Synthesis and Evaluation of *S*-Acyl-2-thioethyl Esters of Modified Nucleoside 5′-Monophosphates as Inhibitors of Hepatitis C Virus RNA Replication

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Several triphosphates of modified nucleosides (**1**−**6**) were identified as inhibitors (IC_{50} = 0.08−3.8 μM) of hepatitis C virus RNA-dependent RNA polymerase (RdRp). Although the initial SAR developed by determining the ability of the triphosphates to inhibit the in vitro activity of the HCV RdRp identified several potent inhibitors, none of the corresponding nucleosides exhibited significant inhibitory potency in a cell-based replicon assay. To improve upon the activity, bis(*t*Bu-*S*-acyl-2-thioethyl) nucleoside 5′-monophosphate esters (**7**−**12**) were synthesized, and these derivatives exhibited improved potency compared to the corresponding nucleosides in the cell-based assay. Analysis of the intracellular metabolism demonstrated that the *S*-acyl-2-thioethyl (SATE) prodrug is metabolized to the 5′-triphosphate 40- to 155-fold more efficiently compared to the corresponding nucleoside. The prodrug approach involving bis(*t*BuSATE)cytidine 5′-monophosphate ester significantly reduced the deamination of cytidine derivatives by cellular deaminases. Additionally, chromosomal aberration studies with the SATE prodrug in cells showed no statistically relevant increase in aberrations compared to the concurrent controls.

Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus that was unambiguously identified in 1989.[1] The HCV RNA genome is approximately 10 kb in length and shares similarities with the genomes of flaviviruses and pestiviruses. HCV is responsible for most cases of non-A and non-B hepatitis, a chronic disease that often leads to liver cirrhosis and hepatocellular carcinoma. Approximately 170 million infected individuals are at risk of developing significant morbidity and mortality.[2] HCV infection has become the primary reason for liver transplantations among adults in Western countries.[3] Thus, HCV has attracted a tremendous amount of attention from academic and pharmaceutical industry research.[4] Current therapies for HCV infection based on administering combinations of interferon-α and ribavirin are suboptimal, especially in patients infected with HCV genotype 1, and are poorly tolerated and have less than 50% overall response rates.[5] These drugs do not act directly on the virus, and their mode of action is poorly understood. Therefore, there is an urgent medical need for more efficient and better tolerated antiviral agents for HCV.

Detailed accounts of the HCV genome and gene products including processing into multiple viral proteins have been recently reported.[6,7] RdRp (RNA-dependent RNA polymerase) is essential for viral replication and proliferation. Hence, designing specific inhibitors of HCV RdRp has become one of the important approaches for developing novel drugs against HCV infection. Several non-nucleoside inhibitors of RdRp have been identified as anti- HCV agents.[8−11] Recently, a series of nucleoside analogues have also been developed as RdRp inhibitors.[12−15] Though triphosphates of several of these nucleoside analogues exhibited excellent RdRp inhibitory potency, only few nucleoside derivatives have exhibited biological activity in cell culture assays.[12,15] This might be due to poor cellular penetration coupled with insufficient metabolism to 5′-triphosphates of these nucleoside derivatives. Here, we report the results of the prodrug approach that resulted in improved activity of HCV inhibitors in a cell-based replicon assay.

Previously we reported that 2′-*O*-Me-C was able to inhibit HCV viral RNA replication in the context of a cell-based replicon[12] assay. Here, we report the synthesis and potency evaluation of *S*-acyl-2-thioethyl (SATE) ester prodrugs of 2′-*O*-Me-C and several closely related nucleoside monophosphates.

Results and Discussion

Several triphosphates of modified nucleosides (Figure 1) were designed and synthesized and were evaluated for the ability to inhibit HCV RdRp.[12] This effort yielded some very potent inhibitors (Table 1). However, when the cell-based replicon assay became available,[7] none of these nucleosides exhibited comparable intracellular potency (Table 1).

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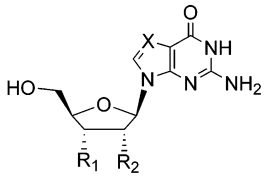
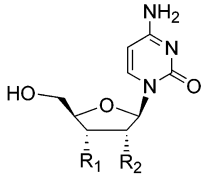
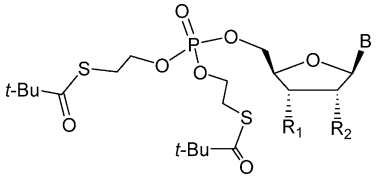


1: R_1 = OH, R_2 = OCH_3, X = N
2: R_1 = OH, R_2 = OCH_3, X = CH
3: R_1 = H, R_2 = OH, X = N
4: R_1 = H, R_2 = OH, X = CH

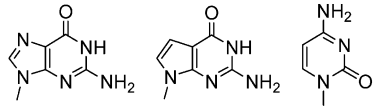


5: R_1 = OH, R_2 = OCH_3
6: R_1 = H, R_2 = OH

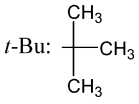
Figure 1. HCV RNA inhibitors.



7: R_1 = OH, R_2 = OCH_3, B = Gua
8: R_1 = OH, R_2 = OCH_3, B = 7-deaza-Gua
9: R_1 = H, R_2 = OH, B = Gua
10: R_1 = H, R_2 = OH, B = 7-deaza-Gua
11: R_1 = OH, R_2 = OCH_3, B = Cyt
12: R_1 = H, R_2 = OH, B = Cyt



Gua 7-deazaGua Cyt



t-Bu:

Figure 2. Bis(*S*-pivaloyl-2-thioethyl]phosphotriester derivatives of HCV RNA inhibitors **1**−**6**.

All these nucleoside analogues have no intrinsic antiviral activity. They must be metabolized to their respective 5′-triphosphate by nucleotidase, kinases, and/or other activating enzymes present naturally in cells to act as RdRp inhibitors.[17] Of the three anabolic steps in the biochemical pathway to convert nucleoside analogues to the active triphosphates, the monophosphorylation step is considered as generally being the rate-limiting step, though there are notable exceptions such as AZT.[18] The presence and the activity of intracellular enzymes involved in the monophosphorylation of nucleoside analogues depend on the host species, the cell type, and the phase in the cell cycle.[19,20] The dependence on phosphorylation for activation of the nucleoside analogues may therefore be problematic in cells where the phosphorylating enzyme activity is known to be low or even lacking. Another factor that may be contributing to the lack of activity of nucleoside analogues in cell-based assays would be their poor cellular uptake. To circumvent these two metabolic steps, Gosselin and Imbach designed the bis(SATE)-bearing monophosphate prodrugs for several nucleoside analogues for HIV and demonstrated impressive antiviral activity compared to corresponding nucleoside analogues.[21−24] These protected neutral monophosphate esters have been shown to enter the cell much more readily than corresponding monophosphate derivatives.[18] After entering the cell, the pronucleotide should metabolize via a sequence of enzymatic thioester hydrolyses, followed by subsequent fragmentation with the simultaneous release of episulfide and its corresponding 5′-monophosphate.[21]

Table 1. Inhibitory Potency (Replicon EC_{50}) of Nucleoside Analogues **1**−**6** and Their 5′-Bis(*t*BuSATE)monophosphate Esters (**7**−**12**) in HCV Replicon Assay in HBI10A Cells and Inhibitory Potency (RdRp IC_{50}) of 5′-Triphosphates of Nucleoside Analogues **1**−**6** on HCV NS5B-Mediated RNA Synthesis[a]

compd	chemistry	replicon EC_{50}, μM	RdRp IC_{50}, μM	toxicity MTS[b] CC_{50}
1	2′-*O*-Me-G	>50	1.2 ± 0.7	
2	2′-*O*-Me-7-deaza-G	>50	0.35 ± 0.08	
3	3′-deoxy-G	>50	0.6 ± 0.3	
4	3′-deoxy-7-deaza-G	>50	0.08 ± 0.01	
5	2′-*O*-Me-C	21 ± 3	3.8 ± 0.3	>100
6	3′-deoxy-C	25	1.2	>50
7	5′-SATE-2′-*O*-Me-G	>50		>50
8	5′-SATE-2′-*O*-Me-7-deaza-G	>50		>100
9	5′-SATE-3′-deoxy-G	23		>50
10	5′-SATE-3′-deoxy-7-deaza-G	7.2		>50
11	5′-SATE-2′-*O*-Me-C	3 ± 1		>100
12	5′-SATE-3′-deoxy-C	1.4 ± 0.9		>100

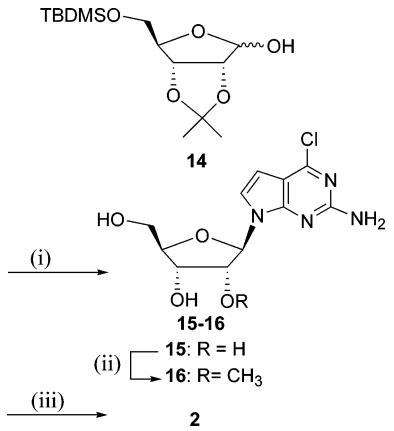
[a] Compounds were incubated in cell culture for 24 h prior to determination of the relative amount of HCV replicon RNA with the in situ ribonuclease protection assay. [b] Compounds cytotoxicity was determined by MTS assay on parallel samples at the same time.

All of the triphosphates of the nucleoside analogues **1**−**6** inhibit the catalytic activity of purified HCV RNA polymerase with IC_{50} values ranging from 0.08 to 3.8 μM (Table 1). However, nucleosides **1**−**6** were very poor inhibitors of RNA replication in cell-based systems (Table 1). The lack of reasonable activity of the nucleosides **1**−**6** in the cell-based assay in comparison to the potency observed in the biochemical enzyme assay may be due to previously noted reasons such as poor cellular penetration or the inability of these nucleosides to be metabolized to the corresponding triphosphates. We envisaged that 5′-bis(*S*-pivaloyl-2-thioethyl) nucleoside monophosphate esters **7**−**12** (*S*-pivaloyl-2-thioethyl abbreviated as *t*BuSATE, Figure 2) should improve cell penetration and would generate the monophosphate intracellularly by following the decomposition pathway previously described for SATE prodrugs.[21]

Chemistry. The 2′-*O*-methylguanosine **1** and 2′-*O*-methylcytidine **5** were procured from commercial sources. The 3′-deoxyguanosine (**3**) and 3′-deoxycytidine (**6**) were synthesized using the reported procedures.[25]

The 2-amino-7-(2-*O*-methyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**2**) was synthesized via the route described in Scheme 1. The 5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribofuranose[26] **14** (Scheme 1) was treated with carbon tetrachloride in hexamethylphosphorus triamide in THF at −76 °C to generate in situ 1-choloro-5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribofuranose. The sodium

Scheme 1[a]

