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THE USE OF LABILE BASE PROTECTING GROUPS IN OLIGORIBONUCLEOTIDE SYNTHESIS

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ABSTRACT: The use of phenoxyacetyl group for the protection of the exocyclic amino function of purine bases and acetyl group for cytosine in oligonucleotide synthesis by the cyanoethylphosphoramidite approach is described. A side reaction - i.e. partial replacement of phenoxyacetyl group of protected guanines by acetyl group - was observed during the capping step. It can be avoided by the use of phenoxyacetic anhydride in place of acetic anhydride.

INTRODUCTION

The chemical synthesis of DNA fragments can be performed very efficiently by the phosphoramidite methodology (1). This is largely due to the convenience of performing the internucleotide coupling reaction with an excellent yield. Curiously, the amino protecting groups currently used, have not been so much improved. They are the same as those proposed by Khorana in 1963 (2). Their elimination at the end of the assembly requires rather drastic experimental conditions : a treatment in concentrated aqueous ammonia for 17 h at 55°C. These conditions are not convenient when fragile bases have to be incorporated in the oligonucleotide chain. To overcome these problems we have introduced a new set of alcali-labile protecting groups which can be used as well in deoxyoligonucleotide synthesis (3) as in the oligoribonucleotide series (4). In this article we describe our results concerning the protection of adenine, guanine and cytosine in the synthesis of RNA fragments.

PREPARATION OF PROTECTED MONONUCLEOTIDES

The aim of this work was to search for a set of amino-protecting groups which could be eliminated in 4h in ammonia/ethanol 1/1 at room temperature. These conditions seem to be acceptable for 5,6-dihydrouridine which is found in t-RNAs. Hence, various protected guanine, adenine and cytosine nucleosides were prepared by the transient protection method (5-7) and the rate of their deprotection in a mixture of ammonia and ethanol was evaluated by HPLC. The values of half time of deprotection show that phenoxyacetyl group is convenient for guanine ($t_{1/2}$ = 15 min) and adenine ($t_{1/2}$ = 10 min) and that acetyl group is adapted to the protection of cytosine ($t_{1/2}$ = 10 min). These results may be compared with those of Ogilvie et al. (8) who suggest the same group for purine bases but keep benzoyl group for cytosine. Thus the final deprotection time is 16 h at room temperature, which is not compatible with our goal.

To protect the 5'hydroxy function of the ribose residues, the 4,4'-dimethoxytrityl group which is more labile in acidic conditions was preferred (9). The protection of the 2' position of the sugar residue was performed with the tertiobutyldimethylsilyl group (10,11). Pure 2' isomers were separated from their 3' isomers and bis-silylated compounds by HPLC on silica gel. They were characterized as free of contaminant by high field proton nuclear magnetic resonance.

For the protection of the phosphorus atom we have preferred the cyanoethyl group as proposed by Köster (12) in DNA synthesis (13). Compounds 1-3 (figure 1) were purified by HPLC and their purity was checked by ^{1}H and ^{31}P NMR and Fast Atom Bombardment mass spectroscopy.

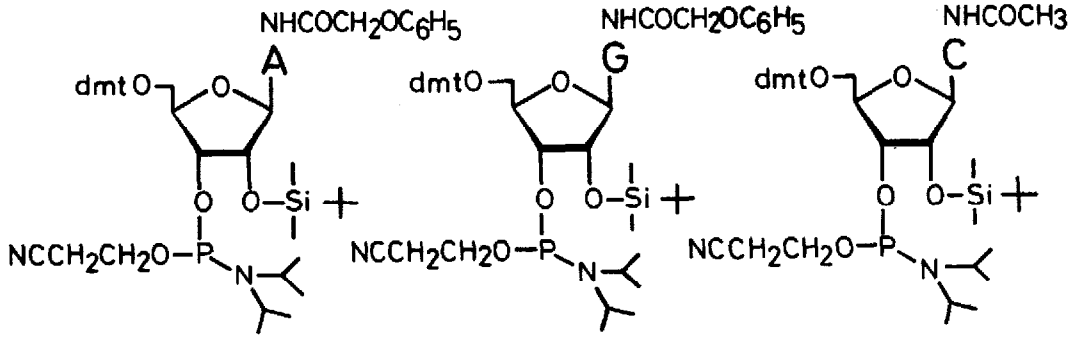

Figure 1 : dmt = 4,4'-dimethoxytrityl-

ASSEMBLY OF THE OLIGONUCLEOTIDES

Four oligoribonucleotides constituting a part of the sequence of *B.subtilis* formylmethionine t-RNA were synthesized :

