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The application of the phosphoramidate ProTide approach confers micromolar potency against Hepatitis C virus on inactive agent 4′-azidoinosine: Kinase bypass on a dual base/sugar modified nucleoside

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ABSTRACT

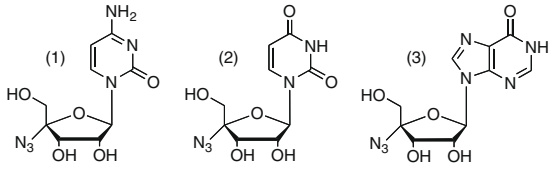
Novel phosphoramidate ProTides derived from 4′-azidoinosine have been prepared and evaluated in the replicon assay against hepatitis C Virus (HCV). The parent nucleoside analogue is inactive in this assay, while the ProTides are active at low μM levels in some cases. This is a rare example of an inosine nucleoside analogue with potent antiviral activity and further supports the notion of ProTides as a drug discovery motif.

According to the World Health Organization (WHO) more than 170 million people are infected with the hepatitis C virus (HCV), representing 3% of the world population.[1] Current therapy consists of ribavirin and pegylated interferon. Both agents are broad spectrum and more specific therapies for HCV are under development in many laboratories.[2]

One of our laboratories has reported extensively on various 4′-substituted ribonucleoside analogues as specific inhibitors of HCV, with 4′-azidocytidine (**1**) emerging as an important lead.[3] The 2′,3′,5′-triester pro-drug of this agent has demonstrated efficacy in phase 2a studies in HCV infected patients. [4–5] Further studies have focused on new nucleoside analogs with improved potency by affecting either intrinsic potency or phosphorylation efficiency. The limitations imposed by host nucleoside kinases can be severe and many nucleoside analogues are inactive due to poor phosphorylation. A good example in the HCV space is 4′-azidouridine (**2**). Thus, while the 5′-triphosphate of (**2**) is a potent, sub-μM, inhibitor of HCV RNA polymerase,[6] the nucleoside itself (**2**) is inactive as an inhibitor of HCV in the cell based replicon assay, presumably due to poor phosphorylation in vitro.[6]

It is considered that the first phosphorylation step of parent nucleoside analogues to their 5′-monophosphates is often rate-limiting, and that the free parent nucleoside monophosphates are of limited direct use in therapy due to poor membrane permeation and instability to dephosphorylation.[7] Thus, we[8] and others[9–12] have developed approaches to the intracellular delivery of preformed nucleotides via pro-drug ('ProTide') approaches. Indeed, application of our phosphoramidate ProTide approach to 4′-azidouridine (**2**) notably conferred sub-μM HCV potency on the inactive parent,[6] this being taken as proof of successful monophosphate delivery in vitro by the ProTide and further supporting the notion that (**2**) (and perhaps other analogues) fail as antivirals due to a poor initial phosphorylation step.

The combination of nucleosides and nucleotides with different bases may increase the overall efficiency of nucleoside analogs when used in combination therapy. In an attempt to identify new series of nucleotides with antiviral potency against HCV, we herein report the application of this technology for the first time to an inosine nucleoside, 4′-azidoinosine (**3**) with positive results emerging.



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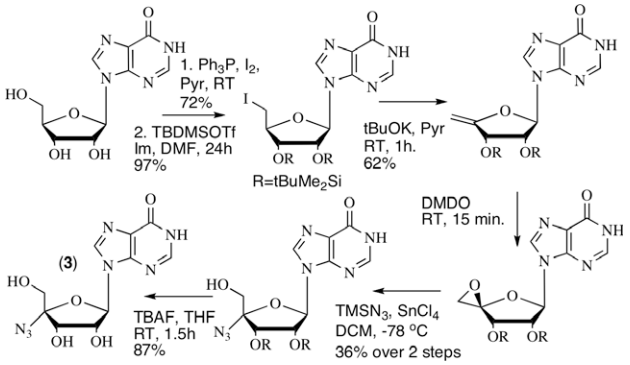


Scheme 1.

