
Synthesis of DNA fragments containing 5,6-dihydrothymine, a major product of thymine gamma radiolysis

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ABSTRACT : 5,6-Dihydrothymine is one of the most important products of base damage by gamma irradiation of DNA in anoxic conditions. This modified base is unstable in the deprotection conditions used for classical synthesis of oligonucleotides. For its incorporation in synthetic DNA fragments, a new set of amino protecting groups has been developed. The 5,6-dihydrothymidine phosphoramidite was successfully employed for the synthesis of two 14-mers and one 17-mer bearing this defect at positions corresponding to restriction enzymes sites. The presence of the modified base still intact in the oligonucleotides was evidenced by mass spectrometry in pyrolytic conditions.

INTRODUCTION

The synthesis of modified oligonucleotides bearing a single defect in a determined position has proved to be of the utmost importance : they are powerful tools to investigate the biological consequences of and to carry out enzymatic or cell repair studies of DNA damage. Scientific literature includes more and more examples of preparation of such oligonucleotides (1-5). These papers describe the incorporation of modified bases such as methylated pyrimidines and purines or nucleobase analogs which are stable under the usual reaction conditions needed for the assembly and deprotection of oligonucleotides. The ammoniacal treatment at 60° C for 17 hours, is necessary to ensure the complete removal of isobutyryl and benzoyl amino-protecting groups from guanine, cytosine and adenine residues. Some bases, such as saturated pyrimidines are unstable in these conditions and therefore cannot be introduced in DNA fragments using the classical methodology. To overcome this problem, several solutions have been suggested. Matteucci and Webb (6,7) proposed to use the 9-fluorenyl-methoxycarbonyl (fmoc) group for the amino protection of nucleic acid bases. Nevertheless, this method was inefficient for the incorporation of fragile bases in DNA fragments containing all four natural nucleosides, mainly because of the difficulty they encountered in adequate protection of guanine residues. Another way (8) is to prepare the

triphosphate corresponding to the modified nucleoside and to incorporate it enzymatically in DNA fragments. This method suffers from lack of selectivity and needs an enzymatic system of replication able to work with the modified triphosphate.

5,6-dihydrothymine (DHT) is the major product formed under oxygen-free gamma irradiation of thymine (9,10). Recently, Furlong et al. proved that this phenomenon also appears with HeLa cells when they are gamma irradiated with a Cobalt 60 source. Both 5R and 5S diastereoisomers are produced with a predominance of the former one (22). The presence of a DNA-Glycosylase corresponding to this defect was evidenced several years ago (11) : An E. coli DNA was irradiated, purified by dialysis to separate small molecules - free bases liberated by radiolysis - and incubated with an E. coli crude extract. After that treatment, 5,6-dihydrothymine was found again in the cell supernatant, pointing out the presence of the corresponding excision system in the cell culture. So we focussed our attention to the preparation of DNA fragments bearing a DHT on chosen sites, as tools for the isolation and mechanism studies of DNA repair systems. The first step was the incorporation of DHT in one determined position of a homopolymer (dT 15). Since this 15-mer only contained dT, no amine protection was required for its assembly and the final drastic 60°C ammonia treatment was omitted in the deprotection step (12). We want to report here the synthesis of three oligonucleotides up to 17 bases in length including the four natural bases and 5,6-dihydrothymine in a central position.

RESULTS

Oxygen-free gamma irradiation of thymidine produces both 5R and 5S diastereoisomers of DHT. They can be separated using reversed-phase liquid chromatography on a C-18 column, eluted with water. However, this separation is useless for any purpose because an isomerisation of these two forms can occur under the basic conditions used for oligonucleotide deprotection. A mixture of 5R and 5S diastereoisomers was used in this work.

It has been shown that DHT was not significantly degraded within four hours of room temperature ammonia treatment (12). However, these conditions are not sufficient to ensure the cleavage of the amide bond formed by the classical isobutyryl and benzoyl blocking groups used either in phosphotriester or phosphoramidite DNA synthesis methodologies (13-15). We have developed a new set of exocyclic amino protecting groups for nucleic bases that allow lowering of the final deblocking step to four hours at room

