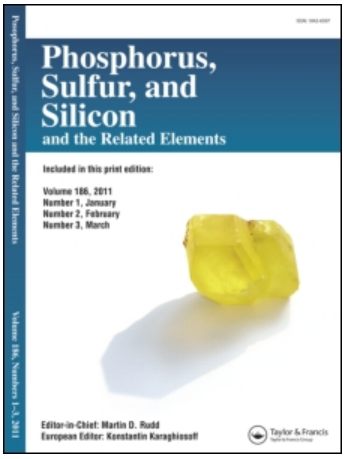


Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

Studies on Modified Oligonucleotides

Li He Zhang[a]; Zhen Jun Yang[a]; Liang Ren Zhang[a]
[a] State Key Laboratory of Natural & Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China

To cite this Article Zhang, Li He , Yang, Zhen Jun and Zhang, Liang Ren(2008) 'Studies on Modified Oligonucleotides', Phosphorus, Sulfur, and Silicon and the Related Elements, 183: 2, 326 — 334
To link to this Article: DOI: 10.1080/10426500701734539
URL: http://dx.doi.org/10.1080/10426500701734539

Phosphorus, Sulfur, and Silicon, 183:326–334, 2008

ISSN: 1042-6507 print / 1563-5325 online
DOI: 10.1080/10426500701734539

Taylor & Francis
Taylor & Francis Group

Studies on Modified Oligonucleotides

Li He Zhang, Zhen Jun Yang, and Liang Ren Zhang
State Key Laboratory of Natural & Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China

A series of isonucleotide incorporated antisense oligodeoxynucleotides have been synthesized, in which isonucleotide was introduced at different positions of the sequences. The results showed that though the incorporated isonucleotides at different position of the sequence interfered in the binding ability in different extent, B-form duplexes were maintained and the binding abilities of the 3′-end modified duplexes were better than the corresponding mismatched duplexes. The DNA / RNA hybrid formed by modified oligodeoxynucleotide and its target RNA could activate the activity of RNase H. The 3′-end modified antisense oligodeoxynucelotides showed the inhibition on S glycoprotein expression of SARS-CoV at the mRNA levels in insect Sf9 cells. In this presentation, a novel class of amino-isonucleoside was synthesized and incorporated into different positions in the sense or antisense strand of siRNA duplexes. The 3′ and 5′ sense strand aminonucleoside modified siRNAs (ssISO-siRNA1 and ssISO-siRNA2) showed the similar duplex thermal and serum stability as natural one and the luciferase activities showed that such modified siRNA is compatible with the intracellular siRNA machinery.

Keywords Isonucleoside; aminonucleoside; siRNA; antisense oligodeoxynucelotide

INTRODUCTION

The rapid and specific inhibition of gene product expression makes antisense technology appealing for therapeutic applications and analysis of the function of newly discovered genes[1] and since the term RNA interference (RNAi) was initially introduced by Fire and coworkers,[2] RNAi has been found to be a very powerful tool for gene regulation.

This work was supported by the National Natural Science Foundation of China (20332010 and SFCBIC 20320130046), the Ministry of Science and Technology of China (2005BA711A04) and Foundation for the Author of National Excellent Doctoral Dissertation of PR China (FANEDD200265).

Address correspondence to Li He Zhang, State Key Laboratory of Natural & Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, 38 Xue-Yan Road, Beijing 100083, China. E-mail: xbna@pku.edu.cn

