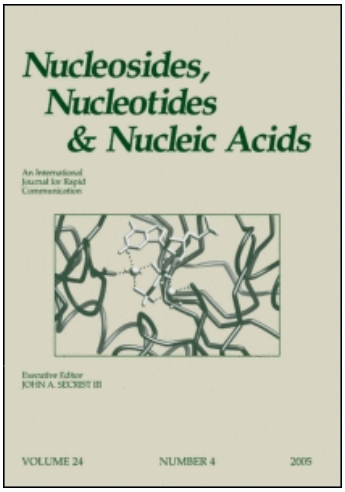

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SYNTHESIS OF CIRCULAR OLIGONUCLEOTIDE CONJUGATES

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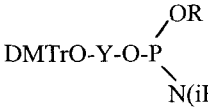
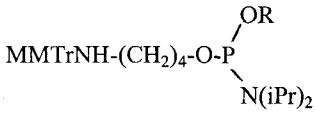
ABSTRACT: A new approach to the non-template synthesis of circular oligodeoxyribonucleotides containing flexible non-nucleotidic linkers has been developed. Using this technique a set of circular molecules representing triple helix forming, antisense and guanosine tetrad containing oligonucleotides has been obtained.

The therapeutic use of oligonucleotides as antisense and antigene agents poses several problems, such as molecular stability and cellular uptake. Various stabilization methods including the change of topological form of oligonucleotides by their circularization have been proposed. Recently, it has been shown that circularized oligonucleotides have increased resistance to degradation by cellular exonucleases and increased binding affinity with their cognate targets[1-4]. In this paper, we report the synthesis of modified circular oligonucleotides with the 3'- and 5'-ends connected using a non-nucleotidic flexible polymeric linker, the size of which corresponds to the length of the target oligonucleotide.

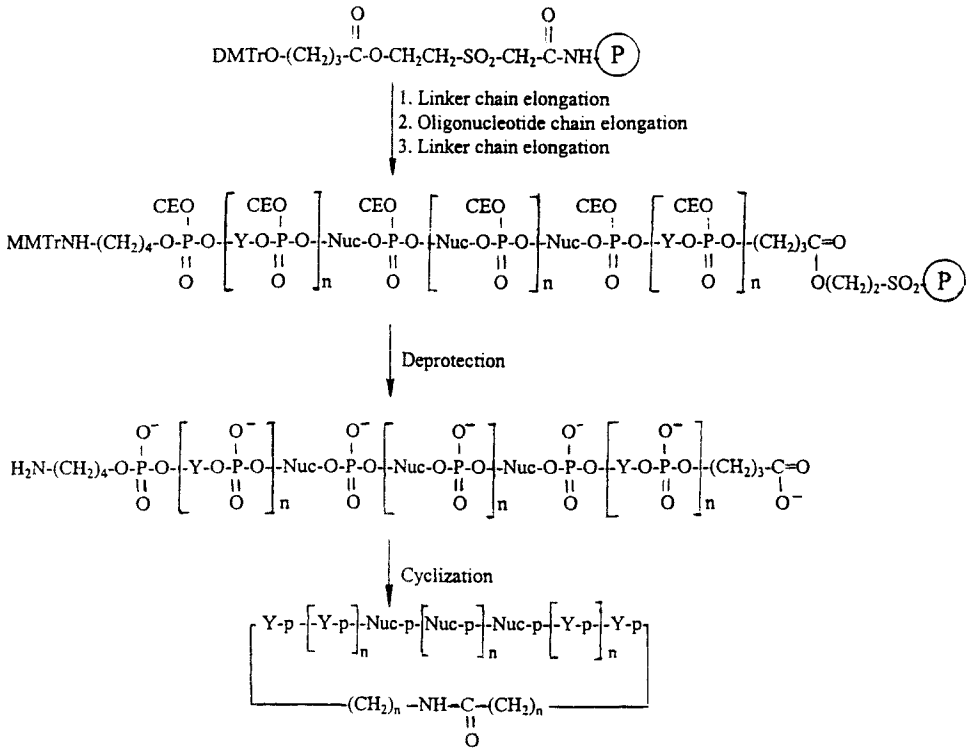
Traditional approaches to the synthesis of single-stranded circular molecules are based on the template-directed ligation of linear oligonucleotides[5]. We have developed an alternative procedure, which includes non-template chemical ligation of a linear precursor containing terminal 5'-amino and 3'-carboxy groups (SCHEME 1).



