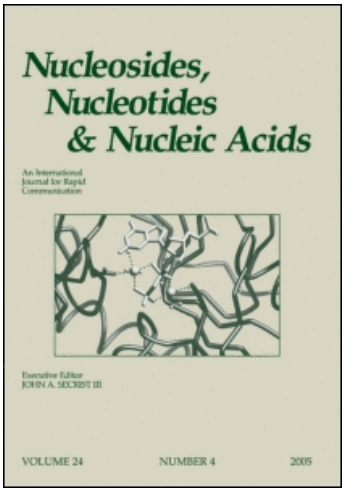

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8-AMINO-2'-DEOXYGUANOSINE INCORPORATION INTO OLIGOMERIC DNA

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ABSTRACT

During the incorporation of 8-amino-dG into oligomeric DNA, the deprotection conditions previously recommended (28% ammonia at room temperature) do not effect complete removal of the dimethylaminomethylene protecting groups. At elevated temperatures oxidative degradation of the oligomer and exchange of ammonia with dimethylamine in the protecting group at C8 occurred. The resolution of these problems and a method to obtain a series of homogenous oligomers in reasonable yield containing 8-amino-dG located site-specifically are described.

2-Nitropropane and other secondary nitroalkanes, as well as acetone oxime, have been shown to modify nucleosides in rat liver DNA and RNA.[1-5] Amongst the modifications produced by these compounds is the amination of guanine residues at the 8-position. It has been proposed that all of the hepatocarcinogens of this class give rise to two common metabolic intermediates, namely hydroxylamine-*O*-acetate and hydroxylamine-*O*-sulfonic acid, which are regarded as the true procarcinogens.[6] Solvolytic decomposition of either moiety is thought to give rise to NH_2^+ ions capable of aminating cellular DNA *in vivo*. This pattern of behavior is common to almost all carcinogenic organic amines.[7-9]

In continuation of previous studies[10-12] on the oxidation and transformation products of DNA bases, we became interested in examining the mutagenic spectrum of 8-amino-2'-

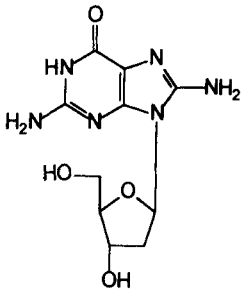
deoxyguanosine (1: 8-amino-dG) when present as a residue in DNA. Our approach involves the synthesis of DNA oligomers, in which are embedded modified deoxynucleosides located site-specifically. These are used for both *in vitro* and *in vivo* studies on duplex DNA. Well-established assay procedures[10] then reveal the extent and nature of any mutagenic events. Here we report on the incorporation of 1 into a series of DNA oligomers that were needed for these biological studies.

Initially, we followed the work of T.S. Rao and his associates who had reported[13] the preparation of a series of oligomers containing 8-amino-dG residues in connection with their studies on triple-strand helical DNA. However, on repeating these synthetic procedures, we found that complete removal of the dimethylaminomethylene (DAM) groups used to protect the 2- and 8-amino groups of 8-amino-dG during DNA synthesis does not occur under the conditions that they describe (concentrated ammonia at ambient temperature; reaction time not given). We have found that one of the protecting groups is removed, while the other survives essentially intact over a period of 48 h. No analysis of individual oligomers was reported[13] so that it is difficult to know whether or not they were working with partially deprotected DNA. However, in our experience when attempts were made to deprotect the DNA fully by raising the temperature for an extended period, decomposition was observed with extensive chain cleavage. Therefore, we initiated a study of the effects of concentrated ammonia on both a short DNA oligomer containing a single *bis*-protected 8-amino-dG and the monomeric *bis*-protected derivative (2) of 8-amino-dG in an attempt to find conditions that would limit the decomposition and accomplish our objective of synthesizing reasonable quantities of DNA oligomers containing this residue.

EXPERIMENTAL PROCEDURES

Chemicals

N^2, N^8-*Bis*[(dimethylamino)methylene]-8-amino-2'-deoxyguanosine [2: 8-amino-dG-$(DAM)_2$] was synthesized as previously described.[13] Phosphoramidites and reagents used in DNA synthesis were purchased from Perkin Elmer/Applied Biosystems (Foster City, CA). HPLC grade acetonitrile and triethylamine were obtained from Fisher Scientific (Springfield, NJ), and the latter chemical was distilled before use. 2-Mercaptoethanol was purchased from



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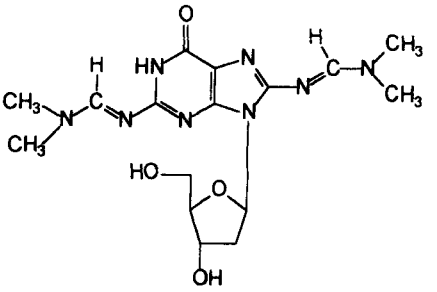

