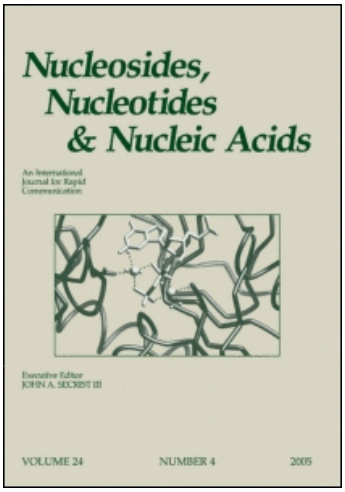

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S. I. Antsypovich[a]; T. S. Oretskaya[a]; E. M. Volkov[a]; E. A. Romanova[a]; V. N. Tashlitsky[a]; M. Blumenfeld[b]; Z. A. Shabarova[a]
[a] Chemical Department, Moscow State University, Moscow, Russia [b] GENSET, 1 Rue R. et S. Delaunay, Paris, France

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SYNTHESIS OF MODIFIED OLIGONUCLEOTIDES AND PRODUCTION OF DUPLEXES WITH COVALENTLY LINKED CHAINS

Sergey I. Antsypovich[1], Tatiana S. Oretskaya[1*], Evgeny M. Volkov[1], Elena A. Romanova[1], Vadim N. Tashlitsky[1], Marta Blumenfeld[2], Zoe A. Shabarova[1]

[1]Chemical Department, Moscow State University, Moscow 119899, Russia, and [2]GENSET, 1 Rue R. et S. Delaunay, 75011 Paris, France

Abstract

A method has been devised to synthesize a DNA-duplex with covalently connected strands. Primary amino group located on one strand is linked to a carboxyl group of the other strand through the agency of a water soluble carbodiimide condensing agent. Conditions for the reaction between chains of a duplex composed of the modified oligonucleotides [1] were optimized. The thermal and hydrolytic stability of the cross linked duplex was examined.

Introduction

Modified oligonucleotides have received wide acceptance in molecular biology and their unique properties continue to be exploited in numerous investigations [2,3]. Oligonucleotides being fragments of the genetic code display essential features of native nucleic acids. The most frequently used are those modified by introducing extra functional groups [4]. This gives several useful properties to oligonucleotides, namely makes them resistant to nuclease degradation and endows them with the capacity to penetrate through the cellular membranes.

Modified oligonucleotides are very promising for the study of protein-nucleic acids interaction, for antisense and sense biotechnology, as well as for creating new drugs. This line of inquiry calls for new substrates with predetermined chemical and physico-chemical properties.

In this work a new strategy is proposed to synthesize a modified duplex with covalently linked strands. Some publications outline the synthesis of DNA-duplexes with covalently linked chains [5-7]. However the linkage is introduced by modifying the heterocyclic bases (cytosine or guanine). This not only discriminates hydrogen bonds at the site of linkage but also greatly restricts the design of target substrates.

The special feature of the substrate proposed is that a linkage can be constructed at any region of the DNA-duplex, including the introduction of multiple cross links. Such structures might find use in studies of enzymes interacted with DNA as well as in sense biotechnology.

The intention of this work is to devise an adequate method to synthesize modified monomer synthons ready to be introduced into oligonucleotides during automatic phosphoramidite synthesis. In addition we want to synthesize complementary oligonucleotides having carboxyl and amino functional groups, to carry out the reaction between chains of the duplex composed of these modified oligonucleotides as well as to optimize conditions of this reaction and to examine the properties of the resultant cross linked duplex.

Results and discussion

Synthesis of modified oligodeoxyribonucleotides

We stated the requirements for the design of the modified oligonucleotide substrate which determine the nature of functional groups introduced into oligonucleotides. The requirements are as follows: (1) modification by introduction of functional groups should be possible at preassigned sites of the oligonucleotide chain; (2) multiple functionalisation should be feasible at preassigned sites in order to obtain an oligonucleotide duplex with several links; (3) a monomeric synthon should be introduced

TABLE 1.

