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To be cited as: *ChemMedChem* 10.1002/cmdc.201800504

Link to VoR: http://dx.doi.org/10.1002/cmdc.201800504

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chemmedchem.org

Symmetrical diamidates as a class of phosphate prodrugs to deliver the 5′-monophosphate form of anticancer nucleoside analogues

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Dedicated to Professor Christopher McGuigan (1958-2016)

Abstract

We herein report on the application of phosphorodiamidate technology to both pyrimidine and purine nucleosides with anticancer activity to potentially overcome the resistance mechanisms associated with parent nucleosides. Sixteen symmetrical phosphorodiamidates prepared from natural amino acids such as L-alanine and glycine were synthesized. All the compounds were evaluated for their cytotoxic activity in a wide panel of solid and leukemic tumour cell lines. In addition, a carboxypeptidase Y assay was performed on a representative phosphorodiamidate in order to reveal the putative bioactivation pathway for the reported phosphorodiamidate-type prodrugs.

Introduction

Current chemotherapeutic treatment of cancer and viral infections is largely based on the use of antimetabolites, in particular modified nucleoside analogues (NA) mimicking natural purine or pyrimidine nucleosides.[1] In addition, there is also strong evidence that NAs have some potential as antibacterial agents.[2,3] These molecules are

often taken up by nucleoside transporters (NT) and then phosphorylated by specific nucleo(s)(t)ide kinases to the corresponding 5′-mono, di-and triphosphate forms. These metabolites interfere with *de novo* synthesis of DNA/RNA precursors leading to inhibition of DNA/RNA synthesis, with subsequent suppression of tumour cell growth or virus replication. However, NA activity is known to be limited by mechanisms associated with (i) poor cellular uptake (i.e. by a down-regulation in the expression of nucleoside transporters), (ii) poor conversion of the drug into an active metabolite (i.e. by a down-regulation of nucleo(s)(t)ide kinases) or (iii) rapid degradation into toxic byproducts (i.e. by up-regulation of deactivating enzymes such as cytidine or cytidylate deaminases).[4,5] Many prodrug strategies aim to overcome such resistance mechanisms associated with NAs to improve the NA's effectiveness. These include phosphate and phosphonate prodrug diesters pivaloyloxymethyl (POM), isopropyloxycarbonyloxymethyl (POC), cyclosaligenyl (*cyclo*Sal), S-acylthioalkyl (SATE), lipid and HepDirect and phosphoramidates in which an amino acid ester promoiety is linked via P–N bond to a nucleoside aryl phosphate.[6-8] This monophosphate prodrug approach, also called ProTide technology, is one of the most successful prodrug strategies, with an established position in the nucleotide prodrug area. This strategy, pioneered by Chris McGuigan's group,[9] led to the discovery and development of the clinically successful drugs sofosbuvir (Sovaldi)[10] and tenofovir alafenamide (TAF)[11] by Gilead Sciences. The first drug is approved for the treatment of chronic HCV infections, whereas the second to treat patients with either HIV or chronic HBV infections (Figure 1). Other phosphoramidates are currently the subject of ongoing clinical trials as antiviral agents. Stampidine is in Phase I for the treatment of HIV infections[12] and GS-5734, more recently developed as a treatment for filovirus infections,[13] was moved forward through a Phase I clinical trial[14] as a consequence of the Ebola outbreak in West Africa in 2013-2016.[15] GS-9131 is an orally bioavailable phosphonoamidate prodrug which has been shown to inhibit HIV-1 reverse transcriptase (RT) in multiple HIV-1 clinical subtypes and has a unique resistance profile toward N(t)RTI resistance mutations. GS-9131 is currently under clinical evaluation.[16]

In the oncology arena, the first phosphoramidate to reach the clinic was thymectacin (NB1011) which was evaluated in a Phase I trial for the treatment of colon cancer.[17] Subsequently, our group designed and synthesised two

phosphoramidate prodrugs named NUC-1031[18-20] and NUC-3373[21-23] (Figure 1). The gemcitabine phosphoramidate NUC-1031 is currently being evaluated in several clinical studies, including a Phase III study for patients with pancreatic cancer, a Phase II study for patients with ovarian cancer and a Phase Ib study for patients with biliary tract cancers. The 5′-fluoro-2′-deoxyuridine (FUdR) ProTide NUC-3373 is currently being evaluated in a Phase I study for the treatment of a variety of solid tumours including colorectal and breast cancers.

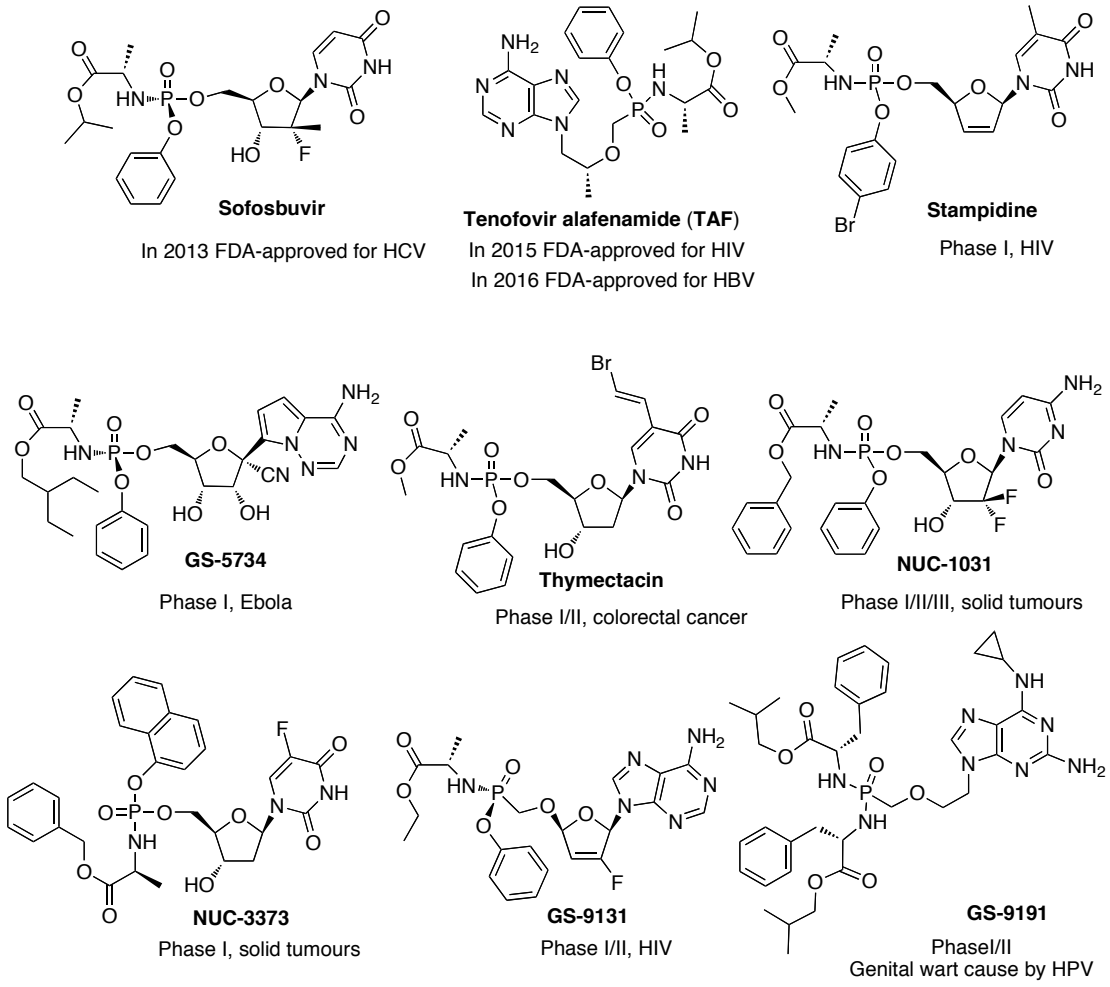


Figure 1. ProTides of antiviral and anticancer nucleoside monophosph(on)ate analogues that have entered clinical studies.

Although less extensively investigated than the ProTide approach, a second phosphoramidate strategy that has been reported by our group as a promising way for the delivery of nucleoside monophosphates inside the cell, is the phosphorodiamidate technology.[24-26] In this approach, two identical amino acid esters are introduced on the monophosphate moiety through a P–N bond in order to mask the negative

charges. Due to their symmetric structure, phosphoro(no)amidates, where no phosphorus chirality arises, offer advantages over the aryloxy monoamidate analogues.

Although stereoisomers have the same chemical structure they may exhibit differences in their pharmacology, toxicology, pharmacokinetics and metabolism. For example, the *S*p diastereoisomer of TAF is 10-fold more potent against HIV than the *R*p diastereoisomer.[27] Likewise, in the HCV replicon assay, the *S*p and *R*p isomers of sofosbuvir demonstrated an approximately 18-fold difference in activity against HCV.[28] Though separation of the ProTide diastereoisomers by crystallization or by chromatography can be achieved in some cases,[29,30] and different approaches to obtain ProTides in a diastereoselective[31-34] and 5′-regioselective fashion[35] have been recently reported, in general most of these methodologies are not very efficient and are high-priced, time consuming and difficult to scale up. In this scenario the diamidate approach, if as effective as the ProTides in terms of activity, could be considered as a valid alternative, as demonstrated by 2′-*C*-methyl-6-*O*-methylguanosine-based phosphorodiamidate agents and their nanomolar anti-HCV activity in replicon assay.[24] Although the diamidate prodrug approach has been validated both *in vitro* and *in vivo,* to our knowledge, only one phosphonodiamidate, GS-9191 (Figure 1), has reached the clinic to date. GS-9191 is a nucleotide analogue under clinical evaluation as a topical ointment in patients with external genital and perianal warts caused by human papilloma virus infection.[36]

Based on our preliminary findings,[24-26] considering the potential advantage of lack of chirality at the phosphorus atom for this class of prodrugs, and taking into account the extraordinary results obtained with gemcitabine and FUdR ProTides, we were interested to investigate the potential of the phosphorodiamidate approach when applied to these two nucleosides. We also took the opportunity to expand our study to other relevant anticancer NAs (**1**-**7**, Figure 2).

Herein, we report the synthesis, and biological activities of these target compounds (**8**-**23**) against a wide panel of cancer cell lines. Moreover, in order to support a putative bioactivation pathway for these prodrugs (in particular regarding the first activation step), we also report on carboxypeptidase Y assays for the gemcitabine phosphorodiamidate **8**.

