*cyclo*Sal-2′,3′-dideoxy-2′,3′-didehydrothymidine Monophosphate (*cyclo*Sal-d4TMP): Synthesis and Antiviral Evaluation of a New d4TMP Delivery System

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The synthesis, hydrolysis, and antiviral evaluation of novel, lipophilic *cyclo*Sal-d4TMP derivatives **3a**−**h** of the anti-HIV dideoxynucleoside 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T, **1**) are reported. This pro-nucleotide concept has been designed to deliver d4TMP (**2**) by selective chemical hydrolysis. All compounds **3a**−**h** were synthesized using phosphorus(III) chemistry in good yields and in somewhat lower yields using phosphorus(V) chemistry starting from substituted salicyl alcohols **6a**−**h**. The phosphotriesters **3** were obtained without stereochemical preference with respect to the configuration at the phosphorus center as 1:1 diastereomeric mixtures. However, a few of the triesters **3** could be separated into the diastereomers by means of semipreparative HPLC. In a 1-octanol/phosphate buffer mixture, all compounds **3** exhibited 9−100-fold higher lipophilicity as judged from their Pa values as compared to d4T (**1**). Furthermore, in hydrolysis studies **3** decomposed under mild aqueous basic conditions releasing solely d4TMP (**2**) and the diols **6** following the designed tandem reaction sequence. A correlation of the electronic properties introduced by the substituents and the half-lives of triesters **3** was observed. Thus, by varying the substituent, the half-lives of **3** could be adjusted over a wide range of compounds still delivering d4TMP (**2**) selectively. Phosphotriesters **3** exhibited considerable activity against HIV-1 and HIV-2 in wild-type human T-lymphocyte (CEM/O) cells as well as mutant thymidine kinase-deficient (CEM/TK$^-$) cells. Surprisingly, we observed a 3−80-fold difference in antiviral activity between the two diastereomers. Our data clearly prove that the *cyclo*Sal-d4TMPs deliver exclusively the nucleotide d4TMP not only under simulated hydrolysis conditions but also under cellular conditions and thus fulfill the thymidine kinase-bypass premise. Therefore, the *cyclo*Sal-nucleotide concept is the first reported pro-nucleotide system that delivers the dideoxynucleotide by a pH-driven, chemically activated, tandem reaction without the requirement of an enzymatic contribution.

Introduction

Due to the ultimately fatal nature of AIDS, an intensive effort has been underway to develop new therapies or to improve existing treatments. Among the various structural classes of antiviral agents investigated for the treatment of HIV infections, nucleoside analogues are among the most effective.[1] Most of these nucleoside analogues are 2′,3′-dideoxynucleosides,[2] e.g., 3′-azido-2′,3′-dideoxythymidine (AZT, Zidovudine, Retrovir)[3] and 2′,3′-dideoxy-2′,3′-didehydrothymidine (**1**) (d4T, Stavudine, Zerit).[4] The general mode of action of these drugs is the inhibition of HIV reverse transcriptase[5,6] and the incorporation into the growing DNA chain which results in DNA chain termination.[7] The dideoxynucleosides are not active as such. After membrane penetration, intracellular conversion of the nucleoside analogues to their 5′-mono-, 5′-di-, and 5′-triphosphates is a prerequisite for the expression of the biological activity. So, formally dideoxynucleosides are prodrugs of the ultimately bioactive triphosphates. This biotransformation is established by host cell kinases in three steps via the nucleoside monophosphate and the nucleoside diphosphate.[2a,4e,8] Although dideoxynucleosides offer great promise for the inhibition of viral replication, several shortcomings limit realization of their full therapeutic efficacy. One problem is the limited ability of some dideoxynucleosides to undergo biotransformation catalyzed by host cell kinases into the triphosphate due to structural differences as compared to natural nucleosides.[6] Furthermore, monocytes and macrophages, which harbor high virus amounts, have very low levels of nucleoside kinases. So, the presence and activity of intracellular enzymes necessary for the phosphorylation of nucleoside analogues are highly dependent on the host species, the cell type, and the stage in the cell cycle. Consequently, the rate and extent to which the nucleoside analogues are converted to their bioactive triphosphates may be at least as important as the affinity of these compounds for the target enzyme. As compared to the approved anti-HIV drug AZT, d4T (**1**) shows comparable selective anti-HIV activity in vitro.[4e,9] Moreover, d4T (**1**) has been found to be less toxic than AZT for bone marrow progenitor cells and to be less inhibitory to mitochondria DNA replication.[10] In contrast to AZT, d4T (**1**) does not interfere with the intracellular dTMP, dTDP, and dTTP

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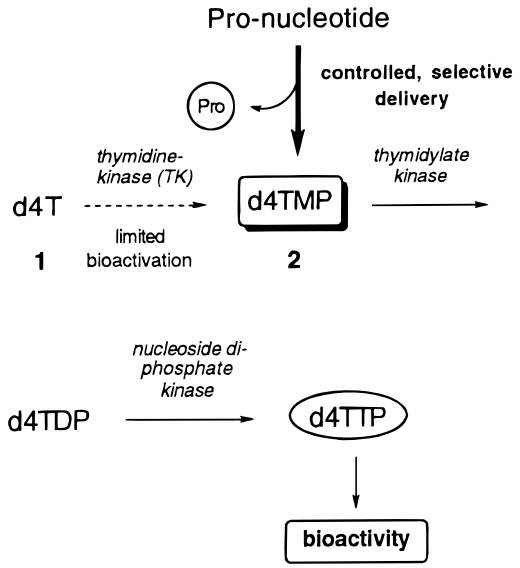
Scheme 1. Principle of a d4TMP *cyclo*Sal-pro-nucleotide Approach

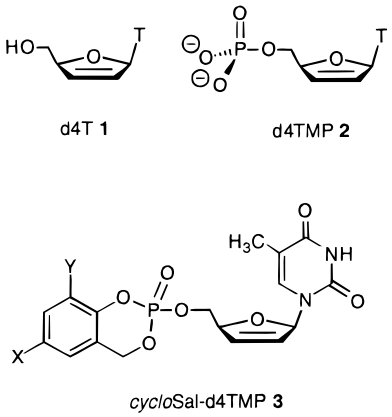


pools, and consequently, d4T does not cause the starvation of the cellular dTTP.[2a] In comparison with AZTTP, d4TTP has a longer half-life in CEM/O cells (3−3.5 h).[8a] However, in contrast to AZT, d4T (**1**) is an example of a dideoxynucleoside analogue with a rate-limiting step in the first phosphorylation catalyzed by the host cell enzyme thymidine kinase (TK) in CEM and MT-4 cells.[8a] The further conversion to the ultimate bioactive drug d4TTP by thymidylate kinase and nucleoside diphosphate kinase is not limiting. Consequently, the direct application of the nucleotide 2′,3′-dideoxy-2′,3′-didehydrothymidine monophosphate **2** (d4TMP) would bypass the metabolization-limiting enzyme TK (TK-bypass) and could thus improve the therapeutic potential of the drug (Scheme 1). But a well-known problem in antiviral therapy is the bioavailability of this type of phosphorylated derivative. This compound does not easily penetrate cellular membranes due to its high polarity. Furthermore, **2** is rapidly dephosphorylated in the blood. Consequently, the delivery of d4TMP (**2**) from neutral, lipophilic prodrugs[12,13] is one effort to achieve the TK-bypass (*pro-nucleotide approach*; Scheme 1).

