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POLYMER SUPPORT OLIGONUCLEOTIDE SYNTHESIS—XV[1,2]

SYNTHESIS OF OLIGODEOXYNUCLEOTIDES ON CONTROLLED PORE GLASS (CPG) USING PHOSPHATE AND A NEW PHOSPHITE TRIESTER APPROACH

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Abstract—Controlled pore glass (CPG) has been functionalized with suitably protected deoxynucleosides and used to synthesize d(TTTATT) and d(CATGAGGAAGT) following the phosphate triester approach with 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) as condensing agent and $ZnBr_2$ as detritylating agent. The efficiency of monomer and dimer additions is discussed. The nonamer d(AACCAGCAC) has been synthesized by a modified phosphite triester approach using suitably protected methyl deoxynucleoside-N-morpholino-phosphoamidites as new active phosphitylating nucleotides. The sequence has been built up by monomer additions after activation of the phosphoamidites with tetrazole, capping with acetic anhydride/DMAP and detritylation with either $ZnBr_2$ (purine nucleotides) or trichloroacetic acid (pyrimidine nucleotides). Deprotection are performed by treatment with thiophenol, aqueous concentrated ammonia and tert. butylamine and the nonamer characterized by reversed phase hplc, polyacrylamide gel electrophoresis and sequence determination. The favourable properties of CPG as carrier for oligonucleotide syntheses are discussed.

The history of polymer support oligonucleotide synthesis started about 18 yr ago by the synthesis of d(CpT) on a low crosslinked popcorn polystyrene. using the phosphate triester approach.[3] Due to the lack of effective activating agents for phosphodiesters at that time and the comprehensive studies of Khorana in the synthesis of oligodeoxynucleotides using the phosphodiester approach the phosphotriester method was not used for many years in polymer support synthesis.

The success of polymer support oligodeoxynucleotide synthesis is mainly dependent on the choice of the polymer support, reliable synthetic strategy and efficient purification methods for the oligonucleotide chains after deprotection and cleavage from the carrier.

In principle, the use of polymer supports which are soluble[4,5] or swellable[6–15] in organic solvents such as benzene and chloroform could not be very successful following the phosphodiester approach due to the solubility problems of a polar oligophosphodiester chain immersed in a chemical environment which is only fully accessible in unpolar solvents.

Various interesting solutions to these problems involving the phosphodiester method have been proposed: highly crosslinked and nonswellable, macroporous (i.e. permanent pores) polystyrene,[16,17] highly crosslinked, nonswellable nonporous polystyrene beads (in this case oligonucleotide synthesis can only be performed on the outer surface of the solid beads),[18] soluble hydrophilic polymers such as polyethyleneglycol[19] and polyvinylalcohol,[20] insoluble and swellable hydrophilic polymers like Sephadex LH 20,[21] crosslinked poly-L-lysine,[22] poly-N,N-dimethylacrylamide[23–25] and polyacrylmorpholide.[26]

As an insoluble and nonswellable hydrophilic polymer silica gel was first introduced for the phosphodiester approach.[27] In the same paper the advantage of glass beads of controlled pore size had been mentioned.

Since condensations at that time using the phosphodiester method were far from being quantitative, a "capping step" was introduced for polymer support oligonucleotide synthesis using acetic anhydride[23,24] or phenyl isocyanate.[22,25,28]

In spite of many efforts using the phosphodiester method polymer support, oligonucleotide synthesis was not very successful. This situation changed dramatically with the improvement of the phosphotriester method,[29,30,31] in particular with the introduction of more effective condensing agents.[32,33] In this approach a non-polar oligonucleotide chain is being synthesized, therefore nonpolar swellable polymers such as polystyrene could be used successfully,[34–36] as well as polystyrene-coated solid teflon beads.[37] However, with these kind of polymers adsorption of oligonucleotides to the polymer due to hydrophobic interaction has to be considered. A lipophilic liquid phase carrier for solution synthesis has also recently been introduced.[38] Although not really necessary for phosphotriester approach hydro-

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philic swellable polymers (e.g. cross-crosslinked poly-N,N-dimethylacrylamide[39] and polyacryl-morpholide)[40,41] and nonswellable polymers(e.g. cellulose[42]) have been used successfully as supports for oligonucleotide synthesis.

Very recently silica gel has been used for polymer support oligonucleotide synthesis following the phosphotriester approach.[43] In the phosphite triester method this carrier is only used.[45–48]

In this paper, we present experimental details on the use of controlled pore glass (CPG) using phosphate[49] and a modified phosphite triester approach.[50] It should be mentioned that CPG is very suitable for the synthesis of oligodeoxymethylphosphonates.[1]

We believe that CPG is the carrier of choice for polymer support oligonucleotide synthesis, because it fulfills the following criteria:

(1) Solvent independent accessibility of functional groups: In contrast to all soluble or swellable polymers, CPG allows the use of aqueous conditions as well as nonpolar organic solvents.

(2) A rigid and incompressible network: This enables column operation including a recycling mode and high flow rates.

(3) Chemically inert under necessary reaction conditions: CPG possesses great stability except in strong alkaline media.

(4) No interaction of surface with reagents and growing oligonucleotide chain: In case of CPG only the silanol groups have to be taken into account. These can be protected by, e.g. trimethylsilylation. Moreover, the surface can be easily cleaned from any adsorbed molecules by simply washing the glass beads.

(5) Controlled pore size: As the total surface area is almost inner surface, reactions are superimposed and strongly influenced by diffusion phenomena. The existence of controlled pore sizes results in homogeneous and reproducible diffusion parameters more or less irrespective of the solvent used. The chain length to be synthesized is only limited by pore size distribution. As the pore size distribution is very narrow all growing chains can be elongated to full length.

(6) Easy functionalibility: There are various standard procedures available to functionalize silanol groups.

