Synthesis and Evaluation of Oligodeoxynucleotides Containing Diphosphodiester Internucleotide Linkages

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1. **General Information.** All reactions were carried out in Bio-Rad polypropylene columns by shaking and mixing using a Glass-Col small tube rotator under extremely dry conditions at room temperature unless otherwise stated. Real-time monitoring of loading of compounds on resin beads was carried out with a Thermo-Nicolet 550 FT-IR spectrophotometer coupled with a Nic-Plan microscope using OMNIC software. The chemical structures of final products were characterized by nuclear magnetic resonance spectrometry determined on a Bruker NMR spectrometer (400 MHz). $^{13}$C NMR spectra are fully decoupled. $^{31}$P NMR spectra for ODNs up to 5mers are decoupled. The chemical structures of final products containing up to 5 bases were confirmed by a high-resolution PE Biosystems Mariner API time-of-flight electrospray mass spectrometer and quantitative phosphorus analysis. The chemical structures of 12-mer ODNs were confirmed by a MALDI-TOF mass spectrometer and quantitative phosphorus analysis. The substitution of the resins for each step was estimated from the weight gain of the resin. Total isolated yields for final products were calculated based on the loading of aminomethyl polystyrene resin-bound bis(2-cyanoethyl diisopropylphosphoramidite (1, 0.74 mmol/g) and the amount of diphosphorylated products. Bis(2-cyanoethyl diisopropylphosphoramidite), [(i-Pr)$_2$NPOCH$_2$CH$_2$CN]$_2$O, and polymer-bound diphosphitylating reagent (1) were prepared according to our previously reported procedure.[21] Unmodified ODNs were purchased from Integrated DNA Technologies, Inc. and were desalted and purified.

2. **General Procedure for the Synthesis of Modified ODNs Containing Diphosphate Diester Internucleotide Linkages Using Unprotected Nucleosides.** The synthesis cycle consists of six chemical reactions that are separated by washing steps designed to remove excess reagents. Steps i-v were carried out under extremely dry conditions and nitrogen. These steps include (i) immobilization of 5'-hydroxyl group of the first unprotected nucleoside (e.g., dT, dA, dG, dC) through the reaction with solid-supported reagent 1 in the presence of 1H-tetrazole to afford $2a$–$d$; (ii) diphosphitylation of the 3'-hydroxyl group with bis(2-cyanoethyl diisopropylphosphoramidite) in the presence of 1H-tetrazole to yield $3a$–$d$; (iii)
repeating steps i and ii (coupling and diphosphitylation reactions) n times (n = 0-4, 11) to produce polymer-bound diphosphite triesters (5a–h); (iv) oxidation with t-butyl hydroperoxide to yield polymer-bound diphosphate triesters (6a–h); (v) removal of 2-cyanoethoxy group in the presence of DBU to produce polymer-bound diphosphate diester (7a–h); and (vi) cleavage of final products (8–22) from the solid support in the presence of DCM/TFA/H₂O/1,2-ethanedithiol. The residues were mixed with Amberlite AG-50W-X8 (100-200 mesh, hydrogen form, 500 mg, 1.7 meq/g). Repetition of this synthesis cycle allowed for assembly of different ODNs. In total, by using different numbers and combinations of nucleosides, 30 compounds were synthesized. The linker remained trapped on the resins, which facilitated the separation of the final products by filtration (Scheme S1).

**Scheme S1.** The cleavage mechanism of 7a-h to modified ODNs containing phosphodiester internucleotide linkages (8–22).

First, ODNs containing 5 bases or less (n = 0-4) were synthesized to examine the feasibility of the synthesis. The crude products had a purity of 66-94% and were purified by using small C₁₈ Sep-Pak cartridges and appropriate solvents to afford ODNs up to five bases containing diphosphodiester bridges (8–12 a–d) in 47-78% (overall yield calculated from 1) (Table S1).
The synthetic cycle was then used to synthesize modified ODNs with 12 bases. The crude products had a purity of 59-91% and were purified first by using small C_{18} Sep-Pak cartridges and appropriate solvents. Selected fractions containing the final products were repurified using reverse phase HPLC to afford ODNs with 12 bases containing diphosphodiester bridges in 32-44% (overall yield calculated from 1) (Table S1). No failure sequences were observed and all 12-mer ODNs were successfully purified by using reverse phase HPLC.

Table S1. Overall isolated yields and purity of crude products for modified ODNs containing diphosphate diester linkage (8–22).

<table>
<thead>
<tr>
<th>Oligomers</th>
<th>Overall yield (%)</th>
<th>Purity of crude products (%)</th>
<th>μmol synthesized</th>
<th>Oligomers</th>
<th>Overall yield (%)</th>
<th>Purity of crude products (%)</th>
<th>μmol synthesized</th>
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<tr>
<td>8a</td>
<td>75</td>
<td>89</td>
<td>75</td>
<td>11d</td>
<td>78</td>
<td>94</td>
<td>20</td>
</tr>
<tr>
<td>8b</td>
<td>68</td>
<td>83</td>
<td>73</td>
<td>12a</td>
<td>74</td>
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<td>65</td>
<td>78</td>
<td>70</td>
<td>12b</td>
<td>70</td>
<td>88</td>
<td>15</td>
</tr>
<tr>
<td>8d</td>
<td>59</td>
<td>66</td>
<td>78</td>
<td>12c</td>
<td>66</td>
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<tr>
<td>9b</td>
<td>62</td>
<td>81</td>
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<tr>
<td>9c</td>
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<tr>
<td>11a</td>
<td>64</td>
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<td>20</td>
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<td>79</td>
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3. Reverse Phase HPLC Purification of Modified ODNs Containing 12 Bases. Modified oligomers were purified (>99%) on a Phenomenex® Prodigy 10 μm ODS reversed-phase column (2.1 × 25 cm) with Hitachi HPLC system using a gradient system of H_{2}O (1 mM ammonium acetate) and acetonitrile. The purification was performed with a gradient of 0-100% CH_{3}CN over 50-60 min and a flow rate of 2.4 mL/min. The product fractions were collected, concentrated under vacuum, and dissolved in filtered (0.22 μm) water. The pH was adjusted with 80% acetic acid to approximately pH 7.2. The solutions were freeze
dried to afford pure 12-mer modified ODNs. Reverse phase HPLC of the crude products showed a broad peak (25-30 min) with only minor contaminants (Figure S1).

![Graph showing retention time versus absorbance for the crude product of d(TTTTTTTTTTT) (14) and d(GGGGGGGGGGGG) (16).]

**Figure S1.** Reverse phase HPLC of the crude product of d(TTTTTTTTTTT) (14) (left) and d(GGGGGGGGGGGG) (16) (right).

**4. Interpretation of $^{31}$P NMR for Modified ODNs.** Fully decoupled $^{31}$P NMR for thymidine 5-mer analogue (12a) displayed 10 non-overlapping peaks corresponding to 10 phosphorus atoms having chemical shifts between -15.78 to 2.29 ppm. The downfield peak at 2.29 ppm was more deshielded compared to other phosphorus atoms and corresponds to the terminal β-phosphorus atom (Figure S2). A similar pattern was observed for all other modified ODNs. It is known that the chemical shifts for phosphorus atoms are pH-dependent. Proton exchange resin was used to exchange all salt anions with hydrogens. In this condition, the terminal phosphorus atom attached to two hydroxyl groups was more deshielded when compared to other phosphorus atoms attached to one hydroxyl group.
Figure S2. Decoupled $^{31}$P NMR of thymidine 5-mer analogue (12a) containing diphosphate diester bridges.