Several prodrug strategies have been developed to deliver nucleotides intracellularly. As a general motive, uncharged nucleotide triesters are used as membrane-permeable nucleotide precursors. The major differences of these approaches are the delivery mechanisms to d4TMP: While almost all approaches based on chemical hydrolysis reported so far were unable to deliver the *nucleotide* selectively and consequently serve only as nucleoside depots,[14] the newer concepts based on *enzymatic activation* processes reported by McGuigan (aryloxyphosphoramidates)[15] and Imbach (bis-SATE phosphotriesters)[16] demonstrated the successful intracellular delivery of free nucleotides from highly lipophilic precursors.[17]

As part of our ongoing program to develop efficient pro-nucleotide delivery systems,[18] we were interested in the design and the synthesis of a completely new pro-nucleotide approach for d4TMP (**2**) based on a pH-driven, selective chemical hydrolysis:[19] the *cyclo*saligenyl-2′,3′-dideoxy-2′,3′-didehydrothymidine monophosphates **3** (*cyclo*Sal-d4TMP).[20] Before hydrolysis takes place, the *cyclo*Sal-nucleotides **3** serve as lipophilic, neutral prodrugs. This concept has been successfully introduced with the anti-HIV nucleosides ddT[21] as well as ddA.[22] Although the general structure and the delivery mechanism were identical, the biological task of the compounds was different: While the ddT phosphotriesters were designed to follow the TK-bypass, the ddA analogues were used for the adenosine deaminase (ADA)-bypass.[23]

Scheme 2. Structures of d4T (**1**), d4TMP (**2**), and *cyclo*Sal-d4TMP Phosphotriesters **3**

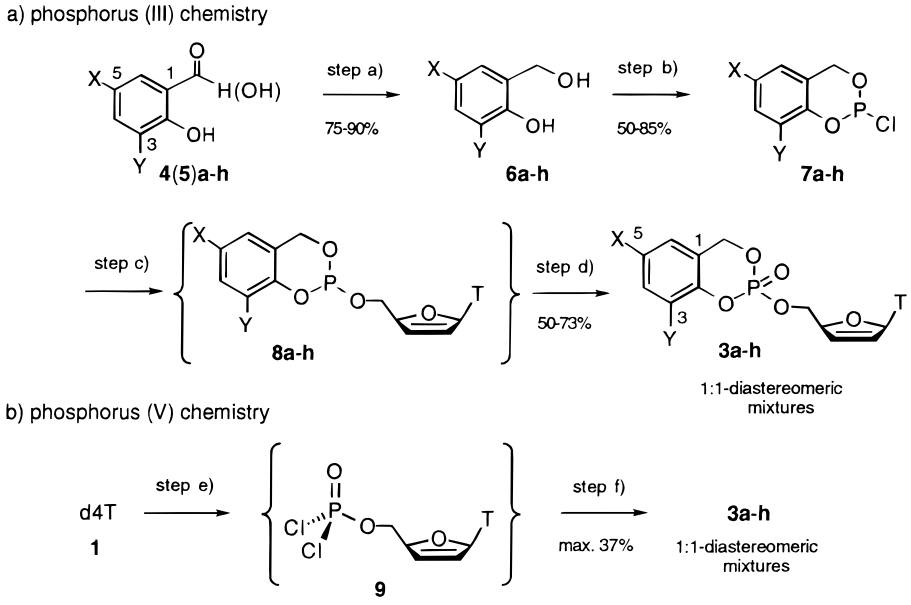


Here we report on the synthesis and the biological evaluation of the *cyclo*Sal-2′,3′-dideoxy-2′,3′-didehydrothymidine monophosphates **3** (*cyclo*Sal-d4TMP; Scheme 2) as potential neutral prodrugs of d4TMP (**2**) of the approved antiviral drug d4T (**1**). Furthermore, we report on their lipophilic properties (partition coefficients, Pa values) and on hydrolysis studies in various aqueous buffers at different pH values as well as on hydrolysis studies under more biologically adapted conditions using RPMI-1640 culture medium containing 10% heat-inactivated fetal calf serum (FCS) at 37 °C.

Results and Discussion

Chemistry. d4T (**1**) was prepared from thymidine following the procedure of Horwitz et al.[24] or using the methodology reported by Mansuri et al.[9] Two approaches were used to yield the title compounds. The first uses phosphorus(III) chemistry, whereas the second involves phosphorus(V) chemistry. In the former methodology, the title compounds **3a**−**h** were synthesized as outlined in Scheme 3a. Differently substituted salicyl aldehydes **4** or salicylic acids **5** were used as starting materials, which were reduced by standard procedures to give the salicyl alcohols **6** in 75−90% yield.[23a] Diols **6** were reacted with phosphorus trichloride to yield the cyclic saligenylchlorophosphanes **7a**−**h**. These were isolated by distillation under argon atmosphere in 50−85% yield.[23a,25] The *cyclo*Sal-d4TMPs **3a**−**h** were accessible via **8a**−**h** in a "one-pot reaction" by reacting d4T (**1**) and 1.5 equiv of chlorophosphanes **7a**−**h** at 0 °C in the presence of dry, distilled diisopropylethylamine (DIPEA) and subsequent in situ oxidation with *tert*-butyl hydroperoxide (TBHP) at ambient temperature as

Scheme 3. Synthetic Pathways to the *cyclo*Sal-d4TMP Triesters **3a**−**h**[a]



3a : X = Nitro, Y = H; **3b** : X = Cl, Y = H; **3c** : X = Y = H; **3d** : X = OMe, Y = H;
3e : X = H, Y = OMe; **3f** : X = Me, Y = H; **3g** : X = H, Y = Me, **3h** : X = Y = Me

[a] Reaction conditions: (a) $NaBH_4$ for **4** ($LiAlH_4$ for **5**); (b) PCl_3, pyridine, Et_2O, −10 °C, 2 h; (c) d4T (**1**), DIPEA, CH_3CN, 0 °C, 20 min; (d) TBHP, CH_3CN, rt, 30 min; (e) $P(O)Cl_3$, DIPEA, THF, 0 °C, 12 h; (f) salicyl alcohol **6**, DIPEA, THF, 0 °C−rt, 12 h.

1:1 diastereomeric mixtures. 3,5-Dimethylsalicylaldehyde (**4h**) was prepared by orthoformylation of the corresponding phenol using dichloromethyl methyl ether in the presence of $TiCl_4$.[26]

An alternative synthetic approach toward the title compounds **3** involved the less reactive phosphorus(V) chemistry shown in Scheme 3b: The reaction of phosphorus oxychloride with d4T (**1**) yielded the 5′-O-d4T phosphodichloridate **9** which was further reacted without isolation with the salicyl alcohols **6** to give the triesters **3**. However, using this approach the yields were markedly lower (∼37%) than obtained according to the first described strategy (50−73% yield). Similar to the phosphorus(III) approach, the phosphorus(V) method gave no diastereoselectivity with respect to the configuration at the phosphorus atom. After chromatographic purification, the *cyclo*Sal-d4TMPs **3a**−**h** were characterized by means of ^1H, ^{13}C, and ^{31}P NMR and UV spectroscopy as well as electrospray mass spectrometry (ESI, negative mode). As expected, the phosphotriesters displayed two closely spaced signals in the ^{31}P NMR spectra (1:1 mixtures, −7.3 to −9.3 ppm), corresponding to the presence of diastereomers, resulting from mixed stereochemistry at the phosphorus center. Similar diastereomeric splitting and phosphorus coupling, where appropriate, were noted in the H-decoupled ^{13}C NMR spectra. The purity of the *cyclo*Sal-d4TMPs **3a**−**h** was checked by analytical HPLC analysis. In almost all cases the two expected peaks of the diastereomers overlapped completely, but in one case two peaks with partial overlap were detected. For the biological evaluation, small amounts of the *cyclo*Sal-d4TMP triesters were additionally purified by semipreparative HPLC using acetonitrile−water eluents in order to be sure to eliminate any trace of the nucleoside d4T (**1**). The second reason for using semipreparative HPLC was the attempt to separate the diastereomers which was achieved for some derivatives. It was noted that the 3-substituted triesters were easier to separate than their 5-substituted counterparts. The configuration at the phosphorus atom of the separated diastereomers was assigned by comparison with the known stereochemistry of a model compound using CD spectroscopy.[27] It should be mentioned that the R_p- or S_p-configuration, respectively, correlated with the elution properties of the diastereomers on a reversed-phase HPLC column. All "slow"-eluting isomers showed R_p-configuration, whereas all "fast"-eluting isomers are S_p-configurated at the phosphorus center. However, in a few cases, the separation of the diastereomers was still unsuccessful. Thus, the diastereomeric mixtures as well as the "slow" (R_p) and the "fast" (S_p) diastereomers of the phosphotriesters **3** were isolated after the HPLC purification followed by lyophilization.

