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SYNTHESIS AND USE OF 4-HYDROXYPHENYL DERIVATIZED PHOSPHORAMIDITES IN THE SELECTIVE RADIOIODINATION OF OLIGONUCLEOTIDE PROBES.

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SUMMARY

Synthetic oligonucleotides probes are nowadays readily available and widely used in the identification of specific gene sequence from bacteria, viruses, in the diagnostic of human genetic diseases as well as in the development of antisense therapy. Much effort has been devoted to the development of non radioactive probes, nevertheless the radioactive labelling is still extensively used for research purposes. In this study we propose a new and simple synthesis of 4-hydroxyphenyl-oligonucleotide conjugates which upon reaction with ^{125}I give rise to radiolabelled probes. We describe two types of phosphoramidite reagents which allow the selective incorporation of a 4-hydroxyphenyl group at a specific site of an oligonucleotide in the course of its chemical synthesis. The radioiodination step is fast and specific of the hydroxyphenyl group and shows a high incorporation yield.

Key words: Iodine-125, Oligonucleotide, Phosphoramidite, Radiolabelling.

INTRODUCTION

Nucleic acids hybridation assays have become a powerful tool for the study of gene structure and in the detection of pathogenic agents (1). Classical methods (Southern, Northern, dot blots) and PCR technology make use of oligonucleotides (ODN) probes which are easily available since the development of automated synthesizers based on the phosphoramidite approach (2). The most popular radiolabelling procedure for ODNs consists in the phosphorylation of their 5'-hydroxyl with [γ-^{32}P] ATP and polynucleotide kinase (3,4). This method gives a high specific activity but the short period and the radiolysis associated with this isotope lead us to use some other isotopes of longer period of activity. Among radioisotopes ^{125}I is readily available, sensitive and highly resolutive; a low induced radiolysis and a long period make it a label of choice either in research or diagnostic area. Among the existing methods for the iodination of nucleic acids some are limited to long DNA probes due to cytosine iodination (5,6) and cannot be applied to ODNs (20-30 bases long) since their hybridization properties are more affected by the perturbation of base pairing. The reported 3'-tailing with ^{125}I labeled deoxycytidine

