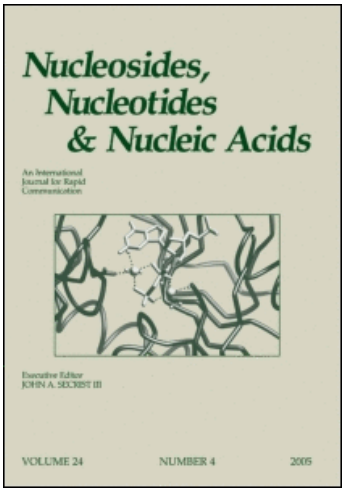

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Polymer Support Oligonucleotide Synthesis XVI: Synthesis of Oligonucleotides Using Suitably Protected Deoxynucleoside-N-morpholinophosphoramidites on Porous Glass Beads

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POLYMER SUPPORT OLIGONUCLEOTIDE SYNTHESIS XVI[1]: SYNTHESIS OF OLIGONUCLEOTIDES USING SUITABLY PROTECTED DEOXYNUCLEOSIDE-N-MORPHOLINOPHOSPHORAMIDITES ON POROUS GLASS BEADS**

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Dedicated to

Professor Friedrich Cramer

on the occasion of his 60th birthday.

ABSTRACT
Several oligodeoxynucleotides had been synthesized on controlled pore glass beads using pure and stable 3'-O(5'-O,N-protected) nucleoside-methyl-N-morpholino phosphoramidites. The average coupling yield at each condensation step was about 94%.

INTRODUCTION

Polymer support syntheses have tremendously reduced the time to obtain oligodeoxynucleotides of defined sequence either by the phosphate or phosphite triester method.

The efficiency of the synthesis carried out on polymer support and the purity of oligodeoxynucleotide sequences obtained depend mainly on the purity and stability of the nucleotide building blocks. This is mainly due to the fact that in solid phase synthesis a large excess of activated nucleotide has to be used to achieve high coupling yields. In the case of phosphate triester approach the monomeric, dimeric or trimeric 5'-O,N-protected deoxynucleoside/deoxyoligonucleotide-3'-O(chlorophenyl)phosphodiesters are obtainable in a pure and stable form. In phosphite triester method, however, normally very reactive 3'-O(5'-O,N-protected)

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deoxynucleoside-methyl-chloro phosphites[2], methyl-N-tetrazolo-phosphoramidites[3] or methyl-N,N-dimethylaminophosphoramidites[4] have been used, which have to be prepared under very stringent conditions. Sometimes it is a problem to obtain a reproducible quality with respect to coupling activity. The storage and handling of these reactive phosphite derivatives need special precautions. This may limit their use especially in automated synthesis where the active phosphite derivatives should be stable in solution for a longer time without influencing the reproducibility of coupling yields. In accordance with the experiences of others[5,6] even 3'-O-nucleoside-methyl-N,N-dimethylaminophosphoramidites[4] do not fulfill the above criteria. Therefore N-morpholino-[5] and N,N-diisopropylaminophosphoramidites[5,6] have been introduced into phosphite triester oligodeoxynucleotide synthesis.

In this paper, we explored the use of 3'-O(5'-O,N-protected deoxynucleoside)methyl-N-morpholinophosphoramidites for the synthesis of several oligomers on a porous glass bead polymeric support which has been successfully used in our group for phosphotriester approach[7]. When our experiments had been completed a paper appeared in which N-morpholino-methoxyphosphine derivatives have been used for oligodeoxynucleotide synthesis on a silica gel support[8].

