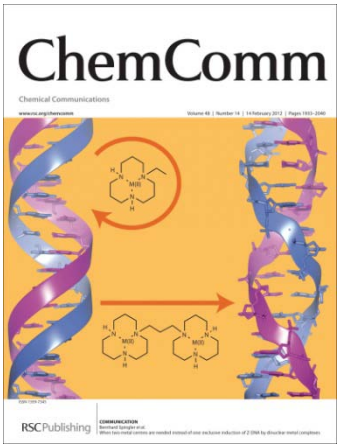

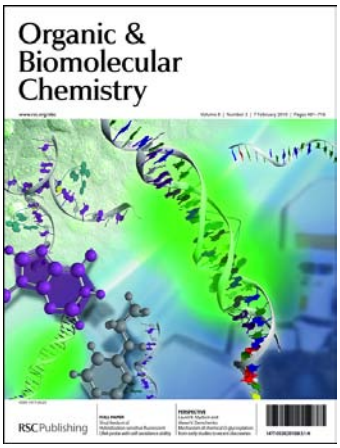

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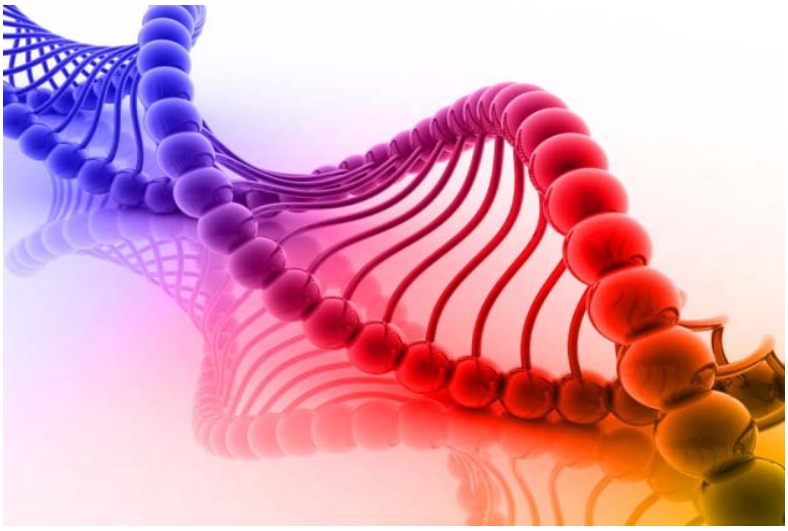

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PAPER

Synthesis and properties of novel L-isonucleoside modified oligonucleotides and siRNAs†‡

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Antisense oligonucleotides and siRNAs are potential therapeutic agents and their chemical modifications play an important role to improve the properties and activities of oligonucleotides. Isonucleoside is a type of nucleoside analogue, in which the nucleobase is moved from C-1 to other positions of ribose. In this report, a novel isonucleoside **5** containing a 5′-CH_2-extended chain at the sugar moiety was synthesized, thus isoadenosine **5a** and isothymidine **5b** were incorporated into a DNA single strand and siRNA. It was found that isonucleoside **5** modified oligonucleotides can form stable double helical structures with their complementary DNA and RNA and the stability towards nuclease and ability to activate RNase H are more promising compared with the unmodified, natural analogues. In siRNA, passenger strand modified with isonucleoside (**5a/b**) at 3′ or 5′ terminal can retain the silencing activity and minimize the passenger strand specific off-target effect.

Introduction

Antisense oligonucleotides (ASONs) and small interfering RNAs (siRNAs) have attracted considerable attention of both biologists and chemists due to their potent therapeutic activities. An antisense oligonucleotide is a single strand of DNA which is able to form a hybridized duplex with its complementary mRNA resulting in the specific inhibition of gene expression by various mechanisms.[1] On the other hand, the later discovered RNAi technique uses double stranded RNAs to inhibit the translation of target gene through an activated RNA-induced silencing complex (RISC*), which includes one single strand of siRNA (the guide strand) and the endonuclease Argonaute 2 (Ago 2) that promotes the cleavage of target mRNAs.[2] These nucleic acids target endogenous mRNA, and modulate gene expression based on the Watson–Crick base pairing.

The main challenge for translating the antisense strategy into clinical applications is how to solve the problems relating to the stability of antisense oligonucleotides in blood and their delivery to a target.[3] Chemically synthesized antisense oligonucleotide has great advantages in tolerating chemical modifications, delivery methods, and dosage changes.[4] Phosphorothiate(PS)-modified ASONs are first generation ASONs, which exhibit high stability against nuclease and promote RNase H activity.[5] However, phosphorothiate modification produces non-specific toxicity. Another modification was reported with elongated phosphodiester backbone, which increases ASONs' stability toward 3′-exonuclease and causes a slightly lower T_m value of duplexes.[6] Other modifications focused on the ribose moiety, that included 2′-*O*-modified ASONs, locked nucleic acid (LNA),[7] peptide nucleic acid (PNA),[8] phosphoramidate morpolino oligomer (PMO).[9] These chemical modifications have also been applied to siRNAs, and proved to be an efficient approach to improve the biophysical and biochemical properties of siRNAs.[10] A significant modification of siRNA was at 2′-*O*-position of the sugar moiety such as 2′-*O*-methyl (2′-*O*-Me)[11] and 2′-deoxy-2′-fluoro (2′-F)[12] substitutions, and locked nucleic acid (LNA).[13,14]

Besides the short half-life time ($t_{1/2}$) in serum and poor penetration through cell membrane, off-target silencing is a fundamental feature of siRNAs and cannot always be predicted and eliminated.[15,16] "Off-target" effect could be the immune responses[17] or knockdown of extra genes usually initiated by passenger strand or 'miRNA' like activities.[15] Appropriately modified sense strand of siRNAs could significantly reduce the "off-target" effect while maintaining silencing activity.[18] It was reported that thermodynamic stability and Watson–Crick base pairing as main determinants of the siRNA-based off-target effects and an siRNA with low thermal stability could show lower off-target effects.[19]

Our previous work indicated that the incorporation of isonucleoside into the oligodeoxynucleotide might lead to some local conformational change of the formed duplexes and lower T_m

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† In memory of Professor Har Gobind Khorana (1922–2011), acknowledging his legacy to the scientific community.

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§ These authors contributed equally to this paper.

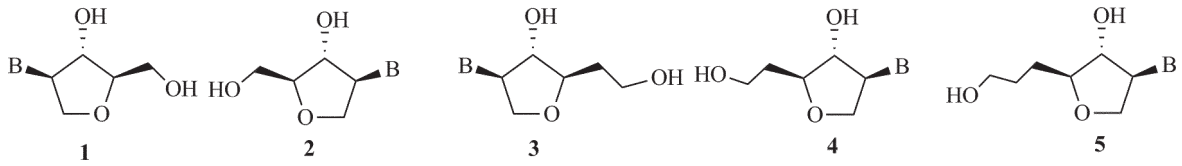


Fig. 1 Isonucleoside **1**,**2**,**3**,**4** and the target compound **5**.

value. Isonucleoside is a novel type of nucleoside analogue, in which the nucleobase is moved from C-1 to other positions of ribose. Oligonucleotides consisting of isonucleosides (**1–4**, Fig. 1) showed greatly increased stability towards nuclease and sufficient affinity toward their complementary DNA or RNA.[20] The DNA/RNA hybrid formed by an isonucleoside modified oligodeoxynucleotide and its target RNA could activate RNase H. The 3′-end modified antisense oligodeoxynucleotides inhibited S-glycoprotein expression of SARS-CoV at the mRNA levels in insect Sf 9 cells.[21] When aminoisonucleoside was incorporated into a luciferase gene-targeting siRNA, it was found that sense strand modifications with aminoisonucleoside at the 3′ or 5′ terminal have less effect on RNA duplex thermal and serum stabilities, and their functional activities are also comparable to their native siRNAs. In contrast, antisense strand modifications with aminoisonucleoside at the corresponding positions brought a strikingly negative effect on RNA duplex stability but still maintained around 40–50% of gene knockdown.[22]

The novel isonucleoside **5** contains a 5′-CH_2-extended chain at the sugar moiety and the oligonucleotides incorporated with isonucleoside **5** would provide an extended phosphate linkage which may modulate the thermodynamic stability of the formed duplex and initiate to reduce the off-target silencing in an unintended target. Here we report the synthesis of a novel type of isonucleoside **5a/b** (Fig. 1) from D-xylose. Isoadenosine **5a**/isothymidine **5b** modified ODNs and siRNA were synthesized, their stabilities against nuclease, ability to activate RNase H and silencing activity of siRNA modified with isonucleosides (**5a/b**) in either strand and at different positions were investigated. A fragment of cdc2 gene was used as the target.[23] The sequence of siCdc2 was chosen from many variants because each strand of this siRNA had considerable activity for its respective target mRNA in an artificial RNAi assay. To evaluate the influence on silencing activity and off-target effect, we constructed two systems: each of this system contained one strand of siRNA as target *via* siQuant vector, matched with the siRNA A strand (sense strand) or B strand (antisense strand), and chemical modification on each one strand of siRNA was carried out and tested by these two systems respectively.

Results

1 Synthesis of isonucleoside 5a/5b and 5a/5b modified oligodeoxynucleotides[24a]

Compound **6a** obtained from D-xylose[24b,25] was benzoylated and then hydrolyzed to yield aldehyde **7**. Compound **8** was obtained from **7** under Wadsworth–Horner–Emmons reaction condition in an overall 75% yield, **8a** (*E*-isomer, J = 15.5 Hz) was yielded as the major product with minor **8b** (*Z*-isomer, J = 11.5 Hz). Catalytic hydrogenation of **8** gave **9**, which was then converted to epoxide **10** in high yield. Epoxide **10** was converted to the desired isoadenosine **11** in 35% yield along with **12** in 10% yield. It was presumed that compound **12** could be obtained by the reaction of compound **11** and dimethylamine, which may be formed by DMF in the presence of DBU at 110 °C (Scheme 1).

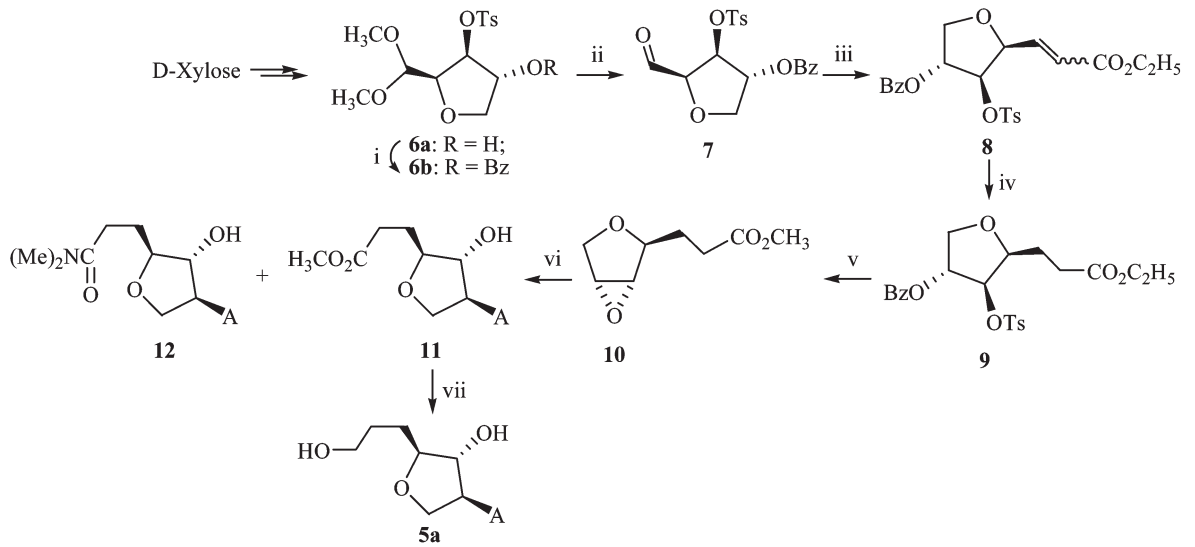
To solve this side reaction, reduction of the ester in **9** was carried out using $LiAlH_4$ to give **13** in 96% yield. After protection of the primary hydroxyl in **13** with TBDMSCl, the key intermediate **14** was treated with nucleobases (adenine or thymine) and DBU in dry DMF to obtain the desired isonucleosides, isonucleoside **15a** was separated with its C3-regioisomer **16a** at 8 : 1 ratio. After deprotection of TBDMS group, **5a** was afforded in reasonable yield. **15b** and its regioisomer **16b** (6 : 1) could not be separated by silica column chromatography directly. After deprotection, pure **5b** was separated (Scheme 2). The structures of the two desired isonucleosides **5a** and **5b** were also confirmed by ^{1}H NMR, COSY and NOESY spectra.

