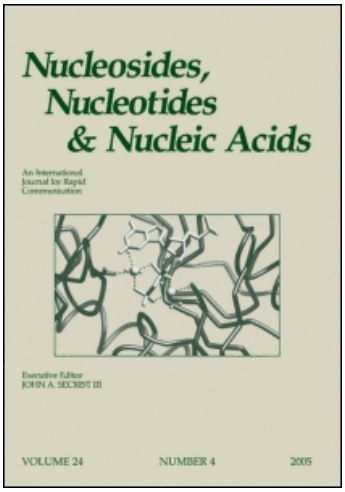

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OLIGODEOXYRIBONUCLEOTIDES WITH INTERNUCLEOTIDIC OR TERMINAL PHOSPHOROTHIOATE GROUPS: DIFFERENT PATHWAYS IN THE REACTION WITH WATER-SOLUBLE CARBODIIMIDE

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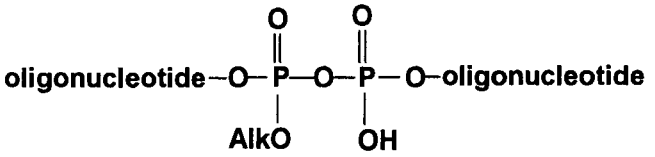
ABSTRACT: Different 'thiophilicity' of water-soluble carbodiimides to sulfhydryl anions in mono- and diesters of phosphorothioic acid was demonstrated. The EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) treatment of oligonucleotides containing phosphorothioate diesters with non-bridging sulfur leads to formation of stable adducts which can be isolated by PAGE or HPLC. In contrast, oligomers with terminal phosphorothioate monoesters form with EDC highly reactive intermediates which react easily with media nucleophiles (*e.g.* water or methanol, phosphomonoester anions).

Water-soluble carbodiimides are widely used in postsynthetic oligonucleotide chemistry, namely, to produce various oligonucleotide conjugates with compounds bearing nucleophilic groups,[1] to ligate oligonucleotides on a complementary template.[1-4] It was clearly shown that natural internucleotidic phoshate groups (phosphodiesters) do not form reactive compounds with water-soluble carbodiimides in aqueous media[4] contrary to terminal phosphates (phosphomonoesters). In the latter case phosphorylisourea is formed which is active not only to strong nucleophiles (such as amines, alcohols), but also to phosphodiester anions or other weak nucleophiles.[5] In our laboratory trisubstituted pyrophosphates of general formula **1** were synthesized by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)-induced ligation of oligonucleotide-p(=O)(OAlk)(OH) and $(OH)_2$p(=O)oligonucleotide on the complementary template.[5,6] These unique compounds have a great potential as highly specific affinity



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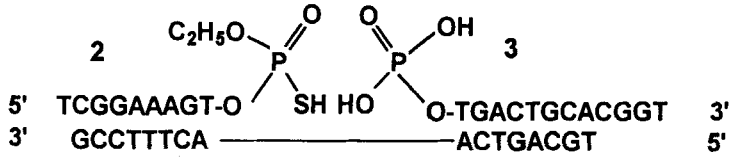

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reagents.[7-9] In an extension of this approach, an effort was made to design species more reactive to protein nucleophiles *i.e.* trisubstituted pyrophosphate containing a sulfur atom instead of a bridging oxygen one: TCGGAAAGTp(=O)(OC_2H_5)-S-p(=O)(OH)-TGACTGCACGGT (prefix «d» (deoxy) is omitted everywhere). Taking into account a higher nucleophilicity of the sulfur atom and that, from purely chemical viewpoint, the reactivity of the phosphorothioate diester group should be only marginally lower than that of phosphate diester[10] we have chosen the same synthetic route: coupling oligonucleotides **2** and **3** on the template **4** by EDC.

