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Design, synthesis and biological evaluation of aryloxy thiophosphoramidate triesters of anticancer nucleoside analogues

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ABSTRACT

Aryloxy phosphoroamidate triesters, known as ProTides, are a class of prodrugs developed to enhance the physicochemical and pharmacological properties of therapeutic nucleosides. This approach has been extensively investigated in the antiviral and anticancer areas leading to three prodrugs on the market and several others in clinical stage. In this article we have prepared the P═S analogues of three ProTides that have reached the clinic as anticancer agents. These novel P═S ProTides were tested for their capacity in enzymatic activation and for their cytotoxic properties against a panel of solid and liquid tumor cell lines. As expected, the replacement of the P═O with a P═S bond led to increased metabolic stability albeit concomitant to a decrease in potency. Surprisingly, the intermediate formed after the first activation step of a thiophosphoramidate with carboxypeptidase Y is not the expected P═S aminoacyl product but the corresponding P═O aminoacyl compound.

1. Introduction

Nucleoside analogues (NAs), although the cornerstone of anticancer and antiviral therapy, suffer of several limitations as poor permeability, slow activation, premature breakdown, and unfavorable pharmacokinetic properties.[1] To overcome these limitations several prodrug strategies have been elaborated.[2] Among them the ProTide approach[3] has led to the development and commercialization of three antiviral drugs: the anti-HCV ProTide sofosbuvir,[4] the anti-HIV ProTide tenofovir alafenamide (TAF)[5] and Remdesivir which shows broad activity against RNA viruses and for this reason was approved for the treatment of COVID-19 infections.[6] In the oncology setting three ProTides have reached the clinic: NUC-1031, (Acelarin),[7] a ProTide of gemcitabine, NUC-3373,[8] a ProTide of 2′-deoxy-5-fluoruridine and NUC-7738,[9–10] a ProTide of 3′deoxyadenosine (Fig. 1). NUC-3373 is in Phase II for the treatment of colorectal cancer[11–12] while NUC-7738 is in phase I/II for the treatment of patients with advanced solid tumors.[13] More recently the ProTide technology has been extended to non-nucleoside small molecules for different therapeutic indications.[3].

As a continuation of our interest in the phoshoramidate technology we decided to investigate whether the drug profile of a ProTide could be further improved by replacing one of the phosphate oxygen atoms by a sulphur atom. Substitution of oxygen atoms by sulphur at various nucleobase or backbone locations in the nucleic acid framework have been studied extensively, especially in the context of the development of antisense therapeutics, leading to a wide variety of sulphur-modified nucleosides and nucleotides.[14] Phosphorothioates which have $–CH_2$-O-**PS**O_2^{2-} groups in place of the $–CH_2$-O-PO_3^{2-} groups have be shown to be excellent mimics of DNA with increased resistance to nuclease degradation, better hydrophobicity compared with the natural counterpart.[15].

The P═S analogue of sofosbuvir[16] and the anti-HIV nucleoside stavudine (d4t) were also described.[17] While the first was reported to have an *in vitro* activity lower than 10 μM in HCV replicon essay, the last were instead found significatively less potent than the corresponding P═O analogues against HIV. 5′-Thiophosphoroamidates of other pyrimidine nucleoside analogues were instead reported to show up to 10-fold enhancement in anticancer activity compared with the almost inactive parent nucleoside.[18].

In this work we focus on replacing one oxygen of the non-phosphate bridge of three anticancer ProTides that have reached the clinic with a sulphur atom. The anticancer activity and the metabolic activation of the P═S hybrid nucleotide prodrugs (**1**–**3**) were both evaluated.

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2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds **1**–**3** begins with the preparation of the aryloxy phosphorochlorothioates **5a,b** via aryloxyphosphodichlorothioates **4a,b**. As depicted in Scheme 1, the procedure previously reported for the synthesis of the aryloxy phosphodichloridate[19] was slightly modified with longer time and higher temperature to consider the lower electrophilicity of the sulphur containing compounds. Briefly, commercially available thiophosphoryl chloride was reacted with either phenol or naphthol in the presence of triethyl amine at 0 °C for 4–6 h to yield the corresponding phosphorodichlorothioates **4a** and **4b**.[20] These were then reacted with L-alanine benzyl ester hydrochloride salt in the presence of triethyl amine at 0 °C for 4–6 h to obtain the phosphorylating reagents **5a,b.**[21].

As first attempt to synthesize the sulfur analog of NUC1031, **5a** was reacted with 3′-*O* O-*tert*-butyloxycarbonyl (Boc) protected gemcitabine (**6**) in the presence of 1 M *t*buMgCl in THF (Entry 1, Table 1).[7] The 3′-Boc protected gemcitabine **6** was chosen to avoid potential side products due to the 3′-OH competing with the 5′-OH toward phosphorylation. However, under this set of conditions no product was formed even when the temperature was raised up to 70 °C. We then attempted the synthesis of **1** via *N*-methyl imidazole (NMI) methodology.[19] Also, in this case only traces of phosphorylated compound without Boc protection were formed as evidenced by mass spectrometry analysis (Entry 2, Table 1). Similar results were achieved with dimethylamino pyridine and triethylamine (Entry 3, Table 1).

Considering the low reactivity of the phosphorochlorothioester and reasoning that perhaps the protective group could create steric hindrance, we decided to attempt the phosphorylation with **4a** directly on gemcitabine (**7**), using dimethylamino pyridine and triethylamine at 50 °C for 48 h. Mass spectrometry analysis showed formation of a monophosphorylated product but only in traces (Entry 4, Table 1). Instead, when using NMI at room temperature, the desired compound **1** was isolated after column chromatography although in a very low yield of 2 % only (Entry 5, Table 1). Unfortunately, attempt to increase the yield by raising the temperature at 80 °C, led to the formation of an undesired product, where phosphorylation occurred at the amino group on the nucleobase.

Our next step was the preparation of compound **2**, a prodrug of 2′-deoxy-5-fluoruridine (FUdR, **9**). As depicted in Scheme 3, FUdR was thereby reacted with 3 equivalents of phosphorothioate **5b**, in presence of 6 equivalents of NMI in acetonitrile, at 70 °C (Scheme 2). As no NH_2 group is present in the nucleobase, desired compound **2** was isolated in 28 % yield. This result led us to conclude that the presence of an amino group in the nucleobase could be problematic for the synthesis of this class of compound. For this reason, for the synthesis of phosphorothioamidate of 3′-deoxyadenosine (3′dA, **10**) which has low solubility in acetonitrile, we perform the reaction to prepare compound **3** at rt and in DMF (Scheme 3). The desired compound **3** was obtained only in 2 % yield despite longer reaction time.

2.2. Enzymatic activation

With prodrugs **1**–**3** in hand, we wanted to investigate their activation mechanism as this aspect has been correlated with the prodrug activity.[3] After entering cells, the first step of ProTide activation is the hydrolysis by the intracellular esterase carboxylesterase 1 (CES1) or cathepsin A (CatA) to release their ester moieties,[3,22–23]followed by spontaneous reaction to form the corresponding aminoacyl phosphor(n)amidate (**III**), which undergoes P—N bond cleavage, mediated by an enzyme with phosphoramidase activity (Hint) to eventually release the monophosphorylated parent drug (MP-NA). The nucleoside monophosphate generated then undergoes two successive phosphorylations to generate the active species (TP-NA) (Fig. 3).[3].

