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Nucleoside Mono- and Diphosphate Prodrugs of 2′,3′-Dideoxyuridine and 2′,3′-Dideoxy-2′,3′-didehydrouridine

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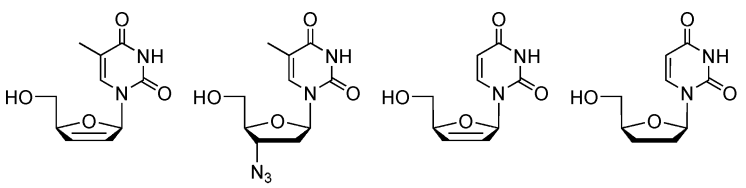
Despite their close structural similarity to nucleoside analogues such as the anti-HIV drugs AZT and d4T, 2′,3′-dideoxyuridine (ddU) and 2′,3′-dideoxy-2′,3′-didehydrouridine (d4U) are entirely inactive against HIV in their nucleoside form. However, it has been shown that the corresponding triphosphates of these two nucleosides can effectively block HIV reverse transcriptase. Herein we report on two types of nucleotide prodrugs (*cyclo*-Sal and Di*PP*ro nucleotides) of ddU and d4U to investigate their ability to overcome insufficient intracellular phosphorylation, which may be the reason behind their low anti-HIV activity. The release of the corresponding mono- and diphosphates from these compounds was demonstrated by hydrolysis studies in phosphate buffer (pH 7.3) and human CD_4^+ T-lymphocyte CEM cell extracts. Surprisingly, however, these compounds showed low or no anti-HIV activity in tests with human CD_4^+ T-lymphocyte CEM cells. Studies of the conversion of ddUDP and d4UDP into their triphosphate metabolites by nucleoside diphosphate kinase (NDPK) showed nearly no conversion of either diphosphate, which may be the reason for low intracellular triphosphate levels that result in low antiviral activity.

Introduction

Analogues of natural nucleosides are widely used in antiviral and anticancer therapy. One major drawback to the use of these compounds for medical purposes is the requirement for their intracellular conversion into the corresponding biologically active nucleoside 5′-triphosphates. Owing to the high substrate specificity of the kinases that catalyze the stepwise phosphorylation, one or more of these phosphorylation steps can be insufficient, resulting in low or no biological activity of the given agent.[1] For example, in the case of the nucleoside HIV reverse transcriptase inhibitor (NRTI) 2′,3′-dideoxy-2′,3′-didehydrothymidine (stavudine, d4T (**1**); Figure 1) the first phosphorylation step (catalyzed by thymidine kinase, TK) is hindered, whereas for the NRTI 3′-azido-3′-deoxythymidine (zidovudine, AZT (**2**)) the second phosphorylation (catalyzed by thymidylate kinase, TMP-K) is the rate-limiting step in the formation of the corresponding nucleoside triphosphate.[2,3]



d4T **1** AZT **2** d4U **3** ddU **4**

Figure 1. Active antiviral nucleoside analogues d4T **1** and AZT **2**, and inactive nucleoside analogues d4U **3** and ddU **4**.

To overcome this hurdle and to provide the active nucleoside 5′-triphosphates after in vivo administration, several nucleoside monophosphate prodrug strategies have been developed based on masking the negative charges of the corresponding nucleoside monophosphate with lipophilic groups (masking units). These masking groups undergo intracellular cleavage, releasing the nucleoside monophosphate. This membrane-permeable prodrug strategy therefore circumvents the first phosphorylation step.[4] An example of this concept is the well-established *cyclo*-Sal approach, in which a substituted salicyl alcohol as the lipophilic masking unit is cleaved intracellularly by chemically driven hydrolysis, releasing the nucleoside monophosphate and the corresponding salicyl alcohol.[5] More recent developments include "lock-in" modified[6] and enzymatically activated *cyclo*Sal pronucleotides.[7] We also recently reported on the diastereoselective synthesis of *cyclo*Sal compounds[8] and applications of the *cyclo*Sal concept for the synthesis of phosphorylated biomolecules.[9]

In contrast to the numerous examples of pronucleotide monophosphate approaches, nucleoside diphosphate prodrugs have been reported only rarely thus far. In this context, we recently reported an efficient nucleoside diphosphate prodrug system, which we termed the Di*PP*ro nucleotide approach.[10] In this approach two 4-acyloxybenzyl groups are used to protect the β-phosphate group of a nucleoside diphosphate, leaving the α-phosphate unprotected to prevent

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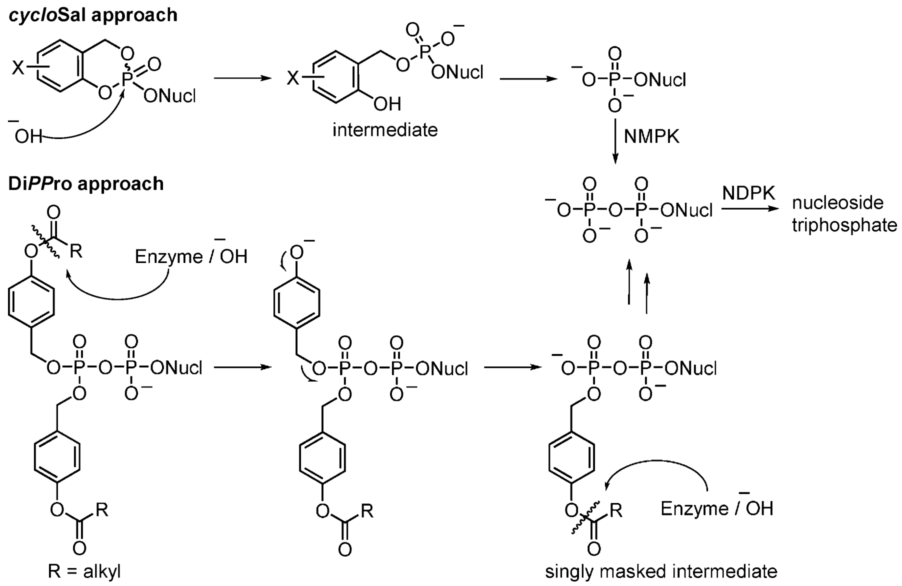
hydrolytic cleavage of the anhydride bond. The concept involves cleavage of the acyl ester bond either by enzymatic or chemical hydrolysis under physiological conditions. As a result, the polarity of the substituent in the *para* position to the benzyloxy group changes from an acceptor to a donor group (*Umpolung*) which destabilizes the benzyl phosphate ester bond and induces a 1,6-elimination of the first masking unit. Repetition of this cleavage mechanism at the second masking unit leads to the release of the nucleoside diphosphate (Scheme 1). The intracellular release of d4TDP and AZTDP from the corresponding DiPPro nucleotides was proven for compounds bearing various alkyl and alkenyl moieties in the masking unit.[10]



Scheme 1. Cleavage mechanisms of *cyclo*Sal pronucleotides and Di*PP*ro nucleotides.

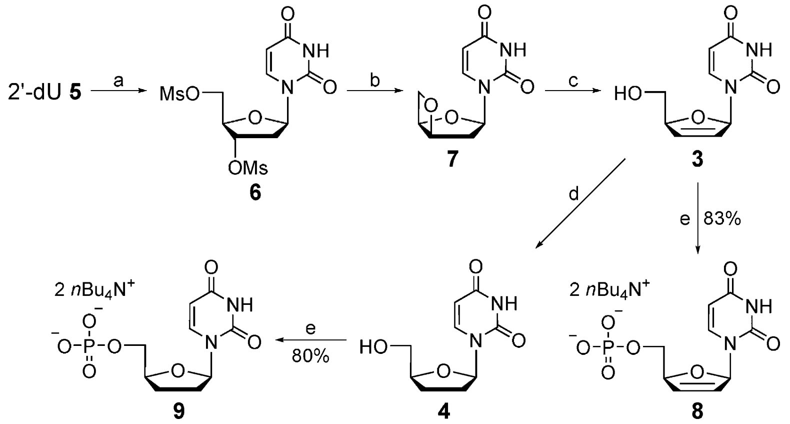
d4T **1** is an anti-HIV nucleoside analogue approved for use in the clinic. Despite their close structural similarity to d4T **1**, d4U **3** and ddU **4** were found to be entirely inactive against HIV in their nucleoside form.[11] However, the corresponding 5′-triphosphates of the latter two nucleosides are highly effective inhibitors of HIV reverse transcriptase.[12] It was concluded from earlier metabolism studies that the first phosphorylation to yield the nucleoside monophosphate is the bottleneck in the activation of ddU and d4U to the corresponding triphosphates. Therefore, several nucleoside monophosphate prodrugs of d4U **3** and ddU **4** were synthesized and evaluated for their antiviral activity, with a particular focus on ddU **4**. Although promising results were reported in a few cases,[13] surprisingly, in the majority of these approaches the pronucleotides showed no or only moderate anti-HIV activity.[14] The release of the monophosphates of both d4U **3** and ddU **4** from phosphoramidate prodrugs was demonstrated in incubation studies using (carboxyl)esterase. However, even this resulted in no (for d4U **3**) or only very moderate (for ddU **4**) anti-HIV activity.[15] These results led us to the synthesis of *cyclo*Sal pronucleotides of **3** and **4** to investigate the potential influence of the type and the delivery mechanism of the prodrug system on anti-HIV activity; the results also led us to the synthesis of nucleoside diphosphate prodrugs (Di*PP*ro compounds) of **3** and **4**, hypothesizing that the second phosphorylation step might be the bottleneck in the formation of the triphosphates of these two nucleoside analogues. As masking units, 4-acyloxybenzyl groups with alkyl substituents of various length (isopropyl to undecyl) were chosen. In addition to the corresponding 3-methyl-*cyclo*Sal phosphate triesters **24** (with d4U) and **25** (with ddU), the nucleoside monophosphates d4UMP **8** and ddUMP **9**, the Di*PP*ro compounds **15**–**22**, and the nucleoside diphosphates d4UDP **26** and ddUDP **27** were synthesized as well.

Results and Discussion

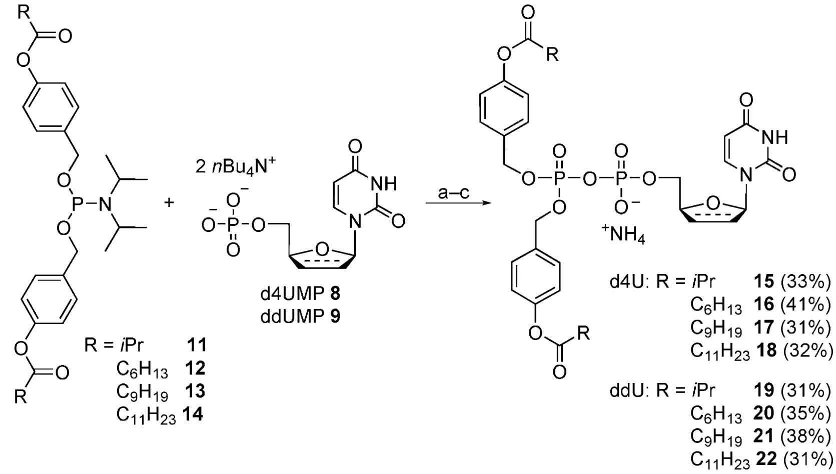
Chemistry

The nucleosides d4U **3** and ddU **4** were synthesized in good yields by following a modified version of the protocol reported by Horwitz et al. (Scheme 2).[16] Briefly, starting from 2′-deoxyuridine **5** the two hydroxy groups were mesylated using methanesulfonyl chloride in pyridine, yielding compound **6**. Treatment of dimesylate **6** with aqueous sodium hydroxide resulted in the formation of 3′,5′-anhydronucleoside **7**, which was converted into d4U **3** in 70% yield using potassium *tert*-butoxide in DMSO. ddU **4** was then obtained in 85% yield after palladium-catalyzed hydrogenation of the double bond.[17] The corresponding nucleoside 5′-monophosphates **8** and **9** were synthesized via the method of Sowa and Ouchi using phosphoryl chloride as phosphorylating agent,[18] followed by cation exchange from ammonium to tetra-*n*-butylammonium in 83 and 80% yields, respectively.

Using a 4,5-dicyanoimidazole (DCI)-mediated coupling reaction of the nucleoside monophosphates **8** or **9** and a phosphoramidite **11**–**14** bearing various acyloxybenzyl masking groups, followed by oxidation of the β-phosphate with *tert*-butylhydroperoxide, the Di*PP*ro compounds **15**–**22** were obtained in yields from 31 to 41% (Scheme 3). The phosphoramidites were synthesized as published before by starting from 4-hydroxybenzyl alcohol.[10] For the coupling reactions, the nucleoside monophosphates were used as tetra-*n*-butylammonium salts in order to increase their reactivity and solubility in acetonitrile. Thorough drying of these hygroscopic salts and the other reactants was crucial for the success of the coupling reactions. Monitoring the reactions by TLC was complicated by the large differences in polarity of the components in the reaction mixture. In contrast, the reactions could be easily followed by RP-



Scheme 2. Synthesis of the nucleosides d4U **3** and ddU **4** and their 5′-monophosphates **8** and **9**. *Reagents and conditions*: a) $MeSO_2Cl$, pyridine, 0 °C, 3 h, 85 %; b) NaOH in H_2O, 100 °C, 3 h, 80 %; c) KO*t*Bu, DMSO, RT, 70 h, 70 %; d) H_2, Pd/C, CH_3OH, RT, 70 h, 85 %; e) 1. $POCl_3$, pyridine, H_2O, CH_3CN, RT, 4 h, 2. NH_4HCO_3, H_2O, 3. Dowex H^+, 4. (nBu_4N)OH.