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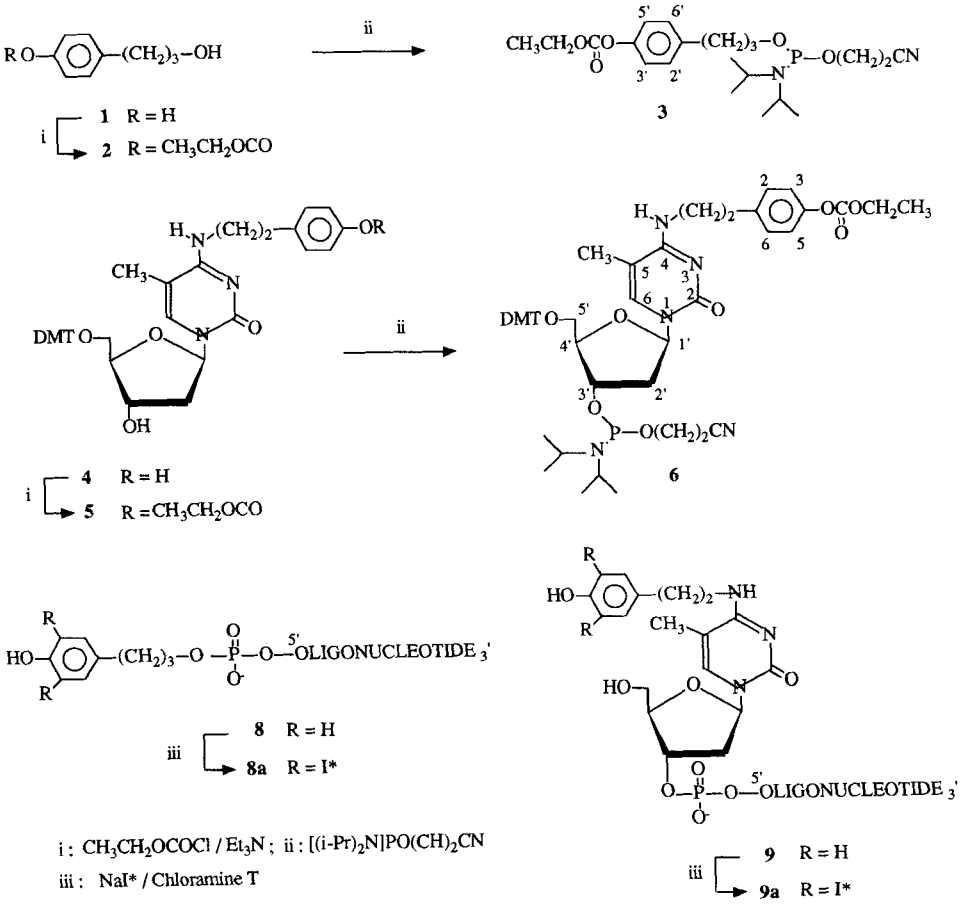
triphosphate (7) is obviously not suitable for the labelling of primers which would be used in a PCR protocole. Introduction of the label at the 5'-end was done through a two step procedure involving the coupling of a 4-methoxyphenyl-isothiocyanate (8) or 4-hydroxyphenylpropanoyl-NHS ester (9) on the amino function linked to a modified ODN. This approach is similar to the post-synthesis labelling which has been used for the introduction of various non radioactive tags as reviewed by M.J Gait (10). Recently we proposed a simpler method based on labelled phosphoramidite which make biotine or fluorescent-labelled ODNs as accessible as current unmodified ones (11). Several of such reagents were developed by other authors (12-16). This last approach makes most of the labelled ODNs readily available. The automated coupling step on a DNA synthesizer takes few minutes with a yield usually better than 90% in comparison with the post-synthesis labelling which takes several hours for a moderate yield. This approach was tested for the synthesis of tyramine-ODN conjugates able to be radioiodinated (17); although the method was successful, the chemical synthesis of the phosphoramidite building block needed to be simplified. Furthermore the new procedure reduces to a minimum the manipulation of radioactivity compared to previous methods.

RESULTS AND DISCUSSION

In order to achieve the site specific iodination of ODN conjugates we selected two types of structure, the former is a nucleoside derivative **3** with a 4-hydroxyphenylethylamine moiety (tyramine) linked to the C-4 position of 5-methyl deoxycytidine, the latter is a 4-hydroxypropanol derivative **6**. The phosphite-phosphoramidite approach commonly in use on commercial automated DNA synthesizers, requires a iodine mediated oxidation of the trivalent phosphite intermediate in order to generate a stable phosphate linkage. Obviously this iodine treatment would affect the 4-hydroxyphenyl residue. Preliminar experiments showed as expected that the phenol function of **1** or **4** was reactive towards phosphitylation reagents such as phosphorodiamidites. Therefore the phenol function must be masked with a protecting group able to stand all the reagents through the synthetic cycle and easily cleaved in the aqueous ammonia treatment required for the final deblocking step of ODN synthesis. Among the known phenol protecting groups (18) we first selected the acetyl group. The selective esterification of the phenolic hydroxyl of **4** in the presence of a secondary alcool by the reported procedure (19) gave only moderate results and was difficult to achieve when scaling-up the reaction. The full protection of the nucleoside hydroxyls (3' and 5') with the monomethoxytrityl groups before the acetylation step required afterwards two extra steps in order to obtain a suitable 5'-protected nucleoside (17). As an alternative the t-butyldimethylsilyl group could be introduced selectively as a phenol protection but it was only partially cleaved in hot aqueous ammonia and thus required a further fluoride treatment .This led us to investigate the use of carbonates for the phenol protection and thus 4-hydroxy-phenylpropanol **1** or the 5'-protected nucleoside **4** were reacted with ethyl chloroformate in the presence of triethylamine and gave cleanly the 4-ethoxycarbonyl derivatives **2** and **5** in 79% and 88% yield, respectively. The attachment of the ethoxycarbonyl group on the phenol was shown

by the downfield shift of the aromatics protons (respectively 0.17 and 0.32 ppm) on the NMR spectra. The phenol-protected derivatives **2** and **5** were converted to their corresponding cyanoethyldiisopropylphosphoramidites **3** , **6** in 73% and 74% yield respectively after purification over short silicagel column. Compound **3** was obtained as an oil upon lyophilisation from benzene and **6** as a powder after precipitation from hexane; they were completely stable after several months of storage (-20°C under argon).



