Modified Oligodeoxynucleotides

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Synthesis and Evaluation of Modified Oligodeoxynucleotides Containing Diphosphodiester Internucleotide Linkages**

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During the past two decades, chemically modified oligodeoxynucleotides (ODNs) have received much attention in the search for potential therapeutic and diagnostic agents and in the study of numerous biochemical and biological processes.[1–5] To a great extent, these modifications have focused on replacing the phosphodiester group by phosphodiester mimics, such as phosphorothioate,[6] methylphosphate,[7] phosphoramidates,[8] boranophosphate,[9] alkylphosphotriesters,[10] phosphorodithioate,[11] phosphonomethyl,[12] propynes,[13] diene,[14] phosphonoformate,[15] and other groups.[16] The phosphate-modified ODNs have been used as inhibitors of gene expression,[1] viral enzymes,[2,3] in vitro messenger-RNA translation,[4] and sequence-specific DNA binding proteins.[5]

As part of our ongoing efforts to design biomolecules containing phosphate groups,[17–22] we became interested in synthesizing modified ODNs containing diphosphate diester internucleotide linkages (Scheme 1). The compounds have a larger distance between the 5′ and 3′ oxygen atoms of two nucleotides across the internucleotide bridge when compared with that in the natural ODNs. Poly(ADP-ribose) (ADP = adenosine diphosphate) is a natural polymer released in response to DNA damage and DNA metabolism.[23] Modified oligonucleotides containing one diphosphodiester mixed with phosphodiester internucleotide linkages have been synthesized.[24,25] To the best of our knowledge, this is the first report of the design and evaluation of modified ODNs containing only diphosphodiester bridges. The potential of modified ODNs for forming a double-stranded DNA molecule by binding with modified and unmodified complementary chains was investigated and compared with natural DNA chains.

Unprotected nucleosides have been previously used for the synthesis of nucleotides and oligonucleotides.[26–29] We recently described the synthesis of nucleoside 5′-*O*-diphosphates by using polymer-bound diphosphitylating reagent **1** and unprotected nucleosides.[21] The aminomethyl polystyrene resin-bound linker of *p*-acetoxybenzyl alcohol was subjected to reaction with a diphosphitylating reagent, $[i\mathrm{Pr_2NPOCH_2CH_2CN}]_2\mathrm{O}$, in the presence of 1*H*-tetrazole to produce **1**, which was used for the synthesis of modified ODNs (Scheme 1).

The 5′-hydroxy group of the first unprotected nucleoside (e.g., dT, dA, dG, dC) was selectively immobilized through the reaction with solid-supported reagent **1** to afford **2a–d**. The selectivity is due to the reaction of the sterically rigid polymer-bound reagent with the most exposed and reactive hydroxy group. In the presence of an excess amount of unprotected nucleosides (4 equivalents), the primary 5′-hydroxy group undergoes the first diphosphitylation reaction in the solid phase. The free 3′-hydroxy group was subjected to the subsequent diphosphitylation in the presence of an excess amount of $[i\mathrm{Pr_2NPOCH_2CH_2CN}]_2\mathrm{O}$ (4 equivalents) to yield **3a–d**. 5′-*O*-coupling and 3′-*O*-diphosphitylation reactions were repeated *n* times ($n = 0$–4, 11) to produce polymer-bound diphosphite triesters (**5a–h**). Oxidation, removal of the 2-cyanoethoxy group, and cleavage reactions afforded 30 modified ODNs containing diphosphodiester internucleotide linkages with up to 12 bases (**8–22**). This strategy offers the advantages of high selectivity for the attachment of a 5′-hydroxy group to the polymer-bound-diphosphitylating reagent, use of unprotected nucleosides, and facile isolation of final products.

