RSC Chemical Biology

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Chem. Biol.*, 2026, 7, 830

Synthesis and properties of L-*lyxo*-thioBsNA-pyrimidine nucleoside: a sulfur-containing analogue of boat-shaped pyranosyl nucleic acid

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Received 30th January 2026,
Accepted 9th March 2026

DOI: 10.1039/d6cb00034g

rsc.li/rsc-chembio

L-*lyxo*-thioBsNA, a boat-shaped nucleic acid (BsNA) analogue designed to enhance hydrophobicity – a property strongly influencing its physical characteristics – was synthesized. Oligonucleotides containing L-*lyxo*-thioBsNA were efficiently synthesized using the phosphoramidite method with levulinyl chemistry, and their hydrophobicity was higher than that achieved using PS-modification, as revealed by analysis using reverse-phase HPLC. The double helix with the complementary RNA strand is highly stable, and the introduction of sulfur atoms with large atomic radii did not adversely affect thermal stability. These characteristics demonstrate that L-*lyxo*-thioBsNA is an ideal nucleic acid modifier for RNA-targeting technologies such as antisense methods, offering a new option for use in situations requiring hydrophobic nucleic acids.

Introduction

Oligonucleotide analogues possess immense potential not only as therapeutic agents but also as recognition units for nanotechnology, driving accelerated research into nucleic acid mimetics over the past decade.[1] Research on 2'-modified RNA and conformationally restricted nucleic acids, which mimic natural RNA structures, has been particularly remarkable, and some of the analogues are now widely recognized as artificial nucleic acids capable of forming stable duplexes and readily available for anyone to use in their research.[1]

Artificial nucleic acids exhibit properties useful for chemical biology, for example furanosyl nucleic acids based on furanoses such as ribose. In particular, since it was reported that the locked nucleic acid (LNA)/2′-O,4′-C-methylene bridged acid (2′,4′-BNA),[2] an RNA mimic that restricts conformational changes during duplex formation, exhibit extremely high duplex formation ability with complementary nucleic acids, numerous studies[3–5] have been conducted aiming to create LNA analogues that exhibit functions unattainable with the original LNA/BNA modification. Similarly, tricyclo-DNA, which can be regarded as a DNA mimic, has been reported as a unique artificial nucleic acid that exhibits high duplex-forming ability and strong resistance to degradation, and is therefore considered a promising candidate for therapeutic applications.[6]

On the other hand, pyranosyl nucleic acids possessing pyranose sugars useful for these purposes have not been found, with the exception of D-altritol nucleic acid (ANA)[7] and the deoxy analogue hexitol nucleic acid (HNA)[8] with a nucleobase at the C2′ position. In this context, it has been reported that pyranosyl nucleic acids bearing a nucleobase at the C1′ position are generally unable to form duplexes with complementary DNA or RNA,[9] except for one example in which an unstable duplex can be formed.[10] These findings suggest that a conformationally restricted design strategy—demonstrated to be highly effective for furanosyl nucleic acids—cannot be readily applied to pyranosyl nucleic acids. Furthermore, the observation that constrained altriol nucleic acid (cANA), which was designed to control the conformation of HNA capable of forming stable duplexes, actually decreases duplex stability further highlighted the inherent difficulty of achieving conformational control in pyranosyl nucleic acids.[11] Meanwhile, we have studied pyranosyl nucleic acids whose conformation is restricted in a boat-shape (BsNAs),[12] and found that isoBsNA and L-*lyxo*-BsNA form stable duplexes with the complementary RNA under physiological conditions.[13] A series of BsNAs are the only analogues that have high affinity with RNA in the pyranosyl nucleic acids having nucleobases at the anomeric C1′ position. Therefore, by creating BsNA analogues with various physical properties, it becomes possible to discover characteristics not present in previously developed artificial nucleic acids.

The most representative example of nucleic acid modification is phosphorothioate (PS) modification of the phosphate

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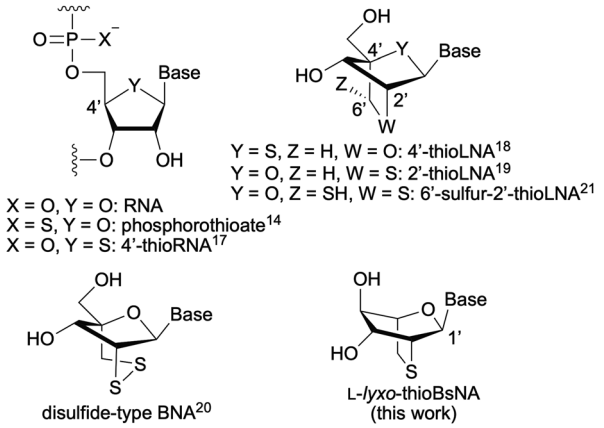


Fig. 1 Examples of sulfur-containing artificial nucleic acids.

backbone, in which one of the oxygen atoms of the phosphate group is replaced by a sulfur atom, which is essential for enhancing *in vivo* stability, such as strong resistance to nucleases and appropriate binding ability to serum proteins (Fig. 1).[14] However, it is also true that reducing or eliminating PS modification has emerged as a major challenge in oligonucleotide-based medicinal chemistry because PS modification has been implicated in toxicity[15] and undesirable tissue distribution.[16]Similarly, interesting properties have been reported for artificial nucleic acids bearing a sulfur-modified sugar moiety. For example, 4′-thio nucleic acids[17] and their LNA analogues[18] exhibit RNA-like structural properties and high duplex forming ability. 2′-thioLNA[19] can also show high duplex forming ability, and the analogues possess the redox sensing feature[20] and post-synthesis modification capabilities[21] in addition to these properties. In particular, the common features of sulfur-modification are increased hydrophobicity and potential resistance to nucleases, suggesting that the use of sugar-modified sulfur-containing artificial nucleic acids has the potential to avoid phosphorothioate modification.

In this study, we synthesized L-*lyxo*-thioBsNA which is a boat-shaped pyranose nucleic acid analogue containing a sulfur atom and evaluated the hybridization properties and the tolerance to the nuclease of L-*lyxo*-thioBsNA oligonucleotides (Fig. 1).

Results and discussion

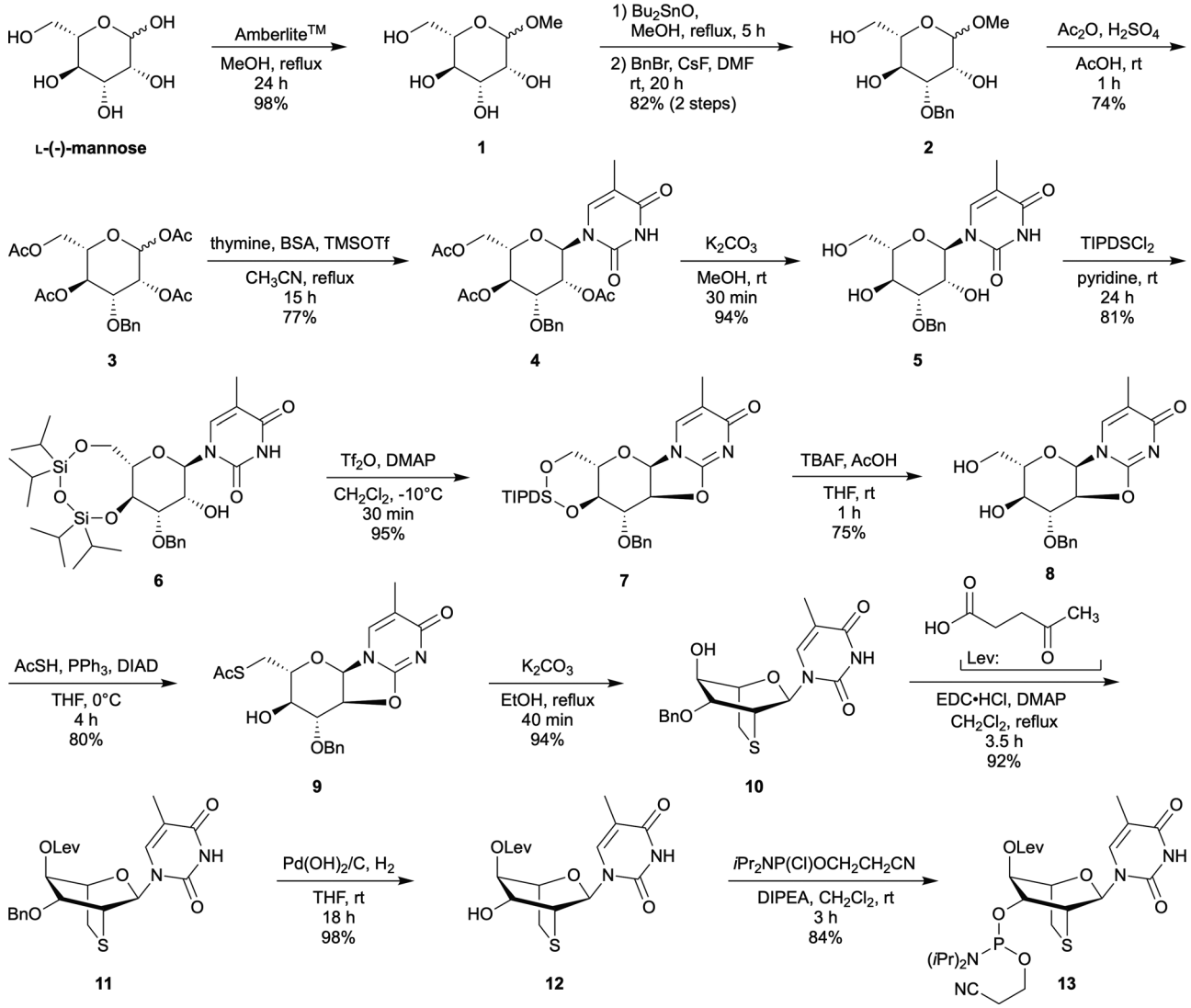
Synthesis of L-*lyxo*-thioBsNA thymine and 5-methylcytosine nucleosides

