label, therefore, has significantly less driving force to photoreduce a DNA base than does a pyrene label. Consequently, the combination of lowered excited state reducing power and lessened label/duplex association for $Ru(bpy)_3^{+2}$ complexes makes them much more suitable as "innocent" labels for DNA duplex attachment than are planar aromatic labels such as pyrene.

Concluding Remarks. The above data demonstrate that for short strands of ruthenium tris(bipyridyl) labeled DNA neither the label's spectroscopic properties nor the duplex's hybridization stability are appreciably impaired. Thus, DNA-based supramolecules related to the ones described here could specifically locate molecular subunits to accomplish particular photochemical and photophysical tasks. To facilitate such research the synthesis of this type of labeled oligomer should be improved. This could be accomplished in several ways. A derivative of tris(2,2'-bipyridine)ruthenium(II) could be prepared that reacts specifically with a functional group of a modified base in an oligonucleotide. This approach contrasts with the semispecific reaction of diaquobis(2,2'-bipyridine)ruthenium(II) used here. Another possibility would be to prepare a ruthenium-labeled nucleoside and convert it to the appropriate reagent for automated oligonucleotide synthesis. Finally, ruthenium and other transition-metal complexes could also be attached to DNA oligomers at an internucleotide phosphate rather than at a modified base.

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(43) Creutz, C.; Chou, M.; Netzel, T. L.; Okamura, M.; Sutin, N. *J. Am. Chem. Soc.* **1980**, *102*, 1309.

DNA Duplexes Covalently Labeled at Two Sites: Synthesis and Characterization by Steady-State and Time-Resolved Optical Spectroscopies

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Abstract: A series of oligonucleotides having the base sequence 5'-GCAC(T*-L)CAG-3' and its complement, 5'-CTGAG-(T*-L)GC-3', were synthesized where T* is a chemically modified thymidine base with a covalently attached linker arm terminating in a primary amine and L is a molecular label: pyrenebutyrate, pyrenesulfonate, or anthraquinone. Melting temperature studies on duplexes of these labeled oligonucleotides established their thermodynamic parameters for duplex formation and suggested strong label/duplex association (perhaps intercalation) for anthraquinone and pyrenebutyrate. Thus, label/label interactions were absent in the duplex with two pyrenebutyrate labels as well as in the one with both pyrenebutyrate and anthraquinone. In contrast, the duplex doubly labeled with pyrenesulfonate showed pronounced label/label quenching. In this case, emission lifetimes were significantly shortened relative to the duplex with a single pyrenesulfonate label. The above results are discussed in terms of the design requirements for constructing duplexes in which label/label interactions dominate label/duplex association.

Duplex formation between oligonucleotides with complementary base sequences is one of the salient features of DNA chemistry. The sequence-specific nature of this interaction for short oligomers allows confidence in the chemical identity and structure of the duplex formed. It is possible to take advantage of this hybridization specificity by using DNA as a template or scaffold to bring together molecular labels covalently attached to individual, complementary strands of DNA. DNA does not naturally contain functional groups to which molecules of interest (or labels) can be covalently attached. However, a number of workers have developed methods for synthesizing chemically modified nucleosides and nucleotides that allow subsequent labeling reactions to proceed. These modifications can be to the heterocyclic bases[1,2] or to phosphorus.[3,4] Additionally, the properties of single labels covalently attached to oligonucleotide single strands and duplexes have been studied by a number of research groups using optical[5-11] and magnetic resonance spectroscopic techniques,[7] as well as thermodynamic analysis based on melting curves.[8,9] However, less attention has been paid to DNA duplexes in which there is more than one label, and in these studies the labels were attached to the 3' or 5' oligonucleotide termini. Several types of such systems have been studied. The first was comprised of a single

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[§] University of Chicago.

(1) Dreyer, G. B.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 968.
(2) Gillam, I. C.; Tener, G. M. *Anal. Biochem.* **1986**, *157*, 199.
(3) Chu, B. C. F.; Wahl, G. M.; Orgel, L. E. *Nucleic Acids Res.* **1983**, *11*, 6513.
(4) Letsinger, R. L.; Bach, S. A.; Eadie, J. S. *Nucleic Acids Res.* **1986**, *14*, 3487.
(5) Yamana, K.; Letsinger, R. L. *Nucleic Acids Symp. Ser.* **1985**, *16*, 169.
(6) Asseline, U.; Thuong, N. T.; Helene, C. *C. R. Acad. Sci., Ser. 3* **1983**, *297*, 369. Asseline, U.; Delarue, M.; Lancelot, G.; Toulme, F.; Thuong, N. T.; Montenay-Garestier, T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3297. Asseline, U.; Toulme, F.; Thuong, N. T.; Delarue, M.; Montenay-Garestier, T.; Helene, C. *EMBO J.* **1984**, *3*, 795.
(7) Lancelot, G.; Asseline, U.; Thuong, N. T.; Helene, C. *Biochemistry* **1985**, *24*, 2521. Lancelot, G.; Asseline, U.; Thuong, N. T.; Helene, C. *J. Biomol. Struct. Dyn.* **1986**, *3*, 913. Lancelot, G.; Thuong, N. T. *Biochemistry* **1986**, *25*, 5357.
(8) Helene, C.; Toulme, F.; Delarue, M.; Asseline, U.; Takasugi, M.; Maurizot, M.; Montenay-Garestier, T.; Thuong, N. T. In *Biomolecular Stereodynamics*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: New York, 1986; Vol. III, pp 119-130.
(9) Telser, J., Cruickshank, K. A.; Morrison, L. E.; Netzel. T. L. *J. Am. Chem. Soc.*, preceding article in this issue.
(10) Helene, C.; Le Doan, T.; Praseuth, D.; Thuong, N. T.; Lhomme, J. *Photochem. Photobiol.* **1988**, *47*, 85S. Le Doan, T.; Perrouault, L.; Chassignol, M.; Thuong, N. T.; Helene, C. *Nucleic Acids Res.* **1987**, *15*, 8643.