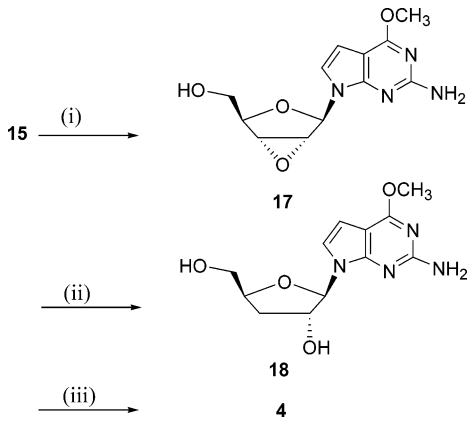


[a] Reagents and conditions. TBDMS: *tert*-butyldimethylsilyl; (i) (a) hexamethylphosphorus triamide, THF, CCl_4, 2-amino-6-chloro-1*H*-pyrrolo[2,3-*d*]pyrimidine, CH_3CN, NaH, −76 to −20 °C; (b) Dowex WX 8-400; (ii) MeI, NaH, DMF; (iii) NaOH, H_2O, reflux.

salt of 2-amino-4-chloro-1*H*-pyrrolo[2,3-*d*]pyrimidine in acetonitrile was coupled to 1-choloro-5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribofuranose to give 2-amino-4-chloro-7-(5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]-pyrimidine and α anomer (α/β 1:3). The two isomers were separated by flash silica gel column chromatography. The β isomer was treated with Dowex WX8-400 resin to deprotect the 5′-silyl and 2′,3′-ispropylidine groups to yield 2-amino-4-chloro-7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (**15**). The treatment of **15** with methyl iodide in the presence of sodium hydride in DMF yielded 2-amino-4-chloro-7-(2′-*O*-methyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (**16**) and the 3′-*O*-methyl derivative. Two regioisomers were separated by column chromatography. ^{1}H NMR spectrum of compound **16** exhibited only one singlet at δ 3.25 for three protons. These data suggested that there was only one methyl group in the molecule. To assign the position of methyl group, two-dimensional (2D) ^{1}H−^{1}H correlated NMR spectroscopy experiments of **16** in DMSO-d_6 were performed. In the 2D correlated NMR spectrum, strong symmetrical cross-peaks due to coupling of hydroxyl protons and 3′- and 5′-protons were observed whereas no coupling between hydroxyl proton and 2′-proton was observed (data not shown). These data indicated that compound **16** was a 2′-*O*-methyl derivative. Compound **16** on saponification with aqueous sodium hydroxide followed by neutralization afforded 2′-*O*-methyl-7-deazaguanosine **2**.

The synthesis of 2-amino-7-(3-deoxy-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**4**) was achieved as described in Scheme 2. Treatment of compound **15** with α-acetoxyisobutyryl bromide in acetonitrile and water gave a mixture of 2′- and 3′-bromoarabino nucleosides.[27] This mixture on treatment with Dowex OH^- resin in methanol gave 2-amino-7-(2,3-anhydro-β-D-ribofuranosyl)-4-methoxy-7*H*-pyrrolo[2,3-*d*]pyrimidine **17** (94% yield).[26] The chlorine at the 6-position of the nucleoside base was displaced with a methoxy group under this condition. Reduction of the 2′,3′-anhydro derivative **17** with triethyllithium borohydride in THF yielded 2-amino-7-(3-deoxy-β-D-ribofuranosyl)-4-methoxy-7*H*-pyrrolo[2,3-*d*]pyrimidine (**18,**

Scheme 2[a]



[a] Reagents and conditions: (i) (a) H_2O/CH_3CN, α-acetoxyisobutyryl bromide; (b) MeOH, Dowex OH^-; (ii) $LiEt_3BH$ in THF, 0 °C; (iii) 2 N NaOH, reflux.

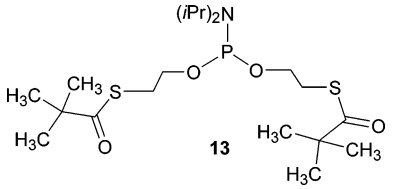


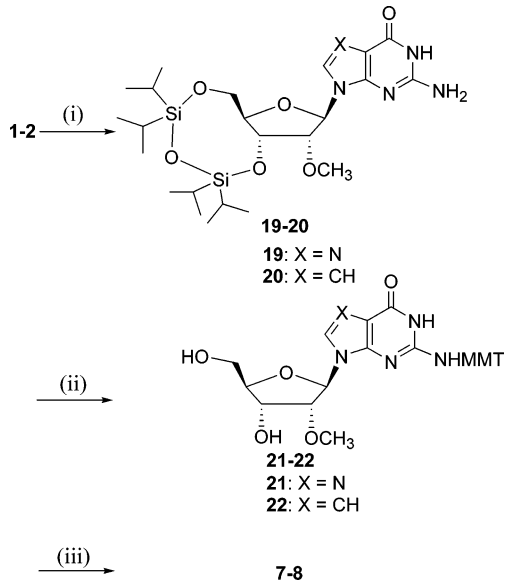
Figure 3. Bis(*S*-pivaloyl-2-thioethyl) *N*,*N*-diisopropylphosphoramidite.

60%). To assign the position of the hydroxyl group, 2D correlation NMR spectroscopy experiments of **18** in DMSO-d_6 were performed. The presence of strong symmetrical cross-peaks (data not shown) in the 2D NMR spectrum, due to the coupling of the hydroxyl proton and proton at the 2′-position as well as the absence of cross-peaks from the coupling of hydroxyl proton and 3′-proton, confirms that the hydroxyl group was at the 2′-position of **18**. Compound **18** was refluxed with 2 N aqueous sodium hydroxide and neutralized with 2 N aqueous hydrochloric acid to give **4** in 80% yield.

The P(III) chemistry involving phosphoramidite intermediates is the most efficient method to prepare phosphate derivatives, and this approach has been used to synthesize several SATE prodrugs.[21] We used similar strategy to synthesize nucleoside-5′-bis(*t*BuSATE) monophosphate esters **7**−**12** (Figure 2). The phosphitylation reagent bis(*t*BuSATE)-*N*,*N*-diisopropylphosphoramidite **13** (Figure 3) with thioester group was synthesized according to the literature procedure.[21] Coupling of the appropriately protected nucleosides with **13** in the presence of 1*H*-tetrazole followed by in situ oxidation yielded the nucleoside-5′-bis(*t*BuSATE) monophosphate esters.

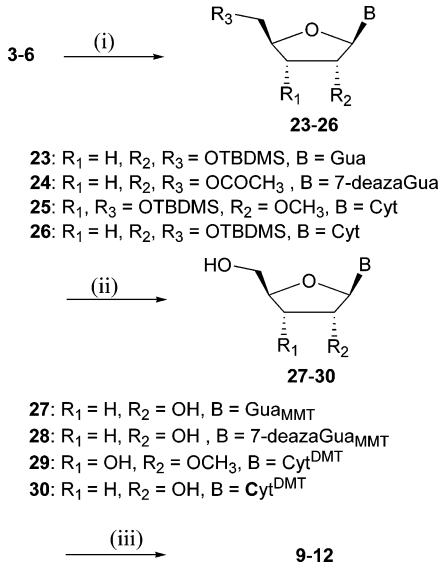
The 2′-*O*-methylguanosine 5′-[bis(*t*BuSATE)]phosphate **7** and 2-amino-7-(2-*O*-methyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one 5′-[bis(*t*BuSATE)]-phosphate **8** were synthesized from 2′-*O*-methylguanosine (**1**) and 2-amino-7-(2-*O*-methyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**2**), respectively (Scheme 3). Compounds **1** and **2** were silylated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in anhydrous pyridine to yield **19** and **20** (80−85%), respectively. These were subsequently treated with 4-monomethoxytrityl chloride in the presence of DMAP in anhydrous

Scheme 3[a]



[a] Reagents and conditions. MMT: *p*-anisyldiphenylmethyl; (i) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, pyridine, room temperature; (ii) (a) *p*-anisylchlorodiphenylmethane, DMAP, pyridine; (b) triethylamine trihydrofluoride, triethylamine, THF, room temperature; (iii) (a) **13**, 1*H*-tetrazole, CH_3CN; (b) 3-chloroperbenzoic acid, CH_2Cl_2, −40 to −10 °C; (c) acetic acid/H_2O/MeOH (3:1:6), 55 °C.

Scheme 4[a]



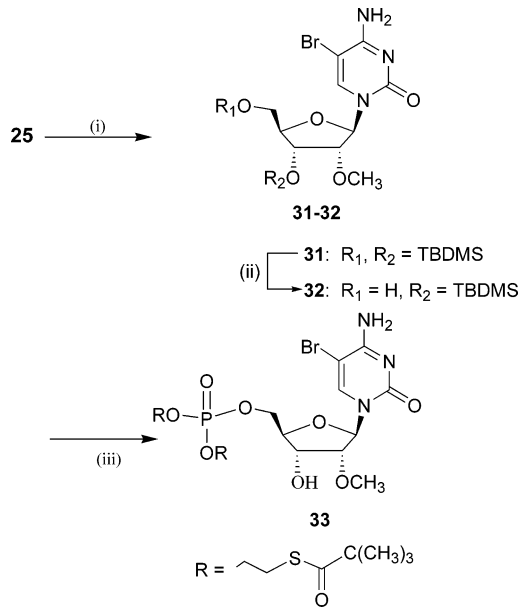
[a] Reagents and conditions. DMT: 4,4′-dimethoxytrityl. (i) for **23**, **25**, and **26**, *tert*-butyldimethylsilyl chloride, imidazole, DMF, room temperature; for **24**, acetic anhydride, triethylamine, acetonitrile, DMAP; (ii) (a) *p*-anisylchlorodiphenylmethane (for **27**−**28)** or 4,4′-dimethoxytrityl (for **29**, **30**), DMAP, pyridine; (b) for **27**, **29**, and **30**, triethylamine trihydrofluoride, triethylamine, THF, room temperature; for **28**, 2 N aqueous NaOH, dioxane, room temperature; (iii) (a) **13**, 1*H*-tetrazole, CH_3CN; (b) 3-chloroperbenzoic acid, CH_2Cl_2, −40 to −10 °C; (c) acetic acid/H_2O/MeOH, 3:1:6, 55 °C.

pyridine to protect the exocyclic amino group at the 2-position of the nucleosides. The protected nucleosides were desilylated on treatment with triethylamine trihydrofluoride and triethylamine in THF to yield **21** and **22** (72−75% yield). The coupling of **21** and **22** with phosphoramidite **13** in the presence of 1*H*-tetrazole followed by oxidation with 3-chloroperbenzoic acid gave a mixture of 5′- and 3′-bis(*t*BuSATE) monophospates of nucleosides in a 3:2 ratio. The mixture of 5′- and 3′-regioisomers were then heated with a solution of acetic acid/water/methanol (3:1:6) at 55 °C to remove the trityl protecting group from the exocyclic amino group. The 5′-regioisomers (**7** and **8**, 28−39% yield) were separated from 3′-regioisomers by high-pressure liquid chromatography (HPLC). The structure of the two isomers was assigned using two-dimensional $^1H-^1H$ correlated NMR spectroscopy experiments in DMSO-d_6. The 2D NMR spectra showed coupling of the proton at the 3′-hydroxyl group and the proton at the 3′-position (data not shown), and no coupling was observed between the hydroxyl proton and the 5′-proton. These data suggested that phosphate ester group was at 5′-position of the compounds **7** and **8**.