Thus, we envisaged a similar activation pathway for the novel thiophosphoroamidate herein synthesized. The activation should first lead to the formation of the corresponding aminoacyl thiophosphoramidate intermediate which, by the action of hint-1, is then converted to the nucleoside monothiophosphate as postulated earlier by Zhao et *al in* 2013.[18].

To investigate whether our compounds are activated with the same mechanism reported for the analogues ProTides,[3] we carried out an enzymatic study on **1** and **2** using a carboxypeptidase Y assay and monitoring the reaction via [31]P-NMR according to our reported protocol.[7].

According to the results reported in Fig. 4, the compound **1** (δ_P 67.77,

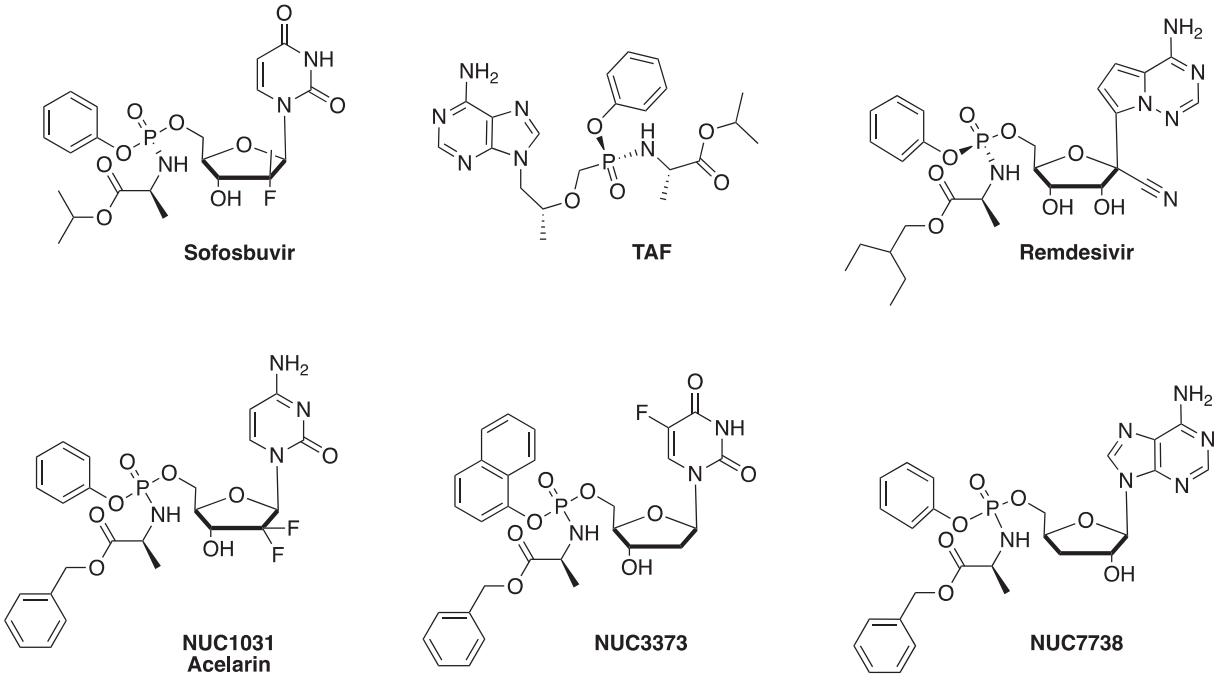


Fig. 1. FDA-approved and clinical candidate ProTides.

67.24 ppm) was slowly (~5 h) hydrolysed to a first metabolite **1-I** which, as indicated by the presence of two signals at a quite downfield chemical shifts (δ_P 57.6 and 57.2 ppm), we assume would be still a chiral phosphoramidate with a P═S bond. Noteworthy, the two diastereoisomers of **1** were processed at different rate with the most upfield diastereoisomer being the most stable. Further processing of **1-I** led to the formation of another metabolite (**1-IV**), shown as a single peak at δ_P 6.83 ppm in the [31]P-NMR spectra (Fig. 4). Intrigued by this drastic change in the chemical shift (consistent with a change from P═S to a P═O) we carried out a mass analysis of the reaction mixture. We indeed found a peak at *m/z* 412, that was consistent with the structure **1-IV** ([M−H$^+$]).

The data gathered confirmed that this metabolite is the same intermediate formed in the activation of NUC1031 (P═O ProTide) mediated by carboxypeptidase Y. This was surprising as we expected the P═S metabolite (**1-III**) to be formed rather than P═O (**1-IV**). We therefore postulated that the attack by a water molecule to the putative cyclic intermediate **1-II** must take place leading to the formation of **1-IV** via H_2S elimination (Fig. 5).

When compared to the activation of NUC1031 (both diastereoisomers disappeared after 10 h)[7] compound **1** is activated at much slower rate with one of the diastereoisomer still present after 48 h. Both steps, hydrolysis of the ester and release of the aryloxy moiety are slower for the P═S derivative than for the P═O prodrug.

Similar results were obtained with compound **2** (Fig. 6), which during the enzymatic process was fully converted via metabolite **2-I** to a metabolite **2-IV** within approximately 48 h. Once more the chemical shift (δ_P 6.83) and the mass analysis (*m/z* 396) of the final metabolite were consistent with structure **2-IV**. Again, when compared to FUdR phosphoramidates (complete conversion to the corresponding metabolite II in 45 min),[8] the corresponding thiophosphoroamidate was activated much slower.

Overall, our results strongly support the fact that the ProTide metabolic pathway is also the actual carboxypeptidase activation route for the phosphorothioamidates, although the rate for the enzymatic activation appears much slower for the P═S phosphoramidates. These findings agree with the anticipated higher stability of the P═S versus the P═O nucleotide analogues.

2.3. Biological activity

Having proved that phosphorothioamidates are indeed activated we were keen to investigate their biological activities against cancer. Compounds **1**–**3**, described above were tested for their cytostatic activity against several established solid tumour cell lines, as presented in Tables 2–5. The parent nucleosides and the three clinical candidates NUC3373, NUC1031 and NUC7738, were also included as positive controls and for comparison. Compound **1**, the phosphorothioamidate analogue of NUC1031, showed to be less active then NUC1031 and gemcitabine in almost all the cell lines tested with IC_{50} values 20 to 300 folds higher than NUC1031. Similar results were also obtained with the thiophosphoramidate **2,** the sulphur analogue of NUC3373, which had lower or similar activity then NUC3373 and FUdR. Compound **3**, the P═S analogue of NUC7738, although was more potent then 3′dA against all the cell lines, it was in general not more cytotoxic then NUC7738. The renal A498, ACHN-luc2, CAKI-2 cell lines were the only cell lines in which the IC_{50} values of **3** were slightly lower than NUC3378 (Table 4).

These results are consistent with the previous knowledge that the phosphoramidate potency is closely connected with their metabolic activation.[3] These findings are also in accord with the study on stavudine thiophosphoroamidates.[17].

