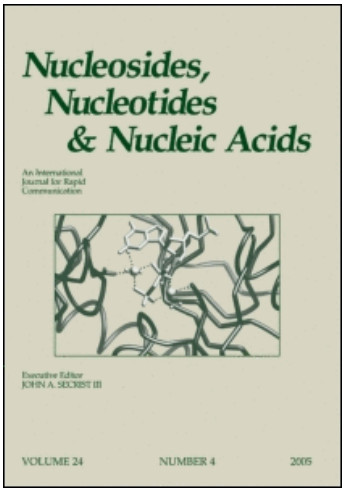

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Vukadinović, D. , Böge, N. P. H. , Balzarini, J. and Meier, C.(2005) '"Lock-in" Modified *Cyclo*Sal Nucleotides—The Second Generation of *Cyclo*Sal Prodrugs', Nucleosides, Nucleotides and Nucleic Acids, 24: 5, 939 — 942
To link to this Article: DOI: 10.1081/NCN-200059298
URL: http://dx.doi.org/10.1081/NCN-200059298

Nucleosides, Nucleotides, and Nucleic Acids, 24 (5–7):939–942, (2005)

ISSN: 1525-7770 print/ 1532-2335 online
DOI: 10.1081/NCN-200059298

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''LOCK-IN'' MODIFIED *CYCLO*SAL NUCLEOTIDES—THE SECOND GENERATION OF *CYCLO*SAL PRODRUGS

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▫ *A new generation of cycloSal-pronucleotides is presented. CycloSal-d4TMPs have been modified by introduction of an esterase-cleavable site in order to trap them inside cells. Hydrolysis studies in different media (PBS, CEM/0- and liver extracts) and anti-HIV evaluation of separated diastereomers revealed unexpected differences between the isomers.*

Keywords Prodrugs, Pronucleotides, *Cyclo*Sal, Antiviral Activity

INTRODUCTION

The *cyclo*Sal prodrugs have been developed as an intracellular delivery system for therapeutically active nucleotides and have already been applied to different nucleoside analogues leading to improved biological activities.[1] However, due to the lipophilic nature of the *cyclo*Sal pronucleotides, it cannot be excluded that a concentration equilibrium is formed through the membrane. Therefore, the intention was to modify the phosphate triesters in order to trap them inside cells. To achieve this goal, triesters bearing esterase-cleavable sites in the *cyclo*Sal-moiety were synthesized. Ester groups were introduced into the 3-position of the masking unit which should lead to the much more polar *cyclo*Sal-NMP-carboxylate or -alcohol after intracellular enzymatic cleavage. The increased polarity should inhibit the efflux and so achieve the intracellular "lock-in" of the prodrug.[2]

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RESULTS

In order to use the synthetic approach that had been used previously for the synthesis of the *cyclo*Sal prodrugs, the appropriate ester-group bearing salicyl alcohols were needed.[3] For the synthesis of the 3-AcEt- and 3-LevEt-phenols transesterfication reactions of ethyl- or levulinlylacetate and 2-[2-hydroxyphenyl]-ethanol were carried out.[4] Methanolysis of dihydrocoumarine yielded 3-MePr phenol (Figure 1).

Esterfication of 3-[2-hydroxyphenyl]-propionic acid with *N*,*N*-dimethylformamide-neopentylacetale and *t*-BuOH gave the desired 3-*t*-BuPr phenol.[5] These ester-modified phenol derivatives were hydroxymethylated following our previously reported protocol to yield the corresponding salicyl alcohols.[3] These diols were reacted with PCl_3 to yield cyclic chlorophosphanes which were then used as phosphitylation agents of d4T. The resulting phosphites were oxidized to give the *cyclo*Sal-triesters **1–6**. The reaction of 3-*t*-BuPr-*cyclo*Sal-d4TMP **3** with TFA led to 3-HPr-*cyclo*Sal-d4TMP **1** and the levulinyl group of 3-LevEt-*cyclo*Sal-d4TMP **6** was cleaved by hydrazine · hydrate to yield 3-OHEt-*cyclo*Sal-d4TMP **4**. Separation of the diastereomers of 3-MePr- (**2**) and 3-AcEt-*cyclo*Sal-d4TMP **5** by preparative RP-HPLC gave four isomers **2** ***fast***, **2** ***slow*** and **5** ***fast***, **5** ***slow***, respectively. They were analysed according to their hydrolysis half-lives in different media (PBS, CEM/0- and liver extracts), their anti-HIV activities.

