2370–2376 *Nucleic Acids Research, 2001, Vol. 29, No. 11*

Solid-phase synthesis of oligopurine deoxynucleic guanidine (DNG) and analysis of binding with DNA oligomers

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Received December 29, 2000; Revised and Accepted April 12, 2001

ABSTRACT

The first stepwise solid-phase synthesis of deoxynucleic guanidine (DNG), a positively charged DNA analog, using controlled pore glass as the solid support is reported. For the first time, purine bases have been incorporated into the DNG oligomer and DNG has been synthesized using a solid-phase method, proceeding in the 3′→5′ direction, that is compatible with the cleavage conditions used in the solid-phase synthesis of DNA. A DNG sequence containing a pentameric tract of adenosine nucleosides has been synthesized and the thermal denaturation temperature of its complexes with complementary thymidyl DNA oligomers was 79°C. Binding of thymidyl DNA oligomers to adenyl DNG oligomers is 2:1, as seen in thymidyl and adenyl DNA triplexes. No binding of adenyl DNG with octameric cytidyl DNA was observed, indicating that the positive charge does not overcome base pairing fidelity.

INTRODUCTION

Oligonucleoside analogs capable of arresting cellular processes at the translational and transcriptional levels via recognition and binding to complementary RNA or DNA are known as antisense and antigene agents, respectively (1–3). Important goals in designing antisense compounds include increasing the binding affinity to the base sequence while maintaining fidelity of recognition, resistance to degradation by nucleases and effective membrane permeability. A recurring theme in many of these antisense compounds is the incorporation of neutral internucleoside linkages that eliminate the mutual repulsion between the negatively charged phosphodiester backbone in duplex DNA. These unnatural linkages are likely to be resistant to nucleases and may also be membrane permeable. Oligonucleosides linked by amides (4), phosphonates (5), carbamates (6), methylenemethylimino (7), heterocycles (8) and acetals (9) are representative of this approach. Another approach is to replace the phosphodiester backbone entirely, such as in the cases of PNA (10,11), PHONA (12) or PNAA (13).

In recent years, approaches involving the incorporation of positive charges in antisense oligomers have been developed. Positive charges can be added to the bases (14) or the sugar rings to give zwitterionic DNA (15,16). The phosphate backbone can be alkylated with alkylamines to produce positively charged phosphate triester linkages between the nucleosides (17–19). Another promising approach is to replace the internucleoside phosphate linkage with a positively charged, achiral guanidinium group (20). The guanidinium linkage is resistant to nucleases (21) and the positive charges of the backbone may give rise to cell membrane permeability through electrostatic attraction of the oligonucleoside to the negatively charged phosphate groups of the cell surface. Recently, micelles featuring positively charged surfaces have been reported to carry impermeable compounds such as DNA oligomers through the lipid bilayer of cells (22).

Pentameric thymidyl deoxynucleic guanidine (DNG) (**1**), a polycationic DNA analog, has been demonstrated to have a high affinity for, and binds in a 2:1 stoichiometry with, complementary adenyl DNA oligomers (20,23–28). The solid-phase synthesis of octameric thymidyl DNG (**2**) has been accomplished using resins and chemistries compatible with peptide synthesis (29). These longer thymidyl oligomers demonstrated sequence specificity despite their large overall positive charge (30). The guanidinium internucleoside linkage has been shown to be compatible with the chemistries used in standard solid-phase synthesis of DNA (21,31). Inclusion of neutral urea internucleoside linkages in a DNG oligomer to reduce the overall positive charge was demonstrated to modulate the binding affinity of the DNG-urea oligomers for complementary DNA (32).

