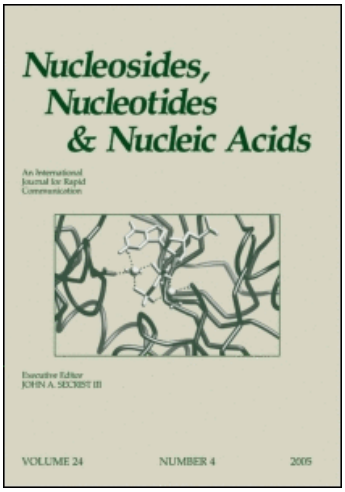

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Convenient Approaches to the Synthesis of Oligonucleotide Macrocycles Containing Non-nucleotide Linkers

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CONVENIENT APPROACHES TO THE SYNTHESIS OF OLIGONUCLEOTIDE MACROCYCLES CONTAINING NON-NUCLEOTIDE LINKERS#

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ABSTRACT: Two convenient, practical routes to the synthesis of non-nucleotide bridged cyclic oligonucleotides have been developed. The first procedure included circularization of oligonucleotides by template-directed ligation on solid phase, while the second procedure involved preparation of a circular oligomer by non-template chemical ligation of a linear precursor in solution. Using these approaches, a series of single- and double-stranded cyclic oligonucleotides with non-nucleotide bridges has been synthesized.

INTRODUCTION

The therapeutic use of oligonucleotides as sense, antisense, and antigene agents poses several problems, including molecular stability and cellular uptake. To overcome these problems, different oligonucleotide modifications have been proposed [1]. Among the more recent studies, circular oligonucleotides have been evaluated as antisense agents, triple helix forming compounds, specific inhibitors of transcription, as well as aptamers, for therapeutic and diagnostic applications [2-8]. The construction of circular hybrid molecules which contain oligonucleotide domains bridged by several types of non-nucleotide chains has also been reported [8-10]. It was shown that these molecules bind with high affinity to complementary strands of RNA and DNA and display increased resistance to degradation by nucleases. Because of these observations, ethyleneglycol and propanediol chains have been successfully used as simple linking groups which replace nucleotide units in linear oligonucleotide chains, in hairpin-shaped oligonucleotides and in circular structures [4,9,10,12]. In the present report, we describe two approaches to the synthesis of modified circular oligonucleotides

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#This paper is dedicated to the memory of Professor Tsujiaki Hata.

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with 3'- and 5'-ends connected by a flexible polymeric linker, which represents a linear aliphatic polymer consisting of oligoethylene glycol phosphate or propanediol phosphate units as the simplest variant. In principal, a such linker can consist of several different elements, each responsible for a different useful function. Thus, recognition domain, binding enhancer, target delivery elements, labels, cross-linker, etc. could be incorporated into the same molecule providing a combination of capabilities to act as a powerful antigene, or antisense reagent. Our basic idea was to develop convenient procedures for the synthesis of such macrocyclic molecules in preparative amounts. The first approach used by us rely on the template-directed ligation of linear precursors of linker-derived oligonucleotides on solid phase, whereas the second approach is based on non-template chemical ligation of a linear precursor. To demonstrate the utility of these approaches, a set of modified circular hybride oligodeoxyribonucleotides (ODNs) representing triple helix forming oligonuleotide sequences (TFO), antisense oligonucleotides (ASO), transcription decoy oligonucleotide sequences (DCO) as well as G-tetrad containing molecules has been constructed.

EXPERIMENTAL

Functionalization of a solid support for the synthesis of 3'-COOH containing oligomers. The 2-O-dimethoxytrityloxyethylsulfonylalkyl-CPG (ES-CPG) (2.5 g, 50 μmol of DMTr-groups per 1 g) prepared from aminopropyl CPG-550 Å (Fluka) as described earlier[13] was treated with 3% trichloroacetic acid in dichloromethane (20 ml) for 5 min to remove DMTr groups, and then washed successively with dichloromethane, pyridine, and acetonitrile and dried by evaporation with pyridine. The triethylammonium salt of 4-(dimethoxytrityloxy)butyric acid (2 mmol) obtained as described by Haralambidis et al.[14] was dissolved in pyridine - acetonitrile (1:1, v/v, 20 ml), and 2.1 mmol of 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) was added for 3 min preactivation. The reaction mixture obtained was combined with the ES-CPG support, and shaken for 5 h at room temperature. The support was then removed by filtration and washed with pyridine and acetonitrile. Non-reacted OH-groups on the support were capped using pyridine - acetonitrile - 1-methylimidazole - acetic anhydride mixture (10 : 10 : 2.5 : 2.5, v/v/v/v) for 1 h, and the support was washed successively with pyridine, acetonitrile, ether and then dried in vacuo.
The DMTr content was determined to be 15-20 μmol/g CPG.

Synthesis of a template-containing support. 3'-O-Pentafluorophenyl carbonate of 5'-O-DMTrT (0.05 mmol) was obtained by the addition of dipentafluorophenyl carbonate to 5'-DMTr-T as previously described [15]. The product was allowed to react with 1 g of long chain alkylamine controlled-pore glass CPG-500 (Pierce, pore size 500 Å, 50 μmol of amino groups/g) or CPG-2000 (pore size 2000 Å, 10 μmol of amino groups /g, prepared from CPG-2000, Sigma,

as described by Kume et al. [16]), in 5 ml of dry dimethylformamide in the presence of triethylamine (0.1 mmol) for 5 h. The support was isolated by filtration, sequentially washed with dimethylformamide, acetonitrile and ether, and residual unreacted amino groups were capped by the action of pyridine - acetonitrile - 1-methylimidazole - acetic anhydride mixture (10 : 10 : 2.5 : 2.5 v/v/v/v) for 1 h. After washing with pyridine, acetonitrile, methanol and ether, the support was dried in vacuo. The DMTrT-containing CPG-2000 (2-5 μmol/g) was used as a support in the solid phase synthesis of a dT_{10} - T_{15} sequence, which was followed by the sequence of a template ODN. The carbamate linkage stability to the action of 25% ammonia solution before and after ODN synthesis was determined essentially as described by Kumar [17]. To remove blocking groups, a polymer support linked oligo-dT - ODN was treated with concentrated ammonia for 16 h at room temperature, washed with aqueous dioxane and water, and then it was used for the template-directed circularization experiments. To remove template ODN from the support, the latter was treated with 0.25 M NaOH solution in 50% aqueous ethanol for 1.5 h at room temperature. To check the authenticity of the template ODN the PAGE analysis was carried out.