First, ODNs of up to five bases containing diphosphodiester bridges (**8–12a–d**) were synthesized and characterized by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, high-resolution TOF electrospray mass spectrometry, and quantitative phosphorus analysis. For example, fully decoupled ^{31}P NMR spectra for the thymidine 5-mer analogue (**12a**) displayed 10 nonoverlapping peaks corresponding to 10 phosphorus atoms. The synthetic cycle was then used to synthesize modified ODNs with 12 bases (**13–22**). The final products were characterized by ^{1}H and ^{31}P NMR spectroscopy, MALDI-TOF mass spectrometry, and quantitative phosphorus analysis. Coupled ^{31}P NMR spectra of the 12-mer analogues **14** and **19** displayed peaks with chemical shifts between $\delta = -18.23$ and 1.33 ppm, corresponding to 24 phosphorus atoms in each compound.

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 (**a** and **b**; Scheme 2) that were prepared according to the previously reported procedures.[30–34] The deprotection of 3′-*O*-*tert*-butyldimethylsilyl (TBDMS) and 9-fluorenylmethyloxycarbonyl (Fmoc) groups was performed with tetrabutylammonium fluoride (TBAF) in THF and 20% piperidine in DMF, respectively, when required. Comparison of the NMR spectrodcopic data and high-resolution TOF

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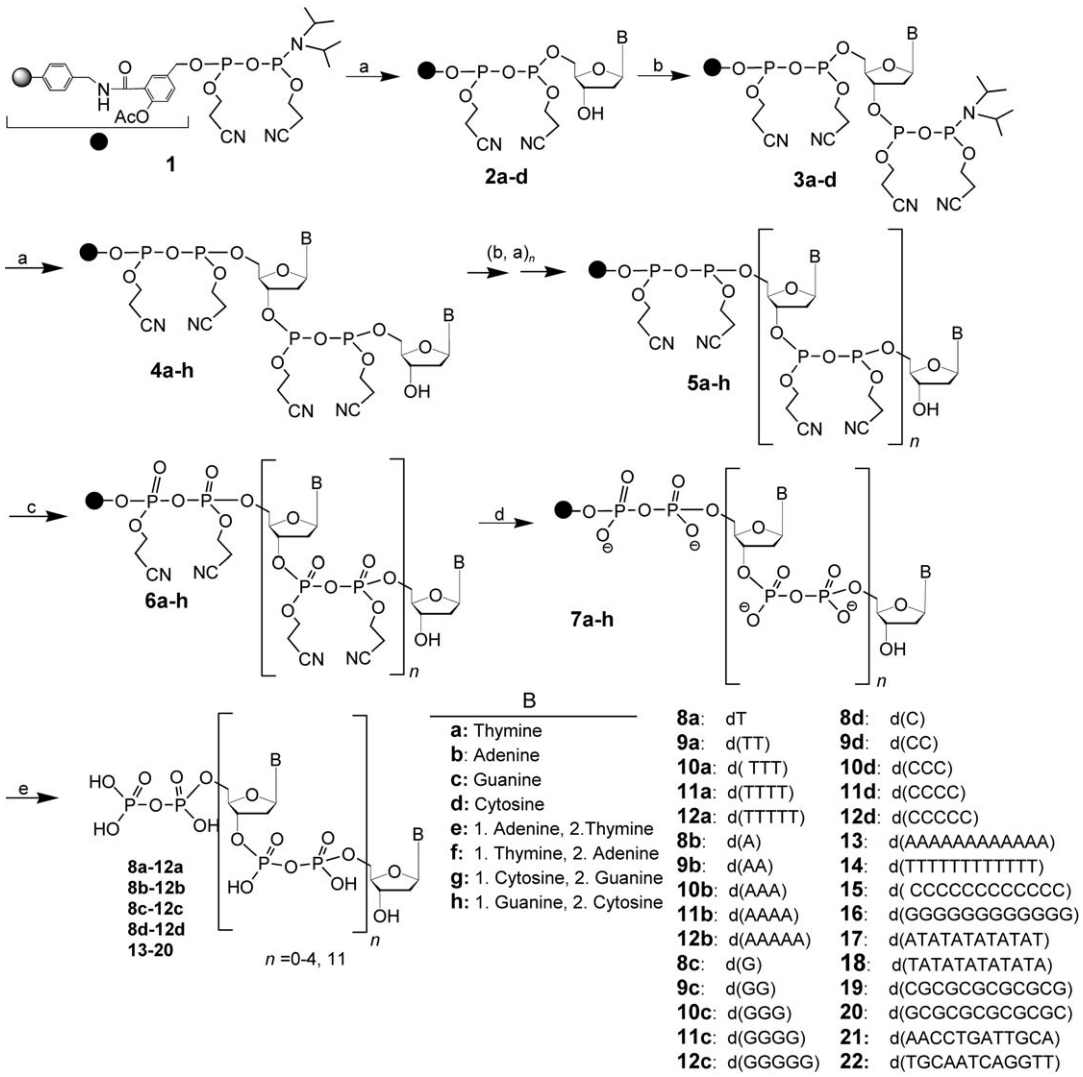
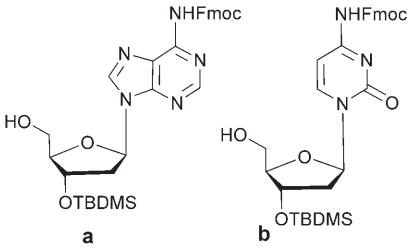
Supporting information (including full experimental procedures and spectral data) for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 1. a) Nucleoside (ROH), THF/DMSO, 1*H*-tetrazole; b) $[i\mathrm{Pr_2NPOCH_2CH_2CN}]_2\mathrm{O}$, THF or THF/DMSO, 1*H*-tetrazole; c) *t*BuOOH, THF; d) DBU, THF; e) DCM/TFA/water/1,2-ethanedithiol. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DCM = dichloromethane, DMSO = dimethyl sulfoxide, THF = tetrahydrofuran, TFA = trifluoroacetic acid.



