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Phosphorodiamidates as a Promising New Phosphate Prodrug Motif for Antiviral Drug Discovery: Application to Anti-HCV Agents

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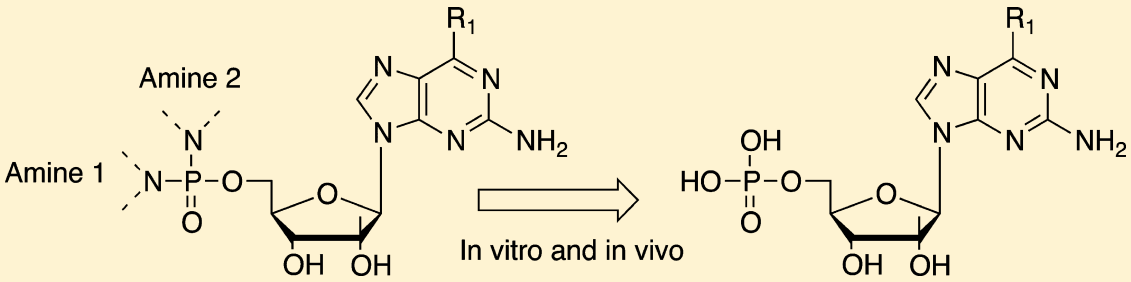
Supporting Information



ABSTRACT: We herein report phosphorodiamidates as a significant new phosphate prodrug motif. Sixty-seven phosphorodiamidates are reported of two 6-*O*-alkyl 2′-*C*-methyl guanosines, with significant variation in the diamidate structure. Both symmetrical and asymmetric phosphorodiamidates are reported, derived from various esterified amino acids, both D and L, and also from various simple amines. All of the compounds were evaluated versus hepatitis C virus in replicon assay, and nanomolar activity levels were observed. Many compounds were noncytotoxic at 100 μM, leading to high antiviral selectivities. The agents are stable in acidic, neutral, and moderately basic media and in selected biological media but show efficient processing by carboxypeptidases and efficiently yield the free nucleoside monophosphate in cells. On the basis of in vitro data, eight leads were selected for additional in vivo evaluation, with the intent of selecting one candidate for progression toward clinical studies. This phosphorodiamidate prodrug method may have broad application outside of HCV and antivirals as it offers many of the advantages of phosphoramidate ProTides but without the chirality issues present in most cases.

INTRODUCTION

There is an urgent ongoing need for improved therapeutic agents for hepatitis C Virus (HCV) infection, with an increasing emphasis on direct acting antivirals (DAAs), and in particular, inhibitors of the viral NS5B RNA polymerase.[1] Nucleoside-based inhibitors of the polymerase are considered particularly valuable on the basis of the high genetic barrier to resistance.[2] Thus, a number of nucleoside modifications (**1**−**4**, Figure 1) have emerged with anti-HCV activity in vitro, and several have progressed to clinical evaluation.

One issue common to all nucleoside analogues, either antiviral or anticancer, is an absolute need for nucleoside kinase-mediated activation to their 5′-monophosphate forms. In some cases, as in the present anti-HCV field, further phosphorylation to the 5′-triphosphate is also required, and in general the first phosphorylation step is considered rate-limiting.[3] With this in mind, and given that the free phosphate derivatives are not considered useful drug entities due to poor cell uptake, a number of phosphate (nucleotide) prodrug motifs have emerged.[4] These have included our aryl phosphoramidate ("ProTide") approach,[5] the amidate diester method of Wagner[6] and McKenna,[7] the lipid diester approach of Hosteller,[8] thioester approaches of Gosselin,[9] cytochrome based methods,[10] and the chemical driven cycloSAL method of Meier.[11] Each of these methods has its strengths and weaknesses. In general, fully blocked prodrugs (ProTides, CycloSAL, etc.) may give better delivery but do often generate a chiral phosphate center leading to isomer issues. Phosphate diester methods avoid the chirality issue but may have delivery challenges.[4] Despite this, several phosphate prodrugs of antiviral nucleosides have progressed into clinical trials. This includes Inhibitex's INX-189[12] (**5**, Figure 2) and Pharmasset's PSI-7977[13] (**6**), both based directly on phosphoramidate ProTides, and Idenix's

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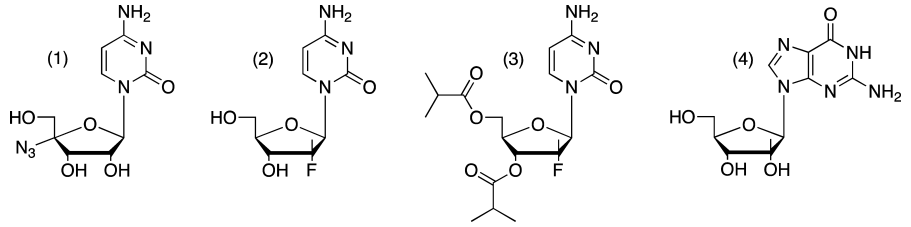


Figure 1. Some anti-HCV nucleoside analogues.

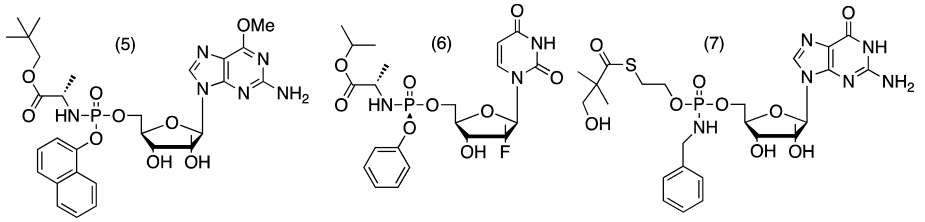
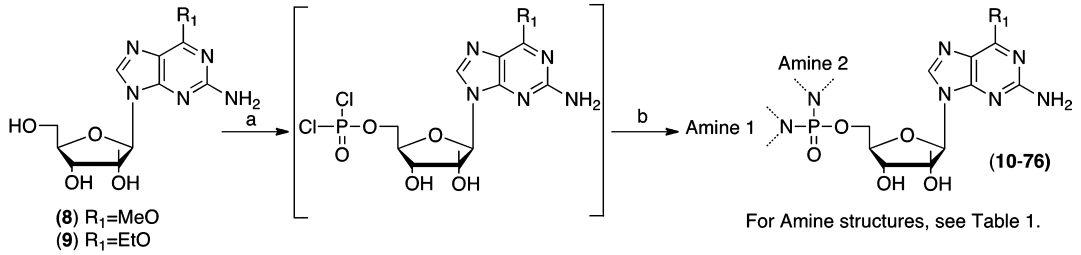


Figure 2. Some anti-HCV phosphate prodrug compounds in clinical trials.

Scheme 1. Synthetic Route to Phosphorodiamidates[a]



[a]Reagents and conditions: (a) Et_3N (1 equiv), $POCl_3$ (1 equiv), dry THF; (b) for symmetrical diamidates, amine (5 equiv), Et_3N (10 equiv), dry DCM; for asymmetric diamidates, amine 1 (1 equiv), Et_3N (2 equiv) then amine 2 (5 equiv), Et_3N (10 equiv), dry DCM.

IDX184[14] (**7**), which is a hybrid phosphoramidate/SATE prodrug. Each of these is now in human trials for efficacy versus HCV.

Most chiral nucleotide prodrugs that have entered into the clinic to date have been progressed as diastereoisomeric mixtures at the phosphate center. NewBiotics' anticancer agent NB1011 is a further example in this category.[15] In general, when tested, both stereoisomers often display similar biological activity in vitro and always release the same pharmacophore after initial metabolism.[16] One exception to this in the HCV field to date is Pharmasset, who has developed a large scale separation technique for their mixed compound PSI-7851, now pursued as the single stereoisomer **6** as above.[13]

With this in mind, we sought to revisit the notion of an achiral phosphate prodrug motif with a phosphoramidate core. In addition, we particularly wanted to formulate a prodrug whose promoieties were nontoxic and preferably natural. Thus our attention turned to phosphorodiamidates. Indeed, over 20 years ago, our group was among the first to report phosphorodiamidate-based nucleotide prodrugs. Thus, seeking to deliver the 5′-monophosphate of the anti-HIV agent AZT into cells, we prepared a series of L-amino acid phosphorodiamidates with a variety of esters.[17] There have since been reports of this motif on phosphonates[18] and non-nucleosides[19] but little further work on nucleosides.[20−22]

In this paper, we report the very promising profile of a family of such phosphorodiamidates of 2′-*C*-methylguanosine and its derivatives, leading toward selection of a clinical candidate.

CHEMISTRY

Following the success of **5** in early clinical trials for HCV[23] and our recent disclosure that alternative 6-alkoxy (and other) groups may be equally effective,[24] we used the 6-alkoxy derivatives of 2′-*C*-methylguanosine as our starting point. These were prepared by methods we have described.[12] Following our much earlier reports on phosphoramidates of AZT,[17] we used a single synthetic route based on treating the unprotected nucleoside with phosphoryl chloride to generate the intermediate dichloridate which was not isolated (Scheme 1). In the first instance, we used the 6-methoxy analogue **8**, the base nucleoside behind **5**. Reaction with $POCl_3$ in THF in the presence of Et_3N for 45 min (when ^{31}P NMR showed no presence of $POCl_3$) followed by addition of an excess of various amino acid esters (or other amines), lead to compounds **10**−**76** as shown in Table 1.

