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ProTides of *N*–(3-(5-(2'-deoxyuridine))prop-2- ynyl) octanamide as potential anti-tubercular and anti-viral agents

Christopher McGuigan[a,*], Marco Derudas[a], Blanka Gonczy[a], Karen Hinsinger[a], Sahar Kandil[a], Fabrizio Pertusati[a], Michaela Serpi[a], Robert Snoeck[b], Graciela Andrei[b], Jan Balzarini[b], Timothy D. McHugh[c], Arundhati Maitra[d], Ernest Akorli[d], Dimitrios Evangelopoulos[c,d], Sanjib Bhakta[d]

[a]*School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, King Edward VII Avenue, Cardiff CF10 3NB, UK*
[b]*Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, Leuven B-3000, Belgium*
[c]*Centre for Clinical Microbiology, Research Department of Infection, Royal Free Campus, University College London, Pond Street, London NW3 2QG, UK*
[d]*Mycobacteria Research Laboratory, Institute of Structural and Molecular Biology, Department of Biological Sciences, Birkbeck, University of London, Malet Street, London WC1E 7HX, UK*

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ABSTRACT

The flavin-dependent thymidylate synthase X (ThyX), rare in eukaryotes and completely absent in humans, is crucial in the metabolism of thymidine (a DNA precursor) in many microorganisms including several human pathogens. Conserved in mycobacteria, including *Mycobacterium leprae*, and *Mycobacterium tuberculosis*, it represents a prospective anti-mycobacterial therapeutic target. In a *M. tuberculosis* ThyX-enzyme inhibition assay, *N*–(3-(5-(2'-deoxyuridine-5'-phosphate))prop-2-ynyl)octanamide was reported to be the most potent and selective 5-substituted 2'-deoxyuridine monophosphate analogue. In this study, we masked the two charges at the phosphate moiety of this compound using our ProTide technology in order to increase its lipophilicity and then allow permeation through the complex mycobacterial cell wall. A series of *N*–(3-(5-(2'-deoxyuridine)) prop-2-ynyl)octanamide phosphoroamidates were chemically synthesized and their biological activity as potential anti-tuberculars was evaluated. In addition to mycobacteria, several DNA viruses depend on ThyX for their DNA biosynthesis, thus these prodrugs were also screened for their antiviral properties.

1. Introduction

Tuberculosis (TB) is an infectious disease, caused by an extremely slow growing bacterial pathogen belonging to the *Mycobacterium tuberculosis* complex.[1] Among them, *M. tuberculosis* is responsible for the majority of human deaths.[2] It is estimated that at least one-third of the world's population is latently infected with the bacteria that causes TB, while in 2012, WHO reported 8.6 million new cases of the disease and 1.3 million deaths due to it, worldwide.[3] Lengthy treatment regimens have as a consequence, the rise of drug resistant TB-causing strains. This has set the alarm for controlling the disease, which necessitates the discovery of new therapeutic targets and synthesis of novel inhibitors.

In order to specifically inhibit mycobacterial growth, alternative metabolic pathways, exclusive to the bacteria, need to be targeted for an efficient anti-TB drug development programme.[4] In this regard, we focused our interest on the genome of *M. tuberculosis*, which has been reported to contain a *thyX* gene (Rv2754c), encoding the ThyX protein, a flavin-dependent thymidylate synthase responsible for the *de novo* synthesis of 2' –deoxythymidine 5'-monophosphate (dTMP), a key precursor of DNA.[5] ThyX, present primarily in prokaryotes and viruses, is rare in eukaryotes and absent in humans, where the corresponding metabolic function is carried out by the conventional ThyA protein. Both the enzymes catalyze the reductive methylation of 2'-deoxy-uridine-5'–monophosphate (dUMP) to dTMP in the presence of methylenetetrahydrofolate; however, based on their structural and catalytic dissimilarities, different enzymatic mechanisms have been suggested for each.[6] In contrast to ThyA, where methylenetetrahydrofolate acts both as the carbon donor and reducing agent, ThyX uses methylenetetrahydrofolate as the one-carbon donor molecule and reduced flavin adenine dinucleotide ($FADH_2$) as a cofactor that serves as the hydride donor.[7]

Micro-organisms contain either ThyA or ThyX; however, mycobacteria encode both ThyA and ThyX. However, a sequence similarity search yielded no significant matches between ThyX of mycobacteria to the ThyA of other eukaryotic cells. Recently, functional studies, devoted to investigate the biological role of both of these enzymes, showed that ThyX is essential for the survival of *M. tuberculosis* even in the presence

of ThyA and exogenous thymidine.[8,9] *M. tuberculosis* cannot utilize exogenous thymidine sources because it lacks thymidine kinase, the essential enzyme for the conversion of thymidine into dTMP.[5] All these factors support the existence of ThyX enzyme as a prospective therapeutic target for the development of a new selective anti-tubercular drug treatment.[10]

The chemical class of C5-alkynyl substituted 2'-deoxyuridine-5'-monophosphate (dUMP) has been reported as selective inhibitor of ThyX in *M. tuberculosis*.[11] In this study, among several compounds, *N*–(3-(5-(2'-deoxyuridine-5'-monophosphate))prop-2-ynyl)octanamide **1** (figure 1) was identified as the most potent and selective analogue with an IC_{50} value of 0.91 μM versus recombinant ThyX from *M. tuberculosis*. However, the polarity of this compound constitutes a major obstacle for its penetration through the complex mycobacterial cell wall. Indeed one of the major challenges for chemotherapeutic drugs is associated with difficulties in crossing the thick, lipid-rich cell wall of the mycobacteria, which prevents their permeation, with a consequential poor biological response. In particular, the permeation of hydrophilic compounds is not very efficient due to the presence of low numbers and the exceptional length of porins, water-filled open channels, responsible for mediating the diffusion of hydrophilic nutrients.[12] Moreover, the existence of active drug efflux pumps reduces the concentration of active molecules inside the bacterial cell and their up-regulation has been suggested to contribute to the emergence of drug resistance.[13] To the contrary more lipophilic drugs are more likely to diffuse through the lipid-rich environment of the mycobacterial cell wall. In addition to high polarity, the instability of **1** in biological media, a well-known drawback for free nucleoside phosphates, may also limit its therapeutic potential, with dephosphorylation likely exceeding cell permeation.

To improve stability, permeability and therefore antibacterial activity, lipophilic prodrugs of **1** are thus required. Among several strategies developed to overcome these issues, our phosphoramidate ProTide approach was selected for this study.[14,15] This technology consists of masking the negative charges of the phosphate group with an aromatic moiety and an amino acid ester. To date, this approach has been widely applied mainly to antiviral[16] and anticancer nucleoside analogues[17] and more recently also to N-acetyl glucosamine to treat osteoarthritis.[18] Typically, two enzymatic cleavages are involved in the cellular bio-activation of antiviral and anticancer ProTides, either in the viral infected or human cancer cell.[19] Firstly, a carboxypeptidase-type enzyme may mediate the cleavage of the ester moiety. This step is followed by a spontaneous intra-molecular cyclisation with the subsequent release of the aryl moiety and formation of an unstable mixed cyclic anhydride which undergoes ring-opening mediated by water to release a mono ester phosphate prodrug. In the last step, a phosphoramidase-type enzyme, most probably a human Hint-1, may be responsible for the cleavage of the phosphorus-nitrogen bond with the consequent release of the monophosphate.

Since we wanted to investigate whether phosphoramidates of **1** are capable of crossing the cell wall of mycobacteria and to be bio-converted into the monophosphate once inside, herein we report the synthesis of several derivatives and their biological evaluation against the TB vaccine strain, *M. bovis* BCG and the virulent TB causing lab strain, *M. tuberculosis* H37Rv, using HT-SPOTi, a rapid but gold standard whole-cell phenotypic assay. This is the first time that the application of the ProTide technology has been reported for improving the antimicrobial activity of inhibitors of a protein target of *M. tuberculosis*.

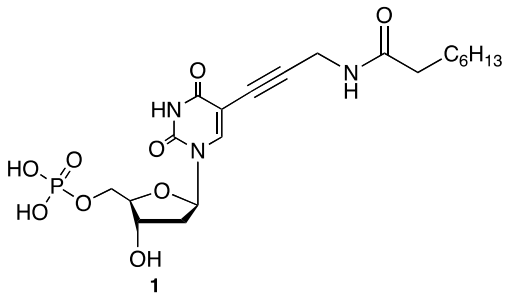


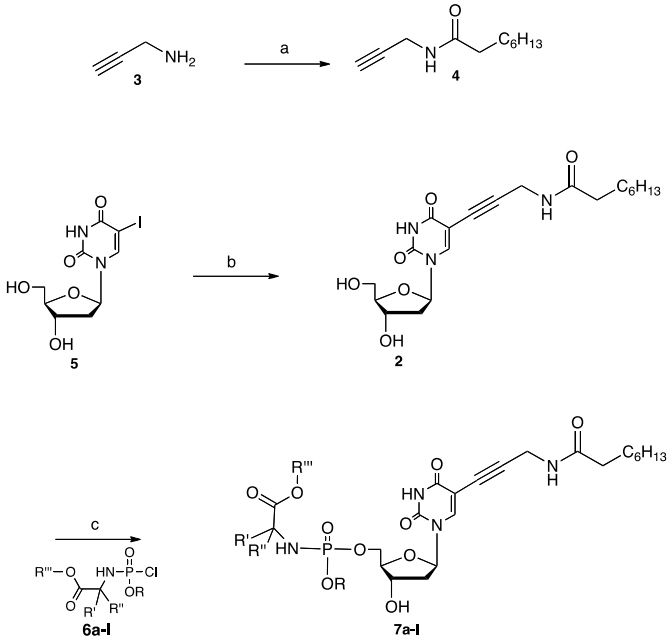
Figure 1. *N*-(3-(5-(2'-deoxyuridine-5'-monophosphate))prop-2-ynyl)octanamide **1**.

