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# Synthesis and Properties of Fluorescent *cyclo*Sal Nucleotides Based on the Pyrimidine Nucleoside $m^5K$ and Its 2′,3′-Dideoxy Analog $dm^5K$

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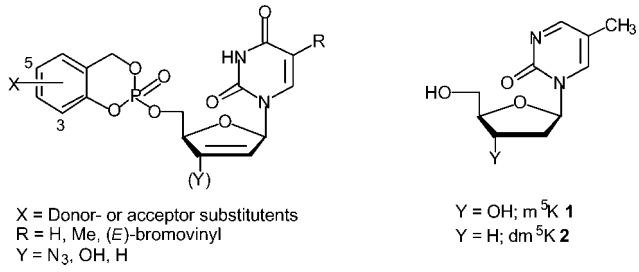
The synthesis of the fluorescent thymidine nucleoside analog 5-methyl-pyrimidin-2-one nucleoside **1** ($m^5K$) and that of its 2′,3′-dideoxy derivative **2** ($dm^5K$) are described. Moreover, the conversion of **1** and **2** into the corresponding 3-methyl-*cyclo*Sal-phosphate triesters **11** and **12** or an enzyme-cleavable prodrug 5-acetoxymethylpropionate-*cyclo*Sal-phosphate triester **13**, and the fluorescence and hydrolysis properties of these new lipophilic triesters are reported. Finally, the suitability of these *cyclo*Sal pronucleotides as probes is demonstrated in a cell-extract hydrolysis experiment and a model study for cell uptake.



## Introduction

Nucleoside analogs are widely used as antiviral or antitumor agents.[1] A severe limitation of these compounds is that they often needed to be 5′-phosphorylated by host cell enzymes into the 5′-triphosphates. The triphosphates act as competitive inhibitors of DNA-polymerases, like the reverse transcriptase of HIV, or they cause chain termination in DNA-synthesis after incorporation.[2] Often, the initial phosphorylation into the nucleoside monophosphate (nucleotide) is the metabolic limiting step. In some cases, the nucleoside analog is entirely inactive in the nucleoside form, but it is found to be highly active as the nucleoside triphosphate (e.g., 2′,3′-dideoxyuridine). However, applying nucleotides in chemotherapy has no benefit because nucleotides are unable to penetrate the cell membrane and are substrates of unspecific blood phosphatases. In order to overcome this hurdle, lipophilic precursors have been developed that release the nucleotides after membrane passage.[3,4] In this context, we developed the *cyclo*Sal-pronucleotide approach.[5] It was shown with several antiviral active nucleoside analogs that the use of their corresponding *cyclo*Sal-phosphate triesters led to improved biological activity due to a successful bypass of phosphorylation limitations (Scheme 1).[6,7] Recently, the approach was extended by attaching an enzyme-cleavable group to the *cyclo*Sal moiety with the aim of trapping the compounds intracellularly.[8,9]

To study the intracellular fate of the *cyclo*Sal derivatives and the subsequent cell uptake in more detail, we were looking for a suitable probe. Radiolabeling of the *cyclo*Sal nucleotides is very expensive and experimentally demanding; fluorescence labeling may thus be the method of choice. Despite the possibility of selecting specific fluorescence properties, attachment of a high-molecular-weight fluorescent dye such as rhodamine, fluoresceine, or the dansyl group would change the size, lipophilicity, and/or hydrolysis properties of *cyclo*Sal compounds considerably. The antiviral nucleoside analogs attached to the *cyclo*Sal moiety are pyrimidine- and purine-type structures. Thus, a fluorescent analog that would be structurally as close as possible to these nucleosides was needed. For the purine series, the strong fluorescent 2-aminopurine 2′-deoxyriboside (2AP) would be appropriate. However, as a surrogate for the pyrimidine-type nucleoside analogs, for example, 2′,3′-dideoxythymidine (ddT) or 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T), the highly fluorescent 5-methylpyrimidin-2-one nucleoside **1** ($m^5K$), the deoxygenation product of thymidine, may be appropriate.[10] Chemically, this compound is a thymidine analog, while functionally, it behaves like a cytidine analog. The first synthesis of pyrimidin-2-one nucleoside was carried out by direct reduction of the corresponding thymidine or uridine derivative with sodium amalgam in low yields.[11] The approach has been modified subsequently, and $m^5K$ was successfully prepared by oxidation of 4-hydrazinopyrimidinone nucleosides and rearrangement of the intervening diazene with $Ag_2O$ in refluxing aqueous




Scheme 1.

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dioxane or EtOH.[12] Holy published a method using 4-thiopyrimidines and deactivated Raney-Ni.[13] Both methods led to $m^5K$ in about 40–50% yield, although the yields were found to be better with ribonucleosides. However, all methods use metals, which are not desirable for syntheses of compounds to be used in biological systems because of possible toxic side effects. Recently, Singleton et al. published a metal-free strategy by a mild, homogeneous oxidation with *N*-phenyltriazolinedione (PTAD).[14] However, in our hands this method led only to low yields of $m^5K$, most probably due to the labile triethylsilyl protecting groups (TES) used for the 2′-deoxyribose.

Herein, we report on a slightly modified but reliable synthetic protocol for the preparation of $m^5K$ (**1**), the application of this protocol to the synthesis of the so far unknown dideoxy analog $dm^5K$ (**2**), the conversion of **1** and **2** into the *cyclo*Sal nucleotides, and the fluorescence and hydrolysis properties of the latter compounds. Additionally, the new fluorescent *cyclo*Sal-phosphate triesters were used as probes for cell-extract hydrolysis studies, demonstrating the strength of the approach. Finally, a transport study with an "aqueous phase-organic solvent-aqueous phase" system was used as a model for cell uptake.

## Results and Discussion

### Chemistry

In contrast to a previous report,[14] the more stable *tert*-butyldimethylsilyl (TBDMS) group for the protection of the glycon hydroxy groups was used in our $m^5K$ synthesis protocol. Conversion of thymidine into 3′,5′-di-TBDMS-dT is straightforward and proceeds in 90% yield. This compound was treated with $P(O)Cl_3$/triazole to give the 4-(triazol-1-yl)-derivative **3** in quantitative yield. Compound **3** was treated with fresh anhydrous hydrazine (1 M in THF), which led to a substitution of the triazole ring to give the hydrazino intermediate **4**.[15] This material was immediately oxidized with *N*-phenyltriazolinedione (PTAD, 1.2 equiv.) to give 3′,5′-TBDMS-$m^5K$ (**5**) in 60% yield (Scheme 2). It is worth mentioning that the yields obtained in the last step strongly depend on the quality of PTAD. The best results were obtained when PTAD was prepared just prior to the reaction. In order to evaluate which of the two steps is the yield-limiting step, we trapped the intermediate hydrazino derivative **4** by methylation. After substituting the triazol-1-yl ring by hydrazine, 4 equiv. methyl iodide was added, leading to a stable double-methylation product **6** that was isolated in 88% yield. NMR spectroscopic analysis revealed that both methyl groups were attached to the terminal amino group of compound **6**. In addition, we isolated 4% of unreacted 4-(triazol-1-yl)-thymidine **3** and the *N*,*N*-dimer of 5-methyl-cytidine **7** in 8% yield. Consequently, the substitution reaction proceeded well, and the yield-limiting step was clearly the oxidation with PTAD. Finally, the TBDMS groups were cleaved with tetrabutylammonium fluoride (TBAF) or triethylamine trihydrofluoride ($NEt_3$·3HF) to give $m^5K$ **1** in quantitative yield in the case of TBAF in THF.[16]

