Phosphorus, Sulfur and Silicon, 1999, Vol. 144-146, pp. 363-366
Reprints available directly from the publisher
Photocopying permitted by license only

Mixed-Backbone Oligonucleotides Containing Segments of Deoxynucleosides Phosphorothioate and 2'-*O*-Methylribonucleosides Methylphosphonate: Synthesis and Properties

MAOJUN GUO, DONG YU and SUDHIR AGRAWAL

Hybridon Inc., 620 Memorial Drive, Cambridge, MA 02139

Synthesis of mixed-backbone oligonucleotides containing segments of deoxynucleoside phosphorothioate and 2'-*O*-methylribonucleoside methylphosphonate have been achieved by using *N*-Pent-4-enoyl (PNT) heterocyclic base-protected 2'-O-methylribonucleoside methylphosphonamidites. Use of PNT for heterocyclic base-protection allows the deprotection to be carried out under milder conditions without affecting base labile methylphosphonate internucleotide linkages. New MBOs have reduced phosphorothioate internucleotide linkages while maintaining affinity with target RNA and are stable towards nucleases.

Keywords: Phosphorothioate; methylphosphonate; methylphosphonamidite; phosphoramidite; oligonucleotide; antisense

Introduction

Oligodeoxynucleoside phosphorothioates (PS-oligos) have been studied extensively as antisense agents[1a]. To further improve the therapeutic potential of these compounds, mixed backbone oligonucleotides (MBOs) have been developed and studied extensively. MBOs have yielded promising results and are being evaluated in human clinical trials as second-generation antisense oligonucleotides[1b]. MBOs contain a segment of PS-oligo, which provides nuclease stability and RNase H activity, and segments of 2'-*O*-methylribonucleoside at both the 5'- and 3'- ends, which provides increased affinity with the target RNA and a further increase in nuclease stability compared to PS-oligo. As well as having improved biological activity, MBOs are less toxic *in vivo* than are PS-oligos[1c], depending on the sequences and base compositions. In addition, MBOs can be delivered orally [1d].

MBOs containing 2'-*O*-alkylribonucleosides (2'-*O*-methyl, 2'-*O*-propyl) along with phosphodiester linkages at both the 3'- and 5'- ends of PS-oligos have a lower number of phosphorothioate linkages, but these MBOs were found to be less stable against nucleases than were PS-oligos[1e-f].

In continuing our effort to optimize the therapeutic potential of oligonucleotides, our goal is to further minimize phosphorothioate-related side effects (complement activation and

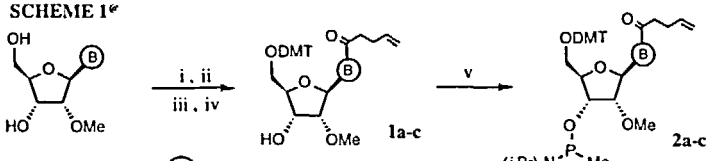
prolongation of activated partial thromboplastin time, aPTT), while maintaining aqueous solubility, affinity to target RNA, RNase H-mediated cleaving efficiency of complementary RNA, and stability against nucleases.

Earlier studies have indicated that non-ionic oligonucleotide methylphosphonates are readily taken up by cells and are resistant to nucleases, but have a lower aqueous solubility and affinity towards target RNA[2]. To improve the aqueous solubility and affinity to complementary RNA, we[3a] and others[3b] have also studied 2'-*O*-methyloligoribonucleoside methylphosphonates. In earlier studies, synthetic methodology to obtain 2'-*O*-methyloligoribonucleoside methylphosphonate required the use of nucleoside methylphosphonamidite monomer, in which heterocyclic bases were protected with isobutyryl (C, G) or benzoyl (A) groups, and relatively harsh deprotection conditions were used[3a-b]. In order to obtain milligram quantities of oligonucleotides containing 2'-*O*-methylnucleoside methylphosphonates that do not require strong deprotection conditions, we have employed PNT as a protecting group for nucleobases[4]. We report here the synthesis of PNT-protected 2'-*O*-methylribonucleoside methylphosphonamidites, the use of these new monomers for the synthesis of MBOs containing segments of PS-oligo and 2'-*O*-methylribonucleoside methylphosphonates.