The Prodrugs were prepared by chemical modification of nucleoside **2** rather than directly modifying compound **1** (*see* Scheme 1). As ThyX proteins are also found in several double-stranded DNA viruses[5] and considering that some 5-alkynyl-2′-deoxyuridine analogues, in addition to their anti-TB activity[20-22] showed significant antiviral activity[23] we also report the antiviral evaluation of the parent nucleoside of **2** and its prodrug monophosphate derivatives.

2. Results and Discussion

2.1. Chemistry

The phosphoramidates **7a–l** described in this study are shown in Table 1 with the reaction sequence for their synthesis summarized in Scheme 1.



Scheme 1. Synthetic method to obtain compounds **7a-l.** *Reagents and Conditions:* (a) Octanoyl chloride, DIPEA, anhydrous CH_2Cl_2, 0°C to 20°C, 2h; (b) Propargyloctanamide (**3**), $Pd(Ph_3)_4$, CuI, DIPEA, anhydrous DMF, 20°C ; (c) Phosphorochlorides **6a-l** (for R, R', R'' and R''' *see* table 1), NMI, anhydrous THF, 20°C, 12h.

Our synthetic efforts started with the synthesis of the propargyloctylamide **4** from propargylamine **3** and octanoyl chloride.[11] Then, Sonogashira cross coupling[24] of the commercially available 5-iodo-2'-deoxyuridine **5** with alkyne **4** afforded the desired nucleoside **2**, isolated in excellent yield (91%) without column chromatography purification. It is

noteworthy to mention that our synthetic procedure to the preparation of **2** was made less time-consuming than the previously reported procedure, with great improvement in terms of yield achieved.[11] Following the general synthetic procedure for compounds **7a-l**, nucleoside **2** was reacted with the appropriate phosphorochloridate **6a-l** using N-methylimidazole as activator, according to a previously described method.[25] The desired phosphoroamidates **7a-l** were obtained from 5 to 39% yield. Yield optimization was beyond the scope of the work as we were in the early stages of establishing biological activity and potential lead molecules.

Table 1
Substituent pattern, yields and ^{31}P NMR shifts of phosphoramidates **7a-l**.

Cpds	R	R'	R''	AA	R'''	Yield (%)	^{31}P NMR (ppm) [a]
7a	Naph	H	CH_3	(*L*)-Ala	CH_3	20	4.36; 4.07
7b	Naph	H	CH_3	(*L*)-Ala	CH_2CH_3	5	4.36; 4.09
7c	Naph	H	CH_3	(*L*)-Ala	$CH_2CH(CH_3)_2$	39	4.34; 4.07
7d	Naph	H	CH_3	(*L*)-Ala	CH_2Ph	14	4.36; 3.98
7e	Ph	H	CH_3	(*L*)-Ala	CH_3	16	3.98; 3.57
7f	Ph	H	CH_3	(*L*)-Ala	CH_2CH_3	29	3.99; 3.58
7g	Ph	H	CH_3	(*L*)-Ala	$(CH_2)_2CH_3$	12	4.00; 3.59
7h	Ph	H	CH_3	(*L*)-Ala	CH_2Ph	12	4.01; 3.51
7i	Ph	CH_3	H	(*D*)-Ala	CH_2Ph	16	3.98; 3.50
7j	Ph	H	H	Gly	CH_2Ph	29	5.02; 4.93
7k	Ph	CH_3	CH_3	DMG	CH_2Ph	9	2.39; 2.10
7l	Ph	H	$CH(CH_3)_2$	(*L*)-Val	CH_2Ph	14	4.95; 4.30

[a] Recorded at 202 MHz in MeOH-d_4 with 85% H_3PO_4 as reference

2.2. Biological activities

2.2.1 Antimycobacterial specificity

All synthesized compounds **7a-l** and nucleoside **2** were evaluated *in vitro* against *M. bovis* BCG and, *M. tuberculosis* H37Rv using the HT-SPOTi assay[26] at 1-250 mg/L concentrations. This whole-cell assay was indicative toward inherent resistance, such as cell wall impermeability. In order to evaluate a specific endogenous mechanism of anti-mycobacterial action for these compounds via ThyX inhibition, we also tested their biological activity against *E. coli*, a Gram-ve bacterium where a functional ThyX homologue is missing from the genome. The minimum inhibitory concentrations (MIC) of compounds that completely inhibited growth of both mycobacterial strains are shown in Table 2.

The highly hydrophilic nucleoside **2** (ClogP = -0.21) did not exhibit antimycobacterial activity with a MIC> 250 mg/L (Table 2). For the first series of phosphoroamidates bearing a naphthyl group as an aromatic part (**7a-d**), a structure-activity relationship (SAR) was found with the activity depending on the size of the ester group of the L-alanine amino acid moiety. Methyl and ethyl derivatives (**7a** ClogP = 1.89; **7b** ClogP = 2.42) showed better inhibitory activity (**7a** MIC = 62.5 mg/L; **7b** MIC = 125 mg/L), whereas more hindered and lipophilic benzyl analogue (**7d** ClogP = 3.60) was found to be significantly less inhibitory (MIC = 250 mg/L). A possible reason for this finding could be due to the higher size of compound **7d** that may prevent its entry inside the bacteria.

The second series of compounds bearing a phenyl aromatic moiety (**7e-l**) showed a different SAR. The smallest alkyl esters (**7e** ClogP = 0.71; **7f** ClogP **=** 1.24) did not inhibit mycobacteria growth, while propyl and benzyl derivatives (**7g** ClogP = 1.77; **7h** ClogP= 2.43) were slightly active (MIC = 125 mg/L). Further investigation on the amino acid part highlighted a certain preference toward alanine with the D-alanine analogue (**7i**) showing a MIC of 62.5 mg/L.

Table 2
Antimycobacterial specificity of nucleoside **2** and phosphoroamidates **7a-l**.

Cpds	Clog P[a]	MIC^{BCG}(mg/L)	MIC^{H37Rv}(mg/L)
2	-0.21	>250	>250
7a	1.89	62.5	62.5
7b	2.42	125	125
7c	3.74	125	62.5
7d	3.6	250	250
7e	0.71	>250	>250
7f	1.24	250	250
7g	1.77	125	125
7h	2.43	125	125
7i	2.43	62.5	125
7j	2.31	>250	>250
7k	2.73	125	>125
7l	3.35	125	31.25

[a] Clog P values were calculated using CambridgeSoft ChemDraw® software. MIC^{BCG} and MIC^{H37Rv} state the minimum inhibitory concentration

of the compounds tested against *M. bovis* BCG and *M. tuberculosis* H37Rv respectively.

Glycine derivative **7j**, showed no activity against *M. bovis* BCG, whereas dimethylglycine or L-valyl derivatives (**7k** and **7l**) in the same assay were slightly active. (MIC = 125 mg/L). However, results for **7i** and **7l** differed in the case of *M. tuberculosis* H37Rv as can be inferred from the table.

Therefore, we postulate that the bioactivity against *M. bovis* BCG and *M. tuberculosis* H37Rv of phosphoroamidates **7a**, **7b**, **7c, 7g**, **7h, 7i** and **7l** might result from better permeation through the mycobacterial cell wall due to an improved balance between their lipophilicity and molecular size. Potencies are moderate in this first series of compounds and subsequent work is required to optimize their biological activity; however, there is a clear antimycobacterial effect for some of these molecules. Notably, the parent nucleoside **2** was devoid of activity showing the crucial importance of the ProTide motif.

No homologues with significant sequence similarity with the protein ThyX of *M. tuberculosis* were found in *E. coli*. This could be a possible explanation for the inactivity of the compounds towards this organism.

2.2.2 Antiviral Activity

Parent nucleoside **2** and all synthesized phosphoramidates were also evaluated for their antiviral activity against varicella zoster virus (VZV), herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), and human cytomegalovirus (HCMV), vaccinia virus (VV) according to previously described methods.[27] Phosphoroamidate **7h** exhibited anti-VZV and anti-HSV activities in the low micromolar range (Table 3).

However, although quite potent against a thymidine kinase positive (TK^+) strain of VZV, **7h** was not active *versus* the thymidine kinase-deficient (TK^-) strain. This showed the importance of this enzyme in the bioactivation of this class of molecules to its active species. Moreover, the lead compound, the L-alanine benzyloxy ester phosphoramidate with a phenyl aromatic group **7h**, presented similar activity (EC_{50} = 2.0 μM) to acyclovir (EC_{50} = 2.6 μM) against VZV. **7h** was also found to be active against HSV-1 and HSV-2 (EC_{50} of 2 and 4 μM) but not against a TK^- strain of HSV-1. In the case of **7a**, **7c**, **7d**, the antiviral activity observed against VZV may be rather due to their underlying cytotoxicity. The compounds were not active against HCMV and VV (data not shown) in HEL cell cultures.

Table 3
Antiviral activity and cytotoxicity of phosphoramidates **7a**, **7c**, **7d** and **7h.**

Compounds	EC_{50}[a] (μM)					CC_{50}[b] (μM)	MCC[c] (μM)
	VZV		HSV-1		HSV-2		
	TK^+ OKA	TK^- 07-1	TK^+ KOS	TK^-KOS	G		
2	>100	>100	>100	>100	>100	>100	-
7a	37	46	>100	>100	>100	64	>100
7c	20	>20	>100	>100	>100	15	>100
7d	8.6	>20	>100	>100	>100	39	>100
7h	2.0	>20	2	>100	4	48	>100
acyclovir	2.6	140	0.2	50	0.1	440	>440

[a]Effective concentration required to reduce virus plaque formation by 50%. VZV, HSV-1 and HSV-2 represent EC_{50} values for varicella zoster virus, herpes simplex virus strains respectively. [b]Cytotoxic concentration required to reduce cell growth by 50%. [c]Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.

