



The phosphoramidate ProTide approach greatly enhances the activity of β -2'-C-methylguanosine against hepatitis C virus

Christopher McGuigan^{a,*}, Plinio Perrone^a, Karolina Madela^a, Johan Neyts^b

^a Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, UK

^b Rega Institute for Medical Research Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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ABSTRACT

β -2'-C-Methyl purines (**1**, **2**) are known inhibitors of hepatitis C virus (HCV). We herein report the synthesis, biological and enzymatic evaluation of their 5'-phosphoramidate ProTides. Described herein are seven L-alanine phosphoramidate derivatives with variations to the amino acid ester. The 1-naphthyl phosphoramidate of β -2'-methylguanosine containing the benzyl ester (**20**) was the most active at 0.12 μ M, an 84-fold of increase in activity compared to the parent nucleoside (**2**) with no increase of cytotoxicity. The carboxypeptidase mediated hydrolysis of several ProTides showed a predictive correlation with their activity versus HCV in replicon.

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The hepatitis C virus (HCV) was identified in 1989 as a member of the family of the Flaviviridae.¹ An estimated 180 million people are chronically infected with HCV and thus at increased risk of developing life threatening liver disease (including cirrhosis and hepatocellular carcinoma). HCV infections are the major reason for liver transplantation in industrialized countries. The current therapy for HCV (pegylated interferon and ribavirin) has limited efficacy and major side-effects.² Several examples of modified nucleosides have already been reported with potential anti-HCV activity.^{3,4} Modified nucleosides need to be phosphorylated to their corresponding 5'-triphosphates by the host cell kinases. In many cases, however, nucleoside analogues are poor substrates for the kinases and the pharmacologically active triphosphate species cannot be considered as possible drug candidates due to their high instability and poor cellular permeation.⁵ In many cases, the limiting step in this process is represented by the conversion to the corresponding 5'-monophosphate. Our group has developed in the past the aryloxy-phosphoramidate ProTide approach which allows the delivery of the monophosphorylated nucleoside analogue into the cell, bypassing the need of the first phosphorylation step.⁵ We have previously reported the successful application of the ProTide approach to different nucleoside analogues^{6,7,10,11} 2'-Methylpurines (adenosine and guanosine) have been shown to

be potent anti-HCV agents.³ β -2'-Methyladenosine (**1**) (Fig. 1) showed EC_{50} = 0.3 μ M against HCV in replicon assay, and its corresponding 5'-triphosphate inhibited HCV RNA polymerase at 1.9 μ M. β -2'-Methylguanosine (**2**), instead, showed IC_{50} = 0.13 μ M (inhibition of RdRp), EC_{50} = 3.5 μ M against HCV, but most importantly the detected level of its corresponding 5'-triphosphate was rather poor.³ In the case of β -2'-methylguanosine (**2**), the low level of its intracellular 5'-triphosphate may be an indication that this nucleoside is a poor substrate for nucleoside kinases, limiting its antiviral efficacy.

We decided to apply the aryloxy-phosphoramidate approach to these two nucleoside analogues in order to explore the possibility of further increasing their activity against HCV. In the first instance, β -2'-methyladenosine (**1**) and β -2'-methylguanosine (**2**) were synthesized and evaluated for their effect on HCV (sub genomic) replicon replication.

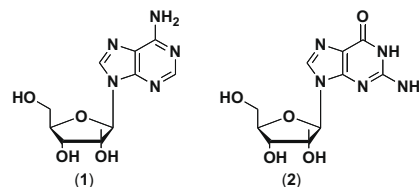


Figure 1. β -2'-Methyladenosine (**1**) and β -2'-methylguanosine (**2**).

* Corresponding author. Tel./fax: +44 0 2920874537.

E-mail address: mcguigan@cardiff.ac.uk (C. McGuigan).

