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SOLID-PHASE PREPARATION OF 5',3'-HETEROBIFUNCTIONAL OLIGODEOXYRIBONUCLEOTIDES USING MODIFIED SOLID SUPPORTS

Ulysse ASSELINE, Edwige BONFILS, Robin KURFURST, Marcel CHASSIGNOL, Victoria ROIG and Nguyen Thanh THUONG*

Centre de Biophysique Moléculaire, C.N.R.S., 1A, Avenue de la Recherche Scientifique 45071 - ORLEANS Cedex 2, France

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Abstract : The solid-phase preparation of oligodeoxyribonucleotides attached to intercalator or reactive groups through their 5'- and (or) 3'-ends is reported. These syntheses implicate the introduction of suitable masked functional groups at the 5'-end of the oligonucleotide by the intermediate of their phosphoramidite derivatives or at the 3'-end of the oligonucleotide using modified solid supports. After full deblocking, the functional groups (phosphate, thiophosphate, primary amine or thiol) can be reacted with the suitable reactive group involved in the chosen ligand. These methods allow the preparation of heterobifunctional derivatized oligodeoxyribonucleotides.

Since the Watson-Crick base pairing discovery, a fundamental property for the replication and inheritance of the genetic information in DNA and its transcription into RNA and translation into proteins, there has been an increasing use of modified oligodeoxyribonucleotides. Oligodeoxyribonucleotides tagged with various ligands have been widely used as research tools for genetic analysis, to assign gene function and to elucidate mechanisms in molecular biology[1,2]. In addition to their use as hybridization probes[3], they have been used in diagnostic procedures[4]. Synthetic oligodeoxyribonucleotides carrying a reporter group have had widespread use for automated sequencing[5-7], fluorescence microscopy[8] and hybridization affinity chromatography[9]. For the past decade, antisense oligodeoxyribonucleotides covalently linked to intercalators, chain cleavers or alkylating agents have been proven efficient as gene expression regulators[10,11]. These systems can be improved using oligodeoxyribonucleotides substituted by multiple molecules of the same[12-16] or different reporters[17], reactive groups[18] or different molecules such as an intercalator and reactive group at each end of the oligodeoxyribonucleotide[19,20]. In general the functionalization of the oligonucleotides for substituting intercalator, reporter or reactive groups can be achieved using the following methods: a) modified nucleosides containing a masked primary amino group on the heterocyclic base are incorporated into the oligonucleotide during the synthesis[21-27]; b) suitably protected chemical moities are coupled at the 5'-[28-32] or 3'-terminus[32] of the protected oligonucleotide; c) deprotected oligonucleotides are functionalized through their 5'-phosphate which can be

*To whom to address correspondence

introduced by enzymatic reactions[33-35]. Another way to attach a substituent group at specific sites in an oligonucleotide is to use suitably functionalized phosphate backbone such as phosphoramidate[36-38] or phosphorothioate[39-40] analogues. Recently, site-specific functionalization of oligonucleotides at two internucleotide linkages using a phosphorothioate analogue and a phosphoramidate substituted by a free primary amino-containing linker has been reported[17].

In most cases substitutions carried out at the nucleic bases level decrease the hybridization properties of the modified oligonucleotide either by steric hindrance of the substituent group or by modification occurring at one atom involved in the base pair recognition process. In the same way substitution at the level of an internucleotide phosphate introduces chirality at the phosphorus atom. Two oligonucleotide diastereoisomers were obtained possessing different hybridization stabilities.

Previous studies carried out with oligodeoxyribonucleotides covalently linked to an acridine derivative at various positions of the oligodeoxyribonucleotide chain have shown that maximum stabilization occurred when the substitution was performed at the termini of the oligomers[41]. For these reasons we focused our work on the solid-phase preparation of oligonucleotides covalently linked through their 5'-or 3'-end to various intercalator, chemically or photochemically reactive groups. In some cases we introduced the substituent group during the oligonucleotide synthesis[42-44]. But some of the reactive groups used in our studies were either difficult to protect or unstable under the chemical conditions required for the synthesis and deprotection of the oligodeoxyribonucleotide and thus needed to be coupled to the unblocked oligomer.

In this way masked functional groups were incorporated into the oligonucleotide during the chain assembly. After the deblocking step these groups were reacted with the appropriate ligand. A great deal of approaches have been reported in the 5'-end modifications of the protected oligonucleotides at the chain elongation end on solid support. Various functional groups such as the following were used: primary amino via a linker[28,45-50], phosphate[46,51-55], phosphorothioate[32,53,55], thiol via a linker[51,56], and carboxy via a linker[57]. The direct incorporation of the 5' substituent group[15] using various phosphorylating procedures (phosphotriester[46,52], phosphoramidite[49], phosphonate[48,49] or carbamate linkage formation[47]) was also described.

Solid-phase modifications of the oligonucleotide 3'-end are more difficult to develop and need the preparation of a modified solid support. Only a few examples of 3'-phosphate containing oligonucleotide preparations have been reported[59-61]. One procedure introducing a primary amino group at the 3'-end via a short and branched linker[62] and two methods used to obtain a 3'-thiol containing oligonucleotide[63,64] via modified supports were described.

RESULTS AND DISCUSSION

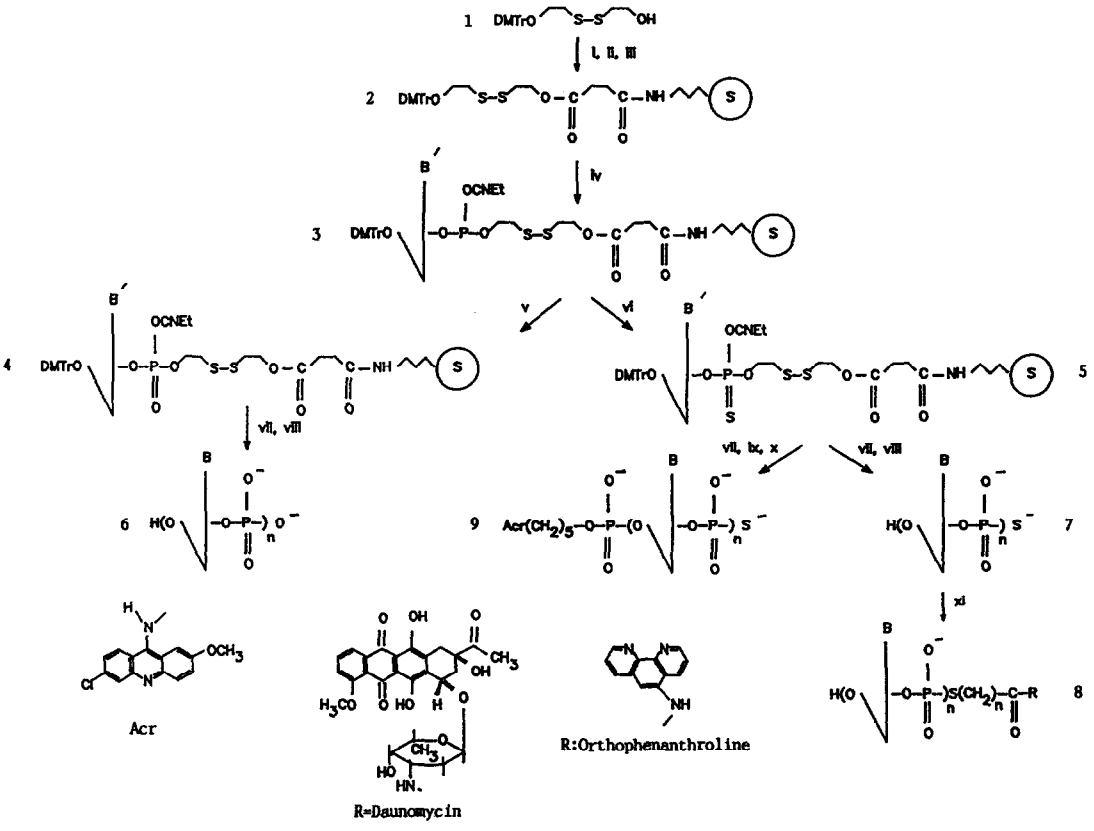
We report here the use of modified solid supports briefly described in our preliminary work which enabled us to obtain oligodeoxyribonucleotides substituted at their 3'-ends by various functional groups such as phosphate[58], phosphorothioate[65], primary amino via a linear aliphatic linker[66], thiol via an oligoethylene glycol arm[67]. The use of these modified supports which involve a disulfide bond stable under acidic and alkali medium opens the possibility of combining a great number of modifications at the nucleotide chain level and also of obtaining oligonucleotides substituted by two different ligands at their 5' and 3'-ends.

