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Phosphoramidates of 2′-β-D-arabinouridine (AraU) as phosphate prodrugs; design, synthesis, in vitro activity and metabolism

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ABSTRACT

2′-β-D-Arabinouridine (AraU), the uridine analogue of the anticancer agent AraC, was synthesized and evaluated for antiviral activity and cytotoxicity. In addition, a series of AraU monophosphate prodrugs in the form of triester phosphoramidates (ProTides) were also synthesized and tested against a range of viruses, leukaemia and solid tumour cell lines. Unfortunately, neither the parent compound (AraU) nor any of its ProTides showed antiviral activity, nor potent inhibitory activity against any of the cancer cell lines. Therefore, the metabolism of AraU phosphoramidates to release AraU monophosphate was investigated. The results showed carboxypeptidase Y, hog liver esterase and crude CEM tumor cell extracts to hydrolyse the ester motif of phosphoramidates with subsequent loss of the aryl group, while molecular modelling studies suggested that the AraU L-alanine aminoacyl phosphate derivative might not be a good substrate for the phosphoramidase enzyme Hint-1. These findings are in agreement with the observed disappearance of intact prodrug and concomitant appearance of the corresponding phosphoramidate intermediate derivative in CEM cell extracts without measurable formation of araU monophosphate. These findings may explain the poor antiviral/cytostatic potential of the prodrugs.

1. Introduction

Cytarabine (AraC, Fig. 1) is an anticancer drug used for the treatment of acute myeloid leukaemia and lymphomas, but has limited activity against solid tumors.[1] It is a prodrug that requires conversion to the active triphosphate derivative by cellular kinases in order to exert its cytotoxic effect. Upon the incorporation of AraCTP into the growing DNA chain by DNA polymerases, the chain elongation process is terminated.[2] In this work, the uridine derivative of AraC, that is, AraU (Fig. 1), was synthesized and investigated for anticancer activity, and in particular its activity versus several leukaemia cell lines was examined. AraU is in fact a metabolite of AraC in vivo as a result of the action of cytidine deaminase,[12] and this is generally regarded as a deactivation mechanism of AraC. In parallel to the mechanism of action of AraC, AraU would most likely have to be phosphorylated to its triphosphate derivative in order to exert any therapeutic effect and this may be inefficient. The phosphorylation of nucleoside analogues, in particular the first step, is believed to be the rate-limiting process for many antiviral and anticancer nucleosides. Thus, a series of AraU triester

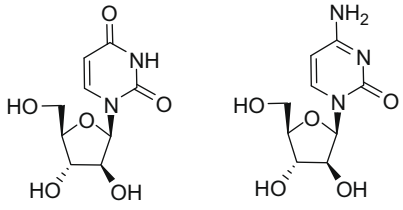

Figure 1. Structures of AraU (left) and AraC (right).

phosphoramidates (ProTides), which would by-pass the first phosphorylation step, were synthesized and evaluated for antiviral activity and cytotoxic potential against a selection of leukaemia cell lines.

The triester phosphoramidate approach is a pronucleotide approach that delivers the nucleoside analogue and carbohydrate monophosphates into cells.[3,4] In this approach, both charges of the phosphate motif are fully masked to allow good passive membrane diffusion. Upon entering the cell, the masking groups can be hydrolyzed to release the nucleoside analogue/carbohydrate monophosphate, which, in the case of nucleosides, may be further phosphorylated to their di- and then triphosphate derivatives. The metabolism of phosphoramidates proceeds in fours steps; two

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spontaneous and two involve enzymes. In this work, the two-enzymatic metabolic steps of AraU phosphoramidates were investigated. The first enzymatic step is the hydrolysis of the ester motif of the phosphoramidate, which in this case was studied using carboxypeptidase Y that has been shown to be able to perform the hydrolysis of the ester moiety present in phosphoramidates.[5] Hog liver esterase and crude human lymphocyte CEM cell extracts have also been used in the hydrolysis studies. Secondly, molecular modeling studies were carried out to investigate whether or not AraU phosphoramidates lacking the ester and aryl motifs may be substrates for the Hint-1 enzyme, which is an enzyme that belongs to the phosphoramidase family and has been described to process such compounds to their free monophosphates.[6]

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of AraU

AraU was synthesized in two steps (Scheme 1) with the first reaction being the formation of the 2,2′-anhydrouridine (**2**). Thus, uridine (**1**) and diphenyl carbonate were suspended in DMF and the slurry was heated to 100 °C.[7] At this point, potassium carbonate (K_2CO_3) was added and the reaction mixture was heated to 137 °C. After 1.5 h, the reaction mixture was allowed to cool to room temperature and then filtered. The white solid obtained was washed with methanol to afford the desired compound (**2**) in 78% yield.

The second reaction was the opening of the 2,2′-anhydro bond to generate a β-hydroxyl at the C-2′. This was achieved by suspending compound **2** in water and 2 N HCl was then added and the reaction mixture was heated to 80 °C for 2 h.[8] Upon neutralization with aqueous sodium hydroxide, the solvent was removed and the residue obtained was purified by column chromatography to give the desired compound in 68% yield.

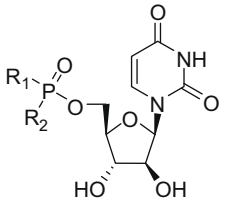
2.1.2. Synthesis of AraU phosphoramidates

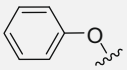
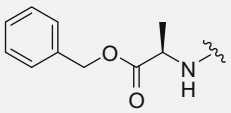
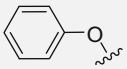
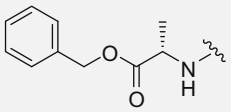
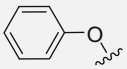
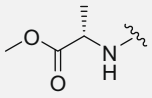
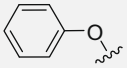
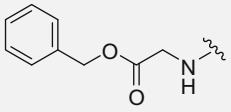
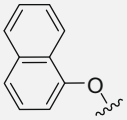
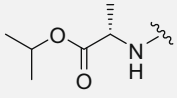
AraU phosphoramidates were synthesised according to the previously reported synthetic routes developed by McGuigan et al.[9–11] Aryl phosphorochloridates were prepared by the reaction of phenyl or 1-naphthyl dichlorophosphate with the appropriate amino acid ester hydrochlorides and tosylate salts. The obtained phosphorochloridates were allowed to react with AraU in THF/pyridine (7:3) and 1-methylimidazole (NMI) to give the target phosphoramidates in moderate yields. ^{31}P NMR investigations of the phosphoramidates displayed two closely spaced signals, corresponding to two diastereoisomers resulting from mixed phosphate stereochemistry. A series of AraU phosphoramidates bearing different ester, amino acid and aryl moieties were prepared (Table 1).

