NMR-based conformational analysis of 2',6-disubstituted uridines and antiviral evaluation of new phosphoramidate prodrugs.

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Abstract

Six novel phosphoramidate prodrugs of uridine analogues, with structural modifications introduced at the 6- and 2',6-positions, has been prepared and was evaluated for selective antiviral activity against hepatitis C virus, as well as some other positive-stranded RNA viruses. An analysis of the conformational properties of the parent nucleosides was carried out using two-dimensional NMR spectroscopy based experiments, highlighting a 3′-*endo* (North) sugar puckering preference and *syn* orientation.

1. Introduction

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus of which the replication is exclusively located in membrane-associated replication complexes in the cytoplasm of infected cells.[1] In contrast to HIV and HBV, for which the chronic infection is caused respectively by integration of the viral genome into the host cell DNA or the establishment of the viral extrachromosomal cccDNA, antiviral treatment of a chronic HCV infection can successfully result in a sustained virological response (SVR). In the past, the immunotherapeutic regimen with IFN/RBV allowed to achieve an SVR of 50% (genotype 1) to 70-90% (genotypes 2 and 3). Recently, SVR's have significantly improved with the clinical approval of combination regimens with selective antiviral inhibitors (Harvoni®, Gilead; Viekira Pak®, AbbVie).

The introduction of structural variation at multiple positions of both the base and sugar moieties of natural ribonucleos(t)ides has yielded numerous analogues with the potential to interfere with viral replication i.e. by competitive binding to the RNA-dependent RNA polymerase (RdRp) catalytic site and/or by termination of the growing RNA chain.[4] The insight into the SAR of these compounds has significantly increased, for example allowing to conclude that the presence of a methyl group at the 2'-*β*-position on the ribose ring is an important structural element necessary to achieve good inhibitory activity on the HCV RNA polymerase activity without interfering with the function of host cell RNA-dependent polymerases (and thus less cytotoxicity).[5]

A well-known concept in the development of new biologically active nucleoside derivatives is the importance of their conformational preference in view of their possible interaction with target enzymes. The introduction of a substituent at the 6-position of a pyrimidine base is described in the literature as giving rise to *syn*-conformationally constrained ribonucleosides, some of which have shown interesting anti-plasmodial[6] and anti-cancer[7] activities. Their scope as antivirals has largely remained uninvestigated.

The present study was designed to evaluate the antiviral activity of a series of 6-substituted uridine analogues, with a particular interest in combining such modification with the familiar 2'-*C*-methylribose sugar pharmacophore, and this to investigate whether compounds exhibiting both structural motifs would maintain the preferred conformation and biological profile of their parent ribonucleosides. Furthermore, a 5'-monophosphate prodrug strategy was pursued as many synthetic nucleotides that prove to be active in *ex cellulo* enzymatic assays, often fail to inhibit viral RNA replication in cell culture due to

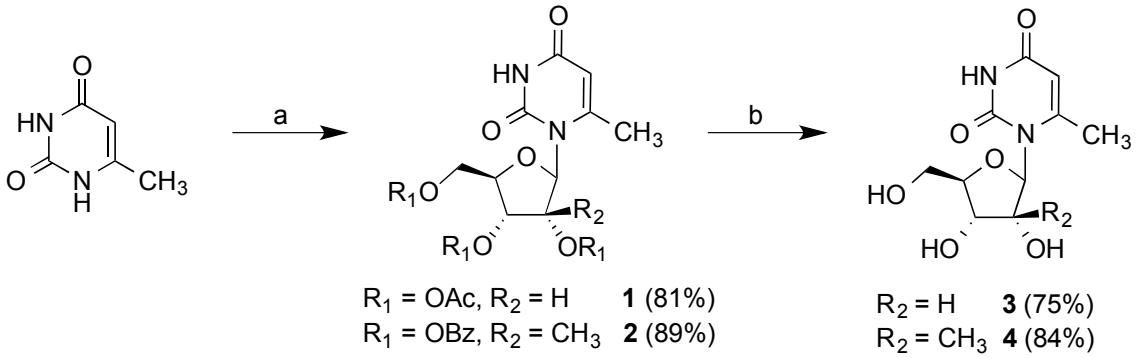
poor phosphorylation in the first rate-limiting step required for intracellular triphosphate activation by kinases.[8] And last but not least, the introduction of aryl phosphoramidates to enhance the pharmacokinetic properties of nucleotides has shown fruitful in anti-HCV drug discovery,[9] eventually leading to the FDA approval of Sufosbuvir for the treatment of chronic hepatitis C.

2. Results and discussion

2.1. Chemical synthesis

Standard preparative routes were followed for the synthesis of a variety of 6-substituted and 2',6-disubstituted uridine analogues, which were then used as platforms for the generation of an entire novel set of phosphoramidate prodrugs.

