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Solid-Phase Synthesis of Positively Charged Deoxynucleic Guanidine (DNG) Oligonucleotide Mixed Sequences

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Abstract—Positively charged DNG oligonucleotide mixed sequences containing A/T bases were prepared by solid-phase synthesis. Synthesis proceeds in 3′→5′ direction and involves coupling of 3′-Fmoc protected thiourea in the presence of $HgCl_2$/TEA with the corresponding 5′-amine of the growing oligo chain. DNG binding characteristics with complementary DNA and with itself have been evaluated.

Oligonucleotide analogues are of interest in antisense/antigene technology to selectively inhibit gene expression at the translational and transcriptional levels.[1,2] The ideal antisense/antigene agents should have high-affinity while maintaining fidelity of recognition, stability towards enzymatic hydrolysis, and efficient membrane permeability. In search of potential antisense and antigene agents, numerous structural analogues of DNA and RNA have been reported in which the sugar-phosphodiester backbone has been replaced by many alternative neutral and anionic backbones.[3] A recurring theme in many of these compounds is modification of internucleoside linkages that eliminates the mutual repulsion between the negatively charged phosphodiester backbones in duplex DNA. We find the replacement of the negatively charged phosphodiester linkages with positively charged guanidinium linkages (DNG) (Fig. 1) enhances the oligonucleotide complex stability due to the electrostatic attraction between the complementary DNA and DNG.[4] Poly-thymidyl[4b] and poly-adenyl[4a] DNGs have been shown to have high affinity towards complementary DNA while maintaining the fidelity of base pairing. In order to fully investigate the properties of DNG oligomers, it is necessary to synthesize DNG oligomers with mixed base sequences and study their binding properties. In this paper, we report the solid-phase synthesis (SPS) of DNG oligomers containing purine and pyrimidine nucleoside bases.

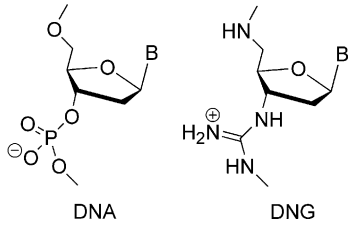

Figure 1. Structures of DNA and DNG.

The building blocks, for SPS of positively charged DNG oligonucleotides, were synthesized as shown in Schemes 1–3. The loading monomers, monomethoxytrityl (MMTr) protected 5′-amino-2′-deoxyadenosine (**7**) and thymidine (**8**), were synthesized from 2′-deoxyadenosine **1** and 2′-deoxythymidine **2** respectively (Scheme 1). Initially **1** was converted into *N,N*-dibenzoyl-5′-*O*-mesyl-2′-deoxyadenosine **3** in a one pot reaction by modifying the transient protection method.[5] This method is facile and gives higher yields than the traditional two-step method,[6] *N*-monobenzoylation by the transient protection method and then mesylation of 5′-OH of 2′-deoxyadenosine (**1**). Also compound **3** is ideal for conversion to **5** in higher yield because a considerable amount of debenzoylated product was isolated along with the desired product **5** on treatment of *N*-monobenzoylated compound with LiN_3 at 80 °C. Hence treatment of *N,N*-dibenzoylated **3** with LiN_3 gave **5** in higher yield by eliminating one of the *N*-benzoyl groups. Further reduction of **5** with 10% Pd/C gave the 5′-amino derivative,[6] which was then selectively protected with MMTr to afford **7**. MMTr protected

