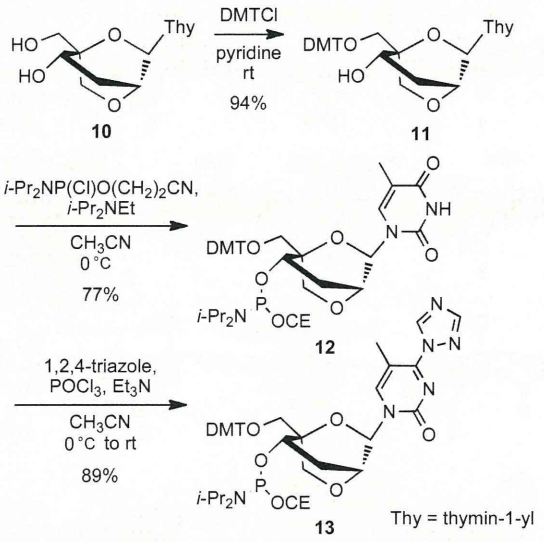
Scheme 2. Synthesis of Phosphoramidite Building Blocks



4′-hydroxy group with 2-cyanoethyl *N*,*N*-diisopropylaminochlorophosphoramidite afforded the desired phosphoramidite building block **12** (Scheme 2). A portion of the thymine phosphoramidite **12** was converted to the triazolyl derivative **13**, which is a convertible phosphoramidite, by the treatment of 1,2,4-triazole in the presence of triethylamine and phosphoryl chloride.[17]

The conformation of the sugar in BsNA was determined from the crystal structure of compound **10**[19] (Figure 2). Compared to the X-ray structure of 2′,4′-BNA analogues,[10a,11,18] the thymine base of **10** leans more, as we designed; C5′/C6′–C1′–N1 angles, e.g., of 2′,4′-BNA^{COC}, 2′,4′-BNA^{NC}[NMe], 2′,4′-BNA/LNA, and **10** were 106°, 111°, 112°, and 125°, respectively.

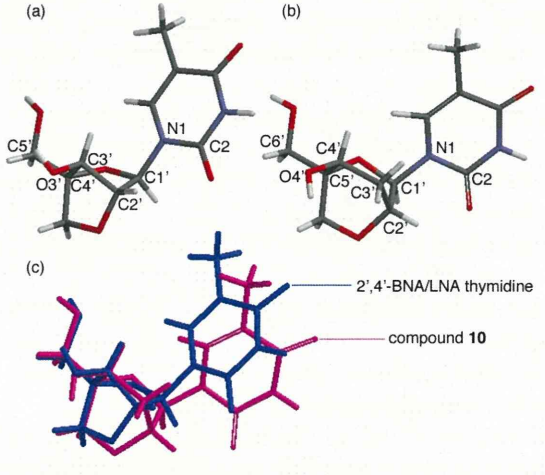


Figure 2. X-ray structures of 2′,4′-BNA/LNA thymidine (a)[18] and **10** (b),[19] and their superimposed images (c).

BsNA-phosphoramidites **12** and **13** were introduced into ONs using an automated DNA synthesizer (Table 1).

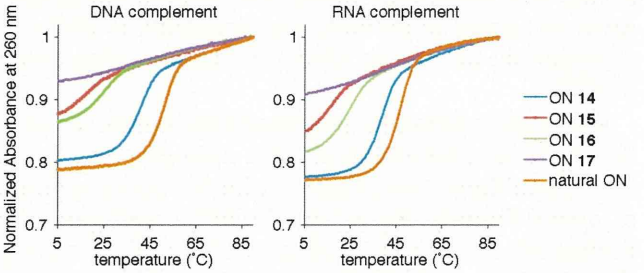
Table 1. Yields and MALDI-TOF-MS Data for the Oligonucleotides

oligonucleotide[a]	yield (%)[b]	calcd [M-H]$^-$	found [M-H]$^-$
5′-d(GCGTTTTTTGCT)-3′ (**14**)	46	3675.4	3676.2
5′-d(GCGTTTTTTTGCT)-3′ (**15**)	55	3759.5	3755.6
5′-d(GCGTTTTTTTGCT)-3′ (**16**)	29	3759.5	3756.7
5′-d(GCGTTTTTTTGCT)-3′ (**17**)	34	3885.6	3883.9
5′-d(TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT)-3′ (**18**)	14	4539.0	4536.9
5′-d(TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT)-3′ (**19**)	6	4539.0	4539.0

[a] Underlined bold characters indicate the modified residues. Superscript m shows that the following C is a 5-methylcytidine derivative. [b] The isolation yields for ON **14**–**19** were calculated from the UV absorbance at 260 nm.

The sequences were the same as those in our previous studies, and cytidines of ONs **18** and **19** were replaced by 5-methylcytidines for the stable triplex formation.[7] Each coupling reaction of modified monomers was accomplished using 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole as an activator over 6 min. Coupling yields were checked by trityl monitoring and were estimated to be over 95%. Synthesized ONs were cleaved from the solid supports and deprotected by treatment with concentrated ammonium hydroxide solution. Simultaneously, the triazole group of ON **19** was converted to an amino group to give a BsNA-5-methylcytosine-modified oligonucleotide.

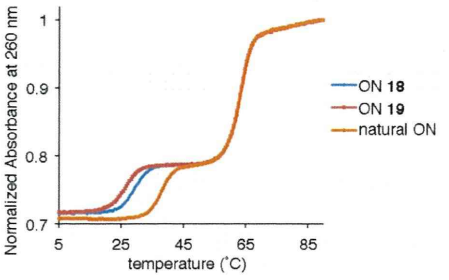
Table 2. Evaluation of Thermal Denaturation Temperatures (T_m Values) of Duplexes[a]



	T_m (°C)[b]	
oligonucleotide	DNA complement	RNA complement
Natural	51	48
14	41	39
15	ND[c]	ND[c]
16	25	24
17	ND[c]	ND[c]

[a] UV melting curves for the duplexes formed by ONs and the target strand, 5′-AGCAAAAAACGC-3′, were measured under the following conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; each strand concentration = 4 μM; scan rate of 0.5 °C min^{-1} at 260 nm. [b] T_m was determined by taking the first derivative of the melting curve. The number is the average of three independent measurements. [c] ND = not detected.

Table 3. Evaluation of Thermal Denaturation Temperatures (T_m Values) of Triplexes[a]



oligonucleotide	T_m (°C)[b]
Natural	37
18	29
19	26

[a] UV melting curves for the triplexes formed by ONs and the target strand, 5′-d(GCT*AAAAAGAAAGAGAGA*TCG)-3′/3′-d(CGA*TTTTTC-TT-TCTCTCT*AGC)-5′, were measured under the following conditions: 7 mM sodium cacodylate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$; each strand concentration = 1.5 μM; scan rate of 0.5 °C min^{-1} at 260 nm. The italic portions indicate the target site for triplex formation. [b] T_m was determined by taking the first derivative of the melting curve. The number is the average of three independent measurements.

We evaluated the affinity of the synthesized ONs with complementary single-stranded RNA (ssRNA) and DNA (ssDNA) and double-stranded DNA (dsDNA) through UV melting experiments. The UV melting profiles and thermal denaturation temperatures (T_m values) are summarized in Tables 2 and 3. BsNA formed unstable duplexes with ssRNA and ssDNA and a triplex with dsDNA. When a larger number of BsNA monomers were introduced into ONs, a smaller hyperchromicity was observed. This is perhaps explained by steric repulsion or destabilization of the hydrogen bonds between the base pairs due to a too large lean of the BsNA nucleobase. This indicates that the lean of the nucleobase may be an important factor in the duplex-forming ability.

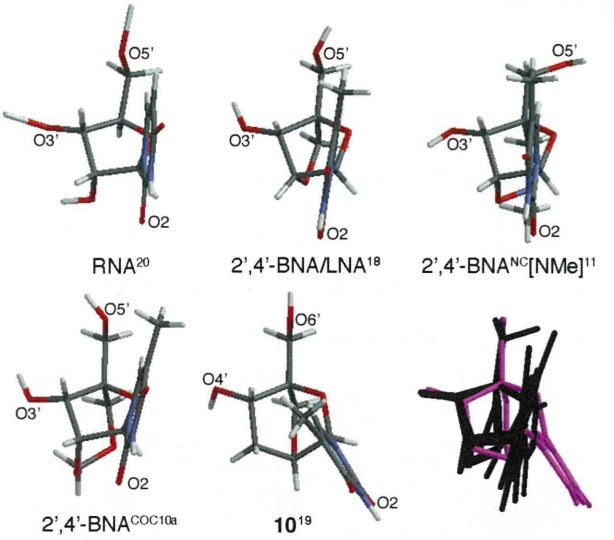


Figure 3. Nucleobase orientations of a typical A-type RNA duplex, BNA analogues, and **10**, and their superimposed images. Hydrogen atoms are omitted in the superimposed images.

However, other factors can be attributed to the destabilization of the duplex and triplex. As shown in Figure 3, the nucleobase orientation of **10** (magenta) differs from those of a typical A-type RNA duplex or other BNA analogues (black), and moreover, the rotation of the C1′−N1 bond axis in **10** may be restricted. Therefore, in the hybridization, the nucleobase orientation of the target strand perhaps needs to be altered to form hydrogen bonds, which is an unfavorable process. In addition, the axial H3′ has the potential to inhibit π-stacking between neighboring bases. Investigation of these possibilities is currently underway in our laboratory.

Acknowledgment. This work was supported by a Grant-in-Aid for Challenging Exploratory Research (22651076) from JSPS, for Young Scientists (B) (20790010) and for Scientific Research on Innovative Area (22136006) from MEXT, and the Program for Promotion of Fundamental Studies in Health Sciences of NIBIO.

Supporting Information Available. Experimental details and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

(17) (a) Scerr, M.; Klebba, C.; Hanner, A.; Ganser, A.; Engels, J. W. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1791. (b) Sung, W. L. *J. Chem. Soc., Chem. Commun.* **1981**, 1089.

(18) Morita, K.; Takagi, M.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.; Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. *Bioorg. Med. Chem.* **2003**, *11*, 2211.

(19) Crystallographic data for **10** can be found in the Supporting Information. Deposition no. CCDC 840597.

(20) RNA structure was obtained using Spartan '06, Wavefunction, Inc., Irvine, CA.

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Synthesis and Properties of a Bridged Nucleic Acid with a Perhydro-1,2-oxazin-3-one Ring

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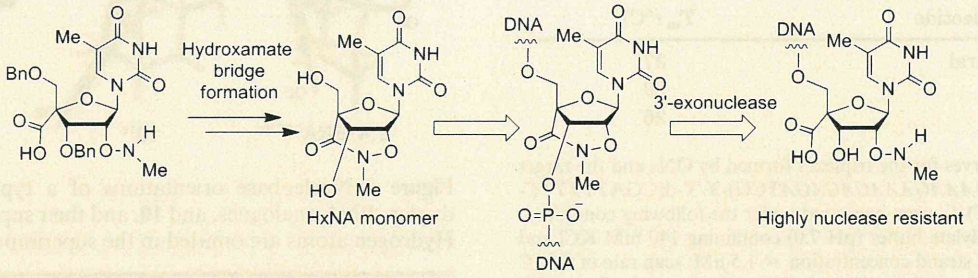
Supporting Information



ABSTRACT: A novel derivative of 2′,4′-bridged nucleic acid, named hydroxamate-bridged nucleic acid (HxNA), containing a six-membered perhydro-1,2-oxazin-3-one ring, was designed and synthesized. The introduction of a carbonyl function along with an N−O linkage in the six-membered bridged structure is the unique structural feature of the novel 2′,4′-bridged nucleic acid analogue. The design was carried out to restrict the flexibility of the sugar moiety through the trigonal planarity of carbonyl function, which would improve the properties of the modification. The synthesized monomer was incorporated into oligonucleotides, and their properties were examined. The HxNA-modified oligonucleotides exhibited selectively high affinity toward complementary ssRNA. Furthermore, the nuclease resistance of the HxNA-modified oligonucleotide was found to be higher than that of the corresponding natural and 2′,4′-BNA/LNA-modified oligonucleotides. Interestingly, exposure of HxNA modified oligonucleotide to 3′-exonuclease resulted in gradual opening of the bridge, which stopped further digestion. Moreover, ring-opening of only one modification at the 3′-end of the oligonucleotides was observed, even if two or three HxNA modifications were present in the sequence. The results demonstrate the strong potential of the HxNA modification as a switch for the generation of highly nuclease-resistant RNA selective oligonucleotide in situ, which could have potential applications in antisense technology.

■ INTRODUCTION

The approach of using chemically modified nucleic acids for the selective control and regulation of gene expression has attracted much attention because of its potential for the development of highly potent therapeutics.[1−10] Conformationally restricted nucleic acids are one of the most interesting and promising candidates which could exhibit many of the desired properties of an ideal oligonucleotide.[11−15] It is well-known that a nucleic acid with its sugar conformation locked in *N*-type, termed 2′,4′-bridged nucleic acid (2′,4′-BNA),[16,17] or locked nucleic acid (LNA)[18] (**NA-1**, Figure 1) can exhibit unprecedented hybridizing affinity to complementary strands (RNA and DNA), sequence selectivity,[19] aqueous solubility, and potential for in vivo application.[20,21] On the basis of the structure of 2′,4′-BNA/LNA, many interesting modifications have been reported by other laboratories[22−26] and us[27,28] in the search for bridged nucleic acids with improved properties (Figure 1). It has been found that the properties of the 2′,4′-bridged nucleic acids are directly related to the size of the bridged structure and the heteroatom in the bridge. Increasing the size of the bridge, in general, increases the nuclease resistance at the expense of hybridizing affinity,[18,22,27] and the presence of heteroatom in the bridge apparently improves binding affinity.[22,25,27]

Recently, we have reported a modification with a six-membered bridged structure, 2′,4′-BNA^{NC27d} (**NA-2**), which exhibited interestingly high binding affinities toward ssRNA and dsDNA along with high nuclease resistance. 2′,4′-BNA^{NC} has the unique structural feature of N−O bond in the bridge of the six-membered ring system, which is attributable for the improved properties of the 2′,4′-BNA^{NC}-modified oligonucleotides. Very recently, we have developed another bridged nucleic acid with a seven-membered ring system having a cyclic urea structure (**NA-3**).[27e] The modification was developed to enhance the hydrophilicity of the modified oligonucleotide through the introduction of N−H and C=O groups in the structure as a proton donor and acceptor, respectively. The introduction of a carbonyl function in the bridge is an interesting approach for restricting the flexibility of the sugar conformation of a bridged nucleic acid with a larger ring system, which could result improved properties of the nucleic acid. In the case of the bridged nucleic acid with cyclic urea structure, highly RNA selective binding affinity was obtained along with promisingly high nuclease resistance.