The triester **2** and **5** were studied concerning their stability in 25 mM aqueous phosphate buffer (PBS) at pH 7.3. The half-lives $t_{1/2}$ are summarized in Table 1. The obtained stability values for compounds **5** clearly revealed differences between $t_{1/2}$ for the *cyclo*Sal-isomer **5** ***fast*** and **5** ***slow*** and the corresponding diastereomic mixture **5**. Triester **5** ***slow*** isomer (18.1 h) was approximately twice as stable as its **5** ***fast*** isomer (7.9 h) while the mixture of **5** ***fast*** and **5** ***slow*** showed a $t_{1/2}$ of 13.6 h. Obviously, the configuration at the phosphorus atom had a great influence on hydrolysis half-lives. As expected the diastereomeric mixture 3-OHEt-*cyclo*Sal-d4T **4** showed the same chemical stability as the mixtures of esters **5**. The $t_{1/2}$ values

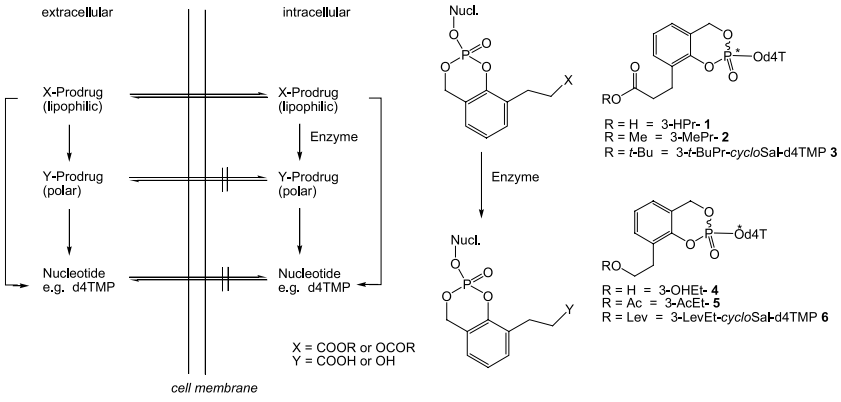
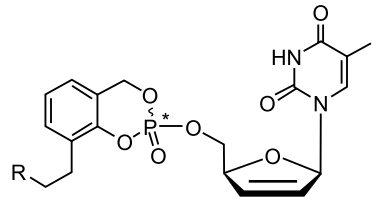


FIGURE 1 Scheme of the "lock-in" concept and nomenclature of the *cyclo*Sal-d4TMPs.