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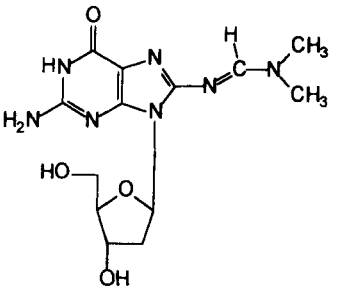

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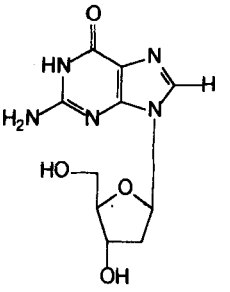

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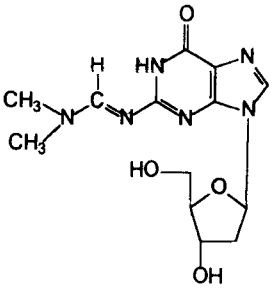

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the Aldrich Chemical Co. (Milwaukee, WI). Bacterial alkaline phosphatase and snake venom phosphodiesterase were supplied by Pharmacia Biotech (Piscataway, NJ).

Synthesis and purification of oligomers containing 8-amino-dG

Oligomers were synthesized on a Perkin Elmer/ABI 394 DNA/RNA synthesizer (Foster City, CA) using standard phosphoramidite protocols. The 8-amino-dG(DAM)$_2$ (**2**) was introduced into oligomers containing 6,10,15, 24, and 38 bases, respectively. After each synthesis the product was deprotected by incubation in 1 mL of 28% ammonium hydroxide containing 2-mercaptoethanol (0.1 M, 7 μL) for 20 h at 55 °C. The solution was evaporated to dryness in a vacuum centrifuge (Savant Instruments, Farmingdale, NY), and the crude product was then resuspended in water. Purification was accomplished by means of a Waters HPLC (Millford, MA) system equipped with a 990 photodiode array detector. For DMT-protected DNA, a Waters μBondapak C_{18} column was employed using a gradient of 16-35% acetonitrile in triethylammonium acetate buffer (0.1 M, pH 7.2) over 30 min. Collections were pooled, dried, and the DMT group was removed from the oligomer by treatment with 80% acetic acid at ambient temperature for 30 min. Samples were then rechromatographed using the same column and mobile phase, but the gradient was changed to 5-20% acetonitrile over 30 min for collection of the DNA. Aliquots were taken at each stage of purification and dried for mass analysis.

Spectrometric Studies

Electrospray (ESI) mass analysis was performed on either a Quattro LC or a TRIO-2000 mass spectrometer system from Micromass (Beverly, MA). Quattro LC parameters are shown in parentheses. Samples were diluted with 60% acetonitrile/water containing 1% triethlyamine and loop injected into the ESI source. The source was supplied with a flow of 60% acetonitrile/water at 12 μL/min (5 μL/min) from a syringe pump (Harvard Apparatus, South Natick, MA). Nitrogen was used as the drying gas at 250 L/h (900 L/h) and for nebulization at 15 L/h (75 L/h).

Oligodeoxynucleotides were analyzed in the negative ion mode with the source voltage set to -3.3 kV and the source temperature at 70 °C, whereas the monomers were analyzed in the positive ion mode with the probe voltage set to +3.0 kV and the source temperature at 150 °C. The sampling cone and lenses were adjusted to maximize transmission. The data system acquired data over a *m/z* range of 200 - 1500 in 8 sec.

Approximately 8 scans were collected in the multi-channel averaging mode. Data were digitally filtered, and the baseline was adjusted to produce each spectrum. ^{1}H and $^{13}CNMR$ spectra were obtained from a Bruker AC 250 or a General Electric QE 300 instrument.

RESULTS

The 8-amino-dG$(DAM)_2$ was converted to the corresponding 5'-*O*-DMT-3'-phosphoramidite and incorporated into oligodeoxynucleotides exactly as described by Rao *et al.*[13] The dimethoxytrityl (DMT)-protected oligomer 5'-(DMT)-TTCXTT, where X is a residue containing 8-amino-dG, was subjected to HPLC analysis (FIG. 1) and gave essentially a single peak (15.50 min) corresponding to a DMT-protected product. When this was collected and analyzed by ESI mass spectrometry (FIG. 2), the apparent molecular mass for this oligomer was found to be 2145.9 Da, whereas the expected molecular mass was 2090.6 Da. The difference is exactly the mass of a single DAM protecting group. This unexpected retention of one of the protecting groups led us to investigate the conditions necessary for the complete removal of these groups at the level of both the oligomer and the protected deoxynucleoside.