An L-*lyxo*-thioBsNA thymine analogue, L-*lyxo*-thioBsNA-T, was synthesized from L-mannose as shown in Scheme 1. In the synthesis process, all reaction conditions were optimized using methyl α-D-mannoside as the starting material (Scheme S1), and an established synthetic route for D-*lyxo*-thioBsNA was applied. First, methylation of L-mannose was performed under acidic conditions to obtain a 1 : 1 anomeric mixture of methyl L-mannoside (**1**). Despite compound **1** being a stereoisomeric mixture, selective benzylation at the 3-position *via* stannylene acetalization of **1** proceeded efficiently, yielding compound **2**. The conversion from **2** to tetra-acetate **3** was achieved under sulfuric acid acidic conditions in moderate yield. The NMR spectrum of the resulting **3** was consistent with that of the D-isomer. A thymine base was introduced under TMSOTf acidic conditions, leading to the formation of a protected L-mannopyranosylthymine analogue **4**. Compound **6** was obtained by deacetylation of **4** followed by TIPDS protection of resulting **5**. Compound **6** was subjected to trifluoromethylation conditions to promote cyclization at the C2 and C2′ positions, giving compound **7**, which was subsequently converted to **8** by desilylation using TBAF-AcOH. The introduction of sulfur atoms into the compound was achieved by a Mitsunobu reaction with thioacetic acid, yielding 6-thiocyclonucleoside **9**. The thioBsNA skeleton of **10** was quantitatively constructed by treating **9** with potassium carbonate in ethanol under reflux conditions. Initially, we considered protecting the hydroxyl group of the obtained **10** with a DMTr group suitable for oligonucleotide synthesis; however, we opted for the levulinyl group instead, since the DMTr group was simultaneously removed under the debenzylation conditions, preventing the synthesis of the tritylated compound (see Scheme S1). Therefore, the hydroxyl group of **10** was esterified with levulinic acid, and the resulting compound **11** was subjected to hydrogenolysis in the presence of palladium–carbon to give compound **12**. Finally, phosphitylation at the 3′-hydroxy group of **12** with 2-cyanoethyl *N*,*N*-diisopropylaminochlorophosphoramidite afforded the desired phosphoramidite building block **13**.

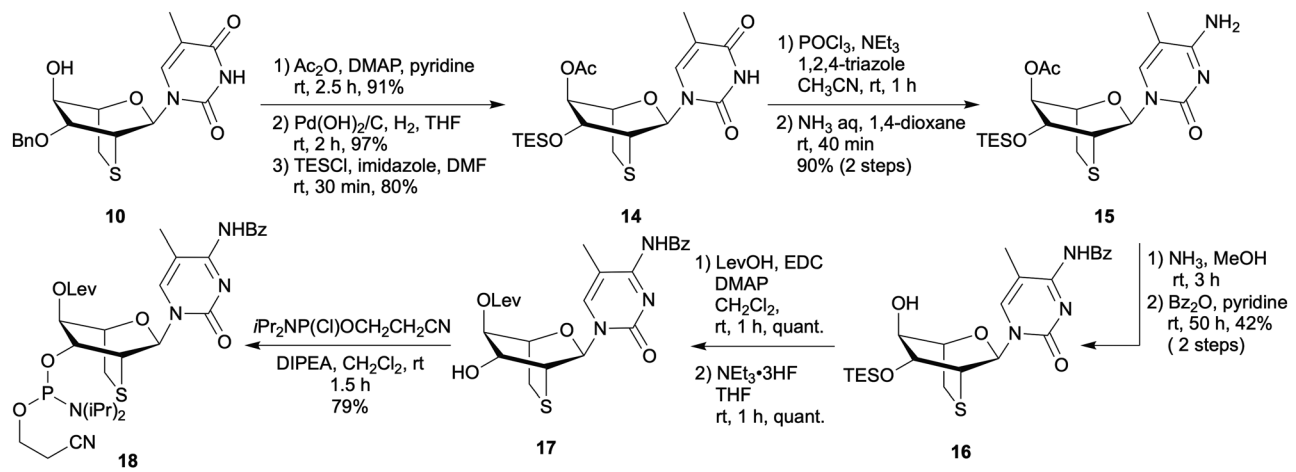
A 5-methylcytidine analogue of L-*lyxo*-thioBsNA, L-*lyxo*-thioBsNA-mC, was synthesized from intermediate **10** as shown in Scheme 2. Since debenzylation did not proceed after converting thymine to 5-methylcytosine (Scheme S2), the protecting group of **10** was first replaced with the TES group to obtain compound **14**. The obtained compound **14** was converted to 5-methylcytosine derivatives **15** in high yield *via* triazolylation at the 4-position, followed by ammonolysis. After deacetylation of **15** with ammonia-methanol solution, the resulting compound was converted to compound **16** by protecting its cytosine base with a benzoyl group. Obtained **16** was then esterified with levulinic acid, followed by desilylation with fluoride ions to yield compound **17**. Finally, the phosphoramidite building block **18** was obtained by phosphitylating the 3′-hydroxy group of **17** with 2-cyanoethyl *N*,*N*-diisopropylaminochlorophosphoramidite.

Synthesis of L-*lyxo*-thioBsNA-containing oligonucleotides

To evaluate the properties of L-*lyxo*-thioBsNA in oligonucleotides (ONs), phosphoramidites **13** and **19** were incorporated into ONs using an automated DNA synthesizer. Each coupling reaction was accomplished using 5-benzylthio-1*H*-tetrazole as an activator, and the coupling time was prolonged to 12 min for L-*lyxo*-thioBsNA. Hydrazine and trichloroacetic acid were used as deblocking reagents for L-*lyxo*-thioBsNA and natural nucleoside units, respectively. The standard iodine oxidation conditions were applicable to the oxidation of the phosphorus atom, and under these conditions the sulfur atom of L-*lyxo*-thioBsNA



Scheme 1 Synthesis of L-*lyxo*-thioBsNA-T phosphoroamidite.



Scheme 2 Synthesis of L-*lyxo*-thioBsNA-meC phosphoroamidite.

Table 1 Sequences of oligonucleotides[ab]

	Sequences (5′–3′)	MALDI-TOF-MS Calc.	MALDI-TOF-MS Found
ON1	GCG TTT TTT GCT	3633.4	3632.7
ON2	GCG **T**TT TTT GCT	3677.5	3677.1
ON3	GCG **TT**T TTT GCT	3721.5	3721.3
ON4	GCG TTT **T**TT GCT	3721.5	3721.2
ON5	GCG **TTT** TTT GCT	3765.6	3764.3
ON6	GCG TTT **TT**T GCT	3765.6	3764.2
ON7	GCG TTC TTT GCT	3616.7	3617.6
ON8	GCG TTC TTT GCT	3676.5	3675.9
ON9	TTT TTT TTT T	2980.0	2979.6
ON10	TTT TTT TTT^T	2996.4	2994.3
ON11	TTT TTT TTT **T**	3024.1	2023.1
ON12	TTT TTT T**T**T T	3024.1	3022.4

[a] Capital letters and bold letters indicate DNA and L-*lyxo*-thioBsNA, respectively. [b] ^ = phosphorothioate.

