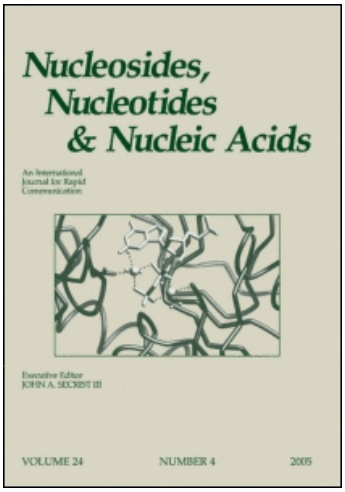

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New Non Nucleosidic Phosphoramidites for the Solid Phase Multi-Labelling of Oligonucleotides: Comb- and Multifork-Like Structures

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NEW NON NUCLEOSIDIC PHOSPHORAMIDITES FOR THE SOLID PHASE MULTI-LABELLING OF OLIGONUCLEOTIDES: COMB- AND MULTIFORK-LIKE STRUCTURES

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Abstract: Comb- and multifork-like structures have been designed for multiple labelling of oligonucleotides. They rely on the synthesis of new non nucleosidic phosphoramidites which can be used in automatic DNA synthesis. The new compounds allowed the 5' and/or 3' tagging of any oligonucleotide and provided reactive primary amino groups for non isotopic labelling. Seventeen polybiotinylated oligonucleotides have been prepared and tested in hybridization assays using a corresponding complementary sequence covalently linked to polystyrene tubes. Detection limits, measured by chemiluminescence, ranged from 10^5 to 3.10^6 molecules, depending on the number of biotins. Optimal detection was achieved either with a 5' multifork-like derivative carrying 8 biotins or with a comb-like structure carrying 4 biotins on both 5' and 3' ends.

INTRODUCTION

Increased interest in non radioactively labelled oligonucleotides has prompted the development of chemical methods to modify oligonucleotides. Recently, a number of procedures have been reported which use the phosphoramidite chemistry to introduce a primary aliphatic amino[1-11] or sulphydryl[12-14] group at either the 3' or, more commonly, the 5' end of the oligonucleotide, or an internal primary aliphatic amino group. The reactive moiety was then used for the subsequent attachment of a variety of labels such as fluorophores, biotin, chemiluminescent groups or

enzymes. This approach is limited because probes often do not have the required sensitivity since only a single label can be attached to the oligonucleotide.

Among the numerous phosphoramidites proposed in the literature for the introduction of a primary amino group into an oligonucleotide, nucleoside phosphoramidite reagents, containing a masked primary amino group on the base heterocycle, have been widely described[6-11]. They offer the advantage of allowing both the 3'-phosphoramidite and the 5'-hydroxyl to participate in solid phase oligonucleotide synthesis. Therefore, such an amino modified nucleosidic phosphoramidite can be incorporated at any position of a synthetic oligonucleotide. Another advantage is that multiple amines can be incorporated through repetitive coupling cycles. However, the preparation of such amino nucleosidic phosphoramidites requires many synthetic steps and introduction of modified nucleotide bases may change the hybridization properties of the oligonucleotide. For these reasons, several non nucleosidic phosphoramidites have been proposed recently.[15-19]

In this paper, we describe new non-nucleosidic phosphoramidite reagents that allow, during the solid phase oligonucleotide synthesis, either the incorporation of multiple primary aliphatic amino groups or the introduction of spacer arms, either between the primary amines or between the oligonucleotide and the detection moiety. Following this strategy, we have prepared a series of polybiotinylated oligomers. To evaluate their respective sensitivity, we used a chemiluminescent detection system wherein these probes underwent hybridization with a target DNA chemically linked to polystyrene surfaces. In this respect, we describe an original method to chemically bind oligonucleotides onto polystyrene tubes or microwells bearing aldehyde functions.

MATERIALS AND METHODS

All chemicals were purchased from Aldrich except the d-biotin-N-hydroxysuccinimide ester and the biotinamidocaproate N-hydroxysuccinimide ester which were from Sigma (U.S.A.) and the alkylamine controlled pore glass support from Pierce (Belgium). 1-N-(N-DMT-biotinyl)-6-O-(cyanoethoxydiisopropylaminophosphinyl)-1-amino-6-hexanol was from Cambridge Research Biochemicals (U.K.). Reagents for automatic DNA synthesis were from Eurogentec (Belgium); aldehyde tubes and microwells were from Bio-Products (England); silica gel 60 (n°9385) was from Merck (Germany) and the chemiluminescent detection kit was from Tropix (U.S.A.).

The oligonucleotides were synthesized on a 394 automatic DNA synthesizer from Applied Biosystems (U.S.A.). Capillary electrophoresis was performed on a 270A apparatus from Applied Biosystems; the capillary column was a Micro-Gel$_{100}$ (internal diameter 50µm, length 50 cm). The luminometer was a Digene DCR-1 (U.S.A.). NMR spectra were run on a Jeol EX90 spectrometer (Japan).

Preparation of 1-O-(4,4'-dimethoxytrityl)-1,3-butanediol (compound 5)

32.5 mmol of 4,4'-dimethoxytrityl chloride were added to a stirred solution of 1,3-butanediol (25 mmol) in 40 ml of dry pyridine and under argon. The reaction mixture was stirred for 1.5 hours at room temperature. 10 ml of methanol were then added to the reaction mixture, it was then diluted with 150 ml of methylene chloride. The resulting organic solution was washed with a 5% $NaHCO_3$ aqueous solution (3 x 100 ml), dried over $MgSO_4$ and filtered. Solvent evaporation gave a crude residue which was purified by chromatography on a silica gel column using methylene chloride as eluent. Compound 5 was obtained with 69% yield.
^1H NMR($CDCl_3$-TMS): 1.1 ppm, d:3H $-CH_3$; 1.7 ppm, m:2H $-CH_2-CH_2-CHOH-$; 3.3 ppm, m:2H DMT-O-CH_2-; 3.65 ppm, s:6H CH_3O-; 3.9 ppm, m:1H CHOH; 6.7-> 7.5 ppm, m:13H aromatic hydrogens.

