Application of the Phosphoramidate ProTide Approach to 4′-Azidouridine Confers Sub-micromolar Potency versus Hepatitis C Virus on an Inactive Nucleoside

Plinio Perrone,[†] Giovanna M. Luoni,[†] Mary Rose Kelleher,[†] Felice Daverio,[†] Annette Angell,[†] Sinead Mulready,[†] Costantino Congiatu,[†] Sonal Rajyaguru,[‡] Joseph A. Martin,[‡] Vincent Levêque,[‡] Sophie Le Pogam,[‡] Isabel Najera,[‡] Klaus Klumpp,[‡] David B. Smith,[‡] and Christopher McGuigan*[,†]

Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3XF, UK, and Roche Palo Alto, 3431 Hillview Avenue, Palo Alto, California 94304

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We report the application of our phosphoramidate ProTide technology to the ribonucleoside analogue 4′-azidouridine to generate novel antiviral agents for the inhibition of hepatitis C virus (HCV). 4′-Azidouridine did not inhibit HCV, although 4′-azidocytidine was a potent inhibitor of HCV replication under similar assay conditions. However 4′-azidouridine triphosphate was a potent inhibitor of RNA synthesis by HCV polymerase, raising the question as to whether our phosphoramidate ProTide approach could effectively deliver 4′-azidouridine monophosphate to HCV replicon cells and unleash the antiviral potential of the triphosphate. Twenty-two phosphoramidates were prepared, including variations in the aryl, ester, and amino acid regions. A number of compounds showed sub-micromolar inhibition of HCV in cell culture without detectable cytotoxicity. These results confirm that phosphoramidate ProTides can deliver monophosphates of ribonucleoside analogues and suggest a potential path to the generation of novel antiviral agents against HCV infection. The generic message is that ProTide synthesis from inactive parent nucleosides may be a warranted drug discovery strategy.

Introduction

The hepatitis C virus (HCV[a]) was identified for the first time in 1989 as a single-stranded positive sense RNA virus of the Flaviviridae family.[1] According to the World Health Organization (WHO), more than 170 million people are estimated to be chronically infected by this virus, which is a major cause of severe liver disease.[2]

At present, treatment options comprise immunotherapy using recombinant interferon (often pegylated) in combination with ribavirin. The clinical benefit of this treatment is limited, and a vaccine has not yet been developed. The development of selective inhibitors of essential viral enzymes such as the serine protease NS3 or the RNA-dependent RNA polymerase NS5b are expected to improve the potency and tolerability of future treatment options for HCV infected patients.[3,4]

Nucleoside analogues have already been validated as an important class of polymerase inhibitors of other viral targets, such as HCMV, HSV, HIV, and HBV.[5] All antiviral agents acting via a nucleoside analogue mode of action need to be phosphorylated, most of them to their corresponding 5′-triphosphates, by cellular and/or viral enzymes. The nucleotide triphosphate analogues will then inhibit the requisite polymerase and/or compete with natural nucleotide triphosphates as substrates for incorporation into viral nucleic acid during viral replication.[5]

Recently, 4′-azidocytidine was discovered as a potent inhibitor of HCV replication in cell culture. The corresponding 5′-triphosphate was described as a competitive inhibitor of cytidylate incorporation by HCV polymerase and a potent inhibitor of native, membrane-associated HCV replicase in vitro.[6]

Interestingly, the corresponding uridine analogue, 4′-azidouridine (1), was inactive as an inhibitor of HCV replication in the cell-based replicon system.[7]

It was hypothesized that (1) (1, Figure 1) may be a poor substrate for phosphorylation by cellular enzymes. The first phosphorylation step to produce the 5′-monophosphate has often been found to be the rate-limiting step in the pathway to intracellular nucleotide triphosphate formation, suggesting that nucleoside monophosphate analogues could be useful antiviral agents. However, as unmodified agents, nucleoside monophosphates are unstable in biological media and they also show poor membrane permeation because of the associated negative charges at physiological pH.[8,9]