Synthesis of 2'-*O*-methylribonucleoside methylphosphonamidites

The PNT heterocyclic base-protected 5'-dimethoxytrityl (DMT)-2'-*O*-methylribonucleoside **1** were synthesized by transient silyl protection of the 3'- and 5'- hydroxyl group of the corresponding 2'-*O*-methylribonucleosides, followed by acylation of the exocyclic amino group using pent-4-enoic anhydride (Scheme 1)[4]. After working up and without further purification, the PNT heterocyclic base-protected 2'-*O*-methylribonucleosides in the crude reaction mixture were reacted with dimethoxytrityl chloride to obtain the PNT heterocyclic base-protected 5'-DMT-2'-*O*-methylribonucleoside **1**.

SCHEME 1[@]



[@] Keys: i) TMSCl/Py.; ii) ; iii) NH_3/H_2O; iv) DMTCl/Py.; v) $MePCl_2$/DIPEA.

The requisite 2'-*O*-methylribonucleoside methylphosphonamidite monomers **2a-c** were prepared by the initial addition of *N,N*-diisopropylamine to dichloromethylphosphine followed by addition of the appropriate 2'-*O*-methylribonucleosides **1a-c** in a one-pot reaction. Each of the PNT 2'-*O*-methylribonucleoside methylphosphonamidite monomers thus obtained was purified by column chromatography and analyzed by ^{31}P NMR.

Synthesis of MBOs

MBOs containing PS-oligo and 2'-*O*-methylribonucleoside methylphosphonate linkages were synthesized using appropriate PNT-protected deoxynucleoside β-cyanoethylphospho-

ramidites[4] and 2'-*O*-methylribonucleoside methylphosphonamidites **2a-c** on an automated synthesizer (Expedite 8909, PerSeptive Biosystems). The sequence chosen for this study was an 18mer oligonucleotide complementary to RIα regulatory subunit of the protein kinase A[5]. Oligonucleotides **1** to **6** listed in Table 1 were synthesized. To synthesize the PS oligo segment, coupling was carried out using appropriate PNT-protected deoxynucleoside β-cyanoethylphosphoramidites and 1-H tetrazole, followed by oxidation with 3H-1,2-benzodithiole-3-one-1,1-dioxide. To synthesize segments with 2'-*O*-methylribonucleoside methylphosphonate and phosphodiester linkages, coupling was carried out using PNT-protected 2'-*O*-methylribonucleoside methylphosphonamidites **2a-c** and PNT-protected 2'-*O*-methylribonucleoside β-cyanoethylphosphoramidites, followed by oxidation with *t*-butyl hydroperoxide (*t*-BuOOH, 1M in toluene). DMT was removed at the end of the synthesis cycles of the required sequence. Both the cleavage of oligonucleotides from solid support and the removal of protecting group (PNT) were achieved by using dry gaseous ammonia under 100 psi at room temperature overnight[6]. The deprotected oligonucleotides were purified by preparative polyacrylamide gel electrophoresis. The purity and integrity of the oligonucleotides were analyzed by ^{31}P NMR, MALDI-TOF mass spectrometry and capillary gel electrophoresis (data not shown).

The design and thermodynamic duplex stability of MBOs

Thermodynamic duplex stability (Tm) of the duplexes formed between the MBOs and the corresponding complementary RNA were determined by measurements of the absorbance at λ260 nm vs temperature. The Tms, which represent the average of at least two determinations are summarized in Table 1.

TABLE 1. Thermodynamic duplex stability of MBOs

No.	Oligo Sequences and Modification (5'-3')	Tm (°C)	Δ Tm (°C)#
1	GCG TGC CTC CTC ACT GGC	63.5	
2	GCG TGC CmUmCm CmUmC ACT GGC	56.2	- 7.3
3	GCG TGC CmUoCm CoUmC ACT GGC	67.2	+ 3.7
4	GmCmG TGC CTC CTC ACUm GmGmC	64.2	+ 0.7
5	GmCoG TGC CTC CTC ACoUm GoGmC	70.3	+ 6.8
6	GmCoGm UoGC CTC CTC ACoUm GoGmC	72.4	+ 8.9

#: Compared to oligo 1;
Normal letters represent 2'-deoxynucleosides; Bold letters represent 2'-O-methylribonucleosides; . Lower case letters o and m represent phosphodiester and methylphosphonate linkages respectively. rest of the internucleotide linkages are phosphorothioate.

