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Phosphoramidate ProTides of 2′-C-Methylguanosine as Highly Potent Inhibitors of Hepatitis C Virus. Study of Their in Vitro and in Vivo Properties

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Hepatitis C virus infection constitutes a serious health problem in need of more effective therapies. Nucleoside analogues with improved exposure, efficacy, and selectivity are recognized as likely key components of future HCV therapy. 2′-C-Methylguanosine triphosphate has been known as a potent inhibitor of HCV RNA polymerase for some time, but the parent nucleoside is only moderately active due to poor intracellular phosphorylation. We herein report the application of phosphoramidate ProTide technology to bypass the rate-limiting initial phosphorylation of this nucleoside. Over 30 novel ProTides are reported, with variations in the aryl, ester, and amino acid regions. L-Alanine compounds are recognized as potent and selective inhibitors of HCV in replicon assay but lack rodent plasma stability despite considerable ester variation. Amino acid variation retaining the lead benzyl ester moiety gives an increase in rodent stability but at the cost of potency. Finally L-valine esters with ester variation lead to potent, stable compounds. Pharmacokinetic studies on these agents in the mouse reveal liver exposure to the bioactive triphosphate species following single oral dosing. Systemic exposure of the ProTide and parent nucleoside are low, indicating possible low toxicity in vivo, while liver concentrations of the active species may be predictive of efficacy in the clinic. This represents one of the most thorough cross-species studies of ProTides to date.

Introduction

An estimated 200 million people, or ca. 3.5% of the world's population, are chronically infected with the hepatitis C virus (HCV[a]) and at risk of developing life threatening liver disease such as cirrhosis or liver carcinoma. HCV infection is the major cause of liver transplantation in industrialized countries. The current therapy for HCV consists of pegylated interferon and ribavirin, neither of which are specific inhibitors of HCV, and a regimen which has side effects and limited efficacy in at least half of the patient population.[1]

The HCV genome offers several clear targets for specific anti-HCV therapy; these include an RNA polymerase and a serine protease, both of which have attracted considerable academic and industrial attention.[2]

The RNA dependent HCV polymerase is considered to be an attractive target for therapy on account of a high degree of conservation across the six HCV genotypes, and agents targeted at the active site, such as nucleoside analogues, may be particularly advantageous with respect to the barrier to resistance.[3]

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[a] Abbreviations: HCV, hepatitis C virus; TLC, thin layer chromatography; HPLC, high performance, liquid chromatography; ClogP, calculated logarithm of octanol−water partition coefficient; PK, pharmacokinetics; SI, selectivity index; DNAUC, dose normalized area under the curve; NTP, nucleoside triphosphate; THF, tetrahydrofuran.

Several families of nucleoside analogues have emerged with apparent selectivity for HCV, these include 4′-modified agents such as 4′-azidocytidine (R1479, **1a**, Figure 1) developed as an oral pro-drug by Roche[4] and 2′-C-methyl adenosine (**1b**, Figure 1) and related deaza compounds developed by Merck.[5]

Interestingly, the Merck team observed that the guanine analogue **1c** was more potent (> 10-fold) as an inhibitor of the HCV RNA polymerase, as its bioactive 5′-triphosphate form, than was the triphosphate of the adenosine lead **1b**. However, poor cell entry by **1c** coupled with apparently poor phosphorylation lead to it being > 10-fold less active than **1b** in HCV replicon (**1b** EC_{50} 0.26 μM, **1c** 3.5 μM).[5]

Unfortunately, despite the reasonable in vitro potency and selectivity of **1b**, the compound could not be progressed due to it being a good substrate for metabolic enzymes (adenosine deaminase and purine nucleoside phosphorylase), leading to low oral bioavailability.[5]

To overcome these PK liabilities of **1b**, the Merck team progressed with several unnatural base modifications, such as the 7-deaza family, leading to their late preclinical candidate MK-0608. As recently highlighted, a lack of data on this family for over three years now may place a query over their progression.[3] We were concerned that such base modifications could carry with them the possibility of toxicity, and we sought to retain a natural base and to seek to overcome the limitations of the guanosine compound **1c** in particular. Because the first phosphorylation of **1c** was considered to be rate-limiting, we

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wondered if a pro-drug of the 5′-monophosphate of **1c** may be active, and thus we applied our phosphoramidate ProTide approach[6] to this nucleoside. We first introduced this ProTide motif in 1996[7] and have exemplified it considerably,[6] including very recently to **1a**[8] and briefly to **1b**−**c**.[9] Our approach has also been adopted by Pharmasset for their 2′-modified nucleoside HCV family[10] and adapted to acyclic nucleoside phosphonates by Gilead.[11] The Pharmasset nucleoside is also 2′-*C*-methyl based, but is a uridine compound, also with a 2′-α-fluorine. This company are using a ProTide based on isopropylalanine phenyl phosphate,[12] rather parallel to our early work on anti-HIV ProTides[13] Idenix have also pursued a

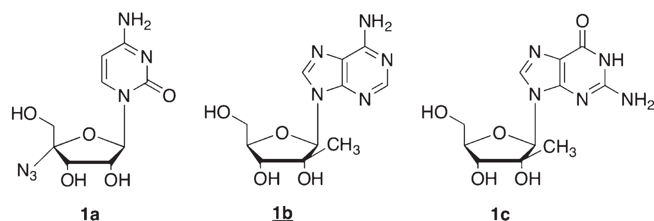


Figure 1. Some anti-HCV nucleosides.

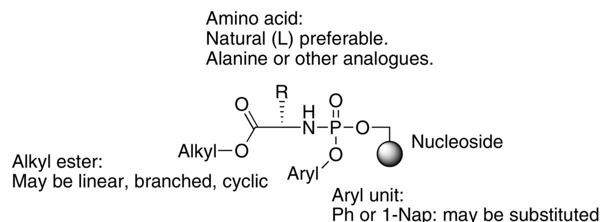


Figure 2. A general ProTide structure.