As L-alanine is often a preferred motif in phosphoramidate ProTides and the final putative activating step for these diamidates would be the same loss of amino acid, we thought that L-alanine may be beneficial here, so our first SAR family was symmetrical substituted L-alanines with linear esters methyl-(**10**), ethyl-(**11**), *n*-propyl-(**12**), *n*-butyl-(**13**), and *n*-pentyl-(**14**); branched esters isopropyl-(**15**) and (*R*,*S*)-2-butyl-(**16**), and 3,3-dimethylbutyl-(**17**); followed by cyclic esters cyclobutyl-(**18**), cyclopentyl-(**19**) cyclohexyl-(**20**), and 2-tetrahydropyranyl-(**24**). We have often reported that benzyl esters can be rather effective in ProTides, so we prepared the benzyl-(**21**), *S*-phenethyl-(**22**), 2,4-difluorobenzyl-(**23**), and 2-indanyl-(**25**) L-alanine esters. Given the extremely high potency

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Table 1. Replicon Data and Cytotoxicity of Nucleoside Analogues and Phosphorodiamidates

				EC_{50} (μM)		EC_{90} (μM)		
compd	AA ester/amine	AA ester/amine	nucleoside	av	SD	av	SD	CC_{50} (μM)
4			2′CMeG	2.2	1.4	8.3	7.5	>100
8			6OMe2′CMeG	4.4	2.2	19.4	10.2	>100
9			6OEt2′CMeG	9.5	1.84	24.5	1.78	>100
5	L-Ala OCH_2tBu	NaphO	6OMe2′CMeG	0.01	0.01	0.04	0.02	7.01
10	L-Ala OMe	L-Ala OMe	6OMe2′CMeG	5.90	0.76	>10		>100
11	L-Ala OEt	L-Ala OEt	6OMe2′CMeG	1.20	0.08	4.10	0.09	>100
12	L-Ala OnPr	L-Ala OnPr	6OMe2′CMeG	0.28	0.05	1.20	0.53	>100
13	L-Ala OButyl	L-Ala OButyl	6OMe2′CMeG	0.07	0.01	0.23	0.02	>100
14	L-Ala OPentyl	L-Ala OPentyl	6OMe2′CMeG	0.03	0.01	0.11	0.02	65
15	L-Ala OiPr	L-Ala OiPr	6OMe2′CMeG	0.49	0.18	2.10	0.09	>100
16	L-Ala *O*-(*R*,*S*)-2Bu	L-Ala *O*-(*R*,*S*)-2Bu	6OMe2′CMeG	0.15	0.00	0.53	0.10	>100
17	L-Ala *O*-3,3-dimethylbutyl	L-Ala *O*-3,3-dimethylbutyl	6OMe2′CMeG	0.02	0.01	0.09	0.07	71
18	L-Ala OcBu	L-Ala OcBu	6OMe2′CMeG	0.32	0.05	0.80		>100
19	L-Ala OcPentyl	L-Ala OcPentyl	6OMe2′CMeG	0.06	0.01	0.22	0.03	>100
20	L-Ala OcHx	L-Ala OcHx	6OMe2′CMeG	0.05	0.02	0.19	0.08	>100
21	L-Ala OBn	L-Ala OBn	6OMe2′CMeG	0.49		1.60		>100
22	L-Ala OSPhEt	L-Ala OSPhEt	6OMe2′CMeG	0.49		1.70		88
23	L-Ala *O*-2,4-diFBn	L-Ala *O*-2,4-diFBn	6OMe2′CMeG	0.26	0.21	1.07	0.92	54
24	L-Ala OTHP	L-Ala OTHP	6OMe2′CMeG	13.33	13.02	29.30	15.14	>100
25	L-Ala OIndanol	L-Ala OIndanol	6OMe2′CMeG	0.58	0.39	1.30	0.10	70
26	L-Ala OCH_2tBu	L-Ala OCH_2tBu	6OMe2′CMeG	0.06	0.04	0.20	0.12	>100
27	L-Ala OCH_2iPr	L-Ala OCH_2iPr	6OMe2′CMeG	0.07	0.01	0.26	0.01	>100
28	L-Ala OCH_2cPropyl	L-Ala OCH_2cPropyl	6OMe2′CMeG	0.21	0.00	0.66	0.001	>100
29	D-Ala OCH_2tBu	D-Ala OCH_2tBu	6OMe2′CMeG	0.11	0.03	0.50	0.19	>100
30	L-Asp OMe	L-Asp OMe	6OMe2′CMeG	10.18	3.17	>40		>100
31	L-Asp OBn	L-Asp OBn	6OMe2′CMeG	0.61	0.08	1.90	0.14	>100
32	L-Gly OBn	L-Gly OBn	6OMe2′CMeG	0.60	0.13	1.80	0.09	>100
33	L-Gly OCH_2tBu	L-Gly OCH_2tBu	6OMe2′CMeG	0.13	0.02	0.60	0.20	>100
34	L-Leu OcHx	L-Leu OcHx	6OMe2′CMeG	0.45	0.52	2.00	2.15	20
35	L-Leu OBn	L-Leu OBn	6OMe2′CMeG	0.38	0.02	1.31	0.21	39
36	L-Leu OCH_2tBu	L-Leu OCH_2tBu	6OMe2′CMeG	0.47	0.15	2.40	0.71	27
37	L-Ile OMe	L-Ile OMe	6OMe2′CMeG	5.17	1.94	29.5	2.66	>100
38	L-Ile OcHx	L-Ile OcHx	6OMe2′CMeG	4.00	0.47	7.10	1.16	14
39	L-Ile OBn	L-Ile OBn	6OMe2′CMeG	0.40	0.11	1.56	0.10	24
40	L-Ile OCH_2tBu	L-Ile OCH_2tBu	6OMe2′CMeG	2.90	0.33	6.30	1.36	15
41	L-Met OcHx	L-Met OcHx	6OMe2′CMeG	0.60	0.17	2.60	0.11	51
42	L-Met OBn	L-Met OBn	6OMe2′CMeG	0.25	0.04	0.77	0.24	>100
43	L-Met OCH_2tBu	L-Met OCH_2tBu	6OMe2′CMeG	2.22		5.00		>100
44	L-Phe OcHx	L-Phe OcHx	6OMe2′CMeG	0.50	0.59	2.00	2.20	25
45	L-Phe OBn	L-Phe OBn	6OMe2′CMeG	0.32	0.14	1.35	1.07	67
46	L-Phe OCH_2tBu	L-Phe OCH_2tBu	6OMe2′CMeG	0.05	0.02	0.21	0.12	24
47	L-Pro OBn	L-Pro OBn	6OMe2′CMeG	0.52	0.21	1.80	0.23	>100
48	L-Pro OCH_2tBu	L-Pro OCH_2tBu	6OMe2′CMeG	0.81	0.05	2.00	0.21	56
49	L-Val OcHx	L-Val OcHx	6OMe2′CMeG	2.50	0.35	10.00	0.72	20
50	L-Val OBn	L-Val OBn	6OMe2′CMeG	0.12	0.04	0.66	0.35	49
51	L-Val OCH_2tBu	L-Val OCH_2tBu	6OMe2′CMeG	0.72		2.50		32
52	L-Tyr (tBu) OMe	L-Tyr (tBu) OMe	6OMe2′CMeG	0.11	0.04	0.50	0.10	89
53	L-PhG OcHx	L-PhG OcHx	6OMe2′CMeG	0.32	0.00	1.80	0.04	18
54	L-PhG OCH_2tBu	L-PhG OCH_2tBu	6OMe2′CMeG	0.27	0.03	1.50	0.09	24
55	L-Val-L-Ala OCH_2tBu	L-Val-L-Ala OCH_2tBu	6OMe2′CMeG	0.54	0.26	2.40	0.46	>100
56	*β*-Ala OBn	*β*-Ala OBn	6OMe2′CMeG	3.80	2.03	>10		>100
57	butylamine	butylamine	6OMe2′CMeG	37.80	3.80	>40		>100
58	morpholine	morpholine	6OMe2′CMeG	>100		>100		>100
59	L-Ala OcPentyl	L-Ala OcHx	6OMe2′CMeG	0.04	0.01	0.16	0.03	>100
60	L-Ala OCH_2tBu	L-Ala OBn	6OMe2′CMeG	0.15	0.04	0.40	0.07	>100
61	L-Ala OCH_2tBu	L-Ala OtBu	6OMe2′CMeG	0.15	0.01	0.57	0.08	>100
62	L-Ala OCH_2tBu	L-Ala OcHx	6OMe2′CMeG	0.04	0.01	0.15	0.02	>100
63	L-Ala OCH_2tBu	L-Pro OMe	6OMe2′CMeG	0.49		2.20		>100
64	L-Ala OCH_2tBu	L-Val OMe	6OMe2′CMeG	0.17	0.06	0.69	0.15	>100

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Table 1. continued

compd	AA ester/amine	AA ester/amine	nucleoside	EC_{50} (μM) av	EC_{50} (μM) SD	EC_{90} (μM) av	EC_{90} (μM) SD	CC_{50} (μM)
65	L-Ala OBn	butylamine	6OMe2′CMeG	0.44	0.28	1.68	0.94	>100
66	L-Ala OCH_2tBu	butylamine	6OMe2′CMeG	0.41	0.28	1.50	0.75	>100
67	L-Ala OCH_2tBu	pentylamine	6OMe2′CMeG	0.11	0.02	0.52	0.05	>100
68	L-Ala OCH_2tBu	cyclopropylamine	6OMe2′CMeG	0.87	0.11	4.70	0.45	>100
69	L-Ala OCH_2tBu	BnNH	6OMe2′CMeG	0.43	0.02	1.90	0.11	>100
70	L-Ala OCH_2tBu	PhNH	6OMe2′CMeG	0.27	0.01	0.82	0.00	>100
71	L-Ala OCH_2tBu	NaphNH	6OMe2′CMeG	0.13	0.05	0.56	0.18	>100
72	L-Ala OCH_2tBu	diethylamine	6OMe2′CMeG	3.70		>10		>100
73	L-Ala OCH_2tBu	pyrrolidine	6OMe2′CMeG	0.87	0.02	4.00	0.16	>100
74	L-Ala OcHx	L-Ala OcHx	6OEt2′CMeG	0.05	0.00	0.15	0.02	69
75	L-Ala OBn	L-Ala OBn	6OEt2′CMeG	0.29	0.04	1.00	0.29	62
76	L-Ala OCH_2tBu	L-Ala OCH_2tBu	6OEt2′CMeG	0.04	0.01	0.11	0.04	72

of the neopentyl alanine compound as a naphthyl ProTide (**5**),[12] we were keen to explore the methylene bridged family starting with the neopentyl-(**26**) and 2-methylpropyl-(**27**) compounds and also the methylene cyclopropyl-(**28**).

