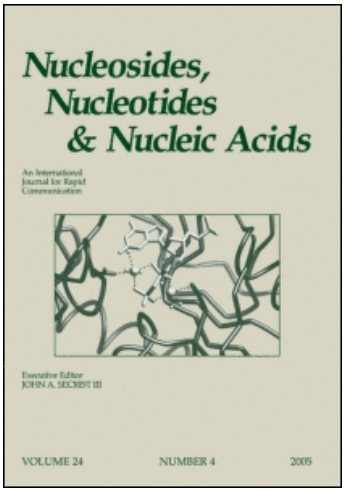

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Synthesis, Decomposition Pathways and "*In Vitro*" Evaluation of Bioreversible Phosphotriesters of Azt

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SYNTHESIS, DECOMPOSITION PATHWAYS AND " IN VITRO " EVALUATION OF BIOREVERSIBLE PHOSPHOTRIESTERS OF AZT.

Isabelle Lefebvre[a], Alain Pompon[a], Christian Périgaud[a], Jean-Luc Girardet[a], Gilles Gosselin[a], Anne-Marie Aubertin[b], André Kirn[b] and Jean-Louis Imbach[a]*.

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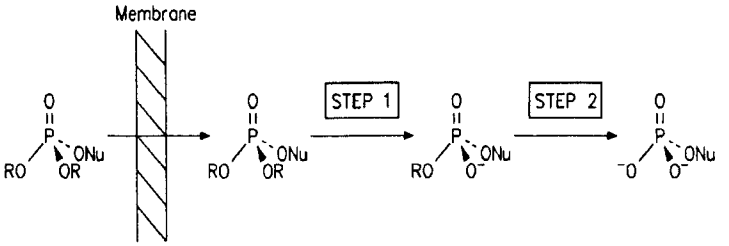
Abstract: The synthesis, pharmacokinetic data and biological evaluation of a series of phosphotriesters containing S-acyl-2-thioethyl groups as enzyme-labile phosphate protecting groups and AZT as a model are described. A comparison of pharmacokinetic data and "in vitro" experiments show that such bioreversible phosphotriesters of AZT are able to cross cell membranes and deliver the corresponding nucleoside monophosphate inside the cell. Moreover, kinetic data show that modification of the protecting groups can allow to modulate both the extracellular stability of the parent compond and the delivery of nucleoside monophosphate inside the cell.

INTRODUCTION:

In the search for effective agents against human immunodeficiency virus (HIV), a large number of dideoxynucleoside (ddN) analogues have emerged as efficient drugs. Antiretroviral effects of ddNs involve their conversion, through cellular enzymes, to the corresponding triphosphate metabolites, which competitively inhibit HIV reverse transcriptase (RT)[1], or terminate the newly synthesized viral DNA chain. One of the ways to improve the efficiency of ddNs would be to bypass the phosphorylation steps. Unfortunately, nucleotidic forms cannot be used as chemotherapeutic agents because they are unable to cross the cell membrane efficiently due to their polar nature. Moreover, they are readily dephosphorylated on cell surfaces and in extracellular fluids by non-specific phosphohydrolases. To overcome these problems, various neutral 5'-nucleotide esters have been prepared in the hope that they penetrate cells by passive diffusion and then revert back to the nucleoside 5'-monophosphate inside the cell[2-4].

Many strategies have previously been envisaged to deliver nucleoside monophosphates inside the cells. To be effective, such masked nucleotide approaches should incorporate the following features:(i) the phosphotriester must be sufficiently lipophilic to enter the cell by passive diffusion;

(ii) the nucleoside phosphotriester must be stable under the experimental cell culture conditions usually used, i.e. RPMI containing 10% heat inactivated fetal calf serum (pH≈7) at 37°C; otherwise, extracellular decomposition to the parent nucleoside could lead to ambiguous biological results; (iii) inside the cell, the nucleoside phosphotriester must be selectively transformed to the nucleoside monophosphate during the time period when virus replication is sensitive to the tested compound.



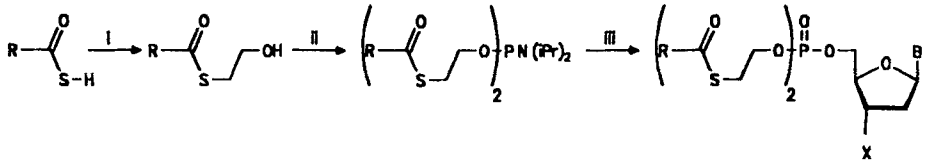
The first step must: (i) be an "initiated chemical process", since no phosphotriesterase activity has been reported; (ii) not involve attack on the phosphorus atom (to ensure selective elimination and to avoid any concomitant nucleoside release through P-O bond breakage); (iii) be locally selective, i.e. slow (or ineffective) in culture media and fast (or exclusive) in cells. The "chemical process" could be initiated by enzymes which are significantly more active in cells than in culture media. The second step must be an enzyme-mediated process, since phosphodiesters are very resistant to chemical hydrolysis.