Coupled $^{31}$P NMR of modified 12-mer analogues 14 and 19 displayed peaks with chemical shifts between -18.23 and 1.33 ppm corresponding to 24 phosphorus atoms in each compound. The doublet downfield doublet peaks centered at 1.29 ppm ($J = 14.6$ Hz) and 1.77 ppm ($J = 16.2$ Hz) for 14 and 19, respectively, correspond to the terminal phosphorus atoms (Figure S3). The peak integrations show the presence of approximately 24 phosphorous atoms in both 12-mer analogues.
5. MALDI-TOF Mass Spectroscopy. MALDI-TOF analysis was performed on a Ciphergen Workstation. Modified ODNs were purified by reverse phase HPLC utilizing volatile salts, concentrated to dryness, and dissolved in acetonitrile/water (1:1) to a final concentration of 200 μM. The matrix contained 2,4,6-trihydroxyacetophenone monohydrate (THAP) and was prepared by adding of THAP (0.2 mmol, 45 mg) and ammonium citrate (8.2 μmol, 2 mg) to acetonitrile/water (500 μL, 1:1), which forms a supersaturated solution (a cloudy suspension). The THAP matrix suspension (1 μL) was spotted on a gold plate. The oligomer solution (1 μL) was pipetted onto the same location. The mixture was carefully dried. The plate was inserted into the MALDI-TOF Workstation. All measurements were performed using a positive detection mode (Table S2).
Table S2. Characterization of 12-mer modified ODNs containing diphosphodiester linkage.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Structure</th>
<th>Calcd Mass (g/mol)</th>
<th>Found Mass (g/mol)</th>
<th>Anal. Calcd. P (%)</th>
<th>Anal. Found P (%)</th>
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<tr>
<td>13</td>
<td>d(AAAAAAAAAAAAAA)</td>
<td>4734.3</td>
<td>4734.2 [M⁺]</td>
<td>15.70</td>
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<td></td>
<td></td>
<td></td>
<td>4757.0 [M + Na⁺]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>d(TTTTTTTTTTTTTTTTTTTTTTTT)</td>
<td>4626.2</td>
<td>4626.9 [M + 1⁺]</td>
<td>16.06</td>
<td>15.87</td>
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<tr>
<td>15</td>
<td>d(CCCCCCCCCCCCCCCCCCC)</td>
<td>4446.2</td>
<td>4446.9 [M + 1⁺]</td>
<td>16.71</td>
<td>16.63</td>
</tr>
<tr>
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<td>d(GGGGGGGGGGGGGGGGGGGGGGGGGG)</td>
<td>4926.2</td>
<td>4927.8 [M + 1⁺]</td>
<td>15.08</td>
<td>15.27</td>
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<tr>
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<td>d(ATATATATATATATATATATATAT)</td>
<td>4680.2</td>
<td>4681.0 [M + 1⁺]</td>
<td>15.88</td>
<td>15.72</td>
</tr>
<tr>
<td>18</td>
<td>d(TATATATATATATATATATATATAT)</td>
<td>4680.2</td>
<td>4681.0 [M + 1⁺]</td>
<td>15.88</td>
<td>16.09</td>
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<tr>
<td>19</td>
<td>d(CGCGCGCGCGCGCGCGCGCGCGCGCG)</td>
<td>4686.2</td>
<td>4687.2 [M + 1⁺]</td>
<td>15.86</td>
<td>15.74</td>
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<tr>
<td>20</td>
<td>d(GCGCGCGCGCGCGCGCGCGCGCGCG)</td>
<td>4686.2</td>
<td>4687.3 [M + 1⁺]</td>
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<tr>
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<td>4668.6 [M + 1⁺]</td>
<td>15.92</td>
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<tr>
<td>22</td>
<td>d(TGCAATCAGGTTT)</td>
<td>4698.2</td>
<td>4698.9 [M + 1⁺],</td>
<td>15.82</td>
<td>15.94</td>
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6. Preparation of ODNs Containing Phosphodiester Internucleotide Linkages (8–22) Using Unprotected Nucleosides

Preparation of Polymer-Bound Diphosphate Triesters (5a–h, n=0-4, 11). Nucleosides (a–d) (1.28 mmol, 4 eq) and 1H-tetrazole (45 mg, 0.64 mmol, 2 eq) were added to polymer-bound diphosphitylating reagent (1, 433 mg, 0.74 mmol/g, 1eq) in anhydrous THF (1 mL) and DMSO (4 mL) under extremely dry conditions and nitrogen. The mixture was shaken for 24 h at room temperature. The resin was collected by filtration and washed with DMSO (3 × 20 mL), THF (2 × 15 mL), and MeOH (3 × 20 mL), respectively, and dried under vacuum to give 2a–d. Stepwise diphosphitylation of 3’-hydroxyl group in 2a–d with the diphosphitylating reagent (4 eq), bis(2-cyanoethyl diisopropylphosphoramidite) ([i-Pr]₂NPOCH₂CH₂CN)₂O), was carried out in anhydrous THF (15 mL) in the presence of 1H-tetrazole (45 mg, 0.64 mmol, 2 eq). The mixture was shaken at room temperature for 24 h under extremely dry conditions and nitrogen. The resin was collected by filtration and washed with THF (2 × 15 mL) and MeOH (3 × 20 mL), respectively, and dried under vacuum to afford 3a–d. Polymer-bound 3’-phosphitylating reagent was subjected with reactions with nucleosides (a–d) in anhydrous THF (1 mL) and DMSO (4 mL) in the presence of 1H-tetrazole (45 mg, 0.64 mmol, 2 eq) under extremely dry
conditions and nitrogen. The mixture was shaken for 24 h at room temperature. The resin was collected by filtration and washed with DMSO (3 × 20 mL) and THF (2 × 15 mL), and MeOH (3 × 20 mL), respectively, and dried under vacuum to afford 4a–h. Diphosphitylation and coupling reactions were repeated several times with the same or different nucleosides to yield polymer-bound oligonucleotide diphosphate ditiester derivatives 5a–h (n=0–4, 11). IR (cm⁻¹): 5a (n=0): 3378 (OH), 2258 (CN), 1772 (C=O ester), 1644 (C=O, amide), 1030 (P-O-C); 5b (n=0): 3344 (OH), 2258 (CN), 1738 (C=O ester), 1644 (C=O, amide), 1026 (P-O-C); 5c (n=0): 3353 (OH), 2260 (CN), 1703 (C=O ester), 1646 (C=O, amide), 1025 (P-O-C); 5d (n=0): 3349 (OH), 2263 (CN), 1715 (C=O ester), 1650 (C=O, amide), 1029 (P-O-C); 5a (n=1): 3328 (OH), 2261 (CN), 1777 (C=O ester), 1647 (C=O, amide), 1029 (P-O-C); 5b (n=1): 3322 (OH), 2258 (CN), 1735 (C=O ester), 1649 (C=O, amide), 1033 (P-O-C); 5c (n=1): 3341 (OH), 2266 (CN), 1734 (C=O ester), 1648 (C=O, amide), 1026 (P-O-C); 5d (n=1): 3329 (OH), 2255 (CN), 1734 (C=O ester), 1641 (C=O, amide), 1030 (P-O-C); 5a (n=2): 3349 (OH), 2259 (CN), 1771 (C=O ester), 1643 (C=O, amide), 1025 (P-O-C); 5b (n=2): 3302 (OH), 2257 (CN), 1744 (C=O ester), 1650 (C=O, amide), 1029 (P-O-C); 5c (n=2): 3316 (OH), 2265 (CN), 1709 (C=O ester), 1649 (C=O, amide), 1027 (P-O-C); 5d (n=2): 3317 (OH), 2247 (CN), 1741 (C=O ester), 1658 (C=O, amide), 1030 (P-O-C); 5a (n=3): 3357 (OH), 2253 (CN), 1774 (C=O ester), 1646 (C=O, amide), 1029 (P-O-C); 5b (n=3): 3359 (OH), 2255 (CN), 1739 (C=O ester), 1653 (C=O, amide), 1030 (P-O-C); 5c (n=3): 3357 (OH), 2260 (CN), 1722 (C=O ester), 1646 (C=O, amide), 1025 (P-O-C); 5d (n=3): 3328 (OH), 2252 (CN), 1738 (C=O ester), 1646 (C=O, amide), 1033 (P-O-C); 5a (n=4): 3322 (OH), 2255 (CN), 1723 (C=O ester), 1648 (C=O, amide), 1025 (P-O-C); 5b (n=4): 3341 (OH), 2258 (CN), 1745 (C=O ester), 1651 (C=O, amide), 1033 (P-O-C); 5c (n=4): 3333 (OH), 2254 (CN), 1775 (C=O ester), 1648 (C=O, amide), 1029 (P-O-C); 5d (n=4): 3329 (OH), 2254 (CN), 1737 (C=O ester), 1648 (C=O, amide), 1026 (P-O-C); 5a (n=11): 3339 (OH), 2257 (CN), 1779 (C=O ester), 1646 (C=O, amide), 1031 (P-O-C); 5b (n=11): 3367 (OH), 2262 (CN), 1749 (C=O ester), 1646 (C=O, amide), 1024 (P-O-C); 5c (n=11): 3318 (OH), 2253 (CN), 1733 (C=O ester), 1661 (C=O, amide), 1027 (P-O-C); 5d (n=11): 3350 (OH), 2251 (CN), 1744 (C=O ester), 1649
Preparation of ODNs Containing Diphosphate Diester Derivatives (8–22) from Polymer-Bound Diphosphite Triester Precursors (5a–h, n=0–4, 11). 

