garner, R. C.; Martin, C. N.; Lindsay Smith, J. R.; Coles, B. F.; Tolson, M. R. *Chem.-Biol. Interact.* **1979**, *26*, 57–73. (b) Coles, B. F.; Lindsay Smith, J. R.; Garner, R. C. *J. Chem. Soc., Perkin Trans. 1* **1979**, 2664–2671. (c) Gorst-Allman, C. P.; Steyn, P. S.; Wessels, P. L. *J. Chem. Soc., Perkin Trans. 1* **1977**, 1360–1364.

0002-7863/94/1516-8863$04.50/0

Scheme 1



be a thermally stable, crystalline substance but reacted rapidly with protic solvents. In neutral aqueous solution, it undergoes hydrolysis within seconds to give the corresponding diol. In spite of this sensitivity to water, treatment of aqueous solutions of DNA with the epoxide gives high yields of a guanine adduct which has been demonstrated to be the *trans* adduct at the N7 position. This species represents at least 95% of all DNA adducts of AFB_1.

The question has frequently been raised whether this is the exclusive adduct. There have been reports of formation of trace adducts of undefined structure on cystosine[7] and adenine,[8] but these claims have been difficult to confirm. Indeed, a report in 1990 from Stark's laboratory found no evidence for adducts with adenine or thymine but a possible very minor adduct with cytosine following reaction of photochemically generated **2** with DNA containing radiolabeled nucleosides.[9] The potential biological importance of minor aflatoxin adducts in DNA should not be dismissed out of hand. The mutagenic potential of minor adducts has in some cases been found to significantly outweigh their relative concentration.[10] The ready availability of the AFB epoxide now facilitates the study of DNA adducts. The search for minor adducts, their identification, and the subsequent evaluation of their biological significance are greatly facilitated by access to authentic samples. The structures of independently prepared adducts can, if prepared in sufficient quantity, be rigorously established. The samples are then available as chromatographic and spectroscopic standards for identification of adducts arising *in vivo* from DNA.

With many carcinogens, adducts can be prepared by direct reaction with the nucleosides and nucleotides and then used to compare with samples derived from hydrolytic degradation of adducted DNA. However, the reactions of aflatoxin epoxide with DNA are strongly dependent on B duplex structure.[11] We have demonstrated that the adduction reaction involves intercalation of the aflatoxin moiety prior to adduction.[4b] Reactions occur poorly or not at all with other DNA duplex conformations, with single-stranded material, and with nucleosides and nucleotides. In the absence of intercalation, the extraordinarily rapid reaction with water precludes significant reaction with DNA. Thus, nucleoside adducts of aflatoxin have been inaccessible.

Table 1. Comparison of the Proton Chemical Shifts of AFB–N7-Guanine (5) and AFB–N7-Adenine (7)[a]

proton	guanine adduct (5)	adenine adduct (7)
	Purine	
H2		8.14 s
H8	7.33 s	7.74 s
NH_2	6.10 br s	6.86 br s
	Aflatoxin	
H6a	6.87 d ($J_{6a,9a}$ = 5.6 Hz)	6.93 d ($J_{6a,9a}$ = 6.0 Hz)
OH	6.38 d ($J_{9,9\text{-OH}}$ = 4.4 Hz)	6.52 d ($J_{H9,9\text{-OH}}$ = 4.4 Hz)
H8	6.24 s	6.42 s
H5	6.62 s	6.38 s
H9	5.23 d (s with D_2O) ($J_{H9,9\text{-OH}}$ = 4.4 Hz)	5.32 d (s with D_2O) ($J_{H9,9\text{-OH}}$ = 4.4 Hz)
H9a	4.15 d ($J_{6a,9a}$ = 5.6 Hz)	4.26 d ($J_{6a,9a}$ = 6.0 Hz)
OMe	3.90 s	3.79 s
H3	3.30 m	3.30 m
H2	2.57 m	2.50 m

[a] Spectra were recorded in DMSO-d_6. Chemical shifts are in ppm relative to TMS. Values for **5** are from Essigmann et al.[5a]

A solution to the synthetic problem had now been found which involves nonaqueous reaction conditions. Reactions of 2′-deoxynucleosides with aflatoxin B_1 epoxide in most non-hydroxylic organic solvents are effectively precluded by poor solubility of the nucleosides; however, solubilization can be improved by derivatization of the 3′ and 5′ hydroxyl groups with lipophilic ethers or esters. Condensations with the epoxide then will occur in good yields although at rates much lower than observed with duplexed DNA in aqueous solution. In this paper we describe use of a 3′,5′-bis-protected derivative of deoxyadenosine for preparation of adenine adduct **7**. Sufficient amounts were prepared to permit NMR spectroscopic studies which revealed that adduction had occurred at the N7 position of the base by *trans* opening of the epoxide. This authentic sample has been used to establish that the reaction of aflatoxin B_1 epoxide with DNA produces **7** along with much larger quantities of the well-characterized guanine N7 adduct.