3. Conclusion

A phosphoroamidate prodrug (ProTide) approach was used to seek to deliver the pre-formed bioactive monophosphate of a new compound recently reported to be a potent and selective inhibitor of thymidylate synthase X, an enzyme essential for the survival of *M. tuberculosis*. The lipophilic ProTide motif was designed to improve permeability trough the mycobacterial cell wall. We have successfully synthesized twelve phosphoroamidate derivatives of *N*–(3-(5-(2'-deoxyuridine-5'-monophosphate))prop-2-ynyl)octanamide **1**, a potent in vitro ThyX inhibitors. Biological tests of our prodrugs showed antimycobacterial activity against *M. tuberculosis* H37Rv and *M. bovis* BCG in contrast to inactivity from the parent nucleoside. We considered that the increased lipophilicity together with the correct molecular size of phosphoroamidate derivatives **7a**, **7b**, **7c 7g**, **7h 7i** and **7l** allowed them to penetrate through the mycobacterial cell wall liberating the monophosphate intracellulary and targeting their proposed biological target, ThyX protein.

Moreover, we found interesting activities against VZV for some of our compounds, showing the necessity of the pro-moiety to help transport of the drug into cells. We identified the L-alanine benzyl ester phosphoramidate with the phenyl aromatic group **7h** as a potent antiviral agent, showing similar activity to acyclovir against VZV in this assay.

The results obtained highlight the possibility that the ProTide methodology could be used for the development of active molecules against TB and reveal the importance of improving lipophilicity to efficiently pass the mycobacteria wall barrier. To the best of our knowledge this is the first application of the ProTide technology to anti-mycobacterial agents. Further work is

currently underway to enhance the potency of the new agents we herein report for the first time.

4. Experimental

4.1 Synthesis

Solvents and Reagents. The following anhydrous solvents were purchased from Sigma-Aldrich: dichloromethane (CH_2Cl_2), 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. Propargylamide and octanoyl chloride were purchased from Aldrich whereas 5-iodo-2'-deoxyuridine from Berry & Associates.

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) or by burning using the following TLC indicators: (i) molybdate ammonium cerium sulfate; (ii) potassium permanganate solution. Preparative TLC plates (20 cm × 20 cm, 500-2000 μm) were purchased from Merck.

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

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

Nuclear Magnetic Resonance (NMR). ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and ^{31}P NMR (202 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 (δ 7.26 ^{1}H NMR, δ 77.36 ^{13}C NMR) or external 85 % H_3PO_4 (δ 0.00 ^{31}P NMR). Coupling constants (J) are measured in Hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), dt (doublet of triplet), app (apparent). The assignment of the signals in ^{1}H NMR and ^{13}C NMR was done based on the analysis of coupling constants and additional two-dimensional experiments (COSY, HSQC, HMBC, PENDANT).

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

4.1.1. Synthesis of N–(3-(5-(2'-deoxyuridine))prop-2-ynyl)octanamide **2**

***Propargyloctanamide* (4).** Propargylamine **3** (2.5 mL, 45.38 mmol) was dissolved in anhydrous CH_2Cl_2 (113 mL) DIPEA (10.4 mL, 59.46 mmol) was added and the solution was cooled to 0 ^{o}C. Octanoyl chloride (8.52 mL, 49.92 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 2 h. The reaction was quenched through dropwise addition of H_2O (5 mL) then diluted with CH_2Cl_2 and washed with saturated solution of $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to obtain **4** as an orange solid (99%, 8.3 g) which was used in the subsequent Sonogashira-coupling step without further purification. ^{1}H NMR (500 MHz, $CHCl_3$- d): δ_H 5.68 (brs, 1H, NH) 4.04 (dd, 2H, J = 7.5 and 2.5 Hz, CH_2C≡C), 2.22 (t, 1H, J = 2.8 Hz, HC≡C), 2.20 (t, 2H, J = 8.0 Hz, CH_2CO), 1.69-1.61 (m, 2H, CH_2CH$_2$CO), 1.31-1.28 (m, 8H, 4 x CH_2), 0.88 (t, 3H, J = 6.8 Hz, CH_3).

4.1.2. N–(3-(5-(2'-deoxyuridine))prop-2-ynyl)octanamide **(2).**

A solution of 5-iodo-2'-deoxyuridine **5** (4.00 g, 11.29 mmol), alkyne **4** (6.14 g, 33.89 mmol), tetrakis Pd(PPh_3)$_4$ (1.30 g, 1.13 mmol), Cu(I)I (0.43 g, 2.26 mmol) and anhydrous DIPEA (3.93 mL, 22.58 mmol) in anhydrous DMF (75 mL) was stirred under an argon atmosphere at room temperature overnight. After this period, the solvent was removed under reduced pressure and the residue was suspended in CH_2Cl_2 and stirred at room temperature for 2 h. The suspension was filtered and the solid was washed with CH_2Cl_2 to give the desired compound as a light brown solid (91%, 4.20 g). For biological testing, the compound **2** was purified by preparative TLC using CH_2Cl_2/MeOH 9/1 as eluent. ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 11.59 (s, 1H, NH), 8.26 (t, 1H, J = 5.4 Hz, NH amide), 8.15 (s, 1H, H-6), 6.12 (t, 1H, J = 6.7 Hz, H-1'), 5.23 (d, 1H, J = 4.3 Hz, 3'-OH), 5.07 (t, 1H, J = 5.0 Hz, 5'-OH), 4.25-4.22 (m, 1H, H-3'), 4.06 (d, 2H, J = 5.5 Hz, NHCH_2), 3.80 (q, 1H, J = 3.3 Hz, H-4'), 3.62-3.56 (m, 2H, H-5'), 2.13-2.07 (m, 4H, H-2', COCH_2), 1.52-1.46 (m, 2H, COCH$_2$CH_2), 1.28-1.21 (m, 8H, 4 x CH_2), 0.86 (t, 3H, J = 6.9 Hz, CH_3). ^{13}C (125 MHz, DMSO-d_6): δ_C 171.83 (CONH), 161.57 (C-4), 149.39 (C-2), 143.57 (C-6), 98.14 (C-5), 89.76 (CH$_2$C≡C), 87.61 (C-4'), 84.69 (C-1'), 74.18 (CH$_2$$C$≡C), 70.23 ($C$-3'), 61.02 ($C$-5'), 40.06 ($C$-2'), 35.06 (CO$CH_2$), 31.12, 28.58, 28.47, 28.39, 25.09, 22.01 (6 x CH$_2$), 13.89 (CH$_2$$CH_3$). MS (ES$^-$) m/z 442 (M+Cl$^-$, 100%), 406 (M-H$^+$, 39%). Reverse-phase HPLC, eluting with H_2O/MeOH from 90/10 to 0/100 in 40 min, Flow = 1 mL/min, λ = 280 nm, t_R= 25.71 min

4.1.3. Synthesis of phosphoramidates 7a-l.

General procedure. To a solution of nucleoside **5** (1 mol/eq) and the appropriate phosphorochloridate **6a-l** (3-5 mol/eq) in anhydrous THF, anhydrous NMI (5 mol/eq) was added dropwise and the reaction mixture was stirred at room temperature overnight. After this period, the solvent was removed under reduced pressure and the residue dissolved in CH_2Cl_2. The organic phase was washed with 0.5 M aqueous solution of citric acid, water and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated. The crude was purified by column chromatography using different eluting systems. Some of the compounds were further purified by preparative TLC using different eluting systems. All the compounds were recovered as a mixture of R_P and S_P diastereoisomers.

4.1.3.1 N-{3-[5-(2'-Deoxy-5'-O-(1-naphthyl(methyloxy-L-alaninyl)phosphate-uridine))] prop-2-ynyl)octanamide (7a).

Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and naphthyl(methyloxy-L-alaninyl)phosphorochloridate **6a** (0.80 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH

from 98/2 to 95/5. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 95/5 to give **7a** as a white solid (20%, 0.07 g). ^{1}H NMR (500 MHz, MeOH-*d*$_4$): δ_H 8.19 (d *J* = 7.5 Hz, 1H, Naph), 7.95 (s, 0.5H of one diastereoisomer, *H*-6), 7.93 (s, 0.5H of one diastereoisomer, *H*-6), 7.92-7.89 (m, 1H, Naph), 7.84 (d, *J* = 7.5 Hz, 0.5H of one diastereoisomer, Naph), 7.74 (d, *J* = 7.5 Hz, 0.5H of one diastereoisomer, Naph), 7.60-7.53 (m, 3H, Naph), 7.47-7.43 (m, 1H, Naph), 6.17 (t, *J* = 5.5 Hz, 0.5H of one diastereoisomer *H*-1'), 6.15 (t, *J* = 6.0 Hz, 0.5H of one diastereoisomer *H*-1'), 4.44-4.33 (m, 3H, *H*-3', *H*-5'), 4.16-4.07 (m, 2H, *H*-4' and C*H*CH$_3$), 3.97, 3.96, 3.93 (3s, 2H, NHC*H*$_2$C≡), 3.68, 3.67 (s, 3H, OCH$_3$), 2.22 (ddd, *J* = 14.0, 6.5 and 3.0 Hz, 0.5H of one diastereoisomer, *H*-2'), 2.16 (ddd, *J* = 14.0, 6.0 and 3.0 Hz, 0.5H of one diastereoisomer, *H*-2'), 2.13 (t, *J* = 7.5 Hz, 1H, COC*H*$_2$CH$_2$), 2.09 (t, *J* = 7.5 Hz, 1H, COC*H*$_2$CH$_2$), 1.89-1.83 (m, 0.5H of one diastereoisomer, *H*-2'), 1.80-1.76 (m, 0.5 H of one diastereoisomer, *H*-2') 1.57-.151 (m, 2H, COCH$_2$C*H*$_2$-), 1.39 (d, *J* = 8.5 Hz, 1.5H of one diastereoisomer, CHC*H*$_3$), 1.38 (d, *J* = 8.5 Hz, 1.5H of one diastereoisomer, CHC*H*$_3$), 1.31-1.21 (m, 8H, 4 x C*H*$_2$), 0.89 (t *J* = 7.0 Hz, 3H, C*H*$_3$). ^{13}C (125 MHz, MeOH-*d*$_4$): δ_C 175.71, 175.68 (*C*ONH), 175.23 (d, $^3J_{C\text{-}P}$= 4.6 Hz, *C*OOCH$_3$), 175.10 (d, $^3J_{C\text{-}P}$= 3.7 Hz, *C*OOCH$_3$), 164.46 (*C*-4), 151.96, 15141 (*C*-2), 148.04 (d, $^2J_{C\text{-}P}$ = 8.0 Hz, '*ipso*' *Ph*O), 144.59 (*C*-6), 136.32, 137.30, 128.98, 128.95, 127.92, 127.72, 127.62, 126.63, 126.56, 126.25, 126.19, 122.72, 122.66 (C-1 '*ipso*' Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 116.47 (d, $^3J_{C\text{-}P}$ = 2.9 Hz, C-2 Naph), 116.41 (d, $^3J_{C\text{-}P}$ = 3.4 Hz, C-2 Naph), 100.26 (*C*-5), 90.36, 90.32 (CH$_2$C≡*C*), 87.44, 87.36 (*C*-1'), 87.16 (d, $^3J_{C\text{-}P}$ = 8.2 Hz, *C*-4'), 86.95 (d, $^3J_{C\text{-}P}$ = 8.2 Hz, *C*-4'), 75.42, 75.37 (CH$_2$C≡C), 72.24, 72.13 (*C*-3'), 67.85 (d, $^2J_{C\text{-}P}$ = 5.5 Hz, *C*-5'), 52.85 (O*C*H$_3$), 51,80, 51.69 (*C*HCH$_3$), 41.34, 41.27 (*C*-2'), 36.90, 36.87 (CO*C*H$_2$), 32.91, 30.54, 30.48, 30.26, 30.13, 26.84, 26.82, 23.67 (*C*H$_2$NHCO, 6 x CH$_2$), 20.59 (d, $^3J_{C\text{-}P}$ = 6.1 Hz, *C*H*C*H$_3$), 20.45 (d, $^3J_{C\text{-}P}$ = 7.2 Hz, *C*H*C*H$_3$), 14.42 (*C*H$_3$).^{31}P NMR (202 MHz, MeOH-*d*$_4$): δ_P 4.36, 4.07. MS (ES+) *m/z*: 721 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/ACN from 90/10 to 0/100 in 30 min, Flow = 1mL/min, λ = 280 nm, t_R= 17.86 min.