In standard chemical ligation conditions (when all three oligonucleotides were incubated overnight with EDC) we got only a new unknown compound **5**. Its electrophoretic mobility was much different from the mobility of the expected 21-mer oligonucleotide (Fig. 1). The same compound **5** was obtained in the control experiments when template or template and oligonucleotide **3** were excluded from the reaction mixture. Moreover, the analogous results were observed in experiments with another oligonucleotide TGGGAATTp(=O)(OC_2H_5)(SH) containing the same 3′-terminal group as in oligonucleotide **1**, as well as with oligonucleotide TGCCGCAGCCACp*G containing one internucleotidic phosphorothioate diester group [p* = -O-P(=O)S^--O-] (FIG. 2). To elucidate the structure of compound **5** we have studied its stability at different conditions (incubation during three days at neutral pH and 3 hours in 0.01 M NaOH at room temperature, 5 min in 15% acetic acid at 95°C; treatment with 0.02 M silver nitrate)

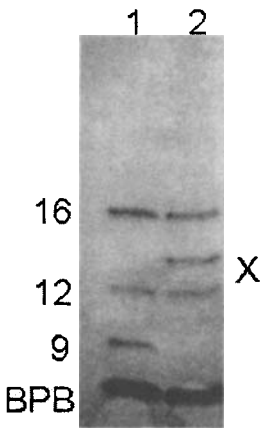


Figure 1. Denaturing PAGE analysis of the reaction mixture containing oligonucleotides **2**, **3** and **4** before (lane 1) and after (lane 2) EDC treatment. See **Experimental** for details. The chain length of oligonucleotides is indicated. X corresponds to new compound **5**. BPB is bromophenol blue marker.

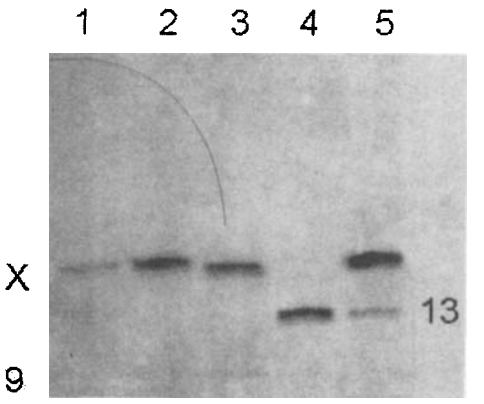
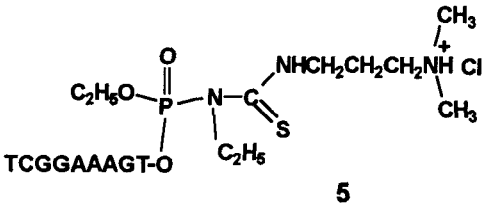


Figure 2. Denaturing PAGE analysis of compound **5** treated with 0.01 M NaOH (lane 2) or 0.02 M silver nitrate (lane 3); control compound **5** (lane 1). Oligonucleotide TGCCGCAGCCACp*G before (lane 4) and after (lane 5) EDC treatment.

(Fig. 2). Compound **5** is stable at neutral and basic pH, stays unchanged after silver nitrate treatment under conditions of P-S bond cleavage,[11] but it transforms to TCGGAAAGTp(=O)(OC_2H_5)(OH) (oxygen-containing analog of oligomer **2**) being subjected to acetic acid treatment under conditions which induce splitting of phosphoramidate bond in oligonucleotide derivatives.[12] This EDC-induced adduct is inert to nucleophilic reagents. Observed mass-spectra data for oligonucleotide **2** and compound **5** (2890.1 and 3047.3 respectively) are very close to expected molecular weights of oligonucleotide **2** and its conjugate with EDC (2886.97 and 3042.2).

All above data and electrophoretic and chromatographic mobilities of compound **5** are consistent with the following structure.



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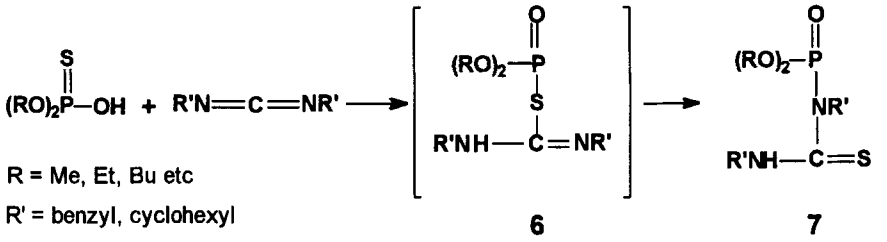
The same regularities were observed when EDC was substituted for another widely used water-soluble carbodiimide, N-cyclohexyl-N'-[β-(N-methylmorpholino)-ethyl]carbodiimide *p*-toluenesulfonate (CMEC).