On the basis of our recent report that the 6-ethoxy group can well substitute for the purine 6-methoxy as a core nucleoside for anti-HCV ProTides,[21] we applied the diamidate method to the *O*6-ethyl-2′-*C*-methylguanosine nucleoside, preparing the symmetrical cyclohexyl-(**74**), benzyl-(**75**), and neopentyl-(**76**) L-alanine analogues.

In aryl phosphoramidate ProTides, we have reported on several occasions the sometimes strong preference, for L-amino acids over D-analogues,[25] and all of the first examples reported here are L-amino acid derived. However, we did synthesize the neopentyl-D-alanine analogue **29** of **8**.

Although L-alanine is generally strongly preferred, other natural and unnatural amino acids are also effective as ProTides,[26] and so we did vary the amino acid motif itself. An attempt was made to consistently select from the same three ester groups, neopentyl, cyclohexyl, and benzyl, for each new amino acid to facilitate comparison. Thus, symmetrical diamidates were prepared from dimethyl-(**30**) and dibenzyl-(**31**) L-aspartic acid, benzyl-(**32**) and neopentyl-(**33**) glycine, cyclohexyl-(**34**), benzyl-(**35**), and neopentyl-(**36**) L-leucine, methyl-(**37**), cyclohexyl-(**38**), benzyl-(**39**), and neopentyl-(**40**) L-isoleucine, cyclohexyl-(**41**), benzyl-(**42**), and neopentyl-(**43**) L-methionine, cyclohexyl-(**44**), benzyl-(**45**), and neopentyl-(**46**) L-phenylalanine, benzyl-(**47**) and neopentyl-(**48**) L-proline, and cyclohexyl-(**49**), benzyl-(**50**), and neopentyl-(**51**) L-valine. The tyrosine methyl ester diamidate was also prepared as its *para*-*O*-*tert*-butyl ether-(**52**). Diamidates of the unnatural amino acid L-phenylglycine were prepared as its cyclohexyl-(**53**) and neopentyl-(**54**) esters. In a prior program on ProTides of d4T for HIV, we reported a complete loss of activity on extending from alanine to β-alanine and beyond,[27] but it was unclear whether similar restrictions would apply here, hence we prepared the symmetrical benzyl-β-alanine diamidate-(**56**).

Finally, in this series, we wondered if the chemistry methodology would extend to a dipeptide and whether such an adduct might be active and so we successfully incorporated an L-valyl-L-alanine neopentyl ester (**55**).

Simple amines have not been found to be useful as the amino component of aryl phosphoramidates, although Idenix has successfully incorporated benzylamine into their clinical analogue IDX184.[14] However, for diamidates, the SAR was unknown, and so we prepared the symmetrical butylamine-(**57**) and morpholinyl-(**58**) diamidates. Notably, from a synthetic perspective, none of the above amino acid and amine variations presented particular challenges although the yields from these reactions were not high and remain unoptimized.

Besides seeking to probe the SAR and potential advantages of symmetrical diamidates, as above, we also wondered if the synthetic method would allow access to asymmetrically mixed diamidates. To do this, we slightly adapted the synthetic route (Scheme 1) to allow the stepwise introduction of two separate amino acids or one amino acid and one amine. To limit the large number of possible combinations, and to facilitate interpretation of the data, one of the amines was generally kept constant as neopentyl L-alanine and variations were made in the second amine. Initially, different L-alanine esters, benzyl-(**60**), *tert*-butyl-(**61**), and cyclohexyl-(**62**), were combined with neopentyl L-alanine, followed by different amino acids methyl L-proline-(**63**) and methyl L-valine-(**64**), and then a number of different simple amines (**65**−**73**). Synthetically, it did not matter much which amine was introduced first. Each of these asymmetric diamidates was isolated as a roughly 1:1 mixture of phosphate diastereoisomers as revealed by ^{31}P NMR and HPLC. No attempt was made to separate the diastereoisomers, and they were tested as mixtures.

In this way, a substantial set of symmetrical and asymmetric phosphorodiamidates of 6-*O*-methyl-2′-*C*-methylguanosine and three derivatives of the 6-*O*-ethyl analogue were prepared and fully characterized. As expected, all of the symmetrical diamidates reported above were observed as one peak by ^{31}P NMR and one signal on analytical HPLC. The ^{31}P NMR shifts of the symmetrical amino aryl diamidates were ca. 13 ppm, being rather downfield of our usual aryloxy phosphoramidate ProTides.[28] The asymmetric diamidates gave two peaks by ^{31}P NMR and for HPLC, in roughly 1:1 ratios, as typically observed for phosphoramidate ProTides. Other spectroscopic and analytical data fully confirmed the structure and purity of the diamidates herein reported.

BIOLOGICAL ACTIVITY IN REPLICON

Each of the diamidates described above were tested for HCV inhibition in a replicon assay, with the clinical anti-HCV ProTide (**5**) as positive control (Table 1). Both EC_{50} and EC_{90} values are reported, along with standard deviations, in a 72 h HCV replicon assay. In general EC_{90} values were 2−5-fold higher than EC_{50} values, and the discussion below will focus on

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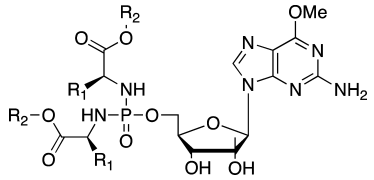
Table 2. Replicon Activity for Symmetrical Phosphorodiamidates



		replicon EC_{50} (μM) (R_2)			
amino acid	compd	cyclohexyl	benzyl	neopentyl	CC_{50} in Huh7 cells (μM)
L-alanine	**20**	0.05			>100
	21		0.49		>100
	19			0.06	>100
D-alanine	**29**			0.11	>100
glycine	**32**		0.60		>100
	33			0.13	>100
L-leucine	**34**	0.45[a]			20
	35		0.38		39
	36			0.47	27
L-isoleucine	**38**	4.0			14
	39		0.40		24
	40			2.9	15
L-valine	**49**	2.5			20
	50		0.12		49
	51			0.72[b]	32
L-proline	**47**		0.52		>100
	48			0.81	56
L-methionine	**41**	0.60			51
	42		0.25		>100
	43			2.22[b]	>100
L-phenylglycine	**53**	0.32			18
	54			0.27	24
L-phenylalanine	**44**	0.50[a]			25
	45		0.32		67
	46			0.05	24
L-aspartic (OBn)	**31**		0.61		>100

[a]Large standard deviation. [b]Single assay result.

the EC_{50} numbers. In addition, the cell cytotoxicities (CC_{50}) in the replicon cell line (Huh7) are reported.

As noted in Table 1, the three parent nucleosides, 2′-C-methyl guanosine **4** and the 6-methoxy **8** and 6-ethoxyl **9** analogues, display only modest anti-HCV activity, with EC_{50} values in the 2−10 μM range. By comparison, **5** is active at nanomolar levels, with an EC_{50} of 10 nM and an EC_{90} of 40 nM, representing a ca. 400-fold potency boost over the respective nucleoside.[12] Compound **5** does show some cytotoxicity to Huh7 cells in this assay at 7 μM, but its high potency still leads to a significant SI of ca. 700.

Examining the potency of the first family of symmetrical L-alanine phosphorodiamidates, we note a consistent and clear increase in potency for the *n*-alkyl esters as they extended from methyl (**10** EC_{50} = 6 μM) to *n*-pentyl (**14** EC_{50} = 0.03 μM). The *n*-pentyl L-alanine **14** is thus about 3 times less active than **5**, but notably it is also about 10 times less cytotoxic. It is interesting to note that the calculated lipophilicity (ClogP) values for this series range from 0.5 (methyl) to 4.7 (*n*-pentyl). It maybe that lipophilicity and potency correlate, however, there are exceptions. Clearly, the *n*-pentyl **14** is an interesting compound with a very attractive SI.

Branching the amino acid ester at the α (Table 1, **15**, **16**), β (**26**, **27**), or γ (**17**) position does not significantly change activity or toxicity relative to the straight chain compounds. Thus, the isopropyl ester **15** and the *n*-propyl ester **12** have

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similar activities, and the β branched neopentyl ester **26** is similar to the *n*-pentyl ester **14**. Indeed, several of these branched esters such as compounds **17** and **27** are very potent, with excellent SI values. We were particularly interested in the neopentyl L-alanine analogue **26** given its similarity to our clinical agent **5**. This compound reveals an EC_{50} of 0.06 μM and EC_{90} of 0.2 μM and is only 5-fold less active in this assay than **5**. However, in common with many of the phosphorodiamidates, this compound is significantly less cytotoxic, with CC_{50} >100 μM. Thus, the SI for **26** is >1600, which exceeds that of **5**.

Cyclic esters of L-alanine such as cyclopentyl-(**19**) and cyclohexyl-(**20**) are also very potent in the replicon assay and show no Huh7 cell toxicity at 100 μM. However, the cyclobutyl derivative **18** is about 5-fold less active with an EC_{50} of 0.32 μM, and the tetrahydropyranyl ester **24** is more than 200-fold less active compared to **20**.

Interestingly, the benzyl ester derivatives of L-alanine (Table 1, **21**−**23**) are somewhat less active than many of the alkyl ester analogues, being 5−10-times less active than the cyclohexyl compound for example. This is in marked contrast to the phosphoramidate ProTides SAR and much of our prior experience.[28]

An observation from our phosphoramidate SAR is that the purine C-6-substituent can be varied considerably and that the 6-ethoxy may be particularly effective.[24] As noted in Table 1, the same applies to this series, with the cyclohexyl-(**74**), benzyl-(**75**), and neopentyl-(**76**) purine C-6-ethoxy derivatives all being equipotent with their C-6-methoxy analogues. Indeed, with an EC_{90} of 110 nM, the neopentyl L-alanine analogue of the 6-ethoxy nucleoside **76** emerged as one of the most potent compounds in the present study.

The SAR next turned to symmetrical phosphorodiamidates with amino acids other than L-alanine. The subtlest change was to make the D-alanine analogue as its neopentyl ester **29**. In contrast to our earlier work on D-amino acids in aryl phosphoramidate ProTides,[25] here we see only a slight (ca. 2-fold) loss of activity. This again points to quite a new and separate SAR for these diamidates as compared to aryloxy phosphoramidates.