The Tm of oligo **1** (PS-oligo) with complementary RNA is 63.5°C, substitution of five deoxynucleoside phosphorothioates in the middle of oligo **1** with five 2'-*O*-methylribonucleosides methylphosphonate (oligo **2**) resulted in lowering of thermodynamic stability, almost by 1.5°C per substitution. Substitution of six deoxynucleoside phosphorothioates

(four at 3'-end and two at 5'-end) with six 2'-*O*-methylribonucleoside methylphosphonate (oligo **4**) shows no compromise in Tm compared to oligo **1**. In our earlier studies, we have made similar observations that incorporation of non-ionic linkages in the center of the oligonucleotide affects the thermodynamic stability more than if non-ionic internucleotide linkages are incorporated at the 3'- and 5'-ends[7]. Our objective in this study was to reduce the number of phosphorothioate linkages in the oligonucleotide while maintaining its thermodynamic stability. We achieved this goal with oligo **4**. To further improve the thermodynamic stability, we introduced alternative methylphosphonate and phosphodiester linkages in the 2'-*O*-methylribonucleoside segment. Oligo **3** and oligo **5** provided us an increase in the Tm of 3.7 °C and 6.8°C respectively, compared with oligo **1**. The increase in Tm was dependent on the number of 2'-*O*-methylribonucleosides, as demonstrated by oligo **6** which has nine 2'-*O*-methylribonucleosides, compared to seven in oligo **5**, and showed Tm of 8.9°C higher than oligo **1**. Oligo **5** and Oligo **6** were found to have stabilities towards snake venom phosphodiesterase similar to that of oligo **4** (data not shown).

In summary, the MBOs synthesized here, which contain segments of oligodeoxynucleoside phosphorothioates and 2'-*O*-methyloligoribonucleoside methylphosphonate or alternated methylphosphonate and phosphodiester linkages provide us a means by which number of phosphorothioate internucleotide linkages can be reduced. These MBOs have higher thermodynamic stability and increased resistance towards nucleases. Further studies are ongoing to explore the use these MBOs as antisense agents.

References

[1] (a) *Antisense Therapeutics,* S. Agrawal, Ed. Humana Press, Totowa (New Jersey) (1996); (b) S. Agrawal, *Trends in Biotechnology,* **14,** 376 (1996); (c) S. Agrawal, and Q. Zhao, *Antisense & Nucleic Acid Drug Dev.* **8,** 135 (1998); (d) S. Agrawal,; *et al Biochem. Pharm.* **50,** 571 (1995); (e) D. Yu, R. P. Iyer, and S. Agrawal, *Bioorg. & Med. Chem,* **4,** 1685 (1996); (f) S. T. Crooke, *et al J. Pharm. Exp. Ther.* **277,** 923 (1996).

[2] S. Agrawal, M. P. Civiera, J. Goodchild, T. Ikeuchi, and P. C. Zamecnik, *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7448 (1988).

[3] (a) E. R. Kandimalla, J. Temsamani, and S. Agrawal, *Nucleosides and Nucleotides,* **14,** 1031 (1995); (b) J. M. Kean, S. A. Kipp, P. S. Miller, M. Kulka, and L. Aurelian, *Biochemistry,* **34,** 14617 (1995).

[4] R. P. Iyer, D. Yu, I. Habus, N. Ho, S. Johnson, T. Devlin, Z. Jiang, W. Zhou, J. Xie, and S. Agrawal, *Tetrahedron,* **53,** 2731 (1997).

[5] Y. S. Cho-Chung, M. Nesterova, S. Agrawal, and K. Noguchi, *Proc. Am. Assoc. Cancer Res.* 351 (1996).

[6] R. P. Iyer, D. Yu, J. Xie, W. Zhou, and S. Agrawal, *Bioorg & Med. Chem. Lett.* **7,**1443 (1997).

[7] S. Agrawal, Z. Jiang, Q. Zhao, D. Shaw, Q. Cai, A. Roskey, L. Channavajjala, C. Saxinger, and R. Zhang, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2620 (1997).