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β-D-2′-C-Methyl-2,6-diaminopurine Ribonucleoside Phosphoramidates are Potent and Selective Inhibitors of Hepatitis C Virus (HCV) and Are Bioconverted Intracellularly to Bioactive 2,6-Diaminopurine and Guanosine 5′-Triphosphate Forms

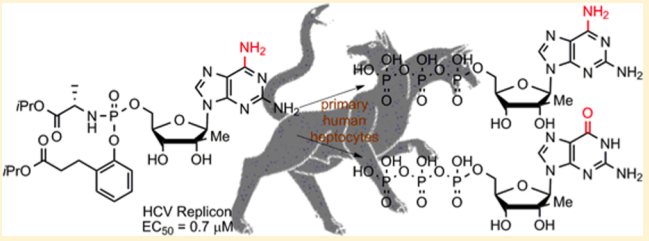
Longhu Zhou,[†] Hong-wang Zhang,[†] Sijia Tao,[†] Leda Bassit,[†,‡] Tony Whitaker,[§] Tamara R. McBrayer,[‡,§] Maryam Ehteshami,[†] Sheida Amiralaei,[†] Ugo Pradere,[†] Jong Hyun Cho,[†] Franck Amblard,[†,‡] Drew Bobeck,[§] Mervi Detorio,[†,‡] Steven J. Coats,[§] and Raymond F. Schinazi*[,†,‡]

[†]Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30322, United States

[‡]Veterans Affairs Medical Center, Decatur, Georgia 30033, United States

[§]CoCrystal Pharma, Inc., Tucker, Georgia 30084, United States

ABSTRACT: The conversion of selected β-D-2,6-diaminopurine nucleosides (DAPNs) to their phosphoramidate prodrug (PD) substantially blocks the conversion to the G-analog allowing for the generation of two bioactive nucleoside triphosphates (NTPs) in human hepatocytes. A variety of 2′-C-methyl DAPN-PDs were prepared and evaluated for inhibition of HCV viral replication in Huh-7 cells, cytotoxicity in various cell lines, and cellular pharmacology in both Huh-7 and primary human liver cells. The DAPN-PDs were pan-genotypic, effective against various HCV resistant mutants, and resistant variants could not be selected. 2′-C-Me-DAPN-TP and 2′-C-Me-GTP were chain terminators for genotype 1b HCV-pol, and single nucleotide incorporation assays revealed that 2′-C-Me-DAPN-TP was incorporated opposite U. No cytotoxicity was observed with our DAPN-PD when tested up to 50 μM. A novel, DAPN-PD, **15c**, has been selected for further evaluation because of its good virologic and toxicologic profile and its ability to deliver two active metabolites, potentially simplifying HCV treatment.



INTRODUCTION

Over the past 5 decades nucleoside analogs have played an ever-increasing role in the treatment of viral infections. To date there are more than a dozen nucleoside analogs approved by the FDA for the treatment of viral infections. Approximately 170 million people worldwide are infected with the seven major genotypes of hepatitis C virus (HCV), and 3−4 million individuals become newly infected each year.[1] HCV infection often leads to reduced liver function, cirrhosis, and cancer and is the leading cause of liver transplantation.[2] The first FDA-approved treatment for chronic HCV was limited to pegylated interferon-α alone or in combination with ribavirin,[3] which was effective in 40−60% of patients and is often associated with significant side effects, such as fatigue, nausea, depression, and thinning hair.[4] In May 2011 the introduction of protease inhibitors (PI) boceprevir and telaprevir to the above standard of care marked the introduction of the first direct acting antiviral agents (DAAs) for HCV.[5,6] Unfortunately, these treatments have limited efficacy for the genotype 1 virus, the most prevalent in the U.S. and China. They are also associated with various side effects, especially when combined with pegylated interferon-α and ribavirin.[7]

Three major drug targets have reached evaluation in humans for chronic HCV infection:[8,9] the NS3/4A serine protease, the large phosphoprotein NS5A, and NS5B RNA-dependent RNA polymerase (RdRp) NS5B (nucleoside[10] and non-nucleoside inhibitors[11]). The HCV NS5B RNA-dependent RNA polymerase (RdRp) is essential for HCV replication, and both nucleoside and non-nucleoside antiviral agents targeting this enzyme are in preclinical and clinical development. For a nucleoside analog to be incorporated into a growing RNA chain by a polymerase, it must first enter the cell by either passive or active transport and undergo three separate phosphorylation steps to ultimately provide the nucleoside analog in its triphosphate form (NTP). It has been reported that many nucleoside analogs are poor substrates for the phosphorylation kinases, which convert the nucleosides to the corresponding nucleoside monophosphate.[12] The initial phosphorylation step is often rate limiting en route to the formation of the active NTP and in many cases does not occur with sufficient efficiency to provide the active NTP form at therapeutically effective concentrations. In order to bypass this rate-limiting step, many

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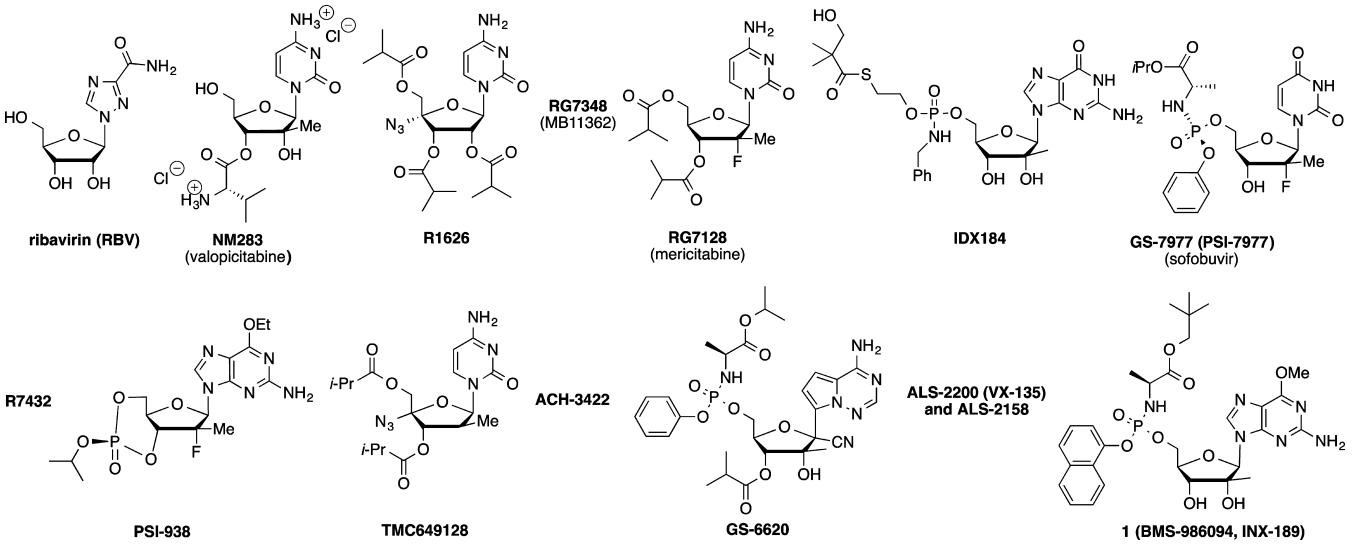
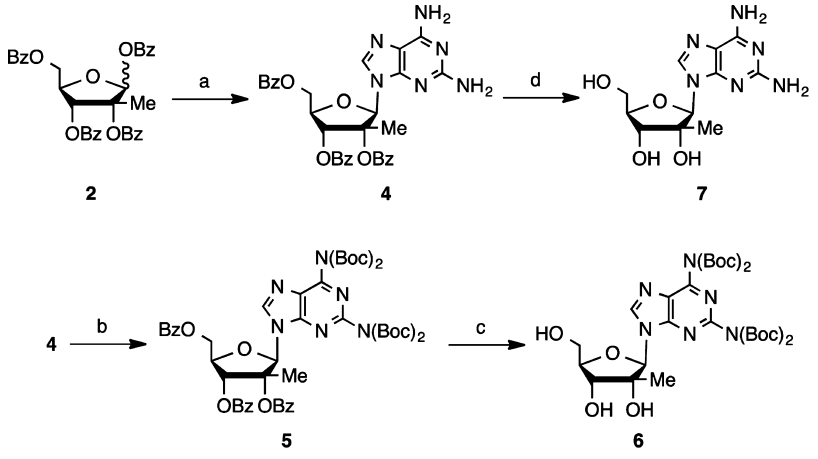


Figure 1. Chemical structures (where available) of nucleoside analog inhibitors that have entered human clinical trials for HCV therapy.

Scheme 1[a]



[a]Reagents and conditions: (a) 2,6-diaminopurine (**3**), DBU, TMSOTf, CH_3CN, −40 to 65 °C, 5 h, 92%; (b) $(Boc)_2O$, DMAP, THF, rt, 90%; (c) NaOMe/MeOH, rt, 30 min, 92%; (d) NH_3/MeOH, rt, 93%.

nucleoside prodrug analogs have been developed as more lipophilic nucleoside monophosphate prodrugs (NMP-PDs).[10,13,14] NMP-PDs antiviral drugs have been reported to increase the oral bioavailability, liver exposure, and brain penetration of parent drugs. The most advanced nucleoside NMP-PDs are the aryloxy phosphoramidates ("ProTide" or "McGuigan PD"),[15] 1-aryl-1,3-propanyl esters ("HepDirect"),[16] and lipid conjugates[17] which have all have been evaluated in humans. Pronucleotide (ProTide), first introduced in 1992,[15] is the most investigated NMP-PD and demonstrated improved pharmacological activity of many parent nucleosides both in vitro and in vivo.[18] Currently the most advanced NMP-PDs have the following limitations: (1) intracellular delivery of potentially toxic agents; (2) a racemic phosphorus center; (3) the nucleoside-MP phosphorus−oxygen bond being weak and therefore prone to cleavage; (4) low to very low yields for the preparation of NMP-PDs.[14]

Nucleoside analog polymerase inhibitors are anticipated to become the backbone of HCV therapy, and to date, at least 14 have entered clinical trials but only one has been approved (Figure 1).[10] Sofosbuvir, which received FDA approval in December 2013, has highly potent clinical antiviral activity across all genotypes in combination with pegylated interferon and/or ribavirin or an NS5A inhibitor (e.g., Harvoni). Sofosbuvir containing regimens with other DAAs can be shorter in duration and achieve very high-sustained virologic response (SVR). However, the continued high cost of DAAs will preclude their prompt worldwide use especially when more than one DAA is needed to cure an HCV-infected person. In addition, the non-nucleoside inhibitors, unlike the nucleoside analogs, are prone to have a low genetic barrier to resistance. Therefore, there is an urgent need for more cost-effective treatments that are pan-genotypic that can be globally utilized.[19]

Early clinical trials of direct acting antiviral agents for HCV treatment[20] made clear the need for combination DAA therapy in order to achieve high SVR and as the ultimate solution for pan genotypic treatment of HCV. The ability to deliver more than one active metabolite from a single drug could help increase potency for inhibition of HCV replication and potentially inhibit the formation of viral mutants.[21] Like Orthrus, the fearsome two-headed dog of Greek mythology and guardian of Geryon's herd of red cattle, an antiviral drug that delivers two potent metabolites should increase potency