To more fully investigate the properties of DNG oligomers it has been a goal to synthesize DNG oligomers with mixed base sequences. In this report we present, for the first time, a DNG oligomer containing purine and pyrimidine nucleoside bases. The synthesis was based on the standard support for the solid-phase of DNA, long-chain alkylamine controlled pore glass (CPG). In the original solid-phase synthesis of DNG, the linker to the resin support was an acid-labile chlorotrityl group (29), which could be cleaved from the resin support with mild acids then deprotected in subsequent steps. The CPG approach was

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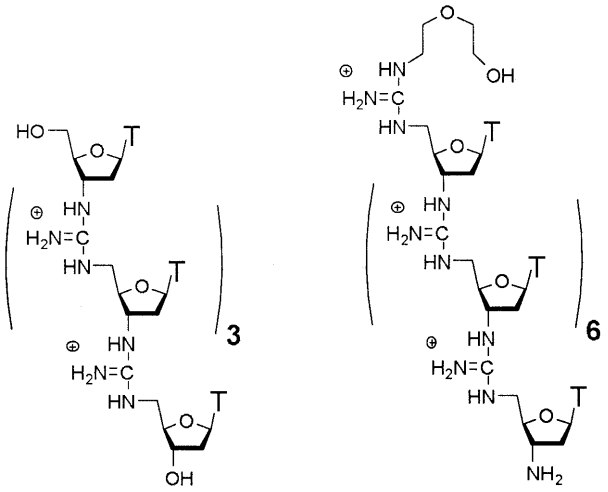
deemed more suitable as deprotection of the oligomer and cleavage from the solid support could be accomplished in a single step followed by the trityl-on purification, ultimately simplifying product isolation. After synthesis and purification of the mixed base DNG oligomer (**3**), its affinity for complementary DNA was investigated.



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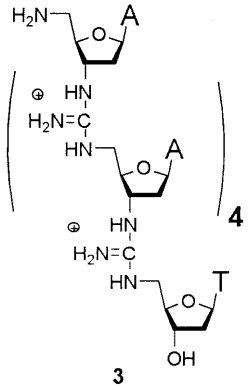

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MATERIALS AND METHODS

HPLC grade solvents and sodium carbonate were obtained from Fisher Scientific. Anhydrous solvents, triethylamine (TEA), mercury(II) chloride, monomethoxytrityl chloride, dichloroacetic acid (DCA) and acetic anhydride were purchased from Aldrich and used without further purification. The anhydrous solvents dimethylformamide (DMF) and dichloromethane (DCM) were purchased in a pure form from Aldrich. Long chain alkylamine CPG was obtained from Sigma and washed with methanol and dry DMF before use. Compound **4** (23) was synthesized from literature procedures. 9-Fluorenylmethoxy-carbonylisothiocyanate (9) was synthesized as described previously (33,34). DNA oligomers **6**, **7** and **8** were purchased pre-purified from the Biological Resource Center at UCSF.

Analytical reverse-phase HPLCs were performed on a Hewlett Packard 1050 system equipped with a quaternary solvent delivery system and UV detector set at 260 nm and a 2.1 × 250 mm C18 ODS-hypersil reverse-phase column purchased from Altech. A gradient from 100% eluent A (100 mM triethylammonium acetate, pH 7.0) to 50% eluent A, 50% eluent B (acetonitrile) over 15 min with a flow rate of 1.3 ml/min was used. Preparative reverse-phase HPLC was performed on the same system with a 10 × 250 mm C8 ODS-hypersil reverse-phase column purchased from Altech. A gradient from 66% eluent A (100 mM triethylammonium acetate, pH 7.0), 33% eluent B (acetonitrile) to 20% eluent A, 50% eluent B over 15 min with a flow rate of 3.0 ml/min was used. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Unity 400 spectrometer at 400 and 100 MHz, respectively. UV spectra were obtained on a Cary 100 Bio UV/vis spectrophotometer equipped with a temperature programmable cell block. IR spectra were in KBr pellets with a Perkin Elmer 1300 spectrophotometer. TLC was carried out on silica gel (Kieselger 60 F_{254}) glass-backed commercial plates and visualized by UV light.

Melting studies

Thermal denaturation (T_m) plots were obtained by observing the absorbance at 260 nm of a solution of the oligomers in 1 cm path length quartz cuvettes as the temperature was raised 0.2°C/min from 15 to 95°C. All samples had previously been annealed by cooling from 90 to 15°C at 0.2°C/min and stored at 20°C for 2 days. Samples consisted of solutions of 2.0 μM DNG and 2.0 μM DNA oligomer in a 10 mM potassium phosphate buffer at pH 7.0 and 0.1 M KCl for ionic strength control. Hyperchromicity was used for T_m determinations. Data were recorded every 0.5°C and the samples were covered with mineral oil to prevent evaporation.