This procedure has been applied to the synthesis of an analog of antivirally active 2′,3′-dideoxypyrimidine nucleosides, the so far unknown 3′-deoxy-5-methyl-pyrimidine-2-one nucleoside **2** ($dm^5K$) via intermediates **8**–**10** (Scheme 2). All steps proceeded with yields comparable to those of $m^5K$ (**1**). The comparable yields, particularly in the PTAD reaction step, are worth mentioning because Holy reported that electron-acceptor groups should be introduced into the glycon to stabilize the glycosidic bond in the reactions with metal oxides. Therefore, they introduced *O*-benzoyl groups. Nevertheless, the yield of ribosylpyrimidin-2-one was 80%, whereas that of 2′-deoxyribosylpyrimidin-2-one ($m^5K$) was 40–50%.[12a]

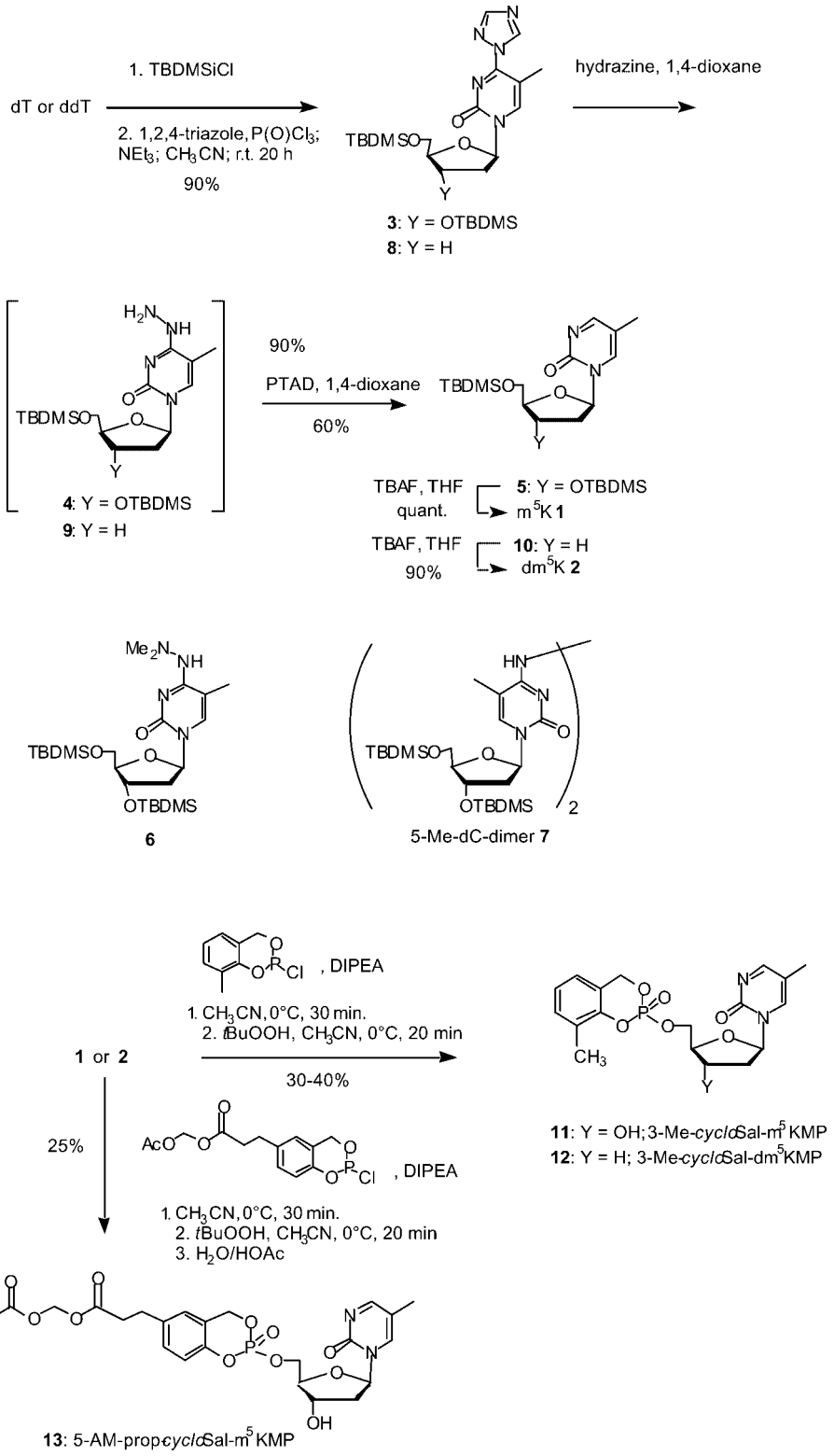
Both, $m^5K$ **1** and $dm^5K$ **2** were subsequently converted into their 5′-*cyclo*Sal-phosphate triesters by the protocols described previously.[6] The two 3-methyl-*cyclo*Sal-phosphate triesters **11** and **12** were obtained in 30–40% yield. In addition, one *cyclo*Sal-$m^5K$MP triester **13** bearing the esterase-cleavable 5-acetoxymethylpropionate (5-AM-propionate) group was prepared starting from the corresponding chlorophosphite.[9] This triester was obtained in a non-optimized yield of 28%. After cell uptake, this compound should be rapidly cleaved by esterases to acetic acid and formaldehyde, releasing a highly polar carboxylate group, which is negatively charged under physiological conditions. As a consequence, the compound should be trapped intracellularly.

### Fluorescence Studies

The absorption, excitation, and emission spectra for $m^5K$ (**1**) and $dm^5K$ (**2**) were observed to be similar. The UV spectra showed maxima at $\lambda_{max}$ = 220 and 314 nm. Fluorescence excitation was achieved at 314 nm, while the fluorescence maximum was at 380 nm (Figure 1). Although the fluorescence of both pyrimidin-2-one nucleosides is 4 to 5-fold weaker than that of the purine analog 2-aminopurine 2′-deoxyriboside **14** (2AP), the advantage of these nucleosides is the red shift of the fluorescence band ($\lambda_{em}$ = 380 nm) with respect to the emission band for 2AP ($\lambda_{em}$ = 352 nm). Thus, the fluorescence of the two nucleosides interferes only weakly with that of tryptophan ($\lambda_{em}$ = 337 nm). Moreover, at the excitation wavelength ($\lambda_{ex}$ = 314 nm) neither tryptophan nor NAD(P)H are excited. These spectral properties may have considerable advantages in studies carried out in biological media, for example, in cell extracts.

Interestingly, although the heterocycle was not modified, the fluorescence intensity of the triesters **11** and **12** was found to be 40% lower than that of the nucleosides (Figure 1, Table 1). It has been reported that the fluorescence of $m^5K$ is strongly sensitive to its microenvironment.[10] Here, an interaction of the pyrimidin-2-one ring with the *cyclo*Sal aromatic ring system led to a partial quenching of the fluorescence. If this is true, the fluorescence should in-




Scheme 2.

crease during chemical hydrolysis because of nucleotide delivery.