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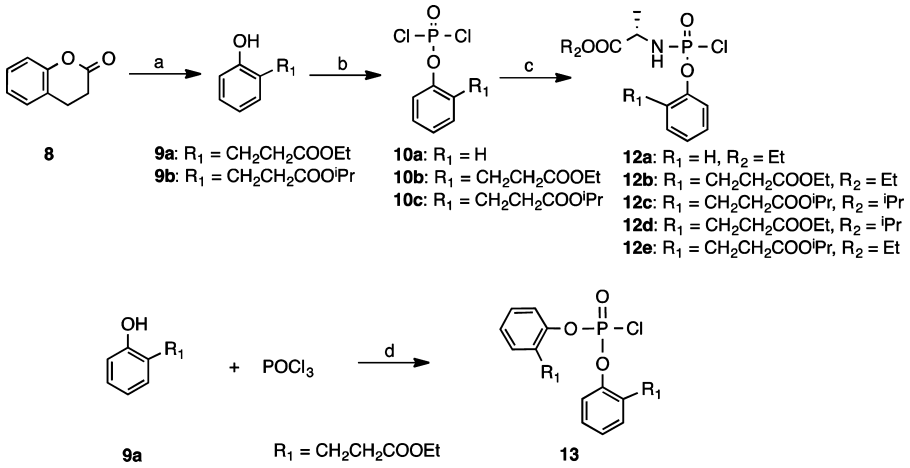
Scheme 2[a]



[a]Reagents and conditions: (a) ROH, cat. H_2SO_4, reflux, overnight, for **9a**, 94%; for **9b**, 89%; (b) $POCl_3$, Et_3N, diethyl ether, −70 °C to rt, overnight; (c) amino acid esters hydrochloride (**11**), Et_3N, DCM, −70 °C to rt, two steps for **12a**, 72%; for **12b**, 75%; for **12c**, 70%; for **12d**, 69%; for **12e**, 70%; (d) Et_3N, diethyl ether, −70 °C to rt, 35%.

and potentially suppress the formation of viral mutants. The typical utilization of the aryloxy phosphoramidate prodrug approach, such as in the case of sofosbuvir, involves the intracellular delivery of phenol. While phenol is used in some over-the-counter products as an oral anesthetic/analgesic and is commonly used to temporarily treat pharyngitis (inflammation of the throat), it has an LD_{50} of 270 mg/kg in mice and phenol may cause harmful effects to the central nervous system and heart, resulting in dysrhythmia, seizures, and coma. Chronic exposure to phenol in humans has been linked to harmful effects to the liver and kidneys.[22] We sought to overcome these potential liabilities by the introduction of a propyl ester side chain at the ortho position of the phenol portion of our prodrugs. Cellular pharmacology in human peripheral blood mononuclear (PBM) cells showed that unmasking to the monophosphate from this substituted phosphoramidate results in the formation of hydroxyphenylpropanoic acid, which is a known nontoxic metabolite[23] of dihydrocoumarin, a common flavoring agent widely used in food[24] and cosmetics.[25]

Herein we report the synthesis and biological evaluation of β-D-2′-*C*-methyl-2,6-diaminopurine ribonucleoside phosphoramidate prodrugs that intracellularly deliver two active nucleoside triphosphates along with nontoxic prodrug metabolites.

CHEMISTRY

The 2,6-diaminopurine tribenzoylated nucleoside **4** was synthesized from known sugar **2**[26] and 2,6-diaminopurine **3** with TMSOTf in the presence of DBU. The unprotected 2,6-diaminopurine nucleoside **7** was prepared by reaction of **4** with ammonia in methanol. Boc protected nucleoside **6** was necessary to significantly improve the yield of the phosphoramidate forming reaction and prepared by reaction of **4** with $(Boc)_2O$ at rt, followed by careful debenzylation with sodium methoxide at rt (Scheme 1). The crude debenzylation reaction was cautiously neutralized with Dowex resin (H^+ form) during workup to avoid unwanted deprotection of the Boc groups.

Hydrolysis of dihydrocoumarin, **8**, in anhydrous ethanol or isopropanol gave esters **9a** and **9b**, respectively. The phenyl dichlorophosphates **10a**, **10b**, and **10c** were prepared from the appropriate phenol and phosphorus oxychloride. Aryloxy phosphorochloridates **12** were freshly prepared by treatment of L-alanine esters **11** with readily prepared aryloxy phosphorochloridates **10** (Scheme 2). Diaryloxy phosphorochloridate **13** was freshly synthesized with the same reaction conditions (Scheme 2). Since the phosphoryl chlorides **12** were produced as an approximate 1 to 1 mixture of the two possible diastereomers at the phosphorus center, the subsequent phosphoramidates **15** (Scheme 3) were also obtained as a diastereomeric mixture. This is due to the often clean inversion of configuration at the phosphorus atom during the S_NP reaction (similar to the inversion at electrophilic carbon centers in the more well-known S_N2 reaction). The phosphorochloridates **12** and **13** could be stored for extended periods when diluted in dry THF to 1 M and stored in a well-sealed container at −80 °C under nitrogen or argon.

Phosphoramidate prodrugs derived from the tetra-Boc nucleoside **6** were prepared initially following literature nucleoside phosphoryl chloride displacement methods, which conveniently afforded the desired products as a mixture of two diastereomers at the phosphorus center (**14a**−**e**).[27] For example, compound **15b** was prepared by reacting **6** in the presence of *N*-methylimidazole with the phosphoryl chloride **12b** in THF/ACN to produce protected prodrug **14b**. Subsequent deprotection with 80% aqueous TFA gave phosphoramidate **15b** in high overall yield from this two-step process (Scheme 3). Acetonitrile was used as a cosolvent to improve the solubility of the reactants, which resulted in a more complete reaction and much better isolated yields. Similar reaction conditions were employed for the synthesis of prodrug **17**, which contains an achiral phosphorus center. For the more lipophilic and potentially better absorbed 2′,3′-carbonate prodrug, **16**,[28] compound **13c** was simply treated with carbonyldiimidazole.

The diastereomeric mixtures **15b**−**e** were separated by careful column chromatography on silica gel to obtain the two single diastereomers. However, preparation of **15b**(R_p) in larger quantities required a more practical method. A single diastereomeric phosphoramidating reagent containing a *p*-nitrophenyl ester was used as an alternative to the chloridate **12b** for the stereospecific synthesis of **15b**(R_p).[28,29] Accordingly, the diastereomeric mixture **19**(R_p) and **20**(S_p) was prepared and subjected to fractional crystallization to obtain the

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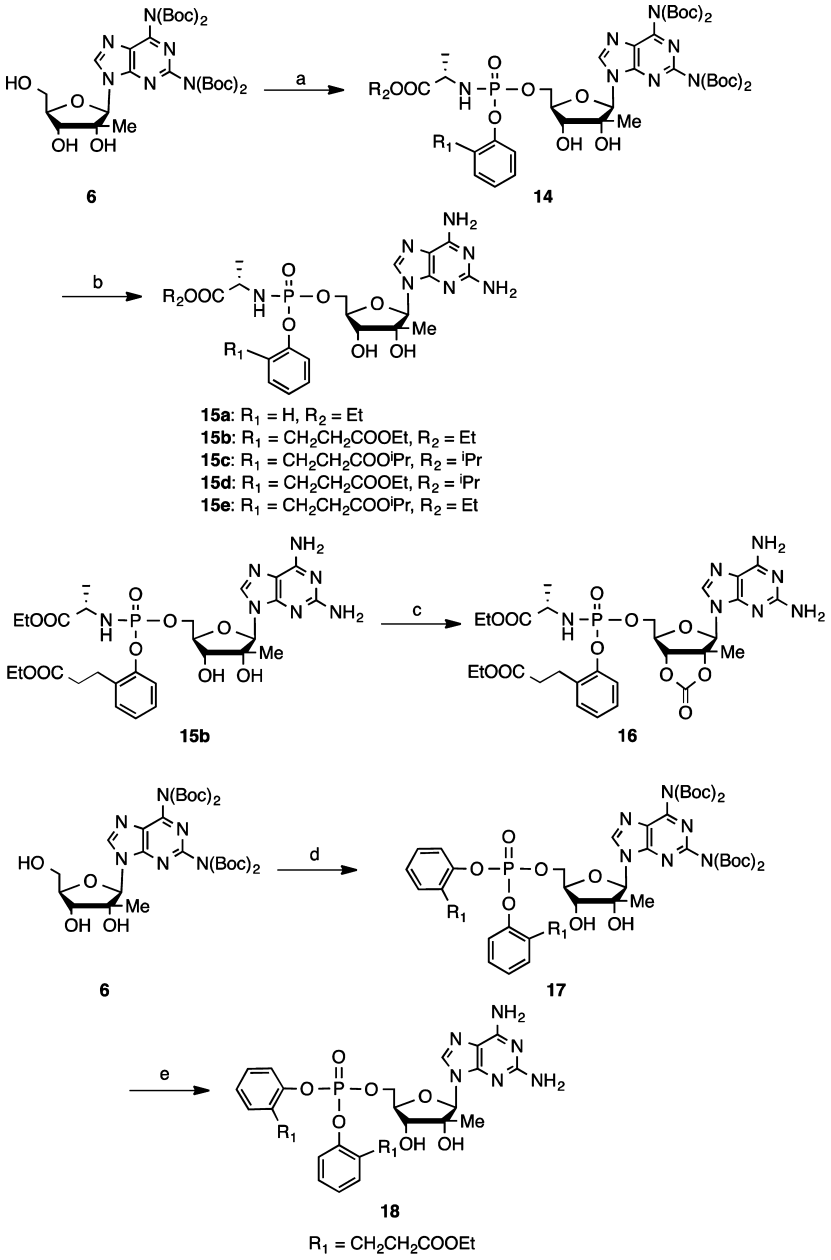
Scheme 3[a]



[a]Reagents and conditions: (a) **12a–e**, *N*-methylimidazole, THF–CH_3CN, rt; (b) 80% TFA, 0 °C to rt, two steps for **15a**, 38%; for **15b**, 56%; for **15c**, 40%; for **15d**, 41%; for **15e**, 43%; (c) *N*,*N*′-carbonyl diimidazole, CH_3CN, rt, 71%; (d) **13**, *N*-methylimidazole, THF–CH_3CN, rt; (e) 80% TFA, 0 °C to rt, two steps for **22**, 55%.

single isomers **19**(R_p) and **20**(S_p) (see Experimental Section) (Scheme 4). Their absolute stereochemistry was determined by single crystal X-ray analysis (Figure 2). Considering the change in stereochemical nomenclature priorities, it is the reagent having the S_p configuration that would lead, after inversion through a nucleophilic displacement reaction with **6**, to the desired product **15b**(R_p). Thus, nucleoside **6** was treated with *tert*-butylmagnesium chloride in THF, and subsequent treatment of the resulting suspension with **20**(S_p) at ambient for 3 days afforded the single diastereomer R_p, which was deprotected with aqueous TFA to afford **15b**(R_p) (Scheme 4). The R_p reagent **19** gave the phosphoramidate product **15b**(S_p) in similar yield (Scheme 4). The absolute configurations of the phosphorus diastereomers of **15c–e** were assigned by comparison to **15b**(S_p) and **15b**(R_p).