Preparation of 1-O-(4,4'-dimethoxytrityl)-3-O-(cyanoethoxydiisopropylamino phosphinyl)-1,3-butanediol (compound 2)

23 mmol of 2-cyanoethoxy-N,N'-diisopropylaminochlorophosphine were added to a solution of 17.3 mmol of compound 5 and 52 mmol of diisopropylethylamine in 52 ml of tetrahydrofuran, under argon. The reaction mixture was stirred for 0.5 hour at room temperature. 100 ml of ethyl acetate were then added to the mixture and the resulting solution was washed with a 5% $NaHCO_3$ aqueous solution (3 x 100 ml), dried over $MgSO_4$ and filtered. Solvent evaporation gave a crude product which was purified by chromatography on a silica gel column using a solution of ethyl acetate 4- hexane 6 as eluent (Yield= 90%).
^{31}P NMR($CDCl_3$): 146.600 ppm (external reference H_3PO_4)

Preparation of 1-O-(4,4'-dimethoxytrityl)-3-buten-1-ol (compound 6)

The synthesis of 6 was performed according to the procedure followed for the synthesis of 5.

Preparation of 1-O-(4,4'-dimethoxytrityl)-3,4-epoxy-1-butanol (compound 7)

Commercial 3-chloroperoxybenzoic acid contained 50% of water. The peracid was dissolved in methylene chloride and the aqueous layer was

removed. To a solution of 17 mmol of 6 in 40 ml of methylene chloride under argon, were added 24.5 mmol of 3-chloroperoxybenzoic acid in 65 ml of methylene chloride. The reaction mixture was stirred for 8 hours, 3-chlorobenzoic acid was filtered and the filtrate was washed with a 5% $NaHCO_3$ aqueous solution (4 x 75 ml), dried over $MgSO_4$, filtered and evaporated to dryness. The crude residue was purified by chromatography on a silica gel column using a solution of hexane 2-methylene chloride 8 as eluent. Compound 7 was obtained with a 80% yield.
^1H NMR ($CDCl_3$-TMS): 1.65->2 ppm, dt:2H CH-$C\underline{H}_2$-CH_2; 2.35->2.90 ppm, m: 3H CH_2-CH-; 3.2 ppm, t:2H CH_2-ODMT; 3.7 ppm, s:6H CH_3O-; 6.65->7.5 ppm, m:13H aromatic hydrogens.

Preparation of 1-O-(4,4'-dimethoxytrityl)-4-amino-1,3-butanediol (compound 8)

In a sealed tube, 7.6 mmol of 7 were heated for 8 hours at 60°C with 25 ml of ammonium hydroxide (32% W/V in water) and 10 ml of acetonitrile. The reaction mixture was then evaporated to dryness and the residue was purified on a silica gel column using a 2 to 10% gradient of methanol in methylene chloride. The yield of 8 was 75%.
^1H NMR ($CDCl_3$-TMS) : 1.7 ppm, m: 2H -$C\underline{H}_2$-CHOH; 2.65 ppm, m: 2H -$C\underline{H}_2$-NH_2; 3.2 ppm, t: 2H DMT-O-CH_2-; 3.7 ppm, s: 6H CH_3-O-; 3.85 ppm, m: 1H C$\underline{H}$-OH; 6.6->7.6 ppm, m: 13H aromatic hydrogens.

Preparation of 6-trifluoroacetamidocaproate-N-hydroxysuccinimide ester (compound 11)

The synthesis of 11 was performed according to a known procedure[20].

Preparation of 1-O-(4,4'-dimethoxytrityl)-4-N-(6-trifluoroacetamidocaproyl)-4-amino-1,3-butanediol (compound 12)

6 mmol of compound 8 and 9 mmol of compound 11 in 25 ml of dry N,N'-dimethylformamide were stirred under argon for 16 hours. The solvent was then evaporated and the residue was purified by chromatography on a silica gel column, the first eluent was a solution of hexane 5- methylene chloride 95, then a 2 to 10% methanol gradient in methylene chloride. The yield of compound 12 was 84%.
^1H NMR ($CDCl_3$-TMS):1.2->1.7 ppm, m:6H -CH_2-; 1.8 ppm, m:2H -CH_2-$C\underline{H}_2$-CHOH-; 2.15 ppm, t:2H -$\overset{O}{\overset{\|}{C}}$-$CH_2$-; 3->3.5 ppm, m:6H -$CH_2$-ODMT, CH_2-NH-; 3.75 ppm, s: 6H CH_3O-; 3.85 ppm, m:1H -C$\underline{H}$OH-; 6.7-7.4 ppm, m: 13H aromatic hydrogens.

Preparation of 1-O-(4,4'-dimethoxytrityl)-3-O-(cyanoethoxydiisopropylaminophosphinyl)-4-N-(6-trifluoroacetamidocaproyl)-4-amino-1,3-butanediol (compound 1)

4,1 mmol of 2-cyanoethoxy-N,N'-diisopropylaminochlorophosphine were added to a solution of 3 mmol of compound 12 and 1.6 ml of diisopropylethylamine in 17 ml of tetrahydrofuran, under argon. The reaction mixture was stirred for 0.5 hour at room temperature. Then, 25 ml of ethyl acetate were added to the mixture and the resulting solution was washed with a 5% $NaHCO_3$ aqueous solution (3 x 20 ml), dried over $MgSO_4$ and filtered. Solvent evaporation gave a crude product which was purified by

chromatography on a silica gel column using a solution of ethyl acetate 85 - hexane 15 as eluent (Yield= 62%).
^{31}P NMR ($CDCl_3$): 147.48 ppm and 148.73 ppm (external reference H_3PO_4).

