New effective method for the synthesis of oligonucleotides via phosphotriester intermediates

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ABSTRACT

A rapid and convenient method for the synthesis of deoxyribonucleotides has been developed using the phosphotriester approach. The advantage of this methodology for work in solution was successfully demonstrated in synthesis of a number of DNA fragments up to 32-long. Adaptation of the presented method to solid-phase synthesis allows a pentadecamer to be assembled in 4-5 hours using dinucleotides as coupling units.

INTRODUCTION

The current phosphotriester approach to oligonucleotide synthesis has been shown to be successful both in solution and on polymer supports, and its effectiveness was proved in the chemical synthesis of various biologically active DNA fragments\(^1\)\(^-\)\(^3\). Despite these achievements, there remain some unsolved problems in this approach. One of them is a relatively low rate of internucleotide condensations, especially as compared with the phosphite methodology\(^4\),\(^5\). In the hope of overcoming this problem, we have undertaken a search for new condensing agents for phosphotriester bond formation which would be more powerful than the now commonly used 1,2,4-triazole-, tetrazole-\(^6\) and 3-nitro-1,2,4-triazole-based\(^7\) reagents. As a result of these studies, a convenient phosphotriester method which enables to increase substantially the rate of the synthesis in solution and on solid-phase and to simplify the procedure has been developed. The basic features of our method are the use of highly effective coupling reagents, arylsulfonyl chlorides in the presence of N-methylimidazole\(^8\), for phosphotriester bond formation and the performance of internucleotide condensations in different organic solvents, such as acetonitrile, methylene chloride, pyridine,
dioxane, etc. It also includes: (i) a simple procedure for the preparation of protected mononucleotides; (ii) reversed phase column chromatography in solution variant of the synthesis for better purification of fully protected oligomers starting from 8-mers; and (iii) the one-solvent procedure for rapid solid-phase oligonucleotide synthesis. In the present paper, we report this modified triester method and its application to the efficient synthesis, in solution and on polymer carriers, of several deoxyribopolynucleotides of 15-32 nucleotides in length.

RESULTS AND DISCUSSION
Preparation of Protected Monomers

In the modern triester approach to the synthesis of deoxyribopolynucleotides the essential monomeric units are the N-protected 5'-dimethoxytrityl-2'-deoxynucleoside 3'-O-chlorophenyl phosphates (VII) and deoxynucleoside-3'-O-chlorophenyl-α-cyanoethyl phosphates (VIII). Usually for preparing triethylammonium salts of nucleoside phosphodiesters (VII) we utilized a method, similar to that suggested by G.R.Gough et al., in which an excess of bis(triazolyl)-p-chlorophenyl phosphate was used for the introduction of the p-chlorophenyl phosphate residue at the nucleoside 3'-OH group. Now we have found that this bifunctional phosphorylating reagent can be successfully replaced by highly reactive monofunctional phosphorylating reagent, the chlorophenyl phosphorylpyridinium derivative (IV), being prepared in situ by the addition of equimolar amount of water to p-chlorophenyl phosphodichloridate (III) in pyridine (Scheme 1).

It is known that the active phosphorylating compound (IV) is formed under the action of arylsulfonyl chlorides on phosphomonoesters (I) in pyridine or under the action of pyridine on arylmethylchlorophosphates (II)\(^{11,12}\). Recently we have revealed that the addition of equimolar amount of water to aryldichlorophosphate solution in pyridine also brings to formation of the activated phosphomonoester (IV). The identity of forming derivative with compound obtained by the two above-mentioned methods was confirmed by \(^{31}\)P NMR spectroscopy. The \(^{31}\)P NMR spectra of the compounds obtained in all three cases were identical and represents a singlet with \(S_p = 10.8 \text{ ppm}\). When 5'-dimethoxytrityl
thymidine (0.5 equiv.) is added to 1 equiv. of (IV) obtained according to the latter procedure, the signal vanishes and a new signal appears characteristic of a three-substituted pyrophosphate (IV) (a multiplet with a centre at $\delta_p$ 17.6 ppm).
Subsequent addition of water to the reaction mixture leads to a rapid decomposition of (VI) to form p-chlorophenyl phosphate ($\delta_p 4.1$ ppm), a small amount of symmetric p-chlorophenyl pyrophosphate ($\delta_p 16.2$ ppm) and 3'-p-chlorophenyl phosphate of 5'-dimethoxytritylthymidine (VII) ($\delta_p 6.5$ ppm). Thus in order to provide a quantitative nucleoside 3'-O-phosphorylation with (IV) it is necessary to take no less than 2 equiv. of the phosphorylating reagent per 1 equiv. of 5'-dimethoxytrityl nucleoside.