4.1.3.2 N-{3-[5-(2'-Deoxy-5'-O-(1-naphthyl(ethyloxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7b). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and naphthyl(ethyloxy-L-alaninyl)phosphorochloridate **6b** (0.84 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of ethyl acetate/MeOH = 100/0 to 98/2. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 95/5 to give **7b** as a white solid (5%, 0.02 g). ^{1}H-NMR (500 MHz; MeOH-*d4*): δ_H 8.20-7.42 (m, 8H, Naph, H-6), 6.17-6.14 (m, 1H, H-1'), 4.45-4.32 (m, 3H, H-3', H-5'), 4.17-4.07 (m, 4H, H-4', COOC*H*$_2$CH$_3$, C*H*CH$_3$), 3.97, 3.93, 3.91 (3s, 2H, NHC*H*$_2$C≡C), 2.23-2.17 (m, 1H, H-2'), 2.16-2.08 (m, 2H, COC*H*$_2$CH$_2$), 1.87-1.74 (m, 1H, H-2'), 1.58-1.51 (m, 2H, COCH$_2$C*H*$_2$), 1.43-1.21 (m, 14H, 4 x CH$_2$, COOCH$_2$C*H*$_3$, CHC*H*$_3$), 0.89-0.84 (t, 3H, *J* = 6.7 Hz, CH$_2$CH$_2$C*H*$_3$). ^{13}C NMR (125 MHz, MeOH-*d4*): δ_C 175.69, 175.68 (CH$_2$*C*O), 175.05, 174.73 (*C*OOCH$_2$CH$_3$), 164.36 (*C*-4), 151.05, 150.93 (*C*-2), 148.00 (d, $^2J_{C\text{-}P}$ = 7.4 Hz, C-1 '*ipso*' Naph), 147.97 (d, $^2J_{C\text{-}P}$ = 7.2 Hz, C-1 '*ipso*' Naph), 144.55 (*C*-6), 136.29, 136.28, 128.95, 128.92, 127.91, 127.90, 127.84, 127.68, 127.58, 126.61, 126.60, 126.53, 126.52, 126.22, 126.17, 126.53, 126.52 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 116.42 (d, $^3J_{C\text{-}P}$ = 3.4 Hz, C-2 Naph), 116.37 (d, $^3J_{C\text{-}P}$ = 3.2 Hz, C-2 Naph), 100.24 (*C*-5), 90.33 (CH$_2$C≡*C*), 87.40, 87.37 (*C*-1'), 87.15 (d, $^3J_{C\text{-}P}$ = 8.4 Hz, *C*-4'), 86.91 (d, $^3J_{C\text{-}P}$ = 8.1 Hz, *C*-4'), 75.40 (CH$_2$C≡C), 72.22, 72.11 (*C*-3'), 67.86 (d, $^2J_{C\text{-}P}$ = 5.4 Hz, *C*-5'), 62.47 (COO*C*H$_2$CH$_3$), 51.87, 51.77 (*C*HCH$_3$), 41.33, 41.25 (*C*-2'), 36.87, 36.84 (CO*C*H$_2$), 32.85, 30.53, 30.44, 30.23, 30.09, 26.80, 23.64 (C*H*$_2$NHCO, 6 x *C*H$_2$), 20.61 (d, $^3J_{C\text{-}P}$ = 6.6 Hz, CH*C*H$_3$), 20.47 (d, $^3J_{C\text{-}P}$ = 7.7 Hz, CH*C*H$_3$), 14.46, 14.39 (COOCH$_2$*C*H$_3$, *C*H$_3$). ^{31}P-NMR (202 MHz; MeOD-*d4*): δ_P 4.36, 4.09. MS (ES$^+$) *m/z*: 735 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/ MeOH from 90/10 to 0/100 in 40 min, Flow = 1 mL/min, λ = 280 nm, t_R= 34.49 min (99.05%).

4.1.3.3 N-{3-[5-(2'-Deoxy-5'-O-(1-naphthyl(neopentyloxy-L-alaninyl)phosphate-uridine))] prop-2-ynyl)octanamide (7c). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and naphthyl(neopentyloxy-L-alaninyl)phosphorochloridate **6c** (0.94 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98/2 to 95/5. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH = 95/5 to give **7c** as a white solid (39%, 0.13 g). ^{1}H NMR (500 MHz, MeOH-*d*$_4$): δ_H 8.22-8.18 (m, 1H, Naph), 7.93 (s, 0.65H of one diastereoisomer, *H*-6), 7.92 (s, 0.35H of one diastereoisomer, *H*-6), 7.90-7.88 (m, 1H, Naph), 7.72 (d *J* = 8.5 Hz, 0.35H of one diastereoisomer, Naph) 7.71 (d, *J* = 8.5 Hz, 0.65H of one diastereoisomer, Naph) 7.58-7.52 (m, 3H, Naph), 7.46-7.42 (m, 1H, Naph), 6.16-6.12 (m, 1H, *H*-1'), 4.45-4.32 (m, 3H, *H*-3', *H*-5'), 4.16-4.07 (m, 2H, *H*-4', C*H*CH$_3$), 3.99, 3.97, 3.95, 3.94 (4s, 2H, NHC*H*$_2$C≡), 3.87, 3.86, 3.76, 3.75 (2x AB, 4H, J_{AB} = 10.5 Hz, 2x OC*H*$_2$C(CH$_3$)$_3$), 2.21-2.14 (m, 1H, *H*-2'), 2.13 (t, *J* = 7.5 Hz, 1H, COC*H*$_2$CH$_2$), 2.08 (t, *J* = 7.5 Hz, 1H, COC*H*$_2$CH$_2$), 1.83-1.73 (m, 1H, *H*-2'), 1.56-1.51 (m, 2H, COCH$_2$C*H*$_2$), 1.43 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$), 1.29-1.19 (m, 8H, 4 x C*H*$_2$), 0.94, 0.93 (2s, 9H, OC*H*$_2$C(CH$_3$)$_3$), 0.87 (t, *J* = 6.5 Hz, 3H, C*H*$_3$). ^{13}C (125 MHz, MeOH-*d*$_4$): δ_C 175.68, 175.64 (*C*ONH), 175.16 (d, $^3J_{C\text{-}P}$= 5.0 Hz, *C*O$_2$CH$_2$C(CH$_3$)$_3$), 174.83 (d, $^3J_{C\text{-}P}$= 4.7 Hz, *C*O$_2$CH$_2$C(CH$_3$)$_3$), 164.42 (*C*-4), 151.00, 150.97 (*C*-2), 148.00 (d, $^2J_{C\text{-}P}$ = 7.3 Hz, C-1 '*ipso*' Naph), 147.90 (d $^2J_{C\text{-}P}$ = 7.2 Hz, C-1 '*ipso*' Naph), 144.64, 144.60 (*C*-6), 136.32, 136.30, 128.99, 128.97, 127.93, 127.73, 127.63, 126.67, 126.58, 126.28, 126.21, 122.77, 122.69 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 116.48 (d, $^3J_{C\text{-}P}$ = 5.7 Hz, C-2 Naph), 100.30, 100.27 (*C*-5), 90.36, 90.33 (CH$_2$C≡*C*), 87.43, 87.41 (*C*-1'), 87.19, (d, $^3J_{C\text{-}P}$ = 8.5 Hz, *C*-4'), 86.95 (d, $^3J_{C\text{-}P}$ = 8.1 Hz, *C*-4'), 75.55 (O*C*H$_2$C(CH$_3$)$_3$), 75.10 (CH$_2$*C*≡C), 72.30, 72.14 (*C*-3'), 67.97 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, *C*-5'), 67.92 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, *C*-5'), 52.03, 51.89 (*C*HCH$_3$), 41.36, 41.31 (*C*-2'), 36.94, 36.89 (CO*C*H$_2$), 32.90 (*C*H$_2$), 32.39 (*C*(CH$_3$)$_3$), 30.65, 30.55, 30.30, 30.15, 26.86, 26.85 (*C*H$_2$NHCO, 4 x *C*H$_2$), 26.81 (OCH$_2$C(*C*H$_3$)$_3$), 26.75, 23.69 (*C*H$_2$), 20.90 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, CH*C*H$_3$), 20.70 (d, $^3J_{C\text{-}P}$ = 7.6 Hz, CH*C*H$_3$), 14.48 (*C*H$_3$).^{31}P NMR (202 MHz, MeOH-*d*$_4$): δ_P 4.34, 4.07. MS (ES+) *m/z*: 777 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min, Flow = 1mL/min, λ = 280 nm, t_R= 22.51, 22.68 min.

