A new general approach for the simultaneous chemical synthesis of large numbers of oligonucleotides: segmental solid supports

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ABSTRACT

A new approach is described which will allow the simultaneous synthesis of large numbers of pre-defined oligonucleotide chains. No machine aid is needed. The simultaneous syntheses can be performed by one person and do not require much more time than is currently needed for the synthesis of just one oligonucleotide in existing strategies. The general idea is the following: One uses noninterchangeable polymeric entities from each of which enough OD units can be isolated after completion of the syntheses. Whenever growing chains on different entities have to be elongated with the same building block these entities are gathered in the same reaction vessel. After such a common reaction cycle the entities are separated and now combined according to the next common building blocks etc. The practicability of this approach is demonstrated by the synthesis of d(T-A-A-T-A-T-T-A) and d(T-A-G-T-A-C-T-A) on cellulose filter disks following the phosphotriester approach.

INTRODUCTION

Recently the polymer supported syntheses of ribo- and deoxyribo-oligonucleotides have been improved extensively using both the phosphotriester and the phosphite methods. They have been built into semi- and totally automated procedures\(^1,2\). All these improvements have drastically reduced the time needed to assemble an oligonucleotide chain. The rapid synthesis of double helical DNA, more than 500 base pairs long, has become possible. Various materials have been tested as solid supports. Polyacrylamide, polystyrene and silica gel are now most commonly used. All synthetic strategies reported so far use these and a few more materials in a granulated form (beads, fibres etc.) placed into a reaction vessel, preferably a column-type one. Automation may then be achieved by a suitable solvent and reagent delivery system. However, all oligonucleotides are still synthesized sep-
arately, either one after the other or parallel in different reaction vessels. Thus, for the synthesis of n oligonucleotide chains that are m plus a chain starter nucleotide units long, n x m elongation cycles still have to be performed if only monomer building blocks are used, or n x m/2 cycles if dimer building blocks are used. In this communication we wish to report on a different approach which will cut down drastically the number of reaction cycles n x m. It makes use of segmental solid supports. All synthetic manipulations can be performed manually by one person. There is practically no limit concerning the number of chains that can be synthesized simultaneously.

**MATERIALS AND METHODS**

Pyridine and triethylamine were purified by refluxing for 2h with tosylchloride. The distillates were refluxed for 2h with KOH and redistilled. Pyridine was stored over 4 Å molecular sieves.

The 2'-deoxyribonucleosides dA, dC, dG and dT were purchased from P-L Biochemicals and Pharma Waldhof, 4-methoxy-trityl chloride, 2-chlorophenyl phosphodichloridate and 1-(mesitylene-sulphonyl)-3-nitro-1,2,4,-triazole (MSNT) from Cruachan Chemical Co., ZnBr₂ from J.T. Baker.

The other reagents and solvents were of the highest purity commercially available.

All reactions performed in solution were monitored by t.l.c. on E. Merck silica gel 60 F254 or silanized (C2) silica gel 60 F254 pre-coated plates. The substances were detected first by their UV-absorption and then by their characteristic colour after spraying with E. Merck sugar reagent. Product quality was routinely checked by ¹H- and ³¹P-n.m.r. spectroscopy.

General procedures for the preparation of starting compounds

Selective aminoacylation of the 2'-deoxyribonucleosides dA, dC and dG was achieved by the method of transient O-silylation. The yields of 75 mmol scale preparations were consistently higher than 90%.

4-Methoxy-tritylations were carried out on a 25 mmolar scale, using 1.1 eq. of the chloride. After work up the 5'-O-tritylated compounds were separated from their by-products by short
column chromatography over E. Merck silica gel 60 H using 1 to 3 % (v:v) methanol in chloroform as eluent. The yields ranged from 60 to 80%. 3'-O-Succinylations of 5'-O-(4-methoxytrityl)-N-acyl-2'-deoxyribonucleosides were performed as published by Chow et al.6 with yields of 85 - 95%.