D-Butyl hydroperoxide in decane (5-6 M, 4 eq) was added to the resins 5a–d (n=0–4, 11) in anhydrous THF (1 mL) and DMSO (4 mL) under extremely dry conditions and nitrogen. After 2 h shaking at room temperature, the resins were collected by filtration and washed with DMSO (3 × 15 mL), THF (3 × 15 mL) and MeOH (3 × 15 mL), respectively, and were dried overnight at room temperature under vacuum to give polymer-bound diphosphate triester 6a–h (n=0–4, 11). To the swelled resins 6a–h (n=0–4, 11) in anhydrous THF (1 mL) and DMSO (4 mL) was added DBU (2 eq) under extremely dry conditions and nitrogen. After 50 h shaking of the mixture at room temperature, the resins were collected by filtration and washed with DMSO (3 × 15 mL), THF (3 × 15 mL) and MeOH (3 × 15 mL), respectively, and were dried overnight at room temperature under vacuum to give polymer-bound diphosphate diester 7a–h (n=0–4, 11). FT-IR C≡N peaks were disappeared for all the polymer-bound diphosphodiesters. To the swelled resins 7a–h (n=0–4, 11) in anhydrous DCM (1 mL) was added DCM/TFA/water/1,2-ethanedithiol (23:72.5:2.5:2 v/v/v/v, 5 mL). After 25 min shaking of the mixtures at room temperature, the resins were collected by filtration and washed with DCM (2 × 10 mL), THF (2 × 10 mL), and MeOH (10 mL), respectively. The solvents of filtrate solutions were evaporated at -20 °C. The residues were mixed with Amberlite AG-50W-X8 (100-200 mesh, hydrogen form, 500 mg, 1.7 meq/g) in water:dioxane (75:25 v/v/v/v, 5 mL) for 30 min at -20 °C. After filtration, the solvents were evaporated and the crude products were purified using a C18 Sep-Pak cartridge and appropriate solvents. For 12-mer analogues, the solvents were evaporated and the crude products were repurified on HPLC as
explained above. After evaporation of organic solvents, the residues were lyophilized to yield **8-22**. The purity and total isolated yields for products are shown in Table S1.

**5’-O-Thymidine Diphosphate (dT, 8a).** HR-MS (ESI-TOF) (m/z): calcd, 402.0229; found, 402.0224 [M]+, 403.037 [M + 1]+; Anal. calcd, P 15.40%; found, 15.31%.

**5’-O-(2’-Deoxyadenosine) Diphosphate (dA, 8b).** HR-MS (ESI-TOF) (m/z): calcd, 411.0345; found 411.0577 [M]+, 411.9469 [M + 1]+; Anal. calcd, P 15.06%; found 14.89%.

**5’-O-(2’-Deoxyguanosine) Diphosphate (dG, 8c).** HR-MS (ESI-TOF) (m/z): calcd, 427.0294; found, 426.0298 [M - 1]; Anal. calcd, P 14.50%; found, 14.63%.

**5’-O-(2’-Deoxycytidine) Diphosphate (dC, 8d).** HR-MS (ESI-TOF) (m/z) calcd, 387.0233; found, 388.0219 [M + 1]+; Anal. calcd, P 16.00%; found, 15.92%.

**Modified d(TT) Containing Diphosphodiester Internucleotide Linkage (9a).** Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, δ ppm): -11.81 (s), -10.34 (s), -8.55 (s), 3.12 (s, terminal phosphorus); HR-MS (ESI-TOF) (m/z): calcd, 786.0353; found, 786.3072 [M]+, 787.4440 [M + 1]+; Anal. calcd, P 15.76%; found, 15.71%.

**Modified d(AA) Containing Diphosphodiester Internucleotide Linkage (9b).** Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, δ ppm): -17.36 (s), -15.84 (s), -12.24 (s), 1.96 (s, terminal phosphorus); HR-MS (ESI-TOF) (m/z): calcd, 804.0584; found, 805.0576 [M + 1]+; Anal. calcd, P 15.40%; found, 15.28%.
Modified d(GG) Containing Diphosphodiester Internucleotide Linkage (9c). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -14.31 (s), -13.77 (s), -12.98 (s), 2.86 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 836.0483; found, 836.9737 [M + 1]$^+$; Anal. calcd, P 14.81%; found, 15.03%.

Modified d(CC) Containing Diphosphodiester Internucleotide Linkage (9d). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -17.61 (s), -14.21 (s), -10.56 (s), 2.56 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 756.036; found, 756.7475 [M + 1]$^+$; Anal. calcd, P 16.38%; found, 16.33%.

Modified d(TTT) Containing Diphosphodiester Internucleotide Linkage (10a). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -13.71 (s), -12.23 (s), -11.77 (s), -10.29 (s), -9.30 (s), 2.37 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 1184.0633; found, 1185.0637 [M + 1]$^+$; Anal. calcd, P 15.69%; found, 15.87%.

Modified d(AAA) Containing Diphosphodiester Internucleotide Linkage (10b). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -18.93 (s), -18.51 (s), -15.35 (s), -10.10 (s), -9.69 (s), 1.79 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 1211.0980; found, 1228.0290 [M + H$_2$O -1]$^-$. Anal. calcd, P 15.34%; found 15.46%.

Modified d(GGG) Containing Diphosphodiester Internucleotide Linkage (10c). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -15.16 (s), -15.00 (s), -14.98 (s), -14.98 (s), -12.79 (s), -11.89 (s), 3.27 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$) calcd, 1259.0828; found 1258.0618 [M - 1]$^-$; Anal. calcd, P 14.75%; found, 15.67%.
Modified d(CCC) Containing Diphosphodiester Internucleotide Linkage (10d). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -18.10 (s), -16.06 (s), -14.00 (s), -12.16 (s), -11.04 (s), 2.08 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 1139.0643; found, 1138.0715 [M - 1]*; Anal. calcd, P 16.31%; found, 16.22%.

Modified d(TTTT) Containing Diphosphodiester Internucleotide Linkage (11a). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -14.23 (s), -12.92 (s), -12.02 (s), -10.72 (s), -10.37 (s), -10.10 (s), -9.06 (s), 3.20 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 1554.0600, found, 1553.0285 [M - 1]*; Anal. calcd, P 15.94%; found, 15.87%.

Modified d(AAAA) Containing Diphosphodiester Internucleotide Linkage (11b). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -17.71 (s), -17.00 (s), -16.32 (s), -14.15 (s), -12.60 (s), -12.17 (s), -9.97 (s), 3.00 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 1590.1063; found, 1589.1103 [M - 1]*; Anal. calcd, P 15.58%; found, 15.72%.

Modified d(GGGG) Containing Diphosphodiester Internucleotide Linkage (11c). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -17.46 (s), -15.09 (s), -14.20 (s), -11.81 (s), -11.67 (s), -11.47 (s), -10.77 (s), 2.10 (s, terminal phosphorus); HR-MS (ESI-TOF) ($m/z$): calcd, 1654.0860; found, 1652.9015 [M - 1]*; Anal. calcd, P 14.97%; found, 15.12%.

