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Discovery of 4′-azido-2′-deoxy-2′-*C*-methyl cytidine and prodrugs thereof: A potent inhibitor of Hepatitis C virus replication

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ABSTRACT

4′-Azido-2′-deoxy-2′-methylcytidine (**14**) is a potent nucleoside inhibitor of the HCV NS5B RNA-dependent RNA polymerase, displaying an EC_{50} value of 1.2 μM and showing moderate in vivo bioavailability in rat (F = 14%). Here we describe the synthesis and biological evaluation of 4′-azido-2′-deoxy-2′-methylcytidine and prodrug derivatives thereof.

Hepatitis C virus (HCV) was first discovered in 1989,[1] and is an infectious agent affecting about 170 million people worldwide.[2] HCV establishes a chronic infection in a majority of affected people, and of these approximately 20% will develop cirrhosis of the liver over a 20–25 year period, and with increased risk of developing end-stage liver disease and hepatocellular carcinoma.[3] Standard of care (SoC) for HCV infection involves treatment with pegylated α-interferon in combination with the nucleoside analogue ribavirin and results in, after a 48 week treatment, approximately 40–50% sustained virological response (SVR) in the HCV genotype 1 population.[4] Recently two HCV NS3/4A protease inhibitors (PIs), Telaprevir[5] (VX-950) and Boceprevir[6] (SCH 503034) were approved by the FDA, which are expected to improve the outcome of HCV treatment when added to SoC in genotype 1 patients. However, in difficult to treat patients, for example, prior partial and null responders to SoC, the new triple therapies of Telaprevir or Boceprevir added to SoC provide only moderate SVR rates.[7,8] Nucleoside analogues have become cornerstone components in treatment of viral diseases and have contributed to a significant reduction in morbidity, mortality and suffering caused by viral infections, including human immunodeficiency virus (HIV), hepatitis B virus and herpes virus infections. The lessons learned from the development of treatment regimens for human immunodeficiency virus (HIV) suggest that drug combinations addressing different targets of viral replication will be required to combat HCV infections, underpinning the need for new and more efficacious treatment options in HCV. The use of nucleoside analogs as one component of a combination of two or three direct acting antiviral (DAA) agents for the treatment of HCV shows emerging promise, as in particular their broad genotypic coverage[9] and the relatively high barrier to resistance are attractive features.[10]

The antiviral properties of 4′-substituted nucleosides in HIV, and in particular 4′-azido nucleosides, has been described by Maag et al.[11] Figure 1 depicts structures of compounds **1**–**6** which are 2′-modified 4′-azidocytidines. The discovery of 4′-azidocytidine, R1479[12] (**1**), as a potent and non-cytotoxic inhibitor of HCV replication (replicon IC_{50} = 1.28 μM, cytotoxicity in Huh7-cells >2000 μM), and its 2′,3′,5′-triisobutyryl ester prodrug, R1626 (**2**) have indeed inspired further modifications in the 2′-position of

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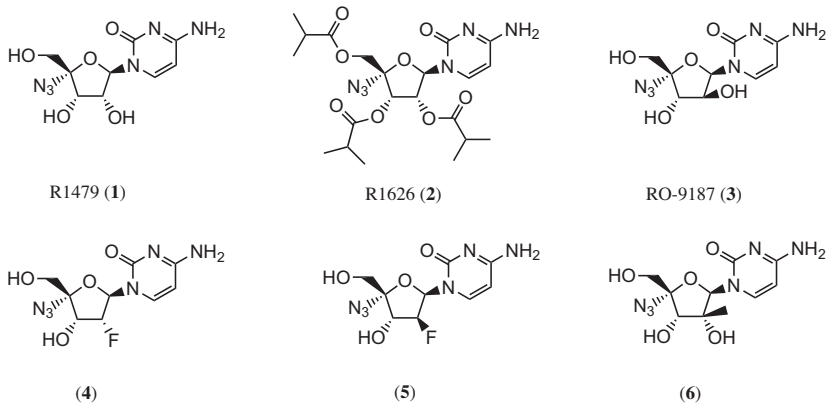
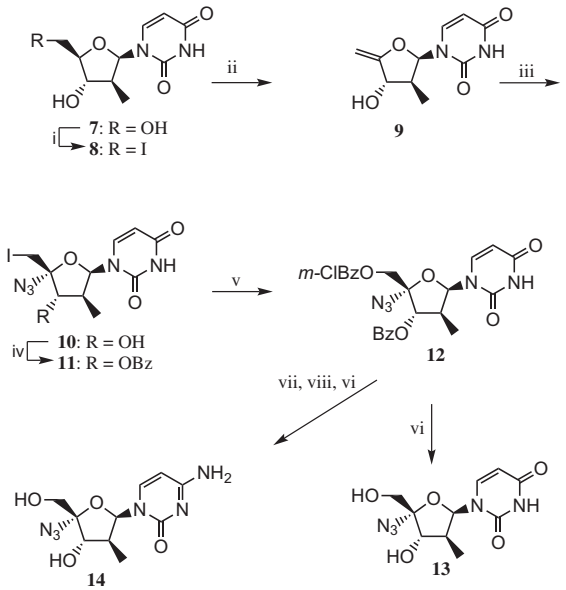