General procedure for the preparation of nucleoside-3'-O-(2-chlorophenyl)-phosphates

This protocol is a variation of a previously published procedure7. To a magnetically stirred solution of 2-chlorophenyl phosphodichloridate (9.29g, 38 mmol) in pyridine (50 ml) was added 1,2,4-triazole (7.87 g, 114 mmol), followed, after 5 min, by 20 mmol of the 5'-O-(4-methoxy-trityl)-N-acyl-2'-deoxyribonucleoside and more pyridine (20 ml). After 45 min the reaction mixture was carefully hydrolyzed by addition of 100 ml 1 M triethylammonium bicarbonate buffer of pH 7.5 (TEAB) and the product extracted into 600 ml of chloroform. The organic layer was washed three times with 0.1 M TEAB, dried over Na$_2$SO$_4$ and concentrated under reduced pressure. Pyridine was removed from the resulting gum by coevaporating twice with 100 ml of chloroform: toluene (7:3, v:v). The product was dissolved in chloroform (150 ml), precipitated into petroleum ether (b.p. 60-80°C, 1600 ml), collected by filtration, and dried overnight in a desiccator. The yields were consistently higher than 90%. In case of inhomogeneity, the phosphates were purified by short column chromatography over E. Merck silica gel 60 H, with 1% triethylamine and 2-4% methanol in chloroform (v:v) as eluent.

Loading of the cellulose support

1 g of cellulose paper disks (Whatman 3MM, Ø=2 cm) is treated with 2 mmol of a protected deoxynucleoside-3'-succinate and 10 mmol MSNT in 5 ml dry pyridine overnight at room temperature. The cellulose disks are separated from the dark supernatant by filtration and washed extensively with pyridine. Remaining free hydroxyl groups on the cellulose are blocked by reaction with 2 ml of acetic acid anhydride in 8 ml pyridine for 1h at room temperature. The disks are washed with pyridine, tetrahydrofurane (THF), CH$_2$Cl$_2$ and diethylether and dried. Detritylation is performed as described below (steps 12 to 18). Yields are determined by measurement of the trityl absorption
General procedure for the assembly of oligonucleotide chains

Condensation reactions are performed in glass vials (20ml) stoppered with rubber septums. For drying of the support a syringe needle is pierced through the septum and the vial is placed into a desiccator under the vacuum of an oil pump. The phosphate components are dried by three times coevaporation with dry pyridine and the flask is stoppered with a septum. MSNT is weighed out in a septum stoppered vial. Reagents and solvent are combined with the aid of a syringe fitted with a platinum needle. During condensation the vials are gently shaken. For capping, detritylation and washings a glass filter funnel (Ø = 2 cm) with a volume of 25 ml is used. This 'capping vessel' is equipped with a teflon stoppered outlet and a ground joint stopper on top.

The manipulations of a complete elongation cycle are listed below. Volumes and amounts are given for 1g support (20 µmol starting nucleoside) per reaction. These values can be reduced down to 0.2 µmol/reaction. The cellulose disks are marked with a pencil.

1) Distribution of the disks into the reaction vials
2) Drying
3) Reaction with 10 equivalents of phosphate component and 30 equivalents of MSNT in dry pyridine for 60 to 90 min at room temperature. The solution should be 0.1 M with respect to the phosphate component
4) Transfer to capping vessel
5) Pyridine-wash (3 times 25 ml)
6) Reaction with 10 ml of acetic acid anhydride in pyridine (1:4, v:v) for 20 min at room temperature
7) Pyridine-wash (4 times 20 ml)
8) THF-wash (4 times 20 ml)
9) CH₂Cl₂-wash (2 times 20 ml)
10) Diethylether-wash (2 times 20 ml)
11) Short drying of disks and vessel in a desiccator
12) Reaction with 20 ml of 0.4 M ZnBr₂ in nitromethane (1% water) for 10 min
13) Wash with the same ZnBr₂-solution until the eluent is colourless
14) THF wash (2 times 20 ml)
15) Quench with 20 ml of THF/collidine/n-butanol (5:1:4, v:v:v) for 5 min
16) THF-wash (5 times 20 ml)
17) Diethylether-wash (2 times 20 ml)
18) Short drying in a desiccator
19) Go to 1

The supernatants from step 3 are hydrolyzed with a few drops of water and stored at 4°C for recovery of excess phosphate components.

Isolation of products

The final elongation cycle is performed only up to step 5 and terminated by a short wash with diethylether. The dry cellulose disks are placed separately into small flasks each containing 5 ml of conc. aqueous ammonia/pyridine (9:1, v:v) and kept at room temperature for three days. The supernatants are collected by filtration and the disks washed two times each with 5 ml of ethanol/0.1 M TEAB (1:1, v:v). The combined filtrates are evaporated to dryness and the residues dissolved in 10 ml 50 mM TEAB. These solutions are extracted two times with ether.