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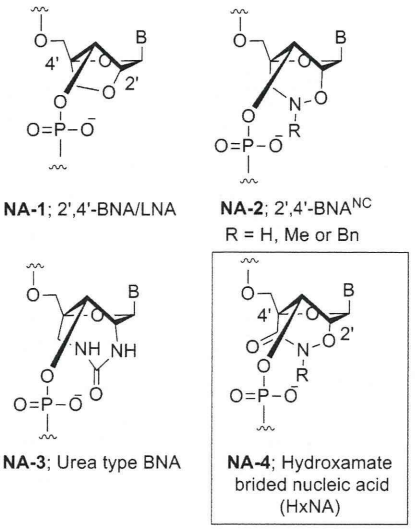


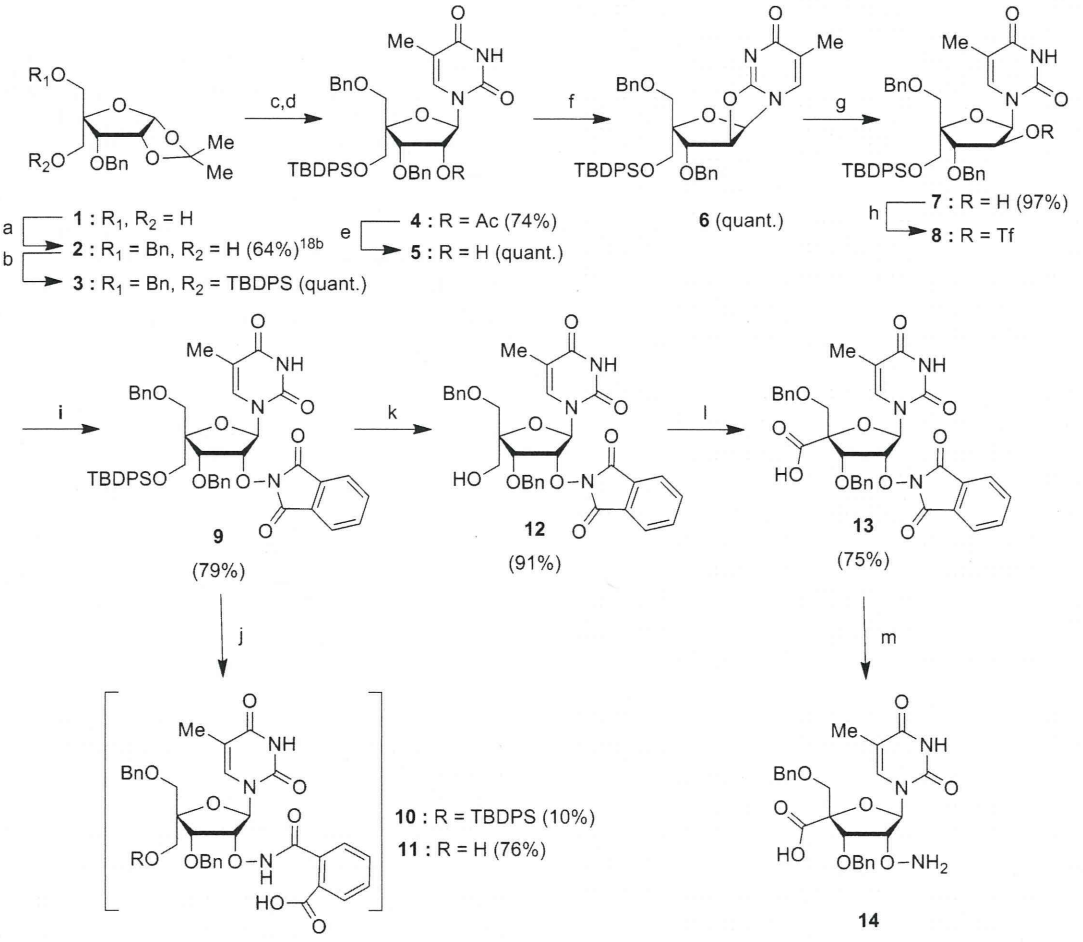
Figure 1. Structures of 2′,4′-bridged nucleic acids.

We herein report the design, synthesis, and properties of a novel bridged nucleic acid analogue with a perhydro-1,2-oxazin-3-one ring system (**NA-4**). The modification was designed as a six-membered bridged structure with hydroxamate (N–O linkage and carbonyl function) moiety in the bridge. The design thus includes the important structural features of 2′,4′-BNA^{NC} and the bridged nucleic acid with cyclic urea structure, which made it promising modification with potentially improved properties. We have accomplished the synthesis of the hydroxamate bridged nucleic acid (HxNA) with a thymine nucleobase, HxNA-T, using a condensation reaction between the aminooxy moiety at C2′ and the carboxyl moiety at C4′. The synthesized HxNA was introduced into oligonucleotides, and their hybridizing affinities and nuclease resistance were investigated.

■ RESULTS AND DISCUSSION

Synthesis of HxNA. Considering the labile nature of N–O moiety, the construction of the bridge of the target monomer was strategically carried out at a late stage in the synthesis. Starting from the common precursor of 2′,4′-BNA/LNA, i.e., 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-ribofuranose **1**, the target monomers were synthesized in 14 and 16 synthetic steps. The regioselective benzylation of **1** afforded **2** in good yield.[18b,27d] The primary hydroxyl moiety of **2** was protected by silylation using TBDPSCl as a protecting agent to

Scheme 1[a]



[a]Reagents and conditions: (a) NaH, BnBr, DMF, 0 °C to rt, 64%; (b) TBDPSCl, imidazole, DMF, rt, quant; (c) Ac_2O, concd H_2SO_4, AcOH, rt; (d) thymine, BSA, TMS-triflate, MeCN, reflux, 74% (two steps); (e) 40% $MeNH_2$ (aq), THF, rt, quant; (f) TfCl, DMAP, CH_2Cl_2, rt, quant; (g) 1 N NaOH (aq), THF, rt, 97%; (h) Tf_2O, pyridine, CH_2Cl_2, 0 °C; (i) *N*-hydroxyphthalimide, DBU, MeCN, rt 79%, two steps; (j) 1 N TBAF/THF, THF, reflux; (k) TEA·3HF, THF, reflux, 90%; (l) PDC/DMF, MS 4A, rt, 75%; (m) $NH_2NH_2{\cdot}H_2O$, EtOH, rt.

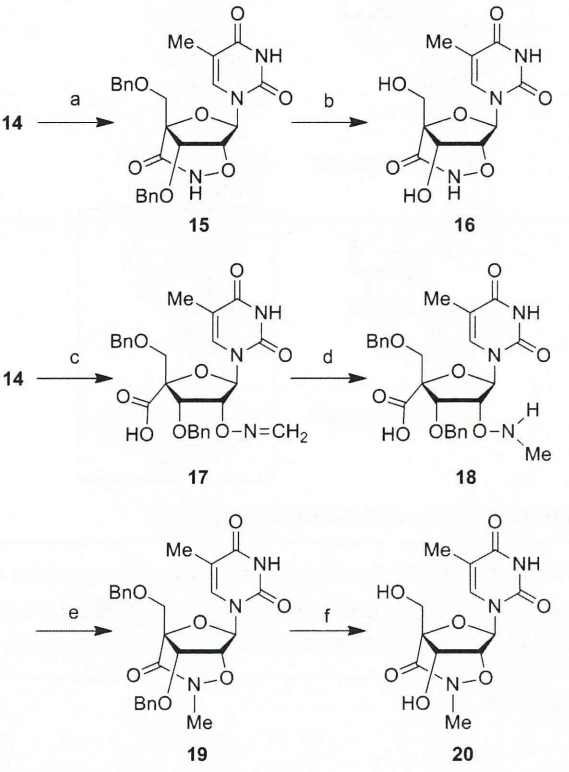
afford **3**. Acetolysis and acetylation of **3**, followed by stereoselective reaction with silylated thymine, afforded **4** in 74% yield. Subsequently, **4** was deacetylated by 40% methylamine to afford **5** in quantitative yield. The configuration of the 2′-carbon of **5** was successfully inverted to afford **7** in excellent yield by reacting **5** with TfCl to yield the 2,2′-anhydro derivative **6**, followed by alkaline hydrolysis. The N−O moiety was introduced into the structure by an S_N2 type substitution reaction with *N*-hydroxyphthalimide in presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), yielding **9**. Desilylation of the TBDPS group was conducted using triethylamine trihydrofluoride (TEA·3HF) yielded **12** in excellent yield. It has been observed that the common desilylating agent, TBAF, was not appropriate because ring-opening reaction occurred at the base labile phthalimide moiety at 2′-position yielding **10** and **11**. Oxidation of the free hydroxyl moiety of **12** with PDC in DMF afforded the carboxylic acid **13** in good yield. Reaction of **13** with hydrazine monohydrate in ethanol yielded **14** with a free aminooxy moiety, which was used as the reactive key intermediate for the synthesis of the HxNA monomers (Scheme 1).

The aminooxy and carboxyl moieties of the key intermediate **14** were coupled using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI·HCl) in DMF as the coupling agent and hydroxybenzotriazole monohydrate (HOBt·H_2O) as the condensation additive to obtain the cyclized product **15**. The cyclized structure of **15** was confirmed by ^{1}H NMR spectroscopy where the 1′-H signal appeared as a singlet ($J_{1',2'}$ = 0 Hz), similar to 2′,4′-BNA/LNA, suggesting that the sugar moiety was in *N*-conformation.[16a] Debenzylation of **15** by catalytic hydrogenolysis with Pearlman's catalyst [20% Pd(OH)$_2$/C] under a hydrogen atmosphere yielded the target nucleoside monomer **16** (Scheme 2). It is noteworthy that hydrogenolysis for debenzylation was not possible for the synthesis of 2′,4′-BNA^{NC}, as bond cleavage occurred at the N−O moiety,[27d] whereas the reaction occurred smoothly with no cleavage reaction in the case of HxNA, presumably due to the electron-withdrawing effect of the carbonyl function toward the N−O moiety.

Starting from the key intermediate **14**, the synthesis of the *N*-methyl congener of HxNA was accomplished in four consecutive steps (Scheme 2). The free aminooxy moiety of the key intermediate **7** was methylated by reductive amination; reacting **14** with formaldehyde yielded the imine **17** which was reduced by sodium cyanoborohydride in the presence of pyridinium *p*-toluenesulfonate to afford **18**. Cyclization of **18**, employing the coupling agent EDCI·HCl in DMF in the presence of HOBt·H_2O, yielded the product **19**. Debenzylation of **19** by catalytic hydrogenolysis afforded the target nucleoside monomer **20**.

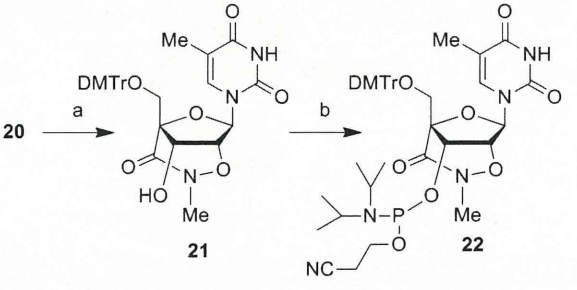
The [NH] derivative of HxNA, having an unsubstituted nitrogen in the bridge, was found to be less stable under basic conditions, and it was difficult to synthesize desired phosphoramidite monomer of the derivative. Therefore, we have used the *N*-substituted HxNA, i.e., HxNA[NMe], for the preparation of modified oligonucleotides in this study. The synthesized HxNA monomer was incorporated into oligonucleotides using the standard phosphoramidite protocol except for a prolonged coupling time of 30−45 min with 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42: Act42) as an activator. For the synthesis of the desired phosphoramidite **22**, the primary hydroxyl group of the nucleoside **20** was selectively protected with the 4,4′-dimethoxytrityl (DMTr) group to give **21**. The secondary hydroxyl group was then phosphitylated with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite to yield the HxNA[NMe]-thymine phosphoramidite **22** as a mixture of two diastereoisomers (Scheme 3).

Scheme 2[a]



[a]Reagents and conditions: (a) EDCI·HCl, HOBt·H_2O, DMF, 77%, two steps; (b) H_2, Pd(OH)$_2$/C, EtOH/$CHCl_3$, 96%; (c) HCHO, MeOH, rt, 65%, two steps; (d) $NaBH_3CN$/1 M PPTS, MeOH, rt; (e) EDCI·HCl, HOBt·H_2O, DMF, rt, 80%, two steps; (f) H_2, Pd(OH)$_2$/C, EtOH/$CHCl_3$, rt, 90%.

Scheme 3[a]



[a]Reagents and conditions: (a) DMTrCl, TEA, pyridine, rt, 60%; (b) 2-cyanoethyl *N,N,N′,N′*-tetraisopropyl phosphorodiamidite, 4,5-dicyanoimidazole, MeCN, rt, 70%.

During postsynthetic processing, it was found that ammonia treatment for deprotection and removal of oligonucleotides from the solid support was not possible because of ammonolytic cleavage of the bridge. Therefore, the synthesized oligonucleotides were treated with 50 mM potassium carbonate in methanol to obtain the desired oligonucleotide without any bond cleavage. The oligonucleotides were purified by reversed-phase HPLC (RP-HPLC) and characterized by MALDI-TOF

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Table 1. T_m (°C) Values of Duplex Formed by HxNA-Modified Oligonucleotides with Complementary ssRNA and ssDNA[a]

	complementary RNA	complementary DNA	RNA selectivity
oligonucleotides	T_m (ΔT_m/mod) (°C)	T_m (ΔT_m/mod) (°C)	$\Delta T_{m(ssRNA-ssDNA)}$ (°C)
5′-d(TTTTTTTTTT)-3′ (**23**)	18	19	−1
5′-d(TTTTTXTTTT)-3′ (**24**)[b]	20 (+2)	12 (−7)	+8
5′-d(TTTXTXTTTT)-3′ (**25**)	24 (+3)	14 (−2.5)	+10
5′-d(TTTXTXTXTT)-3′ (**26**)	29 (+3.7)	21 (+0.7)	+8

[a]Target ssRNA: 5′-r(AAAAAAAAAA)-3′(**32**). Target ssDNA: 5′-d(AAAAAAAAAA)-3′(**33**). Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; strand concentration = 4 μM. [b]T_m values of **24**/5′-r(AAAACAAAAA)-3′ and **24**/5′-r(AAAAUAAAAA)-3′were less than 10 °C.

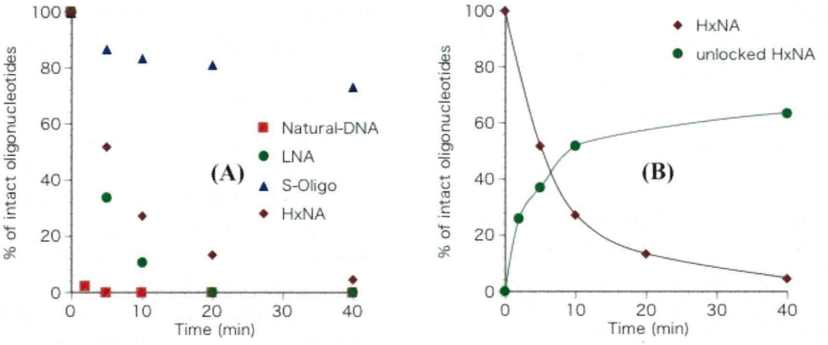

Figure 2. (A) Nuclease resistance of 5′-d(TTTTTTTTXT)-3′ against CAVP. X = HxNA (maroon solid diamond) (**27a**); natural DNA-T (red solid square) (**23**); 2′,4′-BNA/LNA-T (green solid circle) (**27b**); phosphorothioate-T (blue solid triangle) (**27c**). Experiments were performed at 37 °C in 100 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, CAVP (0.175 μg), and 7.5 μM of oligonucleotide. (B) Nuclease resistance of **27a** with HxNA modified singly at 2nd position from the 3′-end. The T-10 mer **27a** (maroon solid diamond) gradually degraded to the $n-1$ fragment from the 3′-end, the bridge of the HxNA modification opened, and the resulting T-9 mer (green solid circle) was highly stable toward nuclease degradation.

mass spectroscopy (mass spectral data and yields of the oligonucleotides are provided in the Supporting Information).

Thermal Stability of the Duplex Formed by HxNA. The thermal stability of the duplex formed by HxNA with complementary RNA, r(A_{10}) (**32**), and single-stranded DNA, d(A_{10}) (**33**), was monitored by UV melting experiments (T_m experiments). The results were compared with those obtained with natural d(T_{10}) oligonucleotide (**23**) and summarized in Table 1. The T_m value of the duplex formed between r(A_{10}) and singly modified oligonucleotide (**24**) was 2 °C higher than compared to that of natural d(T_{10})/r(A_{10}) duplex. An increase in the number of modifications increased the T_m value. For doubly (**25**) and triply (**26**) modified oligonucleotides, the increase in T_m was +3 and +3.7 °C per modification, respectively. In summary, the increase in T_m value per modification (ΔT_m/mod) of the HxNA ranged from 2.0 to 3.7 °C. Thus, the RNA binding affinity of HxNA was sufficiently higher than that of natural oligonucleotide and comparable to that of ENA;[22] however, it was lower than that of 2′,4′-BNA^{NC27d} (Table SI-3, Supporting Information). As regards RNA binding affinity, the effect of the hydroxamate bridge in HxNA was limited and less than what we expected in this case. In the case of duplex formation with complementary 10-mer DNA [d(A_{10})] the T_m values for singly (**24**) and doubly (**25**) modified oligonucleotides decreased by 7 and 2.5 °C per modification, respectively. Increasing the number of modifications to three (**26**) increased the T_m value to 0.7 °C per modification compared to the natural oligonucleotide.

