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Cyclic Saligenyl Phosphotriesters of 2',3'-Dideoxy-2',3'-didehydrothymidine (d4T) - A New Pro-Nucleotide Approach[1] -

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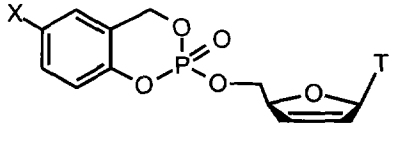
Abstract : The synthesis of a new pro-nucleotide approach for d4TMP **2** based on cycloSal-d4TMP **3a-d** is described. Phosphotriesters **3** release d4TMP **2** selectively by a controlled, chemically induced tandem reaction. CycloSal-phosphotriesters **3** exhibited high biological activity against HIV-1/ HIV-2 in CEM cells which was retained in CEM TK$^-$ cells.

Nucleoside analogues, e.g. 2',3'-dideoxy-2',3'-didehydrothymidine **1** (d4T, Stavudine, Cerit®), are used as antiviral agents in the treatment for AIDS and the AIDS related complex (ARC). As compared to the anti-HIV drug AZT, d4T **1** shows comparable selective anti-HIV activity *in-vitro*[2]. Moreover, d4T **1** has been found to be less toxic than AZT for bone marrow stem cells and to be less inhibitory to mitochondrial DNA replication[3]. After penetration through cell membranes, intracellular conversion of **1** into the 5'-triphosphate is essential for the expression of the biological activity. Because of the structural differences of d4T as compared to natural nucleosides the first thymidine kinase catalyzed phosphorylation into d4T-monophosphate **2** (d4TMP) is the limiting step of the metabolization in CEM and MT-4 cells[4]. Consequently, the direct introduction of d4TMP **2** should bypass the limiting step and have advantages for the biological activity. Unfortunately, the highly polar monophosphate **2** is not able to penetrate membranes or the blood brain barrier due to low lipophilicity. One attempt to improve the therapeutic potential of d4T **1** is the use of neutral, lipophilic prodrugs[5] of d4TMP 2[6] (Pro-Nucleotide-Approach).

In this work we present the synthesis and properties of a new class of cyclosaligenyl d4T-monophosphate **3** (cycloSal-d4TMP) as neutral prodrugs of d4TMP **2**. This prodrug concept was designed to release the nucleotide **2** selectively by controlled, chemically induced hydrolysis following a tandem-mechanism[7].



*cyclo*Sal-d4TMP **3a** : NO_2
b : Cl
c : H
d : Me

In contrast to other prodrug concepts, the approach reported here leads to d4TMP **2** after a coupled cleavage of the phenyl- and the benzylester bond of the phosphotriester **3**. The difference in hydrolytic stability

