Synthesis, purification and characterization of oligonucleotides

Synthesis of 0.2 μmol scale of oligonucleotides **ON-6-44** modified with AmNA[*N*-Me] was performed using Oligonucleotide Synthesizer (Gene Design, ns-8) according to the standard phosphoramidite protocol with Activator 42™ (Proligo) as the activator. Dry MeCN was used to dissolve AmNA[*N*-R]. The standard synthesis cycle was used for assembly of the reagents and synthesis of the oligonucleotides, except that the coupling time was extended to 16 minutes for AmNA[*N*-R] monomers. (The coupling time for AmNA[*N*-Me] was 32 seconds.) The synthesis was carried out in trityl on mode and was treated with concentrated ammonium hydroxide at room temperature for 1 h to cleave the synthesized oligonucleotides from the solid support. The oligonucleotides were initially purified by Sep-pack Plus C_{18} Environmental Cartridge. The separated oligonucleotides were further purified by reverse-phase HPLC with Waters Xbridge™ Shield RP_{18} 2.5 μm (10 mm × 50 mm) columns with a linear gradient of MeCN (7–13% over 42 min for **ON-3–6**, **ON-11**, **ON-12**, **ON-16**, 8–15% over 42 min for **ON-7**, **ON-8**, 13–40% over 42 min for **ON-13**, **ON-17**) in 0.1 M triethylammonium acetate (pH 7.0). The oligonucleotides were analyzed for purity by HPLC and characterized by MALDI-TOF mass spectroscopy.

UV melting experiments and melting profiles

The UV melting experiments were carried out using a Shimadzu UV-1650 spectrometer equipped with a T_m analysis accessory. Equimolecular amounts of the target RNA or DNA strand and oligonucleotide were dissolved in buffer A (10 mM phosphate buffer at pH 7.2 containing 100 mM NaCl) to give a final strand concentration of 4 μM. The samples were annealed by heating at 95 °C followed by slow cooling to room temperature. The melting profile was recorded at 260 nm from 0 to 70 °C (for **ON-3–8**), from 5 to 100 °C (for **ON-11–13**, **16**, **17**) at a scan rate of 0.5 °C min^{-1}. The T_m was calculated as the temperature of the half-dissociation of the formed duplexes, determined by the midline of the melting curve.

Enzymatic digestion study

The sample solutions were prepared by dissolving 0.75 μmol of oligonucleotides in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. To each sample solution, 0.175 μL *Crotalus adamanteus* venom phosphodiesterase (CAVP) was added and the cleavage reaction was carried out at 37 °C. A portion of each reaction mixture was removed at timed intervals and heated to 90 °C for 5 min to deactivate the nuclease. Aliquots of the timed samples were analyzed by RP-HPLC to evaluate the amount of intact oligonucleotides remaining. The percentage of intact oligonucleotide in each sample was calculated and plotted against the digestion time to obtain a degradation curve with time (Fig. S5†).

In vivo knockdown study

All animal procedures were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan). All animal studies were approved by an Institutional Review Board. C57BL/6J mice were obtained from CLEA Japan. All mice were male, and studies were initiated when animals were 8 weeks of age. Mice (n = 3 per arm) were maintained on a 12 h light/12 h dark cycle and fed *ad libitum*. Mice were fed a normal chow (CE-2, CLEA Japan) for 2 weeks before and during treatment. Mice received single treatment of saline-formulated AONs intravenously. At the time of sacrifice after 72 hours of injection, mice were subjected to blood collection from tail veins and then anesthetized with isoflurane (Forane, Abbott Japan) under an overnight fasting condition. Livers were harvested and snap frozen until subsequent analysis. Whole blood was collected and subjected to serum separation for subsequent analysis.

mRNA quantification

Frozen liver tissue was collected in a 2 mL tube with 1 mL of TRIzol Reagent (Life Technologies, Japan) and a zirconia ball (∅ 5 mm, Irie) and mechanically homogenized for 2 min at 30 oscillations per second by a TissueLyser II apparatus (Qiagen). Total RNA was isolated from the resulting suspensions according to the manufacturer's procedure. Gene expression was evaluated by a 2-step quantitative RT-PCR method. Reverse-transcription of RNA samples was performed by using a High Capacity cDNA Reverse-Transcription Kit (Applied Biosystems), and quantitative PCR was performed by TaqMan(R) Fast Universal PCR Master Mix (Applied Biosystems). The mRNA levels of target genes were normalized to the GAPDH mRNA level. For murine apoC-III and GAPDH mRNA quantitation, TaqMan Gene Expression Assay IDs of Mm00445670_m1 and Mm99999915_g1 were used.

ELISA method for AON quantification in liver

Materials and reagents. The template DNA was a 25-mer DNA (5′-gaa tag cga taa taa agc tgg ata a-3′), which is complementary to **ON-9S** to **ON-15S**, with biotin at the 3′-end. The ligation probe DNA was a 9-mer DNA (5′-tcgctattc-3′) with phosphate at the 5′-end and digoxigenin at the 3′-end. The template DNA and the ligation probe DNA were purchased from Japan Bio Service. Reacti-Bind NeutrAvidin-coated polystyrene strip plates were purchased from Thermo Fisher Scientific (Nunc immobilizer streptavidin F96 white, 436015). The template DNA solution (100 nM) was prepared in hybridization buffer containing 60 mM Na_2HPO_4 (pH 7.4), 0.9 M NaCl, and 0.24% Tween 20. The ligation probe DNA solution (200 nM) was prepared in 1.5 units per well of T4 DNA ligase (TaKaRa) with 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT and 0.1 mM ATP.

