Arylsulfonyltetrazoles, new coupling reagents and further improvements in the triester method for the synthesis of deoxyribooligonucleotides

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ABSTRACT

The modified triester approach has been further improved and refined to the synthesis of defined sequences of deoxyribooligonucleotides. Improvements include arylsulfonyltetrazoles as faster and milder condensing agents, benzenesulfonic acid to avoid depuration during deblocking of trityl protecting groups and improved chromatographic procedures for purification of triester intermediates and purification of the final product containing 3'–5' phosphodiester linkages.

The efficiency of these new modifications in terms of yields and time has been illustrated by the synthesis of various defined sequences related to the lactose-operator region of E. coli, its analogues and two restriction enzyme recognition regions.

INTRODUCTION

The synthetic availability of various deoxyribooligonucleotides containing the sequence of various control regions of a gene could be of great importance in the study of the mechanism of sequence specific DNA–protein interaction. The triester chemical method\textsuperscript{3} seems to offer the first opportunity for large scale synthesis of oligonucleotides. Previously, we reported on the use of modified triester method developed in our laboratory for the chemical synthesis of a 21-mer duplex with demonstrated biological activity of the lactose operator gene of E. coli\textsuperscript{4,1}. During these studies we developed arylsulfonyl-triazoles\textsuperscript{5} as new coupling reagents which were found to give much higher yields of condensation products especially in the synthesis of oligonucleotides containing guanine base. However, these reagents were rather slow in completing the condensation reactions (3–5 days)\textsuperscript{1}. In this paper we wish to report on:
(i) arylsulfonyltetrazoles as new and more reactive coupling reagents; (ii) further improvements in the triester approach to remove unreacted starting material; and (iii) purification of the final diester products by PEI-TLC plate. The usefulness of these modifications has been demonstrated by the efficient synthesis of various deoxyribooligonucleotides constituting the duplex sequence of the lac-operator region containing Eco RI restriction endonuclease recognition sequence (A), symmetrical lac-operator (B), its bromouracil analogues (C), decanucleotide containing the Bam I restriction enzyme sequence (D), and second decanucleotide containing the Hind III restriction enzyme recognition sequence (E) as outlined in Figure 1. The sequence of these oligonucleotides was confirmed by electrophoresis homochromatography mobility-shift analysis.

A. LACTOSE-OPERATOR CONTAINING Eco RI RECOGNITION SEQUENCE

|   | I          | II         |
|---|------------|------------|
| 5' | A-A-T-T-C-A-A-T-T-G-T-G-A-G-C-G-G-A-T-A-A-C-A-A-T-T-3' |           |
| 3' | T-T-A-A-C-A-C-T-C-G-C-C-T-A-T-T-G-T-T-A-A-C-T-T-A-A-5' |           |

B. SYMMETRICAL LACTOSE OPERATOR

|   | IV         |
|---|------------|
| 5' | A-A-T-T-G-T-G-A-G-C-G-G-C-T-C-A-A-C-A-A-T-T-3' |
| 3' | T-T-A-A-C-A-C-T-C-G-C-C-T-A-T-T-G-T-T-A-A-5' |

C. LACTOSE-OPERATOR (BROMO-URACIL ANALOGS)

| VI  | a-c       |
|-----|-----------|
| 5'  | HEXAMER   |
| 3'-T-T-A-A-C-A-C-T-C-G-C-G-A-G-T-G-T-T-A-A-5' |

VI a = 5'-A-A-BrU-BrU-G-BrU-3'
VI b = 5'-A-A-T-BrU-G-BrU-3'
VI c = 5'-A-A-T-T-G-BrU-3'

D. RESTRICTION ENZYME RECOGNITION SEQUENCES

| VII | 5'-C-C-G-G-A-T-C-C-G-G-3' |
|-----|---------------------------|
|     | (Bam H-I)                 |
| VIII| 5'-A-C-A-A-G-C-T-G-T-3'   |
|     | (Hind III)                |

dThese sequences are self-complimentary.

Figure 1. The nucleotide sequence of the lac operator and restriction enzyme recognition sites.
RESULTS AND DISCUSSION

Arylsulfonyletetrazoles Coupling Reagents

Triisopropylbenzenesulfonyltetrazole (TPS-tetr) and mesitylenesulfonyltetrazole (MS-tetr) were directly prepared in 80% yield by treating the respective arylsulfonyl chloride with tetrazole in dioxane solution containing triethylamine at 5° for 2 hr. Benzenesulfonyltetrazole (BS-tetr) was obtained in 60-70% yield after purification in crystalline form by shaking its benzene solution with silica-gel, filtration and evaporation in vacuo to dryness. All these compounds (Figure 2) and especially the benzenesulfonyltetrazole were found to decompose on storage within 20 days. Comparison of the time periods for the completion of condensation reaction indicated that the rate of condensation was dependent upon the substitution in the benzene ring[13], i.e. phenyl > 2,4,6-trimethylphenyl > 2,4,6-trisopropylphenyl groups and all these reagents were more reactive than triazoles and even triisopropyl benzenesulfonyl chloride[14].