To our knowledge, the formation of stable phosphoramidate-linked adduct of oligodeoxyribonucleotide with thiourea in aqueous solutions has not been clearly demonstrated earlier. It is known that in non-aqueous media (in ether) dicyclohexyl- or dibenzyl-carbodiimide reacts with O,O-dialkylesters of phosphorothioic acid through formation of S-(O,O-dialkylphosphoryl)isothiourea **6** which rearranges to the thiourea **7**.[13]



We can suppose that the same rearrangement takes place when water-soluble carbodiimides react with sulfhydryl anions in oligodeoxyribonucleotide phosphorothioate diesters. The conjugation of EDC to oligonucleotide (**2**) leads to reduction of total negative charge of molecule on two units (the phosphoramidate group is neutral and nitrogen in dimethylaminopropyl moiety is protonated under conditions used). Incubation of synthetic oligodeoxyribonucleotides containing all or partially thiolated internucleotidic groups (e.g., Tp*Gp*Gp*Gp*Ap*Cp*Cp*Ap*Cp*Cp*Gp*Cp*Gp*Cp*Tp*A-p*Cp*G and TpGpGpGpApCpCpApCp*Cp*Gp*Cp*Gp*Cp*Tp*Ap*Cp*G) with EDC results in a complex mixture of reaction products badly separable by PAGE, their mobilities being lower than that of original species (Fig. 3). Furthermore, the analysis of multimodified compounds obtained was complicated by reduction in their solubility in aqueous solutions.

In contrast, the final product of EDC reaction with oligodeoxyribonucleotide-3'- or -5'-phosphorothioate is not an oligonucleotide-EDC adduct. Oligodeoxy-ribonucleotide-3′-phosphorothioate TCGGAAAGTp(=O)(OH)(SH) was found to transform during

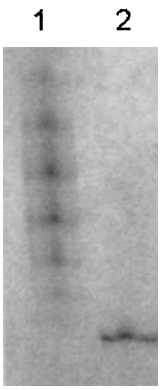


Figure 3. Denaturing PAGE analysis of the reaction mixture containing oligomer TpGpGpGpApCpCpApCp*C-p*Gp*p*Gp*Cp*Tp*Ap*Cp*G before (lane 2) and after (lane 1) EDC treatment.

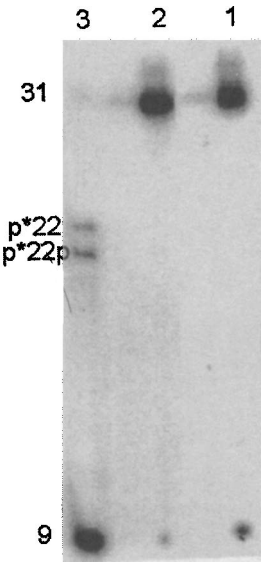


Figure 4. Denaturing PAGE analysis (autoradiogram) of the reaction mixture after EDC-induced nick-sealing in duplex **I** (lane 1) and **II** (lane 2). Control mixture of starting oligonucleotides (lane 3). The chain length of oligonucleotides is indicated. p*22 and *p22p correspond to 5′-^{32}P-labeled 22-mer and 5′-^{32}P-labeled 22-mer containing 3′-phosphate group, respectively.

overnight incubation with EDC to TCGGAAAGTp(=O)$(OH)_2$ (extent of transformation is 85-90%, Fig. 5A). The treatment of TCGGAAAGTp(=O)(OH)(SH) with EDC in 35% methanol leads to formation of corresponding methyl ester with the same yield. For comparison, the oligodeoxyribonucleotide-3'-phosphate TCGGAAAGTp(=O)$(OH)_2$ converts quantitatively to its methyl ester under the same conditions (Fig. 5B).

The comparative template-directed chemical ligation of two couples of oligonucleotides differing in 3′-terminal group facing the nick, phosphorothioate in hairpin duplex **I** and phosphate in identical duplex **II**, was carried out under the action of EDC (Fig. 4). It was demonstrated that the yield of condensation product with pyrophosphate linkage in duplex **I** is only somewhat less efficient (about 95%) than that in duplex **II** approaching 99%.

