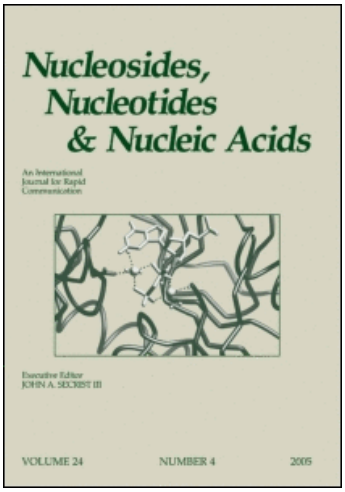

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Solid Phase Non Isotopic Labelling of Oligodeoxynucleotides Using 5'-Protected Aminoalkyl Phosphoramidites: Application to the Specific Detection of Human Papilloma Virus Dna

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To cite this Article De Vos, Marie-Joelle , Cravador, Alfredo , Lenders, Jean-Pierre , Houard, Sophie and Bollen, Alex(1990) 'Solid Phase Non Isotopic Labelling of Oligodeoxynucleotides Using 5'- Protected Aminoalkyl Phosphoramidites: Application to the Specific Detection of Human Papilloma Virus Dna', Nucleosides, Nucleotides and Nucleic Acids, 9: 2, 259 — 273
To link to this Article: DOI: 10.1080/07328319008045137
URL: http://dx.doi.org/10.1080/07328319008045137

NUCLEOSIDES & NUCLEOTIDES, 9(2), 259-273 (1990)

SOLID PHASE NON ISOTOPIC LABELLING OF OLIGODEOXYNUCLEOTIDES USING 5'-PROTECTED AMINOALKYL PHOSPHORAMIDITES : APPLICATION TO THE SPECIFIC DETECTION OF HUMAN PAPILLOMA VIRUS DNA.

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Abstract: Phosphoramidites of thymidine or 2'-deoxyinosine, modified in 5' by the addition of an aminoalkylcarbamate function, were prepared. The derivatized nucleotides can be used in automatic DNA synthesis to tag any oligodeoxynucleotide chain and provide a convenient reactive group for labelling with non radioactive reporters. As an example of application, we show the specific detection of Human Papilloma Virus DNA using a biotin-labelled 29-mer oligodeoxynucleotide entirely prepared on solid support.

INTRODUCTION

Non radioactive systems to label oligodeoxynucleotidic probes received considerable interest for the last few years, especially in view of their impact on diagnostic applications. Well known procedures include direct labelling of DNA with fluorochromes[1-4] and enzymes[5-8] or indirect labelling which requires the detection of biotinylated[9-17] or hapten-tagged[18-21] probes by protein complexes. Most of these strategies rely on the covalent coupling of a reporter group onto DNA. Since both primary 5'-hydroxyl group and aromatic amino groups present on nucleotidic bases are rather unreactive, it is necessary to develop methods which introduce into single stranded DNA adequate and flexible chemical functions allowing the subsequent labelling of the molecules with a wide variety of markers. One of the best choices undoubtedly consists of a primary aliphatic amino group[22-30] which displays a high nucleophilicity. Ideally, the incorporation of such reactive groups into nucleic acids must be done at sites which do not interfere with the hybridization properties of the molecule. In this respect, the 5' end of an oligomer constitutes one logical target for coupling a nucleophilic function, particularly if a spacer alkylated chain separates the oligomer from the reactive function.

Along these lines, we describe a novel method for preparing 5'- aminoalkyl oligodeoxynucleotides. The approach is based on the synthesis of a 5'-N -protected aminoalkyl phosphoramidite of thymidine or 2'-deoxyinosine, which is subsequently condensed to matrix-bound oligodeoxynucleotides of any length. After deprotection of the terminal amino group, a free 5'-primary aminoalkyl group becomes available for the attachment of any reporter molecule, such as biotin, fluorescein, etc. As an example of application, we describe the specific detection of Human Papilloma Virus DNA (HPV16) with a biotin-labelled 29-mer oligodeoxynucleotide entirely prepared by automatic DNA synthesis.

MATERIALS AND METHODS

4,4'-Dimethoxytrityl chloride, tert-butyldimethylsilyl chloride and 1,1'-carbonyldiimidazole were purchased from Janssen Chimica. 1,6-Diaminohexane and 2-(4-biphenylyl)prop-2-yl 4-methoxycarbonylphenyl carbonate were from Fluka. Tetrabutylammonium fluoride trihydrate was purchased from Aldrich, tetrazole was from Cruachem and sulfosuccinimidyl 6-(biotin-amido) hexanoate was from Pierce. Trichloroacetic acid was obtained from Merck, thymidine and 2'-deoxyinosine were purchased from Yamasa Shoyo Co, Ltd. 1,4-dioxane and tetrahydrofuran (Janssen Chimica) were distilled from lithium aluminium hydride; acetonitrile and methylene chloride (Janssen Chimica) were distilled from calcium hydride. Silica gel 60 (n°9385) was from Merck. Thin layer chromatography (tlc) and preparative layer chromatography (plc) were carried out respectively on Merck analytical silica gel plates (n°5719) and on Merck preparative silica gel plates (n°13895).

^{31}P NMR spectra were run on a BRUKER WP 80 NMR spectrometer operating at 32.44 MHz and measuring downfield from 5% H_3PO_4 in DMSO-d6 as external reference, downfield values being positive. FAB mass spectra were recorded on a VG-70S spectrometer.

