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Synthesis of the human insulin gene. Part III[1]. Chemical synthesis of 5'-phosphomonoester group containing deoxyribooligonucleotides by the modified phosphotriester method. Its application in the synthesis of seventeen fragments constituting human insulin C-chain DNA

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ABSTRACT

A method for phosphorylating a protected deoxyribooligonucleotide containing phosphotriester linkages is described. The modified phosphotriester method of chemical synthesis is further refined in terms of (i) better final deblocking conditions and (ii) new chromatography solvent systems containing acetone-water-ethyl acetate to yield pure oligomers. The effectiveness of these improvements has been demonstrated in the rapid and efficient synthesis of seventeen fragments constituting the sequence of human insulin C-chain DNA.

INTRODUCTION

The synthesis of well-defined sequences of DNA are of great importance in the study of a gene function and its regulation. These can either be prepared by (i) synthesis of the complementary DNA of a messenger RNA using the reverse transcriptase enzyme or (ii) isolation of a restriction enzyme digested fragment of a genome. An alternative approach is the chemical-enzymatic synthesis of a well-defined tailor-made DNA sequence. The most consuming and laborious part in this approach is the chemical synthesis of short deoxyribooligonucleotides. In 1973, we reported modified phosphotriester approach[3]. Along with the development of various new coupling and phosphorylating reagents[4,5], this method has become a rapid and efficient one as an alternative to the phosphodiester method developed by Khorana and his coworkers[6].

In this paper, we now wish to report (i) a chemical method to introduce a 5'-phosphomonoester group to a deoxyribooligonucleotide containing phosphotriester linkages. These phosphorylated compounds are important for various enzymatic reactions such as DNA ligation and also for physico-chemical studies; (ii) two-step ammonia treatment as a final deblocking step to improve

the yields of the final products; (iii) new solvent systems containing acetone-water-ethyl acetate for the better purification of fully protected deoxyribooligonucleotides; (iv) the synthesis of seventeen fragments which constitute a human C-chain insulin DNA (Figure 1) and confirmation of their sequences by mobility shift method (Figure 2).

RESULTS AND DISCUSSION

Objective of synthesizing human C-chain DNA

Human proinsulin consisting of 86 amino acid residues is a biosynthetic precursor protein. Biologically active insulin is produced by folding the proinsulin to form disulfide bridges between the A and B chains followed by the