Scheme 2. Protected nucleosides used for the synthesis of modified ODNs, d(AAAAA) (**12b**) and d(CCCCC) (**12d**), containing a diphosphate diester internucleotide linkage.

electrospray mass spectrometry of the synthesized ODNs by using the unprotected and protected nucleosides indicated that the compounds produced by both methods were identical (see the Supporting Information for experimental details). These data confirm that it is possible to use unprotected nucleosides for the synthesis of modified ODNs.

The ability of a number of modified ODNs to form a duplex with complementary chains of modified and unmodified ODNs was examined by UV melting-point measurements. The sequences of duplexes and T_m values are summarized in Table 1. As expected, no melting transition was observed for 5-mer modified ODNs. These ODNs are too short to create a meaningful duplex. However, all 12-mer modified ODNs had duplex-forming ability with complementary ODNs as shown by UV melting experiments. Mixtures (1:1) of two complementary strands yield characteristic sigmoidal melting curves. Control noncomplementary modified chains did not show any sigmoidal melting transitions (see Figure S4 in the Supporting Information), suggesting that the T_m values were dependent on formation and melting of double-stranded structures of complementary oligomers in a bimolecular process.

ODN duplexes that incorporate non-self-complementary modified (mod.) 5′-d(AAAAAAAAAAAA) (**13**) + mod. 3′-d(TTTTTTTTTTTT) (**14**; $\Delta T_m = 0.3\,^{\circ}\mathrm{C}$) and mod. 5′-

Table 1: Thermal denaturation studies (T_m values) of modified (mod.) and unmodified (unmod.) ODNs (1 μM).

