Synthesis and Biological Evaluation of 6-Substituted-5-Fluorouridine ProTides.

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In memory of, and dedicated to Professor Christopher McGuigan (1958-2016).

Abstract

A new family of thirteen phosphoramidate prodrugs (ProTides) of different 6-substituted-5-fluorouridine nucleoside analogues were synthesized and evaluated as potential anticancer agents. In addition, antiviral activity against Chikungunya (CHIKV) virus was evaluated using a cytopathic effect inhibition assay. Although a carboxypeptidase Y assay supported a putative mechanism of activation of ProTides built on 5-fluorouridine with such C6-modifications, the Hint docking studies revealed a compromised substrate-activity for the Hint phosphoramidase-type enzyme that is likely responsible for phosphoramidate bioactivation through P-N bond cleavage and free nucleoside 5′-monophosphate delivery. Our observations may support and explain to some extent the poor *in vitro* biological activity generally demonstrated by the series of 6-substituted-5-fluorouridine phosphoramidates (ProTides) and will be of guidance for the design of novel phosphoramidate prodrugs.

Keywords: Orotidine-5′-monophosphate decarboxylase (ODCase), nucleoside analogue (NA), phosphoramidate (ProTide) approach, anticancer, human Hint enzyme

Introduction

Antimetabolites such as nucleoside analogues (NAs) play a crucial role in the clinical treatment of patients with cancer and viral infections.[1-3] Nucleoside analogues

are chemically-modified synthetic compounds that have been developed with the aim to closely mimic their natural counterparts. Hence, they have a good chance to be able to be taken-up by cells, metabolized and incorporated into nucleic acids to subsequently inhibit cellular division and/or viral replication. At the molecular level, the biological effect of NAs is usually exerted following their metabolic conversion into corresponding 5′-mono-, di- and tri-phosphate forms. In addition to their incorporation into newly synthesized DNA or RNA, NAs can target and inhibit intracellular enzymes like for example viral or human polymerases, ribonucleotide reductase[4] (RNR), thymidylate synthase[5] (TS) or orotidine-5′-monophosphate decarboxylase[6a,b] (ODCase). All these enzymes are involved either in the DNA or RNA synthesis, DNA repair or *de novo* pyrimidine nucleotide synthesis. Because *de novo* synthesis of pyrimidine nucleotides is upregulated during abnormal cell growth or during the replication of cells, when the demand for pyrimidine nucleotides is high, the ODCase can be considered as one of the potential anticancer targets.[7] In fact, in the last decades a significant interest has been given to ODCase as a drug target for a number of modified nucleos(t)ide analogues, in particular C6-substituted UMP derivatives also in the antiviral and antimalarial arena.[7-10] This is due to a pivotal role that ODCase plays in the *de novo* synthesis of pyrimidine nucleotides such as uridine-5′-*O*-monophosphate (UMP, **2**) from orotidine-5′-*O*-monophosphate (OMP, **1**, Figure 1) via decarboxylation[6a] and its extraordinary reaction rate enhancement (over 17 orders of magnitude) in comparison with spontaneous uncatalyzed decarboxylation of OMP observed in water at neutral pH and ambient temperature.[11a,b] Present in most species except viruses, ODCase exists as a monofunctional enzyme in bacteria and parasites and as a part of the bifunctional enzyme UMP synthase in human and other high-developed organisms.[12a,b] A number of nucleoside-like and non-nucleoside ODCase inhibitors have been developed and reported in the literature[13] with 6-azauridine[14a-c] (**3**) and pyrazofurin[15] (**4**) as representative examples of the nucleoside class of compounds being effective at the monophosphate level (Figure 2). In 2009, Kotra and co-workers reported in cell-based assays a variety of modified 5-fluorouridine (Figure 2) nucleoside derivatives bearing at the 6-position of the pyrimidine nucleobase small groups like iodo, azido, amino, or ethyl as potent anticancer agents, and their corresponding monophosphate analogues as inhibitors of human (*Hs)* and *Methanobacterium thermoautotrophicium* (*Mt*) ODCase.[16] Later, the

same group disclosed novel N-modified cytidine-based (CMP) orotidine-5′-monophosphate decarboxylase inhibitors with anti-parasitic activity, improved inhibition of the catalytic enzyme activity and binding conformations studies.[17]

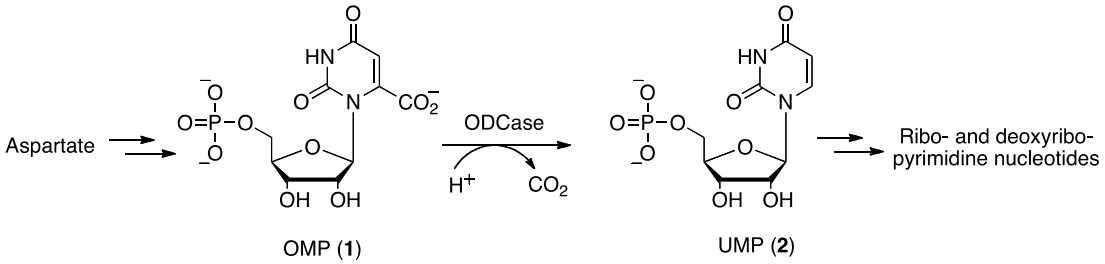


Figure 1. *De novo* synthetic pathway of OMP (**1**) to UMP (**2**) catalysed by ODCase.

Nucleoside analogues, including the above-mentioned 6-substituted-5-fluorouridines are biologically effective after being phosphorylated into their 5′-monophosphate and further to their 5′-di- and 5′-triphosphate forms. The effectiveness of NAs can be impaired as a consequence of limited cellular uptake via nucleoside-specific transporters, down-regulation of nucleoside kinases responsible for the phosphorylation step (activation) and up-regulation of deactivating enzymes (i.e nucleo(s)(t)ide deaminases, purine/pyrimidine nucleoside phosphorylase…).[18] To overcome these limitations, and thus improving their effectiveness, several prodrug approaches have been developed and reviewed over the last years. Notably, phosphate and phosphonate prodrugs such as phosphodiesters (HepDirect, CycloSal, SATE) or phosporamidates in which a nucleoside analogue is linked to the prodrug entity by either a phosphorus-oxygen or a phosphorus-nitrogen bond, respectively, have been extensively studied.[19-21] One of the prominent strategies applied in the modulation of the activity of many nucleoside analogues and potentially overcoming the NA limitations accounting on both innate and acquired resistance of cancer cells to nucleoside analogues, is the ProTide technology pioneered by McGuigan and colleagues.[22] Designed to mask the negative charges in a “monophosphate moiety” of a ProTide template, the ProTide approach introduces an amino acid ester and aryloxy entities as two lipophilic and biolabile groups linked to the phosphate part of the molecule. These two groups increase membrane permeability thereby circumventing the need for nucleoside-transporters and after their intracellular metabolism delivering a nucleoside monophosphate form suitable for further phosphorylation and exertion of its eventual biological activity.

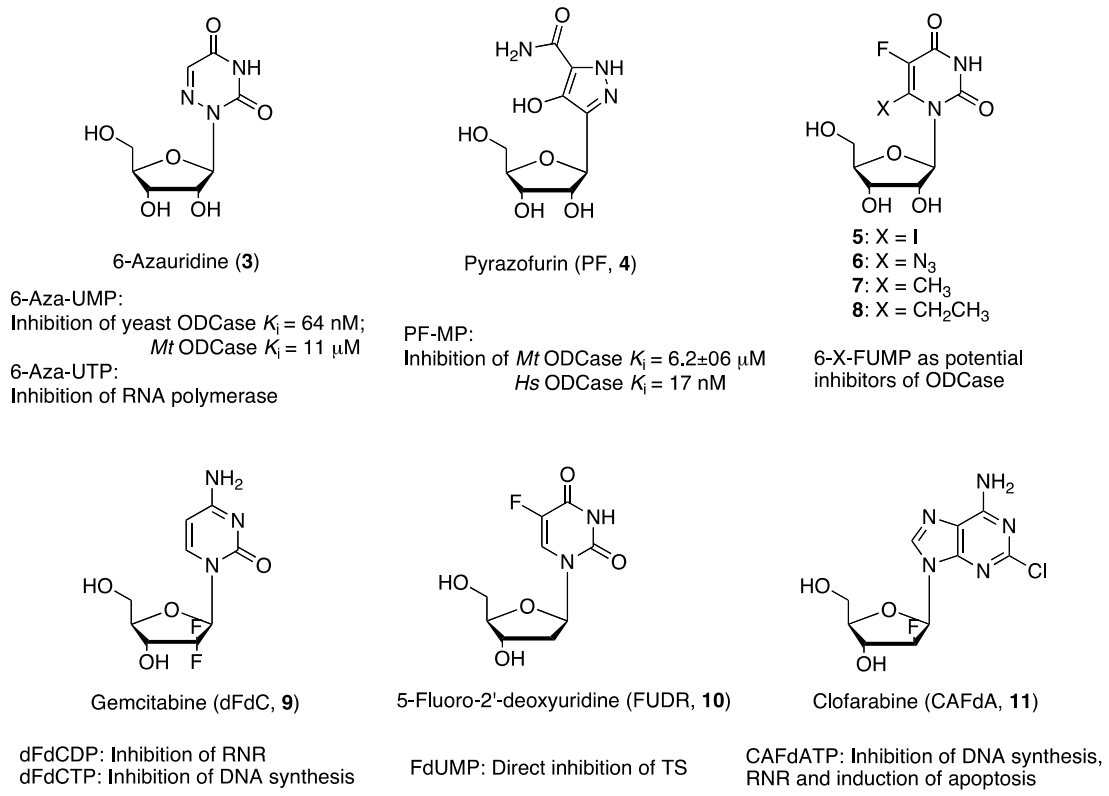


Figure 2. Examples of anticancer nucleoside analogues (**3**-**11**) targeting different intracellular enzymes to exert their eventual biological activity.

The phosphoramidate (ProTide) technology has now an established position in the nucleotide prodrug field and led to the discovery of clinically successful drugs such as Sofosbuvir[23] (FDA approval in 2013 for the treatment of HCV, **12**) and others being subject of currently ongoing clinical trials as exemplified in Figure 3 for antiviral (i.e. stampidine[24] (phase I for HIV treatment, **13**), and anticancer (i.e. thymectacin)[25] (phase I/II, **14**), NUC-1031[26] (phase II, **15**) and NUC-3373[27] (phase I, **16**) activity. In our laboratory we have extensively investigated and applied the ProTide technology to a number of antiviral[28-30] and anticancer agents such as for example 5-fluoro-2′-deoxyuridine (FdUrd),[31] and gemcitabine.[32] As part of our anticancer program and driven by our continuous interests in the discovery of novel anticancer agents we decided to apply the ProTide approach also to 6-substituted-5-fluorouridine analogues. In addition, a new class of 2′-fluoro-6-substituted uridine derivatives reported in the literature[7] as potential inhibitors of ODCase revealed the lack of

cellular anticancer activities most likely due to their poor activation to the corresponding 5′-monophosphate forms. Herein, we report the ProTide technology approach employed to 6-substituted-5-fluorouridine analogues (**5**-**8**) to design novel nucleoside phosphoramidates (**25**-**37**) as potential anticancer agents. These compounds were prepared with the aim to improve cellular uptake of their parent nucleoside analogues and intracellular delivery of their corresponding monophosphate forms. All compounds were preliminary tested for their IC_{50} activity in a panel of cancer cell lines including tumor cell lines of hematopoietic origin as these cell lines were found to be strongly inhibited by 6-substituted-5-fluorouridine analogues.[16]

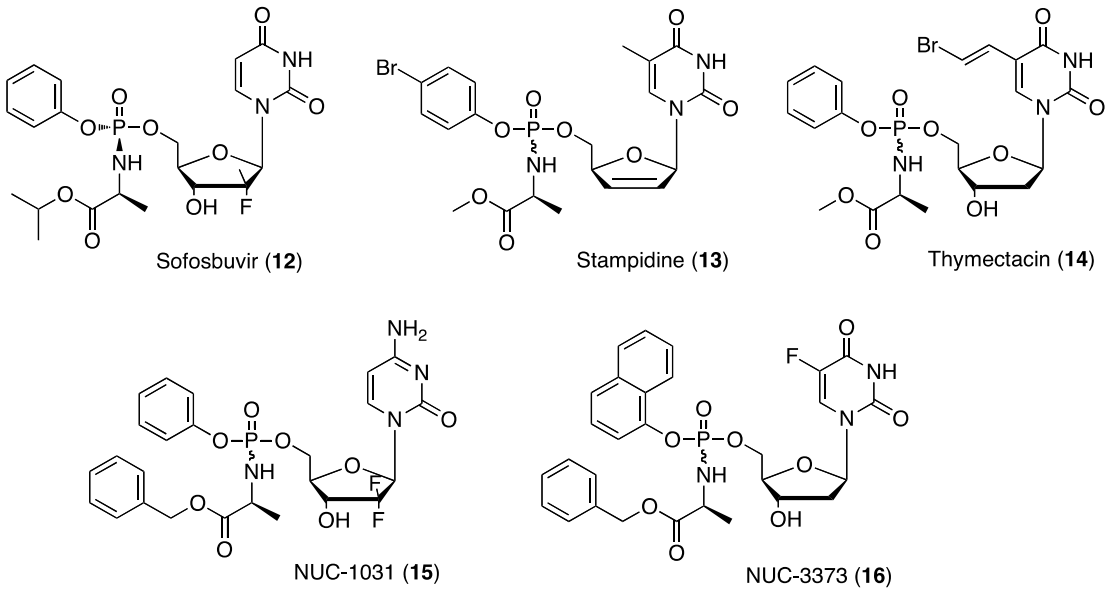


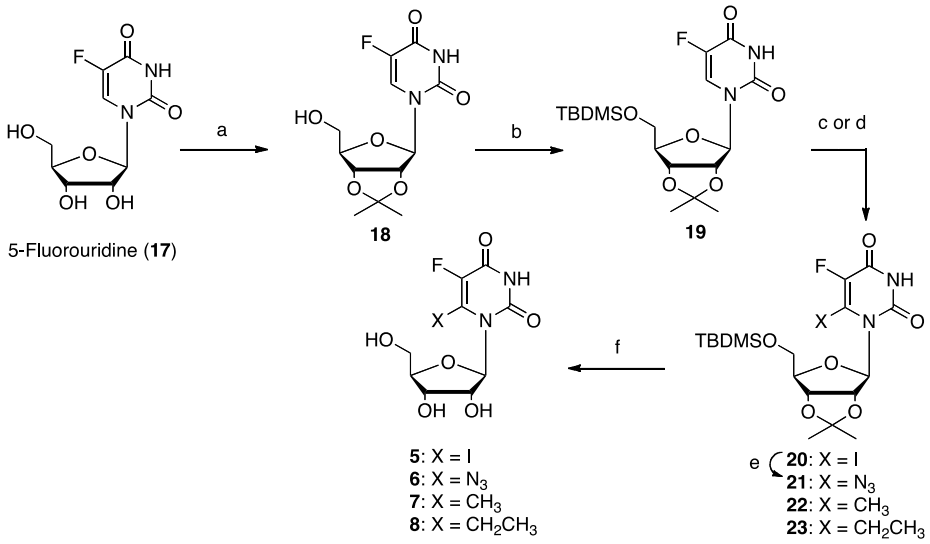
Figure 3. Examples of antiviral and anticancer phosporamidate-type prodrugs.