Deoxyoligonucleotides were synthesized according to published procedure[31] with fully protected home made β-cyanoethyl phosphoramidites[32] on an Applied Biosystems 380A DNA synthesizer. The nitrocellulose filters were purchased from Amersham. Bovine serum albumin, fraction V (BSA), and polyvinylpyrrolidone (PVP) were from Sigma Chemical Co. Ficoll was from Pharmacia Fine Chemicals. The DNA detection system kit and sodium dodecyl sulfate (SDS) were purchased from Gibco Bethesda Research Laboratories (BRL). Herring sperm DNA and triton X-100 were from Boehringer Mannheim.

Synthesis of 5'-O-(4,4'-dimethoxytrityl) nucleosides was performed according to a described procedure[33].

Synthesis of 5'-O-(4,4'-dimethoxytrityl) 3'-O-(tert-butyldimethylsilyl) nucleoside 2a or 2b.

A solution of 16 mmol of tert-butyldimethylsilyl chloride in 40 ml of dry N,N-dimethylformamide was added to a mixture of 8 mmol of 5'-O-(4,4'-dimethoxytrityl) nucleoside and 32 mmol of imidazole under Argon. The reaction mixture was stirred for 5 hours at 20°C and then poured into iced water (400 ml). The white precipitate was filtered and dissolved in methylene chloride (100 ml). The resulting solution was washed twice with a 5% $NaHCO_3$ aqueous solution and dried over Na_2SO_4. Filtration and solvent evaporation yielded compounds 2a or 2b which were sufficiently pure to be used in the next chemical transformation. Yield : 97% for 2a and 80% for 2b.

Synthesis of 3'-O-(tert-butyldimethylsilyl) nucleoside 3a or 3b.

8 mmol of 2a or 2b were treated for 1 hour at room temperature with 100 ml of a 3% trichloroacetic acid methylene chloride solution. The reaction mixture was then washed with a 5% $NaHCO_3$ aqueous solution until neutralization. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated and the residue was purified by chromatography on a silica gel column using a 0 to 30% gradient of ethyl acetate in methylene chloride. Yield : 88% for 3a and 75% for 3b.

Preparation of 2-(4-biphenylyl)propyl-2-oxycarbonylamido-6-aminohexane 5.

10 mmol of 1,6 -diaminohexane and 2.5 mmol of 2-(4-biphenylyl) prop-2-yl 4-methoxycarbonylphenyl carbonate in 40 ml of dry dioxane were stirred for 20 hours at room temperature. The reaction mixture was then diluted with 60 ml of methylene chloride, washed twice with water and twice with a 5% $NaHCO_3$ aqueous solution. The solution was then dried over Na_2SO_4, filtrated and evaporated. The residual viscous oil was purified by chromatography on a silica gel column using a 0 to 5% gradient of methanol in methylene chloride and 0.1% triethylamine. Compound 5 was obtained with 82% yield.

Preparation of 5'-O-[2-(4-biphenylyl) propyl-2-oxycarbonylamido 6-hexylamidocarboxy] 3'-O-(tert-butyldimethylsilyl) nucleoside.

3.3 mmol of 1,1'-carbonyldiimidazole in 5 ml of dioxane were added to a stirred solution of 3a or 3b (3 mmol) in 12 ml of dry dioxane under argon. The reaction mixture was stirred for 5 hours at 50°C and then cooled to room temperature. 4 mmol of monoprotected 1,6 -diaminohexane 5 in 7 ml dry N'N-dimethylformamide were added to the reaction mixture. It was stirred for 20 hours at room temperature and then diluted with 100 ml methylene chloride. The solution, after washing with a 5% $NaHCO_3$ aqueous solution, was dried over Na_2SO_4 and filtered. Solvent evaporation gave a crude residue which was purified by chromatography on a silica gel column using a 0 to 50% gradient of ethyl acetate in methylene chloride. The thymidine derivative was obtained with 94% yield and the inosine derivative with 66% yield.

Preparation of 5'-O-[2-(4-biphenylyl) propyl-2-oxycarbonylamido-6-hexylamidocarboxy] nucleoside 4a or 4b from the corresponding 3'-O-(tert-butyldimethylsilyl) derivative.

5.6 mmol of tetrabutylammonium fluoride trihydrate were added to a solution of 2.8 mmol of 5'-O-[2-(4-biphenylyl) propyl-2-oxycarbonylamido-6-hexylamidocarboxy] 3'-O-(tert-butyldimethylsilyl) derivative of thymidine or 2'-deoxyinosine in 50 ml of tetrahydrofuran. The reaction mixture was stirred for one hour at room temperature, then diluted with 50 ml of methylene chloride and washed twice with a 5% $NaHCO_3$ aqueous solution. The organic layer was then dried over Na_2SO_4 and filtered. Evaporation of the solvents yielded an oily solid which was further purified by chromatography on a silica gel column using a 0 to 10% gradient of methanol in methylene chloride. Yield : 90% for 4a and 93% for 4b.

Preparation of 5'-O-[2-(4-biphenylyl) propyl-2-oxycarbonylamido-6-hexylamidocarboxy] thymidine 4a from thymidine.

