Solid-Phase Synthesis of Oligonucleotides Containing Site-Specific *N*-(2′-Deoxyguanosin-8-yl)-2-(acetylamino)fluorene Adducts Using 9-Fluorenylmethoxycarbonyl as the Base-Protecting Group†

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ABSTRACT: Eight oligodeoxyribonucleotides containing a site-specific *N*-(2′-deoxyguanosin-8-yl)-2-(acetylamino)fluorene (dG-C8-AAF) adduct were prepared successfully by solid-phase DNA synthesis using the 2-cyanoethyl *N,N*-diisopropylphosphoramidites of dA, dC, dG, dT, and dG-C8-AAF, with 9-fluorenylmethoxycarbonyl (Fmoc) as the base-protecting group. The oligonucleotides were deprotected and released from the support by 1:9 piperidine/MeOH at room temperature for 22–36 h or by 1:1 diisopropylamine in MeOH at 55 °C for 15 h, purified by HPLC, and fully characterized. About 6 mg of HPLC-purified d[GTGGCG$^{(C8-AAF)}$CCAAGT] and 7 mg of d[GTGATG$^{(C8-AAF)}$ATAAGT] were obtained from the 10-μmol-scale synthesis, and their 1D ^{1}H NMR spectra were consistent with the presence of a dG-C8-AAF adduct. The dG-C8-AAF oligonucleotides were also deacetylated to afford the corresponding dG-C8-AF oligonucleotides. d[GTGGCG$^{(C8-AAF)}$CCAAGT] formed stable 1:1 duplexes with both the fully complementary 12-mer and a GC-deleted (across the adduct) 10-mer complement, and identical melting temperatures were observed for both duplexes. The multidimensional NMR study of these duplexes is presently under investigation.

It is generally accepted that the first step in the induction of a neoplastic transformation by a chemical carcinogen is the reaction of an electrophilic agent or a metabolically activated species with DNA to produce a covalent adduct that alters the structure of the DNA and induces a mutation (Cairns, 1981; Singer & Grunberger, 1983). It is also becoming increasingly clear that there is a relationship between the structure an adduct induces in a DNA molecule and the type of mutation that is produced (Loechler, 1989). *N*-Acetyl-2-aminofluorene (AAF[1]) has served as a model carcinogen in the study of this relationship. Following AAF treatment of experimental animals, two major DNA adducts have been observed bound to the C8 position of guanine, *N*-(2′-deoxyguanosin-8-yl)-2-(acetylamino)fluorene (dG-C8-AAF) and *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (King, 1985), although a minor adduct, *N*-(deoxyguanosin-N^2-yl)-2-(acetylamino)fluorene (Westra et al., 1976), is also formed. The dG-C8-AAF adduct has been shown to induce a major distortion in the DNA due to the steric crowding produced by the presence of the acetyl group, while the dG-C8-AF adduct is believed to be relatively nondistorting (Fuchs et al., 1976; Grunberger & Weinstein, 1976; Singer & Grunberger, 1983; Norman et al., 1989; O'Handley et al., 1993). Interestingly, these adducts show very different mutagenic properties: the dG-C8-AAF adduct is much more prone to induce frame-shift mutations in bacteria, while the dG-C8-AF adduct produces almost exclusively base substitution mutations (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985). Moreover, the mutations induced by the dG-C8-AAF adduct are very dependent on the surrounding DNA sequence, and recent data suggest that there is a correlation between the local structure and the sequence where the adduct is positioned (Belguise-Vallandier & Fuchs, 1991; Veaute & Fuchs, 1991; Lambert et al., 1992).

We have designed a chemical synthesis of oligodeoxyribonucleotides containing site-specific dG-C8-AAF or dG-C8-AF adducts, which will allow the production of multimilligram quantities of modified oligonucleotides having any desired sequence.[2] The key feature of this method is the use of 9-fluorenylmethoxycarbonyl (Fmoc) as the base-protecting group. We have previously reported the synthesis of the 2-cyanoethyl *N,N*-diisopropylphosphoramidites of Fmoc-protected dA, dC, dG, and dG-C8-AAF (**1–4**).[2] Our studies indicate that the Fmoc group in these compounds can be quantitatively deprotected under conditions where the dG-C8-AAF structural unit is stable.[2] We report here the use of these Fmoc-protected phosphoramidites in the successful large-scale solid-phase synthesis and rigorous characterization of eight oligonucleotides, each containing a site-specific dG-C8-AAF adduct. The multidimensional NMR study of these duplexes is presently under investigation.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. 5′-*O*-(4,4′-Dimethoxytrityl)-N^6-(9-fluorenylmethoxycarbonyl)-2′-deoxyadenosine 3′-[2-cyanoethyl *N,N*-diisopropylphosphoramidite] (**1**), 5′-*O*-(4,4′-dimethoxytrityl)-N^4-(9-fluorenylmethoxycarbonyl)-2′-deoxycytidine 3′-[2-cyanoethyl *N,N*-diisopropylphosphoramidite] (**2**), 5′-*O*-(4,4′-dimethoxytrityl)-N^2-(9-fluorenylmethox-

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[1] Abbreviations: dG-C8-AAF, *N*-(2′-deoxyguanosin-8-yl)-2-(acetylamino)fluorene; Fmoc, 9-fluorenylmethoxycarbonyl; AAF, *N*-acetyl-2-aminofluorene; dG-C8-AF, *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene; CED, (2-cyanoethyl)-*N,N*-diisopropyl; CPG, controlled pore glass; DMTr, dimethoxytrityl; TFA, trifluoroacetic acid; TEAA, triethylammonium acetate; *i*Bu, isobutyryl; DCA, dichloroacetic acid; dH_2O, distilled, deionized water.

[2] Y. Zhou and L. J. Romano, manuscript submitted for publication.

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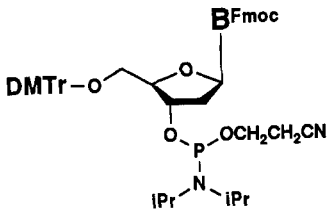
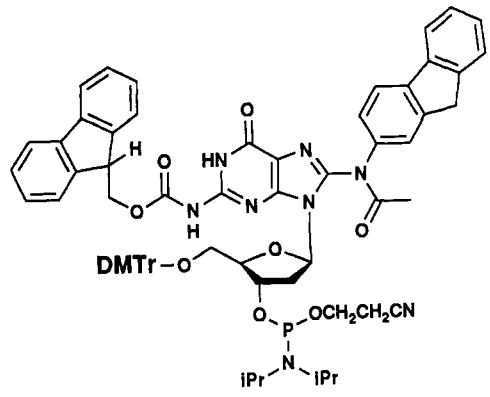
Chart I



B^{Fmoc} = N^6-Fmoc-Ade (1)
N^4-Fmoc-Cyt (2)
N^2-Fmoc-Gua (3)
N^2-Fmoc-Gua-C8-AAF (4)



(4)

ycarbonyl)-2′-deoxyguanosine 3′-[2-cyanoethyl *N,N*-diisopropylphosphoramidite] (**3**), 5′-*O*-(4,4′-dimethoxytrityl)-N^2-(9-fluorenylmethoxycarbonyl)-C^8-(*N*-fluoren-2-ylacetamido)-2′-deoxyguanosine 3′-[2-cyanoethyl *N,N*-diisopropylphosphoramidite] (**4**), and *N*-(guanin-8-yl)-2-(acetylamino)-fluorene (Gua-C8-AAF) were synthesized as described.[2] *N*-Acetoxy-*N*-acetyl-2-aminofluorene was prepared as described (Cramer et al., 1960). 5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxythymidine 3′-[2-cyanoethyl *N,N*-diisopropylphosphoramidite] and other reagents used in oligonucleotide synthesis were purchased from Beckman. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxythymidine succinyl-controlled pore glass (30–54 μmol/g) was purchased from Sigma. Triethylamine (gold label), anhydrous dichloromethane, HPLC-grade acetonitrile and methanol, 1,1,3,3-tetramethylguanidine, 2-nitrobenzaldoxime, and diisopropylamine were purchased from Aldrich. Chloroacetic acid was from MC/B. Anhydrous piperidine was prepared by refluxing with and then distilling over CaH_2. Anhydrous trifluoroacetic acid was from EM Science. [γ-^{32}P]ATP (4500 Ci/mmol) was purchased from ICN Radiochemicals. T4 polynucleotide kinase and phosphodiesterase I (*Crotalus adamanteus* venom) were from USB. Alkaline phosphatase (bovine intestinal mucosa, Type VII-s) was from Sigma. Nensorb Prep nucleic acid purification columns were from Du Pont.