This procedure for preparation of the phosphodiesters (VII), the simplest one if compared with the methods described earlier, was employed by us in the synthesis of protected nucleotides. Large scale phosphorylation of 5'-dimethoxytrityl nucleosides was carried out using 4-5-fold excess of phosphorylating reagent (IV) within 20-30 min. The reaction was terminated by the adding an aqueous solution of triethylammonium bicarbonate, and the nucleoside phosphodiester (VII) was separated from the excess of arylphosphate by chloroform extraction. According to this procedure p-chlorophenyl esters of all the four 5'-dimethoxytrityl nucleoside 3'-phosphates were obtained on 10-20 mmole scale with the yields of 90-95%. No removal of the dimethoxytrityl group from the protected nucleosides was observed under these conditions. No side reactions involving the guanine moiety or other bases could be also detected. With the aid of proposed method the experimental procedure becomes easier, and the yields of the nucleoside phosphodiesters (VII) are not inferior to those obtained with bis(triazolyl)-p-chlorophenyl phosphate.

It should be noted that arylmethylchlorophosphates in pyridine are also remarkable phosphorylating reagents for preparation of compound (VII). However, due to certain difficulties in large scale preparation of pure (II), this source of activated derivative (IV) is less available than compound (III).

The fully blocked nucleoside-3'-O-phosphates were rapidly obtained from corresponding 5'-dimethoxytritylnucleoside 3'-O-chlorophenyl phosphates (VII) under the action of excess β-cyanoethanol in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCI) and N-methylimidazole (Melm) as was described by us previously. In dry pyridine, or acetonitrile, the reac-
tion was complete in 5 min. Phosphotriesters obtained were de-
tritylated without any isolation under the action of benzenesul-
fonic acid in chloroform-methanol solution, and the resulting
monomeric 5'-hydroxyl components (VIII) were isolated by a sil-
ica gel column chromatography (see Scheme 1).

Internucleotide Coupling Reactions

Recently, we have shown that arylsulfonyl chlorides are high-
ly efficient condensing reagents for the formation of phospho-
triester internucleotide linkage in the presence of a nucleophi-
lic catalyst - N-methylimidazole$^{8,14}$. Addition of the latter to
the reaction mixture containing a 3'-phosphodiester component
(VII), 5'-hydroxyl component (VIII) and TPSC1 in pyridine dras-
tically accelerates the rate of internucleotide condensation.
The reaction goes to a completion in 10-20 min, and oligonucleo-
tide yields vary from 50 to 95% depending on their length and
composition. At the same time in the absence of a nucleophilic
catalyst the oligonucleotide yields with the use of TPSC1 rarely
exceed 25-50% in 20 hours$^{15}$. The latter presumably results from
the insufficient reactivity of the activated derivative (IX)
which is formed through the interaction of (VII) with arylsulfo-
nyl chloride in pyridine and represents a tetrasubstituted pyro-
phosphate$^{16}$(Scheme 2). The acceleration of the condensation re-

\[
\begin{align*}
RO-P-OH & \quad \text{OR'} \quad \text{(VII)} \\
\downarrow \text{ArSO}_2\text{Cl} & \quad \downarrow \text{(IX)} \\
\text{RO-P-OSO}_2\text{Ar} & \quad \text{RO-P-OR'} \\
\text{OR'} & \quad \text{RO-P-N(+)CH}_3 \quad \text{X^-} \\
R & = -\text{C}_6\text{H}_4\text{Cl} \\
R', R'' & = \text{protected nucleoside} \\
\text{ArSO}_2\text{Cl} & = \text{TPSC1, or MSOCl} \\
X & = \text{Cl}^-, \text{ArSO}_2\text{O}^- \text{ or } \text{R'}\text{OR}(0)\text{PO}^- \text{.}
\end{align*}
\]

\[\text{SCHEME 2}\]
action under the action of MeIm may be accounted for the intermediate formation of the methylimidazolium cation (X) possessing a high phosphorylating capacity.

