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 deoxyri-booligonucleotides has been developed using the phosphotriester approach. The advantage of this methodology for work in solu-tion 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 syn-thesis has been shown to be successful both in solution and on polymer supports, and its effectiveness was proved in the che-mical 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 inter-nucleotide condensations, especially as compared with the phos-phite methodology[4,5]. In the hope of overcoming this problem, we have undertaken a search for new condensing agents for phos-photriester 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 sub-stantially the rate of the synthesis in solution and on solid-phase and to simplify the procedure has been developed. The ba-sic features of our method are the use of highly effective coup-ling reagents, arylsulfonyl chlorides in the presence of N-me-thylimidazole[8], for phosphotriester bond formation and the per-formance 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 re-
port 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 deoxyri-
booligonucleotides the essential monomeric units are the N-pro-
tected 5'-dimethoxytrityl-2'-deoxynucleoside 3'-O-chlorophenyl
phosphates (VII) and deoxynucleoside-3'-O-chlorophenyl-β-cyano-
ethyl 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.[9], in which an ex-
cess of bis(triazolyl)-p-chlorophenyl phosphate was used for the
introduction of the p-chlorophenyl phosphate residue at the nuc-
leoside 3'-OH group[10]. Now we have found that this bifunctional
phosphorylating reagent can be successfully replaced by highly
reactive monofunctional phosphorylating reagent, the chlorophe-
nyl 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 phosphomo-
noesters (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 aryldichloro-
phosphate solution in pyridine also brings to formation of the
activated phosphomonoester (IV). The identity of forming deri-
vative 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 δ_p 10.8 ppm. When 5'-dimethoxytrityl
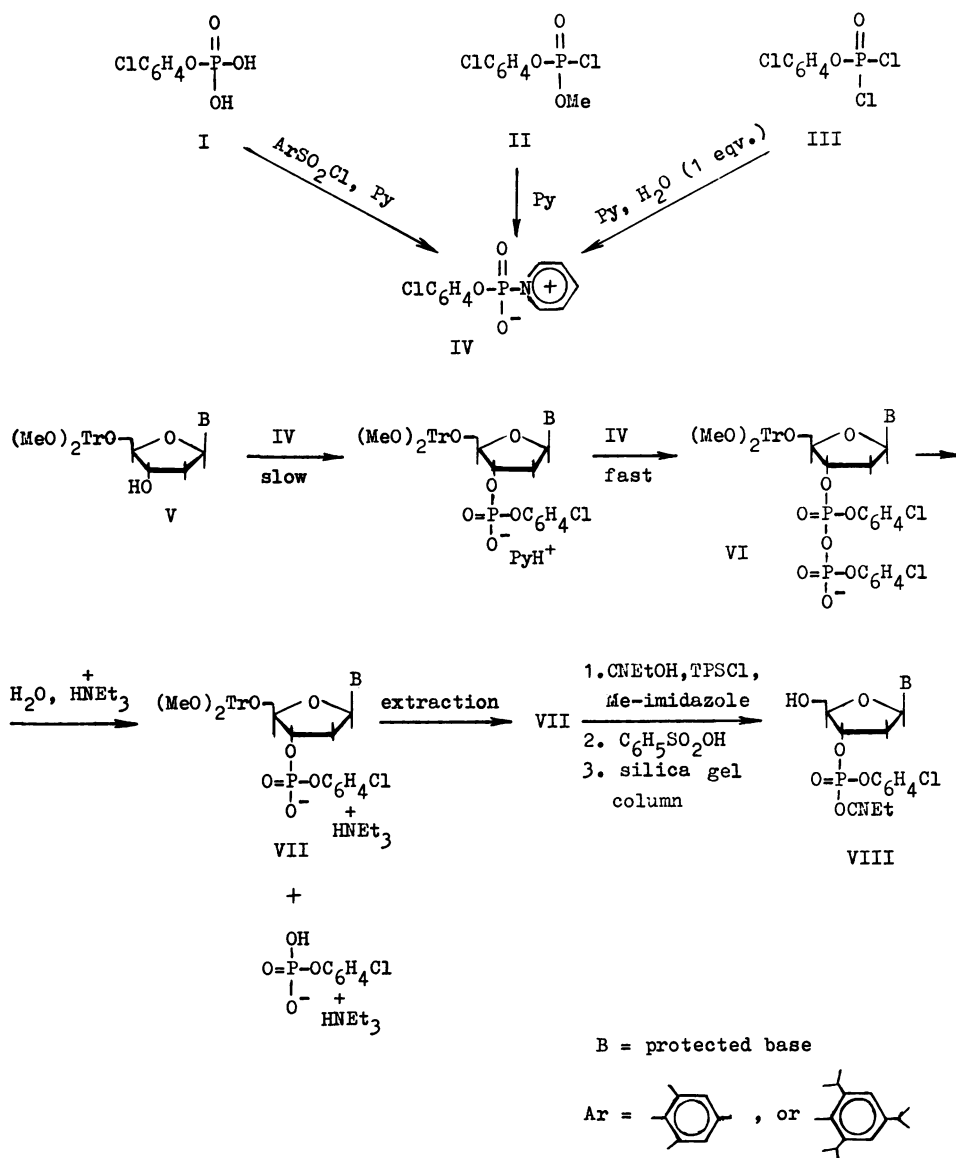
SCHEME 1

