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Synthesis and Purification of Oligonucleotides Containing Sulfur Substituted Nucleobases : 4-Thiouracil, 4-Thiothymine and 6-Mercaptopurine

Pascale CLIVIO, Jean-Louis FOURREY, Jeannette GASCHE

Institut de Chimie des Substances Naturelles, C.N.R.S., 91198 Gif sur Yvette, France.

Annie AUDIC, Alain Favre, Catherine PERRIN and Anne Woisard

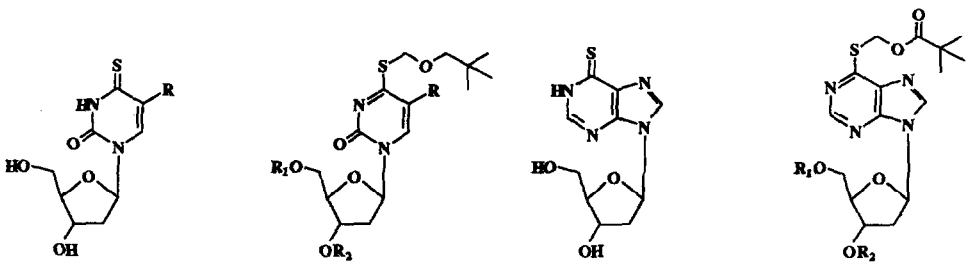
Laboratoire de Photobiologie Moléculaire, Institut Jacques Monod, C.N.R.S., 2, Place Jussieu 75251 Paris Cedex 05, France.

Key words: oligonucleotide; 4-thiouracil; 6-mercaptopurine; gel electrophoresis.

Abstract: A procedure to obtain oligodeoxynucleotides containing sulfur modified nucleobases by standard application of the current phosphoramidite methodology is described as well as their purification technique..

At the present time there is a widespread interest in the design of synthetic oligonucleotide analogues to be used in fundamental research and hopefully as therapeutical agents[1]. Among the sulfur containing oligonucleotide analogues, the most extensively studied are the phosphorothioates and the phosphorodithioates[2]. Surprisingly, oligonucleotides containing, at selected positions, bases in which an oxygen atom has been replaced by sulfur such as in 4-thiouracil, 4-thiothymine or 6-mercaptopurine, which are known to manifest ubiquitous chemical properties[3], have been rarely prepared[4]. We report here that the synthesis of such oligonucleotides can be accomplished, starting from the sulfur containing (deoxy)nucleosides, by application of the current phosphoramidite method of oligonucleotide synthesis[5]. This method requires an appropriate protection for the nucleophilic positions of the corresponding (deoxy)nucleosides to be incorporated in the sequence. Accordingly, for thio-modified (deoxy)nucleosides one should develope a suitable protecting group for the thioamide function that has to be fully compatible with the experimental conditions of the synthetic procedure.

In our hands, the pivaloyloxymethyl group gave the most satisfactory results when using the H-phosphonate chemistry[6] to prepare dinucleotides in solution having such sulfur modified residues. We report here that this protection can be conveniently employed in solid phase synthesis, using the phosphoramidite chemistry, to incorporate 4-thiouracil, 4-thiothymine as well as 6-mercaptopurine at selected positions of an oligonucleotide.



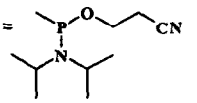
1 (R= H) —i→ 2 (R= H, R_1= R_2= H) —ii→ 3 (R= H, R_1= Dmt, R_2= H) —iii→ 4 (R= H, R_1= Dmt, R_2= X)

5 (R= CH_3) —iii→ 7 (R= CH_3, R_1= Dmt, R_2= X)

6

8 (R_1= Dmt, R_2= X)

Dmt = 4,4-dimethoxytrityl

X = 

i: pivaloyloxymethyl chloride, K_2CO_3, acetone, 90%

ii: DmtCl, pyridine,85%

iii: 1-cyanoethyltetraisopropylphosphorodiamidite, tetrazole,CH_2Cl_2, 70%

Scheme

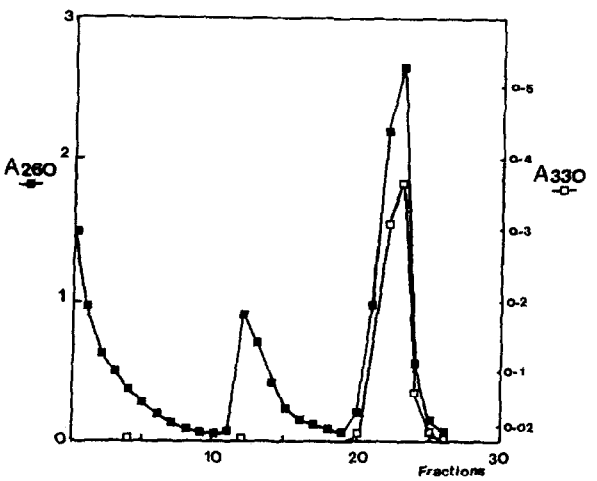


Figure 1 :(Affinity chromatography)