The sequence-selective gene inhibition by siRNA and its rapid adoption as a powerful tool in cell culture has generated the expectation for its use to improve target therapeutics for cancer, metabolic, inflammatory, infectious, neurological and other types of diseases.[3] Although siRNA holds promise for combating human diseases, siRNA therapeutics is hindered by poor intracellular uptake, limited blood stability and non-specific immune stimulation. Several requirements must be fulfilled by a potential antisense oligonucleotide or siRNA including rapid penetration into cells, stability against the degradation by nuclease, delivery methods and dosing changes.[4–7] To improve the biostability of oligonucleotides, chemical modification is widely used. Among the modifications for antisense oligonucleotides, the phosphorothioate oligodeoxynucleotide is most frequently used, which is resistant to nuclease digestion, is recognized by RNase H, and is easily mass-produced. Though phosphorothioate oligodeoxynucleotides are currently used as the standard choice of chemically modified oligodeoxynucleotides, its non-sequence-specific protein binding leads to significant side effects, including complement activation, thrombocytopenia, inhibition of cell-matrix interaction, or reduction of cell proliferation. To overcome these limitations, intensive efforts were focused on the development of other kinds of chemically modified oligonucleotides, for example, modifications of the 2′-position of riboses with electronegative substituents such as the 2′-*O*-(2-methoxy)ethyl (MOE) group[8] or a 2′-*O*,4′-*C*-methylene bridge (locked nucleic acid; LNA) were performed.[9] But on the other hand, the RNA-like conformation leads to the disappearance of the oligonucleotide′s ability to activate RNase H, which is considered to be an important pathway of antisense action. In siRNA applications, modification of the base moiety is relatively less attempted. Most of the chemical modifications of oligonucleotide were done on the 2′-OH group of the ribose moiety. It was found that modifications at the 2′-position of pentose sugars in siRNAs are in general well tolerated.[10] Since such ribose modifications confer an RNA-like conformation to the oligonucleotide, they increase the target RNA binding affinity and cellular uptake.

STUDIES ON THE SYNTHESIS AND HYBRID PROPERTIES OF ANTISENSE OLIGONUCLEOTIDE CONTAINING ISONUCLEOSIDES

Isonucleosides represent a novel class of nucleoside analogues in which the nucleobase is linked to various positions of ribose other than C-1. Because of the shift of N-glycosidic bond, the chemical and enzymatic

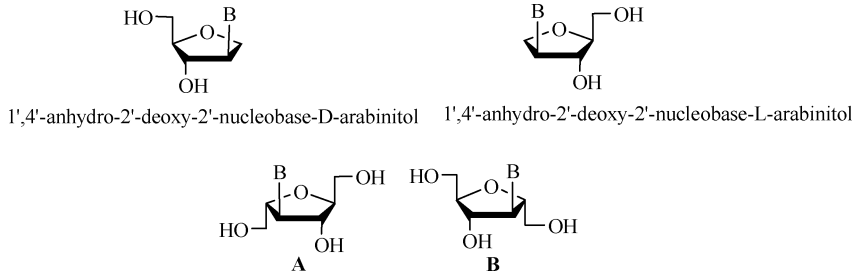


FIGURE 1 The structures of 1′,4′-anhydro-2′–deoxy-2′-nucleobase-L-arabinitol, 1′,4′-anhydro-2′-deoxy-2′–nucleobase -D-arabinitol, and 4-deoxy-4-nucleobase-2,5-anhydro-L- mannitols (A) 4-deoxy-4-nucleobase-2,5-anhydro-D-mannitols (B).

stabilities of the nucleoside are increased. Nair reported the synthesis of isonucleoside, 1′,4′-anhydro-2′-deoxy- 2′-nucleobase-D-arabinitol (Figure 1), and found that oligodeoxynucleotides containing such isonucleoside could form a duplex with complementary DNA sequence and antagonize the degradation of enzyme.[11]

We reported the synthesis of 1′,4′-anhydro-2′-deoxy-2′-nucleobase-L-arabinitol (Figure 1), the stereoisomer of 1′,4′-anhydro-2′-deoxy-2′-nucleobase-D-arabinitol. However, the oligonucleotide containing 1′,4′-anhydro-2′-deoxy-2′-nucleobase-L-arabinitol could not form regular base pair as described for its D-form partner. Since an RNA-like conformation of the modified oligonucleotide could increase the target RNA binding affinity and cellular uptake, we designed and synthesized 4-deoxy-4-nucleobase-2,5-anhydro-D-mannitols (B) and 4-deoxy-4-nucleobase-2,5-anhydro-L-mannitols (A) which contain an additional hydroxymethyl group at the sugar moiety.(Figure 1). The hybrid properties of the oligodeoxynucleotides incorporated with A or B with the complementary DNA were investigated.