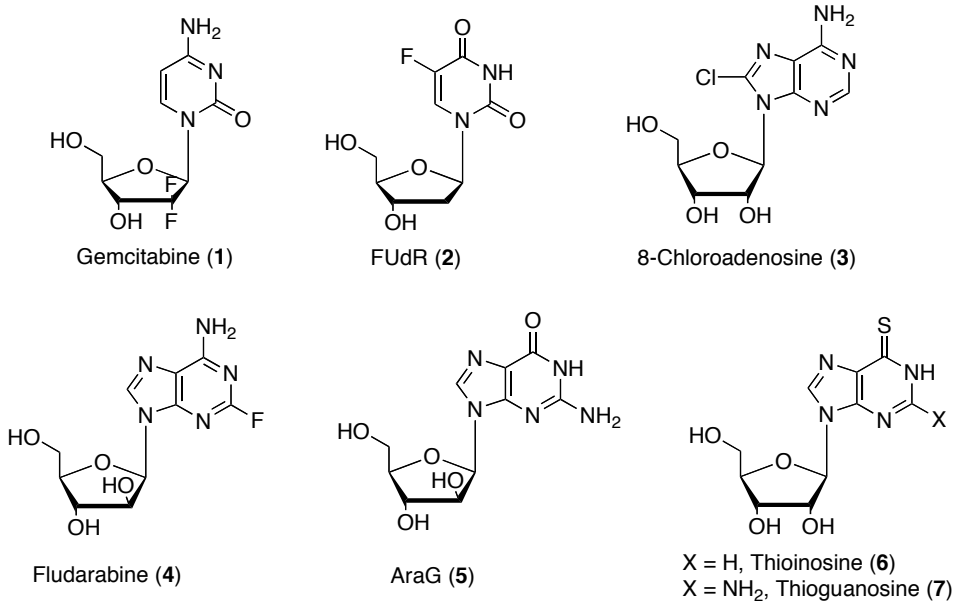


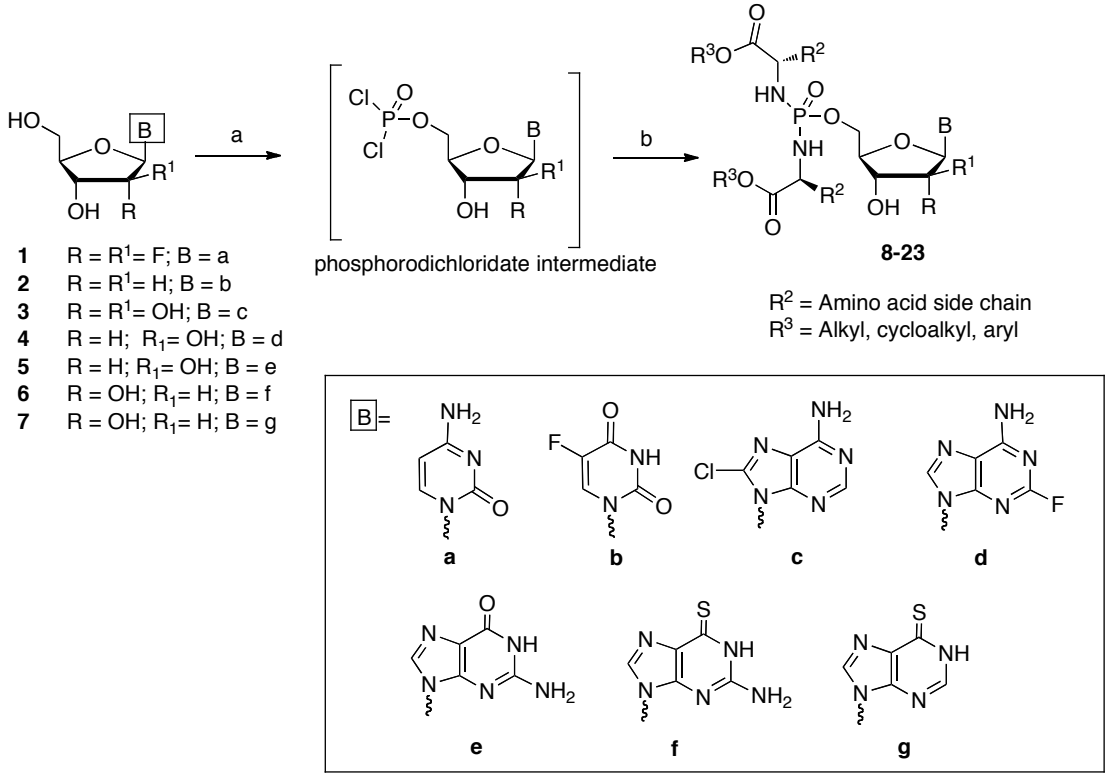
Figure 2. Anticancer nucleosides considered for this study.

Results and discussion

Chemistry

The phosphorodiamidate prodrugs of gemcitabine (**1**), FUdR (**2**), 8-chloroadenosine (**3**), fludarabine (**4**), AraG (**5**), thioinosine (6-MP, **6**), and thioguanosine (6-TG, **7**), were prepared according to reported procedure.[37] This modified method, based on a Yoshikawa procedure to synthesize monophosphate species,[38] was successfully applied previously to a number of antiviral and anticancer nucleosides and led to identification of phosphorodiamidates, with L-alanine bearing either benzyl or 2,2-dimethoxypropyl as the most promising compounds. [24-26] Thus, in this work, we selected the above-mentioned amino acid esters as preferred entities and synthesized in total sixteen phosphorodiamidate prodrugs of seven anticancer NAs. Moreover, for gemcitabine and FUdR, cyclohexyl, pentyl and cyclopentyl esters were also used, since these moieties afforded molecules with low submicromolar anticancer activity when applied in the ProTide approach.[18,19] Additionally, for 8-chloroadenosine, another natural amino acid such as glycine was also included. In the first step of a general one-pot-two-stages strategy, unprotected nucleoside analogue was treated with trimethylphosphate (TMP) and 1 equivalent of $POCl_3$, leading to the formation of the phosphorodichloridate intermediate as depicted in Scheme 1, the formation of the latter intermediate (not isolated) was monitored by ^{31}P NMR (δ_p = ~ 7-8 ppm) and was accomplished within 4-12 hours. Following this, addition of the

appropriate amino acid ester salt (5 equiv.) in the presence of DIPEA (10 equiv.) at –78 °C afforded the desired phosphorodiamidates **8-23** with the yields between 3% and 49%.



Scheme 1. General synthetic route to phosphorodiamidates **8-23**. *Reagents and conditions:* (a) $(Me)_3PO_4$ (1 mL), $POCl_3$ (2 equiv.), 0-5 °C, 16 h, (b) anhydrous DCM, amino acid ester salt (5 equiv.), DIPEA (10 equiv.), -78 °C (30 min.), then 0-5 °C, 72h.

Anticancer Activity

The anti-proliferative activities of nucleoside analogues and their related phosphorodiamidates derived from gemcitabine (**1**; **8-10**), FUdR (**2**; **11-14**), 8-chloroadenosine (**3**; **15**, **16**), fludarabine (**4**, **17**, **18**), AraG (**5**; **19**), 6-MP (**6**; **20**, **21**), 6-TG (**7**; **22**, **23**) are represented by their absolute EC_{50} (μM) and maximal inhibition at the highest compound concentration (%) and are reported in Table 1 (solid tumour cell lines) and Table 2 (hematologic cell lines). Gemcitabine was found to be the most effective compound in Mia-Pa-Ca-2 cell cultures at an EC_{50} of 1.04 μM. However, its prodrugs **8-10** were shown to be less active with EC_{50} ranging between 1.45-7.68 μM. In the same cell line, such loss of activity was also observed for the diamidates of FUdR **2**, with the prodrugs **11** and **13** showing EC_{50} of 3.54 μM and

57.77 μM, respectively, *versus* an EC_{50} of 0.18 μM for the nucleoside **2**. Conversely, the diamidate of 8-ClA **16** showed a 2-fold increase in anticancer potency compared with the parent compound **3** in the Mia-Pa-Ca-2 cell line. A similar trend and a 2-fold boost in activity for prodrug over nucleoside analogue were noticed for 8-ClA **3** and the phosphorodiamidate **16** in the MCF-7 cell line, which is generally (along with HepG2) the least sensitive tumour cell line to our tested compounds. In a panel of leukemia cell lines, while AraG **5** showed micromolar activity (EC_{50} of 1.35 μM for CCRF-CEM and 1.79 μM for MOLT-4), its phosphorodiamidate **19** was found ineffective (>198 μM) in the same cancer cell lines. This may be suggestive of a poor cellular uptake and/or metabolic bioactivation and conversion of the phosphorodiamidate **19** into the corresponding nucleoside monophosphate form. The cytostatic activities of 6-MP **6** and 6-TG **7** were similar at submicromolar level (for **6**: EC_{50} of 0.64 μM in CCRF-CEM and 0.83 μM in MOLT-4; for **7**: EC_{50} of 0.71 μM in HL-60). The two L-Ala-OBn phosphorodiamidates **20** and **22** were ~3-fold less active than the parent **6** and 5-fold less active than the parent **7**, respectively. A similar trend was noted for the two L-Ala-Oneopentyl prodrugs **21** and **23**, however with markedly higher loss of activity in respect to the nucleosides **6** and **7** (55-fold in CCRF-CEM, 46-fold in MOLT-4 for **21**; 54-fold in HL-60 for **23**). This was also observed for AraG phosphorodiamidate **19**. Thus, as already mentioned above, the significant reduction of potency of the prodrugs containing the neopentyl ester may indicate either poor cellular uptake and/or metabolic activation and/or higher susceptibility of L-Ala-Oneopentyl phosphorodiamidates as well as their metabolites to drug efflux pumps.[22] Generally, the maximal inhibition observed at the highest compound concentration (198 μM) for all tested compounds ranged between 79-100% except for **19**, the diamidate of AraG (22% in CCRF-CEM and 25% in MOLT-4).

Table 1. [a]EC_{50} (μM) and Max Inhibition (%) ($MI_{\%}$) of selected phosphorodiamidates for solid tumour cell lines.

Comp	NA	AA	Ester (R^3)	Ar	Cell lines							
					MCF-7		HepG2		HT29		Mia-Pa-Ca-2	
					EC_{50}	$MI_{\%}$	EC_{50}	$MI_{\%}$	EC_{50}	$MI_{\%}$	EC_{50}	$MI_{\%}$
Gem	**1**				>198	51	51.98	50	2.23	57	1.04[18]	80
8	**1**	L-Ala	Bn		>198	52	>198	39	>198	47	1.45	74
9	**1**	L-Ala	CH_2tBu		>198	49	178.12	55	84.96	54	7.68	75
10	**1**	L-Ala	cHex		>198	51	>198	53	70.54	56	4.48	77
NUC-1031	**1**	L-Ala	Bn	Ph	-	-	-	-	2.89	-	0.44[18]	-
FUdR	**2**				9.82	78	94.16	65	0.20	84	0.18	90
11	**2**	L-Ala	CH_2tBu		>198	52	>198	25	8.58	78	3.54	82
12	**2**	L-Ala	Pnt		-	-	-	-	-	-	-	-
13	**2**	L-Ala	cPnt		>198	48	>198	38	>198	47	57.77	74
14	**2**	L-Ala	Bn		-	-	-	-	-	-	-	-
NUC-3373	**2**	L-Ala	Bn	Naph	-	-	-	-	0.42	91	-	-
8-ClA	**3**				2.15	74	0.79	82	0.87	79	1.0	93
15	**3**	L-Ala	Bn		-	-	-	-	-	-	-	-
16	**3**	Gly	Bn		1.08	87	1.24	87	1.64	87	0.52	94

[a]EC_{50} Values (50% effective concentration on inhibition of cell viability), and $MI_{(\%)}$ (maximum inhibitory effect of the drug at the range of concentration considered). The compounds were added to the cell in duplicate and tested in 9 serial concentrations from 198 μM to 0.0199 μM.

Table 2. [a]EC_{50} (μM) and Max Inhibition (%) ($MI_{\%}$) of selected phosphorodiamidates for hematologic cell lines.