Oligonucleotide synthesis and purification. ODNs were synthesized on an Applied Biosystems Synthesizer (Model 381A) using phosphoramidite chemistry. Phosphoramidite monomers were purchased from Millipore. DMTr-O-Hexaethylene glycol phosphoramidites **1**, 3-(O-dimethoxytrityl)propyl-1-[alkyl-(N,N-diisopropyl)]-phosphoramidites **2** and 4-(N-monomethoxytrityl)aminobutyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite **3** were prepared as described by Giovannangeli et al.[18].

Incorporation of the non-nucleotide monomers in ODNs was performed as described [19] using 1-phenyl-5-mercapto-tetrazole (Fluka), or 1-methyl-5-mercapto-tetrazole (Aldrich) as an acidic activator of the coupling reaction and a 5-10 min reaction time. ODNs with a 3'-terminal phosphate group were obtained as described [13]. Introduction of the 5'-phosphate group into ODNs was carried out as the last step of chain elongation with a commercially available 2-[2-(4.4'-dimethoxytrityl-oxy)-ethylsulfonyl]-ethyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (Glen Research, or Cruachem). Deblocking and isolation procedures for ODNs excluding 3'-COOH containing oligomers were carried out according to standard phosphoramidite protocols. The removal of 3'-COOH containing modified oligomers from the CPG support was carried out by the action of 0.2 M NaOH in 60% aqueous dioxane (5 ml/300 mg of the support) for 10-12 min at room temperature. After removal of the support by filtration, the solution obtained was neutralized by 0.2 M HCl and evaporated to dryness. The blocking groups were removed from the ODN by the action of ethanolamine - ethanol (1:1 v/v, 2 ml) at 70°C for 40 min, and after dilution with an

equal volume of water the ODN was isolated by gel-filtration on Pharmacia NAP-10 columns for use in the following experiments.

Anion-exchange separations were performed using Pharmacia Mono-Q column / FPLC system and a linear gradient of NaCl (0 - 1.2 M) in 0.02 M NaOH (pH 12) with a flow rate of 1 ml/min. Polyacrylamide gel electrophoresis (PAGE) was performed using 10-20% gels in natural or denaturing (7 M urea) conditions with 0.1 M Tris-borate/EDTA buffer (pH 8.3), ODNs bands were detected by UV shadowing, or by staining with ethidium bromide.

Template-dependent circularization of linear oligonucleotide precursors. A linear precursor and a complementary template oligomer attached to CPG-2000 support were combined in a buffer containing 500 mM imidazole - HCl (pH=7), 100 mM $NiCl_2$ [4, 20] (20 ml/g CPG). The concentration of the linear precursor was 10^{-4} - 10^{-5} M. The reaction mixture was incubated at 95°C for 1 min and slowly cooled to 0-4°C with agitation. Non-bounded ODN precursor was removed by filtration. The support was washed and mixed with the same buffer (10 ml/g) cooled to 4°C, and 3 M BrCN in acetonitrile was added (1/20 of the total volume) to this mixture. After a 5-10 min incubation at 0-4°C with shaking, the solution was removed by filtration, the support was washed with the buffer, and the circular oligomer was eluted from the support using a 0.01 M NaOH solution (or by hot de-ionized water). After neutralisation of eluates with 0.1 M acetic acid, the ODN was precipitated with 5 volumes of a 2% lithium perchlorate solution in acetone. After 2 h, the precipitate was collected by centrifugation. The reaction products were purified by denaturing 15% PAGE.

Circularization of linear 3'-phosphorylated precircled ODNs in the presence of EDC (0.25 M), was performed in a manner similar to the BrCN procedure in 50 mM sodium MES buffer (pH 6.0) containing 20 mM $MgCl_2$. The reaction mixture was shaken at 10°C for 16 h. The ligation of a linear precursor with the help of T4 DNA ligase (Pharmacia) was carried out similar to the above described procedures in a buffer containing 0.05 M Tris-HCl (pH 7.5); 0.01 M $MgCl_2$; 5 mM ATP and 1 mM DTT during 5-6 h at 10°C. The reaction was terminated by the addition of EDTA to 10 mM concentartion. Isolation of the circular products was carried out as described above. Circularity was confirmed by resistance both to 3'-exonuclease cleavage by T4 DNA polymerase and to 5'-dephosphorylation by T4 polynucleotide kinase as described by Dolinnaya et al.[4].

Non-template circularization of oligonucleotides. Circularization of linear precursors (10^{-4} M) was carried out in a buffer containing 50 mM MES-NaOH (pH 6); 20 mM $MgCl_2$ (or 50 mM KCl for G-tetrad), and 2.5 mM EDTA. For DCOs and G-tetrad, the preliminary annealing was performed in the same buffer. The solution of ODN was treated with 0.2 M EDC for 6-10 h at

10°C. The reaction was stopped by the addition of 5 volumes of a 2% lithium perchlorate solution in acetone. After 2 h, the precipitate formed was collected by centrifugation. The reaction products were desalted by gel-filtration and analysed by a 12-15% denaturing PAGE. The circular products typically migrate in a gel at 0.8-0.9 times the rate of their linear precursors. The bands corresponding to the cyclic products were excised from the gel and eluted using 0.25 M TEAB during 16 h. After removal of the gel, the solution obtained was desalted by gel-filtration on Sephadex G-25 PD-10 column (Pharmacia) (control by UV-monitoring) in 0.05 M TEAB. After evaporation, the residue was dissolved in water and stored at -20°C.