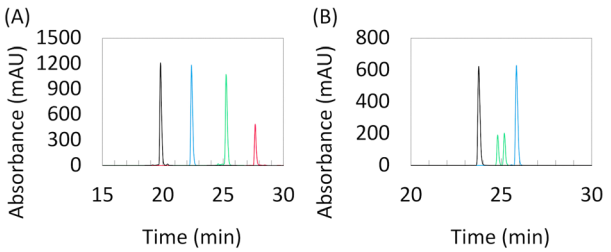


Fig. 2 Overlaid reverse-phase HPLC charts of ONs. (A) **ON1:** black, **ON2:** blue, **ON3:** green and **ON5:** red. (B) **ON9:** black, **ON10:** green and **ON12:** blue.

was not oxidized to sulfoxide or other higher oxidation states. Synthesized ONs were cleaved from the solid supports and deprotected by treatment with ammonium hydroxide solution. The obtained ONs were purified by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry. Sequences are shown in Table 1. Comparing the retention times of synthesized L-*lyxo*-thioBsNA-modified oligonucleotides by reverse-phase HPLC, the retention time increases as L-*lyxo*-thioBsNA modification increases, clearly demonstrating the high hydrophobicity of L-*lyxo*-thioBsNA (Fig. 2A).[22] Furthermore, it was found that L-*lyxo*-thioBsNA-modified oligonucleotides exhibit higher hydrophobicity than PS-oligonucleotides (Fig. 2B). **ON10** exhibited two peaks in the HPLC spectrum due to the presence of stereoisomers on the phosphorothioate moiety.

Effect of L-*lyxo*-thioBsNA on the thermal stability of the oligonucleotide duplex

To evaluate the properties of L-*lyxo*-thioBsNA in oligonucleotides, the thermal stability of L-*lyxo*-thioBsNA-modified ONs with complementary single-stranded DNA and RNA was first evaluated using UV melting experiments. The UV melting curves are shown in Fig. 3 for RNA and in Fig. S1 for DNA and their 50% thermal denaturation temperatures (T_m values) are summarized in Table 2. A single L-*lyxo*-thioBsNA-T modification in oligonucleotides resulted in a 3.1 °C decrease for the DNA complement and in a 2.4 °C increase for the RNA

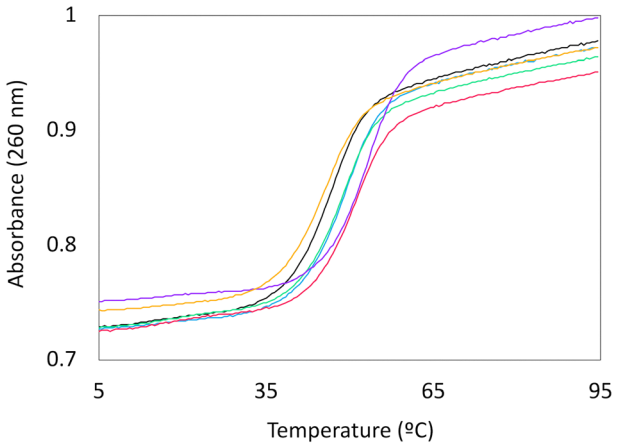


Fig. 3 Representative UV melting curves of the L-*lyxo*-thioBsNA-RNA duplex, **ON1:** black, **ON2:** blue, **ON3:** green, **ON4:** violet, **ON5:** pink, and **ON6:** orange and the complementary RNA: 5′-r(AGC AAA AAA CGC)-3′. Conditions: 10 mM sodium phosphate (pH 7.2), 100 mM NaCl, and 4.0 μM of each oligonucleotide.

Table 2 T_m values of duplexes containing L-*lyxo*-thioBsNA

	T_m (ΔT_m/mod.), °C ssDNA[a]	T_m (ΔT_m/mod.), °C ssRNA[a]	RNA selectivity T_m (RNA)–T_m (DNA)
ON1	50.7	46.1	−4.6
ON2	47.5 (−3.1)	48.4 (+2.4)	+0.9
ON3	41.2 (−4.7)	48.2 (+1.1)	+7.1
ON4	49.8 (−0.4)	52.4 (+3.2)	+2.6
ON5	34.6 (−5.3)	50.4 (+1.5)	+15.8
ON6	27.3 (−7.8)	45.0 (−0.4)	+17.6
ON7	52.9	52.6	−0.3
ON8	53.2 (+0.3)	57.2 (+4.5)	+4.0

[a] For **ON1** to **ON6**, 5′-d/r(AGC AAA AAA CGC)-3′, for **ON7** and **ON8**, 5′-d/r(AGC AAA GAA CGC)-3′. Conditions: 10 mM sodium phosphate buffer (pH = 7.2), 100 mM NaCl solution, and 4 μM each oligonucleotide. The melting profiles were recorded at 260 nm from 5 to 95 °C at a scan rate of 0.5 °C min^{-1}. Each number represents the average of T_m values from three independent experiments.

complement in the T_m value. The influence of multiple L-*lyxo*-thioBsNA-T modification varied depending on their modification pattern. When the modifiers were spaced apart, a strong stabilizing effect on the duplex with complementary RNA was observed. However, this effect diminished as the modifiers were placed closer together and was completely lost when three modifiers were positioned consecutively. Regarding duplex stability with complementary DNA, L-*lyxo*-thioBsNA-T modification generally caused destabilization, and furthermore, it was found that the extent of this destabilization depended on the modification pattern, just as it did for the duplex stability with RNA. Also, the effect of L-*lyxo*-thioBsNA-meC on T_m was similar to that of L-*lyxo*-thioBsNA-T, selectively stabilizing the duplex with RNA complement. Eschenmoser and colleagues reported that when homopyrimidine sequences were fully modified with L-α-threofuranosyl nucleic acid (TNA)[23] or L-α-lyxopyranosyl oligonucleotides,[10] the T_m values decreased markedly. Their observations are consistent with the findings of our study.

Table 3 Mismatch recognition ability

	T_m (ΔT_m = ΔT_m [mismatch] − ΔT_m [match]), °C			
	X = A	X = G	X = C	X = T
ON1/DNA	50.7	39.0 (−11.4)	35.6 (−15.1)	36.5 (−14.1)
ON2/DNA	47.5	34.0 (−13.6)	30.9 (−16.6)	34.0 (−13.0)
ON1/RNA	46.1	43.1 (−3.0)	29.9 (−16.2)	32.6 (−13.5)
ON2/RNA	48.4	40.3 (−8.1)	33.0 (−15.4)	34.0 (−14.5)

*The numbers indicate the T_m values of the duplex formed by **ON1** or **ON2** with the DNA or RNA sequence of 5′-AGC AAA XAA CGC-3′, where X represents the position where T in **ON1** or L-*lyxo*-thioBsNA-T in **ON2** forms a base pair. The experimental conditions are the same as those shown in Table 2.

Considering that TNA is a furanosyl nucleic acid with a similar chemical structure to that of L-*lyxo*-thioBsNA, it is possible that BsNA has comparable properties to TNA. They also reported that the full modification of homopurine sequences or mixed sequences with TNA leads to an increase in T_m values. Therefore, synthesizing L-*lyxo*-(thio)BsNA-purine analogues and elucidating their properties may enable us to uncover even more intriguing characteristics of L-*lyxo*-(thio)BsNA series in the future.

The base-recognition ability of L-*lyxo*-thioBsNA was then examined using single-mismatch DNA and RNA strands (Table 3 and Fig. S2 to S9). As shown in Table 3, any mismatched L-*lyxo*-thioBsNA-T base pairs in duplex with DNA or RNA strands were shown to cause significant destabilization of the duplex. This result suggests that L-*lyxo*-thioBsNA forms typical Watson–Crick base pairs appropriately.

Resistance to exonucleolytic degradation of L-*lyxo*-thioBsNA

Next, we compared the stability of the L-*lyxo*-thioBsNA moiety in a single-stranded thymidine decamer against nucleases with that achieved using phosphorothioate modification. **ON10**, **ON11** and **ON12** were treated with snake venom phosphodiesterase I (SVPDE), 5′-exonuclease, and samples collected at each time point were analysed by reverse-phase HPLC. Fig. 4 shows the results of plotting the ratio of the UV area for full-length ONs against time in HPLC (Fig. S10). As can be seen in the results, under conditions where 60% of PS modifications degraded within 40 minutes, only about 30–40% of L-*lyxo*-thioBsNA degraded, indicating that L-*lyxo*-thioBsNA exhibited higher resistance to the nuclease than that observed in PS modification.[24] Among the many artificial nucleic acids that exhibit degradation resistance at the 3′-terminal side, it was interesting that this modifier showed degradation resistance at both the 3′- and 5′-sides. This result indicates that high resistance to enzymatic degradation can be achieved even without sequential modification, a feature that sufficiently compensates for the potential decrease in duplex stability that L-*lyxo*-thioBsNA may exhibit when sequentially modified, making it suitable for drug discovery applications.