The washing buffer used throughout the assay contained 25 mM Tris-HCl (pH 7.2), 0.15 M NaCl and 0.1% Tween 20. Anti-digoxigenin-AP antibody (Fab fragments conjugated with

alkaline phosphatase) was obtained from Roche Diagnostics. A 1 : 2000 dilution of the antibody with 1 : 10 super block buffer in TBS (Pierce) was used in the assay. The alkaline phosphatase luminous substrate was prepared in 250 μM CDP-Star (Roche) with 100 mM Tris-HCl (pH 7.6) and 100 mM NaCl.

Assay procedures. Frozen liver tissue was collected in a 2 mL tube with 1 mL of PBS and a zirconia ball (∅ 5 mm, Irie) and mechanically homogenized for 2 min at 30 oscillations per second by a TissueLyser II apparatus (Qiagen). Total protein concentrations were measured using a detergent compatible assay kit (Bio-Rad) and adjusted to 8 mg L^{-1} with PBS. The assay was performed at the concentration range of 128 pM to 1000 nM in duplicate. For the standard curve, 7 standard solutions were prepared. To AON-untreated mice liver homogenates were added **ON-10S**, **ON-11S**, **ON-12S**, **ON-13S**, **ON-14S**, and **ON-15S** solutions to prepare 7 standard samples at a range of 128 pM to 1000 nM. Next, the template DNA solution (100 μL) and standard solution (10 μL) or liver homogenates (10 μL) containing **ON-10S**, **ON-11S**, **ON-12S**, **ON-13S**, **ON-14S**, and **ON-15S** were added to Reacti-Bind Neutr Avidin-coated polystyrene strip 96-well plates and incubated at 37 °C for 1 h to allow the binding of biotin to streptavidin-coated wells and hybridization. After hybridization, the plate was washed three times with 200 μL of washing buffer. Then, ligation probe DNA solution (100 μL) was added, and the plate was incubated at room temperature (15 °C) for 3 h. The plate was then washed three times with the washing buffer. Subsequently, 200 μL of a 1 : 2000 dilution of anti-digoxigenin-AP was added, and the plate was incubated at 37 °C for 1 h. After washing three times with the washing buffer, the CDP-Star solution was added to the plate, and finally the luminescence intensity was determined by using a Centro XS^3 luminometer (Berthold) one second after the addition of CDP-Star. The linear range of 128 pM to 1000 nM in this ELISA system was determined as $r > 0.97$.

Serum chemistry

Serum from the blood collected from the inferior vena cava upon sacrifice was subjected to serum chemistry. Assay kits (WAKO) were used to measure serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are biomarkers for hepatic toxicity.

Statistics

Statistical comparisons were performed by Dunnett's multiple comparison tests. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered to be statistically significant in all cases. N.S. indicates no statistical significance.

Acknowledgements

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Organic LETTERS

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Sulfonamide-Bridged Nucleic Acid: Synthesis, High RNA Selective Hybridization, and High Nuclease Resistance

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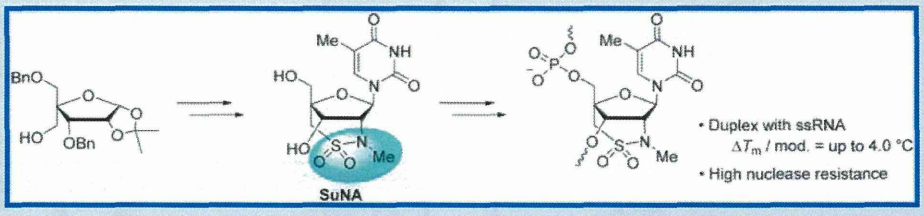
Supporting Information



ABSTRACT: 2′-*N*,4′-*C*-(*N*-Methylamino)sulfonylmethylene-bridged thymidine (SuNA), which has a six-membered linkage including a sulfonamide moiety, was synthesized and introduced into oligonucleotides. The oligonucleotides containing SuNA exhibited excellent nuclease resistance, a high affinity toward single-stranded RNA, and a low affinity toward single-stranded DNA compared to the natural oligonucleotide.

The first systemic antisense drug, Kynamro, was approved by the FDA in 2013,[1] and many other antisense oligonucleotides are in clinical trials. For the practical application of antisense methodology, chemical modification is essential to achieve a strong interaction with single-stranded RNA (ssRNA) in a sequence-specific manner. In addition, high resistance against enzymatic degradation is also required for in vivo applications. Among numerous chemical modifications, the introduction of a bridged structure between the 2′- and 4′-positions generally increases affinity toward ssRNA and improves resistance to nuclease degradation.[2] Since the discovery of the 2′-*O*,4′-*C*-methylene-bridged nucleic acid (2′,4′-BNA[3]/LNA[4]), which is a typical example of these bridged compounds, many bridged nucleic acids have been developed.[5−11] Previous studies have revealed that nuclease resistance can be enhanced by increasing the ring size of the bridge moieties because of increasing steric hindrance (i.e., 2′,4′-BNA/LNA < ENA,[5] 2′,4′-BNA^{NC6} < 2′,4′-BNA^{COC7}) (Figure 1). However, this decreases binding affinity because of insufficient restriction of the sugar conformation (i.e., 2′,4′-BNA/LNA > ENA, 2′,4′-BNA^{NC} > 2′,4′-BNA^{COC}). Thus, a balance between these two properties would be very important for the development of practical antisense oligonucleotides.