Thus 4′-azidoadenose was prepared from inosine in a 5-step route similar to that we have published for the corresponding adenosine analogue.[13] Briefly, inosine was 5′-iodinated using triphenyl phosphine and iodine, the product protected as its 2′,3′-bis (TBDMS) ether and the 4′,5′-olefin generated under basic conditions (Scheme 1).

Stereo- and regio-specific reaction of the olefin with DMDO afforded the key epoxide intermediate[13,14] which was ring opened with TMS azide and tin(IV) chloride to give the protected 4′-azido nucleoside. Desilylation with TBAF give 4′-azidoinosine (**3**). As we have previously noted, protection of the 2′,3′-diol with a cyclopentylidene group enhances the yield and regio selectively of 5′-ProTide formation,[6,13] and so (**3**) was thus protected. Appropriate phosphorylating agents were prepared by methods we have extensively reported.[6,13] Thus, phenyl or 1-naphthyl phosphorodichloridates were allowed to react with various alanine ester hydrochlorides to give the target phosphorochloridates, which were reacted with 2′,3′-*O*-cyclopentylidene-4′-azidoinosine in THF in the presence of *t*-butyl magnesium chloride, to yield protected ProTides **4a–h** (Scheme 2).

In both phenyl and 1-naphthl series the esters prepared were ethyl, *i*-propyl, *t*-butyl and benzyl. Following coupling, the protected ProTides were deprotected to yield target compounds **5a-h**.[15] Coupling yields ranged from 48% to 100% while deprotection yields were in general ca. 70–80% except for the *t*-butyl esters **5c** and **5g** which were 20–30%. The reason for the reduced yield on the deprotection step in these cases is unclear. In each case the ProTides were isolated as roughly 1:1 mixtures of phosphate diastereoisomers as evidenced by two closely spaced ^{31}P NMR signals as ca. 3.3–3.7. Proton NMR signals for the base moiety (H2, H8) also showed splitting due to the diastereomers. Each of the ProTides **5a–h** were evaluated for their ability to inhibit the proliferation of HCV in the replicon assay as described.[3]

Data are presented in Table 1 as EC_{50} values in μM, representing the concentration of compound reducing HCV replication by 50%. All of the compounds showed cytotoxicity (CC_{50}) values >100 μM. The parent compound (**3**) did not inhibit HCV replication (EC_{50} >100 μM).

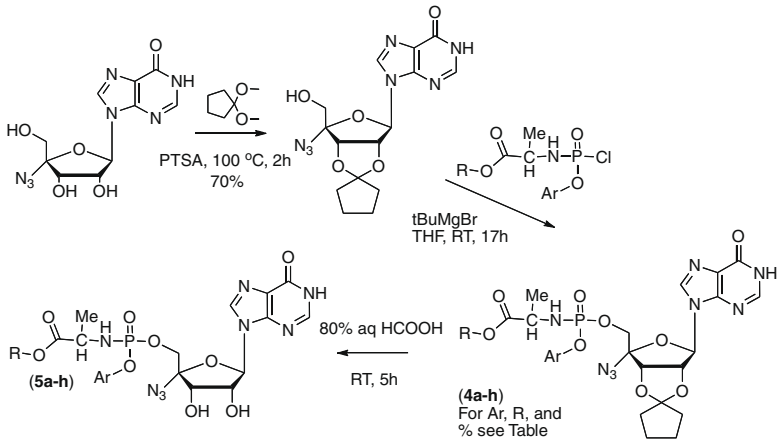
In contrast several of the ProTides did inhibit HCV replication at low μM levels. These data support the notion that (**3**) is inactive due to poor phosphorylation in vitro, that it is the first phosphorylation step that is rate limiting, and that some of the ProTides can successfully deliver the 5′-monophosphate of (**3**) intracellularly.