Thymidine was first coevaporated twice with pyridine (10 ml). A solution of 10 mmol of anhydrous thymidine in 20 ml of dry N,N-dimethylformamide under argon was cooled at -10°C (ice-water-NaCl bath). 10 mmol of 1,1'-carbonyldiimidazole in 10 ml dry dimethylformamide were added. The reaction mixture was stirred at -10°C for 3 hours, and then allowed to reach room temperature.

10 mmol of N-monoprotected 1,6-diaminohexane 5 in 25 ml N,N-dimethylformamide were added to the reaction mixture which was further stirred for 20 hours at room temperature. The reaction solution was then diluted with a 1-1 methylene chloride-tetrahydrofuran solution and washed with a 5% $NaHCO_3$ aqueous solution. The resulting organic solution was dried over Na_2SO_4, filtrated and evaporated, yielding crude compound which was purified by preparative layer chromatography on silica gel plates (eluent 10% methanol in methylene chloride RF: 4a: 0.4; 6a: 0.55; 7a: 0.65). Yield : 4a: 60%; 6a+7a: 6%.

Preparation of 5'-O-[2-(4-biphenylyl) propyl-2-oxycarbonylamido-6-hexylamidocarboxy] 3'-O-(2-cyanoethyl N,N-diisopropylaminophosphoramidite) nucleotides 1a and 1b.

Phosphitylation of 4a and 4b was carried on according to a described procedure[32].

Compound 1a analytical data : ^{31}P n.m.r. spectrum (DMSO-d6) singlets at +148.22 and +148.10 ppm. Anal. Calcd for $C_{42}H_{59}O_9N_6P$: C, 61.30; H, 7.23; N, 10.21. Found : C, 60.85; H, 7.53; N, 9.58. FAB mass spectra using Xe at 8 kV accelerating voltage: FAB(-) : (matrix glycerol-DBU): 821 $(M-H)^-$, 583, 441, 275, 241, 183, 164. FAB(+) : (matrix thioglycerol) 195 : biphenylylisopropyl cation.

Compound 1b analytical data : ^{31}P n.m.r. spectrum (DMSO-d6) singlet at +147.81 ppm. Anal. Calcd for $C_{42}H_{57}O_8N_8P$: C, 60.56; H, 6.90; N, 13.45. Found : C, 60.01; H, 7.49; N, 13.27.

FAB mass spectra using Xe at 8 kV accelerating voltage. FAB(-) : (matrix glycerol-DBU): 831 (M-H), 593, 451, 233, 135. FAB(+) : (matrix thioglycerol) 195 : biphenylylisopropyl cation.

Deprotection reaction of 5'-O-(4,4'-dimethoxytrityl)thymidine and 5'-O-[2-(4-biphenylyl) propyl-2-oxycarbonylamido-6-hexylamidocarboxyl thymidine 1a.

Both 5' protected nucleosides were respectively stirred in a 3% trichloroacetic acid solution in methylene chloride. Both deprotections were followed by TLC silica gel plates with a 10% methanol-methylene chloride solution as eluent. After 10 minutes, both deprotections were complete.

Kinetic studies in liquid phase of the coupling reactions[34] between 3'-O-(levulinyl) thymidine and phosphoramidites 1a or 8.

The two reactions were run in parallel : 0.03 mmol of 3'-O-levulinyl thymidine in 1 ml dry acetonitrile was added to a solution of 0.045 mmol of phosphoramidite 1a or 8. 0.09 mmol of tetrazole in 0.5 ml acetonitrile was then added to the reaction mixture. The condensations were followed by TLC silica gel plates with a 5% methanol-methylene chloride solution as eluent. (RF: 1a or 8 : 0.85 ; 3'-O-(levulinyl) thymidine : 0.45 ; dimers: 0.55). After 15 minutes, both reactions were complete.

Solid phase coupling of phosphoramidite 1a and 8 onto a T derivatized CPG support.

The synthesis of both dimers 5'-O-(aminoalkyl)TT and TT were carried out on a 0.2 µmole scale according to standard procedures[31]. After phosphoramidite condensation, capping and phosphorus oxydation, the 5'-O-protecting group was removed for both synthesis and the respective dimers were cleaved from the solid support with a 25% ammonium hydroxide solution and treated for 16 hours at 55°C. Dimers were analysed by HPLC before and after treatment at 55°C.

Preparation of the 5'-O-(aminoalkyl) oligonucleotides specific for HPV16 DNA and their biotinylation.

The synthesis were carried out on a 0.2 µmole scale. Both sequences were identical up to the twenty-eighth nucleotide; the last phosphoramidite coupling involved either the thymidine derivative 1a or the 2'-deoxyinosine derivative 1b.

In the case of the 5'-aminoalkylthymidine oligomer, the synthesizer was programmed "DMT off Manu" and the 5' terminal primary amino group was biotinylated on solid phase with sulfosuccinimidyl-6-(biotinamido) hexanoate following a described procedure[24]. Cleavage

from the support and removal of protecting groups were achieved with concentrated ammonia (16 hours at 50°). After lyophilization, the crude oligomer was desalted on a 10 ml Sephadex G50 column (eluent triethyl ammonium bicarbonate 10 mM) and was further purified on a 1.5 mm thick denaturating 20% polyacrylamide gel. The biotinylated oligomer was visualized under short wave UV light; the product was cut out of the gel, recovered by electroelution (8mA; 15 mM Tris-HCl pH 8.3) and lyophilized.