Chemistry

The 6-modified nucleosides **5**-**8** were prepared according to previously reported methods[16] starting from a two-steps full protection of 5-fluorouridine (**17**) to give an intermediate (**19**) as shown in Scheme 1. The treatment of compound **19** with LDA followed by iodination with I_2 led to the formation of the key nucleoside **20** that was further deprotected under acidic conditions to yield 6-I-FUR (**5**). In addition the iodo-derivative **20** was used to form three other 6-substituted nucleoside analogues, such as 6-azido-, 6-methyl- and 6-ethyl-FUR (**21**-**23**). The 6-azido-derivative **21** was

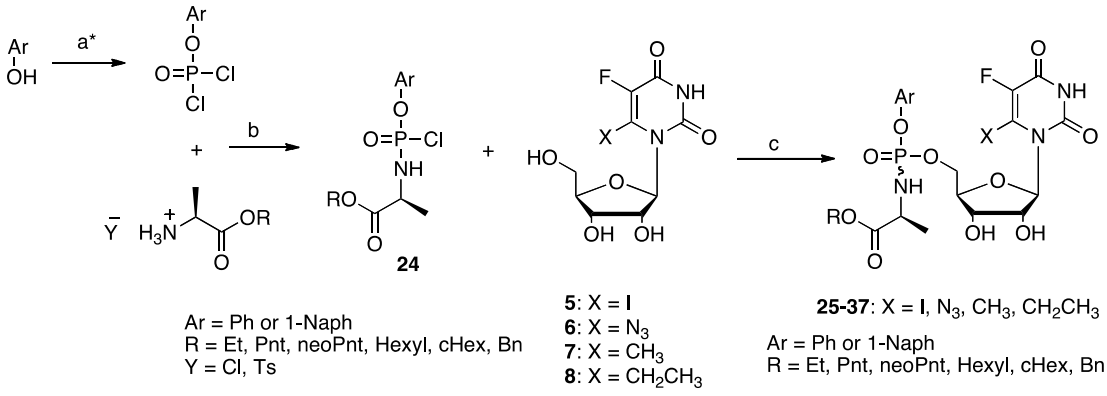
prepared by treating **20** with NaN_3 followed by removal of its silyl and isopropylidine moieties to give compound **6**. The 6-methyl (**22**) and 6-ethyl (**23**) derivatives were successfully prepared using a modified procedure[16] in one-pot reaction upon the treatment of **19** with LDA and subsequent addition of 2.0 eq. of CH_3I. Interestingly, both derivatives **22** and **23**, the latter compound obtained as a side product upon the additional methylation of monomethylated derivative **22**, were isolated and further submitted to the standard acidic deprotection conditions to furnish nucleosides **7** and **8**, respectively. Next, the ProTide technology was applied to the four nucleoside analogues **5**-**8** leading to the formation of 6-substitited-5-fluorouridine ProTides **25**-**37** in moderate yields. The key reagents used in the synthesis of ProTides are the arylaminoacyl phosphorochloridates represented by the general structure **24**, formed by allowing the aryl phosphorodichloridates, either phenyl or 1-naphthyl, to react with L-alanine esters in the presence of triethylamine at low temperature (Scheme 2). The formation of phosphorochloridates **24**, each obtained as a pair of diastereoisomers at the phosphate centre (1:1 mixture), was monitored and confirmed by ^{31}P NMR. Due to their limited stability, **24** were used in the ProTide syntheses as crude materials or after rapid silica gel chromatography. Finally, the four 6-modified nucleosides **5**-**8** were reacted with the key reagents **24** in the presence of NMI to give a number of ProTides **25**-**37** isolated as diastereisomeric mixtures (4-17% yield), as evidenced by ^{31}P NMR, HPLC (two peaks), ^{1}H NMR, and ^{13}C NMR (splitting of many nucleoside signals). Given the low yield of ProTides formation and requirement for an extensive and repeated chromatographic purification, the final compounds **25**-**37** were submitted to *in vitro* evaluation as diastereoisomeric mixtures. Because a primary goal of the following study was to establish preliminary biological activity, at this stage a coupling reaction conditions and methods of isolation of final ProTides as two separate diastereoisomeres were not optimized in the present report.

Scheme 1. Synthesis of 6-substituted-5-fluorouridine nucleoside analogues **5**-**8**[a].



[a]Reagents and conditions: (a) acetone, H_2SO_4, 0 °C, 2 h, 96%; (b) TBDMSCl, imidazole, DCM, 0 °C then room temp 3 h, 90%; (c) LDA, I_2, THF, –78 °C, 7 h, 54%; (d) LDA, CH_3I, THF, –78 °C, 5 h, 20-42%; (e) **1**, NaN_3, DMF, room temp, 3 h, 74%; (f) 50% TFA/H_2O, 0 °C to room temp, 2 h, 80-92%.

Scheme 2. General synthesis of 6-modified ProTides **25**-**37**[a].



[a]Reagents and conditions: (a[*]) for the synthesis of 1-naphthyl phosphorodichloridate $POCl_3$, Et_3N, anhydrous Et_2O, –78 °C for 1 h, then room temp for 1 h, 91%; phenyl phosphorodichloridate commercially available; (b) phenyl or 1-naphthyl phosphorodichloridate, Et_3N, anhydrous DCM, –78 °C for 1 h, then room temp for 1 – 3 h, 70-90%; (c) NMI, anhydrous THF, room temp, 16 h, 4-17%.

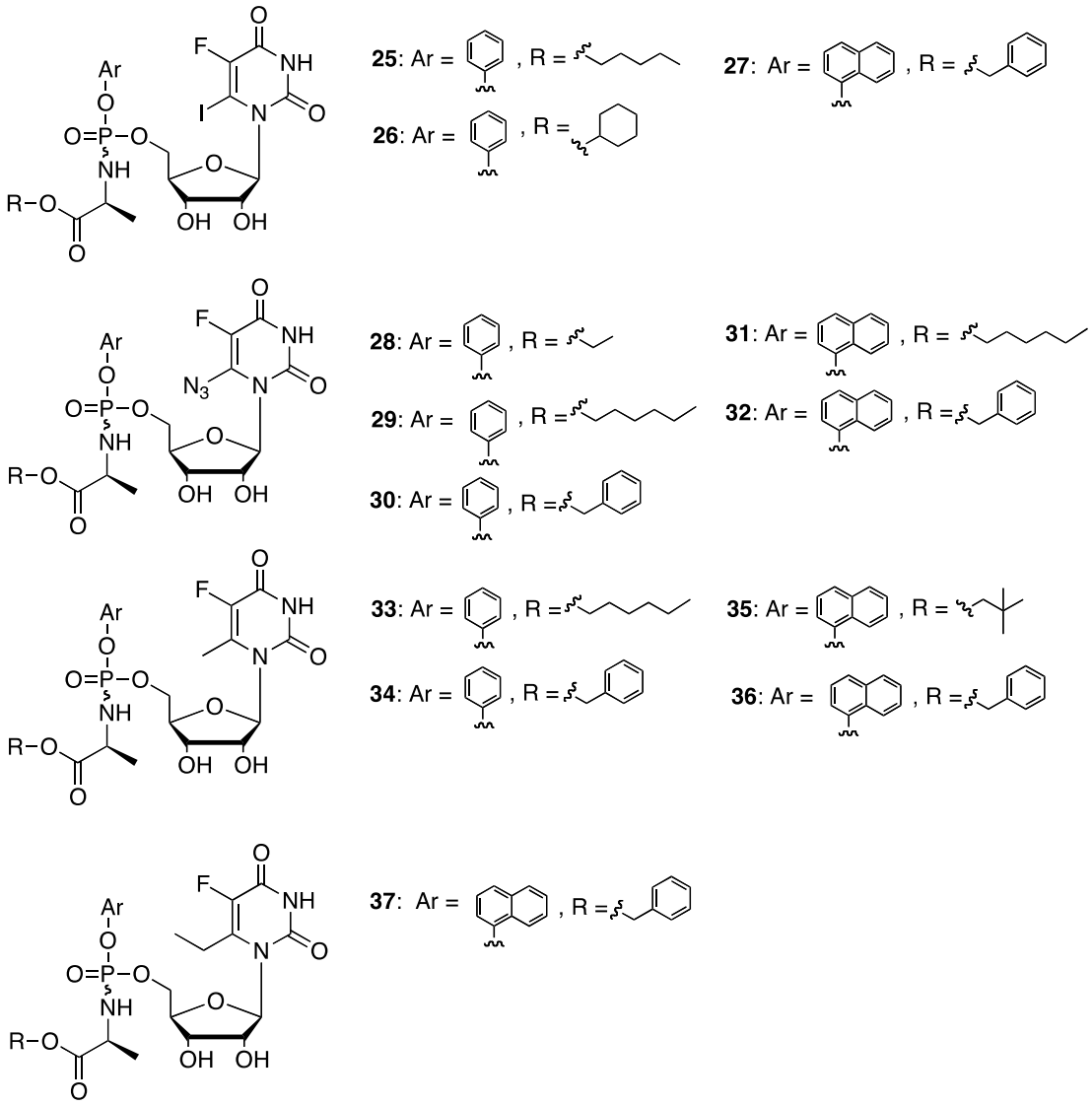


Figure 4. Four classes of 6-substituted-5-fluorouridine target ProTides (**25**-**37**).

Biology

The cytostatic activities of 6-substitited-FUR ProTides **25**-**37** (Figure 4) were evaluated against various cancer cell lines including human breast adenocarcinoma (MCF-7), colon (SW620), pancreatic (Mia-Pa-Ca), acute myeloid leukaemia (MV4-11), erythroleukaemia (HEL92.1.7), non-Hodgkin's lymphoma (RL) and Hodgkin's lymphoma (HS445). The L-alanine-Bn-Naph 6-I-FUR prodrug **27** was among the most active in the series of all 6-modified-FUR ProTides with submicromolar IC_{50} values ranging between 0.08 μM (MV4-11) and 3.9 μM in (MCF-7) cell cultures. The ProTide **27** proved to be equipotent as a cytostatic agent as its parent nucleoside **5** in SW620 cell cultures (0.52 vs 0.50 μM) and 1.8–5.8-fold less potent against HS445 (0.71 vs 0.38 μM) and MCF-7 cell cultures (3.9 vs 0.67 μM), respectively. The loss of

inhibitory activity of the prodrug *versus* **5** was more significant for two other L-alanine 6-I-FUR ProTides, the phenyl-pentyl **25** and phenyl-cyclohexyl **26**, and the activity loss ranged between 2–65-fold and 9–228-fold, respectively.

In the 6-N_3-FUR series, the cytostatic activities for nucleoside analogue **6** were in the lower micromolar range (6–14 μM), whereas for three 6-N_3-FUR ProTides (**30**, **31**, **32**) the IC_{50} values ranged between 6.0 and 44 μM. The ProTides **31** and **32** turned out to be relatively equipotent in three cell lines of hematopoietic origin (MV4-11, RL and HS445) with a 2.3-fold boost in activity for **31** *versus* the parent nucleoside **6** (6.0 μM vs 14.0 μM in the HS445 cell line). In general, the solid tumour cell lines were markedly less sensitive to the 6-N_3-FUR ProTides in particular to the L-alanine phenyl hexyl (**29**) and phenyl ethyl (**28**) derivatives with the latter compound even being inactive up to 50 μM in six out of seven cancer cell lines.

Similar to nucleoside **6**, the 6-Me-FUR nucleoside **7** exerted micromolar activity across all tumor cell lines tested ranging between 2.0 μM (MV4-11) and 16 μM (RL). In the 6-Me-FUR series, no cytostatic activity was detected for ProTides **33**-**36** up to 50 μM in the panel of cancer cell lines. A somewhat similar trend was noted for 6-Et-FUR (**8**) *versus* its ProTide derivative **37**, although the latter compound showed micromolar activity in three out of five tumour cell lines (IC_{50}'s for SW620 10 μM; for RL 27 μM; and for HS445 10 μM).

Table 1. Cytotoxicity of 6-modifed-FUR ProTides **25**-**37** reported as $^{a}IC_{50}$ (μM) Values.

				IC_{50} (μM)							
				Cell lines							
Comp	Nucleoside	AA	Ester (R)	Aryl	MCF-7	SW620	Mia-Pa-Ca	MV4-11	HEL92.1.7	RL	HS445
6-I-FUR	**5**	**-**	**-**	-	0.67	0.50	0.02	0.04	0.1	0.1	0.38
25	**5**	L-Ala	Pnt	Ph	7.83	1.12	1.31	0.20	0.5	0.8	2.6
26	**5**	L-Ala	cHex	Ph	8.12	4.84	4.57	0.74	4.1	2.6	3.8
27	**5**	L-Ala	Bn	Naph	3.93	0.52	0.63	0.08	0.4	0.4	0.71
6-N_3-FUR	**6**	-	-	-	6	8	11	6.6	10	10	14
28	**6**	L-Ala	Et	Ph	>50	>50	>50	>50	>50	>50	44
29	**6**	L-Ala	Hex	Ph	>50	38	>50	9.0	39	20	18
30	**6**	L-Ala	Bn	Ph	20	13	22	8.0	17	21	23
31	**6**	L-Ala	Hex	Naph	31	21	24	6.2	10	9.0	6.0
32	**6**	L-Ala	Bn	Naph	44	28	27	6.3	29	12	15
6-Me-FUR	**7**	**-**	**-**	-	5.4	8.5	12	2.0	14	16	7.0
33	**7**	L-Ala	Hexyl	Ph	>50	>50	>50	>50	>50	>50	>50
34	**7**	L-Ala	Bn	Ph	>50	>50	>50	>50	>50	>50	>50
35	**7**	L-Ala	neoPnt	Naph	>50	>50	>50	>50	>50	>50	>50
36	**7**	L-Ala	Bn	Naph	>50	>50	>50	>50	>50	>50	>50
6-Et-FUR	**8**	-	-	-	>50	>50	ND	ND	>50	>50	>50
37	**8**	L-Ala	Bn	Naph	>50	12	ND	ND	>50	27	10

Cytotoxicity data reported as [a]IC_{50} (μM) values (50% inhibitory concentration of cell viability). The compounds were added to the cell in duplicate and tested in 9 serial concentrations from 198 μM to 0.0199 μM.