Oligonucleotide sequences

Number	Sequences of the oligonucleotides
I	5'-TTC^{n}TT-3'
II	5'-AAAAAAAC^{n}T-3'
III	5'-ATCCTATAATGCGC^{n}CCTGCA-3'
IV	5'-GCCACTCGGAAAGTCCCCTCU^{n}ACCG-3'
V	5'-TNTTT-3'
VI	5'-TTTTTNT-3'
VII	5'-GGTNTGGTTAATGATCTACANTTAAT-3'
VIII	5'-TGCAGGNCGCATTATAGGAT-3'
IX	5'-CGGTNGAGGGGACTTTCCGAGTGGC-3'
X	5'-ATCCTATAATGCGC^{c}CCTGCA-3'
XI	5'-TGCAGGN^{c}CGCATTATAGGAT-3'
XII	5'-CGGTN^{c}GAGGGGACTTTCCGAGTGGC-3'
XIII	5'-ATCCTATAATGCGCCCTGCA-3'
XIV	5'-TGCAGGGCGCATTATAGGAT-3'

C^{n} - 2'-amino-2'-deoxycytidine

U^{n} - 2'-amino-2'-deoxyuridine

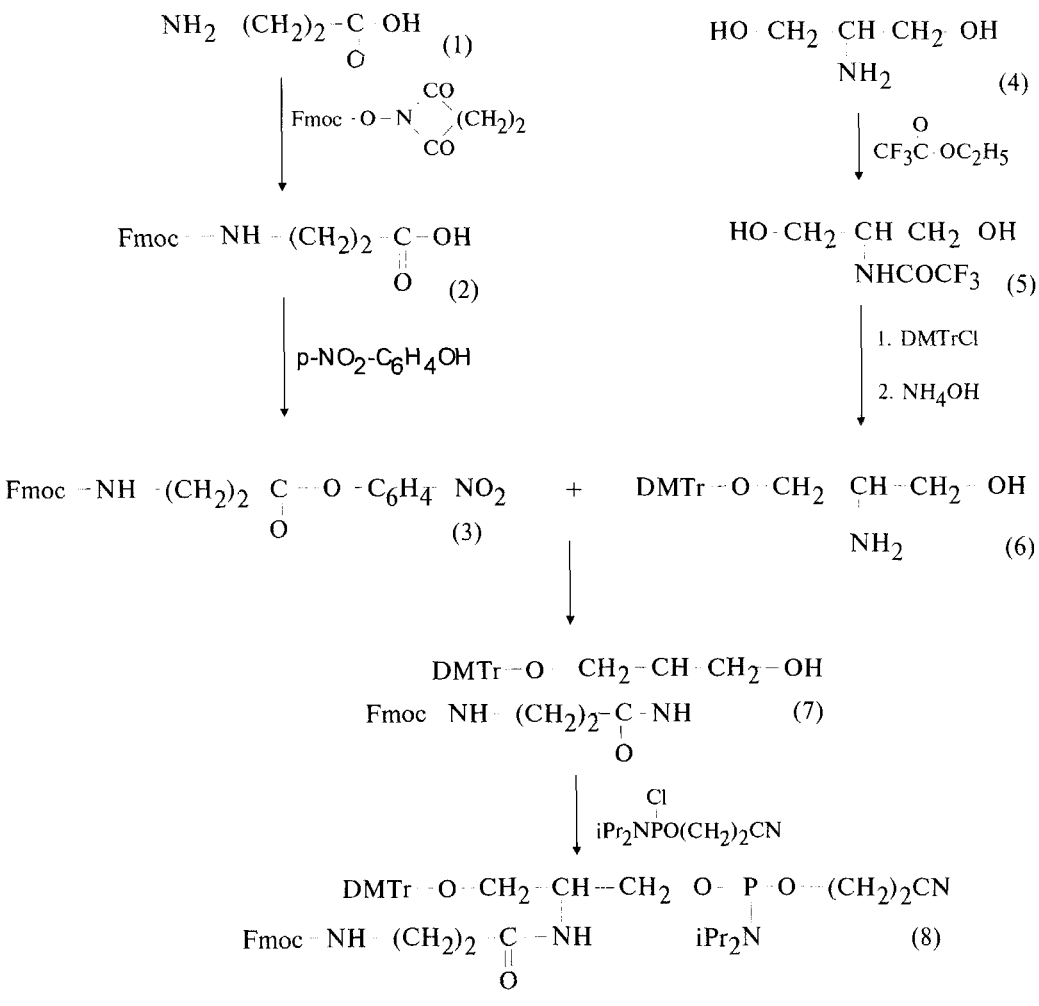
N - nonnucleoside insert

C^{c} - carboxylated 2'-amino-2'-deoxycytidine

N^{c} - nonnucleoside insert with carboxyl group

into oligonucleotide during automated synthesis following standard synthetic procedures.