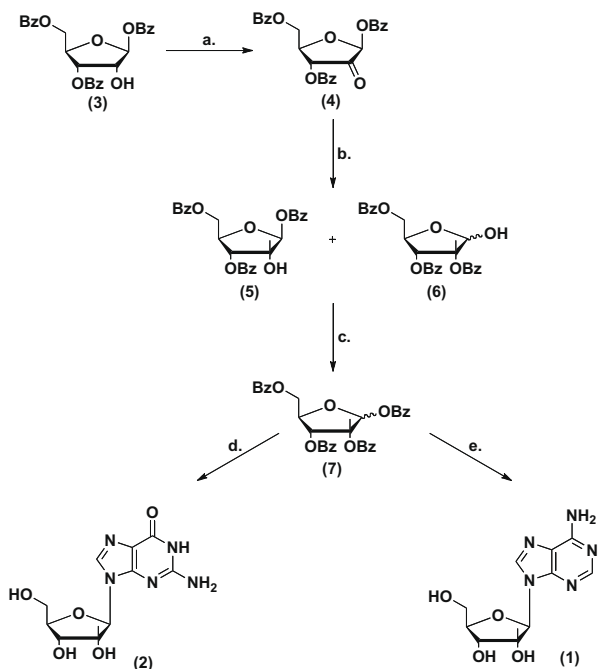


Figure 2. Synthesis of β -2'-methyladenosine (**1**) and β -2'-methylguanosine (**2**). Reagents: (a) Dess–Martin reagent, CH_2Cl_2 dry; (b) CH_3TiCl_3 , Et_2O dry; (c) BzCl , DMAP, Et_3N ; (d) (1) N2-acethylguanine, HMDS, TMS-triflate, *p*-xylene; (2) $\text{CH}_3\text{OH}/\text{NH}_3$; (e) (1) N6-pivaloyladenine, DBU, TMS-triflate, CH_3CN ; (2) $\text{CH}_3\text{OH}/\text{NH}_3$.

The synthesis of β -2'-methyladenosine (**1**) and β -2'-methylguanosine (**2**) was planned following the reported procedure (Fig. 2).³

The oxidation in the 2-position of the tri-benzoylated ribose (**3**) was performed in the presence of the synthesized Dess–Martin reagent.⁷ The stereoselective addition of the methyl group in the β -2-position was performed by the addition of **4** to a solution of methyl titanium trichloride, synthesized in situ from an anhydrous solution of titanium tetrachloride and methylmagnesium bromide in diethyl ether, to give a mixture of **5** and **6** (Fig. 2).⁸ The next benzoylation reaction was performed under standard conditions to give **7**.³ In the case of β -2'-methyladenosine (**1**), in order to avoid a problem of selectivity in N^7 - and N^9 -positions, it was necessary to protect the NH_2 with the pivaloyl group. The following coupling reaction was performed in the presence of DBU and trimethylsilyl trifluoromethanesulfonate (Fig. 2). Heteronuclear multiple bond correlation (HMBC) showed the correlation between C4 and H1', confirming the presence of the N^9 -regioisomer. Nuclear Overhauser Enhancement Spectroscopy (NOESY), showed no correlation between the proton of H1' and the three protons of the methyl group in the 2'-position confirming the presence of the β -nucleoside; instead, the correlation between H2' and the three protons of the methyl group in the 2'-position confirmed the presence of the methyl group in the β -position. The final step was the removal of the pivaloyl group using $\text{NH}_3/\text{CH}_3\text{OH}$ at room temperature in a sealed tube (Fig. 2). The fully benzoylated β -2'-methyl sugar (**7**) was also the substrate for the synthesis of 2'-methylguanosine (**2**). In order to avoid N^9 - and N^7 -regioisomers, the coupling reaction was performed in two steps: synthesis of the totally silylated acetylguanine and coupling reaction in the presence of the appropriate 2-methylribose sugar (**7**) and trimethylsilyl trifluoromethanesulfonate (TMS-triflate) using *para*-xylene as solvent (Fig. 2).⁹ The N^9 -regioisomer was isolated without any traces of N^7 -isomer. Also in this case the final step required the use of $\text{NH}_3/\text{CH}_3\text{OH}$ to give the desired product (**2**) in quantitative yield (Fig. 2). In our previous work,^{10,11} the phosphoramidate synthesis was greatly improved with the presence of a protecting group in the 2'- and