RESULTS AND DISCUSSION

Antiviral Activity and Cytotoxicity. 2′-*C*-Methyl-2,6-diaminopurine phosphoramidate prodrugs (DAPN-PD) were evaluated for inhibition of HCV RNA replication in Huh-7 cells using a subgenomic HCV replicon system.[30] Cytotoxicity in Huh-7 cells were determined simultaneously by extraction and amplification of both HCV RNA and cellular ribosomal RNA (rRNA) (Table 1).[31] DAPN-PD compounds showed greater potency against HCV replication in Huh-7 cells when compared to the parent DAPN nucleoside **7** (up to 14-fold; e.g., EC_{50} = 0.2 for **15b**(R_p) vs 5.4 μM for parent compound **7**.

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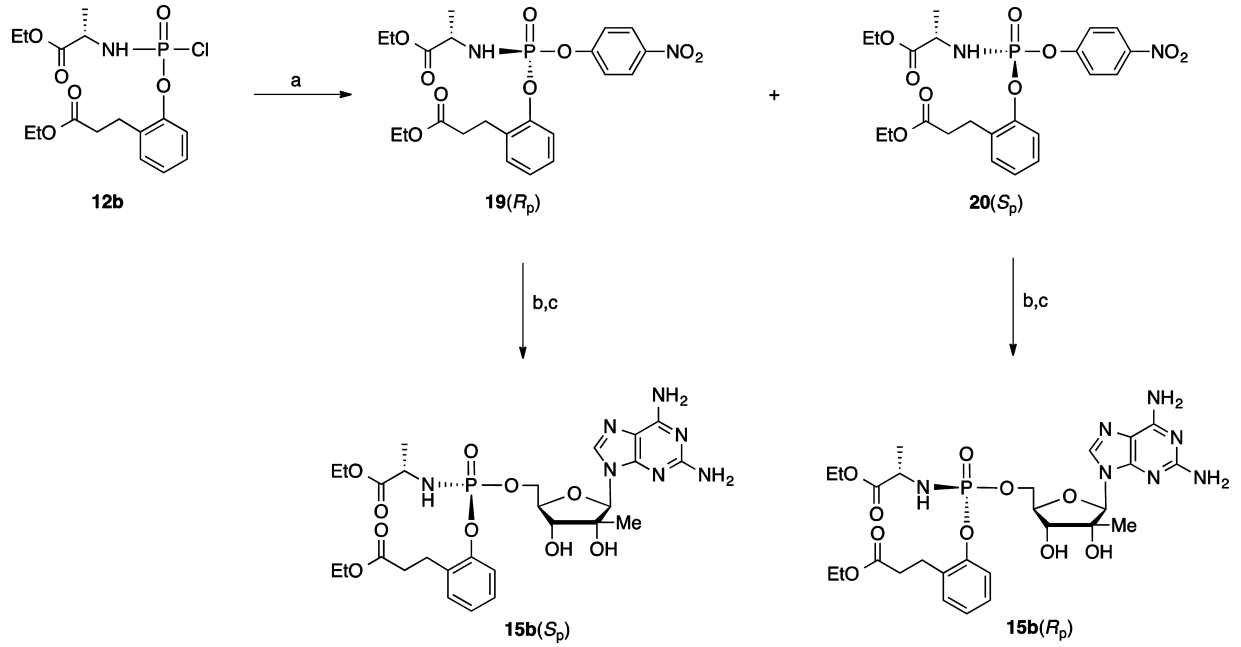
Scheme 4[a]



[a]Reagents and conditions: (a) *p*-nitrophenol, Et_3N, diethyl ether, rt, 85% (**19** + **20**, 1:1 ratio); (b) **6**, *t*-BuMgCl, THF, −78 °C, then rt 3 days; (c) 80% TFA, 0 °C, then rt 4 h. **15b**(R_p): 41% two steps. **15b**(S_p): 39% two steps.

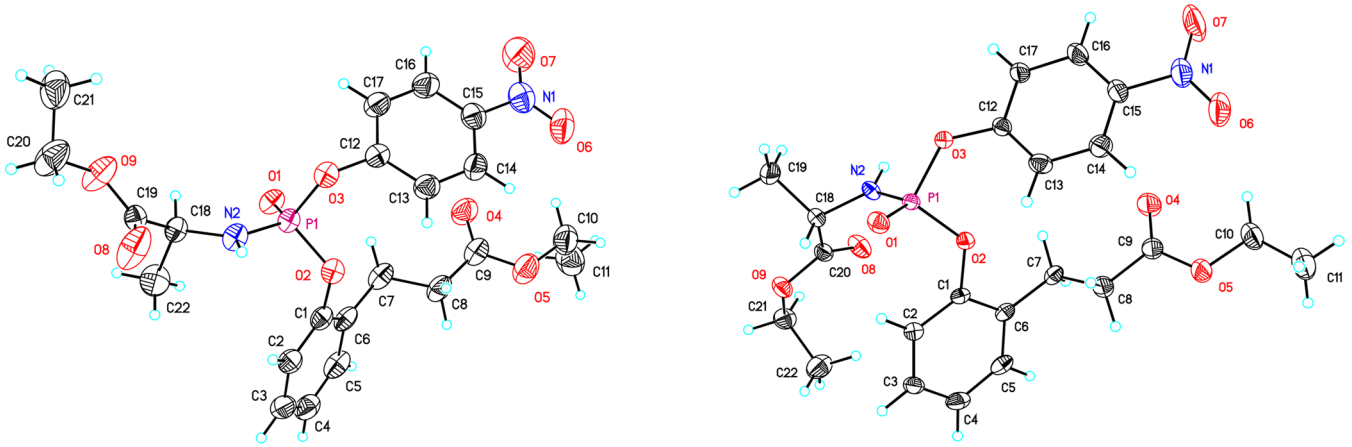


Figure 2. X-ray crystal structures of **19**(R_p) and **20**(S_p).

The difference in activity between the various pairs of phosphorus diastereomers could be due to different rates of deesterification that lead to different levels of unmasked monophosphate. In general one of the diastereomers was producing the majority of the antiviral activity when compared with the diastereomeric mixture. This was not the case for **15c**, as the two phosphorus diastereomers had very similar anti-HCV potency. The 2′,3′-carbonate phosphoramidate **16** retained reasonable activity (EC_{50} = 2.8 μM), but later it was found that the carbonate group falls off quickly in simulated intestinal fluid ($T^{1/2}$ < 5 h). The symmetrical phenolic ester **18** was inactive and presumed to be too stable in cell culture to unmask to the monophosphate. **1** (BMS-986094) was included as a positive control and, consistent with the literature,[32] demonstrated nanomolar potency. Conversely, this compound was found to be cytotoxic in Huh-7 replicon cells as indicated by significant reductions in cellular rRNA levels (CC_{50} = 0.8 μM). No cytotoxic effect was observed with up to 10 μM with the DAPN compounds in the same cell line.

DAPN compounds were further evaluated for cytotoxicity at higher concentrations in human PBM cells, human lymphoblastoid CEM, and African Green monkey Vero cells (Table 1).[33] In contrast to **1** (BMS-986094) that showed cytotoxicity in all cell types tested, we did not observe a cytotoxic effect with the majority of DAPN compounds. Prototype DAPN-PD with the highest antiviral activity, **15b**(R_p), was also further evaluated for toxicity in PC3 cells (a human prostate cancer cell line) as well as its effect on cellular GAPDH levels and thymidine uptake. At 100 μM **15b**(R_p) we did not observe any toxicity, effect on GAPDH levels, or effect on thymidine uptake (data not shown).

It has previously been reported that some nucleotide analog compounds may also inhibit Huh-7 cell growth[34] and exhibit mitochondrial toxicity.[35] In order to address this issue, compounds **7**, **15a**, **15b**(R_p), and **15c** were evaluated for

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Table 1. HCV Replicon Activity and Cytotoxicity of Phosphoramidate Prodrugs

	replicon Huh-7 (μM)[a]			cytotoxicity, CC_{50} (μM)[b]		
compd	EC_{50}	EC_{90}	CC_{50}	PBM	CEM	Vero
7	5.4	>10	>33	>100	>100	>100
15a	0.9 ± 0.3	4.0 ± 2.8	>10	>100	>100	>100
15b	2.3	7.3	>10	>100	>100	>100
15b(R_p)	0.2 ± 0.1	0.7 ± 0.3	>10	>100	>100	>100
15b(S_p)	2.7	8.7	>10	>100	>100	>100
15c	0.7 ± 0.2	2.5 ± 0.2	>10	>100	>100	>100
15c(R_p)	1.0	2.7	>10	>100	>100	>100
15c(S_p)	0.9	2.8	>10	>100	40	>100
15d	2.2	9.0	>10	>100	>100	>100
15d(R_p)	2.4	8.2	>10	>100	>100	>100
15d(S_p)	4.7	9.7	>10	>100	>100	>100
15e	1.7	8.0	>10	>100	>100	>100
15e(R_p)	1.4	6.9	>10	>100	>100	>100
15e(S_p)	9.1	26	>10	>100	>100	>100
16	2.8	8.5	>10	>100	>100	>100
18	>10	>10	>10	64	25 ± 9.9	90
1	0.02 ± 0.2	0.04 ± 0.2	0.8	4.7 ± 3.3	8.7 ± 9.1	14 ± 5.1

[a]EC_{50}, EC_{90}, and CC_{50} values obtained from Huh-7 replicon were generated in triplicate from one or two separate experiments (±SD where applicable). [b]CC_{50} values of compounds reported for PBM, CEM, and Vero cells were generated from one to four separate experiments (±SD where applicable).

Table 2. Mitochondrial Toxicity and Lactic Acid Production with DAPN Prodrugs

	CC_{50} (μM)[a]		lactic acid levels (% of β-actin control)[b]		
compd	mtDNA	β-actin DNA	1 μM	10 μM	50 μM
7	>50	>50	110 ± 0.9	170 ± 3.7	110 ± 6.1
15a	>50	>50	100 ± 13	140 ± 2.0	90 ± 4.5
15b(R_p)	>50	>50	150 ± 1.7	120 ± 5.6	110 ± 1.9
15c	>50	>50	90 ± 5.1	81 ± 0.1	80 ± 3.0
1	3.4, 5.9, <1[c]	<1, 2.3, 0.86[c]	190 ± 20	UM[d]	UM[d]
3TC	>10	>10	ND[e]	91 ± 2.5	ND[e]
ddC	<10	<10	ND[e]	220 ± 11	ND[e]

[a]CC_{50} values obtained from HepG2 cells were generated from two separate experiments (±SD where applicable). mtDNA was measured and compared to nuclear β-actin DNA. [b]Lactic acid measurements are the mean of triplicates from a single 96-well plate (±SD). [c]Determined in three separate experiments. [d]UM: unmeasurable at 100 and 10 μM, as the percent of cells at these concentrations was <1. [e]ND: not determined

their effects on mitochondrial DNA levels. HepG2 cells were propagated in the presence of nucleotide analogs (up to 50 μM) for 14 days prior to quantification of mitochondrial COXII DNA (mtDNA) and β-actin DNA using real-time PCR. Lamivudine (3TC) and β-D-2′,3′-dideoxycytidine (ddC) (at 10 μM) were included as negative and positive controls, respectively (Table 2). Neither the parent nor the DAPN prodrug compounds tested showed measurable mitochondrial toxicity up to 50 μM in HepG2 cell line. On the other hand, **1** (BMS-986094) showed mitochondrial toxicity with a CC_{50} value of 4.7 ± 1.8 μM. Lactic acid levels were also measured in the culture supernatant after 14 days of incubation with each prodrug. The total amount of lactic acid produced was determined for each sample. Increased production of lactic acid (generally above 100% when normalized to β-actin control) is associated with mitochondrial toxicity.[36] We did not observe increased lactic acid production with up to 50 μM parent DAPN or DAPN-PD compounds. As expected, treatment with ddC resulted in increased lactic acid production (Table 2). We were unable to accurately measure lactic acid levels with **1** (BMS-986094) because treatment with 10 μM of this compound led to excessive cell death.