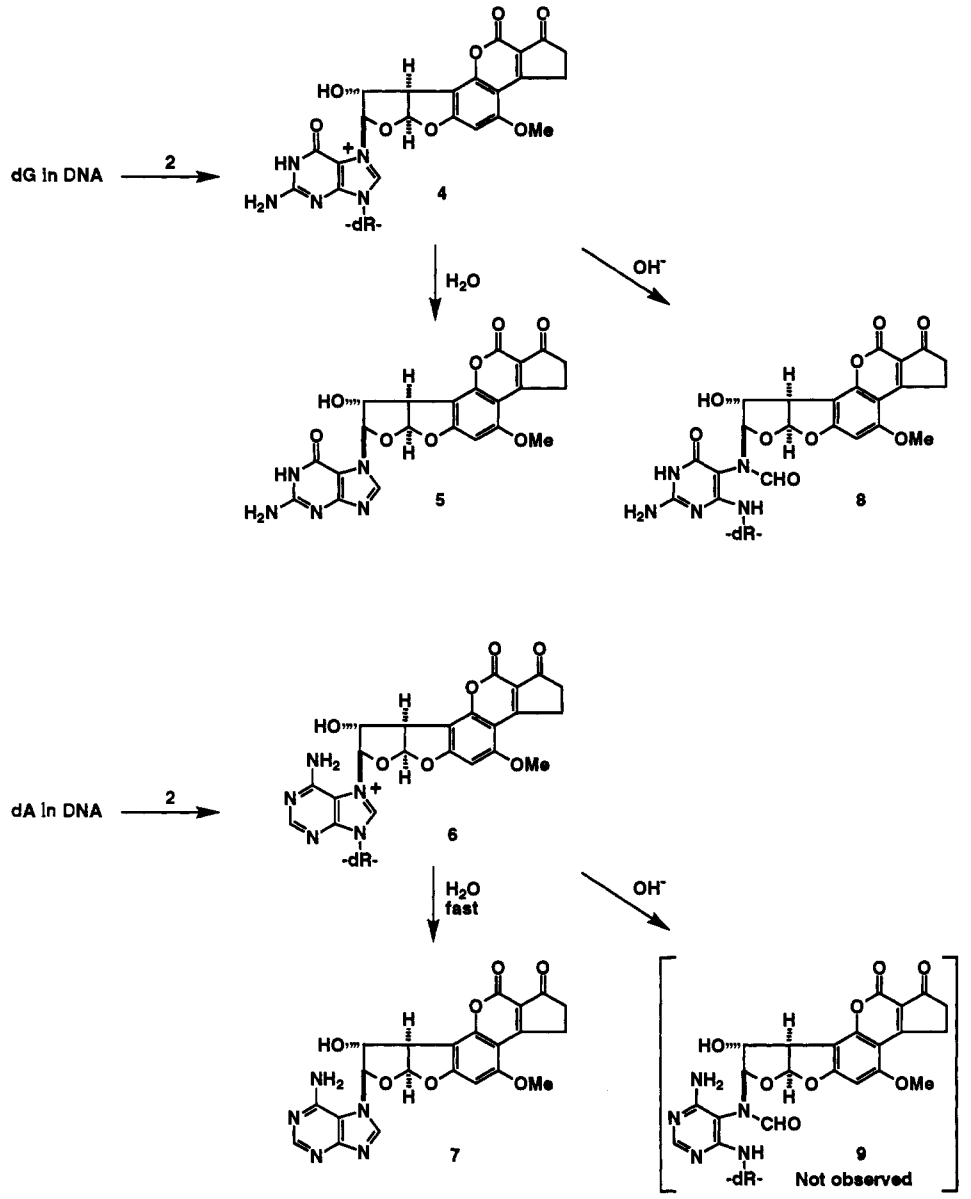
Results and Discussion

The condensation of AFB epoxide with the 3′,5′-dibutyrate of 2′-deoxyguanosine proceeded efficiently in THF to give a high yield of the N7 adduct of the nucleoside within 6 h at ambient temperature. Details of that study will be reported elsewhere. A similar reaction of the epoxide with the 3′,5′-bis-*O*-(*tert*-butyldimethylsilyl) derivative of 2′-deoxyadenosine was much slower, giving ~18% conversion to the adducted base after 10 days at ambient temperature; reaction at 55 °C yielded 23–25% of the adduct after 7 days. Cleavage of the glycosidic linkage had occurred during the course of the reaction, presumably due to the long reaction time and the presence of adventitious moisture. Mass spectroscopy of the adduct gave a molecular ion corresponding to a 1:1 adduct of adenine with aflatoxin epoxide. The mass spectrum provided no evidence as to the site of adduction.

A comparison of the ^1H NMR spectrum of the adduct with that of the N7 guanine–aflatoxin adduct shown in Table 1 reveals strong similarities for the aflatoxin signals in the two spectra, indicating that adenine adduction had occurred at aflatoxin C8 and that the epoxide had been attacked from the *endo* face to give the *trans* adduct. The C8 position of aflatoxin epoxide is the normal site of attack by nucleophiles due to charge stabilization in the transition state by O7. Under conditions promoting S_N2 reactions, attack by nucleophiles occurs from the *endo* face of the epoxide leading to *trans* ring-opened products. In the present case, C8 was confirmed as the site of reaction by the presence of a secondary hydroxyl group at C9 which produced coupled

(7) Yu, F.-L.; Huang, J. X.; Bender, W.; Wu, Z. R.; Chang, J. C. *Carcinogenesis* **1991**, *12*, 997–1002.
(8) (a) Garner, R. C. *Chem.-Biol. Interact.* **1973**, *6*, 125–129. (b) Shieh, J. C.; Song, P.-S. *Cancer Res.* **1980**, *40*, 689–695. (c) D'andrea, A. D.; Haseltine, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4120–4124.
(9) Shaulsky, G.; Johnson, R. L.; Shockcor, J. P.; Taylor, L. C. E.; Stark, A.-A. *Carcinogenesis* **1990**, *11*, 519–527.
(10) (a) Vousden, K. H.; Bos, J. L.; Marshall, C. J.; Phillips, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1222. (b) Dipple, A.; Pigott, M.; Moschel, R. C.; Costantino, N. *Cancer Res.* **1983**, *43*, 4132.
(11) Raney, V. M.; Harris, T. M.; Stone, M. P. *Chem. Res. Toxicol.* **1993**, *6*, 64–68.