0002-7863/89/1511-7226$01.50/0

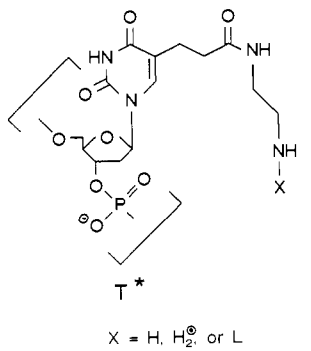

Figure 1. Structure of the modified thymidine base as a unit in an oligonucleotide. The site of label attachment is indicated by X (X = H or H_2^+ for a modified, unlabeled oligomer and X = L for an attached label).

oligonucleotide labeled at both its 3′ and 5′ termini and hybridized to a complementary strand.[10] However, this system was not designed to produce label/label interactions; rather each label had a specific, separate function. One label (acridine) stabilized the duplex by intercalation, while the other (a metalloporphyrin) effected strand scission. Two other systems were designed to promote label/label interactions. In one of these, fluorescent labels were attached to the 3′ terminus of one strand and to the 5′ terminus of a complementary strand.[11] Thus, hybridization led to label/label interactions near one of the terminal base pairs of the duplex. In another system, these interactions were promoted by hybridization of two short oligonucleotides to approximately contiguous regions of a third longer strand.[11] In this case, the short strands were each labeled at a terminal site (one at 3′ and the other at 5′) such that hybridization to the third strand positioned the labels adjacent to each other. However, in none of these cases was there controlled flexibility in positioning the labels: they were either approximately adjacent, across from each other, or at opposite ends of a common strand. A broadly useful molecular scaffold should allow molecular labels to be located in incrementally varied steps. This possibility is demonstrated in the present study by attaching labels to bases internal to each of two complementary oligonucleotides. This work uses a generic eight-base sequence with internal modified thymidines[1] to show that DNA-based systems with multiple labels can be prepared and characterized. As such, this study is a first step on the path to converting DNA duplexes into molecular scaffolds for the construction of macromolecules with specifically located molecular subunits.

Experimental Section

Synthesis. A modified thymidine nucleoside was prepared as described by Dreyer and Dervan[1] and converted to a phosphoramidite for automated oligonucleotide synthesis by standard procedures.[12,13] An Applied Biosystems Model 380B DNA synthesizer was used to prepare oligomers either without or with incorporation of a modified thymidine (T*, see Figure 1) with the protocol and reagents supplied by Applied Biosystems. Labeled oligomers containing pyrenebutyrate or pyrenesulfonate were prepared as described previously.[9] The labeled oligomer containing anthraquinone was prepared by using the same labeling reaction as used above and a reactive derivative of anthraquinone, anthraquinone-2-carboxylic acid (9,10-dihydro-9,10-dioxo-2-anthracenecarboxylic acid, Aldrich). This acid was converted to its *N*-hydroxysuccinimide (NHS) ester by using dicyclohexylcarbodiimide (DCC) as a coupling agent. The anthraquinone and NHS (each 1 mmol) were dissolved in 15 mL of dry *N*,*N*-dimethylformamide (DMF) under nitrogen. The solution was cooled to −20 °C, and DCC (1 mmol in 2 mL of DMF) was added dropwise with stirring. The reaction was allowed to reach room temperature and stir overnight. TLC (1:4 methanol/chloroform) indicated the reaction was nearly complete at that time. The dicyclohexylurea byproduct was removed by filtration, the solvent was pumped off, and the crude solid was recrystallized from methylene chloride to give the NHS ester in 50% yield. The purity and yield are not crucial, since only very small amounts of the NHS ester are needed for the labeling reaction and any starting material contaminant will not react with the T*-modified DNA. Pyrene fluorophores for labeling reactions and standards were obtained from Molecular Probes, Eugene, OR, and used as received.

Purification. The crude oligonucleotides and the products of the labeling reactions were purified by reverse-phase, liquid chromatography on a Pharmacia FPLC system as described previously.[9] The anthraquinone-labeled oligomer was easily purified by using the same procedure as for the pyrene-labeled oligomers and was distinguishable from unreacted DNA by the presence of a weak, broad absorption band extending from 300 to 380 nm.

Thermodynamic Measurements. Melting curves were measured and analyzed by using an automated system and techniques described previously.[9,14,15] The buffer used was 10 mM dibasic sodium phosphate, 0.1 mM disodium EDTA, and 1 M NaCl, adjusted to pH 7. Values for $\Delta H°$ and $\Delta G°$ are accurate to ±5% and for $\Delta S°$ to ±10%. Concentrations of duplex solutions were determined by using standard values for estimating extinction coefficients[16] and correcting for absorption by the labels.[9] The ratio of the absorbance at 340 nm to that at 260 nm in the free anthraquinone was used to approximate the contribution of anthraquinone to the DNA absorption at 260 nm in the labeled oligomer.

Spectroscopic Measurements. Steady-state absorption and emission spectra as well as emission quantum yield measurements were obtained as described previously.[9] All samples for spectroscopic measurements were dissolved in the T_m buffer solution described below and no attempt was made to deaerate the solutions. These conditions may lead to spectroscopic properties different from those reported elsewhere. Consequently, emission quantum yield values are reported relative to free pyrenebutyrate or pyrenesulfonate for the corresponding labeled oligomers. Time-resolved emission measurements were made using a time-correlated single photon counting system described in detail elsewhere.[17] The emission decay measurements reported here were recorded and analyzed as follows. Excitation pulses of 8–10 ps (full width at half-maximum, FWHM) were generated by a cavity-dumped DCM dye laser (repetition rate of 3.8 MHz) synchronously pumped by a mode-locked argon ion laser. The dye laser pulse at 640 nm was frequency doubled to 320 nm by a lithium iodate crystal. A reverse counting configuration was used in which the start input to the time-to-amplitude converter was produced by the fluorescence while the stop signal was provided by part of the excitation beam, which drove a fast photodiode (Telefunken BPW28). Polarized fluorescence at 400 nm was collected at 90° to the excitation beam at the magic angle and detected through a monochromator (JY 7/667 IR, 6-nm bandwidth) by a microchannel plate (Hamamatsu R2809U). The instrument response function had a FWHM of 55 ps and a full width at tenth maximum of 120 ps. The measured fluorescence decays were fitted to a weighted sum of exponentials through convolution with the instrument response function. The fluorescence decay impulse response was described by

$$F(t) = \sum_i A_i \exp(-t/\tau_i) \text{ with } \sum_i A_i = 1.0$$

where τ_i is the lifetime of each decay component and A_i is the corresponding preexponential amplitude. The quality of a fit was judged by a reduced χ^2 criterion, a runs test, and a plot of weighted residuals. The fluorescence decay of anthracene in cyclohexane was used as a standard and was in exact agreement with the literature value.[18]

Results and Discussion

Our previous study demonstrated the feasability of preparing singly labeled oligonucleotides and duplexes with a variety of internal labels.[9] Furthermore, the labels were spectroscopically detectable, and the oligomers exhibited normal melting curve behavior. The latter property was generally true only if the linker arm was not attached directly to a group involved in Watson–Crick base pairing. This limited our quantitative thermodynamic studies