Oligodeoxyribonucleotide-3'-phosphate

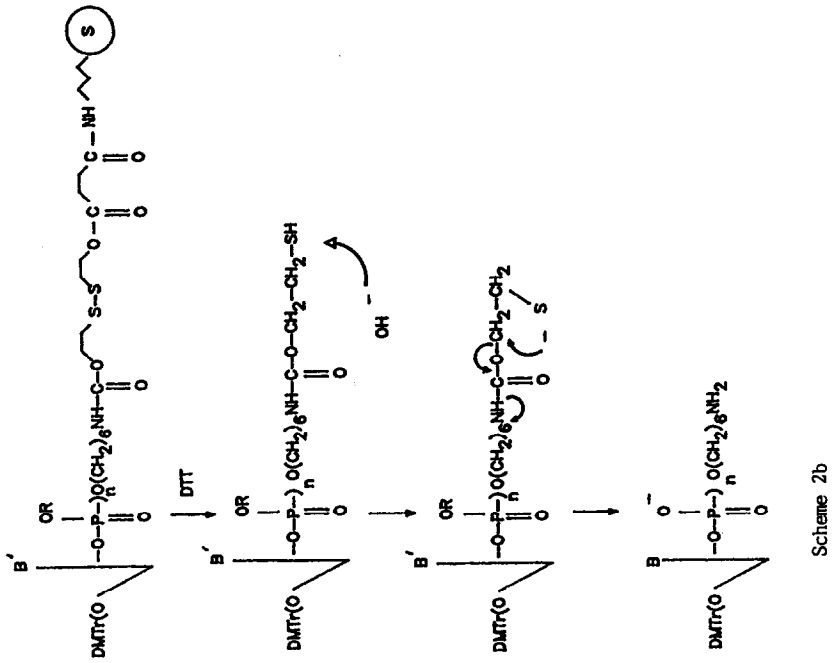
The first system consists in the preparation of a modified support involving the 2,2'-dithiodiethyl group **2**. This was achieved from fractosil 500 [(0.063-0.125 mm) commercially available from Merck] as described in ref[68] by replacing 5'-dimethoxytrityl nucleoside with 1-dimethoxytrityl-2,2'-dithiodiethanol **1**. The use of this support allows us to directly obtain, after the assembly of the oligonucleotide chain via the classical phosphoramidite chemistry and the deprotection steps, oligodeoxyribonucleotides bearing a phosphate group at their 3'-end **6** (Scheme 1). The deprotection procedure using a mixture of dithiothreitol and concentrated ammonia (or sodium hydroxide 0.4 N) allows the cleavage of the disulfide bridge followed by the elimination of ethylene sulfide (scheme 2a) together with the removal of the cyanoethyl group from phosphates and the acyl groups from the nucleic bases[65]. Ion exchange analysis of the crude deblocked oligonucleotide 3'-phosphate d(TTTTCTTTTCCCCCCT)p **6b** shows essentially one main peak (Figure 1) (Rt = 22 min 22 sec) [which is higher than that of the unmodified oligomer d(TTTTCTTTTCCCCCCT) previously prepared (Rt = 19 min 7 sec)]. Other modified supports used to obtain oligonucleotide-3'-phosphate, involving either a β-sulfone linker[60,61] or a ribonucleoside moiety[59] (which can be oxidized with $NaIO_4$ followed by a β-elimination reaction) are more difficult to carry out because the former is unstable under alkali conditions and the latter needs the protection of the 2'-OH and an oxidation step.

Oligodeoxyribonucleotide-3'-phosphorothioate

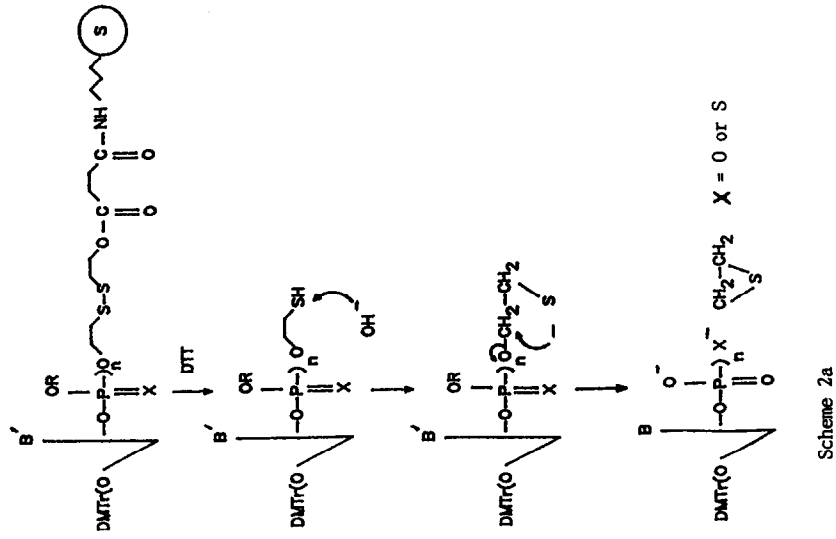
Starting from the support above-described involving the 2,2'-dithiodiethyl group **3**, the replacement of the iodine oxidation step of the first nucleoside-3'-phosphite attached to the support **2** by a sulfurization step leads to the nucleoside-phosphorothioate support **5** (Scheme 1). This step is performed mannually in order to control the loading which is approximately 40-50 μmol of deoxyribonucleoside-3'-phosphorothioate per gram.



Scheme 1 : DMTr = Dimethoxytrityl ; (S) -NH-=aminopropyl-Fractosil 500 ; B'=protected nucleic base ; B=Nucleic base ; CNEt=2-cyanoethyl ; i = succinic anhydride, 4-dimethylaminopyridine, pyridine ; ii = p-nitrophenol, pyridine, dicyclohexylcarbodiimide, dioxane ; iii = aminopropyl Fractosil 500, NEt_3, DMF ; iv = 5'-O-dimethoxytrityl-N-acyl-2'-deoxynucleoside-3'-O-(2-cyanoethyl)-diisopropylamidophosphite, tetrazole ; v = iodine in CH_3CN/H_2O/collidine (65-6-30, V/V); vi = S_8 in CS_2/Pyridine (50/50 V/V) ; vii = elongation of the oligodeoxyribonucleotide chain ; viii = dithiothreitol (0.1 M) in concentrated ammonia and acetic acid ; ix : [2-methoxy-6-chloro-9(ω-pentylamino)acridinyl]-(2-cyanoethyl)-diisopropylamidophosphite/tetrazole and I_2 in CH_3CN/H_2O/collidine (65-6-30, V/V) ; x = dithiothreitol (0.1 M) and NaOH (0.4 M) in H_2O/CH_3CN (4:1 V/V) ; xi = $Br(CH_2)_5C(O)$ Daunomycin or $ICH_2C(O)$ Orthophenanthroline.



Scheme 2b



Scheme 2a

The treatment of an amount of the support **5** with a solution of dithiothreitol (0.1 M) in concentrated ammonia releases the 5'-dimethoxytrityl-deoxynucleoside-3'-phosphorothioate as the sole product. The chain elongation is then performed using the classical phosphoramidite procedure including the oxidation step by iodine. After full deprotection by successive treatment with dithiothreitol in concentrated ammonia and acetic acid, ion exchange analysis of the crude oligomer d(CACACCGACGGC)ps **7b** in Mono P column (Figure 1) shows only one main peak (this compound containing the phosphorothioate group gives a pink-colored spot on TLC silica plates by spraying DBPNC followed by heating). After desalting, reverse-phase analysis on C18 column of the major component give a single peak (Rt= 11 min 6 sec syst A, 5 % to 50 % CH_3CN in 30 min). The 3'-phosphorothioate 20-mer 5'd(TCATCCACCTGGCATTGGAC)ps **7c** was also obtained with a yield of 50 %. These results show that the loss of the sulfur atom during the oxidation step is very low.

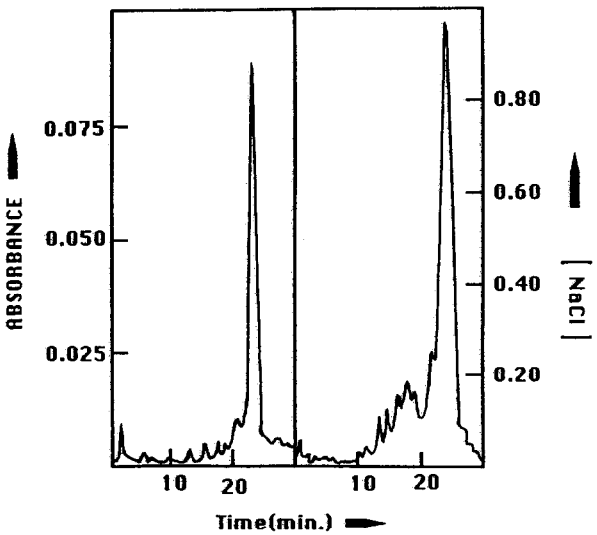


Figure 1 : Elution profile on anion exchange column (Mono P HR 5/5 from Pharmacia) of crude 5'd(TTTTCTTTTCCCCCCT)p **6b** (left) and 5'd(CACACCGACGGC)ps **7b**(right). Elution was performed using NaCl (0 to 1 M) in NaH_2PO_4 0.01 M buffer pH 6 in the presence of acetonitrile 20 %, with a flow rate of 1 ml/min.