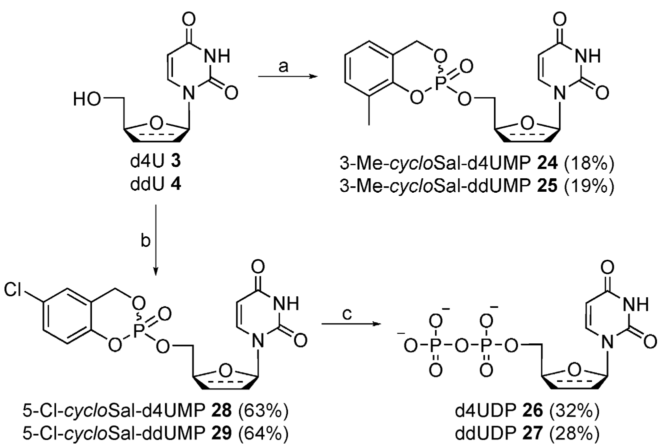
Scheme 3. Synthesis of Di*PP*ro compounds **15**–**22**. *Reagents and conditions*: a) DCI, CH_3CN, RT, 20 h; b) *t*BuOOH, −20 °C→RT, 30–60 min; c) ion exchange: 1. DOWEX 50WX8 (NH_4^+), 2. RP-18 chromatography.

Both 3-methyl-*cyclo*Sal phosphate triesters **24** and **25** of the two nucleosides and the corresponding nucleoside diphosphates d4UDP **26** and ddUDP **27** were synthesized as reference compounds as well. For the synthesis of the 3-methyl-*cyclo*Sal phosphate triesters **24** and **25**, nucleosides **3** and **4** were coupled with 3-methyl saligenylchlorophosphite followed by oxidation with *tert*-butylhydroperoxide (Scheme 4).[5] The diphosphates **26** and **27** were obtained from the corresponding 5-chloro-*cyclo*Sal phosphate triesters **28** and **29** by reaction with tetra-*n*-butylammonium phosphate. The acceptor-substituted 5-chloro-*cyclo*Sal triesters **28** and **29** were prepared as described before.[9c]

Hydrolysis studies

The *cyclo*Sal triesters **24** and **25** and Di*PP*ro compounds **15**–**22** were studied first for their hydrolytic stability at physiological pH 7.3 in aqueous 25 mM phosphate buffer. The enzymatic cleavage was then examined in human CD_4^+ T-lymphocyte CEM cell extracts. Hydrolysis products were identified by analytical RP-HPLC, and complete consumption of the corresponding nucleoside monophosphate was confirmed (Figure 2).

The coupling reactions were usually complete within minutes after the addition of DCI to the mixture of nucleotide **8** or **9** and the phosphoramidite. The subsequent oxidation of the P-III/P-V intermediate **23** could be followed by HPLC as well (Figure 2). After completion of the oxidation reaction, the crude products were purified by RP-18 chromatography using mixtures of methanol and water as eluent. After an initial purification to separate the remaining DCI, cations were changed from tetra-*n*-butylammonium to ammonium, and further purification steps were performed to separate the phosphate diester and the nucleotide that were supposedly formed by partial cleavage of the anhydride bond. To determine the point at which these cleavage products were formed, the coupling reactions were monitored for 20 h, showing no re-formation of the monophosphate. Also, no monophosphate was formed during oxidation. It was therefore assumed that cleavage of the anhydride bond occurs during purification.



Scheme 4. Synthesis of *cyclo*Sal triesters **24**, **25** and **28**, **29** and nucleoside diphosphates **26** and **27**. *Reagents and conditions*: a) 1. 3-methyl saligenylchlorophosphite, DIPEA, CH_3CN, −20 °C→RT, 2.5 h, 2. *t*BuOOH, −20 °C→RT, 2 h; b) 1. 5-chloro saligenylchlorophosphite, DIPEA, CH_3CN, −20 °C→RT, 45 min, 2. oxone, −20 °C→RT, 15 min; c) tetra-*n*-butylammonium phosphate, DMF, RT, 16–18 h.

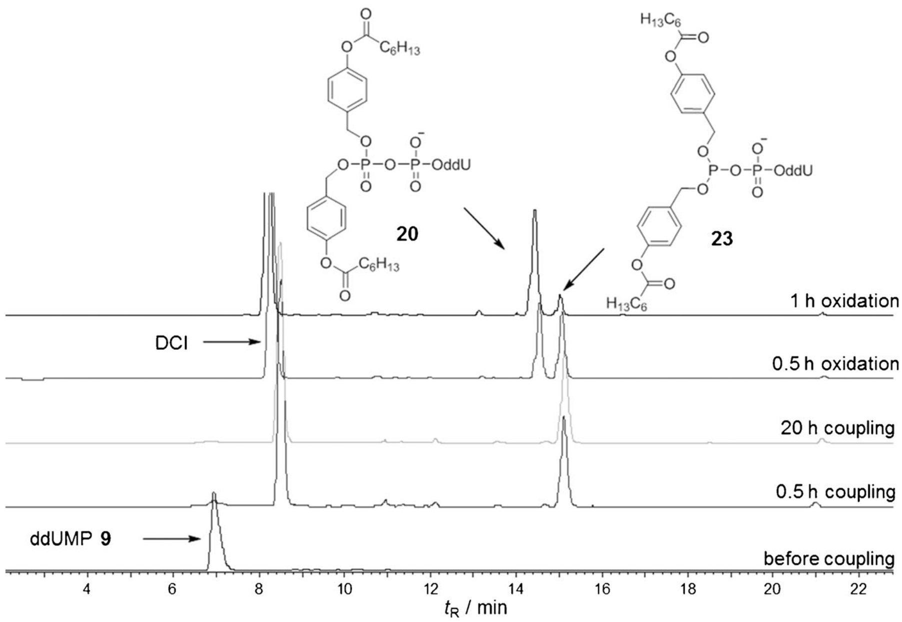


Figure 2. Monitoring of the synthesis of C_6-ddUDP **20** by RP-HPLC.

HPLC methods. The calculated half-lives refer to the degradation of the starting prodrug ($t_1{}^1/_2$) or, in case of the Di*PP*ro compounds, of the singly masked intermediate ($t_2{}^1/_2$) as well. The latter half-lives ($t_2{}^1/_2$) were determined after complete consumption of the starting Di*PP*ro compound.

Chemical hydrolysis

In general, the hydrolysis behavior of compounds **15**–**22** was in good agreement with the data published previously for Di*PP*ro compounds of d4T **1** and AZT **2**.[10] Hydrolysis half-lives of the potential prodrugs increased with increasing alkyl chain length, with the exception of isopropyl-substituted compounds **15** and **19** (Table 1). Their half-lives are in the same range as those of the C_6-substituted compounds **16** and **20**. In accordance with the hydrolysis mechanism shown in Scheme 1, degradation of the prodrugs led to the formation of the desired nucleoside diphosphate, with formation of a mono-masked intermediate. In contrast, for the undecyl-substituted Di*PP*ro nucleotides **18** and **22** only a small amount of the corresponding intermediate was observed. This has been determined earlier for other Di*PP*ro nucleotides with long alkyl chains in the masking unit as well.[10] The half-life of the intermediate formed was always significantly higher than that of the original prodrugs, supposedly caused by repulsive interaction between the negative charges of the intermediate and the nucleophile. As a result of hydrolytic cleavage of the anhydride bond, a significant amount of nucleoside monophosphate was formed as well. The fraction of monophosphate increased with longer half-lives of the starting prodrug, as we proved that the monophosphates were only formed from the original prodrug but were not formed from the intermediate.[10] However, due to partial hydrolysis of the mono- and diphosphates after long hydrolysis periods, their exact ratio could not be determined (Figure 3). In contrast, the hydrolysis of *cyclo*Sal compounds **24** and **25** showed, as expected, selective formation of the corresponding nucleoside monophosphates via a benzyl phosphate diester intermediate.

Table 1. Hydrolysis half-lives of Di*PP*ro nucleotides **15**–**22** and *cyclo*Sal triesters **24** and **25**.

Compd	Nucl	R	PBS, pH 7.3 $t_1{}^1/_2$ [h][b]	PBS, pH 7.3 $t_2{}^1/_2$ [h][c]	CE[a] $t_1{}^1/_2$ [h][b]
15	d4U	*i*Pr	40	275	0.3
16	d4U	C_6	30	326	0.4
17	d4U	C_9	63	223	1.1
18	d4U	C_{11}	76	NA[d]	1.9
19	ddU	*i*Pr	36	255	0.3
20	ddU	C_6	28	337	0.5
21	ddU	C_9	66	290	0.7
22	ddU	C_{11}	88	NA[d]	1.7
24	d4U	–	9	NA[d]	11
25	ddU	–	7	NA[d]	16

[a] CEM/0 cell extract. [b] Half-life of the first hydrolysis step. [c] Half-life of the second hydrolysis step. [d] Not applicable.

Hydrolysis in cell extracts

Hydrolysis of the Di*PP*ro compounds in human $CD_4{}^+$ T-lymphocyte CEM cell extracts led to markedly lower half-lives than the chemical hydrolysis half-lives due to enzymatic cleavage of the ester group. This enzymatic cleavage also took place for the singly masked intermediates that did not accumulate for this reason (Figure 4). The release of nucleoside mono- and diphosphates was proven, although the ratio of these compounds could not be determined, as they were rapidly hydrolyzed under these conditions as well. As observed in the chemical hydrolysis studies, half-lives increased with increasing alkyl chain length in the masking group.

Antiviral evaluation

All Di*PP*ro nucleotides **15**–**22** as well as the *cyclo*Sal compounds **24** and **25** were investigated for their in vitro anti-HIV activity. As anticipated, the monophosphate-releasing *cyclo*Sal prodrugs **24** and **25** showed no activity. Unexpectedly, although the release of the corresponding nucleoside diphosphates was proven by the hydrolysis studies, compounds **15**–**22** exhibited very moderate or no antiviral activity as well (Table 2). This result could be explained by insufficient permea-

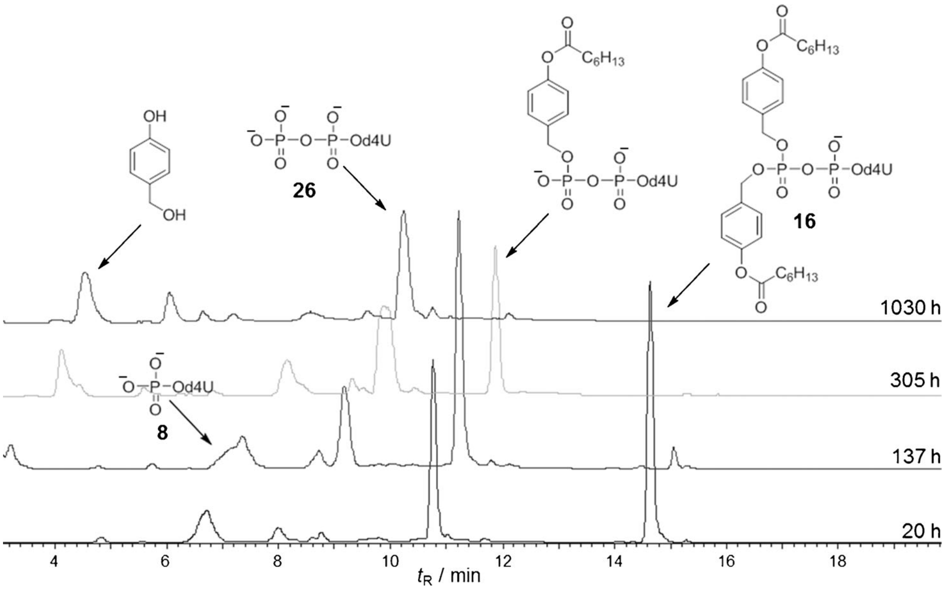


Figure 3. pH-dependent hydrolysis of C_6-d4UDP **16** monitored by RP-HPLC.

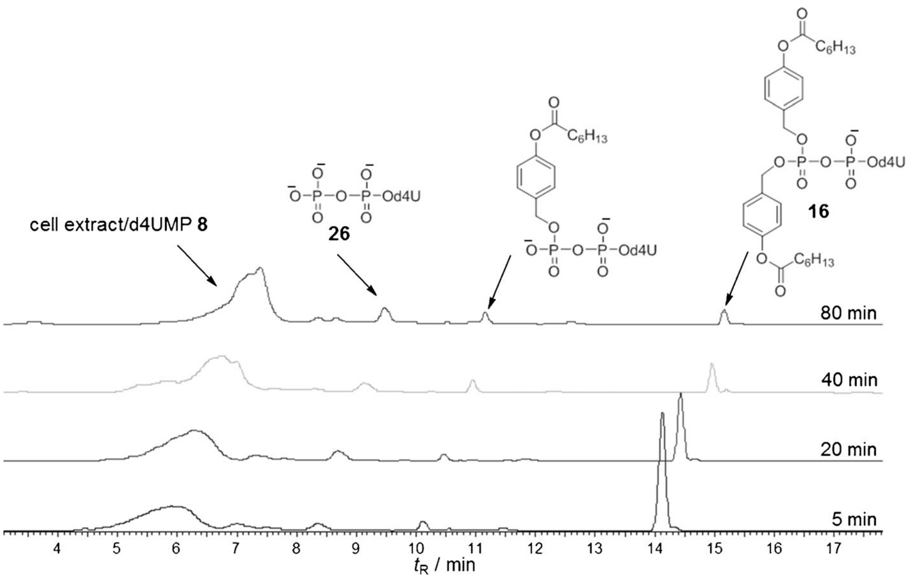


Figure 4. Enzymatic hydrolysis of C_6-d4UDP **16** monitored by RP-HPLC.

tion of the prodrugs into the cells, inefficient intracellular release of the diphosphates, or poor conversion of the diphosphates to the corresponding triphosphates.