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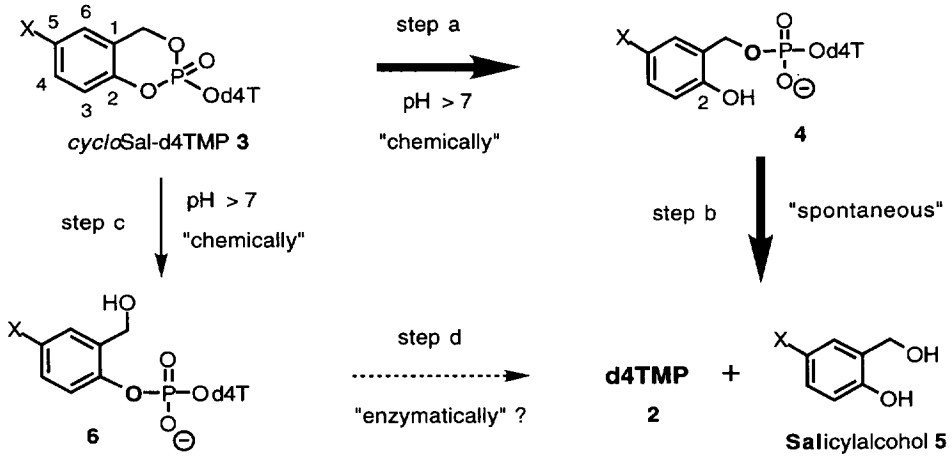
of phenyl-[8] and benzylphosphotriesters[9] has already been reported. Whereas acceptor substituted *bis*-phenylphosphotriesters are rapidly hydrolyzed under alkaline conditions, a fast hydrolysis of *bis*-benzylphosphotriesters was observed only in the case of donor substituted derivatives. In general, both resulting phosphodiesters are very stable against non-enzymatic hydrolysis (step d, scheme 1)[10]. The rational of our new prodrug concept introduced here with **3** is based on this difference in stability of the phenyl- and the benzyl phosphate ester: this allows to discriminate between the different phosphate ester bonds and results in the design of a tripartate prodrug system. The phenyl ester bond should be the most labil one while the concurrent cleavage of the benzyl ester in **3** to yield the 2-hydroxymethylphenylphosphodiester **6** is unfavorable (step c, scheme 1), because the phosphate ester in the ortho position of the benzyl ester in **3** stabilizes this bond. Hence, the hydrolysis should proceed as follows: in the initial step the phenyl ester bond is cleaved selectively (step a, scheme 1), because the negative charge could be delocalized in the aromatic ring leading to the 2-hydroxybenzylphosphodiester **4**. As a consequence, the substituent ortho to the benzyl group switch from an acceptor (phosphate) to a donor (hydroxyl) in this cleavage ("Umpolung") and this induces the cleavage of diester **4** releasing d4TMP **2** and the diol **5** spontaneously[11] (tandem reaction; step b, scheme 1). Moreover, the initial activation step leading to diester **4** should be controlled by substituents in the para position (position 5) of the phenolic phosphate ester bond: Salicylalcohols **5** bearing electron withdrawing substituents like a nitro group or the chloro atom should accelerate step a, whereas electron donating substituents like the methyl group should stabilize the phenol ester bond. To study this effect, the substituted diols **5a-d** were introduced. Before hydrolysis takes place the cycloSal-d4TMP's **3** should serve as lipophilic, neutral prodrugs.

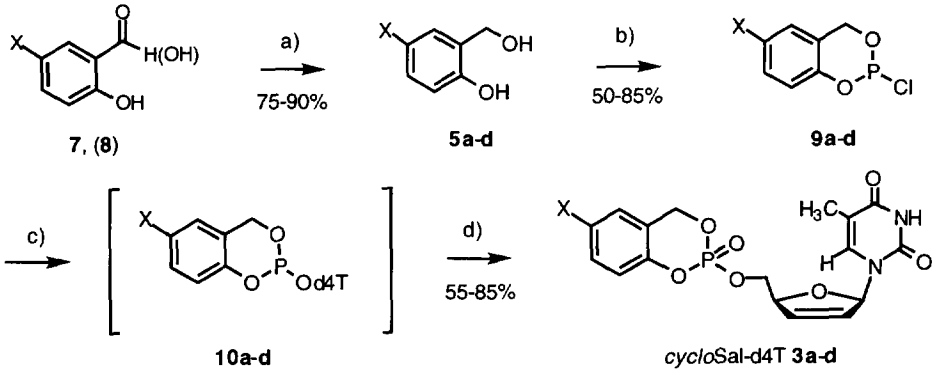
Scheme 1: The hydrolysis pathways of cycloSal-d4TMP phosphotriesters **3**



Substituted salicylaldehydes **7** or salicylic acids **8** were used as starting materials for the synthesis of the cycloSal-d4TMP derivatives **3**. Reduction of **7** and **8** by standard procedures lead to the salicylalcohols **5** in 75-90% yield. Diols **5** were reacted with phosphorustrichloride under anhydrous conditions to yield the cyclic saligenylchlorophosphanes **9**[12], which were isolated by distillation under argon atmosphere in 50-85 % yield. The target compounds **3a-d** were obtained in an "one-pot" reaction by reacting d4T **1**[13] with 1.5 equiv. of the chlorophosphanes **9a-d** at O °C in the presence of dried, distilled diisopropylethylamine (DIPEA) using dry acetonitrile as solvent via the cyclic phosphites **10a-d** and subsequent *in-situ* oxidation with *t*-butylhydroperoxide (TBHP; scheme 2)[7]. After purification on a chromatotron (60-85% yield) the cycloSal-d4TMP's **3**