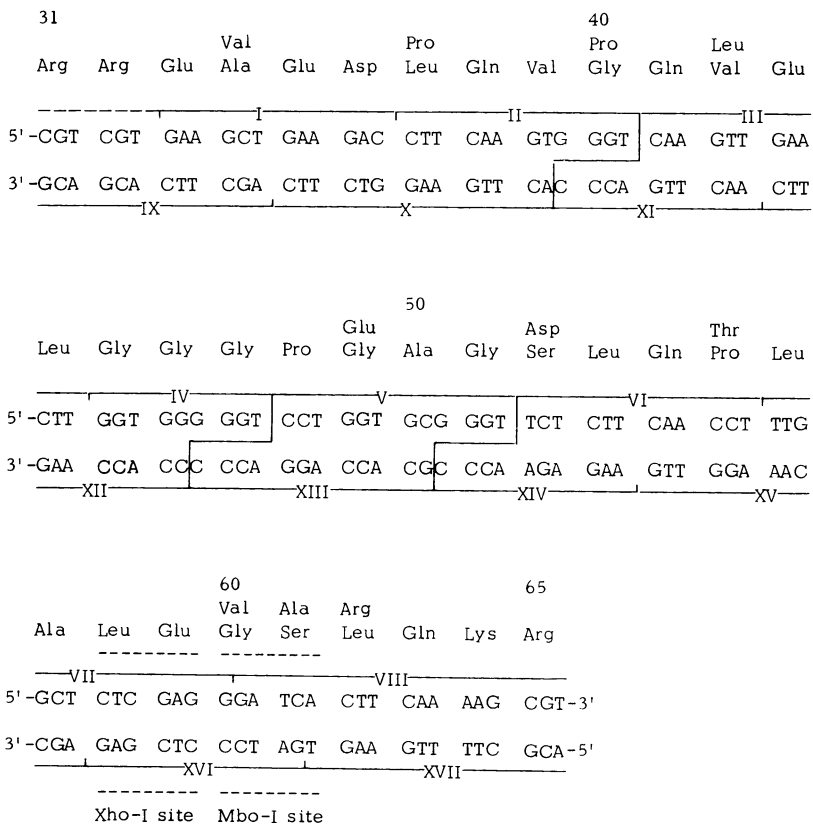
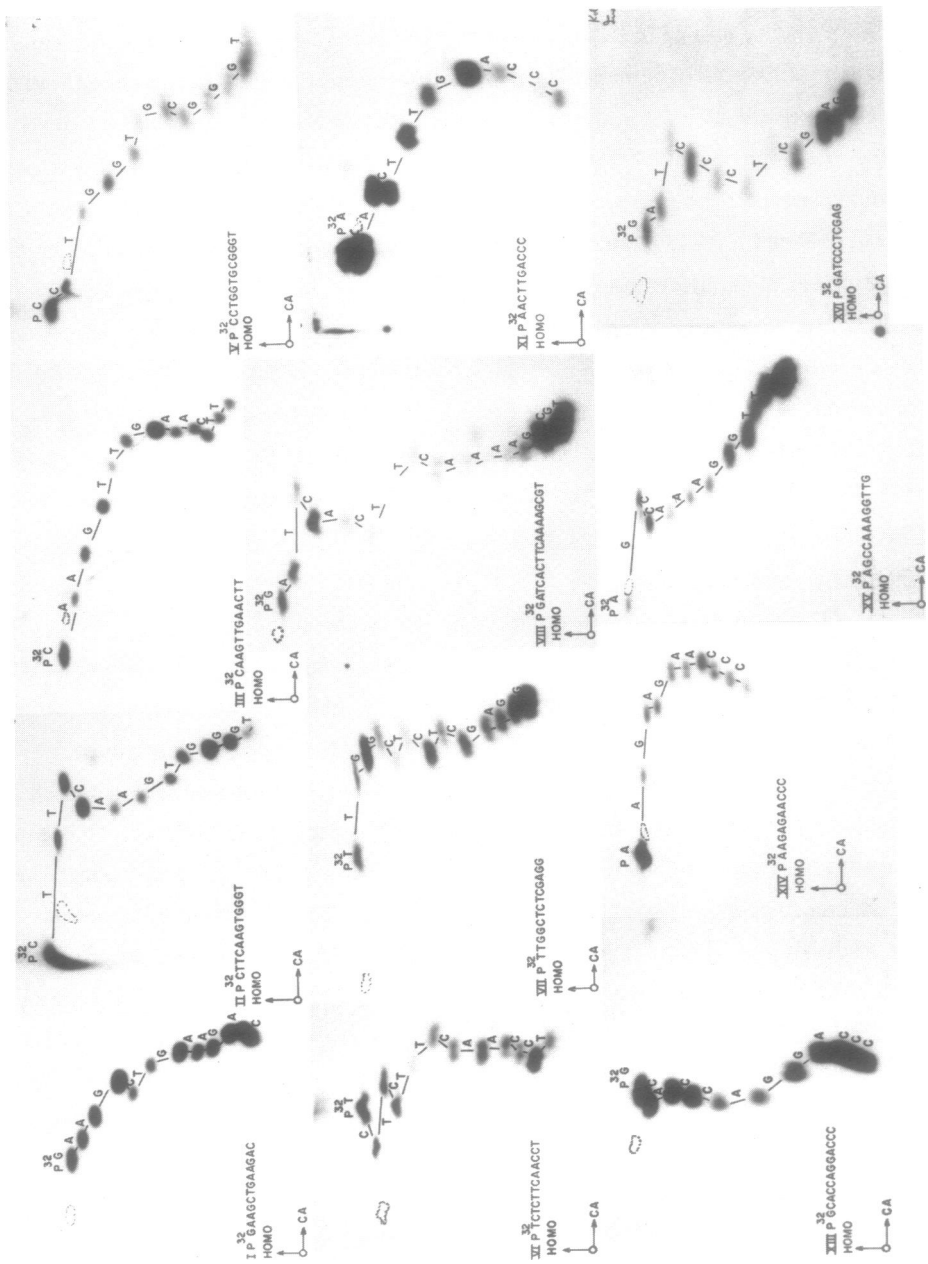


Figure 1. Amino acid sequence of human-like insulin C-chain DNA.

