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SOLID PHASE SYNTHESIS OF 5' NON RADIOACTIVE MULTIPLE LABELLED OLIGODESOXYRIBONUCLEOTIDES.

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Abstract : The convenient solid phase synthesis of oligodesoxyribonucleotides carrying multiple amine groups at their 5' end was described using a branching lysine core. The possibility of attachment of non radioactive label was demonstrated by synthesis of a 5'-tetrathymidilate. The identity of the derivatives was proved by Plasma Desorption Mass Spectrometry.

Labelled synthetic oligonucleotides are widely used in molecular biology as probes for detection of specific genes and as primers for DNA sequencing[1-9]. To avoid the use of the ^{32}P radio-isotope as a labelling technique much effort has recently gone into the development of non radioactive tags such as biotin or fluorophores. Most procedures involve the addition of a single primary aliphatic amino or sulphydryl group at either the 3' or the 5' end of the oligonucleotide or attached to the nucleotide heterocyclic base through a linker arm. This moiety is then used for the subsequent attachment of the reactive reporter group : isothiocyanate[10] or activated ester[11].To achieve a sensitivity equivalent to a radioactive label a possible approach is to link several reporter groups onto each single probe molecule. Thus the preparation of oligonucleotides containing a peptidic moiety at the 3' end was recently published by Haralambidis et al[12] ; in this procedure the peptidic structure can be designed to accommodate any number of labels but nevertheless can just lead to linear amplification. Here is described the use of a branching lysine core to obtain a more efficient amplification of the amine group at the 5' end, in a classical solid phase methodology.

The general synthesis scheme of 5' non radioactive multiple labelled oligonucleotides is shown in figure 1. The first step was the synthesis of a 5'amino-oligonucleotide using carbonyldiimidazole and hexamethylenediamine to obtain NH_2-oligonucleotide-polymer[11]. The convenient doubling of the amine moieties was realized by coupling the commercially available N^{α}-N^{ε}-di-Fmoc lysine in large excess (125 equivalents) with the currently used coupling reagent in peptide synthesis : benzotriazolyl N-oxytrisdimethylamino phosphonium hexafluorophosphate (BOP)[13] (1-5h). Both terminal N^{α} and N^{ε} Fmoc protecting groups were then rapidly removed with 0.1M 1,8-diazabicyclo (5,4,0) undec-7-en (DBU) in acetonitrile (3 min).

The coupling and deprotection can be repeated several times to obtain the desired number of amino groups. To label the oligonucleotide, biotin or fluorophore can be introduced on each free amino group.

To validate our approach this protocol was used to synthesize a 5'-tetrabiotinylated-tetrathymidilate chosen for an easy analytical control. After each coupling step a sample of the resin was cleaved using ammonia and the ammoniacal cleavage solution was directly analyzed by reversed phase HPLC[14] proving complete transformations (figure 2).

