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Note

Purification of fully protected oligonucleotide phosphotriester intermediates by gel filtration on Sephadex LH-60

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In the last few years, much time and effort has been directed towards the synthesis of oligonucleotides via phosphotriester intermediates[1–8]. Basically, the method consists in coupling two partially protected phosphotriester fragments (*e.g.*, 1 and 2 in Scheme 1), in the presence of an activating agent (3) derived from 2,4,6-triisopropylbenzenesulphonic acid, to give the required product (*e.g.*, 4). The fully protected DNA fragment thus obtained has to be separated from excess of first component (*e.g.*, 1) and, if not fully consumed, second component (*e.g.*, 2).

Further, a typical side-product formed in this coupling process is a 5′-O-sulphonylated product (*e.g.*, 5), the formation of which could not be prevented[9] by using different[10–16] activating agents (3a–f).

We consistently observed in our purification procedure, using short column chromatography[17] on silica gel with chloroform–methanol as the eluting solvent, losses of required products (*e.g.*, 4) and unsatisfactory resolution of side- and main products.

The decrease in yield of required products (*e.g.*, 4) and the loss in resolving power became more significant when fully protected DNA fragments of relatively high molecular weight (*e.g.*, 4, $n + m \geq 2$) were purified by this technique, and especially for oligonucleotides with a relatively high content of the bases thymine or uracil and guanine.

Further, the purification of fully protected DNA fragments of high molecular weight (*e.g.*, 4, $n + m = 12$) was virtually impossible by short column chromatography[17] on silica gel, even when water, which inactivated the adsorbent, was added to the eluting solvent.

Therefore, in order to obtain pure coupling products (*e.g.*, 4) which can either be completely deblocked to give oligonucleotides, or selectively deblocked and as such be used for further coupling reactions, it is essential to have an effective purification procedure.

We report here that fully protected oligonucleotide phosphotriester inter-

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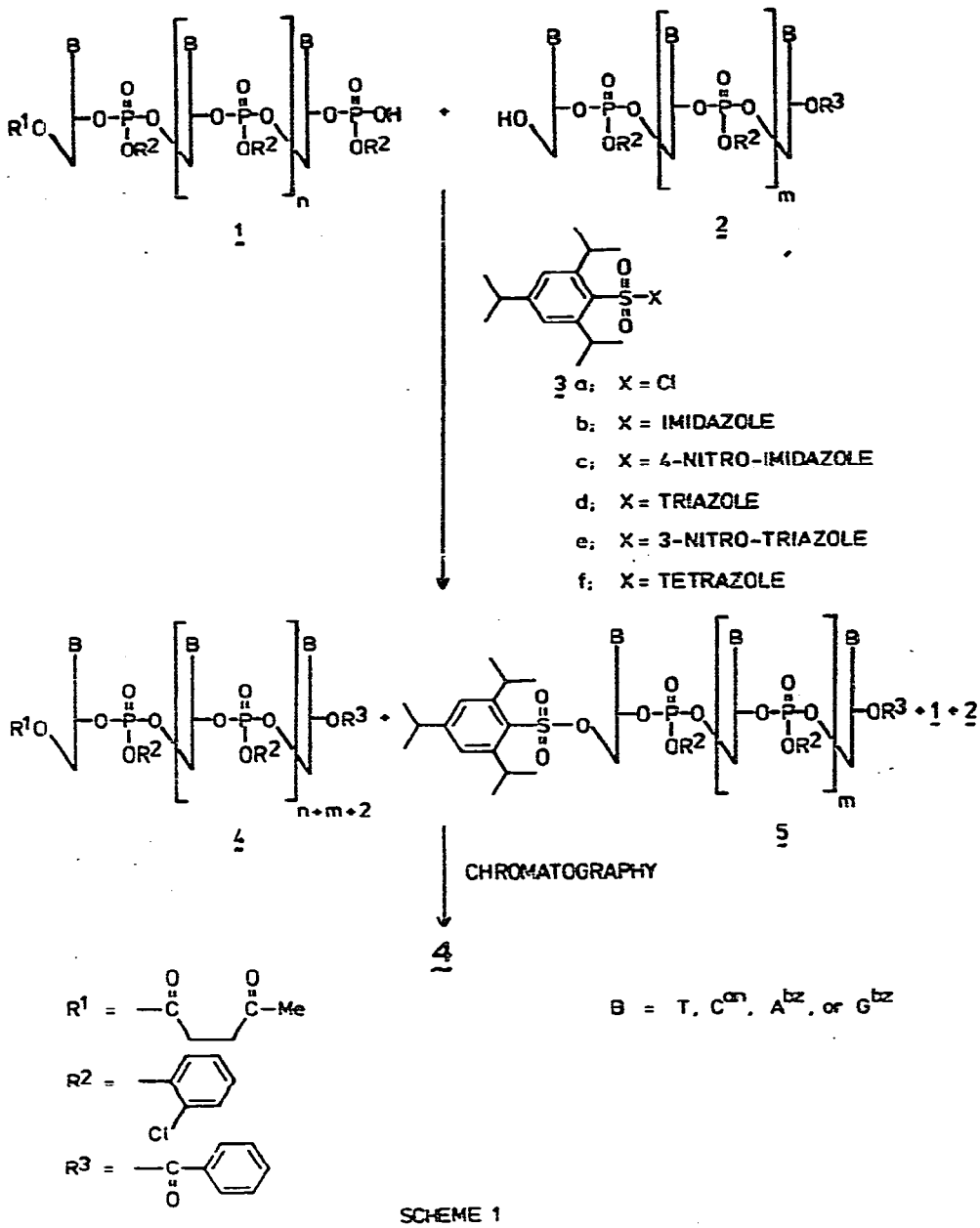

