Synthesis of mixed oligodeoxyribonucleotides following the solid phase method

Anette Elmblad, Staffan Josephson and Gunnar Palm

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

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ABSTRACT

A method for the synthesis of mixed dimers, trimers and oligonucleotides on a solid support using monomeric protected nucleoside phosphochloridites (1a-d) has been developed and studied. No difference in reaction rate was observed when mixing the different nucleoside reagents, and the results show that yields of different oligomers in a mixture could be directly correlated to the concentration of the four reagents. Separation of mixed oligomers on a reversed phase C18 column has also been studied.

INTRODUCTION

In order to make a DNA copy of an amino acid sequence it is usually necessary to synthesise several different oligomers. This is due to the fact that almost every amino acid has several codons. To facilitate this synthesis we now propose a rapid method for producing mixed DNA sequences, based on the solid phase method introduced by Caruthers et al.[1] and Chow et al.[2] This method was used to make mixed dimers, trimers and oligonucleotides. The synthesis was carried out by an automatic DNA-synthesising machine developed by KabiGen AB. Earlier attempts to synthesise mixed sequences on solid support involved introducing the mixture into the middle of a trimer block.[3] In our approach monomer blocks of 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-O-methylphosphochloridite were used which simplifies the whole process. It is only necessary to determine the concentration of the four nucleoside reagents (1a-d) and prepare the appropriate mixtures. The method was studied by varying the composition of the mixtures and the nucleoside on the solid support, initially in the synthesis of different dimers then of trimers and lastly

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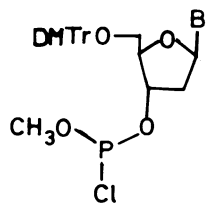
of mixed oligomers. Three 11-mers, one 12-mer and three 14-mers were made and their separation and purification studied, using reversed phase C18 column HPLC. This showed that in some cases it was possible to separate all the different oligomers in a mixture (see table III and figure 2). The total preparation time for the 11-mers was 9.7 hours, the 12-mer 10.7 hours and the 14-mers 12.7 hours. Each cycle takes an hour and includes detritylation, addition of the nucleoside reagent or the mixed nucleoside reagents, oxidation and capping with acetic anhydride[2].

RESULTS AND DISCUSSION

The solid support used was aminated silica Porasil C (37-75 µ) (Waters)[1,4] to which the different 5'-O-dimethoxytrityl-N-protected-2'-deoxynucleoside-3'-O-succinic acids[5] had been coupled. The nucleoside phosphite reagents (1a-d) were prepared according to Chow et al.[2] with some modifications suggested by Caruthers.[6] and their concentration measured immediately before use. When freshly prepared, A-, C- and T-reagents showed a concentration of 0.08 - 0.12 M and the G-reagent 0.04 - 0.06 M. The concentration of the reagents tended to decrease slowly with time even when stored at -20°C, which accounts for different values in tables I and II. Concentrations were measured by reacting known volumes of reagent with 5'-O-dimethoxytrityl thymidine, and calculating the amount of 5'-O-dimethoxytrityl compound used in the formation of 3'→3' dimers. Concentrations for each new reagent were determined in three separate experiments and showed very minor variations.



1a B = T
1b B = bzC
1c B = bzA
1d B = ibG

When the concentration of the G-reagent was calculated by this method the value was consistantly higher than it should have been. This was probably due to the presence of some unreacted

Table I

Solid support	Mixture of reagents	Concentration of reagents in mixture mM	Ratio of concentration	Dimers formed	Ratio between Dimers	Retention* time, min
T	A	73	2.8	AT	2.8	19.3
	T	26	1	TT	1	16.4
T	A	55	1.4	AT	1.3	19.3
	T	39	1	TT	1	16.4
T	C	45	1.2	CT	1.2	7.2
	T	39	1	TT	1	16.4
T	A	55	2.5	AT	2.4	19.3
	G	22	1	GT	1	10.1
T	A	55	1.2	AT	1.1	19.3
	C	45	1	CT	1	7.2
T	C	45	2.0	CT	2.0	7.2
	G	22	1	GT	1	10.1
T	A	28	2.5	AT	2.4	19.3
	C	23	2.1	CT	2.0	7.2
	T	20	1.8	TT	1.8	16.4
	G	11	1	GT	1	10.1
T	T	39	1.8	TT	1.9	16.4
	G	22	1	GT	1	10.1
C	T	39	1.8	TC	1.9	12.8(6.5% CH_3CN)
	G	22	1	GC	1	8.2
A	T	39	1.8	TA	1.8	13.4
	G	22	1	GA	1	8.0
G	T	39	1.8	TG	1.8	17.6(5.5% CH_3CN)
	G	22	1	GG	1	12.4
G	T	26	0.9	TG	0.9	17.6(5.5% CH_3CN)
	G	29	1	GG	1	12.4

