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Phosphoramidate Prodrugs of 2′-*C*-Methylcytidine for Therapy of Hepatitis C Virus Infection

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The application of a phosphoramidate prodrug approach to 2′-*C*-methylcytidine (NM107), the first nucleoside inhibitor of the hepatitis C virus (HCV) NS5B polymerase, is reported. 2′-*C*-Methylcytidine, as its valyl ester prodrug (NM283), was efficacious in reducing the viral load in patients infected with HCV. Several of the phosphoramidates prepared demonstrated a 10- to 200-fold superior potency with respect to the parent nucleoside in the cell-based replicon assay. This is due to higher levels of 2′-*C*-methylcytidine triphosphate in the cells. These prodrugs are efficiently activated and converted to the triphosphate in hepatocytes of several species. Our SAR studies ultimately led to compounds that gave high levels of NTP in hamster and rat liver after subcutaneous dosing and that were devoid of the toxic phenol moiety usually found in ProTides.

Introduction

An estimated 2% of the world's population is infected with the hepatitis C virus.[1] Presently there is no vaccine to prevent the infection and the combination of weekly injections of pegylated interferon-α (PEG-IFN) with daily oral administration of ribavirin is the recommended therapy.[2] This regimen gives a sustained viral response (SVR), which is defined as undetectable viral load 6 months after the end of treatment, in only 50% of the patients. The response rate is lower in patients infected with genotype 1 of the virus, where treatment can last up to 12 months, and considerably higher in infections associated with genotypes 2 and 3 where 6 months of treatment can be sufficient to achieve SVR. The current therapy is often associated with severe side effects such as depression, fatigue, flulike symptons, and hemolytic anemia that force many patients to discontinue treatment.[3,4] On the basis of the successful paradigm established for HIV, the development of agents that target specific viral enzymes is an attractive way to arrive at new and better tolerated treatment regimens.[5−10]

The HCV[a] nonstructural protein 5B (NS5B) is an RNA dependent RNA-polymerase (RdRp) which is at the core of the HCV replication complex. Inhibitors of the polymerase can be assigned to two broad categories based on their mechanism of action and their chemical structure: nucleoside analogues (NI) and allosteric non-nucleoside inhibitors (NNI). Nucleoside inhibitors are especially attractive and are expected to play a prominent role in future therapies. They target the active site of NS5B, which is highly conserved across the six major HCV genotypes, and are expected to be broadly effective against infections with HCV genotypes 1−6.[11] Another advantage of nucleoside inhibitors is that they show a higher barrier to the emergence of resistance with respect to allosteric polymerase inhibitors, whose binding sites display a considerable polymorphism.[9,12] This has been confirmed in studies using the standard subgenomic replicon system[13−15] and also on HCV isolates from patients, where resistance mutations to NNIs were observed at a low frequency within the clinical isolates' viral quasispecies. This was not the case for the known mutations against nucleoside inhibitors.[16]

Three nucleoside inhibitors, all of them cytidine analogues with a modified ribose unit, have shown anti-HCV activity as single agents in clinical trials (Figure 1). Valopicitabine **1** (NM283) was the first polymerase inhibitor to achieve proof of concept for HCV NS5B inhibitors in the clinic. About 1.2 $\log_{10}$ reduction of plasma HCV RNA was observed in 14 days upon an oral dose of 800 mg b.i.d. In similar settings **2** (R7128) achieved 2.7 $\log_{10}$ (1500 mg, bid) and **3** (R1626) achieved 1.2 $\log_{10}$ (1500 mg q.d.). Of these three nucleosides only **2** is still under development.[17]

Nucleoside analogues need to be converted via cellular kinases to the active triphosphate (NTP), which in the cases of **1**−**3** act as a nonobligate chain-terminators, causing inhibition of viral replication.[12,18,19]

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[a] Abbreviations: HCV, hepatitis C virus; SAR, structure−activity relationship; NTP, nucleoside triphosphate; DCM, dichloromethane; THF, tetrahydrofuran; *p*TsOH, *p*-toluenesulfonic acid; RP-HPLC, reverse phase high performance liquid chromatography; Py, pyridine; TFA, trifluoroacetic acid; EC_{50}, compound concentration that returns 50% of inhibition; CC_{50}, compound concentration that returns 50% of cytotoxicity; SI, selectivity index defined as CC_{50}/EC_{50}; AUC, area under the curve; PK, pharmacokinetic; BLQ, below the limit of quantification; RT, room temperature; DMSO, dimethyl sulfoxide; DMA, dimethylacetamide; ACN, acetonitrile; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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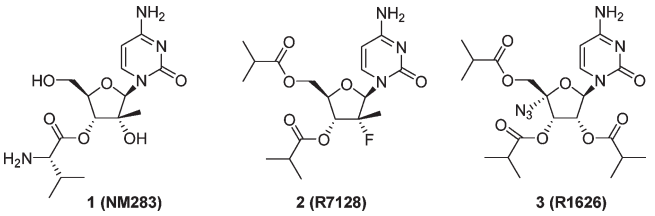


Figure 1. Structures of **1** (NM283, valopicitabine), **2** (R7128), and **3** (R1626).

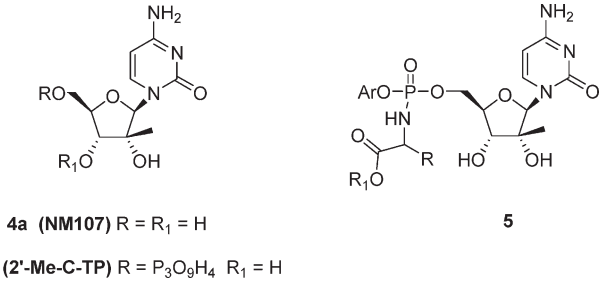


Figure 2. Structures of 2′-*C*-methylcytidine **4a** (NM107), the corresponding NTP **4b**, and the targeted aryl phosphoramidate prodrugs **5**.

The efficacy of the chain termination depends on the affinity of the modified NTP for the polymerase, the ratio of the NTP analogue to endogenous NTP's, and the relative rate of incorporation of the polymerase for different nucleotides. Other factors that potentially limit the activity of the NIs are poor cell permeability, unwanted metabolic reactions, or poor conversion to the pharmacologically active triphosphate. In the latter case the first phosphorylation step that produces the 5′-monophosphate has often been found to be rate-limiting.[20]

This might also be the case for 2′-*C*-methylcytidine. It was noted by us and others that the triphosphate of 2′-*C*-methylcytidine **4b** (2′-Me-C-TP, Figure 2) is highly active on the isolated NS5B enzyme (IC_{50} = 0.025 μM), but the parent nucleoside **4a** (NM107) shows only modest potency in the cell-based assay (2−7 μM).[21] Recent results from the literature indicated that poor conversion of **4a** to its NTP is the reason. Among a variety of modified nucleosides investigated, **4a** was the poorest substrate for conversion to its monophosphate by human 2′-deoxycytidine kinase.[19]

To circumvent this problem, several monophosphate prodrug strategies can be applied, where the charged, non-cell-penetrable phosphate group is masked to give a more lipophilic membrane permeable compound.[20,22] Recent successful examples applied to HCV include liver-targeted prodrugs (HepDirect) of 2′-*C*-methyladenosine, where the nucleoside was protected from rapid metabolism to the inactive inosine derivative,[23] or phosphoramidate (ProTide) prodrugs of 4′-azidouridine, which turned the inactive nucleoside, which was not monophosphorylated in cells, into a potent inhibitor of HCV in cell culture.[24]

The ProTide approach has also been successful with derivatives of ddA,[25] d4T,[26,27] LCd4A,[28] and d4A.[25,29]

We started a program with the objective to discover a monophosphate prodrug of 2′-*C*-methylcytidine **4a**, hoping that by bypassing the initial kinase dependence of **4a**, we could generate high levels of NTP **4b** in the liver. If successful, this could lead to a potential new agent for the treatment of HCV infection or improve the therapeutic index of **4a**. As our starting point, we chose the ProTides **5** (Figure 2).

In the current study we also address issues such as the replacement of the potentially toxic aryl moiety present in **5**.

Results and Discussion

Chemistry. The synthesis of aryl phosphoramidate prodrugs of 2′-*C*-methylcytidine was carried out as depicted in Scheme 1. The previously described chemistry for the synthesis of ProTide prodrugs, using 1-methylimidazole as the coupling agent, failed under various conditions. Uchiyama's method was investigated next. The nucleoside **4a**, prepared as described in the literature,[30,31] was used as such or protected as its 2′,3′-isopropylidene ketal **9** to increase solubility and was then phosphorylated at the 5′ position with the appropriate aryloxyphosphorochloridate **8**,[24,32] using a strong organometallic base such as *tert*-butylmagnesium chloride to form the corresponding alkoxide.

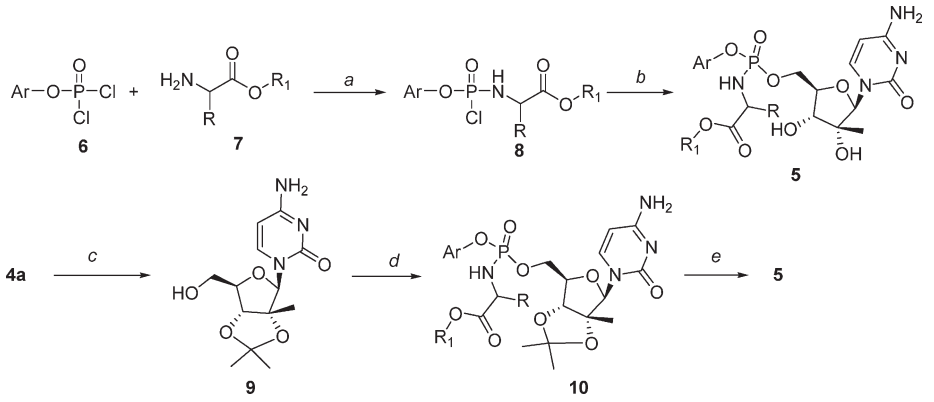
When the ketal **9** was used, the deprotection of **10** was performed with a solution of 80% formic acid in water for 4 h at room temperature. Because of the stereochemistry at the phosphorus center, the final compounds **5** were isolated as mixtures of two diastereoisomers that, whenever possible, were separated by reverse phase high performance liquid chromatography. The absolute stereochemistry of the diastereomers at phosphorus has not been elucidated, but HPLC retention times were used as parameters to discriminate between the two isomers.

For the preparation of benzyloxy phosphoramidates **14a** and **14b** and the *des*-phenol derivative **16** a different chemical approach based on a final oxidative step (Atherton−Todd reaction)[33] was followed (Scheme 2). Diphenylphosphite was treated with fluorenylmethyl alcohol (FmOH) or benzyl alcohol to give the intermediates **12** which were then treated with the nucleoside **4a**, in pyridine at 0 °C. Pyridine was removed from the solution under reduced pressure, and the resulting bisphosphites **13** were subsequently reacted with alanine ethyl ester in the presence of CCl_4 and Et_3N to give the desired products **14** and **15**. Because of solubility problems, this step had to be carried out in a mixture of isopropanol and *N*,*N*-dimethylacetamide in order to achieve good yields. The fluorenylmethyl derivative **15** was submitted to piperidine deprotection to give the phosphoramidate **16**.

Following a literature procedure,[34] the alanyl phosphoramidate **18** was efficiently prepared by treating a 1:1 diastereomeric mixture **17** with Et_3N in water (Scheme 3). Compound **18** is unstable at room temperature, but it could be stored at −80 °C for several weeks.