SCHEME 1

mediates can be purified in relatively large amounts by gel filtration chromatography on Sephadex LH-60 in tetrahydrofuran–methanol (95:5).

EXPERIMENTAL

Fully protected DNA fragments

The fully protected oligonucleotides used are listed in Table I. They were prepared by block condensation of the partially protected fragments 1 and 2 (see

TABLE I

FULLY-PROTECTED OLIGONUCLEOTIDES (*e.g.*, 4 IN SCHEME 1) PURIFIED BY GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX LH-60

THF = Tetrahydrofuran; MeOH = methanol.

No.	*DNA fragment**	*Mol. weight*	*Weight of crude fragment (mg)*	*Eluting solvent*	*Yield*** *(%)*
1	AAAAAAAA	4249	1000	THF	76
2	TTTTAGGAT	4245	450	THF	82
3	CCGTCGACGG	5279	200	THF	60
4	CCGGATCCGG	5279	150	THF–MeOH	60
5	TATCAAGTTG	4890	500	THF	60
6	AAAACCGGAAAA	6403	200	THF–MeOH	65
7	ATCCTATAGGAT	5953	350	THF	78
8	ATCCTATTAGGAT	6367	500	THF–MeOH	68
9	ATCCTATTTAGGAT	6782	500	THF–MeOH	68
10	ATCCTATTTTAGGAT	7196	500	THF–MeOH	75
11	ATCCTATTTTTAGGAT	7601	120	THF	75
12	AAAACCGGAAAAAAAA	8513	200	THF–MeOH	60

* All fragments are protected in the same way as indicated in Scheme I.

** Yields of the precipitated products were based on the second component (*e.g.*, 2 in Scheme 1) of the coupling reaction.

Scheme 1) under the influence of the coupling agent 3e[14,15]. Full details of the synthesis of these compounds will be published elsewhere.

Separation procedure

Sephadex LH-60 (Pharmacia, Uppsala, Sweden) was swollen in freshly distilled "Bakers Grade" tetrahydrofuran (Baker, Deventer, The Netherlands) for 2 h. The gel was packed into the column (150 × 3 cm) under gravity flow and then allowed to equilibrate for several hours under flow conditions. Samples were applied on to the column as 20–25% solutions of the crude fragments in the eluting solvent. The column was eluted with the solvent under gravity flow. Elution was monitored by UV transmission at 254 nm (Uvicord II, LKB, Stockholm, Sweden) and fractions of 7.5–10 ml were collected in an LKB fraction collector.

It was found that the gel performed well at relatively high flow-rates (45–60 ml/h) with good resolution.

The column parameters are given in Table II. The time required for a typical run (*e.g.*, experiment no. 8, Table I) was *ca.* 8 h.

The best results were obtained when the column was eluted with the same solvent between separations until 100% UV transmission was obtained (*ca.* 20 h). In

TABLE II

COLUMN PARAMETERS

Eluting solvent	*Bed volume of Sephadex LH-60 (ml/g)*	*Column diameter (cm)*	*Total bed volume (ml)*	*Flow-rate (ml/h)*
THF	9.7	2.7	630	45–60
THF–MeOH	10.0	2.7	650	45–60

this way, many separations could be carried out on the same column with the maintenance of good resolution.

Thin-layer chromatography (TLC) was carried out on silica gel TLC-Ready Plastic Sheets F 1500 LS 254 (Schleicher & Schüll, Dassel, G.F.R.).

Short column chromatography[17] was carried out on Kieselgel 60 (E. Merck, Darmstadt, G.F.R.).