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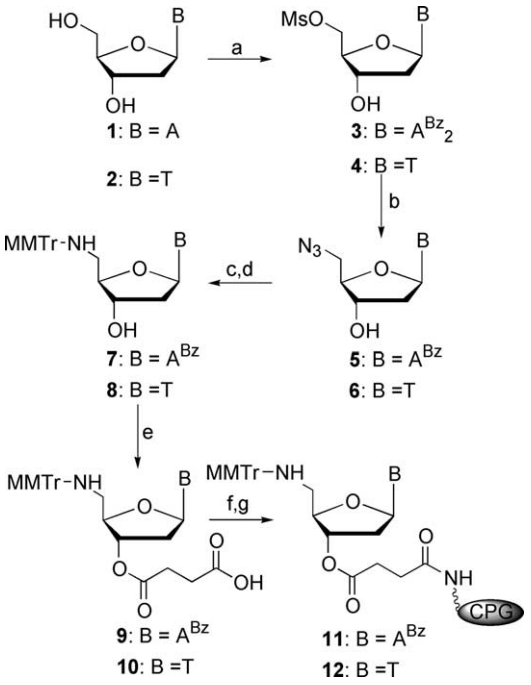
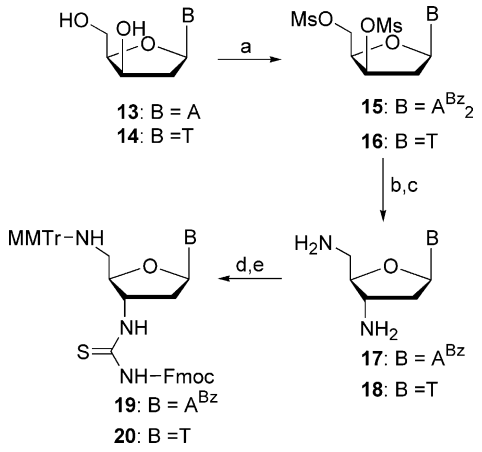


Scheme 1. Reagents and conditions: (a) for **1**→**3** (i) CH_3SO_2Cl 1.0 equiv, pyridine, 0 °C–rt, 9 h; (ii) TMSCl 2.5 equiv, 0 °C, 30 min; (iii) BzCl 5 equiv, 0 °C–rt, 3 h; for **2**→**4** CH_3SO_2Cl 1.0 equiv, pyridine, 0 °C–rt, 9 h; (b) LiN_3 10.0 equiv, DMF, 80 °C, 4 h; (c) 10% Pd/C, H_2, EtOH, 4 h; (d) pyridine, MMTrCl 1.0 equiv; (e) succinic anhydride 0.95 equiv, DMAP, pyridine, 12 h; (f) 4-nitrophenol, DCC, pyridine, 1,4-dioxane; (g) CPG, TEA, DMF, 12 h.

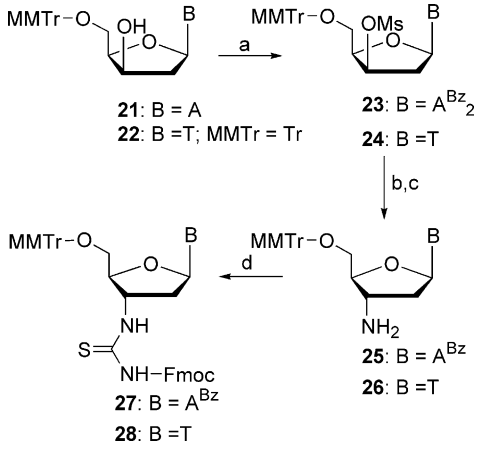
thymidine **8** was also synthesized via **6** as shown in Scheme 1.[7] MMTr protected 5′-amino-3′-OH monomers, **7** and **8** were loaded on to the long chain alkylamine Control Pore Glass (CPG) solid support as their succinyl derivatives.