The ODN **7** (see ODN synthesis in the experimental part) corresponding to a sequence specific of the human papilloma virus type 16 was synthesized on a controled pore glass (CPG) support and either **3** or **6** were coupled at the 5'-end of the oligonucleotide giving the fully protected ODN **8** or **9**, respectively. The coupling yield for **6** was 96% as determined photometrically (498 nm) from the released trityl cation (0.1 M toluene sulfonic acid in acetonitrile). Owing to the lack of DMT group in the compound **3** the coupling yield was determined as follows: the coupling cycle was performed omitting the acetic anhydride capping step, the synthesis column was then treated with dimethoxytrityl chloride (20). After washing with pyridine and acetonitrile the column

was submitted to a detritylation step on the synthesizer. The trityl cation released (10%) corresponded to the unreacted 5'-hydroxyl functions, and thus the coupling efficiency for **3** was 90%. The ODN-conjugates **8** and **9** were deprotected and purified over reverse-phase HPLC by taking advantage of their increased lipophilicity compared to the corresponding unmodified ODN **7**. These ODNs were homogeneous on polyacrylamide gel electrophoresis . The ODN- conjugates **8** and **9** were radioiodinated with 200 µCi of carrier-free [^{125}I]-NaI according to the reported chloramine-T oxidation procedure (21). The radioactive yield was ca 15% and the specific activity was respectively 2.5 and 2.7 Ci /µmol. The radiochemical purity estimated from thin layer chromatography was 94%. In contrast the unmodified ODN **7** submitted to the same conditions was radiolabelled with an efficiency of only 0.8% demonstrating the specificity of the labelling. The labelled ODNs **8a** and **9a** gave sharp radioactive bands on polyacrylamide gel electrophoresis (PAGE) with mobility very close to **8** and **9** respectively, in contrast the PAGE of radiolabelled **7** gave no definite band. The chemical structure of the labelled ODN **9a** was shown to be mainly a 3,5-diiodo-4-hydroxyphenyl derivative. The radiolabelled ODN **9a** after digestion with snake venom phospho-diesterase (22) and HPLC gave a main radioactive peak which coeluted with authentic 4-N-(3,5-diiodo-4-hydroxyphenyl-2-ethyl)-5-methyl-2'-deoxycytidine prepared by an alternate route (data not shown). Various ODN probes have been routinely labelled with the nucleoside phosphoramidite **6** according to the above procedure, giving highly reproducible level for coupling, iodine incorporation and hybridation efficiency, showing the real advantage of the method.

EXPERIMENTALS .

Analytical grade solvents were used and dichloromethane stabilized with 2-methyl-2-butene was used for synthesis. The ^{1}H-NMR and ^{31}P-NMR spectra were recorded respectively on AC200 and WM250 Bruker spectrometer, coupling constants are quoted in hertz and chemical shifts in ppm (δ scale) downfield from TMS or H_3PO_4. For protected nucleosides only the relevants shifts are given. UV spectra were recorded on a Beckman UV 530, maxima are quoted in nm and molar extinction coefficients (ε) in $cm^{-1}.M^{-1}$.The mass spectra were recorded on a Fisons-VG type ZAB2-SEQ spectrometer using a caesium gun and NBA (3-nitrobenzyl alcool) containing sodium iodide as a matrix. Short column chromatography were run on silica G60 (Merck) and TLC analyses were performed on plastic sheets 60F(254) 0.2 mm layer (Merck). The PAGE was run on denaturing 20% cross-linked gel, the R_m value is the ratio of distance of migration relative to bromophenol blue. The HPLC analyses were performed on a Varian 5000 equipped with a Lichrospher RP-18 (5 µm) column (Merck) working at a flow rate of 1 ml/min; gradient 1: from 10% to 50% B in 10 min, then 50% to 100% B in 20 min ; gradient 2: from 10% to 20% B in 20 min; A= 5% CH_3CN in 50 mM triethylammonium acetate (TEAA); B= 50% CH_3CN in 50 mM TEAA. Nordion IPG-I-125-L iodine (549 mCi / ml) was used in labelling experiments. A Kontron MDA 312 spectrometer was used for the ^{125}I counting.