K-Methylimidazole, unlike such nucleophilic catalysts as tetrazole and 3-nitro-1,2,4-triazole, along with nucleophilic catalysis can also provide the general basic catalysis and serve as an acceptor of arylsulfonic acid and HCl which are released in the course of the reaction. This enables to carry out the condensation reactions in the presence of MeIm not only in pyridine but also in some neutral polar solvents without the addition of other bases. In our experiments chloroform, methylene chloride, dioxane, tetrahydrofurane (THF), nitromethane and acetonitrile were tested in coupling reactions under the action of arylsulfonyl chlorides and MeIm. The results on comparison of the reaction rates in the synthesis of [(MeO)\textsubscript{2}Tr] T-T-T(Bz)\textsuperscript{17} in these solvents are shown in Fig. 1. As it follows from these data, the highest rate of phosphotriester bond formation is provided by acetonitrile and nitromethane. The condensations in these polar ap-

![FIGURE 1. Comparison of the coupling rates in the synthesis of [(MeO)\textsubscript{2}Tr] T-T-T(Bz) in pyridine(○), acetonitrile(■), nitromethane(□), THF(Δ), methylene chloride(▲), chloroform (●) and dioxane (×). The coupling reactions were carried out using 0.125 M concentration of [(MeO)\textsubscript{2}Tr] T-(ClPh) and 0.1 M concentration of T-T(Bz) in the presence of 0.25 M arylsulfonyl chloride and 0.5 M nucleophilic catalyst: MeIm (solid lines), 3-nitro-1,2,4-triazole (dashed line) and tetrazole (dotted line). The aliquots were taken at various times, and the yields were estimated by HPLC analysis of the reaction products.](image-url)
rotic solvents in the presence of TPSC1 and MeIm are complete within 5-8 min. In the other, less polar solvents the reaction proceeds slower, but in all cases it goes to a completion in 15-20 min. As it could be expected, the coupling rates in the presence of mesitylenesulfonyl chloride (MSC1) are 2-3-times higher than those with the use of TPSC1. In the same conditions the condensation reactions under the action of TPSC1 + tetrazole or MSC1 + nitrotriazole in pyridine were complete only in 40 and 30 min, respectively.

It should be noted that with the use of arylsulfonyl chlorides and MeIm a minimum of side-products, 5'-0-sulfonated compounds and modifications of heterocyclic bases, was detected. During the condensation reactions in such solvents as acetonitrile, dioxane or CH$_2$Cl$_2$, the reaction mixture remained clear and lacked of darkly colored products usually observed with the use of pyridine. Moreover, the lower viscosity of oligonucleotide solutions in CH$_2$Cl$_2$ and CHCl$_3$ than in pyridine enables to perform the condensation reactions at higher concentration of the reagents, and thus to increase the reaction rate. This is of practical value for solid-phase synthesis.

The utilization of arylsulfonyl chlorides in the presence of 4-N,N-dimethylaminopyridine (DMAP) brings to similar results. However, according to our experiments which are in agreement with the data from, this nucleophilic catalyst can not be used for the preparation of oligomers with a 3'-terminal phosphate blocked by the β-cyanoethyl group, as this group is partly eliminated (up to 40% within 1 h) in the presence of DMAP. Furthermore, the extent of side-reaction of 5'-hydroxyl component sulfonation was higher in the case of DMAP (10% in hour) than in the case of MeIm (2% in hour).

Synthesis of Oligonucleotides in Solution

The feasibility of this methodology for the synthesizing of deoxyribopolynucleotides in solution was proved in synthesis of a number of DNA fragments ranging from 10- to 32-mers. The overall yields of the 16-mer d(GAGTCTTCAGCCCTTG), 24-mer d(GGGTTCCTCGAGCCAGGGTGCGG) and 32-mer d(GCAGTCAGGAGGTTCCCGAGTGGCAT) are given in Table 1. Full details of the use of these polynucleotides representing the fragments of a synthetic gene for
TABLE 1. Overall yields of oligonucleotides

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Coupling yield, %</th>
<th>Isolated yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(GAGTCTTCAGCCCTTG)</td>
<td>35</td>
<td>12</td>
</tr>
<tr>
<td>d(GGGTTCCCCTGCAAGAGGGTGCGCAT)</td>
<td>20.5</td>
<td>6</td>
</tr>
<tr>
<td>d(GCACTGGAGGGTTCCCTCAAGACGGTGGCAT)</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>d(TGCAAGACTGGCAT)</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>d(AGGCAGGACAAAACCAT)</td>
<td>37</td>
<td>9</td>
</tr>
</tbody>
</table>

a The synthesis was carried out in solution starting from 50-100 μmole of dinucleotides. Yields are based on 3'-terminal dinucleoside monophosphate.

b The synthesis was carried out on solid-phase. Yields are based on the first 5'-dimethoxytritylnucleoside attached to resin.

c After HPLC on Zorbax C-8 column.

human proinsulin²⁰, and of other oligomers presented in this paper will be published separately.