Comp	NA	AA	Ester (R^3)	Cell lines							
				CCRF-CEM		MOLT-4		HEL92.1.7		HL-60	
				EC_{50}	$MI_{\%}$	EC_{50}	$MI_{\%}$	EC_{50}	$MI_{\%}$	EC_{50}	$MI_{\%}$
8-ClA	**3**			0.46	89	0.53	99	0.52	96	0.52	99
16	**3**	Gly	Bn	0.41	93	0.5	100	0.35	96	0.39	100
AraG	**5**			1.35	101	1.79	100	-	-	-	-
19	**5**	L-Ala	CH_2tBu	>198	22	>198	25	-	-	-	-
6-MP	**6**			0.64	98	0.83	100	-	-	-	-
20	**6**	L-Ala	Bn	1.85	98	2.58	99	-	-	-	-
21	**6**	L-Ala	CH_2tBu	35.4	76	38.5	84	-	-	-	-
6-TG	**7**	-	-	-	-	-	-	-	-	0.71	95
22	**7**	L-Ala	Bn	-	-	-	-	-	-	3.70	92
23	**7**	L-Ala	CH_2tBu	-	-	-	-	-	-	38.6	79

[a]EC_{50} Values (50% effective concentration on inhibition of cell viability) and $MI_{(\%)}$ (maximum inhibitory effect of the drug at the range of concentration considered). The compounds were added to the cell in duplicate and tested in 9 serial concentrations from 198 μM to 0.0199 μM.

The IC_{50} values of FUdR, 8-ClA and fludarabine and their respective phosphorodiamidates *versus* mouse lymphocytic leukemia (L1210), human CD_4^+ T-lymphocyte cells (CEM), and human cervical carcinoma (HeLa) cells are reported in Table 3. FUdR (**2**) and its phosphorodiamidate prodrugs (**11-14**) showed activity in a submicromolar range with IC_{50} of 0.0046-0.073 μM for FUdR and 0.01-0.40 μM for the FUdR phosphorodiamidates against the wild-type cell lines. The L-alanine pentyl prodrug **12** proved ~2-fold more active against HeLa cell cultures and ~2-fold less active against L1210 and CEM cell cultures than FUdR. The loss of inhibitory

activity for compound **14** *versus* FUdR ranged between 3-13-fold, and for compound **11** between ~3-15-fold.

The cytostatic activity of FUdR strongly depends on the thymidine kinase (TK) enzyme responsible for the first phosphorylation step of FUdR to FdUMP. Therefore the marked loss of activity for **2** was expected in the TK-deficient tumour cell lines and was 10-fold against HeLa/TK$^-$, 100-fold against CEM/TK$^-$ and 217-fold against L1210/TK$^-$. Although the potency of the four FUdR phosphorodiamidates was also diminished in the TK-deficient tumour cell lines, the difference in fold- reduction was less dramatic (11- to 51-fold instead of 217-fold for the parent FUdR).

However, for the CEM/TK$^-$ and HeLa/TK$^-$ cell lines the loss in activity found for the FUdR phosphorodiamidates **11**-**14** was significantly higher than for FUdR. These data clearly indicate that the phosphorodiamidates **11**-**14** require TK to eventually exert biological activity, as they most likely release FUdR instead of the desired 5'-monophosphate metabolite FdUMP. Strikingly, the cytostatic activity spectrum of L-Ala-OBn-ONaph substituted FUdR prodrug, NUC-3373, that is not a phosphorodiamidate but contains the traditional ProTide structure, clearly differed from the phosphorodiamidate prodrugs. NUC-3373 not only showed pronounced cytostatic activity against the wild-type tumour cells (IC_{50}: 0.011-0.068 μM), but it remarkably retained considerable cytostatic potential against the TK-deficient tumour cell lines, in particular for the L1210/TK- and CEM/TK- cell lines.

The drugs **3** and **4** along with their phosphorodiamidates **15**, **16**, **17** and **18** were also investigated. Unlike for FUdR, TK activity is not a prerequisite for 8-Cl-adenosine and fludarabine. 8-Cl-adenosine and fludarabine showed micromolar IC_{50} and both were more active than their phosphorodiamidates. However, the difference in activity was less significant in case of the 8-chloroadenosine series with the benzyl L-alanine diamidate **15** being 2.6-3.4-fold less active than parent nucleoside **3**. The benzyl glycine diamidate **16** was found to be only ~1-5 to 2-fold less active than the parent 8-chloroadenosine.

Table 3. Cytostatic Activity ($^a IC_{50}$ in μM) reported for FUdR (**2**), 8-chloroadenosine (**3**) and fludarabine (**4**), and phosphorodiamidate prodrugs (**11**-**14**, **15**, **16**, **17** and **18**) against tumour cell lines.

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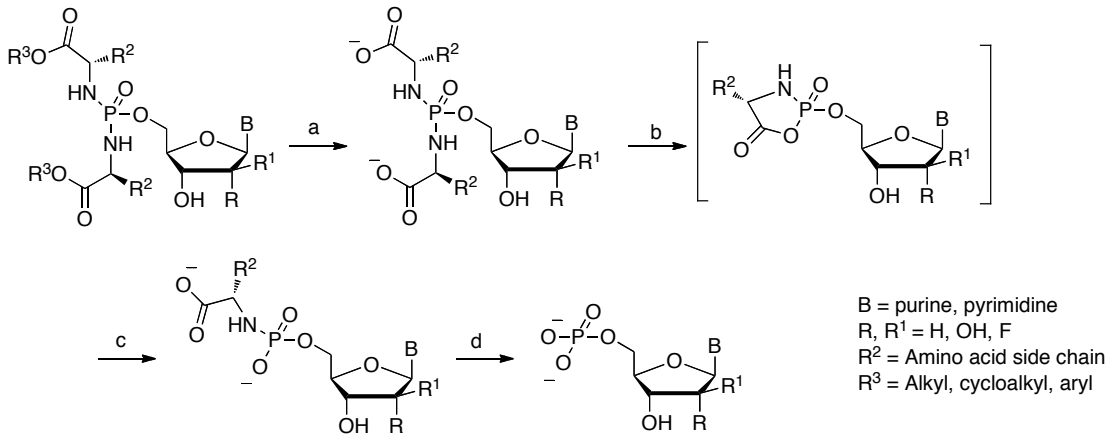
Comp	NA	AA	Ester (R^3)	Ar	IC_{50} (μM)								
					L1210	L1210/ TK^-	Ratio TK^+/TK^-	CEM	CEM/ TK^-	Ratio TK^+/TK^-	HeLa	HeLa/ TK^-	Ratio TK^+/TK^-
FUdR	**2**				0.0046± 0.0027	1.0±0.3	217	0.013±0.008	1.3±0.1	100	0.073±0.032	0.73± 0.16	10
11	**2**	L-Ala	CH_2tBu		0.069± 0.006	0.76±0.06	11	0.16± 0.11	15±8	94	0.19± 0.03	64±6	337
12	**2**	L-Ala	Pnt		0.01± 0.001	0.47±0.0	47	0.028±0.019	4.2±1.0	150	0.037±0.029	3.4±1.1	92
13	**2**	L-Ala	cPnt		0.16± 0.01	2.5±0.1	16	0.24± 0.02	56±21	233	0.4± 0.05	68±9	170
14	**2**	L-Ala	Bn		0.045± 0.013	2.3±0.6	51	0.17± 0.07	38±8	223	0.24± 0.05	16±8	67
NUC-3373	**2**	L-Ala	Bn	Naph	0.011± 0.007	0.045± 0.027	4	0.068±0.035	0.31± 0.06	4	0.065±0.013	2.5±1.3	38[19]
8-ClA	**3**				3.2±0.7	-	-	1.1±0.6	-	-	0.89± 0.5	-	-
15	**3**	L-Ala	Bn		10±4	-	-	3.8±0.2	-	-	2.3±1.0	-	-
16	**3**	Gly	Bn		4.6±0.0	-	-	3.4±0.4	-	-	1.8±0.4	-	-
Fludarabine	**4**				11±9	-	-	2.1±1.4	-	-	8.0±3.3	-	-
17	**4**	L-Ala	CH_2tBu		55±36	-	-	15±2	-	-	47±3	-	-
18	**4**	L-Ala	Bn		32±5	-	-	16±14	-	-	24±11	-	-

[a] IC_{50} or compound concentration required to inhibit tumour cell proliferation by 50%. Data are the mean (±SD) of at least two to four independent experiments.

Enzymatic Studies

The general putative mechanism of the bioactivation pathway for phosphorodiamidates is depicted in Scheme 2 and is similar to the metabolic route postulated for the phosphoramidates[39,40] involving four (a-d) consecutive steps: a) esterase or carboxypeptidase-mediated hydrolysis of one of the two esters; b) spontaneous intracellular displacement of the second amino acid through an intramolecular attack of the carboxylate anion on the phosphorus and further formation of a mixed five-membered ring anhydride; c) spontaneous hydrolysis of the anhydride intermediate; and finally d) P–N bond cleavage mediated by a phosphoramidase-type enzyme.[41,42]



Scheme 2. General putative activation pathway of phosphorodiamidate prodrugs: a) carboxypeptidase-mediated ester hydrolysis; b) spontaneous intracellular displacement; c) spontaneous hydrolysis; d) enzyme-mediated P–N bond cleavage.

Here, we report the carboxypeptidase Y assay performed on the gemcitabine phosphorodiamidate **8** in the presence of d_6-acetone and TRIZMA buffer (pH = 7.6), showing ester hydrolysis of one of the amino acid ester moieties during the bioactivation process. Upon the addition of carboxypeptidase Y, within the first 10 minutes of incubation, phosphorodiamidate **8** (δ_p = 14.14 ppm, Figure 2) was converted to the intermediate **8-A** lacking both ester moieties (peak at 14.82 ppm). Finally, the formation of the metabolite **8-C** lacking one of the amino acid (thought to be mediated by spontaneous cyclisation and hydrolysis) (**8-B**) was confirmed by the presence of the single peak at δ_p 7.04 ppm. This is analogous to results reported in our previous study.[24] No direct ^{31}P-NMR evidence was obtained for the formation of the

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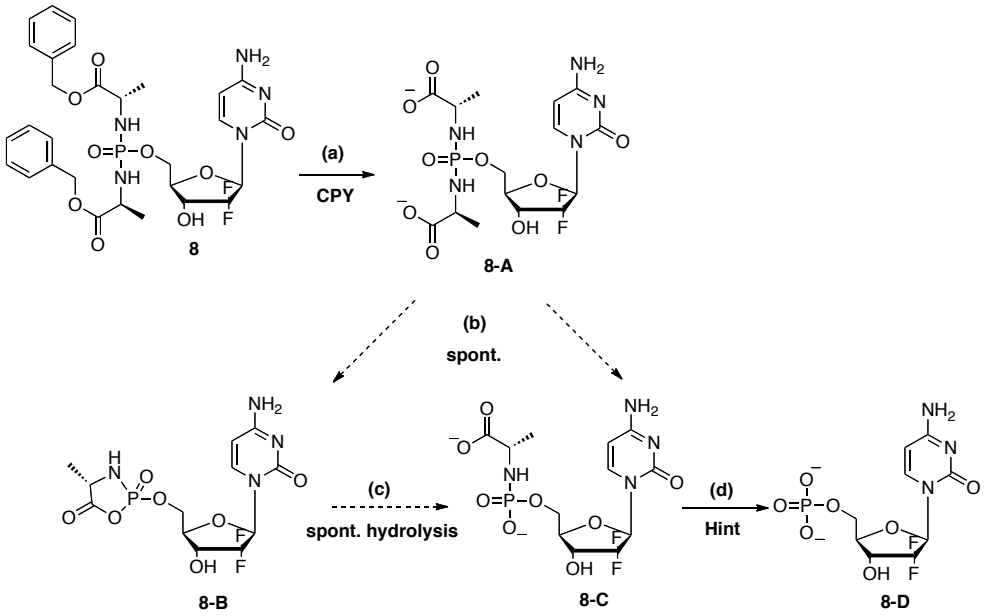
cyclic compound **8-B**, consistent with it being a short-lived metabolite. Another possibility could be that the metabolite **8-C** is formed spontaneously from the metabolite **8-A**, without a cyclisation step being involved. Compound **8** was fully processed to the aminoacyl metabolite **8-C** within approximately 3 h, with an estimated half-life of 6 minutes.