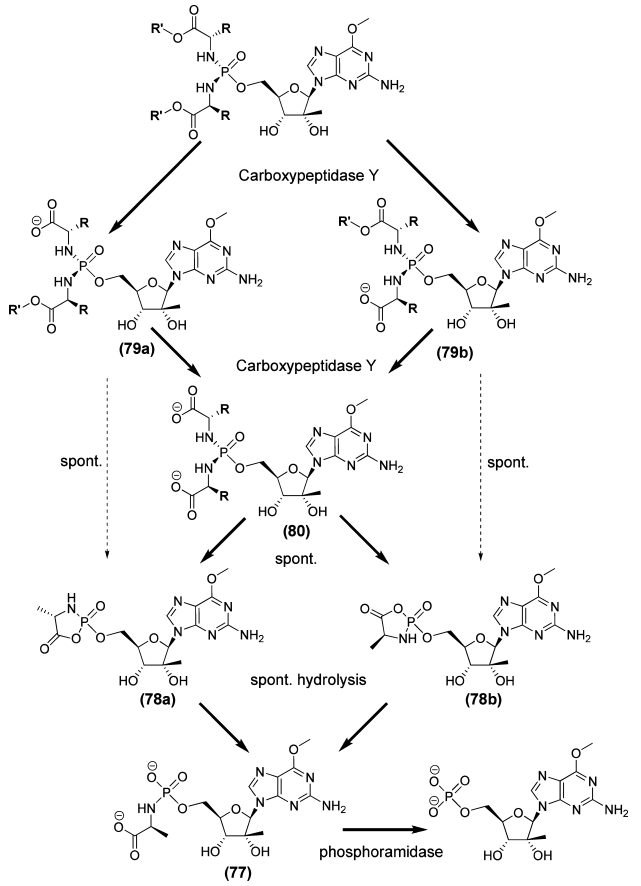
In generating the amino acid SAR in this series of symmetrically substituted phosphorodiamidates, effort was made to use three similar esters for each different amino acid derivative to facilitate comparison. The three ester groups selected were the cyclohexyl, benzyl, and neopentyl. Table 2 shows the compiled data.

A number of observations can be made from the data in Table 2. First, the benzyl esters do not greatly distinguish the different amino acids. The replicon activities for nine amino acids with benzyl ester groups range from 0.25 to 0.61 μM, only 2- to 3-fold, which is similar to the variability in the replicon assay.

The neopentyl and cyclohexyl ester groups distinguish the different amino acids and give a similar rank order. Thus, the best amino acid for both the cyclohexyl and the neopentyl esters is the L-alanine, whereas the worst is L-isoleucine.

Overall evaluation of the amino acid SAR leads to conclusion that small amino acid substituents like glycine or alanine are preferred and that increasing the size of the amino acid R_1 group as for L-leucine and L-methionine is detrimental to replicon activity, as is branching of the amino acid as for L-valine, L-isoleucine, and L-phenylglycine.

Scheme 2. Putative Initial Activation Route of Phosphorodiamidates



The exception to this rule is that the neopentyl L-phenylalanine derivative **46** is very potent (EC_{50} = 0.05 μM) in the replicon assay. The cyclohexyl L-phenylalanine derivative **44** was tested twice, and one value was 10-fold higher than the other (EC_{50} = 0.083 and 0.92). It is possible that further testing would refine these data. Further supporting this surprising L-phenylalanine SAR, the L-tyrosine derivative **52**, as the *para*-*O*-*tert*-butyl ether, also had good activity and limited toxicity in the replicon assay (EC_{50} = 0.11 μM, CC_{50} = 89 μM).

A further observation in this series of symmetrical phosphorodiamidates is that some cytotoxicity is observed in Huh7 cells for the amino acids other than alanine and glycine. In fact, the cyclohexyl L-isoleucine **38** demonstrates that not all phosphorodiamidates are equivalent. Compound **38** has an EC_{50} of 4 μM and a CC_{50} of 14 μM, for an SI of only ca. 4, and being the most cytotoxic diamidate studied.

As an extension of the growing symmetrical phosphorodiamidate SAR, a more complicated, dipeptide, L-Val-L-Ala neopentyl ester **55**, in which the L-valine nitrogen forms the phosphoramidate linkage, was prepared. Testing in the replicon assay shows that **55** has similar replicon activity (Table 1, EC_{50} = 0.54 μM) as the neopentyl L-valine derivative **51**. It is unclear how this dipeptide would fit in with the putative metabolic process (see Scheme 2) and may suggest the existence of another metabolic pathway leading to the triphosphate formation for this compound.

Working the SAR in the opposite direction, simple amine substituted phosphorodiamidates were tested in the replicon assay. The *n*-butyl amine analogue **57**, which may be

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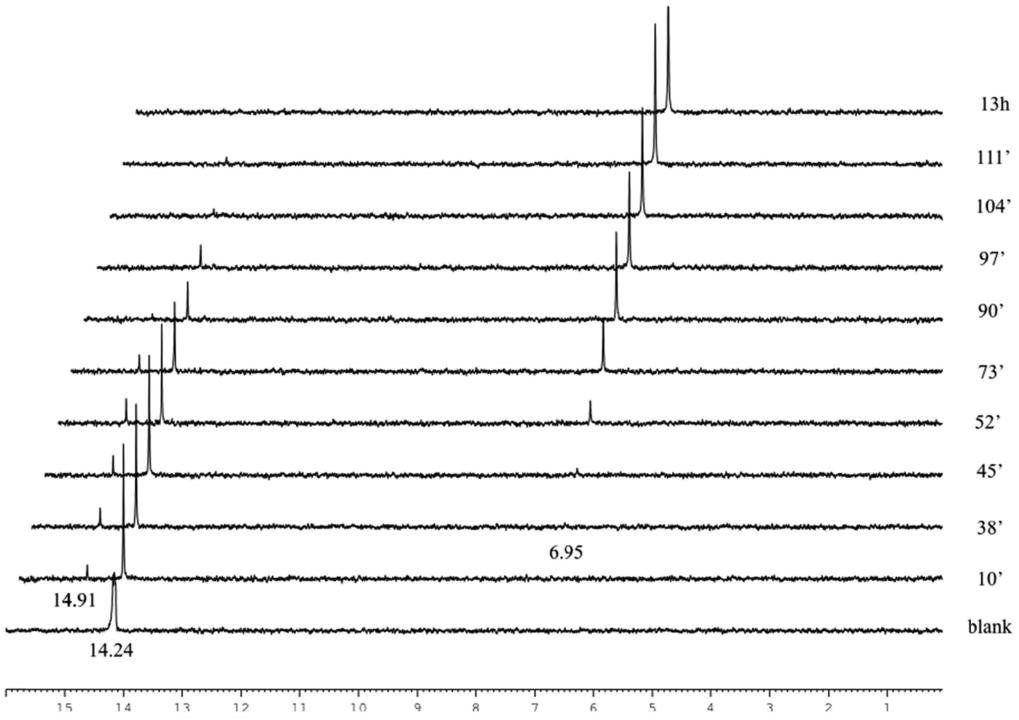

Figure 3. ^{31}P NMR kinetic study of **26** in the presence of carboxypeptidase Y. Conditions: 5.0 mg of **26** in 200 μL of acetone-d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3−0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4).

considered similar to the L-leucine derivatives, but without the α carboxylate, has a replicon EC_{50} of 38 μM, which is about 100-fold less active than the L-leucine derivatives. This is consistent with the proposed metabolic route (Scheme 2) where the carboxylate anion plays a key role. The positioning of the carboxylate β to the amine, as for the β-alanine analogue **56**, results in moderate activity in the replicon assay (Table 1, EC_{50} = 3.8 μM). The morpholino phosphorodiamidate **58**, an example of a secondary amine, is inactive even at 100 μM in the replicon assay. Both of these simple amine phosphorodiamidates **57** and **58** are much less active than the parent nucleoside **8** in the replicon assay, supporting our understanding that there is a limited direct cleavage of the P−O bond, leading to free nucleoside, in the replicon assay.

Although one of the key motivations for this new ProTide motif was to generate an achiral phosphate center, we were interested in understanding some aspects of the asymmetric diamidate SAR. Our synthetic route (Scheme 1) did allow the stepwise addition of two different amines. Each of these asymmetric diamidates were isolated as a roughly 1:1 mixture of phosphorus diastereoisomers, and no attempt was made to separate them and they were tested as mixtures. To facilitate comparison, generally, one of the two amines was maintained as the L-alanine neopentyl ester (Table 1).

From this basis, our SAR study was focused in three areas. First, we explored compounds **60**−**62** in which the second amine was also L-alanine but with different ester groups. The replicon results indicated that the presence of the L-alanine neopentyl ester provides good antiviral potency even in the presence of difficult to cleave[29] *tert*-butyl L-alanine ester **61**. The presence of two easily cleavable esters such as for derivatives **59** and **62** slightly increased the replicon activity comparing to **61** but one "cleavable" group was sufficient.

Next, the SAR of asymmetrical phosphorodiamidates, where the neopentyl L-alanine is combined with different amino acids was briefly explored. The methyl L-proline **63** and the methyl L-valine **64** amino acids had moderate replicon activity (Table 1), more similar to the symmetrical L-prolines and L-valines than the very potent symmetrical neopentyl L-alanine **26**. It might be that compounds **63** and **64** have different metabolic intermediates (**78**, Scheme 2) than does **26**.

Third, the phosphorodiamidate SAR of simple amines combined with neopentyl L-alanine was extensively studied (**66**−**73**, Table 1). In general, these compounds had moderate to good activity, with EC_{50} values ranging from 0.1 to 0.9 μM for monosubstituted amines, more active than the symmetrical simple amines, but less active than the symmetrical neopentyl L-alanine derivative (**26**). Presumably, conversion of compounds **66**−**73** involves cleavage of the L-alanine neopentyl ester followed by the elimination of the simple amine to form the key metabolic intermediate (**78**, Scheme 2). Thus, it might be expected that the amines that make the best leaving groups such as phenylamine **70** and naphthylamine **71**, would be the most active (EC_{50} = 0.27 and 0.13 μM) and that a poor leaving group such as diethyl amine, in derivative **72**, would be the least active (EC_{50} = 3.7 μM, Table 1).