Here we report the synthesis, pharmacokinetic data and biological evaluation of a series of phosphotriesters which incorporated S-acyl-2-thioethyl groups as enzyme-labile phosphate protecting groups and AZT as a model. Such O,O'-bis [S-acyl-2-thioethyl][3] phosphotriesters were designed to facilitate the intracellular release of free nucleoside 5'-monophosphates through a carboxylate esterase-mediated activation process.

SYNTHESIS:

The thiol esters 2a-c have been prepared (respective yields: 65,78 and 55%) by the reaction of 2-iodoethanol and the corresponding thiol acids 1 (commercialy available except for 1b which was prepared by slight modification of a published procedure[4]). The P III phosphorylating agents 3a-c were obtained by treatment of N,N-diisopropylphosphorodichloridite[5] with two equivalents of the appropriate thiol ester in THF in the presence of triethylamine. They were purified by flash column chromatography (respective yields: 46, 38 and 17%). Coupling of the nucleoside analogue (AZT) with 3a-c in the presence of 1H-tetrazole, followed by *in situ* oxidation with 3-

chloroperoxybenzoic acid, gave the desired mononucleoside phosphotriesters 4a-c in moderate to good yields after purification by column chromatography (respective yields: 74,45 and 27%). The target compounds 4a-c were characterized by UV, ^1H and ^{31}P NMR, FAB mass spectrometry, and HPLC, all data being consistent with their structure and purity.

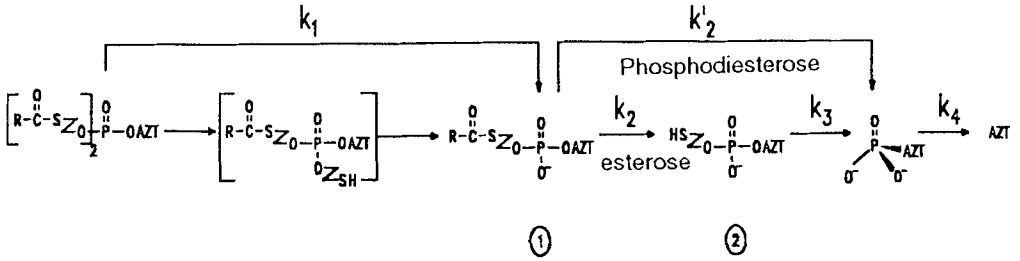


1a R= CH_3 2a R= CH_3 3a R= CH_3 4a R= CH_3, X= N_3, B= Thymin-1-yl
1b R= iPr 2b R= iPr 3b R= iPr 4b R= iPr, X= N_3, B= Thymin-1-yl
1c R= tBu 2c R= tBu 3c R= tBu 4c R= tBu, X= N_3, B= Thymin-1-yl

Reagents and Conditions: i, ICH_2CH_2OH, DBU/$C_6H_5CH_3$, ii, $Cl_2PN(iPr)_2$, $N(C_2H_5)_3$/THF, iii, nucleoside analogue (AZT), ^1H-tetrazole/THF, then $ClC_6H_4CO_3H/CH_2Cl_2$.

RESULTS AND DISCUSSION:

KINETICS: By using a modified "on-line internal surface reversed phase cleaning" HPLC method previously described for the degradation studies of oligonucleotides[6], the kinetics of decomposition of 4a-c were determined under three experimental conditions: (i) RPMI containing 10% heat-inactivated fetal calf serum (culture medium, CM), (ii) CEM cell extracts (CE), (iii) Human Serum (HS).



These media were chosen as models of the intra- and extracellular media. In each medium, the three compounds were first degraded to their corresponding O-[S-acyl-2-thioethyl] phosphodiesters ① (carboxylate esterase activity, then spontaneous elimination of thiirane). The degradation rates strongly differed according to the medium and the nature of the protecting groups: 4a very fast in CE and HS, slow in CM; 4b fast in CE and HS, slow in CM; 4c slow in CE, very slow in HS and CM. In turn, phosphodiesters ① were degraded at various rates (very fast for 4a, slow for 4b and

4c in CE; slow for 4a and 4b, very slow for 4c in HS and CM). More, the degradation mechanisms differed according to the medium: (i) in cell extract, intermediate phosphodiesters ② resulting from carboxylate esterase activity were observed. In turn, diesters ② were slowly converted to AZTMP (phosphodiesterase activity); (ii) in human serum and culture medium, intermediates ② were not observed. Kinetic data strongly suggest that ① was directly converted to AZTMP (phosphodiesterase activity). At last, AZTMP was dephosphorylated to AZT (1-4 hr in CE and HS, very slow in CM).

ACTIVITY: In the CEM-SS, PBMC and MT4 cell lines, compounds 4a-c produced an anti-HIV effect similar to that of AZT. One cannot conclude if the observed activity is due to the intracellular delivery of AZTMP or to the release of AZT. However, in the CEM-TK$^-$ cell lines, compounds 4a-c produced an anti-HIV effect, proving that AZTMP was delivered inside the cells (AZT itself is inactive in the same experiment).

There is a reasonable chance that the rational design of other enzyme-mediated bioreversible phosphate protecting groups may permit modulation of cellular uptake and bioavailability of nucleotide analogues.

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