Modified d(CCCC) Containing Diphosphodiester Internucleotide Linkage (11d). Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -17.68 (s), -14.84
Modified d(TTTTT) Containing Diphosphodiester Internucleotide Linkage (12a). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 1.75–1.79 (br s, 5-CH$_3$, 15H), 2.20–2.44 (m, H-2' and H-2'', 10H), 3.49–3.70 (m, H-5' and H-5'', 10H), 3.74–3.84 (m, H-4', 5H), 4.20–4.30 and 4.35–4.45 (m, H-3', 5H), 4.95–5.09 (m, OH, 2H), 5.15–5.31 (m, OH, 5H), 6.07–6.14 and 6.15–6.22 (m, H-1', 5H), 7.68–7.70 and 7.70–7.72 (br s, H-6, 5H), 11.14–11.40 (br s, NH, 5H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz, $\delta$ ppm): 13.73 (5-CH$_3$), 41.20 (C-2'), 63.27 (C-5'), 72.50 (C-3'), 89.41, 85.99 (C-4', C-1'), 111.84 (C-5), 138.80 (C-6), 153.27 (C-2 C=O), 166.74 (C-4 C=O); Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -15.78 (s), -14.68 (s), -13.58 (s), -12.80 (s), -11.75 (s), -10.65 (s), -9.87 (s), -8.80 (s), -8.29 (s), 2.29 (s, terminal phosphorus); HR-MS (ESI-TOF) (m/z): calcd, 1952.0881; found, 1953.8401 [M + 1]$^+$; Anal. calcd, P 15.86%; found, 16.05%.

Modified d(AAAAA) Containing Diphosphodiester Internucleotide Linkage (12b). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 2.25–2.43 (m, H2', 5H ), 2.70–2.85 (m, H2'', 5H), 3.50–3.75 (m, H-5', 10H), 3.95–4.05 (m, H-4', 5H), 4.45–4.55 (m, H-3', 5H), 5.40–5.65 (m, OH, 10H), 6.35–6.50 (m, H-1', 5H), 7.45–7.60 (br s, 6-NH$_2$, 10H), 8.19–8.23 (br s, H-2, 5H), 8.36–8.40 (br s, H-8, 5H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz, $\delta$ ppm): 40.40 (C-2'), 85.07 (C-1'), 62.75 (C-5'), 71.87 (C-3'), 88.87 (C-4'), 120.11 (C-5), 140.65 (C-8), 149.65 (C-4), 153.29 (C-2), 156.88 (C-6); Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -20.20 (s), -17.36 (s), -17.07 (s), -16.11 (s), -16.07 (s), -12.18 (s), -11.79 (s), -11.18 (s), -11.18 (s), 2.07 (s, terminal phosphorus); HR-MS (ESI-TOF) (m/z) calcd, 1997.1459; found, 1998.1764 [M + 1]$^+$; Anal. calcd, P 15.50%; found, 15.39%.
Modified d(GGGGG) Containing Diphosphodiester Internucleotide Linkage (12c). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 2.15–2.25 (m, H2’, 5H), 2.45–2.58 (m, H2”, 5H), 3.50–3.60 (m, H-5 and H-5”, 10H), 3.75–3.85 (m, H-4’, 5H), 4.30–4.40 (m, H-3’, 5H), 4.95–5.05 (m, OH, 5H), 5.27–5.30 (m, OH, 5H), 6.05–6.15 (m, H-1’, 5H), 6.40–6.60 (br s, 6-NH$_2$, 10H), 7.85–8.00 (br s, H-8, 5H), 10.70–10.75 (br s, NH, 5H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz, $\delta$ ppm): 40.39 (C-2’), 62.54 (C-5’), 71.59 (C-3’), 83.42 (C-1’), 88.41 (C-4’), 117.44 (C-5), 136.22 (C-8), 151.75 (C-4), 154.50 (C-2), 157.67 (C-6); Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -19.46 (s), -19.33 (s), -16.30 (s), -16.20 (s), -15.23 (s), -11.67 (s), -10.91 (s), -10.84 (s), -10.20 (s), 2.78 (s, terminal phosphorus); HR-MS (ESI-TOF) (m/z) calcd, 2077.1205; found, 2076.6367 [M - 1]; Anal. Calcd, P 14.91%; found, 15.13%.

Modified d(CCCCC) Containing Diphosphodiester Internucleotide Linkage (12d). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 2.10–2.50 (m, H-2’ and H-2”, 10H), 3.45–3.65 (m, H-5’ and H-5”, 10H), 3.75–3.86 (m, H-4’, 5H), 4.15–4.27 (m, H-3’, 5H), 5.15–5.75 (m, OH, 10H), 5.98–6.05 (m, H-1’, 5H), 6.17–6.24 (m, H-5, 5H), 8.17–8.32 (m, H-6, 5H), 8.70–8.83 (m, NH, 5H), 9.85–10.10 (br s, NH, 5H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz, $\delta$ ppm): 41.08 (C-2’), 61.49 (C-5’), 70.54 (C-3’), 88.86, 86.67 (C-4’, C-1’), 94.55 (C-5), 145.30 (C-6), 147.64 (C-2), 160.25 (C-4); Decoupled $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -17.46 (s), -17.06 (s), -14.48 (s), -14.40 (s), -14.33 (s), -13.19 (s), -11.57 (s), -11.50 (s), -11.09 (s), 2.86 (s, terminal phosphorus); HR-MS (ESI-TOF) (m/z): calcd, 1877.0897; found, 1878.0569 [M + 1]$^+$; Anal. calcd, P 16.49%; found, 16.61%.

Modified d(AAAAAAAAAAAAAA) Containing Diphosphodiester Internucleotide Linkage (13). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 2.10–2.35 (m, H2’, 12H), 2.60–2.80 (m, H2”, 12H), 3.50–3.70 (m, H-5’, H5”, 24H), 3.75–3.95 (m, H-4’, 12H), 4.25–4.45 (m, H-3’, 12H), 5.10–5.40 (m, OH, 24H),
6.20–6.50 (m, H-1', 12H), 7.20–7.50 (br s, 6-NH₂, 24H), 8.05–8.20 (br s, H-2, 12H), 8.30–8.40 (br s, H-8, 12H); \(^{31}\)P NMR (in DMSO-\(d_6\) and H₃PO₄ 85\% in water as external standard, 162 MHz, \(\delta\) ppm): -16.20 to -13.00 (m, 23P), 1.41 (d, \(J = 16.2\) Hz, terminal phosphorus, 1P); MALDI-TOF (\(m/z\)): calcd, 4734.3; found, 4734.2 [M]+, 4757.0 [M + Na]+; Anal. calcd, P 15.70\%; found, 15.91\%.

**Modified d(TTTTTTTTTTTTT) Containing Diphosphodiester Internucleotide Linkage (14).** \(^1\)H NMR (DMSO-\(d_6\), 400 MHz, \(\delta\) ppm): 1.75–1.79 (br s, 5-C₃H₃, 36H), 2.20–2.44 (m, H-2' and H-2'', 24H), 3.49–3.70 (m, H-5' and H-5'', 24H), 3.74–3.84 (m, H-4', 12H), 4.20–4.30 and 4.35–4.45 (m, H-3', 12H), 4.95–5.09 (m, OH, 5H), 5.15–5.31 (m, OH, 5H), 6.07–6.14 and 6.15–6.22 (m, H-1', 12H), 7.67–7.71 and 7.69–7.73 (br s, H-6, 12H), 11.14–11.40 (br s, NH, 12H); \(^{31}\)P NMR (in DMSO-\(d_6\) and H₃PO₄ 85\% in water as external standard, 162 MHz, \(\delta\) ppm): -15.2 to -12.90 (m, 23P), 1.29 (d, \(J = 14.6\) Hz, terminal phosphorus, 1P); MALDI-TOF (\(m/z\)): calcd, 4626.2; found, 4626.9 [M+1]+; Anal. calcd, P 16.06\%; found, 15.87\%.

**Modified d(CCCCCCCCCCCCC) Containing Diphosphodiester Internucleotide Linkage (15).** \(^1\)H NMR (DMSO-\(d_6\), 400 MHz, \(\delta\) ppm): 2.09–2.40 (m, H-2' and H-2'', 24H), 3.45–3.65 (m, H-5' and H-5'', 24H), 3.79–3.86 (m, H-4', 12H), 4.18–4.24 (m, H-3', 12H), 4.95–5.80 (m, OH, 24H), 5.96–6.08 (m, H-1', 12H), 6.16–6.23 (m, H-5, 12H), 8.20–8.30 (m, H-6, 12H), 8.70–8.83 (m, NH, 12H), 9.85–10.10 (br s, NH, 12H); \(^{31}\)P NMR (in DMSO-\(d_6\) and H₃PO₄ 85\% in water as external standard, 162 MHz, \(\delta\) ppm): -16.70 to -14.85 (m, 23P), 1.64 (d, \(J = 16.2\) Hz, terminal phosphorus, 1P); MALDI-TOF (\(m/z\)): calcd, 4446.2; found, 4446.9 [M + 1]+; Anal. calcd, P 16.71\%; found, 16.63\%.