4.1.3.4 N-{3-[5-(2'-Deoxy-5'-O-(1-naphthyl(benzyloxy-L-alaninyl)phosphate-uridine))] prop-2-ynyl)octanamide (7d). Prepared according to standard procedure from nucleoside **2** (0.30 g, 0.75 mmol) and naphthyl(benzyloxy-L-alaninyl)phosphorochloridate **6d** (0.91 g, 2.25 mmol) in

anhydrous THF (25 mL) and anhydrous NMI (0.30 mL, 3.80 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98/2 to 95/5. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 95/5 to give **7d** as a white solid (14%, 0.08 g). ^{1}H-NMR (500 MHz; MeOD-*d4*) δ_H 8.19-8.17 (m, 1H, Naph), 7.93-7.90 (m, 2H, Naph, H-6), 7.74-7.69 (m, 1H, Naph), 7.58-7.52 (m, 3H, Naph), 7.40-7.30 (m, 6H, Naph, Ph), 6.13-6.10 (m, 1H, *H*-1'), 5.18-5.10 (m, 2H, COOC*H*$_2$Ph), 4.39-4.32 (m, 1H, H-5'), 4.31- 4.25 (m, 2H, H-3', *H*-5'), 4.18-4.07 (m, 2H, H-4', C*H*CH$_3$), 3.92 (s, 2H, NHC*H*$_2$C≡), 2.19-2.09 (m, 3H, H-2', COC*H*$_2$CH$_2$), 1.80-1.75 (m, 1H, H-2'), 1.59-1.50 (m, 2H, COCH$_2$C*H*$_2$), 1.40 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$), 1.36-1.21 (m, 8H, 4 x C*H*$_2$), 0.89-0.87 (m, 3H, C*H*$_3$). ^{13}C-NMR (125 MHz; MeOH-*d4*): δ_C 175.23 (NH*C*O), 174.57 (*C*OOCH$_2$Ph), 164.32 (*C*-4), 150.87 (*C*-2), 148.60 (d, $^2J_{C-P}$ = 7.3 Hz, C-1 '*ipso*' Naph), 144.57, 144.52 (*C*-6), 137.14, 136.29 ('*ipso*' OCH$_2$*Ph*), 129.80, 129.74, 129.64, 129.61, 129.42, 129.37, 128.97, 127.91, 127.72, 127.62, 126.64, 126.55, 126.27, 126.18, 122.74, 122.65 (C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, COOCH$_2$*Ph*), 116.43 (d, $^3J_{C-P}$ = 2.5 Hz, C-2 Naph), 100.26 (*C*-5), 90.40 (CH$_2$C≡*C*), 87.32, 87.19 *(C*-1'), 86.91 (d, $^3J_{C-P}$ = 8.8 Hz, *C*-4'), 75.43 (CH$_2$*C*≡C), 72.25, 72.07 *(C*-3'), 68.14, 68.11 (*C*H$_2$OPh), 67.87 (d, $^2J_{C-P}$ = 5.0 Hz, *C*-5'), 52.00, 51.85 (*C*HCH$_3$), 41.33, 41.29 *(C*-2'), 36.90 (CO*C*H$_2$), 32.86, 30.56, 30.48, 30.25, 30.11, 26.83, 23.66 (*C*H$_2$NHCO, 6 x CH$_2$), 20.55 (d, $^3J_{C-P}$ = 6.25 Hz, CH*C*H$_3$), 14.48, 14.41 (*C*H$_3$). ^{31}P NMR (202 MHz, MeOD-*d4*): δ_P 4.36, 3.98. MS (ES+) *m/z*: 797 (M+Na$^+$, 100%). Reverse HPLC, eluting with H_2O/ CH_3CN from 90/10 to 0/100 in 30 minutes, Flow = 1 mL/min, λ = 280 nm, t_R = 16.63 min.

4.1.3.5 N-{3-[5-(2'-Deoxy-5'-O-(phenyl(methoxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7e). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(methoxy-L-alaninyl)phosphorochloridate **6e** (0.68 g, 2.45 mmol) in anhydrous THF (18 mL) and with NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98/2 to 94/6 to give **7e** as a white solid (16%, 0.05 g). ^{1}H NMR (500 MHz, MeOH-d_4) δ_H 7.99, 7.96 (2s, 1H, H-6), 7.40-7.36 (m, 2H, Ph), 7.30-7.27 (m, 2H, Ph), 7.23-7.20 (m, 1H, Ph), 6.25-6.20 (m, 1H, H-1'), 4.44-4.41 (m, 1H, H-3'), 4.39-4.37 (m, 1H, H-5'), 4.34-4.31 (m, 1H, H-5'), 4.15-4.12 (m, 1H, H-4'), 4.09 (s, 1H, NHC*H*$_2$), 4.06-4.01 (m, 2H, C*H*CH$_3$, NHC*H*$_2$), 3.71, 3.69 (2s, 3H, COOCH$_3$), 2.34 (ddd, *J* = 13.7 Hz, *J* = 6.0 Hz, *J* = 3.1 Hz, 0.5H of one diastereoisomer), H-2', 2.27 (ddd, *J* = 13.7 Hz, *J* = 6.0 Hz, *J* = 2.9 Hz, 0.5H of one diastereoisomer, H-2'), 2.19-2.15 (m, 2H, COCH$_2$-), 2.14-2.08 (m, 0.5H of one diastereoisomer, H-2'), 2.01-1.95 (m, 0.5H of one diastereoisomer, H-2'), 1.60-1.56 (m, 2H, COCH$_2$C*H*$_2$-), 1.38 (d, *J* = 7.1 Hz, 3H, CHC*H*$_3$), 1.34-1.25 (m, 8H, 4 x CH$_2$), 0.91 (t, *J* = 6.9 Hz, 3H, -CH$_2$CH$_3$). ^{13}C-NMR (125 MHz, MeOH-d_4): δ_C 175.75, 175.55, 175.27, 175.23 (*C*OOCH$_3$, *C*ONH), 164.43 (C-4), 152.15 (C-2), 151.03 ('ipso' *Ph*), 144.72, 144.68 (C-6), 130.91, 130.89, 126.38, 126.32, 121.53, 121.49 (*Ph*O), 100.23 (C-5), 90.46, 90.42 (CH$_2$C≡*C*), 87.47, 87.26 (C-1'), 87.13 (d, $^3J_{C-P}$ = 8.1 Hz, C-4'), 87.13 (d, $^3J_{C-P}$ = 8.3 Hz, C-4'), 75.42, 75.37 (CH$_2$*C*≡C), 72.23, 72.14 (C-3'), 67.69 (d, $^2J_{C-P}$ = 5.5 Hz, *C*-5'), 67.57 (d, $^2J_{C-P}$ = 5.3 Hz, *C*-5'), 51.70, 51.53 (COO*C*H$_3$), 50.22 (*C*HCH$_3$), 41.38, 41.32 (C-2'), 36.92 (CO*C*H$_2$), 32.89, 30.56, 30.53, 30.27, 30.13, 26.85, 23.67 (NH*C*H$_2$CO, 6 x CH$_2$), 20.62 (d, $^3J_{C-P}$ = 6.0 Hz, CH*C*H$_3$), 20.44 (d, $^3J_{C-P}$ = 7.1 Hz, CH*C*H$_3$), 14.43 (*C*H$_3$). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 3.98, 3.57. MS (EI) *m/z*: 671 (M+Na$^+$, 100%). Reverse HPLC, eluting with H_2O/CH_3CN from 100/0 to 0/100 in 30 min, Flow = 1 mL/min, λ = 254 nm, t_R = 18.29 min.

4.1.3.6 N-{3-[5-(2'-Deoxy-5'-O-(phenyl(ethoxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7f). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(ethoxy-L-alaninyl)phosphorochloridate **6f** (0.71 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH = 98/2 to 94/6 to give **7f** as a white solid (29%, 0.09 g). ^{1}H NMR (500 MHz, MeOH-d_4) δ_H 7.99, 7.96 (2s, 1H, H-6), 7.40-7.36 (m, 2H, Ph), 7.30-7.27 (m, 2H, Ph), 7.22-7.19 (m, 1H, Ph), 6.25-6.20 (m, 1H, H-1'), 4.44-4.41 (m, 1H, H-3'), 4.39-4.37 (m, 1H, H-5'), 4.35-4.30 (m, 1H, H-5'), 4.20-4.12 (m, 3H, H-4', COOC*H*$_2$CH$_3$), 4.10, 4.05 (2s, 2H, NHC*H*$_2$), 4.04-3.97 (m, 1H, C*H*CH$_3$), 2.34 (m, *J* = 13.7 Hz, *J* = 6.0 Hz, *J* = 3.1 Hz, 0.5H of one diastereoisomer, H-2'), 2.27 (m, *J* = 13.8 Hz, *J* = 6.0 Hz, *J* = 3.0 Hz, 0.5H of one diastereoisomer, H-2'), 2.19-2.15 (m, 2H, COCH$_2$-), 2.13-2.07 (m, 0.5H of one diastereoisomer, H-2'), 1.99-1.94 (m, 0.5H of one diastereoisomer, H-2'), 1.62-1.56 (m, 2H, COCH$_2$C*H*$_2$-), 1.38 (d, *J* = 7.2 Hz, 3H, CHC*H*$_3$), 1.34-1.24 (m, 11H, 4xCH$_2$, COOCH$_2$C*H*$_3$), 0.90 (t, J = 6.9 Hz, 3H, -CH$_2$CH$_3$). ^{13}C-NMR (125 MHz, MeOH-d_4): δ_C 175.76, 175.11, 175.07 (*C*OOCH$_2$CH$_3$, *C*ONH), 164.39 (C-4), 152.20, 152.14 (C-2), 151.02 ('ipso' *Ph*), 144.71, 144.67 (C-6), 130.92, 130.90, 126.40, 126.33, 121.53, 121.50 (*Ph*O), 100.28 (C-5), 90.48, 90.45 (CH$_2$C≡*C*), 87.43, 87.24 (C-1'), 87.12 (d, $^3J_{C-P}$ = 8.3 Hz, C-4'), 86.94 (d, $^3J_{C-P}$ = 8.2 Hz, C-4'), 75.47, 75.43 (CH$_2$*C*≡C), 72.21, 72.12 (C-3'), 67.71 (d, $^2J_{C-P}$ = 5.5 Hz, *C*-5'), 67.59 (d, $^2J_{C-P}$ = 5.4 Hz, *C*-5'), 62.52, 62.51 (COO*C*H$_2$CH$_3$), 51.81, 51.63 (*C*HCH$_3$), 41.42, 41.37 (C-2'), 36.96 (CO*C*H$_2$), 32.90, 30.63, 30.59, 30.30, 30.15, 26.87, 23.69 (NH*C*H$_2$CO, 6 x CH$_2$), 20.74 (d, $^3J_{C-P}$ = 6.2 Hz, CH*C*H$_3$), 20.55 (d, $^3J_{C-P}$ = 7.1 Hz, CH*C*H$_3$), 14.57, 14.55, 14.47 (COOCH$_2$*C*H$_3$, *C*H$_3$). ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 3.99, 3.58. MS (EI) *m/z*: 685 (M+Na$^+$, 100%). Reverse HPLC, eluting with H_2O/ CH_3CN from 100/0 to 0/100 in 30 min, Flow = 1 mL/min, λ = 254 nm, t_R = 19.10 min.