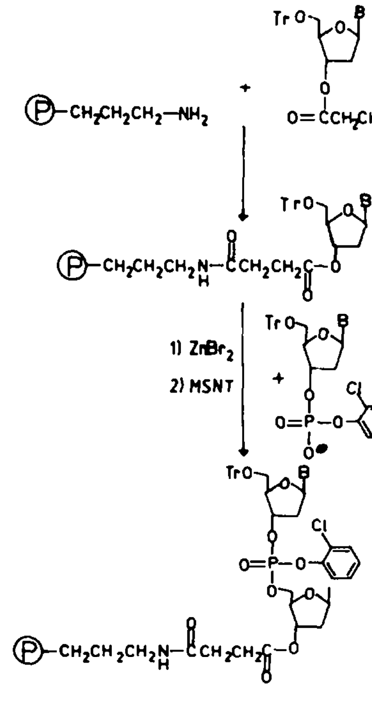
(7) Commercial availability of reproducible qualities ranging in mean pore size from 75 to 3000 Å.

RESULTS AND DISCUSSION

Functionalization of CPG (Scheme 1). The silanol groups of the glass beads are reacted with triethoxyaminopropylsilane under standard conditions with the formation of aminopropyl groups on the surface. They can be directly used for anchoring of the first deoxynucleoside or an extension of the spacer length can be mediated by two (or more) additions of glycine residues (with e.g. fluorenylmethoxycarbonylglycine = Fmoc/DCC followed by piperidine treatment[52]). All non-reacted silanol groups are then protected by trimethylsilylation.

Without further elongation of the aminopropyl groups we found when using phosphate triester approach that formation of a dinucleotide on the polymer is less effective thus reducing significantly the overall yield of an oligonucleotide synthesis. Figure 1 shows that the yields of a dinucleotide synthesis is also dependent on the phosphate component used. When the polymer loaded with deoxythymidine ("T-polymer") was condensed with 5′-O-trityldeoxythymidine-3′-O-(2-chlorophenyl) phosphate in presence of 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) the yield of d(T*p*T-P)† was 89%, whereas when using 5′-O-trityl-N^2-isobutyryldeoxyguanosine-3′-O-(2-chlorphenyl) phosphate under exactly the same conditions yield of d(G*p*T-P) was only 67%. These results may reflect some sterical hindrance of the polymer backbone exerted on the activated phosphate components of differing sizes. At later stages of synthesis these effects are less pronounced apparently due to the fact that a short oligonucleotide chain serves as an effective spacer. This effect is also less significant in the phosphite triester approach probably due to a smaller size of the phosphitylating agent.

The 5′-O-tritylated deoxynucleosides were succinylated and the succinic acid half ester (after purification by silica gel short column chromatography) transformed into a 4-nitrophenylester using 4-nitrophenol and DCC. The active esters are coupled to the amino groups on the polymer. It is important to add triethylamine to prevent a protonation of the primary amino group by the resulting 4-nitrophenol. Residual amino groups are protected by "capping" with acetic anhydride in presence of 4-N,N-dimethylaminopyridine (DMAP). The absence or presence of primary amino groups has been detected by ninhydrine assay.[52]



Scheme 1. B = Thymin, N^6-Benzoyladenin, N^2-Isobutyrylguanin, N^4-(2-methyl)-benzoylcytosin; Ⓟ = Controlled pore glass (CPG).

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†Abbreviations according to,[53,55] *p* represents a protected internucleotidic linkage (e.g. 2-chlorophenyl phosphotriester, methoxyphosphotriester).

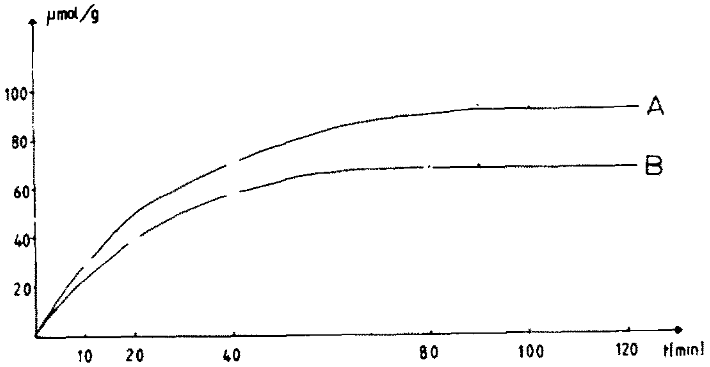


Fig. 1. Kinetics of condensation of 0.2 g detritylated "T-carrier" with $(Tr)T_dp^{\ominus}$(A) and $(Tr)ib^2G_dp^{\ominus}$(B) with MSNT under conditions used for oligomer synthesis. Yields obtained by spectrophotometric determination of trityl cation by treatment with 10% trifluoracetic acid in CH_2Cl_2 ($\epsilon_{410} = 28{,}560$).

The amount of aminopropyl groups has been determined by a reaction with (^{3}H) acetic anhydride and determination of radioactive labelled acetate ions after alkaline hydrolysis. The amount is dependent on pore size (Fig. 2).

The same holds for loading with deoxynucleosides. In the case of CPG with 3000 Å loading with 5′-O-trityldeoxythymidine is about 2 µmole/g, with 1400 Å 9.4 µmole/g, with 240 Å loading is 99 µmole/g for 5′-O-trityldeoxythymidine, 97 µmole/g for 5′-O-trityl-N^6-benzoyldeoxyadenosine, 107 µmole/g for 5′-O-trityl-N^4-(2-methyl)-benzoyldeoxycytidine and 82 µmole/g for 5′-O-trityl-N^2-isobutyryldeoxyguanosine.