In the case of the two octanucleotides described here, 2ml of each of the two solutions were evaporated again to dryness and the residues treated with 2 ml of 80% acetic acid for 30 min at room temperature. The acid was removed by 2 times coevaporation with 10ml of ethanol. The residues were dissolved in 10 ml 50 mM TEAB, followed by 2 times extraction with ether. The aqueous layers were evaporated to dryness and dissolved in 100 μl of 10 mM Tris·HCl (pH 8.0). 25 μl aliquots of each of the two solutions were subjected to preparative electrophoresis on a 1 mm thick denaturing 20% polyacrylamide-gel. The bands were visualized under UV-light, sliced out and eluted with excess of water overnight. The products were desalted by passing through a Sephadex-G15 column (0.9x30 cm) and stored in 10 mM Tris·HCl (pH 8.0) at -20°C.

Characterization of products

HPLC analyses were performed on a Nucleosil-7C₁₈ (Macherey-
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Nagel) column (0.4x25 cm) using a pump module from Dupont Instruments. The flow rate was 2 ml/min. A linear gradient of CH$_3$CN in 0.1 M triethyl ammonium acetate (pH 7.0) was applied to elute the oligonucleotides.

$^{32}$P-labelling of oligonucleotides and polyacrylamide-gel electrophoresis was performed as described$^8$). 'Wandering spot' sequence analyses followed a protocol given in Ref.9. FAB-MS analyses were carried out as described$^{10}$).

RESULTS

In our attempts to increase the number of oligonucleotides that can be synthesized simultaneously we followed the basic idea: design of segmental solid supports. Such segments should meet the following criteria:

- mechanical stability throughout all manipulations involved in a complete synthetic pathway,
- chemical inertness of matrix after loading with first nucleoside,
- efficient flow-through of solvents and reagents,
- can be marked easily.

Paper-like flat material was expected to be the shape of choice. We started our investigations using glass fibre filters, because we believed this material to be compatible with the phosphotriester as well as the phosphite method. For functionalisation of the supports and loading of the first nucleoside we applied established procedures$^6$). However, the specific surface provided by the glass fibres was too small resulting in rather poor loadings (5µmol/g). We then tried cellulose paper. Successful syntheses on cellulose have been described using the phosphotriester method$^{11}$). We coupled protected nucleoside-3'-succinates to paper disks by condensation of their carboxylic functions with the hydroxyl groups of the cellulose in the presence of MSNT. This procedure consistently yields loadings of 30 µmol/g on the average. More chemical details of the oligonucleotide synthesis on this support are shown in Figure 1, and a complete elongation cycle is described under methods. For the simultaneous synthesis of two octanucleotides, d(T-A-A-T-A-T-T-A) (chain A) and d(T-A-G-T-A-C-T-A) (chain B), two paper disks
each loaded with 2.3 μmoles dA were labelled with a pencil. This marking proved stable throughout the whole synthesis. Assembly of the chains was performed by monomer additions. The disks were treated together during cycles 1, 3, 4, 6 and 7 but were treated separately for elongation during cycles 2 (C or T) and 5 (G or A). After the last cycle N-acyl and phosphate protecting groups as well as carrier were cleaved from the products by concentrated ammonia (10% pyridine). Subsequent detritylations with 80% acetic

acid were done without intermediate purification. The resulting crude mixtures were extracted with ether and then subjected directly to RP-C18 HPLC analysis\textsuperscript{12}). The profiles are shown in Figure 2.

Samples of the crude mixtures were also \textsuperscript{32}P-labelled and subjected to gel-electrophoresis. The resulting autoradiogram is shown in Figure 4A. The different band patterns of the truncated chains indicated a successful simultaneous synthesis of two different sequences\textsuperscript{8}). Aliquots of the crude mixtures were then purified by preparative electrophoresis on a 1 mm thick 20\% denaturing polyacrylamide-gel. 1.14 O.D.\textsubscript{260} (chain A) and 0.97 O.D.\textsubscript{260} (chain B) were recovered from the gel. This corresponded to an overall yield of 11.3 and 10.4\% respectively. Purity and correct sequences of the two octanucleotides were proven by HPLC (Figure 3), gel-electrophoresis after \textsuperscript{32}P-labelling (Figure 4B), and 2D-fingerprinting (Fig. 5). The correct sequences have further been revealed by fast atom bombardment mass spectrometry, a recently published method for ultrafast sequence determination\textsuperscript{10}).