(11) Heller, M. J.; Morrison, L. E. In *Rapid Detection and Identification of Infectious Agents*; Kingsbury, D. T., Falkow, S., Eds.; Academic Press: New York, 1985; pp 245–256. Morrison, L. E. European Patent Application, Publication No. 0232967, 1987.
(12) Caruthers, M. *Science* **1985**, *230*, 281.
(13) Gait, M. J., Ed. *Oligonucleotide Synthesis: A Practical Approach*; IRL Press: Oxford, UK, 1984; pp 35–81.
(14) Applequist, J.; Damle, V. *J. Am. Chem. Soc.* **1965**, *87*, 1450.
(15) Marky, L. A.; Breslauer, K. J. *Biopolymers* **1987**, *26*, 1601.
(16) Fasman, G. D., Ed. *Handbook of Biochemistry and Molecular Biology: Nucleic Acids*, 3rd ed.; CRC Press: Cleveland, OH, 1985; Vol. I, p 589.
(17) Fleming, G. R. *Adv. Chem. Phys.* **1982**, *49*, 1. Chang, M. C.; Courtney, S. H.; Cross, A. J.; Gulotty, R. J.; Petrich, J. W.; Fleming, G. R. *Anal. Instrum. (N. Y.)* **1985**, *14*, 433.
(18) Berlman, I. B., Ed. *Handbook of Fluorescence Spectra of Aromatic Molecules*, 2nd ed.; Academic Press: New York, 1971.

Table I. Thermodynamic Parameters for Duplex Formation[a]

duplex	$-\Delta H°$ (±5%), kcal/mol	$\Delta S°$ (±10%), cal/mol·K	$\Delta G°$ (±5%), kcal/mol
GCACTCAG + CTGAGTGC	−56[b]	−150	−10.9
GCAC(T*)CAG + CTGAGTGC	−54[b]	−150	−9.9
T*-pyrenebutyrate + CTGAGTGC	−54[b]	−140	−11.4
T*-pyrenesulfonate + CTGAGTGC	−44[b]	−110	−10.0
GCACTCAG + CTGAG(T*)GC	−54	−150	−10.0
GCACTCAG + T*-anthraquinone	−56	−150	−11.4
GCAG(T*)CAG + CTGAG(T*)GC	−52	−140	−9.5
T*-pyrenebutyrate + T*-pyrenebutyrate	−57	−150	−12.5
T*-pyrenesulfonate + T*-pyrenesulfonate	−47	−120	−10.4
T*-pyrenebutyrate + T*-anthraquinone	−52	−130	−12.3
T*-pyrenesulfonate + T*-anthraquinone	−41	−100	−10.7

[a] Parameters at 25 °C. All duplexes were in the T_m buffer: 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, 1 M NaCl, pH 7. [b] Reference 9.

to oligomers with internal labels covalently attached at a modified thymidine (T*, Figure 1), rather than a modified cytidine. The sequences used here, 5′-GCACTCAG-3′, and its complement, 5′-CTGAGTGC-3′, both contain internal thymidines. Thus, labels were attachable at a central site on each oligomer to give duplexes containing two molecular labels capable of interacting or reacting with each other as a result of duplex formation. The labels used here were 1-pyrenebutyrate, 1-pyrenesulfonate, and anthraquinone-2-carboxylate. Pyrene derivatives were good label choices for several reasons. They are simple molecules with good emission properties, have a high-energy excited state that can act as an electron or energy donor, and form an excimer with characteristic emission.[19] A difficulty, however, is that they can associate with (or intercalate into) the DNA duplex and thus quench their emission. This was seen in the previous study, particularly for pyrenebutyrate.[9] In the case of pyrenesulfonate, label/duplex association appeared to be much weaker. Thus this label could potentially exhibit pyrene–pyrene or pyrene–anthraquinone interactions. Quinone derivatives have been widely used as oxidative quenchers,[20] and the one used here is stable in aqueous media. Thus it is a potential oxidative quencher of the lowest excited state of pyrene. The results are discussed in terms of the design requirements for using DNA duplexes as templates or molecular scaffolds to organize multiple, molecular labels. Two constraints that must be satisfied are (1) label/label interactions must dominate label/duplex association and (2) stable duplex formation must still occur after attachment of both labels.

Duplex Modification. To confirm that hybridization occurred for the doubly modified system as it did for the corresponding unmodified one, a series of melting curves as a function of concentration was obtained, and the resulting thermodynamic parameters are presented in Table I. For comparison purposes some parameter values reported earlier[9] for singly modified and singly labeled duplexes are also included. Representative melting curves are presented in Figure 2. The duplex containing a single modified thymidine, 5′-GCAC(T*)CAG-3′ + 5′-CTGAGTGC-3′, shows destabilized duplex formation, as measured by $\Delta G°$, of 1 kcal/mol relative to the unmodified duplex. It was also important to make

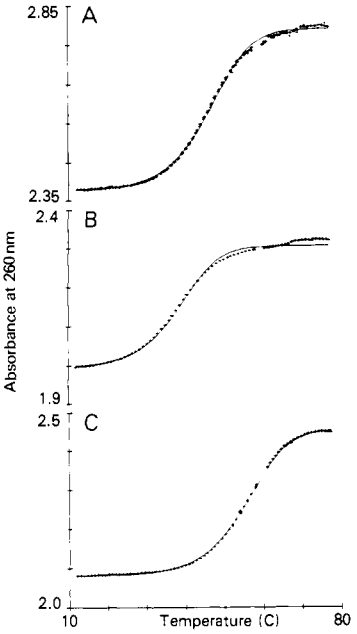

Figure 2. Melting curves of octanucleotide duplexes: (A) unmodified duplex; (B) duplex containing T* on each strand; (C) duplex labeled with pyrenebutyrate at T* on each strand. The high temperature to low temperature experimental curve is shown (plus signs) together with a best nonlinear least-squares fit (solid line) fit using the "all-or-none" model. The absorbance was monitored at 260 nm in a 1 cm path length cell and the temperature was ramped at 10 °C/h.

certain that this modification behaved symmetrically; that is, the effect of a T* modification on duplex stability was independent of which strand contained the modification. The thermodynamic parameters in Table I show that this is the case here. A consequence is that the effects of attaching various labels can be analyzed without regard to which strand is labeled. Finally, adding a modified thymidine to each strand of the duplex increases the $\Delta G°$ of duplex formation ~0.5 kcal/mol relative to either singly modified duplex. However, this magnitude of $\Delta G°$ increase is not significant relative to our experimental errors. A melting curve for this doubly modified duplex is shown in Figure 2B. These measurements, therefore, show that doubly labeled octameric duplexes will be stable provided the particular labels themselves are not a source of duplex instability.