2.2. Antiviral data

The araU prodrugs have been evaluated for their potential inhibitory action against a wide variety of DNA and RNA viruses in cell culture. Unfortunately, none of them showed significant antiviral activity.

Table 1
Structures and yields of AraU phosphoramidates synthesized in this work

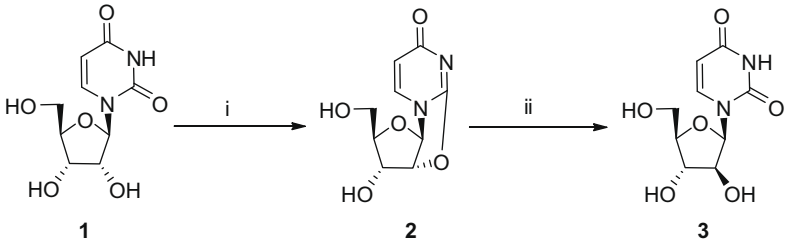


Compound	R_1	R_2	Yield (%)
4			30
5			33
6			28
7			37
8			20

2.3. Cytotoxicity data

The obtained AraU phosphoramidates as well as the parent nucleoside (AraU) were tested for activity against a selection of myeloid leukaemia cell lines (NB4, HL60, KG1, U937, L1210 and CEM) (Table 2). AraU showed low (25–>500 μM) activity versus all the cell lines tested. The best cytostatic activity was shown against the KG1 cell line, IC_{50} = 24 μM. As for its phosphoramidate derivatives they also exhibited rather poor activity against the six tumor cell lines tested against. Notably, AraU and its phosphoramidates were clearly most cytostatic against NB4 and KG1 cells, compared with the other cell lines evaluated. The reason behind such activity profiles remains unclear.

The AraU phosphoramidate derivatives were also screened for activity against solid tumour cell lines (A549, LoVo, PC3, MCF7) with data being shown in Table 3. No antitumor activity was observed at the maximum concentration tested (100 μM), except



Scheme 1. Reagents and conditions: (i) DMF, diphenyl carbonate, 100 °C, K_2CO_3, 137 °C, 1.5 h; (ii) H_2O, HCl (2 N), 80 °C, 2 h.

Table 2
Biological evaluation of AraU phosphoramidates in leukaemia cell lines (IC_{50} μM)

Compound	Ar	AA	Ester	NB4	HL60	KG1	U937	L1210	CEM
AraU (3)	—	—	—	69	90	25	>100	170	>500
4	Ph	D-Ala	Bn	88	92	90	>100	470	313
5	Ph	L-Ala	Bn	38	>100	63	92	184	188
6	Ph	L-Ala	Me	53	>100	32	>100	>500	>500
7	Ph	Gly	Bn	52	>100	39	>100	256	196
8	1-Naph	L-Ala	*i*Pr	77	84	67	>100	250	181

Table 3
Cytostatic evaluation of AraU phosphoramidates in solid tumour cell lines (IC_{50} μM)

Compound	LoVo	PC3	A549	MCF7
4	>100	>100	>100	50
5	>100	>100	>100	>100
6	>100	>100	>100	>100
7	>100	>100	>100	>100
8	>100	>100	>100	>100

for (**4**) which was active against human breast carcinoma MCF7 at 50 μM.

The failure of AraU phosphoramidates to achieve any significant antiviral and anticancer activity may be a result of three factors. Firstly, AraU phosphoramidates were not metabolized (activated) to the monophosphate derivative. Secondly, once AraU monophosphate has been released inside the cell, it was not phosphorylated further to its triphosphate counterpart. Thirdly, AraU triphosphate might not be inhibitory to the antiviral/cytostatic target enzyme (i.e., DNA or RNA polymerase).

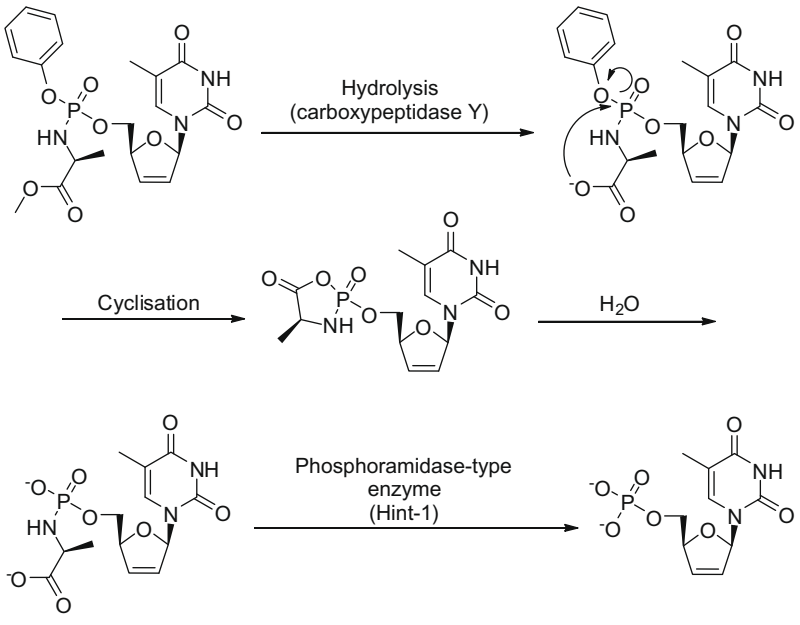
2.4. Metabolism of AraU phosphoramidates

The proposed metabolism of aryl phosphoramidates proceeds in four steps to eventually release the nucleoside analogue monophosphate as shown in Scheme 2.

