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Deoxynucleic guanidine: synthesis and incorporation of purine nucleosides into positively charged DNG oligonucleotides

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Abstract—The synthesis of purine nucleosides capable of making the guanidinium linkage is described for the first time starting from the corresponding 2′-deoxynucleosides. The positively charged mixed base DNG oligomer containing guanine was synthesized on solid-phase using CPG as support from 3′ to 5′ direction using the precursor building block nucleosides.

1. Introduction

The approval of ISIS fomivirsen (Vitravene™, marketed by Novartis Ophthalmics) as an antisense drug for the treatment of HIV-associated CMV retinitis is very promising for the field of antisense therapy. Several groups are in active pursuit of oligonucleotide analogues capable of arresting cellular processes at the translational and transcriptional levels by binding to complementary RNA or DNA target sequence.[1–3] Unraveling the human genome has been an added motivation for discovery of therapies at the molecular level. With the concurrent advances in molecular diagnostic techniques, several companies are developing a huge database of possible target sequences for any particular disease. The recurring concerns in designing an efficient antisense/antigene oligonucleotide are: (1) higher binding affinity to the complementary sequence while maintaining fidelity of recognition, (2) resistance to degradation by cellular nucleases, and (3) improved cell membrane permeability. Innumerable oligonucleotide analogues have been reported in the past decade, for example, phosphorothioate,[4] phosphonates,[5] carbamates,[6] methylenemethylimino,[7] locked nucleic acids[8] where the backbone linkages have been modified. Another approach is where the entire sugar-phosphodiester backbone has been replaced to give PNA,[9] PHONA[10] or PNAA.[11]

It is logical to design a complementary oligonucleotide with net positive charge so as to enhance the binding affinity to the negatively charged natural DNA sense oligonucleotide. Examples of positively charged oligonucleotides include modifications made to the base, sugar ring or backbone linkage.[12–14] Prior reports from this laboratory have demonstrated that replacement of the internucleoside phosphate linkages with positively charged guanidinium group renders an achiral oligonucleotide with enhanced binding affinity to DNA oligonucleotides.[14–21] These molecules, termed as deoxynucleic guanidines (DNG, Fig. 1) bind to complementary DNA sequences with high affinity without compromising the specificity of binding. For example, a DNG thymidyl pentamer sequence (T_5-DNG) binds in a 2:1 stoichiometry with complementary adenyl DNA oligomers, while the DNG adenyl oligomers form 1:2 complexes with the thymidyl DNA oligomers. Mismatch studies of DNG sequences resulted in lowering of melting values, a measure of binding affinity, suggesting weakened DNA association. Also, it was demonstrated that T_5-DNG does not bind to poly dG, poly dC or poly T but binds only to poly dA indicative of its fidelity of binding. The guanidinium linkage has been shown to be resistant to nucleases[19] and it is possible that the positive charges of the DNG backbone may improve cell permeability through electrostatic attraction of the oligomer to the negatively charged phosphate groups on the cell surface.

In order to fully realize the potential of the promising properties of DNG, it is important to be able to synthesize

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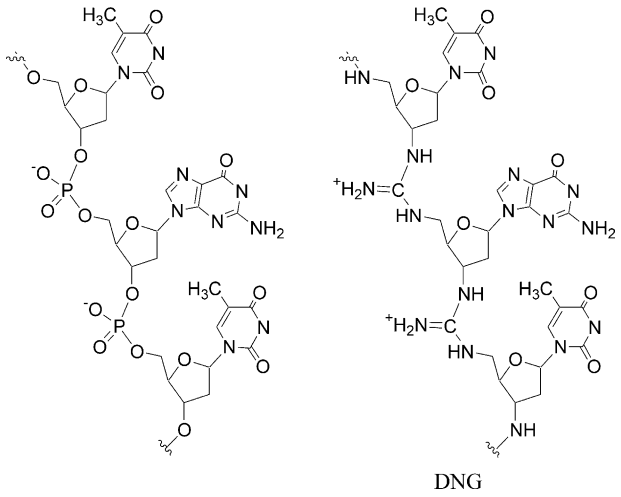
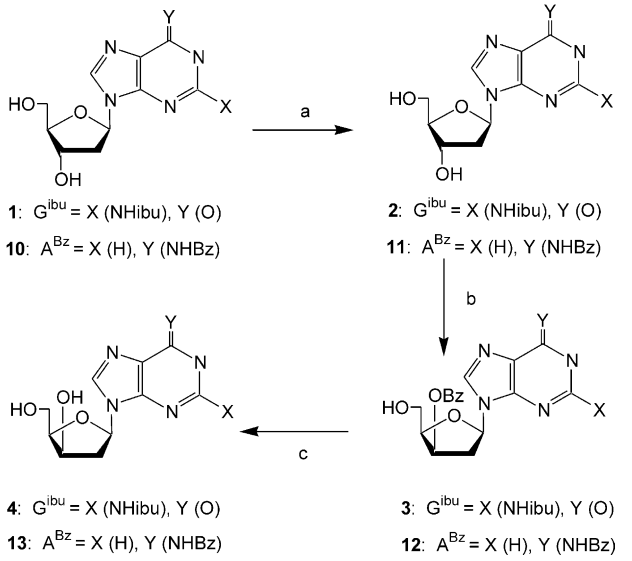


Figure 1.

