HELP (High Efficiency Liquid Phase) new oligonucleotide synthesis on soluble polymeric support

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Received March 22, 1990; Revised and Accepted May 9, 1990

ABSTRACT

A simple, rapid and high-yielding method for the synthesis of oligonucleotides by the phosphotriesters approach is described. The use of polyethylene glycol (PEG) as soluble polymeric support preserves some convenient features of the solid-phase synthesis with new interesting advantages.

Short oligonucleotides in hundred milligrams scale can be obtained from few grams of functionalised PEG.

INTRODUCTION

One of the fundamental development in the oligonucleotide synthesis has been the introduction of the solid-phase method (1–5). The liquid-phase method which adds interesting advantages to some convenient features of the solid-phase synthesis was first proposed for the peptide production (6). Preliminary experiments toward the oligonucleotide synthesis were described in the past (7–11) without any further application.Recently a study on this subject appeared in the literature (12).

This method uses a supporting polymer soluble in the reaction media; excess reagents and coupling components are eliminated by a simple diafiltration (13) or crystallization (14). Making use of polyethylene glycol (PEG), products are easily purified by a rapid crystallization; in fact, PEG differs from other polymers for its marked tendency to crystallize (15) avoiding the formation of gelatinous precipitates (16) and inclusions. Hence the total time required for the synthesis of an oligomer is drastically shortened.

While synthesis on a solid support meets most of the present needs of molecular biology, it is as yet unsatisfactory when relatively large quantities of pure oligonucleotides are required. Therefore there still exists a need for rapid, simple and less expensive methods that allow the high scale production of the desired oligonucleotides.

We propose herein a technique for the synthesis of oligonucleotides based on the liquid-phase method (called **H.E.L.P.**= **H**igh **E**fficiency **L**iquid **P**hase) that permits the obtainment of the desired products in a cheap, rapid and high-yielding way.

RESULTS AND DISCUSSION

Functionalisation of PEG

The first nucleoside was attached by coupling the PEG with the symmetrical anhydride derivative of the four 5′-O-DMT-2′-deoxynucleoside-3′-O-succinates in a way analogous to the solid phase technique (17). The nominal loading value of nucleoside (μmol/g) obtainable depends from the molecular weight of the polymer. In the present case a monomethylether polyethylene glycol 5000 was used that corresponds to 200 μmoles of free OH groups/g. The OH groups have been esterified in a 90–95% yield (Table 1) as determined by measuring the DMT absorption at 498 nm.

The PEG-nucleosides were analysed by ^{1}H-NMR spectroscopy to confirm the complete removal of the reagents (preliminarly ascertained by TLC) and to unequivocally characterize the products (Fig.1). Any unreacted PEG molecule was capped by acetic anhydride treatment; when DMAP was added as catalyst some modification of bases occurred as determined by ^{1}H-NMR investigation (mainly N,N-bis substitution at the N_6 of dA and displacement of isobutyrryl group at the N_2 of dG; data not shown). The removal of DMT was achieved by TCA 3% in methylene chloride without any detectable depurination.

Chain assembly

Synthesis of the homo-dinucleotides

To test the efficiency of the synthesis on the PEG the four homo-dimers d(TpT), d(CpC), d(ApA) and d(GpG) were first prepared.All the common condensing agents were tested (19) as well as the effect of NMI as catalyst (20) and the influence of concentration and excess of nucleoside monophosphates. The effect of the presence of 2,6-lutidine (21) in the solvent mixture was also checked. All the results confirmed the data reported in the literature. The number and solvent composition of the crystallization steps were optimized in order to obtain a rapid and efficient purification. From all these studies the standard synthetic protocol reported in Table 2 was set-up.

The coupling yields, as judged from UV and NMR, range from 90 to 95% and the crude deprotected products result very pure from HPLC analysis (Fig 2). The compounds were identified by comparison with the same homo-dimers independently

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synthesized. In the case of d(GpG) the higher than 100% yield observed indicates the formation of side-products, as confirmed by HPLC; the use of a bulky protecting group like diphenylacetyl(DPA) slightly reduces this side-reaction. From these results it appears advisable to use a guanine protected also at the 6-oxygen position in the synthesis of sequences containing dG stretches.