The phosphorothioate containing oligonucleotides **7** can then be reacted in water, various mixtures of water-organic solvent, or even directly in organic medium (in the presence of crown ether to solubilize the oligodeoxyribonucleotide) with halogenoalkyl ligands [$Br(CH_2)_5$ C(0)Daunomycin, $ICH_2C(0)$Orthophenanthroline] to afford the 3'-conjugated oligodeoxyribonucleotides : 5'd(TTTCCTCCTCT)ps$(CH_2)_5$C(0)Daunomycin **8a**, 5'd(TTTCCTCCTCT)psCH_2C(0)Orthophenanthroline **8b**, 5'd(CACACCGACGGC)psCH_2C(0)Orthophenanthroline **8c** and 5'd(CACACCGACGGC)ps$(CH_2)_5$C(0)Daunomycin **8d**.

This reaction of S-alkylation occurs without any activation in a wide pH range. Usually only excess of a few equivalents of the halogenoalkyl compound are required. After incubation times varying from a few to 24 hours the yield for the coupling reaction is nearly quantitative. Reverse-phase analysis of the crude mixture obtained by coupling the oligomer **7b** with $Br(CH_2)_5C(0)$Daunomycin shows a main peak corresponding to the 3'-conjugated oligomer **8d** which has a higher retention time (Rt=15 min 27 sec) than that of 3'-phosphorothioate **7b**(Rt=9min 33 sec) (Figure 2). The absorption spectrum of the compound **8b** (Figure 3) exhibits the expected absorbance radio at $\lambda \simeq 260$ nm and $\lambda \simeq 500$ nm in accordance with the published value of 10,000 M^{-1} cm^{-1} for the λ max of the daunomycin [69]. It must be noted that, when the daunomycin is linked to the oligonucleotide, a slight red-shift of the absorption maxima is observed.

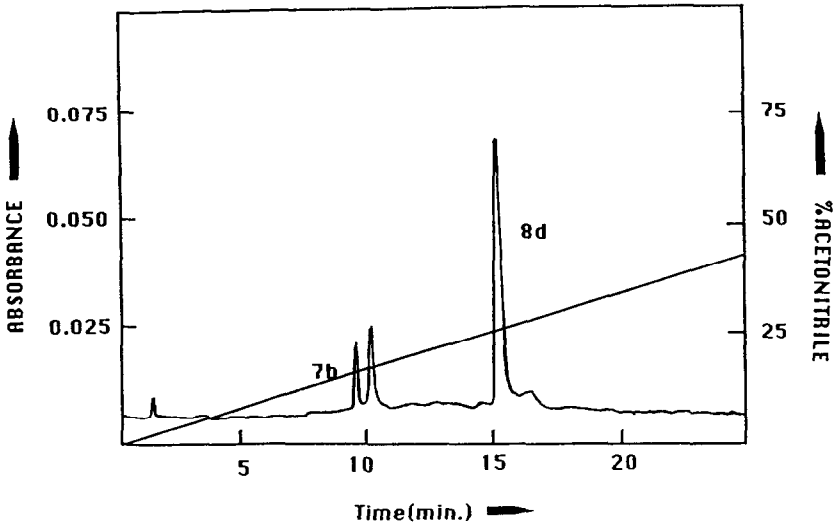


Figure 2 : Reverse phase analysis on a lichrospher 100 RP 18 (5 µm) column (125 mm x 4 mm) (using a linear gradient of CH_3CN in 0.1 M aqueous triethyl ammonium acetate, pH 7, with a flow rate of 1 ml/min) of the crude mixture obtained after coupling of 5'd(CACACCGACGGC)ps **7b**: Rt=9min.33 sec with Daunomycin-C(0)$(CH_2)_5$Br, 5'd(CACACCGACGGC)ps$(CH_2)_5$C(0)Daunomycin **8d**: Rt=15 min-27 sec. A side-product with Rt = 10 min. 10 sec. was identified to [5'd(CACACCGACGGC)ps]$_2$.

Another product can sometimes be observed, beside the starting oligodeoxyribonucleotide-3'-phosphorothioate **7** and the expected oligodeoxyribonucleotide-conjugate (Figure 2). This compound was identified to the corresponding oligodeoxyribonucleotide phosphorothioate dimer $[5'd(CACACCGACGCC)ps]_2$ obtained by disulfide bond formation. Treatment of the latter with dithiothreitol leads to the starting material **7b**.

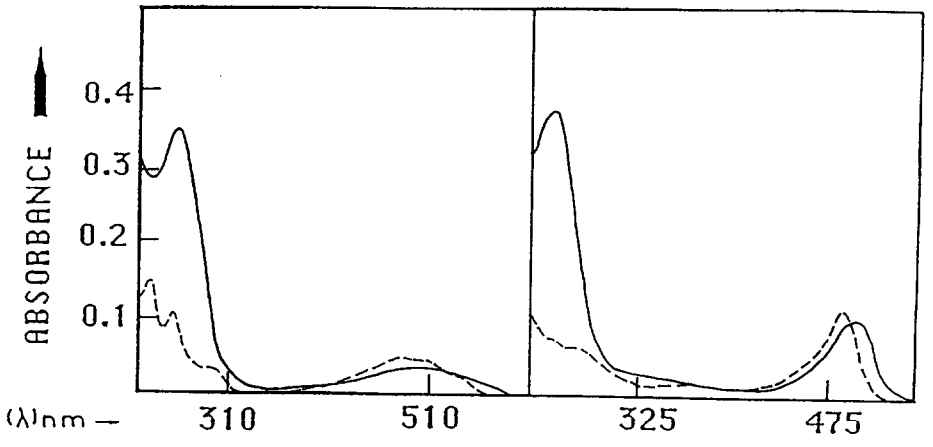
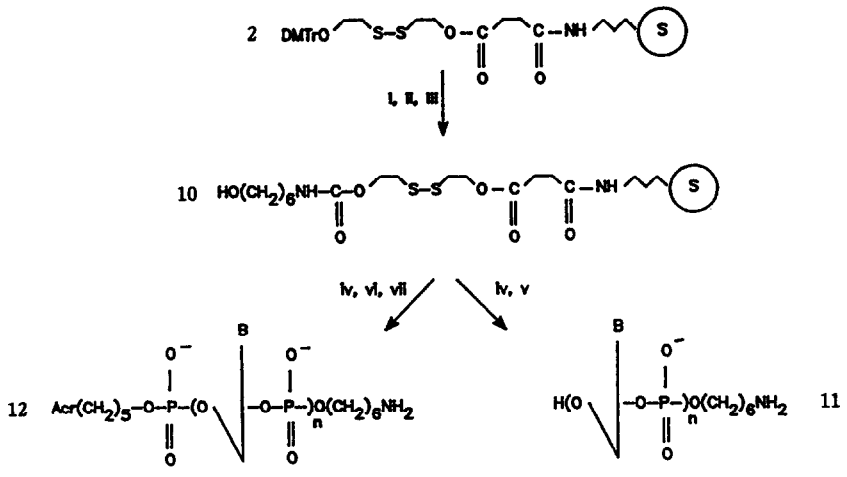


Figure 3 : Absorption spectra of compounds **8d** : 5'd(CACACCGACGGC)ps$(CH_2)_5$C(0)Daunomycin (——) and Daunomycin (---) in water solution (left) and **23** : FluoresceinC(0)CH_2spd^5(AAGCTTTATTGAGGCTTAA)-p$CH_2CH_2(OCH_2CH_2)_2SS(CH_2CH_2O)_2CH_2CH_2OH$ (——) and Fluorescein (---) in hydrogen sodium carbonate buffer 0.1 M pH 9 (right).

Oligodeoxyribonucleotide-3'-aminoalkyl

The 2,2'-dithiodiethyl modified support **2** can be further derivatized in order to obtain oligodeoxyribonucleotides involving a 3'-linker with terminal primary amino group **11** and **12** (scheme 3). This method consists in the immobilizing of an aliphatic amino-alcohol on the 2,2'-dithiodiethyl derivatized support via a carbamate linkage to afford the support **10** carrying a hydroxyl group.