DNG sequences containing all four purine and pyrimidine bases. This would allow versatility in choice of target sequences in determining the efficacy of DNG as an antisense agent. Thus far, there have been several reports on sequences containing guanidinium linkages involving thymidyl monomers or DNG/DNA,[20] DNG/PNA,[21] DNmt/DNA[19] chimera. More recently, DNG sequences containing both adenyl and thymidyl bases have been reported.[18] In this report we present, for the first time, the synthesis of guanyl base for incorporation into DNG sequences to give rise to mixed base DNG oligomers using the standard solid-phase chemistry. An alternative method for the synthesis of adenyl base compatible with the standard solid-phase synthesis of DNG is also described.

2. Results and discussion

The building blocks for the solid-phase synthesis of positively charged DNG oligonucleotides with guanidinium linkages, instead of the natural phosphodiester linkages of the DNA backbone, were synthesized as shown in Schemes 1–3. The key repetitive unit, **9**/**18**, was synthesized starting from 2′-deoxyguanosine or 2′-deoxyadenosine respectively. The first step in the synthesis involves inversion of configuration at the 3′-position to give the 2′-deoxy-threo isomer (Scheme 1). This was achieved by adaptation of the previously reported procedure by Herdewijn and Van Aerschot.[22] The 5′-benzoyl purine nucleosides, with the exocyclic amino functionalities of the bases protected by isobutyryl for guanine (**2**) and benzoyl for adenine (**11**), were treated with 1.5 equiv of trifluoromethanesulfonic anhydride in dichloromethane containing 10% of anhydrous pyridine to activate the 3′-hydroxyl group followed by addition of water to cleave the cyclic hemi-orthoester intermediate, formed by the attack of 5′-*O*-carbonyl group on the activated 3′-position, to yield the 3′-*O*-benzoate product (**3**, **12**). The 3′-*O*-benzoate was then quantitatively converted to the 3′-OH-2′-deoxyxylonucleosides (**4**, **13**) by selective alkaline hydrolysis. The synthesis of **13** was previously achieved starting from adenosine.[18] An analogous synthesis of **4** starting from guanosine



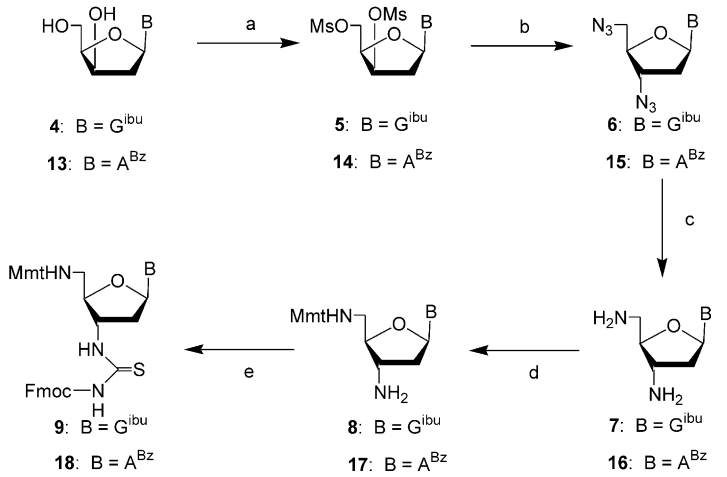
Scheme 1. Reagents and conditions: (a) benzoyl chloride, anhydrous pyridine, rt; (b) trifluoromethanesulfonic anhydride, 10% v/v pyridine in DCM, −35 °C to rt; (c) aqueous NaOH (2M), 5:4:1 dioxane/methanol/water, 0 °C.

was not undertaken due to the literature precedence of reportedly low yields and laborious procedures.[22]

