Comparing In Vitro and In Vivo Activity of 2′-*O*-[2-(Methylamino)-2-oxoethyl]- and 2′-*O*-Methoxyethyl-Modified Antisense Oligonucleotides

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A number of 2′-*O*-modified antisense oligonucleotides have been reported for their potential use in oligonucleotide-based therapeutics. To date, most of the in vivo data has been generated for 2′-*O*-MOE (2′-*O*-methoxyethyl)- and 2′-*O*-Me (2′-*O*-methyl)-modified ASOs (antisense oligonucleotides). We now report the synthesis and biological activity of another 2′-*O*-modification, namely 2′-*O*-[2-(methylamino)-2-oxoethyl] (2′-*O*-NMA). This modification resulted in an increase in the affinity of antisense oligonucleotides to complementary RNA similar to 2′-*O*-MOE-modified ASOs as compared to first-generation antisense oligodeoxynucleotides. The ASO modified with 2′-*O*-NMA reduced expression of PTEN mRNA in vitro and in vivo in a dose-dependent manner similar to 2′-*O*-MOE modified ASO. Importantly, toxicity parameters such as AST, ALT, organ weights, and body weights were found to be normal similar to 2′-*O*-MOE ASO-treated animal models. The data generated in these experiments suggest that 2′-*O*-NMA is a useful modification for potential application in both antisense and other oligonucleotide-based drug discovery efforts.

Introduction

The first marketed antisense drug fomivirsen was a 21-mer phosphorothioate oligodeoxynucleotide (PS-DNA).[1,2] This first-generation chemistry offered a number of advantages, such as ease of synthesis, sufficient nuclease resistance for parenteral administration, activation of RNase H for cleaving the target RNA, and desired binding to cellular and serum proteins for uptake, absorption and distribution.[2] However, upon rigorous analysis, PS-DNAs were found to have some limitations with regard to their broad use as antisense therapeutics because of their relatively short in vivo half-life, low binding affinity to RNA (ca. 1 °C < DNA per modified nucleotide), and nonspecific binding to some cellular proteins.

In order to improve the in vivo profile of antisense oligonucleotides (ASOs), hundreds of chemical modifications have been synthesized and evaluated for biophysical and biochemical properties.[3–5] Antisense molecules modified at the 2′-*O*-position of the sugar either at one or both termini (gapmers, Figure 1) emerged as leading second-generation chemistries for clinical applications.[1–8] In particular, 2′-*O*-methoxyethyl (2′-*O*-MOE, Figure 1)-modified gapmer ASOs successfully demonstrated effective inhibition of many viral and cellular gene products, both in vitro and in vivo.[2] Therefore, the 2′-*O*-MOE chemistry became one of the most successful modifications to advance to clinical use because of significant improvement in metabolic stability, cellular absorption, and protein binding properties. A 2′-*O*-MOE ASO targeting ApoB (mipomersen) is in the final stage of clinical trials for treatment of cardiovascular disease.[9]

Among a number of other 2′-*O*-modifications tested, 2′-*O*-NMA (Figure 1) was selected for further evaluation because of its interesting biophysical properties. In our previous report, we showed favorable hybridization properties and nuclease stability of 2′-*O*-[2-(methylamino)-2-oxoethyl] (2′-*O*-NMA)-modified oligonucleotides and their analogues.[10] The 2′-*O*-NMA-modified oligonucleotide, when hybridized to its complementary RNA, exhibited improved T_m compared to unmodified DNA. Interestingly, the T_m enhancement was found to be similar to the corresponding 2′-*O*-MOE-modified oligonucleotide.[10] Furthermore, the oligonucleotides having 2′-*O*-NMA modification at the 3′-end exhibited enhanced exonuclease stability compared to 2′-*O*-MOE-modified oligonuclotide.[10] The high-resolution crystal structure of an oligonucleotide with 2′-*O*-NMA substituents revealed an extended $C_{3'}$-endo conformation, either the carbonyl oxygen or the amino nitrogen trap water molecule between the phosphate group and the sugar. This conformation allows high affinity binding to mRNA and protects against nuclease degradation.[11] These attributes warranted further investigation of this chemistry in pharmacologically active sequence models.

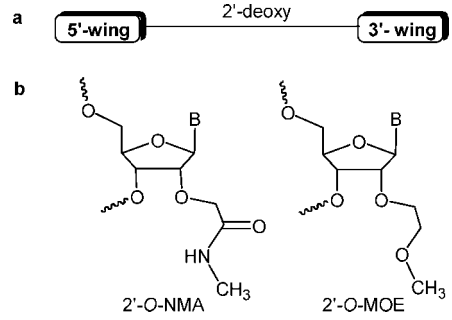

Figure 1. (a) Antisense oligonucleotides with gapmer design having 2′-modified "wings" at the 3′ and 5′-end flanking a central 2′-deoxy gap region for RNase H activity. (b) 2′-*O*-NMA- and 2′-*O*-MOE-modified RNA structures.

The aim of this study was to investigate the in vitro and in vivo potency of 2′-*O*-NMA gapmer ASO and compare its potency with the 2′-*O*-MOE gapmer chemistry. For this purpose, we first developed an efficient method to synthesize 2′-*O*-NMA-modified adenosine, guanosine, 5-methylcytidine, and 5-methyluridine nucleosides and the corresponding 3′-phosphoramidites (see below). We then successfully incorporated these

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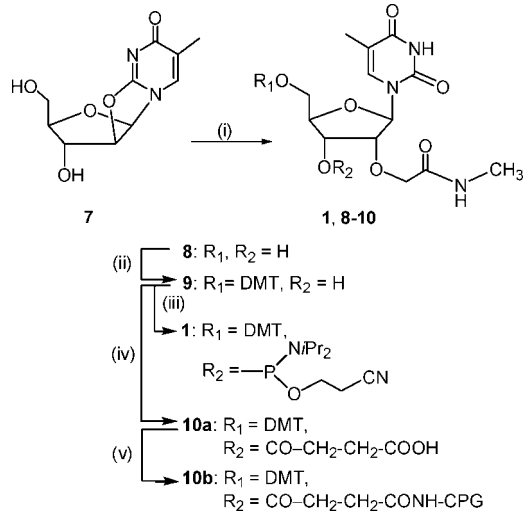
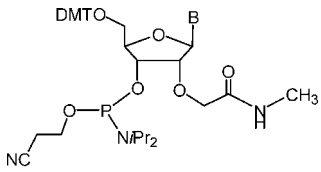
Scheme 1[a]



[a] Key: (i) **6**, BH_3−THF, $NaHCO_3$, 150 °C; (ii) DMTCl, pyridine, DMAP, rt; (iii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 4,5-dicyanoimidazole, CH_3CN, rt; (iv) succinic anhydride, pyridine, DMAP, rt; (v) 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), 4-methylmorpholine, DMF, CPG, rt.

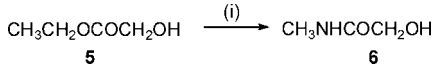
monomers into gapmer ASOs targeting PTEN mRNA. Activity of the 2′-*O*-MOE modification in this sequence was reported.[12] For comparison, we also synthesized 2′-*O*-MOE-modified ASO. These were then evaluated for their ability to reduce PTEN mRNA in vitro and in vivo, and the data from this study are discussed in this report.



1: B = ^{5Me}Ura, **2**: B = bz^{5Me}Cyt,
3: B = bzAde, **4**: B = ibuGua
bz = benzoyl, ibu = isobutyryl

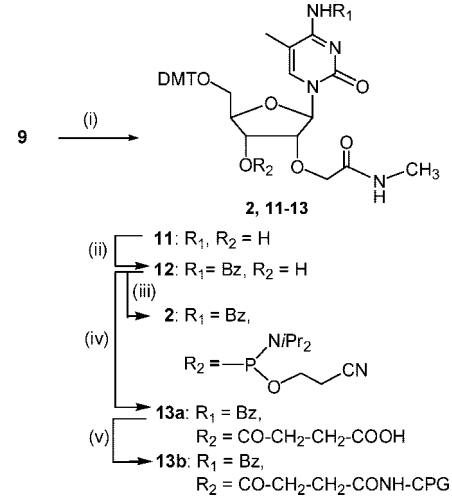
Results and Discussion

Chemistry. The synthesis of 2′-*O*-NMA-5-methyluridine and its phosphoramidite was achieved as described in Scheme 1. The synthesis started with preparation of 2-hydroxy-*N*-methylacetamide (**6**) from ethyl glycol and methylamine in 97% yield (eq 1; key: (i) aqueous CH_3NH_2/THF). The ring opening[13] of the 2,2′-anhydro-5-methyluridine **7** with a borate ester of 2-hydroxy-*N*-methylacetamide **6** at 150 °C furnished 2′-*O*-[2-(methylamino)-2-oxoethyl] 5-methyluridine (2′-*O*-NMA-^{5Me}U) **8** in 32% isolated yield. The compound **8** was then selectively protected at the 5′-position by reaction with 4,4′-dimethoxytrityl chloride (DMTCl) in pyridine to yield compound **9** (94% yield). Phosphitylation of compound **9** at the 3′-position with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of 4,5-dicyanoimidazole in CH_2Cl_2 afforded the corresponding phosphoramidite building block **1** in 84% yield. The compound **9** was also converted to the 3′-*O*-succinyl derivative **10a** and loaded on to the aminoalkyl controlled pore glass (CPG, Scheme 1) according to the standard synthetic procedure[14] to yield the functionalized solid support **10b** (55 μmol/g).



(1)

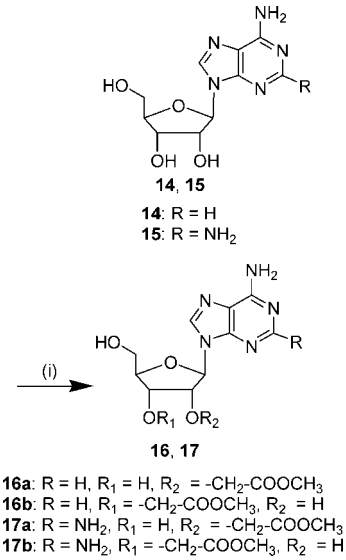
Scheme 2[a]



[a] Key: (i) (a) triethylamine, TMSCl, 1,2,4-triazole, CH_3CN, $POCl_3$, (b) NH_4OH-dioxane; (ii) benzoic anhydride, DMF; (iii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 4,5-dicyanoimidazole, CH_3CN, rt; (iv) succinic anhydride, pyridine, DMAP, rt; (v) TBTU, 4-methylmorpholine, DMF, CPG, rt.

The synthesis of the 5-methylcytidine phosphoramidite **2** is illustrated in Scheme 2. Compound **9** was transiently silylated at the 3′-*O*- position with TMSCl and triethylamine in anhydrous acetonitrile at −10 to 0 °C. The trimethylsilyl derivative was converted to the triazole derivative with 1,2,4-triazole, $POCl_3$, and Et_3N.[15] Subsequent treatment with aqueous NH_3 in dioxane at ambient temperature for 1 h gave the 5-methylcytidine analogue **11** in 69% yield. The exocyclic amino group was protected with a benzoyl group[16] with benzoic anhydride in anhydrous DMF to afford **12** (89% yield). Finally, phosphitylation at the 3′-*O*- position gave the phosphoramidite **2** (85%) in good overall yield. A portion of compound **12** was converted in to the functionalized solid support **13b** (53 μmol/g) as described above.