The different linkage of the phosphate backbone in the oligonucleotide incorporated with isonucleoside B could influence the hybrid property with the complementary strand (Figure 2). Oligomer II (with $6' \rightarrow 4'$ linkage) failed to hybridize with $d(A)_{14}$, nevertheless, a stable

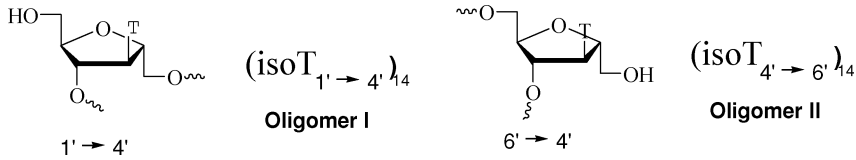


FIGURE 2 The different linkage of the phosphate backbone in the oligonucleotide incorporated with isonucleoside B.

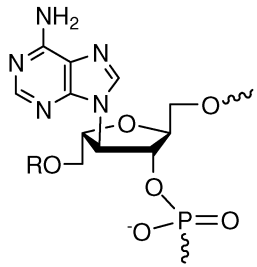
duplex of oligomer I (with $1' \rightarrow 4'$ linkage) with $d(A)_{14}$ was formed. Computer simulation showed that in I/$d(A)_{14}$ the C′6 hydroxyl group of each unit is located in the groove area when hybridized to the complementary strand where it can form hydrogen bonds with water in the medium, while in II/ $d(A)_{14}$ most of C′1 hydroxyl groups are directed to the inside of the duplex.[12] In the case of the oligonucleotide incorporated with isonucleoside A, the existence of hydroxymethyl group on the isonucleoside could stabilize the formed duplex with its complementary sequence, moreover, the octaoligoisonucleotide $(isoT)_2(isoG)_4(isoT)_2$ consisting of 4-deoxy-4-nucleobase-2,5-anhydro-L-mannitols (A) could form a parallel intermolecular G-quadruplex structure, K^+, Na^+ and Li^+ ($K^+ > Na^+ > Li^+$) can prompt the formation of G-quartet structures and stabilize them.[13]

The building block containing 4-deoxy-4-nucleobase-2,5-anhydro-L-mannitols (A) was synthesized and then formed the oligoisonucleotides I–IV as shown in Figure 3.

The hybrid properties of oligomers I–IV showed that when the isonucleotide was located at 3′-end, the duplex was more stable than the corresponding mismatched sequences, whereas when isonucleotide was located at the center of the sequence, the duplex was less stable than the corresponding single-nucleoside-mismatched sequence but was more stable than multinucleoside-mismatched sequence (Table I). Circular dichroism (CD) spectra showed that the hybrid duplex formed by modified oligodeoxynucleotides and their complementary sequence possessed very similar conformations, the single isonucleotide modified oligodeoxynucleotides formed typical

TABLE I Tm Value of the Duplexes of Isonucleoside Modified Oligonucleotides with the Complementary DNA Sequence

Duplexes	Tm (°C)	ΔTm(°C)
I/VII	59.3	−2.2
II/VI	54.2	−7.7
III/VI	58.6	−2.9
IV/VI	54.1	−7.4
V/VI	61.5	—
VII/VI	58.0	−3.5
VIII/VI	58.0	−3.5
IX/VI	49.1	−12.4
X/VI	56.2	−5.3
XI/VI	44.3	−17.2
XII/VI	37.1	−24.4



A_1: R = H;
A_2: R = Allyl

I	5'-d(AAC ATC TCC TGA GGG A**A_1**G)-3'
II	5'-d(AAC ATC TCC TG**A_1** GGG AAG)-3'
III	5'-d(AAC ATC TCC TGA GGG A**A_2**G)-3'
IV	5'-d(AAC ATC TCC TG**A_2** GGG AAG)-3'
V	5'-d(AAC ATC TCC TGA GGG AAG)-3'
VI	5'-d(CTT CCC TCA GGA GAT GTT)-3'
VII	5'-d(AAC ATC TCC TGA GGG A**T**G)-3'
VIII	5'-d(AAC ATC TCC TGA GGG A**TC**)-3'
IX	5'-d(AAC ATC TCC TGA GGG **TTC**)-3'
X	5'-d(AAC ATC TCC TG**T** GGG AAG)-3'
XI	5'-d(AAC ATC TCC T**CT** GGG AAG)-3'
XII	5'-d(AAC ATC TCC T**CT C**GG AAG)-3'

FIGURE 3 The sequences of isonucleotide-incorporated oligodeoxynucleotides.

