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*cyclo*Sal Phosphates as Chemical Trojan Horses for Intracellular Nucleotide and Glycosylmonophosphate Delivery − Chemistry Meets Biology

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Dedicated to Prof. Dr. Dr. h.c. mult. Wittko Francke on the occasion of his 65th birthday

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Pronucleotides represent a promising alternative to improve the biological activity of nucleoside analogs in antiviral and cancer chemotherapy. In addition, pronucleotides are valuable tools for studies regarding the nucleoside/nucleotide metabolism. The aim is to achieve nucleotide delivery into cells and thereby bypass limitations during intracellular formation of nucleotides from their nucleoside precursors. The *cyclo*Sal approach is one of several conceptually different pronucleotide systems known but is the only approach in which a pronucleotide is cleaved successfully by simple but selective chemical hydrolysis. The basic concept, chemistry, different structural modifications, and their effects on the antiviral potency of the *cyclo*Sal d4TMP triesters are briefly discussed first. Then, the application of the approach to various biologically active nucleoside analogs against different targets is summarized. In the second part, the results of a conceptual extension of the *cyclo*Sal approach are presented: once *cyclo*Sal pronucleotides have passed the membrane, they should be trapped inside the cells after an enzyme-catalyzed process and then release the nucleotide. Finally, results are summarized that demonstrate that the *cyclo*Sal approach is not restricted to the delivery of bioactive nucleotides but is also applicable to the intracellular delivery of hexose-1-phosphates. Chemical synthesis, biophysical studies, and biological evaluation will be discussed in combination throughout this paper to demonstrate the strength of the *cyclo*Sal approach.

Introduction

The design and synthesis of nucleoside analogs as potential bioactive agents is a very attractive field of research. Since the discovery of the AIDS drug 3′-azido-3′-deoxythymidine (AZT), considerable efforts have been made to develop new nucleoside analogs that would be more active and less toxic inhibitors of HIV reverse transcriptase (RT).[1,2] These analogs differ from the natural nucleosides with regard to modifications of the glycon and/or the aglycon residue.[3] Today, synthetic nucleoside mimetics represent a highly valuable source of compounds that contribute significantly to the arsenal of agents for the treatment of viral diseases (e.g. HIV, herpes, and hepatitis virus infec-

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Chris Meier was born in 1962 in Berlin, Germany. He studied Chemistry at the University of Marburg/Lahn, where he received his diploma degree in 1987 and his PhD in 1989 with Professor G. Boche. During that time he became interested in bioorganic chemistry. During his postdoctoral studies at the Pasteur Institute in Paris, France, he got involved in the chemistry of nucleosides, oligonucleotides, and prodrugs. In 1991 he returned to the University of Frankfurt/Main and finished his habilitation in 1996. In 1994 he received the Adolf Messer Award for interdisciplinary research. In 1997 he became Associate Professor of Organic Chemistry at the University of Würzburg and in 1999 he moved to the University of Hamburg as a Full Professor of Organic Chemistry. His research interests are pronucleotide design for antiviral nucleoside analogs, stereoselective synthesis of carbocyclic nucleoside analogs, antisense oligonucleotides, DNA-damage induced by arylamine carcinogens, and new syntheses of activated sugar nucleotides.

MICROREVIEWS: This feature introduces the readers to the authors′ research through a concise overview of the selected topic. Reference to important work from others in the field is included.

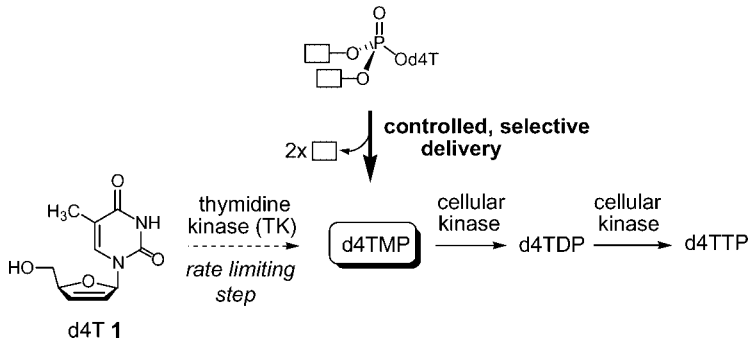


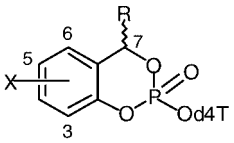
Figure 1. Bioactivation of 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T, **1**) and the principle of the pronucleotide approach.

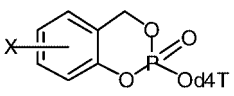
tions) and cancer. In the UK about 55% of the approved antivirals are nucleoside based, while 15% of the anticancer drugs are nucleoside related. The general mode of action of most of the nucleoside analogs is through the inhibition of DNA polymerases, including reverse transcriptase, by acting as competitive inhibitors and/or as DNA chain terminators. To act as DNA chain termination agents/polymerase inhibitors, intracellular conversion of the nucleoside analogs into their 5′-mono-, 5′-di-, and finally 5′-triphosphates is a prerequisite after cell penetration.[2,4] However, the efficient anabolism to the corresponding nucleoside triphosphates is often a major hurdle due to limited anabolic phosphorylation or catabolic processes such as deamination of the aglycon or cleavage of the glycosidic bond. Therefore, their therapeutic efficacy is compromised.[2] For example, the first phosphorylation step of the anti-HIV active dideoxynucleoside analog 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) **1**[5,6] (Figure 1) into d4T 5′-monophosphate (d4TMP) catalyzed by thymidine kinase (TK) is the critical rate-limiting step in human cells. For these reasons the rational design of nucleoside drugs is complicated.

In contrast to d4T, the intracellular fate of the majority of nucleoside analogs has not been studied in detail. These compounds are often exclusively tested as nucleosides and discarded if found to be inactive. However, a lack of information about where the metabolic blockade exists prevents further successful development. On the other hand, knowing the limitations upon phosphorylation of a nucleoside offers a chance to develop derivatives with improved biological potential. In principle, the direct administration of nucleotides like d4TMP should bypass the limiting step in the thymidine kinase-based anabolism of some nucleosides and thus should improve their biological activity. Unfortunately, nucleotides are very polar molecules that do not easily pass through cellular membranes and are dephosphorylated in the serum by unspecific phosphorylases. However, this difficulty can be surmounted by linking suitably degradable lipophilic carrier groups to the phosphate moiety that leads to neutral, membrane-permeable nucleotide delivery systems (the "pronucleotide approach"; Figure 1).[7–9]

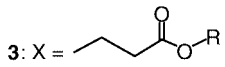
It should also be mentioned that strategies have been studied to improve the uptake and the efficiency of antiviral and anticancer nucleoside analogs. Examples are valacyclovir, famcyclovir, and 5′-lipid-, cholesterol- or simple alkyl esters of the nucleosides. However, these drugs work as prodrugs of the parent nucleoside and not of the nucleotides, hence they cannot help in the bypass of a metabolic limitation during phosphorylation. Therefore, these compounds will not be discussed further in this Microreview because the focus is on a nucleotide releasing system.

Nucleotides are charged compounds under physiological conditions (phosphate monoester pK_a values are about 1.6 and ca. 6.6). Therefore, two masking groups are necessary in order to obtain a neutral, lipophilic phosphate triester. For efficient intracellular nucleotide delivery from a pronucleotide, a specific delivery mechanism is required. Several strategies using different delivery mechanisms have been developed to achieve this goal. Among these, simple dialkyl-, diphenyl-, and dibenzyl phosphate triesters based on pure chemical hydrolysis have proved to be unsuccessful in vitro and in vivo.[8] More recent pronucleotide approaches are based on selective enzymatic or chemical activation of the masking group (tripartate prodrug system). These approaches utilize carboxyesterase activity and specific pH conditions. The delivery mechanisms of the enzyme-cleavable compounds have been summarized recently.[7,8] These enzyme-triggered approaches have proved that a successful intracellular delivery of nucleotides is possible in vitro. However, the only successful, pH-driven nucleotide delivery strategy is the *cyclo*Sal approach.[10] This approach, which is the topic of this Microreview, has been developed in our laboratories and belongs to the group of tripartate prodrug delivery systems. Three groups of derivatives will be summarized. First, the so-called *cyclo*Sal nucleotide prototype **2** will be discussed regarding its hydrolytic behavior, leading to mechanistic insights into the designed cascade cleavage mechanism and the antiviral potential (first generation *cyclo*Sal pronucleotides). A second series of triesters (**3**) that has been designed for intracellular trapping of the triesters ("lock-in" concept; second generation *cyclo*Sal pronucleotides) is then considered, and finally the application of the *cyclo*Sal approach to the delivery of pyranosyl-1-phosphates (**4**), which demonstrates that the approach is not restricted to the application with nucleotides (Figure 2).





2a: X = 5-Cl; R = H
2b: X = 6-Cl; R = H
2c: X = R = H
2d: X = 3-Me; R = H
2e: X = 3,5-Me; R = H
2f: X = 3-*t*Bu; R = H
2g: X = 3,5-*t*Bu; R = H
2h: X = 3,5-*t*Bu, 6-F; R = H
2i: X = H; R = Me
2j: X = 6-Cl; R = Me



a: R = H
b: R = Me
c: R = *i*Pr
d: R = *t*Bu
e: R = $CH_2OC(O)Me$ (AM)
f: R = $CH_2OC(O)tBu$ (POM)
g: R = $CH_2OC(O)OiPr$ (POC)

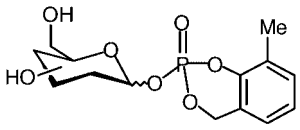


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Figure 2. Prototype *cyclo*Sal d4TMP triesters **2**, "lock-in"-*cyclo*Sal d4TMP triesters **3**, and *cyclo*Sal pyranosyl-1-phosphates **4**.

The Design of a Chemical Trojan Horse (First Generation *cyclo*Sal Pronucleotides)

The aim was to develop a selective nucleotide-delivery mechanism based on an exclusively chemically induced cascade mechanism.[10] However, the chemically driven release of the nucleotide from a lipophilic phosphate triester precursor is not as easy as it seems because a charged phosphate diester is formed as an intermediate. Such diesters are extremely resistant to further chemical hydrolysis. Therefore, simple bis(alkyl), bis(phenyl) (**5**), or bis(benzyl) nucleotide triesters (**6**) are unable to deliver the nucleotide.[11,12] No formation of d4TMP (**9**) was observed because the chemical hydrolysis always stops at the phosphate diester level (**7** and **8**, respectively). Interestingly, the influence of substituents in the aromatic rings of **5** or **6** on the hydrolysis is just the opposite: while *acceptors* in bis(phenyl) esters **5** cause a fast hydrolysis, *donors* in bis(benzyl) esters **6** result in a fast cleavage to yield the diester and (finally) benzyl alcohol (Figure 3).

A combination of these two ester-bond types as part of a cyclic bifunctional masking unit forms the basis of our pronucleotide approach. Salicyl alcohols fulfill the requirements and have been attached through the phenyl and the benzyl ester bond while the nucleoside analog is attached through an alkyl ester bond. The introduction of these three

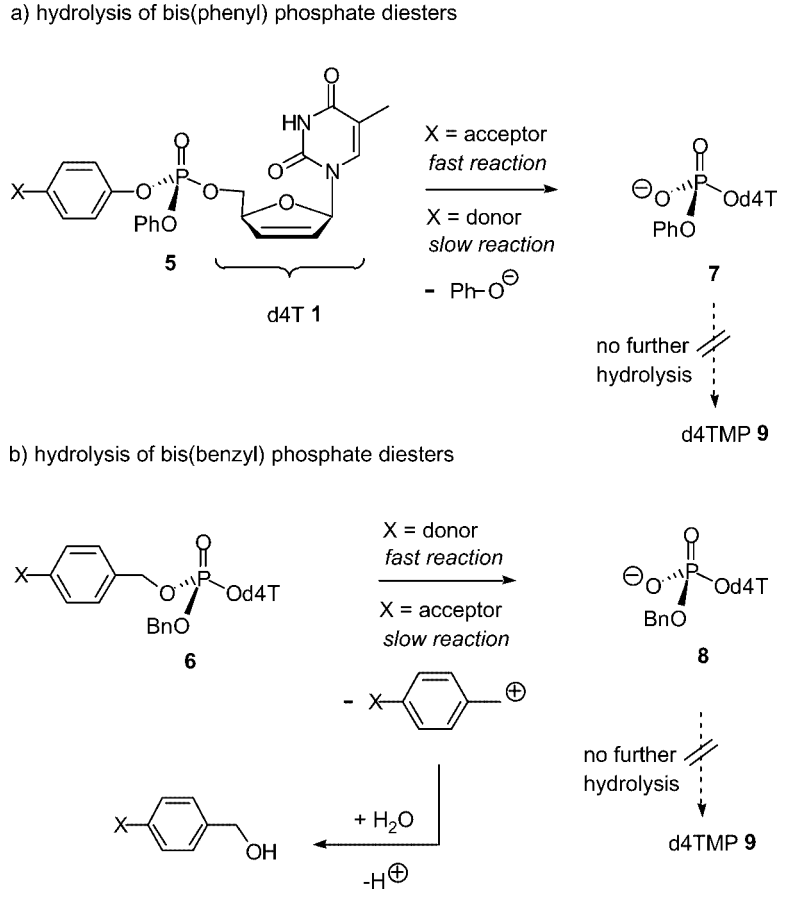

Figure 3. Hydrolysis of bis(phenyl) and bis(benzyl) phosphate triesters **5** and **6**.

different ester bonds allows sufficient discrimination in the hydrolysis process. The designed chemically induced coupling process (tandem or cascade mechanism) is as follows:[10,13] after a nucleophilic attack of hydroxide at the phosphorus atom of *cyclo*Sal triester **2**, the phenolate is displaced preferentially in an S_NP reaction − it is the best leaving group − that leads to 2-hydroxybenzylphosphate diester **10** (Figure 4, step a). As a consequence, the *ortho* substituent of the benzyl ester is changed from a very weak electron-donating group (phosphate) to a strong electron-donating group (hydroxy). This electronic change activates the remaining masking group and induces a spontaneous rupture of diester **10** to yield the nucleotide and salicyl alcohol **11** (cascade reaction; steps b_1 and b_2). The bond cleavage presumably proceeds after intramolecular proton transfer (intermediate **12**) via zwitterion **13** or 2-quinone methide **14**. In this pathway a cleavage mechanism occurs that takes place within the masking group only and thus prevents a pseudorotation process.[14] Pseudorotation at the phosphorus atom would lead to unselective liberation of the nucleotide and the nucleoside.