3. Conclusion

In conclusion we have prepared thiophosphoroamidates analogues of three anticancer nucleoside analogues that have reached clinical trials. These compounds proved to be activated by carboxypeptidase type of enzyme following the same route of the ProTides and surprisingly forming the same P═O aminoacyl intermediate, although a slower rate due to higher energy of activation for hydrolysis during the formation of the active metabolites. The thiophosphoramidates **1**–**3** in general did not prove to be superior for their *in vitro* cytotoxic activity when compared to their corresponding anticancer ProTides. Only compound **3** showed a slightly improved cytotoxic activity when compared to 3′dA and NUC7738 in kidney cell lines.

Although the potency of the prodrugs is lower than the corresponding P═O analogues the higher stability of thiophosphoramidate motif might be exploited to tune the activation of this type prodrugs.

4. Experimental

4.1. Chemistry

4.1.1. General

All commercially available chemicals were supplied by either Sigma-Aldrich or Fisher and used without further purification. All solid reagents were dried for several hours under high vacuum prior to use. For analytical thin-layer chromatography (TLC), precoated aluminium-backed plates (60F-54, 0.2 mm thickness; supplied by E. Merck AG, Darmstadt, Germany) were used and developed by an ascending elution method. After solvent evaporation, compounds were detected by quenching of the fluorescence, at 254 nm upon irradiation with a UV lamp. Column chromatography purifications were carried out by means of automatic Biotage Isolera One. Fractions containing the product were identified by TLC, pooled and the solvent was removed in vacuo. ^{1}H, ^{13}C, ^{31}P and ^{19}F NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. All ^{13}C, ^{31}P and ^{19}F NMR spectra were proton-decoupled. Chemical shifts are given in parts per million (ppm) and coupling

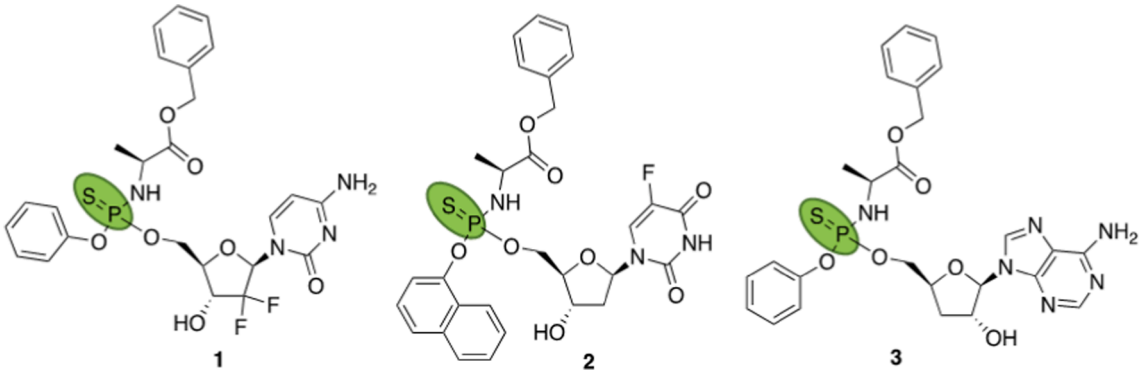
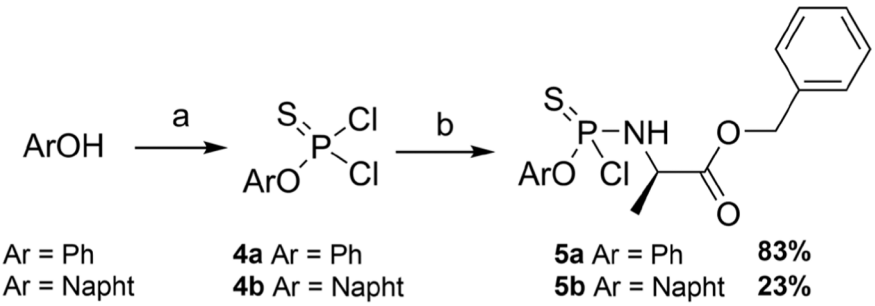


Fig. 2. Thiophosphoramidate prodrugs of gemcitabine (**1**) FuDR (**2**) and 2′-deoxyadenosine (**3**).

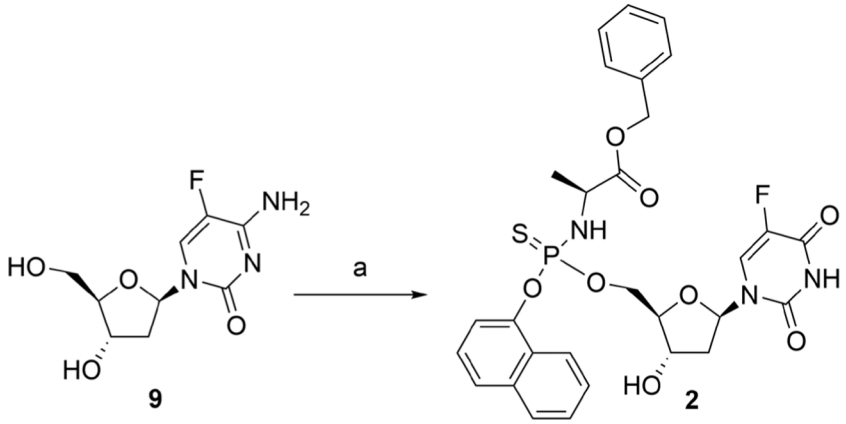


Scheme 1. Synthesis of the phosphorothioates **5a** and **5b**. *Reagents and conditions*: (a) $PSCl_3$, Et_3N, Et_2O, 0 °C to rt, 4 h; (b) L-AlaOBn.HCl, Et_3N, CH_2Cl_2, 0 °C to rt, 4 h.

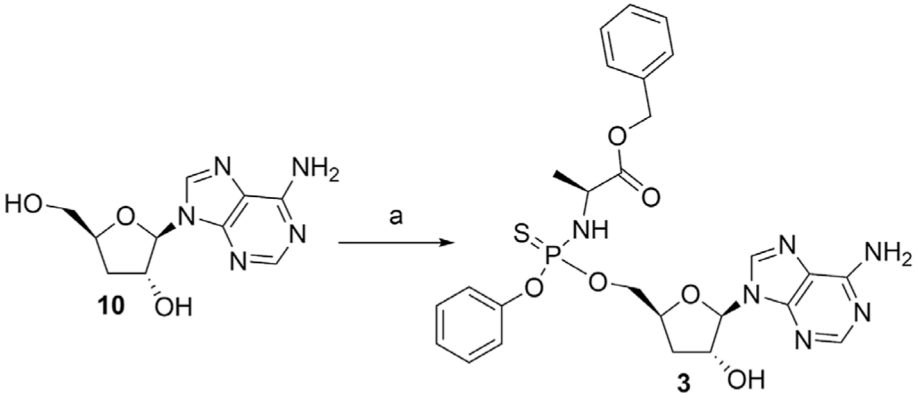
Table 1
Reaction conditions explored for the synthesis of Phosphorothioamidate prodrugs.

