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Automatic Synthesis of Oligodeoxynucleotides and Mixed Oligodeoxynucleotides using the Phosphoamidite Method

STAFFAN JOSEPHSON, EVA LAGERHOLM and GUNNAR PALM

Department of Chemistry, KabiGen AB, S-112 87 Stockholm, Sweden

A simplified procedure for making and using the deoxynucleoside phosphoamidites (*1a–d*) in the automated synthesis of oligodeoxynucleotides has been developed. The reagents prepared show good stability in solution. The reactivity of the reagents was found to be directly proportional to their concentrations, which makes it possible to synthesize mixed oligomers in desired ratios. Oligomers up to 45-mer have been synthesized using a coupling cycle of twenty min.

When using base-protected 5′-*O*′-dimethoxytritylnucleoside-3′-*O*-methylphosphochloridites[1,2] (Fig. 1) in automated solid phase synthesis of oligodeoxynucleotides, one has to take into consideration some disadvantages *e.g.* the reagents are not stable at room temperature, the guanosine reagent gives low yield (about 70 %) and the coupling reaction gives a precipitate of collidine hydrochloride, which can clog the pump and the filter in the machine.

An improvement in phosphite chemistry was made when Caruthers *et al.*[3] introduced the bifunctional chloro-*N,N*-dimethylaminomethoxyphosphine, since nucleoside phosphoamidites are relatively stable against hydrolysis and can be stored as a powder. When nucleoside phosphoamidites are activated with 1*H*-tetrazole they give rapid coupling reactions in high yields.

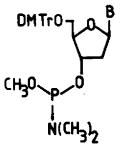

Fig. 1. 1a B=9-(*N*-6-Benzoyladeninyl); *1b* B=1-(*N*-4-Benzoylcytosinyl); *1c* B=9-(*N*-2-Isobutyrylguaninnyl); *1d* B=1-Thyminyl.

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However, we found it difficult to reproduce the procedure[3] of preparing and using the nucleoside phosphoamidites.

Coupling yields varied from batch to batch, due to incomplete drying of the powder and to the instability of the reagents in acetonitrile solution.

We therefore introduced improvements by which the phosphoamidites are obtained in a completely dry state and the problem of the reagent's instability in solution is overcome.

When making probes derived from an amino acid sequence for screening gene banks[4] or for Northern blotting[5] one often has to make several oligomers in order to get one with complete homology to the DNA or the m-RNA sequence, as is also the case when making primers for c-DNA synthesis[6] or DNA sequencing by Sanger's dideoxy method.[7] To overcome this problem we have developed a method for making mixed oligomers with equal amounts of each oligomer, which can be used both as probes and primers.

MATERIALS AND METHODS

The oligomers were synthesized automatically by a machine (Fig. 2) developed by KabiGen AB. The machine is built from commercially available parts except for the chassi where pneumatic and electric valves are mounted.

All parts which are in contact with liquids are made of teflon, stainless steel or glass. Solvents and reagents which must be kept free from

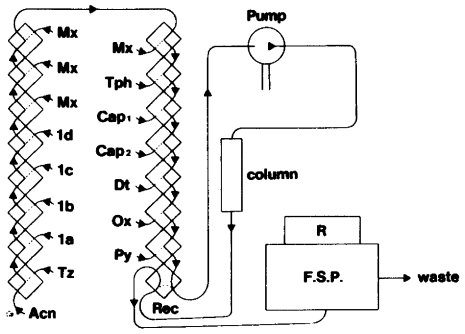

Fig. 2. The computerized machine with fifteen three-way valves which are connected to the following reagents an solutions: acetonitrile (Acn), 1*H*-tetrazole (Tz), nucleoside phosphoamidites (1a–d), mixtures of 1a–d (Mx), thiophenoxide (Tph), capping reagents (Cap_1 and Cap_2), detritylation reagent (Dt), Oxidation (Ox) and Pyridine water mixture (Py). A recycling valve (Rec) and HPLC pump are also controlled by the computer. A flow spectrophotometer (F.S.P.) with a recorder (R) monitors the detritylation yield.

moisture are stored under predried argon at atmospheric pressure in serum bottles (Supelco, Inc. US) seald with teflon septum. All reagents and solvents are connected via a stainless steel cannula and teflon tubing to a three-way valve (Cheminert valves from Laboratory Data Controll LDC, US) with 0.8 mm inner diameter (I.D.).