The problem of N-protecting groups for the heterocyclic bases has been discussed in detail.[53]

As 5′-O-protecting group either the unsubstituted trityl group[54] or 4,4-dimethoxytrityl group has been used. With respect to a high overall yield conditions for detritylation are very critical: detritylation should be quantitative after every condensation step without any depurination. Using protic acids depurination in our hands cannot be excluded quantitatively.[56]

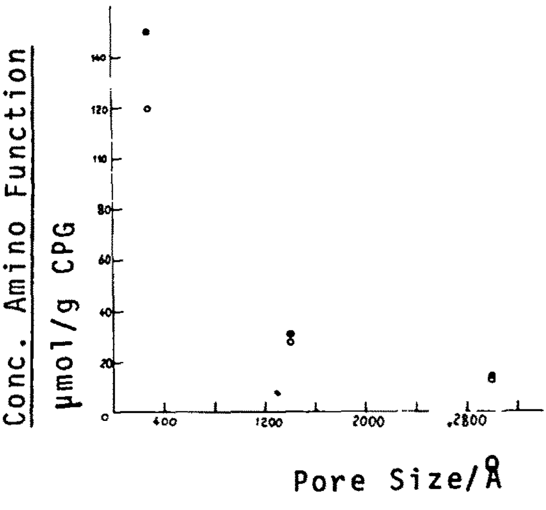


Fig. 2. Amount of aminopropyl groups linked to CPG with respect to mean pore size. Determination after acetylation (capping conditions) with (^{3}H) acetic anhydride, alkaline hydrolysis and measurement of tritium-labeled acetate ions in a β-scintillation counter. ●○, Duplicate experiments.

No detectable depurination takes place when using Lewis acids such as $ZnBr_2$[54] or dialkylaluminium chloride.[57] With $ZnBr_2$ effective detritylation can be afforded with chloroform/methanol (7:3) or nitromethane (1% water). In this case it is important to remove and to avoid traces of pyridine, as insoluble complexes can clog the polymer. In case of diethylaluminium chloride, 1,2-dichloroethane or dichloromethane can be favourably used as solvents. Insoluble complexes are avoided by a treatment with acetylacetone pyridine/dichloromethane.[56]

Synthesis of d(TTTATT) and d(CATGAGGAAGT) via phosphate triester method. 0.2 g (19.8 µmole) "T-polymer" is subjected to detritylation with $ZnBr_2$ ($CHCl_3$/MeOH = 7:3, v/v) and after careful washing dried in vacuo. After condensation, the yield has been determined by detritylation of a sample with 60% aqueous perchloric acid. If condensation yields are satisfactory, capping of unreacted 5′-OH-groups are performed using acetic anhydride, followed by careful washing to remove traces of pyridine or DMAP. The different steps on one reaction cycle are shown in Table 1. Condensation time is 60 min for monomer and 90 min for dimer additions. Total cycle time is about 2 hr. All operations are performed manually in a column with a sintered glass frit and appropriate solvent deliveries using argon pressure.

After the last condensation step the 5′-O-trityl group is removed and the polymer is treated with tetramethylguanidinium pyridine-2-aldoximate[33] followed by aqueous concentrated ammonia at 50°. It has to be taken into account that during oximate treatment oligonucleotide chains are released from the polymer to a considerable extent. The total amount of nucleotide material is purified by preparative DEAE-cellulose anion exchange chromatography in the presence of 7 M urea.[55] Distinct fractions are phosphorylated with T4 polynucleotide kinase and characterized by polyacrylamide gel elec-

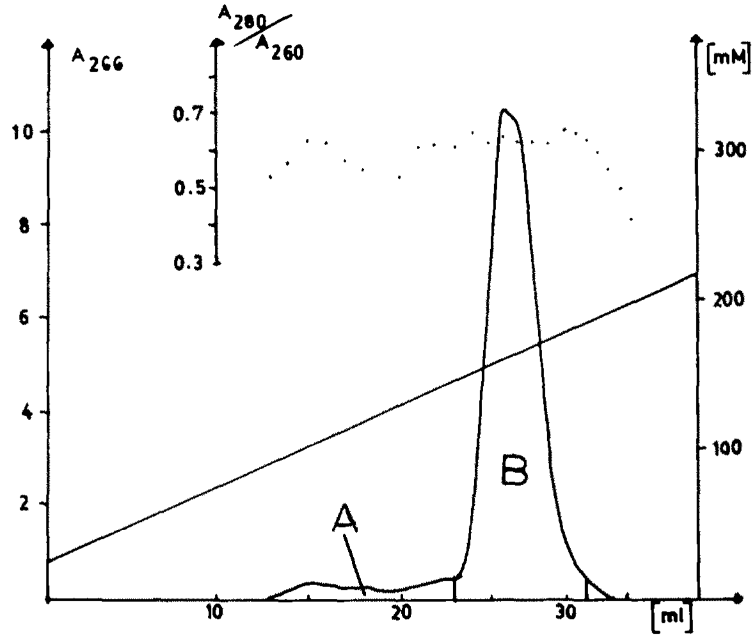


Fig. 3(a).

Fig. 3(b)

Fig. 3(a). Elution profile of deprotection mixture of d(T-T-T-A-T-T) on DEAE-cellulose in presence of 7 M urea. Column: 1 × 17 cm, fractions of about 3 ml are collected; flow rate: 2 ml/min. *d(pT)$_3$.

Fig. 3(b). Polyacrylamide gel electrophoresis (20%) in presence of 7 M urea. Electrophoresis runs from top to bottom. Lane a: fraction A, lane B: fraction B of desalted and phosphorylated material from Fig. 3(a). Right lane: Homo-oligo-dT chain length standard.

