JOC The Journal of Organic Chemistry

Featured Article

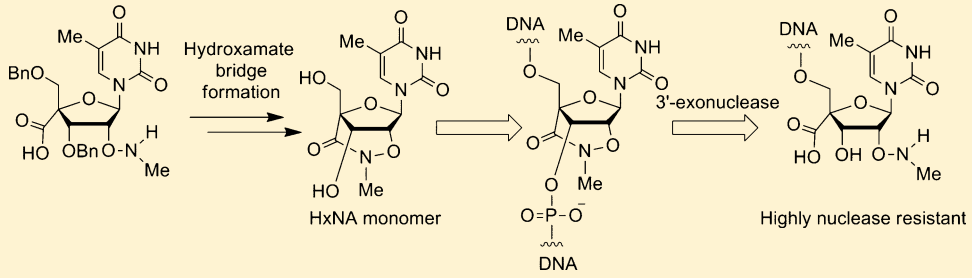
pubs.acs.org/joc

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

Ajaya R. Shrestha, Yoshiyuki Hari, Aiko Yahara, Takashi Osawa, and Satoshi Obika*

Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan

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^{NC}[27d] (**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.

Received: August 4, 2011
Published: November 7, 2011

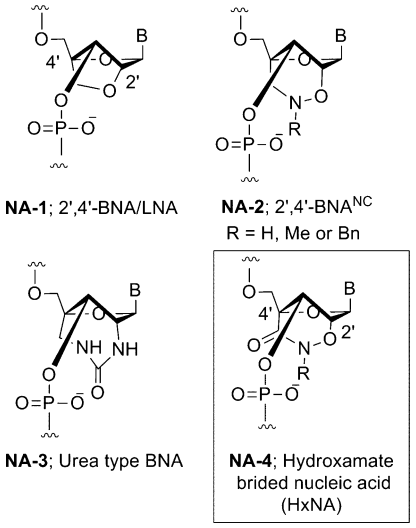


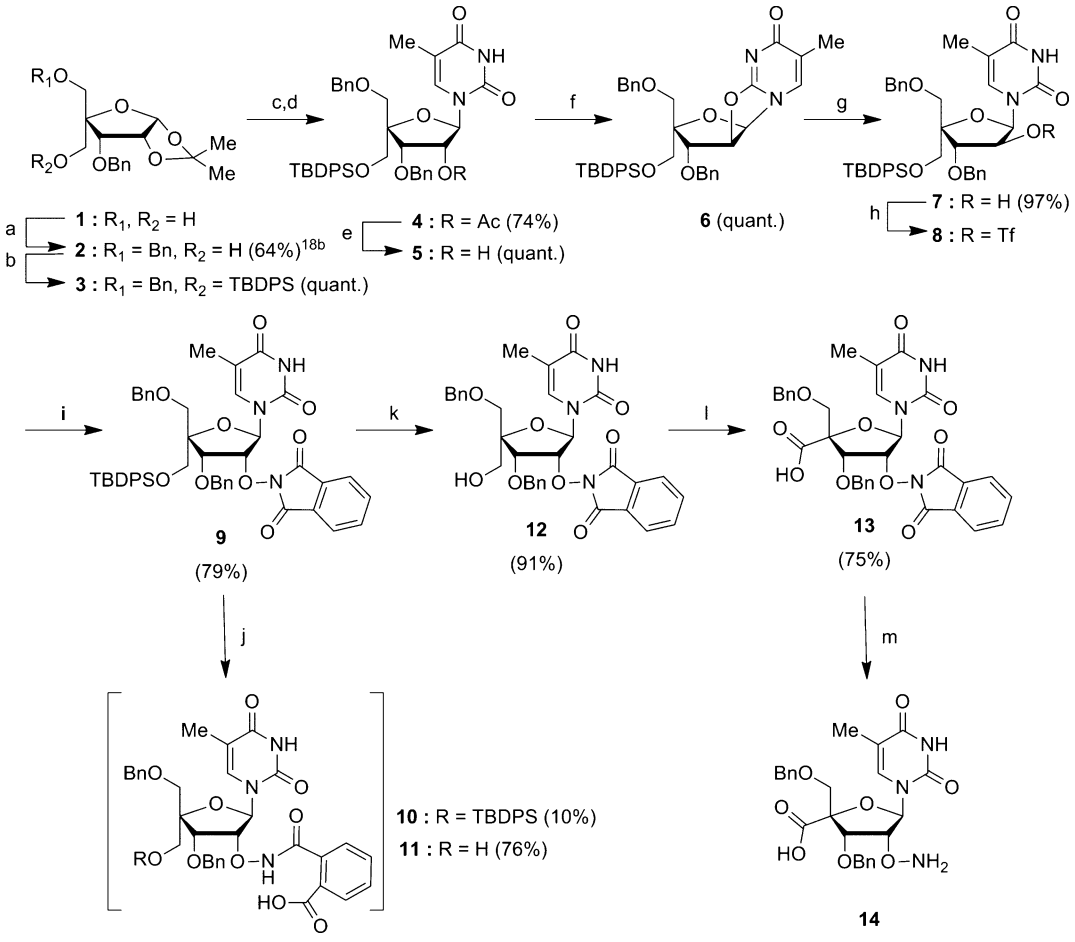
Figure 1. Structures of 2′,4′-bridged nucleic acids.