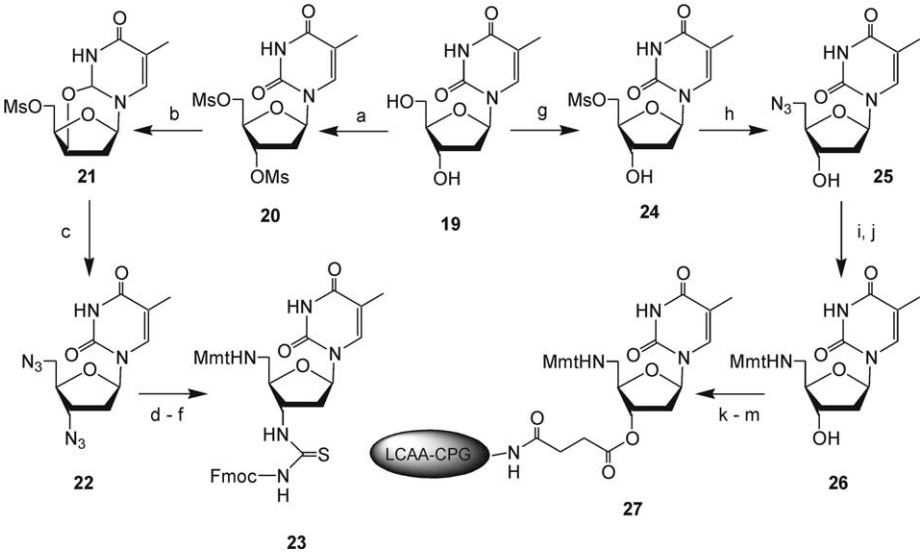
Treatment of the 3′-OH-2′-deoxyxylonucleosides (**4**, **13**) with methanesulfonyl chloride in pyridine at room temperature for 4 h afforded the 3′,5′-disulfonates (**5**, **4**) (Scheme 2).[23] These compounds were then transformed into their respective 3′,5′-diazido derivatives (**6**, **15**) using LiN_3 as nucleophile at 90 °C in DMF. After purification by silica gel chromatography, the 3′,5′-diazido compounds were reduced by catalytic hydrogenation with palladium over carbon to the respective 3′,5′-diamino-2′-deoxynucleosides (**7**, **16**). Selective protection of the 5′-amino group with the acid-labile Mmt group, followed by protection of the 3′-amino group with base-labile Fmoc group yielded the final precursors (**9** and **18**) that can be used for solid-phase synthesis of DNG.

The analogous thymidine precursor, **23**, was also prepared via the 3′,5′-diamino[24] route (Scheme 3), thus reducing the number of synthetic steps from the previously reported[15–17] procedures. Treatment of thymidine with methanesulfonyl chloride afforded the disulfonate (**20**), which upon reflux under alkaline conditions yielded the anhydronucleoside (**21**). The 3′,5′-diamino derivative (**22**) was obtained by treatment of **21** with LiN_3 followed by catalytic hydrogenation in presence of 10% palladium over carbon. Selective protection of first the 5′-amino and then the 3′-amino groups of **22** with MmtCl and Fmoc-isothiocyanate, respectively, yielded the desired thymidine repetitive precursor unit (**23**).

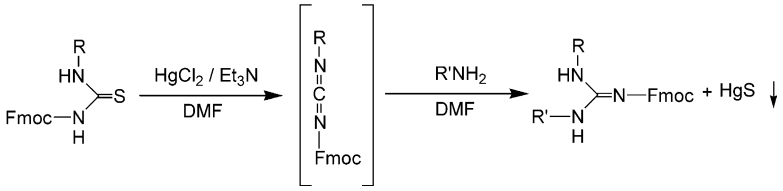
Solid-phase synthesis of DNG tetramer mixed base sequence, 5′-TgGgTgT-3′, was performed on CPG support from 3′ to 5′ direction as in the standard DNA solid-phase synthesis. The 5′-methoxytrityl protected thymidine monomer required for loading onto the LCAA-CPG support was synthesized as previously



Scheme 2. Reagents and conditions: (a) methanesulfonyl chloride, anhydrous pyridine, rt, 4 h; (b) LiN_3, anhydrous DMF, 90 °C, 4 h; (c) H_2, 10% Pd/C, EtOH, rt, 6 h; (d) Mmt-Cl, Et_3N, DCM, −30 °C to rt; (e) Fmoc-NCS, DCM, rt.

