Bioorganic & Medicinal Chemistry 19 (2011) 4338–4345

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design, synthesis and biological evaluation of 2′-deoxy-2′,2′-difluoro-5-halouridine phosphoramidate ProTides

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ARTICLE INFO

Article history:
Received 15 February 2011
Revised 13 May 2011
Accepted 19 May 2011
Available online 26 May 2011

Keywords:
Antiviral
Herpes virus
Feline herpes
Nucleoside
Nucleotide
ProTide
Phosphoramidate
Pro-drug

ABSTRACT

We report the synthesis of a series of novel 2′-deoxy-2′,2′-difluoro-5-halouridines and their corresponding phosphoramidate ProTides. All compounds were evaluated for antiviral activity and for cellular toxicity. Interestingly, 2′-deoxy-2′,2′-difluoro-5-iodo- and -5-bromo-uridines showed selective activity against feline herpes virus replication in cell culture due to a specific recognition (activation) by the virus-encoded thymidine kinase.

1. Introduction

Modified nucleosides containing fluorine in the sugar moiety have attracted attention as antiviral and anticancer agents with therapeutic utility.[1–8] Substitution of fluorine for hydrogen has been associated with an enhancement of biological activity and increase of chemical and metabolic stability. Important factors in fluorine substitution are the comparable size of hydrogen and fluorine atoms, the strong electron withdrawing properties of fluorine relative to hydrogen, and the increased stability of the carbon–fluorine bond compared to the carbon hydrogen bond. Due to their similar van der Waals radius, replacement of hydrogen by fluorine does not greatly affect the steric properties of a fluorine-containing molecule.[9] Among fluorinated nucleosides, 2′-deoxy-2′,2′-difluoro-D-nucleosides have shown antiviral and antineoplastic activities.[10,11] Gemcitabine[12–14] (2′-deoxy-2′,2′-difluorocytidine, Gemzar, Fig. 1) is a well-known drug widely used to treat ovarian, pancreatic, and breast cancers.[15–17] Gemcitabine phosphates interfere directly with DNA replication and can inhibit ribonucleotide reductase.[18,19] Chu and co-workers have reported the synthesis and anti-HIV activities of some enantiomeric 2′-deoxy-2′,2′-difluoro-β-L-*erythro*-pentofuranosyl nucleosides.[20] None of the compounds showed any significant activities against HIV-1, HBV, HSV-1, HSV-2, and lack of appreciable toxicity in Vero, CEM, and PBM cells up to 100 μM, except for the adenine derivative which showed a moderate activity against HIV-1 in PBM cells (3.4 μM). Recently, we have described the synthesis of 2′-deoxy-2′,2′-difluoro-D-5-iodouridine as an intermediate for a 2′,2′-difluoro derivative of FV-100, the most potent anti-varicella zoster (VZV) virus agent reported to date.[21,22]

As a part of our program to develop biologically active nucleosides, here we describe the synthesis of two other novel 2′-deoxy-2′,2′-difluoro-D-5-halo-uridines, containing bromine and chlorine, in order to investigate the influence of the halogen at

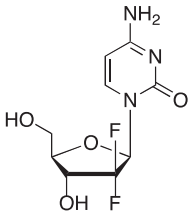

Figure 1.

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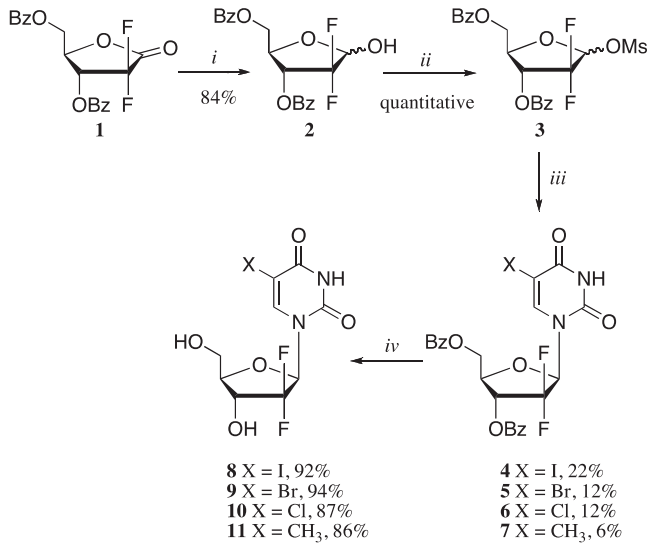
doi:10.1016/j.bmc.2011.05.037

the 5-position on the biological activity of these compounds. In addition, we report the synthesis of 2′-deoxy-2′,2′-difluoro-D-5-methyluridine for a comparison study. Also, since uracil-based nucleoside analogues are often poorly phosphorylated to their active phosphate species, we present the synthesis and biological study of a series of 2′,2′-fluoro-5-halouridine phosphoramidate ProTides,[23] to bypass the nucleoside kinase-mediated first phosphorylation step.

2. Results and discussion

2.1. Chemistry

2′-Deoxy-2′,2′-difluoro-5-bromo-, 5-chloro- and 5-methyl-uridines **9–11** were prepared according to the procedure used to synthesise the 5-iodo derivative **8**,[22] starting from commercially available lactone **1** (Scheme 1). The coupling of intermediate **3** with the silylated bases gave a mixture of the α and β anomers. The desired β anomer was obtained by precipitation from dichloroethane and the corresponding solid formed was filtered and washed with methanol to remove the trace of the α anomer. The anomeric configuration was assigned by 2D NOESY experiments. The final deprotection of **4–7** using sodium methoxide in methanol gave compounds **8–11**.

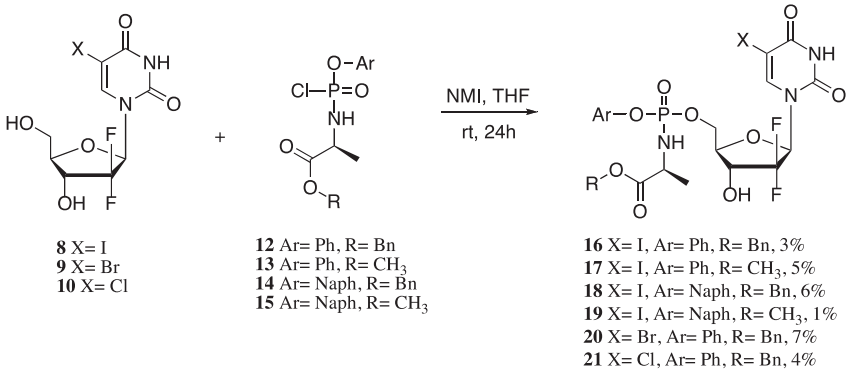


Scheme 1. Reagents and conditions: (i) LiAl(*t*-BuO)$_3$H, THF/Et$_2$O, −78 °C, 1 h; (ii) mesyl chloride, anhydrous TEA, anhydrous DCM, 0 °C to rt, 18 h; (iii) silylated base, TMSOTf, DCE, 90–100 °C, 12 h; (iv) MeONa, anhydrous MeOH, rt, overnight.

Phosphoramidates of the 2′,2′-difluoro-5-halouridines were synthesised according to the previously reported synthetic routes developed by McGuigan et al. (Scheme 2).[23–26] Aryl phosphorochloridates were prepared by the reaction of phenyl or 1-naphthyl dichlorophosphate with the appropriate L-alanine ester hydrochlorides and tosylate salts. The obtained phosphorochloridates **12–15** were allowed to react with **8–10** in THF and 1-methylimidazole (NMI) to give the target phosphoramidates **16–21**. The free 3′-hydroxy group led to the formation of side-products, including 3′ phosphoramidate, which required repeated purification and was reflected by poor yields. ^{31}P NMR investigations of the phosphoramidates displayed two closely spaced signals, corresponding to two diastereoisomers resulting from mixed phosphate stereochemistry.