proteolytic cleavage release of the C-chain. In our studies on the synthesis of biologically active insulin, we also wished to study the effect of the size of C-chain on the proper folding of A- and B-chain of insulin in generating a biologically active insulin molecule. With this objective in mind, we synthesized fragments II, IV and V containing a common tetranucleotide 5'-GGGT at the 3'-end in the upper strand. Similarly, the fragments XI, XIII and XIV in the lower strand contained the common tetranucleotide complementary sequence 5'-ACCC-3' at their 5'-ends. The DNA corresponding to various sizes of C-chain could be constructed by ligating the appropriate fragments. After cloning the family of synthetic genes with different lengths of C-chain coding sequence, proinsulin protein molecules with different sized C-chain will be isolated from the transformed cells. We also introduced the two restriction sites, Xho-I and Mbo-I, in C-chain DNA duplex for their potential use in studying the role of intervening sequence in processing to messenger RNA in various biological systems.

Phosphorylation of 5'-hydroxyl group of a deoxyribooligonucleotide containing phosphotriester linkages

Oligonucleotides containing 5'-phosphomonoester group are useful intermediates for generating longer DNA by DNA-ligase reaction. Although 5'-phosphomonoester can be introduced by T_4-polynucleotide kinase[7] reaction, a chemical method is still desirable for producing large amounts for their physico-chemical studies. However, various phosphorylating reagents such as phosphoryl chloride in pyridine-water[8], aryl phosphoramidochlorides[9], O-aryl S-methyl phosphorochloridothioates[10] and 5-chloro-8-quinolyl phosphate[11] in the presence of 2,2'-dipyridyl diselenide [$(PySe)_2$] and triphenylphosphine [Ph_3P] have been reported in the literature but their preparations as well as deblocking conditions were tedious. With our aim to develop a simple procedure we tried β-cyanoethyl phosphate in the presence of mesitylenesulfonyl tetrazole[5] to phosphorylate dodecamer AGCCAAAGGTTG (XV) and found that the yield of the desired product was poor because of the formation of various side products. However, the desired phosphorylation reaction was achieved by treating protected oligonucleotide containing free 5'-hydroxyl group with β-cyanoethyl phosphate and mesitylenesulfonyl triazole in overnight time period. The completion of phosphorylation reaction was indicated by the retention of all


I P GAAGCTGAAGAC
HOMO
CA
II P CTTCAAGTGGGT
HOMO
CA
III P CAAGTTGAACTT
HOMO
CA
V P CCTGGTGCGGGT
HOMO
CA
VI P TCTCTTCAACCT
HOMO
CA
VII P TTGGCTCTCGAGG
HOMO
CA
XI P AACTTGACCC
HOMO
CA
XIII P GATCACTTCAAAAGCGT
HOMO
CA
XIV P AAGAGAACCC
HOMO
CA
XV P AGCCAAAGGTTG
HOMO
CA
XVI P GATCCCTCGAG
HOMO
CA
XIII P GCACCAGGACCC
HOMO
CA

the uv absorbing material at the origin of silica gel tlc plates because of the presence of cyanoethyl phosphodiester function group. The presence of 5'-phosphate was established by completely deblocking the compound with concentrated ammonia followed by PEI-tlc chromatography. In almost every case the mobility of 5'-phosphate containing oligomer was about half the starting material. On treatment with bacterial alkaline phosphatase, it generates the corresponding 5'-hydroxyl oligomer identical to the starting material. The isolated yield of the phosphorylated product is about 75%.

New solvent systems for the purification of fully protected deoxyribooligonucleotides

In our present studies, we further improved deactivated silica gel tlc by introducing new solvent systems such as acetone-water-ethyl acetate in the purification of fully protected intermediates. This solvent was found to resolve oligomers based on the pyrimidine and purine content. For example, an oligomer containing a pyrimidine-rich sequence moved faster than one rich in purine bases. All the compounds ranging from dinucleotides to pentadecanucleotides were purified by this new solvent system.