ProTide approach on **1C**, although the precise structure of their lead IDX-184 has yet to issue.[14]

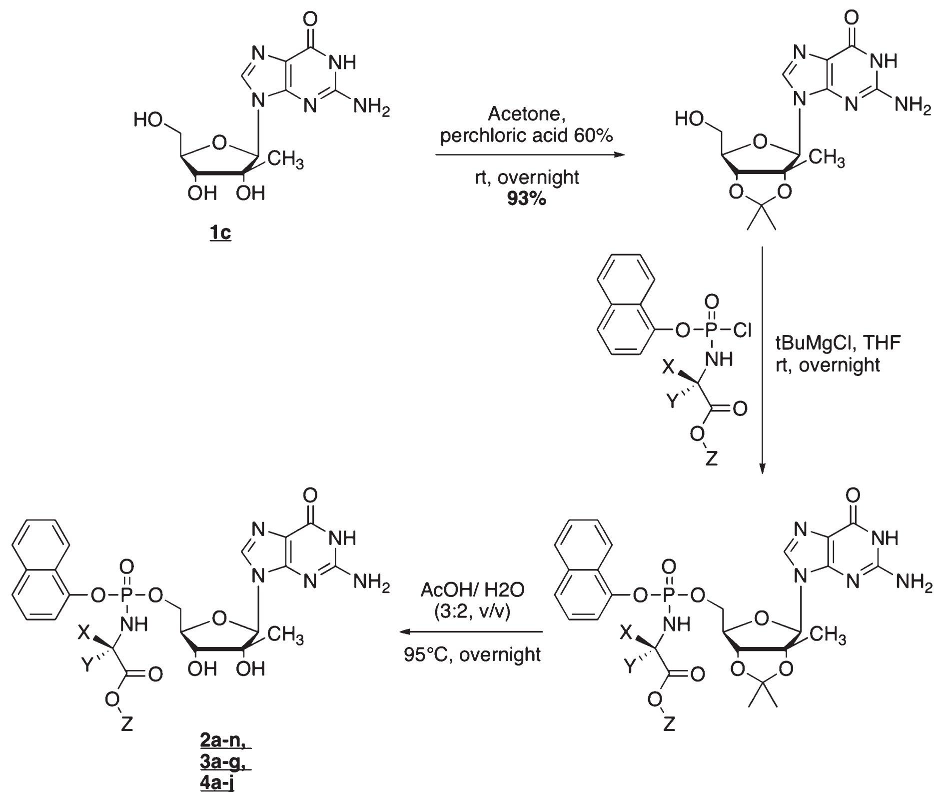
Following an early indication that the ProTides may successfully impact on **1c**,[9] we herein report an extensive study thereof. We report over 30 novel analogues with extensive variation in the aryl, ester, and amino acid regions of the ProTide, and we study these extensively across species for their stability. We note that the benzyl alanine motif continues[7−9] to exhibit good potency in replicon, but there is poor stability and PK in this series, which can only be solved by a novel combined variation in both the amino acid and ester regions as we will describe. The basic structure of a phosphoramidate motif is shown in Figure 2.

Chemistry

The target ProTides of **1c** were prepared using phosphorochloridate chemistry as we have extensively reported.[6−9] To aid in both the 5′-regioselectivity of phosphorylation and the general organic solubility of the nucleoside, we investigated various protecting groups for the 2′,3′-diol unit. One key issue is the ability to remove this protecting group after construction of the 5′-ProTide unit, and the relative chemical fragility of this group dictates the nature of the diol protecting group that might be acceptable. Indeed, the somewhat surprising acid-stable nature of ProTides[15] means that acid sensitive diol protection may be acceptable, and this led us to the use of isopropylidene protection.

Thus **1c** was allowed to react with acetone and perchloric acid at ambient temperature overnight to give **1d** (Scheme 1) in 93% yield. This proved to be significantly more soluble than **1c** and readily reacted with the appropriate phosphorochloridate reagents[6−9,15] in organic solvents such as THF (Scheme 1). Various bases could be used to aid the reaction; the Grignard

Scheme 1[a]



[a] For **2a**−**n**, **3a**−**g**, **4a**−**j**, see Tables 1, 3, 5.

Table 1. HCV Replicon Activity of L-Alanines Ester Derivatives

ProTide	aryl	ester	isomers[a]	$EC_{50}/\mu M$[b]	$CC_{50}/\mu M$
1c				3.5	> 100
2a	1-Nap	Me	38:62	0.21	> 50
2b	1-Nap	nBu	43:57	0.10	> 100
2c	1-Nap	nPnt	37:63	0.091	ND
2d	1-Nap	iPr	40:60	0.17	> 100
2e	1-Nap	2-Bu	38:62	0.12	> 100
2f	1-Nap	cPnt	48:52	0.07	> 100
2g	1-Nap	cHx	34:66	0.045	> 100
2h	1-Nap	$tBuCH_2$	31:69	0.057	> 100
2i	1-Nap	Bn	45:55	0.062	> 100
2j	2-Nap	Bn	43:57	0.17	> 50
2k	1-Nap	$BnCH_2$	44:56	0.09	> 100
2l	1-Nap	$BnCH_2CH_2$	40:60	0.17	> 100
2m	1-Nap	R/S PhEt	33, 14, 35, 18[c]	0.095	> 100
2n	1-Nap	S PhEt	42:58	0.08	> 100

[a] Isomer ratio from HPLC; polar:nonpolar. [b] Replicon data for genotype 1b in HUH7 cells with 48 h exposure. [c] Four stereoisomers.

reagent *t*BuMgCl was among the most effective, and preferably used in ca. 2 molar equivalence, along with the phosphorochloridate. Reaction overnight at ambient temperature followed by flash column chromatography on silica gave the isopropylidene protected intermediates (Scheme 1) in 30−88% yield. These were deprotected using 60% acetic acid at 95 °C overnight. The relative stability of the ProTide motif under these conditions was taken as further support of their surprising acid stability and may be taken as further evidence of their possible compatibility with oral dosing. The final compounds **2a−n**, **3a−g**, and **4a−j** (Tables 1, 3, and 5) were each isolated after column chromatography, sometimes followed by preparative TLC and/or HPLC. Standard methods are given below and spectroscopic and analytical data on **2a** only; full detail on other analogues is given as Supporting Information. In each case, the final products were isolated as mixtures of diastereoisomers at the phosphate, as evidenced by multiple (in most cases 2) peaks in the P-31 NMR and also in the HPLC. The approximate ratio of peaks (fast eluting on reverse phase, i.e., polar to nonpolar) as determined by HPLC is given in Tables 1, 3, and 5, where peaks were sufficiently clearly resolved. The approximate ratios from P-31 NMR roughly mirrored those determined by HPLC and allowed a correlation between HPLC mobility/polarity and NMR shift, such that the most predominant isomer in most cases was the downfield NMR peak, corresponding to the more nonpolar stereoisomer. The HPLC retention time data confirmed that the ProTides were in every case considerably more lipophilic than parent **1c**. Calculated lipophilicity (ClogP) values[16] for **1c** and **2a** are −2.6 and −0.52, respectively, indicating a roughly 100-fold lipophilicity enhancement for **2a**. Because Merck had noted apparently poor cell uptake by **1c**,[5] we considered the enhancement in this parameter for the ProTides potentially useful in vivo. However, the figure for the methylalanine ester **2a** was still somewhat lower than that considered optimal for passive cell uptake, and thus we varied the ester moiety to enhance lipophilicity further and also to probe the putative ester cleavage step considered to be key to the in vivo activation of these agents.[6,15] In the L-alanine series **2a−n**, the lengthening, branching, and aromatization of the ester moiety lead to agents such as the benzyl ester **2i** with a ClogP of 1.2 and branched analogues of this, such as **2m** and **2n**, with perhaps near ideal values of 1.5. Notably, in the case of **2m**, the introduction of a further variable chiral center in the ester led to a mixture of four diastereomers, as evidenced by P-31 NMR and HPLC.