Key to the synthesis of the 2′-*O*-NMA purine nucleosides involved the regioselective alkylation of the 2′-hydroxyl group of unprotected nucleosides. There are many published reports on this subject involving activated[17–20] and inactivated[21,22] electrophiles. The degree of selectivity encompasses a wide range and appears to depend on the nature of the substrate and/or the electrophile. For our purposes, methyl 2-bromoacetate provided the two carbon synthon which could be transformed into the desired 2′-*O*-functionality. The esters of 2-bromoacetate have been used as electrophiles where the sugar moiety of nucleosides has been selectively protected.[23,24] When we reacted unprotected adenosine or 2-,6-diaminopurin-9-yl-riboside (eq 2; key: (i) DMF, methyl 2-bromoacetate, NaH) with methyl 2-bromoacetate under basic conditions at low temperatures (−40 °C), we observed a highly regioselective alkylation of the 2′- vs 3′-hydroxyl group in 9:1 or better ratios. This regioselective alkylation afforded 2′-*O*-(methoxycarbonylmethylene)adenosine (**16a**, eq 2) or 2′-*O*-(methoxycarbonylmethylene)-2-aminoadenosine (**17a**, eq 2) in about 75% yield. The regioselectivity in both cases was confirmed by 2D NMR (TOCSY). In contrast, when alkylation of 2,6-diaminopurin-9-ylriboside with methyl iodide was carried out under similar experimental conditions, a 2:1 mixture of the 2′-*O*- and 3′-*O*-methyl regioisomers was obtained. It appears that the highly regioselective alkylation of the unprotected purine ribosides with methyl 2-bromoacetate is somehow influenced by the effects of the carbonyl group adjacent to the reactive site in this S_N2 reaction.[25]



(2)

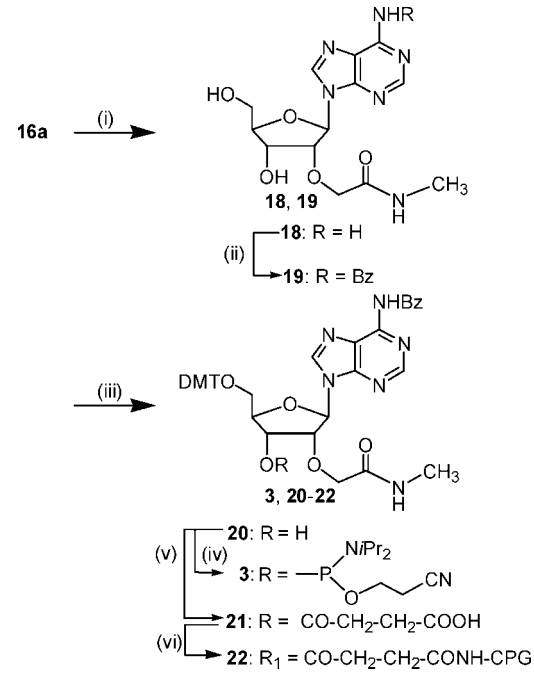
The alkylation reaction was reproducible on a multigram scale (25 g) and the small amounts of 3′-*O*-isomer formed as a side product were easily separated by chromatography at this stage or the subsequent step. The development of the robust large-scale synthetic method for purine precursors **16a** and **17a** in a cost-effective manner was helpful to the successful in vivo evaluation of this chemistry.

The 2′-*O*-(methoxycarbonylmethylene)adenosine (**16a**) was treated with aqueous *N*-methylamine in THF to yield **18** (Scheme 3, 95% yield). The exocyclic amino group of compound **18** was benzoylated under transient protection conditions[26] to obtain **19** (Scheme 3, 80%). The nucleoside **19** was then selectively protected at the 5′-position by reaction with DMTCl in pyridine to furnish **20** in 65% yield. Phosphitylation of compounds **20** at the 3′-position with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of *N*,*N*-diisopropylamine tetrazolide in CH_3CN afforded the corresponding phosphoramidite **3** in 70% yield. A portion of compound **20** was converted to functionalized solid support **22** (53 μmol/g) in a manner similar to that described earlier for other derivatives.

The compound **17a** was treated with 40% aqueous methylamine to yield 2′-*O*-[2-(methylamino)-2-oxoethyl]-2-aminoadenosine, which was subsequently converted to 2′-*O*-[2-(methylamino)-2-oxoethyl]guanosine **23** (Scheme 4) on treatment with adenosine deaminase[27–30] in aqueous buffer at pH 7.5 in good yield (61%). The exocyclic amino group of compound **23** was protected with isobutyryl group under transient protection conditions[26] to yield **24** (Scheme 3, 81%). It was then converted to 5′-*O*-DMT derivative **25** (64% yield). Phosphitylation of compound **25** at the 3′-position with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of *N*,*N*-diisopropylamine tetrazolide in CH_3CN afforded the phosphoramidite **4** in 80% yield. A portion of compound **25** was loaded on solid support **26b** (51 μmol/g).

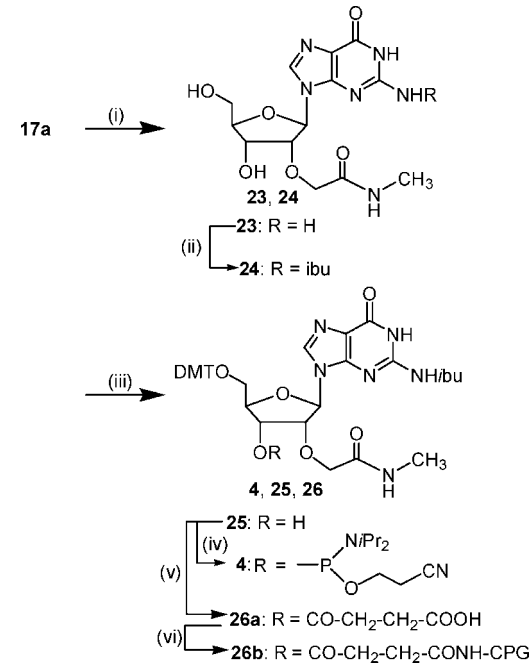
2′-*O*-NMA-Modified PTEN Gapmer ASO Synthesis. The 2′-*O*-MOE ASOs **27** and **29** (Table 1) were synthesized according to literature procedure.[31] The oligonucleotides **28** and **30** (Table 1) were synthesized on a solid-phase DNA synthesizer using the phosphoramidites **1**−**4**. A 0.1 M solution of the phosphoramidites in anhydrous acetonitrile was used for the synthesis. Phenylacetyl disulfide (PADS)[32] was used as the sulfurization agent for the syntheses.

Scheme 3[a]



[a] Key: (i) aqueous CH_3NH_2, THF; (ii) TMSCl, pyridine, benzoyl chloride, NH_3OH, H_2O; (iii) DMTCl, pyridine; (iv) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, *N*,*N*-diisopropylammonium tetrazolide, CH_3CN, rt; (v) succinic anhydride, CH_2Cl_2, DMAP, $(C_2H_5)_3N$, rt; (vi) *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), 4-methylmorpholine, DMF, CPG, rt.

Scheme 4[a]



[a] (i) (a) aqueous CH_3NH_2, THF, (b) adenosine deaminase, 100 mM sodium phosphate, pH 7.5, rt; (ii) TMSCl, Py, isobutyryl chloride, H_2O; (iii) DMTCl, pyridine; (iv) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, *N*,*N*-diisopropylammonium tetrazolide, CH_3CN, rt; (v) succinic anhydride, pyridine, DMAP, rt; (vi) HATU, 4-methylmorpholine, DMF, CPG, rt.

Solid supports were treated with 4% methylamine in aqueous ammonia (28–30 wt %) and kept at room temperature for 24 h to release the ASO from the solid support as well as remove all protecting groups from exocyclic amino groups of the bases

Table 1. 2′-*O*-NMA- and 2′-*O*-MOE-Modified ASOs[a]

no.	sequences	chemistry	target
27	5′ C$^{\&}$T$^{\&}$G$^{\&}$ C$^{\&}$T$^{\&}$A GCC TCT GGA T$^{\&}$T$^{\&}$T$^{\&}$ G$^{\&}$A$^{\&}$ 3′	2′-*O*-MOE	PTEN
28	5′ C*T*G* C*T*A GCC TCT GGA T*T*T* G*A* 3′	2′-*O*-NMA	PTEN
29	5′ T$^{\&}$C$^{\&}$C$^{\&}$ A$^{\&}$G$^{\&}$C ACT TTC TTT T$^{\&}$C$^{\&}$C$^{\&}$ G$^{\&}$G$^{\&}$ 3′	2′-*O*-MOE	control
30	5′ T*C*C* A*G*C ACT TTC TTT T*C*C* G*G* 3′	2′-*O*-NMA	control

[a] Backbone chemistry = phosphorothioates, C = 2′-deoxy-5-methylcytidine, A* = 2′-*O*-NMA-adenosine, C* = 2′-*O*-NMA-5-methylcytidine, G* = 2′-*O*-NMA-guanosine, U* = 2′-*O*-NMA-5-methyluridine, A$^{\&}$ = 2′-*O*-MOE-adenosine, C$^{\&}$ = 2′-*O*-MOE-5-methylcytidine, G$^{\&}$ = 2′-*O*-MOE-guanosine, U$^{\&}$ = 2′-*O*-MOE-5-methyluridine. Control: target mFAS.

Table 2. T_m, IC_{50}, and Mass Spectral Data of 2′-*O*-NMA- and 2′-*O*-MOE-Modified ASOs

no.	T_m[a] (°C)	IC_{50} (nM)	calcd mass	found mass	chemistry
27	67.9	43	7215.2	7213.9	2′-*O*-MOE
28	68.2	38	7345.2	7342.8	2′-*O*-NMA
29	66.8		7154.2	7153.1	2′-*O*-MOE
30	68.5		7284.2	7282.4	2′-*O*-NMA

[a] T_m conditions: see the Experimental Section.

and phosphorothioate linkages. This modified deprotection procedure was used instead of the traditional method (aqueous ammonia at 55 °C) to prevent any transamidation reaction at 2′-position of 2′-*O*-NMA ASOs. The ASOs were then purified by high-pressure liquid chromatography (HPLC) on a strong anion-exchange column and desalted using HPLC on a reversed-phase column. The ASOs were characterized by HPLC coupled mass spectrometry (Table 2).

Biology. 2′-*O*-NMA- and 2′-*O*-MOE-Modified ASOs Exhibited Similar Binding Affinity to Target RNA. In order to evaluate the activity of inhibition of RNase H mediated gene expression, a 2′-*O*-NMA gapmer ASO **28** (Table 1) targeting mouse PTEN mRNA expression[12] was synthesized. For comparison, previously characterized[12] 2′-*O*-MOE gapmer ASO **27** was also synthesized (Table 1). Additionally, we also synthesized 2′-*O*-NMA and 2′-*O*-MOE modified control ASOs **29** and **30** (Table 1) which targeted mouse FAS mRNA.

First, we wanted to know the effect of 2′-*O*-NMA modification on the binding affinity of gapmer ASOs to target RNA. We therefore determined the T_m of ASOs **28** and **30** (Table 2) using a 20-mer complementary RNA. For comparative analysis, T_m of 2′-*O*-MOE-modified ASOs **27** and **29** (Table 2) were also determined. In an earlier report, we had shown that 2′-*O*-NMA and 2′-*O*-MOE modification improves the affinity of oligonucleotide to complementary RNA compared to unmodified oligonucleotides.[10] The 2′-*O*-NMA- (**28**, **30**) and 2′-*O*-MOE-modified (**27**, **29**) ASOs (Table 2) showed similar T_m when duplexes with sequence matched RNA. These data suggest that ASOs with 2′-*O*-NMA and 2′-*O*-MOE modifications were expected to have similar binding affinity to target mRNA.

