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Synthesis and evaluation of 2′-dihalo ribonucleotide prodrugs with activity against hepatitis C virus

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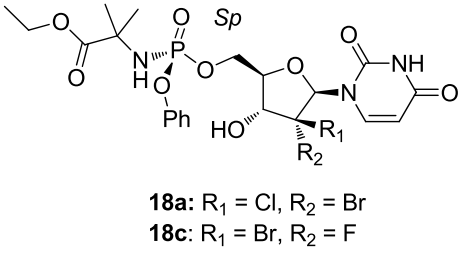
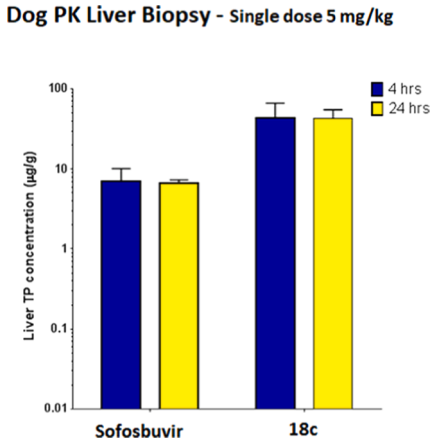
ABSTRACT



18a: R_1 = Cl, R_2 = Br
18c: R_1 = Br, R_2 = F



Hepatitis C virus (HCV) nucleoside inhibitors have been a key focus of nearly 2 decades of HCV drug research due to a high barrier to drug resistance and pan-genotypic activity profile provided by molecules in this drug class. Our investigations focused on several potent 2′-halogenated uridine-based HCV polymerase inhibitors, resulting in the discovery of novel 2′-deoxy-2′-dihalo-uridine analogs that are potent inhibitors in replicon assays for all genotypes. Further studies to improve in vivo performance of these nucleoside inhibitors identified aminoisobutyric acid ethyl ester (AIBEE) phosphoramidate prodrugs **18a** and **18c**, which provide high levels of the active triphosphate in dog liver. AIBEE prodrug **18c** was compared with sofosbuvir (**1**) by co-dosing both compounds by oral administration in dog (5 mg/kg each) and measuring liver concentrations of the active triphosphate metabolite at both 4 and 24 h post dosing. In this study, **18c** provided liver triphosphate concentrations that were 6-fold higher than sofosbuvir (**1**) at both biopsy time points, suggesting that **18c** could be a highly effective agent for treating HCV infected patients in the clinic.

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1. Introduction

Hepatitis C virus (HCV) infection continues to be a global health problem, with current estimates ranging from 71 to 80 million chronically infected individuals at risk of serious and potentially life-threatening liver disease.[1] Of these chronically infected people, 10–20% will develop cirrhosis, a very serious condition that greatly increases the odds of developing hepatocellular carcinoma. In the United States the most recent statistics show that HCV related mortality is on the rise with close to 20,000 deaths listing HCV as the cause in 2014.[2] HCV also exhibits an extraordinarily high degree of genetic diversity, substantially greater than that of the HIV-1 pandemic. Currently there exist six different major HCV genotypes (1–6) found throughout the world. HCV genotype 1, predominant in North America, Europe, and Japan, accounts for over half of the global infections. Genotype 2 infections are most prevalent in North America, Europe, and Japan, while genotypes 3 and 6 infections are predominant within various parts of Southeast Asia. In Egypt, HCV infections are almost exclusively genotype 4, while genotype 5 is common in South Africa.[3]

In the last decade treatment of HCV has been revolutionized with the approval of direct acting antivirals (DAAs), which provide high functional cure rates. This functional cure is considered to be achieved when virus RNA levels in blood remain undetectable for 12 weeks after completion of treatment, commonly noted as a sustained virologic response at 12 weeks (SVR_{12}). Advances in clinical research have provided three different drug combination therapies that have been approved for HCV infections encompassing genotypes 1–6 with very high functional cure rates.[4,5]

Clinical studies have investigated over 3 dozen HCV drug candidates that act on multiple viral drug target proteins. One of these proteins is the HCV RNA-dependent RNA polymerase, NS5B. Nucleoside polymerase inhibitors have been of particular interest, with over a dozen different compounds entering clinical studies. Nucleoside drug candidates are attractive NS5B inhibitors because their polymerase binding site is highly conserved across the HCV genotypes. Consequently, they are effective against all HCV genotypes, and present a high barrier against the development of viral resistance. In spite of the particular attention that nucleoside analogs have received in HCV drug research, sofosbuvir (**1**) is the only nucleoside HCV polymerase inhibitor that has been approved for HCV therapy.[6] Sofosbuvir (**1**) (Fig. 1) is a uridine analog that is dosed as the 5′-phosphoramidate, using the ProTide prodrug strategy for nucleoside drug delivery.[7] Early clinical studies with nucleoside inhibitors focused on cytidine and guanosine analogs, such as NM283 (**2**), the first HCV nucleoside to enter the clinic, and BMS-986094 (**3**), one of the more recent compounds to be studied in clinical trials. These and several other structurally related analogs are potent inhibitors of viral replication, but clinical studies were discontinued due to poor tolerability and/or toxicity.[8]

2. Theory

Antiviral activity requires formation of the nucleoside 5′-triphosphate metabolite, which is required for RNA incorporation by polymerase. Because uridine nucleosides are not efficiently phosphorylated in hepatocytes, uridine-based nucleosides must be delivered to the hepatocyte with a 5′-phosphate group in place. A 5′-phosphoramidate is an effective prodrug approach for delivering the 5′-monophosphate to the cell, which is then converted to the active 5′-triphosphate by cellular kinases. Thus, the phosphoramidate group plays a critical role in the potency of HCV nucleoside inhibitors, particularly in uridine analogs like sofosbuvir. The 2′-beta methyl substituent on nucleoside HCV inhibitors plays a critical role in the ability of these molecules to block viral RNA replication. Following incorporation of the HCV nucleoside into the RNA chain, the 2′-methyl group prevents RNA chain elongation by steric interference with the subsequent incoming nucleotide.[9]

Our research program has explored 2′-beta halogenated uridine analogs as a novel class of HCV polymerase inhibitors. Inhibitors in this structural class had not been described at the time our investigations began, although several reports have recently appeared.[10] During this research, attention was focused on 2′-dihalogenated analogs, which proved to be challenging compounds to assemble. Our initial designs included both chlorinated and brominated analogs, as both groups were expected to provide the necessary steric bulk required for activity. We report herein the synthesis and characterization of the potency, cellular toxicity and pharmacokinetics for phosphoramidate prodrugs of several new 2′-dihalogenated analogs and provide a comparison to additional 2′-substituted analogs, including those previously reported. Bioactivation of 2′-halogenated uridine analogs was found to differ from sofosbuvir (**1**) in the observed triphosphate concentrations and half-lives in human hepatocytes *in vitro* and dog liver biopsy studies in vivo. Thus, we explored a variety of 5′-phosphoramidate prodrugs to identify analogs that efficiently deliver the active triphosphate.

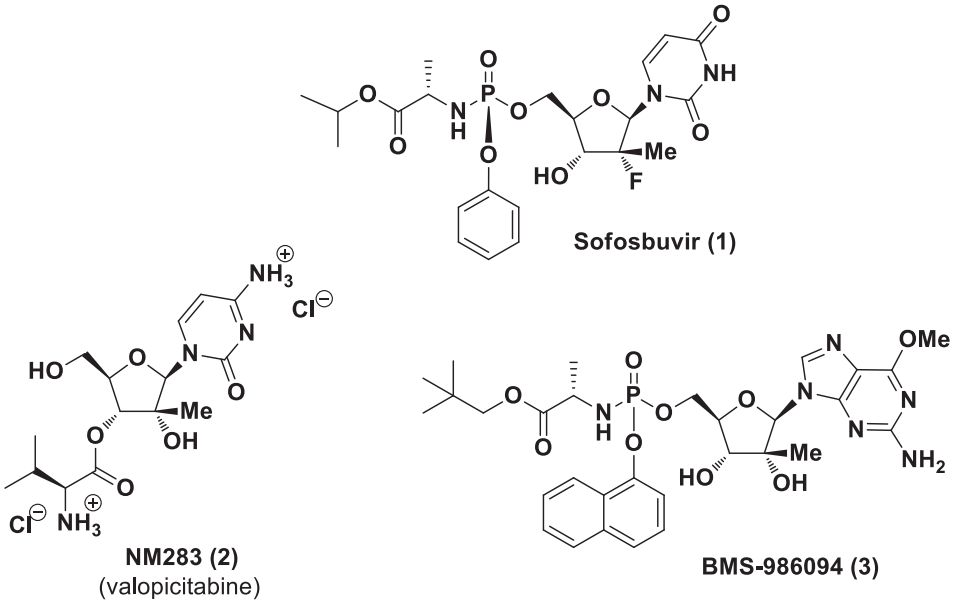
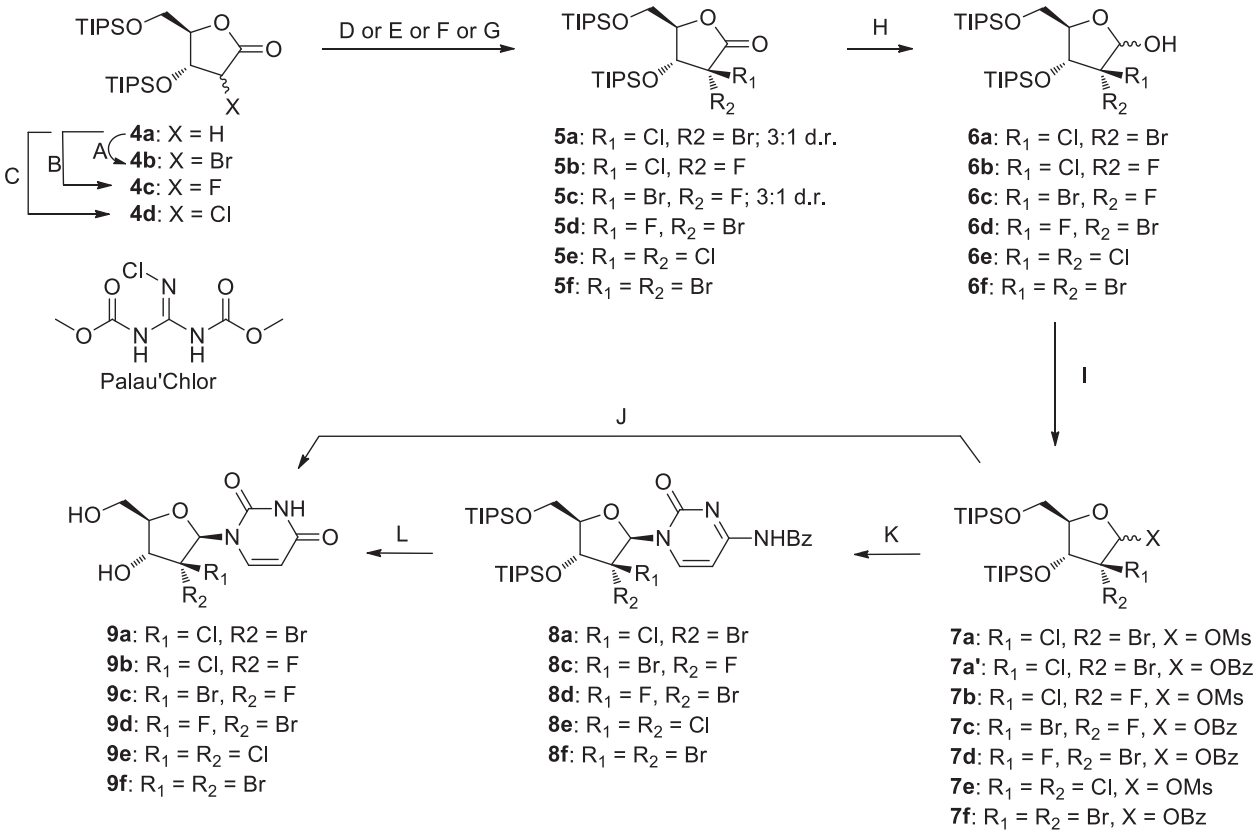


Fig. 1. Examples of HCV drug candidates that contain different nucleoside bases.