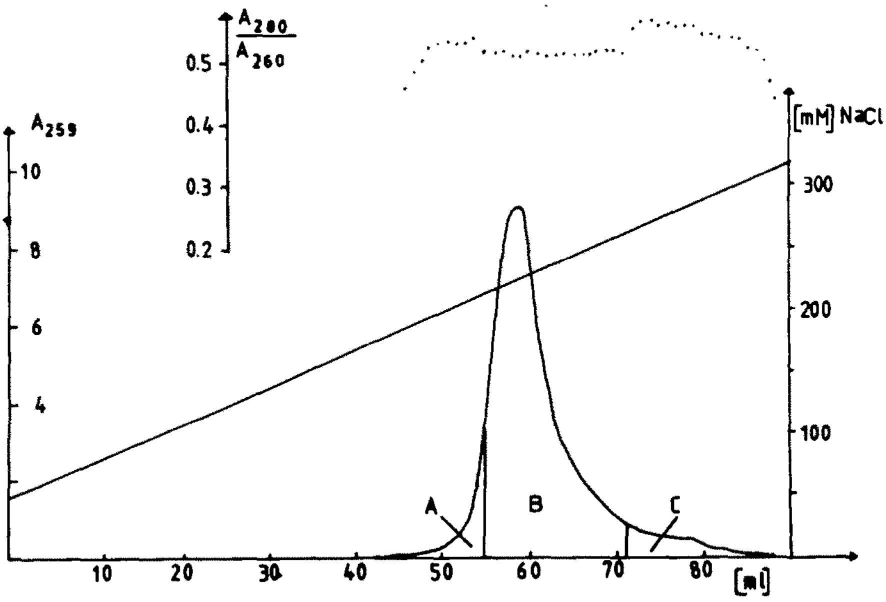


Fig. 4(a)

Fig. 4(b)

Fig. 4(a). Elution profile of deprotection mixture of d(C-A-T-G-A-G-G-A-A-G-T) on DEAE-cellulose (see legend to Fig. 3a).

Fig. 4(b). Polyacrylamide gel electrophoresis (20%) in presence of 7 M urea (see legend to Fig. 3b). Lane A: fraction A, lane B: fraction B, lane C: fraction C from Fig. 4(a), lane 3: Homo-oligo-dT chain length standard. *d(pT)$_3$.

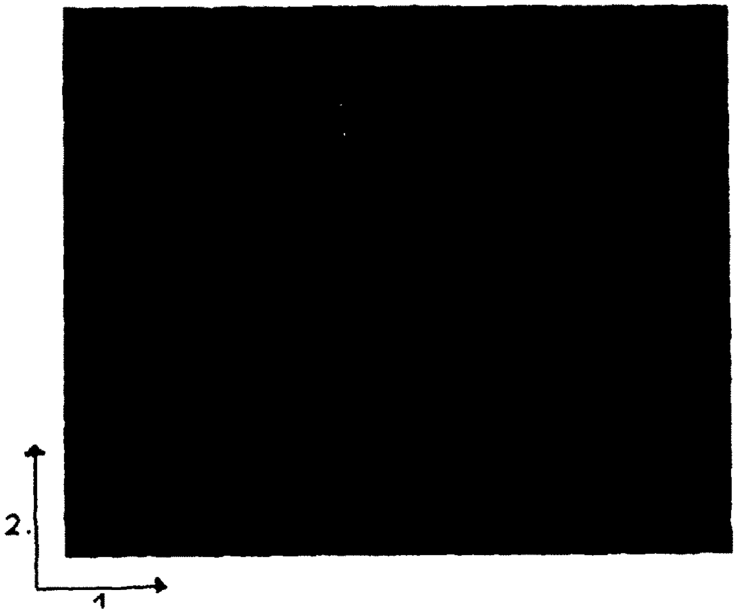


Fig. 3(c)

Fig. 3(c). Sequence determination of fraction B of Fig. 3(a) according to the "mobility shift" method. +, Blue marker.

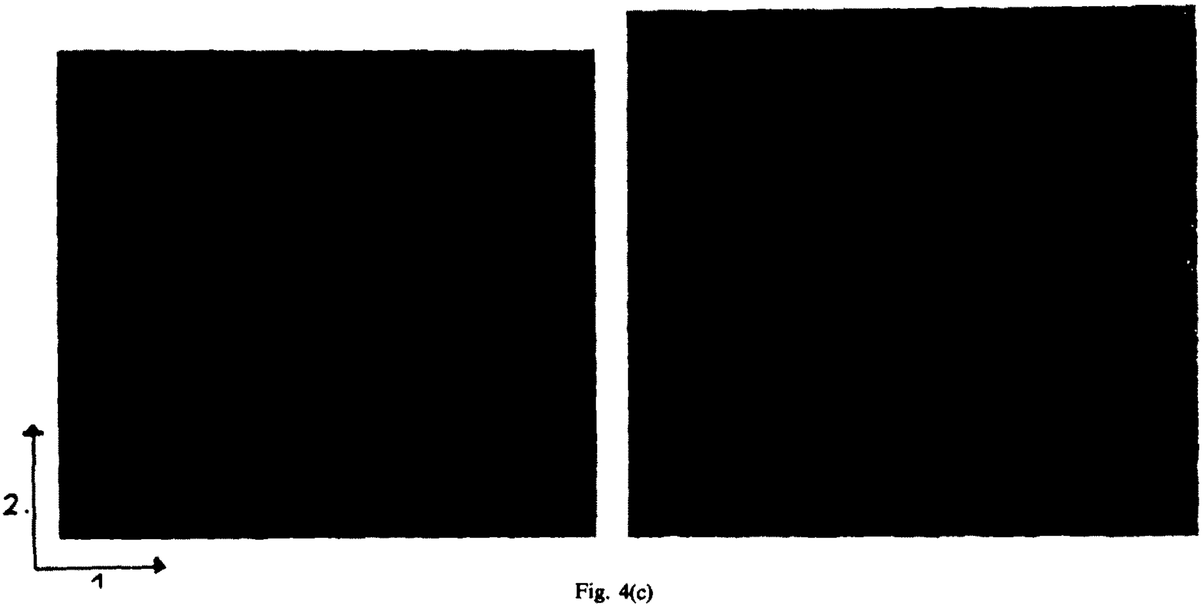


Fig. 4(c)

Fig. 4(c). Sequence determination of fraction B of Fig. 4(a) according to the "mobility-shift" method. +, Blue marker.