The 5'-aminoalkyl 2'-deoxyinosine 29 mer was automatically cleaved from the solid support by concentrated ammonia. The resulting solution was treated for 16 hours at 50° Lyophilization gave a crude oligomer which was desalted and purified as described above. Biotinylation of the resulting 5'-aminoalkyl oligomer by sulfosuccinimidyl 6-(biotinamido) hexanoate was carried out in solution following a described procedure[24].

Hybridization and detection procedures

DNA derived from HPV6b, 11, 16, 18 and 33 clones was mixed with 1 µg of herring sperm DNA, denaturated in 0.5 M NaOH for 15 minutes and spotted onto nitrocellulose filters using a Bio-Dot filtration apparatus (Bio-Rad). The filters were then air dried and baked for 2 hours under vacuum.

The filters were prehybridized for 2 hours at 45°C in 5XSSPE (1XSSPE = 0.18 M NaCl, 10 mM Na_2HPO_4, 1 mM EDTA pH 8), 0.1% Ficoll, 0.1 % BSA, 0.1% PVP, 0.5% SDS, 0.2 mg/ml herring sperm DNA. The filters were hybridized with 1 µg of biotinylated oligomer per ml of fresh prehybridization solution at 45°C for at least 14 hours. The membranes were washed twice for 15 minutes in 3XSSC (1XSSC = 0.15 M NaCl, 0.015 M trisodium citrate pH 8), 0.1 % SDS at room temperature and for 10 minutes in 6XSSC, 0.1 SDS at 45°C.

Colorimetric detection

The colorimetric detection of the bound biotinylated probes with streptavidin and biotinylated calf intestinal alkaline phosphatase were performed with the BRL DNA detection system according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Synthesis of 5'-N-protected aminoalkyl phosphoramidites of thymidine and 2'-deoxyinosine.

A flexible method has been developed for the non-radioactive labelling of oligodeoxynucleotides. It was designed to be compatible with modern methods of automatic DNA synthesis and to provide maximal versatility in the use of reporter groups. The first part of the methodology consists of the synthesis of 5'-N-protected aminoalkyl

phosphoramidites which can be used in the last condensation step of automatic oligonucleotidic synthesis to generate 5'-aminoalkyl oligomers. Two building blocks were synthesized (Fig.1); the first one, 1a, is a phosphoramidite derivative of thymidine, and the second one, 1b, is a phosphoramidite of 5'-modified 2'-deoxyinosine. This last compound was chosen because 2'-deoxyinosine presents no or little destabilizing effects during DNA/DNA hybridization procedures[35].

The routes for the synthesis of both thymidine and 2'-deoxyinosine phosphoramidite derivatives were rigorously similar and are illustrated in figure 2. Both nucleosides did not require any protection on the base moiety. They were first converted into corresponding 5'-dimethoxytrityl derivatives[33] which were then protected on their 3'-secondary hydroxyl group by t-butyldimethylsilyl chloride. This protecting agent was chosen because it resisted efficiently subsequent reaction steps[36]. 3' protected nucleosides 3a and 3b were then generated by detritylation. Activation of the 5'-hydroxyl group with 1,1'-carbonyldiimidazole produced 5'-imidazolyl derivatives which were shown to undergo a clear nucleophilic displacement by reaction with N-monoprotected 1,6-diaminohexane (compound 5, Fig. 3). It is interesting to note the selective monoprotection of 1,6-diaminohexane when treated with 0.25 eq of 2-(4-biphenylyl)prop-2-yl 4-methoxycarbonyl-phenyl carbonate in 1,4-dioxane at room temperature for 24 hours. Under this conditions, compound 5 was obtained with 82% yield without any trace of N,N'-diprotected derivative.

At last, the silyl protecting group was cleaved with tetrabutyl ammonium fluoride in tetrahydrofuran and the products 4a and 4b were phosphitylated[31] to yield the desired 5'-modified 3'-O-(2-cyanoethyl N,N-diisopropylamino phosphoramidite) derivatives of thymidine 1a and 2'-deoxyinosine 1b. Phosphoramidites 1a and 1b were identified by ^{31}P NMR, by FAB Mass Spectrometry and by elemental analysis (see Materials and Methods). The synthetic route described in Fig. 2 can be improved if one takes into account the difference of reactivity between the 5'-primary hydroxyl group and the 3'-hydroxyl group of the starting nucleosides. As seen in Fig. 4, thymidine can be efficiently 5'-aminoalkylated directly without protection of the 3'-hydroxyl group. Compound 4a was obtained with a 60% yield; no conditions however, could be found to eliminate the formation of compounds 6a and 7a. Nevertheless, this one step synthesis of 4a from thymidine offers a serious improvement compared to the one described in Fig.2 in which the synthesis of 4a was performed with a similar overall yield but required 5 steps from thymidine.

Chemical stability of 5'-N-protected aminoalkyl phosphoramidite derivatives.