Thus, the HxNA-modified oligonucleotides exhibited very high selectivitiy toward RNA similar to the urea-type bridged nucleic acid,[27e] indicating that introduction of a carbonyl function may help increase RNA selectivity of the bridged nucleic acid modified oligonucleotides. The T_m value of the duplex formed by singly modified HxNA oligonucleotide (**24**) with complementary ssRNA was +8 °C higher than that obtained with complementary ssDNA. The difference in T_m value increased to +10 °C when the oligonucleotide was modified doubly with HxNA (**25**) and +8 °C higher in the case of triply modified oligonucleotide (**26**) (Table 1). This result showed that the RNA selectivity of HxNA was more or less constant, in contrast with the urea-type bridged nucleic acid where the RNA selectivity increased as the number of modifications increased.[27e]

Nuclease Resistance of HxNA. The resistance of oligonucleotides modified with HxNA toward 3′-exonuclease (*Crotalus adamanteus* venom phosphodiesterase, CAVP, Pharmacia) was examined and compared with that of natural oligonucleotide, 2′,4′-BNA/LNA, and phosphorothioate (PS)-modified oligonucleotides, respectively. All of the oligonucleotides used in this study were 10-mers; those bearing modifications were modified singly at the second position from the 3′-end. The 10-mer HxNA-modified oligonucleotide (**27a**) was found to be less resistant to enzymatic degradation than the PS-modified oligonucleotide (**27c**) but exhibited better resistance than 2′,4′-BNA/LNA (**27b**) and natural d(T_{10}) oligonucleotide (**23**) (Figure 2A).

Interestingly, following the enzymatic removal of the first nucleotide, enzymatic digestion completely stopped. The progress of the enzymatic reaction was monitored by RP-HPLC; as the peak of **27a**, [5′-d(TTTTTTTTXT)-3′, where X = HxNA[NMe]-T], slowly diminished in size (peak **A**) a new peak (peak **B**) appeared and increased in size. The intensity of peak **B** increased with reaction time, and no other peaks appeared except **C** which is the peak of the T_1 monomer

(Figure 2B and 3). This result showed the high resistance of the degraded oligomer **28** toward enzymatic digestion. The

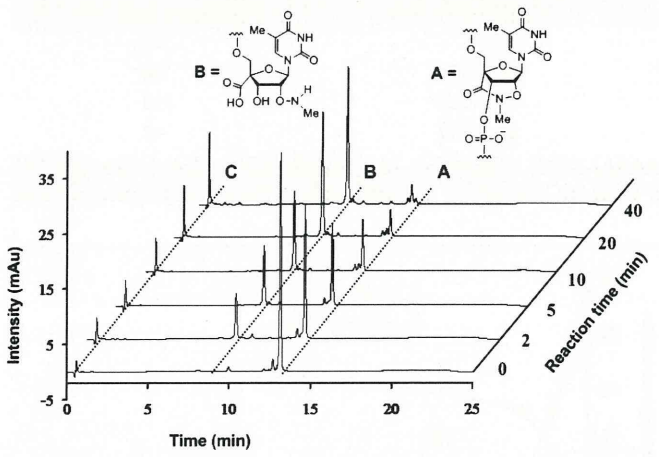

Figure 3. HPLC profile of the enzymatic degradation of HxNA-modified oligonucleotide **27a** with respect to time. Column: XBridge RP18 (3.0 × 50 mm). Mobile phase: Linear gradient of CH_3CN (7 to 14% over 20 min) in 0.1 M triethylammonium acetate (pH 7.0). Flow rate: 0.8 mL/min. Detection: absorbance at 260 nm.

degraded oligomers were isolated by RP-HPLC, and analyzed by MALDI-TOF mass spectrometry. Mass analysis confirmed that the degraded fragment **28** was the 9-mer oligonucleotide, but the bridged structure of the modification opened (Figure SI-43, Supporting Information). To check whether the buffer solution contributed to the ring-cleavage reaction, dT_8**X**T HxNA-modified oligonucleotide (**27a**) was incubated in the buffer solution for same length of time as in the enzymatic degradation experiment (40 min). This control experiment confirmed that the buffer solution played no role in either the degradation or the ring-opening of the modification.

The nuclease digestion experiment was conducted on three other 10-mer HxNA-modified oligonucleotides: modified singly at the fifth position [5′-d(TTTTT**X**TTTT)-3′ (**24**)], doubly at the fifth and seventh positions [5′-d(TTT**X**T**X**TTTT)-3′ (**25**)], and triply at the third, fifth, and seventh positions [5′-d(TT**X**T**X**T**X**TT)-3′ (**26**)] from their 3′-end. In the case of the singly modified oligomer **24**, enzymatic digestion continued up to the fifth position from the 3′-end, as evidenced by the appearance of five peaks in the RP-HPLC profile. The fifth peak of these peaks, peak **B**, intensified with respect to time. Isolation and analysis (Figure SI-44, Supporting Information) of the degraded oligomer confirmed that the sequence of the highly nuclease resistant oligomer fragment was **29** [5′-d(TTTTT**X**′)-3′, where X′ = unlocked HxNA] with the ring of the modification opened. This result suggested that the HxNA modification has potential for generating highly nuclease-resistant oligomers in situ of the enzymatic reaction. If the enzyme cleaves only the modified moiety located at the extreme 3′-end of an oligonucleotide with multiple HxNA modifications, the modification could be developed into a switch to generate highly nuclease-resistant RNA selective oligonucleotides. To study the effect of the enzyme on the HxNA modification at positions other than the 3′-end, doubly (**25**) and triply (**26**) modified oligonucleotides were exposed to the 3′-exonuclease. As expected, degradation stopped at the first modified site from the 3′-end of both **25** and **26**, after opening of the modification, similar to the results of the previous experiments. Gratifyingly, no further digestion was observed, and the remaining modifications were not affected by the nuclease. MALDI-TOF mass analysis confirmed that the rest of the bridged structures remained intact (Figure SI-45 and Figure SI-46, Supporting Information). The HPLC profiles obtained during these experiments are shown in Figures SI-36–SI-38 in the Supporting Information. The MALDI-TOF masses of the degraded oligonucleotides with an unlocked HxNA modification are summarized in Table SI-2 in the Supporting Information. These results demonstrate the significant potential of the HxNA modification to act as a switch for generating highly nuclease-resistant monomer in situ of the enzymatic reaction, which may have interesting applications in the development of antisense oligonucleotides.

■ CONCLUSION

We have designed and synthesized a novel 2′,4′-bridged nucleic acid, HxNA, which contains a six-membered perhydro-1,2-oxazin-3-one ring system. The synthesis was accomplished in 14 and 16 synthetic steps using a condensation reaction between an aminooxy and a carboxyl moiety to close the ring. The synthesized HxNA[NMe]-T monomer was incorporated into oligonucleotides, and their properties were investigated. The HxNA[NH] monomer was not used in this study because of its instability under basic conditions and the difficulty of synthesizing its phosphoramidite derivative. The HxNA[NMe]-modified oligonucleotides exhibited highly selective hybridizing affinity toward complementary ssRNA with an increase in T_m values of 2–3.7 °C per modification. The nuclease resistance of HxNA-modified oligomer was higher than that of 2′,4′-BNA/LNA. Most interestingly, oligomers containing the unlocked derivative of the modification were found to be highly resistant toward enzymatic digestion: exposure of the HxNA-modified oligonucleotide to nuclease resulted in opening of the ring, with the ring-opened derivative resisting further degradation. Furthermore, only the modification located at the extreme 3′-end was affected by the nuclease; the other modifications remained unaffected. Thus, this modification has considerable potential to be developed into an in situ switch for generating highly nuclease-resistant antisense oligonucleotides with high RNA selectivity.

■ EXPERIMENTAL SECTION

General Methods. All moisture-sensitive reactions were carried out in well-dried glassware under a N_2 atmosphere. Anhydrous dichloromethane, DMF, MeCN, and pyridine were used as purchased. ^1H NMR spectra were recorded at 300 and 400 MHz, ^{13}C NMR were recorded at 75 and 100 MHz, and the ^{31}P spectrum was recorded at 161 MHz. Chemical shift values are expressed in δ values (ppm) relative to tetramethylsilane (TMS) as internal standard and residual solvents for ^1H NMR, and $CHCl_3$ (δ = 77.00 ppm), methanol (δ = 49.00 ppm), and DMSO (39.50 ppm) for ^{13}C NMR, and 85% H_3PO_4 (δ = 0 ppm) for ^{31}P NMR. Fast atom bombardment mass spectra (FAB-MS) were recorded in positive-ion mode. For column chromatography, silica gel PSQ 100B was used. The progress of the reaction was monitored by analytical thin-layer chromatography (TLC) on precoated aluminum sheets.

*3,5-Di-O-benzyl-4-C-tert-butyldiphenylsiloxymethyl-1,2-O-isopropylidene-α-D-ribofuranose (**3**).* To a solution of compound **2** (15.0 g, 37.4 mmol) in DMF (150 mL) were added imidazole (5.8 g, 85.1 mmol) and TBDPSCl (15.0 mL, 57.3 mmol) at 0 °C and the mixture stirred for 15 h at room temperature. After completion of the reaction, ice–water was added, and the product was extracted with diethyl ether. The organic phase was washed with water and brine solution and dried (Na_2SO_4). The product was purified by column

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chromatography (*n*-hexane/ethyl acetate = 9:1) to afford **3** as a clear oil (23.8 g, quant). $[\alpha]^{25}_{D}$ = +84.8 (*c* 1.00, $CHCl_3$). IR (KBr): 1457, 1372, 1105, 1025, cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ_H 1.07 (9H, s), 1.29 (6H, s), 3.67 (1H, d, *J* = 10.2 Hz), 3.76 (1H, d, *J* = 10.2 Hz), 4.09 (1H, d, *J* = 11.1 Hz), 4.14 (1H, d, *J* = 11.1 Hz), 4.33 (1H, d, *J* = 5.1 Hz), 4.46–4.59 (4H, m), 4.79 (1H, d, *J* = 12.3 Hz), 5.78 (1H, d, *J* = 3.9 Hz), 7.27–7.76 (20H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C 19.2, 26.2, 26.5, 26.7, 64.6, 71.9, 72.2, 73.5, 76.6, 78.1, 79.5, 87.5, 104.1, 113.1, 127.4, 127.5, 127.5, 127.5, 127.6, 128.1, 128.2, 129.4, 133.1, 133.4, 134.7, 135.6, 135.7, 137.8, 138.0. MS (FAB): *m/z* 661 ($M + Na^+$). Anal. Calcd for $C_{39}H_{46}O_6Si$: C, 73.32; H, 7.26. Found: C, 73.44; H, 7.32.

2′-O-Acetyl-3′,5′-di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyluridine (4). To a stirring solution of compound **3** (23.0 g, 36.0 mmol) in acetic acid (26.0 mL, 460 mmol) were added acetic anhydride (45.0 mL, 480 mmol) and concd sulfuric acid (200 μL) at 0 °C. The reaction mixture was stirred at room temperature for 5 h. After completion of the reaction, the solution was neutralized with satd $NaHCO_3$, and the product was extracted with ethyl acetate. The organic phase was washed with water and brine solution and dried (Na_2SO_4). After concentration, the crude product (25.7 g) was obtained as yellow syrup which was used for the next reaction without purification.

The crude product (25.7 g, 37.6 mmol) was dissolved in acetonitrile (200 mL), and thymine (14.2 g, 110 mmol) and bis(trimethylsilyl)-acetamide (BSA) (47.0 mL, 190 mmol) were added. The solution was heated at 40 °C until all the substrate dissolved and then was cooled to 0 °C. TMS-triflate (10.5 mL, 57.7 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 1 h. After completion of the reaction, ice–water was added, and the product was extracted with dichloromethane. The organic phase was washed with water and brine solution, and dried (Na_2SO_4). The solution was concentrated to afford the crude product which was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **4** as a white solid (20.0 g, 74%, two steps). Mp: 55–59 °C. $[\alpha]_D^{24}$ = −11.7 (*c* 0.800, $CHCl_3$). IR (KBr): 1747, 1693, 1232, 1113 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ_H 1.03 (9H, s), 1.52 (3H, s), 1.96 (3H, s), 3.68 (1H, d, *J* = 10.8 Hz), 3.71 (1H, d, *J* = 10.5 Hz), 3.75 (1H, d, *J* = 10.5 Hz), 3.94 (2H, d, *J* = 10.8 Hz), 4.40 (1H, d, *J* = 5.7 Hz), 4.55 (2H, m), 5.37 (1H, t, *J* = 6.0 Hz), 6.15 (1H, d, *J* = 6.0 Hz), 7.18–7.62 (20H, m), 7.87 (1H, s). ^{13}C NMR (75 MHz, $CDCl_3$): 12.0, 19.2, 20.6, 26.9, 63.8, 72.2, 73.7, 74.6, 74.9, 77.7, 85.5, 87.8, 111.3, 127.6, 127.7, 127.7, 127.7, 127.8, 128.1, 128.3, 128.6, 129.7, 129.8, 132.6, 132.9, 135.5, 135.7, 135.7, 137.2, 137.5, 150.4, 163.6, 170.2. MS (FAB): *m/z* 749 ($M + H^+$). Anal. Calcd for $C_{43}H_{48}N_2O_8Si$: C, 68.96; H, 6.46; N, 3.74. Found: C, 68.92; H, 6.45; N, 3.74.

3′,5′-Di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyluridine (5). To a solution of compound **4** (20.0 g, 26.7 mmol) in THF (100 mL) was added 40% aqueous methylamine solution (62.1 mL, 800 mmol) and stirred for 30 min at room temperature. After completion of the reaction, the product was extracted with ethyl acetate. The organic phase was washed with water and brine solution and dried (Na_2SO_4). The product was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **5** as a white solid (18.3 g, quant). Mp: 61–63 °C. $[\alpha]^{25}_{D}$ = −12.2 (*c* 0.750, $CHCl_3$). IR (KBr): 3403, 3175, 1688, 1468, 1272, 1113 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.05 (9H, s), 1.60 (3H, s), 3.55 (1H, d, *J* = 10.4 Hz), 3.63 (1H, d, *J* = 10.4 Hz), 3.75 (1H, d, *J* = 10.8 Hz), 3.81 (1H, d, *J* = 10.8 Hz), 3.84 (1H, d, *J* = 10.4 Hz), 4.30 (1H, d, *J* = 5.6 Hz,) 4.41 (1H, ddd, *J* = 4.8 Hz, 5.6 Hz, 10.8 Hz), 4.49 (2H, s), 4.64 (1H, d, *J* = 11.2 Hz), 4.75 (1H, d, *J* = 11.2 Hz), 5.95 (1H, d, *J* = 5.0 Hz), 7.21–7.66 (20H, m), 9.04 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): 12.1, 19.0, 26.8, 64.2, 72.2, 73.6, 74.1, 74.5, 78.5, 87.9, 90.9, 110.9, 127.6, 127.8, 127.8, 127.8, 128.9, 128.0, 128.0, 128.5, 129.9, 132.2, 132.2, 135.6, 136.5, 137.2, 137.2, 150.5, 163.8. MS (FAB): *m/z* 707 ($M + H^+$). Anal. Calcd for $C_{41}H_{46}N_2O_7Si$: C, 69.66; H, 6.56; N, 3.96. Found: C, 69.59; H, 6.59; N, 3.93.

2,2′-Anhydro-3′,5′-di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyluridine (6). To a solution of compound **5** (17.0 g, 24.0 mmol) in dichloromethane (250 mL) was added DMAP (11.7 g, 95.7 mmol). The reaction mixture was placed in an ice bath, TfCl (7.6 mL, 71.2 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 1 h. After completion of the reaction, the reaction was quenched with ice-cold water, and the product was extracted with dichloromethane. The organic phase was washed with water and brine solution, and dried (Na_2SO_4). The product was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **6** as white solid (16.5 g, quant.). Mp: 51–54 °C. $[\alpha]^{26}_{D}$ = −33.5 (*c* 1.00, $CHCl_3$). IR (KBr): 1667, 1650, 1563, 1482, 1112 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.04 (9H, s), 1.98 (3H, s), 3.33 (2H, m), 3.70 (1H, d, *J* = 10.8 Hz), 3.83 (1H, d, *J* = 10.8 Hz), 4.31 (1H, d, *J* = 12 Hz), 4.32 (1H, d, *J* = 8.8 Hz), 4.38 (1H, d, *J* = 12 Hz), 4.60 (1H, d, *J* = 11.5 Hz), 4.60 (1H, d, *J* = 11.5 Hz), 5.52 (1H, dd, *J* = 5.2, 8 Hz), 6.27 (1H, d, *J* = 6.0 Hz), 7.09–7.67 (21H, m). ^{13}C NMR (75 MHz, $CDCl_3$): 13.9, 18.9, 26.7, 63.9, 69.4, 73.4, 83.9, 87.1, 88.7, 89.9, 118.9, 127.4, 127.6 127.7, 127.8, 128.1, 128.3, 128.4, 128.5, 129.8, 130.1, 131.9, 132.3, 132.3, 135.3, 135.5, 136.4 136.9, 159.1, 172.3. MS (FAB): *m/z* 689 ($M + H^+$). Anal. Calcd for $C_{41}H_{44}N_2O_6Si$: C, 71.48; H, 6.44; N, 4.07. Found: C, 71.38; H, 6.49; N, 4.08.

