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Antisense Oligonucleotides Containing Conformationally Constrained 2′,4′-(*N*-Methoxy)aminomethylene and 2′,4′-Aminooxymethylene and 2′-*O*,4′-*C*-Aminomethylene Bridged Nucleoside Analogues Show Improved Potency in Animal Models

Thazha P. Prakash,* Andrew Siwkowski, Charles R. Allerson, Michael T. Migawa, Sam Lee, Hans J. Gaus, Chris Black, Punit P. Seth, Eric E. Swayze, and Balkrishen Bhat†

Department of Medicinal Chemistry and Antisense Core Research, Isis Pharmaceuticals Inc., 1896 Rutherford Road, Carlsbad, California 92008. †*Current address: Regulus Therapeutics, 1896 Rutherford Road, Carlsbad, California 92008.*

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To identify chemistries and strategies to improve the potency of MOE second generation ASOs, we have evaluated gapmer antisense oligonucleotides containing BNAs having N−O bonds. These modifications include *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, 2′,4′-BNA^{NC}[NMe], and 2′,4′-BNA^{NC} bridged nucleoside analogues. These modifications provided increased thermal stability and improved in vitro activity compared to the corresponding ASO containing the MOE modification. Additionally, ASOs containing *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, and 2′,4′-BNA^{NC}[NMe] modifications showed improved in vivo activity (> 5-fold) compared to MOE ASO. Importantly, toxicity parameters, such as AST, ALT, liver, kidney, and body weights, were found to be normal for *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, and 2′,4′-BNA^{NC}[NMe] ASO treated animals. The data generated in these experiments suggest that *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, and 2′,4′-BNA^{NC}[NMe] are useful modifications for applications in both antisense and other oligonucleotide based drug discovery efforts.

Introduction

ASOs[a] can modulate gene expression by binding to a specific mRNA through Watson−Crick base pairing. Upon binding, the oligonucleotide can modulate RNA processing, inhibit translation, or promote degradation. Mechanisms of degradation include recruitment of RNase H, which cleaves the RNA strand of a DNA−RNA duplexes, and activation of the RNAi pathway utilizing siRNA or shRNA.[1] Currently there is one approved antisense product[2] and multiple drugs employing various chemical designs in active development. The first generation of ASO therapeutics was 2′-DNA oligomers uniformly modified with the phosphorothioate backbone substitution which work predominantly through an RNase H dependent mechanism. The substitution of a sulfur for oxygen in the backbone ester increases stability to nucleolytic degradation and improves pharmacokinetics via increasing plasma protein binding and tissue uptake.[3,4]

To improve upon the first generation ASO drugs, many different modifications to the core nucleoside monomer unit of the ASO have been evaluated for their effects on affinity for complementary RNA, nuclease resistance, and ASO potency.[5−8] Most of the modifications that enhance affinity and nuclease resistance, in particular, the 2′-substituted nucleosides, also limit the ability of the ASO to support RNase H mediated cleavage of the targeted RNA.[9] Optimization of ASO design has led to the development of chimeric "gapmer" designs (Figure 1A) in which a central DNA region of 7−14 nucleotides is flanked on the 5′- and 3′-ends by two to six 2′-modifications.[10] The most advanced of these second generation antisense designs are MOE gapmer oligonucleotides.[11] MOE modified ASOs show increased affinity to a complementary RNA and are highly resistant toward degradation by nucleases.[12] These improvements resulted in a substantial (> 20-fold) increase in oligonucleotide potency in cell culture, relative to first generation ASOs.[13,14] MOE ASOs have been shown to possess both excellent pharmacokinetic properties[15−17] and robust pharmacological activity in animals[18,19,19−23] and in human clinical trials.[24] To date, MOE ASOs have an excellent safety record in human clinical trials.[24−26]

The improvement in potency of MOE ASOs has, in part, been attributed to the increased affinity for target mRNA

*To whom correspondence should be addressed. Phone: 760-603-2590. Fax: 760-603-3891. E-mail: tprakash@isisph.com.

[a] Abbreviations: EtOAc, ethyl acetate; DMAP, 4-di(methylamino)pyridine; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; DMTCl, 4,4′-dimethoxytrityl chloride; Nap, 2-(methyl)-naphthalene; rt, room temperature; ASO, antisense oligonucleotide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PS-DNA, DNA phosphorothioates; PTEN, phosphatase and tensin homologue; PBS, phosphate buffered saline; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; DMT, 4,4′-dimethoxytrityl ; MOE, 2′-*O*-(2-methoxyethyl); BNA, 2′,4′-bridged nucleic acid; LNA, locked nucleic acid; *N*-Me-aminooxy BNA, 2′-*N*-(methyl)-4′-*C*-aminooxymethylene 2′,4′-bridged nucleic acid; *N*-Me-aminooxy 2′,4′-bridged nucleic acid, 2′-*N*-(methyl)-4′-*C*-aminooxymethylene 2′,4′-bridged nucleic acid; 2′,4′-BNA^{NC} [NMe], 2′-*O*,4′-C-(*N*-methyl) aminomethylene 2′,4′-bridged nucleic acid; 2′,4′-BNA^{NC}, 2′-*O*,4′-C-aminomethylene 2′,4′-bridged nucleic acid; RNAi, RNA interference; siRNA, short RNA duplexes; shRNA, short hairpin RNA; Ms, methanesulfonyl; TBDPS, *tert*-butyldiphenylsilyl; TBDMS, *tert*-butyldimethylsilyl; Ura, uracil; bzCyt, N^4-benzoylcytosine.

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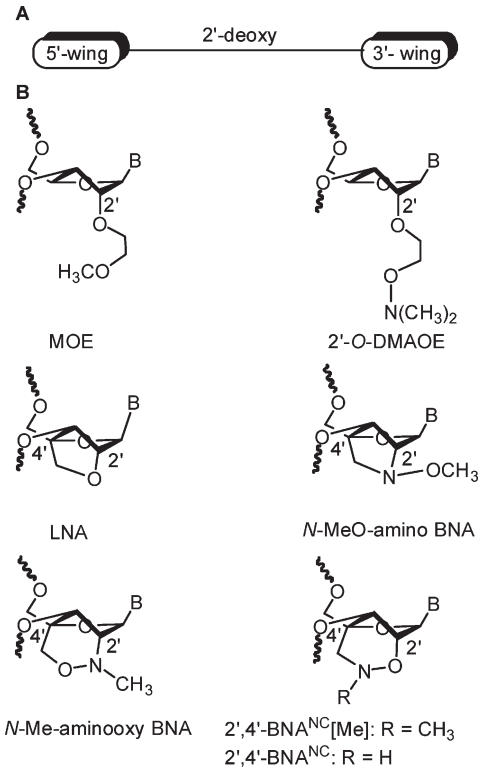


Figure 1. (A) Antisense oligonucleotides with gapmer design having 2′-modified "wings" at the 3′- and 5′-end flanking a central 2′-deoxy gap region for RNase H activity. (B) Chemical structures of MOE RNA, 2′-*O*-DMAOE RNA, and 2′,4′-linkage bridged nucleic acids.

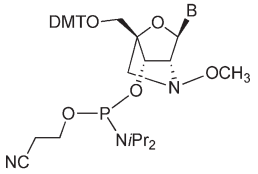
conferred by the MOE modification. While MOE provides a substantial improvement in affinity, bicyclic nucleoside modifications such as 2′,4′-methylene bridged nucleic acids[27,28] commonly called LNA[29,30] have been shown to provide a further increase in affinity. The improved binding affinity of these conformationally constrained oligonucleotides has been attributed to conformational preorganization and improved stacking.[29] The LNA containing chimeric ASOs have recently been shown to inhibit growth in human tumor xenograft models[31] and are entering early human clinical trials for oncology indications. In a comparative study of gapmer ASOs containing LNA and MOE modifications in the wings, it was demonstrated that LNA substitution improved the potency of some of the ASOs in animals.[32] However, this potency was unfortunately accompanied by an increase in the risk of hepatotoxicity.[32] This led us to explore structural analogues around the bicyclic nucleoside scaffold, which resulted in several modifications that showed a promising profile in early animal studies.[33,34]

Recently Rahman et al. reported that 2′-*O*-4′-*C*-aminomethylene bridged (Figure 1, 2′,4′-BNA^{NC}[NMe] and 2′,4′-BNA^{NC}) containing oligonucleotides have similar binding affinity for RNA and improved exonuclease stability compared to LNA.[35] However, there was no report of in vitro and in vivo activity of ASOs containing 2′,4′-BNA^{NC}[NMe] and 2′,4′-BNA^{NC} modifications. This class of BNA modification was particularly intriguing to us, as we have previously reported that ASOs containing 2′-*O*-DMAOE (Figure 1) and MOE modifications exhibited similar in vitro and in vivo activity and toxicity profiles.[36] As a part of our on going effort to identify BNA analogues with improved antisense properties, we prepared certain analogues reported by Rahman et al., as well as the related *N*-MeO-amino BNA (Figure 1) and *N*-Me-aminooxy BNA (Figure 1) bridged nucleic acid structures. These nucleosides incorporate elements of both 2′-*O*-DMAOE RNA and LNA, and we envisaged that introducing aminooxy functionality in the BNA scaffold could alter the hydration properties of nucleic acids. We rationalized that, similar to the MOE modification,[12] the altered hydration and increased steric bulk may improve the toxicity profile of ASOs containing these modifications. In this study we have prepared and evaluated the in vitro and in vivo activity of ASOs containing several "aminooxy" BNA modifications and compared their activities to LNA and MOE benchmarks.

Results and Discussion

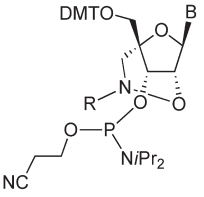
First, we developed efficient routes for the synthesis of *N*-MeO-aminomethylene and *N*-Me-aminooxymethylene bridged nucleosides and their 3′-phosphoramidites (structures shown in blocks 1 and 3). The 2′-*O*-4′-*C*-aminomethylene bridged nucleoside 3′-phoshoramidites (block 2) were synthesized according to reported procedures.[35] We also synthesized gapmer ASOs containing *N*-MeO-aminomethylene, *N*-Me-aminooxymethylene, and 2′-*O*-4′-*C*-aminomethylene bridged nucleoside residues. The hybridization properties of these ASOs with complementary RNA and in vitro and in vivo activities were investigated.

Block 1



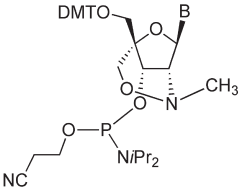
1: B = Ura, **2**: B = bzCyt

Block 2



16: B = Ura, R = H **17**: B = bzCyt, R = H
18: B = Ura, R = CH_3 **19**: B = bzCyt, R = CH_3

Block 3

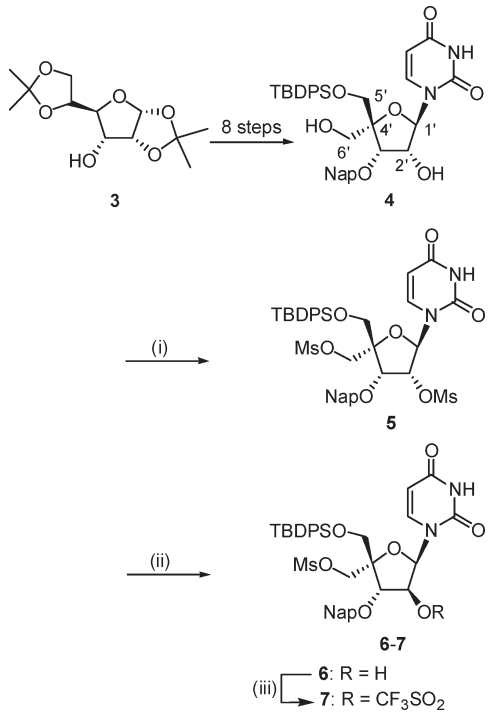


20: B = Ura, R = H **21**: B = bzCyt, R = H

Chemistry

1. Synthesis of *N*-MeO-amino BNA U and C Amidites 1 and 2. Synthesis of *N*-MeO-amino BNA U phosphoramidite **1** started from a known nucleoside (**4**, Scheme 1), which was synthesized from diacetone glucofuranose (**3**) in eight steps.[37] Compound **4** was treated with methanesulfonyl

Scheme 1[a]

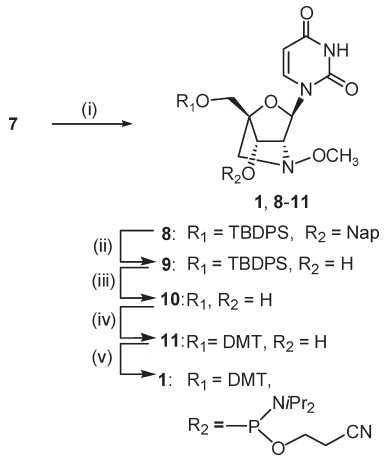


[a] (i) methanesulfonyl chloride, pyridine, rt, 93%; (ii) (a) CH_3CN, DBU; (b) 0.4 M NaOH H_2O/dioxane, rt, 85%; (iii) $(CF_3SO_2)_2O$, DMAP, CH_2Cl_2, −15 to −10 °C, 59.5%.

chloride in pyridine to afford bis-methanesulfonyl derivative **5** (93%), which was cyclized with DBU in acetonitrile to yield the 2,2′-anhydrouridine derivative. Opening of the 2,2′-anhydro ring on treatment with 0.4 M aqueous NaOH in dioxane for 45 min to 1 h gave arabino uridine derivative **6** in good yield (85%). It is noted that prolonged treatment with 0.4 M aqueous NaOH in dioxane resulted in cleavage of the TBDPS group. Reaction of **6** with triflic anhydride and DMAP in CH_2Cl_2 at −15 to −10 °C provided the desired nucleoside **7** in 60% yield.

