Synthesis of Oligonucleotides Containing Site-Specific Carcinogen Adducts. Preparation of the 2-Cyanoethyl *N,N*-Diisopropylphosphoramidite of *N*-(2'-Deoxyguanosin-8-yl)-2-(acetylamino)fluorene with Fmoc as the Base-Protecting Group

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A 9-fluorenylmethoxycarbonyl (Fmoc) group was used to protect the exocyclic amine on the modified guanine of *N*-(2'-deoxyguanosin-8-yl)-2-(acetylamino)fluorene (dG-C_8-AAF) so that oligonucleotides containing a site-specific AAF adduct could be prepared. Reaction of Fmoc-Cl with dG-C_8-AAF gave *N*-[N^2-(9-fluorenylmethoxycarbonyl)-2'-deoxyguanosin-8-yl]-2-(acetylamino)fluorene (N^2-Fmoc-dG-C_8-AAF) and *N*-[O^6-(9-fluorenylmethoxycarbonyl)-2'-deoxyguanosin-8-yl]-2-(acetylamino)fluorene (O^6-Fmoc-dG-C_8-AAF). The 5'-OH of N^2-Fmoc-dG-C_8-AAF was protected using 4,4'-dimethoxytrityl chloride to yield 5'-DMT-N^2-Fmoc-dG-C_8-AAF which was then reacted with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite to obtain the corresponding phosphoramidite. The phosphoramidites of Fmoc-protected dA, dC and dG were also prepared similarly. The stability of Fmoc-protected C_8-AAF-modified deoxyguanosine was studied under different conditions to establish the utility of the prepared phosphoramidite in solid-phase DNA synthesis.

(Acetylamino)fluorene is one of the best studied metabolically activated mutagenic chemical carcinogen.[1] The key event leading to cancer induction by this compound is believed to be the mutagenesis that results following the covalent modification of DNA by highly reactive electrophilic species generated through *in vivo* metabolic activation.[1] Administration of this carcinogen *in vivo* produces *N*-(2'-deoxyguanosin-8-yl)-2-aminofluorene (dG-C_8-AF) and *N*-(2'-deoxyguanosin-8-yl)-2-(acetylamino)-fluorene (dG-C_8-AAF) as the major carcinogen-DNA adducts.[1] dG-C_8-AF and dG-C_8-AAF can also be introduced into DNA *in vitro* by modification of the DNA with the activated carcinogens, *N*-hydroxy-2-aminofluorene (*N*-HO-AF) or *N*-acetoxy-*N*-(trifluoroacetyl-2-aminofluorene (*N*-AcO-*N*-TFA-AAF), and *N*-acetoxy-*N*-acetyl-2-aminofluorene (*N*-AcO-AAF), respectively.[2]

Randomly-modified AF or AAF template DNA were initially used in mutagenesis studies to relate the observed mutagenic effects to the presence of the major adducts,[3] despite the fact that these DNA modifications invariably produced several minor adducts.[2b,4] The development of methods allowing the construction of viral DNAs containing a single site-specific dG-C_8-AF or dG-C_8-AAF adduct made this a potentially powerful tool to investigate the mutagenic outcome of a specific carcinogen adduct.[5] Using this approach it is possible to much more precisely correlate DNA modification and mutagenesis because the adduct structure and local sequence can be specifically controlled. Several recent mutagenesis studies using target DNAs containing a site-specific dG-C_8-AAF adduct or dG-C_8-AF adduct have revealed a diversity in the mutation spectrum induced by these adducts,[6] which is in agreement with the postulation that the production of mutations and their specificity may not be solely dependent on the adduct structure itself.[3,7] It seems likely that such diversity in mutation spectrum for a specific adduct in a given host cell is a reflection of this inherent structure–mutation relationship. The differences between the AAF and AF-adduct-induced mutation spectra have been explained by the proposed dramatic structural differences that are present in the DNA containing these adducts, while the influence of local DNA sequences on the mutation spectrum may be due to the subtle differences in DNA structure that alter the mechanism by which the adduct-containing nucleotide is copied during DNA replication.[3,6,7]

Oligonucleotides containing a single site-specific AF or AAF adduct are currently prepared by modifying a normal oligonucleotide containing only one guanine residue with

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N-AcO-*N*-TFA-AAF or *N*-AcO-AAF followed by purification to obtain the oligonucleotide containing the desired adduct and sequence.[6] Modification of an oligomer containing three guanine residues has also been carried out giving rise to a mixture of products which were separated by extensive HPLC purification.[6e] Because of the low yield of these reactions, especially when the oligonucleotide contains multiple reactive centers, it is difficult to obtain large amounts of suitable pure materials for spectroscopic and biochemical analysis. There has been only one report of a direct chemical synthesis of oligonucleotides containing dG-C_8-AF adduct using automated solid-phase DNA synthesis.[8] This method failed to give the desired dG-C_8-AAF-containing oligonucleotides because of the harsh deprotection conditions that were required to remove the exocylic amine protecting groups.

Studies on the relationship between DNA structure and mutagenesis would be greatly facilitated if methods were available that allowed the preparation of oligonucleotides containing site-specific adducts by direct solid-phase chemical synthesis using phosphoramidites bearing the desired adduct. This method of preparation has numerous advantages over currently used modification procedures. First, the precise structure of the adduct can be easily confirmed prior to incorporation using NMR and mass spectrometry of the precursor nucleotide. Second, unlike procedures where the oligonucleotide is modified with a reactive electrophile, there are no limitations on the sequence of the oligonucleotide. Products containing multiple Gs can be prepared in amounts equivalent to oligonucleotides containing a single G. Third, sufficient quantities of pure oligonucleotides can be easily obtained for spectroscopic structural studies. This then would allow the direct correlation of an adduct-induced conformational change in the context of a specific DNA sequence with the mutation produced, since these same modified oligonucleotides can be incorporated into a vector DNA for mutational analysis.

We have been conducting extensive studies on the chemical synthesis of oligodeoxyribonucleotides containing site-specific carcinogen adducts. One approach involves the protection of all exocyclic amino groups on both the normal and the adducted bases with 9-fluorenylmethoxycarbonyl (Fmoc) groups[9-11] and the development of suitable strategies to use them in solid-phase oligonucleotide synthesis.[12] We report here the synthesis and structural identification of the 2-cyanoethyl N,N-diisopropylphosphoramidites of Fmoc-protected dG-C_8-AAF, dA, dC and dG, as well as the stability studies of Fmoc protected dG-C_8-AAF and the related AAF derivatives. These phosphoramidites have been successfully used in the syntheses of several oligonucleotides containing site-specific dG-C_8-AAF adduct.[13]

Results and Discussion

Protecting Groups. The selection of protecting groups is a critical decision in successful solid-phase oligonucleotide synthesis containing unusual structural units. The highly developed standard solid-phase DNA synthesis employs benzoyl and isobutyryl protection for the exocyclic amino groups on the normal bases (benzoyl for adenine and cytosine and isobutyryl for guanine).[12] Upon completion of the solid-phase synthesis, these two base-protecting groups are removed from the oligonucleotide product under standard deprotection conditions (ammonium hydroxide treatment at 60 °C for 10–12 h). These standard deprotection conditions may not be used if the target oligonucleotide contains abnormal structural units, especially the base-sensitive ones, for these structures are unlikely to be stable under this deprotection condition.

Stöhrer *et al.* reported an attempt to synthesize a dG-C_8-AAF-containing oligonucleotide using a solid-phase approach.[8] These studies used isobutyryl and benzoyl as base-protecting groups which required final base deprotections be performed by treatment of the fully protected oligonucleotide product with concentrated ammonium hydroxide at 50 °C for 4 h, either under aerobic condition or under anaerobic condition. Not surprisingly, these deprotection conditions deacetylated the dG-C_8-AAF adduct incorporated into the oligonucleotide, giving a dG-C_8-AF-containing oligonucleotide under anaerobic condition and the oxidized species under aerobic condition. The base-catalyzed deacetylation of dG-C_8-AAF to dG-C_8-AF and the oxidative degradation of dG-C_8-AF under alkaline condition has been reported previously.[14] Recently, Shibutani *et al.* have shown that the oxidative degradation pathway of dG-C_8-AF resembles that of uric acid in alkali, and the degradation products have been structurally identified.[4c,d]

A successful solid-phase synthesis of AAF-containing oligonucleotides will therefore be determined by the selection of protecting groups that are more base-labile than the adduct. These not only include the base-protecting groups, but also the phosphate-protecting group and the linkage to anchor the oligonucleotide to the solid support. In the present study, we have chosen 9-fluorenylmethoxycarbonyl (Fmoc) as the base-protecting group, and 2-cyanoethyl *N,N*-diisopropylphosphoramidite approach as the method for solid-phase oligonucleotide assembly.