A Hewlett-Packard 8452A diode array spectrophotometer equipped with a variable-temperature cell holder was used to obtain the UV spectra, UV mixing curves, and UV melting curves, with the cell temperature regulated by a Neslab RTE-8 circulating water bath. The temperatures were measured using a Fluke 51 digital thermometer by placing an immersible probe between the cell wall and the holder. For thermal melting experiments, a temperature increase of about 1 °C/min was used, and the temperature and absorbance were read simultaneously at 1-deg intervals. Normalized melting curves ($1 - \alpha$ vs T, the fraction of coil state as a function of temperature) were derived from the 260-nm absorbance data by applying the upper and lower base-line adjustments as described previously (Breslauer, 1986). T_m values were taken as the temperature at which $1 - \alpha = 0.5$. For UV spectra and UV mixing experiments, the temperatures of the cells were regulated to ±0.1 °C and a 10-min equilibration was allowed before each spectrum or absorbance was taken. The solvent used in all of these UV measurements and NMR recordings was 0.1 M NaCl containing 10 mM NaH_2PO_4 and 0.5 mM EDTA (pH 7).

All HPLC experiments used a Varian 5000 HPLC system with a Polychrom 9060 diode array detector channel setting at both 254 and 302 nm. Data were output to a Macintosh SE for further treatment using Dynamax HPLC controller software. Analytical HPLC was conducted using Beckman Ultrasphere C18 250 × 4.6 mm and Hamilton PRP-1 250 × 4.1 mm columns at a flow rate of 1 mL/min. Preparative HPLC of AAF oligonucleotides was performed on a Hamilton PRP-1 350 × 7 mm column with a flow rate of 3 mL/min.

Polyacrylamide gel electrophoresis was performed using standard procedures (Sambrook et al., 1989). Samples were 5′-^{32}P-labeled, electrophoresed on 20% denaturing gel, and autoradiographed with Kodak X-OMAT AR film.

^{1}H NMR spectra of oligonucleotides were recorded on a Varian Unity 500-MHz NMR spectrometer in D_2O buffer at different temperatures, using a preset pulse sequence and the transmitter for residual water suppression. To prepare the NMR samples, about 5 mg of HPLC-purified oligonucleotide was desalted using a 3-mL C18 Sep-Pak cartridge (Water), exchanged to the sodium form, passed through a Chelex column (Bio-Rad 100, sodium form) to remove paramagnetic impurities, and then dissolved in 0.6 mL of H_2O containing 0.1 M NaCl, 10 mM Na_2HPO_4, and 0.5 mM EDTA (pH 7). The solutions were carefully neutralized to pH 7 by adding NaOH or HCl, exchanged with 100% D_2O (Aldrich) three times, redissolved in 0.6 mL of D_2O, and carefully degassed using N_2 after transfer into the NMR tubes.

Solid-Phase Assembly of AAF Oligonucleotides. The AAF oligonucleotides were assembled from the Fmoc (**1–4**) and dT phosphoramidites on a Beckman System 1 Plus DNA synthesizer with a built-in four-step standard CED cycle for a 1-μmol-scale synthesis: deblocking (5% dichloroacetic acid in CH_2Cl_2), condensation (activator gold containing 3–8% 5-methylthiotetrazole in CH_3CN), oxidation (aqueous iodine), and capping (acetic anhydride with (dimethylamino)pyridine as catalyst) (Gait, 1984). For our 10-μmol-scale syntheses, we created a nonstandard CED cycle based on the built-in 10-μmol-scale methylphosphoramidite cycle by extending the condensation time from 105 s (7 loops × 15 s) to 640 s (40 loops ×16 s) while keeping the rest of the cycle unchanged. The solid support was the functionalized long-chain aminoalkyl-controlled pore glass (CPG) with the first nucleoside dimethoxytrityl (DMTr)-dT attached via a succinyl linker. AAF-adducted oligonucleotides were assembled using our prepared CED phosphoramidites with Fmoc as the base-protecting group (**1–4**) together with the commercial dT CED phosphoramidite. Phosphoramidite solutions were made in base diluent from Beckman, except for **3** which was dissolved in anhydrous CH_2Cl_2 instead. The concentrations of the phosphoramidites were 0.25 M for 10-μmol-scale synthesis

Table I: Solid-Phase Synthesized dG-C8-AAF Oligonucleotides

oligomer	sequence	synthesis scale (μmol)
AAF-6-mer	TTT G(AAF)TT	2 × 1
AAF-12-mer-1	GTG GCG(AAF) CCA AGT	2 × 10
AAF-12-mer-3	GTG ATG(AAF) ATA AGT	10
AAF-12-mer-4	GTC GGG(AAF) GGA AGT	10
AAG-12-mer-5	GTC GCG(AAF) CGA AGT	10
AAF-12-mer-2	TCG GCC G(AAF)TC GTT	1
AAF-12-mer-6	TGG GGC G(AAF)TG GTT	1
AAF-9-mer	GGC G(AAF)TC GTT	1

and 0.1 M for the 1-μmol-scale synthesis. The deblocking effluents, after passing through the internal photometer, were collected in a Gilson 203 microfraction collector and assayed for dimethoxytrityl cation by measuring absorbances at 504 or 416 nm of the sample solutions[3] in 5% dichloroacetic acid in CH_2Cl_2. The 5'-DMTr group was unremoved in all assembled oligonucleotides to allow easy purification by Nensorb and reversed-phase HPLC. Table I lists the solid-phase assembled AAF oligonucleotide sequences.

Deprotection of AAF Oligonucleotides. The dried CPG anchoring the AAF-6-mer was treated at room temperature for 8 h with 1 mL of oximate solution made by mixing 50 μL of 1,1,3,3-tetramethylguanidine with 70 mg of 2-nitrobenzaldoxime in 1 mL of 1:1 dioxane/water (Reese & Zard, 1981). The CPG was then filtered off, and the filtrate was extracted with 4 × 5 mL of diethyl ether and 4 x 5 mL of CH_2Cl_2 until no UV-absorbing species was present in the organic extracts. The aqueous phase containing the AAF-6-mer was then dried in a rotary evaporator for further purification.

A typical procedure for large-scale deprotection of the AAF-12-mer-1 (Table I) used 1:9 anhydrous piperidine in methanol. The DMTr-AAF-12-mer-1-CPG (150 mg) was mixed with 2 mL of anhydrous piperidine and 18 mL of anhydrous methanol in a dry flask at room temperature for 36 h, with occasional shaking every few hours. Alternatively, a 1:1 mixture of diisopropylamine/MeOH at 55 °C for 15 h gave comparable results. The supernatant was then separated from the CPG, and the CPG was washed twice with 2 mL of methanol. The remaining CPG was assayed for the DMTr group upon treatment with 5% dichloroacetic acid in dichloromethane. This gave a CPG cleavage yield of greater than 98%. The supernatant and the washing were combined, 1 mL of 1 M aqueous potassium phosphate (pH 7) was then added, and the resulting mixture was dried on a rotary evaporator to give an almost white solid residue. The residue was extracted with a mixture of 5 mL of dH_2O and 10 mL of H_2O-saturated ether, and the aqueous phase containing the fully deprotected AAF oligonucleotide was further extracted three times with 5 mL of H_2O-saturated ether. After brief rotatory evaporation to remove the ether, the aqueous phase was then ready for purification by Nensorb and HPLC.