An intermediate, such as **7**, could potentially lead to double substitution at C-2′ and C-6′ positions. We reasoned that treatment of nucleoside **7** with a highly reactive methoxyamine should first give nucleophilic substitution at the 2′-position to provide the methoxyamino derivative. This methoxyamino substituted nucleoside should initiate an intramolecular ring closing reaction and displace the leaving group at the 6′-position to provide the desired *N*-MeO-aminomethylene bridged nucleoside analogue. As expected, heating compound **7** with *N*-methoxyamine in the presence of *N*,*N*-diisopropylethylamine at 60 °C afforded *N*-methoxy-aminomethylene bridged uridine derivative **8** (Scheme 2) in 84% isolated yield. The side reaction resulting from nucleophilic substitution of *N*-methoxyamine at both C-2′ and C-6′ positions was not detected. This clearly suggests that once nucleophilic substitution occurs at either the C-2′ or C-6′ position with *N*-methoxyamine, spontaneous intramolecular ring closure occurs. High resolution mass spectral analysis and ^1H and ^{13}C NMR experiments were used to prove the structural identity of the cyclized nucleoside. We observed a significant upfield shift of the 6′-proton (2.94 ppm and 3.47) and 2′-proton (4.17 ppm) in the fused nucleoside **8** relative to nucleoside **7** (4.40 and 4.56 ppm 6′-proton and 5.50 ppm 2′-proton). This upfield shift is probably due to a change

Scheme 2[a]



[a] (i) *N*-Methoxyamine, DMF, *N*,*N*-diisopropylethylamine, 60 °C, 84%; (ii) 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, CH_2Cl_2, H_2O, rt, 85.5%; (iii) triethylamine trihydrofluoride, triethylamine, THF, 85%; (iv) DMTCl, pyridine, 99%; (v) 2-cyanoethyl-*N*,*N*,*N*′*N*′-tetraisopropyl-phosphorodiamidite, 1*H*-tetrazole, *N*-methylimidazole, DMF, 89%.

from the more electronegative 6′-*O*-methanesulfonyl or 2′-*O*-trifluoromethanesulfonyl group to the *N*-methoxyamino functionality. A similar upfield shift for C-6′ (61.4 ppm) and C-2′ (68.4 ppm) of compound **8** in the ^{13}C spectra was also observed confirming the structural connectivity of this constrained nucleoside. The appearance of resonances corresponding to the methyl moiety of *N*-MeO group also provided additional evidence for the formation of the bicyclic nucleoside **8**.

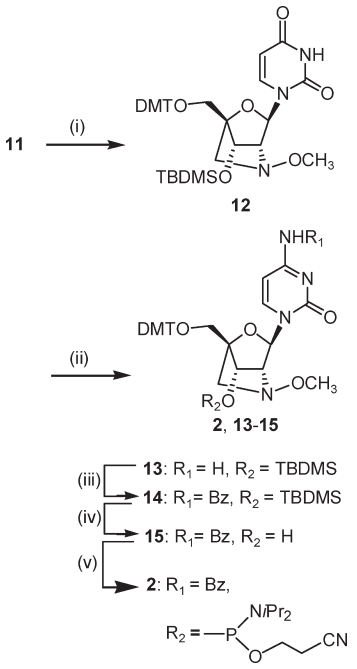
Compound **8** was then treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone to remove the Nap group from the 3′-position to yield compound **9** (86%). Subsequent treatment with triethylamine trihydrofluoride and triethylamine in THF gave fully deprotected constrained nucleoside **10** (85% yield). The 5′-position of compound **10** was then selectively protected with DMT group to yield compound **11**. Phosphitylation of compound **11** at the 3′-position with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of 1*H*-tetrazole and 1-methylimidazole in DMF afforded the corresponding phosphoramidite **1** in 89% yield.

The synthesis of the *N*-MeO-aminomethylene bridged bicyclic cytidine phosphoramidite **2** is illustrated in Scheme 3. Compound **11** on treatment with *tert*-butyldimethylsilyl chloride and imidazole in anhydrous DMF at room temperature yielded 3′-*O*-TBDMS derivative **12** (85% yield). The bicyclic uridine derivative (**12**) was converted into cytidine derivative (**13**, 95% yield) according to literature procedure.[38] The exocyclic amino group of bicyclic cytidine nucleoside (**13**) was protected with the benzoyl group on treatment with benzoic anhydride in anhydrous DMF, affording **14** (99% yield).[39] Compound **14** treated with triethylamine trihydrofluoride and triethylamine in THF to remove TBDMS group from 3′-position yielded **15**. Finally, phosphitylation of compound **15** at the 3′-position gave the phosphoramidite **2** (84%) in good overall yield.

2. Synthesis of 2′,4′-BNA^{NC} and 2′,4′-BNA^{NC}[NMe] U and C Amidites 16−19. The 2′,4′-BNA^{NC} and 2′,4′-BNA^{NC}-[NMe] uridine and cytidine phosphoramidites (**16**−**19**) were synthesized according to the reported procedures.[35]

3. Synthesis of *N*-Me-aminooxy BNA U and C Phosphoramidites 20 and 21. The *N*-Me-aminooxymethylene bridged

Scheme 3[a]

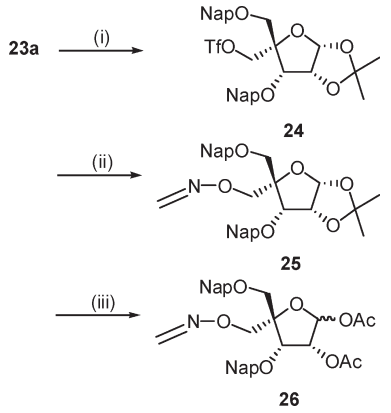


[a] (i) *tert*-Butyldimethylsilyl chloride, imidazole, DMF, rt, 85.4%; (ii) (a) triethylamine, 1,2,4-tetrazole, CH_3CN, $POCl_3$; (b) NH_4OH−dioxane, 95%; (iii) benzoic anhydride, DMF, rt, 99%; (iv) triethylamine trihydrofluoride, triethylamine, THF, rt, 91%; (v) 2-cyanoethyl-*N*,*N*,*N*′*N*′-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, *N*-methylimidazole, DMF, rt, 84%.

uridine phosphoramidite (**20**) was synthesized as described in Schemes 4−6. The 6′-methyleneaminooxy arabinonucleoside (**29**, Scheme 5), with a facile living group at the 2′-position, served as a key intermediate. Reduction of the 6′-methyleneaminooxy group at 6′-position of nucleoside **29** provides a very powerful nucleophile which should attack the 2′-position, provided the leaving group trajectory is positioned correctly.

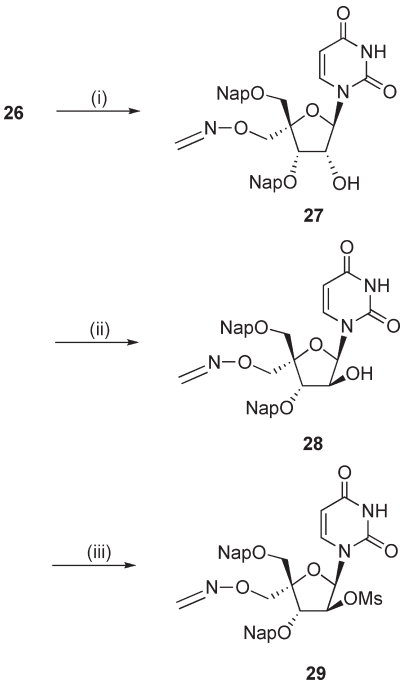
The synthesis started from a known sugar **22** (Scheme 8).[37] Compound **22** was treated with NaH and 2-(bromomethyl)-naphthalene to yield 3,5-*O*-bis(Nap) derivative **23a** (64%) as a major product (Scheme 8). The other regioisomer **23b** was also formed as a minor product (7%). The two isomers were separated by silica gel column chromatography. The 3,5-*O*-bis(Nap) sugar **23a** was converted to 6-*O*-trifluoromethanesulfonyl derivative **24** (Scheme 4, 88% yield). Sugar **24** was first subjected to a nucleophilic substitution reaction with *N*-hydroxyphthalimide and *N*,*N*-diisopropylethylamine in *N*,*N*-dimethylacetamide at 90 °C to afford the 6-phthalimido derivative. The 6-phthalimido derivative was stirred with *N*-methylhydrazene in CH_2Cl_2 at −10 to 0 °C to afford the 6-aminooxy sugar.[40] The byproduct, 2-methyl-1-oxophthalozine, was readily separated by filtration, and excess *N*-methylhydrazene was removed by evaporation. The 6-aminooxy sugar, on treatment with formaldehyde in methanol, afforded methyleneaminooxy derivative **25**. Attempted acetolysis of sugar **25** using acetic anhydride, acetic acid, and a catalytic amount of concentrated sulfuric acid resulted in the formation of an unidentified mixture of products. However, deprotection of the isopropyledine with strong acidic resin in a mixture of water and 1,4-dioxane proceeded smoothly to afford the 1,2-dihydroxysugar, which was subsequently treated with acetic

Scheme 4[a]



[a] (i) Trifluoromethanesulfonic anhydride, DMAP, pyridine, 88%; (ii) (a) *N*-hydroxyphthalimide, *N*,*N*-diisopropylethylamine, DMF, 80 °C; (b) *N*-methylhydrazine, CH_2Cl_2, −10 to 0 °C; (c) CH_3OH, formaldehyde (37 wt % solution in water), 46.1%; (iii) (a) Amberlite IR-120, dioxane, H_2O, rt; (b) acetic anhydride, pyridine, rt, 75%.

Scheme 5[a]

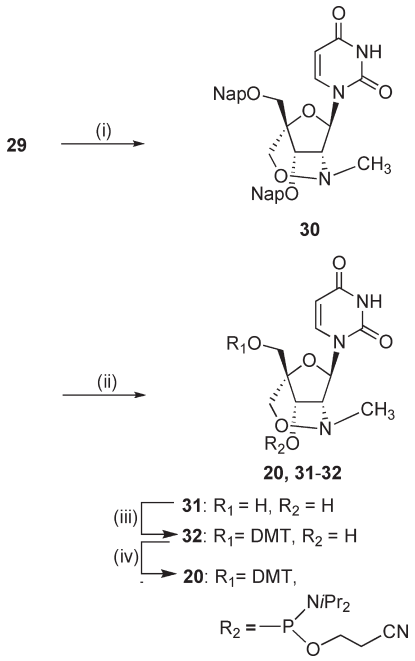


[a] (i) (a) Uracil, trimethylsilyl trifluoromethanesulfonate, bis(trimethylsilyl)acetamide, CH_3CN; (b) NH_3, CH_3OH, rt, 86%; (ii) (a) methanesulfonyl chloride, CH_2Cl_2, triethylamine, rt; (b) DBU, CH_3CN; (c) NaOH, dioxane, 58.9%; (iii) methanesulfonyl chloride, CH_2Cl_2, triethylamine, rt, 86%.

anhydride in pyridine to afford 1,2-*O*-bis-acetyl derivative **26** (75% yield) as an anomeric mixture.

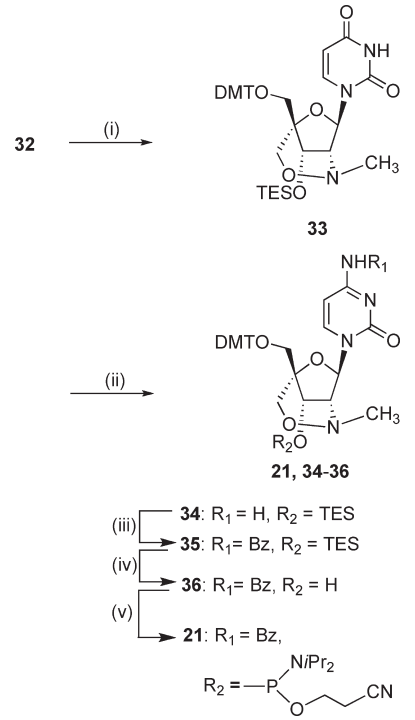
Acetylated sugar **26** was subjected to a modified Vorbrüggen[41] reaction using in situ silylated uridine mediated by trimethylsilyl trifluoromethanesulfonate to yield the desired 2-*O*-acetyl-β-D-ribofuranosyluridine derivative. The removal of the acetyl group was achieved by treatment with methanolic ammonia at room temperature to yield **27** (86% yield). Inversion of configuration of the 2′-hydroxyl group was accomplished in a three-step process. First, **27** was mesylated and then subsequently converted to the 2,2′-anhydro derivative on treatment with DBU in

Scheme 6[a]



[a] (i) (a) Pyridinium *p*-tolunesulfonate, CH_3OH, $NaBH_3CN$, rt; (b) *N*,*N*-diisopropylethylamine, *N*,*N*-dimethylacetamide, 80 °C, 84%; (ii) 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, CH_2Cl_2, H_2O, rt, 86%; (iii) DMTCl, pyridine, 79%; (iv) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, *N*-methylimidazole, DMF, 79%.

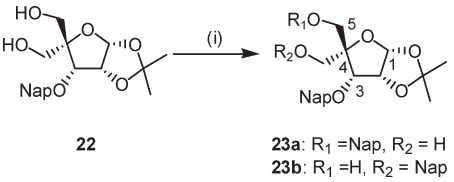
Scheme 7[a]



[a] (i) TESCl, imidazole, DMF, rt, 84%; (ii) (a) triethylamine, 1,2,4-tetrazole, CH_3CN, $POCl_3$; (b) NH_4OH−dioxane, 95%; (iii) benzoic anhydride, DMF, rt, 84%; (iv) triethylamine trihydrofluoride, triethylamine, THF, rt, 92%; (v) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, *N*-methylimidazole, DMF, rt, 87%.

acetonitrile. Opening of the 2,2′-anhydro derivative with aqueous NaOH in 1,4-dioxane gave arabino nucleoside derivative **28** (59% yield). Finally, compound **28**, on treatment with methanesulfonyl chloride and triethylamine in CH_2Cl_2, yielded compound **29** (86%).

Scheme 8[a]



[a] (i) 2-(Bromomethyl)naphthalene, NaH, DMF.

The key ring closure reaction was accomplished in a two-step process. First, the 6′-*N*-methyleneaminooxy derivative **29** was reduced with $NaBH_3CN$ in 1 M PPTS in MeOH.[40] The 6′-*N*-methylaminooxy derivative on heating at 80 °C in *N*,*N*-dimethyl acetamide and *N*,*N*-diisopropylethylamine underwent an intramolecular nucleophilic substitution at 2′-position, giving the desired bicyclic nucleoside **30** (Scheme 6) in good yield. The structure of the target nucleoside **30** was confirmed by NMR and mass spectrometry. The two Nap groups were removed on treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in CH_2Cl_2 and water to afford *N*-Me-aminooxymethylene bridged uridine **31**. The 5′-hydroxyl group of nucleoside **31** was then selectively protected with the DMT group to yield **32** (79%), which was subsequently phosphitylated at the 3′-position to afford phosphoramidite **20**.

