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THE PRO-OLIGONUCLEOTIDE APPROACH. V: INFLUENCE OF THE PHOSPHORUS ATOM ENVIRONMENT ON THE HYDROLYSIS OF ENZYMOLABILE DINUCLEOSIDE PHOSPHOTRIESTERS

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Abstract : Six dithymidine phosphotriester analogs bearing an enzymolabile phosphate proctecting group were synthesized in order to select the most appropriate model for the prooligonucleotide approach. Series **1** and **3** were chosen on the basis of their stability in culture medium and their capacity, when incubated in total cell extract, to deliver selectively dithymidine phosphodiester and phosphorothioate diester respectively.

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We previously demonstrated that chimeric pro-oligonucleotides containing a gap of 3 or 6 phosphorothiolate triesters bearing a bioreversible protecting group (*i.e.* POM, SATM, SATE) could be selectively deprotected to the corresponding chimeric phosphorothioate oligonucleosides in total cell extract [1,2]. Such chimeric pro-oligonucleotides were obtained through selective alkylation of the corresponding phosphorothioate oligonucleosides with the iodo derivative of the bioreversible protecting group. However, we recently showed that a desulfurization side-reaction occurs during the alkylation reaction [2]. Therefore pure chimeric phosphorothiolate pro-oligonucleosides can not be easily prepared by the post synthesis alkylation, hence the necessity to consider a solid phase approach. As the *S*-AcylThioEthyl (SATE) bioreversible protecting group shows better pharmacokinetic parameters than the POM one[3], we decided to synthesize a series of various *tert*-Butyl-SATE dithymidine phosphotriester in order to select the best model in terms of stability in biological media and selectivity of deprotection. Moreover, this study will open the way to the selection of the most appropriate pro-oligonucleotide series, which could be reached with a solid phase strategy from the corresponding SATE phosphoramidite synthons.

The six dithymidine models are presented in scheme 1, they were easily obtained using previously described procedures. Briefly, compounds **1**, **3**, **5**, **6** were synthesized from the corresponding 3',5'-*O,O*-dimethoxytrityldithymidine-*N*-diisopropylphosphoramidite[4] through condensation with 2-hydroxyethyl *S*-thiopivaloate (tBu-COS-$(CH_2)_2$-OH) or 2-thioethyl pivaloate (tBu-CO_2-$(CH_2)_2$-SH) followed by *in situ*

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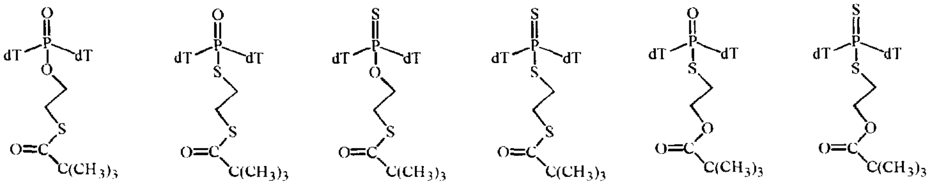
oxidation with terbutyl hydroperoxide or elemental sulfur, and final deprotection with 80% acetic acid / H_2O. Compounds 2, 4 were respectively obtained through direct alkylation of dithymidine phosphorothioate or phosphorodithioate diester with the 2-iodoethyl *S*-thiopivaloate (*t*Bu-COS-$(CH_2)_2$-I) in the presence of 2,6-lutidine[5].


1 2 3 4 5 6

dT : Thymidine

Scheme 1 :Structure of the six dithymidine phosphotriester analogs

The stabilities of 1-6 (table 1) were determined after incubation ($5.10^{-5}M$) in culture medium (RPMI 1640 + 10% heat inactivated calf fetal serum) and in total cell extract (as a rough model of the intracellular contents) using HPLC on-line cleaning method[6].

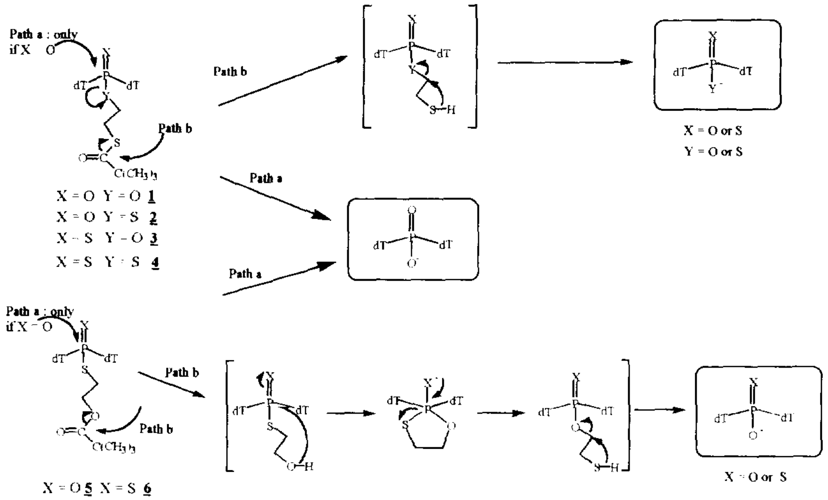
Table 1 : $t_{1/2}$ of the six dithymidine models and identification of the hydrolysis products