Scheme 2



doublets in the NMR spectrum at δ 6.52 (aflatoxin 9-OH) and 5.32 (aflatoxin H9). The δ 5.32 doublet collapsed to a singlet upon exchange of the hydroxyl proton with D_2O. A singlet at δ 6.42 was assigned to aflatoxin H8. As with the guanine adduct, the absence of spin–spin coupling between H8 and H9 and between H9 and H9a is observed and establishes *trans* relationships between H8 and H9 and between H9 and H9a. The adenine adduct shows a strong NOE (15–20%) between H9 of the aflatoxin moiety and a carbon-bound proton of adenine at δ 7.74, but the NMR spectrum provided no evidence as to whether the δ 7.74 proton was adenine H8 or H2.

The positions on deoxyadenosine where substitution could conceivably occur are N1, N3, N7, and the exocyclic amino group. The presence of a broad two-proton singlet in the ^1H NMR spectrum (DMSO-d_6) at δ 6.86 suggested that the exocyclic amino group was not the site of attachment. To distinguish among the remaining three potential sites of adduction, unambiguous assignments of the signals for the adenine H2 and H8 protons were required.

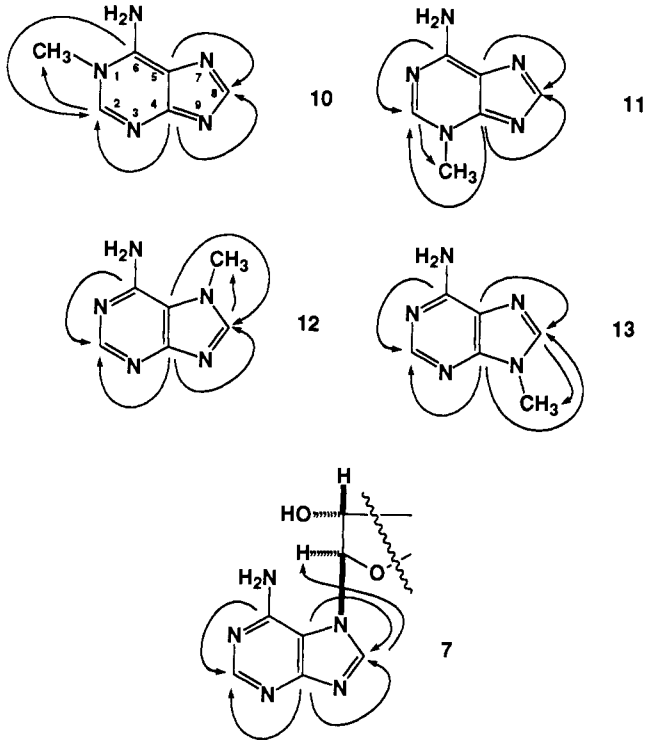
Due to the simplicity of the proton spectrum of the adenine fragment, a solution to this assignment problem using ^1H NMR was not immediately apparent. However, heteronuclear NMR spectroscopy offered possibilities. We first carried out an investigation of four model compounds, the 1-, 3-, 7-, and 9-methyladenines (**10–13**). 1-, 3-, and 7-methyladenines are available from commercial sources. The 9-methyl isomer is easily prepared by using a published procedure.[12]

Heteronuclear correlation spectra were employed to make definitive and complete assignments of the proton and carbon spectra of the methyladenines. Proton-decoupled ^{13}C spectra of the *N*-methyladenines comprise signals for C2, C4, C6, and C8 in the δ 140–160 region, a signal for C5 in the region of δ 110–120, and a signal for the methyl group near δ 35.[13] Carbons 2 and 8 give more intense signals than carbons 4, 5, and 6 due to enhanced relaxation rates and the NOEs arising from the attached protons. Carbon 5 lies upfield of carbons 4 and 6 because it has only one nitrogen substituent, whereas the others have two.

Distinction between C2 and C8 and between C4 and C6 was made using two-dimensional ^1H–^{13}C correlation spectra with ^1H detection optimized for three-bond coupling constants. Results of the studies are summarized in Chart 1. These long-range couplings permitted assignment of the signal for C4 in each case since it is the only carbon with detectable coupling to both H2

(12) Hedayatullah, M. *J. Heterocycl. Chem.* **1982**, *19*, 249–251.
(13) Thorpe, M. C.; Coburn, W. C., Jr.; Montgomery, J. A. *J. Magn. Reson.* **1974**, *15*, 98–112.

Chart 1[a]



[a] Arrows indicate three-bond carbon–hydrogen connectivities. Arrow head indicates location of proton.

Table 2, Carbon and Proton Chemical Shifts in *N*-Methyladenines[a]

	1-methyl-adenine	3-methyl-adenine	7-methyl-adenine	9-methyl-adenine
C2	144.7	146.4	153.7	153.6
C4	158.3	151.3	159.4	150.6
C5	119.7	120.8	113.2	119.5
C6	150.0	156.0	153.3	156.6
C8	155.5	153.9	148.0	144.2
N-*CH*$_3$	38.0	37.7	35.1	31.3
H2	8.07	8.14	8.08	8.08
H8	7.88	7.82	8.01	7.94
N-C*H*$_3$	3.67	3.83	3.90	3.64

[a] Chemical shifts are in ppm with respect to TMS. The spectra were recorded in DMSO-d_6 at 323 K.

and H8. The spectra also permitted H8 to be assigned since it, but not H2, was coupled to C5. The assignments of C6 and H2 follow from these assignments. The $^1J_{C-H}$ couplings permitted assignments to be made for C2 and C8 on the basis of assignments for H2 and H8. With the adenine ring protons and carbons all assigned, the location of the *N*-methyl group could be assigned from three-bond couplings between the protons of the methyl groups and the ring carbon atoms. Proton and carbon chemical shift assignments for the four *N*-methyladenines are given in Table 2.

