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Synthesis and biological evaluation of 5′-*C*-methyl nucleotide prodrugs for treating HCV infections

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ABSTRACT

Nucleotide prodrugs are of great clinical interest for treating a variety of viral infections due to their ability to target tissues selectively and to deliver relatively high concentrations of the active nucleotide metabolite intracellularly. However, their clinical successes have been limited, oftentimes due to unwanted *in vivo* metabolic processes that reduce the quantities of nucleoside triphosphate that reach the site of action. In an attempt to circumvent this, we designed novel nucleosides that incorporate a sterically bulky group at the 5′-carbon of the phosphoester prodrug, which we reasoned would reduce the amounts of non-productive P—O bond cleavage back to the corresponding nucleoside by nucleotidases. Molecular docking studies with the NS5B HCV polymerase suggested that a nucleotide containing a 5′-methyl group could be accommodated. Therefore, we synthesized mono- and diphosphate prodrugs of 2′,5′-*C*-dimethyluridine stereoselectively and evaluated their cytotoxicity and anti-HCV activity in the HCV replicon assay. All four prodrugs exhibited anti-HCV activity with IC_{50} values in the single digit micromolar concentrations, with the 5′(*R*)-*C*-methyl prodrug displaying superior potency relative to its 5′(*S*)-*C*-methyl counterpart. However, when compared to the unmethylated prodrug, the potency is poorer. The poorer potency of these prodrugs may be due to unfavorable steric interactions of the 5′-*C*-methyl group in the active sites of the kinases that catalyze the formation of active triphosphate metabolite.

There is an ongoing need for new clinical entities to treat newly emerging viral diseases, as well as those associated with resistant viral mutants that have developed in response to selective drug pressure.[1–3] While nucleoside analogs are generally considered frontline therapy for viral infections, their ability to inhibit viral encoded polymerases is highly dependent on the individual anabolic pathway required to transform a given nucleoside analog into its active triphosphate metabolite. This can sometimes dramatically limit their therapeutic potential, as activation requires a three-step sequence of kinase-mediated phosphorylations.[4,5] The first phosphorylation, in particular, is typically mediated by cellular or virus-specific enzymes that are very substrate specific and selective. Development of nucleoside monophosphate prodrugs to promote intracellular delivery of the monophosphate metabolite in high concentrations has thus become a useful alternative to overcome this limitation.[6,7] Moreover, their low systemic toxicity, their ability to selectively target tissue and their high membrane permeability due to the presence of lipophilic masking groups can provide many advantages for the modified nucleoside analogs.[8,9]

Despite the significant number of nucleotide prodrugs that have been developed with potent *in vitro* and *in vivo* antiviral activities, only a few have attained clinical success.[10–13] The lack of clinical efficacy of many of these prodrugs can in many cases be attributed to their low metabolic stability, in particular non-productive metabolic cleavage of the prodrug by cellular enzymes such as 5′-nucleotidases or phospholipase D (PLD).[14–19] For example, a metabolism study by Congrong Niu et al. revealed the formation of a significant amount of dephosphorylated nucleoside metabolite from its corresponding nucleotide prodrug within 2 h in an *in vitro* assay using primary human hepatocytes.[14] The general metabolism of a nucleotide prodrug to its active and inactive metabolites is illustrated in Fig. 1A, while cleavage of the phosphodiester bond attached to the lipophilic masking group releases the desired active metabolite (*e.g.,* a nucleoside monophosphate), cleavage of the other phosphoester bond (*i.e.,* the one attached to the nucleoside) generates the poorly-anabolized parent nucleoside.

Strategies that minimize this undesired ‘P—O’ bond cleavage have the potential to enhance the therapeutic efficacy of such prodrugs. One strategy includes replacement of the ‘P—O’ bond with ‘P-CH_2’ bond, *i.e.*, phosphonate prodrugs, which have primarily been utilized with acyclic nucleotides.[20,21] Another potentially interesting strategy involves the introduction of steric bulk at or near the 5′-carbon of the nucleoside. This has been widely explored in the development of “locked”