Job plots

Job (35) or continuous variation plots were obtained by mixing samples at various ratios of DNG oligomer to DNA oligomer while maintaining the total concentration of nucleoside base at 2 μM. All solutions contained 10 mM potassium phosphate buffer at pH 7.0 and 0.1 M KCl. Solutions were mixed and left to equilibrate at room temperature for 24 h.

DNA oligomer concentrations were determined spectrophotometrically using extinction coefficients provided by the manufacturer. The extinction coefficient of the 3′-HO-TgAgAg-AgAgA-NH_2-5′ DNG **3** oligomer was calculated to be 84 400 M^{-1} by the nearest neighbor method (36).

N-benzoyl-5′-methoxytritylamino-3′-(*N*′-9-fluorenylmethoxycarbonylthiourea)-3′,5′-deoxyadenosine (5)

This was synthesized from the starting material **4** in a two step, one pot process. To a solution of *N*-benzoyl-3,5-diamino-3,5-deoxyadenosine (**4**) (0.96 g, 2.7 mM) in 50 ml DCM containing 1% TEA was slowly added 0.84 g (1.0 equiv.) of monomethoxytritylchloride over 5 min. This adds to the 5′-amino group to give *N*-benzoyl-5′-methoxytritylamino-3′-amino-3′,5′-deoxyadenosine. The reaction was complete immediately, and 9-fluorenylmethoxycarbonylisothiocyanate (**9**) (0.76 g, 1.0 equiv.) was then added. The reaction was complete by the time **9** was dissolved, as monitored by TLC. The DCM solvent was removed by rotary evaporation and the residue was dissolved in a small amount of methanol (1 ml) and 20 ml of ether was added to precipitate the product. The product was collected by

filtration, washed with ether and then dried under vacuum to give 2.1 g of white powder (84% yield) TLC (3:1, EtOAc:Hexanes) R_f = 0.75; ^{1}H NMR (400 MHz DMSO-d_6): δ (p.p.m.) 2.35 (m, 1H; 2′-H), 2.47 (m, 1H; 2′-H), 2.65 (m, 1H; 5′-H), 3.31 (m, 1H; 5′-H), 3.10 (t, *J* = 8 Hz, 1H; 4′-H), 4.20 (dd, *J* = 10 Hz, 4Hz, 1H; 3′-H), 4.30 (t, *J* = 8 Hz, 1H; Fmoc-CH), 4.38 (m, 2H; Fmoc-CH_2), 5.59 (p, *J* = 7 Hz, 1H; 5′-NH), 6.47 (t, *J* = 7 Hz, 1H; 1′-H), 6.80 (d, *J* = 8 Hz, 2H; MMTr-m-CH on OMe ring), 7.13 (td, *J* = 8 Hz, 1 Hz, 2H; MMTr-p-CH on phenyl rings), 7.22 (t, *J* = 8 Hz, 4H; MMTr-m-CH on phenyl ring), 7.27 (d, *J* = 8 Hz, 2H; MMTr-o-CH on OMe ring), 7.35 (m, 2H; Fmoc-2″-H), 7.39 (d, *J* = 8 Hz, 4H; MMTr-o-CH on phenyl ring), 7.44 (t, *J* = 8 Hz, 2H; Fmoc-3″-H), 7.54 (t, *J* = 8 Hz, 2H; m-CH Bz), 7.64 (t, *J* = 8 Hz, 2H; p-CH Bz), 7.55 (s, 1H; 6-H), 7.85 (m, 2H; Fmoc-1″-H), 7.91 (d, *J* = 7 Hz, 2H; Fmoc-4″-H), 8.04 (d, *J* = 8 Hz, 1H; p-CH Bz), 8.31 (s, 1H, 2-H), 8.65 (s, 1H; 8-H), 10.18 (d, *J* = 8 Hz, 1H; 3′-NH), 11.44 (s, 1H; Fmoc-NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 12.1, 36.0, 46.7, 51.7, 65.5, 73.8, 80.7, 83.3, 94.9, 109.9, 120.1, 125.1, 127.1, 127.6, 136.2, 140.8, 143.8, 150.4, 152.0, 155.8, 163.7, 179.6; MS (FAB) *m/z*: 907 $(M + H)^+$; HRMS *m/z*: 907.338300 $(M + H)^+$, calculated for $C_{53}H_{46}N_8O_5S$ 907.339013.