The first metabolic step is mediated by esterase or carboxypeptidase enzymes, which cleave the ester motif of the aryl phosphoramidate molecule. This step is essential for the eventual release of the nucleoside analogue monophosphate, as failure at this stage would not lead to the release of the monophosphate. To probe whether the ester motif of AraU phosphoramidates is cleaved off or not, carboxypeptidase Y also known as cathepsin A, which has been reported to catalyze this hydrolysis was used in this study.[5] Phenyl-L-alanine benzyl ester AraU phosphoramidate (**5**) was chosen to be tested. The experiment was carried out for 3 h at room temperature, and the progress of the reaction was followed by ^{31}P NMR (Fig. 2).

As shown in Figure 2, the phenyl-L-alanine benzyl ester phosphoramidate of AraU (**5**) appeared as two ^{31}P NMR peaks (δ 3.5–3.9) corresponding to the two diastereoisomers (oblong highlight). Upon the addition of cathepsin A, the compound was quickly hydrolyzed to intermediate **I** ($t_{1/2}$ ~15 min), which lacks the ester motif (triangle highlight). However, this intermediate did not persist as it was in turn quickly metabolized to intermediate **ii** via the loss of the aryl motif (circle highlight). Intermediate **ii** was then consistently present throughout the 3 h of the assay. This suggests that within 3 h cathepsin A was able to hydrolyse the ester and aryl moieties of phenyl-L-alanine benzyl ester phosphoramidate of AraU, but not the P–N bond as no peaks corresponding to the monophosphate were detected.

The cleavage of the P–N bond of aryl phosphoramidates is thought to proceed via the involvement of a phosphoramidase type enzyme.[6] Hint-1 has been identified as a possible enzyme that can cleave such a bond. Thus, in this study, Hint-1 was used for the docking of intermediate **ii** to probe the possible hydrolysis of the P–N bond to release AraU monophosphate in silico. Upon docking (see Section 4), the analysis of the different predicted poses showed intermediate **ii** as having a conformation that would allow the interactions of the phosphate moiety with the key residues in the catalytic site. Although the sugar hydroxyl groups did not correctly interact with the aspartic acid residues, the pyrimidine base did fit in the hydrophobic pocket (Fig. 3). Other conformers that retained the hydrogen bonding interactions of the sugar moiety with the aspartic residues showed the sugar ring distorted, and consequently, the base was pushed outside of the hydrophobic pocket, in comparison to AMP (thin red line, Fig. 3). Thereby, the hydrolysis of the P–N bond may not be as efficient as with purine and ribo nucleoside analogues.



Scheme 2. Postulated mechanism of action of aryl triester phosphoramidates.

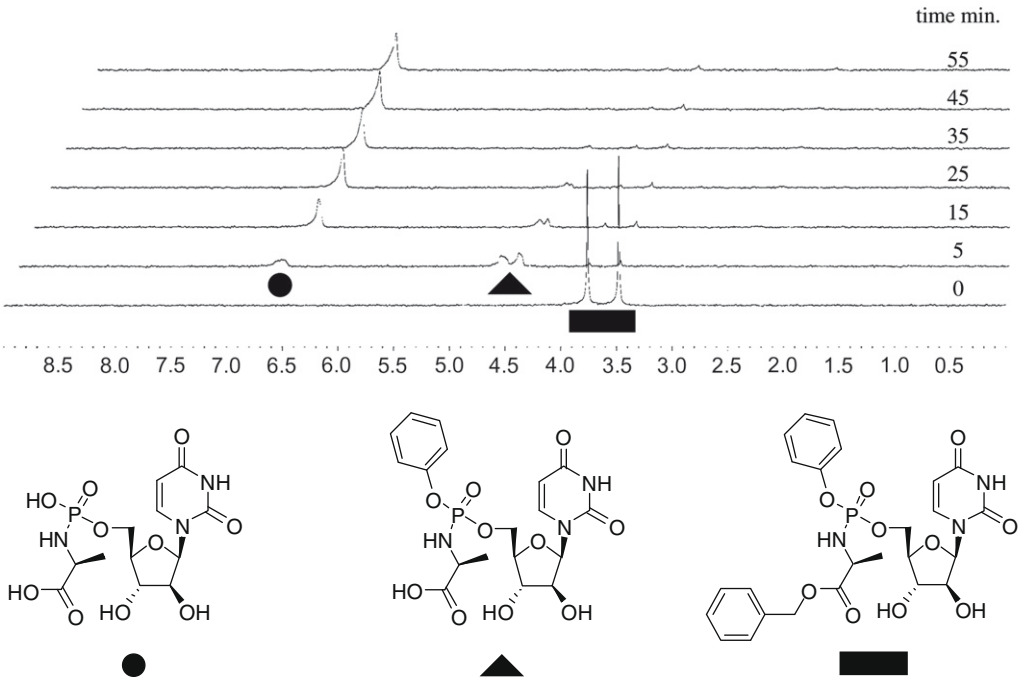


Figure 2. ^{31}P NMR spectra measured at different times during hydrolysis of compound.

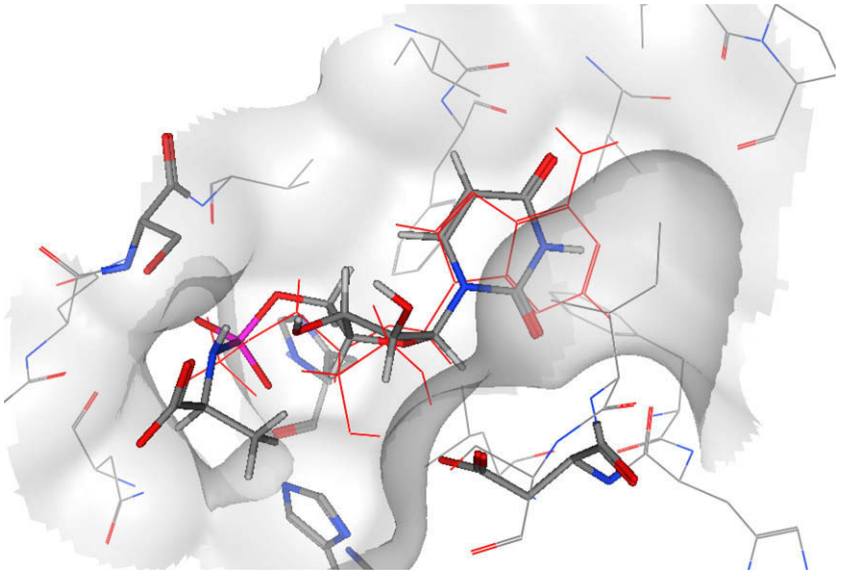


Figure 3. Docking of AMP (thin red line) and AraU L-alanine aminoacyl phosphate derivative.