Table 1. Reaction cycle using modified phosphate triester approach

Step	Operation	Solvent/Reagent	Volume (ml)	Time (min)
1	Washing	Pyridine	18	10
2	Condensation	Phosphate component/MSNT in pyridine	1	60 - 90[a)]
3	Washing	Pyridine	9	10
4	Capping	Ac_2O/pyridine	9[c)]	10
5	Washing	Pyridine	9	10
6	Washing	CH_2Cl_2/MeOH (7:3)	18[b)]	20
7	Detritylation	$ZnBr_2$ in CH_2Cl_2/MeOH (7:3)	9	10
8	Washing	CH_2Cl_2/MeOH (7:3)	18[b)]	10
9	As step 1			

[a)] Condensation time for mononucleotides 60 minutes, for dimer blocks 90 minutes.

[b)] An extended washing procedure is necessary to ensure complete removal of pyridine, which gives an insoluble complex with $ZnBr_2$.

[c)] 2.4 M solution.

trophoresis (20%/7 M urea) using a homooligo-dT chain length standard,[58] which allows a comparison of the calculated mobility of a given sequence with the mobility observed, and by sequence analysis following the "mobility shift" method.[59] Results are shown for the hexamer in Fig. 3 and for the undecamer in Fig. 4.

In case of the hexamer 12-fold excess of phosphate-component (0.12 M) has been used. It can be seen in Fig. 3(a) (fraction A) and Fig. 3(b) (lane A), small amounts of truncated sequences are present (4-mer, 5-mer); shorter sequences have been apparently eluted during loading and subsequent washing with 50 mM NaCl/7 M urea/25 mM Tris·HCl, pH 7.5. Peak A contains a small amount of the hexamer and mainly pentamer. The main peak B contains almost pure hexamer. Both peaks contain traces of slower moving compounds, most probably hexamers with one or two intact triester linkages.

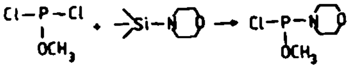
To reduce the amount of truncated sequences the concentration of phosphate component has been increased to 0.24 M for the synthesis of the undecamer, resulting in almost no trunketed sequences (Fig. 3b). Furthermore oximate treatment has been repeated to achieve complete deprotection. Constantly high coupling yields have been obtained under these conditions, even during the use of dimer block AG in a most unfavourable coupling of AG to G following the synthetic route d(GATG$\underline{\text{AG}}$GA$\underline{\text{AG}}$T).

The main fractions of DEAE-cellulose chromatography were desalted on DEAE-cellulose and lyophilized. When using 0.2 g carrier 115 O.D.$_{max}$ pure hexamer and 152 O.D.$_{max}$ pure undecamer were obtained. This is 9.5% and 6.3% overall yield of pure material with respect to polymer-bound deoxythymidine.

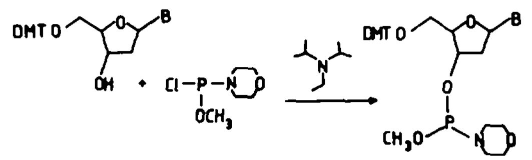
Synthesis of d(AACCAGCAC) via modified phosphite triester method. During our studies on using the phosphite triester approach as described,[44-48] we observed that preparation, handling and storage of 5′-O-dimethoxytrityl-N-acyl deoxynucleoside-3′-O-(N,N-dimethylamino)methylphosphoamidites are difficult to reproduce. We therefore undertook a study to use the more stable morpholinomethylphosphoamidites. Independently to our work, similar observations and conclusions have been made very recently.[60]

The synthesis of chloro-methoxymorpholinophosphine and of the appropriate protected deoxynucleoside phosphoamidites is shown in Schemes 2 and 3. The monochloridite could be obtained after distillation *in vacuo* and shown to be pure by proton-NMR, ^{31}P NMR and mass spectrometry. The deoxynucleoside phosphoamidites are precipitated into hexane and lyophilized from benzene and appeared to be rather stable when stored under argon and under exclusion of moisture at −20°. Only the deoxyguanosine derivative seems to be more sensitive than the other.

To show the reliability of this new reagent we synthesized the nonadeoxynucleotide d(AACCAG-



Scheme 2.



Scheme 3.

Table 2. Reaction cycle using modified phosphite triester approach

Step	Operation	Solvent/Reagent	Volume (ml)	No. of time/Duration
1	Detritylation	a) 3% CCl_3COOH or b) $ZnBr_2$	a) 2 or b) 3	5 times for 1 minute or 3 times for 3 minutes
2	Washing	a) CH_3NO_2 [a)] b) n-Bu-OH/Lutidine/THF [b)]	a) 5 b) 2	3 times for 1 minutes 2 times for 2 minutes
3	Washing	a) CH_3CN b) CH_2Cl_2	a) 5 b) 10	3 times 2 times
4	Drying	High vacuum	-	10 minutes
5	Condensation	Active nucleotides and tetrazole in CH_3CN	2	15 - 30 [c)] minutes
6	Washing	CH_3CN	3	10 times
7	Oxidation	0.1 M J_2 in THF/Pyridine/ H_2O (80:40:2, v/v)	2	2 times for 1 minute
8	Washing	a) Pyridine b) THF	a) 2 b) 5	2 times 2 times
9	Capping	Ac_2O/DMAP/Lutidine/THF	2 x 5	2 times for 2 minutes
10	As step 1			

a) When CCl_3COOH is used. b) When $ZnBr_2$ is used.

c) 15 min for pyrimidine and 30 min for purine nucleotides.