Our aryloxy phosphoramidate ProTide approach allows bypass of the initial kinase dependence by intracellular delivery of the monophosphorylated nucleoside analogue as a membrane permeable "ProTide" form.[10,11] This technology greatly increases the lipophilicity of the nucleoside monophosphate analogue with a consequent increase of membrane permeation and intracellular availability. Previously we have demonstrated the success of our approach with the aryloxy-phosphoramidate derivatives of ddA,[10] d4T,[11,12] LCd4A,[13] and d4A.[10,14] These nucleotide monophosphate analogues were shown to exhibit greatly enhanced activity against HIV compared to the parent nucleoside analogues in vitro. In contrast to the parent nucleosides, full antiviral activity of the monophosphate analogues was retained in kinase-deficient cell lines, which was consistent with an efficient bypass of the first phosphorylation step in HIV infected cells. Aryloxy-phosphoramidates are considered to be efficient lipophilic prodrugs of the corresponding 5′-monophosphate species in which the two masking groups are represented by an amino acid ester and an aryl moiety. After passive diffusion

* Corresponding author: Tel. +44 29 20874537; fax: +44 29 20874537; mcguigan@cardiff.ac.uk.
[†] Cardiff University.
[‡] Roche Palo Alto.

[a] Abbreviations: NS3, nonstructural protein 3; NS5B, nonstructural protein 5B; HSV, herpes simplex virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; d4A/d4T, 2′,3′-dideoxy-2′,3′-didehydroadenosine/thymidine; ddA, 2′,3′-dideoxyadenosine; LCd4A, (1*R*,*cis*)-4-(6-Amino-9*H*-purin-9-yl)-2-cyclopentene-1-methanol; BVdU, *E*-5-(2-bromovinyl)-2′-deoxyuridine; HCMV, human cytomegalovirus; AZU, 4′-azidouridine; NMI, *N*-methylimidazole.

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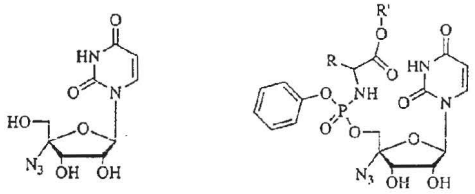


AZU (1) 2

Figure 1. Structure of AZU and its corresponding phenyl-phosphoramidate ProTide.

through cell membranes, the suggested activation pathway[15] involves initial enzyme-catalyzed cleavage of the carboxylic ester, followed by the internal nucleophilic attack of the acid residue on the phosphorus center, displacing the aryloxy group. The putative transient, cyclic mixed-anhydride is then rapidly hydrolyzed to the corresponding amino acid phosphomonoester. Last, a suggested phosphoramidase activity catalyzes the cleavage of the P−N bond to free the nucleoside monophosphate intracellularly. In the current study we tested the possibility to apply our ProTide approach to the inactive 4′-azidouridine (**1**) in order to achieve bypass of the first phosphorylation step and thereby generate novel antiviral agents (**2**) with potent activity against HCV.

Results and Discussion

Chemistry. The synthesis of **1** has been previously described.[16] To prepare monophosphate prodrugs (**2**) of **1** we initially followed the previously described phosphorochloridate chemistry for the synthesis of ProTides developed in our laboratory, using 1-methylimidazole (NMI) as the coupling agent.[14,17,18] Several attempts were performed using different conditions (different amino acid esters, different reaction conditions) without successful isolation of the corresponding aryloxy-phosphoramidate. These initial unsuccessful attempts might be explained considering the presence of a bulky group (azido) at the 4′-position adjacent to the coupling site at the 5′-position; in all previously published ProTide examples the 4′-position was unsubstituted.