	Half-lives (h)					
	1	2	3	4	5	6
Culture medium	32 / 34 POO⁻	17 /18 POO⁻/POS⁻	114 /169 POS⁻	82 / 86 PSS⁻	28 / 33 POO⁻	153 / 169 POS⁻
Cell extract	15.4 / 15.4 POO⁻	5.5 / 8.8 POS⁻	6.9 / 7.6 POS⁻	3.5 / 5.4 PSS⁻	9.9 / 11.8 POO⁻	11.9 / 13.3 POS⁻

POO^- : dithymidine phosphodiester; POS^- : dithymidine phosphorothioate diester; PSS^- : dithymidine phosphorodithioate diester;

Identification of the decomposition products was performed by HPLC, by co-injection of authentic samples. Interestingly, compounds 1-6 were diastereoisomeric mixtures (roughly 1/1 as shown by HPLC) and each half-life is reported on table 1. As such, $t_{1/2}$ reflects the affinity of the molecule for the carboxyesterases, it was not surprising that two diastereoisomers showed different behavior[7].

All the observed data can be mechanistically described as shown in the scheme 2, but as expected the degree to which decomposition pathway is followed, depends strongly on the phosphorus environment and on the media.



Scheme 2 : Different ways of decomposition of compounds 1 to 6

In culture medium, the observed half-life depends mainly on the capacity of the phosphorus atom to be attacked by nucleophiles (*i.e.* water, path a). But some residual esterase activity coming from the fetal calf serum may participate to the hydrolysis process (path b). This is shown by POS^- formation (30% POS^- *versus* 70% of POO^-) from compound 2. As expected from literature data[8], thiono derivatives 3, 4, 6 are by far less susceptible to nucleophilic attack on the phosphorus atom than the corresponding oxo analogs 1, 2, 5, and displayed half-lives higher than 80h, whilst oxo derivatives (1, 2, 5) showed half-lives lower than 33h. In addition, the bond energies (E_{P-S} = 45-50 kcal/mol, E_{P-O} = 95-100 kcal/mol)[9] are consistent with the difference of half-life obtained between 1 and 2. Hence, the observed stability order 6 > 3 > 4 > 5 ≈ 1 > 2, the thiono series being more stable than the oxo one.

In cell extracts where the carboxyesterase activity is predominant, the observed half-life essentially depends on the affinity of the substrate for the enzyme. One can assume that the decomposition process is mediated only by carboxyesterases (path b), which is corroborated by exclusive formation of POS^- from compound 2. Variations in the half-lives of compounds 1 to 4 represent a factor 1.8 to 4.4 even though the bioreversible group is the same for the four compounds. These results show that even small changes in molecular structure may alter the affinity of the compound for the enzyme [10] Substitution of an atom linked to the phosphorus atom by an other is sufficient to influence the hydrolytic reaction mediated by esterases and in consequence the kinetic rates.

Comparison of compounds 2 and 5, and 4 and 6 shows that substitution of the sulfur atom linked to the carbonyl by an oxygen influences the rate of the esterasic reaction. We observed that compounds bearing a thioester are more rapidly hydrolyzed than compounds bearing an ester. The first step of hydrolysis being an attack on the carbonyl by the serine alcohol function of the enzyme, this may be explained on the basis of the nature of the leaving group (pKa_{thiol} = 10-11, $pKa_{alcohol}$ = 15-17). In such medium, the stability order is 1 > 6 > 5 > 2 ≈ 3 > 4, esters being less rapidly hydrolyzed than thioesters for the same phosphorus environment. Noteworthy that in cell extract we observed selective phosphodiester (for 1, 5), phosphorothioate diester (for 2, 3, 6) and phosphorodithioate diester (4) formation according to the nature of the compound.

The aim of the prooligonucleotide approach is to proceed to *in vitro* (and ultimately to *in vivo*) experiments, the stability of the compounds in culture medium is then of prime importance for the choice of the prooligonucleotide series to be evaluated. Considering compounds 2, 3, 6 which selectively go to POS^- in cell extracts, we first rejected compound 2 on the basis of its $t_{1/2}$ in culture medium when compared with the two others. Then, we considered the ease of synthesizing both series 3 and 6 in a solid phase approach strategy as we have already shown a post synthesis alkylation procedure can not easily be used[2]. This implies that one must consider a phosphoramidite (for 3) or a phosphorothioamidite (for 6) precursor. In this last case, it is already known that such derivatives are prone to oxidation and are much less reactive than the former[11]. It follows that series 3 was selected for phosphorothioate delivery. For phosphodiester delivery, (compounds 1 and 5) series 1 was selected on the basis of the ease of synthesis and the reactivity of the corresponding phosphoramidite since the stability of 1 and 5 in culture medium are similar. In addition, the same phosphoramidite synthons could be used as precursors of both series 1 (POO^- delivery) and 3 (POS^- delivery), the differentiation occurring at the oxidation step (I_2/water or terbutyl hydroperoxide *versus* elemental sulfur or Beaucage reagent[12]).

In conclusion, the data reveals that further developments in the prooligonucleotide chemistry based on a solid phase approach will focus on series 1 and 3 for selective delivery of phosphodiester and phosphorothioate oligonucleotides respectively.

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