ODN mixtures (12 mers)	T_m [°C][a]	ΔT_m [°C][b]
mod. 5′-d(AAAAAAAAAAAA) (**13**) + mod. 3′-d(TTTTTTTTTTTT) (**14**)	39.7	+0.3
mod. 5′-d(ATATATATATAT) (**17**) + mod. 3′-d(TATATATATATA) (**17**)	35.5	+1.8
mod. 5′-d(TATATATATATA) (**18**) + mod. 3′-d(ATATATATATAT) (**18**)	34.9	+3.2
mod. 5′-d(GGGGGGGGGGGG) (**16**) + mod. 3′-d(CCCCCCCCCCCC) (**15**)	40.2	n.d.[c]
mod. 5′-d(CGCGCGCGCGCG) (**19**) + mod. 3′-d(GCGCGCGCGCGC) (**19**)	72.5	+3.1
mod. 5′-d(GCGCGCGCGCGC) (**20**) + mod. 3′-d(CGCGCGCGCGCG) (**20**)	75.4	+7.1
mod. 5′-d(AACCTGATTGCA) (**21**) + mod. 3′-d(TTGGACTAACGT) (**22**)	44.3	+1.8
mod. 5′-d(AAAAAAAAAAAA) (**13**) + unmod. 3′-d(TTTTTTTTTTTT)	39.7	+0.3
mod. 5′-d(TTTTTTTTTTTT) (**14**) + unmod. 3′-d(AAAAAAAAAAAA)	39.5	+0.1
mod. 5′-d(GGGGGGGGGGGG) (**16**) + unmod. 3′-d(CCCCCCCCCCCC)	28.1	n.d.[c]
mod. 5′-d(AACCTGATTGCA) (**21**) + unmod. 3′-d(TTGGACTAACGT)	43.8	+1.3
mod. 5′-d(TGCAATCAGGTT) (**22**) + unmod. 3′-(ACGTTAGTCCAA)	44.1	+1.6
unmod. 5′-d(AAAAAAAAAAAA) + unmod. 3′-d(TTTTTTTTTTTT)	39.4	0
unmod. 5′-d(ATATATATATAT) + unmod. 3′-d(TATATATATATA)	33.7	0
unmod. 5′-d(TATATATATATA) + unmod. 3′-d(ATATATATATAT)	31.7	0
unmod. 5′-d(CGCGCGCGCGCG) + unmod. 3′-d(GCGCGCGCGCGC)	69.4	0
unmod. 5′-d(GCGCGCGCGCGC) + unmod. 3′-d(CGCGCGCGCGCG)	68.3	0
unmod. 5′-d(AACCTGATTGCA) + unmod. 3′-d(TTGGACTAACGT)	42.5	0

[a] Data measured with 1 μM + 1 μM ODNs at 260 nm in bisphosphate buffer solution containing EDTA (BPE buffer) (pH 7.2, 10 mM NaH_2PO_4, 21 mM Na_2HPO_4, and 0.20 mM EDTA) containing NaCl (200 mM). [b] ΔT_m [°C] values are the difference in T_m relative to the relevant unmodified reference ODNs. [c] ΔT_m was not determined as unmodified $d(G)_{12}$ forms either cruciform structures or a guanine tetraplex.[35] EDTA = ethylenediaminetetraacetic acid.

d(AACCTGATTGCA) (**21**) + mod. 3′-d(TTGGACTAACGT) (**22**; $\Delta T_m = 1.8$ °C) were found to have slightly higher melting temperatures relative to those containing unmodified–unmodified chains. Mod. self-complementary d(ATATATATATAT) (**17**; $\Delta T_m = +1.8$ °C), d(TATATATATATA) (**18**; $\Delta T_m = +3.2$ °C), d(CGCGCGCGCGCG) (**19**; $\Delta T_m = +3.1$ °C), and d(GCGCGCGCGCGC) (**20**; $\Delta T_m = +7.1$ °C) displayed enhanced hybridization affinity toward their complementary chains relative to the unmodified–unmodified ODN duplex controls. These data suggest that modified ODNs can form stable structures with complementary modified chains, but the binding affinity is slightly different when comparing self-complementary duplexes with those of non-self-complementary duplexes.