PHOSPHORAMIDITES SYNTHESIS:

3-(4'-Ethoxycarbonyloxyphenyl)-1-propanol (2): To a solution of 4-hydroxyphenyl-propanol (2 g , 13.1 mmol) in dichloromethane (100 ml) containing triethylamine (2.2 ml , 15.7 mmol) was added under stirring a solution of ethyl chloroformate (1.4 ml , 14.5 mmol) in dichloromethane (20 ml) at 0°C. The reaction was left under stirring at room temperature for 30 min and then washed with 10% aqueous $NaHCO_3$ (50 ml) and water (50 ml). The product was coevaporated with toluene and purified on a short column. The elution was performed with a gradient from 40% petroleum ether to pure dichloro-methane and then to 5% methanol in dichloromethane. Compound **2** was obtained as an oil which solidified upon standing at -20°C. Yield 2.58 g (88%). R_f= 0.63 in chloroform-methanol (90:10), Rf= 0.2 in hexane:ethyl acetate:triethylamine (75:15:5). UV(MeOH): λ_{max}= 270.8 (ε = 370) and 264.2 (ε = 420) at pH 7; 270.8 (ε = 370) and 264.2 (ε = 420) at pH 2. λ_{max} = 295.0 (ε = 2200) after 30 min at pH 12. ^{1}H-NMR($CDCl_3$): 7.20 (d, J= 8.7, 2H) 2',6'-H; 7.10 (d, 2H) 3',5'-H; 4.32 (q, J= 7.1, 2H) CH_2-CH_3; 3.65 (t, J=6.4, 2H) 1-H; 2.70 (t, J=7.7, 2H) 3-H; 1.88 (m, 2H) 2-H, 1.40 (t, 3H) CH_3. MS(FAB$^+$): (MH^+)= 225.1, Calc. for $C_{12}H_{15}O_4$.

3-(4'-Ethoxycarbonyloxyphenyl)-1-propyl-2-cyanoethyl-N,N-bis-diisopropylaminophos-phoramidite (3): Compound **2** (346 mg , 1.54 mmol) was dried by coevaporation with carbon tetrachloride (3X10 ml) and dissolved in dry dichloromethane (20 ml). To this solution was added with stirring under argon diisopropylammonium tetrazolide (130 mg , 0.76 mmol) and N,N,N',N'-(bis-diisopropylamino)-2-cyanoethyl-phosphine (0.52 ml,1.6 mmol). After 90 min the reaction mixture was diluted with dichloromethane (30 ml), washed with 10% aqueous $NaHCO_3$ (2X50 ml) and brine (2X50 ml). The organic layer was dried ($MgSO_4$) and evaporated; the residue was purified over a short column using a gradient of dichloromethane (10 to 50%) in hexane containing 1% triethylamine. The title compound was evaporated to an oil which was lyophilized from benzene in several serum flasks. Yield 480 mg (73%). Rf = 0.7 in hexane:ethyl acetate:triethylamine (75:15:5). ^{1}H-NMR ($CDCl_3$): 7.19 (d, J=8.4, 2H) 2',6'-H; 7.08 (d, 2H) 3',5'-H; 4.28 (q, 2H) CH_2-CH_3; 3.80 (m, 2H) CH_2-CH_2CN; 3.63 (m, 4H) 1-CH_2 and 2X$CH(CH_3)_2$; 2.70 (t,J=7.4, 2H) 3-CH2; 2.60 (t, J=6.4 , 2H) CH_2-CH_2CN; 1.91 (m, 2H) 2-CH_2; 1.36 (t, 3H) CH_2-CH_3; 1.18 (2d, J=6.6 , 6H) $(CH_3)_2$. ^{31}P-NMR(CD_3CN): 148.5 . MS(FAB$^+$): (MH^+)= 425.2, Calc. for $C_{21}H_{33}N_2O_5P$.

