Bioorganic & Medicinal Chemistry Letters 21 (2011) 6007–6012

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Dual pro-drugs of 2′-*C*-methyl guanosine monophosphate as potent and selective inhibitors of hepatitis C virus

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ARTICLE INFO

Article history:
Received 10 May 2011
Revised 3 June 2011
Accepted 4 June 2011
Available online 17 August 2011

Keywords:
HCV
ProTide
Phosphoramidate
Nucleoside
Nucleotide
Polymerase
NS5B

ABSTRACT

We have previously reported the power of combining a 5′-phosphoramidate ProTide, phosphate pro-drug, motif with a 6-methoxy purine pro-drug entity to generate highly potent anti-HCV agents, leading to agents in clinical trial. We herein extend this work with the disclosure that a variety of alternative 6-substituents are tolerated. Several compounds exceed the potency of the prior 6-methoxy leads, and in almost every case the ProTide is several orders of magnitude more potent than the parent nucleoside. We also demonstrate that these agents act as pro-drugs of 2′-C-methyl guanosine monophosphate. We have also reported the novel use of hepatocyte cell lysate as an ex vivo model for ProTide metabolism.
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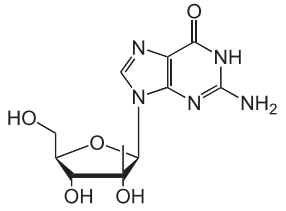
Nucleoside analogues continue to play a vital role in the search for improved therapies for hepatitis C Virus (HCV) infection.[1] Several families of modified nucleosides have been reported as inhibitors of the HCV NS5B RNA polymerase, including 4′-modified[2] and 2′-modified[3] nucleosides. All of these nucleosides act following sequential phosphorylation to their bioactive 5′-triphosphate forms. As with many nucleoside analogues this phosphorylation, and particularly the first, nucleoside kinase-mediated, step may be limiting to their bio-activity.[3] In these circumstances one of a number of phosphate pro-drug methods may be used.[4] We have developed a phosphoramidate ProTide approach[5] and applied it extensively to a variety of bioactive nucleosides. Recently we reported its effectiveness when applied to the anti-HCV agent 2′-C-methylguanosine (**1**).[6] We further reported that combining the



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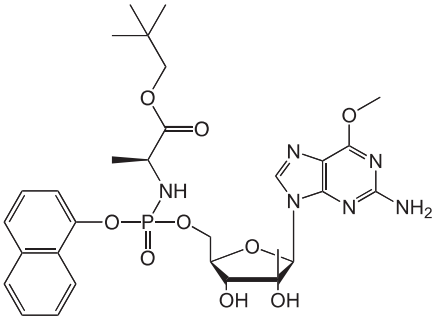

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doi:10.1016/j.bmcl.2011.06.013