The main building units for construction of polynucleotides were dimers. Polynucleotide chains were elongated in the 3'-5' direction by a successive addition of di-, tetra- and octanucleotide blocks to 3'-terminal dinucleoside monophosphate. Usually, 1.2-1.5-fold molar excess of 3'-P-component over 5'-OH component and 2-fold excess of TPSC1 (MSC1) and 4-fold excess of Melm relative to P-component were used in condensation reactions. Acetonitrile and nitromethane were selected as solvents in preparation of short oligonucleotides. The coupling reactions in these cases were carried out for 5-10 min and terminated by the addition of water. In view of poor solubility of octa- and longer oligonucleotides in pure acetonitrile and nitromethane, a mixture of acetonitrile with CH₂Cl₂ or CHCl₃ (up to 50%) was utilized in preparation of higher oligomers, and the coupling time was about 10-20 min. As we have shown earlier⁸,¹⁴, pyridine and dioxide are also good solvents for preparation of oligonucleotides with the aid of arylsulfonyl chlorides and Melm, and the coupling reactions in these solvents are complete within 20 min.

The desired oligonucleotides at initial stages of the synthesis were isolated by a silica gel column chromatography using a methanol concentration gradient in chloroform as an eluent.
ever, when a chain length of the oligonucleotide is increased, the resolution of this method tends to lower. It is already difficult to separate quantitatively the fully protected octanucleotide from the unreacted starting 5'-OH component and the reaction by-products by a conventional silica gel chromatography. This diminishes the yields at subsequent steps of the synthesis and complicates the purification of the final compound. Earlier it was shown that TLC on RP-2 and RP-18 plates in systems containing aqueous acetone and ethylacetate provides good separation of the reaction components. We have found that the effective purification of the fully protected oligonucleotides can be also achieved by a reversed phase column chromatography on Nucleosil 30 C18 under the conditions similar to those described by H.-J. Fritz et al. The isolation of oligonucleotides starting from 8-mers was carried out in a linear gradient of acetonitrile in 0.1 M triethylammonium acetate, or in the same system containing 25% dioxane. In all cases the last peak contained the essentially pure desired product. The elution patterns in the purification of the fully protected hexadecanucleotide d(GAGTCTTC-AGCCCTTG) and the intermediate octanucleotide d(AGCCCTTG) are shown in Fig. 2.

The removal of the protecting groups from the final oligonucleotides after the completion of the synthesis was carried out in three separate steps by the treatment with: (i) a mixture of dioxane and aqueous ammonia; (ii) concentrated ammonia; and (iii) 80% aqueous acetic acid. The deprotected oligonucleotides were isolated by anion exchange HPLC (Fig. 2) or by preparative electrophoresis on 20% polyacrylamide gel slabs. The purity of each oligomer was checked by a reversed phase HPLC on Zorbax C-8 column in methanol concentration gradient in 0.1 M ammonium acetate. After the introduction of a 5'-32P-label, the primary structure of the synthesized DNA fragments was confirmed by the standard sequence analysis procedures. The patterns obtained for the 16-, 24- and 32-mers are shown in Figures 2 and 3. They clearly demonstrate that the oligonucleotides obtained by the proposed procedure are essentially pure, and thus, these substances can be directly used in the biochemical reactions without any additional purification right after the anion-ex-
FIGURE 2. Chromatography of the reaction products formed in the synthesis of d(AGCCTTGTG)(A) and d(GAGTTCAGCCCTTG) (B) on silica gel (1,3) and Nucleosil 30 C-18(2,4) columns. The fractions were pooled as shown by vertical dashed lines. (C) - Ion-exchange HPLC analysis of the 16-mer isolated from (B3)(solid line) and from (B4)(dashed line) after deprotection. Inset. - Zorbax C-8 profile of the purified 16-mer. (D) - Two-dimensional sequence analysis of the hexadecanucleotide.

change chromatography (or after the gel electrophoresis). Due to the minimal contamination of these oligonucleotides with by-products caused by the modification of guanine and other bases, the treatment with aldoximate reagents is not necessary.