Building blocks **19** and **21**, suitable for solid-phase oligonucleotide synthesis, were prepared from **5a/b** respectively (Scheme 3). The exocyclic amino and primary hydroxyl groups in isoadenosine **5a** were protected by benzoyl group using a one-pot procedure[26] to give **17**, and then by dimethoxytrityl group to give **18**, which was converted to the phosphoramidite **19**. The corresponding phosphoramidite **21** was prepared from isothymidine **5b** by a similar procedure (Scheme 3).

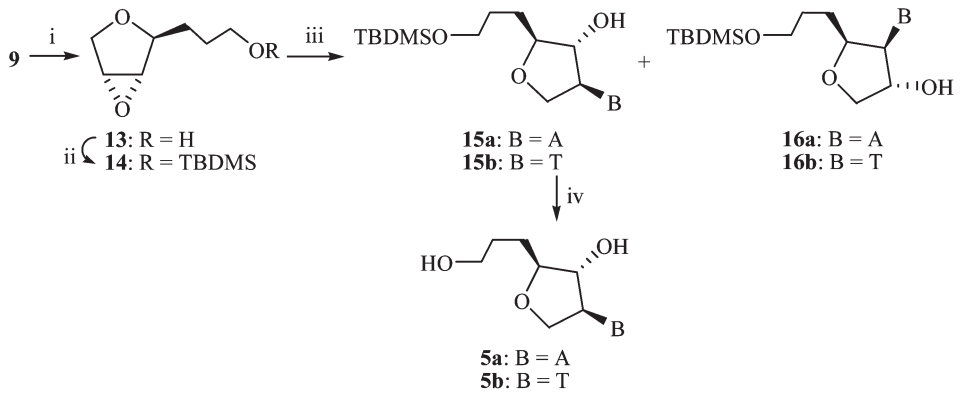
Oligodeoxynucleotides (**I–IV**) containing **5a** were synthesized by solid phase phosphoramidite chemistry (DMT off) using an *Applied Biosystems model 381* DNA Synthesizer. For convenience the synthesis was started with commercially available controlled pore glass with cytidine-loaded or universal CPG (used for **III**) and followed the standard protocol except for a prolonged isonucleosides' coupling time of 900s to ensure adequate coupling yields. The coupling efficiency was determined by the release of DMT. Total yield of each oligonucleotide was around 40%. The isonucleoside coupling yield was about 93%, which was slightly lower than native nucleoside coupling yield (~96% in our experiment). The structures of the modified oligonucleotides were determined by MALDI-TOF mass spectrometry: **I**. calcd 5945.9, found 5940.7; **II**. calcd 5945.9, found 5940.9; **III**, calcd 6259.1, found 6253.9; **IV**, calcd 5974.0, found 5976.9.

2 Stabilities of the formed DNA/DNA, DNA/RNA duplexes and activation of RNase H

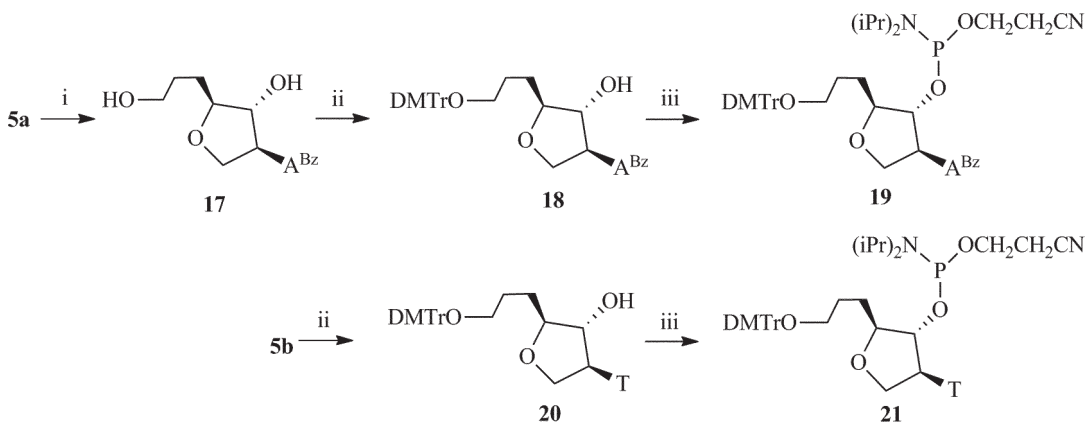
The thermal stabilities of the formed DNA/DNA and DNA/RNA duplexes are listed in Table 1. The results show that all the



Scheme 1 Reagents and Conditions: i. BzCl, DMAP, Py, 98.1%; ii. TFA–H_2O (v : v = 7 : 1); iii. $(Et_2O)_2P(O)$-CH_2COOEt, NaH, THF, −30 °C; iv. Pd-C, H_2(0.4 MPa), THF, 67.9% from **7**; v. K_2CO_3, MeOH, 96.0%; vi. Adenine, DBU, DMF, 34.7% for **11**; 10% for **12**; vii. $LiAlH_4$, THF, 80.4%.



Scheme 2 Reagents and conditions: i. $LiAlH_4$, THF, room temp, 96.2%; ii. TBDMSCl, imidazole, DMF, 94.6%; iii. Adenine or thymine, DBU, DMF, 50.8% for **15a**, 6.4% for **16a**; 41.3% for **15b** and **16b**; iv. TBAF, THF, 96.9% for **5a**, 93.2% for **5b**.



Scheme 3 Reagents and Conditions: i. a. TMSCl, Py; b. BzCl, Py; c. $NH_3.H_2O$ (pH 8–9); 94% for **17**; ii. DMTrCl, Py; 79% for **18**; 68% for **20**; iii. Tetrazole, $NCCH_2CH_2OP[N(iPr)_2]_2$, CH_2Cl_2; 91% for **19**; 50% for **21**.

oligomers can form the stable duplexes with their complementary DNA or RNA and give the very similar CD spectra (data not shown). When **5a** was incorporated in the center of oligomers (**I**, **IV**), the T_m value of DNA/DNA duplexes decreased significantly compared to their native counterpart, and the T_m value of DNA/RNA duplexes decreased by 6 °C.

Table 1 ODNs (**I–IV**) with isonucleoside **5a** modified at 3′ or 5′ terminal or middle position, ODNs (**M1**, **M2**, **M3**) with single mismatched nucleoside, ODN (**Ds**) with phosphorothiate modification. Thermal denaturation studies of these duplexes with complementary sequences (**C1**: 20-*mer* complementary DNA of **D1**, **C2**: 21-*mer* complementary DNA of **D2**, **Cr**: 20-*mer* complementary RNA for natural and modified **D1**)

ODNs	ODN sequence synthesized	DNA/DNA T_m (°C)	ΔT_m (°C)	DNA/RNA T_m (°C)	ΔT_m (°C)
D1	5′- d(ACA TCT CCC GCA TCC CAC TC) -3′	68.0[a]	—	75.0[c]	—
D2	5′- d(ACA TCT CCC GCA TCC CAC TCA) -3′	69.0[b]	—	—	—
I	5′- d(ACA TCT CCC GC***5a***TCC CAC TC) -3′	59.1[a]	−8.9	69.1	−5.9[c]
II	5′- d(***5a***CA TCT CCC GCA TCC CAC TC) -3′	67.2[a]	−0.8	75.1	0.1[c]
III	5′- d(ACA TCT CCC GCA TCC CAC TC***5a***) -3′	68.3[a], 69.1[b]	0.3[a], 0.1[b]	76.1	1.1[c]
IV	5′- d(AC***5a***TCT CCC GCA TCC C***5a***C TC) -3′	57.1[a]	−10.9	69.2	−5.8[c]
Ds	5′-d(ACA TCT CCC GCA TCC CACTC)-3′ (**PS**)	59.1[a]	−8.9	67.0	−8.0[c]
M1	5′-d(***T***CA TCT CCC GCA TCC CAC TC)-3′	67.1[a]	−0.9	76.1	1.1[c]
M2	5′-d(ACA TCT CCC GC***T*** TCC CAC TC)-3′	62.1[a]	−5.9	71.1	−3.9[c]
M3	5′-d(AC***T*** TCT CCC GCA TCC C***T***C TC)-3′	58.2[a]	−9.8	70.2	−4.8[c]
C1	5′- d(GAG TGG GAT GCG GGA GAT GT)-3′	—	—	—	—
C2	5′- d(T GAG TGG GAT GCG GGA GAT GT)-3′	—	—	—	—
Cr	5′- GAG UGG GAU GCG GGA GAU GU -3′	—	—	—	—

[a] T_m value of ODN/D1 duplexes. [b] T_m value of ODN/D2 duplexes. [c] ΔT_m : Compared to **D1**/**Cr** duplex's Tm value (75.0 °C)

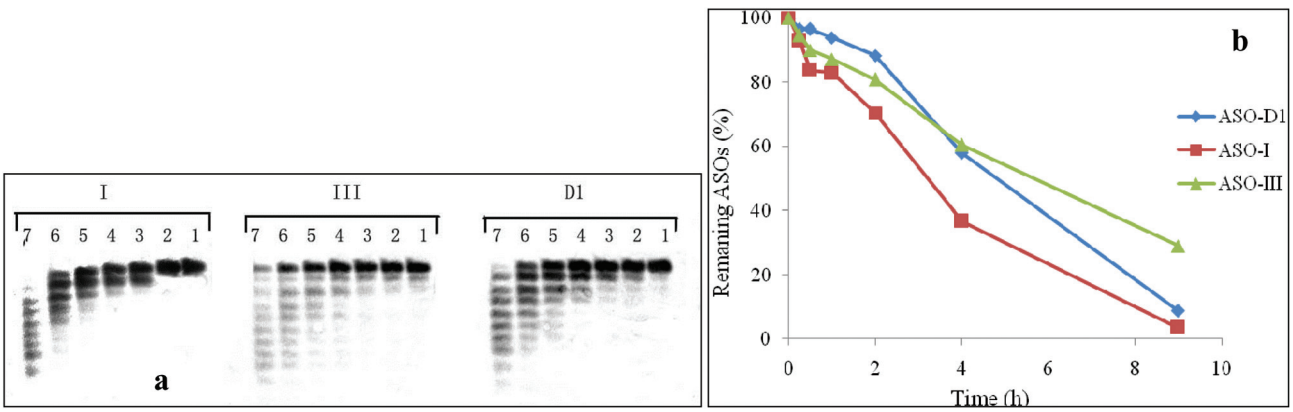


Fig. 2 **a**. PAGE analysis of the degradation of ODN **I**, **III** and **D1** in 80% fetal bovine serum. Lanes 1 to 7 indicate the time points after 0, 15 and 30 min, 1, 2, 4 and 9 h of reaction, respectively. The percentage % of ODNs left after 9 h of incubation: 0% of **D1**, 0% of **I** and 18% of **III**; **b**. Time course of ODN **D1**, **I**, **III** stability in 80% fetal bovine serum.