The three-way valves, total fifteen, are connected in one line to a recycling valve which is connected to an HPLC pump (Constametric III, 0–10 ml/min from LDC, US). The column, which is packed with 70 mg of solid support is a HPLC precolumn 50 mm long, 2 mm I.D. with a 10 micrometer filter in the outlet (Alltec Associates, US) and is connected to the pump by stainless steel tubing.

The valves are pneumatically controlled via electric solenoids (Concordia, from Hugo Tillqvist AB Sweden) connected to a relay card (Pamux PB16P1, Opto 22, US). A micro computer (Metric 850 which is a Compucord computer modified by Scandia Metric AB, Sweden) is connected via a parallel interface to the relays and via a digital-analog interface to the pump (the interfaces are accessories to the computer). The machine is programmed in the BASIC system in such a way that the opening time for each valve and the pump speed are typed into a stored file called cycle. The DNA sequence is typed in and the machine is ready to start with the chosen cycle file.

The syntheses are followed on a recorder connected to an ordinary spectrophotometer with a microflow cell.

Kination and 2-dimensional homochromatography were carried out according to TU and WU.[8]

Base-protected 5′-*O*-dimethoxytritylated nucleosides were prepared acording to published procedures.[9,10]

Solvents used in the synthesis were dried for 24 h before use with molecular sieves (Merck 1.6 mm rods), one volume to five volumes of solvent and without further purification: acetonitrile (Rathburn Chemicals Ltd, HPLC Grade, 0.2 % H_2O) were dried with 3Å sieves, tetrahydrofuran THF (Merck, *p.a.*, 0.1 % H_2O) and toluene (Merck, *p.a.*, 0.03 % H_2O) were dried with 4Å sieves. The bases used, ethyl diisopropylamine (Fluka, *purum*) and sym. collidine (Fluka, *puriss*) were dried over 4Å sieves.

General procedure for the preparation of base-protected 5′-O-dimethoxytrityl-2′-deoxynucleoside-3′-O-methylphosphodimethylamidites.[11] The base-protected 5′-*O*-dimethoxytritylnucleoside (2.0 mmol) was predried with P_2O_5 at 40 °C in vacuo for 12 h and then dissolved in tetrahydrofuran (20 ml) with addition of ethyldiisopropylamine (5 mmol). The solution was dried over sieves 4Å (4 ml) at room temperature for 24 h, then decanted into a predried serum bottle (50 ml) and capped with a teflon septum.

A solution of ethyldiisopropylamine (5 mmol) and chloro-*N,N*-dimethylaminomethoxyphosphine (2 mmol) in THF (8 ml) was added with a syringe to the stirred 5′-*O*-dimethoxytritylnucleoside solution at room temperature over 10 min. After 1 h stirring, the bottle was centrifuged (5 min, 2000 r/min) and the supernatant was decanted into a round bottomed flask. The solution was evaporated at 30 °C on a rotaevaporator connected to a high vacuum pump and then coevaporated with toluene (2×50 ml). The resulting foam was dissolved in acetonitrile (20 ml). A sample diluted one hundred times was analysed on TLC (Silica-gel, 5 % MeOH in $CHCl_3$) which showed that all starting material had been consumed. Two main spots were

formed, one major spot migrating faster than the corresponding nucleoside and a minor spot appearing at the point of origin. R_f values for the migrating spots are presented below. The values for the corresponding 5′-*O*-dimethoxytritylated nucleosides appearing in parentheses: *1a* 0.30 (0.24), *1b* 0.30 (0.25), *1c* 0.23 (0.16) and *1d* 0.34 (0.28).

Synthesis on automated machine. The solid support was Waters LC Porasil C (37–75 μm) aminated with triethoxysilylpropylamine and coupled with 5′-*O*-dimethoxytrityl-*N*-protected-2′-deoxynucleoside-3′*O*-succinic acid as described by Chow *et al.*[2]

The support, 70 mg, corresponding to about 7 mmol of the desired 3′-attached nucleoside, was loaded into the column.

The following 20-minute cycle was used.