**Modified d(GGGGGGGGGGGGG) Containing Diphosphodiester Internucleotide Linkage (16).** \(^1\)H NMR (DMSO-\(d_6\), 400 MHz, \(\delta\) ppm): 2.15–2.23 (m, H₂', 12H ), 2.43–2.54 (m, H₂'', 12H), 3.50–3.60 (m,
Modified d(ATATATATATAT) Containing Diphosphodiester Internucleotide Linkage (17). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 1.60–1.84 (br s, 5-CH$_3$, dT, 18H), 2.20–2.80 (m, H-2' and H-2'', dA, dT, 24 H), 3.49–3.74 (m, H-5' and H-5'', dA, dT, 24H), 3.74–3.79 (m, H-4', dT, 6H), 3.79–3.92 (m, H-4', dA, 6H), 4.20–4.27 (m, H-3', dT, 6H), 4.40–4.45 (m, H-3', dA, 6H), 5.02–5.09 (m, OH, dT, 6H), 5.22–5.40 (m, OH, dA, dT, 16H), 6.14–6.21 (m, H-1', dT, 6H), 6.27–6.40 (m, H-1', dA, 6H), 7.20–7.40 (br s, 6-NH$_2$, dA, 12H), 7.69–7.73 (br s, H-6, dT, 6H), 8.12–8.17 (br s, H-2, dA, 6H), 8.33–8.38 (br s, H-8, dA, 6H), 11.20–11.40 (br s, NH, dT, 6H); $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -18.11 (s), -17.36 (s), -18.05 to -16.54 (m, 23P), 1.93 (d, $J = 14.6$ Hz, terminal phosphorus, 1P); MALDI-TOF (m/z): calcd, 4680.2; found, 4681.0 [M+1]$^+$; Anal. calcd, P 15.88%; found, 15.72%.

Modified d(TATATATATATA) Containing Diphosphodiester Internucleotide Linkage (18). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 1.60–1.84 (br s, 5-CH$_3$, dT, 18H), 2.20–2.80 (m, H-2' and H-2'', dA, dT, 24 H), 3.49–3.74 (m, H-5' and H-5'', dA, dT, 24H), 3.74–3.79 (m, H-4', dT, 6H), 3.79–3.92 (m, H-4', dA, 6H), 4.20–4.27 (m, H-3', dT, 6H), 4.39–4.45 (m, H-3', dA, 6H), 5.02–5.09 (m, OH, dT, 6H), 5.20–5.40 (m, OH, dA, dT, 16H), 6.13–6.21 (m, H-1', dT, 6H), 6.27–6.40 (m, H-1', dA, 6H), 7.19–7.40 (br s, 6-NH$_2$, dA, 12H), 7.63–7.75 (br s, H-6, dT, 6H), 8.10–8.17 (br s, H-2, dA, 6H), 8.31–8.38 (br s, H-8, dA, 6H), 11.20–11.40 (br s, NH, dT, 6H); $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -17.36 (s), -18.05 to -16.54 (m, 23P), 1.39 (d, $J = 16.2$ Hz, terminal
phosphorus, 1P); MALDI-TOF (m/z): calcd, 4680.2; found, 4681.0 [M + 1]+; Anal. calcd, P 15.88% found, 16.09%.

**Modified d(CGCGCGCGCGCG) Containing Diphosphodiester Internucleotide Linkage (19).** $^1$H NMR (DMSO-$d_6$, 400 MHz, δ ppm): 2.10–2.58 (m, H-2' and H-2", dG, dC, 24H), 3.45–3.65 (m, H-5'and H-5", dG, dC, 24H), 3.75–3.90 (m, H-4', dG, dC, 12H), 4.18–4.29 (m, H-3', dC, 6H), 4.30–4.40 (m, H-3', dG, 6H), 4.60–5.80 (m, OH, dG, dC, 20H), 5.98–6.20 (m, H-1', H-5, dC; H-1', dG, 18H), 6.40–6.80 (br s, 6-NH$_2$, dG, 12H), 7.85–8.10 (br s, H-8, dG, 6H), 8.10–8.32 (m, H-6, dC, 6H), 8.50–8.85 (m, NH, dC, 6H), 9.40–9.80 (br s, NH, dC, 6H), 10.90–11.20 (br s, dG, NH, 6H); $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, δ ppm): -18.25 to -14.71 (m, 23P), 1.77 (d, $J = 16.2$ Hz, terminal phosphorus, 1P); MALDI-TOF (m/z): calcd, 4686.2; found, 4687.2 [M + 1]+; Anal. calcd, P 15.86%; found, 15.74%.

**Modified d(GCGCGCGCGCGC) Containing Diphosphodiester Internucleotide Linkage (20).** $^1$H NMR (DMSO-$d_6$, 400 MHz, δ ppm): 2.10–2.58 (m, H-2' and H-2", dG, dC, 24H), 3.45–3.65 (m, H-5'and H-5", dG, dC, 24H), 3.75–3.90 (m, H-4', dG, dC, 12H), 4.18–4.29 (m, H-3', dC, 6H), 4.30–4.40 (m, H-3', dG, 6H), 4.60–5.80 (m, OH, dG, dC, 20H), 5.98–6.21 (m, H-1', H-5, dC; H-1', dG, 18H), 6.58–6.80 (br s, 6-NH$_2$, dG, 12H), 7.85–8.10 (br s, H-8, dG, 6H), 8.10–8.32 (m, H-6, dC, 6H), 8.50–8.85 (m, NH, dC, 6H), 9.40–9.80 (br s, NH, dC, 6H), 10.89–11.19 (br s, dG, NH, 6H); $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, δ ppm): -19.38 to -14.05 (m, 23P), 1.83 (d, $J = 16.2$ Hz, terminal phosphorus, 1H); MALDI-TOF (m/z): calcd, 4686.2; found, 4687.3 [M + 1]+; Anal. calcd, P 15.86%, found, 16.12%.
Modified d(AACCTGATTGCA) Containing Diphosphodiester Internucleotide Linkage (21). $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 1.72 (s, 5-CH$_3$, dT, 3H), 1.74–1.80 (br s, 5-CH$_3$, dT, 6H), 2.00–2.12, 2.12–2.35, 2.60–2.82 (m, H-2' and H-2'', dA, dC, dT, dG, 24H), 3.42–3.69 (m, H-5'and H-5''), dG, dC, dA, dT, 24H), 3.70–3.93 (m, H-4', dG, dC, dA, dT, 12H), 4.16–4.29 (m, H-3', dC, dT, 6H), 4.30–4.43 (m, H-3', dG, dA, 6H), 4.60–5.90 (m, O-H, dG, dC, dT, dA, 27H), 6.00–6.21 (m, H-1', H-5, dC; H-1', dG, dT, 9H), 6.21–6.43 (m, H-1', dT, dA, 6H), 6.57–6.78 (m, 6-NH$_2$, dG, 4H), 7.42–7.62 (br s, 6-NH$_2$, dA, 8H), 7.62–7.75 (m, H-6, dT, 3H), 7.86–8.00 (m, H-8, dG, 2H), 8.07–8.25 (m, H-6, dC; H-2, dA, 7H), 8.30–8.33 (br s, H-8, dA, 4H), 8.34–8.50 (m, NH$_2$, dC, 6H), 9.20–9.48 (br s, NH, dC), 10.75–11.00 (br s, dG, NH), 11.20–11.45 (m, NH, dT, 3H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz, $\delta$ ppm): 12.86 (5-CH$_3$, dT), 40.01–41.01 (C-2', dA, dC, dG, dT), 61.40, 61.90, 62.41 (C-5', dA, dC, dG, dT), 70.41, 71.02, 71.29, 71.50 (C-3', dA, dC, dG, dT), 83.22, 84.32, 84.55, 86.26, 87.84, 88.16, 88.48, 88.61 (C-4' and C-1', dA, dC, dG, dT), 94.41 (C-5, dC), 109.95 (C-5, dT), 119.75 (C-5, dG, dA), 135.86 (C-8, dG), 136.73 (C-6, dT), 140.46 (C-8, dA), 144.41 (C-6, dC), 149.06 (C-2, dC), 149.35 (C-4, dA), 151.06 (C-4, dG), 151.42 (C-2, dA), 152.08 (C-2 C=O, dT), 154.40 (C-2, dG), 155.98 (C-6, dA), 157.30 (C-6, dG), 161.01 (C-4, dC), 164.36 (C-4 C=O, dT); $^{31}$P NMR (in DMSO-$d_6$ and H$_3$PO$_4$ 85% in water as external standard, 162 MHz, $\delta$ ppm): -15.09 to -11.05 (m, 23P), 2.11-2.19 (m, terminal phosphorus, 1H); MALDI-TOF (m/z): calcd, 4667.2; found, 4668.6 [M + 1]$^+$; Anal. calcd, P 15.92%, found, 16.21%.