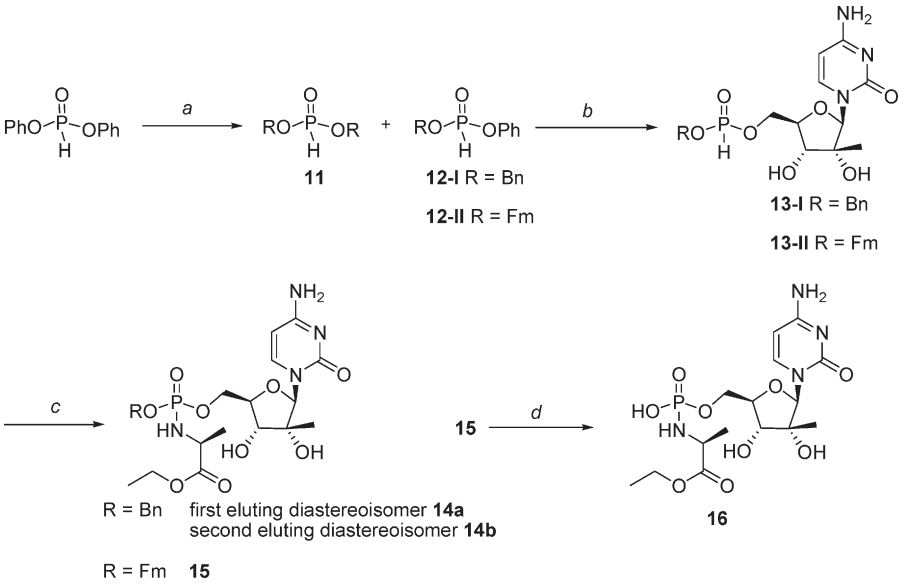
For the preparation of the phosphoramidate monoesters **50** and **51** we worked out conditions for a multigram scale synthesis that are reported in Scheme 4. The protected nucleoside **9** was treated with biphenyl phosphite and then with benzyl alcohol to give compound **19**. The order of addition of the benzyl alcohol and the nucleoside is critical, since treating the phosphite first with benzyl alcohol leads mainly to addition of two molecules of BnOH to the phosphite and consequently low yields of **19**. Compound **19** was then coupled to esterified aminoacid **21-I** or aminoalcohol **21-II** under oxidative conditions, followed by deprotection of the acetonide and removal of the benzyl group. Hydrogenation in the presence of Pd/C 5% was the method of choice to avoid overreduction of the cytidine residue and to obtain the final compounds **50** and **51** in excellent purity.

Scheme 1. General Synthetic Pathway for the Synthesis of 2′-*C*-Methylcytidine Aryloxy Phosphoramidates **5**[a]



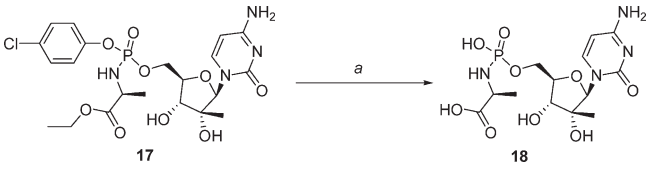
[a] Reagents and conditions: (a) Et_3N, DCM; (b) **4a**, *t*-BuMgCl, dry THF; (c) acetone, *p*TsOH, 71−87% yield; (d) **8**, *t*-BuMgCl, dry THF; (e) HCOOH 80%, water. For details of the structures **5**, see Tables 1−3.

Scheme 2. General Synthetic Pathway for the Synthesis of 2′-*C*-Methylcytidine Aryloxy Phosphoramidates **14a**, **14b**, **15** and Monoester **16**[a]



[a] Reagents and conditions: (a) BnOH or FmOH, Py, 0 °C; (b) **4a**, Py, 0−40 °C, 1 h; (c) L-AlaCOOEt·HCl, Et_3N, CCl_4, *i*-PrOH, *N*,*N*-dimethylacetamide, 10 min, 0 °C; (d) piperidine, DCM.

Scheme 3. Preparation of the Metabolic Product **18**[a]



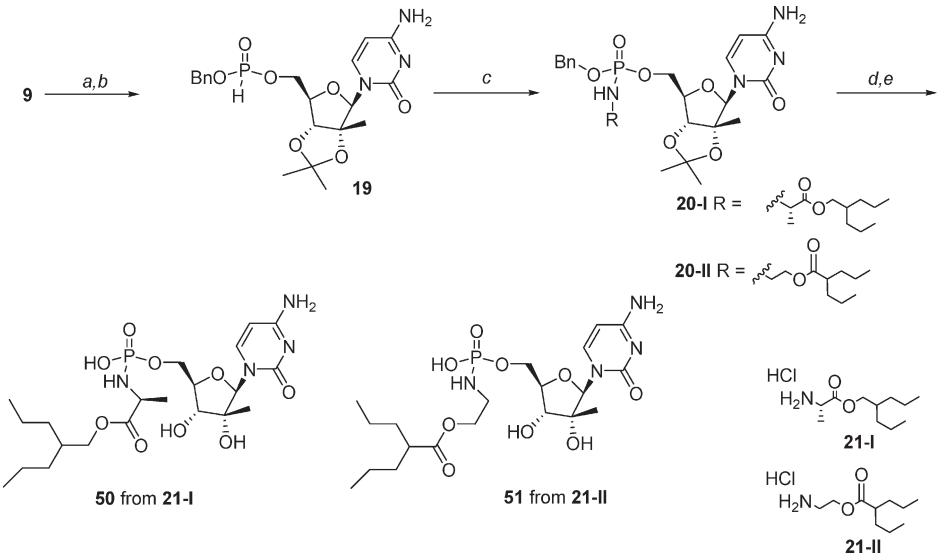
[a] Reagents and conditions: (a) Et_3N/water, yield 54%.

SAR, Antiviral Activity, and in Vivo NTP Formation. On the basis of the extensive studies reported in the literature by McGuigan and co-workers,[25,27] we performed a comprehensive SAR study. The amino acid, its stereochemical variation, the ester functionality of the amino acid, and the aryloxy moiety were explored by preparing about 250 prodrugs. We were aware that a separate ProTide motif optimization process is needed for each nucleoside analogue versus a given target,[24] since cell line dependent enzyme expression may determine different phosphoramidate SAR. The phosphoramidates were characterized as in vitro inhibitors of HCV replication in the subgenomic HCV replicon assay and compared to the parent nucleoside **4a** or its valyl ester **1**. Both reference compounds **1** and **4a** had an EC_{50} of about 8 μM in our hands. In the first round of SAR we kept the *p*-chlorophenyl moiety constant and investigated the amino acid portion using the corresponding methyl or ethyl esters. Table 1 shows the most representative results. Compound **17**, as a 1:1 mixture of diastereoisomers, containing L-alanine ethyl ester already showed a 4-fold improvement in potency with respect to **1** and no sign of cytotoxicity up to 20 μM.

Other amino acids such as glycine methyl ester (**22**, EC_{50} = 0.8 μM) or L-norleucine (**23**, EC_{50} = 1.5 μM) gave also efficient prodrugs. Compound **24**, containing β-alanine was inactive, while compounds with D-alanine (**25**) or other amino acids (**26**−**29**) offered no advantage with respect to **1**. All the potent compounds ($EC_{50} \leq 1$ μM) showed $CC_{50} > 20$ μM.

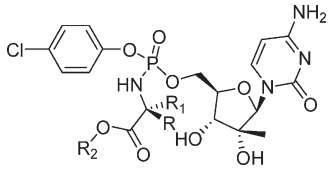
Compound **17** was separated into its diastereoisomers using reversed phase HPLC, giving **17a**, the first eluting,

Scheme 4. Multigram Scale Synthesis of Compounds **50** and **51**[a]



[a] Reagents and conditions: (a) $(PhO)_2POH$, Py, anhydrous conditions; (b) BnOH; (c) **21-I** or **21-II**, Et_3N, CCl_4, CH_3CN, *i*-PrOH, THF, 50% yield over three steps; (d) TFA−H_2O (8:2); (e) H_2, Pd/C 5%, *i*-PrOH.

Table 1. Cell-Based Activity and Cytotoxicity of **1** and Prodrugs **17**, **17a**, **17b**, and **22**−**29**



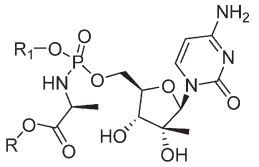
compd	R	R_1	R_2	EC_{50} (μM)[a]	CC_{50} (μM)[b]	SI CC_{50}/EC_{50}
1 (NM283)				7.6	> 100	> 13
17 (1:1)	Me	H	Et	1.8	> 20	> 11
17a (fe)	Me	H	Et	7.9	> 20	> 2.5
17b (se)	Me	H	Et	1.0	> 20	> 20
22 (1:1)	H	H	Me	0.8	> 20	> 25
23 (1:0.8)	*n*-Bu	H	Et	1.5	> 20	> 13
24 (1.5:1)	β-Ala	β-Ala	Me	> 20	> 20	
25 (2:1)	H	Me	Me	7.7	> 20	> 2.5
26 (1:1.5)	Bn	H	Me	> 20	> 20	
27 (1:1)	*i*-Bu	H	Et	7	> 50	> 7
28 (1:0.8)	Ph	H	Et	7.4	> 20	> 2.5
29 (1:0.9)	3-indolyl-CH_2-	H	Et	17	> 50	> 3

[a] EC_{50} values are quoted for $n = 2-9$ independent determinations. [b] Concentration of inhibitor reducing the cell viability by 50%.

and **17b**, the more lipophilic second eluting diastereomer. Interestingly, they showed an 8-fold difference in activity, the first eluting **17a** being the less active. This trend in activity was observed for all the pairs of diastereoisomers whenever we were able to separate them. The higher activity of the second eluting, more lipophilic diastereoisomer is not unprecedented[35] and could be due to a better diffusion through cell membranes or to a more efficient stereoselective metabolism of this diastereoisomer. Because of this trend in the activity, we report from now on only the activity of the second eluting diastereoisomer. A full set of replicon data for the first eluting diastereoisomers can be found in the Supporting Information. Compound **17b** was used as a lead to develop further SAR on the phenol residue with particular focus on more hydrophobic moieties (Table 2).

The SAR around the phenolic moiety showed that beyond 4-Cl, also 2-Cl and even 2-Me were very good substituents (compounds **32b** and **33b**). Substituents with higher electron withdrawing or electron donating effects gave compounds (**30**−**31**) less active than **17**. Very interesting results were obtained by replacing the phenyl ring with much more lipophilic groups such as naphthyls (compounds **34b**−**36**) or an extensively substituted phenyl ring (**37b**) leading to submicromolar inhibitors. Similar results were seen with potential anticancer phosphoramidates of BVdU.[24,35] In the case of compounds **34b** and **35b**, the contribution (2-fold) from a more lipophilic ester group, a butyl versus an ethyl, also needs to be appreciated. All these derivatives showed EC_{50} below 0.2 μM, while salicylate derivative (**38**), nonaromatic (**39b**), or benzylic (**14b**) moieties offered no advantage. Efforts to release a natural amino acid such as

Table 2. Cell-Based Activity of Prodrugs **30**−**42**



Compd	R_1	R	EC_{50} (μM)[a]	CC_{50} (μM)[b]	SI CC_{50}/EC_{50}
17b	4-Cl-Ph	Et	1.0	> 20	> 20
30 (1:1)	4-CF_3-Ph	Me	9.6	> 50	> 5
31 (1:1)	4-OMe-Ph	Me	3.7	> 50	> 13
32b	2-Cl-Ph	Bu	0.87	> 20	> 23
33b	2-CH_3-Ph	Et	0.18	9	50
34b	1-Naphthyl	Et	0.20	15	75
35b	1-Naphthyl	Bu	0.09	9	100
36 (1:2.5)	4-Cl-1-Naphthyl	Bu	0.16	> 20	125
37b	4-Cl-2-*i*Pr-5-Me-Ph	Et	0.19	9	47
38 (1:1)	2-COOMePh	Et	11	> 50	> 5
39b	2-CCl_3-CH_2	Et	10	> 50	> 5
14b	Bn	Et	> 20	> 20	-
40b	TyrCOOMe	Et	> 20	> 20	-
41b		Et	0.50	> 20	40
42b	Ph	Et	0.45	> 20	44

[a] EC_{50} values are quoted for $n = 2-9$ independent determinations. [b] Concentration of inhibitor reducing the cell viability by 50%.

tyrosine instead of phenol (**40b**) failed, probably due to poor processing by cellular esterases in the presence of a sterically more hindered substituent.[36] The release of an indole instead is possible as shown by the activity of prodrug **41b** that is equipotent to the unsubstituted phenol derivative **42b**, which is itself more active than **17b**. All the most active compounds ($EC_{50} \leq 0.5$ μM) demonstrated a selectivity index SI (CC_{50}/EC_{50}) of more than 40-fold. This suggests that inhibition of the replicon is due to conversion of the phosphoramidate prodrugs to the triphosphate species which acts as a nonobligate chain terminator of NS5B polymerase.[12] To assess the selectivity toward other unrelated targets with different mechanism of action, the prodrug **42b** and the active species, the triphosphate **4b**, were also tested in the Pan Laboratories screening against 150 different enzymes, channels, and receptors; the two agents showed no significant activity up to 10 μM.