Pyrenebutyrate Labels. The thermodynamic results for the pyrenebutyrate labeled duplexes are also shown in Table I, and a melting curve for the duplex doubly labeled with pyrenebutyrate is shown in Figure 2C. The higher T_m for this duplex relative to that for both the unmodified duplex (2A) and the duplex with two T* bases (2B) is readily apparent. Quantitatively, labeling with a single pyrenebutyrate gives a stabilization of 1.5 kcal/mol compared to the duplex containing a single T*, and the duplex with two pyrenebutyrate labels is stabilized by 3.0 kcal/mol compared to the duplex with two T* modifications only. Thus, the $\Delta G°$ decrease or duplex stabilization found for adding a single pyrenebutyrate label is doubled by the addition of a second one. This result would occur if each label were associated with the duplex in a similar manner and independently of the other. Label intercalation into different sites within the duplex base-pair stack, for example, could accomplish this.

Pyrenesulfonate Labels. The results for the pyrenesulfonate-labeled duplexes are less easily characterized. The singly labeled duplex has the same $\Delta G°$ for duplex formation as does the duplex with only a single T* modification, −10.0 versus −9.9 kcal/mol. Strikingly, however, both the $\Delta H°$ and $\Delta S°$ values are less negative for the duplex singly labeled with pyrenesulfonate. It is difficult to give a microscopic interpretation of these facts, but it is worth noting the following points. First, the thermodynamic parameters reflect the difference between initial and final states and not absolute values of stability for the final state. Apparently, entropic differences in this case are important enough to offset a loss of

(19) Turro, N. J. *Modern Molecular Photochemistry*; Benjamin/Cummings: Menlo Park, CA, 1978; pp 141–143.

(20) Kong, J. L. Y.; Spears, K. G.; Loach, P. A. *Photochem. Photobiol.* **1982**, *35*, 545. Holten, D.; Gouterman, M.; Parson, W. W.; Windsor, M. W.; Rockley, M. G. *Photochem. Photobiol.* **1976**, *23*, 415. Huppert, D.; Rentzepis, P. M.; Tollin, G. *Biochim. Biophys. Acta* **1976**, 440, 356. Harriman, A.; Porter, G.; Searle, N. *J. Chem. Soc., Faraday Trans. 2* **1979**, *75*, 1514.

enthalpic stability and produce a $\Delta G°$ value which is the same as that for the duplex with only a single T* modification. Second, the pyrenesulfonate linker arm consists of eight atoms (from C-5 on the pyrimidine to C-1 on the pyrene). This is three atoms shorter than the linker arm of the pyrenebutyrate-labeled oligomer and may make label intercalation into the duplex less favorable for pyrenesulfonate. Also, a sulfonyl group at C-1 on pyrene will hinder label intercalation more than a methylene group at the same position. 2D NMR experiments may furnish additional insights into these questions. The $\Delta G°$ values, however, leave no doubt that pyrenesulfonate labels show much reduced label/duplex interactions than do pyrenebutyrate labels. The case of the duplex doubly labeled with pyrenesulfonate also bears this out. Here the $\Delta G°$ decrease is 0.9 kcal/mol relative to the duplex with a T* modification alone on each strand. The same comparison for the duplex doubly labeled with pyrenebutyrate showed a $\Delta G°$ decrease of 3.0 kcal/mol. It is also notable that the $\Delta H°$ and $\Delta S°$ values for the duplexes with both one and two pyrenesulfonate labels are very similar.

Anthraquinone Label. As discussed above, it would be desirable to use DNA hybridization as a method for bringing together interactive molecules. A basic reaction of widespread importance is electron transfer, and pyrene molecules are good electron donors from their lowest energy, electronic excited states. Since anthraquinone is a reversible electron acceptor, it is reasonable to examine first the thermodynamics of duplex formation for the case of a single, covalently attached anthraquinone label and then to discuss duplexes labeled with both pyrene and anthraquinone labels.

The duplex containing only an anthraquinone label was prepared, and its thermodynamic parameters were obtained from a series of melting curves. This duplex showed significant stabilization and had parameters that were nearly the same as those of the duplex with a single pyrenebutyrate label (see Table I). This suggests that intercalation by the 9,10-anthraquinone-2-carboxylate label into the duplex base-pair stack is likely, just as it is also likely for pyrenebutyrate. The antitumor agent doxorubicin contains an anthraquinone moiety that is central to the drug's action on DNA.[21] As a result, extensive studies have been made on the interaction of a number of anthraquinone derivatives with natural DNA.[21] These compounds showed label/duplex association (possibly intercalation), as indicated by large shifts in the absorption bands of the anthraquinones and increases in the T_m values of the DNA duplexes. However, these compounds generally contained 1,4-dihydroxy substituents as well as substitution at the 2-position. The specific compounds of interest here, 9,10-anthraquinone-2-carboxylic acid and its amide derivative, have not been studied in this manner. Nevertheless, given the strong association between the multiply substituted anthraquinones and DNA when both are free in solution, it is not surprising that the duplex with this anthraquinone attached shows a 1.5 kcal/mol decrease in $\Delta G°$ relative to the duplex with only a single T* modification. Unfortunately, the near-UV absorption band due to anthraquinone in the labeled duplex is too weak to allow detailed comparison to that of free anthraquinone. It does, however, appear to be broader. Given the pharmacological effectiveness of related anthraquinone derivatives, anthraquinone-labeled oligonucleotides such as this one may also be of use for medical and biological studies as well as for the macromolecular assembly interests of this work.

Combined Pyrene and Anthraquinone Labels. The similarity of behavior between anthraquinone and pyrenebutyrate labels was confirmed in the duplex containing both of these labels. A duplex stabilization of 3.0 kcal/mol was seen for the duplex with two pyrenebutyrate labels and of 2.8 kcal/mol for the one with pyrenebutyrate and anthraquinone; both values are relative to the duplex with two T* modifications only. For the duplex containing pyrenesulfonate with anthraquinone, the situation was only slightly more complex. A decrease in $\Delta G°$ of 1.2 kcal/mol was seen for this duplex relative to the one with two T* modifications alone. This can be compared to the sum of the individual $\Delta G°$ decreases for each label relative to a duplex with only one T* modification. In particular, adding a pyrenesulfonate label had nearly zero effect on $\Delta G°$, while adding a single anthraquinone label produced a duplex stabilization of 1.4 kcal/mol. Thus, the sum of the individual stabilizations (0 + 1.4 kcal/mol) is in agreement with the observed dual-label stabilization of 1.2 kcal/mol.