Studies of DNA Oligomer 5'-(DMT)-TTC[8-amino-dG$(DAM)_2$]TT

Under the conditions usually used for the deprotection (28% ammonia, 55 °C for 16 h) of DNA oligomers, 5'-(DMT)-TTC[8-amino-dG$(DAM)_2$]TT showed two peaks in the DMT-DNA region of the HPLC chromatogram (FIG. 3). Fractions corresponding to these were collected and analyzed by mass spectrometry. The major peak (23.78 min) contained three components as can be seen from FIG. 4. The dominant component (A) was the desired product having an observed mass of 2090.5 Da (calculated mass of 2090.6 Da). Two minor species (B and C) were also apparent at M+16 and M+57, respectively. The mass of the latter corresponded to that of the monoprotected product containing a single DAM group (calculated mass 2145.4 Da). Component B can be accounted for as an oxidation product having a mass of 2106.6 Da. Interestingly, this product is absent when an antioxidant is added to the ammonium hydroxide solution, a pattern of behavior which we had found in the past to be common to other 8-amino-derivatives of dG[14-16] and to 8-oxo-2'-deoxyguanosine.[17] The second peak in the HPLC chromatogram (FIG. 3) corresponds to two oligomer cleavage fragments formed after the oxidative loss of the modified base.[14,15,17] The resulting apurinic

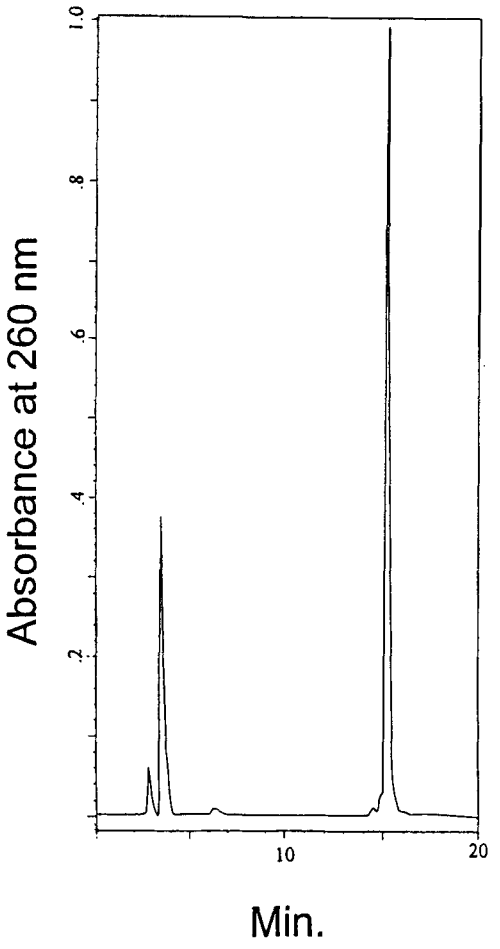


FIG. 1. HPLC analysis of products from the room temperature NH_3 hydrolysis of 5'-TTCXTT from the solid substrate (x = 8-amino-dG-related nucleoside residue).

site is unstable in the basic deprotection medium and leads to the products 5′-(DMT)TTC-PO_3H_2, (1218.1 Da) and 5′-(DMT)TTC-PO_2H-3-amino-deoxyribose (1333.1 Da).[18]

Although the addition of mercaptoethanol at the deprotection step inhibited both oxidation and cleavage, the resultant oligomer still contained approximately 6% of the mono-protected product after a 16 h digestion period. However, extension of the ammonia treatment to 20 h produced a clean product (after DMT removal) in which no monoprotected species could be detected by ESI mass spectrometry.