The synthesis of the *N*-Me-aminooxymethylene bridged cytidine phosphoramidite **21** is illustrated in Scheme 7. The bicyclic nucleoside **32**, on treatment with triethylsilyl chloride and imidazole in DMF at ambient temperature, yielded 3′-*O*-triethylsilyl derivative **33**. Compound **33** was converted to the 4-(1,2,4-triazo-1-yl) derivative with 1,2,4-triazole, $POCl_3$, and Et_3N.[38] Subsequent treatment with aqueous NH_3 in dioxane at ambient temperature for 6 h gave the cytidine analogue **34** in 95% yield. The exocyclic amino group of **34** was protected with a benzoyl group on treatment with benzoic anhydride in anhydrous DMF, affording compound **35**.[39] The triethylsilyl group was removed with triethylamine trihydrofluoride and triethylamine in THF at ambient temperature to yield **36**. Finally, phosphitylation of compound **36** at the 3′-position gave the required phosphoramidite **21** (87% yield).

Antisense Oligonucleotides

To evaluate the effect of *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{NC}[NMe], and *N*-Me-aminooxy BNA modifications in antisense constructs, gapmer ASOs **41**−**44** (Table 1) targeting mouse PTEN mRNA expression[42] were designed. These sequences were derived from a previously identified 14-mer sequence.[34] For comparison, previously characterized[34,43] MOE gapmer ASOs **37** and **39** and LNA gapmers **40** and **45** were also evaluated (Table 1). Additionally, we also synthesized MOE modified control ASO **38** (Table 1).

1. Synthesis of PTEN Gapmer ASO Containing *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{NC}[NMe], and *N*-Me-aminooxy BNA. The MOE ASOs **37**−**39** and LNA ASOs **40** and **45** were synthesized according to literature procedure.[22,34] The ASOs **41**−**44** (Table 1) were synthesized on a solid phase DNA synthesizer using phosphoramidites **1**, **2**, **16**−**21**. The standard phosphoramidites were used for the incorporation of dA, T, dG, and dC nucleotides. A 0.1 M

Table 1. ASOs Containing LNA, MOE, *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{NC}[NMe], and *N*-Me-aminooxy BNA Nucleotide Residues[a]

compd	sequence (5′−3′)	chemistry	target
37	d($^{m}C_eT_eG_e{}^{m}C_eT_eA$ $G^{m}C^{m}CT^{m}CTGG$ $AT_eT_eT_e$ G_eA_e)3′	2′-*O*-MOE	PTEN
38	d($^{m}C_e{}^{m}C_eT_eT_e{}^{m}C_e{}^{m}C^{m}CTG$ A AGGTT $^{m}C_e{}^{m}C_e$ $T_e{}^{m}C_e{}^{m}C_e$)	2′-*O*-MOE	Control
39	d($C_eU_eTAGCACTGGCC_eU_e$)	2′-*O*-MOE	PTEN
40	d($^{m}C_LT_LTAGCACTGGC^{m}C_LT_L$)	LNA	PTEN
41	d($C_{B1}U_{B1}TAGCACTGGCC_{B1}U_{B1}$)3′	*N*-MeO-amino BNA	PTEN
42	d($C_{B2}U_{B2}TAGCACTGGCC_{B2}U_{B2}$)3′	2′,4′-BNA^{NC}	PTEN
43	d($C_{B3}U_{B3}TAGCACTGGCC_{B3}U_{B3}$)3′	2′,4′-BNA^{NC}[NMe]	PTEN
44	d($C_{B4}U_{B4}TAGCACTGGCC_{B4}U_{B4}$)3′	*N*-Me-aminooxy BNA	PTEN
45	d($C_LU_LTAGCACTGGCC_LU_L$)	LNA	PTEN
46	d(CTTAGCACTGGCCT)	DNA	PTEN

[a] All internucleosidic linkages are phosphorothioate. $^{m}C_L$ = LNA 5-methylcytidine. T_L = LNA thymidine. C_L = LNA cytidine. U_L = LNA uridine. C_e = 2′-*O*-MOE-cytidine. U_e = 2′-*O*-MOE-uridine. A_e = 2′-*O*-MOE-adenosine. G_e = 2′-*O*-MOE-guanosine. $^{m}C_e$ = 2′-*O*-MOE-5-methylcytidine. ^{m}C = 2′-deoxy-5-methylcytidine. C_{B1} = *N*-MeO-amino BNA cytidine. U_{B1} = *N*-MeO-amino BNA uridine. C_{B2} = 2′,4′-BNA^{NC} cytidine. U_{B2} = 2′,4′-BNA^{NC} uridine. C_{B3} = 2′,4′-BNA^{NC}[NMe] cytidine. U_{B3} = 2′,4′-BNA^{NC}[NMe] uridine. C_{B4} = *N*-Me-aminooxy BNA cytidine. U_{B4} = *N*-Me-aminooxy BNA uridine.

solution of the phosphoramidites in anhydrous acetonitrile was used for the synthesis of ASOs. A solution of phenylacetyl disulfide in 3-picoline and acetonitrile (1:1 v/v)[44] was used as sulfurizing reagent. "UnyLinker" solid support[45,46] was used for the synthesis of ASOs. It has been reported that during the deprotection, oligonucleotide phosphodiseters were released 1.2−1.7 times more quickly than the corresponding phosphorothioates from "UnyLinker" solid support.[45] Therefore, we used a phosphodiester linkage between "UnyLinker" solid support and the growing ASO to facilitate faster release of ASOs during deprotection.

In order to remove the cyanoethyl group from the phosphorothioate linkages, solid support bearing oligonucleotides were treated with 50% triethylamine in acetonitrile for 45 min.[47] Subsequently, solid supports were suspended in aqueous ammonia (28−30 wt %) and heated at 55 °C for 14 h to release the ASOs from the solid support and to remove all protecting groups from the exocyclic amino groups of the bases. The ASOs were then purified by HPLC on a strong anion exchange column and desalted using HPLC on a reverse phase column. The ASOs were characterized by HPLC coupled mass spectrometry (Supporting Information).

2. Thermal Denaturation Studies of *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{NC}[NMe], and *N*-Me-aminooxy BNA Modified ASOs RNA Duplexes. First, we investigated the effect of *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{NC}[NMe], and *N*-Me-aminooxy BNA modifications on binding affinity of gapmer ASOs to target RNA. We therefore determined the T_m of duplexes formed between ASOs **41**−**44** (Table 2) and 14-mer complementary RNA. For comparative analysis, the T_m of 2′-*O*-MOE modified ASOs **37** and 3**9** and LNA modified ASOs **40** (with 5-methyl C and T), **45** (with U and C), and DNA **46** (Table 2) were also determined. All BNA ASOs showed significantly higher T_m values relative to DNA phosphorothioates **46** (Table 2, 48.5 °C). The T_m values of the BNA modified ASOs were 4 to 7.3 °C higher than corresponding 14-mer MOE ASO **39** (Table 2, 51.3 °C). Comparison of the T_m values of ASOs containing BNA analogues and LNA suggested that the nature and position of the substituent have significant influence on the T_m values. *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, and 2′,4′-BNA^{NC}[NMe] ASOs (Table 2, **41**−**43**, 57.7−58.6 °C) showed similar T_m values. These T_m values were close to T_m of LNA ASO (Table 2, **45**, 58.0 °C).

These data suggested that BNA ASOs **41**−**43** and LNA ASO **45** were expected to have similar binding affinity to target mRNA. These data also suggested that hydrophilic substitutions (OMe or O) in the minor groove (as in *N*-MeO amino BNA **41** or 2′,4′-BNA^{NC} **42**) and hydrophobic substitution in the edge of the minor groove (Me in 2′,4′-BNA^{NC}[NMe] **43**) have no effect on the thermal stability of the BNA ASO/RNA duplex. In contrast, *N*-Me-aminooxy BNA ASO **44** T_m (Table 2, 55.4 °C) was approximately 2.6 °C lower than for 2′,4′-BNA^{NC} ASO **42**. These data suggest that hydrophobic substitutions, such a methyl group, in the minor groove have a destabilizing effect on the BNA ASO/RNA duplex. Similar observation was made recently with carb-LNA analogues.[48] Water surrounding the nucleic acids plays an important role in minimizing phosphate−phosphate electrostatic repulsion.[49] The lower *T*m of BNA ASO **44** with methyl substitution in the minor groove may be attributed to the decreased number of water molecules expected to be present around the hydrophobic methyl group compared to the hydrophilic OMe group or oxygen.

Table 2. *T*m, IC_{50}, and ED_{50} of LNA, MOE, *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{NC}[NMe], and *N*-Me-aminooxy BNA Containing ASOs[a]

compd	chemistry	T_m (°C)	IC_{50} (nM)	ED_{50} (mg/kg)
37	2′-*O*-MOE	67.9	1.0	10
39	2′-*O*-MOE	51.3	34.7	>45
40	LNA	60.6	1.3	3
41	*N*-MeO-amino	57.7	3.1	8
42	2′,4′-BNA^{NC}	57.7	3.2	>37
43	2′,4′-BNA^{NC}[NMe]	58.6	2.5	6
44	*N*-Me-aminooxy	55.4	3.4	11
45	LNA	58.0		
46	DNA	48.5		

[a] For T_m conditions, see Experimental Section.

The LNA ASO **40** showed a higher T_m (60.6 °C) but has 5-methyl LNA thymidine and 5-methyl LNA cytidine residues. It has been reported that 5-methyl substitution on the pyrimidine residues provide around 0.5 to 0.8 °C per residue enhancement in T_m relative to DNA.[50] The observed higher T_m value of **40** relative to **45** could be attributed to methylated cytidine LNA residues in **40** (Table 2). As expected 20-mer ASO **37** with 10 2′-*O*-MOE modified nucleotides exhibited higher T_m (Table 2, 67.9 °C) than 14-mer BNA ASOs.

In Vitro Studies

All the PTEN ASOs containing bicyclic nucleic acids (**40**−**44**, Table 2) reduced the expression of PTEN mRNA

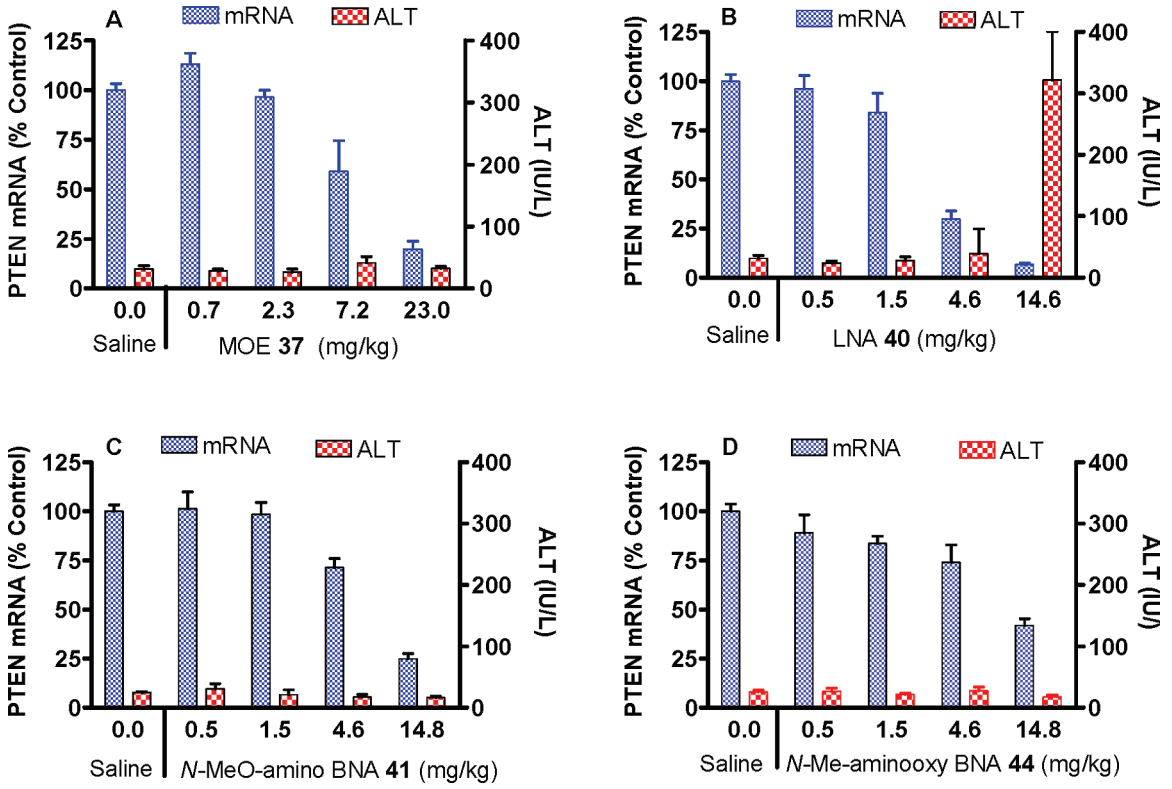


Figure 2. PTEN mRNA reduction in liver and ALT levels after 3-week multiple administration study. Mice (6-week-old male Balb/c) were injected (ip) twice a week for 3 weeks: 0.7, 2.3, 7.2, 23.0 mg kg^{-1} MOE ASO **37**; 0.5, 1.5, 4.5, 14.6 mg kg^{-1} LNA ASO **40**; 0.5, 1.5, 4.6, 14.8 mg kg^{-1} *N*-MeO-amino ASO **41** and *N*-Me-aminooxy ASO **44**. The animals were sacrificed 72 h after administration of the last dose, and ALT levels and liver mRNA were quantified. All errors are ±SD.

in a concentration-dependent manner when transfected into bEND.3 cells (Supporting Information). The IC_{50} values ranged from 1.3 to 3.4 nM (Table 2) for the BNA containing ASOs, and the observed potency was significantly higher than the corresponding MOE gapmer, ASO **39** (IC_{50} = 34.7 nM, Table 2). The T_m of the BNA ASOs in this study was significantly higher than that of the corresponding 14-mer MOE T_m. This roughly correlated with in vitro potency, as BNA ASOs **40**−**44** showed significantly improved potency relative to the lower T_m MOE ASO **39** (Table 2). The in vitro potency of 14-mer BNA ASOs was slightly lower than 20-mer MOE gapmer ASO **37** (IC_{50} = 1.0 nM, Table 2), which also has a higher T_m due to increased length and MOE content. The control ASOs **38** with MOE modifications did not reduce PTEN expression (Supporting Information). These data suggest that the intrinsic activity of short antisense oligonucleotides are improved on incorporation of conformationaly constrained bicyclic nucleotide residues relative to length matched MOE modified residues. This could in part be attributed to improved binding affinity of BNA ASOs to target mRNA.