### Hydrolysis Studies

The fluorescent *cyclo*Sal triesters were hydrolyzed in 25 mM phosphate buffer pH 7.3; UV- and fluorescence detection were used. In this context, it is worth mentioning that the use of the fluorescent *cyclo*Sal triesters led to a more than 1000-fold increase in sensitivity relative to UV detection. The effect of this dramatic increase in sensitivity on the detection of the hydrolysis products can be seen in Figure 2.

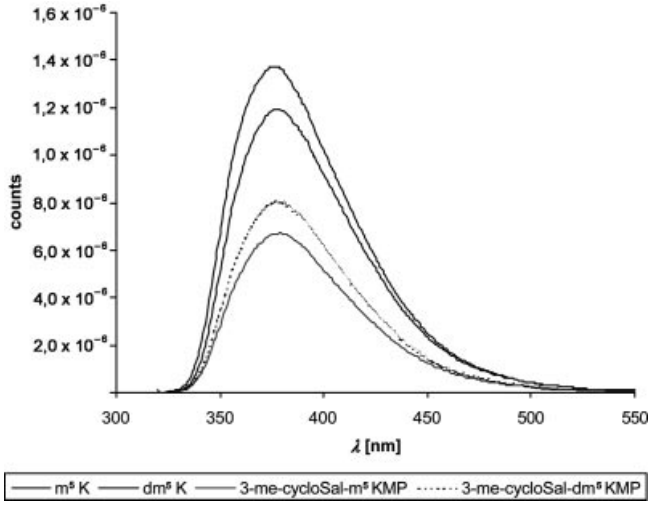



Figure 1. Fluorescence spectra of m$^5$K (**1**), dm$^5$K (**2**) and their pronucleotide derivatives 3-Me-*cyclo*Sal-m$^5$KMP (**11**) and 3-Me-*cyclo*Sal-dm$^5$KMP (**12**). Spectra were recorded in aqueous solution and concentrations of 3.8 μM (**11**), 3.2 μM (**1**), 3.5 μM (**12**), and 4.0 μM (**2**) with an excitation at 314 nm. At this wavelength, the absorption of all compounds was identical.

Table 1. Chemical hydrolysis to yield the nucleotides and fluorescence properties of *cyclo*Sal triesters **11**–**13**.[a]

| | $t_{1/2}$ (h)[b] | $\lambda_{ex}$ | $\lambda_{em}$ | $\Theta_{rel}$[c] | $\Theta_{rel}$[d] |
|---|---|---|---|---|---|
| **11** | 6.8 | 314 | 380 | 0.47 | 0.1 |
| m$^5$K **1** | n.a.[g] | 314 | 380 | 0.87 | 0.19 |
| **15**[e] | 8.6 | n.a.[g] | n.a.[g] | n.a.[g] | n.a.[g] |
| **12** | 15.4 | 314 | 380 | 0.57 | 0.12 |
| dm$^5$K **2** | n.a.[g] | 314 | 380 | 1 | 0.21 |
| **16**[f] | 14.9 | n.a.[g] | n.a.[g] | n.a.[g] | n.a.[g] |
| **13** | 2.0 | 314 | 380 | 0.65 | 0.14 |
| 2AP **14** | n.a.[g] | 305 | 355 | n.a.[g] | 1 |

[a] The corresponding data of the parent nucleosides **1**, **2** and 2-aminopurine 2′-deoxyriboside (2AP) **14** and *cyclo*Sal triesters **15**, **16** are also given for comparison. [b] Phosphate buffer (25 mM, pH 7.3, 37 °C). [c] Quantum yield relative to nucleoside **2**. [d] Quantum yield relative to 2AP **14**. [e] 3-Me-*cyclo*Sal-dTMP **15**. [f] 3-Me-*cyclo*Sal-ddTMP **16**. [g] Not available.

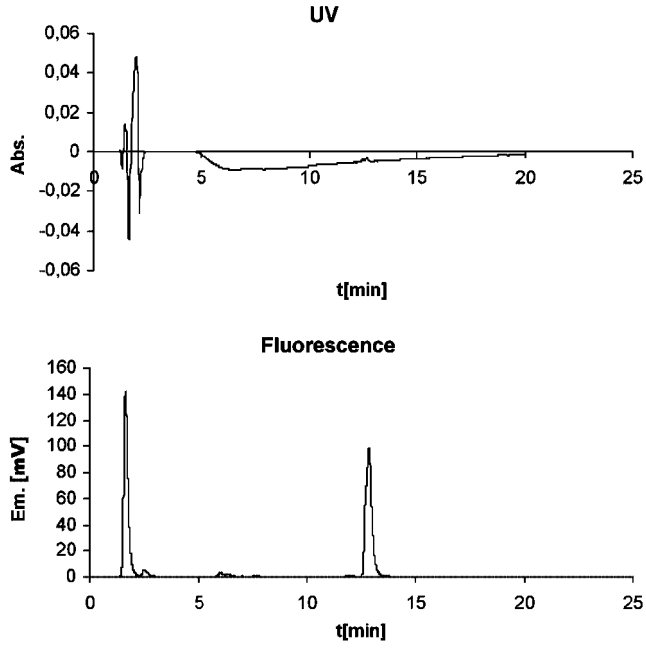



Figure 2. Detection of the hydrolysis mixture of 3-Me-*cyclo*Sal-dm$^5$KMP using either UV detection (a) or fluorescence detection (b): the sample was analyzed using an in-line setup of a UV detector and a fluorescence detector within an HPLC apparatus. $R_t$: 2.3 min, dm$^5$KMP; $R_t$: 13.0 min, 3-Me-*cyclo*Sal-dm$^5$KMP.

As expected, all triesters released the corresponding monophosphate derivatives of the two parent nucleoside analogs. The half-lives at pH 7.3 were found to be in the same stability range as the references 3-methyl-*cyclo*Sal-dTMP **15** or 3-methyl-*cyclo*Sal-ddTMP **16** ($t_{1/2}$ 8.6 h and 14.9 h, respectively; Table 1).

Moreover, the expected increase of the total fluorescence during hydrolysis due to the release of the nucleosides was observed (Figure 3).

Chemical hydrolysis of 5-acetoxymethylpropionate-*cyclo*Sal-m$^5$KMP (5-AM-propionate-*cyclo*Sal-m$^5$KMP) (**13**)

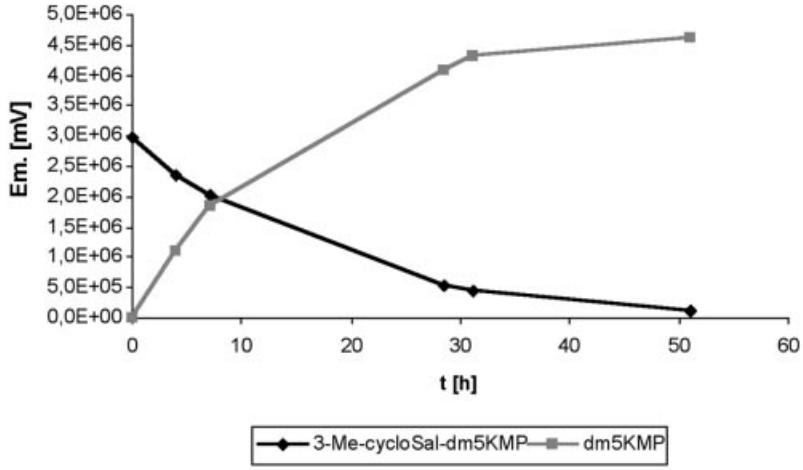



Figure 3. Hydrolysis study using 3-Me-*cyclo*Sal-dm$^5$KMP (**12**) in phosphate buffer, pH 7.3 using fluorescence detection.

had a half-life of 2.0 h, which is lower than previously observed for 5-alkyl-substituted *cyclo*Sal-2′-deoxynucleotides ($t_{1/2}$ = 4.1 h). Moreover, the fluorescence increased more than twofold on hydrolysis of the AM ester **13**.

