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Synthesis and properties of morpholino chimeric oligonucleotides

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Abstract

Chimeric oligonucleotides with the novel morpholino modification and the phosphoramidate linkers have been synthesized and characterized. These oligonucleotides showed moderate thermal stability with complementary RNA and DNA, and enhanced resistance toward the nuclease ($t_{1/2} > 10$ h). The phosphoramidate linker made the synthesis of such oligonucleotides applicable on a DNA synthesizer. Under the acidic condition (pH 3.0), the phosphoramidate linkers were readily cleaved, and such property might be useful for the DNA-sequence determination.

Keywords: Morpholino oligonucleotides; Thermal stability; Nuclease resistance; Duplex conformation; Acid-catalyzed hydrolysis

Chemical-modified oligonucleotides are widely used in biotechnology and gene therapy areas,[1] acting as antisense oligonucleotides, ribozymes, and siRNAs.[2] An applicable modified oligonucleotide needs high binding affinity to target RNA or DNA, high nuclease stability, high cellular uptake efficiency, and low toxicity.[2,3] Morpholino oligonuloetides (Fig. 1a), with morpholino rings in subunits instead of the ribose and a non-ionic intersubunit linkage instead of the phosphodiester bond, have become one of the promising candidates for in vivo and in vitro gene function study.[4] Morpholino oligonuloetides have many advantages: (1) they inhibit the translation of target mRNA by steric block, (2) they have superior binding affinity to RNA; and (3) they have high resistance to enzymatic degradations.[5] However, the morpholino oligonucleotides have several limitations. Firstly, they cannot be transfected by liposome and thus special methods have to be carried out

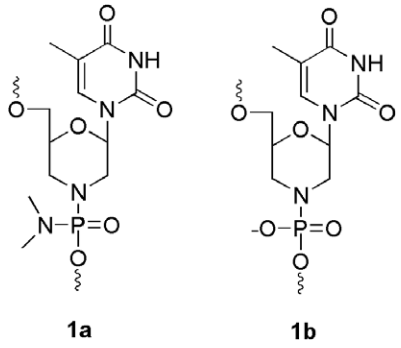

Fig. 1. Morpholino oligonucleotide with a neutral backbone (**1a**) and with an anionic backbone (**1b**).

to deliver them into cells, such as microinjection,[6] hybridization of DNA with the morpholino oligonucleotide and delivery with ethoxylated polyethylenimine,[7] and conjugation with peptides.[8] Secondly, the duplex formed by the morpholino oligonucleotide and target RNA is not a substrate for RNase H due to the heteroduplex conformation change compared to normal DNA/RNA. Lastly, the morpholino nucleoside monomer can not be incorporated into

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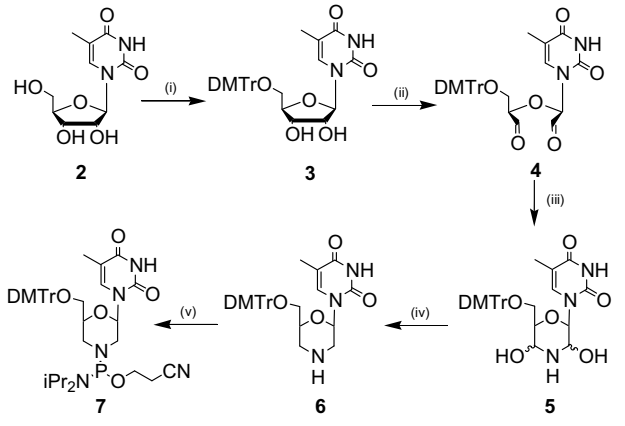
chimeric oligonucleotide on a DNA synthesizer, which limits its applications in the 'gapmer' approach[9] and the chemical-modified siRNA.

Here we report our novel design of chimeric oligonucleotides with anionic phosphate backbones containing morpholino nucleoside analogues (Fig. 1b) and phosphoramidate linkers, which was aimed to maintain the good enzymatic stability as well as to incorporate the modification into oligonucleotides on a DNA synthesizer. Also reported is their affinity toward DNA and RNA, resistance to nuclease, and hydrolysis under acidic conditions.

