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Synthesis and biological evaluation of pyrimidine nucleoside monophosphate prodrugs targeted against influenza virus

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Abstract

Uridine-based nucleoside analogues have often been found to have relatively poor antiviral activity. Enzymatic assays, evaluating inhibition of influenza virus RNA polymerase, revealed that some uridine triphosphate derivatives displayed inhibitory activity on UTP incorporation into viral RNA. Here we report the synthesis, antiviral activity and enzymatic evaluation of novel ProTides designed to deliver the activated (monophosphorylated) uridine analogues inside the influenza virus-infected cells. After evaluation of the activation profile we identified two ProTides with moderate antiviral activity in MDCK cells (**23a**, EC_{99}=49 ± 38 μM and **23b**, EC_{99}≥81 μM) while the corresponding nucleoside analogue (2'-fluoro-2'-deoxyuridine) was inactive. Thus, at least in these cases the poor antiviral activity of the uridine analogues may be ascribed to poor phosphorylation.

Keywords

Uridine-based nucleoside analogues; ProTide; RNA polymerase; Carboxypeptidase Y.

1. Introduction

Most antiviral nucleoside compounds inhibit viral genome replication by acting as mimetics of the natural nucleosides. Nucleoside analogues (NAs) can either act as chain terminators after being incorporated into growing DNA/RNA strands and/or inhibit the viral polymerase function by competition with the natural nucleoside 5'-triphosphate substrate (Wagner, 2000). Since most NAs exert their antiviral activities as their 5'-triphosphate form, phosphorylation is critical to activate these compounds. In most cases, the first step of phosphorylation represents the rate-limiting step of the bioactivation and its inefficiency may limit the therapeutic potential of the NA (Jones, 2005). Bypassing this rate-limiting activation step may improve or extend the biological activity of the NAs and may be a good strategy to broaden their spectrum of activity. However, nucleoside 5'-monophosphates cannot be delivered directly into cells because of their instability in biological media and poor diffusion through cell membranes due to their negative charges. One approach to overcome these issues is to mask the negative charges of the monophosphate moiety with lipophilic substituents. A variety of monophosphate prodrug approaches have been developed based upon intracellular cleavage of the masking group to release the monophosphate inside the cell (Jones, 2005; Meier, 1998; Hecker, 2008). The aryloxy phosphoramidate approach, known as the ProTide concept, has emerged as a versatile method to obtain intracellular levels of nucleoside 5'-monophosphates and several studies have yielded a detailed insight into their intracellular activation pathway (Mehellou, 2010).
Most antiviral pyrimidine analogues reported in the literature act as mimetics of either thymidine or cytidine (Field, 2008; De Clercq, 2008). Uridine-based nucleoside analogues have often been found to have relatively poor antiviral activity, which may be related to the inefficient phosphorylation of uridine-based compounds.
In the study reported here, enzymatic assays with influenza virus polymerase revealed that some uridine 5'-triphosphate (UTP) derivatives display inhibitory activity to incorporation of UTP into influenza virus RNA. In particular, 5-bromouridine 5'-triphosphate and 2'-deoxy-2'-fluorouridine 5'-triphosphate demonstrated 50% inhibitory concentrations below 10 μM. Based on these intriguing enzymatic inhibition data, we applied the phosphoramidate approach to a selection of modified uridines, with the purpose to deliver the nucleoside 5'-monophosphate inside the cell thereby potentially increasing the activity of the nucleoside analogues against

influenza virus. Notably, in each case the parent NA was devoid of anti-influenza activity.

2. Materials and methods

2.1. Experimental chemistry

All anhydrous solvents were bought from Aldrich and all reagents commercially available were used without further purification. Thin Layer Chromatography (TLC): precoated, aluminium backed plates (60 F_{254}, 0.2 mm thickness, Merck) were visualized under both short and long wave ultraviolet light (254 nm and 366 nm). Preparative TLC plates (20x20 cm, 500-2000 μm) were purchased from Merck. Column chromatography processes were carried out using silica gel supplied by Fisher (35-70 μm). Glass columns were slurry packed using the appropriate eluent and samples were applied either as a concentrated solution in the same eluent or pre-adsorbed on silica gel. Analytical and semi-preparative HPLC were conducted using a Varian Prostar system while the software used was Galaxie Chromatography Data System. Only compounds with purity ≥95% were considered in this study. Nuclear Magnetic Resonance (NMR): ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), ^{31}P- NMR (202 MHz) and ^{19}F-NMR (470 MHz) were recorded on a Bruker Avance 500MHz spectrometer at 25 °C. Spectra were calibrated to the residual signal of the deuterated solvent used. Chemical shifts are given in parts per million (ppm) and coupling constants (*J*) in Hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), m (multiplet), bs (broad singlet), dd (doublet of doublet). Mass Spectroscopy (MS): high resolution mass spectroscopy was performed at the School of Chemistry, Cardiff University, using electrospray (ES).

2.1.1. Standard procedure A: synthesis of phosphorochloridates ***4a-b, 6a-b***

Anhydrous triethylamine (2.00 mol equiv) was added dropwise at -78 °C to a stirred solution of the appropriate phosphorodichloridate (1.00 mol equiv) and the appropriate amino acid ester salt (1.00 mol equiv) in anhydrous DCM (30-40 mL) under an argon atmosphere. After 1 h the reaction was allowed to slowly warm to

room temperature and stirred for 2-3 h. The formation of the desired compound was monitored by ^{31}P-NMR. The solvent was removed under reduced pressure and the crude residue purified by flash column chromatography (ethyl acetate/hexane 1/1) to give the product as oil.

2.1.1.1. Synthesis of 1-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate ***4a***

Prepared according to *Standard procedure A*, using **2** (1.86 g, 7.11 mmol), **3a** (2.50 g, 7.11 mmol), anhydrous triethylamine (1.98 mL, 14.22 mmol) in anhydrous DCM (40 mL). The reaction mixture was stirred at -78 °C for 1 h, then at room temperature for 2h to give the product as a pale yellow oil (75%, 2.16 g). ^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.23, 8.02. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.10-7.30 (12H, m, *Naph*O, OCH_2*Ph*), 5.21, 5.19 (2H, m, O*CH*$_2$Ph), 4.70-4.64 (1H, m, NH), 4.42-4.33 (1H, m, C*H*CH_3), 1.59-1.57 (3H, 2d, *J*=7.0 Hz, CH_3).

2.1.1.2. Synthesis of phenyl-(benzoxy-L-alaninyl)-phosphorochloridate ***6a***

Prepared according to *Standard procedure A*, using **5** (1.06 mL, 7.11 mmol) **3a** (2.50 g, 7.11 mmol), anhydrous triethylamine (1.98 mL, 14.22 mmol) in anhydrous DCM (40 mL). The reaction mixture was stirred at -78 °C for 1 h, then at room temperature for 2h to give the product as a pale yellow oil (90%, 2.27 g). ^{31}P-NMR ($CDCl_3$, 202 MHz): δ 7.90, 7.58. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.44-7.25 (10H, m, *Ph*O, OCH_2*Ph*), 5.24, 5.23 (2H, m, O*CH*$_2$Ph), 4.40 (1H, bs, NH), 4.34-4.21 (1H, m, C*H*CH_3), 1.54, 1.53 (3H, 2d, *J*=7.0 Hz, CH_3).

2.1.2. Standard procedure B: synthesis of 2',3'-O-protected nucleosides ***15, 16***

To a solution of the appropriate nucleoside in anhydrous cyclopentanone, $HClO_4$ (3.00 to 4.00 mol equiv) was added and the reaction mixture was stirred at room temperature for 5 h. The reaction was then neutralized with a saturated solution of $NaHCO_3$. The solvent was removed under reduced pressure and the crude residue purified by flash column chromatography (gradient elution of DCM/MeOH=98/2 then 96/4).

2.1.3. Standard procedure C: synthesis of phosphoramidates ***17a-c, 18a-c, 21a-c, 22a, 23a-c***

^{t}BuMgCl (1.0 M solution in THF, 1.10 to 2.00 mol equiv) was added to a suspension/solution of the appropriate nucleoside (1.00 mol equiv) in anhydrous THF (10 mL) under an argon atmosphere and stirred at room temperature for 30 min. The appropriate phosphorochloridate (2.00 mol equiv) dissolved in anhydrous THF was added dropwise and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the crude residue purified by flash column chromatography (gradient elution of DCM/MeOH) to give the desired product.