RESULTS AND DISCUSSIONS

Sephadex LH-60, formed by hydroxypropylation of Sephadex G-50, has both hydrophilic and lipophilic properties. As the beads are known to swell in a wide variety of organic solvents[18], we tested some of these solvents as eluents for the purification of fully protected oligonucleotides.

In our studies the mobile phase tetrahydrofuran–methanol (95:5), which can easily be removed *in vacuo* at 30°, proved to be most generally applicable for this purification procedure. This solvent system provides excellent solubility properties and good chromatographic results, without causing any detectable deprotection or transesterification of the protecting groups. The latter phenomenon, transesterification of the aryl protecting groups (R^2 in Scheme 1), has been observed during purification of fully protected oligonucleotides (*e.g.*, 4) on silica gel, and is presumably due to the relatively high percentage of methanol (up to 10%) in the eluting solvent chloroform–methanol in combination with the catalysing effect of the silica gel surface.

Whereas fully protected oligonucleotides (*e.g.*, 4), obtained in coupling reactions as outlined in Scheme 1, often exhibit, in chromatographic systems involving silica gel, a similar R_F value as the second component (*e.g.*, 2), they behaved differently in chromatographic systems with Sephadex LH-60 in tetrahydrofuran–methanol (95:5). In the latter systems, separation of the various components of the reaction mixture seems to be due mainly to the difference in molecular weight (gel filtration) and adsorption effects. Although methanol was added to the eluting solvent mainly for reasons of solubility of the fully protected oligonucleotides in tetrahydrofuran, it even enhanced the resolution of these molecules.

Table I lists the fully protected DNA fragments employed in this study using tetrahydrofuran–methanol (95:5) as eluents.

For instance, it was found that the crude fragment (experiment no. 1, Table I) on application to Sephadex LH-60 in tetrahydrofuran gave a chromatographically pure product in an isolated yield of 76%. Purification of the same amount of this crude fragment on silica gel in chloroform–methanol (96:4), however, gave only 60% of impure product. The same unsatisfactory results were obtained during the purification of the other DNA fragments on silica gel.

An example of the purification of a fully protected oligodeoxyribonucleotide (experiment no. 8, Table I) on Sephadex LH-60 in tetrahydrofuran–methanol (95:5) is given in Fig. 1. About 3 mg of this crude fragment, obtained by coupling two partially protected fragments (1, $n = 4$, and 2, $m = 5$ in Scheme 1), was applied to Sephadex LH-60 in tetrahydrofuran–methanol (95:5) to give a well resolved peak of the desired product (Fig. 1a). The products eluted from the column were identified by TLC on silica gel in chloroform–methanol (90:10) as indicated in Fig. 1b.

Purification, on a preparative scale, of the same crude fragment (experiment

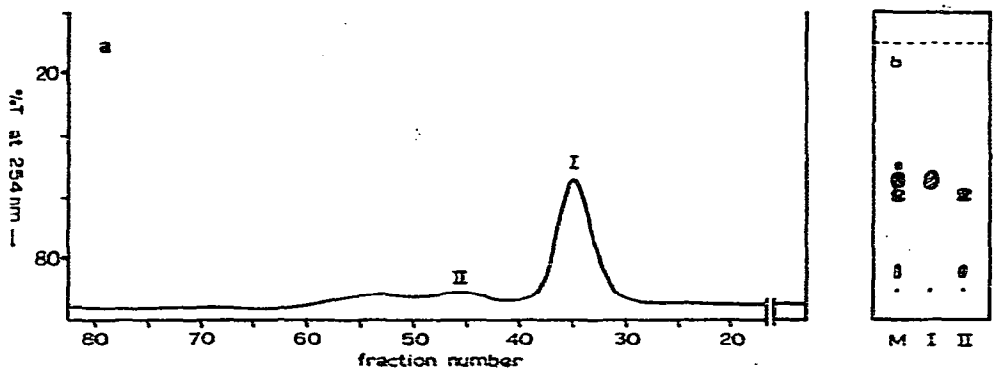

Fig. 1. (a) Purification of *ca.* 3 mg of oligodeoxyribonucleotide fragment no. 8 (Table I) by chromatography on Sephadex LH-60. (b) TLC analysis of the products I and II (Fig. 1a). M refers to the crude fragment; I refers to the desired product.

no. 8, Table I) is demonstrated in Fig. 2. About 500 mg of crude fragment was loaded on to the column, and analysis of the fractions by TLC (Fig. 2b) indicated that the components were eluted from the column without significant loss of resolution compared with the analytical purification (Fig. 1a).

High-performance liquid chromatographic (HPLC) analysis[19] (Fig. 3) of the product (dATCCTATTAGGAT), obtained after removal of the protecting groups of the purified fragment (experiment no. 8, Table I) with fluoride ion[20] followed by aqueous ammonia, confirmed the purity of the isolated fragment. This was indicated by the absence (see Fig. 3) of peaks from the deprotected reaction components dATCCTApOR$_2$ and dTTAGGAT (*e.g.*, unblocked compounds 1 and 2 in Scheme 1).