5'-UGAGCAGUUC-3' sequence 1
5'-AAUCCUGCCC-3' sequence 2
5'-GGGCUCAUAAC-3' sequence 3
5'-GAGCAGUUC-3' sequence 4

The assembly was realized with an Applied Biosystems Model 381A synthesizer from 0.2 µmoles of protected nucleoside attached at its 3' site to the support (long chain alkylamino controlled-pore glass). The usual cycle proposed by the manufacturer was used with only one modification, an increase of the condensation time to 20 min (14). The average coupling yield determined from the 4,4'-dimethoxytrityl cation release was about 95 %.

DEPROTECTION AND PURIFICATION OF THE OLIGONUCLEOTIDES

When the chains were assembled, the deprotection of the oligonucleotides was performed in two steps. The cleavage of the sequence from the support, the elimination of the protecting groups from the bases and the triester phosphates were made simultaneously by treatment in ammonia/ethanol 1/1 for 4 hours at room temperature. In a second step, the 2'tertiobutyldimethylsisyl groups were removed by reaction with 1M tetrabutylammonium fluoride in THF.

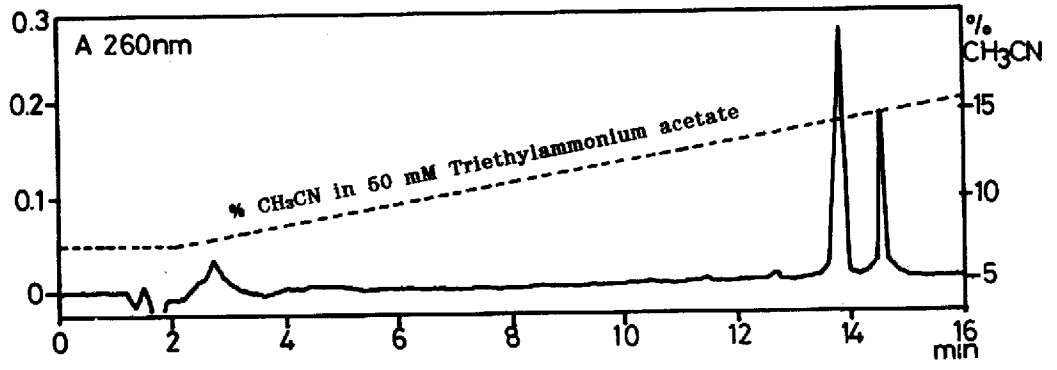

Figure 2 : Reversed phase chromatography analysis of sequence 2 after electrophoretic purification. Column Merck Li-Chrospher RP18e 5 µm. ϕ 4 mm. Length 125 mm. Flow-rate 1 ml/min. Peak 1 retention time 13.7 min. Peak 2 retention time 14.5 min.

The oligonucleotides were desalted on Sephadex G-25 and separated on preparative gel polyacrylamide gel electrophoresis. Each oligonucleotide gave a major band with the expected mobility corresponding to its size. The products were eluted from the gel and analysed by reversed phase HPLC. Inspection of the chromatogram depicted in figure 2 showed two peaks in the major electrophoresis band of the oligonucleotide 10-mer bearing only one guanine (sequence 2). The other oligonucleotides bearing three guanines in their sequence showed four peaks or more in their chromatogram which gave an idea about the role played by this base. These findings were surprising because preliminary results showed that phenoxyacetyl guanine was totally deprotected under the basic conditions used (3). No rational explanation could be given till H. Bazin (15) showed evidence for an exchange reaction between the guanine phenoxyacetyl group and the acetyl group by action of acetic anhydride in the presence of DMAP. It was inferred that this reaction could take place during the capping step in the assembly of the RNA fragments on the support. At room temperature, the deprotection rate of acetylguanine being slower, the acetyl group was retained in the final product. This hypothesis has been confirmed by enzymatic hydrolysis of the compounds collected in peaks 1 and 2 during the analysis depicted in figure 2. The action of exonuclease and alkaline phosphatase on these compounds followed by HPLC analysis of the nucleosides obtained showed the presence of acetylguanosine in compound 2 in contrast to compound 1 (data not shown). Due to the importance of this side reaction and the number of cycles, the protection of guanosine by the phenoxyacetyl group precludes the use of acetic anhydride as the capping agent for medium and long oligonucleotides.