2.1.3.1. Synthesis of 2',3'-O,O-cyclopentylidene-5-iodouridine-5'-O-naphthyl-(benzoxy-L-alaninyl)-phosphate ***17a***

Prepared according to *Standard Procedure C*, using **15** (0.20 g, 0.46 mmol) in anhydrous THF (10 mL), ^{t}BuMgCl (1.0 M solution in THF, 0.92 mL, 0.92 mmol) and **4a** (0.37 g, 0.92 mmol) and the reaction mixture was stirred at room temperature overnight. The crude was purified by column chromatography (gradient elution of DCM/MeOH=98/2, then 96/4) to give a white solid (81%, 0.30 g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.00, 3.77. ^{1}H-NMR (MeOD, 500 MHz): δ 8.26-7.13 (13H, m, *Naph*, OCH$_2$*Ph*, H-6), 5.92-5.80 (1H, m, H-1'), 5.14-5.01 (2H, m, O*CH$_2$*Ph), 4.78-4.66 (1H, m, H-3'), 4.66-4.52 (1H, m, H-2'), 4.42-4.25 (3H, m, H-4', H-5'), 4.21-4.06 (1H, m, *CH*CH$_3$), 2.06-1.51 (8H, m, CH$_2$-cyclopentyl), 1.45-1.26 (3H, m, CH*CH$_3$*).

2.1.3.2. Synthesis of 2',3'-O,O-cyclopentylidene-5-bromouridine-5'-O-naphthyl-(benzoxy-L-alaninyl)-phosphate ***18a***

Prepared according to *Standard Procedure C*, using **16** (0.20 g, 0.51 mmol) in anhydrous THF (10 mL), ^{t}BuMgCl (1.0 M solution in THF, 1.00 mL, 1.00 mmol) and **4a** (0.42 g, 1.03 mmol) and the reaction mixture was stirred at room temperature overnight. The crude was purified by column chromatography (gradient elution of DCM/MeOH=98/2, then 96/4) to give a white solid (63%, 0.24 g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.07, 3.84. ^{1}H-NMR (MeOD, 500 MHz): δ 8.19-7.25 (13H, m, *Naph*O, OCH$_2$*Ph*, H-6), 5.70-5.69 (1H, m, H-1'), 5.13-5.07 (2H, m, O*CH$_2$*Ph), 4.62-4.64 (1H, m, H-2'), 4.51-4.53 (1H, m, H-3'), 4.41-4.26 (3H, m, H-4'. H-5'), 4.18-4.04 (1H, m, *CH*CH$_3$), 2.04-1.83 (2H, m, CH$_2$-cyclopentyl), 1.80-1.58 (6H, m, CH$_2$-cyclopentyl), 1.43-1.31 (3H, m, CH*CH$_3$*).

2.1.3.3. Synthesis of 5-methyl-uridine-5'-O-naphthyl-(benzoxy-L-alaninyl)-phosphate ***21a***

Prepared according to *Standard Procedure C*, using **12** (0.30 g, 1.16 mmol) in anhydrous THF (10 mL), ^{t}BuMgCl (1.0 M solution in THF, 1.40 mL, 1.40 mmol) and **4a** (0.94 g, 2.32 mmol) and the reaction mixture was stirred at room temperature overnight. Then ^{t}BuMgCl (1.0 M solution in THF, 1.40 mL, 1.40 mmol) was added, and after 3 h the solution was concentrated. The crude was purified by column chromatography (gradient elution of DCM/MeOH=98/2, then 96/4). The product was further purified by preparative TLC (gradient elution of DCM/MeOH=98/2, then 96/4) to give a white solid (3%, 0.02 g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.12, 4.09. ^{1}H-NMR (MeOD, 500 MHz): δ 8.27, 7.31 (12H, m, *Naph*O, OCH$_2$*Ph*), 7.48 (1H, m, H-6), 5.93-5.90 (1H, m, H-1'), 5.13-5.05 (2H, m, O*CH*$_2$Ph), 4.47-4.29 (2H, m, H-3', H-4'), 4.23-4.11 (3H, m, H-2', H-5'), 4.10-4.04 (1H, m, *CH*CH$_3$), 1.67-1.65 (3H, m, CH$_3$), 1.34-1.33 (3H, m, CH*CH$_3$*). ^{13}C NMR (MeOD, 126 MHz): δ 12.34, 12.42 (*C*H$_3$-Uridine), 20.27 (d, $J_{C\text{-}P}$=7.5 Hz, *C*H$_3$-Ala), 20.39 (d, $J_{C\text{-}P}$=6.6 Hz, *C*H$_3$-Ala), 51.80, 51.88 (CH-Ala), 67.57 (d, $J_{C\text{-}P}$=5.7 Hz, C-5'), 67.69 (d, $J_{C\text{-}P}$=5.2 Hz, C-5'), 68.03 (O*C*H$_2$Ph), 71.13 (C-2'), 74.89, 74.94 (C-3'), 83.83, 83.88, 83.85, 83.89, 83.93 (C-4'), 90.50, 90.62 (C-1'), 111.98 (C-5), 116.15, 116.18, 116.21, 122.58, 122.74, 126.08, 126.11, 126.41, 126.49, 126.52, 127.08, 127.46, 127.57, 127.86, 127.88, 128.52, 128.91, 128.96, 129.13, 129.31, 129.34, 129.36, 129.46, 129.59 (C-6, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH$_2$*Ph*), 136.33, 137.14, 137.29 (C-4a Naph, "ipso" OCH$_2$*C*-Ph), 147.82, 147.88 ("ipso" *C*-Naph, C-2), 147.95, 147.92 (C-4), 174.53, 174.57, 174.83, 174.85 (*C*OOCH$_2$Ph). HRMS= ($C_{30}H_{33}N_3O_{10}P$) calculated 626.1905, found 625.1907. HPLC= H$_2$O/MeOH from 90/100 to 0/100 in 30 min= retention time 25.61, 25.88 min.

2.1.3.4. Synthesis of 5-bromo-2'-deoxyuridine-5'-O-phenyl-(benzoxy-L-alaninyl)-phosphate ***22a***

Prepared according to *Standard Procedure C*, using **13** (0.20 g, 0.65 mmol) in anhydrous THF (10 mL), ^{t}BuMgCl (1.0 M solution in THF, 0.78 mL, 0.78 mmol) and **6a** (0.46 g, 1.30 mmol) and the reaction mixture was stirred at room temperature

overnight. Then ^{t}BuMgCl (1.0 M solution in THF, 0.78 mL, 0.78 mmol) was added, and after 2 h the solution was concentrated. The crude was purified by column chromatography (gradient elution of DCM/MeOH=98/2, then 96/4) to give a white solid (23%, 0.10 g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.06, 3.60. ^{1}H-NMR (MeOD, 500 MHz): δ 7.99, 7.98 (1H, 2s, H-6), 7.40-7.17 (10H, m, *Ph*O, OCH_2*Ph*), 6.23-6.16 (1H, m, H-1'), 5.15-5.14 (2H, m, O*CH*$_2$Ph), 4.40-4.36 (1H, m, H-3'), 4.33-4.23 (2H, m, H-5'), 4.10-4.07 (1H, m, *CH*CH_3), 4.06-3.96 (1H, m, H-4'), 2.37-1.85 (2H, m, H-2'), 1.38-1.36 (3H, m, CH*CH*$_3$). ^{13}C-NMR (MeOD, 126 MHz): δ 19.56 (d, J_{C-P}=7.1Hz, *C*H_3-Ala), 29.54 (d, J_{C-P}=6.4Hz, *C*H_3-Ala), 41.03, 41.38 (C-2'), 50.82, 50.96 (CH-Ala), 66.83, 66.96 (2d, J_{C-P}=5.5Hz, C-5'), 67.22 (O*CH*$_2$Ph), 71.22, 71.37 (C-3'), 86.49, 86.57 (C-4'), 96.64 (C-5), 105.34 (C-1'), 120.60, 120.63, 120.64, 120.66, 125.41, 125.48, 128.50, 128.56, 128.58, 128.64, 128.78, 128.84, 129.97, 129.99 (2 Ph), 136.29, 136.36 ('ipso' OCH_2*C*-Ph), 140.40, 140.46 (C-6), 150.49, 150.53, 150.60 ('ipso', *C*-Ph), 151.19, 151.24, 151.29 (C-2), 160.64, 160.72 (C-4), 173.71 (d, J_{C-P}=4.9Hz, *C*$OOCH_2$Ph), 173.97 (d, J_{C-P}=4.4Hz, *C*$OOCH_2$Ph). HRMS= ($C_{25}H_{28}N_3O_9PBr$) calculated 624.0747, found 624.0753. HPLC= H_2O/MeOH from 90/100 to 0/100 in 30 min= retention time 24.28, 24.49 min.

