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Rational design of a new series of mixed anti-HIV pronucleotides

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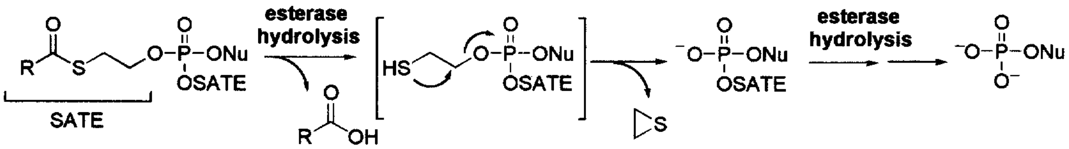
Abstract: MonoSATE aryl phosphotriesters of AZT are able to deliver intracellularly the corresponding 5'-mononucleotide. This process requires activation by an esterase followed by a phosphodiesterase. This finding opens the way to the design of new pronucleotide series.

Keywords: Nucleotides, Antivirals, Phosphoric acids and derivatives

Several reports have recently demonstrated that the *in vitro* anti-HIV activity of antiviral nucleoside analogs can be enhanced by intracellular delivery via mononucleotide prodrugs (pronucleotides) [1-4]. We have previously reported that mononucleoside phosphotriesters incorporating two *S*-acyl-2-thioethyl (SATE) moieties as biolabile phosphate protecting groups allow the delivery of the corresponding nucleoside 5'-monophosphate (mononucleotide) inside the cells [5,6]. The mechanism of decomposition of this type of pronucleotides is well established, involving two successive esterase-mediated hydrolysis steps (in total cell extract, which was used as a model for the intracellular medium) [5], the second one being slower than the first one due to the anionic charge of the resulting phosphodiester (Scheme 1).



Scheme 1: Decomposition pathway of SATE pronucleotides in total CEM cell extract

If the resulting phosphodiesters show low substrate properties towards esterases, the elimination of the second protecting group might also be performed by an intracellular phosphodiesterase activity. In this case, the observed anti-HIV effects might be greatly influenced by the selectivity of the phosphodiesterase activity, since the hydrolysis by this class of enzyme could lead not only to the desired 5'-mononucleotide but also to the nucleoside analogue. Hence, we decided to evaluate the potential of pronucleotides incorporating only one

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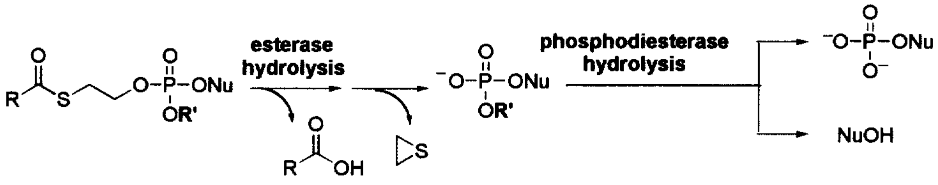
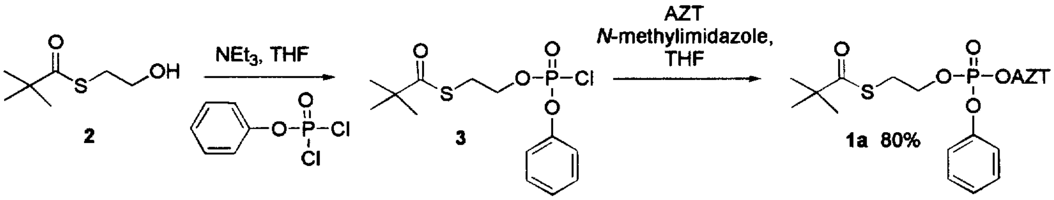
SATE group and an alternative phosphate protecting group (R', Scheme 2). Therefore, we postulated that decomposition of these compounds would proceed first by release of the corresponding phosphodiester via esterase mediated hydrolysis of the SATE group followed by release of the mono-nucleotide by an intracellular phosphodiesterase. The choice of the R' group will be of prime importance, in terms of phosphotriester stability and of its selectivity of elimination from the phosphodiester in intracellular medium.