The 5′-bis(*t*BuSATE) monophosphate esters of 3′-deoxyguanosine (**9**), 2-amino-7-(3-deoxy-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**10**), 2′-*O*-methylcytidine (**11**), and 3′-deoxycytidine (**12)** were synthesized (Scheme 4) using a similar synthetic strategy described for **7** and **8**. The nucleosides **3**, **5**, and **6** were treated with *tert*-butyldimethylsilyl chloride in DMF in the presence of imidazole to yield respective silylated nucleosides **23**, **25**, and **26** (52−98% yield). The nucleoside **4** was treated with acetic anhydride in acetonitrile in the presence of triethylamine to yield 2′,5′-*O*-bis(acetyl) derivative **24** in 95% yield. The exocyclic amino group of the nucleosides **23**−**26** was protected with trityl groups on treatment with *p*-anisylchlorodiphenylmethane (MMT-Cl) or 4,4′-dimethoxytrityl chloride (DMT-Cl) in anhydrous pyridine. The fully protected nucleosides **23**, **25**, and **26** were then treated with triethylamine trihydrofluoride and triethylamine in THF at room temperature to remove the *tert*-butyldimethyl silyl group to yield **27**, **29**, and **30**. The 2′,5′-*O*-bis(acetyl)-N^2-(*p*-anisyldiphenylmethyl) protected nucleoside **24** was treated with 2 N aqueous sodium hydroxide at room temperature to yield **28**. Compounds **27**−**30** were phosphitylated with **13** and 1*H*-tetrazole. The nucleoside phosphites thus formed were oxidized to phosphate using 3-chloroperbenzoic acid in dichloromethane followed by deprotection of the trityl group at the exocyclic amine, yielding the nucleoside 5′-bis(*t*BuSATE)phosphates **9**−**12.** The mixture of 5′ and 3′ isomers were separated by HPLC. The 5′ regioisomers were identified using 2D NMR correlation spectroscopy (COSY) experiments in DMSO-d_6. The 2D NMR spectra showed coupling of proton at the 2′- or 3′-hydroxyl group and the proton at the 2′- or 3′-position, whereas no coupling was observed between hydroxyl proton and 5′-proton. These data indicated that phosphate ester group was at the 5′-position of compounds **9**−**12**.

To evaluate the intracellular metabolism of SATE prodrugs, we have synthesized the tritiated version of **11** using 5-bromo-2′-*O*-methylcytidine 5′-[bis(*t*BuSATE)-monophosphate] **33**. Compound **33** was synthesized according to the route described in Scheme 5. The 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-*O*-methylcytidine **25** was treated with lithium bromide and ceric(IV) ammonium nitrate in acetonitrile to yield the 5-bromo derivative **31** (83% yield).[28] The *tert*-butyldimethylsilyl group at the 5′-postion of **31** was selectively removed on heating with 80% acetic acid in water at 50 °C for 6 h to yield

Scheme 5[a]



[a] Reagents and conditions. (i) LiBr, ammonium ceric(IV) nitrate, CH_3CN; (ii) 80% acetic acid in water, 50 °C; (iii) (a) **13**, 1*H*-tetrazole, CH_3CN; (b) 3-chloroperbenzoic acid, CH_2Cl_2, −40 to −10 °C; (c) triethylamine trihydrofluoride, triethylamine, THF.

5-bromo-3′-*O*-[(*tert*-butyldimethyl)silyl]-2′-*O*-methylcytidine (**32**, 40%). Compound **32** was coupled with phosphoramidite **13** in the presence of 1*H*-tetrazole followed by oxidation, giving the 5-bromo-3′-*O*-[(*tert*-butyldimethyl)silyl]-2′-*O*-methylcytidine 5′-[bis(*t*BuSATE)phosphate. This, on treatment with triethylamine trihydrofluoride to remove *tert*-butyldimethylsilyl group, yielded **33** (37%). To develop a catalytic hydrogenation method for the conversion of **33** into the tritiated version of **11**, compound **33** and 10% palladium on carbon in methanol were stirred under a normal atmosphere of hydrogen at room temperature for 24 h. The product formed was analyzed by spectroscopic methods (^{1}H and ^{31}P NMR and mass spectrometry) and was identical to compound **11**. Compound **33** was subjected to similar reduction conditions in the presence of tritium to synthesize the tritiated version of **11**.

All compounds were characterized by ^{1}H and ^{13}C NMR and mass spectroscopic analysis. The purity of all the compounds tested in biological assay was determined by HPLC and was >95%.

Antiviral Activity. The triphosphates of modified nucleosides **1**−**6** were screened against the purified HCV RdRp for their ability to inhibit HCV NS5B mediated RNA synthesis (RdRp, Table 1). The corresponding nucleosides were screened in a cell-based, subgenomic replicon assay for their ability to inhibit RNA replication in a stable Huh-7 human hepatoma cell line designated HBI10A, which supports the replication of HCV RNA and protein. The effect of the nucleosides upon RNA replication was directly detected by an in situ ribonucleoside protection assay.[12] Thus, in the cell-based assay, activity would be subject to cellular uptake and metabolism to the nucleoside triphosphate. The replicon data indicated that none of the modified nucleosides (**1**−**6**) demonstrated significant activity in the cell-based assay, with EC_{50} values ranging from 20 to >50 μM (replicon, Table 1), in contrast to their corresponding nucleoside triphosphates that proved to be submicromolar to low micromolar ($IC_{50} = 0.08-3.8\ \mu$M) inhibitors of HCV NS5B mediated RNA synthesis.

The lack of activity of the nucleosides **1**−**6** in the replicon assay, in comparison to the enzyme assay, may be due to the inability of these nucleosides to be metabolized to triphosphates or poor cellular penetration or a combination of both. To address these issues, bis(*t*BuSATE) monophosphate esters **7**−**12** (Figure 2) were synthesized and were tested for inhibitory activity in the cell-based replicon assay.

The *t*BuSATE derivatives of 2′-*O*-methylcytidine-5′-monophosphate (**11**, Table 1) and 3′-deoxycytidine-5′-monophosphate (**12**, Table 1) showed improved activity in the assay at 24 h with EC_{50} values of 3 and 1.4 μM, respectively, which was comparable to the corresponding IC_{50} values of triphosphates in enzymatic assay. The replicon potencies of these prodrugs were 7- to 18-fold greater than the corresponding nucleosides**.** These results suggested that the poor activity of nucleosides (**5** and **6** Table 1) in the cell-based assay was due to inefficient conversion of these nucleosides into active triphosphates. The improved activity of prodrugs also suggested that the *t*BuSATE monophosphate esters were delivered intracellularly and that the monophosphates are released resulting in accumulation of phosphorylated forms of the nucleoside derivatives. In contrast, the *t*BuSATE derivative of 2′-*O*-methylguanosine-5′-monophosphate (**7**) and 2′-*O*-methyl-7-deazaguanosine-5′-monophosphate (**8**) were inactive in the cell-based assay whereas 3′-deoxyguanosine-5′-monophosphate (**9**) and 3′-deoxy-7-deazaguanosine-5′-monophosphate (**10**) showed improved activity compared to the corresponding nucleosides (**3** and **4**). The poor activities of compounds **7** and **8** may be due to poor cellular penetration of guanosine nucleosides or inefficient phosphorylation of the 2′-*O*-methylguanosine 5′-monophosphates generated after the intracellular metabolism of **7** and **8**.

To investigate the intracellular metabolism of nucleoside analogues, we chose compound **11** as our model compound for further studies. Therefore, a tritiated derivative of **11** was synthesized to understand the metabolic fate of its *t*BuSATE nucleoside prodrug.

Intracellular Metabolism of the 2′-*O*-methylcytidine 5′-Bis(*t*BuSATE)-phosphate (11). The intracellular metabolism of the tritiated version of 5′-bis(*t*BuSATE)-2′-*O*-methylcytidinephosphate **11** was studied in Huh-7 and HBI10A replicon cell lines. [5-^{3}H]-2′-*O*-Methylcytidine and the [5-^{3}H]-*t*BuSATE prodrug **11** were prepared using 5-bromo-2′-*O*-methylcytidine and the 5-bromo derivative of prodrug **33 (**Scheme 5) by the Tritium Custom Preparation group at Amersham Pharmacia Biotech Ltd. (Cardiff, Wales). The Huh-7 and HBI10A cells were incubated with compounds for 3 or 23 h at 2 μM prior to extraction and HPLC analysis. The results are summarized in Table 2. We observed that the 5′-bis(*t*BuSATE)-2′-*O*-methylcytidine monophosphate ester was more efficiently converted to the corresponding triphosphate compared to 2′-*O*-methylcytidine nucleoside. No detectable amounts of 2′-*O*-methyluridine 5′-triphosphate (2′-*O*-Me UTP), cytidine 5′-triphosphate (CTP), or uridine 5′-triphosphate (UTP) were formed when Huh-7 or HBI10A cells were incu-

Table 2. Intracellular Metabolism 5-[^{3}H] Derivative of 2′-*O*-Methylcytidine 5 and 5′-Bis(*t*BuSATE)-2′-*O*-methylcytidine Monophosphate Ester **11** in Cell Culture[a]

	compd	cell culture	incubation time, h	triphosphate, pmol/million cells[b]	major metabolic byproducts observed
5[c]	2′-*O*-Me-C	Huh-7	3	0.17	CTP and UTP
			23	0.61	CTP, UTP, 2′-*O*-MeUTP
		HBI10A	3	0.05	CTP, UTP, 2′-*O*-Me UTP
			23	0.18	CTP, UTP, 2′-*O*-Me UTP
11	5′-SATE-2′-*O*-Me-C	Huh-7	3	7.02	none
			23	1.88	none
		HBI10A	3	15.9	none
			23	5.8	none

[a] Compounds were incubated in cell culture for times indicated, prior to extraction and analysis by ion pair reverse-phase HPLC. Data are averages of at least three separate experiments. [b] Passage number for Huh-7 is 27; for HBI10A, it is 15. [c] See ref 12.

Table 3. Evaluation of Genotoxicity of 5′-Bis(*t*BuSATE)-2′-*O*-methylcytidine Monophosphate Ester **11**. Assay for Chromosomal Aberrations in Vitro in Chinese Hamster Ovary Cells[a]

treatment/sampling time, h		20/20		20/30		20/42	
treatment type	dose, μM	growth	% aberrant cells	growth	% aberrant cells	growth	% aberrant cells
DMSO,	1% v/v	100	1.5	100	1.5	100	2.0
2′3′-dideoxycytidine	10000	65	9.5[b]	101	8.0[b]	105	3.0
11	550	59	0.0	82	3.5	95	
	600	51	2.0	57	1.5	88	1.5
	650	27	Ns	46	4.5	90	3.0
	700	21	Ns	6	Ns	47	3.5
	800	6	Ns	0	Ns	0	Ns

[a] Ns = not scored because of toxicity. Growth is measured as population doublings from the beginning of the experiment as a percentage of controls, with 200 cells scored per point. [b] $P < 0.01$ compared with concurrent controls.

bated with the SATE prodrug of 2′-*O*-methylcytidine (Table 2). In contrast, when cells were incubated with 2′-*O*-methylcytidine, very little triphosphate was formed (Table 2) and considerable amounts of 2′-*O*-Me UTP, CTP, and UTP were detected.[12]

Cytidine deaminase (CDA) is a widely distributed enzyme that catalyzes the hydrolytic deamination of cytosine nucleosides to the corresponding uracil derivatives.[29,30] The observed formation of 2′-*O*-methyl UTP during incubations with 2′-*O*-methylcytidine is possibly due to CDA activity. It has been reported that 5′-monophosphates of cytidine nucleoside derivatives are poor substrates for the deaminases.[31,32] The *t*BuSATE monophosphate ester generates monophosphate intracellularly, which probably helps to protect the cytidine derivative from deamination by CDA. The fact that no 2′-*O*-methyl UTP was formed when radiolabeled **11** was incubated with cells suggests that the *t*BuSATE monophosphate ester is metabolized into 2′-*O*-methylcytidine 5′-monophosphate and at the same time the 5′-monophosphate nucleoside is stable to CDA activity. The 2′-*O*-methylcytidine was metabolized extensively to UTP and CTP because of base swapping in the cells.[12] However, the *t*BuSATE derivative of 2′-*O*-methylcytidine (**11**) was not metabolized to CTP or UTP (Table 3). This suggests that **11** and the subsequently generated 5′-monophosphate are poor substrates for the nucleoside pyrophosphorylase responsible for these conversions. These observations suggest that the *t*BuSATE derivative enhanced the half-life of 2′-*O*-methylcytidine and may in part account for the improved potency of prodrug **11** ($EC_{50} = 3$ μM, Table 1) in the cellular assay compared to 2′-*O*-methylcytidine (**5**, $EC_{50} = 21$ μM).