3′,5′-Di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyl-arabinouridine (7). To a solution of compound **6** (16.5 g, 23.4 mmol) in THF (200 mL) was added 1 N NaOH solution (70.0 mL, 70.0 mmol), and the mixture was stirred at room temperature for 16.5 h. The solution was neutralized with NH_4Cl solution, and the product was extracted with dichloromethane. The organic phase was washed with water and brine solution, dried (Na_2SO_4), and concentrated. The crude product thus obtained was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **7** as white solid (16.4 g, 97%). Mp: 67–70 °C. $[\alpha]^{26}_{D}$ = +24.5 (*c* 0.840, $CHCl_3$). IR (KBr): 3347, 3184, 1690, 1471 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.99 (9H, s), 1.54 (3H, s), 3.42 (1H, d, *J* = 10.0 Hz), 3.52 (1H, d, *J* = 10.5 Hz), 3.60 (1H, d, *J* = 10.0 Hz), 3.79 (1H, d, *J* = 10.5 Hz), 4. 34 (1H, d, *J* = 6.4 Hz), 4.43 (1H, d, *J* = 11.6 Hz) 4.51 (1H, d, *J* = 11.6 Hz), 4.62 (1H, d, *J* = 11.6 Hz), 4.90 (1H, d, *J* = 6.4 Hz), 4.93 (1H, d, *J* = 11.6 Hz), 5.10 (1H, dd, *J* = 6.4, 12.4 Hz), 6.36 (1H, d, *J* = 6.0 Hz), 7.19–7.68 (20H, m), 7.77 (1H,s), 9.95 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): 12.1, 18.9, 26.5, 64.1, 69.6, 72.6, 73.7, 75.4, 81.7, 85.4, 85.5, 109.7, 127.6, 127.8, 127.8, 127.8, 127.9, 128.1, 128.3, 128.5, 129.6, 129.7 132.5, 135.6, 135.7, 137.1, 137.4, 138.2, 151.4, 164.7. MS (FAB): *m/z* 707 ($M + H^+$). Anal. Calcd for $C_{41}H_{46}N_2O_7Si$: C, 69.66; H, 6.56; N, 3.96. Found: C, 69.42; H, 6.54; N, 3.97.

3′,5′-Di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-2′-O-(1,3-dihyro-1,3-dioxo-2H-isoindol-2-yl)-5-methyluridine (9). To a solution of compound **7** (3.00 g, 4.24 mmol) in dichloromethane (15 mL) were added pyridine (1.70 mL, 21.2 mmol) and trifluoromethanesulfonic anhydride (1.50 mL, 8.91 mmol) at 0 °C. The reaction mixture was stirred in an ice bath for 40 min. After completion of the reaction, ice-cold water was added, and the product was extracted with dichloromethane. The organic phase was washed with water and brine, dried (Na_2SO_4), and concentrated. The crude product **8** (4.10 g) was used for the next reaction without further purification.

The crude triflate **8** (4.60 g. 5.48 mmol) was dissolved in acetonitrile (20 mL), and *N*-hydroxyphthalimide (4.00 mg, 24.5 mmol) and DBU (3.70 mL, 24.7 mmol) were added. The reaction mixture was stirred at room temperature for 24 h. After completion of the reaction, the solution was diluted with dichloromethane, and water was added. The product was extracted with dichloromethane. The organic phase was washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (*n*-hexane/ethyl acetate = 2:1) to produce **9** as a white amorphous solid (2.80 g, 79% two steps). Mp: 78–80 °C. $[\alpha]^{25}_{D}$ = +43.0 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3188, 3067, 2934, 2862, 1791, 1730, 1692, 1465, 1427, 1421, 1366, 1267, 1189, 1106, 973 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.08 (9H, s), 1.38 (3H, s), 3.65 (1H, d, *J* = 10.4 Hz), 4.02 (1H, d, *J* = 11.6 Hz), 4.09 (1H, d, *J* = 10.4 Hz), 4.22 (1H, d, *J* = 11.6 Hz), 4.48 (1H, d, *J* = 11.2 Hz), 4.52 (1H, d, *J* = 5.2 Hz), 4.55 (1H, d, *J* = 11.2 Hz), 4.73 (1H, d, *J* = 11.2 Hz), 4.85 (1H, dd, *J* = 3.2 Hz, 2.8 Hz), 5.13 (1H, d, *J* = 11.2 Hz), 6.40 (1H, d, *J* = 3.2 Hz), 7.16 – 7.86 (26H, m), 8.37 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.8, 19.3, 26.9, 64.6, 70.5, 73.1, 73.6, 75.1, 87.2, 88.3, 88.5, 110.4,

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123.6, 127.6, 127.7, 127.8, 128.3, 128.5, 129.6, 129.6, 132.9, 133.3, 134.5, 135.6, 135.7, 137.2, 137.4, 150.0, 163.1. MS (FAB) m/z 852 (M + H^+). HRMS (FAB) calcd for $C_{49}H_{49}N_3O_9Si$ (M + H^+): 852.3316, found 852.3284.

*3′,5′-Di-O-benzyl-2′-O-(1,3-dihyro-1,3-dioxo-2H-isoindol-2yl)-4′-C-hydroxymethyl-5-methyluridine (**12**).* To a solution of compound **9** (2.80 g, 3.28 mmol) in THF (15 mL) was added TEA·3HF (5.60 mL, 34.3 mmol), and the solution was refluxed for 18 h. The reaction mixture was then cooled and ice-cold water was added. The product was extracted with ethyl acetate, washed with satd sodium hydrogencarbonate and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to produce **12** as white amorphous solid (1.80 g, 90%). Mp: 96–98 °C. $[\alpha]^{25}_D$ = +39.1 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr): 3504, 3181, 3062, 2881, 1789, 1733, 1689, 1466, 1375, 1272, 1187, 1105, 1057, 974 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.36 (3H, s), 2.64 (1H, br s), 3.83 (1H, d, *J* = 10.4 Hz), 3.92 (1H, d, *J* = 7.2 Hz), 3.96 (1H, d, *J* = 10.4 Hz), 4.12 (1H, d, *J* = 7.2 Hz), 4.49 (1H, d, *J* = 11.2 Hz), 4.54 (1H, d, *J* = 11.2 Hz), 4.63 (1H, d, *J* = 6 Hz), 4.74 (1H, d, *J* = 12 Hz), 4.93 (1H, dd, *J* = 6 Hz, 1.6 Hz), 5.16 (1H, d, *J* = 11.6 Hz), 6.34 (1H, d, *J* = 1.2 Hz), 7.15–7.84 (15H, m), 8.59 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.7, 64.1, 70.0, 73.0, 73.6, 75.3, 87.2, 87.8, 88.6, 110.4, 123.7, 127.7, 128.0, 128.1, 128.3, 128.5, 128.6, 128.6, 134.6, 135.5, 136.8, 137.2, 149.8, 163.4. MS (FAB): m/z 614 (M + H^+). HRMS (FAB): calcd for $C_{33}H_{31}N_3O_9$ (M + H^+) 614.2138, found 614.2155.

*3′,5′-Di-O-benzyl-4′-carboxyl-2′-O-(1,3-dihyro-1,3-dioxo-2H-isoindol-2yl)-5-methyluridine (**13**).* To a solution of compound **12** (1.80 g, 2.93 mmol) in DMF (20 mL) was added 4A molecular sieves (2.00 g), and the mixture was stirred for 10 min. To the solution was added PDC (11.6 g, 30.8 mmol), and the mixture was stirred at room temperature for 16 h. The reaction was quenched with ice-cold water. The product was extracted with ethyl acetate, washed with water, and purified by column chromatography (*n*-hexane/ethyl acetate = 1:1 to ethyl acetate/methanol 10:1). The product **13** was obtained as white amorphous solid (1.40 g, 75%). Mp: 137–139 °C. $[\alpha]^{26}_D$ = +23.9 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr): 3178, 3066, 3032, 2873, 1790, 1736, 1468, 1376, 1275, 1187, 1125, 967 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.59 (3H, s), 3.90 (1H, d, *J* = 10.4 Hz), 4.10 (1H, d, *J* = 10.4 Hz), 4.59 (1H, d, *J* = 11.6 Hz), 4.63 (1H, d, *J* = 11.6 H z), 4.68 (1H, d, *J* = 4.4 Hz), 4.91 (1H, d, *J* = 11.2 Hz), 5.06 (1H, t, *J* = 5.6 Hz) 5.27 (1H, d, *J* = 11.2 Hz), 6.70 (1H, d, *J* = 6.4 Hz), 7.27 - 7.86 (16 H, m), 9.16 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.9, 71.4, 73.9, 74.6, 74.6, 74.7, 78.1, 87.7, 88.4, 89.0, 111.4, 123.7, 127.6, 127.7, 127.8, 128.1, 128.2, 128.3, 128.4, 128.5, 134.8, 136.2, 136.3, 136.7, 150.6, 162.9, 164.0, 170.6. MS (FAB) m/z 628 (M + H^+). HRMS (FAB): calcd for $C_{33}H_{29}N_3O_{10}$ (M + H^+) 628.1931, found 628.1938.

*2′-O-Amino-3′,5′-di-O-benzyl-4′-carboxyl-5-methyluridine (**14**).* To a solution of compound **13** (400 mg, 0.637 mmol) in ethanol (2 mL) was added hydrazine monohydrate (40.0 μL, 0.823 mmol), and the mixture was stirred at room temperature for 10 min. After completion of the reaction, the reaction solution was concentrated and ethyl acetate was added. The precipitate was filtered, and the filtrate was extracted with ethyl acetate, washed with water and brine, and dried (Na_2SO_4). The crude compound **14** (380 mg) was used for the next reaction without further purification.

*(1S,5R,6R,8S)-3-Aza-8-benzyloxy-1-benzyloxymethyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one. (**15**).* The crude key intermediate **14** (320 mg, 0.643 mmol) was dissolved in DMF (5 mL), and EDCI·HCl (150 mg, 0.782 mmol) and HOBt·H_2O (106 mg, 0.784 mmol) were added at room temperature. The reaction mixture was stirred at room temperature for 11 h, and then ice-cold water was added. The product was extracted with ethyl acetate. The organic phase was washed with water and brine and dried (Na_2SO_4). The product was purified by column chromatography (*n*-hexane/ethyl acetate = 2:1 to 1:1) to yield **15** as a white solid (240 mg, 77% two steps). Mp: 96–98 °C. $[\alpha]^{23}_D$ = +89.53 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr): 3190, 3064, 3033, 2926, 2877, 1699, 1494, 1455, 1392, 1362, 1268, 1203, 1109, 1053, 1018, 984, 916 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.19 (3H, s), 3.98 (1H, d, *J* = 11 Hz), 4.28 (1H, d, *J* = 11 Hz), 4.51 (1H, d, *J* = 11 Hz), 4.57 (1H, s), 4.58 (1H, d, *J* = 11 Hz), 4.73 (1H, d, *J* = 12.4 Hz), 4.88 (1H, d, *J* = 12.4 Hz), 5.18 (1H, s), 6.25 (1H, s), 7.21–7.38 (10 H, m), 7.57 (1H, s), 7.75 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.8, 64.4, 69.3, 72.2, 73.7, 78.2, 80.1, 87.6, 110.3, 127.8, 127.9, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 135.1, 137.5, 137.6 150.8, 163.3, 167.9. MS (FAB): m/z 480 (M + H^+). HRMS (FAB): calcd for $C_{25}H_{25}N_3O_7$ (M + H^+) 480.1771, found 480.1779.

*(1S,5R,6R,8S)-3-Aza-8-hydroxy-1-hydroxymethyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**16**).* To a solution of compound **15** (110 mg, 0.229 mmol) in ethanol/chloroform 5:1 (5 mL) was added palladium hydroxide on carbon (110 mg). The reaction vessel was degassed several times with hydrogen gas, and the reaction mixture was stirred at room temperature under a hydrogen atmosphere overnight. After completion of the reaction, the solution was filtered, and the filtrate was concentrated. The product was further purified by column chromatography (*n*-hexane/ethyl acetate = 1:1 to 100% ethyl acetate) to produce **16** as a white solid (55.0 mg, 80%). Mp: 261–263 °C dec. $[\alpha]^{24}_D$ = +31.81 (*c* 1.00, MeOH). IR ν_{max} (KBr): 3474, 3406, 3237, 3056, 2979, 2932, 2819, 1693, 1481, 1422, 1386, 1359, 1282, 1207, 1104, 1052, 997, 967, 916 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ_H 1.86 (3H, s), 3.88 (1H, d, *J* = 12.8 Hz), 4.20 (1H, d, *J* = 12.8 Hz), 4.54 (1H, *J* = 3.6 Hz), 4.67 (1H, *J* = 3.6 Hz), 6.07 (1H, s), 7.94 (1H, d, *J* = 1.4 Hz). ^{13}C NMR (100 MHz, DMSO): δ_C 12.4, 48.6, 55.3, 61.9, 79.8, 80.5, 86.9, 108.6, 135.0, 149.9, 163.8, 167.1. MS (FAB): m/z 300 (M + H^+). HRMS (FAB): calcd for $C_{11}H_{13}N_3O_7$ (M + H^+) 300.0831, found 300.0830.

*3′,5′-Di-O-benzyl-4′-carboxyl-2′-O-(N-methyleneamino)-5-methyluridine (**17**).* Formalin (37 wt % in H_2O, 80.0 μL, 0.986 mmol) was added to a stirring solution of compound **14** (380 mg, 0.763 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature for 2 h. The solution was concentrated, and then water was added. The product was extracted with ethyl acetate, washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1 to 1:2) to produce **17** as a white solid (250 mg, 65% two steps). Mp: 81–83 °C. $[\alpha]^{26}_D$ = −29.3 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr): 3172, 3064, 2944, 2872, 1699, 1469, 1366, 1274, 1127, 1070, 916 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.53 (3H, s), 3.77 (1H, d, *J* = 10.8 Hz), 4.07 (1H, d, *J* = 10.8 Hz), 4.45 (1H, d, *J* = 4.8 Hz), 4.54 (1H, d, *J* = 11.2 Hz), 4.60 (1H, d, *J* = 12 Hz), 4.63 (1H, d, *J* = 12 Hz), 4.69 (1H, *J* = 11.2 Hz), 5.04 (1H, dd, *J* = 4.4 Hz, 3.2 Hz), 6.44 (1H, d, *J* = 7.2 Hz), 6.53 (1H, d, *J* = 8 Hz), 7.03 (1H, d, *J* = 7.2 Hz), 7.19–7.34 (10H, m), 7.52 (1H, s), 9.56 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 12.0, 72.0, 73.9, 74.7, 79.8, 83.6, 86.2, 89.7, 111.8, 125.2, 127.7, 127.7, 127.7, 127.8, 128.2, 128.3, 128.7, 128.9, 136.2, 136.6, 137.2, 139.9, 150.7, 164.2, 170.8. MS (FAB): m/z 510 (M + H^+). HRMS (FAB): calcd for $C_{26}H_{27}N_3O_8$ (M + H^+) 510.1876. found 510.1880.

*3′,5′-Di-O-benzyl-4′-carboxyl-2′-O-(N-methylamino)-5-methyluridine (**18**).* To a solution of compound **17** (250 mg, 0.488 mmol) in a methanolic solution of pyridinium *p*-toluenesulfonate (1M, 4.90 mL, 4.89 mmol) was added sodium cyanoborohydride (62.0 mg, 0.986 mmol) at 0 °C, and the mixture was stirred for 10 min. Then the reaction mixture was allowed to come to room temperature and stirred for 2 h. After completion of the reaction, the solvent was evaporated, and the product was diluted with ethyl acetate. The product was washed with water and brine, dried (Na_2SO_4), and concentrated. The crude product **18** (235 mg) was used for the next reactions without further purification.

