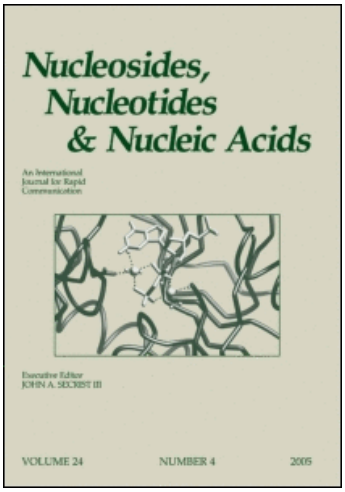

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Study of Different Substituted Cyclic and Acyclic Benzylpronucleotides of d4T Relative to Their Hydrolytic Stability and Antiviral Activity

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ABSTRACT

*Cyclo*Sal-d4TMP and two different bis(benzyl) phosphate triesters of the antivirally active nucleoside analog d4T were studied with regard to their chemical hydrolysis behavior at pH 7.3, in CEM/0 cell extracts, and their anti-HIV activity. In contrast to triesters **2**–**4**, bis-(*o*-AB)-d4TMP **1** was found to be chemically exquisitely stable. All compounds led to the formation of d4TMP in cell extracts and all triesters achieved the TK-bypass.

INTRODUCTION

The *cycloSal*-pronucleotide concept differs from other nucleotide prodrug approaches in that it was designed to selectively deliver the nucleoside 5′-monophosphate by a controlled, chemically induced hydrolysis.[1] The different stability of phenyl-, benzyl- and an alkyl phosphate ester allows a chemical discrimination of these ester

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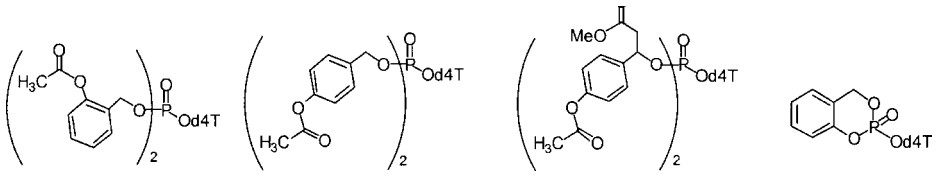
bonds. Only one activation step without any enzymatic triggering is needed.[2,3] Here, we investigated bis(acetyloxybenzyl (AB)-d4TMP **1**, the acyclic analog of the *cyclo*-Sal-d4TMP **4**, in comparison with the bis(*p*-AB)-counterpart **2** and bis(α-methoxycarbonylmethyl (MCM)-*p*-AB)-d4TMP **3** with respect to its chemical and hydrolysis behavior in cell extracts.

RESULTS

The synthesis of the bis(*o*-AB)-d4TMP **1** was performed using *ortho*-hydroxybenzaldehyde as starting material. *Ortho*-acetoxybenzaldehyde was obtained in 45% yield by acetylation with acetic acid anhydride. The subsequent reduction by $NaBH_4$in *i*-propanol led to *ortho*-acetoxybenzyl alcohol in 87% yield. The synthetic approach towards triester **1** via the formation of the corresponding phosphoramidite



Bis(*o*-AB)-d4TMP **1** Bis(*p*-AB)-d4TMP **2** Bis(α-MCM-*p*-AB)-d4TMP **3** *cyclo*Sal-d4TMP **4**

was conducted as published before.[4] The synthesis of the para-substituted counterpart bis(*p*-AB)-d4TMP **2** and (α-MCM-*p*-AB)-d4TMP **3** has been published before.[4]

We have investigated the hydrolytic stability in aqueous buffered medium (PBS/pH 7.3) and the metabolic fate of the four prodrugs of d4T in CEM/0 cell extract (Table 1). To our surprise, the bis(*o*-AB) -d4TMP **1** was found to be extremely stable under chemical hydrolyzing conditions ($t_{1/2} > 100$ h), whereas the *para* substituted counterpart **2** showed a 14-fold decrease of the $t_{1/2}$-value. More intriguing, the bis(α-MCM-*p*-AB) triester **3** showed only a half-life of 1.8 h (55-fold decrease) for the first chemical hydrolysis step.

This decrease in stability can be attributed to the presence of a secondary carbon-atom due to the substitution with the MCM group. We assume a side reaction in which a spontaneous C-O-bond cleavage results in a benzyl cation and a phosphate leaving group. Unfortunately, the resulting products are identical to those formed by the designed pathway. It was interesting to note that the latter two triesters led to the release of d4TMP, even under chemical hydrolysis conditions. *Cyclo*Sal-d4TMP **4** showed a half-life of 4.4 h and released d4TMP directly. The mechanistic interpretation is summarized in Fig. 1.

In addition, a NMR study in an imidazole/HCl buffer was carried out with triester **2** using the presence of the phosphorus nucleus. Figure 2 shows the spectra in dependence of time. Clearly the intermediate phosphate diester could be detected.

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Table 1. Hydrolytic stability and anti-HIV activity.

	Hydrolysis		Anti-HIV activity EC_{50} [μM]			
			CEM/O		CEM/TK^-	
Compound	$t_{1/2}$ [h] (PBS) (First step/Second step)	$t_{1/2}$ [h] (CEM/O)	HIV-1	HIV-2	HIV-2	CC_{50} [μM] CEM/O
1	>100/n.d.	1.4/5	0.12	0.22	0.48	21
2	7.2/17.2	<0.15/4	0.17	0.33	0.13	58
3	1.8/27	<0.15/25	0.25	0.30	0.20	19
4	4.4/n.d.	5.0/n.d.	0.20	0.22	0.15	50
d4T	–	–	0.33	0.25	>10	80

n.d.: not detectable.