Solid-phase synthesis

Loading. 200 mg of long-chain alkylamine-controlled pore glass was placed in a vial with a fritted filter and soaked in DMF for 2 h, then 5′-monomethoxytritylamino-5′-deoxythymidine was loaded onto the CPG as its succinyl ester using standard methods (37). After loading and washing the unreacted loading sites were capped by adding 1 ml each of 0.15 M solutions of acetic anhydride and TEA in DMF. The CPG mixture was agitated for 10 min and then washed with DMF, methanol and DCM. The monomethoxytrityl group was cleaved by the addition of 3% DCA in DCM. The mixture was agitated for 10 min and then washed with DCM, methanol and finally 0.1% TEA in DMF. Now the CPG support had been loaded with a base-cleavable nucleoside to which the oligoadenyl DNG tract could be added. Analysis of the filtrate from the acidic deblocking step showed the loading to be 50 μequiv./g. The CPG was washed with methanol and DCM, vacuum dried and stored at 5°C.

Coupling. An aliquot of 80 mg of the 5′-aminothymidyl derived CPG was placed in a reaction vial with a coarse fritted bottom and stopcock. This will give a solid-phase synthesis on a 4 μmol scale. An aliquot of 20 mg (0.022 mmol, 5 equiv.) of **5** was dissolved in 1 ml of DMF and poured over the beads. Then 0.5 ml of a 100 mM $HgCl_2$ solution in DMF and 0.5 ml of a 200 mM TEA solution in DMF were added rapidly and simultaneously to the monomer/CPG mixture in the reaction tube via two syringes. A fine white precipitate formed immediately and the solution turned pale yellow in color. The tube was capped tightly and agitated for 2 h at room temperature. The supernatant in the reaction tube was removed by filtration. Most of the fine white precipitate in this mixture passed through the filter. Repeated washing with DMF (3 × 2 ml) was able to remove all visible precipitate but the resin beads were darkened from the precipitate contained within. A solution of 20% thiophenol in DMF was poured over the beads and the reaction vial was agitated for 1 min. This step removes any remaining mercury sulfide precipitate. The CPG beads were washed with copious amounts of DMF and the coupling reaction was repeated twice more.

Capping. After the third coupling reaction, unreacted sites were capped by addition of 1 ml of 100 mM acetic anhydride solution in DMF and 1 ml of 200 mM TEA in DMF. The mixture was allowed to sit over the beads for 5 min then was filtered off and the beads were washed with 10 ml of DMF followed by methanol and DCM.

Deblocking. An aliquot of 2 ml of 3% DCA in DCM solution was poured over the beads and allowed to pass through the frit by gravity into a collection flask. This was repeated twice until no more yellow color was present in the filtrate. This step was completed in <3 min. The beads were then washed with DCM and 1% TEA in DMF. The filtrate was diluted to a known volume and analyzed by UV for the monomethoxytrityl cation.

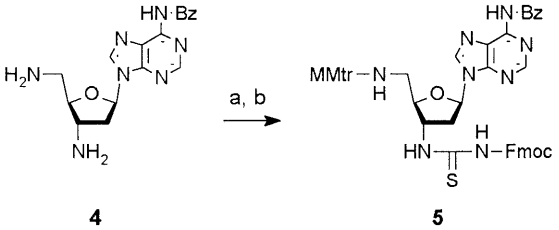
Cycle Repeated. The coupling/capping/deblocking cycle was repeated four more times. For the last coupling, the capping and deblocking steps were skipped to allow the trityl group to remain on the oligomer. Coupling yields were found to be 100% in the first coupling cycle after three additions of reagents, 99% in the second cycle, 95% in the third cycle and 90% in the fourth cycle. This gave an average coupling yield of 96%. The yield for the fifth and final coupling cycle could not be calculated as the methoxytrityl blocking group was not removed but was left to allow trityl-on purification.