Based on the above requirements we chose the following types of oligonucleotide modifications. 2'-Amino-2'-deoxyuridine or 2'-amino-2'-deoxycytidine was introduced into the one chain of the oligonucleotide duplex. The corresponding nucleoside-3'-phosphoramidites were synthesized and modified oligonucleotides I-IV, containing the 2'-amino



SCHEME 1.

group (TABLE 1) were prepared as described previously [8]. Oligonucleotides I and II were used as models and oligonucleotides III, IV as the target products.

Initial aliphatic amino group, localized on a nonnucleoside insert was introduced into the second strand of the target duplex. The corresponding phosphoramidite (8) was synthesized from N-Fmoc-blocked active ester of β-alanine (3) and the dimethoxytrityl derivative of 2-amino-1,3-propanediol (6) (SCHEME 1).

Target compound (8), containing the aliphatic amino group, was directionally introduced at a precisely defined site of the oligonucleotide chain in the process of the standard phosphoramidite procedure. Notice that the design of this nonnucleoside analog retained the three carbon atoms distance (characteristic feature for natural nucleosides) between hydroxyls forming the internucleotide linkage.

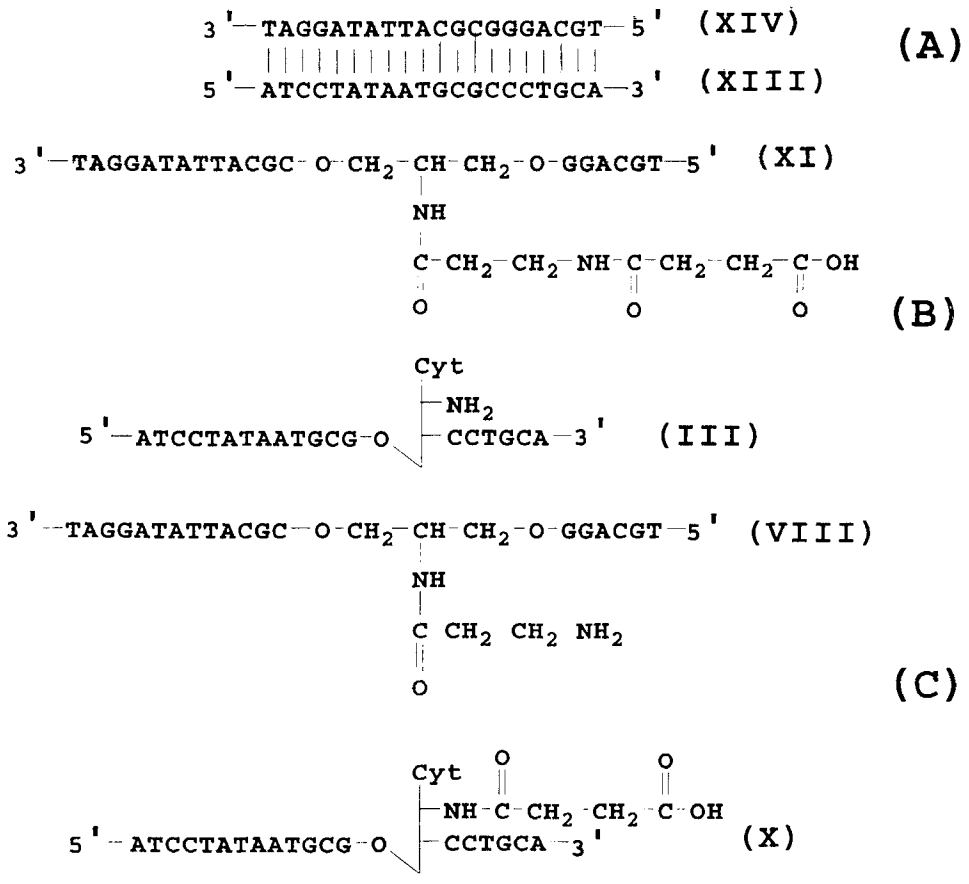
Using compound 8, we obtained five modified oligonucleotides V-IX. Their sequences are given in TABLE 1. Oligonucleotides V-VII were used as models in perfecting techniques for synthesis, isolation and analysis of modified oligonucleotides. Oligonucleotides VIII, IX served as the target products to carry out further experiments towards a covalently linked DNA-duplexes.

