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Synthesis of nuclease-resistant siRNAs possessing benzene-phosphate backbones in their 3′-overhang regions

Yoshihito Ueno [a,b,c,e,*], Takumi Inoue [a], Mahito Yoshida [a], Kayo Yoshikawa [a], Aya Shibata [a], Yoshiaki Kitamura [a], Yukio Kitade [a,b,c,d,*]

[a] Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[c] Center for Emerging Infectious Diseases, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[d] Center for Advanced Drug Research, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[e] PRESTO, JST (Japan Science and Technology Agency), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

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ABSTRACT

We describe the synthesis and silencing activities of siRNA possessing N^1-[3,5-bis(hydroxymethyl)phenyl]thymine (**b**$^{\mathbf{t}}$) in their 3′-overhang regions. We found that an siRNA possessing **b**$^{\mathbf{t}}$ in the 3′-overhang region was more effective than an siRNA with natural nucleosides and the siRNA possessing 1,3-bis(hydroxymethyl)benzene (**b**) without a nucleobase at the 3′-overhang region in in vitro experiment using HeLa cells system. Furthermore, the RNA possessing **b**$^{\mathbf{t}}$ at its 3′-end was more resistant to nucleolytic hydrolysis by snake venom phosphodiesterase (a 3′-exonuclease) than the RNA possessing the natural nucleoside 2′-deoxythymidine at the 3′-end. Thus, the compound **b**$^{\mathbf{t}}$ will be a novel 3′-overhang moiety that can enhance the silencing activity and nuclease-resistant property of siRNAs.

Introduction. Short interfering RNA (siRNA) molecules have drawn considerable attention since it was demonstrated that they mediate potent gene knock-down in a variety of mammalian cells without triggering non-specific RNA degradation and translation inhibition based on interferon response.[1,2] siRNA has considerable potential as a new therapeutic drug for intractable diseases because siRNAs can be rationally designed and synthesized if the sequences of the disease-causing genes are known.[2] Improved nuclease stability of siRNAs is of prime importance for the efficient therapeutic application of synthetic siRNAs. Thus far, many types of siRNAs modified at the base, sugar, or phosphate moieties have been synthesized, and their nuclease-resistant properties and RNAi-inducing activities have been studied.[3,4]

Argonaute2, a key component of the RNA-induced silencing complex (RISC), is responsible for mRNA cleavage in the RNAi pathway.[5,6] It is composed of PAZ, Mid, and PIWI domains. X-ray structural analysis and NMR studies have revealed that the 2-nucleotide 3′-overhang region of the guide strand (antisense strand) of siRNA is recognized by the PAZ domain and is accommodated into its hydrophobic binding pocket.[7–10]

Recently, we have designed and synthesized siRNAs possessing the aromatic compound 1,3-bis(hydroxymethyl)benzene (**b**) in their 3′-overhang regions (Fig. 1).[11,12] We found that these modified siRNAs are more effective than the siRNAs without the 3′-overhang regions in in vitro experiment using HeLa cells system.

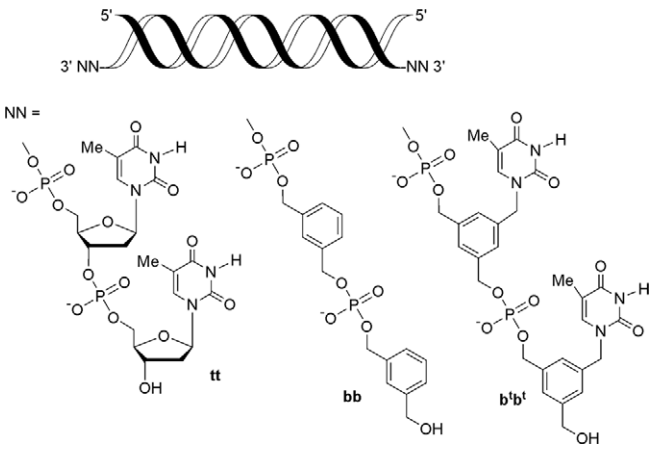

Figure 1. Structures of modified siRNAs.

* Corresponding authors. Tel.: +81 58 293 2639; fax: +81 58 293 2749 (Y.U.).
E-mail addresses: uenoy@gifu-u.ac.jp (Y. Ueno), ykkitade@gifu-u.ac.jp (Y. Kitade).

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Further, the silencing activities of the modified siRNAs are very similar to those of normal siRNAs with natural nucleosides in their 3′-overhang regions. Moreover, RNAs possessing the aromatic groups at their 3′-ends were more resistant to nucleolytic degradation by snake venom phosphodiesterase (SVPD; a 3′-exonuclease) than normal RNAs with natural nucleosides at their 3′-ends.