The decision to use Fmoc as the base-protecting group relies on the fact that it can be easily removed from the protected bases and nucleosides by tertiary amines like triethylamine within 3 h at room temperature.[9b] Chládek and co-workers have demonstrated successful solution synthesis of a series of aminoacylated RNA trimers by using *N*-Fmoc-protected ribonucleotide building blocks and an oximate reagent for the final deprotection of

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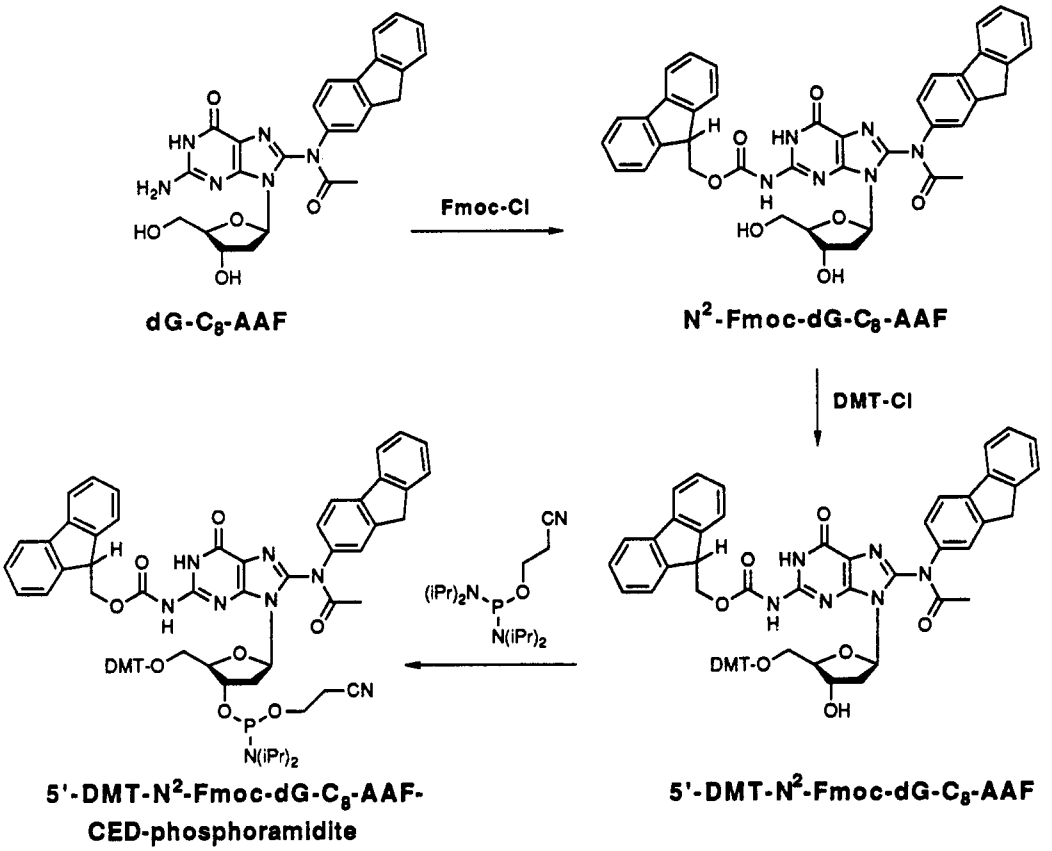
Scheme 1



Fmoc.[15] The work by Koole *et al.* have also shown that Fmoc can be removed by triethylamine in dry pyridine while the methyl phosphate remains intact.[11c] In solid-phase syntheses, Fmoc has been used as both base-protecting group and 5′-OH protecting group.[10,11] The protection of 5′-OH with Fmoc has allowed the successful development of acid-free protocols for both solid-phase DNA synthesis[10d] and solid-phase RNA synthesis.[10e] The use of Fmoc as the base-protecting group in the solid-phase synthesis of oligodeoxyribonucleotides containing base-sensitive functionalities has also been demonstrated.[11a,b]

We decided to use the 2-cyanoethyl *N,N*-diisopropylphosphoramidite approach because of its high yield of internucleotide coupling and the more base-labile 2-cyanoethyl phosphate protection. This makes possible a one-step mild final deprotection which not only removes the Fmoc group but also deprotects the phosphate and cleaves the oligonucleotide anchoring linkage. Therefore, the building block for dG-C_8-AAF to be used in our solid-phase synthesis is 5′-DMT-N^2-Fmoc-dG-C_8-AAF 3′-CED-phosphoramidite the synthesis of which is detailed in Scheme 1.

Synthesis. Our study requires the synthesis of 3′-CED-phosphoramidites of Fmoc-protected dA, dC, dG, and dG-C_8-AAF. The synthesis of dG-C_8-AAF[16] and the Fmoc protection of dA, dC and dG[11c] were achieved according to the published procedures. Fmoc protection of the 2-NH_2 of dG-C_8-AAF was carried out by transient trimethylsilyl protection of the 3′- and 5′-OH groups followed by reaction with 9-fluorenylmethoxy chloroformate (Fmoc-Cl) and subsequent aqueous hydrolysis.[11c] Analytical TLC was used to monitor the progress of the reaction and column chromatography on silica gel was used to separate and purify the Fmoc-protected dG-C_8-AAF products. We found that the reaction of trimethylsilyl protected dG-C_8-AAF with Fmoc-Cl went to completion only after it had run overnight, whereas the similar reactions for normal nucleosides take only 1–2 hours to finish.[11c] Another interesting finding was that this procedure for Fmoc protection, when used to protect dG, gave exclusively N^2-Fmoc-dG, whereas both N^2 and O^6-Fmoc isomers were obtained when applied to dG-C_8-AAF. These two isomers migrated close to each other on TLC but could be completely separated by analytical HPLC. Their isomeric nature was suggested by spectral analysis and was confirmed by removal of Fmoc from the purified isomers which converted both species to the starting material, dG-C_8-AAF, as shown by TLC and HPLC (Figure 1). The ratio of N^2- to O^6-isomer was found to be approximately 5 to 1. Pure N^2-Fmoc-dG-C_8-AAF was obtained directly by silica gel chromatography from the crude product mixtures but pure O^6-Fmoc-dG-C_8-AAF was unable to be obtained in this way, even upon repeated chromatography. We obtained the purified O^6-Fmoc-dG-C_8-AAF by first converting a mixture of N^2- and O^6-Fmoc-dG-C_8-AAF to their corresponding 5′-DMT derivatives and then detritylating the chromatographically purified 5′-DMT derivative of O^6-Fmoc-dG-C_8-AAF. The dimethoxytritylations were carried out by reacting the Fmoc-protected nucleosides with 4,4′-dimethoxytrityl chloride in dry pyridine based on the monomethoxytritylation procedures of Koole *et*

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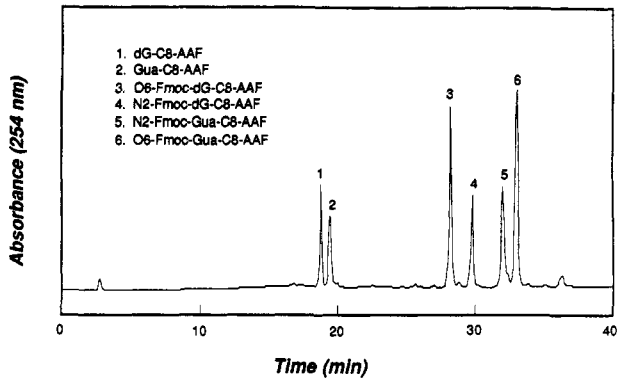

Figure 1. Analytical HPLC profile showing the retention times of AAF-containing compounds. Sample was prepared by mixing the compounds in 50% acetonitrile/50 mM TEAA, pH 7. Elution conditions: linear 10–75% acetonitrile in 50 mM triethylammonium acetate (TEAA), pH 7 over 30 min followed by an isocratic 75% acetonitrile in 50 mM TEAA, pH 7 over 20 min at flow rate of 1 mL/min.

al.[11c] The Fmoc-protected unmodified nucleosides gave exclusively 5′-DMT products, whereas two DMT products were formed from N^2-Fmoc-dG-C_8-AAF. The major product, as expected, was a 5′-DMT derivative; the minor product was identified as a 3′,5′-diDMT derivative. Both isomers were obtained in pure form by silica gel column chromatography.