Table 2. Plasma Stability of L-Alanine Esters[a]

			% remaining in plasma at 2−4 °C				
ProTide	ester	time	human	cyno	canine	rat	mouse
2a	Me	30 min	ND	ND	ND	ND	1.7
2b	nBu	30 min	100	98	95	0.4	0
		4 h	97	81	90	0	0
2c	nPnt	30 min	100	94	98	0	0
		4 h	96	94	98	0	0
2d	iPr	30 min	ND	ND	ND	ND	14
2e	2-Bu	30 min	ND	ND	ND	ND	10
2g	cHx	30 min	100	100	100	2.5	2.9
		4 h	100	100	100	0.5	0
2h	$tBuCH_2$	30 min	ND	ND	ND	ND	0.3
2i	Bn	30 min	58	54	67	1.3	1.1
2j[b]	Bn	30 min	ND	ND	ND	ND	0.7
2m	(*R*/*S*)-1-PhEt	30 min	86	100	97	0.7	2.2
		4 h	86	88	93	0.3	0
2n	*S*-PhEt	30 min	100	100	97	0.8	3.1
		4 h	99	100	92	0	0

[a] ND: not determined. [b] 2-Naphthyl.

Besides ester variation, we prepared one analogue, **2j**, with 2-naphthyl as the aryl moiety in place of 1-naphthyl. We also varied the amino acid unit from L-alanine (**2a−n**), to valine (**3a**), leucine (**3b**), methionine (**3c−d**), phenylalanine (**3e**), isoleucine (**3f**), and the unnatural achiral amino acid dimethylglycine (**3g**) (Table 3). In most cases, these were prepared as the benzyl ester, except for **3d**, being the iPr ester of Met.

As will be discussed below, L-valine emerged as a promising amino acid from these earlier studies. This is notable, as it had previously been observed by us to be among the least effective amino acids when applied to several families of ProTides.[6,17] However, in this case, promising biological data necessitated the preparation of a family of L-valine esters (**4a−j**, Table 5). These included some unusual esters not previously widely reported for ProTides: the substituted benzyl family (**4f−i**) and the neopentyl compound (**4j**).

Activity in Replicon Assay and Plasma Stability

As is common in anti-HCV drug development, we used subgenomic HCV replicon as a primary biological readout. Data are reported for the initial L-alanine family **2a−n** in Table 1, along with comparator data for **1c** in this assay. Each data point represents the mean of at least three independent assays. As noted in Table 1, all of the L-alanine ProTides were active sub-μM in the replicon assay, while **1c** was only active at 3.5 μM. Thus, the initial family of esters was 4−50-fold more potent than **1c**. This was taken as an early indication that the ProTides successfully improved the intracellular delivery of the eventual 5′-triphosphate pharmacophore of **1c**, presumably by a combination of enhanced passive diffusion of the lipophilic ProTides into cells and the delivery of the monophosphate therein by a nucleoside kinase-independent process. There was no clear correlation between ProTide lipophilicity and biological potency in this family, with several diverse esters showing activity in the 40−60 nM range.

All the ProTides displayed minimal toxicity in the Huh7 cells expressing the HCV genotype 1b bicistronic subgenomic replicon. When replicon 1b cells were incubated with the different ProTides for 72 h and cell viability was measured using the CellTiter-Glo assay (Promega, Madison, WI), CC_{50} values greater than 100 μM were routinely observed. This was true even for the most potent compounds like **2g**, thus leading

Table 3. HCV Replicon Activity of Different Amino Acid Derivatives[a]

ProTide	aryl	ester	AA	isomers[b]	$EC_{50}/\mu M$[b]	$CC_{50}/\mu M$
3a	1-Nap	Bn	L-Val	32:68	0.76	> 100
3b	1-Nap	Bn	L-Leu	NA[c]	0.12	> 50
3c	1-Nap	Me	L-Met	31:69	0.28	> 100
3d	1-Nap	iPr	L-Met	36:64	0.34	> 100
3e	1-Nap	Bn	L-Phe	34:66	1.2	> 100
3f	1-Nap	Bn	L-Ile	NA[c]	0.9	> 50
3g	1-Nap	Bn	Me_2Gly	31:69	1.5	ND

[a] NA: not available. ND: not determined. [b] See Table 1. [c] Peaks merged on HPLC.

to high selectivity indexes in these cells. Several compounds were also tested against MT4, CEM, HepG2, Huh7, HEL, 293, IEC-6, and CaCo2 cells. All cell lines tested were in log phase of growth throughout the cell cytotoxicity experiments (data not shown). A similar lack of toxicity was observed in these diverse cell lines. For example, compound **2n** had CC_{50} values > 100 μM for all the above cell lines except MT4 (CC_{50} = 87 μM) and CEM (CC_{50} = 82 μM). This gives **2n** an SI of ca. ≥1000.

Thus, to progress hit to lead selection, we sought other assays that may be informative of in vivo PK. Prior to investigating the PK of the various ProTides, we examined the plasma stability of the L-alanine esters in mouse, rat, dog, monkey, and human plasma (Table 2). The plasma stability studies were carried out at 2−4 °C because they were intended to confirm sufficient stability for calibration curves, not necessarily for understanding stability at the physiologically more relevant 37 °C. Perhaps not surprisingly, the various esters of the L-alanine ProTides all were very unstable in rodent plasma even at these lower temperatures. The data are reported as % remaining after either 30 min or 4 h. All the L-alanine esters synthesized were essentially completely cleaved in 30 min. The most stable esters were the branched isopropyl and 2-butyl esters, which still were greater than 80% consumed in 30 min even at this lower temperature. Because of this profound plasma instability, it became clear we would not be able to generate calibration curves in rodent plasma and as a result we would not be able to measure ProTide plasma concentrations in either mouse or rat. On the other hand, the L-alanine esters for the most part had sufficient plasma stability in other species to generate calibration curves and measure exposure. The benzyl ester **2i** was the notable exception, with nearly 40% degradation in 30 min in human, monkey, and dog plasma. However, substitution at the benzylic position, as in the case of the 2-phenylethyl derivative **2m** and **2n**, led to substantially improved plasma stability in these three species with up to 100% remaining even after 4 h.