were obtained as 1 : 1 diastereomeric mixtures with respect to the configuration at the phosphorus atom. The two diastereomers could be distinguished by ^{1}H-, ^{13}C- and ^{31}P nmr spectroscopy[14] but could not be separated by common chromatographic methods. Furthermore, **3** were characterized by means of UV, MALDI-TOF (positive mode), electrospray (ESI; negative mode) and HR mass spectrometry. The purity was checked by means of analytical HPLC analysis. Small quantities of compounds **3** were additionally purified by semi-preparative HPLC for the in vitro assays in order to be sure to eliminate even traces of impurities.

Scheme 2: Synthesis of the title compounds **3**



X = Nitro, Chloro, H, Methyl

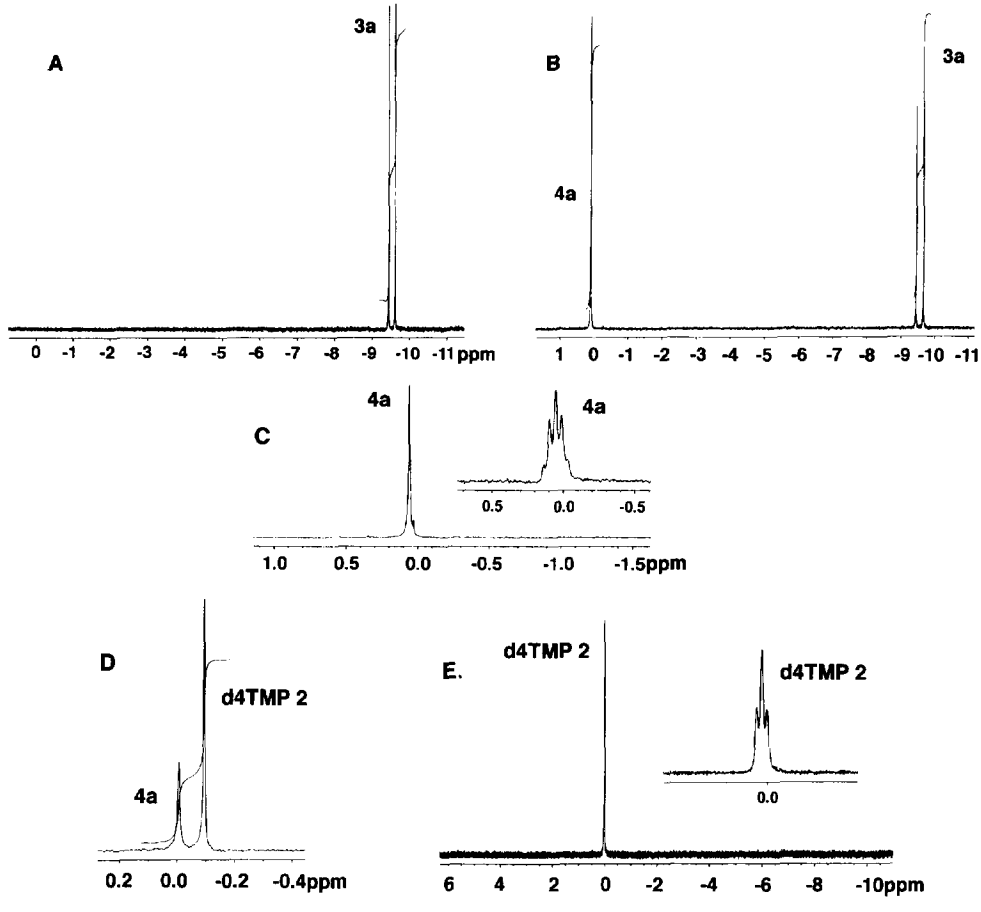
a) $NaBH_4$ ($LiAlH_4$), iPr-OH; b) PCl_3, pyridine, Et_2O, -10°C, 2h; c) d4T **1**, DIPEA, CH_3CN, 0°C, 20 min; d) TBHP, CH_3CN, rt, 30 min.