NDPK studies

To investigate the efficiency of the conversion of nucleoside diphosphates **26** and **27** to the corresponding triphosphates, both compounds were subjected to an enzyme assay using purified nucleoside diphosphate kinase (NDPK). Both d4U and ddU nucleoside diphosphates showed no conversion into the corresponding triphosphates under these conditions (using ATP as the phosphate donor), whereas the reference compound uridine diphosphate (UDP) was very efficiently converted into its 5′-triphosphate derivative (Figure 5).

We conclude that under conditions in which the vast majority of UDP has been converted into UTP in the presence of NDPK after 10 min (70% conversion reached under equilibrium reaction conditions), there were no traces at all on the HPLC chromatogram to indicate any conversion of ddUDP or d4UDP after 60 min under similar experimental conditions. This means that the compounds must act as extremely poor substrates for NDPK (if substrates at all). Therefore, k_{cat}/K_M values could not be determined. Moreover, given the pronounced intracellular levels of the natural substrate UDP (and UTP) for NDPK, it is very unlikely that this enzyme will convert the compounds at a physiologically relevant concentration. However, it should be kept in mind that enzymes other than NDPK might still be able to convert the diphosphates of ddU and d4U into their triphosphate derivatives.

Given its unique mechanism of action that does not require simultaneous binding of acceptor and donor in its active site, NDPK is significantly less substrate specific than nucleoside kinases and nucleoside monophosphate kinases (NMPKs). However, the interaction of the 3′-hydroxy group of the acceptor nucleoside diphosphate with a Mg^{2+} ion in the active site is necessary to stabilize the transition state.[19] Therefore, most probably the lack of a 3′-hydroxy group combined with conformational changes in the glycon due to modifications can result in a significant decrease in catalytic efficiency of NDPK.

Although other possible reasons for the low antiviral activity of the tested Di*PP*ro nucleotides cannot be excluded, it can be assumed that the insufficient formation of the nucleoside triphosphates from the corresponding diphosphates might be the main reason for the poor, or absent, antiviral activity in cell culture.

The anti-HIV activity of other dideoxynucleoside analogues such as d4T, ddC, and AZT, which are also expected to be poor NDPK substrates in their diphosphate form, can be much

Table 2. Antiviral activity and cytotoxicity of nucleoside analogues **3** and **4**, and prodrug compounds **15–22** and **24** and **25**.

Compd	Nucl	R	EC_{50} [μм][a] CEM/0 HIV-1	EC_{50} [μм][a] CEM/0 HIV-2	EC_{50} [μм][a] CEM/TK^- HIV-2	CC_{50} [μм][b] CEM/0
15	d4U	*i*Pr	32±16	23±1.4	≥50	110±1.4
16	d4U	C_6	35±2.1	30±4.2	24±2.8	109±2.1
17	d4U	C_9	36±20	20±3.5	>10	95±3.5
18	d4U	C_{11}	>50	>50	>50	116±2.8
24	d4U	–	>50	>50	>10	93±7.1
d4U	–	–	>250	>250	>250	>250
19	ddU	*i*Pr	>50	>50	>50	>250
20	ddU	C_6	26±9.9	30±4.9	>50	124±11
21	ddU	C_9	≥50	42±12	>50	99±3.5
22	ddU	C_{11}	>50	>50	>50	144±7.1
25	ddU	–	>50	>50	>50	104±3.5
ddU	–	–	>250	>250	>250	>250
d4T	–	–	0.52±0.32	0.72±0.35	250	>250

[a] Antiviral activity in CD_4^+ T-lymphocytes: 50% effective concentration; values are the mean±SD of $n=2$–3 independent experiments. [b] Cytotoxicity: 50% cytostatic concentration or compound concentration required to inhibit CEM cell proliferation by 50%; values are the mean±SD of $n=2$–3 independent experiments.

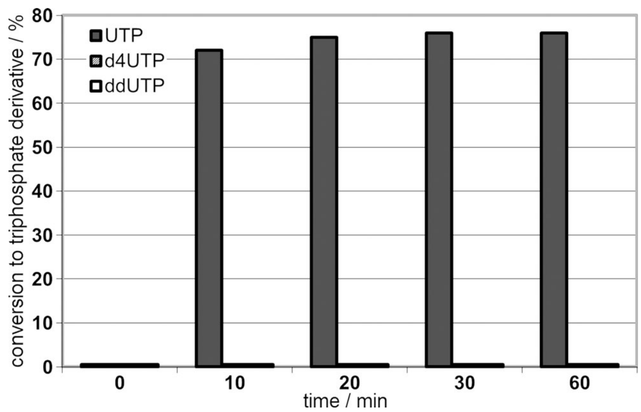


Figure 5. Enzyme assay to evaluate the conversion of nucleoside diphosphates **26** and **27** by NDPK.

better substrates for the activating enzymes (i.e., thymidine kinase or dCyd kinase) than ddU or d4U, explaining antiviral efficacy for these other drugs. It has, in fact, been shown that AZT is a very good substrate for thymidine kinase, and d4T and ddC have been shown to have only a hundredfold less substrate affinity for their activating enzymes than the natural substrates.[1,11,12]

Conclusions

In summary we report on the successful synthesis of two different classes of potential nucleotide prodrugs of the two uridine nucleoside analogues d4U and ddU. The significant inhibitory activity of the triphosphates of these nucleosides to block HIV reverse transcriptase has been reported previously. In contrast, the nucleoside analogues themselves proved to be entirely inactive, and the hypothesis was made that phosphorylation into the monophosphate metabolite was the rate-limiting step in their activation into the triphosphates. Therefore, for both nucleosides, nucleoside monophosphate prodrug strategies have been described, although with no or very limited success. We applied our recently reported nucleoside diphosphate prodrug system, the Di*PP*ro compounds, as well as the well-established *cyclo*Sal pronucleotide approach for comparison, to these two nucleoside analogues to determine if the intracellular delivery of the nucleoside diphosphates could overcome the lack of activity. However, from the data reported herein it became apparent that neither the *cyclo*Sal nor the Di*PP*ro approach was able to solve the problem; however, we could prove that both systems deliver the nucleoside monophosphate and the nucleoside diphosphate, respectively. Separate studies using nucleoside diphosphate kinase showed that this enzyme is practically unable to form the triphosphate starting from both nucleoside diphosphates, which might explain the poor antiviral activity. As a consequence, for these two nucleoside analogues only the intracellular delivery of the corresponding triphosphate could overcome the metabolic hurdles. We are currently working on strategies to deliver nucleoside triphosphates from lipophilic, membrane-permeable precursors. This approach will then be applied to ddU and d4U.

Experimental Section

Chemistry

General: Pyridine and CH_3CN were distilled from CaH_2 under nitrogen. *N*,*N*-Diisopropylethylamine (DIPEA) was distilled from sodium prior to use. $POCl_3$ was distilled under nitrogen prior to use. All commercially available reagents were used without further purification. Thin-layer chromatography (TLC): Merck pre-coated 60 F_{254} plates (0.2 mm layer of silica gel), or pre-coated plates with a 0.2 mm layer of silica gel (Macherey & Nagel Xtra Sil UV_{254}) were used; sugar-containing compounds were visualized with sugar spray reagent (0.5 mL 4-methoxybenzaldehyde, 9 mL EtOH, 0.5 mL conc. H_2SO_4, and 0.1 mL glacial AcOH). All preparative TLC was performed on a Chromatotron (Harrison Research, Model 7924T) using glass plates coated with 1-, 2-, or 4-mm-thick layers of Merck 60 PF_{254} silica gel containing a fluorescent indicator. Flash column chromatography: Merck silica gel 60, 230–400 mesh or Merck RP-18 silica gel was used. Glass columns were slurry packed using the appropriate eluent. Fractions containing the product were identified by TLC, pooled, and the solvent was removed in vacuo. Water as eluent was removed by freeze-drying.

Analytical HPLC was performed on a VWR–Hitachi LaChromElite HPLC system (L-2130, L-2200, L-2455) equipped with an EcoCART 125-3 column containing reversed-phase silica gel Lichrospher 100 RP-18 (5 mm; VWR-Merck, Darmstadt, Germany). The solvents for HPLC were obtained from Sigma–Aldrich or VWR (CH_3CN, HPLC grade). The software used was EZChromElite. Method A: 0–20 min: TBAH ion buffer/CH_3CN gradient (5→90%); 20–24 min: buffer/CH_3CN (90%); 24–29 min: buffer/CH_3CN gradient (90→5%); 30–35 min: buffer/CH_3CN (5%); flow: 1 $mL\,min^{-1}$, TBAH ion buffer: 0.55 mм tetra-*n*-butylammonium hydroxide in H_2O. Method B: 0–

25 min: H_2O/CH_3CN gradient (5→95%); 25–35 min: H_2O/CH_3CN (95%); flow: 0.5 mL min^{-1}.

NMR spectra were recorded with Bruker AMX 400, AV I 400, AV II 400, or Bruker DRX 500 Fourier transform spectrometers. All ^{1}H and ^{13}C NMR chemical shifts (δ) are quoted in parts per million (ppm) downfield from tetramethylsilane and calibrated on solvent signals. The ^{31}P NMR chemical shifts (proton decoupled) are quoted in ppm using H_3PO_4 as the external reference. The spectra were recorded at room temperature in automation mode. Mass spectra were obtained with a VG Analytical VG/70S Xenon FAB [FAB, (double focusing), matrix: *m*-nitrobenzyl alcohol] instrument; ESI mass spectra were recorded with an Agilent 6224 ESI-TOF spectrometer in positive or negative modes. IR spectra were recorded on a Bruker Alpha P FT-IR spectrometer at room temperature in the range of 400–4000 cm^{-1}. Melting points are uncorrected, and ≥95% purity for the final compounds **15**–**22**, **24**–**27** was confirmed by HPLC analysis.

General procedure 1: Synthesis of 3-methyl-*cyclo*Sal triesters. The reaction was carried out under nitrogen and with dry solvents and reagents. A solution of the nucleoside in CH_3CN was cooled to −20 °C and *N*,*N*-diisopropylethylamine (1.6 equiv) followed by 3-methyl saligenylchlorophosphite (2.0 equiv) dissolved in CH_3CN were added. The reaction mixture was stirred at room temperature until the coupling reaction was completed and subsequently cooled to −20 °C before addition of *tert*-butylhydroperoxide (5.5 M in *n*-decane, 3.0 equiv). The solution was stirred at room temperature until completion of the oxidation reaction, poured into ice water and immediately extracted with EtOAc. The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by preparative TLC, dissolved in CH_3CN and H_2O (1:1 *v*/*v*) and freeze-dried.

General procedure 2: Synthesis of 5-chloro-*cyclo*Sal triesters. The reaction was carried out as described above in general procedure 1, except that for the oxidation, oxone (1.0 equiv) dissolved in cold H_2O was used.

General procedure 3: Synthesis of nucleoside monophosphates (bis(tetra-*n*-butylammonium) salts). The reaction was carried out under nitrogen and with dry solvents and reagents. $POCl_3$ (4.4 equiv) was dissolved in CH_3CN and cooled to 0 °C. Then, pyridine (4.4 equiv) and H_2O (2.2 equiv) were carefully added to the stirred solution. After 10 min the nucleoside was added in portions and the solution was stirred at room temperature for 4 h. Ice water was then added, and the solution was stirred for 1 h at 0 °C. After neutralization by the addition of solid ammonium hydrogen carbonate the solution was freeze-dried and the crude product was purified by RP-18 chromatography with H_2O as eluent. The ammonium salt thus obtained was dissolved in H_2O and eluted over Dowex 50WX8 cation-exchange resin (H^+ form). The resulting acidic solution was titrated with tetra-*n*-butylammonium hydroxide solution (40% *w*/*w* in H_2O) to pH 6–7 and the solvent was removed by freeze-drying.

General procedure 4: Synthesis of nucleoside diphosphates (ammonium salts). The reaction was carried out under nitrogen and with dry solvents and reagents. Tetra-*n*-butyl ammonium phosphate (2.0 equiv) was vigorously dried under vacuum, dissolved in *N*,*N*-dimethylformamide and stirred over molecular sieves (4 Å) for 1 h. The corresponding 5-chloro-*cyclo*Sal triester was dissolved in *N*,*N*-dimethylformamide and added dropwise over 1 h. The reaction mixture was stirred at room temperature until completion of the reaction. The solvent was removed under reduced pressure and the residue was dissolved in H_2O and EtOAc (1:1). The layers were separated and the organic layer was extracted with H_2O. The combined aqueous layers were freeze-dried and the crude product thus obtained was purified by RP-18 chromatography. The cations were changed to ammonium ions using a DOWEX 50WX8 cation-exchange resin (NH_4^+ form).