The screening of the compounds was performed on a broad variety of at least 6 different cancer cell lines to cover different metabolic properties that may exist between different types of cancer cells that may result in a different outcome of the eventual cytostatic activity of the compounds. In fact, it was observed that the cytostatic activity for some of the 6-substituted-FUR derivatives and their corresponding prodrugs (i.e. 6-I-FUR, 6-Me-FUR) may significantly differ depending on the nature of the prodrug part in the molecule and/or the nature of the tumor cell line investigated. Although the molecular basis of the observed differences are not further investigated in detail yet, the cytostatic differences may be most likely due to i) differences in drug uptake (i.e. depending on the presence and activity of different nucleoside carriers in the tumor cell membrane) and/or efflux of the drugs by the different tumor cell lines, ii) differences in levels and activity of metabolic enzymes that intracellularly convert the particular nucleotide prodrug to the parent nucleotide

5′-monophosphate, and/or iii) differences in nucleotide kinases and other enzymes that may convert the nucleoside 5′-monophosphate to the eventual 5′-triphosphate (i.e. nucleoside 5'-monophosphate kinases and nucleoside 5′-diphosphate kinases) or to the free nucleoside or nucleobase (i.e. 5′-nucleotidases, thymidine phosphorylase). Thus, a complex interplay of a broad variety of enzymes in the particular tumor cell lines will play a determining role in the eventual cytostatic activity of the nucleotide prodrugs. It would therefore be of importance to identify these different factors to clarify and understand the underlying metabolic processes that lead to the cytostatic activity of the different ProTides.

Moreover, it has been suggested that anticancer activity of 6-substituted-FUR analogues might be exerted via inhibition of thymidylate synthase (TS). These 6-modified nucleosides would be intracellularly degraded to their nucleic bases by thymidine phosphorylase (TPase) and further transformed to the corresponding 2′-deoxynucleosides as acceptable substrates for TS.[16] In the studies of 5-fluoro-2′-deoxyuridine (FdUrd) ProTides, we have previously reported that FdUrd ProTides are completely stable in the presence of TP and uridine phosphorylase (UP).[33] In this view, it might be speculated that the ProTide promoiety introduced into the 6-substituted-5-fluorouridine analogues can potentially compromise activity of such compounds as it would prevent their conversion to 6-substituted-5-fluorouracil. Thus, a formation of 6-substituted-2′-deoxy-5-fluorouridine derivatives and their corresponding monophosphate forms would be impaired resulting in potential lack of TS inhibition.

Antiviral Activity *In vitro*

ODCase has been considered as a potential target for agents directed against RNA viruses such as flaviviruses, and togaviruses.[34] Numerous pyrimidine-nucleosides and their derivatives including the phosphoramidate prodrug 6-aza-uridine-5′-(ethyl-methoxyalaninyl)phosphate were shown to exhibit antiviral (RNA) activity *in vitro* and *in vivo.*[14c] In addition, 6-azauridine was also reported as an *in vitro* inhibitor of Chikungunya virus (CHIKV) via inhibition of host ODCase rather than by inhibiting viral specific enzymes.[35a,b] CHIKV as a re-emerging RNA virus for which currently there is no approved treatment or vaccination,[36] is considered as a global health concern. Although, the recent expanding knowledge about the CHIKV genome allows

design of inhibitors that would target individual viral enzymes,[37a-c] there is continuous need for the discovery of novel anti-CHIKV agents. In this view, we evaluated selected nucleosides (**6**, **7**) and their ProTides **32**, **33**, **35-37** against Chikungunya virus in cell culture. Most of the compounds tested in the cytopathicity (CPE)-based assay were devoid of antiviral activity (EC_{50} of >200 μM in comparison with the control 6-azauridine EC_{50} = 0.468 μM). However, five compounds (**6**, **32**, **7**, **35** and **37**) proved to be cytotoxic in a CC_{50} range of 9.4-82 μM. A significant difference in cytotoxicity was observed for the 6-alkylated nucleoside analogues 6-methyl-FUR (**7**, 9.0 μM) *versus* 6-ethyl-FUR (**8**, >200 μM) as well as their corresponding ProTides, in particular **36** (>200 μM) and **37** (53 μM), respectively.

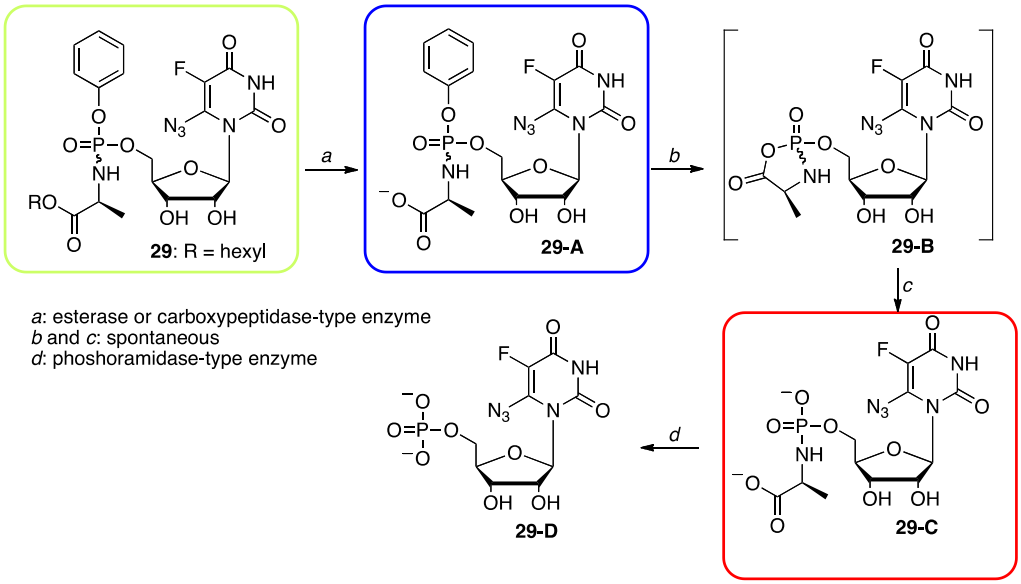
Table 2. Antiviral activity and cytotoxicity in Vero cells of nucleosides **6**, **7** and **8** and ProTides **32**, **33**, **35**-**37**.

Comp	Nucleoside	AA	Ester (R)	Aryl	EC_{50} (μM)	CC_{50} (μM)
6-N_3-FUR	**6**	-	-	-	>200	47.02
32	**6**	L-Ala	Bn	Naph	>200	81.80
6-Me-FUR	**7**	-	-	-	>200	9.04
33	**7**	L-Ala	Hexyl	Ph	>200	>200
35	**7**	L-Ala	neoPnt	Naph	>200	81.29
36	**7**	L-Ala	Bn	Naph	>200	>200
6-Et-FUR	**8**	-	-	-	>200	>200
37	**8**	L-Ala	Bn	Naph	>50	53.10
6-Azauridine	**3**	-	-	-	0.468	>10

EC_{50} or compound concentration required to inhibit the Chikungunya virus-induced cytopathic effect of 50%. The compounds were added to the cells in triplicate and tested in 6 serial concentrations from 198 μM to 0.0196 μM.

Prodrugs such as phosphoramidates exert their biological activity after metabolic activation[38] and intracellular release of the monophosphate form, which would next be phosphorylated to their di- and -triphosphates. In general, the first step in the activation pathway for ProTides is believed to be a hydrolysis of an ester moiety in the amino acid part of the prodrug to form the intermediate **29**-**A** (Scheme 3). This step is mediated by a carboxyesterase-type enzyme and is followed by a spontaneous cyclization leading to a displacement of an aryl moiety *via* an internal nucleophilic attack of the carboxylate residue on the phosphorus to yield an unstable cyclic intermediate **29-B**. In the following two final steps, the cyclic anhydride **29-B** is hydrolyzed to the intermediate **29-C**, which further give rise to the corresponding monophosphate **29-D** upon P-N bond cleavage mediated by the phosphoramidase-type enzyme.[39] In order to assess whether also 6-substituted-FUR ProTides act as a good substrate for the carboxyesterase-type enzyme and hence would be activated by the same common pathway, we performed the carboxypeptidase Y assay on one of the 6-N_3-FUR ProTide, the compound **29** using a reported assay procedure.[40] Thus, compound **29** dissolved in acetone-d_6 in the presence of Trizma buffer (pH 7.6) was treated with carboxypeptidase Y and submitted to ^{31}P NMR analysis over 14 h (Figure 5). Two peaks recorded in the blank spectrum at δ_P 3.64 and 3.82 ppm, correlate to the diastereoisomers of the parent ProTide **29**. Within the first 10 min of the experiment the prodrug **29** was rapidly hydrolysed to the first metabolite **29-A**, lacking the ester moiety (represented as two signals at δ_P 4.68 and 4.87 ppm), which further was processed to the corresponding metabolite **29**-**C** (single peak at δ_P 7.17 ppm). A complete conversion of the ProTide **29** to **29**-**C** occurred within approximately 60 min with an estimated half-life of less than 5 min.

Scheme 3. Proposed Activation Route of 6-N_3-FUR ProTide **29**.



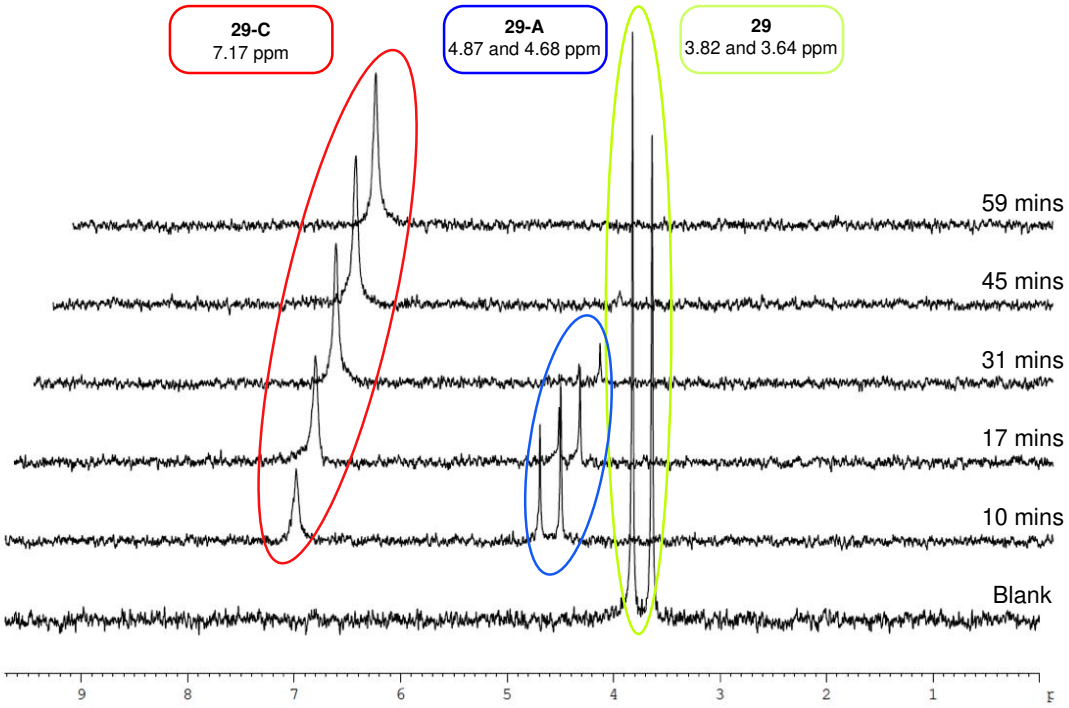


Figure 5. Carboxypeptidase Y-mediated cleavage of ProTide **29** as monitored by ^{31}P NMR.

Docking Studies: Human Hint Enzyme

The last enzymatic step required for the activation of phosphoramidate prodrugs is the cleavage of the P-N bond catalysed by the phosphoramidase-type enzyme, which belongs to the human histidine triad nucleotide-binding protein (Hint).[41] The efficiency of this step determines the eventual activity of ProTides since during this step the free nucleoside monophosphate form is intracellularly delivered. In order to investigate the potential interaction between Hint protein and metabolite **29-C** and to asses its potentiality as a substrate of this enzyme, a series of molecular docking studies using the human Hint enzyme (PDB id: 1KPF)[42] were performed. The catalytic active-site is well-defined by the co-crystallized adenosine monophosphate (AMP), with the three histidine residues interacting with the substrate and Ser107, an important amino acid reported as playing a central role in catalysing the P-N bond cleavage through an acid-base catalysis,[43] making an interaction with the phosphate head. Figure 6 shows the metabolite **29-C** proposed binding mode into the Hint enzyme active pocket. The nucleobase and the sugar are oriented in a different manner if compared to the AMP substrate thus forcing the phosphate moiety in a not-ideal position for the cleavage of the P-N bond (lacking direct interaction with Ser107). This results in a binding that could be considered as being not optimal for the proper enzyme catalytic activity. The docking results seem to suggest that **29-C** and, as a consequence, the other members of this new ProTide series, might not be optimal substrates for Hint. Therefore, the release of the monophosphate form could be drastically reduced or even completely impeded. These findings are in line with previously reported data in which the Hint enzyme has been found to have a lower affinity for pyrimidine than purine derivatives[43,44] and could potentially explain the substantial reduction or even the total lack of cytostatic activity of 6-substituted-FUR ProTides in general when compared with the parent nucleoside.