2.1.3.5. Synthesis of 2'-deoxy-2'-fluorouridine-5'-O-naphthyl-(benzoxy-L-alaninyl)-phosphate ***23a***

Prepared according to *Standard Procedure C*, using **14** (0.30 g, 1.22 mmol) in anhydrous THF (10 mL), ^{t}BuMgCl (1.0 M solution in THF, 1.46 mL, 1.46 mmol) and **4a** (0.98 g, 2.44 mmol) and the reaction mixture was stirred at room temperature overnight. Then **4a** (0.49 g, 1.22 mmol) and ^{t}BuMgCl (1.0 M solution in THF, 1.02 mL, 1.02 mmol) were added, and after 2 h the solution was concentrated. The crude was purified by column chromatography (gradient elution of DCM/MeOH=98/2, then 96/4). The product was further purified by preparative TLC (gradient elution of DCM/MeOH=98/2, then 96/4) to give a white solid (4%, 0.03 g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.26, 4.08. ^{19}F-NMR (MeOD, 470 MHz): δ -203.46, -203.88. ^{1}H-NMR (MeOD, 500 MHz): δ 8.19-7.29 (13H, m, *Naph*-O, OCH_2*Ph*, H-6), 5.92 (0.5H, dd, J_{H-H}=1.9 Hz, J_{H-F}=18.5 Hz, H-1' of one diastereoisomer), 5.91 (0.5H, dd, J_{H-H}=1.9 Hz, J_{H-F}=18.8 Hz, H-1' of one diastereoisomer), 5.43, 5.42 (1H, 2s, H-5), 5.09-5.11 (2H, m, O*CH*$_2$Ph), 4.99-4.87 (1H, m, H-2'), 4.54-4.34 (1H, m, H-5'), 4.33-4.29 (1H, m, H-

3'), 4.15-4.14 (1H, m, H-4'), 4.12-4.07 (1H, m, *CH*CH_3), 1.37-1.29 (3H, m, CH*CH_3*). ^{13}C-NMR (MeOD, 126 MHz): δ 20.28 (d, J_{C-P}=7.6 Hz, *C*H_3-Ala), 20.45 (d, J_{C-P}=6.6 Hz, *C*H_3-Ala), 51.79, 51.88 (CH-Ala), 66.30 (d, J_{C-P}=4.7 Hz, C-5'), 66.72 (d, J_{C-P}=5.3 Hz, C-5'), 68.04 (O*CH_2*Ph), 69.54 (d, J_{C-P}=16.6 Hz, C-3'), 69.73 (d, J_{C-P}=16.6 Hz, C-3'), 83.43, 82.50 (C-4'), 90.49 (d, J_{C-F}=35.4 Hz, C-1'), 90.67 (d, J_{C-F}=35.4 Hz, C-1'), 94.28 (d, J_{C-F}=187.3 Hz, C-2'), 94.30 (d, J_{C-F}=187.3 Hz, C-2'), 103.01, 103.10 (C-5), 116.20, 116.28, 122.61, 122.72, 126.12, 126.59, 127.61, 127.90, 128.95, 128.99, 129.28, 129.33, 129.59 (C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH$_2$*Ph*), 136.34 (C-4a Naph, 'ipso' OCH$_2$*C*-Ph), 142.32, 142.34 (C-6), 147.86, 147.92 ('ipso', *C-Naph*, C-2), 151.84 (C-4), 174.57, 174.59 (*C*OOCH$_2$Ph). HRMS= ($C_{29}H_{29}N_3O_9PFNa$) calculated 636.1643, found 636.1650. HPLC= H_2O/MeOH from 90/100 to 0/100 in 30 min= retention time 20.39, 21.09 min.

2.1.4. Standard procedure C: deprotection of 2',3'-sugar protected phosphoramidates ***19a-c, 20a-c***

A solution of the appropriate 2',3'-sugar protected phosphoramidate in 60% formic acid was stirred at room temperature overnight. The solvent was removed under reduced pressure and the crude residue was purified by column chromatography (gradient elution of DCM/MeOH=98/2, then 96/4).

2.1.4.1. Synthesis of 5-iodouridine-5'-O-naphthyl-(benzoxy-L-alaninyl)-phosphate **19a**

Prepared according to *Standard Procedure C*, using **17a** (0.30 g, 0.37 mmol) in 60% formic acid (30 mL) and the reaction mixture was stirred at room temperature overnight to give a white solid (22%, 0.06 g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.21, 3.95. ^{1}H-NMR (MeOD, 500 MHz): δ 8.32-7.20 (13H, m, *Naph*, OCH$_2$*Ph*, H-6), 5.86-5.81 (0.5H, m, H-1' of one diastereoisomer), 5.80 (0.5H, d, *J*=3.5 Hz, H-1' of one diastereoisomer), 5.14-5.00 (2H, m, O*CH_2*Ph), 4.50-4.19 (3H, m, H-3', H-2', H-4'), 4.19-4.03 (3H, m, *CH*CH_3, H-5'), 1.42-1.26 (3H, m, CH*CH_3*). ^{13}C NMR (MeOD, 126 MHz): δ 20.44 (d, J_{C-P}=7.3 Hz, CH_3-Ala), 20.57 (d, J_{C-P}=6.4 Hz, CH_3-Ala), 51.87, 51.90 (CH-Ala), 67.65 (d, J_{C-P}=5.3 Hz, C-5'), 67.43 (d, J_{C-P}=5.2 Hz, C-5'), 68.10, 68.11 (O*C*H$_2$Ph), 69.44, 69.47 (C-5), 70.80, 70.95 (C-2'), 74.95, 75.07 (C-3'), 84.07,

84.13, 84.19 (C-4'), 91.41 (d, J_{C-P}=19.8 Hz, H-1'), 91.42 (d, J_{C-P}=19.8 Hz, H-1'), 116.21, 116.24, 116.34, 116.36, 122.73, 122.93, 126.09, 126.55, 127.56, 127.84, 128.93, 129.19, 129.53, 129.60 (C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2*Ph*), 136.32, 137.11 (C-4a Naph, 'ipso' OCH_2*C*-Ph), 146.67, 146.74 (C-6), 147.87, 147.94, 148.01 ('ipso' Naph), 152.09 (C-2), 162.60 (C-4), 174.48, 174.61, 174.78, 174.84 (*C*$OOCH_2$Ph). HRMS= ($C_{29}H_{29}N_3O_{10}$PINa) calculated 760.0510, found 760.0515. HPLC= H_2O/MeOH from 90/100 to 0/100 in 30 min= retention time 25.75, 26.16 min.

2.1.4.2. Synthesis of 5-bromouridine-5'-O-naphthyl-(benzoxy-L-alaninyl)-phosphate ***20a***

Prepared according to *Standard Procedure C*, using **18a** (0.24 g, 0.32 mmol) in 60% formic acid (30 mL) to give a white solid (25%, 0.05 g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.23, 4.01. ^{1}H-NMR (MeOD, 500 MHz): δ 8.23-7.20 (13H, m, *Naph*O, OCH_2*Ph*, H-6), 5.86 (0.5H, d, *J* =1.7 Hz, H-1' of one diastereoisomer), 5.85 (0.5H, d, *J*=1.6 Hz, H-1' of one diastereoisomer), 5.11-4.99 (2H, m, O*C*H_2Ph), 4.48-4.30 (3H, m, H-4', H-5'), 4.20-4.06 (3H, m, H-2', H-3', *CH*CH_3), 1.43-1.32 (3H, m, CH*C*H_3). ^{13}C NMR (MeOD, 126 MHz): δ 20.40 (d, J_{C-P}=7.3 Hz, CH_3-Ala), 20.51 (d, J_{C-P}=6.6 Hz, CH_3-Ala), 51.84, 51.89 (CH-Ala), 67.36 (d, J_{C-P}=4.9 Hz, C-5'), 67.57 (d, J_{C-P}=5.3 Hz, C-5'), 68.09 (O*C*H_2Ph), 70.90, 70.96 (C-2'), 75.06, 75.19 (C-3'), 84.05, 84.11, 84.18 (C-4'), 91.32, 91.50 (H-1'), 97.81(C-5), 116.20, 116.23, 116.26, 116.29, 122.67, 122.85, 126.10, 126.51, 127.50, 127.55, 127.83, 127.85, 128.91, 128.94, 129.31, 129.34, 129.38, 129.59 (C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2*Ph*), 136.32 (C-4a Naph), 137.10, 137.13 ('ipso' OCH_2*C*-Ph), 141.51, 141.63 (C-6), 147.89, 147.95 ('ipso' *C*-Naph), 151.68 (C-2), 161.39 (C-4), 174.60 (d, J_{C-P}=4.6 Hz, *C*$OOCH_2$Ph), 174.80 (d, J_{C-P}=4.3 Hz, *C*$OOCH_2$Ph). HRMS= ($C_{29}H_{29}N_3O_{10}$PBrNa) calculated 712.0672, found 712.0694. HPLC= H_2O/MeOH from 90/100 to 0/100 in 30 min= retention time 25.59, 26.01 min.