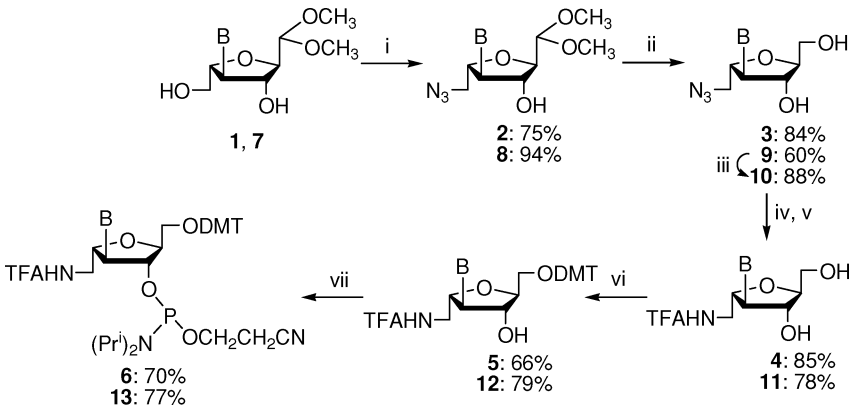
B-form duplexes such as natural oligodeoxynucleotides. The computer simulation results also showed that B-form duplexes were maintained during the entire course of simulation, the helical parameters for the modified oligonucleotide duplexes were very similar to the native DNA duplex. The averaged structure of the native DNA duplex was overlapped with the isonucleotide-modified duplexes, respectively.

The above result indicated that the incorporation of isonucleotide into the oligodeoxynucleotide might only lead to the local conformational change of the duplex. The DNA/RNA hybrid formed by a modified oligoisonucleotide and its target RNA could activate RNase H. The 3′-end modified antisense oligoisonucelotides I and III inhibited

S-glycoprotein expression of SARS-CoV at the mRNA levels in insect Sf9 cells.[14]

STUDIES ON THE SYNTHESIS, HYBRID AND SILENCING PROPERTIES OF SIRNA CONTAINING AMINO-ISONUCLEOSIDES IN SENSE OR ANTISENSE STRAND

For aminoisonucleotide-modified oligonucleotides, introduction of amino groups may increase their thermal stability via the strong binding of cationic amino groups with negative charged phosphate backbones of the complementary sequences. Building blocks **7** and **13** for solid phase oligonucleotide synthesis were synthesized from starting materials **1** and **2** (22), respectively (Scheme 1). We developed a one-step method that at room temperature compound **1** or **2** was directly reacted with sodium azide in the presence of triphenyl phosphine and carbon tetrabromide, giving the key intermediate **3** or **8** in good yield.[15] Incubation of compound **3** or **8** with 1% HCl followed by sodium hydroxide and sodium borohydride in second one-pot reaction at 0°C gave unprotected azido isonucleoside **4** or **9** at yield of 84% and 76%, respectively. After the sequential protection and deprotection, the obtained building blocks **7** and **13** were finally incorporated into the sequences of oligonucleotides according to the solid phase protocols. Amino-isonucleoside building blocks **7** and **13** were incorporated into the sense or antisense strand of a designed siRNA.



1 - **6**: B = thymin-1-yl; **7** - **9**: B = adenin-9-yl; **10** - **13**: B = N^6-benzoyl-adenin-9-yl

SCHEME 1 Synthesis of amino-isonucleoside building block.