The method of Uchiyama was investigated next.[19] This approach is based on the treatment of a nucleoside with 1 equiv of a strong organometallic base, such as a solution of *tert*-butylmagnesium chloride (tBuMgCl), to form the corresponding metal alkoxide. In the case of (**1**), this reaction was observed to be very rapid and gave yields between 3% and 20% of desired products. In the first instance, we synthesized 4′-azidouridine phosphoramidates starting from an unprotected nucleoside. The apparent reactivity at the 2′- and 3′-positions was low, suggesting high regioselectivity for the reaction at the 5′-position. In this way it was also possible to synthesize compounds **13**, **21**, and **26**. In order to achieve higher solubility in the reaction solvent (tetrahydrofuran) and increase reactivity at the 5′ position, the 2′- and 3′-positions of **1** were protected with a cyclopentyl group.[20] The final synthetic pathway (Scheme 1) involves the coupling of phenyl dichlorophosphate with different amino acid ester salts (**5**) to give the corresponding phenyloxy-phosphorochloridates (**6**), which were purified by flash chromatography and then coupled with the 2′,3′-*O*,*O*-cyclopentylidene derivative **7** of (**1**) in the presence of tBuMgCl (1 M solution in THF).

The deprotection step was performed with a solution of 80% formic acid in water for 4 h at room temperature (Scheme 2). Due to the stereochemistry at the phosphorus center, the final compounds were always isolated as mixtures of two diastereoisomers. The presence of these diastereoisomers in the final preparations was confirmed by ^{31}P (two peaks), ^{1}H, and ^{13}C NMR. A total of 22 phenyl phosphoramidates were synthesized as reported in Table 1.

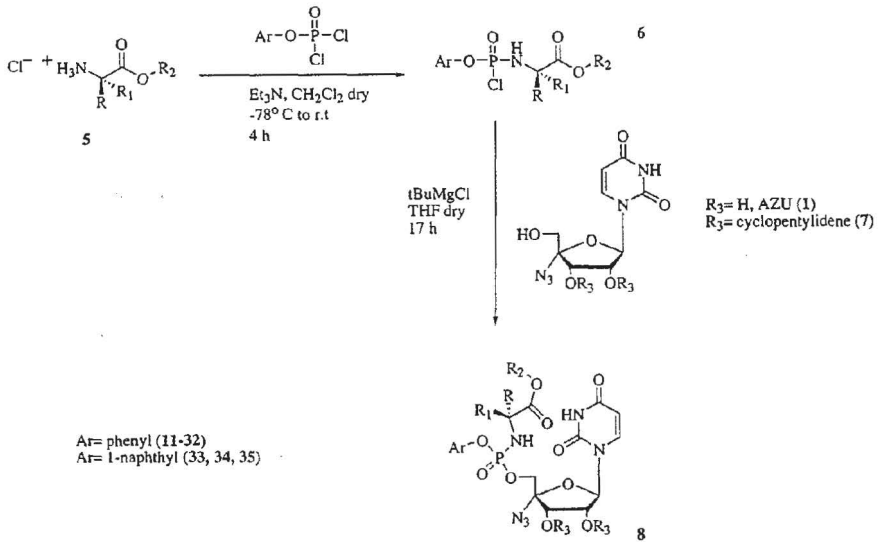
We have previously reported extensive structure−activity relationship (SAR) studies of anti-HIV phosphoramidates exploring the amino acid region, including natural amino acid variation,[21] un-natural α,α-dialkyls,[22] stereochemical variation,[23] and amino acid extensions[24] and replacements.[25] In general, L-alanine and the unnatural amino acid α,α-dimethylglycine showed the best activity for the d4T parent molecule versus HIV.[11,12]

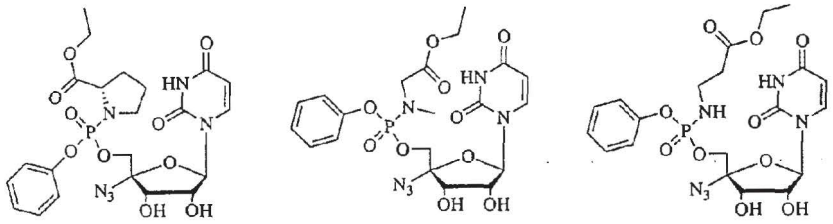
Using the previously described method (Scheme 1), we synthesized the L-alanine (**12**), α,α-dimethylglycine (**18**), cyclopentylglycine (**20**), L-phenylalanine (**22**), L-leucine (**27**), L-methionine (**29**), ethyl L-glutamate (**31**), and L-proline (**28**) phenylphosphoramidates of **1**, each bearing an ethyl ester. Further investigations on the amino acid variation were conducted on a series of benzyl esters: L-alanine (**17**), α,α-dimethylglycine (**19**), cyclopentylglycine (**21**), L-phenylalanine (**23**), L-valine (**24**), and glycine (**25**). We further compared the importance of the stereochemistry at the amino acid position by preparing a D-alanine benzyl ester phosphoramidate (**26**). On the basis of the L-alanine phenyl phosphoramidate backbone, we also explored the SAR of different esters including methyl (**11**), ethyl (**12**), butyl (**13**), 2-butyl (**14**), isopropyl (**15**), *tert*-butyl (**16**), and benzyl (**17**). In order to have an indirect proof of phenyl phosphoramidate metabolism, we synthesized the *N*-methylglycine (**30**) and *β*-alanine (**32**) analogues, which were considered unfavorable substrates according to the postulated mechanism of activation.[15]