Cleavage and Deprotection. The beads were washed with methanol and DCM and dried in vacuum. A mixture of saturated ammonia in methanol and concentrated ammonia (1:1) was made and 5 ml was poured over the beads. The container was tightly sealed and the mixture was placed in a 60°C air bath for 12 h. The supernatant was then collected and evaporated to give a white residue. HPLC analysis revealed a major peak at 30 min that was purified by preparative reverse-phase HPLC. The fractions containing the desired peak were collected and concentrated to give a white residue. This residue was dissolved in 0.2 ml of 3% DCA in DCM solution and, after 3 min, 5 ml of cold ether was added to precipitate the product mixture. HPLC analysis showed a peak corresponding to the starting material and a peak at 18.5 min that was the detritylated oligomer **3**. The detritylation reaction proceeded to ~75% completion in the 3 min allowed. The mixture was purified by HPLC and the fractions containing **3** were collected and evaporated to produce a white residue. Electrospray mass spectrometry gives the expected signals at 807.4 (M + 2H), 538.4 (M + 3H); calculated for $C_{113}H_{137}Cl_{21}N_{40}O_{41}$ 807.3 (M + 2H), 358.6 (M + H).

RESULTS AND DISCUSSION

Solid-phase synthesis of DNG

The adenyl monomer for the solid-phase synthesis was synthesized according to Scheme 1. The synthesis of **5** is described in the Materials and Methods. The diaminoadenosine starting material, **4**, is available from a literature procedure (38,39).



Scheme 1. (a) Monomethoxytrityl chloride, TEA in DCM, 5 min; (b) 9-fluorenylmethoxycarbonylisothiocyanate (**9**), TEA in DCM, 5 min.

Commercially available long-chain alkylamine-controlled pore glass was used as the solid support. The methoxytrityl protected 5′-amino-5′-deoxythymidine was synthesized as reported previously (20,29) and was used to load the CPG support as its 3′-succinyl ester using standard techniques (29,40). After deblocking, this provided a 5′-terminal amine on a base-cleavable nucleoside that was now available for the guanidinium coupling reaction. The coupling reaction to extend the oligomer involves the formation of a 9-fluorenylmethoxy-carbonyl (Fmoc) protected guanidinium group from a corresponding urea group and a 5′-primary amine at the terminus of the oligomer on the solid support. This coupling reaction proceeds through the abstraction of the sulfur atom on the thiourea by mercury(II) to give a carbodiimide which is electronically activated to nucleophilic attack as a result of the electron withdrawing carboxyl group of the Fmoc protecting group (29). The amine attacks the carbodiimide and the result is an asymmetrically disubstituted and protected guanidinium linkage (Scheme 2). In previous solid-phase syntheses of DNG, the trichloroethoxycarbonyl (troc) group was used as the activating/protecting group (30). This group gave excellent coupling yields but required a further deprotection step after the cleavage reaction. Recently, the Fmoc group was shown to be suitable for the solid-phase synthesis of thymidyl ribonucleic guanidinium oligomers on controlled pore glass (33). Since the Fmoc group is removed by the cleavage conditions, thus avoiding the need for a separate deprotection step, it is the preferred protecting group for this synthesis.

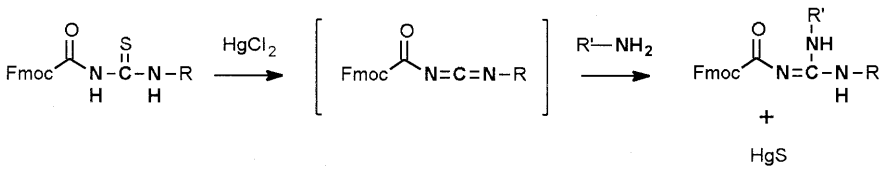
The overall coupling and deblocking cycle is outlined in Scheme 3. The guanidinium remains protected throughout the entire synthesis until it is unmasked in the basic conditions of the cleavage step. As in the solid-phase synthesis of DNA, the coupling efficiency can be followed by colorimetric analysis of the methoxytrityl chromophore after the acid deblocking step. In this particular synthesis the coupling efficiency was ~75% in the presence of 4 equivalents of monomer per coupling site on the support. By repeating this coupling reaction twice more, the overall coupling yield could be driven to <95%. Before the trityl-deblocking of the terminal amine groups, a capping step with acetic anhydride was used to terminate any unextended oligomers.