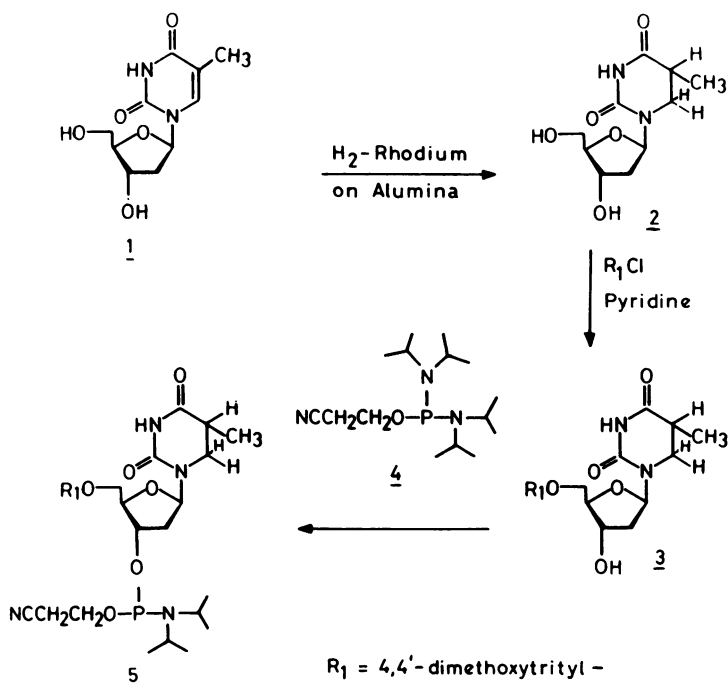


Figure 1 : Reactional scheme used for the preparation of the phosphoramidite derivative of 5,6-dihydrothymidine.

temperature in concentrated aqueous ammonia (16). This treatment is compatible with the stability of DHT under basic conditions.

Synthesis of the cyanoethyl phosphoramidite derivative of DHT.

The preparation of the monomeric unit intended for the phosphoramidite method (15) is described in Figure 1. The mixture of 5R and 5S diastereoisomers of DHT was obtained by catalytic hydrogenation of thymidine, according to Cohn and Doherty (17). The 5'-end of the modified nucleoside was then protected by a 4,4'- dimethoxytrityl group and compound 3 was purified by silica-gel chromatography. For the introduction of the cyanoethyl diisopropylamino phosphite group on the 3' position of the tritylated nucleoside, a mixture of reagent 4 and diisopropylammonium tetrazolidate was used (18). The fully protected mononucleotide 5 was precipitated in cold hexane (-78°C) and purified by silica-gel chromatography with a mixture of dichloromethane, ethyl acetate and triethylamine as the eluent. The identity and purity of the compound

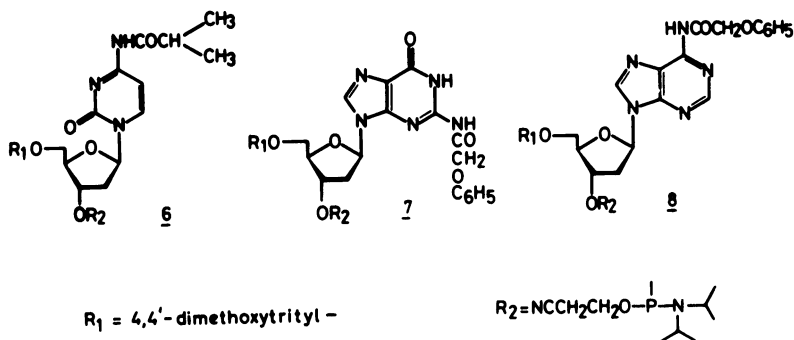


Figure 2 : Labile protected monomers used for the assembly of oligonucleotides containing DHT.

obtained in this way were then checked by nuclear magnetic resonance and FAB mass spectrometry.

Phosphoramidite assembly of oligonucleotides containing DHT.

Compound 5 and the monomers bearing the new amino protecting groups (16) (Compounds 1-4 in figure 2) were used for the assembly of oligonucleotides containing 5,6-dihydrothymidine.

The heptadecamer d(AATTCADHTATCTGATCAT) was synthesized manually according to the syringe method (19). The assembly was started with Controlled Pore Glass functionalised with thymidine. The coupling yield was measured at each step and reached an average value of 95% , showing no difference between the DHT monomer and the four natural nucleotides.

Two tetradecamers bearing restriction enzyme sites : d(CATGACGGADHTCCTT) containing a single modification and d(CATGAGAADHTDHTCACT) twice modified, were synthesized using an Applied Biosystems 381A DNA synthesiser. In the standard coupling conditions used with that apparatus, the average coupling yield reached 98% .

Deprotection, purification and analysis of the oligonucleotides.