5'-O-(4,4'-Dimethoxytrityl)-4-N-(4-hydroxyphenyl-2-ethyl)-5-methyl-2'-deoxycytidine (4): To 5'-dimethoxytrityl-4-thiothymidine (27.1 g, 48.2 mmol) dissolved in warm absolute ethanol (250 ml) was added tyramine (26.4 g, 193 mmol). The resulting suspension was kept in a sealed flask (screw cap) for 64 h at 55-60°C. The reaction mixture was evaporated to dryness and the residue shaken with ethyl acetate, the resulting suspension was filtered, the combined filtrate (400 ml) was washed with 10% aqueous $NaHCO_3$ (2X200 ml) and water (200 ml). The organic layer was evaporated and the residue was loaded on a silicagel column in a dichloromethane-hexane mixture (90:10). The column was eluted wih a gradient of methanol in dichloromethane and the title compound was eluted between 3 to 5% methanol. Yield 27g (84%). UV(MeOH): λ_{max}= 275.8 (ε = 13700) pH 7, 285.0 (ε = 13500) pH 2, 281.7 (ε = 13600) pH 12. ^{1}H-NMR ($CDCl_3$): 6.96 (d, J= 8.6, 2H) 2',6'-H(TYR); 6.88 (d, 2H) 3',5'-H(TYR); 6.44 (t, J= 6.4 , 1H) 1'-H; 4.96 (t, 1H) NH; 4.55 (m, 1H) 3'-

H; 3.67(dd, J= 6.8 , 2H) 1-CH_2(TYR); 2.77 (t, 2H) 2-CH_2(TYR) . MS (FAB^+): (BH_2^+) = 246.2 ; (MH^+) = 664.4 , Calc for $C_{39}H_{41}N_3O_7$.

5'-O-(4,4'-Dimethoxytrityl)-4-N(4-ethoxycarbonyloxyphenyl-2-ethyl)-5-methyl-2'-deoxycytidine (5): Compound **4** (3 g, 4.5 mmol) was treated with ethyl chloroformate (0.5ml, 5.2mmol) in the presence of triethylamine (1.3 ml, 9 mmol) as described above. The crude compound was purified over a short column (40 g silicagel) using a gradient of methanol in dichloromethane. The title compound was eluted with 3% methanol. After evaporation the white foam was dissolved in dichloromethane (10 ml) and precipitated from a mixture of hexane (100 ml) and diethyl ether (50 ml). Yield 2.63 g (79%). Rf= 0.55 in chloroform-methanol (90:10). UV(MeOH): λ_{max}= 275.8 (e = 11000) pH 7, 294.2 (ε = 11800) pH 2, 275.8 (ε = 11400) pH 12. ^{1}H-NMR($CDCl_3$): 7.20 (d, J= 8.5, 2H) 2',6'-H (TYR); 7.10 (d, 2H) 3',5'-H (TYR); 5,12 (t, J= 5.7, 1H) NH; 4.57 (m, 1H) 3'-H; 4.32 (q, J= 7.2 , 2H) CH_2-CH_3; 1.39(s, 3H) CH_2-CH_3. MS(FAB^+): (BH_2^+)= 318, (MH^+)= 736.4 , Calc. for $C_{42}H_{45}N_3O_9$.