* HPLC on reverse-phase C18. Ultracil 10 µm analytical column, Altex system, with 7.5% acetonitrile in 0.1 M triethylammonium acetate pH 7.0.

methylphosphodichloridate that had not coevaporated during its preparation and which reacts with the 5'-O-dimethoxytrityl thymidine. The concentration of this reagent was therefore calculated by comparing the ratio between the 3'-,5'-dimers formed

Table II

Solid support	Mixture	Concentration of reagents in mixture in mM	Con. ratio of reagents	Dimers formed	Ratio of dimers	Reagent added after dimers	Trimers formed	Ratio of trimers	Retention* time, min.
T	A	25	2.5	AT	2.7	T	TAT	2.7	15.8
	G	10	1	GT	1		TGT	1	9.7
T	C	18	1.5	CT	1.5	T	TCT	1.4	12.4
	T	12	1	TT	1		TTT	1	24.8
T	A	25	1.4	AT	1.4	T	TAT	1.4	15.8
	C	18	1	CT	1		TCT	1	12.4
T	A	25	2.5	AT	2.5	T	TAT	2.6	15.8
	C	18	1.8	CT	1.7		TCT	1.7	12.4
	T	12	1.2	TT	1.2		TTT	1.2	24.8
	G	10	1	GT	1		TGT	1	9.7
A	A	25	1	AA		T	TAA	1	12.0
	T	27	1.1	TA			TTA	1	18.4
A	A	25	1	AA		C	CAA	1	7.0
	T	27	1.1	TA			CTA	1.2	8.6
A	A	25	1	AA		G	GAA	1	7.6
	T	27	1.1	TA			GTA	1	10.2
A	A	25	1	AA		A	AAA	1	11.2
	T	27	1.1	TA			ATA	1.1	18.0

* Run on a reverse-phase C18 column Ultracil 10 μm with 8.5% CH_3CN in 0.1 M triethylammonium acetate pH 7.0

in the mixed preparations (see table I). This unreacted methylphosphodichloridate accounts for the drastic drop in total yields observed when making G-rich oligonucleotides. When mixed dimers were made the nucleoside on the solid support was first detritylated, washed and reacted with the mixed nucleoside reagents and then oxidized. The resulting dimer mixtures were deprotected, hydrolised from the solid support and purified on a reversed phase C18 column as DMTr-dimers. The purified dimers were detritylated and injected again on to the HPLC. The peak areas for the different dimers were integrated, corrected according to their extinction coefficients and the ratio between the dimers calculated. In order to identify peaks, the different dimers were also prepared by solution chemistry following the standard triester approach[7]. The ratios between dimers and their retention times are shown in table I. Results in table I show that ratios between dimers can be directly correlated to concentrations; neither different mixtures of reagents nor changed nucleosides on the solid support alters this correlation.

A new set of mixed dimers was synthesised and one more nucleotide added to see if any changes occurred in the ratios of the dimers. However, the results again showed that the ratios of trimers in the mixtures could be directly correlated to the reagent concentrations, indicating that the addition of one other nucleotide does not alter the ratios (table II). The trimers were also synthesised by solution chemistry following the triester approach, in order to identify each trimer in the mixture. HPLC separation of the dimer and trimer mixtures are shown in figure 1.

