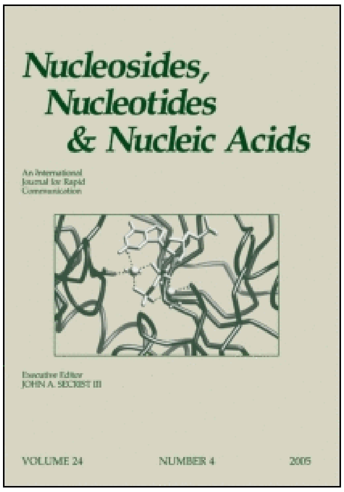

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# Use of Bioreversible Phosphate Protecting Groups for the Intracellular Delivery of Antisense Oligodeoxyribonucleotides

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# USE OF BIOREVERSIBLE PHOSPHATE PROTECTING GROUPS FOR THE INTRACELLULAR DELIVERY OF ANTISENSE OLIGODEOXYRIBONUCLEOTIDES.

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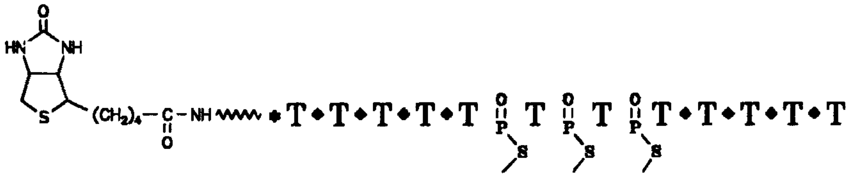
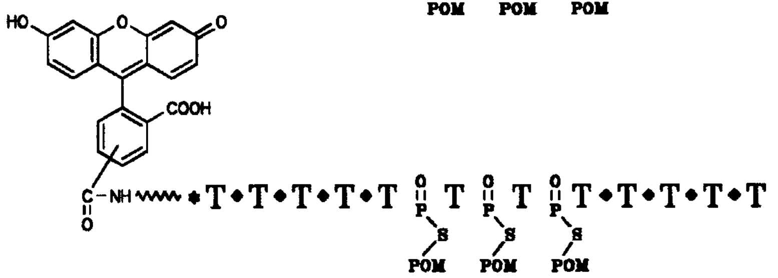
**Abstract:** The aim of this work was to design phosphorothioate oligonucleotides, the negative charges of which are temporarily masked by a group which facilitates the cellular uptake of these analogues. Then, selective removal of this group by enzymes normally present in the cells will deliver intracellularly the intact charged phosphorothioate oligomers able to hybridize to their complementary RNA targets and to elicit RNase H activity.

The use of synthetic antisense oligonucleotides for specific gene inhibition represents a new strategy for drug development in case of diseases which are due to expression of genes of known sequences. However, to be usable as potential therapeutics, antisense oligonucleotides have to be able to reach intact their targets. For this purpose, they have to overcome two mains hurdles: their rapid degradation by nucleolytic enzymes present in cell or culture medium and their poor cellular uptake. Many structural modifications have been designed to improve the properties of antisense oligonucleotides. Among these modifications, phosphorothioate[1] and methylphosphonate[2] oligonucleotides are respectively the most studied anionic and neutral analogues. Although they present both increased nuclease resistance and enhanced cellular uptake compared to unmodified oligonucleotides, methylphosphonates are poor antisense effectors, because they are not able to elicit the activity of RNase H[3], an ubiquitous enzyme which is thought to play a key role in antisense activity through the cleavage of the RNA strand of RNA / DNA duplexes. In contrast, phosphorothioates are able to elicit RNase H activity, but they exhibit a poor cellular uptake comparable to that of unmodified oligonucleotides. In this context, we decided to explore the "pro-oligo" approach. The concept is based on the use of temporary masking groups for the conversion of polyanionic oligonucleotides into non-ionic analogues which are expected to be taken up more efficiently by cells than charged oligonucleotides. Then, cellular enzymes will hydrolyze this masking group to release intracellularly the active antisense oligonucleotides. Such non-ionic oligonucleotides could be obtained through alkylation of phosphorothioate or phosphorodithioate diester linkages with suitable halogenated reagents containing an enzymatically labile moiety.

We have first synthesized a chimeric dodecathymidylate, with methylphosphonate flanks and a central stretch of three phosphorothioate diester linkages. We have shown that it is possible to selectively alkylate these three linkages to generate phosphorothioate triesters bearing a biologically reversible phosphate protective group, *i.e.* S-acetylthiomethyl group. The study of the behavior and the fate of this neutral chimeric pro-oligonucleotide in CEM total cellular extract using the HPLC on-line Internal Surface Reverse Phase cleaning method[4] had demonstrated that upon cellular enzymatic activation, it selectively reversed back to the parent charged structure. However, stability studies in a culture medium composed of RPMI containing 10% of heat inactivated foetal calf serum of this pro-oligonucleotide revealed that the S-acetylthiomethyl group was not stable enough under cell culture conditions to be used as an efficient bioreversible phosphate protecting group (the half-life of the entirely neutral oligonucleotide in this medium was ≈ 40 min.).



(1)



(2)

•: methylphosphonate linkages ✱ : phosphodiester linkages

We have then synthesized model dimeric phosphorothioate and phosphorodithioate triesters bearing various bioreversible groups and studied their stabilities in cellular extract and culture medium. For all the masking groups studied, it was shown that in presence of cellular enzymes, the negatively charged parent structure, *i.e.* the phosphorothioate or the phosphorodithioate diester, was selectively recovered. From both reactivity and culture medium stability studies, we concluded that two possiblities could be envisaged: the masking of a central stretch of phosphorothioate diester linkages by pivaloyloxymethyl groups (half-life of the corresponding dimer ≈ 6 hr) or the masking of a central stretch of phosphorodithioate diester linkages by S-acetylthioethyl groups (half-life of the corresponding dimer ≈ 8 hr).

Finally, we have synthesized two chimeric dodecathymidylates with methylphosphonate flanks and a central stretch of three phosphorothioates masked by pivaloyloxymethyl groups (POM), one 5'-end labelled by biotin **(1)** and the other by fluorescein **(2)**, for forthcoming cellular uptake studies.

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