*(1S,5R,6R,8S)-3-Aza-8-benzyloxy-1-benzyloxymethyl-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**19**).* To a solution of compound **18** (235 mg, 0.459 mmol) in dimethylformamide (4 mL) were added EDCI·HCl (105 mg, 0.547 mmol) and HOBt·H_2O (75.0 mg, 0.555 mmol). The reaction mixture was stirred at room temperature overnight. After completion of the reaction, water was added, and the product was extracted with ethyl acetate, washed with water and brine, dried (Na_2SO_4), and concentrated. The compound was purified by column chromatography (*n*-hexane/ethyl acetate = 4:1 to 1:1) to produce **19** as a white solid (190 mg, 80% two steps). Mp: 90–92 °C. $[\alpha]^{26}_D$ = +62.3 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr): 3164, 3029,

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2926, 2878, 1698, 1456, 1392, 1362, 1274, 1215, 1155, 1094, 1065, 983 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.41 (3H, s), 3.25 (3H, s), 3.95 (1H, d, J = 11.6 Hz), 4.25 (1H, d, J = 11.6 Hz), 4.34 (1H, d, J = 3.2 Hz), 4.56 (1H, d, J = 10.8 Hz), 4.59–4.64 (3H, m), 4.73 (1H, d, J = 10.8 Hz), 6.08 (1H, s), 7.23–7.37 (10 H, m), 7.62 (1H, d, J = 1.6 Hz), 9.22 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.9, 33.7, 64.7, 69.5, 72.6, 73.9, 78.5, 80.2, 87.9, 110.8, 127.6, 127.9, 128.2, 128.3, 128.5, 134.5, 136.5, 137.2, 149.9, 163.9. MS (FAB): m/z 494 (M + H^+). HRMS (FAB): calcd for $C_{26}H_{27}N_3O_7$ (M + H^+) 494.1927, found 494.1931.

(1S,5R,6R,8S)-3-Aza-8-hydroxy-1-hydroxymethyl-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**20**). To a solution of compound **19** (190 mg, 0.385 mmol) in ethanol/chloroform 5:1 (5 mL) was added palladium hydroxide on carbon (190 mg). The reaction vessel was degassed several times with hydrogen gas, and the reaction mixture was stirred at room temperature under a hydrogen atmosphere overnight. After completion of the reaction, the solution was filtered, and the filtrate was concentrated. The product was further purified by column chromatography (n-hexane/ethyl acetate = 1:1 to 100% ethyl acetate) to produce **20** as a white solid (105 mg, 90%). Mp: 232–234 °C dec; $[\alpha]^{26}_D$ = +14.9 (c 1.00, EtOH). IR ν_{max} (KBr): 3444, 3226, 3070, 2941, 1678, 1469, 1412, 1281, 1199, 1078,988 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ_H 1.86 (3H, s), 3.22 (3H, s), 3.87 (1H, d, J = 12.8 Hz), 4.18 (1H, d, J = 12.8 Hz), 4.53 (1H, J = 3.6 Hz), 4.71 (1H, J = 3.6 Hz), 6.04 (1H, s), 7.95 (1H, d, J = 1.2 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ_C 12.6, 33.7, 57.3, 63.9, 82.1, 82.8, 89.1, 111.0, 136.9, 151.8, 166.4. MS (FAB): m/z 314 (M + H^+). HRMS (FAB): calcd for $C_{12}H_{15}N_3O_7$ (M + H^+) 314.0988, found 314.0981.

(1S,5R,6R,8S)-3-Aza-1-(4,4′-dimethoxytrityloxymethyl)-8-hydroxy-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**21**). To a stirring solution of compound **20** (50.0 mg, 0.159 mmol) in pyridine (3 mL) were added DMTrCl (65 mg, 0.191 mmol) and triethylamine (100 μL, 0.727 mmol). The reaction mixture was stirred at room temperature for 8 h. Saturated $NaHCO_3$ was added, and the product was extracted with ethyl acetate. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (1% triethylamine in n-hexane/ethyl acetate = 2:1 to 100% ethyl acetate) to produce **21** as a yellowish white solid (58.0 mg, 60%). Mp: 136–138 °C. $[\alpha]^{28}_D$ = −21.1 (c 1.00, $CHCl_3$). IR ν_{max} (KBr): 3339, 3189, 3062, 2926, 2850, 1693, 1608, 1509, 1464, 1395, 1253, 1177, 1080, 1033, 978 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.36 (3H, s), 3.18 (3H, s), 3.72 (1H, d, J = 12 Hz), 3.76 (6H, s), 3.95 (1H, d, J = 12 Hz), 4.70 (1H, d, J = 3.6 Hz), 4.76 (1H, d, J = 3.6 Hz), 6.03 (1H, s), 6.81–6.84 (4H, m), 7.21 – 7.42 (9H, m), 7.71 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 12.0, 33.7, 55.2, 57.9, 64.5, 81.0, 87.3, 87.5, 111.3, 113.4, 127.1, 127.3, 127.7, 127.8, 128.0, 128.1, 129.1, 130.1, 130.1, 134.2, 135.0, 139.4, 144.1, 149.7, 158.7, 163.7. MS (FAB): m/z 638 (M + Na^+). HRMS (FAB): calcd for $C_{33}H_{33}N_3O_9Na$ (M + Na^+) 638.2109, found 638.2097.

(1S,5R,6R,8S)-3-Aza-8-(2-cyanoethoxy(diisopropylamino)-phosphinoxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**22**). To a stirring solution of compound **21** (50.0 mg, 0.081 mmol) in anhydrous acetonitrile were added 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (30.0 μL, 0.094 mmol) and 4,5-dicyanoimidazole (10.0 mg, 0.058 mmol). The reaction mixture was stirred at room temperature for 7 h. After completion of the reaction, satd $NaHCO_3$ was added, and the product was extracted with ethyl acetate. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (1% triethylamine in n-hexane/ethyl acetate = 2:1 to 1:1) to produce **22** as a white solid (46.0 mg, 70%). Mp: 117–119 °C. ^1H NMR (400 MHz, $CDCl_3$): δ_H 0.97–1.26 (15H, m), 2.39 (1H, t, J = 6 Hz), 2.40–2.63 (1H, m), 3.18 (3H, s), 3.53–3.62 (4H, m), 3.79 (6H, s), 3.95–4.04 (1H, m), 4.88–4.99 (2H, m), 6.08 (1H, s), 6.80–6.85 (4H, m), 7.23–7.43 (9H, m), 7.78 (1H, s), 8.66 (1H, br s). ^{31}P NMR (161 MHz, $CDCl_3$): δ 150.9, 151.5. MS (FAB): m/z 816 (M + H^+). HRMS (FAB): calcd for $C_{42}H_{50}N_5O_{10}P$ (M + H^+) 816.3373, found 816.3376.

Oligonucleotide Synthesis. Oligonucleotides **23**–**27** were synthesized on a 0.2 μmol scale using an Expedite 8909 Nucleic Acid Synthesis System according to the standard phosphoramidite protocol. 5-[3,5-Bis(trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42: Act42) was used as the activator, and Cap mix A (10% acetic acid in tetrahydrofuran) and Cap mix B (10% 1-methylimidazole in tetrahydrofuran/pyridine) were used as the capping agents. The standard synthesis cycle (trityl off mode) was used for assembly of the reagents and synthesis of the oligonucleotides, except that the coupling time was extended to 30–45 min for the HxNA monomers. The synthesized HxNA-phosphoramidite was dissolved in anhydrous acetonitrile. Standard CPG-solid supports from Glen Research were used. After synthesis, the synthesized oligonucleotides were cleaved from the solid support by treatment with 50 mM K_2CO_3 in methanol solution at room temperature for 90 min. The extract was treated with 1 M triethylamine acetic acid (TEAA), and the oligonucleotides were purified by Nap-10 column and reversed-phase HPLC (RP-HPLC) and then characterized by MALDI-TOF mass spectrometry.

UV Melting Experiments. The UV melting experiments were carried out on Shimadzu UV-1800 and Shimadzu UV-1650 instruments. To determine the T_m of the duplexes, equimolar amounts of target RNA/DNA strands and modified oligonucleotides were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to provide a final strand concentration of 4 μM. The samples were annealed at 90 °C and slowly cooled to room temperature. The melting experiment was monitored at 260 nm from 0 to 80 °C at a scan rate of 0.5 °C/min. T_m was calculated as the temperature at which the duplexes were half dissociated, determined by taking the first derivative of the melting curve.

Nuclease Resistance 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 $MgCl_2$. In each sample solutions, 0.175 μL 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.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MALDI-TOF-MS data and yields of oligonucleotides **23** to **27a**, and MALDI-TOF-MS data of oligonucleotides **28** to **31** with an unlocked modification, ^1H and ^{13}C spectra of all new compounds (**3**–**7**, **9**, **12**, **13**, **15**–**17**, **19**–**21**), ^1H and ^{31}P NMR spectrum of **22**, UV melting curves for the duplexes between oligonucleotides **23**–**26** and DNA or RNA complement, CD spectra of duplexes formed by HxNA-modified oligonucleotides, Job plot experiment of **24** and **32**, HPLC profiles of enzymatic degradation of singly (**24**), doubly (**25**), and triply (**26**) modified HxNA oligonucleotides with respect to time, and MALDI-TOF-MS spectra for **24**–**31**. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Zamecnik, P. C.; Stephenson., M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280−284.
(2) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543−584.
(3) Cohen, J. S. *Trends Biotechnol.* **1992**, *10*, 87−91.
(4) Opalinska, J. B.; Gewirtz, A. M. *Nat. Rev. Drug Discov.* **2002**, *1*, 503−514.
(5) Stein, A.; Cheng, Y. C. *Science* **1993**, *261*, 1004−1012.
(6) Gewirtz, A. M.; Sokol, D. L.; Ratajczak, M. Z. *Blood* **1998**, *92*, 712−736.
(7) Buchini, S.; Leumann, C. J. *Curr. Opin. Chem. Biol.* **2003**, *7*, 717−726.
(8) Crooke, S. T. *Annu. Rev. Med.* **2004**, *55*, 61−95.
(9) Yamamoto, T.; Nakatani, M.; Narukawa, K.; Obika, S. *Future Med. Chem.* **2011**, *3*, 339−365.
(10) Agrawal, S.; Kandimalla, E. R. *Mol. Med. Today* **2000**, *6*, 72−81.
(11) Herdewijn, P. *Liebigs Ann.* **1996**, 1337−1348.
(12) (a) Tarköy, M.; Bolli, M.; Schweizer, B.; Leumann, C. *Helv. Chim. Acta* **1993**, *76*, 481−510. (b) Stauffiger, A.; Leumann, C. J. *Eur. J. Org. Chem.* **2009**, 1153−1162. (c) Scheidegger, S. P.; Leumann, C. J. *Chem.—Eur. J.* **2006**, *12*, 8014−8023. (d) Ittig, D.; Gerber, A.-B.; Leumann, C. J. *Nucleic Acids Res.* **2011**, *39*, 373−380.
(13) (a) Saneyoshi, H.; Deschamps, J. R.; Marquez, V. E. *J. Org. Chem.* **2010**, *75*, 7659−7669. (b) Terrazas, M.; Ocampo, S. M.; Perales, J. C.; Marquez, V. E.; Eritja, R. *ChemBioChem* **2011**, *12*, 1056−1065.
(14) (a) Christensen, N. K.; Petersen, M.; Nielsen, P.; Jacobsen, J. P.; Olsen, C. E.; Wengel, J. *J. Am. Chem. Soc.* **1998**, *120*, 5458−5463. (b) Kvaerno, L.; Wengel, J. *J. Org. Chem.* **2001**, *66*, 5498−5503.
(15) (a) Wang, G.; Gunic, E.; Girardet, J. L.; Stoisavljevic, V. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1147−1150. (b) Wang, G. *Tetrahedron Lett.* **1999**, *40*, 6343−6346.
(16) (a) Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735−8738. (b) Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401−5404.
(17) Imanishi, T.; Obika, S. *Chem. Commun.* **2002**, 1653−1659.
(18) (a) Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455−456. (b) Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607−3630. (c) Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301−310.
(19) (a) Singh, S. K.; Wengel, J. *Chem. Commun.* **1998**, 1247−1248. (b) Koshkin, A. A.; Nielsen, P.; Meldgaard, M.; Rajwanshi, V. K.; Singh, S. K.; Wengel, J. *J. Am. Chem. Soc.* **1998**, *120*, 13252−13253. (c) Birte, Vester; Wengel, J. *Biochemistry* **2004**, *43*, 13233−13241.
(20) Wahlestedt, C.; Salmi, P.; Good, L.; Kela, J.; Johnsson, T.; Hökfelt, T.; Broberger, C.; Porreca, F.; Lai, J.; Ren, K.; Ossipov, M.; Koshkin, A.; Jakobsen, N.; Skouv, J.; Oerum, H.; Jacobsen, M. H.; Wengel, J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5633−5638.
(21) (a) Elmén, J.; Lindow, M.; Schütz, S.; Lawrence, M.; Petri, A.; Obad, S.; Lindholm, M.; Hedtjärn, M.; Hansen, H. F.; Berger, U.; Gullans, S.; Kearney, P.; Sarnow, P.; Straarup, E. M.; Kauppinen, S. *Nature* **2008**, *452*, 896−899. (b) Straarup, E. M.; Fisker, N.; Hedtjärn, M.; Lindholm, M. W.; Rosenbohm, C.; Aarup, V.; Hansen, H. F.; Ørum, H.; Hansen, J. B. R.; Koch, T. *Nucleic Acids Res.* **2010**, *38*, 7100−7111. (c) Gupta, N.; Fisker, N.; Asselin, M.-C.; Lindholm, M.; Rosenbohm, C.; Ørum, H.; Elmén, J.; Seidah, N. G.; Straarup, E. M. *PLoS One* **2010**, *5*, No. e10682.
(22) Morita, K.; Takagi, M.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.; Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. *Bioorg. Med. Chem.* **2003**, *11*, 2211−2226.
(23) (a) Kumar, R.; Singh, S. K.; Koshkin, A. A.; Rajwanshi, V. K.; Meldgaard, M.; Wengel, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2219−2222. (b) Rosenbohm, C.; Christensen, S. M.; Sørensen, M. D.; Pedersen, D. S.; Larsen, L. E.; Wengel, J.; Koch, T. *Org. Biomol. Chem.* **2003**, *1*, 655−663.
(24) Albæk, N.; Petersen, M.; Nielsen, P. *J. Org. Chem.* **2006**, *71*, 7731−7740.
(25) (a) Srivastava, P.; Barman, J.; Pathmasiri, W.; Plashkevych, O.; Wenska, M.; Chattopadhyaya, J. *J. Am. Chem. Soc.* **2007**, *129*, 8362−8379. (b) Varghese, O. P.; Barman, J.; Pathmasiri, W.; Plashkevych, O.; Honcharenko, D.; Chattopadhyaya, J. *J. Am. Chem. Soc.* **2006**, *128*, 15173−15187. (c) Zhou, C.; Plashkevych, O.; Chattopadhyaya, J. *Org. Biomol. Chem.* **2008**, *6*, 4627−4633.
(26) Prakash, T. P.; Siwkowski, A.; Allerson, C. R.; Migawa, M. T.; Lee, S.; Gaus, H. J.; Black, C.; Seth, P. P.; Swayze, E. E.; Bhat, B. *J. Med. Chem.* **2010**, *53*, 1636−1650.
(27) (a) Hari, Y.; Obika, S.; Ohnishi, R.; Eguchi, K.; Osaki, T.; Ohishi, H.; Imanishi, T. *Bioorg. Med. Chem.* **2006**, *14*, 1029−1038. (b) Mitsuoka, Y.; Kodama, T.; Ohnishi, R.; Hari, Y.; Imanishi, T.; Obika, S. *Nucleic Acids Res.* **2009**, *37*, 1225−1238. (c) Miyashita, K.; Rahman, S. M. A.; Seki, S.; Obika, S.; Imanshi, T. *Chem. Commun.* **2007**, 375−3767. (d) Rahman, S. M. A.; Seki, S.; Obika, S.; Yoshikawa, H.; Miyashita, K.; Imanishi, T. *J. Am. Chem. Soc.* **2008**, *130*, 4886−4896. (e) Nishida, M.; Baba, T.; Kodama, T.; Yahara, A.; Imanishi, T.; Obika, S. *Chem. Commun.* **2010**, 5283−5285.
(28) (a) Rahman, S. M. A.; Imanishi, T.; Obika, S. *Chem. Lett.* **2009**, *38*, 512−517. (b) Obika, S.; Rahman, S. M. A.; Fujisaka, A.; Kawada, Y.; Baba, T.; Imanishi, T. *Heterocycles* **2010**, *81*, 1347−1392.