Because modification of the ester group did not readily provide rodent plasma stability, and because being able to do initial pharmacokinetic studies in rodents was considered desirable, the amino acid core of the ProTides was varied to investigate both potency and plasma stability. Thus, as noted in Table 3, we prepared several ProTides, mainly as their benzyl esters, of six different amino acids. Benzyl esters were selected because we believed they provided the best chance of showing potency in the replicon assay. We particularly selected bulky amino acids, partly to enhance lipophilicity and partly to seek to induce some steric hindrance to degradation of the ProTide. As noted in Table 3, this lead to a significant (2−10-fold) loss of activity in the best cases and a 20-fold decrease in the worst case. Disubstitution of the α carbon of the amino acid lead to a 20-fold decrease in activity, while

Table 4. Plasma Stabilities of Amino Acid Derivatives

				% remaining in plasma at 2−4 °C				
ProTide	ester	AA	time	human	cyno	canine	rat	mouse
3a	Bn	L-Val	30 min					89
			4 h					66
3b	Bn	L-Leu	30 min					2
3c	Me	L-Met	30 min					34
3d	iPr	L-Met	30 min	77	96	100	2.3	76
			4 h	79	93	100	0.5	32
3e	Bn	L-Phe	30 min					1
3f	Bn	L-Ile	30 min					100
			4 h					95
3g	Bn	Me_2Gly	30 min	100	92	100	0	0
			4 h	41	83	100	0	0

Table 5. HCV Replicon Activity of L-Valine Ester Derivatives[a]

ProTide	aryl	ester	AA	isomers[b]	$EC_{50}/\mu M$[b]	$CC_{50}/\mu M$
3a	1-Nap	Bn	Val	32:68	0.76	> 100
4a	2-Nap	Bn	Val	56:44	1.7	> 100
4b	1-Nap	Bn	D-Val	68:32	> 3	> 100
4c	1-Nap	cHx	Val	65:35	1.0	> 100
4d	1-Nap	(*R*/*S*)-1-PhEt	Val	NA[c]	> 3	ND
4e	1-Nap	$BnCH_2CH_2$	Val		1.7	> 100
4f	1-Nap	*o*-ClBn	Val	33:67	0.43	> 100
4g	1-Nap	*m*-ClBn	Val	31, 25, 44	0.68	ND
4h	1-Nap	*p*-ClBn	Val	35:65	0.62	ND
4i	1-Nap	*o*-MeBn	Val	36:64	1.0	100
4j	1-Nap	$tBuCH_2$	Val	NA[c]	1.5	> 100

[a] NA: not available. ND: not determined. [b] See Table 1. [c] See Table 3.

amino acids such as the L-leucine derivative **3b** were the most potent EC_{50} = 120 nM. Branching at the β carbon of the amino acid as in the L-valine and L-isoleucine derivatives **3a** and **3f**, resulted in a 10-fold decrease in activity, however, these ProTides retained sub-μM activity and thus were still more active than **1c**. Compound **3b** was ca. 30 times more active than **1c**.

The plasma stability of these alternate amino acid ProTides was examined in the hope of finding improved rodent plasma stability. The results of testing in mouse plasma for compounds **3a**−**3g** are reported in Table 4. Disubstitution of the α carbon of the amino acid, **3g**, did not noticeably improve mouse plasma stability relative to the L-alanine derivative **2i**. Longer chain amino acids also failed to provide plasma stability. However, branching at the β carbon did provide significantly improved plasma stability. Thus, the Val and Ile derivatives **3a** and **3f** provided 66% and 95% stability in mouse plasma even after 4 h. This stability was measured at 2−4 °C and clearly would be worse at 37 °C. However, this level of stability would at least allow generation of calibration curves and subsequent measurement of plasma ProTide levels in rodents. These branched ProTides maintained their stability in the plasma from other species, but their activity in the replicon assay was somewhat less than that of the L-alanine analogues.

Thus, we subsequently prepared a small family of L-valine analogues with ester variations to examine whether we could improve antiviral activity and maintain rodent plasma stability. The derivatives made are listed in Table 5 and are compared to compound **3a**. Table 5 includes a 2-naphthyl derivative **4a** and the D-valine derivative **4b**, both of which are

Table 6. Plasma Stability of L-Valine Esters

			% remaining in plasma at 2−4 °C				
ProTide	ester	time	human	cyno	canine	rat	mouse
4a	Bn[a]	30 min	93	100	95	49	99
		4 h	100	95	99	22	76
4b	Bn[b]	30 min	70	68	69	82	51
		4 h	82	80	61	56	8
4c	cHx	30 min	97		95		95
4d	(*S*)-1-PhEt	30 min	97		100		100
4f	*o*-ClBn	30 min	77		100		69
4g	*m*-ClBn	30 min	100		90		79
4h	*p*-ClBn	30 min	95		89		94
4i	*o*-MeBn	30 min	100		100		67
4j	$tBuCH_2$	30 min	91		79		77

[a] 2-Naphthol derivative. [b] D-Val derivative.

less active in replicon assay. The other ester derivatives, **4c−4j**, are similar in activity to the lead compound **3a**, with simple aliphatic esters perhaps falling off slightly in potency and benzyl esters with an electron withdrawing group ortho, meta, or para, all being somewhat more potent. However, branching at the benzylic position, as in the (*R*/*S*)-1-phenylethyl derivative **4d**, resulted in a substantial decrease in activity, which was not observed in the L-alanine series.

Plasma stabilities were examined across this series and are listed in Table 6. A number of interesting observations can be made from the rodent plasma stability data. While the 2-naphthol derivative **4a** has similar stability to the 1-naphthol derivative, the D-valine benzyl ester **4b** was observed to be less stable than the L-valine analogue in rodent plasma. The other L-valine esters maintained rodent, and other species, plasma stabilities with the possible exceptions of the ortho chloro and ortho methyl substituted benzyl esters **4f** and **4i**, which showed minor decreases in stability relative to the para-substituted and unsubstituted benzyl esters.