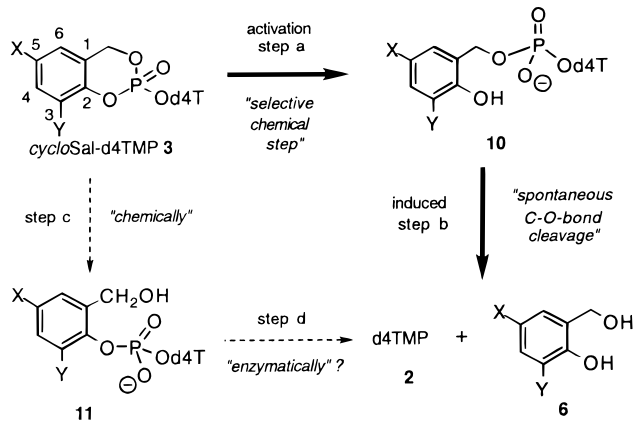
Determination of the Partition Coefficients (Pa Values). The partition coefficients (Pa values) of the *cyclo*Sal-d4TMPs **3a**−**h** were determined in 1-octanol/sodium phosphate buffer (pH 6.8). Triesters **3a**−**h** were partitioned between 1-octanol and phosphate buffer for 5 min, and after centrifugation the relative concentration in each solvent phase was determined by reverse-phase HPLC analysis. This method for the determination of the partition coefficient is a very simple procedure that gives a qualitative estimation of the lipophilicity of the title compounds **3a**−**h**.[28] Like most nucleosides and in contrast to AZT, which enters mammalian cells by passive, nonfacilitated diffusion readily,[29] d4T (**1**) is taken up by the cells only to a limited extent by passive, nonfacilitated diffusion. There seem to be also contributions of active mechanisms such as nucleoside and nucleobase carriers. Consequently, the Pa value of AZT (1.06[30]) was used as a reference for the potential ability of compounds **3** to diffuse passively through cellular membranes. As can be seen in Table 1, all Pa values

Table 1. Hydrolysis in Different Aqueous Buffers and Pa Values of *cyclo*Sal-d4TMPs **3a**−**h**

	hydrolysis ($t_{1/2}$, h)[b] in aqueous buffers at 37 °C			hydrolysis ($t_{1/2}$, h)[b] in CM[e] at 37 °C		
compd	pH 6.9[a]	pH 7.3[c]	pH 8.9[d]	without FCS[f]	with FCS[e]	Pa value[g]
3a	4.1	0.15	0.06	nd[h]	0.12	1.5
3b	6.4	0.7	0.3	2.5	1.0	7.6
3c	24.5	4.5	1.1	7.2	3.9	1.9
3d	28.3	7.2	1.07	7.2	4.3	2.3
3e	9.5	1.4	0.4	3.2	2.0	1.4
3f	28.3	8.0	1.3	8.9	8.4	5.4
3g	68.5	10.2	1.5	16.0	9.1	5.1
3h	98.2	16.1	3.4	17.5	10.8	15.3
1	na[i]	na[i]	na[i]	na[i]	na[i]	0.15

[a] pH 6.9: 30 mM TRIS buffer. [b] Half-lives are given in h. [c] pH 7.3: 30 mM sodium phosphate buffer. [d] pH 8.9: 30 mM sodium borate buffer. [e] CM: RPMI-1640 culture medium, 37 °C. [f] FCS: 10% heat-inactivated fetal calf serum. [g] Pa: partition coefficient. [h] Not determined. [i] Not available.

Scheme 4. Two Possible Hydrolysis Pathways of *cyclo*Sal-d4TMP Phosphotriesters **3a**−**h**



were found to be >1 and the log(Pa) was always positive. The observed Pa values were 9−102-fold higher (Pa = 1.4−15.3) as compared to the value of d4T (**1**) and 1.3−14-fold higher compared to that of AZT. It should be added that the possibility of active transport has not been investigated so far. Nevertheless, improved cellular uptake by a passive way should be possible.

Kinetic Studies. In contrast to other prodrug concepts,[15−17] the pro-nucleotide approach reported here has been designed to selectively yield d4TMP (**2**) and the two-component masking group after a controlled, chemically induced tandem reaction involving a successive *coupled* cleavage of the phenyl and benzyl esters of the *cyclo*Sal-phosphotriester. The dideoxynucleoside is connected via an alkyl ester to the phosphate moiety and this is the most stable bond. The difference in hydrolytic stability of phenyl and benzyl phosphotriesters has already been reported: Whereas *acceptor*-substituted bis-phenyl phosphotriesters are rapidly hydrolyzed under alkaline conditions,[31] a fast hydrolysis of bis-benzyl phosphotriesters was observed only in the case of *donor*-substituted derivatives.[32] In general, both resulting phosphodiesters are very stable against non-enzymatic hydrolysis (step d, Scheme 4).[33,34] Sometimes even the enzymatic hydrolysis[35] of phosphodiesters may be difficult. Consequently, the critical point is the second hydrolysis reaction after the first cleavage of the triester to yield the diester. The rationale of our new prodrug concept is based on the above-mentioned difference in stability of the phenyl and benzyl phosphate esters: This allows to discriminate between the different phosphate ester bonds and results in a tripartate prodrug system.[36] The phenyl ester bond should be the most labile one because the negative charge could be delocalized in the aromatic ring leading to the 2-hydroxy*benzyl* phosphodiester **10**. The alternative cleavage of the benzyl ester in **3** to yield the 2-(hydroxymethyl)*phenyl* phosphodiester **11** is unfavorable (step c, Scheme 4), because the phosphate residue in the ortho position of the benzyl ester stabilizes this bond. Hence, the hydrolysis should proceed as follows: In the initial hydrolysis step, the phenyl ester is cleaved selectively (step a, Scheme 4). As a consequence, the ortho substituent to the benzyl group has switched from an acceptor group (phosphate) to a donor group (hydroxyl) with the result of activation of the remaining masking group caused by the "umpolung" of the 2-substituent. This induces the spontaneous cleavage of diester **10** to yield the nucleotide **2** and the diol **6** (tandem reaction; step b, Scheme 4).

Thus, the major difference between our approach and other concepts based on enzymatic activation[15−17] is that a second activation step (e.g., by enzymes) is not required because we have an intrinsic activation by the first reaction. The hydrolysis pathway has already been verified by NMR spectroscopy.[20] Furthermore, we have already shown for the *cyclo*Sal-AZTMP derivatives that the initial hydrolysis step can be controlled by substituents in the para position (position 5) of the phenolic phosphate ester: the stronger the electron-withdrawing activity of the substituent, the faster the degradation reaction of triesters **3**.[23a]

To prove the selective delivery of d4TMP (**2**), hydrolysis experiments of the *cyclo*Sal-d4TMPs **3a**−**h** were carried out in different aqueous buffers and RPMI-1640 culture medium with or without heat-inactivated fetal calf serum (FCS). First, triesters **3** were hydrolyzed in 30 mM phosphate buffer, pH 7.3, at 37 °C as a model of the physiological milieu. Furthermore, the pH dependence of the hydrolysis was studied in borate buffer (30 mmol, pH 8.9) and TRIS buffer (30 mM, pH 6.9). The degradation of **3a**−**h** was followed by means of reversed-phase HPLC analysis. The half-lives were determined by quantifying the decreasing peaks of **3** vs time. The half-lives are summarized in Table 1.

Using the mentioned buffers, *cyclo*Sal-d4TMPs **3a**−**h** were degraded following pseudo-first-order kinetics to yield exclusively d4TMP (**2**) and the salicyl alcohols **6** in all studied media. At pH < 7, only the 3,5-dimethyl-substituted derivative **3h** yielded also in small amounts the "wrong" phenyl-d4T-phosphodiester **11h**, which is, as expected, stable to further hydrolysis. As can be seen in Table 1, the stability of the phosphotriesters **3a**−**h** increased considerably under mild acidic conditions (see hydrolysis at pH 6.9 vs pH 7.3), whereas the half-lives decreased markedly at more basic pH (see hydrolysis at pH 8.9) and hence confirmed the anticipated pH dependence of the hydrolysis. Furthermore, the triesters were hydrolyzed in acetate buffer (30 mM, pH 4.6). As for the studies at pH 6.9, the half-lives were largely increased with respect to the studies at pH 7.3 indicating a high increase in stability under acidic

conditions (data not shown). As for the corresponding *cyclo*Sal-AZTMP derivatives, we observed a clear correlation between the electronic properties caused by the substituent of the salicyl alcohol and the hydrolysis half-lives in different aqueous buffers.[23a] These results clearly show that we are able to control the hydrolysis kinetics of phosphotriesters **3** by varying the substituents in the aryl ring. From the results summarized in Table 1, especially the donor-substituted *cyclo*Sal-d4TMPs **3d,f**−**h**, which exhibited half-lives of 7−16 h in phosphate buffer at pH 7.3 and high hydrolytic stability at acidic conditions, seem to be interesting candidates for biological evaluation. Furthermore, we observed a change in the rate-limiting step in dependence of the substituent. If an electron-withdrawing substituent is placed in the aromatic ring of the starting phosphotriesters **3**, the initial cleavage to the phosphodiester **10** is the rate-limiting step, whereas in the case of electron-donating substituents in **3** the second step from diester **10** to d4TMP (**2**) is rate-determining. All the mentioned results are in complete agreement with the designed hydrolysis pathway via 2-hydroxybenzyl phospho*di*ester **10** to d4TMP (**2**) and salicyl alcohols **6** (Scheme 4).