Synthesis of Oligonucleotides on Polymer Supports

In order to investigate the effectiveness of our methodology for solid-phase synthesis a number of oligonucleotides have been synthesized using two types of supports - polystyrene (2% cross-linked by divinylbenzene) and pore glass, which recently were shown to be useful with phosphotriester route25,26. The
FIGURE 3. The HPLC analysis on Zorbax C-8 column after isolation by preparative electrophoresis on polyacrylamide gel (A) and the Maxam-Gilbert sequencing (B) of the synthetic 24-mer (I) and 32-mer (II).

procedures for derivatization of supports and the basic strategy of the synthesis were similar to those described earlier (Scheme 3). The growing oligonucleotide chain was elongated from the 3'-to 5'-end by a successive addition of the appropriately protected mono- and dinucleotides containing p-chlorophenyl-protected 3'-phosphates. In the synthesis of oligomers 0.1 M concentration of nucleotide component (3-4-fold excess over the resin capacity), 1.5-2 equivalents of arylsulfonyl chloride and 3-4 equivalents of MelM with respect to a nucleotide component were used in the coupling reactions. Different organic solvents, such as pyridine, CH₂Cl₂, CHCl₃ and acetonitrile, were utilized for performance of internucleotide condensations on nonswellable
glass matrix. The same solvents, except acetonitrile, were employed in conjunction with polystyrene support. Acetonitrile is unsuitable with polystyrene carrier due to its poor swelling properties in this polar solvent. The reactions on the polystyrene support were carried out in a vessel similar to that described by H. Ito et al. 

It has been found that the use of TPSCl + MeIm provides 80-95% yields in the coupling reactions on polystyrene support within 30 min, whereas in the presence of MSCl + MeIm the coupling reactions were complete in 20 min. It should be noted that the rate of condensation reactions on the glass support was slightly higher than that on the solvent-swollen carrier. The time required for a nucleotide unit attachment to a support-bound oligonucleotide with the use of the glass matrix is reduced to 20 and 10 min in the case of TPSCl + MeIm and MSCl + MeIm, respectively. It is almost 3-5 times faster than with the use of mesitylenesulfonyl 3-nitro-1,2,4-triazolide (MSNT) 

We have also examined the rate and the yields in condensation reactions in the presence of TPSCl and DMAP. In our hands, we have observed diminished yields (65-75%) with the use of this nucleophilic catalyst. This is probably due to the involvement of the 5'-hydroxyl of a nucleoside component on the polymer support in the side reaction of sulfonation which extent is higher.
FIGURE 4. Comparison of the coupling rates on polystyrene (Ps) and glass (G) resins. The thymidine supports (200 mg) were reacted in 0.6 ml of pyridine(-○-), methylene chloride (-△-) or acetonitrile(-●-) with 0.1 M [(MeO)$_2$Tr]T-(ClPh) in the presence of 0.2 M arylsulfonyl chloride (TPSCl-solide line or MSCL-dashed line) and 0.4 M MeIm, or in the presence of MSNT (dotted line). The aliquots were taken, and the yields were estimated from dimethoxytrityl release.

in the presence of arylsulfonyl chlorides and DMAP than in the case of arylsulfonyl chlorides and MeIm$^{14}$.

In order to reduce a number of manipulations during the oligonucleotide assembly, we have also investigated the possibility of performing all the reactions on solid-phase in the same solvent. Therefore, we have reexamined the other reactions in a synthetic cycle. It was found that the unreacted 5'-hydroxyl groups on a polymer can be rapidly masked by the treatment with 10% solution of acetic anhydride in different solvents (THF, CH$_3$CN, CHCl$_3$, CH$_2$Cl$_2$, dioxane) in the presence of MeIm. Under these conditions, the acetylation reaction on solid-phase was over within 3-5 min. It was also revealed that the removal of 5'-dimethoxytrityl group can be rapidly accomplished by the reaction with 2% trifluoroacetic acid (TFA) in acetonitrile (or its mixture with CH$_2$Cl$_2$, 7:3 v/v) at room temperature. 5-7 min treatment was sufficient to complete the reaction, and the control experiments in solution have shown that the detritylation of the fully protected derivatives of deoxyadenosine under the conditions described above does not cause any detectable depurination within 10 min. Moreover, good results were obtained with the use of 1% TFA in CH$_2$Cl$_2$ (or CHCl$_3$) containing 25% acetonitrile.