Recently we noted an increase of *in vitro* potency of a 1-naphthyl-phosphoramidate analogue compared to the corresponding phenyl derivative while investigating the anticancer activity of BVdU phosphoramidates.[26] Therefore, similar phosphoramidate analogues were also generated for (**1**). The synthesis of the 1-naphthyl phosphoramidate (**33**) was performed by reacting 1-naphthol with phosphorus oxychloride in an almost quantitative reaction to give the corresponding phosphorodichloridate (Scheme 3), which was then coupled to an amino acid ester and the nucleoside analogue according to our standard procedures. In this case, the separation of the two phosphate diastereoisomers (**34** and **35**) was achieved by using a semi-preparative HPLC purification with elution conditions of 70% water/30% acetonitrile. The ^{31}P NMR spectrum showed the presence of only one peak for the first of the two fractions separated, and the ^{1}H NMR spectrum supported the suggestion of a single diastereoisomer in this case. The second fraction contained an excess of the second diastereoisomer together with a minor proportion (estimated at 7% by ^{31}P NMR integration) of the first diastereoisomer (see Supporting Information for data).

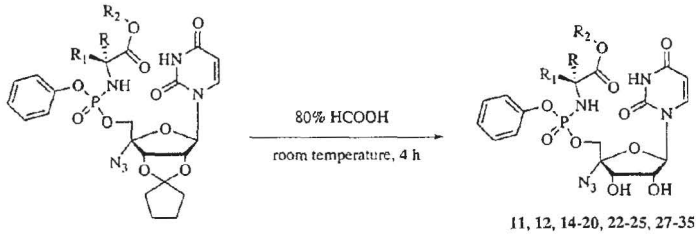
Antiviral Activity. The phenyl phosphoramidates described above (**11**−**32**) were characterized *in vitro* as inhibitors of HCV replication in a HCV replicon assay as previously reported.[6,7] Data are presented in Table 1 as EC_{50} values (representing the concentration of compounds reducing HCV replication by 50%) and CC_{50} values (representing the concentration of compounds reducing cell viability by 50%) as determined using the WST assay. All compounds showed CC_{50} values greater than 100 μM. The parent compound **1** did not inhibit HCV replication significantly in the replicon system (EC_{50} > 100 μM). In contrast, a number of phosphoramidate derivatives showed

Scheme 1. General Synthetic Pathway for the Synthesis of AZU Aryloxy-phosphoramidates





Scheme 2. General Synthetic Pathway for the Deprotection of 2′-3′-cyclopentylidene AZU Phenyl-phosphoramidates



potent inhibition of HCV replication. Assuming that 4′-azidouridine-5′-triphosphate is the active HCV polymerase inhibitor, these results support the notion that the active phosphoramidates successfully delivered 4′-azidouridine monophosphate intracellularly, that 4′-azidouridine (**1**) is inefficiently phosphorylated to the monophosphate in replicon cells, and that 4′-azidouridine monophosphate can be phosphorylated to the 5′-triphosphate in replicon cells. As shown in Table 2, 4′-azidouridine triphosphate notably inhibited recombinant HCV polymerase NS5b in vitro, and did so with similar sub-micromolar potency, like that of the previously described NS5b inhibitor R1479-TP (4′-azidocytidine triphosphate).[6] Therefore, the application of our phosphoramidate approach shown to be a successful tool in overcoming the phosphorylation block of **1** and converting an inactive nucleoside analogue to a potent inhibitor of HCV replication, thus accessing the full potential of the 4′-azidouridine triphosphate.