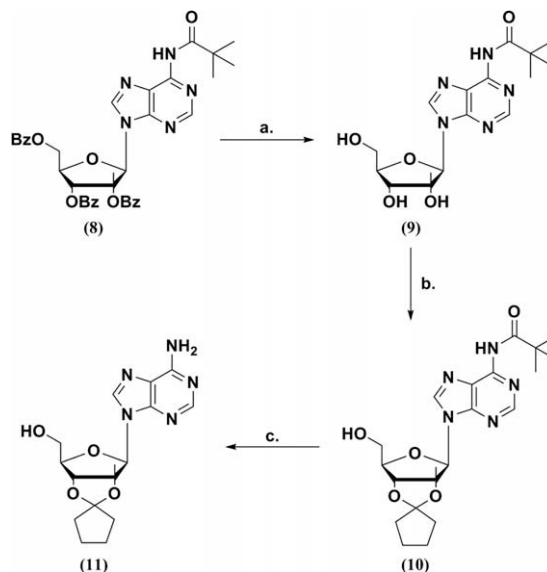


Figure 3. Synthesis of 2',3'-protected- β -2'-methyladenosine (**11**). Reagents: (a) 1 M NaOH, ethanol, pyridine; (b) 1,1-dimethoxypentane, *p*-TSA; (c) NH_3 , CH_3OH .

3'-position of the nucleoside. Consequently, the N^6 -pivaloyl-2',3',5'-tribenzoate- β -2'-methyladenosine (**8**) was selectively deprotected in the 2', 3'-, and 5'-position whilst keeping the pivaloyl group in the N^6 -position, followed by introduction of the cyclopentylidene group in the 2'- and 3'-positions in the presence of 1,1-dimethoxypentane and *p*-TSA. The final deprotection of the 6-amino group was performed in the presence of $\text{NH}_3/\text{CH}_3\text{OH}$ at room temperature in a sealed tube (Fig. 3).

The introduction of the cyclopentylidene group was attempted also in the case of β -2'-methylguanosine: this attempt was unsuccessful and consequently the use of isopropylidene as protecting group was considered. The synthesis was performed in the presence of a catalytic amount of perchloric acid in a solution of dry acetone (Fig. 4).

The synthesis of the phosphoramidate was performed following the Uchiyama procedure,¹² in the presence of *t*-butyl magnesium chloride (Figs. 5 and 6).¹³

The sugar deprotection in the case of the cyclopentylidene group (synthesis of β -2'-methyladenosine phosphoramidates, **15**–**17**) required an acidic hydrolysis using 80% formic acid at room temperature (Fig. 5).¹⁴

The hydrolysis of isopropylidene group (synthesis of β -2'-methyladenosine phosphoramidates, **20**–**23**), instead, was performed using acetic acid at 90 °C (Fig. 6).¹⁵ The yields of the coupling reaction for the synthesis of β -2'-methyladenosine phosphoramidates (**15**–**17**) were 40–50%, whilst the deprotection reaction yields were 50–60%. Instead, in the case of the synthesis of β -2'-methylguanosine phosphoramidates (**20**–**23**) the yields for the coupling reaction ranged from 20–40%, while the yields for the deprotection in

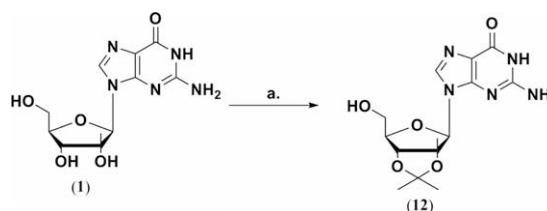


Figure 4. Synthesis of 2',3'-protected- β -2'-methylguanosine (**12**). Reagents: HClO_4 , acetone.