Deprotection and isolation of oligonucleotide products were performed according to [8]. Notice that the nonnucleoside insert was introduced twice into oligonucleotide VII. This confirms that multiple incorporation of proposed modified fragments into oligonucleotide structure is possible.

It was found that the primary aliphatic amino group fitted into modified oligonucleotides I-IX is present and highly reactive in reactions with electrophilic reagents. We carried out the reaction with acetic anhydride followed by the ion-pair HPLC analysis of the reaction products as well as the reaction with fluoresceinisothiocyanate (FITC) according to [9], followed by the spectrophotometrical analysis of the products. The spectrum of the product resulting from the reaction with FITC revealed two absorption peaks at 260 and 495 nm (the ratio 6:1) corresponding to the oligonucleotide and fluorescein parts, respectively.

Further the carboxyl group was introduced into modified oligonucleotides II-IV, VI, VIII by the reaction with succinic anhydride [10].

It should be noted that the retention time of the modified oligonucleotides analyzed by ion-pair HPLC varied with the nature of functional groups introduced. Thus the retention time of oligonucleotides with the amino group was less than that of natural oligonucleotides with an analogous primary structure. This was due to the fact that these oligonucleotides had an extra positive charge. The retention time of



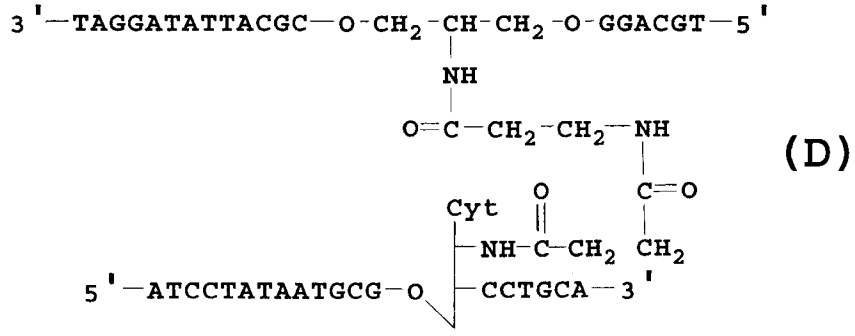
SCHEME 2.

oligonucleotides with the carboxyl group on the contrary was greater owing to an extra negative charge.

Preparation of covalently cross linked duplexes

The modified complementary oligonucleotides obtained III, XI and VIII, X were used to construct two DNA-duplexes each having the amino group in one chain and the carboxyl group in the other (SCHEME 2).

The fundamental difference between these duplexes was that duplex B formed by oligonucleotides III and XI had a free 2'-amino group of



SCHEME 3.

deoxycytidine, whereas duplex C, formed by oligonucleotides VIII and X had a free amino group localized on the nonnucleoside insert.

Water soluble CDI served as a condensing agent in the reaction between chains. The coupling reaction was carried out in a water buffer solution, pH 6.7 for 6 days at 4°C. The reaction products were examined by ion-pair HPLC and 20% PAGE in 7M urea. The yield of the covalently linked duplex D (SCHEME 3) was 2% and 16% for duplexes C and B respectively. This essential difference in the yield of the target product reflects the unique features of the 2'-aminogroup in the deoxynucleoside ribose residue. This group has a much lower pK than the primary aliphatic amino group does (7,4 [8] and 11, respectively). As a consequence, at reaction pH it remains mostly in a free nonprotonated form thus remaining nucleophilic.

We optimized the cross linking reaction conditions using the system of oligonucleotides III and XI to obtain a maximum yield of the target product. The yield of the reaction product grew by the reaction time increasing from 1 to 5 days. The yield of the linked product increased with increasing CDI concentration. Carbodiimide concentration of 0.4 M was taken to be optimal. The yield of the product dropped when shifting reaction pH both to acid and alkaline regions. pH 6.7 was taken to be optimal. The reaction temperature was also varied (see FIG. 1).