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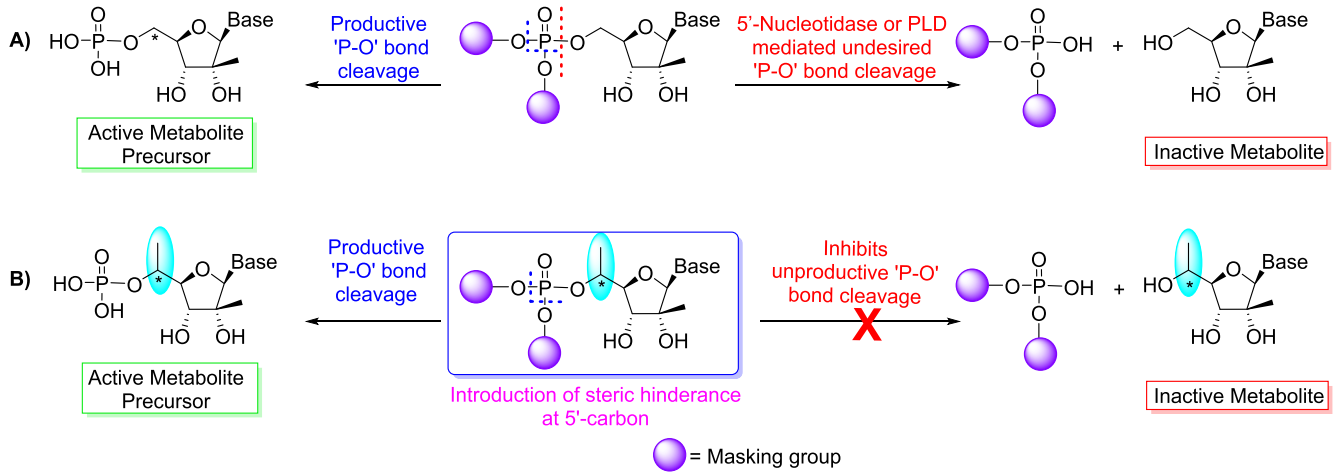


Fig. 1. Nucleotide prodrug design strategy for promoting the formation of the active metabolite; A) Without the 5′-C-methyl, metabolism to the dephosphorylated adduct is problematic; B) A 5′-C-methyl is introduced to inhibit dephosphorylation.

nucleoside analogs for oligonucleotide-based therapeutics.[22,23] For small molecule nucleotide antiviral agents substitution at the 5′-carbon, either with a deuterium or methyl group has been reported. In these studies, the 5′-deuterated prodrugs showed enhanced stability and comparable efficacy.[24] However, whether the incorporation of a 5′-methyl group on these structures confers enhanced stability was not elucidated.[25] We believe the 5′-substitution strategy has the potential to improve the stability of selected nucleotide prodrugs by reducing 5′-nucleotidase or PLD-mediated prodrug cleavage as a consequence of local steric hindrance, thereby increasing intracellular concentrations of nucleoside triphosphates (Fig. 1B). If this hypothesis is correct, it could, in principle, streamline the metabolic profile of selected nucleoside-based antiviral therapeutics and, in the best case scenario, permit the development of dose-sparing drug regimens.

To test the hypothesis, we have designed and synthesized phosphoramidate prodrugs of 2′,5′-*C*-dimethyl uridine **1a** and **1b** (Fig. 2) and evaluated for their anti-HCV efficacy *in vitro*. We chose 2′-*C*-methyl uridine **3** as the test nucleoside of interest due to its commercial availability and synthetic tractability.[26] Of particular interest is the fact that the nucleoside **3** is known to show poor anti-HCV activity (EC_{50} = 15.2 μM), whereas its phosphoramidate prodrug and its triphosphate metabolite exhibit high potency against HCV (EC_{50} = 0.03 μM and EC_{50} = 1.9 μM, respectively).[27–29] However, despite its high *in vitro* potency as the phosphoramidate prodrug, it was not developed into a clinical agent, possibly due to its instability towards plasma and cellular enzymes.

For the 5′-moiety, we chose a methyl group as a preferred substituent since we hypothesized that it possesses sufficient steric bulk to inhibit nucleotidase activity, while being sufficiently distal from the site of the second phosphorylation to not significantly impact the efficiency of the phosphorylation process. A key concern, however, was whether the incorporation of the methyl group would adversely affect the binding of the newly designed nucleotide to the HCV NS5B polymerase. To address this, we employed molecular docking studies using a conveniently available co-crystal structure of HCV NS5B containing sofosbuvir as the diphosphate (PDB ID 4WTG). We docked our envisaged *R*- and *S*-5′-methyl nucleotides (also in the diphosphate form, **2a** and **2b**). These studies suggested that the methyl in the *R*-configuration **2a** could indeed be well accommodated with a very similar conformation to the co-crystal structure of sofosbuvir diphosphate (Fig. 3).