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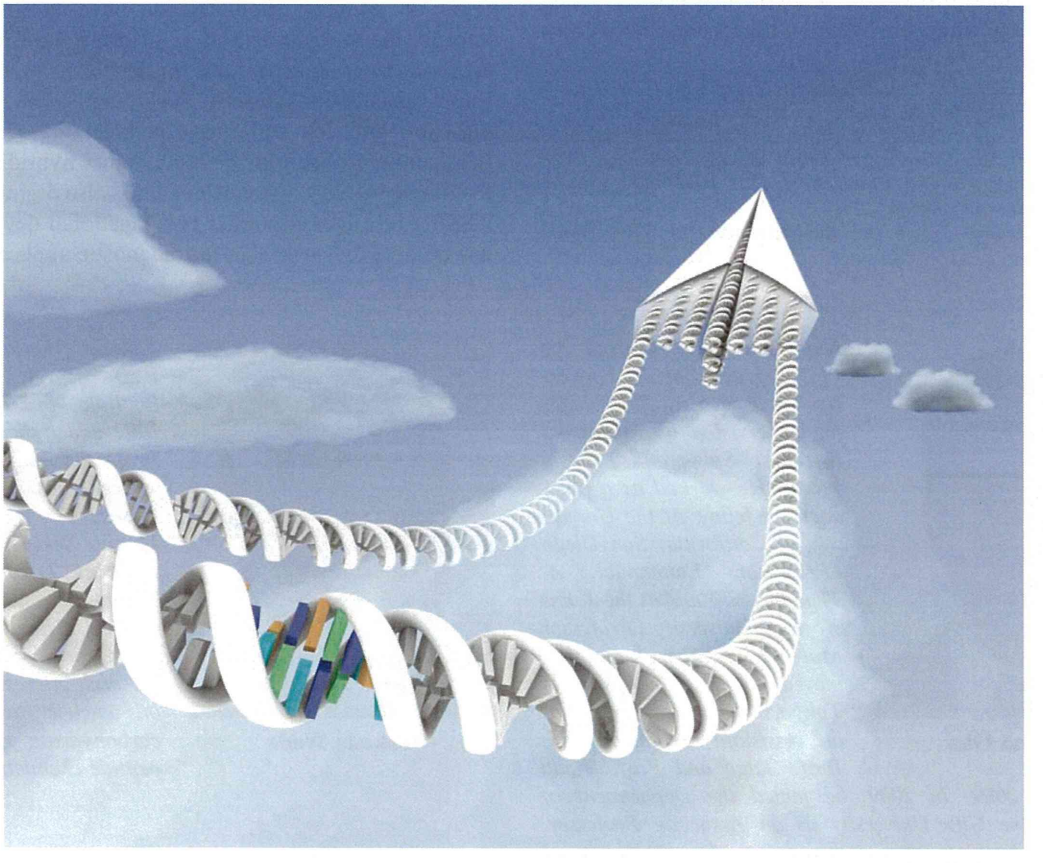

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CRITICAL REVIEW

Stereocontrolled synthesis of oligonucleotide analogs containing chiral internucleotidic phosphorus atoms†

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Oligonucleotides, in which one of the two nonbridging oxygen atoms of internucleotidic phosphates is replaced by a different type of atom or a substituent, are useful as therapeutic agents and probes to elucidate mechanisms of enzymatic reactions. The internucleotidic phosphorus atoms of these oligonucleotides are chiral, and the properties of these oligonucleotides are affected by the absolute configuration of the chiral phosphorus atoms. In order to address the issue of chirality, various methods have been developed to synthesize these *P*-chiral oligonucleotide analogs in a stereocontrolled manner. This *critical review* focuses on the recent progress in this field (123 references).

1. Introduction

Oligonucleotides form rigid double helices in a predictable manner through A–T(U) and G–C base pairings. Because of this capability, oligonucleotides have found numerous applications, such as in gene function analysis[1] and gene-targeting therapy,[2,3] both of which are based on the specific knockdown of target gene expression, genetic diagnosis,[4,5] and assembly of nanostructures.[6–9] However, because of some undesirable properties, such as susceptibility to nucleases and poor cell membrane permeability, natural oligonucleotides are not suitable for many of these applications. Therefore, various types of chemical modifications have been developed to improve their properties and/or integrate new functions, which are desirable or indispensable for the above applications.[4,10–14] Improvements in property, including stability to nuclease digestion and cell membrane permeability, are needed for therapeutic applications,[13,14] and higher hybridization affinity to complementary oligonucleotides is also desirable. Chemical labeling of oligonucleotides is a functional development that has become indispensable for diagnostic applications.[4]

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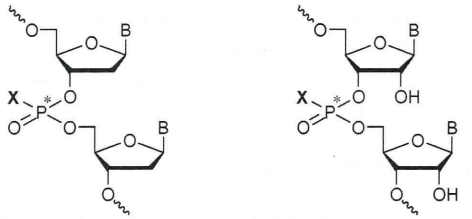


Fig. 1 Oligodeoxyribonucleotide and oligoribonucleotide analogs containing chiral internucleotidic phosphorus atoms. B = nucleobase, X = Me, S^-, Se^-, NR^1R^2, BH_3^-, H, *etc.*

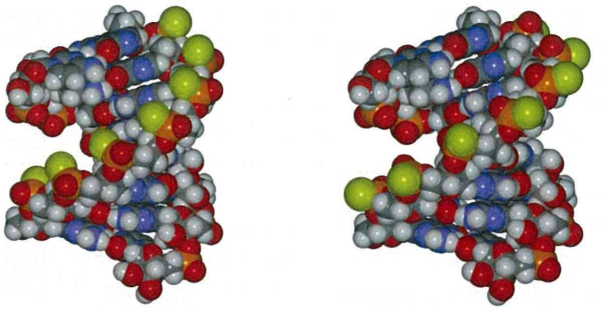


Fig. 2 NMR structure of all-(*Rp*)-PS-5′-d[GCGTCAGG]-3′·3′-r[CGCAGUCC]-5′ (left)[30] and model structure of all-(*Sp*)-PS-5′-d[GCGTCAGG]-3′·3′-r[CGCAGUCC]-5′ in which internucleotidic nonbridging oxygen and sulfur atoms are exchanged (right). Black, carbon; blue, nitrogen; orange, phosphorus; red, oxygen; white, hydrogen; yellow, sulfur.

Because some of the properties of natural oligonucleotides, such as good aqueous solubility and the ability to form sequence-specific duplexes, are necessary for their applications, an ideal modification should improve their properties or expand their functional capabilities without compromising their inherent advantages. To achieve this objective, one approach is to minimize structural changes to the molecule. Replacement of one of the two nonbridging oxygen atoms of internucleotidic phosphate diesters[15–22] is suitable in this regard. Moreover, such modifications greatly improve the stability of the resulting derivatives to nuclease digestion.

Various oligonucleotide analogs with such modified phosphates have been developed to date. Representative analogs are shown in Fig. 1. Methylphosphonate (X = Me)[15,19] and phosphorothioate (X = S^-)[17,20,22] are among the oldest examples and have been extensively studied for gene-targeting therapeutic applications. In particular, the latter has significantly improved cellular uptake and stability to nucleases while maintaining water solubility and the ability to form duplexes with strict base sequence specificity. Therefore, phosphorothioates have been incorporated into many of the therapeutic oligonucleotides in clinical trials, including the one which has been approved for marketing.[3,13,23] In addition to their therapeutic applications, phosphorothioate analogs as well as methylphosphonates have been used as probes for exploring the functions of internucleotidic phosphates in enzymatic reactions.[10,21] Moreover, the P–S^- group is potentially useful for further modifications of oligonucleotides through S-alkylation.[10] Phosphoroselenoates (X = Se^-) have attracted attention with respect to their applicability to multiwavelength anomalous diffraction phasing for X-ray structural studies.[24,25] Phosphoramidates (X = NR^1R^2), which can be synthesized from an *H*-phosphonate derivative (X = H) *via* the Atherton–Todd reaction,[26–28] are useful for the preparation of a variety of oligonucleotide analogs by applying various primary and secondary amines to this reaction. Boranophosphates (X = BH_3^-)[29] have been developed relatively recently and have been reported to show exceptional stability to nucleases compared to the corresponding phosphorothioates, and short interfering RNAs having this type of modification have shown a promising RNA interference activity. Boranophosphate is also expected to be less cytotoxic than the corresponding phosphorothioate.[29] *H*-Phosphonates (X = H) function as precursors of a variety of phosphate-modified oligonucleotide analogs, including those shown in Fig. 1, although the *H*-phosphonate itself is not suitable for the applications mentioned above because of its instability.[26]

The oligonucleotide analogs represented in Fig. 1 have chiral centers on their phosphorus atoms. When they form double helices with complementary oligonucleotides, the substituents on the chiral phosphorus atoms are oriented either "inward" toward the major groove or "outward" from the duplex depending on the absolute configuration of each phosphorus atom[16] (Fig. 2).[30] As a result, the properties of their duplexes with complementary oligonucleotides, such as thermal stability, are affected by the configuration of the phosphorus atoms. For this reason, their stereocontrolled synthesis has been a subject of great importance over the past few decades.[16–21] This *critical review* covers the recent progress on this subject.

2. Diastereomeric separation by chromatography

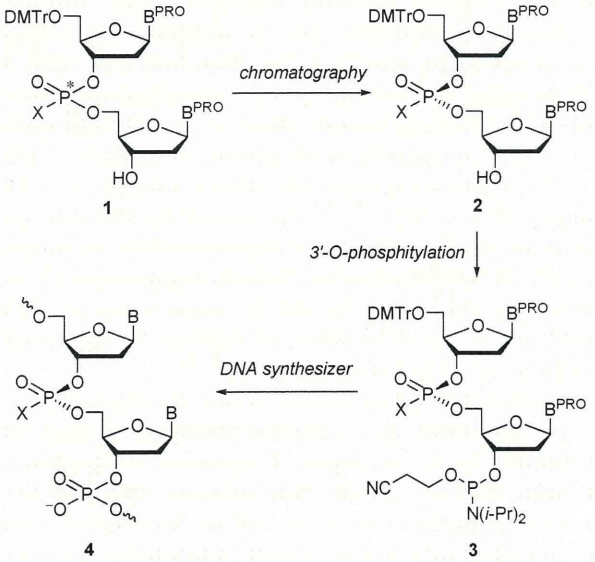
The problems introduced by the chirality of phosphorus atoms have been recognized from the early stages of research on *P*-chiral nucleotide analogs.[15] However, because diastereopure isomers of these analogs were unobtainable by stereocontrolled chemical synthesis, chromatographic separation has played an important role in obtaining stereodefined *P*-chiral nucleotide analogs. It has often been used since the 1970s to separate *P*-chiral dinucleoside phosphate analogs into (*Rp*)- and (*Sp*)-diastereomers, which in turn enabled studies such as their configurational assignment, comparative studies on their stability to nucleases, and affinity to single- or double-stranded DNA or RNA.[31–33] The method has also been used to separate the diastereomers of oligonucleotides containing a single chiral phosphorus atom. Purified diastereomers are used to probe the roles of pro-*Rp* and *Sp* oxygen atoms of specific phosphates in the stabilization of duplexes or higher-order structures and in enzymatic reactions.[34–36]

The advantage of this method is that the oligonucleotides can be synthesized in a nonstereocontrolled manner. The disadvantage is that the degree of separation is dependent on the target structure (length, base sequence, and position of the chiral phosphorus atom) as well as the conditions used for chromatography, and satisfactory separation is not always guaranteed.[37] Furthermore, separation of diastereomers becomes increasingly difficult as the number of chiral phosphorus atoms increases, because the number of diastereomers increases

exponentially. In other words, an oligonucleotide analog with n chiral phosphorus atoms would have 2^n different *P*-diastereomers. For example, an oligodeoxyribonucleoside phosphorothioate (PS-ODN) 10mer, which contains nine chiral phosphorus atoms, is obtained as a mixture of 2^9 (= 512) *P*-diastereomers by a nonstereocontrolled synthesis. The individual diastereomers are very similar to each other with respect to the retention time and thus practically inseparable. Therefore, in cases of oligonucleotide analogs with predominantly chiral phosphorus atoms, only short oligomers (generally up to 5mers) can be separated into their *P*-diastereomers even with high-performance liquid chromatography (HPLC).[38,39]

3. Use of diastereopure dimer building blocks

To obtain diastereopure *P*-chiral dinucleoside phosphate analogs by chromatography, fully deprotected 2mers are separated using reversed-phase HPLC in most cases, but those bearing protecting groups can be separated into diastereomers by either normal- or reversed-phase chromatography. When protected 2mers in which only the 3′-OH group is free are available in a diastereopure form, they can be derivatized into the corresponding 3′-phosphoramidite dimer building blocks, which are applicable to solid-phase syntheses of oligonucleotide analogs by the conventional phosphoramidite method (Scheme 1).[40,41] This method can produce oligonucleotides containing multiple stereodefined chiral phosphate analogs, which are difficult to obtain by chromatographic separation. Another advantage of this method is that a stereocontrolled synthetic method is unnecessary, although stereocontrolled reactions can also be used to synthesize the dimer building blocks. There are also several disadvantages: chromatographic separation is not always assured. Up to 32 types of dimer building blocks may be necessary to synthesize the desired oligonucleotides (four types of nucleosides for the 3′- and 5′-nucleosides and two *P*-diastereomers). The coupling efficiency of the dimer building blocks is generally lower than that of the regular monomer units. Oligonucleotides containing consecutive *P*-chiral nucleotides are not available.



Scheme 1 Synthesis of ODNs containing stereodefined *P*-chiral phosphate analogs **4** using diastereopure dimer building blocks **3**. B^{PRO} = protected nucleobase.

4. Enzymatic synthesis

Enzymatic synthesis has also been used since as early as the 1960s to obtain *P*-chiral oligonucleotide analogs.[16,18,24,25,42–48] Four types of *P*-chiral nucleotide analogs, including nucleoside 5′-phosphorothioates,[42–44] phosphoroselenoates,[24,25] methylphosphonates[45,46] and boranophosphates,[47,48] have been enzymatically incorporated into oligonucleotides. The enzymatic synthesis uses an enzyme, such as *E. coli* DNA polymerase I or T7 RNA polymerase, ribo- or 2′-deoxyribonucleoside 5′-triphosphates (rNTPs, dNTPs), and template and primer oligonucleotides. The enzyme extends the primer from the 5′- to the 3′-end by incorporating nucleotides in a template-dependent manner. To incorporate *P*-chiral nucleotide analogs into oligonucleotides, α-*P*-modified NTPs (Fig. 3) are used in place of natural NTPs. For example, oligodeoxyribonucleotides in which all of the thymidine 5′-phosphate residues are replaced by their phosphorothioate counterparts are synthesized by using α-*P*-thiothymidine 5′-triphosphate (TTPαS) in place of natural TTP in conjunction with the other three types of natural dNTPs. Fully modified PS-ODNs are synthesized by using four types of dNTPαSs. Unlike the approach employing chromatographic separation, enzymatic synthesis can provide oligonucleotides containing multiple *P*-chiral phosphate analogs as well as fully modified *P*-chiral oligonucleotides. The products are always diastereopure. Because stereocontrolled chemical syntheses of *P*-chiral oligonucleotide analogs still have some limitations as described below, particularly in the synthesis of long oligomers, an enzymatic synthesis may still be the method of choice to obtain stereoregulated oligonucleoside phosphorothioates, phosphoroselenoates, methylphosphonates, and boranophosphates longer than 10–30mers. However, the enzymatic synthesis has its own limitations: (1) the enzymes only use the (*Sp*)-isomers of NTPαSs, (*Rp*)-isomers of α-*P*-borano-NTPs and α-*P*-methyl-dNTPs as substrates. As a result, only (*Rp*)-phosphorothioate, (*Sp*)-boranophosphate, and (*Sp*)-methylphosphonate linkages can be incorporated into oligonucleotides. Surprisingly, both (*Rp*)- and (*Sp*)-α-*P*-seleno-TTPs (TTPαSes) are used as substrates by the Klenow fragment of *E. coli* DNA polymerase I, and both (*Rp*)- and (*Sp*)-phosphoroselenoate linkages can be incorporated into oligonucleotides using this system.[24] (It has been reported that the recognition of rATPαSe by T7 RNA polymerase was stereospecific, and only one isomer of rATPαSe was incorporated into oligoribonucleotides.[25]) (2) Modification sites cannot be freely selected

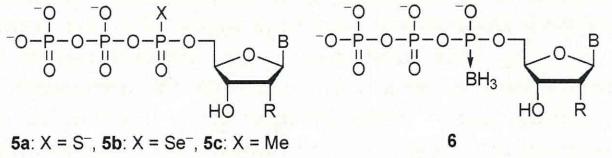


Fig. 3 α-*P*-Modified nucleoside 5′-triphosphates for incorporation of nucleoside phosphorothioate (**5a**), phosphoroselenoate (**5b**), methylphosphonate (**5c**) and boranophosphate (**6**).

because altered residues are universally incorporated, for example, the use of α-*P*-modified TTP results in complete replacement of thymidine 5′-phosphates throughout the oligomer. (3) Because of the substrate recognition specificity of the enzymes, only the above four types of *P*-chiral nucleotides can be incorporated at the current time. Base and sugar modifications are also limited. (4) The product is covalently linked to the primer. (5) The method is not suitable for large-scale syntheses. (6) Undesired byproducts, such as oversized transcripts, are concomitantly produced.[47,48]

5. Stereocontrolled chemical synthesis of *P*-chiral oligonucleotide analogs

As described above, *P*-chiral oligonucleotide analogs which can be prepared by the aforementioned methods are limited. For this reason, the stereocontrolled chemical synthesis of *P*-chiral oligonucleotide analogs has been extensively studied over the past few decades, and a variety of stereoselective and stereospecific reactions have been developed. This review classifies these studies into four categories as outlined below.