Synthesis of d(TAGCGCTA)

The feasibility of this method was tested in the synthesis of the octanucleotide d(TAGCGCTA) following the reported protocol (Table 2). The identity of the final product was confirmed by comparison with the same oligomer independently synthesized (22–23) in solution by a modified phosphotriester method (24).

During the synthesis anhydrous conditions are obviously essential. After each cycle, TLC analyses confirmed the complete removal of the reagent in excess and the soluble by-products.

The spectroscopic data of the PEG-bound oligomers were always in agreement with the expected composition. Moreover, the oligonucleotides released from the polymer, both DMT-protected and OH-free, were analyzed by reverse-phase HPLC: a main peak was observed in every case (data not shown).

Starting from the PEG-pentanucleotide some changes in the physical characteristics of the product were observed: the solid tended to be amorphous and assumed a brownish colour. This fact did not lead to difficulties in the precipitation steps; some small losses of material (2–4%) only occurred, mainly during the crystallization step from ethanol. The single step, the overall and the average yield are reported in Table 3. The measured values are in good agreement with those previously reported for the solid-phase synthesis via phosphotriesters (25). A higher than 100% yield is still measured when a dG residue is the 5'-OH partner of the coupling reaction, but this effect is less considerable than in the homo-dimer synthesis.

Table 1. Degree of functionalisation of PEG

deoxynucleoside	μmol/g PEG	yield %
dT	170	96
dC(Bz)	165	95
dA(Bz)	165	94
dG(Ibu)	158	90
dG(Dpa)	162	94

The yield was calculated by taking into account the average molecular weight of the whole PEG-nucleoside.This value,higher than the starting PEG, decreases the number of μmol/g of DMT corresponding to 100% yield.

Deprotection and purification

At the end of the synthesis the oligomer was removed from the polymer during the standard deprotection procedure (26). The oximate treatment released most of the product from PEG; the complete hydrolysis was achieved during the subsequent reaction with concentrated ammonia. The partially deprotected 5'-O-DMT- oligonucleotide was analyzed by HPLC using a C_{18} column (Fig.3). Finally, after complete deprotection with acetic acid, the product was purified by ion-exchange chromatography. The elution profile is shown in Fig.4, together with the HPLC of the crude and purified product. Its identity was confirmed by co-chromatography with the same oligomer independently synthesized. From 980 mg of PEG-octamer, 85 mg of pure lyophilized TEA salt of d(TAGCGCTA) were obtained.

Concluding remarks

The method described in this paper preserves some convenient features of the solid-phase synthesis with the following further advantages: i) the synthesis is performed in an *homogeneous* solution where a *lower excess of reagents* is required and appreciable *cost savings* are obtained; ii) *large amount* of oligonucleotides can be obtained from only one synthetic run; iii) the synthesis is easily monitored by non-destructive spectrophotometric methods.