Based on these observations, we have designed syntheses of the phosphoramidate prodrugs of 2′-*C*-methyl uridine with a methyl substitution at the 5′-carbon (Fig. 2). Since the 5′-methylation produces a new chiral center, the resulting two isomeric prodrugs **1a** and **1b** were synthesized stereoselectively from their corresponding chiral alcohols **8a** and **8b.** The stereoselective syntheses of the 5′-*C*-methyl alcohols **8a** and **8b** are illustrated in Scheme 1. Commercially available 2′-*C*-methyluridine **3** was protected to its acetonide **4** in 77% yield,[30,31] and then subjected to IBX oxidation in acetonitrile to afford the crude aldehyde **5.** We observed that the aldehyde **5** was unstable when subjected to work-up or silica gel chromatography, as it readily transformed into its hydrate form (structure not shown). Instead, the crude aldehyde **5** was directly treated with excess methylmagnesium bromide in THF to generate the 5′-*C*-methyl alcohol **6** as a diastereomeric mixture (3:1 dr ratio) in a 61% crude yield over two steps.[32] The two diastereomers of **6** were inseparable by silica gel chromatography, and only after repeated recrystallizations (70% EtOAc/hexanes) were we able to isolate the 5′(*R*)-alcohol in pure form, albeit in very low yields. Therefore, we turned to a stereoselective synthesis approach and obtained high diastereoselectivity using a two-step reaction sequence of oxidation, followed by stereoselective reduction. For this sequence, oxidation of the alcohol **6** with IBX in acetonitrile gave the methyl ketone **7** in 94% yield. Initially, the attempted reduction of the ketone **7** to stereoselectively generate alcohol **8** was unsuccessful using a variety of reducing agents including $NaBH_4/CeCl_3$, L-selectride and LiAlH (^{t}OBu)$_3$ as they resulted in incomplete conversions, and poor

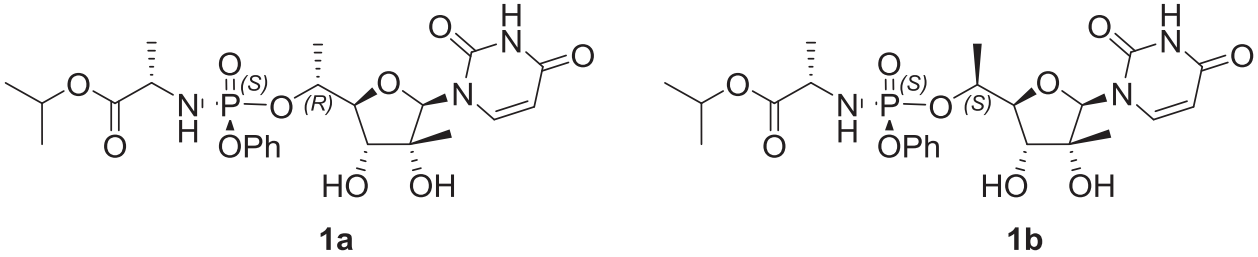


Fig. 2. Structures of phosphoramidate prodrugs of the 2′,5′-C-dimethyluridine epimers.

