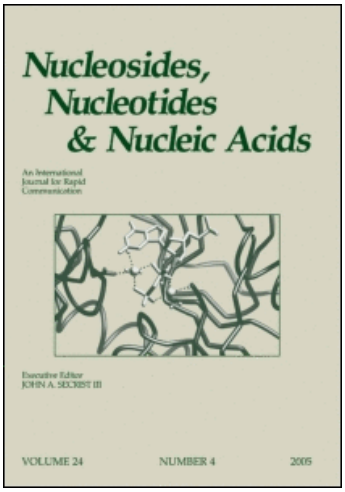


**Nucleosides, Nucleotides and Nucleic Acids**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Beatriz G. de la Torre[a]; Anna Maria Aviñó[a]; Mónica Escarceller[a]; Miriam Royo[b]; Fernando Albericio[b]; Ramon Eritja[a]
[a] Dpt of Molecular Genetics, CID-CSIC, Barcelona, Spain [b] Dpt of Organic Chemistry, University of Barcelona, Barcelona, Spain

**To cite this Article** de la Torre, Beatriz G. , Aviñó, Anna Maria , Escarceller, Mónica , Royo, Miriam , Albericio, Fernando and Eritja, Ramon(1993) 'Use of a Base-Labile Protected Derivative of 6-Mercaptohexanol for the Preparation of Oligonucleotides Containing a Thiol Group at the 5'-End', Nucleosides, Nucleotides and Nucleic Acids, 12: 9, 993 — 1005
**To link to this Article: DOI:** 10.1080/07328319308018569
**URL:** http://dx.doi.org/10.1080/07328319308018569

NUCLEOSIDES & NUCLEOTIDES, 12(9), 993-1005 (1993)

# USE OF A BASE-LABILE PROTECTED DERIVATIVE OF 6-MERCAPTOHEXANOL FOR THE PREPARATION OF OLIGONUCLEOTIDES CONTAINING A THIOL GROUP AT THE 5'-END.

Beatriz G. de la Torre[1], Anna Maria Aviñó[1], Mónica Escarceller[1], Miriam Royo[2], Fernando Albericio[2] and Ramon Eritja*[1].

[1]Dpt of Molecular Genetics. CID-CSIC. Jordi Girona 18-26. 08034 Barcelona. Spain.

[2]Dpt of Organic Chemistry. University of Barcelona. 08028 Barcelona. Spain.



**Abstract:** The preparation of a base-labile (Dnpe) protected derivative of 6-mercaptohexanol is described. The use of the phosphoramidite derivative of this compound improves both yields and the time needed for the preparation of oligonucleotides containing a thiol group at the 5'-end.

Modified oligonucleotides containing a thiol group at the 5'-end have been shown to be useful intermediates for attaching a variety of products to oligonucleotides including fluorescent compounds, biotin and enzymes such as alkaline phosphatase[1]. The usual method to produce 5'-thiol containing oligonucleotides involves the preparation of a thiol derivative protected with the trityl group that is incorporated, at the end of the chemical synthesis of the oligonucleotide, to the 5'-OH[1-3]. The *S*-trityl group is removed after deprotection and purification of the *S*-trityl oligonucleotide with silver nitrate in mild acidic conditions. Alternatively, it has been described that 5'-thiol oligonucleotides can be obtained by reaction of 5'-amino oligonucleotides with protein modification reagents like SPDP[4] or *N*-acetyl-D,L-homocysteine lactone[5]. We have recently described the preparation of oligonucleotide-peptide hybrids by coupling of 5'-thiol oligonucleotides (obtained with S-trityl-6-mercaptohexanol phosphoramidite) with maleimido containing

peptides[6]. When we scaled up the preparation of such hybrids to the milligram scale needed for antisense inhibition studies, we found that the main part of the 5'-thiol oligonucleotide co-precipitated with the DTT-silver complex. So, very low yields of 5'-thiol oligonucleotides were obtained even after washing the DTT-$Ag^+$ precipitate several times. In order to circumvent this problem, we have developed an alternative method that avoids the use of silver nitrate and it is based on the use of the 2,4-dinitrophenylethyl (Dnpe)[7] protecting group for the thiol function. This protecting group, like the *S*-fluorenylmethyl (Fm) group[8], is stable to iodine and acids and they are easily removed giving free thiol derivatives with bases in the presence of reducing agents like DTT.