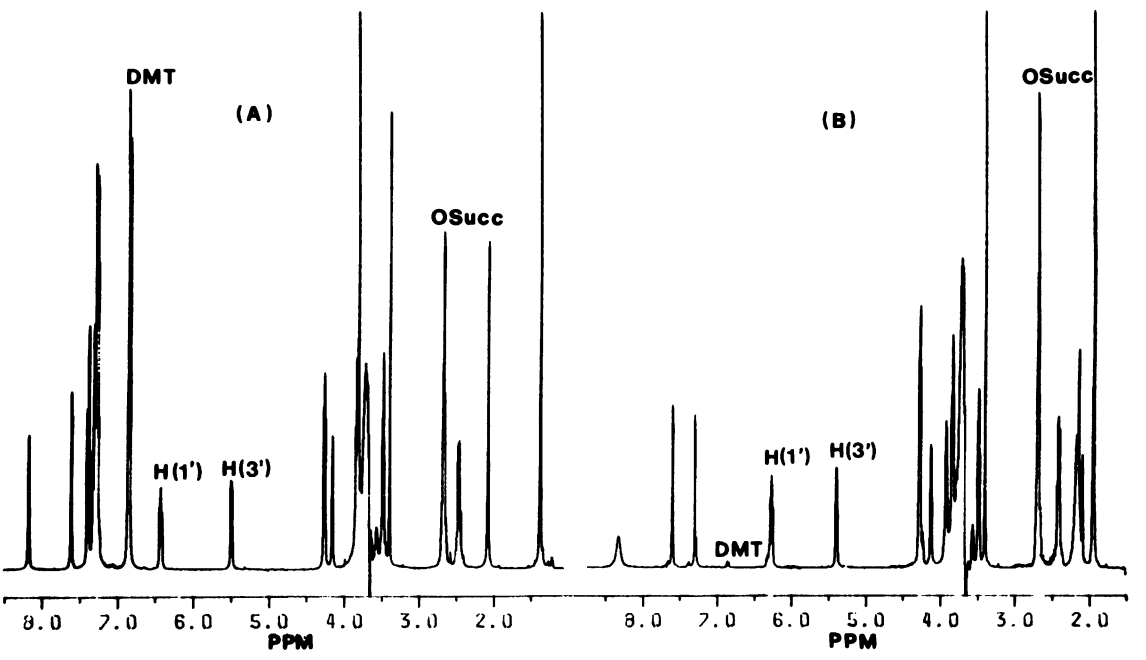

Figure 1. ^{1}H-NMR spectra of: A) DMT-dT-PEG and B) OH-dT-PEG in $CDCl_3$-10 mg in 0.5 ml.

In conclusion with the HELP method it is possible to obtain short oligonucleotides in hundred milligrams scale without any special equipment, at low cost (compared to the solid-phase) and in a short time (compared to the 'classical' solution procedure).

Further investigations on the synthesis of longer oligonucleotides and application of more effective synthetic methodologies (e.g. phosphoroamidites, H-phosphonates) are under way and will be reported in forthcoming papers.

EXPERIMENTAL

Materials and Methods

Polyethylene glycol monomethylether (PEG; average molecular weight=5000, hydroxyl number 0.20 meq/g) was purchased from Fluka and purified by crystallization from methylene chloride/diethyl ether. Pyridine, 2,6-lutidine, methylene chloride and N-methylimidazole were treated as reported (27) and stored under argon over 4 Å molecular sieves. Diethyl ether was dried over Na_2SO_4. All four mononucleotides were prepared by the method of Narang et al.(28). 3'-O-succinate protected nucleosides were synthesized following the standard method (29) with minor modifications. Thin-layer chromatography (TLC) was performed on precoated silica gel sheets 60 F_{254} (Merck). HPLC analyses were carried out on a Nova-Pak C_{18} column (300 Å, 4.5×250 mm, 5 μ) using a Perkin-Elmer 3B system with UV detector LC-75. Ion-exchange was carried out on DEAE-Sephacell (Pharmacia). UV spectra were recorded on a Perkin-Elmer Lambda 5. ^{1}H-NMR spectra were recorded on a Bruker 400 AM spectrometer in $CDCl_3$-99.96% d (Stohler). The 2'-deoxynucleosides and the coupling reagent 2-(mesitylbenzenesulphonyl)-3-nitro-1,2,4-triazole(MSNT) were obtained from Cruachem. All other solvents and reagents,of the highest commercially available purity, were obtained from Aldrich or Jannsen and used as such.

Glass equipment was dried in an oven and stored in a dessicator. PEG-oligonucleotides and nucleoside monophosphates were always coevaporated at least twice from dry pyridine before the coupling reaction.

