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Synthesis and biophysical studies of oligonucleotides containing hydroxamate linkages

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Abstract

Novel oligonucleotide dimers containing hydroxamate linkages (**15** and **16**) were synthesized, incorporated into oligonucleotide sequences and studied for their hybridization properties with complementary DNA and RNA targets. The modified oligonucleotides showed similar binding properties and enhanced resistance to exonucleases compared to natural oligonucleotides. © 2000 Elsevier Science Ltd. All rights reserved.

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Inhibition of gene expression with antisense oligonucleotides by binding to mRNA in a sequence specific manner has become an attractive method for the treatment of viral diseases, cancer, and for the study of genetic disorders.[1–3] As drug candidates, they should inhibit the translation process via duplex formation and/or able to activate RNase H to cleave the target message after hybridization. In addition, antisense oligonucleotides should possess in vivo nuclease stability, adequate binding affinity to the target mRNA, and the ability to reach target cells and tissues. So far, chemical modifications of oligonucleotides have resulted in increased solubility, nuclease stability, cellular uptake, binding properties and RNase H activation.[4] However, the quest for new and novel modified oligonucleotides with improved properties is growing.

Our interest to design novel class of oligonucleotides has led us to envision hydroxamate nucleic acids (HONA)[5] (i.e. oligonucleotides having hydroxamate internucleotide linkages, Fig. 1, **2**). Hydroxamate nucleic acids offer several advantages over previously reported modifications.[6] The hydroxamate unit (-CO-NOH-) is stable under physiological conditions and chelates with ferric ion, which can generate hydroxyl radicals.[7] Thus, in addition to potential RNase-H cleavage process, oligonucleotides having hydroxamate linkages might cleave the target through radical reactions. Furthermore, the solubility of oligonucleotides with hydroxamate linkages

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might be increased by coordination with metal ions and also the charge would be reduced, which should facilitate penetration through negatively charged cell membranes. As a prototype, herein we report our preliminary data on the syntheses of novel thymidine dimers **15** and **16**, their incorporation into oligonucleotide sequences and measurement of hybridization of the duplex formed between HONA and natural DNA/RNA.

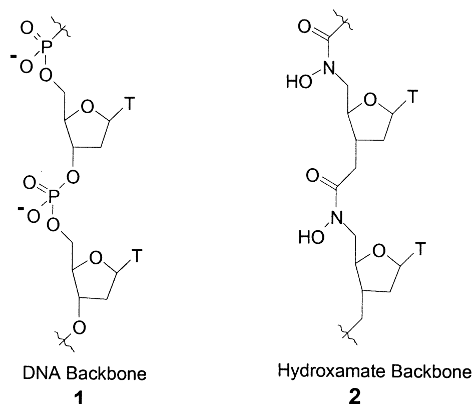
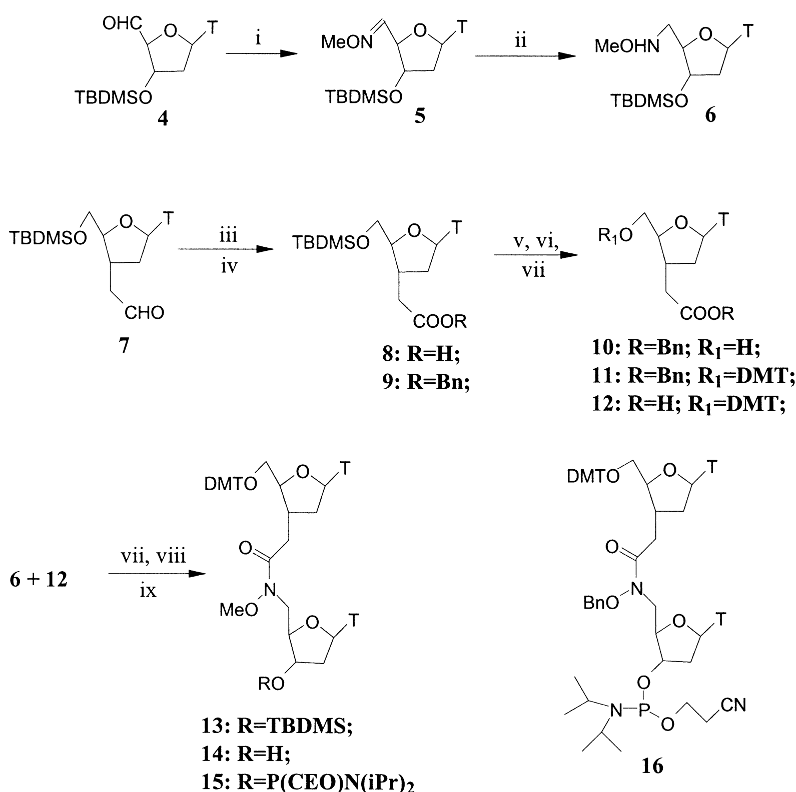