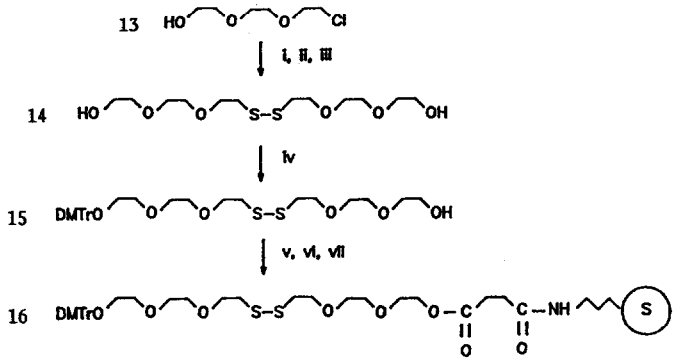
Scheme 3 : (S)-N(H)-=aminopropyl - Fractosil 500 ; i = $H^{\oplus}$; ii = 1,1'-carbonyldiimidazole, dioxane ; iii = $H_2N(CH_2)_6OH$, iv = elongation of the oligodeoxyribonucleotide chain ; v = dithiothreitol (0.1 M) in concentrated ammonia and acetic acid ; vi = [2-methoxy-6-chloro-9(ω-pentylamino)acridinyl]-(2-cyanoethyl)-diisopropylamidophosphite/tetrazole, I_2 in CH_3CN/H_2O/collidine (65-6-30, V/V); vii = dithiothreitol (0.1 M) and NaOH (0.4 M) in H_2O/CH_3CN (4:1 V/V).

The preparation of the carbamate linkage containing support **10** was performed by a two-step procedure. First the hydroxyl group of 2,2'-dithiodiethyl containing support **2** was activated by treatment with 1,1'-carbonyldiimidazole to give the imidazolide derivative which was then reacted with 6-amino-1-hexanol. After the chain elongation, the deprotection procedure was carried out by successive treatments with dithiothreitol in concentrated ammonia and acetic acid to give the compounds d(TTTCCTCCTCT)p$(CH_2)_6NH_2$ **11a** and d(TCATCCACCTGGCATTGGAC)p$(CH_2)_6NH_2$ **11b**. The DTT-NH_4OH treatment allows both the cleavage of the disulfide bridge followed by ethylenesulfide and CO_2 elimination (scheme 2b), to afford the free amino group together with the removal of the cyanoethyl group from the internucleotide phosphate and the acyl groups from the nucleic base.

Oligodeoxyribonucleotide-3'-thiol

The preparation of 3'-modified oligodeoxyribonucleotides with phosphate, phosphorothioate or amino linker needs the use of modified supports involving the 2,2'-diethyldisulfide linker which can be easily eliminated during the unblocking process. Contrary to the above-mentioned modifications, the preparation of oligodeoxyribonucleotides modified at their 3'-end with a thiol linker needs the use of a longer chain to avoid ethylene sulfide elimination [29]. This is why we chose the ω, ω'-dithiodi-[2-[2-(2-ethoxy) ethoxy] ethyl] linker **14**, which moreover possesses hydrophilic property.

The synthesis of the disulfide support **16** was achieved according to Scheme 4. The 2-[2-(2-chloroethoxy)ethoxy]ethanol **13** (commercially available) was first transformed into an iodinated derivative by sodium iodide treatment, then converted into a thiol derivative by reaction with sodium hydrogen sulfide in methanol. The thiol compound was dimerized into the disulfide derivative **14** in the presence of air. The latter was dimethoxytritylated at one end to afford compound **15** which was then immobilized on Fractosil 500 support by using the same procedure as for the preparation of the 2,2'-dithiodiethyl derivatized support described above.



Scheme 4 : DMTr = Dimethoxytrityl ; (S) -NH-=aminopropyl Fractosil 500 ; i = INa, $NaHCO_3$, acetone ; ii = NaSH, MeOH ; iii = diluted ammonia pH 8, air ; iv = dimethoxytritylchloride, pyridine ; v = succinic anhydride, 4-dimethylaminopyridine, pyridine ; vi = p-nitrophenol, pyridine ; dicyclohexylcarbodiimide, dioxane ; vii = aminopropyl Fractosil 500, NEt_3/DMF.

Starting from the modified support involving the ω,ω'-disulfide linker **16** (scheme 5) the chain elongation was performed using the classical phosphoramidite procedure. The fully protected oligodeoxyribonucleotides were then unblocked by ammonia treatment which afforded the oligonucleotides bearing the disulfide arm at their 3'-ends d(TTTCCTCCTCT)p $CH_2CH_2(OCH_2CH_2)_2SS(CH_2CH_20)_2CH_2CH_2OH$ **17a** and d(AAGCTTTATTGAGGCTTAA)p$CH_2CH_2(OCH_2CH_2)_2$S-$S(CH_2CH_20)_2CH_2CH_2OH$ **17b** easily purified by liquid chromatography.

The oligomer-3'-disulfide **17a** and **17b** were then reduced by treatment with aqueous dithiothreitol to afford the 3'-thiol oligomers d(TTTCCTCCTCT)p$CH_2CH_2(OCH_2CH_2)_2SH$ **18a** and d(AAGCTTTATTGAGGCTTAA)p$CH_2CH_2(OCH_2CH_2)_2SH$ **18b** which respectively have shorter retention time, on reverse phase chromatography, than the corresponding disulfide **17a** and **17b.**

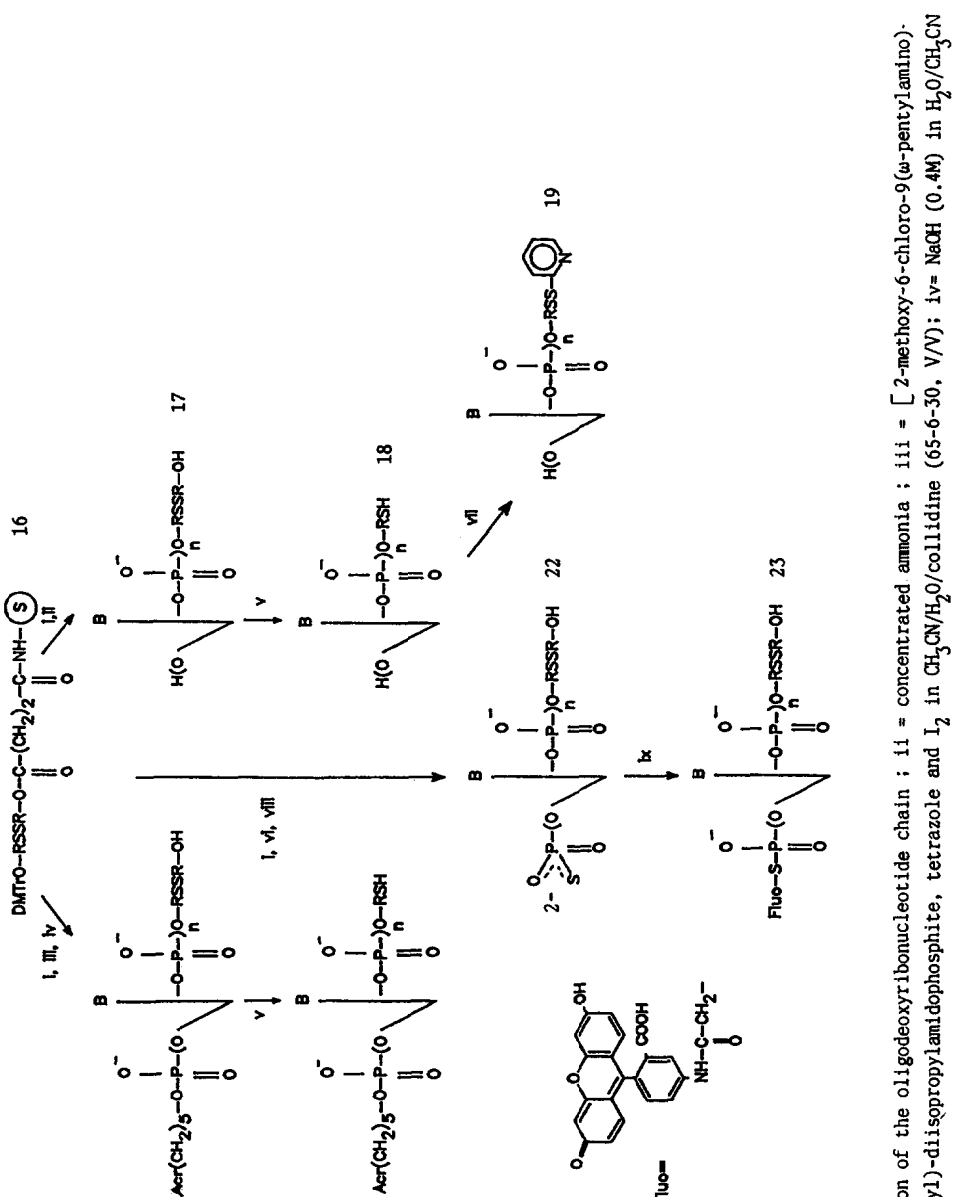
The 3'-thiol containing oligodeoxyribonucleotide **18a** can then be activated by transformation into its 3'-dithiopyridyl derivative d(TTTCCTCCTCT)p$CH_2CH_2(OCH_2CH_2)_2$S-S-  **19a** [Rt **19a** = 7 min 48 sec (syst A, 14 % CH_3CH isochratic)] by reaction with dithiodipyridine. This disulfide exchange reaction can be conveniently followed by monitoring the 2-pyridinethione formation (λ max=343 nm, ε =7,060 M^{-1} cm^{-1}) spectrophotometrically[63].