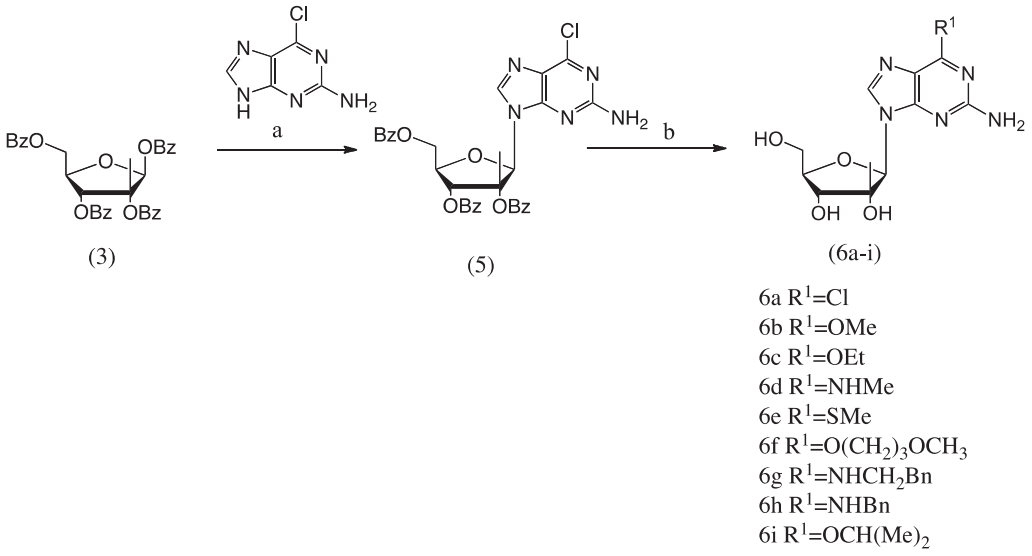


Figure 1. Reagents and conditions: (a) DBU, TMSOTf, ACN, 65 °C, 4–6 h, 76%; (b) NH_3/MeOH, 12 h, rt, 90% (for **6a**); NaOMe, MeOH, rt, 12 h, 78% (for **6b**); NaOEt/EtOH, 50 °C, 5 h, 84% (for **6c**); $MeNH_2$/Et_3N/EtOH, 85 °C, sealed tube, 92% (for **6d**); NaSMe/EtOH, rt, 18 h, 87% (for **6e**); NH_3/MeOH, rt, 12 h, 90%, NaH, 3-methoxy-1-propanol, 50% (for **6f**); $BnCH_2NH_2$, EtOH, reflux, 16 h, 55%, NH_3/MeOH, rt, 94% (for **6g**); $BnNH_2$, EtOH, reflux, 16 h, 98%, NH_3/MeOH, rt, 89% (for **6h**); $NaOCH(Me)_2$, rt, 18 h then NaOMe, rt, 18 h, 70% (for **6i**).

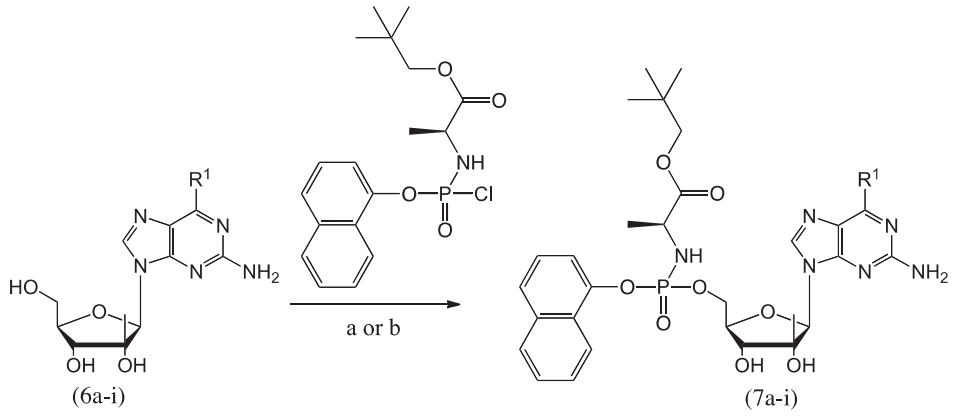


Figure 2. Reagents and conditions: (a) *N*-methylimidazole, THF, 16 h, rt, 11–28%; (b) *t*BuMgCl, THF, 22-25%.

5′-phosphate ProTide moiety with a C6 methoxy pro-drug entity further significantly boosted the potency of the compound to give the clinical candidate INX-189 (**2**) with nanomolar activity against HCV.[7] This agent has recently successfully completed phase 1b clinical trial in HCV infected patients.

Several reasons were suggested for the potency boost presented on C6 modification. Amongst them was the ca 2-log increase in lipophilicity upon this modification, and potential consequential enhancements in passive cell uptake.[7]

Since the ProTide motif is thought to function by intracellular 5′-monophosphate release it was considered that the 5′-monophosphate from (**2**) was a substrate for adenylate deaminase to liberate 2′-methylguanosine monophosphate as an essential precursor to the bioactive 5′-triphosphate. Given the known wide substrate specificity of adenylate deaminase[8] we wondered if alternative 6-substituents might substitute for the 6-methoxy and might further boost lipophilicity and enhance potency. We herein report the notable success of this approach.