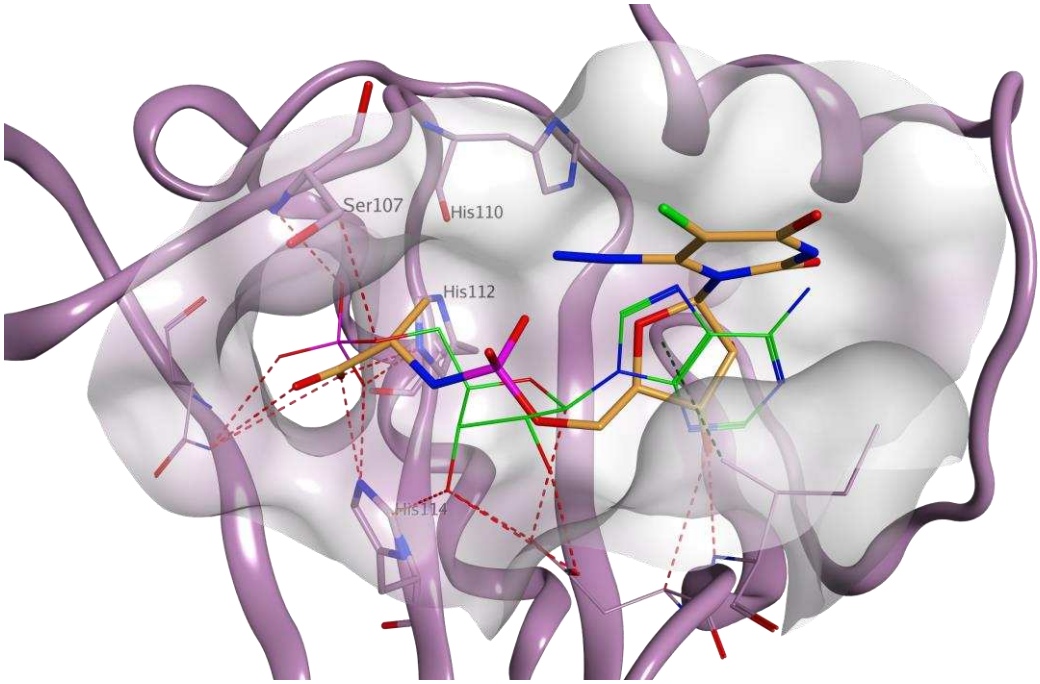


Figure 6. Proposed binding mode of metabolite **29-C** (carbon atoms in orange) in the Hint-1 enzyme. The nucleobase and the sugar occupy the binding site in a different manner when compared with AMP (carbon atoms in green), forcing the P-N bond away from the catalytic Ser107.

Conclusion

In conclusion, we report on the application of the ProTide approach to 6-substituted-5-fluorouridine nucleoside analogues bearing at the C6-position of the 5-fluorouridine scaffold small substituents such as iodo, azido, methyl and ethyl with the aim to prepare potential anticancer and anti-viral agents. It is worth mentioning that during the one-pot synthesis of the 6-alkyl substituted-5-fluorouridine analogues using methyl iodide and LDA, two derivatives were formed and isolated, being the target 6-methyl- and 6-ethyl-5-fluorouridine (as a side-product), respectively. In general, in our *in vitro* studies, 6-substituted-5-fluorouridine ProTides revealed to be less active in comparison with the parent nucleosides. Among four different 6-substituted-5-fluorouridine ProTide series, only the 6-iodo- (**27**) and 6-azido- (**31** and **32**) compounds were equipotent to the parent nucleosides **5** and **6**. The differences in and/or lack of anticancer and anti-CHIKV activity in most cases may indicate either differences in drug uptake depending on the tumor cell, differences in levels and activity of metabolic enzymes and/or poor bioactivation and thus inefficient delivery of a free nucleoside 5′-monophosphate form, respectively. Although carboxypeptidase

Y was able to efficiently activate these phosphoramidate prodrugs to their nucleoside aminoacid phosphate key metabolite, the ProTides seem to be poor, if any substrate for the Hint enzyme. The HINT docking studies performed with 6-azido-flurouridine metabolite indeed showed that structures bearing modifications at the C6-position in a nucleobase moiety are not recognized as good substrates for the phosphoramidase-type Hint enzyme. Overall, the application of the phosphoramidate approach to 6-substituted-5-fluorouridine nucleosides was only modestly successful since none of the ProTides tested showed a significant boost in cytostatic activity against a broad panel of cancer cell lines in comparison with the parent nucleoside analogues. These finding should be kept in mind for the design of novel phosphoramidate ProTides.

Experimental Section

MTS Cell Viability Assay. The assay was contracted and carried-out by WuXi AppTec (Shanghai) Co., Ltd. The tumour cell lines MCF-7, SW620, Mia-Pa-Ca, MV4-11, HEL92.1.7, RL and HS445 were seeded at cell densities of 0.5 to 100 x 10^3 cells/well in a 96-well plate the day before drug incubation. Then the plates were incubated for 72 hours with the different concentrations of compound to be tested. After the incubation period, 50 μL of MTS was added and the tumour 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.

Chikungunya CPE-based EC_{50} Assay. The assay was contracted and carried-out by IBT Bioservices. Vero cells were seeded in 96-well plates and incubated overnight. The next day 6 serial dilutions (starting at 198 μM with 6.32-fold dilutions) of the test compounds and a control compound (6-azauridine) were prepared in culture medium. The growth medium was aspirated from the cells and the compound dilutions were added to the cells in triplicate for a one-hour incubation period. Thereafter, the virus was added at a predetermined MOI (0.01) and the cells were incubated for 3 days. The cell cultures were then fixed and stained with crystal violet in glutaric dialdehyde solution. The optical density was determined and the EC_{50}'s were calculated using the uninfected (cell only) control as 0% CPE and the controls without compound (virus only) as 100% CPE using a 4-PL curve fit of the OD.

Cytotoxicity Assay. The assay was contracted and carried-out by IBT Bioservices. Vero cells were seeded in 96-well plates and incubated overnight. The next day serial dilutions of the test compounds and a control compound (6-azauridine) were prepared. The growth medium was aspirated from the cells and the compound dilutions were added in triplicate. Cells that were incubated with medium only were used for generating the 0% cytotoxicity data. Medium was aspirated and cells were lysed for evaluation of the ATP content using Promega's CelltiterGlo kit on day 3. The resulting luciferase luminescence was quantified and used to calculate the CC_{50} using a 4-PL curve fit of the OD.

Carboxypeptidase Y (EC 3.4.16.1) Assay. The experiment was carried-out by dissolving ProTide **29** (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 analyses (at 25 °C). The spectra were recorded every 7 minutes and followed 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), 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 aluminum backed plates (60 F254, 0.2 mm thickness, Merck) were visualized under both short- and long-wave ultraviolet light (254 and 366 nm). 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 preadsorbed onto silica gel. Fractions containing the product were identified by TLC, 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 analytical column) or ii) Varian Prostar (LC Workstation-Varian Prostar 335 LC detector), Thermo SCIENTIFIC Hypersil Gold C18, 5⌈, 150 x 4.6 mm (as an analytical column). For the method of elution, see the experimental part.

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 MeOH-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 the 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.

Purity of final compounds. The ≥95% purity of all the final compounds was confirmed using HPLC analysis.

The following compounds 2′,3′-*O*-isopropylidene-5-fluoro-uridine (**18**), 5′-*O*-(*t*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-uridine (**19**), fully protected 6-substituted nucleosides **20**-**23** and their deprotected analogues **5**-**8** were prepared according to the slightly modified procedures previously reported in the literature and the experimental data are in agreement with the data reported.[16]

5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-uridine (19). To a stirred suspension of 5-fluorouridine (3.0 g, 11.4 mmol) in anhydrous acetone (120 mL) H_2SO_4 (1.5 mL) was added dropwise at 0°C. The reaction mixture was stirred for 2 h at room temperature. The mixture was then neutralized with 6N NH_4OH and

evaporated to afford crude product which was purified by column chromatography with gradient of MeOH in DCM (5 to 8%) to yield 2′,3′-*O*-isopropylidene-5-fluoro-uridine (**18**) as a white solid (3.30 g, 96%). ^{1}H NMR (500 MHz, MeOD): δ 8.18 (1H, d, *J* = 7.0 Hz, *H*-6), 5.92 (1H, d, *J* = 2.5 Hz, *H*-1′), 4.89 (1H, dd, *J* = 6.5, 2.5 Hz, *H*-2'), 4.87 – 4.85 (1H, apparent m, *H*-3′), 4.25 (1H, apparent q, *J* = 3.0 Hz *H*-4′) 3.83 (1H, dd, *J* = 12.0, 3.0 Hz, 1 x *H*-5′), 3.75 (1H, dd, *J* = 12.0, 3.0 Hz, 1 x *H*-5′), 1.56 (3H, s, CH_3), 1.37 (3H, s, CH_3). A solution of **18** (3.30 g, 10.91 mmol) in anhydrous DCM (130 mL) was treated with imidazole (1.48 g, 21.83 mmol), and TBDMSCl (1.64 g, 10.91 mmol) at 0°C. The reaction mixture was allowed to warm to room temperature and was stirred for 3 h. After solvent evaporation, the crude residue was re-dissolved in EtOAc (60 mL), washed with water (30 mL), brine (30 mL), and dried with Na_2SO_4. Evaporation of the solvent followed by purification by column chromatography with EtOAc/Hexane (3:7) gave 5′-*O*-(*t*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-uridine as a foam (4.08 g, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.99 (1H, d, *J* = 7.0 Hz, *H*-6), 5.87 (1H, d, *J* = 1.5 Hz, *H*-1′), 4.84 – 4.79 (2H, m, *H*-2′, *H*-3′), 4.36 (1H, apparent q, *J* = 3.0 Hz *H*-4′), 3.98 (1H, d, *J* = 11.5, 3.0 Hz, 1 x *H*-5′), 3.87 (1H, d, *J* = 11.5, 3.0 Hz, 1 x *H*-5′), 1.56 (3H, s, CH_3), 1.37 (3H, s, CH_3), 0.94 (s, 9H, C(CH_3)$_3$), 0.14 (6H, s, Si(CH_3)$_2$); ^{13}C NMR (125 MHz, MeOD): δ_C 158.27 (d, $^2J_{C\text{-}F}$ = 23.5 Hz, *C*-4), 149.40 (*C*-2), 140.30 (d, $^1J_{C\text{-}F}$ = 231.0 Hz, *C*-5), 125.31 (d, $^2J_{C\text{-}F}$ = 34.5 Hz, *C*-6), 113.45 (*C*(CH$_3$)$_3$), 91.81 (*C*-1′), 87.23 (*C*-4′), 85.17 (*C*-2′), 80.72 (*C*-3′), 63.27 (*C*-5′), 26.08 (*C*H$_3$), 24.96 (C(*C*H$_3$)$_3$), 24.07 (*C*H$_3$), -6.81 (Si(*C*H$_3$)$_2$).

5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-iodouridine (20). 5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-uridine **19** (3.1 g, 7.44 mmol) was dissolved in anhydrous THF (40 mL) and at -78 °C was treated with dropwise addition of LDA (11.16 mL, 22.32 mmol, 2.0 M solution in THF). After stirring for 1 h, iodine (2.83 g, 11.2 mmol) dissolved in anhydrous THF (50 mL) was added slowly at -78 °C and the resulting mixture was stirred for an additional 6 h in dark. The reaction was quenched with water and brought to room temperature and diluted with EtOAc (80 mL). The organic layer was washed with water (40 mL), brine (40 mL), and dried over Na_2SO_4. The solvent was evaporated and further purified by column chromatography using EtOAc/Hexane (3:7) as an eluent to afford the product as an yellow foam (2.17 g, 54%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.87

(1H, bs, N*H*), 6.10 (1H, d, *J* = 1.5 Hz, *H*-1′), 5.22 (1H, dd, *J* = 6.5, 1.5 Hz, *H*-2′), 4.83 (1H, dd, *J* = 6.0, 4.0 Hz, *H*-3′), 4.21 – 4.18 (1H, m, *H*-4′) 3.84 – 3.77 (2H, m, 2 x *H*-5′), 1.58 (3H, s, CH_3), 1.37 (3H, s, CH_3), 0.90 (s, 9H, C(CH_3)$_3$), 0.07 (6H, s, Si(CH_3)$_2$).

5-Fluoro-6-Iodouridine (5) A stirred solution of 5′-*O*-(*t*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-iodouridine **20** (2.0 g, 3.68 mmol) in water (6 mL) was treated with 50% aqueous (6 mL) at 0°C, brought to room temperature, and stirred for an additional 2 h in dark. The reaction mixture was concentrated under vacuum and was purified by column chromatography with a gradient of MeOH in DCM (5% to 10%) to give a product as a yellowish solid (1.24 g, 87%). ^{1}H NMR (500 MHz, MeOD): δ 5.96 (1H, d, *J* = 3.5 Hz, *H*-1′), 4.76 (1H, dd, *J* = 6.5, 3.5 Hz, *H*-2′), 4.36 (1H, t, *J* = 6.5 Hz, *H*-3′), 3.92 (1H, td, *J* = 6.0, 3.0 Hz, *H*-4′) 3.83 (1H, dd, *J* = 12.0, 3.0 Hz, 1 x *H*-5′), 3.70 (1H, dd, *J* = 12.0, 6.0 Hz, 1 x *H*-5′); ^{13}C NMR (125 MHz, MeOD): δ_C 156.05 (d, $^2J_{C\text{-}F}$ = 29.0 Hz, *C*-4), 148.74 (*C*-2), 145.29 (d, $^1J_{C\text{-}F}$ = 228.0 Hz, *C*-5), 105.17 (d, $^2J_{C\text{-}F}$ = 39.0 Hz, *C*-6), 103.16 (*C*-1′), 86.29 (*C*-4′), 73.42 (*C*-2′), 71.32 (*C*-3′), 63.71 (*C*-5′); MS (ES+) *m/z*: 387.96 (M + Na^+, 100%), Accurate mass: $C_9H_{10}FIN_2O_6$ required 387.96 found 410 (M + Na^+).

5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-azidouridine (21). The compound **20** (1.86 g, 3.43 mmol) dissolved in anhydrous DMF (15 mL) was treated with NaN_3 (0.22 g, 3.43 mmol). The reaction mixture was stirred for 3 h in dark. After that time, the solvent was evaporated, and the residue was re-dissolved in EtOAc (30 mL), washed with brine, and dried over Na_2SO_4. The combined organic layers were evaporated and to afford a yellowish residue which was purified by column chromatography using DCM/MeOH (99:1) as an eluent to give compound 2 as a light-yellow solid (1.16 g, 74%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.40 (1H, s, N*H*), 6.08 (1H, d, *J* = 1.5 Hz, *H*-1′), 5.15 (1H, dd, *J* = 6.5, 1.5 Hz, *H*-2′), 4.80 (1H, dd, *J* = 6.5, 4.5 Hz, *H*-3′), 4.16 – 4.12 (1H, m, *H*-4′), 3.84 – 3.77 (2H, m, 2 x *H*-5′), 1.56 (3H, s, CH_3), 1.35 (3H, s, CH_3), 0.90 (s, 9H, C(CH_3)$_3$), 0.07 (6H, s, Si(CH_3)$_2$). MS (ES+) *m/z*: 480.2 (M + Na^+, 20%), Accurate mass: $C_{18}H_{28}FN_5O_6Si$ required 457.52 found 480.2 (M + Na^+), 937.4 (2 x M + Na^+).