The ^{13}C assignments for 7- and 9-methyladenine have previously been reported.[14] Those assignments were not on as rigorous a basis as the present ones but are consistent with them. ^{13}C spectra of 3-methyl- and 1-methyladenine have not been previously assigned, but our assignments for 3-methyladenine are consistent with assignments reported for 3-butyladenine.[15] It is noteworthy that H8 is upfield of H2 in all four of the methyladenines. The alkyl substituent has a larger effect on carbon shifts, causing increased shielding when close to the *N*-methyl group, i.e., ring carbons adjacent to alkylated nitrogens experience upfield shifts.[16]

Table 3, Carbon–Hydrogen Coupling Constants (Hz) of *N*-Methyladenines at 50 °C[a]

J_{H-C}	1-methyl-adenine	3-methyl-adenine	7-methyl-adenine	9-methyl-adenine
$^1J_{H2-C2}$	213.2	209.3	202.6	202.1
$^1J_{H8-C8}$	201.1	202.8	211.7	213.8
$^3J_{H8-C5}$	9.8	10.2	5.2	11.0
$^3J_{H8-C4}$	10.5	10.4	12.4	6.0
$^3J_{H2-C4}$	12.0	6.5	12.0	12.2
$^3J_{H2-C6}$	6.8	11.7	11.6	11.5
$^3J_{CH3-H2}$	3.3	3.3		
$^3J_{CH3-H8}$				
$^3J_{CH3-C2}$	4.3	4.6		
$^3J_{CH3-C4}$		3.3		3.3
$^3J_{CH3-C5}$			3.5	
$^3J_{CH3-C6}$	3.3			
$^3J_{CH3-C8}$			5.0	3.9

[a] $^1J_{H-C}$ determined from HMBC 2D spectra and $^3J_{H-C}$ determined from 1D SIMBA spectra.

Thus, C2 is downfield of C8 in the 7- and 9-methyl isomers and upfield in the 1- and 3-methyl species. C5 is upfield by more than 5 ppm in 7-methyladenine relative to the other isomers. Proximal substitution also moves C2, C6, and C8 upfield. For C4, the 9-methyl as well as the 3-methyl groups cause increased shielding (~8 ppm upfield shift). Of these relationships, the upfield shift of C5 is of particular significance for assignment of the site of aflatoxin adduction.

Correlations between the methyl group and ring positions were examined. In all four cases, strong NOEs were observed between the methyl protons and the proximal ring proton; three-bond couplings were observed between the methyl protons and proximal ring carbons. The reverse correlation, i.e., between ring protons and the methyl carbon, could be seen in the 1- and 3-methyladenines but was not detected in 7- and 9-methyladenines. The correlation of methyl protons with proximal ring carbons provided unambiguous assignment of the location of methyl groups. No four-bond coupling was detected between methyl protons and proximal ring protons.

The three-bond coupling constants (Table 3) were measured using selective carbon excitation by the 1D selective inverse multiple bond analysis (SIMBA) technique; the digital resolution in the 1D mode gives more accurate measurements than two-dimensional spectra, where practical limitations on the size of data sets preclude high digital resolution. The magnitudes of the three-bond coupling constants between ring protons and ring carbons, i.e., $^3J_{H8-C5}$, $^3J_{H8-C4}$, $^3J_{H2-C4}$, and $^3J_{H2-C6}$, of the four methyladenines are of interest. All of these three-bond coupling constants, i.e., $^3J_{H-C-N-C}$, are in the range 9–12 Hz with the exception of those across nitrogens bearing methyl substituents, where the value is reduced to 4–6 Hz. This observation, albeit empirical, provides confirmatory evidence of the site of substitution.

With the assignments for the methyladenines complete, attention was directed to identification of the aflatoxin adduct. The compound gave a well-resolved ^{1}H spectrum in DMSO-d_6 which was qualitatively similar to that of the aflatoxin–guanine adduct (Figure 1). A two-dimensional ^{1}H–^{13}C correlation spectrum optimized for one-bond coupling constants was employed to observe the protonated carbons. Those in the aflatoxin fragment could immediately be assigned on the basis of the proton spectrum due to assignments of the attached protons being secure. The protonated carbons of the adenine fragment, i.e., C2 and C8, appeared at δ 141.8 and 151.8 but could not be individually assigned. A spectrum was then acquired with a longer mixing time to emphasize small, i.e., long-range, coupling constants. The experiment was carried out several times employing different mixing times to optimize sensitivity for coupling constants of

(14) Chenon, M.-T.; Pugmire, R. J.; Grant, D. M.; Panzica, R. P.; Townsend, L. B. *J. Am. Chem. Soc.* **1975**, *97*, 4627–4636.

(15) Ishino, M.; Sakaguchi, T.; Morimoto, I.; Okitsu, T. *Chem. Pharm. Bull. (Japan)* **1981**, *29*, 2403–2407.

(16) Pugmire, R. J.; Grant, D. M.; Townsend, L. B.; Robins, R. K. *J. Am. Chem. Soc.* **1973**, *95*, 2791–2796.