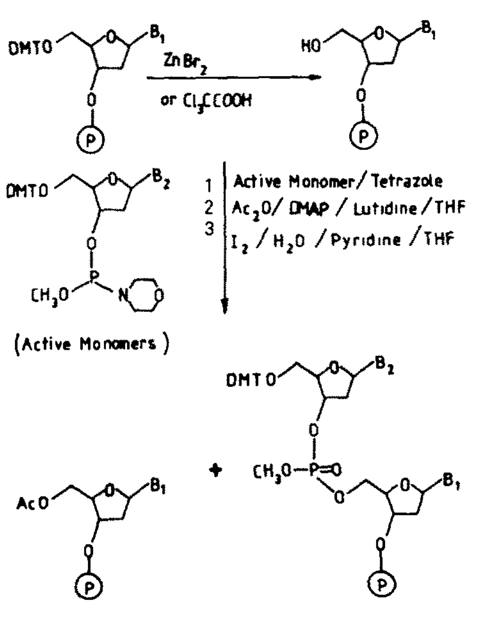
CAC) on CPG as polymer support following the reactions outlined in Scheme 4. The different steps of one cycle are shown in Table 2.

For the activation of the morpholidite sublimed tetrazole has been used, capping has been performed using acetic anhydride/DMAP, detritylations have been mediated by using 3% trichloroacetic acid in nitromethane (1% MeOH) in case of pyrimidine moieties and with $ZnBr_2$ in nitromethane (1% water) in case of purine nucleotides. Coupling yields are determined from samples before capping by measurement of the trityl cation–overall yield after last condensation step 67%. After the last coupling step the triester protecting groups are removed by treatment with thiophenol,[61] concentrated aqueous ammonia at 50° and tert. butylamine. To remove low molecular weight material the resulting mixture is subjected to silica gel TLC using $CHCl_3$/MeOH (6:4, v/v) and checked by reversed phase (RP-18) HPLC (Fig. 5a). The dimethoxytrityl containing peak is detritylated with 80% aqueous acetic acid, phosphorylated with T4 polynucleotide kinase and characterized by polyacrylamide gel electrophoresis using a homo-oligo-dT chain length standard[58] (Fig. 5b) and by sequence analysis using the mobility-shift method[59] (Fig. 5c, d).

The results demonstrate that controlled pore glass (CPG) is a highly suitable carrier for polymer support oligonucleotide synthesis. Since preparation of this manuscript a 13-mer, 15-mer and 16-mer have been synthesized by the same method in excellent yields and within a short time.

EXPERIMENTAL

Unless otherwise mentioned materials and methods are as previously described.[53-55,58] Deoxynucleosides are from Papierwerke Waldhof (Mannheim), CPG from Serva (Heidelberg), $ZnBr_2$ from Riedel de Haen (Seelze, Hannover), all reagents as 4-N,N-dimethylaminopyridine, trityl chloride, succinic anhydride, aminopropyltriethoxysilane, tetrazole, pyridine-2-aldoxime are from EGA (Steinheim). 2,6-Lutidine is purified according to.[66] 2-Chlorophenyl phosphorodichloridate has been prepared as reported.[63] N-acylated 5′-O-tritylated deoxynucleosides are prepared as described.[53-55] Pyridinium 5′-O-trityldeoxynucleoside-3′-O-succinates are basically prepared according to,[64] yields between 70 and 85%. CPG is aminopropylated according to



Scheme 4.