Although disfavored, a cleavage of the benzyl ester bond should also be taken into account (step c). Benzyl esters are cleaved preferably by an S_N1-type C_{benzyl}–O bond breakage that leads to the formation of a stabilized benzyl cation and an anionic phosphate ester group (intermediate **15**). Cation **15** is rapidly trapped by water to yield a phenyl phosphate diester like **16** (Figure 4). However, no further chemical hydrolysis of the phosphate diester takes place at physiologic pH due to the negative charge at the phosphorus atom, which prevents a further nucleophilic attack. Moreover, the charge decreases the leaving group properties of the 5′-nucleoside phosphate fragment in benzyl phosphate diesters in a possible S_N1 or S_N2 reaction at the benzyl position,[15] and even enzymatic cleavage is difficult. Consequently, the formation of phenyl phosphate diester **16** ends in a dead end.

In previous studies, salicyl alcohols **11** have been tested for their biological potency but showed neither antiviral activity nor cytotoxicity.[13] In vivo studies in mice did not show toxic side-effects.[16] It should be added that salicyl alcohol (saligenin) is used as part of the antirheumatic and

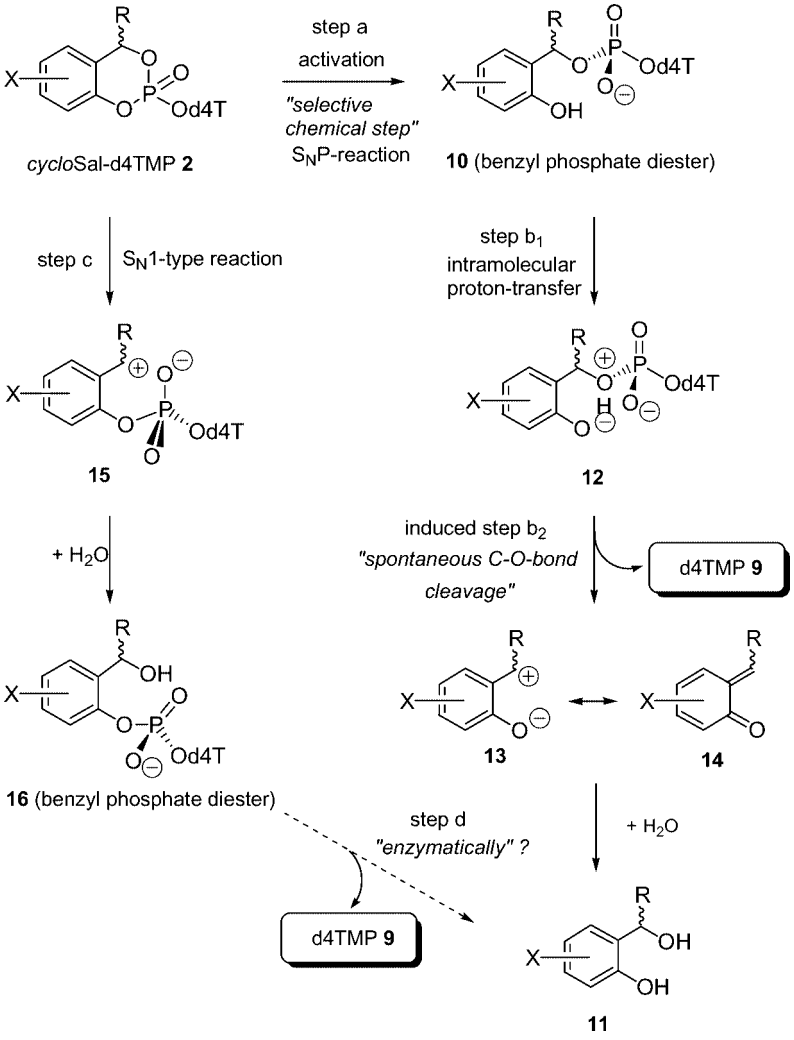

Figure 4. Two possible hydrolysis pathways of the *cyclo*Sal d4TMP triesters **2**.

analgetic drug Salicin [2-(hydroxymethyl)phenyl-β-D-glucopyranoside; Assalix®].[17] β-Glucosidases hydrolyze Salicin to D-glucose and saligenin and the latter is then slowly oxidized by cytochrome P450 to salicylic acid in the blood and in the liver.

In summary, the *cyclo*Sal concept needs only one bifunctional masking unit per equivalent nucleotide and this is cleaved chemically in a coupled manner leading immediately to the nucleotide. In contrast, enzymatically triggered pronucleotides need mask/nucleotide ratios of up to 4:1.[7–9]

Chemistry

For the synthesis of the prototype *cyclo*Sal d4TMP triesters **2**, salicyl alcohols **11** had to be prepared first from the corresponding salicylic aldehydes, acids, or esters by reduction ($NaBH_4$ or $LiAlH_4$; Figure 5) in high yields. However, in most cases the aldehydes/acids/esters were not commercially available. Diols **11** therefore had to be synthesized from appropriately substituted phenols. Selective *ortho*-formylations were achieved according to the Casiraghi[18] or Rieche formylation protocols.[19] Both methods led to salicyl aldehydes, which could then be reduced to the corresponding diols **11**. Alternatively, mild hydroxymethylation according to Nagata et al.[20] or using formaldehyde in aqueous basic medium[21] was used.

Generally, these methods are highly efficient and suitable for the synthesis of the prototype *cyclo*Sal derivatives without a substituent in the benzyl position. 7-Methylated salicyl alcohol **11i** was prepared by alkylation of salicyl aldehyde with methyllithium. Other syntheses of the required salicyl alcohols will be described later in this Microreview.

The synthesis of the *cyclo*Sal pronucleotides was carried out using reactive phosphorus(III) reagents (Figure 6). Thus, diols **11** were treated with phosphorus trichloride to give chlorophosphites **17**, which were treated with the nucleoside analog (e.g. d4T) in the presence of diisopropylethylamine (DIPEA; Hünig's base) to yield the cyclic phosphite triesters. These were oxidized in a one-pot-reaction with *tert*-butylhydroperoxide (TBHP) or dimethyldioxirane. Generally, the phosphate triesters **2** were obtained in reasonable yields (50–73%) as mixtures of stereoisomers.[10]

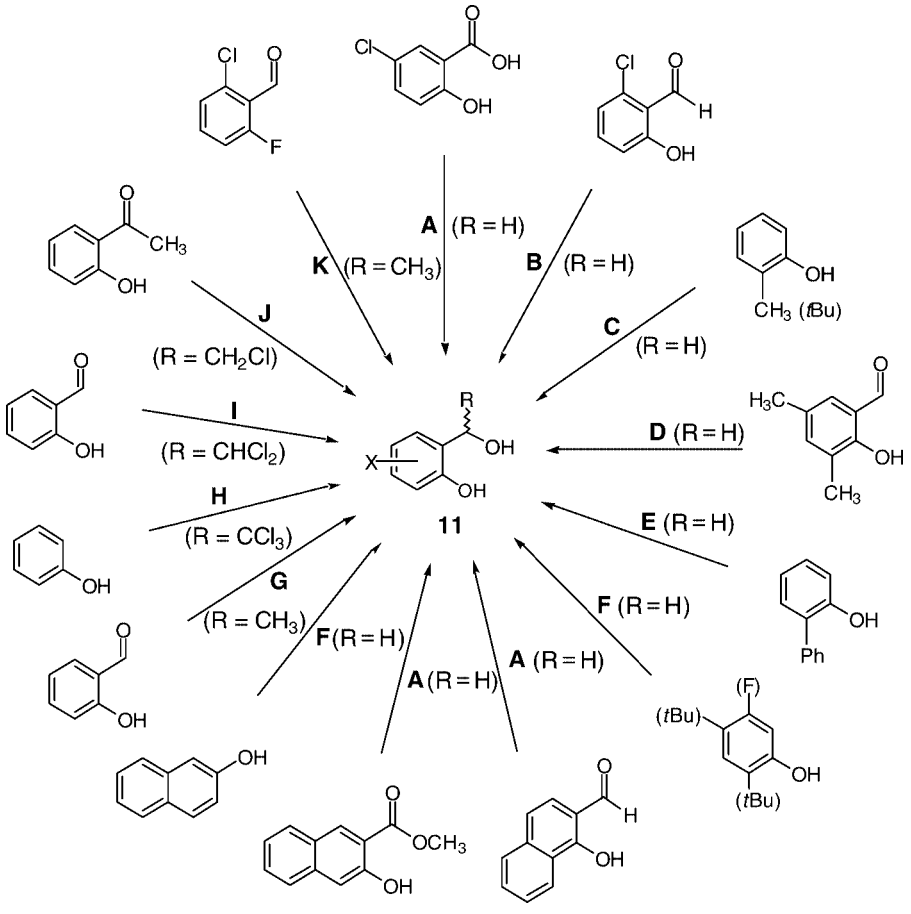

Figure 5. Synthetic pathways to the salicyl alcohols **11**. Method **A**: $LiAlH_4$ or $NaBH_4/I_2$, THF, room temp., 5 h; method **B**: $NaBH_4$, EtOH, room temp., 4 h; method **C**: i) $(HCHO)_n$, $SnCl_4$, 2,6-lutidine, toluene, 110 °C, 16 h; ii) $NaBH_4$, 2-propanol, room temp., 5 h; method **D**: $NaBH_4$, 2-propanol, room temp., 5 h; method **E**: i) $(HCHO)_n$, $PhB(OH)_2$, propionic acid (cat)., toluene, reflux, 10 h; ii) 30% H_2O_2, THF, 0 °C, 35 min; method **F**: 37% HCHO/H_2O, KOH, room temp., 24 h; method **G**: methyllithium, Et_2O, 0 °C, 1.5 h; method **H**: i) CCl_3CHO, $PhB(OH)_2$, propionic acid (cat.), toluene, reflux, 10 h; ii) 30% H_2O_2, THF, 0 °C, 35 min; method **I**: CH_2Cl_2, methyllithium, Et_2O, –78 °C, 1.5 h; method **J**: i) 2,3,4,5,6,6-hexachloro-2,4-cyclohexadien-1-one, EtOH, reflux, 7 h; ii) 2-propanol, room temp., 5 h; method **K**: i) NaOH, 75 °C, 45 h; ii) methyllithium, Et_2O, 0 °C, 1.5 h.

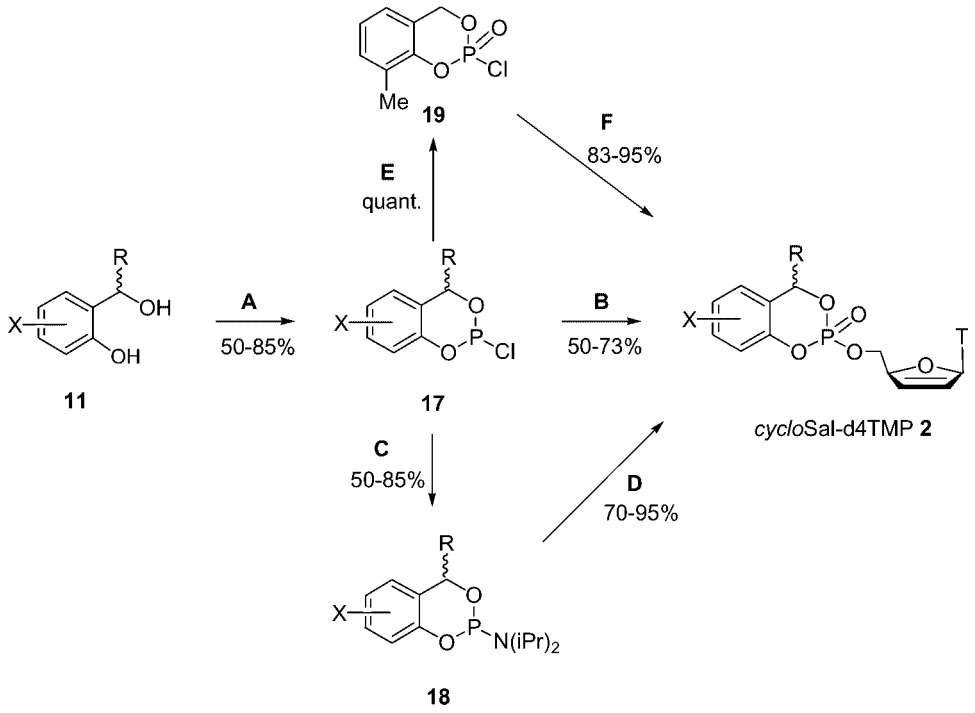

Figure 6. Synthesis of the *cyclo*Sal d4TMP triesters **2**. Method **A**: PCl_3, pyridine, Et_2O, –10 °C, 2 h; method **B**: i) d4T **1**, DIPEA, CH_3CN, –20 °C to room temp., 1 h; ii) TBHP, CH_3CN, –20 °C to room temp., 1 h; method **C**: diisopropylamine (2 equiv.), Et_2O, 0 °C, 30 min; method **D**: i) d4T **1**, pyridinium chloride, tetrazole or imidazolium triflate, CH_3CN, 0 °C, 30 min; ii) TBHP, CH_3CN, room temp., 1 h; method **E**: O_2, toluene, room temp., 16 h; method **F**: d4T **1**, pyridine, –50 °C, 4 h.

Alternatively, chlorophosphites **17** were treated with diisopropylamine to yield the phosphoramidites **18**. The coupling with the nucleoside analog was carried out in acetonitrile in the presence of pyridinium chloride or imidazolium triflate as coupling activator. In some cases the latter procedure resulted in yields of more than 90% using imidazolium triflate as activator.[22] Recently, we obtained very high yields (85–93%) by using the corresponding phosphorochloridates. Thus, chlorophosphites **17** were oxidized with oxygen in toluene overnight to yield the phosphorochloridates **19**. These were treated with the nucleosides in pyridine at –50 °C. However, the starting chloridites should be distilled and be very pure.