However, when **5a** was incorporated in 5′-end (**II**) or 3′-end of oligomers (**III**), the T_m values of DNA/DNA duplexes are similar as the native oligomer, and the T_m value of DNA/RNA duplexes increased by 1 °C. For comparison, nucleoside-mismatched oligomers (**M1**, **M2**, **M3**) were used for the investigation of T_m value of DNA/DNA or DNA/RNA duplexes. It was found that mismatched oligomer at 3′ or 5′ or middle position (**M1**, **M2** and **M3**) makes the decrease of T_m value of the formed double helix. It would indicate that the extended phosphodiester linkage in the modified strand might be more flexible and could not influence the formation of DNA/RNA duplexes.

The tolerance of the modified ODNs **I** and **III** toward an exonuclease (snake venom phosphodiesterase, SVPDE) was also investigated. It is expected that **5a** at 3′-end of ODN makes the oligomer more stable: the native **D1** was completely degraded in 20 min of incubation, while 100% of ODN-**III** was left after 20 min incubation with the enzyme (date not shown). Stabilities of ODN **I** and **III** in serum were also tested. It was found that ODN **III** showed a better stability than that of ODN **D1** (native) and **I** in 80% fetal bovine serum (Fig. 2).

RNase H is an endogenous enzyme, which hydrolyses the RNA strand in an RNA/DNA hybrid in a catalytic manner.[27] Activation of RNase H activity by ODN **I** and ODN **III** was investigated in comparison with native ODN **D1** and PS-modified ODN **Ds**. In Fig. 3-a, the RNA in duplex **III**/RNA had almost the same susceptibility to RNase H compared to native duplex. It seemed that RNA in **I**/RNA showed a little more susceptibility than in Ds/RNA (Fig. 3-b). These results show that duplex of RNA and ODN **I** with **5a** in the middle is a good substrate for RNase H. It may be that the enhancement of RNase H activity arises due to distortion of the conformation of ODN **I**, which makes the enzyme to hydrolyze the RNA strand.

3 Synthesis of 5a/5b modified siRNAs

Using standard phosphoramidite method (DMT-off), building blocks **19** and **21** were incorporated into the sense or antisense strand of the designed siRNA. Controlled pore glass with thymine-loaded CPG was used for the synthesis of each strand,

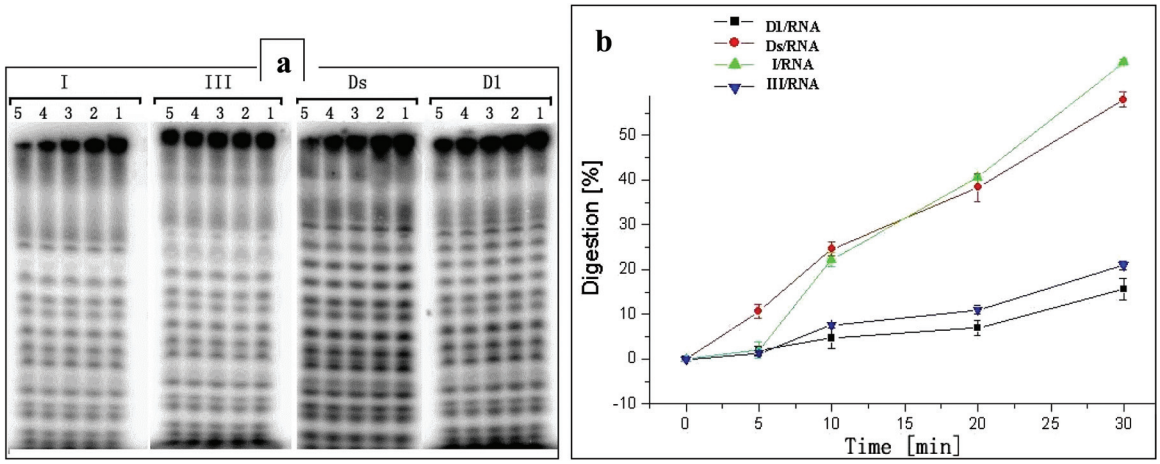


Fig. 3 (a) Autoradiograms of 20% denaturing PAGE, showing the cleavage kinetics of 5′-^{32}P-labeled target RNA by *E. coli* RNase H1 in the native 20 *mer* **D1** /RNA, the native 20 *mer* **Ds**/RNA, **III**/RNA and **I**/RNA hybrid duplexes. Lanes 1 to 5 indicate the aliquots of the digest taken after 0, 5, 10, 20, and 30 min, respectively. Conditions of cleavage reaction: RNA (0.8 μM) and the oligodeoxynucleotides (4 μM) in buffer, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$ and 0.1 mM DTT at 21 °C; 0.8 U of RNase H. Total reaction volume was 11 μL. (b) Time course of RNase H cleavage of hybrid duplexes. The digestion (%) shows the extent of cleavage at time points.

Table 2 Cdc2-siRNA and modified single RNA strand

	Synthesized RNAs	MW (calcd)	MW (found)
Native	**A**: 5′- UCG GGA AAU UUC UCU AUU Att -3′ **B**: 3′- tt AGC CCU UUA AAG AGA UAA U -5′		
***A** strand modification*:			
AI	5′-***5b***CG GGA AAU UUC UCU AUU Att -3′	6604.99	6601.12
AII	5′- UCG GGA AAU UUC UCU AUU***5a***tt -3′	6604.99	6604.42
***B** strand modification*:			
BI	3′- tt AGC CCU UUA AAG AGA***5b***AA U -5′	6674.11	6673.03
BII	3′- tt***5a***GC CCU UUA AAG AGA UAA U -5′	6674.11	6672.21
BIII	3′- tt AGC CCU UUA AAG AGA UA***5a***U -5′	6674.11	6673.03

t = dT.

and coupling time for isonucleoside building block was increased to 15 min to ensure satisfactory reaction yields. The yield of each single RNA strand was determined according to the release of DMT. For comparison, oligonucleotides incorporated by **5a** and **5b** at 3′ or 5′ terminal of both strands were synthesized respectively (Table 2, *t* = dT). After the synthesis and purification, 5 single stranded RNAs were obtained and identified by MALDI-TOF MS.

4 Silencing activities of L-isonucleoside modified siRNAs on guide strand specific and passenger strand specific targets

Isonucleoside **5a/b** modified ODNs could increase the stability of oligomers and alter the local conformation of hybridized duplexes. It would also be interesting to investigate the silencing activity of these modified siRNAs. A siQuant^{TM} vector, the fusion luciferase reporter plasmids carrying the target site of the functional cdc2-siRNA (target sequence complementary to B strand in Fig. 4, **upper**), was constructed, and the dual-luciferase assay was carried out to determine the percentage of intact target mRNA. According to the normalized FL/RL signals in our experiment, it was found that sense strand (passenger strand) of siRNAs modified with isonucleoside at the 3′ or 5′ terminal position showed comparative silencing activity (**AI/B**; **AII/B**, 93–95%) as the native one (95%) (Fig. 4, **upper**). It was confirmed that the passenger strand generally had better toleration toward isonucleoside modification.[22] Isonucleoside incorporating in antisense strand (guide strand) adversely affected the siRNAs silencing activity to various degrees. With single isonucleoside at the 5′-terminal position of guide strand, siRNAs (**A/BI**, **A/BIII**) silencing activity dropped, but the 3′-terminal modified guide strand (antisense) siRNA(**A/BII**) remained similar silencing activity (91%) toward *cdc2* mRNA as native siRNA. This indicated that isonucleoside **5a/5b** modification on guide strand has a lesser influence on the silencing activity.

The main off-target silencing is mediated by a guide strand and the passenger strand of a siRNA can assemble into RISC and serve as the guide strand, thus initiating passenger strand-specific off-targeting. It would be interesting to test these modified siRNAs on the off-target silencing activities mediated by a guide strand. A siQuant^{TM} vector containing luciferase reporter and the target sequence complementary to strand A in *cdc2*-siRNA was also constructed (Fig. 4, **bottom**). Here the normalized FL/RL signals indicated the inhibitory efficiency of siRNA toward to passenger strand specific off-target effect. Interestingly, **AI/B** and **AII/B** showed only less than 15% and 35% activity, respectively, in comparison with native siRNA which showed 80% silencing activity. Obviously, **AI/B** and **AII/B** show a significant selectivity between the siRNA silencing and off-target effect mediated by guide strand. On the contrary, **A/BII** and **A/BIII** still retain about 90% silencing activity. The