Effect of 2′-*O*-NMA-Modified ASO (28) on PTEN mRNA Expression in α Mouse Liver 12 (AML-12) Cells. In order to evaluate the in vitro activity, the 2′-*O*-NMA-modified gapmer ASO **28** (Table 1) targeting PTEN mRNA expression[12] was synthesized. The oligonucleotide **28**, when transfected to the AML-12 cell, efficiently reduced the expression of PTEN mRNA in a concentration-dependent manner (Figure 2) with an IC_{50} of 38 nM (Table 2), and potency was similar to 2′-*O*-MOE gapmer **27** (IC_{50} 43 nM, Table 2). The control ASOs **29** and **30**, with 2′-*O*-MOE and 2′-*O*-NMA modifications, respectively, did not reduce PTEN expression.

Inhibition of PTEN mRNA and Protein Expression in Mouse Liver after Systemic Administration of 2′-*O*-NMA-Modified ASO (28). In order to compare the activity of 2′-*O*-MOE and 2′-*O*-NMA ASOs in animal models, mice were treated either with ASO **27** or **28** at 10, 25, and 50 mg kg^{-1} once a week for 3 weeks. Treatment of mice with 2′-*O*-MOE- and 2′-*O*-NMA-modified ASO reduced the PTEN mRNA expression in a dose-dependent manner, whereas the control ASOs **29** and **30** did not reduce the mRNA expression even at high dose (Figure 3). Consistent with cell culture data, no significant difference in antisense potency was observed between 2′-*O*-NMA- (**28**, ED_{50} 30 mg kg^{-1}) and 2′-*O*-MOE-modified (**27**, ED_{50} 24 mg kg^{-1}) ASOs (Figure 3). However, efficacy of 2′-*O*-MOE ASO was slightly better than 2′-*O*-NMA ASO (Figure 3). More work will be needed to see if the observed difference in efficacy is real or an experimental error. When analyzed by Western blot, a similar dose dependent decrease in protein level was found in liver samples treated with 2′-*O*-NMA- and 2′-*O*-MOE-modified ASOs (Figure 4).

In order to asses the liver function in animals treated with 2′-*O*-NMA and 2′-*O*-MOE ASOs, plasma transaminase levels were examined after the last dose. The AST and ALT levels were within the normal range for all animals (Figure 5). No heaptotoxic events were observed in the H & E staining of the liver section from the animals treated with 2′-*O*-NMA or 2′-*O*-MOE ASOs at 50 mg/kg dose (Figure 6). There was no apoptosis or mitotsis or infiltrates seen with animals treated with these ASOs. The organ weights of the animals treated with 2′-

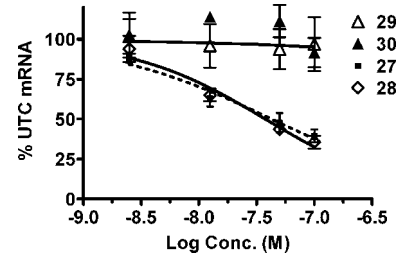


Figure 2. Reduction of PTEN mRNA in AML12 cells after trasfection with 2′-*O*-MOE- (**27**) and 2′-*O*-NMA-modified (**28**) ASOs and 2′-*O*-MOE- (**29**) and 2′-*O*-NMA-modified (**30**) control (mFAS) ASOs.

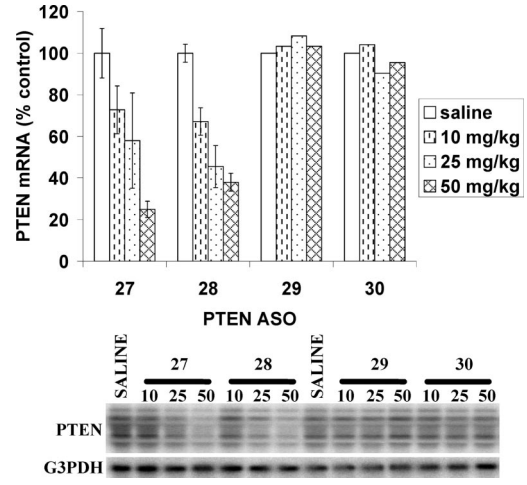


Figure 3. Inhibition of PTEN mRNA expression with 2′-*O*-MOE- (**27**) and 2′-*O*-NMA-modified (**28**) ASOs and control ASOs with 2′-*O*-MOE (**29**) and 2′-*O*-NMA (**30**) modifications in mouse liver.

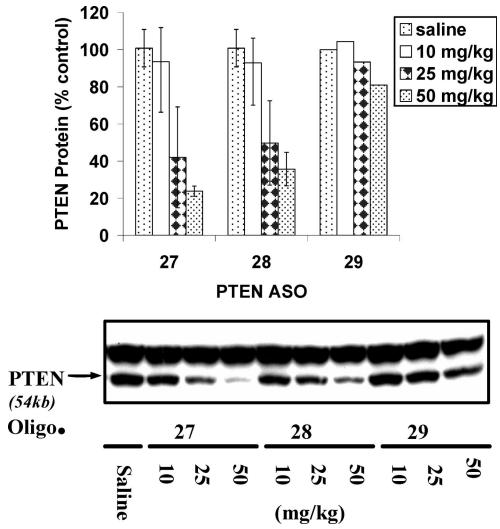


Figure 4. Inhibition of PTEN protein expression with 2′-*O*-MOE- (**27**) and 2′-*O*-NMA-modified (**28**) ASO and control ASOs with 2′-*O*-MOE (**29**) and 2′-*O*-NMA (**30**) modifications in mouse liver.

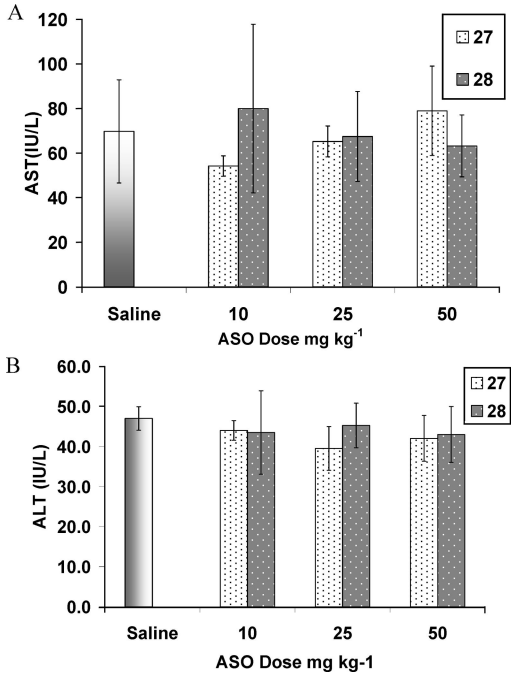


Figure 5. Plasma transaminase level for 2′-*O*-MOE (**27**) and 2′-*O*-NMA (**28**) ASOs treated mice.

O-NMA and 2′-*O*-MOE ASOs were normal at the doses used in this study.

Conclusions

In conclusion, we have developed an efficient method for the synthesis of 2′-*O*-NMA-modified adenosine, guanosine, 5-methylcytidine, and thymidine phosphoramidites.

We also demonstrate that these synthetic routes could be used for multigram scale synthesis of 2′-*O*-NMA nucleosides and the corresponding phosphoramidites. The ASO synthesis bearing 2′-*O*-NMA-modified nucleotides proceeded smoothly using normal solid-phase conditions. This chemistry was then evaluated for in vitro and in vivo potency. The potency of the 2′-*O*-NMA-modified gapmer ASO was similar to the second-generation 2′-*O*-MOE ASO. Our study demonstrates that 2′-*O*-NMA is a suitable modification for RNase H based antisense therapeutics.[33] This modification may also be useful for newly emerging areas such as siRNA and micro RNA based therapeutics, diagnostics, and gene target validation.

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 from Glen Research, Inc., Sterling, 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, Gibbstown, NJ) and visualized with UV light and spraying with a solution of *p*-anisaldehyde (6 mL), H_2SO_4 (8.3 mL), CH_3COOH (2.5 mL) in C_2H_5OH (227 mL) followed by charring. ^1H 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 the College of Chemistry, University of California, Berkeley, CA.

[5′-*O*-(4,4′-Dimethoxytrityl-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methyluridine]-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (1). 5′-*O*-(4,4′-Dimethoxytrityl)- 2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methyluridine **9** (33.60 g, 53.20 mmol) was coevaporated with anhydrous acetonitrile (150 mL). To the residue were added anhydrous CH_2Cl_2 (200 mL), 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (16.83 g, 55.90 mmol) and dicyanoimidazole (1.9 g, 16.00 mmol), and the solution was stirred at ambient temperature for 24 h under argon atmosphere. The reaction mixture was diluted with CH_2Cl_2 (400 mL) and washed with saturated aqueous $NaHCO_3$ (500 mL). The aqueous layer was back-extracted with CH_2Cl_2 (100 mL). The combined organic layer was dried (Na_2SO_4) and concentrated. The residue was dissolved in CH_2Cl_2 (100 mL) and purified by silica gel column chromatography. The product was eluted with a gradient starting with ethyl acetate–

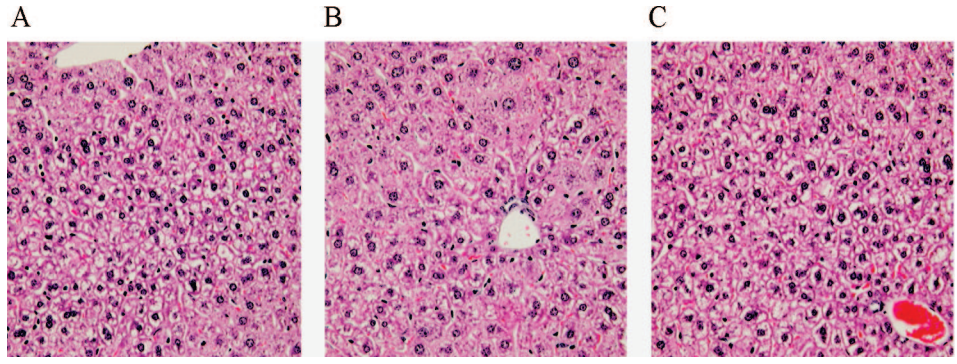


Figure 6. Histopathology of liver sections from mice treated 2′-*O*-MOE (B, **27**) and 2′-*O*-NMA (C, **28**) at 50 mg kg^{-1}. Panel A shows histopathology of liver treated with saline. H & E stain shows that livers from the animals treated with 2′-*O*-MOE (B, **27**) or 2′-*O*-NMA (C, **28**) ASOs did not exhibit any significant hepatotoxicity.

hexanes−triethylamine (80:20:1), then straight ethyl acetate, and then ethyl acetate−MeOH (9:1) to yield **1** (37.0 g, 84%): ^{31}P NMR (80 MHz, $CDCl_3$) δ 149.7, 150.7; HRMS (FAB) *m/z* calcd for $C_{43}H_{55}N_5O_{10}P^+$ 832.3687, found 832.3696.