Scheme 3. Putative mechanism of bioactivation for phosphorodiamidate **8**.

The same activation pathway with a similar estimated half-life was obtained with inactive compound **18**, the phosphorodiamidate of AraG (data not shown). These results indicate that discrimination between active and inactive compounds might be ascribed to the last step in the activation pathway, catalysed by Hint, a phosphoramidase type enzyme.[41-43]

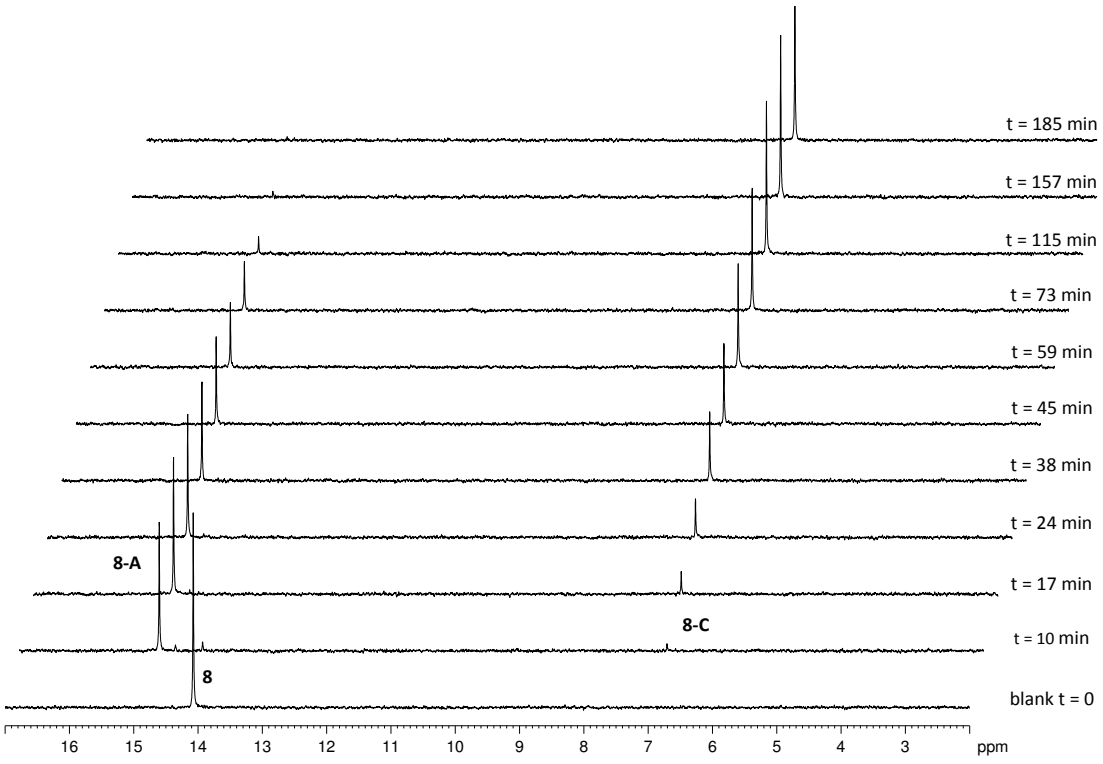


Figure 3. Carboxypeptidase Y-mediated cleavage of compound **8** as monitored by ^{31}P NMR.

Conclusion

In conclusion, we report on the application of the phosphorodiamidate approach leading to sixteen prodrugs derived from amino acids and seven anticancer nucleoside analogues. The biological *in vitro* screening of the phosphorodiamidates against a wide panel of tumour cell lines revealed a potential of this approach in case of FUdR and 8-ClA, since some of their corresponding phosphorodiamidate prodrugs showed similar or improved inhibitory activities when compared with the parent nucleosides. The carboxypeptidase Y assay supported the postulated bioactivation pathway as reported for one gemcitabine phosphorodiamidate **8**, for which a similar activity *versus* its parent compound was observed for the Mia-Pa-Ca-2 tumour cell line. The lack of activity found in the other families of compounds may indicate either poor bioactivation of the phosphorodiamidate motif with a subsequent poor release of the free 5′-monophosphate form (last step) or susceptibility of inactive phosphorodiamidates and their corresponding metabolites to drug efflux pump mechanisms. Overall, the wide range of *in vitro* data presented for the selected

phosphorodiamidates may demonstrate that the biological properties and activities of nucleoside analogues and their prodrugs are unpredictable due to the complexity of the nature of distinct cell systems and differences in levels and activity of enzymes involved in the uptake and conversion of the prodrugs to the eventual intracellular active metabolite.

Experimental Section

MTS Cell Viability Assay. The solid and hematologic tumour cell lines were seeded at a range of density 0.5 to 100 x 10^3 cells/well in the wells of a 96-well plate the day before compound incubation, then incubated for 72 hours with the different concentrations of the test compound. Then 50 μL of MTS was added and the cells were incubated for 4 h at 37 °C. The data were read and collected by a Spectra Max 340 Absorbance Microplate Reader. The compounds were tested in duplicate with 9 serial concentrations (3.16-fold titrations with 198 μM as the highest concentration), and the data were analyzed by XL-fit software.

Cell cultures and Cytostatic Activity Assays. Murine leukemia L1210, human lymphocyte CEM, human cervix carcinoma HeLa and their corresponding TK-deficient congeners L1210/TK$^-$, CEM/TK$^-$ and HeLa/TK$^-$ were seeded at –50 to 75 x 10^3 cells/200 μl in the wells of a 96-well microtiter plate that contained a variety of drug concentrations. After 48 hours (L1210) or 72-96 hours (CEM; HeLa) the cell number was determined by counting with a Coulter Counter. The IC_{50} (50% inhibitory concentration) was determined as the drug concentration required to inhibit tumor cell proliferation by 50%.

Carboxypeptidase Y (EC 3.4.16.1) Assay. The experiment was carried out by dissolving diamidate **8** (5.0 mg) in acetone-d_6 (0.15 mL) followed by addition of 0.30 mL of Trizma buffer (pH 7.6). After recording the control ^{31}P NMR at 25 °C, a previously defrosted carboxypeptidase Y (0.1 mg dissolved in 0.15 mL of Trizma) was added to the sample, which was then immediately submitted to the ^{31}P NMR analysis (at 25 °C). The spectra were recorded every 7 minutes for over 14 hours. ^{31}P NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program.

Chemistry. General Procedures. Solvents and Reagents. The following anhydrous

solvents were purchased from Sigma-Aldrich: dichloromethane (CH_2Cl_2), trimethylphosphate ($(Me)_3PO_4$), triethylphosphate ($(Et)_3PO_4$), diethyl ether (Et_2O), tetrahydrofuran (THF), dimethylformamide (DMF), and any other reagents used. Amino acid esters commercially available were purchased from Novabiochem. All reagents commercially available were used without further purification.

Thin Layer Chromatography (TLC). Precoated aluminium-backed plates (60 F254, 0.2 mm thickness, Merck) were visualized under both short and long wave ultraviolet light (254 and 366 nm) or by burning using the following TLC indicators: (i) molybdate ammonium cerium sulfate; (ii) potassium permanganate solution. Preparative TLC plates (20 cm × 20 cm, 500-2000 μm) were purchased from Merck.

Flash Column Chromatography. Flash column chromatography was carried out using silica gel supplied by Fisher (60A, 35-70 μm). Glass columns were slurry packed using the appropriate eluent with the sample being loaded as a concentrated solution in the same eluent or pre-adsorbed onto silica gel. Fractions containing the product were identified by TLC, and pooled and the solvent was removed *in vacuo*.

High Performance Liquid Chromatography (HPLC). The purity of the final compounds was verified to be >95% by HPLC analysis using either I) ThermoSCIENTIFIC, SPECTRA SYSTEM P4000, detector SPECTRA SYSTEM UV2000, Varian Pursuit XRs 5 C18, 150 x 4.6 mm (as an analytic column) or II) Varian Prostar (LC Workstation-Varian Prostar 335 LC detector), Thermo SCIENTIFIC Hypersil Gold C18, 5 μm, 150 x 4.6 mm (as an analytic column). For the method of elution see the experimental part. The purification of one final compound was achieved on Pursuit XRs 5 C18, 150 x 21.2 mm (as the semi-preparative column), Varian Prostar (LC Workstation-Varian Prostar 335 LC detector).

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 500 MHz spectrometer at 25 ºC. Chemical shifts (δ) are quoted in parts per million (ppm) relative to internal CH_3OH-d_4 (δ 3.34 ^{1}H-NMR, δ 49.86 ^{13}C-NMR) and $CHCl_3$-d_4 (δ 7.26 ^{1}H NMR, δ 77.36 ^{13}C NMR) or external 85 % H_3PO_4 (δ 0.00 ^{31}P NMR). Coupling constants (J) are measured in Hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), dt (doublet of triplet), app

(apparent). The assignment of the signals in ^{1}H NMR and ^{13}C NMR was done based on the analysis of coupling constants and additional two-dimensional experiments (COSY, HSQC, HMBC, PENDANT).

Mass spectromethry (MS). Low resolution mass spectra were performed on Bruker Daltonics microTof-LC, (atmospheric pressure ionization, electron spray mass spectroscopy) in either positive or negative mode.

Standard procedure A: synthesis of phosphorodiamidates. To a solution of nucleoside (1 eq) in trimethyl phosphate (1 mL per 1 mmol of nucleoside, unless otherwise stated), phosphoryl chloride (1-2 eq) was added dropwise at - 5 °C and the reaction mixture was left stirring for 4 - 12 hours. The formation of the intermediate was monitored by ^{31}P-NMR. A suspension of the appropriate amino acid ester (5 eq) in dichloromethane (4 mL per 1 mmol of amino acid ester) was added followed by diisopropyl ethyl amine (10 eq) at - 78 °C. After stirring at room temperature for 20 hours, water (10 mL per mmol of nucleoside) was added and the layers were separated. The aqueous phase was extracted with CH_2Cl_2 (10 mL per mmol of nucleoside) and the organic phase washed with brine (10 mL per mmol of nucleoside). The combined organic layers were dried over Na_2SO_4 and concentrated. The residue was purified unless otherwise stated either by column chromatography or Biotage Isolera One to give the title compound as a white solid.