To avoid this inconvenience, acetic anhydride was replaced by phenoxyacetic anhydride in the capping mixture. The sequence bearing only one G was synthesized again and showed after the same work-up a single peak by HPLC analysis. The results must be extended to the DNA series.

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5) G. S. Ti, B. L. Gaffney & R. A. Jones : J. Amer. Chem. Soc. 1982,104,1316.
6) F. Benseler & L. W. McLaughlin : Synthesis 1986,45.
7) The nucleoside (10 mmol)was transiently protected by trimethylchlorosilane in pyridine (50 ml) and acylated by a mixture of phenoxyacetyl chloride and 1-hydroxybenzotriazole (15 mmol each) in acetonitrile (For cytidine acetyl chloride was used alone). After 17 hours at room temperature, the excess acylating agent is hydrolysed, the precipited salts are filtered and the solvent is evaporated to dryness. The residue is taken up in 300 ml water, washed by chloroform (2 X 150 ml) and the aqueous phase is evaporated. Pure base protected nucleosides are obtained by cristallisation in absolute ethanol.
8) T. Wu, K. K. Ogilvie & R. T. Pon : Tetrahedron Lett. 1988,29,4249.
9) The protected nucleoside (5 mmol) is reacted with 4,4'-dimethoxytrityl chloride in pyridine (50 ml) overnight at 5° C. The excess tritylating agent is hydrolysed by methanol and the reaction mixture is evaporated to dryness. The gum is dissolved in chloroform (150 ml), washed with saturated sodium hydrogenocarbonate and water.The organic phase is evaporated to dryness and fractionated by preparative HPLC on silica gel. The desired compound is obtained in 50-70 % yield.
10) K. K. Ogilvie, A. L. Schifman & C. L. Penney : Can. J. Chem. 1979,57,2230.
11) The 5' and base protected nucleoside (2.5 mmol) is dissolved in 40 ml pyridine. Imidazole (3 eq) and t-butyldimethylchlorosilane (1.5 eq) are added. After 1-3 days at room temperature the excess TBDMS chloride is hydrolysed by water (1 ml) and the solvents are evaporated. The residue is taken up in chloroform (100 ml) and washed with saturated aqueous sodium bicarbonate and water. Pure 2' isomers are obtained in 30 - 50% yield by preparative HPLC on silica gel columns.
12) N. D. Sinha, J. Biernat, J. McNamus & H. Köster : Nucleic Acids Res. 1984,12,4539.
13) For the phosphitylation of nucleosides, cyanoethyl diisopropylamino chlorophosphine (2 eq), diisopropylethylamine (4 eq) and DMAP (0.2 eq) were used in dry THF under argon atmosphere. After 3 hours at room temperature, water (1 ml) was added and the solvant evaporated to dryness. The residue was dissolved in ethyl acetate, washed with saturated aqueous bicarbonate and water. Homogeneous protected nucleoside phosphoramidites were obtained by HPLC on silica gel columns. Their purity was checked by ^{31}P and ^{1}H NMR. This showed that pure 3' phosphoramidite with the exclusion of 2' isomers were obtained.
14) Detritylation (Trichloracetic acid 3% in dichloromethane - 2 min.) - Coupling (Mononucleotide 0.1 M in anhydrous acetonitrile , tetrazole 0.5 M in dry acetonitrile - 20 min.) - Capping (Acetic anhydride or phenoxyacetic anhydride) 0.1 M in lutidine / THF 1/8 + Diméthylaminopyridine 6.5 % wt/vol in THF - 0.5 min.) - Oxidation (Iodine 0.1 M in lutidine / THF / water 10 / 40 / 1 - 0.5 min.).
15) H. Bazin : Unpublished results.

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