Fluorescence detection was used in the cell-extract hydrolysis studies. Here, concentrations that were 500–1000-fold lower relative to UV-detection studies were used. As before, the increase in total fluorescence was observed again during the hydrolysis of the *cyclo*Sal triesters.

In cell extracts, 5-AM-propionate-*cyclo*Sal-m$^5$KMP (**13**) was rapidly cleaved at the acylal site first and subsequently hydrolyzed to give the nucleotide m$^5$KMP. These findings were confirmed in experiments in which pig liver esterase (PLE) was used in aqueous phosphate buffer, pH 7.3. The acylal site was cleaved completely within 5 minutes to give the propionate.

Finally, fluorescence detection has been used in model studies for cellular uptake. In these experiments, the migration of the triesters from an aqueous donor phase to an aqueous acceptor phase via an organic phase ($CH_2Cl_2$) was monitored. Two different experimental setups of this U-tube experiment with triesters **11** and **12** were used: (i) the pH was set to 6.8 in both aqueous phases; and (ii) the pH of the donor phase was 6.8 while that of the acceptor phase was 8.7. In the first experiment, the expected formation of an equilibrium between both phases was observed (data not shown). However, in the second setup, an accumulation in the acceptor phase was observed because the triester was hydrolyzed to the nucleotide under these conditions. The nucleotide formed was too polar to enter the organic phase again (Figure 4).

For 5-AM-propionate-*cyclo*Sal-m$^5$KMP (**13**) a different setup was used: The donor phase was again phosphate buffer, pH 6.8; however, the acceptor phase was phosphate buffer, pH 7.3, containing PLE. As expected, an accumulation of fluorescent material in the acceptor phase was detected (Figure 5).

The compounds found in the acceptor phase by HPLC were 5-propionate-*cyclo*Sal-m$^5$KMP and its hydrolysis product m$^5$KMP. No 5-AM-propionate-*cyclo*Sal-m$^5$KMP (**13**) was detected because of its very high susceptibility to enzyme degradation. This experiment supports the idea of a possible intracellular lock-in or trapping of the *cyclo*Sal-phosphate triesters.

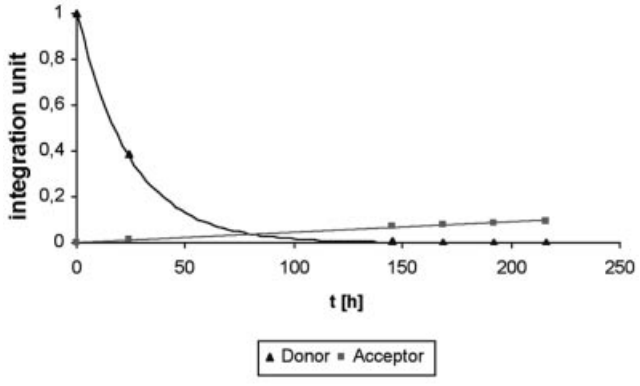



Figure 4. Diffusion experiment of 5-AM-propionate-*cyclo*Sal-dm$^5$K **13** from a donor phase (pH 6.8) to an acceptor phase (pH 8.7).

## Conclusions

A reliable synthetic procedure for the synthesis of two fluorescent thymidine analogs and their conversion into the corresponding *cyclo*Sal-phosphate triesters is described. Hydrolysis data and the fluorescence properties of the *cyclo*Sal-phosphate triesters proved that these compounds are ideal equivalents for *cyclo*Sal-pyrimidine monophosphates bearing antiviral nucleoside analogs like ddT or d4T, which may be used as probes of important physiological processes. The new phosphate triesters were used in cell-extract hydrolysis studies in which the products were detected by fluorescence as well as in a first model study for a cell-uptake process. These studies proved that an accumulation in the acceptor phase could be achieved by pH difference and by

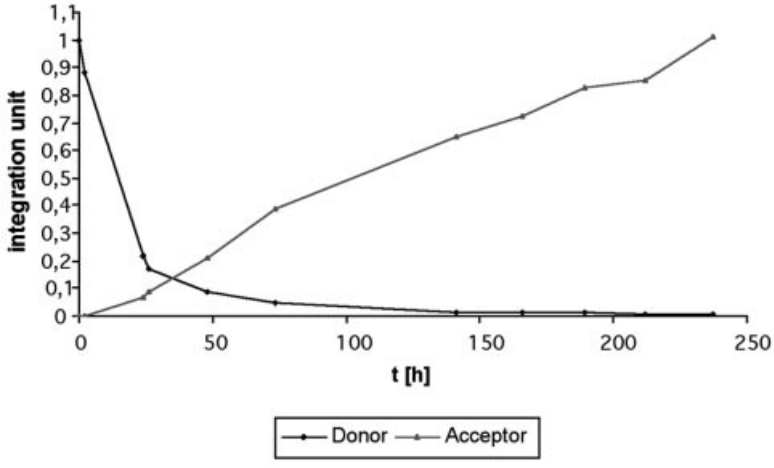



Figure 5. Enzyme-driven transport of 5-AM-propionate-*cyclo*Sal-dm$^5$KMP **13** from an aqueous donor phase into an aqueous acceptor phase containing PLE.

enzyme-driven trapping. Further studies on the use of these fluorescent probes, e.g. in cell incubation studies, are continuing in our laboratories.

## Experimental Section

All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions (under argon). Solvents: Anhydrous dichloromethane ($CH_2Cl_2$), tetrahydrofuran (THF), and acetonitrile ($CH_3CN$) were obtained in Sure/Seal bottles from Fluka and stored over 4 Å molecular sieves; ethyl acetate, dichloromethane, and methanol employed in chromatography were distilled prior to use. *N,N*-Diisopropylethylamine (DIPEA) was distilled from Na prior to use. Solvents for HPLC were obtained from Merck ($CH_3CN$, HPLC grade). Ion-pairing buffer solution was prepared by mixing tetrabutylammonium hydroxide (6.6 mL) with water (1000 mL). The pH value was adjusted to 3.8 by adding concentrated phosphoric acid (buffer I). Buffer I (60 mL) was diluted with water (1000 mL) to form buffer II.