Proof-of-Principle − D4TMP Release from *cyclo*Sal d4TMP Pronucleotides

Extensive studies were performed in order to investigate the designed delivery mechanism of d4TMP from the *cyclo*Sal triesters.[10,23] Chemical hydrolysis studies in different buffer solutions at different pH values proved that all prototype compounds released d4TMP selectively in a pH-dependent manner. As second product, the salicyl alcohols **11** were detected by means of HPLC analysis. D4TMP was identified unequivocally by using ion-pairing eluents and the co-elution technique. The half-lives of the triesters were determined in these studies (Table 1). Mechanistically, the second step of the degradation pathway is an S_N1-type reaction or a nucleophilic attack at the benzyl position and not a further nucleophilic reaction at the phosphorus center. This was proven in a hydrolysis experiment with ^{18}O-labeled water. Electrospray-mass spectrometric analysis of the hydrolysis products showed mass peaks of [M + 2] for d4TMP and [M + 2] for the salicyl alcohol **11**. In the case of a second nucleophilic reaction at phosphorus, the d4TMP peak should have a mass of [M + 4].

As expected, the half-lives depend on the substitution pattern of the aromatic ring. Generally, acceptor substituents like a 5- or 6-chloro atom cause a decrease in hydrolytic stability while donor substituents like 3-methyl-, 3,5-dimethyl-, and, in particular, 3-*tert*-butyl or 3,5-di-*tert*-butyl group(s) lead to an increased stability with respect to the unsubstituted prototype triester **2c**.

The presence of *tert*-butyl groups has a surprisingly pronounced influence on the hydrolysis stability. Although the electron-donating property of a *tert*-butyl group differs only slightly from the +I effect of a methyl group, triesters **2f** (3-*t*Bu) and **2g** (3,5-*t*Bu) have considerably higher half-lives than their methyl counterparts **2d** and **2e**. We attribute this to differences in the lipophilicity and steric reasons, both of which affect the accessibility of the phosphate group.

In addition to these HPLC-based studies, we were able to study the delivery mechanism by ^{31}P NMR spectroscopy. However, phosphate buffer is not an appropriate buffer system for this experiment. The compounds were therefore dissolved in an imidazole/HCl buffer adjusted to pH 7.3. A similar NMR experiment using only a DMSO/water mixture has been performed earlier in the hydrolysis of 5-nitro-

Table 1. Half-lives, product ratio, and antiviral data of the prototype *cyclo*Sal d4TMP triesters **2**.

Compound	Modification X and/or R	$t_{1/2}$ at 37 °C[a] and pH 7.3[d]	Product ratio d4TMP/**16**[e]	EC_{50} [μM][b] CEM/O HIV-1	CEM/O HIV-2	CEM/TK$^-$ HIV-2	CC_{50} [μM][c]
2a	5-Cl	1.1	100:0	0.42	1.40	2.67	49
2b	6-Cl	0.9	100:0	0.087	0.15	0.8	36
2c	5-H	4.4	99:1	0.20	0.22	0.15	50
2d	3-Me	17.5	94:6	0.057	0.07	0.048	32
2e	3,5-Me	29	92:8	0.09	0.17	0.08	21
2f	3-*t*Bu	96	92:8	0.18	0.65	0.33	35
2g	3,5-*t*Bu	73	66:34	1.1	1.2	2.0	27
2h	3,5-*t*Bu; 6-F	6.2	100:0	0.12	0.33	0.6	41
2i	7-Me	0.25	17:83	0.22	0.34	35	152
2j	6-Cl,7-CH_3	2.2	100:0	0.19	0.25	7	42
d4T (**1**)	–	–	–	0.18	0.55	28	35

[a] Hydrolysis half-lives in hours. [b] Antiviral activity: 50% effective concentration. [c] Cytotoxic concentration: 50% cytostatic/toxic activity. [d] 25 mM Sodium phosphate buffer. [e] Ratio of d4TMP: phenyl phosphate diester **16** determined by ^{31}P NMR spectroscopy.

*cyclo*Sal d4TMP.[13] In that case d4TMP was detected as the sole hydrolysis product, which points to an entirely selective delivery reaction.

The prototype *cyclo*Sal d4TMP **2c** led to d4TMP in 99% yield in the imidazole/HCl buffer. Moreover, 1% of phenyl phosphate diester **16** (Figure 4) was detected. As expected, diester **16** proved to be entirely stable for several weeks in the NMR tube at 37 °C. This diester was found in amounts of 8% in the cases of 3,5-dimethyl triester **2e** or 3-*tert*-butyl triester **2f**. The worst case was found for the introduction of two *tert*-butyl groups in the 3- and 5-positions (compound **2g**; 34% of diester **16**). Here, a concurrent S_N1-type reaction plays a considerable role in the degradation. In contrast, 5-chloro- (**2a**), 6-chloro- (**2b**), and 6-fluoro (data not shown) substituents allowed delivery of d4TMP exclusively.

Surprising properties, namely an enormous decrease in chemical stability, were observed for 7-methyl-*cyclo*Sal d4TMP (**2i**). The half-life dropped to 0.25 h (compared to 4.4 h for prototype triester **2c**) and even the product distribution changed dramatically. NMR studies confirmed that the major product was diester **16** (83%; Table 1). Thus, the S_N1-type reaction is now preferred! This may be due to the formation of a secondary benzyl cation intermediate as compared to a primary benzyl cation in the prototype cases. The additional alkyl residue stabilizes the cation by a +I effect. This interpretation was further confirmed by density functional theory (DFT) calculations at the B3LYP/6-311G(d,p) level on model compounds of the prodrug systems. The activation energy barrier for the initial S_N1 reaction is considerably lower for 7-methyl-*cyclo*Sal methylphosphate than for the prototype triester analog[24] (unpublished results). The amount of the phenyl phosphate diester **16** can be further increased by introduction of methyl groups into the *cyclo*Sal ring. These stabilize the phenyl ester bond of the *cyclo*Sal triesters **2** and so favor the benzyl-C–O bond cleavage. Indeed, 3,7-dimethyl- and 3,5,7-trimethyl-*cyclo*Sal triesters yielded 94% and 99% of diester **16**, respectively.

On the other hand, this predominant S_N1-type reaction should be prevented by introduction of an electron-withdrawing substituent like a halogen atom in the 6-position of the aromatic ring as this would decrease the stability of the intermediate benzyl cation and favor again the phenyl ester bond cleavage. Hence, 6-chloro-7-methyl-*cyclo*Sal triester **2j** was prepared. ^{31}P NMR studies proved that the degradation led exclusively to benzyl phosphate diester **10** and subsequently to d4TMP. Obviously, the introduction of the 6-chloro atom compensates the stabilizing effect of the 7-methyl group entirely. At the same time, this compound displays an increased chemical stability [0.9 h (**2b**) vs. 2.2 h (**2j**)]. In conclusion, the hydrolysis pathway and half-life can be fine-tuned by introduction of appropriate substituent in the aryl moiety.

No evidence of an enzymatic degradation in RPMI-1640 medium containing 10% fetal calf serum (pH 7.3) was observed. Studies in CEM-cell extracts showed that the hydrolysis half-lives decreased slightly compared to hydrolysis in buffer. Further studies of the prototype triesters in human serum (10% serum in phosphate buffer) exhibited no difference in stability. Again, no enzymatic contribution could be detected, which confirmed the initial idea of a delivery mechanism that is independent of enzymatic activation.

All data obtained from hydrolysis and NMR studies are in perfect agreement with the designed degradation cascade reaction mechanism and show convincingly that the mechanism shown in Figure 4 can be controlled efficiently by structural modification of the *cyclo*Sal moiety.

Antiviral Evaluation

The in vitro antiviral potency of the *cyclo*Sal nucleotides against HIV-1 and -2 in CEM cells was assessed. It became apparent that the unsubstituted prototype **2c** (0.20 μM) and 6-chloro- (**2b**, 0.087 μM), 3-methyl- (**2d**, 0.057 μM), and 3,5-dimethyl-*cyclo*Sal d4TMP **2e** (0.09 μM) showed comparable or even higher antiviral potency in a wild-type T-lymphocytic cell-line (CEM/O) compared to d4T **1** (0.18 μM, Table 1). Moreover, the complete retention of the antiviral potency of triesters **2c**, **2d** (3-methyl), **2e** (3,5-dimethyl), **2f**

(3-*tert*-butyl), and **2h** (3,5-di-*tert*-butyl-6-fluoro) in mutant thymidine kinase-deficient cells (CEM/TK$^-$) is particularly striking. The antiviral data and the hydrolysis half-lives clearly point to the fact that a certain degree of stability is needed, but beyond this point no further improvement of activity could be observed. The short half-life of the 5-chloro triester **2a** ($t_{1/2}$ = 1.1 h) seems to be responsible for a considerable loss of antiviral activity in the CEM/TK$^-$ cell assay, although its antiviral activity in the TK-competent cells (CEM/O cells) is comparable to that of d4T. Obviously, the compound hydrolyzes extracellularly to yield d4TMP, which cannot migrate into the cells. After extracellular dephosphorylation, d4T is taken up and converted into the triphosphate. Similar results were obtained for the 6-chloro derivative **2b**. It should be added that *cyclo*Sal triesters, as such, have no inhibitory effect on DNA synthesis, which is consistent with a mechanism of action for the *cyclo*Sal triesters that relies on the formation of free d4TTP. This was proven in experiments using an isolated recombinant RT/RNA/DNA template primer.

In summary, these results confirm i) the cellular uptake of the compounds, ii) the highly selective intracellular delivery of d4TMP, and iii) the independence of the biological activity on cellular thymidine kinase activation for some of the described *cyclo*Sal phosphate triesters.

Nevertheless, the in vitro anti-HIV assay gives only an indirect proof of the intracellular delivery of d4TMP. Therefore, incubation experiments of wild-type CEM/O and CEM/TK$^-$ cells with tritium-labeled 3-Me-*cyclo*Sal d4TMP (**2d**) were carried out.[25] The amount of d4TMP in CEM/O cells was considerably higher (15-fold after 6 h incubation) than the d4TMP concentration resulting from the nucleoside d4T. In addition, an increase in the concentration of d4TTP was observed (16-fold), which may explain the higher activity of the prototype *cyclo*Sal d4TMPs in wild-type CEM cells compared to d4T. These results confirm that *cyclo*Sal d4TMPs successfully bypass thymidine kinase and release d4TMP inside the cells.

Furthermore, the *cyclo*Sal d4TMP triesters demonstrated significant antiviral activity in AZT-resistant H9^{r}AZT^{250} cells.[26] This resistance is concomitant with a fivefold lower expression of the TK gene in comparison to parental H9 cells. The consequence is that d4T also shows reduced antiviral potency due to insufficient phosphorylation. In contrast, prototype *cyclo*Sal d4TMP **2c** proved to be equipotent in parental and in H9^{r}AZT^{250} cells (EC_{50} = 0.3 and 0.5 μM), proving again the independence of the expressed TK levels.

Interaction of *cyclo*Sal Compounds and Human Acetyl- (AChE) and Butyrylcholinesterase (BChE)

Because *cyclo*Sal phosphate triesters are reactive organophosphates, they may act as potential inhibitors of human acetylcholine esterase (AChE). A large number of *cyclo*Sal nucleotide triesters bearing different nucleoside analogs and substitution patterns in the aromatic ring have been studied concerning their ability to inhibit cholinesterases of different origins. It was shown that *none* of the tested triesters show any inhibitory effect against the physiologically relevant human acetylcholinesterase (AChE; isolated enzyme) or against AChE from beef erythrocytes, calf serum, and electric eel (*electrophorus electricus*).[27]

In contrast, inhibition of butyrylcholinesterase (BChE) has been observed for some triester derivatives in human and mouse serum.[28] The physiological role of this enzyme is not known, although it is not related to nerve signal transfer. The *cyclo*Sal pronucleotides show strong competitive inhibition with respect to the substrate acetylcholine chloride ($K_i/K_m \approx 2\times10^{-5}$) and act by time-dependent irreversible inhibition of the human serum BChE. Detailed studies have demonstrated that the inhibitory effect against BChE is highly dependent on the nature of the nucleoside analog, the substitution pattern in the *cyclo*Sal moiety, and particularly on the stereochemistry at the phosphorus atom (Figure 7).

A few *cyclo*Sal NMP triesters have been separated into their diastereoisomers by semi-preparative HPLC. The separated diastereomers were subjected to the assays with AChE from electric eel and BChE from human serum. Again, *no* inhibitory activity was found against AChE for either diastereoisomer. In contrast, a marked difference in the inhibition of the triesters against human BChE was observed. In all cases, and independent of the nucleoside attached, the S_p diastereoisomer was found to be inhibitory towards the enzyme while the R_p-isomers were entirely non-inhibitory. The difference in IC_{50} between the two diastereomers of the *cyclo*Sal nucleotide prodrugs was found to be up to >200-fold.

Moreover, an interesting inverse correlation between the inhibitory potency and the antiviral activity was observed.

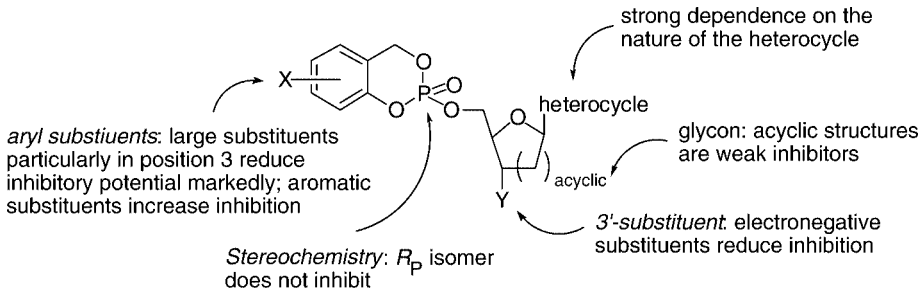

Figure 7. Structure–activity relationship of the interaction of *cyclo*Sal triesters and butyrylcholinesterase.

As a rule, the most antivirally active diastereomer corresponds to the least BChE-inhibitory diastereomer. Thus, the R_p diastereomer of 3-Me-*cyclo*Sal d4TMP (**2d**), which is not inhibitory to human BChE at 50 μM, is fivefold more anti-HIV active (0.087 μM) than the S_p diastereomer. In contrast, the S_p diastereomer is quite inhibitory against BChE (0.24 μM). In deciding which *cyclo*Sal anti-HIV prodrug(s) should be chosen as possible clinical candidate(s), clearly the correct phosphorus configuration is R_p. However, separation of the diastereomers is a laborious task.