Rapid and large-scale synthesis of sixteen dinucleotides

From the practical point of view, we found that the availability of all sixteen dinucleotide blocks enabled the rapid build-up of any deoxyribooligonucleotide sequence. The large-scale purification of all the dinucleotides was performed using deactivated silica gel chromatography using new solvent systems as described above except TG, AA, GA and AG. Excellent purification of these four dimers was achieved by reversed phase[12] fast column chromatography on silanized silica gel using acetone-water system. The desired compound was

Figure 2. Two-dimensional chromatographic fingerprint of synthetic deoxyribooligonucleotides after partial snake venom phosphodiester digestion of (I) d^{32}PG-A-A-G-C-T-G-A-A-G-A-C; (II) d^{32}PC-T-T-C-A-A-G-T-G-G-G-T; (III) d^{32}PC-A-A-G-T-T-G-A-A-C-T-T; (V) d^{32}PC-C-T-G-G-T-G-C-G-G-G-T; (VI) d^{32}PT-C-T-C-T-T-C-A-A-C-C-T; (VII) d^{32}PT-T-G-G-C-T-C-T-C-G-A-G-G; (VIII) d^{32}PG-A-T-C-A-C-T-T-C-A-A-A-A-G-C-G-T; (XI) d^{32}PA-A-C-T-T-G-A-C-C-C; (XIII) d^{32}PG-C-A-C-C-A-G-G-A-C-C-C; (XIV) d^{32}PA-A-G-A-G-A-A-C-C-C; (XV) d^{32}PA-G-C-C-A-A-A-G-G-T-T-G; (XVI) d^{32}PG-A-T-C-C-C-T-C-G-A-G. The first dimension is electrophoresis on cellulose acetate strip at pH 3.5 and the second dimension is homochromatography on 20 x 20 cm DEAE-cellulose plate.

isolated in high yields by extracting the eluent with methylene dichloride.

Improved final deblocking reaction condition of fully protected deoxyribooligonucleotides

The final and complete removal of all the protecting groups from the fully protected oligomers containing phosphotriester linkages is the most critical step in successful application of the phosphotriester method. In our earlier studies, we investigated various conditions such as (i) sodium hydroxide-dioxane[13]; (ii) tetra-alkylammonium fluoride in tetrahydrofuran-pyridine-water[13]; and (iii) concentrated ammonia treatment[14] for 5 hr at 50°C. Out of all these, concentrated ammonia treatment was found to give reasonable yields (70-80%) of the desired product. In some cases, it was found to cause incomplete deblocking of the p-chlorophenyl group of longer oligomers. In our present studies, we have modified this procedure to a two-step method. The protected oligonucleotide was treated with concentrated ammonia for 1-2 days at room temperature followed by heating at 50°C for 5 hours. Under these conditions, unprotected oligomer was achieved in 80-90% yield. Recently, Reese et al.[15] reported that oximate ion caused complete deblocking of protected dinucleotides with minimum formation of side products. We applied this reagent in our larger size oligonucleotides (12-19-mers) and found that syn-pyridine-2-aldoxime followed by concentrated ammonia treatment does virtually remove all the protecting group with minimum side products[16]. Although the oximate ion does give somewhat purer product, it involves a tedious work-up and does not seem to be practical for routine deblocking step as compared to our revised method with ammonia treatment (see experimental section).

Isolation and characterization of oligomers containing 3'→5'-phosphodiester linkages

The unprotected compounds were purified by PEI-tlc and preparative electrophoresis on 20% gel as described previously[12]. Each of the fragments was labelled with [γ-^{32}P]-ATP and T_4-polynucleotide kinase and isolated by the precipitation procedure[17]. The base sequence of each fragment was confirmed by the mobility shift method[18] and radioaudiograms of the fingerprintings of various fragments are shown in Figure 2.

EXPERIMENTAL SECTION

General methods and materials

The materials used in the present studies have been reported earlier[1].

New solvent systems for deactivated silica gel tlc chromatography

The following new solvent systems on silica gel tlc plates are used in the preparative scale purification of fully protected dimers to pentadecamer.

I. Acetone-water-ethyl acetate (6:1.5:92.5 v/v) for di- and trimers.

II. Acetone-water-ethyl acetate (18:5:77 v/v) for tetra-heptamers.

III. Acetone-water-ethyl acetate (24:6:70 v/v) for octa-pentadecamers.

General method of large-scale rapid synthesis of twelve fully protected dinucleotides containing TT, TC, TA, CT, CC, CA, CG, AT, AC, AG, GT, GC sequences

