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Solid Phase Synthesis of 5', 3'-Bifunctional Oligodeoxyribonucleotides Bearing a Masked Thiol Group at the 3'-End

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Key Words: bifunctional oligonucleotides; solid phase synthesis; 3'-thiol; 5'-thiophosphate; 5'-acridine.

Abstract: Immobilization of the "disulfide arm" 8 on a solid support via a succinyl linkage allows the automated synthesis of modified oligonucleotides bearing a disulfide or a thiol function at the 3'-end and other modifications at the 5'-end.

Synthetic oligonucleotides have been successfully used to control gene expression in various systems (see Ref 1, 2). Two major limitations to the potential use of antisense oligonucleotides as therapeutic agents are their poor penetration into cells and their susceptibility to degradation by nucleases. To increase the uptake of oligonucleotides by cells, we decided to couple sequences bearing a thiol group with a proteic carrier *via* a disulfide bridge.

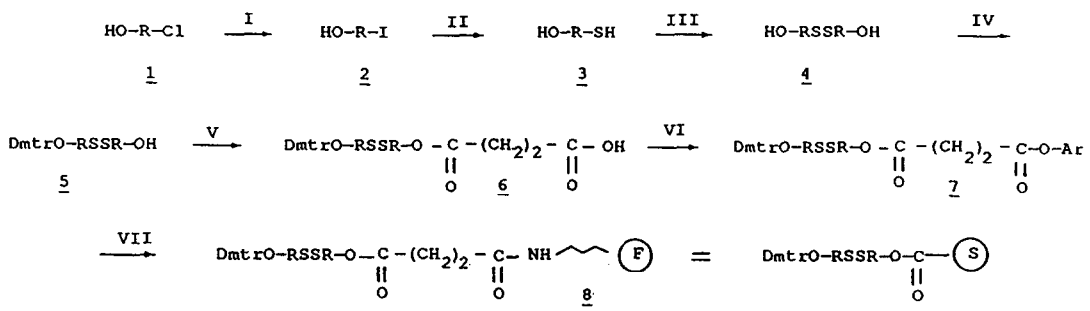
In order to follow these compounds during *in vitro* and *in vivo* experiments, oligonucleotides were modified with an acridine ring or a fluorescein residue by reaction of a sequence bearing a thiophosphate group with iodoacetamidofluorescein. Since the coupling of an acridine residue or a thiophosphate function is easier at the 5'-end than at the 3'-end of an oligonucleotide, the masked thiol group was introduced at the sequences' 3'-end *via* a derivatized solid support.

Solid phase synthesis of 3'-thiol (SH) oligonucleotides involves the preparation of four different polymer supports[3]. More recently, a solid support for the universal synthesis of 3'-thiol containing oligonucleotides has been described[4]. The release of the oligonucleotide from the support was carried out by treatment with dithiotreitol (DTT) leading to the SH form sequence.

We report here a universal solid support allowing the automated synthesis of oligonucleotides containing a 3'-disulfide function and a 5'-acridine or a thiophosphate group. The latter group can be alkylated with iodoacetamidofluorescein. These sequences may then be converted into oligonucleotides containing a 3'-thiol function after purification.

The preparation of the solid support 8 consists in coupling the activated ester of the "disulfide arm" 7 (Scheme 1) with the aminopropyl derivatized Fractosil 500 *via* an amidic linkage. The *p*-nitrophenyl ester 7 was obtained in 6 steps from 2-[2-(2-chloroethoxy) ethoxy] ethanol 1 (Scheme 1). Chloride 1 (1 eq) was converted into the 2-[2-(2-iodoethoxy) ethoxy] ethanol 2 [Rf= 0.58][5] by reaction with NaI (5 eq) in the presence of $NaHCO_3$ in acetone at 60°C for 20 h. Iodine was then displaced by NaSH (1 eq) in methanol to give the 2-[2-(2-mercaptoethoxy) ethoxy] ethanol 3 [Rf= 0.51][5] which dimerized into 4 [Rf= 0.36][5] in the presence of air oxygen. This "disulfide arm" 4 was then dimethoxytritylated at