In this respect, both the hydrolytic properties and the inhibitory potential of the *cyclo*Sal triesters can be modulated by changing the substitution pattern of the aromatic ring. In particular, bulky alkyl substituents in the 3-position lead to a considerable reduction of the inhibitory potential. This effect is even more pronounced if two substituents are introduced in the 3- and 5-positions. Thus, both diastereomers of 3,5-dimethyl-*cyclo*Sal d4TMP (**2e**) and 3,5-di-*tert*-butyl-*cyclo*Sal d4TMP (**2g**) are non-inhibitory to BCHE! However, two properties of these triesters are unfavorable: their chemical stability is very high (29 h and 73 h, respectively) and the phenyl phosphate diesters **16** (8% and 34%, respectively, Table 1) and benzyl phosphate diesters **10** form concomitantly. However, a new *cyclo*Sal group, namely 3,5-di-*tert*-butyl-6-fluoro-*cyclo*Sal d4TMP (**2h**), that has four desirable properties has been developed by combining different properties of various *cyclo*Sal phosphate triesters reported in this Microreview.[29] The two *tert*-butyl groups in this compound avoid the interaction with BChE. The combination with the fluorine atom in the 6-position was done for two reasons. Firstly, in previous studies with 6-chloro-*cyclo*Sal d4TMP (**2b**) the acceptor group prevents the formation of the corresponding phenyl phosphate diester **16**. Thus, although two *tert*-butyl groups are present, hydrolysis of **2h** should lead to a highly selective delivery of d4TMP. Secondly, in the same study the halogen atom in the 6-position reduces the chemical stability of the *cyclo*Sal triester (Table 1). So, a considerable decrease in hydrolysis stability for triester **2h** compared to the *tert*-butyl- (**2f**) and di-*tert*-butyl (**2g**) triesters was expected. The target triester **2f** was prepared starting from commercially available 3-fluorophenol. First, an acid-catalyzed alkylation with isobutene was carried out to give 4,6-*tert*-butyl-3-fluorophenol in 70% yield. This compound was treated with formaldehyde in a basic solution to give the formation of the corresponding salicyl alcohol **11h** in 71% yield. This diol was treated with PCl_3 and subsequently with d4T to give *cyclo*Sal triester **2h** in 47% yield. As expected, the chemical hydrolysis of triester **2h** at pH 7.3 showed a significantly reduction in stability ($t_{1/2}$ = 6.2 h; Table 1). In ^{31}P NMR studies in imidazole/HCl buffer at pH 7.3, only the formation of d4TMP was observed from triester **2h** which proves that the introduced fluorine atom completely prevents the formation of phenyl phosphate diester **16** (Figure 4). Incubation studies with human AChE showed that the diastereomeric mixture of compound **2h** is not inhibitory to this enzyme. Moreover, incubations with human serum confirmed that this triester is almost not inhibitory towards BChE ($IC_{50(BChE)}$ = 48 μM). Finally, in antiviral tests using HIV-1- and HIV-2-infected wild-type CEM/O cells, triester **2h** (0.16 μM) exhibits pronounced antiviral potency that is even better than the activity of d4T (**1**). Most importantly, the antiviral activity is fully retained in HIV-2 infected CEM/TK$^-$ cells. Thus, salicyl alcohol **11h** is the best masking group so far.

*cyclo*Sal Pronucleotides of Different Nucleoside Analogs

The *cyclo*Sal approach has also been applied to different nucleoside analogs (Figure 8). In the case of derivatives of 2′-deoxyadenosine (dA), the metabolism into the bioactive triphosphates is more complicated than for thymidine analogs. Prior to phosphorylation, adenosine deaminase (ADA) converts the adenine heterocycle rapidly into hypoxanthine. The 2′-deoxyinosine derivative thus formed is then phosphorylated to give the monophosphate, which is reaminated in two enzymatic steps to yield the corresponding dAMP derivative. Thus, a total of four metabolic processes are needed to get to the monophosphate, and one of these steps is often inefficient. Therefore, the *cyclo*Sal approach was applied to 2′,3′-dideoxyadenosine (ddA; **20**) and 2′,3′-dideoxy-2′,3′-didehydroadenosine (d4A; **21**). The chemical synthesis of these *cyclo*Sal derivatives is more complicated than before due to the exocyclic amino group of the adenine heterocycle. Protecting group chemistry can not be used efficiently because of the low chemical stability of the nucleoside analog (against acidic conditions) or the *cyclo*Sal triesters (against acidic and basic conditions). Finally, the target triesters were prepared without amino protection at low temperature using the chlorophosphite method in moderate yields (ca. 40–50%). The anti-HIV activity is increased 100-fold compared to the parent nucleoside analog ddA (**20**), while the activity of **21** is increased 600-fold after *cyclo*Sal mask introduction.[30] Thus, both *cyclo*Sal derivatives achieved the adenosine deaminase-bypass. However, in the BChE inhibition assay these adenosine analog phosphate triesters proved to be the most inhibitory compounds of all nucleosides studied so far. In the same project, two fluorinated ddA derivatives (**22** and **23**) were also used.[31] The activity of 2′-*ara*-F-ddA (**22**) is 10-fold higher but, more interestingly, the entirely inactive nucleoside 2′-*ribo*-F-ddA (**23**) was converted into an anti-HIV active compound after modification with the 3-methyl-*cyclo*Sal moiety. The reason for this unexpected difference in bioactivity of the two nucleosides may be related to a conformational difference caused by the fluorine atom. This was the first example of the conversion of an inactive nucleoside into a bioactive derivative by the *cyclo*Sal approach.

Recently, the purine nucleoside analogs carbovir **24** and abacavir **25** were also studied. No improvement against HIV and HSV-1/2 was observed for carbovir compared to the parent nucleoside. However, the antiviral potency of abacavir was improved after *cyclo*Sal modification against HIV and antiviral activity was also observed against HSV-1/2, whereas abacavir is non-inhibitory against HSV-1/2.[32]

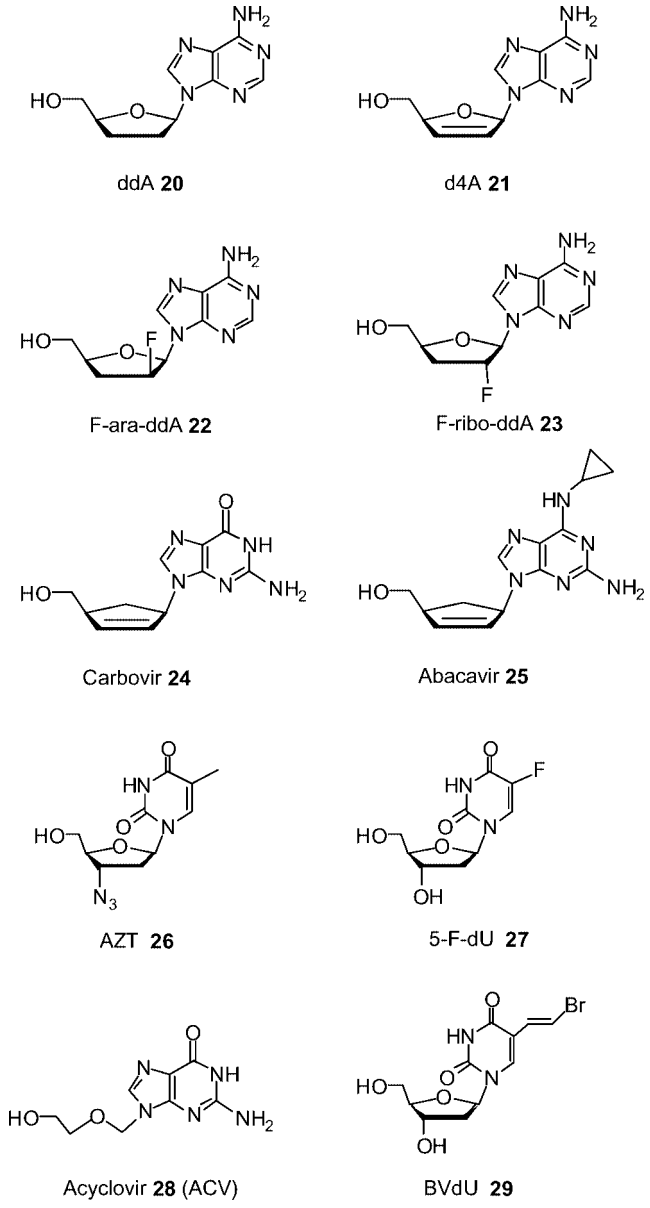

Figure 8. Nucleoside analogs used with the *cyclo*Sal approach.

The Failure of the *cyclo*Sal AZTMP and 5-Fluoro-2′-deoxyuridine (FdU) Triesters

This approach was also applied to the delivery of the monophosphate of the clinically used anti-HIV drug 3′-azidothymidine (AZT, **26**; Figure 8). The preparation of these compounds was achieved by the chlorophosphite method. Biophysical studies confirmed that these triesters behave like the corresponding *cyclo*Sal d4TMP triesters: they release AZTMP selectively with half-lives slightly higher than the *cyclo*Sal d4TMP triesters. The anti-HIV test of these compounds showed pronounced activity in the wild-type CEM cells against both HIV-1 and HIV-2 (EC_{50} = 0.006 μM vs. 0.006 μM for AZT). However, *cyclo*Sal AZTMP derivatives lost most of their antiviral activity in mutant thymidine kinase-deficient CEM/TK$^-$ cells. Nevertheless, 3-methyl- and 3,5-dimethyl-*cyclo*Sal AZTMP were still considerably more active than the parent nucleoside AZT (EC_{50} = 7 μM vs. >100 μM for AZT).[33] This was a surprising result because the mutant cell-line is unable to phosphorylate AZT into its monophosphate and the retention of antiviral activity has been expected again. The metabolic bottleneck in the activation of AZT is the conversion of AZTMP into AZTDP.[34] To investigate the reasons for the failure of the *cyclo*Sal AZTMP derivatives, a study was conducted using radiolabeled 3-methyl-*cyclo*Sal AZTMP.[35] In CEM/O cells the concentration of AZTMP found after incubation with the *cyclo*Sal AZTMP triester was considerably *lower* than the AZTMP concentration found after incubation with AZT. However, after only 48 h of incubation the AZTMP levels formed from the triester reached the same level as those formed from the nucleoside AZT. In contrast, AZT formed extremely low levels of AZTMP in CEM/TK$^-$ cells (slightly over detection limit), while the levels formed by hydrolysis of the *cyclo*Sal AZTMP triester were considerably higher. This at least explains why the compounds keep some antiviral activity in contrast to AZT. Nevertheless, a comparison of the AZTTP levels formed from the triester in CEM cells and in mutant CEM/TK$^-$ cells after six hours showed a 100-fold lower AZTTP level in the mutant cell-line. This may explain the 100-fold decrease in antiviral activity in the mutant cell-line as compared to wild-type CEM cells. Our interpretation of the failure of the AZTMP triesters is as follows (Figure 9): because the conversion of AZTMP into AZTDP is the bottleneck in the activation to the triphosphate, an increase of the AZTMP level by release from a pronucleotide is counterproductive for the forward phosphorylation. Moreover, assuming an efficient enzymatic dephosphorylation of AZTMP into AZT, the intracellular delivery of AZTMP cannot improve the activity. In wild-type cells, AZT formed by this catabolic reaction is readily rephosphorylated into AZTMP by cellular thymidine kinase (TK) and, thus, no effect on the antiviral activity will be observed. In contrast, in mutant CEM/TK-deficient cells this process has a severe consequence: if the released AZTMP from the pronucleotide is readily dephosphorylated, the intracellular pool of AZTMP is dramatically reduced because no TK phosphorylation restores the AZTMP pool. For AZT, this dephosphorylation/rephosphorylation metabolism plays a significant role due to rate-limiting phosphorylation into AZTDP and the non-phosphorylation to AZTMP in TK$^-$ cells. A similar process does not occur in the case of d4T because the metabolism bottleneck is the formation of d4TMP by cellular TK, while d4TMP released from the pronucleotide is readily phosphorylated into d4TDP.

An enzyme that may be involved in the dephosphorylation of AZTMP is 3′,5′-(deoxy)nucleotidase.[35,36] The relative efficacy of the dephosphorylation of dTMP, d4TMP, and AZTMP by this enzyme is 1, 0.08, and 2, respectively. This shows that AZTMP is a better substrate than dTMP while d4TMP is dephosphorylated about 20-fold less efficiently. This may point to an important factor when pronucleotides are used in order to bypass metabolic limita-

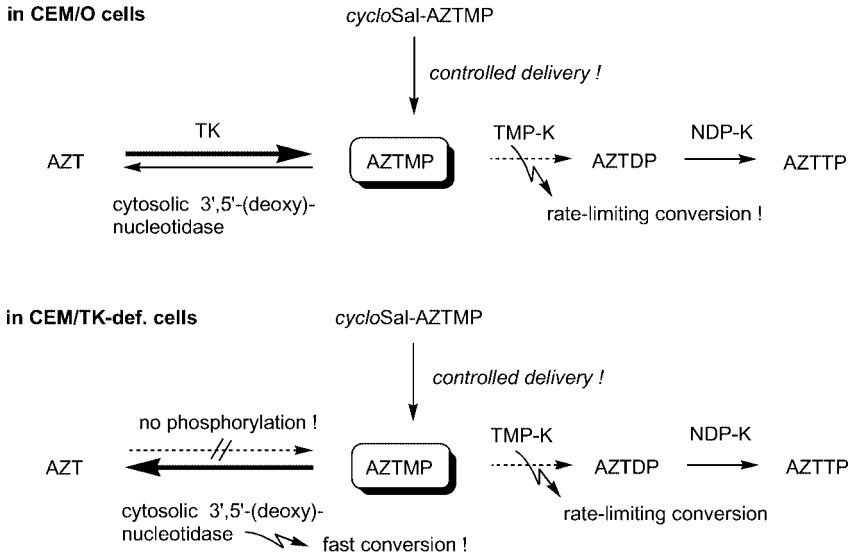

Figure 9. Comparative metabolism of *cyclo*Sal AZTMP in CEM/O$^-$ and CEM/TK$^-$ cells.

tions: although a selective release of the nucleotide occurs, this alone may not be sufficient to overcome a limitation if catabolic processes are also involved.