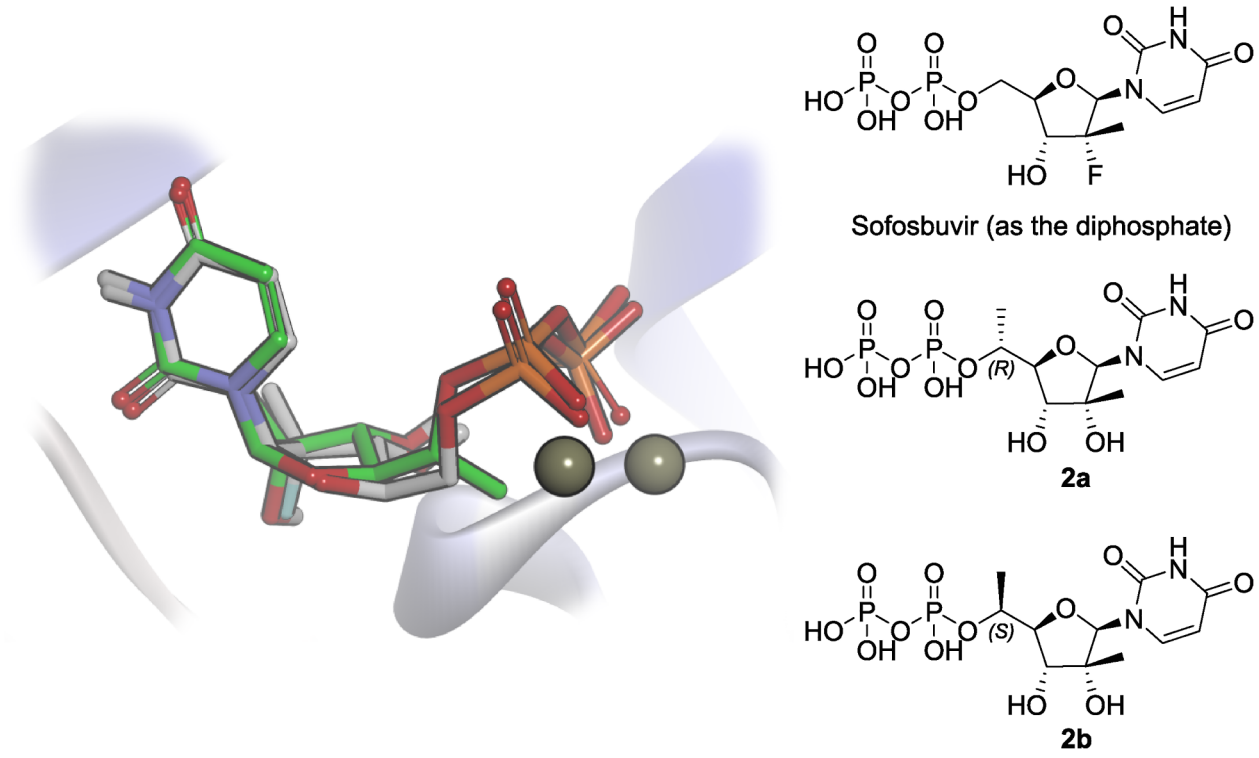
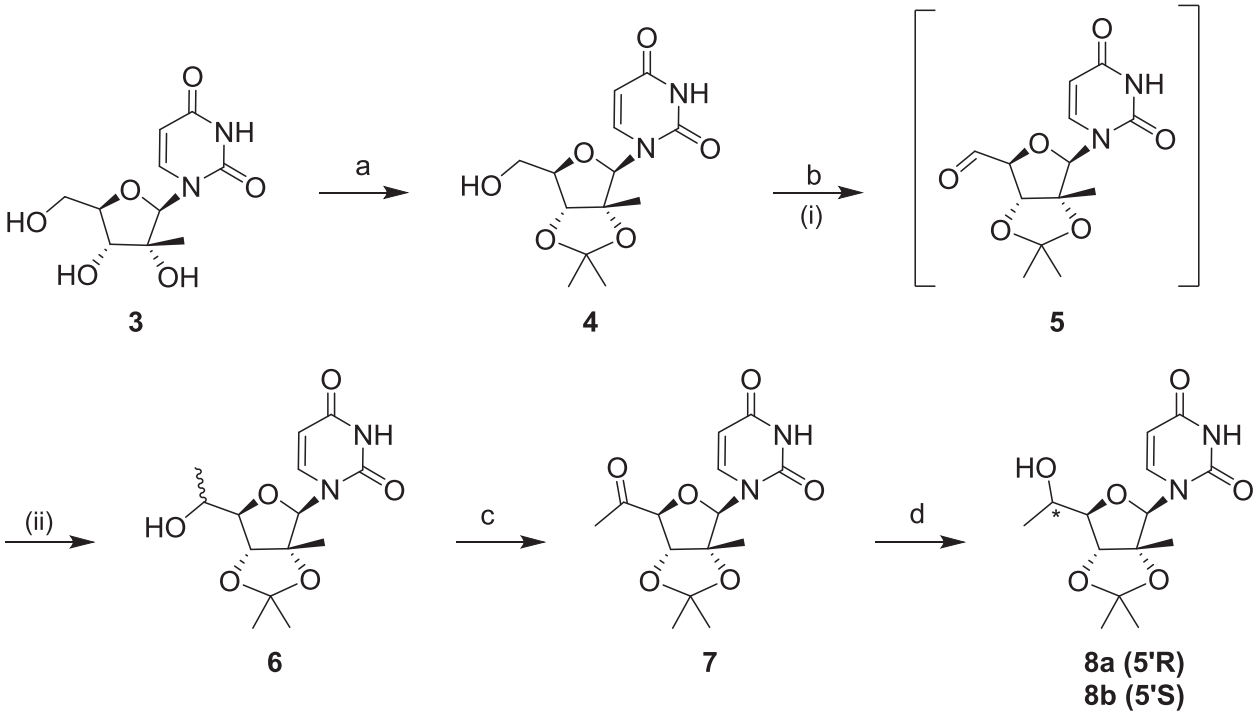