As shown in Figure 1 we used the 6-chloro nucleoside as a route into various 6-substituted analogues. Thus, tetrabenzoyl 2′-*C*-methyl-D-ribose (**3**) was condensed with 2-amino-6-chloropurine (**4**) in the presence of TMS triflate to give the protected beta nucleoside (**5**) in 76% yield. This was deprotected with methanolic ammonia to give (**6a**) in 78% yield. Use of alternatives deprotection/substitution conditions gave the various 6-substituted analogues (**6b–i**) as shown in Figure 1. Thus, sodium methoxide and ethoxide respectively gave (**6b–c**), methylamine (**6d**), and thiomethoxide gave (**6e**). Other analogues were similarly prepared.

The anti-HCV activity of (**6a–i**) was studied in sub-genomic replicon assay and the data are reported in Table 1. In general the compounds are active at μM levels; activity decreasing for larger C6 substituents.

Following the discovery of the power of the naphthyl neopentylalanine ProTide motif in (**2**) we now applied this moiety to (**6a–i**) as shown in Figure 2. Thus, 1-naphthyl neopentylalaninyl phosphorochloridate was allowed to react with (**6a–i**) to generate the corresponding ProTides (**7a–i**) in moderate yield.

All compounds were isolated as roughly equimolar mixtures of phosphorus diastereoisomers as evidenced by ^{31}P NMR and HPLC.[9]

The ProTides were tested as inhibitors of HCV as noted above, with data being shown in Table 2.

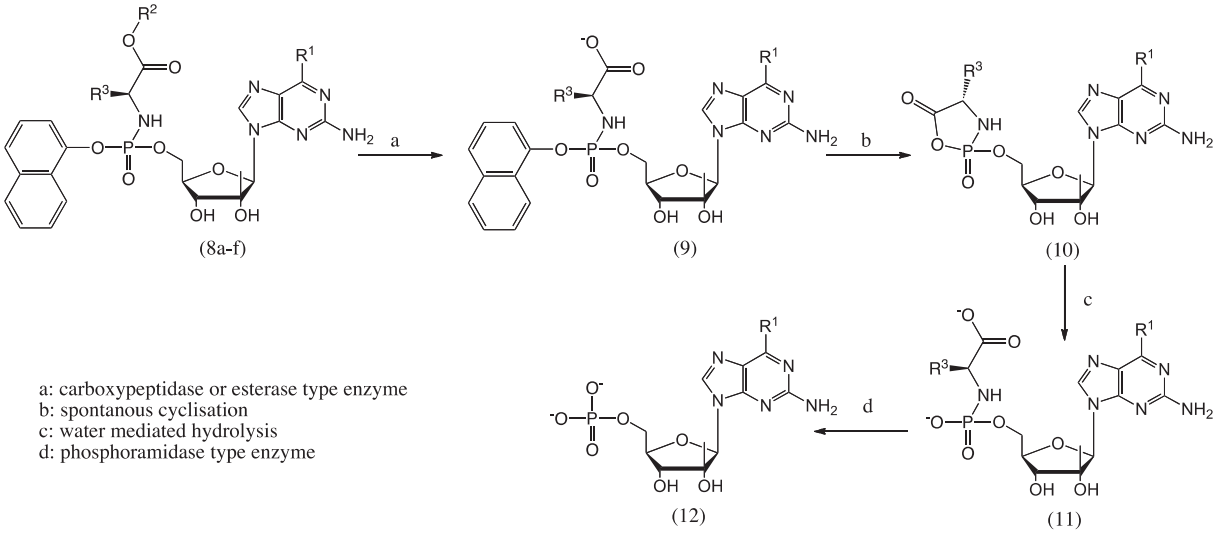


Figure 3. Putative mechanism of ProTide activation.