In conclusion, all but one of the 67 phosphorodiamidates tested in the replicon assay were active at or below micromolar concentrations, with 55 of them active below 1 μM and 12 active below 100 nM. Many are noncytotoxic at high μM levels, some at 100 μM, giving attractive SI values for many compounds. Clearly several of these compounds were worthy of advancing to in vivo studies.

■ STABILITY ASSAYS

Given the new structural motif we are reporting and the very promising replicon data (as described above), we sought to establish some outline stability data under a variety of conditions. To begin with, the lead symmetrical neopentylalanine compound **26** was dissolved in pH 7 phosphate buffer at

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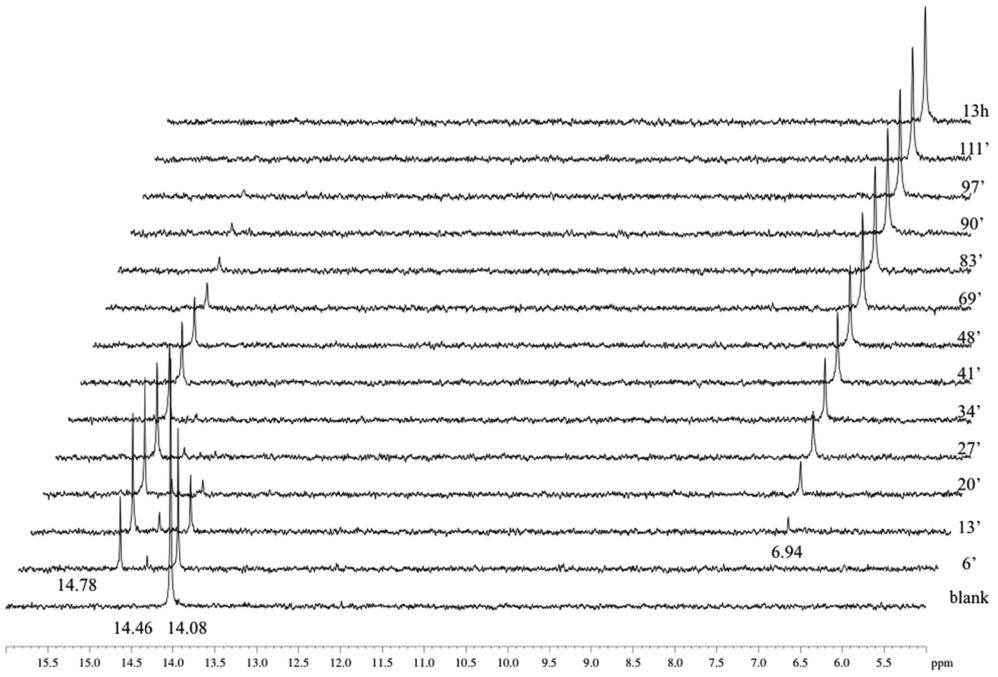

Figure 4. ^{31}P NMR kinetic study of **21** in the presence of carboxypetidase Y. Conditions: 5.1 mg of **21** in 200 μL of acetone-d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3−0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4).

25 °C and monitored by ^{31}P NMR over 14 h. Compound **26** was observed at 13.2 ppm, and no additional signal(s) appeared over the course of the experiment (see Supporting Information for stability spectra). HPLC also indicated no detectable decomposition. We have previously reported acid stability data[27] for several acyclovir aryloxy phosphoramidate ProTides. As a representative example, the phosphorodiamidate (**26**) was dissolved in citric acid−HCl buffer at pH 2 and maintained at either 37 or 47 °C while being monitored by ^{31}P NMR over 14 h. Despite the acidic pH and elevated temperatures, the samples remained entirely stable over the course of the assay. A base stability study was conducted on compound **26** at pH 8.5/37 °C and pH 11/37 °C, and these studies revealed slow decomposition. After 17 h at pH 11, the majority species was still unchanged (**26**), with only minor peaks at 6.9 ppm and −4.3 ppm. The peak at 6.9 ppm corresponds to an amino acid phosphoramidate derivative similar to compound **77** (see Scheme 2 below), suggesting one phosphorodiamidate P−N bond on compound **26** had been hydrolyzed under the aqueous basic conditions. However, the decomposition is rather slow, with a half-life exceeding 100 h (if first order). From this initial stability data, it seems possible that the phosphorodiamidates have a pH stability profile that is consistent with oral dosing.

Next, we studied the stability of compound **26** with human serum at 37 °C. As before, the solution was monitored at 1 h intervals over by ^{31}P NMR over 12 h. The phosphorodiamidate **26** gave a peak at 14.5 ppm under these conditions, and no sign of degradation was observed by ^{31}P NMR. Thus, as noted for aryloxy phosphoramidates,[30] the present phosphorodiamidates seem essentially stable in human serum, certainly for periods of hours appropriate for human dosing.

As we have reported previously,[12,30] the initial step of the conversion of aryloxy amino acid ester phosphoramidates (ProTides) to phosphates is thought to involve an enzyme-mediated cleavage of the amino acid ester group. We have also reported on the use of ^{31}P NMR and a buffered solution of the enzyme carboxypeptidase Y as an in vitro model for studying the initial steps of ProTide[30] activation. We sought to apply this method to our new family of anti-HCV phosphorodiamidates. Thus, compounds of interest were dissolved in acetone-d_6 and TRIZMA buffer at pH 7.4, and the ^{31}P NMR spectrum was recorded as the baseline. Then carboxypeptidase Y (cathepsin) was added and spectra recorded at intervals up to 13 h (Figure 3). The data from these experiments were used to map a possible phosphorodiamidate metabolic pathway (Scheme 2).

The first compound studied was the symmetrical neopentyl ester of L-alanine **26**, which has a ^{31}P NMR shift of 14.2 ppm at baseline (Figure 3). After 10 min, a small downfield metabolite peak is observed at 14.9 ppm, which is consistent with cleavage of the neopentyl ester. The peak at 14.9 ppm builds up for approximately one hour, then diminishes, with a new peak emerging at 6.95 ppm. This is first observed at ca. 40 min and continues to grow through the course of the experiment. The peak at 6.95 corresponds to the key amino acid phosphate derivative **77** (R = CH_3) (Scheme 2). This was determined by synthesizing compound **77** (R = CH_3) and using it as an analytical reference standard (data not shown). Intermediate **77** could form by intramolecular attack of the amino acid carboxylate anion onto the phosphorus, with elimination of the second amino acid, followed by spontaneous hydrolysis of the cyclic, mixed anhydride intermediate **78a**,**b**. No direct ^{31}P NMR evidence (Figure 3) was obtained for this cyclic compound, consistent with it being a short-lived metabolic intermediate. As discussed in our publications on aryloxy amino acid phosphoramidates, final conversion to the nucleoside monophosphate is generally thought to be effected by enzymes of the histidine triad nucleotide binding (Hint) family (Scheme 2).[30,33]

This initial evidence indicates that nucleoside phosphorodiamidates are converted to nucleoside monophosphates in a similar fashion as aryl phosphoramidates, that is, via metabolite **77**. To further support this hypothesis, a second phosphor-

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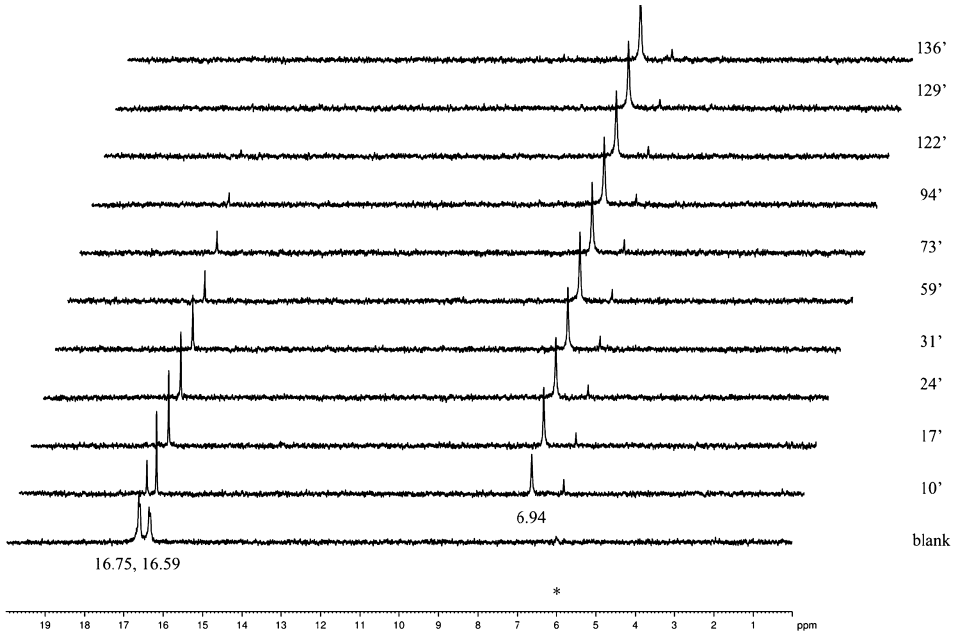

Figure 5. ^{31}P NMR kinetic study of **65** in the presence of carboxypetidase Y. Conditions: 4.9 mg of **65** in 200 μL of acetone-d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3−0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4). * Phosphate impurity in Trizma buffer; in blank and remains unchanged throughout.