These results prompted us to investigate the silencing activities of the modified siRNAs possessing the aromatic compound N^1-[3,5-bis(hydroxymethyl)phenyl]thymine (**b^t**), which has a nucleobase capable of forming hydrogen bonds. We predicted that the silencing activities of the siRNAs possessing **b** in their 3′-regions would be enhanced by introducing the nucleobase capable of forming hydrogen bonds in their 3′-overhang regions. In this paper, we report the synthesis and silencing properties of siRNAs possessing **b^t** in their 3′-overhang regions (Table 1).

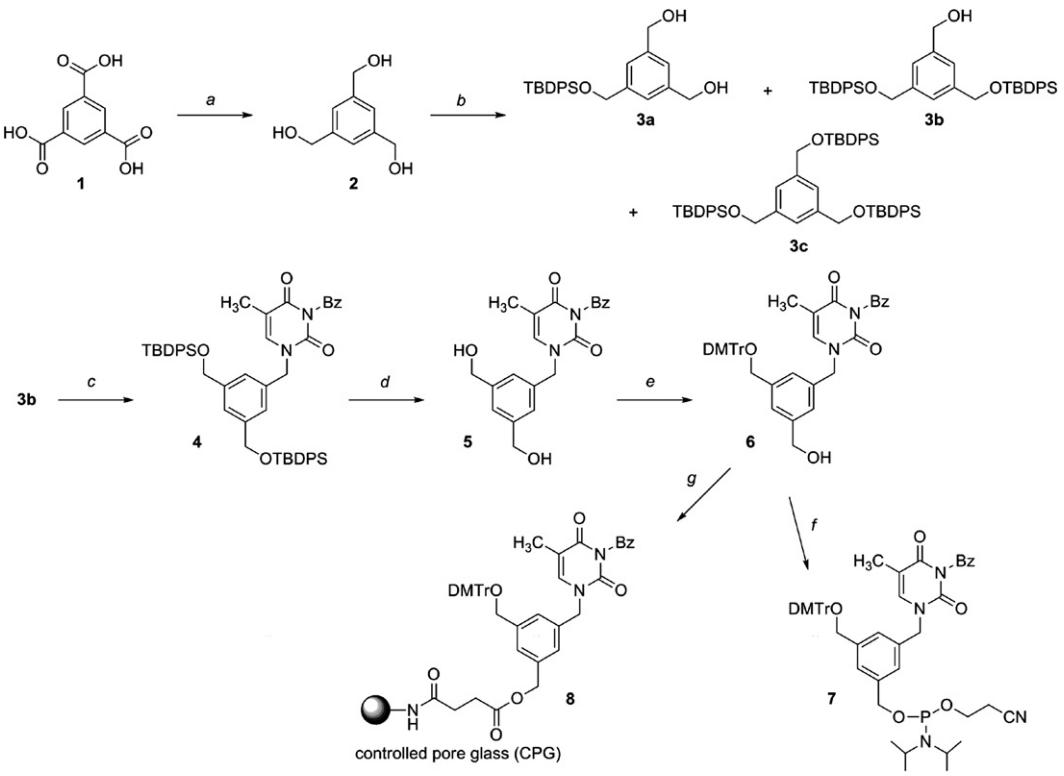
Table 1
Sequences of ONs and siRNAs used in this study

No. of siRNA	No. of ON	Sequence
siRNA **9**	ON **13**	5′-GGCCUUUCACUACUCCUAC-3′
	ON **14**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **10**	ON **15**	5′-GGCCUUUCACUACUCCUACtt-3′
	ON **16**	3′-ttCCGGAAAGUGAUGAGGAUG-5′
siRNA **11**	ON **17**	5′-GGCCUUUCACUACUCCUAC**b^{t}b^t**-3′
	ON **18**	3′-**b^{t}b^t**CCGGAAAGUGAUGAGGAUG-5′
siRNA **12**	ON **19**	5′-GGCCUUUCACUACUCCUAC**bb**-3′
	ON **20**	3′-**bb**CCGGAAAGUGAUGAGGAUG-5′

Capital letters represent ribonucleosides and small letters represent 2′-deoxyribonucleosides. The compounds **b** and **b^t** are 1,3-bis(hydroxymethyl)benzene and N^1-[3,5-bis(hydroxymethyl)phenyl]thymine, respectively.