Scheme 2: Expected decomposition pathway for mixed pronucleotides

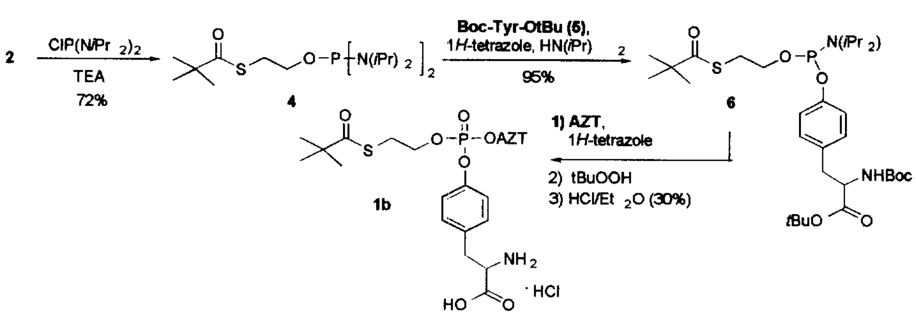
5'-Nucleotide phosphodiesterases or phosphodiesterases of type I (EC 3.1.4.1) catalyze the hydrolysis of a wide range of synthetic phosphoesters in addition to naturally occurring nucleotide substrates [7-9]. Phosphodiesterase I activity degrades nucleic acids at the 3' end to liberate the 5'-nucleotides, in contrast to 3'-nucleotide phosphodiesterase activity (phosphodiesterases of type II, EC 3.1.4.18), which leads to the formation of 3'-nucleotides. It has been postulated that the active site of some phosphodiesterases may include two separate binding domains, one having a much greater affinity for aromatic groups than the other one [9]. It has also been shown that *p*-nitrophenyl thymidine 5'-phosphate is a very good substrate for phosphodiesterases I, giving rise to the selective formation of 5'-dTMP [7]. Thus, we decided to evaluate the use of SATE phosphotriesters incorporating different aryl substituents. We report herein the synthesis, antiviral activity and stability study of aryl (*S*-pivaloyl-2-thioethyl) phosphotriester **1a,1b** (Scheme 3,4) of 3'-azido-3'-deoxythymidine (AZT) in culture medium and cell extracts.



Scheme 3: Synthesis of the aryl phosphotriester 1a

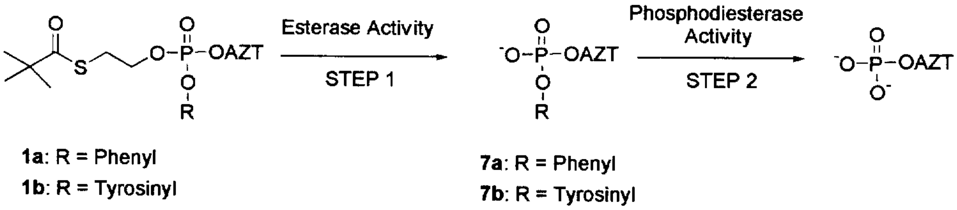
Synthesis of the phenyl phosphotriester **1a** (Scheme 3) started with the commercially available phenyl phosphorodichloridate, which was coupled with *S*-pivaloyl-2-thioethanol **2** [5]. The crude reaction product **3** was further reacted with AZT in presence of *N*-methylimidazole to give the desired aryl phosphotriester **1a** [10] in 80% overall yield.

The tyrosinyl phosphotriester **1b** [10] (Scheme 4) was obtained by coupling the appropriate phosphoramidite **6** with AZT upon 1*H*-tetrazole activation, followed by *in situ* oxidation of the intermediate phosphitetriester with *tert*-butyl hydroperoxide and deprotection of the amino acid by an acidic treatment (overall yield for **6**→**1b**: 52%).



Scheme 4: Synthesis of the tyrosinyl phosphotriester 1b

In order to validate our starting hypothesis, the decomposition pathway of compounds **1a** and **1b** was determined upon incubation in culture medium (extracellular medium used for antiviral evaluation) and total cell extract. These studies were performed using the HPLC "on-line internal-surface reversed-phase (ISRP) cleaning" method previously described [11], which allows the direct analysis of biological samples without any pretreatment. Identification of the decomposition metabolites was performed using HPLC/MS coupling and confirmed by coinjection with authentic samples. The phosphodiesterase activity of the cell extract was evaluated using *p*-nitrophenyl thymidine phosphate (50 µM). This compound was hydrolyzed in cell extract with a half-life of 2h, giving rise selectively to 5'-dTMP. In the same conditions, p-nitrophenyl AZT 5'-phosphate is decomposed to AZTMP ($t_{1/2}$= 36 min). However, it is noteworthy that in the presence of EDTA, a well established phosphodiesterase inhibitor [12], its half-life is 2.7 days.



Scheme 5: Observed decomposition products in cell extract

In cell extract (Table I) formation of AZTMP was observed from **1b** and **7b** (LC/MS identification) but not from **1a** and **7a**. However in both cases, AZT was the final metabolite. For compounds **1a** and **7a**, absence of detected AZTMP as intermediate metabolite may come from a rapid phosphatase hydrolysis of AZTMP ($t_{1/2}$ = 4h) compared to the slow decomposition of **7a** ($t_{1/2}$ estimated to be about 4 days).