The synthesis of the building block, phosphoramidite monomer **7**, is shown in Scheme 1. 5-Methyluridine (**2**) was treated with DMTr–Cl in anhydrous pyridine overnight under argon atmosphere to afford 5′-O-DMTr-5-methyluridine (**3**) in 86% yield.[10] Following published procedures[11] with improvements, compound **3** was then subjected to the construction of the morpholino ring in one step by treating it with sodium periodate and then ammonium biborate to afford 2′,3′-dihydroxyl-5-methyl-morpholino-uridine (compound **5**). Reduction of 2′ and 3′ hydroxyl groups on compound **5** gave compound **6**. The total yield of the 3 steps above was 84%. The phosphoramidite building block **7** was then obtained by the phosphitilation of compound **6** under the conditions with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite and 4,5-dicyanoimidazole (DCI) in hydrous CH_2Cl_2 for 4 h under argon atmosphere in 90% yield.[12] All the compounds were characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and HRMS.

The building block **7** was incorporated into oligonucleotides (Table 1) on a DNA synthesizer, with 5-ethylthio-1*H*-tetrazole (ETT) as the activator in 5 min coupling time. After being removed from solid support, crude oligonucleotides were purified by HPLC. The isolated yield of modified oligonucleotides was approximately 30%. Oligonucleotides were analyzed by HPLC and MALDI-TOF mass spectroscopy.[13]



Scheme 1. Reagents and conditions: (a) DMTr–Cl, NEt_3, Py, rt, Ar; (b) and (c) $NaIO_4$, $(NH_4)_2B_4O_7$, CH_3OH, rt; (d) $NaCNBH_3$, CH_3OH, rt; (e) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, DCI, CH_2Cl_2, rt, Ar.

Table 1
Oligonucleotides sequence and corresponding T_m values of complexes with complementary DNA and RNA[a]

No.	Sequence (5′-3′)	T_m/°C	
		DNA	RNA
8	d(GCGTTTTTTGCT)	61.5	58.9
9	d(GCGTT**T**TTTGCT)	59.6	58.6
10	d(GCGTT**TT**TTGCT)	57.9	57.9
11	d(GCGT**TTT**TTGCT)	57.2	57.0
12	d(TTTTTTTTTT)	nd	nd
13	d(TTTTTTTTT**T**)	nd	nd
14	d(TTTTTTTT**TT**)	nd	nd
15	d(TTTTTTT**TTT**)	nd	nd

[a] Morpholino modifications are in bold.

Thermal stability of modified oligonucleotides **9**, **10**, **11** toward DNA and RNA was studied by UV-T_m experiments. As a result, the morpholino nucleosides modification caused distabilization with RNA and DNA only to a very limited extent. Specifically, the modification in oligonucleotide unstabilized the duplex with DNA by −1.7 °C per modification, whereas it only distabilized the duplex with RNA by −0.5 °C per modification. The thermal stability of our morpholino-modified oligonucleotides toward RNA is similar to that of the literature reported phosphorothioate oligonucleotides.[14] It is believed that such slight distabilization effect will not affect the further application of morpholino-modified oligonucleotides.

The conformation of DNA/RNA duplex was reported to activate RNase H which degrades target RNA.[15] The CD spectroscopy was used to study the conformation of duplexes formed by the modified oligonucleotide **9**, **10**, and **11** with complementary RNA. As shown in Figure 2, the spectra of the duplexes containing different numbers of modified subunits are quite similar to that of the natural DNA/RNA duplex, indicating that such modification did not affect the helical conformation of the duplex.

We synthesized unmodified oligonucleotide **12** and three modified oligonucleotides **13**, **14** and **15** with the modifications on the 3′-terminal ends to investigate the stability of our morpholino-modified oligonucleotides against 3′-exo-

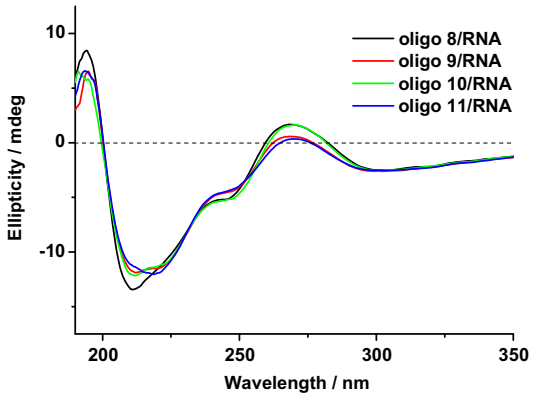


Fig. 2. CD spectra of the duplexs between modified oligonucleotides with complementary RNA.

nuclease (snake venom phosphodiesterase, SVPDE), which is the major cause of oligonucleotides degradation in serum.[16] Hydrolysis was carried out at near physiological conditions, and the degradation of oligonucleotides was analyzed by HPLC at several time points. As shown in Figure 3, the unmodified oligonucleotide **12** degraded rapidly with a half-life $t_{1/2}$ of 20 min, whereas in contrast, half-lives of modified oligonucleotides **13**, **14** and **15** are 10 h, 12 h, and 15 h, respectively. As a result, all the modified oligonucleotides demonstrated significantly improved stability, which was gradually enhanced with the increasing number of modifications.