Other modified supports, described in literature, to obtain 3'-thiol modified oligodeoxyribonucleotides involve the preparation of four different polymer supports[60]. More recently an universal solid support for the 3'-thiol containing oligonucleotides preparation has been reported[64]. However the releasing of the oligodeoxyribonucleotide from the support needed treatment with dithiothreitol which did not allow the preservation of the 3'-thiol masked group.

5',3'-heterobifunctional oligodeoxyribonucleotides

The use of 2,2'-diethyldisulfide **5**, **10** and ω, ω'-disulfide derivatized support **16** allows, after the chain elongation, further modification of the 5'- end of the oligodeoxyribonucleotide. Direct incorporation of an acridine derivative [2-methoxy-6-chloro-9-(ω-pentylamino)-acridine] at the 5'- end of oligodeoxyribonucleotides immobilized on the support **5**, **10** and **16** can be automatically performed via its phosphoramidite derivative[44] on a DNA synthesizer. The introduction of a masked phosphorothioate group at the 5'-termini of an oligodeoxyribonucleotide synthesized on the support **16** was achieved by condensation of the 5'-hydroxyl group with the bis(2-cyanoethyl)-diisopropylamidophosphite followed by a sulfurization step[32].

Special conditions for the deprotection of the obtained heterobifunctional oligonucleotides are required depending on the 5'-substituent. The deprotection of bifunctional



Scheme 5 : i = elongation of the oligodeoxyribonucleotide chain ; ii = concentrated ammonia ; iii = [2-methoxy-6-chloro-9(ω-pentylamino)-acridinyl]-(2-cyanoethyl)-diisopropylamidophosphite, tetrazole and I_2 in CH_3CN/H_2O/collidine (65-6-30, V/V); iv= NaOH (0.4M) in H_2O/CH_3CN (4:1 V/V) ; v = dithiothreitol, H_2O ; vi = bis(2-cyanoethyl)-diisopropylamidophosphite, tetrazole and S_8 in CS_2/pyridine (50:50 V/V); vii= 2,2' dithiodipyridine (4 equiv.) in $NaHCO_3$ pH 4.5,0.1 N NaCl ; viii = concentrated ammonia ; ix = iodoacetamidofluorescein.

oligomers involving an acridine derivative at their 5'-end and a thiophosphate **9** or an amino group **12** at their 3'-end was performed by treatment with a solution of 0.1 N dithiothreitol and 0.4 N sodium hydroxide. Sodium hydroxide was used instead of concentrated ammonia because of the instability of the bond between the C_9 atom of the acridine ring and the N atom of the linker in the presence of the latter. The oligonucleotides with a 5'-acridine and a 3'-disulfide arm **20** were deprotected using only 0.4 N sodium hydroxide in order to obtain the masked thiol group. It must be noted that in this case some cleavage of the disulfide bridge occurred which generated the oligomer-3'-thiol, the latter can be separated from the disulfide **20** by HPLC. Whereas heterobifunctional oligonucleotides involving 5'-phosphorothioate and 3'-masked thiol group **22** can be deblocked by treatment with concentrated ammonia. The 5'-phosphorothioate of the oligomer carrying a 3'-disulfide arm **22** was then reacted with the chosen halogenoalkyl derivative such as the iodoacetamidofluorescein to afford the 5'-fluorescein-thiolophosphate **23** which can be reduced to the corresponding 3'-thiol by a dithiothreitol treatment. The absorbance spectrum of the compound **23** shown in Figure 3 is in agreement with the max value of the fluorescein ε 489 $\simeq$ 77,700 [70].

CONCLUSION

Oligodeoxyribonucleotides carrying at their 3'-end a phosphate, a phosphorothioate an aminoalkyl, a disulfide or a thiol group were easily synthesized on the supports **2**, **5**, **10** and **16**. These oligomers can be used to prepare the 3'-conjugated oligodeoxyribonucleotides by condensation respectively with amino, halogenoalkyl, activated ester or thiol containing compounds. The use of the above described supports **2**, **5**, **10** and **16** involving a disulfide bond stable under acidic and alkali medium allows moreover various modifications of the 5'-end oligodeoxyribonucleotide chain. Oligodeoxyribonucleotides involving an acridine group at their 5'-termini and a phosphorothioate **9** or an amino **12** or a masked thiol group **20** at their 3'- end were automatically synthetized on supports **5**, **10** and **16**.

The last modified support described **16** allows the synthesis of bifunctional oligodeoxyribonucleotides substituted at their 3'-end by a thiol protected group and at their 5'-termini by a functional group such as a phosphorothioate which can be further conjugated with various ligands.

The characterization of the oligomers substituted at their 5',3' or both ends has been achieved in different ways. By using both exchange and reverse-phase chromatography analysis the obtained retention times for oligomers of same sequence but with different end modifications are different (see part "Materials and Methods for HPLC results).

After full purification of the oligomers, the nucleoside composition and the position of the substituent group in the oligonucleotide chain were ascertained by nuclease hydrolysis followed by HPLC or TLC analysis of the hydrolysate by using previously described methods[42,43]. The oligonucleotides covalently linked to chromophores were also characterized by their absorption spectra (Figure 3).

The above described conjugated oligonucleotides can bind to single stranded or double stranded nucleic acids. The covalent linking of an intercalating agent such as an acridine or a daunomycin derivative to the oligodeoxyribonucleotide chain has been proven efficient to increase the stability of the duplexes[71]. The orthophenanthroline oligomer conjugates have been successfully used to selectively cleave single and double stranded DNA[72].

MATERIALS AND METHODS

General :

The following chemicals were obtained from commercial sources : Fractosil 500 (particle size 63-125 µm, pore diameter 500 Å) ; 5'-O-(4,4'-dimethoxytriphenylmethyl)-N-protected-2'-deoxynucleoside-3'-O-(2-cyanoethyl)-diisopropylamidophosphites ; succinic anhydride ; 4-dimethylaminopyridine ; dichloroacetic acid ; p-nitrophenol ; acetic anhydride ; 2,4,6-trimethylpyridine ; iodine (Merck). Ninhydrine ; 3-aminopropyltriethoxysilane ; dicyclohexylcarbodiimide ; dithiothreitol ; diisopropylethylamine, dimethylethylamine ; 2,2'-dithiodiethanol ; 2-cyanoethyl-N,N-diisopropylchlorophosphite ; 6-bromo-1-hexanol ; dimethoxytritylchloride ; 1,1'-carbonyldiimidazole ; 2-[-2-(2-chloroethoxy) ethoxy]-ethanol ; 18-crown-6 ; 15-crown-5 ; (Aldrich). S_8 ; triethylamine ; sodium sulfate ; 2,6-dibromoparabenzoquinone-N-chloroimine (DBPNC) (Prolabo). Pyridine ; Dichloromethane ; ethylacetate (SDS). 5-iodoacetamidofluorescein (Molecular Probes). Daunomycin (Rhône-Poulenc). 5-(iodoacetamido)-1,10-phenanthroline was a gift from Prof. D. Sigman. All the solvents were dried and distilled before use. Preparation of 3-aminopropyl derivatized Fractosil 500 was achieved according to standard published method[68]. Oligodeoxyribonucleotides described in this report were synthesized on a Pharmacia synthesizer using the phosphoramidite method[73]. Analytical TLC was carried out on Merck 5554 Kieselgel 60F 254 plates and eluted with various solvents noticed in the experimental procedures. Merck 9387 Kieselgel 60 or Merck 7734 Kieselgel 60 were used for column chromatography.