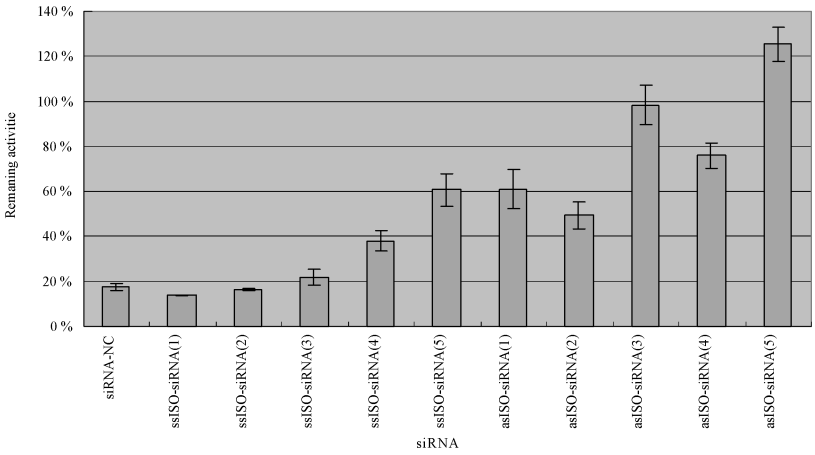
TABLE II Sequences and Tm Values of Native siRNA (siRNA-NC) and Amino-Isonucleoside Modified Sense Strand (ssISO-siRNA 1-5)

Name	Sequence	T_m(°C)
siRNA-NC	5′-CUGGUAAAGCAUUCAGUAUtt-3′ 3′-ttGACCAUUUCGUAAGUCAUA-5′	66
ssISO-siRNA(1)	5′-CUGGUAAAGCAUUCAGUA**T**tt-3′ 3′-ttGACCAUUUCGUAAGUCAUA-5′	66
ssISO-siRNA(2)	5′-C**T**GGUAAAGCAUUCAGUAUtt-3′ 3′-ttGACCAUUUCGUAAGUCAUA-5′	66
ssISO-siRNA(3)	5′-CUGGUAA**A**GCAUUCAGUAUtt-3′ 3′-ttGACCAUUUCGUAAGUCAUA-5	62
ssISO-siRNA(4)	5′-C**T**GGUAAAGCAUUCAGUAU**T**t-3′ 3′-ttGACCAUUUCGUAAGUCAUA-5′	56
ssISO-siRNA(5)	5′-C**T**GGU**A**AAGCAUUC**A**GUA**T**t-3′ 3′-ttGACCAUUUCGUAAGUCAUA-5′	36

It was found that incorporation of one amino-isonucleoside at 3′ or 5′ terminal of modified oligonucleotides exhibited no effect on the duplex thermal stability which is equal to native siRNA form, siRNA NC. However, internal incorporation of one amino-isonucleoside into siRNA duplexes decreased the thermal stability. Furthermore, when two or more amino-isonucleosides were incorporated into siRNAs, Tm values decreased strikingly (Table II).

Incubation of modified siRNAs with 50% FBS at 37°C indicated that siRNAs with a single amino-isonucleoside at the sense strand, such as ssISO-siRNA1, ssISO-siRNA2, and ssISO-siRNA3, exhibited the similar serum stability as their native siRNA with all degraded in 3 h. Modified siRNAs with two or more amino-isonucleosides at the sense strand, such as ssISO-siRNA4 and ssISO-siRNA5, showed further weaker serum stability compared with the native siRNA and rapidly degraded within 1.5 h. The firefly luciferse-targeting modified siRNAs with amino-isonucleosides in either the sense strand or antisense strand were evaluated based on a dual-luciferase assay that renillar luciferase acts as an internal control. We found that sense strand-modified siRNAs with a single amino-isonucleoside at the 3′- or 5′-end, such as ssISO-siRNA1 and ssISO-siRNA2, exhibited comparative and even better activity than the native siRNA NC (Table III). Alternative modifications with amino-isonucleoside at the siRNA internal region, such as ssISO-siRNA3, bring some negative effect on siRNA activity compared with the terminal modification, and such negative effect become further apparent when internal and terminal modification in the sense strand were combined together (Table III). In contrast to

TABLE III *Firefly Renilla* Luciferase Activities of siRNA, Different ssISO-siRNA and asISO-siRNA



All experiments were performed in triplicates and repeated at least twice.

sense strand modifications, antisense strand modification led to more negative effect on the silencing activity of siRNA duplex. A single nucleotide modification at the 3′ or 5′-end of the antisense strand reduced the siRNA silencing activity to 40%–50%. Internal modification of the antisense strand of siRNA has more negative effect on the silencing activity than the terminal modification. Terminal modification at both the 3′- and 5′-end of the antisense strand reduced the silencing activity of the siRNA significantly but does not abolish the silencing activity completely. However, combination of the terminal and internal modifications on antisense strand totally abolish the silencing activity.