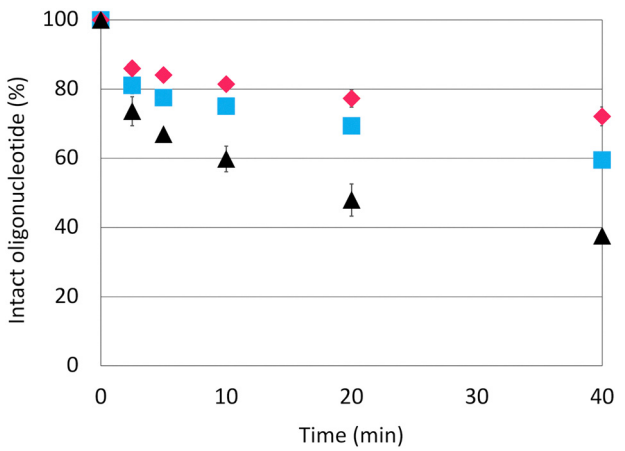


Fig. 4 Stability of **ON10**–**ON12** against SVPDE. **ON10:** black triangles, **ON11:** pink diamonds, and **ON12:** blue squares. Conditions: 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 4.0 μM of each oligonucleotide, and 25 mU PDE at 37 °C. This experiment was conducted in triplicate (n = 3 per group). Data are presented as mean ± SD.

Conclusions

We successfully synthesized thymidine and 5-methylcytidine analogs of L-*lyxo*-thioBsNA, a sulfur-containing boat-shaped nucleic acid that we designed as a highly hydrophobic artificial nucleic acid. Upon incorporation of L-*lyxo*-thioBsNA into oligodeoxynucleotides, the resulting duplex with its complementary RNA was thermally stabilized, whereas the duplex formed with its complementary DNA was markedly destabilized. This result indicates that the large atomic radius of the sulfur atom did not exert a significant influence on duplex stability. In contrast, it was found by reverse-phase HPLC that the hydrophobicity of ODNs bearing L-*lyxo*-thioBsNA was as high as that of PS-modified ODN, confirming that the sulfur atom had a significant impact on hydrophobicity as intended in the design. Furthermore, L-*lyxo*-thioBsNA exhibited high resistance to degradation by the exonuclease homolog known to be abundant in serum, and hydrolysis at both its 3' and 5' sides required a longer time than that observed for the hydrolysis of the PS-modified moiety. The various properties elucidated in this study demonstrate that L-DNA is an ideal nucleic-acid material for RNA-targeting technologies—particularly those intended for use in biological environments—such as RNA detection platforms and antisense-based therapeutic approaches. Given that its pronounced hydrophobicity is expected to influence the *in vivo* distribution and pharmacokinetic behavior of nucleic acids, we are synthesizing various nucleobase analogs to elucidate their fundamental properties in greater detail, aiming to apply this research to drug discovery.

Experimental

Synthesis of L-*lyxo*-thioBsNA phosphoramidites

General. All moisture-sensitive reactions were performed under an argon atmosphere in well-dried glassware. Anhydrous acetonitrile (MeCN), dichloromethane (CH_2Cl_2), and tetrahydrofuran (THF) were used as received. The other anhydrous

solvents were purified as follows: ethanol by drying over activated molecular sieves (3 Å); methanol by distillation; and pyridine and DMF by distillation from KOH and CaH_2, respectively. All purified solvents were stored over activated molecular sieves (3 Å for methanol, and 4 Å for pyridine and DMF) under an argon atmosphere until use. ^{1}H NMR (400 MHz), ^{13}C NMR (101 MHz) and ^{31}P NMR (162 MHz) spectra were recorded on a JEOL JNM-ECS400 or a JNM-ECZ400 spectrometer. Chemical shifts are reported in parts per million referenced to internal tetramethylsilane (0.00 ppm), residual $CHCl_3$ (7.26 ppm), CH_3OH (3.31 ppm), or DMSO (2.50 ppm) for ^{1}H NMR, and $CHCl_3$ (77.06 ppm), CH_3OH (49.03 ppm) or DMSO (39.53 ppm) for ^{13}C NMR. H_3PO_4 (0.00 ppm) was used as an external standard for ^{31}P NMR. High-resolution mass spectra (HRMS) were obtained on a JEOL JMS-T100LP AccuTOF LC-plus spectrometer equipped with an electrospray ionization (ESI) source in positive ion mode. For column chromatography, the Fuji Silysia PSQ-60B silica gel was used. The progress of the reactions was monitored by analytical thin-layer chromatography on precoated glass plates (silica gel 60 F254, Merck).

Methyl L-mannopyranoside ***1***. To a solution of L-(-)-mannopyranose (12.61 g, 70.0 mmol) in dry methanol (140 mL) was added Amberlite IRC120 H-form (25 g) at room temperature under an argon atmosphere. After refluxing for 24 h, the reaction mixture was filtered and concentrated under reduced pressure to obtain **1** (13.36 g, 68.8 mmol, 98%) as a pale yellow solid. ^{1}H-NMR (400 MHz, DMSO-D6) δ 4.71 (d, J = 1.4 Hz, 1H), 4.69 (d, J = 2.8 Hz, 1H), 4.55 (d, J = 6.0 Hz, 1H), 4.48 (d, J = 1.4 Hz, 1H), 4.45 (t, J = 6.0 Hz, 1H), 3.65 (qd, J = 6.0, 2.3 Hz, 1H), 3.59–3.57 (m, 1H), 3.46–3.35 (m, 3H), 3.24 (s, 3H); ^{13}C-NMR (101 MHz, DMSO-D6) δ 101.0, 73.9, 71.0, 70.2, 67.0, 61.3, 53.9; HRMS (ESI) m/z: $[2M + Na]^+$ calcd for $C_{14}H_{28}NaO_{12}^+$ 411.1478; found 411.1461.

Methyl 3-O-benzyl-L-mannopyranoside ***2***. To a solution of **1** (12.68 g, 65.3 mmol) in dry methanol (130 mL) was added dibutyltin oxide (19.50 g, 78.3 mmol) at room temperature under an argon atmosphere. After refluxing for 5 h, the reaction mixture was concentrated under reduced pressure. To the residue in dry DMF (130 mL) were added cesium fluoride (11.95 g, 78.7 mmol) and benzyl bromide (11.6 mL, 97.7 mmol) at room temperature under an argon atmosphere. After stirring at room temperature for 20 h, the reaction mixture was filtered and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography ($CHCl_3$, then $CHCl_3$/MeOH = 50 : 1 to 15 : 1) to obtain **2** (15.17 g, 53.4 mmol, 82%) as a brown oil. ^{1}H-NMR (400 MHz, $CDCL_3$) δ 7.38–7.30 (m, 5H), 4.76 (d, J = 1.4 Hz, 1H), 4.69 (d, J = 11.4 Hz, 1H), 4.58 (d, J = 11.5 Hz, 1H), 3.98–3.94 (m, 2H), 3.80 (dd, J = 6.4, 3.7 Hz, 2H), 3.67 (dd, J = 9.2, 3.2 Hz, 1H), 3.56 (dt, J = 9.6, 3.7 Hz, 1H), 3.35 (s, 3H), 3.02 (d, J = 2.8 Hz, 1H), 2.86 (d, J = 3.2 Hz, 1H), 2.69 (t, J = 6.7 Hz, 1H); ^{13}C-NMR (101 MHz, $CDCL_3$) δ 137.6, 128.7, 128.3, 128.1, 100.6, 79.7, 71.9, 71.7, 67.7, 66.2, 62.1, 55.0; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{14}H_{20}NaO_6^+$ 307.1158; found 307.1131.