Furthermore, modified ODNs showed the ability to bind to the complementary chains of unmodified (unmod.) ODNs. Generally, ΔT_m values were in the range of 0 to +0.5 when modified ODNs (e.g., **13**, **21**) annealed with modified ODNs and their T_m values were compared to the analogous duplexes containing modified–unmodified ODNs. Mod. 5′-d(GGGGGGGGGGGG) (**16**) exhibited a higher binding affinity towards mod. 3′-d(CCCCCCCCCCCC) (**15**) than that of unmod. 3′-d(CCCCCCCCCCCC).

The effects of ionic strength on T_m values were determined for a number of ODNs in the presence of different concentrations (50 to 800 mM) of sodium chloride solutions at pH 7.2 (Figure 1). T_m values were dependent on the salt concentration and were lower at low ionic strength, possibly owing to the presence of higher columbic repulsions under these conditions. Mod.–mod. self-complementary 5′-d(ATATATATATAT) (**17**) and 5′-d(TATATATATATA) (**18**) displayed a similar pattern and slope, suggesting that hybridization in these modified ODNs is probably identical. Non-self-complementary oligomers, such as mod. 5′-d(AAAAAAAAAAAA) (**13**) + mod. 5′-d(TTTTTTTTTTTT) (**14**), mod. 5′-d(AACCTGATTGCA) (**21**) + mod. 3′-d(TTGGACTAACGT) (**22**), and mod. 5′-d(GGGGGGGGGGGG) (**16**) + unmod. 3′-d(CCCCCCCCCCCC) also exhibited direct correlations between T_m values and ionic strength, confirming the effects of ionic strength on duplex formation of modified ODNs with the complementary chains of modified and unmodified ODNs. Further studies are required to determine the nature of thermal transitions of modified ODNs with the complementary chains of modified and unmodified ODNs to duplex or denatured strands in different ionic strengths.

For subsequent analysis, CD measurements were used to investigate the conformational changes of modified ODNs in the presence of their complementary modified and unmodified chains and to compare with unmodified–unmodified analogues.

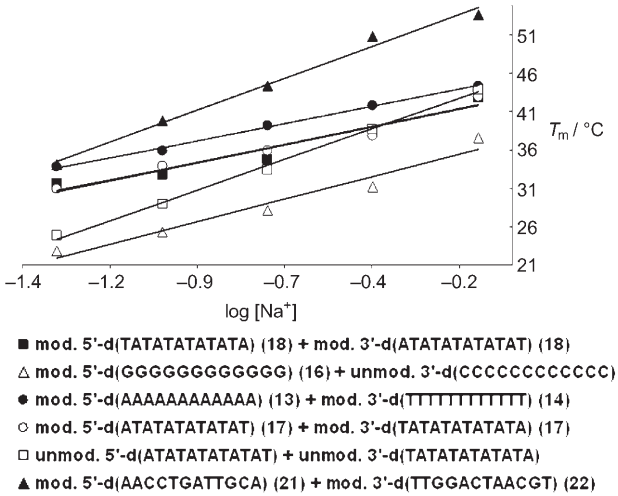


Figure 1. Apparent T_m values of the duplex to a single-strand transition at different ionic strengths and pH 7.2 in BPE buffer solution. The concentration of ODNs was 1 μM for each strand.

The CD spectra of the self-complementary and non-self-complementary strands showed significant difference between the modified and natural sequences. Most modified 12-mer ODNs (**14**, **15**, **17**–**20**) showed a fairly intense negative band between 217–219 nm and a positive band between 270–283 nm. In comparison, unmodified self-complementary and non-self-complementary 12-mer ODNs exhibited a negative band centered near 247–253 nm and a positive band centered

near 268–285 nm (Figure 2 and Figure S5 in the Supporting Information).