Animal Studies

Next we evaluated the activity of MOE, *N*-MeO-amino BNA, 2′,4′-BNA^{NC}, 2′,4′-BNA^{NC}[NMe], *N*-Me-aminooxy BNA, and LNA ASOs in animal models. The ASOs containing *N*-MeO-amino BNA **41**, 2′,4′-BNA^{NC}[NMe] **43**, and *N*-Me-aminooxy BNA **44** reduced PTEN mRNA expression in a dose-dependent manner (Figures 2 and 3), whereas the 2′,4′-BNA^{NC} ASO **42** did not reduce the mRNA expression even at the highest dose tested (Supporting Information). The

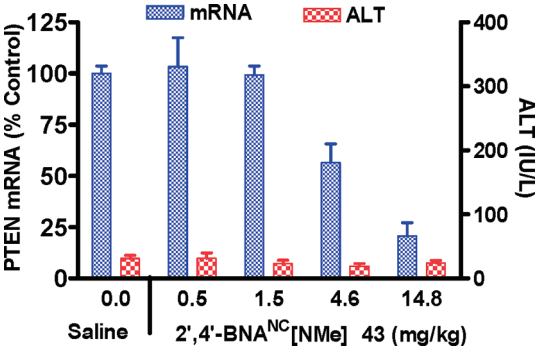


Figure 3. PTEN mRNA reduction in liver and ALT levels after 3-week multiple administration of 0.5, 1.5, 4.6, 14.8 mg kg^{-1} 2′,4′-BNA^{NC}[NMe] ASO **43**. The animals (6-week-old Balb/c mice) were sacrificed 72 h after administration of the last dose, and ALT levels and liver mRNA were quantified. All errors are ±SD.

N-MeO-amino BNA ASOs **41** (ED_{50} = 8 mg kg^{-1}, Table 2) and 2′,4′-BNA^{NC}[NMe] ASO **43** (ED_{50} = 6 mg kg^{-1}, Table 2) showed similar potency. In contrast, the *N*-Me-aminooxy BNA ASO **44** was slightly less potent (ED_{50} = 11 mg kg^{-1}, Table 2). The corresponding 14-mer MOE ASO was significantly less active (ED_{50} > 45 mg kg^{-1}, Table 2). The LNA ASO **40** was most active (ED_{50} = 3 mg kg^{-1}, Table 2) in this study. The potencies of the 14-mer LNA ASO **40**, the *N*-MeO-amino BNA ASO **41**, and the 2′,4′-BNA^{NC}[NMe] ASO **43** were all improved relative to the reference 20-mer MOE ASO **37** (ED_{50} = 10 mg kg^{-1}, Table 2). However, the 14-mer *N*-Me-aminooxy BNA ASO **44** demonstrated reduced in vivo potency relative to 20-mer MOE ASO **37** (Table 2). These data clearly suggest that the nature and position of substitution on

the BNA scaffold influence the in vivo activity of BNA modified ASOs, despite showing similar activity in cell culture.

It was interesting that the 2′,4′-BNA^{NC}[NMe] ASO **43** worked well in vivo while the corresponding 2′,4′-BNA^{NC} ASO **42** was inactive, despite both ASOs having similar potencies in cell culture (Table 2). In order to determine if this was a pharmacokinetic difference, we measured tissue concentration of ASO **42** in the liver of animals treated with the highest dose. To our surprise we could not detect any drug or metabolites in the tissues of animals treated with ASO **42**. In contrast, active ASOs such as the LNA **40** showed the expected levels of drug in liver tissue ($\sim$30 μg g^{-1} at the high dose). ASO **42** has reactive oxyamino functionality, and it is conceivable that it reacts with plasma protein components which prevent it from being delivered to tissues. The monosubstituted oxyamino functionality could also be rapidly metabolized and degraded in tissues. In any case, it is clear that pharmacokinetic differences explain the loss of activity for oxyamino ASO **42**.

In order to assess the toxicity of the ASOs, plasma transaminase levels of animals treated with MOE ASO **37**, LNA ASO **40**, *N*-MeO-amino **41**, 2′,4′-BNA^{NC}[NMe] ASO **43**, and *N*-Me-aminooxy ASO **44** were examined. The ALT levels were within the normal range for all animals (Figures 2 and 3) except LNA ASO **40** treated animals, which showed a modest elevation at the high dose group, consistent with our previous reports (ALT, Figure 2B). The liver, kidney, and body weights of the animals treated with MOE ASO **37**, *N*-MeO-amino BNA ASO **41**, 2′,4′-BNA^{NC}[NMe] ASO **43**, *N*-Me-aminooxy ASO **44**, and 2′-*O*-MOE ASO were normal at the doses used in this study. However, the spleen weights of the animals treated with *N*-MeO-amino BNA **41** and 2′,4′-BNA^{NC}[NMe] ASO **43** ASO were higher than saline treated animals, indicating a proinflamatory effect. The LNA ASO **40** increased liver weight and reduced body weight gain, and this was consistent with the modest ALT elevations we observed at the high dose and indicates an onset of liver toxicity.

Conclusions

Efficient methods for the synthesis of *N*-MeO-aminomethylene and *N*-Me-aminooxymethylene bridged uridine and cytidine nucleosides and their 3′-phosphoramidites were developed. The synthesis of *N*-MeO-aminomethylene bridged nucleoside includes a one pot nucleophilic substitution and intramolecular cyclization in very good yield. A convenient route to prepare 4′-(methyleneaminooxy)methyl nucleosides was also developed. A one pot intramolecular ring closure reaction involving reductive amination and nucleophilic substitution afforded the *N*-Me-aminooxymethylene bridged nucleoside. For comparative evaluation we synthesized 2′-*O*,4′-*C*-aminomethylene (2′,4′-BNA^{NC} and 2′,4′-BNA^{NC}-[NMe]) bridged nucleosides and corresponding phosphoramidites according to recently reported literature procedures.[35] The synthesis of ASOs containing *N*-MeO-aminomethylene and *N*-Me-aminooxymethylene bridged nucleotides was achieved using traditional solid phase DNA synthesis procedures.

N-MeO-amino BNA, 2′,4′-BNA^{NC}, and 2′,4′-BNA^{NC}-[NMe] containing ASOs (14-mer 2−10−2 gapmer) showed high affinity to target RNA, significantly higher than the corresponding MOE ASO but similar to the LNA ASO. The T_m of *N*-Me-aminooxy BNA ASO was higher than the corresponding MOE ASO but reduced compared to LNA ASO. The in vitro activities of *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, 2′,4′-BNA^{NC}, and 2′,4′-BNA^{NC}[NMe] containing ASOs were very similar and were significantly higher than those of the corresponding MOE ASOs. This is likely a result of the improved binding affinity for RNA targets due to the conformational constraint of the bicyclic nucleoside and is not unexpected. The in vivo results showed a separation of both potency and toxicities of the BNA containing ASOs. *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, and 2′,4′-BNA^{NC}-[NMe] containing ASOs showed significantly improved activity compared to the corresponding 14-mer MOE ASO but were 2- to 4-fold reduced compared to the LNA 14-mer. The 2′,4′-BNA^{NC} ASO was inactive because of a lack of accumulation in liver, presumably because of rapid metabolism and/or elimination. The in vivo activities of 14-mer (2−10−2 design) *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, and 2′,4′-BNA^{NC}[NMe] containing ASOs were similar or slightly improved relative to that of a 20-mer 5−10−5 MOE gapmer. Our study demonstrates that nucleoside modifications containing "aminooxy" functionality such as *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, and 2′,4′-BNA^{NC}[NMe] are potentially useful modifications for RNase H based antisense therapeutics. While they appear slightly less potent than LNA and recently reported carbon containing analogues,[34,51] they also appear to reduce the transaminase increases seen with the LNA ASO having the same sequence. Since the potency was also reduced, whether this translates into an increase in therapeutic index for the "aminooxy" containing ASOs is unknown and will have to be determined via extensive evaluation in multiple sequences and species.

Experimental Section

General Procedures. Solvents used were of anhydrous grade and were stored under nitrogen at all times. The 2′-deoxynucleoside phosphoramidites and reagents for oligonucleotide synthesis were procured form Glen Research Inc., VA. All other starting materials and reagents were purchased from Aldrich Chemical Co. and were used without further purification. Thin-layer chromatography was performed on precoated plates (silica gel 60 F254, EM Science, NJ) and visualized with UV light and spraying with a solution of *p*-anisaldehyde (6 mL), H_2SO_4 (8.3 mL), and CH_3COOH (2.5 mL) in C_2H_5OH (227 mL) followed by charring. ^{1}H NMR spectra were referenced using internal standard $(CH_3)_4Si$ and ^{31}P NMR spectra using external standard 85% H_3PO_4. Mass spectra were recorded by College of Chemistry, University of California, Berkeley, CA. The purities of all the compounds tested in biological assay were assessed by HPLC and capillary gel electrophorisis and were $\geq$95%.

5′-*O*-(DMT)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]uridine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite 1. A mixture of compound **11** (1.31 g, 2.22 mmol) and 1*H*-tetrazole (0.14 g, 2.00 mmol) was dried over P_2O_5 overnight under reduced pressure. To the solution of the mixture in anhydrous DMF (5.44 mL), 2-cyanoethyl-*N*,*N*,*N*′*N*′-tetraisopropylphosphorodiamidite (1.03 mL, 3.25 mmol) and 1-methylimidazole (0.052 mL, 0.65 mmol) were added. The reaction mixture was stirred at room temperature for 6 h under argon atmosphere. The reaction mixture was poured into EtOAc (50 mL), and the organic layer was washed with aqueous $NaHCO_3$ (5% by wt, 100 mL), brine (60 mL), dried (Na_2SO_4), and evaporated. The residue was purified by silica gel column chromatography (1:1 EtOAc/hexane) to yield compound **1** (1.57 g, 89% yield) as a white foam. ^{31}P NMR (121 MHz, $CDCl_3$) δ 148.57, 148.00; HRMS (FAB) calcd for $C_{41}H_{51}N_5O_9P^+$ 788.3424, found 788.3428.

N^4-Benzoyl-5′-*O*-(DMT)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene)cytidine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite 2. Compound **2** (1.17 g, 84% yield) was synthesized from compound **15** (1.08 g, 1.57 mmol), 1*H*-tetrazole (0.1 g, 1.4 mmol), DMF (4.3 mL), 2-cyanoethyl-*N*,*N*,*N*′*N*′-tetraisopropylphosphorodiamidite (0.75 mL, 2.35 mmol), and 1-methylimidazole (0.032 mL, 0.47 mmol) according to the procedure used for the synthesis of compound **1**. ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.91, 149.00; HRMS (TOF MS ES) calcd for $C_{48}H_{56}N_6O_6P^+$ 891.3846, found 891.3832.

2-*O*-(Methanesulfonyl)-4-*C*-(*O*-methanesulfonyl)hydroxymethyl-3-*O*-(Nap)-5-*O*-(TBDPS)-β-D-ribofuranosyluracil 5. Compound **4** (14 g, 21.46 mmol) was dried over P_2O_5 under reduced pressure. Methanesulfonyl chloride (7.51 mL, 96.68 mmol) was added to a cold (0 °C) solution of compound **4** in anhydrous pyridine (118 mL). After being stirred at room temperature for 3 h, the reaction mixture was poured into EtOAc and the organic layer was sequentially washed with saturated aqueous $NaHCO_3$ (400 mL), brine (400 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The residue obtained was purified using flash silica gel column chromatography and eluted with 5% CH_3OH in CH_2Cl_2 to provide compound **5** (16.21 g, 93% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (s, 1 H), 7.86−7.79 (m, 4 H), 7.58−7.28 (m, 14 H), 6.13 (d, J = 3.6 Hz, 1 H), 5.38 (m, 1H), 5.31 (d, J = 8.1 Hz, 1 H), 4.96 (d, J = 11.5 Hz, 1 H), 4.65 (d, J = 11.3 Hz, 1 H), 4.57−4.53 (m, 2 H), 4.23 (d, J = 11.5 Hz, 1 H), 3.98 (d, J = 11.3 Hz, 1 H), 3.77 (d, J = 11.3 Hz, 1 H), 3.18 (s, 3H), 2.84 (s, 3 H), 1.05 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.6, 150.1, 142.3, 135.8, 135.7, 135.5, 134.5, 133.8, 133.5, 132.6, 131.8, 130.6, 130.5, 129.9, 128.8, 128.2, 128.1, 127.9, 127.8, 127.2, 126.7, 126.5, 126.1, 103.2, 89.5, 87.0, 78.7, 76.1, 74.3, 68.8, 64.6, 39.1, 37.7, 27.7, 19.5; MS (ES) m/z 806.9 $[M - H]^-$; HRMS (ES-FT Orbit-Trap) calcd for $C_{39}H_{44}N_2O_{11}S_2SiNa^+$ 831.2048, found 831.2041.