Recently, we synthesized several 2′,4′-BNAs possessing amide or urea moieties in their bridged structure (Figure 1). They possessed increased nuclease resistance and/or RNA selectivity compared to analogues with the same-membered bridged structure, maintaining high affinity toward ssRNA (i.e., 2′,4′-BNA/LNA vs AmNA,[8] ENA vs six-membered AmNA,[9] 2′,4′-BNA^{COC} vs urea-BNA[10]). These properties suggest that

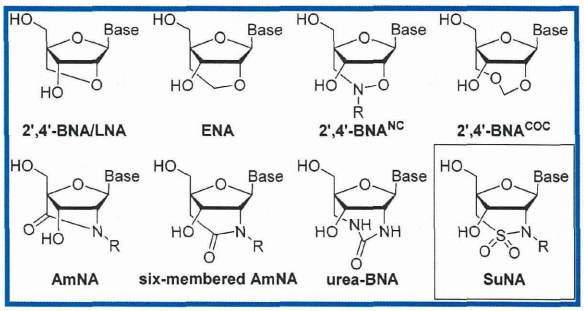


Figure 1. Structures of 2′,4′-BNA/LNA, ENA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{COC}, AmNA, six-membered AmNA, urea−BNA, and SuNA designed in the present study.

the exocyclic carbonyl groups inhibit the interaction between the oligonucleotides and nuclease and destabilize the duplex formed with single-stranded DNA (ssDNA). These are expected to derive from steric and electronic properties of the exocyclic carbonyl groups. However, how the bridged moiety itself affects the hybridization properties and the nuclease resistance of the oligonucleotides remains unknown.

This study used a sulfonamide structure, which is often seen in bioactive compounds or drugs,[12] to evaluate the relationship between ring size and hybridization properties with ssRNA and between bulkiness of the bridge structure and nuclease resistance. Ring size of the bridge structure containing a

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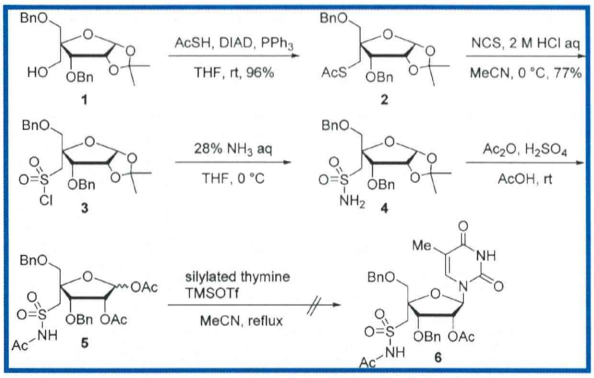
sulfonamide moiety should be larger than that of a same-membered bridged structure because sulfur is larger than oxygen, carbon, or nitrogen. Moreover, a sulfonamide moiety is more bulky than an amide or urea structure. Thus, 2′-*N*,4′-*C*-(*N*-methylamino)sulfonylmethylene-bridged thymidine (SuNA-T) (Figure 1) was synthesized, and the properties of the SuNA-T-modified oligonucleotides were examined.

Initially, the construction of a sulfonamide-bridged structure from **1**,[13] a common precursor for the synthesis of 2′,4′-BNA/LNA, was attempted without any *N*-substituents (Scheme 1). A

Scheme 1. Synthesis of Intermediates and Introduction of Base

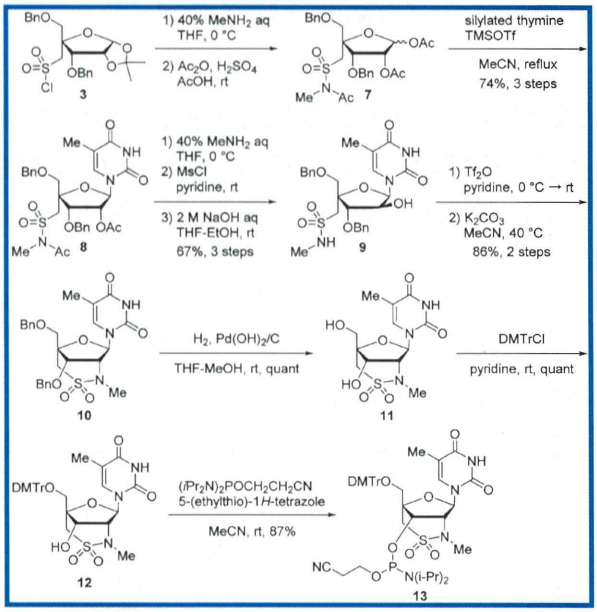


thioacetyl group was introduced into **1** through a Mitsunobu reaction to afford **2**, which was then converted to the sulfonyl chloride derivative **3**.[14] Treatment of **3** with ammonia gave the sulfonamide derivative **4**. In the acetolysis of **4**, the sulfonamide and two hydroxy groups were acetylated to afford triacetate **5**. Although the coupling reaction of **5** with silylated thymine, prepared in situ from thymine and *N*,*O*-bis(trimethylsilyl)-acetamide, was attempted, a complex mixture resulted instead of the desired product **6**. Reactivity of the acylsulfonamide group of **5** may cause many side reactions. This result implied that the construction of a sulfonamide-bridged structure without any *N*-substituents was difficult via this synthetic route.