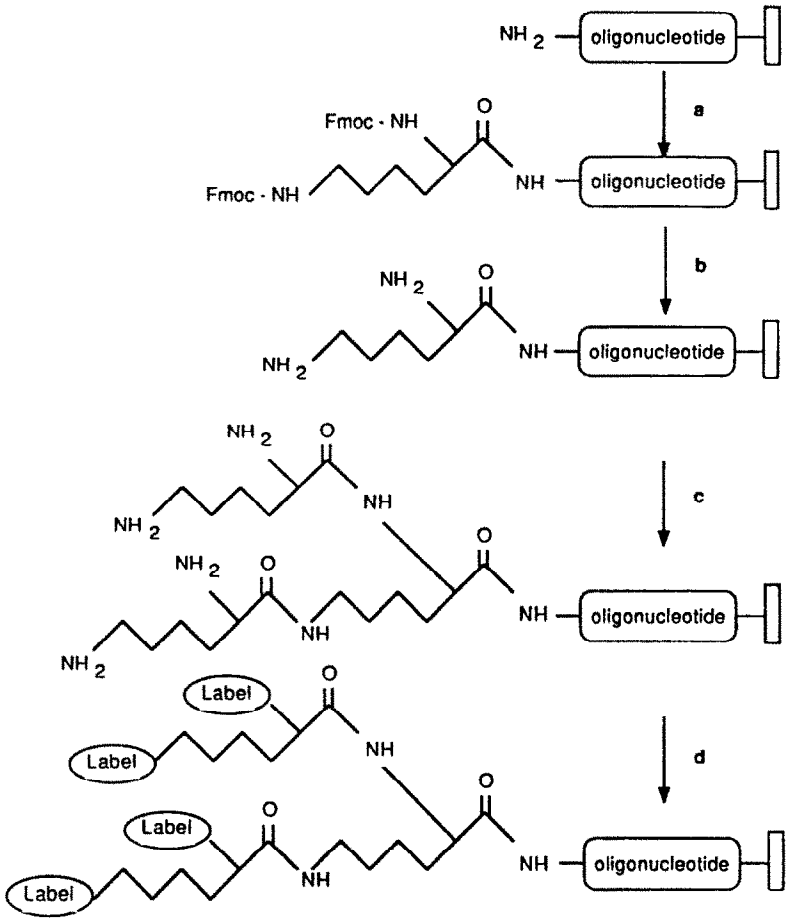

Fig. 1. **GENERAL REACTION SHEME FOR THE SYNTHESIS OF 5' MULTI-LABELLED OLIGONUCLEOTIDES.**

a) di-Fmoc lysin, BOP, DIPEA, DMF.
b) DBU, CH3CN.
c) Repeat steps a) and b) .
d) (biotin or fluorophore carboxylic form), BOP, DIPEA, DMF.
or (fluorophore isothiocyanate form) , DIPEA, DMF.

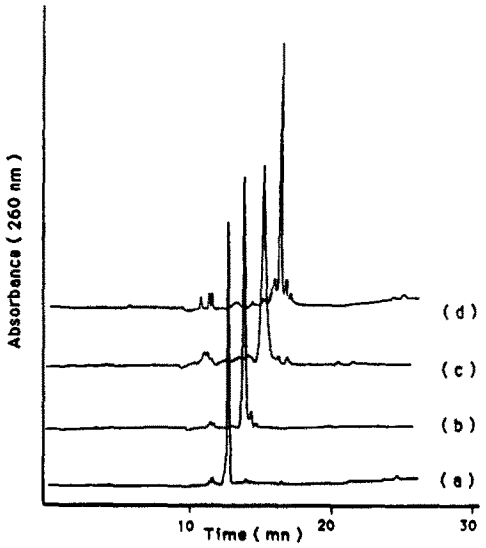

Fig. 2. Reversed phase HPLC of crude:

(a) NH_2 - T_4 , (b) (lysin) - NH -T_4, (c) $(lysin)_2$ - (lysin) - NH - T_4,
(d) $(biotin)_4$ - $(lysin)_2$ - (lysin) - NH -T_4

Identity of the different products was simultaneously checked by Plasma Desorption Mass Spectrometry on a BIO-ION 20 apparatus (figure 3). Briefly 10 µl of a water solution of modified oligonucleotide (ammonium salt 1µg/10µl) was slowly deposited on the nitro-cellulose of a mylar foil with simultaneous solvent evaporation. The accelerating voltage was -12kV and the spectrum was accumulated for 10^6 fission events.