Further Modification in the Triester Approach

Previously, we have modified the triester synthetic approach[4] by introducing two key steps, i.e. (i) the starting material was a fully protected mononucleotide-3'-phosphotriester in order to eliminate the phosphorylation step before each condensation, and (ii) the chain was grown from the 3'-end towards 5'-terminus thus requiring the deblocking of an acid labile protecting group (monomethoxy- or dimethoxytrityl) instead of a base labile β-cyanoethyl group. Increasingly stronger basic conditions were required to deblock the β-cyanoethyl group in

\[ R_1 \text{SO}_2 \text{Cl} + \text{N}_3 \text{N} \rightarrow R_1 \text{SO}_2 \text{N}_3 \text{N} \]

\[ \begin{align*}
\text{a. BS-Tetr.} & \quad (R_1 = R_2 = R_3 = H) \\
\text{b. MS-Tetr.} & \quad (R_1 = R_2 = R_3 = CH_3) \\
\text{c. TPS-Tetr.} & \quad (R_1 = R_2 = R_3 = \text{iso-propyl})
\end{align*} \]

Figure 2. Synthesis of various arylsulfonyltetrazoles.
longer chain oligonucleotides. Using the modified triester approach, we occasionally observed that the pure fully protected product could not be separated quantitatively from the unreacted starting component containing 5'-primary hydroxyl groups, especially in the case of longer chains containing several guanine bases. Phosphorylation of the 5'-hydroxyl component followed by rechromatography on silica-gel should cause the oligonucleotide component containing the negatively charged phosphate group to be retained more strongly on silica-gel. We first tried phosphorus-oxychloride for phosphorylation - but obtained a neutral product, probably a phosphodichloridate. Attempted phosphorylation with β-cyanoethyl phosphate in the presence of benzenesulfonyltetrazole yielded again a triester but in the presence of benzenesulfonyltriazole, the desired phosphodiester group was introduced quantitatively.

Benzenesulfonic Acid, A New Detritylating Reagent

The removal of trityl protecting groups from fully protected deoxyribooligonucleotides with 80% acetic acid causes extensive depurination and the production of numerous side products during synthesis by the modified triester approach. In order to overcome this problem we searched for a new deblocking reagent which would be able to hydrolyze the ether bond of trityl protecting groups much faster than the glycosidic bond in fully protected deoxyribooligonucleotides containing purine bases. We reasoned that an aromatic acid would have an affinity for the aromatic rings of the trityl group this localizing the acid catalysis to this part of the molecule. Indeed, we found that (Table I) benzenesulfonic acid as 2% solution in chloroform selectively removed monomethoxy or dimethoxytrityl groups at 0°C in 20 min and 3 min respectively with a minimum of depurination. Under the above conditions, all the other protecting groups were found to be stable. Comparative data with classical reagent for detritylation, 80% acetic acid is given in Table I.

Synthesis of Deoxyribooligonucleotides

The general plan for the synthesis is outlined in Figure 3. The 3'-hydroxyl of a starting nucleoside was protected with the benzoyl instead of the acetyl group because of the high solubility of the fully protected compounds in chloroform, faster mobility
Table I. Removal of Mono- and Dimethoxytrityl Group with Benzenesulfonic Acid (BSA) and Its Comparison with 80% Acetic Acid*

| Compounds | [(MeO)Tr]BzdA-OH | [(MeO)₂Tr]BzdA-OH | [(MeO)₂Tr]BzdA-p (CE, ClPh) |
|-----------|-----------------|-----------------|-----------------|
| Reagent   | Condition       | Depur. †        | Prod. †         | Condition       | Depur. †        | Prod. † |
| 2% BSA in CHCl₃/MeOH (7:3) | 0°C, t = 20 min | 12% | 88% | 0°C, t = 3 min | 4% | 96% | 0°C, t = 2.5 min | a) 100% nil |
| 1% BSA in CHCl₃/MeOH (7:3) | room temp., t = 3.5 min | 20% | 80% | room temp., t = 1.5 min | 15% | 85% | room temp., 45 sec | b) 100% nil |
| 80% AcOH | room temp., 2 hr | 55% | 45% | room temp., 30 min | 36% | 64% | room temp., 30 min | 15% | 85% |

* Yield was estimated on silica-gel plates.
† Depurinated product found.
‡ Expected detritylated intact product found.

a) After 2 hr just a trace of depurination; after 6 hr less than 3%.
b) After 20 min depurination less than 3%.