Evaluation of Genotoxicity of *t*BuSATE Prodrug: In Vitro Chromosomal Aberrations in Chinese Ovary Cells. To assess the genotoxicity of the *t*BuSATE prodrug, we evaluated its potential to cause chromosomal aberrations in Chinese hamster ovary (CHO) cells. The *t*BuSATE prodrug **11** was tested with and without a microsomal enzyme activation system (S-9) prepared from the livers of xenobiotic-treated rats. A solution of **11** in dimethyl sulfoxide was used for the study. Known chromosomal breaking agents, cyclophosphamide (CP) with S-9 activation and mitomycin C (MMC) without S-9 activation, were tested as positive controls. 2′,3′-Dideoxycytidine was also used as a positive control. The doses selected were based on limiting solubility in culture medium and on suppression of cell growth. The protocol used was previously shown to be suitable for detection of aberration induction by nucleoside analogues and included long treatment (20 h) with and without recovery times of 10 and 22 h. The 3 h treatments were also done with sampling at 20 h.

The 3 h treatments with **11** with and without S-9 did not induce aberrations at 700 and 800 μM, respectively (data not shown). These were the highest does that could be scored and had little growth suppression, whereas 1 mM doses suppressed growth completely. Positive controls cyclophosphamide at 5 μM and mitomycin C and 1.5 μM induced 25% and 48% of cells with aberrations, respectively. The results from the long (20 h) treatment are shown in Table 3. The top doses selected for scoring reduced cell growth by about 50%. There were no statistically significant increases in aberrations over concurrent controls. Dideoxycytidine induced an increase in aberrations after 20 h of treatment, at 20 and 30 h (Table 3).

Conclusions

We have identified several nucleoside analogues as inhibitors of RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. Bis(*t*BuSATE) nucleoside 5′-monophosphate ester derivatives **7**−**12** improved the activity of these nucleosides in a cell-based assay. Analysis of the intracellular metabolism of a radiolabeled *t*BuSATE prodrugs demonstrated that the prodrugs were metabo-

lized to the 5′-triphosphate 40- to 155-fold more efficiently compared to the corresponding nucleosides. The prodrug approach involving bis(*t*BuSATE)cytidine 5′-monophosphate ester helped to protect the cytidine derivative from deamination. The chromosome aberration studies of cells treated with bis(*t*BuSATE)cytidine 5′-monophosphate ester showed no statistically significant increase in aberrations over the concurrent controls.

Experimental Section

General Procedures. All the reagents and anhydrous solvents were purchased from Aldrich and were used without further purification. Compounds **1** and **5** were procured from R. I. Chemical Inc., CA. 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. ^1H NMR spectra were referenced using internal standard $(CH_3)_4Si$, and ^{31}P NMR spectra were referenced using external standard 85% H_3PO_4. Mass spectra were recorded by Mass Consortium, San Diego, CA, and the College of Chemistry, University of California, Berkeley. Combustion analyses were performed by Quantitative Technologies Inc., NJ.

2-Amino-7-(2-*O*-methyl-*β*-D-ribofuranosyl)-7*H*-pyrrolo-[2,3-*d*]pyrimidin-4(3*H*)-one (2). A solution of the compound **16** (2.0 g, 6.65 mmol) in NaOH/H_2O (4.09 g/51.1 mL) was heated at reflux for 7 h. The pH of the solution was adjusted to 7 with 1 N HCl, and the solvent was removed under reduced pressure. The residue obtained was purified by flash silica gel column chromatography and eluted with 20% MeOH in ethyl acetate to afford **2** (1.79 g, 86%) as a white solid. ^1H NMR (200 MHz, DMSO-d_6) δ 3.25 (s, 3H), 3.52 (m, 2H) 3.81 (m, 1H), 4.00 (m, 1H), 4.19 (m, 1H), 5.10 (br s, 2H), 5.95 (d, J = 2.5 Hz, 1H), 6.27 (d, J = 4 Hz, 1H), 6.33 (br s, 2H), 6.95 (d, J = 4 Hz, 1H), 10.55 (br s, 1H); ^{13}C NMR (100 MHz, D_2O/CD_3OD, 9:1) δ 57.8, 60.5, 69.2, 71.7, 82.3, 85.2, 100.9, 102.9, 119.3, 151.5, 153.9, 161.7; HRMS (FAB) calcd for $C_{12}H_{17}N_4O_5^+$ 297.1193, found 297.1199.

2-Amino-7-(3-deoxy-*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (4). Compound **18** (0.4 g, 1.4 mmol) was refluxed in 2 N aqueous NaOH (40 mL) for 3 h. The solution was cooled in an ice bath, neutralized with 2 N aqueous HCl, and evaporated to dryness. The residue obtained was suspended in MeOH and mixed with silica, and the solvent was evaporated to give a dry powder. The dry powder was loaded onto a flash silica gel column and eluted with 10−20% MeOH in CH_2Cl_2 to yield **4** (0.3 g, 80%) as white solid. ^1H NMR (400 MHz, DMSO-d_6) δ 1.85 (m, 1H), 2.12 (m, 1H), 3.46 (m, 1H), 3.54 (m, 1H), 4.18 (m, 1H), 4.28 (m, 1H), 4.85 (t, J = 5.6 Hz, 1H), 5.42 (d, J = 4.4 Hz, 1H), 5.82 (d, J = 2.4 Hz, 1H), 6.19 (s, 2H), 6.23 (d, J = 3.6 Hz, 1H), 6.87 (d, J = 3.6 Hz, 1H), 10.31 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 36.0, 63.8, 75.4, 80.1, 90.3, 110.7, 102.8, 117.7, 151.2, 153.6, 159.3; HRMS (FAB) calcd for $C_{11}H_{15}N_4O_4^+$ 267.1043, found 267.1239.

2′-*O*-Methylguanosine 5′-[Bis(*S*-pivaloyl-2-thioethyl)-phosphate] (7). N^2-(4-Monomethoxytrityl)-2′-*O*-methylguanosine **21** (0.6 g, 1.05 mmol) was mixed with 1*H*-tetrazole (0.05 g, 0.7 mmol) and dried over P_2O_5 in vacuo overnight. The mixture was suspended in anhydrous acetonitrile (13.8 mL), bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphoramidite **13** (0.32 g, 0.7 mmol) was added, and the reaction mixture was stirred at ambient temperature for 8 h under an inert atmosphere. Solvent was removed under reduced pressure. The residue was cooled to −40 °C, and a solution of 3-chloroperbenzoic acid (0.2 g, 1.4 mmol) in CH_2Cl_2 (10 mL) was added. The solution was allowed to warm to room temperature over 1 h. Aqueous sodium hydrogen sulfite (10 wt %, 2 mL) was added to reduce the excess of 3-chloroperbenzoic acid. The organic phase was separated, diluted with CH_2Cl_2 (20 mL), washed with saturated aqueous $NaHCO_3$ (10 mL) and water (10 mL), dried over Na_2SO_4, and evaporated to dryness. The residue obtained was purified by flash silica gel column chromatography and eluted with 5% MeOH in CH_2Cl_2. A mixture of 5′- and 3′-[bis(*S*-pivaloyl-2-thioethyl)]phosphate derivative (6:4 ratio) was eluted together (0.18 g, 28% yield). ^{13}P NMR (80 MHz, $CDCl_3$) δ −0.70, −1.37; MS (API-ES) *m/z* 936.32 $[M - H]^-$. The mixture (0.18 g, 0.19 mmol) was dissolved in acetic acid/MeOH/H_2O, 3:6:1 (10 mL), and was heated at 55 °C for 24 h. Solvent was removed, and the residue was purified by HPLC on a reverse-phase column (Hamilton PRP-1, 250 mm × 22 mm, A = acetonitrile, B = H_2O, 20−100% B in 65 min, flow rate of 10 mL min^{-1}). Fractions containing 3′- and 5′-isomers were pooled together and evaporated. The 3′- and 5′-isomers were characterized by $^1H-^1H$ COSY experiments in DMSO-d_6. The 5′-isomer **7** was isolated in 16% yield (0.11 g). HPLC retention time was 18.64 min (Phenomenex aqua, C-18, 150 mm × 4.6 mm, 5 μm, A = 100 mM triethylammonium acetate, pH 7, B = 90% acetonitrile in A, 2−100% B in 25 min, flow rate of 1 mL min^{-1}, λ = 260 nm). ^1H NMR (DMSO-d_6) δ 1.17 (s, 18H), 3.07 (m, 4H), 3.33 (s, 3H), 3.95−4.06 (m, 6 H), 4.14−4.21 (m, 2H), 4.3 (m, 1H), 5.42 (d, J = 5.4 Hz, 1H), 5.81 (d, J = 5.8 Hz, 1H), 6.49 (br s, 2H), 7.86 (s, 1H), 10.66 (br s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 27.2, 27.3, 28.5, 46.4, 46.5, 58.8, 66.4, 68.02, 68.8, 69.7, 82.3, 83.1, 84.6, 87.6, 116.5, 135.8, 151.4, 154.1, 160.0, 205.7; ^{13}P NMR (80 MHz, DMSO-d_6) δ −0.71; MS (API-ES) *m/z* 664.2 $[M - H]^-$; HRMS (FAB) calcd for $C_{25}H_{41}N_5O_{10}PS_2^+$ 666.7232, found 666.7283.