2.2. Biological assay

The 5-substituted 2′-deoxy-2′,2′-difluorouridine nucleosides **8–11** and the corresponding ProTides **16–21** have been evaluated for their potential inhibitory action against a wide variety of DNA and RNA viruses in cell culture. The compounds did not show appreciable antiviral activity against a broad panel of RNA viruses. However, anti-DNA virus activity, in particular, anti-herpes virus activity was observed for several compounds, in particular the nucleoside derivatives **8** to **11** (Tables 1 and 2). Whereas no activity was observed for human cytomegalovirus (HCMV), notable inhibitory activity was recorded for herpes simplex virus type 1 (HSV-1) (EC_{50}: 12–33 μM), varicella-zoster virus (VZV) (EC_{50}: 5.8–50 μM) and in particular feline herpes virus (FHV) (EC_{50}: 1.1 and 2.4 μM for **8** and **9**, and 13 and 28 μM for **10** and **11**). The preferential antiviral activity against herpes (HSV-1 and VZV) viruses and the decreased antiviral activity against HSV-2 and the TK-deficient HSV and VZV strains point to the virus-encoded thymidine kinase as the activating enzyme to afford eventual antiviral activity (Table 1). When evaluated for their inhibitory activity against [^{3}H]dThd phosphorylation by purified recombinant HSV-1, VZV and FHV TK, compounds **8** to **11** were most inhibitory to HSV-1 TK-catalysed dThd phosphorylation, followed by VZV TK and FHV TK (Table 3). Thus, the compounds are endowed with a higher affinity for HSV-1 TK (and also VZV TK) than FHV TK. However, they were markedly more inhibitory to FHV than to HSV-1 and VZV replication in cell culture. This apparent contradiction may point to a preferential and better inhibition of the presumed antiviral target enzyme, DNA polymerase derived from FHV than the DNA polymerase derived from HSV-1 or VZV. Indeed, whereas virus-encoded TK acts as the activating (phosphorylating) enzyme, the DNA polymerase could be considered as the most likely target for the compounds after metabolic activation to their 5′-triphosphate derivatives. The lower affinity for FHV TK than for HSV-1 and



Scheme 2.

Table 1
Cytotoxicity and anti-herpes virus activity of compounds in HEL and CRFK cell cultures

Compound	EC_{50}[a] (μM)					Minimum cytotoxic concentration[b] (MCC)(μM)	CC_{50}[c] (μM)
	Herpes simplex virus-1 (KOS) (HEL)	Herpes simplex virus-1 TK^- KOS ACV^r (HEL)	Feline herpes virus (CRFK)	Herpes simplex virus-2 (G) (HEL)	Vaccinia virus (HEL)	(HEL)	(CRFK)
8	16 ± 6.9	20	1.2 ± 0.4	64 ± 33.4	>100	>100	>100
9	15 ± 7.8	73 ± 38.9	2.4 ± 1.1	>100	>100	>100	>100
10	33 ± 17.7	78 ± 29.7	13 ± 10	⩾100	>100	>100	>100
11	12 ± 0	20	>100	39 ± 7.8	⩾100	>100	>100
16	27 ± 9.9	⩾100	4.4 ± 3.6	50 ± 0	58 ± 0	>100	⩾70
17	>100	>100	28 ± 4.5	>100	>100	>100	>100
18	>20	>20	>20	>20	>20	>100	36
19	>100	>100	>20	>100	>100	>100	92
20	>100	>100	11.6 ± 0.7	>100	>100	>100	>100
21	>100	>100	>100	>100	>100	>100	>100
Brivudin	0.08	250	30	250	10	>250	>100
Cidofovir	2	2	1.3	2	10	>250	>100
Acyclovir	0.4	50	1.0	0.4	>250	>250	>100
Ganciclovir	0.03	0.8	—	0.03	>100	>100	—

CRFK cells: Crandell-Rees Feline Kidney cells.
HEL cells: human embryonic lung fibroblast cells.
[a] Compound concentration affording 50% inhibition of the virus-induced cytopathic effect.
[b] Minimum cytotoxic concentration required to cause a microscopically detectable alteration of normal cell morphology.
[c] 50% Cytotoxic concentration, or compound concentration required to reduce cell viability as determined with the colorimetric formazan-based MTS assay.

Table 2
Activity of compounds against varicella-zoster virus (VZV) and cytomegalovirus (HCMV) in human embryonic lung (HEL) cells

Compound	EC_{50}[a] (μM)						Minimum cytotoxic concentration[b] (μM)	CC_{50}[c] (μM)
	TK^+ VZV strains		TK^- VZV strains		HCMV strains			
	YS	OKA	07-1	YS/R	AD-169	Davis	(HEL)	(HEL)
8	13 ± 1	9.1 ± 0.8	>50	>50	>50	>50	>50	>100
9	23	11 ± 4	>50	>50	>50	>50	>50	>100
10	>50	⩾50	>50	>50	>50	>50	>50	>100
11	10	5.8 ± 3.4	>50	>50	>50	>50	>50	100
17	—	>50	>50	—	>50	>50	>50	51
18	—	8.5 ± 3.5	37 ± 7	—	>50	>50	>50	49
19	29 ± 3	25 ± 9	>50	>50	>50	>50	>50	⩾100
20	—	39	>50	—	>50	>50	>50	⩾50
21	—	>50	>50	—	>50	>50	>50	⩾100
Acyclovir	4.9	1.4	43	111	—	—	>400	>400
Brivudin	0.011	0.030	⩾60	>150	—	—	⩾300	512

[a] Effective concentration required to reduce virus plaque formation by 50%. Virus input was 20 plaque forming units (PFU) for VZV or 100 PFU for HCMV.
[b] Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
[c] 50% Cytostatic concentration or compound concentration required to reduce cell growth by 50%.

VZV TK but higher activity for FHV in cell culture than observed for HSV-1 and VZV in cell culture, is strongly suggestive for a superior affinity of the 5′-triphosphate derivatives for FHV DNA polymerase than HSV-1/VZV DNA polymerase to explain the data. Alternatively, although somewhat less likely, the compounds may far better be phosphorylated in the FHV-infected feline cells than the HSV-1/VZV-infected human cells leading to a substantial accumulation of the 5′-triphosphate metabolite in the feline but not the human cells. Direct affinity measurements of the 5′-triphosphates with the herpes DNA polymerases and/or metabolic experiments with the test compounds in feline versus human cells should reveal which assumption is correct.

No antiviral activity was found for the corresponding ProTides. The observation that the phosphoramidate prodrugs of **8, 9** and **10** were devoid of marked antiviral activity is suggestive of a very limited release of the 5′-monophosphate and/or the parent derivative from the prodrugs in cell culture (Tables 1 and 2). Kinetic experiments of drug release in the intact virus-infected cells should reveal this issue.

The 2′,2′-difluoro 5-substituted uridine nucleoside derivatives **8–11** and the corresponding ProTides **16–21** have also been evaluated for their cytostatic activity against a selection of tumor cell lines (L1210, FM3A, CEM and HeLa) and found to be virtually non-toxic with the exception of 8 that proved cytostatic in the middle micromolar range against several cell lines (Table 4).

In conclusion, several 5-substituted 2′-deoxy-2′,2′-difluorouridine derivatives have been identified as specific and selective inhibitors of herpes virus replication in cell culture, in particular against feline herpes virus. They are herpes virus TK-dependent for antiviral activity. It would be of particular interest to further investigate the prototype compounds for antiviral activity in cats that suffer from an FHV infection in the eye.