Discussion of in Vitro S9 Stability Studies

Human, dog, cynomolgus monkey, and rat liver and intestinal S9 stability data, as measured by disappearance of parent compound, were obtained for selected compounds (Table 7). Mouse data were not collected because of difficulty in obtaining mouse liver S9 preparations. All samples were run with and without cofactor (NADPH) and expressed as % remaining compound after 60 min incubations. Because NADPH is a cofactor for P450 isozymes, metabolism in the absence of cofactor was considered non-P450 dependent metabolism. We were interested in identifying which species compared closest to human S9 data and which compounds gave the most desirable profile in human intestinal and liver S9 experiments.

Compounds were compared across species mainly by looking at the ratios of liver vs intestinal metabolism as opposed to absolute numbers in each category. When considered this way, it became clear that rat and monkey were reasonable comparators to the human data, but that the dog in vitro data was the outlier. As an example of this, compound **2d** (Table 7) shows more liver cleavage than intestinal cleavage in human, monkey, and rat, but in the dog it is reversed.

Human S9 data were collected for the majority of project compounds but only a portion of the data are reported here. We were looking for compounds with good stability in human intestinal S9 preparations, but with rapid cleavage in liver, in the presence of cofactor. In addition to looking at the ratio between liver and intestinal cleavage, we also paid attention to the overall degree of cleavage. The majority of compounds

Table 7. Multiple Species Liver and Intestinal S9 Data for Selected Compounds with 60 min Incubations (% Remaining)

ProTide	S9% remaining	human	dog	monkey	rat
2a	liver ±	20/40	67/85	ND	ND
	intestine ±	60/63	50/54	ND	ND
2d	liver ±	27/41	81/91	0/86	25/28
	intestine ±	65/85	66/68	48/87	77/76
2e	liver ±	16/50	53/80	ND	ND
	intestine ±	42/48	82/100	ND	ND
2h	liver ±	7/59	49/87	16/88	5/31
	intestine ±	28/49	42/56	20/68	57/86
2i	liver ±	27/41[a]	16/53	0/32[a]	0/0[a]
	intestine ±	0/49[a]	8/29	0/30[a]	30/46[a]
2j	liver ±	14/29	33/28	ND	ND
	intestine ±	24/33	23/27	ND	ND
3a	liver ±	0/78	56/75	0/77[a]	8/62
	intestine ±	24/70	56/72	25/77[a]	65/81
3b	liver ±	0/44[a]	14/48	ND	ND
	intestine ±	8/28[a]	12/33	ND	ND
3c	liver ±	3/14	27/40	ND	ND
	intestine ±	42/59	57/86	ND	ND
3e	liver ±	0/20[a]	4/15[a]	ND	ND
	intestine ±	0/5[a]	0/0[a]	ND	ND
3f	liver ±	29/83	64/84	0/89[a]	37/78
	intestine ±	52/74	87/96	29/95	71/70

[a] Incubated for 30 min. ±refers to with/without cofactor.

demonstrated greater stability in intestinal S9 preps than in liver, as was desired (Table 7). In this general sense, these compounds could be considered as liver targeting. The lone exception was the benzyl ester of L-alanine, compound **2i**, which was highly cleaved in the intestine. Stability in intestinal cells was considered important because we wanted the ProTide to pass through the gut and intestine and be absorbed and cleaved by the liver. The intestinal stabilities, as measured by % remaining after 60 min incubations, ranged from 0% to 65%. The most stable of this set of compounds was **2d**, the isopropyl ester of L-alanine.

As noted above, the compounds in Table 7 were all incubated with and without cofactor. Looking across the data in the table, there is a trend toward greater cofactor dependence in the liver than in the intestine. Because P450 isozymes require a cofactor, and because P450 levels are high in liver, this is taken as a further subtle indication of liver targeting. The benzyl ester derivatives such as compound **3a** showed a large degree of cofactor dependent liver cleavage, as did the neopentyl ester derivative compound **2h**.

Mouse DMPK Results and Discussion

A series of ester and amino acid derivatives were selected for in vivo studies based on a combination of factors including potency, plasma stability, and SAR. Primarily L-valine derivatives were investigated because they had the most rodent plasma stability. The in vivo work focused on oral dosing not only because it is a desired route of administration in general but also because HCV is a liver disease and absorbed ProTide would pass directly into the liver via the portal vein. For HCV, the general problem of first pass metabolism may work in our favor to produce high levels of the active agent. The overall goal would be to find a compound with low systemic nucleoside exposure but good therapeutic liver triphosphate levels.

For all the ProTides, systemic levels of the parent ProTides were not detectable in the mouse (data not shown). Even for the L-valine derivatives, which demonstrated much improved

Table 8. Mouse Systemic Nucleoside Levels from 50 mg/kg PO Dose of ProTide

ProTide	C_{max} ng/mL	C_{last} ng/mL (h)	$T_{1/2}$ h	AUC_{0-t} ng·h/mL	AUC_{0-inf} ng·h/mL	DNAUC
1c	5203	22 (24 h)	5.4	20390	20567	411
2h	262	103 (6 h)	5.2	1008	1920	38
2n	1380	222 (6 h)	1.8	3722	4269	85
3a	504	130 (6 h)	2.3	1611	2012	40
4c	185	1.5 (24 h)	3.0	1664	1670	33
4f	518	202 (6 h)	3.5	1600	2447	49
4g	388	388 (6 h)		1650		> 33
4h	339	43 (24 h)	6.9	3990	4420	88
4i	668	688 (6 h)	> 6	2590		52
4j	652	244 (6 h)	3.0	2269		> 45

rodent plasma stability, ProTides were not observed in systemic circulation.

Table 8 reports the plasma levels of parent nucleoside (**1c**) that result from a po dose of ProTide. Mice were orally dosed at 50 mg/kg in an experimental formulation of 5% DMA, 20% Solutol HS 15, 20% PEG 400, 55% 50 mM sodium acetate, pH 4.0. Plasma nucleoside levels were measured at 0.25, 0.5, 1, 2, 4, 6, and 24 h and are compared to the oral PK of the parent nucleoside itself. The parent nucleoside (**1c**) demonstrates only modest bioavailability from a 50 mg/kg oral dose. However, the percent oral bioavailability, calculated from the po and iv (data not shown) DNAUCs values, is quite high (F = 85%). The dose normalized AUC (DNAUC) for the nucleoside **1c** is 411 ng·h/mL/mg/kg, which is higher than the DNAUCs for any of the different ProTides. It should be noted that the oral doses of the different compounds were not molar equivalent doses, so the reported values are not exact comparisons. However, it is clear from the Table 8 data that all the ProTides result in lower systemic exposure of parent nucleoside. The ProTides all have nucleoside DNAUCs that are all 5−10-fold lower than parent nucleoside. This is consistent with the substantially lower nucleoside C_{max} values that were also observed. It is considered possible that these lower systemic exposures to the parent nucleoside may lead to lower in vivo toxicity.