2′-Deoxy-2′,2′-difluoro-D-cytidine-5′-*O*-bis(benzoxy-L-alaninyl)] phosphate (8): Prepared according to the standard procedure A from gemcitabine (0.25 g, 0.95 mmol), $POCl_3$ (0.29 g, 0.17 mL, 1.89 mmol), followed by addition of L-Ala benzyl ester tosylate salt (1.67 g, 4.75 mmol), DIPEA (1.23 g, 1.65 mL, 9.50 mmol). Column purification followed by preparative TLC purification gave the product **8**, as a white solid (0.079 g, 12%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.87; ^{19}F NMR (470 MHz, CD_3OD): δ = – 118.14 (d, *J* = 239 Hz), –119.70 (d, broad signal, *J* = 239 Hz); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.67 (d, *J* = 7.53 Hz, 1H, *H*-6), 7.38 – 7.31 (m, 10H, *H*-Ar), 6.27 (t, *J* = 8.05 Hz, 1H, *H*-1′), 5.97 (d, *J* = 7.51 Hz, 1H, *H*-5), 5.18 – 5.10 (m, 4H, 2 x C*H*$_2$Ph), 4.31 – 4.17 (m, 3H, *H*-5′, *H*-3′, *H*-5′), 4.02 – 3.95 (m, *H*-4′, 3H, 2 x NHC*H*CH$_3$), 1.38 (d, *J* = 7.14 Hz, 3H, NHCHC*H*$_3$), 1.36 (d, *J* = 7.14 Hz, 3H, NHCHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.43 (d, $^{3}J_{C\text{-}P}$ = 5.40 Hz, *C*=O, ester), 167.68 (*C*-2), 157.81 (*C*-4), 142.63 (*C*-6), 137.29, 137.28 (*C*-Ar), 129.85,

129.66, 129.65, 129.40, 129.39, 129.32, 127.02 (*C*H-Ar), 123.69 (apparent t, $^{1}J_{C\text{-}F}$ = 258 Hz, *C*F_2), 96.87 (*C*-5), 86.02 (apparent t, broad signal, $^{2}J_{C\text{-}F}$ = 27.0 Hz, *C*-1′), 80.46 (apparent t, $^{3}J_{C\text{-}F}$ = 8.20 Hz, *C*-4′), 71.28 (apparent t, $^{2}J_{C\text{-}F}$ = 23.44 Hz, *C*-3′), 68.00, 67.99 (*C*H_2Ph), 64.52 (d, $^{2}J_{C\text{-}P}$ = 4.78 Hz, *C*-5′), 51.15 (d, $^{2}J_{C\text{-}P}$ = 5.41 Hz, NH*C*HCH$_3$), 20.78, 20.67 (2 x d, $^{3}J_{C\text{-}P}$ = 5.53 Hz, NHCH*C*H$_3$); MS [ES+] *m/z*: found 666.22 [M + H^+], 688.21 [M + Na^+], $C_{29}H_{34}F_2N_5O_9P$ required 665.58 [M]. HRMS [ESI] *m/z*: calculated for $C_{29}H_{34}F_2N_5O_9P$: 665.2062; found 666.2058 [M + H]$^+$. Reverse-phase HPLC eluting with H_2O/CH_3OH from 100/0 to 0/100 in 35 min), F = 1 mL/min, λ = 254, t_R = 24.15 min.

2′-Deoxy-2′,2′-difluoro-D-cytidine-5′-*O*-bis(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (9): Prepared according to the standard procedure A from gemcitabine (0.25 g, 0.95 mmol), $POCl_3$ (0.29 g, 0.17 mL, 1.89 mmol), and $(Et)_3PO$ (1.0 mL), followed by addition of L-Ala neopentyl ester tosylate salt (1.57 g, 4.75 mmol), DIPEA (1.23 g, 1.65 mL, 9.50 mmol). Column purification followed by preparative TLC purification gave the compound **9** as a white solid (0.041 g, 7%); ^{31}P NMR (202 MHz, CD_3OD): δ = 13.93; ^{19}F NMR (470 MHz, CD_3OD): δ = –118.1 (d, *J* = 245 Hz), –119.6 (d, *J* = 245 Hz); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.70 (d, *J* = 7.58 Hz, 1H, *H*-6), 6.29 (t, *J* = 8.20 Hz, 1H, *H*-1′), 6.01 (d, *J* = 7.58 Hz, 1H, *H*-5), 4.37 – 4.33 (m, 1H, *H*-5′), 4.30 – 4.23 (m, 2H, *H*-5′, *H*-3′), 4.08 – 4.06 (m, 1H, *H*-4′), 4.03 – 3.96 (m, 2H, 2 x NHC*H*CH$_3$), 3.90, 3.93 (AB system, J_{AB} = 10.55 Hz, 2H, CH_2C(CH$_3$)$_3$), 3.79, 3.73 (AB system, J_{AB} = 11.0 Hz, 2H, CH_2C(CH$_3$)$_3$), 1.45 (d, *J* = 7.08 Hz, 3H, NHCHCH_3), 1.43 (d, *J* = 7.08 Hz, 3H, NHCHCH_3), 0.97 (s, 18H, 2 x CH_2C(CH_3)$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.71 (d, $^{3}J_{C\text{-}P}$ = 3.44 Hz, *C*=O, ester), 175.67 (d, $^{3}J_{C\text{-}P}$ = 3.44 Hz, *C*=O, ester), 167.70 (*C*-2), 157.79 (*C*-4), 142.68 (*C*-6), 123.65 (apparent t, $^{1}J_{C\text{-}F}$ = 258 Hz, *C*F_2), 96.84 (*C*-5), 86.04 (apparent t, $^{2}J_{C\text{-}F}$ = 26 Hz, *C*-1′), 80.48 (apparent t, $^{3}J_{C\text{-}F}$ = 8.51 Hz, *C*-4′), 75.46, 75.43 (*C*H_2C(CH$_3$)$_3$), 71.30 (t, $^{2}J_{C\text{-}F}$ = 23.0 Hz, *C*-3′), 64.57 (d, $^{2}J_{C\text{-}P}$ = 4.73 Hz, *C*-5′), 51.17 (d, $^{2}J_{C\text{-}P}$ = 7.78 Hz, NH*C*HCH$_3$), 32.35 (CH$_2$*C*(CH$_3$)$_3$), 32.33 (CH$_2$*C*(CH$_3$)$_3$), 26.7 (CH$_2$C(*C*H$_3$)$_3$), 21.08 (d, $^{3}J_{C\text{-}P}$ = 6.10 Hz, NHCH*C*H$_3$), 20.96 (d, $^{3}J_{C\text{-}P}$ = 6.10 Hz, NHCH*C*H$_3$); MS [ES+] *m/z*: found 626.28 [M + H^+], 648 [M + Na^+], $C_{25}H_{42}F_2N_5O_9P$ required 625.60 [M]. Reverse-phase HPLC eluting with H_2O/CH_3CN from 100/0 to 0/100 in 35 min), F = 1 mL/min, λ = 254, t_R = 17.27 min.

2′-Deoxy-2′,2′-difluoro-D-cytidine-5′-*O*-bis(cyclohexoxy-L-alaninyl)]-phosphate (10): Prepared according to the standard procedure A from gemcitabine (0.25 g, 0.95 mmol), $POCl_3$ (0.29 g, 0.17 mL, 1.89 mmol), followed by addition of L-Ala cyclohexyl ester HCl salt (0.99 g, 4.75 mmol), DIPEA (1.23 g, 1.65 mL, 9.50 mmol). Column purification followed by preparative TLC purification gave the product **10**, as a white solid (0.029 g, 5%); ^{31}P NMR (202 MHz, CD_3OD): δ = 13.94; ^{19}F NMR (470 MHz, CD_3OD): δ = –118.30 (d, *J* = 241 Hz), –119.78 (d, broad signal, *J* = 245 Hz); ^{1}H NMR (500 MHz, CD_3OD): δ = 7.70 (d, *J* = 7.15 Hz, 1H, *H*-6), 6.29 (t, *J* = 7.97 Hz, 1H, *H*-1′), 6.01 (d, *J* = 7.51 Hz, 1H, *H*-5), 4.79 – 4.73 (m, 2H, 2 x OC*H*-cyclohexyl), 4.37 – 4.33 (m, 1H, *H*-5′), 4.32 – 4.22 (m, 2H, *H*-3′, *H*-5′), 4.08 – 4.06 (m, 1H, *H*-4′), 3.93 – 3.89 (m, 2H, 2 x NHC*H*CH_3), 1.87 – 1.84 (m, 4H, 2 x CH_2-cyclohexyl), 1.77 – 1.74 (m, 4H, 2 x CH_2-cyclohexyl), 1.58 – 1.56 (m, 2H, CH_2-cyclohexyl), 1.48 – 1.33 (m, 16H, 5 x CH_2-cyclohexyl, 2 x NHCHCH_3); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.64 (d, $^{3}J_{C\text{-}P}$ = 5.37 Hz, *C*=O, ester), 167.69 (*C*-2), 157.79 (*C*-4), 142.66 (*C*-6), 123.66 (apparent t, $^{1}J_{C\text{-}F}$ = 259 Hz, *C*F_2), 96.85 (*C*-5), 86.00 (apparent t, broad signal, $^{2}J_{C\text{-}F}$ = 30 Hz, *C*-1′), 80.48 (apparent t, $^{3}J_{C\text{-}F}$ = 8.50 Hz, *C*-4′), 74.66, 74.56 (2 x *C*H-cyclohexyl), 71.29 (apparent t, $^{2}J_{C\text{-}F}$ = 25.0 Hz, *C*-3′), 64.54 (d, $^{2}J_{C\text{-}P}$ = 4.23 Hz, *C*-5′), 51.13 (d, $^{2}J_{C\text{-}P}$ = 13.0 Hz, NH*C*HCH_3), 32.55, 32.53, 32.48, 32.34, 26.58, 26.44, 24.79, 24.69 (*C*H_2-cyclohexyl), 21.15 (d, $^{3}J_{C\text{-}P}$ = 5.52 Hz, NHCH*C*H_3), 20.93 (d, $^{3}J_{C\text{-}P}$ = 5.52 Hz, NHCH*C*H_3); MS [ES+] *m/z*: found 650.28 [M + H^+], 672.26 [M + Na^+], $C_{27}H_{42}F_2N_5O_9P$ required 649.62 [M]. Reverse-phase HPLC eluting with H_2O/CH_3CN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, t_R = 17.72 min.

5-Fluoro-2′-deoxyuridine-5′-*O*-bis(2,2-dimethylpropoxy-L-alaninyl) phosphate (11): Prepared according to the standard procedure A from FUdR (0.25 g, 1.01 mmol), $POCl_3$ (0.18 mL, 2.03 mmol), the tosylate salt of 2,2-dimethylpropoxy-L-alanine (1.68 g, 5.07 mmol) and DIPEA (1.77 mL, 10.15 mmol). After work-up and silica gel column chromatography phosphorodiamidate **11** was obtained as an off-white solid (0.020 g, 3%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.94; ^{19}F NMR (470 MHz, CD_3OD): δ = –167.40; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.86 (d, *J* = 6.2 Hz, 1H, *H*-6), 6.35 (t, *J* = 5.2 Hz, 1H, *H*-1′), 4.48 – 4.45 (m, 1H, *H*-3′), 4.26 – 4.20 (m,

2H, *H*-5′), 4.18 – 4.15 (m, 1H, *H*-4′), 4.08 – 4.00 (m, 2H, 2 x NHC*H*CH_3), 3.86 – 3.78 (m, 4H, 2 x CH_2C$(CH_3)_3$), 2.38 – 2.35 (m, 1H, *H*-2′), 2.33 – 2.29 (m, 1H, *H*-2′), 1.48 – 1.43 (m, 6H, 2 x NHCHCH_3), 1.00 (s, 18H, 2 x CH_2C(C$H_3)_3$); ^{13}C NMR (125 MHz, CD_3OD): δ_C 175.6 (d, $^3J_{C–P}$ = 3.7 Hz, *C*=O, ester), 159.8 (d, $^2J_{C–F}$ = 25.0 Hz, *C*-4), 151.0 (*C*-2), 141.7 (d, $^1J_{C–F}$ = 232.5 Hz, *C*-5), 125.9 (d, $^2J_{C–F}$ = 33.75 Hz, *C*-6), 86.8 (*C*-1′), 86.6 (d, $^3J_{C-P}$ = 8.75 Hz, *C*-4′), 75.4, 75.3 (*C*H_2C$(CH_3)_3$), 71.9 (*C*-3′), 66.4 (d, $^2J_{C–P}$ = 5.0 Hz, *C*-5′), 51.2 (d, $^2J_{C–P}$ = 7.5 Hz, *C*HCH_3), 40.7 (*C*-2′), 32.4 (CH_2*C*$(CH_3)_3$), 26.8 (CH_2C(*C*$H_3)_3$), 20.7, (2 x d, $^3J_{C–P}$ = 5.0 Hz, NHCH*C*H_3). MS [ES+] *m/z*: found 631.64 [M + Na^+], $C_{25}H_{42}FN_4O_{10}P$ required 608.59 [M]. Reverse HPLC, eluting with H_2O/CH_3OH from 100/0 to 0/100 in 30 min, F = 1 mL/min, λ = 254, t_R = 22.59.