These findings are in agreement with the hydrolysis studies that were performed using crude human CEM lymphocyte extracts (Fig. 4) and hog liver esterase (data not shown). Indeed, the diastereomers of each of the prodrugs could be separated by HPLC analysis. When the compounds were exposed to crude CEM cell extracts for 30 and 120 min, the L-alaninyl and glycinyl araU phosphoramidate prodrugs were converted to their corresponding intermediate phosphoramidate derivatives (after release of their ester and aryl moieties), except for compound **4** (the D-alaninyl derivative) that remained unaltered during the incubation period (Fig. 4). Also, the speed of conversion of the prodrug compounds **5**–**8** to their corresponding phosphoramidate intermediate was different depending the nature of the ester/aryl part of the prodrug. In particular, compound **5** was most efficiently converted, followed by **6** and **8**. The glycinyl prodrug **7** was the least efficiently converted (Fig. 4). With the use of purified hog liver esterase, it was ascertained that the metabolites that appeared in the CEM cell extracts were indeed the expected L-alaninyl (for **5, 6, 8**) and glycinyl (for **7**) intermediates of the prodrugs (data not shown). Interestingly, whereas CEM cell extracts did not hydrolyze the ester moiety from prodrug **4**, the hog liver esterase did, although at a much lower efficiency than for the other prodrugs.

As the application of the phosphoramidate technology to widely diverse ribo- and deoxyribonucleoside analogues has mostly led to improvements in biological activity.[13–18] In this case, its application to an arabinonucleoside did not improve the biological activity. The type of the nucleoside analogue seems to affect the cleavage of the P–N bond and thus the outcome of the use of such a pronucleotide technology.

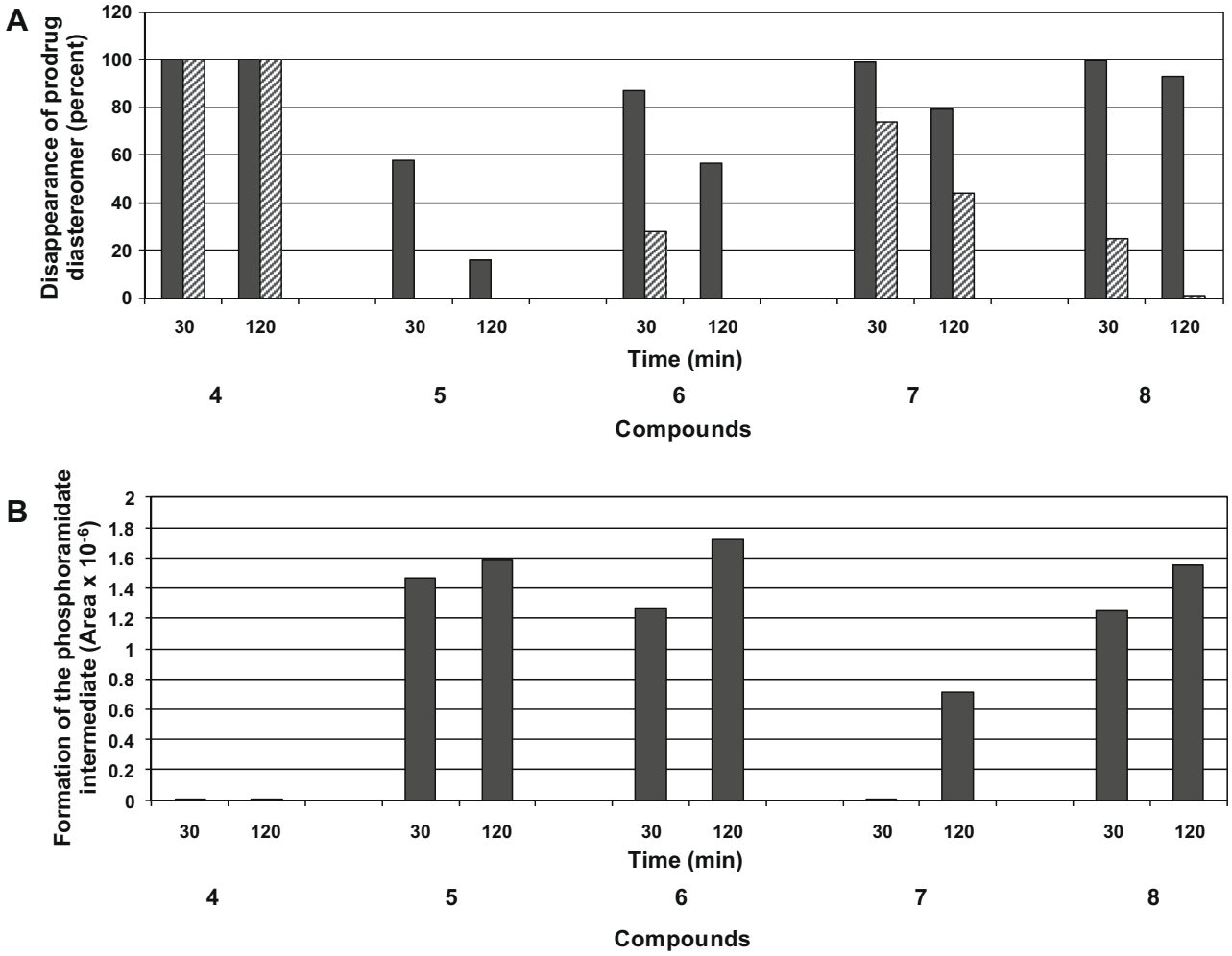


Figure 4. Conversion of prodrugs **4**–**8** in the presence of human T-lymphocyte CEM cell extracts. Panel A. Disappearance (hydrolysis) of prodrug. The individual diastereomers are represented by the black and dashed bars. The levels of the parent prodrug molecules and their conversion products are given as percentage of total amounts of parent compound and metabolites. Panel B. Appearance of the aminoacyl phosphoramidate intermediates (expressed as area under the curve).