Entry	Cpd	Solvent	Temp.	Base	Time	Remarks	Yield (%) **8**	**1**
1	**3**	THF	rt to 70 °C	1 M *t*BuMgCl in THF	48 h	–	–	–
2	**3**	THF	70 °C	NMI	18 h	traces of phosphorylated compound[1]	–	–
3	**3**	THF	70 °C	DMAP/Et_3N	18 h	traces of phosphorylated compound[1]	–	–
4	**4**	THF	70 °C	DMAP/Et_3N	48 h	traces of phosphorylated compound[1]	–	–
5	**4**	CH_3CN	rt	NMI	18 h	–	–	2 %[2]
6	**4**	CH_3CN	80 °C	NMI	18 h	*N*-phosphorylated compound	–	–

[1] m/z 597 $[M + H]^+$ detected by mass spectrometry; [2]isolated yield.



Scheme 2. Synthesis of 5′*O*-aryloxy thiophosphoroamidates **2.** *Reagents and conditions:* compound **5b**, NMI, CH_3CN, 70 °C, 18 h, 28 %.



Scheme 3. Synthesis of 5′-*O* aryloxy thiophosphoroamidate **3.** *Reagents and conditions:* compound **5a**, NMI, DMF, rt, 3 days, 2 %.

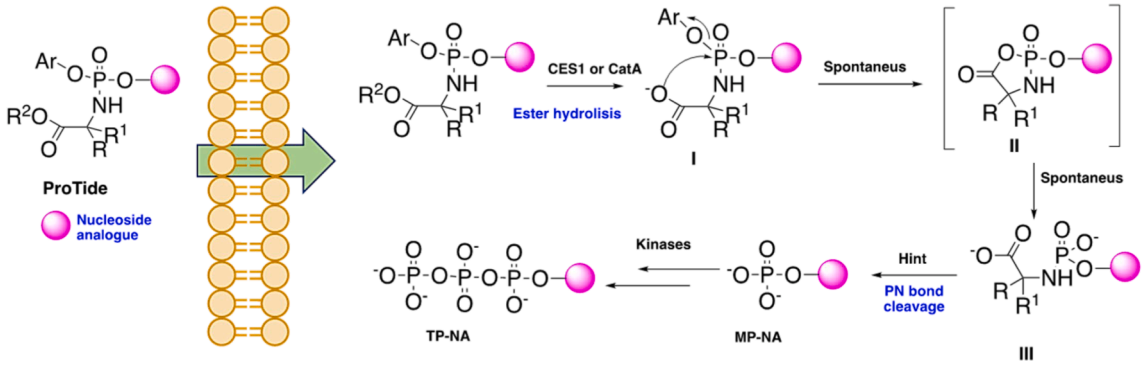


Fig. 3. Metabolic activation of phosphoramidate nucleoside analogues.

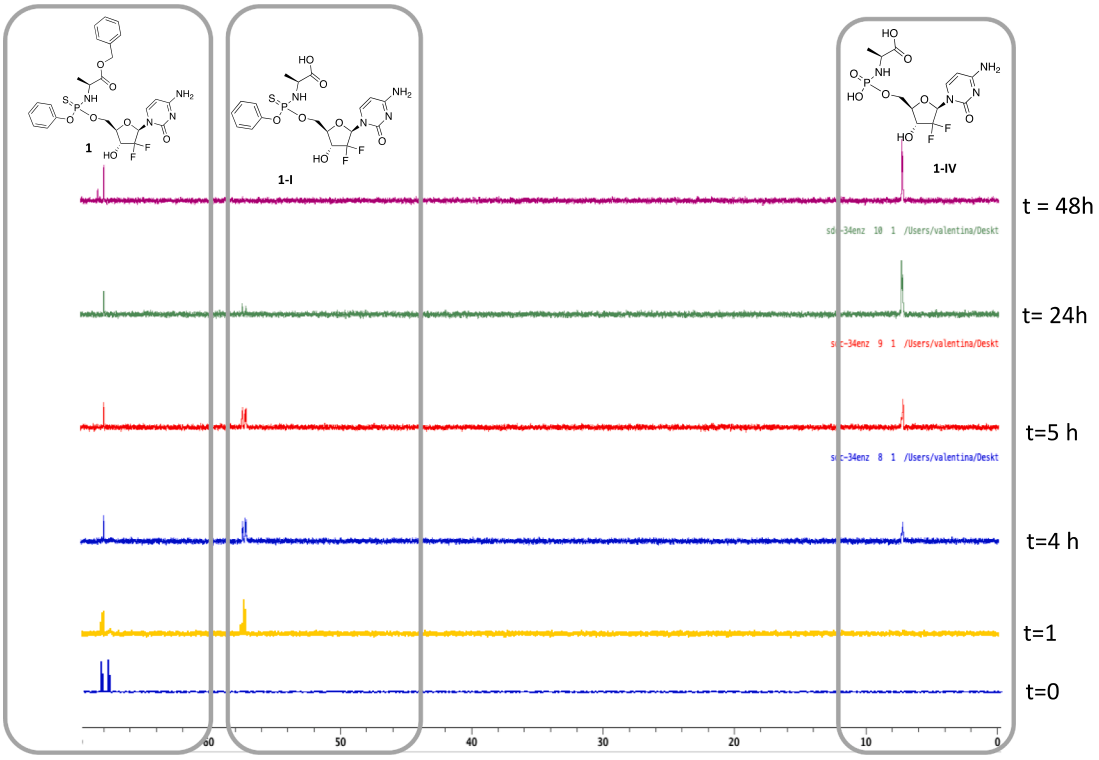


Fig. 4. Fig. 2. ^{31}P NMR stack spectra (500MHz, $(CD_3)_2CO$) of ProTide 6 over time after carboxypeptidase Y digestion at 25°C showing the signals of metabolites 1-I and 1-IV.

constants (*J*) are measured in Hertz (Hz). The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), m (multiplet), br (broad). The assignment of the signals was done based on the analysis of coupling constants and additional two-dimensional experiments (COSY, HSQC). Analytical High-Performance Liquid Chromatography (HPLC) analysis was performed using Spectra System SCM (with X-select-C18, 5 mm, 4.8 x 150 mm column), Varian Prostar system (LC Workstation- Varian Prostar 335 LC detector). Preparative HPLC was performed with Varian Prostar (with pursuit XRs C18 150 x 21.2 mm column). Low and high-resolution mass spectrometry was performed on a Bruker Daltonics MicroTof-LC system (atmospheric pressure ionization, electron spray mass spectroscopy) in positive mode.

4.1.2. Synthesis and characterization

4.1.2.1. General Procedure A. A solution of phenol or naphthol (1 eq) and triethylamine (2 eq) in chloroform (40 ml) was added dropwise to the solution of thiophosphoryl chloride (1 eq) in chloroform at 0–10 °C, stirred at room temperature for 4 h and left for 24 h. The solution was washed twice with water, dried, filtered and evaporated to yield the desired compound, which was in some cases used without further purification in the next step.

4.1.2.2. O-phenyl phosphorodichloridothioate ***(4a)***. Prepared according to the general procedure A using phenol (5.00 g, 0.053 mol), thiophosphoryl chloride (5.3 ml, 0.053 mol) and Et_3N (13.9 ml, 0.106 mol). ^{31}P NMR (202 MHz, $CDCl_3$) δ_P 53.7. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.47–7.44 (m, 2H, Ar), 7.36–7.32 (m, 3H, Ar).