Comparing the ProTides to each other, the highest nucleoside C_{max} and DNAUC values belong to the L-alanine derivative **2n**, while the lowest come from the L-valine derivative **4c**. The selection of the ester group also makes a significant difference in C_{max} and DNAUC. For the two L-alanine derivatives **2h** and **2n**, the (S)-1-phenylethyl ester **2n** resulted in a 5-fold increase in C_{max} and 2-fold increase in DNAUC over the neopentyl ester **2h**. A similar comparison in the L-valine series is not possible because the (S)-1-phenyl ethyl ester in the L-valine series was inactive and not tested. Comparison of the neopentyl esters in the L-alanine, and L-valine series shows a higher nucleoside C_{max} and DNAUC for the L-valine (**4j**) than the L-alanine (**2h**). The benzyl ester derivatives **3a**, **4f**, **4g**, **4h**, and **4i**, were the most potent compounds in the L-valine series. These compounds along with the primary alcohol ester **4j**, produced the highest C_{max} values for parent nucleoside. Interestingly, the secondary alcohol derivative **4c** has a much lower C_{max} and over all nucleoside exposure than the other L-valine esters.

Although the very potent neopentyl L-alanine derivative **2h** showed low systemic levels of nucleoside, as was desired, because of their inherent rodent plasma instability, the L-alanine derivatives were not further studied in the mouse. Several of the L-alanines were later studied in cynomolgous monkeys, and their data will be reported elsewhere.

Although maintaining low systemic nucleoside levels is desirable, it only makes sense if sufficient liver triphosphate levels are achieved. Table 9 reports the 2′-C-methyl guanosine triphosphate levels in mouse liver. Liver levels were determined at 0.25, 0.5, 1, 2,4, and 6 h. For three compounds, **1c**, **4c**, and **4h**, the 24 h liver levels were also measured. The NTP DNAUC values were calculated based on actual values not projections to 24 h. This results in significantly lower DNAUC values for several of the compounds with only 6 h data points. Once again, these doses are nonequivalent on a molar basis, and as a result the triphosphate levels of **1c** are approximately 2-fold higher than if dosed at an equivalent level.

Table 9. Comparison of 2′-C-Methyl Guanosine Triphosphate Liver Levels from 50 mg/kg PO Dose of ProTide

ProTide	C_{max} ng/g (h)	C_{last} ng/mL (h)	AUC_{0-6} h·ng/g	AUC_{0-24} h·ng/g	DNAUC
1c	3470 (2)	390 (24)	16510	36868	737
2h	370 (6)	370 (6)	1584		32
2n, no. 1	1950 (4)	1158 (6)	6980		140
2n no. 2	670 (4)	134 (16)	2596	5390	108
3a	512 (4)	470 (6)	1940		97
4c	275 (4)	74 (24)	815	3860	77
4f	290 (6)	290 (6)	880		18
4g	216 (6)				
4h	225 (4)	166 (6)	920		18
4i	91 (6)				
4j					

As mentioned above, we have observed the nucleoside **1c** to have excellent oral bioavailabilty in the mouse (F = ∼85%). It is rapidly absorbed, and its metabolism in mouse liver is modest, leading to similar exposure from an oral dose as from an iv dose. On the other hand, oral bioavailability in the cynomolgus monkey is poor (F ∼ 10%, data not shown). Clearly, there are major differences in how the mouse and nonhuman primates absorb and metabolize 2′-C-methylguanosine and its ProTides, and the mouse may not be an appropriate model for studying 2′-C-methylguanosine ProTide pharmacokinetics. However, liver triphosphate levels can be measured in the mouse at multiple time points so very valuable data can be obtained.

The liver triphosphate levels from the parent nucleoside in the mouse were substantially higher than those from the ProTides, mimicking the systemic nucleoside levels. Even accounting for dosing differences, and extrapolating out the 6 h DNAUC values to 24 h, all ProTides provide 2−5-fold less liver triphosphate, as measured by DNAUC, than does the nucleoside itself.

However, in considering the ratio of the triphosphate DNAUC to the nucleoside DNAUC, several ProTides have similar, to perhaps better, ratios than the nucleoside **1c**. The ProTides **2n**, **4a**, and **2c** have triphosphate DNAUC to nucleoside DNAUC ratios of about 2, similar to **1c**. For the L-alanine derivative **2h**, or the L-valine derivative **3a**, the DNAUC values are calculated only using 6 h data. If the DNAUC values are

extrapolated out to 24 h, it is quite possible that the triphosphate to nucleoside ratio for these two compounds would be better than that obtained for the nucleoside.

The best overall triphosphate exposure appears to come from the (*S*)-1-phenyl ethyl ester of L-alanine phosphoramidate (**2n**). This compound was examined in an additional experiment out to 16 h. A certain amount of interexperiment variation was observed due to the extreme difficulty of measuring triphosphate levels in the liver. The DNAUC from the second experiment was 140 ng·h/mL/mg/kg. Nucleoside systemic exposure was also measured in the second experiment, and the DNAUC for nucleoside was 71 ng·h/mL/mg/kg.

In summary of the mouse DMPK work, the ProTides produce significantly lower liver triphosphate levels than the parent nucleoside, however, several compounds could be identified with improved ratios of liver triphosphate to systemic nucleoside levels. The mouse is considered a nonoptimal model for studying ProTides of 2′-*C*-methyl guanosine because of the observed rapid breakdown of the ProTides in the mouse and because of the good absorption and metabolic stability of the resulting 2′-*C*-methyl guanosine. However, very useful data were obtained comparing different ProTides to each other and parent nucleoside. Further work on ProTides and other pro-drugs of 2′-*C*-methyl guanosine is underway in the cynomolgus monkey, and will be reported elsewhere.

