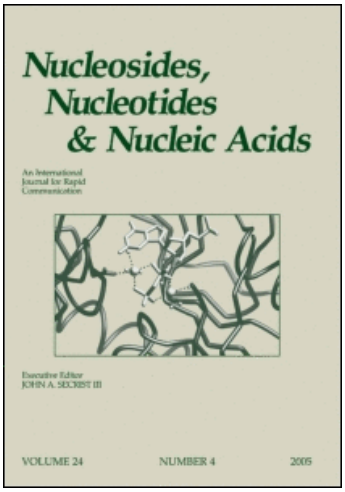

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Synthesis and Evaluation of Oligonucleotides of High Purity

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SYNTHESIS AND EVALUATION OF OLIGONUCLEOTIDES OF HIGH PURITY

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ABSTRACT

We have developed and evaluated methods for the production of highly pure oligonucleotides.

Presently the solid phase synthesis in an automated DNA synthesiser applying the phosphoramidite chemistry can be regarded as a standard. During the synthesis several undesirable by-products arise:

- incomplete coupling (1%) leads to 5'-truncated sequences. These sequences are acetylated at their 5'-hydroxyl group to prevent further elongation in subsequent coupling steps, but this "capping step" is incomplete, the capping-yield is 90%, leading to accumulation of sequences of the length n-1 with internal deletions.
- the glycosidic bond to N-protected purines, especially adenine, is susceptible to acid leading to depurination and subsequently to strand scission during alkaline deprotection of the oligonucleotide. This gives rise to 3'- and to 5'-truncated sequences. The 3'-truncated sequences will not be removed by standard RP HPLC as they are tritylated.
- the reactions involved in synthesis and deprotection may cause base modifications (full length product with damaged bases).
- insufficient deprotection procedures may result in incomplete removal of protecting groups, especially from the bases (full length products with altered bases).

We have set up two different schemes (Fig. 1 and Fig. 2) for synthesis and purification, which should provide highly pure oligonucleotides with the potential of adapting to large scale production:

- accumulation of n-1 sequences (failure of capping) will be avoided by a double capping procedure using phosphite in the first capping step and an acetic anhydride capping reagent in the second capping step, as described in the literature[1].
- 3'-truncated sequences are removed by different methods in the two schemes. In scheme I (Fig. 1) the 3'-truncated sequences can be washed off, as the 3'-full length product still is anchored to the solid support after deprotection. In scheme II (Fig. 2) the 3'-truncated sequences are digested by snake venom phosphodiesterase. The 3'-full length product is protected against digestion by a 3' - 3'-inverted end. An oligo with a correct 3'-end is, in both schemes, eventually obtained by cleaving with RNase between the ribo unit and the requested DNA-sequence.
- 5'-truncated sequences are removed by RP HPLC using the DMTr group of the last coupling step (trityl-on synthesis) as a hydrophobic tag.

Very labile protecting groups will be used to avoid problems with deprotection.

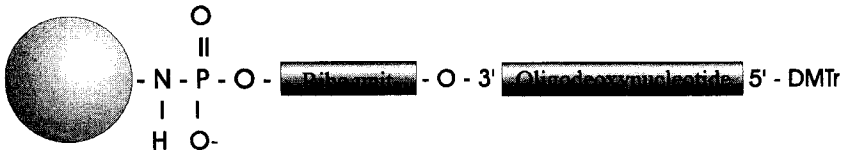

FIG. 1 The product after deprotection according to scheme I, the oligonucleotide still anchored to the solid support, and the 3'-truncated sequences can be washed off before cleaving with RNase.