4.1.2.3. O-(naphthalen-1-yl) phosphorodichloridothioate ***(4b)***. Prepared according to the general procedure A using Naphthol (3.00 g, 0.021 mol), thiophosphoryl chloride (2.13 ml, 0.021 mmol) and Et_3N (5.5 ml, 0.042 mmol). ^{31}P NMR (202 MHz, $CDCl_3$) δ_P 52.8. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.15 (d $J = 8.0$ Hz, 1H, Ar), 7.92 (d $J = 5.0$ Hz, 1H, Ar), 7.83 (dd $J = 8.5$, 2.0 Hz, 1H, Ar), 7.65–7.57 (m, 3H), 7.51–7.48 (dd $J = 8.0$, 8.0 Hz, 1H).

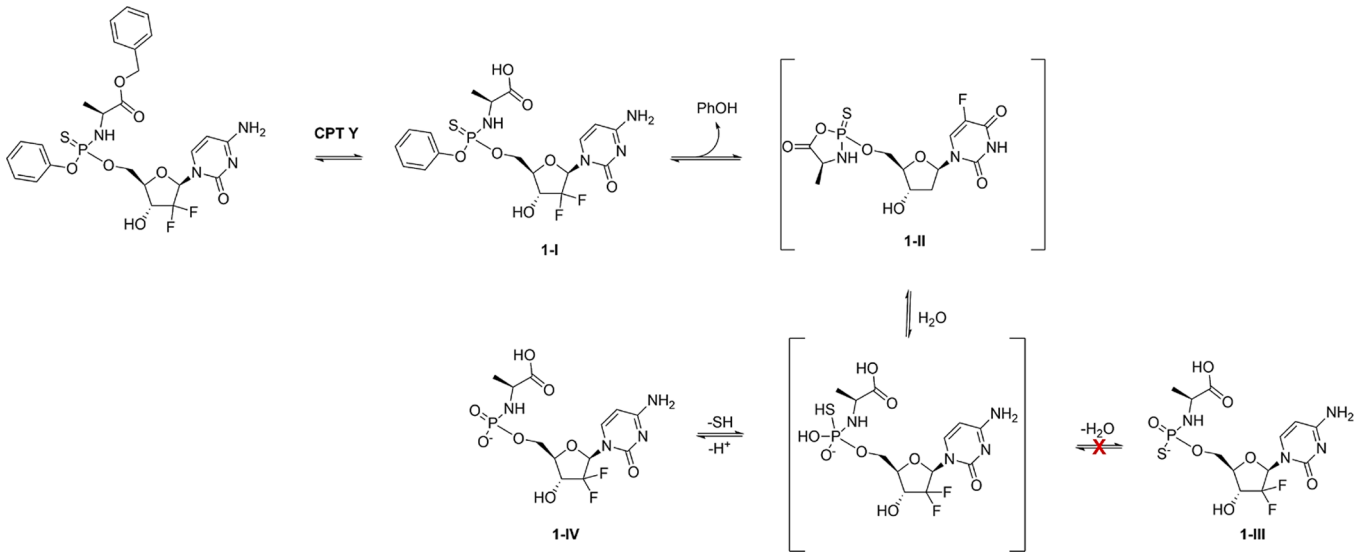


Fig. 5. Possible mechanism of activation of thiophosporamidates of nucleoside analogues by carboxypeptidase Y.

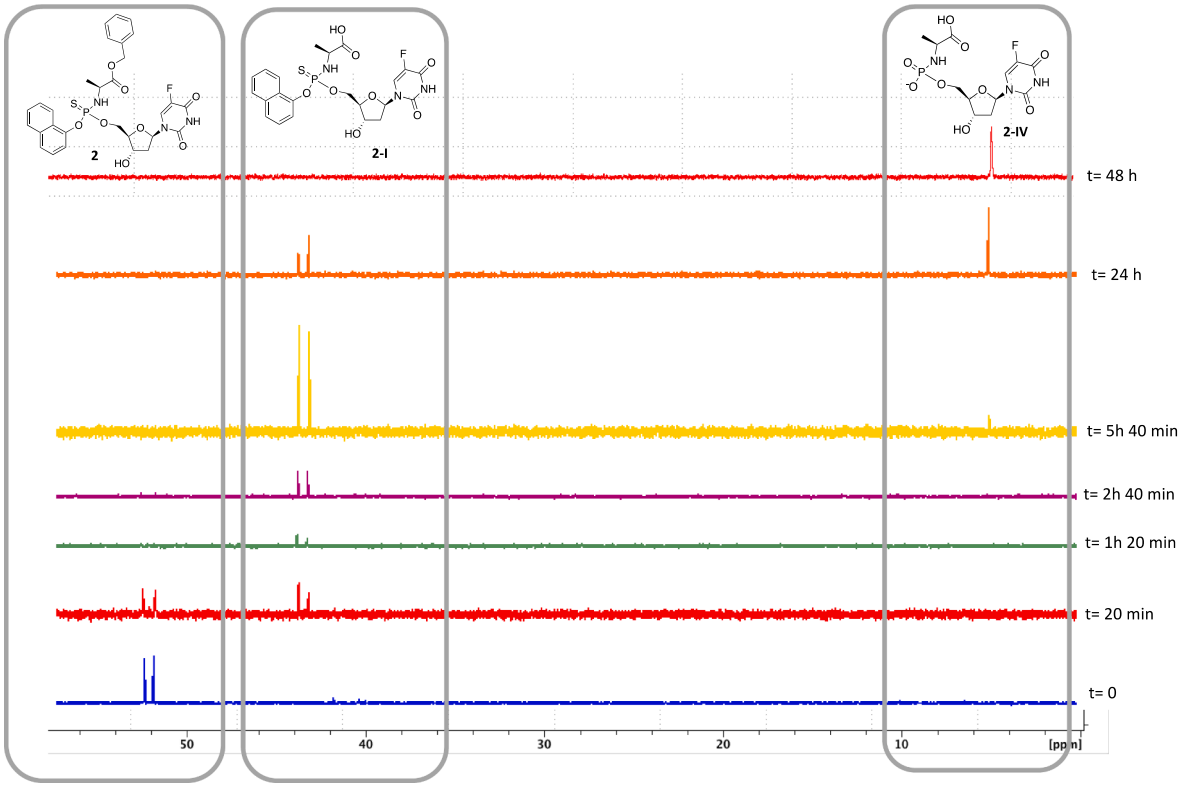


Fig. 6. ^{31}P NMR stack spectra (500 MHz, $(CD_3)_2CO$) of ProTide **2** over time after carboxypeptidase Y digestion at 25 °C showing the signals of metabolites **2-I** and **2-IV**.

4.1.2.4. General Procedure B. L-Alanine benzyl ester hydrochloride salt (1 eq) was dissolved in CH_2Cl_2 (4 ml per mmol of amino acid ester) under Argon. A solution of the appropriate phosphorothioate **4a** or **4b** (1 eq) in CH_2Cl_2 was added and the mixture cooled to 0 °C. Et_3N (2 eq) was added dropwise, and the reaction mixture was stirred at 0 °C for 20 min and thereafter at rt for 5 h. The solvent was evaporated under reduced pressure and the resulting solid was triturated with Et_2O and filtered. The filtrate was reduced to dryness to give the desired products **5a,b** as oil, which were used without further purification in the next step.