1,2,4,6-Tetra-O-acetyl-3-O-benzyl-L-mannopyranoside ***3***. To a solution of **2** (11.44 g, 40.2 mmol) in acetic acid (28.6 mL), acetic anhydride (71 mL, 752 mmol) and concentrated sulfuric acid (1.07 mL, 20.0 mmol) were added dropwise at room temperature under an argon atmosphere, and the reaction mixture was stirred at room temperature for 1 h. After the addition of water, the resulting mixture was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (n-hexane, then n-hexane/AcOEt = 1 : 1) to obtain **3** (13.02 g, 29.7 mmol, 74%) as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37–7.26 (m, 5H + $CHCl_3$), 6.10 (d, J = 1.8 Hz, 1H), 5.36 (t, J = 2.8 Hz, 1H), 5.29 (t, J = 9.9 Hz, 1H), 4.68 (d, J = 12.4 Hz, 1H), 4.45 (d, J = 12.4 Hz, 1H), 4.23 (dd, J = 12.4, 5.0 Hz, 1H), 4.09 (dd, J = 12.4, 2.3 Hz, 1H), 3.94 (ddd, J = 10.1, 5.0, 2.3 Hz, 1H), 3.86 (dd, J = 9.9, 3.4 Hz, 1H), 2.17 (s, 3H), 2.12 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 170.8, 170.0, 169.6, 168.1, 137.4, 128.5, 128.0, 127.8, 91.1, 74.1, 71.5, 70.8, 67.1, 66.9, 62.4, 20.9, 20.9, 20.8, 20.8; HRMS (ESI) m/z: [M + Na]+ calcd for $C_{21}H_{26}NaO_{10}$ 461.1424; found 461.1440.

1-(2,4,6-Tri-O-acetyl-3-O-benzyl-α-L-mannopyranosyl)thymine ***4***. To a solution of **3** (14.40 g, 32.8 mmol,) in dry acetonitrile (320 mL) were added thymine (8.12 g, 64.4 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (32.0 mL, 131 mmol) at room temperature under an argon atmosphere. After refluxing for 1 h, to the reaction mixture was added trimethylsilyl trifluoromethanesulfonate (12.0 mL, 64.4 mmol) at room temperature, and the reaction mixture was refluxed for 15 h. After the addition of saturated aqueous $NaHCO_3$ at 0 °C, the resulting mixture was extracted with AcOEt. The obtained organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (n-hexane/AcOEt = 2 : 3) to obtain **4** (12.78 g, 25.3 mmol, 77%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.48 (brs, 1H), 7.39–7.30 (m, 5H), 7.15 (brs, 1H), 6.26 (d, J = 9.6 Hz, 1H), 5.19 (dd, J = 9.6, 3.2 Hz, 1H), 5.03 (d, J = 3.7 Hz, 1H), 4.75–4.64 (m, 3H), 4.31–4.27 (m, 2H), 4.02 (t, J = 3.2 Hz, 1H), 2.16 (s, 3H), 2.07 (s, 3H), 1.93 (s, 6H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 170.6, 169.8, 169.7, 163.4, 150.3, 136.7, 135.6, 128.6, 128.4, 128.3, 111.5, 76.4, 75.0, 73.9, 73.6, 68.4, 67.6, 61.0, 21.1, 20.8, 20.7, 12.6; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{24}H_{28}N_2NaO_{10}^+$ 527.1642; found 527.1639.

1-(3-O-Benzyl-α-L-mannopyranosyl)thymine ***5***. To a solution of **4** (11.20 g, 22.2 mmol) in dry methanol (222 mL) was added potassium carbonate (9.20 g, 66.6 mmol) at room temperature under an argon atmosphere. After stirring at room temperature for 30 min, the reaction mixture was filtered. The filtrate was neutralized with Amberlite IRC120 H-form. The mixture was filtered and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (AcOEt, then $CHCl_3$/MeOH = 9 : 1) to obtain **5** (7.90 g, 20.9 mmol, 94%) as a white solid. ^{1}H-NMR (400 MHz, DMSO-

d6) δ 11.27 (s, 1H), 7.54 (s, 1H), 7.40–7.27 (m, 5H), 5.81 (d, J = 9.6 Hz, 1H), 5.26 (d, J = 5.5 Hz, 1H), 5.22 (d, J = 6.4 Hz, 1H), 4.71–4.64 (m, 3H), 4.14–4.09 (m, 1H), 3.89–3.69 (m, 4H), 3.64–3.59 (m, 1H), 1.81 (s, 3H); ^{13}C-NMR (101 MHz, DMSO-D6) δ 163.8, 151.1, 138.7, 137.0, 128.2, 127.5, 127.4, 109.3, 81.3, 80.6, 76.9, 72.5, 66.4, 65.1, 59.4, 48.7, 12.1; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{18}H_{22}N_2NaO_7^+$ 401.1325; found 401.1344.

1-{3-O-Benzyl-4,6-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-α-L-mannopyranosyl}thymine ***6***. To a solution of **5** (7.01 g, 18.5 mmol) in dry pyridine (92.5 mL), 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (6.1 mL, 19.5 mmol) was added dropwise at 0 °C under an argon atmosphere, and the reaction mixture was stirred at room temperature for 24 h. After the addition of water at 0 °C, the resulting mixture was extracted with AcOEt. The obtained organic layer was washed with 1M HCl, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 3 : 2 to $CHCl_3$/MeOH = 9 : 1) to obtain **6** (9.33 g, 15.0 mmol, 81%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.58 (brs, 1H), 7.36–7.29 (m, 5H), 7.08 (d, J = 0.9 Hz, 1H), 5.36 (d, J = 3.2 Hz, 1H), 4.80 (d, J = 11.9 Hz, 1H), 4.68–4.64 (m, 2H), 4.33 (dd, J = 7.3, 4.1 Hz, 1H), 4.25 (dd, J = 9.4, 7.6 Hz, 1H), 4.08 (dd, J = 12.8, 1.8 Hz, 1H), 3.87 (dd, J = 12.6, 1.6 Hz, 1H), 3.64–3.61 (m, 1H), 2.96 (d, J = 3.2 Hz, 1H), 1.90 (d, J = 1.4 Hz, 3H), 1.11–0.96 (m, 28H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 163.8, 150.7, 139.9, 137.6, 128.6, 128.1, 128.0, 111.4, 89.4, 80.9, 76.4, 73.0, 67.4, 66.7, 60.9, 17.4, 17.3, 17.3, 17.1, 13.7, 13.3, 12.7, 12.5, 12.4; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{30}H_{48}N_2NaO_8Si_2^+$ 643.2847; found 643.2859.

2,2′-Anhydro-1-{3-O-benzyl-4,6-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-α-L-glucopyranosyl}thymine ***7***. To a solution of **6** (8.76 g, 14.1 mmol) in dry dichloromethane (141 mL) was added 4-dimethylaminopyridine (8.62 g, 70.6 mmol) at room temperature and trifluoromethanesulfonic anhydride (4.6 mL, 28.0 mmol) at −10 °C under an argon atmosphere, and the reaction mixture was stirred at −10 °C for 30 min. After the addition of water at −10 °C, the resulting mixture was extracted with AcOEt. The obtained organic layer was washed with 1M HCl, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure to obtain **7** (8.08 g, 13.4 mmol, 95%) as a pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.36–7.28 (m, 5H), 7.14 (d, J = 1.4 Hz, 1H), 6.00 (d, J = 7.4 Hz, 1H), 4.90 (dd, J = 7.3, 3.7 Hz,1H), 4.81 (d, J = 11.5 Hz, 1H), 4.75 (d, J = 11.9 Hz, 2H), 4.14–4.06 (m, 2H), 3.95–3.91 (m, 2H), 3.32 (brd, J = 9.6 Hz, 1H), 1.98 (d, J = 0.9 Hz, 3H), 1.09–0.92 (m, 28H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 172.2, 159.1, 136.9, 129.1, 128.6, 128.2, 128.0, 120.3, 84.7, 82.7, 79.5, 73.7, 73.3, 67.3, 60.3, 17.3, 17.3, 17.3, 17.2, 17.2, 17.1, 14.3, 13.5, 13.2, 12.6, 12.6; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{30}H_{46}N_2NaO_7Si_2^+$ 625.2741; found 625.2735.