As shown in Table 1, L-alanine derivatives represented a series of active antiviral phosphoramidates (**11**−**17**). Low or sub-micromolar activity was noted in marked contrast to the *inactive* nucleoside parent (**1**). The *tert*-butyl ester (**16**) was the least active of the series, which was in agreement with the SARs previously obtained in the d4T series[24] and may relate to the relative stability of tertiary esters to enzyme-mediated hydrolysis. The isopropyl ester (**15**) showed high potency and represented one of the most active phosphoramidates prepared. Similarly, the 2-butyl ester (**14**) was highly active in our assay in contrast to previous observations with other nucleoside analogues. Together with the benzyl analogue (**17**), these three esters provided the most potent compounds of HCV replication

Table 1. Anti HCV Activity and Cytotoxicity Data for (1) and Phenyl Phosphoramidate Nucleotide Analogues

compound	amino acid	ester	EC_{50} (μM)	CC_{50} (μM)
11	L-Ala	Me	3.1	>100
12	L-Ala	Et	1.3	>100
13	L-Ala	Bu	1.2	>100
14	L-Ala	2-Bu	0.63	>100
15	L-Ala	iPr	0.77	>100
16	L-Ala	tBu	5.1	>100
17	L-Ala	Bn	0.61	>100
18	Me_2Gly	Et	10.3	>100
19	Me_2Gly	Bn	3.4	>100
20	cPntGly	Et	>100	>100
21	cPntGly	Bn	<100	>100
22	Phe	Et	1.37	>100
23	Phe	Bn	<100	>100
24	Val	Bn	<100	>100
25	Gly	Bn	1.6	>100
26	D-Ala	Bn	1.2	>100
27	Leu	Et	2.3	>100
28	Pro	Et	6.0	>100
29	Met	Et	14	>100
30	N-MeGly	Et	>100	>100
31	EtGlu	Et	>100	>100
32	β-Ala	Et	>100	>100
4′-azidouridine (1)	–	–	>100	>100

Scheme 3. Synthetic Pathway for the Synthesis of 1-naphthylphosphorodichloridate

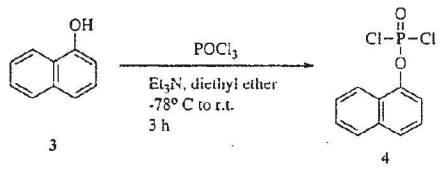


Table 2. Inhibition of HCV Polymerase (NS5B) Activity in Vitro

	IC_{50} [μM]	
enzyme	R1479-TP (4′-azido CTP)	4′-azido UTP
NS5B570n-BK	0.29 ± 0.13	0.23 ± 0.01
NS5B570-Con1	0.32 ± 0.11	0.22 ± 0.02

inhibitors in the L-alanine series, all having μM inhibition of HCV. The antiviral activity of these three phosphoramidates was exceptional if compared to the parent compound 1 (EC_{50} > 100 μM), providing strong support for the notion of ProTide-mediated kinase bypass.

In the benzyl ester family, L-alanine (**17**) provided the most active compound with D-alanine (**26**) and glycine (**25**) being only slightly less potent. These results were striking when compared to the 60−70 fold reduction in anti-HIV potency for d4T ProTides with an L-alanine to glycine replacement and a 20−40 fold reduction for the corresponding abacavir ProTides.[21,27] This reinforces our earlier conclusion that a separate ProTide motif optimization process is needed for each nucleoside analogue versus a given target. It may be that cell line dependent enzyme expression may determine different phosphoramidate SARs.

The presence of a methyl (D- and L-alanine, **26** and **17**) or α,α-dimethyl (**19**) enhanced the activity if compared to larger and hydrophobic amino acid side chain residues such as L-valine (**24**), L-phenylalanine (**23**), and cyclopentylglycine (**21**), which were weakly active in the replicon assay.