Under optimal conditions (20°C, 120h, CDI concentration 0.4M, pH 6.7, concentration of the oligonucleotides 1mM per nucleotide) the

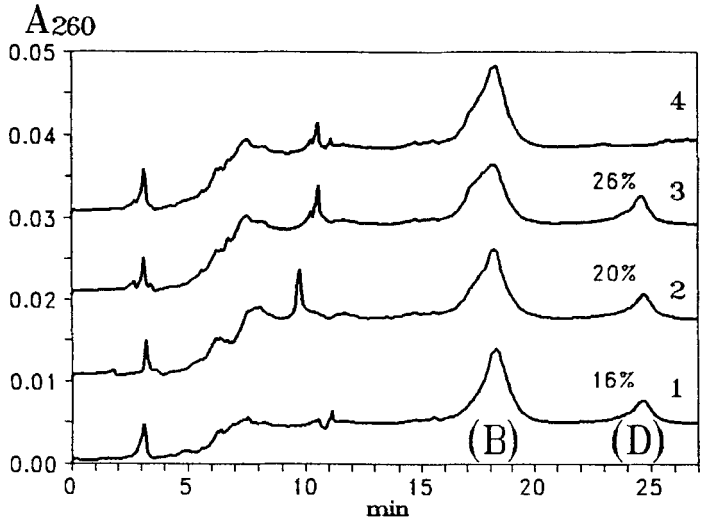


FIG.1. Temperature influence on the yield of the cross linked duplex D. HPLC analysis of reaction mixture under 1 - 4°C, 2 - 10°C, 3 - 20°C, 4 - mixture of oligonucleotides III and XI without CDI.

yield of the linked duplex D (see SCHEME 3) in the reaction between oligonucleotides III and XI was 26% (FIG. 2).

The efficiency of the cross linking reaction using another oligonucleotide duplex obtained from IV and XII having the same type of modifications but higher thermal stability reached 70%.

Properties of the duplex with covalently linked chains

The resulting duplex with covalently linked strands is one of a new unknown class of compounds. Therefore research on its properties is of great interest in determining possible areas of application of oligonucleotide substrates of such structure.

The thermal stability of the cross linked duplex was studied by UV-spectroscopy. Natural duplex A and duplex B were used as standards. Modified duplex B was marked by a decrease in thermal stability as compared to natural duplex A (34°C and 63°C respectively). This fact is

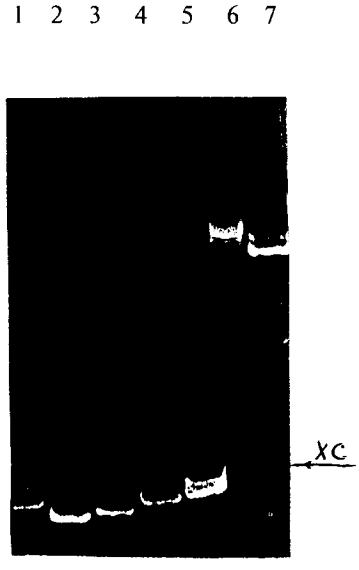


FIG.2. Electrophoresis on the 20% polyacrylamide gel in 7M urea of: nonmodified oligonucleotides: lane 1 - XIII, lane 2 - XIV; modified oligonucleotides: lane 3 - III, lane 4 - XI; lane 5 - modified duplex B; lane 6 - cross linked duplex D (material purified by HPLC); lane 7 - 40-mer oligonucleotide (control).

due to the destabilizing action of the modifications introduced (the decrease of thermal stability by introducing 2'-amino-2'-deoxypyrimidines was shown earlier in [8]), as well as to the elimination of one G-C pair at the site of introduction of the nonnucleoside insert. However the opposite situation occurred with the linked duplex D.

The melting temperature increased considerably (68°C). Its value proved to exceed not only that for the modified duplex B but also that for nonmodified duplex A. The higher Tm of the cross linked duplex D confirmed the presence of covalent linkage between the two strands.

Hydrolytic resistance of the resultant compound to the action of a mixture of snake venom phosphodiesterase (SVP) and alkaline phosphatase was studied under conditions of complete enzymatic hydrolysis (50°C, 180 min) followed by reversed-phase HPLC of the hydrolysate. Under these

conditions natural duplex A and mixture of modified oligonucleotide-precursors III and XI were hydrolysed completely whereas linked duplex D was hydrolyzed by 30% only. This low hydrolysis under such severe conditions is undoubtedly a result of the covalent bond present between the oligonucleotides - components of the linked duplex D.