Finally, activity of candidate DAPN-PD compound **15b**(R_p) was also tested against a panel of genetically diverse HCV replicons. We found that **15b**(R_p) was equally active against all HCV genotype replicons tested (Table 3). Furthermore, this compound remained effective against a panel of HCV genotype 1b replicons harboring mutations associated with drug resistance (Table 4).

Table 3. Pan-Genotypic Activity of DAPN pProdrug 15b(R_p) Tested in Cell-Based Assays

Huh-7 replicon	EC_{50} (μM)	EC_{90} (μM)
GT 1a	0.35	0.74
GT 1b	0.26	0.39
GT 2a	0.26	0.70
GT 2b (chimera)[a]	0.29	0.42
GT 3a (chimera)[a]	0.23	0.39
GT 4a (chimera)[a]	0.39	0.89
GT 5a (chimera)[a]	0.32	0.64

[a]Chimeric replicons harbored NS5B gene from indicated genotypes in backbone of HCV GT 1b con1 strain.

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Table 4. Cell-Based Antiviral Activity of 15b(R_p) against Mutant HCV GT 1b Strains Commonly Associated with Drug Resistance

Huh-7 replicon[a]	EC_{50} (μM)	EC_{90} (μM)
NS5A Y93H	0.30	3.0
NS3 R155K	0.32	1.3
NS5B S282T	0.10	0.23
NS5B C316Y	0.27	0.77
NS5B C445F	0.12	0.25
NS5B M414I	0.28	1.1
NS5B P495A	0.08	0.14

[a]Values averaged from two to three experiments except GT 1a Y93H (n = 1).

Cellular Pharmacology and Biochemical Evaluation. It has been well established that 6-modified nucleosides can be substrates for human deaminases.[37] We therefore set out to evaluate the intracellular metabolism of DAPN-PD in both Huh-7 cells and primary human hepatocytes.[38] Parent DAPN compound 7 and DAPN-PD **15b**(R_p) were incubated in Huh-7 cells at 50 μM for 4 h at 37 °C. Intracellular metabolites were extracted with 70% ice-cold methanol in water and subsequently identified by LC−MS/MS. Incubation of the parent DAPN in Huh-7 revealed the generation of two triphosphate metabolites, namely, 2′-*C*-Me-DAPN-TP and 2′-*C*-Me-GTP at a 1:3 ratio (Figure 3A). Conversely, incubation of DAPN-PD **15b**(R_p) increased the intracellular levels of 2′-*C*-Me-DAPN-TP in Huh-7 cells over 13-fold compared to the levels achieved with parent nucleoside DAPN (0.74 ± 0.3 pmol/10^6 cells and 9.9 ± 5.5 pmol/10^6 cells, respectively). The same trend was observed in primary human hepatocytes where 7-fold higher triphosphate metabolites were observed when compared to Huh-7 cells (Figure 3B). Very similar results were obtained in Huh-7 cells and primary human hepatocytes for **15c**. Together, these data indicate that DAPN-PD was capable of delivering two nucleoside triphosphate metabolites intracellularly.

In Vitro Inhibition of Viral RNA Synthesis by NTP Metabolites Generated by DAPN-PD. 2′-*C*-Me-GTP is a known inhibitor of HCV NS5B polymerase.[39] On the other hand, 2′-C-Me-DAPN-TP has not been previously examined for its effect on RNA polymerization. In order to determine whether 2′-C-Me-DAPN-TP alone can inhibit NS5B-mediated RNA polymerization, we separately synthesized each of the 5′-triphosphate analogs and evaluated them in cell-free assays using purified recombinant NS5B enzyme (Figure 4). The IC_{50} values for 2′-*C*-Me-GTP and 2′-*C*-Me-DAPN-TP were determined to be 5.6 ± 1.6 and 3.4 ± 1.1, respectively. Under the conditions tested, we found that both NTP metabolites inhibited polymerization as indicated by the reduction in full-length RNA product formation with increased TP inhibitor concentrations. As expected, distinct pausing was observed at sites of GMP incorporation for 2′-*C*-Me-GTP. Conversely, incubation with 2′-*C*-Me-DAPN-TP resulted in the appearance of distinct pausing bands at AMP incorporation sites. These data suggest that 2′-*C*-Me-DAPN-TP acts as an A-analog. This finding was consistent with previous observations that nucleotides with the 2,6-diaminopurine modification are incorporated opposite a template T or U.[40] Further biochemical characterization is underway to evaluate the kinetic properties of 2′-*C*-Me-DAPN-TP in vitro.

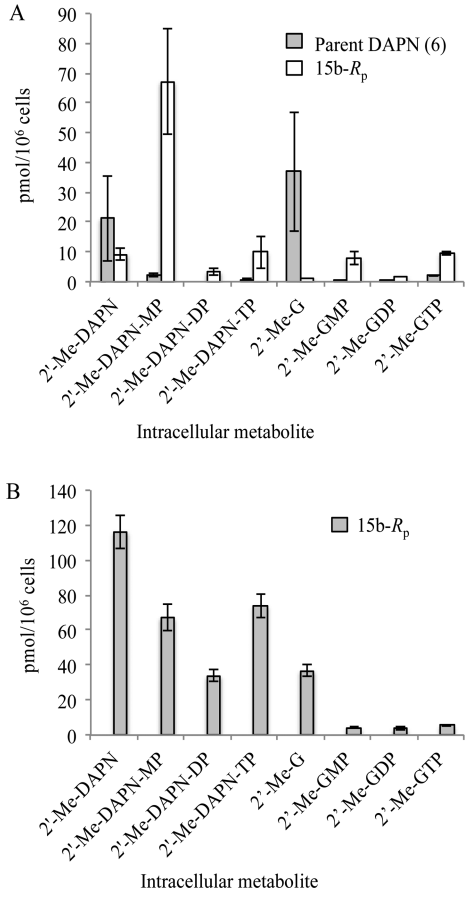

Figure 3. Intracellular metabolism of parent DAPN and DAPN-PD in one donor hepatocytes. (A) An amount of 50 μM of either parent DAPN (gray bars) or DAPN-PD (**15b**(R_p)) (white bars) was incubated with Huh-7 cells for 4 h at 37 °C. Intracellular generation of parent nucleotide, NI-MP, NI-DP, and NI-TP was analyzed using LC−MS/MS. (B) An amount of 50 μM DAPN-PD (**15b**(R_p)) was incubated with primary human hepatocytes (PHH, gray bars) cells for 4 h at 37 °C. Generation of intracellular metabolites was determined as described above. Error bars represent standard deviation from three separate replicates from a single 12-well plate.

CONCLUSIONS

The chemical synthesis, antiviral activity, cytotoxicity, and cellular metabolism of DAPN prodrug compounds were described. The intracellular metabolism of DAPN-PD resulted in the generation two NI-TP metabolites, namely, 2′-C-Me-DAPN-TP and 2′-*C*-Me-GTP, both of which inhibited HCV NS5B polymerase-mediated RNA synthesis in vitro. Furthermore, DAPN-PDs did not exhibit any of the in vitro toxicities observed with **1** (BMS-986094) and appear to be differentiated in their toxicity profile additionally with the release of nontoxic prodrug metabolites. Further studies are underway to evaluate to enzymatic properties of each of the nucleoside 5′-triphosphate metabolites, as well as the intracellular metabolism of DAPN-PD in various cell lines such as cardiomyocytes. On the basis of these results, compound **15c** has been selected for further in depth preclinical studies.

EXPERIMENTAL SECTION

Anhydrous solvents were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Reagents were purchased from commercial sources. Unless noted otherwise, the materials used in the examples were obtained from readily available commercial suppliers or synthesized by

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J. Med. Chem. XXXX, XXX, XXX−XXX

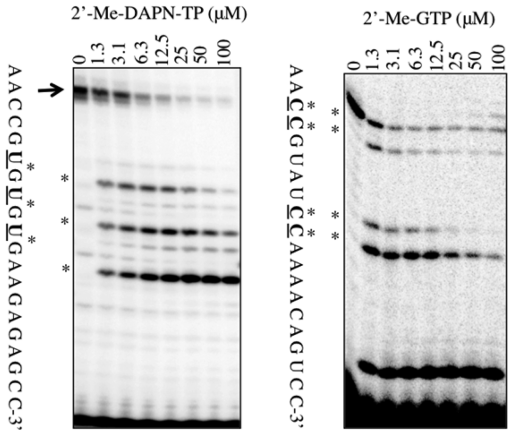

Figure 4. Incorporation of NI-TPs by NS5B RNA-dependent RNA polymerase. Increasing amounts of either 2′-*C*-Me-DAPN-TP or 2′-*C*-Me-GTP were incubated with NS5B polymerase, RNA template, radiolabeled GpG primer, and rNTP nucleotide mix. Amount of RNA product formed was measured after a 2 h incubation and visualized on a 20% denaturing polyacrylamide gel. The RNA template sequence is indicated, and sites of either A analog or G analog incorporation are highlighted with an asterisk. Arrow represents full length RNA product formed in the absence of inhibitor.

standard methods known to one skilled in the art of chemical synthesis. ^{1}H and ^{13}C NMR spectra were taken on a Varian Unity Plus 400 spectrometer at rt and reported in ppm downfield from internal tetramethylsilane. Deuterium exchange, decoupling experiments were done to confirm proton assignments. Signal multiplicities are represented by s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quadruplet), br (broad), bs (broad singlet), m (multiplet). All *J*-values are in Hz. Purity of final compounds was determined to be >95%, using analytical HPLC analyses performed on a Hewlett-Packard 1100 HPLC instrument with a Phenomenex Gemini-NX column (2 mm × 50 mm, 3 μm, C18, 110 Å) and further supported by clean NMR spectra. Mobile phase flow was 0.5 mL/min with a 3.5 min initial hold, a 6.5 min gradient from 96% aqueous media (0.05% formic acid) to 96% CH_3CN (0.05% formic acid), and a 15 min total acquisition time. Photodiode array detection was from 190 to 360 nm. Mass spectra were determined on a Micromass Platform LC spectrometer using electrospray ionization. Analytic TLC was performed on Whatman LK6F silica gel plates, and preparative TLC was performed on Whatman PK5F silica gel plates. Column chromatography was carried out on silica gel or via reverse-phase high performance liquid chromatography.