Three mixed 11-mers, one mixed 12-mer and three mixed 14-mers were also made (see table III). The mixed 11-mers were completely resolved on HPLC (see figure 2) as were two of the 14-mer mixtures, the third 14-mer mixture (compound 7) could only be partially resolved. In the case of the oligomers the separation of the mixtures was carried out in two stages, firstly as the DMTr-oligomers and then as the fully deprotected oligomers. Compound 3 (table III) could not be resolved on HPLC so the ratio was calculated by first radiolabelling the 5'-end of the purified mixture, then running a 2-dimensional homochromatograph and scraping

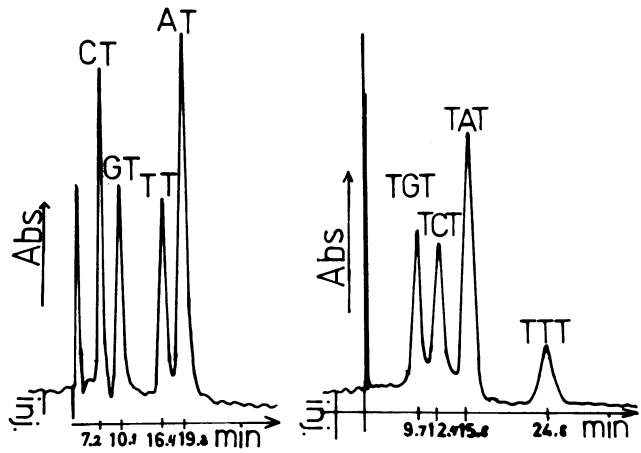


Fig 1. Separation of dimers and trimers on C18 reversed-phase column with 7.5% acetonitrile (dimers) and 8.5% acetonitrile (trimers) in 0.1 M triethylammonium acetate pH 7.0

out the spots belonging to the different oligomers to compare their radioactivity. Compound 7 could just be separated into two peaks on the HPLC containing oligomers with either A or G in the third nucleotideposition, but when the 2-dimensional homochromatograph was run on the two fractions, four oligomers were seen in each case. Separation and purification might be a slight problem with mixtures containing several oligomers and consequently it is necessary to collect the peaks from the HPLC with broad margins so as not to loose any oligomers present in smaller amounts.

EXPERIMENTAL SECTION

The oligomers were synthesised by an automatic DNA-synthesising machine developed by KabiGen AB. Separations were done on an Ultracil reversed phase C18 10 µm column, Altex HPLC, and a spherisorb reversed phase C18 5µ column, LDC HPLC system. All dimers, trimers and oligomers were synthesised and deblocked according to published procedures[2]. Kination and 2-dimensional homochromatography were carried out according to Tu and Wu[8].

Table III

Compound No.	Oligomer mixtures	Ratio between oligomers	DMTr-oligomers* Retention time min.	Fully deprotected oligomers. Retention time min.	% CH_3CN in 0.1 M triethyl ammonium acetate
2	5'CCCCATAC$^{C}/_{T}$TT^{3}'	1.5(T) : 1(C)[a]	21.6	16.5(C) 19.5(T)	10.7
3	5'CCA$^{C}/_{T}$TGCCA$^{G}/_{A}$TC^{3}'	1.6(T) : 1(C)[a]	24.6	27.2	10 ⟶ 15 30 min
4	5'TG$^{A}/_{G}$TATATCAT^{3}'	2.0(A) : 1(G)[b]	19.4(G) 21.4(A)	16	11
5	5'A$^{A}/_{G}$ $^{C}/_{T}$TCCCACCA^{3}'	1.9(A) : 1(G)[b] 1(T) : 1.2(C)	23.2(G) 26.0(A)	18.5(G,C) 21.5(G,T) 32.5(A,C) 37.5(A,T)	11
6	5'TG$^{A}/_{G}$TAGATCAT$^{C}/_{T}$TG^{3}'	1.6(A) : 1(G)[c] 1(T) : 1(C)	23.0(A) 25.6(G)	8.5(A,C) – 11.0(A,T) / 28(G,C) – 32.5(G,T)	– – – 11.5 – – – – – / 13
7	5'TG$^{A}/_{G}$TA$^{T}/_{A}$ATCAT$^{C}/_{T}$TG^{3}'	1.5(A) : 1(G)[c]	22.8(A) 27.2(G)	20(A) 21.5(G)	10 ⟶ 15 30 min
8	5'TC$^{C}/_{T}$TG$^{A}/_{G}$TACATCAT^{3}'	2.4(A) : 1.0(G)[d] 1.0(T) : 1.2(C)	18.5	13.0(A,C) 15.0(G,C) 19.0(G,T) 21.0(A,T)	11.5