Scheme 1. Synthesis of halogenated nucleosides **9**. (A) TMSOTf, Et_3N; NBS; (B) LHMDS, NFSI; (C) TMSOTf, Et_3N; Palau'Chlor; (D) LHMDS, NCS; (E) LHMDS, NCS for **5b** or NBS for **5c** and **5d**; (F) LHMDS, NCS; (G) LHMDS, $(BrCl_2C)_2$; (H) DIBAL; (I) MsCl, Et_3N; or BzCl, DMAP, pyridine; (J) (i) 2,4-bis((trimethylsilyl)oxy)pyrimidine, TMSOTf; (ii) NH_4F; (K) di-TMS-Bz-cytonsine, $SnCl_4$; (L) (i) H_2O, AcOH; (ii) NH_4F.

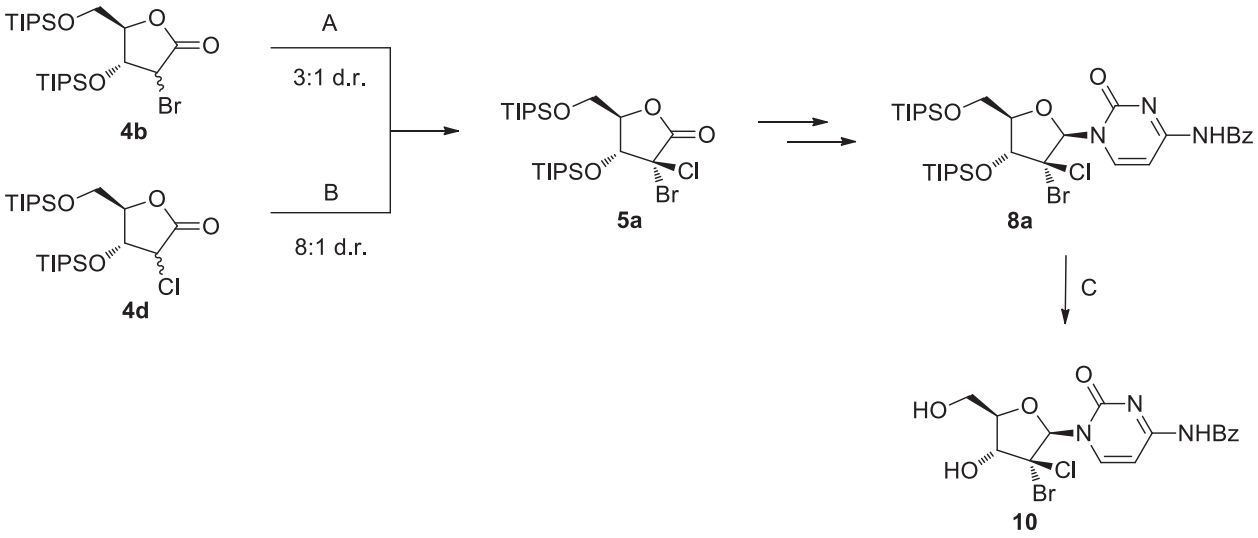
3. Chemistry

The synthesis of the halogenated nucleosides was carried out by halogenation of a 2-deoxyribonolactone protected by bulky silyl groups to provide flexible access to a variety of halogenated nucleosides. As shown in Scheme 1, the two key steps involved halogenation of lactone **4a**[11] and subsequent stereoselective glycosylation of the activated 2-deoxy-2,2-dihalo-*D*-furanose **7**.

Lactone **5a** was prepared by bromination[11] of compound **4a** followed by treatment with lithium bis(trimethylsilyl)amide and *N*-chlorosuccinimide.[12] The stereochemistry of the major isomer was preliminarily assigned as **5a**, based on the assumption that the chlorine electrophile would approach *anti* to the bulky silyl protecting group. Reduction using diisobutylaluminium hydride and subsequent activation with methanesulfonyl chloride provided intermediate **7a**, which glycosylates in the presence of tin tetrachloride with bis(trimethylsilyl)-*N*-benzoylcytosine, generated from *N*,*O*-bis(trimethylsilyl)acetamide and *N*-(2-oxo-1,2-dihydropyrimidin-4-yl)benzamide to give a 2:1 diastereomeric mixture of the nucleosides favoring the desired β-anomer. Column chromatography yielded an inseparable mixture of the desired compound **8a**, containing, by ^{1}H NMR, 12% of the corresponding 2,2-dichloro compound **8e**. De-silylation of **8a** with ammonium fluoride (Scheme 2) gave compound **10** as a crystalline solid. X-ray crystallography of **10** (Fig. 2) confirmed the C2′-stereochemistry of compound **8a** and verified the stereochemistry established by chlorination of 2-deoxy-2-bromolactone **4b**. Hydrolysis of the *N*-benzoylcytosine and desilylation of **8a** gave a mixture containing mostly the desired α-Br- β -Cl-nucleoside **9a**,[13] which was isolated by chiral supercritical fluid chromatography (SFC).

Based on the stereochemical outcomes of the halogenation sequence leading to **5a**, it was envisioned that the corresponding α -Cl- β -Br isomer could be derived as the major product from bromination of the chlorolactone **4d**. Treatment of the silyl ketene acetal of **4a** with Palau'Chlor, followed by bromination with lithium bis(trimethylsilyl) amide and *N*-bromosuccinimide, gave an inseparable mixture of products in a ratio of 8:1. Interestingly, the major isomer of the mixture was identical by ^{1}H NMR to be compound **5a** (Scheme 2). The synthetic approach was further evaluated in order to produce the quantity needed to fully characterize compound **9a**. This unexpected stereochemical outcome was examined under a variety of conditions and led to an improved synthesis involving chlorination of lactone **4a**, followed by treatment with potassium bis(trimethylsilyl)amide in toluene in combination with 1,2-dibromo-1,1,2,2-tetrachloroethane in a 2:1 ratio,[14] and quenching the reaction with methanol at −78 °C before warming. The resulting compound **5a** was stable and no decomposition or structural changes occurred after standing at room temperature for several weeks. Activation of lactol **6a** as the benzoate and carrying out the Vorbruggen reaction at 65 °C with 3 equivalents of tin tertachloride and 2.5 equivalents of bis(trimethylsilyl)-*N*-benzoylcytosine provided the improved stereoselectivity for the glycosylation.

Fluorination of lactone **4a**,[11] followed by chlorination with lithium bis(trimethylsilyl)amide and *N*-chlorosuccinimide, produced the desired β -chloro intermediate **5b**. On the other hand, generation of the silyl ketene acetal of the fluorolactone **4c** and treatment with Palau'-Chlor resulted in the opposite stereoselectivity yielding the corresponding α -Cl- β -F-lactone in 26% yield. Diisobutylaluminium hydride reduction and subsequent acylation of lactone **5b** gave compound **7b**, which was reacted with bis-trimethylsilyl protected pyrimidine-di-one in the presence of trimethylsilyl trifluoromethanesulfonate. The resulting mixture of products was de-protected to give a 1:3 mixture of β: α anomers. Chiral SFC purification provided the desired β-anomer **9b**.[15,10c]



Scheme 2. Unexpected stereoselectivity from chlorination/bromination of lactone **4a**. (A) LHMDS, NCS; (B) LHMDS, NBS; (C) NH_4F.

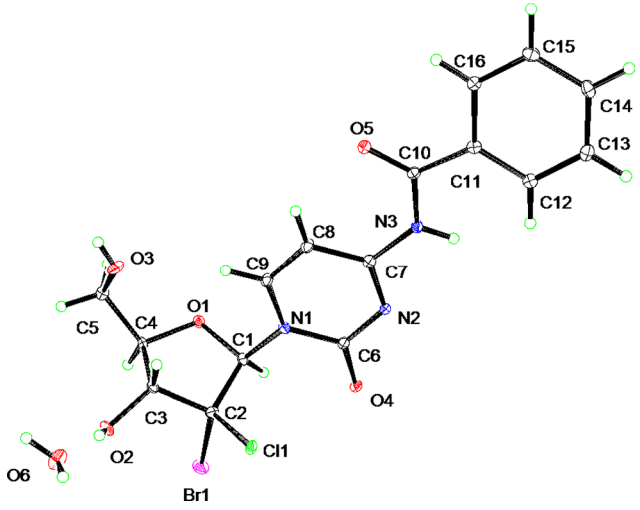
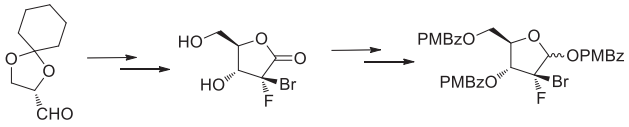


Fig. 2. X-ray structure of compound **10**.