Removal of classical protecting groups like dimethoxytrityl from 5' positions on nucleotides is usually followed by monitoring absorbance increases at 498 nm. This procedure cannot

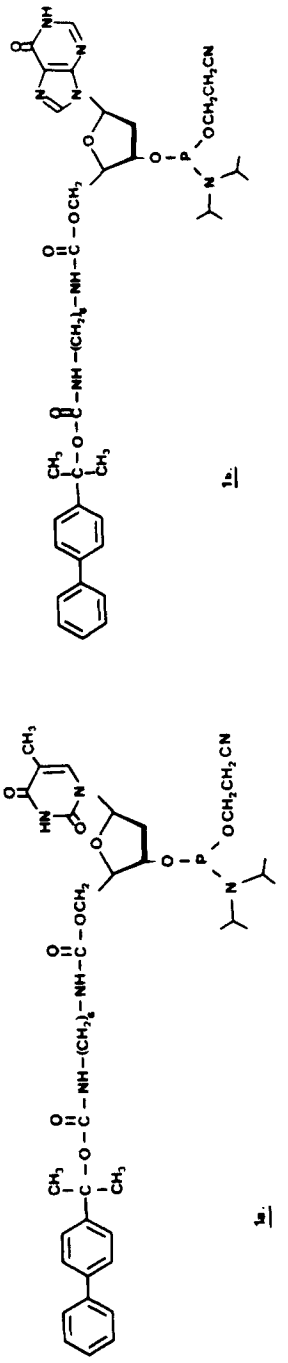

Figure 1: Building blocks for the synthesis of 5'-aminoalkyl oligomers.

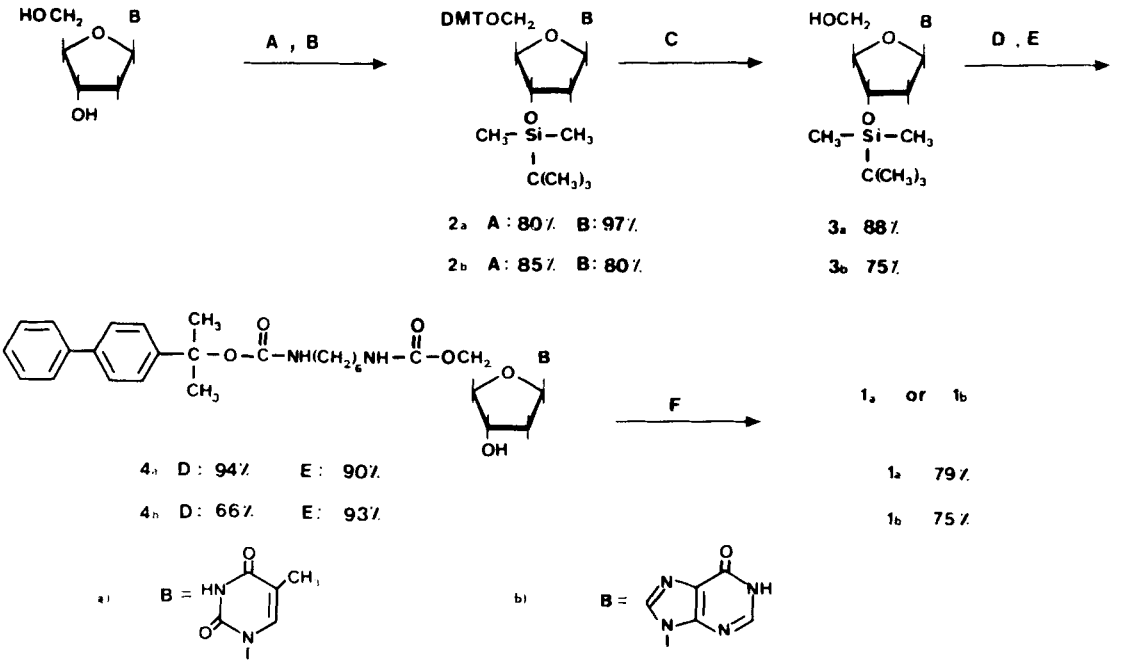

Figure 2: Preparation of the 5'-modified phosphoramidites of thymidine and 2'-deoxyinosine.
Reagents : A, 4,4'-dimethoxytrityl chloride/pyridine;
B, tert-butyldimethylsilylchloride/imidazole/THF;
C, trichloroacetic acid 3% in CH_2Cl_2;
D, 1,1'-carbonyldiimidazole/dioxane/50° then N-[(p-biphenylyl) isopropyloxycarbonyl]-1,6- diaminohexane 5/DMF/RT;
E, tetrabutyl ammonium fluoride/THF;
F, 2- cyanoethoxy N,N-diisopropylaminochlorophosphine/N,N-diisopropyl ethylamine/ CH_2Cl_2.

be used for the amino protecting moiety of phosphoramidites 1a or 1b since no sufficient chromogenic effect is generated by the release of the (p-biphenylyl) isopropyloxycarbonyl group. Therefore, we relied on mobility parameters in thin layer chromatography to follow, in solution, the deprotection step. We found that under classical acidic deprotection conditions, such as 3% CCl_3COOH in CH_2Cl_2, the kinetics of deprotection reaction of the 5' hydroxyl group protected by DMTr and of the alkylaminogroup protected by (p-biphenylyl) isopropyloxycarbonyl are similar. Consequently, (p-biphenylyl) isopropyloxycarbonyl protecting group can be removed in the automatic DNA synthesizer using the same deprotecting conditions as for dimethoxytrityl. We also compared the condensation kinetic in liquid phase of the phosphoramidite derivative of thymidine (compound 1a, Fig.1) and of 5'-