RESULTS AND DISCUSSION

In order to determine the utility and compatibility of the active 3'-O(5'-O,N-protected)deoxynucleoside-methyl-N-morpholinophosphoramidites on porous glass beads, we synthesized the following oligonucleotides:

I) d(CGGATCCG)

II) d(TCAGTTGCAGTAG)

III) d(TTTGTCAACCAGCAC)

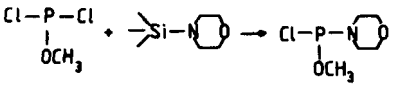
IV) d(CTTTGTGGTTCTCACC)

5'-O,N-protected deoxynucleosides were anchored via activated succinate esters to the aminopropylated porous glass beads according to standard methods[7]. Chloro-N-morpholino-methoxyphosphine was prepared according to Scheme 1. When morpholine was used for the preparation of monochloromethoxy-N-morpholino phosphine, we found the complete removal of the voluminous precipitate of morpholine hydrochloride not to be

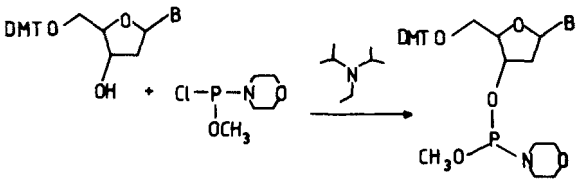
easy. Moreover, there is substantial loss of material (40 to 50%). In contrast, trimethylsilyl-N-morpholine as intermediate[5] does not lead to such voluminous precipitate and the yield of product was always found to be more than 75% after distillation. Chloromethoxy-N-morpholino phosphine was found to be quite stable and easy to handle; no formation of white fumes or white solids as compared to chloro-N,N-dimethylaminomethoxyphosphine have been observed when exposed to air during transfer to reaction vessels. The monochlorophosphine was characterized by ^{1}H-NMR, ^{31}P-NMR and mass spectra.

The active nucleosides were prepared by the action of monochloromethoxy-N-morpholinophosphine on suitably protected deoxynucleosides in THF at room temperature following Scheme 2. Neither oxidation of P(III) to P(V) nor hydrolysis of the reactive N-P bond during the work-up process was observed. We found that when solvents and reagents are kept at inert atmosphere and reaction is carried out under the same conditions the resulting deoxynucleoside phosphoramidites are more than 95% pure. For the sake of good coupling yields, no further purification on silica gel columns[5,8] seems to be necessary. In order to check their stability, the ^{31}P-NMR of these compounds were measured after six months. Only in the guanosine derivative some degradation could be seen by ^{31}P-NMR due to hydrolysis of the P-N bond and oxidation of P(III) to P(V). The reason for this special instability of the guanosine derivative is not clear yet.

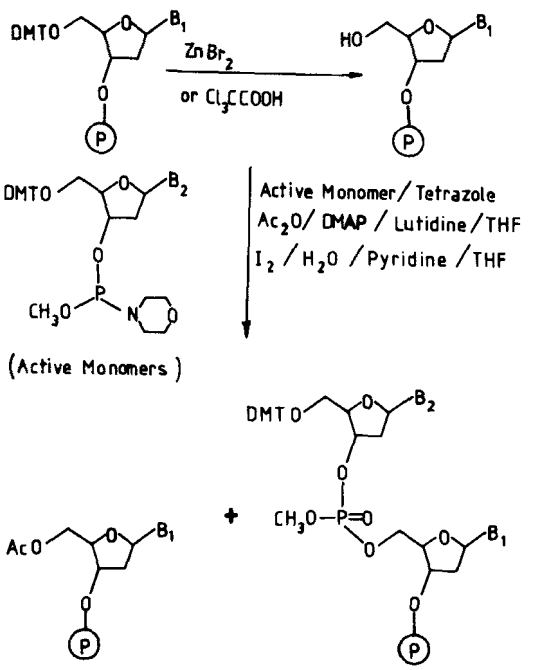
The syntheses of different oligodeoxynucleotides were carried out according to Scheme 3 by using 100 mg of the CPG bound nucleoside (7 to 7.5 µmole) in a column type reactor fitted with a sintered glass funnel and capped airtight with a serum cap. The 5'-DMTr group was removed either using 3% CCl_3COOH in 1% methanol-nitromethane or saturated $ZnBr_2$ in nitromethane-water (1%), in order to avoid undesirable depurination of the adenosine moiety. After proper washing and drying of the glass beads in vacuo, the active nucleoside was added in powdered form (25-30 equivalents), followed by addition of a solution of tetrazole in acetonitrile with sy-



Scheme 1



Scheme 2



Scheme 3

B, B_1, B_2 = Nucleobase, a: thymine, b. N-benzoyladenine, c: N-isobutyrylguanine, d: N-(2-methyl)benzoylcytosine[18].