During these studies we have found that methylene chloride (or chloroform) is a convenient solvent for operating with both types of carriers, whereas acetonitrile (or its mixture with CH$_2$Cl$_2$) can be successfully used in conjunction with the non-
swellable glass support. The addition of methylene chloride to acetonitrile increases the solvation of the oligomer attached to the resin. Table 2 lists the operations per one cycle of an oligonucleotide unit addition to the resins. As outlined in the table, it takes about 45 min to complete one cycle of the synthesis with the use of polystyrene support. Whereas in the synthesis on glass support in a flow system, the overall time for the addition of each block to a growing chain is about 35 min\(^3\). The synthetic cycle was repeated with the appropriate blocks until the required sequence was obtained, and the oligonucleotides were cleaved from the supports, deprotected and purified by the preparative electrophoresis on polyacrylamide gel slabs. In most cases the reversed phase HPLC on Zorbax C-8 revealed

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent or solvent</th>
<th>Time(min)</th>
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<tbody>
<tr>
<td><strong>Polystyrene support</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1% TFA in CH(_2)Cl(_2)-CH(_3)CN (15:5 v/v, 3ml)</td>
<td>2 x 3</td>
</tr>
<tr>
<td>2</td>
<td>CH(_2)Cl(_2) (5 ml)</td>
<td>2 x 0.5</td>
</tr>
<tr>
<td>3</td>
<td>coupling mixture in CH(_2)Cl(_2)(^a)</td>
<td>20 or 30(^b)</td>
</tr>
<tr>
<td>4</td>
<td>CH(_2)Cl(_2) (5 ml)</td>
<td>2 x 0.5</td>
</tr>
<tr>
<td>5</td>
<td>CH(_2)Cl(_2)-Ac(_2)O-Melm (8:1:1 v/v, 1 ml)</td>
<td>3-5</td>
</tr>
<tr>
<td>6</td>
<td>CH(_2)Cl(_2) (5 ml)</td>
<td>2 x 0.5</td>
</tr>
<tr>
<td><strong>Glass support</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2% TFA in CH(_3)CN-CH(_2)Cl(_2) (7:3 v/v)</td>
<td>5-7</td>
</tr>
<tr>
<td>2</td>
<td>CH(_3)CN-CH(_2)Cl(_2) (7:3 v/v)</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>coupling mixture in CH(_3)CN-CH(_2)Cl(_2) (7:3 v/v)(^a)</td>
<td>10 or 20(^b) (recycle)</td>
</tr>
<tr>
<td>4</td>
<td>CH(_3)CN-CH(_2)Cl(_2) (7:3 v/v)</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>CH(_3)CN-Ac(_2)O-Melm (8:1:1, v/v)</td>
<td>3-5 (recycle)</td>
</tr>
<tr>
<td>6</td>
<td>CH(_3)CN-CH(_2)Cl(_2) (7:3 v/v)</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) The nucleotide component (30 \(\mu\)mole) is dried by coevaporation with pyridine or acetonitrile, then a solution of arylsulfonyl chloride (45-60 \(\mu\)mole) and Melm (90-120 \(\mu\)mole) in dry solvent (0.3 ml) is added and the reaction solution is injected into the reaction vessel.

\(^b\) The first reaction time is given for the use of MSCl, the second - for TPSCl.
that the oligonucleotides obtained were essentially homogeneous and thus further purification was unnecessary. The HPLC profiles of hexadecanucleotide d(TGCAGAAGCGTGGCAT) obtained with the use of polystyrene support and pentadecanucleotide d(AGGCAGGACAACCAT) obtained on the glass carrier are shown in Fig. 5. The sequence of the 16-mer represents a part of a synthetic gene for human proinsulin. The 15-mer was designed as a primer for preparation of cDNA for human immune interferon (INF-γ). The overall coupling and isolated yields of these oligonucleotides are outlined in Table 1. After the isolation, the material gave the expected pattern in a mobility shift analysis$^{23}$(Fig. 5).