To avoid the side reactions promoted by the acylsulfonamide group of **5**, a methyl group was introduced into the nitrogen atom of the acylsulfonamide group (Scheme 2). Treatment of **3** with methylamine and subsequent acetolysis afforded triacetate **7**. As expected, the coupling reaction of **7** with silylated thymine was successful and provided the desired product **8** in good yield (74% in three steps), indicating **7** is a good precursor for coupling reactions with silylated nucleobases. After removal of the acetyl groups of **8**, the 2′-hydroxyl group was inverted by mesylation, followed by treatment with NaOH to afford compound **9**. Triflation of **9** and subsequent treatment with K_2CO_3 resulted in intramolecular cyclization to give the desired product **10**. Benzyl groups were removed by hydrogenolysis to afford SuNA-T monomer **11**. Finally, dimethoxytritylation of **11** with 4,4′-dimethoxytrityl chloride followed by phosphitylation gave the phosphoramidite **13**.

The structure of SuNA-T monomer **11**[15] was confirmed by X-ray crystallography (Figure 2a, Table 1). The crystal structure of **11** revealed that the pseudorotation phase angle *P* was 16°, which supports its N-type sugar pucker. Moreover, the ν_{max} and δ values of **11** were 44° and 80°, respectively. The ν_{max} values, which represent the maximum degree of the sugar puckering mode (N/S-type), indicated that the sugar conformation of

Scheme 2. Synthesis of Phosphoramidite 13



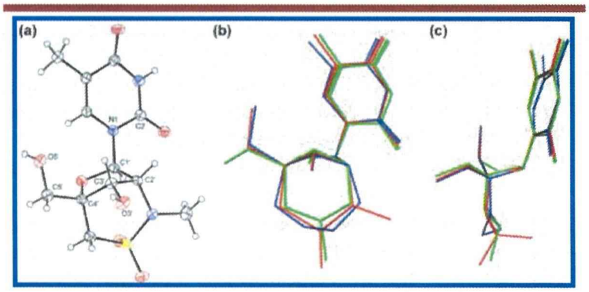


Figure 2. (a) X-ray structure of SuNA-T (**11**). (b, c) Superpositions of X-ray structure of SuNA-T (**11**) (red), 2′,4′-BNA^{NC}[NMe] (green), and 2′,4′-BNA^{COC} (blue).

Table 1. Selected Parameters from X-ray Analysis

		δ (deg)	ν_{max} (deg)	*P* (deg)
2′,4′-BNA		66	57	17
ENA		76	48	15
2′,4′-BNA^{NC}[NMe]		75	49	23
SuNA-T (**11**)	this work	80	44	16
2′,4′-BNA^{COC}		78	38	17

SuNA (ν_{max} 44°) was between the six-membered bridge (ENA and 2′,4′-BNA^{NC}) and the seven-membered bridge (2′,4′-BNA^{COC}) (Figure 2b,c). This may be due to the large sulfur atom. The bridge structure of SuNA is more bulky than that of 2′,4′-BNA^{NC}[NMe], which has the same six-membered bridged structure, because of the two oxygen atoms and methyl group of the sulfonamide moiety (Figure 2b,c).

Phosphoramidite **13** was incorporated into oligonucleotides using an automated DNA synthesizer with standard phosphoramidite chemistry, except for a prolonged coupling time of 16 min with 5-(ethylthio)-1*H*-tetrazole as an activator, conditions similar to those for 2′,4′-BNA^{COC} (see the Supporting Information). The sulfonamide bridge was stable under conventional conditions, that is, aqueous ammonia and

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methylamine at room temperature, for cleavage from the resin and removal of protecting groups.

The duplex-forming abilities of the modified oligonucleotides **15**–**19** with ssDNA and ssRNA were evaluated by UV melting experiments and compared with those of the corresponding natural DNA **14** (Table 2). The T_m values for duplexes formed

Table 2. T_m Values (°C) of Oligonucleotides with Complementary DNA and RNA[a]

	T_m (ΔT_m/mod.)		
oligonucleotides	ssDNA	ssRNA	$T_{m\,(RNA)} - T_{m\,(DNA)}$
5′-GCGTTTTTTGCT-3′ (**14**)	53	49	−4
5′-GCGTTT̲TTTGCT-3′ (**15**)	49 (−4.0)	51 (+2.0)	+2
5′-GCGTTT̲TT̲TGCT-3′ (**16**)	47 (−3.0)	54 (+2.5)	+7
5′-GCGT̲TT̲TT̲TGCT-3′ (**17**)	46 (−2.3)	60 (+3.7)	+14
5′-GCGTTT̲TT̲TGCT-3′ (**18**)	48 (−1.7)	57 (+2.7)	+9
5′-GCGT̲TTTTT̲GCT-3′ (**19**)	53 (0.0)	73 (+4.0)	+20