2.2. Biological experiments

2.2.1. Purification of viral ribonucleoprotein complexes

The procedure for purification of viral vRNP complexes from disrupted influenza virions was developed many years ago (Plotch, 1981; Rochovansky, 1976). Influenza A/X-31 [A/Aichi/2/68 (H3N2) x A/PR/8/34 (H1N1)] virus was inoculated in 10-day-old embryonated chicken eggs and incubated for 48 h at 32°C. After clarification of the allantoic fluid (total volume: 150 ml) by centrifugation (1800 g; 30 min; 4 °C), the virus was pelleted by ultracentrifugation (35,000 g; 3 h; 4 °C) in a sucrose/NTE buffer, consisting of 30% sucrose, 0.1 M NaCl, 0.01 M Tris HCl, 0.001 M EDTA at pH 7.4. The virus pellet was disrupted by incubation at 31 °C for 25 min in a buffer containing 0.1 M Tris pH 8.1, 0.1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 1.5% triton-N 101, 10 mg/ml lysolecithin and 1.5 mM dithiothreitol (DTT). Then, the viral lysate was subjected to ultracentrifugation (288,000 g; 6 h; 4 °C) on a discontinuous (33 - 40 - 50 -70 %) glycerol gradient in a buffer with 0.15 M NaCl and 48.5 mM Tris pH 7.8. The polymerase-containing fractions were identified by Western blot, using an anti-PB2 antibody (Tebu-Bio) and a horseradish peroxidase-labeled secondary antibody. Pooled fractions were dialyzed overnight at 4 °C against storage buffer (50 mM Tris pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT and 50% (V/V) glycerol) with a Slide-A-Lyzer Dialysis Cassette MW 10,000 (Pierce), and stored at -80 °C.

2.2.2. Enzymatic assay with influenza virus polymerase

The modified UTP analogues were purchased from TriLink Biotechnologies, whereas [5-^{3}H]-UTP (specific activity: 23.1 Ci/mmol) was from Moravek. The influenza virus vRNP (2 µL) was incubated for 60 min at 30 °C in a reaction mixture (final volume: 25 µL) consisting of 50 mM Tris pH 8.0, 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 0.4 U/µL Recombinant RNasin (Promega), 200 µM ApG primer [adenyl (3'-5') guanosine, obtained from Sigma], 100 µM GTP, 100 µM CTP, 500 µM ATP, 2 µM UTP and 1.7 µM [5-^{3}H]UTP, with or without test compounds. The enzyme reaction was terminated by the addition of 1.0 mL of a mixture containing 10% trichloroacetic acid (TCA) and 20 mM $Na_4P_2O_4$. After 30 min incubation on ice, precipitates were spotted onto glass microfibre filters and washed 10 times with 5% TCA, and once with denaturated ethanol. Then, the filters were dried during 2 h and incorporated radioactivity was determined by liquid scintillation counting. The IC_{50} was calculated by extrapolation, and defined as the compound concentration showing 50% reduction in incorporated radioactivity, as compared to the condition receiving no inhibitor.

2.2.3. Antiviral assay in influenza virus-infected MDCK cells

Details on virus strain, cells and media can be found elsewhere (Vanderlinden 2010). One day prior to infection, Madin Darby canine kidney (MDCK) cells were seeded in 96-well plates at 25 000 cells per well. Cells were infected with influenza A virus (strain A/X-31) at a multiplicity of infection of 0.0004 plaque-forming units per cell. After adding serial dilutions of the test compounds, the plates were incubated during 24 hr at 35 °C, when supernatants were collected and stored at -80 °C. An aliquot (4 µl) was mixed with 22 µL lysis reagent to disrupt the virus particles, following the instructions inserted in the Cellsdirect One-step qRT-PCR kit from Invitrogen. After 10 min heating at 75°C, 10µL lysate was transferred to a qPCR plate containing the RT-PCR enzymes and buffer, and influenza virus M1-specific primers and probe (as described in Vanderlinden 2010). The RT-PCR program consisted of: 15 min at 50 °C; 2 min at 95 °C; and 40 cycles of 15 sec at 95 °C followed by 90 sec at 60 °C. Absolute quantitation of vRNA copies was performed by including an M1-plasmid standard. The EC_{99} was calculated by extrapolation and defined as the compound concentration causing a 2-$\log_{10}$ reduction in the amount of vRNA copies, as compared to untreated virus control. In parallel, compound cytotoxicity was estimated from the morphological changes in MDCK cells observed by microscopy after three days incubation with the test compounds.

2.2.4. Enzymatic assays using carboxypeptidase Y and cell lysate

The carboxypeptidase Y experiment was performed using ^{31}P-NMR and recording 1 experiment every 7 minutes within 14 h. The ProTides were dissolved in *d*6-acetone and Trizma buffer (pH=7.6) and a solution of carboxypeptidase Y in Trizma buffer was added after recording the blank for each sample (Derudas, 2009).

The cell lysate experiment was performed using a concentrated cell lysate prepared from 10^7 HuH7 hepatocyte cells, which was incubated with the ProTides in deuterium oxide and dimethylsulfoxide at 37 °C. ^{31}P-NMR spectra were recorded every 30 minutes within 14 h.

2.2.5. Metabolism in MDCK cells

MDCK cells were seeded at 1,500,000 cells per 25 cm^2 flask and incubated at 35°C for 24 hr. 5-Bromouridine (**11**) or its Protide **20a** were added to a final concentration of 250 µM, and the cells were further incubated at 37°C for 24 or 48 hr. To prepare

the HPLC extracts, the cells were rinsed twice with DMEM and detached by trypsinization at 37°C for 10 min. After collection of the cell pellet by centrifugation (290 x g, 10 min, 4°C), extraction was performed with 200 µl ice-cold methanol 66%. After 10 min incubation on ice, followed by centrifugation (15,700 x g, 10 min, 4°C), the clarified extracts were subjected to HPLC analysis. Therefore, 100 µl extract was injected onto an anion-exchange Partisphere SAX column (dimensions: 4.6 mm x 125 mm) from Whatman (Maidstone, UK), and separated with two phosphate buffers (A: 5 mM and B: 0.3 M ammonium dihydrogen phosphate; both at pH 5), and the following gradient (flow: 2 ml per min): 100% A (5 min); linear gradient to 100% B (15 min); 100% B (20 min); linear gradient to 100% A (5 min) and 100% A (5 min). UV absorbance was measured with a photodiode-array detector, allowing simultaneous detection at 260 nm (λmax for UTP) and 280 nm (λmax for 5-Br-UTP).

3. Results and Discussion

3.1 Chemistry

The synthesis of the first synthon, the requisite phosphorochloridate, involves the phosphorylation of the aromatic alcohol and subsequent coupling with the appropriate amino acid ester salts to give the desired compounds **4a-b** and **6a-b** (Scheme 1).
All the nucleosides were purchased, with the exception of 5-iodouridine **10**, which was synthesised in a three-step synthesis starting from uridine **7**. The first step involved the acetylation of the 2', 3' and 5' positions using dimethylaminopyridine (DMAP), anhydrous triethylamine (TEA) and acetic anhydride in acetonitrile to yield **8**. Selective iodination took place using iodine and cerium (IV) ammonium nitrate (CAN) in acetonitrile under anhydrous conditions to obtain compound **9** in 98% yield (Asakura, 1990). Finally, the deprotection step was carried out with sodium methoxide and methanol to yield compound **10** (Scheme 2).
Compounds **10** and **11** were protected on the sugar level (2'-3' positions), before performing the coupling reaction with the appropriate phosphorochloridate, using an excess of cyclopentanone, while the deprotection of the blocked ProTide was carried out using 60% formic acid in water. The coupling reactions between the phosphorochloridates (**4a-b** and **6a-b**) and the unprotected and protected nucleosides

(**12-16**) were carried out in anhydrous tetrahydrofuran, using 1.1-2 equivalents of ^{t}BuMgCl as a base (Scheme 3 and 4) (Uchiyama, 1993).

Based on our previous insights in the ProTide structure-activity relationships (Cahard et al., 2004), we chose to synthesise ProTides with either phenyl or 1-naphthyl as the aromatic moiety. L-alanine was the preferred amino acid substituent while benzyl and methyl were chosen as the amino acid ester substituents. In this way, our SAR study was mainly focused on how base- or sugar-modifications in the uridine part of these ProTides would impact their bioactivation and antiviral activity against influenza virus. All compounds were characterized and isolated as equimolar mixtures of phosphorus diastereoisomers as judged by ^{31}P-NMR, and each displayed purity above 95% as determined by analytical HPLC.