1. Detritylation 5 min, flow rate (f.r.) 3 ml/min with saturated $ZnBr_2$ 1 % water in nitromethane v/v.
2. Acetonitrile 2 min, f.r. 5 ml/min.
3. Coupling reagents were stored in two bottles, 1*H*-tetrazole (0,2 M) in acetonitrile, and nucleoside phosphoamidite (nominally 0.1 M). The two solutions were successively pumped into the column for 6 s each, then repeated once, and finally for 6 s more with 1*H*-tetrazole. The activated nucleoside phosphoamidite was then recycled through the column for 4 min 30 s, f.r. 3 ml/min.
4. Oxidation for 1 min, f.r. 5 ml/min with 0,01 M I_2 in a mixture of tetrahydrofuran–water–lutidine 10:5:1 v/v.
5. Acetonitrile 2 min, f.r. 5 ml/min.
6. Capping of unreacted 5′-hydroxy groups by a reagent stored in two bottles, acetic anhydride 0,8 M in a mixture of tetrahydrofuran; collidine 6:1 v/v, 4-dimethylaminopyridine 0,8 M in tetrahydrofuran. The two solutions were successively pumped into the column for 10 s each and then repeated twice, total 1 min. The activated capping reagent was then recycled for 2 min 30 s, f.r. 3 ml/min.
7. Acetonitrile 1 min 30 s, f.r. 5 ml/min.

Deprotection of the methyl group on the internucleotidephosphotriester was performed by the machine with an end program.

1. Removal of 1*H*-tetrazole with a mixture of pyridine: water 1:1 v/v, from the support 6 min, f.r. 5 ml/min.
2. Acetonitrile 2 min, f.r. 5 ml/min.
3. A mixture of Et_3N: dioxan: thiophenol, 2:2:1 v/v, was run through the column for 2 min and then recycled for 15 min, f.r. 3 ml/min.
4. Pyridine: water 1:1 v/v 2 min, f.r. 5 ml/min.
5. Acetonitrile 3 min, f.r. 5 ml/min.

Removal of the oligonucleotide from the support and removal of the base protecting groups was done by concentrated ammonia solution at 50 °C for 4 h. The silica support was filtered off and 0.5 ml of 0.1 M trisbuffer pH 7.5 was added. The ammonia solution was evaporated to dryness and the residue redissolved in 0.5 ml water.

The 5′-*O*-dimethoxytrityloligonucleotides up to 24-mers were purified on a semi preparative Licrosorb 5 μm C-18 HPLC column, using a gradient of 20–30 % acetonitrile in 0.1 M triethylammonium acetate pH 7.0 over 30 min for oligomers up to 20-mers and a gradient of 15–25 % for oligomers up to 24-mers. Deprotection of the 5′-*O*-dimethoxytrityl group was done with 80 % acetic acid for 15 min at room temperature. The fully deprotected oligomer was isolated either on an analytical Licrosorb 5 μm C-18 HPLC column using a gradient of 10–15 % acetonitrile in 0.1 M triethylammonium acetate pH 7.0, over 30 min, or by gel-electrophoresis.

Longer oligomers were isolated as fully deprotected by gel-filtration (sephadex G-25) followed by gel-electrophoresis.

The electrophoresis was performed on a 20 % polyacrylamide gel (7 M urea) in 50 mM Trisborate, 0.8 mM EDTA pH 8.3 for 1 h at 400 V. The oligomer was extracted from the acrylamide gel with 20 mM ammonium acetate pH 7 and then purified on a Whatman DE-52 cellulose ion-exchange column (using 1.5 M ammonium acetate pH 7 as eluent). The isolated oligomers were then lyophilized and ready for use.

RESULTS AND DISCUSSION

The four 5′-*O*-dimethoxytrityl-*N*-protected-2′-deoxynucleoside-3′-*O*-methylphosphodimethylamidites (*1a–1d*) solutions are connected each to one valve and four valves are intended for reagent mixtures (Fig. 2). Coupling yields ranging from 85 to 100 % (based on the amount of detritylation) were obtained except for the first coupling which usually varies from 50 to 90 %. We also found that the rate of detritylation was dependent on the base at the coupled nucleotide, using the flow spectrophotometer at 550 nm we

can estimate the yields and determine the sequence of the made oligomer. Detritylation of deoxyadenosine gives a narrow peak about 0.4 min, deoxycytidine gives a broad peak 1.0 min, deoxyguanosine and thymidine give a medium peak 0.6 min.

When the oligomers were isolated it was found that it was necessary to store the 5′-*O*-dimethoxytritylated oligomer after the ammonia treatment in a trisbuffer with pH>7; if dissolved in water, the solution becomes acidic, (about pH 3), and detritylation is observed.

The stability of the phosphoamidite reagents (*1a–1d*) was found to be good; after four weeks in acetonitrile solution at room temperature the coupling yields were found to be only slightly lower than that from fresh reagents. ^{1}H NMR studies on the reagents (*1a–1d*) showed that some of the ethyldiisopropylamine still remained after coevaporation with toluene, up to 50 % based on the dimethoxytrityl group, which might explain the good stability of the reagents.