Conclusions

We have reported herein an extensive ProTide study of 2′-*C*-methylguanosine, with variations in the aryl, ester, and amino acid region of the ProTide. In almost every case, the ProTides are more active in the HCV replicon assay, than the parent nucleoside, often by 10−30-fold. We have extensively studied the compounds for stability in plasma from multiple species and also in liver and intestinal preparations from several species. A combination of ester and amino acid changes gives rodent plasma-stable compounds. Several analogues were further evaluated for oral PK in mouse and reveal rapid uptake and metabolism to triphosphate in the liver. The data are somewhat suggestive of liver targeting by these ProTides. This, and their inherent potency and selectivity against HCV in vitro, suggests that their continued preclinical evaluation is warranted.

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, ^{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 (system 1, 100/0 to 0/100 v/v in 30 min) or water/methanol (system 2, 100/0 to 0/100 v/v in 30 min). High resolution mass spectra was performed as a service by Cardiff University, using electrospray (ES). CHN microanalysis were performed as a service by the School of Pharmacy at the University of London and by MEDAC Ltd., Surrey. 2′-*C*-Methyl guanosine 5′-triphosphate was purchased from Carbosynth, Berkshire, UK.

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.

Replicon Potency. Huh7 cells expressing the HCV genotype 1b bicistronic subgenomic relicon (Apath, LLC, Brooklyn, NY) were seeded into white 96-well plates (Nunc/VWR) at a density of 2×10^4 cells/well in medium without G-418. A Stacker multidrop liquid dispenser (MTX Lab Systems, Vienna, VA) was employed to ensure uniform and fast cell seeding into multiple plates. Then 18−24 h after cell plating, inhibitors were added and cells were incubated for additional 48 h. Compounds were tested in triplicates and quadruplicates at 3× or 4× serial dilutions over a range of 0.0001−10 μM concentrations. HCV replication was monitored by Renilla luciferase reporter activity assay using Renilla luciferase reporter (Promega, Madison, WI) and a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). Then 50% inhibitory concentration (EC_{50}) values were calculated as the concentration of compound that results in 50% decrease in the reporter expression as compared to untreated cells. The values were determined by nonlinear regression (four-parameter sigmoidal curve fitting) analysis. For any one IC_{50} determination, the replicon assay was run in triplicate and standard deviation was calculated from the three repeats and this standard deviation is usually less than 20% of the IC_{50}. A second standard deviation is determined when multiple IC_{50}s are obtained from different batches of replicon cells. When more than three independent IC_{50} values have been obtained, this second standard deviation is calculated. Because of the differences in the different batches of replicon cells, this standard deviation is often ca. 100% of the IC_{50}.

Cytotoxicity Assays. The same cells used in the replicon assay were seeded into 96-well plates at a density of 2×10^4 cells per well. Twenty-four h after cell plating, 11 serial 2× compound dilutions, starting with 100 μM, were applied to the testing plates (three repeats per compound dilution). Each testing plate was run with a "no-compound" control. Incubation with compounds was continued at 37 °C in a CO_2 incubator for 72 h. The cells were in log phase of growth throughout the cell cytotoxicity experiments. To determine cell viability, the CellTiter-Glo assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. The compound concentration resulting in 50% luminescent signal was reported as the CC_{50} concentration.

Plasma Stability. Stability experiments were performed in duplicate using human, cynomolgus monkey, canine, rat, and mouse plasma (Bioreclamation, Inc., Long island, NY). ProTides were added to a final concentration of 1 μg/mL in 1 mL of plasma preincubated at 2−4 °C. The reaction mixture was maintained at 2−4 °C, and 50 μL samples were taken at 30 or 240 min of incubation and transferred to a 96-well V-bottom plate. Then 200 μL of ice cold acetonitrile was added to each sample. The precipitated samples were centrifuged at 2500 rpm, 4 °C, for 20 min in a Sorvall RT6000S centrifuge (Thermo Scientific, Waltham MA). Then 50 μL of supernatant from each sample was transferred to a 96-deep well plate followed by the addition of 50 μL of H_2O to each sample. Samples were covered, mixed well by vortexing, and maintained at 2−8 °C before and during analysis. Fifteen μL of each test sample was analyzed for compound concentrations by LC-MS/MS. Liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with a Synergi 4 μm Polar-RP, 30 mm × 2.0 mm column (Phenomenex, Torrance, CA). Linear gradient (100% mobile phase A (H_2O + 0.1% HCOOH) to 100% B (acetonitrile + 0.1% HCOOH) over 3 min, flow rate 1.0 mL/min) was used for the analyte elution. The HPLC system was coupled to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectrometry analysis was performed in positive ion mode (MH^+ 659.2, transition 166.1).

Data was analyzed using Analyst v1.4.2 software (Applied Biosystems, Framingham, MA), Microsoft Office Excel 2007, and GraphPad Prism 5 (GraphPad Software, La Jolla, CA

S9 Clearance Assay. All liver and intestinal S9 tissue fractions were purchased from Xenotech (Lenexa, KS) and stored at −80 °C until time of use. NRS (NADPH-Regenerating System) was purchased from BD Gentest (BD Biosciences, San Jose, CA) and was used as cofactor. Reagents including NRS components and liver or intestinal S9 tissue fractions were thawed and immediately placed on ice. S9 fractions, 50 mM potassium phosphate buffer with or without NRS were placed in wells of a 96-well deep-well plate for final concentrations of 1 mg/mL S9, 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride. The plate was preincubated in an orbital shaker at 37 °C for 10 min. The reaction was then initiated by addition of compound at a final concentration of 10 μM. The suspensions were thoroughly mixed by pipetting and an initial sample was withdrawn and added to equal volume of cold acetonitrile (for a calculation of starting concentration). The plate was placed back in the 37 °C shaker, and at 60 min a sample was withdrawn and added to equal volume cold acetonitrile. Following quenching by acetonitrile, samples were kept on ice and precipitate was pelleted in all samples by centrifugation at 3000 rpm for 15 min. Supernatant was removed and transferred to vials for HPLC analysis.

Pharmacokinetic Studies. Compounds were formulated in 5% dimethylacetamide, 20% Solutol HS15, 20% polyethylene glycol 400, 55% 50 mM sodium acetate, (pH 4) (D/S/P) and administered by oral gavage to female ICR mice (Taconic Farms, Germantown, NY). Blood and tissue collections were performed at various time points as terminal procedures. Blood was collected into EDTA containing tubes (1.6 mg EDTA/mL blood, Sarstedt, Inc., Newton, NC), and the plasma was separated by centrifugation within 30 min of collection. Liver samples were immediately frozen upon collection in liquid nitrogen. Plasma and liver samples were stored frozen at ≤ -80 °C prior to analysis.