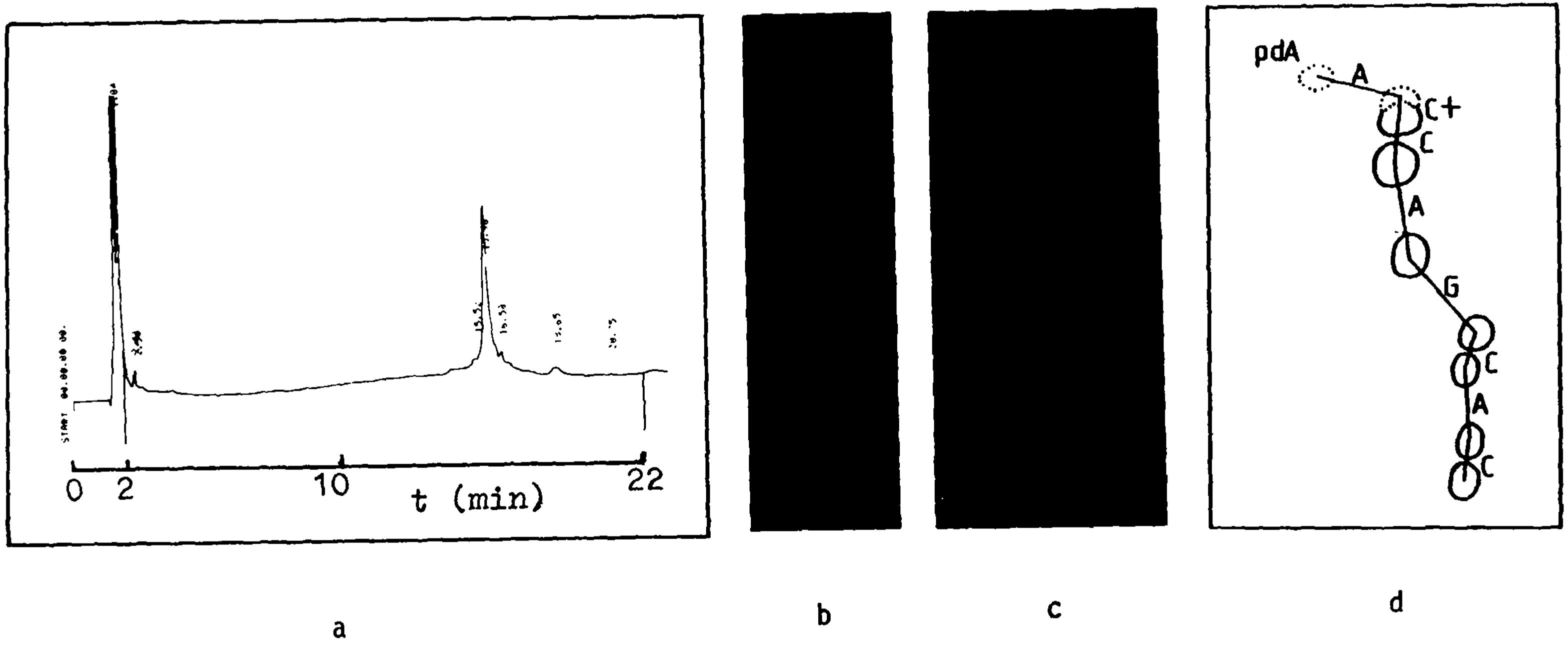


Fig. 5(a). Purification of the resulting nonamer after deprotection from the polymer support by HPLC as DMT-d(AACCAGCAC) on reversed phase C18 (details see Experimental). (b) Electrophoresis of the nonamer (lane a) on a 20% polyacrylamide gel containing 7 M urea before HPLC and after, detritylation and phosphorylation using (γ-^{32}P)ATP and T4 polynucleotide kinase. Lane b: homo-oligo-dT chain length standard. The nonamer, rich in dA and dC nucleosides has a relative mobility similar to d(pT)$_8$; *: d(pT)$_3$. (c) Two-dimensional fingerprint of a partial snake venom phosphodiesterase digest of the nonamer, d(AACCAGCAC); 1, cellulose acetate electrophoresis; 2, homochromatography; +, xylene cyanol (blue marker). (d) Schematic representation of the two-dimensional fingerprint shown in Fig. 5(c).

known methods.[65] The unreacted silanol groups are trimethylsilylated with trimethylsilyl chloride and pyridine. 4-Nitrophenylesters of 5′-O-trityldeoxynucleoside-3′-O-succinates are prepared in 5 ml 1,4-dioxane/pyridine (20:1) with dicyclohexylcarbodiimide and 4-nitrophenol for 12 hr at room temp. Dicyclohexylurea is removed by centrifugation, the supernatant evaporated, taken up in CH_2CL_2, filtered to remove traces of the urea, evaporated with toluene to a foam and finally lyophilized from 1,4-dioxane, yields are more than 95%.

Attachment of 5′-O-trityl-deoxynucleoside-3′-O-succinate derivatives to the carrier. 3 g aminopropylated CPG (240 Å pore size) with apiroximately 0.52 mmole amino groups are suspended in 9 ml N,N-DMF/Et_3N (8:1) and a soln of 1.3 mmole of 5′-O-N-protected deoxynucleoside-3′-O-succinyl-(4-nitro)phenylester in a mixture of 3 ml 1,4-dioxane/pyridine (10:1) is added. After shaking for one day at room temp, 0.6 ml Ac_2O is added and after further shaking for 1 hr, the polymer is washed with 500 ml each of N,N-DMF, EtOH, 1,4-dioxane, ether and dried *in vacuo* at 45°.

Deprotection, cleavage and purification from the resin

Phosphate triester method. After having performed step 8 (Table 1) from the last reaction cycle 1 ml (per 0.2 g polymer) of 0.3 M tetramethylguanidinium pyridinine-2-aldoximate in dioxane/water (1:1, v/v) is added to the reactor. It is allowed to react for 24 hr and is replaced by the same amount of freshly prepared oximate reagent. After further 24 hr, this soln is substituted by 1 ml of NH_4OH and treated for 24 hr at 50°. The polymer is then washed with further 5 ml conc NH_4OH, the total amount of eluent and washing is evaporated, the residue is taken up in bidistilled water and extracted with ether (5 ml, 2-fold) and $CHCl_3$ (5 ml, 2-fold). The aqueous phase is diluted to 60 ml with 10 mM Tris·HCl, pH 7.5/7 M urea and applied to a DEAE-cellulose (DE-52, Whatman) column (1 cm × 17 cm). After washing with 100 ml 25 mM NaCl/10 mM Tris·HCl, pH 7.5/7 M urea, a gradient from 25 mM NaCl (125 ml) to 300 mM NaCl (125 ml) in above buffer is followed. Desired fractions are collected, diluted to about 10-fold its volume with 10 mM triethylammonium bicarbonate (TBC) and applied to a DEAE-cellulose column. After washing with 10 mM TBC (eluent free of urea and chloride anions) oligomer is eluted with 1 M TBC. The oligomer containing fractions are collected and lyophilized, taken up in 10 mM Tris·HCl, pH 7.5/1 mM EDTA to get 1 mM concentration of oligomer and stored at −35°.

Phosphite triester method. After final condensation and oxidation the polymer (0.1 g) is treated 4 times with 5 ml of a mixture of thiophenol/1,4-dioxane/Et_3N (1:2:1, v/v) at room temp for 25 min. Polymer is then washed with MeOH (4 times 5 ml) and ether (2 times 10 ml). The dried polymer is subjected to conc NH_4OH (10 ml) at 50° for 24 hr. After removal of the supernatant the polymer is washed with 10 ml bidistilled water and the combined supernatants and washings are evaporated. The residue is treated with 10 ml t-$BuNH_2$/MeOH (1:1, v/v) for 24 hr at 45–50°. After evaporation the residue is taken up in conc NH_4OH (1 ml) and applied to silica gel plate (Merck, 0.5 mm silica gel layer with fluorescent indicator, 254 nm) and developed with $CHCl_3$/MeOH (6:4, v/v). The trityl positive band is eluted with EtOH/conc NH_4OH (1:1, v/v) and evaporated.