Table I: Summary of the different steps performed in one elongation cycle

Step	Operation	Solvent/Reagent	Volume (ml)	No. of times/Duration
1	Detritylation	a) 3% CCl_3·COOH or b) $ZnBr_2$	a) 2 or b) 3	5 times/1 minute 3 times/3 minutes
2	Washing	a) CH_3NO_2 [a] b) n-BuOH/Lutidine/THF [b]	a) 5 b) 2	3 times 2 times
3	Washing	a) CH_3CN b) CH_2Cl_2	a) 5 b) 10	2 times 2 times
4	Drying	High Vacuum		5 minutes
5	Condensation	Active nucleosides and tetrazole in CH_3CN	1.5	15-30 minutes [c]
6	Washing	CH_3CN	3	10 times
7	Oxidation	0.1 M I_2 in THF/Pyridine/H_2O (80:40:2, v/v)	2	2 times/1 minute
8	Washing	a) Methanol b) THF	a) 2 b) 5	2 times 2 times
9	Capping	Ac_2O/DMAP/Lutidine/THF	2.5	2 times/2 minutes
10	As Step 1			

[a] When CCl_3COOH is used.

[b] When $ZnBr_2$ is used.

[c] 15 minutes for pyrimidine and 30 minutes for purine nucleosides.

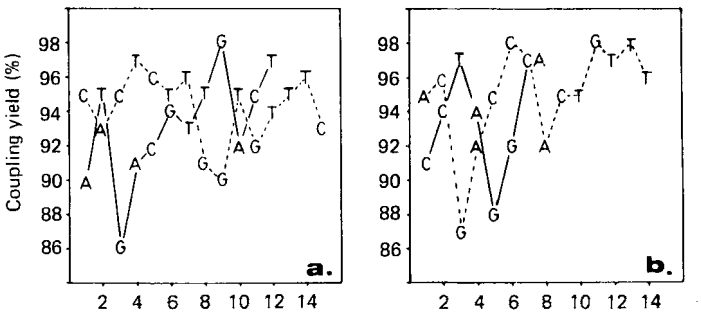


Figure 1: Representation of the coupling yields at each condensation step, a: d(CTTTGTGGTTCTCACC), ----, d(TCAGTTGCAGTAG), ———. b: d(TTTGTCAACCAGCAC), ----, d(CGGATCCG), ———. Overall yields were found to be for 16-mer: 42%, 15-mer: 50%, 13-mer: 43% and 8-mer: 62% according to dimethoxytrityl cation determination (experimental error ± 0.5%).

ringe under argon atmosphere. Condensation yield was checked after each cycle by the determination of dimethoxytrityl cation.

In the case of purine derivatives, coupling was found to require slightly longer time or more concentrated mixture than in the case of pyrimidine derivatives in order to afford more than 95% coupling. The oligonucleotides of desired sequences were synthesized following the steps described in Table 1. The coupling yields at each step and overall yield of the synthesized oligonucleotides are represented in Figure 1. The average yield per condensation was found to be about 94%.

It is noteworthy that the yield of the first coupling of an activated nucleotide (irrespective of purine or pyrimidine derivative) depends on the nucleoside attached to the polymer (Fig. 1).

The oligomers synthesized on the CPG beads were partially deprotected with a mixture of thiophenol : dioxane : triethylamine[9] (1:2:1) and finally cleaved from the polymer by concomitant de-N-acylation by incubating the CPG beads in concentrated aqueous NH_3 for 24 hours at 50° C. The aqueous solutions were concentrated and applied to a silica gel TLC plate prior to further purification of oligomers.