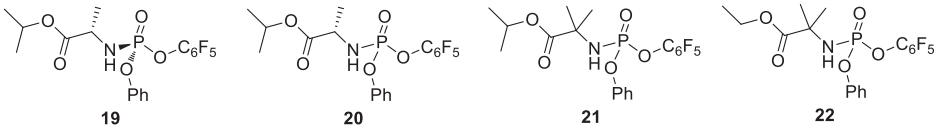
Although chlorination of 2-deoxy-2-fluorolactone **4c** with lithium bis(trimethylsilyl)amide and *N*-chlorosuccinimide gave **5b** as a single isomer, bromination of compound **4c** using lithium bis(trimethylsilyl) amide and *N*-bromosuccinimide gave a 3:1 inseparable mixture of diastereomers favoring the β -bromo compound **5c**. A mixture of compounds **6c** and **6d** was generated from diisobutylaluminium hydride reduction of **5c**, and could be separated by silica gel column chromatography. The C2 stereochemistry of **6c** and **6d** was determined by H—H ROESY and H-F NOE NMR techniques. Lactols **6c** and **6d** were activated as the corresponding benzoates **7c** and **7d**, respectively. The unfavorable diastereoselectivity observed in the conversion of **7b** was exacerbated by the bulky β-bromo of **7c**, and treatment with protected cytosine in the presence of tin tetrachloride led to a 20:1 mixture of diastereomers favoring the α-anomer. The desired compound **8c** was isolated in 5% yield. Given the need for *in vitro* and *in vivo* characterization of compound **9c**,[16,10d] it was necessary to optimize the current route due to poor stereoselectivity in two key steps. Collaboration between the discovery and process chemistry teams enabled two synthetic approaches with improved diastereoselectivity and efficiency to deliver compound **9c** in kilogram scale, which included an X-ray structure of this intermediate compound (Scheme 3).[17] Nucleosides **9e** and **9f** were prepared in similar fashion from bis-chlorination or bis-bromination of the lactone **4a** followed by transformation of the corresponding glycol donors **7e** or **7f**, respectively.[10a,10b]

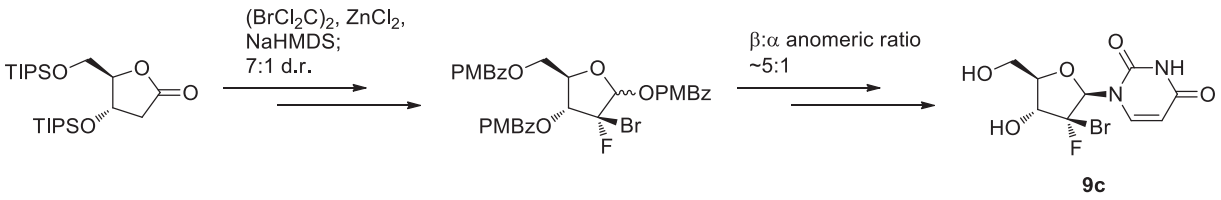
Third-Generation Route



The β-bromonucleoside **15** was synthesized from lactone **11**, obtained as a minor product from bromination of the corresponding 2-chlorolactone using potassium bis(trimethylsilyl)amide and 1,2-dibromo-1,1,2,2-tetrachloroethane (Scheme 4). Diisobutylaluminium hydride reduction of **11**, activation of the resulting lactol with *para*-methoxybenzoyl (PMBz) chloride, and protecting group transformation produced intermediate **13**. The *para*-methoxybenzoyl protection of the 3-hydroxyl group has demonstrated superior selectivity in the glycosylation of activated 2- β -bromo-D-furanose and improved β -anomer selectivity through anchimeric assistance.[17] Executing the Vorbruggen reaction upon **13**, followed by hydrolysis of the *N*-benzoylcytosine with 80% aqueous acetic acid, and deprotection of the *para*-methoxybenzoyl groups with ammonia in methanol gave nucleoside **15**.

Prodrugs **16a-k** and **17** were prepared by treating the corresponding nucleosides with *tert*-butylmagnesium chloride and phosphoramidate **19**[18] or **21**, whereas the prodrugs **16l**, **18a**, **18c**, and **18l** started from racemic phosphoramidates **20** or **22**, followed by chiral SFC separation to provide the diastereomerically pure prodrugs (Scheme 5).





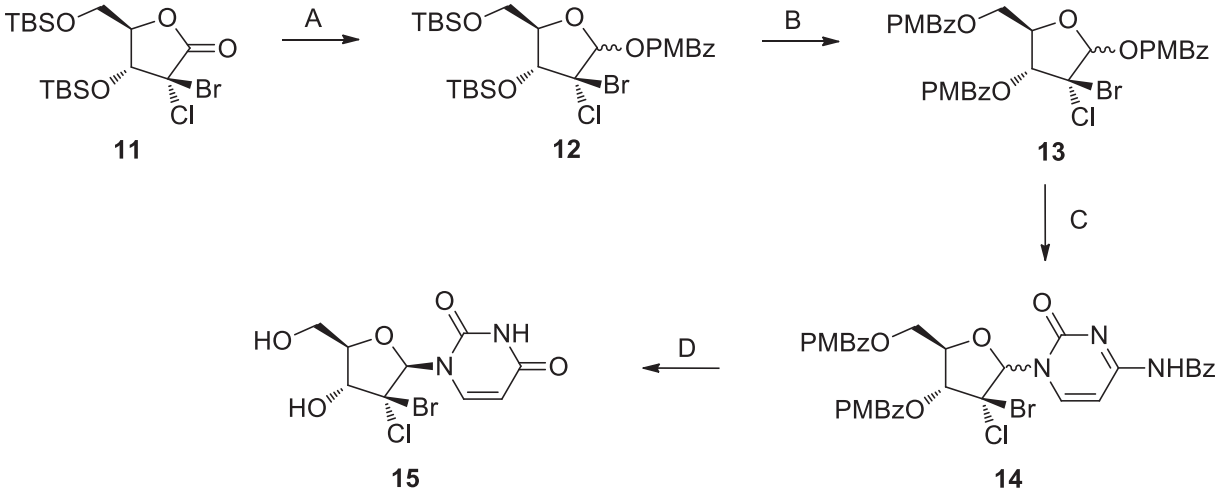
Scheme 3. Alternative synthetic approaches to compound **9c** second generation route.

4. Results and discussion

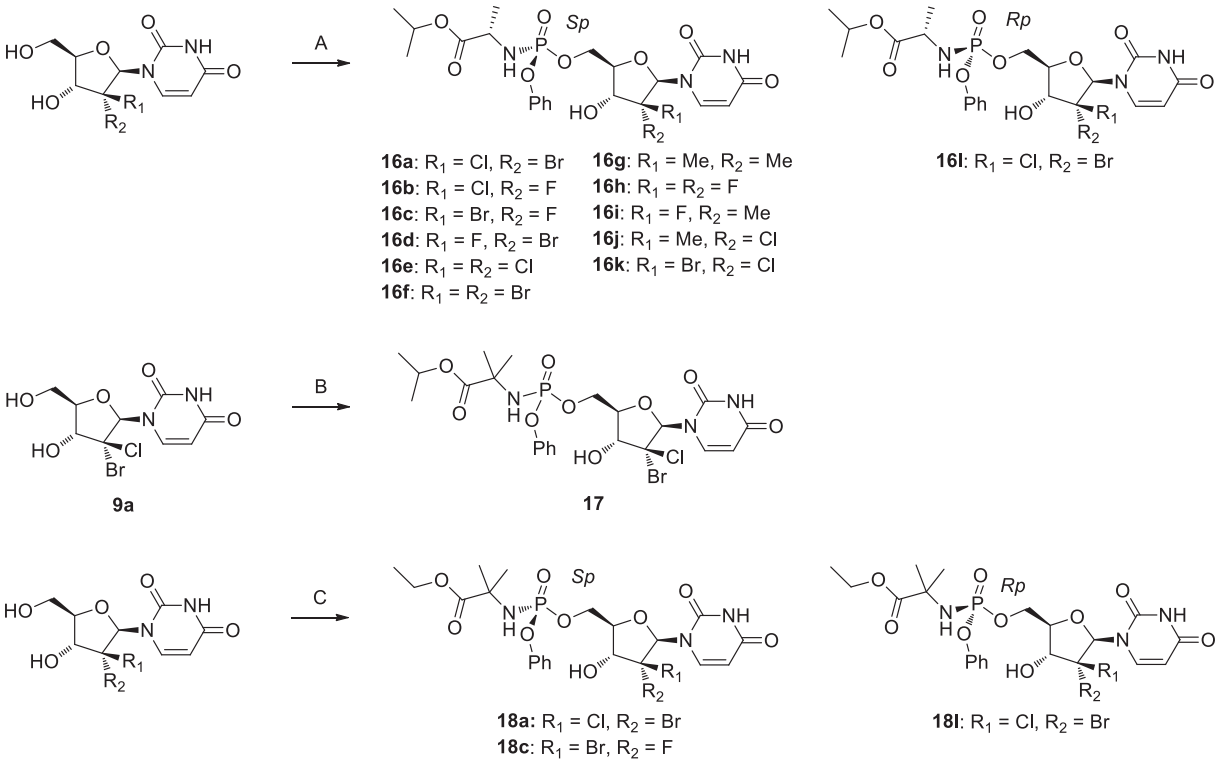
Several phosphoramidate prodrug analogs were tested bearing modifications at the 2′ position. Inhibition of HCV genotypes 1a and 1b RNA replication in Huh-7 cells using a subgenomic HCV replicon cell culture system are shown in Table 1.[19] As a comparator, our assay conditions provided 50% inhibition values of 0.155 μM and 0.230 μM for sofosbuvir (**1**) against genotypes 1a and 1b respectively, along with no measured cellular toxicity in Huh-7 cells. Replacement of the polarizable 2′-α-fluoro group in **1** with a methyl group yielded the corresponding 2′-dimethyl analog **16g**. This led to a surprising reduction in replicon inhibitory potencies. On the other hand, the moderate to excellent replicon potency of analogs having two polar functional groups on the 2′ position, such as the symmetrical 2′-dihalo analogs **16h**, **16e**, and **16f**, demonstrated the feasibility of halogen substitiution on C2′. The difluoro analog **16h** demonstrated a significant amount of toxicity in Huh-7 cells in contrast to **1**, **16e** and **16f**. The 2′-dichloro analog **16e** was more potent than **1**, with EC_{50} values 3- to 4-fold lower against genotype 1 replicons. Compound **16e** was subsequently reported elsewhere.[10a] While the 2′-dibromo analog **16f** was not cytotoxic in Huh-7 cells, it was nevertheless approximately 10-fold less active than **16e**. Our attention then turned to unsymmetrical substitutions at the 2′-position.