An unexpected correlation was found between amino acid and ester function. While the L-phenylalanine derivative was substantially inactive as a benzyl ester (**23**), the corresponding ethyl ester (**22**) showed a significantly increased antiviral activity, displaying an EC_{50} value of 3.4 μM. Therefore, matrix-based optimization of amino acid and ester functions may be preferred over stepwise approaches.

Table 3. Anti HCV Activity and Cytotoxicity Data for (1) and 1-Naphthyl Nucleotide Analogues

compound	phosphorus configuration	amino acid	ester	EC_{50} (μM)	CC_{50} (μM)
33	*S/R*	L-Ala	Bn	0.22	>100
34	R	L-Ala	Bn	0.39	>100
35	S	L-Ala	Bn	0.43	>100
17 (Phenyl ProTide)	*S/R*	L-Ala	Bn	0.61	>100
4′-azidouridine (1)				>100	>100

The inactivity of the β-alanine (**32**) and of the *N*-methyl glycine (**30**) compounds might underline the presence of an α-amino acid and a free NH as a minimum requirement in the amino acid structure to enable the metabolic activation of aryloxy-phosphoramidates. However, the proline compound (with a blocked NH) did show modest (**28**) activity, pointing to a complex amino acid SAR.

In conclusion, ester variation was widely tolerated except for the *tert*-butyl which gave a slight reduction in potency in the L-alanine series (**16**) and the benzyl in the case of the L-phenylalanine derivative (**23**). L-Alanine remained the most effective amino acid, with glycine and D-alanine showing only slightly reduced potency. Dimethylglycine, L-leucine, and L-proline also provided compounds with antiviral potencies in a low micromolar range. It therefore appears that the amino acid core could be considerably varied to give antiviral agents with potencies within a 10-fold range in replicon cells. Importantly, potency optimization requires consideration of both amino acid and ester moieties as most clearly shown for the ethyl and benzyl esters of the L-phenylalanine analogues. Moreover, quite distinct SARs emerged from this family versus HCV as compared to our prior studies in other families.

We also explored the possibility to replace the phenyl substituent on the phosphate with a more hydrophobic moiety, 1-naphthyl. Previously, we noted an increase of *in vitro* potency of 1-naphthyl-phosphoramidates compared to the corresponding phenyl phosphoramidates when investigating BVdU phosphoramidates in an anticancer assay.[26] We synthesized **33**, the 1-naphthyl analogue of **17** (L-alanine benzyl ester). As shown in Table 3, compound **33** inhibited HCV replication with an EC_{50} of 0.22 μM, leading to a further increase in antiviral activity (>450-fold) in comparison to 4′-azidouridine (Table 3). One of the two phosphorus diastereoisomers could be purified using a C-18 reverse phase semipreparative HPLC. One of the two main fractions obtained showed only one ^{31}P NMR peak. The second fraction was less pure, although the second diastereoisomer appeared as the major component of the mixture. We have previously reported a method for the prediction of the phosphorus configuration of such diastereoisomers based on a different ^{1}H NMR profile of the methylene protons of the benzyl ester.[26] Applying this concept to compounds **34** and **35**, we noticed that in one case (more polar, **35**) a clear AB-system was observed while, for the other diastereoisomer (less polar, **34**), the two protons displayed an apparent doublet. Conformational studies were performed using the Sybyl 7.0 software package. The lowest energy conformation found for each diastereoisomer is shown in Figure 2. These differences in proton profiles can be explained by the ability of one, but not the other, diastereoisomer to form $\pi-\pi$ interactions between the naphthyl and the phenyl group of the benzyl ester resulting in a constrained conformation. This interaction can only occur with the *S* phosphorus configuration (**35**) with the two methylene

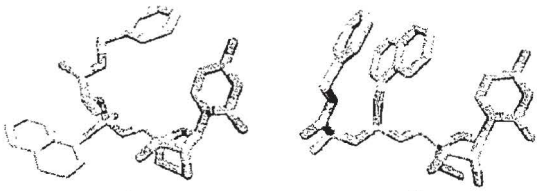


Figure 2. Lowest energy conformations of compounds 34 and 35.

protons becoming nonmagnetically equivalent (AB system). For the diastereoisomer with *R* phosphorus configuration (**34**), this interaction does not occur and the higher degree of flexibility around the methylene renders its protons more magnetically similar (apparent doublet). The biological activities of the separated diastereoisomers (**34** and **35**) were comparable to each other and to the mixture (**33**) (Table 3).