Table II. Emission Data[a]

sample	relative quantum yield	lifetime, %
1-pyrenebutanoic acid (P-32)	1.0[b,c]	83 ns (100)
sodium 1-pyrenesulfonate (P-80)	1.0[d,e]	50 ns (100)
N-(1-pyrenesulfonyl)ethylenediamine (P-168)	0.7[d]	3.5 ns (6) 9.3 ns (94)
P-32 + GCACTCAG + CTGAGTGC	0.7[b,f]	1.85 ns (30) 71 ns (70)
P-80 + GCACTCAG + CTGAGTGC	0.9[d,f]	2.2 ns (11) 51 ns (89)
P-168 + GCACTCAG + CTGAGTGC	0.7[d,f]	1.5 ns (4) 9.1 ns (96)
T*-pyrenebutyrate single strand	0.02[b]	200 ps (50) 2.1 ns (29) 15.8 ns (21)
T*-pyrenebutyrate duplex	≤0.002[b]	70 ps (78) 715 ps (15) 6.3 ns (7)
T*-pyrenebutyrate + T*-pyrenebutyrate	≤0.002[b]	80 ps (−80) 460 ps (18) 6.1 ns (2)
T*-pyrenebutyrate + T*-anthraquinone	0.004[b]	60 ps (85) 490 ps (11) 6.9 ns (4)
T*-pyrenesulfonate single strand	0.24[d]	230 ps (21) 2.0 ns (13) 8.2 ns (66)
T*-pyrenesulfonate duplex	0.06[d]	140 ps (36) 2.0 ns (22) 10.1 ns (42)
T*-pyrenesulfonate + T*-pyrenesulfonate	0.04[d]	40 ps (53) 785 ps (12) 7.7 ns (35)
T*-pyrenesulfonate + T*-anthraquinone	0.05[d]	90 ps (38) 2.6 ns (21) 9.9 ns (41)

[a] All samples in air-saturated T_m buffer at 20 °C. [b] Using 1-pyrenebutanoic acid as a standard; excitation = 320 nm, emission = 400 nm for lifetime measurements, 350–500 nm for quantum yield measurements. [c] Absolute quantum yield = 0.6 using quinine sulfate (0.05 M H_2SO_4, QY = 0.70) as a standard. [d] As in *a*, except with sodium 1-pyrenesulfonate as a standard. [e] Absolute quantum yield = 0.8 using the standard in *b*. [f] 1:1:1.

General Label/Label Interactions. In our previous study[9] steady-state emission spectra and emission quantum yield measurements were made on duplexes singly labeled with pyrene. Here we describe the results of similar measurements on the just discussed doubly labeled duplexes as well as time-resolved emission measurements on both doubly and singly pyrene-labeled duplexes. The photochemical data are summarized in Table II. It is important to note that the melting curves indicated that for all the duplexes described here, the oligonucleotides were >98% in the duplex form at 20 °C, the temperature at which emission measurements were made. Substantial emission quenching has been used as an indication of intercalation,[5,6] but it actually demonstrates only strong association. In particular, pyrene and other polycyclic aromatic hydrocarbons have been shown to associate with both native[22] and denatured DNA[23] as evidenced by

(21) Bennett, S.; Sharples, D.; Brown, J. R. *J. Med. Chem.* **1982**, *25*, 369, and references therein.

(22) Geacintov, N. E.; Prusik, T.; Khorofian, J. M. *J. Am. Chem. Soc.* **1976**, *98*, 6444.

(23) Wolfe, A.; Shimer, G. H., Jr.; Meehan, T. *Biochemistry* **1987**, *26*, 6392.

extensive emission quenching. The nearly complete quenching of pyrenebutyrate emission for the singly labeled duplex provides further support for strong pyrene/duplex association in agreement with the thermodynamic results, which showed a $\Delta G°$ decrease of 1.5 kcal/mol. Importantly, at least 30 times less quenching was seen for the duplex singly labeled with pyrenesulfonate. This also was consistent with the thermodynamic data, which showed almost no change in $\Delta G°$. Of particular interest here is whether or not the doubly labeled duplexes showed any evidence of label/label interactions. Two processes are of prime interest in this context. The first is excimer formation, which could occur in duplexes containing two pyrene molecules. Pyrene forms a characteristic excimer with emission at 480 nm, and pyrene molecules linked by aliphatic chains of varying length show this emission dramatically.[24] If the two labels did interact, then such excimer emission might be observed. The second process of interest is oxidative quenching via electron transfer from the lowest energy, excited state of pyrene to a nearby anthraquinone label. It is possible to make rough estimates of the free energies involved in this process to show that it is thermodynamically allowed. The labeled duplexes studied here have excited-state energies of 3.35 and 3.40 eV, respectively, for pyrenesulfonate and pyrenebutyrate additions. Electrochemical data exist for similar pyrene and quinone molecules: for example, 2-(*p*-ethylbenzoyl)-9,10-anthraquinone has a reduction potential of −0.5 V,[25] and the reduction potential of pyrene cation ranges from 1.2 to 1.6 V.[26] Thus, the production of a pyrene cation and a quinone anion can be estimated to require about 1.7–2.1 eV of free energy. Comparison with the excited-state free energy of pyrene shows that over 1 eV (23 kcal/mol) of excess free energy is available, and thus the oxidative quenching reaction is highly favorable. However, for both oxidative quenching and excimer formation to occur, it is necessary for the excited state of pyrene to interact with the other label (either another pyrene or anthraquinone) during its excited-state lifetime. It is therefore useful to measure the excited-state lifetimes of the pyrene labels both when they are free and when they are attached to DNA.