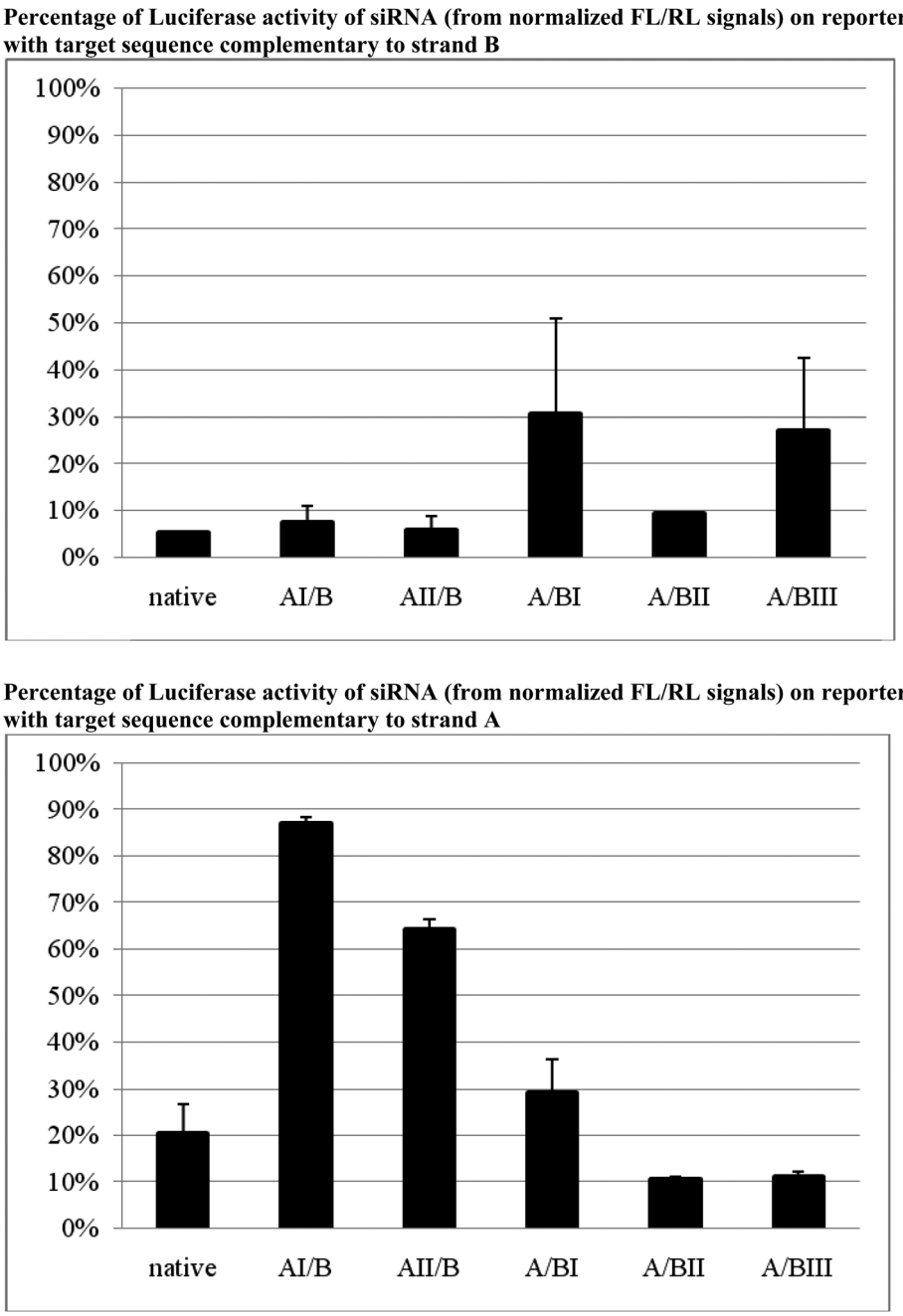


Fig. 4 Inhibitory effects of HEK-293 cells were expressed in normalized ratios between the Renilla luciferase and the firefly luciferase activities. (Upper: target sequence complementary to strand B, Bottom: target sequence complementary to strand A.).

data indicate that L-isonucleoside **5a**/**5b** modified at 3′ or 5′ terminal of passenger strand of siRNA could retain silencing activities and reduce the off-target effect mediated by guide strand.

Discussion

A number of studies have indicated that flexibility and minor groove width are important structural factors governing DNA/RNA hybrid duplex recognition by RNase H.[28] The hybrid DNA/RNA duplex in the vicinity of the RNase H active site exhibits irregularities in base pairing, which suggest that the helical conformation of the hybrid is very flexible. Furthermore, the minor groove width for this region of the hybrid is greatly diminished and the depth is increased compared to canonical A-conformation, which facilitates contacts between RNase H residues and the hybrid duplex. Our data show that duplex of **5a** modified ODN **I** with RNA is a good substrate for RNase H. According to computer simulation, the averaged structures over the last 1500 ps of MD for hybrid **D1**/RNA, **Ds**/RNA, **I**/RNA, and **III**/RNA duplexes show conformational characteristics ranging between the canonical A-form and B-form, and more close to the A-form. CURVES program was used to

calculate the minor groove width of the four DNA/RNA duplexes studied. The results indicate that the minor groove width of the duplexes is greatly reduced compared to A-conformation and lie intermediate between A-form and B-form geometries (ESI‡). In **I**/RNA duplex, the minor groove width in the modified region becomes narrow, the incorporating of isonucleoside **5a** in ODN **I** caused large deviations in some helical parameters and makes the duplex structure bent. This modified area of the duplex may correspond to the region interacting with the RNase H. The high variability of helical parameters is also found in the structure of **Ds**/RNA duplex.

In general, the loading of the siRNA into the RISC is accomplished by the formation of RISC-loading complex (RLC), and during the activation of the RISC, the designated sense strand is cleaved.[29] On the other hand, the antisense strand played vital role for the RISC formation and activity, and its modification may seriously influence the silencing activity, especially the 5′-terminal position which is important for the 5′-phosphorylation. Off-target effects are strictly sequence-specific and are caused either by near-perfect complementary between the central region of the siRNA and its targets or by seed-sequence complementary between the siRNA and the target 3′-UTR.[30–32] It would be a competition between passenger strand and guide strand to the number of B-RISC*.[33] It was proposed that the thermodynamic characteristics of the siRNA, that is, the relative stability of the two ends, determine the asymmetric incorporation of the two strands into the RISC.[34] Many approaches were reported to reduce the off-target effect mediated by guide strand including 2′-OMe, 5′-OMe-modified siRNA, DNA substitution in the seed region of siRNA.[16,35,36] Recently, unlocked nucleobase analogs modification on siRNA was reported and showed a significant reduction on global off-target events.[37,38] Our experiments show that as expected the sense strand is much better tolerated toward L-isonucleoside **5a/5b** modification and 3′-terminal L-isonucleoside **5a/5b** modified antisense strand seems to produce no adverse effect on the silencing activity. In our experiment, isonucleoside modified at 3′ or 5′ terminal of oligonucleotide can maintain or slightly increase the thermodynamic stability (Table 1); L-isonucleoside modified at 3′ or 5′ terminal of passenger strand of siRNA could retain silencing activities and reduce the off-target effect mediated by guide strand. From the experimental data reported here, it is proposed that a 5′–CH_2– extended chain isonucleoside makes the modified passenger strand more flexible and thereby could not influence the potency of guide strand to form the RISC, however this modification could inhibit the entrance of passenger strand and minimize the passenger specific off-target effect.

Conclusion

A novel isonucleoside **5** containing a 5′–CH_2 extended chain at the sugar moiety was synthesized and several oligonucleotides incorporated with **5** have been shown to form stable double helical structures with complementary DNA and RNA. The stability of these modified oligonucleotides towards nuclease and their ability to activate RNase H are more promising compared with the natural analogues. In siRNA, passenger strand modified with isonucleoside (**5a/b**) can retain the silencing activity and minimize the passenger strand specific off-target effect.

Experimental section

Chemistry

All solvents were dried and distilled prior to use. Thin layer chromatography (TLC) was performed using silica gel GF-254 (Qing-Dao Chemical Co., China) plates with detection by UV or dyed by phosphomolybdic acid. Column chromatography was performed on silica gel (200–300 mesh, Qing-Dao Chemical Co.). NMR spectra were recorded on Varian Inova VXR-500 or Avance 500 Bruker spectrometer with TMS as an internal standard. Mass spectra (ESI-TOF MS) were obtained on a MDS SCLEX QSTAR instrument. High-resolution mass spectra (ESI-TOF HRMS) were obtained on a Bruker DALTONICS APEX IV 70e, and the data were reported in m/e. MALDI-TOF mass spectra was recorded on Bruker BIFLEX III instrument. Elemental analyses were obtained by Vario EL III instrument. UV spectra were recorded with a Pharmacia LKB Biochrom 4060 spectrophotometer. Optical rotations were recorded on a Perkin-Elmer 243B polarimeter. All the intermediates were dried in vacuum over P_2O_5 at room temperature, for expoxide opening reaction, adenine or thymine was dried in vacuum over P_2O_5 at 78 °C.

[2*S*-(2-Ethoxycarbonyl-vinyl)-3*R*-*p*-toluenesulfonyl-4*R*-benzoxy]-tetrahydrofuran (8). Compound **6b** (3.00 g, 6.87 mmol) was dissolved in trifluoroacetic acid (7 mL) and water (1 mL), and the solution was stirred for 5 h at room temperature. After evaporation of most of solvent, the residue was dissolved in CH_2Cl_2, and the solution was washed with saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4. Solvent was removed to afford white foam (crude aldehyde). After dried in vacuum for 2 days at room temperature, NaH (60%, 275 mg, 6.88 mmol) was suspended in anhydrous THF (19 mL) under an inert atmosphere at −30 °C and triethyl phosphonoacetate (1.43 mL, 6.99 mmol) was added. After the mixture was stirred for 30 min at −30 °C, crude aldehyde was added. The solution was stirred for 4 h at room temperature. Saturated NH_4Cl solution was added to quench the reaction, and the aqueous system was extracted with EtOAc. After drying over anhydrous Na_2SO_4, the organic extracts were evaporated and the residue was purified by silica gel column chromatography (P.E.–EtOAc) to give pure **8a** and a mixture of **8a/b** (2.36 g, 74.6%) as a white solid.

Compound **8a**. $[\alpha]_D^{20}$ −82.00° (c = 0.050, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.32 (t, J = 7.0 Hz, 3H, –CH_3), 2.41 (s, 3H, Ts-CH_3), 3.94 (dd, $J_{5a,5b}$ = 11.0 Hz, $J_{5a,4}$ = 2.0 Hz, 1H, 5a-H), 4.22 (q, 2H, –$COOCH_2$–), 4.41 (dd, $J_{5b,4}$ = 5.0 Hz, 1H, 5b-H), 4.76 (m, 1H, 2-H), 5.11 (dd, $J_{2,3}$ = 4.0 Hz, $J_{3,4}$ = 1.5 Hz, 1H, 3-H), 5.48 (m, 1H, 4-H), 6.09 (dd, $J_{1',2'}$ = 15.5 Hz, $J_{2',2}$ = 2.0 Hz, 1H, 2′-H), 6.73 (dd, $J_{1',2}$ = 5.0 Hz, 1H, 1′-H), 7.31 (d, 2H, Bz-H), 7.44–7.47 (m, 2H, Bz-H), 7.59–7.62 (m, 1H, Bz-H), 7.80 (d, 2H, Ts), 7.97–7.99 (m, 2H, Ts); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.2, 21.7, 60.6, 71.5, 77.6, 78.8, 83.3, 124.4, 128.1, 128.5, 128.8, 129.8, 130.0, 133.7, 139.7, 145.5, 165.0, 165.4; Anal. Calcd for $C_{23}H_{24}O_8S$: C, 59.99; H, 5.25. Found: C, 59.93; H, 5.27.

[2*S*-(2-Ethoxycarboxyethyl)-3*R*-*p*-toluenesulfonyl-4*R*-benzoxy]-tetrahydrofuran (9). The solution of **8** (661 mg, 1.44 mmol) in THF (15 mL) was stirred under hydrogen (0.4 MPa) in the presence of 10% Pd-C at room temperature overnight, and the catalyst was filtered and the solution was evaporated to residue. Recrystallization from EtOAc and petroleum ether (60–90 °C) provided pure **9** (313 mg, 96.0%) as a white crystalline solid.

$[\alpha]_D^{20}$ −35.71° (*c* = 0.154, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.26 (t, J = 7.0 Hz, 3H, –CH_3), 1.87–1.94 (m, 1H, 1′–CH_2–), 1.97–2.02 (m, 1H, 1′–CH_2–), 2.38 (s, 3H, Ts-CH_3), 2.40–2.46 (m, 2H, 2′–CH_2–), 3.72 (dd, $J_{5a,5b}$ = 11.0 Hz, $J_{5a,4}$ = 3.0 Hz, 1H, 5a-H), 4.10–4.17 (m, 3H, 2-H, –$COOCH_2$–), 4.33 (dd, $J_{5b,4}$ = 5.5 Hz, 1H, 5b-H), 5.05 (dd, $J_{3,4}$ = 1.0 Hz, $J_{2,3}$ = 3.5 Hz, 1H, 3-H), 5.30 (m, 1H, 4-H), 7.31 (d, 2H, Bz-H), 7.44–7.47 (m, 2H, Bz-H), 7.58–7.61 (m, 1H, Bz-H), 7.84 (d, 2H, Ts), 7.94–7.96 (m, 2H, Ts); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.2, 21.6, 23.9, 30.4, 60.4, 71.0, 77.9, 78.9, 79.3, 82.4, 83.0, 128.0, 128.5, 128.9, 129.7, 130.0, 133.1, 133.6, 145.4, 165.0, 172.9; Anal. Calcd for $C_{23}H_{26}O_8S$: C, 59.73; H, 5.67. Found: C, 59.73; H, 5.63.