5-Fluoro-6-azidouridine (6) A stirred solution of 5′-*O*-(*t*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-azidouridine **21** (1.10 g, 2.18 mmol) in water (10 mL) was treated with 50% aqueous (10 mL) at 0°C, brought to room temperature, and stirred

for an additional 2 h in dark. The reaction mixture was concentrated under vacuum and was purified by column chromatography with a gradient of MeOH in DCM (6% to 12%) to give a product as a yellowish solid (0.56 g, 85%). ^{1}H NMR (500 MHz, MeOD): δ 5.95 (1H, d, *J* = 3.5 Hz, *H*-1′), 4.65 (1H, dd, *J* = 6.5, 3.5 Hz, *H*-2′), 4.32 (1H, t, *J* = 6.5 Hz, *H*-3′), 3.86 – 3.81 (2H, m, *H*-4′, 1 x *H*-5′), 3.70 – 3.67 (1H, m, 1 x *H*-5′); ^{13}C NMR (125 MHz, MeOD): δ_C 156.54 (d, $^{2}J_{C\text{-}F}$ = 26.0 Hz, *C*-4), 148.40 (*C*-2), 137.20 (d, $^{1}J_{C\text{-}F}$ = 213.0 Hz, *C*-5), 132.8 (d, $^{2}J_{C\text{-}F}$ = 24.0 Hz, *C*-6), 91.60 (*C*-1′), 86.29 (*C*-4′), 84.50 (*C*-2′), 71.94 (*C*-3′), 69.0 (*C*-5′); MS (ES+) *m/z*: 326.20 (M + Na^+, 100%), Accurate mass: $C_9H_{10}FN_5O_6$ required 303.06 found 326.20 (M + Na^+).

5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-methyluridine (22) and 5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-ethyluridine (23). 5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-uridine **19** (3.5 g, 8.40 mmol) was dissolved in anhydrous THF (40 mL) and at -78 °C was treated with dropwise addition of LDA (12.6 mL, 25.20 mmol, 2.0 M solution in THF). After stirring for 1 h, CH_3I (1.04 mL, 16.80 mmol) dissolved in anhydrous THF (5 mL) was added and, and the mixture was stirred for 5 h at -78 °C. The reaction was quenched with water (7 mL) and allowed to warm to rt and then dissolved in ethyl acetate (100 mL). The organic layer was washed with water (40 mL), brine (40 mL), and dried over $NaSO_4$. The solvent was evaporated and the residue was purified by column chromatography using hexane/EtOAc (7:3) as an eluent to give the product **23** (fast eluting fraction, 0.75 g, 20%), and the product **22** (slow eluting fraction, 1.53 g, 42%), both as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.40 (1H, s, N*H*), 5.63 (1H, d, *J* = 1.0 Hz, *H*-1′), 5.18 (1H, dd, *J* = 6.0, 1.0 Hz, *H*-2′), 4.78 (1H, dd, *J* = 6.0, 4.5 Hz, *H*-3′), 4.13 – 4.10 (1H, m, *H*-4′), 3.80 – 3.74 (2H, m, 2 x *H*-5′), 2.31 (3H, d, $J_{H\text{-}F}$ = 3.5 Hz, C-6-CH_3), 1.50 (3H, s, CH_3), 1.30 (3H, s, CH_3), 0.83 (s, 9H, C(CH_3)$_3$), 0.02 (6H, s, Si(CH_3)$_2$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 156.70 (d, $^{2}J_{C\text{-}F}$ = 28.0 Hz, *C*-4), 149.20 (*C*-2), 138.40 (d, $^{1}J_{C\text{-}F}$ = 228.0 Hz, *C*-5), 137.85 (d, $^{2}J_{C\text{-}F}$ = 24.3 Hz, *C*-6), 113.78 (*C*(CH$_3$)$_3$), 91.89 (*C*-1′), 89.58 (*C*-4′), 84.12 (*C*-2′), 81.70 (*C*-3′), 64.10 (*C*-5′), 27.20 (*C*H$_3$), 25.87 (C(*C*H$_3$)$_3$), 25.31 (*C*H$_3$), 11.92 (d, $J_{C\text{-}F}$ = 2.9 Hz, C-6-*C*H$_3$), -5.31 (Si(*C*H$_3$)$_2$).

5′-*O*-(*t*-Butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-ethyluridine (23). δ 9.70 (1H, s, N*H*), 5.57 (1H, d, *J* = 1.5 Hz, *H*-1′), 5.16 (1H, dd, *J* = 6.5, 1.5 Hz, *H*-2′), 4.77 (1H, dd, *J* = 6.5, 4.5 Hz, *H*-3′), 4.14 – 4.10 (1H, m, *H*-4′), 3.81 – 3.75 (2H, m, 2

x *H*-5′), 2.82 – 2.65 (2H, m, C-6-C*H*$_2$CH$_3$), 1.51 (3H, s, C*H*$_3$), 1.30 (3H, s, C*H*$_3$), 1.27 (3H, t, *J* = 8.0 Hz, C-6-CH$_2$C*H*$_3$), 0.83 (9H, s, C(C*H*$_3$)$_3$), 0.04 (6H, s, Si(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 156.90 (d, $^2J_{C\text{-}F}$ = 27.8 Hz, *C*-4), 149.28 (*C*-2), 143.00 (d, $^2J_{C\text{-}F}$ = 23.9 Hz, *C*-6), 137.64 (d, $^1J_{C\text{-}F}$ = 229.3 Hz, *C*-5), 113.91 (*C*(CH$_3$)$_3$), 91.88 (*C*-1′), 89.68 (*C*-4'), 84.26 (*C*-2′), 81.93 (*C*-3′), 64.19 (*C*-5′), 27.24 (*C*H$_3$), 25.92 (C(*C*H$_3$)$_3$), 25.31 (*C*H$_3$), 18.79 (C-6-*C*H$_2$CH$_3$), 12.44 (C-6-CH$_2$*C*H$_3$), -5.23 (Si(*C*H$_3$)$_2$).

5-Fluoro-6-methyluridine (7). A stirred solution of 5′-*O*-(*t*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-methyluridine **22** (1.43 g, 3.32 mmol) in water (10 mL) was treated with 50% aqueous (10 mL) at 0°C, brought to room temperature, and stirred for an additional 2 h. The reaction mixture was concentrated under vacuum and was purified by column chromatography with a gradient of MeOH in DCM (6% to 10%) to give a product as a yellowish solid (0.84 g, 92%). ^{1}H NMR (500 MHz, MeOD): δ 5.53 (1H, d, *J* = 4.0 Hz, *H*-1′), 4.78 (1H, dd, *J* = 6.5, 4.0 Hz, *H*-2′), 4.33 (1H, t, *J* = 6.5 Hz, *H*-3′), 3.93 – 3.90 (1H, m, *H*-4′), 3.82 (1H, dd, *J* = 12.0, 3.0 Hz, 1 x *H*-5′), 3.69 (1H, dd, *J* = 12.0, 5.5 Hz, 1 x *H*-5′), 2.38 (3H, d, $J_{H\text{-}F}$ = 4.0 Hz, C-6-C*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 158.75 (d, $^2J_{C\text{-}F}$ = 27.8 Hz, *C*-4), 150.77 (*C*-2), 140.20 (d, $^2J_{C\text{-}F}$ = 24.5 Hz, *C*-6), 139.71 (d, $^1J_{C\text{-}F}$ = 225.5 Hz, *C*-5), 94.12 (*C*-1′), 86.32 (*C*-4′), 72.81 (*C*-2′), 71.40 (*C*-3′), 63.62 (*C*-5′), 12.02 (d, $^3J_{C\text{-}F}$ = 3.80 Hz, C-6-*C*H$_3$); MS (ES+) *m/z*: 299.2 (M + Na^+, 100%), Accurate mass: $C_{10}H_{13}FN_2O_6$ required 276.22 found 299 (M + Na^+). Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, t_R = 5.01 min.

5-Fluoro-6-ethyluridine (8). A stirred solution of 5′-*O*-(*t*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene-5-fluoro-6-ethyluridine **23** (0.75 g, 1.68 mmol) in water (10 mL) was treated with 50% aqueous (10 mL) at 0°C, brought to room temperature, and stirred for an additional 2 h. The reaction mixture was concentrated under vacuum and was purified by column chromatography with a gradient of MeOH in DCM (6% to 10%) to give a product as a yellowish solid (0.38 g, 80%). ^{1}H NMR (500 MHz, MeOD): δ 5.34 (1H, d, *J* = 4.0 Hz, *H*-1′), 4.64 (1H, dd, *J* = 6.5, 4.0 Hz, *H*-2′), 4.19 (1H, t, *J* = 6.5 Hz, *H*-3′), 3.79 – 3.76 (1H, m, *H*-4′), 3.67 (1H, dd, *J* = 12.0, 3.0 Hz, 1 x *H*-5′), 3.59 (1H, dd, *J* = 12.0, 5.5 Hz, 1 x *H*-5′), 2.73 – 2.60 (2H, m, C-6-*C*H$_2$CH$_3$), 1.18 (3H, t, *J* = 7.5 Hz, C-6-CH$_2$C*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 157.50 (d, $^2J_{C\text{-}F}$ = 28.8 Hz, *C*-4), 149.52 (*C*-2), 143.68 (d, $^2J_{C\text{-}F}$ = 23.8 Hz, *C*-6), 137.62 (d, $^1J_{C\text{-}F}$ = 225.0 Hz, *C*-

5), 92.71 (*C*-1′), 85.01 (*C*-4′), 71.38 (*C*-2′), 70.06 (*C*-3′), 62.38 (*C*-5′), 18.25 (d, $^{3}J_{C\text{-}F}$ = 2.5 Hz, C-6-*C*H_2CH$_3$), 11.31 (C-6-CH$_2$*C*H$_3$); MS (ES+) *m/z*: 313.25 (M + Na^+, 100%), Accurate mass: $C_{11}H_{15}FN_2O_6$ required 290 found 313 (M + Na^+). Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, t_R = 6.05 min.

General Method for the Preparation of phosphorochloridates (24).[45] Anhydrous triethylamine (2.0 mol eq.) was added dropwise at -78 ºC to a stirred solution of the appropriate aryl dichlorophosphate (1.0 mol eq.) and an appropriate amino acid ester (1.0 mol eq.) in anhydrous DCM under argon atmosphere. Following the addition, the reaction mixture was allowed to slowly warm to room temperature and stirred for 1-2 hours. A formation of a desired compound was monitored by ^{31}P NMR. After the reaction was completed, the solvent was evaporated under reduced pressure and the resulting residue was re-dissolved in anhydrous Et_2O and filtered. The filtrate was reduced to dryness to give a crude product as an oil, which was in some cases used without further purification in the next step. Most of aryl phosphorochloridates, in particular those obtained from the amino acid tosylate salt were purified by flash column chromatography using EtOAc/Hexane (7:3) as an eluent.

General method for the preparation of phosphoramidates.

To a stirring solution of nucleoside analogue (1.0 mol/eq.) in anhydrous THF, an appropriate phosphorochloridate (3.0 mol/eq.) dissolved in anhydrous THF was added dropwise under an argon atmosphere. To that reaction mixture NMI (5.0 mol/eq.) was added dropwise over 5 minutes at –78° C under an argon atmosphere. After 15 minutes, the reaction mixture was let to rise to room temperature and stirred overnight (16 - 18h). The solvent was removed under reduced pressure and the residue was re-dissolved in DCM and washed with 0.5 M HCl (3 x 3 mL). The organic layer was dried over $MgSO_4$, filtered, reduced to dryness and purified by column chromatography with gradient of eluent (DCM/MeOH 99:1 to 97:3 to 95:5) followed by preparative TLC purification (DCM/MeOH 95:5).

5-Fluoro-6-iodouridine-5′-*O*-[phenyl-(pentoxy-L-alaninyl)] phosphate (25). Prepared according to the standard procedure from 5-fluoro-6-iodouridine **5** (0.20 g, 0.51 mmol), NMI (0.20 mL, 2.57 mmol), phenyl-(pentoxy-L-alaninyl)-

phosphorochloridate (0.43 g, 1.03 mmol). After column purification on silica gel **25** was obtained as a yellowish solid (0.021 g, 6%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.52, 3.46; ^{1}H NMR (500 MHz, MeOD): δ_H 7.36 – 7.33 (2H, m, *H*-Ar), 7.24 – 7.22 (2H, m, *H*-Ar), 7.19 – 7.16 (1H, m, *H*-Ar), 5.96 (1H, apparent t, J = 3.0 Hz, *H*-1′), 4.73, 4.70 (1H, 2 x dd, J = 6.0, 2.5 Hz, *H*-2′), 4.53 – 4.34 (2H, m, *H*-3′, 1 x *H*-5′), 4.27 – 4.22 (1H, m, 1 x *H*-5′), 4.18 – 4.02 (2H, m, OCH_2), 3.98 – 3.94 (1H, m, *H*-4′), 3.64 – 3.59 (1H, m, NHC*H*CH$_3$), 1.71 – 1.60 (6H, m, 3 x CH_2 ester), 1.39 – 1.32 (3H, m, NHCHCH_3), 0.96 – 0.91 (3H, m, CH_3); ^{13}C NMR (125 MHz, MeOD): δ_C 173.59, 172.90 (*C*=O ester), 156.56 (*C*-4), 151.75, 151.26 (*C*-2), 149.19 (*C*-Ar), 145.28 (d, $^{1}J_{C\text{-}F}$ = 205.0 Hz, *C*-5), 129.28, 127.57, 124.61, 120.12 (*C*H-Ar), 108.98 (*C*-6), 102.19 (*C*-1′), 83.02, 82.51 (*C*-4′), 72.29 (*C*-2′), 70.59, 69.89 (*C*-3′), 65.08 (*C*-5′), 64.24 (O*C*H$_2$), 50.06 (NH*C*HCH$_3$), 28.00, 21.96, 21.06 (*C*H$_2$), 19.37 (NHCH*C*H$_3$), 12.90 (O(CH$_2$)$_4$*C*H$_3$); MS (ES+) *m/z*: 708.0 (M + Na$^+$, 100%), Accurate mass: $C_{23}H_{30}FIN_3O_{10}P$ required 685.38 found 708.0 (M + Na$^+$); Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 17.11, 17.43 min.