Lack of Excimer Formation. The steady-state emission spectra of the duplexes doubly labeled with either pyrenebutyrate or pyrenesulfonate failed to show any excimer emission. As mentioned above, over 98% of the oligonucleotides were in the duplex conformation at 20 °C. Thus absence of excimer emission was not due to lack of duplex formation. It is not surprising that the duplex doubly labeled with pyrenebutyrate did not produce pyrene excimers in view of its 3.0 kcal/mol stabilization by the attached labels. This large decrease in $\Delta G°$ suggests that each pyrene label is strongly associated with and possibly intercalated into the duplex. However, the duplex stabilization by attached pyrenesulfonate labels is much less, only 0.9 kcal/mol. In spite of this reduced label/duplex association, the two pyrenes were still unable to approach each other during the lifetime of a pyrene excited state and form a reasonably long-lived excimer. The attached duplex may have relaxed the pyrene excited state sufficiently fast that there was not enough time for excimers to form. Alternatively, the attached duplex could have quenched the excimers that were produced. To learn about the excited-state lifetimes of the pyrene labels attached to DNA duplexes, time correlated photon-counting measurements were made on the emission from these samples. The results are listed in Table II and representative emission kinetics are shown in Figure 3.

Duplex Emission. Relatively concentrated (>1 OD at 260 nm) solutions of the unmodified DNA duplex excited at 320 nm gave very weak steady-state emission from 400 to 500 nm. For quantum yield measurements on the weakly emitting pyrenebutyrate duplexes, this contribution was subtracted out. Such a correction was not necessary for the other samples. Time-resolved emission from the unmodified duplex was predominantly (70%) characterized by a 60-ps lifetime, and the anthraquinone-labeled duplex behaved similarly with no indication of any additional emission arising from the label. Geacintov et al.[22] made time-resolved emission measurements on polycyclic aromatic hydrocarbons in the presence of natural DNA and also observed a similar weak background signal. The background DNA emission is ~100 times weaker than most of the pyrene emission observed in this study, but it may make a small contribution to the short (< 100 ps) lifetime emission signals.

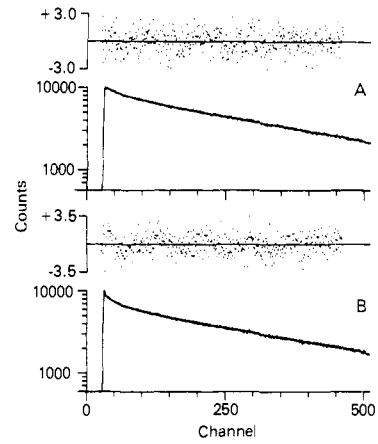

Figure 3. Emission decay curves of DNA samples in the air-saturated T_m buffer at 20 °C: (A) doubly labeled duplex containing T*-pyrenesulfonate and T*-anthraquinone; (B) doubly labeled duplex containing T*-pyrenesulfonate and T*-pyrenesulfonate. For both (A) and (B), upper trace: plot of residuals; lower trace: experimental points (dots) together with a best fit (solid line). For all traces, the ordinate scale is 21 ps/channel. The excitation was at 320 nm and the emission was monitored at 400 nm. See text for the lifetime values for these fits and further experimental details.

Free Label Emission. The free pyrene labels studied here all have large emission quantum yields, >0.5, which are dependent upon solvent and exogenous quenchers. The T_m buffer used here is an unusual solvent system for studying the excited-state lifetimes of pyrenes, and the solutions were not deoxygenated. Thus, the quantum yield and lifetime values for the free pyrene labels reported in Table II may differ from those reported elsewhere. For example, 1-pyrenebutanoic acid has a lifetime of 115 ns in an air-saturated pH 8 Tris buffer,[27] compared to 83 ns found here. The significant comparison is among the samples within this study, where the experimental conditions were held constant. Both pyrene acids studied here, 1-pyrenebutanoic acid (P-32) and 1-pyrenesulfonic acid (P-80), have reduced emission quantum yields when each is present in solution with an equal amount of octameric DNA duplex. Several studies have already described pyrene-saturated aqueous solutions (as well as solutions of other polycyclic aromatic hydrocarbons) in the presence of both natural DNA[22,23,28] and synthetic polynucleotides.[28] These studies report a red shift in the pyrene absorption bands of 10 nm,[22,23,28] and emission quenching relative to pyrene in the absence of DNA.[22,23] Geacintov et al.[22] found no change in the lifetime of pyrene in the presence of DNA, but could only measure lifetimes greater than 10 ns. Benzopyrenes did, however, show lifetime reductions under their conditions. Our work used more water-soluble pyrene derivatives and short synthetic DNA duplexes. The concentrations here were also lower than those used in most previous studies, and lifetimes as short as 50 ps could be measured. Under these conditions, the absorbance spectra of the free pyrene derivatives in the presence of DNA were indistinguishable from solutions without added DNA. However, emission measurements showed evidence for pyrene–DNA interactions. Free pyrenebutyrate and pyrenesulfonate both showed single-exponential emission decays with lifetimes of 83 and 50 ns, respectively. In contrast, when 1 equiv of the octameric duplex was also present, a double ex-

(24) Snare, M. J.; Thistlewaite, P. J.; Ghiggino, K. P. *J. Am. Chem. Soc.* **1983**, *105*, 3328.
(25) Funt, B. L.; Hoang, P. M. *J. Electrochem. Soc.* **1984**, *131*, 2295.
(26) Meites, L.; Zuman, P. *Handbook of Organic Electrochemistry*; CRC Press: Cleveland, OH, 1977; Vol. I.
(27) Chen, R. F. *Anal. Biochem.* **1974**, *57*, 593.
(28) Chen, F.-M. *Nucleic Acids Res.* **1983**, *11*, 7231.

ponential was needed to fit the data for both labels. The major emission components (70% for pyrenebutyrate and 89% for pyrenesulfonate) had roughly the same values as the lifetimes of the free labels. However, the minor components had considerably shorter lifetime values (~2 ns). Pyrene–DNA interactions were also manifested in decreased quantum yields relative to pyrene solutions without added DNA. Despite the lower sample concentrations used here and the relatively higher water solubilities of these pyrene acids relative to pyrene itself, pyrene/duplex association still appears to occur, but it is not dominant over dissociation. Covalent attachment between pyrene and DNA will strongly favor association.

Emission from Duplexes Singly Labeled with Pyrene. Internal covalent attachment of pyrenebutyrate to the octanucleotide 5′-GCACTCAG-3′ leads to a large decrease (50 times) in emission quantum yield and multiple emission lifetimes all much shorter than that of the free label. It is striking that 50% of the pyrenebutyrate excited states in the labeled oligomer have an emission lifetime of only 200 ps compared to lifetimes of at least 50 ns for the free-label models. While three lifetimes of 200 ps, 2 ns, and 16 ns characterize the emission decay of this single strand, it is not necessarily true that there are only three distinct excited-state decay processes. It is possible that there are many times this number of such processes, and we are not proposing a microscopic model to explain these decays. Rather, we view the emission lifetimes in Table II as qualitative indicators of label/duplex and possibly label/label interactions. Their significance here lies in comparison of the patterns of lifetimes found for systematically varied samples.