5'-Dimethoxytrityl N-protected deoxymononucleoside 3'-p-chlorophenylphosphate (5.0 mmoles) and 5'-hydroxyl deoxymononucleoside 3'-p-chlorophenyl β-cyanoethyl phosphate (4.5 mmoles) in anhydrous pyridine (30 ml) was reacted with mesitylenesulfonyl tetrazole (9 mmoles) at room temperature for 1 hr. The mixture was then decomposed with cold water (5 ml) and the resultant solution was evaporated to a gum in vacuo. The gum was dissolved in ice cold chloroform (200 ml) and washed with 5% sodium bicarbonate solution (2 x 100 ml) and then water (1 x 100 ml). The organic layer was dried over sodium sulfate. The desired product was isolated by fast column chromatography on silica gel type 60 (230-400 mesh) tightly packed in methylene chloride (weight ratio between silica gel to the material applied is 5 to 1) and eluted with solvent I: acetone-water-ethyl acetate (6:1.5:92.5 v/v) (3 litres) at a flow rate of 30 ml/min. The pure fully protected dimers were isolated in (60-75%) yields in less than 2 hr.

The large-scale purification of all the dinucleotides was achieved using deactivated silica gel chromatography system described above except TG, AA, GA and GG. In these cases, excellent purification was accomplished by reversed phase column chromatography as described below.

Rapid and large-scale purification of fully protected dinucleotides on reversed phase column chromatography

The crude reaction mixture of fully protected dinucleotide [$(MeO)_2Tr$]-

dIsoG^{i-Bu}bzA^{bz}CE (8 g) in acetone solution (15 ml) was added to a column (4 cm diameter) of lightly packed RP-2 silica gel (E. Merck, 70-320 μ mesh) (30 g) in 40% water-acetone solvent. It was next followed by the addition of water (15 ml) which precipitated the crude product. The solution was drained into the absorbent under mild air pressure. The column was then eluted with 40% water-acetone solvent (3 litres) at a flow rate of 30 ml/min. Fractions of 100 ml were collected. The separation pattern was followed by analytical tlc on silica gel (solvent I: acetone-water-ethyl acetate (6:1.5:92.5 v/v)) and reversed phase tlc (30% water-acetone). The fractions containing pure product were combined and extracted with equal volume of dichloromethane. Subsequent evaporation of the dichloromethane solvent in vacuo yields 5.3 g of pure [$(MeO)_2$Tr]-dIsoG^{i-Bu}bzA^{bz}CE.

Similar procedure was also used in the purification of fully protected TG, AA and AG.

General method of synthesis and purifying fully protected deoxyribooligonucleotides

The synthesis of fully protected hexamer [$(MeO)_2$Tr]dT^{i-Bu}IsobG^{i-Bu}bzA^{bz}bzC^{bz}bzC^{bz}bzC-OBz was achieved by condensing [$(MeO)_2$Tr]dT^{i-Bu}IsobG-ClPh (100 μmoles) with dbzA^{bz}bzC^{bz}bzC^{bz}bzC (OBz) (60 μmoles) in anhydrous pyridine solution (4 ml) using mesitylenesulfonyl tetrazole (300 μmoles) for 1 hr at room temperature. After the usual work-up as described above, the desired fully protected hexanucleotide was isolated by preparative silica gel tlc using solvent II: acetone-water-ethyl acetate (18:5:77 v/v) in 65% yield.

Similarly, the purification of all the seventeen fragments in their fully protected stage as reported in Table I was carried out by preparative silica gel tlc using solvent III: acetone-water-ethyl acetate (24:6:70 v/v). The mixture was resolved into well-defined bands. The desired band was eluted with methylene chloride-methanol (8:2 v/v) in 38-60% yield. The reaction conditions and yields are documented in Table I.

General method of phosphorylating 5'-hydroxyl group of a protected deoxyribooligonucleotide

The 5'-dimethoxytrityl group of a fully protected dodecamer having AGCCAAAGGTTG (XV) sequence was removed by 2% benzenesulfonic acid in chloroform solution according to the published procedure[14]. After purification

on silica gel tlc, the resultant material (0.005 mmole) was mixed with the pyridinium salt of β-cyanoethyl phosphate (0.05 mmole) and co-evaporated to dryness with anhydrous pyridine. The residue was redissolved in anhydrous pyridine (0.5 ml) and treated overnight with mesitylenesulfonyl triazole (0.12 mmole). After the completion of reaction as indicated by the appearance of a single spot at the origin on silica gel tlc using methylene chloride-10% methanol, the reaction mixture was then diluted with chloroform (1 ml). The unreacted β-cyanoethyl phosphate was removed by washing with water (0.5 ml). The chloroform layer was then concentrated to a gummy residue and deblocked by treatment with concentrated ammonia by a two-step method as described in the next section. The 5'-phosphorylates component containing 3'→5'-phosphodiester linkage was purified by PEI-tlc plate using 0.7 M LiCl-7 M urea at 60°C and the slower moving predominant band (R_f 0.5 with respect to the starting material) was isolated in 75% yield. The presence of 5'-phosphate group was demonstrated by its complete susceptibility to bacterial alkaline phosphatase digestion and the generation of the starting material as checked by PEI-tlc as well as its sequence analysis.