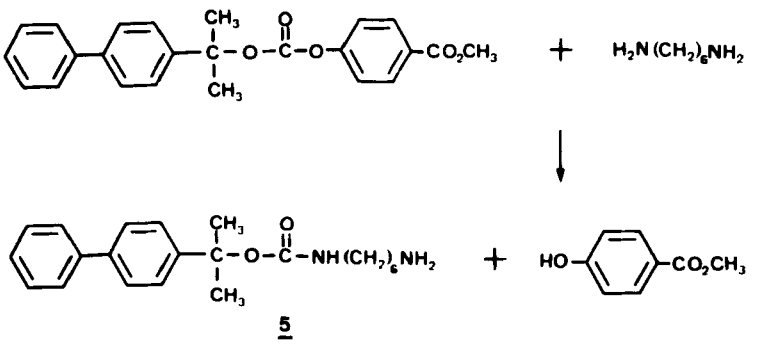

Figure 3 : Preparation of N-(p-biphenylyl) isopropyloxycarbonyl 1,6-diaminohexane. 5

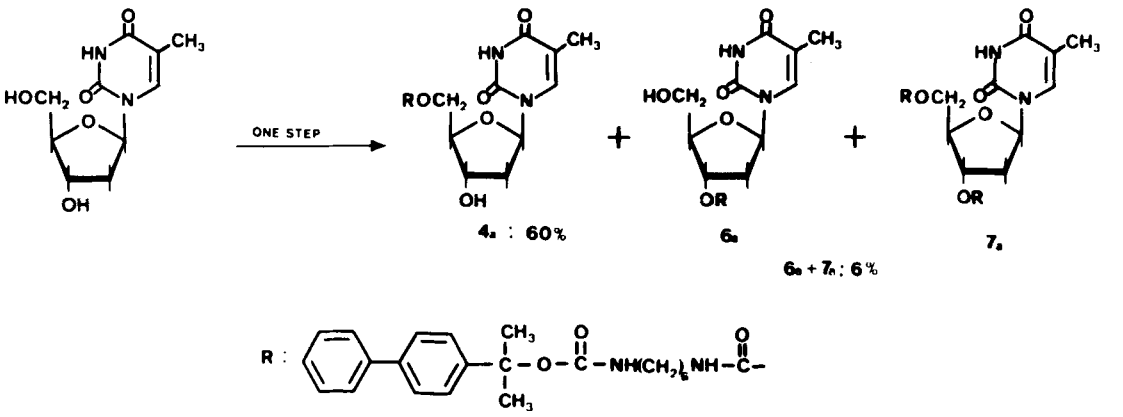

Figure 4 : Direct 5'-aminoalkylation of thymidine.
Reagents: 1,1'-carbonyldiimidazole/DMF/- 10°/3 hours, then 5/DMF/RT/16 hours.

O-dimethoxytrityl 3'-O-(2-cyanoethyl N,N-diisopropylamino phosphoramidite) thymidine (compound 8, Fig.5) on a 3'-protected thymidine nucleoside. In reaction conditions which are routinely used in automatic DNA synthesis (tetrazole-CH_3CN), both compounds 1a and 8 condensed to 3'-protected thymidine with similar efficiencies. These experiments were repeated in an automatic DNA synthesizer, using a CPG matrix functionalized with thymidine. After condensation, acidic deprotection of 5' protecting groups was performed automatically and the dimers were cleaved from the matrix by ammonium hydroxide (25% w/v). The products, TT dimer (10) and 5'-aminoalkyl TT dimer (9), were then analyzed by HPLC. As seen in Figure 5, the 5'-aminoalkyl TT dimer (9) is essentially free of any TT