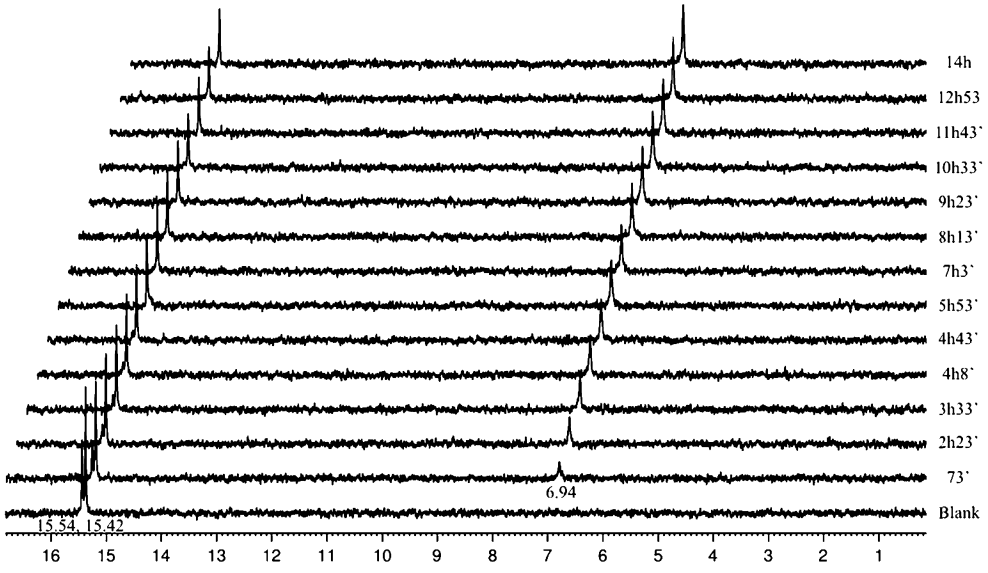

Figure 6. ^{31}P NMR kinetic study of **73** in the presence of carboxypetidase Y. Conditions: 5.3 mg of **73** in 200 μL of acetone-d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3−0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4).

odiamidate, the symmetrical benzyl L-alanine derivative **21**, was studied in the same system (Figure 4). In this case, a similar ^{31}P NMR pattern, with the formation of the same key metabolite at 6.94 ppm (**77**, R = CH_3) is observed, but there are subtle differences. Parent **21** shows one phosphorus ^{31}P NMR peak at 14.08 ppm at baseline, which disappears within the first 30 min upon incubation with enzyme. However, in this case, two downfield singlet peaks are observed, one small and transient at 14.46 ppm and the other larger and longer lived at 14.78 ppm. Three possibilities exist to explain these two new downfield peaks: (a) one specific benzyl ester is cleaved by carboxypeptidase Y to give either **79a** or **79b**, but not both, followed by a second ester cleavage to give **80**; (b) both pro-pR and pro-pS benzyl esters are cleaved by carboxypeptidase Y and both **79a** and **79b** are observed, but cleavage of both benzyl esters does not occur; (c) the peak at 14.46 ppm represents mono ester cleavage, where the ^{31}P NMR signals of **79a** and **79b** overlap, and the peak at 14.78 represents diester cleavage to give **80**.

Regardless of how the amino acid ester groups are cleaved by this particular enzyme, the important information from the ^{31}P NMR experiments is that compound **21** goes through the same common intermediate **77** (R = CH_3) as does compound **26**.

In Supporting Information, we present further detail of the kinetics of appearance of each species in the carboxypeptidase Y mediated cleavage of **21**.

It should be noted that **26** is nearly 10-fold more active than **21** in the HCV replicon assay (Table 1), however comparison

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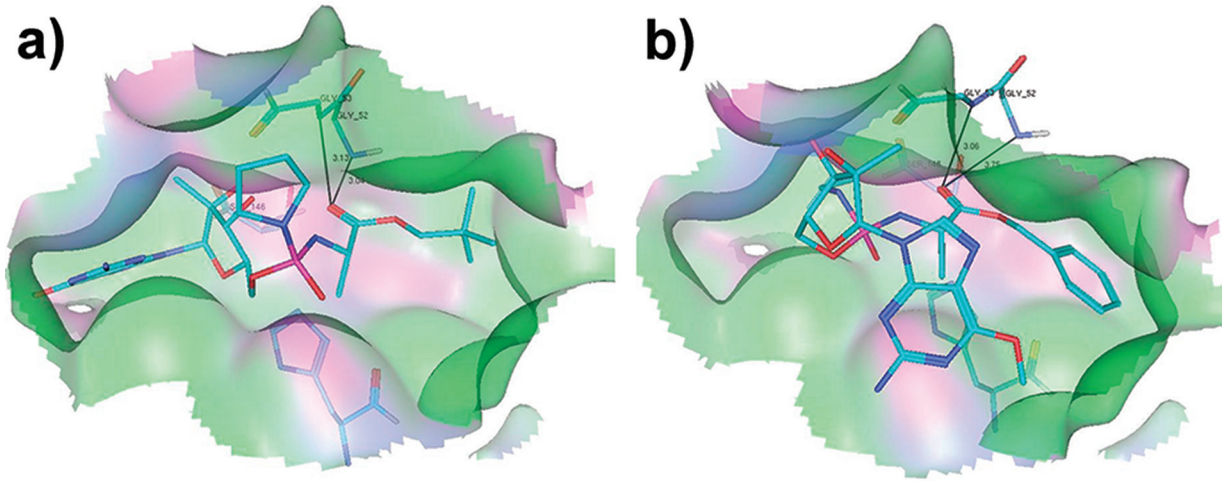

Figure 7. (a) Docking of compound **65−Rp** isomer within the catalytic site of carboxypeptidase Y. (b) Docking of compound **65−Sp** isomer within the catalytic site of carboxypeptidase Y.

Table 3. Rat Liver Triphosphate Levels from PK Analysis of Phosphorodiamidates

compd	AA ester	replicon EC_{50} (μM)	C_{max}[a] (ng/g)	C_{last}[b] (ng/g)	T_{max}[c] (h)	AUC_{0-t}[d] (ng·h/g)
5[e]	L-Ala OCH_2tBu	0.01	1446.7	983.3	8.0	24557
26	L-Ala OCH_2tBu	0.06	1580.0	800.0	8.0	25147
13	L-Ala *O*-*n*-butyl	0.07	1553.3	719.7	8.0	27671
14	L-Ala *O*-*n*-pentyl	0.03	1042.3	506.0	8.0	18600
16	L-Ala O-(*R*,*S*)-2-butyl	0.15	907.7	538.7	8.0	16252
19	L-Ala *O*-cyclopentyl	0.06	1523.3	610.0	8.0	24546
25	L-Ala *O*-2-indanol	0.58	632.0	632.0	24.0	10642
29	D-Ala OCH_2tBu	0.11	598.3	441.7	8.0	11608
50	L-Val OBn	0.12	359.5	73.6	4.0	4478

[a] C_{max}: Maximum observed concentration. [b] C_{last}: Concentration at the last measurable time-point. [c] T_{max}: Time at which maximum concentration was observed. [d] AUC_{0-t}: area under the concentration−time curve from time 0 to the last measurable concentration. [e] Compound **5** is a the phosphoramidate INX-189 (see Figure 2).

of Figures 3 and 4 shows that the speed of carboxypeptidase Y processing is very similar for the two prodrugs, and thus the replicon and enzyme assays do not correlated directly, but we do regard enzyme mediated ester cleavage as a useful tool to study our prodrugs in vitro.

If a carboxylate anion intermediate such as **79a**, **79b**, or **80** can eliminate an amino acid moeity to give intermediate **77**, we wondered if simple primary or secondary amines could likewise be eliminated from asymmetric phosphorodiamidates such as **65** and **73**. Figure 5 shows the ^{31}P NMR traces for an experiment with the benzyl L-alanine, *n*-butyl amine derivative **65**, and carboxypeptidase Y.

Because **65** is asymmetrical, two peaks are seen in the baseline ^{31}P NMR (16.59 and 16.75 ppm). Both peaks disappear within 120 min, but the diastereomer at 16.75 is cleaved faster. Once again. a peak at 6.94, identified as compound (**77**, R = CH_3), is observed growing in magnitude during the course of the experiment. A very similar pattern is observed in Figure 6, when the neopentyl L-alanine, pyrrolidine asymmetrical phosphoramidate **73** was incubated with carboxypeptidase Y. Again compound **77** (R = CH_3) is observed at 6.94 ppm and grows in over 14 h. Interestingly, in this case, only one of the two diastereomers of compound **73** (^{31}P NMR δ = 15.54 ppm) is cleaved by carboxypeptidase Y over the course of the experiment.

Summarizing this portion or our work, we have built on our previous understanding that aryloxy groups are eliminated from asymmetrical phosphoramidates containing an amino acid carboxylate by showing that a second amino acid or a primary amine or a secondary amine can also be eliminated to give the key intermediate **77**. The data on **21**, **26**, **65**, and **73** supports the notion that only one ester cleavage is necessary and that the second amine loss is rapid following the first ester cleavage.

Docking Studies. To further support the above, we conducted some docking studies on several asymmetric diamidates, using published[31] crystal structure of carboxypeptidase Y. Thus, as shown in Figure 7a,b the Sp diastereomer of **65** binds significantly better than the Rp diastereomer.

In the case of the Sp isomer, the stabilization by H-binding of two glycine residues (Gly52, 53) is notable and the nucleophilic active site of Ser146 is also well positioned. These docking data would then suggest that one diastereoisomer of **65** might be processed better than the other. On the basis of the clear kinetic difference noted above (Figure 5), perhaps the more downfield species in ^{31}P NMR of **65** is the Sp isomer. It is interesting to wonder if the differing kinetics of metabolism here may lead to a difference in biological potency as we have noted in some cases for aryl phosphoramidates.[5] However, we were unable to separate the compound **65** diastereomers to test this hypothesis.

Pharmacokinetics. The HCV replicon, stability, and carboxypeptidase Y data suggests that our new phosphorodiamidate prodrug strategy may be a promising means of delivering 2′-*C*-methylguanosine triphosphate into cells. The

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next hurdle was to determine if this prodrug strategy works in vivo in a rodent. Because of our growing experience with phosphoramidates such as **5** in rats, we decided to continue with the rat as our initial PK model for the phosphorodiamidates. Eight symmetrical derivatives were selected for rat PK studies (**13**, **14**, **16**, **19**, **25**, **26**, **29**, and **50**, Table 3) based on activity in the replicon assay and structural considerations. We focused on symmetrical diamidates because they are represented by single diastereoisomers, which would simplify further development. All but two were L-alanine derivatives because we wanted to fully explore the ability of these relatively simple derivatives to provide sufficient liver triphosphate levels.