[4-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methylcytidine]-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (2). Compound **2** (41.6 g, 85%) was synthesized from compound **12** (38.5 g, 52.4 mmol), CH_2Cl_2 (150 mL), 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (16.58 g, 55.00 mmol) and dicyanoimidazole (1.87 g, 15.70 mmol) using the procedure used for the synthesis of compound **1**: ^{31}P NMR (80 MHz, $CDCl_3$) δ 149.8, 151.2; HRMS (FAB) *m/z* calcd for $C_{50}H_{60}N_6O_{10}P^+$ 935.46520, found 935.4638.

[6-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl-2′-*O*-[2-(methylamino)-2-oxoethyl]adenosine]-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (3). 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (2.41 mL, 7.59 mmol) was added dropwise into a solution of the nucleoside **20** (3.76 g, 5.06 mmol) and diisopropylamine tetrazolide (0.87 g, 5.06 mmol) in anhydrous CH_3CN (25 mL). The reaction mixture was stirred at room temperature under argon atmosphere for 10 h. The reaction mixture was diluted with EtOAc (200 mL), extracted with saturated aqueous $NaHCO_3$ (2 × 100 mL), and then washed with brine (100 mL). The organic phase dried over $MgSO_4$, filtered, and concentrated. The residue was dissolved in CH_2Cl_2 (5 mL) and added to a vigorously stirred hexane (250 mL), and precipitate formed was collected by decanting the supernatant. The precipitation processes was repeated once more to yield **3** (3.34 g, 70%): ^{31}P NMR (80 MHz, $CDCl_3$) δ 151.08 and 151.41; HRMS (FAB) *m/z* calcd for $C_{50}H_{58}N_8O_9P^+$ 945.4064, found 945.4054.

[5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-2-*N*-isobutyrylguanosine]-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (4). Compound **25** (25.0 g, 34.5 mmol) and diisopropylamine tetrazolide (1.77 g, 10.00 mmol) were coevaporated with anhydrous acetonitrile (150 mL). To the resulting oil were added anhydrous acetonitrile (170 mL) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′- tetraisopropylphosphorodiamidite (11.46 g, 38.00 mmol), and the mixture was stirred at ambient temperature for 24 h under argon atmosphere. The reaction mixture was concentrated to an oil and then partitioned between CH_2Cl_2 (300 mL) and saturated aqueous $NaHCO_3$ (300 mL). The aqueous layer was back-extracted with CH_2Cl_2 (100 mL). The combined organic layer was dried (Na_2SO_4) and concentrated to an oil under reduced pressure. The residue was purified by silica gel column chromatography and eluted with a gradient (0–10%) of methanol in EtOAc−triethylamine (99:1). Appropriate product-containing fractions were combined, concentrated under reduced pressure, and coevaporated with CH_3CN (200 mL). The foam was dissolved in a mixture of EtOAc−CH_2Cl_2 (1:1, 40 mL), and then hexanes were slowly added until cloudiness was maintained. After 20 min, a precipitate formed which was collected, washed with hexanes−EtOAc−CH_2Cl_2 (8:1:1), and dried to yield **4** (14.46 g, 45.35%). The filtrate was concentrated to yield 10 g (31.36%) of 4 as a foam: ^{31}P NMR (80 MHz, $CDCl_3$) δ 146.9, 149.5; HRMS (FAB) *m/z* calcd for $C_{47}H_{60}N_8O_{10}P^+$ 927.4170, found 927.4160.

2-Hydroxy-*N*-methylacetamide (6). Aqueous methylamine (40 wt %, 825 mL, 10630.00 mmol) was added to THF (1 L), and the solution was cooled to 10 °C. Ethyl glycolate **5** (500 g, 4800.00 mmol) was added slowly with stirring at a rate so as to maintain the internal temperature between 15 and 20 °C (ca. 1 h). The reaction mixture was concentrated under reduced pressure to an oil. The oil was coevaporated with anhydrous acetonitrile. Diethyl ether (1 L) was added to the oil, which caused a white solid to form exothermically (Caution: add slowly to prevent ether from boiling over). After stirring, the slurry was filtered and the resulting solid was washed with diethyl ether (500 mL) and then hexanes (2 × 500 mL) and then dried to yield **6** (417 g, 97%) as white irregular crystals: mp 64–66 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.80 (d, J = 37 Hz, 2 H), 4.00 (s, 3 H), 4.4 (br s, 1 H), 7.0 (br s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.6, 61.9, 173.4; HRMS (EI) *m/z* calcd for $C_3H_7NO_2^+$ 89.0477, found 89.0477.

2′-*O*-[2-(Methylamino)-2-oxoethyl]-5-methyluridine (8). To an 8 L Parr stainless steel pressure reactor were added borane in tetrahydrofuran (720 mL, 1.5 M, 1070.00 mmol), $NaHCO_3$ (6.45 g, 76.00 mmol), and 2-hydroxy-*N*-methylacetamide **6** (400 g, 4490.00 mmol). The mixture was stirred uncovered until the evolution of hydrogen gas subsided (ca. 5 min). 2,2′-Anhydro-5-methyluridine **7** (215.90 g, 899.00 mmol) was added, and the vessel was sealed and heated with stirring to 150 °C (internal temperature with pressure ca. 110 psi). The reaction was heated for 48 h more and then allowed to cool to ambient temperature. The dark solution was decanted and concentrated under reduced pressure (50 °C, 20 mm) to dark oil. Water (1 L) was added, and the mixture was heated to 100 °C for 30 min to hydrolyze the borate ester. The mixture was concentrated, and the residue was dissolved in a mixture of CH_2Cl_2−acetone−CH_3OH (1 L, 40:10:1) and applied onto a silica gel column (2.5 kg). The product was eluted with a gradient of 1−6% CH_3OH in CH_2Cl_2−acetone (40:10). After concentration, the residue was crystallized from CH_3OH to yield **8** (95.5 g, 32%) as a white solid: mp 193–194 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.77 (s, 3 H), 2.65 (d, J = 4.7 Hz, 3 H), 3.45–3.82 (m, 2 H), 3.83–4.15 (m, 5H), 5.22 (t, J = 5.0 Hz, 1 H), 5.40 (d, J = 6.3 Hz, 1 H), 5.85 (d, J = 3.8 Hz, 1 H), 7.82 (s, 1 H), 7.87 (d, J = 4.6 Hz, 1 H), 11.37 (br s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.2, 25.6, 60.0, 68.2, 69.3, 82.2, 84.1, 86.4, 109.1, 136.1, 150.5, 163.8, 169.7; MS (ES) *m/z* 328.1 [M − H]$^-$; HRMS (TOF MS ES) *m/z* calcd for $C_{13}H_{18}N_3O_7^-$ 328.1145, found 328.1132.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methyluridine (9). 2′-*O*-[2-(Methylamino)-2-oxoethyl]-5-methyluridine **8** (93.0 g, 282.00 mmol) was coevaporated with anhydrous pyridine (500 mL) and then dissolved in the same (1 L). 4,4′-Dimethoxytrityl chloride (97 g, 286.00 mmol) was added in four portions over 2 h. The reaction mixture was stirred for an additional 1 h at room temperature under argon atmosphere. TLC indicated a complete reaction (R_f of starting nucleoside 0.25, product 0.60, in CH_2Cl_2−acetone−methanol, 20:5:3). The reaction was concentrated under reduced pressure to thick oil and then redissolved in a mixture of EtOAc and saturated aqueous $NaHCO_3$ (1 L each). The organic layers were separated. The aqueous layer was extracted with more EtOAc (200 mL), and the combined organic layer was washed with more $NaHCO_3$ solution (200 mL). The organic layer was dried (Na_2SO_4), concentrated, and then redissolved in CH_2Cl_2 (200 mL). The solution purified by silica gel column chromatography (2.5 kg) and eluted with a gradient of CH_3OH (0−10%) in EtOAc to yield **9** (167.5 g, 94%) as a white foam: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41 (s, 3 H), 2.64 (d, J = 4.7 Hz, 3 H), 3.20–3.40 (m, 2 H), 3.74 (s, 6 H), 4.0–4.4 (m, 5H), 5.45 (d, J = 7.1 Hz, 1 H), 5.84 (d, J = 3.2 Hz, 1 H), 6.90 (d, J = 8.8, 4 H), 7.3–7.5 (m, 9 H), 7.50 (s, 1 H), 7.86 (q, J = 4.3, 4.7 Hz, 1 H), 11.43 (br s, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.0, 26.0, 55.4, 61.9, 68.6, 70.0, 83.6, 84.2, 86.0, 88.6, 111.4, 113.5, 127.3, 128.2, 128.3, 130.3, 135.3, 135.5, 135.6, 144.5, 151.4, 158.9, 164.4, 170.8; MS (ES) *m/z* 630.2 [M − H]$^-$; HRMS (TOF MS ES) *m/z* calcd for $C_{34}H_{36}N_3O_9^-$ 630.2452, found 630.2448.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methyluridine 3′-*O*-Succinate (10a). Compound **9** (5 g, 7.92 mmol) was mixed with succinic anhydride (1.58 g, 15.8 mmol) and DMAP (0.483 g, 3.95 mmol). The mixture was dried over P_2O_5 in vacuo overnight at 40 °C. The dried material was dissolved in anhydrous pyridine (30 mL), and the reaction mixture was stirred at room temperature under argon atmosphere overnight. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed with 20% citric acid (ice cold, 100 mL). The citric acid layer was then washed with 20 mL of CH_2Cl_2. The combined organic phase was dried over Na_2SO_4 and evaporated to foam. It was then coevaporated with 50 mL of CH_3CN and 3 mL of triethylamine to give **10a** (5.79 g, 97%) as a yellow foam: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37 (s, 3 H), 2.61 (m, 4 H), 2.82, (d, J = 4.8 Hz, 3H), 3.22–3.36 (m, 1 H), 3.62–3.55 (m, 1 H), 3.80 (s, 6 H), 4.16 (d, J = 2.1 Hz, 2H), 4.31 (m, 2H), 5.45 (t, J = 4.3 Hz, 1 H), 6.07 (d, J = 5.5 Hz, 1 H), 6.85 (d, J = 10.7, 4 H), 7.03 (q, J = 4.8 Hz, 1 H), 7.23–7.38 (m, 9 H), 7.60 (s, 1 H), 10.00 (br s, 1 H); ^{13}C NMR (75

MHz, $CDCl_3$) δ 11.9, 26.0, 29.0, 29.4, 55.4, 62.7, 70.7, 71.4, 81.1, 81.4, 86.5, 87.6, 112.2, 113.6, 127.5, 128.3, 130.3, 135.1, 135.2, 144.0, 151.2, 159.0, 164.6, 170.0, 171.6, 175.5; MS (ES) *m*/*z* 730.2 $[M - H]^-$.