Figure 3 shows the CD spectra of DNA–DNA hybrids with and without diphosphodiesters. Modified double-

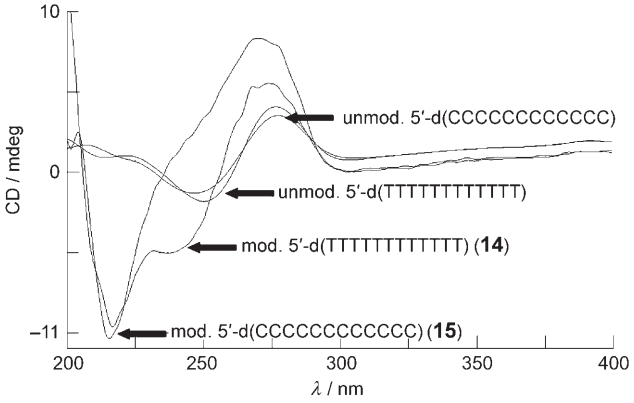


Figure 2. Comparison of the CD spectra for modified and unmodified d(TTTTTTTTTTTT) and d(CCCCCCCCCCCC).

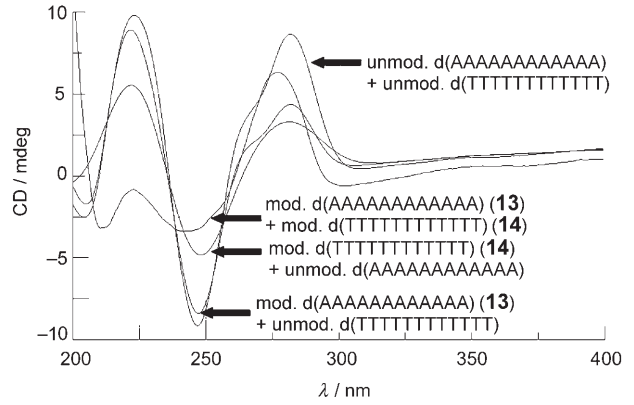


Figure 3. Comparison of CD spectra of modified–modified, modified–unmodified, and unmodified–unmodified ODNs.

stranded ODNs consisted of one or two modified strands. CD spectra showed a pattern similar to right-handed B-DNA for solutions containing the non-self-complementary chains of modified–unmodified ODNs. Characteristic of the B-form, CD spectra show a positive band centered near 275 nm and a negative band centered near 240 nm. The spectra of mod. 5′-d(TTTTTTTTTTTT) (**14**) + unmod. 3′-d(AAAAAAAAAAAA) and mod. 5′-d(AAAAAAAAAAAA) (**13**) + unmod. 3′-d(TTTTTTTTTTTT) in a 1:1 fashion were very similar in terms of the pattern with their negative and positive bands centered near 248 and 281 nm, respectively (Figure 3). A similar pattern was observed for unmodified–unmodified duplexes with negative and positive bands centered near 248 and 282 nm, respectively, providing evidence that the structural effect of the diphosphodiester modification was relatively insensitive to which strand contained the modification and confirming the absence of changes in the nucleic acids chromophore. This demonstrated that the diphosphodiester modification in one strand did not affect the conformation of the hybrids of modified–unmodified duplexes. The diphosphate diester bridges are flexible and can fold to occupy a position similar to that of a phosphodiester group, allowing appropriate folding of the backbone for duplex formation with unmodified oligomers. A relatively similar pattern was observed for ODNs containing all four bases. Mod. 5′-d(AACCTGATTGCA) (**21**) + unmod. 3′-d(TTGGACTAACGT) and mod. 5′-d(TGCAATCAGGTT) (**22**) + unmod. 3′-(ACGTTAGTCCAA) showed negative and positive bands centered near 230–233 and 282–285 nm, respectively (see Figure S6 in the Supporting Information).