Preparation of 1,6-O-(4,4'-dimethoxytrityl)-1,2,6-hexanetriol (compound 13)

40 mmol of 4,4'-dimethoxytrityl chloride were added to a stirred solution of 1,2,6-hexanetriol (20 mmol) in 20 ml of dry pyridine and under argon. The reaction mixture was stirred for 2 hours at room temperature. 10 ml of methanol were then added to the reaction mixture, it was further diluted with 100 ml of methylene chloride. The resulting organic solution was washed with a 5% $NaHCO_3$ aqueous solution (3 x 50 ml), dried over $MgSO_4$ and filtered. Solvent evaporation gave a crude residue which was then purified by chromatography on a silica gel column using a 0 to 40% ether gradient in hexane. The yield was almost quantitative.
^1H NMR ($CDCl_3$-TMS): 1.3->1.7 ppm, m:6H $-CH_2-$; 2.95->3.2 ppm, m:4H $-CH_2-O-$DMT; 3.7->3.85 ppm, m:1H -CHOH; 3.75 ppm, s:12H CH_3-O-; 6.7->7.5 ppm, m: 26H aromatic hydrogens.

Preparation of 1,6-O-(4,4'-dimethoxytrityl)-2-O-(cyanoethoxydiisopropyl aminophosphinyl)-1,2,6-hexanetriol (compound 3)

2 mmol of 2-cyanoethoxy-N,N'-diisopropylaminochlorophosphine were added to a solution of 1.5 mmol of compound 13 and 0.8 ml of diisopropylethylamine in 6 ml of tetrahydrofuran under argon. The mixture was stirred for 0.5 hour. 60 ml of methylene chloride were then added to the reaction mixture and the organic solution was washed with a 5% $NaHCO_3$ aqueous solution (3 x 20 ml), dried over $MgSO_4$ and filtered. Solvent evaporation gave a crude product which was purified on a silica gel column, the eluent was a solution of ethyl acetate 3 - hexane 7 (Yield= 84%).
^1H NMR ($CDCl_3$-TMS): 1.15 ppm, d: 12H $CH(CH_3)_2$; 1.3->1.8 ppm, m: 6H $-CH_2-$; 2.4 ppm, m: 2H CH_2-CN; 3.05 ppm, m:4H $-CH_2-O-DMT$; 3.75 ppm, s: 12H CH_3-O-; 3.4->4.1 ppm, m: -CH-O-P-, -CH-N, $-CH_2-O-P$; 6.7->7.6 ppm, m: 26H aromatic hydrogens.

Preparation of 1-O-(4,4'-dimethoxytrityl)-3-O-succinate-1,3 butanediol (compound 14)

6 mmol of 5, 6.1 mmol of 4-dimethylaminopyridine and 6.4 ml of succinic anhydride in 22 ml of dry pyridine were stirred under argon for three days. Toluene was then added to remove pyridine azeotropically (rotavapor p = 20 mm Hg). The residue was then dissolved in 75 ml of methylene chloride. The organic solution was washed twice with a 4 x 10^{-2} M aqueous solution of citric acid, dried over $MgSO_4$ and filtered. After solvent evaporation, the crude mixture was purified on a silica gel column using first a 15 to 0% gradient of hexane in methylene chloride as eluent, followed by a methanol 3 - methylene chloride 7 elution (Yield= 34%).

Preparation of 1-O-(4,4'-dimethoxytrityl)-3-O-(pentachlorophenylsuccinate) -1,3-butanediol (compound 15)

A solution of 2.2 mmol of pentachlorophenol and 3 mmol of 1,3-dicyclohexylcarbodiimide in 14 ml of dry N,N'-dimethylformamide was added under argon to 2 mmol of acid 14. After 24 hours stirring at room

temperature, the precipitated 1,3-dicyclohexylurea was filtered, the reaction solution was evaporated to dryness and the residue was purified on a silica gel column using a hexane 15 - methylene chloride 85 solution as eluent. The yield was almost quantitative.

Preparation of a controlled pore glass solid support bearing 1-O-(4,4'-dimethoxytrityl)-1,3-butanediol residues (compound 16)

The CPG solid support used (Pierce Ref.24875) had a 500Å porosity, it beared primary amine alkyl chains on its surface. 0.5 g of amino CPG solid support, 0.2 ml of triethylamine and 0.385 g of ester 15 in 13 ml of dry N,N'-dimethylformamide were stirred at 37°C for 24 hours. 70µl of acetic anhydride were then added to the reaction mixture to block the remaining primary amine functions. After 10 minutes stirring at 37°C, the solid support was filtered, it was successively washed with 20 ml of N,N'-dimethylformamide, 20 ml of ethanol, 20 ml of dioxane and 20 ml of ether. The support was then dried under reduced pressure (20 mm Hg) for several hours in the presence of phosphorus pentoxide. To determine the number of residue 15 fixed on the support, it was treated with p-toluene sulfonic acid: 10 ml of a 0.1 M p-toluene sulfonic acid solution in dry acetonitrile were added to 8.1 mg of CPG solid support. The orange colour intensity of the liberated 4,4'- dimethoxytrityl cation was determined by spectroscopy (497 nm, ε = 7 10^4). The solid support 16 has fixed 30.2 µmole/g of compound 15.

Solid phase automatic coupling of phosphoramidites 1, 2 & 3

All the trials were performed on a 0.2 µmole scale using a CPG matrix functionalized with a 5'-O-(4,4'-dimethoxytrityl) thymidine. The first condensation was always carried out with a thymidine phosphoramidite, the following ones were performed either with phosphoramidite 1, 2 or 3. The standard procedures were used for the condensations of 1, 2 and 3 except the condensation time which was ten minutes for 1 and 3, and the concentration of phosphoramidite 3 which was 0.4 M.

Chemical condensation of oligonucleotides onto polystyrene microwells or tubes

* amination of aldehyde tubes or aldehyde microwells with 1,6-hexanediamine

Each tube or microwell was treated for 5 hours at room temperature with 250 µl of a 1 M solution of 1,6-hexanediamine in PBS 0.5x (PBS 5x = NaCl 0.15 M, KH_2PO_4 0.02M in water). 50 µl of a 2M aqueous solution of sodium cyanoborohydride were then added to each cup. After 2 hours of incubation at room temperature, the tubes or wells were emptied and washed five times with milli-Q water.

* chemical condensation of 5'-phosphate oligonucleotides onto the aminated microwells or tubes.

This condensation has been performed following the procedure described by S.R. Rasmussen *et al*[21]. The average amount of bound oligonucleotide (30 mer) was 1.4 picomole.