Figure 1.

The synthetic route to the hydroxamate dimers **15** and **16** is shown in Scheme 1. The known aldehyde[8] **4** was heated with *O*-methylhydroxylamine hydrochloride in the presence of pyridine at 100°C for 12 h to give oxime **5** in 99% yield. Interestingly, **4** did not react with *O*-methylhydroxylamine hydrochloride at room temperature under different conditions. Reduction of **5** with sodium cyanoborohydride in dry methanol containing acetic acid (pH below 6) for 6 h at room temperature provided one of the building blocks **6**. For the building block **12**, aldehyde **7**[9] was oxidized to the corresponding carboxylic acid **8** with sodium chlorite using 2-methyl-2-butene as HOCl scavenger.[10] Esterification of **8** with benzyl alcohol using water soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC)[11] afforded benzyl ester **9** in 87% yield. Desilylation of **9** followed by dimethoxytritylation[12] and base hydrolysis provided second building block **12**. Carboxylic acid **12** was activated with *O*-(1*H*-benzotriaol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoroborate (HBTU) and *N*-hydroxybenzotriazole[13] and then coupled with hydroxylamine **6** at room temperature to give a dimer **13** in 63% yield. Removal of the silyl protective group from **13** with tetra-*n*-butylammonium fluoride and subsequent phosphitylation[14] gave the target phosphoramidite dimer **15**.[15] The second dimer **16** was also prepared by using the same methodology depicted in Scheme 1 (60% overall yield) and substituting *O*-benzylhydroxylamine hydrochloride for *O*-methylhydroxylamine hydrochloride during the formation of **5**.

Incorporation of the dimers **15** and **16**[15] into oligonucleotide sequences was accomplished using ABI 394 DNA synthesizer and protocol,[16] and the coupling efficiency was found to be higher than 95%. The binding behavior of the modified oligonucleotides was assayed by examining their ultraviolet (UV) absorbance versus temperature profiles. An 18-mer oligonucleotide (5′-TTCCTGCTTGATGGCTTC-3′) was modified with **15** and **16** at different locations and hybridized to complementary DNA or RNA. Melting temperatures of the duplexes formed between oligonucleotides containing the hydroxamate linkages and their DNA and RNA complementary



Scheme 1. [a](i) MeOHN·HCl/Py/100°C; (ii) $NaCNBH_3$/ACOH/MeOH; (iii) $NaClO_2$/2-methyl-2-butene/*t*-BuOH/H_2O; (iv) BnOH/EDC/*N*,*N*-dimethylaminopyridine; (v) TBAF/THF/H_2O/Py; (vi) DMTCl/TEA/CH_2Cl_2; (vii) 1N NaOH/THF/H_2O/H^+; (viii) HBTU/H-hydroxybenzotriazole/MeCN; (ix) $(i\text{-Pr})_2NP(Cl)OCH_2CH_2CN$/*i*-$Pr_2NEt$/$CH_2Cl_2$ T = thymine