Previous studies showed that two hours at room temperature in concentrated aqueous ammonia was a sufficient treatment to ensure both cleavage of the chain from the support and deblocking of the new exocyclic amide functions of the bases. Thus the CPGs carrying the synthetic oligonucleotides were treated under these conditions and the supernatants containing the oligonucleotides were desalted on disposable Sephadex G 25 cartridges (Pharmacia PD 10 : 5 X 1.5 cm).

The crude oligomers were run by electrophoresis on preparative

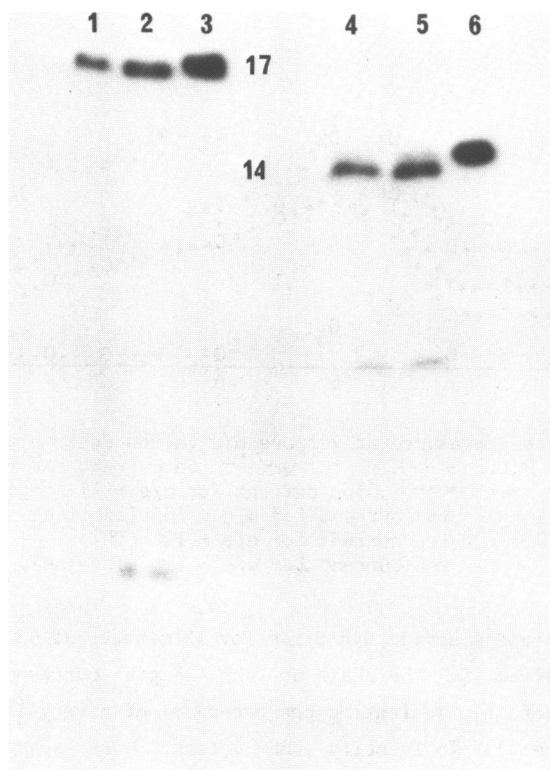


Figure 3 : Characterization of DHT in the 17-mer (lanes 1-3) and in the 14-mer bearing one modified base (lanes 4-6). Lanes 1 & 4 : Oligonucleotides treated in 0.2 M NaOH at 90° C for 30 minutes and then in piperidine according to Maxam & Gilbert procedure. Lanes 2 & 5 : Oligonucleotides treated in 0.2 M NaOH at 45° C for 30 minutes and then in piperidine. Lanes 3 & 6 : Untreated products.

polyacrylamide denaturing gels (20 % urea). The oligonucleotides were seen (by U.V. shadowing) to be single predominant products with the correct mobilities. They were extracted from the gel to give the following yields : 65 % and 70 % for the two tetradecamers and 35 % for the heptadecamer manually synthesised.

The analysis of the modified oligonucleotides by the Maxam and Gilbert sequencing method (20) was attempted. Unfortunately, the positions corresponding to the DHT gave a response with every sequencing reaction and consequently a band in each channel. We took advantage of the sensitivity of saturated pyrimidines towards alkaline conditions and developed an additional channel in which the radioactive oligonucleotide was simply

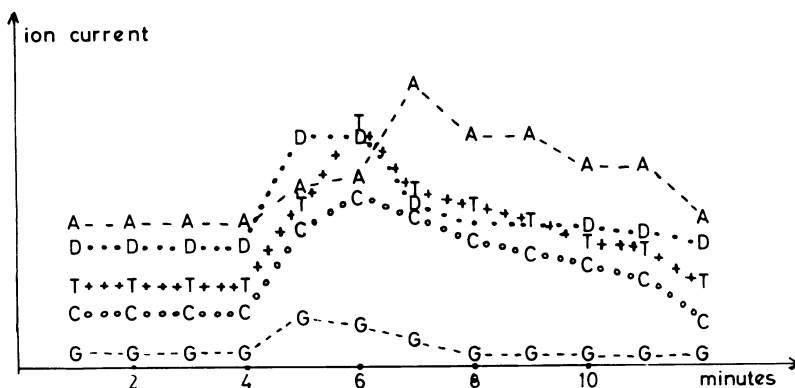


Figure 4 : Ion current measured at various m/e values under pyrolysis of the 14-mer bearing two DHT.: ---G--- ion current for $m/e = 151$ (guanine). $\circ\circ\circ$ C $\circ\circ\circ\circ$ C $\circ\circ\circ\circ\circ$ ion current for $m/e = 111$ (cytosine). --- A --- ion current for $m/e = 135$ (adenine). $\cdot\cdot\cdot$ D $\cdot\cdot\cdot\cdot\cdot$ D $\cdot\cdot$ ion current for $m/e = 128$ (DHT) $+++$ T $++++$ T $++$ ion current for $m/e = 126$ (Thymine).

treated in 0.2 M aqueous sodium hydroxide for 30 minutes at 45° C. A partial cleavage was observed in the chain at the length corresponding to the position of the defect, indicating the presence of a very labile base at that place (Figure 3). To identify the defect, mass spectrometry under pyrolytic conditions was used (21) .