The coupling cycle was carried out a total of five times with the adenyl monomer **5**. To maximize coupling yields, the coupling step in each cycle was repeated three times before the capping step was performed. This resulted in the DNG sequence of 3′-HO-TgAgAgAgAgA-NH_2-MMtr-5′ (lowercase g indicates the guanidinium linkage) which is the oligomer **3a**.

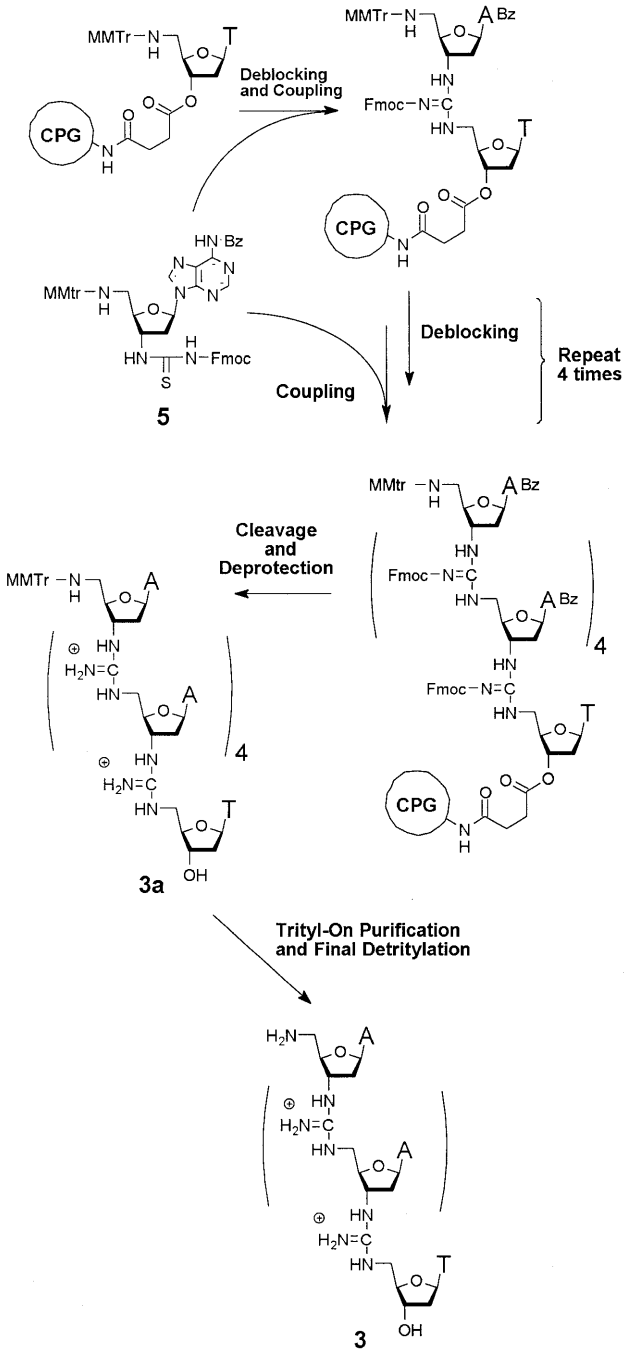
As shown in Scheme 3, the resulting oligomer on the solid support is cleaved and the guanidinium linkages deprotected in the same step. This involves soaking the beads in a solution composed of equal volumes of methanol saturated with ammonia and commercial ammonium hydroxide and keeping the mixture at 60°C for 12 h. When the trityl group on the 5′-N-terminus of the oligomer is not de-blocked after the final coupling step, the purification of the product mixture is simplified. Like 'Trityl-on' purification in solid-phase DNA synthesis, only the oligomer of the desired length will have a trityl-blocking group on it. This occurs because capping with acetic anhydride after each set of coupling reactions terminates any unreacted oligomers. The overall coupling yields, after three additions of the monomer, were measured to be 96% on average by trityl cation colorimetric analysis. The overall yield of the desired 5′-tritylated oligomer **3** is therefore expected to be 80%. After cleavage and precipitation of the reaction mixture, HPLC analysis indicated a large single peak among the other reaction products. Purification of this compound by reverse-phase semi-prep HPLC yielded the trityl protected form of the product, **3**. Electrospray mass spectral analysis shows the expected *m/z* of 1887.0 (1887 expected for M + $2H^{2+}$ ion) for the trityl protected form of oligomer **3**. This oligomer was detritylated by 1 min treatment with 3% DCA in DCM solution followed by precipitation by ether. HPLC analysis shows a single peak due to the detritylated oligomer **3** and a later peak that is the starting material, tritylated **3**. After a 1 min acid treatment, the oligomer was approximated 75% detritylated. The reaction time was not extended to prevent depurination. The oligomer was purified by HPLC and stored as a solid. Electrospray mass spectral analysis shows the expected *m/z* of 1614.86 (1615 expected for M + $2H^{2+}$ ion) for the HPLC purified oligomer **3**.