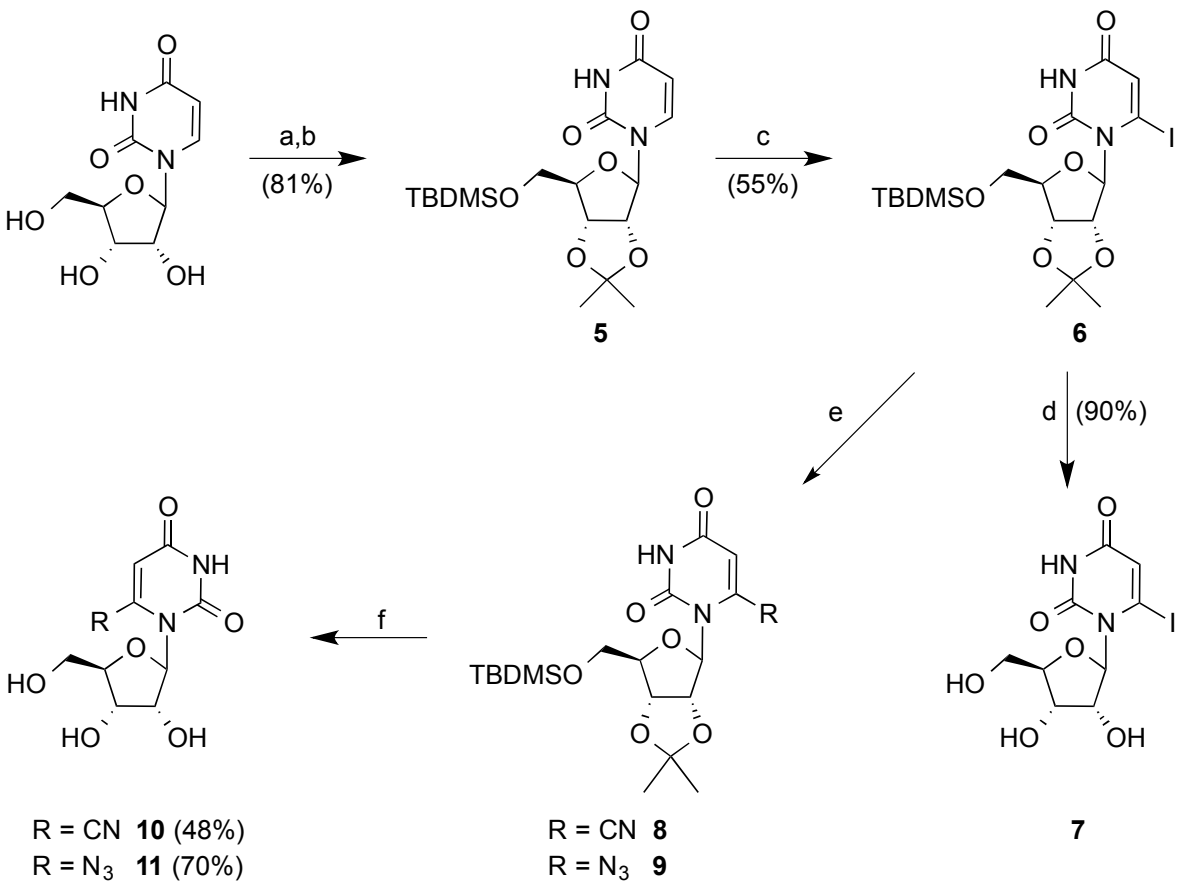
Methyl-substituted uridine analogues **3** and **4** were readily obtained in two steps from commercially available 6-methyluracil and peracetylated ribofuranose or 1,2,3,5-tetra-*O*-benzoyl-2'methyl-β-D-ribofuranose, respectively, under optimized Vorbruggen condensation reaction conditions (Scheme 1), followed by total deprotection of intermediates **1** and **2**. In both cases, the outcome of the glycosylation step showed to be highly dependent on the reaction conditions, since the undesired N^1,N^3-bis-riboside was preferentially formed at room temperature. At lower temperatures, however, the exclusive formation of a N^1-glycosidic bond rather that a N^3-bond was confirmed by NOESY experiments, as selective irradiation of the 6-methyl group of compounds **3** and **4** showed a strong interaction with H-1'. These results were found to be in agreement with ^{1}H correlations to C2 and C6 observed in HMBC spectra.



Scheme 1. (a) BSA, sugar, TMSOTf, -20 ºC to 10-15 ºC, 6-48 h; (b) 7N NH_3 in MeOH, 5-48 h.

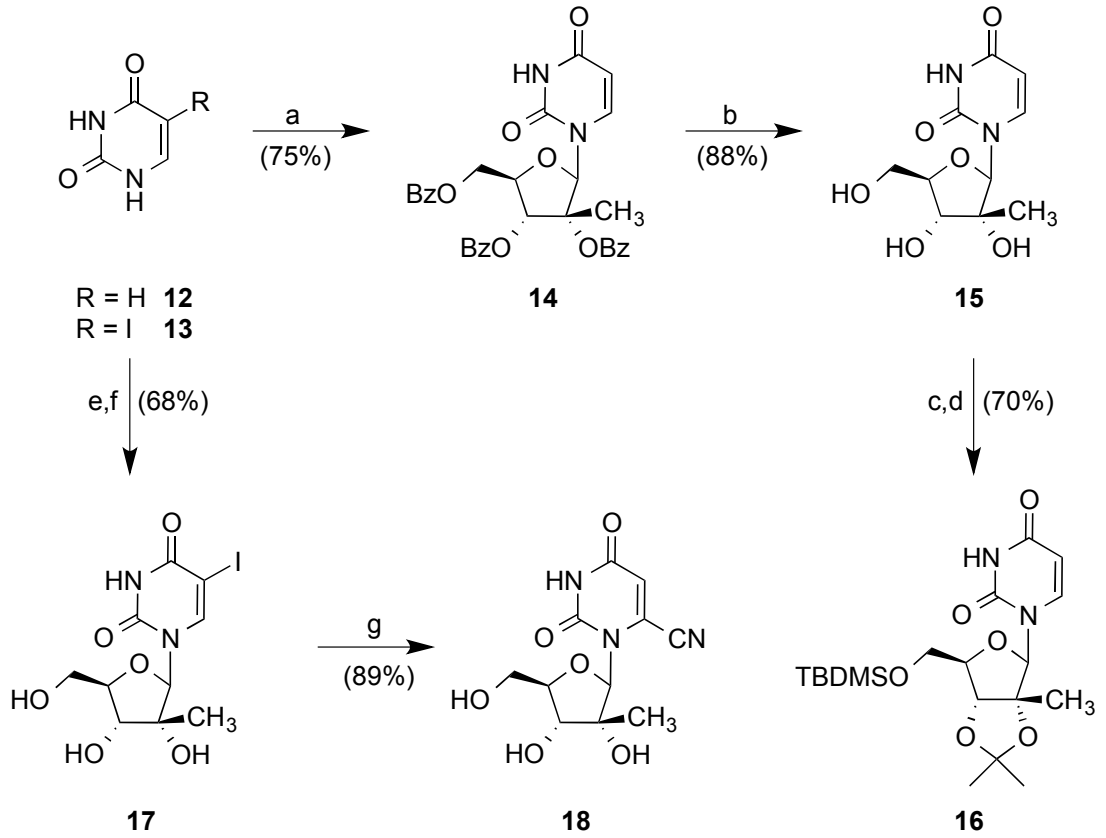
Initial attempts to form analogues starting from nucleobases substituted with a 6-functional group other than methyl by a similar condensation approach were unsuccessful, leading to the preferential formation of a glycosidic bond at the N^3-position (HMBC). This can be attributed to an undesired silylation reaction taking place at 6-position, thus leading to an increased steric hindrance at the N^1-position in the pyrimidine ring which impedes the coupling between the silylated base and the electrophilic sugar cation required to form the glycosidic bond.

Thus, a literature route was pursued to access 6-modified ribouridines, which relies upon the initial introduction of an iodine atom at the 6-position of the base, followed by an addition-elimination step. As shown in Scheme 2, the hydroxyl groups at the 2'- and 3'-positions of uridine were simultaneously protected with acetone in the presence of a catalytic amount of sulfuric acid, followed by conventional silylation at the 5'-position. The resulting protected compound **5** was then treated with LDA and iodine at low temperature under dry conditions to give compound **6** in 55% yield.[6a] Removal of the TBDMS and isopropylidene groups in the presence of TFA proceeded in excellent yield to afford 6-iodouridine **7**. In addition, treatment of compound **6** with $(Et)_4NCN$ or NaN_3 in dry DMF,[10] afforded the corresponding 6-cyanouridine **10** and 6-azidouridine **11** in moderate yields, after a deprotection step.