on silica-gel TLC plates and their tendency to solidify. Thus its structural analysis was much easier. Condensation of this 3'-O-benzoyl-N-protected mono- or oligodeoxynucleoside with a 5'-dimethoxytrityl-N-protected mono- or oligodeoxynucleoside-3'- (p-chlorophenyl) phosphate (about equimolar amounts) was carried in the presence of benzenesulfonfyltetrazole (2-3 molar equivalent based on 5) in anhydrous pyridine for 5-12 hr at room temperature. The reaction mixture was then decomposed with aqueous pyridine followed by extraction with chloroform which was washed with 0.1 M triethylammonium bicarbonate pH 7.5. The chloroform solution was then co-evaporated with toluene to a gum which was chromatographed on a silica-gel column with chloroform:methanol (1-10% v/v) as eluent. The fractions displaying a trityl positive color test on TLC were pooled, concentrated to dryness and further treated overnight with excess of β-cyanoethyl phosphate (pyridinium salt) in the presence of benzenesulfonyltriazole in anhydrous pyridine solution. After the usual work-up fully protected oligonucleotide was obtained after silica-gel chromatography. The trityl group was now removed by treating with 2% chloroform.
Figure 3. Synthesis of fully protected pentadecanucleotides of the defined sequence by the improved triester approach.

Solution of benzenesulfonic acid at 0° and after the completion of the reaction the chloroform solution was washed with 5% sodium bicarbonate solution and water. The reaction conditions, isolated yields of the triester intermediates and yields of diester products after deblocking for various oligonucleotides appears in Table II.

Removal of the Protecting Groups

In our earlier studies \(^1,^{16}\) alkali labile protecting groups such as 3'-O-acetyl and p-chlorophenyl on the phosphotriester functions were removed by 0.1 N sodium hydroxide-dioxane-water treatment for 3-6 hr at room temperature, N-acetyl groups with concentrated ammonia at 50° and finally 5'-O-dimethoxytrityl groups with 80% acetic acid at room temperature. During these steps, we generally detected various impurities such as degradation products of the oligonucleotides, oligonucleotides containing 3'-3' phosphodiester bonds, cyclic dinucleotides \(^4\), unknown faster moving compound due to the degradation of the cytosine ring \(^17\) and depurinating products. Recently we have observed that treatment with concentrated ammonia at 50°C for
4-6 hr removed the p-chlorophenyl group from the internucleotidic phosphotriester function with minimum formation of side products. In the present studies the dimethoxytrityl group was removed with a 2% solution of benzenesulfonic acid in chloroform at 0°C followed by treatment with concentrated ammonia at 50°C for 4-6 hr. After removal of ammonia, the concentrated solution was chromatographed on Sephadex G-75 (superfine) column. The fractions containing the desired phosphodiester compound were pooled and further fractionated on a polyethylenimine-PEI-cellulose TLC plate as described below.

**Fractionation of Synthetic Oligonucleotides on PEI-Cellulose TLC Plates**

The final purification of the desired oligonucleotides containing 3'-5' phosphodiester functions were carried out on PEI-Cellulose TLC UV 254 plates with eluent containing different concentrations of lithium chloride-7 M urea at 60°C or lithium acetate-7 M urea (pH 3.5) at room temperature. This is a modification of an earlier procedure. A preparative scale (up to 200 A 260) separation of oligonucleotides up to 20 bases long could be achieved in 4 hr. The relative mobility of various compounds are given in Table III.

**Characterization of Oligonucleotides by Two-Dimensional Procedure**

In the present studies the base sequence of each synthetic oligomer was determined and confirmed by using the mobility shift analysis of Tu et al. This involves the labeling by phosphorylation of the 5'-hydroxyl group with polynucleotide kinase enzyme and [γ-32P] ATP followed by controlled snake venom phosphodiesterase digestion to obtain a sequential population of all the intermediate oligonucleotides. The total mixture containing all the intermediate products was fractionated by the two-dimensional electrophoresis-homochromatography system which was based upon the charge and size difference of each oligonucleotide. The number of radioactive spots on the two-dimensional maps denote the number of bases in a given oligonucleotide and the mobility shift value between the neighboring nucleotides could be interpreted to assign the particular base difference between adjacent oligonucleotides. Figure 4 shows the two-dimensional maps for the sequence analysis of P32 (CGGATAACAATT) and Figure 5...
Table II. Reaction conditions and yields of various deoxyriboligonucleotides using benzene-sulfonylethrazole as the condensing reagent.