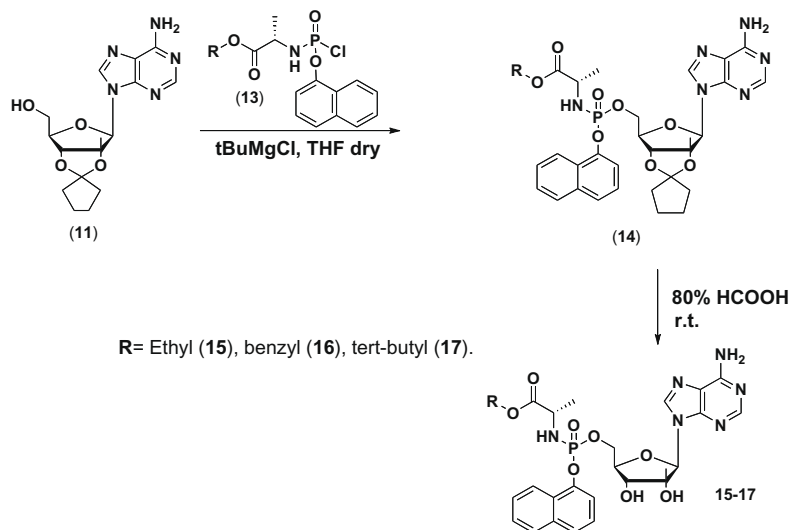


Figure 5. Synthesis of β -2'-methyladenosine phosphoramidates (15–17).

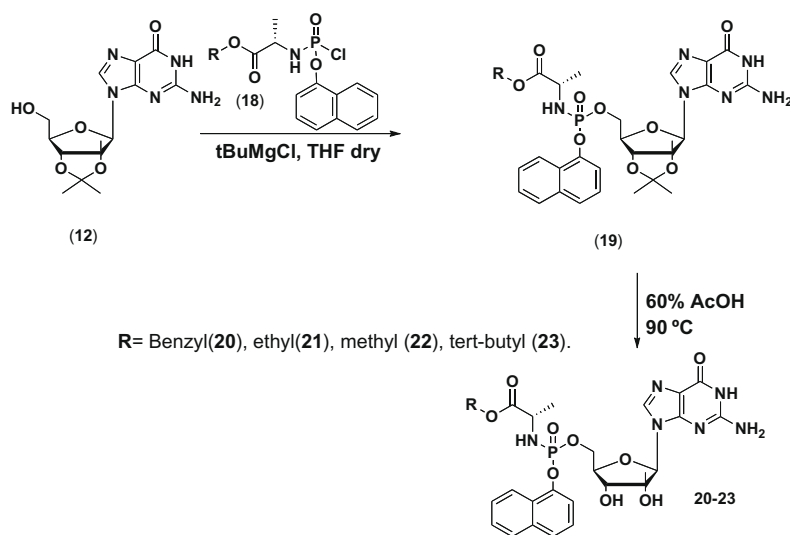


Figure 6. Synthesis of β -2'-methyladenosine phosphoramidates (20–23).

2'- and 3'-positions were 30–50%. In most of the cases, for both nucleosides, purification by column chromatography and a semi-preparative HPLC were required to obtain the pure products. In each case, the phosphoramidates were isolated as mixtures of phosphorus diastereoisomers (ratio 1:1) with two signals present in the ^{31}P NMR spectra.

Each of the phosphoramidates (15–17 and 20–23) and their parent nucleosides (1 and 2) were tested in vitro as inhibitors of HCV replication in the HCV replicon assay using similar conditions as described (Table 1).¹⁶

None of the compounds showed toxicity at 50 μM concentration. The two nucleosides (1 and 2) inhibited HCV replication with EC_{50} values of at 0.25 and 10.1 μM , respectively. The phosphoramidate approach in the case of 1 was unsuccessful, with at best a maintenance of biological activity compared to the parent nucleoside. This result confirms that the conversion of β -2'-methyladenosine (1) to its corresponding 5'-monophosphate in the cell is efficient; consequently, the ProTide approach does not improve this process. However, compound 15 and 16 showed a biological activity similar to their parent nucleoside (1), indicating that in this case, the phosphoramidate approach does successfully deliver the

