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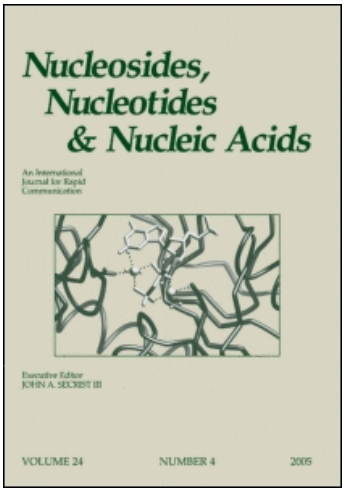

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Synthesis of Anti-Sense Phosphorothioate Analogues for Pharmacokinetic and Pre-Clinical Studies

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Abstract. Nuclease-resistant oligonucleotides (11- to 28-mers) containing stereorandom phosphorothioate linkages have been recently reported to exhibit potent anti-HIV III effects and sequence-specific inhibition of protein synthesis. Relatively large amounts (100 mg - 1 g) of these analogues, which are needed for further biological testing and initial pharmacokinetic and pre-clinical studies, were readily obtained by automated hydrogen phosphonate chemistry followed by reversed-phase HPLC and further processing. This chemistry features 1-adamanetanecarbonyl chloride as the activator, capping with isopropyl phosphite, and more complete sulfurization in only one-step following chain assembly. An automated, quantitative, picomole method for analysis of the analogues in blood samples has been developed.

Anti-sense oligonucleotide analogues are of interest as a new class of potential therapeutic agents that function by preventing the synthesis of a specific protein target. These compounds should be resistant to nucleases, taken up by cells, bind to complementary sequences of mRNA, and interfere with protein synthesis by either physical blockage or inducing RNase H-mediated cleavage of mRNA. Among the possible phosphate-modified linkages, a phosphorothioate of the type 3'0-P(0)S$^-$-05' seemed attractive considering that unmodified oligodeoxyribonucleotides, despite their negative charge, had been reported (1) to function by an anti-sense mechanism in cell culture at relatively low concentration, and that these phosphorothioate linkages were known (2) to be resistant to nucleases. In addition, the automated solid-phase phosphoramidite method for DNA synthesis was available and lent itself to introduction of one or more phosphorothioate linkages anywhere in the chain (2), albeit stereorandomly, thus generating a mixture of 2^n diastereomers (n=no. of phosphorothioate linkages). On the other hand, an 11-mer with 10 such chiral centers (1024 diastereomers) when studied with its complementary DNA showed a Tm lowering of only ca. 10°C, without flattening of the Tm curve, as did a 28-mer (3).

In any event, poly-phosphorothioate analogues at ca. 1-μM concentrations were found to protect receptor-positive, sensitive cells against the cytopathic effect of human immunodeficiency virus (HIV) but in a sequence non-specific manner (4). By contrast, more recent results (M. Matsukura, private communication) obtained with infected cells in culture indicate 90% inhibition of the expression of HIV by ca. 25-μM concentrations of an anti-art/trs (rev) poly-phosphorothioate in a sequence-specific manner. Evidently there may be two independent mechanisms operating at different stages of the HIV life-cycle, namely, non-specific inhibition of reverse transcriptase (J. S. Cohen, private communication) and anti-sense interference with protein synthesis.

Results. Preliminary pharmocokinetic and other pre-clinical investigations of poly-phosphorothioates are facilitated by simplified preparations of 100-mg to 1-g amounts of material. In this context, we are further investigating the H-phosphonate method for DNA synthesis reported by Andrus et al (5). This chemistry features in situ activation of the monomer (triethylammonium salt) by reaction with adamanetanecarbonyl chloride in pyridine-acetonitrile. Unlike pivaloyl chloride, which is very unstable in pyridine-acetonitrile, this new activator remains stable for months. Cleaner crude products are obtained by capping with isopropyl phosphite, which is similarly activated with adamanetanecarbonyl chloride. Sulfurization involves only one step, which is performed either manually or automatically after chain assembly. This allows for convenient to ^{35}S-labeling.

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Products were isolated by HPLC as their DMT derivatives using injections of 30- to 100-μmol of crude material. Purity was assessed by polyacrylamide gel electrophoresis and ^{31}P NMR analyses. On an ABI Model 380B DNA Synthesizer, which can perform either 1, 2, or 3 10-μmol syntheses, comparison of the standard cycles for phosphoramidite and H-phosphonate chemistry indicated that the H-phosphonate method uses 5-times less monomer (2-equivalents), and requires half the time per cycle (25 minutes). These advantages are, however, offset by the lower yield of isolated product, which could be due to various factors that are currently under investigation. Nevertheless, this currently available methodology affords ca. 100-150 mg of crude ca. 20-mer poly-phosphorothioate every ca. 5 h, which is an adequate starting point for further process development.

Fluorescently labeled poly-phosphorothioates were desired for studies of cell uptake, to be reported elsewhere, and development of a convenient method for quantitative analysis of anti-sense poly-phosphorothioates (and their metabolites) in blood and tissue samples derived from pre-clinical studies. The preparation of these compounds was accomplished by sequential H-phosphonate synthesis, sulfurization, tetrazole-catalyzed coupling of an aminohexylphosphoramidite linker, sulfurization, deprotection with conc. NH_4OH, and then reaction with a dye having a reactive NHS ester moiety. The HPLC isolated product was added to human blood, which was then processed with an ABI Model 340A Nucleic Acid Extractor. HPLC analysis of all fractions showed, by means of absorbance (A_{260}) and fluorescence (E_{550}) detection, that this fluorescently labeled oligomer was retained in the final aqueous layer. Repetition of the experiment with an unlabeled poly-phosphorothioate indicated that ca. 10 pmole/injection was adequate for HPLC detection (A_{260}).

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