3.2. RNA polymerase assays

Various base- or sugar-modified UTP derivatives were selected for inhibition studies in an enzymatic assay with influenza virus polymerase (Table 1). The assay is based on incorporation of radiolabeled UTP into viral cRNA upon ApG-primed RNA synthesis by purified influenza virus ribonucleoprotein (vRNP) complexes, which contain the heterotrimeric influenza virus polymerase and the viral RNA template bound to nucleoprotein (Vreede 2007). Under our experimental conditions, the obligate chain terminator 3'-deoxy-UTP yielded an IC_{50} value of 68 µM, as shown in the table. Compared to this reference compound, several UTP derivatives were considerably more active, the two most potent inhibitors being 5-bromo-UTP (IC_{50}: 7.1 µM) and 2'-fluoro-2'-deoxy-5-methyl-UTP (IC_{50}: 9.5 µM). Intermediate inhibitory activity was noted for 5-methyl-UTP, 5-iodo-UTP and 2'-fluoro-2'-deoxy-UTP (IC_{50}: 20, 20 and 14 µM, respectively), whereas weak inhibitory activity was obtained with 5-fluoro-UTP, 4-thio-UTP and 2'-ara-UTP (IC_{50}: 36, 45 and 68 µM, respectively). The inhibitory effect of 5-bromo-UTP, 5-iodo-UTP and 5-methyl-UTP on the incorporation of radiolabeled UTP by the influenza virus polymerase could indicate that these modified nucleotides may act as alternative substrates for the natural UTP substrate. Likewise, many eukaryotic or prokaryotic polymerases are known to be relatively tolerant to pyrimidine modifications at the 5 position (Hocek 2008). This explains the common use of 5-bromo-2'-deoxyuridine and 5-bromouridine (or liposomal forms of their 5'-triphosphates), for intracellular labeling of nascent cellular or viral nucleic acids (Vecchio 2008). Many years ago, Nakayama

and Saneyoshi (1984) studied whether base-substituted UTP analogues can serve as alternative substrates for UTP incorporation by cellular RNA polymerase I and II. Incorporation of 5-methyl-UTP was at least as efficient as that of UTP. Incorporation of 5-Br-UTP was more efficient than that of UTP for RNA polymerase I, and slightly less efficient for RNA polymerase II. Both 2-thio-UTP and 4-thio-UTP were less efficiently used than UTP by both RNA polymerase I and II. Our ongoing biochemical studies should reveal which of these base substitutions are indeed tolerated by the influenza virus polymerase, and whether there is a difference for the three viral RNA species [(+)mRNA, (+)cRNA or (-)vRNA] that are synthesized by this enzyme. Given that the vRNP has a highly compact structure (Resa-Infante 2011), we hypothesised that a bulky 5-bromo substituent may impede the RNA elongating activity of the PB1 polymerase subunit. Unfortunately, insight into the protein structure of the catalytic site of PB1 is currently lacking. Incorporation of 5-bromo, 5-iodo or 5-methyl-UTP in nascent influenza virus RNA is expected to result in non-functional viral RNA, thus impeding its use for subsequent genome replication or synthesis of viral proteins. This provides a rationale to incorporate these base modifications when designing NAs for selective inhibition of influenza virus. Regarding the sugar-modified analogues, we observed strong inhibition by the 2'-fluoro-2'-deoxy-derivatives of UTP and TTP. The 2'-fluoro-2'-deoxy-GTP analogue has been reported to cause nonobligate chain termination in an enzymatic influenza virus polymerase assay similar to ours (Tisdale 1995). Likewise, 2'-methoxy- and 2'-C-methyl-substituted nucleotides have been extensively investigated for their inhibitory effect on the hepatitis C virus polymerase (Deval 2007).

3.3. Antiviral assays

The base- or sugar-modified UMP ProTides (**19a-c**, **20a-c**, **21a-c**, **22a**, **23a-c**) were evaluated for their anti-influenza virus activity in cell culture, using a novel PCR-based virus yield assay (Table 2). With this method, the number of virus particles produced by infected MDCK cells at 24 h p.i. was quantitated by real-time RT-PCR (replacing virus titration on cells, as is done in the classical virus yield assay). The reference compound ribavirin produced a concentration-dependent reduction in virus yield. Its EC_{99} value was 8 μM, which is very similar to the 50% antiviral effective concentrations that we previously obtained for ribavirin in CPE reduction experiments (Vanderlinden 2010). Two ProTides, **23a** and **23b**, both containing 2'-deoxy-2'-

fluorouridine, were found to have weak activity against influenza virus in cell culture and their EC_{99} values were 49 ± 38 μM and ≥81 μM, respectively. The naphthyl ProTide **23a** showed a higher activity than the phenyl analogue **23b**, and this may be due to the higher lipophilicity and therefore permeability through the cell membrane (ClogP **23a**= 2.37, ClogP **23b**=1.20).

3.4. Metabolism study

In an attempt to explain the lack of antiviral activity of 5-bromouridine and its prodrug **20a**, a metabolic experiment was performed in MDCK cell cultures. MDCK cells were exposed to 250 μM of 5-bromouridine (**11**) or its phosphoramidate prodrug **20a** and incubated for 24 hr or 48 hr. Extracts of drug-exposed cells were then prepared and analyzed by anion-exchange HPLC. It was ascertained that any 5-bromo-UTP that may have sufficiently been formed intracellularly could be UV-detected at 280 nm. The standard 5-bromo-UTP (R_t: 20.0 min) eluted on the chromatograms between UTP (R_t: 19.0 min) and CTP (R_t: 20.5 min). Unfortunately, no formation of 5-bromo-UTP could be observed in the cell cultures exposed to either 5-bromouridine (**11**) or the protide **20a**. It should be mentioned, however, that the UV_{280nm} detection limit for non-radiolabeled 5-bromo-UTP was 440 pmol, corresponding to an intracellular 5-bromo-UTP concentration of ~100 μM. The failure to detect measurable 5-bromo-UTP levels in cell cultures exposed to 250 μM 5-bromouridine may be not surprising given the fact that 5-bromouridine is much less efficiently phosphorylated by uridine/cytidine kinase than uridine (Van Rompay et al., 2001). Also, the 5'-mono- or 5'-diphosphate metabolites of 5-bromouridine could not be detected on the chromatograms. Moreover, limitation in the solubility of the prodrug of 5-bromouridine (**20a**) prevented us to apply higher prodrug concentrations than 250 μM to the MDCK cell cultures. Thus, these metabolic experiments could unfortunately not reveal the basis for the failure of the 5-bromouridine phosphoramidate prodrug as a potential antiviral agent in cell culture.

3.5. Enzymatic studies

3.5.1. Carboxypeptidase Y assay

The putative mechanism of bioactivation of the ProTides proceeds in four steps starting with the cleavage of the ester moiety mediated by an esterase or carboxypeptidase type enzyme, followed by the spontaneous intracellular displacement of the aryloxy group which leads to the formation of an unstable five-membered cyclic mixed anhydride. The last step involves the cleavage of the P-N bond by an intracellular phosphoramidase-type enzyme with consequent release of the nucleoside 5'-monophosphate (Saboulard, 1999).

An enzymatic study using carboxypeptidase Y enzyme was carried out with the intent to study the first step of the activation pathway yielding the aminoacyl intermediate (Birkus, 2007). The experiment was performed using ^{31}P-NMR, following the NMR spectra shift of the compounds during the process for 14 h (Figure 1). Compound **20a** (5 mg) was dissolved in *d*6-acetone (0.15 mL) and Trizma buffer (0.30 mL) and a ^{31}P-NMR was recorded. Then a solution of carboxypeptidase Y (0.1 mg) in Trizma buffer (0.15 mL) was added and ^{31}P-NMR experiments were performed every 7 min for 14 h. The spectra show a relatively rapid hydrolysis of the starting material (δp= 4.06, 3.83 ppm) to the metabolite **i** (δp= 4.93, 4.86 ppm) and the metabolite **ii** (δp= 6.66 ppm). Metabolite **i** has been identified as the ProTide without the ester in the amino acid moiety and bearing one negative charge, while metabolite **ii** as the intermediate bearing two negative charges, after the loss of the aromatic moiety. The two diastereoisomers appear to be processed with slightly different efficacy with one diastereoisomer being hydrolised faster than the other. Table 3 contains the data collected over the 14 h to show the different metabolites.

3.5.2. Cell lysate assay

An enzymatic study using cell lysate from HuH7 hepatocytes was carried out at 37 °C and the formation of the monophosphate species was followed by ^{31}P-NMR. Compound **19a** was considered for this study.

From the experiment shown in Figure 2, there was no evidence of formation of the aminoacyl derivative (**ii**) and this suggested a quick enzymatic hydrolysis of the intermediate, but an overall slow bioactivation of the ProTide to the 5'-monophosphate. The deconvoluted spectra clearly showed only 2 species (compound **19a** and its monophosphate nucleotide) after 14 h in a ratio of 1 to 2 (Figure 3).