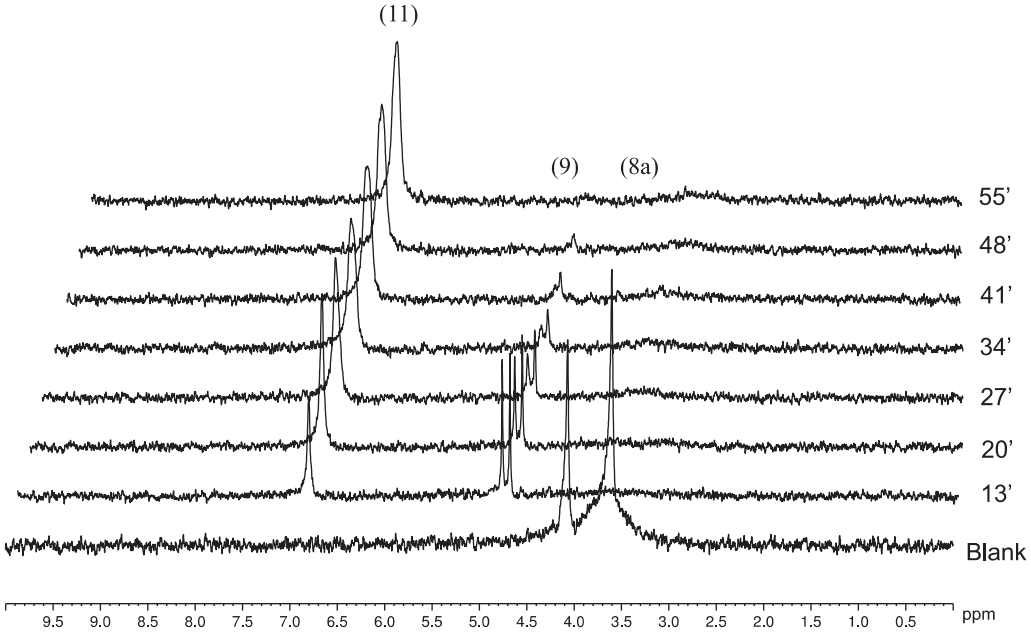


Figure 4. Carboxypeptidase Y mediated hydrolysis of (8a) followed by ^{31}P NMR.

It is striking that while all of the parent nucleosides (**6a–i**) are poorly active, with EC_{50} values of ca 5–30 μM, all of the ProTides are active sub-μM. Particularly active are the 6-ethoxy, 6-methoxy and 6-chloro compounds with EC_{90}values of <100 nM. The largest C6 substituted compounds such as (**7h**) seem to lose some activity, implying some size restrictions at C6, but even here the ProTide remains 30-fold more active than the parent (**6h**). The ProTides are cytotoxic at μM levels but their high potency still leads to therapeutic index values in the 1000 range. Given the extremely high potency of the 6-*O*-ethoxy analogue we decided to vary the ProTide motif on (**7c**). Following the same procedures as in Figure 2 but varying the phosphorochloridate we converted (**6c**) to (**8a–f**) (Table 3).

All of these compounds show sub-μM activity in replicon but none are as active as (**7c**). In general, the alanine compounds are more active than the valine ones as we have previously noted.[6]

To confirm the possible mode of action of these compounds we conducted a number of assays. In the first instance we performed a Carboxypeptidase Y assay, an in vitro probe of ProTides activation pathway (Fig. 3).

The data shown in Figures 4 and 5 represent ^{31}P NMR spectra recorded every 7 min during 14 h incubation period of (**8a**) and (**8e**) with Carboxypeptidase Y in acetone-d_6 and Trizma buffer (pH 7.6).

In case of the naphthyl benzylalanine compound (**8a**) (Fig. 4), the experiment showed fast hydrolysis of starting material (δ_P = 3.62, 4.11 ppm) to the intermediate lacking the ester group (δ_P = 4.69, 4.83 ppm). Both diastereoisomers of (**8a**) appear to be processed with roughly similar efficacy as far as the data allow us to discern. The single peak at 6.95 ppm corresponds to the final product of the hydrolysis—the achiral aminoacyl phosphate. The estimated half-life of (**8a**) was less than 5 min. In case