4-*C*-(*O*-Methanesulfonyl)hydroxymethyl-3-*O*-(Nap)-5-*O*-(TBDPS)-β-D-arabinofuranosyluracil 6. To a solution of compound **5** (16 g, 19.74 mmol) in anhydrous CH_3CN (135 mL) was added DBU (5.46 mL, 39.48 mmol). After being stirred at room temperature for 3 h, the mixture was diluted with EtOAc (300 mL), washed with 1% (v/v) aqueous acetic acid (1 × 400 mL) and brine (2 × 400 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to foam. The foam was redissolved in 1,4-dioxane (216 mL), and 2 M aqueous NaOH (54 mL) was added. After 45 min, the mixture was neutralized with acetic acid, diluted with EtOAc (400 mL), washed with saturated aqueous $NaHCO_3$ (1 × 300 mL) and brine (300 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. Purification by silica gel column chromatography (5% CH_3OH in CH_2Cl_2) yielded compound **6** (12.25 g, 85% yield) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.33 (s, 1 H), 7.91 (br s, 4 H), 7.59−7.34 (m, 14 H), 6.22 (d, J = 4.9 Hz, 1 H), 6.02 (d, J = 4.7 Hz, 1 H), 5.16 (d, J = 8.1 Hz, 1 H), 4.97 (d, J = 12.2 Hz, 1 H), 4.79 (d, J = 12.2 Hz, 1 H), 4.58−4.50 (m, 2 H), 4.40 (d, J = 10.4 Hz, 1 H), 4.34 (br s, 1 H) 3.84 (m, 2 H), 3.16 (s, 3 H), 0.85 (s, 9 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.2, 150.5, 143.2, 135.8,134.5, 133.4, 132.7, 132.6, 130.1, 130.0, 128.7, 128.2, 128.0, 127.9, 127.5, 126.4, 126.4, 126.2, 100.5, 86.9, 86.1, 83.8, 73.2, 72.2, 68.3, 62.3, 37.3, 26.8, 19.2; MS (ES) m/z 731.2 $[M + H]^+$; HRMS (ES-FT Orbit-Trap) calcd for $C_{38}H_{42}N_2O_9SSiNa^+$ 753.2267, found 753.2272.

4-*C*-(*O*-Methanesulfonyl)hydroxymethyl-3-*O*-(Nap)-5-*O*-(TBDPS)-2-*O*-trifluoromethanesulfonyl)-β-D-arabinofuranosyluracil 7. Compound **6** (11.76 g, 16.11 mmol) was mixed with DMAP (11.79 g, 96.62 mmol) and dried over P_2O_5 under reduced pressure overnight. The dried mixture was dissolved in anhydrous CH_2Cl_2 (94 mL). The reaction mixture was cooled to −15 °C (dry ice/ethanol bath). To the chilled solution was added a solution of trifluoromethanesulfonic anhydride (6.59 mL, 32.22 mmol) in anhydrous CH_2Cl_2 (70 mL). After being stirred for 1.5 h at −15 to −10 °C under argon atmosphere, the mixture was diluted with ice cold CH_2Cl_2 (200 mL). The resulting solution was washed with ice-cold saturated aqueous $NaHCO_3$ (200 mL) and brine (200 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated to a pale-yellow oil. Purification by silica gel column chromatography (1:1 hexanes/EtOAc) yielded compound **7** (8.26 g, 60%) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (s, 1H), 7.85−7.78 (m, 4H), 7.56−7.21 (m, 14 H), 6.36 (d, J = 3.6 Hz, 1 H), 5.57 (m, 1 H), 5.50 (br s, 1 H), 4.98 (d, J = 11.9 Hz, 1 H), 4.70−4.56 (m, 3 H), 4.40 (d, J = 11.1 Hz, 1 H), 3.81 (d, J = 10.6 Hz, 1 H), 3.66 (d, J = 11.1 Hz, 1 H), 2.87 (s, 3H), 0.89 (s, 9 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.4, 149.5, 145.0, 135.6, 135.3, 133.4, 133.2, 132.8, 132.7, 131.7, 130.2, 130.1, 128.9, 128.1, 128.0, 127.8, 127.6, 126.6, 125.4, 124.6, 120.3, 116.1, 102.4, 86.0, 85.4, 83.7, 82.1, 73.7, 67.2, 62.8, 37.9, 26.6, 19.0; ^{19}F NMR (282 MHz, $CDCl_3$) δ −74.22; MS (ES) m/z 863.0 $[M + H]^+$; HRMS (TOF MS ES) calcd for $C_{39}H_{41}F_3N_2O_{11}S_2SiNa^+$ 885.1771, found 885.1769.

3′-*O*-(Nap)-5′-*O*-(TBDPS)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]uridine 8. To a solution of compound **7** (7.86 g, 9.12 mmol) in anhydrous DMF (12 mL) in a pressure bottle was added *N*,*N*-diisopropylethylamine (15.66 mL, 89.90 mmol) and *N*-methoxyamine (4.23 g, 90 mmol). The reaction mixture was heated at 60 °C for 18 h. The reaction mixture was poured into EtOAc (300 mL) and washed sequentially with aqueous $NaHCO_3$ (5 wt %, 2 × 300 mL) and brine (300 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue obtained was purified by silica gel column chromatography (1:1 hexanes/EtOAc) to yield compound **8** (5.09 g, 84% yield) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.39 (s, 1 H), 7.93−7.81 (m, 4 H), 7.73−7.32 (m, 14 H), 5.98 (br s, 1 H), 5.15 (d, J = 8.1 Hz, 1 H), 4.86−4.69 (m, 2 H), 4.31 (s, 1H), 4.17 (s, 1 H), 3.96−3.86 (m, 2 H), 3.53 (s, 3 H), 3.47 (d, J = 11.9 Hz, 1 H), 2.94 (br s, 1 H), 0.95 (s, 9 H); ^{13}C NMR (75 MHz, CD_3OD) δ 166.2, 151.7, 141.4, 136.7, 136.5, 135.8, 134.6, 134.1, 133.9, 133.5, 133.2, 131.2, 131.1, 130.0, 129.5, 129.4, 129.0, 128.9, 128.8, 128.0, 127.5, 127.4, 127.3, 127.2, 126.9, 101.8, 89.5, 84.1, 77.3, 73.2, 68.4, 61.4, 60.8, 27.5, 20.2; MS (ES) m/z 664.2 $[M + H]^+$; HRMS (TOF MS ES) calcd for $C_{38}H_{41}N_3O_6SiNa^+$ 686.2662, found 686.2657.

5′-*O*-(TBDPS)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]uridine 9. To a solution of compound **8** (4.98 g, 7.5 mmol) in CH_2Cl_2 (77 mL), water (0.3 mL, 16.54 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (9.76 g, 43 mmol) were added. The dark-brown solution was stirred at room temperature for 18 h. The reaction mixture was diluted with EtOAc (200 mL) and washed sequentially with aqueous $NaHCO_3$ (5 wt %, 2 × 200 mL) and brine (200 mL). The organic phase was dried (Na_2SO_4), filtered, and evaporated. The residue obtained was purified by silica gel column chromatography and eluted with 5% CH_3OH in CH_2Cl_2 containing 1% triethylamine to yield **9** (3.36 g, 86% yield) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.37 (s, 1 H), 7.79 (d, J = 8.9 Hz, 1 H), 7.70−7.42 (m, 10 H), 5.95 (br s, 1 H), 5.61 (d, J = 3.8 Hz, 1 H), 5.29 (d, J = 8.1 Hz, 1 H), 4.09 (br s, 1 H), 3.89 (br s, 2 H), 3.85 (s, 1 H), 3.49 (s, 3 H), 3.43 (d, J = 11.7 Hz, 1 H), 2.83 (br s, 1 H), 1.03 (s, 9 H); ^{13}C NMR (75 MHz, CD_3OD) δ 166.4, 151.8, 141.8, 137.0, 136.7, 134.4, 133.9, 131.3, 129.2, 101.8, 89.9, 83.5, 71.7, 68.5, 61.3, 61.2, 54.9, 27.5, 20.3; MS (ES) m/z 524.1 $[M + H]^+$; HRMS (TOF MS ES) calcd for $C_{27}H_{33}N_3O_6SiNa^+$ 546.2036, found 546.2029.

2′-*N*,4′-*C*-[(*N*-Methoxy)aminomethylene]uridine 10. To a stirred solution of compound **9** (3.30 g, 6.31 mmol) in THF (63 mL), triethylamine (2.18 mL, 15.66 mmol) and triethylamine trihydrofluoride (5.10 mL, 31.31 mmol) were added. The resulting reaction mixture was stirred at room temperature for 18 h. Solvent was removed under reduced pressure to get an oil, and this oily residue was purified by silica gel column chromatography and eluted with 5% MeOH in CH_2Cl_2 containing 1% triethylamine to yield **10** (1.53 g, 85%) as a white solid. ^{1}H NMR

(300 MHz, DMSO-d_6) δ 11.32 (s, 1 H), 7.82 (d, J = 7.9 Hz, 1 H), 5.89 (br s, 1 H), 5.62 (d, J = 8.3 Hz, 1 H), 5.45 (d, J = 4.35 Hz, 1 H), 5.09 (t, J = 5.5 Hz, 1 H), 3.93 (br s, 1 H), 3.79 (s, 1 H), 3.70−3.57 (m, 2 H), 3.48 (s, 3 H), 3.41 (d, J = 11.7 Hz, 1 H), 2.78 (br s, 1 H); ^{13}C NMR (75 MHz, CD_3OD) δ 166.6, 151.9, 141.9, 101.9, 90.1, 83.4, 71.7, 68.6, 61.2, 58.8; MS (ES) m/z 286 [M + H]$^+$; HRMS (TOF MS ES) calcd for $C_{11}H_{16}N_3O_6^+$ 286.1039, found 286.1046.

5′-*O*-(DMT)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]uridine 11. Compound **10** (1.48 g, 5.19 mmol) was mixed with DMTCl (2.50 g, 7.38 mmol) and dried over P_2O_5 under reduced pressure overnight. The dried mixture was dissolved in anhydrous pyridine (14 mL), and the resulting solution was stirred at room temperature for 8 h under argon atmosphere. The reaction mixture was poured into CH_2Cl_2 (150 mL) and washed sequentially with aqueous $NaHCO_3$ (5 wt %, 150 mL) and brine (150 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue obtained was purified by silica gel column chromatography and eluted with 0−5% MeOH in CH_2Cl_2 containing 1% triethylamine to yield compound **11** (3.02 g, 99% yield) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.38 (s, 1 H), 7.81 (d, J = 8.1 Hz, 1 H), 7.41−7.25 (m, 9 H), 6.91 (d, J = 8.5 Hz, 4 H), 5.94 (br s, 1 H), 5.59 (d, J = 4.0 Hz, 1 H), 5.41 (d, J = 7.7 Hz, 1 H), 4.15 (br s, 1 H), 3.84 (s, 1 H), 3.75 (s, 6 H), 3.48 (s, 3 H), 3.40−3.29 (m, 2 H), 3.21 (d, J = 10.7 Hz, 1 H), 2.87 (br s, 1 H); ^{13}C NMR (75 MHz, CD_3OD) δ 166.5, 160.4, 151.7, 150.2, 146.3, 141.9, 137.1, 136.8, 131.5, 129.5, 129.0, 128.2, 114.4, 101.9, 88.9, 88.1, 83.6, 72.2, 68.4, 61.2, 60.5, 55.9; HRMS (TOF MS ES) calcd for $C_{32}H_{32}N_3O_8^-$ 586.2189, found 586.2190.

5′-*O*-(DMT)-3′-*O*-(TBDMS)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]uridine 12. To a solution of compound **11** (1.4 g, 2.38 mmol) and imidazole (1.62 g, 23.8 mmol) in anhydrous DMF (5.3 mL), *tert*-butyldimethylsilyl chloride (1.79 g, 11.90 mmol) was added. The reaction mixture was stirred at room temperature for 24 h under argon atmosphere. The reaction was quenched with aqueous $NaHCO_3$ (60 mL), and extraction was performed with EtOAc (2 × 50 mL). The combined organic phase was washed with brine (100 mL) and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by silica gel column chromatography and eluted with 80% EtOAc in hexane to yield **12** (1.43 g, 85%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.39 (s, 1 H), 7.89 (d, J = 8.1 Hz, 1 H), 7.39−7.251 (m, 9 H), 6.91 (d, J = 8.9 Hz, 4 H), 5.92 (br s, 1 H), 5.44 (d, J = 8.3 Hz, 1 H) 4.27 (s, 1H), 3.86 (s, 1 H), 3.74 (s, 6 H), 3.45 (s, 3 H), 3.32−3.29 (m, 2 H), 3.22 (d, J = 11.7 Hz, 1 H), 2.92 (br s, 1 H) 0.71 (s, 9 H), 0.03 (s, 3 H), −0.06 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.7, 159.0, 149.9, 144.6, 140.4, 135.6, 135.5, 130.3, 128.3, 128.2, 127.3, 113.5, 101.7, 88.3, 86.8, 83.4, 71.7, 67.0, 61.0, 60.6, 58.9, 55.5, 25.7, 18.1, −4.6, −5.0; MS (ES) m/z 698.8 [M − H]$^-$.

5′-*O*-(DMT)-3′-*O*-(TBDMS)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]cytidine 13. A suspension of 1,2,4-triazole (4.65 g, 67.27 mmol) in anhydrous CH_3CN (25.4 mL) was cooled in an ice bath for 5−10 min under an argon atmosphere. To this cold suspension, $POCl_3$ (1.47 mL, 60 mmol) was added slowly over 10 min and stirring continued for an additional 5 min. Triethylamine (11.00 mL, 79.20 mmol) was added slowly over 10 min, keeping the bath temperature around 0−2 °C. The reaction mixture was stirred at 0−2 °C for an additional 30 min. Compound **12** (1.39 g, 1.98 mmol) in anhydrous CH_3CN (12.7 mL) was added in one portion and stirred for 10 min, and the reaction mixture was removed from the ice bath and stirred at room temperature for 4 h under an argon atmosphere. The mixture was concentrated to one-third of its volume, diluted with EtOAc (100 mL), and washed with water (2 × 100 mL) and brine (100 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting residue was dissolved in a solution of aqueous NH_3 (12.7 mL, 28−30 wt %) and dioxane (30.5 mL). The reaction mixture was stirred in a pressure bottle for 6 h at room temperature. The solvent was removed under reduced pressure, and the resulting residue was purified by flash silica gel column chromatography and eluted with 5% CH_3OH in CH_2Cl_2 containing 1% triethylamine to yield **13** (1.32 g, 95%) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (d, J = 7.4 Hz, 1 H), 7.51−7.31 (m, 9 H), 6.92−6.88 (m, 4 H), 6.18 (br s, 1 H), 5.63 (d, J = 7.38 Hz, 1 H), 4.33 (s, 1 H), 4.11 (s, 1 H), 3.86 (s, 6 H), 3.63 (s, 3 H), 3.52 (d, J = 10.6 Hz, 1 H), 3.43 (d, J = 11.4 Hz, 1 H), 3.52 (d, J = 10.6 Hz, 1 H), 3.27 (d, J = 10.5 Hz, 1 H), 3.04 (br s, 1 H), 0.81 (s, 9 H), 0.09 (s, 3 H), 0.06 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.9, 158.7, 155.5, 144.5, 141.7, 135.6, 135.5, 130.2, 130.1, 128.2, 127.9, 127.0, 113.2, 93.6, 87.7, 86.4, 71.5, 67.1, 66.4, 60.9, 59.0, 55.3, 25.5, 17.9, −4.6, −5.1; MS (ES) m/z 698.9 [M − H]$^-$; HRMS (TOF ES MS) calcd for $C_{38}H_{49}N_4O_7Si^+$ 701.3356, found 701.3356.