| 5'-Protected component (mmol) | 5'-Hydroxyl component (mmol) | Condensing reagent (mmol) | Reaction time (hr) | Product (yield %) | Deblocked product (yield %) |
|-------------------------------|-------------------------------|--------------------------|--------------------|------------------|-----------------------------|
| Undecamer (I)                |                               |                          |                    |                  |                             |
| [(MeO)₂ Tr]dbzA₁-Th₇dbzC₁-Th₇dbzC₁-CIPh (0.44) |                               |                          |                    |                  |                             |
| Pentadecamer (II)            |                               |                          |                    |                  |                             |
| [(MeO)₂ Tr]dbzC₁-Th₇isoG₁-Th₇isoG₁-CIPh (0.6) | dbzA₁-Th₇dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                               | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
| Pentadecamer (III)           |                               |                          |                    |                  |                             |
| [(MeO)₂ Tr]dbzC₁-Th₇isoG₁-Th₇isoG₁-CIPh (0.2) | dbzA₁-Th₇dbzA₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.15)                        |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.15)                        |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.15)                        |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.15)                        |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.15)                        |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.15)                        |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.15)                        |                          |                    |                  |                             |
| Pentadecamer (IV)            |                               |                          |                    |                  |                             |
| [(MeO)₂ Tr]dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁-CIPh (0.6) | dbzA₁-Th₇dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
| Pentadecamer (V)             |                               |                          |                    |                  |                             |
| [(MeO)₂ Tr]dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁-CIPh (0.15) | dbzA₁-Th₇dbzA₁ dbzA₁ dbzA₁ dbzA₁ dbzA₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |
|                              | dbzC₁-Th₇dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ dbzC₁ |                          |                    |                  |                             |
|                               | (0.5)                         |                          |                    |                  |                             |

A. LACTOSE-OPERATOR CONTAINING Eco RI RECOGNITION SEQUENCE

| Reaction conditions and yields of various deoxyriboligonucleotides using benzene-sulfonylethrazole as the condensing reagent.

B. SYMMETRICAL LACTOSE-OPERATOR

(continued)
Table II (contd)

| 5'-Protected \(^a\) component (mmol) | 5'-Hydroxyl component (mmol) | Condensing reagent (mmol) | Reaction time (hr) | Product (yield %) | Deblocked product (yield %) |
|-------------------------------------|-----------------------------|--------------------------|-------------------|------------------|---------------------------|
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (0.25)\) | \(\text{dBrU}-\text{CI} \text{Ph} (0.22)\) | 0.6                      | 0.5               | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (76)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (76)\) |
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (0.05)\) | \(\text{dBrUisoG+BrU(Obs)} (0.045)\) | 0.11                     | 1                  | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (80)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (80)\) |
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (0.05)\) | \(\text{dBrUisoG+BrU(Obs)} (0.045)\) | 0.15                     | 1                  | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (78)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (78)\) |
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (0.03)\) | \(\text{diisoG+BrU(Obs)} (0.15)\) | 0.9                      | 1                  | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (82)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-BrU-CI} \text{Ph} (82)\) |

D. RESTRICTION ENZYMES RECOGNITION SEQUENCES

Decamer (VII)

| 5'-Protected \(^a\) component (mmol) | 5'-Hydroxyl component (mmol) | Condensing reagent (mmol) | Reaction time (hr) | Product (yield %) | Deblocked product (yield %) |
|-------------------------------------|-----------------------------|--------------------------|-------------------|------------------|---------------------------|
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (0.25)\) | \(\text{dbzC\#bxC\#isogG\#isog(Obs)} (0.21)\) | 0.75                     | 1                  | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (71)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (71)\) |
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (0.09)\) | \(\text{dbzC\#bxC\#isogG\#isog(Obs)} (0.06)\) | 0.27                     | 12                 | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (65)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (65)\) |

Decamer (VII)

| 5'-Protected \(^a\) component (mmol) | 5'-Hydroxyl component (mmol) | Condensing reagent (mmol) | Reaction time (hr) | Product (yield %) | Deblocked product (yield %) |
|-------------------------------------|-----------------------------|--------------------------|-------------------|------------------|---------------------------|
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (0.45)\) | \(\text{dTr isoG+OBS} (0.35)\) | 1.2                      | 1                  | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (75)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (75)\) |
| \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (0.15)\) | \(\text{diisoG+bxC\#isogG\#isog(Obs)} (0.11)\) | 0.45                     | 12                 | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (60)\) | \([\text{MeO}_2\text{Tr}]\text{dbza}^\text{Arbza-CI} \text{Ph} (60)\) |

\(^a\)Abbreviations are as suggested by the IUPAC-IUB. Biochemistry 9, 4022 (1970). A phosphodiester linkage is represented by hyphen and phosphotriester linkage is represented by (\(^\circ\)) symbol. Each internal internucleosidic phosphate is protected with p-chlorophenyl group (CIPh).
Table III. $R_f$ Values* of Deoxyribonucleotide on PEI-Cellulose TLC Plates