The partition coefficients (PC values) in 1-octanol / phosphate buffer (pH 6.8) are a qualitative estimation of the lipophilic properties of cycloSal-d4TMP's **3**[15]. The calculated PC values are listed in table 1. As can be seen, all phosphotriesters **3** have partition coefficients that are by a factor of 10 to 50 higher as compared to d4T **1** and all higher than 1. In principle, an improved cellular uptake as compared to d4T should to be possible.

The most important question was the verification of the designed hydrolysis pathway (step a and b, scheme 1). First, a nmr experiment was carried out. In this study the 5-nitro substituted cycloSal-d4TMP **3a** was dissolved in DMSO, containing 1% water (pH ~7.01). It should be mentioned that this experiment was used only as an model in order to prove the degradation pathway. After several intervals the ^{1}H, ^{13}C and ^{31}P nmr spectra were recorded. The result of this study is shown in figure 1: at the beginning (t = 0) only the two ^{31}P nmr resonance signals of the two diastereomers of cycloSal-d4TMP's **3a** (~-9.5 ppm; section A, figure 1) were observed. Following the hydrolysis, a new ^{31}P nmr signal at +0.05 ppm appears and increases while the signals of **3a** are disappearing (section B, figure 1). Recording a proton-coupled ^{31}P nmr spectra, this new signal splits into a quintet (section C, figure 1). Hence, the phosphorus is coupled with four protons: the two protons at the C5'-atom of d4T and the two protons of the benzyl C-atom. This splitting of the ^{31}P nmr signal fits only with the structure of the 2-hydroxybenzylphosphodiester **4a**. This interpretation was confirmed by the ^{1}H and the ^{13}C nmr spectra. When the hydrolysis proceeded, again one additional ^{31}P nmr signal at -0.1 ppm was observed (section D, figure 1). Again in the proton couple spectra, a splitting of this ^{31}P signal into a triplet was observed (section E, figure 1). The splitting into a triplet is in accordance with the structure of the d4TMP **2** (P-coupling with two protons of the C5'-atom). Again, this result was confirmed by the ^{1}H and ^{13}C nmr spectra. The half-lifes of the two steps were $t_{1/2}$ = 6 h and $t_{1/2}$ = 4 d, respectively. It should be stressed that at

the end of the hydrolysis d4TMP **2** and 2-hydroxy-5-nitrobenzylalcohol **5a** were the sole products. Under the reported conditions the designed tandem reaction undoubtedly took place.

Figure 1: ^{31}P NMR detected hydrolysis of cycloSal-d4TMP **3a**



As a model for the physiological milieu hydrolysis studies of the cycloSal-d4TMP's **3** were carried out in 30 mmol phosphate buffer, pH 7.5 at 37 °C and followed by means of HPLC analysis. In all cases the cycloSal-d4TMP's **3** were degraded following pseudo-first order kinetics to give d4TMP **2** as well as the salicylalcohols **5** (Table 1). Furthermore, the identification of **2** and **5** was accomplished directly from the hydrolysis mixture by electrospray mass spectrometry. In subsequent hydrolysis studies the degradation of the triesters **3** was investigated in TRIS buffer (30 mmol, pH 6.8) and borate buffer (30 mmol, pH 8.9) in order to investigate the pH dependence of hydrolysis (table 1). As expected a clear pH dependence was observed: slowest degradation was observed at pH 6.8 whereas the hydrolysis of **3** was fastest at pH 8.9. Furthermore, in addition to the selective hydrolysis to d4TMP **2**, the expected effect of the substituents of the aromatic ring on the $t_{1/2}$ values was observed. All these results are in fully agreement with the postulated hydrolysis pathway shown in scheme 1. Consequently, in accordance to their hydrolytic stability ($t_{1/2}$ value) the donor substituted derivatives of **3** should act as suitable pro-nucleotides (table 1).