2,2′-Anhydro-1-(3-O-benzyl-α-L-glucopyranosyl)thymine ***8***. To a solution of **7** (7.89 g, 13.1 mmol) in dry tetrahydrofuran (131 mL) were added acetic acid (3.0 mL, 52.5 mmol) and 1 M tetrabutylammonium fluoride in tetrahydrofuran (26.2 mL, 26.2 mmol) at room temperature under an argon atmosphere. After stirring at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography ($CHCl_3$/MeOH = 10 : 1) to obtain **8** (3.52 g, 9.77 mmol, 75%) as a white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ 7.70 (d, J = 1.4 Hz, 1H), 7.42–7.28 (m, 5H), 6.17 (d, J = 6.4 Hz, 1H), 5.11–5.08 (m, 1H), 4.79 and 4.77 (each d J = 11.9 Hz, each 1H), 3.95 (t, J = 4.1 Hz, 1H), 3.83–3.77 (m, 2H), 3.67 (dd, J = 11.9, 6.0 Hz, 1H), 3.29–3.26 (m, 1H), 1.96 (d, J = 1.4 Hz, 3H); ^{13}C-NMR (101 MHz, CD_3OD) δ 175.4, 161.8, 139.0, 134.0, 129.5, 129.1, 129.0, 119.8, 85.3, 80.1, 79.6, 76.3, 73.6, 67.9, 63.0, 14.0; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{18}H_{20}N_2NaO_6^+$ 383.1219; found 383.1202.

2,2′-Anhydro-1-(6-acetylthio-3-O-benzyl-6-deoxy-α-L-glucopyranosyl)thymine ***9***. To a solution of triphenylphosphine (3.30 g, 12.6 mmol) in dry tetrahydrofuran (110 mL), 1.9 M diisopropyl azodicarboxylate in toluene (6.6 mL, 12.5 mmol) was added dropwise over 15 min at 0 °C under an argon atmosphere. After stirring at 0 °C for 30 min, **8** (3.24 g, 8.99 mmol) was added, followed by another 30 min of stirring before the slow addition of thioacetic acid (900 μL, 12.7 mmol) in dry tetrahydrofuran (2.5 mL). The reaction mixture was then stirred at room temperature for 4 h and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography ($CHCl_3$/MeOH = 30 : 1 to 20 : 1) to obtain **9** (3.00 g, 7.17 mmol, 80%) as a pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.38–7.29 (m, 5H), 7.16 (d, J = 0.9 Hz, 1H), 5.96 (d, J = 6.8 Hz, 1H), 4.85 (dd, J = 6.9, 4.1 Hz, 1H), 4.81 (d, J = 11.4 Hz, 1H), 4.77 (d, J = 11.4 Hz, 1H), 3.96 (dd, J = 6.0, 4.1 Hz, 1H), 3.74 (d, J = 4.6 Hz, 1H), 3.69–3.60 (m, 2H), 3.37–3.27 (m, 2H), 2.37 (s, 3H), 1.98 (d, J = 0.9 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 196.5, 172.4, 159.4, 137.0, 129.8, 128.7, 128.3, 128.1, 119.8, 83.7, 79.6, 79.2, 73.2, 72.8, 69.9, 30.7, 30.6, 14.2; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{20}H_{22}N_2NaO_6S^+$ 441.1096; found 441.1084.

1-(2,6-Anhydro-3-O-benzyl-2-deoxy-2-thio-α-L-mannopyranosyl)thymine ***10***. To a solution of **9** (2.86 g, 6.83 mmol) in dry ethanol (137 mL) was added potassium carbonate (1.89 g, 13.7 mmol) at room temperature under an argon atmosphere, and the reaction mixture was refluxed for 40 min. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with H_2O twice and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography ($CHCl_3$, then $CHCl_3$/MeOH = 30 : 1) to obtain **10** (2.42 g, 6.43 mmol, 94%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.15 (brs, 1H), 7.65 (s, 1H), 7.33–7.30 (m, 5H), 6.17 (d, J = 1.4 Hz, 1H), 4.69 (d, J = 11.4 Hz, 1H), 4.48 (d, J = 11.9 Hz, 1H), 4.38 (brs, 1H), 3.90 (brs, 1H), 3.67 (dd, J = 2.8, 2.7 Hz, 1H), 3.32 (brs, 1H), 3.23 (dd, J = 11.4, 3.2 Hz, 1H), 2.85 (dd, J = 11.4, 1.8 Hz, 1H), 2.69 (d, J = 3.2 Hz, 1H), 1.86 (s, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.0, 150.2, 136.8, 136.0, 128.7, 128.3, 109.4, 86.4, 77.8, 75.5, 74.4, 71.1, 37.0, 29.3, 12.6; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{18}H_{20}N_2NaO_5S^+$ 399.0991; found 399.0998.

*1-(2,6-Anhydro-3-O-benzyl-2-deoxy-4-O-levulinyl-2-thio-α-L-mannopyranosyl)thymine **11**.* To a solution of **10** (565 mg, 1.50 mmol) in dry dichloromethane (7.5 mL) were added levulinic acid (308 μL, 3.00 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (865.1 mg, 4.51 mmol) and 4-dimethylaminopyridine (92.5 mg, 0.76 mmol) at room temperature under an Ar atmosphere, and the reaction mixture was stirred at room temperature for 3.5 h. After the addition of 1M HCl, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 1 : 2) to obtain **11** (658.1 mg, 1.39 mmol, 92%) as a pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.52 (brs, 1H), 7.51 (s, 1H), 7.33–7.25 (m, 5H + $CHCl_3$), 6.14 (d, J = 1.8 Hz, 1H), 4.92 (d, J = 3.7 Hz, 1H), 4.59 (d, J = 11.9 Hz, 1H), 4.50–4.45 (m, 2H), 3.77 (t, J = 3.2 Hz, 1H), 3.34 (t, J = 2.3 Hz, 1H), 3.24 (dd, J = 11.9, 3.7 Hz, 1H), 2.97 (dd, J = 11.9, 1.8 Hz, 1H), 2.92–2.75 (m, 2H), 2.64–2.49 (m, 2H), 2.21 (s, 3H), 1.87 (s, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 206.4, 171.8, 163.7, 150.0, 136.5, 135.1, 128.5, 128.2, 109.5, 86.3, 77.6, 74.0, 71.6, 71.1, 38.0, 37.0, 29.7, 28.8, 28.0, 12.8; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{23}H_{26}N_2NaO_7S^+$ 497.1358; found 497.1356.

*1-(2,6-Anhydro-2-deoxy-4-O-levulinyl-2-thio-α-L-mannopyranosyl)thymine **12**.* To a mixture of 20% $Pd(OH)_2$/C in 50% H_2O (260.2 mg) in dry tetrahydrofuran (11.0 mL) was added compound **11** (261.4 mg, 0.55 mmol) at room temperature. The reaction mixture was stirred under a H_2 atmosphere at room temperature for 18 h, filtered and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 1 : 3) to obtain **12** (206.7 mg, 0.54 mmol, 98%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.40 (brs, 1H), 7.65 (d, J = 0.9 Hz, 1H), 6.24 (d, J = 1.8 Hz, 1H), 4.62 (brd, J = 2.7 Hz, 1H), 4.53 (m, 1H), 4.03 (dt, J = 7.8, 3.0 Hz, 1H), 3.37 (dd, J = 3.2, 1.8 Hz, 1H), 3.34 (d, J = 7.3 Hz, 1H), 3.29 (dd, J = 11.9, 3.7 Hz, 1H), 2.93–2.78 (m, 3H), 2.70–2.55 (m, 2H), 2.20 (s, 3H), 1.94 (d, J = 1.4 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 206.4, 173.0, 163.6, 150.1, 135.2, 109.8, 85.9, 80.4, 70.9, 69.1, 40.1, 38.1, 29.7, 28.8, 28.0, 12.9; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{16}H_{20}N_2NaO_7S^+$ 407.0889; found 407.0896.

*1-{2,6-Anhydro-3-O-{2-cyanoethoxy(diisopropylamino)phosphanyl}-2-deoxy-4-O-levulinyl-2-thio-α-L-mannopyranosyl}thymine **13**.* To a solution of **12** (402 mg, 1.05 mmol) in dry dichloromethane (21.0 mL) were added *N*,*N*-diisopropylethylamine (712 μL, 4.19 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (467 μL, 2.09 mmol) at room temperature under an Ar atmosphere, and the reaction mixture was stirred at room temperature for 3 h. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 1 : 2) to obtain 13 (514.3 mg, 0.88 mmol, 84%) as a white solid. ^{31}P-NMR (162 MHz, $CDCl_3$) δ 150.3, 147.7; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{25}H_{37}N_4NaO_8PS^+$ 607.1967; found 607.1940.