Additionally, an interesting question arises concerning the second cleavage step: is the second reaction an intermolecular or intramolecular reaction? Thus, is there a second nucleophilic attack of a water molecule or a hydroxide at the phosphorus center or is the intermediate phosphodiester cleaved via (i) a *Grob fragmentation* to give a neutral quinone methide and after water quenching the diol **6** or (ii) a spontaneous C−O bond cleavage and a subsequent water quenching of the formed ion pair?[37] This question was addressed by hydrolysis experiments of the 5-chloro-substituted *cyclo*Sal-derivative **3b** in [^{16}O]water and [^{18}O]water. In the hydrolysis with [^{16}O]water, we detected the intermediate phosphodiester **10b** (M = 443/445 in 3:1 ratio ^{35}Cl/^{37}Cl), d4TMP (**2**) (M = 303), and the diol **6** (M = 157/159 in 3:1 ratio ^{35}Cl/^{37}Cl) in the electrospray mass spectra (negative mode) with the corresponding mass peaks. The first reaction of the degradation is obviously a nucleophilic attack at the phosphorus atom. Consequently, in the experiment in [^{18}O]water, the intermediate phosphodiester **10b** should be detected with a mass (M + 2 with a 3:1 ratio for ^{35}Cl/^{37}Cl). In the case of a second nucleophilic attack at the phosphorus center, the resulting d4TMP would have a mass (M + 4) and the salicyl alcohol should have only a mass (M with a 3:1 ratio for ^{35}Cl/^{37}Cl) without ^{18}O. If the reaction involves a spontaneous C−O bond cleavage yielding d4TMP with a mass (M + 2) and an intermediate benzyl cation/quinone methide, which is rapidly quenched by water, then the final salicyl alcohol should also have a mass (M + 2 with a 3:1 ratio for ^{35}Cl/^{37}Cl). An alternative is a nucleophilic reaction at the benzylic carbon atom releasing d4TMP (M + 2) and directly the diol (M + 2). Thus, the difference is a mass spectrometric analysis of M + 4 and M in the former scenario or a situation with M + 2 and M + 2 in the latter case for the nucleotide and the salicyl alcohol, respectively. Running the reaction and analyzing the mixture by ESI mass spectrometry, we observed the second situation with *both* products bearing one ^{18}O-isotope only. At the same time, this result made it clear that a second, separate activation process for the delivery of the nucleotide is obviously not required. However, this experiment gives no further information if the reaction proceeds via a benzyl carbocation or a Grob fragmentation (quinone methide). The ^{18}O-isotope distribution found for **3b** was additionally verified in the hydrolysis corresponding to the 3-methoxy-substituted *cyclo*Sal-triester **3e**.

Table 2. Inhibitory Effects of *cyclo*Sal-d4TMPs **3a**−**h** as Well as Representative Salicyl Alcohols **6** on the Replication of HIV-1 and HIV-2 and Toxicity in Wild-Type CEM/O Cells and Mutant Thymidine Kinase-Deficient CEM/TK$^-$ Cells

		antiviral activity EC_{50} (μM)[a]			
compd	subst X	CEM/O (HIV-1)	CEM/O (HIV-2)	CEM/TK$^-$ (HIV-2)	cytotoxicity CC_{50} (μM)[b]
3a	5-NO_2	0.29	0.40	40.0	75
3b	5-Cl	0.42	1.40	2.67	49
3c	5-H	0.28	0.62	0.50	47
3d	5-OMe	0.18	0.70	0.80	39
3e	3-OMe	0.17	0.42	0.17	35
3f	5-Me	0.18	0.34	0.18	38
3g	3-Me	0.087	0.12	0.093	21
3h	3,5-Me	0.09	0.17	0.08	21
6c	5-H	>250	>250	nd[c]	>250
6d	5-OMe	>250	>250	nd[c]	158
6g	3-Me	>250	>250	nd[c]	>250
d4T (**1**)		0.18	0.26	15.0	56
AZT		0.007	0.006	>100	>100

[a] 50% effective concentration. [b] 50% cytotoxic concentration. [c] Not determined.

Further hydrolysis studies were carried out in RPMI-1640 culture medium with and without 10% heat-inactivated FCS at 37 °C. This medium is normally used for the cultivation of human T-lymphocytes used in the in vitro HIV tests. The hydrolysis products in RPMI-1640 medium were again exclusively d4TMP (**2**) and the diols **6a**−**h**, but the half-lives were slightly shorter than in phosphate buffer. The shorter half-lives in the RPMI-1640 medium most likely result from its more basic pH value (pH 7.8). In the RPMI-1640 medium supplemented with 10% FCS, we also detected the dephosphorylation of d4TMP to d4T by remaining phosphatases in the serum. As judged from the obtained half-lives, the donor-substituted derivatives of **3** should still be stable enough to serve as intracellular depots of d4TMP (**2**). Finally, it should be mentioned that we have not found any evidence for a contribution of an enzymatic degradation of the *cyclo*Sal-phosphotriesters in the RPMI-1640/FCS hydrolyses. This points so far to a purely chemical degradation of our pronucleotide. Additional hydrolysis studies in sera, plasma, and cell extracts will be the subject of further work in our laboratories and will be reported in due course.

Antiviral Evaluation. The *cyclo*Sal-ddAMP phosphotriesters have already demonstrated the effectiveness of our new pro-nucleotide system in anti-HIV assays in rapidly dividing human T-lymphoblastic leukemia cells (CEM/O).[22] Moreover, particularly striking was the structure-bioactivity correlation: The stronger the electron-donating activity of the substituent, the better was the antiviral activity against HIV-1 and HIV-2 in CEM/O cells.[22] These results confirmed the highly selective intracellular delivery of ddAMP and demonstrated the efficient ADA-bypass.

Table 3. Antiviral Evaluation of the Separated Diastereomers of *cyclo*Sal-d4TMPs **3** in Wild-Type CEM/O Cells and Mutant Thymidine Kinase-Deficient CEM/TK$^-$ Cells

				antiviral activity EC_{50} (μM)[a]			cytotoxicity	SI
compd	subst X	HPLC[d]	config	CEM/O (HIV-1)	CEM/O (HIV-2)	CEM/TK$^-$ (HIV-2)	CC_{50} (μM)[b]	value[c]
3e	3-OMe	slow	R_p	0.14	0.17	0.12	38	320
3e	3-OMe	fast	S_p	0.47	1.03	9.67	84	9
3g	3-Me	slow	R_p	0.08	0.067	0.063	11	190
3g	3-Me	fast	S_p	0.42	1.1	0.70	76	108
3h	3,5-Me	slow	R_p	0.093	0.17	0.08	18	218
3h	3,5-Me	fast	S_p	0.50	0.80	0.38	22	57
1				0.18	0.26	15.0	56	4

[a] 50% effective concentration. [b] 50% cytotoxic concentration. [c] Selectivity index: ratio of 50% cytotoxic concentration/50% effective concentration. [d] Eluting properties of the compound on reversed-phase HPLC column.

For these reasons, the parent nucleoside d4T (**1**) and *cyclo*Sal-d4TMPs **3** were evaluated for their ability to inhibit the replication of HIV-1 and HIV-2 in wild-type CEM/O cells. Furthermore, the compounds were evaluated in HIV-2-infected CEM/TK$^-$ cells. The use of the TK-deficient cells should prove the TK-bypass. The test compounds were free of the parent nucleoside **1**, which was verified by means of analytical HPLC. The results obtained are displayed in Table 2.