General procedure for functionalisation of PEG

1.0 g of PEG (0.200 mmoles of free OH groups), was rendered anhydrous by coevaporation with pyridine and dissolved in 10 ml of methylene chloride (+ 0.5% of pyridine). 0.600 mmoles of 5'-O-DMT-2'-deoxynucleoside-3'-O-succinate, dried by coevaporation with pyridine were dissolved in 5 ml of methylene chloride (+ 0.5 % of pyridine) and 0.300 mmoles of DCC were added under stirring at 0°C. After 15 min the solution was filtered, poured into the reaction vessel containing the PEG solution and 0.600 mmoles of 4-dimethylaminopyiridine (DMAP) were added. The reaction volume was reduced to 1/3 by evaporation and the final solution was left to react under stirring under argon atmosphere for 3 days at room temperature. The solution was filtered, and the PEG-nucleoside precipitated by slow addition of an excess of diethyl ether (10−20 volumes) at 0°C and under vigorous stirring. The final product was filtered and extensively washed with ether. The complete removal of the unreacted excess of reagents was ascertained by TLC (eluant: ethyl acetate/acetone/water=5/10/1) : only the PEG-nucleoside has a zero Rf value under these conditions. If higher migrating compounds are present, the product can be purified by redissolving in methylene chloride (10 ml/g) and reprecipitating with diethyl ether (100 ml); alternatively recrystallization from absolute ethanol (100 ml/g) is often effective (dissolution at 35°C and filtration after cooling at 0°C for at least 30 min). The degree of functionalisation was estimated spectrophotometrically. 1.0 mg of PEG-nucleoside was dissolved in 10 ml of a 60% aqueous perchloric acid/ethanol (3:2 v/v) and the absorbance at 498 nm was measured. The nucleoside loading was calculated (in μmol/g) from the equation : $[A_{498}(10\text{ mm cell})\times 10\times 14.3]$/mg of weighed support. An internal control of this value was possible

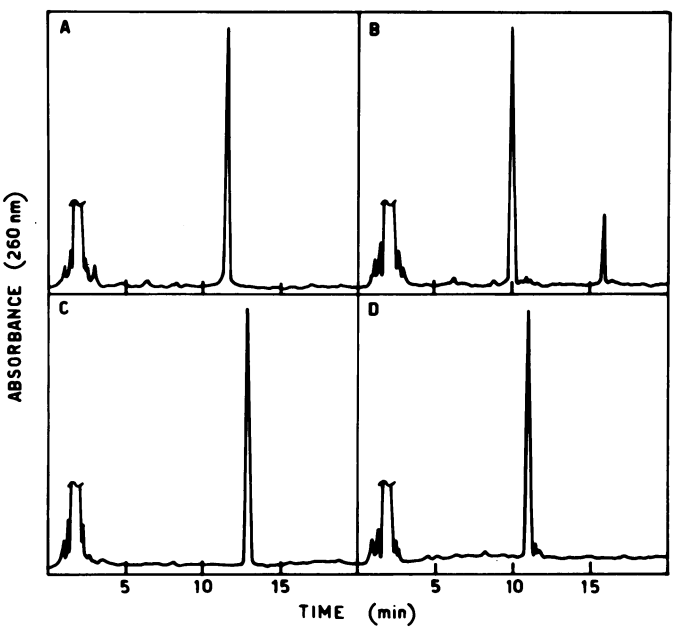

Figure 2. Reverse-phase HPLC profiles of crude DMT-homo-dinucleotides: A) 5'-DMT-d(ApA); B) 5'-DMT-d(GpG); C) 5'-DMT-d(TpT); D) 5'-DMT-d(CpC).

Table 2. Chain elongation cycle

step	solvent or reagent	time(min)
detritylation	3% TCA in CH_2Cl_2 (10 ml)	15
recrystallization	CH_2Cl_2/Et_2O 2× (10/90 ml)	60
condensation	5'-DMT-O-nucleoside (3 equiv.) MSNT (6 equiv.) NMI (10 equiv.) in pyr/2,6-lutidine (5 ml)	60
recrystallization	EtOH abs 2× (100 ml)	60
capping	Ac_2O 10% in pyr (10 ml)	60
recrystallization	CH_2Cl_2/Et_2O 1× (100 ml)	30

Volumes are for 1.0 g of PEG-oligonucleotide.

Table 3. PEG-supported synthesis of d(TAGCGCTA)

samples	weighed amount (g)	yield (%)
PEG-d(A)	1.00	–
PEG-d(AT)	1.05	95
PEG-d(ATC)	1.10	87
PEG-d(ATCG)	1.18	105
PEG-d(ATCGC)	1.13	97
PEG-d(ATCGCG)	1.08	106
PEG-d(ATGCGCA)	1.04	92
PEG-d(ATGCGCAT)	0.98	97
Overall yield = 79% Average yield = 97%		

Yields were calculated from both UV and NMR data.