Nensorb Purification of AAF Oligonucleotides. The standard Nensorb purification procedure was modified to separate the full-length AAF-containing oligonucleotides from the failure sequences. For purification of the AAF-6-mer, a 60-mL (instead of 10 mL) wash with 0.1 M triethylammonium acetate (TEAA) (pH 7) was used following sample loading in order to remove the oximate contaminants. After removal the failure sequences by 10% CH_3CN/TEAA and on-column detritylation by 0.5% aqueous trifluoroacetic acid (TFA), the full-length AAF-6-mer was recovered by 35% aqueous MeOH elution. For purification of AAF-12-mers, all TEAA-containing buffers were replaced by 0.1 M potassium phosphate buffers (pH 7). The crude AAF-12-mers in 4 mL of 0.1 M potassium phosphate buffers (pH 7) were loaded onto the column. A 20-mL 0.1 M potassium phosphate wash was conducted followed by a 20-mL 10% MeCN/0.1 M potassium phosphate (pH 7) wash to remove the failure sequences. At this stage, it is preferable that the full-length DMTr-containing AAF-12-mers be eluted by 35% aqueous MeOH and the DMTr-containing AAF-12-mers be purified further by reversed-phase HPLC. Alternatively, the Nensorb column can be detritylated first and the full-length detritylated AAF-12-mers then recovered by 35% aqueous MeOH elution. In either case, it is desirable to add about 1 mL of 0.1 M potassium phosphate buffer (pH 7) to the 35% MeOH fractions before attempting to dry them, so that the dried AAF-12-mers are easily redissovable in dH_2O.

Modification of d(TTTGTT) with AcO-AAF. The unmodified 6-mer d(TTTGTT) was prepared on the solid-phase synthesizer using standard CED phosphoramidites and was deprotected with concentrated ammonium hydroxide at 55 °C overnight. The 6-mer was purified by Nensorb column and reverse-phase HPLC. About 2 OD units of the 6-mer was modified with excess *N*-acetoxy-2-(acetylamino)fluorene (AcO-AAF) in 2 mM sodium citrate buffer (pH 7) containing 20% ethanol at 37 °C under nitrogen for 2 h (Kriek et al., 1967). Unreacted AcO-AAF was removed by repeated ether extraction, and the remaining aqueous solution was then vacuum-dried and dissolved in 5% acetonitrile/50 mM TEAA (pH 7) for HPLC purification.

TFA Digestion of Oligonucleotides. The HPLC-purified adducted oligonucleotides (0.4–1 OD_{260} unit) were vacuum-dried and incubated with 100 μL of anhydrous trifluoroacetic acid at 70 °C for 30 min as described (Tang & Lieberman, 1983). The hydrolysates were vacuum-dried and analyzed by HPLC. Chromatograms were obtained by monitoring the absorbances at 254 and 302 nm. Coinjections with standard Gua-C8-AAF and Gua-C8-AF were performed to confirm the identity of the AAF(AF)-modified base released by TFA treatment.

Enzymatic Digestion of Oligonucleotides. Enzymatic digestion of AAF oligonucleotides was performed by treatment of HPLC-purified oligonucleotides with phosphodiesterase I at 37 °C for 3 h in a reaction buffer containing 10 mM mercaptoethanol followed by another 3 h with alkaline phosphatase (Shibutani et al., 1991). The reaction mixtures were directly injected into reverse-phase HPLC to analyze the normal four deoxynucleosides. The insoluble portions were then dissolved using 1:1:1 MeOH/AcOEt/10 mM K_2HPO_4 (pH 7) and analyzed for the AAF-adducted nucleoside by reverse-phase HPLC.

RESULTS

Solid-Phase Synthesis of d(TTTGTT) and d[TTTG$^{(C8-AAF)}$TT]. To test the feasibility of N^2-Fmoc-dG phosphoramidite (**3**) and N^2-Fmoc-dG-C8-AAF phosphoramidite (**4**) for the solid-phase synthesis of AAF oligonucleotides, we first synthesized an unmodified 6-mer, d(TTTGTT), and an AAF-adducted 6-mer, d[TTTG$^{(C8-AAF)}$TT], using a standard 1-μmol CED cycle on a Beckman System 1 Plus DNA synthesizer, using **3** and **4** prepared by phosphitylation of DMTr-N^2-Fmoc-dG and DMTr-N^2-Fmoc-dG-C8-AAF with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite.[2]

[3] The absorbances of the deblocking fractions at 504 and 416 nm are strongly dependent on the acidity of the medium. The absorbances reached their maxima when the DCA concentration increased to 2%; they were 3-fold higher than in 0.5% DCA and remained unchanged when the DCA concentration was increased up to 10%.

The assembled 6-mers were deprotected by oximate, and the full-length 6-mers containing a 5′-DMTr group were separated from the failure sequences by Nensorb Prep columns. After detritylation, the full-length 6-mers were then purified by reverse-phase HPLC.

During our initial solid-phase synthesis, we also used a slightly modified 1-μmol CED cycle by replacing the standard detritylating reagent, 5% dichloroacetic acid in CH_2Cl_2, with 5% chloroacetic acid in CH_2Cl_2. This was because 5% dichloroacetic acid in CH_2Cl_2 caused fast depurination to DMTr-N^2-Fmoc-dG-C8-AAF, but treatment with 5% chloroacetic acid in CH_2Cl_2 for 35 min could give quantitative detritylation without any detectable depurination.[2] However, our solid-phase syntheses indicated no dG-C8-AAF depurination, even when detritylation was conducted by the dichloroacetic acid treatment. Therefore, all subsequent solid-phase syntheses used the standard synthesis cycle only.

The coupling efficiencies for the Fmoc phosphoramidite materials used in these initial studies were quite low: 81–86% for **3** and 50% for **4**. We found that this was due to the impurities present in these phosphoramidite preparations. As will be demonstrated later, when we used highly pure Fmoc phosphoramidites, coupling efficiencies were dramatically increased, ranging up to greater than 98%.

As part of these initial studies, we also investigated the effect of the capping step on solid-phase assembly using the Fmoc phosphoramidites, since other studies reported an instability of Fmoc to (dimethylamino)pyridine (Ma & Sonveaux, 1989; Webb & Matteucci, 1986; Kuijpers et al., 1990), the capping catalyst used in our synthesizer. We conducted additional syntheses of the unmodified 6-mer and the AAF-6-mer by excluding the capping step from the standard CED cycle. No difference in trityl response was observed between the two methods. After deprotection under the same conditions (oximate, room temperature, 8 h), the crude oligomers and the Nensorb-separated full-length sequences were compared by PAGE with those obtained from synthesis with capping. Our results showed that the crude oligomers from both methods had identical oligonucleotide compositions, but the full-length sequences showed noticeably higher levels of failure sequence contamination when the capping step was deleted (data not shown). These results indicated that the consequence of possible Fmoc cleavage during capping is insignificant and that the capping step is necessary in order to synthesize highly pure oligonucleotides with the desired sequences.

Characterization of d[TTTG$^{(C8\text{-}AAF)}$TT]. To characterize the 6-mers synthesized from the Fmoc phosphoramidites, a standard, [d(TTTGTT)] was prepared using the commercial N^2-*i*Bu-dG CED phosphoramidite, deprotected by concentrated ammonium hydroxide treatment at 55 °C overnight, and purified by HPLC. The identity of the 6-mer synthesized from **3** to the standard 6-mer synthesized from N^2-*i*Bu-dG CED phosphoramidite was established by HPLC coinjection and PAGE comigration.

The HPLC-purified standard [d(TTTGTT)] was then reacted with *N*-acetoxy-*N*-acetyl-2-aminofluorene (Kriek et al., 1967) to prepare the standard AAF-modified 6-mer, d[TTTG$^{(C8\text{-}AAF)}$TT]. Analysis of the reaction mixture revealed the formation of a major AAF adduct, together with several minor components, as indicated by HPLC (Figure 1C, 23 min) and PAGE (Figure 2, lane h). This major AAF adduct was then assigned to be the desired dG-C8-AAF adduct, d[TTTG$^{(C8\text{-}AAF)}$TT], since the formation of this major adduct has been well-established when this modification is applied to single-stranded DNA fragments (Kriek et al., 1967; Johnson et al., 1986). This species was then isolated and purified by reverse-phase HPLC.