Internal covalent attachment of pyrenesulfonate to the above octanucleotide results in 4 times less emission than was found for free pyrenesulfonate. For this labeled single strand, 21% of the pyrene excited states have an emission lifetime of ~200 ps. This decrease in the fraction of emission with the shortest lifetime relative to that seen in the single strand labeled with pyrenebutyrate is paralleled by a similar decrease in the fraction of short lifetime (~2 ns) emission in the experiments with equal amounts of free labels and added DNA. There the pyrenebutyrate label has 30% of its emission shortened, while the pyrenesulfonate label has only 11% shortened.

Both pyrenes show reduced emission quantum yields in the singly labeled duplexes, ≥500 times less emission for pyrenebutyrate and 17 times less for pyrenesulfonate relative to free pyrenebutyrate and pyrenesulfonate, respectively. Both singly labeled duplexes also showed a significant reduction in the lifetime of the short component and a greater than 50% increase in its relative share of the duplex's total emission compared to the corresponding labeled octanucleotides. It is striking to note that for the pyrenebutyrate-labeled duplex 93% of the emission decays with lifetimes less than 750 ps, while for the pyrenesulfonate-labeled duplex 64% of the emission lifetimes are 2 ns or longer. Clearly the best chance of observing label/label interactions will be found in pyrenesulfonate-labeled duplexes. The above emission yield and lifetime patterns are also consistent with the earlier described thermodynamic results for duplex formation. The pyrenebutyrate-labeled duplex showed a $\Delta G°$ decrease of 1.5 kcal/mol relative to the corresponding modified but unlabeled duplex, while the pyrenesulfonate labeled one showed no difference in $\Delta G°$ relative to the same unlabeled duplex.

Emission from Doubly Labeled Duplexes. Comparison of the emission yields and lifetimes for duplexes doubly and singly labeled with pyrenebutyrate shows remarkable similarity. This is reasonable if one views each label as interacting strongly with the duplex and independently of the other. Relevant here is the earlier finding that the duplex stabilization for one label was 1.5 kcal/mol and for two labels was 3.0 kcal/mol. Also, comparison of these same results with those for the duplex labeled with both pyrenebutyrate and anthraquinone shows no appreciable differences. The fact that it makes no difference whether the second label is pyrenebutyrate or anthraquinone is consistent with the earlier observation that both labels produce the same duplex stabilization, 1.5 kcal/mol, and have nearly the same $\Delta H°$ and $\Delta S°$ values for duplex formation. Thus, no evidence is seen for either excimer formation or oxidative quenching in doubly labeled duplexes containing pyrenebutyrate, but the constancy of the emission results for these three duplexes is remarkable and provides valuable reference data as a case without label/label interactions.

Since the thermodynamic results for a single anthraquinone label indicate that it is strongly associated with (or intercalated into) the duplex, this label is not likely to be free to quench significant amounts of pyrene emission. In fact the quantum yield and lifetime results for the duplexes labeled with either pyrenebutyrate or pyrenesulfonate together with anthraquinone are very similar to the results for the duplexes singly labeled with the corresponding pyrene derivative. This similarity extends even as far as the relative amounts of each lifetime component, indicating that the presence of the anthraquinone label has little spectroscopically detectable effect on either pyrene label.

The case of the duplex containing two pyrenesulfonate labels is the most interesting. In contrast to the results involving pyrenebutyrate-labeled duplexes, the duplex doubly labeled with pyrenesulfonate shows major differences in emission yield and lifetimes relative to both the duplex with a single pyrenesulfonate label and the duplex containing pyrenesulfonate and anthraquinone. This doubly labeled duplex has less total emission, and all three emission lifetime components are shortened (see Table II). This can also been seen graphically in Figure 3, which shows the emission decay spectra for the duplexes labeled with pyrenesulfonate and anthraquinone (3A) and with two pyrenesulfonate labels (3B). Additionally, the magnitudes of both of the longer lifetime components decrease in the duplex with two pyrenesulfonate labels, while the shortest lifetime component shortens 3.5 times and increases 50% in fractional weight, relative to the singly labeled duplex. These differences strongly indicate that a new pyrene emission–decay path was created by the addition of a second pyrenesulfonate label.

The objective of attaching two pyrene labels to a duplex was to observe pyrene excimer formation as an indication of label/label interaction. While increased pyrene emission decays were seen for the duplex with two pyrenesulfonate labels, no excimer emission was found. However, it is likely that the bases of a DNA duplex can quench pyrene excimer emission as well as they quench that of the pyrene monomers. If this is true, it accounts for our failure to detect excimer emission. Nevertheless, excimer emission has recently been reported in a related system by Eriksson et al.,[29] who prepared duplexes of poly(dG-dC) and $(dG\text{-}dC)_6$ in which (+)-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-7,8-diol [(+)-*anti*-BPDE] was covalently bound to DNA duplexes at multiple locations. This molecule binds specifically at N-2 of guanine, facing the minor groove of DNA.[30] These workers observed excimer emission for both poly and dodecamer duplexes, which arose from the interaction between (+)-*anti*-BPDE molecules attached to nearby guanine bases on the same duplex.[29] This demonstrates that it is possible to observe excimer emission from polycyclic aromatic hydrocarbon chromophores covalently bound to DNA duplexes, provided the chromophores are not associated with the bases and yet are positioned proximally to each other.

Conclusion

The results reported here show that octanucleotides with covalently attached internal labels, one on each strand, hybridize to produce a duplex in which the labels are capable of interacting with each other. These results also make it clear that in order to maximize label/label, rather than label/duplex interactions, an appropriate molecular design strategy must be chosen. This study used relatively long, flexible linker arms to attach the labels to modified thymidines. This linkage allowed the labels, themselves significantly hydrophobic, to associate with the duplex so that neither pyrene excimer emission nor oxidative quenching by anthraquinone was observed. Nevertheless, an important first step was made. In addition, we have shown that melting curve analysis

(29) Eriksson, M.; Norden, B.; Jernstrom, B.; Graslund, A.; Lycksell, P.-O. *J. Chem. Soc., Chem. Commun.* **1988**, 211.
(30) Meehan, T.; Straub, K. *Nature* **1979**, *277*, 410.

is a useful tool for assessing whether or not label/duplex interactions are present and whether or not label attachment has significantly affected the ability of the complementary strands to hybridize. The combination of thermodynamic measurements and steady-state and time-resolved emission studies has been shown to provide a consistent and complementary view of label/label and label/duplex interactions.