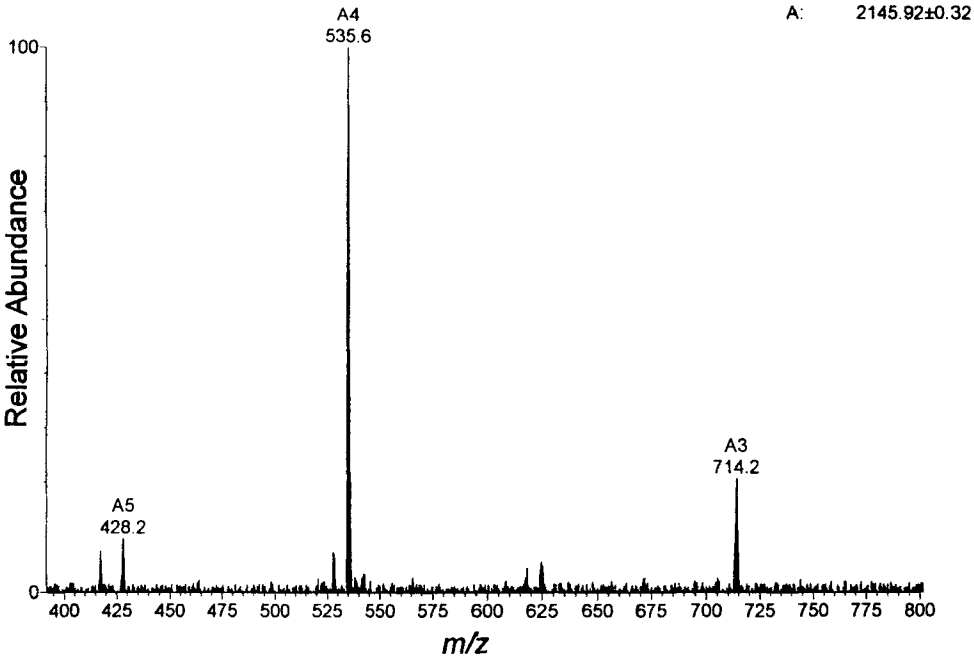


FIG. 2. Negative ion ESI mass spectrum of the main product corresponding to the peak at 15.5 min in FIG. 1. Molecular ions appear with 3, 4, and 5 positive charges from which the molecular mass was determined to be 2145.9 ± 0.3 Da.

Studies on 8-Amino-2'-deoxyguanosine (1)

Investigations on this deoxynucleoside paralleled those presented for the oligomer above. HPLC was employed for the separation of products, initially using 100% triethylammonium acetate buffer (0.1 M, pH 7.2) for 5 min. Acetonitrile was then added linearly during the next 20 min to attain a final concentration of 40%. Each chromatographic peak was collected and dried for ESI/MS analysis. When 8-amino-dG(DAM)$_2$ (**2**) was exposed to ammonia at ambient temperature for 16 h, the DAM group at N^2 was easily removed, whereas the DAM moiety on the 8-amino group was unaffected (NMR proof given below). The HPLC chromatogram of the reaction mixture obtained from **2** under these reaction conditions is shown in FIG. 5. A small amount of starting material is still evident (19.90 min), but two new peaks can be seen at 15.93 and 18.23 min, respectively. Collection of the material corresponding to the latter peak and analysis by ESI/MS confirmed that this was the monoprotected product **5** (mass 337.3 Da).

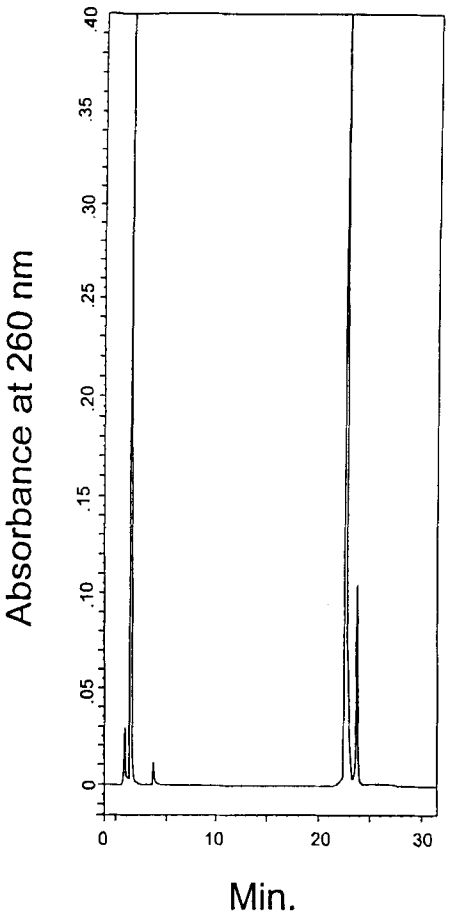


FIG. 3. HPLC analysis of the hydrolysis product of 5'-TTC[8-amino-dG(DAM)$_2$]TT under standard conditions (28% ammonia, 55 °C, 16 h).

The position of the DAM group in this material was inferred to be on the 8-amino group by comparative ^{13}CNMR spectroscopy. When 2'-deoxyguanosine (**3**) was converted[13] to the N^2-DAM derivative (**4**), the absorption position of the C2 carbon (157 ppm) in the ^{13}CNMR spectrum showed a down field shift of 4 ppm relative to the C2 (153 ppm) of (**3**). A very similar down field shift difference of 3.5 ppm was seen between these same carbon atoms in the ^{13}CNMR spectra of **2** (156.0 ppm) and **5** (152.5 ppm). On the other hand, the C8 carbon atoms in both **2** and **5** showed peaks at ~152 ppm, indicating a very similar magnetic environment in each of these materials. On this basis, we believe that the structure assigned to **5** is correct for the mono-hydrolysis product.