Y = $-(CH_2CH_2O)_6-$ **(1)** or $-(CH_2)_3-$ **(2)**



(3)



SCHEME 1

To obtain linear oligonucleotide precursors, the synthesis of non-nucleotidic synthons (1-3) has been accomplished. The chemistry employed was chosen to be compatible with standard phosphoramidite methododology. Each synthon contained dimethoxytrityl and the reactive phosphitilating groups. The synthons were used in the solid phase automated synthesis of linear oligonucleotide conjugates containing 16-18-mer nucleotide sequences and non-nucleotidic chains on the 3'- and 5'-ends. To introduce the 5'-terminal amino group, the phosphoramidite (3) was used on the last step of chain elongation. A support for the synthesis of oligomers having a 3'-carboxyl terminus has been prepared from the alkylsulfonylethyl CPG[6] by the introduction of a 4-O-dimethoxytrityloxybutyric acid residue with the help of 2,4,6- triisopropylbenzenesulfonyl 3-nitro-1,2,4-triazolide. In these experiments, 1-phenyl-5-mercapto-, or 1-methyl-5-mercapto-tetrazole, was used as an acidic catalist for the internucleotide bond formation in the phosphoramidite methodology[7]. With the use of these catalysts, the coupling time for the nucleoside

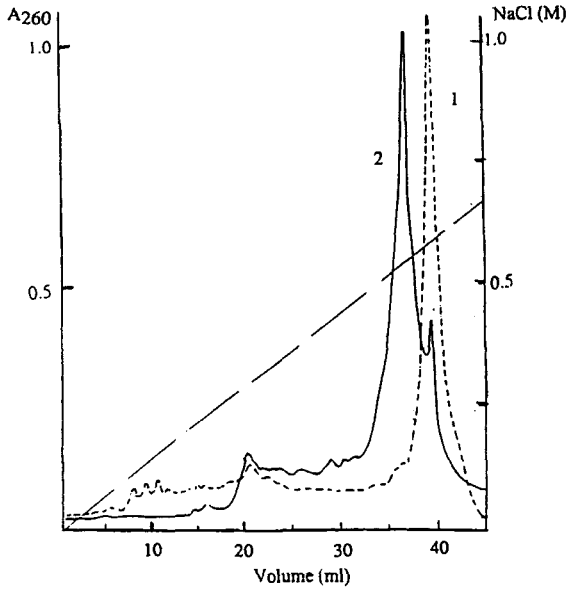


FIG.1. Isolation of the linear (1) and circular (2) the 3'-carboxy and 5'-amino groups containing conjugates of the 18-mer ASO d(ATTTTAGTGTATGTACAA), repesenting the sequence complementary to the TNF-alfa receptor translation initiator region[8], by the anion-exchange FPLC on a Pharmacia Mono-Q column at pH=12.

phosphoramidites was less than 0.5 min, whereas for non-nucleotidic synthons it was on the order of 5-10 min.

After the last elongation step and the removal of the MMTr-group from the 5'-amino function (5% dichloroacetic acid in dichloromethane, 2 min), the modified oligomer was cleaved from the support using 0.2 M NaOH in 60% aqueous dioxane for 15 min at room temperature followed by neutralization and evaporation of the solvent. Other blocking groups were removed using ethanolamine - ethanol (1:1 v/v) at 70°C for 40 min[9]. The crude linear oligonucleotides were isolated by gel-filtration and purified by electrophoresis on a 10-20% polyacrylamide gel containing 7 M urea, or by anion-exchange FPLC (FIG.1), and subsequently desalted.

The non-template chemical ligation of the linear precursors was performed at room temperature for 6-10 h in the presence of 0.2 M N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide in a buffer containing 25 mM MES (pH 6)/ 50 mM KCl/ 2.5 mM EDTA[10]. The concentration of the linear oligonucleotide in the reaction mixture was in the range of 10^{-3} - 10^{-4} M. The reaction was stoped by precipitation of the oligonucleotide using a 2% lithium perchlorate solution in acetone followed by centrifugation, and the reaction

products were desalted by gel-filtration. The circular oligonucleotides were isolated by gel electrophoresis, or FPLC. (FIG. 1). The circular nature of the oligonucleotides obtained was confirmed by gel-electrophoresis mobility, HPLC analysis and and by the absence of a free amino group[11].

Using this approach a set of circular hybride molecules representing triple helix forming and antisense oligonucleotide sequences as well as anti-HIV1 G-tetrad containing molecules[12] has been obtained and their physico-chemical properties investigated. Experimental determination of the thermal stability of the duplexes formed by the modified linear, or circular oligomers and and their complementary deoxyribo- and ribooligonucleotide targets demonstrated an enhanced stability of the heteroduplexes formed using the circular oligonucleotides compared to natural duplexes (data not shown). The *in vitro* and *in vivo* biological effect of the circular oligonucleotides obtained is currently under investigation.

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