Here, we conclude that siRNA sense strand modification has less effect on siRNA activity while antisense strand modification significantly reduces siRNA activity. Our data also suggests: 1) the bases in modified siRNAs, despite of their lower activity, retain their hybridization properties with complementary sequences; 2) the torsion angles in the sugar-phosphate backbones would not perturb the entry of the modified siRNA into RISC; and 3) the two strands of the siRNA duplex are not equally eligible for assembly into RISC.

Isonucleoside modified oligonucleotide is superior to other chemical modifications since such modified RNAs are good substrates of RNase H and isonucleoside triphosphates can be recognized by many different DNA polymerases.[16] Furthermore, siRNA sense strand modification with amino-isonucleoside at the 3′ or 5′-end showed comparable

gene-silencing activity and serum stability as their native siRNA. Taken together, isonucleoside modified oligonucleotide represents a novel direction and is a good mimic of natural oligonucleotide with the required biological and chemical properties.

REFERENCES

[1] E. Uhlman and A. Peyman, *Chem. Rev.*, **90,** 543 (1990).
[2] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello, *Nature*, **391,** 806 (1998).
[3] D. Zhou, Q. S. He, C. Wang, J. Zhang, and F Wong-Staal, *Current Topics in Medicinal Chemistry*, **6,** 901 (2006).
[4] R. W. Wagner, *Nature*, **372,** 333 (1994).
[5] S. T. Crooke, *Curr. Opin. Biotechnol.*, **3,** 656 (1992).
[6] V. V. Vlasov, L. A. Balakireva, and L. A. Yakubov, *Biochim. Biophys. Acta*, **1197,** 95 (1997).
[7] A. M. Krieg, J. Tonkinson, S. Matson, Q. Zhao, M. Saxon, L. Zhang, U. Bhanja, L. Yakubov, and C. A. Stein, *Proc. Natl. Acad. Sci. USA*, **90,** 1048 (1993).
[8] B. F. Baker, S. S. Lot, T. P. Condon, S. Cheng-Flournoy, E. A. Lesnik, H. M. Sasmor, and C.F. Bennett, *J. Biol. Chem.*, **272,** 11994 (1997).
[9] C. Wahlestedt, P. Salmi, L. Good, J. Kela, T. Johnsson, T. Hokfelt, C. Broberger, F. Porreca, J. Lai, K. Ren, M. Ossipov, A. Koshkin, N. Jakobsen, J. Skouv, H. Oerum, M. H. Jacobsen, and J. Wengel, *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 5633 (2000).
[10] C. R. Allerson, N. Sioufi, R. Jarres, T. P. Prakash, N. Naik, A. Berdeja, L. Wanders, R. H. Griffey, E. E. Swayze, and B. Bhat, *J. Med. Chem.*, **48,** 901 (2005).
[11] T. Wenzel, V. Nair, *Bioorg. Med. Chem. Lett.*, **7,** 3195 (1997); (b) T. Wenzel and V. Nair, *Bioconjugate Chem.*,**9,** 683 (1998).
[12] L. Zhen, L. Zhang, L. R. Zhang, J. Chen, J. M. Min, and L. H. Zhang, *Nucleic Acids Research*, **29,** 1470 (2001).
[13] J. Cheng, L. R. Zhang, J. M. Min, and L. H. Zhang, *Nucleic Acids Research*, **30,** 3005 (2002).
[14] Z. L. Wang, J. F. Shi, H. W. Jin, L. R. Zhang, J. F. Lu, and L. H. Zhang, *Bioconjugate Chemistry*,**16,** 1081 (2005).
[15] Z. S. Li, R. P. Qiao, Z. J. Yang, L. R Zhang, and L. H. Zhang, *Tetrahedron:Asymmetry*, **17,** 1056 (2006).
[16] C. W. Jiang, B. C. Li, Z. Guan, Z. J. Yang, L. R. Zhang, and L. H. Zhang, *Bioorg. & Med. Chem.*, **15,** 3019 (2007).