* All compounds run on a reversed-phase C18 column with a 20%→30% gradient of acetonitrile in 0.1 M ammonium acetate
compound 2. and 3 on a preparative 10 µm column
compound 4 and 8 on a analytical 5 µm column
compound 5-7 on an analytical 10 µm column

+ All compounds run on a reverse-phase C18 column
compound 2,4-8 on an analytical 5 µm column
compound 3 on a preparative 10 µm column

Separation of mixture in brackets.
a) same reagents used b) same reagents used c) same reagents used d) same reagents used as in table I

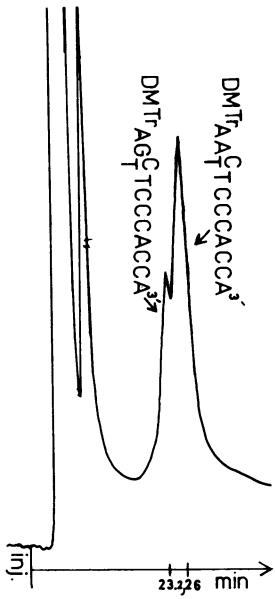
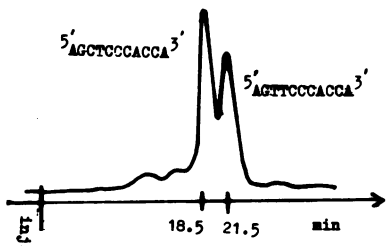
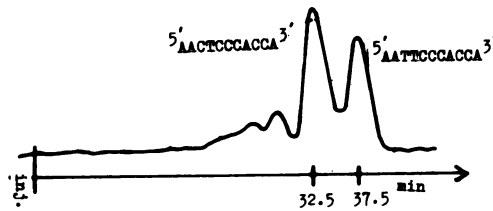


Figure 2

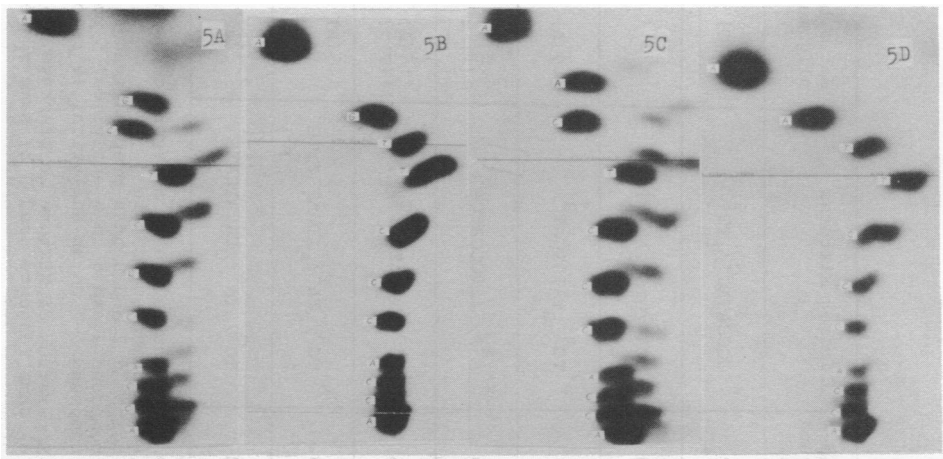
Separation of a mixed 11 mer $^{5'}A^{A}/_{G}{}^{C}/_{T}CCCACCA^{3'}$

30 min
Gradient 20 ⟶ 30% acetonitrile in 0.1 M triethylammonium acetate pH 7.0





11% acetonitrile in 0.1 M triethylammonium acetate pH 7.0



2-dimensional homochromatography of the four separated oligomers: 5A AGCTCCCACCA, 5B AGTTCCCACCA, 5C AACTCCCACCA and 5D AATTCCCACCA