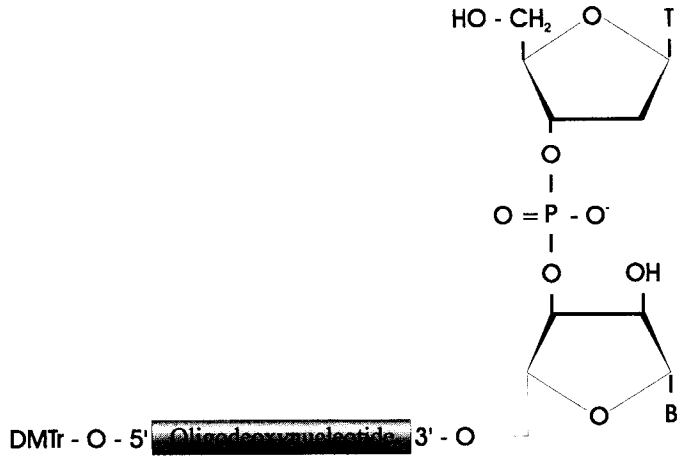

FIG. 2 The product after deprotection according to scheme II, the anchoring to the solid support is ammonia labile and is cleaved during deprotection. The 3'-truncated sequences are digested by snake venom phosphodiesterase befor the correct 3'-end is obtained by cleaving with RNase between the ribo unit and the requested DNA-sequence.

A 42mer (5'- CGC TCC AGA GGA TCC GGA AGA GCA GAT GCA GCC CAG CCT TCU -3') was used to evaluate the purification methods, The oligonucleotide was synthesised on Pharmacia Gene Assembler 4 Primers using phosphoramidite chemistry in 40 nmol scale.

For scheme I several different solid supports were tested in order to find one which does not disturb the RNase cleavage (core-shell beads[2], CPG 500 (a gift from Pierce Chemical Co.), Merckogel (Merck), Pierce (Pierce Chemical Co.) and Rapp Tenta Gel (RAPP Polymere GmbH)). The following supports were also tested to improve the yield (Argo Gel OH (Argonaut Technologies Inc.), Biosynthes Polymerträger (Merck), Fractogel EMD Amino (Merck), Fractogel EMD Diol (Merck), LiChrospher 4000 Diol (Merck), Plex EMD Diol (Merck), Polymerträger VA-Hydroxy (Riedel-de Haen), Aminomethyl Polystyrene Resin (Sigma), 4% Cellulose beads (Pharmacia), magnetic core shell beads[2], functionalised polypropylen film[3], and CPG 3000 (a gift from Pierce Chemical Co.)). After synthesis the base protecting groups were removed with 32% ammonia at 55°C overnight. At this point the oligonucleotide of scheme I is