Concentration of the reagents was estimated from ^{31}P NMR with *N,N*-dimethylaminodimethoxyphosphine as internal standard. The concentrations varied between 60 to 70 % of the nominal value of 0,1 M and the purity was about 85 % (Table 1).

Several oligomers have been synthesized by the method described, from 8-mer to 45-mer.

Table 1. NMR-data of nucleosid phosphoamidites.

Compound	$-^{31}$P (ppm) 20 % $CDCl_3$ in Acetonitrile	Isolated yield[a] %	Purity %
1a	147.4 (m)	59	88
1b	146.5 (m) 147.1 (m)	60	85
1c	146.8 (m)	68	83
1d	146.3 (m) 146.9 (m)	64	82

[a] Estimated yield *N,N*-dimethylaminodiethoxyphosphine as internal standard.

Table 2. Sequence and yield of made oligomers.

No.	Compound	Functionalisation of silica with 3′ nucleoside mmol/g	Amount of silica used (mg)	Yield % dMTr+	Isolated fully deprotected O.D. 260 and (yield %)
2	TTTTTTTT	0.075	77	51	79 (17)
3	CGCAAGAACGAAAG	0.090	70	20	6 (0.7)
4	CAGCCTCCCCATCA	0.094	70	25	10 (1.0)
5	TC$^{C}_{T}$TG$^{G}_{A}$TACATCAT	0.075	70	34	9 (1.2)
6	TCGTCCCTCTG-AAACTCTGT	0.075	70	32	4 (0.4)
7	CCAAGCTTTTAC-TTAGCGATTTGG	0.08	70	30.1	5.6 (0.4)
8	AGCCACCCAAAT-CGCTAAGTAAAA-GCTTGG	0.08	70	8.2	2 (0.1)
9	AACCACGGTCACC-GCAAACAAACTGG-AGAGTGTCAACCA-GTTCAC	0.07	70	8.6	4.7 (2.2)

