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SOLID-PHASE SYNTHESIS OF MODIFIED OLIGODEOXYRIBONUCLEOTIDES WITH AN ACRIDINE DERIVATIVE OR A THIOPHOSPHATE GROUP AT THEIR 3'END

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Summary : Use of a derivatized support involving the 2,2'-diethyldithio-group 7 allows the automated synthesis of oligodeoxyribonucleotide bearing acridine derivative (via nucleoside-3'-acridinylphosphoramidite 3) or 3'phosphorothioate group (including the sulfurization step for attachment of the first nucleoside to the support).

Introduction

Oligodeoxyribonucleotides covalently linked to an acridine derivative via a polymethylene linker interact specifically and strongly with the complementary sequences (1-2). A few of those bearing the intercalating agent at their 3'end, have recently been demonstrated to inhibit, in vitro or in cell culture, the expression of previously chosen genes (3).

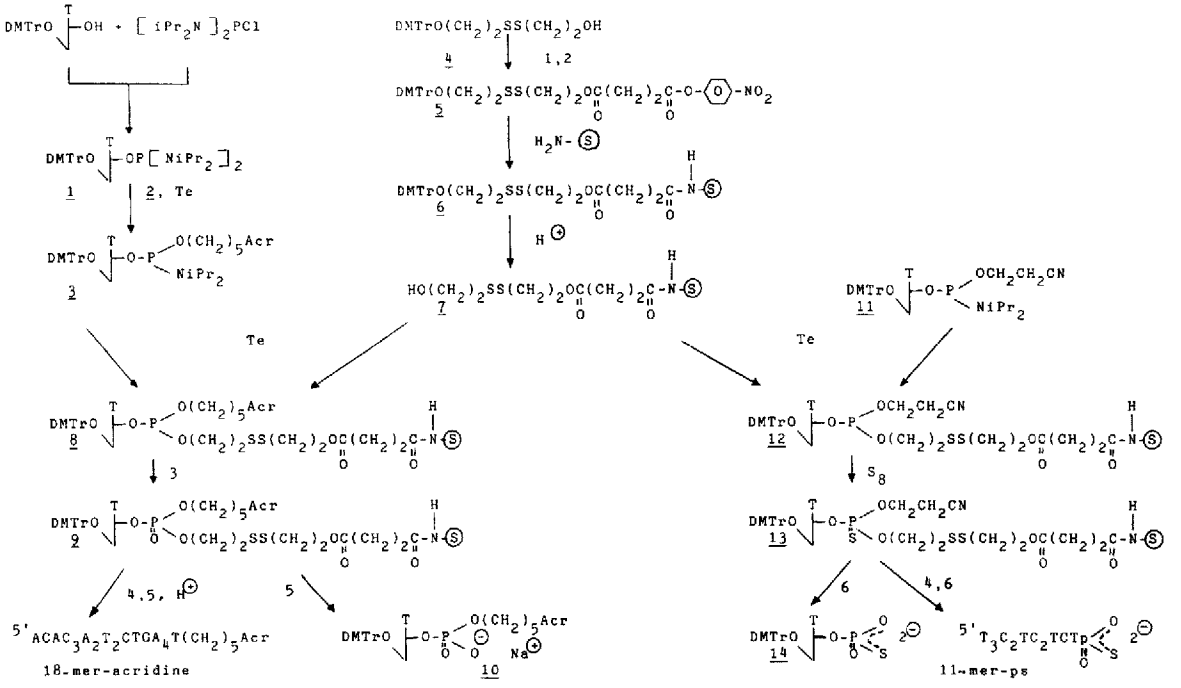
In order to induce irreversible damage to the complementary nucleic acid sequence, another set of oligodeoxyribonucleotides bearing various chemically or photochemically reactive groups has been developed (3). Many of these reactive groups are not stable under the chemically reactive conditions required for deprotection of oligodeoxyribonucleotides and need to be coupled with unblocked oligonucleotides via a thiophosphate group. Until the present time, preparation of these two families of molecules has been carried out by the phosphotriester method in solution (4,5). In order to easily obtain a great number of various sequences, we have automatized the synthesis of these molecules. We report here the solid-phase preparation of an oligodeoxyribonucleotide covalently linked through its 3'-phosphate to 2-methoxy-6-chloro-9-pentylaminoacridine (18 mer-acridine) and an oligodeoxyribonucleotide bearing a phosphorothioate group at its 3' end (11-mer-(ps)).