We found that the 2′-α-methyl-2′-β-fluoro analog **16i** (the 2′ inverse stereochemical combination in **1**) was inactive as the polarizability at this position is quite different than in **1** and the natural substrate which has a 2′-α-hydroxy group. Likewise **16g** was also sub optimal. We also made the analog, 2′-α-chloro-2′-β-methyl (**16j**) for comparison.[20] Under our assay conditions, analog **16j** had similar genotype 1a and 1b potency to **1** along with no associated cellular toxicity. However analog **16b** displayed roughly 3 to 4 fold better potency as compared to **1**, but also displayed some level of cellular toxicity, with a TD_{50} of 11 μM. Further investigations were directed towards 2′,2′-chloro,bromo and 2′,2′-fluoro,bromo analogs. Reasoning that a chlorine or a bromine atom possesses roughly the same Van der Waal's radius as a methyl group, we postulated that it would provide not only sufficient steric interference but also polarizability to terminate the RNA chain elongation for viral replication. We found that the 2′-α-bromo-2′-β-chloro analog **16a**, and the 2′-α-chloro-2′-β-bromo analog **16k** both had potencies that were similar to **1** with very little or no measured cellular toxicity. Finally the 2′-α-fluoro-2′-β-bromo analog **16c** displayed excellent potencies of 0.056 μM and 0.087 μM which were roughly 2 to 3 fold better than **1** with no associated cellular toxicity, while analog **16d**, with the opposite stereochemistry at the 2′ position, was about 30 to 50 fold less potent. Further studies focused on promising analogs **16a** and **16c**, which were evaluated for their ability to inhibit replication of a variety of HCV genotypes, using both wild-type and variant replicon constructs containing the S282T mutation. Activities against genotypes 2a, 3a, 4a, and 6a are compared to genotype 1 in Table 2. Both **16a** and **16c** provided potent inhibition of replication in replicon assays for all of these genotypes, with measured EC_{50} values that were significantly better than **1**. Additionally, the loss in potency for both **16a** and **16c** against the S282T resistant variants was similar to **1**.

Pharmacokinetic studies in dog were designed to assess the ability of promising 2′-bromo-uridine phosphoramidate prodrugs **16a** and **16c** to deliver the active 5′-triphosphate (TP) in liver. Each compound was co-dosed with sofosbuvir (**1**) (5 mg/kg p.o. for both test compounds) and the concentration of the active TP for both drugs was measured by liver biopsy at 4 and 24 h post dosing (Table 3). This method provided a direct comparison of active TP levels for the 2′-bromo-uridine nucleoside drugs with those produced by dosing a clinically effective agent. Including sofosbuvir (**1**) as an internal standard also helped to correct for variability between experiments, thus providing a more reliable comparison of different prodrugs. Phosphoramidate **16a** provided liver TP concentrations that were > 20-fold lower than the concentrations of sofosbuvir TP (shown in brackets in the table) at both 4 and 24 h post-dosing. This finding was disappointing given the similar replicon potency of **16a** in comparison to sofosbuvir (**1**). Results from this study suggest that a clinically efficacious dose for **16a** would be much greater than the approved dose of sofosbuvir (**1**) (400 mg/day).



Scheme 4. Synthesis of compound **15**. (A) (i) DIBAL, PhMe; (ii) PMBzCl, DMAP, Et_3N; (B) (i) AcOH, TBAF; (ii) PMBzCl, DMAP, Et_3N; (C) di-TMS-Bz-cytonsine, $SnCl_4$; (D) (i) 80% aq. AcOH; (ii) NH_3/MeOH.



Scheme 5. Synthesis of phosphoramidate prodrugs **16**, **17**, and **18.** (A) *t*-BuMgCl, **19** or **20**, THF/DMPU; (B) *t*-BuMgCl, **21**, THF/DMPU; (C) PhMgCl, **22**, THF/DMPU.

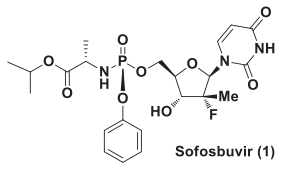
Phosphoramidate **16c** provided TP concentrations that were similar to the concentrations of sofosbuvir (**1**) TP at both biopsy time points, evidence that clinical success with this compound might be achievable at a similar dose.

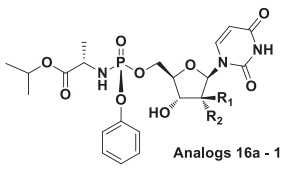
We investigated alternative 5′-phosphoramidate prodrugs for the 2′-α-bromo-β-chloro uridine nucleoside in an effort to identify compounds that deliver high TP levels in comparison to **16a**.[21] Physical properties for **16a** that distinguish it from sofosbuvir include much higher permeability in the PAMPA assay, and relatively poor aqueous solubility (Table 4). The 2′-halogens on **16a** make it more liphophilic than sofosbuvir (**1**), as evidenced by the higher LogD, which likely contributes to the improved permeability. However, the low solubility (23 μM at pH 7.4) is believed due primarily to the crystalline nature of **16a**, and was suspected to be a contributing factor in the poor in vivo performance, possibly due to limiting effects on drug absorption. The R*p* isomer **16l**, which has the opposite phosphate stereochemistry, was less crystalline and much more soluble. However, both **16l** and **16a** provided similar TP levels in dog liver, indicating that solubility was not a significant contributor to the poor in vivo performance of these isomeric prodrugs.

Further prodrug modifications were focused on the amino-acid portion of the phosphoramidate. A key compound made in this effort was the α-amino-isobutyric acid analog **17**, which provided TP concentrations in dog liver that were several fold higher than **16a**. Further improvements were made by replacing the isopropyl ester with an ethyl ester group to give **18a**, which provided dog liver TP concentrations that were roughly 10-fold higher than **16a** at 4 and 24 h post dosing after adjusting for the sofosbuvir TP internal standard levels. The R*p* isomer **18l** gave similar results in a single dose PK study. When **18a** and **18l** were co-dosed with sofosbuvir (**1**) (5 mg/kg for both test compounds) for 4 days to achieve near steady state TP concentrations, the *Sp* isomer **18a** provided higher TP concentrations at 24 h following the final dose. The amino-isobutyric acid ethyl ester (AIBEE) prodrug provided dog liver TP concentrations for **18a** that were similar to sofosbuvir (**1**) in the single dose PK studies, although sofosbuvir (**1**) TP concentrations were higher in the repeat dosing study.

Due to the dramatic effect on triphosphate levels observed in **18a**, we also prepared the amino-isobutyric acid ethyl ester (AIBEE) prodrug **18c** in an effort to deliver higher level TP concentrations of the 2′-α-

Table 1
HCV genotype 1a and 1b replicon activity, and cytotoxicity, in Huh7 cells for 2′-halogenated uridine phosphoramidate prodrugs.





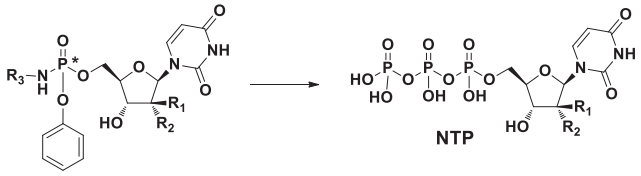
Compound #		1	16 g	16 h	16e	16f	16i	16j	16b	16a	16 k	16c	16d
R_1 =		Me	Me	F	Cl	Br	F	Me	Cl	Cl	Br	Br	F
R_2 =		F	Me	F	Cl	Br	Me	Cl	F	Br	Cl	F	Br
Assay	GT1a EC_{50} (μM)	0.155	5.63	0.226	0.043	0.408	> 10	0.139	0.053	0.114	0.200	0.056	3.03
	GT1b EC_{50} (μM)	0.230	4.75	0.286	0.048	0.522	> 10	0.167	0.061	0.147	0.258	0.087	2.50
	Huh-7 TD_{50} (μM)	> 100	31.6	0.384	> 100	> 100	> 32	> 100	11	80	> 100	> 100	26.5

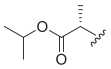
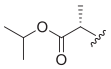
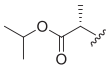
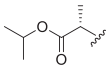
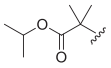
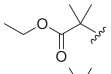
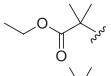
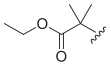
Table 2
Activities of **16a**, **16c**, and **1** in transient replicon assays for various HCV genotypes, with and without the S282T mutation.[a]

Cpd #	EC_{50} μM							
	1b-Con1-WT	1b-S282T	2a-WT	2a-S282T	3a-WT	3a-S282T	4a-WT	6a-WT
16a	0.0304	0.1606	0.1882	0.2617	0.0314	0.1418	0.0299	0.0227
16c	0.0152	0.1343	0.1888	0.3082	0.0127	0.0753	0.0257	0.0145
1	0.0620	0.6376	0.6813	1.002	0.0751	0.3301	0.0785	0.0867

[a] WT refers to Wild Type virus.

Table 3
Concentrations of the active 5′-triphosphate of 2′-bromo-uridine nucleosides.



Compound	Structure				Nucleotide triphosphate concentration (μM)				
					Dog liver[†]			Human hepatocytes[‡]	
	R_1	R_2	R_3	*	4 h	24 h	4 day SS[a]	4 h	24 h
Sofosbuvir (**1**)	Me	F		S_p	[see below][b]	[see below][b]	[see below][b]	97	122
16a	Cl	Br		S_p	0.088 [1.59]	0.049 [1.02]	NT	110	22
16c	Br	F		S_p	11.5 [7.39]	6.61 [8.87]	NT	192	91
16l	Cl	Br		R_p	0.143 [1.76]	0.036 [1.26]	NT	102	24
17	Cl	Br		S_p/R_p	0.713 [4.20]	0.244 [3.43]	NT	157	40
18a	Cl	Br		S_p	8.83 [15.03]	2.10 [5.44]	2.89 [11.1]	2818	203
18l	Cl	Br		R_p	7.21 [5.35]	2.64 [4.03]	1.43 [6.05]	NT	NT
18c	Br	F		S_p	43.3 [7.0]	42.7 [6.64]	8.18 [10.7][c]	3600	736

[†] Concentration of nucleotide triphosphate (TP) in dog liver following a 5 mg/kg p.o. dose of the prodrug.
[‡] Concentration of TP in human hepatocytes following a 4 h incubation with the prodrug (100 μM).
[a] TP concentrations following once daily p.o. dosing for 4 days to achieve near steady-state TP levels (liver biopsy taken 24 h following dose 4).
[b] Bracketed values are the concentration of sofosbuvir TP in liver (co-dosed with the test compound at 5 mg/kg p.o.).
[c] **18c** (1 mg/kg) and sofosbuvir (5 mg/kg) co-dosed p.o.; NT = not tested.

fluoro-β-bromo uridine nucleoside. In a single-dose dog PK study, prodrug **18c** provided TP concentrations that were over 5-fold higher than **16c** and about 6-fold higher than sofosbuvir (**1**) TP at the 24 h biopsy time point. Importantly, liver TP concentrations were the same at both 4 and 24 h following dosing with **18c**, demonstrating excellent TP persistence with this prodrug. In a repeat dosing study in dog, **18c** (1 mg/kg) was co-dosed with sofosbuvir (**1**) (5 mg/kg) for four days. The liver TP concentration provided by **18c** (8 μM) was similar to sofosbuvir (**1**) TP (12 μM) at 1/5 the dose.