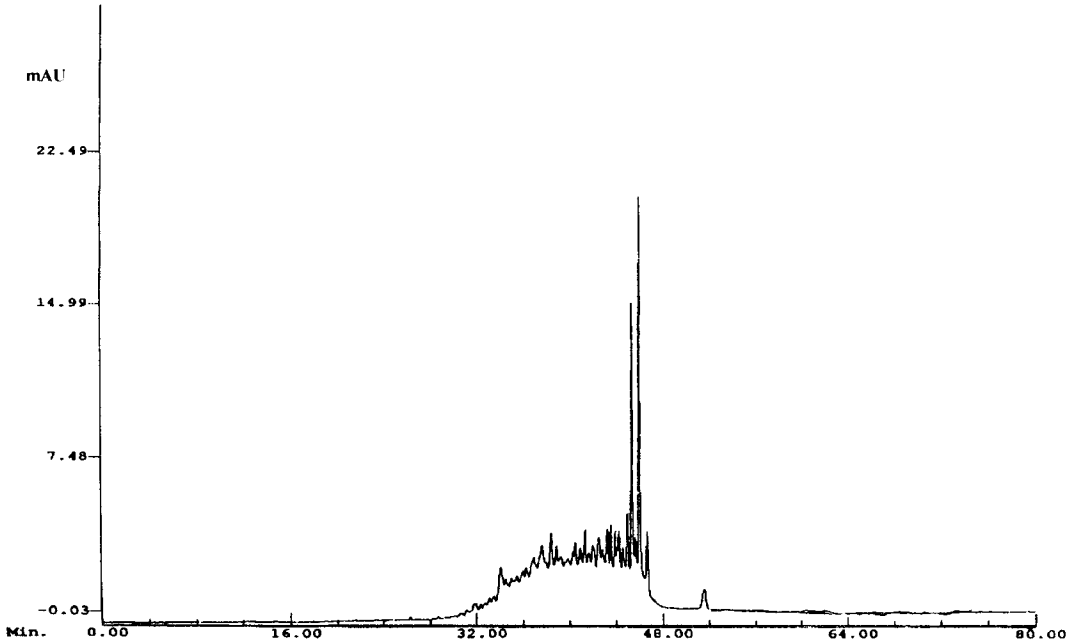

FIG. 3 The crude product after synthesis on Rapp Tenta Gel, synthesis conditions were modified to give a high percentage of impurities. The separation was made by gel capillary electrophoresis as described in the text. The diagram shows Absorbtion (260 nm) vs retention time.

still anchored to the solid support and the 3'-truncated sequences can be washed off. The support is dried before the 2' - O protecting silyl groups were removed by adding 1.0 M TBAF in THF at room temperature for 24 hours. After washing the support first with THF and then with water, the 3'-full length product is cleaved from the solid support by adding 100 U RNase-It^{TM} Ribonuclease Cocktail, Stratagene in H_2O, 4 hours at 37°C.

For scheme II, CPG was used as solid support. After deprotection of the bases, as described earlier, the ammoniacal solution was desalted on a NAP column and dried. The 2' - O protecting silyl groups were removed as described in the literature[4]. To digest the 3'-truncated sequences 100 U phosphodiesterase, Boehringer Mannheim, in CIP buffer (500mM Tris HCl, pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$) were added at 37°C for 1 hours. The phosphodiesterase was inactivated by heating 5 minutes to 95°C before 100 U RNase-It^{TM} Ribonuclease Cocktail, Stratagene in H_2O were added to the sample.

5'-full length sequences were collected after separation by HPLC using a LiChrospher RP 18 (5μm) column and the DMTr-group as a hydrophobic tag. The gradient was linear, solvent A: 0.01M ammoniumacetate, solvent B: 0.01M ammoniumacetate and 50% acetonitrile. Finally the DMTr-group was removed by adding 300μl 80% acetic acid for 30 minutes at room temperature. The analysis of the oligonucleotides was performed by a Biorad Biofocus Gel Capillary electrophoresis system equipped with a μPage (J&W Scientific) polyacrylamide gel capillary (75 μm x 50 cm). The electrophoresis was done at constant voltage of 12kV in a μPage Buffer Solution (J&W Scientific); 100mM Tris borate, 7M urea, pH 8.3.