5-Fluoro-2′-deoxyuridine-5′-*O*-bis(pentoxy-L-alaninyl) phosphate (12): Prepared according to the standard procedure A from FUdR (0.35 g, 1.42 mmol), $POCl_3$ (0.26 mL, 2.84 mmol), the tosylate salt of pentoxy-L-alanine (1.39 g, 7.10 mmol) and DIPEA (2.47 mL, 14.20 mmol). After work-up and silica gel column chromatography phosphorodiamidate **12** was obtained as an off-white solid (0.050 g, 6%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.91; ^{19}F NMR (470 MHz, CD_3OD): δ = – 167.35; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.90 (d, *J* = 6.2 Hz, 1H, *H*-6), 6.30 (t, *J* = 5.5 Hz, 1H, *H*-1′), 4.49 – 4.45 (m, 1H, *H*-3′), 4.20 – 4.15 (m, 7H, 2 x O*CH*$_2$, 2 x *H*-5′, *H*-4′), 3.90 – 3.85 (m, 2H, 2 x NHC*H*CH_3), 2.38 – 2.35 (m, 1H, *H*-2′), 2.29 – 2.20 (m, 1H, *H*-2′), 1.72 – 1.60 (m, 4H, 2 x CH_2), 1.43 – 1.30 (m, 14H, 4 x CH_2, 2 x NHCHCH_3), 1.00 – 0.96 (m, 6H, 2 x CH_2CH_3); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.70, 175.67 (2d, $^3J_{C–P}$ = 3.7 Hz, *C*=O, ester), 160.10 (d, $^2J_{C–F}$ = 26.0 Hz, *C*-4), 151.30 (*C*-2), 141.78 (d, $^1J_{C–F}$ = 235.0 Hz, *C*-5), 125.85 (d, $^2J_{C–F}$ = 35.0 Hz, *C*-6), 86.90 (*C*-1′), 86.78 (d, $^3J_{C–P}$ = 7.5 Hz, *C*-4′), 71.95 (*C*-3′), 66.48, 66.47 (O*C*H_2), 66.38 (d, $^2J_{C–P}$ = 5.0 Hz, *C*-5′), 51.15, 50.64 (2d, $^2J_{C–P}$ = 8.5 Hz, NH*C*HCH_3), 40.79 (C-2′), 29.43, 29.20 (*C*H_2), 23.61, 23.40 (*C*H_2), 20.95, 20.26 (2 x d, $^3J_{C–P}$ = 6.25 Hz, NHCH*C*H_3). MS [ES+] *m/z*: found 631.59 [M + Na^+], $C_{25}H_{42}FN_4O_{10}P$ required 608.59 [M]. Reverse HPLC, eluting with H_2O/CH_3CN from 100/0 to 0/100 in 30 min, F = 1 mL/min, λ = 254, t_R = 15.88.

5-Fluoro-2′-deoxyuridine-5′-*O*-bis(cyclopentoxy-L-alaninyl) phosphate (13): Prepared according to the standard procedure A from FUdR (0.35 g, 1.42 mmol),

$POCl_3$ (0.26 mL, 2.84 mmol), the tosylate salt of cyclopentoxy-L-alanine (2.34 g, 7.10 mmol) and DIPEA (2.47 mL, 14.20 mmol). After work-up and silica gel column chromatography phosphorodiamidate **13** was obtained as an off-white solid (0.075 g, 9%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.99; ^{19}F NMR (470 MHz, CD_3OD): δ = –167.47; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.00 (d, *J* = 6.5 Hz, 1H, *H*-6), 6.26 (t, *J* = 5.5 Hz, 1H, *H*-1′), 5.24 – 5.15 (m, 2H, OC*H*), 4.48 – 4.45 (m, 1H, *H*-3′), 4.32 – 4.29 (m, 2H, *H*-5′), 4.15 – 4.08 (m, 1H, *H*-4′), 3.95 – 3.86 (m, 2H, 2 x NHC*H*CH_3), 2.38 – 2.35 (m, 1H, *H*-2′), 2.29 – 2.24 (m, 1H, *H*-2′), 2.00 – 1.89 (m, 4H, 2 x C*H*$_2$), 1.72 – 1.59 (m, 10H, 5 x C*H*$_2$), 1.59 – 1.52 (m, 2H, C*H*$_2$), 1.48 – 1.30 (m, 6H, 2 x NHCHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.45, 175.41 (2d, $^{3}J_{C–P}$ = 2.5 Hz, *C*=O, ester), 159.76 (d, $^{2}J_{C\ F}$ = 25.0 Hz, *C*-4), 151.06 (*C*-2), 142.0 (d, $^{1}J_{C–F}$ = 232.5 Hz, *C*-5), 125.95 (d, $^{2}J_{C–F}$ = 34.0 Hz, *C*-6), 86.86 (*C*-1′), 86.78 (d, $^{3}J_{C–P}$ = 7.5 Hz, *C*-4′), 79.50 (O*C*H), 71.95 (*C*-3′), 66.42 (d, $^{2}J_{C–P}$ = 5.0 Hz, *C*-5′), 51.20, 50.80 (2d, $^{2}J_{C–P}$ = 8.5 Hz, NH*C*HCH$_3$), 40.75 (*C*-2′), 33.84, 33.70, 33.52, 33.49 (*C*H$_2$), 24.70, 24.69 (*C*H$_2$), 20.94, 20.85 (2d, $^{3}J_{C–P}$ = 6.25 Hz, CH*C*H$_3$). MS [ES+] *m/z*: found 627.222 [M + Na^+], $C_{25}H_{38}FN_4O_{10}P$, required 604.56 [M]. HRMS [ESI] *m/z*: calculated for $C_{25}H_{38}FN_4O_{10}P$: 604.2310; found 605.2314 $[M + H]^+$. Reverse HPLC, eluting with H_2O/CH_3CN from 100/0 to 0/100 in 30 min, F = 1 mL/min, λ = 254, t_R = 13.39.

5-Fluoro-2′-deoxyuridine-5′-*O*-bis(benzoxy-L-alaninyl) phosphate (14): Prepared according to the standard procedure A from FUdR (0.25 g, 1.01 mmol), $POCl_3$ (0.18 mL, 2.03 mmol), the tosylate salt of benzoxy-L-alanine (1.78 g, 5.07 mmol) and DIPEA (1.76 mL, 10.15 mmol). After work-up and silica gel column chromatography phosphorodiamidate **14** was obtained as an off-white solid (0.030 g, 5%). ^{31}P NMR (202 MHz, CD_3OD): δ =13.82; ^{19}F NMR (470 MHz, CD_3OD): δ = –167.51; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.90 (d, *J* = 6.5 Hz, 1H, *H*-6), 7.38 – 7.31 (m, 10 H, Ar-*H*), 6.25 – 6.22 (m, 1H, *H*-1′), 5.22 – 5.10 (m, 4H, 2 x C*H*$_2$Ph), 4.39 – 4.36 (m, 1H, *H*-3′), 4.16 – 4.07 (m, 2H, *H*-5′), 4.01 – 3.95 (m, 3H, *H*-4′, 2 x NHC*H*CH_3), 2.31 – 2.26 (m, 1H, *H*-2′), 2.20 – 2.14 (m, 1H, *H*-2′), 1.42 – 1.34 (m, 6H, 2 x NHCHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ_C = 175.3 (*C*=O, ester), 159.3 (*C*-4), 150.5 (*C*-2), 141.5 (d, $^{1}J_{C–F}$ = 238.0 Hz, *C*-5), 137.3, 137.1 (2 x *C* ipso-Ph), 129.7, 129.6, 129.5, 129.4, 129.3, 129.2, 128.0 (*C*H-Ar), 125.8 (d, $^{2}J_{C–F}$ = 33.75 Hz, *C*-6), 122.5, 116.3 (*C*H-Ar), 86.8 (*C*-1′), 86.7 (d, $^{3}J_{C–P}$ = 8.7 Hz, *C*-4′), 71.3 (*C*-3′), 68.0, 67.9 (*C*H$_2$Ph), 66.4 (d, $^{2}J_{C–P}$ =

5.0 Hz, *C*-5′), 51.2, 51.1 (NH*C*HCH$_3$), 40.7 (C-2′), 20.7, 20.6 (2 x d, $^3J_{C-P}$ = 6.2 Hz, NHCH*C*H$_3$). MS [ES+] *m/z*: found 671.20 [M + Na$^+$], $C_{29}H_{34}FN_4O_{10}P$ required 648.57 [M]. Reverse HPLC, eluting with H_2O/CH_3CN from 100/0 to 0/100 in 30 min, F = 1 mL/min, λ = 254, t_R = 13.88.

8-Chloroadenosine 5′-*O*-bis(benzoxy-L-alaninyl)phosphate (15): Prepared according to the general procedure A, using 8-chloroadenosine (0.25 g, 0.83 mmol), in $(CH_3O)_3PO$ (5 mL), $POCl_3$ (77 μL, 0.83 mmol), L-alanine-*O*-benzyl ester tosylate salt (1.46 g, 4.15 mmol) in CH_2Cl_2 (5 mL) and DIPEA (1.45 mL, 8.3 mmol). The crude was purified by column chromatography (gradient elution of 4-7% CH_3OH/CH_2Cl_2) to give the title compound **15** as a white solid (0.24 g, 42%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.54; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.21 (s, 1H, *H*-2), 7.36 – 7.26 (m, 10H, *H*-Ph), 6.02 (d, *J* = 4.8 Hz, 1H, *H*-1′), 5.30 (t, *J* = 5.5 Hz, 1H, H2′), 5.14 – 5.02 (m, 4H, 2 x C*H*$_2$Ph), 4.62 (dd, *J* = 7.5, 5.0 Hz, 1H, *H*-3′), 4.31 – 4.24 (m, 1H, *H*-5′), 4.18 – 4.11 (m, 2H, *H*-5′, *H*-4′), 3.95-3.82 (m, 2H, NHC*H*CH$_3$), 1.28 (d, *J* = 7.2 Hz, 3H, CHC*H*$_3$), 1.23 (d, *J* = 7.2 Hz, 3H, CHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.34 (d, $^3J_{C-P}$= 5.0 Hz, *C*=O), 175.30 (d, $^3J_{C-P}$= 5.0 Hz, *C*=O), 156.35 (*C*-6), 154.22 (*C*-2), 151.71 (*C*-4), 139.83 (*C*-Ar), 137.32 (*C*-Ar), 137.27 (*C*-Ar), 129.58 (*C*H-Ar), 129.56 (*C*H-Ar), 129.32 (*C*H-Ar), 129.28 (*C*H-Ar), 119.55 (*C*-5), 91.28 (*C*-1′), 84.50 (d, $^3J_{C-P}$= 7.3 Hz, *C*-4′), 72.65 (*C*-2′), 71.47 (*C*-3′), 67.91 (*C*H$_2$Ph), 67.86 (*C*H$_2$Ph), 66.17 (d, $^2J_{C-P}$= 4.4 Hz, *C*-5′), 51.01 (NH*C*HCH$_3$), 50.98 (NH*C*HCH$_3$), 20.81 (d, $^3J_{C-P}$= 6.0 Hz, NHCH*C*H$_3$), 20.53 (d, $^3J_{C-P}$= 6.0 Hz, NHCH*C*H$_3$). MS [ES+] m/z: found 704.1 [M + H$^+$], 726.1 [M + Na$^+$], $C_{30}H_{35}ClN_7O_9P$ required 704.07 [M]. HRMS [ESI+] *m/z*: calculated for $C_{30}H_{35}ClN_7O_9P$: 703.1922; found 704.0612 [M + H]$^+$. Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 35 minutes, F = 1 mL/min, λ = 254, t_R 24.98 min.