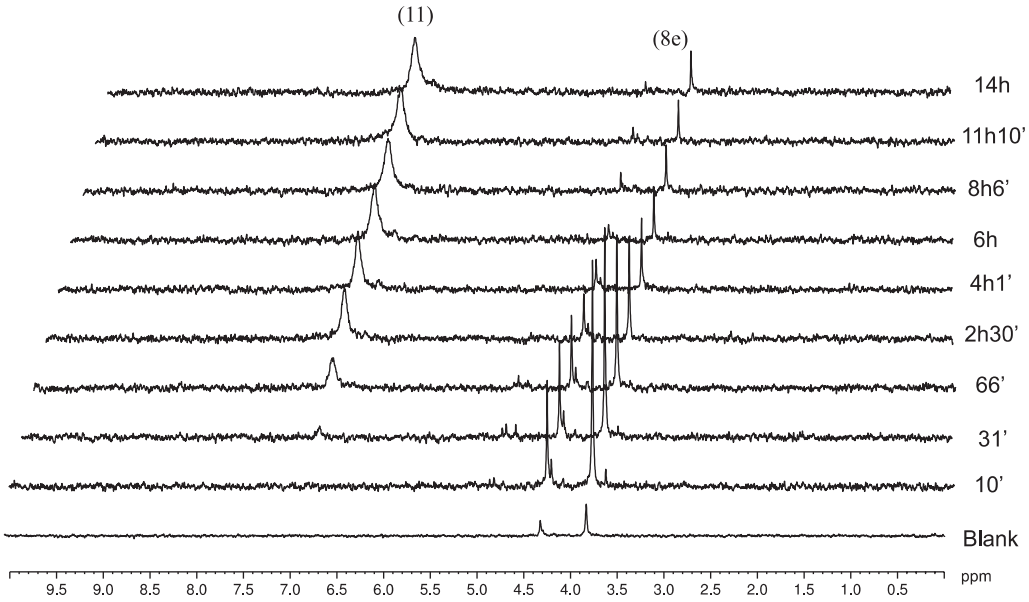


Figure 5. Carboxypeptidase Y mediated hydrolysis of (8e) followed by ^{31}P NMR.

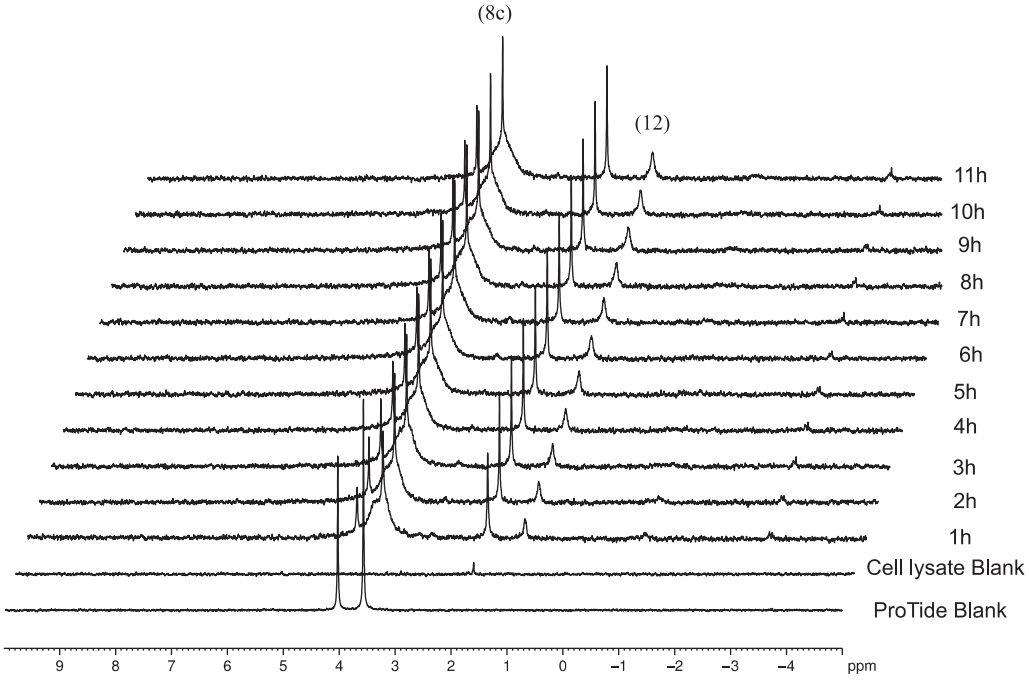


Figure 6. Metabolism of (8c) in HuH7 cell lysate followed by ^{31}P NMR.

of the isopropyl analogue (**8e**) (Fig. 5) conversion of the starting material was slower, with a half-life of 4 h and in this case one of the diastereoisomers of (**8e**) seems to be processed rather more rapidly.