3. Conclusions

A series of 2′-deoxy-2′,2′-difluoro-5-halouridines and their corresponding phosphoramidates were synthesized and investigated for antiviral activity and for cytotoxicity. 2′-Deoxy-2′,2′-difluoro-5-iodo- and 5-bromo-uridines showed significant activity against feline herpes virus, while none of the ProTides were endowed with antiviral activity. No appreciable antiproliferative activity against any of the cancer cell lines evaluated was found. Our findings point

Table 3
Inhibitory activity of compounds **8** to **11** against thymidine kinase-catalysed phosphorylation of 1 μM [^{3}H]dThd

Compound	IC_{50}[a] (μM)		
	HSV-1 TK	VZV TK	FHV TK
8	3.5 ± 0.0	19 ± 1	38 ± 6
9	7.5 ± 5.3	26 ± 2	34 ± 10
10	22 ± 5	35 ± 3	93 ± 63
11	2.8 ± 0.0	21 ± 2	85 ± 65

[a] 50% Inhibitory concentration or compound concentration required to inhibit phosphorylation of 1 μM dThd by 50%.

Table 4
Inhibitory effects of compounds on the proliferation of murine leukemia (L1210), murine mammary carcinoma (FM3A), human T-lymphocyte (CEM) and human cervix carcinoma (HeLa) cells

Compound	IC_{50}[a] (μM)			
	L1210	FM3A	CEM	HeLa
8	302 ± 22	409 ± 129	154 ± 42	230 ± 16
9	>500	>500	>500	458 ± 59
10	>500	>500	>500	⩾500
11	>500	>500	>500	>500
17	226 ± 18	258 ± 2	176 ± 20	126 ± 24
18	23 ± 1	nd	21 ± 3	29 ± 12
19	134 ± 23	46 ± 13	60 ± 21	133 ± 37
20	50 ± 32	91 ± 18	104 ± 47	148 ± 28
21	175 ± 11	226 ± 11	196 ± 44	200 ± 25

[a] 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%.

to the 5-iodo- and 5-bromo- derivatives of 2′-deoxy-2′,2′-difluorouridine as selective anti-FHV compounds that should be further explored in vivo.

4. Experimental methods

4.1. Chemistry

Anhydrous solvents were purchased from Aldrich and used without further purification. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on Silica Gel 60-F254 precoated aluminium plates and visualized using UV lamp (254 nm) and/or with ^{31}P NMR spectra. Column chromatography was performed on silica gel (35–70 μM). Proton (^{1}H), carbon (^{13}C), and phosphorus (^{31}P) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak, and all ^{13}C NMR, ^{31}P NMR were proton-decoupled. Analytical and semipreparative HPLC were conducted by Varian Prostar (LC Workstation-Varian prostar 335 LC detector) using Varian Polaris C18-A (10 μM) as an analytic column and Varian Polaris C18-A (10 μM) as a semipreparative column; elution was performed using a mobile phase consisting of water/methanol in gradient. Low and high resolution mass spectra were performed as a service by Cardiff University, using electrospray (ES). Compound purity was assured by a combination of high field multinuclear NMR (H, C, P) and HPLC. Purity by the latter was always >95% for all final products.

4.1.1. Standard procedure A: synthesis of phosphorochloridates

Anhydrous TEA (2.00 mol/equiv) was added dropwise to a stirred solution of the appropriate aryl dichlorophosphate (1.00 mol/equiv) and the appropriate amino acid ester salt (1.00 mol/equiv) in anhydrous DCM at −78 °C. Following the addition, the reaction mixture was stirred at −78 °C for 30 min, then at room temperature for 2–3.5 h. Formation of the desired compound was monitored by ^{31}P NMR. After this period the solvent was removed under reduced pressure and the residue triturated with anhydrous diethyl ether. The precipitate was filtered under nitrogen and the solution was concentrated to give an oil. Most of the aryl phosphorochloridates synthesised were purified by flash column chromatography (eluting with ethyl acetate/petroleum ether 6:4).

4.1.2. Standard procedure B: synthesis of phosphoramidates

Anhydrous 1-methylimidazole (NMI, 5 mol/equiv) was added dropwise to a stirring solution of the appropriate nucleoside (1.00 mol/equiv) and the appropriate phosphorochloridate (3.00 mol/equiv) in anhydrous THF (10 mL) and the reaction was stirred at room temperature for 24 h. Upon the removal of the solvent, the crude was purified by column chromatography (DCM/MeOH 98:2) and then preparative HPLC to give the desired product as a white solid.

4.1.3. 2-Deoxy-D-*erythro*-2,2-difluoro-ribofuranose-3,5-dibenzoate (2)

A solution of 2-deoxy-D-*erythro*-pentafuranos-1-ulose-3,5-dibenzoate **1** (5.00 g, 0.013 mmol) in anhydrous THF (40 mL) and anhydrous diethyl ether (10 mL) was cooled to −78 °C and lithium tri(*tert*-butoxy)aluminium hydride (14.58 mL, 1.0 M in THF) was added dropwise. The reaction mixture was stirred for 1 h at −78 °C and was quenched by the slow addition of methanol (3.2 mL). The reaction mixture was allowed to warm to rt and then ethyl acetate (162 mL) was added. The organic phase was washed with equal volumes of saturated $NaHCO_3$ solution and brine, and the organic layer was dried on Na_2SO_4 and concentrated to give a thick oil as a mixture of anomers (4.14 g, 84%). ^{19}F NMR ($CDCl_3$, 471 MHz): δ −108.97, −109.50, −123.19, −123.70, −124.93, −125.46. ^{1}H NMR of major (∼55%) anomer ($CDCl_3$, 500 MHz): δ 8.14–7.99 (4H, m, Bz), 7.66–7.55 (m, 2H, Bz), 7.51–7.39 (m, 4H, Bz), 5.81–5.73 (m, 1H, H-3), 5.52–5.49 (m, 1H, H-1), 4.82–4.58 (m, 3H, H-4, H-5), 3.66 (s, 1H, OH). ^{1}H NMR of minor (∼45%) anomer ($CDCl_3$, 500 MHz): δ 8.14–7.99 (m, 4H, Bz), 7.66–7.55 (m, 2H, Bz), 7.51–7.39 (m, 4H, Bz), 5.55–5.49 (m, 1H, H-3), 5.40–5.35 (m, 1H, H-1), 4.77–4.60 (m, 2H, H-5), 4.51–4.45 (m, 1H, H-4), 3.93 (s, 1H, OH). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 63.46, 64.57 (C-5), 71.52 (dd, $J_{1C\text{-}F}$ = 16.4 Hz, $J_{2C\text{-}F}$ = 28.4 Hz, C-3), 72.15 (dd, $J_{1C\text{-}F}$ = 17.9 Hz, $J_{2C\text{-}F}$ = 36.3 Hz, C-3), 76.82, 79.25 (C-4), 95.84 (dd, $J_{1C\text{-}F}$ = 23.7 Hz, $J_{2C\text{-}F}$ = 37.0 Hz, C-1), 96.10 (dd, $J_{1C\text{-}F}$ = 23.2 Hz, $J_{2C\text{-}F}$ = 42.1 Hz, C-1), 121.29 (dd, $J_{1C\text{-}F}$ = 254.4 Hz, $J_{2C\text{-}F}$ = 263.9 Hz, C-2), 121.77 (dd, $J_{1C\text{-}F}$ = 248.4 Hz, $J_{2C\text{-}F}$ = 272.4 Hz, C-2), 127.15, 127.63, 128.45, 128.52, 128.62, 128.66 (Ph), 129.38 ('*ipso*' Ph), 129.80, 130.07, 130.11, 133.37, 133.43, 133.91, 134.00 (Ph), 165.31, 165.59, 166.59, 166.65 (C═O).