5′-*O*-Dimethoxytrityl-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methyluridine 3′-*O*-Succinyl CPG (10b). Compound **10a** (0.2 g, 0.27 mmol) was dissolved in DMF (0.60 mL). 2-(1*H*-Benzotriazole-1-yl)-1,2,3-tetramethyluronium tetrafluoroborate (TBTU, 0.81 g, 0.25 mmol) and 4-methylmorpholine (0.50 g, 0.49 mmol) were added. The mixture was vortexed until it became a clear solution. Anhydrous DMF (2.2 mL) and activated CPG (1.11 g, 115.2 μmol g^{-1}) were added and allowed to shake on a shaker for 18 h. The mixture was filtered and the functionalized CPG washed thoroughly with DMF, CH_2Cl_2, and diethyl ether. The functionalized CPG was dried over P_2O_5 in vacuo. To cap the unfunctionalized sites, it was mixed with the capping reagents (2 mL of 10% pyridine, 10% acetic anhydride in THF and 2 mL of *N*-methylimidazole in THF) and allowed to shake for 2 h. It was then filtered and washed with acetonitrile and diethyl ether to give the functionalized CPG. After drying in vacuo, the loading capacity is determined (53 μmol g^{-1}) using standard dimethoxytrityl assay.[34]

5′-*O*-(4,4′-Dimethoxytrityl-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methylcytidine (11). A 10 L jacketed reaction vessel was equipped with a mechanical stirrer, thermometer, argon line, and addition funnel and connected to a recirculating heater/chiller. Compound **9** (112.40 g, 178.00 mmol) was coevaporated with anhydrous acetonitrile (1 L) and then the residue dissolved in the same (4 L). Triethylamine (396 mL, 2850.00 mmol) was added, and the solution was cooled to –10 °C. Trimethylsilyl chloride (113 mL, 890.00 mmol) was dropped in over 15 min. The solution was warmed to 0 °C and maintained for 1 h. TLC indicated a complete reaction (R_f of starting material 0.20, TMS intermediate 0.65, EtOAc−CH_3OH, 95:5). 1,2,4-Triazole (123 g, 1780.00 mmol) was added as a solid. The reaction mixture was cooled to –12 °C, and phosphorus oxychloride (49.7 mL, 534.00 mmol) was dropped in slowly so as to maintain the temperature below –10 °C (ca. 30 min). The reaction was allowed to warm to 35 °C over 2 h to give a reddish suspension. TLC indicated a ca. 95+% complete reaction (R_f of TMS U intermediate 0.65, TMS triazole intermediate 0.35 with a glow in long UV light, EtOAc−CH_3OH, 95:5). Water (2 L) and EtOAc (3 L) were added. After the mixture was stirred and allowed to separate, the aqueous layer was removed and the organic layer was washed with more water (3 L) as before. The organic layer was concentrated under reduced pressure to foam. The foam was dissolved in dioxane (1 L). Aqueous ammonium hydroxide (50 mL, 28–30 wt %) was added in two portions over 1 h. (Note: To reduce the possibility of transamidation, a minimum amount of ammonia was used and the reaction progress was followed by TLC). TLC indicated a ca. 95+% complete reaction (R_f of TMS triazole intermediate 0.65, unblocked product 0.25, CH_2Cl_2−acetone−CH_3OH, 20:5:3). The reaction mixture concentrated to foam and dissolved in a minimum amount of CH_2Cl_2 and purified by silica gel column chromatography. The product was eluted with a 0–15% CH_3OH in CH_2Cl_2−acetone 4:1 to yield **11** (77.3 g, 69%) as a white foam: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.43 (s, 3H), 2.65 (d, J = 4.7 Hz, 3H), 3.20–3.40 (m, 2H), 3.74 (s, 6H), 3.84–3.86 (m, 1H,), 4.04–4.13 (m, 2H), 4.19–4.32 (m, 2H), 5.41 (d, J = 7.6 Hz, 1H), 5.80 (d, J = 1.8 Hz, 1H), 6.81 (br s, 2H), 6.92 (d, J = 8.9 Hz, 4H), 7.21–7.52 (m, 9H), 7.49 (s, 1H), 7.93 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 26.1, 55.4, 61.9, 68.9, 70.3, 83.0, 84.5, 86.9, 89.6, 102.2, 113.5, 127.3, 128.2, 128.4, 130.3, 130.4, 135.7, 135.8, 138.3, 144.7, 156.2, 158.8, 158.9, 165.7, 171.1; MS (ES) *m*/*z* 629.2 $[M - H]^-$.

4-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methylcytidine (12). Compound **11** (77.3 g, 123.00 mmol) and benzoic anhydride (33.25 g, 147.00 mmol) were dissolved in anhydrous DMF (350 mL). The resulting solution was stirred at ambient temperature for 20 h under argon atmosphere. TLC indicated a complete reaction (R_f of starting material 0.15, product 0.80, EtOAc−CH_3OH, 9:1). The reaction mixture was diluted with EtOAc (1.2 L) and washed with saturated aqueous $NaHCO_3$ (2 L). The organic layer was separated, dried (Na_2SO_4), and concentrated under reduced pressure to white foam. The residue was purified by silica gel column chromatography and eluted with EtOAc−hexanes 4:1 to yield **12** (80.20 g, 89.4%): ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.63 (s, 3H), 2.67 (d, J = 4.7 Hz, 3H), 3.20–3.40 (m, 2H), 3.77 (s, 6H), 4.0–4.5 (m, 5H), 5.57 (d, J = 7.4 Hz, 2H), 5.90 (d, J = 2.2 Hz, 1H), 6.94 (d, J = 8.0 Hz, 4H), 7.23–7.62 (m, 12 H), 7.80 (br s, 1H), 7.95 (s, 1H), 8.18 (br s, 2 H), 13.07 (br s, 1H); ^{13} C NMR (75 MHz, $CDCl_3$) δ 12.5, 25.4, 54.9, 61.4, 68.5, 69.6, 82.8, 83.4, 86.5, 88.1, 111.7, 112.9, 126.8, 127.8, 128.8, 129.4, 129.8, 132.1, 134.9, 135.0, 136.4, 136.3, 144.0, 148.0, 158.4, 158.3, 159.4, 170.2, 170.9; HRMS (TOF MS ES) *m*/*z* calcd for $C_{41}H_{41}N_4O_9^-$ 733.2717, found 733.2718.

4-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methylcytidine 3′-*O*-Succinate (13a). Compound **13a** (0.2 g, 71%) was synthesized from compound **12** (0.25 g, 0.38 mmol), succinic anhydride (0.057 g, 0.57 mmol), DMAP (0.023 g, 0.19 mmol), CH_2Cl_2 (1 mL), and triethylamine (0.106 mL, 0.76 mmol) using the procedure used for the synthesis of compound **10a**: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.56 (s, 3H), 2.63 (m, 4H), 2.83 (d, J = 4.8 Hz, 3H), 3.34–3.64 (m, 2H), 3.73 (s, 3H), 3.79 (s, 4H), 4.00–4.34 (m, 3H), 5.41 (t, J = 4.4 Hz, 1H), 6.16 (d, J = 5.6 Hz, 1H), 6.94 (d, J = 8.4 Hz, 4H), 7.24–7.56 (m, 12 H), 7.74 (s, 1H), 8.25–8.28 (m, 2 H); ^{13} C NMR (50 MHz, $CDCl_3$) δ 12.8, 25.8, 28.7, 29.0, 55.2, 62.0, 69.3, 70.4, 71.1, 81.4, 81.5, 86.9, 87.4, 113.0, 113.3, 127.3, 128.1, 129.9, 130.0, 132.5, 134.8, 135.8, 136.8, 143.9, 148.4, 158.8, 159.3, 169.7, 171.5, 174.7, 179.4; MS (API-EI) *m*/*z* 857.2 $[M + Na]^+$.

4-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-5-methylcytidine 3′-*O*-Succinyl CPG (13b). Compound **13b** (55 μmol g^{-1}) was synthesized from compound **13a** (0.2 g, 0.24 mmol), DMF (0.64 mL), 2-(1*H*-benzotriazole-1-yl)-1,2,3-tetramethyluronium tetrafluoroborate (TBTU, 0.83 g, 0.26 mmol), 4-methylmorpholine (0.53 g, 0.52 mmol), anhydrous DMF (2.2 mL), and activated CPG (1.13 g, 115.2 μmol/g) using the procedure used for the synthesis of compound **10b**.

2′-*O*-(Methoxycarbonylmethylene)adenosine (16a). Adenosine **14** (25.00 g, 93.50 mmol) was dissolved in anhydrous DMF (900 mL). The solution was cooled to –50 °C, and NaH (60% dispersion in mineral oil, 4.86 g, 122.00 mmol) was added in two portions. The reaction mixture was allowed to warm to –30 °C and stirred for 30 min under argon atmosphere. After the reaction mixture was cooled to –50 °C again, methyl 2-bromoacetate (11.5 mL, 122.00 mmol) was added dropwise, and the reaction mixture was allowed to warm to ambient temperature. After the mixture was stirred at ambient temperature for 1 h, MeOH (100 mL) was added, the reaction mixture was stirred for 10 min, and the solvent was evaporated in vacuo to give a foam. The product was coevaporated with EtOAc to afford **16a** (14.23 g, 45%) as a crude solid which was of sufficient purity for use in the following reaction. A small portion of the product was purified by column chromatography and characterized: ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.48 (s, 3H), 3.56–3.64 (m, 2H), 4.0 (m, 1H), 4.11–4.24 (dd, J = 16.6, 11.2 Hz, 2H), 4.38 (m, 1H), 5.63 (m, 1H), 5.29 (d, J = 4.8 Hz, 1H), 5.40 (m, 2H), 6.02 (d, J = 6.4 Hz, 1H), 7.37 (m, 2H), 8.12 (s, 1H), 8.28 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 52.7, 63.5, 69.4, 71.2, 84.9, 88.0, 89.3, 121.4, 141.3, 148.7, 152.6, 156.4, 172.4; HRMS (FAB) *m*/*z* calcd for $C_{13}H_{17}N_5O_6 + H^+$ 340.1257, found 340.1257. 2D-^{1}H NMR (TOCSY) confirmed 2′-*O*-alkylation.

2′-*O*-(Methoxycarbonylmethylene)-2,6-diaminopurin-9-yl Riboside (17a). 2,6-Diaminopurin-9-yl riboside **15** (25.00 g, 88.60 mmol) was dissolved in anhydrous DMSO (90 mL) with gentle heating to give a brown solution which was diluted with anhydrous DMF (355 mL). The reaction mixture was cooled to 5 °C, NaH (60% oil dispersion, 6.20 g, 155.00 mmol) was added, and the reaction mixture was allowed to warm to ambient temperature and stirred for 1 h. The suspension was cooled to –40 °C in an CH_3CN-CO_2 bath, and methyl 2-bromoacetate (14.29 mL, 155.00 mmol) was added slowly. The reaction mixture was allowed to slowly warm to ambient temperature over 1 h, and after 17 h, glacial AcOH (6 mL) was added dropwise to give a solution with pH 4.

The solvent was evaporated in vacuo (1 Torr) at 45 °C to give an amorphous mass to which CH_2Cl_2 (400 mL) was added. The mixture was mechanically stirred for 15 min to give a suspension to which hexanes (400 mL) was slowly added. The suspension stood for 15 min, after which time the solids settled. The supernatant was decanted, and the solid was washed with hexanes (3 × 200 mL) and then dissolved in H_2O (250 mL). The aqueous layer was extracted with diethyl ether (3 × 100 mL), which resulted in an emulsion within the organic phase. The organic phase was then washed with H_2O (2 × 100 mL), the aqueous portions were combined, and the H_2O was evaporated in vacuo at 45 °C to give a residue which was utilized for the following reaction. A small portion of product was dissolved in MeOH, adsorbed onto silica and was purified by column chromatography using 15% MeOH in CH_2Cl_2 to yield **17a** as a white solid: ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.53 (s, 3H), 3.55 (m, 2H), 3.92 (m, 1H), 4.20 (q, J = 16.8 Hz, 2H), 4.29 (m, 1H), 4.52 (m, 1H), 5.21 (d, J = 2.5 Hz, 1H), 5.39 (m, 1H), 5.73 (br s, 2H), 5.87 (d, J = 6.8 Hz, 1H), 6.76 (br s, 2H), 7.91 (s, 1H); ^{13}C NMR (50 MHz, CD_3OD) δ 52.4, 63.4, 68.6, 71.1, 83.4, 88.0, 88.7, 115.0, 139.1, 152.0, 157.7, 161.3, 172.6; HRMS (FAB) m/z calcd for $C_{13}H_{18}N_6O_6^+$ 355.1362, found 355.1366.