These results are consistent with the replicon data, showing that (**8a**), which is processed faster, is also found to be more active (EC_{50} = 0.04 μM vs 0.14 μM for (**8e**)). The carboxypeptidase assay is thus a potentially useful predictive model for the in vitro bio-activation of these agents.

HuH7 cell lysates have been prepared to examine monophosphate formation during the ProTide hydrolysis. An NMR based assay was performed on (**8c**) using HuH7 cell lysate (10^7 cells) in acetone-d_6 and Trizma buffer (pH 7.6) at 37 °C. ^{31}P NMR spectra were recorded every 1 h for 11 h. After 1 h of incubation, the presence of newly formed peak at 0.98 ppm was observed suggesting successful liberation of the monophosphate species (Fig. 6). This signal had the same chemical shift as an authentic monophosphate under these conditions. Given that its release is considered to be a pre-requisite for antiviral action, via the triphosphate, we view these data as encouraging and consistent with the replicon data in Tables 1–3 above.

As far as we are aware, this is the first time that P-31 NMR of hepatocyte lysate has been used as an ex vivo assay of anti-HCV ProTide metabolism.

Since we regard each of these 6-modified nucleotides as prodrugs of the guanine nucleotides, we also conducted an adenosine deaminase (ADA) assay on (**6b**), with spectra recorded each minute.[10] The results are shown in Fig 7.

Table 1
HCV Replicon activity and cytotoxicity of 6-substituted 2′-C-methylguanosine nucleosides

Compound	R^1	EC_{50}/ μM[a]	CC_{50}/ μM[b]
6a	Cl	8.5	>100
6b	OMe	4.6	>100
6c	OEt	8.8	>100
6d	NHMe	13	>100
6e	SMe	11	>100
6f	$O(CH_2)_3$ OMe	12	>100
6g	$NHCH_2$ Bn	24	>100
6h	NHBn	30	ND
6i	$OCH(CH_3)_2$	9.3	>100

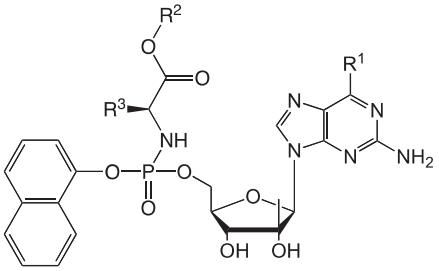
For structures see Figure 1.
[a] 50% effective concentration. Replicon data for genotype 1b in HUH7 cells with 48 h exposure.
[b] 50% cytotoxic concentration in HUH7 cells.

Table 2
HCV Replicon activity and cytotoxicity of 1-naphthyl neopentyl alanine ProTides of 6-substituted 2′-C-methylguanosine nucleosides

Compound	R^1	EC_{50}[a]/μM	EC_{90}[b]/μM	CC_{50}[c]/μM
7a	Cl	0.012	0.028	16
7b	OMe	0.010	0.038	7
7c	OEt	0.008	0.032	10
7d	NHMe	0.035	0.103	38
7e	SMe	0.037	0.16	41
7f	$O(CH_2)_3OMe$	0.043	0.12	31
7g	$NHCH_2Bn$	0.270	1.30	42
7h	NHBn	0.79	2.7	15
7i	$OCH(CH_3)_2$	0.023	0.065	11

For structures see Figure 1.
[a] 50% effective concentration. Replicon data for genotype **1b** in HUH7 cells with 48 h exposure.
[b] 90% effective concentration. Replicon data for genotype **1b** in HUH7 cells with 48 h exposure.
[c] 50% cytotoxic concentration in HUH7 cells.