Table 4. ^1H–^{13}C Connectivities for AFB–N7-Adenine (7) Established by 2-Dimensional NMR Heterocorrelation Spectra

		AFB									ADE	
	$^{13}C(\delta)^a$	H2	H3	OMe	H5	H6a	H8	H9	9-OH	H9a	H2	H8
AFB												
C1	201.4	HCC										
C2	34.9	HC										
C3	28.7		HC									
C3b	104.1				HCCC							
C4	162.4			HCOC	HCC							
OMe	57.0			HC								
C5	90.7				HC							
C5a	165.0				HCC	HCOC				HCCC		
C6a	114.3					HC	HCOC	HCCC				
C8	93.8					HCOC	HC	HCC		HCCC		
C9	75.6						HCC	HC		HCC		
C9a	52.0									HC		
C9b	103.5				HCCC			HCCC		HCC		
C9c	no.[b]											
C11	no.[b]											
C11a	no.[b]											
ADE												
C2	152.9										HC	
C4	160.1										HCNC	HCNC
C5	110.9											HCNC
C6	151.9										HCNC	
C8	141.8						HCNC					HC

[a] Chemical shifts for ^{13}C signals observed in the 2D spectra are in ppm relative to TMS. Spectra were recorded in DMSO-d_6. [b] Not observed.

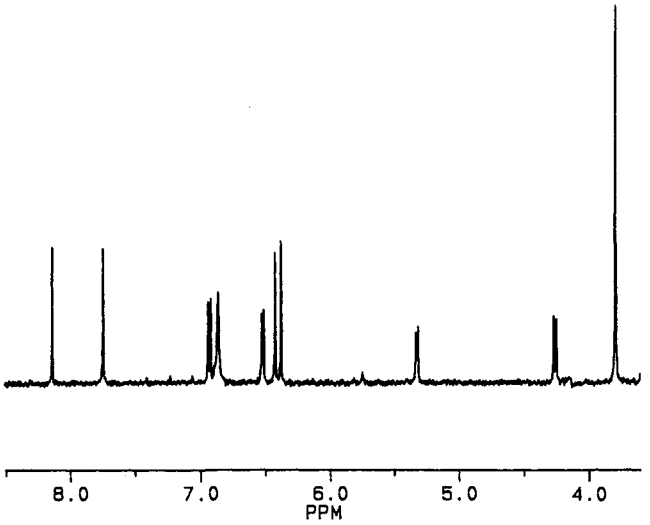


Figure 1. ^1H NMR spectrum (300 MHz) of AFB–adenine (7) recorded in DMSO-d_6.

different magnitudes. These spectra allowed assignment of all the adenine ring carbons and protons using arguments similar to those detailed above for the model *N*-methyladenines. The results are summarized in Chart 1 and Table 4. In particular, C8 and C2 of the adenine fragment could be assigned as δ 141.8 and 151.8, respectively. Consequently, H8 and H2 of the adenine fragment could respectively be assigned as δ 7.74 and 8.14. Therefore, the NOE observed between aflatoxin H9 and an adenine proton involves adenine H8, which places the aflatoxin moiety at the N7 position of adenine.

Additional features of the spectra served to confirm the N7 assignment for the site of attachment. A three-bond coupling could be observed between aflatoxin H8 and adenine C8; a similar correlation was observed with 7-methyladenine. A comparison of the chemcial shifts of the purine ring carbons of the adduct with those for the methyladenines supported adduction at this site with shielding of adenine C5 and C8 by the bulky aflatoxin moiety being observed. A strong NOE observed from adenine H8 to aflatoxin H9 (but not to aflatoxin H8) is a particularly important piece of evidence. A similar NOE can be observed with the aflatoxin adduct of guanine.

A study was made of the reaction of aflatoxin B_1 epoxide with poly(dAdT). The reaction yielded 2–3% of adenine adduct **7**. An important difference between this reaction and the reaction carried out with the diTBDMS derivative in THF is that the aqueous reaction was very rapid, i.e,, any epoxide which had not reacted with adenine within a few seconds underwent hydrolysis to the unreactive dihydrodiol. It was discovered that the reaction, when carried out in deuteriated buffer, pH 7.2, led to replacement of adenine H8 by deuterium. The exchange undoubtedly occurred on the adducted nucleoside **6**. H8 and H2 of adenine and its derivatives are known to undergo H/D exchange, but the reaction is slow except under vigorous conditions.[17] Control experiments with poly(dAdT) and with the aflatoxin–adenine adduct **7** showed that neither one undergoes significant H/D exchange under the conditions of the reaction, pointing to exchange having occurred on the transient nucleoside adduct **6**. Facile H/D exchange reaction is also observed with the adduct of deoxyguanosine in oligonucleotides.[18] Exchange in N7-adducted nucleosides is rapid due to the positive charge.

The reaction of aflatoxin B_1 epoxide with a 33-mer deoxyoligonucleotide containing 10 guanines and 12 adenines was found to yield a ~50:1 mixture of the guanine and adenine adducts, The oligonucleotide, d(5′-CGG ACA AGA AGA ATT CGT CGT GAC TGG GAA AAC), is non-self-complementary; however, the existence of unidentified secondary structures favoring formation of adducts cannot be excluded. Formation of the adenine adduct was also detected in the reaction of epoxide with calf thymus DNA. The guanine and adenine adducts were assayed twice, once immediately after reaction with the epoxide and then again after the mixture was heated for 15 min at 95 °C. The initial assay showed a 10:1 mixture of the guanine and adenine adducts **5** and **7**. After heat treatment the ratio dropped to 200:1. The implication is that adenine adduct **7** had been released prior to heating. Deoxyguanosine adduct **4** is more stable thermally, and only a small fraction had depurinated to **5** prior to heat treatment. The absolute yield of **7** was not determined accurately but was clearly higher than that in the reaction with the oligonucleotide showing the importance of duplex structure in the transition state of the adduction reaction.