Final deblocking reaction

1. Two-step concentrated ammonia treatment

A protected deoxyribooligonucleotide containing free 5'-hydroxyl group (5 mg) was treated with concentrated ammonia (1.0 ml) containing pyridine (0.1 ml) for 1-2 days at room temperature followed by heating to 50°C for 5 hr. After removing ammonia carefully by evaporation to dryness in vacuo, the residue was suspended in water (0.1 ml), washed with ether and the aqueous solution was chromatographed on PEI-tlc plate. The major band was isolated in 80-90% yield.

2. Treatment with oximate ions

A protected deoxyribooligonucleotide containing free 5'-hydroxyl group (3 mg) was treated with 2-pyridine carboxaldoxime[15] in dioxane (0.6 M, 0.2 ml) and 1, 1, 3', 3'-tetramethylguanidine in water (0.6 M, 0.2 ml) with occasional stirring at room temperature. After 4 hr additional 1, 1, 3', 3'-tetramethylguanidine (0.6 ml) was added and the solution kept for 16 hr. It was next treated with concentrated ammonia (1 ml) for another 16 hr at room temperature and then heated for 5 hr at 50°C. The reaction mixture was concentrated to dryness

Table I. Summary of the reaction conditions and yields of deoxyribooligonucleotide synthesis constituting human-like insulin-C DNA

5'-Protected component[a] (μmole)	5'-Hydroxyl component (μmole)	Condensing reagent[b] (μmole)	Reaction time (min)	Product isolated[c] (yield %)	Deblocked product
[(MeO)$_2$Tr]dIsobG-bzA-bzA-IsobG-ClPh (35)	dbzC-T-IsoG-bzA-bzA-IsobG-bzC-T-IsobG-bzA-bzA-IsobG-bzA-bzC-OBz (30)	150	30	[(MeO)$_2$Tr]dIsobG-bzA-bzA-IsobG-bzC-T-IsobG-bzA-bzA-IsobG-bzA-bzC-OBz (45)	dG-A-A-G-C-T-G-A-A-G-A-C (I)
[(MeO)$_2$Tr]dbzC-T-T-bzC-ClPh (31)	dbzA-bzA-IsobG-T-IsobG-IsobG-IsobG-T-OBz (27)	100	30	[(MeO)$_2$Tr]dbzC-T-T-bzC-bzA-bzA-IsobG-T-IsobG-IsobG-IsobG-T-OBz (55)	dC-T-T-C-A-A-G-T-G-G-G-T (II)
[(MeO)$_2$Tr]dbzC-bzA-bzA-IsobG-ClPh (25)	dT-T-IsobG-bzA-bzA-bzC-T-T-OBz (24)	150	30	[(MeO)$_2$Tr]dbzC-bzA-bzA-IsobG-T-T-IsobG-bzA-bzA-bzC-T-T-OBz (60)	dC-A-A-G-T-T-G-A-A-C-T-T (III)
[(MeO)$_2$Tr]dIsobG-IsobG-T-IsobG-ClPh (60)	dIsobG-IsobG-IsobG-IsobG-T-OBz (40)	350	30	[(MeO)$_2$Tr]dIsobG-IsobG-T-IsobG-IsobG-IsobG-IsobG-IsobG-T-OBz (35)	dG-G-T-G-G-G-G-G-T (IV)
[(MeO)$_2$Tr]dbzC-bzC-T-IsobG-ClPh (23)	dIsobG-T-IsobG-bzC-IsobG-IsobG-IsobG-T-OBz (20)	150	30	[(MeO)$_2$Tr]dbzC-bzC-T-IsobG-IsobG-T-IsobG-bzC-IsobG-IsobG-IsobG-T-OBz (42)	dC-C-T-G-G-T-G-C-G-G-G-T (V)
[(MeO)$_2$Tr]dT-bzC-T-T-bzC-ClPh (32)	dT-T-bzC-bzA-bzA-bzC-bzC-T-OBz (16)	120	30	[(MeO)$_2$Tr]dT-bzC-T-T-bzC-T-T-bzC-bzA-bzA-bzC-bzC-T-OBz (58)	dT-C-T-C-T-T-C-A-A-C-C-T (VI)
[(MeO)$_2$Tr]dT-T-IsobG-IsobG-ClPh (47)	dbzC-T-bzC-T-bzC-IsobG-bzA-IsobG-IsobG-OBz (30)	150	30	[(MeO)$_2$Tr]dT-T-IsobG-IsobG-bzC-T-bzC-T-bzC-IsobG-bzA-IsobG-IsobG-OBz (46)	dT-T-G-G-C-T-C-T-C-G-A-G-G (VII)
[(MeO)$_2$Tr]dIsobG-bzA-T-bzC-bzA-ClPh (36)	dbzC-T-T-bzC-bzA-bzA-bzA-bzA-bzA-IsobG-bzC-IsobG-T-OBz (18)	180	30	[(MeO)$_2$Tr]dIsobG-bzA-T-bzC-bzA-bzC-T-T-T-bzC-bzA-bzA-bzA-bzA-IsobG-bzC-IsobG-T-OBz (38)	dG-A-T-C-A-C-T-T-C-A-A-A-A-G-C-G-T (VIII)
[(MeO)$_2$Tr]dbzA-IsobG-bzC-T-ClPh (160)	dT-bzC-bzA-bzC-IsobG-bzA-bzC-IsobG-OBz (178)	900	45	[(MeO)$_2$Tr]dbzA-IsobG-bzC-T-T-bzC-bzA-bzC-IsobG-bzA-bzC-IsobG-OBz (70)	dA-G-C-T-T-C-A-C-G-A-C-G (IX)
[(MeO)$_2$Tr]dbzA-bzC-T-T-IsobG-bzA-ClPh (10)	dbzA-IsobG-IsobG-T-bzC-T-T-bzC-OBz (9)	50	50	[(MeO)$_2$Tr]dbzA-bzC-T-T-T-IsobG-bzA-bzA-IsobG-IsobG-T-bzC-T-T-T-bzC-OBz (42)	dA-C-T-T-G-A-A-G-G-T-C-T-T-C (X)