Human hepatocyte studies were used to measure TP concentrations produced using phosphoramidate prodrugs of 2′-bromo uridine nucleosides in the target cell. In this assay, sofosbuvir (**1**) provided higher TP levels at 24 h (140 μM) than at 4 h (95 μM). The remarkable persistence of sofosbuvir (**1**) TP in hepatocytes undoubtedly contributes to its efficacy, and is an important characteristic for an HCV nucleoside prodrug. Although **16a** provided acceptable concentrations of TP in hepatocytes at 4 h, TP concentration dropped 4-fold by 24 h. **16c** provided much higher TP concentration at 4 h, and better TP persistence through 24 h. The AIBEE prodrugs **18a** and **18c** both provided very high TP concentrations in hepatocytes at 4 h, 30-fold to 40-fold higher than sofosbuvir (**1**) TP, although TP concentrations at 24 h were much lower. **18c** provided better TP persistence, with a 5-fold reduction in TP concentration from 4 h to 24 h in comparison to over 12-fold reduction from 4 to 24 h for **18a**.

The high concentrations of nucleoside TP provided by AIBEE phosphoramidate prodrugs of HCV active nucleosides observed both in human hepatocytes and in dog liver biopsy studies, allude to great potential for these compounds as potent inhibitors of HCV replication *in vivo*. Yet, activity studies in genotype 1 HCV replicon assays found that AIBEE prodrug analogs are less potent than sofosbuvir (**1**), **16a**, and **16c**, all of which have the "standard" L-alanine isopropyl ester phosphoramidate prodrug (Table 4). These results are consistent with a

Table 4
In vitro potency and ADME properties of nucleoside prodrugs described in Table 3.

Compound id	Replicon EC_{50} (μM)			ADME properties								
	GT1a	GT1b	GT1b CES1[a]	LogD HT-LCMS	PAMPA	aq sol (μM) (pH 7.4)	Plasma stability[b]		fu_p		Hep Cl_{int} (L/h/kg)	
							Dog	Human	Dog	Human	Dog	Human
Sofosbuvir (**1**)	0.168	0.188	0.053	3.02	0.36	> 200	93%	96%	0.323	0.563	2.4	4.3
16a	0.114	0.147	NT	3.51	2.22	23.4	92%	92%	0.213	0.113	6.8	16.4
16c	0.056	0.095	0.056	3.07	0.97	> 200	86%	100%	0.367	0.430	NT	15.4
16l	0.412	0.515	0.218	3.21	1.68	> 200	NT	NT	NT	NT	NT	12.1
17	1.09	1.38	0.162	3.30	2.86	> 200	100%	100%	0.130	0.193	8.0	11.5
18l	2.72	2.56	NT	3.07	3.46	> 200	100%	92%	0.187	0.310	5.9	9.3
18a	0.644	0.952	0.154	3.11	3.16	> 200	86%	100%	0.133	0.240	12.0	16.1
18c	0.259	0.434	0.069	2.86	0.71	519	100%	98%	0.317	0.473	7.4	7.9

[a] EC_{50} in GT1b replicons that overexpress CES1.
[b] Percent remaining following a 4 h incubation at 37 °C in dog or human plasma; NT = not tested.

theory that the carboxyesterase 1 (CES1) enzyme is principally responsible for metabolism of prodrugs having an AIB ester group. The CES1 enzyme is poorly expressed in Huh-7 cells used in standard HCV replicon assays, which have been shown to rely on cathepsin A (CatA) to initiate metabolic activation of sofosbuvir (**1**).[22] We established a modified replicon assay using genotype 1b replicon cells that transiently express higher levels of CES1 that are more similar to levels in human hepatocytes (Table 4, CES1 EC_{50}). EC_{50} values in this modified assay for AIBEE prodrugs **18a** and **18c** are equivalent to EC_{50} values for the alanine isopropyl ester phosphoramidate prodrugs of the corresponding nucleosides (**16a** and **16c**) in the standard genotype 1b replicon assay. Interestingly, **16c** has better activity than **18c** in the CES1 modified genotype 1b replicon assay. Since TP concentrations drive cellular potency through inhibition of HCV NS5B polymerase, this result would indicate that AIBEE prodrugs do not provide higher TP concentrations in replicon cells.

A comparison of ADME properties of phosphoramidate prodrugs does not provide an explanation for the difference in TP levels provided by AIBEE prodrugs. All of the prodrugs (with the exception of **16a**) have good aqueous solubility in phosphate buffer, and all are stable in dog and human plasma, with little or no degradation observed following incubation at 37 °C for 4 h. A significant difference in unbound fraction of drugs in plasma was observed, with higher protein binding contributing to a lower free fraction for the more lipophilic 2′- bromo,-chloro uridine prodrugs. However, the plasma free fraction difference did not contribute to an observable difference in plasma stability, and importantly, measured unbound fractions were no different for prodrugs of the same nucleoside (ie, **16c** and **18c**). The measured intrinsic clearance in human and dog hepatocytes offered no help to understand the large increase in TP concentrations observed for the AIBEE phosphoramidate prodrugs.

5. Conclusions

A research program to discover novel 2′-halogenated uridine-based nucleoside HCV polymerase inhibitors identified **16a** and **16c**, which are potent inhibitors of HCV replication in replicon assays.[23] These unique bromine-containing 2′-dihalogenated nucleosides are synthetically challenging molecules due to the difficulty in controlling stereoselectivity during the halogenation and heteroarylation steps. While both compounds have *in vitro* antiviral potency equal to or better than sofosbuvir against multiple genotypes, oral dosing studies in dog found that **16a** provided nucleoside TP concentrations in dog liver that were much lower than **16c** and sofosbuvir (**1**). Given the importance of the 5′-phosphoramidate group for delivering the nucleoside to the liver in a form that can be efficiently converted to the active TP metabolite, alternative phosphoramidate prodrugs were investigated, resulting in the discovery of aminoisobutyric acid ethyl ester (AIBEE) phosphoramidate analogs **18**. When AIBEE prodrug **18a** was co-dosed with sofosbuvir (**1**) in dog (5 mg/kg p.o. for both compounds), this compound provided liver TP concentrations that were > 10-fold higher than those obtained with **16a** at the same dose, and similar to TP concentrations observed for sofosbuvir (**1**). When AIBEE prodrug **18c** was co-dosed with sofosbuvir (**1**) in dog (5 mg/kg p.o. for both compounds), this compound provided liver TP concentrations that were 5-fold higher than sofosbuvir (**1**) TP concentrations. With regards to TP persistence in liver, the 2′-bromo,fluoro nucleosides **16c** and **18c** are superior to the 2′-bromo,chloro nucleosides **16a** and **18a**. In order to confirm the advantage provided by AIBEE prodrugs in vivo, a head to head repeat dosing study in dogs for 4 days to assess near steady-state liver TP levels, with **18c** dosed at 1 mg/kg, and sofosbuvir (**1**) dosed at 5 mg/kg. **18c** provided TP concentrations at 24 h following the final dose that were similar to sofosbuvir (**1**) TP concentrations, confirming the results from the single dose study. This data suggests that **18c** could be a highly effective agent for treating HCV infected patients in the clinic.

6. Experimental section

6.1. Single crystal X-ray diffraction studies

A single crystal of compound **10** was mounted on loop. Intensity data were collected on a Bruker APEX2. Data were collected at 100 K with graphite-monochromated MoK_α radiation ($\lambda = 0.71073$ Å) and triumph optics. Data were collected in four sets using omega-phi scans with omega steps of 0.3° and phi steps of 90°. A total of 2350 frames were collected with 20 s frame exposures. Data processing was carried out using the APEX software package.[24] Corrections for Lorentz-polarization effects and absorption were applied. All structures were solved using direct methods that yielded the non-hydrogen atoms. All presented hydrogen atoms were located in Fourier-difference electron density maps. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms associated with carbon atoms and the amide nitrogen atom were refined in geometrically constrained riding positions. The hydrogen atoms associated with alcohol groups and the water molecule were refined isotropically. Refinement was carried out with the use of SHELX-97.[25] Absolute stereo-chemistry was determined by anomalous dispertion.

6.2. Replicon assays

All compounds were tested against 1a-H77 (GenBank accession number NC004102) and genotype 1b-Con1 (GenBank accession number AJ238799) stable subgenomic replicon cell lines containing the luciferase reporter activity as described previously.[19] In order to express liver carboxylesterase 1(CES1) (NM_001266) in the replicon cell lines, CES1 expressing plasmid was purchased from OriGene Technologies

(Rockville, MD). Approximately 6 million genotype 1b-Con1 replicon cells were incubated overnight in the absence of antibiotics in Dulbecco's Modified Eagle Mediun (DMEM) containing 5% fetal bovine serum (FBS). The CES1 transfection mixture was prepared by incubating 800 μL DMEM, 24 μL turbofectin-TF (OriGene Technologies Rockville, MD), and 8 μg CES1 DNA for 25 min at room temperature. Media on cells incubated overnight was replaced with CES1 transfection mixture in 15 mL DMEM without antibiotics containing 5% FBS for 24 h resulting in transient expression of CES1. The inhibitory effect of compounds on HCV replication in replicon cells with or without CES1 overexpression was determined in DMEM containing 5% FBS. The cells were incubated for 3 days in the presence or absence of compounds, and were subsequently lysed and processed according to the manufacturer's instructions (Promega, Madison, WI) to measure the luciferase reporter activity using an EnVision instrument (Perkin-Elmer, Waltham, MA). Previously described replicon shuttle vectors were used to generate constructs encompassing NS5B derived from HCV genotypes 2a, 3a, 4a, or 6a -infected patient samples.[26] Mutagenesis was performed using the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (USB), or an NS5B gene containing the mutation was synthesized (IDT) and inserted into the appropriate shuttle vector plasmid. Transfection of Huh-7 cells and measurement of the inhibition of HCV replication by compounds with the luciferase assay were conducted as described previously.[27] EC_{50} was calculated using nonlinear regression curve fitting to the 4-parameter logistic equation and GraphPad Prism 5 software.

6.3. Hepatocyte studies

Cryopreserved primary human hepatocytes were obtained from BIOIVT, thawed and plated (~55,000 viable cells per well) on collagen-coated 96 well culture dishes. Cells were incubated at 37 °C (5% CO_2 incubator), then plating medium was replaced after 4–6 h by Incubation Medium (Williams E Medium without phenol red, containing Hepatocyte Maintenance Supplements, Gibco product CM4000) and Geltrex™ (Life Technologies) 0.35 mg/mL. Following overnight incubation, two separate plates of hepatocytes were incubated with prodrug compounds (100 μM) for 4 h. Prodrug containing media was removed and washed from hepatocytes after 4hrs. One plate from each experiment was further incubated in incubation media only for a further 20 h (washout period). Following the 4 or 24 h incubation period, plates were washed with ice-cold HBSS and stored at −80 °C until extraction for analysis. Samples were analyzed using LC-MS/MS.