NMR spectra were recorded with a Bruker AMX 400 ($^1$H NMR, 400 MHz; $^{13}$C NMR, 101 MHz; $^{31}$P NMR, 162 MHz) or Bruker DMX 500 ($^1$H NMR, 500 MHz; $^{13}$C NMR, 123 MHz; $^{31}$P NMR, 202 MHz) spectrometer, with $CDCl_3$ or DMSO as internal standard for $^1$H and $^{13}$C NMR spectroscopy and with $H_3PO_4$ as external standard for $^{31}$P NMR spectroscopy. All $^1$H and $^{13}$C NMR chemical shifts ($\delta$) are quoted in parts per million (ppm) downfield from tetramethylsilane; $(CD_3)(CD_2H)SO$ was set at $\delta_H = 2.49$ as a reference. $^{31}$P NMR chemical shifts are quoted in ppm by using $H_3PO_4$ as external reference. All spectra were recorded at room temperature, and $^{13}$C and $^{31}$P NMR spectra were recorded in the proton-decoupled mode. Mass spectra were obtained with a Finnigan electrospray MAT 95 Trap XL (ESI) or a VG Analytical VG/70–250 F spectrometer [FAB, (double-focusing), matrix: *m*-nitrobenzyl alcohol]. FAB high-resolution (HR) mass spectra were recorded by using a VG Analytical 70–250S spectrometer with the MCA method and polyethylene glycol as support. UV spectra were recorded with a Varian Cary 1E UV/Vis spectrometer. Infrared spectra were recorded with a Perkin–Elmer 1600 Series FT-IR or a ATI Mattson Genesis Series FT-IR spectrometer; samples were prepared in KBr pellets. Fluorescence spectra were recorded with a Fluorolog 3 instrument; Jobin Yvon. Solvent: water (dist.); cuvette: 1 × 1 cm; excitation wavelength: 314 nm; spectral bandwidth: 5 nm; stepwidth: 1 nm; temp.: 25 °C. In the HPLC system, a Merck Hitachi fluorescence detector L-7480 was used. Chromatography: All preparative TLCs were performed with a Chromatotron (Harrison Research 7924) using glass plates coated with 1- or 2-mm layers of Merck 60 $PF_{254}$ silica gel containing a fluorescent indicator and UV detection at 254 nm. Column chromatography: Merck silica gel 60, 230–400 mesh was used. TLC: analytical thin layer chromatography was performed on Merck precoated aluminum plates 60 $F_{254}$ with a 0.2 mm layer of silica gel containing a fluorescence indicator; sugar-containing compounds were visualized with the sugar spray reagent (0.5 mL 4-methoxybenzaldehyde, 9 mL ethanol, 0.5 mL concentrated sulfuric acid, and 0.1 mL glacial acetic acid) by heating with a fan or a hot plate. Analytical HPLC: Merck-Hitachi HPLC system ($^d$-7000) equipped with a LiChroCART 125–3 column containing reverse-phase silica gel Lichrospher 100 RP 18 (endcapped, 5 μM) (Merck, Darmstadt, Germany). HPLC method: 0–25 min water or ion pairing (buffer/$CH_3CN$ gradient 5–100%); isocratic $CH_3CN/H_2O$, 5:95 v/v 25–35 min, flow: 0.5 mL/min; UV detection at 250 nm or fluorescence detection at 380 nm. The *cyclo*Sal compounds **11**–**13** were isolated as mixtures of diastereomers arising from the mixed stereochemistry at the phosphate center. The resulting lyophilized triesters did not give useful microanalytical data, most probably because of incomplete combustion of the compounds or varying amounts of water. However, they were found to be pure by rigorous HPLC analysis (gradient of 5–100% $CH_3CN$ in $H_2O$ within 25 min, flow 0.5 mL/min), high-field multinuclear NMR spectroscopy and mass spectroscopy.

**Preparation of the Triazolo Compounds 3 and 8**

To a suspension of 1,2,4-triazole (100 mmol) in anhydrous $CH_3CN$ (75 mL) at 0 °C was slowly added $P(O)Cl_3$ (23 mmol) and then triethylamine (100 mmol). After 30 min at 0 °C, 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxythymidine or 5′-*O*-*tert*-butyldimethylsilyl-2′,3′-dideoxythymidine (6.50 mmol) in $CH_3CN$ (40 mL) was added dropwise over 30 min. The reaction mixture was stirred for a further 20 h. It was then filtered, evaporated, and diluted with EtOAc. The organic phase was washed with sat. $Na_2CO_3$ and with brine. It was dried with $Na_2SO_4$ and concentrated under reduced pressure. Purification was carried out using a Chromatotron with $CH_2Cl_2$ and a MeOH-gradient (0–2% v/v).

**3′,5′-Bis(*O*-*tert*-butyldimethylsilyl)-4-(1,2,4-1H-triazol-1-yl)-thymidine (3):** Yield: 98% (colorless crystals; m.p. 104–107 °C). $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 9.28 (s, 1 H), 8.24 (d, $^4J_{H,H}$ = 0.5 Hz, 1 H), 8.10 (s, 1 H), 6.28 (dd, $^3J_{H,H}$ = 6.3, $^3J_{H,H}$ = 6.3 Hz, 1 H), 4.39 (ddd, $^3J_{H,H}$ = 6.6, $^3J_{H,H}$ = 3.6, $^3J_{H,H}$ = 3.6 Hz, 1 H), 4.06 (ddd, $^3J_{H,H}$ = 2.8, $^3J_{H,H}$ = 3.4, $^3J_{H,H}$ = 3.4 Hz, 1 H), 3.96 (dd, $^2J_{H,H}$ = 11.5, $^3J_{H,H}$ = 2.5 Hz, 1 H), 3.79 (dd, $^2J_{H,H}$ = 11.5, $^3J_{H,H}$ = 2.5 Hz, 1 H), 2.64 (ddd, $^3J_{H,H}$ = 13.6, $^3J_{H,H}$ = 6.1, $^3J_{H,H}$ = 3.7 Hz, 1 H), 2.44 (d, $^3J_{H,H}$ = 0.7 Hz, 3 H), 2.07 (ddd, $^3J_{H,H}$ = 13.4, $^3J_{H,H}$ = 6.2, $^3J_{H,H}$ = 6.2 Hz, 1 H), 0.91 (s, 9 H, *t*Bu), 0.90 (s, 9 H), 0.12 (s, 3 H, $CH_3$-Si), 0.11 (s, 3 H), 0.08 (s, 3 H), 0.07 (s, 3 H) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 158.1, 153.9, 153.4, 146.6, 145.1, 105.2, 88.8, 87.8, 71.6, 62.6, 42.6, 25.9, 25.7, 18.4, 18.0, 17.3, –4.6, –4.9, –5.3, –5.4 ppm. MS (HR-FAB): calcd. 522.293 [M + H$^+$]; found 522.294.

**5′-*O*-*tert*-Butyldimethylsilyl-4-(1,2,4-1H-triazol-1-yl)-2′,3′-dideoxythymidine (8):** Yield: 96% (colorless oil). $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 9.28 (s, 1 H), 8.39 (s, 1 H), 8.10 (s, 1 H), 6.08 (dd, $^3J_{H,H}$ = 3.2, $^3J_{H,H}$ = 6.6 Hz, 1 H), 4.30–4.25 (m, 1 H), 4.12 (dd, $^2J_{H,H}$ = 11.7, $^3J_{H,H}$ = 2.5 Hz, 1 H), 3.77 (dd, $^2J_{H,H}$ = 11.7, $^3J_{H,H}$ = 2.5 Hz, 1 H), 2.68–2.58 (m, 1 H), 2.45 (s, 3 H), 2.22–2.15 (m, 1 H) 1.99–1.88 (m, 2 H), 0.93 (s, 9 H), 0.13 (s, 3 H), 0.12 (s, 3 H) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 155.1, 153.3, 147.1, 145.5, 145.0, 104.8, 88.5, 83.0, 63.9, 33.6, 26.0, 24.3, 18.6, 17.3, –5.2, –5.3 ppm. MS (HR-FAB): calcd. 392.2118 [M + H$^+$]; found 392.2119.