HPLC was performed on Varian 5000 liquid chromatography equipped with a Varian UV 50 detector operating at λ=254 nm, λ =425 nm for acridine containing compounds, λ =495 nm for fluorescein derivative and λ=500 nm for the daunomycin derivatives) or by using a Waters 600 E (system controller) equipped with a photodiode array detector Waters 990. The reverse-phase column Lichrocart (125 mm x 4 mm) packed with 5 µm Lichrospher RP 18 from Merck was used with a linear gradient of CH_3CN in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 ml/min (syst A) and the reverse-phase column (150 mm x 3 mm)packed with Lichrospher RP 18 from Merck with a linear gradient of CH_3CN in 0.12 M aqueous ammonium acetate, pH 5.9, with a flow rate of 0.8 ml/min (syst B) for analysis. Reverse-phase purifications were carried out on a 250 mm x 10 mm column packed with 10 µm lichrospher RP 18 from Merck. The elution system employed was the same as used in the syst A, with a flow rate of 4 ml/min. Analysis and purification by ion exchange chromatography were performed with a FPLC apparatus (Pharmacia). The following systems were used: polyanions SI column HR 5/5 with a linear gradient of KH_2PO_4 in water, pH-6, with a flow rate of 1 ml/min (syst C) or MonoP HR 5/5 with a linear gradient of NaCl in NaH_2PO_4 0.01 M pH 6.8, 20 % CH_3CN using a flow rate of 1 ml/min (syst D) for analysis. Purification was performed by using the systeme D on Mono P HR 5/5 column or polyanions SI column HR 10/10 with the syst C by using a flow rate of 4 ml/min. All buffers used with phosphorothioate containing oligonucleotides must be treated with chelex 100 resin (Biorad) to remove the divalent cations.

Synthesis of the 2,2'-dithiodiethyl derivatized support **2**

This protocol is a variation of a previously published procedure[68]

- Preparation of 1-dimethoxytrityl-2,2'-dithiodiethanol **1**

Dimethoxytritylchloride (1.37 g, 0.4 mmol) was added to a magnetically stirred solution of 2,2'-dithiodiethanol (0.6 g, 3.8 mmol) in anhydrous pyridine (15 ml). The reaction was monitored by TLC analysis on silica gel plates using CH_2Cl_2/MeOH (90:10 V/V) as eluent. When the reaction was completed, the solution was concentrated under reduced pressure and the obtained gum was dissolved in dichloromethane (80 ml). The organic layer was washed with a 5 % aqueous solution of $NaHCO_3$ (10 ml), and then with H_2O (3x30 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness under vacuo. The residue was purified by flash chromatography using increasing concentrations of methanol in dichloromethane (1:99 V/V to 3:97 V/V) as eluent. Yield 75 %. TLC analysis on silica was performed using dichloromethane/methanol (90:10 V/V) as eluent Rf 1 0.66.

- Synthesis of the p-nitrophenylester of the 1-dimethoxytrityloxyethyl-1'-succinylethyl-2',2'-disulfide

This compound was obtained as described in ref[68] by replacing the dimethoxytritylnucleoside with the compound **1**. The succinylated derivative was obtained with a yield of 80 % after flash chromatography purification [Rf ≃ 0.26 CH_2Cl_2/MeOH (90:10 V/V)] and the p-nitrophenylester of the 1-dimethoxytrityloxyethyl-1'-succinylethyl-2,2'-disulfide with a yield of 70 % after purification [Rf ≃ 0.84 CH_2Cl_2/MeOH (90:10 V/V)].

- Loading of the aminopropyl derivatized support **2**

Reaction of the p-nitrophenyl ester of the 1-dimethoxytrityloxyethyl-1'-succinylethyl-2,2'-disulfide with the aminopropyl derivatized support was achieved as described in ref. 68 with a loading of approximately 55 µmol of dithiodiethyl derivative per gram by determination of the amount of dimethoxytrityl cation released by acidic treatment of a sample of the derivatized support. Following this the capping step of the unreacted aminogroup of the support was achieved by acetylation as described in ref. 68.

Synthesis of oligodeoxyribonucleotides containing a 3'-phosphate group **6**

Chain elongation was performed by using the classical phosphoramidite procedure on the modified support **2** (1 µmole or 10 µmole scale). The oligonucleotide bound to the support was treated with a 0.1 N dithiothreitol solution in concentrated ammonia for 6 h at 55°C. Following this, after removal of the solid support by filtration, the ammonia solution was concentrated to dryness and the residue kept in acetic acid for 30 min at room temperature. The acetic acid was removed by evaporation and the residue was taken up in water (3 ml) then extracted with ethylacetate (3 times). After filtration by using 0.45 µm disposable filter the water oligodeoxyribonucleotides solution was purified by HPLC.

5'd(TTTCCTCCTCT)p **6a**, Rt = 18 min 15 sec (syst D, 0 to 1 M NaCl in 25 min)

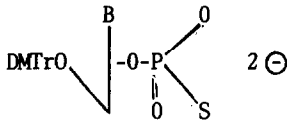
5'd(TTTTCTTTTCCCCCCT)p **6b**, Rt = 22 min 22 sec (syst D, 0 to 1 M NaCl in 25 min), Rt = 9 min 56 sec (Syst A, 5 % to 50 % of CH_3CN in 30 min)

Preparation of the derivatized support with nucleoside-3'-phosphorothioate **5**

A mixture of 5'-O-dimethoxytrityl-N-acyl-deoxynucleoside-3'-(β-cyanoethyl)-diisopropylamidophosphite (116 mg, 82 µmol) and tetrazole (11.5 mg, 165 µmol) in anydrous acetonitrile was shaked by hand with the detritylated dithiodiethyl derivatized support **2** (300 mg, 16.5 µmol) for 5 min under nitrogen atmosphere. The excess of phosphoramidite monomer was removed and the solid support washed with acetonitrile. 2 ml of a S_8 solution (5 % by weight) in CS_2/pyridine (50:50 V/V) mixture were added to the solid-phase and shaked from time to time for 30 min. The support was washed with CS_2/pyridine (50:50 V/V) mixture and then with CH_3CN. The deoxyribonucleoside phosphorothioate loading is determined by spectrophotometry determination of the amount of dimethoxytrityl cation released by the acid treatment of a sample of the support (see ref. 68). The support should have a loading of approximately 40-50 µmol of deoxyribonucleoside phosphorothioate per gram. If the support has a lower loading, it can be rederivatized using another sample of the phosphoramidite monomer followed by the sulfurization step. When the loading was satisfactory the unreacted hydroxyl groups were capped by acetylation as described in ref. 68.

Verifying the derivatized nucleoside-3'-phosphorothioate **5**

An analytical amount of each derivatized support **5** was treated with dithiothreitol in concentrated NH_4OH (50 mg/ml) for 48 h. TLC analysis on silica gel of the obtained product using $iPrOH/NH_4OH/H_2O$ (65:9:15 V/V) as eluent allows to verify the purity of the loaded modification. Usually the 5'-O-dimethoxytritylnucleoside-3'-phosphorothioate was obtained as the sole product detected as an orange-colored or a pink-colored spot by spraying respectively the perchloric acid or DBPNC.



B = A Rf = 0.65 B = T Rf = 0.55

B = G Rf = 0.60 B = C Rf = 0.60

Synthesis of oligodeoxyribonucleotides involving a 3'-phosphorothioate group **7**

The synthesis were carried out by using the modified solid support **5** (1 µmole or 10 µm scale) instead of support **2** and operating as described for the preparation of compounds **6** with a 3'-phosphate group. In this case it was necessary to use buffer treated with chelex 100 resin. The oligodeoxyribonucleotides **7** involving a phosphorothioate group at their 3'-end were obtained with good yields (Figure 2).

5'd(TTTCCTCCTCT)ps **7a**, Rt = 12 min 15 sec (syst B, 0 to 60 % 1 M KH_2PO_4 in 25 min)
Rt = 19 min 36 sec (syst D, 0 to 1 M NaCl in 25 min)

5'd(CACACCGACGGC)ps **7b**, Rt = 24 min 15 sec (syst D, 0 to 1 M NaCl in 25 min)
Rt = 9 min 33 sec (syst A, 5 % to 50 % CH_3CN in 30 min)

5'd(TCATCCACCTGGCATTGGAC)ps **7c**, Rt = 10 min 34 sec (syst A, 5 % to 50 % CH_3CN in 30 min)

5'd(CCCAATTCTGAAAAT)ps **7d**, Rt = 24 min 18 sec (syst D, 0 to 1 M NaCl in 30 min)
Rt = 11 min 6 sec (syst A, 5 % to 50 % CH_3CN in 30 min)

Preparation of the derivatized support including masked primary amino group **10**

The solid support **2** (500 mg, 30 µmol) was detritylated and then shaken by hand with a solution (10 ml) of 1,1'-carbonyldiimidazole in dioxane (50 mg/ml, 3 mmol) under nitrogen atmosphere for 5 min. After removal of the excess 1,1'-carbonyldiimidazole solution, the activated support was treated with an excess of 6-amino-1-hexanol in dioxane/H_2O (9 : 1 V/V) solution (10 mg/ml) (40 equiv.) for 10 min. Then the solid support was washed with dioxane and then with acetonitrile. Treatment of a sample of the support **10** with a solution of 0.1 M dithiothreitol in concentrated aqueous ammonia, gave a product identified by TLC through comparison with a sample of 6-amino-1-hexanol (positive ninhydrin test).