Initially, the N^2-Fmoc-protected dG and dG-C_8-AAF 3′-CED phosphoramidites were prepared by phosphitylation of the Fmoc and DMT-protected nucleosides with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in the presence of diisopropylethylamine according to the previous procedures.[12b] The product mixtures were difficult to purify even upon repeated chromatography and hexane precipitation. As a result, the N^2-Fmoc-dG phosphoramidite showed a low coupling efficiency (81–86%) during solid-phase synthesis.[13] The coupling efficiency for N^2-Fmoc-dG-C_8-AAF phosphoramidite was even lower, only about 50%.[13] The reason for these low yields is not known but may involve the loss of the Fmoc group by the amine used during the preparation of the phosphoramidites.

The highly pure Fmoc-protected phosphoramidites were obtained using a different procedure which gave a much cleaner phosphitylation. As an example, the 5′-DMT-N^2-Fmoc-dG-C_8-AAF 3′-CED phosphoramidite was prepared by the reaction of 5′-DMT-N^2-Fmoc-dG-C_8-AAF with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite in dry CH_2Cl_2, using diisopropylethylammonium 1*H*-tetrazolide as the coupling agent.[17] The product was purified by flash chromatography on silica gel and repeated hexane precipitation. The purified phosphoramidite was a white powder with a yield of 96% and was estimated to be 95% pure by analytical TLC and ^{31}P NMR. The 2-cyanoethyl *N,N*-diisopropylphosphoramidites of the Fmoc-protected dA, dC, and dG were prepared similarly with comparable yields and purities. The phosphoramidites were stable at −20 °C under nitrogen for up to 2 months. Several oligodeoxyribonucleotides containing a dG-C_8-AAF adduct were successfully synthesized using these phosphoramidites, and the coupling efficiency of these phosphoramidite preparations reached to as high as 99%.

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Stability of dG-C_8-AAF Compounds. To determine suitable conditions that can be used in oligonucleotide syntheses using the dG-C_8-AAF phosphoramidite, we have conducted a series of TLC and HPLC studies on the stability of dG-C_8-AAF and its derivatives (summarized in Table 1). TLC was used to monitor the reactions and HPLC was used to identify the products. Several AAF compounds have been prepared and structurally identified to serve as the TLC and HPLC standards: N^2-Fmoc-Gua-C_8-AAF, O^6-Fmoc-Gua-C_8-AAF, and Gua-C_8-AAF. N^2- and O^6-Fmoc-Gua-C_8-AAF were prepared from depurination of N^2- and O^6-Fmoc-dG-C_8-AAF in 3% dichloroacetic acid in dichloromethane. Gua-C_8-AAF was prepared from N^2-Fmoc-Gua-C_8-AAF by deprotection of Fmoc in 1:1 triethylamine in 1:4 methanol–dichloromethane and was identical to the depurination product of dG-C_8-AAF by TLC and HPLC. The typical HPLC profiles showing the retention times of these AAF compounds are shown in Figure 1.

(1) Base-deprotection and oligonucleotide release: Concentrated ammonium hydroxide treatment at 50 °C had been shown to be unsuitable for deprotection of AAF-containing oligonucleotides.[8a] To find a milder base deprotection condition, we analyzed the stabilities of corresponding AAF compounds in oximate, a 0.5 M solution of the N^1,N^1,N^2,N^2-tetramethylguanidinium salt of *syn*-2-nitrobenzaldoximate in 1:1 water–dioxane, which has been used extensively in the phosphotriester method to remove the aryl protection on phosphate and to cleave the succinate linkage, all of which were done at room temperature.[12c] Our TLC and HPLC experiments showed that oximate treatment completely removed Fmoc from N^2-Fmoc-dG-C_8-AAF and N^2-Fmoc-Gua-C_8-AAF in about 15 min and that dG-C_8-AAF and Gua-C_8-AAF are fairly stable to this treatment for about 5 h, although prolonged treatment for 20 h caused about 50% degradation.

(2) Detritylation: Three percent dichloroacetic acid in dichloromethane is one of the widely used detritylation agents in oligonucleotide synthesis.[12b] We examined the detritylation of DMT-N^2-Fmoc-dG-C_8-AAF under this condition and found that the glycosidic bond had undergone rapid cleavage, resulting in quantitative depurination of the starting materials in less than 10 min. The depurinated product was identified as N^2-Fmoc-Gua-C_8-AAF. Similar treatment produced depurination of DMT-O^6-Fmoc-dG-C_8-AAF, N^2(and O^6)-Fmoc-dG-C_8-AAF, and dG-C_8-AAF. The rates of detritylation and depurination correlated with the strength of the acid used. Thus, the fastest detritylation and instant depurination was observed in 3% benzenesulfonic acid in dichloromethane, while no depurination was detected in 3% acetic acid in dichloromethane after 3 days treatment, although under these conditions detritylation required 3 h. An ideal reagent was found to be 3% chloroacetic acid in dichloromethane. The latter gave quantitative detritylation in 30–40 min without significant depurination and was chosen as an alternative detritylation reagent in our solid-phase oligonucleotide synthesis. We attribute this enhanced rate of depurination to the weakened glycosidic bond due to AAF modification on C8 of guanine. It should be noted that we have found that the stability of glycosidic bond toward depurination for a nucleoside differs to some extent from that when it is present in an oligonucleotide and that 3% dichloroacetic acid worked

Table 1. Stability of dG-AAF to Oligonucleotide Synthesis Deprotection Conditions

compound	treatment	moitey remaining[a] (%)		
		dG-AAF	Fmoc	DMT
N^2-Fmoc-dG-C_8-AAF	oximate, 15 min	100	0	-
N^2-Fmoc-dG-C_8-AAF	oximate, 20 h	50	0	-
N^2-Fmoc-dG-C_8-AAF	5:4:1 TEA:CH_2Cl_2:MeOH, 2h	100	0	
DMT-N^2-Fmoc-dG-C_8-AAF	3% benzenesulfonic acid, 2 min	0	100	0
DMT-N^2-Fmoc-dG-C_8-AAF	3% dichloroacetic acid, 10 min	0	100	0
DMT-N^2-Fmoc-dG-C_8-AAF	3% chloroacetic acid, 30 min	98	100	0
DMT-N^2-Fmoc-dG-C_8-AAF	3% acetic acid, 3 h	100	100	0
DMT-O^6-Fmoc-dG-C_8-AAF	3% dichloroacetic acid, 10 min	20	100	0
DMT-N^2-Fmoc-dG-C_8-AAF	capping reagent,[b] 90 min	100	80	100
DMT-N^2-Fmoc-dG-C_8-AAF	capping reagent, 20 min	100	98	100

[a] Amount of protecting group remaining or integrity of the guanosine and AAF structures as determined by TLC analysis. [b] Capping reagent was a mixture of (dimethylamino)pyridine, 2,6-lutidine, and acetic anhydride in THF.

well for detritylation during the solid-phase oligonucleotide synthesis.[13]

(3) Capping conditions: Several previous studies showed that (dimethylamino)pyridine (DMAP), the effective capping catalyst in our solid-phase DNA synthesizer (Beckman System 1 Plus), induced Fmoc cleavage to the Fmoc-protected nucleosides and phosphoramidites.[10c,11a,b] We conducted a TLC study on the stabilities of DMT-N^2-Fmoc-dG-C_8-AAF and several other Fmoc-protected nucleosides to this capping reagent. Our result indicated that Fmoc cleavage is noticeable only after 30 min treatment, and C8-AAF modification did not result in enhanced instability of the Fmoc protection to the capping condition. Ma and Sonveaux used DMAP as the capping catalyst in the synthesis of a 17mer using 5'-O-Fmoc-3'-O-phosphoramidites and still obtained a good solid-phase synthesis with apparent coupling yield of 95% per cycle.[10c] We therefore concluded that it is still desirable to include the capping step into the oligonucleotide chain assembly because of its importance in oligonucleotide synthesis and the brief contact period (less than 1 min) of the solid support to the capping catalyst.