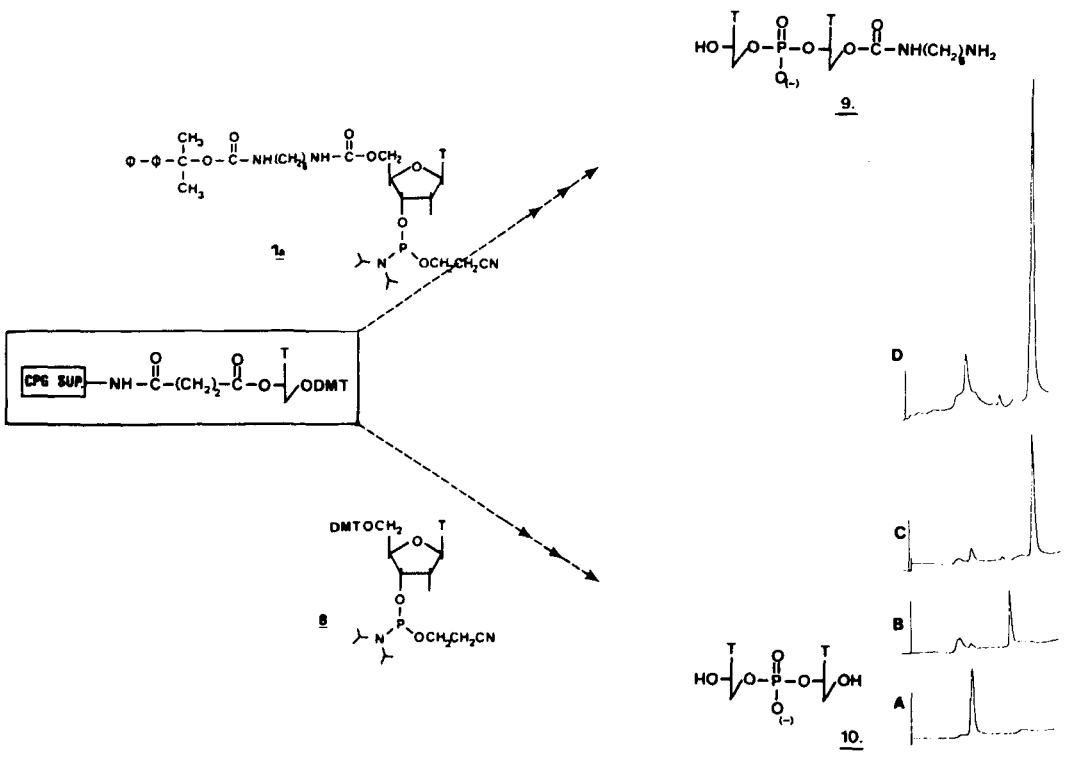

Figure 5 : Phosphoramidite 1a condensation study on solid support. Stability of the resulting dimer: HPLC C18 Reverse Phase elution profiles on a PRP-1 column:
A. Thymidine R.T.9';
B. thymidine-thymidine dimer 10 R.T. 15';
C. 5'-aminoalkyl thymidine-thymidine dimer 9 after ammoniacal cleavage from the solid support R.T. 18.4';
D. dimer 9 after 10 hours in NH_4OH 25% at 55°C.
The initial conditions of the chromatograms shown are 100% solvent A and a linear gradient to 40% of solvent B over 25' at a flow rate of 1ml/min. Solvent A: triethylammonium acetate 0.1 M pH 7.5. Solvent B: 50% triethylammonium acetate 0.1 M pH 7.5, 50% CH_3CN.

dimer. This indicates that the carbamate function present in the final product resists the conditions used to cleave the dimer from the matrix; it is still stable after ten hours at 55°C in 25% NH_4OH.

Automatic synthesis and labelling of 5'-aminoalkyl oligomers specific for HPV16 DNA.

As an application of our methodology to diagnostic purposes, we synthesized a 29-mer oligodeoxynucleotidic probe hybridizing specifically with Human Papilloma Virus DNA, type

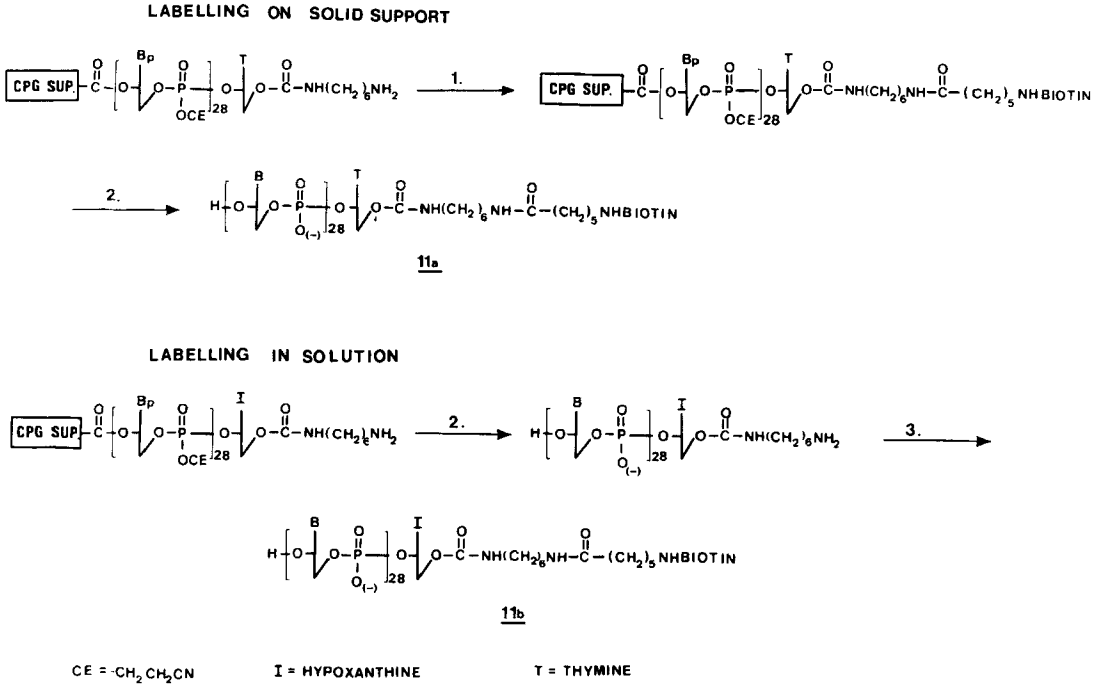

Figure 6 : Labelling of the 5'- aminoalkyl icosanonamers specific to HPV16:
5'-TACGCACAACCGAAGCGTAGAGTCACACT-3' 11a and
5'-IACGCACAACCGAAGCGTAGAGTCACACT-3' 11b.
Reagents: 1. sulfosuccinimidyl 6-(biotinamido)hexanoate 0.1M in dry DMF/ 10 hours.
2. NH_4OH 25%/classical conditions.
3. sulfosuccinimidyl 6-(biotinamido)hexanoate 0.1M in HEPES buffer 0.2M pH 7.6