| Oligonucleotides containing phosphodiester groups | Solvent system |
|--------------------------------------------------|---------------|
|                                                  | E (pH 8.0)    | F (pH 3.5) |
| A-A-T-G-G-T                                      | 2.35          | 6.50       |
| A-A-BrU-BrU-G-BrU                                 | 2.20          | 6.60       |
| A-A-T-BrU-BrU-G-BrU                               | 2.25          | 6.55       |
| A-A-T-G-BrU                                      | 2.30          | 6.50       |
| A-A-T-C-A-A-T-T-G-T                               | 1.60          | 5.30       |
| C-G-T-C-A-C-A-A-T-T                               | 1.40          | 5.40       |
| T-A-T-C-C-G-C-T-C-A-C-A-A-T-T                     | 0.46          | 4.9        |
| C-G-G-A-T-A-A-C-A-A-A-T-T                         | 1.0           | 5.30       |
| G-A-G-C-G-G-G-G-A-T-A-A-C-A-A-A-T-T               | 0.30          | 4.5        |
| C-T-C-A-C-A-A-A-T-T                               | 2.00          | 5.8        |
| C-C-G-T-C-A-C-A-A-A-T-T                           | 1.05          | 5.30       |
| G-A-G-C-G-C-T-C-A-C-A-A-A-T-T                     | 0.41          | 4.8        |
| C-G-G-T-C-A-A-A-A-A-T-T                           | 0.90          | 5.4        |
| G-A-G-C-G-C-T-C-A-C-A-A-A-T-T                     | 0.40          | 4.9        |
| C-C-G-G-A-T-C-C-G-G                             | 0.9           | 5.0        |
| A-A-A-A-G-C-T-G-T                                 | 1.2           | 5.2        |

* All $R_f$ values are with respect to yellow dye.

for $^{32}$P (CGGCTCACAATT). These maps unambiguously confirmed the entire sequence from the mobility shifts as mentioned above.

CONCLUDING REMARKS

Thus the present studies have clearly demonstrated that these improvements in condensing agents, deblocking conditions and purification procedures have increased the effectiveness of the triester approach. It is a rapid method for the synthesis of long oligonucleotides of defined sequence in high yields.

EXPERIMENTAL SECTION

General Methods and Materials

Thymidine, deoxyadenosine and deoxycytidine (Calbiochem), deoxyguanosine (Nutritional Biochemical Corp.), 5-bromodeoxyuridine, $l$(H)-tetrazole, triisopropylbenzenesulfonyl chloride, mesitylenesulfonyl chloride, benzenesulfonyl chloride, $\beta$-cyanoethylphosphate, benzenesulfonic acid, monomethoxytrityl and
Figure 4. Two-dimensional fingerprinting of a partial snake venom phosphodiesterase digest of the dodecanucleotide P*CGGATAACAATT.

dimethoxytrityl chloride (Aldrich), Avicel-cellulose TLC plates containing fluorescence indicator, polyethylenimine (PEI/UV<sub>254</sub>) and silica gel F<sub>254</sub> thin layer plates, silica-gel H grade for column chromatography (Brinkman) and cellogram strip (Kalex Scientific Co., N.Y.) were purchased commercially.

Snake venom phosphodiesterase was purchased from Worthington Biochemical Co. and purified before use. T<sub>4</sub>-poly-nucleotide kinase (20,000 units/mg) was purchased from Biogenics Research Co. (Chagrin Falls, Ohio).

Homo-Mix and DEAE-cellulose plates were purchased or prepared according to previously reported procedures<sup>12,20</sup>.

N-Benzoyl-5'-O-dimethoxytrityldeoxyadenosine and N-benzoyl-5'-O-dimethoxytrityldeoxycytidine<sup>21</sup> and fully protected deoxymononucleotides containing 3'-(p-chlorophenyl)-6-cyanoethyl phosphate group<sup>4</sup> were prepared by the published procedures.

Solvent Systems

Oligonucleotides containing phosphotriester groups were analysed on silica-gel F<sub>254</sub> TLC plates by developing in solvent A, chloroform-methanol (1-10% v/v); for oligonucleotides containing phosphodiester groups Avicel-cellulose TLC plates were developed in solvent systems B [isopropyl alcohol-concentrated ammonium
hydroxide-water (7:1:2), C [isobutyric acid-1 M ammonium hydroxide-0.1 M EDTA (100:60:1.6)], and D [n-propyl alcohol-concentrated ammonium hydroxide-water (55:10:35)]. PEI/UV\textsubscript{254} TLC plates were developed in solvent E, 0.6 M LiCl-7 M urea-0.025 M Tris (pH 8.0) at 60°C, or F, 0.6 M LiOAc-7 M urea (pH 3.5) at room temperature.

Figure 5. Two-dimensional fingerprinting of partial snake venom phosphodiesterase digest of the dodecanucleotide P*CGGCTCACATT.

General Method for the Preparation of Arylsulfonyltetrazoles

A dioxane (20 ml) solution of triethylamine (10.1 g, 0.1 mol) was added to a stirred suspension of arylsulfonyl chloride (0.1 mol) and 1 H-tetrazole (7.0 g, 0.1 mol) in dioxane (200 ml) at 5°C). After 2 hr the precipitate was filtered off and the dioxane solution was evaporated to dryness under vacuum. The crystalline residue was dissolved in chloroform (50 ml) which was
washed with water (2 x 20 ml), dried over anhydrous sodium sulfate, filtered and evaporated to provide a residue. Recrystallization from benzene produced the pure product in 70-80% yield. In the case of benzene sulfonyltetrazole the benzene solution was shaken with silica-gel for 1 hr and after removing silica-gel, the solution was evaporated under vacuum to dryness and recrystallized from benzene (60% yield). Each of the compounds were characterized by their elemental analysis and NMR spectra as detailed below (see Figure 2).