On the basis of the encouraging results obtained in the cell based assay, we wanted to confirm that these prodrugs generate high levels of triphosphate in the replicon cells (HuH7/HB1 10a cells) and in hepatocytes. As shown in Figure 3, in the replicon HuH7/HB1 10a cells, NTP formation from prodrug **17b** was much higher than from the valyl ester **1** and the 10-fold more potent prodrug **35b** gave even higher NTP levels.

The same behavior was observed in human hepatocytes between prodrugs **17b** and **35b** and reference compound **1** (see Figure 4). In human hepatocytes, the amount of NTP formed is dose dependent (see Table 3). When compound **17b** was incubated at 10 μM, the amount of NTP formed expressed as AUC_{0-4h} was 62 μM·h, 10-fold lower than when the same compound was incubated at 100 μM, $AUC_{0-4h} = 698$ μM·h. A good dose dependence was also observed with compound **35b**. With it AUC_{0-4h} of 106, 343, and 553 μM·h were obtained at incubation concentrations of 10, 30, and 100 μM, respectively.

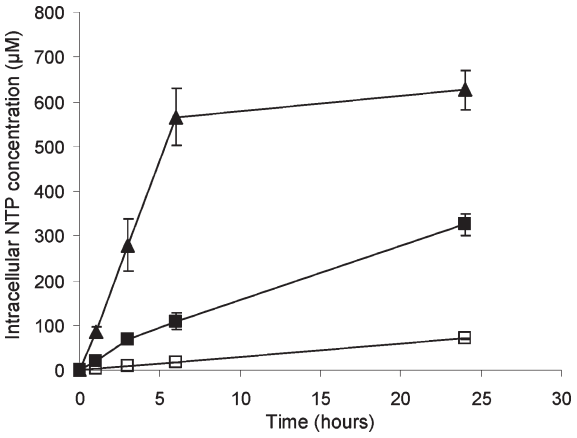


Figure 3. Time course of triphosphate formation in HBI10A cells. HBI10A cells were incubated with 100 μM compound **1** (empty square), compound **17b** (filled square), and compound **35b** (triangle). Intracellular NTP was quantified at the indicated time points, considering that 10^6 hepatoma cells is 2 μL in volume. Each point is the mean ± half-range of duplicate determinations.

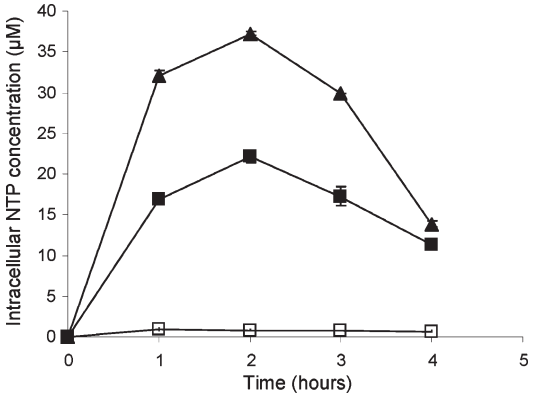


Figure 4. Kinetics of phosphorylation in primary human hepatocytes. Cryopreserved primary human hepatocytes were incubated with 10 μM compound **17b** (filled square), compound **35b** (triangle), and compound **1** (empty square). The intracellular concentration of NTP is estimated considering that 10^6 hepatocytes is 4 μL in volume. Results are the average ± half-range, $n = 2$.

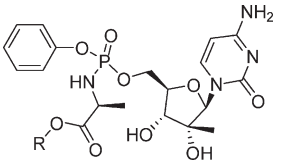
Table 3. NTP AUC of Compounds **1**, **17b**, and **35b** in HuH7 Cells and in Human and Rat Hepatocytes[a]

compd [concn (μM)]	HuH7/HB1 NTP AUC_{0-24h} (μM·h)[b]	human hepatocytes NTP AUC_{0-4h} (μM·h)[c]	rat hepatocytes NTP AUC_{0-4h} (μM·h)[d]
1 [10]	70	3	7
1 [100]	830		
17b [10]		62	517
17b [100]	4265	698	
35b [10]		106	
35b [30]		343	
35b [100]	12404	553	

[a] Summary of results reported in Figures 3−5. [b] Intracellular 2′-*C*-methylcytidine triphosphate (NTP) after incubation in replicon HuH7/HB1 10a cells. [c] Intracellular NTP after incubation with human hepatocytes. [d] Intracellular NTP after incubation with rat hepatocytes.

To validate these prodrugs in vivo, we screened them in rat hepatocytes, and also in this preclinical species, compound **17b** gave high NTP level (see Figure 5).

Table 4. Antiviral Activity and Conversion to NTP in Human Hepatocytes of Prodrugs **42b**−**49b**



compd[a]	R	EC_{50} (μM)[b]	CC_{50} (μM)[c]	SI CC_{50}/EC_{50}	[NTP], AUC_{0-2h} (μM·h)[d]
42b	Et	0.45	> 20	> 44	65
43b	*n*Pr	0.97	> 20	> 20	57
44b	*i*-Pr	0.21	10	47	89
45b	$MeO(CH_2)_3$	1.9	> 20	> 10	115
46b	2EtBu	0.22	7	31	460
47b	1-Hep	0.10	6	60	551
48b	*c*-Hep	0.10	9	90	638
49b	2-PrPen	0.037	2	54	107
1 (NM283)		7.6	> 100	13	16

[a] Second eluting diastereoisomer. [b] EC_{50} values are quoted for $n = 2-9$ independent determinations. [c] Concentration of inhibitor reducing the cell viability by 50%. [d] Intracellular 2′-*C*-methylcytidine triphosphate (NTP) after incubation at 10 μM with cryopreserved human hepatocytes (10^6 cells/mL), $n = 2-8$.

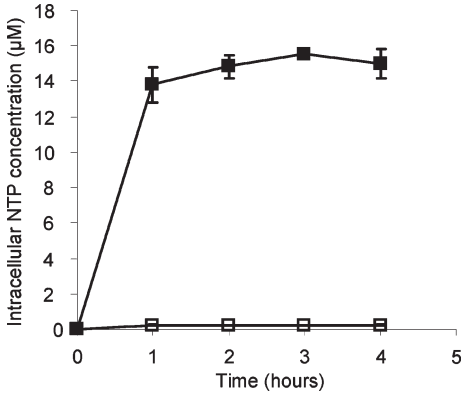


Figure 5. Time course of triphosphate formation in rat hepatocytes. Rat hepatocytes were incubated with 10 μM compound **1** (empty symbol) and compound **17b** (filled symbol), and NTP levels were quantified at the indicated time points. Data are the average ± half-range from duplicate experiments.

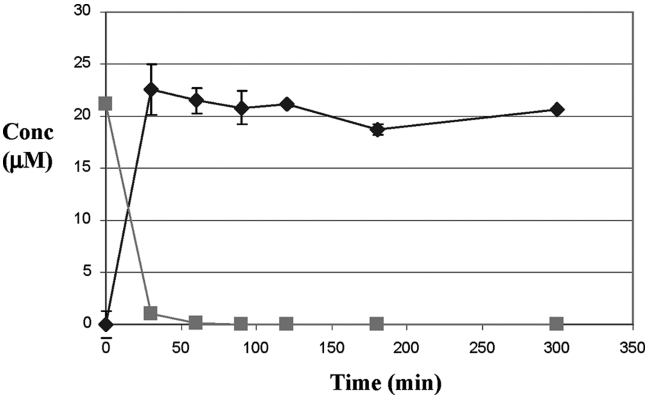


Figure 6. Conversion of compound **17b** (square) to the metabolic product **18** (rhombus) in rat plasma.

These results were very encouraging and consistent with the observations in the replicon assay, indicating that the prodrugs successfully deliver the monophosphate to the replicon cells and confirming that a kinase bypass approach can circumvent the poor conversion of **4a** to its NTP **4b**.

The plasma stability of prodrug **17b** was tested in several species. No significant degradation occurred in human and dog plasma, while significant degradation was observed after 5 min of incubation time in rat and mouse plasma. To facilitate the identification of the degradation products of these pronucleotides, the hypothesized metabolite **18** was prepared,[34] and it proved to be the main hydrolysis product of compound **17b** in rat plasma (Scheme 2 and Figure 6).

Having shown that these prodrugs deliver NTP in human and rat hepatocytes, we turned our attention to the releasing moiety: 1 equiv of phenol is released by each molecule of pronucleotide administered. Since *p*-chlorophenol toxicity is less documented, the studies were focused on the unsubstituted phenol derivatives **42b**−**49b**, which are also more potent (Table 4). An extensive SAR study was conducted, and a very wide range of esters were tested. From the results in Table 4, it was clear that ester variation was widely tolerated: not only were the usually reported esters active (**42b**−**44b**) but also longer residues such as compounds **45b**−**49b**, with the 2-Pr-Pen derivative **49b** being one of the most potent phosphoramidates in the replicon assay with $EC_{50} = 0.037$ μM. The reported compounds were all tested for their capacity to generate NTP in human hepatocytes (see Table 4). All the reported prodrugs were more active than the reference compound **1** in the replicon assay with $EC_{50} \leq 1$ μM, and these results were confirmed by the values of NTP measured after incubation of the prodrugs at 10 μM with cryopreserved human hepatocytes. In every case the NTP concentration was much higher than with compound **1**. The correlation between replicon activity and NTP concentration in human hepatocytes is good. Very potent compounds such as **46b**−**48b** with $EC_{50} < 250$ nM generally form very high NTP concentrations in human hepatocytes with AUC_{0-2h} above 450 μM·h.

On the basis of the in vitro potency and of the data collected in the hepatocytes, compound **46b** was profiled further. The stability of prodrug **46b** was measured in plasma samples of several species. No significant degradation occurred in dog and human plasma, while a significant degradation occurred after 5 min of incubation in rat plasma samples. The poor stability in rat plasma of these prodrugs complicates their

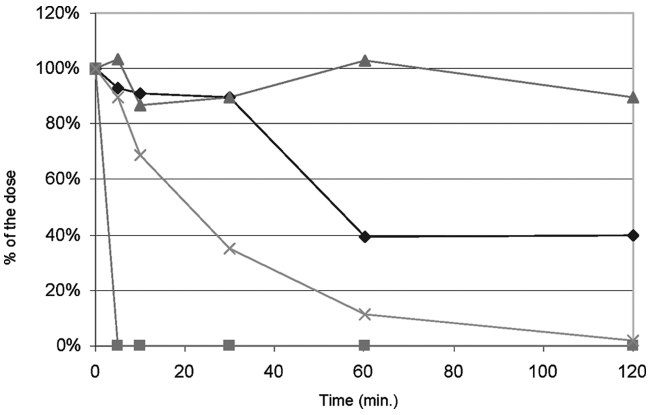


Figure 7. Disappearance of compound **46b** in human (rhombus), rat (square), dog (triangle), and hamster (cross) plasma.