Synthesis of poly-primaryamino probes

The syntheses were performed in a DNA synthesizer on a 0.2 µmole scale. For the synthesis of COMB-like probes (17->30), the labelling units were introduced by condensations of phosphoramidites 1 and 2; the condensation time for 1 was ten minutes. For the oligonucleotides tagged in 3'(OH) (23->30), the modified CPG matrix 16 was used.

Multifork-like probes were prepared by x condensations of phosphoramidite 3 at the 5'(OH) end of the nucleotidic chain followed by a final condensation of phosphoramidite 4. For both phosphoramidites 3 and 4, the condensation time was ten minutes using a 0.4 M solution.

All other conditions were as recommended by Applied Biosystems. After synthesis and classical deprotection, the aminated oligomers were desalted on a 10 ml sephadex G50 column prior to biotinylation.

Biotinylation of the poly primary amino oligonucleotides

20 OD_{260} of aminated oligonucleotide in 120 µl of 0.01 M phosphate buffer pH 7.5 were treated for 16 hours at room temperature with 20 mg of biotinamidocaproate N-hydroxysuccinimide ester in 240 µl of N,N'-dimethylformamide. The crude oligomers were desalted on a 10ml sephadex G50 column and were further purified on a 1.5 mm thick denaturing 20% polyacrylamide gel. The biotinylated oligomers were visualized under short wave UV light, the products were cut out of the gel, and recovered by electroelution (8mA, 15 mM tris-HCl pH 8.3).

Detection limit of polybiotinylated oligonucleotides

Hybridizations were performed in polystyrene tubes coated with a 30 mer oligonucleotide complementary on its 3' end to the 23 mer sequence of the polybiotinylated oligonucleotides, each test was run in duplicate. For each biotinylated oligonucleotide to be tested, a series of eight trials was performed: 1 picog (10^8 copies), 300 femtog, 100 femtog, 30 femtog, 10 femtog, 3 femtog, 1 femtog and 0.3 femtog. A series of 8 hybridizations at the above concentrations was run with a non complementary biotinylated oligonucleotide. A series of 10 blank hybridizations was performed without any biotinylated probe. The detection limit in each series of biotinylated probes corresponded to the smallest oligonucleotide concentration for which the emitted light was superior to the average of the 10 blanks RLU values plus 3 standard deviations. The tubes were saturated for 1 hour at 37°C with a 5% nonfat dried milk TBS 1x solution (TBS 1x = 0.05 M tris pH 7.5, 0.15 M NaCl). The saturating solution was then removed and each tube was prehybridized for 2 hours at 50°C with 100 µl of binding buffer (binding buffer: 5x Denhardt's, 6xSSC, 0.1% SDS, 100 µg/ml herring sperm DNA). After the 2 hours of pre- hybridization, the tube were hybridized for 2 hours at 50°C with 100 µl of binding buffer containing the biotinylated probe. At that time, the tubes were first washed three times with SSC 6x for 10 minutes at 50°C and then once with buffer I (0.1M tris, 2mM $MgCl_2$, 0.05% triton, 1M NaCl). The tubes were then incubated for 30 minutes at room temperature with 100 µl of a streptavidin-alkaline phosphatase solution prepared by dilution of 1 µl of the streptavidin-alkaline phosphatase solution provided by Tropix in 5000 µl of buffer I. Tubes were then washed six times with buffer I. Finally tubes were incubated during 45 minutes with 100 µl of the revelation solution:

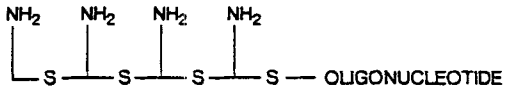
80 µl of CSPD (Tropix), 500 µl emerald (Tropix), 4.42 ml buffer II. (Buffer II: 0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide). Light emission was then red in a luminometer.

RESULTS AND DISCUSSION

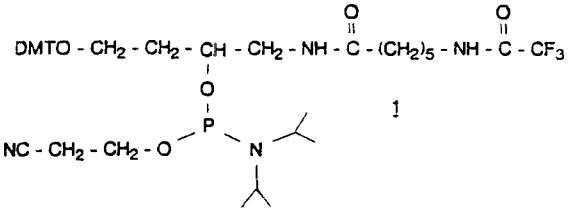
The general aim of this work was to develop non-nucleosidic labelling synthons bearing suitable chemical functions, allowing their introduction,under automatic DNA synthesis conditions, in an oligonucleotidic growing chain or in a non-nucleotidic polymer.

Two types of labelling structures have been designed:

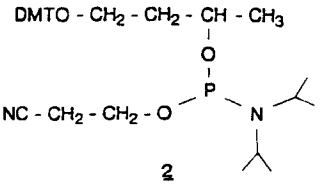
- Comb-like structures wherein several primary amine units



were introduced by condensation of a phosphoramidite 1 derived from -amino-1,3-butanediol, Spacer arms, either between the primary amine units or between the oligonucleotide and the labelling moiety, were then introduced by condensation of phosphoramidite 2, derived from 1,3-butane diol.



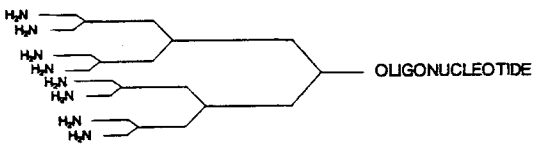
1



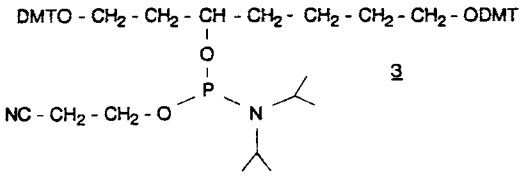
2

In the comb approach, the phosphoramidites 1 and 2 were introduced on both 5' and 3' ends of the synthetic oligonucleotide.

- Multifork-like structures in which the ramification of the

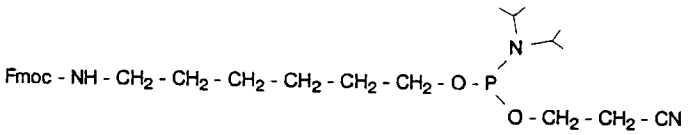


chain was created by condensation of a phosphoramidite 3 derived from 1,2,6-hexanetriol.