The synthesized oligomers were purified as 5'-DMTr derivative by reversed phase HPLC using a gradient of acetonitrile in 0.1 M TEAA buffer. The hydrophilic material (without dimethoxytrityl group) eluted at lower percentage of acetonitrile in 0.1 M TEAA buffer and the product was col-

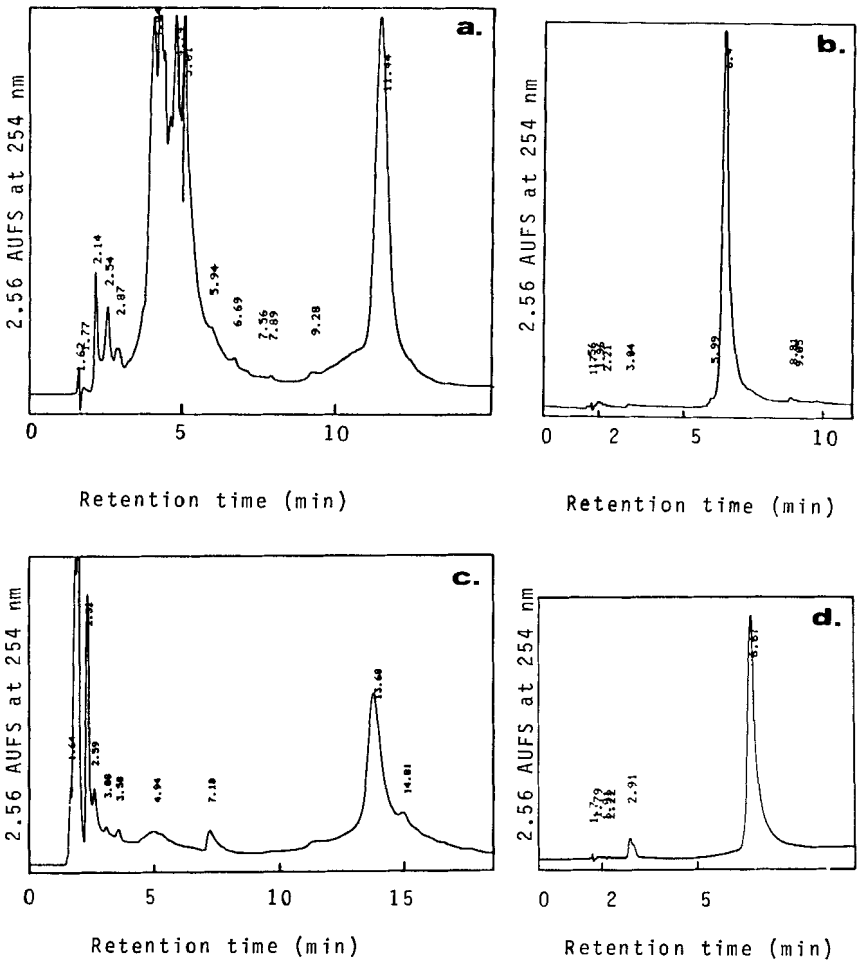


Figure 2: HPLC chromatograms of the final reaction mixture of 16-mer and 15-mer. Figures (a) (16-mer) and (c) (15-mer) show the separation of dimethoxytritylated oligomers from truncated sequences and Figures (b) (16-mer) and (d) (15-mer) show the analysis of desired fully deprotected oligonucleotides. The conditions for purification have been described in Experimental Section. The notated yields by HPLC were 16% (16-mer) and 25% (15-mer) respectively.

lected as a well-separated peak (Figure 2a, c). We found that purification was less effective when using an isocratic elution system. The pooled product after lyophilization was detritylated with 80% acetic acid in 45 minutes. An aliquot of this was labelled at the 5'- end with (γ-^{32}P)ATP and found to be usually more than 90% pure as estimated by 20% polyacrylamide gel electrophoresis (Figure 3).

All deoxyoligonucleotides were further chromatographed on the same reversed phase column in the detritylated form