In order to prove this assumption cycloSal-d4TMP's **3** were evaluated concerning their antiviral activity in HIV-1 and HIV-2 infected CEM/O cells as well as in HIV-2 infected CEM-thymidine kinase deficient (TK^-) cells. The results are summarized in table 1. A clear correlation of the biological activity with the half lives of the cycloSal-d4TMP's **3** in the hydrolysis studies was observed.

Table 1: Hydrolysis in different buffers, PC values, and antiviral activity of cycloSal-d4TMP's **3**

3 or d4T **1**	Hydrolysis ($t_{1/2}$) in buffers			Antiviral Activity EC_{50} (µg/ml)			PC value
X	TRIS pH 6.9 [h]	phosphate pH 7.5 [h]	borate pH 8.9 [h]	CEM/O HIV-1	CEM/O HIV-2	CEM/TK^- HIV-2	
3a (NO_2)	4.1	0.15	0.06	0.29	0.40	40	1.5
3b (Cl)	6.5	0.6	0.3	0.18	0.25	20	7.7
3c (H)	24.5	4.0	1.1	0.14	0.16	**0.35**	1.9
3d (Me)	28.3	7.1	1.3	0.074	0.16	**0.45**	5.4
1	--	--	--	0.40	1.45	45	0.15

The nitro- as well as the chloro-substituted derivative **3a** and **3b** showed at least equal activity in the wild type cell line (CEM/O) as the parent nucleoside d4T **1** and a loss of activity in the TK^- cell line. This loss of activity in the TK^- cell line could be explained with the short half-lives of these two acceptor substituted derivatives **3a,b** as compared to **3c,d** in the buffer solutions (table 1): it seems that these phosphotriesters are hydrolyzed outside the cells to give finally d4T **1**. In contrast, the unsubstituted and the methyl substituted triesters **3c** and **3d** exhibited a three to six-fold higher activity already in the CEM/O cell line as **1**. Moreover, this activity was retained in the TK^- cell line. As compared to d4T **1**, the cycloSal-d4TMP's **3c** and **3d**, respectively, were by a factor of 100 (two logs) more antiretrovirally active. This correlates with the markedly higher hydrolytic stability of these triesters as compared to **3a** and **3b** in the buffer hydrolyses and shows the ability to deliver d4TMP **2** inside the cells[16].

In summary, for the first time the described pro-nucleotide concept is suitable to deliver selectively d4TMP **2** from the cycloSal-d4TMP's **3** by a controlled, non-enzymatic mechanisms at physiological pH according to the designed tandem-reaction in two coupled steps. The donor substituted derivatives **3c, d** were highly active in CEM/O cells as well as in CEM/TK^- cells confirming the intracellular delivery of d4TMP **2**. Further work is currently in progress in our laboratory in order to explore this pro-nucleotide concept.

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EXPERIMENTAL

General Procedure : To a solution of 0.64 mmol of dried d4T **1** in 10 ml acetonitrile 1.27 mmol diisopropylethylamine (DIPEA) was added at 0 °C. To this solution 2.0 equiv. (1.28 mmol in 1 ml acetonitrile) of the cyclic chlorophosphanes **9a-d** solubilized in 2 ml dry acetonitrile were slowly added and stirring was continued for 20 min. The formed cyclophosphites **10a-d** were oxidized *in-situ* using 200 µl *t*-

butylhydroperoxide (TBHP) within 30 min. After evaporation of the solvent the residue was purified by chromatography on a Chromatotron using a gradient of ethyl acetate / methanol (0-30%) or of dichloromethane / methanol (0-20%). For the biological evaluation small samples were additionally purified by semi-preparative HPLC (isocratic elution in water / acetonitrile) and isolated by lyophilisation.

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14. In all cases the resonance signals of the two diastereomers of **3** were found to be well separated in the ^{31}P nmr spectra. The chemical shifts correlate with the electronic properties of the substituents: as stronger the electron withdrawing properties of the substituents as highfield-shifted the resonance signals were observed: **3a** (NO_2; σ = 0.81; -9.40/-9.64 ppm); **3b** (Cl; σ = 0.24; -8.59/8.66 ppm); **3c** (H; σ = 0.0; -8.13/-8.15 ppm); **3d** (Me; σ = -0.14; -8.04/-8.11).
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