General procedure 5: Synthesis of Di*PP*ro nucleotides. The reaction was carried out under nitrogen and with dry solvents and reagents. The nucleoside monophosphate (tetra-*n*-butylammonium salt) was freeze-dried, co-evaporated with CH_3CN and dissolved in CH_3CN. The corresponding bis(acyloxybenzyl)phosphoroamidite (1.7–3.2 equiv) and DCI (1.8–3.3 equiv) were added, and the reaction mixture was stirred at room temperature for 20 h. The solution was subsequently cooled to −20 °C, and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 1.8–3.2 equiv) was added. The solution was stirred for 30–60 min at room temperature and the solvent was removed under reduced pressure. The crude product thus obtained was purified by a first RP-18 flash column chromatography (MeOH/H_2O gradient) and the cations were changed to ammonium by elution over Dowex 50WX8 cation-exchange resin (NH_4^+ form). The ammonium salts of the products were purified a second and third time if necessary by RP-18 flash column chromatography using different MeOH/H_2O gradients. Product containing fractions were pooled, and the MeOH was removed under reduced pressure. The remaining aqueous solution was freeze-dried and the product was obtained as a colorless foam or syrup.

3′,5′-Di-*O*-methanesulfonyl-2′-deoxyuridine 6. The reaction was carried out under nitrogen and with dry solvents and reagents. A suspension of 2′-deoxyuridine **5** (1.00 g, 4.40 mmol) in pyridine (18 mL) was cooled to 0 °C, and methanesulfonyl chloride (0.85 mL, 1.3 g, 11 mmol, 2.5 equiv) was added dropwise. The mixture was stirred at 0 °C for 3 h, then poured into ice water and stirred for an additional hour at 0 °C. Subsequently, the precipitate was filtered, washed with cold H_2O and dried in vacuum. The remaining aqueous phase was extracted with EtOAc until no product could be detected in the aqueous phase by TLC. The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The obtained solid was combined with the one obtained by filtration to yield a colorless solid (1.44 g, 3.74 mmol, 85%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 11.41 (s, 1 H, NH), 7.66 (d, $^{3}J_{H,H}$ = 8.1 Hz, 1 H, H-6), 6.19 (dd, $^{3}J_{H,H}$ = 7.0 Hz, $^{3}J_{H,H}$ = 7.0 Hz, 1 H, H-1′), 5.68 (dd, $^{3}J_{H,H}$ = 8.1 Hz, $^{4}J_{H,H}$ = 2.3 Hz, 1 H, H-5), 5.31–5.25 (m, 1 H, H-3), 4.50–4.34 (m, 3 H, H-4′, H-5′), 3.31, 3.24 (2×s, 2×3 H, 2×CH_3), 2.55 ppm (dd, $^{3}J_{H,H}$ = 7.1 Hz, $^{3}J_{H,H}$ = 5.1 Hz, 2 H, H-2′); ^{13}C NMR (101 MHz, [D$_6$]DMSO): δ = 162.9 (C-4), 150.3 (C-2), 140.6 (C-6), 102.2 (C-5), 84.6 (C-1′), 80.6 (C-4′), 79.2 (C-3′), 68.2 (C-5′), 37.7, 36.8 (2×CH_3), 36.0 ppm (C-2′); IR: $\tilde{\nu}$ = 3034, 1685, 1328, 1169, 919, 805, 524 cm^{-1}; mp: 142 °C; TLC: R_f = 0.59 (CH_2Cl_2/MeOH 9:1 *v*/*v*); HRMS (ESI$^+$): *m*/*z* calcd: 407.0189 $[M+Na]^+$, found: 407.0175.

3′,5′-Anhydro-2′-deoxyuridine 7. At room temperature, 3′,5′-di-*O*-methanesulfonyl-2′-deoxyuridine **6** (1.44 g, 3.74 mmol) was added in portions to a solution of NaOH (447 mg, 11.2 mmol, 3.0 equiv) in H_2O (80 mL). The reaction mixture was stirred at reflux for 3 h and after cooling to room temperature neutralized by addition of 1 M HCl. Half of the solvent was removed under reduced pressure and the product crystallized over night at 4 °C. The crystalline product was filtered, washed with cold H_2O and dried under vacuum. The filtrate was extracted with EtOAc until no product could be detected in the aqueous phase by TLC. The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The obtained solid was combined with the one obtained by filtration to yield a colorless solid (630 mg, 3.00 mmol, 80%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 11.36 (s, 1 H, NH), 8.16 (d,

$^3J_{H,H}$ = 8.0 Hz, 1H, H-6), 6.51 (dd, $^3J_{H,H}$ = 5.2 Hz, $^3J_{H,H}$ = 5.2 Hz, 1H, H-1′), 5.71 (d, $^3J_{H,H}$ = 8.0 Hz, 1H, H-5), 5.50–5.46 (m, 1H, H-3′), 4.91 (ddd, $^3J_{H,H}$ = 4.0 Hz, $^3J_{H,H}$ = 4.0 Hz, $^3J_{H,H}$ = 2.2 Hz, 1H, H-4′), 4.68 (dd, $^2J_{H,H}$ = 8.1 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, H-5′a), 4.01 (dd, $^2J_{H,H}$ = 8.1 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, H-5′b), 2.49–2.45 ppm (m, 2H, H-2′); ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 163.2 (C-4), 151.2 (C-2), 141.2 (C-6), 102.2 (C-5), 88.5 (C-1′), 86.9 (C-3′), 80.1 (C-4′), 75.2 (C-5′), 37.2 ppm (C-2′); IR: $\tilde{\nu}$ = 3039, 1714, 1682, 1461, 1269, 1087, 802, 549, 525 cm^{-1}; mp: 183 °C; TLC: R_f = 0.57 (CH_2Cl_2/MeOH 9:1 *v/v*); HRMS (ESI$^+$): *m/z* calcd: 233.0533 [M + Na]$^+$, found: 233.0549.

2′,3′-Dideoxy-2′,3′-didehydrouridine 3. The reaction was carried out under nitrogen and with dry solvents and reagents. To a solution of 3′,5′-anhydro-2′-deoxyuridine **7** (630 mg, 3.00 mmol) in 6 mL DMSO KO*t*Bu (537 mg, 4.80 mmol, 1.6 equiv) was added in portions. The solution was stirred at room temperature for 70 h and the solvent was removed under reduced pressure. The residue was dissolved in H_2O, neutralized by addition of 1 M HCl and freeze-dried. The crude product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 9:1 *v/v*) to yield a colorless solid (441 mg, 2.10 mmol, 70%). The analytical data were identical to those reported earlier.[15]

2′,3′-Dideoxyuridine 4. The reaction was carried out with dry solvents and reagents. 2′,3′-Dideoxy-2′,3′-didehydrouridine **3** (1.97 g, 9.37 mmol) was dissolved in MeOH (80 mL) and palladium on carbon (10% Pd, 100 mg) was added. The solution was stirred under hydrogen atmosphere at room temperature for 70 h, filtered and the solvent was removed under reduced pressure. The product was obtained as colorless solid (1.70 g, 8.01 mmol, 85%). The analytical data were identical to those reported earlier.[15]

3-Methyl-*cyclo*Sal-2′,3′-dideoxy-2′,3′-didehydrouridine monophosphate 24. General procedure 1 with 2′,3′-dideoxy-2′,3′-didehydrouridine **3** (200 mg, 952 µmol) dissolved in CH_3CN (25 mL), *N,N*-diisopropylethylamine (0.30 mL, 0.22 g, 2.2 mmol, 2.3 equiv) and 3-methylsaligenyl chlorophosphite (474 mg, 2.34 mmol, 2.5 equiv) dissolved in CH_3CN (8 mL) were stirred for 2.5 h. *tert*-butylhydroperoxide (5.5 M in *n*-decane, 500 µL, 2.73 mmol, 2.9 equiv) was added and the solution was stirred for 2 h. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 0–5%). The product was obtained as a colorless foam as a mixture of two diastereomers (1:1) (65 mg, 0.17 mmol, 18%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 11.33 (s, 2H, 2×NH), 7.30–7.20 (m, 4H, 2×H-6″, 2×H-6), 7.13–7.06 (m, 4H, 2×H-4″, 2×H-5″), 6.80–6.76 (m, 2H, 2×H-1′), 6.43 (ddd, $^3J_{H,H}$ = 6.1 Hz, $^3J_{H,H}$ = 1.6 Hz, $^4J_{H,H}$ = 1.6 Hz, 1H, 1×H-3′), 6.39 (ddd, $^3J_{H,H}$ = 6.1 Hz, $^3J_{H,H}$ = 1.6 Hz, $^3J_{H,H}$ = 1.6 Hz, 1H, 1×H-3′), 6.04–5.97 (m, 2H, 2×H-2′), 5.51–5.32 (m, 4H, 4×H-7″), 5.29 (dd, $^3J_{H,H}$ = 8.1 Hz, $^4J_{H,H}$ = 2.3 Hz, 1H, 1×H-5), 5.15 (dd, $^3J_{H,H}$ = 8.1 Hz, $^4J_{H,H}$ = 2.1 Hz, 1H, 1×H-5), 5.00–4.92 (m, 2H, 2×H-4′), 4.36–4.23 (m, 4H, 4×H-5′), 2.21 (s, 3H, 1×CH_3), 2.20 ppm (s, 3H, 1×CH_3); ^{13}C NMR (101 MHz, [D$_6$]DMSO): δ = 163.0 (1×C-4), 162.9 (1×C-4), 150.7 (1×C-2), 150.6 (1×C-2), 140.1 (1×C-6), 140.0 (1×C-6), 133.1 (1×C-3′), 133.1 (1×C-3′), 131.0 (2×C-6″), 127.0 (2×C-2′), 126.9 (2×C-2″), 124.0 (2×C-5″), 123.6 (2×C-4″), 121.0 (2×C-1″), 101.7 (1×C-5), 101.6 (1×C-5), 89.4 (1×C-1′), 89.3 (1×C-1′), 84.3 (d, $^3J_{C,P}$ = 5.5 Hz, 1×C-4′), 84.2 (d, $^3J_{C,P}$ = 5.8 Hz, 1×C-4′), 68.4–68.1 (m, 2×C-5′, 2×C-7″), 14.9 (1×CH_3), 14.8 ppm (1×CH_3); ^{31}P NMR (162 MHz, [D$_6$]DMSO): δ = −8.78, −8.97 ppm; IR: $\tilde{\nu}$ = 1685, 1459, 1376, 1287, 1243, 1188, 1086, 994, 940, 844, 720, 654, 433 cm^{-1}; TLC: R_f = 0.50 (CH_2Cl_2/MeOH 9:1 *v/v*); HRMS (ESI$^+$): *m/z* calcd: 415.0666 [M + Na]$^+$, found: 415.0630.

3-Methyl-*cyclo*Sal-2′,3′-dideoxyuridine monophosphate 25. General procedure 1 with 2′,3′-dideoxyuridine **4** (74 mg, 0.35 mmol) dissolved in CH_3CN (10 mL), *N,N*-diisopropylethylamine (0.10 mL, 72 mg, 0.71 mmol, 2.0 equiv) and 3-methylsaligenyl chlorophosphite (140 mg, 0.691 mmol, 2.0 equiv) dissolved in CH_3CN (2.2 mL) were stirred for 3 h. *tert*-butylhydroperoxide (5.5 M in *n*-decane, 190 µL, 1.00 mmol, 2.9 equiv) was added and the solution was stirred for 2.5 h. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 19:1). The product was obtained as a colorless foam as a mixture of two diastereomers (1:0.9) (27 mg, 68 µmol, 19%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 11.28 (s, 2H, 2×NH), 7.55 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, 1×H-6), 7.52 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, 1×H-6), 7.29–7.22 (m, 2H, 2×H-6″), 7.13–7.06 (m, 4H, 2×H4″, 2×H-5″), 6.00–5.92 (m, 2H, 2×H-1′), 5.54–5.35 (m, 6H, 4×H-7″, 2×H-5), 4.44–4.14 (m, 6H, 2×H-4′, 4×H-5′), 2.35–2.24 (m, 2H, 2×H-2′a), 2.22 (s, 3H, 1×CH_3), 2.20 (s, 3H, 1×CH_3), 2.07–1.86 (m, 4H, 2×H-2′b, 2×H-3′a), 1.84–1.71 ppm (m, 2H, 2×H-3′b); ^{13}C NMR (101 MHz, [D$_6$]DMSO): δ = 163.0 (2×C-4), 150.3 (2×C-2), 140.1 (1×C-6), 140.1 (1×C-6), 130.8 (1×C-6″), 130.8 (1×C-6″), 126.9 (1×C-2″), 126.8 (1×C-2″), 123.9 (2×C-5″), 123.5 (1×C-4″), 123.5 (1×C-4″), 120.9 (2×C-1″), 101.4 (1×C-5), 101.4 (1×C-5), 85.2 (1×C-1′), 85.1 (1×C-1′), 78.1 (d, $^3J_{C,P}$ = 3.8 Hz, 1×C-4′), 78.0 (d, $^3J_{C,P}$ = 3.8 Hz, 1×C-4′), 68.8 (d, $^2J_{C,P}$ = 5.2 Hz, 1×C-5′), 68.7 (d, $^2J_{C,P}$ = 5.2 Hz, 1×C-5′), 68.4 (d, $^2J_{C,P}$ = 7.4 Hz, 1×C-7″), 68.3 (d, $^2J_{C,P}$ = 7.4 Hz, 1×C-7″), 30.6 (1×C-2′), 30.5 (1×C-2′), 25.1 (1×C-3′), 25.0 (1×C-3′), 14.8 (1×CH_3), 14.8 ppm (1×CH_3); ^{31}P NMR (162 MHz, [D$_6$]DMSO): δ = −9.05, −9.21 ppm; IR: $\tilde{\nu}$ = 1680, 1464, 1377, 1265, 1188, 1090, 993, 937, 771, 720, 522, 422 cm^{-1}; TLC: R_f = 0.52 (CH_2Cl_2/MeOH 9:1 *v/v*); HRMS (ESI$^+$): *m/z* calcd: 417.0822 [M + Na]$^+$, found: 417.0718.