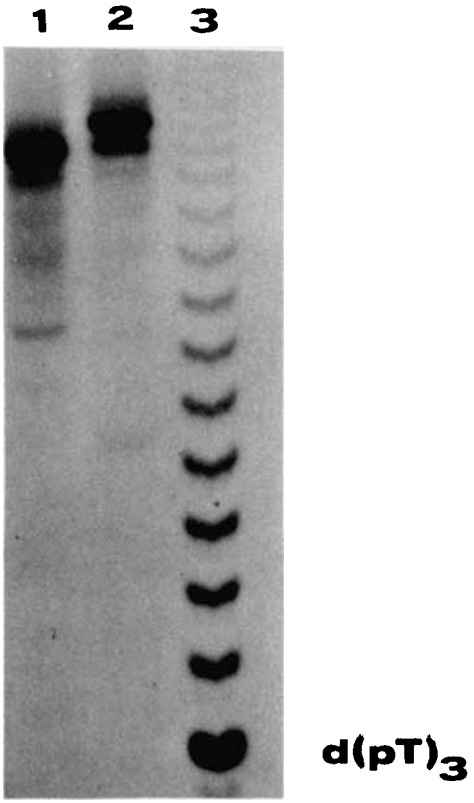


Figure 3: Electrophoresis of the pentadecamer and hexadecamer in lanes 1 and 2, respectively, on 20% polyacrylamide gel containing 7 M urea, after HPLC (Figure 2a, c), detritylation and phosphorylation with (γ-^{32}P)ATP and T4 polynucleotide kinase. Lane 3: homo-oligo-dT chain length standard[19].

using a different gradient of acetonitrile in 0.1 M TEAA buffer according to the literature[2b, 3, 10, 11].

The chromatographic performance at this stage was excellent, as indicated by essentially a single peak in the chromatograms for 16-mer and 15-mer (Figure 2b, d). There was, however, not much improvement in the purity of these chromatographed materials as demonstrated by 20% polyacrylamide gel electrophoresis (not shown). The materials obtained by eluting from the gel (Figure 4) were subjected to sequence analysis following "mobility shift"[12] or Maxam-Gilbert[13] method (Figure 5).

It could be suggested from our chromatographic studies that even a sharp single peak in the reversed phase HPLC

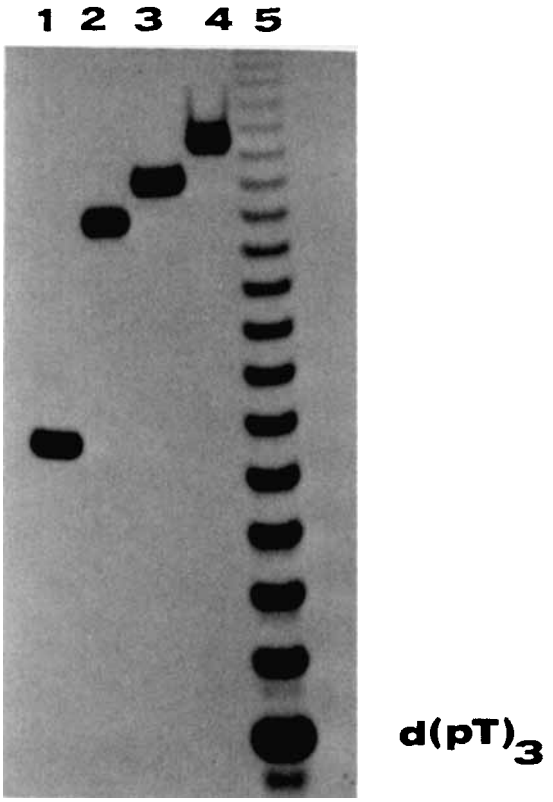


Figure 4: Electrophoretic representation of the oligomers on 20% polyacrylamide gel used for the sequence analysis. Octamer, tridecamer, pentadecamer, hexadecamer and homo-oligo-dT chain length standard in lanes 1,2,3,4 and 5, respectively.

chromatogram might not always represent 100% homogeneity or purity of the material collected from the single peak fraction.

CONCLUSION

(i) The active nucleosides prepared from chloro-N-morpholino-methoxyphosphine are obtainable in pure form and can be stored at low temperature under argon atmosphere for about six months.

(ii) The coupling yield per elongation step is comparable to other phosphite reagents.