[a]UV melting profiles were measured in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl at a scan rate of 0.5 °C/min at 260 nm. The concentration of the oligonucleotide was 4 μM for each strand. T̲ = SuNA-T. The sequences of target DNA and RNA complements were 5′-d(AGCAAAAAACGC)-3′ and 5′-r(AGCAAAAAACGC)-3′.

by **15**–**19** with ssRNA were higher than that of the duplex formed by the natural DNA **14** and ssRNA. Changes in ΔT_m/modification values ranged from +2.0 °C to +4.0 °C. This stabilization is between the six-membered bridge (ENA; +3.5 °C to +5.2 °C, 2′,4′-BNA^{NC}; +4.7 °C to +5.8 °C, the six-membered AmNA; +1.0 °C to +4.7 °C) and the seven-membered bridge (2′,4′-BNA^{COC}; +1.0 °C to +2.0 °C and urea-BNA; +1.0 °C to +2.3 °C). This tendency seems to correlate the ν_{max} values of the sugar conformations. In contrast, the oligonucleotides **15**–**19** destabilized the duplex with ssDNA. In the case of **17** and **19**, the differences in T_m values with ssRNA and with ssDNA were 14 and 20 °C, respectively. The oligonucleotides modified by SuNA-T monomer **11** exhibited greater RNA selective hybridization ability than the six-membered AmNA and urea–BNA. This result indicates that a bulky bridge structure destabilized the duplex with ssDNA more efficiently than a small bridge structure, because the bulky bridge would make a steric clash with the C5′ atom of the 3′-neiboring residue when it is located in the narrow minor groove of the B-form DNA duplex.

The enzymatic stability of the modified oligonucleotides was evaluated using a 3′-exonuclease. A comparison of oligonucleotides **20**–**24** is shown in Figure 3. Under the conditions used in this experiment, natural oligonucleotide **20** and the 2′,4′-BNA(LNA)-modified oligonucleotide **21** were completely degraded within 2 and 10 min, respectively. In contrast, the SuNA-modified oligonucleotide **24** significantly enhanced stability against the 3′-exonuclease. This ability was comparable to that of the 2′,4′-BNA^{COC}-modified oligonucleotide **23**, which had a seven-membered bridge structure and was better than that of the 2′,4′-BNA^{NC}[NMe]-modified oligonucleotide **22**, which had a six-membered bridge structure. These results revealed that the six-membered bridged structure possessing a

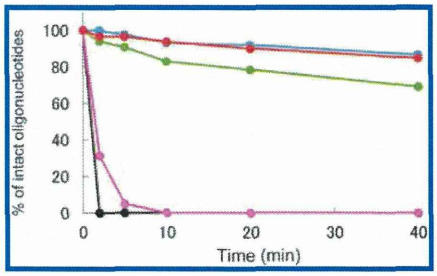

Figure 3. Hydrolysis of oligonucleotides (750 pmol) conducted at 37 °C in buffer (100 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and phosphodiesterase I (4.0 μg/mL). Sequences: 5′-d(TTTTTTTTT̲T)-3′, T̲ = natural (black, **20**), 2′,4′-BNA/LNA (pink, **21**), 2′,4′-BNA^{NC}[NMe] (green, **22**), 2′,4′-BNA^{COC} (blue, **23**), SuNA (red, **24**).

sulfonamide moiety inhibited degradation by a 3′-exonuclease as well as the seven-membered bridge structure did. Previous modeling studies suggested that the appropriate bridged structure between the 2′- and 4′-positions causes a steric challenge to nuclease binding, and a steric clash with the metal ion in the active site of the nuclease and consequently this provides high nuclease resistance.[16] We suppose that the sulfonamide bridge can emphasize the steric clash with the nuclease surface and the metal ion and lead to high enzymatic stability.

For the practical application of antisense methodology, the degradation of the complementary RNA through the RNase H mechanism is very important.[17,18] Hence, the SuNA-modified gapmer **25**, which is 16-mer length having 7-mer central DNA gap and fully modified with the phosphorothioate linkages, was synthesized (Table S1, Supporting Information), and the degradation of complementary RNA in the **25**/RNA hetereoduplex was examined in the presence of RNase H (Figure 4).[11g] Under the conditions used in this experiment,

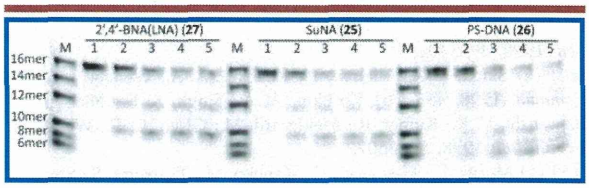

Figure 4. *E. coli* RNase H activity analysis of 5′-Cy3-labeled RNA forming duplexes with phosphorothioated DNA (PS-DNA) **26**, the 2′,4′-BNA(LNA)-modified gapmer **27**, and the SuNA-modified gapmer **25** using 25% denaturing PAGE containing 7 M urea. Lanes 1–5 represent digestion time at 0, 5, 15, 30, and 60 min, respectively. Conditions of cleavage reaction: 5′-Cy3-labeled RNA (0.5 μM) and **25**-**27** (10 μM) in reaction buffer containing 40 mM Tris-HCl (pH 7.2), 150 mM NaCl, 4 mM $MgCl_2$, and 1 mM DTT at 37 °C; 0.01 U/μL of RNase H. M: Marker.

degradation of RNA in the **25**/RNA hetereoduplex was observed. A similar degradation was shown in the phosphorothioated DNA (PS-DNA) **26**/RNA and the 2′,4′-BNA(LNA)-modified gapmer **27**/RNA hetereoduplex.