4.1.4. 2-Deoxy-D-*erythro*-2,2-difluoro-ribofuranose-3,5-dibenzoate-1-methanesulfonate (3)

A solution of **2** (4.14 g, 10.9 mmol) in anhydrous DCM (52 mL) and anhydrous TEA (2.4 mL) was cooled to 0 °C and methane sulfonylchloride (1.23 mL, 15.8 mmol) was added dropwise. The reaction was stirred at rt for 18 h. The mixture was partitioned between DCM (140 mL) and a saturated solution of $NaHCO_3$ (56 mL). The organic phase was dried over Na_2SO_4 and concentrated to give an oil as a mixture of anomers (5.03 g, quantitative). ^{19}F NMR ($CDCl_3$, 471 MHz): δ −107.70, −108.22, −120.65, −121.17, −122.21, −122.73, −123.76, −124.45. ^{1}H NMR of major (∼60%) anomer ($CDCl_3$, 500 MHz): δ 8.13–8.04 (m, 4H, Bz), 7.65–7.54 (m, 2H, Bz), 7.50–7.41 (m, 4H, Bz), 6.17 (d, J = 5.6 Hz, 1H, H-1), 5.62 (dd, J_1 = 4.2 Hz, J_2 = 16.4 Hz, 1H, H-3), 4.91 (q, J = 3.9 Hz, 1H, H-4), 4.81–4.61 (m, 2H, H-5), 3.17 (s, 3H, CH_3). ^{1}H NMR of minor (∼40%) anomer ($CDCl_3$, 500 MHz): δ 8.13–8.04 (4H, m, Bz), 7.65–7.54 (m, 2H, Bz), 7.50–7.41 (m, 4H, Bz), 6.09 (d, J = 6.4 Hz, 1H, H-1), 5.98 (dt, J_1 = 7.3 Hz, J_2 = 15.0 Hz, 1H, H-3), 4.81–4.61 (m, 3H,

H-4, H-5), 3.03 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 40.09, 40.20 (CH_3), 62.52, 63.08 (C-5), 69.61 (dd, $J_{1C\text{-}F}$ = 15.7 Hz, $J_{2C\text{-}F}$ = 26.0 Hz, C-3), 71.04 (dd, $J_{1C\text{-}F}$ = 17.4 Hz, $J_{2C\text{-}F}$ = 36.4 Hz, C-3), 79.68, 79.75, 82.59 (C-4), 98.81 (dd, $J_{1C\text{-}F}$ = 25.0 Hz, $J_{2C\text{-}F}$ = 41.8 Hz, C-1), 99.52 (dd, $J_{1C\text{-}F}$ = 24.5 Hz, $J_{2C\text{-}F}$ = 46.3 Hz, C-1), 120.61 (dd, $J_{1C\text{-}F}$ = 253.5 Hz, $J_{2C\text{-}F}$ = 269.8 Hz, C-2), 120.91 (dd, $J_{1C\text{-}F}$ = 249.3 Hz, $J_{2C\text{-}F}$ = 276.3 Hz, C-2), 128.42, 128.58, 128.63, 128.70, 128.76, 128.79 (Ph), 129.18, 129.25 ('*ipso*' Ph), 129.76, 130.07, 130.14, 133.51, 133.63, 134.19, 134.26 (Ph), 164.89, 165.03, 165.81, 165.90 (C=O).

4.1.5. 1-(3,5-Di-*O*-benzoyl-2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-5-iodouracil (4)

5-Iodouracil (3.61 g, 15.2 mmol) was treated with an excess of hexamethyldisilazane (100 mL) in the presence of $(NH_4)_2SO_4$ (0.10 g, 0.76 mmol) and refluxed at 125–130 °C for 4 h. Excess solvent was evaporated under reduced pressure, and the resulting syrup was dissolved in anhydrous dichloroethane (57 mL). A solution of **3** (3.46 g, 7.59 mmol) in anhydrous dichloroethane (86 mL) was added, and the mixture was stirred for 10 min. Trimethylsilyl trifluoromethanesulfonate (2.95 mL, 16.30 mmol) was added to the mixture slowly while stirring, and the reaction was refluxed at 90–100 °C for 10 h. The reaction mixture was cooled to rt and washed with equal volumes of saturated solution of $NaHCO_3$ and brine. The β-anomer was obtained by precipitation from the organic solvent and washed with methanol to remove traces of α-anomer as a white solid (1.00 g, 22%). ^{19}F NMR (DMSO, 471 MHz): δ −111.37, −111.90, −114.17 (broad). ^1H NMR (DMSO, 500 MHz): δ 12.03 (s, 1H, NH), 8.17 (s, 1H, H-6), 8.08–7.94 (m, 4H, Bz), 7.77–7.63 (m, 2H, Bz), 7.61–7.46 (m, 4H, Bz), 6.36 (t, J = 9.1 Hz, 1H, H-1′), 5.93–5.84 (m, 1H, H-3′), 4.83–4.73 (m, 3H, H-4', H-5′). ^{13}C NMR (DMSO, 126 MHz): δ 63.13 (C-5′), 71.10 (C–I), 71.19, 71.37, 71.56 (C-3′), 75.79 (C-4′), 84.04 (C-1′), 121.50 (t, $J_{C\text{-}F}$ = 258.2 Hz, CF_2), 127.91 ('*ipso*' Ph), 128.79, 128.92 (Ph), 128.97 ('*ipso*' Ph), 129.19, 129.66, 133.61, 135.25 (Ph), 144.73 (C-6), 149.96 (C-2), 160.35 (C-4), 164.34, 165.37 (C=O). MS (ES+) *m/z*: 598.01 (M). HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 28.63 min.

4.1.6. 1-(3,5-Di-*O*-benzoyl-2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-5-bromouracil (5)

5-Bromouracil (4.82 g, 25.2 mmol) was condensed with **3** (5.76 g, 12.6 mmol) as described above to obtain β-anomer as a white solid (0.82 g, 12%). ^{19}F NMR (DMSO, 471 MHz): δ −111.26, −111.77, −114.49 (broad). ^1H NMR (DMSO, 500 MHz): δ 12.16 (s, 1H, NH), 8.20 (s, 1H, H-6), 8.08–7.93 (m, 4H, Bz), 7.77–7.62 (m, 2H, Bz), 7.61–7.45 (m, 4H, Bz), 6.38 (t, J = 8.9 Hz, 1H, H-1′), 5.93–5.85 (m, 1H, H-3′), 4.83–4.72 (m, 3H, H-4′, H-5′). ^{13}C NMR (DMSO, 126 MHz): δ 63.19 (C-5′), 71.20, 71.37, 71.58 (C-3′), 75.74 (C-4′), 84.46 (C-1′), 97.15 (C-Br), 121.49 (t, $J_{C\text{-}F}$ = 261.6 Hz, CF_2), 127.91 ('*ipso*' Ph), 128.75, 128.92 (Ph), 128.98 ('*ipso*' Ph), 129.27, 129.66, 133.60, 134.25 (Ph), 140.19 (C6), 149.56 (C-2), 158.89 (C-4), 164.32, 165.36 (C=O). MS (ES+) *m/z*: 550.02 (M). HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 28.44 min.

4.1.7. 1-(3,5-Di-*O*-benzoyl-2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-5-chlorouracil (6)

5-Chlorouracil (3.51 g, 0.024 mol) was condensed with **3** (5.53 g, 0.012 mol) as described above to obtain the β-anomer as a white solid (0.68 g, 12%). ^{19}F NMR (DMSO, 471 MHz): δ −111.20, −111.74, −113.72 (broad). ^1H NMR (DMSO, 500 MHz): δ 12.20 (s, 1H, NH), 8.14 (s, 1H, H-6), 8.08–7.93 (m, 4H, Bz), 7.77–7.62 (m, 2H, Bz), 7.62–7.44 (m, 4H, Bz), 6.38 (t, J = 8.8 Hz, 1H, H-1′), 5.94–5.84 (m, 1H, H-3′), 4.85–4.71 (m, 3H, H-4′, H-5′). ^{13}C NMR (DMSO, 126 MHz): δ 63.19 (C-5′), 71.20, 71.37, 71.58 (C-3′), 75.74 (C-4′), 84.19 (C-1′), 108.51 (C–Cl), 121.49 (t, $J_{C\text{-}F}$ = 262.5 Hz, CF_2), 127.88 ('*ipso*' Ph), 128.73, 128.92 (Ph), 128.97 ('*ipso*' Ph), 129.18, 129.27, 129.65, 133.61, 134.26 (Ph), 137.79 (C-6), 149.35 (C-2), 158.76 (C-4), 164.33, 165.37 (C=O). MS (ES+) *m/z*: 506.07 (M). HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 28.35 min.