8-Chloroadenosine 5′-*O*-bis(benzoxy-glycinyl) phosphate (16): Prepared according to the general procedure A, using 8-chloroadenosine (0.10 g, 0.33 mmol), in $(CH_3O)_3PO$ (2.5 mL), $POCl_3$ (31 μL, 0.33 mmol), glycine-*O*-benzyl ester hydrochloride salt (0.33 g, 1.65 mmol), in CH_2Cl_2 (5 mL) and DIPEA (0.57 mL, 3.3 mmol). The crude was purified by column chromatography (gradient elution of 4-7%

CH_3OH in CH_2Cl_2) to give the title compound as a white solid (0.06 g, 27%). ^{31}P NMR (202 MHz, CD_3OD): δ = 15.96; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.23 (s, 1H, H2), 7.35 – 7.26 (m, 10H, *H*-Ph), 6.03 (d, *J* = 5.1 Hz, 1H, *H*-1′), 5.31 (dd, *J* = 5.5, 5.1 Hz, 1H, *H*-2′), 5.11 – 5.08 (m, 4H, 2 x C*H*$_2$Ph), 4.59 (dd, 5.5 Hz, 5.1 Hz, 1H, *H*-3′), 4.34 – 4.28 (m, 1H, *H*-5′), 4.27 – 4.16 (m, 2H, *H*-5′, *H*-4′), 3.70 – 3.64 (m, 4H, 2 x NHC*H*$_2$); ^{13}C NMR (125 MHz, CD_3OD): δ = 172.91 (d, $^{3}J_{C\text{-}P}$= 4.7 Hz, *C*=O), 172.89 (d, $^{3}J_{C\text{-}P}$= 4.7 Hz, *C*=O), 156.24 (C6), 154.08 (*C*-2), 151.69 (*C*-4), 139.88 (*C*-Ar), 137.22 (*C*-Ar), 129.57 (*C*H-Ar), 129.56 (*C*H-Ar), 129.33 (*C*H-Ar), 129.31 (*C*H-Ar), 119.56 (*C*-5), 91.16 (*C*-1′), 84.70 (d, $^{3}J_{C\text{-}P}$= 7.2 Hz, *C*-4′), 72.62 (*C*-2′), 71.58 (*C*-3′), 67.86 (*C*H$_2$Ph), 67.84 (*C*H$_2$Ph), 66.17 (d, $^{2}J_{C\text{-}P}$= 4.6 Hz, *C*-5′), 43.56 (NH*C*H$_2$), 43.47 (NH*C*H$_2$). MS [ES+] m/z: found 704.1 [M + H$^+$], 726.1 [M + Na$^+$], $C_{28}H_{31}ClN_7O_9P$ required 676.01 (M). Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 35 minutes, F = 1 mL/min, λ = 254, t_R 23.23 min.

2-Fluoroadenine-9-β-D-arabinofuranoside-5′-*O*-bis(2,2-dimethylpropoxy-L-alaninyl) phosphate (17): Prepared according to the standard procedure A from fludarabine (0.25 g, 0.88 mmol), $POCl_3$ (0.16 mL, 1.75 mmol), the tosylate salt of 2,2-dimethylpropoxy-L-alanine (1.45 g, 4.38 mmol) and DIPEA (1.52 mL, 8.76 mmol). After work-up and silica gel column chromatography phosphorodiamidate **17** was obtained as an off-white solid (0.052 g, 9%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.84; ^{19}F NMR (470 MHz, CD_3OD): δ = –53.28; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.25 (s, 1H, *H*-8), 7.40 – 7.20 (m, 10 H, Ar-*H*), 6.32 (d, *J* = 4.3 Hz, 1H, *H*-1′), 4.46 – 4.25 (m, 4H, *H*-2′, H-3′, 2 x *H*-5′), 4.20 – 4.15 (m, 1H, *H*-4′), 3.98 – 3.94 (m, 2H, 2 x NHC*H*CH$_3$), 3.87, 3.84 (AB system, J_{AB} = 10.5 Hz, 2H, C*H*$_2$C(CH$_3$)$_3$), 3.74, 3.72 (AB system, J_{AB} = 10.5 Hz, 2H, C*H*$_2$C(CH$_3$)$_3$), 1.44 – 1.39 (m, 6H, 2 x NHCHC*H*$_3$), 0.99 (s, 18H, CH$_2$C(C*H*$_3$)$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.74, 175.66 (2d, $^{3}J_{C–P}$ = 5.0 Hz, *C*=O, ester), 160.70 (d, $^{1}J_{C–F}$ = 207.5 Hz, *C*-2), 158.90 (d, $^{3}J_{C–F}$ = 20.0 Hz, *C*-6), 152.26 (d, $^{3}J_{C–F}$ = 18.8 Hz, *C*-4), 142.69 (*C*-8), 117.84 (d, $^{4}J_{C–F}$ = 3.75 Hz, *C*-5), 86.07 (*C*-1′), 84.06 (d, $^{4}J_{C–P}$ = 7.5 Hz, *C*-3′), 77.31, 77.15 (*C*-2′, *C*-4′), 75.42 (*C*H$_2$C(CH$_3$)$_3$), 66.35 (d, $^{2}J_{C–P}$ = 5.0 Hz, *C*-5′), 51.2 (d, $^{2}J_{C–P}$ = 8.75 Hz, *C*HCH$_3$), 32.34, 32.32 (CH$_2$*C*(CH$_3$)$_3$), 26.82 (CH$_2$C(*C*H$_3$)$_3$), 21.15 (d, $^{3}J_{C–P}$ = 6.30 Hz, NHCH*C*H$_3$), 21.02 (d, $^{3}J_{C–P}$ = 6.30 Hz, NHCH*C*H$_3$). [ES+] *m/z*: found 670.61 [M +

Na^+], $C_{26}H_{43}FN_7O_9P$ required 647.63 [M]. HRMS [ESI+] *m/z*: calculated for $C_{26}H_{43}FN_7O_9P$: 647.2844 found 647.2839 $[M + H]^+$. Reverse HPLC, eluting with H_2O/CH_3OH from 100/0 to 0/100 in 30 min, F = 1 mL/min, λ = 254, t_R = 25.52.

2-Fluoroadenine-9-β-D-arabinofuranoside-5′-*O*-bis(benzoxy-L-alaninyl) phosphate (18): Prepared according to the standard procedure A from fludarabine (0.25 g, 0.88 mmol), $POCl_3$ (0.16 mL, 1.75 mmol), the tosylate salt of benzoxy-L-alanine (1.50 g, 4.38 mmol) and DIPEA (1.52 mL, 8.76 mmol). After work-up and silica gel column chromatography phosphorodiamidate **18** was obtained as an off-white solid (0.016g, 3%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.76; ^{19}F NMR (470 MHz, CD_3OD): δ = –53.30; ^1H NMR (500 MHz, CD_3OD): δ = 8.25 (s, 1H, *H*-8), 7.40 – 7.20 (m, 10 H, Ar-*H*), 6.32 (d, *J* = 4.4 Hz, 1H, *H*-1′), 5.22 – 5.15 (m, 4H, 2 x CH_2Ph), 4.40 – 4.36 (m, 1H, *H*-2′), 4.32 – 4.24 (m, 3H, 2 x *H*-5′, *H*-4′), 4.19 – 4.00 (m, 1H, *H*-3′), 3.98 – 3.94 (m, 2H, NHC*H*CH_3), 1.45 – 1.38 (m, 6H, 2 x NHCHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.56, 175.48 (2d, $^3J_{C–P}$ = 5.0 Hz, *C*=O, ester), 160.60 (d, $^1J_{C–F}$ = 207.5 Hz, *C*-2), 158.85 (d, $^3J_{C–F}$ = 20.0 Hz, *C*-6), 152.18 (d, $^3J_{C–F}$ = 20.0 Hz, *C*-4), 142.69 (*C*-8), 137.26, 137.21 (2 x *C* ipso-Ph), 129.86, 129.63, 129.61, 129.38, 129.33, 129.32, 129.29, 129.25 (*C*H-Ar), 117.86 (*C*-5), 86.02 (*C*-1′), 83.96 (d, $^4J_{C–P}$ = 7.5 Hz, *C*-3′), 77.25 (C-4′), 77.15 (C-2′), 67.98, 67.85 (*C*H_2Ph), 66.30 (d, $^2J_{C–P}$ = 5.0 Hz, *C*-5′), 51.2 (d, $^2J_{C–P}$ = 8.75 Hz, NH*C*HCH$_3$), 20.85 (d, $^3J_{C–P}$ = 6.30 Hz, NHCH*C*H$_3$), 20.72 (d, $^3J_{C–P}$ = 6.30 Hz, NHCH*C*H$_3$). [ES+] *m/z*: found 710.22 [M + Na^+] $C_{30}H_{35}FN_7O_9P$ required 687.61 [M]. Reverse HPLC, eluting with H_2O/CH_3OH from 100/0 to 0/100 in 30 min, F = 1 mL/min, λ = 254, t_R = 23.08.

Guanine-9-β-D-arabinofuranoside-5′-*O*-bis(2,2-dimethoxypropoxy-L-alaninyl) phosphate (19): Prepared according to general procedure A from AraG (0.40 g, 1.41 mmol), $POCl_3$ (0.216 g, 0.131 mL), the tosylate salt of 2,2-dimethoxypropoxy-L-alanine (2.33 g, 7.05 mmol) and DIPEA (2.45 mL, 14.1 mmol). After the reaction was completed the solvent was removed under vacuum and the crude material was purified by column chromatography on silica gel followed by preparative HPLC (reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 20 minutes, F = 20 ml/min, l = 254 nm, t_R = 10.72 min) to afford phosphorodiamidate **19** as a white solid (0.050 g, 6%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.88; ^1H NMR (500 MHz,

CD_3OD): δ_H 7.90 (s, 1H, *H*-8), 6.37 (d, J = 4.6 Hz, 1H, *H*-1′), 4.33 – 4.27 (m, 4H, 2 x *H*-5′, *H*-4′, *H*-2′), 4.07 – 4.05 (m, 2H, *H*-3′), 3.99 – 3.95 (m, 2H, 2 x NHC*H*CH_3), 3.88, 3.78 (AB system J_{AB} = 10.50 Hz, 2H, C*H*$_2$C$(CH_3)_3$), 3.87, 3.77 (AB system, J_{AB} = 10.50 Hz, 2H, C*H*$_2$C$(CH_3)_3$), 1.42 (d, J = 7.5 Hz, 3H, NHCHC*H*$_3$), 1.40 (d, J = 7.5 Hz, 3H, NHCHC*H*$_3$), 0.98 (s, 18H, CH_2C(C*H*$_3)_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.70, 175.65 (*C*=O, ester), 159.35 (*C*-6), 155.36 (*C*-2), 152.99, (*C*-4), 139.61 (*C*-8), 117.09 (*C*-5), 85.82 (*C*-1′), 84.00 (d $^3J_{C\text{-}P}$ = 7.36 Hz, *C*-4′), 77.59 (*C*-2′), 77.25 (*C*-3′), 75.39, 75.37 (*C*H_2C$(CH_3)_3$, 66.49 (d, $^2J_{C\text{-}P}$ = 5.26 Hz, *C*-5′), 51.14 (*C*HCH_3), 32.31 (CH_2*C*$(CH_3)_3$), 26.66 (CH_2C(*C*$H_3)_3$), 21.09 (d, $^3J_{C\text{-}P}$ = 5.97 Hz, NHCH*C*H_3), 20.95 (d, $^3J_{C\text{-}P}$ = 6.44 Hz, NHCH*C*H_3); MS [ES+] *m/z:* found 646 [M + H^+], 669 [M + Na^+], $C_{26}H_{44}N_7O_{10}P$ required: 645.28 (M). HRMS [ESI+] m/z: calculated for $C_{26}H_{44}N_7O_{10}P$: 645.2887; found 646.2881 [M + H]$^+$. HPLC Reverse-phase HPLC eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, F = 1ml/min, l = 254 nm, t_R = 14.93 min.