Thermal denaturation and enzyme degradation experiments Absorbance (260 nm) versus temperature curves of duplexes were measured using a Gilford 250 UV-VIS spectrophotometer equipped with a Gilford 2527 thermocontroller. Solutions contained 3 μM of each ODN in 100 mM NaCl / 10 mM Tris-HCl (pH 7.0) / 5 mM EDTA. After heating at 100°C for 2 min, the solution was allowed to cool slowly to 5°C before data collection. The changes in absorbance at 260 nm versus temperature were measured with a heating rate 0.5°C/min from 5-95°C. Melting temperatures (T_m) were taken to be the temperature of half-dissociation and were obtained from a plot of the derivative of $1/T$ vs absorbance at 260 nm. Snake venom phosphodiesterase and S_1 nuclease assays were performed as described [4, 21]. The digestion products were analysed using a 20% denaturing PAGE.

Biological assays . The human monocytic cell line, THP-1 was grown in RPMI 1640 supplemented with L-glutamine (GIBCO BRL, Life Technologies, Inc.), 10% heat inactivated fetal bovine serum (GIBCO, BRL) and penicillin (100 U/ml) /streptomycin (100 μg/ml). Cellfectin was obtained from Life Technologies (Gaithersberg, MD). The TNF-α level in culture medium was determined by enzyme linker immunosorbent assays (ELISA) using a commercially available kit obtained from R&D Systems (Minneapolis, MN).

THP-1 cells ($3x10^6$) were seeded in 48-well plates in 0.2 ml of Opti-MEM medium (GIBCO). In a 96-well plate, varying concentrations of ODNs (20 μl) were each added to 20 μl of Cellfectin in Opti-MEM (2.4 μl of 1 mg/ml solution added to 17.6 μl of Opti-MEM) and incubated for 15 min at room temperature. The mixture was then added to the cells dropwise to the 10 μg/ml final concentration of the Cellfectin in 0.24 ml of Opti-MEM. After 4 h at 37 °C, 0.48 ml of 15% RPMI medium was added to each well to inactivate the Cellfectin. The final concentration of cell was ~ 1 x 10^6 cells/ml in 10% RPMI. Then, the cells were stimulated with a combination of 100 ng/ml of PMA and 300 U/ml of IFN-γ. After 6 and/or 18 h post-stimulation, the culture supernatants were

collected and stored at -80°C until evaluated by ELISA for levels of TNF-α in the culture medium. The data were expressed as percent inhibition of TNF-α compared to control (no ODN treatment) cells.

The analysis of the inhibition of HIV-1 integrase by a series of G-tetrad ODNs, including the extent of 3'-processing and strand transfer, was performed using 5'-end labeled substrates and recombinant HIV-1 intergrase as described previously [22].

RESULTS AND DISCUSSION

Solid phase approach

Several procedures have been described for the synthesis of cyclic ODNs, including direct chemical synthesis in solution [23, 24] and on solid phase [25], non-template circularization by disulfide bridge formation [11, 26] and a template-directed approach [2, 4, 5, 9, 10]. The latter approach usually utilizes chemical, or enzymatic circularization of an open chain oligonucleotide precursor via connection of its 3'- and 5'-ends, which are held together by base pairing to a complementary oligonucleotide functioning as a template.

The efficiency of such ligation depends on many factors, including the length of adjoining ODNs and the linker spacer between them which should be sufficient to provide a stable duplex formation with the template bringing the reactive ends adjacent to one another, the concentration of ODNs in the reaction mixture, and the influence of secondary structures of the linear precursor and the template ODN. In the case of chemical ligation, the nature of the nucleotide residues to be ligated and the conditions of chemical coupling including the choice of condensing agent are very important. One of the main drawbacks of this method is the necessity to carry out the circularization reaction using low ODN concentrations (usually about 10^{-6} M) to minimize the side reaction of dimerisation, the other one is the problem of the circular product purification from a template ODN and an excess of the corresponding linear precursor.

One of possible solutions of these problems is to attach the template ODN to a solid phase. Thus, we have developed a simple solid phase assembling procedure depicted in SCHEME 1. A linker-bridged linear ODN precursor, complementary to the template sequence, is added to a template containing support at some excess. After annealing, the unbound ODN precursor is washed away, and a template-bounded duplex is treated with a condensing agent (water soluble carbodiimide, or BrCN), or with T4 DNA ligase to seal the nick. After denaturation, the circular oligomer is removed from the template containing support. It should be noted that solid phase template-depended ligation of ODNs, as any other solid-phase approach, has the potential for