N^4-Benzoyl-5′-*O*-(DMT)-3′-*O*-(TBDMS)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]cytidine 14. Compound **13** (1.34 g, 1.91 mmol) was dissolved in anhydrous DMF (5 mL), and benzoic anhydride (0.65 g, 2.88 mmol) was added. After being stirred for 18 h at room temperature, the reaction mixture was diluted with EtOAc (100 mL). The resulting organic phase was washed with saturated aqueous $NaHCO_3$ (2 × 100 mL) and brine (100 mL). The EtOAc layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue obtained was purified by flash silica gel column chromatography and eluted with 80% EtOAc in hexane to yield **14** (1.52 g, 99%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.33 (s, 1H), 8.43 (d, J = 7.5 Hz, 1 H), 8.02 (d, J = 7.7 Hz, 2 H), 7.65−7.24 (m, 13 H), 6.92 (d, J = 8.7 Hz, 4 H), 6.02 (br s, 1 H), 4.31 (s, 1 H), 3.98 (s, 1 H), 3.76 (s, 6 H), 3.50 (s, 3 H), 3.39−3.25 (m, 3 H), 2.96 (br s, 1 H), 0.70 (s, 9 H), −0.01 (s, 3 H), −0.09 (s, 3 H); ^{13}C NMR (75 MHz, CD_3CN) δ 168.4, 164.0, 160.0, 155.4, 146.0, 145.6, 136.8, 136.6, 134.6, 134.0, 131.2, 130.7, 129.7, 129.6, 129.2, 129.0, 128.2, 114.3, 97.0, 88.9, 87.4, 84.6, 72.6, 67.2, 61.7, 61.3, 60.1, 56.0, 26.1, 18.6, −4.3, −4.7; MS (ES) m/z 802.9 [M − H]$^-$; HRMS (ES-Orbit-Trap) calcd for $C_{45}H_{53}N_4O_8Si^+$ 805.3627, found 805.3620.

N^4-Benzoyl-5′-*O*-(DMT)-2′-*N*,4′-*C*-[(*N*-methoxy)aminomethylene]cytidine 15. In a 100 mL round-bottom flask, triethylamine trihydrofluoride (1.52 mL, 9.33 mmol) was dissolved in anhydrous THF (18.7 mL). Triethylamine (0.65 mL, 4.67 mmol) was added to this solution, and the mixture was quickly poured onto compound **14** (1.5 g, 1.87 mmol). The resulting mixture was stirred at room temperature for 48 h. The reaction mixture was poured into EtOAc (50 mL). The organic phase was washed sequentially with water (50 mL), 5% aqueous $NaHCO_3$ (50 mL), and brine (50 mL). The EtOAc layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography and eluted with 50% EtOAc in hexane to afford **15** (1.17 g, 91%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.32 (s, 1 H), 8.36 (d, J = 7.4 Hz, 1 H), 8.02 (d, J = 8.1 Hz, 2 H), 7.66−7.25 (m, 13 H), 6.93 (d, J = 8.9 Hz, 4 H), 6.05 (br s, 1H), 5.61 (d, J = 3.8 Hz, 1 H), 4.20 (d, J = 3.6 Hz, 1 H), 3.96 (s, 1 H), 3.77 (s, 6H), 3.53 (s, 3 H), 3.43−3.23 (m, 3 H), 2.90 (br s, 1 H); ^{13}C NMR (75 MHz, CD_3CN) δ 168.0, 163.7, 159.6, 155.3, 145.8, 145.5, 136.8, 136.5, 134.3, 133.7, 130.9, 130.8, 129.5, 129.0, 128.9, 128.8, 127.9, 114.0, 96.7, 88.4, 87.2, 84.0, 71.8, 67.1, 61.1, 60.8, 60.0, 55.8; MS (ES) m/z 691.2 [M + H]$^+$; HRMS (TOF ES MS) calcd for $C_{39}H_{38}N_4O_8Na^+$ 713.2587, found 713.2573.

5′-*O*-(DMT)-2′-*N*,4′-*C*-(*N*-methyl)aminooxymethyleneuridine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite 20. Compound **20** (0.85 g, 79% yield) was synthesized from compound **32** (0.8 g, 1.36 mmol), 1*H*-tetrazole (0.09 g, 1.29 mmol), DMF (4.00 mL), 2-cyanoethyl-*N*,*N*,*N*′*N*′-tetraisopropylphosphorodiamidite (0.67 mL, 2.12 mmol), and 1-methylimidazole (0.033 mL, 0.40 mmol) according to procedure used for the synthesis of compound **1**. ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.42, 149.18; HRMS (TOF MS ES) calcd for $C_{41}H_{49}N_5O_9P^-$ 786.3268, found 786.3283.

N^4-Benzoyl-5′-*O*-(DMT)-2′-*N*,4′-*C*-(*N*-methyl)aminooxymethylene)cytidine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite 21. Compound **21** (0.76 g, 87% yield) was synthesized from compound **36** (0.68 g, 0.99 mmol), 1*H*-tetrazole (0.06 g, 0.88 mmol), DMF (2.90 mL), 2-cyanoethyl-*N*,*N*,*N*′*N*′-tetraisopropylphosphorodiamidite (0.49 mL, 1.53 mmol), and 1-methylimidazole (0.023 mL, 0.29 mmol) according to procedure used for the synthesis of compound **1**. ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.58, 149.06; HRMS (TOF MS ES) calcd for $C_{48}H_{56}N_6O_9P^+$ 891.3846, found 891.3836.

3,5-*O*-Bis(Nap)-1,2-isopropylidene-4-*C*-hydroxymethyl-α-D-ribofuranose 23a. NaH (60% dispersion in mineral oil, 7.94 g, 198.16 mmol) was added to nitrogen flushed round-bottom flask and washed with hexanes (2 × 50 mL) to remove the mineral oil. After the hexanes were decanted, DMF (490 mL) was added and the mixture was cooled in an ice bath. 3-*O*-(Nap)-1,2-isopropylidene-4-*C*-hydroxymethyl-α-D-ribofuranose **22** (44.6 g, 123.90 mmol) was added to the reaction mixture, and the mixture was stirred for 30 min. 2-(Bromomethyl)naphthalene (30.08 g, 136.08 mmol) was slowly added to the reaction mixture, and the stirring was continued at room temperature for another 6 h. The reaction mixture was cooled in an ice bath, and CH_3OH (20 mL) was added. The reaction mixture was poured into ice cold water (1.5 L). The resulting aqueous phase was extracted with EtOAc (2 × 400 mL), and the organic layers were combined, washed with brine (1 L), and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to yield **23a** (39.32 g, 64%) as white solid. A minor amount (4.12 g, 6.65%) of compound **23b** was also isolated. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14−7.08 (m, 14 H), 5.78 (d, J = 3.6 Hz, 1 H), 4.91 (d, J = 11.9 Hz, 1 H), 4.77−4.44 (m, 4 H), 4.31 (d, J = 5.3 Hz, 1 H), 4.06−3.76 (m, 2 H), 3.72−3.42 (m, 2 H), 2.42 (t, J = 6.8 Hz, 1 H), 1.67 (m, 3 H), 1.34 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) 135.4, 134.8, 133.2, 133.0, 128.4, 128.1, 127.9, 127.7, 126.9, 126.5, 126.3, 126.2, 126.1, 125.9, 125.7, 113.6, 104.4, 86.4, 78.8, 78.5, 73.7, 72.8, 71.6, 63.2, 26.8, 26.1; MS (ES) m/z 523 $[M + Na]^+$; HRMS (ES-Orbit-Trap) calcd for $C_{31}H_{32}O_6Na^+$ 523.2091, found 523.2086.

3,5-*O*-Bis(Nap)-1,2-isopropylidene-4-*C*-(trifluoromethanesulfonyloxymethyl-α-D-ribofuranose 24. A mixture of compound **23a** (30.40 g, 60.72 mmol) and DMAP (7.40 g, 60.72 mmol) was dried over P_2O_5 under reduced pressure. The mixture was dissolved in anhydrous pyridine (152 mL) and chilled to −5 °C. To this trifluoromethanesulfonic anhydride (20.44 mL, 121.44 mmol) was added dropwise in 20 min. After being stirred at −5 to 0 °C for 15 min under argon atmosphere, the reaction mixture was allowed to warm to room temperature. Stirring continued for 3 h under argon atmosphere. The reaction mixture was diluted with EtOAc (300 mL) and poured into an iced cold aqueous $NaHCO_3$ (300 mL). The organic phase was separated, washed with brine (300 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography (1:1 hexanes/EtOAc) to afford compound **24** (33.64 g, 88.00%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03−7.27 (m, 14 H), 5.72 (d, J = 3.4 Hz, 1 H), 5.04 (d, J = 10.5 Hz, 1 H), 4.97−4.82 (m, 2 H), 4.79−4.48 (m, 4 H), 4.25 (d, J = 4.9 Hz, 1 H), 3.69−3.40 (m, 2 H), 1.67 (s, 3 H), 1.34 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 134.9, 134.4, 133.2, 133.2, 133.1, 133.1, 133.0, 128.4, 128.2, 127.9, 127.9, 127.7, 127.7, 127.7, 127.1, 126.6, 126.3, 126.2, 126.2, 126.2, 126.0, 125.7, 125.6, 120.8, 116.5, 113.9, 104.3, 84.0, 79.2, 78.6, 76.2, 73.9, 72.8, 70.5, 26.5, 25.9; ^{19}F NMR (282 MHz, $CDCl_3$) δ −74.28; MS (ES) m/z 655.1 $[M + Na]^+$.

3,5-*O*-Bis(Nap)-1,2-isopropylidene-4-*C*-(methyleneaminooxymethyl)-α-D-ribofuranose 25. Compound **24** (29.80 g, 47.15 mmol) was dissolved in anhydrous *N*,*N*-dimethylacetamide (88 mL). To this, *N*-hydroxyphthalimide (60.77 g, 377.18 mmol) and *N*,*N*-diisopropylethylamine (65.70 mL, 377.18 mmol) were added. The dark-colored suspension thus obtained was heated at 90 °C for 18 h under argon atmosphere. The reaction mixture was diluted with EtOAc (400 mL) and washed with aqueous $NaHCO_3$ (3 × 600 mL) and brine (400 mL) and dried (Na_2SO_4) and concentrated under reduced pressure. The residue obtained was purified by by silica gel column chromatography (30−40% EtOAc in hexane), and the product obtained (23.84 g, 36.78 mmol) was dissolved in CH_2Cl_2 (272 mL). The reaction mixture was chilled to −10 °C, and *N*-methylhydrazine (2.60 mL, 48.84 mmol) was added. The reaction mixture was stirred at −10 to 0 °C for 2 h. The precipitate formed was filtered, and the precipitate was washed with ice cold CH_2Cl_2. The combined filtrate and the washing were concentrated, and the residue obtained was dissolved in CH_3OH (272 mL). To this, formaldehyde (37 wt % in H_2O, 3.24 mL, 39.94 mmol) was added, and the mixture was stirred at room temperature for 4 h. Solvent was removed under reduced pressure and the residue dissolved in EtOAc (250 mL) and washed with water (150 mL) and brine (150 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography and eluted with 30−40% EtOAc in hexane to yield **25** (11.48 g, 46.1%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90−7.27 (m, 14 H), 7.05 (d, J = 8.1 Hz, 1 H), 6.39 (d, J = 8.1 Hz, 1 H), 5.78 (d, J = 3.6 Hz, 1 H), 4.91 (d, J = 12.2 Hz, 1 H), 4.83−4.60 (m, 4 H), 4.59−4.50 (m, 1 H), 4.44 (d, J = 12.1 Hz, 1 H), 4.37 (d, J = 5.1 Hz, 1 H), 3.75−3.62 (m, 1 H), 3.61−3.52 (m, 1 H), 1.66 (s, 3 H), 1.35 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.0, 135.5, 135.2, 133.1, 132.8, 128.1, 128.0, 127.8, 127.6, 126.6, 126.2, 125.9, 125.7, 125.6, 113.7, 104.2, 86.4, 79.5, 78.2, 74.5, 73.7, 72.6, 71.5, 26.8, 26.4; MS (ES) m/z 550.2 $[M + Na]^+$; HRMS (ES-Orbit-Trap) calcd for $C_{32}H_{33}NO_6Na^+$ 550.2194, found 550.2200.