	Culture medium[a]		Total CEM cell extract	
	STEP 1	STEP 2	STEP 1	STEP 2
1a	3 days	ND[b]	1.2 h	ND[b]
1b	3.6 days	3.7 h[c]	1.4 h	25 h

[a] RPMI 1640 containing 10% of heat inactivated fetal calf serum. [b]not determined because of the slow hydrolysis of **1a** and of the low accumulation of the metabolites. [c] this value was obtained from a separately synthesized phosphodiester **7b**.

Table 1: Half-lives of phosphotriesters 1a and 1b in culture medium and total cell extract upon incubation at 37°C (c= 50µM)

Both compounds **1a** and **1b** are quite stable in culture medium ($t_{1/2} \geq 3$ days), while they are easily decomposed in cell extract, which is in agreement with the pronucleotide concept.

The anti-HIV effects of phosphotriesters **1a**,**1b** were evaluated as previously described [13] in comparison with the parent nucleoside AZT on two HIV-1 infected T-cell lines, including the cell line CEM/TK$^-$. This cell line is deficient in cytosol thymidine kinase and should be considered as an ideal mean to investigate the antiviral activities of pronucleotides. As shown in Table 1, the aryl phosphotriesters exhibited in the CEM-SS cells a potent antiviral effect in the same range as AZT. In contrast, the pronucleotides **1a** and **1b** proved to be inhibitory against HIV-1 replication in CEM/TK$^-$, whereas AZT was completely inactive at concentrations up to 100 μM. These results clearly demonstrate that the aryl phosphotriesters are able to deliver the 5'-mononucleotide inside the cells, circumventing the first intracellular phosphorylation step.

	CEM-SS		CEM/TK$^-$	
	IC$_{50}$ (μM)	CC$_{50}$ (μM)	IC$_{50}$ (μM)	CC$_{50}$ (μM)
1a	0.001	> 10	3.5	> 10
1b	0.006	> 90	29	90
AZT	0.006	> 100	> 100	> 100

[a] IC$_{50}$: inhibitory concentration or concentration required to inhibit the replication of HIV-1 by 50%.

[b] CC$_{50}$: cytotoxic concentration or concentration required to reduce the viability of uninfected cells by 50%.

Table 2: Antiviral activity of the phosphotriesters 1a,1b compared to AZT in two cell lines infected with HIV-1

In conclusion, we have shown for the first time that a pronucleotide strategy involving two different enzymatic hydrolysis steps, performed successively by an esterase and presumably a phosphodiesterase activity, can lead to the delivery of the corresponding 5'-mononucleotide. Such finding opens the way to the conception of various monoSATE pronucleotides which will be further developed in forthcoming papers with the hope of obtaining mononucleotide prodrugs presenting optimized pharmacokinetic parameters.

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[10] **1a**: ^{1}H NMR (CDCl$_3$, 200 MHz) δ 9.02 (bs, 1H, NH), 7.18-7.42 (m, 6H, H-6, H$_{aryl}$), 6.22-6.29 (m, 1H, H-1'), 4.19-4.53 (m, 5H, H-3', H-5', H-5", OCH_2CH$_2$S), 4.05-4.10 (m, 1H, H-4'), 3.11-3.20 (m, 2H, OCH$_2$CH_2S), 2.18-2.53 (m, 2H, H-2', H-2"), 1.89 (m, 3H, CH$_3$-thymine), 1.25, 1.24 (2s, 9H, C(CH_3)$_3$). ^{31}P NMR (CDCl$_3$, 81 MHz) δ -5.30, -5.45.
1b: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.47, 7.46 (2s, 1H, H-6), 7.25 (d, 2H, J = 8.4, H$_\delta$), 7.09 (m, 2H, H$_\epsilon$), 6.12-6.15 (m, 1H, H-1'), 4.45-4.49 (m, 1H, H-3'), 4.28-4.37 (m, 2H, H-5', H-5"), 4.12-4.17 (m, 2H, OCH_2CH$_2$S), 4.01 (m, 1H, H-4'), 3.36-3.40 (m, 1H, partly covered by HDO peak, H$_\alpha$), 3.07-3.16 (m, 3H, H$_{\beta a}$, OCH$_2$CH_2S), 2.80-2.86 (m, 1H, H$_{\beta b}$), 2.31-2.44 (2m, 2H, H-2', H-2"), 1.73 (m, 3H, CH$_3$-thymine), 1.16, 1.15 (2s, 9H, C(CH$_3$)$_3$). ^{31}P NMR (DMSO-d_6, 81 MHz) δ -5.4, -5.5.

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