It should be mentioned that other AZTMP pronucleotides also lose most of their activity in the mutant TK-deficient cell-line.[7,8,37] This clearly point to a general problem during AZT metabolism and not to a specific problem of the *cyclo*Sal pronucleotide approach.

A comparable failure in the enhancement of bioactivity was observed in the case of the antitumor agent 5-fluoro-2′-deoxyuridine (FdU, **27**; Figure 8). 5-Fluorouracil (5-FU) is used frequently in antitumor chemotherapy. The mechanism of action of this compound includes the intracellular formation of 5-fluoro-2′-deoxyuridine monophosphate (FdUMP). FdUMP does not need to be phosphorylated because it acts as such as an inhibitor of thymidylate synthase, which catalyzes the methylation of dUMP into dTMP. To avoid the lengthy metabolism of 5-FU to FdUMP we used the *cyclo*Sal approach to the intracellular delivery of FdUMP.[38]

The chemical synthesis was first carried out using the chlorophosphite method. However, the regioselectivity could be controlled only moderately: we obtained a 2:1 selectivity for the 5′-O-phosphorylated product and the 3′,5′-O-bisphosphorylated side product. Using *cyclo*Sal phosphorchloridates **19** instead of the phosphorchloridites **17**, the regioselectivity increased to 5:1 in favor of the 5′-O-phosphorylated compound. Interestingly, only very minor amounts of the 3′-O-phosphorylated *cyclo*Sal FdUMP triester could be isolated. *cyclo*Sal FdUMP triesters were easily separated into the diastereomers by semi-preparative HPLC. Chemical hydrolysis studies proved that FdUMP was released. Surprisingly, both diastereomers were found to be non-inhibitory to BChE. Biological evaluation of the separated diastereomers of *cyclo*Sal FdUMP phosphate triesters was performed with several tumor cell-lines. Sometimes FdU and the *cyclo*Sal triesters were found to be equipotent but often the biological activity of the *cyclo*Sal triesters were found to be even lower (IC_{50} = 0.057 μM) than FdU (IC_{50} = 0.009 μM). Additionally, the anti-thymidylate synthase activity was determined in a specific inhibition assay. As before, *cyclo*Sal FdUMP triesters proved to be 10-fold less efficient in L1210/O cells or mutant L1210/TK$^-$ cells than FdU. Nevertheless, compared to the clinically used 5-FU (IC_{50} = 0.7 μM), the *cyclo*Sal compounds are 23-fold more active.

Taking all these results together, an inefficient cellular nucleotide delivery obviously takes place. One possibility may be the formation of 3′,5′-cyclic FdUMP (cFdUMP) instead of FdUMP because of the hydroxy group at the 3′-position. The cyclic diester does not act as an inhibitor of thymidylate synthase. However, all data obtained from chemical hydrolysis studies definitively exclude the formation of cFdUMP. Another possibility is a limitation of the cellular uptake of the triesters. Evidence for this assumption may be taken from the following experiment: the aforementioned thymidylate synthase assay was repeated in permeabilized L1210/O cells, with FdU, FdUMP, and 5-Cl-*cyclo*Sal FdUMP as test compounds. The IC_{50} values obtained were 2.5, 0.15, and 0.3 μM, respectively. Here, the triester clearly shows a comparable biological activity to FdUMP and a higher potency than FdU. Moreover, this experiment confirms that FdUMP and not FdU is released from the pronucleotide.

Finally, a competing intracellular dephosphorylation of FdUMP, as for AZTMP, cannot be excluded. Evidence for that interpretation can be taken from the fact that FdUMP is a very good substrate for 3′,5′-(deoxy)nucleotidase.[36]

As for AZTMP pronucleotides, the FdUMP pronucleotides reported by other authors also lose their activity in the mutant TK-deficient cell-lines. Again, this clearly points to an intrinsic problem in FdU metabolism.

Summarizing this part, the given results are excellent examples for demonstrating that *cyclo*Sal triesters may be

used as biochemical tools to study nucleoside metabolism and allows new insights into biosynthetic pathways.

Application of the *cyclo*Sal Pronucleotides Against DNA Viruses

DNA viruses are also attractive targets for the application of pronucleotides. In contrast to RNA viruses like HIV, these viruses do not rely on reverse transcription of their genome prior to replication. Therefore, the target is not reverse transcriptase but a viral DNA polymerase. Moreover, some of the known antivirals against DNA viruses are not monophosphorylated by a cellular kinase but a virus-encoded thymidine kinase (TK).[39] Drug-resistant virus strains are often selected in vivo. One reason for this resistance seems to be associated with a down-regulation of the expression of viral thymidine kinase.[40] However, not all the virus types belonging to the herpes-virus family express viral thymidine kinase activity, e.g. Epstein–Barr virus (EBV) and cytomegalovirus (CMV). Therefore, the *cyclo*Sal approach was applied to the broad-spectrum acyclic purine nucleoside acyclovir (ACV, **28**) and to the pyrimidine-modified nucleoside 5-[(*E*)-2-bromovinyl]-2′-deoxyuridine (BVDU or Brivudin, **29**; Figure 8).

cycloSal Pronucleotides of Acyclovir (ACV)

One of the most active and most broadly applicable nucleoside antivirals in this area is the guanine-bearing acyclic nucleoside analog acyclovir (ACV).[41] Acyclovir acts in its triphosphate form as a chain terminator and/or as an inhibitor of HSV-DNA polymerase. Although ACV is preferentially phosphorylated by a viral thymidine kinase, it is not a particularly good substrate for HSV-TK and the rate of phosphorylation is reckoned to be slow. The most common mutation related to resistance against ACV is the selection of virus mutants deficient in TK activity or in mutants that express TKs with altered substrate specificity.

The chemical synthesis of the 3-methyl-*cyclo*Sal ACVMP triester can be carried out using the chlorophosphite method.[42] However, better results were obtained when the exocyclic amino group of the guanine residue was protected by dimethoxytritylation prior to the phosphitylation reaction using the phosphoramidite strategy using pyridinium chloride as activating agent. Reproducible yields of 80% were obtained. The protecting group was cleaved by acid treatment of the N^2-blocked *cyclo*Sal triester. Chemical hydrolysis again proved selective delivery of ACVMP, and ACV derivatives were found to be non-inhibitory to BChE.

Antiviral evaluation showed an EC_{50} for the parent nucleoside ACV of 0.62 μM against herpes simplex type-1 (HSV-1/TK^+) in Vero cells. However, ACV lost its activity in Vero cells infected with ACV-resistant HSV-1/TK^- (EC_{50} = 58 μM). Strikingly, 3-methyl-*cyclo*Sal ACVMP shows identical activity values of 0.47 and 0.51 μM against both viruses strains (HSV-1 TK^+ and TK^-) without an increase of toxicity.

A comparable result was obtained for the antiviral activity against varicella zoster virus (VZV). 3-Methyl-*cyclo*Sal ACVMP showed an antiviral activity of EC_{50} = 4.1 and 1.2 μM against two wild-type virus strains. This activity was completely retained in cells infected with VZV/TK^- (EC_{50} = 7.6 μM) without changing the cell morphology (MCC > 200 μM). As expected, ACV showed activity against the wild-type viruses *only*. Moreover, 3-methyl-*cyclo*Sal ACVMP proved to be antivirally active at 10 μM against cytomegalovirus (CMV) while ACV itself is entirely inactive. This example clearly shows the potential of the *cyclo*Sal phosphate triester approach: it converts a non-antivirally active nucleoside analog into a bioactive compound whilst at the same time broadening the spectrum of antiviral activity.

Application of the cycloSal Approach to the Anti-Herpes Drug Brivudin (BVdU)

The nucleoside analog 5-[(*E*)-2-bromovinyl]-2′-deoxyuridine (BVdU or Brivudin, **29**) is a very potent and highly selective inhibitor of HSV-1 and particularly VZV replication.[43] Its selectivity as an inhibitor primarily depends upon a specific activation by HSV-encoded thymidine kinase to the mono- *and* diphosphate. The triphosphate is formed by cellular enzymes. BVdU-triphosphate (BVdUTP) can act either as an inhibitor of the cellular DNA polymerase or alternate substrate that leads to formation of nonsense-DNA or renders DNA more prone to degradation after incorporation into DNA.[44] In contrast, BVdU is not active against HSV-2 or Epstein–Barr virus (EBV). EBV does not express an HSV-1-like thymidine kinase and therefore BVdU is inactive to inhibit EBV replication. Here, the *cyclo*Sal approach was used to broaden the application of BVdU against infections caused by Epstein–Barr virus (EBV).[45] EBV infections play a significant role as secondary infection in AIDS patients, for example, and may be responsible for cancer formation.

Thus, *cyclo*Sal BVdUMP triesters **30** were synthesized with different substituents in the aromatic residue.[46] The target compounds were prepared using 3′-O-levulinylated BVdU, which was phosphorylated using the phosphoramidite/oxidation method. The levulinyl protection group was removed by treatment of the triester with hydrazine hydrate.

First, chemical hydrolysis studies proved the selective delivery of BVdUMP (**31**) as sole product without formation of 3′,5′-cyclicBVdUMP (**32**; Figure 10). Furthermore, the 3-methyl-*cyclo*Sal triester was hydrolyzed in P3HR-1 cell extracts to BVdUMP with a half-life comparable to that observed in chemical hydrolysis studies ($t_{1/2}$ = 8.9 h). Thus, the degradation is chemically rather than enzymatically driven. As before, cBVdUMP (**32**) was not detected.[47] In contrast to the chemical hydrolyses, BVdU was also observed to a minor extent after 4 h (5%) and 8 h (22%), which is due to an enzymatic dephosphorylation of BVdUMP by phosphatases/nucleotidases (Figure 10). Comparable data were obtained in the CEM/O cell extract incubation. The major difference was that the triesters yielded higher amounts of BVdUMP after 8 h, as in the P3HR-1 extracts. Moreover, dephosphorylation of BVdUMP proceeded to a minor extent in CEM/O cell ex-

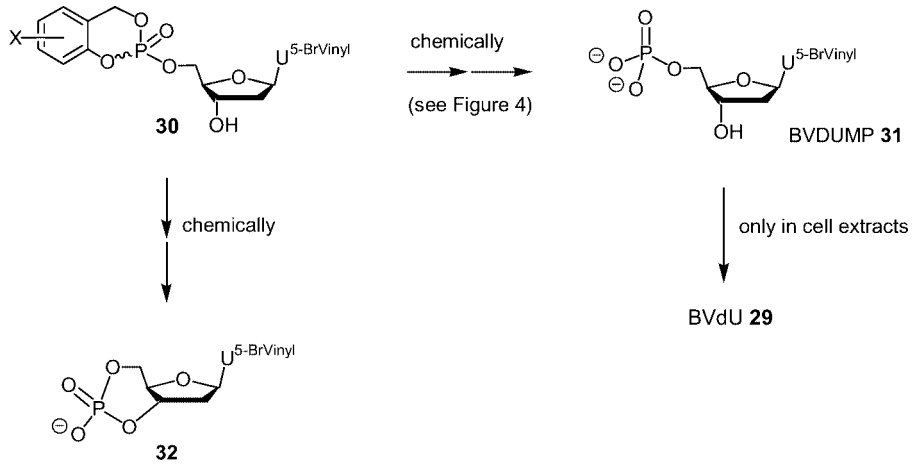

Figure 10. Hydrolysis pathways of *cyclo*Sal BVDUMPs **36**.

tracts, as in P3HR-1 cell extracts (13% vs. 26%, respectively).

3-Methyl-*cyclo*Sal BVdUMP was tested for inhibition of VZV replication. BVdU proved to be highly active against VZV/TK$^+$ with an EC_{50} of 0.033 μM. As expected, this activity was completely lost when a thymidine-kinase deficient VZV/TK$^-$ strain was used (EC_{50} > 200 μM). 3-Methyl-*cyclo*Sal BVdUMP triesters showed comparable anti-VZV activity as compared to the parent. However, the *cyclo*Sal triesters lost all their antiviral activity against the VZV/TK$^-$ strains (EC_{50} > 50 μM). This led to the conclusion that only the VZV/TK-associated viral thymidylate kinase activity is involved in the intracellular formation of BVdUDP and that cellular enzymes are unable to phosphorylate BVdUMP.

*cyclo*Sal BVdUMP triesters were then tested for inhibition of EBV replication in P3HR-1 cells. BVdU itself was found to be entirely inactive (EC_{50} > 100 μM in the EBV DNA synthesis assay as well as the EB-VCA expression assay) due to the missing HSV-1-like thymidine kinase. Strikingly, some of the *cyclo*Sal BVdUMP triesters exhibited pronounced anti-EBV activity. The most active compound was 5-methoxy-*cyclo*Sal BVDUMP (EC_{50} = 1.8 μM), which is more than 166-fold more active than BVdU (EC_{50} > 300 μM) and even fourfold more active than the reference compound ACV (EC_{50} = 7.2 μM). Obviously, BVdUMP released from the *cyclo*Sal pronucleotide leads to the antiviral activity and thus the phosphorylation of BVdUMP into BVdUTP seems to be achieved by cellular enzymes. Again, the *cyclo*Sal approach converts the inactive nucleoside analog into an anti-EBV active agent.

Application of the *cyclo*Sal approach to Antiviral Acyclic Nucleoside Phosphonates

Nucleoside phosphonates represent a further class of interesting antiviral agents.[48] The most prominent compounds are 9-(2-phosphonylmethoxyethyl)adenine (**33**, PMEA, adefovir), (*R*)-9-(2-phosphonylmethoxypropyl)adenine (**34**, PMPA, tenofovir), and (*S*)-9-(3-hydroxyphosphonylmethoxypropyl)cytosine (**35**, HPMPC, cidofovir; Figure 11).