one end by 4,4'-dimethoxytritylchloride (0.25 eq) in pyridine to give **5** [Rf= 0.66][5]. The *p*-nitrophenyl ester **7** [Rf= 0.9][5] was prepared as described for the 5'-dimethoxytrityldeoxynucleoside derivative[6] by succinylation of the hydroxyl function of **5** with succinic anhydride followed by coupling the carboxylic acid of **6** with *p*-nitrophenol in the presence of DCCI. The reaction of the activated ester **7** with the aminopropyl functions of the derivatized Fractosil 500 gave the support **8**. The amount of dimethoxytrityl cation released by acidic treatment showed a loading of 70 μmol/g. Underivatized NH_2 functions of the support **8** were capped by acetylation with acetic anhydride[6].



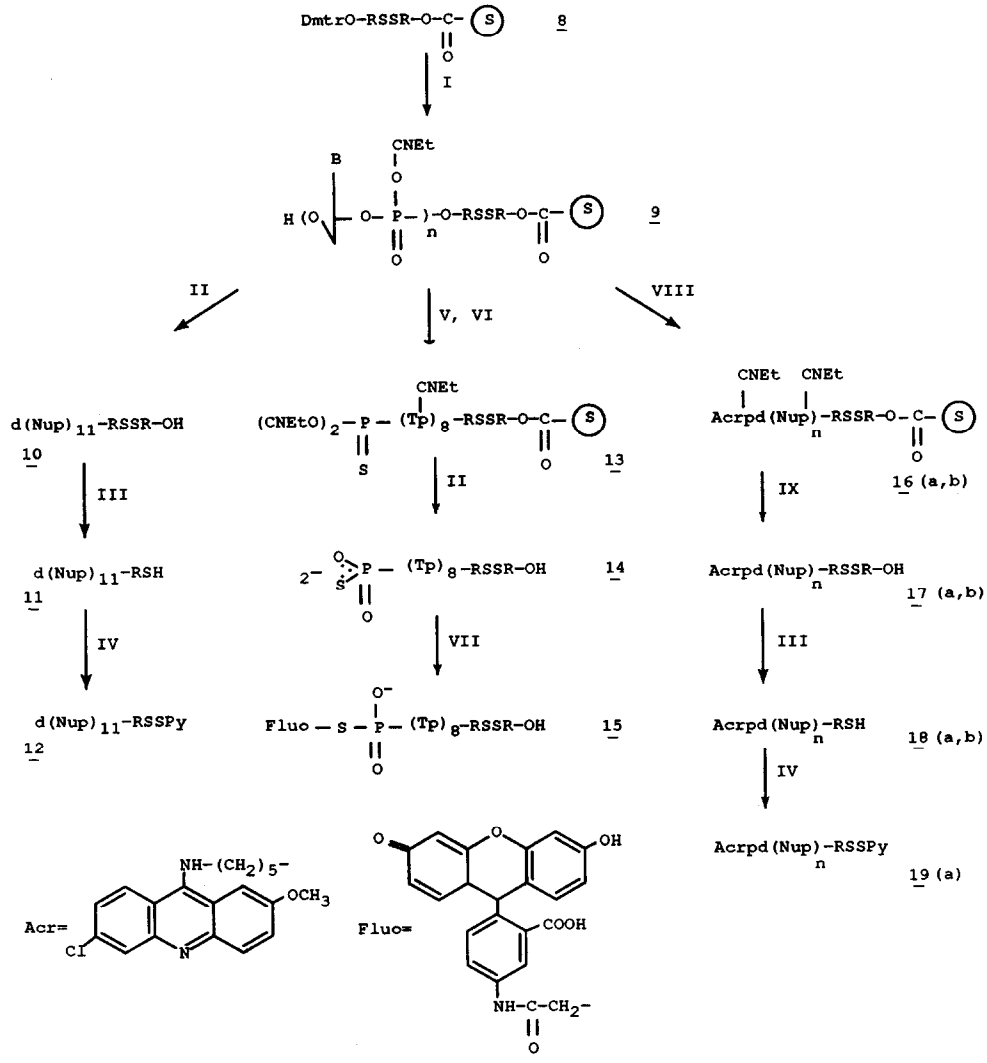
Scheme 1: Ar= 4-nitrophenyl; R= $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)-$; I: NaI, $NaHCO_3$, acetone; II: NaSH, MeOH; III: O_2; IV: DmtrCl, pyridine; V: succinic anhydride, 4-dimethylaminopyridine, dioxane; VI: *p*-nitrophenol, DCCI; VII: H_2N~~~(F) = aminopropyl-Fractosil 500.