(iii) Controlled pore glass (CPG) is a very suitable polymeric carrier for the synthesis of oligodeoxynucleotides

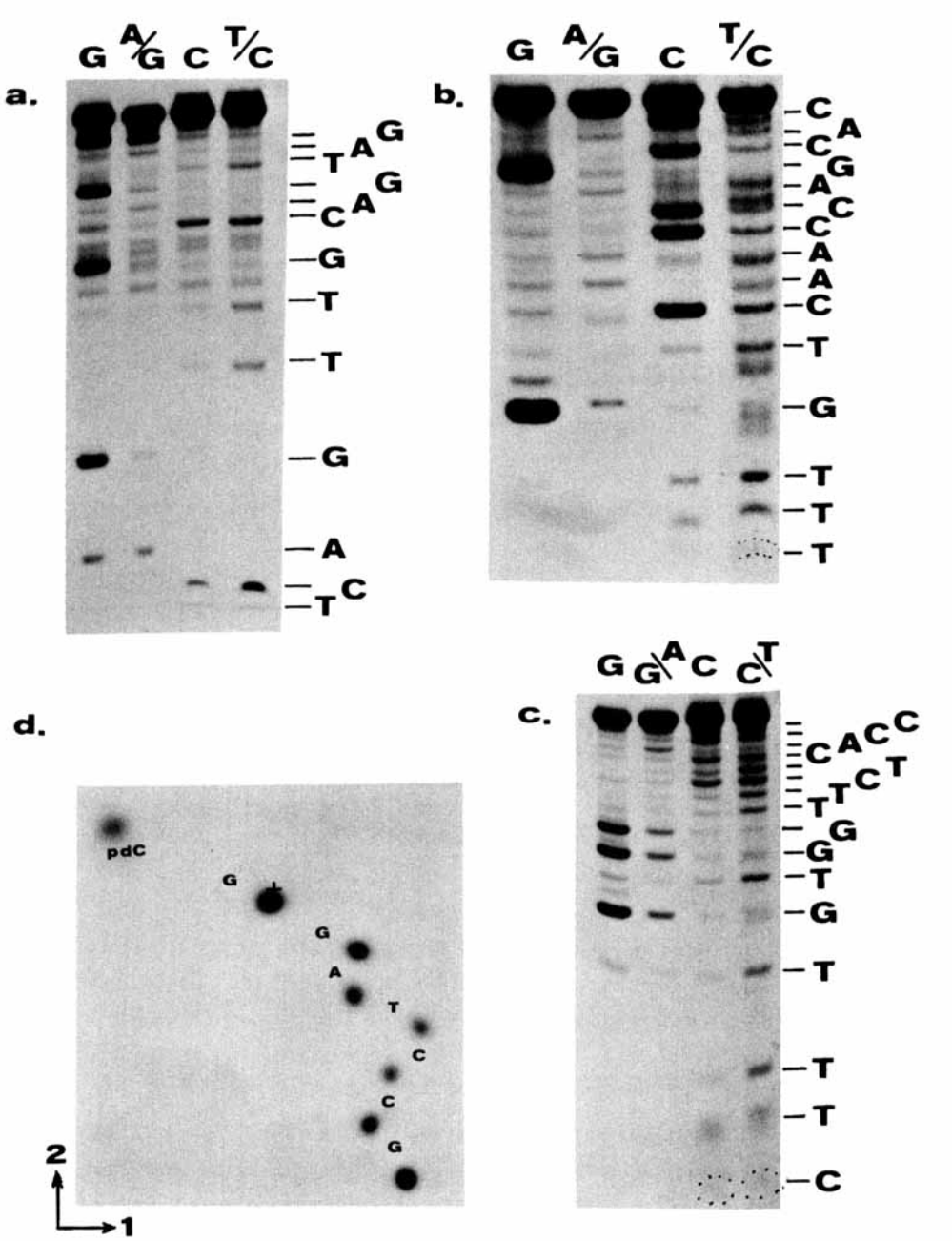


Figure 5: Sequence analysis of: a, d(TCAGTTGCAGTAG), b, d(TTTGTCAACCAGCA), c, d(CTTTGTGGTTCTCACC) by Maxam-Gilbert procedure and d, d(CGGATCCG) by "mobility shift" method. +: xylene cyanol marker. 1. dimension: electrophoresis, 2. dimension: homochromatography.

using the N-morpholinophosphoramidites as active deoxynucleosides.