Figure 1. 2′-Modified 4′-azidocytidines.

the 4′-azidocytidines for the exploration of SAR and ultimately optimization of phosphorylation efficiency and antiviral potency. The 2′-modified 4′-azidocytidines **3**, **4**, **5** and **6** have been reported.[13,14] Nucleoside analogs **3**, **4** and **5** are all potent inhibitors of HCV replication in the HCV replicon system,[15] however compound **6** was found to be inactive, most likely due to being a poor substrate for kinases involved in the first phosphorylation step leading up to its corresponding active 5′-triphosphate, as indicated by Schinazi and co-workers.[16] We now report on the discovery of 4′-azido-2′-deoxy-2′-methylcytidine (**14**) and the identification of the clinical candidate TMC649128 (**16**). The synthesis of 4′-azido-2′-deoxy-2′-methyluridine (**13**) and 4′-azido-2′-deoxy-2′-methylcytidine (**14**) are outlined in Scheme 1 and were prepared from compound **7**[17,18] similar as described for analogous compounds.[13,14]

In order to overcome the observed moderate bioavailability of **14**, a selected set of prodrugs were synthesized according to Schemes 2–5 with the intention to compare their pharmacokinetic properties to those of the parent nucleoside.

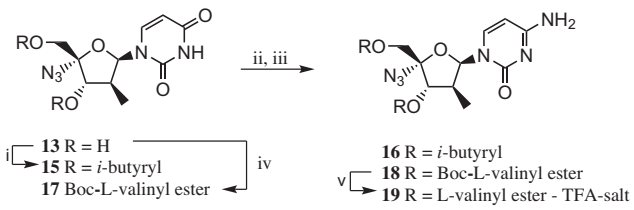


Scheme 1. Reagents and conditions: (i) I_2, PPh_3, imidazole, THF, 76%; (ii) NaOMe, MeOH, 75%; (iii) $[Bn(Et)_3]NN_3$:I_2, NMO, THF; (iv) BzCl, DMAP, NMP, THF, 81% over two steps; (v) m-CPBA, m-CBA, $(NH_4)HSO_4$, CH_2Cl_2, 68%; (vi) NH_3, MeOH, 85% for **13**, 61% for **14** over three steps; (vii) pyridine, 2-chlorophenyldichlorophosphate, 1-*H*-tetrazole; (viii) dioxane, NH_3.

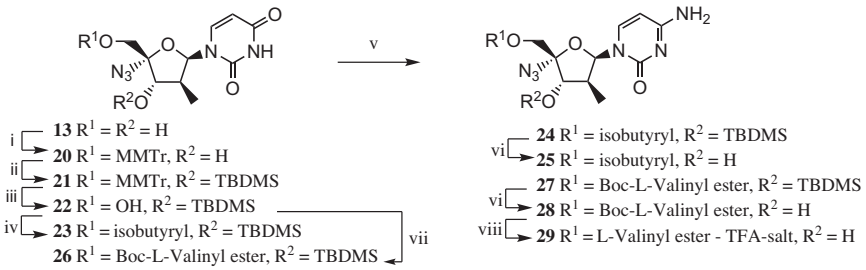
The di-isobutyryl ester **16** was obtained by treating 4′-azido-2′-deoxy-2′-methyluridine (**13**) with isobutyric anhydride in pyridine, which yielded crude compound **15**. This was subsequently treated with 4-chlorophenylchlorophosphate and 1-*H*-tetrazole in pyridine, followed by ammonia in dioxane, which afforded final product **16** in 35% yield. For the synthesis of 3,5-di-*O*-Boc-valinyl ester **19**, compound **13** was treated with *N*,*N*′-diisopropylcarbodiimide (DipCDI) and L-Boc-valine which gave diester **17** in 50% yield. The conversion of **17** into the cytidine derivative **18** was effected in 40% yield over two steps, as described, vide supra. Subsequent N-deprotection of **18** using trifluoroacetic acid in dichloromethane afforded compound **19** as its trifluoroacetic acid salt in 98% yield.