4. Conclusion

The inhibitory effect of several base- or sugar-modified UTP derivatives observed in our influenza polymerase assays provided a rationale to design innovative nucleoside analogues for selective inhibition of influenza virus. The phosphoramidate ProTide technology was applied to the modified uridines, with the aim of improving their intracellular bioavailability and activation, and achieving an interesting antiviral activity. The uridine ProTides showed efficient processing in the enzymatic carboxypeptidase Y assay, and the cell lysate experiments suggested that these ProTides release their monophosphate metabolite inside the cells, although they are not completely metabolized. After antiviral evaluation in influenza virus-infected cells, we identified two ProTides derived from 2'-fluoro-2'-deoxyuridine showing moderate antiviral activity, **23a** (EC_{99} = 49 μM) and **23b** ($EC_{99} \geq 81$ μM), while the parent nucleoside analogue showed no activity in cell culture. In the viral polymerase assay, the corresponding triphosphate derivative had an inhibitory activity (IC_{50}) of 14 μM. Based on the kinetic results obtained with the cell lysate, we hypothesize that these uridine phosphate ProTides may be slowly processed to release the active monophosphate species. Another possible explanation for their weak antiviral activity could be the inefficient di- or triphosphorylation of the substituted uridine 5'-monophosphate after its release inside the cells, resulting in poor levels of the active 5'-triphosphate metabolite.

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Compound	IC_{50} (μM)[a]
Base-modified analogues	
2-Thio-UTP	>400
4-Thio-UTP	45 ± 8
6-Aza-UTP	>400
5-Fluoro-UTP	36 ± 4
5-Bromo-UTP	7.1 ± 0.4
5-Iodo-UTP	20 ± 1
5,6-Dihydro-UTP	389
5-Methyl-UTP	20 ± 4
Sugar-modified analogues	
3'-Deoxy-UTP	68 ± 9
3'-O-Methyl-UTP	>400
3'-Azido-2',3'-dideoxy-UTP	>400
2'-O-Methyl-UTP	345
2'-Fluoro-2'-deoxy-UTP	14 ± 3
2'-Azido-2'-deoxy-UTP	220
2'-Amino-2'-deoxy-UTP	205
2'-Ara-UTP	72 ± 49
Base- and sugar-modified analogues	
2'-Deoxy-5-bromo-UTP	>400
3'-Deoxy-5-methyl-UTP	>400
2'-O-Methylpseudo-UTP	>400
2'-O-Methyl-5-methyl-UTP	>400
2'-Fluoro-2'-deoxy-5-methyl-UTP	9.5 ± 1.8

[a]Compound concentration causing 50% inhibition of [5-^{3}H]-UTP incorporation during INF vRNP-mediated RNA synthesis. Values are the mean ± SEM of two to four independent experiments.

Table 1. Inhibitory effect of base- or sugar-modified UTP analogues on RNA incorporation of radiolabeled UTP by INF vRNP.

Compound	Nucleoside	Aryl	Amino acid	Ester	Antiviral activity (EC_{99}) µM[a]	Cytotoxicity (MCC) µM[b]
19a	5-IU	Naph	L-Ala	Bn	>100	>100
19b	5-IU	Phe	L-Ala	Bn	>100	>100
19c	5-IU	Naph	L-Ala	Me	>100	>100
20a	5-BrU	Naph	L-Ala	Bn	>100	>100
20b	5-BrU	Phe	L-Ala	Bn	>100	>100
20c	5-BrU	Naph	L-Ala	Me	>100	>100
11	5-BrU				>100	>100
21a	5-MeU	Naph	L-Ala	Bn	>100	>100
21b	5-MeU	Phe	L-Ala	Bn	>100	>100
21c	5-MeU	Phe	L-Ala	Me	>100	>100
12	5-MeU				>100	>100
22a	5-Br-2'dU	Phe	L-Ala	Bn	>100	>100
13	5-Br-2'dU				>100	>100
23a	2'F2'dU	Naph	L-Ala	Bn	49 ± 38	>100
23b	2'F2'dU	Phe	L-Ala	Bn	≥81	>100
23c	2'F2'dU	Naph	L-Ala	Me	>100	>100
14	2'F2'dU				>100	66
Ribavirin					8.0 ± 0.8	100

[a]The EC_{99} represents the compound concentration producing a 2-log_{10} reduction in the number of virus particles released from MDCK cells at 24 hr p.i.
Values are the mean ± SEM of two independent experiments.
[b]Minimum cytotoxic concentration, or compound concentration causing alterations in MDCK cell morphology after three days incubation.

Table 2. Inhibitory effect of base- or sugar-modified UMP ProTides on influenza virus replication in MDCK cells.

Time (min)	Phosphoramidate **20a** (%) δp 4.06, 3.83	Metabolite **i** (%) δp 4.93, 4.86	Metabolite **ii** (%) δp 6.66
0	100	0	0
7	45	36	19
14	21	37	42
21	10	24	66
28	8	7	75
56	6	3	91
60	0	0	100

Table 3. Data collected from the enzymatic experiment with carboxypeptidase Y in **Figure 1**.

Scheme 1. Reagents and conditions: (*i*) phosphorus oxychloride, dry triethylamine, dry diethyl ether, -78 °C, 1h, then rt overnight (*ii*) anhydrous triethylamine, anhydrous DCM, -78 °C, 1h, then rt 2h.

Scheme 2. Reagents and conditions: (*i*) acetic anhydride, anhydrous triethylamine, DMAP, anhydrous CH_3CN, rt, 1h; (*ii*) I_2, CAN, CH_3CN, 80 °C, 1h; (*iii*) NaOMe, anhydrous MeOH, rt, 2h.

Scheme 3. Reagents and conditions: (*i*) cyclopentanone, $HClO_4$, rt, overnight, (*ii*) ^{t}BuMgCl, anhydrous THF, appropriate phosphorochloridate, rt, overnight; (*iii*) 60% formic acid, rt, overnight.

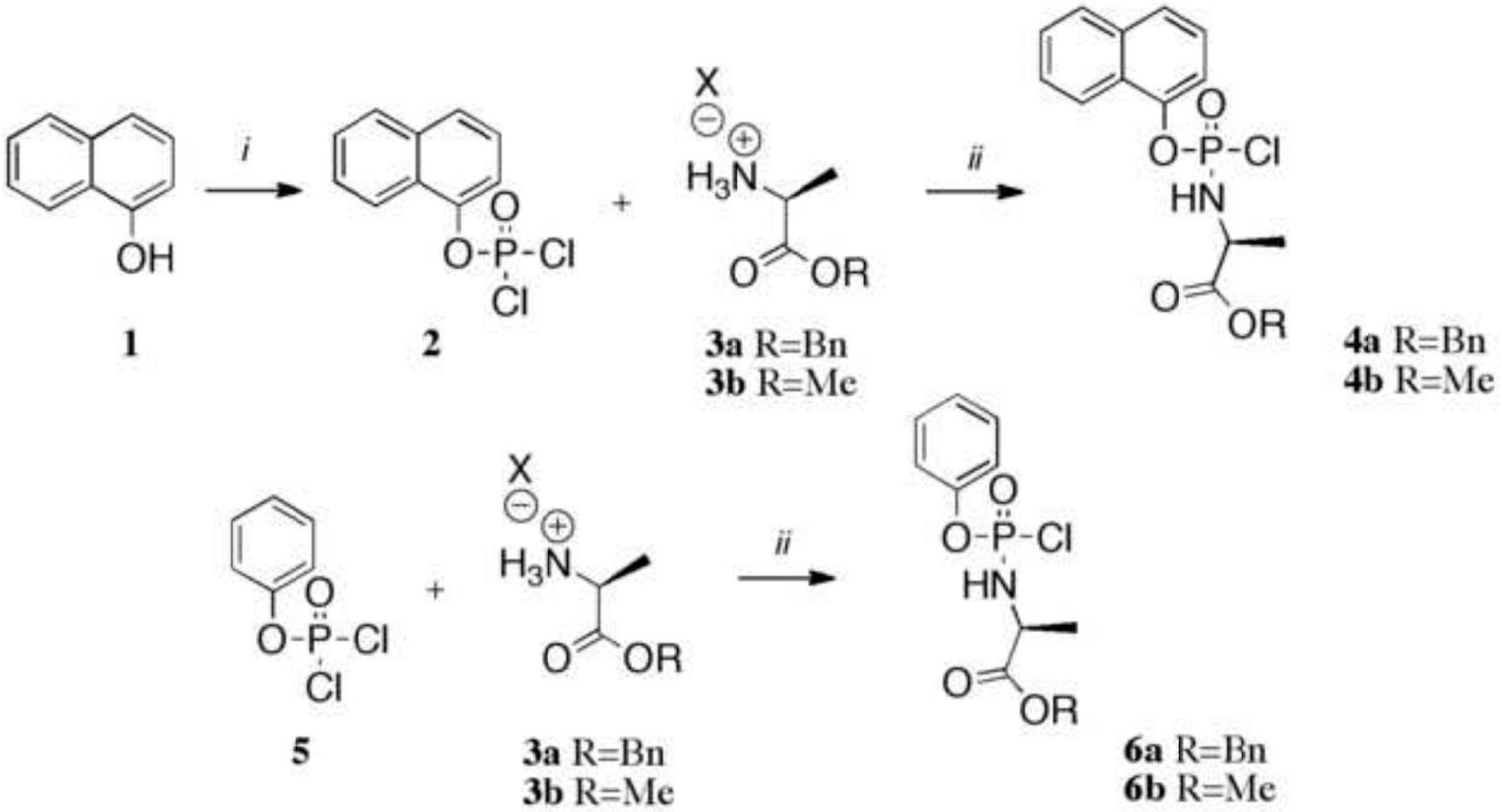
Scheme 4. Reagents and conditions: (*i*) ^{t}BuMgCl, anhydrous THF, appropriate phosphorochloridate, rt, overnight.