1,2-Di-*O*-acetyl-3,5-*O*-bis(Nap)-4-*C*-(methyleneaminooxymethyl)-α-D-ribofuranose 26. To a solution of compound **25** (10.85 g, 20.59 mmol) in 1,4-dioxane (71 mL), water (63.5 mL) and strong acidic resin (Amberlite IR-120, 25.1 g) were added. The reaction mixture was heated at 80 °C for 3 days. Resin was filtered and washed with EtOAc (4 × 50 mL). The the filtrate and washing were combined and concentrated to dryness under reduced pressure to get yellowish oil. The oil was coevaporated with pyridine (2 × 50 mL), and the residue was dissolved in anhydrous pyridine (54 mL). To this acetic anhydride (7.79 mL, 82.36 mmol) was added, and the mixture was stirred at room temperature for 3 h under argon atmosphere. Solvent was removed under reduced pressure, and the residue was dissolved in EtOAc (100 mL) and washed with aqueous $NaHCO_3$ (100 mL) and brine (100 mL) and dried (Na_2SO_4) and concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography and eluted with 30% EtOAc in hexane to yield **26** (8.85 g, 75%). ^{1}H NMR (300 MHz, $CDCl_3$, mixture of isomers) δ 8.18−7.27 (m, 14 H), 7.12−6.84 (m, 1 H), 6.62−6.27 (m, 1 H), 6.21 (s, 1 H), 5.54−5.24 (m, 1 H), 4.99−4.37 (m, 7 H), 3.84−3.40 (m, 2 H), 2.31−1.95 (m, 3 H), 1.93−1.74 (m, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$, mixture of isomers) δ 169.9, 169.7, 169.0, 137.2, 135.5, 135.0, 133.1, 133.0, 132.9, 128.2, 128.1, 128.0, 127.8, 127.8, 127.6, 126.5, 126.3, 126.3, 126.2, 126.1, 125.9, 125.9, 125.7, 125.6, 125.5, 125.5, 125.5, 113.8, 97.7, 94.6, 88.0, 86.1, 78.6, 74.6, 73.5, 73.5, 73.5, 70.9, 21.3, 20.8, 20.8, 20.6; MS (ES) m/z 594.2 $[M + Na]^+$; HRMS (ES-Orbit-Trap) calcd for $C_{33}H_{33}NO_8Na^+$ 594.2098, found 594.2095.

3,5-*O*-Bis(Nap)-4-*C*-(methyleneaminooxymethyl)-β-D-ribofuranosyluracil 27. To a stirred solution of anomeric mixture of **26** (11.75 g, 20.57 mmol) and uracil (5.19 g, 46.31 mmol) in anhydrous acetonitrile (125 mL) was added *N*,*O*-bis(trimethylsilyl)acetamide (37.8 mL, 154.54 mmol). After heating at 67 °C for 2 h under an argon atmosphere, the reaction mixture was cooled in an ice bath. To this, trimethylsilyl trifluoromethanesulfonate (8.54 mL, 47.24 mmol) was added dropwise. The reaction mixture was heated at 65 °C for 2 h under an argon atmosphere. The reaction was quenched with an ice cold aqueous $NaHCO_3$ (saturated, 200 mL). The mixture was extracted

with EtOAc (2 × 100 mL). The organic phase was washed with brine (200 mL) and dried (Na_2SO_4) and concentrated under reduces pressure. The residue obtained was dissolved in methanolic NH_3 (7 M, 150 mL) and stirred at room temperature for 18 h. Solvent was removed under reduced pressure and the residue obtained was purified by silica gel column chromatography and eluted with 5% CH_3OH in CH_2Cl_2 to yield **27** (10.33 g, 86%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.33 (s, 1 H), 8.01−7.76 (m, 8 H), 7.70 (d, J = 8.1 Hz, 1 H), 7.60−7.37 (m, 6 H), 7.05 (d, J = 7.4 Hz, 1 H), 6.59 (d, J = 7.4 Hz, 1 H), 5.92 (d, J = 6.6 Hz, 1 H), 5.68 (d, J = 5.9 Hz, 1 H), 5.34 (d, J = 8.1 Hz, 1 H), 5.07−4.96 (m, 1 H), 4.80−4.61 (m, 3 H), 4.47−4.26 (m, 3 H), 4.25−4.18 (m, 1 H), 3.78−3.65 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.0, 150.6, 140.4, 137.9, 134.4, 133.1, 133.0, 128.4, 128.1, 127.9, 127.8, 127.7, 127.3, 127.1, 126.9, 126.5, 126.3, 126.2, 125.8, 125.6, 102.4, 90.0, 87.1, 78.6, 74.8, 74.5, 74.2, 73.9, 71.9 ; MS (ES) m/z 582.1 $[M + H]^+$; HRMS (ES-Orbit-Trap) calcd for $C_{33}H_{31}N_3O_7Na^+$ 604.2054, found 604.2047.

3,5-*O*-Bis(Nap)-4-*C*-(methyleneaminooxymethyl)-β-D-arabinofuranosyl)uracil 28. Compound **27** (10.20 g, 17.56 mmol) was dissolved in anhydrous CH_2Cl_2 (175 mL), and triethylamine (29.35 mL, 210.57 mmol) was added. The reaction mixture was chilled in an ice bath and methanesulfonyl chloride (2.73 mL, 35.07 mmoL) was added dropwise. The mixture was stirred for 10 min under an argon atmosphere. The reaction mixture was allowed to come to room temperature. After being stirred for 3 h under an argon atmosphere, reaction mixture was poured into an ice cold aqueous $NaHCO_3$ (saturated, 150 mL). The organic phase was separated and washed with brine (150 mL) and dried (Na_2SO_4) and concentrated under reduces pressure. The residue obtained was purified by silica gel column chromatography and eluted with 50−70% EtOAc in hexane to yield methanesulfonyl derivative (8.10 g, 70%). To a solution of methanesulfonyl derivative (8.10 g, 12.29 mmol) in anhydrous CH_3CN (97.50 mL) was added DBU (4.16 mL, 27.80 mmol). After being stirred at room temperature for 3 h, the mixture was diluted with EtOAc (200 mL), washed with 1 M aqueous HCl (200 mL), water (200 mL), aqueous $NaHCO_3$ (200 mL), and brine (200 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to a foam. The foam was redissolved in 1,4-dioxane (270 mL), and 2 M aqueous NaOH (68 mL) was added. After 45 min, the mixture was diluted with EtOAc (200 mL), washed with 1 M aqueous HCl (200 mL), water (200 mL), saturated aqueous $NaHCO_3$ (200 mL), and brine (200 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. Purification by silica gel column chromatography (70% EtOAc in hexane) yielded **28** (6.01 g, 59%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.24 (s, 1 H), 8.02- 7.72 (m, 8 H), 7.68 (s, 1 H), 7.62−7.43 (m, 7 H), 7.32 (m, 1 H), 7.11 (d, J = 7.3 Hz, 1 H), 6.63 (d, J = 7.3 Hz, 1 H), 6.11 (d, J = 6.0 Hz, 1 H), 5.90 (d, J = 5.3 Hz, 1 H), 5.17 (m, 1 H), 4.92−4.75 (m, 2 H), 4.67−4.47 (m, 3 H), 4.44−4.32 (m, 1 H), 4.28−4.17 (m, 2 H), 3.75- 3.59 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 150.7, 142.1, 137.7, 134.9, 134.2, 133.2, 133.1, 128.4, 128.2, 128.0, 127.8, 127.7, 126.9, 126.8, 126.4, 126.2, 126.2, 126.0, 125.7, 125.6, 100.8, 85.6, 85.0, 83.0, 74.9, 73.8, 73.7, 72.7, 70.2; MS (ES) m/z 582.2 $[M + H]^+$; HRMS (ES-Orbit-Trap) calcd for $C_{33}H_{31}N_3O_7Na^+$ 604.2054, found 604.2050.

3,5-*O*-Bis(Nap)-4-*C*-(methyleneaminooxymethyl-2′-*O*-methanesulfonyl-β-D-arabinofuranosyluracil 29. Compound **28** (5.99 g, 10.33 mmol) was dissolved in anhydrous CH_2Cl_2 (55 mL), and triethylamine (18.27 mL, 31.06 mmol) was added. The reaction mixture was cooled to 0 °C, and methanesulfonyl chloride (1.70 mL, 21.76 mmol) was added dropwise and warmed to room temperature and stirred for 3 h under an argon atmosphere. The reaction mixture was diluted with CH_2Cl_2 (50 mL), cooled to 0°C, and quenched with saturated aqueous $NaHCO_3$ (10 mL). The reaction mixture was washed with saturated aqueous $NaHCO_3$ (100 mL) and brine (100 mL) and dried (Na_2SO_4) and concentrated under reduces pressure. The residue obtained was purified by silica gel column chromatography and eluted with 70% EtOAc in hexane to yield **29** (5.84 g, 86%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.38 (brs, 1 H), 8.01−7.76 (m, 9 H), 7.65−7.42 (m, 6 H), 7.16 (d, J = 6.0 Hz, 1 H), 6.67 (d, J = 6.0 Hz, 1 H), 6.29 (d, J = 6.0 Hz, 1H), 5.51 (t, J = 6.3 Hz, 1 H), 5.27 (d, J = 6.2 Hz, 1 H), 4.86 (dd, J = 12.0 Hz, 24.1 Hz, 2 H), 4.71−4.40 (m, 4 H), 4.25 (d, J = 12.0 Hz,1 H), 3.77−3.61 (m, 2 H), 3.20 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.8, 150.1, 140.9, 138.2, 134.4, 134.2, 133.2, 133.1, 133.0, 128.4, 128.3, 128.0, 127.8, 127.7, 127.7, 127.7, 127.4, 126.8, 126.4, 126.3, 125.9, 125.6, 102.0, 84.7, 82.3, 81.5, 80.4, 73.6, 73.4, 73.3, 68.7, 38.2; MS (ES) m/z 660.0 $[M + H]^+$; HRMS (TOF MS ES) calcd for $C_{34}H_{33}N_3O_9SNa^+$ 682.1830, found 682.1822.

2′-*N*,4′-*C*-[(*N*-Methyl)aminooxymethylene]-3′,5′-bis-*O*-(Nap)-uridine 30. Compound **29** (5.74 g, 8.71 mmol) was dissolved in a solution of 1 M pyridinium p-tolunesulfonate in anhydrous CH_3OH (87 mL). The reaction mixture was cooled to 10 °C in an ice bath, and $NaBH_3CN$ (1.09 g, 17.42 mmol) was added. After being stirred for 15 min at 10 °C, the reaction mixture was allowed to come to room temperature and stirred for an additional 2 h. The reaction mixture was concentrated to get an oil, diluted with EtOAc (150 mL), washed with water (150 mL), aqueous $NaHCO_3$ (5%, 100 mL), and brine (100 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue obtained was dissolved in DMF (57.4 mL), and N,N-diisopropylethylamine (57.4 mL) was added. The reaction mixture was heated at 80 °C for 18 h. The reaction mixture was cooled and diluted with EtOAc (300 mL). The solution was washed with aqueous $NaHCO_3$ (saturated, 300 mL) and brine (300 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography and eluted with 50% EtOAc in hexane to yield **30** (4.13 g, 84%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.33 (s, 1 H), 7.91−7.75 (m, 9 H), 7.56−7.43 (m, 6 H), 6.18 (s, 1 H), 5.22 (d, J = 8.1 Hz, 1 H), 4.82−4.62 (m, 4 H), 4.12 (d, J = 11.1 Hz, 1 H), 4.03 (m, 1 H), 3.79−3.64 (m, 3 H), 3.47 (d, J = 10.9 Hz, 1 H), 2.70 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.6, 150.0, 139.8, 134.8, 134.7, 133.3, 133.2, 128.6, 128.5, 128.0, 127.9, 127.1, 126.7, 126.6, 126.5, 126.4, 125.9, 125.5, 101.7, 83.0, 82.8, 73.9, 72.5, 70.8, 68.4, 67.7, 66.6, 43.7; MS (ES) m/z 566.2 $[M + H]^+$; HRMS (TOF MS ES) calcd for $C_{33}H_{31}N_3O_6Na^+$ 588.2111, found 588.2127.

2′-*N*,4′-*C*-[(*N*-Methyl)aminooxymethylene]uridine 31. To a solution of compound **30** (4.11 g, 7.27 mmol) in CH_2Cl_2 (73 mL), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (9.37 g, 41.27 mmol) and H_2O (0.29 mL, 16.16 mmol) were added. The reaction mixture was stirred at room temperature for 18 h. Solvent was removed under reduced pressure. The residue obtained was dissolved in 10% CH_3OH in CH_2Cl_2 (20 mL) and purified by silica gel column chromatography and eluted with 5−10% CH_3OH in CH_2Cl_2 containing 2% triethylamine to yield **31** (1.78 g, 86%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.01 (d, J = 8.1 Hz, 1 H), 6.16 (s, 1 H), 5.62 (d, J = 8.1 Hz, 1 H), 4.16 (d, J = 11.3 Hz, 1H), 4.09 (d, J = 4.8 Hz, 1 H), 3.68 (d, J = 5.8 Hz, 1 H), 3.47−3.41 (m, 2 H), 3.15−3.12 (m, 1 H), 2.78 (s, 3 H). ^{13}C NMR (75 MHz, MeOD) 167.8, 153.2, 142.8, 103.1, 85.8, 84.7, 72.2, 70.7, 66.7, 60.9, 44.8; MS (ES) m/z 286.1 $[M + H]^+$; HRMS (TOF MS ES) calcd for $C_{11}H_{16}N_3O_6^+$ 286.1039, found 286.1034.

5′-*O*-(DMT)-2′-*N*,4′-*C*-[(*N*-methyl)aminooxymethylene]uridine 32. Compound **32** (2.82 g, 79% yield) was synthesized from compound **31** (1.72 g, 6.03 mmol), DMTCl (2.99 g, 8.83 mmol), and anhydrous pyridine (17 mL) according to the procedure used for the synthesis of compound **11** except that the residue obtained after workup was purified by silica gel column chromatography and eluted with 70% EtOAc and 2% triethylamine in hexane to yield a white foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.36 (s, 1 H), 7.87 (d, J = 8.1 Hz, 1 H), 7.53−7.06 (m, 9 H),

6.91 (d, J = 8.5 Hz, 4 H), 6.16 (s, 1 H), 5.26 (d, J = 7.6 Hz, 2 H), 4.17 (m, 1 H), 3.96 (d, J = 11.5 Hz, 1 H), 3.75 (s, 6 H), 3.49−3.34 (m, 2H), 3.27−3.05 (dd, J = 11, 26.4 Hz, 2 H), 2.67 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.8, 159.2, 159.1, 150.4, 144.8, 139.9, 135.4, 135.2, 130.3, 130.2, 128.0, 127.3, 113.5, 102.6, 87.1, 83.4, 82.4, 70.1, 68.9, 65.4, 60.1, 55.5, 43.5; MS (ES) m/z 586.2 $[M - H]^-$; HRMS (TOF MS ES) calcd for $C_{32}H_{33}N_3O_8Na^+$ 610.2166, found 610.2162.