(17) Brush, C. K.; Stone, M. P.; Harris, T. M. *Biochemistry* **1988**, *27*, 115–122.
(18) Gopalakrishnan, S.; Harris, T. M.; Stone, M. P. *Biochemistry* **1990**, *29*, 10438–10448.

In summary, we have established that deoxyadenosine adduct **6** is formed in the reaction of aflatoxin B_1 epoxide with DNA and that adduction involves attack by the N7 position of deoxyadenosine on the C8 position of aflatoxin epoxide. Attack occurs from the *endo* face of the aflatoxin epoxide and causes inversion of configuration at C8. Reaction of electrophilic reagents at the N7 position of deoxyadenosine is uncommon; electrophiles usually react at N1, N3, or the exocyclic amino group. In the present case, as with deoxyguanosine adduction, attack is probably directed to the N7 position by a transition state involving intercalation in duplexed DNA and stacking in single-stranded DNA and nucleosides.

Does the AFB–deoxyadenosine adduct **6**, in spite of its low yield, have any biological significance? More study will be required to answer this question. AFB–deoxyguanosine adduct **4** depurinates readily but several orders of magnitude more slowly than **6**. Adduct **4** also undergoes base-catalyzed cleavage of the guanine ring system to form an aflatoxin-linked formamidopyrimidine (i.e., FAPY) adduct **8**. The FAPY adduct is highly persistent *in vivo* and, consequently, is likely to be an important contributor to the genotoxicity of aflatoxin in eukaryotic systems. The corresponding FAPY adduct **9** derived from **6** has not been observed. Indeed, the rapid deglycosylation of **6** may preclude significant formation of this lesion *in vivo*.

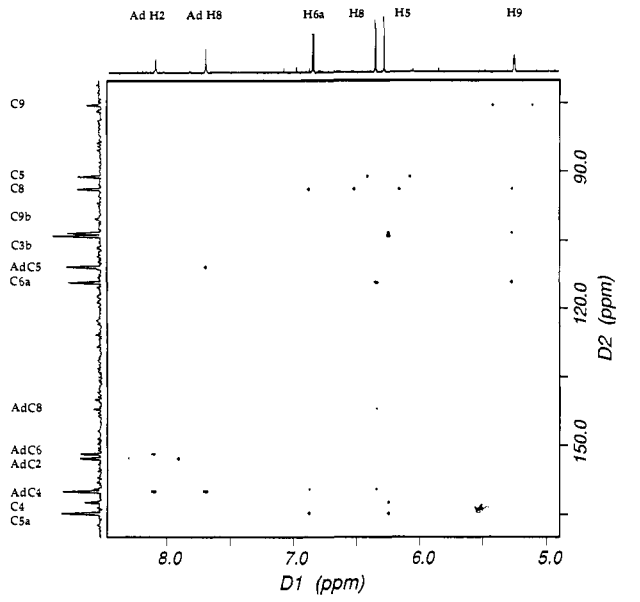


Figure 2. 2D HMBC spectrum of adenine adduct **7** in DMSO-d_6 showing $^3J_{H-C}$.

Experimental Procedures

Biological Hazards. *Aflatoxin B_1 and many of its derivatives are potently carcinogenic. Great care should be exercised to avoid personnel exposure. Crystalline material presents an inhalation hazard because the crystals develop electrostatic charge and cling to dust particles. The dust produced by the scrapping of preparative TLC plates should be regarded as particularly hazardous.*

Chemicals. Aflatoxin B_1 was purchased from Aldrich Chemical Co. (Milwaukee, WI). Aflatoxin concentration was measured spectrophotometrically using an extinction coefficient of 2.18×10^4 M^{-1} cm^{-1} at 362 nm. Calf thymus DNA (type I) and poly(deoxyadenylic thymidylic acid) (pdAdT) were purchased from Sigma Chemicals, Inc. Calf thymus DNA concentrations (expressed as base pairs) were determined spectrophotometrically by using an extinction coefficient of 1.32×10^4 M^{-1} cm^{-1} at 260 nm. Reagents for oligodeoxynucleotide synthesis were purchased from Pharmacia P. L. Biochemicals, Inc., and Fisher Scientific. d(5′-CGG ACA AGA AGA ATT CGT CGT GAC TGG GAA AAC) was synthesized by standard solid-phase phosphoramidite chemistry with an automated synthesizer. Dimethyldioxirane was synthesized as described by Murray and Jeyaraman[19] and Adam et al.[20] Solutions of dioxirane were stored over anhydrous $MgSO_4$ at −20 °C and were used within 1 month of preparation. AFB epoxide was prepared as previously described.[4a] Standards of AFB–N7-guanyl adduct and AFB dihydrodiol were prepared according to standard methods.[3a] 3′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyadenosine was synthesized according to a published procedure.[21]

Dimethyl sulfoxide-d_6 and D_2O were obtained from Cambridge Isotope Laboratories (Woburn, MA). 1-Methyladenine and 3-methyladenine were obtained from Aldrich Chemical Co. 7-Methyladenine was obtained from Chemsyn Science Laboratories (Lenexa, KS). 9-Methyladenine was synthesized according to published procedures and recrystallized from water.[12]