Scheme 2. (a) Acetone, H_2SO_4, 3 h; (b) TBDMSCl, Im, dry DMF, overnight; (c) LDA, I_2, AcOH, dry THF, 5 h; (c) 50% aq. TFA; (e) $(Et)_4NCN$, dry DMF, overnight or NaN_3, dry DMF, overnight; (f) 50% aq. TFA.

In a similar fashion, we aimed to generate another group of compounds that incorporate in the sugar moiety the key feature necessary for HCV inhibitory activity. Glycosylation of 2'-methyl substituted ribose with uracil proceeded selectively at the N^1-position to give **14**, which, after debenzoylation, underwent the same protecting steps previously described for uridine. However, it was found that, when compound **16** was subjected to the iodination reaction, no product was formed. This result was attributed to the poor stabilization of the anionic intermediate forming at the 6-position under the applied reaction conditions. Steric factors linked to the presence of the 2'-methyl group on the sugar ring might be accountable for the absence of the formation of mixed aggregate derived stabilizing the intermediate species.[11]

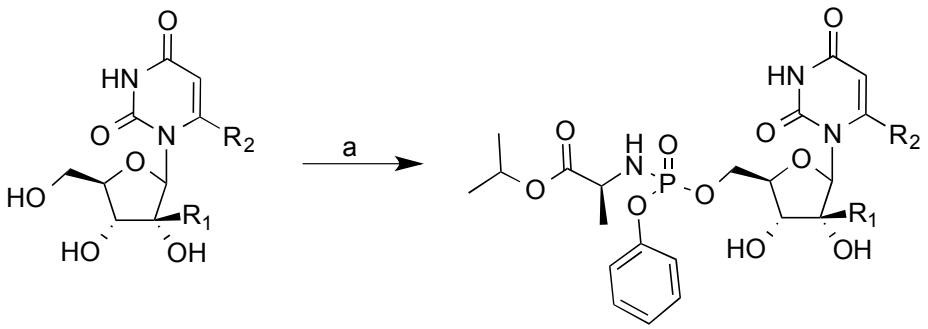


Scheme 3. (a) BSA, sugar, TMSOTf, -20 °C to 70 °C, 3 h; (b) 7N NH_3 in MeOH, 5-48 h; (c) Acetone, H_2SO_4, 3 h; (d) TBDMSCl, Im, dry DMF, overnight; (e) BSA, sugar, $SnCl_4$, CH_2Cl_2, rt; (f) 7N NH_3 in MeOH; (g) (*i*) TBDMSCl, Im, dry DMF, overnight, (*ii*) $(Et)_4$ NCN, dry DMF, overnight, (*iii*) TBAF in THF.

An alternative route, however, provided an additional 2',6-modified substrate, compound **18**. The formation of 2'-methyl-5-iodouridine **17** was readily realized as reported in the literature[12], followed by an addition-elimination reaction which converted compound **17** to 2'-methyl-6-cyanouridine **18** in 89% yield after a final deprotection step using TBAF.[13] The presence of the new chemical bond C6-C7 (C_{Sp2}-C_{Sp3}) was confirmed by HMBC correlation.

The substitution of the phosphate function with an amino acid ester residue and a lipophilic aromatic group to mask the negative charge allows hepatic delivery of higher concentrations of monophosphate nucleosides, which are then liberated following an intracellular cascade of metabolic steps and further converted into their active triphosphate forms. The conversion of all of the prepared nucleosides to their

corresponding 5'-phosphoramidates was achieved by a common methodology. The phosphoramidating reagent was readily prepared by reacting alanine isopropyl ester hydrochloride and phenyl dichlorophosphate in the presence of triethylamine at low temperature. Addition of the obtained reagent mixture for each of the modified nucleosides provided the relative phosphoramidates as diastereomeric mixtures, with moderate to good yields.



R_1 = H, R_2 = CH_3 **3**	R_1 = H, R_2 = CH_3 **19** (60%)
R_1 = CH_3, R_2 = CH_3 **4**	R_1 = CH_3, R_2 = CH_3 **20** (55%)
R_1 = H, R_2 = I **7**	R_1 = H, R_2 = I **21** (39%)
R_1 = H, R_2 = CN **10**	R_1 = H, R_2 = CN **22** (42%)
R_1 = H, R_2 = N_3 **11**	R_1 = H, R_2 = N_3 **23** (22%)
R_1 = CH_3, R_2 = CN **18**	R_1 = CH_3, R_2 = CN **24** (60%)

Scheme 4. (a) (i) $PhOP(O)Cl_2$, L-Alanine isopropyl ester hydrochloride, dry CH_3CN, -40 °C, TEA, (ii) Nucleoside, *N*-methylimidazole, 5 h.

2.2. Conformational analysis of 2'6-dimethyluridine and 2'-methyl-6-cyanouridine

Subsequently, the predominant conformation of unprecedented 2',6-disubstituted analogues **4** and **18** in solution was determined by NMR spectroscopy techniques. An *anti*-relationship across the glycosidic bond is common in most pyrimidine nucleosides as a result of repulsive interactions between the sugar ring and the carbonyl group at the 2-position. Nevertheless, the insertion of a bulky substituent at the 6-position sterically restrains the molecule into a *syn*-conformation. We were interested in studying the effect played by the additional presence of a methyl group at the 2'-*beta*-position on both the glycosidic bond orientation and sugar puckering.

Although an infinite number of conformations is theoretically possible, the pucker position of five-membered rings of nucleosides in the pseudorotation cycle is commonly confined to either of two antipodal regions, C3′-*endo* or "North" and C2′-*endo* or

"South".[14] An estimated ratio of North and South conformers in solution can be determined on the basis of the values of vicinal homonuclear coupling constants,[15] and in our specific case exclusively from the coupling between H3' and H4' protons.