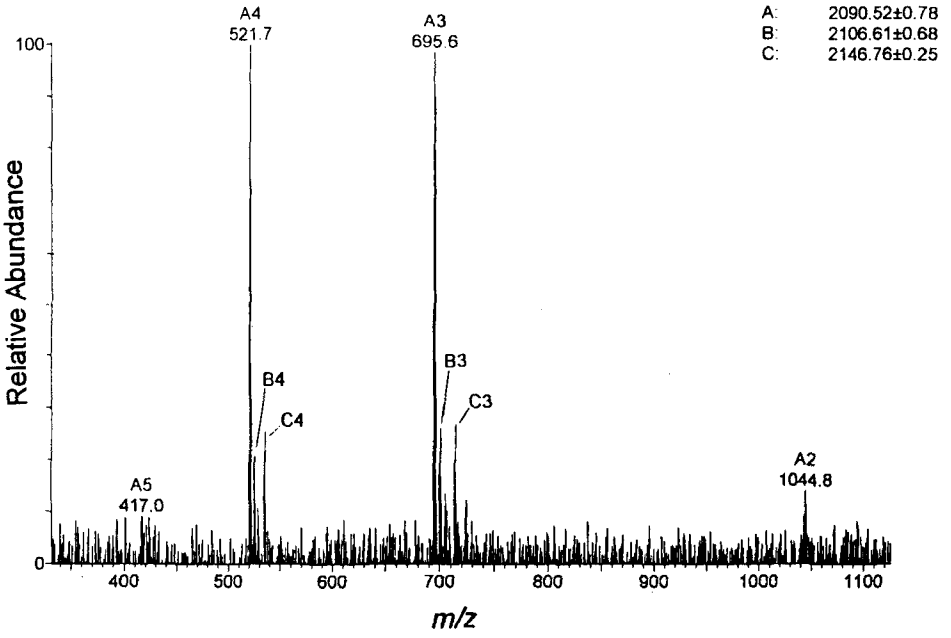


FIG. 4. Negative ion ESI mass spectrum of the major hydrolysis products found in the peak at 23.78 min in FIG. 3. Three distinct molecular ion series are present. The measured molecular mass for each product is shown in the upper right corner.

Isolation of the significant minor component represented by the second HPLC peak (FIG. 5; elution time: 15.93 min) and analysis by ESI/MS indicated a molecular mass of 309.2 Da. This is 28 Da less than the mass of **5** and undoubtedly is the lower homologue **6** in which the dimethylamino group of **5** has been replaced by an amino group. Interestingly, this compound is also quite insensitive to these hydrolysis conditions because even prolonged treatment at ambient temperature failed to reduce its level substantially. Nevertheless, when the *bis*-protected 8-amino-dG (**2**) was subjected to concentrated ammonia at 55 °C for 22 h in the presence of 2-mercaptoethanol as an antioxidant, a 95% yield of 8-amino-2'-deoxyguanosine (**1**) could be recovered, demonstrating that given a sufficient length of time both amidino groups are cleaved.

Synthesis of Oligomers and Enzyme Degradation Studies

For each oligomer (Table 1), the DMT-containing product was collected by HPLC, dried, and the DMT group was removed by treatment with 80% acetic acid (200 μL) for 30

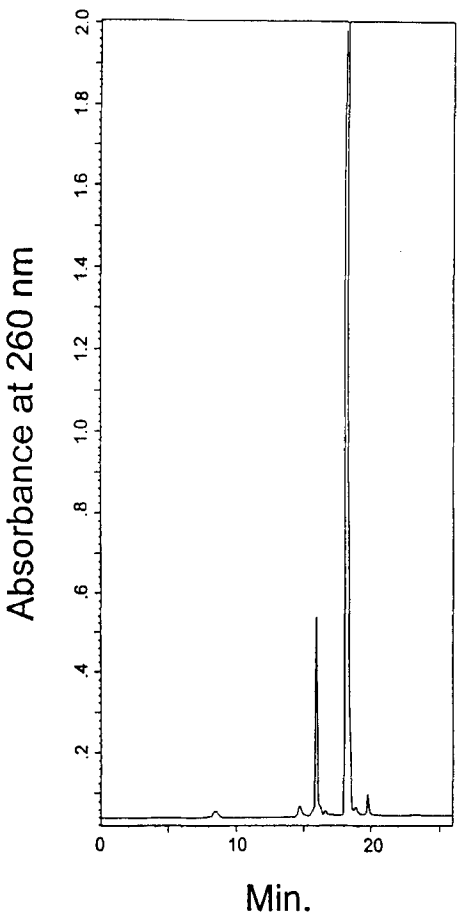


FIG. 5. HPLC analysis of the products from the treatment of 8-amino-dG(DAM)$_2$ (**2**) with ammonia at room temperature for 16 h.