[2*S*-(2-Methoxycarboxyethyl)-3*S*,4*R*-epoxy]-tetrahydrofuran (10). To the solution of **9** (870 mg, 1.88 mol) in dry methanol (25 mL) was added anhydrous K_2CO_3 (1.10 g, 7.96 mmol), and the resulting suspension was stirred for 1 h at room temperature. The mixture was neutralized with acetic acid and evaporated. The residue was purified by silica gel column chromatography (P.E.–EtOAc) to give **10** (313 mg, 96.0%) as a colorless syrup.

$[\alpha]_D^{20}$ −32.72° (*c* = 0.162, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ1.64–1.72 (m, 1H, 1′–CH_2–), 1.76–1.83 (m, 1H, 1′–CH_2–), 2.39–2.53 (m, 2H, 2′–CH_2–), 3.61 (d, $J_{3,4}$ = 3.0 Hz, 1H, 3-H), 3.68 (s, 3H, –$COOCH_3$), 3.69 (dd, 1H, 5a-H), 3.77 (d, 1H, 4-H), 3.97 (d, $J_{5a,5b}$ = 11.0 Hz, 1H, 5b-H), 4.08 (dd, $J_{2,1'a}$ = 10.0 Hz, $J_{2,1'b}$ = 3.5 Hz, 1H, 2-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 25.9, 29.7, 30.1, 51.7, 55.6, 58.7, 65.9, 173.6; Anal. Calcd for $C_8H_{12}O_4$: C, 55.81; H, 7.02. Found: C, 55.94; H, 6.97.

[5*S*-(2-Methoxycarboxyethyl)-4*R*-hydroxy-3*S*-(adenin-9-yl)]-tetrahydrofuran (11) {5*S*-[2-(*N*,*N*-dimethyl amino-carboxy-ethyl)]-4*R*-hydroxy-3*S*-(adenin-9-yl)}-tetrahydrofuran (12). To a solution of **10** (971 mg, 5.64 mmol) and adenine (1.54 g, 11.40 mmol) in DMF (32 mL) was added DBU (2.53 mL, 16.92 mmol), and the mixture was heated to 105 °C for 46 h. After the mixture cooled to room temperature, the solvent was removed and residue purified by silica gel column chromatography (CH_2Cl_2–MeOH) to yield **11** (600 mg, 34.7%) and **12** (55 mg, 10.0%) as a white solid, respectively.

Compound **11**, $[\alpha]_D^{20}$ +21.57° (*c* = 0.102, MeOH); UV (MeOH): λ_{max} = 260.5 nm (ε 11 754). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.78–1.86 (m, 1H, 1′–CH_2–), 1.92–1.98 (m, 1H, 1′–CH_2–), 2.42–2.47 (m, 2H, 2′–CH_2–), 3.60 (s, 3H, –$COOCH_3$), 3.65 (m, 1H, 5-H), 4.11 (dd, $J_{2a,2b}$ = 9.5 Hz, $J_{2a,3}$ = 6.0 Hz, 1H, 2a-H), 4.16 (dd, $J_{2b,3}$ = 7.5 Hz, 1H, 2b-H), 4.30 (m, 1H, 4-H), 4.86 (m, 1H, 3-H), 5.77 (d, J = 5.5 Hz, 1H, 4-OH), 7.24 (s, 2H, –NH_2), 8.15 (s, 1H, 2-H in adenine), 8.17 (s, 1H, 8-H in adenine); ^{13}C NMR (125 MHz, DMSO-d_6) δ 27.9, 29.8, 51.3, 61.8, 68.7, 78.5, 82.6, 119.0, 139.3, 149.4, 152.3, 156.0, 173.0; Anal. Calcd for $C_{13}H_{17}N_5O_4$: C, 50.81; H, 5.58; N, 22.79. Found: C, 50.97; H, 5.54; N, 22.72.

Compound **12**, ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.71–1.78 (m, 1H, –CH_2–), 1.86–1.93 (m, 1H, –CH_2–), 2.36–2.45 (m, 2H, –CH_2–), 2.80 (s, 3H, –$N(CH_3)_2$), 2.94 (s, 3H, –$N(CH_3)_2$), 3.66 (m, 1H, 5-H), 4.09–4.16 (m, 2H, 2-H), 4.28 (m, 1H, 4-H), 4.84 (m, 1H, 3-H), 5.73 (d, J = 5.5 Hz, 1H, 4-OH), 7.23 (s, 2H, –NH_2), 8.14 (s, 1H, 2-H in adenine), 8.16 (s, 1H, 8-H in adenine); ESI-TOF MS *m*/*z*: 321.2 $(M + H)^+$, 343.25 $(M + Na)^+$.

[2*S*-(3-Hydroxypropyl)-3*S*,4*R*-epoxy]-tetrahydrofuran (13). The solution of **9** (180 mg, 0.39 mmol) in dry THF (5 mL) was cooled to 0 °C, and $LiAlH_4$ (25 mg, 0.62 mmol) was added in batches. The mixture was stirred for 10 h at room temperature, quenched with water, stirred overnight, and then filtered. After removing the solvents the mixture was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give **13** (54 mg, 96.2%) as a colorless syrup.

$[\alpha]_D^{20}$ −24.66 (*c* = 0.073, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.41–1.48 (m, 1H, 1′–CH_2), 1.55–1.62 (m, 1H, 1′–CH_2), 1.64–1.75 (m, 2H, 2′–CH_2), 2.53 (brs, 1H, –OH), 3.61 (d, $J_{3,4}$ = 3.0 Hz, 1H, 3-H), 3.65 (m, 2H, 3′–CH_2–), 3.73 (d, $J_{5a,5b}$ = 10.5 Hz, 1H, 5a-H), 3.78 (d, 1H, 4-H), 3.98 (d, 1H, 5b-H), 4.10 (dd, $J_{2,1'a}$ = 4.5 Hz, $J_{2,1'b}$ = 8.0 Hz, 1H, 2-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 27.4, 28.7, 55.7, 58.7, 62.2, 65.8, 77.4; Anal. Calcd for $C_7H_{12}O_3$: C, 58.32; H, 8.39. Found: C, 58.24; H, 8.12.

[2*S*-(3-*O*-*t*-Butyldimethylsilyl-propyl)-3*S*,4*R*-epoxy]-tetrahydrofuran (14). To a solution of compound **13** (458 mg, 3.18 mmol) in dry DMF (15 mL) was added TBDMSCl (764 mg, 5.09 mmol) and imidazole (75 mg, 11.07 mol), and the mixture was stirred for 3 h at room temperature. Water (~30 mL) was added to quench the reaction, and the solvent was evaporated. The residue was purified by silica gel column chromatography (P.E.–EtOAc) to yield **14** (777 mg, 94.6%) as a colorless syrup.

$[\alpha]_D^{20}$ −13.21° (*c* = 0.106, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.05 (s, 6H, TBDMS-CH_3), 0.89 (s, 9H, TBDMS-^{t}Bu), 1.40–1.71 (m, 4H, 1′,2′–CH_2–), 3.60 (d, $J_{3,4}$ = 3.0 Hz, 1H, 3-H), 3.62–3.68 (m, 2H, 3′–CH_2–), 3.71 (d, $J_{5a,5b}$ = 10.5 Hz, 1H, 5a-H), 3.76 (d, 1H, 4-H), 3.97 (d, 1H, 5b-H), 4.09 (dd, $J_{2,1'a}$ = 5.5 Hz, $J_{2,1'b}$ = 7.5 Hz, 1H, 2-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ −5.4, 18.3, 25.9, 27.3, 28.7, 55.8, 58.9, 62.6, 65.9, 77.4; Anal. Calcd for $C_{13}H_{26}O_3Si$: C, 60.42; H, 10.14. Found: C, 60.13; H, 10.32.

[5*S*-(3-*O*-*t*-Butyldimethylsilyl-propyl)-4*R*-hydroxyl-3*S*-(adenin-9-yl)]-tetrahydrofuran (15a). To a solution of compound **14** (491 mg, 1.90 mmol) and dry adenine (515 mg, 3.79 mmol) in dry DMF (18 mL) was added DBU (0.87 mL, 5.70 mmol), and the solution was heated to 110 °C for 5d. After the mixture cooled to room temperature, the solvent was evaporated and the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give **15a** (376 mg, 50.8%) and **16a** (37 mg, 6.4%) as a white solid, respectively.

Compound **15a**, $[\alpha]_D^{20}$ +18.18° (*c* = 0.055, MeOH); UV (MeOH): λ_{max} = 260.5 nm (ε 11 684). ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.01 (s, 6H, TBDMS-CH_3), 0.84 (s, 9H, TBDMS-^{t}Bu), 1.52–1.69 (m, 4H, –CH_2CH_2–), 3.57–3.64 (m, 3H, 5-H, –CH_2–O–), 4.12 (m, 2H, 2-H), 4.23 (m, 1H, 4-H), 4.83 (m, 1H, 3-H), 5.71 (d, J = 5.5 Hz, 1H, 4-OH), 7.22 (br s, 2H, –NH_2), 8.13 (s, 2H, 2-H, 8-H in adenine); ^{13}C NMR (125 MHz,

DMSO-d_6) δ −5.3, 17.9, 25.8, 28.8, 29.1, 62.0, 62.4, 68.6, 78.9, 83.6, 119.0, 139.2, 149.4, 152.3, 156.0; Anal. Calcd for $C_{18}H_{31}N_5O_3Si$: C, 54.93; H, 7.94; N, 17.80. Found: C, 54.94; H, 7.75; N, 17.73.

Compound **16a**, ^{1}H NMR (500 MHz, DMSO-d_6) δ −0.18 (d, 3H, TBDMS-CH_3), −0.16 (d, 3H, TBDMS-CH_3), 0.70 (s, 9H, TBDMS-^{t}Bu), 1.30–1.65 (m, 4H, –CH_2CH_2–), 3.45–3.65 (m, 2H, –CH_2–O–), 4.07 (m, 1H, 2a-H), 4.46–4.50 (m, 2H, 2b-H, 3-H), 4.82 (m, 1H, 4-H), 5.77 (d, J = 3.5 Hz, 1H, 3-OH), 7.22 (s, 2H, –NH_2), 8.13 (s, 2H, 2-H, 8-H); ESI-TOF MS m/z: 394.34 $(M + H)^+$, 416.33 $(M + Na)^+$.

[5*S*-(3-O-*t*-Butyldimethylsilyl-propyl)-4*R*-hydroxyl-3*S*-(thymin-1-yl)]-tetrahydrofuran (15b). To the solution of **14** (514 mg, 1.99 mmol) and dry thymine (559 mg, 4.39 mmol) in anhydrous DMF (27 mL) was added DBU (0.9 mL, 5.90 mmol), and the solution was heated to 110 °C for 5d. After the mixture cooled to room temperature, the solution was evaporated and the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give mixture of **15b** and **16b** (6 : 1 by ^{1}H NMR, 316 mg, 41.3%) as a white foam, respectively.