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)[13](a multiplet with a centre at δ_p 17.6 ppm).

Subsequent addition of water to the reaction mixture leads to a rapid decomposition of (VI) to form p-chlorophenyl phosphate (δ_p4.1 ppm), a small amount of simmetric p-chlorophenyl pyrophosphate (δ_p 16.2 ppm) and 3'-p-chlorophenylphosphate of 5'-dimethoxytritylthymidine (VII)(δ_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'-dimethoxytritylnucleoside.

This procedure for preparation of the phosphodiesters (VII), the simpliest one if compared with the methods described earlier[9,13], was employed by us in the synthesis of protected nucleotides. Large scale phosphorylation of 5'-dimethoxytritylnucleosides 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'-dimethoxytritylnucleoside 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 (TPSCl) and N-methylimidazole (MeIm) as was described by us previously[14]. 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 sili-
ca 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 TPSCl 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 TPSCl 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-

$$R = -C_6H_4Cl$$
R',R" = protected nucleoside
$ArSO_2Cl$ = TPSCl, or MSCl

$X = Cl^-$, $ArSO_2O^-$ or
$R'ORO(O)PO^-$

SCHEME 2

action under the action of MeIm may be accounted for the inter-
mediate formation of the methylimidazolium cation (X) possessing
a high phosphorylating capacity.