## RESULTS

We have prepared the *S*-Dnpe protected derivative of 6-mercapto hexanol using two different synthetic methods as shown in FIG. 1. In the first method Dnpe-S-$(CH_2)_6$-OH (**6**) was obtained by reaction of HS-$(CH_2)_6$-O-benzoate (**4**) with 2-(2,4-dinitrophenyl)ethyl bromide[7] (Dnpe-Br) and subsequent acid hydrolysis of the benzoyl ester. Compound **3** was prepared similarly to what has been described in ref. 9 for the DMT-derivative. Alternatively, compound **6** could be obtained directly by reaction of 6-mercapto hexanol[10] (**8**) with Dnpe-Br. 6-mercaptohexanol (**8**) was prepared from 6-chlorohexanol (**1**) via 6-iodohexanol (**7**) as described in ref. 10. This second method is recommended because it is shorter and compound **6** is obtained in higher yields.

$\beta$-Cyanoethyl phosphoramidite derivative was obtained using standard protocols[11]. The corresponding phosphoramidite was coupled to the 5'-end of the following oligonucleotide sequences on an automatic DNA synthesizer:

A: 5' Dnpe-S-$(CH_2)_6$-phosphate-GCATGC 3' 6 mer

B: 5' Dnpe-S-$(CH_2)_6$-phosphate-AACGTTGAGGGGCAT 3' 15 mer

C: 5' Dnpe-S-$(CH_2)_6$-phosphate-ACCCCAGCTCAGCTC 3' 15 mer

Coupling yields with the new phosphoramidite were 80 to 92%. Treatment of oligonucleotide-support with concentrated ammonia containing 0.1 M DTT gave directly the desired 5'-thiol oligonucleotides together with a product with a strong UV absorption (U.V. max 283) coming from the Dnpe group. Alternatively, the S-