3

The primary amine functions were introduced by a final condensation with the traditional phosphoramidite N-Fmoc-O-cyanoethoxydiisopropylamino-phosphinyl-6-amino-1-hexanol 4 (21,3).



4

Synthesis of non-nucleosidic phosphoramidites

Phosphoramidites 1 and 2 have been designed keeping in mind the chemical structure of nucleosidic phosphoramidites, i.e. the binding of the dimethoxytrityloxy group to a primary carbon atom while preserving the attachment of the phosphoramidite function to a secondary carbon atom and the 1,3 space between the two functions.

The synthetic scheme for preparing 1-O-DMT-3-O-cyanoethoxydiiso-propylaminophosphinyl-1,3-butanediol 2 is shown in FIG. 1.
The primary alcohol of 1,3-butanediol was selectively protected, the resulting 1-O-DMT-1,3-butanediol 5 was phosphitylated to yield the desired phosphoramidite 2.
The preparation of phosphoramidite 1 is presented in FIG.2.

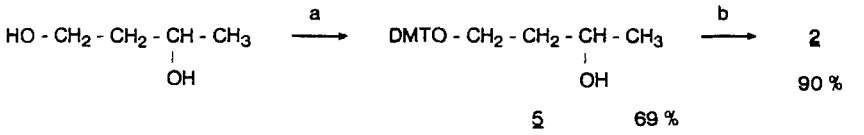


FIG.1: Synthesis of phosphoramidite 2
a: 4,4'-dimethoxytrityl chloride/pyridine
b: 2-cyanoethoxyN,N'-diisopropylaminochlorophosphine/N,N'-diisopropyl ethylamine/THF

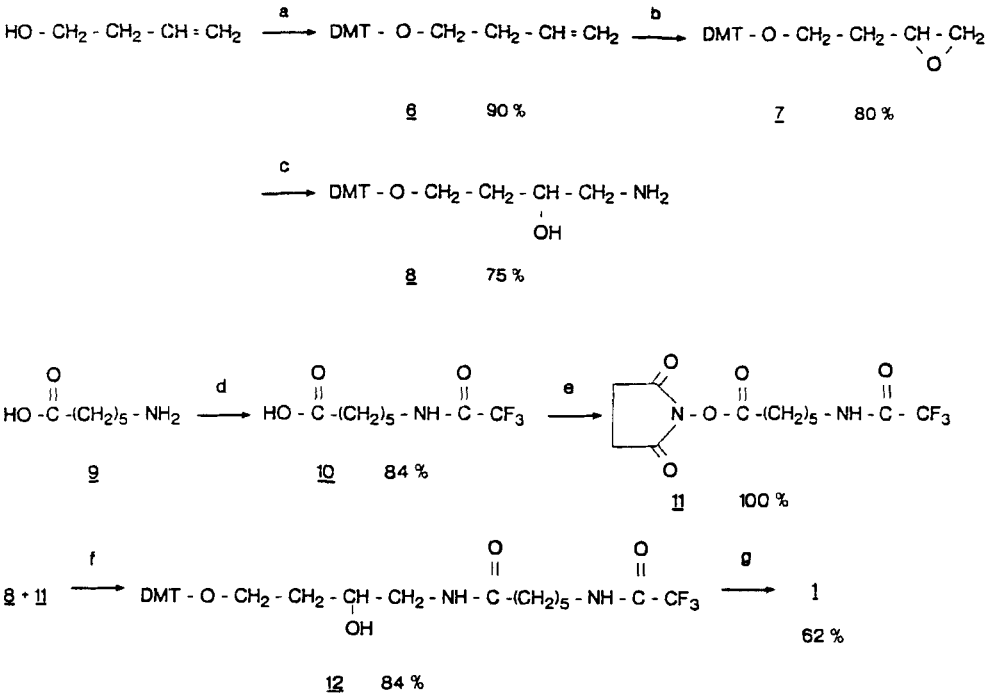


FIG.2 : Synthesis of phosphoramidite 1
a: 4,4'-dimethoxytrityl chloride/pyridine
b: 3-chloroperoxybenzoic acid/CH_2Cl_2
c: ammonium hydroxide 32%(W-V)/CH_3CN/60°C
d: S-ethyl trifluoroacetate/DMF
e: N-hydroxysuccinimide/1,3-dicyclohexylcarbodiimide/CH_2Cl_2
f: DMF
g: 2-cyanoethoxy N,N'-diisopropylaminochlorophosphine/ N,N'-diisopropylethylamine/THF

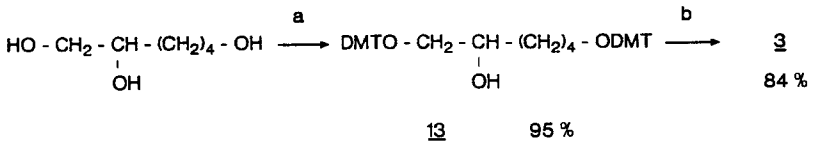


FIG.3 : Synthesis of phosphoramidite 3
a: 4,4'-dimethoxytrityl chloride/pyridine
b: 2-cyanoethoxy N,N'-diisopropylaminochlorophosphine/ N,N'-diisopropylethylamine /THF.

The primary alcohol of 3-buten-1-ol was protected, the resulting O-DMT-3-buten-1-ol 6 was epoxidated and the epoxide 7 was then opened by ammonium hydroxide. On the other hand, the primary amine of 6-aminocaproic acid 9 was protected by condensation with S-ethyl trifluoroacetate to give N-trifluoroacetamido-6-aminocaproic acid 10 which was further activated by the N-hydroxysuccinimide to the corresponding ester 11[20]. Condensation between 8 and 11 followed by phosphitylation yielded the desired phosphoramidite 1.

For the multifork-like structures, the phosphoramidite 3 has been synthesized as described in FIG.3.

The two primary alcohol functions of 1,2,6-hexanetriol were selectively protected, the resulting 1,6-O-DMT-1,2,6-hexanetriol 13 was phosphitylated to yield the phosphoramidite 3.