4.1.2.5. (2S)-benzyl 2-((chloro(phenoxy)phosphorothioyl)amino)propanoate (5a). Prepared according to the general procedure **B** using L-Alanine benzyl ester hydrochloric salt (3 0.00 g, 0.14 mol), 4a (3.15 g, 13.95 mmol) and Et_3N (3.9 ml, 0.28 mol). The product was obtained as an oil (4.28 g, 83 %).^{31}P NMR (202 MHz, $CDCl_3$) δ_P 64.6, 64.4. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.37–7.13 (m, 10*H*, Ar), 5.15 (s, 1H, CH_2PH), 5.13 (s, 1H, CH_2PH), 4.58–4.53 (m, 0.5H, C*H*CH_3 L-Ala), 4.46–4.41 (m, 0.5H, C*H*CH_3 L-Ala), 4.37–4.24 (m, 1H, NH), 1.48–1.44 (m, 3H, CHC*H*$_3$ L-Ala).

Table 2
Cytotoxic activity of Gemcitabine, FUDR, 3́-dA, NUC1031, NUC373, NUC7738 and compounds 1–3.

Cpd	Pancreas						Bladder						Prostate	
	PANC-1		Mia-Pa-Ca		BxPC-3		RT112/84		T24/83		5637		PC-3	
	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$
Gemcitabine	0.68	43	0.04	68	1.81	79	1.22	88	<0.02	–	–	–	0.46	61
31	3.96	45	0.12	69	0.33	64	0.11	79	0.05	–	–	–	1.09	65
1	76.43	99	3.83	99	8.16	95	0.92	94	0.32	–	–	–	43.85	96
FUdR	–	–	0.3	85	–	–	0.09	67	–	–	–	–	–	–
373	–	–	1.47	96	–	–	0.32	77	–	–	–	–	–	–
2	–	–	2.69	100	–	–	2.49	97	–	–	–	–	–	–
3′dA	–	–	76.78	87	–	–	–	–	99.07	96	71.56	44	85.34	70
378	–	–	7.41	95	–	–	–	–	31.44	92	47.45	100	51.18	74
3	–	–	12.56	100	–	–	–	–	42.56	100	82.62	100	62.77	97
Paclitaxel	0.003	56	0.003	83	0.005	85	0.01	77	0.004	97	0.002	95	0.01	62

Cytotoxicity data reported as μM IC_{50} values (concentration of drug causing 50% inhibition of cell viability) and MI% values (maximum inhibitory effect of the drug at the range of concentrations considered). PTX: paclitaxel (control); (-) not tested.

Table 3
Cytotoxic activity of Gemcitabine, FUDR, 3́-dA, NUC1031, NUC373, NUC7738 and compounds 1–3.

Cpd	Colon				Breast				Ovarian			
	HT29		Colo205		MDA MB231		MCF-7		A2780		OVCAR 3	
	IC_{50}	$MI_{\%}$	IC_{50}	MI%	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	MI%	IC_{50}	$MI_{\%}$
Gemcitabine	0.07	64	0.11	86	3.7	74	–	–	0.32	85	67.18	35
NUC1031	0.48	64	1.82	72	1	71	–	–	0.27	76	119.05	16
1	11.5	99	22.32	97	11.64	91	–	–	3.58	100	97.44	46
FUdR	0.13	83	0.02	88	–	–	–	–	–	–	0.74	58
NUC373	0.52	81	0.14	0.14	–	–	–	–	–	–	6.91	92
2	2.81	100	4	100	–	–	–	–	–	–	21.5	100
3′dA	26.33	90	107.93	24	78.63	59	45.88	93	–	–	–	–
NUC738	9.47	96	27.75	87	19.42	79	3.69	90	–		–	–
3	57.92	100	41.82	99	50.64	96	40.35	100	–	–	–	–
paclitaxel	0.03	79	0.01	90	0.003	70	0.02	75	0.008	72	0.004	59

Cytotoxicity data reported as μM IC_{50} values (concentration of drug causing 50% inhibition of cell viability) and MI% values (maximum inhibitory effect of the drug at the range of concentrations considered). PTX: paclitaxel (control); (-) not tested.

Table 4
Cytotoxic activity of Gemcitabine, FUDR, 3́-dA, NUC1031, NUC373, NUC7738 and compounds 1–3.

Cpd	Lung				Kidney						Liver	
	SK-MES-1		NCI-H1975		A498		ACHN-luc2		CAKI-2		HepG2	
	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$
Gemcitabine	1.22	88	–	–	–	–	–	–	–	–	16.7	53
NUC1031	0.11	79			–	–	–	–	–	–	1.16	57
1	0.92	94	–	–	–	–	–	–	–	–	27.06	98
FUdR	0.09	67	91	91	–	–	–	–	–	–	53.9	67
NUC373	0.32	77	72	72	–	–	–	–	–	–	24.5	62
2	2.49	97	100	100	–	–	–	–	–	–	25.5	100
3′dA	–	–	–	–	85.36	18	77.05	14	94.2	25	77.19	71
NUC378	–	–	–	–	58.05	82	111.92	62	102.45	78	25.24	80
3	–	–	–	–	44.46	95	33.89	97	76.04	78	25.27	100
Paclitaxel	0.01	77	75	75	0.02	74	0.02	47	0.01	78	0.06	54

Cytotoxicity data reported as μM IC_{50} values (concentration of drug causing 50% inhibition of cell viability) and MI% values (maximum inhibitory effect of the drug at the range of concentrations considered). PTX: paclitaxel (control); (-) not tested.

4.1.2.6. (2S)-benzyl 2-((chloro(naphthalen-1-yloxy)phosphorothioyl) amino)propanoate (5b). Prepared according to the general procedure **B** using L-Alanine benzyl ester hydrochloric salt (3.00 g, 0.139 mol), **4b** (3.85 g, 0.14 mmol) and Et_3N (3.9 ml, 0.28 mol). Purification by silica gel flash chromatography using EtOAc/hexane (5:5) as an eluent to afford the title compound (1.36 g, 23 %) as an oil.^{31}P NMR (202 MHz, $CDCl_3$) δ_P 64.3, 64.1.^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.12–8.07 (m, 1H, Ar), 7.90–7.86 (m, 1H, Ar), 7.78–7.72 (m, 1H, Ar), 7.71–7.64 (m, 1H, Ar), 7.58–7.50 (m, 2H, Ar), 7.47–7.28 (m, 6H, Ar), 5.27 (s, 1H, CH_2PH), 5.24–5.18 (m, 1H, CH_2PH), 4.77–4.72 (m, 0.5H, C*H*CH_3 L-Ala), 4.65–4.56 (m, 0.5H, C*H*CH_3 L-Ala), 4.56–4.45 (m, 1H, NH), 1.62–1.57 (m, 3H, CHC*H*$_3$ L-Ala).