3. Conclusions

A series of AraU phosphoramidates were synthesized and investigated for antiviral activity and for cytotoxicity versus six leukaemia cell lines and four solid tumour cell lines. Unfortunately, none of the phosphoramidates or their parent nucleoside (AraU) showed significant activity. Metabolic assays using carboxypeptidase Y and hog liver esterase showed the ability of these enzymes to metabolize the ester motif of AraU phosphoramidates, with subsequent spontaneous phenyl loss. Molecular modelling studies suggested AraU L-alanine aminoacyl phosphate may not to be a good substrate for Hint1. In crude CEM cell extracts, formation of the phosphoramidate intermediate was seen for the L-alaninyl and glycinyl prodrugs, but not for the D-alaninyl prodrug. No formation of AraU monophosphate was observed. Thus both hydrolysis studies and docking suggest that the efficiency of the P–N bond cleavage to release AraU monophosphate might be a contributor to the poor cytotoxic activity of AraU ProTides. Taken together, these results suggest that the poor cytostatic activities of AraU phosphoramidates could be a result of inefficient P–N cleavage, though poor phosphorylation to the di- and triphosphates could also be reasons for poor biological activity. This study does suggest that phosphoramidate ProTides may have limited efficacy for arabino nucleosides, although the exact scope of this conclusion needs confirmation.

4. Experimental

4.1. General

All experiments involving water-sensitive compounds were carried out under dry conditions. The solvents used were dry and used as purchased from Aldrich. All the glassware was oven-dried at 130 °C for several hours and allowed to cool under a steam of dry nitrogen.

Thin-Layer Chromatography (TLC) was performed using precoated, aluminium-backed silica gel plates (60 F-54, 0.2 mm thickness; supplied by E Merck AG, Darmstad, Germany). Visualisation of the plates was achieved using an ultra-violet (UV) lamp.

Glass columns were slurry-packed in the appropriate eluant under pressure with silica gel, 60A, 40–60 μm, (Phase Separations Ltd, Deeside, Clwyd, UK). Samples were applied as a concentrated solution in the same eluant or pre-absorbed on silica gel. Fractions containing the product were identified by TLC, pooled and the solvent was removed in vacuo.

^{1}H, ^{13}C, ^{31}P NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer and autocalibrated to the deuterated solvent reference peak. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet).

HPLC analytical investigations were conducted on a Varian Prostar instrument (LC work station, Varian prostar 355 LC detector) using a Polaris C18-A 10 μm column; eluant was performed using a mobile phase consisting of a water/acetonitrile gradient.

Electrospray mass spectra were obtained using a Waters LCT time of flight (TOF) mass spectrometer coupled to a Waters M600 HPLC pump. Data was collected in the continuum mode over the mass range 100–2000 amu and processed using Masslynx 4.1 software.

4.2. Chemistry

4.2.1. 1-((2*R*,4*S*,5*R*)-3,4-Anhydro-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidin-4(1*H*)-one (2)

Uridine (5.00 g, 20.47 mmol) and diphenyl carbonate (4.88 g, 22.80 mmol) were suspended in DMF (40 mL). The slurry was heated to 100 °C and sodium bicarbonate (150 mg) was then added and the reaction mixture was heated up to 137 °C for 1.5 h. After completion, the reaction was cooled down to room temperature, filtered and washed with methanol (60 mL). Following drying under vacuum for 3 h the title compound obtained as a white solid in 78% yield (3.60 g). ^{1}H NMR (DMSO-d_6): δ 7.83 (1H, d, J = 8.1 Hz, H-6), 6.31 (1H, m, H-1′), 5.90 (1H, d, J = 8.1 Hz, H-5), 5.83 (1H, s, H-3′), 5.21 (1H, s, H-4′), 5.06 (1H, s, H-5′), 4.37 (1H, s, 5′-OH), 4.09 (1H, s, 3′-OH), 3.15–3.24 (2H, m, H-2′). ^{13}C NMR (DMSO-d_6): δ 171.32 (CO), 159.79 (CO), 136.84 (C6), 108.56 (C5), 89.99 (C1′), 89.18 (C2′), 88.74 (C4′), 74.72 (C3′), 74.79 (C5′).

4.2.2. 1-((2*R*,3*S*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione (3)

Compound **2** (3.50 g, 15.47 mmol) in 20 mL of 2 N HCl was heated to 80 °C for 2 h. The reaction mixture was cooled down and neutralized to pH ~7 with sodium hydroxide. The solvent was removed and the crude was purified by column chromatography employing 15% methanol in DCM as an eluant. Pooling and evaporation of the appropriate fractions gave the title compound (2.55 g, 68% yield) as a white solid. ^{1}H NMR (DMSO-d_6): δ 11.24 (1H, br s, NH), 7.64 (1H, d, H-6, J = 8.1), 5.98 (1H, m, H-1′), 5.63 (1H, m, 2′-OH), 5.57 (1H, d, J = 8.1 Hz, H-5), 5.53 (1H, m, 3′-OH), 5.10 (1H, s, 5′-OH), 4.04 (1H, m, H-2′), 3.92 (1H, m, H-3′), 3.75 (1H, m, H-4′), 3.56–3.62 (2H, m, H-5′). ^{13}C NMR (DMSO-d_6): δ 163.41 (CO), 150.40 (CO), 142.28 (C-6), 99.86 (C-5), 85.05 (C-1′), 84.59 (C-4′), 75.28 (C-3′), 75.13 (C-2′), 60.67 (C-5′).

4.3. Standard procedure for the synthesis of phosphoramidates

AraU (1 equiv) was dissolved in 10 mL of THF/pyridine (7/3) under argon. 1-Methylimidazole (NMI, 5 equiv) was added to the reaction flask and this was followed by the addition of the appropriate phosphorochloridate (3 equiv) and the mixture was stirred at room temperature for 16 h. Upon the removal of the solvent, the crude was purified by column chromatography (CH_2Cl_2/CH_3OH 95/5) and then preparative HPLC to give the desired product as a white solid.