Synthesis of the AFB–N7-Adenine Adduct. Aflatoxin epoxide (5 mg, 15 μmol) and 3′,5′-bis-*O*-(TBDMS)-2′-deoxyadenosine (36 mg, 75 μmol) were dissolved in anhydrous THF (3 mL). The mixture was sealed in an ampule which was then heated at 50–55 °C for 7 days. Unreacted epoxide was destroyed by treatment with methanol (1 mL) for 4 h at room temperature. Solvents were then removed *in vacuo*, and the residue was resuspended in 1:10 methanol/water (5 mL). The resulting suspension was centrifuged and the supernatant fractionated by HPLC using a reverse-phase column (Econosphere ODS, 5 μm C-18, 250 mm × 4.6 mm, Alltech Associates) eluted isocratically with 30% CH_3CN/MeOH (1:1 v/v) in 20 mM ammonium acetate buffer, pH 3.9, at a flow rate of 1.5 mL/min. The adenine adduct eluted at 9.6–10.3 min. The adduct was collected and lyophilized. The residue was desalted by dissolving in water (30 mL) and loading directly onto a C18 column. The column was then eluted with a methanol–water gradient and the major peak collected to afford pure adduct **7** in 23–25% isolated yield. When the reaction was carried out at room temperature, using 10 equiv of 3′,5′-bis-O-(TBDMS)-2′-deoxyadenosine, adduct **7** was obtained in 18% yield after 10 days of reaction.

^{1}H NMR (300 MHz, DMSO-d_6): δ (adenine signals) 8.14 (1H, s, H2), 7.74 (1H, s, H8), 6.86 (2H, br s, $-NH_2$); (aflatoxin signals) 6.93 (1H, d, J = 6.0 Hz, H6a), 6.52 (1H, d, J = 4.4 Hz, –OH), 6.42 (1H, s, H8), 6.38 (1H, s, H5), 5.32 (1H, d, s with D_2O, J = 4.4 Hz, H9), 4.26 (1H, d, J = 6.0 Hz, H9a), 3.79 (3H, s, –OMe), ~3.30 (2H, m, H3), ~2.50 (2H, m, H2). MS (FAB$^+$): m/z calcd for $C_{22}N_5O_7H_{18}$ (MH^+), 464.1206; found 464.1214.

Formation of the AFB–N7-Adenine Adduct by Reaction of Epoxide 2 with Poly(dAdT) and with 33-Mer. A solution of aflatoxin epoxide (2 mg, 6.1 μmol) in CH_2Cl_2 (250 μL) was added to poly(dAdT) (10 A_{260} units) dissolved in 600 μL of 0.01 M sodium phosphate buffer, pH 7, with vigorous stirring. After 1.5 h at ambient temperature, aliquots of the aqueous layer were analyzed by reverse-phase HPLC [Econosphere C18, 5μm, 250 mm × 4.6 mm column, Alltech Associates, eluted isocratically with 30% CH_3CN/CH_3OH (1:1 v/v) in 20 mM ammonium acetate buffer, pH 3.9, at a flow rate of 1.5 mL/min; UV detection at 360 nm]. Retention times of AFB dihydrodiols, the AFB–N7-guanine adduct, and the AFB–N7-adenine adduct were 4–5.5, 7.3, and 7.8 min, respectively. Peak areas were estimated using a Hitachi D-2500 Chromato-Integrator. Analyses were repeated after acid hydrolysis of purine–deoxyribose linkages (0.1 M HCl, 60 °C, 1 h). The adduct from the reaction was isolated by preparative HPLC with conditions identical to those used for the product from the nonaqueous reaction. The ^{1}H NMR spectrum, UV spectrum, and MS of the adduct isolated from this reaction were identical to those of the previously synthesized material. Reaction of the 33-mer d(5′-CGG ACA AGA AGA ATT CGT CGT GAC TGG GAA AAC) with aflatoxin epoxide was carried out similarly.

Adduct **7** with deuterium replacement at the 8 position in adenine was prepared in the following manner. Poly(dAdT) (~13 A_{260} units) was lyophilized twice from 5 mL of D_2O and then redissolved in 300 μL of D_2O. To this solution was added 0.1 M sodium phosphate buffer in D_2O (500 μL), pD 7.2, followed by a solution of aflatoxin epoxide (5 mg, 15.2 μmol) in CH_2Cl_2 (500 μL). The two-phase reaction mixture was stirred vigorously for 90 min. The organic layer was discarded and the adenine adduct isolated from the aqueous solution by HPLC as described above. The ^{1}H NMR spectrum of the product was identical to that from the previous reaction except for the absence of the H8 singlet at 7.74 ppm. As a control experiment, the undeuteriated adduct was stored overnight in an NMR tube in DMSO-d_6/D_2O. No change was detected in the

(19) Murray, R. W.; Jeyaraman, R. *J. Org. Chem.* **1985**, *50*, 2847–2853.
(20) (a) Adam, W.; Chan, Y.-T.; Cremer, D.; Gauss, J.; Scheutzow, D.; Schindler, M. *J. Org. Chem.* **1987**, *52*, 2800–2803. (b) Adam, W.; Bialas, J.; Hadjiarapoglou, L. *Chem. Ber.* **1991**, *24*, 2377.
(21) Ogilvie, K. K.; Thompson, E. A.; Quilliam, M. A.; Westmore, J. B. *Tetrahedron Lett.* **1974**, 2865–2868.

intensity of the adenine H8 proton or elsewhere in the NMR spectrum. The sample was then heated for 2 h at 60 °C. Still no exchange was detected.