1-(Benzenesulfonyl)-tetrazole: m.p. 86-92° (Benzene). Anal. Calcd. for C_{7}H_{6}N_{4}O_{2}S: C, 36.36; H, 3.05; N, 28.27. Found: C, 36.50; H, 3.00; N, 28.24. NMR (CDCl₃, ppm from Me₄Si) 9.25 (1H, s, CH in tetrazole); 8.0 (5H, m, aromatic H's).

1-(Mesitylenesulfonyl)-tetrazole: m.p. 108-119° (Benzene). Anal. Calcd. for C_{10}H_{12}N_{4}O_{2}S: C, 47.61; H, 4.79; N, 22.21. Found: C, 47.69; H, 4.84; N, 22.30. NMR (CDCl₃, ppm from Me₄Si) 9.21 (1H, s, CH in tetrazole), 7.5 (2H, s, aromatic H's), 2.7 (6H, s, ortho CH₃), 2.36 (3H, s, para CH₃).

1-(2,4,6-Triisopropylbenzenesulfonyl)-tetrazole: m.p. 95-97° (Benzene-petroleum ether). Anal. Calcd. for C_{16}H_{24}N_{4}O_{2}S: C, 57.12; H, 7.19; N, 16.65. Found: C, 57.25; H, 7.20; N, 16.69. NMR (CDCl₃, ppm from Me₄Si) 9.28 (1H, s, CH in tetrazole), 7.4 (2H, s, aromatic H's), 4.15 (2H, m, ortho CH), 3.14 (1H, m, para CH), 1.28 (18H, pseudoquartet CH₃).

3'-O-Benzoyl 5-bromodeoxyuridine: To an anhydrous pyridine solution (60 ml) of 5'-dimethoxytrityl 5-bromodeoxyuridine (16.35 g, 30 mmol) was added benzoyl chloride (4.62 g, 33 mmol). After 2.5 hr at room temperature the reaction mixture was poured into ice water (500 ml) and extracted with chloroform (2 x 500 ml). The chloroform was washed with water (1 x 200 ml) and dried over anhydrous sodium sulfate, filtered and evaporated to dryness (free from pyridine) and the residue dissolved in 80% acetic acid (150 ml). After removing the acetic acid by evaporation under vacuum, the residue was coevaporated with toluene and on crystallization from ethanol, 3'-O-benzoyl, 5-bromouridine was obtained in 80% yield: m.p. 195-196°. UV spectrum (MeOH) λ_max 276 nm (ε 11900); λ_min 250 nm (ε 7500). Anal. Calcd. for C_{16}H_{15}N_{2}O_{6}Br: C, 46.7; H, 3.67; N, 6.81. Found: C, 46.6;
Using the above 3' -O-benzoyl, N-isobutryldeoxyguanosine was also prepared in 70% yield: m.p. 145-147°. Anal. Calcd. for C_{21}H_{23}N_{5}O_{6}: C, 57.14; H, 5.25; N, 15.87. Found: C, 57.25; H, 5.38; N, 15.77.

5' -O-Monomethoxytrityl-N-isobutryldeoxyguanosine-3'-p-chlorophenyl-ß-cyanoethyl Phosphate

Deoxyguanosine (50 mmol, 14.0 g) was made anhydrous by repeated (3 x 100 ml) evaporation under vacuo of its suspension in anhydrous pyridine. To the residue suspended in chloroform (300 ml) containing anhydrous pyridine (36 ml) was added dropwise isobutryl chloride (40 ml) in chloroform (200 ml) with stirring and cooling in ice bath. The reaction mixture became clear as the reaction proceeded and after 2 hr it was decomposed with water (100 ml). The organic layer was evaporated to dryness, the residue dissolved in ethanol (100 ml), treated with 2 N sodium hydroxide (100 ml) and kept at 0°C for 10 min. After neutralizing with excess Dowex resin (pyridinium form) and filtration the filtrate was evaporated to dryness in the presence of pyridine. The anhydrous pyridine solution (250 ml) was treated with monomethoxytrityl chloride (50 mM, 15.4 g), shaken for 20 hr, decomposed by adding cold water (100 ml) and after 20 min the solution was extracted with chloroform (3 x 200 ml). The combined organic layers were further washed with water (3 x 100 ml), evaporated to dryness in vacuo and then with toluene to provide a residue which was dissolved in chloroform (30 ml) and chromatographed on silica-gel column (500 g, 4 cm diameter) and eluted with chloroform-methanol (3.5% v/v). The product was isolated in 70% yield.