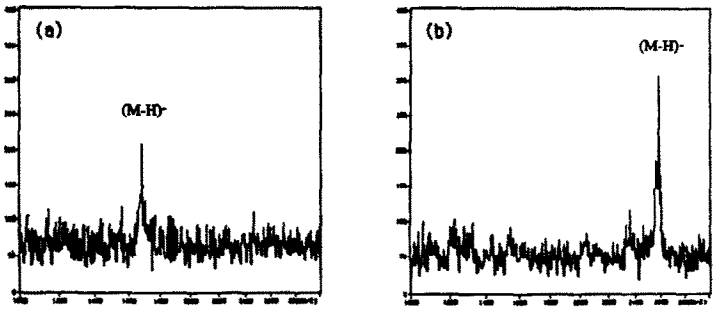

Fig. 3. Plasma Desorption Mass Spectrometry of :

(a) $(lysin)_2$ - (lysin) - NH -T_4 . M. W. Calcd (1680). Found (1680)
(b) $(biotin)_4$ - $(lysin)_2$ - (lysin) - NH -T_4 . M. W. Calcd (2584). Found (2587)

Conclusion : We described here a convenient and fast method to obtain multi-labelled oligonucleotides. Solid phase synthesis was chosen to get the desired compounds for it allows the use of large excesses of di-Fmoc lysine[15] and biotine and therefore leads reactions to completion. In addition, the innovating use of BOP as a coupling reagent strongly reduced the coupling reaction times. The efficiency of the coupling and deprotection steps is demonstrated by the reversed phase HPLC of the different crude products and can allow an easy PAGE or HPLC purification. The identity of a 5' tetrabiotinylated-tetrathymidilate prepared using this protocol was checked by Plasma Desorption Mass Spectrometry and NMR.

References and notes :

1 Agrawal, S., Christodoulou, C., Gait, M.J. *Nucl. Acids. Res.*, **1986**, *14*, 6227

2 Cardullo, R.A., Agrawal, S., Flores, C., Zamecnik, P.C., Wolf, D.E. *Proc. Natl. Acad. Sci. (USA)*, **1988**, *85*, 8790.

3 Agrawal, S., Cardullo, R.A., Zamecnick, P.C., Wolf, D.E. *J. Cell. Biol.*, **1988**, *107*, 468.

4 François, J.C., Saison-Behmoaras, T., Chassignol, M., Thoung, N.T., Helene, C. *Biochemistry*, **1988**, *27*, 2272.

5 Emson, P.C., Arai, H., Agrawal, S., Christodoulou, C., Gait, M.J. *Methods in Enzymology*, **1989**, *168*, 753.

6 Arai, H., Emson, P.C., Agrawal, S., Gait, M.J. *Mol. Brain. Res.*, **1988**, 63.

7 Beck, S., O'Keffe, T., Coulls, J.M., Kster, H. *Nucl. Acids. Res.*, **1988**, *17*, 5115.

8 Kaiser, R.J., Mackellar, S.L., Vinayak, R.S., Sanders, J.Z., Saavedra, R.A., Hood, L.E. *Nucl. Acids. Res.*, **1989**, *17*, 6087.

9 Wilchek, M., Bayer, E.A. *Anal. Biochem.*, **1989**, *171*, 1.

10 Smith, L.M., Kaiser, R.J., Sanders, J.Z., Leroy, E.H. *Methods in Enzymology*, **1987**, 15, 260.

11 Wachter, L., Jablonsky, J.A., Ramachandran, K.L. *Nucl. Acids. Res.*, **1986**, *14*, 7985.

12 Haralambidis, J., Duncan, L., Angus, K., Tregear, g.W. *Nucl. Acids Res.*, **1990**, *14*, 493.

13 Castro, B., Dormoy, J.R., Evin, G., Selve, C. *Tetrah. Letters.*, **1975**, *14*, 1219.

14 HPLC was realized on a reversed phase column (Vydac 12.5 cm x 0.4 cm)
Solvent conditions : Solvent A for 5 min, then linear gradient to 100% solvent B over 15 min at a flow rate of 0.7 ml/min.
Solvent A : 0.1 M NH_4Ac
Solvent B : 50% CH_3CN-50% 0.1 M NH_4Ac.

15 di-FMOC lysine was purchased from Bachem (Switzerland).