**Conversion of 3 and 8 into the Hydrazino Compounds and PTAD Reduction (One-Pot Reaction)**

3′,5′-Bis(*O*-*tert*-butyldimethylsilyl)-4-(1,2,4-triazol-1-yl)-thymidine (**3**) (2.53 mmol) or 5′-*O*-*tert*-butyldimethylsilyl-4-(1,2,4-triazol-1-yl)-2′,3′-dideoxythymidine (**8**) (2.53 mmol) was dissolved in anhydrous dioxane (35 mL) and degassed. The solution was cooled until dioxane began to solidify. After removal from the ice bath, anhydrous hydrazine/THF solution (5.0 mL, 1 M, 5.00 mmol) was added at once. The solution was stirred for 1 h at room temp. and was then reduced to 5 mL. Fresh, anhydrous dioxane (35 mL) was added and the solution was warmed to 60 °C. A solution of *N*-phenyltriazolidinedione (PTAD; 3.70 mmol) in anhydrous dioxane (30 mL) was added dropwise, and the mixture was stirred for one additional hour. The solvent was then removed under reduced pressure. Purification was carried out with a Chromatotron with $CH_2Cl_2$ and a MeOH-gradient (0–2% v/v).

**3′,5′-Bis(*O*-*tert*-butyldimethylsilyl)-*N*$^1$-(β-D-2′-deoxyribosyl)-5-methyl-pyrimidin-2-one (5):** Yield: 57% (yellow oil). $^1$H NMR

(400 MHz, $CDCl_3$): $\delta$ = 8.44 (d, $^4J_{H,H}$ = 3.3 Hz, 1 H), 8.04 (dd, $^4J_{H,H}$ = 3.3, $^4J_{H,H}$ = 0.8 Hz, 1 H), 6.23 (dd, $^3J_{H,H}$ = 6.4, $^3J_{H,H}$ = 6.4 Hz, 1 H), 4.38–4.34 (m, 1 H), 4.01 (ddd, $^3J_{H,H}$ = 3.4, $^3J_{H,H}$ = 2.5, $^3J_{H,H}$ = 3.6 Hz, 1 H), 3.93 (dd, $^2J_{H,H}$ = 11.5, $^3J_{H,H}$ = 2.7 Hz, 1 H), 3.77 (dd, $^2J_{H,H}$ = 11.5, $^3J_{H,H}$ = 2.7 Hz, 1 H), 2.61 (ddd, $^3J_{H,H}$ = 4.0, $^3J_{H,H}$ = 6.4, $^2J_{H,H}$ = 13.5 Hz, 1 H), 2.10 (d, $^4J_{H,H}$ = 0.8 Hz, 3 H), 2.03 (ddd, $^3J_{H,H}$ = 6.4, $^3J_{H,H}$ = 6.4, $^2J_{H,H}$ = 13.5 Hz, 1 H), 0.91 (s, 9 H), 0.90 (s, 9 H), 0.11 (s, 3 H), 0.10 (s, 3 H), 0.07 (s, 3 H), 0.06 (s, 3 H) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 167.4, 155.1, 140.8, 112.4, 88.4, 87.4, 71.4, 62.4, 42.5, 25.9, 25.7, 18.4, 18.0, 14.5, –4.6, –4.9, –5.4, –5.6 ppm. MS (FAB): calcd. 455.3 [M + H$^+$]; found 455.0.

**5′-*O*-*tert*-Butyldimethylsilyl-*N*$^1$-(β-D-2′,3′-dideoxyribosyl)-5-methylpyrimidin-2-one (10):** Yield: 48% (yellow foam). $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 8.44 (d, $^4J_{H,H}$ = 2.8 Hz, 1 H), 8.17 (dd, $^4J_{H,H}$ = 3.0, $^4J_{H,H}$ = 0.8 Hz, 1 H), 6.03 (dd, $^3J_{H,H}$ = 3.3, $^3J_{H,H}$ = 6.6 Hz, 1 H), 4.26–4.20 (m, 1 H), 4.10 (dd, $^2J_{H,H}$ = 11.7, $^3J_{H,H}$ = 2.3 Hz, 1 H), 3.74 (dd, $^2J_{H,H}$ = 11.7, $^3J_{H,H}$ = 2.3 Hz, 1 H), 2.65–2.54 (m, 1 H), 2.16–2.08 (m, 1 H), 2.11 (d, $^4J_{H,H}$ = 0.6 Hz, 3 H), 1.95–1.86 (m, 2 H), 0.92 (s, 9 H), 0.12 (s, 3 H), 0.10 (s, 3 H) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 167.3, 155.3, 141.3, 112.2, 88.2, 82.7, 63.9, 33.5, 25.9, 24.3, 18.5, 14.6, –5.3 ppm. MS (HR-FAB): calcd. 325.195 [M + H$^+$]; found 325.193.

**Deprotection of the Silylated Nucleosides 5 and 10**

3′,5′-Bis(*O*-*tert*-butyldimethylsilyl)-*N*$^1$-(β-D-2′-deoxyribosyl)-5-methyl-pyrimidin-2-one (**5**) or 5′-*O*-*tert*-butyldimethylsilyl-*N*$^1$-(β-D-2′,3′-dideoxyribosyl)-5-methylpyrimidin-2-one (**10**) (140 mg, 0.43 mmol) was dissolved in THF (5 mL). TBAF/THF solution (1.08 mL, 1 M; 2.0 equiv. in the case of **10** and 3.0 equiv. in the case of **5**) was added and the reaction mixture was stirred for 20 min. The solvent was removed under reduced pressure. Purification was achieved with a Chromatotron by using EtOAc and a MeOH-gradient (0–10% v/v) which yielded a yellow, sticky solid (**2**) or colorless crystals (**1**).

***N*$^1$-(β-D-2′-Deoxyribosyl)-5-methyl-pyrimidin-2-one (m$^5$K) (1)** was prepared as described above and isolated in quantitative yield. Analytical data were identical with those published previously.[12,13]

Starting with di-TBDMS-protected m$^5$K **5** (500 mg, 1.1 mmol), m$^5$K **1** (247 mg, 1.1 mmol, 100%) was isolated.

***N*$^1$-(β-D-2′,3′-Dideoxyribosyl)-5-methyl-pyrimidin-2-one (dm$^5$K) (10):** Yield: 72% (light yellow solid, m.p. 139–140 °C). The spectra did not show any traces of remaining $(n\text{Bu})_4N^+$ ions. $^1$H NMR (500 MHz, [D$_6$]DMSO): $\delta$ = 8.46 (s, 2 H), 5.91 (dd, $^3J_{H,H}$ = 2.5, $^3J_{H,H}$ = 6.9 Hz, 1 H), 5.20 (t, $^3J_{H,H}$ = 5.4 Hz, 1 H), 4.17–4.12 (m, 1 H), 3.82 (ddd, $^2J_{H,H}$ = 12.3, $^3J_{H,H}$ = 3.2, $^3J_{H,H}$ = 5.6 Hz, 1 H), 3.62 (ddd, $^2J_{H,H}$ = 12.3, $^3J_{H,H}$ = 3.4, $^3J_{H,H}$ = 4.9 Hz, 1 H), 2.46–2.38 (m, 1 H), 2.06 (s, 3 H), 1.99–1.93 (m, 1 H), 1.90–1.77 (m, 2 H) ppm. $^{13}$C NMR (100 MHz, [D$_6$]DMSO): $\delta$ = 167.3, 154.2, 142.1, 111.8, 87.3, 83.1, 61.4, 33.1, 23.7, 14.3 ppm. UV ($H_2O$): $\lambda_{max}$ = 311, 213 nm. IR (KBr): $\tilde{\nu}$ = 3395, 2928, 1654, 1532, 1395, 1254, 1104, 797 cm$^{-1}$. MS (HR-FAB): calcd. 211.108 [M + H$^+$]; found 211.109.