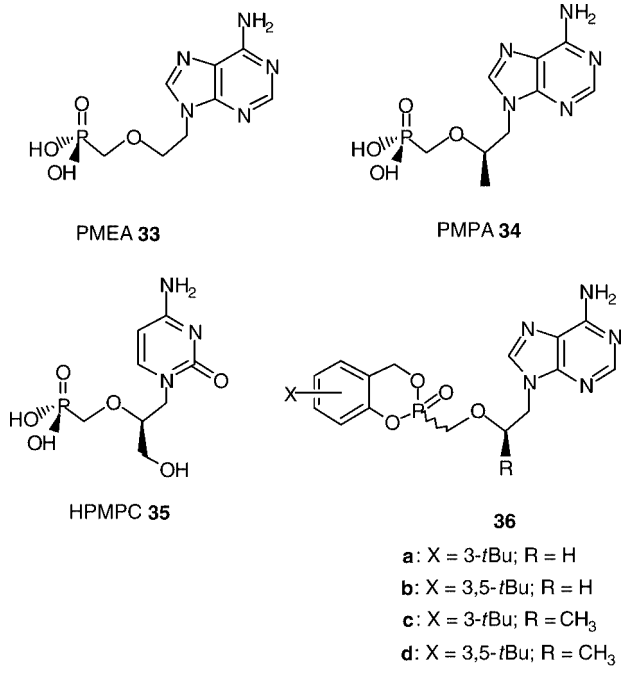

Figure 11. Structural formulae for acyclic nucleoside phosphonates PMEA, PMPA, and HPMPC and the *cyclo*Sal derivatives of PMEA and PMPA.

In 1986, De Clercq and Holy reported on the antiviral activity of such compounds against DNA viruses, which started a tremendous effort in order to find highly potent derivatives of this class of antiviral agents.[49] These nucleoside phosphonates often exhibit a broad antiviral activity spectrum. HPMPC shows a broad spectrum of activity against DNA viruses such as the whole herpes family, pox virus, and papillomavirus. The mechanism of action of these nucleoside phosphonates is identical to that of the nucleoside analogs: they have to be converted into the corresponding nucleoside phosphonate diphosphates, the nucleoside triphosphate analog. However, due to the presence of the phosphonate group, the parent compounds are analogs

of the nucleoside monophosphates and thus do not need the first kinase-catalyzed conversion. On the other hand, the compounds are highly polar because of the charged phosphonate group and the bioavailability and cellular uptake is limited. Thus, the aim of prodrugs of these nucleoside phosphonates is to enhance membrane uptake and not an enzyme-bypass. A few prodrugs of nucleoside phosphonates are known: the bis(POM)-PMEA (adefovir dipivoxil, Hepsera®), bis(POC)-PMPA (tenofovir diisoproxil, Viread®), bis(SATE)-PMEA and bis(AB)-PMEA, and arylphosphoramidate-prodrugs of PMEA and PMPA.[50–54] The first two lipophilic prodrugs have been approved by the FDA for clinical use against HBV and HIV. In all given examples, the parent drug is released by enzymatic activation.

The application of the *cyclo*Sal approach to this class of compounds (*cyclo*Sal phosphonates **36**) deserves an entirely different chemical synthesis pathway. Therefore, the *cyclo*Sal group was attached to the nucleoside phosphonates directly. However, numerous attempts failed but finally the in situ conversion of the dialkyl esters of the parent phosphonates into the dichloride and subsequent one-pot esterification with the salicyl alcohol leading to **36** was successful. In the case of PMPA, direct esterification with salicyl alcohols using intermediately formed active esters was possible.[55]

Studies regarding the hydrolysis properties of the *cyclo*Sal PMEA and PMPA derivatives showed a surprising effect: the half-lives of compounds **36** were found to be 13-fold lower than the corresponding *cyclo*Sal d4TMP triesters (3,5-di-*tert*-butyl-*cyclo*Sal PMPA: $t_{1/2}$ = 5.91 hM 3,5-di-*tert*-butyl-*cyclo*Sal d4TMP: $t_{1/2}$ = 73 h in phosphate buffer, pH 7.3 at 37 °C). However, the hydrolysis in the case of the PMPA derivatives proceeded selectively to PMPA without any trace of the formation of phenyl phosphonate monoester. In comparable hydrolysis studies, 35% of the corresponding phenyl phosphate diester was formed starting from 3,5-di-*tert*-butyl-*cyclo*Sal d4TMP. Further hydrolysis studies in CEM cell extracts proved that the compounds are not cleaved by enzymes.

Moreover, it was interesting to note that the *cyclo*Sal nucleoside phosphonates were entirely non-inhibitory to human butyrylcholinesterase: 3-*tert*-butyl-*cyclo*Sal PMPA: $IC_{50} > 50$ μM and 3-*tert*-butyl-*cyclo*Sal d4TMP: IC_{50} = 4.2 μM after incubation for five minutes. In addition, both compounds proved to be non-inhibitory to human acetylcholinesterase (AChE).

Finally, the anti-HIV activity of the *cyclo*Sal nucleoside phosphonates was determined in wild-type CEM cells as well as in CEM/TK-deficient cells. *cyclo*Sal nucleoside phosphonates show considerable anti-HIV activity against HIV-1 and HIV-2 in wild-type CEM cells as well as in the thymidine kinase-deficient cells. The EC_{50} values observed were two- to fourfold lower than those of the parent nucleoside phosphonates PMEA and PMPA. Bis(POM)-PMEA and bis(POC)-PMPA were tested for comparison. Both compounds were found to be more active than the *cyclo*Sal derivatives but also showed a considerable increase in the toxicity (CC_{50} value). A possible reason for the lower activity of the *cyclo*Sal derivatives might be the lower chemical stability of these derivatives. In comparative hydrolysis studies, bis(POM)-PMEA showed a half-life of 16 h while the most stable *cyclo*Sal PMEA derivative showed a half-life of only four hours.

"Lock-in" *cyclo*Sal d4TMP Triesters – A Conceptual Extension of the Trojan Horse Concept (Second Generation *cyclo*Sal Triesters)

The compounds described so far belong to the first-generation compounds of the *cyclo*Sal concept. Although these first-generation *cyclo*Sal triesters led to convincing antiviral results, the use of a pure chemical hydrolysis mechanism may also have some limitations. So far we can clearly exclude an enzymatic contribution to the hydrolysis of the *cyclo*Sal triesters in cell extracts and in human serum. Moreover, lipophilic *cyclo*Sal triesters are able to penetrate cell membranes and deliver nucleotides *inside* the cell. Therefore, it cannot be excluded that the chemical hydrolysis also takes place extracellularly. In addition, it cannot be excluded that the triesters can also be exported from the cells. This would lead to the formation of a membrane equilibrium. In order to avoid such export the triesters should be converted inside the cell into a more polar compound by an enzymatic reaction ("lock-in" mechanism; Figure 12).[56]

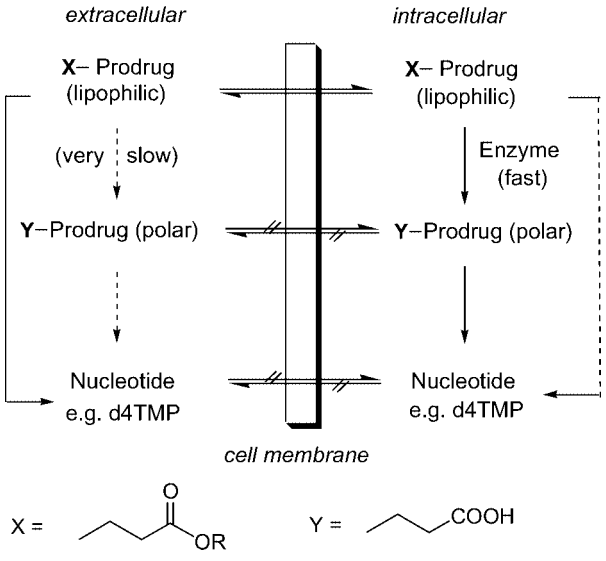

Figure 12. "Lock-in" principle with the *cyclo*Sal approach.

First a(n) (carboxy)esterase reaction on a carboxylic ester attached to the *cyclo*Sal aromatic ring via a C2-alkyl chain linker was investigated. The ethylene spacer was introduced and should separate the ester group efficiently from the aromatic ring to avoid an electronic effect on the hydrolysis of the phosphate triester moiety (Figure 13).

Under physiological pH conditions, alkylpropionate *cyclo*Sal triesters **3b–d** should lead to the formation of deprotonated propionate-*cyclo*Sal d4TMP after enzymatic cleavage. The synthesis of these compounds was performed as

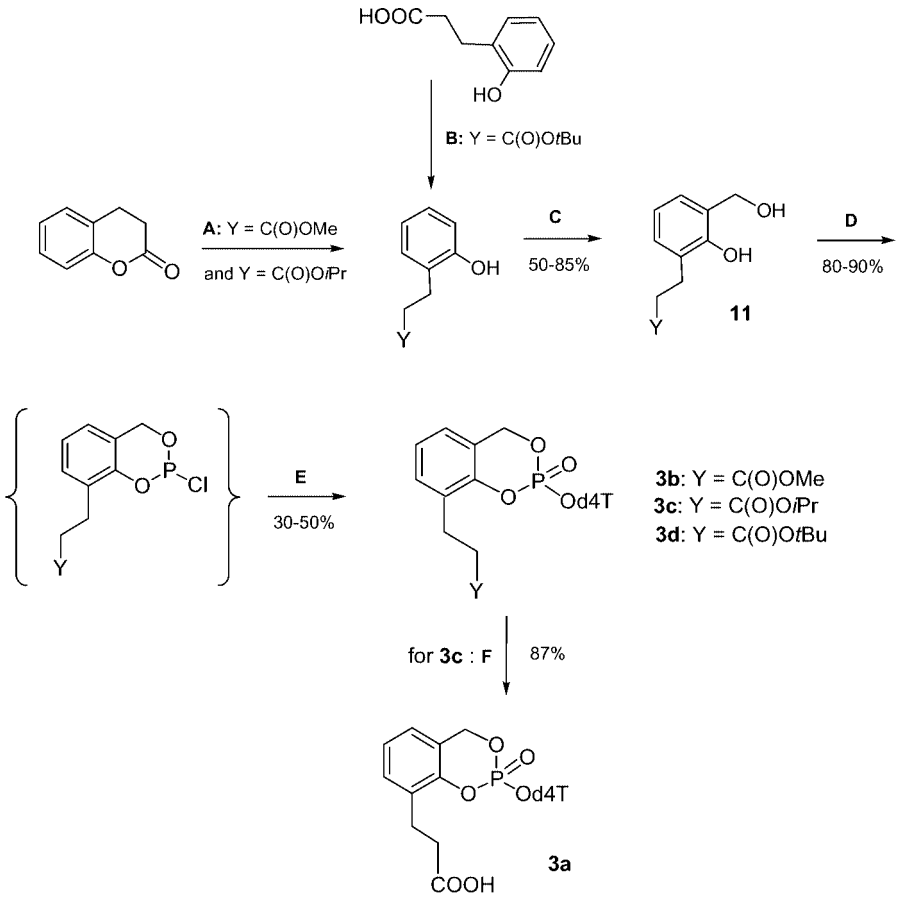

Figure 13. Target *cyclo*Sal phosphate triesters for the "lock-in" approach. Method **A**: methanol or isopropanol, H_2SO_4, reflux, 5–8 h; method **B**: $(CH_3)_2NCH(OCH_2tBu)_2$, *tert*-butyl alcohol, toluene, reflux, 5 h; method **C**: i. phenylboronic acid, propionic acid (cat.), *p*-formaldehyde, toluene, reflux, 6–8 h; ii) H_2O_2, THF, 0 °C, 30 min:; method **D**: PCl_3, pyridine, Et_2O, 0–21 °C, 1 h; method **E**: i. d4T **1**, CH_3CN, DIPEA, 0–20 °C; ii. *t*BuOOH, CH_3CN, room temp., 30 min; method **F**: TFA (10 equiv.), CH_2Cl_2, room temp., 1 h.

for compounds **2** using reactive phosphorus(III) reagents, and phosphate triesters **3** were obtained in reasonable yields as diastereomeric mixtures.[56] Diols **11** needed to be synthesized from the phenols by selective *ortho*-hydroxymethylation according to Nagata.[20] Unfortunately, there is no generally applicable method for the preparation of substituted phenols bearing a propionate ester and so each one was prepared differently (Figure 13). All new triesters were cleaved chemically to yield d4TMP at pH 7.3 in 25 mM phosphate buffer. Half-lives were found to be between 7.3 h and 13.5 h. A comparison with 3-methyl-*cyclo*Sal d4TMP (**2d**; $t_{1/2}$ = 17.5 h) proved that the ethylene spacer separates the electron-withdrawing ester group and the *cyclo*Sal aromatic ring fairly sufficiently. Interestingly, propionate-*cyclo*Sal d4TMP triester (**3a**) has a twofold higher half-life than the neutral ester-modified *cyclo*Sal triesters. A possible explanation for this effect may be the presence of an overall negative charge on the molecule due to the carboxylate formed at pH 7.3, which slows down the nucleophilic reaction necessary for the initial cleavage step.

The hydrolysis of 3-methylpropionate-*cyclo*Sal d4TMP (**3b**), which was followed by ^{31}P NMR spectroscopy, showed that the phenyl phosphate diester was formed to a minor extent (approx. 2%). However, this amount is lower than the situation found for 3-methyl-*cyclo*Sal d4TMP (**2d**; 5.5%). In both chemical hydrolysis experiments no cleavage of the ester group was observed. Further studies in 25 mM phosphate buffer (pH 7.3) containing 50 units of pig liver esterase (PLE) and studies in CEM/O cell extracts for 10 h at 37 °C were carried out. It was observed that the half-lives of *cyclo*Sal triesters **3b**–**d** were slightly lower than the situation in pure phosphate buffer. However, no trace of *cyclo*Sal triester propionate **3a** was detected and an enzymatic ester cleavage has consequently to be excluded. In conclusion, the results of these enzyme-driven cleavage studies were disappointing, particularly because experiments using the methyl ester of the 3-propionate-*cyclo*Sal mask showed an extremely fast de-esterification under the same conditions. The reason for the insusceptibility of the alkyl esters is surprising because alkyl esters are often used in prodrug strategies.[57]

Nevertheless, the triesters were tested for their anti-HIV potency in HIV-1- and HIV-2-infected CEM/O cells and HIV-2-infected CEM/TK$^-$ cells. All *cyclo*Sal triesters proved to be active in the wild-type cell-line against both virus types and all of them retained their antiviral activity in the CEM/TK$^-$ cells (EC_{50} = 1–2 μM), thus proving at least the TK-bypass. However, propionate-*cyclo*Sal triester **3a** lost all

its antiviral activity in this mutant cell-line due to the charge at the carboxylate, which prevents a transmembrane passage.