The parent nucleoside d4T (**1**) proved active against HIV-1- and HIV-2-induced cytopathicity in CEM/O cells (EC_{50} 0.18 μM and 0.26 μM). However, as anticipated, d4T (**1**) virtually lost its antiviral potential when evaluated against HIV-2 in CEM/TK$^-$ cells due to the lack of its bioactivating enzyme thymidine kinase (EC_{50} 15.0 μM). As can be seen in Table 2, the *cyclo*Sal-d4TMPs **3c**−**h** proved to be at least as active as d4T (**1**) against HIV-1- as well as HIV-2-induced cytopathicity in the wild-type cell line. Moreover, a correlation of the antiviral activity with the electronic properties introduced by the substituents and the hydrolytic stability in the aqueous buffers and RPMI-1640 culture medium was observed: the stronger the electron-donating activity of the substituent and the more stable the triester (Table 1), the better is the antiviral activity against HIV-1 and HIV-2 in CEM/O cells (Table 2). The 3-methyl- and the 3,5-dimethyl-*cyclo*Sal-d4TMPs (**3g**,**h**, respectively) exhibited at least equal or even a slightly higher antiviral potency in the wild-type cell line than d4T (**1**). As for d4T, all phosphotriesters **3** were slightly less active against HIV-2-induced cytopathicity. It should be mentioned that no correlation of the biological activity with the lipophilicity of **3a**−**h** (Pa values) could be observed. Furthermore, particularly striking is the complete retention of the biological activity in thymidine kinase-deficient CEM/TK$^-$ cells for most of the compounds. Only the most labile 5-nitro-*cyclo*Sal-d4TMP (**3a**) showed a considerable loss in antiviral activity (Table 2). This loss in activity in the mutant TK$^-$ cell line for **3a** could be explained by the short half-life of this acceptor-substituted derivative as compared to the donor-substituted derivatives (having much longer half-lives) in the buffer solutions (Table 2). Presumably, this phosphotriester is predominantly hydrolyzed *outside* the cells to give after dephosphorylation d4T (**1**), which permeates into the cells by passive diffusion and is rephosphorylated only in the wild-type cells. In contrast, the most active derivatives **3g**,**h** were 180-fold more active than d4T in the TK-deficient cells. As compared to AZT, which is inherently more potent in thymidine kinase-competent CEM cells than d4T or d4TMP but completely inactive in TK$^-$ cells, the *cyclo*Sal-d4TMP triesters are >1250 times more active in the thymidine kinase-deficient CEM cell line. The almost equal antiviral activity of the donor-substituted phosphotriesters in the wild-type cells and the thymidine kinase-deficient cells demonstrates the highly selective intracellular delivery of d4TMP making these compounds entirely independent of the TK. This clearly proves the TK-bypass concept. The selectivity indices (SI) of the *cyclo*Sal-d4TMPs **3b**−**h** in CEM/TK$^-$-deficient cells showed a 50-fold increase compared to that of the parent nucleoside d4T, while the SI value in the wild-type cells was approximately the same. In addition to the *cyclo*Sal derivatives, three representative salicyl alcohols **6** were tested in vitro. As shown in Table 2, the diols used as masking group obviously did not contribute to the antiviral effect, nor did they result in any toxicity (EC_{50} and CC_{50} > 250 μM).

An interesting effect was observed for the antiviral potency of the separated diastereomers of **3**. The results are summarized in Table 3. As can be seen, the "slow"-eluting (R_p) diastereomer always proved much more active than the "fast"-eluting (S_p) diastereomer. Moreover, in all cases the selectivity index was found to be much higher for the R_p-stereoisomer compared to the S_p-isomer. Again, the antiviral activity was retained in the TK-deficient cells except for (S_p)-3-methoxy-*cyclo*Sal-d4TMP (**3e**). One possible explanation for this behavior could be a difference in hydrolysis stability dependent on the configuration at the phosphorus center. But only a 1.5-fold enhancement of hydrolysis from the R_p- to the S_p-diastereomer was detected in independent hydrolysis studies which seems not to be sufficient to explain the above-examined difference in bioactivity (data not shown). Other possibilities that should also be taken into account are (i) a different uptake of the cyclic phosphotriesters dependent on the stereochemistry at the phosphorus atom and (ii) a different enzymatic contribution in the hydrolysis of the diastereomers of the phosphotriesters in the in vitro cell system. How these effects influence the selectivity index of the diastereomers remains unclear. Further studies in order to explain the different properties of the diastereomers are currently in progress in our laboratories.

Finally, the high biological activity of the donor-substituted *cyclo*Sal-d4TMP derivatives was also observed in MT-4 cells (Table 4) and Molt4/C8 cells (data not shown). As in the in vitro tests in CEM cells, a difference in biological activity with respect to the configuration at the phosphorus atom was observed.

Table 4. Antiviral Evaluation of the *cyclo*Sal-d4TMPs **3** and the Separated Diastereomers in MT-4 Cells

				antiviral activity EC_{50} (μM)[a]	
compd	subst X	config	HPLC[b]	MT-4 (HIV-1)	MT-4 (HIV-2)
3b	5-Cl	R_p/S_p	mixture	0.031	0.038
3f	5-Me	R_p/S_p	mixture	0.021	0.029
3g	3-Me	R_p/S_p	mixture	0.028	0.033
3g	3-Me	R_p	slow	0.012	0.011
3h	3,5-Me	R_p	slow	0.025	0.026
3h	3,5-Me	S_p	fast	0.066	0.080
1				0.018	0.022

[a] 50% effective concentration. [b] Eluting properties of the compound on reversed phase HPLC column.

Again, the "slow"-eluting compound (R_p) was more active than both the "fast"-eluting (S_p) diastereomer and the mixture of the two diastereomers. This is shown in the case of the 3,5-dimethyl-substituted derivative **3h**, while the R_p-isomer of the 3-methyl-substituted derivative **3g** was even more active than the diastereomeric mixture (Table 4).

Conclusion

In summary, as for the previously reported *cyclo*Sal-ddAMP triesters, the described pro-nucleotide concept is suitable to deliver d4TMP (**2**) selectively from *cyclo*Sal-d4TMPs **3a**−**h** by a controlled, nonenzymatic mechanism at physiological pH according to the tandem reaction. The half-lives of hydrolysis could be controlled by the substituents in the aromatic ring. All compounds exhibited considerably higher partition coefficients than the parent nucleoside d4T (**1**). Antiviral evaluation of the compounds proved that all *cyclo*Sal-d4TMP derivatives exhibited high antiviral activity in the wild-type CEM cells that exceeded in a few cases even the activity of the parent nucleoside d4T. Most important is the complete retention of the biological activity in thymidine kinase-deficient CEM cells for most of the compounds confirming the selective delivery of d4TMP (**2**) inside the cells and the considerably higher selectivity indices of some compounds. The presented phosphotriesters will be investigated further in cell lines for their hydrolysis behavior and monophosphate-releasing mechanism in order to explore this *pro-nucleotide concept*.

Experimental Section

All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions (argon atmosphere). 2′,3′-Dideoxy-2′,3′-didehydrothymidine (d4T) (**1**) was prepared according to the method of Mansuri.[9b]

Solvents. Anhydrous acetonitrile was obtained in a Sure/Seal bottle from Fluka and stored over 4-Å molecular sieves; ethyl acetate, methylene chloride, and methanol for chromatography were distilled before use. Triethylamine was distilled from CaH_2 prior to use. The solvents for the HPLC were obtained from Merck (acetonitrile, HPLC grade) and Riedel-de-Haen (water, HPLC grade). Evaporation of solvents was carried out on a rotary evaporator under reduced pressure or using a high-vacuum pump.

Chromatography. Chromatotron (Harrison Research 7924) and silica gel 60_{PF} (Merck, gipshaltig) were used; UV detection at 254 nm. Column chromatography: Merck silica gel 60 (40−60 μm). 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 fluorescent indicator; sugar-containing compounds were visualized with the sugar spray reagent (0.5 mL of 4-methoxybenzaldehyde, 9 mL of ethanol, 0.5 mL of concentrated sulfuric acid, and 0.1 mL of glacial acetic acid) by heating with a fan or a hot plate. HPLC: (Merck-Hitachi) semipreparative HPLC—LiChroCART 250-10 containing LiChrospher 100 RP18 (10 μm); analytical HPLC—LiChroCART 250-4 or 250-3 with LiChrospher 100 RP18 (5 μm). Gradient I (standard gradient): 18−100% CH_3CN (0−16 min), 100% CH_3CN (16−20 min), 18% CH_3CN (19−31 min); flow, 0.5 mL; UV detection at 265 nm. Gradient II: 0−100% CH_3CN (0−18 min), 100% CH_3CN (18.1−20 min), 0% CH_3CN (20.1−33 min); flow, 0.5 mL; UV detection at 265 nm. Gradient III: 11−100% CH_3CN (0−18 min), 100% CH_3CN (18−20 min), 11% CH_3CN (20.1−32 min); flow, 0.5 mL; UV detection at 265 nm. Distillation: Büchi Kugelrohr apparatus.