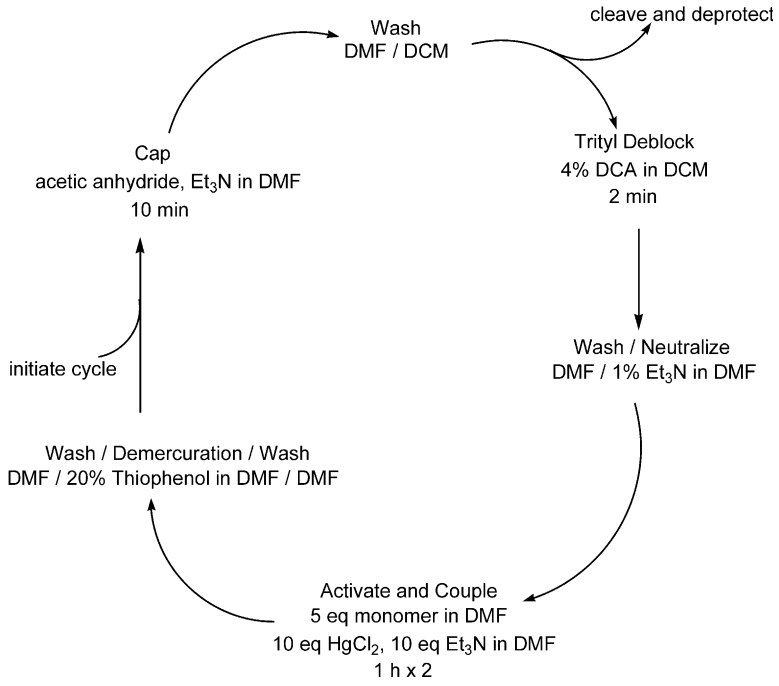


Scheme 3. Reagents and conditions: (a) methanesulfonyl chloride, anhydrous pyridine, rt, 4 h; (b) aqueous NaOH (2M), EtOH, reflux, 1 h; (c) LiN_3, anhydrous DMF, 90 °C, 4 h; (d) H_2, 10% Pd/C, ethanol, rt, 4 h; (e) Mmt-Cl, Et_3N, DCM, −30 °C to rt; (f) Fmoc-NCS, DCM, rt; (g) methanesulfonyl chloride, Et_3N, DCM, 0 °C; (h) LiN_3, anhydrous DMF, 90 °C, 4 h; (i) H_2, 10% Pd/C, EtOH, rt, 6 h; (j) Mmt-Cl, Et_3N, DMAP, anhydrous pyridine, rt; (k) succinic anhydride, DMAP, pyridine; (l) 4-nitrophenol, DCC, anhydrous pyridine, dioxane, rt; (m) CPG, Et_3N, anhydrous DMF, rt.



Scheme 4. Activation and coupling reaction.

reported and loaded onto the CPG as its 3′-succinyl ester using standard protocols (Scheme 3).[25] After loading, the unreacted sites were capped and the loading yield was determined spectrophotometrically from the amount of Mmt cation released. A typical solid-phase synthesis cycle is illustrated in Scheme 5. Upon deblocking the acid-labile trityl group of the growing chain, the 5′-amino functionality is now available to couple with the incoming precursor for the formation of the guanidinium linkage. The coupling reaction is accomplished in the presence of $HgCl_2$ and TEA (Scheme 4), whereby the 3′-Fmoc protected thiourea of the incoming precursor is converted into an activated carbodiimide intermediate through the abstraction of the



Scheme 5. Solid phase synthesis (3′ to 5′) cycle for DNG oligos on CPG support.

sulfur atom on the thiourea by mercury (II). The carbodiimide intermediate then reacts with the unmasked 5′-NH_2 of the growing chain to yield an Fmoc protected guanidinium linkage. This Fmoc protecting group remains on the guanidinium linkage until the end of the synthesis when it is easily deprotected during cleavage of the oligomer from the CPG support. The HgS precipitate formed during the coupling reaction is removed by demercuration using 20% thiophenol in DMF. Then, capping with acetic anhydride and TEA to ensure inertness towards subsequent chain elongation reactions blocks the unreacted 5′-NH_2 sites. This completes one cycle of the synthesis. The coupling efficiency at the end of each cycle can be monitored by colorimetric analysis of the Mmt-cation released at the acid deblock step. The coupling yield in each cycle was 90–95%, thus the overall yield of the 5′-protected DNG mixed base tetramer is expected to be ~80%. After the final coupling reaction, the Mmt protecting group was retained on the oligomer to simplify the purification of the crude product. The Mmt-on DNG oligomer was then cleaved off the CPG support using methanolic ammonia solution at 60 °C for 16 h, when the Fmoc protection on the guanidinium linkage and the isobutyryl groups on the exocyclic guanine base were also deprotected simultaneously. The crude product containing the desired oligomer along with the failure sequences was purified on reverse-phase HPLC (Altech C8 column) using 100 mM TEAA buffer as solvent A with a gradient of 5% to 95% CH_3CN as solvent B in 30 min. The HPLC purified trityl-on oligomer was then detritylated using 1 mL of 80% acetic acid (1 h, rt), lyophilized, redissolved in water, and further purified by RP-HPLC. The final detritylated and HPLC purified oligomer was analyzed by mass spectrometry (ESI) to be the desired tetramer: $m/z = 1061.80$, 1083.83 $(M+H/Na)^+$; calculated for $C_{43}H_{59}N_{21}O_{12}(M+H)^+$ 1062.07. Thus, the precursor building block **9** was successfully assembled into mixed base DNG oligomer.

3. Conclusion

The first stepwise solid-phase synthesis of DNG oligomer containing guanine purine nucleoside has been accomplished. The tetrameric DNG mixed base oligomer 3′-OH-TgTgGgT-NH_2-5′ was synthesized in 3′ to 5′ direction with average coupling yields of 95% on CPG solid support as used for DNA solid-phase synthesis. The orthogonally protected precursor guanyl monomer with acid-labile 5′-amino and base-labile 3′-amino functionalities is described for the first time and the analogous adenyl precusor synthesis is also detailed, both starting from the 2′-deoxy purine nucleosides. The described methods enable the synthesis of DNG mixed base sequences for evaluating their interactions with specific DNA target sequences.