The CD spectra of the duplexes with both strands modified differed slightly from those of the unmodified DNA duplexes and DNA duplexes containing one modified strand. The CD spectra of mod. d(AAAAAAAAAAAA) (**13**) + mod. d(TTTTTTTTTTTT) (**14**; Figure 3) and mod. 5′-d(AACCTGATTGCA) (**21**) + mod. 3′-d(TTGGACTAACGT) (**22**; see Figure S6 in the Supporting Information) showed the spectral shift toward the shorter wavelengths with a negative band near 241–244 nm when compared with those of modified–unmodified and unmodified–unmodified duplexes between 247–249 nm. The difference may be due to significant conformational change when both strands are modified, an increase in the flexibility of diphosphodiester-modified DNA strands, and/or different stacking. CD spectra for some modified ODNs (e.g., **13** + **14**) generally showed (Figure 3) decreased negative-band intensities throughout the spectral region studied. This was interpreted as reflecting the fact that the bases of the modified–modified ODNs were less stacked. Further structural analysis is required to determine and compare the nature of stacking interactions of modified–modified or modified–unmodified duplexes with those of the unmodified–unmodified duplexes.

The reported CD spectra of a triplex of poly(dA·dT·dT) and poly(dT·dA·dA)[36,37] were different from CD spectra shown in Figure 3, suggesting that these hybrid ODNs are not triplex structures of poly(dA·dT,dT) or poly(dA·dA·dT). Furthermore, triplex DNA are formed in a higher molar ratio of dA or dT and at a high concentration of NaCl (e.g., ≥ 2.6 M).[36,37]

The presence of the additional phosphodiester group altered the optical properties of modified ODNs. The changes in hydration patterns of phosphate groups and/or a larger sugar–sugar separation appear to modify the conformation of modified ODNs. The phosphate–phosphate separation is 6.6 A° on average in B-DNA. In the B form of DNA, the phosphate groups are located far apart and as such, they are independently hydrated.[38,39] The presence of additional phosphodiester groups provides an opportunity for water molecules that not only bridge neighboring phosphate groups, but also both phosphate groups in the diphosphodiester linkage, and thus stabilize the hybrid. Phosphate–phosphate electrostatic repulsion is also diminished in water. Further structural analysis may provide insights about other contributing factors in the conformation of modified ODNs.

A number of modified ODNs were incubated with DNase I and 3′-exonuclease I to determine their nuclease stability compared with the corresponding unmodified

oligomers. Figure 4 shows results of the degradation of the modified and unmodified ODNs by DNase I. The modified oligomers were resistant to degradation by DNase I under

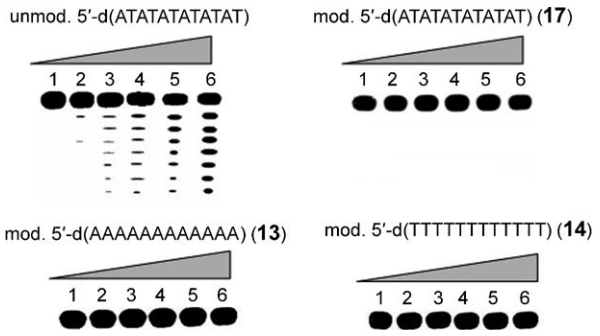


Figure 4. DNase I incubation with the modified and unmodified ODNs followed by gel electrophoresis analysis. Incubation time: lane 1, 0 h; lane 2, 0.5 h; lane 3, 1 h; lane 4, 2 h; lane 5, 3 h; lane 6, 4 h.

conditions in which unmodified ODNs were degraded and showed multiple bands. Similar results were obtained with 3′-exonuclease I (see Figure S7 in the Supporting Information).

These studies established a new family of chemically modified ODNs. These data indicate that modified ODNs are able to form duplexes with their complementary modified or unmodified chains based on thermal denaturation studies and CD analysis. To the best of our knowledge, this is the first reported design of chemically modified ODNs containing only diphosphodiester bridges. The modified ODNs have the ability to bind to complementary unmodified strands, suggesting that these compounds may have potential applications in nucleic acid research for developing inhibitors against specific sequences of oligonucleotides.

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