Results and discussion. *Synthesis*. Modified siRNAs were synthesized by the standard phosphoramidite method. In order to incorporate the aromatic compound in the 3′-overhang regions of the siRNAs, a solid support carrying the aromatic compound **6** and a phosphoramidite of **6** were synthesized according to the route shown in Scheme 1. Trimesic acid (**1**) was treated with $LiAlH_4$ to give tris(hydroxymethyl)benzene (**2**) in a 91% yield. The 3 hydroxyl groups of **2** were protected with *tert*-butyldiphenylsilyl (TBDPS) groups to afford a mono-TBDPS derivative **3a**, a di-TBDPS derivative **3b**, and a tri-TBDPS derivative **3c**, in 10%, 34%, and 44% yields, respectively. The **3b** derivative was coupled with N^3-benzoylthymine under the Mitsunobu conditions to give the N^1-substituted thymine derivative **4** in a 92% yield. The silyl groups of **4** were removed by treatment with tetra-*n*-butylammonium fluoride (TBAF). One of the 2 hydroxyl groups of **5** was protected with a 4,4′-dimethoxytrityl (DMTr) group to afford a mono-DMTr derivative **6** in a 69% yield. The mono-DMTr derivative **6** was phosphitylated by the standard procedure[13] to produce the corresponding phosphoramidite **7** in an 80% yield. In order to incorporate **6** at the 3′-ends of RNAs, **6** was further modified to afford the corresponding 3′-succinate, which was then reacted with controlled pore glass (CPG) to afford a solid support containing **6** (42 μmol/g).

All the oligoribonucleotides (ONs) were synthesized using a DNA/RNA synthesizer. Fully protected ONs (1.0 μmol each) linked to solid supports were treated with concentrated NH_4OH:EtOH (3:1, v/v) at room temperature for 12 h and then with 1.0 M TBAF/THF at room temperature for 12 h. The ONs released after the treatment were purified by denaturing 20% polyacrylamide gel electrophoresis (20% PAGE) to afford deprotected ONs 17 and 18 in 12 and 9 OD_{260} absorbance units, respectively.[14] These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and observed molecular weights were in agreement with their structures.[16]



Scheme 1. Reagents and conditions: (a) $LiAlH_4$, THF, rt, 91%; (b) TBDPSCl, imidazole, DMF, rt, 10% for **3a**, 34% for 3b, and 44% for **3c**; (c) N^3-benzoylthymine, PPh_3, diethylazodicarboxylate, THF, rt, 92%; (d) TBAF, THF, rt, 87%; (e) DMTrCl, pyridine, rt, 69%; (f) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, *i*-Pr_2NEt, CH_2Cl_2, rt, 80%; (g) (1) succinic anhydride, DMAP, pyridine, rt; (2) CPG, WSCI, DMF, rt, 42 μmol/g.

Dual-luciferase assay. The ability of modified siRNAs to suppress gene expression was studied by a dual-luciferase assay using a psiCHECK-2 vector (Promega), which contained the *Renilla* and firefly luciferase genes. The siRNA sequences were designed to target the *Renilla* luciferase gene. HeLa cells were co-transfected with the vector and indicated amounts of siRNAs, and the signals of *Renilla* luciferase were normalized to those of firefly luciferase.[17]

As shown in Figure 2, the silencing activities of siRNAs **10**, **11**, and **12**, which possessed overhang moieties, were markedly greater than that of siRNA **9**, which had no overhang moiety. The silencing activity of the siRNA **12**, which had **b** in its overhang region, was almost equal to that of the unmodified siRNA **10**, which had thymidine in its overhang region, at each concentration,[11] whereas the activity of the siRNA **11**, which possessed $\mathbf{b}^{\mathbf{t}}$ in its overhang region, was greater than that of the siRNAs **10** and **12** at each concentration. Thus, it was found that the siRNA **11**, which had thymine base in the overhang region, was more potent than the siRNA **12**, which had no base in the overhang region.

Nuclease-resistant property. Next, the susceptibility of the ONs to snake venom phosphodiesterase (SVPD), a 3′-exonuclease, was examined. The unmodified ON 16 and the modified ON 18 possessing the benzene derivative $\mathbf{b}^{\mathbf{t}}$ at its 3′-end, were labeled with [γ-^{32}P]ATP and incubated with SVPD. The reactions were analyzed by PAGE under denaturing conditions (data not shown). Densities of radioactivity of the gel were visualized by a Bio-imaging analyzer. As shown in Figure 3, the ON 18 possessing $\mathbf{b}^{\mathbf{t}}$ was more resistant to SVPD than the unmodified ON 16. The half-lives ($t_{1/2}$s) of the ONs 16 and 18 were 3.7 min and 11.4 min, respectively. Thus, it was found that the ON 18 possessing $\mathbf{b}^{\mathbf{t}}$ was 3 times more resistant to SVPD than the unmodified ON 16.