Table 3
HCV Replicon activity and cytotoxicity of various ProTides of 6-ethoxy-2′-C-methylguanosine



Compound	R^1	R^2	R^3	EC_{50}/μM[a]	CC_{50}/μM[b]
8a	OEt	Bn	L-Ala	0.04	11
8b	OEt	NeoPnt	L-Val	0.17	24
8c	OEt	S-PhEt	L-Ala	0.085	12
8d	OEt	THP	L-Ala	0.08	47
8e	OEt	*i*Pr	L-Ala	0.14	40
8f	OEt	$2,4F_2Bn$	L-Ala	0.034	17

[a] 50% effective concentration. Replicon data for genotype **1b** in HUH7 cells with 48 h exposure.
[b] 50% cytotoxic concentration in HUH7 cells.

These data demonstrate that (**6b**) is an excellent substrate for adenosine deaminase-mediated deamination. The final product has an identical UV spectrum to (**1**) as expected. Compound (**6c**) was found to be suitable substrate for the enzyme, however the conversion rate to (**1**) was much slower than for (**6b**). We expect that the monophosphates of (**6a–c**) released from the ProTide in vitro, may similarly be substrates for adenylate deaminase, giving the 2′-*C*-methylguanosine monophosphate in each case. ADA assay performed on (**6d**) and (**6e**) showed no transformation within the reaction time of 24 h which may suggest that broader substrate specificity might operate at the adenylate deaminase level or that the deamination of these nucleoside analogues and their corresponding monophosphates is catalysed by a different type of deaminase for example, human abacavir monophosphate deaminase.[11] To further pursue this notion we conducted the replicon anti-viral assay in the presence and absence of pentostatin, a known inhibitor of both adenosine and adenylate deaminase (data not shown) and noted that each of the ProTides herein described lost entirely their activity in the presence of pentostatin, clearly confirming an absolute need for 'deamination' to be active.

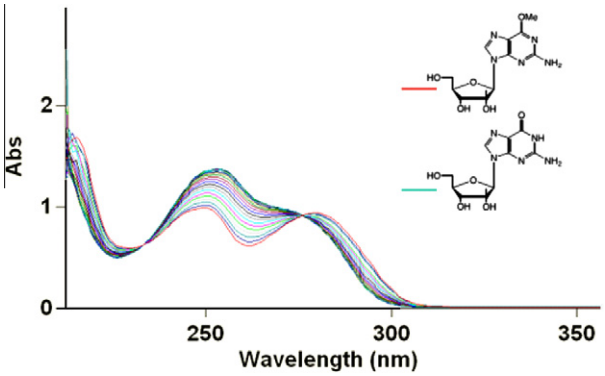


Figure 7. Adenosine deaminase mediated deamination of (6b) followed by UV spectroscopy.

In conclusion, we herein report that a wide range of 6-substituents is acceptable at the ProTide level of 2′-*C*-methyl guanosine based anti-HCV agents. In every case, the parent nucleosides are poorly active, being active at only high μM levels. In every case their ProTides are active sub-μM; in most cases the potency of the nucleoside is enhanced >100 fold. Alanine emerges again as the preferred amino acid, and several ester variants lead to very potent compounds. In some cases the potency and selectivity in vitro exceeds that of the clinical candidate (**2**), INX-08189. In every case 'deamination' at the C6-position, presumably at the monophosphate level, is implicated as essential for activity, and thus these compounds are dual pro-drugs. We have also reported the novel use of hepatocyte cell lysate as an ex vivo model for ProTide metabolism.

Acknowledgements

This study was supported by a grant from Inhibitex, Inc. to C.M. C.M. is a board member and shareholder of Inhibitex, Inc. All authors from Inhibitex, Inc. own options and are shareholders. We would like to thank Helen Murphy for secretarial assistance.