Interestingly, application of similar ProTide methods to the active 4′-azidocytidine gave little or no boost in anti-HCV activity (data not shown), implying a rather efficient phosphorylation of this nucleoside analogue, with which ProTide methods presumably cannot compete.

Conclusion

A series of phosphoramidate ProTides of 4′-azidouridine were prepared and evaluated as inhibitors of HCV replication *in vitro*. The phosphoramidate approach provided novel compounds with highly increased potency in the replicon assay when compared to the inactive parent compound, corresponding to boosts in anti-HCV potency of >450-fold. All phosphoramidates tested were nontoxic in the replicon assay (CC_{50} > 100 μM). The most active compound prepared in the series was the 1-naphthyl L-alanine benzyl ester phosphoramidate with an EC_{50} of 0.22 μM in the replicon assay. The diastereoisomers of this compound were separated by HPLC and their absolute phosphorus configurations predicted by modeling and NMR. However, they did not show any differences in biological activity. This report demonstrates the ability of the ProTide approach to successfully bypass the rate limiting initial phosphorylation of a ribonucleoside analogue and thus confer significant antiviral activity on an inactive parent nucleoside.

Experimental Section

Biology. HCV replicon assay was performed in the stable replicon cell line 2209-23 derived from Huh-7 cells stably transfected with a bicistronic HCV replicon (genotype 1b) expressing the renilla luciferase reporter gene, as described.[7] The RNA synthesis activity of recombinant HCV polymerase proteins was measured as incorporation of radiolabeled UMP into acid-insoluble RNA products using HCV genome derived cIRES RNA as a template in a primer-independent RNA synthesis assay.[7] Recombinant proteins used were truncated at amino acid position 570 and derived from genotype 1b strain BK (NS5B570n-BK) or Con1 (NS5B570-Con1).

Chemistry. General Procedures. All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Anhydrous tetrahydrofuran and dichloromethane were purchased from Aldrich. Proton, carbon, and phosphorus Nuclear Magnetic Resonance (^{1}H, ^{13}C, ^{31}P NMR) spectra were recorded on a Bruker Avance spectrometer operating at 500, 125, and 202 MHz, respectively. All ^{13}C and ^{31}P spectra were recorded proton-decoupled. All NMR spectra were recorded in CD_3OD at room temperature (20 °C ± 3 °C). Chemical shifts for ^{1}H and ^{13}C spectra are quoted in parts per million downfield from tetramethylsilane. Coupling constants are referred to as *J* values. Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), broad signal (br), doublet of doublet (dd), doublet of triplet (dt), or multiplet (m). Chemical shifts for ^{31}P spectra are quoted in parts per million relative to an external phosphoric acid standard. Many proton and carbon NMR signals were split due to the presence of (phosphate) diastereoisomers in the samples. The mode of ionization for mass spectroscopy was fast atom bombardment (FAB) using MNOBA as matrix. Column chromatography refers to flash column chromatography carried out using Merck silica gel 60 (40−60 μM) as stationary phase.

For convenience, standard procedures have been given, as similar procedures were employed for reactions concerning the synthesis of precursors and derivatives of ProTides. Variations from these procedures and individual purification methods are given in the main text. Preparative and spectroscopic data on individual precursor, blocked nucleosides are given as Supporting Information only (see below), excluding only the first example.

Standard Procedure 1: Preparation of 2′,3′-*O*,*O*-Cyclopentylidene-4′-azidouridine Phosphoramidates. ^{t}BuMgCl (2.0 mol equiv) and 2′,3′-*O*,*O*-cyclopentylidene-4′-azidouridine (1.0 mol equiv) were dissolved in dry THF (31 mol equiv) and stirred for 15 min. Then a 1 M solution of the appropriate phosphorochloridate (2.0 mol equiv) in dry THF was added dropwise and then stirred overnight. A saturated solution of NH_4Cl was added, and the solvent was removed under reduced pressure to give a yellow solid, which was consequently purified by chromatography.