Using support **8** and the classical phosphoramidite procedure[7], the assembly of the oligonucleotide chain was performed on 2 and 10μmol scales in a DNA synthesizer to afford the protected oligomer which was detritylated to give **9** (Scheme 2). The synthesis of a model 11 mer d^5'(TTTCCTCCTCT)$^{3'}$-RSSR-OH was first carried out to test the support **8**. Treatment of **9** with concentrated ammonia led to the 11 mer **10** bearing the disulfide tail at the 3'-end. Figures 1a and 1b show the ion-exchange chromatogram of crude 11 mer **10** [Rt= 8.7 min] and the reverse phase chromatogram of purified 11 mer **10** [Rt= 4.9 min], respectively. This disulfide was kept during purification to avoid oligonucleotide dimerization. The disulfide bridge was then reduced with DTT to give the 11 mer 3'-thiol **11** [Rt= 2.5 min, Fig 1b] which was converted into the 3'-thiol activated group **12** [Rt= 6.8 min, Fig 1b] by reaction with the 2,2'-dithiodipyridine. This modified oligonucleotide released 1 mol of 2-pyridine thione by mol of 11 mer after treatment with DTT.

We then synthetized the bifunctional octathymidilate 5'-thiophosphate 3'-disulfide **14**. The bis (cyanoethyl) thiophosphate group was introduced at the 5'-end of **9** as described[8]. The modified sequence **13** was deprotected with concentrated ammonia and purified by ion exchange chromatography in its 3'-disulfide form **14** [Rt= 5.6 min][9a]. The resulting oligonucleotide 5'-thiophosphate reacted with iodoacetamidofluorescein to give the 5'-fluorescein octathymidilate-RSSR-OH **15** [Rt= 11.7 min][9a].

Other bifunctional sequences ^{5}'Acrpd$(Tp)_8$-RSSR-OH$^{3'}$ **17a** and 19 mer ^{5}'Acrpd(AAGCTTTATTGAGGCTTAA)p-RSSR-OH$^{3'}$ **17b**) bearing the 3'-disulfide bridge and an acridine group were synthetized. The intercalant agent was coupled at the 5'-terminus of the oligonucleotides *via* its phosphoramidite as described[10]. Modified octathymidylate **16a** and 19 mer **16b** were then deprotected by treatment at room temperature with NaOH 0.5 M in H_2O/ CH_3OH for 1 h and 15 h respectively. Sodium hydroxide was used in order to avoid the acridine elimination. The $^{5'}$Acrp$(Tp)_8$-RSSR-OH$^{5'}$ **17a** was purified by ion exchange chromatography and then analyzed on a reverse phase column [Rt= 6.1 min][9b]. The disulfide function was reduced by DTT and the resulting 3'-thiol function of **18a** [Rt= 5.3 min][9b] was converted into the 3'-dithiopyridinyl derivative **19a** [Rt= 12.0 min][9b] with 2,2'-dithiodipyridine. The deprotection step of the

19 mer **16b**, longer than that of derivative **16a**, led to a mixture of three products: two nonadecamers bearing a 3'-disulfide bridge **17b** [Rt= 9.2 min][9c] or a 3'-thiol function **18b** [Rt= 7.8 min][9c] and a 38 mer [Acr-d(AAGCTTTATTGAGGCTTAA)p-RS-]$_2$ [Rt= 8.1 min][9c] (**18b** dimer) due to a partial rupture of the disulfide tail in 0.5 M NaOH . Treatment of the mixture with DTT gave exclusively the oligonucleotide **18b**.