Due to the failure of the delivery of the *cyclo*Sal nucleotide carboxylates from simple alkyl esters, a more sophisticated system was developed.[58] Acyloxymethyl groups have been used in the past to mask carboxylic acid groups in penicillins and also in nucleotides [see bis(POM)-nucleotides or -nucleoside phosphonates]. Therefore, we introduced such a masking unit to the carboxylates in the *cyclo*Sal side-chain. Three different groups were studied, namely the acetoxymethyl group (AM, **3e**), the pivaloxymethyl group (POM, **3f**), and the isopropyloxycarbonyloxymethyl group (POC, **3g**). The preparation of the target acylal-*cyclo*Sal phosphate triesters was achieved by alkylation of the *cyclo*Sal phosphate triester presenting the carboxylic acid moiety in the side-chain **38** in about 50% yield (Figure 14). *cyclo*Sal triester **38** was prepared from the corresponding *tert*-butyl ester **37** by acid treatment. This approach allows a fast excess to a variety of different acylal structures of the *cyclo*Sal triester of interest.

In addition, we prepared a masking unit containing the acylal group already attached to the *cyclo*Sal chlorophosphite. This enables the fast excess of acylal-*cyclo*Sal phosphate triesters of different nucleosides.

As in the case of the ester-modified *cyclo*Sal triesters, the stability of the acylal-*cyclo*Sal nucleotides towards chemical hydrolysis (pH 7.3) was found to be more or less identical to those observed for alkylated *cyclo*Sal triesters, thus pointing to a sufficient separation of the electron acceptor (acylal) by the ethylene spacer. Only the cleavage of the *cyclo*Sal triester moiety was detected, leading to d4TMP, while the acylal group proved to be stable. This was unambiguously proven by hydrolysis studies followed by ^{31}P NMR spectroscopy (Figure 15).

However, in CEM cell extracts and liver extracts, but not in human serum, the acylal group was efficiently cleaved to give the free propionate. In the case of 5-AM-*cyclo*Sal d4TMP (**3e**), 50% of the starting acylal triester was cleaved into the propionate-*cyclo*Sal derivative within 15 minutes (Figure 16).

There is a clear correlation between the stability and the acylal structure. The AM-acylal is the least stable compound ($t_{1/2}$ = 0.25 h) while the POC-acylal (**3g**) is the most stable group ($t_{1/2}$ = 0.9 h). The POM-acylal (**3f**) has a half-life of 0.38 h. Thus, the stability difference between the chemical hydrolysis of the *cyclo*Sal ester and the acylal cleavage in the cell extracts is up to 16-fold. As before, the acylal-*cyclo*Sal d4TMP triesters were tested against HIV. Interestingly, in contrast to the POM and the POC derivatives, which retained their antiviral activity in TK-deficient CEM cells, the AM-acylal lost all the activity in that mutant cell-line. One explanation of the loss of activity may be a rapid cleavage of the AM-acylal in the cell-culture me-

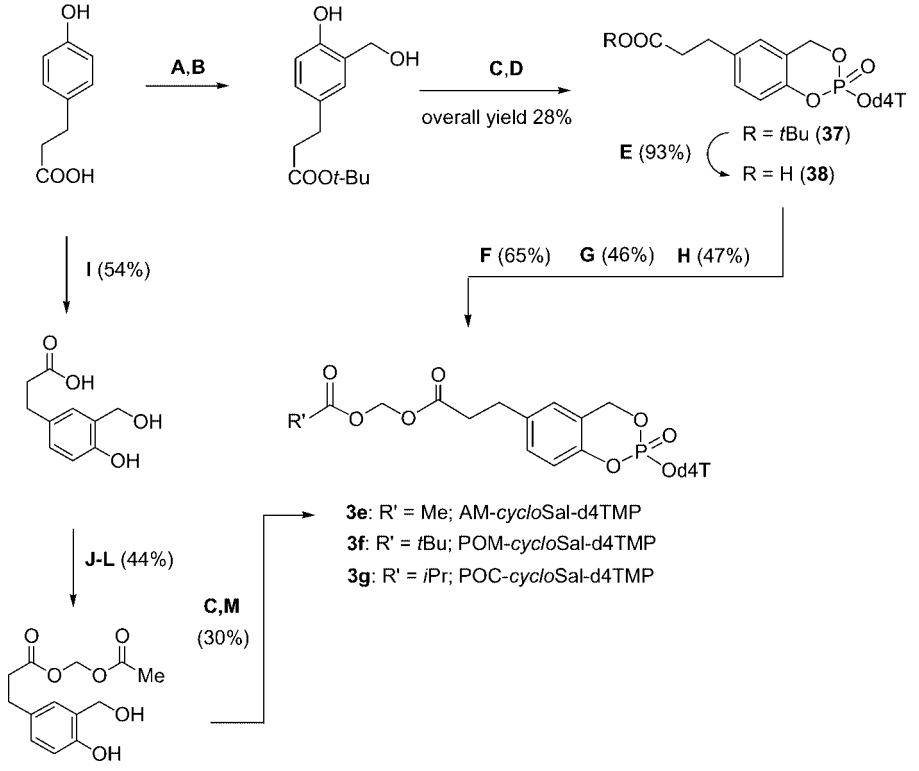

Figure 14. Acylal-modified *cyclo*Sal phosphate triesters for the "lock-in" approach. Methods **A**–**E**: see ref.[15]; method **F**: bromomethyl acetate, DIPEA, CH_3CN, 10 °C, 3 h; method **G**: chloromethyl pivalate, DIPEA, CH_3CN, room temp., 9 d; method **H**: isopropyl chloromethylcarbonate, DIPEA, CH_3CN, 50 °C, 7 d; method **I**: see ref.[16]; method **J**: benzaldehyde dimethyl acetal, *p*-TosOH, THF, room temp., 3 d; method **K**: bromomethyl acetate, DIPEA, DMF, room temp., 5 h; method **L**: cat. HCl, CH_3CN/H_2O (7:3), 1 min, reflux; method **M**: i. m^5K **3**, CH_3CN, DIPEA, 0–20 °C; ii. *t*BuOOH, CH_3CN, room temp., 2 h.

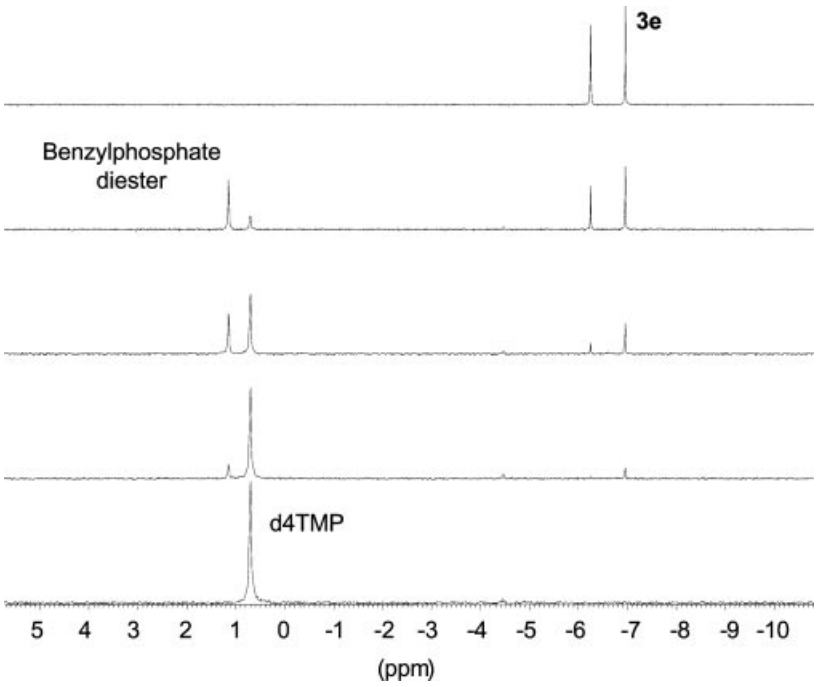

Figure 15. Chemical hydrolysis of enzyme labile AM-*cyclo*Sal triester **3e**. Chemical hydrolysis followed by ^{31}P NMR spectroscopy (solvent: [D_6]DMSO/50 mM imidazole·HCl buffer, pH 7.3 1:1(v/v); spectra were recorded once a week; H_3PO_4 was used as external reference).

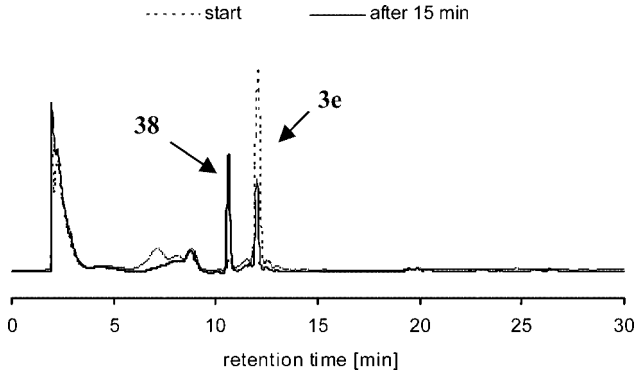

Figure 16. Cell extract hydrolysis of enzyme labile *cyclo*Sal triester **3e** (HPLC analysis).

dium, which contains 10% heat-inactivated fetal calf serum (FCS). Therefore, the AM-acylal was incubated in RPMI culture medium as well as in RPMI medium containing FCS. It was proven that the AM-acylal is stable in the culture medium but labile in the mixture with FCS. In contrast, the POM- and POC-acylals were found to be stable in the FCS-containing medium. For further exploration of the "lock-in" concept, we recently introduced intrinsically fluorescent nucleoside analogs as analytical tools to study membrane uptake processes.

Fluorescent Probes of *cyclo*Sal Nucleotides

To study the intracellular fate of the *cyclo*Sal derivatives in more detail, and later the cell uptake, a suitable probe is needed. In principal, radiolabeling *cyclo*Sal nucleotides is one option, although this is very expensive and experimentally demanding. Therefore, we became interested in fluorescence labeling. Despite the possibility of selecting specific fluorescence properties, attachment of a high molecular weight fluorescent dye like rhodamine, fluoresceine, or the dansyl group would change the size, lipophilicity, and/or hydrolysis properties of *cyclo*Sal compounds considerably. Antiviral nucleoside analogs attached to the *cyclo*Sal moiety belong to the class of pyrimidines and purines. Thus, a fluorescent analog was needed that is structurally as close as possible to the nucleosides used before. As a surrogate for the pyrimidine-type nucleoside analogs, for example ddT, ddC, or d4T, the highly intrinsic fluorescent 5-methylpyrimidin-2-one nucleoside **39** (m^5K; Figure 17), the deoxygenation product of thymidine, was used. A few synthetic protocols have already been published for the synthesis of m^5K. However, they use heavy-metal-containing reagents and give low yields of the target nucleoside.[59] Recently, we reported a slightly modified but reliable synthetic protocol for m^5K[60] that uses a mild, homogeneous oxidation with *N*-phenyltriazolinedione (PTAD).[61] In addition, we applied the protocol to the synthesis of the dideoxy analog dm^5K (**40**). Thus, both nucleosides were converted into the *cyclo*Sal nucleotides of the first generation **41a**,**b**. In addition, the 5-AM-*cyclo*Sal m^5KMP triester **41c**, which bears the esterase-cleavable 5-acetoxymethyl-(AM) propionyl group, was prepared (Figure 17). The latter triester was obtained in a poor non-optimized yield of 25%.

All fluorescent *cyclo*Sal triesters released the corresponding monophosphates of the two parent fluorescent nucleoside analogs in phosphate buffer, and their half-lives at pH 7.3 were found to be in the same range as 3-methyl-

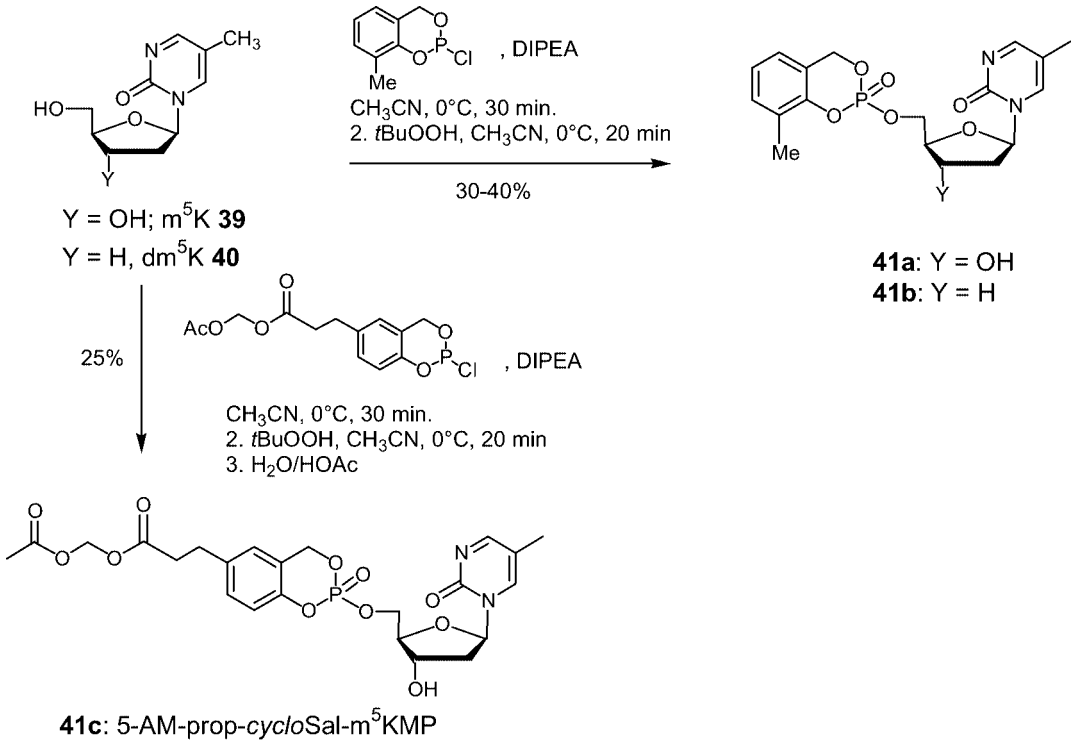

Figure 17. Fluorescent nucleoside analogs and their *cyclo*Sal phosphate triesters.