**Modified d(TGCAATCAGGTT) Containing Diphosphodiester Internucleotide Linkage (22).** $^1$H NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 1.80 (s, 5-CH$_3$, dT, 3H), 1.82–1.88 (br s, 5-CH$_3$, dT, 6H), 2.08–2.20, 2.20–2.42, 2.70–2.85 (m, H-2' and H-2'', dA, dC, dT, dG, 24H), 3.52–3.78 (m, H-5'and H-5''), dG, dC, dA, dT, 24H), 3.78–4.20 (m, H-4', dG, dC, dA, dT, 12H), 4.22–4.40 (m, H-3', dC, dT, 6H), 4.40–4.58 (m, H-3', dG, dA, 6H), 4.90–5.95 (m, O-H, dG, dC, dT, dA, 27H), 6.10–6.32 (m, H-1', H-5, dC; H-1', dG, dT, 10H), 6.35–6.52 (m, H-1', dT, dA, 4H), 6.68–6.88 (m, 6-NH$_2$, dG, 4H), 7.58–7.72 (br s, 6-
NH₂, dA, 6H), 7.72–7.90 (m, H-6, dT, 4H), 8.00–8.15 (m, H-8, dG, 3H), 8.21–8.40 (m, H-6, dC; H-2, dA, 5H), 8.44–8.48 (br s, H-8, dA, 3H), 8.48–8.54 (m, NH₂, dC, 4H), 9.40–9.53 (br s, NH, dC), 10.90–11.15 (br s, dG, NH), 11.32–11.52 (m, NH, dT, 4H); ¹³C NMR (DMSO-d₆, 100 MHz, δ ppm): 12.88 (5-CH₃, dT), 40.01–41.01 (C-2', dA, dC, dG, dT), 61.71, 62.21, 62.73 (C-5', dA, dC, dG, dT), 70.77, 71.39, 71.66, 71.87 (C-3', dA, dC, dG, dT), 83.66, 84.76, 84.99, 86.71, 88.31, 88.64, 88.95, 89.08 (C-4' and C-1', dA, dC, dG, dT), 94.92 (C-5, dC), 110.55 (C-5, dT), 120.41 (C-5, dG, dA), 136.62 (C-8, dG), 137.49 (C-6, dT), 141.24 (C-8, dA), 145.22 (C-6, dC), 149.89 (C-2, dC), 150.19 (C-4, dA), 151.91 (C-4, dG), 152.93 (C-2, dA), 155.27 (C-2 C=O, dT), 156.86 (C-2, dG), 156.96 (C-6, dA), 158.18 (C-6, dG), 161.92 (C-4, dC), 165.28 (C-4 C=O, dT); ³¹P NMR (in DMSO-d₆ and H₃PO₄ 85% in water as external standard, 162 MHz, δ ppm): -18.30 to -7.05 (m, 23P), 1.90–1.97 (m, terminal phosphorus, 1H); MALDI-TOF (m/z): calcd, 4698.2; found, 4698.9 [M + 1]⁺, 4700.1 [M + 2]⁺; Anal. calcd, P 15.82%, found, 15.94%.

7. Preparation of Modified ODNs, d(AAAAA) (12b) and d(CCCCC) (12d), Containing Diphosphate Diester Internucleotide Linkages Using Protected Nucleosides. To further confirm the chemical structures of the synthesized compounds, two representative modified ODNs, d(AAAAA) (12b) and d(CCCCC) (12d), were also synthesized by using differentially protected nucleoside building blocks, 3’-O-(1,1-dimethyl)dimethylsilyl-N6-(9-fluorenylmethyloxycarbonyl)-2’-deoxyadenosine (a) and 3’-O-(1,1-dimethyl)dimethylsilyl-N4-(9-fluorenylmethyloxycarbonyl)-2’-deoxycytidine (b) (Scheme S2). The hydroxyl groups (5’- and 3’-hydroxyl groups) in 2’-deoxyadenosine and 2’-deoxycytidine were protected by tert-butyldimethylsilyl (TBDMS) by the reaction of the unprotected nucleosides with tert-butyldimethylsilyl chloride in the presence of imidazole in DMF as described previously. The protection of free exocyclic amino groups with 9-fluorenylmethyloxycarbonyl chloride (Fmoc-chloride)
in the presence of sodium carbonate in dioxane,\cite{33} followed by selective removal of 5′-O-TBDMS group in the presence of AcOH/H₂O/THF (13:7:3 v/v/v)\cite{34} afforded a and b.

The protected nucleosides (a and b) were attached to polymer-bound diphosphitylating reagent 1 through 5′-hydroxyl group in the presence of 1H-tetrazole under extremely dry conditions and nitrogen to afford 21a and 21b. The deprotection of 3′-O-TBDMS group in 21a and 21b with tetrabutylammonium fluoride (TBAF) in THF at room temperature, followed by diphosphitylation of 3′-hydroxyl group with diphosphitylating reagent in the presence of 1H-tetrazole under extremely dry conditions and nitrogen afforded 23a and 23b. The coupling, deprotection of TBDMS group, and diphosphitylation reactions were repeated several times to yield polymer-bound Fmoc-protected oligonucleotide diphosphate triester derivatives 25a and 25b. Oxidation with t-butyl hydroperoxide produced polymer-bound diphosphate triesters 26a and 26b. The removal of Fmoc and 2-cyanoethoxy groups was carried out in the presence of 20% piperidine and DBU, respectively, to produce polymer-bound diphosphate diester (28a and 28b). After the cleavage of final products from the solid support in the presence of DCM/TFA/H₂O/1,2-ethanedithiol, the residues were desalted with Amberlite AG-50W-X8 (100-200 mesh, hydrogen form) and purified by HPLC to afford 12b and 12d. Comparison of the NMR data (\(^{31}\)P NMR, \(^{1}\)H NMR, and \(^{13}\)C NMR) and high-resolution time-of-flight electrospray mass spectrometry of the synthesized ODNs using the unprotected and protected nucleosides indicated that the compounds produced by both methods were identical (See pages 34-36 and 39-42 for comparing NMR spectra).
Scheme S2. Synthesis of modified ODNs containing diphosphate diester internucleotide linkages using protected nucleosides. Reagents: (a) Protected nucleosides (a or b), THF/DMSO, 1H-tetrazole; (b) TBAF, THF (c) [(i-Pr)₂NPOCH₂CH₂CN]₂O, THF/DMSO, 1H-tetrazole; (d) tBuOOH, THF; (e) 20% piperidine/DMF; (f) DBU, THF; (g) DCM/TFA/water/1,2-ethanedithiol (23:72.5:2.5:2 v/v/v/v).