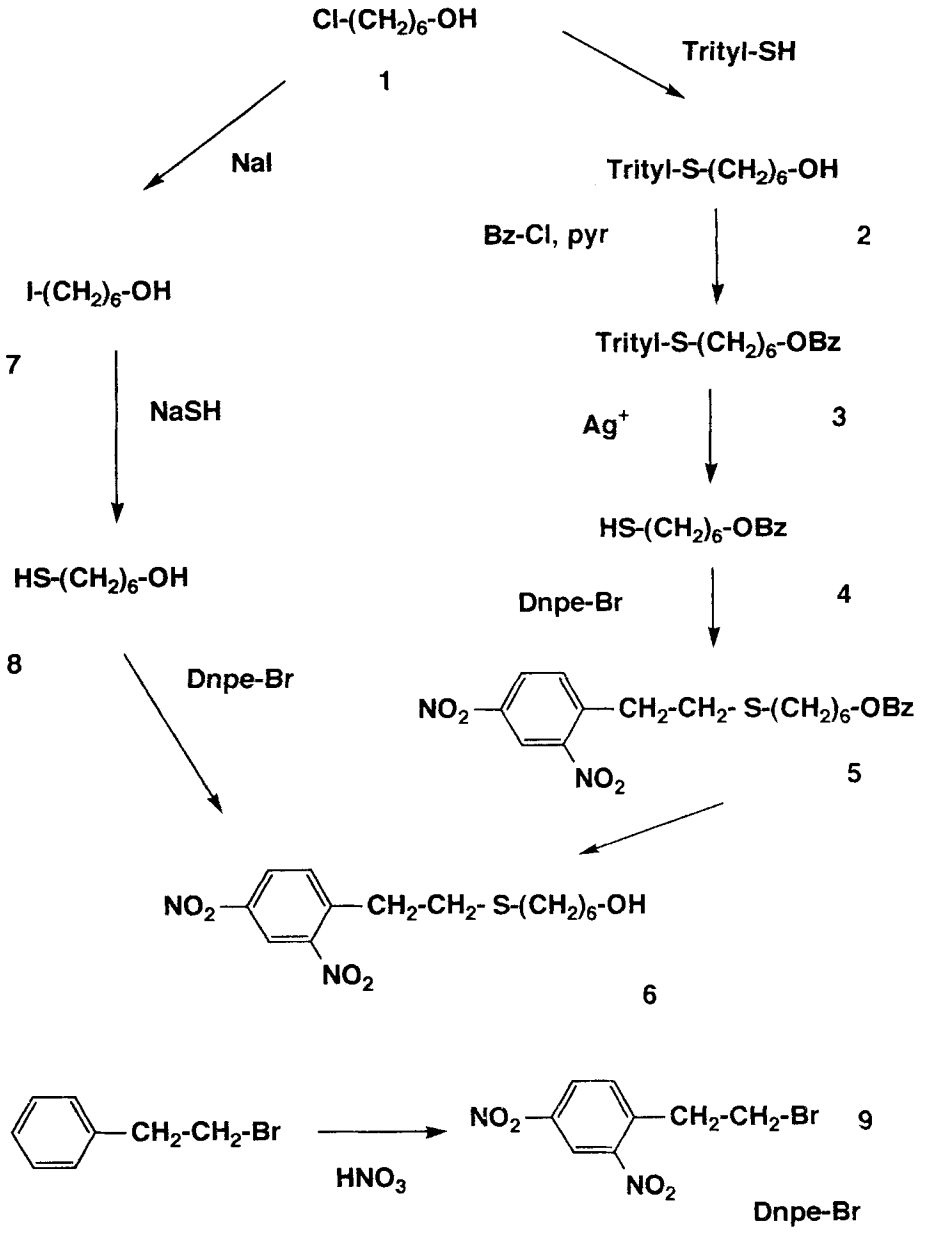


FIGURE 1: Preparation of the Dnpe derivative of 6-mercaptohexanol.

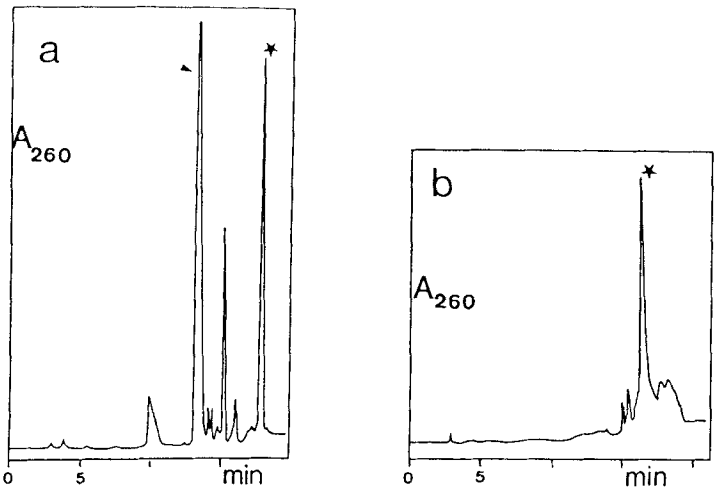


FIGURE 2: HPLC purification of a) 5'-thiol-hexamer A after ammonia treatment and before Sephadex desalting and b) 5'-thiol 15 mer B after Sephadex desalting. * indicates the desired thiol-oligonucleotide, ► indicates the product coming from the deprotection of the Dnpe Group.



Dnpe group can be eliminated before ammonia treatment by treating oligonucleotide-supports with a 0.3 M solution of DBU (1,8-diazabicyclo[5.4.0] undec-7-ene) in acetonitrile for 5-10 minutes. During this treatment a strong green colour due to the Dnpe deprotection is observed. Following the DBU treatment, the supports were treated with conc. ammonia containing 0.1 M DTT obtaining a much cleaner product but still with the presence of the product coming from the deprotection of the Dnpe (see FIG. 2a). This product and the excess of DTT were removed using a Sephadex G-10 column. 5'-Thiol oligonucleotides were further purified by HPLC (see FIG. 2) and the resulting products were reacted with a maleimido-peptide (maleimido-AlaAlaProLysLysLysArgLysVal-$NH_2$, prepared as described in ref. 6) and a derivative of digoxigenin with a maleimido group. In both cases, a new compound was obtained quantitatively (see FIG. 3). The presence of the peptide molecule linked to the oligonucleotide was confirmed by hydrolysis and amino acid analysis. The presence of the digoxigenin molecule was confirmed by