(2*R*,3*R*,4*R*,5*R*)-5-((Benzoyloxy)methyl)-2-(2,6-diamino-9*H*-purin-9-yl)-3-methyltetrahydrofuran-3,4-diyl Dibenzoate, 4. To a stirred suspension of (3*R*,4*S*,5*R*)-5-((benzoyloxy)methyl)-3-methyltetrahydrofuran-2,3,4-triyl tribenzoate **2** (2.9 g, 5 mmol) and 2,6-diaminopurine **3** (830 mg, 5.5 mmol) in anhydrous acetonitrile at −78 °C was added DBU (2.3 mL, 15 mmol), followed by a slow addition of TMSOTf (3.8 mL, 20 mmol). The reaction mixture was stirred at −78 °C for 20 min and then raised to 0 °C. After it was stirred for 30 min at 0 °C, the reaction mixture was heated gradually to 65 °C and stirred overnight. The reaction mixture was cooled, diluted with CH_2Cl_2 (200 mL), and washed with saturated $NaHCO_3$. The layers were separated, and the resulting aqueous layer was extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layers were dried over Na_2SO_4. After removal of the solvent, the residue was purified by silica gel column chromatography (0−10% MeOH in EtOAc). 2.8 g of compound **4** was obtained (92% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15−8.17 (m, 2H), 7.97−8.02 (m, 4H), 7.77 (s, 1H), 7.45−7.61 (m, 5H), 7.32−7.37 (m, 4H), 6.62 (s, 1H), 6.53 (d, 1H, *J* = 6.6 Hz), 5.91 (s, 2H), 5.04−5.10 (m, 3H), 4.82−4.86 (m, 1H), 4.70−4.74 (m, 1H), 1.62 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 17.5, 64.0, 76.0, 79.4, 85.8, 88.5, 114.9, 128.3, 128.4, 128.5, 128.9, 129.5, 129.7, 129.8, 129.9, 133.1, 133.5, 133.6, 137.4, 151.3, 155.9, 159.7, 165.3, 165.4, 166.3. LC/MS calcd for $C_{32}H_{29}N_6O_7$ $(M + 1)^+$, 609.2; observed, 609.2.

(2*R*,3*R*,4*R*,5*R*)-5-((Benzoyloxy)methyl)-2-(2,6-bis(bis(*tert*-butoxycarbonyl)amino)-9*H*-purin-9-yl)-3-methyltetrahydrofuran-3,4-diyl Dibenzoate, 5. A solution of **4** (1.4 g, 2.3 mmol), Boc anhydride (3.0 g, 14 mmol), and DMAP (56 mg, 0.46 mmol) in THF (12 mL) was stirred at rt for 30 h. After the reaction was complete, the solvent was removed under reduced pressure and the residue was purified by flash column chromatography (0−40% EtOAc in hexane). 2.1 g of white solid **5** was obtained (90% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.39 (s, 1H, H_8), 8.07−8.20 (m, 4H, Ar−H), 7.87−7.89 (m, 2H, Ar−H), 7.42−7.61 (m, 7H, Ar−H), 7.25−7.30 (m, 2H, Ar−H), 6.74 (s, 1H, H_1'), 6.00 (d, 1H, *J* = 4.8 Hz), 4.95−4.96 (m, 2H), 4.72−4.76 (m, 1H), 1.58 (s, 3H), 1.44 (s, 18H), 1.38 (s, 18H). ^{13}CNMR (100 MHz, CD_3Cl) δ 17.8, 27.7, 27.7, 63.6, 76.0, 81.2, 83.3, 83.9, 84.2, 89.2, 127.7, 128.4, 128.5, 128.6, 128.7, 129.3, 129.4, 129.7, 129.75, 130.0, 133.4, 133.6, 133.7, 143.7, 150.0, 150.6, 151.4, 152.4, 153.3, 165.1, 165.2, 166.3.

Di-*tert*-butyl (9-((2*R*,3*R*,4*R*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)-3-methyltetrahydrofuran-2-yl)-9*H*-purine-2,6-diyl)bis(*tert*-butoxycarbonylcarbamate), 6. To a solution of **5** (1.7 g, 1.7 mmol) in anhydrous methanol (50 mL) was added a solution of sodium methoxide (4.4 M, 0.3 mL, 1.3 mmol) at rt for 30 min (monitored by TLC and LC−MS). After the reaction was complete, Dowex resin (H^+ form) was added portionwise to adjust the pH to 7.0. The resin was filtered and washed with methanol, the filtrate was concentrated, and the residue was purified by flash column chromatography (0−10% MeOH in CH_2Cl_2) to afford 1.1 g of white solid **6** (92% yield). ^{1}H NMR (400 MHz, CD_3OD): 9.09 (s, 1H), 6.19 (s, 1H), 4.22 (d, 1H, *J* = 8.8 Hz), 4.03−4.11 (m, 2H), 3.89 (dd, 1H, *J* = 2.8 Hz, *J* = 12.4 Hz), 1.41 (s, 18H), 1.40 (s, 18H), 0.92 (s, 3H). ^{13}C NMR (CD_3OD): 20.2, 27.9, 28.1, 60.9, 73.1, 80.2, 84.6, 84.9, 85.4, 93.3, 128.8, 147.0, 151.2, 151.9, 152.0, 152.9, 155.0. LC/MS calcd for $C_{31}H_{49}N_6O_{12}$ $(M + 1)^+$, 697.3; observed, 697.4.

(2*R*,3*R*,4*R*,5*R*)-2-(2,6-Diamino-9*H*-purin-9-yl)-5-(hydroxymethyl)-3-methyltetrahydrofuran-3,4-diol, 7. Nucleoside **4** (1.1 g, 1.7 mmol) was treated with saturated ammonia/methanol (100 mL). The reaction mixture was stirred at rt for 36 h to completion. After removal of the solvent under reduced pressure, 150 mL of dichloromethane was added to the residue. The white solid **7** was collected and dried (500 mg, 93%). ^{1}H NMR (400 MHz, DMSO-d_6): 8.01 (s, 1H), 6.73 (s, 2H), 5.81 (s, 2H), 5.76 (d, *J* = 2.8 Hz, 1H), 5.18−5.22 (t, *J* = 6.8 Hz), 5.05 (s, 1H), 0.78 (s, 3H), 3.98−4.02 (m, 1H), 3.78−3.84 (m, 2H), 3.63−3.67 (m, 1H). LC/MS calcd for $C_{11}H_{17}N_6O_2$ $(M + 1)^+$, 297.1; observed, 297.2.

Ethyl 3-(2-Hydroxyphenyl)propanoate, 9a. Dihydrocoumarin (52 g, 350 mmol) was added to 300 mL of anhydrous ethanol. H_2SO_4 (0.5 mL) was added, and the resulting solution was heated overnight at reflux. To the solution was added Ambersep 900 OH resin with stirring until approximately pH 7.0 was reached, and then the solution was filtered. The filtrate was evaporated under reduced pressure, and the residue was dissolved in ether acetate (300 mL), washed with water (3 × 50 mL), brine (50 mL), and dried over $MgSO_4$. The solution was filtered, and the filtrate was evaporated under reduced pressure. The residue was dried to give 64 g of solid product **9a** (94% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (s, 1H), 7.05−7.15 (m, 2H), 6.84−6.90 (m, 2H), 4.14 (q, *J* = 6.8 Hz, 2H), 2.90 (m, 2H), 2.72 (m, 2H), 1.23 (t, *J* = 6.8 Hz, 3H). LC/MS calcd for $C_{11}H_{15}O_3$ $(M + 1)^+$, 195.1; observed, 195.2.

Isopropyl 3-(2-Hydroxyphenyl)propanoate, 9b. Same procedure was employed for the preparation of compound **9b** in 89% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 7.47 (brs, 1H), 7.09−7.27 (m, 2H, Ar−H), 6.87−6.91 (m, 2H, Ar−H), 5.00−5.06 (m, 1H), 2.90−2.93 (m, 2H), 2.68−2.72 (m, 2H), 1.22 (d, 6H, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3Cl) δ 21.7, 24.7, 35.5, 68.9, 117.1, 120.6, 127.4, 127.9, 130.5, 154.4, 175.2. LCMS calcd for $C_{12}H_{17}O_3$ $(M + 1)^+$, 209.1; observed, 209.2.

Ethyl 3-(2-(((Chloro(((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)-phosphoryl)oxy)phenyl)propanoate, 12b. To a solution of phosphorus oxychloride (2.4 mL, 26 mmol) in 70 mL of anhydrous

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J. Med. Chem. XXXX, XXX, XXX−XXX

diethyl ether was added dropwise a solution of **9a** (5.0 g, 26 mmol) and triethylamine (3.6 mL, 26 mmol) in 80 mL of anhydrous diethyl ether at −78 °C under an Ar atmosphere over 2 h. After stirring for 1 h at −78 °C under Ar atmosphere, the solution was additionally stirred for 15 h toward rt, and then the solids were removed by filtration under a N_2 atmosphere. The solid salt was washed with anhydrous diethyl ether. The filtrate was concentrated under reduced pressure and dried under high vacuum overnight at rt. To a mixture of the oil **10b** and dried L-alanine ethyl ester hydrogen chloride **11** (3.9 g, 26 mmol) in 20 mL of anhydrous CH_2Cl_2 was added a solution of Et_3N (7 mL, 51 mmol) in 20 mL of anhydrous CH_2Cl_2 over 2 h at −78 °C under Ar atmosphere. The solution was stirred for 16 h at rt, and the solid was filtered. The filtrate was concentrated under reduced pressure and purified on a silica gel column (EtOAc/hexane; gradient 0−50%, v/v) to give 9.5 g of compound **12b** in 75% yield in two steps. ^{1}H NMR (400 MHz, $CDCl_3$) (1:1 mixture of P diastereomers): δ 7.14−7.49 (m, 4H), 4.70−4.80 (m, 1H), 4.09−4.27 (m, 5H), 2.92−3.08 (m, 2H), 2.61−2.65 (m, 2H), 1.50−1.55 (m, 3H), 1.21−1.32 (m, 6H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 8.88, 8.72.

The same procedure was employed for the preparation of compounds **12a**, **12c**, **12d**, and **12e** in 72%, 70%, 69% and 70% yields, respectively.

12a. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.17−7.40 (m, 1H, 5H), 4.10−4.50 (m, 4h), 1.50−1.53 (m, 3H), 1.28−1.33 (m, 3H). ^{31}PNMR (162 MHz, $CDCl_3$): 8.13, 9.15.

12c:.^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43−7.48 (m, 1H), 7.13−7.26 (m, 3H), 4.97−5.12 (m, 2H), 4.50−4.53 (m, 1H), 4.08−4.23 (m, 1H), 2.93−3.02 (m, 2H), 2.58−2.62 (m, 2H), 1.18−1.29 (m, 12H). ^{31}PNMR (162 MHz, $CDCl_3$): 8.64, 8.87.