The 3′,5′-diamino protected adenyl and thymidyl building blocks **19** and **20**, required for the synthesis of the body of the DNG oligomer, were accomplished from **13** and **14** respectively. The 3′-OH inverted **13** was conveniently prepared by a literature procedure[8] starting from adenosine, whereas **14** was obtained by trityl deprotection of commercially available **22**.[9] *N,N*-Dibenzoyl-3′,5′-dimesyl derivative **15** was conveniently synthesized from **13** in a one-pot reaction. This method is simpler, the product easier to purify in almost quantitative yield, as compared to the traditional two-step reaction,[6] benzoylation of 6-NH_2 by transient protection and then mesylation of 3′- and 5′-OH groups. Treatment of **15** with LiN_3 at 80 °C resulted in its *N*-benzoyl-3′,5′-diazido derivative by removing one of the *N*-benzoyl groups. Further reduction of 3′,5′-azido groups with 10% Pd/C gave 3′,5′-diamino-2′-deoxyadenosine **17**. Selective protection of 5′- and 3′-amino groups of **17** with acid-labile MMTr and base-cleavable Fmoc groups gave **19**. Thymidine monomer **20** was also synthesized in a similar way from **14** via **18**[10] as shown in Scheme 2.

The capping building blocks **27** and **28** were synthesized from **21** and **22**, respectively, as shown in Scheme 3. The 5′-OH of **13** was protected with MMTr to give **21**, which was then converted into **25** via **23** as explained for



Scheme 2. Reagents and conditions: (a) for **13**→**15** (i) CH_3SO_2Cl 2.2 equiv, pyridine, 0 °C–rt, 10 h; (ii) BzCl 5.0 equiv, 0 °C–rt, 3 h; for **14**→**16** CH_3SO_2Cl 5.0 equiv, pyridine, 0 °C–rt, 9 h; (b) LiN_3 20.0 equiv, DMF, 80 °C, 4 h; (c) 10% Pd/C, H_2, EtOH, 4 h; (d) MMTrCl 1.0 equiv, TEA 2.0 equiv, DCM, 2 h; (e) Fmoc-NCS 1.2 equiv, DCM, rt, 2 h.



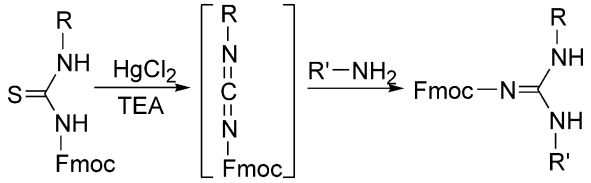
Scheme 3. Reagents and conditions: (a) for **21**→**23** (i) CH_3SO_2Cl 1.1 equiv, pyridine, 0 °C–rt, 10 h, (ii) BzCl 5.0 equiv, 0 °C–rt, 3 h; for **22**→**24** CH_3SO_2Cl 5.0 equiv, pyridine, 0 °C–rt, 9 h; (b) LiN_3 10.0 equiv, DMF, 80 °C, 4 h; (c) 10% Pd/C, H_2, EtOH, 4 h; (d) Fmoc-NCS 1.2 equiv, DCM, rt, 2 h.

15. The 3′-NH_2 of **25** was then protected with Fmoc-NCS[11] to afford the desired capping building block **27**. The thymidine monomer **28** was also synthesized in a similar way as shown in Scheme 3.

The monomers **7** and **8** were loaded on to the CPG as their succinyl derivatives by a standard procedure[12] (Scheme 1). After loading, the unreacted sites were capped with acetic anhydride/TEA, and then 5′-MMTr was deprotected with 3% DCA in DCM solution. The loading yield, 36 μmol/g, was determined spectrophotometricaly from the amount of MMTr cation released. A typical solid-phase synthesis is outlined in Scheme 4. Both DNGs, **29** and **30**, which are complementary to each other, were synthesized on a 5 μmol scale.