Figure 1. Enzymatic experiment of **20a** with carboxypeptidase Y.

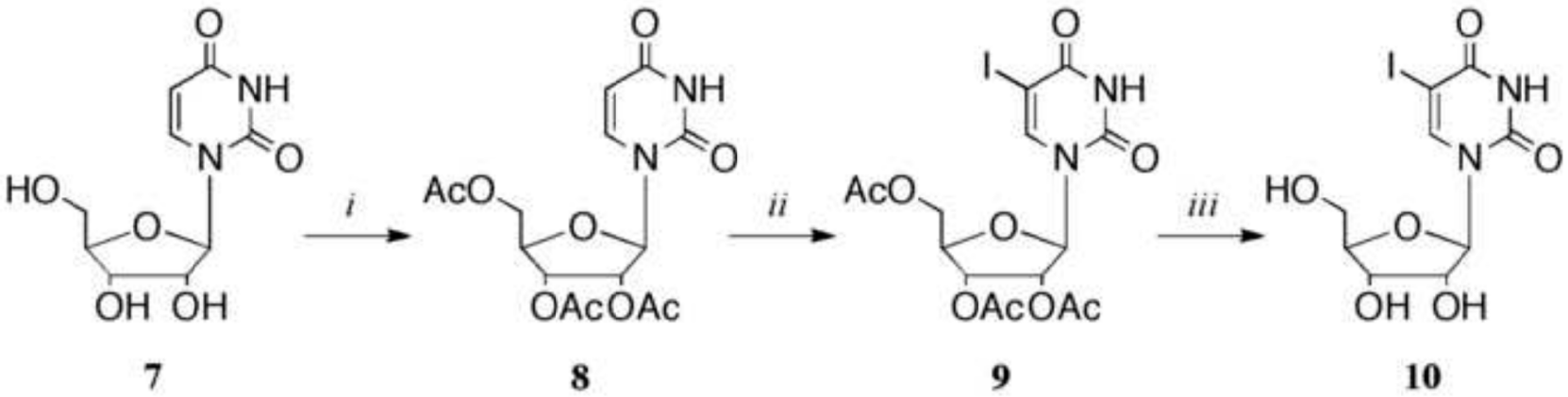
Figure 2. Enzymatic experiment with **19a** incubated with HuH7 cell lysate during 14 h at 37 °C.

Figure 3. Deconvoluted ^{31}P-NMR of the enzymatic experiment in **Figure 3**.

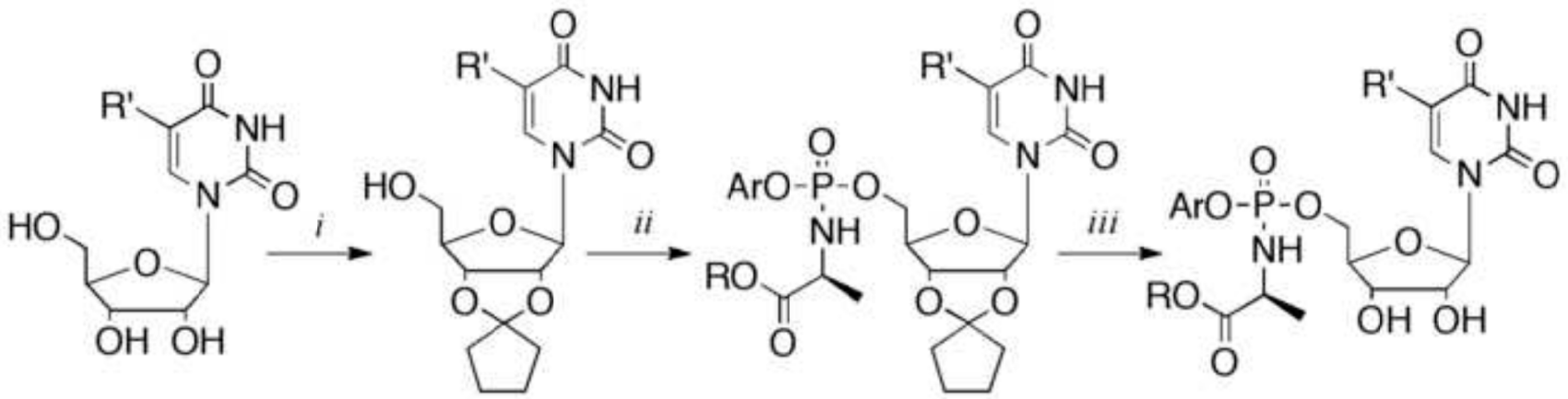
Scheme 1



Scheme 2



Scheme 3



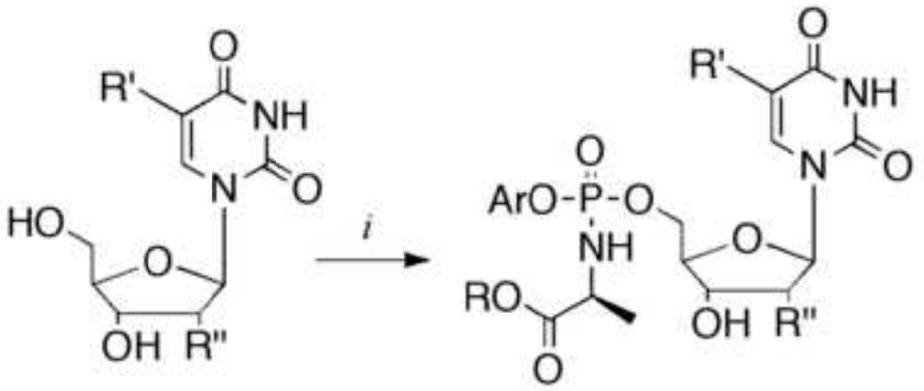
10 R'=I
11 R'=Br

15 R'=I
16 R'=Br

17a-c R'=I
18a-c R'=Br

19a= R=Bn; R'=I; Ar=Naph
19b= R=Bn; R'=I; Ar=Ph
19c= R=Me; R'=I; Ar=Naph
20a= R=Bn; R'=Br; Ar=Naph
20b= R=Bn; R'=Br; Ar=Ph
20c= R=Me; R'=Br; Ar=Naph

Scheme 4



12 R'=Me, R''=OH
13 R'=Br, R''=H
14 R'=H, R''=F

21a= R=Bn; R'=Me; R''=OH; Ar=Naph
21b= R=Bn; R'=Me; R''=OH; Ar=Ph
21c= R=Me; R'=Me; R''=OH; Ar=Ph
22a= R=Bn; R'=Br; R''=H; Ar=Ph
23a= R=Bn; R'=H; R''=F; Ar=Naph
23b= R=Bn; R'=H; R''=F; Ar=Ph
23c= R=Me; R'=H; R''=F; Ar=Naph