Binding with complementary DNA oligomers

In the case of homo-oligomers of DNG, the positive charge attraction for the backbone of target DNA more than compensates for the steric cost of the DNG backbone and very high binding affinities are observed. Octameric thymidyl DNG-8



Scheme 2.



Scheme 3.

(**2**) binds to octameric adenyl DNA in a 2:1 fashion with a T_m of 63°C (30).

The pentameric adenyl tract of the DNG oligomer **3** should bind to complementary pentameric thymidyl DNA (HO-ApApApApA-OH, **6**). In the case of a similar pentameric adenyl DNA oligomer binding to **6**, the T_m is too low to measure. In the case of DNG **3** in the presence of DNA **6** in 2 μM concentration each, the T_m is 69°C (Fig. 1). Continuous variation or Job plot (35) analysis indicates that the stoichiometry of the preferred DNG:DNA complex is 1:2 with two thymidyl DNA penatmers (**6**) bound to each DNG oligomer **3** (Fig. 2). This is the expected behavior of homopolymeric thymidyl and adenyl DNA strands, which can form a triple helical structure with two thymidyl polymers bound to one adenyl polymer. The situation when two adenyl polymers bind in a triple helical fashion to one thymidyl polymer is not favored. The triple helix with a 2:1 thymidyl:adenyl polymer ratio is the preferred complex and it is seen to be the case for DNG binding to DNA as well. Thymidyl DNG **2** binds in a 2:1 fashion to its DNA complement and thymidyl DNA **6** binds in a 2:1 fashion with its DNG complement **3**. Oligomeric pyrimidine and oligomeric purine DNGs behave similarly to their DNA

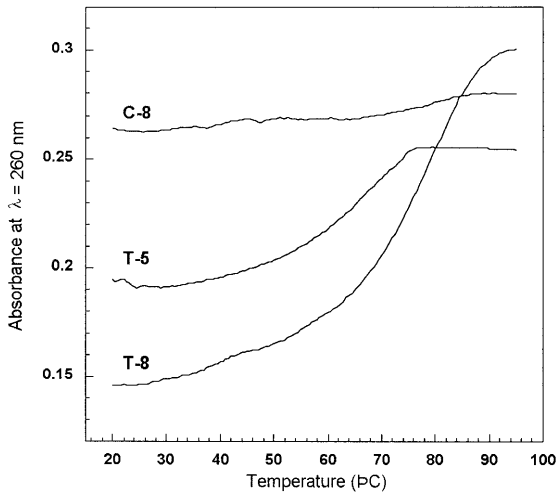


Figure 1. Thermal denaturation plots for DNA:DNG association. Aliquots of 2.0 μM DNA oligomers **T-5** (**6**), **C-8** (**8**) and **T-8** (**7**), 2.0 μM DNG **TAAAAA** (**4**), [$KHPO_4$] = 10 mM, [KCl] = 0.1 M, pH 7.0. Observed at λ = 260 nm. Rate of heating was 0.2°C/min. T_m for DNG TAAAAA association with **T-5** (**6**) is found to be 69°C and T_m for DNG **TAAAAA** (**4**) association with **T-8** (**7**) is found to be 79°C.