5-Chloro-*cyclo*Sal-2′,3′-dideoxy-2′,3′-didehydrouridine monophosphate 28. General procedure 2 with 2′,3′-dideoxy-2′,3′-didehydrouridine **3** (192 mg, 0.913 mmol) dissolved in CH_3CN (12 mL), *N,N*-diisopropylethylamine (0.25 mL, 0.19 g, 1.5 mmol, 1.6 equiv) and 5-chlorosaligenyl chlorophosphite (400 mg, 1.79 mmol, 2.0 equiv) dissolved in CH_3CN (5.0 mL) were stirred for 45 min. Oxone (562 mg, 0.914 mmol, 1.0 equiv) was added and the solution was stirred for 15 min. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 9:1 + 0.1% vol. glacial AcOH). The product was obtained as a colorless foam as a mixture of two diastereomers (1:0.9) (237 mg, 0.574 mmol, 63%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 11.35 (s, 2H, 2×NH), 7.47–7.40 (m, 4H, 2×H-6″, 2×H-4″), 7.28 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, 1×H-6), 7.23–7.16 (m, 3H, 1×H-6, 2×H-3″), 6.81–6.75 (m, 2H, H-1′), 6.43 (ddd, $^3J_{H,H}$ = 6.1 Hz, $^3J_{H,H}$ = 1.6 Hz, $^4J_{H,H}$ = 1.6 Hz, 1H, 1×H-3′), 6.39 (ddd, $^3J_{H,H}$ = 6.1 Hz, $^3J_{H,H}$ = 1.6 Hz, $^4J_{H,H}$ = 1.6 Hz, 1H, 1×H-3′), 6.05–5.97 (m, 2H, 2×H-2′), 5.55–5.31 (m, 5H, 4×H-7″, 1×H-5), 5.26 (dd, $^3J_{H,H}$ = 8.1 Hz, $^4J_{H,H}$ = 2.0 Hz, 1H, 1×H-5), 5.00–4.94 (m, 2H, 2×H-4′), 4.38–4.25 ppm (m, 4H, 4×H-5′); ^{13}C NMR (101 MHz, [D$_6$]DMSO): δ = 163.0 (1×C-4), 163.0 (1×C-4), 150.7 (1×C-2), 150.7 (1×C-2), 148.5 (2×C-1″), 140.2 (1×C-6), 140.1 (1×C-6), 133.1 (2×C-3′), 129.7 (1×C-4″), 129.6 (1×C-4″), 128.4 (1×C-5″), 128.3 (1×C-5″), 127.1 (2×C-2′), 126.1 (1×C-6″), 126.0 (1×C-6″), 123.0 (1×C-2″), 122.9 (1×C-2″), 120.2 (d, $^3J_{C,P}$ = 2.2 Hz, 1×C-3″), 120.1 (d, $^3J_{C,P}$ = 2.2 Hz, 1×C-3″), 101.8 (1×C-5), 101.7 (1×C-5), 89.4 (2×C-1′), 84.3 (d, $^3J_{C,P}$ = 7.7 Hz, 1×C-4′), 84.2 (d, $^3J_{C,P}$ = 7.7 Hz, 1×C-4′), 68.4–68.2 (m, 2×C-5′), 68.0–67.8 ppm (m, 2×C-7″); ^{31}P NMR (162 MHz, [D$_6$]DMSO): δ = −10.05, −10.08 ppm; IR: $\tilde{\nu}$ = 1685, 1481, 1244, 1187, 1086, 1027, 995, 941, 816, 440 cm^{-1}; TLC: R_f = 0.38 (CH_2Cl_2/MeOH 9:1 *v/v*); HRMS (ESI$^+$): *m/z* calcd: 435.0119 [M + Na]$^+$, found: 435.0124.

5-Chloro-*cyclo*Sal-2′,3′-dideoxyuridine monophosphate 29. General procedure 2 with 2′,3′-dideoxyuridine **4** (195 mg, 0.918 mmol) dissolved in CH_3CN (12 mL), *N,N*-diisopropylethylamine (0.25 mL, 0.19 g, 1.5 mmol, 1.6 equiv) and 5-chlorosaligenyl chlorophosphite (400 mg, 1.79 mmol, 2.0 equiv) dissolved in CH_3CN (5.0 mL) were stirred for 45 min. Oxone (560 mg, 0.911 mmol, 1.0 equiv) was added and the solution was stirred for 15 min. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 9:1 + 0.1% vol. glacial AcOH). The product was obtained as a colorless foam as a mixture of two diastereomers (1:0.9) (245 mg, 0.591 mmol, 64%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=11.28$ (s, 2H, 2×NH), 7.56 (d, $^3J_{H,H}=8.1$ Hz, 1H, 1×H-6), 7.52 (d, $^3J_{H,H}=8.1$ Hz, 1H, 1×H-6), 7.47–7.39 (m, 4H, 2×H-6′′, 2×H-4′′), 7.23–7.16 (m, 2H, 2×H-3′′), 6.00–5.93 (m, 2H, 2×H-1′), 5.56–5.34 (m, 6H, 4×H-7′′, 2×H-5), 4.42–4.24 (m, 4H, 4×H-5′), 4.22–4.15 (m, 2H, 2×H-4′), 2.34–2.22 (m, 2H, 2×H-2′a), 2.05–1.88 (m, 4H, 2×H-2′b, 2×H-3′a), 1.84–1.71 ppm (2×H-3′b); ^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta=163.1$ (1×C-4), 163.0 (1×C-4), 150.3 (2×C-2), 148.2 (2×C-1′′), 140.2 (1×C-6), 140.2 (1×C-6), 129.6 (2×C-4′′), 128.3 (2×C-5′′), 126.0 (2×C-6′′), 122.9 (1×C-2′′), 122.8 (1×C-2′′), 120.2 (2×C-3′′), 101.5 (1×C-5), 101.5 (1×C-5), 85.2 (1×C-1′), 85.2 (1×C-1′), 78.1 (d, $^3J_{C,P}=6.7$ Hz, 1×C-4′), 78.0 (d, $^3J_{C,P}=6.7$ Hz, 1×C-4′), 69.0 (d, $^2J_{C,P}=5.9$ Hz, 1×C-5′), 68.9 (d, $^2J_{C,P}=5.9$ Hz, 1×C-5′), 68.0 (d, $^2J_{C,P}=7.5$ Hz, 1×C-7′′), 67.9 (d, $^2J_{C,P}=7.5$ Hz, 1×C-7′′), 30.6 (1×C-2′), 30.5 (1×C-2′), 25.1 (1×C-3′), 25.1 ppm (1×C-3′); ^{31}P NMR (162 MHz, [D$_6$]DMSO): $\delta=-10.20$, -10.31 ppm; IR: $\tilde{\nu}=1680$, 1481, 1460, 1263, 1186, 1028, 994, 939, 865, 810, 718, 435 cm^{-1}; TLC: $R_f=0.38$ (CH_2Cl_2/MeOH 9:1 *v/v*); HRMS (ESI$^+$): *m/z* calcd: 437.0270 [$M+Na$]$^+$, found: 437.0274.

2′,3′-Dideoxy-2′,3′-didehydrouridine-5′-monophosphate (d4UMP, tetra-*n*-butylammonium salt) 8. General procedure 3 with 2′,3′-dideoxy-2′,3′-didehydrouridine **3** (245 mg, 1.17 mmol), $POCl_3$ (0.48 mL, 0.81 g, 5.3 mmol, 4.4 equiv), pyridine (0.42 mL, 0.41 g, 5.2 mmol, 4.4 equiv), H_2O (48 µL, 48 µg, 2.6 mmol, 2.2 equiv) in 11.5 mL CH_3CN. The product was obtained as a hygroscopic, colorless solid (754 mg, 975 µmol, 83%). ^{1}H NMR (400 MHz, D_2O): $\delta=7.88$ (d, $^3J_{H,H}=8.1$ Hz, 1H, H-6), 6.99–6.96 (m, 1H, H-1′), 6.51 (ddd, $^3J_{H,H}=6.2$ Hz, $^3J_{H,H}=1.9$ Hz, $^4J_{H,H}=1.6$ Hz, 1H, H-3′), 5.95 (ddd, $^3J_{H,H}=6.2$ Hz, $^3J_{H,H}=2.2$ Hz, $^3J_{H,H}=1.5$ Hz, 1H, H-2′), 5.89 (d, $^3J_{H,H}=8.1$ Hz, 1H,H-5), 5.13–5.08 (m, 1H, H-4′), 4.01 (dd, $^2J_{H,H}=5.5$ Hz, $^3J_{H,H}=3.3$ Hz, 2H, H-5′), 3.26–3.17 (m, 16H, H-a), 1.74–1.61 (m, 16H, H-b), 1.38 (tq, $^3J_{H,H}=7.3$ Hz, $^3J_{H,H}=7.3$ Hz, 16H, H-c), 0.96 ppm (t, $^3J_{H,H}=7.4$ Hz, 24H, H-d); ^{13}C NMR (101 MHz, D_2O): $\delta=166.4$ (C-4), 152.1 (C-2), 143.0 (C-6), 134.6 (C-3′), 125.0 (C-2′), 102.1 (C-5), 90.1 (C-1′), 86.1 (d, $^3J_{C,P}=8.5$ Hz, C-4′), 65.3 (d, $^2J_{C,P}=5.5$ Hz, C-5′), 58.1, (C-a), 23.1 (C-b), 19.1 (C-c), 12.8 ppm (C-d); ^{31}P NMR (162 MHz, D_2O): $\delta=0.29$ ppm; IR: $\tilde{\nu}=2959$, 2873, 1684, 1458, 1380, 1246, 1175, 1102, 1045, 886, 740, 527, 424 cm^{-1}; TLC: $R_f=0.57$ (*i*PrOH/NH$_4$OAc (1 M) 2:1 *v/v*); HRMS (ESI$^-$): *m/z* calcd: 289.0231 [$M-H$]$^-$, found: 289.0233.

2′,3′-Dideoxyuridine-5′-monophosphate (ddUMP, tetra-*n*-butylammonium salt) 9. General procedure 3 with 2′,3′-dideoxyuridine **4** (130 mg, 613 µmol), $POCl_3$ (0.25 mL, 0.42 g, 2.8 mmol, 4.4 equiv), pyridine (0.22 mL, 0.21 g, 2.7 mmol, 4.4 equiv), H_2O (25 µL, 25 µg, 1.4 mmol, 2.2 equiv) in 6 mL CH_3CN. The product was obtained as a hygroscopic, light yellow solid (380 mg, 490 µmol, 80%). ^{1}H NMR (400 MHz, D_2O): $\delta=8.09$ (d, $^3J_{H,H}=8.1$ Hz, 1H, H-6), 6.12 (dd, $^3J_{H,H}=6.7$ Hz, $^3J_{H,H}=3.5$ Hz, 1H, H-1′), 5.92 (d, $^3J_{H,H}=8.1$ Hz, 1H, H-5), 4.41–4.33 (m, 1H, H-4′), 4.10 (ddd, $^2J_{H,H}=11.6$ Hz, $^3J_{H,H}=4.9$ Hz, $^3J_{H,P}=2.9$ Hz, 1H, H-5′), 3.97–3.90 (m, 1H, H-5′), 3.26–3.16 (m, 16H, H-a), 2.51–2.40 (m, 1H, H-2′a), 2.22–2.07 (m, 2H, H-2′b, H-3′a), 2.05–1.91 (m, 1H, H-3′b), 1.73–1.60 (m, 16H, H-b), 1.37 (tq, $^3J_{H,H}=7.5$ Hz, $^3J_{H,H}=7.5$ Hz, 16H, H-c), 0.96 ppm (t, $^3J_{H,H}=7.3$ Hz, 24H, H-d); ^{13}C NMR (101 MHz, D_2O): $\delta=166.4$ (C-4), 151.6 (C-2), 142.4 (C-6), 101.74 (C-5), 86.5 (C-1′), 81.0 (d, $^3J_{C,P}=8.5$ Hz, C-4′), 65.4 (d, $^2J_{C,P}=5.1$ Hz, C-5′), 58.1 (C-a), 31.6 (C-2′), 24.8 (C-3′), 23.1 (C-b), 19.1 (C-c), 12.8 ppm (C-d); ^{31}P NMR (162 MHz, D_2O): $\delta=2.33$ ppm; IR: $\tilde{\nu}=2959$, 2873, 1683, 1521, 1459, 1381, 1268, 1185, 1101, 1056, 886, 790, 521 cm^{-1}; TLC: $R_f=0.52$ (*i*PrOH/NH$_4$OAc (1 M) 2:1 *v/v*); HRMS (ESI$^-$): *m/z* calcd: 291.0388 [$M-H$]$^-$, found: 291.0451.