12d. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44−7.48 (m, 1H), 7.14−7.26 (m, 3H), 5.04−5.12 (m, 1H), 4.54−4.57 (m, 1H), 4.09−4.56 (m, 3H), 2.95−3.04 (m, 2H), 2.61−2.64 (m, 2H), 1.48−1.52 (m, 3H), 1.20−1.29 (m, 9H). ^{31}PNMR (162 MHz, $CDCl_3$): 7.35, 7.57.

12e. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44−7.48 (m, 1H), 7.16−7.26 (m, 3H), 4.97−5.03 (m, 1H), 4.47−4.53 (m, 1H), 4.16−4.28 (m, 3H), 2.94−3.04 (m, 2H), 2.58−2.62 (m, 2H), 1.50−1.55 (m, 3H), 1.29−1.33 (m, 3H), 1.17−1.22 (m, 6H). ^{31}PNMR (162 MHz, $CDCl_3$): 7.84, 8.08.

Diethyl 3,3′-(((Chlorophosphoryl)bis(oxy))bis(2,1-phenylene))dipropanoate, 13. To a solution of phosphorus oxychloride (0.47 mL, 5.0 mmol) in 30 mL of anhydrous diethyl ether were added dropwise a solution of **9a** (1.9 g, 10 mmol) and triethylamine (1.4 mL, 10 mmol) in 50 mL of anhydrous diethyl ether at −78 °C under an Ar atmosphere in 1.5 h. After stirring for 1 h at −78 °C under Ar atmosphere, the solution was additionally stirred for 15 h toward rt and then the solid was removed by filtration under a N_2 atmosphere. The solid salt was washed anhydrous diethyl ether. The filtrate was concentrated under reduced pressure and purified on a silica gel column (EtOAc/hexane; gradient 0−25%, v/v) to give 815 mg of compound **13** as a colorless oil in 35% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44−7.47 (m, 2H), 7.18−7.31 (m, 6H), 4.09−4.14 (q, J = 12.0 Hz, J = 8.0 Hz, 4H), 3.00 (t, J = 6.0 Hz, 4H), 2.60 (t, J = 8.0 Hz, 4H), 1.22 (t, J = 8.0 Hz, 6H). ^{31}P NMR (162 MHz, $CDCl_3$) δ −5.85.

(2*S*)-Ethyl 2-((((2*R*,3*R*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate, 15a. To a solution of **6** (780 mg, 1.1 mmol) and *N*-methylimidazole (0.45 mL, 5.8 mmol) in THF (5 mL) at 0 °C was added dropwise a 1 M solution of (2*R*)-ethyl 2-(chloro(phenoxy)phosphorylamino)propanoate[1] (5.8 mL, 5.8 mmol). The resulting mixture was stirred overnight at rt. After solvent removal under reduced pressure, the residue was purified by flash column chromatography with a gradient of MeOH (0−10% MeOH in CH_2Cl_2) to afford 580 mg of white solid **14a** (54% yield). A precold solution of TFA (80%, 23 mL) was added to a precold **14a** (550 mg, 0.58 mmol) in an ice bath. The solution was stirred from 0 °C to rt and stirred at rt for 4 h (monitored by TLC and LC/MS). After the reaction was completed, the solvent was removed under reduced pressure and the residue was coevaporated with methanol (4 × 15 mL). The residue was dissolved in methanol (20 mL) and neutralized by saturated $NaHCO_3$. After solvent removal, the residue was purified by flash column chromatography with a gradient of MeOH (0−15% MeOH in CH_2Cl_2) to afford 225 mg of white solid **15a** (71%) (38% yield over two steps). ^{1}H NMR (400 MHz, CD_3OD) (1:1 mixture): 7.86 (s, 2H), 7.14−7.34 (m, 10H), 5.96 (s, 1H), 5.93 (s, 1H), 3.90−4.58 (m, 14H), 1.16−1.31 (m, 6H), 1.13−1.19 (m, 6H), 0.97 (s, 3H), 0.94 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD): 4.77, 4.89. LC/MS calcd for $C_{22}H_{31}N_7O_8P$ $(M + 1)^+$, 552.2; observed, 552.3.

Ethyl 3-(2-(((*S*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)-propanoate, 15b(S_p), and Ethyl 3-(2-(((*R*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)propanoate 15b(R_p). A procedure similar to that used for **15a** was employed for the synthesis of prodrug **15b** (56% over two steps). Two diasteroisomers were separated and identified.

15b(S_p). Optical rotation $[\alpha]^{24}_D$ −7.08 (*c* 0.24, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 7.86 (s, 1H, H8), 7.10−7.39 (m, 4H, Ar−H), 5.93 (s, 1H, H1′), 3.96−4.51 (m, 9H), 2.99 (t, 2H, J = 8.0 Hz, CH_2), 2.62 (t, 2H, J = 8.0 Hz, CH_2), 1.34 (d, 3H, J = 7.2 Hz, CH_3), 1.15−1.17 (m, 6H, 2 × CH_3), 0.97 (s, 3H, CH_3). ^{31}PNMR (162 MHz, CD_3OD) δ 5.03; LRMS calcd for $C_{27}H_{39}N_7O_{10}P$ $(M + 1)^+$ 652.25, found 652.32.

15b(R_p). Optical rotation $[\alpha]^{24}_D$ +12.12 (*c* 0.13, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.38 (d, J = 8.4 Hz, 1H), 7.26 (d, J = 7.6 Hz, 1H), 7.18 (dt, J = 8.0 Hz, J = 1.6 Hz, 1H), 7.12 (t, J = 7.6 Hz, 1H), 5.91 (s, 1H), 4.51−4.48 (m, 2H), 4.25−3.95 (m, 7H), 2.99 (t, J = 8.0 Hz, 2H), 2.62 (t, J = 8.0 Hz, 2H), 1.34 (d, J = 6.8 Hz, 3H), 1.18 (t, J = 7.2 Hz, 3H), 1.16 (t, J = 7.2 Hz, 3H), 0.97 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 4.98. LCMS calcd for $C_{27}H_{39}N_7O_{10}P$ $(M + 1)^+$ 652.3, found 652.3.

Isopropyl 3-(2-(((*S*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)-(((*S*)-1-isopropoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)-phenyl)propanoate 15c(S_p) and Isopropyl 3-(2-(((*R*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(((*S*)-1-isopropoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)propanoate 15c(R_p). A procedure similar to that used for **15a** was employed for the synthesis of prodrug **15c** (40% over two steps). Two diastereoisomers were separated and identified.

15c(S_p). ^{1}H NMR (400 MHz, CD_3OD) δ 7.86 (s, 1H), 7.37 (d, J = 8.4 Hz, 1H), 7.25 (d, J = 7.2 Hz, 1H), 7.17 (dt, J = 8.0 Hz, J = 1.6 Hz, 1H), 7.08 (t, J = 7.2 Hz, 1H), 5.94 (s, 1H), 5.05−4.84 (m, 2H), 4.62−4.46 (m, 2H), 4.22 (s, 2H), 3.95−3.91 (m, 1H), 2.99 (t, J = 8.0 Hz, 2H), 2.60 (t, J = 8.0 Hz, 2H), 1.33 (d, J = 7.2 Hz, 3H), 1.20 (d, J = 6.4 Hz, 3H), 1.19 (d, J = 6.0 Hz, 3H), 1.15 (d, J = 6.0 Hz, 3H), 1.14 (t, J = 6.4 Hz, 3H), 0.96 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 5.08. LRMS calcd for $C_{29}H_{43}N_7O_{10}P$ $(M + 1)^+$ 680.28, found 680.12.

15c(R_p). ^{1}H NMR (400 MHz, CD_3OD) δ 8.03 (s, 1H), 7.38 (d, J = 8.0 Hz, 1H), 7.26 (d, J = 7.6 Hz, 1H), 7.18 (dt, J = 7.6 Hz, J = 1.6 Hz, 1H), 7.10 (t, J = 7.2 Hz, 1H), 5.91 (s, 1H), 4.95−4.79 (m, 2H), 4.53−4.46 (m, 2H), 4.18 (s, 2H), 3.95−3.91 (m, 1H), 2.99 (t, J = 8.0 Hz, 2H), 2.61 (t, J = 8.0 Hz, 2H), 1.35 (d, J = 7.2 Hz, 3H), 1.19−1.16 (m, 12H), 0.99 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 5.02. LRMS calcd for $C_{29}H_{43}N_7O_{10}P$ $(M + 1)^+$ 680.28, found 680.14.

Ethyl 3-(2-(((*S*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(((*S*)-1-isopropoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)-phenyl)propanoate, 15d(S_p), and Ethyl 3-(2-(((*R*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(((*S*)-1-isopropoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)propanoate, 15d(R_p). A procedure similar to that used for **15a** was employed for the synthesis of prodrug **15d** (41% over two steps). Two diasteroisomers were separated and identified.

15d(S_p). ^{1}H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.36 (d, J = 8.4 Hz, 1H), 7.25 (d, J = 7.2 Hz, 1H), 7.16 (dt, J = 8.0 Hz, J = 1.6 Hz, 1H), 7.08 (t, J = 7.2 Hz, 1H), 5.94 (s, 1H), 5.02−4.84 (m, 1H), 4.62−4.49 (m, 2H), 4.21 (s, 2H), 4.07−3.91 (m, 3H), 2.99 (t, J = 8.0 Hz,

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J. Med. Chem. XXXX, XXX, XXX−XXX

2H), 2.62 (t, J = 8.0 Hz, 2H), 1.34−1.14 (m, 12H), 0.96 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 5.04. LRMS calcd for $C_{28}H_{41}N_7O_{10}P$ (M + 1)$^+$ 666.27, found 666.10.

15d(R_p). ^{1}H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.38 (d, J = 8.0 Hz, 1H), 7.25 (d, J = 7.6 Hz, 1H), 7.18 (dt, J = 8.0 Hz, J = 2.0 Hz, 1H), 7.12 (t, J = 7.2 Hz, 1H), 5.91 (s, 1H), 4.62−4.48 (m, 4H), 4.25−3.91 (m, 4H), 2.99 (t, J = 7.6 Hz, 2H), 2.62 (t, J = 8.0 Hz, 2H), 1.33 (d, J = 7.2 Hz, 3H), 1.18 (t, J = 7.2 Hz, 3H), 1.17 (d, J = 6.0 Hz, 3H), 1.14 (d, J = 6.4 Hz, 3H), 0.97 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 4.97. LRMS calcd for $C_{28}H_{41}N_7O_{10}P$ (M + 1)$^+$ 666.27, found 666.08.

Ethyl ((*S*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(2-(3-isopropoxy-3-oxopropyl)phenoxy)phosphoryl)-L-alaninate, 15e(S_p), and Ethyl ((*R*)-(((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(2-(3-isopropoxy-3-oxopropyl)phenoxy)phosphoryl)-L-alaninate, 15e(R_p). A procedure similar to that used for **15a** was employed for the synthesis of prodrug **15e** (41% over two steps). Two diasteroisomers were separated and identified.

