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

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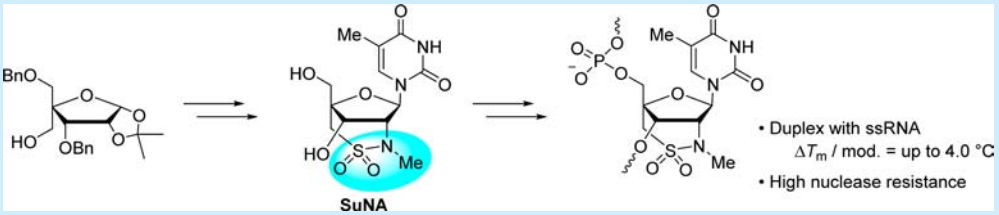
Supporting Information



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 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.

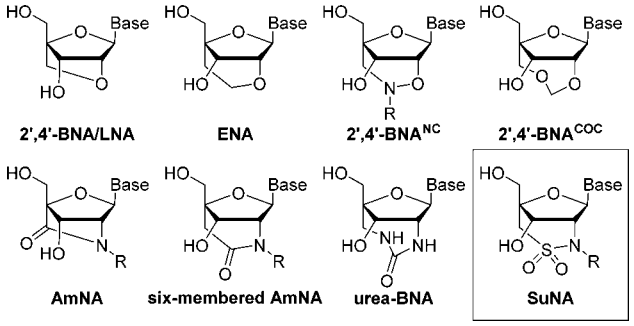


Figure 1. Structures of 2′,4′-BNA/LNA, ENA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{COC}, AmNA, six-membered AmNA, urea−BNA, and SuNA designed in the present study.

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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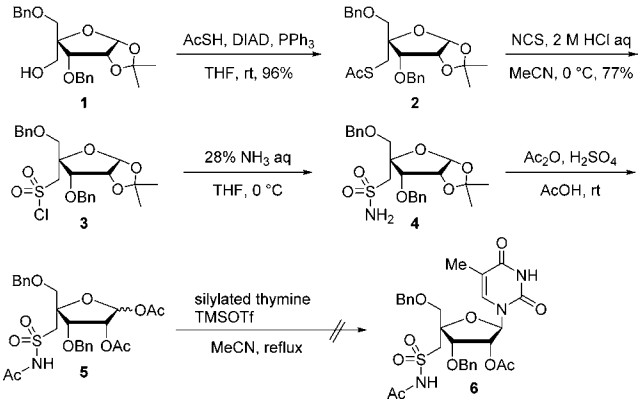
sulfonamide moiety should be larger than that of a same-membered bridged structure because sulfur is larger than oxygen, carbon, or nitrogen. Moreover, a sulfonamide moiety is more bulky than an amide or urea structure. Thus, 2′-*N*,4′-*C*-(*N*-methylamino)sulfonylmethylene-bridged thymidine (SuNA-T) (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

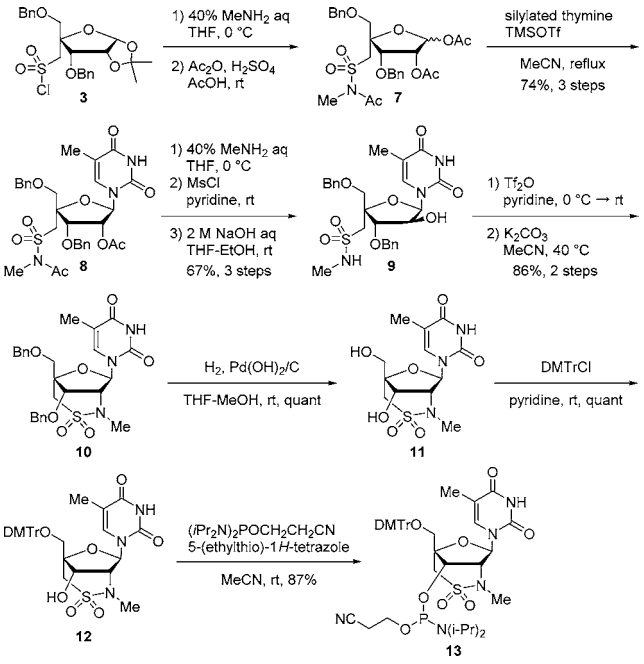


thioacetyl group was introduced into **1** through a Mitsunobu reaction to afford **2**, which was then converted to the sulfonyl chloride derivative **3**.[14] Treatment of **3** with ammonia gave the sulfonamide derivative **4**. In the acetolysis of **4**, the sulfonamide and two hydroxy groups were 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



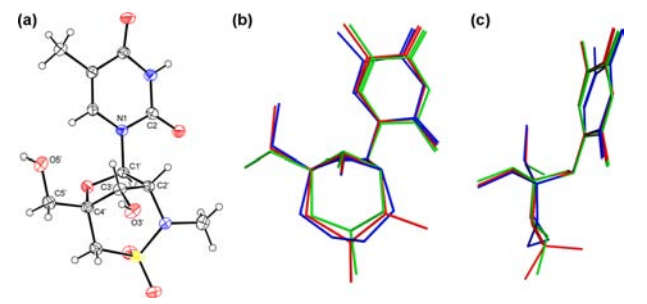


Figure 2. (a) X-ray structure of SuNA-T (**11**). (b, c) Superpositions of X-ray structure of SuNA-T (**11**) (red), 2′,4′-BNA^{NC}[NMe] (green), and 2′,4′-BNA^{COC} (blue).

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′-GCGTTTTTTGCT-3′ (**15**)	49 (−4.0)	51 (+2.0)	+2
5′-GCGTTTTTTGCT-3′ (**16**)	47 (−3.0)	54 (+2.5)	+7
5′-GCGTTTTTTGCT-3′ (**17**)	46 (−2.3)	60 (+3.7)	+14
5′-GCGTTTTTTGCT-3′ (**18**)	48 (−1.7)	57 (+2.7)	+9
5′-GCGTTTTTTGCT-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. T = 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

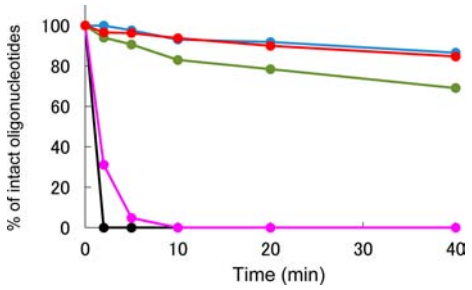

Figure 3. Hydrolysis of oligonucleotides (750 pmol) conducted at 37 °C in buffer (100 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and phosphodiesterase I (4.0 μg/mL). Sequences: 5′-d(TTTTTTTTTT)-3′, T = natural (black, **20**), 2′,4′-BNA/LNA (pink, **21**), 2′,4′-BNA^{NC}[NMe] (green, **22**),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

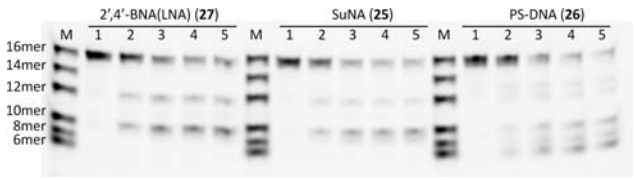

Figure 4. *E. coli* RNase H activity analysis of 5′-Cy3-labeled RNA forming duplexes with phosphorothioated DNA (PS-DNA) **26**, the 2′,4′-BNA(LNA)-modified gapmer **27**, and the SuNA-modified gapmer **25** using 25% denaturing PAGE containing 7 M urea. Lanes 1–5 represent digestion time at 0, 5, 15, 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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