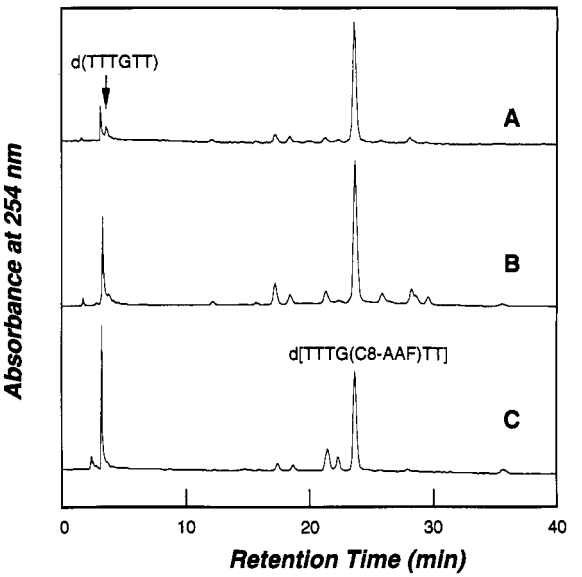


FIGURE 1: HPLC analysis of AAF-6-mer preparations. (A and B) Nensorb-separated full-length d[TTTG$^{(C8\text{-}AAF)}$TT] fractions obtained from solid-phase DNA synthesis. A was synthesized using the standard CED synthesis cycle and B using a modified synthesis cycle employing chloroacetic acid as the detritylation reagent. (C) Reaction mixture from the modification of d(TTTGTT) with *N*-acetoxy-*N*-acetyl-2-aminofluorene. Column: Beckman ultrasphere C18, 250 × 4.6 mm. Elution: linear 20–40% B over 40 min at flow rate of 1 mL/min (buffer A: 5% MeCN in 50 mM TEAA, pH 7; buffer B: 50% MeCN in 50 mM TEAA, pH 7).

The Nensorb-separated, full-length AAF-6-mer preparations from the solid-phase synthesis (using the standard CED cycle and the modified CED cycle) were compared with the reaction mixture of AcO-AAF modification of the unmodified 6-mer. Figure 1 shows the HPLC analysis of the three preparations under elution conditions where the unmodified 6-mer elutes in the void volume and the second detection channel is set at 302 nm to confirm the AAF-containing peaks. It was apparent that the solid-phase preparations contained a major component identical to d[TTTG$^{(C8\text{-}AAF)}$TT], and this identity was further confirmed by their identical UV spectra (not shown) and by HPLC coinjections. The same comparisons were also made by PAGE analysis (Figure 2). As was expected, the AAF-6-mer preparation showed an intense slower moving (relative to the unmodified 6-mer) AAF band, and the HPLC-purified major AAF components from the solid-phase preparations comigrated with the assigned standard d[TTTG$^{(C8\text{-}AAF)}$TT].

As also indicated by PAGE and HPLC analysis, both the solid-phase synthesis and the standard AcO-AAF modification produced multiple AAF-modified oligonucleotide species. We therefore decided to further characterize the major species d[TTTG$^{(C8\text{-}AAF)}$TT] to confirm its dG-C8-AAF identity. Figure 3 shows the conversion of HPLC-purified d[TTTG$^{(C8\text{-}AAF)}$TT] to a later eluting species (panel C) under deacetylation conditions (1 M NaOH containing 0.25 M mercaptoethanol at room temperature), consistent with the formation of the corresponding AF-6-mer. Figure 3B shows the conversion to two earlier eluting species in a 3:2 ratio under oxidation conditions (0.1 M NaOH in the air at 75 °C), indicating the formation of the oxidized 6-mers, OP-I-6-mer and OP-II-6-mer (Shibutani et al., 1991). The dG-C8-AAF identity was also confirmed by standard TFA digestion (Tang

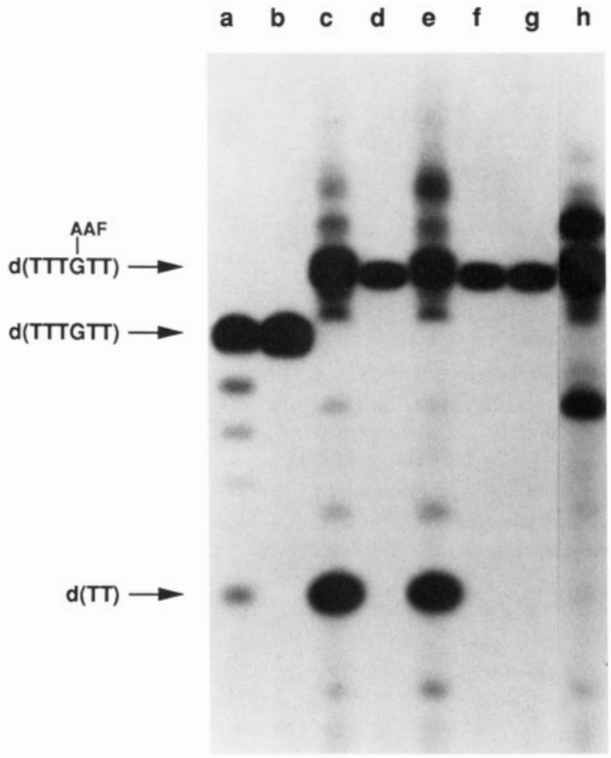


FIGURE 2: ^{32}P PAGE analysis of solid-phase synthesized d[TTTG$^{(C8-AAF)}$TT]. Lanes a and b: Crude and HPLC-purified d(TTTGTT) synthesized by a standard CED phosphoramidite approach. Lanes c and d: Crude and HPLC-purified d[TTTG$^{(C8-AAF)}$TT] synthesized by a standard CED cycle. Lanes e and f: Crude and HPLC-purified d[TTTG$^{(C8-AAF)}$TT] synthesized by a modified CED cycle (with chloroacetic acid as the detritylating agent, see text). Lanes h and g: Crude and HPLC-purified d[TTTG$^{(C8-AAF)}$TT] from the AcO-AAF modification of HPLC-purified d(TTTGTT).

& Lieberman, 1983). Figure 4 shows the HPLC analysis of the TFA hydrolysates of d[TTTG$^{(C8-AAF)}$TT]. As was expected, the hydrolysates showed an early peak (3.5 min) due to thymine with little absorbance at 302 nm and a later peak (19.5 min) due to guanine-C8-AAF, with about the same absorbances at 254 and 302 nm, and which upon HPLC analysis coeluted with an independently prepared guanine-C8-AAF standard.[2]

Large-Scale Synthesis of dG-C8-AAF Oligonucleotides Containing Multiple Guanines. To extend our solid-phase synthesis to larger scale preparations of AAF oligonucleotides containing multiple guanine residues, we reinvestigated the synthesis and purification of Fmoc-CED phosphoramidites. We found that the preparation of highly pure Fmoc phosphoramidites (**1–4**) resulted in dramatically increased coupling efficiencies: usually greater than 98% for **1–3**, and about 95% for **4**. The new phosphoramidites were synthesized by a cleaner phosphitylation reaction with 2-cyanoethyl *N,N,-N′,N′*-tetraisopropylphosphorodiamidite as the phosphitylation reagent, and the products were further purified by flash chromatography.[2] Using these pure phosphoramidites, we synthesized seven additional oligonucleotide sequences on either the 1-μmol or 10-μmol scale, each containing a dG-C8-AAF together with several normal dGs. The sequences of these AAF oligonucleotides are listed in Table I. One of the oligonucleotides (AAF-12-mer-1) contains a dG-C8-AAF in one of the three guanines in the *Nar*I sequence (GGCGCC), which has been shown to be a hot spot for frame-shift mutagenesis (Koffel-Schwartz et al., 1984). The trityl responses for the 10-μmol synthesis of this oligonucleotide are shown in Figure 5.