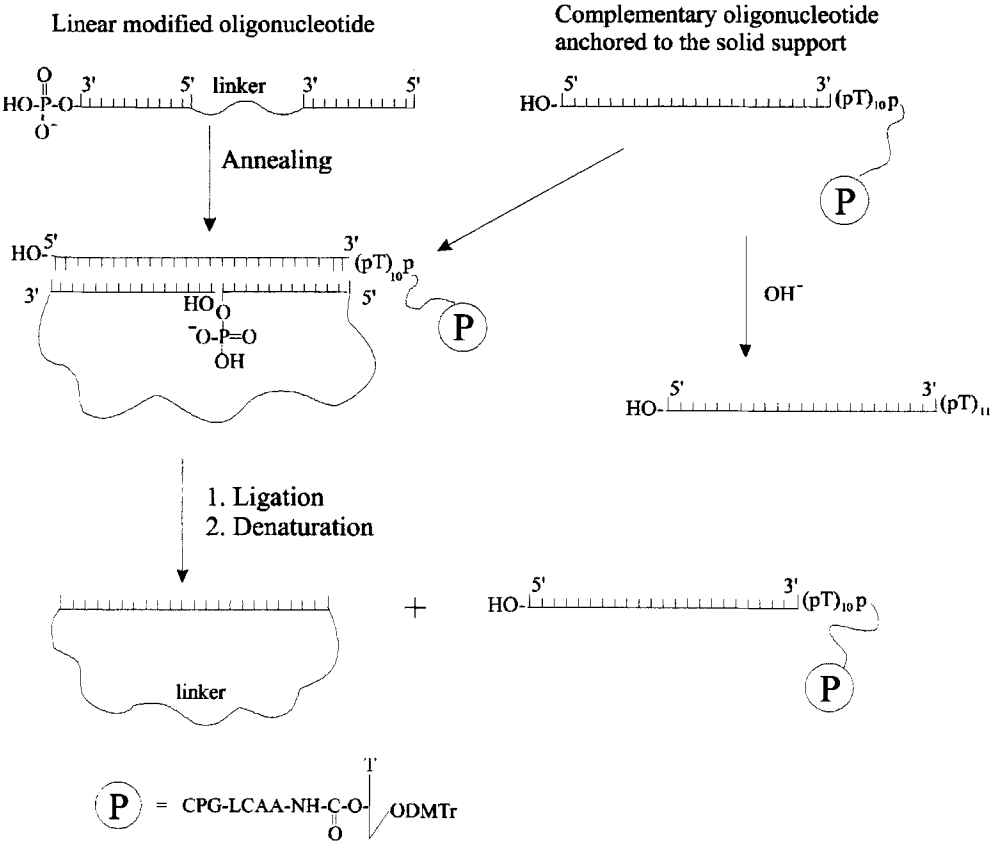
automation. Moreover, the non-circularized excess of a linear precursor as well as a template containing CPG can be used in the next syntheses.

The first step in the solid-phase assembly procedure was the synthesis of a template ODN bound to a solid support at one of its terminus (5'-, or 3'-) as shown in SCHEME 1. The development of a suitable support on the basis of standard CPG beads for the synthesis of ODN templates has been carried out to obtain one, which would provide reliable hybridization in the solid phase annealing process with complementary ODN. From several suitable linkages[17], a carbamate bond was chosen for covalent attachment of template ODNs to a solid support. This bond was fully stable during the elongation of ODN chain, and up to 75% of synthesized ODN was kept on the support after its treatment with concentrated aqueous ammonia solution for 24 h at room temperature. At the same time, the template ODN can be easily reattached from the matrix, on which it was synthesized, by the action of 0.2 M NaOH to control its quality and sequence.

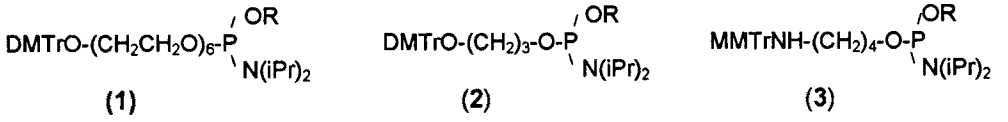
Hybridization experiments with the support-linked 16-18-mer ODN revealed that the hybridization yield depends on the CPG pore size and support capacity. The best results with complementary ODN hybidization having yields in the range of 50-70% were obtained using the support on the base of CPG with a pore size 2000 Å and having a low level of functionalization (2-5 μmol of ODN per 1 g). Whereas with the support on the base of CPG-500 Å (~20 μmol of ODN per 1 g), the hybridization yields were only 25-45%. Furthermore, it was found that low support capacity is important during circularization process to exclude the steric hindrance and the interaction of one molecule of a linear precursor with two molecules of a template ODN on the support, and as a result an increased yield of a circular product was obtained.

The non-nucleotide synthons compatible with the standard phosphoramidite method of automated ODN synthesis, which were integrated into linear precursors of cyclic ODNs during automated synthesis are shown in SCHEME 2. The monomers of type **1**, derived from hexaethyleneglycol, can be used for the generation of neutral (Alk = ethyl), or negatively charged (Alk = 2-cyanoethyl) non-nucleotidic chains. Linkers of type **2**, derived from 1,3-propanediol have the same number of carbon atoms in a chain as a single ribose backbone and can be considered as a "natural" substitute for any individual nucleotide approximately retaining the correct internucleotide spacing.

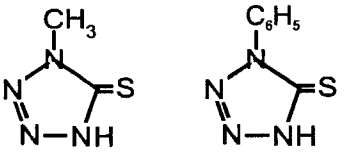
As chemical ligation is most effective for 3'-phosphorylated nicked sites [4], the synthetic route of the circular ODN precursors for chemical ligation involved the synthesis of a 3'-phosphorylated linker bridged oligonucleotide pair. To obtain oligonucleotide 3'-phosphates we derivatized an aminated CPG support by the introduction of alkylsulfonylethyl group as previously described [13]. After the removal of the dimethoxytrityl group from the support, the ODN chain was elongated



SCHEME 1



R = Et, or CNEt



SCHEME 2

using the phosphoramidite chemistry. In the first experiment, 1H-tetrazole was used as a standard acidic catalysts for the phosphite triester bond formation. However, we have found that it was not sufficiently efficient in conjunction with non-nucleotide synthons, especially with synthon **1**. Even when the condensation time was prolonged to 15-20 min the yields on each step of chain elongation were in the range of 50-70%. To overcome this problem, we used the modified phosphoramidite method, in which 1-methyl-5-mercapto-tetrazole **4**, or 1-phenyl-5-mercapto-tetrazole **5** (SCHEME 2), was used as the acidic catalyst for the phosphite triester bond formation during the synthesis of non-nucleotide chains as well as in the synthesis of nucleotide sequences [19]. It was found that on the 0.2 and 1 μmol scales the time necessary for efficient internucleotide bond formation (yields 98-99%) was less than 0.5 min. With the use of non-nucleotide synthons we increased the reaction time to 2 min on 1 μmol scale and 5-10 min on 10 μmol scale for optimum results (yields 97-98%). The removal of the target 3'-phosphorylated ODN from the support was achieved using standard procedures with concentrated ammonia, ethanolamine, or hydrazine [27]. After deblocking and isolation, the linear ODNs were used for the synthesis of circular ODNs.