4.1.3.7 N-{3-[5-(2'-Deoxy-5'-O-(phenyl(propyloxy-L-alaninyl)phosphate-uridine))] prop-2-ynyl)octanamide (7g). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(propyloxy-L-alaninyl)phosphorochloridate **6g** (0.71 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98/2 to 94/6 to give **7g** as a white solid. ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 7.99, 7.97 (2s, 1H, C*H*-6), 7.40-7.35 (m, 2H, *Ph*), 7.31-7.26 (m, 2H, *Ph*), 7.23-7.19 (m, 1H, *Ph*), 6.24-6.19 (m, 1H, *H*-1'), 4.39-4.35 (m, 1H, *H*-3'), 4.34-4.20 (m, 2H, *H*-5'), 4.15-3.99 (m, 6H, *H*-4', OC*H*$_2$, NHC*H*$_2$C≡C, C*H*CH$_3$), 2.34 (ddd, *J* = 14.0, 6.0 and 3.0 Hz, 0.5H of one diastereoisomer, *H*-2'), 2.25 (ddd, *J* = 13.5, 6.0 and 2.5Hz, 0.5H of one diastereoisomer, *H*-2'), 2.19-2.14 (m, 2H, *H*-2', COC*H*$_2$CH$_2$), 2.12-2.00 (m, 1H of one diastereoisomer, *H*-2'), 199-1.96 (m, 1H of one diastereoisomer, *H*-2'), 1.70-1.63 (m, 2H, OCH$_2$C*H*$_2$), 1.62-1.56 (m, 2H, COCH$_2$C*H*$_2$), 1.41-1.37 (d, *J* = 7.5 Hz, 3H, CHC*H*$_3$), 1.34-1.26 (m, 8H, 4 x C*H*$_2$), 0.96, 0.95 (2t *J* = 7.5 Hz, 3H, OCH$_2$C*H*$_3$), 0.90 (t, *J* = 7.0 Hz, 3H, C*H*$_3$). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.75, 175.72 (CONH), 175.16 (d, $^3J_{C-P}$ = 4.5 Hz, *C*O$_2$CH$_2$), 174.88 (d, *J* = 5.4 Hz, *C*O$_2$CH$_2$), 164.40, 164.42 (*C*-4), 152.19 (d, $^2J_{C-P}$ = 7.1Hz 'ipso' *Ph*O), 152.19 (d, $^2J_{C-P}$ = 7.1 Hz

'ipso' *Ph*O), 152.13 (d, $^{2}J_{C\text{-}P}$ = 6.5Hz 'ipso' *Ph*O), 151.04 (*C*-2), 144.70, 144.74, (*C*-6), 130.89, 130.87, 126.36, 126.29, (*Ph*O), 121.52 (d, $^{2}J_{C\text{-}P}$ = 7.1 Hz, *Ph*O), 100.25, 100.28 (*C*-5), 90.46, 90.44 ($CH_2C{\equiv}C$), 87.48, 87.37 (*C*-1'), 86.97 (d, $^{3}J_{C\text{-}P}$ = 8.2 Hz, *C*-4'), 86.16 (d, $^{3}J_{C\text{-}P}$= 8.1 Hz, *C*-4'), 75.41, 75.34 ($CH_2C{\equiv}C$), 72.21, 72.12 (*C*-3'), 68.06, 68.05 (OCH_2), 67.75 (d, $^{2}J_{C\text{-}P}$ = 5.6 Hz, *C*-5'), 67.63 (d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, *C*-5'), 51.88, 51.72 ($CHCO_2$), 41.38, 41.35 (*C*-2'), 36.96 ($COCH_2$), 32.90, 30.59, 30.56, 30.27, 30.09, 26.87, 23.05, 23.03 (-CH_2-), 20.58 (d, $^{3}J_{C\text{-}P}$ = 7.0 Hz, $CHCH_3$), 20.77 (d, $^{3}J_{C\text{-}P}$ = 6.1 Hz, $CHCH_3$), 14.37 (CH_3), 10.64 (OCH_2CH_3); ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 4.00, 3.59. MS (EI) *m/z*: 711 ($M+Cl^-$, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90/10 to 0/100 in 35 min, Flow = 1 mL/min, λ = 280 nm, t_R= 17.77 min.

4.1.3.8 N-{3-[5-(2'-Deoxy-5'-O-(phenyl-(benzyloxy-L-alaninyl)phosphate-uridine))]prop-2-ynyl)octanamide (7h). Prepared according to standard procedure nucleoside **2** (0.30 g, 0.75 mmol) and phenyl(benzyloxy-L-alaninyl)phosphorochloridate **6h** (0.80 g, 2.25 mmol) in anhydrous THF (25 mL) and anhydrous NMI (0.30 mL, 3.80 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH = 98/2 to 95/5. The residue was further purified by preparative TLC, eluting with CH_2Cl_2/MeOH 95/5 to give **7h** as a white solid (11%, 0.06 g). ^{1}H-NMR (500 MHz; MeOH-*d4*) δ_H 7.97-7.93 (m, 1H, H-6), 7.91-7.87 (m, 1H, *Ph*), 7.41-7.32 (m, 6H, *Ph*), 7.29-7.17 (m, 3H, Ph), 6.23-6.17 (m, 1H, H-1'), 5.20-5.11 (m, 2H, OCH_2Ph), 4.41-4.34 (m, 1H, H-3'), 4.33-4.29 (m, 2H, H-5'), 4.11-4.01 (m, 2H, H-4', $CHCH_3$), 3.31 (s, 2H, $NHCH_2C{\equiv}$), 2.32-2.21 (m, 3H, *H*-2', $COCH_2CH_2$), 1.93-1.88 (m, 1H, *H*-2'), 1.63-1.51 (m, 2H, $COCH_2CH_2$), 1.41 (d, *J* = 7.0 Hz, 3H, $CHCH_3$), 1.37-1.21 (m, 8H, 4 x CH_2), 0.92-0.88 (m, 3H, CH_3). ^{13}C-NMR (125 MHz; MeOD-*d4*): δ_C 174.93 ($NHCO$), 171.69 (CO_2CH_2Ph), 164.37 (C-4), 152.25 (d, $^{3}J_{C\text{-}P}$ = 7.4 Hz 'ipso' O*Ph*), 151.17 (C-2), 144.84, 144.70 (C-6), 137.21, 137.18 ('ipso' OCH_2Ph), 131.25, 130.90, 129.80, 129.76, 129.73, 129.65, 129.64, 129.61, 129.44, 129.40, 129.38, 129.18, 126.99, 126.39, 126.30, 121.55, 121.52, 121.47 (*Ph*, OCH_2Ph), 100.22 (C-5), 90.50 ($CH_2C{\equiv}C$), 87.36, 87.32 (*C*-1'), 87.14, 87.07 (d, $^{3}J_{C\text{-}P}$ = 8.8 Hz, *C*-4'), 75.42 ($CH_2C{\equiv}C$), 72.24, 72.06 (*C*-3'), 68.13, 68.09 ($PhCH_2$), 67.65 (d, $^{2}J_{C\text{-}P}$ = 5.8 Hz, *C*-5'), 51.89, 51.69 ($CHCH_3$), 41.37, 40.99 (C-2'), 36.99, 36.93 ($COCH_2$), 32.89, 30.58, 30.54, 30.45, 30.28, 26.85, 23.67 ($NHCH_2CO$, 6 x CH_2), 20.61, 20.56 ($CHCH_3$), 14.43 (CH_3). ^{31}P NMR (202 MHz, MeOH-*d4*): δ_P 4.01, 3.51. MS (ES+) *m/z*: 747 ($M+Na^+$, 100%). Reverse HPLC, eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 minutes, Flow = 1 mL/min, λ = 280 nm, t_R = 16.22 min.