For compound **4**, a $^{3}J_{H3'\text{-}H4'} = 9.06$ Hz was measured in D_2O, which is indicative for a predominant North-type sugar conformation,[16] further corroborated by the presence of a significant NOE enhancement between 2'-OH and H4' and the absence of any interaction between 2'-Me and H5' protons (Figure 1). For comparison, the conformational analysis was also applied to 6-methyluridine **3** and results were consistent with literature report.[7]

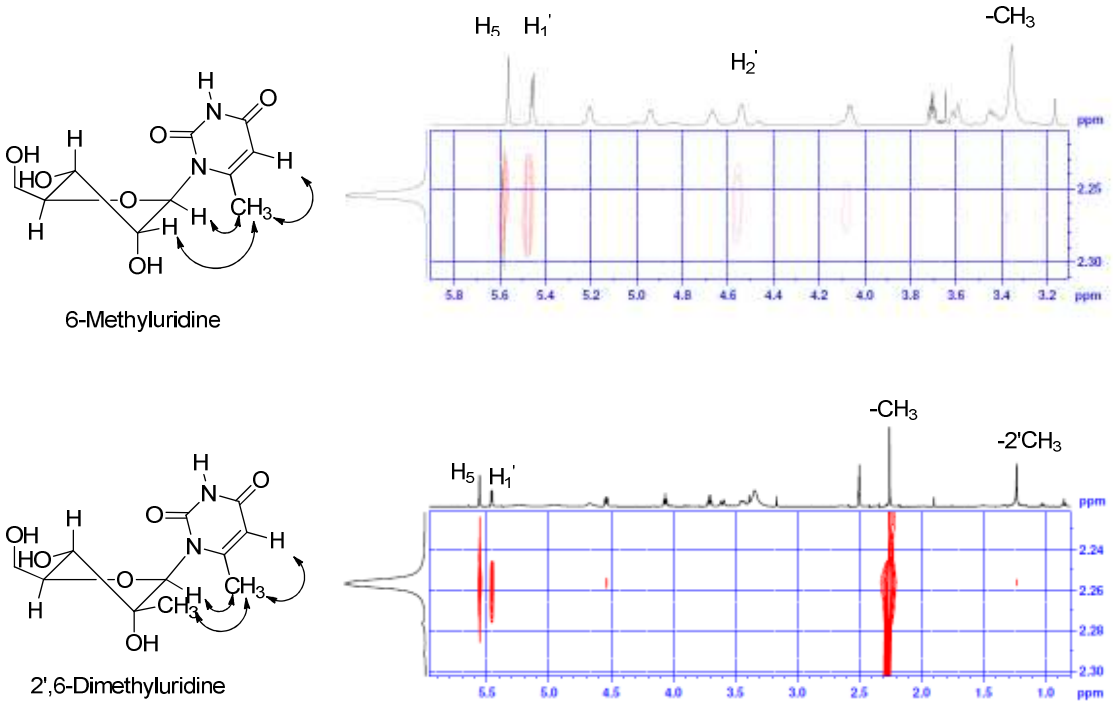


Figure 1. NOESY spectra for 6-dimethyluridine **3** and 2',6-dimethyluridine **4**, run in DMSO-*d6* on Bruker Avance II 500 MHz spectrometer with a TXI HCP probe.

The $^{3}J_{H3'\text{-}H4'}$ values measured at five different temperatures indicates that compound **4** does not undergo a significant temperature dependent conformational change, as shown in Table 1. The percentage of preferred C3′-*endo* ring conformer was obtained by performing a pseudorotational analysis with the aid of a Matlab interface.[17]

Table 1. Pseudorotational analysis data for compound **4**.

T ºC	peak 1	peak 1	$^3J_{H3'-H4'}$	% N-conformer
5	2439.1682	2430.0462	9.122	92.24
10	2474.9359	2465.814	9.1219	92.24
15	2510.2236	2501.1616	9.062	91.36
20	2544.1909	2535.129	9.0619	91.36
25	2577.1981	2568.1361	9.062	91.36

The determination of the prevalent orientation state, derived from the relative positions of sugar and base around the glycosidic bond, was based upon the values of $^3J_{H1'C6}$ and $^3J_{H1'C2}$, the combination of which represents a direct correlation to the *syn*- or *anti*-range for the glycosidic torsion angle χ.[18] Thus, in a 1D ^{13}C NMR spectrum where no ^1H decoupling was applied during signal detection, compound **4** exhibited $^3J_{H1'C6}$ = 4.78 Hz and $^3J_{H1'C2}$ = 6.49 Hz at 600 MHz in D_2O. Since $^3J_{H1'C2} > {}^3J_{H1'C6}$, which allows to concluded that the glycosidic torsion angle χ is in the *syn* domain.

The observed *syn*-relationship of the nucleobase relative to the sugar ring was supported by analysis of NOE interactions. Selective irradiation of the group at the 6-position showed the presence of strong cross peaks between R-6 and H-1' in all compounds, whilst any correlation of R-6 to H5' and H5" was absent.

3. Biological evaluation

Initially, the synthetized nucleoside phosphoramidates **19-24** were evaluated for selective antiviral activity against the following viruses: hepatitis C virus (type 1b, Con1 strain) in the Huh 5-2 replicon system. No selective antiviral activity could be observed. Because of the high conservation of the catalytic amino acid motifs across RNA-dependent RNA polymerases of positive-strand RNA viruses,[19] the compounds were subsequently also evaluated against chikungunya virus and also various species belonging to the genus Enteroviruses, for instance Coxsackie virus (type B3, Nancy strain) in Vero cells (subtype A), Coxsackievirus (type B4, E2 Edwards strain) in Vero cells (subtype A), Poliovirus (type 1, Sabin strain) in BGM cells, enterovirus 71, and rhinovirus (type 14) in Hela cells (subtype Rh). Unfortunately, no selective antiviral activity could be observed either.

4. Conclusion

A new set of phosphoramidate nucleosides, derived from 6- and 2',6-modified uridines, were prepared but found to be devoid of selective antiviral activity on the replication of several single-stranded, positive-sense RNA viruses. Spectroscopic analysis of the conformational properties of the parent nucleosides showed a *syn/N* preference, which is incompatible with recognition by viral replication enzymes and as such provides an explanation for the lack of antiviral activity.

5. Experimental section

5.1 General

NMR spectra were recorded on Bruker Avance I 300 MHz (BBI probe), Avance II 500 MHz (TXI HCP probe) and 600 MHz (TCI HCN cryoprobe) spectrometers using a TXI HCP probe. Chemical shifts (δ) are expressed in parts per million (ppm). 1H and 13C NMR chemical shifts are referenced relative to TMS (δ = 0.00 ppm). Mass spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3uL/min and spectra were obtained in positive (or negative) ionization mode with a resolution of 15000 (FWHM) using leucine enkephalin as lock mass. Thin-layer chromatography (TLC) was performed on Alugram silica gel UV254 mesh 60, 0.20 mm (Macherey-Nagel).

5.1. 1-(2',3',5'-Tri-*O*-acetyl-*β*-D-ribofuranosyl)-6-methyluracil (1).