5'-O-(4,4'-Dimethoxytrityl)-4-N-[(4-ethoxycarbonyloxyphenyl)-2-ethyll-5-methyl-2'-deoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (6) : The protected nucleoside **5** (4 g, 5.4 mmol) was dried by two co-evaporations with a mixture of dichloromethane (15 ml) and acetonitrile (25 ml). The resulting foam was dissolved in dichloromethane (20 ml) and treated with tetrazolide (0.46 g, 2.7 mmol) and phosphine (1.8 ml, 5.9 mmol) for 60 min as described above for **3**. The compound was purified over a short silicagel column packed with 30% hexane in dichloromethane containing triethylamine (0.5 %), the column was eluted with the same solvent and then with dichloromethane. The relevant fractions were evaporated to a glass which was taken up in ethyl acetate (10 ml) and precipitated from hexane (300 ml). Yield 3.75 g (74%). Rf= 0.61 & 0.69 in dichloromethane:ethyl acetate:triethylamine (50:45:5). ^{1}H-NMR (CD_3COCD_3): 7.21(d, J=8.5, 2H) 2',6'-H (TYR); 7.12 (d, 2H) 3',5'-H (TYR); 4.65 (m, 1H) 3'-H; 4.20 (q,J=7.2, 2H) CH_2-CH_3; 3.7-3.5 (m, 6H) 1-CH_2 (TYR), 2X$CH(CH_3)_2$ and CH_2-CH_2CN; 3.14 (t, 2H) 2-CH_2 (TYR); 2.74 and 2.50 (2Xt, 2H) CH_2-CH_2CN; 1.3-1.0 (m, 9H) CH_2-CH_3 and $CH(CH_3)_2$. ^{31}P-NMR (CD_3CN): 149.9 & 149.4. MS(FAB+): (BH^+) = 318, (MH^+) =936.5 , Calc. for $C_{51}H_{62}N_5O_{10}P$.

OLIGONUCLEOTIDE SYNTHESIS: The oligonucleotide **7** (sequence : 5'GAA AGT TAC CAC AGT TAT GC3') was built on a 381B (Applied Biosystems) DNA Synthesizer. An aliquot (ca. 2mg) of the CPG support bearing the fully protected ODN was treated with 25% aqueous ammonia (55°C , 16 h). After evaporation the crude compound (4.58 OD units) was purified over HPLC using gradient 1. The main peak (Rt=14.4 min) was collected and the corresponding compound was detritylated (acetic acid:water 8:2, 20 min). Yield 1.94 OD units (43%). The fully deprotected oligonucleotide **7** showed a single peak (Rt= 12.1 min) on HPLC (gradient 2). The phosphoramidite **3** in 0.1 M acetonitrile solution was coupled on an aliquot (8 mg) of CPG support bearing the oligonucleotide **7** using the 0.2µM scale cycle, omitting the acetic anhydride capping step. The resulting modified oligonucleotide **8** was deprotected in ammonia as above (10.2 OD units crude) and purified on HPLC giving a main peak at Rt=13.8 min. Yield 3.7 OD units (37%). Similarly the amidite **6** was coupled to the oligonucleotide **7** on a 0.2µM column using the

standard cycle. After deprotection the crude oligonucleotide (23 OD units) was purified on HPLC (gradient 2) and the main peak (Rt= 16.5 min) collected. Yield 7.2 OD units (31%). On PAGE the R_m value for **7**, **8** and **9** are 0.59, 0.58 and 0.55 respectively.

OLIGONUCLEOTIDES LABELLING: To the ODN (13 pmol) in 15 µl of 0.25 M phosphate buffer pH 8.5 was added 10 µl (200 µCi) of a [^{125}I]-NaI stock solution (2 µl commercial solution in 50 µl water). Then 10 µl of aqueous chloramine-T (5 mg/ml) were added and after 1min the reaction was quenched with 40 µl of aqueous sodium metabisulfite (3.8 mg/ml) followed by 10 µl of 2M potassium iodide solution. The reaction mixture was loaded on a NAP10 column (Pharmacia) equilibrated in water. After the void volume, the fraction (800 µl) containing the labelled ODN was collected (93% recovery based on optical density). The recovered activity for **7**, **8a** and **9a** were $3.60x10^6$, $6.49x10^7$ and $5.65x10^7$ respectively. The radiochemical purity of the radiolabelled oligonucleotides **8a** and **9a** was checked as followed: the labelled solutions (2 µl) were spotted on silica TLC plate, alone and in admixture with the corresponding unlabelled material (2 µl, 0.05 OD units) or potassium iodide (1 µl, 2 µmol). The TLC plate was eluted with a n-propanol :conc.ammonia : water (55:35:10) mixture. Autoradiography showed one spot (R_f= 0.24) coeluting with the oligonucleotide visualized with UV light. Counting of this spot represented 95% of the total activity. The iodide (R_f= 0.6) gave only a shadow on the autoradiography.

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