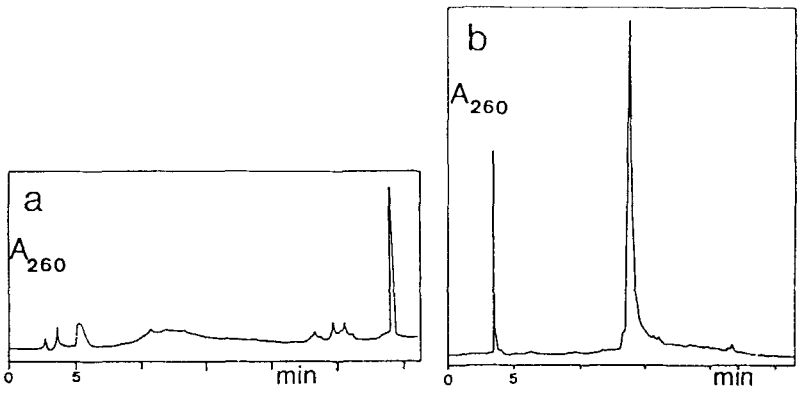


FIGURE 3: HPLC analysis of the conjugation reactions. a) Thiol-hexamer A with the maleimido derivative of digoxigenin and b) thiol-15 mer C with maleimido peptide.



incubation of a nylon filter containing the hybrid molecule with the conjugate anti-digoxigenin-alkaline phosphatase and detection with colorimetric substrates (NBT/BCIP)[12].

Finally, oligonucleotide C was prepared in 1 $\mu$mol scale and it was coupled to the maleimido peptide as described above. The corresponding oligonucleotide-peptide hybrid was purified by HPLC and characterized by enzymatic digestion and amino acid analysis (see FIG. 4). Overall yield (from the first nucleoside attached to the CPG support to purified hybrid molecules) was 15%, that is ten times higher than the yield obtained in a parallel synthesis using the standard S-trityl phosphoramidite.

In conclusion, we have described that the substitution of the trityl protecting group for the thiol function for a base labile protecting group, such as the Dnpe group, improves dramatically both yields and the time needed for the preparation of 5'-thiol containing oligonucleotides. The use of Dnpe-thiol phosphoramidite described here will facilitate not only the preparation of oligonucleotide-peptide hybrids but also the preparation of oligonucleotides labeled non-radioactive reporter groups such us biotine, fluorescent compounds and alkaline phosphatase. Finally, similar S-Dnpe derivatives could be designed for the introduction of thiol groups on the bases or phosphodiester linkages.