The synthesis of 5′-mono ester prodrugs **25** and **29** are outlined in Scheme 3. Monomethoxytritylation of **13** using MMTrCl (4-methoxytrityl chloride) in pyridine afforded compound **20** in 88% yield. Treatment of **20** with TBDMSCl (*tert*-Butyl(chloro)dimethylsilane) and imidazole in DMF resulted in intermediate **21** in 56% yield. Removal of the MMTr protecting group with trichloroacetic acid yielded the common intermediate **22** in 75% yield. For the synthesis of derivative **24**, compound **22** was treated with isobutyric anhydride furnishing **23** in 99% yield followed by conversion into its corresponding cytidine derivative **24,** similar as described, vide supra. Finally, deprotection of the TBDMS group with TBAF (tetrabutylammonium fluoride) gave **25** in 44% yield over three steps. Compound **29** was prepared from **22** by esterification with Boc-L-valine which afforded **26** in 74% yield. Compound **26** was converted into its cytidine derivative **27** similar as above, followed by deprotection of the TBDMS group using TBAF which furnished **28** in 28% yield over three steps. N-Boc-deprotection of **28** yielded the target valine ester **29** in 98%.

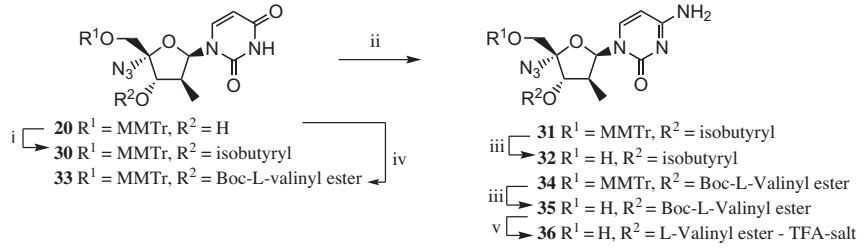
The 3′-prodrug esters **32** and **36** were prepared as outlined in Scheme 4. Treatment of the common intermediate **20** with isobutyric anhydride gave **30** in 96% yield, which subsequently was converted into its corresponding cytidine derivative **31** in 75% yield



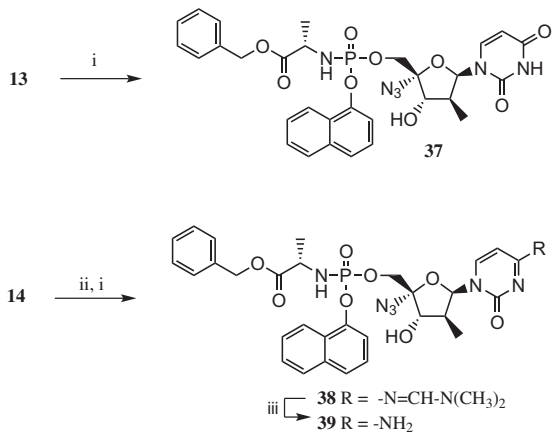
Scheme 2. Reagents and conditions: (i) pyridine, isobutyric anhydride; (ii) pyridine, 4-chlorophenylchlorophosphate, 1-*H*-tetrazole; (iii) dioxane, NH_3, 35% for **16** over 3 steps, 40% for **18** over 2 steps ; (iv) *N*-Boc-Val-OH, DipCDI, DMAP, DMF, 50%; (v) TFA, DCM, 98%.