2′-*O*-[2-(Methylamino)-2-oxoethyl]adenosine (18). Compound **16a** (89.63 g, 155.15 mmol) was dissolved in THF (1300 mL), *N*-methylamine (310 mmol, 350 mL, of 40 wt % solution in water) was added, and the reaction mixture was stirred at ambient temperature for 18 h. The mixture was concentrated to oil which was dissolved in EtOAc (420 mL). The organic phase was washed with water (3 × 350 mL) and brine (1 × 350 mL), dried (Na_2SO_4), and evaporated under reduced pressure to yield **18** (84.80 g, 95%) as a white solid: ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.60 (d, J = 4.7 Hz, 3H), 3.60–3.53 (m, 1H), 3.72–3.66 (m, 1H), 3.97 (s, 2H), 3.99–4.02 (m, 1H), 4.33 (q, J = 4.7, 4.3, 5.1 Hz, 1H), 4.50 (t, J = 5.1 Hz, 1H), 5.35 (t, J = 4.9 Hz, 1H), 5.48 (d, J = 5.3 Hz, 1H), 6.07 (d, J = 5.1 Hz, 1H), 7.34 (s, 2H), 7.83 (q, J = 4.1, 4.5 Hz, 1H), 8.14 (s, 1H), 8.36 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.0, 61.1, 69.2, 69.3, 82.2, 85.4, 86.0, 119.3, 139.5, 148.9, 152.5, 156.1, 169.2; MS (ES) m/z 337.1 [M − H]$^-$; HRMS (TOF MS ES) m/z calcd for $C_{13}H_{17}N_6O_5^-$ 337.1260, found 337.1260.

6-*N*-Benzoyl-2′-*O*-[2-(methylamino)-2-oxoethyl]adenosine (19). Compound **18** (84 g, 146.00 mmol) was dissolved in anhydrous pyridine (500 mL) and cooled in an ice bath. Chlorotrimethylsilane (70 mL, 582.60 mmol) was added dropwise, and the reaction mixture was stirred for 30 min at 0 °C under argon atmosphere. To the reaction mixture benzoyl chloride (68 mL, 582.60 mmol) was added dropwise. The ice bath was removed after 30 min, and the reaction mixture was stirred for 3 h at room temperature under argon atmosphere. The reaction mixture was cooled in an ice bath. Water (100 mL) was added followed with aqueous NH_3 (28–30 wt %, 100 mL). After 1 h, the reaction mixture was partitioned between water and EtOAc (600/600 mL). The aqueous layer was extracted with EtOAc (2 × 500 mL). The EtOAc layer was washed with brine (500 mL), dried (anhydrous $MgSO_4$), filtered, and evaporated in vacuo to oil. The resulting crude material was purified by silica gel column chromatography using Et_2N/MeOH/EtOAc/$CHCl_3$ (2/5/60/33, v/v/v/v) as the eluent to yield **19** (79.32 g, 72.22%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.62 (d, J = 4.7 Hz, 3H), 3.77–3.56 (m, 2H), 4.08–3.99 (m, 3H), 4.39 (q, J = 4.9, 5.4 Hz, 1H), 4.59 (t, J = 4.8 Hz, 1H), 5.16 (t, J = 5.6 Hz, 1H), 5.56 (d, J = 5.8 Hz, 1H), 6.24 (d, J = 4.7 Hz, 1H), 7.68–7.53 (m, 3H), 7.90 (q, J = 4.2, 4.7 Hz, 1H), 8.05 (d, J = 7.2 Hz, 2H), 8.72 (s, 1H), 8.77 (s, 1H), 11.21 (br s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.1, 60.8, 69.0, 69.4, 82.2, 85.3, 85.9, 125.5, 128.8, 132.4, 133.3, 142.9, 150.4, 151.7, 151.9, 165.6, 169.2; MS (ES) m/z 441.1 [M-H]$^-$; HRMS (TOF MS ES) m/z calcd for $C_{20}H_{21}N_6O_6^-$ 441.1523, found 441.1518.

6-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]adenosine (20). Compound **19** (5.7g, 12.90 mmol) was coevaporated with pyridine (3 × 50 mL) and the residue dissolved in anhydrous pyridine (129 mL). 4,4′-Dimethoxytrityl chloride (5.24 g, 15.46 mmol) was added in two portions to the reaction mixture. The reaction mixture was stirred at room temperature for 10 h under argon atmosphere. The solvent was removed under reduced pressure to get an oil. The oil was partitioned between ethyl acetate (200 mL) and water (200 mL). The aqueous phase was extracted with ethyl acetate (2 × 50 mL). Combined ethyl acetate layer dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography and eluted with MeOH/$CHCl_3$ (5/95, v/v) to afford **20** (6.24 g, 65%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.62 (d, J = 4.7 Hz, 3H), 3.27–3.21 (m, 2H), 3.71 (s, 6H), 4.18–4.04 (m, 3H), 4.57 (m, 1H), 4.70 (t, J = 4.8, 3.8 Hz, 1H), 5.53 (d, J = 6.8 Hz, 1H), 6.29 (d, J = 3.5 Hz, 1H), 6.84–6.80 (m, 4H), 7.36–7.16 (m, 9H), 7.67–7.52 (m, 3H), 7.89 (q, J = 3.0, 4.7 Hz, 1H), 8.04 (d, J = 7.2 Hz, 2H), 8.56 (s, 1H), 8.70 (s, 1H), 11.21 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.0, 55.4, 63.3, 70.2, 70.6, 81.7, 84.1, 87.0, 87.7, 113.5, 127.2, 128.0, 128.1, 128.3, 129.2, 129.3, 130.2, 133.1, 133.3, 133.7, 135.7, 141.7, 144.6, 147.6, 151.5, 152.0, 152.3, 158.8, 164.9, 170.0, 170.5; MS (ES) m/z 743.2 [M − H]$^-$; HRMS (TOF MS ES) m/z calcd for $C_{41}H_{39}N_6O_8^-$ 743.2829, found 743.2838.

6-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl-2′-*O*-[2-(methylamino)-2-oxoethyl]adenosine-3′-*O*-succinate (21). Compound **20** (1.5 g, 2.03 mmol) was mixed with succinic anhydride (0.4g, 4.03 mmol) and DMAP (0.13 g, 1.04 mmol). The mixture was dried over P_2O_5 in vacuo overnight at 40 °C. The dried mixture was dissolved in anhydrous CH_2Cl_2 (6 mL) and triethyamine (1.12 mL, 8.66 mmol) was added with stirring at ambient temperature under argon atmosphere for 4 h. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with 10% citric acid (ice cold, 50 mL) followed by water (50 mL). The organic phase was dried (anhydrous Na_2SO_4) and evaporated in vacuo. The resulting foam was purified by silica gel column chromatography and eluted with MeOH/CH_2Cl_2/pyridine (1/8.9/0.1, v/v/v) to yield **21** (1.71 g, 98%) as a white foam: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.63–2.79 (m, 7H), 3.38–3.45 (m, 1H), 3.53–3.60 (m, 1H), 3.79 (s, 6H), 3.90–4.15 (m, 2H), 4.38 (m, 1H), 4.95 (m, 1H), 5.49 (m, 1H), 6.21 (d, J = 6.7 Hz, 1H), 6.83 (d, J = 8.9 Hz, 4H), 6.99 (q, J = 5.3 Hz, 1H), 7.22–7.42 (m, 9H), 7.50–7.63 (m, 3H), 8.04 (d, J = 6.7 Hz, 2H), 8.25 (s, 1H), 8.70 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.6, 29.0, 29.2, 55.1, 62.9, 70.3, 71.7, 80.5, 82.4, 86.6, 87.0, 113.2, 127.0, 128.1, 128.6,129.9, 132.7, 133.3, 135.2, 142.0, 144.1, 149.8, 151.7, 152.0, 152.3, 158.6, 165.0, 169.3, 171.4, 174.8; MS (ES) m/z 842.9 [M − H]$^-$.

6-*N*-Benzoyl-5′-*O*-(4.4′-dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]adenosine 3′-*O*-Succinyl CPG (22). Compound **22** (70.3 μmol g^{-1}) was synthesized from compound **21** (1.0g, 1.18 mmol), DMF (16 mL), HATU (0.45 g, 1.18 mmol), diisopropylethylamine (0.63 g, 0.81 mL,4.84 mmol), anhydrous DMF (33 mL), and activated CPG (7.00 g, 115.2 μmol/g) using the procedure described for the synthesis of compound **10b**.

2′-*O*-[2-(Methylamino)-2-oxoethyl]guanosine (23). Compound **17a** (100 g, 280.0 mmol) was dissolved in water (300 mL) and *N*-methylamine (40 wt % solution in water, 87.6 mL, 1010.00 mmol) was added in one portion with mechanical stirring at ambient temperature. After 4 h, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in a phosphate buffer (0.1 M, 1 L, pH 7.0). Adenosine deaminase (1.5 g) was added, and the mixture was mechanically stirred very slowly at 37 °C for 18 h. TLC indicated about a 60% complete reaction and the pH had risen to 8.5. The pH was adjusted to 7.0 with phosphoric acid and another portion of enzyme (0.5 g) was added. After 24 h, the reaction was complete, and a precipitate formed was collected washed with water and dried to yield **23** (60.9 g, 61% yield): ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.62 (d, J = 4.7 Hz, 3 H), 3.66–3.51 (m, 2H), 3.95–3.91 (m, 1H), 3.97 (s, 2H), 4.25 (m, 1H), 4.34 (t, J = 5.1, 1H), 5.07 (t, J = 5.4 Hz, 1H), 5.44 (d, J = 4.9, 1H), 5.85 (d, J = 5.4, 1H), 6.48 (s, 2H) 7.83 (q, J = 4.6, 1H), 7.95 (s, 1H), 10.59 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.1, 61.0, 69.1, 69.3, 82.4, 84.6, 85.0, 116.7, 135.3, 151.0, 153.8, 156.7, 169.2; MS (ES) m/z 353.1 [M − H]$^-$; HRMS (TOF MS ES) m/z calcd for $C_{13}H_{17}N_6O_6^-$ 353.1210, found 353.1208.