Structural Identification. The structure of dG-C_8-AAF,[18] and those of N^6-Fmoc-dA, N^4-Fmoc-dC, and N^2-Fmoc-dG,[11c] have been well characterized and our ^1H and ^{13}C NMR data were identical to those reported in the literature. The formation of two isomeric Fmoc-protected dG-C_8-AAF from the reaction of dG-C_8-AAF with Fmoc-Cl was clearly shown by ^1H NMR, ^{13}C NMR, and FAB-MS spectral data. ^1H NMR provided valuable information to identify the origin of this isomerization. In $CDCl_3$, the high-R_f isomer did not show the N^2-amino proton signal at 6.40 ppm for dG-C_8-AAF but showed a new exchangeable signal at 10.30 ppm corresponding to one proton, which indicates that Fmoc modification is on the 2-amino position of guanine. The signals for 1-NH of guanine and the two hydroxyls of the sugar are almost unaffected, as well as the signals for the rest of the sugar protons. The low-R_f isomer lost the 1-NH signal for the guanine, which is consistent with O^6-modification by Fmoc. Its 2-amino proton signal was probably shifted into the aromatic region and hence was unobservable. The downfield shift of $H_{2'}$ protons in both isomers to about 3 ppm indicates that the riboses are still in *syn*-conformation relative to the guanine, as in the case for dG-C_8-AAF.[18a,19] Therefore, the isomer formation is not due to the ribose orientation. This was further confirmed by the formation of different guanine derivatives from the depurination of the two isomers, N^2-Fmoc-Gua-C_8-AAF from N^2-Fmoc-dG-C_8-AAF and O^6-Fmoc-Gua-C_8-AAF from O^6-Fmoc-dG-C_8-AAF. Again, as in the dG isomers, the N^2-guanine isomer shows low-field exchangeable signals for both 1-NH and Fmoc-N^2H protons plus an additional 9-NH signal for the guanine, but the O^6-guanine isomer shows only the 9-NH signal. The subsequent removal of Fmoc from both of the guanine isomers gave only one product, Gua-C_8-AAF, which was identical to the depurination product formed directly from dG-C_8-AAF. This again proves that the Fmoc modification gave rise to the isomerization.

FAB mass spectroscopy was also used to identify the compounds involved in this study. N^2- and O^6-Fmoc-dG-C_8-AAF and the DMT-derivatives of N^2-Fmoc-dG-C_8-AAF, all show a unique peak at m/e 595 (BH_2 in positive FAB)[20] or m/e 593 (BH–H in negative FAB)[20] due to the characteristic fragmentation of the molecular ion to that of the corresponding base.[21] These two ions became the molecular ions for N^2-Fmoc-Gua-C_8-AAF, which was prepared by the depurination of Fmoc-protected nucleosides, and is a good indication of the identity of Fmoc-guanine-AAF structural unit in these nucleosides. When N^2-Fmoc-Gua-C_8-AAF was treated with triethylamine, it was quantitatively converted to Gua-C_8-AAF, which showed a strong peak at m/e 373 in FAB^+ (M + H) and at m/e 371 in FAB^- (M–H). Therefore, the guanine-C_8-AAF identity remained intact after going through Fmoc protection, dimethoxytritylation, depurination, and Fmoc deprotection.

The ^{13}C NMR of the AAF compounds are all consistent with their assigned structures. The aliphatic region is particularly useful to ascertain the presence or absence of AAF, Fmoc, DMT, and deoxyribose structural units. The site(s) of dimethoxytritylation in DMT-N^2-Fmoc-dG-C_8-AAF and diDMT-N^2-Fmoc-dG-C_8-AAF were confirmed by comparing the $C_{3'}$ and $C_{5'}$ chemical shifts before and after dimethoxytritylation. Thus, dimethoxytritylation was found to cause a downfield shift of about 3–4 ppm for the carbons at the tritylation sites. The same downfield shift upon dimethoxytritylation was also observed in our ^{13}C NMR spectra of the DMT derivatives of Fmoc-protected normal nucleosides.

The structures of Fmoc-protected CED phosphoramidites were confirmed by both ^1H and ^{31}P NMR. All four

(18) (a) Evans, F. E.; Miller, D. W.; Beland, F. A. *Carcinogenesis* **1980**, *1*, 955. (b) Elfarra, A. A.; Hanna, P. E. *J. Med. Chem.* **1985**, *28*, 1453.

(19) Lipkowitz, K. B.; Chevalier, T.; Widdifield, M.; Beland, F. A. *Chem.-Biol. Interact.* **1982**, *40*, 57.

(20) BH = *N*-[N^2-(9-Fluorenylmethoxycarbonyl)guanin-8-yl]-2-(acetylamino)fluorene (N^2-Fmoc-Gua-C_8-AAF).

(21) (a) Crow, F. W.; Tomer, K. B.; Gross, M. L.; McCloskey, J. A.; Bergstorm, D. E. *Anal. Biochem.* **1984**, *139*, 243. (b) Mitchum, R. K.; Evans, F. E.; Freeman, J. P.; Roach, D. *Int. J. Mass Spectrom. Ion Phys.* **1983**, *46*, 383.

phosphoramidites exhibit a predominant doublet (2 diastereomers) within 148–150 ppm in ^{31}P NMR. This diastereomeric feature was also revealed in the ^{1}H NMR spectra.

Experimental Section

Materials and Methods. 2′-Deoxynucleosides were purchased from USB and ABN. Chlorotrimethylsilane, 9-fluorenylmethyl chloroformate, 4,4′-dimethoxytrityl chloride, 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite, triethylamine (Gold label), 1*H*-tetrazole, dichloroacetic acid, anhydrous pyridine and anhydrous dichloromethane were from Aldrich. *N,N*-Diisopropylethylamine were purchased from Aldrich, dried over KOH pellets, refluxed with ninhydrin for 3 h, distilled, refluxed again with BaO under nitrogen for 3 h and distilled over BaO under N_2. Chloroacetic acid was from MC/B. HPLC grade methanol, dichloromethane, and chloroform used in liquid chromatography and TLC were Fisher reagents. Liquid chromatography were run on columns packed with Kieselgel 60 silica gel (230–400 mesh) from EM Science. Analytical TLC were run on aluminum sheets coated with silica gel 60 F254 from EM Science. Preparative TLC plates used were silica gel GF 20 × 20 cm, 1000 μm, from Analtech. Reverse-phase HPLC analyses were conducted using a 4.6 × 250 mm Beckman ultrasphere reverse-phase C18 column (5 μm) and a diode array detector channel-setting at 254 and 302 nm. For the NMR analyses, sample concentrations of 5–8% and 10000 aquisitions were normally used to obtain ^{13}C spectra. Assignments of ^{13}C chemical shifts were based on comparison of the broad-band decoupled spectra, and APT and DEPT spectra, by comparison of the relative signal intensities, and by reference to the chemical shifts of model compounds from the literature.[18b,22] ^{31}P NMR were recorded in $CDCl_3$. Chemical shifts were obtained from the proton-decoupled experiments and were expressed relative to the external standard, 85% phosphoric acid. Fast atom bombardment mass spectrometry (FAB) was carried out in either positive or negative ion mode using thioglycerol as the matrix material.

***N*-(2′-Deoxyguanosin-8-yl)-2-(acetylamino)fluorene (dG-C$_8$-AAF).** This compound was synthesized from 2′-deoxyguanosine and 2-nitrofluorene according to the published procedures.[16] Purification of the compound was achieved as follows: The impure compound was dissolved in 1:4 (by volume) CH_3OH/CH_2Cl_2 to remove the insoluble materials. The solution was evaporated under reduced pressure to give a yellow solid, which was then chromatographed on a silica gel column, using 1:10 CH_3OH/CH_2Cl_2 for column packing and sample loading. Elution was effected first with 1:10 CH_3OH/CH_2Cl_2 and then with 1:4 CH_3OH/CH_2Cl_2. Fractions containing pure dG-C$_8$-AAF were combined and evaporated in vacuo to give a yellowish solid, which gave a single peak on HPLC at 18.8 min (Figure 1). The room temperature ^{1}H NMR (DMSO-d_6) showed line-broadening for deoxyribose protons:[18a] δ 1.7–2.4 (b, 1H), 2.02 (s, 3H), 2.7–3.1 (b, 1H), 3.50 (m, 1H), 3.60 (m, 1H), 3.82 (m, 1H), 3.92 (s, 2H), 4.46 (m, 1H), 4.93 (s, 1H), 5.19 (s, 1H), 6.09 (m, 1H), 6.40 (s, 2H), 7.27–7.39 (2t, 3H), 7.55–7.59 (d, 2H) and 7.87–7.94 (m, 2H), 10.80 ppm (s, 1H); ^{13}C NMR (DMSO-d_6) δ 23.0, 36.9, 37.4, 62.6, 71.6, 84.5, 88.6, 115.5, 120.7, 120.9, 125.6, 127.3, 127.4, 139.0, 140.6, 141.6, 143.7, 143.8, 144.5, 150.8, 153.9, 156.6, 173.0.