2-Amino-7-(2-*O*-methyl-*β*-D-ribofuranosyl)-7*H*-pyrrolo-[2,3-*d*]pyrimidin-4(3*H*)-one 5′-[Bis-(*S*-pivaloyl-2-thioethyl)phosphate] (8). Compound **8** (0.16 g, 29% yield) was synthesized from 7-deaza-N^2-(4-monomethoxytrityl)-2′-*O*-methylguanosine **22** (0.47 g, 0.82 mmol) according to the procedure used for the synthesis of **7** from **21**. 1*H*-Tetrazole (0.044 g, 0.63 mmol), bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphoramidite **13** (0.29 g, 0.63 mmol), acetonitrile (11 mL), 3-chloroperbenzoic acid (0.21 g, 1.26 mmol, 57−80%) in CH_2Cl_2 (5.2 mL), and a solution of acetic acid/MeOH/H_2O, 3:6:1 (20 mL), were also used for the synthesis. HPLC retention time was 11.23 min (Phenomenex aqua, C-18, 150 mm × 4.6 mm, 5 μm, A = 100 mM triethylammonium acetate, pH 7, B = 90% acetonitrile in A, 2−90% B in 9 min, 90% B till 15 min, flow rate of 1 mL min^{-1}, λ = 260 nm). ^1H NMR (200 MHz, DMSO-d_6) δ 1.14 (s, 18H), 3.06 (m, 4H), 3.31 (s, 3H), 3.96−4.26 (m, 9H), 5.35 (d, J = 2.6 Hz, 1H), 5.99 (d, J = 6.6 Hz, 1H), 6.27 (m, 3H), 6.86 (d, J = 3.6 Hz, 1H), 10.39 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 27.2, 28.4, 28.5, 46.4, 46.5, 58.5, 66.3, 66.4, 67.2, 69.7, 81.9, 82.9, 86.3, 101.3, 103.2, 118.2, 151.5, 152.3, 161.12, 205.6, 205.7; ^{13}P NMR (80 MHz, DMSO-d_6) δ −0.72; MS (API-ES) *m/z* 663.20 $[M - H]^-$; HRMS (MALDI) calcd for $C_{26}H_{42}N_4O_{10}PS_2$ 665.2074, found 665.2071. Anal. ($C_{26}H_{41}O_{10}PS_2$) H, N. C: calcd, 46.98; found, 46.17.

3′-Deoxyguanosine 5′-[Bis(*S*-pivaloyl-2-thioethyl)phosphate] (9). Compound **9** (0.067 g, 30% yield) was synthesized from compound **27** (0.20 g, 0.35 mmol) following a similar procedure used for the synthesis of **7** from compound **21**. 1*H*-Tetrazole (0.02 g, 0.27 mmol), bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphoramidite **13** (0.12 g, 0.27 mmol), 3-chloroperbenzoic acid (0.12 g, 0.7 mmol, 57−80%), CH_2Cl_2 (2.2 mL), and a solution of acetic acid/water/methanol (5 mL, 3:1:6) were also used for the synthesis. HPLC retention time was 10.93 min (Phenomenex aqua, C-18, 150 mm × 4.6 mm, 5 μm, A = 100 mM triethylammonium acetate, pH 7, B = 90% acetonitrile in A, 2−90% B in 9 min, 90% B from 9 to 11 min, flow rate of 1 mL min^{-1}, λ = 260 nm). ^1H NMR (DMSO-d_6) δ 1.15 (s, 18H), 1.92−2.01 (m, 1H), 2.17−2.28 (m, 1H), 3.04 (t, J = 6.2 Hz, 4H), 3.91−4.23 (m, 6 H), 4.37−4.55 (m, 2H), 5.67 (m, 2H), 6.45 (br s, 2H), 7.75 (s, 1H), 10.61 (s, 1H); ^{13}P NMR (80 MHz, DMSO-d_6) δ −0.75; MS (API-ES) *m/z* 634.2 $[M - H]^-$. HRMS (FAB) calcd for $C_{24}H_{39}N_5O_9PS_2^+$ 636.1923, found 636.1913. Anal. ($C_{24}H_{38}N_5O_9PS_2$) H, N. C: calcd, 45.35; found, 44.37.

2-Amino-7-(3-deoxy-*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one 5′-[Bis(*S*-pivaloyl-2-thioethyl)-phosphate] (10). Compound **10** (0.08 g, 23% yield) was

synthesized from 2-(4-monomethoxytrityl)amino-7-(3-deoxy-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one **28** (0.30 g, 0.52 mmol) according to the procedure used for the synthesis of **7** from **21**. 1*H*-Tetrazole (0.028 g, 0.40 mmol), bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphoramidite **13** (0.18 g, 0.40 mmol), 3-chloroperbenzoic acid (0.14 g, 0.8 mmol, 57−80%), and a solution of acetic acid/water/methanol (10 mL, 3:1:6) were also used for the synthesis. HPLC retention time was 34.78 min (Hamilton RP-1, 250 mm × 21.5 mm, 10 μm, A = water, B = acetonitrile, 20−100% B in 65 min, flow rate of 10 mL min^{-1}, λ = 260 nm). ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.16 (s, 18H), 1.91−2.01 (m, 1H), 2.17−2.25 (m, 1H), 3.05 (t, *J* = 6.2 Hz, 4H), 3.92−4.2 (m, 6 H), 4.35 (br s, 2H), 5.56 (d, *J* = 4.2 Hz, 1H), 5.76 (d, *J* = 2.4 Hz, 1H), 6.24 (m, 3H), 6.77 (d, *J* = 3.6 Hz, 1H), 10.36 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 27.2, 28.5, 34.9, 46.5, 46.5, 66.3, 68.9, 75.6, 90.4, 100.0, 102.4, 117.4, 150.8, 152.3, 161.12, 205.6, 205.7; ^{13}P NMR (DMSO-d_6) δ −0.89; HRMS (MALDI) calcd for $C_{25}H_{40}N_4O_9PS_2$ 635.1969, found 635.1964. Anal. ($C_{24}H_{38}N_5O_9PS_2$) C, H, N.

2′-*O*-Methylcytidine 5′-[Bis(*S*-pivaloyl-2-thioethyl)-phosphate] (11). Compound **11** (0.12 g, 22% yield) was synthesized from N^4-(4,4′-dimethoxytrityl)-2′-*O*-methylcytidine **29** (0.48 g, 0.86 mmol) using a similar procedure used for the synthesis of compound **7** from compound **21**. 1*H*-Tetrazole (0.06 g, 0.86 mmol), anhydrous acetonitrile (6 mL), bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphoramidite **13** (0.39 g, 0.86 mmol), 3-chloroperbenzoic acid (0.3 g, 1.72 mmol, 57−80%) in CH_2Cl_2 (5.5 mL), and a solution of acetic acid/water/methanol (10 mL, 3:1:6) were also used for the synthesis. HPLC retention time was 11.12 min (Phenomenex aqua, C-18, 150 mm × 4.6 mm, 5 μm, A = 100 mM triethylammonium acetate, pH 7, B = 90% acetonitrile in A, 2−90% B in 9 min, 90% B from 9 to 12 min, flow rate of 1 mL min^{-1}, λ = 260 nm). ^{1}H NMR (DMSO-d_6) δ 1.18 (s, 18H), 3.12 (t, *J* = 6.4 Hz, 4H), 3.39 (s, 3H), 3.69 (t, *J* = 4.2 Hz, 1H), 3.93−4.3 (m, 8H), 5.29 (d, *J* = 6.2 Hz, 1H), 5.72 (d, *J* = 7.4 Hz, 1H), 5.86 (d, *J* = 4 Hz, 1H), 7.21 (br s, 2H), 7.58 (d, *J* = 7.4 Hz, 1H); ^{13}C NMR (50 MHz, CD_3CN) δ 27.4, 29.2, 47.1, 58.9, 66.4, 67.1,67.4, 69.4, 82.3, 84.0, 89.5, 95.0, 141.9, 156.4, 167.1, 206.4; ^{13}P NMR (80 MHz, CD_3CN) δ −0.64; MS (AP-ES) *m/z* 625.69 $[M + H]^+$; HRMS (MALDI) calcd for $C_{24}H_{40}N_3O_{10}PS_2Na^+$ 648.1785, found 648.1804. Anal. ($C_{24}H_{40}N_3O_{10}PS_2$) H, N. C: calcd, 46.07; found, 45.54.

3′-Deoxycytidine 5′-[Bis(*S*-pivaloyl-2-thioethyl)phosphate] (12). Compound **12** (0.07 g, 22% yield) was synthesized from N^4-(4,4′-dimethoxytrityl)-3′-deoxycytidine **30** (0.3 g, 0.57 mmol) following a similar synthetic procedure used for the synthesis of compound **7** from compound **21**. 1*H*-Tetrazole (0.04 g, 0.57 mmol), acetonitrile (4 mL), bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphoramidite (0.52 g, 1.14 mmol), 3-chloroperbenzoic acid (0.2 g, 1.14 mmol, 57−80%) in CH_2Cl_2 (3.6 mL), and acetic acid/water/methanol (10 mL, 3:1:6) were also used for the synthesis. HPLC retention time was 12.79 min (Phenomenex aqua, C-18, 250 mm × 4.6 mm, 5 μm, A = 100 mM triethylammonium acetate, pH 7, B = 90% acetonitrile in A, 2−80% B in 15 min, flow rate of 1 mL min^{-1}, λ = 260 nm). ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.17 (s, 18H), 1.84 (m, 2H), 3.11 (t, *J* = 6.4 Hz, 4H), 3.93−4.31 (m, 8H), 4.39 (m, 1H), 5.55 (d, *J* = 4.2 Hz, 1H), 5.67 (dd, *J* = 7.4 and 1.8 Hz, 2H), 7.1 (br s, 2H), 7.56 (d, *J* = 7.4 Hz,1H); ^{13}C NMR (50 MHz, CD_3CN) δ 27.5, 29.3, 29.4, 34.0, 47.2, 67.1, 67.2, 69.0, 69.1, 76.7, 79.8, 94.9, 141.7, 157.3, 167.3, 206.5; ^{13}P NMR (80 MHz, CD_3CN) δ −0.71; MS (AP-ES) *m/z* 596.1 $[M + H]^+$; HRMS (MALDI) calcd for $C_{23}H_{38}N_3O_9PS_2Na^+$ 618.1679, found 618.1600.

Bis(*S*-pivaloyl-2-thioethyl) *N,N*-Diisopropylphosphoramidite (13). Bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphramidite was synthesized according to the literature procedure.[21] ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.15−1.28 (m, 30H), 3.10 (t, *J* = 6.5 Hz, 4H), 3.45−3.83 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 24.5, 27.3, 30.0, 42.8, 43.1, 46.3, 62.3, 206.1; ^{13}P NMR (80 MHz, $CDCl_3$) δ 147.12.

2-Amino-4-chloro-7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (15). Hexamethylphosphorous triamide (10.65 mL, 55 mmol) was added portionwise over 30 min to a solution of 5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribofuranose **14** (13.3 g, 44 mmol), anhydrous THF (135 mL), and $CC1_4$ (5.6 mL, 58 mmol) under N_2 at −76 °C. After 30 min, the temperature was raised to −20 °C. In a separate flask, a suspension of 2-amino-4-chloro-1*H*-pyrrolo[2,3-*d*]pyrimidine (15 g, 89 mmol) in CH_3CN (900 mL) was treated with 60% NaH (3.60 g., 90 mmol) at 15 °C. The reaction mixture was stirred for 30 min, whereupon the previous reaction mixture was cannulated with vigorous stirring. The reaction mixture was stirred for 16 h and then concentrated under reduced pressure. To the residue water (200 mL) was added and extracted with ethyl acetate (3 × 200 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated. The resulting oil was purified by flash silica gel column chromatography and eluted with 50% ethyl acetate in hexane to afford an oil. The oil was dissolved in MeOH/H_2O (200 mL/100 mL), Dowex WX8-400 (4.8 g) was added, and the mixture was stirred for 16 h at room temperature. The resin was filtered off and the filtrate was evaporated to yield **15** (10.7 g, 81% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.47−3.60 (m, 2H), 3.83 (q, *J* = 3.6 and 4 Hz, 1H), 4.04 (m, 1H), 4.29 (q, *J* = 6.4 and 5.2 Hz, 1H), 4.95 (t, *J* = 5.6 Hz, 1H), 5.06 (d, *J* = 4.8 Hz, 1H), 5.26 (d, *J* = 6.4 Hz, 1H), 5.97 (d, *J* = 6.4 Hz, 1H), 6.35 (d, *J* = 4 Hz, 1H), 6.67 (s, 2H), 7.37 (d, *J* = 4 Hz, 1H); MS (ES) *m/z* 301.70 $[M + H]^+$.