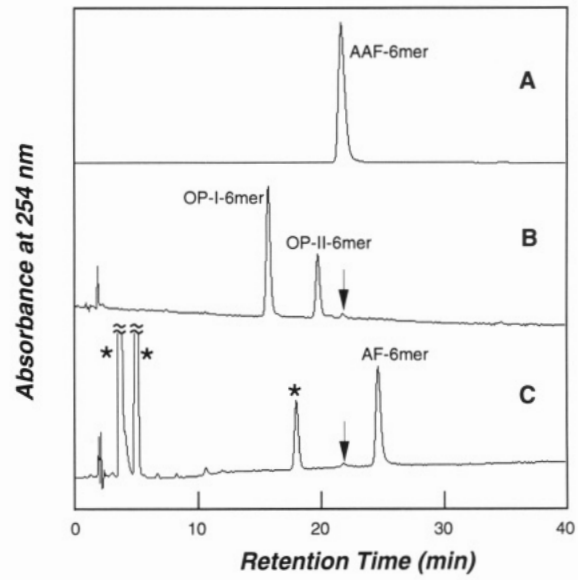


FIGURE 3: HPLC analysis of conversion of d[TTTG$^{(C8-AAF)}$TT] (AAF-6-mer) into d[TTTG$^{(C8-AF)}$TT] (AF-6-mer) and oxidized 6-mers (OP-I-6-mer and OP-II-6-mer). (A) HPLC-purified AAF-6-mer. (B) Oxidation of AAF-6-mer with 0.1 N NaOH at 75 °C in air for 2 h. (C) Deacetylation of AAF-6-mer with 1 N NaOH containing 0.25 N mercaptoethanol at room temperature for 45 min. Column: Beckman ultrasphere C18, 250 × 4.6 mm. Elution: linear 20–40% B over 40 min at a flow rate of 1 mL/min (buffer A: 5% MeCN in 50 mM TEAA, pH 7; buffer B: 50% MeCN in 50 mM TEAA, pH 7). The peaks indicated with an asterisk are due to the mercaptoethanol. The arrow indicates the position where the AAF-6-mer eluted on coinjection.

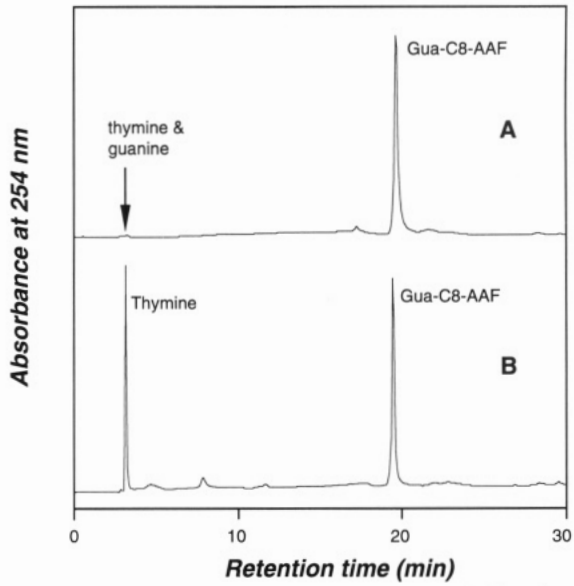


FIGURE 4: HPLC analysis of trifluoroacetic acid digestion of d[TTTG$^{(C8-AAF)}$TT]. (A) Standard guanine-C8-AAF (0.3 OD) in 50 μL of 50% MeCN/50 mM TEAA (pH 7). (B) TFA digested TTTG$^{(C8-AAF)}$TT (0.4 OD) in 50 μL of 50% MeCN/50 mM TEAA (pH 7). Column: Beckman Ultrasphere C18, 250 × 4.6 mm. Elution conditions: linear 10–75% acetonitrile in 50 mM TEAA (pH 7) at a flow rate of 1 mL/min.

Although oximate treatment was effective for the deprotection of the AAF-6-mer, it was unable to completely remove the Fmoc group from the AAF-12-mers (Figure 6A). When AAF-12-mer-1-CPG was treated with oximate (with or without 0.25 M mercaptoethanol) under the same conditions employed for AAF-6-mer deprotection, less than 10% of the released oligonucleotides were free of Fmoc. Extended treatment with oximate was found only to cause more

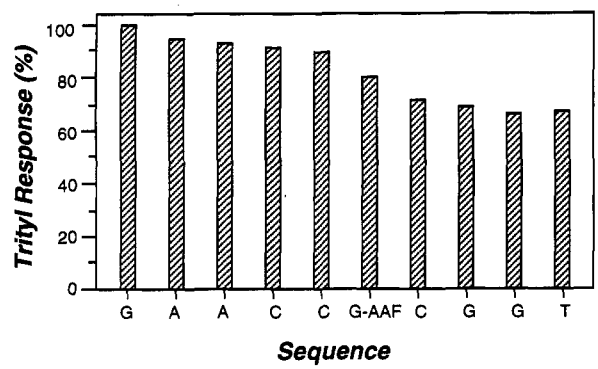


FIGURE 5: Relative trityl responses for the solid-phase synthesis of 5′-DMTr-d[GTGGCG(C8-AAF)CCAAGT] (AAF-12-mer-1) on a 10-μmol scale. Category labels along the horizontal axis represent the 5′-nucleotide of the growing oligonucleotide chain from which the DMTr group was released upon detritylation. The trityl response in the second cycle was set as 100%.

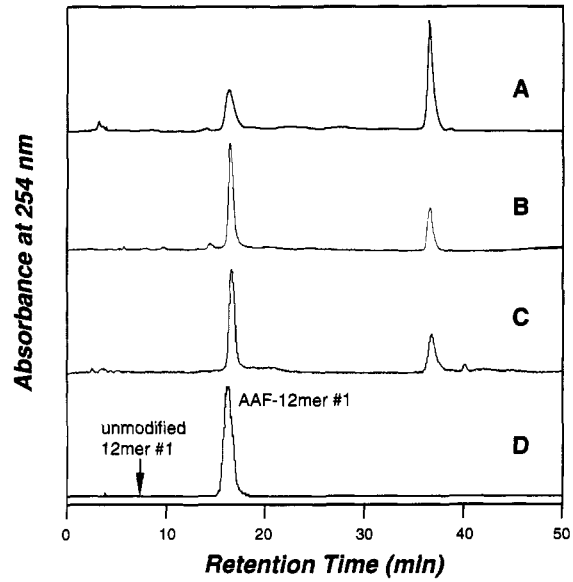


FIGURE 6: HPLC analysis of Nensorb-separated full-length d[GTGGCG(C8-AAF)CCAAGT] (AAF-12-mer-1) fractions. (A) Deprotected by oximate at room temperature for 18 h. (B) Deprotected by 1:9 piperidine/MeOH at room temperature for 22 h. (C) Deprotected by 1:1 diisopropylamine/MeOH at 55 °C for 15 h. (D) HPLC-purified AAF-12-mer-1. HPLC conditions: Hamilton PRP-1, 250 × 4.1 mm column. Elution: 0–10 min, 5–11% B; 10–30 min, 11% B; 30–50 min, 11–100% B. Flow rate: 1 mL/min (buffer A: 10 mM KH_2PO_4, pH 7; buffer B: 7:1:2 ACN/MeOH/A).

degradation of the oligonucleotides. Concentrated ammonium hydroxide (containing 0.25 M mercaptoethanol) at room temperature gave CPG cleavage in about 1 h, but the Fmoc cleavage was inefficient. Prolonged treatment or increased temperature caused extensive deacetylation.

In the effort to search for a satisfactory final deprotection procedure, the CPG containing the assembled AAF-12-mer-1 was subjected to treatments with various reagents under different conditions. Several nonnucleophilic tertiary amines, such as triethylamine, diisopropylethylamine, 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), and 1,4-diazabicyclo[2.2.2]octane, were found to be too slow to remove Fmoc completely and to cleave CPG in solvents such as CH_2Cl_2, CH_3CN, pyridine, or MeOH at temperatures up to 55 °C. Fmoc deprotection by a primary amine such as ethylenediamine in 95% aqueous ethanol (Miller et al., 1991) was efficient but was accompanied by noticeable AAF deacetylation, as was found for 2 M methanolic ammonia. Potassium carbonate in anhydrous MeOH, the favored reagent to remove Fmoc from phosphate-methylated oligonucleotides (Kuijpers et al., 1990), also caused extensive AAF deacetylation together with oxidation.