4.3.1. Synthesis of arabinouridine-5′-[phenyl-(benzoxy-L-alaninyl)]-phosphate (5)

Prepared using AraU (0.20 g, 0.82 mmol), NMI (0.33 mL, 4.09 mmol), phenyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.87 g, 2.46 mmol). The desired product was obtained as a white solid (150.0 mg, 33%). ^{31}P NMR (MeOD, 202 MHz): δ 3.76, 3.69. ^{1}H NMR (MeOD, 500 MHz): δ 7.74 (1H, d, J = 8 Hz, H-6), 7.37–7.17 (10H, m, Ph, Ph), 6.19, 6.20 (1H, 2d, J = 4.0 Hz, H-1′), 5.62, 5.56 (1H, 2d, J = 8.0 Hz, H-5), 5.17–5.12 (2H, m, CH_2Ph), 4.44–4.30 (2H, m, H-5′), 4.25–4.22 (1H, m, H-2′), 4.14–4.02 (3H, m, H-3′, H-4′, *CH*NH), 1.38, 1.37 (3H, 2d, J = 8.0, 7.5 Hz, CH_3CH). ^{13}C NMR (MeOD, 125 MHz): δ 20.45, 20.56 (2d, J = 7.5 Hz, CH_3CH), 51.69, 51.79 (*CH*NH), 67.23, 67.45 (2d, J = 5.0 Hz, C-5″), 68.03, 68.06 (CH_2Ph), 76.74 (C-2′), 77.52, 77.74 (C-3′), 84.18, 84.33 (2d, J = 7.5 Hz, C-4′), 87.87 (C-1′), 101.33 (C-5), 121.46, 121.51, 121.55, 126.21, 126.24, 129.29, 129.33, 129.40, 129.66, 129.98, 130.85, 130.86, 131.20 (Ph), 137.25 (*ipso* Ph), 144.25, 144.36 (C-6), 152.14, 152.26 (C-2), 166.42 (C-4), 174.75, 174.92 (2d, J = 5 Hz, C = O). MS (ES+) *m/e*: 584.4 (MNa^+, 100%); Accurate mass: $C_{25}H_{28}N_3O_{10}NaP$ required 584.1410, found 584.1418. $HPLC_b$ (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): t_R 13.31, 13.41 min.

4.3.2. Synthesis of arabinouridine-5′-[phenyl-(methoxy-L-alaninyl)]-phosphate (6)

Prepared using AraU (0.20 g, 0.82 mmol), NMI (0.33 mL, 4.09 mmol), phenyl-(methoxy-L-alaninyl)-phosphorochloridate (0.68 g, 2.46 mmol). The desired product was obtained as a white solid (110.0 mg, 28%). ^{31}P NMR (MeOD, 202 MHz): δ 3.77, 3.70. ^{1}H NMR (MeOD, 500 MHz): δ 7.77, 7.75 (1H, 2d, J = 8 Hz, H-6), 7.40–7.19 (5H, m, Ph), 6.21, 6.20 (1H, 2d, J = 4.0 Hz, H-1′), 5.64, 5.60 (1H, 2d, J = 8.0 Hz, H-5), 4.46–4.31 (2H, m, H-5′), 4.25–4.22 (1H, m, H-2′), 4.16–4.09 (2H, m, H-3′, H-4′), 4.04–3.98 (2H, m, *CH*NH), 3.70 (3H, s, CH_3O), 1.38 (1.5H, dd, J = 7.5, 0.5 Hz, CH_3CH one d.i.), 1.36 (1.5H, dd, J = 7.0, 0.5 Hz, CH_3CH one d.i.). ^{13}C NMR (MeOD, 125 MHz): δ 20.44, 20.55 (2d, J = 7.5 Hz, CH_3CH), 51.52, 51.63 (*CH*NH), 52.87, 52.90 (CH_3O), 67.23, 67.35 (2d, J = 6.25 Hz, C-5′), 76.74 (C-2′), 77.55, 77.70 (C-3′), 84.31, 84.38 (2d, J = 7.5 Hz, C-4′), 87.86, 87.88 (C-1′), 101.29 (C-5), 121.48, 126.23, 130.85, 131.18 (Ph), 144.29, 144.37 (C-6), 152.17, 152.26 (C-2), 166.42 (C-4), 175.42, 175.59 (2d, J = 5.0 Hz, C═O). MS (ES+) *m/e*: 508.2 (MNa^+, 100%); Accurate mass: $C_{19}H_{24}N_3O_{10}NaP$ required 508.1097, found 508.1107. $HPLC_b$ (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): t_R 10.41, 10.53 min.

4.3.3. Synthesis of arabinouridine-5′-[1-naphthyl-(isopropoxy-L-alaninyl)]-phosphate (8)

Prepared using AraU (0.20 g, 0.82 mmol), NMI (0.33 mL, 4.09 mmol), naphthyl-(isopropoxy-L-alaninyl)-phosphorochloridate (0.87 g, 2.46 mmol). The desired product was obtained as a white solid (102.5 mg, 22%). ^{31}P NMR (MeOD, 202 MHz): δ 4.24, 4.17. ^{1}H NMR (MeOD, 500 MHz): 8.19–7.37 (8H, m, Naph, H-6), 6.14 (1H, d, J = 4.0 Hz, H-1′), 5.49, 5.39 (1H, 2d, J = 8.0 Hz, H-5), 4.94–4.85 (1H, m, *CH*CH_3), 4.48–4.32 (2H, m, H-5′), 4.18–4.15 (1H, m, H-2′), 4.12–4.04 (2H, m, H-3′, H-4′) 3.99–3.92 (2H, m, *CH*NH), 1.29 (3H, dd, J = 7.0, 0.5 Hz, CH_3CH), 1.17–1.11 (6H, m, $(CH_3)_2$CH). ^{13}C NMR (MeOD, 125 MHz): δ 20.47, 20.60 (2d, J = 7.5, 6.25 Hz, CH_3CH), 21.91, 21.95, 21.99 ($(CH_3)_2$CH), 51.83, 51.90 (*CH*NH), 67.42, 67.59 (2d, J = 5.0, 6.25 Hz, C-5′), 70.22, 70.25 ($(CH_3)_2CH$), 76.70 (C-2′), 77.61, 77.77 (C-3′), 84.34, 84.41 (2d, J = 8.75 Hz, C-4′), 87.87 (C-1′), 101.21 (C-5), 116.20, 116.22, 116.28, 116.31, 122.73, 122.85, 125.99, 126.58, 127.45, 127.52, 127.83, 127.91, 128.91 (Naph), 136.33 (*ipso* Naph), 144.22, 144.36 (C-6), 152.21 (C-2), 166.32 (C-4), 174.48, 174.72 (C═O). MS (ES+) *m/e*: 586.4 (MNa^+, 100%); Accurate mass: $C_{25}H_{30}N_3O_{10}$-NaP required 586.1567, found 586.1571. HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): t_R 13.43, 13.60 min.