Preparation of Fmoc-Protected dA, dC, and dG. The protection of the exo-amino groups of the normal 2′-deoxy nucleosides was achieved according to the transient trimethylsilyl procedure of Koole et al.[11c] The products were purified by silica gel chromatography and their ^{1}H NMR data were consistent with those reported.[11c] Yields of purified Fmoc nucleosides are as follows: N^6-Fmoc-dA, 60%; N^4-Fmoc-dC, 95%; and N^2-Fmoc-dG, 70%.

Fmoc Protection of *N*-(2′-Deoxyguanosin-8-yl)-2-(acetylamino)fluorene. An amount of 1.03 g (2.12 mmol) vacuum-dried dG-C$_8$-AAF was coevaporated with 60 mL of anhydrous pyridine and was redissolved in 20 mL of anhydrous pyridine under nitrogen. A volume of 3 mL of chlorotrimethylsilane was then injected into the flask and the mixture was stirred at room temperature for about 2 h. Stirring was then stopped for 5 min to allow the crystalline pyridinium hydrochloride to settle to the bottom of the flask. The solution was then transferred under nitrogen through a stainless steel cannula to another flask containing 2 g (7.63 mmol) of vacuum-dried 9-fluorenylmethoxy chloroformate. The mixture was stirred and the reaction was allowed to proceed at room temperature under nitrogen until the starting dG-C$_8$-AAF had been consumed as judged by TLC.[23] The reaction time was 20 h at which time the reaction was stopped by the addition of 20 mL of water. CH_3OH and CH_2Cl_2 were added to the mixture, and the resulting homogenous solution was stirred at room temperature for 2 h. Solid sodium bicarbonate was then added to the solution until no CO_2 evolution was noticeable. The mixture was then evaporated to dryness under reduced pressure at room temperature. The residues were extracted with a mixture of 50 mL of water and 50 mL of CH_2Cl_2. The organic phase was separated, filtered, and evaporated to give a light brown oil, which was then coevaporated three times with 150 mL of dry toluene, yielding a yellowish solid. The latter consisted of two products migrating very close to each other on the TLC plate (R_f = 0.5 and 0.48, 1:7 CH_3OH/CH_2Cl_2), with the high-R_f species as the major product. To separate these two products, the solid materials were taken up in 10 mL of 1/20 CH_3OH/CH_2Cl_2 and loaded onto a column of 100 g silica gel packed with 1:20 CH_3OH/CH_2Cl_2. The column was eluted with a linear 500 mL gradient of CH_3OH/CH_2Cl_2 from 1:20 to 1:10 to produce 620 mg of pure high-R_f product (HPLC: 29.7 min, Figure 1) together with an unresolved mixture of both. The latter was rechromatographed to give another 142 mg of pure high-R_f product (total 762 mg, yield 48%) and 320 mg of an equimolar mixture of both products as shown by TLC. Structural identification of the high-R_f product showed that it to be ***N*-[*N*^{2}-(9-fluorenylmethoxycarbonyl)-2′-deoxyguanosin-8-yl]-2-(acetylamino)fluorene (*N*^{2}-Fmoc-dG-C$_8$-AAF)**: ^{1}H NMR (DMSO-d_6) δ 1.8–2.3 (vb) and 2.05 (s, 4H), 2.9–3.3 (vb, 1H), 3.51 (m, 1H) and 3.63 (m, 1H), 3.81 (s, 1H), 3.93 (s, 2H), 4.33 (t, J = 6.6 Hz, 1H), 4.46 (s, 1H), 4.56 (d, J = 6.6 Hz, 2H), 4.68 (s, 1H), 5.21(s, 1H), 6.17(m, 1H), 7.25–8.0 (m, 15H), and 11.45 (bs, 2H). In $CDCl_3$, N^{2}H-Fmoc and 1-NH appeared separately at 10.30 and 11.55 ppm but the aliphatic region showed considerable signal overlapping. ^{13}C NMR (DMSO-d_6) δ 22.6, 36.5, 36.7, 46.2, 61.8, 67.2, 70.7, 83.9, 88.0, 118.6, 120.0, 120.3, 120.6, 121.4, 125.2, 126.9, 127.2, 127.3, 127.9, 128.9, 136.1, 140.1, 140.8, 143.2, 143.4, 144.2, 147.2, 148.4, 154.4, 155.3 (in $CDCl_3$), 173.0. FAB^{+}[20] $C_{40}H_{34}N_6O_7$ = 710.1189 (0.6,$(BH)_2$ + H), 818 (4, M + thioglycerol), 711 (2, M + H), 595 (37, BH_2), 552 (5), 399 (9), 373 (16), 357 (9), 331 (16), 330 (15), 223 (19), 197 (19), 179 (100), 165 (42). FAB$^-$ 709 (2, M–H), 593 (2), 513 (14), 487 (80), 471 (5), 445 (6), 444 (4), 427 (2), 397 (5), 371 (5), 355 (11), 329 (11), 328 (6), 249 (18), 165 (6).

The low-R_f product, identified as ***N*-[*O*^{6}-(9-fluorenylmethoxycarbonyl)-2′-deoxyguanosin-8-yl]-2-(acetylamino)-fluorene (*O*^{6}-Fmoc-dG-C$_8$-AAF)**, was purified by converting a mixture of both high- and low-R_f products to their corresponding DMT derivatives and by detritylating the purified DMT derivative of the low-R_f product (see the preparation and detritylation of DMT-*N*^{2}-Fmoc-dG-C$_8$-AAF for details): HPLC 28.2 min, Figure 1; ^{1}H NMR ($CDCl_3$) δ 2.17 (s, 3H), 1.9–2.5 (b, 1H), 2.5–3.0 (b, 1H), 3.55 (bs, 2H), 3.87 (s, 2H), 4.00 (m, 1H), 4.05 (bs, 1H), 4.49 (b, 2H), 4.59 (m, 1H), 6.29 (t, 1H), 6.9–7.9 (m, 17H). ^{13}C NMR ($CDCl_3$–CD_3OD) δ 22.4, 36.9, 39.6, 46.2, 68.8, 62.9, 72.1, 86.2, 88.8. FAB^{+}:[24] 818 (1, M + thioglycerol), 595 (2, BH_2), 399 (7), 373 (5), 331 (7), 330 (6), 179 (100), 165 (50).

Dimethoxytritylation of Fmoc-Protected Nucleosides. Dimethoxytritylation was carried out based on the monomethox-

(22) (a) Nicoletti, M.; Iorio, M. A. *Magn. Reson. Chem.* **1986**, *24*, 221. (b) Evans, F. E.; Miller, D. W. *J. Am. Chem. Soc.* **1983**, *105*, 4863. (c) Chang, C.-J.; Gomes, J. D.; Byrn, S. R. *J. Org. Chem.* **1983**, *48*, 5151.

(23) To monitor the progress of the reaction by TLC, a 0.1-mL portion of the reaction mixture was withdrawn through a dry syringe, treated with a mixture of water and methanol for 2 h to produce a clear solution, and then applied to the TLC plate. The plates were developed using 1/7 methanol–dichloromethane, dried in the oven at 130 °C for 20 min to remove the pyridine, and then visualized under a UV light. If the aqueous methanol treatment of the TLC samples was less then 1 h, some of the unreacted dG-C$_8$-AAF remained in the silylated forms, which was more mobile than dG-C$_8$-AAF on the TLC plate.

(24) BH = *N*-[*O*^{6}-(9-Fluorenylmethoxycarbonyl)guanin-8-yl]-2-(acetylamino)fluorene (*O*^{6}-Fmoc-Gua-C$_8$-AAF).

ytritylation procedures of Koole et al.[11c] Only the 5'-DMT products were formed from N^6-Fmoc-dA, N^4-Fmoc-dC and N^2-Fmoc-dG. N^2-Fmoc-dG-C_8-AAF gave two products, namely, the 5'-DMT and the 3',5'-diDMT product, and its dimethoxytritylation is described here in detail: Dry N^2-Fmoc-dG-C_8-AAF (620 mg, 0.873 mmol) was coevaporated to dryness with 50 mL of anhydrous pyridine under reduced pressure at room temperature and redissolved in 15 mL of anhydrous pyridine. 4,4'-Dimethoxytrityl chloride (640 mg, 1.89 mmol) was then introduced into the flask and the mixture was stirred at room temperature under nitrogen in the dark. The reaction was shown to be complete in 50 min as judged by the disappearance of Fmoc-dG-C_8-AAF by TLC.[25] Two DMT-containing products were formed, the major one with R_f 0.32 and the minor one with R_f 0.76 (1:20 CH_3OH/CH_2Cl_2). The reaction was stopped by the addition of 20 mL of saturated aqueous sodium bicarbonate solution and stirring for another 15 min. The mixture was extracted three times with CH_2Cl_2 (150 mL). The extracts were combined, evaporated under reduced pressure to dryness, and then coevaporated three times with 150 mL of toluene. The red-brown solid residue thus obtained was taken up in 5 mL of CH_2Cl_2, filtered, and then chromatographed twice on a 100-g silica gel column, eluting first with CH_2Cl_2 followed by a linear gradient from 1/40 to 1/10 CH_3OH/CH_2Cl_2. This gave the purified R_f 0.32 species, identified as *N*-[5'-Dimethoxytrityl-N^2-(9-fluorenylmethoxycarbonyl)-2'-deoxyguanosin-8-yl]-2-(acetylamino)fluorene (5'-DMT-N^2-Fmoc-dG-C_8-AAF) (yield 450 mg, 51%), and the purified R_f 0.76 species, identified as *N*-[3', 5'-bis(dimethoxytrityl)-N^2-(9-fluorenylmethoxycarbonyl)-2'-deoxyguanosin-8-yl]-2-(acetylamino)fluorene (3',5'-diDMT-N^2-Fmoc-dG-C_8-AAF).