Synthesis of oligodeoxyribonucleotides with a 3'-primaryamino group **11**

Starting from the modified support **10** and by operating as described for the preparation of oligonucleotides with 3'-phosphate group, oligodeoxyribonucleotides with a 3'-amino hexyl linker **11** were obtained (1 µmol scale).

5'd(TTTCCTCCTCT)p$(CH_2)_6NH_2$ **11a**, Rt = 12 min 12 sec (syst C, 0 to 0.6 M KH_2PO_4 in 20 min),
Rt = 9 min 41 sec (syst B, 11,9% to 18.5% CH_3CN in 30 min)

5'd(TCATCCACCTGGCATTGGAC)p$(CH_2)_6NH_2$ **11b**, Rt = 9 min 51 sec. (syst A, 5 % to 50 % CH_3CN in 30 Min, 0.8 ml/min.)

Preparation of ω,ω'-dithiodi-[2-[(2-ethoxy) ethoxy]ethyl] derivatized support **16**

- Synthesis of ω,ω'-dithiodi-[2-[2-(2-ethoxy) ethoxy] ethanol] **14**

Transformation of 2-[2-(2-chloroethoxy) ethoxy] ethanol **13** into its iodinated derivative.

A solution of 2-[2-(2-chloroethoxy)ethoxy] ethanol **13** ($3.6\ 10^{-2}$ mol, 6g), sodium iodide ($1.77\ 10^{-1}$ mol, 26,5 g) and $NaHCO_3$ ($1.77\ 10^{-1}$ mol, 14,8 g) in acetone 200 ml was heated at 60°C. The reaction was monitored by TLC analysis on silica gel plates using CH_2Cl_2/MeOH (90:10 V/V) mixture as eluent. The iodinated derivative has a higher Rf (Rf ≃ 0.58) than the starting material (Rf ≃0.41). When the reaction was completed, the solid was removed by filtration and the solvent was evaporated under reduced pressure. The excess NaI was precipitated in CH_2Cl_2 (5x10 ml) then in ether (2x20 ml). The iodinated derivative was obtained as a yellowish oil. Yield 86 %.

- Thiol derivative

The iodinated compound ($1.53\ 10^{-2}$ mol, 3g) was dissolved in a methanol solution of 2.5 M NaSH (6 ml, $1.53\ 10^{-2}$ mol) (obtained by bubbling of H_2S through a sodium methoxide (2.5 M). The mixture was kept at room temperature with magnetic stiring. The reaction was monitored by TLC analysis using CH_2Cl_2/MeOH (90:10 V/V) mixture as eluent. After 3 h the iodinated compound (Rf = 0.58) was fully transformed into the thiol derivative (87 %, Rf= 0.51, brown colored spot with DBPNC) and the disulfide derivative (10 %, Rf = 0.36, yellow spot with DBPNC and heating), which can be easily separated by flash chromatogrphy on silica gel (Art. 9385 from Merck) using CH_2Cl_2/MeOH mixture as eluent.

- Disulfide derivative **14**

A solution of thiol compound (9.10^{-3} mol, 1.5 g) in diluted ammonia pH 8 was allowed to react in contact with air under stirring. After 48 h the thiol derivative was fully dimerized. The mixture was concentrated to dryness under reduced pressure. The compound was then purified by flash chromatography on a silica gel column using a CH_2Cl_2/CH_3OH mixture as eluent, yield 85 %.

Preparation of the tritylated compound **15**

The disulfide derivative **14** (3.10^{-3} mol, 1 g) was dried by coevaporation with anhydrous pyridine (three times) and kept in anhydrous pyridine (6 ml). Dimethoxytritylchloride ($7.6\ 10^{-4}$ mol, 0.26 g) was added to the cooled solution. The solution was then allowed to react at room temperature. The reaction was monitored by TLC on silica gel using CH_2Cl_2/MeOH (90:10 V/V) mixture as eluent (Rf **15** ≃ 0.66, orange colored spot with perchloric acid). After 2 h the pyridine was removed by evaporation under reduced pressure. The residue was solubilized in CH_2Cl_2 (10 ml) and twice washed with saturated NaCl solution (2x4 ml) then with distilled water (2x4 ml). After being dried over $MgSO_4$ the organic layer was concentrated to dryness and the obtained residue was purified by flash chromatography, yield 83 %.

Loading of the aminopropyl fractosil support

The ω,ω'-disulfide derivatized support **16** was obtained as described for the preparation of the 2,2'-dithiodiethyl support **2** by using the tritylated compound **15** instead of the compound **1**. By TLC analysis on silica gel, the corresponding succinylated and activated ester give respectively the following Rf ≃ 0.40 and Rf ≃ 0.90 using CH_2Cl_2/MeOH (90:10 V/V) as eluent. The obtained loading on Fractosil 500 was approximately 70 µmol/g.

Synthesis of oligodeoxyribonucleotide with 3'-disulfide arm **17** or 3' thiol function **18**

Starting from the modified support **16** the chain elongation was performed on 1 µmol or 10 µmol scale. The protected oligodeoxyribonucleotide bound to the support was treated

by concentrated ammonia (6 h at 55°C). After this the solid support was removed by filtration and the ammonia solution was concentrated to dryness and additional acetic acid treatment for 30 min was performed. Oligodeoxyribonucleotides with 3'-disulfide arm **17** were purified by ion exchange and reversed-phase liquid chromatography. The 3'-thiol function of oligomers was liberated just prior to use by treatment of the disulfide oligomer **17** (1 mg) with 0.5 M DTT solution in $NaHCO_3$ pH 4.5, 0.1 M NaCl mixture for 5 h, the excess of DTT was extracted with ethylacetate (5 x 10 ml).

17a : 5'd(TTTCCTCCTCT)p$CH_2CH_2(OCH_2CH_2)_2SS(CH_2CH_2O)_2CH_2CH_2OH$, Rt=4 min 54 sec (syst A, 14% CH_3CN isochratic)

17b : 5'd(AAGCTTTATTGAGGCTTAA)p$CH_2CH_2(OCH_2CH_2)_2SS(CH_2CH_2O)_2CH_2CH_2OH$, Rt=14 min 12 sec (syst D,0 to 1.5 M NaCl in 25 Min), Rt=10 min 12 sec (syst A, 5 % to 50 % CH_3CN in 30 min)

18a : 5'd(TTTCCTCCTCT)p$CH_2CH_2(OCH_2CH_2)_2SH$, Rt=2 min 30 sec (syst A,14 % CH_3CN isochratic)

18b : 5'd(AAGCTTTATTGAGGCTTAA)p$CH_2CH_2(OCH_2CH_2)_2SH$, Rt=14 min 30 sec (syst A, 5 % to 27 % CH_3CN in 30 min)

Activation of the thiol containing oligonucleotide **18** to afford the compound **19**

After the extraction of DTT, 2,2'-dithiodipyridine solution (25 µl, 1 mg/ml) was added to the 3'-thiol containing oligonucleotide **18** solution (1.5 ml) ($NaHCO_3$, pH 4.5, 0,1 M NaCl).

The formation of the activated disulfide containing oligomer **19** was monitored by reverse phase analysis by using the syst A described before (14 % CH_3CN isochratic in 0.1 M triethylammonium acetate, pH 7, for the compound **19a**). The excess of 2,2'-dithiodipyridine and 2-pyridine thione were extracted with ethyl acetate (5 x 10 ml) and the compound **19a** was purified by reverse phase HPLC using the syst A.