*1-(4-O-Acetyl-2,6-anhydro-2-deoxy-2-thio-3-O-triethysilyl-α-L-mannopyranosyl)thymine **14**.* To a solution of **10** (938.8 mg, 2.49 mmol) in dry pyridine (83 mL) were added 4-dimethylaminopyridine (143.7 mg, 1.18 mmol) and acetic anhydride (354 μL, 3.74 mmol) at room temperature under an argon atmosphere, and the reaction mixture was stirred at room temperature for 2.5 h. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with 1 M HCl, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure to obtain the corresponding acetate **S25** (946.3 mg) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$)) δ 8.89 (brs, 1H), 7.38 (d, J = 0.9 Hz, 1H), 7.33–7.22 (m, 5H + $CHCl_3$), 6.16 (d, J = 1.4 Hz, 1H), 4.92 (d, J = 3.2 Hz, 1H), 4.60 (d, J = 11.9 Hz, 1H), 4.47–4.42 (m, 2H), 3.73 (t, J = 3.2 Hz, 1H), 3.34 (brq, 1H), 3.24 (dd, J = 11.9, 3.7 Hz, 1H), 3.00 (dd, J = 11.9, 2.3 Hz, 1H), 2.12 (s, 3H), 1.87 (d, J = 0.9 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$)) δ 169.5, 163.5, 149.9, 136.5, 134.8, 128.6, 128.3, 128.2, 109.5, 86.3, 74.0, 72.0, 71.1, 37.0, 28.9, 21.1, 12.8; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{20}H_{22}N_2NaO_6S^+$ 441.1096; found 441.1093. To a mixture of 20% $Pd(OH)_2$/C in 50% H_2O (1.85 g) in dry tetrahydrofuran (44 mL) was added a fraction of the obtained acetate (928.5 mg, 2.22 mmol) at room temperature. The reaction mixture was stirred under a H_2 atmosphere at room temperature for 2 h, filtered and concentrated under reduced pressure. The crude product was subjected to column chromatography, and the corresponding alcohol **S26** (706 mg) was obtained as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.12 (brs, 1H), 7.54 (d, J = 0.9 Hz, 1H), 6.22 (d, J = 1.8 Hz, 1H), 4.66 (d, J = 2.3 Hz, 1H), 4.49 (brt, 1H), 4.01–3.98 (m, 1H), 3.43 (d, J = 7.8 Hz, 1H), 3.37 (q, J = 1.7 Hz, 1H), 3.27 (dd, J = 11.9, 3.7 Hz, 1H), 2.93 (dd, J = 12.4, 2.3 Hz, 1H), 2.20 (s, 3H), 1.95 (d, J = 0.9 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 170.5, 163.7, 150.1, 135.0, 109.7, 85.9, 80.0, 71.1, 69.1, 40.2, 28.8, 21.0, 12.9; HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{13}H_{16}N_2NaO_6S^+$ 351.0627; found 351.0642. To a solution of a fraction of the obtained alcohol (662 mg, 2.01 mmol) in dry DMF (10 mL) were added imidazole (275.8 mg, 4.05 mmol) and chlorotriethylsilane (406 μL, 2.42 mmol, 1.2 equiv.) at room temperature under an Ar atmosphere, and the reaction mixture was stirred at room temperature for 30 min. After the addition of saturated aqueous $NaHCO_3$, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 3 : 2) to obtain **14** (710.1 mg, 1.60 mmol, 80%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.89 (brs, 1H), 7.55 (d, J = 1.4 Hz, 1H), 6.17 (d, J = 1.4 Hz, 1H), 4.79 (d, J = 3.2 Hz, 1H), 4.44 (t, J = 2.5 Hz, 1H), 4.10 (t, J = 3.2 Hz, 1H), 3.23 (dd, J = 11.9, 3.7 Hz, 1H), 3.11 (q, J = 1.5 Hz, 1H), 2.99 (dd, J = 12.1, 2.5 Hz, 1H), 2.18 (s, 3H), 1.98 (d, J = 1.4 Hz, 3H), 0.87 (t, J = 8.0 Hz, 9H), 0.52 (q, J = 7.9 Hz, 6H); ^{13}C-NMR (101 MHz, $CDCl_3$)

δ 169.8, 163.6, 149.9, 135.0, 109.6, 86.5, 80.1, 72.3, 69.5, 41.3, 29.1, 21.0, 12.8, 6.5, 4.6; HRMS (ESI) *m*/*z*: $[M + Na]^+$ calcd for $C_{19}H_{30}N_2NaO_6SSi^+$ 465.1492; found 465.1480.

1-(4-O-Acetyl-2,6-anhydro-2-deoxy-2-thio-3-O-triethysilyl-α-L-mannopyranosyl)-5-methylcytosine ***15***. To a mixture of 1,2,4-triazole (2.67 g, 38.7 mmol) in dry acetonitrile (12 mL), phosphoryl chloride (832 μL, 9.12 mmol) was added dropwise at 0 °C under an Ar atmosphere. After stirring at 0 °C for 5 min, to the reaction mixture was slowly added triethylamine (6.3 mL, 45.4 mmol), and stirred at 0 °C for 20 min. To the reaction mixture was added dropwise a solution of **14** (504 mg, 1.14 mmol) in dry acetonitrile (11 mL) over 5 min, and the reaction mixture was stirred at room temperature for 1 h. After the addition of saturated aqueous $NaHCO_3$, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to obtain the residue. The residue was dissolved in a solution of 28% aqueous ammonia (8 mL) and dioxane (15 mL), and the reaction mixture was stirred at room temperature for 40 min. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to obtain **15** (454 mg, 1.03 mmol, 90%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.54 (brs, 1H), 6.19 (d, J = 1.4 Hz, 1H), 4.77 (d, J = 3.7 Hz, 1H), 4.42 (t, J = 2.8 Hz, 1H), 4.06 (t, J = 3.2 Hz, 1H), 3.27 (q, J = 1.4 Hz, 1H), 3.23 (dd, J = 11.9, 3.2 Hz, 1H), 2.97 (dd, J = 11.9, 2.3 Hz, 1H), 2.17 (s, 3H), 1.98 (s, 3H), 0.84 (t, J = 7.8 Hz, 9H), 0.48 (q, J = 7.9 Hz, 6H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 169.9, 165.7, 155.6, 137.5, 100.8, 86.7, 80.3, 72.2, 69.5, 41.1, 29.1, 21.0, 13.5, 6.5, 4.6; HRMS (ESI) *m*/*z*: $[M + Na]^+$ calcd for $C_{19}H_{31}N_3NaO_5SSi^+$ 464.1651; found 464.1633.

1-(2,6-Anhydro-2-deoxy-2-thio-3-O-triethysilyl-α-L-mannopyranosyl)-4-N-benzoyl-5-methylcytosine ***16***. A solution of **15** (433.1 mg, 0.98 mmol) in 4% ammonia in methanol (20.0 mL) was stirred at room temperature for 3 h and concentrated under reduced pressure to the residue. To the solution of the obtained residue in dry pyridine (11.4 mL) was added benzoic anhydride (268.1 mg, 1.19 mmol) at room temperature under an Ar atmosphere. After stirring at room temperature for 24 h, to the reaction mixture was added benzoic anhydride (103.1 mg, 0.46 mmol), and the reaction mixture was stirred at room temperature for 26 h. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 3 : 1) to obtain **16** (205.5 mg, 0.41 mmol, 42%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.35–8.32 (m, 2H), 7.93 (d, J = 1.4 Hz, 1H), 7.56–7.51 (m, 1H), 7.47–7.43 (m, 2H), 6.23 (d, J = 1.8 Hz, 1H), 4.40 (t, J = 2.5 Hz, 1H), 3.96 (t, J = 3.0 Hz, 1H), 3.83 (brt, 1H), 3.26 (dd, J = 11.7, 3.4 Hz, 1H), 3.13 (q, J = 1.7 Hz, 1H), 2.87 (dd, J = 11.9, 2.3 Hz, 1H), 2.16 (d, J = 0.9 Hz, 3H), 0.90 (t, J = 8.0 Hz, 9H), 0.56 (q, J = 7.9 Hz, 6H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 179.6, 159.9, 147.9, 137.4, 137.1, 132.6, 130.2, 129.9, 128.5, 128.2, 110.7, 87.0, 78.0, 75.0, 72.8, 41.1, 29.4, 13.8, 6.7, 4.8; HRMS (ESI) *m*/*z*: $[M + Na]^+$ calcd for $C_{24}H_{33}N_3NaO_5SSi^+$ 526.1808; found 526.1819.