4.1.2.7. General procedure C. To a stirring suspension of the appropriate nucleoside (1 eq) in anhydrous acetonitrile (for the synthesis of **1** and **2**) or DMF (for the synthesis of **3**) (31 ml per 1 mmol of nucleoside), was added the appropriate phosphorothioate (3 eq) followed by NMI (6 eq) The mixture was stirred at 80 °C (for **1** and **2**) or rt (for **3**) for 24–60 h and the solvent evaporated under vacuum. The obtained crude was was taken up with CH_2Cl_2, washed with 0.1 M HCl. The organic phase was dried with $MgSO_4$, filtered and evaporated and the residue was purified by silica gel flash chromatography using Biotage Isolera One.

Table 5
Cytotoxic activity of Gemcitabine, FUDR, 3́-dA, NUC1031, NUC373, NUC7738 and compounds 1–3.

Cpd	Lymphoma		Myeloma		Leukaemia							
	Non-Hodgkin		MM		CML		AML				ALL	
	RL		RPMI-8226		K562		KG1		HL-60		Molt-4	
	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$	IC_{50}	$MI_{\%}$
Gemcitabine	–	–	–	–	–	–	–	–	–	–	–	–
NUC1031	–	–	–	–	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	–	–	–	–	–	–
FUDR	–	–	11.77	100	–	–	–	–	–	–	–	–
NUC373	–	–	5.90	93	–	–	–	–	–	–	–	–
2	–	–	6.63	100	–	–	–	–	–	–	–	–
3′dA	135.06	4	–	–	94.44	70	88.96	92	145.24	57	85.28	77
NUC378	34.65	94	–	–	10.34	83	23.51	92	38.40	101	4.67	96
3	95.56	113	–	–	64.51	96	59.98	98	73.71	99	9.19	99
paclitaxel	0.007	63	0.002	95	0.013	87	0.068	79	0.012	97	0.004	99

Cytotoxicity data reported as μM IC_{50} values (concentration of drug causing 50% inhibition of cell viability) and MI% values (maximum inhibitory effect of the drug at the range of concentrations considered). PTX: paclitaxel (control); (-) not tested.

5. (2*S*)-benzyl 2-(((((2*R*,3*R*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-4,4-difluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorothioyl)amino)propanoate (1)

Prepared according to the general procedure C using gemcitabine (**7**) (0.20 g, 0.76 mmol) in acetonitrile (25 ml), **5a** (0.84 g, 2.28 mmol) and *N*-methylimidazole (0.37 ml, 4.71 mmol). The reaction mixture was stirred for 48 h at 80 °C. Purification by silica gel flash chromatography using a gradient of CH_2Cl_2/MeOH (98:2) to CH_2Cl_2M/MeOH (90:10) as an eluent afforded the title compound **1** (10 mg, 2 %). ^{31}P NMR (202 MHz, CD_3OD) δ_P 68.7, 68.2. ^{19}F NMR (470 MHz, CD_3OD) δ_F −117.20, −118.24, −118.43, −118.76. ^{1}H NMR (500 MHz, CD_3OD) δ_H 7.55 (d, J = 7.6 Hz, 0.5H, H5), 7.45 (d, J = 7.6 Hz, 0.5H, H5), 7.29–7.04 (m, 10*H*, Ar), 6.19–6.11 (m, 1H, H1′), 5.77 (d, J = 7.6 Hz, 0.5H, H6), 5.72 (d, J = 7.6 Hz, 0.5H, H6), 5.10–5.02 (m, 2H, CH_2PH), 4.38–4.28 (m, 1H, H5′), 4.26–4.07 (m, 3H, H5′, H3′, C*H*CH_3 L-Ala), 4.00–3.93 (m, 1H, H4′), 1.31–1.26 (m, 3H, CHC*H*$_3$ L-Ala). ^{13}C NMR (125 MHz, CD_3OD) δ_C 173.6 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, C═O, ester), 166.1 (C-NH2), 156.3, 156.2 (C═O base), 151.0, 152.03 (d, $^2J_{C\text{-}P}$ = 1.6 Hz, C-Ar), 141.0, 140.9 (CH base), 135.8, 135.8 (C-Ar), 129.0, 129.0, 128.1, 128.0, 127.9, 127.8, 127.8 (CH − Ar), 122.1 (t, $^1J_{C-F}$ = 258 Hz, CF2), 120.9, 120.8, 120.8 (CH − Ar), 95.2, 95.1 (CH base), 84.5 (broad signal, C-1′), 79.0 (broad signal, C-4′), 69.8 (broad signal, C-3′), 66.5, 66.5 (OCH_2Ph), 64.2, 63.8 (2d, $^2J_{C-P}$ = 4.3 Hz, C-5′), 51.3, 50.9 (*C*HCH3), 18.9 (d, $^3J_{C\text{-}P}$ = 6.5 Hz, CH*C*H3), 18.7 (d, $^3J_{C\text{-}P}$ = 7.9 Hz, CH*C*H3). HPLC Reverse-phase eluting with H_2O/CH_3CN from 100/0 to 75/25 in 30 min, F = 1 ml/min, λ = 254 nm, t_R 17.41 min.; $C_{25}H_{28}F_2N_4O_7PS^+$ required *m/z*: 597.1379. [M + H]$^+$, HRMS (ES +) found *m/z* 597.1372 [M + H]$^+$; $C_{25}H_{27}F_2N_4NaO_7PS^+$ required *m/z*: 619.1198 [M + Na]$^+$ HRMS (ES +) found *m/z* 619.1196 [M + Na]$^+$.

5.0.0.1. (2S)-benzyl 2-(((((2R,3S,5R)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(naphthalen-1-yloxy)phosphorothioyl)amino)propanoate (2)