Compounds were formulated in 95% Capmul MCM/5% Tween 80, and doses of 10 mg/kg were administered by oral gavage to male Sprague–Dawley rats. Liver samples were collected up to 24 h postadministration and were snap-frozen in liquid nitrogen. Liver concentrations of 2′-*C*-methylguanosine triphosphate were determined by LC-MS/MS. Results for these eight phosphorodiamidates are compared to **5** in Table 3.

The main observation is that all compounds tested produced significant levels of triphosphate in rat livers from a 10 mg/kg dose, further validating phosphorodiamidates as effective phosphate prodrugs. In addition, several of the phosphorodiamidates provide similar liver triphosphate exposures as the clinical compound **5**, which has shown efficacy against HCV in phase Ib clinical trials.[23] The amount of 2′-*C*-methylguanosine triphosphate necessary to achieve an EC_{90} in the replicon assay can be determined by measuring triphosphate levels in the replicon cells upon incubation with a 2′-*C*-methylguanosine based inhibitor such as **5**. This EC_{90} triphosphate level in cells can be extrapolated to EC_{90} triphosphate levels in the liver, as measured in ng of triphosphate/gram of liver tissue. The value we have calculated is 243 pmol of triphosphate per gram of liver (equal to 131 ng/g).[32] Thus, for all phosphorodiamidates tested, except for **50**, the level of triphosphate 24 h post dose, is several fold above 131 ng/g level necessary to achieve 90% inhibition of HCV replication (Table 3).

An important part of our evaluation of any new prodrug approach for delivering 2′-*C*-methylguanosine triphosphate, including these phosphorodiamidates, is measurement of systemic nucleoside (2′-*C*-methylguanosine, **4**) levels after oral dosing of the prodrug. Our desire is to limit the systemic exposure of this nucleoside. The plasma 2′-*C*-methylguanosine levels for these eight phosphorodiamidates were measured, and all but compound **13** had lower nucleoside C_{max} values than **5** (<100 nM), and it is only slightly higher (data not shown).

The neopentyl L-alanine diamidate **26** has both the highest C_{max} and C_{last} of any diamidate tested. Figure 8 shows rat liver triphosphate levels after a 10 mg/kg dose for the clinical compound **5** and for **26**. Triphosphate levels were measured at eight time points over 24 h. It is clear that both prodrug strategies produce similarly high levels of triphosphate. The *n*-butyl L-alanine ester **13** has the highest overall triphosphate AUC (Table 3), and the cyclopentyl ester **19** also has excellent AUC and C_{last} values. These compounds along with the *n*-pentyl ester **14** were advanced to monkey PK studies. A combination of rat PK, monkey PK, and preliminary rodent toxicology studies will be used to help select a clinical candidate. These additional studies will be reported elsewhere upon completion.

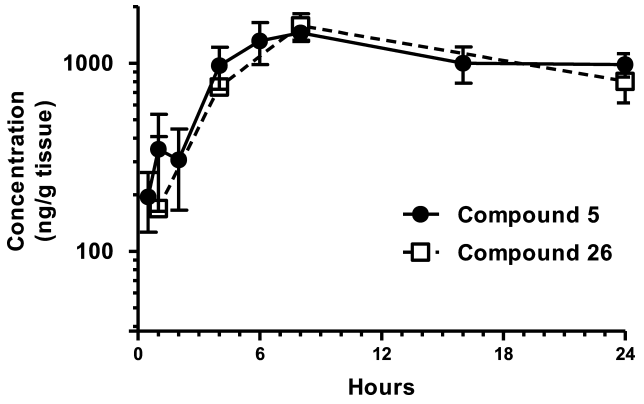

Figure 8. Rat liver 2′-*C*′-methyl guanosine triphosphate levels from compounds **5** and **26**.

CONCLUSIONS

In conclusion, we report on a new family of phosphate prodrugs based on phosphorodiamidates. This type of 5′-monophosphate prodrug has the advantage that it can be designed to be achiral at the phosphate center if desired. We report on a range of novel prodrugs derived from amino acids and simple amines and build a substantial HCV replicon SAR for both symmetrical and asymmetrical phosphorodiamidates of 2′-*C*-methyl-6-*O*-methylguanosine. The replicon data suggests that one aminoacyl ester is essential for potent activity versus HCV. The phosphorodiamidates are stable in acid and mild base and also in human serum. Carboxypeptidase Y is able to activate these compounds to a nucleoside aminoacid phosphate key intermediate, which is also the essential metabolic intermediate for our earlier aryloxy ProTides. Many of the novel compounds in this study show low nanomolar activity versus HCV in replicon coupled with low cytotoxicity in the Huh7 replicon cell line. Eight potent HCV inhibitors were advanced to PK studies in Sprague–Dawley rats, and it was demonstrated that they all provided substantial 2′-*C*-methylguanosine triphosphate levels, in rat livers, that were maintained over a period of 24 h. This body of work has validated phosphorodiamidates as prodrugs for 2′-*C*-methylguanosine both at the in vitro and the in vivo levels. Further in vivo studies are underway that are intended to lead toward selection of a phosphorodiamidate prodrug for HCV clinical studies.

EXPERIMENTAL SECTION

General. Anhydrous solvents were purchased from Aldrich and used without further purification. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminum plates and visualized under UV (254 nm) and/or with ^{31}P NMR spectra. Column chromatography was performed on silica gel (35–70 μM). Proton (^{1}H), carbon (^{13}C), and phosphorus (^{31}P) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak, and all ^{13}C NMR and ^{31}P NMR were proton-decoupled. Analytical and semipreparative HPLC were conducted by Varian Prostar (LC Workstation-Varian prostar 335 LC detector) using Varian Polaris C18-A (10 μM) as an analytic column and Varian Polaris C18-A (10 μM) as a semipreparative column; elution was performed using a mobile phase consisting of water/acetonitrile in gradient (system1, 90/10 to 0/100 v/v in 30 min) or water/methanol (system 2, 90/10 to 0/100 v/v in 30 min). High-resolution mass spectra (HRMS) was performed as a service by Cardiff University, using electrospray (ES). Compound purity was assured by a

combination of high field multinuclear NMR (H, C, P) and HPLC. Purity by the latter was always >95% with no detectable parent nucleoside for all final products.

Standard Procedure A: Synthesis Of Symmetrical Diamidates. To a suspension of the nucleoside (1.0 mol equiv) in anhydrous tetrahydrofuran, triethylamine (1.0−1.2 mol equiv) was added. After stirring for 30 min at room temperature, phosphoryl chloride (1.0−1.2 mol equiv) was added dropwise at −78 °C. The reaction mixture was stirred for 30 min at −78 °C and then allowed to warm to room temperature. Anhydrous dichloromethane was added, followed by amino acid ester (5.0 mol equiv) and triethylamine (10.0 mol equiv) at −78 °C. After stirring at room temperature for 20 h, water was added and the layers are separated. The aqueous phase was extracted with dichloromethane and the organic phase washed with brine. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting residue was purified by silica gel column chromatography using as an eluent a gradient of methanol in dichloromethane or chloroform. In some cases, a subsequent repurification was necessary either by preparative HPLC (gradient of methanol in water) or preparative TLC.

Standard Procedure B: Synthesis of Symmetrical Diamidates. To a solution of the nucleoside (1.0 mol equiv) in anhydrous triethylphosphate was added phosphoryl chloride (2.0 mol equiv) at 0 °C. The reaction mixture was stirred for 24 h at 5 °C. Anhydrous dichloromethane was added to the reaction mixture followed by amino acid ester (5.0 mol equiv) and diisopropylethylamine (10.0 mol equiv) at 0 °C. After stirring at 5 °C for 5 days, water was added and the layers were separated. The aqueous phase was extracted with dichloromethane and the organic phase washed with brine. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting residue was purified by silica gel column chromatography using as an eluent a gradient of methanol in dichloromethane. A subsequent repurification, if necessary, was accomplished either by preparative HPLC (gradient of methanol in water) or preparative TLC.

Standard Procedure C: Synthesis of Asymmetrical Diamidates. To a suspension of the nucleoside (1.0 mol equiv) in anhydrous tetrahydrofuran, triethylamine (1.0 mol equiv) was added. After stirring for 30 min at room temperature, phosphoryl chloride (1.0 mol equiv) was added dropwise at −78 °C. The reaction mixture was stirred for 30 min at −78 °C and then allowed to warm to room temperature. Anhydrous dichloromethane was added, followed by the addition of amino acid ester or amine (1 mol equiv) and anhydrous triethylamine (2 or 1 mol equiv, respectively) at −78 °C. Reaction was warmed to room temperature and monitored by ^{31}P NMR. When NMR indicated completion of the reaction (no starting material, presence of monosubstituted product), a second amino acid ester or amine (5 mol equiv) was added followed by the addition of triethylamine (10 or 5 mol equiv, respectively) at −78 °C. After stirring at room temperature for 16−20 h, water was added and the layers were separated. The aqueous phase was extracted with dichloromethane. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting residue is purified by silica gel column chromatography using as an eluent a gradient of methanol in chloroform.

Example: Synthesis of 2-Amino-6-methoxy-9-(2′-*C*-methyl-β-D-ribofuranosyl) Purine 5′-*O*-Bis(methoxy-L-alaninyl) Phosphate (10). The phosphorodiamidate **10** was prepared according to the standard procedure B.

In a first step, a suspension of 6-*O*-methyl-2′-*C*-methylguanosine (250 mg, 0.803 mmol) in anhydrous tetrahydrofuran (4 mL) was reacted with triethylamine (135 μL, 0.964 mmol) and phosphorus oxychloride (89 μL, 0.964 mmol). In a second step, anhydrous dichloromethane (4 mL), L-alanine methyl ester hydrochloride salt (561.1 mg, 4.02 mmol), and triethylamine (1.12 mL, 8.03 mmol) were added.