Formation of AFB–N7-Adenyl Adduct by Reaction of Aflatoxin Epoxide with Calf Thymus DNA. A solution of calf thymus DNA (1.1 mM in base pairs) was prepared in 0.01 M sodium phosphate buffer, pH 7.0. A solution of aflatoxin epoxide (0.25 mg, 0.76 μmol) in CH_2Cl_2 (500 μL) was added to a 3 mL solution of DNA with vigorous stirring. The reaction was allowed to proceed for 15 min at room temperature. Formation of guanyl-N7 and adenyl-N7 adducts was assayed by reverse-phase HPLC (Econosphere C18, 5μm, 250 mm × 4.6 mm column, Alltech Associates; isocratic elution with 25% CH_3CN in water, 1.5 mL/min, UV detection at 360 nm). Analyses were repeated after aliquots of the reaction mixture were heated at 95 °C for 15 min. Retention times for AFB–N7-guanine and AFB–N7-adenine were 6.8 and 18.4 min, respectively. The relative yield of the N7-guanine adduct to N7-adenine adduct was ~10:1 when assayed immediately after reaction and ~200:1 after thermal depurination.

NMR Studies. Routine spectra were recorded on a Bruker AC-300 instrument at 300.13 MHz. Two-dimensional and SIMBA spectra were recorded on an AMX-500 spectrometer at 500.13 MHz. NMR samples of the methyladenines were prepared as ~0.02 M solutions in mixtures of D_2O/DMSO-d_6 (7:3 v/v). The AFB–adenine adduct was prepared in DMSO-d_6 solution. Proton chemical shifts are reported using the residual ^1H in DMSO-d_6 as the internal reference (δ 2.49). Carbon shifts are referenced to the CD_3 signal of DMSO-d_6 at 49 ppm. All 2D spectra were measured with natural abundance samples and at 50 °C.

Carbon chemical shifts were obtained via HMQC (heteronuclear multiple quantum coherence) or HMBC (heteronuclear multiple bond coherence) experiments.[22] 2D HMQC spectra were run using the BIRD sequence to suppress the center signal and with carbon decoupling during acquisition in the TPPI mode.[23] The applied pulse sequence for the HMBC experiment was as follows: relaxation delay–$90°_x(^1H)$–Δ_1–$90°_{\phi1}(^{13}C)$–$t_1/2$–$180°_x(^1H)$–$t_1/2$–$90°_{\phi2}(^{13}C)$–acquire. HMBC spectra of adduct **7** were recorded using a delay time, Δ_1, of 46 ms and presaturation of the water signal. A $2K \times 0.5K$ spectrum covering 4902 Hz in the f_2 dimension and 13 500 Hz in the f_1 dimension was acquired using 128 scans for each increment. The spectra of methyladenines were measured with Δ_1 of 60 ms for optimization of long-range couplings of ~8 Hz; no presaturation was used. A total of 256 increments for 1-methyladenine and 512 increments for the 3-, 7-, and 9-methyladenines were taken in the t_1 dimension, with an optimized spectral width of 2808 Hz and 64 scans for each increment. The spectral width in the f_1 dimension was 12 828 Hz. The spectral window in the f_2 dimension included all the ^1H signals, whereas the f_1 dimension covered only the range 79–181 ppm. This allowed for a higher digital resolution but folded over the ^{13}C signals for the solvent and methyl peaks. The digital resolutions of the FIDs in the f_2 and f_1 dimensions are 1.4 and 25.0 Hz, respectively. The data were processed by selected regions, applying 8-fold zero-filling in the f_2 dimension with squared sine apodization and 4-fold zero-filling in the f_1 dimension with shifted sine apodization. In this way, $^3J_{C-H}$ coupling constants as small as 4 Hz could be determined from the proton projections.

The pulse sequences used in the SIMBA experiment are similar to those previously reported.[22,24] Modifications were made in the published procedure; in some instances, sinc3-shaped pulses were used for the second and third carbon pulses.[25] In others, the second pulse was half-Gaussian-shaped to obtain symmetry around the refocusing ^1H 180° pulse. The shaped carbon pulses were of 10 ms duration. The delays Δ_1, Δ_2, and Δ_3 were set at 3.5 ms, 55 ms, and 5 μs, respectively. Use of the half-Gaussian pulse minimized distortion by decay of transverse magnetization, and fewer artifacts were observed in the spectrum. The half-Gaussian pulse is preferable if the selected signal is well defined. Loss of sensitivity occurs if the half-Gaussian pulse is applied slightly off resonance. Acquisitions using the sinc3 pulse are less sensitive to these factors. The sinc3 pulse had a selective excitation window with half-height width of 510 Hz for a 10 ms pulse, whereas the 10 ms half-Gaussian pulse covered only 170 Hz but had a broad base. The advantage of the second pulse being shaped rather than the third has been presented for region-selective HMBC spectra.[24] The C–H couplings were determined with a resolution of 0.2 Hz.

Acknowledgment. This research was generously supported by the U.S. Public Health Service via Project Grant ES-03755, Center Grant ES-00267, and Instrumentation Grant RR-05805 (500 MHz NMR spectrometer).

Supplementary Material Available: HMQC spectrum of the AFB–N7-adenine adduct (**7**) and HMBC spectra of 1-, 3-, 7-, and 9-methyladenine (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(22) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson.* **1983**, *55*, 301–315.

(23) Bax, A.; Subrumanian, S. *J. Magn. Reson.* **1986**, *67*, 565–569.

(24) Crouch, R. C.; Spitzer, T. D.; Martin, G. E. *Magn. Reson. Chem.* **1992**, *30*, 595–605.

(25) Crooks, L. E.; Hoenninger, J.; Arakawa, M.; Kaufman, L.; McRee, R.; Watts, J.; Singer, J. R. *SPIE, Rec. Future Dev. Med. Imaging II* **1979**, *206*, 120.