2′,3′-Dideoxy-2′,3′-didehydrouridine-5′-diphosphate (d4UDP, ammonium salt) 26. General procedure 4 with tetra-*n*-butylammonium phosphate (1.5×*n*Bu$_4$N$^+$; 619 mg, 1.35 mmol, 2.9 equiv) in 10 mL *N,N*-dimethylformamide and 5-chloro-*cyclo*Sal-2′,3′-dideoxy-2′,3′-didehydrouridine monophosphate **28** (194 mg, 470 µmol) in 8 mL *N,N*-dimethylformamide. The reaction mixture was stirred at room temperature for 18 h. The crude product was purified using RP-18 flash chromatography (1. H_2O; 2. H_2O/MeOH 9:1 *v/v*). The product was obtained as a hygroscopic, colorless solid (62 mg, 0.15 mmol, 32%). ^{1}H NMR (400 MHz, D_2O): $\delta=7.84$ (d, $^3J_{H,H}=8.0$ Hz, 1H, H-6), 6.99–6.95 (m, 1H, H-1′), 6.53 (d, $^3J_{H,H}=6.4$ Hz, 1H, H-3′), 5.95 (d, $^3J_{H,H}=6.0$ Hz, 1H, H-2′), 5.89 (d, $^3J_{H,H}=8.0$ Hz, 1H, H-5), 5.15–5.10 (m, 1H, H-4′), 4.18–4.08 ppm (m, 2H, H-5′); ^{13}C NMR (101 MHz, D_2O): $\delta=166.3$ (C-4), 151.8 (C-2), 143.0 (C-6), 134.6 (C-3′), 124.9 (C-2′), 102.3 (C-5), 90.1 (C-1′), 86.1 (d, $^3J_{C,P}=8.8$ Hz, C-4′), 66.0 ppm (d, $^2J_{C,P}=6.0$ Hz, C-5′); ^{31}P NMR (162 MHz, D_2O): $\delta=-9.74$ (d, $^2J_{P,P}=19.7$ Hz), -11.29 ppm (d, $^2J_{P,P}=21.5$ Hz); IR: $\tilde{\nu}=2953$, 2927, 1699, 1541, 1250, 1178, 1066, 1037, 963, 902, 833, 776, 702, 668, 515, 419 cm^{-1}; TLC: $R_f=0.17$ (*i*PrOH/NH$_4$OAc (1 M) 2:1 *v/v*); HRMS (ESI$^-$): *m/z* calcd: 368.9894 [$M-H$]$^-$, found: 368.9719.

2′,3′-Dideoxyuridine-5′-diphosphate (ddUDP, ammonium salt) 27. General procedure 4 with tetra-*n*-butylammonium phosphate (1.5×*n*Bu$_4$N$^+$; 300 mg, 653 µmol, 2.0 equiv) in 5 mL *N,N*-dimethylformamide and 5-chloro-*cyclo*Sal-2′,3′-dideoxy-2′,3′-didehydrouridine monophosphate **28** (137 mg, 330 µmol) in 5 mL *N,N*-dimethylformamide. The reaction mixture was stirred at room temperature for 16 h. The crude product was purified using RP-18 flash chromatography (H_2O). The product was obtained as a hygroscopic, colorless solid (39 mg, 92 µmol, 28%). ^{1}H NMR (500 MHz, D_2O): $\delta=8.00$ (d, $^3J_{H,H}=8.0$ Hz, 1H, H-6), 6.11 (dd, $^3J_{H,H}=6.7$ Hz, $^3J_{H,H}=3.5$ Hz, 1H, H-1′), 5.92 (d, $^3J_{H,H}=8.2$ Hz, 1H, H-5), 4.43–4.35 (m, 1H, H-4′), 4.28–4.21 (m, 1H, H-5′a), 4.11–4.05 (m, 1H, H-5′b), 2.50–2.40 (m, 1H, H-2′a), 2.21–2.08 (m, 2H, H-2′b, H-3′a), 2.05–1.94 ppm (m, 1H, H-3′b); ^{13}C NMR (101 MHz, D_2O): $\delta=166.5$ (C-4), 151.7 (C-2), 142.2 (C-6), 101.8 (C-5), 86.5 (C-1′), 80.6 (d, $^3J_{C,P}=8.8$ Hz, C-4′), 66.4 (d, $^2J_{C,P}=5.1$ Hz, C-5′), 31.3 (C-2′), 24.7 ppm (C-3′); ^{31}P NMR (162 MHz, D_2O): $\delta=-9.54$ (bs), -10.94 ppm (bs); IR: $\tilde{\nu}=2987$, 2901, 1666, 1409, 1221, 1055, 900, 809, 715, 523 cm^{-1}; TLC: $R_f=0.17$ (*i*PrOH/NH$_4$OAc (1 M) 2:1 *v/v*); HRMS (ESI$^-$): *m/z* calcd: 371.0051 [$M-H$]$^-$, found: 371.0091.

Bis(4-isobutyryloxybenzyl)-d4UDP 15. General procedure 5 with bis(tetra-*n*-butylammonium)-d4UMP **8** (107 mg, 138 µmol) in 5 mL CH_3CN, bis(4-isobutyryloxybenzyl)-*N,N*-diisopropylaminophosphoroamidite **11** (129 mg, 249 µmol, 1.8 equiv), DCI (30 mg, 0.25 mmol, 1.8 equiv). The reaction mixture was stirred at room temperature for 20 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 45 µL, 0.25 mmol, 1.8 equiv) was added. The mixture was stirred for 30 min at room temperature. The crude product was purified by a first RP-18 flash chromatography (MeOH/H_2O 1:1 *v/v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography (MeOH/H_2O 3:1 *v/v* and MeOH/H_2O 1:1 *v/v*). The product was obtained as a hygroscopic, colorless solid (33 mg, 45 µmol, 33%, 1×NH_4^+). ^{1}H NMR (400 MHz, CD_3OD): $\delta=7.82$ (d, $^3J_{H,H}=8.1$ Hz, 1H, H-6), 7.43–7.37 (m, 4H, H-2′′), 7.08–7.03 (m, 4H, H-3′′), 6.94–6.92 (m, 1H, H-1′), 6.39 (ddd, $^3J_{H,H}=6.1$ Hz, $^3J_{H,H}=1.7$ Hz, $^4J_{H,H}=1.7$ Hz, 1H, H-3′), 5.85 (ddd, $^3J_{H,H}=6.1$ Hz, $^3J_{H,H}=2.2$ Hz, $^3J_{H,H}=1.4$ Hz, 1H, H-2′), 5.75 (d, $^3J_{H,H}=8.1$ Hz, 1H, H-5), 5.11 (dd, $^2J_{H,H}=8.3$ Hz, $^3J_{H,P}=5.1$ Hz, 1H, Bn), 4.98–4.93 (m, 1H, H-4′), 4.22–4.10 (m, 2H, H-5′), 2.82 (sept, $^3J_{H,H}=7.0$ Hz, 2H, CH-*i*Pr), 1.30 ppm (d, $^3J_{H,H}=$

7.0 Hz, 12 H, CH_3-*i*Pr); ^{13}C NMR (101 MHz, CD_3OD): δ = 177.1 (2×C=O), 166.2 (C-4), 152.7 (2×C-4′′), 152.5 (C-2), 143.3 (C-6), 135.5 (C-3′), 134.9 (d, $^3J_{C,P}$ = 7.7 Hz, 2×C-1′′), 130.4 (d, $^4J_{C,P}$ = 2.9 Hz, 4×C-2′′), 127.3 (C-2′), 122.8 (4×C-3′′), 103.2 (C-5), 91.0 (C-1′), 87.0 (d, $^3J_{C,P}$ = 9.6 Hz, C-4′), 70.3 (dd, $^2J_{C,P}$ = 5.9 Hz, $^4J_{C,P}$ = 2.2 Hz, 2×Bn), 68.0 (d, $^3J_{C,P}$ = 5.8 Hz, C-5′), 35.2 (2×CH-*i*Pr), 19.2 ppm (4×CH_3-*i*Pr); ^{31}P NMR (162 MHz, CD_3OD): δ = −12.10 (d, $^2J_{P,P}$ = 20.1 Hz), −12.88 ppm (d, $^2J_{P,P}$ = 20.1 Hz); IR: $\tilde{\nu}$ = 2972, 2877, 1753, 1684, 1509, 1460, 1386, 1244, 1106, 1004, 964, 837, 764, 694, 501 cm^{-1}; TLC: R_f = 0.65 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^-$): *m*/*z* calcd: 721.1569 [*M*−H]$^-$, found: 721.1798.

Bis(4-heptanoyloxybenzyl)-d4UDP 16. General procedure 5 with bis(tetra-*n*-butylammonium)-d4UMP **8** (107 mg, 138 μmol) in 5 mL CH_3CN, bis(4-heptanoyloxybenzyl)-*N*,*N*-diisopropylaminophosphoroamidite **12** (152 mg, 253 μmol, 1.8 equiv), DCI (30 mg, 0.25 mmol, 1.8 equiv). The reaction mixture was stirred at room temperature for 20 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 45 μL, 0.25 mmol, 1.8 equiv) was added. The mixture was stirred for 30 min at room temperature. The crude product was purified by a first RP-18 flash chromatography ($MeOH/H_2O$ 1:1–5:1 *v*/*v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography ($MeOH/H_2O$ 3:1 *v*/*v*). The product was obtained as a hygroscopic, colorless solid (47 mg, 57 μmol, 41 %, 1×NH_4^+). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.82 (d, $^3J_{H,H}$ = 8.1 Hz, 1 H, H-6), 7.42–7.37 (m, 4 H, H-2′′), 7.08–7.03 (m, 4 H, H-3′′), 6.96–6.93 (m, 1 H, H-1′), 6.39 (ddd, $^3J_{H,H}$ = 6.1 Hz, $^3J_{H,H}$ = 1.7 Hz, $^4J_{H,H}$ = 1.7 Hz, 1 H, H-3′), 5.87–5.83 (m, 1 H, H-2′), 5.75 (d, $^3J_{H,H}$ = 8.1 Hz, 1 H, H-5), 5.10 (dd, $^2J_{H,H}$ = 8.2 Hz, $^3J_{H,P}$ = 6.9 Hz, 4 H, Bn), 4.97–4.93 (m, 1 H, H-4′), 4.21–4.11 (m, 2 H, H-5′), 2.57 (t, $^3J_{H,H}$ = 7.4 Hz, 4 H, H-a), 1.73 (tt, $^3J_{H,H}$ = 7.4 Hz, $^3J_{H,H}$ = 7.4 Hz, 4 H, H-b), 1.47–1.33 (m, 12 H, H-c, H-d, H-e), 0.93 ppm (t, $^3J_{H,H}$ = 7.0 Hz, 6 H, H-f); ^{13}C NMR (101 MHz, CD_3OD): δ = 173.8 (2×C=O), 166.2 (C-4), 152.7 (2×C-4′′), 152.4 (C-2), 143.3 (C-6), 135.5 (C-3′), 134.9 (d, $^3J_{C,P}$ = 6.9 Hz, 2×C-1′′), 130.5 (d, $^4J_{C,P}$ = 3.7 Hz, 4×C-2′′), 127.3 (C-2′), 122.9 (4×C-3′′), 103.3 (C-5), 91.0 (C-1′), 87.0 (d, $^3J_{C,P}$ = 8.7 Hz, C-4′), 70.3 (dd, $^2J_{C,P}$ = 5.7 Hz, $^4J_{C,P}$ = 2.8 Hz, 2×Bn), 68.0 (d, $^2J_{C,P}$ = 5.6 Hz, C-5′), 35.0, 32.6, 29.8, 25.9, 23.6 (C-a, C-b, C-c, C-d, C-e), 14.4 ppm (C-f); ^{31}P NMR (162 MHz, CD_3OD): δ = −12.11 (d, $^2J_{P,P}$ = 19.8 Hz), −12.92 ppm (d, $^2J_{P,P}$ = 21.8 Hz); IR: $\tilde{\nu}$ = 2958, 2873, 1754, 1687, 1508, 1461, 1378, 1198, 1103, 1005, 977, 763, 498 cm^{-1}; TLC: R_f = 0.29 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^-$): *m*/*z* calcd: 805.2508 [*M*−H]$^-$, found: 805.2505.