To a solution of 6-methyluracil (604.0 mg, 4.80 mmol) in dry CH_3CN (10 mL), was added *N,O*-bistrimethylsilylacetamide (2.4 mL, 14.0 mmol) and the mixture was stirred for 20 min at room temperature. To this mixture, a solution of 1,2,3,5-tetra-*O*-acetyl-*β*-D-ribofuranose (1.27 g, 4.00 mmol) in dry CH_3CN (15 mL) was then added. After cooling to -20 °C, TMSOTf (2.4 mL, 4.4 mmol) was added and the reaction mixture was allowed to warm slowly to 15-20 °C and stirred for 6 h. The mixture was then poured into CH_2Cl_2 and washed with saturated aq. $NaHCO_3$. The water layer was extracted with CH_2Cl_2. The organic extracts were combined and dried over Na_2SO_4, filtered and evaporated in vacuo. The crude residue was purified by column chromatography (CH_2Cl_2/MeOH 49:1) on silica gel to give product **1** (1.50 g) as an anomeric mixture. The *β*-anomer was isolated in 81% yield (1.24 g) and the *α*-anomer in 11% yield (168.0 mg). The undesired N_1,N_3-bis-riboside was isolated in 11.2 % yield (173.0 mg). All products were oils. Data for **1** : ^{1}H-NMR (600MHz, DMSO-d_6): δ 11.39 (s, 1H, NH), 5.57 (d, 1H, J = 2.2 Hz, H-2'), 5.57-5.55 (m, 2H, H-5 and H-1'), 5.42 (dd, 1H, J = 2.2 Hz and J = 6.6 Hz, H-3'), 4.32 (m, 1H, H-5'), 4.15-4.12 (m, 1H, H-4'), 4.03 (m, 1H, H-5''), 2.25 (s, 3H, 6-CH_3), 2.11 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.09 (s, 3H, CH_3); ^{13}C-NMR (150MHz, DMSO-d_6): δ 170.2 (2 x CO), 169.5 (CO), 162.4 (4-C), 153.0 (2-C), 150.6 (6-C), 102.5 (5-CH), 90.1 (1'-CH), 78.2 (4'-CH), 73.0 (2'-CH), 69.7 (3'-CH), 62.9 (5'-CH_2), 20.6 (CH_3), 20.5 (CH_3), 20.3 (CH_3), 19.5 (6-CH_3); HRMS for $C_{16}H_{20}N_2O_9$ $[M–H]^-$ Calc.: 383.1095, found: 383.1103.

5.2. 1-(2',3',5'-Tri-*O*-benzoyl-2'-methyl-*β*-D-ribofuranosyl)-6-methyluracil (2).

To a solution of 6-methyluracil (302.0 mg, 2.40 mmol) in dry CH_3CN (5 mL), was added *N,O*-bistrimethylsilylacetamide (1.18 mL, 7.00 mmol) and the mixture was stirred for 20 min at room temperature. To this mixture, a solution of 1,2,3,5-tetra-*O*-benzoyl-2'-methyl-*β*-D-ribofuranose (1.16 g, 2.00 mmol) in dry CH_3CN (10 mL) was added, followed by dropwise addition of TMSOTf (1.4 mL) at -20 °C. The reaction mixture was then allowed to warm slowly to 10-15 °C and stirred for 6 h. The mixture was then poured into CH_2Cl_2 and washed with saturated aq. $NaHCO_3$. The organic layer was combined, dried over Na_2SO_4 and filtered. After removal of all the volatiles, the residue was purified by column chromatography (hexane/ethyl acetate 3:1→3:2), to give product **2** (1.08 g) as an oil in 88.5% yield. The undesired N_1,N_3-bis-riboside (66.0 mg) was obtained as an oil in 5.7% yield. ^{1}H-NMR (300MHz, DMSO-d_6): δ 11.56 (s, 1H, 3-NH), 8.07–7.35 (m, 15H, aromatic), 6.34 (s, 1H, H-1'), 6.31 (d, 1H, J = 8.7 Hz, 3'- H), 5.66 (s, 1H, H-5), 4.73-4.66 (m, 1H, H-4'), 4.61–4.59 (m, 2H, H-5'), 2.39 (s, 3H, 6-CH_3), 1.76 (s, 3H, 2'-CH_3); ^{13}C-NMR (75MHz, DMSO-d_6): δ 165.6 (5''-C=O), 165.1 (2''-C=O), 164.9 (3''-C=O), 162.4 (4-C), 154.1 (2-C), 150.9 (6-C), 134.0–128.7 (15 x C-aromatic), 102.9 (5-CH), 91.3 (1'-CH), 88.0 (4'-CH), 77.4 (2'-CH), 77.1 (3'-CH), 64.5 (5'-CH_2), 19.7 (7-CH_3), 18.1 (6'-CH_3); HRMS for $C_{32}H_{28}N_2O_9$ $[M+H]^+$ Calc.:585.1867, found: 585.1897.

5.3. 6-Methyluridine (3).

A dried 50 mL round-bottomed flask was charged with **1** (550.0 mg, 1.43 mmol) and 20 mL of a 7N solution of NH_3 in MeOH was then added at 0 °C. The reaction was allowed to warm slowly to room temperature and stirred for 5 h to give a yellow solution. The solvent was then removed in vacuo and the resulting residue was purified by column chromatography (CH_2Cl_2/ MeOH 9:1) on silica gel to give product **3** (268 mg) as a white solid in 75% yield. UV λ_{max} (MeOH) = 269 nm (ε = 5 179 mol^{-1} cm^{-1}); ^{1}H-NMR (500MHz, DMSO-d_6): δ 11.10 (s, 1H, NH), 6.03 (d, 1H, J = 3.7 Hz, H-1'), 5.45 (s, 1H, H-5), 4.99 (d, 1H, J = 4.8 Hz, OH-2'), 4.82 (d, 1H, J = 6.3 Hz, OH-3'), 4.55 (t, 1H, J = 5.8 Hz, H-2'), 4.46 (d, 1H, J = 5.35 Hz, OH-5'), 4.08 (q, 1H, J = 6.0 Hz, H-3'), 3.69-3.55 (m, 2H, H-5'), 3.68-3.65 (m, 1H, H-4'), 2.02 (s, 3H, 6-CH_3); ^{13}C-NMR (125MHz, DMSO-d_6): δ 162.9 (4-C), 152.4 (2-C), 151.1 (6-C), 98.7 (5-CH), 87.3 (1'-CH), 84.4 (4'-CH), 71.0 (2'-

CH), 70.3 (3'-CH), 62.5 (5'-CH_2), 18.2 (7-CH_3); HRMS for $C_{10}H_{14}N_2O_6$ $[M+H]^+$ Calc.: 259.0924, found: 259.0931.

5.4. 2',6-Dimethyluridine (4).