Fig. 3. Our envisaged 5′-methyl nucleotide **2a** (in the *R* configuration, and as the diphosphate for modeling purposes), docked into the HCV NS5B receptor (PDB ID 4WTG). Compound **2a** (in green) is well accommodated in the active site and is able to adopt a very similar conformation to co-crystallized sofosbuvir (in white, as the diphosphate). Compound **2b** (*S*), required a significant deviation from the co-crystal conformation in order to fit into the pocket (shown in supplementary material).



Scheme 1. Synthesis of 5′(*R/S*)-methyl derivatives of 2′-*C*-methyluridine. Reagents and Conditions: (a) Acetone, cat. H_2SO_4, reflux, 77%; (b) (i) IBX, CH_3CN, 85 °C; (ii) CH_3MgBr (3 M in Et_2O), −78 °C, 61% (crude yield over two steps); (c) IBX, CH_3CN, 80 °C, 94%; (d) RuCl(p-cymene)[(*R,R*)-TsDPEN], HCO_2Na, H_2O, EtOAc, RT, 74% for **8a**; RuCl(p-cymene)[(*S,S*)-TsDPEN], HCO_2Na, H_2O, EtOAc, RT, 72% for **8b**.

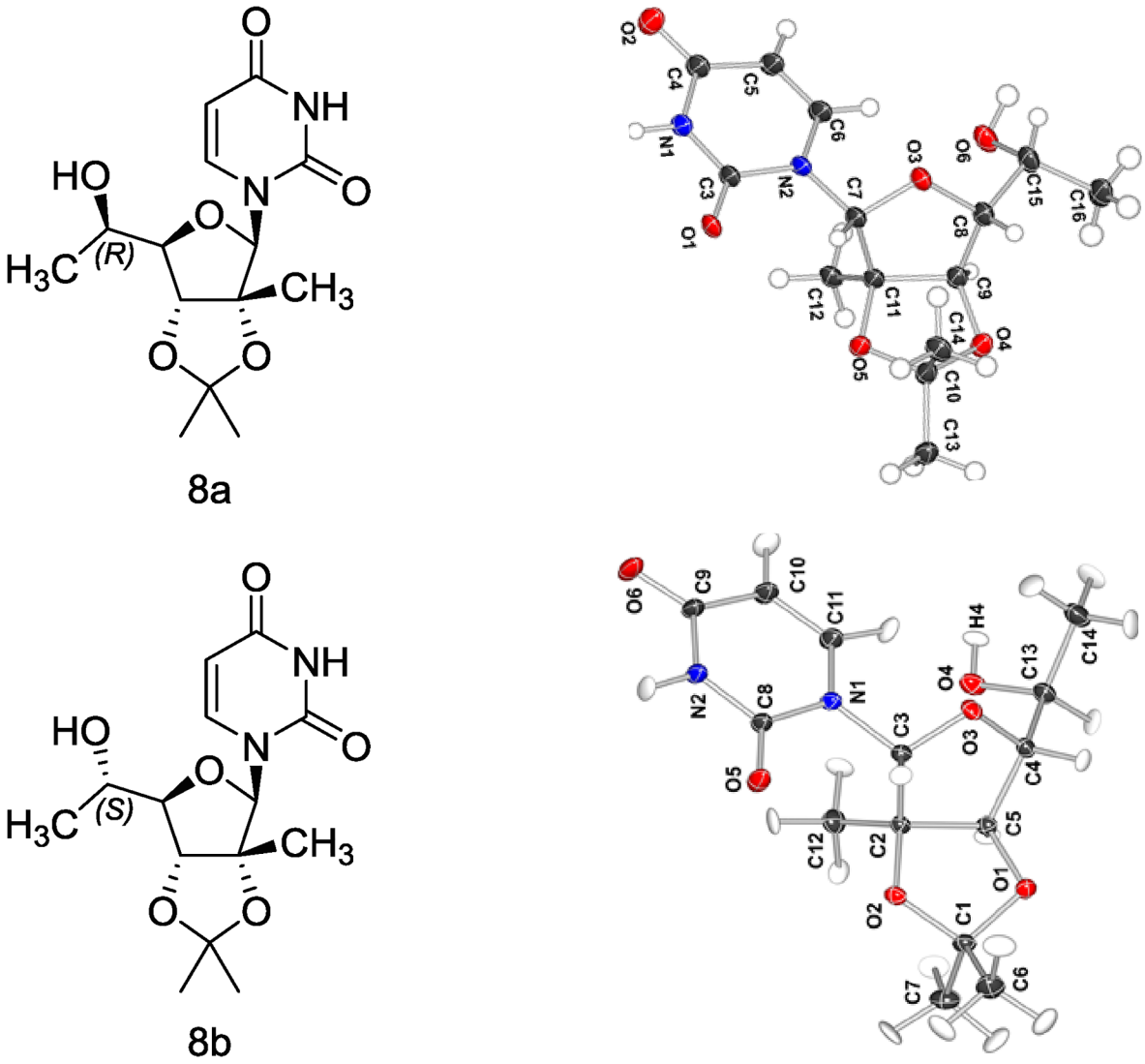


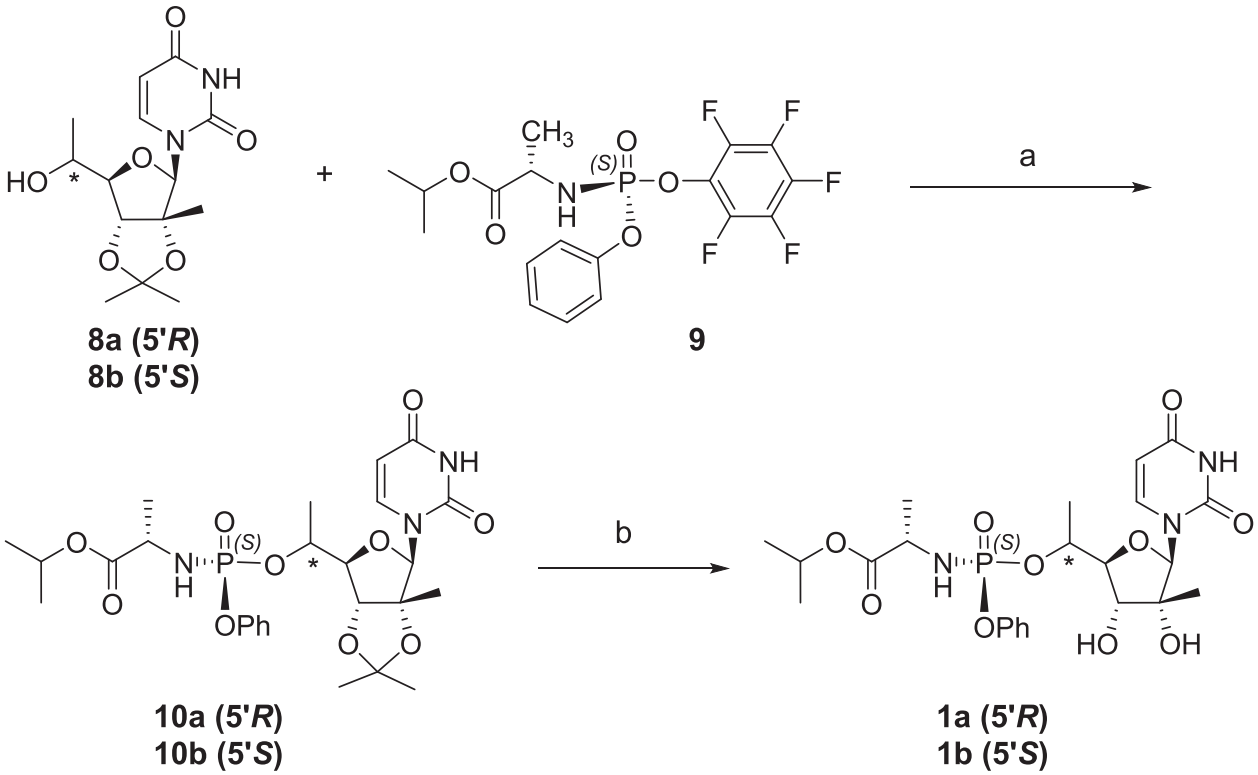
Fig. 4. X-ray crystal structures of 5′-epimers of 2′,5′-*C*-dimethyluridine.