**FIGURE 5.** The HPLC analysis on Zorbax C-8 column (A) and two-dimensional sequence analysis (B) of the synthetic 16-mer d(TGCAGAAGCGTGGCAT)(I) and 15-mer d(AGGCAGGACAACCAT)(II).
The results obtained indicate that the developed method for solid-phase synthesis is sufficiently faster than those employing a conventional phosphotriester route\textsuperscript{25,27,28} and the yields are comparable. On the other hand, the rate of the synthesis by our method is very close to that with the use of phosphite approach\textsuperscript{4,5}. Moreover, the described one-solvent procedure has great potential for automation of the synthetic process.

**EXPERIMENTAL SECTION**

The following reagents were purchased from commercial sources: deoxyguanosine, deoxyadenosine, deoxycytidine and thymidine (Sigma), 4,4'-dimethoxytritylchloride, 2,4,6-triisopropylbenzenesulfonyl chloride, mesitylenesulfonyl chloride, \( \beta \)-cyanoethanol and benzenesulfonic acid (Aldrich), N-methylimidazole, \( N,N \)-dimethylaminopyridine (Merk), silica gel F\textsubscript{254} TLC plates (Eastman Kodak), silica gel LH 40-100 for column chromatography (Chemapol), acrylamide, bis-acrylamide (Merk).

N-methylimidazole was vacuum distilled and stored over molecular sieves 4Å. TPSC\textsubscript{1} and MSC\textsubscript{1} were recrystallized from hexane and dried in vacuo. Protected nucleosides and pyridinium 5'-O-dimethoxytrityl-2'-deoxynucleoside 3'-O-succinates were prepared by standard procedures\textsuperscript{29,30}. Silica gel plates were developed in chloroform-methanol (19:1 v/v). To deblock cyanoethyl group the fully protected oligonucleotides were treated with 20% solution of triethylamine in acetonitrile (20 ml/mmole) at room temperature for 0.5-1 hour.

The chemical shifts in NMR spectra are given in ppm relative to 85% \( \text{H}_3\text{PO}_4 \) as an external standard. The assignment of the signals of all the compounds was accomplished by comparison with \( ^{31} \text{P} \) NMR spectra of the known samples obtained by other methods.

**General Procedure for Preparation of Protected Monomers**

\( N \)-protected 5'-dimethoxytrityl deoxynucleoside (1 mmole) was coevaporated with anhydrous pyridine leaving a final volume of 5-10 ml. In a separate flask p-chlorophenyl phosphodichloridate (5 mmole) was dissolved in dry pyridine (10 ml), water (5 mmole) was added to this solution under cooling and the mixture was left at room temperature for 10 min. After the removing of pyridinium chloride precipitate by filtration, the clear solu-
tion of the phosphorylating reagent (IV) was mixed with protected deoxynucleoside. The mixture was concentrated to a volume of 10 ml and allowed to react for 30 min. Completeness of the phosphorylation reaction was monitored by TLC on silica gel. The reaction was stopped by the addition of 1 M triethylammonium bicarbonate solution (15 ml) cooled to 0°C. After 10 min chloroform (100 ml) was added and the mixture was washed with 0.1 M triethylammonium bicarbonate solution (2 x 50 ml). The organic phase was evaporated to an oil with pyridine, then co-evaporated with toluene and dried in vacuo. The yields were 90-95%.

The triethylammonium 5'-O-dimethoxytrityl deoxynucleoside-3'-p-chlorophenyl phosphate (5 mmole) and β-cyanoethanol (10 mmole) were evaporated two times with dry pyridine, or acetonitrile, to give a final volume of 50 ml. Then TPSCl (10 mmole) and MeIm (12 mmole) were added. After 5 min at room temperature the silica gel TLC showed complete conversion of (VII). The excess of water was added and the reaction mixture was concentrated to a gum. The residue was dried by coevaporation with toluene and dissolved in pre-cooled 2% solution of benzenesulfonic acid in chloroform-methanol (7:3 v/v, 50 ml). After 2-3 min at 0°C, the solution was diluted with chloroform (100 ml) and washed with 0.1 M NaHCO₃ (2 x 100 ml). The organic phase was evaporated to an oil, the residue was dissolved in chloroform. The product (VIII) was isolated by a short column silica gel chromatography in 80-90% yields.

General Method for Synthesis of Fully Protected Oligonucleotides in Solution

1. For preparation of di- and tetranucleotides the nucleotide component (1.2-1.5 mmole) and the OH-component (1 mmole) were dried by evaporation with acetonitrile. Then a solution of TPSCl (2.5-3.0 mmole) and MeIm (5-6 mmole) in 10 ml of anhydrous acetonitrile (or nitromethane) was added, and the mixture was allowed to react for 5-10 min (control by TLC). Then water was added, and the reaction mixture was evaporated to dryness, dissolved in chloroform and the desired product was isolated by chromatography on a silica gel column (100 g) using a linear gradient of methanol in chloroform (0-10%) as an eluent. The
fractions containing the desired product were pooled and evaporated. The yields were 75-95%.