N-Methylimidazole, unlike such nucleophilic catalysts as tet-
razole and 3-nitro-1,2,4-triazole, along with nucleophilic cata-
lysis 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 con-
densation 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 arylsulfo-
nyl chlorides and MeIm. The results on comparison of the reaction
rates in the synthesis of $[(MeO)_2Tr] T\text{-}T\text{-}T(Bz)^{17}$ in these solvents
are shown in Fig.1. As it follows from these data,the highest
rate of phosphotriester bond formation is provided by acetonit-
rile and nitromethane. The condensations in these polar ap-
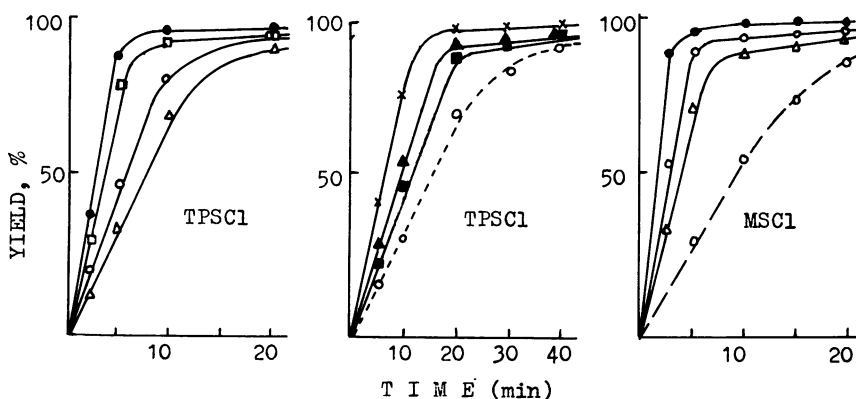
FIGURE 1. Comparison of the coupling rates in the synthesis
of $[(MeO)_2Tr] T\text{-}T\text{-}T(Bz)$ in pyridine(-o-), acetonitrile(-•-), nit-
romethane(-□-), THF (-■-), methylene chloride(-△-), chloroform
(-▲-) and dioxane(-x-). The coupling reactions were carried out
using 0.125 M concentration of $[(MeO)_2Tr] T\text{-}(ClPh)$ and 0.1 M con-
centration 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.

rotic solvents in the presence of TPSCl 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 pre-
sence of mesitylenesulfonyl chloride (MSCl) are 2-3-times higher
than those with the use of TPSCl. In the same conditions the
condensation reactions under the action of TPSCl + tetrazole
or MSCl + nitrotriazole in pyridine were complete only in 40
and 30 min, respectively[18].

It should be noted that with the use of arylsulfonyl chlo-
rides and MeIm a minimum of side-products, 5'-O-sulfonated com-
pounds and modifications of heterocyclic bases, was detected[14].
During the condensation reactions in such solvents as acetonit-
rile, dioxane or CH_2Cl_2, the reaction mixture remained clear
and lacked of darkly colored products usually observed with the
use of pyridine. Moreover, the lower viscosity of oligonucleo-
tide solutions in CH_2Cl_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)[8,16] brings to similar results.
However, according to our experiments which are in agreement
with the data from [19], this nucleophilic catalyst can not be
used for the preparation of oligomers with a 3'-terminal phos-
phate blocked by the β-cyanoethyl group, as this group is part-
ly eliminated (up to 40% within 1 h) in the presence of DMAP.
Furthermore, the extent of side-reaction of 5'-hydroxyl compo-
nent sulfonation was higher in the case of DMAP (10% in hour)
than in the case of MeIm (2% in hour)[14].

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 ove-
rall yields of the 16-mer d(GAGTCTTCAGCCCTTG), 24-mer d(GGGTTC-
CCTGCAGAAGCGTGGCAT) and 32-mer d(GCACTGGAGGGTTCCCTGCAGAAGCGTGG-
CAT) are given in Table 1. Full details of the use of these po-
lynucleotides representing the fragments of a synthetic gene for

TABLE 1. Overall yields of oligonucleotides

Sequence	Coupling yield,%	Isolated yield, %[c]
d(GAGTCTTCAGCCCTTG)[a]	35	12
d(GGGTTCCCTGCAGAAGCGTGGCAT)[a]	20.5	6
d(GCACTGGAGGGTTCCCTGCAGAAGCGTGGCAT)[a]	9	2
d(TGCAGAAGCGTGGCAT)[b]	28	7
d(AGGCAGGACAACCAT)[b]	37	9