6.4. Dog pharmacokinetic studies

Prodrugs (5 mg/kg or 1 mg/kg PO in EtOH: PEG-400: Phosal 53 MCT (10:30:60, w/w)) were cassette dosed with sofosbuvir (5 mg/kg) to beagle dogs (3 dogs/per time point). Liver biopsy samples were collected at 4 or 24 h post single dose or post final dose when compounds were dosed for 4-days. Liver biopsy samples were flash frozen and stored at −80 °C until analysis. The concentrations of triphosphates in liver were analyzed by LC–MS/MS. All animal procedures were approved by the Institutional Animal Care and Use Committee at AbbVie Inc.

6.5. Chemistry

General Procedures. All starting materials were dried by azeotropic distillation of toluene prior to subjecting to reaction conditions. Reagents and solvents, including anhydrous solvents, were obtained from commercial sources and used as supplied. Column chromatography was carried out on silica gel. ^{1}H NMR spectra were measured using either a Bruker 500 MHz or a Varian 400 MHz spectrometer. Chemical shifts are reported in ppm (δ) and referenced to an internal standard of tetramethylsilane (δ 0.00 ppm). ^{1}H − ^{1}H couplings are assumed to be first-order, and peak multiplicities are reported in the usual manner. MS analysis was conducted using a Finnigan SSQ7000 (ESI) mass spectrometer. All final compounds were purified to > 95% purity as determined by reverse phase HPLC performed on a Waters 2695 separation module/Waters 2489 UV/visible detector equipped with a YMC ODS-A, 5.5 μm, 120 Å, 4.6 mm × 150 mm column using a solvent gradient of 10–100% acetonitrile in water (0.1% TFA).

(3*S*,4*R*,5*R*)-3-Chloro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)dihydrofuran-2(3H)-one (4d). To a solution of compound **4a** (376.86 g, 847 mmol) and triethylamine (0.295 l, 2117 mmol) in dichloromethane (1.9 l) at −15 °C was slowly added trimethylsilyl trifluoromethanesulfonate (0.352 l, 1948 mmol) (~45 min). The mixture was stirred for 10 min, and a solution of Palau'Chlor (231.35 g, 1104 mmol) in dichloromethane (1.1 l) was added slowly (~1 h). The mixture was stirred for 30 min after addition, quenched with 2 N HCl (1.8l), and stirred for 20 min. The layers were separated, and the aqueous layer was extracted with dichloromethane (1 l, 0.3 l). The combined organic layers were washed with 1 N HCl (350 mL), 1 M $Na_2S_2O_3$ (400 mL), water (400 mL), and brine. The light yellow organic layer was dried over Na_2SO_4 and concentrated. Copious white solids precipitated during concentration, so heptanes (780 mL) was added, the resulting suspension was spun on the rotovap for 5 min, and decanted carefully, rinsing the sticky white solids with heptanes (3 × 50 mL). The mixture was concentrated to 416 g total mass and purified by column chromatography on silica gel using a solvent gradient of 0–25% dichloromethane in heptanes to give compound **4d** (224.65 g, 55.3% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 1.03 – 1.19 (m, 63H), 3.90 – 3.97 (m, 1.5H), 4.02 – 4.10 (m, 1.5H), 4.29 (dt, J = 5.4, 3.2 Hz, 1H), 4.38 (d, J = 6.1 Hz, 1H), 4.45 (q, J = 2.4 Hz, 0.5H), 4.68 (d, J = 5.4 Hz, 0.5H), 4.71 (dd, J = 5.5, 2.5 Hz, 0.5H), 4.83 (dd, J = 6.2, 5.4 Hz, 1H).

(3*R*,4*R*,5*R*)-3-Bromo-3-chloro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)dihydrofuran-2(3H)-one (5a). A solution of compound **4d** (116.59 g, 243 mmol) in tetrahydrofuran (800 mL) was stirred at room temperature and 1,2-dibromo-1,1,2,2-tetrachloroethane (127.02 g, 390 mmol) was added. Cooled to −115 °C (liquid nitrogen, slurry started to form) and added a 0.5 M toluene solution of potassium bis(trimethylsilyl)amide (1070 mL, 535 mmol) slowly maintaining the internal temperature between −100 to −85 °C (~1 h). The cooling bath was removed, and after 15 min (final temperature of −79 °C), 2 N HCl (650 mL) was added. The mixture was warmed to room temperature and the layers were separated. The organic layer was washed with brine (300 mL), dried over Na_2SO_4, and concentrated to give 274 g of crude product as a dark oil. Another batch of **5a** was prepared using a similar condition starting from compound **4d** (108.06 g, 225 mmol). The material from both reactions were combined, diluted with heptane (1 l), and washed with acetonitrile (100 mL, and 50 mL). The acetonitrile layers were combined and back-extracted with heptanes (700 mL), and the new combined heptane layers were washed with acetonitrile (50 mL). This acetonitrile wash was then back-extracted with heptanes (500 mL), the heptanes layers were combined and concentrated to give compound **5a** (272.33 g, quantitative) which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.32–0.97 (m, 42H), 3.99 (dd, J = 12.5, 1.9 Hz, 1H), 4.24–4.10 (m, 2H), 4.75 (d, J = 7.6 Hz, 1H). MS (DCI) m/z 576 $(M + NH_4)^+$.

(3*R*,4*R*,5*R*)-3-Bromo-3-chloro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)tetrahydrofuran-2-yl benzoate (7a'). A solution of compound **5a** (261 g, 468 mmol) in toluene (1000 mL) was cooled to an internal temperature of −78 °C. A solution of diisobutylaluminium hydride (1 M in toluene, 515 mL, 515 mmol) was added over 45 min so that the internal temperature remained below −65 °C. After stirring for 30 min at −70 °C, pyridine (114 mL, 1410 mmol), 4-dimethylaminopyridine (85.94 g, 703 mmol), and then benzoic anhydride (212.1 g, 938 mmol) were added. The cooling bath was removed, and the mixture was stirred for 45 min (final temperature of −15 °C), and warmed with a water bath to room temperature, and

stirred for 30 min. The reaction was quenched by addition of mL 2 N HCl (150 mL, exothermic to 65 °C), more 2 N HCl was added (1.8 l, temperature to 50 °C), and stirred vigorously for 5 min becoming a mostly biphasic solution. The mixture was diluted with heptane (1 l), washed with 1 N HCl (350 mL), saturated aqueous $NaHCO_3$ (3 × 400 mL), brine (350 mL), dried over Na_2SO_4, and concentrated. This material was dissolved in heptane (2 l), and washed with acetonitrile (5 × 400 mL). The combined acetonitrile layers were back-extracted with heptanes (400 mL), which were extracted with acetonitrile (3 × 75 mL). The combined heptanes layers were concentrated to give compound **7a'** (276.61 g, 89% yield).

***N*-(1-((2*R*,3*R*,4*R*,5*R*)-3-Bromo-3-chloro-4-((triisopropylsilyl) oxy)-5-(((triisopropylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)benzamide (8a).** A suspension of *N*-(2-oxo-1,2-dihydropyrimidin-4-yl)benzamide (224 g, 1041 mmol) and trimethylsilyl *N*-(trimethylsilyl)acetimidate (305 mL, 1247 mmol) in chlorobenzene (1250 mL) was heated to 80 °C for 1 h (becoming an orange solution). The heating mantle was removed, and a solution of compound **7a'** (276.6 g, 416 mmol) in chlorobenzene (1250 mL) was added at 45 °C, then tin tetrachloride (142 mL, 1210 mmol) was added slowly (~20 min, exothermic to 60 °C). The orange-red solution was heated to 65–70 °C. After 13 h, the brown reaction mixture was concentrated to 872 g total mass (of a thick brown solution), then diluted with methyl *tert*-butyl ether (2.8 l) and water (2.8 l). The resulting suspension was filtered through Celite®, and the solid washed with additional methyl *tert*-butyl ether and heptanes. The filtrate was washed with saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated to give 300 g of crude product, which was purified by column chromatography on silica gel using a solvent gradient of 0–20% ethyl acetate in heptane to give the product (128 g, 41% yield). The product was further purified by addition of heptanes (384 mL) and heating to 90 °C, aging 10 min until the thin white slurry was finely dispersed. The suspension was cooled to room temperature over 90 min, and the solid was collected by filtration, washed with heptanes (60 mL), then dried in a vacuum oven at 50 °C to give compound **8a** (81.6 g, 26% yield). ^{1}HNMR (400 MHz, DMSO-d_6) δ 0.98–1.25 (m, 42H), 3.98 (dd, J = 20.0, 10.4 Hz, 2H), 4.20 (d, J = 12.4 Hz, 1H), 4.33 (d, 1H), 6.84 (s, 1H), 7.39 (d, J = 7.6 Hz, 1H), 7.50 (t, J = 7.7 Hz, 2H), 7.62 (t, J = 7.3 Hz, 1H), 7.98 (d, 2H), 8.24 (d, J = 7.7 Hz, 1H), 11.52–11.26 (m, 1H). MS (ESI) m/z 758 $(M + H)^+$.

1-((2*R*,3*R*,4*R*,5*R*)-3-Bromo-3-chloro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (9a). A mixture of compound **8a** (81.4 g, 107 mmol), acetic acid (651 mL), and water (163 mL) was heated to 100 °C. After 4 h, the mixture was concentrated (70 °C rotovap temp), then flushed with toluene (2 × 250 mL) to 89 g of total mass (light yellow semi-solid), then methanol (407 mL) and ammonium fluoride (39.8 g, 1075 mmol) were added and the resulting mixture was heated to 60–65 °C. After stirring for 16 h a slurry was observed, so acetonitrile (400 mL) and methanol (400 mL) were added, keeping the temperature greater than 60 °C and the material went back into solution. The solution was cooled to room temperature and filtered, then the solid was washed with 2:1 methanol/acetonitrile (2 × 120 mL), Silica gel (150 g) was added and the mixture was concentrated carefully then dry-loaded material and purified by column chromatography using a solvent gradient of 0–10% methanol in dichloromethane to give compound **9a** (27.75 g, 76% yield) containing ~ 5% a diastereomeric impurity by HPLC. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 3.65 (ddd, J = 13.2, 5.1, 2.9 Hz, 1H), 3.79–3.87 (m, 2H), 4.03 (dd, J = 8.8, 5.8 Hz, 1H), 5.49 (t, J = 4.7 Hz, 1H), 5.71 (dd, J = 8.2, 0.5 Hz, 1H), 6.62 (s, 1H), 6.83 (d, J = 5.9 Hz, 1H), 8.09 (d, J = 8.2 Hz, 1H), 11.55 (s, 1H). MS (ESI) m/z 341 $(M-H)^-$.