***N*-[5'-Dimethoxytrityl-N^2-(9-fluorenylmethoxycarbonyl)-2'-deoxyguanosin-8-yl]-2-(acetylamino)fluorene (5'-DMT-N^2-Fmoc-dG-C_8-AAF):** ^{1}H NMR (DMSO-d_6) δ 2.08 (s, 3H), 2.2–2.4 (vb, 1H), 2.8–3.2 (vb, 1H), 3.13 (m, 1H), 3.38 (m, 1H), 3.61 (s, 6H), 3.85 (b, 2H), 4.03 (s, 1H), 4.33 (t, J = 6.6 Hz, 1H), 4.52–4.63 (m, 3H), 5.15 (bs, 1H), 6.27 s, 1H), 6.5–8.0 (m, 28H), 11.24 (s, 2H). In $CDCl_3$, N^2H-Fmoc and 1-NH appeared separately at 9.7–10.30 (bs) and 11.2-11.7 (bs) ppm but the aliphatic region showed considerable signal overlapping. ^{13}C NMR ($CDCl_3$) δ 22.8, 36.9, 38.6, 46.3, 54.9, 64.8, 68.2, 70.8, 83.8, 85.9, 87.0, 112.7, 113.0, 118.7, 135.8, 136.2, 146.6, 147.9, 154.2, 155.2, 158.1, 173.0; FAB^{+}[20] $C_{61}H_{52}N_6O_9$ = 1012.6, 595 (2, BH_2), 399 (2), 373(9), 357 (3), 331(7), 330 (6), 303 (92), 179 (13), 165 (15). FAB$^-$: 815 (1), 789 (4, M–Fmoc), 513 (2), 487 (3), 355 (5), 329 (5), 249(6), 165 (11).

***N*-[3', 5'-Bis(dimethoxytrityl)-N^2-(9-fluorenylmethoxycarbonyl)-2'-deoxyguanosin-8-yl]-2-(acetylamino)-fluorene (3',5'-diDMT-N^2-Fmoc-dG-C8-AAF):** ^{1}H NMR ($CDCl_3$) δ 2.33 (bs, 3H), 2.53 (b, 1H), 2.98 (b, 1H), 3.32 (b, 1H), 3.48 (b, 1H), 3.68 and 3.72 (2s, 14H), 4.05 (b, 1H), 4.40 (bs, 4H, reduced to 3 bs upon adding CD_3OD), 6.17 (b, 1H), 6.5–7.8 (m, 41H), 11.3 (b,1H); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 23.0, 36.8, 39.0, 46.6, 55.2, 64.2, 68.4, 74.9, 85.9, 84.8, 87.1, 113.1, 113.3, 119.6, 120.2, 124.8, 136.0, 136.3, 145.4, 149.0, 153.4, 155.4, 158.5, 158.7, 173.0; FAB^{+}:[20] $C_{82}H_{70}N_6O_{11}$ = 1314, 595 (16, BH_2), 553 (3), 552 (4), 399 (2), 373 (11), 331 (13), 330 (10), 304 (78), 303 (100), 197 (13), 179 (70), 165 (33).

5'-*O*-(4,4'-Dimethoxytrityl)-N^6-(9-fluorenylmethoxycarbonyl)-2'-deoxyadenosine (DMT-N^6-Fmoc-dA): ^{1}H NMR ($CDCl_3$) δ 2.48–2.6 (m, 1H), 2.73–2.91 (m, 1H), 3.39 (m, 1H), 3.41 (m, 1H), 3.72 (s, 6H), 4.21 (m, 1H), 4.31 (t, J = 6.6 Hz, 1H), 4.60 (d, J = 6.9 Hz, 2H), 4.72 (m, 1H,), 6.46 (t, J = 6.3 Hz, 1H), 6.76 (d, J = 8.7 Hz, 4H), 7.1–7.3 (m, 9H), 7.36 (t, J = 7.1 Hz, 4H), 7.61 (d, 2H) and 7.74 (d, J = 7.5 Hz, 2H), 8.12 (s, 1H), 8.69 (s, 1H), 9.19 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 40.4, 47.0, 55.3, 63.8, 67.8, 72.4, 84.9, 86.5, 86.7.

5'-*O*-(4,4'-Dimethoxytrityl)-N^4-(9-fluorenylmethoxycarbonyl)-2'-deoxycytidine (DMT-N^4-Fmoc-dC): ^{1}H NMR ($CDCl_3$) δ 2.19–2.30 (m, 1H), 2.71–2.80 (m, 1H), 3.37–3.52 (m, 2H), 3.76 (s, 6H), 4.18 (m, 1H), 4.25 (t, J = 7.2 Hz, 1H), 4.44 (d, J = 7.2 Hz, 2H), 4.53 (m, 1H), 6.29 (t, J = 5.7 Hz, 1H), 6.84 (d, J = 8.1 Hz, 4H), 7.00 (4H,b, 4H), 7.15–7.45 (m, 13H), 7.58 (d, 2H), 7.76 (d, J = 7.5 Hz, 2H), 8.26 (d, J = 7.5 Hz, 1H), 9.0 (vb, 1H); ^{13}C NMR ($CDCl_3$) δ 42.0, 46.6, 55.2, 62.7, 68.0, 70.7, 86.5, 86.8, 87.3.

5'-*O*-(4,4'-Dimethoxytrityl)-N^2-(9-fluorenylmethoxycarbonyl)-2'-deoxyguanosine (DMT-N^2-Fmoc-dG): ^{1}H NMR ($CDCl_3$) δ 2.55–2.80 (m, 2H), 3.46 (m, 2H), 3.62 (s, 6H), 3.90 (t, J = 7.2 Hz, 1H), 4.10–4.45 (m, 3H), 5.19 (m, 1H), 5.63 (m, 1H), 6.24 (t, J = 6 Hz, 1H), 6.64 (d, J = 7.5 Hz, 4H), 6.9–7.31 (m, 13H), 7.38 (d, 2H), 7.49 (d, J = 7.5 Hz, 2H), 7.72 (s, 1H), 10.02 (s, 1H), 11.43 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 40.2, 46.5, 55.2, 64.7, 68.3, 71.1, 84.7, 86.4, 87.1.