4. Experimental

4.1. General

All ^1H and ^{13}C spectra were recorded on 400 and 100 MHz Varian instrument respectively, using $CDCl_3$ or DMSO-d_6 or CD_3OD as solvent. Chemical shifts are reported in δ (ppm) as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). TLC was monitored on aluminum-backed silica gel 60 (F_{254}) 0.25 mm plates (Merck) with fluorescent indicator. Column chromatography was performed using silica gel (particle size 63–200 Å, Selecto Scientific, Georgia, USA). Reversed phase HPLC was performed on a Hewlett Packard 1050 system equipped with a quaternary solvent delivery system, a UV detector set at 260 nm, and an

Altech Macrosphere C8 RP semiprep column (7 μ, 10×250 mm). All solvents were purchased dry from Aldrich.

4.1.1. Synthesis of N^2-isobutyryl-3′-*N*-(9-fluorenylmethyloxycarbonylamino)-5′-*N*-(4-monomethoxytritylamino)-2′,3′-dideoxyguanosine, 9. N^2-Isobutyryl-2′-deoxyguanosine (1).[26] 2′-Deoxyguanosine (5.705 g, 20 mmol) was coevaporated with anhydrous pyridine and then suspended in 200 mL anhydrous pyridine. Trimethylchlorosilane (13 mL, 100 mmol) was added slowly to the suspension cooled in an ice-bath. After 30 min, isobutyric anhydride (17 mL, 100 mmol) was added dropwise and the reaction mixture was stirred for 2.5 h at room temperature. The reaction mixture was then chilled in an ice-bath, 40 mL of cold water was added and let stir for 15 min. Concentrated aqueous NH_4OH was added, let stir for another 30 min and rotovapped to give an oil with salts. Water was added until all salts dissolved and washed once with equal volume of ether. The product crystallizes immediately in the aqueous layer, which was filtered and dried on vacuum until constant weight to give 5.575 g of **1** (82% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.4–12.2 (s, br, 2H), 8.2 (s, 1H), 6.2 (t, 1H), 5.3 (d, 1H), 4.9 (t, 1H), 4.3 (m, 1H), 3.8 (m, 1H), 3.4–3.6 (m, 2H), 2.7 (m, 1H), 2.6 (m, 1H), 2.2 (m, 1H), 1.1 (d, 6H). Anal. calcd for $C_{14}H_{19}N_5O_5$: C, 49.8; H, 5.7; N, 20.8. Found: C, 49.2; H, 5.8; N, 19.7.

4.1.2. N^2-Isobutyryl-5′-*O*-benzoyl-2′-deoxyguanosine (2).[27] To a suspension of **1** (5.04g, 14.9 mmol) in anhydrous pyridine (40 mL) was added dropwise a solution of benzoyl chloride (1.76 mL, 15.2 mmol) in 10 mL anhydrous pyridine over 4 h. After completion of addition, the reaction mixture was allowed to stir for 1 h and then quenched with water and rotovapped to an oil. The oil was dissolved in 100 mL DCM and washed with water until the emulsions began to solidify. At this point, the DCM layer was stored under fresh water for 30 min when the product crystallized out. The product was collected by filtration and dried on vacuum to give 5.33 g of **2** (81% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.1 (s, 1H), 11.6 (s, 1H), 8.2 (s, 1H), 7.5–7.9 (m, 5H), 6.3 (t, 1H), 5.6 (d, 1H), 4.4–4.6 (m, 3H), 4.1 (m, 1H), 2.7–2.8 (m, 2H), 2.4 (m, 1H), 1.1 (d, 6H). Anal. calcd for $C_{21}H_{23}N_5O_6$: C, 57.1; H, 5.3; N, 15.9. Found: C, 55.8; H, 5.3; N, 14.8. *m/z* (HRESI-MS) 442.1743, calcd for $C_{21}H_{23}N_5O_6$ $(M+H)^+$ 442.1726.

4.1.3. N^2-Isobutyryl-3′-*O*-benzoyl-2′,3′-dideoxyguanosine (3). A solution of **2** (6.10g, 13.82 mmol) dissolved in anhydrous 10% v/v pyridine in DCM (100 mL) was cooled to −35 °C in an isopropanol/dry ice bath. Trifluoromethanesulfonic anhydride (3.5 mL, 20.73 mmol) was added slowly while maintaining the reaction temperature at −35 °C. After completion of addition, the reaction mixture was allowed to warm to 0 °C, let stir for 45 min, quenched with 3 mL water and let stir overnight at room temperature. The reaction mixture was then rotovapped to an oil, redissolved in ethyl acetate and washed twice with equal volumes of water. The organic layer was rotovapped to a paste, which upon sitting over DCM for 1 h crystallized the desired product. The product was collected by filtration and dried on vacuum to give 2.67 g of **3** (45% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.0 (s, 1H), 11.7 (s, 1H), 8.1 (s, 1H), 7.5–7.8 (m, 5H), 6.2 (dd, 1H), 5.6 (t, 1H), 4.9 (t, 1H), 4.3 (m, 1H); 3.7–3.8 (m, 2H), 3.0 (m, 1H), 2.7–2.8 (m, 2H), 1.1 (d, 6H). Anal. calcd for $C_{21}H_{23}N_5O_6$: C, 57.1; H, 5.3; N, 15.9. Found: C, 56.9; H, 5.4; N, 15.5. *m/z* (HRESI-MS) 442.1711, calcd for $C_{21}H_{23}N_5O_6$ $(M+H)^+$ 442.1726.