strands are summarized in Table 1. Compared to unmodified DNA, incorporation of a hydroxamate dimer **15** at 3′-end of an oligonucleotide (Table 1, line 3) led to an increase in Tm of 0.2°C against DNA and RNA complements. Substitution of **15** at 5′-end, decreased the Tm with DNA by 0.2°C and the RNA duplex stability is unaffected (Table 1, line 4). Incorporation of **15** at 3′-end and 5′-end formed stable duplexes with DNA/RNA complements. When **15** was substituted in the middle of the oligonucleotide (Table 1, line 6), the Tm was decreased by 0.4°C against DNA/RNA. On the other hand, incorporation of **15** at 3′-end, 5′-end and in the middle (Table 1, line 7), did not disturb duplex stability with neither DNA nor RNA. However, when **15** was substituted at 3′-end and in the middle (Table 1, line 7), the duplex stability is decreased with DNA/RNA by a Tm of 0.2°C (Table 1, line 8). A similar binding pattern (data not shown) was observed when another hydroxamate dimer **16** is incorporated either at the 5′-end or 3′-end or in the middle of the sequence. However, compared to **15**, hydroxamate dimer **16** destabilized both DNA and RNA duplexes (ΔTm of 1.0°C/modification). Interestingly, compared to unmodified oligonucleotides, incorporation of hydroxamate modified dimer at the 3′-end of the oligonucleotides, exhibited tenfold increase in resistance to exonucleases.[18]

This study suggests that a smaller substitution (CH_3 group) on the hydroxamate dimer **15** did not affect duplex stability and is a preferred dimer over **16** for oligonucleotide backbone modifications.

Table 1
Tm values of oligonucleotides containing *O*-methylhydroxamate linkage[a]

Sequence	Tm(°C) DNA	Tm(°C) RNA	ΔTm /mod (°C) DNA	ΔTm /mod (°C) RNA
5' GAA GCC ATC AAG CAG GAA 3'				
5' TTC CTG CTT GAT GGC TTC 3'	61.07	63.88		
5' TTC CTG CTT GAT GGC ttC 3'	61.42	64.13	0.2	0.2
5' ttC CTG CTT GAT GGC TTC 3'	60.75	64.03	-0.2	0.1
5' ttC CTG CTT GAT GGC ttC 3'	61.33	64.18	0.1	0.1
5' TTC CTG Ctt GAT GGC TTC 3'	60.25	63.03	-0.4	-0.4
5' ttC CTG Ctt GAT GGC ttC 3'	60.31	63.07	-0.1	-0.1
5' TTC CTG Ctt GAT GGC ttC 3'	60.26	62.97	-0.2	-0.2

[a]Tm is the temperature at the midpoint of the melting curve; The concentrations are as follows: Oligomer strands, 2 μM each. Melting temperatures (Tm) were determined[17] by measuring change in absorbance at 260 nm (cuvette, 1-mm path length) as a function of temperature in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA. All the values are averaged from at least three experiments. The letters "tt" denote thymidine dimer containing hydroxamate linkages.

On the other hand, dimer **16** with a bulky substitution (benzyl) in oligonucleotide sequence decreases its ability to form stable duplexes with complementary DNA/RNA. The introduction of bulky group such as benzyl in the hydroxamate unit **16** is sterically demanding which may alter the structure and the stability of the duplex formed with DNA/RNA complement. The weaker binding of HONA containing dimer **16** is indicative that the internucleotide hydroxamate linkages will not tolerate larger groups. The presence of bulky groups may force the oligonucleotides to adopt a different conformation than that of oligonucleotides having unmodified phosphate backbone. Furthermore, it seems that the hydroxamate dimer **15** adopts a more favorable geometry in the duplex with DNA/RNA complement than that of **16**.

In summary, oligonucleotides containing hydroxamate dimers have been synthesized for the first time and studied for their ability to form stable duplexes. Interestingly, oligonucleotides containing hydroxamate dimer **15** not only displayed a similar or even slightly higher affinity for RNA target than the natural analogues, but also showed substantial resistance towards 3′-exonucleases.

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