Preparation of CED phosphoramidites. A general procedure is described for the preparation of CED phosphoramidite from Fmoc-protected nucleosides: **5'-*O*-(4,4'-Dimethoxytrityl)-N^2-(9-fluorenylmethoxycarbonyl)-C_8-(*N*-fluoren-2-ylacetamido(-2'-deoxyguanosine 3'-*O*-[2-Cyanoethyl *N,N*-diisopropylphosphoramidite] (DMT-dG-AAF-Fmoc-CED):** In a 25-mL round bottomed flask connected to a constant flow of nitrogen was dissolved 500 mg (0.49 mmol) of vacuum dried 5'-DMT-N^2-Fmoc-dG-C_8-AAF in 5 mL of dry CH_2Cl_2. A mixture of 0.5 mL 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite and 5 mL of 0.2 M diisopropylethylammonium 1*H*-tetrazolide in dry CH_2Cl_2 was then injected into the reaction flask with constant stirring at room temperature.[17] The reaction was monitored by TLC as described in the synthesis of DMT-Fmoc-dG-C_8-AAF. The phosphoramidite product (two diastereoisomers) appeared as two highly mobile spots on TLC. A less-mobile spot, possibly due to the phosphonate species, was also observed with an intensity of less than $^1/_{20}$ that of the major phosphoramidite. The stirring was continued for 2 h to allow the reaction to complete. The mixture was then poured into 80-mL concentrated aqueous $NaHCO_3$ and was then extracted with 3 × 20 mL portions of CH_2Cl_2. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure at room temperature to give a yellow solid residue. The residue was dissolved in 5 mL of toluene (1 mL of CH_2Cl_2 can be added to facilitate dissolving when necessary) and precipitated from 150 mL vigorously stirring hexane at room temperature. The precipitates were then purified by flash chromatography on 70 mL of silica gel using 1/20 CH_3OH/CH_2Cl_2 as eluent (with or without 1% pyridine). Fractions containing the desired phosphoramidite product were combined and evaporated to dryness and then precipitated once more from 150 mL of hexane. The precipitates were vacuum-dried for 1 day to give 576 mg (0.468mmol, yield 96%) of white powders which were stored at −20 °C under nitrogen: R_f 0.18 and 0.30 ($AcOEt/CH_2Cl_2$, 1/1), 0.46 and 0.53 (CH_3OH/CH_2Cl_2, 1/20); ^{1}H NMR ($CDCl_3$) δ 0.9–1.3 (m, 12H), 2.22 (s, 3H), 2.0–2.7 (m, 4H), 3.25–3.95 (m, 8H), 3.75 (s, 6H), 4.25 (t) and 4.32 (m) (2H), 4.53 (d, J = 6.6 Hz, 2H), 4.82 (b, 1H), 6.2–6.3 (m, 1H), 6.6–6.8 (m,4H), 7.1–7.9 (m, 24H), 8.2 and 8.4 (b, 1H), 11.4 (b, 1H); ^{31}P NMR ($CDCl_3$) δ 148.3 and 148.8 ppm.

5'-*O*-(4,4'-Dimethoxytrityl)-N^6-(9-fluorenylmethoxycarbonyl)-2'-deoxyadenosine 3'-*O*-[2-Cyanoethyl *N,N*-diisopropylphosphoramidite] (DMT-dA-Fmoc-CED): reaction time, 5 h, flash chromatography solvent, $AcOEt/CH_2Cl_2$/pyridine (25/75/1); R_f 0.38 and 0.48 ($AcOEt/CH_2Cl_2$, 1/1), 0.63 and 0.68 (CH_3OH/CH_2Cl_2, 1/20); ^{1}H NMR ($CDCl_3$) δ 1.1–1.3 (m, 12H), 2.46 and 2.60 (2t, J = 6.3 Hz, 2H), 2.65 (m, 1H), 2.91 (m, 1H), 3.3–3.9 (m, 6H), 3.76 (s, 6H), 4.31 (m, 2H), 4.62 (d, J = 6.6 Hz, 2H), 4.80 (m, 1H), 6.46 and 6.47 (2t, 1H), 6.78 and 6.79 (2d, J = 8.7 Hz, 4H), 7.1–7.4 (m, 13H), 7.61 and 7.74 (2d, J = 7.5 Hz, 4H), 8.17 and 8.19 (2s, 1H,), 8.73 (s, 1H), 9.06 (bs, 1H); ^{31}P NMR ($CDCl_3$) 149.2 and 149.3 ppm.

5'-*O*-(4,4'-Dimethoxytrityl)-N^4-(9-fluorenylmethoxycarbonyl)-2'-deoxycytidine 3'-*O*-[2-Cyanoethyl *N,N*-diisopropylphosphoramidite] (DMT-dC-Fmoc-CED): reaction time, 1h; flash chromatography solvent: $AcOEt/CH_2Cl_2$/pyridine (33/67/1); R_f 0.30 and 0.42 ($AcOEt/CH_2Cl_2$, 1/1), 0.47 and 0.56 (CH_3OH/CH_2Cl_2, 1/20); ^{1}H NMR ($CDCl_3$) δ 1.0–1.25 (m, 12H), 2.26 (m, 1H), 2.43 and 2.56 (2t, J = 6.3 Hz, 2H), 2.69 (m, 1H), 3.3–3.8 (m, 6H), 3.79 (s, 6H), 4.21 (m, 1H), 4.28 (t, J = 7 Hz, 1H), 4.46 (m, 2H), 4.60 (m, 1H), 6.25 and 6.27 (2t, J = 6 Hz, 1H), 6.85 and

(25) To monitor the progress of the reaction, a portion of the reaction mixture was withdrawn, treated with saturated aqueous sodium bicarbonate solution, and then checked by TLC. The plates were developed using 1/20 methanol–dichloromethane, dried at 130 °C to remove the pyridine, visualized under UV light to detect the UV-absorbing species, and then sprayed with 5% dichloroacetic acid in dichloromethane to reveal the DMT-containing species.

6.86 (2d, J = 8.4 Hz, 4H), 6.95 (m, 1H), 7.2–7.45 (m, 13H), 7.57 and 7.77 (2d, J = 7.5 Hz, 4H), 8.22 and 8.32 (2d, J = 7.5 Hz, 1H), 8.44 (bs, 1H); ^{31}P NMR ($CDCl_3$) 149.2 and 149.7 ppm.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*²-(9-fluorenylmethoxycarbonyl)-2′-deoxyguanosine 3′-*O*-[2-Cyanoethyl *N*,*N*-diisopropylphosphoramidite] (DMT-dG-Fmoc-CED): reaction time: 2 h; flash chromatography solvent: CH_3OH/CH_2Cl_2 (1/25); R_f: 0.13 and 0.20 ($AcOEt/CH_2Cl_2$, 1/1), 0.31 and 0.37 (CH_3OH/CH_2Cl_2, 1/20); ^{1}H NMR ($CDCl_3$) δ 1.1–1.23 (m, 12H), 2.30–2.85 (m, 4H), 3.32 and 3.41 (2m, 2H), 3.5–3.9 (m, 4H), 3.74 and 3.75 (2s, 6H), 4.20–4.32 (m, 1H) and 4.22 (t, 1H), 4.49 and 4.53 (2d, J = 6.6 Hz, 2H), 4.63–4.82 (m, 1H), 6.21 (t, J = 6.6 Hz, 1H), 6.77 and 6.79 (2d, J = 8.7 Hz, 4H), 7.1–7.45 (m, 13H), 7.57 (m, 2H), 7.75 (d, J = 7.5 Hz, 2H), 7.77 and 7.81 (2s, 2H), 8.7 (b, 1H), 11.4 (b, 1H); ^{31}P NMR ($CDCl_3$) 148.6 and 149.0 ppm.

Detritylation and Depurination Studies of dG-C₈-AAF Compounds. (1) Depurination of *N*²-Fmoc-dG-C₈-AAF by 3% Dichloroacetic Acid in Dichloromethane. Preparation of *N*-[*N*²-(9-Fluorenylmethoxycarbonyl)guanin-8-yl]-2-(acetylamino)fluorene (*N*²-Fmoc-Gua-C₈-AAF): An amount of 50 mg (70.4 μmol) of N^2-Fmoc-dG-C_8-AAF was mixed with 10 mL of 3% dichloroacetic acid in CH_2Cl_2 and the resulting solution was stirred at room temperature under nitrogen. TLC showed the disappearance of N^2-Fmoc-dG-C_8-AAF in 15 min and the clean formation of a highly mobile UV-absorbing species. After stirring for another 15 min, the mixture was evaporated to dryness, and the residues were separated by preparative TLC (developing solvent 1:25 CH_3OH/CH_2Cl_2) to give 34 mg of a white solid material, identified as N-[N^2-(9-fluorenylmethoxycarbonyl)-guanin-8-yl]-2-(acetylamino)fluorene (N^2-Fmoc-Gua-C_8-AAF): yield 81%; HPLC 32.0 min (Figure 1); ^{1}H NMR (1/7 $CD_3OD/CDCl_3$) δ 1.89 (s, 3H), 3.73 (s, 2H), 4.03 (t, J = 6.6 Hz, 1H), 4.35 (d, J = 6.6 Hz, 2H), 7.05–7.3 (m, 8H), 7.36 (d, J = 7.2 Hz, 1H), 7.40 (d, J = 7.5 Hz, 2H), 7.54 (d, J = 7.5 Hz, 2H), 7.61 (d, J = 7.2 Hz, 1H), 7.67 (d, J = 7.8 Hz, 1H). The residual peaks for the three exchangeable protons were observed in equal but reduced intensities due to exchange with solvent: 10.7 (bs,1H), 11.5 (s, 1H), 12.35 (bs, 1H); ^{1}H NMR ($CDCl_3$) δ (In $CDCl_3$ the compound exists as two rotamers[22] of 2:3 ratio.) 1.95 and 1.96 (2s overlap, 3H, CH_3CO AAF), 3.67 and 3.76 (2s, 2H, CH_2 AAF), 4.19 (q, 1H, CH Fmoc), 4.44 (d, J = 6.3 Hz) and 4.51 (bs, 2H, CH_2 Fmoc), 6.88–7.75 (m, 15H, aromatic AAF and Fmoc), 9.9 and 10.7(1H, 2b, N^2H-Fmoc), 11.4 (bs, 1H, 1-NH guanine), 11.75 and 11.8 (2bs, 1H, 9-NH guanine); ^{13}C NMR ($CDCl_3/CD_3OD$) δ 24.1, 36.6, 46.4, 67.6, 107.9, 119.8, 120.1, 120.7, 124.6, 124.9, 125.1, 126.8, 126.9, 127.1, 127.3, 127.7, 136.6, 139.9, 141.0 142.6, 142.9, 143.4, 144.9, 146.6, 149.2, 152.7, 154.5, 155.6, 172.4; FAB⁺ $C_{35}H_{26}O_4N_6$ = 594, 595 (25, M + H), 552 (5), 399 (5), 373 (17), 357 (11), 330 (21), 179 (100).