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.

dx.doi.org/10.1021/jo201597e | J. Org. Chem. 2011, 76, 9891–9899

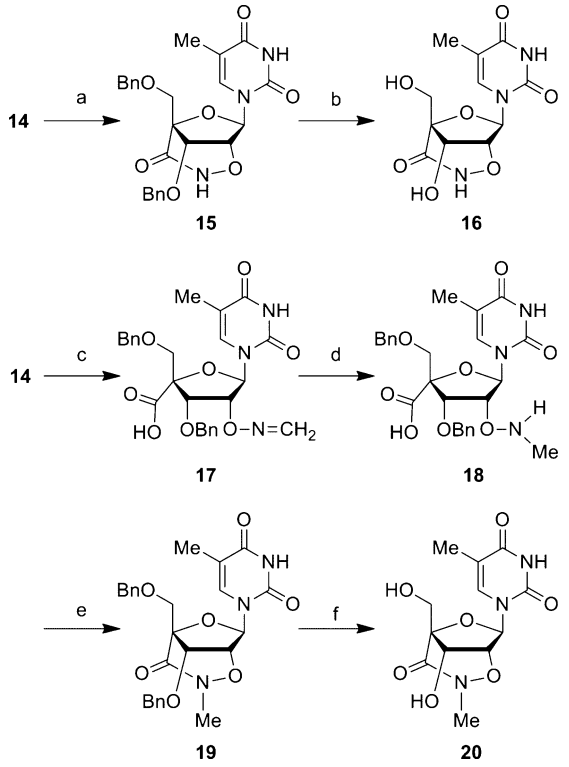
afford **3**. Acetolysis and acetylation of **3**, followed by stereoselective reaction with silylated thymine, afforded **4** in 74% yield. Subsequently, **4** was deacetylated by 40% methylamine to afford **5** in quantitative yield. The configuration of the 2′-carbon of **5** was successfully inverted 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**.

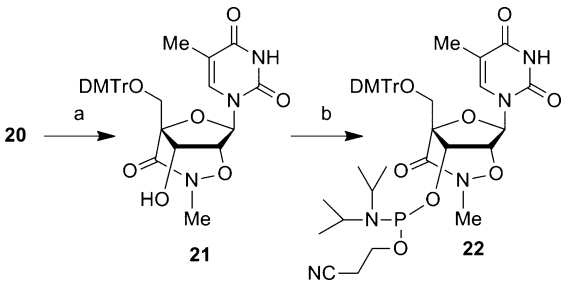
The [NH] derivative of HxNA, having an unsubstituted nitrogen in the bridge, was found to be less stable under basic conditions, and it was difficult to synthesize desired phosphoramidite monomer of the derivative. Therefore, we have used the *N*-substituted HxNA, i.e., HxNA[NMe], for the preparation 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

dx.doi.org/10.1021/jo201597e | J. Org. Chem. 2011, 76, 9891−9899

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(TTTTT**X**TTTT)-3′ (**24**)[b]	20 (+2)	12 (−7)	+8
5′-d(TTT**X**T**X**TTTT)-3′ (**25**)	24 (+3)	14 (−2.5)	+10
5′-d(TTT**X**T**X**T**X**TT)-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.