Scheme 3. Reagents and conditions: (i) MMTrCl, pyridine, 88%; (ii) TBDMSCl, imidazole, DMF, 56%; (iii) TCA, DCM, 75%; (iv) pyridine, isobutyric anhydride, 99%; (v) First pyridine, 4-chlorophenylchlorophosphate, 1-*H*-tetrazole, followed by dioxane, NH_3; (vi) NH_4F, MeOH, 44% for **25** over three steps, 28% for **28** over three steps ; (vii) *N*-Boc-Val-OH, DipCDI, DMAP, DMF, 74%; (viii) TFA, DCM, 98%.



Scheme 4. Reagents and conditions: (i) pyridine, isobutyric anhydride, 96%; (ii) first pyridine, 4-chlorophenylchlorophosphate, 1-*H*-tetrazole, then dioxane, NH_3, 75% of **31** over two steps, 72% of **34** over two steps; (iii) aq. 80% acetic acid, 89% for **32**, 84% for **35**; (iv) *N*-Boc-Val-OH, DipCDI, DMAP, DMF, 95%; (v) TFA, DCM, quantitative.



Scheme 5. Reagents and conditions: (i) *N*-methyl imidazole, THF, 1-naphtyl(benzoxy-L-alaninyl)phosphorochloridate, (ii) *N*,*N*′-dimethylformamide dimethylacetal, methanol; (iii) Acetonitrile–aq HCl, 4% yield over 3 steps.

using standard conditions. Finally, removal of the MMTr group of **31** afforded compound **32** in 89% yield. For the synthesis of compound **36**, compound **20** was esterified with Boc-L-valine yielding **33** in 95% yield. Compound **33** was converted into the cytidine derivative **34** in 72% over two steps, then removal of the MMTr group gave **35** in 84% yield and N-Boc deprotection quantitatively afforded prodrug ester **36**.

The synthesis of a selection of phosphoramidate prodrugs is outlined in Scheme 5. Uridine derivative **37** was obtained in 31% yield by reacting compound **13** with 1-naphthyl(benzoxy-L-alaninyl)phosphorochloridate.[19] The cytidine compound **39** was synthesized from compound **14** by first protection of the amino group using dimethylformamide dimethylacetal, followed by reaction with 1-naphthyl(benzoxy-L-alaninyl)phosphorochloridate which resulted in compound **38**. Finally deprotection of the amino group of **38** under acidic conditions afforded **39** in an unoptimized 4% overall yield. The phosphoramidate prodrug of compounds **37** and **39** was chosen because of its beneficial effect on the anti HCV activity of 4′-azidouridine reported by McGuigan and co-workers.[20]

In Table 1, we present data from the HCV replicon assay.[15] Compound **13** did not display any activity at the concentrations tested, probably due to being a poor substrate for kinases, which was supported by the activity of the corresponding nucleotide prodrug **37** (EC_{50} = 6.2 μM). Compound **14** displayed good activity (EC_{50} = 1.2 μM) with no cytotoxic activity at the concentrations tested. The activity of **14** was not improved by its nucleotide prodrug derivative **39**, indicating that the first phosphorylating step in replicon cells is not rate-limiting. Prodrug derivatives **16**, **19**, **25**, **29**, **32** and **36** showed similar or slightly lower activity compared with compound **14**, reflecting the ease of hydrolysis in the replicon assay, ultimately releasing the parent nucleoside **14**.

In Table 2 a selection of in vitro PK data are presented. The parent nucleoside **14** displayed excellent stability in human plasma (<3 μl/min/mg) and in human liver microsomes (<6 μl/min/mg), however only moderate permeability in Caco-2 cells (Papp = 1.7 cm/s $\times 10^{-6}$). As expected, the diester prodrugs **16**, **19**, **25**, and **29** all displayed low stability in human plasma, most likely related to the facile cleavage of the 5′-esters. Compound **16** showed a good permeability in Caco-2 cells (Papp = 7.7 cm/s $\times 10^{-6}$), possibly related to its increased lipophilicity compared to the parent nucleoside **14**. The 3′-ester prodrugs **32** and **36** both had good stability in human plasma (<3 and 4 μl/min/mg respectively).