$[\alpha]_D^{20}$ −18.75° (c = 0.016, MeOH); UV (MeOH): λ_{max} = 272.0 nm (ε 8918). ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.02 (s, 6H, TBDMS-CH_3), 0.85 (s, 9H, TBDMS-^{t}Bu), 1.48–1.69 (m, 4H, –CH_2CH_2–), 1.77 [2s (6 : 1), 3H, Thymine-CH_3], 3.48 (m, 1H, 5-H), 3.59 (m, 2H, –CH_2–O–), 3.78 (dd, $J_{2a,2b}$ = 10.0 Hz, $J_{2a,3}$ = 5.0 Hz, 1H, 2a-H), 3.86 (m, 1H, 4-H), 3.95 (dd, $J_{2b,3}$ = 7.5 Hz, 1H, 2b-H), 4.75 (m, 1H, 3-H), 5.56 (d, J = 6.0 Hz, 1H, 4-OH), 7.43 (d, J = 1.0 Hz, 1H, 6-H in thymine), 11.26 (s, 1H, –NH–); ^{13}C NMR (125 MHz, DMSO-d_6) δ −5.3, 12.1, 17.9, 25.7, 28.8, 62.4, 62.6, 68.1, 78.7, 83.1, 109.4, 137.7, 151.0, 163.7; HRMS (TOF) calcd for $C_{18}H_{33}N_2O_5Si$ $(M + H)^+$: 385.2080; found: 385.2153.

[5*S*-(2-Hydroxypropyl)-4*R*-hydroxyl-3*S*-(adenin-9-yl)]-tetrahydrofuran (5a). (1) **11a** (56 mg, 0.18 mmol) was dissolved in dry THF (15 mL), and $LiAlH_4$ (20 mg, 0.50 mmol) was added stepwise at 0 °C. After stirring over night at room temperature, the reaction mixture was quenched with water, filtered, and evaporated. The residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to yield **5a** (41 mg, 80.4%) as a white foam.

(2) A solution of **15a** (452 mg, 1.15 mmol) and TBAF (1 M in THF, 2.3 mL, 2.3 mmol) in THF (30 mL) was stirred for 3 h at room temperature. The resulting mixture was evaporated, and the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give **5a** (white foam, 311 mg, 96.9%).

$[\alpha]_D^{20}$ +37.89° (c = 0.095, MeOH); UV (MeOH): λ_{max} = 260.5 nm (ε 12 035). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.48–1.62 (m, 3H, 1′–CH_2–, 2′–CH_2–), 1.66–1.71 (m, 1H, 1′–CH_2–), 3.42 (m, 2H, 3′–CH_2–), 3.62 (m, 1H, 5-H), 4.11 (dd, $J_{2a,2b}$ = 9.5 Hz, $J_{2a,3}$ = 5.5 Hz, 1H, 2a-H), 4.15 (dd, $J_{2b,3}$ = 7.0 Hz, 1H, 2b-H), 4.23 (m, 1H, 4-H), 4.40 (t, J = 5.0 Hz, 1H, 3′-OH), 4.85 (m, 1H, 3-H), 5.72 (d, J = 5.5 Hz, 1H, 4-OH), 7.23 (s, 2H, –NH_2), 8.15 (s, 2H, 2-H, 8-H in adenine). ^{13}C NMR (125 MHz, DMSO-d_6) δ 29.0, 29.4, 60.6, 62.0, 68.6, 79.0, 83.7, 119.0, 139.2, 149.5, 152.4, 156.0; Anal. Calcd for $C_{12}H_{17}N_5O_3$: C, 51.60; H, 6.14; N, 25.08. Found: C, 51.66; H, 6.21; N, 24.96.

[5*S*-(2-Hydroxypropyl)-4*R*-hydroxyl-3*S*-(thymin-1-yl)]-tetrahydrofuran (5b). A solution of mixture **15b** and **16b** (224 mg, 0.58 mmol) and TBAF (1 M in THF, 1.2 mL, 1.2 mmol) in THF (8.5 mL) was stirred for 3 h at room temperature. The resulting mixture was evaporated, and the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give **5b** (147 mg, 93.2%) as a white solid.

$[\alpha]_D^{20}$ +4.17° (c = 0.048, MeOH); UV (MeOH): λ_{max} = 262.0 nm (ε 8040). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.45–1.61 (m, 3H, 1′–CH_2–, 2′–CH_2–), 1.65–1.72 (m, 1H, 1′–CH_2–), 1.79 (s, 3H, thymidinyl-CH_3), 3.41 (m, 2H, 3′–CH_2), 3.48 (m, 1H, 5-H),3.78 (dd, $J_{2a,2b}$ = 10.0 Hz, $J_{2a,3}$ = 5.0 Hz, 1H, 2a-H), 3.87 (m, 1H, 4-H), 3.97 (dd, $J_{2b,3}$ = 8.0 Hz, 1H, 2b-H), 4.39 (t, 1H, 3′–OH), 4.78 (m, 1H, 3-H), 5.55 (d, J = 5.5 Hz, 1H, 4-OH), 7.45 (s, 1H, 6-H in thymine), 11.27 (s, 1H, –NH–); ^{13}C NMR (125 MHz, DMSO-d_6) δ21.1, 29.0, 60.6, 62.6, 68.1, 78.7, 83.2, 109.4, 137.7, 151.0, 163.7; Anal. Calcd for $C_{12}H_{18}N_2O_5$: C, 53.33; H, 6.71; N, 10.36. Found: C, 53.30; H, 6.49; N, 10.24.

[5*S*-(3-Hydroxypropyl)-4*R*-hydroxyl-3*S*-(6′-*N*-benzoyl-adenine-9′-yl)]-tetrahydrofuran (17). A solution of **5a** (90 mg, 0.32 mmol) in pyridine (3 mL) was added TMSCl (0.43 mL, 3.35 mmol). After stirring at room temperature for 3 h, BzCl (0.2 mL, 1.74 mmol) was added at 0 °C, and the mixture was kept on stirring at room temperature for 4 h. Conc. aq. ammonia was added to adjust the solution pH to 8–9 at 0 °C. After another 2 h stirring at room temperature, the solvent was removed, and the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give **17** (90 mg, 93.6%) as a white foam.

$[\alpha]_D^{20}$ +121.67° (c = 0.120, MeOH). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.50–1.61 (m, 3H, 1″–CH_2–, 2″–CH_2–), 1.62–1.73 (m, 1H, 1″–CH_2–), 3.43 (m, 2H, 3″–CH_2–), 3.67 (m, 1H, 5-H), 4.17–4.23 (m, 2H, 2-H), 4.29 (m, 1H, 4-H), 4.40 (t, J = 5.0 Hz, 1H, 3″–OH), 5.01 (m, 1H, 3-H), 5.78 (d, J = 5.0 Hz, 1H, 4-OH), 7.54–7.57 (m, 2H, Bz), 7.63–7.66 (m, 1H, Bz), 8.04–8.05 (m, 2H, Bz), 8.51 (s, 1H, 2′-H), 8.76 (s, 1H, 8′-H), 11.16 (s, 1H, –NH–); ^{13}C NMR (125 MHz, DMSO-d_6) δ 29.0, 29.4, 60.6, 62.3, 68.5, 79.0, 83.7, 125.7, 128.4, 132.4, 133.4, 143.1, 150.2, 151.4, 152.4, 165.6; Anal. Calcd for $C_{19}H_{21}N_5O_4$(0.8 H_2O): C, 57.36; H, 5.73; N, 17.60. Found: C, 57.51; H, 5.66; N, 17.83.

{5*S*-[3-*O*-(4′,4-Dimethoxytriphenylmethyl)-propyl]-4*R*-hydroxyl-3*S*-(6′-*N*-benzoyl-adenine-9′-yl)}-tetrahydrofuran (18). Compound **17** (235 mg, 0.61 mmol) was dissolved in dry pyridine (9 mL), and dimethoxytrityl chloride (234 mg, 0.68 mmol) was added. The solution was stirred at room temperature for 26 h. After evaporation the mixture was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give **18** (333 mg, 79.3%) as a white foam.

^{1}H NMR (500 MHz, DMSO-d_6) δ 1.64–1.78 (m, 4H, 1″–CH_2–, 2″–CH_2–), 3.00 (m, 2H, 3″–CH_2–), 3.64 (m, 1H, 5-H), 3.73 (s, 6H, –OCH_3), 4.16–4.22 (m, 2H, 2-H), 4.32 (m, 1H, 4-H), 5.01 (m, 1H, 3-H), 5.79 (d, J = 5.5 Hz, 1H, 4-OH), 6.88–6.91 (m, 4H, DMT), 7.20–7.26 (m, 5H, DMT), 7.30–7.33 (m, 2H, DMT), 7.37–7.38 (m, 2H, DMT), 7.54–7.57 (m, 2H, Bz), 7.63–7.66 (m, 1H, Bz), 8.04–8.05 (m, 2H, Bz), 8.52 (s, 1H, 2′-H), 8.75 (s, 1H, 8′-H), 11.16 (s, 1H, –NH–); ^{13}C NMR (125 MHz, DMSO-d_6) δ 26.0, 29.6, 55.0, 62.2, 62.7, 68.6, 78.8,

83.4, 85.2, 113.1, 125.6, 126.5, 127.6, 127.8, 128.4, 129.6, 132.4, 133.4, 136.0, 143.1, 145.2, 150.2, 151.4, 152.4, 158.0, 165.5; Anal. Calcd for $C_{40}H_{39}N_5O_6$: C, 70.06; H, 5.73; N, 10.21. Found: C, 70.09; H, 5.78; N, 10.30.

{5*S*-[3-*O*-(4′,4-Dimethoxytriphenylmethyl)-propyl]-4*R*-O-[(2-cyanoethyl-*N*,*N*′-diisopropyl)-phosphoramidite]-3*S*-(6′-*N*-benzoyl-adenine-9′-yl)}-tetrahydrofuran (19). A solution of compound **18** (291 mg, 0.42 mmol) and 1*H*-tetrazole (28 mg, 0.40 mmol) in dry CH_2Cl_2 (8 mL) was added $NCCH_2CH_2OP[N(iPr)_2]_2$ (0.19 mL, 0.60 mmol) under inert atmosphere. The mixture was stirred at room temperature for 2 h, and was diluted with CH_2Cl_2. The organic layer was washed with 5% aq. $NaHCO_3$, followed by saturated aq. NaCl, dried over Na_2SO_4, evaporated, and purified by silica gel column chromatography [P.E. (60–90 °C)–CH_2Cl_2–EtOAc, 0.5% Et_3N] to give **19** (344 mg, 91.4%) as a white foam.

^{31}P NMR (121.5 MHz, DMSO-d_6): δ 148.5, 148.8

{2*S*-[3-*O*-(4′,4-Dimethoxytriyl)-propyl]-3*R*-hydroxyl-4*S*-(thymin-1′-yl)}-tetrahydrofuran (20). Compound **5b** (320 mg, 1.18 mmol) was dissolved in dry pyridine (8 mL), and dimethoxytrityl chloride (400 mg, 1.16 mmol) was added. The solution was stirred at room temperature for 26 h. After evaporation the mixture was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give **20** (461 mg, 68.0%) as a white foam.