4.1.4. N^2-isobutyryl-2′-deoxyxyloguanosine (4). To a solution of **3** (1.34g, 3.02 mmol) in a mixture of dioxane/methanol/water (50 mL, 5:4:1 v/v/v), cooled in an ice-bath, were added 7 mL of aqueous NaOH (2M) and let stir at 0 °C for 40 min. The reaction mixture was then neutralized with Dowex 50W-X8 pyridinium H^+-form cation exchange resin (Fluka). Once the solution was neutral (∼15 min), the resin was filtered and the filtrate was rotovapped to a thick paste which upon trituration with DCM gave 1.00g of **4** (98% yield) as an off-white solid after drying on vacuum. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.0 (s, 1H), 11.7 (s, 1H), 8.2 (s, 1H), 6.1 (dd, 1H), 5.3 (d, 1H), 4.7 (t, 1H), 4.5 (m, 1H), 3.9 (m, 1H), 3.5–3.7 (m, 2H), 2.6–2.8 (m, 2H), 2.2 (m, 1H), 1.1 (d, 6H). Anal. calcd for $C_{14}H_{19}N_5O_5$: C, 49.8; H, 5.7; N, 20.8. Found: C, 49.8; H, 5.7; N, 20.4. *m/z* (HRESI-MS) 338.1463, calcd for $C_{14}H_{19}N_5O_5$ $(M+H)^+$ 338.1464.

4.1.5. N^2-Isobutyryl-3′,5′-dimesyl-2′,3′-dideoxyguanosine (5). To a solution of **4** (1.00g, 2.96 mmol) in anhydrous pyridine was added neat methanesulfonyl chloride (0.9 mL, 11.83 mmol) dropwise over 15 min. The reaction mixture was stirred at room temperature for 4 h, quenched with MeOH and rotovapped to a thick oily paste. The paste was redissolved in ethyl acetate/water and the product extracted into ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, rotovapped to give 1.32 g of **5** (90% yield) as a pale wheatish brittle foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.1 (s, 1H), 11.7 (s, 1H), 8.0 (s, 1H), 6.2 (dd, 1H), 5.5 (m, 1H), 4.5–4.6 (m, 3H), 3.29 (s, 3H), 3.21 (s, 3H), 3.1 (m, 1H), 2.7–2.8 (m, 2H), 1.1 (d, 6H). Anal. calcd for $C_{16}H_{23}N_5O_9S_2$: C, 38.9; H, 4.7; N, 14.2. Found: C, 38.6; H, 4.8; N, 14.0. *m/z* (HRESI-MS) 494.1000, calcd for $C_{16}H_{23}N_5O_9S_2$ $(M+H)^+$ 494.1015.

4.1.6. N^2-Isobutyryl-3′,5′-diazido-2′,3′-dideoxyguanosine (6). To a solution of **5** (1.83 g, 3.7 mmol) in anhydrous DMF (50 mL) was added LiN_3 (3.41 g, 69.7 mmol). The reaction mixture was stirred at 90 °C until all the starting material was consumed as shown by TLC (∼4 h). The reaction mixture was filtered and rotovapped to give a brown oil. The oil was purified by silica gel column chromatography using 4:1 EtOAc/$CHCl_3$ to give 0.84 g of **6** (60% yield) as brittle foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.1 (s, 1H), 11.6 (s, 1H), 8.2 (s, 1H), 6.2 (s, 1H), 4.6 (m, 1H), 4.0 (m, 1H), 3.6 (m, 2H), 3.0 (m, 1H), 2.7 (m, 1H), 2.6 (m, 1H), 1.1 (d, 6H). Anal. calcd for $C_{14}H_{17}N_{11}O_3$: C, 43.4; H, 4.4; N, 39.8. Found: C, 43.6; H, 4.3; N, 38.9. *m/z* (HRESI-MS) 388.1600, calcd for $C_{14}H_{17}N_{11}O_3$ $(M+H)^+$ 388.1594.