All crude oligodeoxyribonucleotide fragments (experiments nos. 1–12, Table I) were purified by gel filtration chromatography on the same column of Sephadex LH-60, and could be isolated in high yields as indicated in Table I.

The purification of two fully protected oligoribonucleotide fragments, rAAGAAG and r-pAGCCUGGp[21], demonstrated the general application of this separation procedure for the purification of fully protected oligoribonucleotide phosphotriester intermediates.

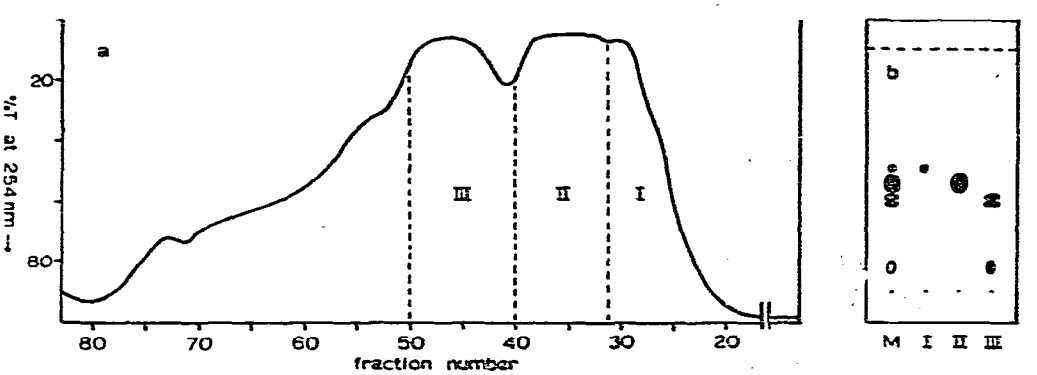

Fig. 2. (a) Purification of *ca.* 500 mg of oligodeoxyribonucleotide fragment no. 8 (Table I) by chromatography on Sephadex LH-60. (b) TLC analysis of the products I, II and III (Fig. 2a). I refers to an unidentified product; II refers to the required product; M refers to the crude fragment.

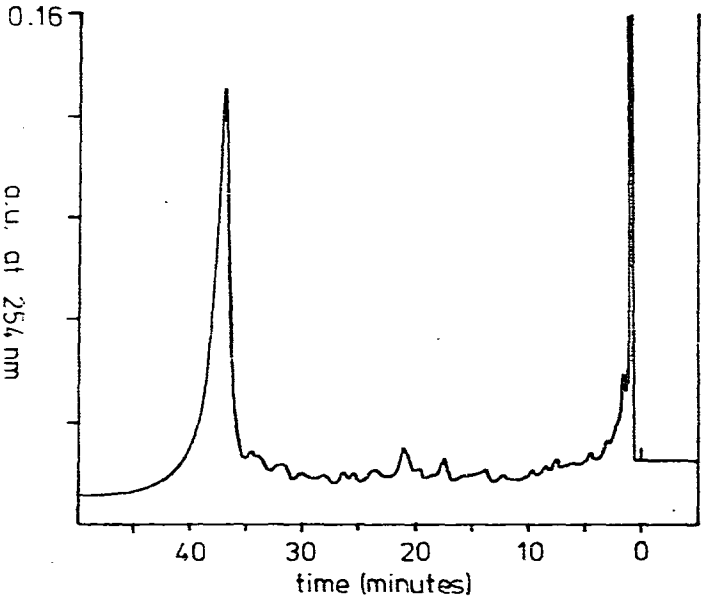

Fig. 3. HPLC analysis of dATCCTATTAGGAT, obtained after removal of the protecting groups of the purified fragment no. 8 (Table I), *i.e.*, product II in Fig. 2a, on Permaphase AAX at 60°. Solvent system: buffer A, 0.005 *M* KH_2PO_4 (pH 4.1); buffer B, 0.5 *M* KCl–0.05 *M* KH_2PO_4. Elution was carried out with a linear gradient of 2% of buffer B per minute, starting from 97% buffer A and 3% buffer B.

CONCLUSION

Gel filtration chromatography on Sephadex LH-60 in tetrahydrofuran–methanol (95:5) is an excellent and economical method for the purification of fully protected oligonucleotide phosphotriester intermediates. The procedure has the following advantages over the previously used method employing short column chromatography on silica gel in chloroform–methanol: (a) excellent resolving power; (b) total recovery of purified product; (c) repeated use of the same column; and (d) low operating costs.

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