5.1. Stereoselective synthesis of *P*-chiral oligonucleotide analogs by utilizing the chirality of ribose

The chirality of ribose and deoxyribose moieties affects the stereoselectivity of the synthesis of *P*-chiral internucleotidic phosphate analogs. For instance, it has been reported that the *Rp* to *Sp* ratios of dinucleoside phosphorothioates are not exactly 50 : 50, but range from *Rp* : *Sp* = 63 : 37 to 43 : 57[38] even when they are synthesized with a "nonstereoselective" conventional phosphoramidite method.[49] This is due to the chirality of deoxyribose. Although this "stereoselectivity" is far from sufficient to synthesize stereoregulated *P*-chiral oligonucleotide analogs, a number of studies designed to enhance this selectivity have been reported to date. Since several reviews[16–18] have covered this topic, here we highlight only representative examples.

Ohtsuka *et al.* developed a 2,4,6-triisopropylbenzenesulfonyl tetrazolide derivative (Fig. 4, **7**) incorporating a 5-(2-pyridyl)tetrazole in place of the typical 1*H*-tetrazole as a nucleophilic catalyst. This condensing agent was used to synthesize dinucleoside phosphate derivatives *via* the phosphotriester method and only the (*Sp*)-isomers were obtained with complete stereoselectivity.[50] The 2-pyridyl group may stabilize the transition states yielding the (*Sp*)-diastereomers. Engels *et al.* designed new 2-trityl-4,5-dicyanoimidazole derivatives (**8**) and

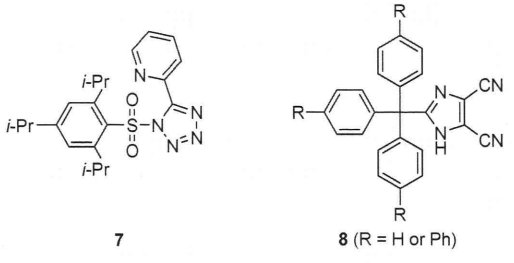


Fig. 4 New condensing agent **7** and azoles **8** used for stereoselective synthesis of *P*-chiral dinucleoside phosphate analogs.

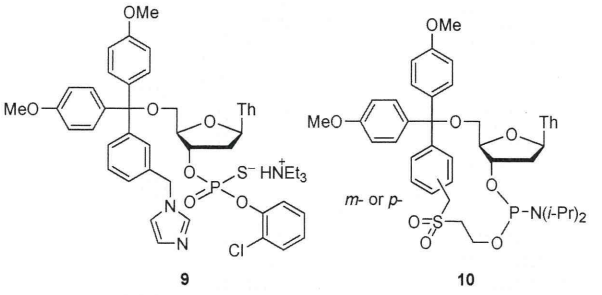
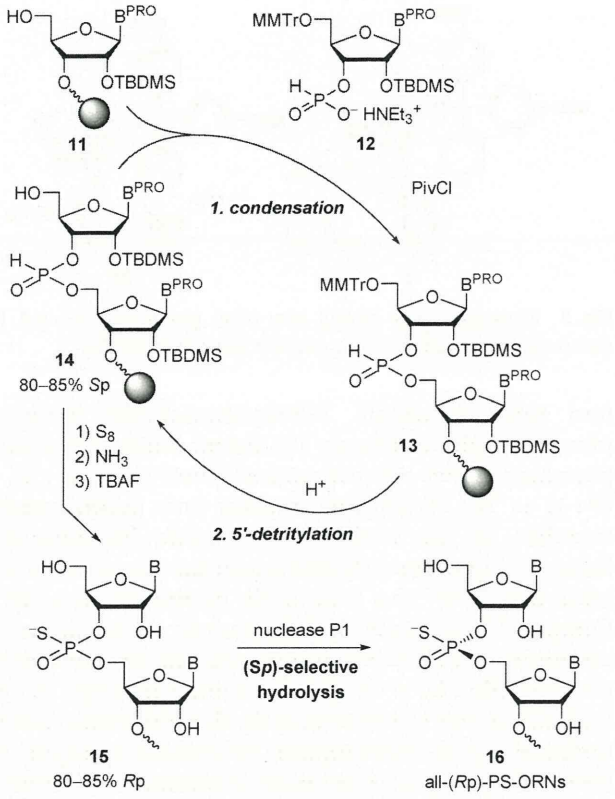


Fig. 5 Monomer units having new trityl group (**9**, **10**) used for stereoselective synthesis of dinucleoside phosphorothioates.

used these to activate 2′-deoxyribonucleoside 3′-methylphosphonamidite monomers. The desired dinucleoside methylphosphonates were obtained stereoselectively (from *Rp* : *Sp* = 89 : 11 to 76 : 24) probably resulting from dynamic kinetic resolution of the methylphosphonazolide intermediates.[51] Sekine *et al.* developed new trityl groups that have an imidazolyl group (Fig. 5, **9**)[52] or a linker to the phosphorus atom (**10**).[53] Compound **9** was used in the phosphotriester method to give the corresponding dithymidine phosphorothioate derivative stereoselectively (*Rp* : *Sp* = 18 : 82). The authors confirmed that the imidazolyl moiety was involved in the diastereoselection process (probably *via* an intramolecular nucleophilic attack on the phosphorus atom) by replacing the imidazolyl–dimethoxytrityl group with a traditional dimethoxytrityl group, resulting in an almost complete loss of stereoselectivity. Compound **10** was used to synthesize dithymidine phosphorothioate *via* the phosphoramidite method. The linker was readily removed from the product by treatment with ammonia. The stereoselectivity of the product varied from 50 : 50 to 86 : 14 depending on the structure of the linker and the activator used. The monomer having a standard dimethoxytrityl group showed little stereoselectivity. The authors attributed the stereoselectivity to the dynamic kinetic resolution of the phosphorazolide intermediate. Steric hindrance around the phosphorus atom of **10** probably hampers one of the two reaction paths.

It has been reported that the stereoselectivity of the synthesis of oligonucleoside *H*-phosphonates can be significantly enhanced by the existence of a bulky substituent at the 2′-position.[54,55] This enhancement of stereoselectivity enables a stereoselective synthesis of *P*-chiral oligoribonucleotide analogs, although the same strategy is not applicable to 2′-deoxyribonucleotide analogs. For example, Strömberg *et al.* developed a method to synthesize diastereopure all-(*Rp*)-oligoribonucleoside phosphorothioates (PS-ORNs) *via* the *H*-phosphonate method (Scheme 2).[55] The condensation of 2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4-monomethoxytrityl)-ribonucleoside 3′-*H*-phosphonate monomers **12** with the 5′-OH of a nucleoside on a solid support **11** and the subsequent 5′-detritylation step give protected oligoribonucleoside *H*-phosphonate **14** in a stereoselective manner (80%–85% *Sp*). Because the sulfurization of *H*-phosphonate diesters proceeds with retention of configuration (according to the Cahn–Ingold–Prelog rules, P–S$^-$ has the highest priority among the four substituents around the phosphorus atom, while P–H has the lowest priority), crude PS-ORN obtained from **14** is enriched with (*Rp*)-PS-linkages (**15**). Finally, crude **15** is treated with nuclease P1, which exclusively



Scheme 2 Synthesis of all-(*Rp*)-PS-ORNs by the *H*-phosphonate method.

hydrolyzes (*Sp*)-PS-diester linkages, to digest all the oligomers containing one or more (*Sp*)-PS-linkages so that the desired all-(*Rp*)-PS-ORN **16** is easily purified by HPLC.

Thus, the chirality of ribose sometimes produces a highly asymmetric environment. However, it is difficult to further enhance the stereoselectivity shown in these examples because the methodology offers little flexibility in designing chiral sources. More importantly, only one of the two *P*-isomers is available through these methods because of the homochirality of ribose.

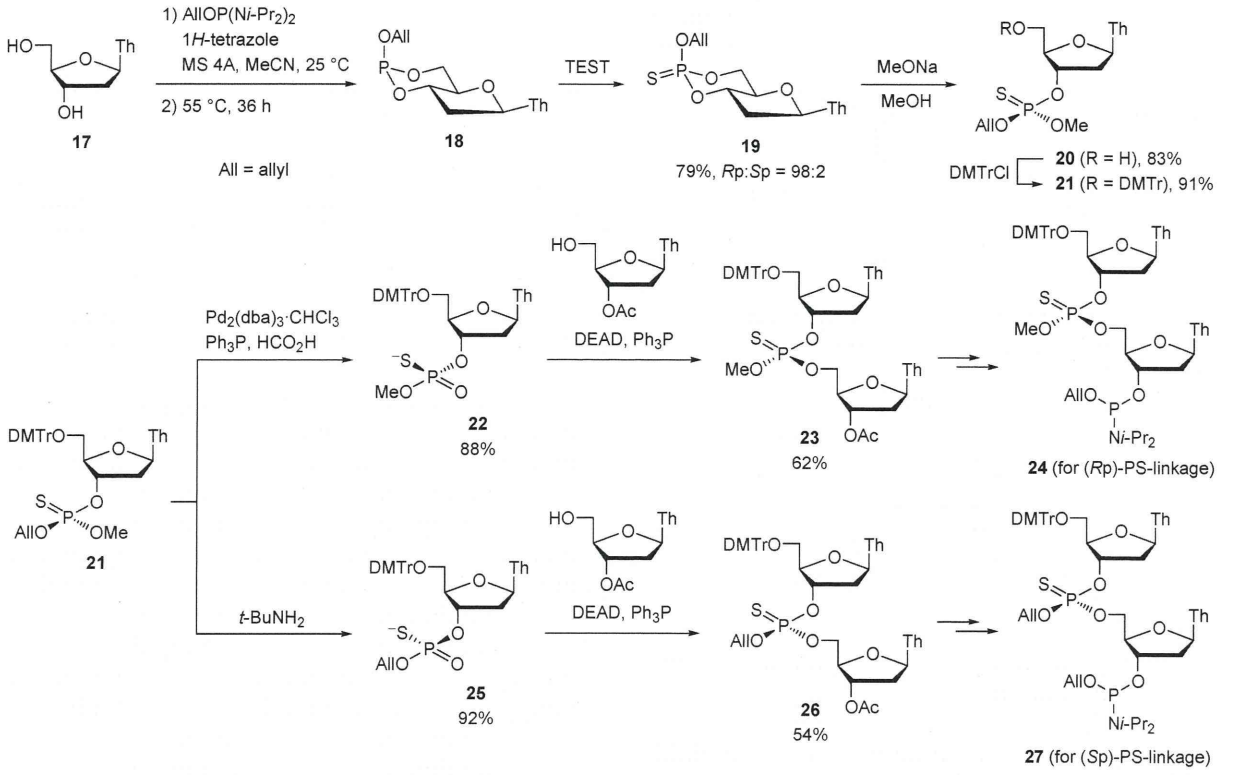
Recently, Hayakawa *et al.* reported a method to solve these problems, in which they successfully transferred the chirality of 2-deoxy-D-ribose to both (*Rp*)- and (*Sp*)-phosphorothioate internucleotide linkages (Scheme 3).[56] First, they synthesized thymidine 3′,5′-cyclic phosphite derivative **18** from thymidine **17** stereoselectively. The more thermodynamically stable isomer having the allyloxy group at the axial position was preferentially generated upon heating the reaction mixture. Subsequent sulfurization with bis[3-(triethoxysilyl)propyl] tetrasulfide (TEST) afforded the 3′,5′-cyclic phosphorothioate derivative **19** (*Rp* : *Sp* = 98 : 2). Treatment of **19** with MeONa preferentially cleaved the P–O-5′ bond to give the thymidine 3′-phosphorothioate triester derivative **20**. A small amount of the 5′-phosphorothioate counterpart was also generated because of P–O-3′ bond cleavage (*ca.* 10%), which was removed by chromatography. 5′-*O*-Dimethoxytritylation of **20** gave **21**. This compound functioned as a common intermediate to synthesize both (*Rp*)- and (*Sp*)-PS-internucleotidic linkages owing to the orthogonality of the allyl and methyl protecting groups. Thus, the allyl and methyl groups were selectively removed by using Pd(0) and *t*-BuNH$_2$, respectively, in a stereoretentive manner. Subsequent Mitsunobu reactions with 3′-*O*-acetyl-thymidine afforded the dithymidine phosphorothioate triesters **23** and **26**, which were converted into the corresponding 3′-phosphoramidite dimer building blocks **24** and **27** and used to incorporate (*Rp*)- and (*Sp*)-PS-linkages into ODNs, respectively. This method is advantageous compared to the other methods described in this section in that both (*Rp*)- and (*Sp*)-PS-linkages can be synthesized by using 2-deoxy-D-ribose as the sole chiral source. However, because the P–S$^-$ groups of **22** and **25** also work as a nucleophile in the Mitsunobu reaction, the yields of the desired products **23** and **26** are relatively low, and thus it is currently not practical to use this reaction for the chain elongation of PS-ODNs. Therefore, this method has the same limitations as those of the other methods using dimer building blocks.

5.2. Use of chiral activators

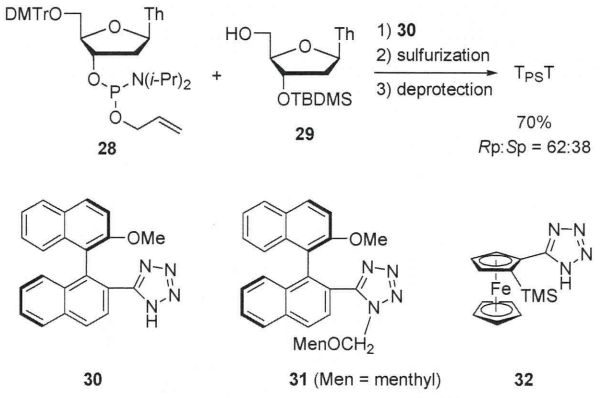
In order to develop a more efficient and versatile method that can equally incorporate both (*Rp*)- and (*Sp*)-*P*-chiral internucleotidic phosphate analogs at any position of oligonucleotides, stereoselective or stereospecific syntheses using chiral sources other than the ribose moieties have been studied. The general strategy described in this section uses nucleophilic acids with a chiral moiety to activate diastereomeric mixtures of monomers having a P^{III} atom. From the viewpoint of synthetic organic chemistry, this may be one of the best strategies if accomplished because diastereomixtures of monomer units can be easily prepared and the chiral components are not covalently linked to the substrates. Hence, additional steps for their introduction and removal are unnecessary. However, there have been only a few reports using this strategy, and all of them resulted in the synthesis of dinucleoside phosphate analogs with low to modest diastereoselectivity.[57,58] For example, Hayakawa *et al.* synthesized tetrazole derivatives having a chiral moiety (Scheme 4, **30–32**) and applied them to the synthesis of dithymidine phosphorothioate, but the level of diastereoselectivity was not sufficient.[57] Schell and Engels also synthesized a camphor-derived tetrazole derivative and used it to synthesize dinucleoside methylphosphonates, but the diastereomer ratio (dr) of the product only reached 64 : 36.[58] Thus, it is still difficult to induce high stereoselectivity with this strategy. It should be noted that this difficulty is not limited to the case of nucleotide analogs. In general, a highly stereoselective synthesis of *P*-chiral phosphite triesters has not yet been achieved without using a chiral auxiliary that is covalently bonded to the starting materials.[59] It is also worth noting that the effects of the chirality of ribose on stereoselectivity further complicate the design process in the case of nucleotide analogs.