[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 proinsulin[20], 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 TPSCl (MSCl) and 4-fold excess of MeIm 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 CH_2Cl_2 or $CHCl_3$(up to 50%) was utilized in preparation of higher oligomers, and the coupling time was about 10-20 min. As we have shown earlier[8,14], pyridine and dioxane are also good solvents for preparation of oligonucleotides with the aid of arylsulfonyl chlorides and MeIm, 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. How-

ever, when a chain length of the oligonucleotide is increased,
the resolution of this method tends to lower. It is already dif-
ficult to separate quantitatively the fully protected octanucle-
otide from the unreacted starting 5'-OH component and the reac-
tion 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 con-
taining aqueous acetone and ethylacetate provides good separa-
tion of the reaction components[21]. We have found that the effec-
tive purification of the fully protected oligonucleotides can be
also achieved by a reversed phase column chromatography on Nuc-
leosil 30 C_{18} under the conditions similar to those described by
H.-J.Fritz et al.[22]. 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 contai-
ning 25% dioxane. In all cases the last peak contained the es-
sentially pure desired product. The elution patterns in the pu-
rification 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 oligonuc-
leotides 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[9,14]. 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'-^{32}P-label, the primary
structure of the synthesized DNA fragments was confirmed by the
standard sequence analysis procedures[23,24]. The patterns ob-
tained 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
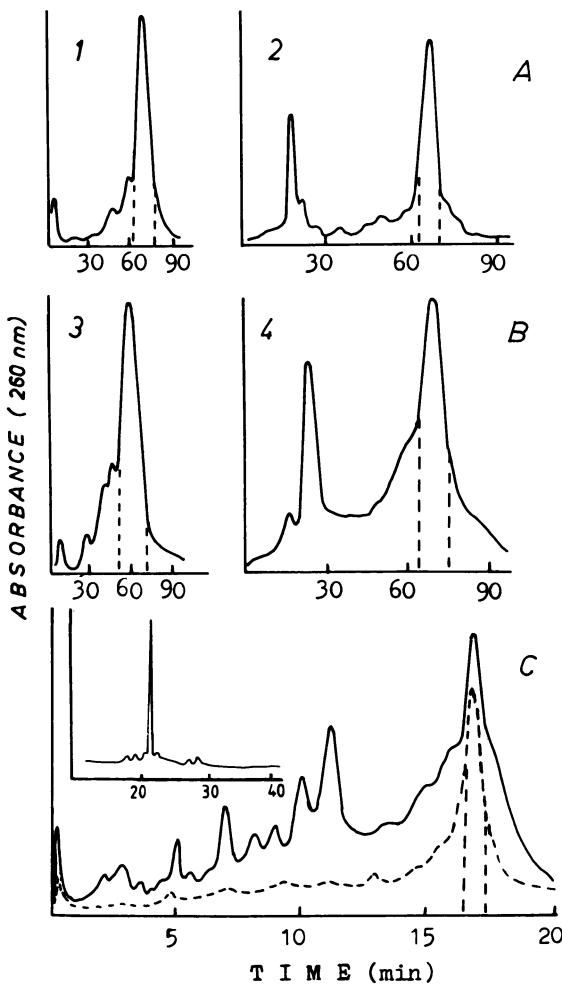without any additional purification right after the anion-ex-