The modified 3'-phosphorylated linear ODN precursors were annealed with the corresponding template ODNs and circularized in the presence of BrCN , or N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (water soluble carbodiimide (EDC) [4, 10]. However, in the agreement with the data reported by Dolinnaya et al.[28], it was found that BrCN was not suitable reagent to obtain the circular form of purine rich ODNs, especially when the joining is between two purine nucleotide residues. EDC gave better results, but even in this case, the yield of circular compounds was only increased to about 50%. At the same time, other modified linear ODNs containing pyrimidine nucleotide residues on 5'- and 3'-ends subjected to chemical ligation were circularized in the presence of their templates and BrCN with yields of approximately 75%. The analysis of the reaction products by gel-electrophoresis in denaturing conditions revealed that a side-product representing the ODN dimer was present in the amount of 10-15% in the reaction mixture if the concentration of oligonucleotide during annealing was in the range of 10^{-4} -10^{-5} M (FIG. 1A). In the case of higher oligonucleotide concentrations ($\geq 10^{-3}$), the yield of a circular oligomer was lower than the yield of the corresponding dimer. At the same time, it was found that the length of a non-nucleotide linker practically has no effect on the yield of ligation.

As reference compounds, several circular ODNs were obtained by the enzymatic ligation. The precursors, having free 3'- and 5'-hydroxyl groups, were 5'-phosphorylated with the help of T4-polynucleotide kinase and ATP. After annealing with the corresponding template on a solid support, the circularization reaction was performed in the presence of T4 DNA ligase. The circular nature of the ODNs obtained by template-directed ligation was also confirmed by their resistance to

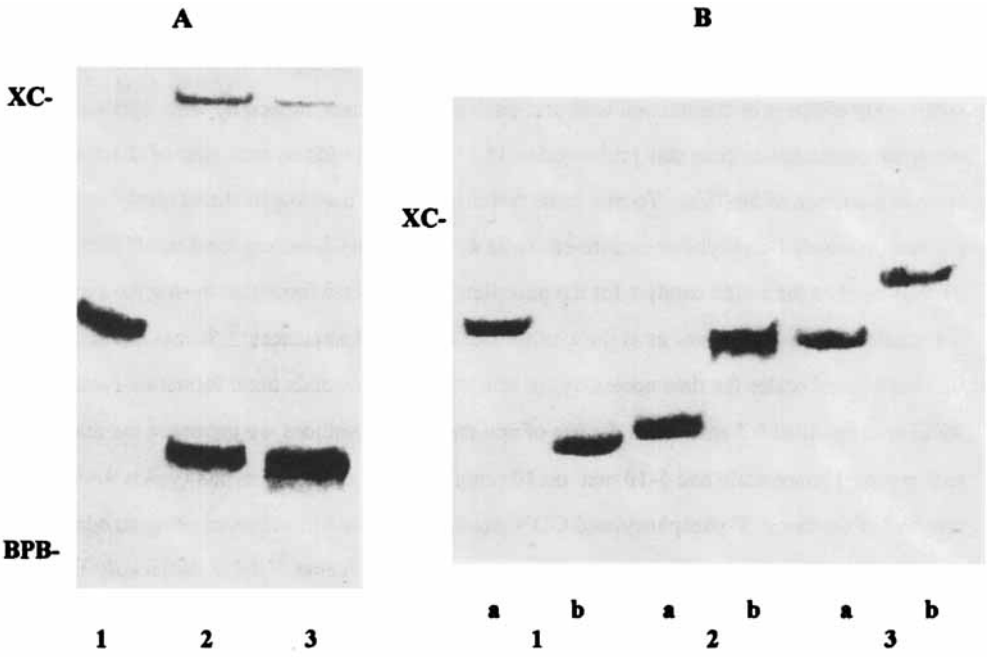
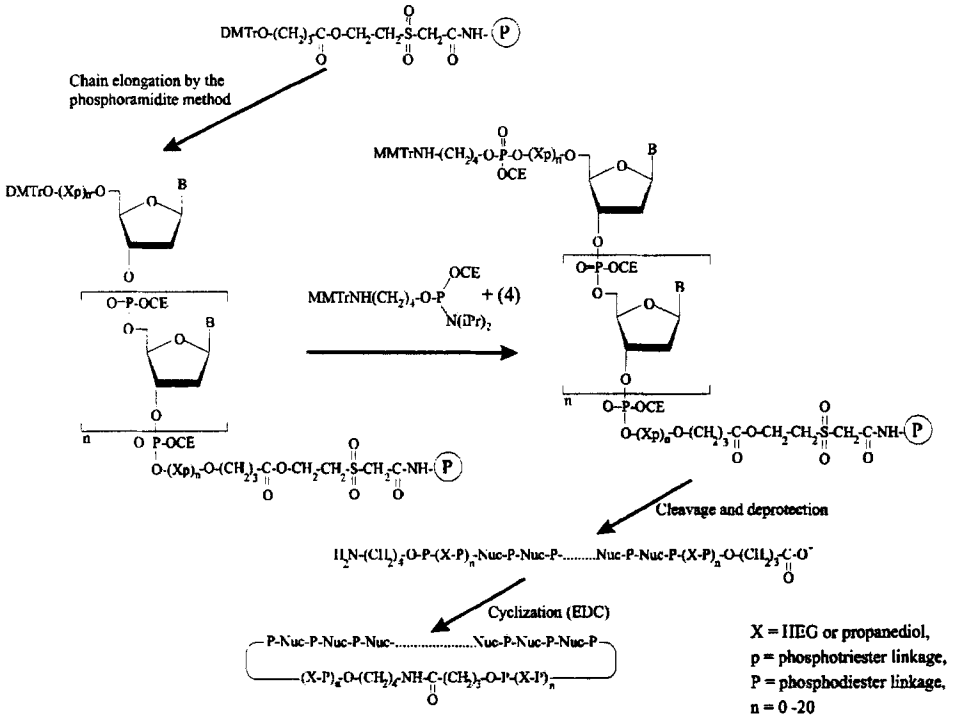


FIG. 1. Analysis of the circular ODNs synthesized with the use of template-dependent solid phase approach by PAGE in denaturing conditions. **(A)** - The reaction products obtained in the chemical ligation of the linear precursor **(1)** of ASO-1 with the help of BrCN using ODN concentration 10^{-4} M **(2)** and 10^{-5} M **(3)** (a 10% gel, staining with ethydium bromide);. **(B)** - Analysis of purified circular ODNs: **(a)** and their linear precursors **(b)**: ASO-2 with 3 HEG-ethylphosphate residues **(1)**; ASO-1 with 7 HEG-phosphate residues **(2)**, and ASO-2 with 7 HEG-ethylphosphate residues **(3)** (a 15% gel containing 7 M urea, staining with "Stains all").