After workup, silica gel column chromatography and preparative HPLC, 19.8 mg of (**10**) was obtained in 4.4% yield as an off-white solid. ^{1}H NMR (500 MHz, MeOD-d_4) δ 7.99 (s, 1H, H-8), 5.99 (s, 1H, H1′), 4.44−4.34 (m, 2H, H5′), 4.31 (d, 1H, *J* = 8.9 Hz, H3′), 4.21−4.16 (m, 1H, H4′), 4.07 (s, 3H, OCH_3), 3.98−3.89 (m, 2H, 2× CHα Ala), 3.70, 3.69 (2s, 6H, 2× OCH_3 ester), 1.33 and 1.32 (2d, 6H, *J* = 7.1 Hz, CH_3 Ala), 1.00 (s, 3H, CH_3). ^{13}C NMR (126 MHz, MeOD-d_4) δ 176.10 (2d, $^3J_{C-C-N-P}$ = 4.8 Hz, 2× *C*═O Ala), 162.75 (C6), 161.95 (C2), 154.21 (C4), 139.33 (C8), 115.54 (C5), 93.19 (C1′), 82.33 (d, $^3J_{C-C-O-P}$ = 7.6 Hz, C4′), 80.02 (C2′), 74.73 (C3′), 66.13 (d, $^2J_{C-O-P}$ = 4.8 Hz, C-5′), 54.21 (OCH_3), 52.72 (2× CH_3 ester), 51.01, 50.93 (2d, $^2J_{C-N-P}$ = 2.3 Hz, 2× *C*H Ala), 20.86, 20.67 (2d, $^3J_{C-C-N-P}$ = 6.2 Hz, 2× CH_3 Ala), 20.27 (2′-CH_3). ^{31}P NMR (202 MHz, MeOD-d_4) δ 14.00. HPLC t_R = 8.86 min (system 1).

Example: Synthesis of 2-Amino-6-methoxy-9-(2′-*C*-methyl-β-D-ribofuranosyl) Purine 5′-*O*-Bis(benzoxy-L-alaninyl) Phosphate (21). The phosphorodiamidate **21** was prepared according to the standard procedure B.

In the first step, a solution of 6-*O*-methyl-2′-*C*-methylguanosine (250 mg, 0.803 mmol) in anhydrous triethylphosphate (1 mL) was reacted with phosphorus oxychloride (148 μL, 1.61 mmol). In the second step, anhydrous dichloromethane (4 mL), the tosylate salt of benzoxy-L-alanine (1.41 g, 4.02 mmol), and diisopropylethylamine (1.40 mL, 8.03 mmol) were added to the previous mixture. After workup, silica gel column chromatography and preparative HPLC, 50.1 mg of **21** was obtained in 8.7% yield as an off white solid. ^{1}H NMR (500 MHz, MeOD-d_4) δ 7.96 (s, 1H, H-8), 7.34−7.25 (m, 10H, 2× Ph), 5.99 (s, 1H, H1′), 5.16−5.02 (m, 4H, 2× CH_2 ester), 4.41−4.31 (m, 2H, H-5′), 4.29 (d, 1H, *J* = 9.0 Hz, H3′), 4.21−4.15 (m, 1H, H4′), 4.04 (s, 3H, OCH_3), 4.02−3.94 (m, 2H, 2× CH Ala), 1.33 (d, 6H, *J* = 7.1 Hz, 2× CH_3 Ala), 0.99 (s, 3H, CH_3). ^{13}C NMR (126 MHz, MeOD-d_4) δ 175.42, 175.36 (2d, 2× C═O, $^3J_{C-C-N-P}$ = 6.3 Hz, ester), 162.72 (C6), 161.91 (C2), 154.56 (C4), 139.31 (C8), 137.32, 137.29 (d, 2× C ipso OCH_2Ph), 129.55, 129.5, 129.24, 129.21 (OCH_2Ph), 115.55 (C5), 93.17 (C1′), 82.37 (C4′), 80.01 (C2′), 74.81 (C3′), 67.89,67.87 (2× OCH_2Ph), 66.26 (C5′), 54.19 (OCH_3), 51.13, 51.08 (2d, 2× Cα Ala), 20.79−20.58 (2d, $^3J_{C-C-N-P}$ = 6.3 Hz, 2× CH_3 Ala), 20.26 (2′CCH_3). ^{31}P NMR (202 MHz, MeOD-d_4) δ 13.93. HPLC t_R = 13.16 min (system 1).

Example: Synthesis of 2-Amino-6-methoxy-9-(2′-*C*-methyl-β-D-ribofuranosyl) Purine 5′-*O*-[(Benzoxy-L-alaninyl)-(2,2-dimethylpropoxy-L-alaninyl)] Phosphate (60). The phosphorodiamidate **60** was prepared according to the standard procedure C.

In the first step, a solution of 6-*O*-methyl-2′-*C*-methylguanosine (250 mg, 0.803 mmol) in anhydrous tetrahydrofuran (5 mL) was allowed to react with triethylamine (110 μL, 0.803 mmol) and phosphorus oxychloride (70 μL, 0.803 mmol). The tosylate salt of benzoxy-L-alanine (282 mg, 0.803 mmol) and triethylamine (110 μL, 0.803 mmol) were added. Anhydrous dichloromethane (4 mL) and the tosylate salt of neopentyloxy-L-alanine (1.33 g, 4.02 mmol) and triethylamine (1.12 mL, 8.03 mmol) were added as described in method C. After workup and silica gel column chromatography, 25 mg of the prodrug was obtained in 4% yield as an off-white solid. ^{1}H NMR (500 MHz, MeOH-d_4) 7.97, 7.96 (2s, 1H, H8), 7.36−7.30 (m, 5H, OCH_2Ph), 5.98, 5.97 (2s, 1H, H1′), 5.18- 5.09 (m, 2H, OCH_2Ph), 4.39−4.33 (m, 2H, H5′), 4.28 (2d, *J*= 8.00 Hz, 1H, H3′), 4.20−4.16 (m, 1H, H4′), 4.06, 4.05 (2s, 3H, 6OCH_3), 4.02−3.94 (m, 2H, 2× CHα Ala), 3.84, 3.82, 3.72, 3.67 (2AB, J_{AB}= 10.50 Hz, 2H, $CH_2C(CH_3)_3$), 1.39−1.32 (m, 6H, 2× CH_3 Ala), 0.97 (s, 3H, 2′CCH_3), 0.93, 0.91 (2s, 9H, $CH_2C(CH_3)_3$). ^{13}C NMR (126 MHz, MeOH-d_4) 175.54, 175.43, 175.39 (C═O ester), 162.73, 162.71 (C6), 161.93, 161.89 (C2), 154.57, 154.55 (C4), 139.32, 139.08 (C8), 137.39 (ipso OCH_2Ph), 129.55, 129.35, 129.25, 129.23, 129.20, 129.16, 128.27, 128.00 (OCH_2Ph), 116.19, 115.54 (C5), 93.34, 93.18 (C1′), 82.39, 82.33 (C4′), 80.01, 79.99 (C2′), 75.34, 75.04 ($CH_2C(CH_3)_3$), 74.84, 74.82 (C3′), 67.88, 67.85 (OCH_2Ph), 67.86 (d, $^2J_{C-O-P}$ = 3.75 Hz, C5′), 66.36 (d, $^2J_{C-O-P}$ = 5.50 Hz, C5′), 54.18, 54.01 (6OCH_3), 49.69, 49.64, 49.52, 49.46 (2× Cα Ala), 32.28, 32.25 ($CH_2C(CH_3)_3$), 26.74, 26.71 ($CH_2C(CH_3)_3$), 21.07, 20.90, 20.79, 20.66 (4*d*, $^3J_{C-C-N-P}$ = 6.25 Hz, 2× CH_3 Ala), 20.39, 20.25 (2′CCH_3). ^{31}P NMR (202 MHz, MeOH-d_4) 13.98, 13.94. HPLC t_R = 16.11, 16.80 min (system 1). MS (TOF ES+) *m*/*z*: 716.28 (M + Na^+, 100%). HRMS $C_{30}H_{44}N_7O_{10}P_1$ calculated, 694.2966; found, 694.2956

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Biological Methods. *Replicon Assays.* The HCV inhibitory activity of compounds was evaluated in an Huh7 cell line expressing a stable, bicistronic subgenomic HCV genotype 1b (Con1) replicon encoding the Renilla luciferase reporter gene (Apath, LLC, Brooklyn, NY) as previously described.[32] Cellular cytotoxicity was evaluated using the CellTiter-Glo Luciferase assay (Promega, Madison WI). A day before testing, 2×10^4 Huh7 cells were seeded in 96-well flat bottom white plates (Nunc, Roskilde, Denmark). Four-fold serial drug dilutions were made in growth medium and added to the cells. No drug controls were included in each plate. The plates were incubated in the presence of test compound for 3 days at 37 °C with 5% CO_2. Luciferase reagent was added to cells and plates were incubated for 20 min before measuring relative luminescent units (RLU) in a luminometer (Veritas, Turner Biosystems, Sunnyvale, CA).

Pharmacokinetic Studies in Rats. Rat studies were conducted at Inhibitex, Inc., in accordance with NIH Guidelines and following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Inhibitex, Inc. Studies were carried out as previously described.[32] Test compounds were formulated in 95% Capmul MCM (ABITEC Corp., Janesville, WI)/5% Tween 80 (Sigma, St. Louis, MO), and doses of 10 mg/kg were administered by oral gavage to male Sprague–Dawley rats (Taconic Farms, Germantown, NY). Liver samples were collected as a terminal procedure up to 24 h postadministration and were snap-frozen immediately upon collection in liquid nitrogen. Liver samples were stored frozen at ≤−80 °C prior to analysis.

Bioanalysis of Pharmacokinetic Samples. The concentration of 2′-*C*-MeGTP in liver samples from rats was measured by LC-MS/MS as described previously.[28] The assay was linear ($r^2 \geq 0.99$) in the concentration range of 100–4000 ng per gram of tissue with ≥85% accuracy and ≤2% CV. Noncompartmental pharmacokinetic analyses were performed on the liver concentration data using WinNonlin v5.2 software (Pharsight, St. Louis, MO) as described previously.[32]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Preparative methods, spectroscopic and analytical data on target compounds plus ^{31}P NMR stability assays and metabolic activation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

DAAs, direct acting antivirals; HCV, hepatitis C virus; AZT, 3′azidothymidine; SAR, structure–activity relationships; TLC, thin layer chromatography; HPLC, high performance/liquid chromatography; ClogP, calculated logarithm of the octanol/water partition coefficient; PK, pharmacokinetics

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