5'd(TTTCCTCCTCT)p$CH_2CH_2(OCH_2CH_2)_2SS$-R **19a**, Rt=7 min 48 sec (syst A,14 % CH_3CN isochratic)

Heterobifunctional oligodeoxyribonucleotides with an acridine derivative at their 5'-end and thiophosphate **9** amino **12** disulfide **20** or thiol **21** group at their 3'-end

- Preparation of the 2-methoxy-6-chloro-9-(ω-pentylamino)-acridinyl-(2-cyanoethyl)-diisopropylamidophosphite

To an acetonitrile solution (5 ml) of 2-methoxy-6-chloro-9-(ω-hydroxypentylamino) acridine (obtained as previously described in ref 42 (200 mg, 0.57 mmol, previously dried by coevaporation with CH_3CN) under argon atmosphere, diisopropylethylamine (88.4 mg, 0.12 ml, 0.68 mmol) and 2-cyanoethyl-N,N-diisopropyl-chlorophosphite (150 mg, 0.15 ml, 0.63 m mmol) were added at room temperature with magnetic stirring. The reaction was monitored by TLC on silica gel with CH_2Cl_2/MeOH (80:20 V/V) as eluent. After 30 min the starting material (Rf:0.35) was transformed into two compounds isomers : (Rf=0.55 and 0.82). When the reaction was completed, CH_2Cl_2 was added (10 ml) and the organic layer washed with 3 ml of an aqueous citric acid (10 % by weigh) then with concentrated NaCl. After being dried over $MgSO_4$ the organic solution was concentrated to dryness. The residue was purified on a silica gel column by using a mixture of ethylacetate and NEt_3 (95:5 V/V). The fractions containing the pure product were pooled and the solvent was removed under reduced pressure. The purified acridine phosphoramidite can be stored at -20°C for a few months.

- Coupling of the acridine derivative to the 5'-end of the oligonucleotide chain bound to each modified solid support **5**, **10** or **16**.

After the chain elongation using the classical phosphoramidite procedure an additional detritylation step was performed. Then a mixture of acridine phosphoramidite obtained above (0.1 ml of 0.1 M solution in CH_3CN), tetrazole (0.5 ml of 0.5. M solution in CH_3CN)

and CH_3CN (0.1 ml) was recycled for 7 min with a flow rate of 1 ml/min on a Pharmacia gene Assembler. This reaction step was performed twice. Then the oxidation step was carried out using iodine solution following the standard procedure.

- Deprotection and purification

Oligodeoxyribonucleotide bound to the supports **5** and **10** were treated with a mixture of 0.1 M dithiothreitol and 0.5 N sodium hydroxide, in water/methanol solution for 1 h. Then the support was removed by filtration and the solution was kept at room temperature for 40 h. After this the solutions were neutralized by Dowex 50 resin (pyridinium form). Methanol was evaporated and the yellow water solution was extracted with ethyl acetate (three times). The crude oligodeoxyribonucleotide **9** and **12** were purified by HPLC. Oligodeoxyribonucleotide bound to the support **16** were treated only with sodium hydroxide (0.5 N) in water methanol instead of DTT and sodium hydroxide mixture following the above described procedure for compounds **9** and **12**. In this case some cleavage of the 3'disulfide bound occurs to afford a mixture of oligomers **20** and **21**.

Acr$(CH_2)_5$p d5'(TCATCCACCTGGCATTGGAC)ps **9**, Rt = 18 min 30 sec (syst C, 0 to 1 M KH_2PO_4 in 25 min)

Acr$(CH_2)_5$p d5'(TCATCCACCTGGCATTGGAC)p$(CH_2)_6$ NH_2 **12**, Rt = 10 min 55 sec (syst A, 5% to 50% CH_3CN in 30 min, 0.8 ml/min)

Acr$(CH_2)_5$p d5'(AAGCTTTATTGAGGCTTAA)p $CH_2CH_2(OCH_2CH_2)_2SS(CH_2CH_2O)_2CH_2CH_2OH$ **20**, Rt=14 min (syst D,0 to 1 M NaCl in 25 min), Rt=9 min 18 sec (syst A, 13 % to 44 % of CH_3CN in 30 min)

Acr$(CH_2)_5$p d5'(AAGCTTTATTGAGGCTTAA)p $CH_2CH_2(OCH_2CH_2)_2SH$ **21**, Rt = 13 min 30 sec (syst D,0 to 1 M NaCl in 25 min), Rt = 7 min 56 sec (syst A, 13 % to 44 % of CH_3CN in 30 min)

Synthesis of heterobifunctional oligodeoxyribonucleotide with a 5'-thiophosphate group and a 3'-disulfide **22**

- Procedure for 5'-thiophosphate incorporation

After the chain elongation using the classical phosphoramidite procedure an additional detritylation step was performed. Then a mixture of bis (2-cyanoethyl)-diisopropylamidophosphite (obtained as described in ref. 44) (0.1 ml of 0.1 M solution in CH_3CN), tetrazole (0.5 ml of 0.5 M solution in CH_3CN) and CH_3CN(0.1 ml) was recycled for 7 min with a flow rate of 1 ml/min on a Pharmacia gene Assembler. This reaction was carried out twice. Then by using a peristaltic pump (out of the synthesizer) a solution of S_8 (5 % by weight) in CS_2/pyridine (50:50 V/V) was passed at 1 ml/min for 90 min, under argon atmosphere, through the support bearing the oligonucleotide. Then, the excess sulfur was removed by washing the solid support with a mixture of CS_2/pyridine for 10 min.

- Deprotection and purification

Unblocking was carried out by treatment with conc. ammonia for 6 hours at 55°C. The solution was concentrated to dryness and the crude material was purified by liquid chromatography.

5'spd(AAGCTTTATTGAGGCTTAA)p $CH_2CH_2(OCH_2CH_2)_2SS$ $(CH_2CH_2O)_2CH_2CH_2OH$ **22**, Rt = 28 min 18 sec (syst D,0 to 1.5 M NaCl in 35 min), Rt = 17 min 42 sec (syst A, 5 % to 80 % CH_3CN in 30 min)

Coupling of halogenoalkyl derivatives with phosphorothioate containing oligonucleotides **7** and **22** to afford compound **8** and **23**

Preparation of halogenoalkyl derivative of daunomycin

A mixture of daunomycin (30 mg, $5.3\ 10^{-5}$ mol), 6-bromohexanoyl-p-nitro-phenylester

(19.2 mg, $5.8\ 10^{-5}$ mol) and diisopropylethylamine (10 µl, $5.8\ 10^{-5}$ mol) in anhydrous DMF (5 ml) was stirred at room temperature. The reaction was monitored by TLC analysis on silica gel plates using CH_2Cl_2/MeOH (90:10 V/V) as eluent (Rf starting material ≃ 0.1, Rf halogenoalkylderivative of daunomycin ≃ 0.53) when the reaction was completed, the solution was concentrated under reduced pressure, the residue was kept in chloroform (10 ml) and the organic layer washed with cold water (2 x 3 ml). After being dried over $MgSO_4$ the organic solution was evaporated and the obtained red gum was purified on preparative silica-gel plates (Art 5717 from Merck) using CH_2Cl_2/MeOH (97:3 V/V) then CH_2Cl_2/MeOH (90:10 V/V) as eluents. Yield 60 %.

Coupling procedures

- in organic medium

Lyophilized oligonucleotide phosphorothioate (sodium salt) (10 OD units) was dissolved in MeOH (0.6 ml) in the presence of 15-crown-5 (or 18-crown-6 in the case of potassium salt) (0.015 g) the daunomycin derivative with halogenoalkyl linker (≃ 1 mg) was added to the oligonucleotide solution and the mixture was incubated with stirring at room temperature for 24 h.

- in aqueous medium

Iodoacetamidofluorescein (1 mg) or 5-iodoacetamido-1,10-phenanthroline (1 mg) was dissolved in DMF (1 ml) and added to a sodium hydrogenocarbonate solution (5 % by weight) (0.6 ml) of oligonucleotide phosphorothioate (30 units in 1 ml). The mixture was incubated with stirring at room temperature for 24 h.

The excess of halogenoalkyl derivative was removed by gel filtration on G10 or G25 from Pharmacia and the conjugated oligonucleotides **8a**, **8b**, **8c**, **8d** and **23** were purified by HPLC. Absorption spectra of the compounds **8d** and **23** are shown in the figure 3.

5'd(TTTCCTCCTCT)ps$(CH_2)_5$C(0)Daunomycin **8a**, Rt = 23 min 15 sec (syst D,0 to 1 M NaCl in 25 min)

5'd(TTTCCTCCTCT)psCH_2C(0)Orthophenanthroline **8b**, Rt = 19 min 15 sec (syst D,0 to 1 M NaCl in 25 min)

5'd(CACACCGACGGC)psCH_2C(0)Orthophenanthroline **8c**, Rt = 24 min (syst D,0 to 1 M NaCl in 25 min)

5'd(CACACCGACGGC)ps$(CH_2)_5$C(0)Daunomycin **8d**, Rt = 15 min 27 sec (syst A, 5 % to 50 % CH_3CN in 30 min)

Fluorescein-C(0)CH_2sp d 5'(AAGCTTTATTGAGGCTTAA)p$CH_2CH_2(OCH_2CH_2)_2SS(CH_2CH_2O)_2CH_2CH_2OH$ **23**,

Rt = 24 min 6 sec (Syst D,0 to 1.5 M NaCl in 35 Min), Rt = 15 min 24 sec (Syst A, 5 % to 80 % CH_3CN in 30 min)

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