A dried 50 mL round-bottomed flask was charged with **2** (1.20 g, 4.41 mmol) and 20 mL of a 7N solution of NH_3 in MeOH was then added at 0 °C. The reaction was allowed to warm slowly to room temperature and stirred for two days. The solvent was then removed in vacuo and the residue was purified by column chromatography (CH_2Cl_2/MeOH 100:7) on silica gel to give product **4** (459.0 mg) as a white solid in 84% yield. ^{1}H-NMR (300MHz, D_2O): δ 5.67 (s, 2H, H-1' and H-5), 4.22-4.19 (m, 1H, H-3'), 3.89 - 3.76 (m, 3H, H-4' and H-5'), 2.30 (s, 3H, 6-CH_3), 1.21 (s, 3H, 2'-CH_3); ^{13}C-NMR (75 MHz, D_2O): δ 165.1 (4-CO), 155.6 (2-CO), 150.7 (5-CH), 102.3 (5-CH), 94.3 (1'-CH), 82.4 (4'-CH), 79.0 (2'-CH), 73.9 (3'-CH), 61.4 (5'-CH_2), 20.0 (7-CH_3), 19.0 (6'-CH_3). MS for $C_{32}H_{28}N_2O_9$ $[M+H]^+$ Calcd.: 273.1089, found: 273.1089.

5.5. 6-Cyanouridine (10).

A dried 100 mL, round-bottomed flask was charged with 5-*O*-(*tert*-butyldimethylsilyl)-6-iodo-2',3'-*O*-isopropylideneuridine **6** (200.0 mg, 0.38 mmol) in anhydrous dimethylformamide (4 mL) and then $(Et)_4NCN$ (150.0 mg, 1.00 mmol) was added at room temperature. The reaction mixture was stirred overnight, poured into water (20 mL) and extracted with EtOAc (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was treated with 50% aq. TFA (10 mL) at 0 ºC and then stirred at room temperature overnight in the dark. The solvent was evaporated and the crude residue was purified by column chromatography (MeOH/CH_2Cl_2, 10:100) on silica gel to give product **10** as a brown solid (50.0 mg) in 48% yield. ^{1}H-NMR (300MHz, D_2O): δ 6.66 (s, 1H, H-5), 5.97 (d, 1H, J = 4.56 Hz, H-1'), 4.73 (q, 1H, J = 4.59, 6.6 Hz, H-2'), 4.34 (t, 1H, J = 6.6 Hz, H-3'), 4.07-4.01 (m, 1H, H-4'), 3.95-3.81 (m, 2H, H-5'); ^{13}C-NMR (75MHz, D_2O): δ 162.7 (4-C), 149.7 (2-C), 126.8 (7-CN) 114.1 (6-C), 110.7 (5-CH), 92.3 (1'-CH), 84.0 (4'-CH), 71.4 (2'-CH), 68.5 (3'-CH), 60.8 (5'-CH_2).

5.6. 6-Azidouridine (11).

5'-*O*-(*tert*-Butyldimethylsilyl)-6-iodo-2',3'-*O*-isopropylideneuridine **6** (300.0 g, 0.57 mmol) in anhydrous DMF (3.4 mL) was treated with sodium azide (60.0 mg, 0.9 mmol). The reaction mixture was stirred overnight at room temperature, concentrated, and redissolved in EtOAc (30 mL). The organic layer was washed with water (20 mL), brine (20 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was then treated with 50% aq. TFA (10 mL) at 0 ºC and stirred at room temperature overnight in the dark. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (MeOH/CH_2Cl_2, 10:100) on silica gel to give product **11** as a yellow solid (146 mg, 90%). ^{1}H-NMR (300MHz, D_2O): δ 5.97 (d, 1H, *J* = 3.51 Hz, H-1'), 5.63 (s, 1H, H-5), 4.65 (q, 1H, *J* = 3.6 Hz, H-2'), 4.32 (t, 1H, *J* = 6.69 Hz, H-3'), 3.85-3.79 (m, 2H, H-5'), 3.70-3.64 (m, 1H, H-4'); ^{13}C-NMR (75MHz, D_2O): δ 163.9 (4-C), 153.4 (2-C), 150.1 (6-C), 89.6 (5-CH), 88.3 (1'-CH), 83.3 (4'-CH), 71.3 (2'-CH), 68.8 (3'-CH), 61.1 (5'-CH_2).

5.7. 1-(2,3,5-Tri-*O*-benzoyl-2'-methyl-*β*-D-ribofuranosyl)-uridine (14).

To a solution of uracil **12** (250.0 mg, 2.20 mmol) in dry CH_3CN (5 mL), was added *N,O*-bistrimethylsilylacetamide (1.18 mL, 7.00 mmol) and the mixture was stirred for 20 min at room temperature. To this mixture, a solution of 1,2,3,5-tetra-*O*-benzoyl-2'-methyl-*β*-D-ribofuranosyl (1.16 g, 2.00 mmol) in dry CH_3CN (10 mL) was added. After cooling to -20 ˚C, TMSOTf (1.4 mL) was added and the reaction mixture was allowed to warm slowly to room temperature and then stirred at 70 °C for 2 h. The mixture was then poured into ethyl acetate and washed with saturated aq. $NaHCO_3$. The water layer was again extracted with ethyl acetate. The organic extracts were combined, dried over Na_2SO_4 and filtered. After removal of all the volatiles in vacuo, the residue was purified by column chromatography (CH_2Cl_2/ MeOH 49:1) on silica gel to give product **14** (983.0 mg) as an anomeric mixture. The β-anomer (946.0 mg) was isolated as an oil in 75% yield. ^{1}H-NMR (300MHz, DMSO-d_6): δ 9.7 (s, 1H, 3-NH), 8.20-7.26 (m, 16H, H-6 and 3 x aromatic), 6.55 (s, 1H, H-1'), 5.79 (d, 1H, *J* = 5.3 Hz, H-3'), 5.75 (d, 1H, *J* = 8.19 Hz, H-5), 4.67-4.63 (m, 1H, 4'- H), 4.93-4.80 (m, 2H, 2H-5'), 1.78 (s, 3H, CH_3); ^{13}C-NMR (75MHz, DMSO-d_6): δ 165.9 (2''-C=O), 164.97 and 164.94 (2'' and 3''-C=O), 162.8 (4-C), 149.7 (2-C), 140.6 (6-C), 133.32-128.09 (aromatic), 102.1 (5-CH), 84.2 (1'-CH), 80.1 (4'-CH), 75.2 (2'-CH), 63.0 (3'-CH), 60.1 (5'-CH_2), 17.7 (CH_3); HRMS for $C_{31}H_{26}N_2O_9$ $[M+Na]^+$ Calc.: 593.1530, found: 593.1541.

5.8. 2'-Methyluridine (15).