Preparation of Polymer-Bound Diphosphate Triesters (25a, 25b). Protected nucleosides (a and b) (1.28 mmol, 4 eq) and 1H-tetrazole (45 mg, 0.64 mmol, 2 eq) were added to polymer-bound diphosphitylating reagent[21] (1, 433 mg, 0.74 mmol/g, 1eq) in anhydrous THF (1 mL) and DMSO (4 mL) under extremely dry conditions and nitrogen. The mixture was shaken for 24 h at room temperature. The resins were collected by filtration and washed with DMSO (3 × 20 mL), THF (2 × 15 mL), and MeOH (3 × 20 mL), respectively, and dried under vacuum to give 21a and 21b. 3′-TBDMS group in 21a and 21b was deprotected with TBAF/THF (0.2 M, 5 mL) at room temperature for 1 h. The resins were collected
by filtration and washed with THF (3 × 15 mL) and MeOH (3 × 20 mL), respectively, and dried under vacuum to yield 22a and 22b. Diphosphitylation of 3′-hydroxyl group with the diphosphitylating reagent (4 eq), bis(2-cyanoethyl diisopropylphosphoramidite) ([[(i-Pr)2NPOCH2CH2CN]2O],[21] was carried out in anhydrous THF (15 mL) in the presence of 1H-tetrazole (45 mg, 0.64 mmol, 2 eq) under extremely dry conditions and nitrogen. The mixtures were shaken at room temperature for 24 h. The resins were collected by filtration and washed with THF (2 × 15 mL) and MeOH (3 × 20 mL), respectively, and dried under vacuum to afford 23a and 23b. Polymer-bound compounds 23a and 23b were subjected with reactions with protected nucleosides (a and b) in anhydrous THF (1 mL) and DMSO (4 mL) in the presence of 1H-tetrazole (45 mg, 0.64 mmol, 2 eq) under extremely dry conditions and nitrogen. The mixtures were shaken for 24 h at room temperature. The resins were collected by filtration and washed with DMSO (3 × 20 mL), THF (2 × 15 mL), and MeOH (3 × 20 mL), respectively, and dried under vacuum to yield polymer-bound protected oligonucleotide diphosphate triester derivatives of 24a and 24b. Deprotection of TBDMS group, diphosphitylation, and coupling reactions were repeated several times to afford 25a and 25b.

Preparation of d(AAAAA) (12b) and d(CCCCC) (12d) from Polymer-Bound Fmoc-protected Diphosphite Triester Precursors (25a and 25b). t-Butyl hydroperoxide in decane (5-6 M, 4 eq) was added to the resins 25a and 25b in THF (1 mL) and DMSO (4 mL) under extremely dry conditions and nitrogen. After 2 h shaking at room temperature, the resin were collected by filtration and washed with DMF (3 × 15 mL), THF (3 × 15 mL), and MeOH (3 × 15 mL), respectively, and were dried overnight at room temperature under vacuum to give polymer-bound diphosphate triester 26a and 26b. To the swelled resins 26a and 26b in DMF was added 20% piperidine in DMF (4 mL) and the mixtures were shaken at room temperature for 5 min. The resins were collected by filtration and washed with DMSO (3 × 15 mL), THF (3 × 15 mL) and MeOH (3 × 15 mL), respectively, and were dried overnight at room temperature under vacuum to give polymer-bound diphosphate triester 27a and 27b. To the swelled resins 27a and
27b in THF (1 mL) and DMSO (4 mL) was added DBU (2 eq). After 50 h shaking of the mixture at room temperature, the resins were collected by filtration and washed with DMSO (3 × 15 mL), THF (3 × 15 mL) and MeOH (3 × 15 mL), respectively, and were dried overnight at room temperature under vacuum to give polymer-bound diphosphate diester 28a and 28b. To the swelled resins 28a and 28b in anhydrous DCM (1 mL) was added DCM/TFA/water/1,2-ethanedithiol (23:72.5:2.5:2 v/v/v/v, 5 mL). After 25 min shaking of the mixtures at room temperature, the resins were collected by filtration and washed with DCM (2 × 10 mL), THF (2 × 10 mL), and MeOH (10 mL), respectively. The solvents of filtrate solutions were evaporated at -20 °C. The residues were mixed with Amberlite AG-50W-X8 (100-200 mesh, hydrogen form, 500 mg, 1.7 meq/g) in water:dioxane (75:25 v/v, 5 mL) for 30 min at -20 °C. After filtration, the solvents were evaporated. The purities of crude products were 84% and 89% for d(AAAAA) and d(CCCCC), respectively. The crude products were purified on HPLC as explained in general information. After evaporation of organic solvents, the residues were lyophilized to yield d(AAAAA) (12b, yield from 181%) and d(CCCCC) (12d, yield from 170%) (See pages 34-36 and 39-42 for comparing NMR spectra).

8. 31P NMR, 1H NMR, and/or 13C NMR spectra of ODNs Containing Diphosphodiester Internucleotide Linkages (9–22).
$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)

$\delta(TT)$

9a

$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)

$\delta(AA)$

9b
$^{31}$P NMR (decoupled) (162 MHz, DMSO-$d_6$)

d(GG)

d(CC)

2.86
-12.98
-13.77
-14.31

2.56
-10.56
-14.21
-17.61
$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)

$d$(TTT)

10a

$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)

$d$(AAA)

10b
$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)
d(GGG)

$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)
d(CCC)

29
$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)

d(TTTT)

11a

$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)

d(AAAA)

11b
$^1$H NMR
(400 MHz, DMSO-$d_6$)

$d($TTTTT$)$

12a

$^{13}$C NMR
(100 MHz, DMSO-$d_6$)

$d($TTTTT$)$

12a
$^{31}$P NMR (decoupled)
(162 MHz, DMSO-$d_6$)

d(TTTTT)

12a
d(AAAAA) (12b) synthesized using unprotected adenosine.

$^1$H NMR (400 MHz, DMSO-$d_6$)

$^1$H NMR
(400 MHz, DMSO-$d_6$)

d(AAAAA)

12b

$^{13}$C NMR (100 MHz, DMSO-$d_6$)

d(AAAAA)

12b

$^{13}$C NMR
(100 MHz, DMSO-$d_6$)
d(AAAAA) (12b) synthesized using unprotected adenosine.

\[ ^{31}P \text{ NMR (decoupled)} \]
\[ (162 \text{ MHz, DMSO-}d_{6}) \]
\[ d(\text{AAAAA}) \]
\[ 12b \]

d(AAAAA) (12b) synthesized using protected nucleoside a.

\[ ^{1}H \text{ NMR} \]
\[ (400 \text{ MHz, DMSO-}d_{6}) \]
\[ d(\text{AAAAA}) \]
d(AAAAA) (12b) synthesized using protected nucleoside a.

$^{13}$C NMR (100 MHz, DMSO-$d_6$)

$^{31}$P NMR (decoupled) (162 MHz, DMSO-$d_6$)
$^{31}$P NMR (decoupled) (162 MHz, DMSO-$d_6$) 

$d$(GGGGG) 

12c
d(CCCCC) (12d) synthesized using the unprotected cytidine.

\[^1\text{H} \text{ NMR} (400 \text{ MHz, DMSO-}d_6)\]

\[^{13}\text{C} \text{ NMR} (100 \text{ MHz, DMSO-}d_6)\]

\(d(\text{CCCCC})\)

12d
d(CCCCC) (12d) synthesized using unprotected cytidine.
d(CCCCC) (12d) synthesized using protected nucleoside b.

$^1\text{H NMR}$

(400 MHz, DMSO-$d_6$)

$^1\text{H NMR}$

(d(CCCCC))

$^1\text{H NMR}$

(400 MHz, DMSO-$d_6$)

$^1\text{H NMR}$

(d(CCCCC))

$^{13}\text{C NMR}$

(100 MHz, DMSO-$d_6$)

$^{13}\text{C NMR}$

(d(CCCCC))

$^{13}\text{C NMR}$

(100 MHz, DMSO-$d_6$)

$^{13}\text{C NMR}$

(d(CCCCC))
d(CCCCC) (12d) synthesized using protected nucleoside b.