Table 1
Inhibition of HCV replication in Huh7-Rep Cells (EC_{50}, Luciferase Assay) and cytotoxicity (CC_{50}) measured in Huh7-CMV-Luc and MT4-LTR-Luc cells

Compound	CC_{50} (μM)		
	EC_{50} (μM)	Huh7-CMV-Luc	MT4-LTR-Luc
13	>50	>100	>100
14	1.2	>98	>98
16	6.6	>100	>50
19	1.9	>100	NA
25	3.6	>100	NA
29	0.79	>100	NA
32	6.5	>100	NA
36	0.72	>100	NA
37	6.2	>100	41
39	3	>100	>32

Table 2
In vitro data: Stability in human plasma, human liver microsomes (HLM) and permeability in Caco-2 cells

Compound	Human plasma Cl_{int} (μl/min/mg)	HLM Cl_{int} (μl/min/mg)	Caco-2 Papp (cm/s × 10^{-6})
14	< 3	< 6	1.7
16	17	> 300	7.7
19	36	ND	ND
25	> 40	ND	ND
29	17	ND	ND
32	< 3	12	1.6
36	4	ND	ND

Table 3
Mean plasma levels ($n = 2$) together with pharmacokinetic parameters after a single intravenous (5 mg base-equiv/kg) administration of the parent nucleoside **14** and oral administration (20 mg base-equiv/kg) of **14**, **16**, **19**, **25**, **29**, **32** and **36** in the fed male Sprague–Dawley rats. PK parameters based on the plasma concentration of **14**

Compound	**14**	**14**	**16**	**19**	**25**	**29**	**32**	**36**
Dose (μmol/kg)	5	20	20	20	20	20	20	20
Administration	IV	PO	PO	PO	PO	PO	PO	PO
AUC_{0-t} (μM × h)	4.97	2.66	12.7	1.22	1.59	1.56	15.3	3.66
AUC_{0-inf} (μM × h)	4.98	2.87	12.9	1.35	1.72	1.68	15.8	4.06
C_{max} (μM)	—	0.61	4.65	0.29	0.53	0.5	4.56	1.14
t_{max}	—	2.00	1	1	1	1	1	1
CL/*F* (L/h/kg)	1.03	7.15	1.55	14.8	11.8	12.0	1.3	4.93
F (%)	100	14	65	7	8	8	78	20

In Table 3 in vivo rat plasma levels and pharmacokinetic parameters of the parent nucleoside **14** and its ester prodrugs are presented. In addition to the in vivo data in Table 3, the stability of **14** in in vitro rat liver microsomes (RLM) was found to be high (Cl_{int} <6 μl/min/mg). Based on IV administration, a clearance of **14**, in line with the in vitro data, was found (CL/F = 1.03 L/h/kg). Following the results of the oral administration, the low to moderate oral bioavailability of **14** ($F = 14\%$) could be calculated. Prodrugs potentially targeting peptidyl transporters, which previously have been shown to have beneficial effect on oral bioavailability for 2′-*C*-Methylcytidine, such as compounds **19**, **29** and **36** all gave lower or similar oral bioavailability ($F = 7\%$, 8% and 20% respectively) compared with the parent nucleoside **14**.[21] For the isobutyryl ester series, compound **25** gave similar oral bioavailability ($F = 8\%$) to the parent nucleoside. However, the isobutyryl esters **16** and **32** both demonstrated greatly improved mean maximum plasma concentrations (C_{max} = 4.65 and 4.56 μM respectively) and hence a larger area under the curve (AUC_{0-t} = 12.7 and 15.3 μM/h respectively) and oral bioavailability ($F = 65\%$ and 78% respectively).

In conclusion, the data reported here supports that the 4′-azido-2′-deoxy-2′-methylcytidine (**14**) is a potent (in the HCV replicon assay) and non-cytotoxic agent (in Huh-7 and MT4 cells). A nucleotide prodrug of **14**, such as compound **39** did not improve the activity of the compound in the HCV replicon assay, indicating that further exploration of nucleotide prodrugs of **14** may be futile. Furthermore, it has been possible to improve the rat PK properties by introducing prodrug esters, that is, compounds **16** and **32**. Further details on the advancement of **16** into clinical phase I studies in our ambition towards improved HCV therapies will be reported elsewhere.

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