Two methods were found that gave high yields of CPG cleavage and high yields of Fmoc deprotection without detectable deacetylation of the deprotected AAF-12-mers. These two methods employed treatment with either 1:9 anhydrous piperidine/MeOH at room temperature for 22–36 h or 1:1 diisopropylamine/MeOH at 55 °C for 15 h. MeOH was found to be the best solvent. Substitution CH_3CN for MeOH resulted in poor CPG cleavage and a low yield of Fmoc deprotection. The yields of CPG cleavage for both methods were more than 98%, as determined by quantifying the DMTr group and measuring the amount of oligonucleotide remaining bound on the CPG after treatment. The HPLC profiles of the Nensorb-separated full-length AAF-12-mer-1 deprotected by the two methods are shown in Figure 6B,C. The high yields of Fmoc cleavage resulted in the high yields of conversion of the Fmoc-protected AAF-12-mer-1 to the fully deprotected AAF-12-mer-1 (17 min). The treatment with 1:9 anhydrous piperidine/MeOH at room temperature was then used for larger scale deprotection of the AAF oligonucleotides synthesized.

The preferred purification method for AAF-modified oligonucleotides is to first separate the full-length DMTr-containing oligonucleotides by Nensorb Prep and to then purify the full-length sequences by reverse-phase HPLC on a semipreparative PRP-1 column using potassium phosphate based elution buffers (Huang & Krugh, 1990). The purified DMTr oligomers were then detritylated by 80% aqueous acetic acid, and the fully deprotected AAF oligomers were again HPLC-purified. Depending on the sequence contexts of the oligonucleotides, re-injections may be required. For AAF-12-mer-1, two reinjections were necessary due to its self-complementarity and high GC content. AAF-12-mer-3 did not need to be reinjected. AAF-12-mer-4 had to be purified on an ion-exchange column using formamide-containing elution buffers due to the presence of the run of five guanines in this sequence (Newton et al., 1983). The HPLC-purified AAF-12-mer-1 (Figure 6D), AAF-12-mer-3, and AAF-12mer-4 were also analyzed by PAGE, as shown in Figures 7 and 8, to confirm their purities. About 6 mg HPLC-purified AAF-12-mer-1 and 7 mg of AAF-12-mer-3 were obtained by the above deprotection and purification procedures.

Characterization of the AAF Oligonucleotides Containing Multiple Guanines. The solid-phase synthesized dG-C8-AAF oligonucleotides containing multiple guanine residues were rigorously characterized to confirm the dG-C8-AAF adduct structure and the site of this adduct in the oligomer sequence. The dG-C8-AAF adduct structure is confirmed by the following evidence: (a) UV spectra showed the presence of a shoulder at around 305 nm without extension to beyond 320 nm (not shown), which is consistent with the AAF structure rather than that of AF (Johnson et al., 1987). (b) The line-broadening effect due to the presence of dG-C8-AAF (Evans et al., 1980; O'Handley et al., 1993) was observed in the room-temperature ^1H NMR spectra (500 MHz) of AAF-12-mer-1 and AAF-12-mer-3. This effect is more pronounced for the resonances arising from the residues closer to the adduct site. When recorded at 60 °C, the line-broadening effect was diminished and better resolved spectra were obtained. Figure 9 is a comparison of the 1D ^1H NMR spectra of AAF-12-mer-3 recorded at 32 and 60 °C. (c) Digestion of the AAF-12-mer-1 with anhydrous TFA (Tang & Lieberman, 1983) generated a guanine species with a UV spectrum identical to that of guanine-C8-AAF and which coeluted with authentic

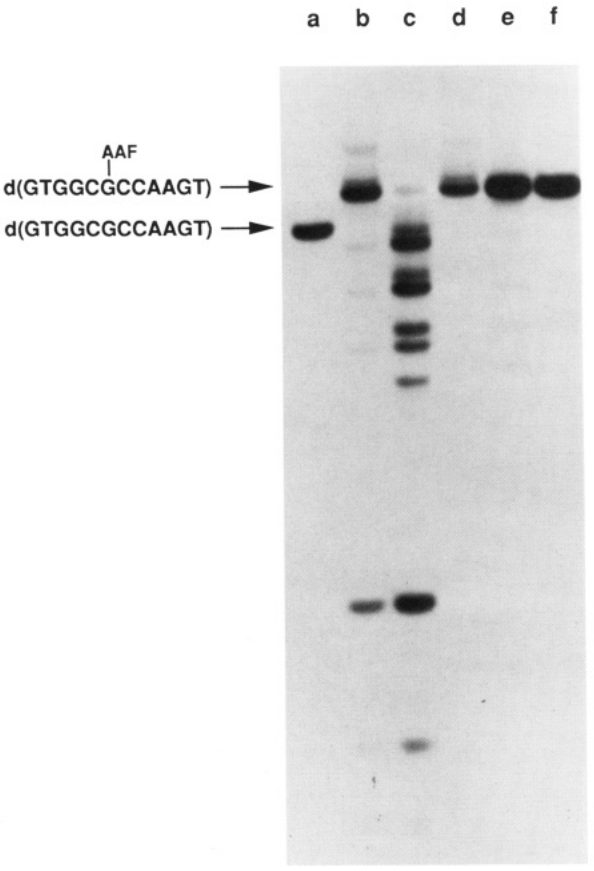


FIGURE 7: ^{32}P PAGE analysis of solid-phase synthesized AAF-12-mer-1. Lane a: HPLC-purified d(GTGGCGCCAAGT) (unmodified 12-mer-1) synthesized using standard CED phosphoramidites. Lanes b–e: Solid-phase synthesized d[GTGGCG$^{(C8-AAF)}$CCAAGT] (AAF-12-mer-1) deprotected by 1:9 piperidine/MeOH at room temperature for 36 h. Lane b, crude deprotection mixture; lane c, Nensorb-separated failure sequences; lane d, Nensorb-separated full-length product; lane e, HPLC-purified AAF-12-mer-1. Lane f, HPLC-purified AAF-12-mer-1 from deprotection by 1:1 diisopropylamine/MeOH at 55 °C for 15 h.

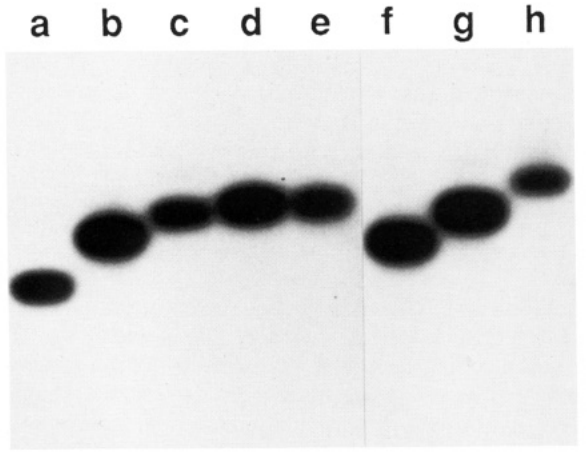


FIGURE 8: ^{32}P PAGE characterization of HPLC-purified AAF-12-mers. Lane a, unmodified 12-mer-1. Lanes b and f, AAF-12-mer-1. Lane c, AF-12-mer-1. Lane d, OP-I-12-mer-1. Lane e, OP-II-12-mer-1. Lane g, d[GTGATG$^{(C8-AAF)}$ATAAGT] (AAF-12-mer-3). Lane h, d[GTCGGG$^{(C8-AAF)}$GGAAGT] (AAF-12-mer-4).

guanine-C8-AAF[2] upon HPLC coinjection (Figure 10A). (d) Digestion of AAF-12-mer-1 using phosphodiesterase and alkaline phosphatase (Shibutani et al., 1991) generated a nucleoside species with UV spectrum identical to that of dG-C8-AAF, which coeluted with the authentic dG-C8-AAF[2] upon HPLC coinjection (Figure 10B). (e) Like the AAF-

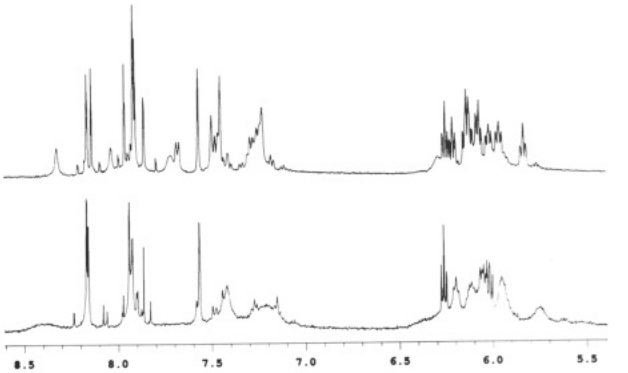


FIGURE 9: Aromatic and H1′ portion of the 500-MHz ^{1}H NMR of d[GTGATG$^{(C8-AAF)}$ATAAGT] (AAF-12-mer-3) in D_2O/0.1 M NaCl/10 mM KH_2PO_4/0.5 mM EDTA at pH 7: bottom, 32 °C; top, 60 °C.