min. The oligomer was then rechromatographed to effect complete purification. By way of example, FIG. 6A shows the chromatogram obtained for the 24 mer. The ESI mass spectrum (panel B) showed multiply-charged negative ions resulting from the loss of 5 to 12 protons. The observed mass, namely 7146.8 ± 1.4 Da, agrees well with the calculated value of 7146.7 Da with an error of 0.002%. After purification and analysis, 7 OD_{260} were obtained starting from 0.2 μmol of resin bound monomer indicating an overall yield of 16%.

Both the oligomer 5'-ACGT(8-amino-dG)CTACA, and a control oligomer with dG at position 5 replacing the modified base, were enzymatically digested[19] and compared by

TABLE 1

Oligomer sequence	Number of bases	Calculated Mass	Observed mass	% error
5'-TTCXTT	6	1788.23	1788.15	0.004
5'-ACGTXCTACA	10	3027.05	3027.5	.015
5'-TCCTCCTXGCCTCTC	15	4445.93	4445.59	0.008
5'-CCTTCXCTACTTTCCTCTCCATTT	24	7146.69	7146.82	0.002
5'-CATGCTGATGAATCCCTTCXCTAC -TTTCCTCTCCATTT	38	11486.50	11487.69	0.010

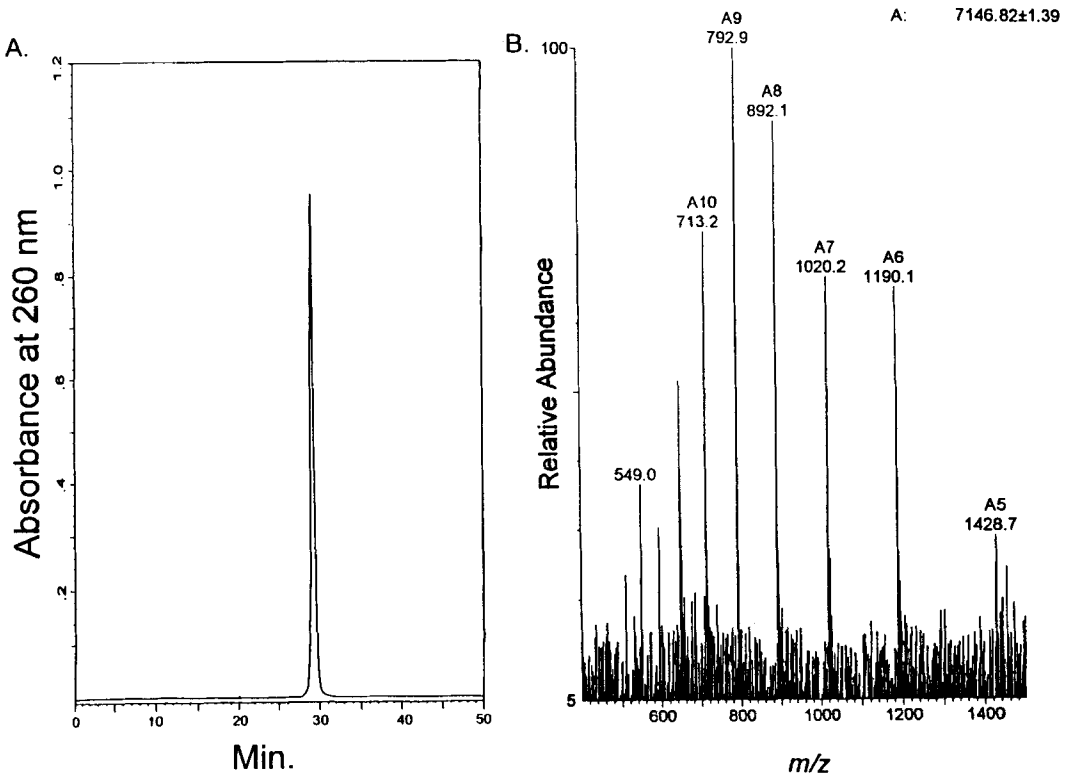


FIG. 6. Panel A: HPLC analysis of 5'-CCTTC(8-amino-dG)CTACTTTCCTCTCCTT after ammonia hydrolysis and DMT removal; Panel B: Negative ion ESI mass spectrum of the HPLC fraction containing the 24-mer in Panel A showing multiply charged (z = 5 - 12) molecular ions. The measured molecular mass is shown in the upper right corner.