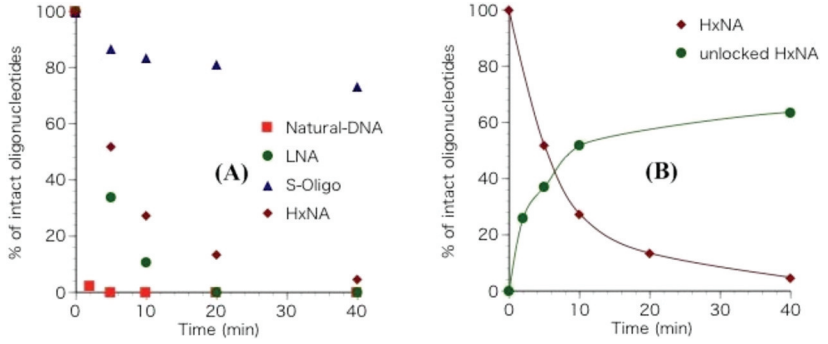


Figure 2. (A) Nuclease resistance of 5′-d(TTTTTTTTT**X**T)-3′ against CAVP. X = HxNA (maroon solid diamond) (**27a**); natural DNA-T (red solid square) (**23**); 2′,4′-BNA/LNA-T (green solid circle) (**27b**); phosphorothioate-T (blue solid triangle) (**27c**). Experiments were performed at 37 °C in 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(TTTTTTTTT**X**T)-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

dx.doi.org/10.1021/jo201597e | J. Org. Chem. 2011, 76, 9891–9899

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

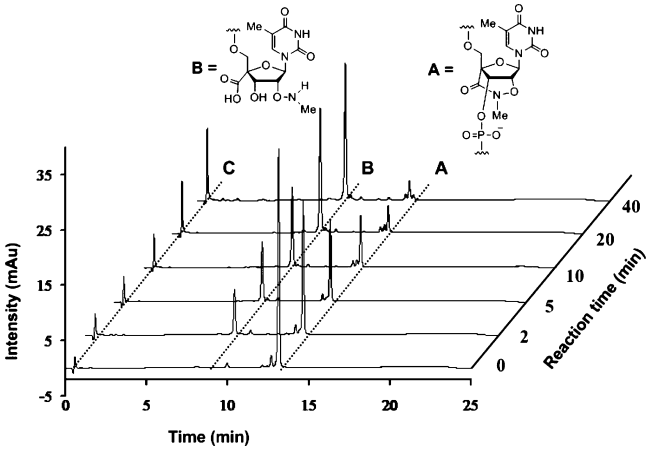


Figure 3. HPLC profile of the enzymatic degradation of HxNA-modified oligonucleotide **27a** with respect to time. Column: XBridge RP18 (3.0 × 50 mm). Mobile phase: Linear gradient of CH_3CN (7 to 14% over 20 min) in 0.1 M triethylammonium acetate (pH 7.0). Flow rate: 0.8 mL/min. Detection: absorbance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

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(TTT**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. ^{1}H 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 ^{1}H 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

dx.doi.org/10.1021/jo201597e | J. Org. Chem. 2011, 76, 9891–9899

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,

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}. ^{1}H 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}. ^{1}H 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}. ^{1}H 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}. ^{1}H 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}. ^{1}H 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,

dx.doi.org/10.1021/jo201597e | J. Org. Chem. 2011, 76, 9891−9899

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.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: obika@phs.osaka-u.ac.jp.

■ ACKNOWLEDGMENTS

A part of this work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Program to Disseminate Tenure Tracking System of the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) and a Grant-in-Aid for Scientific Research on Innovative Areas (No. 22136006) from MEXT.

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.