DISCUSSION

Synthetic DNA plays an important role in many fields of modern bioscience and biotechnology. Scientific and economic prospects of genetic engineering, in particular, have challenged many groups to work on synthetic DNA to such a degree that even specialized boutiques for the commercialisation of such products have been founded. In general, all efforts to improve the metho-

dology aimed at supplying the users of synthetic DNA with increasing reliability and, in shorter times, with enough (sometimes just enough) of purer material. These efforts include:
- simplified procedures for the preparation of suitably protected building blocks,
- faster and cleaner coupling procedures for chain elongation,
- solid supports that are compatible with all necessary chemical reactions and allow automation,
- introduction of partially automated procedures,
- highly selective reactions for removal of protecting groups,
- efficient separation techniques for intermediates and target
Figure 5. 'Wandering spot' sequence analyses (1. high voltage electrophoresis at pH 3.5; 2. homochromatography). For comparison the corresponding theoretical computer graphs are given. These were determined using the rules of Tu et al.. A) d(T-A-A-T-A-T-T-A); B) d(T-A-G-T-A-C-T-A).

- rapid methods for unambiguous product characterization, particularly sequence analysis.

Since in some publications the cycle times for chain elongation seem to approach zero minutes, further progress is commonly expected from better and cheaper "DNA making machines". Contrary to these expectations we report, for the first time, on a completely different approach. Large numbers of oligonucleotides can now be synthesized simultaneously without any
machine aid and very much faster than to date. The basic idea is more concerned about intelligent arrangement of polymer support rather than about another new chemistry.

The syntheses are performed on unambiguously marked segments, one for every oligonucleotide to be synthesized. For the assembly of n oligonucleotides at least n segments must be used, each loaded with the appropriate starting block according to the desired sequences. All segments that have to react with the same nucleotide block will form one group of segments. All members of one group will be treated together in the same reaction vessel. In every elongation cycle there is a maximum of four different groups if monomer building blocks were used, a maximum of 16 groups if dimer building blocks were used etc. After a complete elongation cycle all segments will be grouped again according to their different pre-defined sequences and the next elongation cycle could be performed. Consequently, the synthesis of $n$ oligonucleotides, that are $m$ plus a chain starter nucleotide units long, would never require more than $4 \times m$ monomer additions or $16 \times m/2$ dimer additions etc. In our example, the simultaneous synthesis of two 8mers, we only had to run 9 instead of 14 cycles. It is obvious, that the time-saving becomes more impressive the higher the number of oligomers to be synthesized. A simultaneous synthesis of e.g. sixty 15mers (for a total synthesis of a gene) would never require more than $4 \times 14 = 56$ elongation cycles, compared to $60 \times 14 = 840$ cycles following the traditional route. Moreover, further time-saving will be possible by combining all segments in a simultaneous synthesis except for the coupling reactions and first washings.

We predict that simultaneous synthesis of oligonucleotides via segmental solid supports will prove useful, whatever their need. Mixed probes/primers synthesized with this method can certainly be worked up and used batchwise as before - with the additional option of isolating all of them separately. This would also allow using them separately for priming and probing or in all kinds of groups. For example, one group might be formed by A-T rich, the other by G-C rich oligomers, a third group might be formed by those oligomers which contain the most probable codons. Isolation of every single probe/primer will not
only increase the probability that these oligomers will be re-used one day but also allow a carefully adjusted stoichiometry of the different oligonucleotides in the mixture - sometimes a source of uncertainty or of bad results in traditional syntheses of mixed probes/primers\textsuperscript{14}.

The new approach for the chemical synthesis of oligonucleotides also encourages a more systematical investigation of structure-function relations of DNA by site specific mutagenesis.

The striking advantages of segmental solid supports for the total synthesis of long DNA duplexes have already been mentioned above. More than 90% of the reaction cycles will now be avoided if a DNA double strand is synthesized which is about of the size of an interferon gene. One might ask whether one can really handle about 60 segments in a simultaneous synthesis. The use of monomer building blocks results in the formation of 4 elongation groups, each consisting of 60:4=15 segments on the average. In our initial experiments we found that 30 paper disks in one group can be handled without difficulties. Hence, the assembly of all oligonucleotide chains for a large gene will now be possible in less than two weeks.

The usefulness of the new approach is not necessarily connected to special applications such as probes/primers, site specific mutagenesis or synthesis of genes, but will also be shown whenever a certain general request for oligonucleotides exists. This invites the question: How might other strategies be judged? We believe, there is currently still a niche for solution chemistry if one aims to produce several hundreds or even thousands of optical density units of one oligonucleotide. However, all so-called "gene machines" we have come to know, should now be regarded to be obsolete. It is a paradox: in future, the "gene machines" that are currently on the market will only be used logically if oligonucleotides will be needed now and then and in low numbers. In such cases, however, one will certainly prefer to use low cost manually operated devices\textsuperscript{15} rather than machines that are still very expensive and sometimes not even reliable.

Future developments will include alternative solid supports, increase of loadings per gram, test of the phosphite method in
this approach and syntheses of larger quantities (O.D. units) of oligonucleotides. Experiments are on the way.

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