Measurement of Plasma Nucleoside Concentrations. Plasma samples were prepared for analysis as follows. First, 400 μL of 50 mM ammonium acetate was added to 100 μL of each plasma sample. Calibration curves were prepared by serial dilution of a stock solution of 2′-*C*-methyl guanosine (**1c**) into control plasma. Solid phase extraction of the samples was performed with H_2O-Philic speeddisk columns (J.T. Baker) which were previously solvated with 1 mL of methanol followed by equilibration with 1 mL of 50 mM ammonium acetate. The columns were rinsed with 1 mL of 50 mM ammonium acetate:methanol (95:5, v/v), and samples were eluted with 1 mL of methanol:ammonium hydroxide (95:5, v/v). The samples were dried under nitrogen and reconstituted in 80 μL of H_2O. A volume of 10 μL of each test sample was analyzed for 2′-*C*-methyl guanosine (**1c**) concentrations by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with a Betasil 2.1 mm × 100 mm 5 μm column (Thermo Scientific). Mass spectrometry analysis was in positive ion mode as described above.

Measurement of Liver Triphosphate Concentrations. Liver samples were snap-frozen in liquid nitrogen and pulverized with a steel mortar and pestle on dry ice with liquid nitrogen. Frozen, pulverized tissue samples were weighed and 5 equiv of 70% methanol containing 50 mM thenoyltrifluoroacetone was added. Samples were then homogenized twice in a bead mill (FastPrep Homogenizer) using Silicone-Carbide Sharp Particles (BioSpec Products, Inc., Bartlesville, OK) at 4 °C at an agitating speed of 6 for 30 s. The homogenates were centrifuged at 15000 rpm for 30 min at 4 °C. The supernatants were collected and dried in Masterblock polypropylene deep-well plates (SPEware Corp., Baldwin Park, CA) under a stream of nitrogen. The dried extracts were reconstituted with 100 μL of 10 mM *N*,*N*-dimethylhexylamine, 3 mM ammonium formate in H_2O, vortexed, and centrifuged at 3500 rpm for 30 min at 4 °C. Calibration curves were constructed by spiking varying concentrations of 2′-*C*-methyl guanosine 5′-triphosphate (Carbosynth, Berkshire, UK) into control liver samples prior to homogenization. Fifteen μL of each test sample was analyzed for 2′-*C*-methyl guanosine 5′-triphosphate concentrations by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Reverse phase liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with an. XTerra MS C18, 3.5 μM, 2.1 mm × 50 mm Column (Waters, Milford, MA). Mass spectrometry analysis was performed in negative ion mode and data were analyzed as above. The levels (peak areas) of endogenous adenosine 5′-triphosphate and guanosine 5′-triphosphate were also monitored as internal quality controls for the liver samples.

Pharmacokinetic Analysis. Plasma and liver concentration data was analyzed for standard pharmacokinetic parameters using Win-Nonlin v5.2 software (Pharsight, St. Louis, MO).

Standard Procedure A: ProTide Synthesis. To the 2′,3′-*O*,*O*-isopropylidene-2′-*C*-β-methylguanosine (1 equiv) in anhydrous THF (6 mL/g of nucleoside) was added dropwise a solution of *t*BuMgCl in THF (1M, 2 equiv). After stirring for 20 min, a solution of the phosphorochloridate (2 equiv) in anhydrous THF (6 mL/g of phosphorochloridate) was slowly added. The resulting solution was stirred overnight at room temperature, then the solvent was removed under reduced pressure. The resulting foamy residue was purified by silica gel column chromatography (eluent: $CHCl_3$/MeOH 92:8, v/v) to afford the pure protected ProTide.

Standard Procedure B: ProTide Deprotection. A solution of the protected ProTide in a 60% acetic acid in water solution (~10 mL/200 mg of protected protide) was stirred at 95 °C overnight. The solvent was then removed under reduced pressure to dryness (can also be coevaporated with hexane or toluene to remove traces of AcOH), and the resulting residue was purified by silica gel column chromatography (eluent: $CHCl_3$/MeOH 9:1, v/v) to give after lyophilization the pure ProTide.

Example: Synthesis of 2′-*C*-Methylguanosine-5′-*O*-[naphthyl-(methyloxy-L-alaninyl)]phosphate (2a). Step 1: Synthesis of the protected ProTide

Prepared using standard procedure A. Starting from 181 mg of protected nucleoside. Yield: 37%.

^{31}P NMR (202 MHz, CD_3OD) δ 4.49, 4.40. ^1H NMR (500 MHz, CD_3OD) δ 8.17 (2d, $J = 8.0$, 1H, H_8-napht), 7.85 (m, 2H, H_5-napht and H_8), 7.70 (m, 1H, H_4-napht), 7.58−7.48 (m, 3H, H_7, H_6, H_2-napht), 7.42 (m, 1H, H_3-napht), 6.08 and 6.07 (s, 1H, $H_{1'}$), 4.62−4.54 (m, 2H, $H_{3'}$ and $H_{5'}$), 4.48−4.36 (m, 2H, $H_{4'}$ and $H_{5''}$), 4.09 (m, 1H, Hα), 3.61 (s, 3H, CH_3 ester), 1.59 and 1.57 (2s, 3H, CH_3 isopropylidene), 1.36 (m, 6H, CH_3 isopropylidene and CH_3 Ala), 1.03 and 1.00 (s, 3H, 2′-CH_3).

Step 2: Deprotection of the ProTide

Prepared using standard procedure B. Starting from 70.5 mg. Yield: 45% (30 mg).

^{31}P NMR (202 MHz, CD_3OD) δ 4.36, 4.27. ^1H NMR (500 MHz, CD_3OD) δ 8.18 (m, 1H, H_8-napht), 7.93−7.83 (m, 2H, H_5-napht and H_8), 7.69 (m, 1H, H_4-napht), 7.56−7.49 (m, 3H, H_7, H_6, H_2-napht), 7.40 (m, 1H, H_3-napht), 5.94 (s, 1H, $H_{1'}$), 4.57 (m, 2H, $H_{5'}$ and $H_{5''}$), 4.29−4.23 (m, 2H, $H_{4'}$ and $H_{3'}$), 4.10−3.98 (m, 1H, Hα), 3.56 and 3.55 (2s, 3H, CH_3 ester), 1.30 (m, 3H, CH_3 Ala), 1.01 and 0.99 (2s, 3H, 2′-CH_3). HPLC, system 1, RT 13.00, 13.43 min.

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Supporting Information Available: Preparative methods, spectroscopic and analytical data on target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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