Table 5. Hamster Liver NTP Level (μM) of Compound **46b** and **1** after Different Administration Routes

		hamster liver NTP level (μM)[c]	
compd	administration route	**3h**	**6h**
46b	po[a]		1.1
	im[b]	3.6	2.2
	sc[b]	5.6	10.1
1	po[c]		0.48
	sc[d]		BLQ[e]

[a] 30.2 μmol/kg. [b] 1.5 μmol/kg. [c] $n = 3$. [d] 7.55 μmol/kg. [e] Lower limit of quantification = 0.2 μM.

development. In the literature very interesting findings are reported to overcome these kinds of problems such as conducting the studies in commercially available carboxyesteresase deficient mice[37] or with the support of carboxyesterases inhibitors. However, the compound[38] was found to be moderately stable in hamster plasma (Figure 7).

Because of its acceptable stability in hamster plasma, we could evaluate the pharmacokinetic profile of compound **46b** in this species. Compound **46b** was dosed orally, intramuscularly, and subcutaneously using compound **1** as a reference, and liver levels of NTP **4b** were determined at 3 and 6 h (time of maximum NTP concentration).[39]

As shown in Table 5, after oral administration of 30 μmol/kg compound **46b** to hamsters, the NTP concentration in the liver at 6 h was 1.1 μM. This low NTP concentration, albeit 2-fold higher than that obtained with **1**, is indicative of a very low bioavailability of this prodrug. The administration by intramuscular injection at 20-fold lower dose (1.5 μmol/kg) gave much higher liver NTP concentrations, 3.6 and 2.2 μM at 3 and 6 h, respectively. The administration by subcutaneous injection at the same dose gave even higher liver exposure with NTP concentrations of 5.6 and 10.1 μM at 3 and 6 h, respectively, while **1** gave NTP levels below the lower limit of quantification (0.2 μM). The stability of prodrug **46b** in simulated gastric fluid[40] was measured as well, and the compound was completely stable after 3 h; the reason for the low bioavailability might be poor intestinal absorption of the compound or metabolic degradation in the intestine.

Subcutaneous administration gave a high level of NTP in the liver, and the remaining issue to be faced was the compatibility of this approach with the release of an equivalent amount of phenol. In the literature many of the effects of phenol toxicity are extensively documented;[41] therefore, the

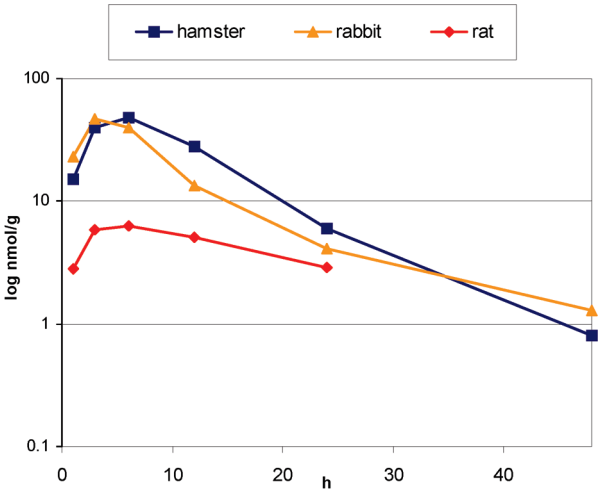


Figure 8. Profile of the liver concentration of NTP **4b** in hamster, rabbit, and rat after subcutaneous administration of **50** at 1 mg/kg.

elimination of the phosphoramidate phenol moiety became the focus of our investigation.

The lesson learned so far was that a more lipophilic residue is necessary to obtain higher NTP levels. With this in mind, supported by the work already present in the literature by Wagner and co-workers,[42−46] two pairs of compounds with and without the phenol and based on a relevant difference in lipophilicity were prepared. The results are reported in Table 6.

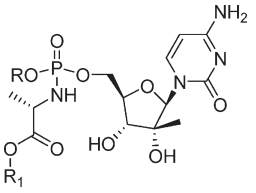
In the case of the less polar pair of compounds **42b** and **16** bearing an alanine ethyl ester, the results were in favor of the phenol derivative **42b**, active in the replicon and more efficient in forming NTP in human hepatocytes. In the case of the more apolar pair of compounds **49b** and **50**, bearing the more lipophilic 2-propylpentyl ester, the behavior was inverted. We were very pleased to see that the monoester prodrug **50**, although not more active in the replicon assay than the parent compound **1**, generated very high NTP levels in human hepatocytes with AUC_{0-2h} = 511 μM·h. The reason for the difference between the replicon cells and the hepatocyte data might be the completely different physicochemical properties of **50** with respect to the ProTide prodrugs. Possibly, nucleoside uptake mechanisms present in hepatocytes but not in the replicon cells could be involved, as well as differences in the expression of hydrolytic enzymes between the two types of cells.[47]

Compound **50** demonstrated a selectivity index SI (CC_{50}/EC_{50}) comparable to that of the parent compound. It was tested in the Pan Laboratories screening as well, and it showed no significant activity up to 10 μM.

Prodrug **50** was stable in human, dog, rabbit, rhesus, and hamster plasma but turned over rapidly in rat plasma, as the corresponding phenol derivative **49b**. Compound **50** was dosed in hamster, rabbit, and rat subcutaneously at 1 mg/kg (Figure 8). Very efficient NTP formation was observed in the liver of all species. In hamster and rabbit, NTP levels were higher than in rat; at 24 h they were 6.0, 4.1, and 2.9 μM for hamsters, rabbit, and rat, respectively.

Compound **50** was also administered at higher doses, 5 and 10 mg/kg in hamster and rat, respectively (Figure 9). After 1 and 5 mg/kg subcutaneous dosing, the AUC_{0-48h} in

Table 6. Antiviral Activity, Cytotoxicity, and Conversion to NTP in Human Hepatocytes of Prodrugs **42b**, **16**, **49b**, and **50**



compd	R	R_1	EC_{50} (μM)[a]	CC_{50} (μM)[b]	SI CC_{50}/EC_{50}	[NTP] $AUC_{0-2\ h}$ (μM·h)[c]
42b	Ph	Et	0.45	> 20	> 44	65
16	H	Et	8.8	> 20	> 2	10
49b	Ph	2-PrPen	0.037	2	54	107
50	H	2-PrPen	8.2	> 100	> 12	511

[a] EC_{50} values are quoted for $n = 2-9$ independent determinations. [b] Concentration of inhibitor reducing the cell viability by 50%. [c] Intracellular NTP after incubation with human hepatocytes.

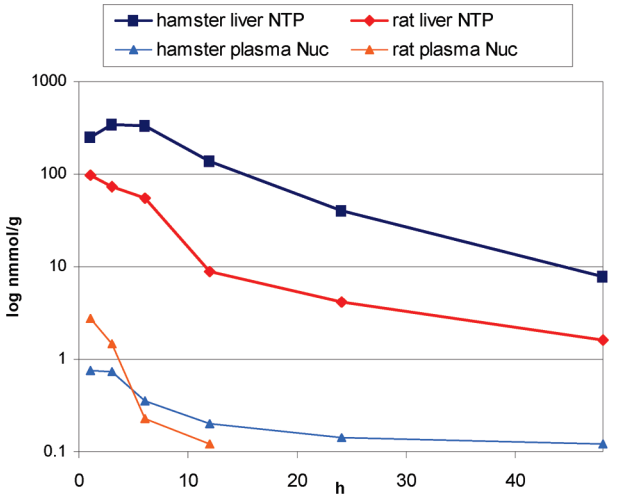


Figure 9. Profile of liver concentration of NTP **4b** and plasma concentration of nucleoside **4a** in hamster and rat after subcutaneous administration of **50** at 5 and 10 mg/kg, respectively.

Table 7. Comparison of Triphosphate Levels (AUC) between Compounds **50** and **51** in Hepatocytes from Several Species

	NTP AUC_{0-4h} (μM·h)	
species	**50**	**51**
dog	150	50
rabbit	330	70
rat	860	170
human	1720	190
rhesus	2000[a]	470[a]
hamster	2040	100

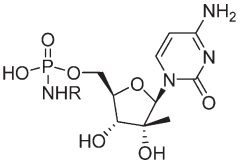
[a] AUC_{0-3h}.

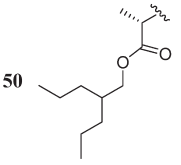
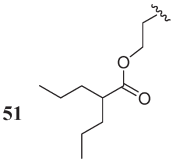
hamster were near dose proportional, 700 and 4600 μM·h, respectively.

Analysis of hamster and rat plasma showed the nucleoside **4a** as the major species, while only traces of **18**, prodrug **50**, and nucleoside monophosphate were detected (Figure 9).

The formation of NTP from compound **50** was studied in hepatocytes of several species and while lower levels of NTP were observed in dog and rabbit hepatocytes with AUC_{0-4h} of 150 and 330 μM·h, respectively, a similar behavior was observed between human and hamster hepatocytes with AUC_{0-4h} of 1720 and 2040 μM·h, respectively (see Table 7, compound **50**).

Table 8. Rat and Hamster Liver NTP Level (μM) of Compounds **50** and **51** after Different Administration Routes



Compd	R	Administration route	Liver NTP level (μM) rat	hamster
50		(s.c.)[a]	5	39
		(p.o.)[b]	-	0.5
51		(s.c.)[a]	36	13
		(p.o.)[b]		0.5

[a] Dose, 1.5 μmol/kg. [b] Dose, 30.2 μmol/kg.

The metabolic stability of compound **50** was studied in rat and human liver S9 fractions in the presence of NADPH, and the prodrug showed a very high metabolic stability in human fractions (rat Cl_{int} = 12 (μL/min)/mg; human Cl_{int} < 1 (μL/min)/mg). On the basis of these data, the good levels of NTP found in human hepatocytes, the high stability in human plasma, and the good correlation between in vitro and in vivo data in preclinical species, we predict a favorable PK of this prodrug in humans.

Based on the work carried on in parallel in our laboratories,[48] we wanted to investigate if the same approach could be used in the acyloxyethylaminophosphoramidate series, where the amino acid was replaced by an amino alcohol, and compound **51** was synthesized following the same synthetic route applied to obtain compound **50**. The potential for NTP formation was assessed in hepatocytes of several species, and although NTP levels were generally lower with respect to compound **50**, significant NTP concentrations were achieved in human and rat hepatocytes, with AUC_{0-4h} of 190 and 170 μM·h, respectively

(Table 7, compound **51**). Compound **51** showed a higher stability in rat plasma, and the compound was tested both in rat and in hamster for comparison (Table 8).

The values obtained both in human hepatocytes (NTP AUC_{0-2h} = 70 $\mu M \cdot h$) and after subcutaneous administration show that also this type of prodrug should be taken into consideration when a kinase bypass needs to be addressed. After subcutaneous administration at 1.5 μmol/kg, high liver exposure of **4b** was obtained in both rat and hamster (36 and 13 μM after 6 h). Compound **51** was also dosed orally in hamster at 30.2 μmol/kg, and at 6 h only 0.5 nmol/g 2′-Me-Cy-TP was observed in the liver, indicating a low oral bioavailability as for the phosphoramidate monoester.

Conclusions

A series of ProTide phosphoramidate prodrugs of 2′-Me-cytidine have been prepared and tested as HCV replication inhibitors. Not only were they tested in the replicon and cytotoxicity assays but their activation to nucleotide triphosphate was measured both in HuH7 cells and in hepatocytes of several species. The phenol release was an issue that was solved by the preparation of more lipophilic phosphoramidate monoesters. The most interesting compound in this series was compound **50**, which gave rise to high NTP concentrations in human hepatocytes. It demonstrated a selectivity index comparable to that of the parent drug. Together with its active anabolite **4b**, it was also tested in the Pan Laboratories screening where both species showed no significant activity up to 10 μM. This compound was also dosed subcutaneously in hamsters, reaching a high NTP exposure in liver, namely, an AUC_{0-48h} of 4600 $\mu M \cdot h$, after 5 mg/kg dosing.