4.1.7. N^2-isobutyryl-3′,5′-diamino-2′,3′-dideoxyguanosine (7). To a solution of **6** (0.84g, 2.17 mmol) in absolute EtOH (100 mL) was added 100 mg of Pd/C (10% wet) and hydrogenated at 45 PSI for 6 h. The reaction mixture was filtered over a pad of Celite and the filtrate was rotovapped and coevaporated with DCM to give 0.71 g of **7** (97% yield) as a pale yellowish solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.2 (s, 1H), 6.2 (m, 1H), 3.5 (m, 3H), 2.7–2.8 (m, 2H), 2.2 (m, 1H), 1.1 (d, 6H), 1.0 (m, 1H). Anal. calcd for $C_{14}H_{21}N_7O_3$: C, 50.1; H, 6.3; N, 29.2. Found: C, 50.3; H, 6.2; N, 29.1. *m*/*z* (HRESI-MS) 336.1769, calcd for $C_{14}H_{21}N_7O_3$ $(M+H)^+$ 336.1784.

4.1.8. N^2-Isobutyryl-3′-amino-5′-*N*-(4-monomethoxytritylamino)-2′,3′-dideoxyguanosine (8). To a suspension of **7** (690 mg, 2.06 mmol) in DCM was added triethylamine (0.6 mL, 4.11 mmol) and cooled to −30 °C in an isopropanol/dry ice bath. A solution of Mmt-Cl in DCM (635 mg, 2.06 mmol) was added slowly and the reaction mixtured was allowed to warm up to room temperature. The reaction was quenched with MeOH and rotovapped to an oil. The oil was purified by silica gel column chromatography using EtOAc with 0–30% MeOH gradient to give 793 mg of **8** (63% yield). ^{1}H NMR (400 MHz, CD_3OD-d_6) δ 12.1 (s, 1H), 11.6 (s, 1H), 7.1–7.5 (m, 12H), 6.8 (d, 2H), 6.1 (dd, 1H), 5.4 (t, 1H), 4.1 (m, 1H), 3.8 (s, 3H), 3.0 (m, 1H), 2.6–2.8 (m, 2H), 2.1 (m, 1H), 1.8 (m, 1H), 1.4 (m, 1H), 1.2 (d, 6H). Anal. calcd for $C_{34}H_{37}N_7O_4$: C, 67.2; H, 6.1; N, 16.1. Found: C, 66.5; H, 6.1; N, 15.7. *m*/*z* (HRESI-MS) 608.3005, calcd for $C_{34}H_{37}N_7O_4$ $(M+H)^+$ 608.2985.

4.1.1.9. N^2-Isobutyryl-3′-*N*-(9-fluorenylmethyloxycarbonylamino)-5′-*N*-(4-monomethoxytritylamino)-2′,3′-dideoxyguanosine (9). To a solution of **8** (340 mg, 559 μmol) in DCM (40 mL) was added a solution of Fmoc-NCS in DCM (157 mg, 559 μmol) and let stir for 1 h. The reaction mixture was rotovapped and the crude product purified by ether precipitation to give 397 mg of **9** (80% yield) as an off-white solid. ^{1}H NMR (400 MHz, CD_3OD-d_6) δ 12.0 (s, 1H), 11.4 (s, 1H), 11.6 (s, 1H), 7.1–7.9 (m, 20H), 6.8 (d, 2H), 6.0 (dd, 1H), 4.4 (m, 2H), 4.3 (t, 1H), 4.0 (m, 1H), 3.7 (s, 3H), 3.1 (m, 1H), 2.6–2.8 (m, 2H), 2.0 (m, 1H), 1.6 (m, 1H), 1.3 (m, 1H), 1.2 (d, 6H). Anal. calcd for $C_{50}H_{48}N_8O_6S$: C, 67.6; H, 5.4; N, 12.6. Found: C, 66.9; H, 5.3; N, 12.4. *m*/*z* (HRESI-MS) 889.3521, calcd for $C_{50}H_{48}N_8O_6S$ $(M+H)^+$ 889.3496.

4.1.10. Synthesis of N^6-benzoyl-3′-*N*-(9-fluorenylmethyloxycarbonylamino)-5′-*N*-(4-monomethoxytritylamino)-2′,3′-dideoxyadenosine (18). N^6-Benzoyl-2′-deoxyadenosine (10). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.2 (s, br, 1H), 8.75 (s, 1H), 8.65 (s, 1H), 8.0 (d, 2H), 7.5–7.6 (m, 3H), 6.5 (t, 1H), 5.4 (d, 1H), 5.0 (t, 1H), 4.4 (m, 1H), 3.9 (m, 1H), 3.5–3.6 (m, 2H), 2.8 (m, 1H), 2.3 (m, 1H). Anal. calcd for $C_{17}H_{17}N_5O_4$: C, 57.5; H, 4.8; N, 19.7. Found: C, 56.7; H, 4.9; N, 18.9. *m*/*z* (HRESI-MS) 356.1365, calcd for $C_{17}H_{18}N_5O_4$ $(M+H)^+$ 356. 1359.