Standard Procedure 2: Deprotection of 2′,3′-Protected 4′-Azidouridine Phosphoramidates. The appropriate 2′,3′-*O*,*O*-cyclopentylidene-4′-azidouridine phosphoramidate was added to a solution 80% of formic acid in water. The reaction was stirred at room temperature for 4 h. The solvent was removed under reduced pressure, and the obtained oil was purified by chromatography.

Standard Procedure 3: Preparation of 4′-Azidouridine Phosphoramidates via Free Nucleoside. ^{t}BuMgCl (2.0 mol equiv) and 4′-azidouridine (1.0 mol equiv) were dissolved in dry THF (31 mol equiv) and stirred for 15 min. Then a 1 M solution of the appropriate phosphorochloridate (2.0 mol equiv) in dry THF was added dropwise and then stirred overnight. A saturated solution of NH_4Cl was added, and the solvent was removed under reduced pressure to give a yellow solid, which was purified by chromatography.

HPLC Method Used for the Separation of Compound 34 and 35. Varian ProStar instrument using a Polaris C18-A 10u column; elution was performed using a mobile phase consisting of water/acetonitrile 70% H_2O/30% CH_3CN, 17 min elution time with a flow of 20 mL/min. Optimal loading on column: 8 mg of phosphoramidate per run.

Synthesis of 2′,3′-*O*,*O*-Cyclopentylidene-4′-azidouridine 5′-*O*-[Phenyl(methyloxy-L-alaninyl)] Phosphate (Methyl *N*-[[1-[(3a*R*,4*R*,6*R*,6a*S*)-4-Azido-tetrahydro-4-(hydroxymethyl)-2,2-cyclopentylfuro[3,4-*d*][1,3]dioxol-6-yl]pyrimidine-2,4(1*H*,3*H*)-dione} (Phenoxy)-phosphoryl]-L-alaninate). Prepared according to the standard procedure 1, from 2′,3′-*O*,*O*-cyclopentylidene-4′-azidouridine (150 mg, 0.427 mmol), ^{t}BuMgCl (0.85 mL, 1 M solution in THF, 0.854 mmol), and phenyl(methyloxy-L-alaninyl) phosphorochloridate (0.85 mL of solution 1 M in THF, 0.854 mmol). The crude product was purified by column chromatography, using as eluent $CHCl_3$/MeOH (95/5). The pure product was a white solid (156 mg, 0.263 mmol, 61%). δ_P (d_4-CH_3OH): 3.14, 3.04; δ_H (d_4-CH_3OH): 7.66 (1H, t, H6-uridine), 7.35 (2H, t, 2 CH-phenyl), 7.28−7.19 (3H, m, 3 CH-phenyl), 5.97 (1H, dd, H1′-uridine), 5.70 (1H, dd, H5-uridine), 5.12−5.04 (2H, m, H2′-uridine, H3′-uridine), 4.31−4.27 (2H, m, H5′-uridine), 4.01 (1H, m, CHα), 3.70 (3H, d, CH_3-methyl), 2.21−2.11 (2H, m, CH_2-cyclopentyl), 1.79−1.73 (6H, m, 3 CH_2-cyclopentyl), 1.37 (3H, t, CH_3-alanine, J = 9.5 Hz).

Synthesis of 4′-Azidouridine 5′-*O*-[Phenyl(methyloxy-L-alaninyl)] Phosphate (Methyl *N*-[[1-(2*R*,3*S*,4*R*,5*R*)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furan-2-yl)pyrimidine-2,4-(1*H*,3*H*)-dione} (Phenoxy)-phosphoryl]-L-alaninate) (11). Prepared according to the standard procedure 2, from 2′,3′-*O*,*O*-cyclopentylidene-4′-azidouridine 5′-*O*-[phenyl(methyloxy-L-alaninyl)] phosphate (135 mg, 0.222 mmol), and a solution 80% of HCOOH in water (10 mL). The crude was purified by column chromatography,

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