(4*R*,5*R*)-3-Fluoro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)dihydrofuran-2(3H)-one (4c). To a solution of **4a** (5.466 g, 12.29 mmol) in tetrahydrofuran (40 mL) was added *N*-fluorobenzenesulfonimide (5.0562 g, 16.03 mmol), and the resulting mixture was cooled to −78 °C. A solution of lithium bis(trimethylsilyl) amide in toluene (1 M, 13.5 mL, 13.50 mmol) was added dropwise over 20 min. After an additional 20 min the reaction mixture was quenched by the dropwise addition of an aqueous solution of HCl (1 N, 20 mL) at −78 °C. The cooling bath was removed, and heptanes (40 mL) and 1 N HC1 (50 mL) were added. The mixture was stirred and warmed to room temperature. The layers were separated. The organic layer was washed with 1 N HC1 (40 mL), water (20 mL) and brine (20 mL). The combined aqueous layers were extracted with heptanes (2 × 30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography on silica eluting with a solvent gradient of 0–60% dichloromethane in heptane to provide compound **4c** (4.31 g, 71.6% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.04–1.12 (m, 42H), 3.94 (dd, J = 12.2, 2.4 Hz, 2H), 4.11 (dt, J = 12.2, 2.1 Hz, 2H), 4.19 (dt, 35 J = 7.0, 2.3 Hz, 2H), 4.91 (t, J = 7.2 Hz, 1H), 4.96 (t, J = 7.2 Hz, 1H), 5.07 (d, J = 7.4 Hz, 1H), 5.20 (d, J = 7.4 Hz, 1H).

(3*S*,4*R*,5*R*)-3-Bromo-3-fluoro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)dihydrofuran-2(3H)-one (5c) and (3*R*,4*R*,5*R*)-3-Bromo-3-fluoro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)dihydrofuran-2(3H)-one (5d). To a solution of compound **4c** (5.8357 g, 12.61 mmol) in tetrahydrofuran (55 mL) was added *N*-bromosuccinimide (2.69028 g, 15.12 mmol), and the resulting mixture was cooled to −78 °C. A toluene solution of lithium bis (trimethylsilyl)amide (1 M, 15.5 mL, 15.50 mmol) was added dropwise over 15 min. After 10 min, thin layer chromatography (TLC) (50% methylene chloride in heptane) showed the reaction was incomplete. Additional lithium bis(trimethylsilyl)amide (12 mL, 12.00 mmol) was added dropwise over 10 min as the light yellow suspension turned light brown–red. After 5 min TLC showed completion and the reaction was quenched with 1 N HCl (30 mL) at −78 °C. The cooling bath was removed, heptanes (50 mL) and additional 1 N HCl (100 mL) was added. After warming to room temperature the layers were separated. The organic layer was washed with water (30 mL) and brine (25 mL). The combined aqueous layers were extracted with heptanes (50 mL × 2). The combined organic layers were dried over $MgSO_4$ and concentrated to give a light yellow oil (7.9 g). Flash chromatography using silica gel eluted with a solvent gradient of 0–5% ethyl acetate in heptanes gave, by ^{1}HNMR, a 3.4:1 mixture of compounds **5c** and **5d** (5.9467 g, 78% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.04–1.21 (m, 58H), 3.94–4.12 (m, 3H), 4.13–4.18 (m, 0.4H), 4.46 (ddd, J = 6.4, 4.9, 2.5 Hz, 1H), 4.79 (dd, J = 14.6, 7.7 Hz, 0.4H), 4.93 (dd, J = 4.5, 2.8 Hz, 1H); MS (ESI) m/z 541.0 $(M + H)^+$.

(3*S*,4*R*,5*R*)-3-Bromo-3-fluoro-4-((tri-isopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)tetrahydrofuran-2-ol (6c) and (3*R*,4*R*,5*R*)-3-Bromo-3-fluoro-4-((tri-isopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)tetrahydrofuran-2-ol (6d). To a solution of the above mixture of compounds **5c** and **5d** (6.0767 g, 11.22 mmol) in toluene (35 mL) at −78 °C was added dropwise a solution of diisobutylaluminium hydride in toluene (1 M, 13 mL, 13.00 mmol). The solution was stirred at −78 °C for 1 h, then quenched with a 10% aqueous solution of sodium potassium tartrate (60 mL) at −78 °C, and the mixture was allowed to warm to room temperature, then filtered through a pad of Celite®, and rinsed with methyl *tert*-butyl ether. The layers of the filtrate were separated. The organic layer was washed with an aqueous solution of 1 N Na_2CO_3 (20 mL), 1 N HCl (50 mL), water (15 mL) and brine (15 mL), dried over $MgSO_4$, concentrated and purified by flash chromatography eluting with a solvent gradient of 0–30% dichloromethane in heptanes to give compound **6c** (3.49 g, 57.2% yield) and compound **6d** (2.8 g, 45.9% yield). For **6c**: ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.06–1.14 (m, 42H), 3.58 (dd, J = 12.1, 1.8 Hz, 1H), 3.82–3.86 (m, 2H), 3.88–3.99 (m, 4H), 4.08 (dt, J = 5.7, 2.3 Hz, 1H), 4.22 (dtd, J = 5.9, 4.1, 1.8 Hz, 1H), 4.75 (dd, J = 6.0,

3.5 Hz, 1H), 4.87 (dd, J = 16.1, 5.7 Hz, 1H), 5.15 (dd, J = 9.7, 5.5 Hz, 1H), 5.39 (dd, J = 12.0, 2.1 Hz, 1H). For **6d**: ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 1.04–1.21 (m, 59H), 3.48 (d, J = 12.7 Hz, 0.4H), 3.77–3.84 (m, 2H), 3.86–3.91 (m, 2H), 3.94 (dt, J = 6.6, 2.0 Hz, 1H), 4.08 (tdd, J = 4.6, 3.5, 1.0 Hz, 0.4H), 4.67 (ddd, J = 11.6, 4.6, 0.9 Hz, 0.4H), 4.72 (dd, J = 12.8, 6.6 Hz, 1H), 5.17 (ddd, J = 12.7, 5.9, 0.9 Hz, OH), 5.34 (dd, J = 9.2, 1.2 Hz, 1H).

(3*S*,4*R*,5*R*)-3-Bromo-3-fluoro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)tetrahydrofuran-2-yl benzoate (7c). To a solution of compound **6c** (3.67 g, 6.74 mmol) in pyridine (18 mL) was added 4-dimethylaminopyridine (80.6 mg, 0.660 mmol) followed by slow addition of benzoyl chloride (1.1 mL, 9.48 mmol). A white solid appeared after a few minutes and the suspension was stirred at room temperature for 1 h. The suspension was concentrated at reduced pressure, diluted with heptanes, and filtered through a pad of Celite® and rinsed with heptanes. The filtrate was concentrated to give an oil which was purified by flash chromatography eluting with a solvent gradient of 0–35% dichloromethane in heptane and gave compound **7c** (4.07 g, 93% yield) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 1.06–1.31 (m, 67H), 3.86 (dd, J = 11.8, 2.4 Hz, 0.6H), 3.95–4.07 (m, 4H), 4.32 (qd, J = 4.3, 1.0.6 Hz, 1.2H), 4.87 (dd, J = 4.2, 2.0 Hz, 1.2H), 4.96 (dd, J = 19.6, 7.4 Hz, 0.6H), 6.42 (d, J = 9.5 Hz, 0.6H), 6.73 (s, 1H), 7.41–7.47 (m, 4H), 7.56–7.61 (m, 2H), 8.02–8.05 (m, 1.2H), 8.08–8.11 (m, 2H).

(*Z*)-*N*-(1-((2*R*,3*S*,4*R*,5*R*)-3-Bromo-3-fluoro-4-((triisopropylsilyl)oxy)-5-(((triisopropylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)benzimidic acid (8c). To a suspension of *N*-(2-oxo-2,3-hydroxypyrimidin-4-yl)benzamide (4.66 g, 21.66 mmol) in chlorobenzene (27 mL) was added trimethylsilyl *N*-(trimethylsilyl)acetimidate (5.5 mL, 22.41 mmol). The suspension was stirred at 80 °C for 45 min then cooled to room temperature. The solution was then added to compound **7c** (4.07 g, 6.29 mmol) via cannula followed by addition of freshly distilled (114–116 °C, 760 mmHg) tin tetrachloride (3.5 mL, 29.8 mmol) at room temperature, and then the solution was heated to 106–114 °C. After 16 h the suspension was slowly poured into an aqueous solution of 1 M $NaHCO_3$ (200 mL) with vigorous stirring at room temperature, rinsing the reaction flask with methyl *tert*-butyl ether (50 mL). The mixture was stirred at room temperature for 1 h, filtered through Celite®, and the solid was rinsed with methyl *tert*-butyl ether (100 mL). The layers of the filtrate were separated, and the organic layer was washed with water (20 mL), 1 N HCl (15 mL) and brine (15 mL). The filter cake and the reaction flasks were rinsed with dichloromethane. The organic layers were combined, dried over $MgSO_4$ and concentrated to give a yellow oil (5.85 g), which was purified by flash chromatography on silica eluting with a solvent gradient of 0–25% ethyl acetate in heptanes to give impure product. The resulting material was re-purified by flash chromatography on silica eluting with a solvent gradient of 0–25% ethyl acetate in dichloromethane to give compound **8c** (230 mg, 0.311 mmol, 4.94% yield) as an off-white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 0.99–1.26 (m, 42H), 3.98 (dd, J = 12.2, 2.9 Hz, 1H), 4.05 (d, J = 8.5 Hz, 1H), 4.17 (dd, J = 12.1, 2.4 Hz, 1H), 4.70 (dd, J = 15.5, 8.2 Hz, 1H), 6.45 (d, J = 16.0 Hz, 1H), 7.41 (s, 1H), 7.52 (t, J = 7.7 Hz, 4H), 7.60–7.66 (m, 2H), 7.88–7.93 (m, 2H), 7.97–8.07 (m, 3H), 11.40 (s, 2H); MS (APCI) m/z 741.36 $(M + H)^+$

1-((2*R*,3*S*,4*R*,5*R*)-3-Bromo-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (9c). Compound **9c** was prepared using a method similar to that described for the synthesis of compound **9a**. Using compound **8c** (230 mg, 0.311 mmol), the reaction mixture, upon completion, was diluted with dichloromethane, filtered through Celite® and rinsed with 10% methanol in dichloromethane. The filtrate was concentrated to a solid, which was purified by flash chromatography on silica eluting with a solvent gradient of 0–10% methanol in dichloromethane and gave an 80% pure product which was further purified by flash chromatography on silica eluting with a solvent gradient of 0–40% of (10% methanol in acetonitrile) in dichloromethane to give pure product (45 mg) and mixed fractions (30 mg), which were further purified by preparative thin layer chromatography in 50% of (10% methanol in acetonitrile) in dichloromethane. The pure product was combined to provide compound **9c** (59 mg, 0.172 mmol, 55.4% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 3.64 (ddd, J = 12.6, 5.2, 3.0 Hz, 1H), 3.79–3.87 (m, 2H), 4.24–4.33 (m, 1H), 5.35 (t, J = 5.1 Hz, 1H), 5.73–5.76 (m, 1H), 6.24 (d, J = 16.9 Hz, 1H), 6.50 (d, J = 7.1 Hz, 1H), 7.84 (d, J = 8.1 Hz, 1H), 11.57 (s, 1H); MS (DCI) m/z 342.1 $(M + NH_4)^+$.