This study demonstrates that it is possible to bypass the rate limiting first phosphorylation of 2′-*C*-methylcytidine by delivering directly into the cell the corresponding monophosphate that is then efficiently converted to the active triphosphate species **4b**, responsible for the antiviral activity.

Experimental Section

Solvents and reagents were obtained from commercial suppliers and were used without further purification. Flash chromatography purifications were conducted using prepacked cartridges on a Biotage system, eluting with petroleum ether/ethyl acetate mixtures. Reactions were carried out under an atmosphere of nitrogen in oven-dried (110 °C) glassware. Organic extracts were dried over sodium sulfate (Na_2SO_4) and were concentrated (after filtration of the drying agent) on rotary evaporators operating under reduced pressure. ^{1}H and ^{31}P NMR spectra were recorded on Bruker AM series spectrometers operating at (reported) frequencies between 300 and 600 MHz. Chemical shifts (δ) for signals corresponding to nonexchangeable protons (and exchangeable protons where visible) are recorded in parts per million (ppm) relative to tetramethylsilane and are measured using the residual solvent peak as reference. As a criterion of purity, two different chromatographic systems with NMR and MS data were employed. Purity of final compounds was more than 95% by area. Preparative scale HPLC separations were carried out on a Waters 2525 pump equipped with a 2487 dual absorbance detector. Compounds were eluted with linear gradients of water and MeCN, with water containing 5 mM ammonium bicarbonate. As stationary phase, the following columns were used: Phenomenex Luna C_{18} (2) 5 μm, 250 mm × 21.20 mm; Waters XBridge Pre C_{18} OBD, 5 μm, 30 mm × 150 mm; Waters XTerra MS C_{18} OBD, 5 μm, 19 mm × 150 mm, and 50 mm × 100 mm. Compound **50** was analyzed by HPLC on the column: Waters XTerra MS C_{18}, 5 μm, 3 mm × 150 mm.

2′-*C*-Methyl-2′,3′-*O*-(1-methylethylidene)cytidine (9). 2′-*C*-Methylcytidine was diluted with acetone (0.04 M), and catalytic *p*-toluensulfonic acid and 2,2-dimethoxypropane were added. The resulting slurry was stirred for 24 h at RT. The solvent was removed under vacuo, the residue was dissolved in MeOH, and AmberliteA-26 (previously washed with 2 N NaOH and H_2O) was added. The resulting mixture was stirred for 2 h. The Amberlite was filtered off, and the solution was evaporated. The crude product was purified by column chromatography, using as eluent DCM/MeOH (9/1), to obtain the pure product **9** as a white solid. ^{1}H NMR (300 MHz, CD_3OD, δ) 7.96 (d, J = 7.56 Hz, 1H), 6.18 (s, 1H), 5.90 (d, J = 7.56 Hz, 1H), 4.51−4.48 (m, 1H), 4.28−4.23 (m, 1H), 3.86 (dd, J = 3.04, 12.12 Hz, 1H), 3.78 (dd, J = 3.52, 12.12 Hz, 1H), 1.59 (s, 3H), 1.43 (s, 3H), 1.25 (s, 3H). MS (ES^+) m/z 298 $(M + H)^+$.

General Procedure. Preparation of 2′-*C*-Methylcytidine Phosphoramidates (17, 22−49). 2′-*C*-Methylcytidine was diluted with dry THF (0.1 M). The resulting slurry was cooled to −78 °C, and then *tert*-butylmagnesium chloride (as 1.0 M solution in THF, 2.2 equiv) was added. The mixture was immediately warmed to 0 °C, stirred for 30 min, and again cooled to −78 °C. Then the appropriate aryloxyphosphorochloridate (as 1.0 M solution in THF, 2.2 equiv) was added dropwise. The mixture was allowed to reach room temperature overnight, and then the reaction was quenched by the addition of water. The aqueous phase was extracted three times with EtOAc, and the combined organic phases were washed with brine and dried over Na_2SO_4. The crude was purified by column chromatography using as eluent DCM/MeOH (from 9/1 to 8/2), and the resulting off-white solid was dissolved in DMSO and purified by RP-HPLC. Fractions containing the pure diastereoisomers were combined and freeze-dried to afford the title compounds as a TFA salt.

5′-*O*-[(4-Chlorophenoxy)[[(1*S*)-2-ethoxy-1-methyl-2-oxoethyl]-mino]phosphinyl]-2′-*C*-methylcytidine. First eluting diastereoisomer (**17a**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.03 (d, J = 7.8 Hz, 1H), 7.41 (d, J = 8.9 Hz, 2H), 7.28 (d, J = 8.9 Hz, 2H), 6.09 (d, J = 7.8 Hz, 1H), 6.03 (s, 1H), 4.66−4.59 (m, 1H), 4.52−4.45 (m, 1H), 4.24−4.16 (m, 3H), 4.04−3.95 (m, 1H), 3.63 (d, J = 9.2 Hz, 1H), 1.39 (d, J = 7.1 Hz, 3H), 1.29 (t, J = 7.1 Hz, 3H), 1.21 (s, 3H). ^{31}P NMR (300 MHz, CD_3OD, δ) 5.21. MS (ES^+) m/z 547 $(M + H)^+$. Second eluting diastereoisomer (**17b**): ^{1}H NMR (300 MHz, CD_3OD, δ) 8.01 (d, J = 7.74 Hz, 1H), 7.43 (d, J = 8.85 Hz, 2H), 7.31 (d, J = 8.85 Hz, 2H), 6.06 (d, J = 7.74 Hz, 1H), 6.02 (s, 1H), 4.58 (ddd, J = 1.82, 6.25, 11.89 Hz, 1H), 4.47−4.38 (m, 1H), 4.24−4.12 (m, 3H), 4.06−3.92 (m, 1H), 3.81 (d, J = 9.28 Hz, 1H), 1.41 (d, J = 6.85 Hz, 3H), 1.27 (t, J = 7.18 Hz, 3H), 1.21 (s, 3H). ^{31}P NMR (300 MHz, CD_3OD, δ) 5.10. MS (ES^+) m/z 547 $(M + H)^+$.

5′-*O*-[(4-Chlorophenoxy)[(2-methoxy-2-oxoethyl)amino]phosphinyl]-2′-*C*-methylcytidine. Mixture 1.2:1 of diastereoisomers at phosphorus [(first eluting, ∗)/(second eluting)] (**22**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.09 (d, J = 7.9 Hz, 1H) and 8.04* (d, J = 7.9 Hz, 1H), 7.44−7.40 (m, 2H, both), 7.31−7.29 (m. 2H, both), 6.09* (d, J = 7.9 Hz, 1H) and 6.08 (d, J = 7.9 Hz, 1H), 6.03 (s, 1H, both), 4.67−4.60 (m, 1H, both), 4.52−4.45 (m, 1H, both), 4.20−4.18 (m, 2H, both), 3.87−3.79 (m, 3H, both), 3.76* (s, 3H) and 3.75 (s, 3H), 1.23 (s, 3H) and 1.21* (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 5.49 and 5.09*. MS (ES^+) m/z 541 $(M + Na)^+$.

5′-*O*-[(4-Chlorophenoxy)[[(1*S*)-1-(ethoxycarbonyl)pentyl]-amino]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:0.8* of diastereoisomers at phosphorus (**23**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.09−8.03 (m, 1H), 7.45−7.39 (m, 2H), 7.33−7.26 (m, 2H), 6.13 and 6.10* (d, J = 7.9 Hz, 1H), 6.02 and 6.01* (s, 1H), 4.65−4.55 (m, 1H), 4.52−4.39 (m, 1H), 4.24−4.11 (m, 3H), 3.91−3.80 (m, 2H), 1.83−1.71 (m, 1H), 1.71−1.57 (m, 1H), 1.40−1.19 (m, 10H), 0.95−0.85 (m, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 4.51, 4.24*. MS (ES^+) m/z 589 $(M + H)^+$.

5′-*O*-[(4-Chlorophenoxy)[(3-methoxy-3-oxopropyl)amino]-phosphinyl]-2′-*C*-methylcytidine. Mixture 1.5:1 of diastereoisomers at phosphorus (**24**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.05 (d, J = 7.9 Hz, 1H) and 8.01* (d, J = 7.9 Hz, 1H), 7.44−7.40 (m, 2H, both), 7.29−7.27 (m, 2H, both), 6.06 (d, J = 7.9 Hz, 1H) and 6.05* (d, J = 7.9 Hz, 1H), 6.01 (s, 1H, both), 4.59−4.53 (m, 1H, both), 4.43−4.38 (m, 1H, both), 4.21−4.18 (m, 1H, both), 3.84 (d, J = 9.2 Hz, 1H) and 3.82* (d, J = 9.2 Hz, 1H), 3.69* (s, 3H) and 3.68 (s, 3H), 3.33−3.28 (m, 2H, both), 2.57−2.54 (m, 2H, both), 1.22 (s, 3H) and 1.21* (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 5.77 and 5.55*. MS (ES^+) m/z 533 $(M + H)^+$.

5′-*O*-[(4-Chlorophenoxy)[[(1*R*)-2-methoxy-1-methyl-2-oxoethyl]amino]phosphinyl]-2′-*C*-methylcytidine. Mixture 1.9:1* of diastereoisomers at phosphorus (**25**). ^{1}H NMR (400 MHz, CD_3OD, δ) 8.12 (d, J = 7.9 Hz, 1H) and 8.00* (d, J = 7.9 Hz, 1H), 7.45−7.41 (m, 2H, both), 7.32−7.27 (m, 2H, both), 6.11 (d, J = 7.9 Hz, 1H) and 6.10* (d, J = 7.9 Hz, 1H), 6.04 (s, 1H) and 6.01* (s, 1H), 4.63−4.56 (m, 1H, both), 4.49−4.37 (m, 1H, both), 4.21−4.16 (m, 1H, both), 4.06−3.98 (m, 1H, both), 3.86 (d, J = 9.2 Hz, 1H) and 3.77* (d, J = 9.2 Hz, 1H), 3.73 (s, 3H) and 3.72* (s, 3H), 1.41* (d, J = 7.2 Hz, 3H) and 1.37 (d, J = 7.2 Hz, 3H), 1.23 (s, 3H) and 1.21* (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 4.39 and 3.60*. MS (ES^+) m/z 533 $(M + H)^+$.

5′-*O*-[(4-Chlorophenoxy)[[(1*S*)-2-methoxy-2-oxo-1-(phenylmethyl)ethyl]amino]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:1.5* of diastereoisomers at phosphorus (**26**). ^{1}H NMR (300 MHz, CD_3OD, 300 K) δ 7.99* and 7.96 (d, J = 7.9 Hz, 1H), 7.40−7.00 (m, 9H), 6.10* and 6.02 (d, J = 7.9 Hz, 1H), 6.01* and 5.97 (s, 1H), 4.38−4.02 (m, 4H), 3.81−3.74 (m, 1H), 3.73* and 3.66 (s, 3H), 3.21−3.10 (m, 1H), 2.97−2.83 (m, 1H), 1.20 (s, 3H). ^{31}P NMR (300 MHz, CD_3OD, 300 K) δ 4.99, 4.79. MS (ES^+) m/z 609 $(M + H)^+$.