Table 1

Biological activity of β -2'-methyladenosine (1) and β -2'-methylguanosine (2) and their corresponding phosphoramidates

Compd	Nucleoside	R	HCV HUH 5-2	
			EC_{50} (μM)	CC_{50} (μM)
1	2'-Me-A	—	0.25	>50
15	2'-Me-A	Ethyl	0.24	>50
16	2'-Me-A	Benzyl	0.27	>50
17	2'-Me-A	<i>t</i> -Butyl	4.18	>50
2	2'-Me-G	—	10.1	>50
20	2'-Me-G	Benzyl	0.12	>50
21	2'-Me-G	Ethyl	0.28	>50
22	2'-Me-G	Methyl	0.23	>50
23	2'-Me-G	<i>t</i> -Butyl	27	>50

5'-monophosphate with a similar efficiency to the nucleoside itself. In contrast, the phosphoramidates of β -2'-methylguanosine (20–22) showed a significant increase in inhibition of HCV replication compared to the parent nucleoside (2). This result supports the notion that: the phosphoramidate approach allows the efficient delivery of the monophosphate of 2 into the replicon cell, 2 is a poor

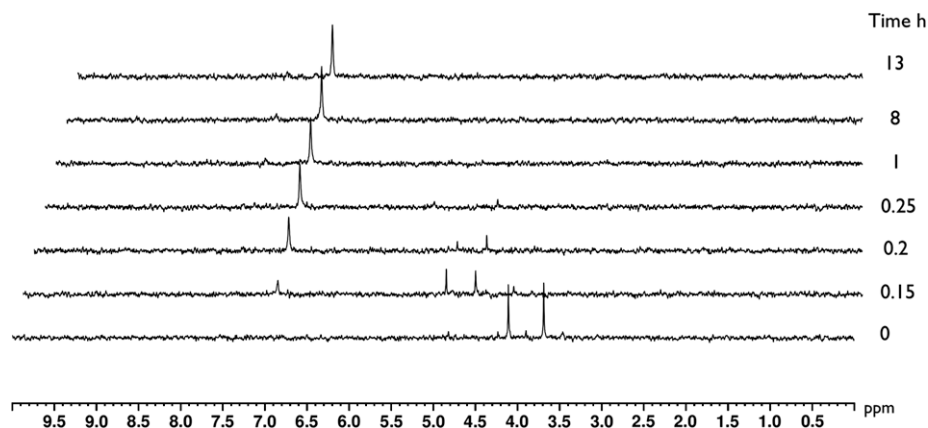


Figure 7. Sequential P-31 NMR scans of (20) incubated with carboxypeptidase Y.¹⁷

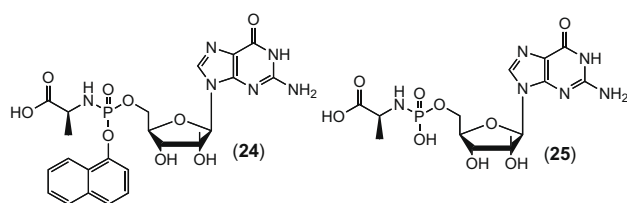


Figure 8. Putative metabolites in the processing of ProTide (20).

substrate for the nucleoside kinases in replicon cells, and the corresponding 5'-monophosphate is efficiently phosphorylated to the corresponding 5'-triphosphate in vitro. Among the four esters, the *t*-butyl (17 and 23) phosphoramidates were found to be less active compared to benzyl, methyl and ethyl esters. This reduced antiviral activity may be related to the relative stability of tertiary esters to enzyme-mediated hydrolysis. The effect is particularly notable for the guanosine series, where the tertiary ester causes a >100-fold loss of activity.