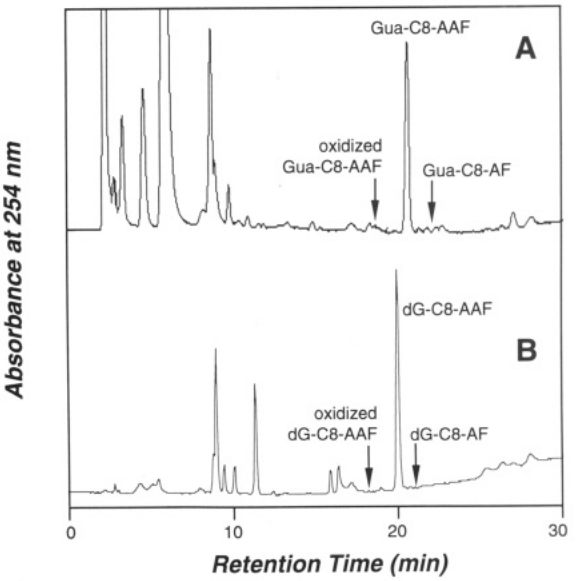


FIGURE 10: HPLC profiles of digested d[GTGGC-G$^{(C8-AAF)}$CCAAGT]. (A) Anhydrous trifluoroacetic acid digestion. (B) Enzymatic digestion with phosphodiesterase and alkaline phosphatase. Shown here is the aqueous insoluble portion of the enzyme-digested sample dissolved in 1:1:1 MeOH/AcOEt/10 mM KH_2PO_4 (pH 7) and then analyzed by HPLC. HPLC conditions: Hamilton PRP-1, 250 × 4.1 mm column, with a linear gradient of 2–100% B over 30 min at a flow rate of 1 mL/min (buffer A: 10 mM KH_2PO_4, pH 7; buffer B: 7:1:2 ACN/MeOH/A).

6-mer, the AAF-12-mer-1 can also be deacetylated to the corresponding AF-12-mer-1 and oxidized to the two oxidized species, OP-I-12-mer-1 and OP-II-12-mer-1. The HPLC profiles for these conversions were similar to those shown in Figure 3.

To confirm the site of dG-C8-AAF in the oligonucleotide sequence containing multiple guanines, the 5′-^{32}P-labeled AAF-12-mer-1 was treated with 1 M aqueous piperidine at 90 °C to induce chain cleavage at the adduct site (Johnson et al., 1987). The resulting fragmented AAF-12-mer-1 was then analyzed by PAGE (Figure 11), which showed the formation of only one ^{32}P fragment (Figure 11, lane d), indicating the presence of a single adduct. This fragment is identified as [5′-^{32}P]d(GTGGCp) by comparison with the Maxam–Gilbert G-cleavage reaction of the 5′-^{32}P-labeled unmodified 12-mer-1 (Figure 11, lane e), confirming the presence of a single C8-AAF modification at the second guanine to the 3′-side of the AAF-12-mer-1.

Duplex Formation from AAF-12-mer-1. Prior to initiation of the multidimensional NMR studies on AAF-12-mer-1, it

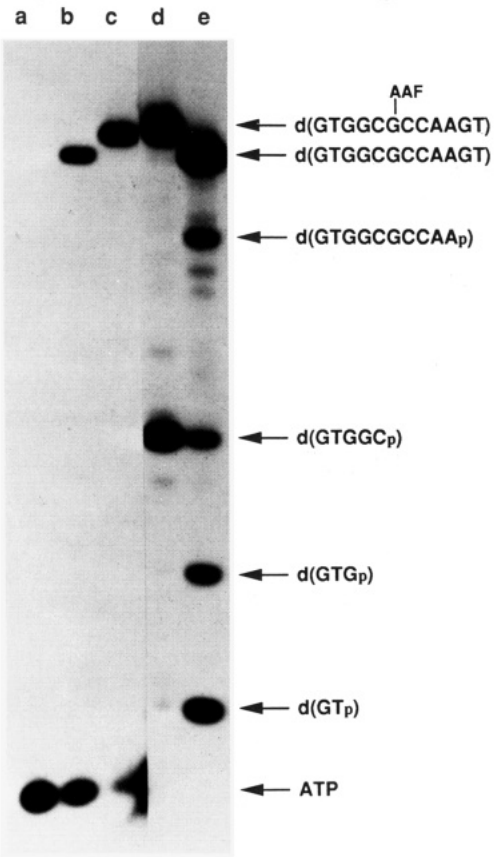


FIGURE 11: Piperidine digestion of AAF-12-mer-1. Lane a, [γ-^{32}P]-ATP. Lane b, unmodified 12-mer-1. Lane c, AAF-12-mer-1. Lane d, 5′-^{32}P-labeled AAF-12-mer-1 treated with 1 M aqueous piperidine at 90 °C for 8 h. Lane e, Maxam–Gilbert G-cleavage reaction of 5′-^{32}P-labeled unmodified 12-mer-1.

was important to establish the ability of this oligonucleotide to form a duplex with its complement. The significance of these experiments comes from the fact that the AAF adduct resides in the center of an eight-nucleotide sequence which is self-complementary. Therefore, whether or not the heteroduplex forms is dependent on the relative stability of the hemimodified heteroduplex, the dimodified self-duplex, and the unmodified self-duplex formed by the complement.

The melting curves for these duplexes were determined by the methods described by Breslaurer (1986), and the heteroduplex curves formed between AAF-12-mer-1 and 12-mer-1 and their complements are shown in Figure 12 (curves a and c, respectively). Also presented is the melting curve for the heteroduplex formed between AAF-12-mer-1 and a complementary 10-mer in which the central GC dinucleotide has been deleted (Figure 12, curve b). This is the structure that would result when the preferred –2 frame-shift occurs during mutagenesis induced by this sequence. T_m's for these experiments were determined from the normalized melting curves and are displayed in Table II.