Considering previous SARs reported by us for ProTides in general[8] and for HCV in particular[6,13] we chose to concentrate this initial study on alanine-based ProTides, we have previously found benzyl esters of alanine to be a particularly effective ProTide motif in vitro, and ethyl and isopropyl esters to substitute for benzyl in many cases.[8] *t*-Butyl esters have in general been found to be poorly effective, and this has been attributed to poor cleavage of these esters in vitro, this cleavage being an essential first step in ProTide activation.[16] Thus, it was not surprising that the phenyl *t*-butylalanine compound **5c** was poorly active (EC_{50}>100 μM). However, it was a surprise that the corresponding ethyl (**5a**), isopropyl (**5b**) and benzyl (**5d**) esters were also inactive. One possible reason for the poor activity of this phenyl family could be their relatively low lipophilicity (Table 1) with Clog*P* values of ca. −1 to −2. While they are still ca. 2− logs more lipophilic than (**3**), their lipophilicity values may predict poor passive diffusion into cells.

Table 1

Compd	Aryl	Ester	%[a]	EC_{50} μM	Clog*P*[b]
3	—	—	—	>100	−3.6
5a	Ph	Et	68, 76	>100	−2.1
5b	Ph	iPr	87, 73	>100	−1.8
5c	Ph	tBu	71, 27	>100	−1.4
5d	Ph	Bn	57, 70	>100	−1.0
5e	Nap	Et	48, 62	16.4	−1.0
5f	Nap	iPr	70, 80	6.1	−0.7
5g	Nap	tBu	100, 18	>100	−0.3
5h	Nap	Bn	59, 66	4.3	0.2

[a] Isolated yield of **5a**–**h** over each of the 2 steps from protected (**3**).
[b] Calculated log*P* (Clog*P*) based on ChemDraw Ultra 11.0.1.



Scheme 2.

With this in mind and also noting our recent observations of the particular efficacy of 1-naphthyl ProTides,[6,13,17] we prepared a parallel series of naphthyl ProTides of alanine esters.

Although the ethyl compound (**5e**) was only active above 10 μM, the isopropyl (**5f**) and benzyl (**5h**) esters were active at low μM levels. Notably, the *t*-butyl ester (**5g**) remained inactive in this system. In each case, the naphthyl phosphates were calculated to be ca 10-times more lipophilic than their phenyl analogues. To some extent there was a tendency towards potency increasing with lipophilicity, which may support the notion that cell entry was limiting, but the benzyl ester in the phenyl series (**5d**) and the ethyl ester in the naphthyl series (**5e**) happened to share a common log*P* and yet only the naphthyl system was active, indicating lipophilicity to be at best only part of the reason for the different profiles. The altered pK_a and leaving group ability of the 1-naphthyl group may be an additional parameter, as loss of the aryl moiety is considered to be the essential second step in ProTide activation. This is the first example that we are aware of where a family of ProTides depends for its activity on the presence of a naphthyl moiety rather than a phenyl group.

In conclusion, we have developed a synthesis of 4′-azidoinosine from inosine and reported the preparation of a family of 8 ProTides thereof.

While the parent nucleoside analogue is inactive against HCV in replicon, some of the ProTides are active at low μM levels. Notably, activity depends on the presence of a naphthyl phosphate, and is lost for *t*-butyl esters. This study represents one of very few highlighting activity from inosine based systems and suggests that the potential of nucleosides based on this base motif may be unleashed by ProTide methods.