NMR. Spectra were recorded using ^{1}H NMR: Bruker AMX 400 at 400 MHz or Bruker WH 270 at 270 MHz (DMSO as internal standard). ^{13}C NMR: Bruker AM 250 at 63 MHz ($CDCl_3$ or DMSO as internal standard). ^{31}P NMR: Bruker AMX 400 at 162 MHz (H_3PO_4 as external standard). All ^{1}H and ^{13}C NMR chemical shifts (δ) are quoted in parts per million (ppm) downfield from tetramethylsilane, $(CD_3)(CDH)SO$ being set at δ_H 2.49 as a reference. The ^{31}P NMR chemical shifts are quoted in ppm using H_3PO_4 as external reference. The spectra were recorded at room temperature, and all ^{13}C and ^{31}P NMR spectra were recorded in the proton-decoupled mode. UV spectra were taken with a Varian Cary 1E UV/vis spectrophotometer. The infrared spectra were recorded with a Perkin-Elmer 1600 series FT-IR spectrometer in KBr pellets. Mass spectra were obtained with a Fisons electrospray VG platform II spectrometer in negative mode (ESI^-). The test compounds 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 probably due to incomplete combustion of the compounds but were found to be pure by rigorous HPLC analysis (three different gradients), high-field multinuclear NMR spectroscopy, and electrospray mass spectrometry.

General Procedure for the Preparation of the *cyclo*Sal-d4TMP Phosphotriesters 3a−h. The reactions were performed in an argon atmosphere under anhydrous conditions. To a solution of d4T (**1**) (0.10 g, 0.37 mmol) in 6.0 mL of CH_3CN, cooled to 0 °C in ice/water, was added DIPEA (0.097 g, 2 equiv, 0.75 mmol). Then the chlorophosphanes **7** (2 equiv, 0.75 mmol) were added within 5 min, and the solutions were stirred for a further 20 min for completion (TLC analysis). For oxidation of the intermediate cyclic phosphites, *tert*-butyl hydroperoxide (0.072 g, 2.1 equiv, 0.80 mmol) was added to the reaction mixture at 0 °C. After the mixture stirred for 1 h and warmed to room temperature, the solvent was removed under reduced pressure. The residues were purified twice by chromatography on silica gel plates on a chromatotron first using a gradient of CH_3OH in ethyl acetate (0−30% methanol) followed by a gradient of CH_3OH in CH_2Cl_2 (0−20% methanol) to yield the title compounds **3**.

***cyclo*-(5-Nitrosaligenyl)-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3a):** yield 55%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H, NH), 8.30 (m, 1H, H4-aryl), 8.24 (2 × d, 1H, H6, $^4J_{HHCH3}$ = 1.1 Hz), 7.37 (2 × d, 1H, H3-aryl, $^3J_{HH4}$ = 9.0 Hz), 7.17 (2 × d, 1H, H6-aryl, $^4J_{HH4}$ = 1.3 Hz), 6.78 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.5 Hz, $^4J_{HH3'}$ = 1.8 Hz, $^4J_{HH4'}$ = 1.6 Hz), 6.40 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.5 Hz, $^4J_{HH1'}$ = 1.8 Hz), 6.03 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.5 Hz, $^4J_{HH4'}$ = 1.4 Hz), 5.34 (2 × ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.4 Hz, $^3J_{HP}$ = 17.4 Hz, $^3J_{HP}$ = 6.6 Hz), 4.94 (m, 1H, H4′), 4.28 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.5 Hz, $^3J_{HP}$ = 7.2 Hz, $^3J_{HP}$ = 6.9 Hz, $^3J_{HH4'}$ = 2.5 Hz), 1.66 (2 × d, 3H, CH_3-thymine, $^4J_{HH6}$ = 1.1 Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ −9.10, −9.35; R_f value 0.48 (CH_2Cl_2/MeOH, 9:1); analytical HPLC t_R 17.60 min (96%, gradient I).

***cyclo*-(5-Chlorosaligenyl)-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3b):** yield 49%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (s, 1H, NH), 7.43 (m, 1H, H6), 7.16 (m, 2H, H6, H3-aryl), 6.79 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.9 Hz, $^4J_{HH3'}$ = 1.7 Hz, $^4J_{HH4'}$ = 1.8 Hz), 6.39 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.4 Hz, $^4J_{HH1'}$ = 1.7 Hz), 6.01 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.9 Hz, $^4J_{HH4'}$ = 1.7 Hz), 5.43 (2

× ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.4 Hz, $^3J_{HP}$ = 17.3 Hz, $^3J_{HP}$ = 6.3 Hz), 4.95 (m, 1H, H4′), 4.30 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.6 Hz, $^3J_{HP}$ = 7.3 Hz, $^3J_{HP}$ = 6.7 Hz, $^3J_{HH4'}$ = 2.3 Hz), 1.66 (2 × d, 3H, CH_3-thymine, $^4J_{HH6}$ = 1.0 Hz); ^{13}C NMR (63 MHz, DMSO-d_6) δ 163.74 (C4), 150.74 (C2), 135.69 (C2-aryl), 132.75 (C3′), 129.59 (C6), 128.30 (C6-aryl), 127.37 (C2′), 126.11 (C5-aryl), 123.31 (C4-aryl), 120.11 (C1-aryl), 119.97 (C3-aryl), 109.68 (C5), 89.24 (C1′), 84.08 (C4′), 68.58 (C5′), 67.75 (CH_2-benzyl), 11.91 (CH_3-thymine); ^{31}P NMR (162 MHz, DMSO-d_6) δ −8.69, −8.75; MS (ESI$^-$) m/z 425.4; UV λ_{max} (CH_3CN) 266 nm; IR (KBr) ν 3504, 3186, 3059, 1690, 1481, 1302, 1248, 1189, 1114, 1029 cm^{-1}; R_f value 0.49 (CH_2Cl_2/MeOH, 9:1); analytical HPLC t_R 16.77 min (99%, gradient I), t_R 19.97 min (98%, gradient II), t_R 18.73 min (98%, gradient III).

***cyclo*Saligenyl-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3c):** yield 61%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.23 (s, 1H, NH), 7.36 (m, 1H, H4-aryl), 7.27 (2 × d, 1H, H6, $^4J_{HHCH3}$ = 1.6 Hz), 7.18 (m, 2H, H5-aryl, H6-aryl), 7.10 (m, 1H, H3-aryl), 6.78 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.5 Hz, $^4J_{HH3'}$ = 1.9 Hz, $^4J_{HH4'}$ = 1.6 Hz), 6.37 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.5 Hz, $^4J_{HH1'}$ = 1.9 Hz), 6.00 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.5 Hz, $^4J_{HH4'}$ = 1.6 Hz), 5.43 (2 × ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.5 Hz, $^3J_{HP}$ = 16.8 Hz, $^3J_{HP}$ = 5.5 Hz), 4.94 (m, 1H, H4′), 4.29 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.7 Hz, $^3J_{HP}$ = 7.0 Hz, $^3J_{HP}$ = 6.7 Hz, $^3J_{HH4'}$ = 2.7 Hz), 1.64 (2 × d, 3H, CH_3-thymine, $^4J_{HH6}$ = 1.6 Hz); ^{13}C NMR (63 MHz, DMSO-d_6) δ 163.74 (C4), 150.74 (C2), 149.54 (C2-aryl), 135.71 (C6), 132.82 (C3′), 129.91 (C6-aryl), 127.34 (C4-aryl), 126.20 (C2′), 124.49 (C5-aryl), 121.32 (C1-aryl), 118.12 (C3-aryl), 109.68 (C5), 89.20 (C1′), 84.12 (C4′), 68.39 (C5′), 68.28 (CH_2-benzyl), 11.94 (CH_3-thymine); ^{31}P NMR (162 MHz, DMSO-d_6) δ −8.22, −8.24; MS (ESI$^-$) m/z 391.4; UV λ_{max} (CH_3CN) 265 nm; IR (KBr) ν 3448, 3198, 3067, 1690, 1490, 1459, 1293, 1246, 1109, 1019 cm^{-1}; R_f value 0.52 (CH_2Cl_2/MeOH, 9:1); analytical HPLC t_R 15.19 min (99%), t_R 18.84 min (99%, gradient II), t_R 17.07 min (98%, gradient III).