diastereoselectivities. A further attempt of asymmetric reduction using the CBS catalyst was also unfruitful. By contrast, Noyori's asymmetric transfer hydrogenation (ATH) using a RuCl(p-cymene) catalyst in presence of varying TsDPEN chiral ligands efficiently generated the alcohols **8a** (5′(*R*), 74%, 95:5 dr) and **8b** (5′(*S*), 72%, 98:2 dr).[33] The absolute configurations of the alcohols **8a** and **8b** were determined by single crystal X-ray analysis (Fig. 4), showing the 5′-carbon in **8a** with the (*R*)-configuration (CCDC# 2002775) and **8b** with the (*S*)-configuration (CCDC# 2002762).

The 5′-*C*-methyl alcohols **8a** and **8b** were then converted to their corresponding phosphoramidate prodrugs as shown in Scheme 2. Coupling of the alcohols **8a** or **8b** with a pre-synthesized, diastereomerically-pure phosphoramidate moiety **9**,[34] generated the protected, diastereomerically-pure prodrugs **10a** or **10b** in 67% and 55% yields, respectively, using ^{t}BuMgCl as a base. Final deprotection of the acetonide in TFA/H_2O afforded the desired 2′,5′-*C*-dimethyl prodrugs **1a** (5′(*R*), 56% yield) and **1b** (5′(*S*), 66% yield).

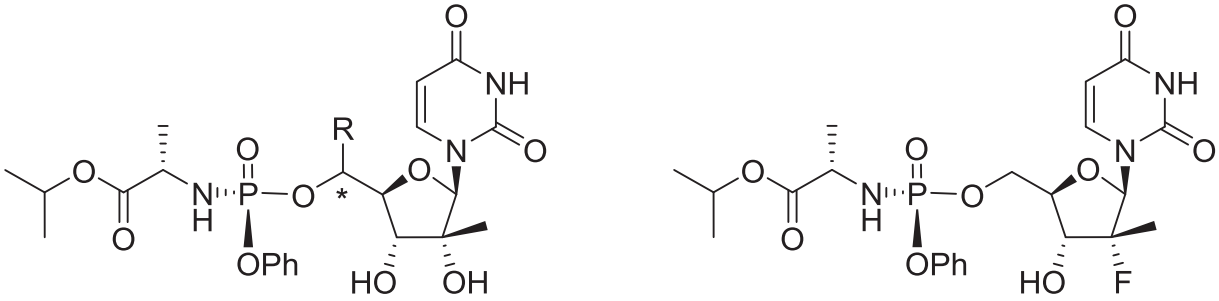
The antiviral activities of the 2′,5′-*C*-dimethyluridine prodrugs **1a** and **1b** were assessed using a replicon assay against HCV genotype 1b and cytotoxicities were established using Huh-7-Luc/Neo ET cells (Table 1). The anti-HCV potency of the phosphoramidate prodrug **1a** (EC_{50} = 1.49 μM) is 5.2 fold greater than its diastereomeric prodrug **1b** (EC_{50} = 7.75 μM), and both exhibited negligible cytotoxicity. The better antiviral activity of prodrug **1a** over **1b** demonstrates that the orientation of the newly installed methyl group at 5′- carbon is an important factor in the observed anti-HCV activities of these compounds. Nevertheless, both of the 5′-*C*-methyl derivatives (**1a** and **1b**) exhibited significantly poorer potency than either the parent unmethylated prodrug (**1c**) or sofosbuvir (control, standard of care for HCV).

We hypothesized that the poorer anti-HCV activity of both **1a** and **1b** relative to the unmethylated parent nucleotide **1c** arises from unfavorable steric interactions of the 5′-*C*-methyl group in the active sites of the kinases that catalyze the second and/or third phosphorylation steps. To test this hypothesis, we prepared the diphosphate prodrugs **11a**, **11b,** and **11c**, assuming that these would circumvent the second kinase blockade and enable efficient triphosphate formation. These syntheses were achieved by following Meier's Di*PP*ro strategy using the *bis*(decyloxybenzyl) moiety as lipophilic masking group (synthesis of these compounds can be found in the accompanying Supplementary Material).[35] The antiviral activity and cytotoxicity of these diphosphate prodrugs were evaluated in replicon cells against HCV genotype 1b using Huh-7-Luc/Neo ET cells (Table 2). To our surprise, the diphosphate prodrugs **11a** and **11b** also showed poor potency compared to their parent diphosphate prodrug **11c**. One potential rationale for this is based on the relative conformational differences between the mono- and diphosphate derivatives of the 5′-*C*-methylated and unmethylated nucleosides. If one assumes that both the nucleobase and the