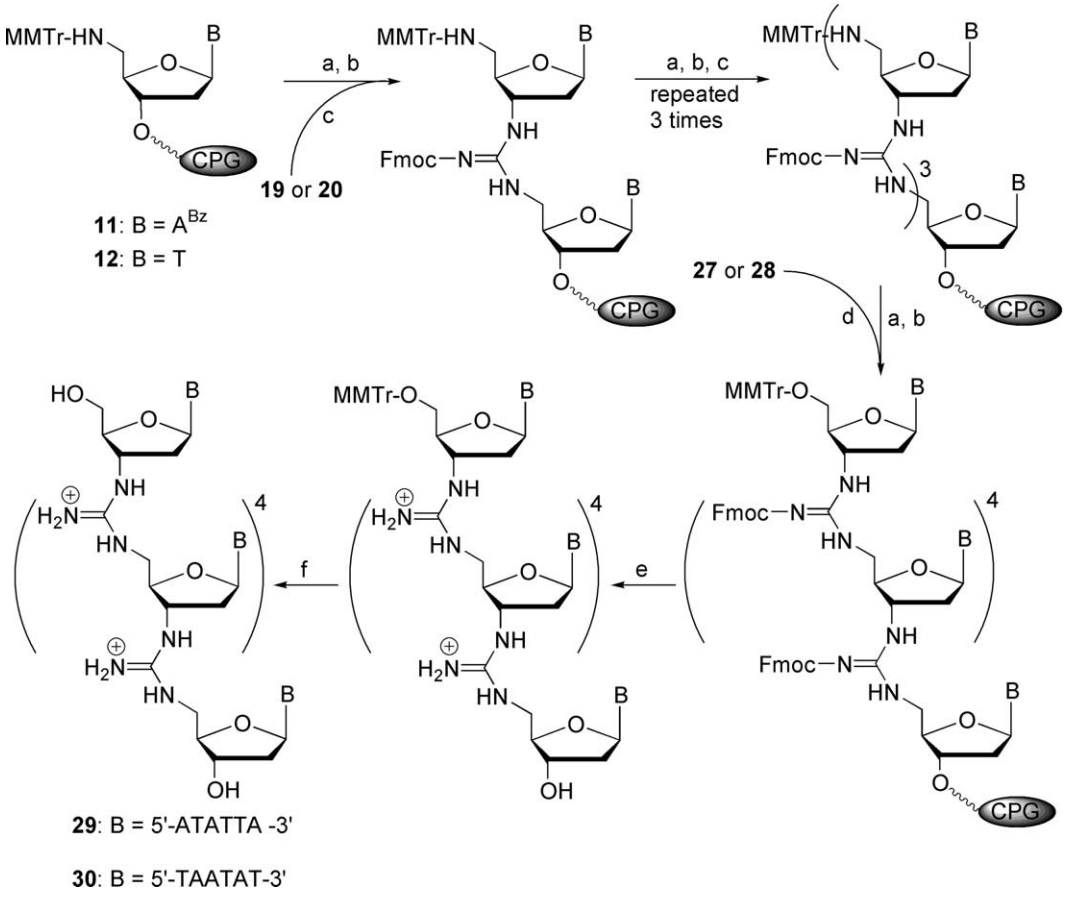
The coupling reaction[4] of MMTr deprotected **11** or **12** with **19** or **20** (Scheme 4), for the formation of guanidinium linkage, was accomplished in the presence of

$HgCl_2$ and TEA. Treatment of **19** or **20** with $HgCl_2$ in the presence of TEA converts the 3′-Fmoc protected thiourea into an intermediate carbodiimide,[13] which reacts in situ with the 5′-NH_2 of CPG loaded monomer to provide an Fmoc protected guanidinium linkage (Scheme 5). This Fmoc protecting group remains in place on the guanidinium linkage until the end of the SPS when it is readily removed during cleavage of the oligomer from CPG. After coupling, the unreacted 5′-NH_2 sites were blocked by capping, rendering them inert towards further chain extension. The terminal 5′-MMTr group was deprotected, and coupling yield for this step was determined to be 98% by UV analysis of absorbance of MMTr cation. The whole coupling cycle, coupling/capping/deprotection, was repeated three more times with **19** or **20** before coupling with capping monomer **27** or **28**. The coupling yield in each cycle was 95–98%, and the overall yield of the 5′-protected oligomers **29** or **30** is therefore expected to be ~85%. After final coupling, the capping and deprotection steps were omitted to simplify the crude product purification. The resulting 5′-MMTr protected oligomers, 5′-AgTgAgTgTgA-3′ and 5′-TgAgAgTgAgT-3′, were cleaved from CPG using 28% NH_4OH solution at 60 °C. The Fmoc protection on guanidinium linkages and benzoyl protection on deoxyadenosine were also deprotected in the same step. At this time only the desired oligomers will have MMTr protecting group on the 5′-terminus