Figure 1

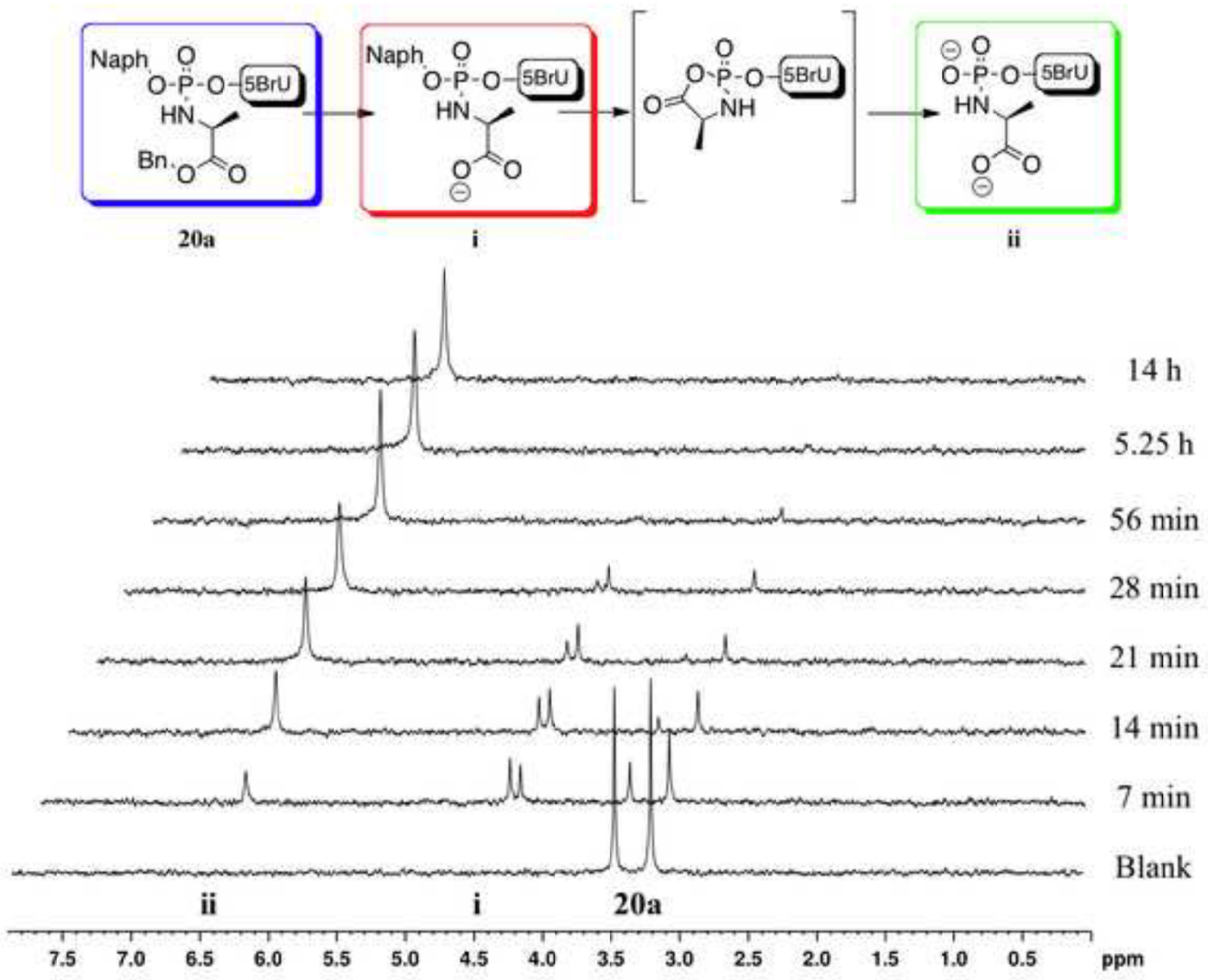


Figure 2

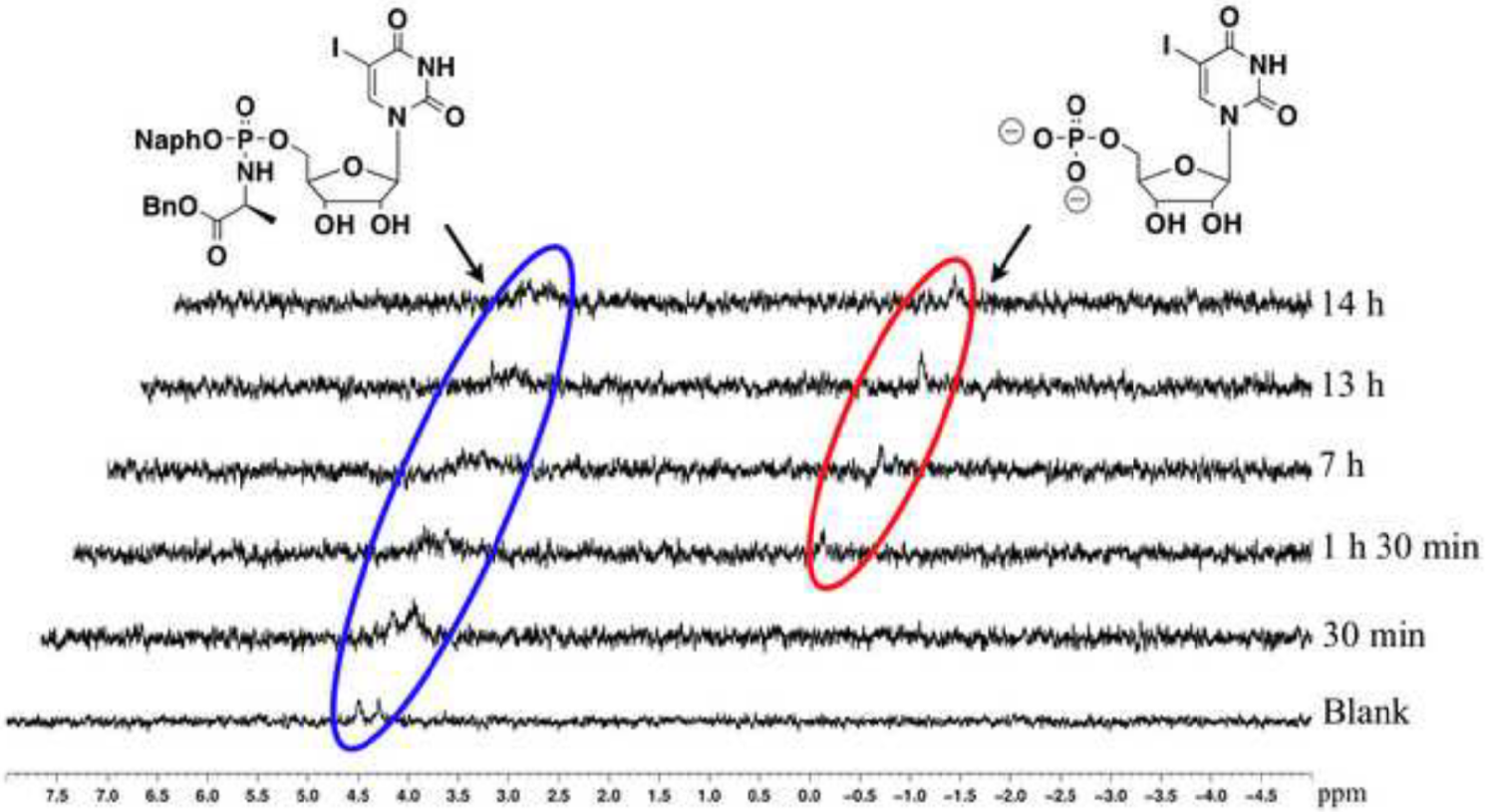
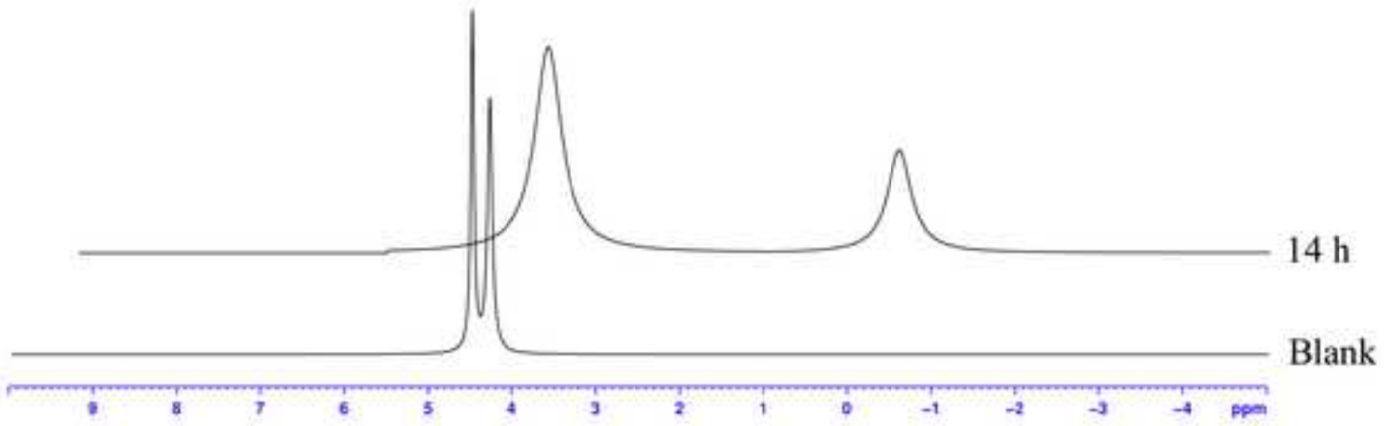


Figure 3



> Uridine 5'-triphosphate derivatives can inhibit influenza RNA polymerase.

> Application of ProTide approach to uridine-based nucleoside analogues is reported.

> Two ProTides derived from 2'-fluoro-2'-deoxyuridine show moderate antiviral activity whilst parent nucleoside is inactive.

> Slow processing of ProTide is causing relatively low antiviral activity.