3'-exonucleolytic degradation activity of phosphodiesterase I and by the absence of the ability to incorporate the ^{32}P-label in the presence of T4 polynucleotide kinase and γ-^{32}P-ATP.

Non-template solution approach

In addition to the template-directed method, an alternative procedure based on effective and convenient method for non-template circularization of ODNs in aqueous solution, has been developed. Recently, it was reported that base pairing is not a prerequisite for circularization, and sequences with no regions of self complementarity can be cyclized by the oxidation of terminal thiol groups [11]. A procedure, which we report in this paper, involves amide bond formation between the oligonucleotide 5'- and 3'-termini in the presence of a condensing agent. To do this we prepared a linear ODN conjugate containing non-nucleotide chains on the 5'- and 3'-ends having terminal reactive amino and carboxyl groups (SCHEME 3).



SCHEME 3

A support for the synthesis of ODNs having a 3'-carboxyl terminus was prepared from 2-hydroxyethylsulfonylacetamido-CPG [19] by the introduction of a 4-O-dimethoxytrityl oxybutyric acid residue with the help of a condensing agent. In these experiments, methyl- (4) and phenyl-mercaptotetrazole (5) (SCHEME 2) were used as acidic catalysts for the internucleotide bond formation by the phosphoramidite method. To introduce the 5'-terminal amino group, the phosphoramidite 3 was used on the last step of chain elongation. After the last chain elongation step and the removal of the monomethoxytrityl group from the 5'-amino function, the modified ODN was cleaved from the support by the action of 0.2 M NaOH in aqueous dioxane. After the removal of the other blocking groups by the action of conc. ammonia, or ethanolamine [27], the crude linear ODN was isolated by gel-filtration and purified by gel-electrophoresis, or by anion-exchange FPLC, and subsequently desalted.

The amino- and carboxyl group containing linear precursor was then circularized by the action of water soluble carbodiimide [28, 29]. A study of the cyclization reaction revealed that it was

essentially complete after 5-6 h at room temperature, or after 10-12 h at 10°C. The examination of the circular product yield dependence from pH showed that a pH of 6 was most preferable for this reaction. We have found that optimal ODN concentrations in the reaction mixture were in the range of 10^{-3} - 10^{-4} M that is significantly higher than in the case of template-dependent cyrcularization in solution. As determined by gel electrophoresis or FPLC analysis of the crude reaction products, the yield of the circular ODNs was more than 80% at these conditions (FIG. 2), and competing dimerization reaction was not significant. This procedure was used to prepare cyclic ODNs of a wide range of sizes starting from 15-16-mers with different non-nucleotide linker sizes, and in all cases the circularization appeared to be highly efficient.

As a variant, ODNs containing terminal 5'-amino- and 3'-phosphate residues, as well as 5'-OH and 3'-phosphate groups were synthesized and subjected to circularization in the presence of EDC. However, as determined by gel electrophoresis, the yield of a circular compound via the reaction of a phosphoramide bond formation was only about 20% after 16 h, and we were not able to see any circular product in the case of interaction of terminal hydroxyl and phosphate groups. For this reason, we concluded that the use of amino and carboxyl groups for non-template circularization is much more preferable.

The circular nature of the ODNs obtained by this method was confirmed by gel electrophoresis mobility, FPLC analysis, and by the absence of free amino groups [30]. The method has been applied in the synthesis of a set of cyclic hybrid ODNs (TABLE 1) representing triple helix forming ODN sequences from the human vascular endothelial growth factor (VEGF) promoter region [31] and various antisense ODN sequences, particularly the sequences complementary to the different regions of the tumor necrosis factor alpha (TNF-α) gene and TNF-α receptor translation initiator region 10 [32, 33], as well as anti-HIV1 G-tetrad containing molecules [34] have been obtained. This procedure was also applied to the synthesis of bridged duplexes representing sequence specific transcription inhibitors (DCO) such as the upstream region of TNF-α gene [35] and upstream region of the IL-1β gene [36].

Some properties of circular compounds obtained

In order to test the method of construction and the effectiveness of the circle formation, we attempted to obtain circular molecules containing the non-nucleotide linkers of varied length. Circles, containing from 0 to 8 non-nucleotide residues were successfully constructed from the linear precursors. In denaturing conditions, it was found that the electrophoretic mobility of the circular ODNs comparatively to that of the corresponding linear precursors depended on the number of non-nucleotide residues in the chain (FIG. 1B). Thus, the circular 18-mer ODNs

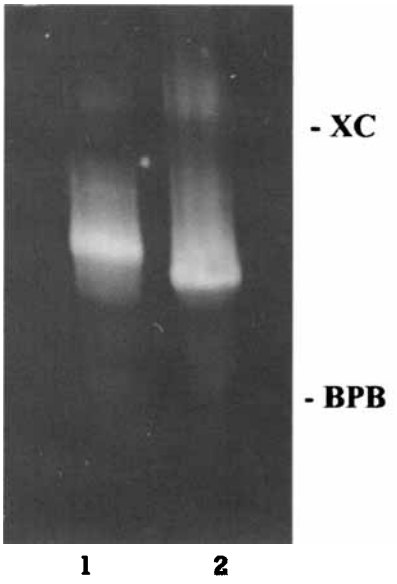


FIG. 2. Analysis of circular ODN ASO-3 (1) synthesized with the use of non-template chemical ligation approach and its linear precursor containing 5'-amino and 3'-carboxyl terminal groups (2) by 12% PAGE in the presence of 7 M urea. Staining with ethydium bromide.

containing more than 6 residues of the type 1 migrated during gel electrophoresis in the presence of 7 M urea faster than their linear precursors, and the circular ODNs containing less than five non-nucleotide residues 1 migrated slower than their linear precursors. Under native conditions, all circular ODNs migrated on a polyacrylamide gel slower than corresponding linear precursors.