5.3. Diastereomeric separation of monomer units and their application to the synthesis of stereoregulated *P*-chiral oligonucleotide analogs

As described in the previous section, there are continuing difficulties related to the development of an efficient method to synthesize stereoregulated *P*-chiral oligonucleotide analogs



Scheme 3 Synthesis of (*Rp*)- and (*Sp*)-phosphorothioate dimer building blocks (**24**, **27**) *via* common intermediate **21**.

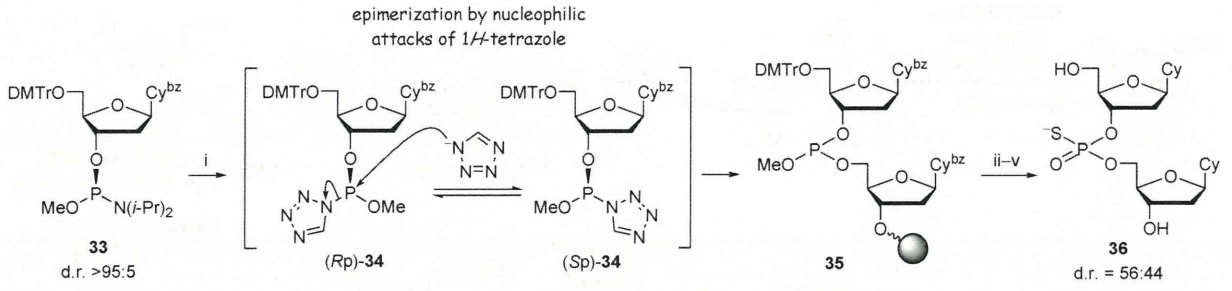


Scheme 4 Synthesis of dithymidine phosphorothioate with optically pure tetrazole derivatives **30–32** as activators.

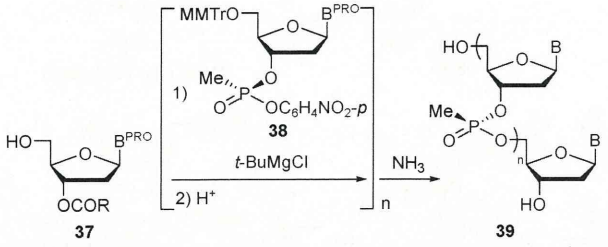
with a stereoselective reaction. In contrast, some reliable methods using stereospecific reactions have been developed in which diastereopure monomers are prepared, and individual isomers are synthetically incorporated into an oligonucleotide containing the corresponding *P*-chiral internucleotidic phosphate analogs without loss of diastereopurity. The diastereopure monomers are prepared by either chromatographic separation of diastereomixtures or stereoselective synthesis. This section highlights examples of the former approach.

As in the case of *P*-chiral dinucleoside phosphate analogs and their oligomers, nucleoside analogs bearing a chiral phosphorus atom have (*Rp*)- and (*Sp*)-diastereomers, and they can theoretically be separated by chromatography. In general, diastereomers have similar chromatographic retention times, and their separation requires a relatively long elution time. Therefore, it is necessary to use monomers with sufficient stability on stationary phase materials (typically silica gel). For this reason, monomers having a P^V atom have been employed in most cases as described below. Conventional nucleoside 3′-phosphoramidite monomers with a P^{III} atom can also be separated into *P*-diastereomers. However, it has been reported that the synthesis of a dinucleoside phosphorothioate using a separated phosphoramidite *P*-diastereomer in the presence of 1*H*-tetrazole was accompanied by nearly complete loss of *P*-diastereopurity.[60] This was attributed to repetitive attacks of 1*H*-tetrazole at the chiral phosphorus atom (Scheme 5).

In contrast, several types of P^V monomers that undergo a base-promoted stereospecific reaction have been developed. For example, nucleoside 3′-methylphosphonate derivatives having a *p*-nitrophenoxy group (Scheme 6, **38**) have been introduced by Stec *et al.* as monomer units for stereocontrolled synthesis of oligodeoxyribonucleoside methylphosphonates (PMe-ODNs) **39**.[19,61,62] Monomers **38** are synthesized nonstereoselectively and separated into (*Rp*)- and (*Sp*)-diastereomers by silica gel column chromatography. A *p*-nitrophenoxy group is displaced by a nucleoside that has been activated by *t*-BuMgCl to yield a methylphosphonate diester linkage stereospecifically. This method was used to synthesize all-(*Rp*)- and (*Sp*)-PMe-ODN 2–5mers in a solution phase. This method, which employs a *p*-nitrophenoxy group and *t*-BuMgCl,[63] was also used to synthesize short PS-ODN[64] and ORN[65] oligomers. Later, the same research group developed new monomers having a SMe[66] or SeMe[67] group instead of the *p*-nitrophenoxy group to synthesize PMe-ODN 5mers in a solution phase. The improved reactivity of these monomers



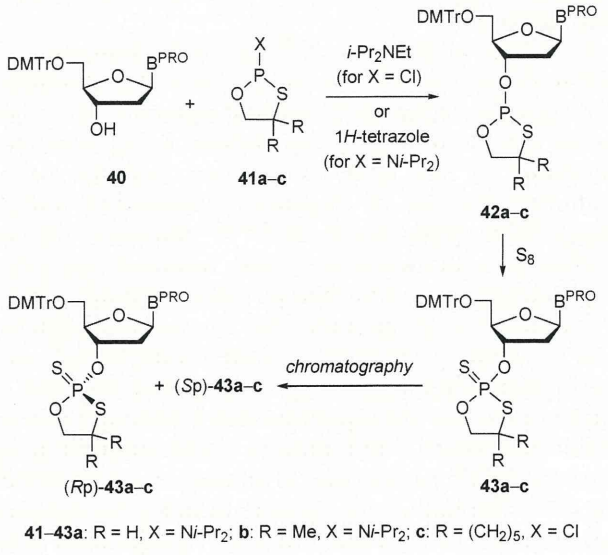
Scheme 5 Solid-phase synthesis of d[C_{PS}C] using diastereopure nucleoside 3′-phosphoramidite monomer and 1*H*-tetrazole. Reagents: (i) support-bound N^4-benzoyl-2′-deoxycytidine, 1*H*-tetrazole; (ii) S_8, 2,6-lutidine; (iii) 3% $CHCl_2CO_2H$ in CH_2Cl_2; (iv) PhSH, Et_3N; (v) NH_3 aq.



Scheme 6 Stereospecific synthesis of oligodeoxyribonucleoside methylphosphonates **39** using *t*-BuMgCl.

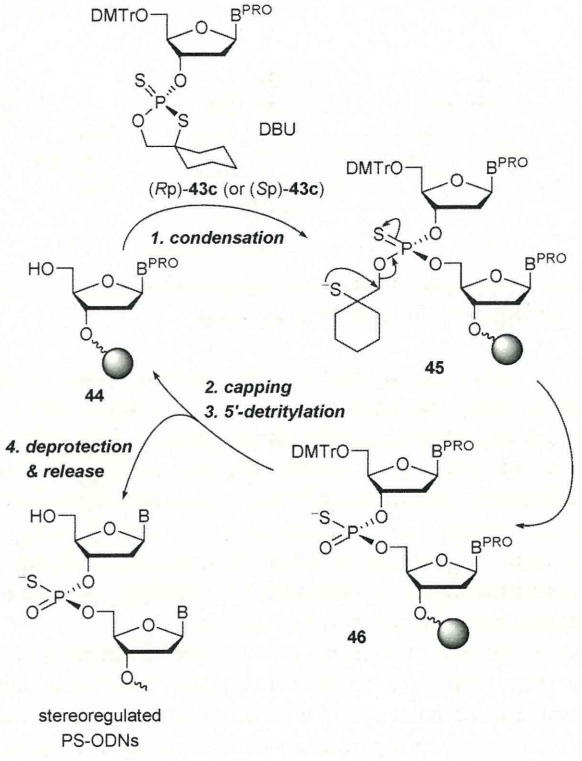
enabled the use of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) with LiCl in place of *t*-BuMgCl to promote the reaction. This reaction is completely stereospecific, but the application of this method is still limited to solution-phase syntheses, and only the synthesis of 2mers has been achieved on a solid support.[68] Further investigation of solid-phase synthesis is necessary to synthesize longer oligomers.

Stec *et al.* have also developed a method to synthesize *P*-chiral oligonucleotide analogs by using a DBU-promoted stereospecific reaction of nucleoside 3′-*O*-oxathiaphospholane monomers. (For more detailed reviews on the oxathiaphospholane method, see ref. 17, 69 and 70 by Stec *et al.*) Scheme 7 shows the synthesis of oxathiaphospholane monomers **43** used for the preparation of PS-ODNs from appropriately protected nucleosides **40** and 2-chloro- or 2-diisopropylamino-1,3,2-oxathiaphospholane derivatives **41a–c**. Nonstereoselectively synthesized monomers **43a–c** were separated into (*Rp*)- and (*Sp*)-isomers by silica gel column chromatography. The 4,4-pentamethylene group of the oxathiaphospholane ring of **43c** was introduced to enhance the asymmetry of the chiral phosphorus atom, resulting in easier chromatographic separation of diastereomers.[71] In fact, *ca.* 70% recovery of diastereopure (*Rp*)- and (*Sp*)-**43c** was achieved by a single passage through a silica gel column, which was a significant improvement compared to the separation of **43a,b**.[72–74]



Scheme 7 Synthesis of 2′-deoxyribonucleoside 3′-*O*-oxathiaphospholane monomers **43**.

Chain elongation of a PS-ODN is carried out by the nucleophilic substitution reaction of diastereopure oxathiaphospholane monomers **43a–c** with the 5′-OH of a support-bound nucleoside or an oligonucleotide **44** in the presence of a strong base (DBU, Scheme 8). The cleavage of the P–S bond by nucleophilic substitution and the subsequent elimination of an



Scheme 8 Synthetic cycle for stereoregulated PS-ODNs by the oxathiaphospholane method.

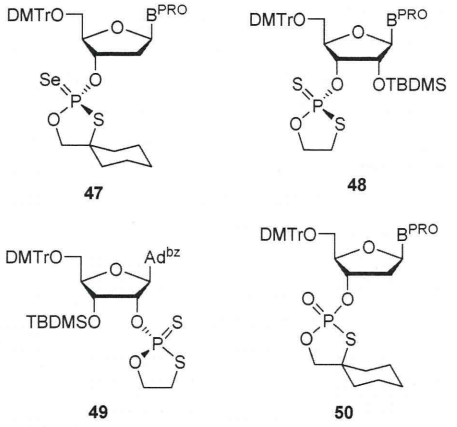


Fig. 6 Nucleoside oxathiaphospholane monomers for stereospecific introduction of 2′-deoxyribonucleoside phosphoroselenoate (**47**), ribonucleoside phosphorothioate (**48**), adenosine (2′–5′) phosphorothioate (**49**) and 2′-deoxyribonucleoside phosphate (**50**).

episulfide yielded the extended PS-ODN **46** stereospecifically. It has been confirmed that the reaction proceeds with retention of configuration. The chain elongation cycle consists of nucleophilic substitution, capping of unreacted 5′-OH by Ac_2O, and 5′-detritylation by dichloroacetic acid in CH_2Cl_2. The efficiency per cycle was typically 92%–96%. Deprotection and cleavage from the solid support can be carried out by standard treatment with ammonia. Synthesis of up to 28mer PS-ODNs was achieved.[71]

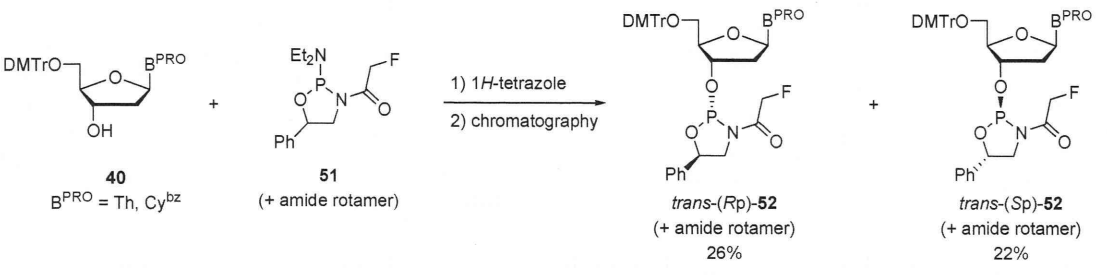
The oxathiaphospholane method is also applied to the synthesis of other *P*-chiral oligonucleotide analogs. The monomers used for these syntheses are shown in Fig. 6. These monomers can be used for stereospecific chain elongation of the corresponding *P*-chiral oligonucleotides through the same synthetic cycle. Stereoregulated oligodeoxyribonucleoside phosphoroselenoates (PSe-ODNs),[75] PS-ORNs,[76] and oligoadenosine (2′–5′) phosphorothioates[77] are synthesized by using **47**, **48**, and **49**, respectively. Monomer **50** has been developed to incorporate achiral phosphate diester linkages into PS-ODNs[71] so that stereoregulated chimeric PS/PO-ODNs can be synthesized by the same cycle. This is advantageous because the oxathiaphospholane method is not compatible with either the phosphoramidite or the *H*-phosphonate method generally used for incorporating these achiral phosphate monomers. The incompatibility is due to the sensitivity of phosphorothioate diester linkages of PS-ODN intermediates (*e.g.* **46** in Scheme 8) toward the oxidizing reagent (I_2–H_2O) used in these two methods.[71]

Thus, the oxathiaphospholane method can produce relatively long stereoregular *P*-chiral oligonucleotide analogs, particularly, PS-ODNs. The resultant oligomers have been used in many studies to identify the effects of the configuration of the phosphorus atoms on their physicochemical and biological properties.[71–84] However, the method has the following disadvantages: (1) chromatographic separation of diastereomixtures of monomers (*ca.* 1 : 1) into individual diastereopure monomers is required. (2) The efficiency of the DBU-promoted condensation is relatively low. As a result, in particular, the synthesis of PS-ORNs by using monomers **48** bearing a bulky 2′-*O*-TBDMS group is limited to 2mers.[76] (3) The method is not compatible with the most widely used phosphoramidite method because the two methods use completely different types of monomers and reactions. The sensitivity of the phosphorothioate diester linkages toward oxidizing reagents is another factor contributing to the incompatibility, as described above.

On the other hand, Beaucage *et al.* have reported the synthesis of stereoregulated PS-ODNs using 2′-deoxyribonucleoside 3′-*O*-(3-*N*-acyl-1,3,2-oxazaphospholidine) derivatives **52** as monomers, which bear a chiral P^{III} atom.[85] Monomers **52** were synthesized as shown in Scheme 9 from the appropriately protected nucleosides **40** and the 2-(diethylamino)-3-*N*-acyloxazaphospholidine derivative **51**, which was prepared from (±)-2-amino-1-phenylethanol. Diastereopure *trans*-(*Rp*)- and *trans*-(*Sp*)-**52**, in which the nucleoside moiety and the phenyl group are oriented on opposite sides of the oxazaphospholidine ring, were isolated from the resultant diastereomixtures by silica gel column chromatography. The isolated yields were relatively low because monomers **52** were unstable on silica gel and partially decomposed during purification.

Monomers **52** undergo a stereospecific condensation with the 5′-OH of a support-bound nucleoside or an oligonucleotide **53** in the presence of a strong base 1,1,3,3-tetramethylguanidine. The synthetic cycle for stereoregulated PS-ODNs using this stereospecific condensation is shown in Scheme 10. The resulting phosphite intermediate **54** is sulfurized with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[86] after capping the unreacted 5′-OH by Ac_2O to give the phosphorothioate triester intermediate **55**. The average coupling yield per cycle estimated by the $DMTr^+$ assay was *ca.* 98%. Deprotection of the phosphorothioate linkages can be carried out together with base deprotection and cleavage from the solid support by treatment with ammonia without affecting diastereopurity. All-(*Rp*)-$[T_{PS}]_{11}T$ and (*Rp*,*Sp*,*Rp*)-$d[C_{PS}C_{PS}C_{PS}C]$ were synthesized using this cycle.

It is worth noting that monomers **52** can be used to synthesize PS/PO-chimeric oligonucleotides by switching the sulfurization/oxidation (with *t*-BuOOH) steps. This is advantageous compared to the oxathiaphospholane method,



Scheme 9 Synthesis of diastereopure 2′-deoxyribonucleoside 3′-*O*-(3-*N*-acyl-1,3,2-oxazaphospholidine) monomers **52**.