***cyclo*-(5-Methoxysaligenyl)-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3d):** yield 73%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.27 (s, 1H, NH), 7.11 (2 × dd, 1H, H6, $^4J_{HHCH3}$ = 1.2 Hz), 6.97 (2 × d, 1H, H3-aryl, $^3J_{HH4}$ = 9.3 Hz), 6.84 (2 × dd, 1H, H4-aryl, $^3J_{HH3}$ = 9.3 Hz, $^4J_{HH6}$ = 2.7 Hz), 6.80 (d, 1H, H6-aryl, $^4J_{HH4}$ = 2.7 Hz), 6.72 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.5 Hz, $^4J_{HH3'}$ = 1.9 Hz, $^4J_{HH4'}$ = 1.6 Hz), 6.30 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.5 Hz, $^4J_{HH1'}$ = 1.9 Hz), 5.94 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.5 Hz, $^4J_{HH4'}$ = 1.6 Hz), 5.32 (2 × ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.4 Hz, $^3J_{HP}$ = 16.8 Hz, $^3J_{HP}$ = 5.5 Hz), 4.87 (m, 1H, H4′), 4.20 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.6 Hz, $^3J_{HP}$ = 7.3 Hz, $^3J_{HP}$ = 6.6 Hz, $^3J_{HH4'}$ = 2.6 Hz), 3.66 (s, 3H, OCH_3), 1.59 (2 × d, 3H, CH_3-thymine, $^4J_{HH6}$ = 1.2 Hz); ^{13}C NMR (63 MHz, DMSO-d_6) δ 163.75 (C4), 155.63 (C5-aryl), 150.73 (C2), 143.03 (C2-aryl), 135.71 (C6), 132.89 (C3′), 127.30 (C2′), 122.08 (C1-aryl), 119.07 (C3-aryl), 115.20 (C6-aryl), 110.81 (C4-aryl), 109.68 (C5), 89.18 (C1′), 84.13 (C4′), 68.29 (C5′), 68.27 (CH_2-benzyl), 55.59 (OCH_3), 11.94 (CH_3-thymine); ^{31}P NMR (162 MHz, DMSO-d_6) δ −8.08; MS (ESI$^-$) m/z 421.5; UV λ_{max} (CH_3CN) 266 nm; IR (KBr) ν 3448, 3164, 3036, 1685, 1498, 1458, 1288, 1198, 1115, 1027 cm^{-1}; R_f value 0.50 (CH_2Cl_2/MeOH, 9:1); analytical HPLC t_R 15.19 min (99%), t_R 19.24 min (99%, gradient II), t_R 17.32 min (99%, gradient III).

***cyclo*-(3-Methoxysaligenyl)-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3e):** yield 70%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.32 (s, 1H, NH), 7.17 (2 × d, 1H, H6, $^4J_{HHCH3}$ = 1.2 Hz), 7.11 (m, 2H, H5-aryl, H6-aryl), 6.83 (m, 1H, H4-aryl), 6.80 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.4 Hz, $^4J_{HH3'}$ = 1.7 Hz, $^4J_{HH4'}$ = 1.5 Hz), 6.36 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.3 Hz, $^4J_{HH1'}$ = 1.7 Hz), 6.00 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.4 Hz, $^4J_{HH4'}$ = 1.4 Hz), 5.43 (2 × ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.4 Hz, $^3J_{HP}$ = 17.1 Hz, $^3J_{HP}$ = 6.7 Hz), 4.97 (m, 1H, H4′), 4.29 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.5 Hz, $^3J_{HP}$ = 7.1 Hz, $^3J_{HP}$ = 6.8 Hz, $^3J_{HH4'}$ = 2.5 Hz), 3.32 (s, 3H, OCH_3), 1.66 (2 × s, 3H, CH_3-thymine); ^{13}C NMR (63 MHz, DMSO-d_6) δ 163.70 (C4), 150.68 (C2), 148.20 (C3-aryl), 138.90 (C2-aryl), 135.52 (C6), 132.79 (C3′), 127.28 (C2′), 124.57 (C1-aryl), 122.24 (C5-aryl), 117.02 (C6-aryl), 112.69 (C4-aryl), 109.66 (C5), 89.11 (C1′), 84.06 (C4′), 68.27 (C5′), 68.12 (CH_2-benzyl), 55.85 (OCH_3), 11.69 (CH_3-thymine); ^{31}P NMR (162 MHz, DMSO-d_6) δ −7.82, −7.81; MS (ESI$^-$) m/z 421.5; UV λ_{max} (CH_3CN) 266 nm; IR (KBr) ν 3448, 3190, 3060, 1686, 1490, 1467, 1284, 1243, 1113, 1023 cm^{-1}; R_f value 0.51 (CH_2Cl_2/MeOH, 9:1); analytical HPLC t_R 14.37 min (96%), t_R 18.85 min (99%, gradient II), t_R 17.03, 17.16 min (98%, gradient III).

***cyclo*-(5-Methylsaligenyl)-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3f):** yield 53%; ^{1}H NMR (250 MHz, DMSO-d_6) δ 11.34 (2 × s, 1H, NH), 7.68 (m, 1H, H6), 7.18 (m, 1H, H6-aryl), 7.16 (m, 1H, H4-aryl), 7.00 (m, 1H, H3-aryl), 6.78 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.5 Hz, $^4J_{HH3'}$ = 1.9 Hz, $^4J_{HH4'}$ = 1.6 Hz), 6.37 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.3 Hz, $^4J_{HH1'}$ = 1.9 Hz), 6.00 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.5 Hz, $^4J_{HH4'}$ = 1.6 Hz), 5.39 (2 × ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.4 Hz, $^3J_{HP}$ = 17.6 Hz, $^3J_{HP}$ = 6.9 Hz), 4.94 (m, 1H, H4′), 4.28 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.3 Hz, $^3J_{HP}$ = 7.2 Hz, $^3J_{HP}$ = 6.9 Hz, $^3J_{HH4'}$ = 2.5 Hz), 2.25 (s, 3H, CH_3-C5-aryl), 1.65 (2 × s, 3H, CH_3-thymine); ^{13}C NMR (63 MHz, DMSO-d_6) δ 163.73 (C4), 150.71 (C2), 147.42 (C2-aryl), 135.68 (C6), 133.73 (C3′), 132.84 (C5-aryl), 130.14 (C6-aryl), 127.28 (C2′), 126.18 (C4-aryl), 120.82 (C1-aryl), 117.83 (C3-aryl), 109.67 (C5), 89.18 (C1′), 84.11 (C4′), 68.43 (C5′), 68.23 (CH_2-benzyl), 20.14 (CH_3-C5-aryl), 11.94 (CH_3-thymine); ^{31}P NMR (162 MHz, DMSO-d_6) δ −8.13, −8.20; MS (ESI$^-$) m/z 405.4; UV λ_{max} (CH_3CN) 265 nm; IR (KBr) ν 3168, 3038, 2929, 1696, 1498, 1466, 1307, 1207, 1115, 1036 cm^{-1}; R_f value 0.52 (CH_2Cl_2/MeOH, 9:1); analytical HPLC t_R 16.59 min (99%), t_R 19.68 min (99%, gradient II), t_R 18.28 min (98%, gradient III).

***cyclo*-(3-methylsaligenyl)-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3g):** yield 60%; ^{1}H NMR (250 MHz, DMSO-d_6) δ 11.34 (2 × s, 1H, NH), 7.25 (m, 1H, H4-aryl), 7.18 (2 × d, 1H, H6, $^4J_{HHCH3}$ = 1.2 Hz), 7.16 (m, 2H, H5-aryl, H6-aryl), 6.78 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.5 Hz, $^4J_{HH3'}$ = 1.7 Hz, $^4J_{HH4'}$ = 1.8 Hz), 6.38 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.5 Hz, $^4J_{HH1'}$ = 1.7 Hz), 6.01 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.5 Hz, $^4J_{HH4'}$ = 1.7 Hz), 5.40 (2 × ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.3 Hz, $^3J_{HP}$ = 17.6 Hz, $^3J_{HP}$ = 5.9 Hz), 4.94 (m, 1H, H4′), 4.28 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.7 Hz, $^3J_{HP}$ = 7.4 Hz, $^3J_{HP}$ = 6.7 Hz, $^3J_{HH4'}$ = 2.7 Hz), 2.25 (s, 3H, CH_3-C5-aryl), 1.65 (2 × s, 3H, CH_3-thymine); ^{13}C NMR (63 MHz, DMSO-d_6) δ 163.19 (C4), 150.18 (C2), 148.50 (C2-aryl), 135.14 (C6), 132.76 (C3′), 130.46 (C4-aryl), 126.78 (C3-aryl), 126.32 (C2′), 123.48 (C5-aryl), 123.15 (C6-aryl), 120.70 (C1-aryl), 109.12 (C5), 89.15 (C1′), 83.65 (C4′), 68.20 (C5′), 67.70 (CH_2-benzyl), 14.22 (CH_3-C3-aryl), 11.33 (CH_3-thymine); ^{31}P NMR (162 MHz, DMSO-d_6) δ −7.48, −7.68; MS (ESI$^-$) m/z 405.3; UV λ_{max} (CH_3CN) 265 nm; IR (KBr) ν 3448, 3189, 3049, 1690, 1473, 1288, 1190, 1113 cm^{-1}; R_f value 0.51 (CH_2Cl_2/MeOH, 9:1); analytical HPLC t_R 16.25 min (99%), t_R 19.40 min (99%, gradient II), t_R 18.09 min (98%, gradient III).