Results : The key step in the preparation of the 18-mer involving the 2-methoxy-6-chloro-9-pentylaminoacridine at its 3' end consists in the synthesis of a nucleoside phosphoramidite bearing acridine derivative 3 which was then reacted with the hydroxyl of 2,2'-dithiodiethanol attached to support 7 (Scheme I). Synthesis of the 11-mer with a 3' end phosphorothioate function was carried out by coupling the same support 7 with 5'-O-dimethoxytritylthymidine-3'-(β-cyanoethyl) diisopropylamidophosphite followed by the addition of sulfur to the intermediate phosphite 12 to obtain the nucleotide 3' thiophosphate derivatized support 13 (Scheme I). The elongation chain was then carried out for both modified oligonucleotides by the phosphoramidite procedure (6) on an automatic solid-phase DNA synthetizer using commercially available 5'-O-dimethoxytrityl-N-acyl-2'deoxyribonucleoside-3'-(β-cyanoethyl) diisopropylamidophosphites.

A) Preparation of 5'-O-dimethoxytritylthymidine-3'-[2-methoxy-6-chloro-9-ω-pentylamino] acridine-diisopropylamidophosphite 3 was carried out by a two-step procedure from bis(diisopropylamido) chlorophosphite (7). To a dry solution of 5'-O-dimethoxytritylthymidine (1 equiv) and triethylamine (1 equiv.) in dry dichloromethane, bis(diisopropylamido) chlorophosphite (1 equiv.) was added at 0°C under nitrogen atmosphere. The reaction mixture was gradually warmed to room temperature and stirred for 10 min. The phosphitylation reaction was monitored by tlc analysis [Rf1 = 0.68 solvent A, (8)]. When the reaction was completed the reaction mixture was diluted with ethylacetate in the presence of triethylamine and washed with 10 % aqueous

sodium carbonate and with saturated aqueous sodium chloride. After the organic phase was dried over anhydrous sodium sulfate, and evaporated under reduced pressure, the residue was purified by flash chromatography on silica gel to give the bis diisopropylamidophosphite derivative 1 (85%). Reaction of 1 and 2-methoxy-6-chloro-9-(ω-hydroxy-pentylamino) acridine 2 (1 equiv.), in the presence of 1 H tetrazole in anhydrous CH_3CN followed by the usual work up and flash chromatography on silica gel gave the acridinyl derivative 3 (80 %) [Rf 2 = 0.33 solvent B, Rf 3 = 0.43 solvent B (8)]. Dithiodiethanol derivatized solid-phase 7 was obtained from Fractosil 500 [(0.063-0.125 mm) commercially available from Merck] according to the described procedure (9) by replacing dimethoxytritylnucleoside with 2-dimethoxytrityl-2,2'-dithiodiethanol 4. This method involves the synthesis of p-nitrophenylester 5 of the 2-dimethoxytrityloxy-2'-succinylethyl-disulfide and the subsequent condensation with primary aminopropyl derivatized silica gel [tlc, Rf 4 = 0.66 solvent C, Rf 5 = 0.84 solvent C (8)]. Determination of the amount of dimethoxytrityl cation released by acidic treatment of a sample of support 6 showed a loading of approximately 55 μmol per gram of the dithiodiethanol derivative. After capping of the unreacted amino groups (9) and detritylation, the reaction of the free hydroxyl group on derivatized "Fractosil 500" 7 with 3 (20 % excess) in the presence of 1 H tetrazole (6) in anhydrous CH_3CN showed a persistent yellow color. Support 8 was then treated with I_2 in CH_3CN/H_2O/collidine (65:6:30) to give the oxidized support 9. This derivatized support has a loading of approximately 25 μmol of the monomer bearing acridinyl derivative per gram. Further treatment with another sample of activated phosphoramidite 3 did not increase the loading of the support. Capping of the unreacted hydroxyl groups was carried out under the described procedure (9).