(cont'd)

Table I (cont'd)

5'-Protected component[a] (μmole)	5'-Hydroxyl component (μmole)	Condensing reagent (μmole)	Reaction time (min)	Product isolated (yield %)	Deblocked product
[(MeO)$_2$Tr]dbzA$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$T-ClPh (50)	dT$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzC-OBz (30)	250	40	[(MeO)$_2$Tr]dbzA$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$T$\bar{+}$T$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzC-OBz (50)	dA-A-C-T-T-G-A-C-C-C (XI)
[(MeO)$_2$Tr]dbzC$\bar{+}$bzC$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC-ClPh (39)	dbzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$T$\bar{+}$T$\bar{+}$bzC-OBz (20)	200	30	[(MeO)$_2$Tr]dbzC$\bar{+}$bzC$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$T$\bar{+}$T$\bar{+}$bzC-OBz (55)	dC-C-A-C-C-A-A-G-T-T-C (XII)
[(MeO)$_2$Tr]dIsobG$\bar{+}$bzC$\bar{+}$bzA$\bar{+}$bzC-ClPh (50)	dbzC$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzC-OBz (30)	250	45	[(MeO)$_2$Tr]dIsobG$\bar{+}$bzC$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzC-OBz (40)	dG-C-A-C-C-A-G-G-A-C-C-C (XIII)
[(MeO)$_2$Tr]dbzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$bzA-ClPh (60)	dIsobG$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzC-OBz (40)	300	30	[(MeO)$_2$Tr]dbzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzC-OBz (60)	dA-A-G-A-G-A-A-C-C-C (XIV)
[(MeO)$_2$Tr]dbzA$\bar{+}$IsobG$\bar{+}$bzC$\bar{+}$bzC-ClPh (35)	dbzA$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$IsobG$\bar{+}$T$\bar{+}$T$\bar{+}$IsobG-OBz (25)	170	40	[(MeO)$_2$Tr]dbzA$\bar{+}$IsobG$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$IsobG$\bar{+}$T$\bar{+}$T$\bar{+}$IsobG-OBz (40)	dA-G-C-C-A-A-A-G-G-T-T-G (XV)
[(MeO)$_2$Tr]dIsobG$\bar{+}$bzA$\bar{+}$T$\bar{+}$bzC-ClPh (50)	dbzC$\bar{+}$bzC$\bar{+}$T$\bar{+}$bzC$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$IsobG-OBz (35)	250	60	[(MeO)$_2$Tr]dIsobG$\bar{+}$bzA$\bar{+}$T$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$bzC$\bar{+}$T$\bar{+}$bzC$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$IsobG-OBz (45)	dG-A-T-C-C-C-T-C-G-A-G (XVI)
[(MeO)$_2$Tr]dbzA$\bar{+}$bzC$\bar{+}$IsobG$\bar{+}$bzC$\bar{+}$T$\bar{+}$T-ClPh (25)	dT$\bar{+}$T$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$T-OBz (20)	125	60	[(MeO)$_2$Tr]dbzA$\bar{+}$bzC$\bar{+}$IsobG$\bar{+}$bzC$\bar{+}$T$\bar{+}$T$\bar{+}$T$\bar{+}$T$\bar{+}$IsobG$\bar{+}$bzA$\bar{+}$bzA$\bar{+}$IsobG$\bar{+}$T-OBz (52)	dA-C-G-C-T-T-T-T-G-A-A-G-T (XVII)