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.56–1.74 (m, 4H, 1′–CH_2–, 2′–CH_2–), 1.76 (s, 3H, T-CH_3), 2.98 (m, 2H, 3′–CH_2–), 3.47 (m, 1H, 2-H), 3.74 (s, 6H, –OCH_3), 3.77 (dd, 1H, $J_{5a,5b}$ = 10.0 Hz, 5a-H), 3.89 (m, 1H, 3-H), 3.95 (dd, 1H, 5b-H), 4.77 (m, 1H, 4-H), 5.58 (br s, 1H, 3-OH), 6.88 (d, 4H, Ph), 7.20–7.38 (m, 9H, Ph), 7.43 (s, 1H, 6-H in thymine), 11.28 (br s, 1H, –NH–); ^{13}C NMR (125 MHz, DMSO-d_6): δ 12.2, 26.0, 29.4, 55.0, 62.5, 62.7, 68.1, 78.5, 83.0, 85.1, 109.3, 113.1, 126.5, 127.6, 127.7, 129.5, 136.0, 137.5, 145.2, 151.4, 158.0, 164.2; HRMS (TOF) calcd for $C_{33}H_{36}N_2O_7$ $(M + Na)^+$: 595.2415; found: 595.2443.

{2*S*-[3-*O*-(4′,4-Dimethoxytriyl)-propyl]-3*R*-*O*-[(2-cyanoethyl-*N*,*N*′-diisopropyl)-phosphoramidite]-4*S*-(thymin-1′-yl)}-tetrahydrofuran (21). A solution of compound **20** (291 mg, 0.42 mmol) and 1H-tetrazole (28 mg, 0.40 mmol) in dry CH_2Cl_2 (8 mL) was added $NCCH_2CH_2OP[N(iPr)_2]_2$ (0.19 mL, 0.60 mmol) under inert atmosphere. The mixture was stirred at room temperature for 2 h, and was diluted with CH_2Cl_2. The organic layer was washed with 5% aq. $NaHCO_3$, followed by saturated aq. NaCl, dried over Na_2SO_4, evaporated, and purified by silica gel column chromatography [P.E. (60–90 °C)–CH_2Cl_2–EtOAc, 0.5% Et_3N] to give **21** (344 mg, 50.0%) as a white foam.

^{31}P NMR (121.5 MHz, DMSO-d_6): δ 148.5

Synthesis of oligonucleotides

Oligonucleotides synthesis was carried out on a 1.0 μM scale by an *Applied Biosystems model 381* DNA Synthesizer with regular phosphoramidite chemistry (DMT off). Cleavage and deprotection of the oligonucleotides (**I**, **II**, **IV**) were performed in conc. aq. ammonia soln. at 55 °C for 12 h, and oligonucleotides (**III**, **V**) were performed in mixed solution of conc. aq. ammonia and 0.5 M NaCl (v : v = 5 : 1) at 55 °C for 12 h. The crude oligomers were purified by HPLC (ZORBAX Bio Series Oligo Column, 6.2 mm ID × 80 mm), gradient eluting with eluants A (MeCN/0.02 M NaH_2PO_4, 1 : 4) and B (1.0 M NaCl in A) and 1.0 ml min^{-1} flow rate. The fraction containing pure oligonucleotides were desalted by a *Sephadex* G-25 column. The pure oligonucleotides were lyophilized and stored at −20 °C. Total yields were about 35% after purification. Other natural oligonucleotides were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.

Synthesis of siRNAs

siRNAs (21 nt, Table 2) were chemically synthesized by the automatic DNA synthesizer (*Applied Biosystems model 381*) using commercial phosphoramidites as building blocks. Isonucleoside-modified siRNAs were synthesized by the same method but replacing the regular **A** and **T** unit with building blocks **19** and **21** at the defined positions (Table 2) (synthesis scale, 1 μmol; coupling time, 15 min). Cleavage and deprotection of RNA oligos were carried out according to the standard protocol (firstly treated with 29% NH_3–H_2O and ethanol (v : v = 3 : 1) in sealed vessel at 55 °C for 12 h, and then treated with TBAF (1.0 M in THF) to remove TBDMS). The synthetic oligoribonucleotides were then purified by HPLC using ion-exchange column (ZORBAX Bio Series Oligo Column, 6.2 mm ID* 80 mm), gradient eluting with eluants A (MeCN/0.02 M NaH_2PO_4, 1 : 4) and B (1.0 M NaCl in A) with 1.0 mL min^{-1} flow rate [Clarity 10u Oligo-WAX Column 300A (150 × 10.0 mm), Phenomenex, gradient eluting with eluants A (10% CH_3CN: 90% 0.02 M Tris-HCl(H_2O), pH = 8.0) and B (2.0 M NaCl in solvent A) with 3.0 mL min^{-1} flow rate], and the pure oligonucleotides were lyophilized and stored at −78 °C in about 20% yield. By mixing complementary sense- and antisense-strand RNAs at equal 20 μM in MQ water, incubating them in boiling water for 1 min, and then gradually decreasing temperature to room temperature and then 4 °C, the siRNA duplexes were efficiently formed. The quality of the RNA duplexes was assessed on a 15% PAGE gel.

UV Melting experiments

Determination of the T_m of the ODNs/DNA or ODNs/RNA hybrids was carried out in the following buffer: 57 mM Tris-HCl (pH 7.5), 57 mM KCl, 1 mM $MgCl_2$. Absorbance was monitored at 260 nm in the temperature range from 20 °C to 90 °C using UV-visible spectrophotometer with the heating rate of 0.5 °C per minute. Prior to measurements, the samples (0.85 μM of ODNs and 0.75 μM of DNA mixture) were preannealed by heating to 90 °C for 5 min followed by slow cooling to 4 °C and keeping this temperature overnight.

CD Spectra

CD Spectra were recorded from 300 to 200 nm in 1 cm path length cuvettes. Spectra were obtained with an ASON/DNA or ASON/RNA duplex concentration of 0.75 μM in buffer containing 57 mM Tris-HCl (pH 7.5), 57 mM KCl and 1 mM $MgCl_2$.

All the spectra were measured at 20 °C with a J715 CD spectrophotometer (*JAC*).

Exonuclease degradation studies

Stability of the ASONs toward 3′-enonuclease was tested using snake venom phosphodiesterase (SVPED). All reactions were performed at 7.1 μM DNA concentration in 56 mM Tris-HCl (pH 7.9) and 4.4 mM $MgCl_2$ at 37 °C. Exonuclease concentration of 28.6 ng μL^{-1} was used for digestion of oligonucleotides. Total reaction volume was 14 μL. Aliquots were taken at 0, 10, 20, 40, 60 min and quenched by addition of the same volume of 50 mM EDTA in 95% formamide. Reaction progress was monitored by 20% denaturing (7 M urea) PAGE and was visualized by staining with SYBR gold and quantified by Model & Storm 860 hardware and Imagequant software (Amersham Biosciences, PKU, China).

Endonuclease degradation studies

Stability of ASONs toward endonuclease was tested using DNase I form bovine pancreas. Reactions were carried out at 10 μM DNA concentration in 100 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ at 37 °C using 15 unit of DNase I. Total reaction volume was 10 μL. Aliquots were taken at 0, 2, 4, 10, 20, 40 and 60 min and quenched with the same volume of 50 mM EDTA in 95% formamide. They were resolved in 20% polyacrylamide denaturing (7 M urea) gel electrophoresis and visualized by staining with SYBR gold and quantified by Model & Storm 860 hardware and Imagequant software (Amersham Biosciences, PKU, China).

Stability studies in bovine fetal serum

To investigate the stability in 80% bovine fetal serum, the AONs (8 μL) at 10 μM concentration were incubated in 32 μL of fetal bovine serum at 37 °C (total reaction volume was 40 μL). Aliquots (5 μL) were taken at 0, 15 and 30 min, 1, 2 and 9 h, and quenched with 10 μL of solution containing 8 M urea and 50 mM EDTA, resolved in 20% polyacrylamide denaturing (7 M urea) gel electrophoresis and visualized by staining with SYBR gold and quantified by Model & Storm 860 hardware and Imagequant software (Amersham Biosciences, PKU, China).

RNase H cleavage

RNAs were 5′-end labeled with ^{32}P using T4 polynucleotide kinase, [γ-^{32}P]ATP and standard procedure. Labeled RNAs were purified by 20% denaturing PAGE and specific activities were measured using Beckman LS 3801 counter. The source of RNase H (obtained from Amersham Bioscience) was *Escherichia coli* containing clone of RNase H gene. The solutions of the oligodeoxynucleotides/RNA duplex: ^{32}P-labeled RNA (0.8 μM) and the oligodeoxynucleotides (4 μM) in buffer, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$ and 0.1 mM DTT at 21 °C; 0.8 U of RNase H. Total reaction volume was 11 μL. The aliquots of the digest were taken after 0, 5, 10, 20, and 30 min, respectively. Loading buffer (30% glycerol with 0.14 nM bromophenol blue, and 0.19 nM xylene cyanol FF) was added, and samples were loaded on a 20% denaturing polyacrylamide gel and run at room temperature and recorded by autoradiography.

Transfection and dual-luciferase assay

Human embryonic kidney cells (HEK293) were grown in DMEM (Life Technologies, Gibco) and seeded in 24-well plates. After the cells reached about 50% confluence, the culture medium was changed to OPTIMEM (Gibco) and then transfected with plasmids and siRNA duplex in the presence of 0.17% Lipofectamine 2000 (Invitrogen). For each well, 0.17 g of recombination plasmid and 0.017 g pRL-TK were used. The final concentration of siRNA is 13 nM. The transfection medium was changed to culture medium (1 mL) after 4 h. All experiments were performed in triplicate and repeated at least twice. Cells were harvested 24 h after transfection and lyzed by passive cell lysis. Dual-luciferase assay was conducted. Luciferase activities were determined with 10 μL cell lysate using the Dual-Luciferase Assay System (Promega) by NOVOStar (BMG Labtechnologies Gmbh, Germany). The ratio between firefly and Renilla luciferase readings was generated for each well, and the inhibition efficiency of each siRNA was calculated by normalizing to respective buffer control.

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References

1 S. T. Crooke, Molecular mechanisms of action of antisense drugs, *Biochim. Biophys. Acta, Gene Struct. Expression*, 1999, **1489**, 31–44.
2 G. Meister and T. Tuschl, Mechanisms of gene silencing by double-stranded RNA, *Nature*, 2004, **431**, 343–349.
3 R. Kretschmer-Kazemi Far and G. Sczakiel, The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides, *Nucleic Acids Res.*, 2003, **31**, 4417–4424.
4 E. Urban and C. R. Noe, Structural modifications of antisense oligonucleotides, *Farmaco*, 2003, **58**, 243–258.
5 F. Eckstein, Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them?, *Antisense Nucleic Acid Drug Dev.*, 2000, **10**, 117–121.
6 (*a*) M. L. Svendsen, J. Wengel, O. Dahl, F. Kirpekar and P. Roepstorff, Oligodeoxynucleotide analogs containing 3′-deoxy-3′-C-threo-hydroxymethylthymine: synthesis, hybridization properties and enzymatic stability, *Tetrahedron*, 1993, **49**, 11341–11352; (*b*) P. N. Jergensen, M. L. Svendsen, C. Scheuer-Larsen and J. Wengel, Incorporation of 3′-C-(hydroxymethyl)thymidine into novel oligodeoxynucleotide analogues, *Tetrahedron*, 1995, **51**, 2155–2164; (*c*) T. Kofoed, P. B. Rasmussen, P. Valentin Hansen and E. B. Pedersen, Oligodeoxynucleotides with extended 3′- and 5′-homologous internucleotide linkages, *Acta Chem. Scand.*, 1997, **51**, 318–324.
7 (*a*) B. Vester and J. Wengel, LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA, *Biochemistry*, 2004, **43**, 13233–13241; (*b*) E. E. Swayze, A. W. Siwkowshi, E. V. Wancewicz, M. T. Migawa, T. K. Wyrzykiewicz, G. Hung, B. P. Monia and C. F. Bennett,

Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals, *Nucleic Acids Res.*, 2007, **35**, 687–701.
8 (*a*) P. E. Nielsen, PNA technology, *Mol. Biotechnol.*, 2004, **26**, 233–248; (*b*) A. Porcheddu and G. Giacomelli, Peptide nucleic acids (PNAs), a chemical overview, *Curr. Med. Chem.*, 2005, **12**, 2561–2599.
9 A. Amantana and P. L. Iversen, Pharmacokinetics and biodistribution of phosphorodiamidate morpholino antisense oligomers, *Curr. Opin. Pharmacol.*, 2005, **5**, 550–555.
10 D. R. Corey, Chemical modification: the key to clinical application of RNA interference?, *J. Clin. Invest.*, 2007, **117**, 3615–3622.
11 M. Rusckowski, T. Ou, A. Roskey and S. Agrawal, Biodistribution and metabolism of a mixed backbone oligonucleotide (GEM 231) following single and multiple dose adminstration in mice, *Antisense Nucleic Acid Drug Dev.*, 2000, **10**, 333–345.
12 A. M. Kawasaki, M. D. Casper, S. M. Freier, E. A. Lesnik, M. C. Zounes, L. L. Cummins and P. D. Cook, Uninformly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides as nuclease resistant antisense compounds with high affinity and specificity for RNA targets, *J. Med. Chem.*, 1993, **36**, 831–841.
13 J. Elmen, H. Thonberg, K. Ljungberg, M. Frieden, M. Westergaard, Y. H. Xu, B. Wahren, Z. C. Liang, H. Urum, T. Koch and C. Wahlestedt, Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality, *Nucleic Acids Res.*, 2005, **33**, 439–447.
14 O. R. Mook, F. Baas, M. B. de Wissel and K. Fluiter, Evaluation of locked nucleic acid-modified small interfering RNA *in vitroin vivo*, *Mol. Cancer Ther.*, 2007, **6**, 833–843.
15 A. L. Jackson, S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet and P. S. Linsley, Expression profiling reveals off-target gene regulation by RNAi, *Nat. Biotechnol.*, 2003, **21**, 635–637.
16 A. L. Jackson, J. Burchard, J. Schelter, B. N. Chau, M. Cleary, L. Lim and P. S. Linsley, Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity, *RNA*, 2006, **12**, 1179–1187.
17 M. Schlee, H. Veit and H. Gunther, siRNA and isRNA: two edges of one sword, *Mol. Ther.*, 2006, **14**, 463–470.
18 A. L. Jackson, J. Burchard, D. Leake, A. Reynolds, J. Schelter, J. Guo, J. M. Johnson, L. Lim, J. Karpilow, K. Nichols, W. Marshall, A. Khvorova and P. S. Linsley, Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing, *RNA*, 2006, **12**, 1197–1205.
19 K. Ui-Tei, Y. Naito, K. Nishi, A. Juni and K. Saigo, Thermodynamic stability and Watson–Crick base pairing in the seed duplex are major determinants of the efficiency of the siRNA-based off-target effect, *Nucleic Acids Res.*, 2008, **36**, 7100–7109.
20 (*a*) Z. Guan, X. B. Tian, L. R. Zhang, J. M. Min and L. H. Zhang, Synthesis and hybridization properties of an oligonucleotide consisting of 1′,4′-anhydro-2′,5′-dideoxy-2′-(thymin-1-yl)-D-altritol, *Helv. Chim. Acta*, 2002, **85**, 1479–1483; (*b*) Z. J. Yang, H. Y. Zhang, J. M. Min, L. T. Ma and L. H. Zhang, Synthesis and enzymatic stability of oligonucleotides consisting of isonucleotides, *Pure Appl. Chem.*, 1999, **71**, 1143–1146; (*c*) Z. J. Yang, H. Y. Zhang, J. M. Min, L. T. Ma and L. H. Zhang, Synthesis and duplex stabilization of oligonucleotides consisting of isonucleotides, *Helv. Chim. Acta*, 1999, **82**, 2037–2043; (*d*) Z. Lei, L. Zhang, L. R. Zhang, J. Chen, J. M. Min and L. H. Zhang, Hybrid characteristics of oligonucleotides consisting of isonucleotide 2′,5′-anhydro-3′-deoxy-3′-(thyminl-yl)-D-mannitol with different linkage modes, *Nucleic Acids Res.*, 2001, **29**, 1470–1475; (*e*) J. Chen, L. R. Zhang, J. M. Min and L. H. Zhang, Studies on the synthesis of a G-rich octaoligoisonucleotide $(isoT)_2(isoG)_4$ $(isoT)_2$ by the phosphotriester approach and its formation of G-quartet structure, *Nucleic Acids Res.*, 2002, **30**, 3005–3014.
21 Z. L. Wang, J. F. Shi, H. W. Jin, L. R. Zhang, J. F. Lu and L. H. Zhang, Properties of isonucleotide-incorporated oligodeoxy-nucleotides and inhibition of expression of spike protein of SARS-Cov, *Bioconjugate Chem.*, 2005, **16**, 1081–1087.
22 Z. S. Li, R. P. Qiao, Q. Du, Z. J. Yang, L. R. Zhang, Z. C. Liang and L. H. Zhang, Studies on aminoisonucleoside modified siRNAs: stability and silencing activity, *Bioconjugate Chem.*, 2007, **18**, 1017–1024.
23 E. Aleem, H. Kiyokawa and P. Kaldis, Cdc2-cyclin E complexes regulate the G1/S phase transition, *Nat. Cell Biol.*, 2005, **7**, 831–836.
24 (*a*) Z. J. Yang, J. Zhang, Z. Wang, Y. Huang, Z. Guan, L. R. Zhang and L. H. Zhang, Process for preparation of isonucleoside compounds or phosphoramidite derivatives thereof. *Chinese Patent*, CN 102212065 A 20111012, 2011; (*b*) Y. C. Liu, J. Zhang, L. Xing, Z. J. Yang, L. R. Zhang and L. H. Zhang, Facile synthesis of 2-(*S*)-(2-hydroxyethyl)-3-(*R*)-hydoxy-4-(*S*)-(thymin-1-yl or adenine-9-yl)-tetrahydrofuran, *Tetrahedron*, 2008, **64**, 9630–9635.
25 H. W. Yu, L. R. Zhang, J. C. Zhou, L. T. Ma and L. H. Zhang, Studies on the synthesis and biological activities of 4′-(*R*)-hydroxy-5′-(*S*)-hydroxymethyl-tetrahydrofuranyl purines and pyrimidines, *Bioorg. Med. Chem.*, 1996, **4**, 609–614.
26 G. S. Ti, B. L. Gaffney and R. A. Jones, Transient protection-efficient one-flask syntheses of protected deoxynucleosides, *J. Am. Chem. Soc.*, 1982, **104**, 1316–1319.
27 E. Zamaratski, P. I. Pradeepkumar and J. Chattopadhyaya, A critical survey of the structure-function of the antisense oligo/RNA heteroduplex as substrate for RNase H, *J. Biochem. Biophys. Methods*, 2001, **48**, 189–208.
28 (*a*) M. Tonelli, N. B. Ulyanov, T. M. Billeci, B. Karwowshi, P. Guga, W. J. Stec and T. L. James, Dynamic NMR structures of [Rp]- and [Sp]-phosphorothioated DNA/RNA hybrids: is flexibility required for RNase H recognition?, *Biophys. J.*, 2003, **85**, 2525–2538; (*b*) B. J. Premraj, S. Raja and N. Yathindra, Structural basis for the unusual properties of 2′,5′ nucleic acids and their complexes with RNA and DNA, *Biophys. Chem.*, 2002, **95**, 253–272; (*c*) S. G. Sarafianos, K. Das, C. Tantillo, A. D. Clark, J. Ding, J. M. Whitcomb, P. L. Boyer, S. H. Hughes and E. Arnold, Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA : DNA, *EMBO J.*, 2001, **20**, 1449–1461.
29 (*a*) C. Matranga, Y. Tomari, C. Shin, D. P. Bartel and P. D. Zamore, Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes, *Cell*, 2005, **123**, 607–620; (*b*) T. A. Rand, S. Petersen, F. Du and X. Wang, Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation, *Cell*, 2005, **123**, 621–629.
30 P. R. Clark, J. S. Pober and M. S. Kluger, Knockdown of TNFR1 by the sense strand of an ICAM-1 siRNA: dissection of an off-target effect, *Nucleic Acids Res.*, 2008, **36**(4), 1081–1097.
31 A. L. Jackson, S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet and P. S. Linsley, Expression profiling reveals off-target gene regulation by RNAi, *Nat. Biotechnol.*, 2003, **21**, 635–637.
32 A. Birmingham, E. M. Anderson, A. Reynolds, D. Ilsley-Tyree, D. Leake, Y. Fedorov, S. Baskerville, E. Maksimova, K. Robinson and J. Karpilow, 3′-UTR seed matches: but not overall identity, are associated with RNAi off-targets, *Nat. Methods*, 2006, **3**, 199–204.
33 J. X. Wei, J. Yang, J. F. Sun, L. T. Jia, Y. Zhang, H. Z. Zhang, X. Li, Y. L. Meng, L. B. Yao and A. G. Yang, Both strands of siRNA have potential to guide posttranscriptional gene silencing in mammalian cells, *PLoS One*, 2009, **4**(4), e5382.
34 (*a*) Y. Shao, C. Y. Chan, A. Maliyekkel, C. E. Lawrence, I. B. Roninson and Y. Ding, Effect of target secondary structure on RNAi efficiency, *RNA*, 2007, **13**, 1631–1640; (*b*) Y. Wang, S. Juranek, H. Li, G. Sheng, S. W. Greg, T. Tuschl and D. J. Patel, Nucleation, propagation and cleavage of target RNAs in argonaute silencing complexes, *Nature*, 2009, **461**, 754–761.
35 P. Y. Chen, L. Weinmann, D. Gaidatzis, Y. Pei, M. Zavolan, T. Tuschl and G. Meister, Strand-specific 5′-*O*-methylation of siRNA duplexes controls guide strand selection and targeting specificity, *RNA*, 2008, **14**, 263–274.
36 K. Ui-Tei, Y. Naito, S. Zenno, K. Nishi, K. Yamato, F. Takahashi, A. Juni and K. Saigo, Functional dissection of siRNA sequence by systematic DNA substitution: modified siRNA with a DNA seed arm is a powerful tool for mammalian gene silencing with significantly reduced off-target effect, *Nucleic Acids Res.*, 2008, **36**, 2136–2151.
37 J. B. Bramsen, M. M. Pakula, T. B. Hansen, C. Bus, N. Langkjaer, D. Odadzic, R. Smicius, S. L. Wengel, J. Chattopadhyaya and J. W. Engels, A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects, *Nucleic Acids Res.*, 2010, **38**, 5761–5773.
38 N. Vaish, F. Chen, S. Seth, K. Fosnaugh, Y. Liu, R. Adami, T. Brown, Y. Chen, P. Harvie, R. Johns, G. Severson, B. Granger, P. Charmley, M. Houston, M. V. Templin and B. Polisky, Improved specificity of gene silencing by siRNAs containing unlocked nucleobase analogs, *Nucleic Acids Res.*, 2011, **39**(5), 1823–1832.