There was no significant difference in antiviral potency considering ethyl, methyl and benzyl esters, indicating that these esters are well tolerated by the enzyme involved in the phosphoramidate conversion. To further understand the high activity of these compounds we carried out an enzyme incubation designed to mimic the first stages of the putative activation of these ProTides in vivo. Thus compound (20) was incubated with carboxypeptidase

Y in TRIZMA buffer and the metabolism of (20) monitored by P-31 NMR.¹⁷ Spectra were recorded at periodic intervals and selected scans are reproduced in Figure 7.

Two P-31 NMR signals are noted for (20) at δ_p ca. 4.0, corresponding to the two phosphate diastereoisomers of the ProTide. After ca. 0.2 h the signals for the parent have disappeared and an intermediate is noted at δ_p ca. 4.5. This material must likely be chiral at the phosphate, as evidenced by the presence of 2 peaks. Parallel work in our laboratories on other compounds indicates this species to be the ester hydrolysed analogue of (20) with a free carboxylate, compound (24).¹⁸ This material is then converted to the final product in this assay, with a single peak at δ_p ca. 7. This is achiral at the phosphate and considered to be the key aminoacyl intermediate, which lacks the ester and aryl moieties; it is the 5'-alaninyl phosphate of (2), compound (25) (Fig. 8).

Our previous work has indicated that this may be a substrate for the HINT family of enzymes, to liberate to free 5'-monophosphate in vivo.¹⁹ As such, its appearance in the enzyme assay is seen as a necessary pre-requisite for antiviral action in vivo. Thus, for comparison purposes we carried out the same study with the poorly active *t*-butyl ester (23). Similar data for the carboxypeptidase assay on (23) are shown in Figure 9.

Several clear differences are noted between the enzyme stability assays of (20) and (23). Most striking is the difference in rate. Thus, while both proceed to give the same putative amino acyl metabolite (25), the *t*-butyl ester (23) is massively slower. The benzyl ester (20) shows only ca. 7% of parent ProTide remaining after 10 min, with a calculated first order half life of 3 min. By

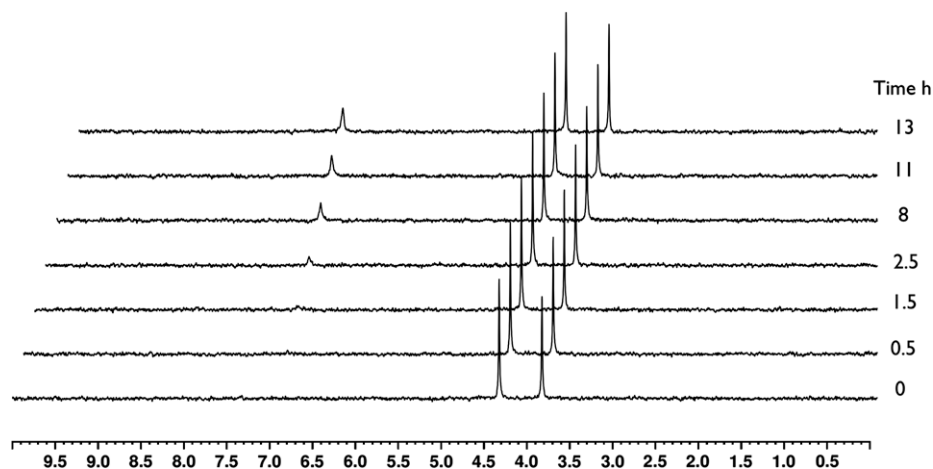


Figure 9. Sequential P-31 NMR scans of (23) incubated with carboxypeptidase Y.

contrast the *t*-butyl ester is only ca. 24% hydrolysed after 13 h, with an estimated half life of 33 h. Thus the (active) benzyl ester is processed ca. 700-fold more efficiently to the key amino acyl metabolite in this assay than is the (poorly active) *t*-butyl ester. Secondly, it is notable that the intermediate (**24**) observed in Figure 7, corresponding to the ester cleaved ProTide, is not observed in Figure 9; presumably the ester cleavage is greatly rate limiting in the case of (**23**) and aryl loss is much more rapid, so intermediate (**24**) never builds up to detectable levels.