FIGURE 2. Chromatography of the reaction products formed in the synthesis of d(AGCCCTTG)(A) and d(GAGTCTTCAGCCCTTG) (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 the 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[7] 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 route[25,26]. The
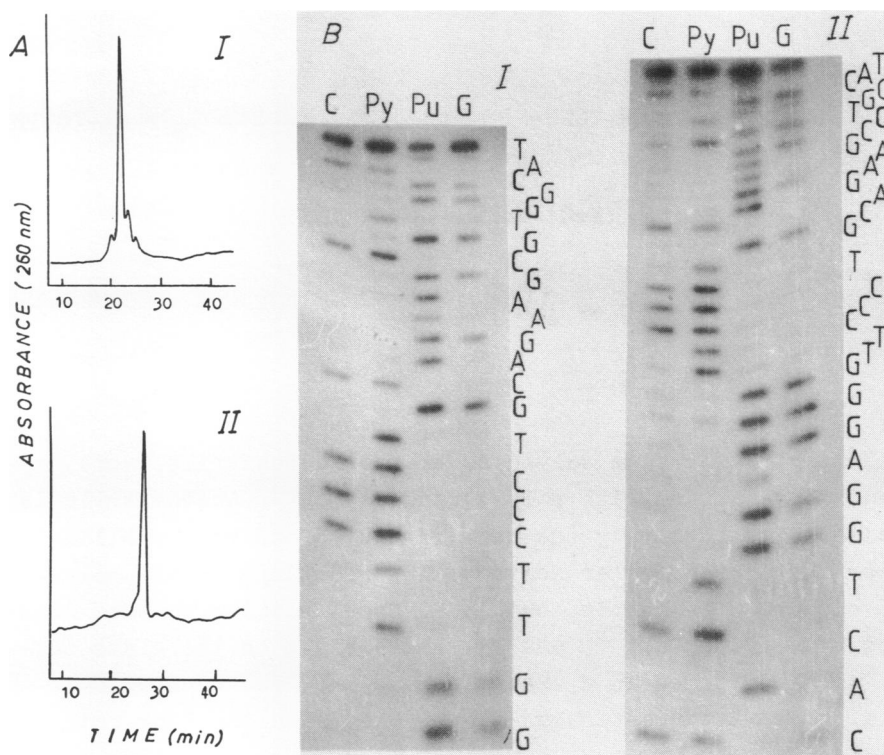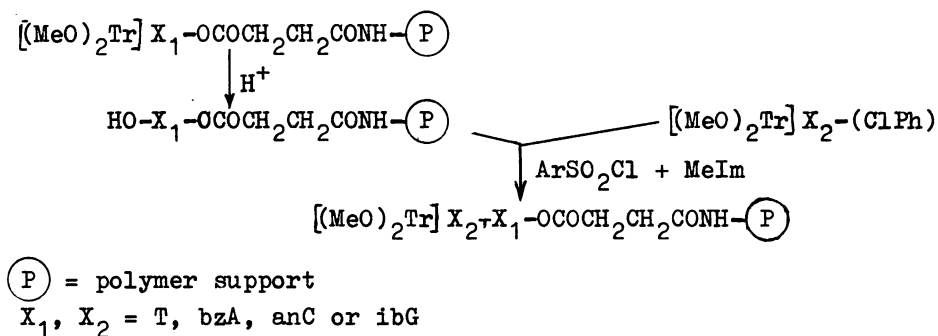
FIGURE 3. The HPLC analysis on Zorbax C-8 column after isolation by preparative electrophoresis on polyacrylamide gel (A) and the Maxam-Gilbert sequencing (B) of the synthetic 24-mer (I) and 32-mer (II).

procedures for derivatization of supports and the basic strategy of the synthesis were similar to those described earlier[25,27] (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 MeIm with respect to a nucleotide component were used in the coupling reactions. Different organic solvents, such as pyridine, CH_2Cl_2, $CHCl_3$ and acetonitrile, were utilized for performance of internucleotide condensations on nonswellable

$$[(\text{MeO})_2\text{Tr}]\,X_1\text{-OCOCH}_2\text{CH}_2\text{CONH-}\textcircled{P}$$

$$\downarrow \text{H}^+$$

$$\text{HO-}X_1\text{-OCOCH}_2\text{CH}_2\text{CONH-}\textcircled{P} \qquad\qquad [(\text{MeO})_2\text{Tr}]\,X_2\text{-(ClPh)}$$

$$\downarrow \text{ArSO}_2\text{Cl + MeIm}$$

$$[(\text{MeO})_2\text{Tr}]\,X_2\text{-}X_1\text{-OCOCH}_2\text{CH}_2\text{CONH-}\textcircled{P}$$

$\textcircled{P}$ = polymer support

X_1, X_2 = T, bzA, anC or ibG

SCHEME 3

glass matrix. The same solvents, except acetonitrile, were emp-
loyed in conjuction with polystyrene support . Acetonitrile is
unsuitable with polystyrene carrier due to its poor swelling
properties in this polar solvent. The reactions on the polysty-
rene support were carried out in a vessel similar to that desc-
ribed by H.Ito et al.[25], and the synthesises on the glass sup-
port were performed in a small column (3x50 mm) connected to a
manually operated continuous-flow system with the flow rate of
1-2 ml/min.