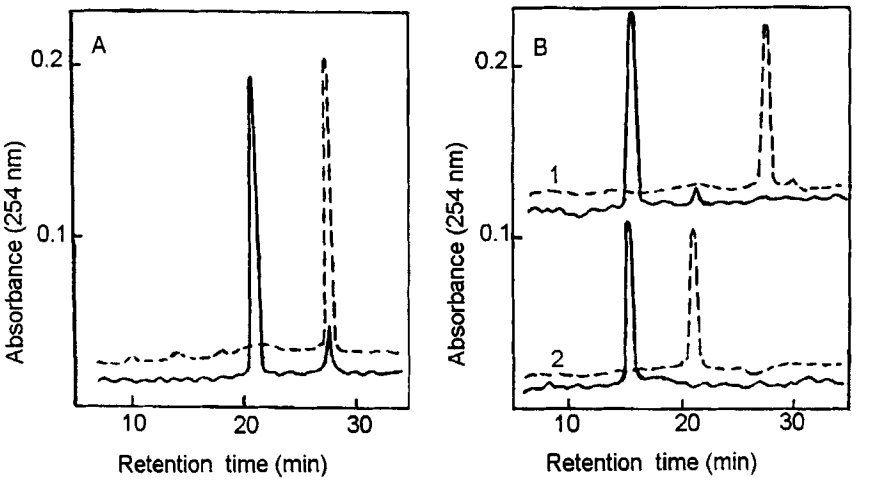
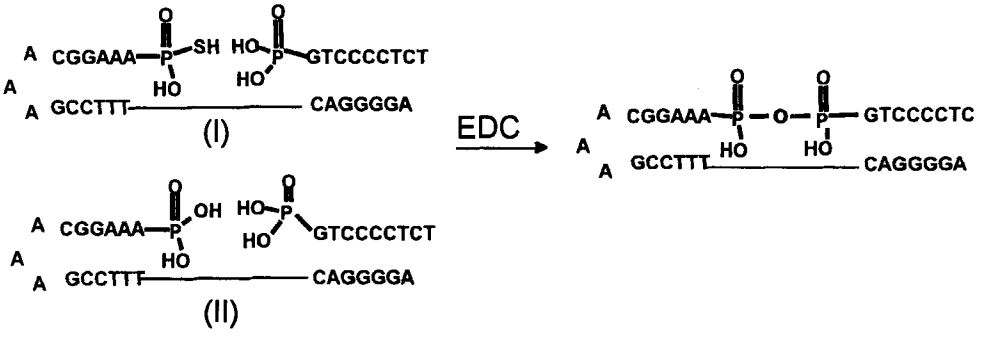


Figure. 5. Ion-exchange HPLC analysis of EDC-induced transformation of oligonucleotide (1) with terminal phosphorothioate group in water solution (A) and in 35% methanol (B, profile 1). For comparison, the same reaction in 35% aqueous methanol was performed for oxygen-containing analog of oligomer (1) with terminal phosphate group (B, profile 2). Initial oligomer (------), post-reaction mixture (———). See **Experimental** for details.

Summarizing above facts, we can propose that under the action of EDC on oligodeoxyribonucleotide-3′-phosphorothioate highly reactive phosphorylisothiourea intermediate is formed initially, it being only somewhat less active than corresponding phosphorylisourea derivative. Further, this intermediate interacts fast with media nucleophile (*e.g.* water or methanol, phosphomonoester anions) without rearrangement to stable phosphoramidates as do phosphorothioate diesters. This conclusion is supported by the data describing CMEC-induced template chemical ligation of GGTAGAGCGTTACCT and (HS)(HO)(p=O)AGGAAGCGCAAGGCCC which leads to formation of new phosphodiester bond.[14]



It worthwhile mentioning here that different behaviour of comparable oligonucleotide sulfhydryl anions in relation to other reagents was demonstrated earlier. 2,2'-Dipyridyldisulfide or Ellman's reagent forms S-S adducts with sulfhydryl anions of terminal 3'- or 5'-phosphorothioate monoesters[15-17] but not with Tp*Gp*Gp*Gp*Ap*-Cp*Cp*Ap*Cp*Cp*Gp*Cp*Gp*Cp*Tp*Ap*Cp*G or other oligodeoxyribonucleotide phosphorothioate diesters as we specially tested (data not shown). Phosphorothioate monoesters interact strongly with mercury (II) in contrast to internucleotidic phosphorothioate diesters which interact poorly.[10] On the other hand, alkylation of sulfhydryl anions of terminal phosphorothioate monoester and internucleotidic phosphorothioate diester, for example, by bromobimane, showed that the former reacted only marginally faster.[16]