Bis(4-decanoyloxybenzyl)-d4UDP 17. General procedure 5 with bis(tetra-*n*-butylammonium)-d4UMP **8** (106 mg, 137 μmol) in 5 mL CH_3CN, bis(4-decanoyloxybenzyl)-*N*,*N*-diisopropylaminophosphoroamidite **13** (171 mg, 249 μmol, 1.8 equiv), DCI (30 mg, 0.25 mmol, 1.8 equiv). The reaction mixture was stirred at room temperature for 18 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 45 μL, 0.25 mmol, 1.8 equiv) was added. The mixture was stirred for 1 h at room temperature. The crude product was purified by a first RP-18 flash chromatography ($MeOH/H_2O$ 1:1–9:1 *v*/*v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography ($MeOH/H_2O$ 5:1 *v*/*v*). The product was obtained as a hygroscopic, colorless solid (39 mg, 43 μmol, 31 %, 1×NH_4^+). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.82 (d, $^3J_{H,H}$ = 8.1 Hz, 1 H, H-6), 7.42–7.35 (m, 4 H, H-2′′), 7.08–7.03 (m, 4 H, H-3′′), 6.96–6.93 (m, 1 H, H-1′), 6.39 (ddd, $^3J_{H,H}$ = 6.0 Hz, $^3J_{H,H}$ = 1.7 Hz, $^4J_{H,H}$ = 1.7 Hz, 1 H, H-3′), 5.88–5.83 (m, 1 H, H-2′), 5.75 (d, $^3J_{H,H}$ = 8.1 Hz, 1 H, H-5), 5.10 (dd, $^2J_{H,H}$ = 8.6 Hz, $^3J_{H,P}$ = 5.4 Hz, 4 H, Bn), 4.98–4.93 (m, 1 H, H-4′), 4.22–4.11 (m, 2 H, H-5′), 2.58 (t, $^3J_{H,H}$ = 7.4 Hz, 4 H, H-a), 1.73 (tt, $^3J_{H,H}$ = 7.5 Hz, $^3J_{H,H}$ = 7.2 Hz, 4 H, H-b), 1.47–1.25 (m, 24 H, H-c, H-d, H-e, H-f, H-g, H-h), 0.91 ppm (t, $^3J_{H,H}$ = 6.8 Hz, 6 H, H-i); ^{13}C NMR (101 MHz, CD_3OD): δ = 173.8 (2×C=O), 166.3 (C-4), 152.7 (2×C-4′′), 152.4 (C-2), 143.3 (C-6), 135.6 (C-3′), 149.9 (d, $^3J_{C,P}$ = 5.9 Hz, 2×C-1′′), 130.5 (d, $^4J_{C,P}$ = 2.9 Hz, 4x C-2′′), 127.3 (C-2′), 122.9 (4×C-3′′), 103.3 (C-5), 91.0 (C-1′), 87.0 (d, $^3J_{C,P}$ = 9.5 Hz, C-4′), 70.3 (dd, $^2J_{C,P}$ = 5.9 Hz, $^4J_{C,P}$ = 2.9 Hz, 2×Bn), 68.0 (d, $^2J_{C,P}$ = 5.9 Hz, C-5′), 35.0, 33.1, 30.6, 30.4, 30.4, 30.2, 26.0, 23.7 (C-a, C-b, C-c, C-d, C-e, C-f, C-g, C-h), 14.4 ppm (C-i); ^{31}P NMR (162 MHz, CD_3OD): δ = −12.12 (d, $^2J_{P,P}$ = 21.1 Hz), −12.93 ppm (d, $^2J_{P,P}$ = 19.6 Hz); IR: $\tilde{\nu}$ = 2921, 2851, 1754, 1687, 1509, 1464, 1379, 1248, 1105, 1005, 968, 914, 837, 764, 695, 504 cm^{-1}; TLC: R_f = 0.38 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^-$): *m*/*z* calcd: 889.3520 [*M*−H]$^-$, found: 889.3448.

Bis(4-dodecanoyloxybenzyl)-d4UDP 18. General procedure 5 with bis(tetra-*n*-butylammonium)-d4UMP **8** (100 mg, 129 μmol) in 5 mL CH_3CN, bis(4-dodecanoyloxybenzyl)-*N*,*N*-diisopropylaminophosphoroamidite **14** (190 mg, 0.256 mmol, 2.0 equiv), DCI (30 mg, 0.25 mmol, 1.8 equiv). The reaction mixture was stirred at room temperature for 24 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 45 μL, 0.25 mmol, 1.8 equiv) was added. The mixture was stirred for 1 h at room temperature. The crude product was purified by a first RP-18 flash chromatography ($MeOH/H_2O$ 1:1–9:1 *v*/*v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography ($MeOH/H_2O$ 9:1 *v*/*v*). The product was obtained as a hygroscopic, colorless solid (40 mg, 41 μmol, 32 %, 1×NH_4^+). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.82 (d, $^3J_{H,H}$ = 8.1 Hz, 1 H, H-6), 7.41–7.35 (m, 4 H, H-2′′), 7.07–7.03 (m, 4 H, H-3′′), 6.96–6.93 (m, 1 H, H-1′), 6.39 (ddd, $^3J_{H,H}$ = 6.0 Hz, $^3J_{H,H}$ = 1.7 Hz, $^4J_{H,H}$ = 1.7 Hz, 1 H, H-3′), 5.87–5.84 (m, 1 H, H-2′), 5.75 (d, $^3J_{H,H}$ = 8.1 Hz, 1 H, H-5), 5.10 (dd, $^2J_{H,H}$ = 8.5 Hz, $^3J_{H,P}$ = 5.2 Hz, 4 H, Bn), 4.98–4.93 (m, 1 H, H-4′), 4.21–4.11 (m, 2 H, H-5′), 2.57 (t, $^3J_{H,H}$ = 7.4 Hz, 4 H, H-a), 1.73 (tt, $^3J_{H,H}$ = 7.3 Hz, $^3J_{H,H}$ = 7.3 Hz, 4 H, H-b), 1.48–1.24 (m, 32 H, H-c, H-d, H-e, H-f, H-g, H-h, H-i, H-j), 0.90 ppm (t, $^3J_{H,H}$ = 7.0 Hz, 6 H, H-k); ^{13}C NMR (101 MHz, CD_3OD): δ = 173.7 (2×C=O), 166.2 (C-4), 152.7 (2×C-4′′), 152.4 (C-2), 143.3 (C-6), 135.5 (C-3′), 134.9 (d, $^3J_{C,P}$ = 6.5 Hz, 2×C-1′′), 130.4 (d, $^4J_{C,P}$ = 2.9 Hz, 4×C-2′′), 127.3 (C-2′), 122.9 (2×C-3′′), 103.3 (C-5), 91.0 (C-1′), 87.0 (d, $^3J_{C,P}$ = 8.8 Hz, C-4′), 70.3 (dd, $^2J_{C,P}$ = 5.9 Hz, $^4J_{C,P}$ = 3.0 Hz, 2×Bn), 67.9 (d, $^3J_{C,P}$ = 6.5 Hz, C-5′), 35.0, 33.1, 30.7, 30.6, 30.5, 30.4, 30.2, 26.0, 23.7 (C-a, C-b, C-c, C-d, C-e, C-f, C-g, C-h, C-i, C-j), 14.4 ppm (C-k); ^{31}P NMR (162 MHz, CD_3OD): δ = −12.10 (d, $^2J_{P,P}$ = 20.8 Hz), −12.91 ppm (d, $^2J_{P,P}$ = 19.7 Hz); IR: $\tilde{\nu}$ = 2917, 2849, 1753, 1686, 1509, 1465, 1380, 1221, 1169, 1107, 977, 917, 837, 764, 720, 506 cm^{-1}; TLC: R_f = 0.34 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^+$): *m*/*z* calcd: 947.4219 [*M*+H]$^+$, found: 947.4212.

Bis(4-isobutyryloxybenzyl)-ddUDP 19. General procedure 5 with bis(tetra-*n*-butylammonium)-ddUMP **9** (66 mg, 85 μmol) in 2.5 mL CH_3CN, bis(4-isobutyryloxybenzyl)-*N*,*N*-diisopropylaminophosphoroamidite **11** (75 mg, 0.14 mmol, 1.6 equiv), DCI (13 mg, 0.11 mmol, 1.3 equiv) and after 1 h stirring at room temperature additional bis(4-isobutyryloxybenzyl)-*N*,*N*-diisopropylaminophosphoramidite **11** (75 mg, 0.14 mmol, 1.6 equiv) and DCI (20 mg, 0.17 mmol, 2.0 equiv). The reaction mixture was stirred at room temperature for 20 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 48 μL, 0.27 mmol, 3.2 equiv) was added. The mixture was stirred for 45 min at room temperature. The crude product was purified by a first RP-18 flash chromatography ($MeOH/H_2O$ 1:1–5:1 *v*/*v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography ($MeOH/H_2O$ 2:1 *v*/*v*). The product was obtained as a hygroscopic, colorless solid (19 mg, 26 μmol, 31 %, 1×NH_4^+). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.97 (d, $^3J_{H,H}$ = 8.4 Hz, 1 H, H-6), 7.45–7.37 (m, 4H, H-2′′), 7.09–7.02 (m, 4H, H-3′′), 6.05–5.99 (m, 1H, H-1′), 5.72 (d, $^3J_{H,H}$ = 8.4 Hz, 1H, H-5), 5.13 (d, $^3J_{H,H}$ = 8.3 Hz, 4H, Bn), 4.27–4.19 (m, 2H, H-5′), 4.11–4.04 (m, 1H, H-4′), 2.81 (sept, $^3J_{H,H}$ = 7.0 Hz, 2H, CH-*i*Pr), 2.38–2.27 (m, 1H, H-2′a), 2.07–1.91 (m, 3H, H-2′b, H-3′), 1.30 ppm (d, $^3J_{H,H}$ = 7.0 Hz, 12H, CH_3-*i*Pr); ^{13}C NMR (101 MHz, CD_3OD): δ = 163.4 (C-4), 152.5 (2×C-4′′), 142.6 (C-6), 134.9 (d, $^3J_{C,P}$ = 6.8 Hz, 2×C-1′′), 130.5 (2×C-2′′), 122.8 (2×C-3′′), 102.5 (C-5), 87.6 (C-1′), 81.2 (d, $^3J_{C,P}$ = 9.4 Hz, C-4′), 70.3 (d, $^2J_{C,P}$ = 5.9 Hz, 2×Bn), 68.3 (d, $^2J_{C,P}$ = 6.2 Hz, C-5′), 35.3 (2×CH-*i*Pr), 33.3 (C-2′), 26.1 (C-3′), 19.2 ppm (4×CH_3-*i*Pr); ^{31}P NMR (162 MHz, CD_3OD): δ = −11.66 (d, $^2J_{P,P}$ = 19.7 Hz), −12.83 ppm (d, $^2J_{P,P}$ = 19.8 Hz); IR: $\tilde{\nu}$ = 2974, 2878, 1753, 1674, 1509, 1461, 1261, 1202, 1166, 1005, 956, 803, 498 cm^{-1}; TLC: R_f = 0.69 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^-$): *m*/*z* calcd: 723.1726 [*M*−H]$^-$, found: 723.1736.

Bis(4-heptanoyloxybenzyl)-ddUDP 20. General procedure 5 with bis(tetra-*n*-butylammonium)-ddUMP **9** (130 mg, 168 µmol) in 5 mL CH_3CN, bis(4-heptanoyloxybenzyl)-*N*,*N*-diisopropylaminophosphoroamidite **12** (170 mg, 0.283 mmol, 1.7 equiv), DCI (36 mg, 0.30 mmol, 1.8 equiv). The reaction mixture was stirred at room temperature for 24 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 55 µL, 0.31 mmol, 1.8 equiv) was added. The mixture was stirred for 1 h at room temperature. The crude product was purified by a first RP-18 flash chromatography (MeOH/H_2O 1:1–5:1 *v*/*v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography (MeOH/H_2O 5:1 *v*/*v*). The product was obtained as a hygroscopic, colorless solid (48 mg, 58 µmol, 41%, 1× NH_4^+). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.96 (d, $^3J_{H,H}$ = 8.2 Hz, 1H, H-6), 7.43–7.37 (m, 4H, H-2′′), 7.08–7.01 (m, 4H, H-3′′), 6.02 (dd, $^3J_{H,H}$ = 6.8 Hz, $^3J_{H,H}$ = 3.6 Hz, 1H, H-1′), 5.73 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, H-5), 5.12 (d, $^3J_{H,H}$ = 8.4 Hz, 4H, Bn), 4.27–4.18 (m, 2H, H-5′), 4.11–4.03 (m, 1H, H-4′), 2.58 (t, $^3J_{H,H}$ = 7.4 Hz, 4H, H-a), 2.38–2.27 (m, 1H, H-2′a), 2.06–1.93 (m, 3H, H-2′b, H-3′), 1.73 (tt, $^3J_{H,H}$ = 7.5 Hz, $^3J_{H,H}$ = 7.3 Hz, 4H, H-b), 1.48–1.28 (m, 12H, H-c, H-d, H-e), 0.93 ppm (t, $^3J_{H,H}$ = 6.8 Hz, 6H, H-f); ^{13}C NMR (101 MHz, CD_3OD): δ = 173.7 (2×C=O), 166.3 (C-4), 152.4 (2×C-4′′), 152.3 (C-2), 142.6 (C-6), 134.9 (d, $^3J_{C,P}$ = 7.4 Hz, C-1′′), 130.5 (4×C-2′′), 122.9 (4×C-3′′), 102.5 (C-5), 87.5 (C-1′), 81.2 (d, $^3J_{C,P}$ = 9.2 Hz, C-4′), 70.3 (d, $^3J_{C,P}$ = 5.5 Hz, 2×Bn), 68.3 (d, $^2J_{C,P}$ = 6.5 Hz, C-5′), 35.0 (C-a), 33.3 (C-2′), 32.6, 29.8 (C-b, C-c), 26.1 (C-3′), 25.9, 23.6 (C-d, C-e), 14.4 ppm (C-f); ^{31}P NMR (162 MHz, CD_3OD): δ = −11.73 (d, $^2J_{P,P}$ = 17.7 Hz), −12.86 ppm (d, $^2J_{P,P}$ = 20.1 Hz); IR: $\tilde{\nu}$ = 2955, 2929, 2858, 1755, 1678, 1509, 1462, 1263, 1198, 1102, 1025, 960, 810, 557 cm^{-1}; TLC: R_f = 0.75 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^-$): *m*/*z* calcd: 807.2737 [*M*−H]$^-$, found: 807.2652.