5-Fluoro-6-iodouridine-5′-*O*-[phenyl-(cyclohexoxy-L-alaninyl)] phosphate (26).

Prepared according to the standard procedure from 5-fluoro-6-iodouridine **5** (0.23 g, 0.59 mmol), NMI (0.23 mL, 2.96 mmol), phenyl-(cyclohexoxy-L-alaninyl)-phosphorochloridate (0.40 g, 1.18 mmol). After column purification on silica gel **26** was obtained as a yellowish solid (0.028 g, 7%). ^{31}P NMR (202 MHz, MeOD): δ 3.68, 3.54; ^{1}H NMR (500 MHz, MeOD): δ 7.37 – 7.33 (2H, m, *H*-Ar), 7.24 – 7.23 (2H, m, *H*-Ar), 7.20 – 7.17 (1H, m, *H*-Ar), 5.97 – 5.95 (1H, m, *H*-1′), 4.51 – 4.34 (2H, m, *H*-2′, OC*H* ester), 4.51 – 4.34 (2H, m, *H*-3′, 1 x *H*-5′), 4.28 – 4.23 (1H, m, 1 x *H*-5′), 4.05 – 4.01 (1H, m, *H*-4′), 3.96 – 3.89 (1H, m, NHC*H*CH$_3$), 1.83 – 1.73 (5H, m, CH_2 ester), 1.58 – 1.54 (1H, m, CH_2 ester), 1.49 – 1.38 (4H, m, CH_2 ester), 1.37, 1.32 (3H, 2 x dd, J = 7.0, 1.0 Hz, NHCHCH_3); ^{13}C NMR (125 MHz, MeOD): δ_C 174.52, 174.4 (2 x d, $^{3}J_{C\text{-}P}$ = 5.4 Hz, *C*=O ester), 156.40 (d, $^{2}J_{C\text{-}F}$ = 32.0 Hz, *C*-4), 152.30, 152.25 (2 x d, $^{4}J_{C\text{-}F}$ = 4.70 Hz, *C*-2), 148.67, 148.52 (*C*-Ar), 145.36, 145.30 (2 x d, $^{1}J_{C\text{-}F}$ = 228.0 Hz, *C*-5), 130.69, 129.34, 126.03 (*C*H-Ar), 121.68 (d, $^{3}J_{C\text{-}P}$ = 4.6 Hz, *C*H-Ar), 104.94, 104.78 (2 x d, $^{2}J_{C\text{-}F}$ = 39.0 Hz, *C*-6), 103.58, 103.43 (*C*-1′), 84.01, 83.55 (d, $^{3}J_{C\text{-}P}$ = 7.10 Hz, *C*-4′), 75.01, 74.99 (O*C*H), 73.70, 73.55 (*C*-2′), 71.28,

70.69 (*C*-3′), 68.52, 67.48 (2 x d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, *C*-5′), 51.63 (d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, NH*C*HCH$_3$), 32.49, 32.47, 32.39, 26.45, 26.43, 26.41, 24.69, 24.62 (*C*H$_2$), 20.85, 20.76 (2 x d, $^{3}J_{C\text{-}P}$ = 5.75 Hz, NHCH*C*H$_3$); MS (ES+) *m/z*: 720.1 (M + Na$^+$, 100%), Accurate mass: $C_{24}H_{30}FIN_3O_{10}P$ required 697.39 found 720.1 (M + Na$^+$); Reverse-phase HPLC, eluting with H$_2$O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 17.60, 17.64 min.

5-Fluoro-6-iodouridine-5′-*O*-[1-naphthyl-(benzoxy-L-alaninyl)] phosphate (27).

Prepared according to the general procedure from 5-fluoro-6-iodouridine **5** (0.18 g, 0.46 mmol), NMI (0.18 mL, 2.32 mmol), 1-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.37 g, 0.93 mmol). After column purification on silica gel **27** was obtained as a yellowish solid (0.017 g, 5%). ^{31}P NMR (202 MHz, MeOD): δ 3.89, 3.74; ^{1}H NMR (500 MHz, MeOD): δ 8.19 – 8.16 (1H, m, *H*-Ar), 7.89 – 7.85 (1H, m, *H*-Ar), 7.69 – 7.65 (1H, m, *H*-Ar), 7.54 – 7.47 (3H, m, *H*-Ar), 7.41 – 7.36 (1H, m, *H*-Ar), 7.33 – 7.25 (5H, m, *H*-Ar), 5.96 – 5.94 (1H, m, *H*-1′), 5.01 – 4.96 (2H, m, C*H*$_2$Ph), 4.73, 4.68 (1H, 2 x dd, *J* = 6.5, 3.0 Hz, *H*-2′), 4.54 – 4.41 (2H, m, *H*-3′, 1 x *H*-5′), 4.33 – 4.27 (1H, m, 1 x *H*-5′), 4.12 – 4.02 (2H, m, *H*-4′, NHC*H*CH$_3$), 1.35, 1.30 (3H, 2 x dd, *J* = 7.0, 0.5 Hz, NHCHC*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 174.40, 174.26 (*C*=O, ester), 156.50 (*C*-4), 152.40, 152.20 (*C*-2), 148.67 (*C*-Ar), 137.20 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, *C*-Ar), 136.10 (*C*-Ar), 135.30, 135.22 (2 × d, $^{1}J_{C\text{-}F}$ = 225.0 Hz, *C*-5), 129.68, 129.58, 129.36, 129.25, 129.26, 128.76, 128.69, 127.75, 127.71, 127.48, 127.46, 126.57, 126.54, 125.90, 122.90, 122.75 (*C*H-Ar), 116.30, 116.21 (2 x d, $^{3}J_{C\text{-}P}$ = 3.4 Hz *C*H-Ar), 108.74 (*C*-6), 103.63, 103.44 (*C*-1′), 84.00, 83.64 (2 × d, $^{3}J_{C\text{-}P}$ = 7.25 Hz, *C*-4′), 74.05, 73.88 (*C*-2′), 71.28, 70.72 (*C*-3′), 68.52 (d, $^{2}J_{C\text{-}P}$ = 5.8 Hz, *C*-5′), 67.97, 67.85 (O*C*H$_2$Ph), 67.71 (d, $^{2}J_{C\text{-}P}$ = 5.4 Hz, *C*-5′), 51.70 (NH*C*HCH$_3$), 20.86, 20.74 (2 × d, $^{3}J_{C\text{-}P}$ = 6.4 Hz, NHCH*C*H$_3$); MS (ES+) *m/z*: 755.42 (M + Na$^+$, 100%), Accurate mass: $C_{29}H_{28}FIN_3O_{10}P$ required 755.42 found 778.0 (M + Na$^+$); Reverse-phase HPLC, eluting with H$_2$O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 17.21, 17.37 min.

5-Fluoro-6-azidouridine-5′-*O*-[phenyl-(ethoxy-L-alaninyl)] phosphate (28).

Prepared according to the general procedure from 5-fluoro-6-azidouridine **6** (0.15 g, 0.49 mmol), NMI (0.19 mL, 2.47 mmol), phenyl-(ethoxy-L-alaninyl)-phosphorochloridate (0.28 g, 0.99 mmol). After column purification on silica gel **28** was obtained as a yellowish solid (0.014 g, 5%). ^{31}P NMR (202 MHz, MeOD): δ_P

3.69, 3.53; ^{1}H NMR (500 MHz, MeOD): δ_H 7.38 – 7.35 (2H, m, *H*-Ar), 7.25 – 7.18 (3H, m, *H*-Ar), 5.95, 5.92 (1H, 2 × d, *J* = 2.80 Hz, *H*-1′), 4.67 – 4.62 (1H, m, *H*-2′), 4.47 – 4.34 (2H, m, *H*-3′, 1 x *H*-5′), 4.28 – 4.22 (1H, m, 1 x *H*-5′), 4.18 – 4.10 (2H, m, OC*H*$_2$CH$_3$), 4.00-3.92 (2H, m, NHC*H*CH$_3$, *H*-4′), 1.36, 1.32 (3H, 2 × d, *J* = 7.17 Hz, NHCHC*H*$_3$), 1.27 – 1.23 (m, 3H, OCH$_2$C*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 175.11, 174.97 (2 × d, $^{3}J_{C\text{-}P}$ = 5.60 Hz, *C*=O, ester), 157.85 (d, $^{2}J_{C\text{-}F}$ = 24.1 Hz, *C*-4), 152.25 (apparent t, $^{2}J_{C\text{-}P}$ = 12.75 Hz, O-*C*-Ar), 149.32, 149.22 (*C*-2), 139.36 (d, $^{2}J_{C\text{-}F}$ = 21.3 Hz, *C*-6), 136.26, 134.45 (2 × d, $^{1}J_{C\text{-}F}$ = 235 Hz, *C*-5), 130.0, 121.47, 121.43, 121.56, 121.53, 121.47, 121.43 (*C*H-Ar), 92.96, 92.84 (*C*-1′), 83.60, 83.33 (2 × d, $^{3}J_{C\text{-}P}$ = 7.12 Hz, *C*-4′), 73.41, 73.30 (*C*-2′), 70.97, 70.50 (*C*-3′), 68.22, 67.38 (2 × d, $^{2}J_{C\text{-}P}$ = 6.10 Hz, *C*-5′), 62.41, 61.37 (O*C*H$_2$CH$_3$), 51.50 (d, $^{2}J_{C\text{-}P}$ = 5.17 Hz, NH*C*HCH$_3$), 20.15, 20.53 (2 × d, $^{3}J_{C\text{-}P}$ = 6.39 Hz, NHCH*C*H$_3$), 14.97 (OCH$_2$*C*H$_3$); MS (ES+) *m/z*: 581.1 (M + Na$^+$, 100%), Accurate mass: $C_{20}H_{24}FN_6O_{10}P$ required 558.1 found 581.1 (M + Na$^+$); Reverse-phase HPLC, eluting with H$_2$O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 14.68, 14.95 min.

5-Fluoro-6-azidouridine-5′-*O*-[phenyl-(hexoxy-L-alaninyl)] phosphate (29).

Prepared according to the general procedure from 5-fluoro-6-azidouridine **6** (0.10 g, 0.33 mmol), NMI (0.13 mL, 1.65 mmol), phenyl-(hexoxy-L-alaninyl)-phosphorochloridate (0.23 g, 0.66 mmol). After column purification on silica gel **29** was obtained as a yellowish solid (0.008 g, 4%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.67, 3.51; ^{1}H NMR (500 MHz, MeOD): δ_H 7.38 – 7.35 (2H, m, *H*-Ar), 7.25 – 7.18 (3H, m, *H*-Ar), 5.95, 5.93 (1H, 2 × d, *J* = 2.75 Hz, *H*-1′), 4.67 – 4.62 (1H, m, *H*-2′), 4.47 – 4.34 (2H, m, *H*-3′, 1 x *H*-5′), 4.28 – 4.22 (1H, m, 1 x *H*-5′), 4.14 – 4.05 (2H, m, OC*H*$_2$), 4.00 – 3.93 (2H, m, NHC*H*CH$_3$, *H*-4′), 1.66 – 1.60 (2H, m, OCH$_2$C*H*$_2$(CH$_2$)$_3$CH$_3$), 1.40 – 1.31 (9H, m, 3 x C*H*$_2$, NHCHC*H*$_3$), 0.93 – 0.90 (3H, m, C*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 175.17, 175.03 (2 × d, $^{3}J_{C\text{-}P}$ = 5.90 Hz, *C*=O, ester), 157.80 (d, $^{2}J_{C\text{-}F}$ = 23.7 Hz, *C*-4), 152.25 (apparent t, $^{2}J_{C\text{-}P}$ = 12.9 Hz, O-*C*-Ar), 149.42, 149.37 (*C*-2), 139.35 (d, $^{2}J_{C\text{-}F}$ = 20.92 Hz, *C*-6), 136.27, 134.47 (2 × d, $^{1}J_{C\text{-}F}$ = 232.9 Hz, *C*-5), 130.70, 126.08, 126.04, 121.58, 121.54, 121.47, 121.43 (*C*H-Ar), 92.97, 92.84 (*C*-1′), 83.65, 83.33 (2 × d, $^{3}J_{C\text{-}P}$ = 7.11 Hz, *C*-4′), 73.42, 73.29 (*C*-2′), 70.96, 70.50 (*C*-3′), 68.24, 67.39 (2 × d, $^{2}J_{C\text{-}P}$ = 5.44 Hz, *C*-5′), 66.49, 66.45 (O*C*H$_2$), 49.87 (NH*C*HCH$_3$), 32.88, 32.57, (OCH$_2$*C*H$_2$), 29.66, 26.63, 26.60, 23.59, 23.58

(CH_2), 20.70, 20.59 (2 × d, $^3J_{C\text{-}P}$ = 6.9 Hz, NHCHCH_3), 14.34 (O$(CH_2)_5CH_3$); MS (ES+) *m/z*: 581.1 (M + Na^+, 100%), Accurate mass: $C_{24}H_{32}FN_6O_{10}P$ required 614.52 found 637.2 (M + Na^+); Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 18.99, 19.23 min.

5-Fluoro-6-azidouridine-5′-*O*-[phenyl-(benzoxy-L-alaninyl)] phosphate (30).