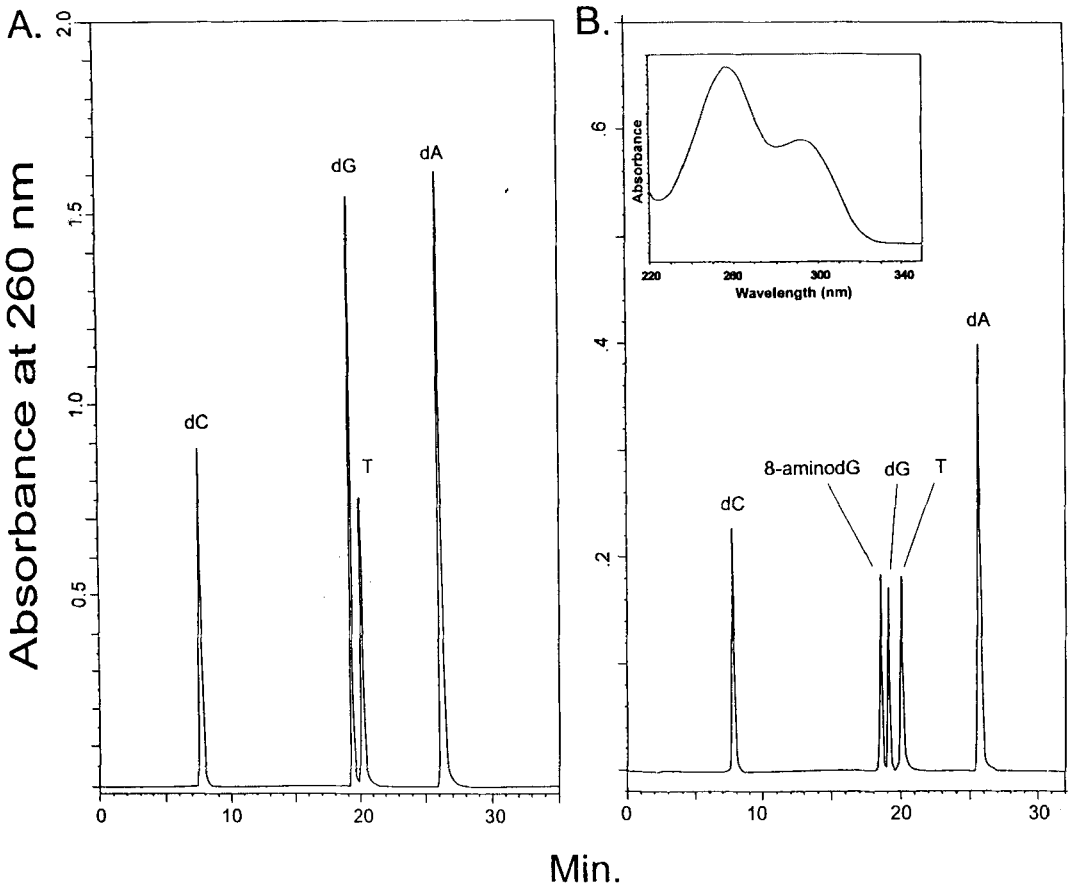
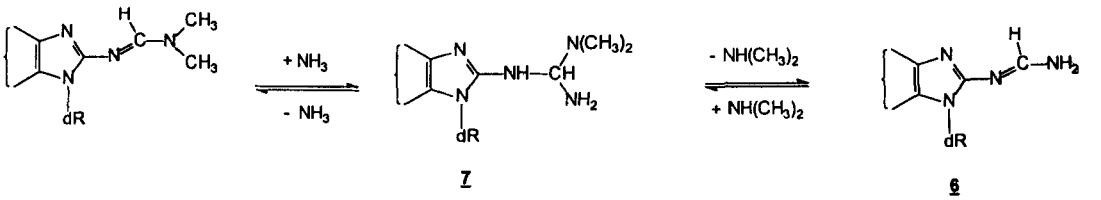


FIG. 7. Panel A: HPLC analysis of the products of enzymatic degradation of the control oligomer 5'-ACGTGCTACA; Panel B: HPLC analysis of the enzymatic degradation products of the oligomer 5'-ACGT(8-amino-dG)CTACA and the UV spectrum (inset) of the material corresponding to the 8-amino-dG peak at 18.5 min.