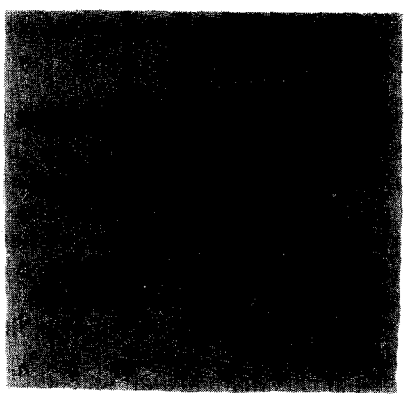


Figure 2 :(Affinity electrophoresis)

In the case of 2'-deoxy-4-thiouridine **1**, the new phosphoramidite **4**, easily prepared in good overall yield by a standard three step procedure (Scheme), did serve for the construction of a number of oligodeoxynucleotides containing thiolated bases in crucial positions of the sequence. As an illustration, we describe the synthesis, purification and characterization of a 14-mer containing a 2'-deoxy-4-thiouridine **1** (s^4U) residue in the central position, namely 5'-GTTCTAAs^4UAATAGC.

This was accomplished by following the usual instructions for oligodeoxynucleotide synthesis[5]. However, since 4-thiouracil derivatives are normally interconverted to the corresponding cytosines by heating in ammonia[7,8], we have preferred to carry on the final deprotection step with ammonia at room temperature over a long period of time (48h). These conditions have been recommended for oligonucleotide analogues which are prone to degradation under a more vigourous ammonia treatment[11]. The final amount of recovered crude oligonucleotide (300 µg for a 0.2 µmole column) was within the range currently obtained for oligonucleotides of the same size.

The UV absorbance spectrum of the crude fraction provided a direct evidence for the presence of 4-thiouracil (λ_{max} 330 nm). However, owing to possible hypochromic effects, it could not be used to estimate its incorporation level. Hence, the non-thiolated and thiolated fractions were separated by agarose affinity chromatography after adapting to oligonucleotides the procedure which was initially developed for RNA containing sulfur modified nucleobases[12]. The crude oligonucleotide mixture was absorbed onto a mercurated agarose column (Affigel 501, Biorad). The non-thiolated fractions were eluted by extensive washing with 0.1 ammonium acetate containing increased concentrations of NaCl. Finally, the desired oligonucleotide was obtained by displacement with 3mM mercaptoethanol; it represented 60% of the recovered material (Figure 1).

To analyse the size distribution of oligonucleotides in the crude sample an aliquot was 5' ^{32}P labelled using polynucleotide kinase[12b] and loaded onto a 15% denaturating gel. After electrophoresis and autoradiography one could observed that the labelled material migrated as a 14 mer. This fraction was eluted and further analysed for its s^4U content by affinity electrophoresis according to Igloi[13]. As shown in Figure 2 the sulfur containing fraction (70%) was more retarded on the gel than a 19 mer control and could be isolated in pure form. The fully purified fraction was then subjected to sequence analysis, following a recently published procedure[14], for complete confirmation.

Having developed a methodology to incorporate 2'-deoxy-4-thiouridine **1** into oligonucleotides by the phosphoramidite approach[15], it was of interest to verify if the results could be extended to other sulfur modified nucleosides such as 4-thiothymidine **5** and 6-mercaptopurinedeoxy-2'-riboside **6**. Their appropriately pivaloyloxymethyl protected derivatives[6] were phosphitylated into their corresponding phosphoramidites **7** and **8**, respectively which proved to be perfectly suitable for the synthesis of oligomers by following the above described procedure.

Moreover in the ribonucleoside series, we have prepared the corresponding 2'-0-*tert*-butyldimethylsilyl protected phosphoramidite of 4-thiouridine (**9**) to be used to prepare RNA containing this naturally occurring residue by automatic synthesis[17].

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