*cyclo*Sal dTMP or 3-methyl-*cyclo*Sal ddTMP ($t_{1/2}$ = 8.6 and about 14.9 h). Thus, these properties prove that the fluorescent *cyclo*Sal triesters are ideal probes of the original *cyclo*Sal pyrimidine nucleotides. A clear advantage of the fluorescent *cyclo*Sal derivatives was observed in cell extract hydrolysis studies because the concentrations needed were 500–1000-fold lower for UV detection.

In cell extracts 5-AM-*cyclo*Sal triester **41c** was rapidly cleaved enzymatically at the acylal site, delivering propionate-*cyclo*Sal m^{5}KMP and, subsequently, the nucleotide m^{5}KMP by chemical hydrolysis. Again, the difference in half-lives was about 16-fold. These conclusions were supported by experiments with pig liver esterase (PLE) in aqueous phosphate buffer (pH 7.3). The acylal site was cleaved within five minutes to the propionate!

In addition, model studies for cellular uptake using fluorescence spectroscopy were performed. 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 set-ups were used: i) in both aqueous phases the pH was set to 6.8 and ii) the pH in the donor phase was 6.8 while that of the acceptor phase was pH 8.7. In the first experiment, the expected formation of an equilibrium between both phases was detected. However, in the second set-up an accumulation in the acceptor phase was observed because the triester was hydrolyzed to give the nucleotide. The nucleotide is too polar to enter the organic phase again. For 5-AM-*cyclo*Sal triester **41c** a different set-up was used: the donor phase was phosphate buffer (pH 6.8) but the acceptor phase was PLE containing phosphate buffer (pH 7.3). As expected, an accumulation of all fluorescent material in the acceptor phase was detected.[61] It was shown by HPLC analysis that the compounds found in the acceptor phase were 5-propionate-*cyclo*Sal m^{5}KMP and its hydrolysis product m^{5}KMP. No 5-AM-*cyclo*Sal triester was detected due to its very high susceptibility to enzyme degradation. This experiment supports the idea of a possible intracellular "lock-in" of the *cyclo*Sal phosphate triesters.

Application of the *cyclo*Sal Strategy to the Delivery of Pyranosyl-1-phosphates

The congenital disorders of glycosylation syndrome (CDGs) are an autosomal recessively inherited disorder first described by Jaeken in 1980.[62,63] CDGs are classified into two types according to the type and intracellular localization of the glycosylation pathways. CDG-I are disorders of the assembly of the lipid-linked oligosaccharide (LLO) at the membrane of the endoplasmic reticulum and the transfer of the oligosaccharide from the dolichol anchor to selected residues of nascent polypeptides. CDG-II involves disorders of the *N*-glycan processing occurring in the endoplasmic reticulum or the Golgi apparatus and disorders affecting the assembly of *O*-glycans. Protein glycosylation plays an important role in the metabolism, function, and structure of glycoconjugates. The hypoglycosylation of several proteins in CDG has severe consequences for the patients. The by far most frequent CDG type is CDG-Ia. This defect leads to a significant reduction in the concentra-

tion of phosphomannomutase 2 (PMM, 2), thus hindering the conversion of mannose-6-phosphate into mannose-1-phosphate. As a consequence, the synthesis of the sugar donor GDP-mannose decreases and a general hypoglycosylation of many glycoconjugates is the consequence. A therapeutic approach may be the intracellular delivery of mannose-1-phosphate. However, as for nucleotides, mannose-1-phosphate does not penetrate cellular membranes and rapid dephosphorylation occurs in the blood. In 2004, we reported the synthesis of acetylated *cyclo*Sal mannose-1-phosphates **4a** and, for comparison, of *cyclo*Sal glucose-1-phosphates **4b**, leading to successful "carbohydrate prodrugs".[64] *cyclo*Sal pyranosyl-1-monophosphates **4** were prepared in a convergent synthesis starting from D-glucose (**42b**) and D-mannose (**42a**), respectively, in yields of up to 62%. The procedure is summarized in Figure 18.

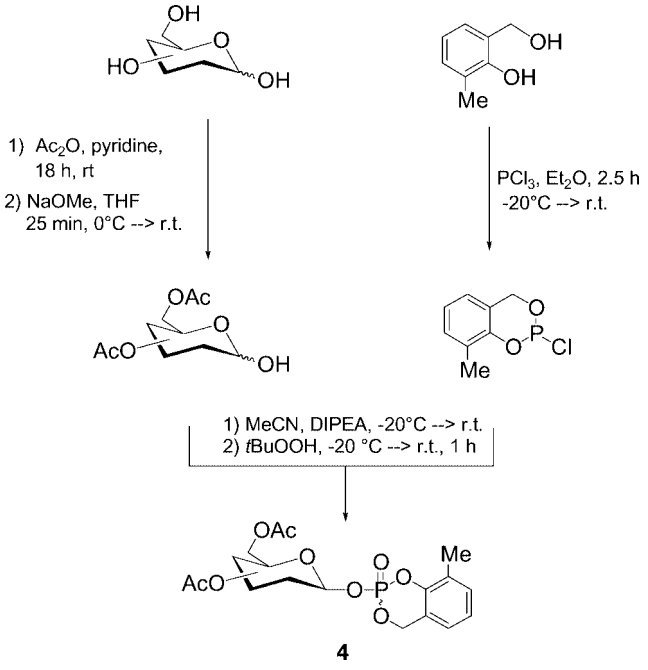

Figure 18. Carbohydrate prodrugs based on the *cyclo*Sal approach.

Two interesting differences between the mannose- and the glucose-1-phosphate esters were observed in hydrolysis assays: i) *cyclo*Sal mannose-1-phosphates were found to be 10-fold less stable than the corresponding glucose-1-phosphate derivative. However, even the glucose-1-phosphate triesters proved to be 10-fold less stable than the corresponding nucleotide triesters. Obviously, the attachment of the neutral *cyclo*Sal residue to the anomeric carbon atom leads to a tremendous decrease in stability compared to the attachment of the group to a primary alcohol in the nucleotides; ii) the selectivity of pyranose-1-phosphate release from the prodrugs is much lower in the case of the mannose-1-phosphate triesters than their glucose counterparts. The interpretation of this is that two reactions take place in concurrence (Figure 19), namely the "normal" cleavage of the *cyclo*Sal phosphate ester leading to the pyranosyl-1-phosphate (S_NP-reaction) and the cleavage of the glycosidic bond by an S_N1-type reaction due to the formation of a stabilized oxocarbenium ion. Moreover, and in addition to the general increase in lability of the glycosidic bond, an anchimeric acceleration takes place for the mannose derivative **4**, leading to acetoxonium ions **B** and/or **C** (pathway 2b). In the case of the glucose derivatives this anchimeric acceleration is less pronounced because the geometry is less

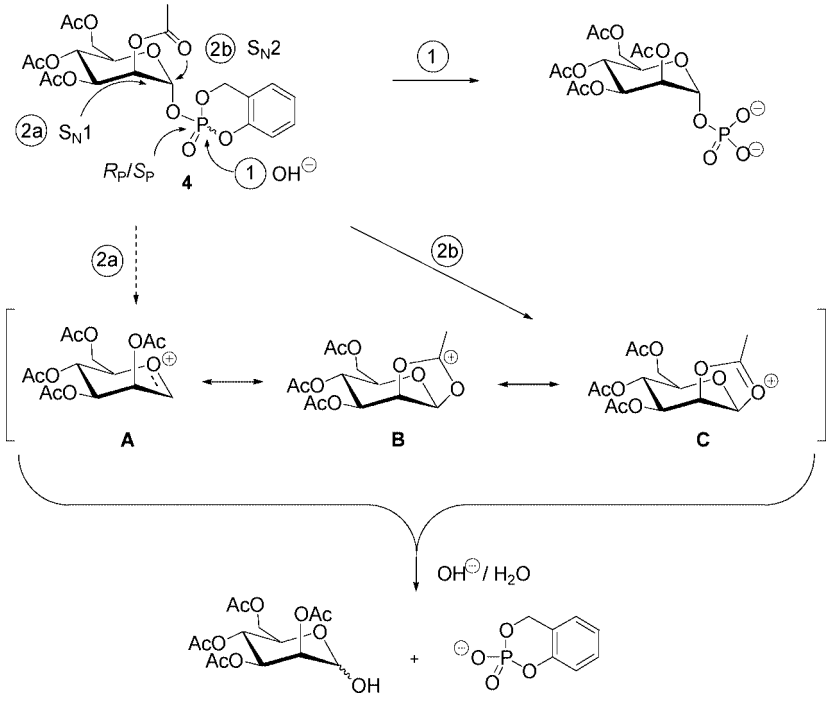

Figure 19. Hydrolysis mechanism of carbohydrate prodrugs leading to hexose-1-phosphazes and the hexoses.

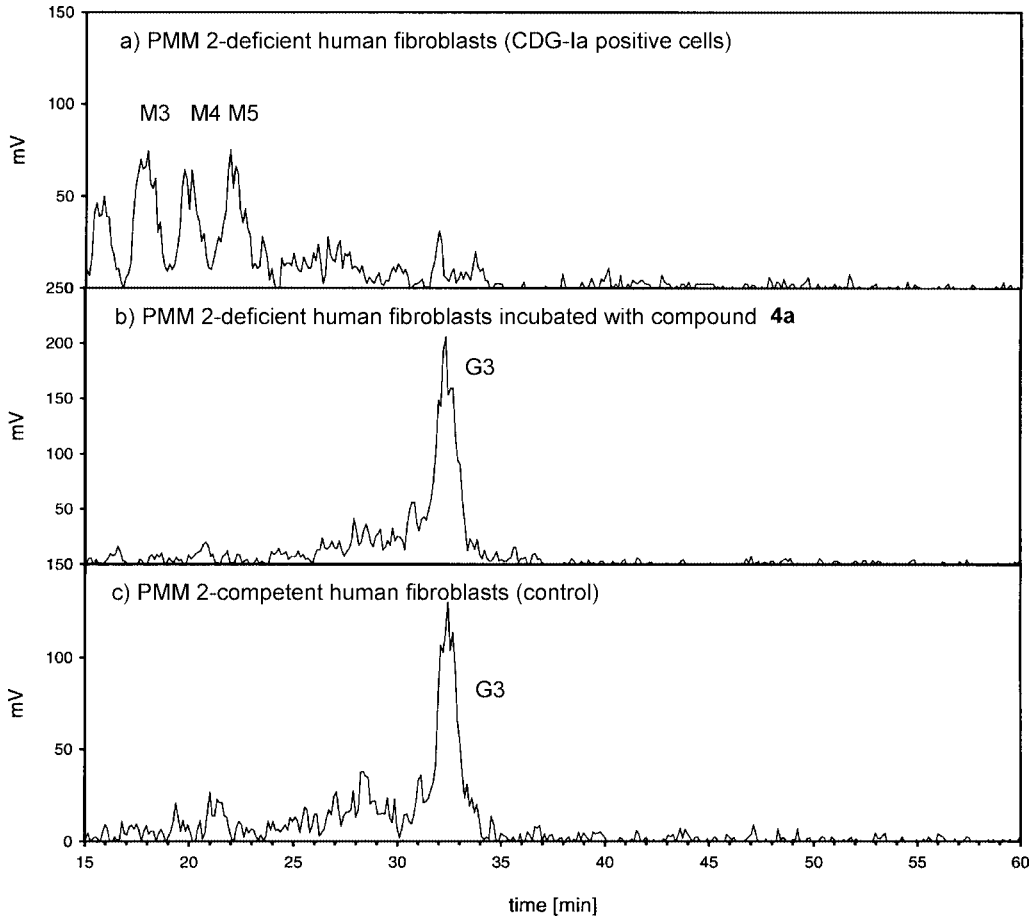

Figure 20. HPLC chromatogram of the LLO analysis of compound **4a** (G3 = $Glc_3Man_9GlcNAc_2$; M3 = $Man_3GlcNAc_2$; M4 = $Man_4GlcNAc_2$; M5 = $Man_5GlcNAc_2$).

favored for an intramolecular reaction. Nevertheless, although the cleavage reaction was found to be not as selective as in the case of the *cyclo*Sal nucleotides, the mannose derivative **4a** releases considerable amounts of mannose-1-phosphate from the lipophilic precursor.

Finally, the biological activity of 3-methyl-*cyclo*Sal mannose-1-phosphate (**4a**) was tested in vitro in fibroblasts. Healthy fibroblasts assemble complete oligosaccharide chains having a $Glc_3Man_9GlcNAc_2$ structure (G3), whereas PMM 2-deficient fibroblasts synthesize only truncated oligosaccharide chains with $Man_4GlcNAc_2$ (M4) and $Man_3GlcNAc_2$ structures (M3; Figure 20).

The 3-methyl-*cyclo*Sal test compound showed a total correction of the hypoglycosylation pattern, thus proving the intracellular mannose-1-phosphate delivery. Moreover, the observed bioactivity also proves that the acetyl groups attached to the glycon are cleaved intracellularly by (carboxy)-esterases.

Conclusions

In summary, the *cyclo*Sal approach convincingly demonstrates the intracellular delivery of nucleotides by a non-enzymatically induced cascade reaction. The *cyclo*Sal pronucleotide system is also an ideal tool for studying biochemical pathways in nucleoside metabolism. Modification of the *cyclo*Sal moiety, and particularly of the benzyl position, leads to considerable differences in the hydrolysis pathway. This allows further insights into the mechanism of degradation and at the same time gives clues to improve the d4TMP delivery. It has considerably improved the antiviral activity of several nucleoside analogs using the first-generation *cyclo*Sal triesters. First attempts have been made to influence the equilibrium formed by a lipophilic phosphate triester through the membrane. This resulted in the development of second-generation *cyclo*Sal triesters having enzyme-cleavable acyloxymethylpropionate esters in the side chain of the *cyclo*Sal aromatic ring. These compounds achieve an efficient intracellular trapping ("lock-in") of the triester.

Finally, the promising total correction of hypoglycosylation found in the biological assays shows that the delivery of biologically active phosphorylated compounds from *cyclo*Sal phosphate triesters may lead to a strong improvement in the bioactivity. At the same time this example proves that the *cyclo*Sal approach is not restricted to nucleotides.

Acknowledgments

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