Scheme 2. Synthesis of phosphoramidate prodrugs of 5′(*R*/*S*),2′-*C*-dimethyluridine. Reagents and Conditions: (a) ^{t}BuMgCl, THF/NMP, −5 °C – RT (**10a**, 67% yield; **10b**, 55% yield); (b) TFA: H_2O (1:1), RT (**1a**, 56% yield; **1b**, 66% yield).

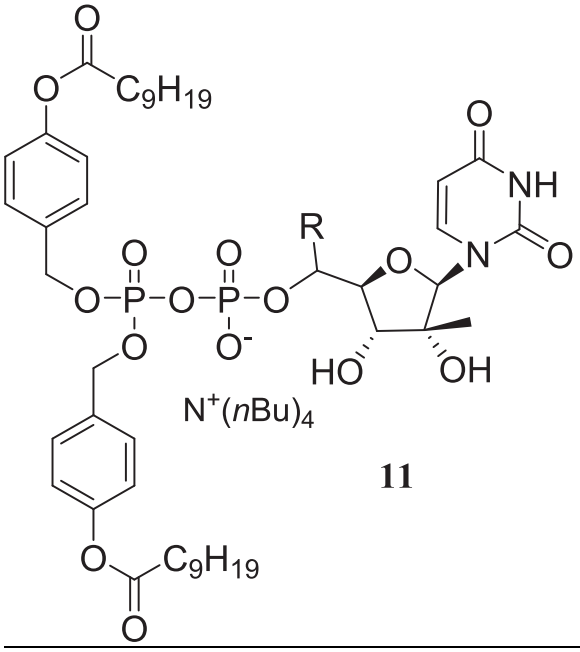
Table 1
Anti-HCV activity and cytotoxicity of 2′,5′-*C*-dimethyluridine prodrugs in the replicon assay.



Compound	R	Replicon[a]		Therapeutic index
		EC_{50} (μM)	TC_{50} (μM)	
1a	(*R*)-Me	1.49	> 100	> 67.1
1b	(*S*)-Me	7.75	> 100	> 12.9
1c	H	0.03	85.6	2779
Sofosbuvir (Control)		0.03	> 1.0	> 29.2

[a] Huh-7 Luc/Neo ET cells bearing HCV genotype 1b were used. EC_{50} and TC_{50} values represent an average of triplicate experiments.

Table 2
Anti-HCV activity and cytotoxicity of diphosphate prodrugs of 2′,5′-*C*-dimethyluridine in replicon assay.



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Compound	R	Replicon[a]		Therapeutic index
		EC_{50} (μM)	TC_{50} (μM)	
11a	(*R*)-Me	2.60	> 100	> 38.5
11b	(*S*)-Me	25.3	> 83.2	3.29
11c	H	0.04	> 100	> 2591
Sofosbuvir (Control)		0.05	> 1.0	> 21.3

[a] Huh-7 Luc/Neo ET cells bearing HCV genotype 1b were used. EC_{50} and TC_{50} values represent an average of triplicate experiments.

nucleoside's 5′-hydroxyl group (or it's 5′-monophosphate and it's 5′-diphosphate) are important recognition sites for nucleoside kinases, the presence of the 5′-methyl group might significantly reduce the concentrations of their bioactive conformations.

In conclusion, 5′-*C*-methylated mono- and diphosphate prodrugs of 2′-*C*-methyluridine were synthesized with high diastereoselectivity and evaluated for their toxicity and anti-HCV activity *in vitro*. The 5′(*R*)-*C*-methyl prodrug **1a** exhibited better anti-HCV activity and therapeutic efficacy than the corresponding 5′(*S*)-analog **1b**. However, the activities of both of these compounds were poorer than the unmethylated parent nucleotide prodrug **1c**. A similar trend was observed for the diphosphate prodrugs (**11a** and **11b**), indicating that 5′-*C*-methylation is impairing the catalytic activity of the nucleotide kinases responsible for di- and triphosphate conversion.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127539.

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