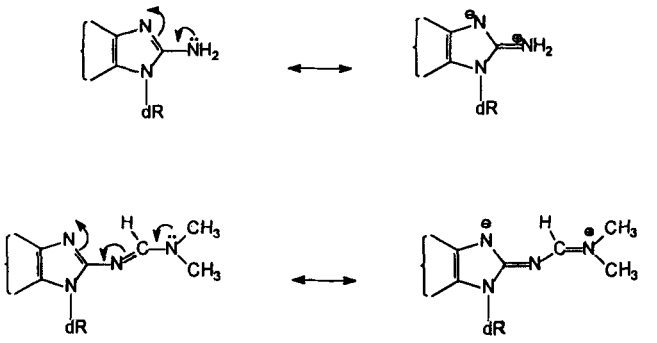
HPLC. FIG. 7, panel A, is the HPLC chromatogram of the deoxynucleosides obtained from the control sequence identifying the four normal bases. Under these conditions, 2'-deoxyguanosine was eluted at room temperature just beyond 19 min. After enzymatic treatment of the modified oligomer, the peak corresponding to deoxyguanosine was reduced in total area and a new peak appeared at 18 min (FIG 7, panel B). The UV spectrum of the new peak (inset) exactly matched the UV spectrum of an authentic sample of 8-amino-dG.

DISCUSSION

When we initiated our chemical studies on the introduction of 8-amino-dG (1) into oligomeric DNA, it appeared that the preparative aspects would be straightforward, given the then-current state of the literature. Accordingly, although during the synthesis[20] of 8-amino-dG (1) itself care must be taken to avoid aerial oxidation of the intermediate 8-hydrazino derivative, none of the published procedures proved troublesome. Conversion of 8-amino-dG to the *bis*-DAM-protected 5′-O-DMT-3′-phosphoramidite also could be accomplished smoothly according to the methods of Rao *et al.*[13] However, after automated synthesis of the DNA, the deprotection step could not be brought to completion using the conditions (28% ammonia at 25 °C) employed by these workers. Unfortunately, when standard conditions (ammonia at 55 °C for 16 h) were utilized, there was significant destruction of the DNA oligomers involving chain cleavage. This type of degradation (under alkaline conditions) is highly characteristic of deoxyguanosine derivatives that have either an arylamino group at the 8-position[14-16] or an 8-oxo group[17,18] and is principally due to aerial oxidation.[14] The addition of 2-mercaptoethanol[15] as an antioxidant to the hydrolysis mixture immediately led to a simplification of the HPLC chromatogram. Nevertheless, this did not completely rectify the situation because complete hydrolysis of both DAM groups required extension of the hydrolysis period to 20-22 h. As the hydrolysis proceeded, it was evident from the HPLC data of a number of these oligomers that, oxidation aside, the deprotection sequence was more complicated than normal (data not shown). To resolve this matter, we examined the action of concentrated ammonia on 8-amino-dG(DAM)$_2$ (2) itself. Hydrolysis for 16 h in the presence of this reagent at 25 °C produced no 8-amino-dG, but led dominantly to the monoprotected product whose structure was assigned as 5 by ^{13}CNMR data. However, a second product also appeared and by mass spectral data was assigned structure 6. This type of transformation of a DAM protecting group by ammonia is generally not observed during DNA deprotection (although, of course, it may be occurring). An explanation for its appearance probably resides in the reluctance of the sp^3-hybridized intermediate 7 to shed the heterocyclic moiety selectively in a system that probably is best viewed as an equilibrium process (Scheme 1). Thus, dimethylamine is lost, and the simplest amidino group is generated at C8. When the temperature was raised to 55 °C in the absence of 2-mercaptoethanol, the second amidine (6) became much more prominent, and 8-amino-dG



Scheme 1.



Scheme 2.

(1) was produced, although reduced in absolute quantity by oxidation. In this case, the oxidation products were not evident in the HPLC chromatogram because they are transparent in the ultraviolet spectral range of 220-400 nm. However, when the hydrolysis was conducted at 55 °C in the presence of 2-mercaptoethanol for 20 h, almost complete conversion of the amidino compounds to 8-amino-dG occurred. Finally, it is remarkable that the amidino derivatives are apparently not susceptible to oxidation despite the fact that they are iminologs of 8-amino-dG. One might expect that electronically, in a resonance sense, they should behave similarly as noted in Scheme 2. It is not clear what contribution simple oxygen or nitrogen substituents at C8 make to the sensitivity of the guanine nucleosides to aerial oxidation. There seems to be a general pattern of oxygenation at C5 (and C4 in the case of uric acid) and the pathways of degradation are related, but the effects of structure variation are not well-understood.

Now, having identified the sources of the impediments to the synthesis of oligomeric DNA containing 8-amino-dG residues, it was possible to apply the above deprotection conditions, namely concentrated ammonia at 55 °C for 20 h or more in the presence of 2-mercaptoethanol, to obtain the desired oligomers as pure entities in good yield.

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