The examination of the circular product's stability to hydrolysis by nucleases revealed that they were completely resistant to 3'-exonucleolytic degradation by snake venom phosphodiesterase under the conditions in which the corresponding unmodified linear oligonucleotides were totally degraded. We further analysed the double-stranded dumbbell structures obtained by incubating them with single-strand-specific S1 nuclease. With this enzyme, degradation was monitored by the loss of the full length oligomer. No significant degradation of the circular modified oligomers was observed over a 30 min interval, whereas under the same conditions, the unmodified double-stranded ODNs and hairpin-like molecules were found to be partially degraded probably due to some extent of the 5'- and 3'-ends deshybridization in the non-ligated structures.

TABLE 1. The yields of modified circular ODNs obtained by solid phase template-directed and non-template ligation procedures..

ODN	*Sequence*	*Meth.*	*Yield,%*	*Ref.*
TFO-1	⟨-AAAAAAAAaaGGGGGGGG-⟩ ⟨------(HEGp)$_7$-------⟩	p	45	[31]
TFO-2	⟨-GGGGGGGGttTTTTTTTT-⟩ ⟨-------(PRp)$_{20}$------⟩	p	72	[31]
ASO-1	⟨-CATGCTTtcAGTGCTCAT-⟩ ⟨------(HEGp)$_7$-------⟩	p	77	[32]
ASO-2	⟨-CATGCTTtcAGTGCTCAT-⟩ ⟨-----(HEGpEt)$_n$------⟩ n=1-8	p	70-78	[32]
ASO-3	⟨-----ATTTTAGTGTATGTACA------⟩ ⟨-(HEGp)$_2$-R'-NHCO-R''-(HEGp)$_2$-⟩	s	83	[33]
ASO-4	⟨----TCATGGTGTCCTTTGCAG-----⟩ ⟨-(HEGp)$_2$R'-NHCO-R''-(HEGp)$_2$-⟩	s	90	[32]
DCO-1	HNR'-HEGp-CGCTTCCTCCAGATGAGCTCATGGGTTTCT-HEGp-R''C=O | | O=CR''-HEGp-GCGAAGGAGGTCTACTCGAGTACCCAAAGA-HEGp-R'NH	s	82	[35]
DCO-2	/-ACTTCTGCTTTTGGG-PRp-R''C=O (PRp)$_3$ | ⟨-TGAAGACGAAAACCC-PRp-R'NH	s	95	[36]
GTO-1	/-GTGGTGGGTGGGTGGGT-⟩ ⟨----R'-NH-CO-R''----/	s	80	[34]
GTO-2	⟨-GGGTGGGTGGGTGGGT-⟩ ⟨----R'-NH-CO-R''---⟩	s	84	[34]

a - Yields of circular oligomers are given relatively to the corresponding linear precursors.
p - Solid phase template-directed method (nucleotides in a place of junction are shown by small letters).
s - Non-template ligation method.
R' = -$(CH_2)_4$- ; PRp = propanediol phosphate;
R''= -$(CH_2)_3$- ; HEGp = hexaethylene glycol phosphate.

The melting behaviors of the duplexes formed between the cyclic compounds described above and their DNA (or RNA) complement were studied in comparison with their unmodified counterparts, and with the corresponding unligated linear precursors at pH 7.0. It was found that duplexes formed by single-stranded circular modified oligomers and DNA, or RNA target are quite stable toward thermal denaturation. From the comparison of the T*m* values, we have found that 3-5 units of type (1) in a loop is the optimal number for single-stranded circular molecules containing 16-18 nucleotide residues, whereas the optimal number of type (2) units is equal to the amount of

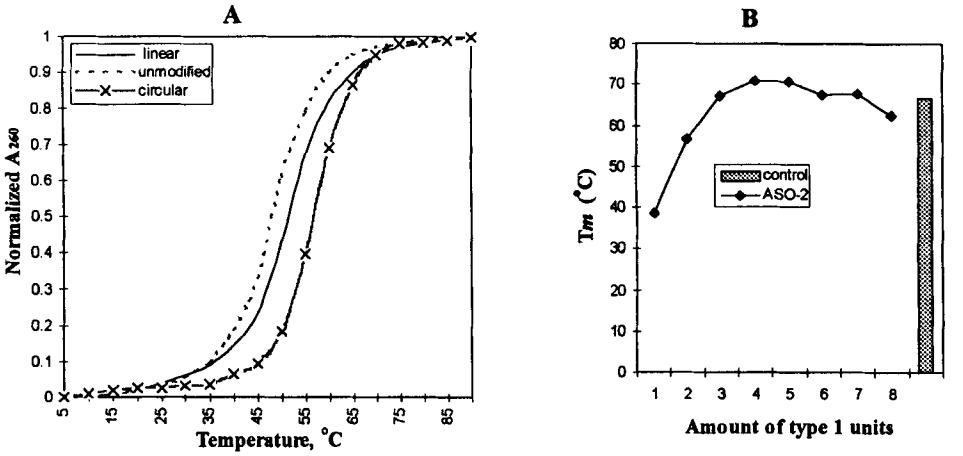


FIG. 3. Thermal stability of duplexes formed by circular ODNs and their complementary targets. **(A)** -Melting temperature (T*m*) curves of duplexes consisting of the linear precursor, or circular ASO-3 and its 18-mer unmodified deoxyribonucleotide target and that of two unmodified complementary oligonucleotides. **(B)** - The comparison of Tm values of duplexes formed by circular ODNs with the same ASO-2 nucleotide sequence and various lengths of non-nucleotide chain and the corresponding unmodified complementary ribooligonucleotide target. The duplex formed by unmodifed 18-mer of the same sequence and its target was used as a control.

nucleoside residues in the molecule plus two. Such duplexes exhibit greater stability than those with unmodified ODNs and duplexes formed by linear ODN precursors with their unmodified complements (FIG. 3A). Decreasing the amount of non-nucleotide residues in the circular molecule leads to decreased thermal transition temperature values if compare to corresponding linear ODNss, whereas increasing of non-nucleotide residues almost does not influence T*m* values (FIG. 3B).