4.1.8. 1-(3,5-Di-*O*-benzoyl-2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-thymine (7)

Thymine (0.83 g, 6.57 mmol) was condensed with **3** (1.00 g, 2.19 mmol) as described above to obtain the β-anomer as a white solid (0.21 g, 6%). ^{19}F NMR (DMSO, 471 MHz): δ −111.75, −112.26, −114.59 (broad). ^1H NMR (DMSO, 500 MHz): δ 11.65 (s, 1H, NH), 8.09–7.95 (m, 4H, Bz), 7.77–7.64 (m, 2H, Bz), 7.61–7.47 (m, 4H, Bz), 7.53 (s, 1H, H-6), 6.38 (t, J = 9.0 Hz, 1H, H-1′), 5.91–5.80 (m, 1H, H-3′), 4.85–4.68 (m, 3H, H-4′, H-5′), 1.71 (s, 3H, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 11.81 (CH_3), 62.97 (C-5′), 71.22, 71.40, 71.60 (C-3′), 75.65 (C-4′), 83.75 (C-1′), 110.26 (*C*–CH_3), 121.60 (t, $J_{C\text{-}F}$ = 263.2 Hz, CF_2), 127.81 ('*ipso*' Ph), 128.79, 128.94, 129.17, 129.64, 133.70, 134.31 (Ph), 136.02 (C-6), 150.15 (C-2), 163.47 (C-4), 164.37, 165.40 (C=O). MS (ES+) *m/z*: 487.14 (M+H), 509.12 (M+Na), 525.09 (M+K). HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 28.15 min.

4.1.9. 1-(2-Deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-5-iodouracil (8)

Sodium methoxide (0.25 g, 4.67 mmol) was added to a stirring solution of **4** (0.93 g, 1.56 mmol) in anhydrous methanol (30 mL), and the reaction mixture was stirred at rt overnight. The reaction was neutralised with amberlite, filtered and concentrated. The residue was purified by flash column chromatography ($CHCl_3$/MeOH 80:10) and obtained as a white solid (0.56 g, 92%). ^{19}F NMR (DMSO, 471 MHz): δ −117.16. ^1H NMR (DMSO, 500 MHz): δ 11.89 (s, 1H, NH), 8.37 (s, 1H, H-6), 6.30 (d, J = 6.5 Hz, 1H, 3′-OH), 6.02 (t, J = 6.8 Hz, 1H, H-1′), 5.45 (m, 1H, 5′-OH), 4.29–4.17 (m, 1H, H-3′), 3.91–3.84 (m, 1H, H-4′), 3.84–3.60 (m, 2H, H-5′). ^{13}C NMR (DMSO, 126 MHz): δ 58.30 (C-5′), 67.76 (t, $J_{C\text{-}F}$ = 21.8 Hz, C-3′), 70.22 (C–I), 80.92 (C-4′), 83.27 (t, $J_{C\text{-}F}$ = 32.2 Hz, C-1′), 122.82 (t, $J_{C\text{-}F}$ = 258.2 Hz, CF_2), 143.70 (C-6), 149.87 (C-2), 160.20 (C-4). MS (ES+) *m/z*: 389.95 (M); Accurate Mass: $C_9H_9N_2O_5F_2I$ required 389.9524, found 389.9524. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 9.44 min.

4.1.10. 1-(2-Deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-5-bromouracil (9)

Compound **5** (0.5605 g, 1.02 mmol) was deprotected with sodium methoxide (0.0970 g, 1.79 mmol) in anhydrous methanol (46 mL) as described above. The residue was purified by flash column chromatography ($CHCl_3$/MeOH 80:10) and obtained as a white solid (0.3296 g, 94%). ^{19}F NMR (DMSO, 471 MHz): δ −117.17. ^1H NMR (DMSO, 500 MHz): δ 12.07 (s, 1H, NH), 8.36 (s, 1H, H-6), 6.33 (s, 1H, 3′-OH), 6.03 (t, J = 7.1 Hz, 1H, H-1′), 5.46 (s, 1H, 5′-OH), 4.30–4.18 (m, 1H, H-3′), 3.93–3.85 (m, 1H, H-4′), 3.85–3.60 (m, 2H, H-5′). ^{13}C NMR (DMSO, 126 MHz): δ 58.39 (C-5′), 67.79 (t, $J_{C\text{-}F}$ = 21.8 Hz, C-3′), 81.02 (t, $J_{C\text{-}F}$ = 4.5 Hz, C-4′), 83.40 (t, $J_{C\text{-}F}$ = 30.9 Hz, C-1′), 96.53 (C-Br), 122.78 (t, $J_{C\text{-}F}$ = 257.9 Hz, CF_2), 139.05 (C-6), 149.50 (C-2), 158.82 (C-4). MS (ES+) *m/z*: 341.97 (M); Accurate Mass: $C_9H_9N_2O_5BrF_2$ required 341.9663, found 341.9659. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 9.53 min.

4.1.11. 1-(2-Deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-5-chlorouracil (10)

Compound **6** (0.3020 g, 0.64 mmol) was deprotected with sodium methoxide (0.0606 g, 1.12 mmol) in anhydrous methanol (35 mL) as described above. The residue was purified by flash column chromatography ($CHCl_3$/MeOH 80:10) and obtained as a

white solid (0.1646 g, 87%). ^{19}F NMR (DMSO, 471 MHz): δ −117.14. ^{1}H NMR (DMSO, 500 MHz): δ 12.11 (s, 1H, NH), 8.29 (s, 1H, H-6), 6.32 (d, J = 5.7 Hz, 1H, 3′-OH), 6.04 (t, J = 7.1 Hz, 1H, H-1′), 5.45 (m, 1H, 5′-OH), 4.29–4.19 (m, 1H, H-3′), 3.90–3.85 (m, 1H, H-4′), 3.85–3.62 (m, 2H, H-5′). ^{13}C NMR (DMSO, 126 MHz): δ 58.47 (C-5′), 67.85 (t, $J_{C\text{-}F}$ = 21.8 Hz, C-3′), 81.04 (t, $J_{C\text{-}F}$ = 4.5 Hz, C-4′), 83.40 (t, $J_{C\text{-}F}$ = 31.8 Hz, C-1′), 107.97 (C–Cl), 122.79 (t, $J_{C\text{-}F}$ = 257.9 Hz, CF_2), 136.66 (C-6), 149.29 (C-2), 158.68 (C-4). MS (ES+) m/z: 298.02 (M); Accurate Mass: $C_9H_9N_2O_5ClF_2$ required 298.0168, found 298.0178. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 8.65 min.

4.1.12. 1-(2-Deoxy-2,2-difluoro-β-D-*erythro*-pentofuranos-1-yl)-thymine (11)

Compound **7** (0.1548 g, 0.32 mmol) was deprotected with sodium methoxide (0.0306 g, 0.57 mmol) in anhydrous methanol (15 mL) as described above. The residue was purified by flash column chromatography ($CHCl_3$/MeOH 80:10) and obtained as a white solid (0.0765 g, 86%). ^{19}F NMR (DMSO, 471 MHz): δ −116.60. ^{1}H NMR (DMSO, 500 MHz): δ 11.54 (s, 1H, NH), 7.66 (s, 1H, H-6), 6.29 (s, 1H, 3′-OH), 6.05 (t, J = 6.8 Hz, 1H, H-1′), 5.30 (s, 1H, 5′-OH), 4.30–4.15 (m, 1H, H-3′), 3.89–3.60 (m, 3H, H-4′, H-5′), 1.79 (s, 3H, CH_3). ^{13}C NMR (DMSO, 126 MHz): δ 12.11 (CH_3), 58.87 (C-5′), 68.42 (t, $J_{C\text{-}F}$ = 22.7 Hz, C-3′), 80.74 (C-4′), 82.96 (t, $J_{C\text{-}F}$ = 32.7 Hz, C-1′), 109.75 (*C*–CH_3), 122.96 (t, $J_{C\text{-}F}$ = 257.9 Hz, CF_2), 135.30 (C-6), 150.23 (C-2), 163.41 (C-4). MS (ES+) m/z: 278.07 (M); Accurate Mass: $C_{10}H_{12}N_2O_5F_2$ required 278.0714, found 278.0713. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 8.63 min.