Thus, the present work describes a synthetic strategy for introducing into oligonucleotides a nonnucleoside insert with an amino group during automatic phosphoramidite method following the standard synthetic procedures. It was shown that the insert retaining the natural length of the sugar-phosphate backbone fragment can be incorporated into any multiply predetermined sites of the oligonucleotide chain.

The method was proposed to synthesize an oligonucleotide duplex with firmly covalently linked strands. The conditions for the reaction between chains were optimized. Under optimal conditions for different duplexes the yield of the target product varied from 26 to 70%.

EXPERIMENTAL

5'-O-(4,4'-Dimethoxytrityl)-N-acyl-2'-deoxynucleoside-3'-O-(β-cyanoethyl-N,N-diisopropylamido)phosphites and controlled pore glass supports derivatized with protected deoxynucleosides were obtained from Applied Biosystems. Fluorescein isothiocyanate, snake venom phosphodiesterase, alkaline phosphatase were purchased from Sigma, β-cyanoethyl-N,N-diisopropylamidochlorophosphite was obtained from Aldrich. N-(9-Fluorenylmethoxycarbonyloxy)succinimide, N'-(3-dimethyl-aminopropyl)-N-ethylcarbodiimid hydrochloride, 2-amino-1,3-propanediol oxalate from Merck were used.

TLC was performed on Kieselgel 60 F_{254} plates (Merck), column chromatography was on Kieselgel 60 (Merck) using one of the following solvent systems: a) $CHCl_3$/EtOH 95:5 (v/v); b) $CHCl_3$/EtOH 9:1 (v/v); c) $CH_2Cl_2/CH_3OH/Et_3N$ 94:5:1 (v/v/v).

NMR spectra were registrated on the spectrometer VXR-400. UV spectra and thermal melting curves were recorded using 150-20 Spectrophotometer (Hitachi, Japan). To determine the Tm values the first derivative was calculated. The melting behaviour of the cross linked

duplexes was determined in the 0.05M MES-buffer (pH 6.7, 0.02M $MgCl_2$).

5'-O-(4,4'-Dimethoxytrityl)-2'-(trifluoroacetamido)-2'-deoxy-uridine-3'-O-(β-cyanoethyl-N,N-diisopropylamido)phosphite and 5'-O-(4,4'-Dimethoxytrityl)-N4-benzoyl-2'-(trifluoroacetamido)-2'-deoxy-cytidine-3'-O-(β-cyanoethyl-N,N-diisopropylamido)phosphite were prepared according to the [8].

N-Fluorenylmethoxycarbonyl-β-alanine (2). N-(9-Fluorenyl-methoxycarbonyloxy)succinimid (0.86 g, 2.55 mmol) was added dropwise under stirring to a solution of β-alanine (1) (0.46 g, 5.16 mmol) in 10 ml of water with 700 μl Et_3N. After 30 min the mixture was concentrated in vacuo and 8 ml 1.5 M HCl was added to the residue. The insoluble material was removed by filtration, washed twice with water and dried under 45°C.

Yield of 2 0.76 g (95%), R_f 0.5 (system a).

N-Fluorenylmethoxycarbonyl-β-alanine-p-nitrophenyl ether (3). p-Nitrophenol (0.40 g, 2.90 mmol) and dicyclohexylcarbodiimide (0.66 g, 3.2 mmol) were added to 2 (0.90 g, 2.9 mmol) dissolved in 5 ml of dry dioxane. The mixture was stirred for 24 h at room temperature. The precipitate was removed by filtration. The filtrate was concentrated in vacuo to dryness.

Yield of 3 1.2 g (95%). R_f 0.8 (system a).

2-amino-1,3-propanediol (4) was prepared from its oxalate salt by calcium hydroxide action. Trifluoracetylation of 4 was carried out according method described in [8]. Tritylation of 2-trifluoroacetamido-1,3-propanediol (5) was carried out using the procedure similar for the nucleosides [11].

1-Dimethoxytrityloxy-2-aminopropanol-3 (6). 1-Dimethoxy-trityloxy-2-trifluoroacetamidopropanol-3 (2.94 g, 6 mmol) was dissolved in 30 ml EtOH and NH_4OH (18 ml) was added dropwise under stirring . The reaction mixture was heated under 45°C during 2 h and solvents were removed in vacuum. The residue was purified by chromatography on a silica gel column eluting with solvent system b to give 1.73 g (73%) 6; R_f 0.25 (system a).