4.1.3.9 N-{3-[5-(2'-Deoxy-5'-O-(phenyl(benzyloxy-D-alaninyl)phosphate-uridine))] prop-2-ynyl)octanamide (7i). Prepared according to standard procedure from nucleoside **2** (0.25 g, 0.61 mmol) and phenyl(benzyloxy-D-alaninyl)phosphorochloridate **6i** (1.10 g, 3.05 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.24 mL, 3.05 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of ethyl acetate/MeOH from 100/0 to 98/2. The residue was further purified by preparative TLC eluting with CH_2Cl_2/MeOH 90/10 to give **7i** as a white solid (16%, 0.07 g). ^{1}H NMR (500 MHz; MeOH-*d4*): δ_H 7.99, 7.82 (2s, 1H, H-6), 7.38-7.20 (m, 10H, PhO, $COOCH_2Ph$), 6.21 (dd, *J* = 7.4 and 6.1 Hz, 0.5H of one diastereoisomer, H-1'), 6.16 (dd, *J* = 7.4 Hz and 6.1 Hz, 0.5H of one diastereoisomer, H-1'), 5.15 (2H, m, OCH_2Ph), 4.41-4.25 (m, 3H, H-3', H-5'), 4.16-4.01 (m, 4H, H-4', $NHCH_2C{\equiv}C$, $CHCH_3$), 2.29 (ddd, *J* = 13.7 Hz, *J* = 6.0 Hz, *J* = 3.0 Hz, 0.5H of one diastereoisomer, H-2'), 2.18-2.06 (m, 3H, H-2', $COCH_2CH_2$), 1.70 (m, 0.5H of one diastereoisomer, H-2'), 1.60-1.57 (m, 2H, $COCH_2CH_2$), 1.43 (d *J* = 7.1 Hz, 1.5H of one diastereoisomer, $CHCH_3$), 1.36 (d, *J* = 7.1 Hz, 1.5H of one diastereoisomer, $CHCH_3$), 1.35-1.26 (m, 8H, 4 x CH_2), 0.90 (t, 3H, *J* = 7.0 Hz, CH_3). ^{13}C NMR (125 MHz; MeOH-d_4): δ_C 175.69 (CH_2CO), 174.87 (d, $^{3}J_{C\text{-}P}$ = 3.9 Hz, $COOCH_2Ph$), 174.80 (d, $^{3}J_{C\text{-}P}$ = 4.3 Hz, $COOCH_2Ph$), 164.37 (*C*-4), 152.20 (d, $^{2}J_{C\text{-}P}$ = 6.6 Hz, '*ipso*' *Ph*O), 152.01 (d, $^{2}J_{C\text{-}P}$ = 6.7 Hz, '*ipso*' *Ph*O), 150.85 (*C*-2), 144.79, 144.41 (*C*-6), 137.18, 137.04 ('*ipso*' $COOCH_2Ph$), 130.93, 130.83, 129.59, 129.56, 129.46, 129.41, 129.37, 129.35, 126.33, 121.60, 121.56, 121.27, 121.23 (*Ph*O, $COOCH_2Ph$), 100.21, 100.12, (C-5), 90.78, 90.26 ($CH_2C{\equiv}C$), 87.24, 86.94 (*C*-1'), 87.05 (d, $^{3}J_{C\text{-}P}$ = 8.6 Hz, *C*-4'), 86.90 (d, $^{3}J_{C\text{-}P}$ = 8.7 Hz, *C*-4'), 75.48, 75.44 ($CH_2C{\equiv}C$), 72.14, 72.03 (*C*-3'), 68.13, 68.07 (OCH_2Ph), 67.70 (d, $^{2}J_{C\text{-}P}$ = 5.5 Hz, C-5'), 67.10 (d, $^{2}J_{C\text{-}P}$ = 4.8 Hz, C-5'), 51.77, 51.56 ($CHCH_3$), 41.36 (*C*-2'), 36.90 (CH_2CO), 32.85, 30.56, 30.47, 30.25, 30.10, 26.82, 23.65 (CH_2NHCO, 6 x CH_2), 20.43 (d, $^{3}J_{C\text{-}P}$ = 6.8 Hz, $CHCH_3$), 20.39 (d, $^{3}J_{C\text{-}P}$ = 7.6 Hz, $CHCH_3$), 14.39 (CH_3). ^{31}P NMR (202 MHz; MeOH-*d4*): δ_P 3.98, 3.50. MS (ES^+) m/z: 747 ($M+Na^+$, 100%). Reverse-phase HPLC, eluting with H_2O/ACN from 90/10 to 0/100 in 20 min, Flow = 1 mL/min, λ = 280 nm, t_R= 14.48 min, 14.86 min.

4.1.3.10 N-{3-[5-(2'-Deoxy-5'-O-(phenyl(benzyloxy-glycinyl)phosphate-uridine))]prop-2-ynyl)octanamide (7j). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(benzyloxy-glycinyl)phosphorochloridate **6j** (0.87 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98/2 to 95/5 to give **7j** as a white solid (29%, 0.10 g). ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 8.09, 7.89 (2s, 1H, *H*-6), 7.38-7.19 (m, 10H, P*h*, OCH_2P*h*), 6.24-6.18 (m, 1H, *H*-1'), 5.23-5.13 (m, 2H, OCH_2Ph), 4.36-4.30 (m, 3H, *H*-3', *H*-5'), 4.13-4.08 (m, 1H, *H*-4'), 4.08, 4.07, 4.02 (3s, 2H, $NHCH_2C{\equiv}C$), 3.91-3.85 (m, 2H, CH_2CO_2Bn), 2.30 (ddd *J* = 14.0, 6.5 and 3.5 Hz, 0.5H of one diastereoisomer, *H*-2'), 2.18-2.09 (m, 3H, *H*-2', $COCH_2CH_2$), 1.80-1.74 (m, 0.5H of one diastereoisomer, *H*-2'), 1.59-1.55 (m, 2H, $COCH_2CH_2$), 1.30-1.27 (m, 8H, 4 x CH_2), 0.91-0.86 (t, *J* = 6.7 Hz, 3H, CH_3). ^{13}C-NMR (125 MHz, MeOH-d_4): δ_C 175.79, 175.77 (CONH), 172.42, 172.38 (d, $^{3}J_{C\text{-}P}$= 4.0 Hz, *C*OOBn), 164.38 (*C*-4), 152.20 (d $^{2}J_{C\text{-}P}$ = 7.0 Hz, '*ipso*' *P*hO), 151.04, 150.97 (*C*-2), 144.84, 144.60 (*C*-6), 137.10, 137.14 ('*ipso*' OCH_2Ph), 130.97, 130.90, 129.63, 129.60, 129.50, 129.44, 126.43, 126.37 (*Ph*O, OCH_2Ph), 121.55 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, *Ph*O), 121.37 (d, $^{3}J_{C\text{-}P}$ = 4.6 Hz, *Ph*O), 100.20, 100.17 (*C*-5), 90.78, 90.44 (*C*≡C), 87.32 (*C*-1'), 87.20 (d, $^{3}J_{C\text{-}P}$ = 8.2 Hz, *C*-4') 87.10 (*C*-1'), 87.03 (d, $^{3}J_{C\text{-}P}$ = 8.2 Hz, *C*-4'), 75.41, 75.34 ($CH_2C{\equiv}C$), 72.28, 72.20 (*C*-3'), 68.08 (OCH_2Ph) 67.79 (d, $^{2}J_{C\text{-}P}$ = 5.7 Hz, *C*-5'), 67.49 (d, $^{2}J_{C\text{-}P}$ = 5.1 Hz, *C*-5'), 44.03, 43.96 (CH_2CO_2), 41.43, 41.40 (*C*-2'), 36.94 ($COCH_2$), 32.89, 32.88, 30.53, 30.48, 30.29, 30.14, 26.85, 23.68 (CH_2NHCO, 6 x CH_2), 14.43 (CH_3).^{31}P NMR (202 MHz, MeOH-d_4): δ_P 5.02, 4.93. MS (ES+) *m/z*: 733 ($M+Na^+$, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 min, Flow = 1mL/min, λ = 280 nm, t_R= 18.78, 19.15 min.

4.1.3.11 N-{3-[5-(2'-Deoxy-5'-O-(phenyl(benzyloxy-dimethylglycine)phosphate-uridine))] prop-2-ynyl) octanamide (7k). Prepared according to standard procedure from nucleoside **2**

(0.20 g, 0.49 mmol) and phenyl(benzyloxy-L-dimethylglycine)phosphorochloridate **6k** (0.86 g, 2.45 mmol) in anhydrous THF (15 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98/2 to 94/6 to give **7k** as a white solid. ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 7.95 (s, 0.3H of one diastereoisomer, *H*-6), 7.86 (s, 0.7H of one diastereoisomer, *H*-6), 7.40 - 7.17 (m, 10H, P*h*, OCH_2Ph), 6.21-6.15 (m, 1H, *H*-1'), 5.20 – 5.12 (m, 2H,OCH_2Ph), 4.32-4.21(m, 3H, *H*-3', *H*-5'), 4.09- 3.99 (m, 3H, *H*-4', $NHCH_2C\equiv$), 2.32-2.24 (m, 0.7H of one diastereoisomer, *H*-2'), 2.20-2.15 (m, 2.3H, *H*-2', one diastereoisomer, $NHCOCH_2$), 2.13-2.05 (m, 0.70H, H-2'), 1.77-1.72 (m, 0.30H of one diastereoisomer, *H*-2'), 1.61-1.48 (m, 6H, $NHCOCH_2CH_2$, $C(CH_3)_2$), 1.36-1.23 (m, 8H, 4 x CH_2), 0.95–0.85 (t, *J* = 6.0Hz, 3H, CH_2CH_3). ^{13}C NMR (125 MHz, MeOH-d_4): δ_c 176.75 (d, $^3J_{C-P}$ = 2.5 Hz, *C*OCH_2), 176.55, 176.53 (CONH), 164.52 (*C*-4), 152.35 (d, $^2J_{C-P}$ = 6.9Hz 'ipso' *Ph*O), 152.22 (d, $^2J_{C-P}$ = 6.8 Hz 'ipso' *Ph*O), 151.11 (*C*-2), 144.86, 144.62 (*C*-6), 137.32 ('ipso' OCH_2Ph), 130.90, 130.81, 129.63, 129.54, 129.35, 129.30, 129.20, 126.31, 126.22 (*Ph*O, OCH_2Ph), 121.70 (d, $^3J_{C-P}$ = 5.0 Hz, *Ph*O), 121.56 (d, $^3J_{C-P}$ = 4.6 Hz, *Ph*O), 90.75, 90.60 ($CH_2C\equiv C$), 87.00 (d, $^3J_{C-P}$ = 10.6 Hz, *C*-4'), 86.79 (d, $^3J_{C-P}$ = 10.6 Hz, *C*-4'), 86.26 (*C*-1'), 75.49, 75.38 ($CH_2C\equiv$C), 72.07, 71.99 (*C*-3'), 68.36, 68.35 (OCH_2), 67.76 (d, $^2J_{C-P}$ = 5.62 Hz, *C*-5'), 67.53 (d, $^2J_{C-P}$ = 5.78 Hz, *C*-5'), 41.27, 41.24 (*C*-2'), 36.99 ($NHCOCH_2$), 32.92, 30.61, 30.59, 30.17, 30.12 (CH_2), 27.93 (d, $^3J_{C-P}$ = 6.4 Hz, CH_3), 27.85 (d, $^3J_{C-P}$ = 6.4 Hz, CH_3), 27.67 (d, $^3J_{C-P}$ = 4.2 Hz, CH_3), 27.57 (d, $^3J_{C-P}$ = 4.2 Hz, CH_3), 23.71, 23.69, 26.90 (CH_2), 14.37 (CH_2CH_3); ^{31}P NMR (202 MHz, MeOH-d_4): δ_P 2.39, 2.1. MS (EI) *m/z*: 773 ($M+Cl^-$, 100%). Reverse-phase HPLC, eluting with H_2O/ CH_3CN from 90/10 to 0/100 in 35 min, Flow = 1 mL/min, λ = 280 nm, t_R= 19.24 min.