2. Synthesis of oligonucleotides from 8-mers were carried out similar to that of di- and tetranucleotides. Condensation reactions were performed during 15-20 min. Acetonitrile containing 25-50% CH₂Cl₂ (or CHCl₃) was used as a solvent. After a short column silica gel chromatography (or without this step) oligonucleotide (500-1000 A₂₈₀ units) was applied to a Nucleosil 30 C₁₈ column (1 x 25 cm). Column was eluted at flow rate of 2 ml/min with a gradient of 60-80% acetonitrile in 0.1 M triethylammonium acetate (or 40-70% acetonitrile in 0.1 M triethylammonium acetate containing 20% dioxane). Oligonucleotide containing fractions were combined and evaporated.

Assembly of Oligonucleotide chains on Polymer Supports

The functionalized polystyrene support (0.08 mmole of nucleoside/g) was prepared as published from chloromethyl polystyrene (2% cross-linked by divinylbenzene, 1.0-1.1 mmole Cl/g, Reanal)²⁵. Glass support (0.09 mmole of nucleoside/g) was derivatized from long chain alkylamine controlled pore glass (0.1 mmole amine/g, Pierce) as described²⁶. The amount of nucleoside attached to the polymer was estimated by the quantitative analysis of dimethoxytrityl group and nucleoside liberated from the resin. The various chemical operations performed for the addition of one coupling unit to the polymer supports, the amount of reagents and the reaction times are listed in Table 2. The synthesis of 16-mer d(TGCAGAAGCGTGGCAT) was carried out starting from 100 mg of polystyrene resin by successive addition of mono- and seven dinucleotides. The average coupling yield per cycle was 85%. The 15-mer d(AGGCAGGACAACCAT) was made by seven consecutive additions of dinucleotides to the glass support (100 mg) with average coupling yield 87%. In both cases TPSCI in the presence of Melm was used as coupling agent. The coupling yields were estimated by the spectroscopic analysis of the dimethoxytrityl function liberated from the resins²⁹.

Deprotection and Isolation of Oligonucleotides

In order to remove the protecting groups from the oligonucleotides synthesized in solution, they were treated with a mixture of dioxane and conc. ammonia (1:1 v/v) at room temperature
for 24 h. After the evaporation, the residue was treated with conc. ammonia at 50°C for 5 h, or at room temperature for 3 days. Then ammonia was removed by evaporation and the residue was treated at room temperature with 80% acetic acid for 15 min and evaporated. Deprotection and cleavage of the oligomers from the resins were accomplished similar to the foregoing procedure. The deprotected oligomers were purified by gel electrophoresis\(^9,14\) or by ion-exchange HPLC. In the first case a sample of oligonucleotide (25-50 A\(_{260}\) units) in 500 µl of 7 M urea was applied in a lot (15 cm wide and 1 cm deep) on a 20% (w/v) polyacrylamide denaturing gel slab (20x20x0.3 cm). Electrophoresis was carried out at 600 V for 4-6 hrs. The bands on the gel were visualized under UV light and the desired band was sliced. The oligomer was eluted from the gel with 0.25 M triethylammonium bicarbonate and desalted. The ion-exchange HPLC was carried out on Pellionex SAX column (4x250 mm). Column was eluted at flow rate of 2 ml/min with a gradient from 0 to 1 M KCl in 0.02 M potassium phosphate (pH 4.5)/40% ethanol over a period of 30 min at 50°C. The peak containing the desired product was collected and desalted. The oligonucleotide homogeneity was checked by the reversed phase HPLC on Zorbax C-8 column (4x250 mm). The column was eluted at flow rate of 1 ml/min with a gradient of 5-35% methanol in 0.1 M ammonium acetate at 45°C.

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REFERENCES

17. The abbreviation (-r) signifies a protected phosphodiester bond, (CIPh) - p-chlorophenyl.
31. Subsequent to preparation of this manuscript, we have successfully accomplished the synthesis of two oligomers (11 and 15-mer) with the use of the similar procedure and HPLC grade silica gel support derivatized as described in (5).