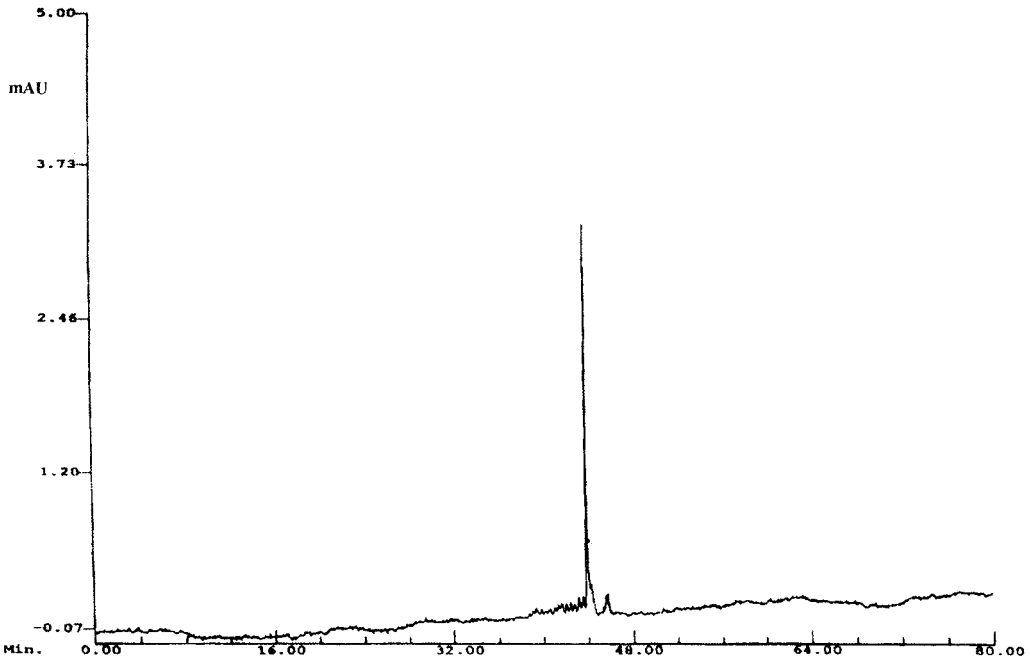

FIG. 4 The same product as in fig. 3, after purification according to scheme I. The separation was made by gel capillary electrophoresis as described in the text. The diagram shows Absorbtion (260 nm) vs retention time.

The principle that involves anchoring of the oligonucleotide to the solid support via a ribo unit with subsequent cleavage by RNase (scheme I) seems to date to be the most feasible approach. We have assessed different solid support systems for their susceptibility to RNase cleavage of the immobilized oligonucleotides. RNase seems to have the best possibility to cleave when Rapp Tenta Gel or Argo Gel OH is used. Fig. 3 shows a separation of the complete crude product obtained from the synthesis of a 42mer on Rapp Tenta Gel (synthesis conditions were modified to give a high percentage of impurities) by combining the nucleotidic material cleaved from the polymer by 1. ammonia plus 2. RNase. Fig. 4 represents the same separation, however, in this case only the product released by RNase according to scheme I has been applied to the column. From this comparision it is obvious that the pre-purification of the support-bound oligonucleotide followed by enzymatic product release removes most of the impurities.

In the case of the second scheme, we found that the oligonucleotide is not sufficiently protected against degradation by SV-PDE by the 3' - 3'-inverted end. This should be overcome by choosing a different exonuclease or eliminating the endonucleolytic side activity from SV-PDE, see the literature[5].

The extent of base modification will be monitored by sequencing after cloning, and synthesis techniques will be adapted accordingly.

A purification scheme using chemical instead of enzymatic modification steps, has been reported[6,7].

REFERENCES

1. Yu, D., Tang, J., Iyer, R. P. and Agrawal, S., Tetrahedron Lett. 35:8565-8568 (1994)
2. Margel, S., Gura, S., Bamnolker, H., Nitzan, B., Tennenbaum, T., Bar-Toov, B., Hinz, M., Seliger, H., „New solid and hollow, magnetic and nonmagnetic, organic-inorganic monodispersed hybrid microsheres: Synthesis, characterization and use“ in Scientific and Clinical Applications of Magnetic Carriers
3. Lee, K.-W. and McCarthy, T. J., Macromolecules 21:304-313 (1988)
4. Vinayak, R., A Companion to Methods in Enzymology 5, 7-18 (1993)
5. Ortigão, J. F. R., Rösch, H., Selter, H., Fröhlich, A., Lorenz, A., Montenarh, M. and Seliger, H., Antisense Res. and Dev. 2:129-146 (1992)
6. Schirmeister, H., Himmelsbach, F., Pfleiderer, W., Helv. Chim. Acta 76(1):385-401 (1993)
7. Kwiatkowski, M., Nilsson, M., and Landegren, U., Collection of Czech. Chem. Commun. 61:307-310 (1996)