A dried 50 mL round-bottomed flask was charged with **14** (3.00 g, 5.26 mmol) and 50mL of a 7N solution of NH_3 in MeOH was then added at 0 ºC. The reaction was stirred at room temperature for 48 h. The solvent was then removed in vacuo and the residue was purified by column chromatography (ethyl acetate/ MeOH 100:5→100:8) on silica gel to give product **15** (1.19 g) as a white solid in 88% yield. ^{1}H-NMR (300MHz, DMSO-*d*$_6$): δ 11.34 (s, 1H, 3-NH), 8.05 (d, 1H, *J* = 8.07 Hz, H-6), 5.80 (s, 1H, H-1'), 5.61 (d, 1H, *J* = 8.07 Hz, H-5),), 5.18 (d, 2H, *J* = 4.08 Hz, H-3', OH-3'), 5.10 (s, 1H, OH-2'), 4.06-3.59 (m, 4H, 2H-5', H-4', OH-5'), 1.01 (s, 3H, CH_3); ^{13}C-NMR (75MHz, DMSO-*d*$_6$): δ 163.1 (4-C), 150.8 (2-C), 140.5 (6-C), 101.5 (5-CH), 91.1 (1'-CH), 82.3 (4'-CH), 78.4 (2'-CH), 71.7 (3'-CH), 58.8 (5'-CH_2), 19.93 (CH_3); HRMS for $C_{10}H_{14}N_2O_6$ $[M+H]^+$ Calcd.: 259.0924, found: 259.0929.

5.9. 2'-Methyl-6-cyanouridine (18).

A dried 100 mL, round-bottomed flask was charged with 2',3',5'-tri-*O*-*tert*-butyldimethylsilyl-5-iodo-uridine **17** (200.0 mg, 0.275 mmol) in anhydrous dimethylformamide (4 mL) and then $(Et)_4NCN$ (75.0 mg, 0.5 mmol) was added at room temperature. The reaction mixture was stirred overnight, it was then poured into $NaHCO_3$ (20 mL) and extracted with EtOAc (100 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was treated with TBAF in THF (3 mL) at room temperature overnight. The solvent was evaporated and the crude residue was purified by column chromatography (MeOH/CH_2Cl_2, 10:100) on silica gel to give product **18** as a brown solid (69 mg, 89%). ^{1}H-NMR (300 MHz, MeOD): δ 6.47 (s, 1H, H-5), 5.97 (s, 1H, H-1'), 4.03 (br s, 1H, H-3'), 3.95-3.91 (m, 2H, H-5', H-5''), 3.89-3.79 (m, 1H, H-4'), 1.26 (s, 3H, CH_3); ^{13}C-NMR (75MHz, MeOD): δ 161.2 (4-C), 149.0 (2-C), 126.7 (7-CN) 113.1 (6-C), 111.2 (5-CH), 96.8 (1'-CH), 83.4 (4'-CH), 77.7 (2'-CH), 74.0 (3'-CH), 61.4 (5'-CH_2), 13.9 (2'-CH_3). HRMS for $C_{11}H_{13}N_3O_6$ $[M+Na^+]$ Calcd.: 306.0696, found: 306.0698.

5.10. General procedure for phosphoramidates synthesis

A dried 50 mL, round-bottomed flask was charged with phosphate dichloride phenol (1 mmol) and alanine isopropyl hydrochloric ester (1 mmol) in dry CH_3CN under inert atmosphere. After cooling to -40 ^oC, Et_3N (2 mmol) was added dropwise and stirred overnight. A portion of this solution was then removed under inert atmosphere and added to another round-bottomed flask containing the relevant nucleoside (0.35 mmol). The mixture was stirred until complete solubilization, then 1-methylimidazole (2 mmol) was added dropwise over 5 min and the stirring was continued for 3 h. The solvent was then removed in vacuo and the residue was purified by column chromatography (DCM/ ethanol/pyridine (50/3/0.2) on silica gel.

5.10.1. (2S)-Isopropyl 2-(2'R,3'R,4'R) (((3',4'-dihydroxy-5-(6-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-)methoxy)(phenoxy)phosphorylamino)propanoate (19)

Yellow oil (54.1 mg, 60% yield). ^{1}H-NMR (300MHz, DMSO-d_6): δ 11.24 (s, 1H, NH), 7.34-7.17 (m, 10H, aromatics), 5.91 (d, 1H, H-1'), 5.57 (s, 1H, H-5), 5.44 (s, 1H, OH-2'), 5.33 (s, 1H, OH-3'), 5.12 (d, 1H, J = 6.6 Hz, H-2'), 4.87-4.81 (m, 1H, H from alanine), 4.22-4.12 (m, 1H, H-3'), 3.91-3.88 (m, 1H, H-4'), 3.76-3.70 (m, 2H, H-5'), 2.24 (s, 3H, 7-CH_3); ^{13}C-NMR (75MHz, $CDCl_3$): δ 172.9 (=CO), 162.6 (4=CO), 152.7 (2=CO), 150.3 (5-C $_{sp^2}$), 102.6 (6-CH), 138.3-119.8 (aromatics), 92.9 (1'-CH), 82.0 (4'-CH), 72.5 (2'-CH), 70.14 (3'-CH), 69.4 (5'-CH_2), 69.4 (CH), 49.9 (CH), 31.6 (CH_3), 21.3 (CH_3), 21.2 (CH_3), 20.2 (7-CH_3). ^{31}P-NMR (121MHz, DMSO-d_6): δ 3.55; HRMS for $C_{22}H_{30}N_3O_{10}P[M+H]^+$ Calcd.: 528.1741, found: 528.1754.

5.10.2. (2S)-Isopropyl 2-(2'R,3'R,4'R)(((3',4'-dihydroxy-2'-methyl-5-(6-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate (20)

Yellow oil (52.3 mg, 55% yield). ^{1}H-NMR (500MHz, DMSO-d_6): δ 11.19 (s, 1H, 3-NH), 7.32-7.09 (m, 5H, aromatic), 5.47 (s, 1H, H-1'), 5.55 (s, 1H, H-5), 5.12 (d, 1H, OH-2'), 4.92 (s, 1H, OH-3'), 4.89-4.80 (m, 1H, 1H), 4.48 (t, 1H, J = 5.0 Hz, H-3'), 4.07 (t, 1H, J = 5.1 Hz H-4'), 3.86-3.62 (m, 3H, 2H-5'overlapped with 1H, CH), 2.25 (s, 3H, 6-CH_3), 1.23 (s, 6H, 2x CH_3), 1.16 (d, 3H, J = 6.15 Hz, CH_3), 1.09 (s, 3H, 2'-CH_3); ^{13}C-NMR

(125MHz, DMSO-d_6): δ 173.4 (CO), 162.5 (4=CO), 153.5 (2=CO), 150.4 (5-CH), 129.3-120.6 (aromatics), 102.3 (6-CH), 94.9 (1'-CH), 82.1 (4'-CH), 77.9 (2'-CH), 74.9 (3'-CH), 68.8 (5'-CH), 67.8 (CH), 49.9 (CH), 29.3 (CH_3), 20.5 (7-CH_3), 20.5 (CH_3), 20.4 (CH_3), 13.9 (6'-CH_3). ^{31}P-NMR (200MHz, DMSO-d_6): δ 3.75; HRMS for $C_{23}H_{32}N_3O_{10}P[M+H]^+$ Calcd.: 542.1897, found: 542.1897.