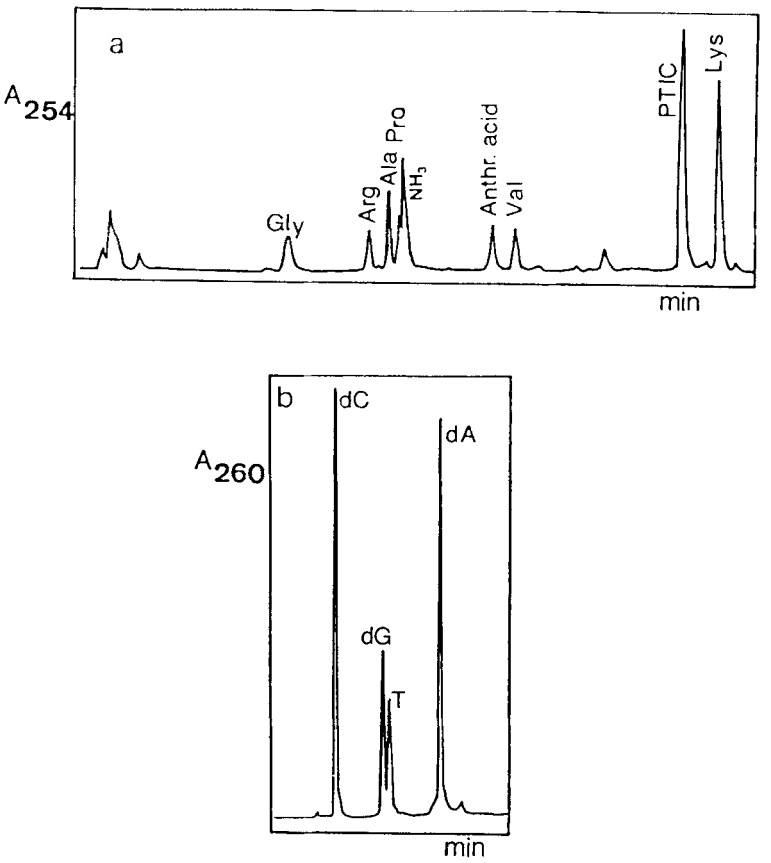


FIGURE 4: Characterization of peptide-oligonucleotide hybrid corresponding to oligonucleotide sequence C (see FIG 3b). a) Amino acid analysis of the acid (HCl 6N) hydrolysate using PICO-TAG. b) HPLC analysis of phosphodiesterase and alkaline phosphatase digestion.

## EXPERIMENTAL PART

Abbreviations used : $A_{260}$: absorbance at 260 nm. BCIP : 5-bromo-4-chloro-3-indolyl phosphate, CPG: controlled-pore glass, DBU : 1,8-diazabicyclo[5.4.0]undec-7-ene, DMF: N,N-dimethylformamide, DMT : dimethoxytrityl, DTT: 1,4-dithiothreitol, NBT: nitro blue tetrazolium salt.

6-S-trityl-6-mercapto-1-hexanol (**2**) was prepared as described in ref. 1. Digoxigenin-3-O-succinyl-[2-(N-maleimido)]ethylamide and the conjugate anti-digoxigenin-alkaline phosphatase were obtained from Boehringer Mannheim (Germany).

*6-S-trityl-6-mercapto-1-hexyl benzoate (3)*

6-S-trityl-6-mercapto-1-hexanol[1] (**2**) (1.5 gr, 3.98 mmol) were dissolved in 10 mL of pyridine. The solution was cooled with an ice bath and 1.46 gr of benzoyl chloride (4.77 mmol) were added. After one hour of magnetic stirring, 3 mL of a 5% $NaHCO_3$ solution were added and the mixture was concentrated to dryness. The residue was dissolved with dichloromethane (50 mL). The solution was washed successively with a 5% $NaHCO_3$ solution (50 mL), water (50 mL), a 5% citric acid solution (50 mL) and water (50 mL) and then dried ($Na_2SO_4$). The crude product was chromatographed on a silica gel column with dichloromethane as the eluent and (**3**) was obtained in 81% yield (1.55 gr, 3.23 mmol). $R_f$ 0,80 (methanol-dichloromethane 5:95). $^1$H-NMR, $\delta$ (90 MHz, $CDCl_3$) : 1,3-2.2 (m, 8H, 4 $CH_2$), 3.55 (t, 2H, $Ph_3C$-S-$CH_2$-), 4.3 (t, 2H, -$CH_2$-OBz), 7.2-8.2 (m, 20H, Ar).

*6-S-(2-(2',4'-dinitrophenyl)ethyl)-6-mercapto-1-hexyl benzoate (5)*

Compound **3** (0.48 gr, 1 mmol) was dissolved with a 2% triethylamine solution in chloroform (20 mL) and 60 mL of a 8 mM silver nitrate solution ethanol was added. After 30 min of magnetic stirring at room temperature 20 mL of a 0.35 M DTT solution in chloroform was added. The silver-DTT complex precipitated was filtered and the solution was concentrated to dryness. The residue was dissolved in chloroform (50 mL) and washed with water. The organic phase was dried ($Na_2SO_4$) and the solution was evaporated to dryness. The resulting product (compound **4**) was used in the following reaction without further purification.