TABLE 1 Hydrolysis Half-Lives and Anti-HIV Activities



		$t_{1/2}$ [h]			EC_{50} [μM]			CC_{50} [μM]
Triester	R	pH 7.3	CEM/O	Liver	CEM/0 HIV-1	CEM/0 HIV-2	CEM/TK$^-$ HIV-2	CEM/0
1	–COOH	20.4	22.9	n.d.	0.19 ± 0.08	1.40 ± 0.60	20.0 ± 0.00	20.0 ± 0.00
2	–COOMe	12.4	8.4	22.9	0.18 ± 0.04	0.22 ± 0.03	0.26 ± 0.14	42.8 ± 13.5
2 Fast	–COOMe	8.1	7.6	2.8	0.30 ± 0.14	0.40 ± 0.00	1.93 ± 1.10	66.0 ± 22.6
2 Slow	–COOMe	16.7	8.2	2.7	0.090 ± 0.018	0.08 ± 0.00	0.077 ± 0.025	12.9 ± 0.92
4	–OH	12.6	15.0	n.d.	0.24 ± 0.01	0.33 ± 0.11	0.49 ± 0.30	96.0 ± 19.9
5	–OAc	13.6	3.8	0.5	0.12 ± 0.05	0.16 ± 0.06	0.17 ± 0.12	13.6 ± 3.32
5 Fast	–OAc	7.9	2.6	0.8	0.19 ± 0.09	0.27 ± 0.13	0.35 ± 0.09	52.1 ± 13.5
5 Slow	–OAc	18.1	5.2	0.3	0.055 ± 0.0035	0.14 ± 0.10	0.063 ± 0.032	15.8 ± 4.81
	d4T	n.a.	n.a	n.a	0.23 ± 0.04	0.24 ± 0.02	15.0 ± 7.71	58.8 ± 24.2

n.d., not determined; n.a., not available.

measured for **2** showed the same effect as observed for diastereomers **5**. The **2** ***slow*** (16.7 h) was approximately twice as stable as **2** ***fast*** (8.1 h) while for the diastereomeric mixture of **2** a $t_{1/2}$ value of 12.4 h was measured. The chemical stability of the acid 3-HPr-*cyclo*Sal-d4T **1** (20.4 h) was 1.3- to 2.8-fold higher compared to the esters. This increased stability may be attributed to the charge of the formed carboxylate. In all cases, the product formed in the hydrolysis was d4TMP and the corresponding salicyl alcohols. No cleavages of the ester groups were observed.

In contrast, in CEM/0 extracts studies acetate ester was cleaved in the case of triester **5** ($t_{1/2}$ = 3.8 h vs. 13.6 h in PBS), **5** ***fast*** (2.6 h vs. 7.9 h), and **5** ***slow*** (5.2 h vs. 18.1 h). As well as in the chemical hydrolysis, the **5** ***slow*** isomer was more stable than **5** ***fast***. In all cases, the product of the enzymatic hydrolysis, the alcohol 3-OHEt-*cyclo*Sal-d4TMP **1**, was detected by HPLC-co-elution experiments. The esters **2** which should release the acid 3-HPr-*cyclo*Sal-d4TMP **1** were found to be resistant to enzymatic cleavage. This difference was unexpected because carboxyl groups are often bioreversibly blocked by esterification.

In liver extracts the stability of the acetates **5** were 26- to 60-fold lower than those obtained in PBS! Here also the alcohol 3-OHEt-*cyclo*Sal-d4TMP **4** was formed in the hydrolysis. Obviously, the concentration of the involved enzymes is much higher in the liver extracts as compared to the CEM extracts. In this case, no difference between the half-lives of the diastereomers **5** ***fast*** and ***slow*** was detectable. The methyl ester in triesters **2** was again not cleaved.

Finally, the "lock-in" modified *cyclo*Sal-d4TMP's were tested for their anti-HIV activity in wild-type CEM- and mutant thymidine-kinase deficient cells (CEM/TK^-; Table 1). The parent nucleoside d4T was found to be active against HIV-1 and HIV-2 but lost its activity in the mutant CEM-cells. The same has been found for the acid 3-HPr-*cyclo*Sal-d4TMP **5**, which can be attributed to an incapability of membrane penetration due to the charged carboxylate. The acetate esters **5** showed good activities against HIV-1 and HIV-2 in wild-type cells. The isomer 3-AcEt-*cyclo*Sal-d4TMP **5** ***slow*** was 2- to 3.5-fold more activity than the isomer **5** ***fast***. In the mutant cell line (CEM/TK^-) full retention of antiviral activity was observed (TK-bypass). For the methyl ester triesters **2** the same was found, except that **2** ***fast*** lost some of its activity in the mutant CEM-cells.

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