Scheme 5.

that facilitate the trityl-on purification as in the case of SPS of DNA oligomers. The crude products were purified on reverse-phase HPLC (C18 column) using solvent A (0.1 M TEAA buffer, pH 7.0) and a gradient of solvent B (acetonitrile) 0% → 90% in 30 min. ESI/TOF+ analysis of MMTr protected product **29** exhibited desired peak at m/z 934.4 (M+2H); calculated 934.41 (M+2H) for $C_{85}H_{102}N_{36}O_{15}$ and trityl protected **30** exhibited m/z 919.3 (M+2H); calculated 919.41 (M+2H) for $C_{84}H_{100}N_{36}O_{14}$. The trityl group on the 5′-terminus of oligomers was deprotected with 3% DCA in DCM solution and precipitated with excess of ether. The precipitated products were centrifuged, dried, and RP HPLC analysis with the same column and solvent system revealed single peaks for both products **29** and **30**. ESI/TOF+ analysis exhibited the expected peaks at m/z 798.35 (M+2H) and 532.4 (M+3H) for both **29** and **30**; calculated 798.36 (M+2H) and 532.57 (M+3H) for $C_{65}H_{86}N_{36}O_{14}$.



Scheme 4. Reagents and conditions: (a) *Capping*: $(CH_3CO)_2O$, TEA, DMF, 10 min; (b) *Deprotection*: 3% DCA in DCM, 1 min, (c) *Coupling*: monomer **19** or **20**, $HgCl_2$, TEA, DMF, 2 h, then 20% PhSH in DMF, 1 min; (d) monomer **27** or **28**, $HgCl_2$, TEA, DMF, 2 h, 20% PhSH in DMF, 1 min; (e) NH_4OH, 60 °C, 15 h; (f) 3% DCA in DCM, 1 min.

Continuous variation or Job plots (Fig. 3) of DNGs **29** and **30** with complementary DNAs revealed 1:1 binding stoichiometry consistent with the formation of a Watson–Crick base paired duplex. Duplex formation experiments of DNGs **29** and **30** were carried out in 10 mM potassium phosphate buffer, pH 7.0, containing 100 mM KCl. (Fig. 4). The DNG sequences **29** and **30** exhibited melting temperatures (T_m) of 40 and 41 °C with complementary DNAs (Fig. 2). This indicates that the mixed sequences demonstrate lower affinity towards complementary DNA than the octameric-thymidyl[4b] (T_m 62 °C) and pentameric-adinyl[4a] (T_m 69 °C) DNGs. This is probably due to the length of the DNG strand or nearest-neighbor base stacking interactions. In comparison, DNA duplex **31**/**32** exhibited no hypochromic shift above 5 °C under similar conditions. Furthermore, no considerable hypochromic shifts were observed for non-complementary DNG/DNA sequences **29**/**32** and **30**/**31**. Complementary pairing experiments of DNG sequences

DNG: 5'-AgTgAgTgTgA-3' (**29**)
DNG: 5'-TgAgAgTgAgT-3' (**30**)
DNA: 5'-TpApApTpApT-3' (**31**)
DNA: 5'-ApTpApTpTpA-3' (**32**)

Figure 2. Sequences of DNGs and DNAs. g, guanidinium linkage in DNG; p, phosphate linkage in DNA.