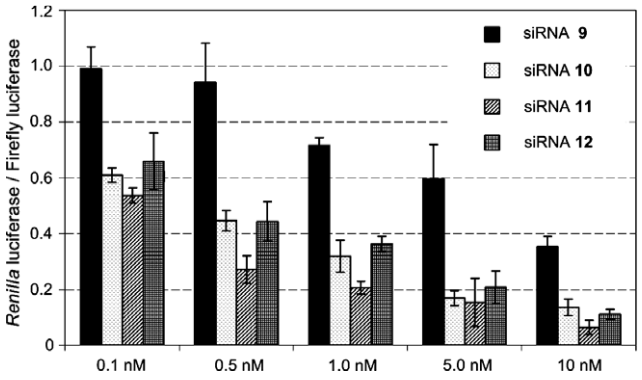


Figure 2. Dual-luciferase assay.[17]

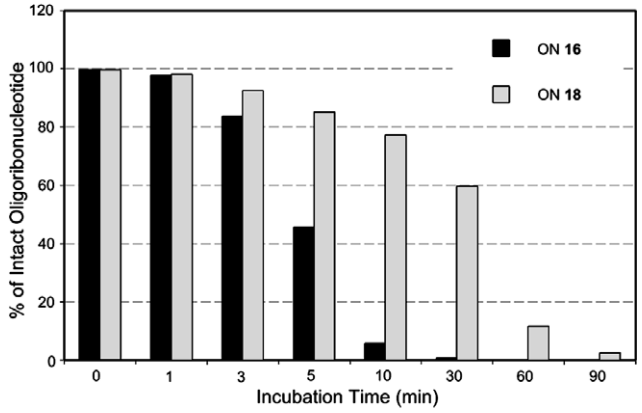


Figure 3. Nuclease resistance of ON **16** and ON **18** against SVPD. Each ON (100 pmol) labeled with ^{32}P at the 5′-end was incubated with snake venom phosphodiesterase (8×10^{-3} U) in a buffer containing 37.5 mM Tris–HCl (pH 7.0) and 7.5 mM $MgCl_2$ (total 40 μL) at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9 M urea (10 μL). The mixtures were analyzed by electrophoresis on 20% PAGE containing 7 M urea. Densities of radioactivity of the gel were visualized by a Bio-imaging analyzer (Bas 2000, Fuji Co., Ltd).

In conclusion, we have demonstrated the synthesis of the siRNAs possessing N^1-[3,5-bis(hydroxymethyl)phenyl]thymine ($\mathbf{b}^{\mathbf{t}}$) in their 3′-overhang regions. The silencing activities of the siRNAs were examined by the dual-luciferase assay. It was found that the siRNA possessing $\mathbf{b}^{\mathbf{t}}$ in the 3′-overhang region was more effective than the siRNA with 2′-deoxythymidine as the natural nucleosides in the 3′-overhang region and the siRNA possessing **b** without the nucleobase in in vitro experiment using HeLa cells system. Furthermore, the RNA possessing $\mathbf{b}^{\mathbf{t}}$ was more resistant to nucleolytic hydrolysis by SVPD than the RNA possessing the natural 2′-deoxythymidine. Thus, $\mathbf{b}^{\mathbf{t}}$ can be a novel overhang unit that enhances the silencing activity and nuclease-resistant properties of the siRNAs.

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16. MALDI-TOF/MS analyses of RNAs. The following spectra were obtained using a time-of-flight mass spectrometer. ON **17**: calculated mass, 6571.0; observed mass, 6567.9. ON **18**: calculated mass, 6880.3; observed mass, 6877.3.
17. Dual-luciferase assay. HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours before transfection, HeLa cells (4×10^4/mL) were transferred to 96-well plates (100 μL per well). They were transfected using TransFast (Promega) according to the manufacturer's instructions, for transfection of adherent cell lines. Cells in each well were transfected with a solution (35 μL) of 20 ng of psiCHECK-2 vector (Promega), the indicated amounts of siRNAs, and 0.3 μg of TransFast in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37 °C. After 1 h, MEM (100 μL) containing 10% FBS and antibiotics was added to each well, and the mixture was further incubated at 37 °C. After 24 h, cell extracts were prepared in Passive Lysis Buffer (Promega). Activities of firefly and *Renilla* luciferases in the cell lysates were determined using a dual-luciferase assay system (Promega) according to the manufacturer's protocol. The results were confirmed by performing at least 3 independent transfection experiments with 2 cultures each and are expressed as the average from 4 experiments as mean ± SD.