Scheme I



2=Acr$(CH_2)_5$OH=2-methoxy-6-chloro-9-ω-hydroxypentylamino acridine ; DMTr=dimethoxytrityl, Te=tetrazole ; T=thymidine. iPr=Isopropyl ; H_2N-Ⓢ=aminopropyl-Fractosil 500. 1=succinic anhydride, 4-dimethylaminopyridine, pyridine. 2=p-nitrophenol, pyridine, dicyclohexylcarbodiimide, dioxane. 3=iodine in CH_3CN/H_2O/collidine (65:6:30,V/V). 4=elongation of the oligodeoxyribonucleotide chain (10). 5:dithiotreitol (0.1 M) and NaOH (0.4 M) in H_2O/CH_3CN 4:1 V/V. 6:dithiotreitol (0.1M) in concentrated NH_4OH.

Treatment of solid support 9 with a solution of dithiotreitol (0.1 M) and NaOH (0.4 M) in H_2O/CH_3CN 4:1 V/V for 20 min gave an ionic product involving nucleotide and the fluorophore of acridine, which was identified by tlc through comparing to a sample of 5'-O-dimethoxytritylthymidine-3'- [2-methoxy-6-chloro-9-ω-pentylamino] acridine phosphate 10 [Rf10 = 0.52 solvent C (8)]. After detritylation, the obtained compound was submitted to nuclease digestion [treatment with endonuclease P1 from Penicillium citrinum followed by alkaline phosphatases degradation] which gave two products identical to thymidine and 2-methoxy-6-chloro-9-ω-hydroxypentylaminoacridine 2. Preparation of an 18-mer acridine [5'ACACCCAATTCTGAAAAT$(CH_2)_5$ Acr] was carried out, using a 1 μmole scale on support 9 and 12 μmol of nucleosidephosphoramidite per cycle with a cycle time of 9.1 min (10), with the dimethoxytrityl efficiency per cycle of about 98.3 %. The efficiency of the first coupling step was not altered with regard to that commonly obtained with commercially available derivatized supports. The deblocking of the polynucleotide was achieved by treatment with a solution of 0,1 M dithiotreitol and 0.4 M NaOH in H_2O/CH_3CN 4:1 V/V. This one-step process allows the removal of classical protective groups (β-cyanoethyl from phosphates and acyl from nucleic bases) together with the cleavage of the disulfide bond by dithiotreitol (11) and the elimination of the β-mercaptoethyl group (12-14). The choice of using NaOH instead of concentrated aqueous ammonia was based upon the stability of the acridine derivative in the presence of NaOH at pH >10 (4). After being released from the support and *full deprotection, the crude 18-mer-acridine was chromatographed on a Polyanion HR* 5/5 column (Fig. 1a). The major product with the highest retention time was collected, desalted and analyzed by HPLC on a reverse phase column C18 (Fig. 2a). Enzymatic digestion by endonuclease P_1 from Penicillium citrinum and alkaline phosphatases gave the expected results.

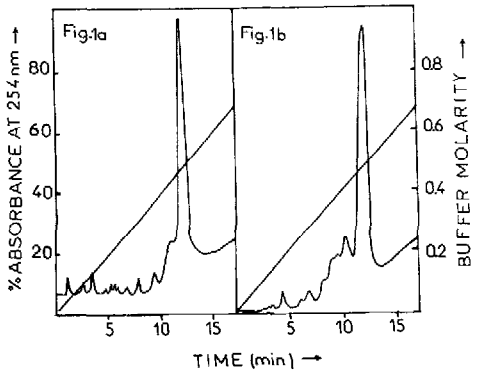


Fig.1: FPLC profiles of crudes 18-mer-acridine: Retention time = 11 min 30 sec (Fig. 1a), and 11 mer-(ps): Retention time = 12 min 15 sec. (Fig 1 b) on a Polyanion HR 5/5 column (Parmacia) ; flow rate of 1 ml/min, eluted with a linear gradient from 0 to 60 % in 15 min of 10^{-3} M KH_2PO4 (pH=6) and 1 M KH_2PO4, (pH6).