A mixture of p-chlorophenylphosphodichloridate (42 mmol), 1H-1,2,4-triazole (87 mmol) and triethylamine (87 mmol) was stirred in anhydrous dioxane (500 ml), first at 0°C for 10 min, and then at room temperature for 1 hr. The precipitate was filtered and the filtrate concentrated (to 250 ml) under reduced pressure, at ≤ 20°C. To this solution was added 5'-O-monomethoxytrityl, N-isobutryldeoxyguanosine (25 mmol), the reaction mixture kept at 20°C for 6 hr then β-cyanoethanol (35 mmol) was added and the reaction maintained overnight. The fully protected
mononucleotide was isolated by silica-gel chromatography in 70-80% yield: m.p. 86-88°. UV spectrum (MeOH) $\lambda_{\text{max}}$ 262 nm (ε 17000), $\lambda_{\text{max}}$ 262 nm (ε 18500), and $\lambda_{\text{min}}$ 250 mμ (ε 15200). Anal. Calcd. for C$_{44}$H$_{44}$N$_{6}$O$_{10}$PCl: C, 59.95; H, 5.03; N, 9.08. Found: C, 59.90; H, 4.98; N, 8.97.

5'-O-Dimethoxytrityl-5-bromodeoxyuridine-3'-p-chlorophenyl-β-cyanoethyl Phosphate

An anhydrous pyridine solution (10 ml) containing 5-bromo-deoxyuridine (3.07 g, 10 mmol) and dimethoxytrityl chloride (3.5 g, 10.3 mmol) was shaken at room temperature for 3 hr. The reaction was stopped by the addition of methanol (1 ml) and this solution evaporated to dryness in vacuo at room temperature. To the residue was added chloroform (30 ml) and water (20 ml), partitioned, the chloroform phase was further extracted with water (2 x 10 ml), dried, evaporated and the residue taken up in chloroform (10 ml) and chromatographed on a silica-gel column (150 g, 4.5 cm diameter). The desired compound was isolated in 95% yield by eluting the column with chloroform-methanol (95.5:4.5 v/v).

A mixture of p-chlorophenylphosphodichloridate (5.6 mmol) 1H-1,2,4-triazole (11 mmole) and triethylamine (11 mmole) was stirred in anhydrous dioxane (50 ml) as described above. The phosphorylation and cyanoethylation reactions were carried out by adding 5'-O-dimethoxytrityl 5-bromouridine (4.6 mmol) followed 6 hrs later by β-cyanoethanol (4.6 mmol) as described above. The desired fully protected compound was isolated by silica-gel chromatography in 80% yield: m.p. 79-81° UV spectra (MeOH) $\lambda_{\text{max}}$ 276 nm (ε 12000), $\lambda_{\text{min}}$ 254 nm (ε 8600). Anal. Calcd. for C$_{39}$H$_{37}$N$_{3}$O$_{10}$PClBr: C, 54.9; H, 4.25; N, 4.92. Found: C, 54.8; H, 4.19; N, 4.90.

General Method for the Synthesis of Fully Protected Deoxyribonucleotides

An anhydrous pyridine solution (5 ml per mmol of the nucleotidic component) containing 5'-O-dimethoxytritylnucleotide (or oligonucleotide)-3'-p-chlorophenyl phosphate plus an appropriate oligonucleotide containing a free 5'-hydroxyl and fully protected 3'-O-phosphate group (1.2 molar equivalent) was treated with benzenesulfonyltetrazole (3 molar equivalent relative to 3'-phosphodiester component) for 30 min to 12 hr at
room temperature. The reaction was then decomposed by addition of water (10 ml per g of the nucleotidic material) with cooling, followed by extraction with chloroform (100 ml per g of the nucleotidic material). The chloroform layer was washed with 0.1 N triethylammonium bicarbonate pH 7.5 (3 x 50 ml), water (1 x 50 ml), dried over anhydrous sodium sulfate, filtered, and under reduced pressure evaporated to a gum in the presence of excess toluene. The gum was dissolved in chloroform and chromatographed by column chromatography on silica gel in chloroform-methanol (1-10% v/v) solvent.

Isolation of the Fully-Protected Deoxyribooligonucleotides

The fractions from the silica-gel chromatography (described above) exhibiting a positive trityl test on TLC were pooled, evaporated to dryness and treated with excess of β-cyanoethylphosphate (5 molar equivalent) in anhydrous pyridine solution (10 ml) in the presence of benzenesulfonyltriazole (3 molar equivalent based on β-cyanoethylphosphate) overnight at room temperature. After the usual work-up as described above, the required fully protected oligonucleotide was isolated by silica-gel chromatography. The reaction conditions and isolated yields are given in Table II.