5′-*O*-[(4-Chlorophenoxy)[[(1*S*)-1-(ethoxycarbonyl)-3-methylbutyl]amino]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:0.9* of diastereoisomers at phosphorus (**27**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.06 and 8.03* (d, J = 7.9 Hz, 1H), 7.45−7.39 (m, 2H), 7.33−7.26 (m, 2H), 6.13 and 6.08* (d, J = 7.9 Hz, 1H), 6.02 and 6.01* (s, 1H), 4.66−4.54 (m, 1H), 4.53−4.38 (m, 1H), 4.25−4.10 (m, 3H), 3.95−3.86 (m, 1H), 3.86−3.79 (m, 1H), 1.81−1.51 (m, 3H), 1.33−1.19 (m, 6H), 0.97−0.81 (m, 6H). ^{31}P NMR (400 MHz, CD_3OD, δ) 4.45, 4.20*. MS (ES^+) m/z 589 $(M + H)^+$.

5′-*O*-[(4-Chlorophenoxy)[[(1*S*)-2-ethoxy-2-oxo-1-phenylethyl]amino]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:0.8* of diastereoisomers at phosphorus (**28**): ^{1}H NMR (400 MHz, CD_3OD, δ) 7.90 and 7.82* (d, J = 7.9 Hz, 1H), 7.42−7.29 (m, 7H), 7.22 (d, J = 7.8 Hz, 1H), 7.15 (d, J = 7.8 Hz, 1H), 6.02* and 5.98 (s, 1H), 5.97 and 5.91* (d, J = 7.9 Hz, 1H), 5.20−4.96 (m, 1H), 4.58−4.30 (m, 2H), 4.22−4.10 (m, 3H), 3.78−3.69 (m, 1H), 1.21−1.18 (m, 3H), 1.16 (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 3.78, 3.71*. MS (ES^+) m/z 609 $(M + H)^+$.

5′-*O*-[(4-Chlorophenoxy)[[(1*S*)-2-ethoxy-1-(1*H*-indol-3-ylmethyl)-2-oxoethyl]amino]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:0.9* of diastereoisomers at phosphorus (**29**): ^{1}H NMR (400 MHz, CD_3OD, δ) 7.91 and 7.82* (d, J = 7.9 Hz, 1H), 7.55−7.49 (m, 1H), 7.42−7.29 (m, 2H), 7.26−7.19 (m, 1H), 7.18−7.06 (m, 3H), 7.05−6.96 (m, 2H), 6.01 and 5.87* (d, J = 7.9 Hz, 1H), 5.95 and 5.91* (s, 1H), 4.41−4.00 (m, 6H), 3.75−3.68 (m, 1H), 3.33−3.24 (m, 1H), 3.16−3.03 (m, 1H), 1.26−1.18 (m, 3H), 1.16 (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 3.75, 3.71*. MS (ES^+) m/z 663 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Methoxy-1-methyl-2-oxoethyl]amino][4-(trifluoromethyl)phenoxy]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:1 of diastereoisomers at phosphorus (**30**): ^{1}H NMR (300 MHz, CD_3OD, δ) 8.05 (d, J = 7.7 Hz, 1H, both), 7.76−7.74 (m, 2H, both), 7.52−7.46 (m, 2H, both), 6.12−6.07 (m, 1H, both), 6.01 (s, 1H, both), 4.65−4.45 (m, 2H, both), 4.25−4.17 (m, 1H, both), 4.07−4.01 (m, 1H, both), 3.85−3.81 (m, 1H, both), 3.74 (s, 3H) and 3.70 (s, 3H), 1.40 (d, J = 6.9 Hz, 3H, both), 1.22 (s, 3H, both). ^{31}P NMR (300 MHz, CD_3OD, δ) 4.85 and 4.78. ^{19}F NMR (300 MHz, CD_3OD, δ) 65.9, 79.5. MS (ES^+) m/z 589 $(M + Na)^+$.

5′-*O*-[[[(1*S*)-2-Methoxy-1-methyl-2-oxoethyl]amino](4-methoxyphenoxy)phosphinyl]-2′-*C*-methylcytidine. Mixture 1:1 of diastereoisomers at phosphorus (**31**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.08 (d, J = 7.9 Hz, 0.5H), 8.07 (d, J = 7.9 Hz, 0.5H), 7.25−7.17 (m, 2H), 6.98−6.91 (m, 2H), 6.1 (d, J = 7.9 Hz, 0.5H), 6.07 (d, J = 7.9 Hz, 0.5H), 6.02 (s, 0.5H), 6.01 (s, 0.5H), 4.65−4.53 (m, 1H), 4.50−4.36 (m, 1H), 4.23−4.15 (m, 1H), 4.05−3.95 (m, 1H), 3.87−3.80 (m, 1H), 3.82 (s, 1.5H), 3.81 (s, 1.5H), 3.75 (s, 1.5H), 3.72 (s, 1.5H), 1.42−1.33 (m, 3H), 1.22 (s, 1.5H), 1.21 (s, 1.5H). ^{31}P NMR (400 MHz, CD_3OD, δ) 4.47, 4.29. MS (ES^+) m/z 530 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Butoxy-1-methyl-2-oxoethyl]amino](2-chlorophenoxy)phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**32b**): ^{1}H NMR (300 MHz, CD_3OD, δ) 8.0 (d, J = 7.9 Hz, 1H), 7.6−7.49 (m, 2H), 7.4−7.31 (m, 1H), 7.29−7.19 (m, 1H), 6.06 (d, J = 7.8 Hz, 1H), 6.01 (s, 1H), 4.67−4.55 (m, 1H), 4.53−4.42 (m, 1H), 4.23−3.98 (m, 4H), 3.82 (d, J = 9.2 Hz, 1H), 1.7−1.57 (m, 2H), 1.48−1.33 (m, 5H), 1.20 (s, 3H), 0.96 (t, J = 7.3 Hz, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 4.0. MS (ES^+) m/z 576−578 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Ethoxy-1-methyl-2-oxoethyl]amino](2-methylphenoxy)phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**33b**): ^{1}H NMR (400 MHz, CD_3OD, δ) 7.96 (d, J = 7.6 Hz, 1H), 7.37−7.12 (m, 4H), 6.02−5.97 (m, 2H), 4.59−4.51 (m, 1H), 4.44−4.37 (m, 1H), 4.19−4.11 (m, 3H), 4.02−3.92 (m, 1H), 3.79 (d, J = 8.8 Hz, 1H), 2.35 (s, 3H), 1.39 (d, J = 6.8 Hz, 3H), 1.27−1.24 (m, 3H), 1.19 (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 3.97. MS (ES^+) m/z 528 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Ethoxy-1-methyl-2-oxoethyl]amino](1-naphthalenyloxy)phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**34b**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.27−8.22 (m, 1H), 7.98−7.94 (m, 1H), 7.92 (d, J = 7.83 Hz, 1H), 7.79 (d, J = 7.83 Hz, 1H), 7.65−7.55 (m, 3H), 7.50 (t, J = 7.95 Hz, 1H), 6.01 (s, 1H), 5.85 (d, J = 7.83 Hz, 1H), 4.65 (ddd, J = 1.83, 6.12, 11.81 Hz, 1H), 4.49 (ddd, J = 11.87 Hz, J = 5.81 Hz, J = 3.53 Hz, 1H), 4.23−4.17 (m, 1H), 4.11 (q, J = 7.07 Hz, 2H), 4.07−3.99 (m, 1H), 3.84 (d, J = 9.09 Hz, 1H), 1.36 (dd, J = 6.69 Hz, J = 0.37 Hz, 3H), 1.22 (t, J = 7.07 Hz, 3H), 1.17 (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 4.35. MS (ES^+) m/z 563 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Butoxy-1-methyl-2-oxoethyl]amino](1-naphthalenyloxy)phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**35b**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.28−8.22 (m, 1H), 7.99−7.92 (m, 2H), 7.79 (d, J = 8.1 Hz, 1H), 7.65−7.69 (m, 2H), 7.59−7.55 (m, 1H), 7.50 (t, J = 8.1 Hz, 1H), 6.00 (s, 1H), 5.86 (d, J = 7.83 Hz, 1H), 4.65 (ddd, J = 1.6, 6.3, 11.7 Hz, 1H), 4.53−4.46 (m, 1H), 4.24−4.17 (m, 1H), 4.10−4.00 (m, 3H), 3.83 (d, J = 9.3 Hz, 1H), 1.62−1.53 (m, 2H), 1.41−1.32 (m, 5H), 1.17 (s, 3H), 0.93 (t, J = 7.4 Hz, 3H). ^{31}P NMR: (300 MHz CD_3OD, δ) 4.33. MS (ES^+) m/z 591 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Butoxy-1-methyl-2-oxoethyl]amino][(4-chloro-1-naphthalenyl)oxy]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:2.5 of diastereoisomers at phosphorus (**36**): ^{1}H NMR (300 MHz, CD_3OD, δ) 8.33−8.24 (m, 2H), 8.05−7.96 (m, 1H), 7.79−7.48 (m, 4H), 6.00−5.91 (m, 2H), 4.71−4.45 (m, 2H), 4.25−4.16 (m, 1H), 4.11−3.96 (m, 3H), 3.82 (d, J = 9.1 Hz, 1H), 1.62−1.46 (m, 2H), 1.44−1.26 (m, 5H), 1.18 (s, 3H), 0.97−0.85 (m, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 4.39, 4.26. MS (ES^+) m/z 626−628 $(M + H)^+$.

5′-*O*-[[4-Chloro-5-methyl-2-(1-methylethyl)phenoxy][[(1*S*)-2-ethoxy-1-methyl-2-oxoethyl]amino]phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**37b**): ^{1}H NMR (300 MHz, CD_3OD, δ) 7.96 (d, J = 7.83 Hz, 1H), 7.37 (s, 1H), 7.33 (s, 1H), 6.02 (s, 1H), 6.00 (d, J = 7.83 Hz, 1H), 4.57 (ddd, J = 1.77, 6.45, 11.88 Hz, 1H), 4.41 (ddd, J = 4.04, 6.45, 11.81 Hz, 1H), 4.20−4.14 (m, 3H), 4.00 (dt, J = 7.33, 17.18 Hz, 1H), 3.80 (d, J = 9.34 Hz, 1H), 2.34 (s, 3H), 1.43 (d, J = 7.08 Hz, 3H), 1.29−1.25 (m, 9H), 1.20 (s, 3H), NH_2, NH, 2 × OH not visible. ^{31}P NMR (300 MHz, CD_3OD, δ) 5.03. MS (ES^+) m/z 625 and 627 $(M + Na)^+$.

5′-*O*-[[[(1*S*)-2-Ethoxy-1-methyl-2-oxoethyl]amino][2-(methoxycarbonyl)phenoxy]phosphinyl]-2′-*C*-methylcytidine. Mixture 1:1 of diastereoisomers at phosphorus (**38**): ^{1}H NMR (300 MHz, CD_3OD, δ) 8.17 (d, J = 7.9 Hz, 0.5H), 8.11 (d, J = 7.8 Hz, 0.5H), 7.99−7.89 (m, 1H), 7.69−7.3 (m, 3H), 6.16 (d, J = 7.8 Hz, 0.5H), 6.09 (d, J = 7.8 Hz, 0.5H), 6.03 (s, 0.5H), 6.01 (s, 0.5H), 4.73−4.44 (m, 2H), 4.27−3.84 (m, 5H), 3.94 (s, 3H), 1.35 (t, J = 7.3 Hz, 3H), 1.3−1.18 (m, 6H). ^{31}P NMR (300 MHz, CD_3OD, δ) 4.78, 4.57. MS (ES^+) m/z 572 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Ethoxy-1-methyl-2-oxoethyl]amino](2,2,2-trichloroethoxy)phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**39b**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.0 (d, J = 8.1 Hz, 1H), 6.15 (d, J = 7.8 Hz, 1H), 6.01 (s, 1H), 4.74−4.70 (m, 2H), 4.58−4.49 (m, 1H), 4.47−4.36 (m, 1H), 4.28−4.13 (m, 3H), 4.05−3.95 (m, 1H), 3.81 (d, J = 9.3 Hz, 1H), 1.47 (d, J = 6.8 Hz, 3H), 1.31 (t, J = 7.1 Hz, 3H), 1.21 (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 7.59. MS (ES^+) m/z 567 $(M + H)^+$.