Compound 7 was prepared from 3 (1.2 g, 2.8 mmol) by the reaction with 6 (1.14 g, 0.3 mmol) in dry dioxane (10 ml). The mixture was stirred for 24 h at room temperature. The solvent was removed under reduced pressure, the residue was purified on a silica gel column with solvent system c.

Yield of 7 1.2 g (63%). R_f 0.4 (system b).

^{1}H-NMR ($CDCl_3$, δ): 6.1 (d, 1H, CH-NH-CO, J 7.9 Hz); 5.53 (t, 1H, CH_2-NH-CO, J 6.1 Hz); 4.35 (d, 2H, O-CH_2-CH, J 7.0 Hz); 4.18 (t, 1H, CH_2-CH, J 7.0 Hz); 4.1 (m, 1H, CH_2-CH-CH_2); 3.75 (s, 6H, CH_3-O); 3.7 (2d, 2H, O-CH_2-CH, J_1 11.3 Hz, J_2 4.8 Hz); 3.48 (m, 2H, CH_2-CH_2-NH); 3.3 (2d, 2H, CH-CH_2-OH, J_1 11.3 Hz, J_2 4.0 Hz); 2.41 (m, 2H, CO-CH_2-CH_2).

Phosphitylation of 7 was carried out according [12].

Oligonucleotide syntheses were performed on an Applied Biosystems 380B DNA synthesizer following standard phosphoramidite protocols and cycles using reagents supplied by the manufacture (Applied Biosystems). When modified phosphoramidites are used, a longer coupling time (5-7 min) was employed. The oligonucleotides were normally synthesized in 0.4 mmol scale in the "Trityl-On" mode. Standard deprotection conditions (30% NH_4OH, 55°C, 16 h) were employed.

Analyses of reaction mixtures and isolation of oligonucleotides, containing dimethoxytrityl group, were done by reversed phase HPLC as described earlier [8].

Hydrolytic stability of the resulting compounds to the action of a mixture of SVP and alkaline phosphatase was studied under conditions of complete enzymatic hydrolysis (50°C, 180 min). Oligonucleotides were incubated with a mixture of enzymes in 0.2M Tris-HCl buffer (pH 8.5, with 0.04M $MgCl_2$). Reaction mixtures were analyzed by reversed phase HPLC according [8].

Reactions with FITC for oligonucleotides containing amino group were performed as described [9].

The carboxylation of the amino group in the oligonucleotides was carried out similar to [10]. 1.0 A_{260} units of amino containing oligonucleotide were dissolved in 500 μl of 0.25M sodium bicarbonate buffer (pH 8) and 500 μl of 0.1M succinic anhydride in dry acetonitrile

were added. The mixture was stirred for 3 h at 37°C. The reaction mixture was isolated by gel filtration using a Sephadex G-25 column. The final product was analyzed using reversed phase HPLC in the ion-pair mode on Waters chromatograph (USA), sorbent Diasorb C-16T (7.5 mkm) of ALSICO (Russia), column size 4x250 mm, in logarithmic 5-40% gradient of acetonitrile concentration in 48 mM potassium-phosphate buffer (pH 7.0) in the presence of 2 mM tetrabutylammonium phosphate, flow rate 1 ml/min. The yield of the products resulting from carboxylation of oligonucleotides is 95%.

The cross linking reaction was carried out in 0.05 M MES-buffer (pH 6.7) with 0.02 M $MgCl_2$ (under conditions of CDI-condensing chemical ligation reaction [13]). 0.1 A_{260} units both oligonucleotides were dissolved in 37.5 ml buffer, the mixture was heated to 95°C, then cooled slowly and 37.5 μl 0.4 M CDI in the same buffer was added. After 120.0 h the reaction mixture was analyzed and the final product was isolated by means of the reversed phase HPLC in ion-pair mode as described above.

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1. Abbreviations used: Prefix "d" (deoxy) is omitted in design of 2'-deoxyribonucleosides and oligodeoxyribonucleotides; Fmoc - fluorenilmethyloxicarbonyl; FITC - fluorescein isothiocyanate; CDI - N'-(3-dimethyl-aminopropyl)-N-ethylcarbodiimid hydrochloride.

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