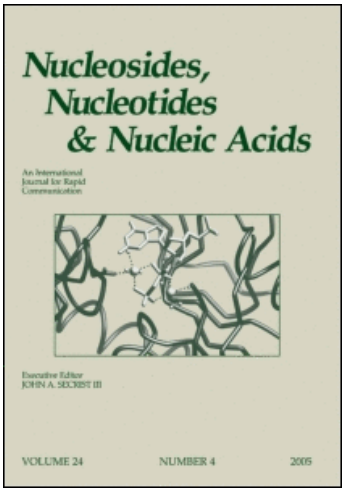

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Stability Studies in Biological Media of Dimer Phosphotriesters Bearing Glucuronic Acid Residue

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STABILITY STUDIES IN BIOLOGICAL MEDIA OF DIMER PHOSPHOTRIESTERS BEARING GLUCURONIC ACID RESIDUE

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ABSTRACT: Hydrolytic stability of dithymidine phosphorothioates and dithioates bearing a glucuronic acid derivative protecting group on the phosphate linkage were studied in various biological media. We found that the enzymatic hydrolysis was accompanied by another reaction resulting in formation of the dithymidine phosphodiesters. We have proposed several possible mechanisms of hydrolysis.

Two main obstacles that limit the use of oligonucleotides as antisense agents in therapeutics are their instability in extra and intracellular media and their poor cellular uptake.

Firstly, the resistance of oligonucleotides to nuclease degradation has been improved by various chemical modifications of the sugar moiety or the phosphate backbone[1]. However, at this time, only phosphorothioate and phosphorodithioate derivatives are known to induce RNase H activity[2]. This intracellular enzyme plays a key role in the antisense action of oligonucleotides because it digests the target RNA strand of the formed RNA / DNA duplex[3].

The anionic nature of oligonucleotides seems to lower in part their membrane crossing ability. To overcome this second problem, we have considered the « pro-oligo approach »[4], based on the transient masking of the negatively charged phosphates with

biocleavable substituents which could be selectively removed by intracellularly located enzymes to yield oligonucleotide phosphodiesters. The transient protective group must be stable enough in biological fluids, but also selectively and rapidly removed upon intracellular enzymatic activation. In the case of carboxyesterase activity, the group must bear an ester or thioester linkage and the nature of the radical should be preferably hydrophilic to increase the solubility of protected oligonucleotides in aqueous media.

In a previous paper, we had described the synthesis of two novel alkylating agents derived from D-glucuronic acid (**1** and **2**)[5]. To evaluate the potential of these new substituents, dithymidine phosphorothioate and phosphorodithioate triester models were used. Masking of the phosphorothioate charge was achieved by a post-synthesis alkylation (FIG. 1).

In this work we report on the stability of various dimer phosphotriesters in different media. Since we had observed difficulties in purifying some dimers by HPLC, we carried out initial stability studies in water (TABLE 1).

Based on the results in TABLE 1, we noticed that compounds **4** and **5** are much more sensitive to hydrolysis than the phosphotriester **3**, and the main product of decomposition from **4** was identified as a phosphorothioate diester. Decomposition of the phosphorodithioate **5** led to the corresponding phosphorodithioate. These observations suggest that an intramolecular reaction via a lactonization of the sugar moiety probably occurred in solution rather than a nucleophilic attack on the phosphorus atom. This intramolecular hydrolysis favoured in **4** and **5** could be explained by the better leaving group ability of a thiolate compared to an alkoxide. The lack of stability of **4** and **5** made their use uncertain in further experiments and convinced us to carry out stability studies in biological media exclusively with the dimer triesters **3** and **7**.

RPMI and Culture Medium are supposed to simulate the activity of biological fluids. By contrast, Cell Extract contains intracellular enzymes, especially carboxyesterase mixtures. Activity of carboxyesterases in cell extract was confirmed by hydrolysis study of a known substrate[6].

As shown on the TABLE, half-live of dimers **3** and **6** in cell extract were longer as compared to culture medium. This may indicate that **3** and **6** are not efficient substrates for the esterases which are present in Cell Extract. This result is confirmed by the