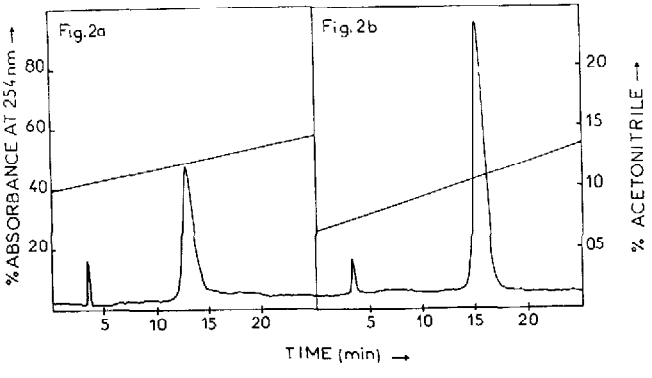


Fig.2: Analysis by HPLC Column Lichrosorb RP 18 (diam 7μmm) Merck L 250 mm, diam 4 mm. Flow rate 1.2 ml/min with a linear gradient of acetonitrile in 0.12 M ammonium acetate buffer, pH 5. Fig 2a: From 10 % to 14.37 % acetonitrile in 25 min (Retention time 18 mer Acr = 13 min 20 sec). Fig. 2b: From 5.3 % to 12.17 % in 25 min (Retention time 11-mer-(ps) = 15 min 45 sec).

B) Synthesis of oligodeoxyribonucleotide bearing a phosphorothioate group at its 3' end was carried out by reaction of phosphoramidite 11 (1.5 equiv.) with derivatized support 7 (1 equiv.) in the presence of 1H tetrazole (2 equiv.) in CH_3CN for 5 min, followed by a sulfurization step by S_8 in CS_2/pyridine mixture for 30 min. Determination of the amount of the dimethoxytrityl group showed a loading of approximately 50 μmol of the nucleoside derivative per gram. After capping of the unreacted hydroxyls an analytical amount of the derivatized support 13 was treated with dithiotreitol 0.1 M in concentrated NH_4OH (16 h at room temperature) to eliminate both the β-cyanoethyl and the β-mercaptoethyl groups. The final product 14 was identified by

tlc through comparison of the same product obtained by the previously described procedure (5) [Rf 14=0.59 solvent D (8)]. This compound contained the trityl and phosphorothioate groups (red-colored spot with 2,6-dibromoparabenzoquinone-N-chloroimine DBPNC). Sequential growth of the oligonucleotide chains was completed by the routinely used phosphoramidite method including the oxidation step by I_2 in CH_3CN/H_2O/collidine. Using a 3 μmole scale on derivatized support 13 and 12 μmole of phosphoramidite per cycle (10), the 11-mer-(ps) 5'TTTCCTCCTCT(ps) was obtained with a dimethoxytrityl efficiency per cycle of about 96.2 %. After being released from the support and fully deblocked by treatment with a mixture of dithiotreitol 0.1 M and concentrated NH_4OH overnight at room temperature, the crude product was chromatographed on a polyanion HR 5/5 column (Fig. 1b). The major component with the highest retention time was collected, desalted and analyzed by HPLC on a reverse phase column C18. Chromatogram (Fig. 2b) showed only one peak. The obtained compound was that expected with the phosphorothioate group (positive test with DBPNC).

Conclusion

These results show the ability of the described derivatized support to synthesize oligodeoxyribonucleotides which are covalently linked to an acridine derivative through their 3'-phosphate or which carry a thiophosphate group at their 3' end. The conditions required for the cleavage of the disulfide bridge and for the elimination of the resultant β-mercaptoethyl group are compatible with those necessary for full deprotection of these modified oligodeoxyribonucleotides. The development of this method with the others nucleic bases is now in progress.

Acknowledgements

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References and Notes

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