***cyclo*-(3,5-Dimethylsaligenyl)-5′-*O*-(2′,3′-dideoxy-2′,3′-didehydrothymidinyl)phosphate (3h):** yield 59%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (2 × s, 1H, NH), 7.16 (2d, 1H, H6, $^4J_{HH}$ = 1.2 Hz), 7.03 (m, 1H, H4-aryl), 6.87 (m, 1H, H6-aryl), 6.77 (2 × ddd, 1H, H1′, $^3J_{HH2'}$ = 3.5 Hz, $^4J_{HH3'}$ = 1.9 Hz, $^4J_{HH4'}$ = 1.6 Hz), 6.37 (2 × ddd, 1H, H3′, $^3J_{HH2'}$ = 5.9 Hz, $^3J_{HH4'}$ = 3.5 Hz, $^4J_{HH1'}$ = 1.9 Hz), 6.00 (2 × ddd, 1H, H2′, $^3J_{HH3'}$ = 5.9 Hz, $^3J_{HH1'}$ = 3.5 Hz, $^4J_{HH4'}$ = 1.6 Hz), 5.34 (2 × ddd, 2H, CH_2-benzyl, $^2J_{HH}$ = 14.4 Hz, $^3J_{HP}$ = 17.6 Hz, $^3J_{HP}$ = 6.4 Hz), 4.94 (m, 1H, H4′), 4.28 (2 × dddd, 2H, H5′, $^2J_{HH}$ = 11.4 Hz, $^3J_{HP}$ = 7.0 Hz, $^3J_{HP}$ = 6.7 Hz, $^3J_{HH4'}$ = 2.7 Hz), 2.22 (s, 3H, CH_3-C5-aryl), 2.14 (2s, 3H, CH_3-C3-aryl), 1.63 (2d, 3H, CH_3-thymine, $^4J_{HH6}$ = 1.2 Hz); ^{13}C NMR (68 MHz, DMSO-d_6) δ 163.68 (C4), 150.68 (C2), 145.89 (C2-aryl), 135.64 (C6), 133.11 (C3′), 132.80 (C5-aryl), 131.41 (C6-aryl), 127.28 (C2′), 126.49 (C4-aryl), 123.69 (C3-aryl), 120.87 (C1-aryl), 109.64 (C5), 89.19 (C1′), 84.12 (C4′), 68.28 (C5′), 68.12 (CH_2-benzyl), 20.09 (CH_3-C5-aryl), 14.70 (CH_3-C3-aryl), 11.94 (CH_3-thymine); ^{31}P NMR (162 MHz, DMSO-d_6) δ −7.32, −7.63; MS (ESI$^-$) m/z 442.6; UV λ_{max} (CH_3CN) 265 nm; IR (KBr) ν 3448, 3186, 3069, 1690, 1482, 1288, 1201, 1113, 1027 cm^{-1}; R_f value 0.51 (CH_2Cl_2/MeOH,

9:1); analytical HPLC t_R 17.08 min (99%), t_R 20.41 min (98%, gradient II), t_R 18.84 min (99%, gradient III).

Determination of the Partition Coefficients. Pa values were determined as follows: A sample of the compounds **3a**−**h** was dissolved in 1.0 mL of 1-octanol. To this solution was added 1.0 mL of aqueous phosphate buffer solution (10 mmol, pH 6.8). After the phases were mixed extensively for 10 min (vortex) and separation by centrifugation (3 min at 9000 rpm), aliquots of each phase were analyzed by analytical HPLC (Merck LiChroCART column filled with LiChrospher 100 reversed-phase silica gel RP 18 (5 μm); gradient, 11−100% CH_3CN in water (0−18 min), 100% CH_3CN (18−20 min), 11% CH_3CN (20−32 min); flow, 0.5 mL; UV detection at 265 nm). By quantification of the peak areas, the Pa values were obtained from the quotient.

Kinetic Data. (a) All Aqueous Buffers: Aqueous buffer (1.0 mL) (30 mM TRIS buffer, pH 6.9; 30 mM sodium phosphate buffer, pH 7.3; 30 mM sodium borate buffer, pH 8.9) was equilibrated at 37 °C and mixed with 0.5 mL of a solution of 1.0 mg of compound **3a**−**h** in 1.2 mL of H_2O (∼1.9 mM) also equilibrated at 37 °C (final concentration of the hydrolysis mixture: ∼0.63 mM). For the kinetic data, aliquots (100 μL) of the hydrolysis mixture were stopped by the addition of 5.0 μL of acetic acid and subsequently analyzed by analytical HPLC (Merck LiChroCART column filled with LiChrospher 100 reversed-phase silica gel RP 18 (5 μm); gradient, 9−100% CH_3CN in 0.6 mM tetrabutylammonium phosphate, pH 3.8 (0−20 min) and 9% CH_3CN (20−30 min); 0.5 mL flow; UV detection at 265 nm). The degradation of **3** was followed by quantification of the peak areas in the HPLC chromatograms. The rate constants k were determined from the slope of the logarithmic degradation curve of the title compounds. The half-lives ($t_{1/2}$) were calculated by using the rate constants k.

(b) *R*oswell *P*ark *M*emorial *I*nstitute (RPMI)-1640 Culture Medium Containing 10% Heat-Inactivated FCS: This medium was used instead of aqueous buffers, and the data were collected in the same way.

Mass Spectrometric Analysis. As for the kinetic studies, ∼0.63 mM solutions of the 5-chloro-*cyclo*Sal-d4TMP (**3b**) or the 3-methoxy-*cyclo*Sal-d4TMP (**3e**) were prepared by solubilizing the *cyclo*Sal derivative in 10 μL of acetonitrile, diluting with HPLC-grade water (Riedel-de-Haen). The solution was stored at 50 °C in a water bath. After several time intervals aliquots were taken and analyzed by means of electrospray mass spectroscopy run in the negative mode. The appropriate mass peaks of the starting materials, the intermediate phosphodiesters, the salicyl alcohols, and d4TMP were detected with their $^{35}Cl/^{37}Cl$ and $^{12}C/^{13}C$ isotope peaks. Subsequently, the same concentrations were prepared in 10% normalized [^{18}O]water (Aldrich). Again, the hydrolysates were analyzed by mass spectrometry, and the amounts of ^{18}O-incorporation were calulated.

Antiretroviral Evaluation. Human immunodeficiency virus type 1 [HIV-1 ($HTLV\text{-}III_B$)] was originally obtained from a persistently HIV-infected H9 cell line as described previously[38] which was kindly provided by Dr. R. C. Gallo (National Institutes of Health, Bethesda, MD). Virus stocks were prepared from the supernatants of HIV-1-infected MT-4 cells. HIV-2 (strain LAV 2) was provided by Dr. L. Montagnier (Pasteur Institute, Paris, France), and virus stocks were prepared from the supernatants of HIV-2-infected MT-4 cells. CEM/O cells were obtained from the American Tissue Culture Collection (Rockville, MD), and CEM/TK^- cells were a gift from Prof. S. Eriksson and Prof. A. Karlsson (Karolinska Institute, Stockholm, Sweden). CEM cells were infected with HIV as previously described.[39] Briefly, 4×10^5 cells/mL were infected with HIV-1 or HIV-2 at ∼100 $CCID_{50}$ (50% cell culture infective dose)/mL of cell suspension. Then 100 μL of the infected cell suspension was transferred to 96-well microtiter plate wells and mixed with 100 μL of the appropriate dilutions of the test compounds. After 4 days giant cell formation was recorded microscopically in the HIV-infected cell cultures.

MT-4 cells (5×10^5 cells/mL) were suspended in fresh culture medium and infected with HIV-1 at 100 times the 50% cell culture infective dose ($CCID_{50}$)/mL of cell suspension (1 $CCID_{50}$ being the dose infective for 50% of the cell cultures). Then 100 μL of the infected cell suspension was transferred to microtiter plate wells, mixed with 100 μL of the appropriate dilutions of compound, and further incubated at 37 °C. After 5 days, the number of viable cells was determined in a blood-cell-counting chamber by Trypan blue staining for both virus-infected and mock-infected cell cultures. The 50% effective concentration (EC_{50}) and the 50% cytotoxic concentration (CC_{50}) of the test compounds were defined as the compound concentrations required to inhibit virus-induced cytopathicity by 50% or to reduce the number of viable cells in mock-infected cell cultures by 50%, respectively.

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Supporting Information Available: ^1H, ^{13}C, and ^{31}P NMR spectral data for the title compounds **3a**−**h** (23 pages). Ordering information is given on any current masthead page.

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