4.1.13. Synthesis of phenyl-(benzoxy-L-alaninyl)-phosphorochloridate (12)

Prepared according to standard procedure A, using phenyldichlorophosphate (0.30 mL, 2.00 mmol), L-alanine benzyl ester tosylate (0.43 g, 2.00 mmol), anhydrous TEA (0.56 mL, 4.00 mmol) in anhydrous DCM (15 mL). The reaction mixture was stirred at −78 °C for 30 min, then at room temperature for 3.5 h. The crude product was obtained as an oil (0.62 g, 87%). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.86, 7.52. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.33–7.28 (m, 10H, PhO, OCH_2Ph), 5.15–5.13 (m, 2H, OCH_2Ph), 4.18–4.13 (m, 1H, C*H*NH), 1.46–1.44 (m, 3H, CH_3).

4.1.14. Synthesis of phenyl-(methoxy-L-alaninyl)-phosphorochloridate (13)

Prepared according to standard procedure A, from phenyldichlorophosphate (2.24 mL, 15.00 mmol), L-alanine methyl ester hydrochloride salt (2.09 g, 15.00 mmol), anhydrous TEA (4.20 mL, 30.00 mmol) and anhydrous DCM (80 mL). The reaction mixture was stirred at −78 °C for 30 min, then at room temperature for 2.5 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane 6:4 to give an oil (3.35 g, 81%). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.95, 7.66. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.32–7.15 (m, 5H, PhO), 4.42–4.34 (m, 1H, NH), 4.17–4.08 (m, H, C*H*NH), 3.72, 3.70 (2s, 3H, C*H*$_3$O), 1.45–1.43 (m, 3H, CHC*H*$_3$).

4.1.15. Synthesis of naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (14)

Prepared according to standard procedure A, from 1-naphthyl phosphorodichloridate (7.43 g, 28.46 mmol), L-alanine benzyl ester tosylate salt (10.00 g, 28.46 mmol), and anhydrous TEA (7.92 ml, 56.91 mmol) in anhydrous DCM (200 mL). The reaction mixture was stirred at −78 °C for 30 min, then at room temperature for 2.5 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane 6:4 to give an oil (9.80 g, 85%). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.16, 7.92. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.15–7.33 (m, 12H, Naph, Ph), 5.29–5.23 (m, 2H, OCH_2Ph), 4.60–4.48, 4.53–4.49 (2 m, 1H, NH), 4.41–4.34 (m, 1H, C*H*CH_3), 1.59, 1.57 (2d, J = 7.5 Hz, 3H, CHCH_3).

4.1.16. Synthesis of naphthyl-(methoxy-L-alaninyl)-phosphorochloridate (15)

Prepared according to standard procedure A, using 1-naphthylphosphodichloridate (4.68 g, 17.91 mmol), L-alanine methyl ester hydrochloride salt (2.50 g, 17.91 mmol), anhydrous triethylamine (3.62 g, 35.82 mmol, 4.99 mL) in anhydrous DCM (40 mL). The reaction mixture was stirred at −78 °C for 30 min, then at room temperature for 2.5 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane 6:4 to give an oil (5.00 g, 85%). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.19, 7.92. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.11–7.28 (m, 7H, NaphO), 4.53–4.41 (2 m, 1H, NH), 4.37–4.28 (m, 1H, CHNH), 3.83, 3.78 (2s, 3H, CH_3O), 1.59–1.55 (m, 3H, CH_3).

4.1.17. 2′-Deoxy-2′,2′-difluoro-D-5-iodouridine 5′-O-phenyl-(benzoxy-alaninyl)-phosphate (16)

Prepared according to standard procedure B, from **8** (0.1851 g, 0.47 mmol), **12** (0.5023 g, 1.42 mmol), anhydrous NMI (0.19 mL, 2.35 mmol) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 98:2). The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH from 90/0 to 0/100 in 30 min) to give a white solid (0.0109 g, 3%). ^{31}P NMR (MeOD, 202 MHz): δ 3.87, 3.64. ^{19}F NMR (MeOH, 471 MHz): δ −117.44 (d, J = 242.2 Hz), −117.50 (d, J = 240.4 Hz), −119.45 (broad), −120.00 (broad). ^{1}H NMR (MeOD, 500 MHz): δ 7. 94, 7.94 (2s, 1H, H-6), 7.40–7.17 (m, 10H, *Ph*O, OCH_2*Ph*), 6.17–6.07 (m, 1H, H-1′), 5.21–5.10 (m, 2H, O*CH*$_2$Ph), 4.49–4.19 (m, 3H, H-5′, H-3′), 4.12–4.02 (m, 2H, H-4′, CH-Ala), 1.42–1.35 (m, 3H, CH_3-Ala). ^{13}C NMR (MeOD, 126 MHz): δ 20.43 (d, $J_{C\text{-}P}$ = 7.6 Hz, CH_3-Ala), 20.56 (d, $J_{C\text{-}P}$ = 6.3 Hz, CH_3-Ala), 51.72, 51.82 (CH-Ala), 65.57 (d, $J_{C\text{-}P}$ = 5.0 Hz, C-5′), 65.97 (d, $J_{C\text{-}P}$ = 3.8 Hz, C-5′), 68.10, 68.11 (OCH_2Ph), 69.88, 69.91 (C–I), 70.88, 71.14, 71.26, 71.51 (C-3′), 80.89 (C-4′), 85.75 (C-1′), 121.53, 121.56, 121.60, 121.64 (Ph), 123.34 (t, $J_{C\text{-}F}$ = 260.3 Hz, CF_2), 126.30, 126.33, 129.30, 129.42, 129.63, 129.65, 130.86 (Ph), 137.16, 137.21 ('*ipso*' OCH_2*Ph*), 145.95, 146.11 (C-6), 151.54 ('*ipso*', Ph), 152.04, 152.10 (C-2), 162.20 (C-4), 174.65 (d, $J_{C\text{-}P}$ = 4.6 Hz, C═O ester), 174.80 (d, $J_{C\text{-}P}$ = 4.6 Hz, C═O ester). MS (ES+) m/z: 708.04 (M+H), 730.03 (M+Na), 746.01 (M+K); Accurate Mass: $C_{25}H_{26}N_3O_9F_2PI$ required 708.0420, found 708.0449. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 24.95, 25.17 min.