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 coup-
ling 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-bo-
und 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)[25,27](Fig. 4).
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 sup-
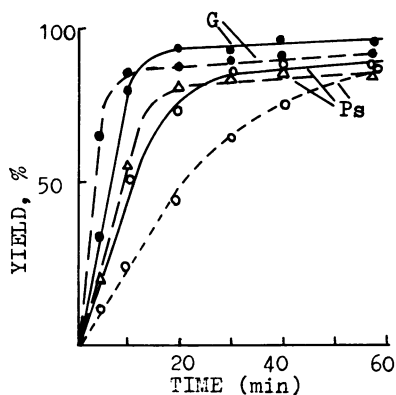port in the side reaction of sulfonation which extent is higher

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(-o-), methylene chloride (-▵-) or acetonitrile(-●-) with 0.1 M $[(MeO)_2Tr]$ T-(ClPh) in the presence of 0.2 M arylsulfonyl chloride(TPSCl-solid 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_3CN, $CHCl_3$, CH_2Cl_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_2Cl_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_2Cl_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_2Cl_2) can be successfully used in conjuction 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 syn-
thesis with the use of polystyrene support.Whereas in the syn-
thesis on glass support in a flow system, the overall time for
the addition of each block to a growing chain is about 35 min[31].

The synthetic cycle was repeated with the appropriate blocks
until the required sequence was obtained, and the oligonucleo-
tides 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 2. One cycle of operation on the polymer supports

Step	Reagent or solvent	Time(min)
Polystyrene support		
1	1% TFA in CH_2Cl_2 -CH_3CN (15:5 v/v, 3ml)	2 x 3
2	CH_2Cl_2 (5 ml)	2 x 0.5
3	coupling mixture in CH_2Cl_2[a]	20 or 30[b]
4	CH_2Cl_2 (5 ml)	2 x 0.5
5	CH_2Cl_2 - Ac_2O - MeIm (8:1:1 v/v, 1 ml)	3-5
6	CH_2Cl_2 (5 ml)	2 x 0.5
Glass support		
1	2% TFA in CH_3CN - CH_2Cl_2 (7:3 v/v)	5-7
2	CH_3CN - CH_2Cl_2 (7:3 v/v)	2
3	coupling mixture in CH_3CN - CH_2Cl_2 (7:3 v/v)[a]	10 or 20[b] (recycle)
4	CH_3CN - CH_2Cl_2 (7:3 v/v)	2
5	CH_3CN - Ac_2O - MeIm (8:1:1, v/v)	3-5 (recycle)
6	CH_3CN - CH_2Cl_2 (7:3 v/v)	2