**Preparation of the *cyclo*Sal-phosphate Triesters 11–13 from m$^5$K and dm$^5$K**

The nucleosides (0.72 mmol) were co-evaporated twice with pyridine and dissolved in anhydrous THF (1 mL) and anhydrous DMF (1 mL). The reaction mixture was cooled to –20 °C. After addition of DIPEA (0.54 mmol), 3-methyl- or 5-acetoxymethylpropionate-saligenylchlorophosphite (0.30 mmol) was added dropwise. The reaction mixture was warmed to room temp. for 1 h. After cooling to –20 °C, *t*BuOOH (0.13 mL) was added (70% in *n*-decane, 0.81 mmol). After stirring for 1 h at room temp., the solvent was removed under reduced pressure. For triester **13**, the reaction mixture was diluted with $CH_2Cl_2$, extracted twice with brine and directly purified. Purification of the residue was carried out with a Chromatotron by using EtOAc and a MeOH-gradient (0–5% v/v) and repeated purification by using $CH_2Cl_2$ and a MeOH-gradient (0–3% v/v). The products were lyophilized to yield white foams.

**3-Methyl-*cyclo*Sal-*N*$^1$-(β-D-2′-deoxyribosyl)-5-methyl-pyrimidin-2-one-5′-monophosphate (3-Me-*cyclo*Sal-m$^5$KMP) (11):** Yield: 40% (colorless, hygroscopic solid). $^1$H NMR (500 MHz, [D$_6$]DMSO): $\delta$ = 8.48 (d, $^4J_{H,H}$ = 3.3 Hz, 1 H, 4-H), 8.45 (d, $^4J_{H,H}$ = 3.3 Hz, 2 H, 4-H), 8.00–7.96 (m, 2 H, 6-H), 7.30–7.24 (m, 2 H, 6-H$_{aryl}$), 7.13–7.10 (m, 4 H, 5-H$_{aryl}$, 4-H$_{aryl}$), 6.11 (2×dd, $^3J_{H,H}$ = 6.6, $^3J_{H,H}$ = 6.6 Hz, 2 H, 1′-H), 5.55–5.37 (m, 6 H, 7-H$_{benzyl}$, 3′-OH), 4.47–4.30 (m, 4 H, 5′-H), 4.26–4.19 (m, 2 H, 3′-H), 4.10–4.05 (m, 2 H, 4′-H), 2.40–2.32 (m, 2 H, 2′-H), 2.21 (s, 3 H, Me$_{aryl}$), 2.20 (s, 3 H, Me$_{aryl}$) 2.08–1.99 (m, 2 H, 2′-H), 2.02 (d, $^4J_{H,H}$ = 0.5 Hz, 3 H, Me$_{het}$), 1.98 (d, $^4J_{H,H}$ = 0.5 Hz, 3 H, Me$_{het}$) ppm. $^{13}$C NMR (100 MHz, [D$_6$]DMSO): $\delta$ = 2×168.0 (2×C$_{het}$-4), 157.1 (2×C$_{het}$-2), 148.1 (2×C$_{aryl}$-2), 141.4, 141.3 (2×C$_{het}$-6), 131.1 (2×C$_{aryl}$-4), 127.1 (2×C$_{aryl}$-3), 124.2 (2×C$_{aryl}$-6), 2×123.8 (2×C$_{aryl}$-5), 121.2, 121.1 (2×C$_{aryl}$-1), 112.1 (2×C$_{het}$-5), 87.1, 87.0 (2×C-1′), 85.4, 85.3 (2×C-3′), 70.0, 69.9 (2×C-4′), 68.6, 68.5 (2×C-7), 67.8, 67.7 (2×C-5′), 40.5 (2×C-2′), 2×15.0 (2×Me$_{aryl}$), 13.8, 13.7 (2×Me$_{het}$) ppm. $^{31}$P NMR ($^1$H-decoupled, 200 MHz, [D$_6$]DMSO): $\delta$ = –7.6, –7.7 ppm. UV ($H_2O$): $\lambda_{max}$ = 314, 213 nm. MS (MALDI-TOF) calcd. 431.1 [M + Na$^+$]; found 431.1. $R_t$ (HPLC) 11.95 min and 12.19 min.

**3-Methyl-*cyclo*Sal-*N*$^1$-(β-D-2′,3′-dideoxyribosyl)-5-methyl-pyrimidin-2-one-5′-monophosphate (3-Me-*cyclo*Sal-dm$^5$KMP) (12):** Yield: 28% (colorless, hygroscopic solid). $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 8.43 (2×d, $^4J_{H,H}$ = 3.5 Hz, 2 H, 4-H), 8.08 (2×d, $^4J_{H,H}$ = 3.5 Hz, 2 H, 6-H), 7.22–7.15 (m, 2 H, 6-H$_{aryl}$), 7.05 (dd, $^3J_{H,H}$ = 7.6, $^3J_{H,H}$ = 7.6 Hz, 2 H, 5-H$_{aryl}$), 6.94 (d, $^3J_{H,H}$ = 7.6 Hz, 4-H$_{aryl}$), 6.05 (2×dd, $^3J_{H,H}$ = 3.2, $^3J_{H,H}$ = 3.2 Hz, 2 H, 1′-H), 5.45–5.25 (m, 4 H, 7-H$_{benzyl}$), 4.60–4.11 (m, 6 H, 5′-H, 4′-H), 2.63 (2×dddd, $^2J_{H,H}$ = 14.2, $^3J_{H,H}$ = 7.3, $^3J_{H,H}$ = 3.2, $^3J_{H,H}$ = 3.2 Hz, 2 H, 2′-H), 2.28, 2.25 (s, 6 H, Me$_{aryl}$), 2.19–2.11 (m, 2 H, 2′-H), 2.06, 2.03 (s, 6 H, Me$_{het}$), 2.11–2.00 (m, 2 H, 3′-H), 1.87–1.78 (m, 2 H, 3′-H) ppm. $^{13}$C NMR (100 MHz, [D$_6$]DMSO): $\delta$ = 2×167.7 (2×C$_{het}$-4), 2×155.1 (2×C$_{het}$-2), 148.2 (2×C$_{aryl}$-2), 141.3, 141.2 (2×C$_{het}$-6), 131.2, 131.1 (2×C$_{aryl}$-4), 127.1 (2×C$_{aryl}$-3), 124.2 (2×C$_{aryl}$-6), 123.9, 123.8 (2×C$_{aryl}$-5), 120.8 (2×C$_{aryl}$-1), 112.0, 111.9 (2×C$_{het}$-5) 87.7, 87.6 (2×C-1′), 2×79.8 (2×C-4′, $^4J_{C,P}$ = 2.2 Hz), 69.0 (2×C-7, $^2J_{C,P}$ = 6.1 Hz), 68.6 (2×C-5′), 32.3, 32.2 (2×C-2′), 24.5 (2×C-3′), 15.1, 15.0 (2×Me$_{aryl}$), 13.8, 13.7 (2×Me$_{het}$) ppm. $^{31}$P NMR ($^1$H-decoupled, 200 MHz, [D$_6$]DMSO): $\delta$ = –7.4, –7.5 ppm. UV ($H_2O$): $\lambda_{max}$ = 314, 271, 213 nm. HRMS (ESI$^+$): calcd. 415.10349; found 415.10344. $R_t$ (HPLC) 13.17 min and 13.31 min.