In conclusion, a novel bridged nucleic acid monomer **11**, 2′-*N*,4′-*C*-(*N*-methylamino)sulfonylmethylene-bridged thymidine (SuNA-T), has been designed and successfully synthesized. This is the first example of a nucleic acid analogue with a sulfonamide-type bridged structure between the 2′- and 4′-positions. The SuNA-modified oligonucleotides produced

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stable duplexes with ssRNA and unstable duplexes with ssDNA. In addition, oligonucleotides containing this analogue have increased stability against nuclease degradation, similar to a seven-membered bridge structure, maintaining high affinity with ssRNA. These investigations reveal that decreasing the ring size, which means increasing the ν_{max} value, increases binding affinity to ssRNA, and that a bulkier bridge structure produces greater nuclease resistance. These results suggest that the SuNA modification provides valuable information that can be applied to antisense technology. In addition, the SuNA-modified gapmer exhibited the degradation of complementary RNA through the RNase H mechanism, and further biological studies are in progress.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental details, representative UV melting data, ^{1}H, ^{13}C, and ^{31}P spectra of all new compounds, and HPLC charts and MALDI-TOF-MS spectra of new oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Design and evaluation of locked nucleic acid-based splice-switching oligonucleotides *in vitro*

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ABSTRACT

Antisense-mediated modulation of pre-mRNA splicing is an attractive therapeutic strategy for genetic diseases. Currently, there are few examples of modulation of pre-mRNA splicing using locked nucleic acid (LNA) antisense oligonucleotides, and, in particular, no systematic study has addressed the optimal design of LNA-based splice-switching oligonucleotides (LNA SSOs). Here, we designed a series of LNA SSOs complementary to the human dystrophin exon 58 sequence and evaluated their ability to induce exon skipping *in vitro* using reverse transcription-polymerase chain reaction. We demonstrated that the number of LNAs in the SSO sequence and the melting temperature of the SSOs play important roles in inducing exon skipping and seem to be key factors for designing efficient LNA SSOs. LNA SSO length was an important determinant of activity: a 13-mer with six LNA modifications had the highest efficacy, and a 7-mer was the minimal length required to induce exon skipping. Evaluation of exon skipping activity using mismatched LNA/DNA mixmers revealed that 9-mer LNA SSO allowed a better mismatch discrimination. LNA SSOs also induced exon skipping of endogenous human dystrophin in primary human skeletal muscle cells. Taken together, our findings indicate that LNA SSOs are powerful tools for modulating pre-mRNA splicing.

INTRODUCTION

Alternative pre-mRNA splicing is an essential system for gene expression in eukaryotes that allows the production of various types of proteins from a limited set of genes (1). However, mutations in splice sites cause mis-splicing, which is followed by genetic diseases (2,3,4). To correct these splicing errors, exon skipping by using antisense oligonucleotides (AONs) has been suggested (5,6). These splice-switching oligonucleotides (SSOs) bind to target sequences in pre-mRNA and prevent the interaction of various splicing modulators (7). Thus, SSOs are able to modulate pre-mRNA splicing and repair defective RNA without inducing the RNase H-mediated cleavage of mRNA (8,9).

To enhance the *in vivo* activity of AONs, many artificial nucleic acids have been synthesized to improve nuclease resistance, binding properties, RNase H activity and serum stability (10,11). Locked nucleic acid (LNA) (also known as 2'-*O*,4'-*C*-methylene-bridged nucleic acid (2',4'-BNA)) is an artificial nucleic acid derivative that was synthesized by us and by Wengel's group independently in the late 1990s (12,13). LNA contains a methylene bridge connecting the 2'-*O* with the 4'-*C* position in the furanose ring, which enables it to form a strictly *N*-type conformation that offers high binding affinity against complementary RNA (14,15,16). LNA also presents enzyme resistance, similar to other nucleic acid derivatives. Given these features, LNA can be used for various gene silencing techniques, such as antisense, short interfering RNA, blocking of microRNA and triplex-forming oligonucleotides. Previous studies also showed that LNA could be used in SSOs (17,18,19,20), and LNA-based SSOs (LNA SSOs) have been shown to be functional *in vivo* in mouse models (21,22).

Recently, SSOs based on 2'-*O*-methyl RNA (2'-OMe) with a full-length phosphorothioate (PS) backbone,

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phosphorodiamidate morpholino oligomer or 2'-*O*,4'-*C*-ethylene-bridged nucleic acids have been applied to clinical trials for the treatment of genetic diseases, particularly Duchenne muscular dystrophy (DMD) (23,24,25,26,27,28). DMD is a severe muscle-weakening disease that arises from mutations in dystrophin, which links the cytoskeleton to the extracellular matrix of muscle fibers. Mutations in the dystrophin gene lead to premature termination of translation and prevent the synthesis of a functional gene product. SSO-mediated exon skipping in dystrophin pre-mRNA can restore the reading frame and allow the expression of a truncated but functional dystrophin similar to that found in Becker muscular dystrophy patients, who have relatively milder symptoms (29). Thus, modulation of splicing using SSOs is an attractive strategy for the treatment of genetic diseases, such as DMD. However, relatively few studies have used LNA SSOs compared to those using SSOs based on other chemistries.