6-Thioinosine-5′-*O*-bis(benzoxy-L-alaninyl) phosphate (20): Prepared according to the standard procedure A from 6-thioinosine (0.15 g, 0.53 mmol), $POCl_3$ (0.05 mL, 0.53 mmol), the tosylate salt of benzoxy-L-alanine (0.88 g, 2.50 mmol) and DIPEA (0.91 mL, 5.01 mmol). After work-up and silica gel column chromatography phosphorodiamidate **20** was obtained as an off-white solid (0.014 g, 4%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.71; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.40 (s, 1H, *H*-2), 8.15 (s, 1H, *H*-8), 7.35 – 7.29 (m, 10 H, Ar-*H*), 6.01 (d, J = 4.5 Hz, 1H, *H*-1′), 5.15, 5.13, 5.10, 5.07 (2 x AB system, J_{AB} = 12.5, 4.5 Hz, 4H, 2 x C*H*$_2$Ph), 4.70 (t, J = 4.5 Hz, 1H, *H*-2′), 4.40 (t, J = 4.5 Hz, 1H, *H*-3′), 4.25 – 4.14 (m, 3H, 2 x *H*-5′, *H*-4′), 3.94 – 3.91 (m, 2H, 2 x NHC*H*CH_3), 1.32, 1.29 (2 x d, J = 7.0 Hz, 2 x NHCHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 178.27 (*C*=S), 175.44, 175.38 (2d, $^3J_{C\text{–}P}$ = 5.0 Hz, *C*=O, ester), 145.03 (*C*-2), 142.90 (*C*-4), 142.80 (*C*-8), 137.31, 137.27 (2 x *C* ipso-Ph), 137.05 (*C*-5), 129.58, 129.57, 129.31, 129.30, 127.17 (*C*H-Ar), 90.42 (*C*-1′), 84.74, 84.70 (d, $^3J_{C\text{–}P}$ = 8.2 Hz, *C*-4′), 75.53 (C-2′), 71.57 (*C*-3′), 67.99, 67.96 (*C*H_2Ph), 66.22, 66.18 (*C*-5′), 51.14, 51.10 (d, $^2J_{C\text{–}P}$ = 6.25 Hz, NH*C*HCH$_3$), 20.85 (d, $^3J_{C\text{–}P}$ = 6.25 Hz, NHCH*C*H_3), 20.62 (d, $^3J_{C\text{–}P}$ = 6.30 Hz, NHCH*C*H_3). [ES+] *m/z*: found 709.71 [M + Na^+] $C_{30}H_{35}N_6O_9PS$ required 686.67 [M].

6-Thioinosine-5′-*O*-bis(2,2-dimethoxypropoxy-L-alaninyl)phosphate (21): Prepared according to the standard procedure A from 6-thioinosine (0.15 g, 0.53 mmol), $POCl_3$ (0.05 mL, 0.53 mmol), the tosylate salt of 2,2-dimethoxypropoxy-L-alanine (0.87 g, 2.50 mmol) and DIPEA (0.91 mL, 5.01 mmol). After work-up and silica gel column chromatography phosphorodiamidate **21** was obtained as an off-white solid (0.017 g, 5%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.84; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.43 (s, 1H, *H*-2), 8.19 (s, 1H, *H*-8), 6.04 (d, *J* = 5.0 Hz, 1H, *H*-1′), 4.72 (t, *J* = 5.0 Hz, 1H, *H*-2′), 4.43 (t, *J* = 5.0 Hz, 1H, *H*-3′), 4.30 – 4.21 (m, 3H, 2 x *H*-5′, *H*-4′), 3.97 – 3.93 (m, 2H, 2 x NHC*H*CH_3), 3.88, 3.86, 3.76, 3.74 (2 x AB system, J_{AB} = 10.5, 4.5 Hz, 4H, 2 x C*H*$_2$C$(CH_3)_3$), 1.39, 1.37 (2 x d, *J* = 7.0 Hz, 2 x NHCHC*H*$_3$), 0.95 (s, 18H, 2 x CH_2C(C*H*$_3)_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 178.44 (*C*=S), 175.07, 175.02 (2d, $^{3}J_{C–P}$ = 6.25 Hz, *C*=O, ester), 146.47 (*C*-2), 145.03 (*C*-4), 142.76 (*C*-8), 137.19 (*C*-5), 90.44 (*C*-1′), 84.82, 84.76 (d, $^{3}J_{C–P}$ = 7.8 Hz, *C*-4′), 79.47 (*C*H_2C$(CH_3)_3$), 74.89 (C-2′), 71.58 (*C*-3′), 66.27, 66.23 (d, $^{2}J_{C–P}$ = 5.0 Hz, *C*-5′), 51.19, 51.09 (d, $^{2}J_{C–P}$ = 6.0 Hz, NH*C*HCH_3), 32.53, 32.50, 32.46, 32.44 (CH_2*C*$(CH_3)_3$), 30.73, 26.44, 24.67 (CH_2C(*C*H$_3)_3$), 21.13 (d, $^{3}J_{C–P}$ = 5.6 Hz, NHCH*C*H$_3$), 20.89 (d, $^{3}J_{C–P}$ = 5.60 Hz, NHCH*C*H$_3$). [ES+] *m/z*: found 669.83 [M + Na^+] $C_{26}H_{43}N_6O_9PS$ required 646.69 [M].

6-Thioguanosine-5′-*O*-bis(benzoxy-L-alaninyl) phosphate (22): Prepared according to the standard procedure A from 6-thioguanosine (0.15 g, 0.50 mmol), $POCl_3$ (0.05 mL, 0.53 mmol), the tosylate salt of benzoxy-L-alanine (0.88 g, 2.50 mmol) and DIPEA (0.91 mL, 5.01 mmol). After work-up and silica gel column chromatography phosphorodiamidate **22** was obtained as an off-white solid (0.010 g, 3%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.70; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.15 (s, 1H, *H*-8), 7.35 – 7.30 (m, 10 H, Ar-*H*), 6.01 (d, *J* = 5.5 Hz, 1H, *H*-1′), 5.16 –5.06 (m, 4H, 2 x C*H*$_2$Ph), 4.68 (t, *J* = 5.5 Hz, 1H, *H*-2′), 4.48 (t, *J* = 5.5 Hz, 1H, *H*-3′), 4.24 – 4.20 (m, 1H, 1 x *H*-5′), 4.16 – 4.14 (m, 2H, 1 x *H*-5′, *H*-4′), 3.97 – 3.91 (m, 2H, 2 x NHC*H*CH_3), 1.32, 1.29 (2 x d, *J* = 7.0 Hz, 2 x NHCHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 177.12 (*C*=S), 175.44, 175.38 (2d, $^{3}J_{C–P}$ = 6.25 Hz, *C*=O, ester), 163.19 (*C*-2), 161.34 (*C*-4), 151.47 (*C*-8), 140.49 (*C*-5), 137.25, 137.20 (2 x *C* ipso-Ph), 129.61, 129.37, 129.34, 129.32, 127.17 (*C*H-Ar), 90.02 (*C*-1′), 84.49, 84.41 (d, $^{3}J_{C–P}$ = 8.2 Hz, *C*-4′), 74.63 (C-2′), 71.69 (*C*-3’), 68.07, 68.02 (*C*H_2Ph), 66.38, 66.32 (d,

$^{2}J_{C-P}$ = 7.5 Hz, *C*-5′), 51.14, 51.08 (d, $^{2}J_{C-P}$ = 3.8 Hz, NH*C*HCH$_3$), 20.85 (d, $^{3}J_{C-P}$ = 5.8 Hz, NHCH*C*H$_3$), 20.62 (d, $^{3}J_{C-P}$ = 5.80 Hz, NHCH*C*H$_3$). [ES+] *m/z*: found 724.75 [M + Na$^+$] $C_{30}H_{36}N_7O_9PS$ required 701.68 [M].

6-Thioguanosine-5′-*O*-bis(2,2-dimethoxypropoxy-L-alaninyl)phosphate (23): Prepared according to the standard procedure A from 6-thiguanosine (0.15 g, 0.50 mmol), $POCl_3$ (0.05 mL, 0.50 mmol), the tosylate salt of 2,2-dimethoxypropoxy-L-alanine (0.83 g, 2.50 mmol) and DIPEA (0.91 mL, 5.01 mmol). After work-up and silica gel column chromatography phosphorodiamidate **23** was obtained as an off-white solid (0.016 g, 5%). ^{31}P NMR (202 MHz, CD_3OD): δ = 13.83; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.05 (s, 1H, *H*-8), 5.87 (d, *J* = 5.0 Hz, 1H, *H*-1′), 4.71 (t, *J* = 5.0 Hz, 1H, *H*-2'), 4.41 (t, *J* = 5.0 Hz, 1H, *H*-3′), 4.30 – 4.19 (m, 3H, 2 x *H*-5′, *H*-4′), 4.00 – 3.95 (m, 2H, 2 x NHC*H*CH$_3$), 3.88, 3.86, 3.76, 3.74 (2 x AB system, J_{AB} = 10.5, 4.5 Hz, 4H, 2 x C*H*$_2$C(CH$_3$)$_3$), 1.38, 1.35 (2 x d, *J* = 7.0 Hz, 2 x NHCHC*H*$_3$), 0.94 (s, 18H, 2 x CH$_2$C(C*H*$_3$)$_3$); ^{13}C NMR (125 MHz, CD_3OD): δ = 177.1 (*C*=S), 175.66, 175.58 (2d, $^{3}J_{C-P}$ = 5.8 Hz, *C*=O, ester), 164.19 (*C*-2), 148.82 (*C*-4), 140.71 (*C*-8), 130.28 (*C*-5), 90.11 (*C*-1′), 84.70, 84.64 (d, $^{3}J_{C-P}$ = 7.5 Hz, *C*-4′), 75.44 (*C*H$_2$C(CH$_3$)$_3$), 75.41 (C-2′), 74.71 (*C*-3′), 66.56, 66.50 (d, $^{2}J_{C-P}$ = 7.70 Hz, *C*-5′), 51.17, 51.09 (d, $^{2}J_{C-P}$ = 10.0 Hz, NH*C*HCH$_3$), 32.38, 32.35 (CH$_2$*C*(CH$_3$)$_3$), 26.79 (CH$_2$C(*C*H$_3$)$_3$), 21.12, 21.10 (d, $^{3}J_{C-P}$ = 5.6 Hz, NHCH*C*H$_3$), 20.89 (d, $^{3}J_{C-P}$ = 5.60 Hz, NHCH*C*H$_3$). [ES+] *m/z*: found 684.78 [M + Na$^+$] $C_{26}H_{44}N_7O_9PS$ required 661.70 [M].

Acknowledgements

We thank NuCana plc for financial support. Part of the cytostatic evaluations were performed by Mrs. Lizette van Berckelaer at the Rega Institute for Medical Research, KU Leuven (GOA 15/19 TBA).

Conflict of interest

The authors (M. Serpi and M. Slusarczyk) receive research funding from NuCana plc, whose two agents NUC-1031 and NUC-3373 are cited here.

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