In summary, we have demonstrated in this work that in aqueous media carbodiimides are thiophilic to internucleotidic and terminal phosphorothioates but the final reaction products are different. The EDC- and CMEC- treatment of oligonucleotides containing phosphorothioate diesters with non-bridging sulfur leads to formation of stable adducts which can be isolated by PAGE or HPLC. We think that this fact can be used for analysis of oligodeoxyribonucleotides with restricted numbers of internucleotidic phosphorothioates as well as for the synthesis of stable adducts of these oligonucleotides with substances containing carbodiimide moiety.

Experimental

Synthesis of oligodeoxyribonucleotides. Oligodeoxyribonucleotides were synthesized on a DNA synthesizer (Applied Biosystems 380B). Assembly of oligonucleotides was performed using standard phosphoramidite chemistry. Phosphorothioate groups were introduced by oxidation of corresponding phosphite with 1% solution of Beaucage thiolating reagent in acetonitrile as described.[18] Full-length oligonucleotides were purified using reverse-phase HPLC followed by dialysis.

Modification of oligodeoxyribonucleotide with phosphorothioate diesters by water-soluble carbodiimides. 0.1 - 10 nmol of oligonucleotide (2) or oligonucleotide containing internucleotidic phosphorothioate linkages was incubated in buffer 1 (0.05 M MES adjusted with triethylamine to pH 6.0, 0.02 M $MgCl_2$) with 0.2 - 0.3 M EDC or

0.15 M CMEC at 0-4°C during 16-48 hours. After ethanol precipitation, stable adduct was isolated using denaturing PAGE and UV shadowing.

Analysis of oligonucleotide (1) - EDC adduct *NaOH treatment.* Gel purified compound **5** (0.1 - 10 nmol) was dissolved in 10 µl of 0.01 M NaOH and incubated at room temperature for 3 hours. Than 1 µl of 1% acetic acid was added and oligonucleotide material was isolated by standard ethanol precipitation.

Acetic acid treatment. Compound **5** dissolved in 10 µl of 15% acetic acid was incubated at 95°C for 5 min. After adding of 8 µl of 2 M NaOH oligonucleotide material was isolated by standard ethanol precipitation.

Silver nitrate treatment. 5-10 nmol of compound **5** dissolved in 110 µl of 0.02 M silver nitrate was incubated at 30°C for 1 hour in the dark. Than 65 µl of 0.1 M DTT were added and oligonucleotide material from supernatant was precipitated by ethanol. Reaction products were analyzed by 20% denaturing PAGE; UV shadowing was used to visualize the oligonucleotide bands.

EDC-induced chemical ligation of oligonucleotides in duplexes I and II. Hairpin and linear (containing 5'-^{32}P label) oligonucleotides were combined in a 1.5:1 ratio and dissolved in buffer 1 to 0.1 mM (per monomer residue) oligonucleotide concentration. After annealing procedure, EDC was added to give a final concentration of 0.3 M. The reaction mixture was incubated overnight at 0-4°C in the dark. The oligonucleotide material was precipitated by adding 2 % $LiClO_4$ in acetone and analyzed by electrophoresis on denaturing 20% gel. The coupling yield was determined after autoradiography of wet gel and scintillation counting of cut bands.

EDC-induced conversion of TCGGAAAGTp(=O)(OH)(SH) in water and water-methanol solution. Oligonucleotide (1-10 nmol) was dissolved in buffer 1 or in buffer 1 containing 35 % methanol. 0.3 M EDC was added and resulting mixtures was incubated during 16-48 hours at 0-4°C in dark. After ethanol precipitation, reaction products were analyzed by ion-exchange HPLC. on a SCOUT WP PEI column (5 µm, 4.6 x 50 mm, J.T. Barker), flow rate 1.0 ml/min, potassium phosphate gradient (0.005 →0.5 M in 60 min), pH 7.0 in 25% acetonitrile.

Oligonucleotide **2** and compound **5** were characterized by MALDI-TOF mass detection on a VISION 2000.

Acknowledgments

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