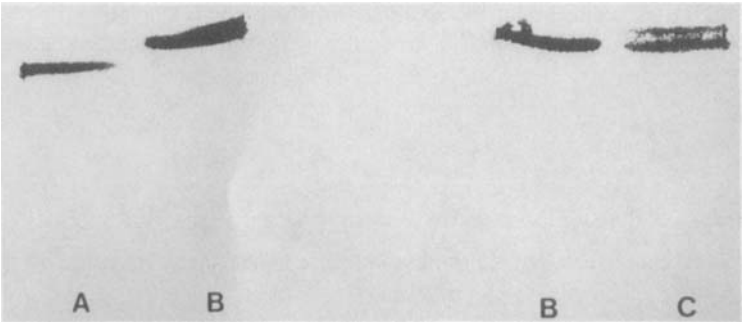

Figure 7 : Electrophoretic mobility of :

A 5'-hydroxyl 28-mer :
5' ACGCACAACCGAAGCGTAGAGTCACACT 3'
B 5'-aminoalkyl 29-mer:
5' H_2N-alkyl-IACGCACAACCGAAGCGTAGAGTCACACT 3'
C 5'-biotinylated 29-mer:
5' biot.NH-alkyl-IACGCACAACCGAAGCGTAGAGTCACACT 3'

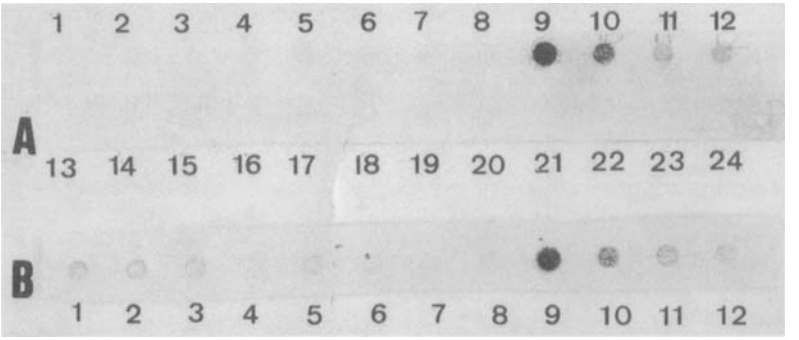

Figure 8 : Hybridization of the biotinylated probes to immobilized HPV cloned DNA. (A) 11a and (B) 11b probe hybridized to 10, 1, 0.1 and 0.01 pmol of HPV6b clone (1,2,3,4), of HPV11 clone (5,6,7,8), of HPV16 clone (9,10,11,12), of HPV18 clone (13,14,15,16) and of HPV33 clone (17,18,19,20). Both probes hybridize exclusively to HPV16 DNA with a sensitivity detection level of 0.1 pmol.

16[37]. The synthesis was performed on a DNA synthesizer under routine conditions. The last condensation step involved either the 5'-aminoalkyl phosphoramidite derivative of thymidine or the derivative of 2'-deoxyinosine (compounds 1a and 1b respectively, Fig.1). After classical acidic deprotection of the 5' primary amine, resulting 5'-aminoalkyl oligomers were biotinylated either on solid phase or in solution[24] (Figure 6). Labelled oligomers were eventually purified by electrophoresis on polyacrylamide gels in denaturing conditions. As seen in Figure 7c, biotinylation decreases the mobility of oligomers to some extent; the mixture of labelled and unlabelled oligomers is clearly resolved and the recovery of the biotinylated species can be done without contaminating the product with unlabelled material such as 5'-aminoalkyl 29-mer or 5'-hydroxyl 28-mer (Fig.7, A and B). We made no attempt for improving the yield of biotinylation.

Specific colorimetric detection of HPV16 DNA with N-biotinylated aminoalkyl oligomers.

Purified biotinylated 29-mer 11a and 11b were used to detect homologous sequences present in HPV DNA. Probes were hybridized in standard conditions to nitrocellulose-bound DNA prepared from different HPV clones[37]. Hybrids were then revealed colorimetrically using the commercial DNA Detection System™. As shown in Fig.8, biotinylated probes recognize exclusively their specific target, HPV16 DNA. This indicates that the additional nucleotide and spacer chain present on the oligomers do not impede the hybridization processus between complementary sequences. In fact, the use of 2'-deoxyinosine, instead of other nucleotides, provides a fairly general method to tag any

probe without destabilizing DNA duplexes in hybridization conditions. Using the conditions described in Materials and Methods, it is possible to detect down to 100 attomoles of HPV DNA within four hours of colour development. This level is similar to the one obtained with ^{32}P-labelled oligomer probes after three hours of autoradiography[37]. Substitution of biotin by a more sensitive reporter group could possibly extend the detection limit to even lower numbers.

Acknowledgements

We thank A. Van Elsen for technical support, Dr. M. Herin, C. Delatour and S. Beuze, from Continental Pharma-Searle (Mont-Saint-Guibert) for providing us FAB Mass Spectra and elemental analysis results. This work was supported by the Walloon Region (Belgium), Improbio S.A. and IRE-Celltarg (Fleurus, Belgium).

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Received September 4, 1989.