The residue was dissolved in dichloromethane (50 mL) and was reacted with 2,4-dinitrophenylethyl bromide (0.27 gr, 1 mmol) together with ethyldiisopropylamine (0.13 gr, 1 mmol). After 4 hours of magnetic stirring, the solvent was evaporated to dryness and the residue was purified by silica gel chromatography. The column was eluted with dichloromethane/hexane (1:1) and dichloromethane. Compound **5** was obtained in a 75% yield (0.5 gr, 0.75 mmols). $R_f$ 0.77 (dichloromethane). $^1$H-NMR,

$\delta$ (90 MHz, $CDCl_3$) : 1.3-1.9 (m, 8H, 4 $CH_2$), 2.65 (t, 2H, S-$CH_2$), 2.8 (t, 2H, $CH_2$-S), 3.28 (t, 2H, $CH_2$-Ar), 4.32 (t, 2H, $CH_2$-OBz), 7.2-8.8 (m, 8H, Ar).

*6-[S-2-(2',4'-dinitrophenyl)ethyl]-6-mercapto-1-hexanol (6) from compound 5.*

Compound **5** (0.5 gr, 0.75 mmol) was dissolved with a 10% solution of *aq.* HCl in methanol (20 mL) and heated to reflux for 3 hours. The solution was evaporated to dryness and the residue was purified by silica gel column chromatography using dichloromethane and dichloromethane/methanol 98:2 as eluents. Compound **6** was obtained in a 73% yield (0.24 gr, 0.73 mmol). TLC: $R_f$ 0.15 (dichloromethane). HPLC: $t_R$ 30.8 min, a linear gradient from 20%B to 100%B in 30 min and isocratic 100%B for 5 min (A: 0.1% TFA in water; B: 0.1% TFA in $CH_3CN$/ water (7:3); column Nucleosil 120-10C18; 25 x 0.4 mm; 1 mL/min). $^1$H-NMR, $\delta$ (200 MHz, $CDCl_3$) : 1.3-1.8 (m, 8H, 4 $CH_2$), 2.56 (t, J= 7.8 Hz, 2H, $CH_2$), 2.85 (t, J= 7.0 Hz, 2H, $CH_2$), 3.30 (t, J= 7.8 Hz, 2H, $CH_2$), 3.65 (t, J= 7.0 Hz, 2H, $CH_2$), 7.64 (d, J= 8.4 Hz, 1H, Ar), 8.40 (dd, J= 2.4 and 8.4, 1H, Ar), 8.81 (d, J= 2.4 Hz, 1H, Ar).



*6-Iodo-1-hexanol (7)*

A mixture of 6-chloro-1-hexanol (2 gr, 14.6 mmol), sodium iodide (10.9 gr, 73.2 mmol) and sodium bicarbonate (6.1 gr, 73.2 mmol) in acetone (125 mL) was refluxed for 3.5 hrs. The reaction mixture was filtered and the filtrate was concentrated to dryness. The residue was treated with dichloromethane (2 x 20 mL) and the remaining solid was removed by filtration. Again, the solution was concentrated to dryness, the residue was treated with ether (2 x 20 mL) and the remaining salts were removed by filtration. The iodinated compound **7** was obtained as a coloured oil (1.9 gr, 8.2 mmol) in a 57% yield. TLC : $R_f$ (dichloromethane) 0.47. $^1$H-RMN, $\delta$ (90 MHz, $CDCl_3$) : 1.3-2.0 (m, 8H, 4 $CH_2$), 3.2 (t, 2H, $CH_2$), 3.7 (t, 2H, $CH_2$).

*6-mercapto-1-hexanol (8)*

A mixture of 6-iodo-1-hexanol **7** (1 gr, 4.4 mmol) and sodium hydrogen sulfide (0.28 gr) in methanol (2 ml) was refluxed for 3 hrs. The reaction mixture was concentrated to dryness and the residue was purified by silica gel column chromatography eluted with dichloromethane yielding compound **8** in 70% yield

(0.41 gr, 3 mmol). TLC : $R_f$ (dichloromethane) 0.45 (colored spot with 2,6-dibromoparabenzoquinone-N-chloroimine and with Ellman reagent). $^1$H-NMR, $\delta$ (90 MHz, $CDCl_3$) : 1.3-2.0 (m, 8H, 4 $CH_2$), 3.5 (t, 2H, $CH_2$), 3.7 (t, 2H, $CH_2$).