15e(S_p). ^{1}H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.25 (d, J = 7.2 Hz, 1H), 7.17 (dt, J = 8.0 Hz, J = 2.0 Hz, 1H), 7.08 (t, J = 7.2 Hz, 1H), 5.94 (s, 1H), 4.93−4.84 (m, 1H), 4.61−4.49 (m, 2H), 4.22 (s, 2H), 4.18−3.91 (m, 3H), 2.99 (t, J = 8.0 Hz, 2H), 2.60 (t, J = 8.0 Hz, 2H), 1.34 (d, J = 6.4 Hz, 3H), 1.20 (t, J = 7.2 Hz, 3H), 1.15 (d, J = 6.0 Hz, 3H), 1.14 (d, J = 6.0 Hz, 3H), 0.97 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 5.02; LRMS calcd for $C_{28}H_{41}N_7O_{10}P$ (M + 1)$^+$ 666.27, found 666.19.

15e(R_p). ^{1}H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.38 (d, J = 8.4 Hz, 1H), 7.25 (d, J = 7.6 Hz, 1H), 7.18 (dt, J = 8.0 Hz, J = 2.0 Hz, 1H), 7.10 (t, J = 7.2 Hz, 1H), 5.91 (s, 1H), 4.93−4.84 (m, 1H), 4.51−4.48 (m, 2H), 4.25−3.95 (m, 5H), 2.98 (t, J = 8.0 Hz, 2H), 2.60 (t, J = 8.0 Hz, 2H), 1.34 (d, J = 7.2 Hz, 3H), 1.18−1.49 (m, 9H), 0.97 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 4.97. LRMS calcd for $C_{28}H_{41}N_7O_{10}P$ (M + 1)$^+$ 666.27, found 666.09.

Ethyl 3-(2-(((((3a*S*,4*R*,6*R*,6a*R*)-6-(2,6-Diamino-9*H*-purin-9-yl)-6a-methyl-2-oxotetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methoxy)(((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)propanoate, 16. To a solution of compound **15b** (110 mg, 0.17 mmol) in acetonitrile (10 mL) was added carbodiimidazole (60 mg, 0.37 mmol), and the reaction mixture was stirred at rt overnight. Water (0.2 mL) was added, and the reaction mixture was stirred for 0.5 h. The mixture was concentrated and portioned between ethyl acetate (100 mL) and water (15 mL). The organic layer was washed with brine, dried over Na_2SO_4, concentrated, and the residue was purified by silica gel column chromatography in a gradient of MeOH (0−10% MeOH in CH_2Cl_2) to afford white solid **16** (84 mg, 71%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.86 (s, 0.5H), 7.81 (s, 0.5H), 7.34−7.39 (m, 1H), 7.06−7.27 (m, 3H), 6.32 (s, 0.5H), 6.30 (s, 0.5H), 5.32−5.36 (m, 1H), 4.71−4.77 (m, 1H), 4.38−4.57 (2H), 3.97−4.16 (m, 5H), 2.93−3.00 (m, 2H), 2.57−2.65 (m, 2H), 1.32−1.39 (m, 6H), 1.13−1.23 (m, 6H). ^{31}P NMR (162 MHz, CD_3OD) δ 4.10, 3.89. LCMS calcd for $C_{28}H_{37}N_7O_{11}P$ (M + 1)$^+$ 678.2, found 678.3.

Ethyl 3-(2-(((((2*R*,3*S*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(2-(3-ethoxy-3-oxopropyl)phenoxy)phosphoryl)oxy)phenyl)propanoate, 17. To a solution of **6** (630 mg, 0.91 mmol) and *N*-methylimidazole (0.35 mL, 4.5 mmol) in THF (3 mL) at 0 °C was added dropwise a 1 M solution of diethyl 3,3′-(((chlorophosphoryl)bis(oxy))bis(2,1-phenylene))dipropanoate, **13** in THF (9 mL, 4.5 mmol). The resulting mixture was stirred overnight at rt. After solvent removal under reduced pressure, the residue was purified by flash column chromatography in a gradient of MeOH (0−10% MeOH in CH_2Cl_2) to afford 540 mg of white solid **17** (53% yield). A precold solution of TFA (80%, 26 mL) was added to a precold **17** (540 mg, 0.58 mmol) in an ice bath. The solution was stirred from 0 °C to rt and stirred at rt for 4 h (monitored by TLC and LC/MS). After the reaction was complete, the solvent was removed under reduced pressure and the residue was coevaporated with methanol (4 × 15 mL). The residue was dissolved in methanol (20 mL) and neutralized by saturated $NaHCO_3$. After solvent removal, the residue was purified by flash column chromatography in a gradient of MeOH (0−15% MeOH in CH_2Cl_2) to afford 270 mg of white solid **18** (77%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.80 (s, 1H), 7.09−7.32 (m, 8H), 5.90 (s, 1H), 4.72−4.93 (m, 1H), 4.26−4.38 (m, 2H), 4.02−4.08 (m, 4H), 2.86−2.92 (m, 4H), 2.46−2.54 (m, 4H), 1.16 (t, 6H), 1.00 (s, 3H). ^{31}P NMR (162 MHz, CD_3OD) δ 12.21. LCMS calcd for $C_{22}H_{31}N_7O_8P$ (M + 1)$^+$ 729.2; observed, 729.3.

Ethyl 3-(2-(((*R*)-(((*S*)-1-Ethoxy-1-oxopropan-2-yl)amino)(4-nitrophenoxy)phosphoryl)oxy)phenyl)propanoate, 19(R_p), and Ethyl 3-(2-(((*S*)-(((*S*)-1-Ethoxy-1-oxopropan-2-yl)amino)(4-nitrophenoxy)phosphoryl)oxy)phenyl)propanoate, 20(S_p). A solution of Et_3N in anhydrous diethyl ether (100 mL) was added dropwise to a solution of **12b** (10 g, 26 mmol) and *p*-nitrophenol (3.8 g, 27 mmol) in diethyl ether (200 mL) at 0 °C in 30 min. The reaction mixture was stirred at 0 °C for 1 h and at rt for 15 h. The solids were removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified on a silica gel column (EtOAc/CH_2Cl_2, EtOAc gradient 0−10%, v/v) to give 10.8 g of a mixture of **19** and **20** in 85% yield in 1:1 ratio. The mixture was recrystallized in 2% CH_3CN in diisopropyl ether. Pure **19** was obtained by seeding the recrystallization solution with pure **19** crystal seeds which were obtained by column chromatography. The resulting solution was stored for 18 h at 0 °C. Filtration of the resulting solid from the mother liquor gave 2.2 g of isomer **19**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22−8.24 (dd, J = 10 Hz, J = 2.0 Hz, 2H), 7.11−7.44 (m, 6H), 4.06−4.20 (m, 6H), 2.88−3.00 (m, 2H), 2.54−2.59 (m, 2H), 1.40 (d, J = 6.8 Hz, 3H), 1.25 (t, J = 7.2 Hz, 3H), 1.23 (t, J = 7.2 Hz, 3H). ^{31}P NMR (162 MHz, $CDCl_3$) δ −2.01. LC−MS, m/z 495 (M + 1)$^+$. A single crystal of **19** was obtained by crystallization in 2% CH_3CN in diisopropyl ether and an X-ray structure of **19** was obtained to unambiguously confirm the configuration of the phosphorus center as R_p (Figure 2).

The filtrate was concentrated under reduced pressure to dryness, and the mixture was stirred in diisopropyl ether (200 mL) with pure **20** seeds. An amount of 510 mg of pure **20** was collected. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22−8.24 (dd, J = 10 Hz, 2H), 7.11−7.43 (m, 6H), 4.00−4.18 (m, 6H), 2.93−2.98 (m, 2H), 2.55−2.60 (m, 2H), 1.43 (d, J = 7.2 Hz, 3H), 1.24 (t, J = 7.2 Hz, 3H), 1.24 (t, J = 7.2 Hz, 3H). ^{31}P NMR (162 MHz, $CDCl_3$) δ −2.07. LC−MS, m/z 495 (M + 1)$^+$. A single crystal of **20** was obtained by crystallization in 2% CH_3CN in diisopropyl ether and an X-ray structure of **20** was obtained to unambiguously confirm the configuration of the phosphorus center as S_p (Figure 2).

Ethyl 3-(2-(((*R*)-(((2*R*,3*R*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)-propanoate, 15b(R_p). To a solution of **6** (100 mg, 0.14 mmol) in THF (0.5 mL) was added 0.5 mL of *t*-BuMgCl solution (1 M, 0.5 mmol) at −78 °C under Ar atmosphere. The reaction mixture was stirred for 30 min at this temperature and then warmed to rt. A solution of **20** (210 mg, 0.42 mmol) in 1 mL of anhydrous THF was added. The reaction mixture was stirred at rt for 3 days under Ar atmosphere to completion. The solvent was evaporated under reduced pressure, and to the residue was added a precooled 80% TFA solution (10 mL) at 0 °C. The reaction mixture was stirred for an additional 4 h toward rt to completion. After evaporation of the solvents under reduced pressure, to the residue was added a small amount of saturated solution of aqueous $NaHCO_3$ to adjust to pH 7.0. The mixture was concentrated under reduced pressure and then purified by a silica gel column (MeOH/DCM, MeOH gradient 0−10%, v/v) to afford 37.5 mg of **15b**(R_p) in 41% yield over two steps. Optical rotation $[\alpha]^{24}_D$ −7.08 (c 0.24, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 0.97 (s, 3H, CH_3), 1.15−1.20 (m, 6H, 2 x CH_3), 1.34 (d, 3H, J = 7.2 Hz, CH_3), 2.62 (t, 2H, J = 8.0 Hz, 2H, CH_2), 2.99 (t, 2H, J = 8.0 Hz, 2H, CH_2), 3.95−4.58 (m, 9H), 5.94 (s, 1H, H_1'), 7.07−7.38 (m, 4H, Ar−H), 7.86 (s, 1H, H_8). ^{13}C NMR (100 MHz, CD_3OD) δ 14.5, 14.6, 20.4, 20.6, 26.8, 35.4, 51.7, 61.7, 62.5, 67.0, 74.4, 80.1, 81.9, 92.8, 114.4, 121.0, 126.2, 128.8, 131.8, 133.2, 137.5, 150.6, 152.7, 157.7, 162.0, 174.8, 175.1. ^{31}P NMR (162 MHz, CD_3OD): 5.03. LC/MS calcd for $C_{27}H_{39}N_7O_{10}P$ (M + 1)$^+$, 652.2; observed, 552.2.

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J. Med. Chem. XXXX, XXX, XXX−XXX

Ethyl 3-(2-(((*S*)-(((2*R*,3*R*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)-propanoate 15b(S_P). A procedure similar to that used for **15a** was employed for the preparation of **15b**(S_P) in 39% yield. Optical rotation $[\alpha]^{24}_D$ +12.12 (*c* 0.13, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 0.97 (s, 3H, CH_3), 1.15−1.17 (m, 6H, 2 x CH_3), 1.34 (d, 3H, *J* = 7.2 Hz, CH_3), 2.62 (t, 2H, *J* = 8.0 Hz, 2H, CH_2), 2.99 (t, 2H, *J* = 8.0 Hz, 2H, CH_2), 3.96−4.51 (m, 9H), 5.93 (s, 1H, H_1'), 7.10−7.39 (m, 4H, Ar−H), 7.86 (s, 1H, H_8). ^{13}C NMR (100 MHz, CD_3OD) δ 14.5, 14.6, 20.4, 20.8, 26.8, 35.4, 51.6, 61.6, 62.4, 67.7, 74.7, 80.0, 82.1, 93.0, 114.4, 121.1, 126.2, 128.8, 131.7, 133.1, 137.7, 150.5, 152.6, 157.6, 161.9, 174.7, 174.8. ^{31}P NMR (162 MHz, CD_3OD): 4.98. LC/MS calcd for $C_{27}H_{39}N_7O_{10}P$ $(M + 1)^+$, 652.2; observed, 552.3.