N^2-Fmoc-Gua-C_8-AAF was also obtained by reaction of DMT-N^2-Fmoc-dG-C_8-AAF with 3% dichloroacetic acid in CH_2Cl_2. Quantitative depurination was found to occur in 5 min.

(2) Depurination of *O*⁶-Fmoc-dG-C₈-AAF by 3% Dichloroacetic Acid in Dichloromethane. Preparation of *N*-[*O*⁶-(9-Fluorenylmethoxycarbonyl)guanin-8-yl]-2-(acetylamino)-fluorene (*O*⁶-Fmoc-Gua-C₈-AAF). This compound was prepared from the reaction of O^6-Fmoc-dG-C_8-AAF with 3% dichloroacetic acid in CH_2Cl_2 and purified by the same method as described for preparing N^2-Fmoc-Gua-C_8-AAF. HPLC 32.9 min (Figure 1); ^{13}C NMR ($CDCl_3/CD_3OD$) δ 24.4, 36.8, 46.1, 69.1, 112, 119.8, 120.3, 121.0, 124.6, 125.1, 125.4, 126.9, 127.0, 127.3, 127.5, 127.6, 136.7, 140.2, 141.0, 142.6, 142.9, 143.1, 143.5, 145.1, 149.7, 172.7. A 4:1 mixture of O^6- to N^2-Fmoc-Gua-C_8-AAF was also prepared by mixing the purified isomers and the ^{1}H NMR was taken ($CDCl_3/CD_3OD$) δ 1.92 (s) and 2.03 (s) (3H), 3.78 (s) and 3.84 (s) (2H), 3.89 (t, J = 6 Hz,) and 4.07 (t, J = 6.6 Hz) (1H), 4.21 (d, J = 6 Hz) and 4.38 (d, J = 6.6 Hz) (2H), 6.9–7.5, 7.57 (d, J = 7.2 Hz), 7.66 (d, J = 6.6 Hz), 7.69 (d, J = 7.5 Hz), 7.74 (d, J = 8.1 Hz), 7.81 (d, J = 8.1 Hz). The exchangeable protons in the low-field range were observed in $CDCl_3$: 11.3 (bs, 1-NH, N^2-isomer), 11.75 and 11.9 (2bs, 9-NH, N^2-isomer), 12.3 (bs, 9-NH, O^6-isomer).

(3) Detritylation of DMT-*N*²-Fmoc-dG-C₈-AAF by 3% Acetic Acid in Dichloromethane. DMT-N^2-Fmoc-dG-C_8-AAF was dissolved in 3% acetic acid in dichloromethane and the course of detritylation was followed by TLC.[25] Detritylation was found to be complete in 4 h and quantitative formation of N^2-Fmoc-dG-C_8-AAF (as a less-mobile non-DMT UV spot) was observed. The detritylated product was found to be stable on prolonged contact with the reagent for at least 3 days without the formation of the depurinated species N^2-Fmoc-Gua-C_8-AAF which would show a more-mobile non-DMT UV spot on the TLC. To isolate the product, the reaction was stopped after running overnight and the mixture was concentrated. Hexane was then added and the precipitate formed was chromatographed on a silica gel column. The purified product was identical with N^2-Fmoc-dG-C_8-AAF as shown by analytical TLC, HPLC, and the spectral data.

Detritylation of diDMT-N^2-Fmoc-dG-C_8-AAF under the same conditions as described above also afforded N^2-Fmoc-dG-C_8-AAF quantitatively. The same procedure was also used to detritylate DMT-O^6-Fmoc-dG-C_8-AAF which lead to the purification and structural identification of O^6-Fmoc-dG-C_8-AAF (see synthesis of Fmoc-dG-C_8-AAF for spectral data).

(4) Detritylation of DMT-*N*²-Fmoc-dG-C₈-AAF by 3% Chloroacetic Acid in Dichloromethane. DMT-N^2-Fmoc-dG-C_8-AAF was dissolved in 3% chloroacetic acid in dichloromethane and the procedure above was repeated. TLC indicated complete detritylation in 35 min as judged by the disappearance of the starting DMT-containing UV spot and the formation of a single less-mobile non-DMT UV spot corresponding to N^2-Fmoc-dG-C_8-AAF. No depurination product was detected. The reaction mixtures were purified as above to give the identical detritylated product N^2-Fmoc-dG-C_8-AAF.

***N*-(Guanin-8-yl)-2-(acetylamino)fluorene (Gua-C₈-AAF).** To a solution of N-[N^2-(9-fluorenylmethoxycarbonyl)guanin-8-yl]-2-(acetylamino)fluorene (N^2-Fmoc-Gua-C_8-AAF) in 10 mL of 1:4 CH_3OH/CH_2Cl_2 was added 10 mL of triethylamine, and the solution was allowed to stand at room temperature for Fmoc deprotection to occur. The solution began to turn cloudy white in about 30 min. TLC showed the disappearance of N^2-Fmoc-Gua-C_8-AAF in 2 h and the clean formation of a single less-mobile product having R_f = 0 in 1:30 $MeOH/CH_2Cl_2$, and 0.5 in 1:4 $MeOH/CH_2Cl_2$. The reaction mixture was evaporated under reduced pressure to give a white solid residue, which was then dissolved in 1:4 $MeOH/CHCl_3$ and applied on a silica gel column. The column was then eluted with 1:4 $MeOH/CHCl_3$ to yield the purified Gua-C_8-AAF as a white powder: HPLC 19.4 min (Figure 1); ^{1}H NMR (DMSO-d_6) δ 2.00 (s, 3H), 3.92 (s, 2H), 6.32 (s, 2H), 7.29 (t, 1H), 7.32 (d, J = 5.1 Hz, 1H), 7.36 (t, J = 7.2 Hz, 1H), 7.51 (s, 1H), 7.56 (d, J = 7.2 Hz, 1H), 7.87 (d, J = 8.8 Hz, 1H), 7.90 (d, J = 8.8 Hz, 1H), 10.59 (s, 1H), 12.6 (bs, 1H); ^{1}H NMR (1:4 $CD_3OD/CDCl_3$) (The compound exists as two rotamers[22] of 1:1 ratio.) δ 1.94 and 1.95 (2s overlap, 3H), 3.82 and 3.84 (2s overlap, 2H), 7.15–7.30 (2t + 1d, 3H), 7.35 and 7.36 (2s, 1H), 7.44, 7.68, and 7.77 (3t, 3H,); ^{13}C NMR (1:4 $CD_3OD/CDCl_3$) δ 24.3 (2s overlap) 36.8, 116.4, 120.4, 120.9, 125.1, 125.4, 127.1, 127.2, 127.6, 137.0, 140.1, 143.0 143.4, 145.0, 172.5; FAB⁺: ($C_{20}H_{16}N_6O_2$ = 372) 395 (2, M + Na), 373 (33, M + H), 331 (9), 330 (6), 245 (33), 240 (100), 223 (38), 207 (3), 181 (7), 165 (5); FAB⁻ 371 (100), 343 (7), 329 (84), 328 (50), 222 (14), 205 (51, 180 (9), 165 (4).

Gua-C_8-AAF was also formed from O^6-Fmoc-Gua-C_8-AAF by the deprotection of Fmoc as described above and from depurination of dG-C_8-AAF with 3% dichloroacetic acid in 1:4 $MeOH/CH_2Cl_2$. Analytical TLC and HPLC experiments showed that Gua-C_8-AAF from the three reactions are identical.

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Supplementary Material Available: A full listing of NMR data and peak assignments and copies of spectra (34 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.