Methods for designing effective SSOs have recently been developed and provide insight into factors that are critical for SSO activity, including the melting temperature (T_m), guanine-cytosine content and secondary structures or sequence motifs that correspond to splicing signals of the target RNA (30,31). Because LNA oligonucleotides possess high binding affinity to complementary RNA, the SSOs that incorporate LNA are considered as promising tools for inducing exon skipping. However, no systematic study has addressed the optimal design of LNA SSOs. Therefore, in this study, we designed a series of LNA SSOs complementary to the human dystrophin exon 58 sequence, and evaluated their ability to induce exon skipping using reverse transcription-polymerase chain reaction (RT-PCR) and a minigene reporter encompassing exons 57–59 of the human dystrophin gene.

MATERIALS AND METHODS

Synthesis of oligonucleotides

All SSOs used in this study are shown in Supplementary Tables S1–S9. Two types of modification, LNA and 2'-OMe, were incorporated into the SSO sequences, in which the phosphodiester linkages were completely replaced by PS linkages (Figure 1). All SSOs were designed to have sequences complementary to human dystrophin gene and were synthesized and purified by Gene Design Inc. (Osaka, Japan).

Plasmid construction

The reporter construct was generated using standard cloning techniques published in a previous study (32). A FLAG (DYKDDDDK)-coding oligonucleotide was constructed by annealing the forward oligonucleotide 5'-AGCTTACCATGGATTACAAGGACGACGAC GACAAGGGGGTAC-3' (including HindIII and KpnI sites, underlined) and reverse oligonucleotide 5'-CCCCTTGTCGTCGTCGTCCTTGTAATCCATGGTA-3'. The annealed oligonucleotide was cloned into the HindIII-KpnI sites of the pcDNA5/FRT vector (Invitrogen, Carlsbad, CA, USA) (termed pcDNA5/FRT-FLAG). The EGFP fragment was obtained by PCR using the forward primer 5'-CCCGGGTGTGAGCAAGGGCGAGGAGCTGT-3' (including a SmaI site, underlined) and reverse primer 5'-ATAGGGCCCTTACTTGTACAGCTCGTCCAT-3' (including an ApaI site, underlined). The obtained EGFP fragment was cloned into the EcoRV-ApaI sites of pcDNA5/FRT-FLAG (termed pcDNA5/FRT-FLAG-EGFP). The DsRed fragment was obtained by PCR from the pDsRed-Express-N1 vector (Clontech, Mountain View, CA, USA) using the forward primer 5'-ATATGGATCCAACCGGTGTGGCC TCCTCCGAGGACGTCA-3' (including BamHI and AgeI sites, underlined) and reverse primer 5'-CGGTCTACAGGAACAGGTGGTGGC-3'. The obtained DsRed fragment was cloned into the BamHI-SmaI sites of the pcDNA5/FRT-FLAG-EGFP vector (termed pcDNA5/FRT-FLAG-DsRed-EGFP). A nuclear localization signal (NLS) was constructed by annealing the forward oligonucleotide 5'-ATGCCCCAAAAAAAAA ACGCAAAGTGGAGGACCCAAAGGTACCAAAG-3' (including a KpnI site, underlined) and reverse oligonucleotide 5'-GATCCTTTGGTACCTTTGGGTCCTCCAC TTTGCGTTTTTTTTTTGGGGCATGTAC-3'. The annealing oligonucleotide was cloned into the KpnI-BamHI sites of pcDNA5/FRT-FLAG-DsRed-EGFP (termed pcDNA5/FRT-FLAG-NLS-DsRed-EGFP).

A human dystrophin minigene containing exons 57–59 was isolated as follows. Because the intron 57 sequence consists of 17 684 bp and is thus too long to insert into a plasmid, we designed a human dystrophin minigene by removing the sequence of intron 57 from position +207 to +17 486. Thus, exon 57, together with a short flanking intronic sequence, was obtained by PCR from the HepG2 genome using the forward primer 5'-AACGGTACCAACGCTGCTGTTCTTTTTCA-3' (including a KpnI site, underlined) and reverse primer 5'-GTGTTTGTAATGGACGATTTCTTAAAGGGTATT-3'. Another fragment containing a short 3' sequence of intron 57 to exon 59 was also obtained by PCR using the forward primer 5'-AAATCGTCCATTACAAACACAGCGCTTTCC-3' and reverse primer 5'-AGACCGGTACTCCTCAGCCTGCTTTCGTA-3' (including an AgeI site, underlined). These two fragments were mixed, and a second round of PCR was performed. Finally, after the second round of PCR, the newly synthesized full-length PCR product was cloned into the KpnI-AgeI sites of the pcDNA5/FRT-FLAG-NLS-DsRed-EGFP vector to generate a dystrophin reporter minigene (termed pcDNA5/FRT-FLAG-NLS-DMD-Exon57_58_59(short-Intron57)-DsRed-EGFP). All constructs were verified by sequencing.