**5-(Acetoxymethylpropionate)-*cyclo*Sal-*N*$^1$-(β-D-2′-deoxyribosyl)-5-methyl-pyrimidin-2-one-5′-monophosphate (5-AM-propionate-*cyclo*Sal-m$^5$KMP) (13):** Yield: 28% (colorless, hygroscopic solid). $^1$H NMR (500 MHz, [D$_6$]DMSO): $\delta$ = 8.48 (d, $^4J_{H,H}$ = 3.2 Hz, 1 H, 4-H), 8.45 (d, $^4J_{H,H}$ = 3.2 Hz, 1 H, 4-H), 7.98 (dd, $^4J_{H,H}$ = 3.2, $^4J_{H,H}$ = 0.7 Hz, 1 H, 6-H), 7.95 (dd, $^4J_{H,H}$ = 3.2, $^4J_{H,H}$ = 0.8 Hz, 1 H, 6-H), 7.27–7.22 (m, 2 H, 4-H$_{aryl}$), 7.15 (2×d, $^4J_{H,H}$ = 2.3 Hz, 2 H, 6-H$_{aryl}$), 7.05 (d, $^3J_{H,H}$ = 8.5 Hz, 1 H, 3-H$_{aryl}$), 7.04 (d, $^3J_{H,H}$ = 8.2 Hz, 1 H, 3-H$_{aryl}$), 6.11 (dd, $^3J_{H,H}$ = 6.5, $^3J_{H,H}$ = 6.5 Hz, 1 H, 1′-H), 6.10 (dd, $^3J_{H,H}$ = 6.4, $^3J_{H,H}$ = 6.4 Hz, 1 H, 1′-H), 5.68, 5.67 (2×s, 4 H, 11-H), 5.52–5.38 (m, 6 H, 3′-OH, 7-H$_{benzyl}$), 4.47–4.30

(m, 4 H, 5′-H), 4.25–4.19 (m, 2 H, 3′-H), 4.09–4.05 (m, 2 H, 4′-H), 2.84 (2×t, $^3J_{H,H}$ = 7.4 Hz, 4 H, 8-H), 2.70 (2×t, $^3J_{H,H}$ = 7.4 Hz, 4 H, 9-H), 2.39–2.31 (m, 2 H, 2′-H), 2.10–1.98 (m, 2 H, 2′-H), 2.07 (s, 3 H, Me), 2.06 (s, 3 H, Me), 2.02 (d, $^4J_{H,H}$ = 0.7 Hz, 6 H, $Me_{het}$), 1.98 (d, $^4J_{H,H}$ = 0.8 Hz, 6 H, $Me_{het}$) ppm. $^{13}$C NMR (100 MHz, [D$_6$]DMSO): $\delta$ = 173.7 (2×C-10), 171.2 (2×C-12), 168.0, 167.9 ($C_{het}$-4), 154.3 (2×$C_{het}$-2), 148.5 (2×$C_{aryl}$-2), 141.4, 141.3 (2×$C_{het}$-6), 136.7 (2×$C_{aryl}$-5), 129.8 (2×$C_{aryl}$-4), 126.0, 125.9 (2×$C_{aryl}$-6), 122.3 (2×$C_{aryl}$-1), 118.5 (2×$C_{aryl}$-3), 112.6, 112.5 (2×$C_{het}$-5), 87.1, 87.0 (2×C-1′), 85.3 (2×C-3′), 82.9 (2×C-11), 69.9, 69.8 (2×C-4′), 68.6 (2×C-7), 67.7 (2×C-5′), 40.4 (2×C-2′), 34.6 (2×C-9), 29.2 (2×C-8), 20.6 (2×Me), 13.8, 13.7 (2×$Me_{het}$) ppm. $^{31}$P NMR ($^1$H-decoupled, 162 MHz, [D$_6$]DMSO): $\delta$ = –9.31, –9.38 ppm. UV ($H_2O$): $\lambda_{max}$ = 315, 271, 213 nm. HRMS (ESI$^+$): calcd. 539.1431; found 539.1434. $R_t$ (HPLC) 12.8 min.

**Kinetic Studies**

**(a) Aqueous Buffers:** DMSO stock solutions (11.4 μL, 50 mM) of the triesters were diluted in water or water/DMSO (300 μL, *c* = 1.9 mM). This solution (0.3 mL) was added to aqueous buffer (0.3 mL; 50 mM phosphate buffer, pH 7.3 or 50 mM phosphate buffer, pH 6.8) containing an aqueous AZT solution (5 μL; AZT used as internal standard) at 37 °C. The final concentrations were 0.96 mM for the triesters and 24.8 mM for the aqueous buffer. Aliquots of the hydrolysis mixture (60 μL) were taken, and the hydrolysis was stopped by freezing the samples in liquid air. After thawing, samples were analyzed by analytical HPLC [Merck LiChroCART column, LiChrospher 100 reverse-phase silica gel RP-18 endcapped (5 μm); UV detection at 310 nm]. The hydrolysis of the compounds **5** and **6** was followed by integration of the decreasing peak areas in the HPLC chromatograms. The rate constants (*k*) were determined from the slope of the logarithmic degradation curve. The half-lives ($t_{1/2}$) were calculated by using the rate constants *k*.

**(b) CEM Cell Extract:** Stock solutions (1.5 mM) of the triesters in DMSO were prepared. The stock solution (20 μL) was mixed with cell extract (100 μL) and magnesium chloride solution (20 μL, 70 mM). The hydrolysis process was stopped by addition of acidic methanol (300 μL) and storage for 5 min at 0 °C. The mixtures were centrifuged at 13000 rpm for 10 min, filtered (Schleicher-Schuell Spartan 13/30, 0.2 μm), and the supernatant was analyzed as mentioned above.

**(c) U-tube Experiments**: DMSO stock solution (38.0 μL, 50 mM) was diluted with deionized water (962 μL, pH = 6.7, Donor solution D). Subsequently, phosphate-buffered saline (990 μL, pH = 7.3, Accepter solution A) was mixed with a freshly prepared PLE stock solution (10 μL, 275 U/mL). A U-tube (inner diameter: 0.7 mm, width: 3.5 cm, length: 6.0 cm) was filled with $CH_2Cl_2$ (1.0 mL). The organic layer was stirred with a small magnetic bar (500 rpm). Both solutions D and A were poured simultaneously into the arms of the U-tube. Samples were taken as follows: solution D or A (1.0 μL) was dissolved in water (499 μL). The solution (40 μL) was analyzed by RP-HPLC with fluorescence detection at 380 nm.

The pH-driven diffusion experiment was carried out with the same experimental setup, except that no PLE was added to the acceptor phase A. Instead, the pH of the phosphate buffer saline was set to 8.7.

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