In all four cases formation of d4TMP was observed in CEM cell extracts. As expected, the $t_{1/2}$ values in CEM cell extracts were markedly lower for all enzymatically activated compounds **1**–**3**. The degradation pathway is summarized in Fig. 3. Remarkable is the marked difference in the $t_{1/2}$ values of compounds **1** and **2**. We assume a steric hindrance of the enzyme-catalyzed ester bond cleavage in the *ortho*-position.

Finally, the antiviral activity of the compounds was evaluated in vitro. Compounds **1**–**4** retained their activity found in wild-type cells in the thymidine-kinase deficient (TK^-) cells. Thus, these compounds function through a thymidine kinase-bypass mechanism.

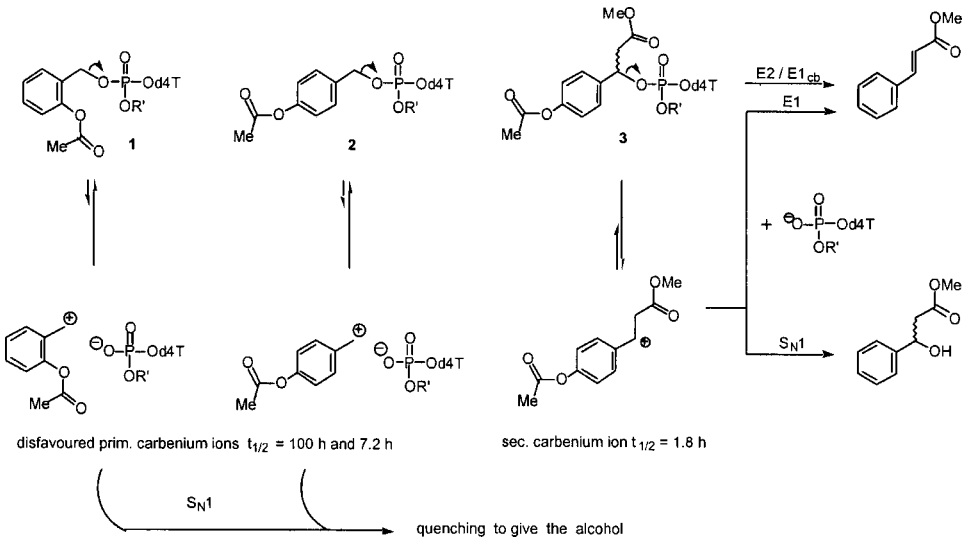

Figure 1. Mechanisms of degradation of the bis(AB)-d4TMP triesters.

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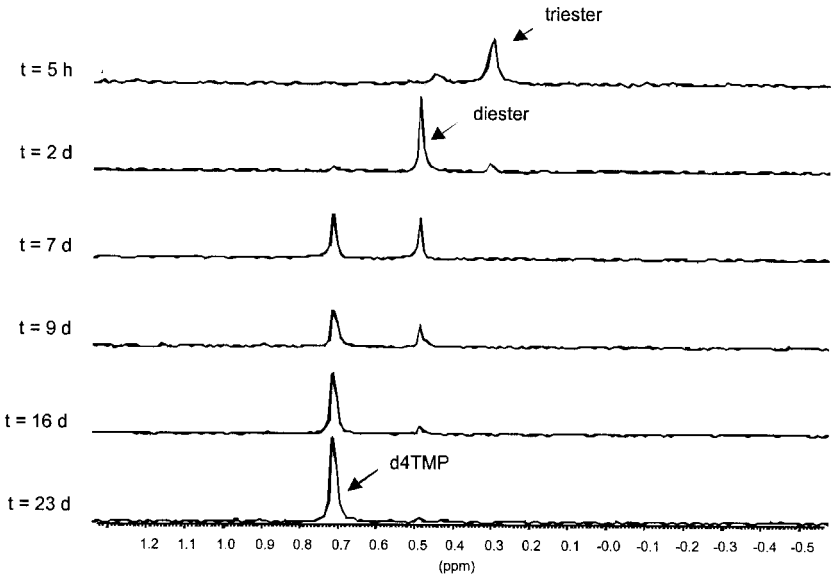

Figure 2. Hydrolysis study of triester **1** followed by ^{31}P-NMR.

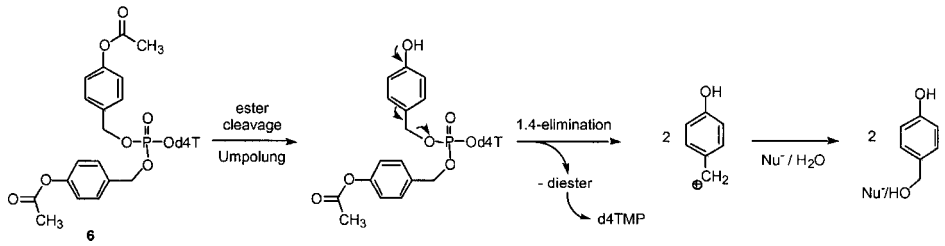

Figure 3. Degradation of bis(*p*-AB)-d4TMP **2** in cell extracts.

Interestingly, the chemically stabile triester **1** was as active as the other triesters and so clearly adheres to enzymatic activation by cellular esterases. No difference was found between the *ortho*- or the *para*-substituted acetoxy derivatives. Even bis(α-MCM-*p*-AB)-d4TMP, which proved to be the least stabile triester, showed identical antiviral potency in the TK-competent and the TK-deficient CEM cells as the most stabile compound. Importantly, the antiviral potency of the prototype *cyclo*-Sal-d4TMP **4** was as good as that of the three enzymatically activated bis(acetoxybenzyl)-d4TMPs **1**–**3**.

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