Scheme 2: B= Protected nucleic base; Dmtr= dimethoxytrityl; C= cytidine; CNEt= cyanoethyl; d= deoxy-; Nu'= protected nucleoside; Nu= nucleoside; p= phosphate; Py= pyridinyl; T= thymidine; R= -$(CH_2)_2$-O-$(CH_2)_2$-O-(CH_2)-; a: d(Nup)$_n$= (Tp)$_8$; b: d(Nup)$_n$= d(AAGCTTTATTGAGGCTTAA); d(Nup)$_{11}$= d(TTTCCTCCTCT); I: elongation of the oligonucleotide chain, trityl off; II: concentrated NH_4OH; III: DTT 1M in 0.1 M acetate buffer 0.1M NaCl; IV: 2,2'-dithiodipyridine; V: bis (cyanoethyl) diisopropylamidophosphite, tetrazole; VI: S_8 in CS_2/pyridine; VII: iodoacetamido-fluorescein; VIII: acridine-phosphoramidite, tetrazole, I_2, H_2O; IX: NaOH 0.5 M in MeOH/H_2O.

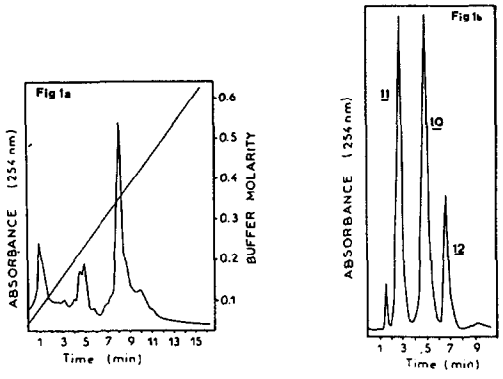


Figure 1: a) Ion-exchange HPLC chromatogram of crude d(TTTCCTCCTCT)-RSSR-OH **10**; column Polyanion HR 5/5 (Pharmacia); flow rate 1 ml/min; eluant 10^{-3} M KH_2PO_4 (pH= 6) and 1 M KH_2PO_4 (pH= 6) linear gradient from 0 to 80% of 1 M KH_2PO_4 in 20 min. b) Reverse phase HPLC profile of d(TTTCCTCCTCT)-RSSR-OH **10**, d(TTTCCTCCTCT)-RSH **11** and d(TTTCCTCCTCT)-RSSPy **12**; column Licrosorb RP-18 (5 μm) Merck 3 X 150 mm; flow rate 1 ml/min; eluant 0.1 M ammonium acetate buffer pH 7 containing 9.6% acetonitrile.

The solid support described above allows the synthesis of bifunctional oligodeoxynucleotides, carrying a disulfide group at their 3'-ends, which are easily purified. The presence of the masked 3'-thiol function permits the coupling of a 5'-thiophosphate group with an alkylated derivative. The 3'-thiol function is then liberated by reduction with DTT and can be used to couple the oligonucleotides with proteins *via* a disulfide bond . These results will soon be published in another publication.

Acknowledgements

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References and Notes

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9. Reverse phase chromatography: a) column Licrosorb RP-18 5μm, 3 X 150 mm (Merck); flow rate 1 ml/min; buffers A: ammonium acetate 0.76%, CH_3CN 17% (pH= 6.9); B: ammonium acetate 0.87%, CH_3CN 24% (pH= 6.9); elution 50% of B; b) column Hibar Licrosorb RP-18, 7μm, 4 X 250 mm (Merck); flow rate 0.8 ml/min; elution 70% of B ; c) column Licrospher 100 RP-18 7μm, 4 X 125 mm (Merck); buffers C: trietylammonium acetate 0.1 M, CH_3CN 5% (pH= 7); D: trietylammonium acetate 0.1 M, CH_3CN 80% (pH= 7); linear gradient from 10 to 50% of D in 30 min.
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