Under pyrolytic conditions free bases are extracted from the sugar-phosphate backbone of the oligonucleotides. It is possible to observe the evolution of the ion-current at various m/e values corresponding to the bases present in the DNA chain and consequently, to identify modified bases. In figure 4 are plotted versus time the ion currents measured at various m/e values corresponding to adenine, guanine, cytosine, thymine and 5,6-dihydrothymine, in the analysis of the 14-mer bearing two DHTs. The respective curves obtained for $m/e=126$ (Thymine) and $m/e=128$ (dihydrothymine) clearly indicate that the latter is due to an independent ion (DHT.+) formed by pyrolysis and not to the $M+2$ isotopic peak of thymine. Thus, the presence of the modified base in the oligonucleotide chain after the assembly and deprotection was demonstrated.

CONCLUSION

The use of the new labile exocyclic amino protecting groups allows the incorporation of alkali sensitive defects of DNA on positions determined in

advance. The modified oligonucleotides obtained contain well defined modifications necessary for further biological studies.

EXPERIMENTAL SECTION

2'-deoxynucleosides were purchased from Pharma-Waldhorf. They were fully protected according to procedures previously described (16). 5'-O-dimethoxytrityl 5,6-dihydrothymidine was prepared as indicated in reference 12.

Preparation of the phosphoramidite derivative of 5,6-dihydrothymidine (compound 5).

5'-O-dimethoxytrityl-5,6-dihydrothymidine (273 mg, 0.5 mmole) was dissolved in 5 ml of anhydrous dichloromethane and kept under inert atmosphere. Through a rubber septum a mixture of 0.25 mmoles of diisopropylamine and 0.25 mmoles of tetrazole in 5 ml of dichloromethane was added, followed by 0.55 mmoles of cyanoethyl bis(diisopropylamino) phosphine. The reaction mixture was then kept under magnetic stirring for 35 minutes. The dichloromethane was removed by evaporation under reduced pressure and the gummy residue was dissolved in 25 ml of ethyl acetate. This organic layer was washed with 10 % sodium carbonate (2 X 25 ml) and water (25 ml) and dried over sodium sulfate. After removal of the solvent, the foamy residue was dissolved in 3 ml of toluene and precipitated in 50 ml of hexane at - 78° C. A second purification was made on silica gel using a mixture of chloroform 60 - hexane 35 - triethylamine 5 as the eluent. The white powder obtained (560 mg) after evaporation of the solvent was dried under vacuum and stored under argon (75 % yield).

Proton NMR in deuterated pyridine : 6.79 ppm H1' (t), 4.72 ppm H3' (m), 4.22 ppm H4' (m), 3.72 ppm methoxyl (s), 3.4-3.25 H5', H5'' (m), 1.05 methyl of the base (d). 31-P NMR in deuterated pyridine 149.1 ppm (s). FAB Mass Spectrometry : m/e = 745 (42 %) molecular peak : m/e = 692 molecular peak - Cyanoethyl.

Oligonucleotide assembly.

Compound 5 was used in the same way as other mononucleotides for the synthesis of DNA fragments by the phosphoramidite approach. The controlled pore glass functionalised with the first 3' end monomer was introduced into a syringe or into the automatic DNA synthesiser and the successive additions of nucleosides were carried out according to previously described procedures (16).

Characterisation of the defect in the DNA chains.

A few nanograms of the oligonucleotides were 5' end ³²P labelled using T4 polynucleotide kinase and gamma ³²P adenosine triphosphate and purified by polyacrylamide gel electrophoresis. After elution from the gel and lyophilisation, they were taken up in 5 μ l of water and treated by 100 μ l of 0.2 molar sodium hydroxyde for 30 minutes at 45 ° C. After precipitation in cold ethanol, the oligonucleotide was treated in 100 μ l of molar aqueous piperidine and run on a sequencing 20 % polyacrylamide gel. The bands corresponding to the labelled oligonucleotides were then revealed by autoradiography.

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