The next step is to refine the molecular design strategy to optimize label/label interactions. An important improvement would be the construction of more rigid linker arms to minimize label/duplex association. This could be done by introducing either a six-membered ring like piperazine or an alkyne into the attachment modification. For the former case, ethylenediamine could be replaced by piperazine in the synthesis of the modified thymidine reported here. For the latter case, linker arms with a terminal primary amine could be derived from propargylamine.[31] Another design improvement would involve attaching labels at internucleotide phosphate sites. Such labeling is less likely to interfere with base pairing than is base labeling, yet flexibility in label location is preserved. Phosphate labeling has been used at terminal sites by several groups to prepare singly labeled duplexes.[5,6] Finally, a good molecular design would employ hydrophilic labels whenever possible to minimize further label/duplex association. When these steps are taken, it will be possible to prepare DNA-based macromolecules with specifically located molecular subunits. Some fundamental processes that these subunits can be arranged to carry out are energy, electron, and proton translocations.

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Registry No. GCACTCAG, 121329-89-3; CTGAGTGC, 121808-78-4; GCAC(T*)CAG, 121351-63-1; CTGAG(T*)GC, 121808-81-9; GCACTCAG + CTGAGTGC, 121844-89-1; GCAC(T*)CAG + CTGAGTGC, 121808-84-2; GCACTCAG + CTGAG(T*)GC, 121844-90-4; GCAC(T*)CAG + CTGAG(T*)GC, 121808-88-6; T*-pyrenebutyrate, 121808-79-5; T*-pyrenebutyrate + CTGAGTGC, 121808-85-3; T*-pyrenesulfonate, 121808-80-8; T*-pyrenesulfonate + CTGAGTGC, 121808-86-4; T*-anthraquinone, 121808-82-0; GCACTCAG + T*-anthraquinone, 121808-87-5; 1-pyrenebutanoic acid, 3443-45-6; sodium 1-pyrenesulfonate, 59323-54-5; *N*-(1-pyrenesulfonyl)-ethylenediamine, 121808-83-1.

(31) Prober, J. M.; Trainor, G. L.; Dam, R. J.; Hobbs, F. W.; Robertson, C. W.; Zagursky, R. J.; Cocuzza, A. J.; Jensen, M. A.; Baumeister, K. *Science* **1987**, *238*, 336.

Preparation and Characterization of an Aflatoxin B_1 Adduct with the Oligodeoxynucleotide d(ATCGAT)$_2$

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Abstract: Preparation of a double-stranded aflatoxin B_1–oligodeoxynucleotide adduct by direct addition of aflatoxin B_1 8,9-epoxide to d(ATCGAT)$_2$ is described. Reaction occurred rapidly at 5 °C to give a high yield of adduct. The reaction reached a limiting stoichiometry of 1:1 aflatoxin B_1 to d(ATCGAT)$_2$. The major product, which exhibited UV absorbance at 360 nm, was identified as 8,9-dihydro-8-{N^7-guanyl[d(ATCGAT)]}-9-hydroxyaflatoxin B_1·d(ATCGAT). Reversed-phase HPLC yielded equimolar quantities of unmodified d(ATCGAT) and 8,9-dihydro-8-{N^7-guanyl[d(ATCGAT)]}-9-hydroxyaflatoxin B_1. Acid hydrolysis followed by reversed-phase HPLC yielded 8,9-dihydro-8-N^7-guanyl-9-hydroxyaflatoxin B_1 [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. Lin, J. K.; Miller, J. A.; Miller, E. C. *Cancer Res.* **1977**, *37*, 4430–4438]. Gentle heating at pH 8 and subsequent acid hydrolysis gave products consistent with formation of formamidopyrimidine (FAPY) aflatoxin B_1 derivatives. Circular dichroism measurements showed negative ellipticity at 360 nm and an increase in positive ellipticity at 260 nm as compared to unmodified d(ATCGAT)$_2$. UV melting curves demonstrated that adduct formation increased duplex stability. Spontaneous depurination of the modified duplex was observed but was sufficiently slow at 5 °C and neutral pH to obtain NMR spectra. ^{1}H NMR spectra exhibited a doubling of oligodeoxynucleotide resonances upon adduct formation due to loss of strand symmetry; strand exchange between modified and unmodified oligodeoxynucleotide duplexes was slow on the NMR time scale. Adduct formation resulted in increased shielding for aflatoxin protons. The C^8 proton of the modified guanine was not observed in the ^{1}H NMR spectrum in D_2O, but an additional signal tentatively assigned as that proton was observed at 9.75 ppm in the spectrum in H_2O. This signal was not observed after mild basic hydrolysis of the duplex cationic adduct. Six hydrogen-bonded NH resonances were observed between 12 and 14 ppm. ^{31}P NMR showed a doubling of resonances and one signal shifted downfield at least 1 ppm. A structure for the adduct is proposed in which the aflatoxin moiety is intercalated above the 5′ face of the modified guanine.

Aflatoxin B_1 (AFB1, **1**) is among the most potent environmental mutagens.[1] It is an established animal carcinogen and is implicated as a human carcinogen. Genotoxicity requires oxidative activation involving the 8,9 double bond in the terminal furan moiety. Although the product of this oxidation, putatively epoxide **2**, has never been detected in biological systems, its existence has been postulated on the basis of in situ reaction of microsomally activated **1** with DNA followed by hydrolysis to give products consistent with structure **2** for the reactive electrophile.[2] Fur-

(1) For review see: (a) Busby, W. F., Jr.; Wogan, G. N. In *Chemical Carcinogens*, 2nd ed.; Searle, C., Ed.; ACS Monograph Series 182; American Chemical Society: Washington, DC, 1984; pp 945–1136. (b) Essigmann, J. M.; Green, C. L.; Croy, R. G.; Fowler, K. W.; Büchi, G.; Wogan, G. N. *Cold Spring Harbor Symp. Quant. Biol.* **1983**, *47*, 327–337. (c) Hsieh, D. P. H.; Wong, J. J. In *Biologically Active Intermediates*; Snyder, et al., Eds.; Plenum: New York, 1982; pp 847–863. (d) Garner, R. C. *Br. Med. Bull.* **1980**, *36*, 47–52.

(2) (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) Garner, R. C.; Miller, E. C.; Miller, J. A. *Cancer Res.* **1972**, *32*, 2058–2066.

0002-7863/89/1511-7232$01.50/0