4.1.18. 2′-Deoxy-2′,2′-difluoro-D-5-iodouridine 5′-O-phenyl-(methoxy-alaninyl)-phosphate (17)

Prepared according to standard procedure B, from **8** (0.1542 g, 0.39 mmol), **13** (0.3292 g, 1.19 mmol), anhydrous NMI (0.16 mL, 1.98 mmol) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 98:2). The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH from 90/0 to 0/100 in 30 min) to give a white solid (0.0117 g, 5%). ^{31}P NMR (MeOD, 202 MHz): δ 3.86, 3.70. ^{19}F NMR (MeOH, 471 MHz): δ −117.27, −117.78, −119.45 (broad), −119.93 (broad). ^{1}H NMR (MeOD, 500 MHz): δ 8.00, 7.98 (2 s, 1H, H-6), 7.42–7.19 (m, 5H, Ph), 6.18–6.11 (m, 1H, H-1′), 4.58–4.24 (m, 3H, H-5′, H-3′), 4.16–4.08 (m, 1H, H-4′), 4.08–3.98 (m, 1H, CH-Ala), 3.71, 3.70 (2 s, 3H, OCH_3), 1.41–1.34 (m, 3H, CH_3-Ala). ^{13}C NMR (MeOD, 126 MHz): δ 20.37 (d, $J_{C\text{-}P}$ = 6.9 Hz, CH_3-Ala), 20.51 (d, $J_{C\text{-}P}$ = 6.4 Hz, CH_3-Ala), 51.59, 51.66 (CH-Ala), 52.83 (OCH_3), 65.56, 65.81 (C-5′), 69.74 (C–I), 70.90, 71.06, 71.28 (C-3′), 80.86 (C-4′), 85.87 (C-1′), 121.53, 121.56, 121.59 (Ph), 123.38 (t, $J_{C\text{-}F}$ = 259.2 Hz, CF_2), 126.28, 130.82 (Ph), 146.04, 146.15 (C-6), 151.62 ('*ipso*' Ph), 152.07, 152.13 (C-2), 162.34 (C-4), 175.30, 175.68 (C═O ester). MS (ES+) m/z: 573.99 (M+Na); Accurate Mass:

$C_{19}H_{22}N_3O_9F_2PI$ required 632.0107, found 632.0123. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 18.92, 18.93 min.

4.1.19. 2′-Deoxy-2′-fluoro-D-5-iodouridine 5′-O-naphthyl-(benzoxy-alaninyl)-phosphate (18)

Prepared according to standard procedure B, from **8** (0.1527 g, 0.39 mmol), **14** (0.4742 g, 1.17 mmol), anhydrous NMI (0.15 mL, 1.95 mmol) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 98:2). The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH from 90/0 to 0/100 in 30 min) to give a white solid (0.0171 g, 6%). ^{31}P NMR (MeOD, 202 MHz): δ 4.15, 4.01. ^{19}F NMR (MeOH, 471 MHz): δ −117.31 (d, J = 242.7 Hz), −117.44 (d, J = 242.1 Hz), −119.53 (broad). ^1H NMR (MeOD, 500 MHz): δ 8.21, 8.19 (2s, 1H, H-6), 7.98–7.26 (m, 12H, *Naph*O, OCH_2Ph), 6.14–6.03 (m, 1H, H-1′), 5.13–5.08 (m, 2H, OCH_2Ph), 4.55–4.36 (m, 2H, H-5′), 4.34–4.20 (m, 1H, H-3′), 4.18–4.05 (m, 2H, H-4′, CH-Ala), 1.40–1.32 (m, 3H, CH_3-Ala). ^{13}C NMR (MeOD, 126 MHz): δ 20.37 (d, $J_{C\text{-}P}$ = 7.3 Hz, CH_3-Ala), 20.47 (d, $J_{C\text{-}P}$ = 6.7 Hz, CH_3-Ala), 51.85, 51.87 (CH-Ala), 66.02 (d, $J_{C\text{-}P}$ = 4.8 Hz, C-5′), 66.19 (d, $J_{C\text{-}P}$ = 4.9 Hz, C-5′), 68.06 (OCH_2Ph), 69.81, 69.85 (C–I), 71.05, 71.23, 71.43, 71.61 (C-3′), 79.47 (C-4′), 80.91 (C-1′), 116.26, 116.28, 116.42, 116.45, 122.69, 122.87 (Ph, Naph), 123.30 (CF_2, t, $J_{C\text{-}F}$ = 259.3 Hz), 126.12, 126.53, 126.55, 127.51, 127.56, 127.81, 127.84, 127.89, 127.94, 128.89, 128.93, 129.28, 129.36, 129.59, 136.33, 136.35 (Ph, Naph), 137.12, 137.15 (‘*ipso*’ OCH_2Ph), 146.07, 146.22 (C-6), 147.90, 147.95 (‘*ipso*’ Naph), 151.51, 151.53 (C-2), 162.20 (C-4), 174.58 (d, $J_{C\text{-}P}$ = 4.8 Hz, C=O ester), 174.78 (d, $J_{C\text{-}P}$ = 4.4 Hz, C=O ester). MS (ES+) *m/z*: 780.04 (M+Na); Accurate Mass: $C_{29}H_{27}N_3O_9F_2NaPI$ required 780.0395, found 780.0416. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 26.68, 26.89 min.

4.1.20. 2′-Deoxy-2′-fluoro-D-5-iodouridine 5′-O-naphthyl-(methoxy-alaninyl)-phosphate (19)

Prepared according to standard procedure B, from **8** (0.2000 g, 0.51 mmol), **15** (0.5040 mg, 1.54 mmol), anhydrous NMI (0.61 mL, 7.70 mmol,) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 98:2). The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH from 90/0 to 0/100 in 30 min) to give a white solid (0.0044 g, 1%). ^{31}P NMR (MeOD, 202 MHz): δ 4.17, 4.08. ^{19}F NMR (MeOH, 471 MHz): δ −117.30 (d, J = 241.1 Hz), −117.44 (d, J = 237.5 Hz), −119.38 (broad). ^1H NMR (MeOD, 500 MHz): δ 8.22, 8.21 (2s, 1H, H-6), 8.03–7.42 (m, 7H, *Naph*O), 6.17–6.07 (m, 1H, H-1′), 4.61–4.41 (m, 2H, H-5′), 4.40–4.25 (m, 1H, H-3′), 4.16–4.02 (m, 2H, H-4′, CH-Ala), 3.64, 3.64 (2s, 3H, OCH_3), 1.38–1.30 (m, 3H, CH_3-Ala). ^{13}C NMR (MeOD, 126 MHz): δ 20.36 (d, $J_{C\text{-}P}$ = 7.2 Hz, CH_3-Ala), 20.48 (d, $J_{C\text{-}P}$ = 6.5 Hz, CH_3-Ala), 51.71, 51.80 (CH-Ala), 52.81 (OCH_3), 65.65, 65.95 (C-5′), 69.83 (C–I), 70.92, 71.06, 71.06 (C-3′), 80.85 (C-4′), 85.70 (C-1′), 116.24, 116.38, 122.59, 122.67, 123.42 (Naph), 123.33 (t, $J_{C\text{-}F}$ = 259.4 Hz, CF_2), 126.18, 126.58, 127.26, 127.56, 127.66, 127.82, 127.90, 127.93, 128.52, 128.96, 128.98, 136.20, 136.34 (Naph), 146.06, 146.18 (C-6), 147.90, 147.95 (‘*ipso*’ Naph), 152.02, 152.10 (C-2), 162.24 (C-4), 174.62, 175.78 (C=O ester). MS (ES+) *m/z*: 704.01 (M+Na); Accurate Mass: $C_{23}H_{23}N_3O_9F_2NaPI$ required 704.0082, found 704.0110. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 23.03, 23.27 min.