General Procedure for the Preparation of 5'-O-Dimethoxytrityl-2'-Deoxynucleoside-3'-O-Methyl Phosphochloridites

The baseprotected 5'-O-dimethoxytritylnucleoside[9,10] (5.5 mmol) in dry tetrahydrofurane (THF) (20 ml) was added with a syringe over a 30 minute period to a stirred cooled solution (-78°C) of methyldichlorophosphite [11,12] (5.0 mmol, 0.48 ml) and sym-collidine (25 mmol 3.3 ml) in THF (20 ml) under argon in a capped serum bottle. After an additional 30 minutes stirring, the temperature was allowed to rise to room temperature and the reaction mixture centrifuged. The clear supernatant was removed from the serum bottle and transferred to an argon filled round bottomed flask. The solution was concentrated to a gum, which was then dissolved in toluene and evaporated again, this was repeated twice[6]. The resulting white foam was dissolved to a concentration of 0.2 M (25 ml) in THF to which sym-collidine (25 mmol 3.3 ml) was added and transferred to a dry serum bottle.

Measurement of reagent concentration

5'-O-dimethoxytrityl thymidine was dried to constant weight in vacuo over P_2O_5 at 40°C and was dissolved to a concentration of 0.04 M in THF, previously distilled over sodium/benzophenone under argon. To 2.5 ml of this solution kept under argon in a serum bottle at room temperature, 0.5 ml of 5'-O-dimethoxytrityl-2'-deoxynucleoside 3'-O-methyl phosphochloridite (theoretical 0.18 M) was added with a syringe. After complete reaction (1h), a solution of 5'-O-dimethoxytrityl-N-benzoyladenosin (2 ml 0.025 M) dissolved in THF was added as an internal standard. The reaction mixture was analysed by HPLC on a reversed phase C18 10 µm column with acetonitril 52%: 0.1 M ammonium acetate in water, pH 7, as eluent; the retention times were 10.9 minutes for 5'-O-dimethoxytrityl thymidine and 14.0 minutes for 5'-O-dimethoxy-N-benzoyladenosine. The concentration of the reagent could then be directly correlated to the consumption of 5'-O-dimethoxytritylthymidine. Whilst the concentration of the A-, C- and T-reagents could be measured directly the concentration of the G-reagent could only be calculated by making a mixed 3'→5' dimer. For example the dimer $^{5'A}/_{G}T^{3'}$ was made by putting DMTr-T-silica (100 mg 5 µmol) in the column and detritylate it

with $ZnBr_2$ in nitromethane (with 1% water) followed by washing with dry THF. A mixture of the A-reagent (of known concentration) and G-reagent was then introduced and recycled through the column. When the reaction was completed, ca 10 minutes, the dimers so formed were washed with THF the phosphite bond oxidized with iodine followed by another washing. The dimers were deprotected firstly by treatment with thiophenol-triethylamine in dioxane[1,13] for 30 minutes at room temperature which removes the methyl protecting group of the internucleotide phosphotriester, and then with concentrated ammonia at 50°C for 4 hours. The silica was removed by filtration and the ammonia solution evaporated. The DMTr-dimers were redesolved in water and purified by HPLC, detritylated with acetic acid (15 minutes) and injected again on to HPLC. The peak areas of the two dimers were measured and the concentration of the G-reagent calculated.

Preparation of oligomers

Oligomers (compound 2-8) were synthesised by just repeating the reaction cycle for the dimer preparation only with one addition that between each cycle a capping reaction with acetic anhydride, collidine and dimethylaminopyridine was done. The nucleoside reagent or the mixed nucleoside reagents were used in each addition in a 4-18 (20-90 µmol) fold excess over the nucleoside hooked on to the silica. The total yield of oligomers 2-8 (table III) varied from 5-20% depending on lenght and base composition.

Preparation of reagent mixtures

Reagents were mixed under argon in small predried serum bottles in a 1:1 ($^{v}/_{v}$) mixture except in the first and last example in table I where 2:1 mixtures were made.

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