4.1.3.12 N-{3-[5-(2'-Deoxy-5'-O-(phenyl(benzyloxy-L-valinyl)phosphate-uridine))]prop-2-ynyl)octanamide (7l). Prepared according to standard procedure from nucleoside **2** (0.20 g, 0.49 mmol) and phenyl(benzyloxy-L-valinyl)phosphorochloridate **6l** (0.93 g, 2.45 mmol) in anhydrous THF (20 mL) and anhydrous NMI (0.19 mL, 2.45 mmol). The crude compound was purified by flash chromatography on silica gel gradient elution of CH_2Cl_2/MeOH from 98/2 to 95/5. The residue was further purified by preparative TLC, eluting with CH_2Cl_2/MeOH 95/5 to give **7l** as a white solid (14%, 0.05 g). ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 7.95 (s, 0.35H of one diastereoisomer, *H*-6), 7.91 (s, 0.65H of one diastereoisomer, *H*-6), 7.40-7.18 (m, 10H, P*h*, OCH_2Ph), 6.21-6.17 (m, 1H, *H*-1'), 5.21-5.09 (m, 2H, OCH_2Ph), 4.39-4.37 (m, 1H, *H*-3'), 4.34-4.22 (m, 2H, *H*-5'), 4.11-4.06 (m, 3H, *H*-4', $NHCH_2C\equiv$), 3.81 (dd, *J* = 9.5, 5.5 Hz, 0.65H of one diastereoisomer, C*H*$CH(CH_3)_2$), 3.76 (dd, *J* = 10.0, 6.0 Hz, 0.35H of one diastereoisomer, C*H*$CH(CH_3)_2$)), 2.30 (ddd, *J* = 13.5, 6.5 and 3.0 Hz, 0.35H of one diastereoisomer, *H*-2'), 2.22 (ddd, *J* = 13.5, 6.0 and 3.0 Hz, 0.65H of one diastereoisomer, *H*-2'), 2.18-2.04 (m, 3.35H, *H*-2' of one diastereoisomer, $COCH_2CH_2$, C*H*$(CH_3)_2$), 1.90-1.64 (m, 0.65H of one diastereoisomer, *H*-2'), 1.61-1.65 (m, 2H, $COCH_2CH_2$), 1.33-1.29 (m, 8H, 4 x CH_2), 0.92-0.86 (m, 9H, -CH_2CH_3, $CH(CH_3)_2$). ^{13}C NMR (125 MHz, MeOH-d_4): δ_C 175.72, 175.69 (*C*ONH), 174.09 (d, $^3J_{C-P}$= 2.7 Hz, CO_2Bn), 173.77 (d, $^3J_{C-P}$= 3.7 Hz, CO_2Bn), 164.46 (*C*-4), 152.25 (d, $^2J_{C-P}$ = 7.2 Hz, *'ipso'* *Ph*O), 150.99 (C-2), 144.71, 144.68 (*C*-6), 137.20, 137.13 ('*ipso*' OCH_2Ph), 130.91, 130.87, 129.73, 129.67, 129.65, 129.51, 126.44, 126.29 (*Ph*, OCH_2Ph), 121.68 (d, $^3J_{C-P}$ = 4.5 Hz, *Ph*O), 121.52 (d, $^3J_{C-P}$ = 5.5 Hz, *Ph*O), 100.28, 100.26 (*C*-5), 90.54, 90.51 (*C*≡C), 87.42 87.27 (*C*-1'), 87.15, 86.85 (d, $^3J_{C-P}$ = 8.1 Hz, *C*-4'), 75.59, 75.53 ($CH_2C\equiv$C), 72.28, 72.04 (*C*-3'), 68.04, 67.96 (OCH_2Ph) 67.87 (d, $^2J_{C-P}$ = 5.7 Hz, *C*-5'), 67.65 (d, $^2J_{C-P}$ = 5.4 Hz, *C*-5'), 58.38 (*C*HCH$(CH_3)_2$), 41.40, 41.38 (*C*-2'), 36.97, 36.96 (CH_2CO), 32.91, 30.68, 30.64, 30.32, 30.17, 26.89, 26.87, 23.70 (CH_2NHCO, 6 x CH_2), 19.70, 19,64, 18.49, 18,43, 18.06, 14.46 ($CH(CH_3)_2$, CH_3).^{31}P NMR (202 MHz, MeOH-d_4): δ_P 4.95, 4.30. MS (ES+) *m/z*: 775 ($M+Na^+$, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 min, Flow = 1 mL/min, λ = 280 nm, t_R= 21.23, 21.49 min.

4.2. Biological evaluation

4.2.1 Assay for antimycobacterial activity

M. tuberculosis H37Rv (ATCC27294) was grown in Middlebrook 7H9 broth supplemented with 0.02% (*v/v*) glycerol, 0.05% (*v/v*) tween-80 and 10% oleic acid, albumin, dextrose and catalase (OADC; BD Biosciences) as a stand culture at 37°C. *M. bovis* BCG (ATCC35734) was grown in Middlebrook 7H9 broth supplemented with 0.02% (*v/v*) glycerol, 0.05% (*v/v*) tween-80 and 10% albumin, dextrose and catalase (ADC; BD Biosciences) in a roller bottle at 2 rpm at 37°C. The antimycobacterial activities of the compounds were tested following the HT-SPOTi.[28,29] The high throughput growth inhibition assay was conducted in a semi-automated 96 well plate format as described previously.[26] Briefly, compounds dissolved in DMSO at a final concentration of 250 mg/L were serially diluted and dispensed in a volume of 2 µL into each well of a 96 well plate to which 200 µL of Middlebrook 7H10 agar medium kept at 55°C supplemented with 0.05% (*v/v*) glycerol and 10% (*v/v*) OADC was added. A well with no compounds (DMSO only) and isoniazid were used as experimental controls. To all the plates, a drop (2 µL) of *mycobacterial culture* containing 2 X 10^3 colony-forming units (CFUs) was spotted in the middle of each well and the plates were incubated at 37°C for up to two weeks. The minimum inhibitory concentrations (MICs) were determined as the lowest concentrations of the compound investigated where mycobacterial growth was completely inhibited by the presence of the compound.

4.2.2 Assay for antibacterial activity

E. coli DH5α (ATCC53868) was grown in Luria-Bertani broth (Oxoid, Thermoscientific) at 180 rpm as shaking culture with an incubation temperature of 37°C. The antibacterial activities of the compounds were tested using the HT-SPOTi as mentioned earlier, using appropriate culture medium and growth conditions. The plates were incubated overnight following which observations were recorded.

4.2.3 Assay for antiviral activity

Cells. Human embryonic lung (HEL) fibroblasts were grown in minimum essential medium (MEM) supplemented with 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 0.3% sodium bicarbonate.

Viruses. The laboratory wild-type varicella zoster virus (VZV) strain Oka, the thymidine kinase-deficient VZV strain 07-1, herpes simplex virus (HSV-1, KOS), (HSV-2, G), the thymidine kinase-deficient (ACV^R) HSV-1 strain B-2006, human cytomegalovirus (HCMV) strains Davis and AD-169, and vaccinia virus (VV) were used in the virus inhibition assays.

Confluent HEL cell cultures grown in 96-well microtiter plates were inoculated with VZV at an input of 20 plaque forming units (PFU) per well or with HCMV at an input of 100 PFU per well. Confluent HEL cell cultures were inoculated with HSV at 100 $CCID_{50}$ (50% cell culture infective dose) per well. After a 1-2 h incubation period, residual virus was removed and the infected cells were further incubated with MEM (supplemented with 2% inactivated FCS, 2 mM L-glutamine, and 0.3% sodium bicarbonate) containing varying concentrations of the compounds. Antiviral activity was expressed as EC_{50} (50% effective concentration), or compound concentration required to reduce viral plaque formation after 5 days (varicella zoster virus (VZV)) or virus-induced cytopathicity (HCMV after 7 days and HSV, VV after 3 days) by 50% compared to the untreated control.

4.2.4 Assay for eukaryotic cell toxicity Confluent monolayers of HEL cells as well as growing HEL cells in 96-well microtiter plates were treated with different concentrations of the experimental drugs. Cell cultures were incubated for 3 (growing cells) or 5 (confluent cells) days. At the indicated time, the cells were trypsinized, and the cell number was determined using a Coulter counter (Beckman, Analis, Suarlée, Belgium). The 50% cytostatic concentration (CC_{50}) was defined as the compound concentration required to reduce the cell number by 50%.

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