Prepared according to the general procedure C using 5-fluorodeoxyuridine (**9**) (0.17 g, 0.71 mmol) in acetonitrile (20 ml), **5b** (0.89 g, 2.13 mmol) and NMI (0.34 ml, 4.26 mmol). The reaction mixture was stirred at rt for 60 h. Purification by silica gel chromatography, using a gradient of CH_2Cl_2/MeOH (99:1) to CH_2Cl_2/MeOH (90:10) as an eluent afforded the title compound **2** (0.12 g, 28 %). ^{31}P NMR (202 MHz, CD_3OD) δ_P 69.4, 68.7. ^{19}F NMR (470 MHz, CD_3OD): δ_F −167.20, −167.37. ^{1}H NMR (500 MHz, CD_3OD) δ_H 8.06–8.03 (m, 1H, Ar), 7.75–7.071 (m, 1H, Ar), 7.45 (d, J = 6.4 Hz, 0.5H, H6), 7.59–7.46 (m, 2.5H, Ar, H6), 7.41–7.32 (m, 2H, Ar), 7.29–7.12 (m, 6H, Ar), 6.07–6.03 (m, 0.5H, H1′), 5.96–5.92 (m, 0.5H, H1′), 5.07–4.93 (m, 2H, CH_2PH), 4.27–4.14 (m, 3.5H, H3′, H5′, C*H*CH_3 L-Ala), 4.12–4.06 (m, 0.5H, H5′), 3.99–3.92 (m, 1H, H4′), 2.06–2.00 (m, 0.5H, H2′), 1.97–1.91 (m, 0.5H, H2′), 1.72–1.65 (m, 0.5H, H2′), 1.47–1.39 (m, 0.5H, H2′), 1.31–1.23 (m, 3H, CHC*H*$_3$ L-Ala). ^{13}C NMR (125 MHz, CD_3OD) δ_C 173.7 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, C═O, ester), 173.3 (d, $^3J_{C\text{-}P}$ = 5.1 Hz, C═O, ester), 158.1, 157.9 (C═O, base), 149.1, 149.0 (C═O, base), 147.0, 147.8 (d, $^2J_{C\text{-}P}$ = 8.0 Hz, C-Ar), 140.3, 140.1 (d, $^1J_{C\text{-}F}$ = 234.1 Hz, C-F), 135.8, 134.8, 134.7 (C-Ar), 128.2, 128.1, 128.9, 128.8, 127.4, 127.3 (CH-Ar), 127.0, 127.0 (C-Ar), 126.2, 125.84, 125.82, 125.0, 124.9, 124.5, 124.4, 121.7, 121.6, 115.9, 115.9, 115.5, 115.5 (CH-Ar), 85.6, 85,6, 85.5, 85.4 (C-1′, C-4′), 71.1, 70.9 (C-3′), 66.6, 66.5 (CH_2Ph), 66.5, 66.4, 66.4 (C-5′), 51.4, 51.2 (*C*HCH$_3$), 39.6, 39.5 (C-2′), 18.9 (d, $^3J_{C-P}$ = 7.0 Hz, CH*C*H$_3$), 18.7 (d, $^3J_{C-P}$ = 8.0 Hz, CH*C*H3). HPLC Reverse-phase eluting with H_2O/CH_3CN from 100/0 to 75/25 in 30 min, F = 1 ml/min, λ = 272 nm, t_R 17.09 min.; $C_{29}H_{30}FN_3O_8PS^+$ required *m/z*: 630.1470. [M + H]$^+$; HRMS (ES +) found *m/z* 630.1485 [M + H]$^+$ $C_{29}H_{29}FN_3NaO_8PS^+$ required *m/z*: 652.1289 [M + Na]$^+$. HRMS (ES +) found *m/z*: 652.1295 [M + Na]$^+$.

5.0.0.2. benzyl ((((2S,4R,5R)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorothioyl)-L-alaninate (3).

Prepared according to the general procedure C using 3′-deoxy adenosine (**10**) (0.15 g, 0.60 mmol) in DMF (20 ml), **5a** (0.66 g, 1.80 mmol) and NMI (0.24 ml, 3.00 mmol). The reaction mixture was stirred at rt for 60 h. Purification by silica gel chromatography, using a gradient of CH_2Cl_2/MeOH (99:1) to CH_2Cl_2/MeOH (90:10) as an eluent afforded the title compound **9** (8 mg, 2 %). ^{31}P NMR (202 MHz, CD_3OD) δ_P 68.9, 67.9; ^{1}H NMR (500 MHz, CD_3OD) δ_H 8.35 s, (0.5H, H-8), 8.30 (0.5H, H-8), 8.23 (0.5H, H-8), 8.22 (0.5H, H-8), 7.35–7.26 (m, 7H, Ar), 7.23–7.19 (m, 2H, Ar), 7.16–7.13 (m, 1H, Ar), 6.02 (d, J = 1.5 Hz, 0.5H, H-1́), 6.00 (d, J = 1.5 Hz, 0.5H, H-1́),5.16–5.07 (m, 2H, C*H*$_2$Ph), 4.72–4.60 (m, 0.5H, H-2′),4.68–4.61 (m, 1.5H, H-2′ and H-4′), 4.4–4.33 (m, 1H, H-5́), 4.25–4.11(m, 2H, C*H*CH_3 and H-5́), 2.35–2.28 (m, 1H, H-3́), 2.07–2.01 (m, 1H, H-3́), 1.36 (d, J = 7.0 Hz, 1.5H, CHC*H*$_3$), 1.30 (d, J = 6.5 Hz, 1.5H, CHC*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD) δ_C 170.1 (C═O, ester), 155.8 (C-6), 152.4 (CH-2), 152.3 (CH-2), 151.0 (C-4), 151.0 (C-4), 148.7 (C-2), 148.7 (C-2), 135.92 (C-Ar), 135.8 (C-Ar), 128.9 (CH-Ar), 128.9 (CH-Ar), 128.1 (CH-Ar), 128.1 (CH-Ar), 127.8 (CH-Ar), 127.7 (CH-Ar), 120.9 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, CH-Ar), 120.7 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, CH-Ar), 91.78 (C-1́), 91.73 (C-1́), 79.1 (d, $^3J_{C\text{-}P}$ = 7.5 Hz, C-4́), 79.0 (d, $^3J_{C\text{-}P}$ = 7.5 Hz, C-4́), 75.34 (C-2́), 75.31 (C-2́), 67.1 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, C-5́), 66.6 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, C-5) 63.4 (CH_2Ph), 51.2 (*C*HCH$_3$), 50.8 (*C*HCH$_3$), 33.4 (C-3́), 33.1 (C-3́), 18.9 (d, $^3J_{C\text{-}P}$ = 7.5 Hz, CH*C*H$_3$), 18.6 (d, $^3J_{C\text{-}P}$ = 7.5 Hz, CH*C*H$_3$). HPLC Reverse-phase eluting with H_2O/CH_3CN from 100/0 to 75/25 in 30 min, F = 1 ml/min, λ = 272 nm, t_R 17.38 min.; $C_{26}H_{30}N_6O_6PS^+$ required *m/z*: 585.1680. [M + H]$^+$. HRMS (ES +) found *m/z* 585.1672 [M + H]$^+$.

5.1. Biological activity

5.1.1. MTS cell viability assay. The assay was contracted and carried-out by WuXi AppTec (Shanghai) Co., Ltd. The tumor cell lines PANC-1, Mia-Pa-Ca, BxPC-3, RT112/84, T24/83, 5637, PC-3, HT29, HepG2, Colo205, MDA MB231, MCF-7, A2780, OVCAR 3, RL, SK-MES-1, NCI-H1975, RPMI-8226, A498, ACHN-luc2, CAKI-2, K562, KG1, Molt-4, HL-60. were seeded at cell densities of 0.5 to 100 Å~ 103 cells/well in a 96-well plate the day before drug incubation. Then the plates were incubated for 72 h with the different concentrations of compound to be tested. After the incubation period, 50 μL of MTS was added and the tumor 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 XLfit software.

5.1.2. Carboxypeptidase Y assay. Phosphorothioamidates **1**or **2** (5 mg, ± 0.008 mmol) was dissolved in 150 μL of acetone-d_6 and 300 μL of TRIZMA buffer (pH 7.6) was added. A ^{31}P NMR (202 MHz, 64–128 scans) was conducted at this stage as a reference (blank, t = 0). To this mixture, 130 μL of a stock solution of carboxypeptidase enzyme (Purchased from Sigma Aldrich, >50 unit/mg, dissolved in TRIZMA buffer 7.6 pH to a concentration of 50 units/mL, EC 3.4.16.1) was added. ^{31}P NMR (128 scans) were carried out with 1 min of delay between experiments for 48 h at 25 °C.

CRediT authorship contribution statement. **Michaela Serpi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Samule di Ciano: . Fabrizio Pertusati:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Michaela Serpi and Fabrizio Pertusati reports financial support was provided by NuCana plc. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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