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15. *Selected synthetic procedures and spectroscopic data*: 2′,3′-*O*-cyclopentandienide-4′-azidoinosine-5′-phenyl(ethoxy-L-alaninyl)phosphate (**4a**) To a solution of 2′,3′-*O*-cyclopentadienide-4′-azidoinosine (55 mg, 0.147 mmol), in THF a 1 M solution of *tert*-butylmagnesium chloride in THF (1 M soln in THF, 366 μL) was added dropwise and the mixture left to equilibrate at rt for 15 min. A 1 M solution of phenyl ethylalaninyl phosphorochloridate in THF (0.366 mmol, 366 μL) was added dropwise. The mixture was stirred at rt for 14 h. and the solvent removed and the crude product purified by silica column chromatography using $CHCl_3$/MeOH (from 95/5) as eluent. The appropriate fractions were collected and the solvent removed under reduced pressure to afford a white solid (63 mg, 68%). ^{31}P NMR (MeOD; 202.5 MHz): δ 3.54, 3.17. ^{1}H NMR (MeOD; 500 MHz): δ 8.26, 8.25 (1H, 2s, H-8), 8.05 (1H, s, H-2), 7.91–7.12 (5H, m, *Ph*O), 6.51–6.50 (1H, m, H-1′), 5.41–5.36 (1H, m, H-2′), 5.27–5.25 (1H, m, H-3′), 4.32–4.26 (2H, m, H-5′), 4.23–4.10 (2H, m, CH_3CH_2), 3.92–3.87 (1H, m, CH_3CH), 2.27–2.13 (2H, m, cyclopentylidene), 1.86–1.71 (6H, m, cyclopentylidene), 1.32, 1.31 (3H, 2d, ^3J = 7.00 Hz, CH_3CH), 1.24 (3H, t, ^3J = 7.15 Hz, CH_3CH_2).
^{13}C NMR (MeOD; 125.7 MHz): δ 14.47 (CH_3CH_2), 20.35, 20.31 (CH_3CH), 24.09, 24.81, 36.27, 36.36, 37.31 (CH_2, cyclopentylidene), 51.64 (CH_3CH), 62.42 (CH_3CH_2), 69.25, 69.29 (C-5′), 83.60, 83.64 (C-3′), 85.38 (C-2′), 90.29, 90.55 (C-1′), 100.58, 100.66 (C4′), 121.28, 121.32, 121.41, 121.45, 126.28, 126.34, 130.75, 130.79 (*Ph*O + C-5), 141.28 (C-8), 147.27 (C-2), 149.36 (C-4), 151.88, 151.93 ('ipso', *Ph*O), 158.85 (C6), 174.97 (*C*OOEt). 4′-azidoinosine-5′-[phenyl(ethoxy-L-alaninyl)] phosphate. (**5a**) 2′,3′-*O*-cyclopentandienide-4′-azidoinosine-5′-phenyl (ethoxy-L-alaninyl)]phosphate (**4a**) (60 mg, 0.095 mmol) was dissolved in HCOOH (80% v/v solution in water, 10 mL) and the mixture stirred at rt for 6 h. The solvent was removed and the crude purified by silica column chromatography using $CHCl_3$/MeOH (gradient elution from 95/5 to 9/1) as eluent to give 41 mg of a white solid (76%). ^{31}P NMR (MeOD; 202.5 MHz): δ 3.50, 3.31. ^{1}H NMR (MeOD; 500 MHz): δ 8.28, 8.26 (1H, 2s, H-8), 8.07, 8.03 (1H, s, H-2), 7.36–7.15 (5H, m, *Ph*O), 6.32–6.30 (1H, m, H-1′), 4.94–4.91 (1H, m, H-2′), 4.69–4.66 (1H, m, H-3′), 4.35–4.20 (2H, m, H-5′), 4.16–4.09 (2H, m, CH_3CH_2), 3.94–3.82 (1H, m, CH_3CH), 1.32, 1.31 (3H, 2d, ^3J = 7.15 Hz, CH_3CH), 1.23 (3H, t, ^3J = 7.10 Hz, CH_3CH_2). ^{13}C NMR (MeOD; 125.7 MHz): δ 14.44, 14.49 (CH_3CH_2), 20.25, 20.31, 20.45, 20.50 (CH_3CH), 51.50, 51.64 (CH_3CH), 62.45 (CH_3CH_2), 68.43, 68.47, 68.70, 68.74 (C-5′), 73.99, 74.23, 74.39 (C-3′ + C-2′), 91.08 (C-1′), 99.06, 99.23, 99.31 (C-4′), 121.31, 121.33, 121.36, 121.41, 121.15, 126.27 130.77, 130.83 (*Ph*O + C-5), 141.08, 141.15 (C-8), 147.06 (C-2), 150.04 (C-4), 151.92, 151.97 ('ipso', *Ph*O), 158.90 (C-6), 174.02, 175.05 (*C*OOEt).
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