4.3.4. Synthesis of arabinouridine-5′-[phenyl-(benzoxy-D-alaninyl)]-phosphate (4)

Prepared using AraU (0.20 g, 0.82 mmol), NMI (0.33 mL, 4.09 mmol), phenyl-(benzoxy-D-alaninyl)-phosphorochloridate (0.87 g, 2.46 mmol). The desired product was obtained as a white solid (140.0 mg, 30%). ^{31}P NMR (MeOD, 202 MHz): δ 4.02, 3.50. ^{1}H NMR (MeOD, 500 MHz): δ 7.77, 7.71 (1H, 2d, J = 8.0 Hz, H-6), 7.37–7.17 (10H, m, Ph, Ph), 6.20, 6.18 (1H, 2d, J = 4.0 Hz, H-1′), 5.61, 5.56 (1H, 2d, J = 8.0 Hz, H-5), 5.19–5.12 (2H, m, CH_2Ph), 4.37–4.27 (2H, m, H-5′), 4.23–4.22 (1H, m, H-2′), 4.11–4.00 (3H,

m, H-3′, H-4′, *CH*NH), 1.38 (1.5H, dd, *J* = 7.0, 0.5 Hz, CH_3CH one d.i.), 1.36 (1.5H, dd, *J* = 7.0, 0.5 Hz, CH_3CH one d.i.). ^{13}C NMR (MeOD, 125 MHz): δ 20.45, 20.50 (CH_3CH), 51.62, 51.76 (*CH*NH), 67.09, 67.43 (2d, *J* = 5.0 Hz, C-5′), 68.02, 68.07 (CH_2Ph), 76.71, 76.76 (C-2′), 77.43, 77.64 (C-3′), 84.15, 84.30 (2d, *J* = 8.75 Hz, C-4′), 87.79 (C-1′), 101.27, 101.30 (C-5), 121.43, 121.47, 121.51, 121.55, 126.21, 129.31, 129.40, 129.64, 129.71, 129.73, 130.85, 131.17 (Ph), 137.25 (*ipso* Ph), 144.27, 144.40 (C-6), 152.17, 152.26 (C-2), 166.44 (C-4), 174.78, 174.94 (2d, *J* = 5.0 Hz, C=O). MS (ES+) *m/e*: 584.3 (MNa^+, 100%); Accurate mass: $C_{25}H_{28}N_3O_{10}NaP$ required 584.1410, found 584.1395. HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): t_R 13.43 min.

4.3.5. Synthesis of arabinouridine-5′-[phenyl-(benzoxy-glycinyl)]-phosphate (7)

Prepared using AraU (0.20 g, 0.82 mmol), NMI (0.33 mL, 4.09 mmol), phenyl-(benzoxy-glycinyl)-phosphorochloridate (0.83 g, 2.46 mmol). The desired product was obtained as a white solid (168.0 mg, 37%). ^{31}P NMR (MeOD, 202 MHz): δ 5.01, 4.78. ^{1}H NMR (MeOD, 500 MHz): δ 7.76, 7.72 (1H, 2d, *J* = 8 Hz, H-6), 7.37–7.17 (10H, m, Ph, Ph), 6.20, 6.19 (1H, 2d, *J* = 4.0 Hz, H-1′), 5.61, 5.57 (1H, 2d, *J* = 8.0 Hz, H-5), 5.19–5.14 (2H, m, CH_2Ph), 4.46–4.34 (2H, m, H-5′), 4.25–4.22 (1H, m, H-2′), 4.13–4.08 (3H, m, H-3′, H-4′), 3.85–3.81 (2H, m, CH_2NH). ^{13}C NMR (MeOD, 125 MHz): δ 43.95, 43.97 (CH_2NH) 67.21, 67.49 (2d, *J* = 6.25, 5.0 Hz, C-5′), 68.03 (CH_2Ph), 76.75 (C-2′), 77.47, 77.66 (C-3′), 84.19, 84.33 (2d, *J* = 7.5 Hz, C-4′), 87.79 (C-1′), 101.32 (C-5), 121.49, 121.53, 126.27, 129.39, 129.41, 129.65, 129.70, 129.72, 129.74, 129.76, 129.98, 130.88 (Ph), 137.17 (*ipso* Ph), 144.26, 144.36 (C-6), 152.12, 152.24 (C-2), 166.40 (C-4), 172.31, 172.35 (C=O). MS (ES+) *m/e*: 570.2 (MNa^+, 100%); Accurate mass: $C_{24}H_{26}N_3O_{10}NaP$ required 570.1254, found 570.1262. HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): t_R 12.77, 12.89 min.

4.4. Leukaemia cell line assays

AraU phosphoramidates were evaluated against six myeloid cell types (NB4, HL60, KG1, U937, CEM, L1210) in vitro. Inhibitory concentrations (IC_{50}) at which 50% of the cell growth was reduced (cell counting assay (CEM, L1210) using a Coulter Counter) or no longer viable (calculated using an MTS assay) were determined. Cells were treated with AraU and its 'ProTides' at concentrations between 100 μM and 0.02 μM by serial dilutions and incubated for 72 h at 37 °C, 5% CO_2 in a final volume of 90 μL. Twenty microliters of MTS reagent (Promega UK Ltd, Southampton, Hants) was added to the tumor cell cultures (NB4, HL60, KG1 and U937) and the reaction incubated for a further 4 h at 37 °C, 5% CO_2. The absorbance of the reaction after this time was read by spectrophotometry at 490 nm and the percentage of viable cells calculated relative to untreated control cells in the same assay.

4.5. Solid tumour cell line assays

Anti-tumour evaluation in MCF7, LoVo, A549 and PC3 cell lines was performed using MTT assay. Protides were prepared as 0.1–100 mM stock solutions dissolved in DMSO and stored at −20 °C. Cells were seeded into 96-well microtitre plates at a density of 5×10^3 cells per well and allowed 24 h to adhere. Decimal protide dilutions were prepared in medium immediately prior to each assay (final concentration 0.1–100 μM). Following 96 h protide exposure at 37 °C, 5% CO_2, MTT reagent (Sigma Aldrich) was added to each well (final concentration 0.5 mg/mL). Incubation at 37 °C for 4 h allowed reduction of MTT by viable cells to an insoluble formazan product. MTT was removed and formazan solubilised by addition of 10% Triton X-100 in PBS. Absorbance was read on a Tecan Sunrise plate reader at 540 nm as a measure of cell viability; thus inhibition relative to control was determined (IC_{50}).