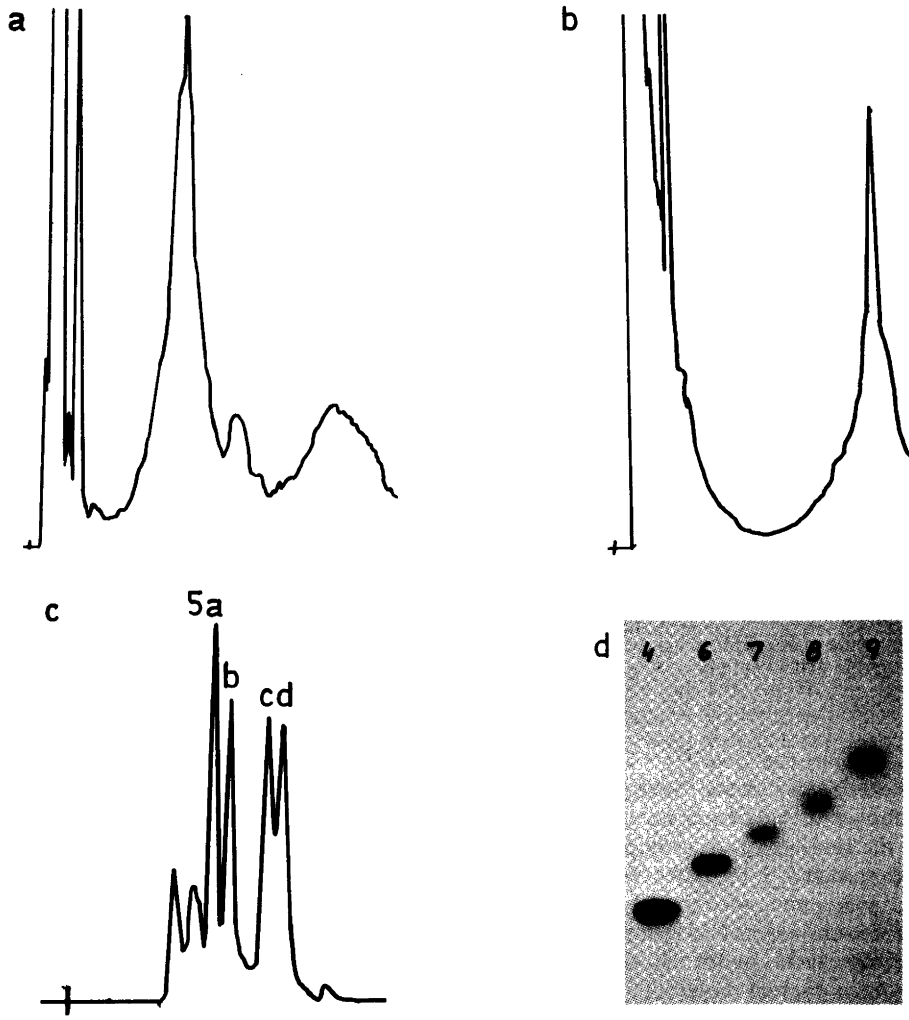

Fig. 3. HPLC profiles of 5′-*O*-dimethoxytritylated compounds *5,6* and fully deprotected compound *5a–5d*. Gel electrophoresis on fully deprotected compounds. a, Gradient 20 $\xrightarrow{30\ \text{min}}$ 30 % acetonitrile in 0.1 M triethylammonium acetate pH 7.0. b, Gradient 15 $\xrightarrow{30\ \text{min}}$ 25 % acetonitrile in 0.1 M triethylammonium acetate pH 7.0. c, Isocratic 11,5 % acetonitrile in 0.1 M ammonium acetate pH 7.0. Compound: *5a* $^{5'}$TCCTGATACATCAT, *5b* $^{5'}$TCCTGGTACATCAT, *5c* $^{5'}$TCTTGGTACATCAT, *5d* $^{5'}$TCTTGATACATCAT. d, Gel electrophoresis 20 % polyacrylamide (7 M urea). Compound: *4* 14-mer, *6* 20-mer, *7* 24-mer, *8* 30-mer and *9* 45-mer.

The yields are shown in Table 2 and the HPLC-profile for compounds 5 and 6 are shown in Fig. 3. Oligomers prepared by this method have been used as probes, primers and in ligation.

For mixed synthesis it is necessary to know the relative reactivity of the reagents *1a–1d* in order to get equal incorporation of the nucleotides in the mixtures.[12] We made 3-mers with thymidine at the ends and mixtures of all four nucleotides in the middle. Three mixtures of *1a:1b:1c:1d* were prepared in three different ratios 1:1:1:1, 2:1:1:2 and 1:2:2:1. The mixtures were analysed as fully deprotected 3-mers on HPLC and identified by

Table 3. Ratio between oligomers in mixtures.

Compound	No.	Mixture v/v	Calculated ratio from trimer	Found ratio in mixture of oligomers[a]	Retention[b] time in min and % acetonitrile
A		1 A	1.5	1.5 A	13.2 (A)
TCT	*10*	1 C	1.6	1.6 C	10.0 (C) 8.5 %
G		1 G	1.0	1.0 G	8.7 (G)
T		1 T	1.7	1.7 T	16.6 (T)
A		2 A	3.1	2.9 A	
TCT	*11*	1 C	1.6	1.6 C	same
G		1 G	1.0	1.0 G	as
T		2 T	3.4	3.5 T	above
A		1 A	1.0	1.0 A	
TCT	*12*	2 C	2.1	1.8 C	same
G		2 G	1.3	1.2 G	as
T		1 T	1.1	1.1 T	above
$TC^{C}_{T}TG^{A}_{G}TACATCAT$	*5*	1 C	1.0	1.0 C	12.2 (AC)
		1 T	1.1	1.1 T	13.6 (GC) 11.5 %
		1 A	1.5	1.4 A	16.8 (GT)
		1 G	1.0	1.0 G	18.0 (AT)

[a] Corrections are made for varying extinction coefficients of the trimers. [b] HPLC on reverse-phase C-18. Licrosorb 5 μm analytical column, using acetonitrile in 0.1 M triethylammonium acetate pH 7.0.

reference substances (Table 3). The relative amounts of every 3-mer in each mixture accorded with the ratio of the prepared mixtures, which shows that the reactivity is directly proportional to the concentration. One tetradecamer with two mixed points was made, one mixture being *1a:1c* and the other with reagents *1b:1d* mixed 1:1 v/v using the same reagents as for the trimers. Four peaks could be separated on HPLC (Fig. 3) and identified by homochromatography. The amount of each oligomer agreed with the calculated ratio from the trimer (Table 3). Some other mixed oligomers have been synthesized which confirm these results.

Thus, by making a mixed trimer and analysing the ratio it is possible to prepare mixed oligomers of desired ratios.

Mixed dimethoxytritylated oligomers from HPLC separation must be collected with a broad margin because the oligomers in the mixture could have different retention times. When collecting with broad margins, traces of shorter oligomers could contaminate the mixture, hence to eliminate the shorter fragments gel-electrophoresis was run and the longest main fragment was cut out.

Mixed oligomers prepared by this method have been used as probes for screening gene banks and in sequencing using Sanger's dideoxy method.[13]

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