Because the T_m for the AAF-12-mer-1 bound to its complement was 6 °C lower than that for the self-duplex formed for the complement, it is possible that the heteroduplex was not forming completely on mixing these two oligonucleotides. However, evidence that the heteroduplex was, in fact, forming is the observation that the melting curve for the heteroduplex displays a fairly sharp transition (Figure 12, curve a) and the fact that the duplex formed with the 10-mer complement is nearly identical (Figure 12, curve b). Also, when the method of continuous variation was used for the AAF-12-mer-1 and its 12-mer complement, two straight lines

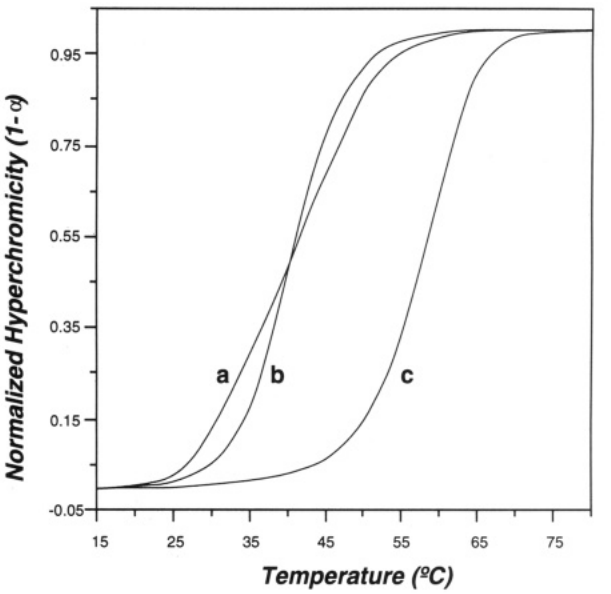


FIGURE 12: UV melting curves at 260 nm of d[GTGGCG$^{(C8-AAF)}$CCAAGT]:d(ACTTGGCGCCAC) (curve a, strand concentration 9.7 μM, T_m = 41 °C), d[GTGGCG$^{(C8-AAF)}$CCAAGT]:d(ACTTGGCCAC) (curve b, strand concentration 15.6 μM, T_m = 41 °C), and d[GTGGCGCCAAGT]:d(ACTTGGCGCCAC) (curve c, strand concentration 10.3 μM, T_m = 58 °C). Solvent: 0.1 M NaCl/10 mM NaH_2PO_4/0.5 mM EDTA, pH 7.

Table II: Melting Temperatures for the Oligonucleotide Duplexes[a]

T_m (°C)	12-mer-1[b]	AAF-12-mer-1	12-mer compt[c]	10-mer compt[d]
46	x			
		x		
47			x	
58	x		x	
41		x	x	
41		x		x
27				x

[a] Melting temperatures were determined using the methods described by Breslauer (1986). [b] This oligonucleotide has the sequence identical to AAF-12-mer-1 but lacks the AAF adduct. [c] This oligonucleotide is the complement to 12-mer-1 (5′-dACTTGGCGCCAC). [d] This oligonucleotide has the sequence 5′-dACTTGGCCAC.

were generated which crossed near 1:1 stoichiometry (Figure 13, curve a), suggesting that the complement self-duplex was not forming. A similar result was obtained for the mixing experiment using the 10-mer complement (Figure 13, curve b), a case where a self-duplex would be highly unlikely.

DISCUSSION

Solid-phase DNA synthesis of oligonucleotides has become a powerful tool in molecular biology. The standard synthetic and deprotection procedures associated with the commercial DNA synthesizer, such as those used for the standard cyanoethyl diisopropylphosphoramidite approach, were developed on the basis of the chemistry of the four normal deoxyribonucleosides (Gait, 1984). It is unlikely that these standard schemes would be amenable for the synthesis of oligonucleotides containing carcinogenic adducts, since many of these structures would not be expected to be stable to the relatively harsh conditions used for deprotection following synthesis. Our goal in this study was to develop a solid-phase synthetic scheme that would be appropriate for DNA lesions, such as dG-C8-AAF, which would not survive the normal deprotection procedure.

In 1983, Stöhrer reported the first attempt at a solid-phase synthesis of a 14-mer containing a site-specific dG-C8-AAF

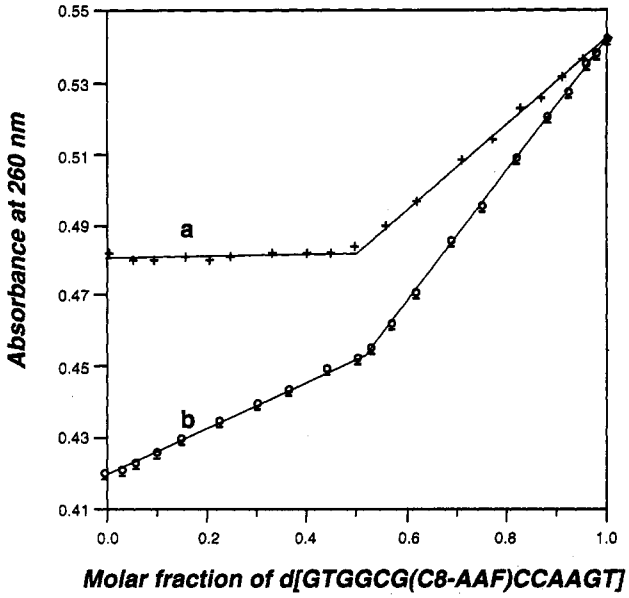


FIGURE 13: UV mixing curves of d[GTGGCG(C8-AAF)CCAAGT] with a 12-mer complement d(ACTTGGCGCCAC) (curve a) and a GC-deleted 10-mer complement d(ACTTGGCCAC) (curve b). The 1:1 stoichiometries indicate duplex formations in both cases. Solvent: 0.1 M NaCl/10 mM NaH_2PO_4/0.5 mM EDTA, pH 7. Strand concentration: 4.9 μM. Temperature: 20 °C.

adduct, using N^2-isobutyryl-protected dG-C8-AAF 3′-(chloromethoxyphosphine) as the building block for the adducted deoxyguanosine nucleotide unit (Stöhrer et al., 1983). Since the *exo*-amino groups of the heterocyclic bases were protected using the standard benzoyl and isobutyryl groups, the hot ammonium hydroxide treatment had to be used in order to deprotect the oligonucleotide product. This stringent alkaline treatment deacetylated all dG-C8-AAF units incorporated into the oligonucleotide (Kriek & Westra, 1980).

We have been conducting an extensive investigation on the chemical synthesis of oligonucleotides containing site-specific base-labile carcinogenic adducts. Throughout this study, we focused on the selection of an *exo*-amino protecting group for the heterocyclic nucleic bases, which should be removable under mild conditions so as not to destroy the base-sensitive dG-C8-AAF moiety. For this purpose, we chose Fmoc in our current study. Fmoc was introduced in 1983 by Heikkilä and Chattopadhyaya to protect the *exo*-amino groups on the nucleic bases (Heikkilä & Chattopadhyaya, 1983). This protecting group has been used in solid-phase DNA synthesis of base-sensitive functions (Webb & Matteucci, 1986; Kuijpers et al., 1990). The Fmoc protecting group in our study has allowed us to select much milder conditions for the final oligonucleotide deprotection, such as 1:9 piperidine in methanol at room temperature or 1:1 diisopropylamine/MeOH at 55 °C. These conditions not only remove Fmoc efficiently but also cleave cyanoethyl groups on the phosphate and the succinyl linkage of the oligonucleotide to the solid support. Moreover, the dG-C8-AAF structural unit is quite stable to such treatments, which is the key to success for our synthesis. Finally, we have also demonstrated the clean conversion of our solid-phase synthesized dG-C8-AAF oligonucleotides to the corresponding dG-C8-AF oligonucleotides under deacetylation conditions as reported by Shibutani et al. (1990, 1991). Therefore, our solid-phase synthesis can be used to obtain both dG-C8-AAF and dG-C8-AF site-specific adducted oligonucleotides, allowing structural comparisons of these two related adducts.

An interesting finding of these studies is the comparison of the melting curves from heteroduplexes generated between the 12-mer containing an adduct in the GC mutation hot spot *Nar*I site and either the 12-mer complement or a complementary 10-mer which is missing the central GC dinucleotide. These curves show similar characteristics and result in identical T_m's, suggesting that the structure of the deleted material may resemble the structure generated when the AAF adduct is situated in this normal sequence. Multidimensional NMR studies on these heteroduplexes are in progress.

To our knowledge, the syntheses reported here are the first successful preparations of base-sensitive oligonucleotides using a complete set of *N*-Fmoc-protected phosphoramidites, including the adduct containing phosphoramidite. This method allows us to incorporate a dG-C8-AAF adduct into any oligonucleotide without sequence limitation. Extension of the synthesis to the 10-μmol scale has given us access to 6–7-mg quantities of pure site-specific dG-C8-AAF-modified oligonucleotide sequences containing multiple guanines, species that would be impossible to make by the commonly adopted postsynthesis modification of the corresponding unmodified oligonucleotides. Multidimensional NMR studies on these oligonucleotides will make possible a correlation between the AAF-induced DNA conformational changes and the biological consequences of these adduct structures.

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