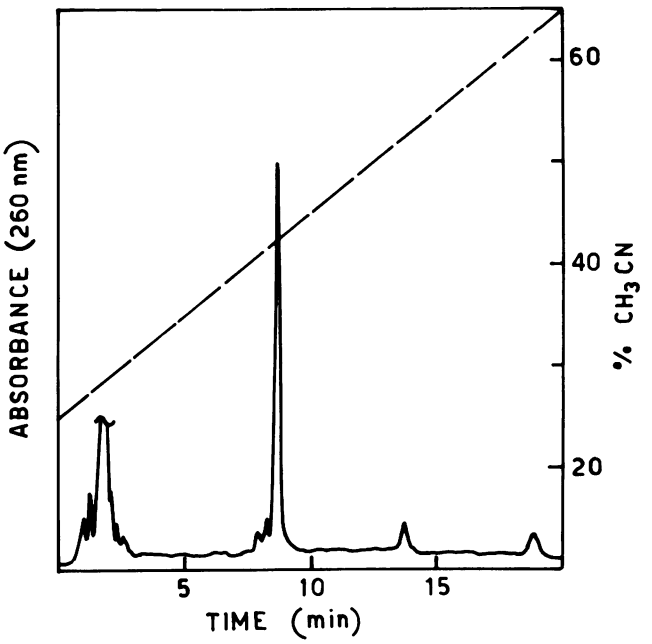

Figure 3. Reverse-phase HPLC profile of crude DMT-octanucleotide.

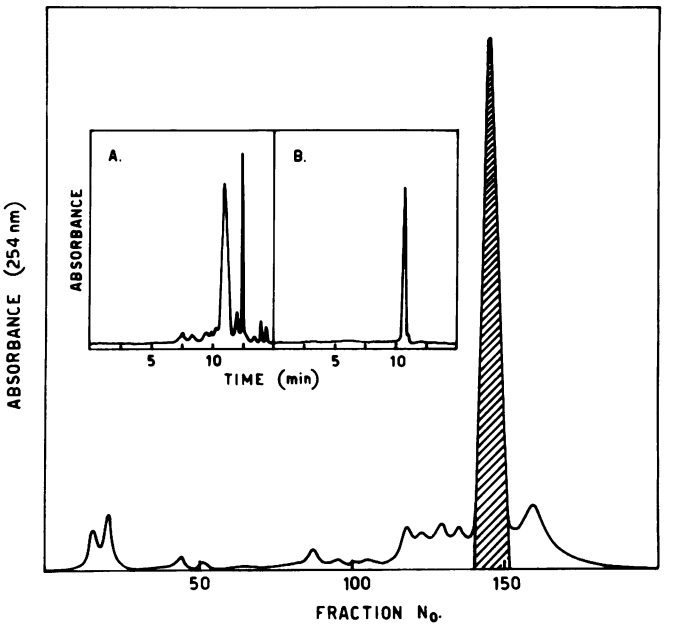

Figure 4. DEAE-Sephacell chromatography of crude d(TAGCGCTA). Insert: reverse-phase HPLC profiles of crude d(TAGCGCTA) (A) and purified (B).

by measuring the A_{max} in the 260 nm region in methanol solution; the extinction coefficent for each protected mononucleoside was previously determined.

The unreacted OH groups of PEG were capped by reacting with a 10% solution of acetic anhydride in pyridine (10 ml/g PEG) for one hour at room temperature, under stirring. The polymer was precipitated with diethyl ether and dried under vacuum over KOH pellets.

General procedure for oligonucleotide assembly

Detritylation

1.0g of 5'-O-DMT-nucleoside-3'-O-PEG was dissolved in 10 ml of methylene chloride. 10 ml of a 6% solution of trichloroacetic acid (TCA) in methylene chloride was added dropwise under vigorous stirring (at 0°C in the case of 5'-terminal purine nucleosides). After 15 min the PEG-nucleoside was precipitated with diethyl ether, washed with ether and filtered. The extent of deblocking was controlled qualitatively by TLC (no orange colour present with acidic spraying) and quantitatively by UV analysis. If some DMT was still present, the TCA treatment was repeated. The final polymer was recrystallized from methylene chloride/diethyl ether and dried under vacuum over KOH pellets.