5′-*O*-[[4-[(2*S*)-2-Amino-3-methoxy-3-oxopropyl]phenoxy][[(1*S*)-2-ethoxy-1-methyl-2-oxoethyl]amino]phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**40b**): ^{1}H NMR (300 MHz, CD_3OD, δ) 8.0 (d, J = 7.8 Hz, 1H), 7.32 (m, 4H), 6.1 (d, J = 9.8 Hz, 1H), 6.02 (s, 1H), 4.59−4.31 (m, 3H), 4.25−4.13 (m, 3H), 4.06−3.94 (m, 1H), 3.85 (s, 3H), 3.81 (d, J = 9.4 Hz, 1H), 3.31−3.14 (m, 2H), 1.42 (d, J = 7.2 Hz, 3H), 1.31 (t, J = 7.2 Hz, 3H), 1.21 (s, 3H). ^{31}P NMR (300 MHz, CD_3OD, δ) 5.23. MS (ES^+) m/z 615 $(M + H)^+$.

5′-*O*-[[[1-[(1,1-Dimethylethoxy)carbonyl]-1*H*-indol-5-yl]oxy][[(1*S*)-2-ethoxy-1-methyl-2-oxoethyl]amino]phosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**41b**): ^{1}H NMR (300 MHz, DMSO-d_6, δ) 8.02 (d, J = 8.91 Hz, 1H), 7.72 (d, J = 3.63 Hz, 1H), 7.57 (d, J = 7.54 Hz, 1H), 7.49 (s, 1H), 7.20 (d, J = 7.60 Hz, 1H), 7.18 (br s, 1H), 7.10 (br s, 1H), 6.71 (d, J = 3.63 Hz, 1H), 6.00 (dd, J = 10.42, 12.34 Hz, 1H), 5.94 (s, 1H), 5.70 (d, J = 7.44 Hz, 1H), 5.23 (d, J = 7.05 Hz, 1H), 5.09 (s, 1H), 4.43−4.32 (m, 1H), 4.30−4.18 (m, 1H), 4.13−3.93 (m, 3H), 3.92−3.80 (m, 1H), 3.62 (t, J = 7.02 Hz, 1H), 1.65 (s, 9H), 1.25 (d, J = 7.02 Hz, 3H), 1.13 (t, J = 7.11 Hz, 3H), 0.96 (s, 3H). ^{31}P NMR (300 MHz, DMSO-d_6, δ) 4.16. MS (ES^+) m/z 653 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Ethoxy-1-methyl-2-oxoethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**42b**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.05 (d, J = 7.9 Hz, 1H), 7.43 (t, J = 7.6 Hz, 2H), 7.32 (d, J = 7.6 Hz, 2H), 7.26 (t, J = 7.6 Hz, 1H), 6.05 (d, J = 7.9 Hz, 1H), 6.01 (s, 1H), 4.62−4.54 (m, 1H), 4.46−4.39 (m, 1H), 4.22−4.13 (m, 3H), 4.04−3.94 (m, 1H), 3.83 (d, J = 9.2 Hz, 1H), 1.40 (d, J = 7.1 Hz, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.21 (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 3.85. MS (ES^+) m/z 513 $(M + H)^+$.

5′-*O*-[[[(1*S*)-1-Methyl-2-oxo-2-propoxyethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**43b**): ^{1}H NMR (400 MHz, CD_3OD, δ) 8.01 (d, J = 7.8 Hz, 1H), 7.45−7.41 (m, 2H), 7.32−7.24 (m, 3H), 6.04−6.02 (m, 2H), 4.60−4.55 (ddd, J = 1.8, 5.9, 11.9 Hz, 1H), 4.45−4.39 (ddd, J = 3.4, 5.9, 11.9 Hz, 1H), 4.19−4.17 (m, 1H), 4.10−4.06 (m, 2H), 4.03−3.98 (m, 1H), 3.83−3.81 (d, J = 9.2 Hz, 1H), 1.72−1.63 (m, 2H), 1.40 (d, J = 7.1 Hz, 3H), 1.21 (s, 3H), 0.97 (t, J = 7.4 Hz, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 3.84. (ES^+) m/z 527 $(M + H)^+$.

5′-*O*-[[[(1*S*)-1-Methyl-2-(1-methylethoxy)-2-oxoethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**44b**): ^{1}H NMR (300 MHz, CD_3OD, δ) 8.06 (d, J = 7.8 Hz, 1H), 7.5−7.19 (m, 5H), 6.06 (d, J = 7.8 Hz, 1H), 6.01 (s, 1H), 5.08−4.93 (m, 1H), 4.64−4.37 (m, 2H), 4.23−4.14 (m, 1H), 4.02−3.88 (m, 1H), 3.83 (d, J = 9.2 Hz, 1H), 1.39 (d, J = 7.0 Hz, 3H), 1.26 (d, J = 6.2 Hz, 6H), 1.22 (s, 3H). ^{31}P NMR (300 MHz, CD_3OD, δ) 5.01. MS (ES^+) m/z 528 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-(3-Methoxypropoxy)-1-methyl-2-oxoethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**45b**): ^{1}H NMR (400 MHz, DMSO-d_6, δ) 7.53 (d, J = 7.2 Hz, 1H), 7.46−7.33 (m, 2H), 7.31−7.14 (m, 4H), 7.09 (br s, 1H), 6.05 (t, J = 11.4 Hz, 1H), 5.94 (br s, 1H), 5.68 (d, J = 7.2 Hz, 1H), 5.23 (d, J = 6.6 Hz, 1H), 5.09 (s, 1H), 4.43−4.30 (m, 1H), 4.29−4.16 (m, 1H), 4.16−3.78 (m, 4H), 3.69−3.52 (m, 1H), 3.43−3.36 (m, 2H), 3.22 (s, 3H), 1.84−1.73 (m, 2H), 1.26 (d, J = 6.5 Hz, 3H), 0.96 (s, 3H). ^{31}P NMR (400 MHz, DMSO-d_6, δ) 3.75. MS (ES^+) m/z 557 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-(2-Ethylbutoxy)-1-methyl-2-oxoethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**46b**): ^{1}H NMR (400 MHz, CD_3OD, δ) 7.70 (d, J = 7.5 Hz, 1H), 7.41−7.37 (m, 2H), 7.30−7.28 (m, 2H), 7.22 (t, J = 7.3 Hz, 1H), 6.06 (s, 1H), 5.85 (d, J = 7.5 Hz, 1H), 4.52 (dd, J = 5.7, 10.3 Hz, 1H), 4.39 (dd, J = 5.3, 9.5 Hz, 1H), 4.12−3.95 (m, 4H), 3.74 (d, J = 9.2 Hz, 1H), 1.54−1.48 (m, 1H), 1.39−1.33 (m, 7H), 1.12 (s, 3H), 0.90 (t, J = 7.5 Hz, 6H). ^{31}P NMR (400 MHz, CD_3OD, δ) 3.79. MS (ES^+) m/z 569 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-(Heptyloxy)-1-methyl-2-oxoethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**47b**): ^{1}H NMR (300 MHz, CD_3OD, δ) 7.72 (d, J = 7.7 Hz, 1H), 7.44−7.39 (m, 2H), 7.32−7.22 (m, 3H), 6.08 (s, 1H), 5.86 (d, J = 7.7 Hz, 1H), 4.58−4.52 (m, 1H), 4.42−4.39 (m, 1H), 4.15−4.07 (m, 3H), 4.03−3.98 (m, 1H), 3.76 (d, J = 9.1 Hz, 1H), 1.67−1.62 (m, 1H), 1.41−1.34 (m, 13H), 1.14 (s, 3H), 0.96−0.91 (m, 3H). ^{31}P NMR (300 MHz, CD_3OD, δ) 3.67. MS (ES^+) m/z 583 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-(Cycloheptyloxy)-1-methyl-2-oxoethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**48b**): ^{1}H NMR (400 MHz, DMSO-d_6, δ) 7.52 (d, J = 7.4 Hz, 1H), 7.39 (t, J = 7.7 Hz, 2H), 7.30−7.14 (m, 4H), 7.09 (br s, 1H), 6.08−5.96 (m, 1H), 5.94 (s, 1H), 5.69 (d, J = 7.4 Hz, 1H), 5.23 (d, J = 6.9 Hz, 1H), 5.09 (s, 1H), 4.87−4.74 (m, 1H), 4.43−4.29 (m, 1H), 4.29−4.14 (m, 1H), 4.03−3.91 (m, 1H), 3.90−3.71 (m, 1H), 3.66−3.52 (m, 1H), 1.88−1.71 (m, 2H), 1.67−1.44 (m, 8H), 1.44−1.32 (m, 2H), 1.24 (d, J = 7.0 Hz, 3H), 0.95 (s, 3H). ^{31}P NMR (400 MHz, DMSO-d_6, δ) 3.73. MS (ES^+) m/z 581 $(M + H)^+$.

5′-*O*-[[[(1*S*)-1-Methyl-2-oxo-2-[(2-propylpentyl)oxy]ethyl]amino]phenoxyphosphinyl]-2′-*C*-methylcytidine. Second eluting diastereoisomer (**49b**): ^{1}H NMR (400 MHz, DMSO-d_6, δ) 7.50 (d, J = 7.4 Hz, 1H), 7.40−7.34 (m, 2H), 7.24−7.15 (m, 4H), 7.11−7.03 (bs, 1H), 6.08−5.99 (m, 1H), 5.94−5.88 (bs, 1H), 5.66 (d, J = 7.4 Hz, 1H), 5.20 (d, J = 7.1 Hz, 1H), 5.10−5.06 (bs, 1H), 4.37−4.29 (m, 1H), 4.23−4.16 (m, 1H), 4.00−3.90 (m, 2H), 3.90−3.81 (m, 2H), 3.60−3.52 (m, 1H), 1.64−1.55 (m, 1H), 1.31−1.15 (m, 11H), 0.93 (s, 3H), 0.87−0.79 (m, 6H). ^{31}P NMR (400 MHz, DMSO-d_6, δ) 3.90. MS (ES^+) m/z 597 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Ethoxy-1-methyl-2-oxoethyl]amino](9*H*-fluoren-9-ylmethoxy)phosphinyl]-2′-*C*-methylcytidine (15). Bisphenyl phosphite was dissolved in pyridine (0.3 M), and a solution of fluorenylmethyl alcohol in pyridine (0.3 M) was added. The mixture was stirred at 0 °C for 20 min. Then a solution of 2′-*C*-methylcytidine in pyridine (0.3 M) was added at 0 °C. The resulting solution was warmed to 40 °C and stirred for 1 h at this temperature. The solvent was evaporated, and the crude **13-II** was dissolved in DMA (0.19 M). The resulting solution was added to a solution of L-alanine ethyl ester hydrochloride (1.2 equiv) and Et_3N (2.0 equiv) in *i*-PrOH/CCl_4 (0.24 M, 10/1). The mixture was stirred for 10 min at 0 °C, and then the solvent was evaporated. The residue was dissolved in EtOAc and water. The aqueous phase was extracted three times with EtOAc, and the combined organic phases were washed with brine and dried over Na_2SO_4. The crude product was purified by RP-HPLC. Fractions containing the pure compound were freeze-dried to afford the title compound **15** as white powder and as a 1:1 mixture of diastereoisomers. ^{1}H NMR (400 MHz, CD_3OD, δ) 8.1 (d, J = 7.9 Hz, 0.5H), 7.95 (d, J = 7.9 Hz, 0.5H), 7.85−7.81 (m, 2H), 7.74−7.64 (m, 2H), 7.45−7.38 (m, 2H), 7.36−7.29 (m, 2H), 6.02 (d, J = 7.8 Hz, 1H), 5.97 (d, J = 9.1 Hz, 1H), 4.56−4.2 (m, 4H), 4.2−4.04 (m, 3H), 3.89−3.73 (m, 2H), 1.37 (d, J = 7.2 Hz, 1.5H), 1.34 (d, J = 7.2 Hz, 1.5H), 1.25 (t, J = 7.2 Hz, 3H), 1.19 (s, 1.5H), 1.19 (s, 1.5H). ^{31}P NMR (400 MHz, CD_3OD, δ) 8.46, 8.14. MS (ES^+) m/z 615 $(M + H)^+$.