[a] The nucleotide component (30 μmole) is dried by coevaporation
with pyridine or acetonitrile, then a solution of arylsulfo-
nyl chloride (45-60 μmole) and MeIm (90-120 μmole) in dry sol-
vent (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 se-
cond - 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(AGGCAGGACAACC-
AT) obtained on the glass carrier are shown in Fig. 5. The sequ-
ence of the 16-mer represents a part of a synthetic gene for hu-
man proinsulin. The 15-mer was designed as a primer for prepara-
tion of cDNA for human immune interferon (INF-ɣ). The overall
coupling and isolated yields of these oligonucleotides are out-
lined in Table 1. After the isolation, the material gave the ex-
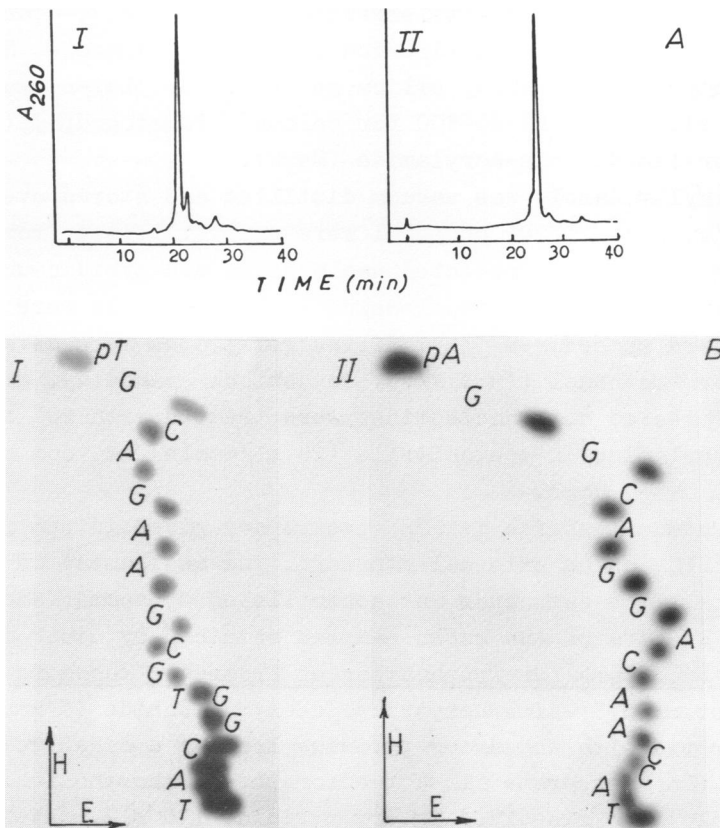pected pattern in a mobility shift analysis[23] (Fig. 5).

FIGURE 5. The HPLC analysis on Zorbax C-8 column (A) and two-
dimentional 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[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[4,5]. Moreover, the described one-solvent procedure has greate potential for automation of the synthetic process.

EXPERIMENTAL SECTION

The following reagents were purchased from commercial soures: deoxyguanosine, deoxyadenosine, deoxycytidine and thymidine (Sigma), 4,4'-dimethoxytritylchloride, 2,4,6-triisopropylbenzenesulfonyl chloride, mesitylenesulfonyl chloride, β-cyanoethanol and benzenesulfonic acid (Aldrich), N-methylimidazole, N,N-dimethylaminopyridine (Merk), silica gel F_{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. TPSCl and MSCl were recrystallized from hexane and dried in vacuo. Protected nucleosides and pyridinium 5'-0-dimethoxytrityl-2'-deoxynucleoside 3'-0-succinates were prepared by standard procedures[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% H_3PO_4 as an external standard. The assignment of the signals of all the compounds was accomplished by comparison with ^{31}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 protec-
ted 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 triethylammoni-
um 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 aceto-
nitrile, 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 convertion of (VII). The ex-
cess of water was added and the reaction mixture was concentra-
ted to a gum. The residue was dried by coevaporation with tolu-
ene and dissolved in precooled 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 $NaHCO_3$ (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 Oligonucleo-
tides in Solution

1. For preparation of di- and tetranucleotides the nucleo-
tide 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 anhydro-
us 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% CH_2Cl_2 (or $CHCl_3$) was used as a solvent. After a short column silica gel chromatography (or without this step) oligonucleotide (500-1000 A_{280} units) was applied to a Nucleosil 30 C_{18} column (1×25 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 described[26]. 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 resine 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 TPSCl in the presence of MeIm was used as coupling agent. The coupling yields were estimated by the spectroscopic analysis of the dimethoxytrityl function liberated from the resins[29].

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 $50^{\circ}C$ 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 $50^{\circ}C$. The peak containing the desired product was collected and desalted. The oligonucleotide homogenity 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 $45^{\circ}C$.

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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).