In conclusion, we have reported the synthesis of **1** and **2** and a small series of their corresponding phosphoramidates. The most potent compounds were the 1-naphthyl derivatives with benzyl (**20**), ethyl (**21**) or methyl (**22**) ester moieties with 84-fold increase in activity against HCV compared to the parent nucleoside (**2**). The application of phosphoramidate approach to **1** did not significantly improve the activity against HCV compared to the parent nucleoside (**1**). We also note the valuable predictive power of a P-31 NMR based enzyme metabolic assay for the activity of these ProTides versus HCV in vitro.

Acknowledgments

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Supplementary data

Supplementary data (additional spectroscopic and analytical data on the target compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.122.

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- Standard procedure for the synthesis of 2',3'-protected phosphoramidates*: BuMgCl (2.0 mol equiv, 1 M solution in dry THF) and the appropriate nucleoside (**11** or **12**, 1.0 mol equiv) were dissolved in dry THF (31 mol equiv) and stirred for 15 min. Then a 1 M solution of the appropriate phosphorochloridate (2.0 mol equiv, **13** or **19**) in dry THF was added dropwise, then stirred for 14 h. A saturated solution of NH₄Cl was added and the solvent was removed under reduced pressure to give a yellow solid, which was purified by column chromatography using CHCl₃/MeOH (from 95/5) as eluent. The appropriate fractions were collected and the solvent was removed under reduced pressure to give a white solid.
- Standard procedure for the deprotection of β-2'-methyladenosine phosphoramidates*: the appropriate 2',3'-O,O-cyclopentylidene-β-2'-methyladenosine phosphoramidate (1.0 mol equiv) was added to a solution of formic acid (80% v/v solution in water). The reaction was stirred at rt for 6 h. The solvent was removed under reduced pressure and the obtained yellow oil was subsequently purified by column chromatography using CHCl₃/MeOH (95:5) as eluent followed by a semipreparative HPLC to give a white solid.
- Standard procedure for the deprotection of β-2'-methylguanosine phosphoramidates*: the appropriate 2',3'-isopropylidene-β-2'-methyladenosine phosphoramidates (1.0 mol equiv) was added to a solution of 60% v/v of acetic acid in water at 90 °C for 15 h. The solvent was removed under reduced pressure and the obtained yellow oil was subsequently purified by column chromatography using CHCl₃/MeOH (95:5) as eluent followed by a semipreparative HPLC to give a white solid. Key spectroscopic data on (**20**) (NMR assignments confirmed by 2D spectra): δ_p (CH₃OH-*d*₄): 4.25, 4.14; δ_H (CH₃OH-*d*₄): 8.17 (1H, m, H8-guanosine), 7.88 (1H, m, CH-naphthyl), 7.79 (1H, m, CH-naphthyl), 7.53 (2H, m, CH-naphthyl, CH-benzyl), 7.42–7.40 (1H, m, CH-naphthyl), 7.36–7.21 (7H, m, CH-naphthyl, CH-benzyl), 6.05 (1H, d, H1'-guanosine, *J* = 8.4 Hz), 5.15–4.90 (2H, m, CH₂-benzyl), 4.58–4.49 (2H, d, H3'-guanosine, H4'-guanosine), 4.44–4.34 (2H, m, H5'-guanosine), 4.17–4.11 (1H, m, CHα), 1.37 (3H, m, CH₃-alanine), 1.00 (3H, s, CH₃-2'-guanosine). MS (ES) *m/z*: 687.2 (MNa⁺, 100%); accurate mass: C₃₁H₃₃N₆O₉NaP required 687.1954, found 687.1944. Spectroscopic and Analytical data on other analogues are listed in SI.
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- Carboxypeptidase Y (Sigma CAS No. 9046-67-7, EC 3.4.16.1, 0.5 mg) was suspended in 200 μL Trizma buffer and added to the ProTide (5 mg) in 200 μL of acetone-*d*₆ and 400 μL of Trizma, and the reaction followed by ³¹P NMR.
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