5′-*O*-(DMT)-3′-*O*-(triethylsilyl)-2′-*N*,4′-*C*-[(*N*-methyl)aminooxymethylene]uridine 33. Compound **33** (1.18 g, 84% yield) was synthesized from compound **32** (1.18 g, 2.01 mmol), imidazole (1.37 g, 20.08 mmol), DMF (5.00 mL), and chlorotriethyl silane (1.69 mL, 10.04 mmol) according to the procedure used for the synthesis of compound **12**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.38 (s, 1 H), 7.99 (d, J = 8.1 Hz, 1 H), 7.49−7.08 (m, 9 H), 6.90 (dd, J = 1.6, 8.8 Hz, 4 H), 6.20 (s, 1 H), 5.30 (d, J = 8.1 Hz, 1 H), 4.28 (d, J = 4.7 Hz, 1 H), 3.87 (d, J = 11.1 Hz, 1 H), 3.74 (s, 6 H), 3.46 (d, J = 4.7 Hz, 1 H), 3.36 (d, J = 11.0 Hz, 1 H), 3.15 (dd, J = 10.8, 32.7 Hz, 2 H), 2.64 (s, 3 H), 0.97−0.68 (m, 9 H), 0.63−0.33 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ163.8, 158.8, 149.9, 144.1, 140.0, 135.1, 134.9, 130.1, 129.1, 128.1, 127.9, 127.8, 127.2, 113.5, 113.4, 101.7, 86.8, 83.2, 82.9, 69.7, 67.9, 65.8, 59.7, 55.2, 43.2, 6.9, 5.1; MS (ES) m/z 702.3 $[M + H]^+$.

5′-*O*-(DMT)-3′-*O*-(triethylsilyl)-2′-*N*,4′-*C*-[(*N*-methyl)aminooxymethylene]cytidine 34. Compound **34** (1.06 g, 95% yield) was synthesized from compound **33** (1.12 g, 1.60 mmol) in anhydrous CH_3CN (10.5 mL) according to the procedure used for the synthesis of compound **13**. 1,2,4-Triazole (3.76 g, 54.4 mmol), CH_3CN (21.00 mL), $POCl_3$ (1.18 mL, 12.69 mmol), triethylamine (8.89.mL, 63.77 mmol), aqueous NH_3 (12.7 mL, 28−30 wt %), and dioxane (30.5 mL) were added. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.09 (d, J = 7.3 Hz, 1 H), 7.52−7.05 (m, 9 H), 6.90 (dd, J = 1.5, 8.7 Hz, 4 H), 6.17 (s, 1 H), 5.59 (d, J = 7.5 Hz, 1 H), 4.28 (d, J = 4.5 Hz, 1 H), 3.85 (d, J = 11.1 Hz, 1 H), 3.75 (s, 6H), 3.35−3.28 (m, 3 H), 3.01 (d, J = 10.8 Hz, 1 H), 2.67 (s, 3 H), 0.90−0.64 (m, 9 H), 0.59−0.32 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.2, 158.9, 155.8, 144.5, 141.8, 135.6, 135.5, 130.4, 128.5, 128.1, 127.3, 113.4, 94.0, 86.9, 83.7, 83.0, 69.4, 68.2, 66.0, 60.3, 55.5, 43.6, 6.9, 5.1; MS (ES) m/z 701.3 $[M + H]^+$; HRMS (TOF ES MS) calcd for $C_{38}H_{49}N_4O_7SiNa^+$ 723.3184, found 723.3179.

***N*^{4}-Benzoyl-5′-*O*-(DMT)-3′-*O*-(triethylsilyl)-2′-*N*,4′-*C*-[(*N*-methyl)aminooxymethylene]cytidine 35.** Compound **35** (0.98 g, 84% yield) was synthesized from compound **34** (1.02 g, 1.46 mmol), anhydrous DMF (6.60 mL), and benzoic anhydride (0.56 g, 2.49 mmol) according to the procedure used for the synthesis of compound **14**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.35 (br s, 1 H), 8.58 (d, J = 7.5 Hz, 1 H), 8.02 (d, J = 7.1 Hz, 2 H), 7.66−7.25 (m, 13 H), 6.93 (d, J = 8.9 Hz, 4 H), 6.21 (s, 1 H), 4.35 (d, J = 4.7 Hz, 1 H), 3.92 (d, J = 11.2 Hz, 1 H), 3.78 (s, 6 H), 3.46 (d, J = 4.7 Hz, 1 H), 3.39 (d, J = 11.0 Hz, 1 H), 3.29 (m, 1H), 3.14 (m, 1 H), 2.73 (s, 3 H), 0.84−0.76 (m, 9), 0.59−0.41 (m, 6H); ^{13}C NMR (75 MHz, CD_3Cl_3) δ 163.0, 159.0, 145.4, 144.1, 135.6, 135.4, 133.3, 130.4, 130.3, 129.1, 128.5, 128.2, 128.0, 127.5, 113.5, 96.6, 87.1, 84.2, 83.5, 69.2, 68.1, 65.8, 60.2, 55.5, 43.6, 6.9, 5.1; MS (ES) m/z 805.3 $[M + H]^+$; HRMS (ES-FT Orbit-Trap) calcd for $C_{45}H_{53}N_4O_8Si^+$ 805.3627, found 805.3622.

***N*^{4}-Benzoyl-5′-*O*-(DMT)-2′-*N*,4′-*C*-[(*N*-methyl)aminooxymethylene]cytidine 36.** Compound **36** (0.74 g, 92% yield) was synthesized from compound **35** (0.94 g, 1.16 mmol), triethylamine trihydrofluoride (1.15 mL, 7.09 mmol), anhydrous THF (13.7 mL), and triethylamine (0.49 mL, 3.55 mmol) according to the procedure used for the synthesis of compound **15**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.31 (s, 1 H), 8.46 (d, J = 7.5 Hz, 1 H), 8.01 (d, J = 7.3 Hz, 2 H), 7.71−7.20 (m, 13 H), 6.93 (d, J = 8.9 Hz, 4 H), 6.22 (s, 1 H), 5.29 (d, J = 7.3 Hz, 1 H), 4.29−4.18 (m, 1 H), 4.08−3.93 (m, 1H), 3.77 (s, 6 H), 3.43−3.38 (m, 2 H), 3.35−3.29 (m, 1 H), 3.19−3.07 (m, 1 H), 2.75 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.1, 162.5, 158.7, 155.0, 144.9, 144.1, 135.6, 135.2, 133.1, 130.1, 130.3, 130.2, 129.2, 128.4, 128.3, 127.8, 127.4, 113.6, 96.8, 87.1, 83.5, 69.6, 68.9, 65.2, 60.4, 55.5, 43.7; HRMS (TOF MS ES) calcd for $C_{39}H_{39}N_4O_8^+$ 691.2768, found 691.2755.

Synthesis of ASOs Containing 2′,4′-*N*-Methoxyaminomethylene and 2′-*O*-4′-*C*-Aminomethylene and 2′,4′-Aminooxymethylene Linked Bicyclic Nucleic Acids. The synthesis of ASOs (**41**−**44**, Table 1) on a 40 μmol scale was performed on an ÄKTA oligopiolot 10 automated DNA/RNA synthesizer (GE Healthcare Bioscience, NJ) using a 1.2 mL column and UnyLinker solid support (loading 160 μmol g^{-1}). The standard phosphoramidites were used for incorporation of dA, T, dG, and dC residues. A 0.1 M solution of the phosphoramidites **1**, **2**, **16**−**21** in anhydrous CH_3CN was used for the synthesis. For the coupling step, the phosphoramidites **1**, **2**, **16**−**21** were delivered 4-fold excess over the solid support and phosphoramidites condensation were carried out for 10 min. The oxidation step was carried out with *tert*-butyl hydroperoxide (20% in CH_3CN containing 6% water, 12 min contact time).[52] A 0.2 M phenylacetyl disulfide solution in 3-picoline/CH_3CN (1:1, v/v) was used as a sulfur-transfer reagent with 3 min of contact time. Dichloroacetic acid (6%) in toluene was used for removing 4,4′-dimethoxytrityl group. A 0.7 M solution of 4,5-dicyanoimidazole in anhydrous CH_3CN was used as an activator. All other steps in the protocol supplied by manufacturer were used. At the end of the synthesis, solid support bearing the oligonucleotides was treated with 50% triethylamine in acetonitrile for 45 min to remove the cyanoethyl group from the phosphorothioate linkages. Subsequently, the solid-support bound ASOs were suspended in aqueous ammonia (28−30 wt %, 40 mL) and heated at 55 °C for 14 h. The solid supports were filtered and washed with water. The combined filtrate and the washing were concentrated, and the residues obtained were purified by HPLC on a strong anion exchange column (GE Healthcare Bioscience, Source 30Q, 30 μm, 2.54 cm × 8 cm, A is 100 mM ammonium acetate in 30% aqueous CH_3CN, B is 1.5 M NaBr in A, 0−60% of B in 60 min, flow rate of 14 mL min^{-1}). The ASOs were desalted by HPLC on a reverse phase column to yield ASOs **41**−**44** in an isolated yield of 25−30% based on the loading of the solid support. ASOs were characterized by ion-pair-HPLC−MS analysis with Agilent 1100 MSD system (Supporting Information).

Cell Culture Assay. Oligonucleotides were transfected into bEnd.3 cells (ATCC, Manassas, VA) to determine IC_{50}. bEnd.3 cells were grown in T-75 flasks incubated at 37 °C in the presence of 10% CO_2 in a complete growth medium of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM and FBS, respectively, Invitrogen, Carlsbad CA) until 70−80% confluent. Once this confluency was reached, the cells were either subcultured or plated at 1 × 10^4 cells per well into 96-well plates for transfection the following day. On the day of transfection 3 μg mL^{-1} of Lipofectin (Invitrogen, Carlsbad CA) was thoroughly mixed into Opti-MEM (Invitrogen, Carlsbad CA) and incubated at 37 °C for 30−50 min. Immediately prior to transfection, concentrated oligonucleotide solutions were added to the lipid/media mixture and serially diluted 1:2 into eight treatment concentrations for each oligonucleotide (40 nM to 312.5 pM). The plated bEnd.3 cells were removed from the incubator and washed once with 1× phosphate buffered saline PBS (Invitrogen, Carlsbad, CA). Then the transfection mixtures were added to individual wells of the 96-well plate. The plates were incubated at 37 °C for 4 h in the presence of 5% CO_2. Then the transfection mixtures were removed and the cells were refed with complete media. After 24 h of incubation, the cells were washed once with 1× PBS and lysed, and total mRNA was isolated using Qiagen 96-well RNeasy plates (RNeasy 96, Qiagen, Germantown, MD). The isolated RNA was analyzed for PTEN and cyclophilin A RNA expression levels using qRT-PCR as described.[53] PTEN RNA expression levels for each well were normalized to those of cyclophillin A and expressed as a percentage of the levels

observed in untreated control wells (% UTC). Each treatment was performed in triplicate on separate 96-well plates. IC_{50} values were determined using GraphPad Prism software by fitting the data to a sigmoidal dose−response curve (variable slopes) using a defined top of 100% and bottom of 0%.

Animal Treatment. Male BALB/c mice (6−8 weeks old, Charles River, Wilmington, MA) were housed four to a cage under conditions meeting National Institutes of Health regulations and AAALAC accreditation.[54] For the multiple administration studies ASOs **37**, **39**−**41**, **43**, and **44** were administered in PBS by intraperitoneal (ip) injection according to the indicated dose levels twice a week for 3 weeks. For the single administration studies the ASOs **40**, **42**, **43** were administered in PBS by injection according to the indicated dose levels once. The animals were maintained at a constant temperature of 23 °C and were fed standard lab diet. Animal weight was recorded prior to each dosing throughout the live phase of the study. Mice were anesthetized and sacrificed 3 days after administration of final dose. Plasma was isolated from whole blood obtained via cardiac puncture. ALT and AST levels were determined using an Olympus AU400e bioanalyzer. Liver and spleen weights were determined. Effect of compound on organ weights, normalized to body weight, was expressed relative to those of the saline treated group.

RNA Analysis. Tissues were homogenized in 4 M guanidine isothiocyanate, 25 mM EDTA, 50 mM Tris-HCl, pH 6, containing 1 M β-mercaptoethanol immediately following sacrifice and homogenization. RNA was extracted using RNeasy columns (Qiagen) according to the manufacturer's protocol. RNA was eluted from the columns with water. RNA samples were analyzed by fluorescence-based quantitative RT-PCR using an Applied Biosystems 7700 sequence detector. Levels of target RNAs as well as those of cyclophilin A, a housekeeping gene, were determined. Target RNA levels were normalized to cyclophilin levels for each RNA sample. Primers used for determination of PTEN RNA level are as follows: FP 5′ ATGACAATCATGTTGCAGCAATTC 3′, RP 5′ CGATGCAATAAATATGCACAAATCA 3′, and PR 5′ 6FAM-CTGTAAAGCTGGAA AGGGACGGACTGGT-TAMRA 3′.

ASO Extraction and HPLC−ES/MS Metabolite Identification. Organs were frozen in liquid nitrogen immediately following the removal and stored at −80 °C until ASO extraction. Tissues were weighed, and ASO was extracted as described previously.[32] Tissue samples were homogenized and subjected to solid phase extraction using phenyl bonded SPE column (Isolute). Concentrations of ASO in tissue were determined by ion pair HPLC coupled mass spectrometry.[55]

T_m Analysis. The thermal stability of the duplexes formed by oligonucleotides with the MOE and bicyclic nucleic acid modified gapmer ASOs **37**−**45**, DNA **46** (Table 2), and complementary RNA was studied by measuring the UV absorbance versus temperature curves as described previously.[56] Each sample contained 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, 4 μM oligonucleotides, and 4 μM complementary length matched RNA. Each T_m reported was an average of two experiments.

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Supporting Information Available: Mass spectral data of oligonucleotides **37**, **39**, **40**−**44**; figures of PTEN mRNA reduction in bend cells after transfection of ASOs **37**−**44**; figure of PTEN mRNA reduction in liver and ALT level after single administration of ASOs **40**, **42**, **43**; ^{31}P NMR and HRMS of compounds **1**, **2**, **20**, **21**; ^{1}H and ^{13}C NMR spectra and HRMS results of compounds **5**−**7**, **10**, **11**, **15**, **23a**, **25**, **27**, **28**, **30**−**32**, **36**; ^{19}F NMR of compound **7**; LC−MS profiles of ASOs **41**−**44**. This material is available free of charge via the Internet at http://pubs.acs.org.

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