5′-*O*-[[[(1*S*)-2-Ethoxy-1-methyl-2-oxoethyl]amino]hydroxyphosphinyl]-2′-*C*-methylcytidine (16). Compound **15** was dissolved in DCM (0.012 M), and piperidine (56 equiv) was added. The resulting solution was evaporated and the residue washed with water. The precipitate was discarded, and the solution was concentrated to give a residue that was purified by RP-HPLC. Fractions containing the pure compound were freeze-dried to afford the title compound **16** as a white powder as an NH_4 salt. ^{1}H NMR (400 MHz, CD_3OD, δ) 8.23 (d, J = 7.6 Hz, 1H), 6.08−6.06 (m, 2H), 4.25−4.15 (m, 3H), 4.10−4.02 (m, 2H), 3.95−3.87 (m, 2H), 1.36 (d, J = 7.1 Hz, 3H), 1.28 (t, J = 7.1 Hz, 3H), 1.15 (s, 3H). ^{31}P NMR (400 MHz, CD_3OD, δ) 6.87. MS (ES^+) m/z 436 $(M + H)^+$.

5′-*O*-[[[(1*S*)-1-Methyl-2-oxo-2-[propylpentyl)oxy]ethyl]amino]-phenylmethoxy)phosphinyl]-2′-*C*-methyl-2′,3′-*O*-(1-methylethylidene)cytidine (20-I). Compound **9** was diluted with pyridine (0.67 M) in the presence of molecular sieves. The resulting solution was cooled to 0 °C, diphenylphosphite (80%, 1.3 equiv) was added, and the mixture was stirred for 1 h at 0 °C. To this solution benzyl alcohol (2.0 equiv) was added, and the mixture was stirred at RT for 1 h. The solvent was evaporated, and the residue **19** was dissolved in THF/CCl_4 (0.08 M, 12/1). The resulting solution was cooled to 0 °C, and Et_3N (7.0 equiv) and a solution of L-alanine, 2-propylpentyl ester hydrochloride **21-I** (1.3 equiv) in *i*-PrOH were added. The mixture was stirred for 30 min at 0 °C and then was quenched by the addition of water. The aqueous phase was extracted three times with EtOAc, and the combined organic phases were washed with brine and dried over Na_2SO_4. The crude product was purified by column chromatography using as eluent DCM/MeOH (95/5) to give **20-I** as a white solid as a 1:1 mixture of diastereoisomers. ^{1}H NMR (400 MHz, CD_3OD, δ) 7.76 (d, J = 7.6 Hz, 0.5H), 7.75 (d, J = 7.6 Hz, 0.5H), 7.46−7.33 (m, 5H), 6.15 (s, 0.5H), 6.13 (s, 0.5H), 5.88−5.82 (m, 1H), 5.11 (d, J = 7.6 Hz, 1H), 5.09−5.05 (m, 1H), 4.54−4.43 (m, 1H), 4.41−4.35 (m, 1H), 4.33−4.21 (m, 2H), 4.14−3.88 (m, 3H), 1.75−1.65 (m, 1H), 1.58 (s, 3H), 1.44−1.27 (m, 8H), 1.41 (s, 6H), 1.20 (d, J = 7.3 Hz, 3H), 0.92 (t, J = 5.9 Hz, 6H). ^{31}P NMR (400 MHz, CD_3OD, δ) 8.68, 8.48. MS (ES^+) m/z 652 $(M + H)^+$.

5′-*O*-[Hydroxy[[(1*S*)-1-methyl-2-oxo-2-[(2-propylpentyl)oxy]-ethyl]amino]phosphinyl]-2′-*C*-methylcytidine (50). Compound **20-I** was dissolved in a solution of TFA/H_2O (0.1 M, 8/2). The resulting solution was warmed to 30 °C and stirred for 20 min. The solvent was evaporated and the residue dissolved in water and EtOAc. The aqueous phase was extracted three times with EtOAc, and the combined organic phases were washed with brine and dried over Na_2SO_4. The crude product was purified by column chromatography using as eluent DCM/MeOH (95/5) to give a white solid as mixture of diastereoisomers. MS (ES^+) m/z 611 $(M + H)^+$. This mixture was dissolved in isopropanol (0.08 M), and Pd/C (5%) (20% w/w) was added. The resulting suspension was stirred under H_2 atmosphere for 2 h at RT. The mixture was filtered, and the solvent was evaporated. The residue was dissolved in acetonitrile and purified by RP-HPLC. Fractions containing the pure compound were freeze-dried to afford the title compound **50** as a white powder. ^{1}H NMR (300 MHz, CD_3OD, δ) 8.26 (d, J = 7.65 Hz, 1H), 6.11 (d, J = 7.47 Hz, 1H), 6.05 (s, 1H), 4.27−4.2 (m, 1H), 4.18−3.90 (m, 6H), 1.75−1.6 (m, 1H), 1.4−1.35 (m, 11H), 1.16 (s, 3H), 0.95−0.9 (m, 6H). ^{13}C NMR (100 MHz, MeOD, δ) 176.99, 165.61, 155.97, 144.15, 96.19, 93.47, 82.73, 82.64, 80.20, 73.09, 69.95, 68.66, 63.07, 51.71, 38.21, 34.74, 34.67, 21.91, 20.93, 20.87, 20.18, 14.71. ^{31}P NMR (300 MHz, CD_3OD, δ) 5.22. MS (ES^+) m/z 521 $(M + H)^+$.

5′-*O*-[[[2-[(1-Oxo-2-propylpentyl)oxy]ethyl]amino]phenylmethoxy)phosphinyl]-2′-*C*-methy-2′,3′-*O*-(1-methylethylidene)cytidine (20-II). Following the same procedure used to obtain compound **20-I** but using a solution of 2-aminoethyl 2-propylpentanoate hydrochloride **21-II**, the desired compound (1.3 equiv) in *i*-PrOH/THF was added. The mixture was stirred for 30 min at 0 °C, and then the salts were filtrated. The resulting solution was evaporated and then diluted with EtOAc and water. The aqueous phase was separated and extracted three times with EtOAc, and the combined organic phases were washed with brine and dried (Na_2SO_4). The crude product was purified by column chromatography using as eluent DCM/MeOH (95/5) to give **20-II**, a white solid as 1:1 mixture of diastereoisomers. ^{1}H NMR (300 MHz, DMSO-d_6, δ) 8.78 (bs, 1H), 8.01 (bs, 1H), 7.95−7.85 (m, 1H), 7.42−7.30 (m, 5H), 6.02 (s, 1H), 5.93 (d, J = 7.26 Hz, 1H), 5.46−5.38 (m, 1H), 5.0 (d, J = 7.44 Hz, 2H), 4.51 (s, 1H), 4.38 (s, 1H), 4.22−4.19 (m, 2H), 4.06−4.01 (m, 2H), 3.09−3.03 (m, 2H), 2.37−2.30 (m, 1H), 1.53 (s, 3H), 1.58−1.21 (m, 8H), 1.39 (s, 3H), 1.21 (s, 3H), 0.86 (t, J = 7.26 Hz, 6H). ^{31}P NMR (300 MHz, DMSO-d_6, δ) 10.2, 10.4. MS (ES^+) m/z 637 $(M + H)^+$.

5′-*O*-[Hydroxy[[2-[(1-oxo-2-propylpentyl)oxy]ethyl]amino]phosphinyl]-2′-*C*-methylcytidine (51). Compound **20-II** was dissolved in a solution of TFA/H_2O (0.1 M, 8/2). The resulting solution was warmed to 30 °C and stirred for 20 min. The solvent was evaporated and the residue dissolved in water and EtOAc. The aqueous phase was extracted three times with EtOAc, and the combined organic phases were washed with brine and dried (Na_2SO_4). The crude product was purified by column chromatography using as eluent DCM/MeOH (95/5) to give a white solid as mixture of diastereoisomers. MS (ES^+) m/z 597 $(M + H)^+$. This mixture was dissolved in methanol (0.08 M), and Pd/C (10%) (20% w/w) was added. The resulting suspension was stirred under H_2 atmosphere for 18 h at RT. The mixture was filtered, and the solvent was evaporated. The residue was dissolved in acetonitrile and purified by RP-HPLC. Fractions containing the pure compound were freeze-dried to afford the title compound **51** as white powder. ^{1}H NMR (400 MHz, DMSO-d_6, δ) 8.76 (s, 1H), 8.08 (d, J = 7.8 Hz, 1H), 6.05 (d, J = 5.88 Hz, 1H), 5.79 (s, 1H), 4.21−4.13 (m, 1H), 4.02−3.95 (m, 4H), 3.6 (d, J = 9.1 Hz, 1H), 2.96−2.9 (m, 2H), 2.35−2.27 (m, 1H), 1.51−1.3 (m, 4H), 1.25−1.16 (m, 4H), 1.03 (s, 3H), 0.82 (t, J = 6.3 Hz, 6H). ^{31}P NMR (300 MHz, DMSO-d_6, δ) 7.87. MS (ES^+) m/z 507 $(M + H)^+$.

Diammonium 5′-*O*-({[(1*S*)-1-Carboxylatoethyl]amino}phosphinato)-2′-*C*-methylcytidine (18). Compound **17** (39 mg, 0.072 mmol; as a 1:1 mixture of isomers **17a** and **17b**) was dissolved in water (3 mL) and triethylamine (3 mL) and stirred at RT for 15 h. All volatiles were removed in vacuo, and the resulting crude was redissolved in ACN (0.5 mL) and water (1 mL) and purified by RP-HPLC (eluent, H_2O (containing 5 mM ammonium bicarbonate)/MeCN). The fractions containing the pure compound were combined and freeze-dried to constant weight to afford the title compound **18** as a bis-ammonium salt (17.2 mg, 54%) as a white powder. ^{1}H NMR (300 MHz, D_2O, δ) 8.05 (d, J = 7.7 Hz, 1H), 6.13 (d, J = 7.6 Hz, 1H), 5.95 (s, 1H), 4.13 (m, 1H), 4.04 (m, 1H), 3.95−3.87 (m, 2H), 3.55 (app t, J = 7.2 Hz, 1H), 1.22 (d, J = 6.7 Hz, 3H), 1.08 (s, 3H). ^{31}P NMR (300 MHz, D_2O, δ) 6.79. MS (ES^+) m/z 409 $(M + H)^+$.

Prodrug Conversion to NTP in Hepatic Cells. HBI10A cells and primary hepatocytes were used for nucleoside triphosphate quantification. Briefly, HBI10A cells were plated at 800 000 cells/well in six-well tissue culture plates and, 24 h after plating, incubated with compounds at 37 °C in 5% CO_2. Primary hepatocytes (10^6 cells/mL) were incubated with compounds in 96-well plates in a shaking water bath at 37 °C, under an atmosphere of 95% O_2/5% CO_2. At different times, cells were then extracted in 70% methanol, 20 mM EDTA, and 20 mM EGTA and centrifuged. Samples were dried, reconstituted, and purified by solid-phase extraction, and triphosphate levels were quantified by LC−MS/MS.

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Supporting Information Available: Analytical data for target compounds and biological data for the first eluting diastereoisomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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