((2*R*,3*R*,4*R*,5*R*)-5-(2,6-Diamino-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methyl Tetrahydrogen Triphosphate Tetratriethylammonium Salt (DAPN-TP) and ((2*R*,3*R*,4*R*,5*R*)-5-(2-Amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl)methyl Tetrahydrogen Triphosphate Tetratriethylammonium Salt (2′-Me-GTP). A mixture of 2,6-diaminopurine ribonucleoside **7** (14 mg, 1 equiv), trimethyl phosphate (0.5 mL), and molecular sieves were stirred at rt overnight. 2,4,6-Collidine (9 μL, 1.5 equiv) was added and the mixture stirred for 10 min before phosphorus oxychloride (9 μL, 2 equiv) was added. The reaction solution was stirred for 1 h at rt. A solution of tributylammonium pyrophosphate (0.26 mL of 1 M solution in DMF, 5.5 equiv) and tributylamine (45 μL, 4 equiv) were added simultaneously. The mixture was stirred for 30 min at rt. The reaction was then quenched by addition of 2 mL of triethylammonium bicarbonate buffer (0.25M) and stirred for 45 min. After extraction with dichloromethane (2 × 3 mL), the aqueous phase was concentrated under reduced pressure. The crude was subjected to HPLC purification using ion-exchange DNAPac PA200 (9 mm × 250 mm) column (Dionex), with water and triethylammnoium bicarbonate (0.5M) as eluent. LC/MS calcd for $C_{11}H_{18}N_6O_{13}P_3$ $(M - 1)^+$, 535.0; observed, 535.0.

The same procedure was applied for 2′-C-Me-GTP, using 2′-C-Me guanosine (20 mg, 1 equiv), trimethylphopsphate (0.65 mL), 2,4,6-collidine (14 μL, 1.5 equiv), phosphorus oxychloride (14 μL, 2.1 equiv), tributylamine (63 μL, 4 equiv), tributylammonium pyrophosphate (0.37 mL of 1 M solution in DMF, 5.5 equiv), triethylammonium bicarbonate buffer (2.5 mL, 0.25 M). LC/MS calcd for $C_{11}H_{17}N_5O_{14}P_3$ $(M - 1)^+$, 536.0; observed, 535.9.

X-ray Crystallography of Diastereomers. Results from X-ray structure determination of compound **19** are the following. Crystal data for $C_{22}H_{27}N_2O_9P$ (M = 494.43): 0.29 × 0.11 × 0.09, triclinic, space group *P*1, a = 5.0028(3) Å, b = 8.9645(7) Å, c = 14.5700(14) Å, β = 90.753(7)°, V = 603.56(8) Å^3, Z = 1, μ(Cu Kα) = 1.484 mm^{-1}, D_{calc} = 1.360 g/mm^3, temperature 173 K. Intensity data were collected on a Bruker APEX II CCD diffractometer with monochromated Cu Kα radiation (λ = 1.541 78 Å) at 173 K in the 2θ range 3.18−64.34°. The user interface Olex2 was used for the crystallographic calculations and crystal structure visualization.[41] The structure was solved with Superflip by charge flipping and refined by least-squares minimization using SHELXL.[42,43] A total of 3668 reflections were measured ($3.18 \leq 2\theta \leq 64.34$), while 2214 unique data (R_{int} = 0.0273) were used in the refinements. The final R_1 was 0.0475 ($I > 2\sigma(I)$) and the weighted R value wR2 was 0.1255 (all data). All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The maximum and the minimum peak on the final difference Fourier map corresponded to 0.46 and −0.27 e/Å^3, respectively.

Results from the X-ray structure determination of compound **20** are the following. Crystal data for $C_{22}H_{27}N_2O_9P$ (M = 494.43): 0.28 × 0.26 × 0.11, monoclinic, space group *P*2(1), a = 4.87400(10) Å, b = 28.8315(6) Å, c = 8.9118(2) Å, β = 104.3380(10)°, V = 1213.32(4) Å^3, Z = 2, μ(Cu Kα) = 1.477 mm^{-1}, D_{calc} = 1.353 g/mm^3, temperature 173 K. Intensity data were collected on a Bruker APEX II CCD diffractometer with monochromated Cu Kα radiation (λ = 1.541 78 Å) at 173 K in the 2θ range 5.12−69.48°. The user interface Olex2 was used for the crystallographic calculations and crystal structure visualization.[41] The structure was solved with Superflip by charge flipping and refined by least-squares minimization using SHELXL.[42,43] A total of 8394 reflections were measured ($5.12 \leq 2\theta \leq 69.48$), while 3469 unique data (R_{int} = 0.0159) were used in the refinements. The final R_1 was 0.0267 ($I > 2\sigma(I)$) and the weighted R value wR2 was 0.0728 (all data). All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The maximum and the minimum peak on the final difference Fourier map corresponded to 0.239 and −0.255 e/Å^3, respectively.

NS5B-Mediated RNA Polymerization Assay. C-terminal His-tagged NS5BΔ21 enzyme was purified as previously described (Powdrill et al., 2010). 1 μM NS5BΔ21 was incubated at 30 °C with synthetic 1 μM 20-mer RNA templates (IDT) and 1 μM ^{32}P-radiolabeled GpG primer (Trilink) in a buffer containing 40 mM Tris, pH 7.5, 6 mM NaCl, and 2 mM $MgCl_2$. Reactions were initiated with the addition of 10 μM NTP mix, 1 μM of competing NTP, and varying concentrations of inhibitor ranging from 0 to 100 μM. Reactions were allowed to proceed for 120 min and subsequently stopped with the addition of 10 mM EDTA and formamide. Samples were visualized on 20% denaturing polyacrylamide gel and quantified using QuantityOne software. IC_{50} values were calculated using KaleidaGraph software.

Intracellular Metabolism. Huh-7 cells and fresh plated human primary hepatocytes (BioreclamationIVT, Baltimore, MD) were seeded at 1 × 10^6 per well in 12-well plates. After attachment (Huh-7 cells) or acclimatation overnight (hepatocytes), cells were exposed to 50 μM compounds, respectively. At 4 h, medium was removed from the cell layers and cells were washed twice with ice-cold phosphate buffered saline (PBS) to remove any residual medium. Cells were resuspended in 70% methanol containing 20 nM ddATP overnight at −20 °C. The supernatants were dried under a flow of air and dried samples stored at −20 °C until LC−MS/MS analysis.

Mitochondrial Toxicity Assa. Human hepatocarcinoma cells (HepG2) were propagated at 37 °C and 5% CO_2 atmosphere in Dulbecco's modified Eagle medium. Media contained 4.5 g/L D-glucose and were supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. All cell reagents were from Invitrogen Corporation (Carisbad, CA, USA), Cellgro/Mediatech, Inc. (Manassas, VA, USA), and Hyclone (Logan, UT, USA). Low-passage-number HepG2 cells were seeded at 5000 cells/well onto collagen-coated 24-well tissue culture plates. After 1 day of cell seeding, test compounds were added to the medium to obtain final concentrations varying from 1 to 50 μM. Media and compounds were replenished every 3−4 days until the end of 14 days. All samples were performed in replicates of two in two independent experiments. On culture day 14, cells and cell culture supernatants were harvested. Total nucleic acid was isolated from cells by using commercially available kits (MagNA Pure LC DNA Isolation Kit, Roche). The mitochondrial cytochrome *c* oxidase subunit II (COXII) gene and the rRNA gene were amplified in parallel by a real-time PCR protocol that uses suitable primers and probes for both target and reference amplifications (Stuyver et al., 2002).[44] Real-time PCR was performed by using a TaqMan 9700 HT sequence detection system (Applied Biosystems, Foster City, CA, USA). The primers and probes for the rRNA gene were purchased from Applied Biosystems. The amount of target (mtDNA) was normalized to the amount of an endogenous control (nuclear DNA) and was relative to the untreated control. The 50% inhibitory concentration (IC_{50}) of test compounds was determined by using CalcuSyn program (Biosoft, Cambridge, U.K.).

Lactate Production Assay. Following a 14-day incubation with compounds at various concentrations, lactic acid quantification from cultured supernatant was performed in a 96-well plate using the D-lactic acid/L-lactic acid test kit (Boehringer Mannheim, Indianapolis, IN; USA/R-Biopharam, South Marshall, MI, USA/Roche). Extracellular levels of lactic acid were measured using a Multiscan Spectrum (Thermo Electron Corporation). The total amount of lactic acid produced and the fold change in lactic acid production (% of lactic acid/% of nuclear DNA) were determined for each sample.

HCV Replicon Assay. Huh-7 clone B cells containing HCV replicon RNA were seeded in a 96-well plate at 3000 cells/well, and

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J. Med. Chem. XXXX, XXX, XXX−XXX

the compounds were added in dose response in triplicate immediately after seeding.[45] Following 5 days of incubation (37 °C, 5% CO_2), total cellular RNA was isolated by using the RNeasy 96-well extraction kit from Qiagen. Replicon RNA and an internal control (TaqMan rRNA control reagents, Applied Biosystems) were amplified in a single step multiplex real time RT-PCR assay. The antiviral effectiveness of the compounds was calculated by subtracting the threshold RT-PCR cycle of the test compound from the threshold RT-PCR cycle of the no-drug control (ΔCt HCV). A ΔCt of 3.3 equals a 1 log reduction (equal to 90% less starting material) in replicon RNA levels. The cytotoxicity of the compounds was also calculated by using the ΔCt rRNA values. 2′-*C*-Me cytidine was used as the positive control. To determine EC_{90} and CC_{50} values, ΔCt values were first converted into fraction of starting material and then were used to calculate the % inhibition.

Toxicity Assays. For detailed descriptions of cytotoxicity assays, see ref 33.

AUTHOR INFORMATION

Corresponding Author

*Telephone: 404-727-1414. E-mail: rschina@emory.edu.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DAPN, β-D-2,6-diaminopurine nucleoside; PD, prodrug; NTP, nucleoside triphosphate; PI, protease inhibitor; DAA, direct acting agent; RdRp, RNA-dependent RNA polymerase; NMPD, nucleoside monophosphate prodrug; SVR, sustained virologic response; PBM, peripheral blood mononuclear; SD, standard deviation; mtDNA, mitochondrial DNA; ND, not determined; NA, not applicable; GT, genotype; NI, nucleoside inhibitor; DBU, 1,8-diazabicycloundec-7-ene; TMSOTf, trimethylsilyl trifluoromethanesulfonate; $(Boc)_2O$, di-*tert*-butyl dicarbonate; DMAP, 4-dimethylaminopyridine; DCM, dichloromethane; THF, tetrahydrofuran

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