EXPERIMENTAL

General methods: The following chemicals were purchased from commercial sources: deoxynucleosides from Pharma Waldhof (Mannheim), CPG from Serva (Heidelberg), $ZnBr_2$ from Riedel de Haen (Seelze), all reagents as 4-N,N-dimethylaminopyridine, aminopropyltriethoxysilane, tetrazole are from EGA (Steinheim). 2,6-lutidine was purified according to literature[14]. Pyridine was purified by distilling over p-toluenesulphonylchloride, potassium hydroxide and finally over CaH_2. Ether and tetrahydrofuran were distilled over sodium and benzophenone under nitrogen. Acetonitrile was first distilled over P_2O_5 and then CaH_2 in nitrogen atmosphere. Other solvents were distilled and stored over molecular sieve 4 Å.

Dimethoxytritylchloride, dichloromethoxyphosphine[15], N-trimethylsilylmorpholine[16] and dimethoxytritylated amino-protected nucleosides[17] were prepared following the standard published methods. Thin layer silica gel plates were developed in chloroform-methanol (9:1, v/v). ^{1}H-NMR spectra were recorded either with T-60 (Varian) or 270 MHz (Bruker). ^{31}Phosphorous-NMR were measured with 80 MHz (Bruker). Visible, UV-measurements were performed with Beckman model 35, HPLC was carried out with RP Ultrasphere ODS (5%) column (4.6 x 240 mm) on Beckman model 344.

Methyl chloro-N-morpholinophosphoramidite:

A two-necked flask fitted with additionfunnel, stirrer and argon delivery system was charged with methyldichlorophosphite (13.4 g, 100 mmoles) and dry ether (60 ml). To this a solution of N-trimethylsilylmorpholine (16.0 g, 100 mmoles) in ether (30 ml) was added at -20° C over 1.5 hours with constant stirring. After addition of the morpholine derivative, the mixture was stirred for additional 20 hours at room temperature under argon atmosphere. The solvent and other volatile material were removed under reduced pressure at 0° C. The residue so obtained was distilled by using a short distillation apparatus to give a clear liquid (13.2 g, 75%) boiling at 65-67° C/0.5 mm Hg. ^{1}H-NMR ($CDCl_3$): doublet

at 3.65 ppm, J_{PH} = 14 Hz (OCH_3); multiplet at 3.75 ppm ($O\langle^{CH_2-}_{CH_2-}$)(total = 7 H) and multiplet at 3.26 ppm ($N\langle^{CH_2}_{CH_2}$, 4 H). ^{31}P-NMR (CH_3CN) at 172.6 ppm. The mass spectrum (CH7, Varian) showed main peaks at m/z (% of relative intensity): 183 ($M^{\cdot+}$, 52), 185 ($M^{\cdot+}$ + 2, 17), 148 ($M^{\cdot+}$ - Cl, 86), 97 ($M^{\cdot+}$ - $C_4H_8NO^{\cdot}$, 62), 86 ($C_4H_8NO^{+}$, 100).

Synthesis of 3'-O(5'-O,N-protected deoxynucleoside)methyl-N-morpholinophosphoramidites

The 5'-O,N-protected deoxynucleosides (3.0 mmoles) were dried by coevaporation with pyridine and finally with toluene. The dried residue was dissolved in dry THF (15 ml) in presence of N,N,N-diisopropylethylamine (12.0 mmoles) and chloro-methoxy-N-morpholino-phosphine (6.0 mmoles) was added dropwise through syringe with constant stirring and under argon atmosphere at room temperature over 2 minutes. After 35 minutes of stirring N,N,N-diisopropylethylamine hydrochloride, which precipitated during the reaction, was filtered under argon atmosphere and the filtrate concentrated to remove THF and excess amine. The residue was dissolved in argon saturated ethylacetate (150 ml), washed with ice cold 10% Na_2CO_3 solution (50 ml, twice) and dried over Na_2SO_4 under argon atmosphere. Concentration of the dried organic extract resulted in a foam which was dissolved in toluene (60 ml) (for pyrimidines) or ethylacetate (60 ml) (for purine), then precipitated into hexane (250 ml) at -70° C and finally lyophilized from benzene to afford 95-98% pure phosphitylated nucleotides (2.7 to 2.9 mmoles) as fine white powder. The purity of these compounds was checked by ^{31}P-NMR as indicated by a singlet or doublet (for diastereomers) in the range 143 to 145 ppm with respect to 80% H_3PO_4 in acetone-d_6. ^{31}P-NMR (acetonitrile) (for Ba in Scheme 2) = 144.43, (Bb) = 144.28, (Bc) = 143.62, 143.78 and (Bd) = 144.38.