(*S*)-Isopropyl 2-(((*S*)-(((2*R*,3*R*,4*R*,5*R*)-4-bromo-4-chloro-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate (16a). To a solution of compound **9a** (1.5 g, 4.39 mmol) in tetrahydrofuran (12.0 mL) and 1,3-dimethyltetrahydropyrimidin-2(1H)-one (1.5 mL) at 0 °C, was added a tetrahydrofuran solution of *tert*-butylmagnesium chloride (1 M, 4.39 mL, 4.39 mmol). The resulting suspension was stirred at 0 °C for 30 min and (*S*)-isopropyl 2-(((*S*)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate (3.98 g, 8.78 mmol) was added. The resultant mixture was allowed to warm to room temperature. After 1.5 h an aqueous solution of half saturated $NaHCO_3$ (75 mL) and then extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography on silica eluting with a solvent gradient of 0–5% methanol in dichloromethane to provide compound **16a** (2.17 g, 81% yield) containing 6 wt% of 1,3-dimethyltetrahydropyrimidin-2(1H)-one by NMR. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.14 (d, J = 6.3 Hz, 6H), 1.21 (d, J = 7.1 Hz, 3H), 3.79 (m, 1H), 4.00 (m, 1H), 4.08 (m, 1H), 4.30 (m, 2H), 4.84 (m, 1H), 5.56 (d, J = 8.2 Hz, 1H), 6.08 (dd, J = 13.1, 10.1 Hz, 1H), 6.62 (s, 1H), 6.99 (d, J = 5.5 Hz, 1H), 7.20 (m, 3H), 7.36 (m, 2H), 7.63 (d, J = 8.1 Hz, 1H), 11.58 (s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 20.26 (d, J = 6.5 Hz), 21.84, 21.89, 50.25, 64.58, 68.52, 77.00, 80.23, 84.59, 102.55, 120.51 (d, J = 4.9 Hz), 125.14, 130.17, 150.72, 151.09 (d, J = 6.5 Hz), 163.09, 173.05; ^{31}P NMR (162 MHz, DMSO-d_6) δ 3.28. MS (ESI) m/z 612.0 $(M + H)^+$.

(*S*)-Isopropyl 2-(((*S*)-(((2*R*,3*R*,4*S*,5*R*)-4-bromo-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate (16c). Compound **16c** was prepared using a method similar to that described for the synthesis of compound **16a**. Using compound **9c** (26 mg, 0.080 mmol), *tert*-butylmagnesium chloride (1 M, 0.080 mL, 0.080 mmol), and (*S*)-isopropyl 2-(((*R*)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate (72.5 mg, 0.160 mmol) provided compound **16c** (31 mg, 62.6% yield) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.14 (d, J = 2.2 Hz, 6H), 1.23 (dd, J = 7.2, 1.0 Hz, 3H), 3.74–3.86 (m, 1H), 4.00–4.05 (m, 1H), 4.22–4.40 (m, 2H), 4.86 (hept, J = 6.3 Hz, 1H), 5.63 (d, J = 8.2 Hz, 1H), 6.06 (dd, J = 13.0, 10.0 Hz, 1H), 6.23 (d, J = 17.7 Hz, 1H), 6.65 (d, J = 7.1 Hz, 1H), 7.16–7.24 (m, 3H), 7.35–7.40 (m, 2H), 7.56 (d, J = 8.1 Hz, 1H), 11.58 (s, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 19.68 (d, J = 6.7 Hz), 21.30, 21.34, 49.69, 64.35, 67.95, 75.60 (d, J = 16.9 Hz), 78.96, 102.38, 109.11 (d, J = 260.9 Hz), 120.01 (d, J = 4.8 Hz), 124.58, 129.62, 150.04, 150.55 (d, J = 6.4 Hz), 162.60, 172.50, 172.54; ^{19}F NMR (376 MHz, DMSO-d_6) δ −118.29; ^{31}P NMR (162 MHz, DMSO-d_6) δ 3.24; MS (ESI) m/z 593.9 $(M + H)^+$.

Ethyl 2-(((*S*)-(((2*R*,3*R*,4*R*,5*R*)-4-bromo-4-chloro-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)-2-methylpropanoate (18a) and Ethyl 2-(((*R*)-(((2*R*,3*R*,4*R*,5*R*)-4-bromo-4-chloro-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)-2-methylpropanoate (18 l). Compounds **18a** and **18l** were prepared using a method similar

to that described for compound **16a**. Using compound **9a** (554 mg, 1.622 mmol), *tert*-butylmagnesium chloride (1 M, 1.784 mL, 1.784 mmol), and ethyl 2-methyl-2-(((perfluorophenoxy)(phenoxy) phosphoryl)amino)propanoate (1103 mg, 2.433 mmol) provided a mixture of the products, which was purified by chiral SFC using a Regis Whelk-O1 (*S*,*S*) column, eluting with 20% methanol in supercritical CO_2 to provide **18a** (417 mg, 42.1% yield) (the second of two stereoisomers to elute) and **18l** (176 mg, 17.8% yield) (the first of two stereoisomers to elute). For **18a**: ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.12 (t, J = 7.1 Hz, 3H), 1.33 (s, 3H), 1.37 (s, 3H), 3.97–4.14 (m, 4H), 4.25–4.40 (m, 2H), 5.52 (dd, J = 8.2, 2.2 Hz, 1H), 5.96 (d, J = 9.6 Hz, 1H), 6.62 (s, 1H), 7.01 (s, 1H), 7.14–7.24 (m, 3H), 7.36 (dd, J = 8.6, 7.3 Hz, 2H), 7.66 (d, J = 8.2 Hz, 1H), 11.58 (d, J = 2.2 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 13.85, 26.87 (d, J = 5.4 Hz), 26.96 (d, J = 6.7 Hz), 56.02, 60.60, 63.87, 76.47, 79.64, 84.15, 101.95, 120.10 (d, J = 4.7 Hz), 124.52, 129.56, 150.19, 150.64 (d, J = 6.5 Hz), 162.55, 174.58; ^{31}P NMR (162 MHz, DMSO-d_6) δ 1.83; MS (ESI) m/z 611.8 $(M + H)^+$. For **18l**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.14 (t, J = 7.1 Hz, 3H), 1.34 (s, 3H), 1.36–1.42 (m, 3H), 3.98–4.09 (m, 4H), 4.26–4.35 (m, 1H), 4.39 (dd, J = 11.8, 5.3 Hz, 1H), 5.53 (dd, J = 8.2, 2.1 Hz, 1H), 6.02 (d, J = 9.7 Hz, 1H), 6.65 (s, 1H), 7.06 (s, 1H), 7.17 (t, J = 7.3 Hz, 1H), 7.22 (dt, J = 8.6, 1.1 Hz, 2H), 7.37 (dd, J = 8.6, 7.3 Hz, 2H), 7.62 (d, J = 8.2 Hz, 1H), 11.59 (d, J = 2.2 Hz, 1H); MS (ESI) m/z 611.8 $(M + H)^+$.

Ethyl 2-(((*S*)-(((2*R*,3*R*,4*S*,5*R*)-4-bromo-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxytetrahydrofuran-2-yl) methoxy)(phenoxy)phosphoryl)amino)-2-methylpropanoate (18c). To a solution of compound **9c** (1.31 g, 4.04 mmol) in tetrahydrofuran (13 mL) and 1,3-dimethyltetrahydropyrimidin-2(1H)-one (DMPU) (1.3 mL) at an internal temperature of 1.1 °C was added a tetrahydrofuran solution of phenylmagnesium chloride (2 M, 2.5 mL, 5.00 mmol) dropwise over 5 min keeping the internal temp less than 3 °C. The viscous off-white slurry was stirred for 30 min, then (*S*)-ethyl 2-methyl-2-(((perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate[18] (2.01 g, 4.44 mmol) was added as a solid. The suspension was allowed to slowly warm to room temperature while stirring for 16 h, and then quenched with 1 N HCl (15 mL, internal temp 18 to 27 °C) and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with brine (2 × 10 mL) and 4:1 brine/1N HCl (10 mL), dried over sodium sulfate, filtered and concentrated to give 4.2 g of a yellow oil which was purified by flash chromatography on silica eluting with a solvent gradient of 30–75% ethyl acetate in dichloromethane to give a white foam (1.9563 g). The impure product was dissolved in isopropyl acetate (20 mL), washed with water (2 × 10 mL), brine (10 mL), then dried over sodium sulfate, filtered and concentrated to give **18c** (1.66 g, 66.9% yield) as a white solid. HPLC showed 96.9 area % desired product at 210 nm, with 0.26 area% DMPU and 0.11 area% *i*-PrOAc. ^{n}H NMR (500 MHz, DMSO-d_6) δ ppm 1.12 (t, J = 7.1 Hz, 3H), 1.33 (s, 3H), 1.37 (s, 3H), 4.02 (q, J = 7.1 Hz, 3H), 4.22–4.41 (m, 3H), 5.58 (d, J = 8.1 Hz, 1H), 5.92 (d, J = 9.6 Hz, 1H), 6.22 (d, J = 17.7 Hz, 1H), 6.66 (s, 1H), 7.13–7.23 (m, 2H), 7.32–7.39 (m, 2H), 7.56 (d, J = 8.1 Hz, 1H), 11.39 (s, 1H).

Declaration of Competing Interest

The authors declare the following competing financial interest(s): All authors were employees of AbbVie at the time of the study. ACK, HC, JTR, BSB, GTH, HRH, TL, CCM, JPS, EAV, DB, CVH, VP, RAC, DS, TD, PK, RFH, RW, and DAD are current employees of AbbVie. MLI is a former AbbVie employee. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.115208.

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