2′-*O*-[2-(Methylamino)-2-oxoethyl]-2-*N*-isobutyrylguanosine (24). Compound **23** (55.85 g, 158.00 mmol) was coevaporated with anhydrous pyridine (2 × 200 mL) and then the residue dissolved in anhydrous pyridine (700 mL). Trimethylsilyl chloride (100 mL, 790.00 mmol) was dropped in at a rate as to maintain the internal temperature between 20 and 25 °C. The reaction was allowed to stir at room temperature for 90 min under argon atmosphere. The reaction was cooled to –15 °C, and isobutyryl chloride (83.25 mL, 790.00 mmol) was added at a rate as to maintain the internal temperature between –10 to 0 °C. The reaction was allowed to stir at ambient temperature for 18 h. The reaction mixture was cooled to 0 °C, and water (50 mL) was added to quench the reactions. After 10 min, the solvent was removed under reduce pressure and the residue was dissolved in a mixture of CH_2Cl_2−acetone−MeOH (500 mL, 40:10:3). A precipitate formed was collected and identified as inorganic salts. The filtrate was loaded on to a silica gel column (800 g) and eluted with CH_2Cl_2−acetone−MeOH (40:10:3 to 4:1:1 ration) to yield **24** (53.5 g, 81%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.12 (d, J = 6.8 Hz, 6H), 2.61 (d, J = 4.7 Hz, 3H), 2.82–2.73 (m, 1H), 3.59 (q, J = 12.0 Hz, 2H), 3.97–3.98 (m, 2H), 4.00 (s, 2H), 4.30 (d, J = 3.7 Hz, 1H), 4.43 (t, J = 5.1 Hz, 1H), 5.08 (s, 1H), 5.53 (d, J = 4.2 Hz, 1H), 5.96 (d, J = 5.6 Hz, 1H), 7.87 (q, J = 4.6 Hz, 1H), 8.27 (s, 1H), 11.65 (s, 1H), 12.09 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 18.8, 19.5, 25.1, 34.7, 60.9, 69.1, 69.3, 82.4, 84.7, 85.3, 120.2, 137.5, 148.2, 154.7, 156.7, 169.2, 180.1; MS (ES) m/z 422.12 [M − H]$^-$.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-2-*N*-isobutyrylguanosine (25). Compound **24** (42.5 g, 101.00 mmol) was coevaporated with anhydrous pyridine (2 × 200 mL) and then redissolved in the same (500 mL). 4,4′-Dimethoxytrityl chloride (37.63, 1110.00 mmol) was added in one portion, and the reaction mixture was stirred at room temperature under argon atmosphere for 18 h. The reaction was quenched by the addition of methanol (20 mL) and then concentrated under reduced pressure to thick oil. The oil was partitioned between EtOAc (500 mL) and 1 M aqueous $NaHCO_3$ solution (500 mL). The organic layer was dried (anhydrous Na_2SO_4) and concentrated and the residue obtained was purified by silica gel column chromatography and eluted with a gradient of MeOH in EtOAc (0 to 10%) to yield **25** (47 g, 64%) as a white foam: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.12 (dd, J = 2.8, 4.0 Hz, 6H), 2.83–2.71 (m, 1H), 2.62 (d, J = 4.7 Hz, 3H), 3.29–3.15 (m, 2H), 3.72 (s, 6H), 4.18–4.06 (m, 2H), 4.41 (q, J = 5.6 Hz, 1H), 4.50 (t, J = 4.7 Hz, 1H), 5.46 (d, J = 6.3, 1H), 6.06 (d, J = 4.1 Hz, 1H), 6.84–6.79 (m, 4H), 7.34–7.08 (m, 9H), 7.84 (q, J = 4.7 Hz, 1H), 8.13 (s, 1H), 11.58 (s, 1H), 12.10 (s, 1H); ^{13}C NMR (75 MHz, DNSO-d_6) δ 19.0, 26.1, 36.3, 37.5, 55.4, 63.5, 69.7, 70.4, 82.4, 83.4, 84.0, 86.7, 87.6, 113.4, 121.7, 127.2, 128.1, 128.3, 130.2, 135.9, 148.3, 156.0, 158.8, 171.3, 179.9; MS (ES) m/z 725.2 [M − H]$^-$; HRMS (TOF MS ES) m/z calcd for $C_{38}H_{41}N_6O_9^-$ 725.2935, found 725.2936.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-2-*N*-isobutyrylguanosine 3′-*O*-Succinate (26a). Compound **25** (5 g, 6.9 mmol) was mixed with succinic anhydride (2.76 g, 27.6 mmol) and DMAP (0.42 g, 3.4 mmol). The mixture was dried over P_2O_5 in vacuo overnight at 40 °C. The mixture was dissolved in anhydrous pyridine (60 mL) and stirred at ambient temperature under argon atmosphere for 4 h. The reaction was diluted with CH_2Cl_2 (300 mL) and washed with 10% citric acid (ice cold, 300 mL) and water (300 mL). The organic phase was dried over anhydrous sodium sulfate and evaporated in vacuo. The resulting foam was purified by silica gel column chromatography using MeOH/CH_2Cl_2/pyridine (1/8.9/0.1, v/v/v) as the eluent to yield **26a** (5.19 g, 71%,): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.91 (d, J = 6.8 Hz, 3H), 1.05 (d, J = 6.8 Hz, 3H), 2.67 (m, 4H), 2.76 (d, J = 4.7 Hz, 3H), 3.20 (dd, J = 3.1, 7.9 Hz, 1H), 3.56–3.49 (m, 2H), 3.77 (s, 6H), 4.23–4.11 (m, 2H), 4.36–4.25 (m, 2H), 5.03 (t, J = 4.9, 5.2, 1H), 5.86 (d, J = 4.9 Hz, 1H), 6.91–6.77 (m, 4H), 7.54–7.08 (m, 10H), 7.84 (q, J = 4.7 Hz, 1H), 7.86 (s, 1H), 8.99 (s, 1H), 12.05 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 19.0, 26.1, 36.3, 55.4, 63.5, 69.7, 70.4, 82.4, 83.4, 84.0, 86.7, 87.6, 113.4, 121.7, 127.2, 128.1, 128.3, 130.2, 135.9, 137.5, 144.8, 148.3, 156.0, 158.8, 171.3, 179.9; MS (ES) m/z 725.2 [M − H]$^-$.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(methylamino)-2-oxoethyl]-2-*N*-isobutyrylguanosine 3′*O*-Succinyl CPG (26b). Compound **26b** (68.3 μmol/g) was synthesized from compound **26a** (1.0g, 1.21 mmol), DMF (16 mL), HATU (0.462 g, 1.21 mmol), diisopropylethylamine (0.63 g, 0.81 mL, 4.84 mmol), anhydrous DMF (35 mL), and activated CPG (7.13 g, 115.2 μmol/g) according to the procedure used for the synthesis of compound **10b**.

2′-*O*-[2-(Methylamino)-2-oxoethyl] (2′-*O*-NMA)-Modified ASO Synthesis. The synthesis of 2′-*O*-NMA ASO was performed on ABI 394 DNA synthesizer or MilliGene/Biosearch 8800 reagent delivery module for 2 μmol scale or 150 μmol scale, respectively. The standard phosphoramidites and solid supports were used for incorporation of A, T, G, and 5-methyl C residues. A 0.1 M solution of the phosphoramidites **1**−**4** in anhydrous CH_3CN was used for the synthesis. The oligonucleotides were synthesized on prederivatized CPG support **22** or **26b**. For the coupling step, the 2′-*O*-NMA phosphoramidites **1**−**4** were delivered 9-fold excess for 2-μmol scale synthesis or 4 fold excess for 150-μmol scale over the solid support and phosphoramidite condensation was carried out for 10 min. All other steps in the protocol supplied by the manufacturer were used. PADS (0.2 M) in 1:1 3-picoline/CH_3CN was used as a sulfurization reagent with 2 min contact time. Solid-support bound ASOs were cleaved in a two-step procedure. In the first step, solid support was suspended in aqueous ammonia (28–30 wt %, 2.7 mL for 2 μmol or 81 mL for 150 μmol scale synthesis) at room temperature for 2 h. In the second step, aqueous methylamine (40 wt % in water, 0.3 mL for 2 μmol or 9 mL for 150 μmol scale synthesis) was added, and the resulting mixture was allowed to stay at room temperature for an additional 24 h. The solid supports were filtered and washed with water. The combined filtrate and the washing were concentrated and the residues obtained were then purified by high-pressure liquid chromatography on a strong anion-exchange column (Amersham Bioscience, Mono Q, 10/100, A = 100 mM ammonium acetate in 30% aqueous CH_3CN, B = 1.5 M NaBr in A, 0–60% of B in 60 min, flow 1.5 mL min^{-1}). The ASOs were desalted by HPLC on a reversed-phase column to yield **28** and **30** in an isolated yield of 30–35% based on the loading of the solid support. ASOs were characterized by ion-pair HPLC−MS analysis with an Agilent 1100 MSD system.

Cell Culture Assay. AML12 (α mouse liver 12) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). AML12 cells were grown in T-75 flasks in complete growth media, a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 medium (DMEM/F12, Invitrogen, Carlsbad, CA) supplemented with 5 μg mL^{-1} of insulin, 5 μg mL^{-1} of transferrin, 5 ng mL^{-1} of selenium, 40 ng mL^{-1} of dexamethasone, and 10% fetal bovine serum (FBS, HyClone) until 70–80% confluent. Cells were washed twice in 10 mL of DMEM. Oligonucleotides **27**–**30** were transfected with the indicated concentration of oligonucleotides using 3 μg mL^{-1} Cytofectin in OptiMEM and then incubated at 37 °C for 4 h followed by replacement of transfection media with complete growth media. After 24 h, cells were harvested, and total mRNA was isolated from tissue culture cells using Qiagen 96-well RNeasy plates. RNA was analyzed for PTEN and cyclophilin A RNA levels. PTEN RNA levels normalized to those of cyclophilin A were express as percent untreated control (% UTC). Each treatment was performed in triplicate. 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.[35] Oligonucleotides **27**−**30** were administered in 0.9% NaCl by intraperitoneal (i.p.) injection according to the indicated dose levels once a week for three weeks. The animals were maintained at a constant temperature of 23 °C and were fed standard laboratory diet. Animal weight was recorded prior to each dosing throughout the live phase of the study. Mice were

anesthetized and sacrificed 2 days after administration of the final dose. Serum was isolated from whole blood obtained via cardiac puncture. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using an Olympus AU400e bioanalyzer. Liver and spleen weight were determined. The effect of the compound on organ weights, normalized to body weight, was expressed relative to those of the saline treated group.

RNA Analysis. Total RNA was extracted from mouse liver by immediate homogenization of the tissue in 4 M guanidinium isothiocyanate/8% β-mercaptoethanol followed by ultracentrifugation through a cesium chloride gradient. RNAs (20–40 μg) were resolved in 1.2% agarose gels containing 1.1% formaldehyde and transferred to nylon membranes. The blots were hybridized with a radiolabeled mouse PTEN cDNA probe as described.[36] Probes hybridized to mRNA transcripts were visualized and quantified using a PhosphorImager (Molecular Dynamics). After the blots of the radiolabeled probe were stripped, they were reprobed with radiolabeled glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA to confirm equal loading. Data was expressed as a percentage of PTEN mRNA level in saline-treated control after normalization to G3PDH.

Western Blot Analysis. Frozen tissue samples were homogenized in RIPA buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing Complete protesase inhibitors (Roche, Palo Alto, CA). Protein concentrations were determined by Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA). Equal quantities of total protein from each sample were separated on a 10% PAGE gel (Invitrogen) and subsequently transferred to a PVDF membrane (Invitrogen). Following transfer, membranes were initially blocked at room temperature in 5% nonfat dry milk in TBS (Tris-buffered saline) and then incubated with PTEN antibody (Cell Signaling, Beverly, MA) at a 1:1000 dilution overnight at 4 °C. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated antirabbit secondary antibody (BD Biosciences/PharMingen, San Diego, CA) at 1:10000 dilution at room temperature. The blot was developed using ECL Plus Western Blotting Detection System (GE Healthcare Amersham, Buckinghamshire, UK). Protein bands were visualized by exposure to film; band intensities were quantified by densitometry.

Hematoxylin and Eosin (H & E) Staining. Tissue samples were fixed in formalin for 24 h and then changed to 70% ethanol until blocked. Further dehydration and processing of tissue samples were conducted using a Leica ASP300 tissue processor. Tissues were embedded in paraffin, and 4 μm sections were mounted on positive charged glass slides. The samples were then stained for hematoxylin and eosin (H & E) using a Leica Autostainer XL.