Prepared according to the general procedure from 5-fluoro-6-azidouridine **6** (0.18 g, 0.59 mmol), NMI (0.24 mL, 2.90 mmol), phenyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.42 g, 1.18 mmol). After column purification on silica gel **30** was obtained as a yellowish solid (0.026 g, 7%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.71, 3.45; ^{1}H NMR (500 MHz, MeOD): δ_H 7.37 – 7.32 (7H, m, *H*-Ar), 7.22 – 7.16 (3H, m, *H*-Ar), 5.96 (1H, 2 x d, *J* = 3.0 Hz, *H*-1′), 5.17 – 5.11 (2H, m, CH_2Ph), 4.65, 4.62 (1H, 2 x dd, *J* = 6.5, 3.0 Hz, *H*-2′), 4.45 – 4.33 (2H, m, *H*-3′, 1 x *H*-5′), 4.26 – 4.21 (1H, m, 1 x *H*-5′), 4.04 – 3.95 (2H, m, NHCHCH_3, *H*-4′), 1.37, 1.33 (3H, dd, *J* = 7.0, 1.0 Hz, NHCHCH_3); ^{13}C NMR (125 MHz, MeOD): δ_C 173.39, 173.26 (2 × d, $^3J_{C\text{-}P}$ = 4.75 Hz, *C*=O, ester), 157.76 (d, $^2J_{C\text{-}F}$ = 24.8 Hz, *C*-4), 149.26, 149.15 (*C*-2), 152.36 (*C*-Ar), 139.31 (*C*-6), 136.18 (*C*-Ar), 135.30, 135.21 (2 × d, $^1J_{C\text{-}F}$ = 224.0 Hz, *C*-5), 130.10, 130.04, 129.58, 129.52, 128.86, 128.79, 127.65, 127.55 (*C*H-Ar), 120.06, 120.0 (*C*H-Ar), 92.90, 92.81 (*C*-1′), 83.45, 83.25 (*C*-4′), 73.45, 73.28 (*C*-2′), 70.94, 70.50 (*C*-3′), 68.50 (d, $^2J_{C\text{-}P}$ = 5.4 Hz, *C*-5′), 68.00, 67.80 (O*C*H_2Ph), 67.70 (d, $^2J_{C\text{-}P}$ = 5.4 Hz, *C*-5′), 51.70 (NH*C*HCH$_3$), 20.70, 20.55 (2 × d, $^3J_{C\text{-}P}$ = 6.4 Hz, NHCHCH_3); MS (ES+) *m/z*: 643.0 (M + Na^+, 100%), Accurate mass: $C_{25}H_{26}FN_6O_{10}P$ required 620.48 found 643.0 (M + Na^+); Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 17.60, 17.76 min.

5-Fluoro-6-azidouridine-5′-*O*-[1-naphthyl-(hexoxy-L-alaninyl)] phosphate (31).

Prepared according to the general procedure from 5-fluoro-6-azidouridine **6** (0.18 g, 0.59 mmol), NMI (0.23 mL, 2.97 mmol), 1-naphthyl-(hexoxy-L-alaninyl)-phosphorochloridate (0.47 g, 1.19 mmol). After column purification on silica gel **31** was obtained as a yellowish solid (0.019 g, 5%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.89; ^{1}H NMR (500 MHz, MeOD): δ_H 8.08 – 8.06 (1H, m, *H*-Ar), 7.78 – 7.74 (1H, m, *H*-Ar), 7.59 – 7.57 (1H, m, *H*-Ar), 7.45 – 7.30 (3H, m, *H*-Ar), 7.32 – 7.28 (1H, m, *H*-Ar), 5.81, 5.79 (1H, 2 × d, *J* = 3.0 Hz, *H*-1′), 4.53, 4.50 (1H, 2 x dd, *J* = 6.5, 3.0 Hz,

H-2′), 4.38 – 4.26 (2H, m, *H*-3′, 1 x *H*-5′), 4.23 – 4.17 (1H, m, 1 x *H*-5′), 3.93 – 3.85 (4H, m, OCH_2, NHCHCH_3, *H*-4′), 1.45 – 1.38 (2H, m, OCH$_2$CH_2(CH$_2$)$_3$CH$_3$), 1.24, 1.20 (3H, 2 x dd, *J* = 7.0, 0.5 Hz, NHCHCH_3), 1.17 – 1.10 (6H, m, 3 x CH_2), 0.78 – 0.73 (3H, m, CH_3); ^{13}C NMR (125 MHz, MeOD): δ_C 175.18, 175.0 (2 × d, $^3J_{C\text{-}P}$ = 4.50 Hz, *C*=O, ester), 157.75 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, *C*-4), 149.25, 149.17 (*C*-2), 148.10 (d, $^2J_{C\text{-}P}$ = 7.0 Hz, O-*C*-Ar), 139.30 (d, $^2J_{C\text{-}F}$ = 19.5 Hz, *C*-6), 136.29 (d, $^3J_{C\text{-}P}$ = 2.0 Hz, *C*-Ar), 135.32, 135.29 (2 × d, $^1J_{C\text{-}F}$ = 225.0 Hz, *C*-5),128.87, 128.79, 127.75, 127.73, 127.42, 127.38 (*C*H-Ar), 126.54, 126.52 (2 x d, $^3J_{C\text{-}P}$ = 1.5 Hz, *C*H-Ar), 125.83, 122.95, 122.78, 116.23, 116.20, 116.19, 116.17 (*C*H-Ar), 92.98, 92.85 (*C*-1′), 83.68, 83.36 (2 × d, $^3J_{C\text{-}P}$ = 7.0 Hz, *C*-4′), 73.46, 73.31 (*C*-2′), 70.95, 70.52 (*C*-3′), 68.51, 67.66 (2 × d, $^2J_{C\text{-}P}$ = 5.5 Hz, *C*-5′), 66.52, 66.45 (O*C*H$_2$), 51.64 (NH*C*HCH$_3$), 32.57, 32.54 (OCH$_2$*C*H$_2$), 29.60, 26.60, 26.57, 23.57, 23.55 (*C*H$_2$), 20.78, 20.64 (2 × d, $^3J_{C\text{-}P}$ = 6.5 Hz, NHCH*C*H$_3$), 14.35, 14.33 (O(CH$_2$)$_5$*C*H$_3$); MS (ES+) *m/z*: 687.58 (M + Na$^+$, 10%), Accurate mass: $C_{28}H_{34}FN_6O_{10}P$ required 664.58 found 687.2 (M + Na$^+$), 1351.4 (2 x M + Na$^+$), Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 23.08, 23.51 min.

5-Fluoro-6-azidouridine-5′-*O*-[1-naphthyl-(benzoxy-L-alaninyl)] phosphate (32). Prepared according to the general procedure from 5-fluoro-6-azidouridine **6** (0.17 g, 0.56 mmol), NMI (0.23 mL, 2.80 mmol), 1-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.45 g, 1.12 mmol). After column purification on silica gel **32** was obtained as a yellowish solid (0.029 g, 8%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.95, 3.79; ^{1}H NMR (500 MHz, MeOD): δ_H 8.19 – 8.17 (1H, m, *H*-Ar), 7.89 – 7.86 (1H, m, *H*-Ar), 7.70 (1H, apparent d, *J* = 12.5 Hz, *H*-Ar), 7.55 – 7.48 (3H, m, *H*-Ar), 7.43 – 7.38 (1H, m, *H*-Ar), 7.32 – 7.29 (5H, m, *H*-Ar), 5.91, 5.90 (1H, 2 x d, *J* = 3.0 Hz, *H*-1′), 5.09 – 5.02 (2H, m, CH_2Ph), 4.65, 4.60 (1H, 2 x dd, *J* = 6.0, 3.0 Hz, *H*-2′), 4.48 – 4.37 (2H, m, *H*-3′, 1 x *H*-5′), 4.33 – 4.28 (1H, m, 1 x *H*-5′), 4.13 – 4.05 (1H, m, NHCHCH$_3$), 4.02 – 3.98 (1H, m, *H*-4′), 1.35, 1.32 (3H, 2 x dd, *J* = 7.0, 1.0 Hz, NHCHCH_3); ^{13}C NMR (125 MHz, MeOD): δ_C 173.39, 173.26 (2 × d, $^3J_{C\text{-}P}$ = 4.75 Hz, *C*=O, ester), 157.76 (d, $^2J_{C\text{-}F}$ = 24.8 Hz, *C*-4), 149.26, 149.15 (*C*-2), 148.07 (d, $^2J_{C\text{-}P}$ = 7.3 Hz, O-*C*-Ar), 139.31, 139.26 (2 x d, $^2J_{C\text{-}F}$ = 19.1 Hz, *C*-6), 137.18 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *C*-Ar), 136.28 (*C*-Ar), 135.28, 135.25 (2 × d, $^1J_{C\text{-}F}$ = 224.8 Hz, *C*-5), 129.58, 129.54, 129.31, 129.23, 129.18, 128.86, 128.79, 127.75, 127.73, 127.46, 127.41,

126.53, 126.51, 125.87, 122.95, 122.75 (*C*H-Ar), 116.31, 116.21 (2 x d, $^{3}J_{C\text{-}P}$ = 3.4 Hz *C*H-Ar), 92.97, 92.81 (*C*-1′), 83.65, 83.35 (2 × d, $^{3}J_{C\text{-}P}$ = 6.8 Hz, *C*-4′), 73.45, 73.28 (*C*-2′), 70.94, 70.50 (*C*-3′), 68.52 (d, $^{2}J_{C\text{-}P}$ = 5.4 Hz, *C*-5′), 68.01, 67.80 (*C*H$_2$Ph), 67.71 (d, $^{2}J_{C\text{-}P}$ = 5.4 Hz, *C*-5′), 51.70 (NH*C*HCH$_3$), 20.58, 20.45 (2 × d, $^{3}J_{C\text{-}P}$ = 6.4 Hz, NHCH*C*H$_3$); MS (ES+) *m/z*: 693.2 (M + Na$^+$, 30%), Accurate mass: $C_{29}H_{28}FN_6O_{10}P$ required 670.54 found 693.2 (M + Na$^+$), 1363.4 (2 x M + Na$^+$), Reverse-phase HPLC, eluting with H$_2$O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 17.20, 17.53 min.

5-Fluoro-6-methyluridine-5′-*O*-[1-phenyl-(hexoxy-L-alaninyl)] phosphate (33). Prepared according to the general procedure from 5-fluoro-6-methyluridine **7** (0.18 g, 0.65 mmol), NMI (0.26 mL, 3.26 mmol), phenyl-(hexoxy-L-alaninyl)-phosphorochloridate (0.45 g, 1.30 mmol). After column purification on silica gel **33** was obtained as an off-white solid (0.034 g, 9%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.56, 3.44; ^{1}H NMR (500 MHz, MeOD): δ_H 7.37 – 7.33 (2H, m, *H*-Ar), 7.25 – 7.22 (2H, m, *H*-Ar), 7.22 – 7.17 (1H, m, *H*-Ar), 5.52, 5.50 (1H, 2 × d, *J* = 2.75 Hz, *H*-1′), 4.77, 4.74 (1H, 2 x d, *J* = 6.0, 3.0 Hz, *H*-2′), 4.49 – 4.34 (2H, m, *H*-3′, 1 x *H*-5′), 4.27 – 4.21 (1H, m, 1 x *H*-5′), 4.14 – 3.93 (4H, m, OC*H*$_2$, NHC*H*CH$_3$, *H*-4′), 2.37, 2.36 (3H, 2 x d, $J_{C\text{-}F}$ = 4.0 Hz, CH$_3$), 1.65 – 1.58 (2H, m, OCH$_2$C*H*$_2$(CH$_2$)$_3$CH$_3$), 1.38 – 1.31 (9H, m, 3 x C*H*$_2$, NHCHC*H*$_3$), 0.93 – 0.89 (3H, m, C*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 175.13, 174.99 (2 × d, $^{3}J_{C\text{-}P}$ = 6.0 Hz, *C*=O, ester), 158.85 (d, $^{2}J_{C\text{-}F}$ = 26.6 Hz, *C*-4), 152.30, 152.26 (2 x d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, O-*C*-Ar), 150.57, 150.45 (*C*-2), 139.95 (d, $^{2}J_{C\text{-}F}$ = 24.4 Hz, *C*-6), 139.72, 139.67 (2 × d, $^{1}J_{C\text{-}F}$ = 225.5 Hz, *C*-5), 130.07, 126.08, 126.03, 121.66, 121.62, 121.50, 121.47 (*C*H-Ar), 94.63, 94.51 (*C*-1′), 83.70, 83.38 (2 × d, $^{3}J_{C\text{-}P}$ = 7.40 Hz, *C*-4′), 73.17, 73.04 (*C*-2′), 71.26, 70.75 (*C*-3′), 68.34, 67.46 (2 × d, $^{2}J_{C\text{-}P}$ = 5.50 Hz, *C*-5′), 66.49, 66.42 (O*C*H$_2$), 51.54 (NH*C*HCH$_3$), 32.88, 32.57 (OCH$_2$*C*H$_2$), 29.67, 26.65, 26.62, 23.59, 23.58 (*C*H$_2$), 20.73, 20.64 (2 × d, $^{3}J_{C\text{-}P}$ = 6.0 Hz, NHCH*C*H$_3$), 14.35 (O(CH$_2$)$_5$*C*H$_3$), 11.96 (d, $^{3}J_{C\text{-}F}$ = 3.6 Hz, C-6-*C*H$_3$); MS (ES+) *m/z*: 610.2 (M + Na$^+$, 100%), Accurate mass: $C_{25}H_{35}FN_3O_{10}P$ required 587.53 found 610.2 (M + Na$^+$), 1197.4 (2 x M + Na$^+$); Reverse-phase HPLC, eluting with H$_2$O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 17.73, 17.91 min.

5-Fluoro-6-methyluridine-5′-*O*-[phenyl-(benzoxy-L-alaninyl)] phosphate (34). Prepared according to the general procedure from 5-fluoro-6-methyluridine **7** (0.15 g,

0.54 mmol), NMI (0.22 mL, 2.72 mmol), phenyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.38 g, 1.08 mmol). After column purification on silica gel **34** was obtained as an off-white solid (0.026 g, 8%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.57, 3.39; ^{1}H NMR (500 MHz, MeOD): δ_H 7.38 – 7.30 (7H, m, *H*-Ar), 7.22 – 7.16 (3H, m, *H*-Ar), 5.51, 5.49 (1H, 2 x d, *J* = 3.0 Hz, *H*-1′), 5.17 – 5.09 (2H, m, C*H*$_2$Ph), 4.76, 4.72 (1H, 2 x dd, *J* = 6.5, 3.0 Hz, *H*-2′), 4.48 – 4.33 (2H, m, *H*-3′, 1 x *H*-5′), 4.25 – 4.18 (1H, m, 1 x *H*-5′), 4.04 – 3.95 (2H, m, NHC*H*CH$_3$, *H*-4′), 2.35, 2.33 (3H, 2 x d, *J* = 4.0 Hz, C*H*$_3$), 1.37, 1.31 (3H, 2 x dd, *J* = 7.0, 1.0 Hz, NHCHC*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 173.37, 173.30 (2 × d, $^{3}J_{C\text{-}P}$ = 5.10 Hz, *C*=O, ester), 157.46, 157.40 (2 x d, $^{2}J_{C\text{-}F}$ = 26.2 Hz, *C*-4), 150.81, 150.76 (*C*-2), 147.96 (*C*-Ar), 139.16, 139.09 (d, $^{2}J_{C\text{-}P}$ = 7.5 Hz, O-*C*-Ar), 138.53, 138.50 (2 x d, $^{2}J_{C\text{-}F}$ = 24.6 Hz, *C*-6), 136.58 (d, $^{1}J_{C\text{-}F}$ = 185.5 Hz, *C*-5), 129.33, 129.27, 128.17, 128.15, 127.91, 127.85, 124.64, 124.62 (*C*H-Ar), 120.23, 120.06 (2 x d, $^{2}J_{C\text{-}P}$ = 4.5 Hz *C*H-Ar), 93.22, 93.06 (*C*-1′), 82.26, 81.92 (2 × d, $^{3}J_{C\text{-}P}$ = 7.2 Hz, *C*-4′), 71.74, 71.60 (*C*-2′), 69.80, 69.27 (*C*-3′), 66.95 (d, $^{2}J_{C\text{-}P}$ = 5.8 Hz, *C*-5′), 66.58, 66.46 (O*C*H$_2$Ph), 66.04 (d, $^{2}J_{C\text{-}P}$ = 5.8 Hz, *C*-5’), 50.16 (NH*C*HCH$_3$), 19.13, 19.05 (2 × d, $^{3}J_{C\text{-}P}$ = 6.4 Hz, NHCH*C*H$_3$), 10.54 (d, $^{3}J_{C\text{-}F}$ = 3.3 Hz, C-6-*C*H$_3$); MS (ES+) *m/z*: 616.1 (M + Na$^+$), Accurate mass: $C_{26}H_{29}FN_3O_{10}P$ required 593.49 found 616.1 (M + Na$^+$), 1209.3 (2 x M + Na$^+$), Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 15.15, 15.35 min.