Bis(4-decanoyloxybenzyl)-ddUDP 21. General procedure 5 with bis(tetra-*n*-butylammonium)-ddUMP **9** (86 mg, 110 µmol) in 5 mL CH_3CN, bis(4-decanoyloxybenzyl)-*N*,*N*-diisopropylaminophosphoroamidite **13** (135 mg, 197 µmol, 1.8 equiv), DCI (25 mg, 0.21 mmol, 1.9 equiv). The reaction mixture was stirred at room temperature for 20 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 36 µL, 0.20 mmol, 1.8 equiv) was added. The mixture was stirred for 30 min at room temperature. The crude product was purified by a first RP-18 flash chromatography (MeOH/H_2O 1:1–9:1 *v*/*v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography (MeOH/H_2O 5:1 *v*/*v*). The product was obtained as a colorless oil (42 mg, 42 µmol, 38%, 0.4×*n*Bu_4N^+, 0.6×NH_4^+). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.97 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, H-6), 7.43–7.37 (m, 4H, H-2′′), 7.07–7.03 (m, 4H, H-3′′), 6.02 (dd, $^3J_{H,H}$ = 6.4 Hz, $^3J_{H,H}$ = 3.6 Hz, 1H, H-1′), 5.73 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, H-5), 5.12 (d, $^2J_{H,H}$ = 8.2 Hz, 4H, Bn), 4.27–4.19 (m, 2H, H-5′), 4.11–4.04 (m, 1H, H-4′), 3.26–3.19 (m, 3H, H-A), 2.57 (t, $^3J_{H,H}$ = 7.4 Hz, 4H, H-a), 2.37–2.25 (m, 1H, H-2′a), 2.07–1.93 (m, 3H, H-2′b, H-3′), 1.78–1.61 (m, 7H, H-b, H-B), 1.47–1.24 (m, 27H, H-c, H-d, H-e, H-f, H-g, H-h, H-C), 1.03 (t, $^3J_{H,H}$ = 7.4 Hz, 5H, H-D), 0.91 ppm (t, $^3J_{H,H}$ = 6.9 Hz, 6H, H-i); ^{13}C NMR (126 MHz, CD_3OD): δ = 173.8 (2×C=O), 152.4 (2×C-4′′), 142.6 (C-6), 134.8 (d, $^3J_{C,P}$ = 7.4 Hz, 2×C-1′′), 130.5 (2×C-2′′), 122.9 (2×C-3′′), 102.5 (C-5), 87.5 (C-1′), 81.2 (d, $^3J_{C,P}$ = 9.2 Hz, C-4′), 70.35 (d, $^2J_{C,P}$ = 9.2 Hz, 2×Bn), 68.3 (d, $^3J_{C,P}$ = 6.0 Hz, C-5′), 59.5 (C-A), 35.0 (C-a), 33.3 (C-2′), 33.0, 30.6, 30.4, 30.4, 30.2 (C-b, C-c, C-d, C-e, C-f), 26.1 (C-3′), 26.0, 24.7 (C-g, C-h), 23.7 (C-B), 20.7 (C-C), 14.4 (C-i), 13.9 ppm (C-D); ^{31}P NMR (162 MHz, CD_3OD): δ = −11.67 (d, $^2J_{P,P}$ = 20.3 Hz), −12.86 ppm (d, $^2J_{P,P}$ = 20.9 Hz); IR: $\tilde{\nu}$ = 2921, 2851, 1755, 1686, 1509, 1464, 1380, 1264, 1200, 1106, 964, 813, 510 cm^{-1}; TLC: R_f = 0.29 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^-$): *m*/*z* calcd: 891.3676 [*M*−H]$^-$, found: 891.3592.

Bis(4-dodecanoyloxybenzyl)-ddUDP 22. General procedure 5 with bis(tetra-*n*-butylammonium)-ddUMP **9** (91 mg, 110 µmol) in 5 mL CH_3CN, bis(4-dodecanoyloxybenzyl)-*N*,*N*-diisopropylaminophosphoroamidite **14** (160 mg, 216 µmol, 2.0 equiv), DCI (25 mg, 0.21 mmol, 1.9 equiv). The reaction mixture was stirred at room temperature for 22 h, cooled to −20 °C and *tert*-butylhydroperoxide (5.5 M in *n*-decane, 40 µL, 0.22 mmol, 2.0 equiv) was added. The mixture was stirred for 30 min at room temperature. The crude product was purified by a first RP-18 flash chromatography (MeOH/H_2O 1:1–10:1 *v*/*v*), counter-ions were changed to ammonium (Dowex 50WX8, NH_4^+ form) and the product was further purified by RP-18 flash chromatography (MeOH/H_2O 9:1 *v*/*v*). The product was obtained as a colorless oil (37 mg, 34 µmol, 31%, 0.6× *n*Bu_4N^+, 0.4×NH_4^+). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.97 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, H-6), 7.42–7.37 (m, 4H, H-2′′), 7.07–7.02 (m, 4H, H-3′′), 6.04–6.00 (m, 1H, H-1′), 5.73 (d, $^3J_{H,H}$ = 8.1 Hz, 1H, H-5), 5.12 (d, $^3J_{H,H}$ = 8.6 Hz, 4H, Bn), 4.27–4.19 (m, 2H, H-5′), 4.11–4.04 (m, 1H, H-4′), 3.26–3.20 (m, 5H, H-A), 2.57 (t, $^3J_{H,H}$ = 7.5 Hz, 4H, H-a), 2.37–2.27 (m, 1H, H-2′a), 2.07–1.94 (m, 3H, H-2′b, H-3′), 1.73 (tt, $^3J_{H,H}$ = 7.4 Hz, $^3J_{H,H}$ = 7.4 Hz, 4H, H-b), 1.70–1.61 (m, 5H, H-B), 1.47–1.24 (m, 37H, H-c, H-d, H-e, H-f, H-g, H-h, H-i, H-j, H-C), 1.03 (t, $^3J_{H,H}$ = 7.3 Hz, 7H, H-D), 0.90 ppm (t, $^3J_{H,H}$ = 6.9 Hz, 6H, H-k); ^{13}C NMR (126 MHz, CD_3OD): δ = 173.7 (2×C=O), 167.8 (C-4), 152.4 (2×C-4′′), 152.3 (C-2), 142.6 (C-6), 134.9 (d, $^3J_{C,P}$ = 6.5 Hz, 2×C-1′′), 130.5 (4×C-2′′), 122.9 (4×C-3′′), 102.5 (C-5), 87.5 (C-1′), 81.2 (d, $^3J_{C,P}$ = 9.1 Hz, C-4′), 70.3 (d, $^2J_{C,P}$ = 6.4 Hz, 2×Bn), 68.3 (d, $^2J_{C,P}$ = 5.6 Hz, C-5′), 59.5 (C-A), 35.0 (C-a), 33.3 (C-2′), 33.1, 30.7, 30.6, 30.5, 30.4, 30.2 (C-b, C-c, C-d, C-e, C-f, C-g, C-h), 26.1 (C-3′), 26.0, 24.8 (C-i, C-j), 23.7 (C-B), 20.7 (C-C), 14.4 (C-k), 13.9 ppm (C-D); ^{31}P NMR (162 MHz, CD_3OD): δ = −13.18 (d, $^2J_{P,P}$ = 19.8 Hz), −14.35 ppm (d, $^2J_{P,P}$ = 22.2 Hz); IR: $\tilde{\nu}$ = 2956, 2916, 2849, 1752, 1685, 1510, 1465, 1382, 1222, 1004, 968, 816, 720, 513 cm^{-1}; TLC: R_f = 0.19 (EE/MeOH 7:3 *v*/*v*); HRMS (ESI$^-$): *m*/*z* calcd: 947.4230 [*M*−H]$^-$, found: 947.4225.

Chemical hydrolysis of *cyclo*Sal and Di*PP*ro compounds 15–22, 24, and 25. Stock solutions (50 mM in [D$_6$]DMSO) of the compounds were prepared. For the hydrolysis solution, 11 µL of the stock solution were added to 189 µL [D$_6$]DMSO and 100 µL Millipore H_2O. In case of the *cyclo*Sal compounds **24** and **25**, 5 µL of an AZT **2** solution (37 mM in Millipore H_2O) were added as internal standard. The hydrolysis was started by the addition of 300 µL of phosphate-buffered saline (PBS, 50 mM, pH 7.3). This solution was incubated at 37 °C in a Thermomixer. An initial aliquot (30 µL) was taken directly after addition of the buffer and further aliquots (30 µL) were taken to monitor the hydrolysis. Of each aliquot 20 µL were directly analyzed by analytical RP-HPLC (method A for Di*PP*ro compounds, method B for *cyclo*Sal compounds; 260 nm). Calculation of exponential decay curves with commercially available software (Microsoft Excel) yielded the half-life ($t^1/_2$) of the prodrug compounds in hours. For each compound, two determinations of $t^1/_2$ were performed, and the resulting values were averaged.

Bioassays

Hydrolysis of *cyclo*Sal and Di*PP*ro compounds 15–22, 24 and 25 in CD_4^+ T-lymphocyte CEM cell extracts: Stock solutions (4.3 mM in [D_6]DMSO) of the compounds were prepared. For the hydrolysis solution, 10 μL of the stock solution were added to 50 μL of the cell extract and 10 μL of magnesium chloride solution (70 mM in Millipore H_2O). For every reading a separate solution was prepared and incubated at 37 °C in a Thermomixer for various periods (depending on the rate of hydrolysis). The reactions were stopped by the addition of 200 μL MeOH, the samples were stored on ice for 10 min followed by ultrasonication for 10 min. The samples were centrifuged at 0 °C (14 000 rpm) for 10 min, the supernatant was filtered using a syringe filter (Schleicher & Schuell Spartan 13/30, 0.2 mm) and stored under liquid nitrogen. For analysis, the samples were defrosted and aliquots of 90 μL were analyzed by analytical RP-HPLC (method A for Di*PP*ro compounds, method B for *cyclo*Sal compounds; 260 nm). Calculation of exponential decay curves with commercially available software (Microsoft Excel) yielded the half-life ($t^1/_2$) of the prodrug compounds in hours. For each compound, two determinations of $t^1/_2$ were performed, and the resulting values were averaged.

Preparation of CEM cell extracts: Human CD_4^+ T-lymphocyte CEM cells were grown in RPMI-1640-based cell culture medium to a final density of $\sim 3\times10^6$ cells mL^{-1}. Then, cells were centrifuged for 10 min at 1250 rpm, 4 °C, washed twice with cold PBS, and the pellet was resuspended at 10^8 cells mL^{-1} and sonicated (Hielsecher Ultrasound Techn., 100 % amplitude, 3×10 s) to destroy cell integrity. The resulting cell suspension was then centrifuged at 10 000 rpm to remove cell debris, and the supernatant divided in aliquots before being frozen at −80 °C and used.

Anti-HIV activity assays: Inhibition of HIV-1(III_B)- and HIV-2(ROD)-induced cytopathicity in CEM cell cultures was measured in microtiter 96-well plates containing $\sim 3\times10^5$ CEM cells mL^{-1} infected with 100 $CCID_{50}$ of HIV per milliliter and containing appropriate dilutions of the test compounds. After 4–5 days of incubation at 37 °C in a CO_2-controlled humidified atmosphere, CEM giant (syncytium) cell formation was examined microscopically. The EC_{50} (50 % effective concentration) was defined as the compound concentration required to inhibit HIV-induced giant cell formation by 50 %.

NDP kinase assays: Nucleoside diphosphate kinase was derived and purified from bovine liver (Sigma–Aldrich, St. Louis, MO, USA). The reaction mixture contained 200 μM ATP in PBS, pH 7.2, 1 mM $MgCl_2$ (Sigma–Aldrich) and 100 μM UDP, ddUDP or d4UDP. The reaction was started by adding the enzyme (134 mU), and the reaction was incubated at 37 °C for 0, 10, 20, 30 or 60 min. At the end of the incubation period, 100 μL aliquots were added to 200 μL ice-cold MeOH, kept on ice for 10 min, centrifuged for 10 min at 4 °C, 13 000 rpm (5415R Eppendorf centrifuge) to remove protein and the cleared supernatants were subjected to anion-exchange HPLC analysis (Partisphere-SAX, Whatman Int., Maidstone, UK). The gradient used consisted of 5 mM $NH_4H_2PO_4$ pH 5.0 (B) and 0.3 M $NH_4H_2PO_4$ pH 5.0 (C) and was run as follows: 5 min 100 % B, 15 min from 100 % B to 100 % C, 20 min 100 % C, 5 min from 100 % C to 100 % B, 5 min 100 % B (equilibration); t_R UDP: 13.0 min, ddUDP: 14.0 min, d4UDP: 14.4 min; t_R UTP: 20.9 min.

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Keywords: antiviral agents · bioreversible protection · drug delivery · HIV · nucleosides · prodrugs

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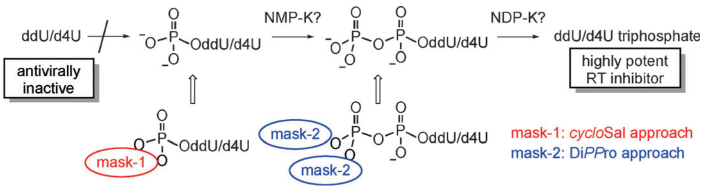
Nucleoside Mono- and Diphosphate Prodrugs of 2′,3′-Dideoxyuridine and 2′,3′-Dideoxy-2′,3′-didehydrouridine



Chemical Trojan horses: Can they rescue antiviral activity? Nucleoside mono- and diphosphate prodrugs of d4U and ddU were prepared by applying the *cyclo*Sal and Di*PP*ro approaches. These compounds underwent successful delivery, but surprisingly showed weak or no antiviral activity. Phosphorylation studies with nucleoside diphosphate kinase showed that this enzyme is practically unable to convert ddUDP and d4DUP to the triphosphate form.