*S-[2-(2,4-Dinitrophenyl)ethyl]-6-mercapto-1-hexanol (6) from compound 8.*

A mixture of 6-mercapto-1-hexanol **8** (0.41 gr, 3 mmol), 2-(2,4-dinitrophenyl)ethyl bromide (0.81 gr, 3 mmol) and ethyldiisopropylamine (0.39 gr, 3 mmol) in dichloromethane was stirred overnight at room temperature. The reaction mixture was concentrated to dryness and the residue was purified by silica gel column chromatography using dichloromethane and a 2% methanol solution in dichloromethane, obtaining compound **6** (0.75 gr, 2.3 mmol) in a 77% yield. Physical and spectroscopical data identical to compound **6** obtained from compound **5**.



*2-(2,4-dinitrophenyl)ethyl bromide (9).*

2-phenylethyl bromide (25 gr, 0.13 mmol) was added dropwise in 1 hour to 125 mL of fuming nitric acid (d 1.52) at -10ºC. After the addition, the reaction was kept at 0ºC for 4 hours. The reaction mixture was added to 300 mL of ice and the product was extracted with eter (3 x 100 mL). The ethereal phase was neutralized to a pH 7 with a sodium carbonate solution. After separation of the aqueous phase, the organic layer was dried ($Na_2SO_4$) and concentrated to dryness obtaining the desired product (33 gr) in a 87% yield. TLC : $R_f$ (ethyl acetate/ hexane 1:1) 0.44. $^1$H-NMR, $\delta$ (90 MHz, $CDCl_3$) : 3.5-3.8 (m, 4H, 2 $CH_2$), 7.7 (d, 1H, Ar), 8.5 (dd, 1H, Ar), 8.9 (d, 1H, Ar). $^{13}$C-NMR ($CDCl_3$) : 31.1 ($CH_2$), 36.6 ($CH_2$), 121.2 (Ar), 127.6 (Ar), 135.1 (Ar).

*O-[S-2-(2,4-dinitrophenyl)ethyl-6-mercapto-1-hexyl]-O-β-Cyanoethyl-N,N-diisopropylphosphoramidite (10).*

The phosphoramidite derivative was prepared essentially as described in ref. 11. S-2-(2,4-Dinitrophenyl)ethyl-6-mercapto-1-hexanol (**6**, 0.5 gr, 1.5 mmol) was reacted with chloro-O-β-cyanoethyl-N,N-diisopropylaminophosphine (0.45 gr, 1.9 mmol) and diisopropyl ethyl amine (0.66 ml, 3.8 mmol) in dry tetrahydrofuran at 0ºC under argon atmosphere. After 30 min of magnetic stirring at 0ºC, the ice bath was

removed and the reaction was continued for 30 more minutes. The reaction mixture was filtered through a sintered glass funnel and the solid amine hydrochloride was washed with dry tetrahydrofuran. The filtrates were combined and evaporated to dryness. The resulting oil was dissolved in ethyl acetate and washed with an ice-cooled 5% aqueous sodium bicarbonate solution followed by a saturated sodium chloride solution. The organic layer was dried with anhydrous sodium sulfate and concentrated to dryness. The residue was dissolved in ethyl acetate/hexane (25:75) and the solution was loaded on a silica gel column previously packed with ethyl acetate/ hexane (25:75) containing 5% pyridine. The column was eluted with ethyl acetate/ hexane (25:75). The fractions containing the desired product were combined and evaporated to dryness yielding 0.4 gr (0.76 mmol, 50%) of a yellowish oil.



*Incorporation of the phosphoramidite on an automated DNA synthesizer.*

Phosphoramidite **10** was dissolved in dry acetonitrile under an argon atmosphere and the solution was placed into a vial on the DNA synthesizer. A mock synthesis was done to evaluate the coupling efficiency of the new phosphoramidite: To a 0.2 $\mu$mol column loaded with DMT-T, phosphoramidite **10** was coupled following a standard 0.2 $\mu$mol synthesis cycle except that no capping was done. To the resulting column a second synthesis cycle was performed with DMT-T phosphoramidite. The DMT colour of this second T was only 8% of the DMT colour obtained during the detritylation of DMT-T-CPG, so the new phosphoramidite **10** coupling efficiency was 92%.