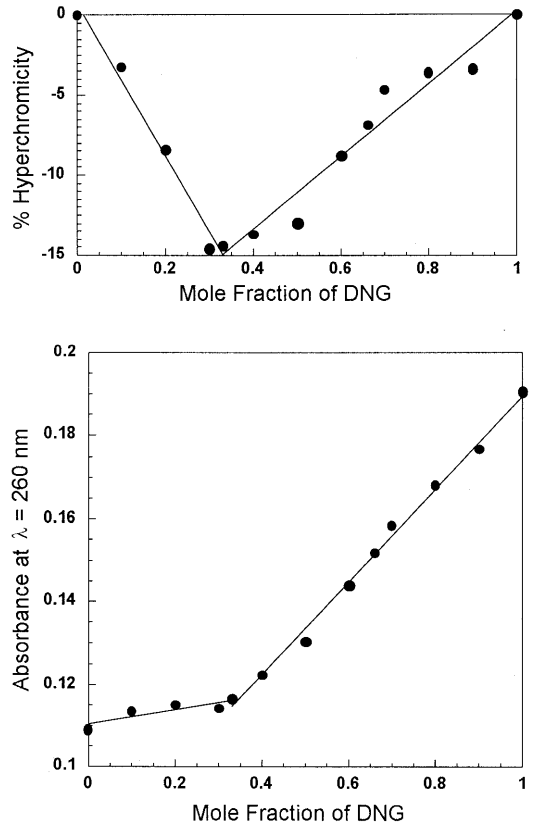


Figure 2. Job plots for DNG **TAAAAA** (**4**) with DNAs **T-5** (**6**), **C-8** (**8**) and **T-8** (**7**) plotted by percent hyperchromicity (top) and absorbance (bottom). [Oligomer] = 2.0 μM, [$KHPO_4$] = 10 mM, [KCl] = 0.1 M, pH 7.0, 25°C, observed at 260 nm.

counterparts with respect to triple helix formation. The large binding affinity of DNG for DNA insures that the triple helix will form even for short oligomers.

As seen in Figure 1, octameric thymidyl DNA (HO-TpTp-TpTpTpTpTpT-OH, **7**) demonstrates even strong affinity for the pentameric adenyl tract of DNG **3**. This is probably due to the ability of the longer DNA strand to adopt a more helical form compared to shorter DNA oligomers. No significant hyperchromic shift is observed for non-complementary octameric cytidyl DNA (HO-CpCpCpCpCpCpC-OH, **8**) indicating that the strong affinity of the positively charged DNG oligomers for negatively charged DNA does not overcome the selectivity due to base pairing interactions.

To summarize, the first stepwise solid-phase synthesis of a DNG oligomer containing purine nucleosides has been accomplished. The hexameric DNG oligomer 3′-HO-TgAgAgAgAgA-NH_2-5′ (3) was synthesized with average coupling yields of 96% on a controlled pore glass solid support. This support and the base cleavage/deprotection chemistry are compatible with standard automated DNA synthesis chemistries. Using this methodology mixed sequences of DNG can now be synthesized for studying their interaction with specific target sequences of DNA. The protected guanidinium linkage has previously been shown to be compatible with DNA synthesis chemistries (31,33) and, using appropriate monomers, a DNA sequence could be incorporated at the end of the DNG oligomer using standard DNA solid phase synthesis techniques.

The pentameric adenyl tract in DNG **3** has been shown to bind very tightly to complementary DNA in a 1:2 adenyl:thymidyl oligomer ratio (Fig. 2). This is consistent with the triple helix structure that exists in associations of homopolymeric adenyl and thymidyl DNA. The DNG backbone did not result in significant changes to the triple helical character of homo-oligomeric T:A associations. The DNG **3** oligomer did not show any detectable association with a mismatched cytidyl oligomer. This indicates that the base pairing fidelity is not overcome by the strong electrostatic attraction between the positively charged DNG backbone and the negatively charged DNA backbone.

ACKNOWLEDGEMENTS

Funding for this research was provided by the National Institutes for Health and Genelabs Technologies Inc. (Redwood City, CA).

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