5.10.3. (2S)-Isopropyl 2-(2'R,3'R,4'R) (((3',4'-dihydroxy-5-(6-iodo-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate (21)

Yellow oil (50.3 mg, 39% yield). ^{1}H-NMR (300MHz, DMSO-d_6): δ 11.54 (s, 1H, NH), 7.65-7.15 (m, 10H, aromatics), 6.35 (s, 1H, H-5), 5.76 (d, 1H, J = 2.31 Hz, H-1'), 5.35 (t, 1H, J = 4.38 Hz), 5.16 (d, 1H, J = 6.81 Hz), 4.88-4.80 (m, 1H), 4.51 (s, 1H), 4.51-3.72 (m, 5H), 1.23-1.13 (m, 18H, 6 x CH_3); ^{13}C-NMR (125MHz, DMSO-d_6): δ 172.7 (=CO), 161.6 (4=CO), 150.8 (2=CO), 147.3 (5-C_{sp^2}), 132.1-120.2 (aromatics), 118.4 (6-C), 115.4 (1'-CH), 82.4 (4'-CH), 71.6 (2'-CH), 69.8 (3'-CH), 68.0 (5'-CH_2), 49.9 (CH), 49.8 (CH), 29.1 (CH_3), 21.5 (CH_3), 21.4 (CH_3); ^{31}P-NMR (121MHz, $CDCl_3$): δ 2.86 and 2.75; HRMS for $C_{21}H_{27}IN_3O_{10}P[M+H]^+$Calcd.: 640.0553, found: 640.0552.

5.10.4. (2S)-Isopropyl 2-(2'R,3'R,4'R) ((((5-(6-cyano-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate (22)

Yellow oil (39.5 mg, 42% yield). ^{1}H-NMR (300MHz, DMSO-d_6): δ 9.51 (s, 1H, NH), 7.30-7.16 (m, 10H, aromatics), 6.43 (s, 1H, H-5), 5.91 (s, 1H, H-1'), 5.30 (s, 1H), 5.05-4.94 (m, 2H), 4.44-3.96 (m, 5H), 3.94-3.59 (m, 4H), 1.23 (s, 9H, 3 x CH_3); ^{13}C-NMR (125MHz, $CDCl_3$): δ 173.1 (=CO), 150.5 (2=CO), 160.8 (4=CO), 147.3 (5-C_{sp^2}), 129.8-129.6 (aromatic), 125.1 (6-CN), 117.1 (6-C), 113.7 (1'-CH), 82.5 (4'-CH), 73.5 (2'-CH), 70.0 (3'-CH), 69.5 (5'-CH_2), 66.5 (CH), 50.2 (CH), 29.7 (CH_3), 21.7 (CH_3), 21.6 (CH_3); ^{31}P-NMR (121MHz, DMSO-d_6): δ 2.91 and 2.81; HRMS for $C_{22}H_{27}N_4O_{10}P[M+H]^+$ calcd.:575.1513; found: 575.1516.

5.10.5. (2S)-Isopropyl 2-(2'R,3'R,4'R) (((5-(6-azido-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate (23)

Pale yellow oil (42.8 mg, 22% yield). ^{1}H-NMR (500MHz, $CDCl_3$): δ 9.50 (s, 1H, NH), 7.35-7.17 (m, 5H, aromatics), 5.93 (s, 1H, H-5), 5.50-5.49 (d, *J* = 4.05 Hz, 1H, H-1'), 5.02-4.98 (m, 1H), 4.75-4.74 (d, *J* = 7.25 Hz, 1H), 4.67-4.65, (d, *J* = 10.5 Hz , 1H), 4.42-4.40 (m, 1H), 4.32 (s, 1H), 4.16-4.13 (m, 1H), 4.94-4.02 (m, 1H), 1.22-1.24 (m, 9H, 3x CH_3); ^{13}C-NMR (200MHz, $CDCl_3$): δ 173.2 (=CO), 161.5 (4=CO), 152.3 (2=CO), 150.6 (5-C $_{sp^2}$), 149.5 (2=CO), 129.7 (aromatic), 125.0 (aromatic), 120.2 (aromatic), 120.1 (aromatic), 91.0 (6-C), 88.8 (1'-CH), 82.3 (4'-CH), 73.1 (2'-CH), 70.5 (3'-CH), 69.5 (5'-CH_2), 66.7 (CH), 63.1 (CH), 50.3 (CH), 29.7 (CH_3), 21.6 (CH_3), 21.6 (CH_3); ^{31}P-NMR (75MHz, $CDCl_3$): δ 2.99 and 2.84; HRMS for $C_{21}H_{27}N_6O_{10}P_1$ $[M+H]^+$ calcd.:555.1598, found: 555.1595.

5.10.6. (2S)-Isopropyl 2-(2'R,3'R,4'R)(((3',4'-dihydroxy-2'-methyl-5-(6-cyano-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate (24)

Yellow oil (33.0 mg, 60% yield). ^{1}H-NMR (500 MHz, DMSO): δ 10.36 (s, 1H, H-3), 7.35-7.11 (m, 5H, aromatic), 6.31 (s, 1H, H-5), 5.96 (s, 1H, H-1'), 5.03-4.95 (q, 1H, *J* = 6.24, H amino ester), 4.54-4.43 (m, 3H), 4.35-4.20 (m, 2H), 4.18-4.11 (m, 2H), 1.37-1.20 (m, 12H, 4 x -CH_3); ^{13}C-NMR (125MHz, $CDCl_3$): δ 172.8 (-CO), 160.0 (4-C), 150.3 (2-C), 148.5 (5-C $_{sp^2}$), 129.4 (aromatic), 124.8 (aromatic), 129.7-119.9 (aromatic), 125.9 (7-CN), 113.1 (6-C), 110.6 (5-CH), 97.5 (1'-CH), 82.1 (4'-CH), 74.5 (2'-CH), 69.9 (3'-CH), 66.7 (-CH), 64.8 (5'-CH_2), 50.0 (CH), 29.4 (CH_3), 21.3 (CH_3), 21.3 (CH_3), 13.8 (CH_3); ^{31}P-NMR (75 MHz, DMSO-*d*$_6$): δ 3.05 and 2.79; HRMS for $C_{23}H_{29}N_4O_{10}P_1$ $[M+Na^+]$ Calcd.: 575.1513, found: 575.1516.

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