Synthesis of a controlled pore glass solid support for the 3'(OH) multilabelling of oligonucleotides

In order to introduce a Comb-like structure either at the 5' or 3'(OH) of a synthetic oligonucleotide, we have synthesized a controlled pore glass solid support (CPG) on which we chemically fixed 1-O-DMT-1,3-butanediol 5 by the classical method presented in FIG.4.

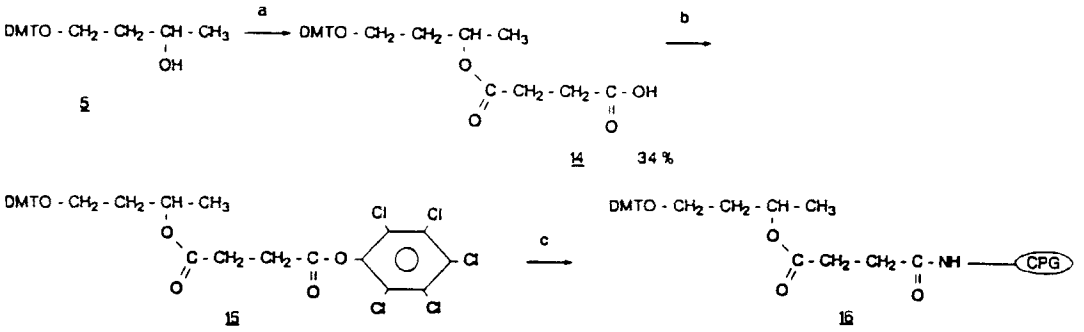


FIG.4 :Preparation of a solid support for the 3'(OH) labelling of oligonucleotides.
a: succinic anhydride/pyridine/4-dimethylaminopyridine
b: pentachlorophenol/1,3-dicyclohexylcarbodiimide/ DMF
c: CPG solid support bearing primary amine functions/Et_3N/DMF

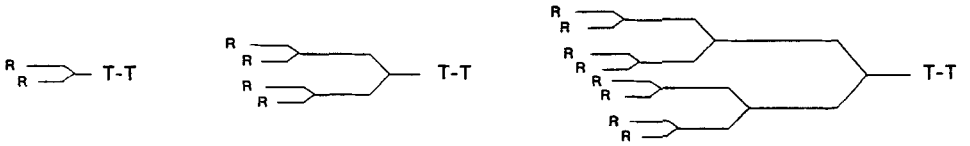
The secondary alcohol derivative 5 was acylated, the resulting carboxylic acid 14 was activated and the ester 15 was later condensed on a controlled pore glass solid support bearing primary amino functions.

Reactivity of phosphoramidites 1, 2 and 3

The reactivity of phosphoramidites 1, 2 and 3 was tested in the DNA synthesizer by condensation of respectively 1, 2 and 3 on a CPG matrix functionalized with a 5' dimethoxytrityl thymidine-thymidine dimer T-T.

The following 5'(OH) modified T-T dimers were successfully prepared: N-T-T, S-T-T, N-S-T-T and S-N-S-T-T in which S (spacer) represents one condensation of phosphoramidite 2 and N (primary amine), one condensation of phosphoramidite 1.

In the case of phosphoramidite 3, the following derivatives (R=OH) were prepared, they corresponded respectively to one, two and three running condensations of 3 on the T-T dimer.



The corresponding primary amino derivatives (R=NH_2) have been prepared by one, two or three condensations of 3 on a T-T CPG solid support followed by a final condensation of phosphoramidite 4.

For all of these condensations, the routinely used coupling parameters of automatic DNA synthesis have been kept. Nevertheless, with phosphoramidites 1 and 3, a best coupling yield was obtained with a condensation time of 10 minutes instead of the classical 25 seconds. Moreover, phosphoramidite 3 was 0.4 M instead of 0.1 M in conventional conditions. Measuring the dimethoxytrityl cation colour intensity after condensations of 3, we observed for the first and second condensation a 95% coupling yield and 90% for the third.

After condensations, the 5' modified T-T dimers were cleaved from the matrix by ammonium hydroxide (32% W/V). The condensation products were then analyzed by capillary electrophoresis; for each condensation, the resulting diastereoisomeric mixture was not resolved. As shown in FIG.5, they all appeared as a single peak, almost pure and essentially free of any T-T dimer.

On the other hand, the new matrix 16 was efficiently used to introduce a label in the 3'(OH) of an oligonucleotide. Indeed, the following polymers were successfully obtained by condensation, in the DNA synthesizer, of 1, 2 or the thymidine phosphoramidite on 16: T-T-S-S, T-T-N-S, N-S-T-T-S-N-S.

Chemical condensation of oligonucleotides onto polystyrene microwells or tubes

The ideal way to immobilize DNA onto a solid phase appears to be formation of a covalent bond at a single point of the molecule,