General coupling procedure on the polymer (CPG)

Usually 100 mg of the appropriate 5'-dimethoxytritylated amino-protected deoxynucleoside suitably anchored to control pore glass beads were detritylated with either 3% trichloroacetic acid in nitromethane (1% methanol) or saturated $ZnBr_2$ in nitromethane (1% water) and after washing according to Table I were dried in vacuo for 5 minutes.

The solid active nucleoside (100 mg, 20 equiv.) was placed into the column type reactor containing the detritylated polymer. The reactor was capped with septum and flushed with argon. To this a solution of sublimed tetrazole (50 mg, 0.7 mmoles) in acetonitrile (1.5 ml) was added through syringe. The suspension of the CPG beads was shaken gently. After either 15 minutes (for active pyrimidine nucleoside) or 30 minutes (for active purine nucleoside) of shaking the excess reagents were removed by washing with suitable solvents. The condensation yield was determined at this stage by taking out a small sample of CPG. When the desired high coupling yield was obtained, oxidation was performed with 2 ml, 0.1 mmoles I_2 solution in a mixture of THF, pyridine and water (80:40:2, v/v) for 2 minutes. The oxidizing reagent was removed by passing argon and washing with methanol followed by dichloromethane and finally CPG beads were dried by argon pressure. Then the polymer was suspended in a mixture of acetic anhydride (0.5 g), 2,6-lutidine (0.55 g) and 4,4-dimethylaminopyridine (0.3 g) in 5 ml THF for 5 minutes to block the unreacted 5'-OH groups of nucleoside or nucleotide linked to CPG. The steps comprising one cycle are summarized in Table I.

Deprotection of oligomers from the CPG-polymer

After the final elongation step of the desired sequence, the CPG beads were dried and treated four times with 5 ml of a mixture of thiophenol: 1,4-dioxane and triethylamine (1:2:1, v/v) at room temperature for a total duration of 2 hours. This mixture was removed, washed with dry methanol (4 x 5 ml) and dry ether (2 x 10 ml). The dried polymer was subjected to concentrated aqueous NH_3 (10 ml) at 50° C for 24 hours. After cooling, the supernatant liquid was removed and the polymer was washed with bidistilled water (3 x 5 ml). The combined supernatant and washings were evaporated under reduced pressure to dryness. The residue was treated with 10 ml tert. butylamine-methanol (1:1, v/v) for 24 hours at 45-50° C and concentrated under reduced pressure. The residue so obtained was dissolved in concentrated aqueous NH_3 (1 ml) and applied to a silica gel plate (Merck, 0.5 mm with fluorescent indicator, 254 nm) and developed to remove short trunketed sequences and non-nucleotidic material. The trityl positive band was eluted

with ethanol/aqueous NH_3 (1:1, v/v) and concentrated to small volume. The eluted product contained the desired oligomer along with some longer trunketed sequences, which was finally purified by reversed phase HPLC.

Purification of the oligomer by HPLC

The eluted and appropriately concentrated oligomer was applied to the reversed phase column (HPLC). The column was eluted at a flow rate of 1.5 ml/min. The purification to separate the 5'-DMTr-oligomer from trunketed non-DMTr-oligomer was performed with step linear gradient from 10-25% CH_3CN in 5 minutes and 25 to 29% CH_3CN in 30 minutes in 0.1 M TEAA, pH 7.0 at room temperature. The peak containing the desired oligodeoxynucleotide was collected, lyophilized and finally detritylated with 80% acetic acid for 45 minutes at room temperature.

The second chromatography was carried out with the detritylated material on the same column with a new step linear gradient form 5 to 15% for 2 minutes and 15-18% CH_3CN in 20 minutes in 0.1 M TEAA, pH 7.0, at room temperature.

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