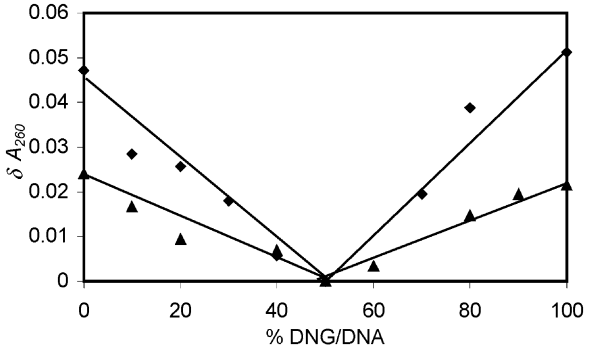


Figure 3. Job plots for **29**/**31** (◆) $c = 3\,\mu M$ and **30**/**32** (▲) $c = 2\,\mu M$ plotted by change in A_{260} in 10 mM potassium phosphate buffer, pH 7.0, 100 mM KCl.

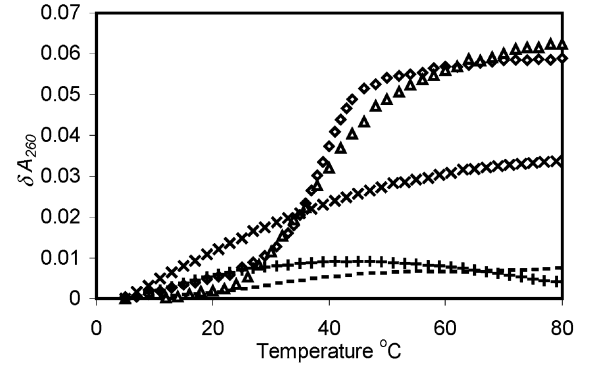


Figure 4. Normalized T_m curves (260 nm) of DNG/DNA and DNG/DNG duplexes: (Δ) **29**/**31**, (◇) **30**/**32**, (−) **29**/**32**, (+) **30**/**31**, (×) **29**/**30** in 10 mM potassium phosphate buffer, pH 7.0, 100 mM KCl and $c = 3\,\mu M$. **31**/**32** exhibited no T_m profile ($T_m = < 5$ °C) above 5 °C under similar conditions.

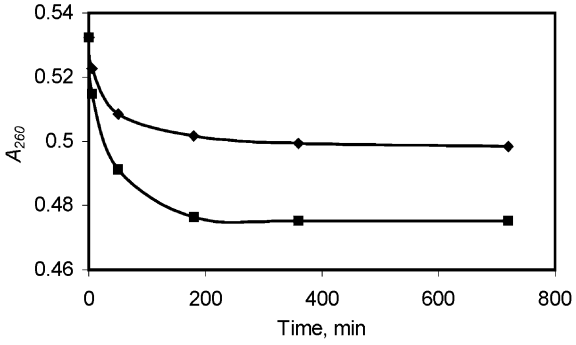


Figure 5. Change in UV absorbance (A_{260}) of **29**/**30** (◆) and **29**/**31** (■) duplex versus time, $c = 3\,\mu M$, 10 mM potassium phosphate buffer, pH 7.0, 100 mM KCl.

29/**30** were also carried out at low (100 mM) and high (750 mM) salt concentrations in 10 mM potassium phosphate buffer, pH 7.0. Although clear transition was not observed from the melting curve for complementary DNG/DNG (**29**/**30**) duplex, considerable decrease (~7%) in absorbance was observed when **29** and **30** were mixed and cooled to 4 °C (Fig. 5). This reveals that, DNG/DNG duplex may have a very broad transition or most likely the six-mer sequences are too short to exhibit a clear transition in the T_m curve.

In conclusion, we have synthesized DNG oligomeric mixed sequences containing adinyl and thymidyl bases for the first time and shown that these hexamers (**29** and **30**) bind sequence specifically to DNAs. The synthesis of longer mixed base sequences on solid phase is under way in order to fully explore the DNG/DNG Watson-Crick base pairing duplex formation properties.

Acknowledgements

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