[a] Abbreviations are as suggested by IUPAC-IUB (1970) Biochemistry 9, 4022. A phosphodiester linkage is represented by hyphen and phosphotriester linkage is represented by ($\bar{+}$) symbol. Each internal internucleotidic phosphate is protected by p-chlorophenyl (ClPh) group.

[b] Mesitylenesulfonyl tetrazole (Ms-Tet).

[c] Each of the fully protected final oligomers was purified by silica gel tlc using solvent III: acetone-water-ethyl acetate (24:6:70 v/v).

under vacuo. The residue was dissolved in water (1 ml) and washed with ether (2 x 1 ml) to remove ether-soluble material. It was next desalted by passing through a short DEAE-cellulose (0.5 ml) column (packed in a disposable pipette). The oligonucleotide material was eluted with triethylammonium bicarbonate (2 M, 0.5 ml). After evacuating the salt in vacuo and purifying on PEI-tlc using appropriate lithium chloride-7 M urea, the desired compound was obtained in about 90% yield.

Characterization of synthetic deoxyribooligonucleotide

1. Purification

The compounds isolated by tlc on PEI-plates were further purified by 20% gel electrophoresis[12] on a 20 x 40 cm slab.

2. 5'-Labelling of synthetic deoxyribooligonucleotide and its isolation by precipitation procedure

Each fragment was labelled with [γ-^{32}P]-ATP and isolated by the precipitation procedure[17] as described below.

Deoxyribooligonucleotide (100-200 pmoles) in 10 μl of solution containing 50 mM Tris-HCl (pH 9.0), 100 mM $MgCl_2$, 5 mM dithiothritol and 60 μM [γ-^{32}P]-ATP was incubated at 37°C with 2-4 units of T_4-polynucleotide kinase for 45 min. The reaction was terminated by adding 100 μl of 4 M ammonium acetate and 2 μl of carrier tRNA (10 mg/μl) and then 0.6 ml of cold ethanol. It was chilled at -70°C for 5 min, centrifuged at 10 x 1000 g for 5 min (Eppendorf Centrifuge) and the supernatant removed. The radioactive precipitate was redissolved in 200 μl of 0.3 M sodium acetate, 0.6 ml of cold ethanol added, mixed, cooled to -70°C/5 min, spun at 10 x 1000 g for 5 min and the supernatant removed. To the precipitate was added once again 0.6 ml of ethanol, spun for 3 min and the supernatant removed. The precipitate oligonucleotide was dried under vacuo, dissolved in 20 μl of loading buffer containing dye markers and electrophoresed on 20% gel.

3. Sequence analysis

The expected band containing 5'-^{32}P-oligomer was isolated from the gel by extracting with 0.25 M triethylammonium bicarbonate. The salt was removed by passing through DEAE-cellulose column (0.1 ml). The purified compound was sequenced by the mobility shift method[18] and in each case the expected pattern was obtained as shown in Figure 2.

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