1-(2,6-Anhydro-2-deoxy-4-O-levulinyl-2-thio-α-L-mannopyranosyl)-4-N-benzoyl-5-methylcytosine ***17***. To a solution of **16** (251.4 mg, 0.50 mmol) in dry dichloromethane (10 mL) were added 4-dimethylaminopyridine (61.4 mg, 0.50 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (265 μL, 1.50 mmol) and levulinic acid (102 μL, 1.00 mmol) at room temperature under an Ar atmosphere, and the reaction mixture was stirred at room temperature for 1 h. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with 1M HCl, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure to obtain the corresponding ester **S27** (298.8 mg, quant.) as a colourless, transparent oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.35–8.32 (m, 2H), 7.85 (d, J = 0.9 Hz, 1H), 7.55–7.52 (m, 1H), 7.47–7.44 (m, 2H), 6.21 (d, J = 1.8 Hz, 1H), 4.78 (d, J = 3.2 Hz, 1H), 4.50 (t, J = 2.5 Hz, 1H), 4.12 (t, J = 3.2 Hz, 1H), 3.27–3.23 (m, 2H), 2.99–2.89 (m, 2H), 2.83–2.67 (m, 2H), 2.58 (td, J = 11.0, 5.5 Hz, 1H), 2.23 (s, 3H), 2.17 (d, J = 0.9 Hz, 3H), 0.87 (t, J = 8.0 Hz, 9H), 0.52 (q, J = 7.9 Hz, 6H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 206.3, 172.1, 159.7, 147.7, 137.1, 136.5, 132.6, 129.9, 128.2, 110.7, 86.9, 80.2, 71.9, 69.4, 40.8, 38.0, 29.8, 29.0, 28.0, 13.9, 6.5, 4.6; HRMS (ESI) *m*/*z*: $[M + Na]^+$ calcd for $C_{29}H_{39}N_3NaO_7SSi^+$ 624.2176; found 624.2164. To a solution of a fraction of the obtained ester (280.5 mg, 0.47 mmol) in dry tetrahydrofuran (4.70 mL) was added triethylamine trihydrofluoride (228 μL, 1.40 mmol) at room temperature under an Ar atmosphere, and the reaction mixture was stirred at room temperature for 1 h. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 1 : 1 to 1 : 2) to obtain **17** (226 mg, 0.46 mmol, quant.) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1H-NMR δ 8.32 (d, J = 7.3 Hz, 2H), 7.82 (d, J = 0.9 Hz, 1H), 7.55–7.51 (m, 1H), 7.45–7.42 (m, 2H), 6.30 (d, J = 1.8 Hz, 1H), 4.63 (d, J = 2.3 Hz, 1H), 4.56 (brs, 1H), 4.02 (dt, J = 7.3, 3.1 Hz, 1H), 3.45 (dd, J = 3.2, 1.8 Hz, 1H), 3.31 (dd, J = 12.4, 3.6 Hz, 1H), 3.27 (d, J = 7.4 Hz, 1H), 2.94–2.79 (m, 3H), 2.73–2.57 (m, 2H), 2.22 (s, 3H), 2.14 (d, J = 0.9 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 206.4, 173.0, 159.7, 147.9, 137.1, 136.4, 132.5, 129.9, 128.2, 110.9, 86.3, 80.4, 70.9, 69.0, 39.9, 38.1, 29.7, 28.8, 28.0, 14.0; HRMS (ESI) *m*/*z*: $[M + Na]^+$ calcd for $C_{23}H_{25}N_3NaO_7S^+$ 510.1288; found 510.1311.

1-{2,6-Anhydro-3-O-{2-cyanoethoxy(diisopropylamino)phosphanyl}-2-deoxy-4-O-levulinyl-2-thio-α-L-mannopyranosyl}-4-N-benzoyl-5-methylcytosine ***18***. To a solution of **17** (146.6 mg, 0.30 mmol) in dry dichloromethane (6.0 mL) were added *N*,*N*-diisopropylethylamine (205 μL, 1.21 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (134 μL, 0.60 mmol) at room temperature under an Ar atmosphere, and the reaction mixture

was stirred at room temperature for 1.5 h. After the addition of H_2O, the resulting mixture was extracted with AcOEt. The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 3 : 2) to obtain **19** (163.8 mg, 0.24 mmol, 79%) as a colorless, transparent oil. ^{31}P-NMR (162 MHz, $CDCl_3$) δ 150.5, 147.9; HRMS (ESI) *m*/*z*: $[M + Na]^+$ calcd for $C_{32}H_{42}N_5NaO_8PS^+$ 710.2389; found 710.2403.

Solid-phase synthesis, purification and characterization of L-*lyxo*-thioBsNA-modified oligonucleotides. Synthesis of oligonucleotides were performed using a phosphoramidite coupling protocol with a syringe only for coupling with L-*lyxo*-thioBsNA, and the others were performed on a 1.0 μmol scale using an automated DNA synthesizer. 0.25 M 5-Benzylthio-1*H*-tetrazole acetonitrile solution was used as the activator. 3% (w/v) Trichloroacetic acid dichloromethane solution was used as the detritylating agent. 0.5 M Hydrazine monohydrate in pyridine/AcOH [3 : 2(v/v)] solution was used as the delevulinoylating agent. 0.02 M Iodine THF/water/pyridine [78 : 20 : 2(v/v/v)] solution was used as the oxidant. The oxidation was performed for a total of 18 s (delivered in two steps of 10 s and 8 s). 10 vol% Acetic anhydride THF solution and 1-methylimidazole/pyridine/THF [1 : 1 : 8(v/v/v)] solution were used as capping reagents. The concentration of each phosphoramidite was 0.075 M, and the coupling times were 10 s (2 cycles) for natural deoxynucleic acids and 12 min for L-*lyxo*-thioBsNA. Cleavage from the solid support and removal of all protecting groups were performed using 28% aqueous ammonia at 55 °C for 16 h. The resulting DMTr-on oligonucleotides were briefly purified using a Sep-Pak Plus C18 cartridge, and the DMTr group was removed using 1% aqueous trifluoroacetic acid in the cartridge. Oligonucleotides were further purified using reversed-phase HPLC (Waters XBridge Oligonucleotide BEH C18 OBD column 2.5 μm, 10 × 50 mm). The purity of the purified oligonucleotides was analyzed by reversed-phase HPLC (Waters XBridge Oligonucleotide BEH C18 column 2.5 μm, 4.6 ×50 mm). For HPLC analysis, Shimadzu DGU-20A3R/20A5R, LC-20AT, SIL-20AC HT, CBM-20A, CTO-20AC, SPD-20A, and FRC-10A instruments were used. The compositions were confirmed by MALDI-TOF mass spectrometry (Bruker ultrafleXtreme) using 3-hydroxypicolinic acid as the matrix. Isolated yields were determined by the UV absorbance at 260 nm using a DeNovix NanoPad DS-11+ spectrophotometer.

UV melting experiments. Melting temperatures (T_m) were determined using a Shimadzu UV-1800 spectrophotometer equipped with a TMSPC-8 T_m analysis accessory. For the UV melting experiments of the duplexes with target single-stranded RNA or DNA, oligonucleotides were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to achieve a final concentration of 4.0 μM for each strand. The samples were annealed by heating at 100 °C, followed by slow cooling to room temperature. The absorbance at 260 nm was recorded at a scan rate of 0.5 °C min^{-1} from 5 to 90 °C. T_m values were determined from the temperatures at which half of the duplexes dissociated according to the sigmoidal melting curves.

Nuclease resistance study. Oligonucleotides (400 pmol) were dissolved in 50 mM Tris-HCl buffer (pH 8.0, 90 μL) containing 10 mM $MgCl_2$. To each sample, 25 mU of phosphodiesterase I from *Crotalus adamanteus* venom in 10 μL of water was added, followed by incubation at 37 °C. Aliquots (10 μL) were taken at 0, 2.5, 5.0, 10, 20, and 40 min, heated at 90 °C for 2 min to deactivate the enzyme, and analyzed using reversed-phase HPLC (Waters XBridge Oligonucleotide BEH C18 column 2.5 μm, 4.6 × 50 mm) to assess the intact oligonucleotide proportion.

Author contributions

MI, SM and NY performed the chemical synthesis supervised by TK. MI and SM performed the evaluation of oligonucleotides supervised by HH and TK. The manuscript was prepared by MI and TK.

Conflicts of interest

There are no conflicts to declare.

Data availability

Supplementary information (SI): synthetic procedures, ^{1}H-, ^{13}C- and ^{31}P-NMR spectra of new compounds, yields and MALDI-TOF-MS data for modified oligonucleotides, and UV-melting curves. See DOI: https://doi.org/10.1039/d6cb00034g.

Acknowledgements

This research was supported by Research Support Project for Life Science and Drug Discovery (Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)) from AMED under Grant Number JP25ama121044.

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