2-Amino-4-chloro-7-(2-*O*-methyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (16). A solution of the compound **15** (3.0 g, 9.9 mmol) in anhydrous DMF (300 mL) at 15 °C was treated with 60% NaH (0.42 g, 10.5 mmol). After 30 min, iodomethane (1.4 g, 9.9 mmol) was added dropwise to the reaction mixture and stirred at room temperature for 16 h. The solvent was removed under reduced pressure. The residue was purified by flash silica gel column chromatography and eluted with 5−10% MeOH in dichloromethane to afford **16** (2.43 g, 80% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.25 (s, 3H), 3.54 (m, 2H), 3.87 (m, 1H), 4.07 (m, 1H), 4.22 (m, 1H), 5.01 (m, 1H), 5.16 (d, *J* = 6.4 Hz, 1H), 6.07 (d, *J* = 6.4 Hz, 1H), 6.37 (d, *J* = 4 Hz, 1H), 6.70 (s, 2H), 7.40 (d, *J* = 4 Hz, 1H); MS (ES) *m/z* 316.00 $[M + H]^+$.

2-Amino-7-(2,3-anhydro-β-D-ribofuranosyl)-4-methoxy-7*H*-pyrrolo[2,3-*d*]pyrimidine (17). To a mixture of compound **15** (1.8 g, 6.0 mmol) in acetonitrile (80 mL) a solution of H_2O/CH_3CN (1:9, 1.08 mL) and α-acetoxyisobutyryl bromide (3.5 mL, 24 mmol) was added. After 2 h of stirring at room temperature, saturated aqueous $NaHCO_3$ (170 mL) was added and the mixture was extracted with ethyl acetate (2 × 200 mL). The combined organic phase was washed with brine (100 mL), dried over anhydrous Na_2SO_4, and evaporated. The pale-yellow residue obtained was suspended in anhydrous MeOH (80 mL) and stirred overnight with 25 mL of DOWEX OH^- resin (previously washed with anhydrous MeOH). The resin was filtered and washed thoroughly with MeOH, and the combined filtrate was evaporated to yield **17** (1.82 g, 94%) as a pale-yellow foam. ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.51 (m, 2H), 3.94 (s, 3H), 4.10 (t, *J* = 5.76 Hz, 1H), 4.19 (d, *J* = 2.6 Hz, 1H), 4.25 (d, *J* = 2.62 Hz, 1H), 5. 02 (t, *J* = 5.4 Hz, 1H), 6.20 (s, 1H), 6.31 (m, 3H), 7.13 (d, *J* = 3.76 Hz, 1H); MS (ES) *m/z* 279.1 $[M + H]^+$.

2-Amino-7-(3-deoxy-β-D-ribofuranosyl)-4-methoxy-7*H*-pyrrolo[2,3-*d*]pyrimidine (18). A solution of $LiEt_3BH$/THF (1 M, 75 mL, 75 mmol) was added dropwise at 0 °C (ice bath) to a deoxygenated (argon, 15 min) solution of compound **17** (1.92 g, 6.90 mmol) under argon atmosphere. Stirring was continued for 4 h at 0 °C. The reaction mixture was acidified with 5% aqueous acetic acid (110 mL), then purged with argon for 1 h and evaporated. The residue obtained was purified by flash silica gel column chromatography and eluted with 10−15% MeOH in CH_2Cl_2 to yield **18** (1.01 g, 60%) as a colorless foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.87 (m, 1H), 2.15 (m, 1H), 3.51 (m, 2H), 3.90 (s, 3H), 4.21 (m, 1H), 4.33 (m, 1H), 4.88 (t, *J* = 5.64 Hz, 1H), 5.44 (d, *J* = 4.44 Hz, 1H), 5.92 (d, *J* = 2.80 Hz, 1H), 6.15 (s, 2H), 6.23 (d, *J* = 3.60 Hz, 1H), 7.05 (d, *J* = 4.0 Hz, 1H); HRMS (FAB) calcd for $C_{12}H_{17}N_4O_4^+$ 281.1244, found 281.1252.

2′-*O*-Methyl-3′,5′-*O*-[1,1,3,3-tetraisopropyldisiloxane-1,3-diyl]guanosine (19). The 2′-*O*-methylguanosine **1** (10 g, 36.46 mmol) was dried over P_2O_5 under reduced pressure overnight. It was then dissolved in anhydrous pyridine (160 mL), 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (17.2 mL, 54.69 mmol) was added, and the reaction mixture was stirred at room temperature for 8 h under an inert atmosphere. The solvent was removed under reduced pressure, and the residue was partitioned between diethyl ether (200 mL) and water (200 mL). The mixture was then stirred for 5 min, and the precipitate formed was filtered. The sticky solid obtained was triturated with dichloromethane (100 mL), and the white solid obtained was filtered and washed with diethyl ether and dried under reduced pressure to yield **19** (16.23 g, 87% yield) as white solid. **19**. R_f = 0.38 (10% MeOH in CH_2Cl_2); ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.05 (m, 28H), 3.35 (s, 3H), 3.90−4.15 (m, 4H), 4.47 (m, 1H), 5.77 (s, 1H), 6.51 (br s, 2H), 7.74 (s, 1H), 10.68 (s, 1H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 12.0, 12.2, 12.3, 12.6, 16.8, 17.1, 17.2, 58.7, 60.1, 69.8, 80.8, 82.7, 86.1, 116.7, 133.8, 150.3, 153.8, 156.6; MS (AP-ES) *m/z* 538.7 [M − H]$^-$. Anal. ($C_{23}H_{41}N_5O_6Si_2$) C, H, N.

2-Amino-7-[2-*O*-(methyl)-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (20). Compound **20** (2.7 g, 85%) was synthesized from 2-amino-7-[2-*O*-(methyl)-*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one **2** (1.7 g, 6.13 mmol), anhydrous pyridine (27 mL), and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (3.85 mL, 12.26 mmol) following the procedure used for the synthesis of **19** except that the residue obtained after workup was purified by flash silica gel column chromatography and eluted with 5−10% MeOH in CH_2Cl_2. R_f = 0.3 (5% MeOH in CH_2Cl_2). ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.00 (m, 28H), 3.30 (s, 3H), 3.81−4.10 (m, 4H), 4.27 (m, 1H), 5.88 (d, J = 1.4 Hz, 1H), 6.25 (d, J = 3.7 Hz, 1H), 6.28 (br s, 2H), 6.75 (d, J = 3.7, 1H), 10.40 (s,1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.5, 13.0, 13.2, 16.9, 17.1, 17.3, 59.2, 60.1, 69.9, 80.8, 84.4, 87.4, 101.3, 102.3, 117.9, 150.5, 152.6, 161.1; MS (AP-ES) *m/z* 537.3 [M − H]$^-$. Anal. ($C_{24}H_{42}N_4O_6Si_2$) C, H. N: calcd, 10.40; found, 9.80.

***N*²-(4-Monomethoxytrityl)-2′-*O*-methylguanosine (21).** Compound **19** (5.32 g, 10.2 mmol) was mixed with DMAP (1.25 g, 10.2 mmol) and dried under reduced pressure over P_2O_5. The mixture was dissolved in anhydrous pyridine (102 mL), *p*-anisylchlorodiphenylmethane (9.44 g, 30.6 mmol) was added, and the reaction mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was removed under reduced pressure, and the residue obtained was co-evaporated with CH_2Cl_2 (3 × 50 mL). To the residue, a mixture of triethylamine trihydrofluoride (8.45 mL, 51 mmol) and triethylamine (3.56 mL, 25.5 mmol) in THF (102 mL) was added. The reaction mixture was stirred at room temperature for 24 h, diluted with ethyl acetate (150 mL), and washed with water (100 mL), aqueous $NaHCO_3$ (5%, 100 mL), and brine (100 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated to dryness, and the residue obtained was dissolved in 5% MeOH in dichloromethane (20 mL) and added dropwise into vigorously stirring hexane (800 mL). White precipitate formed was filtered and dried to yield **21** (7.81 g, 72%). R_f = 0.45 (10% MeOH in CH_2Cl_2). ^{1}H NMR (DMSO-d_6) δ 2.87 (s, 3H), 3.35−3.53 (m, 2H), 3.61−3.68 (m, 2H), 3.71 (s, 3H), 3.91 (q, J = 5.2 and 6.52 Hz, 1H), 4.91 (m, 2H), 5.17 (d, J = 3.3 Hz, 1H), 6.86 (m, 2H), 7.14−7.34 (m, 12H), 7.64 (s, 1H), 7.88 (s, 1H), 10.66 (s, 1H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 55.0, 57.2, 60.4, 68.3, 69.7, 82.6, 83.9, 85.3, 113.0, 117.3, 126.5, 127.4, 127.6, 128.4, 129.8, 135.8, 136.6, 144.3, 144.7, 149.0, 150.9, 156.4, 157.7; MS (AP-ES) *m/z* 568.1 [M − H]$^-$. Anal. ($C_{31}H_{31}N_5O_6$) C, H. N: calcd, 12.30; found, 11.80.

2-[(4-Monomethoxytrityl)amino]-7-[2-*O*-(methyl)-*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (22). Compound **22** (0.72 g, 75% yield) was synthesized from compound **20** (0.9 g, 1.67 mmol) according to the procedure used for the synthesis of **21**. DMAP (0.20 g, 1.67 mmol), anhydrous pyridine (16 mL), *p*-anisylchlorodiphenylmethane (1.03 g, 3.34 mmol), triethylamine trihydrofluoride (1.37 mL, 8.33 mmol), and triethylamine (0.60 mL, 4.20 mmol) in THF (17 mL) were also used. The residue obtained after workup was purified by flash silica gel column chromatography and eluted with 5% MeOH in CH_2Cl_2. R_f = 0.2 (5% MeOH in CH_2Cl_2). ^{1}H NMR (DMSO-d_6) δ 2.85 (s, 3H), 3.32−3.64 (m, 4H), 3.71 (s, 3H), 3.97 (q, J = 6.20 and 5.40 Hz, 1H), 4.84 (m, 2H), 5.28 (d, J = 3.4 Hz, 1H), 6.19 (d, J = 3.6 Hz, 1H), 6.85 (m, 2H), 6.88 (d, J = 3.6 Hz, 1H), 7.14−7.30 (m, 12H), 7.43 (s, 1H), 10.38 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 55.1, 57.8, 62.0, 69.6, 70.2, 82.5, 83.7, 87.2, 101.6,102.9, 113.2, 118.8, 127.0, 127.9, 128.5, 128.6, 129.9, 136.4, 144.4, 144.6, 149.5, 150.1, 158.3, 159.8; MS (AP-ES) *m/z* 567.2 [M − H]$^-$.