4.6. Enzymatic carboxypeptidase Y assay

The phosphoramidate prodrug (5 mg) was dissolved in acetone-d_6 (150 μL), and TRIZMA buffer pH 7.6 (300 μL) was added. The resulting cloudy solution was placed in a NMR tube and a ^{31}P NMR experiment at 25 °C was recorded as the blank experiment.

The frozen enzyme Carboxypeptidase Y [EC 3.4.16.1] (0.3 mg), left out to reach room temperature (at least 20 min), was then solubilised in TRIZMA (150 μL) and added to the solution of phosphoramidate in the NMR tube. Immediately after, the tube was placed in the NMR machine, which was set to run a ^{31}P NMR experiment (64 scans) every 10 min for 3 h at 25 °C. Data were processed and analysed with Bruker TOPSPIN 2.1 program.

4.7. Molecular modelling

All molecular modelling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 8 using Molecular Operating Environment (MOE) 2008.10 and FlexX (Biosolveit FlexX 3; BiosolveIT GmbH An der Ziegelei 75, 53757 Sankt Augustin, Germany; http://www.biosolveit.de/flexx).

Hydrogen atoms were added to the crystal structure (PDB code: 1KPF) and minimised with MOE until a gradient of 0.05 kcal mol^{-1} Å^{-1} was reached, using the MMFF94x forcefield. The partial charges were automatically calculated. Docking experiments were carried out using the MOE GUI of FlexX implemented in MOE. AraU analogues were built in MOE and minimised before the docking.

4.8. Antiviral assays

The compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase-deficient (TK$^-$) HSV-1 KOS strain resistant to ACV (ACV^r), herpes simplex virus type 2 (HSV-2) strain G, vaccinia virus Lederle strain, respiratory syncytial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, Parainfluenza 3, Reovirus-1, Sindbis, Punta Toro, influenza virus type A (H1N1, H3N2) and type B and feline corona virus. The antiviral, other than anti-HIV, assays were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts, African green monkey cells (Vero), human epithelial cervix carcinoma cells (HeLa), Crandel feline kidney cells (CFKC) or Madin-Darby canine kidney cells (MDCK). Briefly, confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures). After a 1–2 h adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations 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. Antiviral activity was expressed as the EC_{50} or compound concentration required to reduce virus-induced cytopathicity by 50%. Cytotoxicity of the test compounds was expressed as the minimum cytotoxic concentration (MCC) or the compound concentration that caused a microscopically detectable alteration of cell morphology.

To examine inhibition of HIV-induced cytopathicity in CEM cells, human CEM cell cultures (~3×10^5 cells mL^{-1}) were infected with ~100 $CCID_{50}$ HIV-1(III_B) or HIV-2(ROD) per mL and seeded in 96-well (200 μL/well) microtiter plates, containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, syncytia formation was examined microscopically in the CEM cell cultures. EC_{50} values were determined as described above.

The cytostatic compound concentration was calculated as the CC_{50}, or the compound concentration required to reduce cell proliferation by 50% relative to the number of cells in the untreated controls. CC_{50} values were estimated from graphic plots of the number of cells (percentage of control) as a function of the concentration of the test compounds.

4.9. Prodrug conversion studies in the presence of crude cell extracts

CEM cell extracts were prepared from 20×10^6 exponentially growing cells, suspended in 1 ml PBS upon sonication (to destroy the integrity of the cells), and subsequent centrifugation (to remove the cell debris). Six hundred microliters incubation medium (containing 200 μl cell extract, 200 μl PBS and 200 μl test compound at 300 μM in 15% DMSO) was prepared and kept at 37 °C for 0, 30 or 120 min. At each time point 100 μl was withdrawn from the incubation medium and added to 200 μl ice-cold methanol 100% (final concentration: 66%). After 10 min at 4 °C, the reaction mixtures were centrifuged at 13,000 rpm for 10 min and 150 μl of the supernatants were analyzed by reverse phase HPLC. The retention times for both enantiomers of **4** were 18.3 and 18.4 min, for **5** the *t*R were 18.3 and 18.4 min, for **6** the *t*R were 15.0 and 15.2 min, for **7** the *t*R were 17.7 and 17.8 min and for **8** the *t*R were 18.5 and 18.7 min. Separation of the parent compounds and the conversion products was performed on a reverse phase (Lichrospher-60 RP-select B) column (Merck, Darmstadt, Germany) using following gradient: 2 min 2% acetonitrile (Buffer A); 8 min linear gradient to 20% Buffer A + 80% Buffer B (50 mM NaH_2PO_4 + 5 mM heptane sulfonic acid); 2 min linear gradient to 25% Buffer A and 75% Buffer B; 2 min linear gradient to 35% Buffer A + 65% Buffer B; 8 min linear gradient to 50% Buffer A and 50% Buffer B; 10 min isocratic flow; 5 min linear gradient to 2% Buffer A + 98% Buffer B; 5 min equilibration by 2% Buffer A + 98% Buffer B.

4.10. Hog liver carboxyesterase studies

The conversion of the prodrugs by hog liver esterase (Boehringer, Mannheim, Germany) (5% in Tris·HCl 25 mM, $MgCl_2$ 10 mM, pH 7.6) was analysed by Anion Exchange HPLC. The retention times for the L- and D-alaninyl phosphoramidate intermediates were 9.36–9.40 min and for the glycinyl phosphoramidate intermediate of **7** was 9.52 min. Following anion exchange (Column Partisphere-Sax, Whatman, Maidstone, UK) gradient was used: 5 min Buffer A (5 mM $NH_4H_2PO_4$, pH 5.0), 15 min linear gradient to Buffer B (300 mM $NH_4H_2PO_4$, pH 5.0), 20 min at Buffer B, 5 min linear gradient to Buffer A, 5 min Buffer A (equilibration).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.059.

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