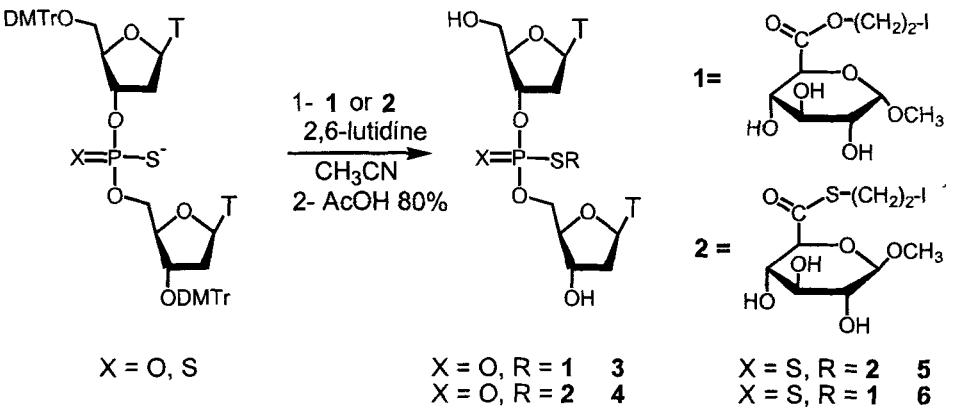
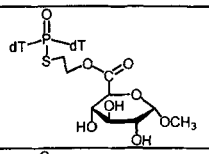
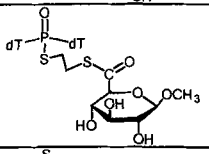
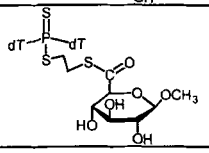


FIGURE 1: Alkylation of phosphorothioate and phosphorodithioate diesters

TABLE 1: Half-lives (h) of phosphotriesters in H_2O and resulting phosphodiesters

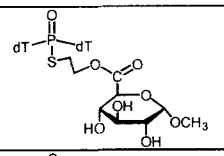
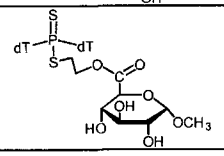
Dimer phophotriesters	$T_{1/2}$ in H_2O
3 	~ 48 $PO(O)^-$
4 	2.30 70% $PO(S)^-$ 30% $PO(O)^-$
5 	3.30 $PS(S)^-$

dT: deoxythymidine

Hydrolysis mixtures contained 5.10^{-5}M of dimers.

Nature of dimer phosphodiesters had been confirmed by co-injection in HPLC with authentical samples.

TABLE 2: Half-lives (h) of phosphotriesters and resulting phophodiesters

	RPMI 1640	CULTURE MEDIUM (CM)	CELL EXTRACT (CE)	PIG LIVER ESTERASE (PLE)
3 	2.30 $PO(O)^-$	2.05 $PO(O)^-$	4.70 $PO(O)^-$	2.20 $PO(O)^-$
6 	4.10 $PO(S)^-$	3.00 $PO(S)^-$	5.00 $PO(S)^-$	1.00 $PO(S)^-$

Culture medium: RPMI 1640 buffer supplemented with 10% heat deactivated foetal calf serum
Cell Extract: CEM-SS cells supernatant
Pig Liver Esterase: 8U/ml PLE From Aldrich in phosphate buffer (20mM; pH 7.4)

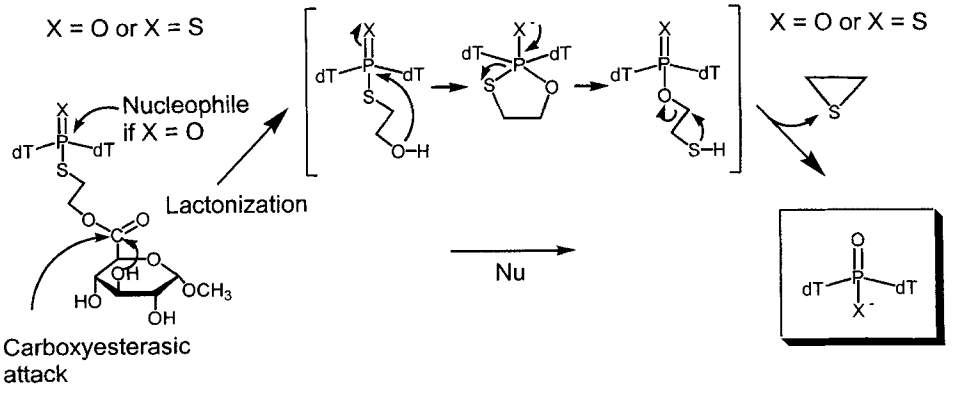


FIGURE 2: proposed competitive mechanisms of hydrolysis of dimer phosphotriesters

observed hydrolysis kinetics upon purified pig liver esterase. Moreover, because the half-lives of **3** and **6** are slightly higher in RPMI buffer than in CM, one can suggest that the observed decomposition is mainly due to a non-enzymatic process. We think, as described on FIGURE 2, that hydrolysis of phosphotriesters bearing glucuronic acid residue essentially goes through lactonization process that primarily occurs in aqueous media, although a complementary esterases-mediated hydrolysis may take place. Hydrolysis via nucleophilic attack on the phophorus atom can only be considered on the phosphorothiolate linkage[6].

Consequently, the glucuronic acid transient phosphate protecting groups cannot be used in a pro-oligonucleotide approach. The corresponding phosphotriesters are neither stable enough in aqueous solutions, nor are they efficient substrates for intracellular esterases. Despite these preliminary results, development of new protecting groups derived from a sugar moiety is still of a great interest for oligonucleotide prodrug approach and must be investigated further.

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