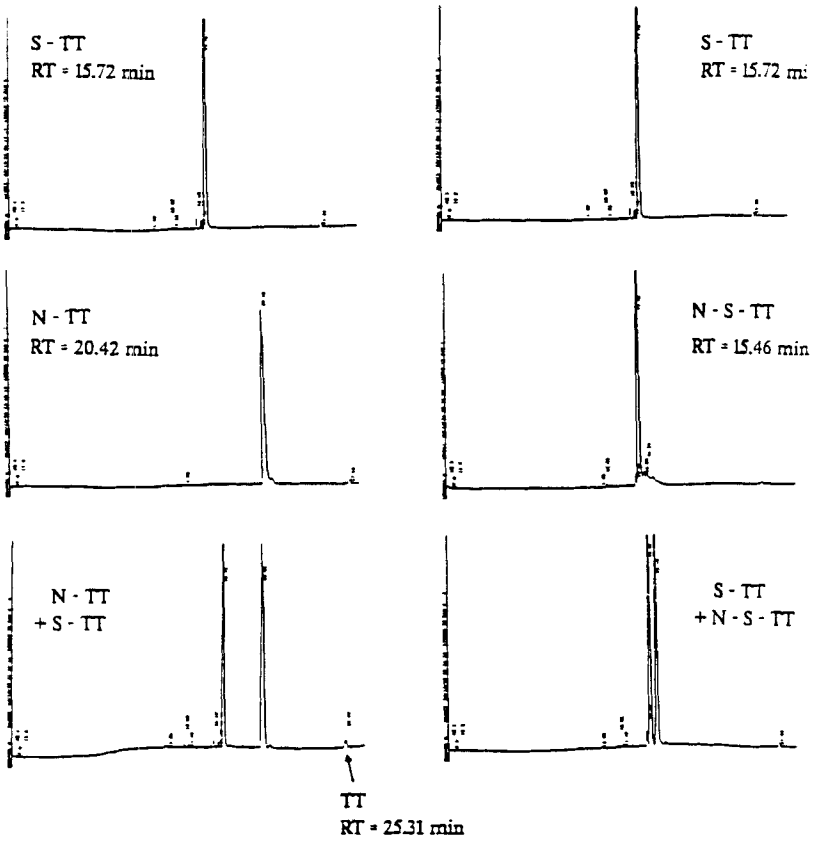


FIG.5 : Reactivity of phosphoramidites 1 and 2
electropherograms of some 5' modified T-T dimers: capillary column MICRO-GEL$_{100}$ - 50µm ID - 50cm, buffer: MICRO-GEL$_{100}$ buffer. Voltage 15kV. Detection 260nm.

preferentially at one extremity. This type of bond has already been achieved using several strategies[21-28]. In our method, a polystyrene surface bearing aldehyde functions was transformed into a primary amino surface by condensation of 1,6-hexanediamine followed by reduction of the resulting imine (FIG.6). Then, carbodiimide-mediated coupling between the primary amine and a 5'-phosphate oligonucleotide (30 mer) was done according to the method described by Rasmussen *et al*[21]. The

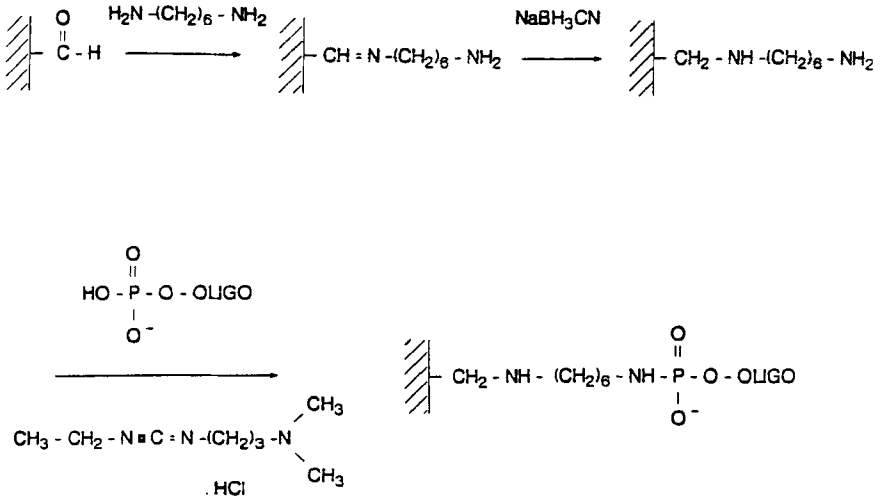


FIG.6 :Chemical condensation of oligonucleotides onto polystyrene microwells or tubes bearing aldehyde functions.

condensation was always performed using 100 µl of oligonucleotide reacting solution; in these conditions, the average amount of bound oligonucleotide was 1.4 picomole.

Synthesis of multi labelled probes; study of their detection limits

In order to demonstrate the validity of our labelling method, we have prepared a series of probes. In the comb labelling approach, several probes 17->22 have been tagged at the 5'(OH) position, others 23->26 at the 3'(OH) position and four of them 27->30 at both 3'(OH) and 5'(OH) extremities. Three other probes 31, 32 and 33 beared a multifork labelling structure at their 5' end (FIG.7).

For all probes, a mono or poly primary amino oligonucleotide was first synthesized and subsequently biotinylated. The conformity of probes was reproducible from one synthesis to another; it was checked by capillary

NSS-OLIGO 17
NSSNSS-OLIGO 18
NSSNSSNSS-OLIGO 19
NSSNSSNSSNSS-OLIGO 20
NSSSSNSSSSNSSSSNSSSS-OLIGO 21
NSSSSSSNSSSSSSNSSSSSSNSSSSSS-OLIGO 22
OLIGO-SSNS 23
OLIGO-SSNSSNS 24
OLIGO-SSNSSNSSNS 25
OLIGO-SSNSSNSSNSSNS 26
NSS-OLIGO-SSNS 27
NSSNSS-OLIGO-SSNSSNS 28
NSSNSSNSS-OLIGO-SSNSSNSSNS 29
NSSNSSNSSNSS-OLIGO-SSNSSNSSNSSNS 30

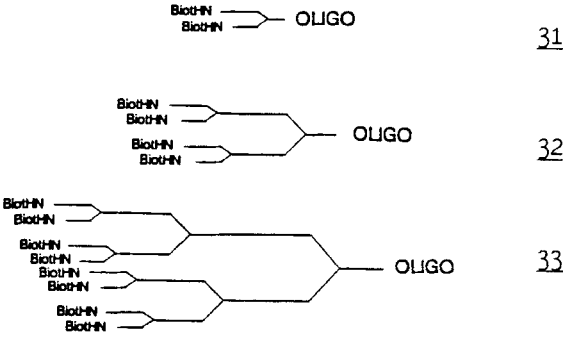


FIG.7 : Comb- and Multifork- labelled probes: S represents the condensation of phosphoramidite 2, N represents the condensation of phosphoramidite 1, followed by biotinylation of the resulting primary amine. The oligonucleotide (OLIGO) is a 23 mer: TTTTCAAAGAAGATGGCAAAACA.

electrophoresis and an example is shown in fig. 8. In the multifork labelling method, the degree of biotinylation was estimated by preparing an "eight" biotins multifork thymidine-thymidine dimer by two different chemical methods: the one described in this paper, i.e. three condensations of phosphoramidite 3 (93%, 96% and 90%), one condensation of phosphoramidite 4 and the biotinylation of the primary amine with d-biotin-N-hydroxysuccinimide ester. The other method consisted of three consecutive condensations of phosphoramidite 3 (89%, 95% and 91%) followed

by a final condensation of 1-N-(N-DMT-biotinyl)-6-O-(cyanoethoxydiisopropylaminophosphinyl)-1-amino-6-hexanol 34 (78%).