The following sequences were assembled on a DNA synthesizer following standard 0.2-1 $\mu$mol synthetic cycles. A: 5' Dnpe-S-$(CH_2)_6$-phosphate-GCATGC 3, 6 mer; B: 5' Dnpe-S-$(CH_2)_6$-phosphate-AACGTTGAGGGGCAT 3', 15 mer; C: 5' Dnpe-S-$(CH_2)_6$-phosphate-ACCCCAGCTCAGCTC 3', 15 mer.

Coupling efficiencies were also estimated after ammonia deprotection by measuring the area of the peaks obtained on the HPLC chromatograms. They were between 80% to higher than 90% depending on the scale of the synthesis.

*Deprotection and Purification of 5'-thiol oligonucleotides.*

Dnpe-S-$(CH_2)_6$-phosphate-oligonucleotide supports were deprotected using two protocols:

a) The supports were treated directly with a 0.1 M DTT solution in concentrated aqueous ammonia (30%) at 55 ºC for at least 5 hours.

b) The supports were placed on a sintered glass funnel and treated three times with a 0.3 M DBU solution in acetonitrile for 2-3 minutes. A strong green colour appears on the support indicating the deprotection of the Dnpe group. The support was succesively washed with acetonitrile, 1/10 diluted ammonia solution (to replace DBU cations for ammonium), and water. The supports were placed on scew-capped tubs and they were treated with 0.1 M DTT solution in conc. $NH_4OH$ at 55ºC for at least 5 hours.

Although method b is longer we recommend it because it gives an indication of the presence of the Dnpe group and the major part of the deprotection product coming from the Dnpe group were washed out of the support.



After deprotection, the supports were filtered out and the solutions were desalted on a Sephadex G-10 or G-25 column eluted with 50 mM triethylammonium acetate. This step removes the excess of DTT and the deprotection product from the Dnpe group. After desalting, the resulting 5'-thiol oligonucleotide crude could be used for conjugation reactions directly or after standard reversed phase HPLC purification. A C-18 Nucleosil 120 (10$\mu$m) column (250 x 4 mmm) was used eluted with a 5-25% gradient of acetonitrile in 10 mM triethylammonium acetate over 20 min (FIG 2). It is important to use 5'-thiol oligonucleotide during the following one or two days to avoid dimerization by disulfide bond formation.

*Conjugation of 5'-thiol oligonucleotide with maleimido derivatives.*

a) Conjugation with a maleimido derivative of Digoxigenin.

5'-Thiol oligonucleotide coming either from HPLC purification or after desalting on Sephadex columns were used. Both products were dissolved in pH 7.5 triethylammonium acetate buffer. The product coming from HPLC purification contained also acetonitrile. The solutions containing 5'-thiol oligonucleotide (approx. 0.5 O.D. units) were mixed with 1 mg of Digoxigenin-3-O-succinyl-[2-(N-maleimido)] ethylamide dissolved in DMF. DMF was added until obtain a 1:1 mixture of DMF/ water to prevent the precipitation of the digoxigenin derivative. After 24 hrs of incubation at room temperature, the mixture was concentrated to dryness and the

resulting product was purified by HPLC (see FIG. 3). HPLC conditions were similar to the conditions described above except that a 5-40% gradient of acetonitrile over 40 min was used. The presence of the digoxigenin molecule was confirmed by fixing a small aliquot of the product in a nylon filter with UV light, incubation of the filter with the conjugate anti-digoxigenin-alkaline phosphatase and detection with colorimetric substrates (NBT/BCIP)[12].

b) Conjugation with a maleimido-peptide. The preparation of the maleimido-peptide (maleimido-AlaAlaProLysLysLysArgLysVal-$NH_2$) and the conjugation with 5'-thiol containing oligonucleotides has been previously described in ref. 6.
In this case, 1 $\mu$mol scale synthesis yield around 80 O.D. units of crude 5'-thiol oligonucleotide after Sephadex desalting that were reacted with the maleimido peptide. After HPLC purification, 18 O.D. units of purified peptide-oligonucleotide hybrid molecule were obtained (15% yield).



## ACKNOWLEDGEMENTS

We are grateful to CICYT (SAL 90-828) for financial support. We thank Dr. Nanda Sinha for helpful suggestions and continued interest in this work.

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Received 3/30/93
Accepted 7/27/93