Earlier study into some properties of DCOs with non-nucleotide bridges demonstrated their high stability toward thermal denaturation (10). Similar results were obtained in this study for self-complementary DCO sequences bridged by aliphatic chains. Covalently closed duplexes had ~10°C higher melting temperatures than do hairpin-like molecules and considerably higher (ΔT_m 25-30 °C) than linear non-modified double-stranded chains, which contained the same sequences.

Circular ODNs with various linker sizes and the same lead sequence motif targeted to a mRNA start site region of the TNF-α gene were tested in biological assays for their ability to inhibit TNF-α expression from stimulated THP-1 cells. As it is follows from the preliminary results of these culture assays, the lead sequence motif, when circularized with a short linker (n=3, compound

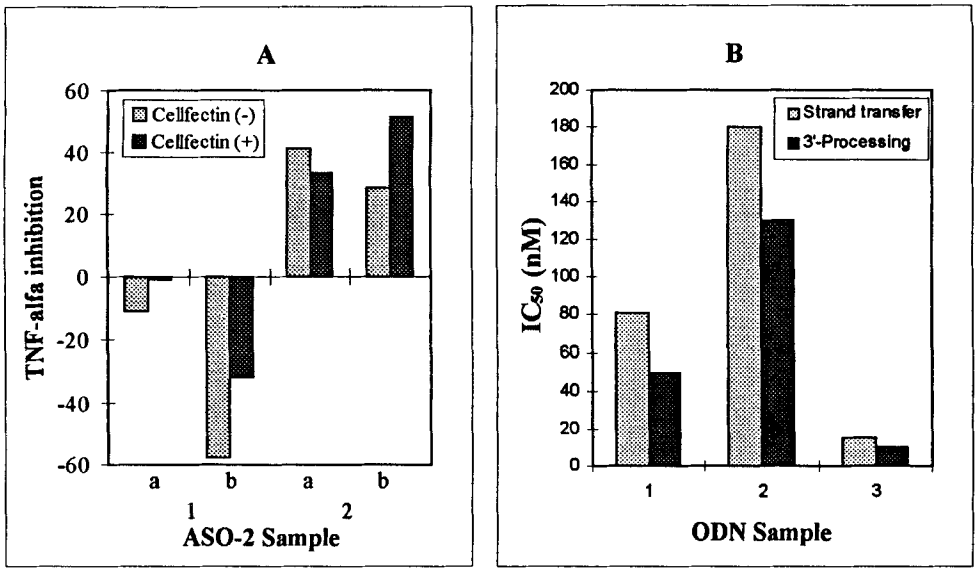


FIG. 4. Functional activity of circular ODNs. **(A)** - Analysis of the inhibition of TNF-α expression in THP-1 cells in the absence or in the presence of the uptake enhancer Cellfectin (10 μg/ml) by circular ASO-2 (n=7) **(1)** and ASO-2 (n=3) **(2)** using ODN in 0.25 μM **(a)** or 0.5 μM **(b)** concentration . **(B)** - Comparison of the inhibition of HIV-1 Integrase 3'-processing and strand transfer (IC_{50}=50% inhibition of control values) by the unmodified guanosine quartet [compound T3017 [22]] **(1)**, the linear precursor of GTO-1 **(2)** and the circular GTO-1 **(3)**.

ASO-2), was able to significantly inhibit TNF-α production when added to cells with or without the cationic lipid uptake enhancer Cellfectin . This is in contrast to the data obtained when the same sequence was circularized with a longer linker (n=7, compound ASO-2), which was unable to inhibit TNF-α production and in fact stimulated TNF-α in this assay (FIG. 4A). At the same time, the ODNs TFO-1 and TFO-2 directed to human VEGF gene , which were used as a control in these experiments, had no effect on TNF production.

Previously, it was shown that an ODN composed entirely of deoxyguanosine and thymidine residues can fold upon itself in the presence of potassium into a highly stable four-stranded DNA structure containing two stacked deoxyguanosine quartets (G-tetrad) and can protect host cells from the cytopathic effects of human immunodeficiency virus type 1 (HIV-1) [34]. It was also reported that G-tetrad is the most potent inhibitor of HIV-1 integrase identified to date [22]. Preliminary experiments on the ability of a circular G-tetrad GTO-1 (TABLE 1) obtained in this study, to inhibit the strand transfer and 3'-nucleotide activities of HIV-1 integrase revealed that the circular

compound was approximately 5 fold more active than the control unmodified ODN and 10 times more active than the linear precursor of GTO-1 containing terminal 5'-amino and 3'-carboxylic groups (FIG. 4B).

CONCLUSION

The methods described above provide effective and convenient routes for circularization of ODNs of varying sizes. It should be noted that for the aim of preparative synthesis of cyclic ODNs, the use of non-template ligation method is more economical, because it does not include the synthesis of a template ODN and makes possible to work with higher concentrations of ODN. In comparison with the earlier described method of non-template cyclization through the formation of alkyl disulfied bridges[11], the above described procedure has the advantage of using more stable amide bond for the formation of cycles. The application perspectives for circular ODNs obtained by both above described procedures are currently under investigation in different tissue culture-based assay systems. Nevertheless, the preliminary experiments on the biological activity of the circular modified ODNs having short linkers show that they may have superior characteristics compared to conventional linear ODNs.

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