Additionally, the acid-catalyzed hydrolysis properties of these modified oligonucleotides were evaluated. Morpholino-modified oligonucleotide **9** was rapidly cleaved at pH 3.0, while it was stable at pH 7.0 (Fig. 4). For example, after 30 min, about 50% modified oligonucleotide **9** was cleaved at pH 3, while no obvious cleavage was observed at pH 7. In addition, no cleavage of unmodified oligonucleotide **8** was detected at pH 3.0. This acid labile phosphoramidate linkage can be used to produce short polynucleotide fragments, which can be easily analyzed by MALDI-TOF mass spectrometry and thereby be applicable for DNA-sequence determination.[17]

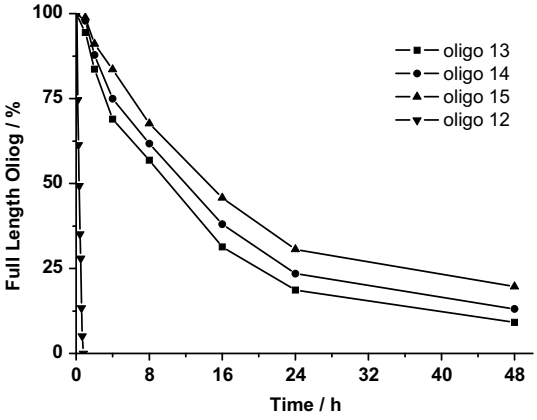


Fig. 3. Enzymatic stability of oligo **12**, **13**, **14**, **15** against SVPDE.

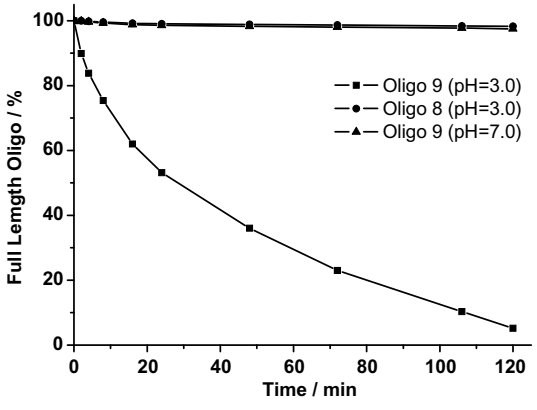


Fig. 4. Acid-catalyzed hydrolysis of morpholino oligonucleotide **9** and unmodified oligonucleotide **8**.

In conclusion, we have synthesized morpholino-modified chimeric oligonucleotides containing anionic phosphate backbones, along with moderate binding affinity to complementary RNA and high resistance of nuclease. Incorporation of modifications into oligonucleotides does not affect the conformation of duplex with complementary RNA. The phosphoramidate linkage can be readily cleaved under acidic condition. These favorable properties make morpholino modification a good candidate for antisense therapeutics and the gene function studies.

Acknowledgments

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Supplementary data

Experimental procedures and spectral data for compounds in Scheme 1, synthesis of oligonucleotides and procedures used for T_m, CD, nuclease study and acid-catayzed hydrolysis are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.04.035.

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13. *MALDI-TOF data of oligonucleotides*: Compound **8** $[M-H]^-$ 3632.30 (calcd 3632.36), **9** $[M-H]^-$3631.39 (calcd 3631.38), **10** $[M-H]^-$ 3630.44 (calcd 3630.40), **11** $[M-H]^-$3629.27 (calcd 3629.41), **12** $[M-H]^-$ 2978.99 (calcd 2978.96), **13** $[M-H]^-$2977.82 (calcd 2977.98), **14** $[M-H]^-$2977.04 (calcd 2976.99), and **15** $[M-H]^-$2975.97 (calcd 2976.01).
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