2′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-3′-deoxyguanosine (23). The 3′-deoxyguanosine **3** (1.45 g, 5.43 mmol) was mixed with imidazole (0.92 g, 13.6 mmol) and dried over P_2O_5 under reduced pressure overnight. The reaction mixture was dissolved in anhydrous DMF (50 mL) and *tert*-butyldimethylsilyl chloride (2.05 g, 27.15 mmol) was added. The resulting mixture was stirred at room temperature for 12 h under argon atmosphere. The reaction mixture was diluted with water (100 mL) and extracted with ethyl acetate (2 × 60 mL). The organic phase was dried over anhydrous Na_2SO_4, and solvent was removed under reduced pressure. The residue obtained was purified by flash silica gel column chromatography and eluted with 5% MeOH in CH_2Cl_2 to yield **23** (1.42 g, 53% yield).

2-Amino-7-[2,5-*O*-bis(acetyl)-3-deoxy-*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (24). Compound **4** (0.5 g, 1.9 mmol) was mixed with DMAP (0.09 g, 0.7 mmol) and dried under reduced pressure overnight. The sample was suspended in acetonitrile (10 mL) and triethylamine (0.63 mL, 3.8 mmol), and acetic anhydride (0.36 mL, 3.8 mmol) was added. The reaction mixture was stirred at room temperature for 5 h under argon atmosphere. The solvent was removed under reduced pressure, and the oily residue obtained was partitioned between water (15 mL) and CH_2Cl_2 (15 mL). The organic phase was separated and extracted with brine (20 mL), dried over anhydrous Na_2SO_4, and evaporated to yield **24** (0.63 g, 95% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.99 (s, 3H), 2.05 (s, 3H), 2.08−2.14 (m, 2H), 4.04 (dd, J = 6.4 and 5.6 Hz, 1 H), 4.23 (dd, J = 2.8 and 9.2 Hz, 1H), 4.35 (m, 1H), 5.34 (d, J = 6.8 Hz, 1H), 5.97 (d, J = 2 Hz, 1H), 6.27 (m, 3H), 6.84 (d, J = 3.6 Hz, 1H), 10.39 (s, 1H); MS (FAB) *m/z* 349.23 [M − H]$^-$.

3′,5′-*O*-Bis(*tert*-butyldimethylsiliyl)-2′-*O*-methylcytidine (25). The 2′-*O*-methylcytidine **5** (3 g, 11.7 mmol) was mixed with imidazole (7.94 g, 116.6 mmol) and dried under reduced pressure overnight. The mixture was dissolved in anhydrous DMF (8 mL), *tert*-butyldimethylsilyl chloride (8.82 g, 58.5 mmol) was added, and the reaction mixture was stirred at room temperature for 18 h under argon atmosphere. The reaction mixture was diluted with water (100 mL) and washed with ethyl acetate (2 × 60 mL). The organic phase separated, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified by flash silica gel column chromatography and eluted with 60% ethyl acetate in hexane to yield **25** (5.52 g, 97.5%). R_f = 0.33 (10% MeOH in CH_2Cl_2). ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.05 (s, 6H), 0.08 (s, 6H), 0.87 (m, 18H), 3.30 (s, 3H), 3.64 (m, 1H), 3.71 (br s, 1H), 3.84−3.95 (m, 2H), 4.17 (t, J = 6.6 and 5 Hz, 1H), 5.68 (d, J = 7.4 Hz, 1H), 5.82 (d, J = 2.4 Hz, 1H), 7.17 (br s, 2H), 7.82 (d, J = 7.4 Hz 1H); ^{13}C NMR (50 MHz,$CDCl_3$) δ −5.6, −5.4, −5.1, −4.6, 18.0, 18.3, 25.6, 25.9, 58.2, 60.3, 67.9, 82.0, 84.1, 88.1, 93.9, 141.2, 155.7, 166.0; MS (ES) *m/z* 484.3 [M − H]$^-$. Anal. ($C_{22}H_{43}N_3O_5Si_2$) C, H, N.

2′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-3′-deoxycytidine (26). Compound **26** (1.27 g, 79% yield) was synthesized from 3′-deoxycytidine **6** (0.8 g, 3.54 mmol), imidazole (2.41 g, 35.4 mmol), DMF (2.5 mL), and *tert*-butyldimethylsilyl chloride (2.68 g, 17.78 mmol) according to the procedure used for the synthesis of compound **25**. R_f = 0.24 (5% MeOH in CH_2Cl_2). ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.05 (m, 6H), 0.09 (s, 6H), 0.80 (s, 9H), 0.84 (s, 9H), 1.6−1.70 (m, 1H), 1.79−1.93 (m, 1H), 3.74 (dd, J = 2.2 and 9.6 Hz, 1H), 4.00 (dd, J = 2.4 and 9.4 Hz, 1H), 4.22 (m, 1H), 4.33 (m, 1H), 5.61 (m, 2H), 7.07 (br s,

2H), 7.91 (d, J = 7.4 Hz, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ −5.6, −5.4, −5.3, −4.5, 17.3, 18.4, 25.7, 25.9, 32.6, 62.8, 77.4, 81.5, 93.1, 141.6, 155.9, 165.9; MS (AP-ES) m/z 454.21 $[M - H]^-$. Anal. ($C_{21}H_{41}N_3O_4Si_2$) C, H, N.

***N*^{2}-(*p*-Anisyldiphenylmethyl)-3′-deoxyguanosine (27).** Compound **23** (0.36 g, 0.73 mmol) was dissolved in anhydrous pyridine (5.0 mL), and *p*-anisylchlorodiphenylmethane (0.25 g, 0.81 mmol) was added. The reaction mixture was stirred at room temperature overnight, evaporated in vacuo, and co-evaporated once with acetonitrile. The resulting residue was purified by flash silica gel column chromatography and eluted with methanol/dichloromethane (1:19). Fractions containing the product were pooled together and evaporated in vacuo to yield the desired intermediate (0.56 g). To this compound in THF (20 mL) was added tetrabutylammonium fluoride on silica (6.62 g, 10 equiv), and the resulting mixture stirred at room temperature for 2 h. The supernatant was decanted and evaporated in vacuo. The crude product obtained was purified by flash silica gel chromatography and eluted with MeOH/CH_2Cl_2 (1:9) to yield **27** (23.4%, 0.65 g). ^{1}H NMR (DMSO-d_6) δ 1.17−1.68 (m, 2H), 3.20−3.50 (m, 2H), 3.71 (s, 3H), 3.92 (br s, 1H), 4.01 (m, 1H), 4.85 (t, J = 5 Hz, 1H), 5.09 (d, J = 3.6 Hz, 1H), 5.12 (s, 1H), 6.86 (d, J = 8.6 Hz, 2H), 7.10−7.4 (m, 11H), 7.62 (s, 1H), 7.83 (s, 1H), 10.59 (br s, 1H); ^{13}C NMR (CD_3OD) δ 34.0, 54.7, 63.2, 70.8, 74.6, 81.2, 91.8, 92.1, 112.9, 117.3, 126.8, 127.7, 128.8, 130.3, 136.8, 137.4, 145.0, 145.1, 149.7, 151.2, 158.15, 158.8; MS (API-ES) m/z 540.0 $[M + H]^+$; HRMS (FAB) calcd for $C_{30}H_{30}N_5O_5^+$ 540.2276, found 540.2247. Anal. ($C_{30}H_{29}N_5O_5 \cdot H_2O$) H, N. C: calcd, 64.64; found, 65.57.

2-[(*p*-Anisyldiphenylmethyl)amino]-7-[3-deoxy-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (28). Compound **24** (0.63 g, 1.8 mmol) was dissolved in anhydrous pyridine (15.0 mL), and *p*-anisylchlorodiphenylmethane (1.2 g, 3.6 mmol) was added. The mixture was stirred at room temperature overnight, evaporated in vacuo, and coevaporated once with acetonitrile. The residue was purified by flash silica gel column chromatography using MeOH/CH_2Cl_2 (1:19) as the eluent. Fractions containing the product were pooled together and evaporated in vacuo to give the desired intermediate (0.94 g, 84%). This intermediate was dissolved in 2 N aqueous NaOH (50 mL) and dioxane (10 mL) and stirred at room temperature for 18 h. The reaction mixture was neutralized with 0.5 N aqueous HCl at 0 °C. The solid separated was filtered and dried under reduced pressure to yield **28** (0.73 g, 74% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.63 (m, 1H), 1.81 (m, 1H), 3.36−3.42 (m, 1H), 3.42−3.50 (m, 1H), 3.70 (s, 3H), 3.87 (br s, 1H), 4.01 (m, 1H), 4.79 (t, J = 5.2 Hz, 1H), 4.91 (d, J = 4.0 Hz, 1H), 5.17 (d, J = 1.6 Hz, 1H), 6.14 (d, J = 3.6 Hz, 1H), 6.84 (m, 3H), 7.15−7.39 (m, 12H), 10.30 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 35.5, 55.6, 63.4, 70.3, 75.5, 80.3, 90.9, 101.4, 102.2, 113.5, 118.5, 127.0, 127.7, 128.1, 128.2, 129.0, 129.1, 130.7, 137.6, 145.8, 146.0, 149.2, 150.2, 158.3, 159.0; HRMS (FAB) calcd for $C_{31}H_{30}N_4O_5Na^+$ 561.2108, found 561.2116.

***N*^{4}-(4,4′-Dimethoxytrityl)-2′-*O*-methylcytidine (29).** Compound **29** (1.8 g, 87%) was synthesized from compound **25** (1.75 g, 3.6 mmol), DMAP (0.06 g, 0.36 mmol), anhydrous pyridine (10. 4 mL), 4,4′-dimethoxytrityl chloride (2.44 g, 7.2 mmol), triethylamine trihydrofluoride (2.90 g, 18 mmol), and triethylamine (0.91 g, 9 mmol) in THF (36 mL) according to the procedure used for the synthesis of compound **21**. The residue obtained after workup was purified by flash silica gel column chromatography and eluted with ethyl acetate/hexane, 60:40, to yield **29**. R_f = 0.41 (5% MeOH in CH_2Cl_2). ^{1}H NMR (DMSO-d_6) δ 3.29 (s, 3H), 3.55−3.60, (m, 2H), 3.69 (s, 6H), 3.70−3.78 (m, 2H), 3.98 (m, 1H), 5.00 (d, J = 6.2 Hz, 2H), 5.69 (d, J = 4.6 Hz, 1H), 6.20 (d, J = 5.2 Hz, 1H), 6.81 (d, J = 8.8 Hz, 4 H), 7.10−7.60 (m, 9H), 7.75 (d, J = 7.2 Hz), 8.32 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 55.1, 58.5, 59.3, 67.3, 70.0, 83.2, 84.1, 89.5, 94.8, 112.9, 113.4, 127.3, 127.6, 128.3, 128.4, 128.7, 135.7, 135.9, 142.0, 144.1, 155.2, 158.5, 165.4; HRMS (FAB) calcd for $C_{31}H_{34}N_3O_7^+$ 560.2325, found 560.2319. Anal. ($C_{31}H_{33}N_3O_7$) C, H, N.