5-Fluoro-6-methyluridine-5′-*O*-[1-naphthyl-(neopentoxy-L-alaninyl)] phosphate (35). Prepared according to the general procedure from 5-fluoro-6-methyluridine **7** (0.10 g, 0.36 mmol), NMI (0.14 mL, 1.81 mmol), 1-naphthyl-(neopentoxy-L-alaninyl)-phosphorochloridate (0.28 g, 0.72 mmol). After column purification on silica gel **35** was obtained as an off-white solid (0.038 g, 17%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.92, 3.78; ^{1}H NMR (500 MHz, MeOD): δ_H 8.04 – 8.0 (1H, m, *H*-Ar), 7.73 – 7.70 (1H, m, *H*-Ar), 7.53 (1H, apparent d, *J* = 8.0 Hz, *H*-Ar), 7.39 – 7.33 (3H, m, *H*-Ar), 7.27 – 7.23 (1H, m, *H*-Ar), 5.33, 5.31 (1H, 2 x d, *J* = 3.0 Hz, *H*-1′), 4.58, 4.55 (1H, 2 x dd, *J* = 6.5, 3.0 Hz, *H*-2′), 4.35 – 4.24 (2H, m, *H*-3′, 1 x *H*-5′), 4.19 – 4.13 (1H, m, 1 x *H*-5′), 3.94 – 3.86 (2H, m, NHC*H*CH$_3$, *H*-4′), 3.63 – 3.50 (2H, m, C*H*$_2$C(CH$_3$)$_3$), 2.15, 2.14 (3H, 2 x d, *J* = 4.0 Hz, C*H*$_3$), 1.22, 1.18 (3H, 2 x dd, *J* = 7.0, 0.5 Hz, NHCHC*H*$_3$), 0.75, 0.73 (9H, 2 x s, CH$_2$C(C*H*$_3$)$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 175.01, 174.87 (2 × d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, *C*=O, ester), 158.83, 158.79 (2 x d,

$^{2}J_{C\text{-}F}$ = 28.3 Hz, *C*-4), 150.55, 150.42 (*C*-2), 148.12, 148.07 (2 x d, $^{2}J_{C\text{-}P}$ = 5.2 Hz, O-*C*-Ar), 140.58, 140.54 (*C*-Ar), 139.92, 139.90 (2 x d, $^{2}J_{C\text{-}F}$ = 24.4 Hz, *C*-6), 139.35 (*C*-Ar), 137.54, 137.52 (2 × d, $^{1}J_{C\text{-}F}$ = 310.0 Hz, *C*-5), 128.85, 128.79, 127.75, 127.72, 127.41, 126.56, 126.52, 125.86, 122.95, 122.77 (*C*H-Ar), 116.35, 116.20 (2 x d, $^{3}J_{C\text{-}P}$ = 2.8 Hz *C*H-Ar), 94.65, 94.51 (*C*-1′), 83.71, 83.45 (2 × d, $^{3}J_{C\text{-}P}$ = 7.3 Hz, *C*-4′), 75.45, 75.35 ($CH_2C(CH_3)_3$), 73.21, 73.03 (*C*-2′), 71.18, 70.76 (*C*-3′), 68.66, 67.90 (2 x d, $^{2}J_{C\text{-}P}$ = 5.8 Hz, *C*-5′), 51.68 (NH*C*HCH$_3$), 32.31 ($CH_2C(CH_3)_3$), 26.73 ($CH_2C(CH_3)_3$), 20.96, 20.79 (2 × d, $^{3}J_{C\text{-}P}$ = 5.6 Hz, NHCH*C*H$_3$), 11.96 (d, $^{3}J_{C\text{-}F}$ = 3.4 Hz, C-6-*C*H$_3$); MS (ES+) *m/z*: 646.2 (M + Na$^+$, 80%), Accurate mass: $C_{28}H_{35}FN_3O_{10}P$ required 623.56 found 646.2 (M + Na$^+$), 1269 (2 x M + Na$^+$); Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 17.72, 17.92 min.

5-Fluoro-6-methyluridine-5′-*O*-[1-naphthyl-(benzoxy-L-alaninyl)] phosphate (36). Prepared according to the general procedure from 5-fluoro-6-methyluridine **7** (0.18 g, 0.65 mmol), NMI (0.26 mL, 3.26 mmol), 1-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.52 g, 1.30 mmol). After column purification on silica gel **36** was obtained as an off-white solid (0.033 g, 8%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.94, 3.75; ^{1}H NMR (500 MHz, MeOD): δ_H 8.13 – 8.09 (1H, m, *H*-Ar), 7.82 – 7.79 (1H, m, *H*-Ar), 7.62 (1H, apparent d, *J* = 8.5 Hz, *H*-Ar), 7.48 – 7.41 (3H, m, *H*-Ar), 7.35 – 7.29 (1H, m, *H*-Ar), 7.25 – 7.19 (5H, m, *H*-Ar), 5.41, 5.39 (1H, 2 x d, *J* = 3.0 Hz, *H*-1′), 5.02 – 4.93 (2H, m, C*H*$_2$Ph), 4.68, 4.65 (1H, 2 x dd, *J* = 6.5, 3.0 Hz, *H*-2′), 4.44 – 4.34 (2H, m, *H*-3′, 1 x *H*-5′), 4.27 – 4.22 (1H, m, 1 x *H*-5′), 4.06 – 3.94 (2H, m, NHC*H*CH$_3$, *H*-4′), 2.21, 2.20 (3H, 2 x d, *J* = 4.0 Hz, C*H*$_3$), 1.28, 1.24 (3H, 2 x dd, *J* = 7.0, 1.0 Hz, NHCHC*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 173.39, 173.26 (2 × d, $^{3}J_{C\text{-}P}$ = 4.75 Hz, *C*=O, ester), 157.45, 157.40 (2 x d, $^{2}J_{C\text{-}F}$ = 26.0 Hz, *C*-4), 149.13, 149.02 (*C*-2), 146.65, 146.63 (2 x d, $^{2}J_{C\text{-}P}$ = 7.0 Hz, O-*C*-Ar), 139.15, 139.09 (*C*-Ar), 138.52, 138.46 (2 x d, $^{2}J_{C\text{-}F}$ = 24.0 Hz, *C*-6), 136.58, 135.53 (2 × d, $^{1}J_{C\text{-}F}$ = 192.4 Hz, *C*-5), 134.84 (d, $^{3}J_{C\text{-}P}$ = 1.6 Hz, *C*-Ar), 128.15, 128.10, 127.88, 127.81, 127.77, 127.42, 127.36, 126.33, 126.30, 126.02, 125.14, 125.10, 124.44, 121.56, 121.34 (*C*H-Ar), 115.01, 114.79 (2 x d, $^{3}J_{C\text{-}P}$ = 3.0 Hz *C*H-Ar), 93.20, 93.06 (*C*-1′), 82.25, 81.87 (2 × d, $^{3}J_{C\text{-}P}$ = 7.0 Hz, *C*-4′), 71.75, 71.30 (*C*-2′), 69.75, 69.20 (*C*-3′), 67.19 (d, $^{2}J_{C\text{-}P}$ = 5.8 Hz, *C*-5′), 66.58, 66.44 (O*C*H$_2$), 66.23 (d, $^{2}J_{C\text{-}P}$ = 5.8 Hz, *C*-5’), 50.25 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, NH*C*HCH$_3$), 19.21, 19.16 (2 × d, $^{3}J_{C\text{-}P}$ = 6.3 Hz, NHCH*C*H$_3$), 10.53 (d, $^{3}J_{C\text{-}F}$ =

3.6 Hz, C-6-*C*H$_3$); MS (ES+) *m/z*: 687.58 (M + Na$^+$, 10%), Accurate mass: $C_{30}H_{31}FN_3O_{10}P$ required 643.55 found 666.2 (M + Na$^+$), 1309.4 (2 x M + Na$^+$), Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 16.89, 17.15 min.

5-Fluoro-6-ethyluridine-5′-*O*-[1-naphthyl-(benzoxy-L-alaninyl)] phosphate (37). Prepared according to the general procedure from 5-fluoro-6-ethyluridine **8** (0.15 g, 0.52 mmol), NMI (0.21 mL, 2.58 mmol), 1-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.42 g, 1.03 mmol). After column purification on silica gel **37** was obtained as an off-white solid (0.027 g, 8%). ^{31}P NMR (202 MHz, MeOD): δ_P 3.88, 3.73; ^{1}H NMR (500 MHz, MeOD): δ_H 8.19 – 8.15 (1H, m, *H*-Ar), 7.88 – 7.87 (1H, m, *H*-Ar), 7.68 (1H, dd, *J* = 8.5, 1.0 Hz, *H*-Ar), 7.55 – 7.47 (3H, m, *H*-Ar), 7.42 – 7.35 (1H, m, *H*-Ar), 7.35 – 7.26 (5H, m, *H*-Ar), 5.45, 5.43 (1H, 2 x d, *J* = 3.0 Hz, *H*-1′), 5.08 – 4.98 (2H, m, C*H*$_2$Ph), 4.75, 4.70 (1H, 2 x dd, *J* = 6.5, 3.0 Hz, *H*-2′), 4.51 – 4.39 (2H, m, *H*-3′, 1 x *H*-5′), 4.37 – 4.28 (1H, m, 1 x *H*-5′), 4.12 – 4.01 (2H, m, NHC*H*CH$_3$, *H*-4′), 2.83 – 2.65 (2H, m, C*H*$_2$CH$_3$), 1.36 – 1.24 (6H, m, NHCHC*H*$_3$, CH$_2$C*H*$_3$); ^{13}C NMR (125 MHz, MeOD): δ_C 174.80, 174.66 (2 × d, $^3J_{C\text{-}P}$ = 3.75 Hz, *C*=O, ester), 159.0 (d, $^2J_{C\text{-}F}$ = 27.5 Hz, *C*-4), 150.71, 150.61 (*C*-2), 148.07 (d, $^2J_{C\text{-}P}$ = 6.25 Hz, O-*C*-Ar), 139.90 (*C*-Ar), 138.13 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, *C*-6), 136.26 (d, $^1J_{C\text{-}F}$ = 220.5 Hz, *C*-5), 130.77 (*C*-Ar), 129.57, 129.53, 129.23, 128.85, 128.78, 128.29, 127.89, 127.74, 127.72, 127.44, 126.57, 126.54, 126.53, 125.88, 122.99, 122.77, 121.91 (*C*H-Ar), 116.44, 116.27 (2 x d, $^3J_{C\text{-}P}$ = 2.5 Hz *C*H-Ar), 94.65, 94.53 (*C*-1′), 83.87, 81.72 (2 × d, $^3J_{C\text{-}P}$ = 7.0 Hz, *C*-4′), 71.64, 71.31 (*C*-2′), 68.71, 68.02 (*C*-3′), 67.84 (d, $^2J_{C\text{-}P}$ = 7.5 Hz, *C*-5′), 65.27 (O*C*H$_2$Ph), 51.65 (d, $^3J_{C\text{-}P}$ = 6.5 Hz, NH*C*HCH$_3$), 20.68, 20.58 (2 × d, $^3J_{C\text{-}P}$ = 6.3 Hz, NHCH*C*H$_3$), 19.64 (C-6-*C*H$_2$CH$_3$), 12.74, 12.65 (C-6-*C*H$_3$); MS (ES+) *m/z*: 680.2 (M + Na$^+$, 100%), Accurate mass: $C_{31}H_{33}FN_3O_{10}P$ required 657.58 found 680.2 (M + Na$^+$), 1337.4 (2 x M + Na$^+$), Reverse-phase HPLC, eluting with H_2O/AcCN from 100/0 to 0/100 in 35 min, F = 1 mL/min, λ = 254, two peaks for two diastereoisomers with t_R = 18.60, 18.77 min.

Molecular Modelling

All molecular modelling studies were performed on a Viglen Genie Intel®Core™ i7-3770 vPro CPU@ 3.40 GHz x 8 running Ubuntu 14.04. Molecular Operating Environment (MOE) 2015.10[46] and PLANTS[47] were used as molecular modelling

software. The human HINT-1 structure was downloaded from the PDB data bank (http://www.rcsb.org/; PDB code 1KPF). Hydrogen atoms were added to the protein, using the Protonate 3D routine of the Molecular Operating Environment (MOE). Ligand structures were built with MOE and minimized using the MMFF94x force field until a RMSD gradient of 0.05 kcal $mol^{-1}/Å^{-1}$ was reached. The docking simulations were performed using PLANTS applying the following parameters: search algorithm: aco_ants 20, aco_evap 0.15, aco_sigma 2.0; binding site: bindingsite_center [10.77 11.16 13.79], binding site_radius 12; cluster algorithm: cluster_rmsd 2.0, cluster_strucures 10; scoring function: chemplp. The reliability of PLANTS docking results has been validated by docking the AMP substrate into HINT-1 catalytic site and then measuring the root mean square deviation (RMSD) of the best docking pose obtained with the co-crystallized AMP giving a RMSD value of 1.0180.

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