4.1.21. 2′-Deoxy-2′,2′-difluoro-D-5-bromouridine 5′-*O*-phenyl-(benzoxy-alaninyl)-phosphate (20)

Prepared according to standard procedure B, from **9** (0.1660 g, 0.48 mmol), **12** (0.5135 g, 1.45 mmol), anhydrous NMI (0.19 mL, 2.4 mmol) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 98:2). The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH from 90/0 to 0/100 in 30 min) to give a white solid (0.0214 g, 7%). ^{31}P NMR (MeOD, 202 MHz): δ 3.89, 3.69. ^{19}F NMR (MeOH, 471 MHz): δ −117.61 (d, J = 241.5 Hz), −117.73 (d, J = 243.1 Hz), −119.71 (broad), −120.21 (broad). ^1H NMR (MeOD, 500 MHz): δ 7.94, 7.90 (2s, 1H, H-6), 7.40–7.20 (m, 10H, *Ph*O, OCH_2Ph), 6.16–6.12 (m, 1H, H-1′), 5.19–5.13 (m, 2H, OCH_2Ph), 4.51–4.22 (m, 3H, H-5′, H-3′), 4.13–4.02 (m, 2H, H-4′, CH-Ala), 1.45–1.34 (m, 3H, CH_3-Ala). ^{13}C NMR (MeOD, 126 MHz): δ 20.31 (d, $J_{C\text{-}P}$ = 7.1 Hz, CH_3-Ala), 20.44 (d, $J_{C\text{-}P}$ = 6.5 Hz, CH_3-Ala), 51.70, 51.82 (CH-Ala), 65.54 (d, $J_{C\text{-}P}$ = 4.4 Hz, C-5′), 65.90 (d, $J_{C\text{-}P}$ = 4.3 Hz, C-5′), 68.05 (OCH_2Ph), 70.79, 70.96, 71.01, 71.06, 71.17, 71.22, 71.28, 71.44 (C-3′), 80.92 (C-4′), 85.58, 85.98 (C-1′), 98.19 (CBr), 121.47, 121.51, 121.56 (Ph), 123.33 (t, $J_{C\text{-}F}$ = 259.4 Hz, CF_2), 126.29, 126.31, 129.26, 129.38, 129.39, 129.62, 130.83 (Ph), 137.19, 137.23 (‘*ipso*’ OCH_2Ph), 140.91, 141.12 (C-6), 151.14 (‘*ipso*’ Ph), 152.04, 152.09 (C-2) 160.97 (C-4), 174.62 (d, $J_{C\text{-}P}$ = 4.9 Hz, C=O ester), 174.79 (d, $J_{C\text{-}P}$ = 4.4 Hz, C=O ester). MS (ES+) *m/z*: 682.04 (M+Na); Accurate Mass: $C_{25}H_{25}N_3O_9F_2NaPBr$ required 682.0378, found 682.0390. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 23.72, 24.21 min.

4.1.22. 2′-Deoxy-2′,2′-difluoro-D-5-chlorouridine 5′-*O*-phenyl-(benzoxy-alaninyl)-phosphate (21)

Prepared according standard procedure B, from **10** (0.1491 g, 0.50 mmol), **12** (0.5295 g, 1.50 mmol), anhydrous NMI (0.2 mL, 2.49 mmol) and anhydrous THF (10 mL). The crude was purified by column chromatography (DCM/MeOH 98:2). The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH from 90/0 to 0/100 in 30 min) to give a white solid (0.0118 g, 4%). ^{31}P NMR (MeOD, 202 MHz): δ 3.92, 3.73. ^{19}F NMR (MeOH, 471 MHz): δ −117.75 (d, J = 234.1 Hz), −117.92 (d, J = 239.3 Hz), −119.83 (broad), −120.34 (broad). ^1H NMR (MeOD, 500 MHz): δ 7.86, 7.83 (2s, 1H, H-6), 7.45–7.19 (m, 10H, *Ph*O, OCH_2Ph), 6.20–6.11 (m, 1H, H-1′), 5.23–5.11 (m, 2H, OCH_2Ph), 4.53–4.21 (m, 3H, H-5′, H-3′), 4.14–4.00 (m, 2H, H-4′, CH-Ala), 1.47–1.34 (m, 3H, CH_3-Ala). ^{13}C NMR (MeOD, 126 MHz): δ 20.30 (d, $J_{C\text{-}P}$ = 7.0 Hz, CH_3-Ala), 20.41 (d, $J_{C\text{-}P}$ = 6.5 Hz, CH_3-Ala), 51.70, 51.83 (CH-Ala), 65.54 (d, $J_{C\text{-}P}$ = 4.6 Hz, C-5′), 65.88 (d, $J_{C\text{-}P}$ = 4.8 Hz, C-5′), 68.04 (OCH_2Ph), 70.74, 70.89, 70.98, 71.02, 71.13, 71.40 (C-3′), 80.89 (C-4′), 85.51, 85.71 (C-1′), 110.36 (C–Cl), 121.45, 121.48, 121.52 (Ph), 123.33 (t, $J_{C\text{-}F}$ = 259.7 Hz, CF_2), 126.29, 129.25, 129.37, 129.61, 130.82 (Ph), 137.21, 137.23 (′*ipso*’ OCH_2Ph), 138.34, 138.56 (C-6), 151.00 (‘*ipso*’ Ph), 152.04, 152.09 (C-2), 160.99 (C-4), 174.61 (d, $J_{C\text{-}P}$ = 4.9 Hz, C=O ester), 174.80 (d, $J_{C\text{-}P}$ = 4.3 Hz, C=O ester). MS (ES+) *m/z*: 638.09 (M+Na); Accurate Mass: $C_{25}H_{25}N_3O_9F_2NaPCl$ required 638.0883, found 638.0898. HPLC (H_2O/MeOH from 90/10 to 0/100 in 30 min): t_R = 24.33, 24.72 min.

4.2. Antiviral assays

The antiviral assays [except anti-human immunodeficiency virus (HIV) assays] were based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1 (HSV-1), HSV-2 (G), vaccinia virus and vesicular stomatitis virus], Vero (parainfluenza-3, reovirus-1, Sindbis, Coxsackie B4, and Punta Toro virus), HeLa (vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus), MDCK (influenza virus A and B) and CRFK (feline corona virus and feline herpes virus) cell cultures. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 cell culture infective dose-50 ($CCID_{50}$) of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures) in the presence of varying concentrations (200, 40, 8, ... μM) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. The methodology of the anti-HIV assays

was as follows: human CEM ($\sim 3 \times 10^5$ cells/ml) cells were infected with 100 $CCID_{50}$ of HIV-1(III_B) or HIV-2(ROD)/mL and seeded in 200 μL wells of a microtiter plate containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, HIV-induced CEM giant cell formation was examined microscopically.

4.3. Antiproliferative assays

Murine leukemia L1210, murine mammary carcinoma FM3A, human T-lymphocyte CEM and human cervix carcinoma HeLa cells were suspended at 300,000–500,000 cells/mL of culture medium, and 100 μL of a cell suspension was added to 100 μL of an appropriate dilution of the test compounds in 200 μL wells of 96-well microtiter plates. After incubation at 37 °C for two (L1210, FM3A) or three (CEM, HeLa) days, the cell number was determined using a Coulter counter. The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

4.4. Thymidine kinase assay using [CH_3-^{3}H]dThd as the natural substrate

The activity of recombinant thymidine kinase of herpes simplex virus-1 (HSV-1) TK, VZV TK and FHV TK and the 50% inhibitory concentration of the test compounds were assayed in a 50-μl reaction mixture containing 50 mM Tris/HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM CHAPS, 3 mg/ml bovine serum albumin, 2.5 mM ATP, 1 μM [methyl-^{3}H]dThd, and enzyme. The samples were incubated at 37 °C for 30 min in the presence or absence of different concentrations (fivefold dilutions) of the test compounds. At this time point, the enzyme reaction still proceeded linearly. Aliquots of 45 μl of the reaction mixtures were spotted on Whatman DE-81 filter paper disks (Whatman, Clifton, NJ). The filters were washed three times for 5 min each in 1 mM ammonium formate, once for 1 min in water, and once for 5 min in ethanol. The radioactivity was determined by scintillation counting.

Acknowledgements

Financial support was provided by the 'Geconcerteerde Onderzoeksacties' (GOA No. 10/014) of the K.U. Leuven. The authors are grateful to Mrs. Lizette van Berckelaer, Frieda De Meyer, Leen Ingels, Anita Camps, Lies Van den Heurck and Mr. Steven Carmans for dedicated technical help and Helen Murphy for clerical support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.037.

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