***N*^{4}-(4,4′-Dimethoxytrityl)-3′-deoxycytidine (30).** Compound **30** (0.36 g, 43% yield) was prepared from **26** (0.67, 1.47 mmol), DMAP (0.18 g, 1.47 mmol), anhydrous pyridine (4 mL), 4,4′-dimethoxytrityl chloride (1.00 g, 2.95 mmol), THF (15 mL), triethylamine trihydrofluoride (1.21 mL, 7.35 mmol), and triethylamine (0.51 mL, 3.68 mmol) according to procedure used for the synthesis of compound **21**. R_f = 0.41 (5% MeOH in CH_2Cl_2). ^{1}H NMR (DMSO-d_6) δ 1.66 (m, 1H), 1.85 (m, 1H), 3.47 (m, 1H), 3.63 (m, 1H), 3.71 (s, 6H), 4.00 (br s, 1H), 4.19 (m, 1H), 4.96 (t, J = 5.2 Hz, 1H), 5.39 (br s, 1H), 5.53 (s, 1H), 6.17 (br s, 1H), 6.83 (d, J = 8.8 Hz, 4H), 7.04−7.22 (m, 9H), 7.77 (d, J = 7.6 Hz, 1H), 8.27 (br s, 1H); ^{13}C NMR (50 MHz,CD_3CN) δ 33.7, 55.9, 63.1, 70.8, 77.0, 82.2, 94.9, 113.8, 114.2, 127.4, 127.7, 128.6, 129.6, 129.0, 130.0, 131.0, 137.58, 138.1, 140.5, 146.0, 156.3, 159.5; HRMS (FAB) calcd for $C_{30}H_{32}N_3O_6^+$ 530.2286, found 530.2291. Anal. ($C_{30}H_{31}N_3O_6$) H. C: calcd, 68.04; found, 67.29. N: calcd, 7.93; found, 7.29.

5-Bromo-3′,5′-*O*-bis[(*tert*-butyldimethyl)silyl]-2′-*O*-methylcytidine (31). Compound **25** (2.76 g, 5.68 mmol) was dissolved in acetonitrile (19.4 mL), LiBr (0.62 g, 7.18 mmol) was added, and the reaction mixture was stirred to get a clear solution. To this, ammonium ceric(IV) nitrate (6.24 g, 11.37 mmol) was added, and the reaction mixture was stirred at room temperature for 3 h. Solvent was removed under reduced pressure. The residue obtained was taken in ethyl acetate (100 mL) and washed with water (80 mL). The organic phase was separated, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified by silica gel column chromatography and eluted with 5% MeOH in CH_2Cl_2 to yield **31** (2.66 g, 83%). R_f = 0.57 (10% MeOH in CH_2Cl_2). ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.00 (s, 6H), 0.05 (s, 6H), 0.79 (s, 9H), 0.84 (s, 9H), 3.29 (s, 3H), 3.61−3.87 (m, 4H), 4.12 (t, J = 5.2 Hz, 1H), 5.74 (d, J = 3.6 Hz, 1H), 6.99 (br s, 1H), 7.84 (br s, 2H); ^{13}C NMR (50 MHz,$CDCl_3$) δ −5.3,−4.9, −4.5, 18.1, 18.8, 25.7, 26.3, 58.2, 60.8, 68.2, 83.4, 84.0, 87.6, 88.4, 141.0, 154.4, 162.6; MS (AP-ES) m/z 565.1 and 566.1 $[M + H]^+$; HRMS (FAB) calcd for $C_{22}H_{43}BrN_3O_5Si_2^+$ 564.1922, found 564.1925. Anal. ($C_{22}H_{42}BrN_3O_5Si_2$) H, N. C: calcd, 46.60; found, 45.64. Br: calcd, 14.15; found, 15.83.

5-Bromo-3′-*O*-[(*tert*-butyldimethyl)silyl]-2′-*O*-methylcytidine (32). Compound **31** (2.66 g, 4.7 mmol) was dissolved in 80% acetic acid in water (44 mL) and heated at 50 °C for 6 h. The solvent was removed under reduced pressure, and the residue obtained was purified by flash silica gel column chromatography and eluted with 5% MeOH in CH_2Cl_2 to yield **32** (0.85 g, 40%). R_f = 0.31 (5% MeOH in CH_2Cl_2). ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.07 (s, 6H), 0.86 (s, 9H), 3.39 (s, 3H), 3.44−3.6 (m, 2H), 3.69−3.9 (m, 2H), 4.24 (m, 1H), 5.29 (t, J = 4.4 Hz, 1H), 5.76 (d, J = 3.2 Hz, 1H), 7.06 (br s,1H), 7.88 (br s, 1H), 8.39 (s, 1H); ^{13}C NMR (50 MHz, CD_3OD) δ −4.9, −4.6, 19.0, 26.2, 58.9, 62.3, 70.2, 73.5, 85.5, 88.6, 90.2, 143.7, 157.3, 164.4; MS (AP-ES) m/z 450.1 and 453.1 $[M + H]^+$.

5-Bromo-2′-*O*-methylcytidine 5′-[Bis(*S*-pivaloyl-2-thioethyl)phosphate] (33). 5-Bromo-3′-*O*-(*tert*-butyldimethyl)-silyl-2′-*O*-methylcytidine **32** (0.09 g, 0.21 mmol) was mixed with 1*H*-tetrazole (0.03 g, 0.42 mmol) and dried over P_2O_5 in vacuo overnight. To this mixture anhydrous acetonitrile (2 mL) and bis(*S*-pivaloyl-2-thioethyl) *N*,*N*-diisopropylphosphoramidite **13** (0.2 g, 0.42 mmol) were added at 0 °C. The reaction mixture was allowed to come to room temperature and stirred for 4 h under inert atmosphere. Solvent was removed in vacuo, the residue obtained was cooled to −40 °C, and a solution of 3-chloroperbenzoic acid (0.07 g, 0.42 mmol, 57−80%) in CH_2Cl_2 (2 mL) was added. The solution was allowed to warm to −10 °C over 2 h. Sodium hydrogen sulfite (10% aqueous solution, 2 mL) was added to reduce the excess of 3-chloroperbenzoic acid. The organic phase was separated, diluted with CH_2Cl_2 (30 mL), washed with saturated aqueous $NaHCO_3$ (20 mL) and water (20 mL), dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved in THF (2.1 mL), and triethylamine trihydrofluoride (0.17 g, 1.1 mmol) was added. The reaction mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure to yield an oil. The oil was dissolved in ethyl acetate (30 mL) and was washed with

water (20 mL), 5% aqueous $NaHCO_3$, and brine (20 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated. The residue was dissolved in 20% MeOH in water and purified by HPLC on C-18 column (Luna C18, 250 mm × 2.12 mm, A = water, B = acetonitrile, 20−100% B in 65 mL, flow rate of 10 mL min^{-1}, λ = 260 nm) to yield **33** (0.054 g, 37% yield). HPLC retention time was 11.55 min (Phenomenex aqua, C-18, 150 mm × 4.6 mm, 5 μm, A = 100 mM triethylammonium acetate, pH 7, B = 90% acetonitrile in A, 2−100% B in 9 min,100% B till 25 min, flow rate of 1 mL min^{-1}, λ = 260 nm). ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.17 (s, 18H), 3.11 (t, J = 6.2 Hz, 4H,), 3.39 (s, 3H), 3.75 (t, J = 4.8 Hz, 1H), 3.93−4.3 (m, 8H), 5.23 (d, J = 6.4 Hz, 1H), 5.8 (d, J = 3.8 Hz, 1H), 7.07 (br s, 1H), 7.89 (s, 1H) 7.94 (br s, 1H); ^{13}C NMR (50 MHz, CD_3CN) δ 27.5, 29.3, 29.4, 47.2, 59.0, 66.8, 66.9, 67.2, 69.0, 82.5, 82.7, 84.2, 87.7, 89.6, 142.5, 155.1, 163.4, 206.5; ^{13}P NMR (80 MHz, CD_3CN) δ −0.34; MS (AP-ES) *m/z* 702.00 and 704.00 $[M - H]^-$; HRMS (MALDI) calcd for $C_{24}H_{39}BrN_3O_{10}PS_2Na^+$ 726.0890 and 728.0890, found 726.0893 and 728.0860.

Inhibition of HCV RNA Replication in Cells. The assay previously described in the literature was used to determine the inhibition of HCV RNA replication in a subgenomic bicistronic replicon in HBI10A cells by in situ RPA.[12]

Inhibition of NS5B Activity in Vitro. Inhibition of the enzymatic activity of HCV NS5BΔ21 by nucleoside analogue triphosphates was determined as previously described.[12]

Cytotoxicity Assay.[33] Cell cultures were prepared by plating at 2.0 × 10^4 cells/well of a 96-well plate in 100 μL of media. The next day 100 μL of cell culture media containing a 2 × level of compound was added per well and the plates were incubated at 37 °C and 5% CO_2 for a specified period of time. After the incubation period, 20 μL of CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent (MTS) (Promega) was added to each well, the plates were incubated at 37 °C and 5% CO_2 for 1 h, and the absorbance at 490 nm was read using a plate reader.

Intracellular Metabolism Studies. The intracellular metabolism of 5-[^{3}H] derivatives of 2′-*O*-methylcytidine **5** and 5′-bis(SATE)-2′-*O*-methylcytidine monophosphates ester **11** in Huh-7 and HBI10A cell lines was studied using the procedure reported in the literature.[12]

Assay for Chromosomal Aberrations in Chinese Hamster Ovary Cells. Compound **11** was evaluated for its potential to cause chromosome aberrations in Chinese hamster ovary (CHO, clone WBL) cells. Compound **11** was tested with and without a microsomal enzyme activation system (S-9) prepared from the livers of xenobiotic-treated rats. A solution of **11** in DMSO was used in this study and diluted 100-fold into culture medium. Exponentially growing monolayers were treated for 3 h with test compound with S-9 (in serum-free medium) or without S-9 (in medium with 10% fetal bovine serum), washed and allowed to recover until harvest at 20 h after beginning of the treatment, when the cells were fixed for analysis of chromosome aberrations (about 1.5 normal cell cycle lengths). Three additional sets of cultures (without S-9) were treated for 20 h and harvested at 20, 30, and 42 h after dosing. Colcemid was added 1−2 h before harvest to arrest cells in metaphase. Cytotoxicity was assessed as reductions in relative cell growth (population doubling) or monolayer confluence, abnormal cell morphology, and inhibition of cell division. Known chromosome-breaking agents, Cyclophosphamide (CP) with S-9 activation and mitomycin C (MMC) without S-9 activation, were tested as positive controls with compound **11**. 2′,3′-Dideoxycytidine (ddC) was also used as a positive control. The doses for the chromosome aberration assay of compound **11** were based on a preliminary solubility study in culture medium, and top doses for scoring aberrations were selected to suppress growth by about 50%.

Supporting Information Available: ^{1}H NMR, ^{31}P NMR, and HPLC data of compounds **7**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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