$^{31}$P NMR (decoupled)  
(162 MHz, DMSO-$d_6$)
$^1$H NMR
(400 MHz, DMSO-$d_6$)

$^3$P NMR
(162 MHz, DMSO-$d_6$)
$^1$H NMR
(400 MHz, DMSO-$d_6$)

d(CCCCCCCCCCCC)

$^{31}$P NMR
(162 MHz, DMSO-$d_6$)

d(CCCCCCCCCCCC)

15
$^1$H NMR
(400 MHz, DMSO-$d_6$)

$d(\text{GGGGGGGGGGGG})$

$^{31}$P NMR
(162 MHz, DMSO-$d_6$)

$d(\text{GGGGGGGGGGGG})$
$^1$H NMR
(400 MHz, DMSO-$d_6$)

d(ATATATATATAT)

17

$^{31}$P NMR
(162 MHz, DMSO-$d_6$)

d(ATATATATATAT)

17
$^1$H NMR
(400 MHz, DMSO-$d_6$)

d(TATATATATATA)

$^{31}$P NMR
(162 MHz, DMSO-$d_6$)

d(TATATATATATA)
$^1$H NMR  
(400 MHz, DMSO-$d_6$)  
d(CGCGCGCGCGCG)  

$^{31}$P NMR  
(162 MHz, DMSO-$d_6$)  
d(CGCGCGCGCGCG)
$^1$H NMR
(400 MHz, DMSO-$d_6$)
d(GCGCGCGCGCGC)

$^{31}$P NMR
(162 MHz, DMSO-$d_6$)
d(GCGCGCGCGCGC)


\[ ^1\text{H NMR} \]
\[ (400 \text{ MHz, DMSO-}\text{d}_6) \]
\[ d(AACCTGATTGCA) \]

\[ ^{13}\text{C NMR} \]
\[ (100 \text{ MHz, DMSO-}\text{d}_6) \]
\[ d(AACCTGATTGCA) \]
9. *T*<sub>m</sub> Measurements and Thermodynamic Parameters. Melting points (*T*<sub>m</sub> values) for the ODN duplexes were determined on a Beckman Coulter DU 800 UV/Visible Spectrophotometer equipped with a Peltier Temperature Controller. The absorbance at 260 nm was measured, while the temperature of the sample was increased or decreased at a rate of 1.0 °C/min. The percent hyperchromicity at 260 nm was plotted as a function of temperature. All modified and control oligomers were separately mixed with target oligomers in a 350 µL quartz cuvette of 1 cm optical path length, and the *T*<sub>m</sub> was determined as the maximum of the first derivative of the melting curve (*A*<sub>260</sub> against temperature). Each *T*<sub>m</sub> is the average of three separate determinations (exp. error: ± 0.5 °C). All duplexes were formed in a 1:1 ratio of the test oligomer with the complementary strand. Mixtures of two complementary strands yield characteristics sigmoidal melting curves with a single cooperative transition (Figure S4). Concentrations were 1 µM in each strand. BPE buffer (pH 7.2) was NaH<sub>2</sub>PO<sub>4</sub> (10 mM), Na<sub>2</sub>HPO<sub>4</sub> (21 mM), and EDTA (0.20 mM). NaCl (50-800 mM) was used for determination of *T*<sub>m</sub> values at different ionic strengths. Prior to analysis, the strands were allowed to anneal by heating briefly (8 min) at 85 °C, followed by equilibrating to room temperature, chilling on ice for 25 min, and then re-equilibrating to room temperature (25 °C). The thermodynamic data of duplex formation was calculated using the program MeltWin 3.5 (J. A. McDowell, D. H. Turner, *Biochemistry* **1996**, **35**, 14077–14089).

Unmodified ODN 5′-d(GGGGGG) was not tested since ODNs containing of six or more guanine bases result in formation of extremely stable self-complementary cruciform structures or guanine tetraplex.[35] Therefore, we were not able to compare the stability of oligomers between 15 + 16 and 16 + unmodified 3′-d(CCCCCCCCCCCC) with their natural counterparts, because of the challenges in the synthesis of unmodified 5′-d(GGGGGG).
**Figure S4.** UV melting curves monitored at 260 nm. Experiments were performed in the buffer (pH 7.2) containing NaH$_2$PO$_4$ (10 mM), Na$_2$HPO$_4$ (21 mM), NaCl (200 mM), and EDTA (0.20 mM).
Table S3. Thermodynamic data of duplex formation of ODNs containing modified (mod.) and unmodified (unmod.) sequences.\textsuperscript{a}

<table>
<thead>
<tr>
<th>ODN mixtures (12 mers)</th>
<th>$\Delta G^\circ$ (kcal/mol, 37 °C)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (cal/K.mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mod. 5'-d(AAAAAAAAAAA) (13) + mod. 3'-d(TTTTTTTTTTT) (14)</td>
<td>-8.7</td>
<td>-56.7</td>
<td>-154.8</td>
</tr>
<tr>
<td>mod. 5'-d(ATAATATAATAT) (17) + mod. 3'-d(TATATATATA) (17)</td>
<td>-9.2</td>
<td>-70.9</td>
<td>-198.7</td>
</tr>
<tr>
<td>mod. 5'-d(TATATATATA) (18) + mod. 3'-d(TATATATATATA) (18)</td>
<td>-9.4</td>
<td>-72.6</td>
<td>-203.7</td>
</tr>
<tr>
<td>mod. 5'-d(GGGGGGGGGGGG) (16) + mod. 3'-d(CCCCCCCCCCCC) (15)</td>
<td>-9.2</td>
<td>-53.7</td>
<td>-143.4</td>
</tr>
<tr>
<td>mod. 5'-d(GGCGCGCGCGCG) (19) + mod. 3'-d(GGCGCGCGCGCG) (19)</td>
<td>-6.9</td>
<td>-45.1</td>
<td>-123.2</td>
</tr>
<tr>
<td>mod. 5'-d(AACCTGATTGCA) (21) + mod. 3'-d(TTTTTTTTTTTTT) (14)</td>
<td>-8.7</td>
<td>-64.4</td>
<td>-179.5</td>
</tr>
<tr>
<td>mod. 5'-d(GGGGGGGGGGGG) (16) + mod. 3'-d(CCCCCCCCCCCC) (15)</td>
<td>-8.6</td>
<td>-37.2</td>
<td>-92.0</td>
</tr>
<tr>
<td>mod. 5'-d(AACCTGATTGCA) (21) + mod. 3'-d(TTTTTTTTTTTTT) (14)</td>
<td>-8.9</td>
<td>-64.1</td>
<td>-178.1</td>
</tr>
<tr>
<td>mod. 5'-d(TGCAATCAGGTT) (22) + unmod. 3'-d(TGCAATCAGGTT) (22)</td>
<td>-10.7</td>
<td>-62.1</td>
<td>-165.7</td>
</tr>
<tr>
<td>unmod. 5'-d(AAAAAAAAAAAA) + unmod. 3'-d(TTTTTTTTTTTTT) (14)</td>
<td>-11.4</td>
<td>-62.5</td>
<td>-173.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data measured with 1 µM + 1 µM ODNs at 260 nm in the 1X BPE buffer (pH 7.2), NaCl (200 mM), and EDTA (0.20 mM).

10. Circular Dichroism Spectra. CD spectra were recorded using a JASCO J-810 spectropolarimeter. All CD experiments were performed at 5 °C in 1 mm path-length cuvettes with a buffer adjusted to pH 7.2 containing 200 mM NaCl, 10 mM NaH$_2$PO$_4$, 21 mM Na$_2$HPO$_4$, and 0.20 mM EDTA. The
concentrations were 10 µM for each strand in a total volume of 350 µL. Prior to CD analysis, hybridization of the duplexes was performed as described for the melting curve analysis.

Figure S5. Comparison of the CD spectra for modified and unmodified self-complementary ODNs: (a) modified ODNs; (b) unmodified ODNs.
11. Stability Studies of ODNs Toward DNase I and 3'-Exonuclease I. Nuclease digestion experiments were performed by using DNase I from bovine pancreas lyophilisate purchased from Amersham bioscience and 3'-exonuclease I from E. Coli strain purchased from USB Corporation. The stability assays were carried out using 50 pmol of ODNs in a buffer containing Tris-HCl (40 mM, pH 7.5) and MgCl₂ (6 mM). DNase I or 3'-exonuclease I (2 μL, 20 Units) was added to the oligomers (3 μL total reaction volume). The reaction was incubated at 37 °C for 0.5-4.0 h and then was quenched by adding urea (7.5 M) in TBE buffer (6 μL). After heating of the samples at 95 °C for 2 min, the mixtures were stored on ice until analysis by gel electrophoresis using Reliant gels (1% SeaKem Gold agarose-1X TBE buffer + ethidium bromide). Imaging was performed using an Amersham Biosciences imager (Typhoon 9410).
Figure S7. 3'-Exonuclease I incubation with the modified and unmodified ODNs followed by gel electrophoresis analysis. Incubation time: lane 1, 0 h; lane 2, 0.5 h; lane 3, 1 h; lane 4, 2 h; lane 5, 3 h; lane 6, 4 h.