4.1.11. N^6-Benzoyl-5′-*O*-benzoyl-2′-deoxyadenosine (11). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.2 (s, br, 1H), 8.7 (s, 1H), 8.6 (s, 1H), 7.5–8.0 (m, 10H), 6.5 (t, 1H), 5.6 (d, 1H), 4.7 (m, 1H), 4.4–4.6 (m, 2H), 4.2 (m, 1H), 3.0 (m, 1H), 2.4 (m, 1H). Anal. calcd for $C_{24}H_{21}N_5O_5$: C, 62.7; H, 4.6; N, 15.2. Found: C, 62.5; H, 4.6; N, 15.1. *m*/*z* (HRESI-MS) 460.1628, calcd for $C_{24}H_{22}N_5O_5$ $(M+H)^+$ 460.1621.

4.1.12. N^6-Benzoyl-3′-*O*-benzoyl-2′,3′-dideoxyadenosine (12). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.2 (s, br, 1H), 8.62 (s, 1H), 8.58 (s, 1H), 7.5–8.0 (m, 10H), 6.5 (dd, 1H), 5.7 (m, 1H), 5.0 (t, 1H), 4.4 (m, 1H), 3.8 (m, 2H), 3.1 (m, 1H), 3.0 (m, 1H). Anal. calcd for $C_{24}H_{21}N_5O_5$: C, 62.7; H, 4.6; N, 15.2. Found: C, 62.8; H, 4.6; N, 14.8. *m*/*z* (HRESI-MS) 460.1631, calcd for $C_{24}H_{22}N_5O_5$ $(M+H)^+$ 460.1621.

4.1.13. N^6-Benzoyl-2′-deoxyxyloadenosine (13). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.2 (s, br, 1H), 8.75 (s, 1H), 8.65 (s, 1H), 8.0 (d, 1H), 7.5–7.6 (m, 4H), 6.4 (dd, 1H), 5.6 (s, br, 1H), 4.7 (s, br, 1H), 4.4 (m, 1H), 4.0 (m, 1H), 3.6–3.7 (m, 2H), 2.8 (m, 1H), 2.3 (m, 1H). Anal. calcd for $C_{17}H_{17}N_5O_4$: C, 57.5; H, 4.8; N, 19.7. Found: C, 56.8; H, 4.8; N, 18.7. *m*/*z* (HRESI-MS) 356.1365, calcd for $C_{17}H_{18}N_5O_4$ $(M+H)^+$ 356.1359.

4.2. Solid phase synthesis

4.2.1. Loading. The 5′-monomethoxytritylamino-5′-deoxythymidine monomer was loaded onto commercially available LCAA-CPG (500 Å, 80–120 mesh size) as its succinyl ester using standard protocols.[25] The efficiency of loading was determined to be 32 μmol/g by treating an aliquot of thymidine-loaded CPG with a solution of 4% DCA in DCM and assaying the trityl cation released by UV spectroscopy. Then the unreacted amino groups on the CPG were capped with acetic anhydride and triethylamine to prevent side reactions. The T-loaded CPG was stored at 5 °C.

4.2.2. Deblocking. An aliquot of 100 mg (4 μmol scale) of the T-loaded CPG was placed in a fritted peptide-synthesis vessel and treated with a deblock solution (4% DCA in DCM) while collecting the solution into a 10 mL volumetric flask as it drips through the frit by gravity. The beads were treated with the deblock solution until no more yellow coloration was apparent (2 min). The beads were then washed with DCM and neutralized with 1% TEA in DMF. The filterate collected in the volumetric flask was assayed for the released monomethoxytrityl cation.

4.2.3. Coupling. To the exposed 5′-amino group on the CPG was added 5 equiv of the fully-protected **9** or **23** dissolved in 1 mL of DMF. Then 10 equiv of $HgCl_2$ and TEA, dissolved separately in 0.5 mL DMF each, were added simultaneously to the reaction vessel when a cloudy yellow-white precipitate was observed. The reaction mixture was agitated for 1 h, the supernatant filtered, beads washed with DMF until no more precipitate was visible, washed with 20% thiophenol in DMF until the beads were clear, washed with copious amounts of DMF and then the coupling reaction was repeated two more times in the same manner.

4.2.4. Capping. The unreacted sites were capped by addition of 1 mL of 100 mM acetic anhydride in DMF

and 1 mL of 200 mM TEA in DMF (5 min×2). The beads were washed copiously with DMF and DCM and then dried on high vacuum.

4.2.5. Elongation. The deblocking/coupling/capping steps were repeated two more times with the appropriate elongation monomer to give the desired tetramer DNG sequence on the LCAA-CPG. The final coupling reaction, the capping and deblocking steps were skipped to allow the trityl group to remain on the oligomer.

4.2.6. Cleavage and deprotection. The desired tetramer DNG sequence was cleaved from the CPG support by treating with methanolic ammonia at 60 °C for 16 h. Simultaneously, the isobutyryl protecting groups on the G-bases were also deprotected. The solution now containing the desired tetramer was filtered and lyophilized to yield a white residue of the crude product mixture.

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