T_m Analysis. The thermal stability of the duplexes formed by oligonucleotides with the 2′-*O*-NMA- and 2′-*O*-MOE-modified gapmer ASOs **27**−**30**, and complementary RNA was studied by measuring the UV absorbance versus temperature curves as described previously.[37] 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: ^{31}P NMR and HRMS (FAB) mass spectra of compounds **1**–**4**. Copies of ^{1}H and ^{13}C NMR spectra and HRMS of compounds **8**, **9**, **12**, **18**−**20**, **23**, and **25**. LC MS profiles of oligonucleotides **28** and **30**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Crooke, S. T., *Antisense Drug Technology*; Marcel Dekker: New York, 2001; pp 92.

(2) Manoharan, M. 2′-Carbohydrate Modifications in Antisense Oligonucleotide Therapy: Importance of Conformation, Configuration and Conjugation. *Biochim. Biophys. Acta* **1999**, *1489*, 117–130.

(3) Freier, S. M.; Altmann, K. H. The Ups and Downs of Nucleic Acid Duplex Stability: Structure-Stability Studies on Chemically-modified DNA:RNA Duplexes. *Nucleic Acids Res.* **1997**, *25*, 4429–4443.

(4) Swayze, E. E.; Bhat, B. The medicinal Chemistry of oligonucleotides. In *Antisense Drug Technology Principles, Strategies, and Applications*, 2nd ed.; Crooke, S. T., Eds.; CRC Press Taylor & Francis Group, LLC: Boca Raton, 2008; pp 143–182.

(5) Prakash, T. P.; Bhat, B. 2′-Modified oligonucleotides for antisense therapeutics. *Curr. Top. Med. Chem.* **2007**, *7*, 641–649.

(6) Kurreck, J. Antisense Technologies Improvement Through Novel Chemical Modifications. *Eur. J. Biochem.* **2003**, *270*, 1628–1644.

(7) Mangos, M. M.; Damha, M. J. Flexible and Frozen Sugar Modified Nucleic Acids Modulation of Biological Activity Through Furanose Ring Dynamics in the Antisense Strand. *Curr. Top. Med. Chem.* **2002**, *2*, 1147–1171.

(8) Egli, M.; Minasov, G.; Tereshko, V.; Pallan, P. S.; Teplova, M.; Inamati, G.; Lesnik, E.; Owens, S.; Ross, B.; Prakash, T. P.; Manoharan, M. Probing the Influence of Stereoelectronic Effects on the Biophysical Properties of Oligonucleotides: Comprehensive Analysis of the RNA Affinity, Nuclease Resistance and Crystal Structure of Ten 2′-O-Ribonucleic Acid Modifications. *Biochemistry* **2005**, *44*, 9045–9057.

(9) Kastelein, J. J.; Wedel, M. K.; Baker, B. F.; Su, J.; Bradley, J. D.; Yu, R. Z.; Chuang, E.; Graham, M. J.; Crooke, R. M. Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. *Circulation* **2006**, *114*, 1729–1735.

(10) Prakash, T. P.; Kawasaki, A. M; Lesnik, E. A.; Owens, S. R.; Manoharan, M. 2′-O-[2-(Amino)-2-oxoethyl] Oligonucleotides. *Org. Lett.* **2003**, *5*, 403–406.

(11) Pattanayek, R.; Sethaphong, L.; Pan, C.; Prhavc, M.; Prakash, T. P.; Manoharan, M.; Egli, M. Structural Rationalization of a Large Difference in RNA Affinity Despite a Small Difference in Chemistry between Two 2′-O-Modified Nucleic Acid Analogues. *J. Am. Chem. Soc.* **2004**, *126*, 15006–15007.

(12) Butler, M.; McKay, R. A.; Popoff, I. J.; Gaarde, W. A.; Witchell, D.; Murray, S. F.; Dean, N. M.; Bhanot, S.; Monia, B. P. Specific Inhibition of PTEN Expression Reverse Hyperglycemia in Diabetic Mice. *Diabetes* **2002**, *51*, 1028–1034.

(13) Ross, B. S.; Springer, R. H.; Tortorici, Z.; Dimock, S. A Novel and Economical Synthesis of 2′-*O*-Alkyl-uridines. *Nucleosides Nucleotides* **1997**, *16*, 1641–1643.

(14) (a) Kumar, P.; Sharma, A. K.; Sharma, P.; Garg, B. S.; Gupta, K. C. Express protocol for functionalization of polymer supports for oligonucleotide synthesis. *Nucleosides Nucleotides* **1996**, *15*, 879–888. (b) Bayer, E.; Bleicher, K.; Maier, M. A. TBTU mediated synthesis of functionalized CPG synthesis. *Z. Naturforsch.* **1995**, *50b*, 1096–1100.

(15) Divakar, K. J.; Reese, C. B. 4-(1,2,4-Triazol-1-yl)-and 4-(3-Nitro-1,2,4-Triazol-1-yl)-1-(β-D-2,3,5-tri-O-Acetylarabinofuranosyl)Pyrimidin-2(1H)-ones. Valuable Intermediates in The Synthesis of Derivatives of 1-(β-D Arabinofuranosyl)Cytosine (ara-C). *J. Chem. Soc., Perkin. Trans. 1* **1982**, *117*, 1–1176.

(16) Bhat, V.; Ugarkar, B. G.; Sayeed, V. A.; Grimm, K.; Kosora, N.; Domenico, P. A.; Stocker, E. A simple and convenient method for the selective N-acylations of cytosine nucleosides. *Nucleosides Nucleotides* **1989**, *8*, 179–183.

(17) Izatt, R. M.; Hansen, L. D.; Rytting, J. H.; Christensen, J. J. Proton ionization from adenosine. *J. Am. Chem. Soc.* **1965**, *87*, 2760–2761.

(18) Takaku, H.; Kamaike, K. Synthesis of oligoribonucleotides using 4-methoxybenzyl group as a new protecting group of the 2′-hydroxyl group of adenosine. *Chem. Lett.* **1982**, *2*, 189–192.

(19) Christensen, L. F.; Broom, A. D. Specific chemical synthesis of ribonucleoside O-benzyl ethers. *J. Org. Chem.* **1972**, *37*, 3398–3401.

(20) Birnbaum, G. I.; Giziewicz, J.; Huber, C. P.; Shugar, D. Intramolecular hydrogen bonding and acidities of nucleoside sugar hydroxyls. Crystal structure and conformation of O2,2′-anhydro-1-α -D-xylofuranosyluracil. *J. Am. Chem. Soc.* **1976**, *98*, 4640–4.

(21) Guinosso, C. J.; Hoke, G. D.; Frier, S.; Martin, J. F.; Ecker, D. J.; Mirabelli, C. K.; Crooke, S. T.; Cook, P. D. Synthesis and biophysical and biological evaluation of 2′-modified antisense oligonucleotides. *Nucleosides Nucleotides* **1991**, *10*, 259–262.

(22) Manoharan, M.; Guinosso, C. J.; Cook, P. D. Novel functionalization of the sugar moiety of nucleic acids for multiple labeling in the minor groove. *Tetrahedron Lett.* **1991**, *32*, 7171–4.

(23) Buhr, C. A.; Matteucci, M. Preparation of 2′-modified nuclease-resistant oligonucleotide. *PCT Int. Appl.* 1991.

(24) Keller, T. H.; Haener, R. A general method for the synthesis of 2′-O-modified ribonucleosides. *Helv. Chim. Acta* **1993**, *76*, 884–992.

(25) Carey, F. A.; Sundberg, R. J. *Advanced Organic Chemistry, Part A: Structures and Mechanisms,* 3rd ed.; Plenum Press: New York, 1990; pp 296–297.

(26) Ti, G. S.; Gaffney, B. L.; Jones, R. A. Transient protection: efficient one-flask syntheses of protected deoxynucleosides. *J. Am. Chem. Soc.* **1982**, *104*, 1316–1319.
(27) Robins, M. J.; Hansske, F.; Bernier, S. E. Nucleic acid related compounds. 36. Synthesis of the 2′-O-methyl and 3′-O-methyl ethers of guanosine and 2-aminoadenosine and correlation of O′-methylnucleoside carbon-13 NMR spectral shifts. *Can. J. Chem.* **1981**, *59*, 3360–3364.
(28) McGee, D. P. C.; Cook, P. D.; Guinosso, C. J. US PCT 92-918362, 1994.
(29) Robins, M. J.; Zou, R.; Hansske, F.; Wnuk, S. F. Nucleic acid related compounds. 96. Synthesis of sugar-modified 2,6-diaminopurine and guanine nucleosides from guanosine via transformations of 2-aminoadenosine and enzymic deamination with adenosine deaminase. *Can. J. Chem.* **1997**, *75*, 762–767.
(30) Yokozeki, K.; Tsuji, T. A novel enzymatic method for the production of purine-2′-deoxyribonucleosides. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 207–213.
(31) Shen, L.; Siwkowski, A.; Wancewicz, E. V.; Lesnik, E.; Butler, M.; Witchell, D.; Vasquez, G.; Ross, B.; Acevedo, O.; Inamati, G.; Sasmore, H.; Manoharan, M.; Monia, B. P. Evaluation of C-5 propynyl Pyrimidine-Containing Oligonucleotides *In Vitro* and *In Vivo*. *Antisense and Nucleic Acid Drug Develop.* **2003**, *13*, 129–142.
(32) Cheruvallath, Z. S.; Carty, R. L.; Moore, M. N.; Capaldi, D. C.; Krotz, A. H.; Wheeler, P. D.; Turney, B. J.; Craig, S. R.; Gaus, H. J.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. Synthesis of antisense oligonucleotides: replacement of 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) with phenylacetyl disulfide (PADS) as efficient sulfurization reagent: from bench to bulk manufacture of active pharmaceutical ingredient. *Org. Process Res. Dev.* **2000**, *4*, 199–204.
(33) Wu, H.; Lima, W. F.; Zhang, H.; Fan, A.; Sun, H.; Crooke, S. T. Determination of the human RNase H1 in the pharmacology of DNA-like antisense drugs. *J. Biol. Chem.* **2004**, *279*, 17181–17189.
(34) Atkinson, T.; Smith, M. Solid-phase Synthesis of Oligodeoxyribonucleotides by Phosphite-triester Method. In *Oligonucleotide Synthesis a Practical Approach*, 2nd ed.; Gait, M. J., Ed.; IRL Press: Oxford, Washignton DC, 1985; pp 35–81.
(35) *Committee on Care and use of Laboratory Animals Guide for the care and use of Laboratory animals*; DHHS Publication No. 85; National Institutes of Health: Bethesda, MD, 1985; pp 23–26.
(36) Swayze, E. E.; Siwkowski, A. M.; Wancewicz, E. W.; Migawa, M. T.; Wyrzykiewicz, T. K.; Hung, G.; Monia, B. P.; Bennett, C. F. Antisense Oligonucleotides Containing Locked Nucleic Acid Improve Potency but Cause Significant Hepatotoxicity in Animals. *Nucleic Acids Res.* **2007**, *35*, 687–700.
(37) Lesnik, E. A.; Freier, S. M. What Affects the Effect of 2′-Alkoxy Modifications? 1. Stabilization Effect of 2′-Methoxy Substitutions in Uniformly Modified DNA Oligonucleotides. *Biochemistry* **1998**, *37*, 6991–6997.

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