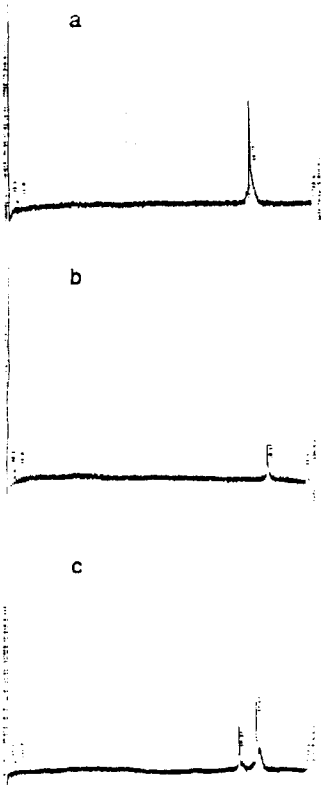


FIG.8 : Electropherogram of probe 19. a) aminated probe; b) corresponding tri biotinylated probe 19; c) a+b mixture. Capillary column MICRO-GEL$_{100}$, 50µmID, 50cm. Buffer: MICRO-GEL$_{100}$ buffer. Voltage 15kV. Detection 260nm.

Analysis of both polybiotinylated T-T dimers by polyacrylamide gel revealed a highest degree of biotinylation by our method. We extended this strategy for the synthesis of a 23 mer oligonucleotide; unfortunately the N-DMT-biotinylated phosphoramidite 34 failed to react on the multifork polyhydroxy network (13%).

On the other hand, the ninhydrin assay[29] performed respectively on the polybiotinylated oligonucleotide 33 and the corresponding poly primary amino oligomer revealed that essentially all the amine functions had been biotinylated (data not shown).

It is interesting to note that phosphoramidites 1, 2 and 3 each contain one asymmetrical carbon; the probes 17->33 are therefore diastereoisomeric. Nevertheless, these probes were not resolved by capillary electrophoresis but the peaks were broadened compared with a classical 23 mer oligonucleotide. In the same way, the diastereoisomeric probes 17->33 did not complicate the polyacrylamide gel purification. To study the detection limits of probes 17 to 33, we performed with each

OLIGO	Number of biotins	Detection limit fg	number of molecules
17	1 5'(OH)	30	3×10^6
18	2 "	30	3×10^6
19	3 "	30	3×10^6
20	4 "	3	3×10^5
21	4 "	10	10^6
22	4 "	3	3×10^5
23	1 3'(OH)	30	3×10^6
24	2 "	10	10^6
25	3 "	3	3×10^5
26	4 "	3	3×10^5
27	2 [3'(OH) + 5'(OH)]	30	3×10^6
28	4 " "	10	10^6
29	6 " "	3	3×10^5
30	8 " "	1	10^5
31	2 5'(OH)	10	10^6
32	4 "	10	10^6
33	8 "	3	3×10^5

FIG. 9: Detection limits of comb- and multifork-labelled probes.

of them a series of hybridizations at different probe concentrations. The hybridizations were carried out in polystyrene tubes coated with a 30 mer chemically linked to the support by its 5' end and complementary to the 23 mer probes on its 3' end. After hybridization, hybrid structures were detected by chemiluminescence. The results are presented in FIG. 9.

It can be seen that probes 33 and 30, bearing eight biotins, provided a detection limit 10 to 30 times higher than that obtained with a single biotin. On the other hand, probes 20, 21 and 22, which differ from each other by the length of the spacer arm (respectively 2, 4 and 6 S units between biotins), presented almost the same detection threshold.

At very low dilution, the RLU values were of course very close to each other but, at higher concentration, the impact of the number of biotins on the light emission was clearly apparent. (FIG.10).

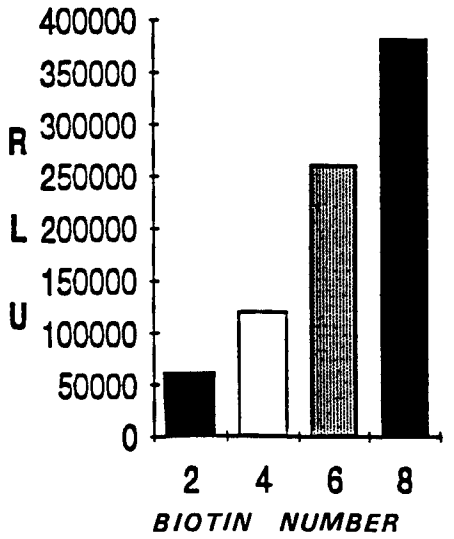


FIG.10: Relative Light Units (RLU) values: the hybridizations were carried out with 3000 femtog. of oligonucleotides ($3x10^8$ copies). The biotinylated oligonucleotides were: 27 (2 biotins), 28 (4 biotins), 29 (6 biotins) and 30 (8 biotins).

CONCLUSIONS

In this paper, we described the very simple and inexpensive synthesis of three phosphoramidites, 1, 2 and 3, for the preparation of 3'(OH) and/or 5'(OH) polyprimary amine oligonucleotides and their subsequent labelling. We showed that the length of spacer arms had no influence on the sensitivity of the probes and that optimal detection could be achieved either with a 5' multifork-like structure carrying 8 biotins or with a comb-like structure carrying 4 biotins on both 5' an 3' ends.

In addition, we described an original method to couple oligonucleotides to polystyrene tubes and used this support to design a chemiluminescent assay for hybridizations. Application of such a capture format may be of interest for the development of specific and sensitive diagnostic kits

based on DNA probes. While writing this paper, we became aware of the paper by Beegs *et al*[18] describing the synthesis of phosphoramidite 3 and its use for the synthesis of tetrabiotinylated oligoribonucleotide probes for antisense affinity selection. The authors, however did not give any comments on the sensitivity of the resulting probes.

ACKNOWLEDGEMENTS

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