Deblocking of Monomethoxy- or Dimethoxytrityl Group from the Fully Protected Deoxyribooligonucleotides

The fully protected compound (100 mg) was dissolved in chloroform-methanol (1 ml, 7:3 v/v) containing 2% benzenesulfonic acid and kept at 0°C. The reaction was over in 20 min (monomethoxytrityl), 3 min (dimethoxytrityl), as checked by silica-gel TLC (chloroform:methanol 10:1 v/v). The reaction mixture was washed with a 5% sodium bicarbonate solution and then water. The chloroform layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The residue was dissolved in chloroform (5 ml), applied to a silica-gel column (50 g, 4 cm diameter) and eluted with chloroform:methanol (97:3 v/v, 500 ml) at room temperature. By monitoring the fractions by silica-gel TLC in chloroform-methanol (9:1 v/v), the product was pooled and obtained in 90% yield.
Complete Deblocking of the Fully Protected Deoxyribooligonucleotides

Removal of the dimethoxytrityl group from the fully protected compound (100 mg) was achieved by treatment with a 2% solution of benzenesulfonic acid in chloroform-methanol at 0°C for 3 min as described above. After work-up the residue was dissolved in concentrated ammonia (20 ml) containing pyridine (0.5 ml) to make an homogeneous solution and the sealed flask was heated at 50°C for 4-6 hr then concentrated to dryness in vacuo. The residue was dissolved in water (50 ml), extracted with ether (2 x 5 ml) and the aqueous layer was concentrated (1-2 ml) in the presence of pyridine and chromatographed on a column of sephadex G-75 (SP) K25/100. The fractions containing the desired compound were pooled, concentrated and further purified on polyethylenimine PEI/UV_254 TLC plate as described below.

Preparative Thin Layer Chromatography on Polyethylenimine-Cellulose (PEI/UV_254) Plates

Before applying the samples, the PEI layers were predeveloped with methanol, dried and redeveloped with distilled water by the ascending technique. The plates were then dried and stored in the refrigerator. This treatment apparently reactivated the ion-exchange capacity and also removed a yellow discoloration material.

The samples of oligonucleotides (~100-150 Å_260 in 100 µl) were applied to the TLC plate as a narrow band along with dye marker^{22} on each side of the sample. The plastic sheet was placed on a glass plate (20 x 20 cm) and a Whatman 3 MM paper was then clipped to the top of the plate and uniformly pressed against the PEI-plate by a plastic strip (2 cm x width of plate). The plate was first developed in water for 3 cm and then in solvent E, 0.6 M lithium chloride-7 M urea-0.025 M Tris (pH 8.0) at 60° till the blue dye was 2 cm from the top, or in solvent F, 0.6 M LiOAc-7 M urea (pH 3.5) at room temperature.

For recovery of materials, the wet plates were washed three times in methanol and then dried. The PEI-cellulose containing the desired band of product was removed and the compound was eluted with 2 M triethylammonium bicarbonate (pH 9.5)
Nucleic Acids Research

Characterization of Completely Unprotected Oligonucleotides Containing 3'-5' Phosphodiester Groups

I. PEI-Cellulose TLC

The mobilities of various oligonucleotides higher than hexamer compounds with respect to the yellow dye are given in Table III.

II. Sequence Analysis

Phosphorylation of Oligonucleotide with T4 Polynucleotide Kinase and [γ-32P] ATP

The oligonucleotide (200 pmol) in 5 μl of solution containing 66 mM Tris-HCl (pH 7.8), 6.7 mM MgCl₂, 15 mM dithiothreitol, and 66 μM [γ-32P] ATP was incubated at 37° with 2-4 units of T₄ polynucleotide kinase for 40 min. The reaction was stopped by the addition of excess EDTA. The 5'-32P labeled oligonucleotides were purified and desalted on a Sephadex G-50 column (fine) using 0.1 M triethylammonium bicarbonate (pH 8.0) as buffer.

Partial Venom Phosphodiesterase Digestion of 32P-labelled Oligonucleotides

The 5' -labeled oligonucleotide was digested at 37° in a 10 μl solution containing 30 μg of RNA carrier (partially hydrolyzed RNA), 50 mM Tris-HCl (pH 8), 5 mM MgCl₂ and 0.2 μg of venom phosphodiesterase. One-microliter samples were withdrawn at intervals of 5, 10, 20, 40, 60, 90, 120 and 180 min and blown into 50 μl of 0.1 M EDTA. The combined digest was dried in a desiccator, dissolved in 2 to 5 μl of water and applied to a cellulose-acetate strip (2.5 x 52 cm). Electrophoresis was carried out in pyridine-acetate buffer, pH 3.5, at 2500 volts for 30 to 60 min. The oligonucleotides were transferred onto a DEAE-cellulose thin layer plate. The plates were developed with water for 30 min and then in 2% partially hydrolyzed RNA containing 7 M urea (Homo-Mix) at 65° until the blue dye marker was within 1 inch of the top. The x-ray fingerprinting of two dodecamers is given in Figures 4 and 5.

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