Condensation

1g of 5'-OH'-nucleoside-3'-O-PEG was coevaporated 3 times with pyridine and dissolved in 2.5 ml of a pyridine/2,6-lutidine mixture (1:1 v/v). In a separate vessel 3 equivalents of the 3'-phosphate nucleoside (calculated on the basis of the PEG-nucleoside loading) was dried by coevaporation with pyridine and dissolved in 2.5 ml of pyridine/2,6-lutidine mixture. The solution was taken up with a syringe and injected through a septum into a vial containing a weighed amount of MSNT (6 equivalents). 10 equivalents of NMI were injected into the same vial. After 5 min the resulting solution was transferred (with a syringe) into the reaction vessel containing the PEG- nucleoside solution and left to react for one hour under stirring (Ar).

The polymer was precipitated at 0°C with diethyl ether, washed with ether and recrystallized twice from absolute ethanol. The yield of the reaction was judged spectrophotometrically as described in the previous paragraph. This value was also independently estimated by ^{1}H-NMR: 10 mg of PEG-oligonucleotide were dissolved in a NMR tube with 0.4 ml of $CDCl_3$ (0.5% of tetramethylsilane as internal standard). Peak-suppression technique was used to eliminate the strong signal due to methylene protons of PEG (3.70 ppm; 2.0 sec of irradiation; 20 L of decoupling power). The reaction yield was estimated from the inspection of the signals of sugar protons at 1' and/or 3' position (between 6.5 and 5.5 ppm), of four protons of succinyl group (2.68 ppm) and four of the protons of DMT group (6.7–6.8 ppm). The integration value of these signals correspond, in a 100% yield reaction, to a n:4:4 ratio, where n is the number of nucleosides units.

Capping

The unreacted 5'-OH groups were acetylated with a 10% acetic anhydride solution obtained by adding dropwise 1 ml of acetic anhydride to a 10 ml/g pyridine solution of PEG-oligonucleotide The mixture was left under stirring for one hour, precipitated at 0°C with diethyl ether and recrystallized from methylene chloride/diethyl ether. The polymer wsa filtered,washed extensively with ether and dried under vacuum over KOH pellets.

Purification and isolation of the final product

After the last coupling the PEG-oligonucleotide (0.2 g) was reacted overnight at 37°C with a solution containing 515 mg of *syn*-pyridine-2-carbaldoxime and 0.5 ml of 1,1,3,3-tetramethylguanidine in 10 ml of dioxane/H_2O (1:1 v/v). The solution was evaporated to dryness *in vacuo* and the residue dissolved in 5 ml of a 30% NH_4OH solution; the reaction mixture was allowed to stand at 60°C for 24 hours. After cooling, the aqueous solution was evaporated, redissolved in 5 ml of water and extracted with ether (5 ml, 3 times). An aliquot was taken

up in 0.05 M TEAB buffer to be analyzed by C_{18} HPLC−(eluent=buffer A:0.1 M TEAAc, pH 7.0;buffer B:0.1 M TEEAc, pH 7.0, 80% acetonytrile; gradient:25−60% buffer B in 20 min; flow 1.5 ml/min). The solution was evaporated to dryness and treated with 80% acetic acid (5 ml) for 30 min at room temp. After evaporation of acetic acid, the residue was dissolved in TEAB buffer 0.05 M.

The final product was isolated by ion-exchange chromatography on a DEAE-Sephacell column (3×13 cm) with a linear gradient of TEAB 0.05−1.50 M , pH 7.0. (flow ratio 1.5ml/min; 5 ml fractions). The fractions corresponding to the desired product were pooled, coevaporated several times with water to remove the buffer and liophylized. The triethylammonium counterions can be replaced by sodium with a Dowex 50 W (Na) column (10×1.5 cm). The product was analyzed by C_{18} HPLC (eluent= buffer A:KH_2PO_4 0.05 M, pH 4.5; buffer B:KH_2PO_4 0.05 M, pH 4.5/acetonytrile 60%; gradient 0−30% B in 10 min; flow 1.5 ml/min).

ACKNOWLEDGEMENTS

The authors are grateful to Prof.J.H.van Boom and Dr.G.van der Marel for their valuable comments and critical reading of the manuscript and to Prof.Z.A.Shabarova for advice and encouragement.This research was supported by MPI and CNR grants.

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