Generation of a stable cell line

Flp-In 293 cells (Invitrogen) were cultured in Dulbecco's modified Eagle Medium (DMEM) (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France), 100 units/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque) and maintained in a 5% CO_2 incubator at 37°C. Flp-In 293 cells were

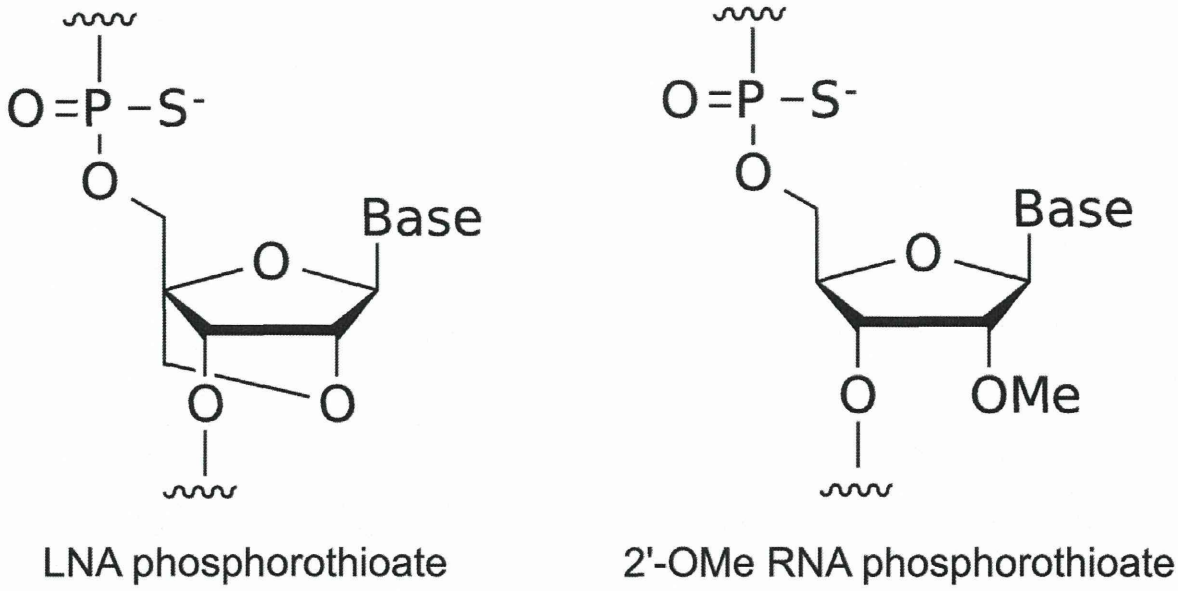

Figure 1. Structures of the building blocks for SSOs. PS LNA and PS 2'-OMe RNA.

co-transfected with pcDNA5/FRT-FLAG-NLS-DMD-Exon57_58_59(short-Intron57)-DsRed-EGFP and pOG44 (the flp recombinase expression plasmid) (Invitrogen). Stable cell lines were selected by 50 μg/ml hygromycin B (Invitrogen).

SSOs transfection

Stable cell lines were seeded one day before transfection at a density of 8.0×10^4 cells/well on 24-well plates. At 30%–40% confluence, SSOs were transfected into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were harvested.

RNA isolation and cDNA synthesis

Total RNA samples were isolated from the cells using the QuickGene 800 and QuickGene RNA cultured cell kit S (KURABO, Osaka, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 150 ng of the total RNA of each cell sample using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions.

Primary myoblast cell culture, SSO transfection and RNA isolation

Primary human skeletal muscle myoblasts (HSMM) derived from healthy Caucasian donor (female aged 17 years) were purchased from Lonza (Walkersville, MD, USA). HSMM cells were cultured in SkBM-2 basal medium (Lonza) supplemented with 10% FBS, epidermal growth factor (EGF), dexamethasone, L-glutamine, gentamycin sulfate and amphotericin B (SingleQuots, Lonza) and maintained in a 5% CO_2 incubator at 37°C. For SSO transfection, cells were seeded 2 days before transfection at a density of 1.0×10^5 cells/well on 24-well collagen type I coated plates. After 24 h, cells were differentiated by changing the growth medium to differentiation medium (DMEM/F-12 (Life Technologies, Carlsbad, CA, USA) containing 2% horse serum (Life Technologies) and antibiotic-antimycotic solution (100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Life Technologies)) for 24 h. Cells were transfected with 500 nM SSOs using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours after transfection, total RNA samples were isolated from the cells using the QuickGene 800 and QuickGene RNA cultured cell kit S according to the manufacturer's instructions. First-strand cDNA was synthesized from 50 ng of the total RNA of each cell sample using the ReverTra Ace qPCR RT Master Mix according to the manufacturer's instructions.

RT-PCR analysis

The cDNA was used as a template for individual PCR reactions using specific primer sets (Supplementary Table S10), which were designed using the Primer3 program written by the Whitehead Institute (33). PCR reactions were conducted using KOD FX Neo DNA polymerase (TOYOBO), and the PCR products were analyzed on a 2% agarose gel stained with ethidium bromide, with specific bands purified for sequence analysis. The intensity of each band was quantified by using ImageJ software (National Institutes of Health; freeware from http://rsb.info.nih.gov/ij/) and normalized according to the nucleotide composition. The exon skipping percentage was calculated as the amount of exon 58-skipped transcript relative to the total amount of the exon 58-skipped and full-length transcripts (34). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.