The eluted product containing the nonamer with DMT-moiety is purified by HPLC. Chromatographic conditions: stationary phase is reversed phase silica gel C-18 (Beckman), Mobile phase: eluent A: aqueous 0.1 M TEAA, pH 7.0, eluent B: acetonitrile. Linear gradient from 10% B to 50% B in 20 min. Detection: UV adsorption (1.28 AUFS at 254 nm). Flow rate 1.5 ml/min.

Characterization

Analysis by polyacrylamide gel electrophoresis,[58] phosphorylation with (γ^{32}P)ATP and T4 polynucleotide kinase and sequencing by the "mobility shift" method have been performed as described earlier.[59]

Synthesis via phosphate triester approach

Synthesis of d(T-T-T-A-T-T). 0.2 g of detritylated "T-beads" (19.8 μmoles deoxythymidine) are washed with dry pyridine (step 1, Table 1), 0.24 mmole of phosphate component is dried by 3-fold evaporation with pyridine, dissolved in 2 ml of pyridine and 0.60 mmole of MSNT are added. This soln is added to the reactor containing the polymer. Condensation, capping and detritylation are carried out according to Table 1. Five cycles of mononucleotide additions using the appropriate monomer units are performed. Deprotection, cleavage from the carrier and purification are carried out as described above. DEAE-cellulose anion-exchange chromatography (Fig. 3a): A (fraction 13–22), 15 O.D. and B (fraction 23–32), 115 O.D.; B contains very pure hexanucleotide (A_{280}/A_{260} calculated 0.59, found 0.62, λ_{max} = 265 nm). Overall yield 1.88 μmol (9.5%).

Synthesis of d(C-A-T-G-A-G-G-A-A-G-T). 0.2 g of detritylated "T-beads" are treated as described above and in Table 1 with appropriate mononucleotides or dimer blocks (0.24 mmole) in 1 ml pyridine according to the synthetic plan. After deprotection, cleavage from the carrier and purification by DEAE-cellulose chromatography (Fig. 4a) three main fractions are obtained: A (fraction 40–54, 13.7 O.D.$_{258}$, $A_{280}/A_{260} = 0.52$), B (fraction 55–71, 152 O.D.$_{258}$, $A_{280}/A_{260} = 0.50$, calculated value: 0.45, λ_{max} = 258 nm), C (fraction 72–86, 18.2 O.D.$_{258}$, $A_{280}/A_{260} = 0.54$). B contained 1.14 μmol pure undecanucleotide (overall yield is 5.8%). Together with almost pure undecamer from A overall yield is 6.3%.

Synthesis via phosphite triester approach

Synthesis of chloro-N-morpholinomethoxyphosphine. Dichloromethoxyphosphine (13.4 g, 100 mmole) is dissolved in absolute ether (60 ml) and cooled to −20°. N-trimethylsilylmorpholine (16.0 g, 100 mmole) dissolved in absolute ether (30 ml) is added dropwise over 1.5 hr and the resulting mixture is stirred at room temp under argon for 20 hr. The mixture is then concentrated under reduced pressure to remove ether and volatile compounds and finally distilled *in vacuo* to give an oil (13.2 g, 75%) b.p. 65–67%/0.5 mm. ^{31}P NMR: $\delta = -172.6$ (CCl_4) with respect to H_3PO_4 in acetone-d_6. ^{1}H NMR ($CDCl_3$): doublet at 3.7 and 3.6 ppm, $J_{PH} = 14$ Hz (OCH_3); multiplet at 3.75 ppm ($0\,{}^{CH_2}_{CH_2}$) (total = 7H) and multiplet at 3.26 ppm ($N\,{}^{CH_2}_{CH_2}$, 4H). The mass spectrum showed main peaks at 183 $(m/e)^+$, 185 $[(m+2)/e]^+$, 148 $[(m-Cl)/e]^+$, 97 $[(m-C_4H_4NO)/e]^+$ and 86 $[(m-CH_3POCl)/e]^+$.

Synthesis of methyl-5′-O,N-protected deoxynucleoside-3′-O-N-morpholino phosphoamidites

5′-O,N-protected deoxynucleosides (3.0 mmole) are dried by coevaporation with pyridine and finally with toluene. The dried residue is dissolved in acid-free absolute $CHCl_3$ (12 ml) in presence of N,N,N-diisopropylethylamine (12.0 mmole). Chloro-N-morpholinomethoxyphosphine (6.0 mmole) is added through syringe with constant stirring and under argon over 2 min. After 20–25 min stirring, the soln is dissolved in argon sat EtOAc (100 ml) and washed with argon saturated ice-cold NaCl soln (3 times 30 ml). The organic extract is dried over Na_2SO_4 and concentrated under reduced pressure to afford phosphorylated deoxynucleosides as foam, which are dissolved in toluene (60 ml) (or 60 ml ethylacetate for guanosine derivative) precipitated in about 250 ml cold hexane (−70°) and finally lyophilized from benzene as fine white amorphous powder (2.75–2.85 mmole, i.e. 92–95%). The purity of these compounds is checked with ^{31}P NMR. The compounds are stable when stored under argon and at −20° for about 3 months.

Synthesis of d(A-A-C-C-A-G-C-A-C). 100 mg of "C-polymer" (7.5 μmole protected deoxycytidine) is detritylated with 3% trichloroacetic acid in nitromethane containing 1% MeOH and after washing according to Table 2 is dried *in vacuo*. The nonamer has been built up by 8 successive cycles with 200 mg of activated nucleotides (about 25-fold excess) and 100 mg sublimed tetrazole (1.42 mmole) in 2 ml acetonitrile according to synthetic plan. After washing and drying, a sample of about 1 mg is withdrawn for trityl cation determination ($\epsilon_{502} = 70.000$ with 10% trifluoracetic acid in dichloromethane). Oxidation and capping follow standard procedures. The overall condensation yield according to final trityl cation measurement is 67%.

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