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9. *Procedure for* **7c**. To nucleoside (1 mol equiv) in anhydrous THF, phosphorochloridate (3 mol equiv) in THF was added, followed by addition of *N*-methyl-imidazole (5 mol equiv). The mixture was stirred overnight under argon atmosphere. Solvent was removed under reduce pressure. To remove the *N*-methyl-imidazole, the phosphoramidate was dissolved in chloroform and washed 3 times with hydrochloric acid (HCl 0.5 N). The organic layer was then dried over sodium sulfate and evaporated under reduce pressure. The residue was then purified on silica gel using $CHCl_3$ to $CHCl_3$/MeOH 95:5 as an eluent, to give the pure phosphoramidate as a white solid. Starting from 500 mg of nucleoside. Yield 28% (285 mg).

^{1}H NMR (500 MHz, MeOD-d_4) δ 8.21–8.14 (m, 1H, H-8 napht), 7.98 and 7.96 (2s, 1H, H-8), 7.86–7.79 (m, 1H, H-5 napht), 7.66 and 7.63 (2d, J = 8.3 Hz, 1H, H-4 napht), 7.56–7.43 (m, 3H, H-7, H-6, H-3 napht), 7.41–7.34 (m, 1H, H-2 napht), 6.03 and 6.02 (2s, 1H, H-1′), 4.67–4.71 (m, 2H, H-5′, H-5″), 4.54–4.47 (2q, J = 7.0 Hz, 2H, O$CH_2$$CH_3$), 4.37-4.25 (m, 2H, H-3′, H-4′), 4.12–4.04 (m, 1H, CH Ala, 3.69 and 3.65 (2 AB systems, J = 10.5 Hz, 2H, CH_2 ester), 1.41 (t, J = 7.1 Hz, 3H, OCH$_2$$CH_3$), 1.33 (d, J = 7.1 Hz, 3H, CH_3 Ala), 0.98 and 0.96 (2s, 3H, CH_3), 0.84 and 0.82 (2s, 9H, 3× CH_3 ester)
^{13}C NMR (126 MHz, MeOD-d_4) δ 175.06, 174.80 (2d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 5.0 Hz, C═O), 162.39 (C-6), 161.87, 161.86 (C-2), 154.57, 154.53 (C-4), 148.01, 147.96 (2d, $^2J_{C\text{-}O\text{-}P}$ = 3.6 Hz, C-1 napht), 139.32, 139.04 (C-8), 136.29, 136.26 (C-10 napht), 128.84, 128.79 (C-5 napht, 127.91, 127.86 (C-6 napht), 127.75, 127.72 (C-7 napht), 126.53, 126.40 (C-9 napht), 125.96, 125.93 (C-3 napht), 122.81, 122.76 (C-4 napht), 116.23, 116.18 (2d, $^3J_{C\text{-}C\text{-}O\text{-}P}$ = 2.9 Hz, C-2 napht), 115.63, 115.62 (C-5), 93.34, 93.20 (C-1′), 82.33, 82.16 (2d, $^3J_{C\text{-}C\text{-}O\text{-}P}$ = 8.0 Hz, C-4′), 79.98, 79.94 (CH_2 ester), 75.37 (C-2′), 74.96, 74.72 (C-3′), 68.14, 67.67 (2d, $^2J_{C\text{-}O\text{-}P}$ = 5.3 Hz, C-5′), 63.59, 63.57 (O$CH_2$$CH_3$), 51.79, 51.72 (CH Ala), 32.26, 32.23 (C(CH_3)$_3$ ester), 26.72, 26.69 (C(CH_3)$_3$ ester), 20.84, 20.64 (2d, $^3J_{C\text{-}C\text{-}N\text{-}P}$ = 6.6 Hz, CH_3 Ala), 20.34, 20.31 (2′-CH_3), 14.89 (OCH$_2$$CH_3$) ^{31}P NMR (202 MHz, MeOD-d_4) δ 4.28 and 4.21. HPLC t_R = 22.92, 23.16 min (Varian Polaris C18-A (10 μM) analytic column; elution was performed using a mobile phase consisting of water/acetonitrile in gradient 90/10 to 0/100 v/v in 30 min).
10. 1 ml of 44 μM solution of nucleoside in phosphate buffer (pH7.4), 30 μL of ADA solution. Spectra recorded in 1 min intervals for 30 min.
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