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Synthesis of the human insulin gene. Part II[1]. Further improvements in the modified phosphotriester method and the synthesis of seventeen deoxyribooligonucleotide fragments constituting human insulin chains B and mini-C DNA

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ABSTRACT

The purification of protected deoxyribooligonucleotides containing phosphotriester internucleotidic linkages has been improved by developing a deactivated silica gel chromatographic technique. The efficiency of this technique as applied in the modified phosphotriester approach has been demonstrated in the rapid synthesis of seventeen pure fragments constituting the sequence of human insulin B and mini-C DNA. The sequence of each oligomer was confirmed by the two dimensional mobility shift method of fingerprinting.

INTRODUCTION

The synthesis of deoxyribooligonucleotides has always been the most time consuming and laborious part of gene synthesis. The modified phosphotriester approach developed in our laboratory[3,4] offers a much simpler and more efficient method of synthesis of these compounds. The main features of our approach are (i) use of 5'-dimethoxytrityldeoxyribomononucleoside 3'-phosphotriester (monomer) as a starting material; (ii) condensation between 5'-dimethoxytrityldeoxyribomononucleoside 3'-phosphodiester with a 5'-hydroxy-containing component yields a product containing internucleotidic phosphotriester linkages as neutral species which are amenable to all the conventional tools of organic chemistry for their isolation; (iii) formation of the desired product in a higher yield because of the absence of side products. In particular, degradation of internucleotidic phosphotriester linkages in 2-4 hr reaction period and the formation of the pyrophosphates as commonly encountered in the case of the phosphodiester approach[5] are avoided. In spite of these advantages, this method still lacked an efficient method of

fractionating the desired product from the starting material. In our previous publication[1], we introduced reversed phase chromatography on silanized silica gel in aqueous acetone solvent systems to purify the desired product from the starting materials. In this paper, we now wish to report a new chromatographic technique, deactivated silica gel chromatography, by which large-scale amounts of desired product can be purified using aqueous organic solvent systems on fast silica gel column chromatography or tlc plates. The efficiency of this technique has been demonstrated by achieving a rapid and efficient synthesis of seventeen deoxyribooligonucleotide fragments constituting the sequence of human insulin B and mini-C chains DNA, as shown in Figure 1.

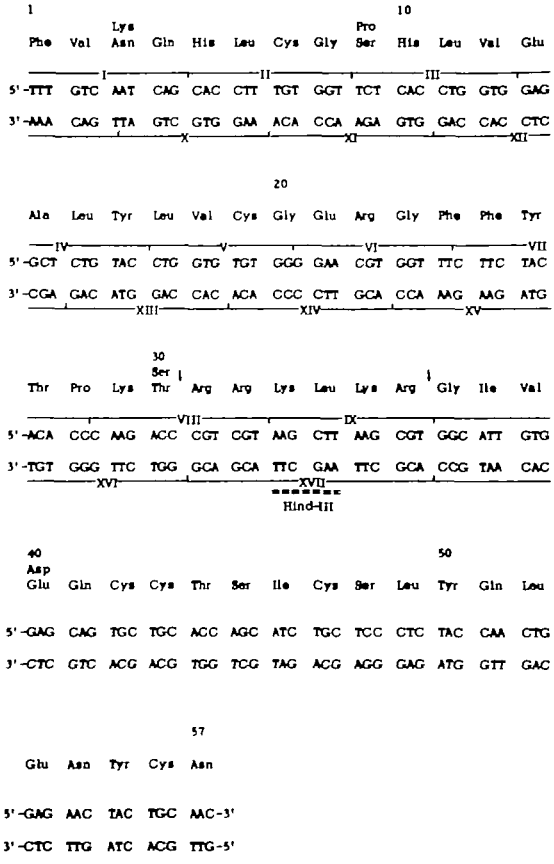

Figure 1. Amino acid sequence of human insulin B and mini-C chains and the corresponding DNA sequence.

The sequence of human-like DNA chain-B was derived from the rat insulin sequence[6] by changing one codon for the amino acid in position (3) from lysine (AAA) to asparagine (AAU), that in position (9) from proline (CCU) to serine (UCU) and that in position (30) from serine (UCC) to threonine (ACC). The sequence of mini-C chain DNA contains only the six amino acid sequence (arg-arg-lys-leu-lys-arg) shown in Figure 1. The basic assumption was that arg-arg at the junction of the B-chain and lys-arg at the A-chain could be sufficient for the mini-C chain to fold human insulin A and B peptides in the proper configuration[7]. The middle dipeptide lys-leu constituting Hind-III DNA site was included to expand the C-chain by adding the appropriate amino acid sequence codons at the Hind-III site. Our rationale of synthesizing sequences closely related to the rat insulin A and B DNA was also to use the synthetic single strand A and B polynucleotides as primer and terminator to copy the insulin C-chain sequence enzymatically from the rat proinsulin gene by the use of the reverse transcriptase enzyme.

Recently the synthesis of human-like insulin A and B DNA derived from the genetic code dictionary has also been reported[8].

RESULTS AND DISCUSSION

The most time-consuming task in the present project was the large-scale synthesis of fully protected monomer, dimer and trimer blocks. To speed up this step in the present studies, we introduced fast column chromatography on deactivated silica gel adsorbent to resolve the complex reaction mixtures while fast column chromatography on silica gel using methylene chloride-methanol was sufficient for the purification of fully protected deoxymononucleotides.

Large-scale synthesis of fully protected mononucleotides

The removal of excess of β-cyanoethanol from the fully protected mononucleotides, especially of adenosine and cytosine prepared by our earlier method[9], was a major problem. In the present study, we overcome this difficulty by precipitating the crude reaction mixture, dissolved in methylene chloride from diethyl ether. The precipitate material was further purified on fast column chromatography on regular silica gel. The absence of β-cyano-

ethanol was monitored by nmr analysis. The main advantage of this procedure is that a large amount of the reaction product (~70 g) can be fractionated on a tightly packed silica gel column (300 g) in less than 2 hr. The fast flow rate was maintained by keeping the solvent level 75 cm above the absorbent. Due to the faster flow rate, the pure desired product was isolated in higher yield because of the decreased contact with the adsorbent. This technique was also found to be more convenient than HPLC because it consumes less solvent.

Large-scale synthesis of fully protected di- and trimer blocks

The general procedure used was to condense a slight excess of 5'-dimethoxytrityldeoxymononucleoside-3'-p-chlorophenyl phosphate (as triethylammonium salt) (1.2 molar equivalent) with the 5'-hydroxyl mono- or dinucleotide component in the presence of mesitylenesulfonyl tetrazole[10] as a coupling reagent. After work-up, the reaction mixture was fractionated by fast column chromatography on tightly packed silica gel using a water-saturated methylene chloride-acetone system (i.e. deactivated silica gel) which was found to give an excellent separation in high isolated yield.

General method of deoxyribooligonucleotide synthesis and isolation by the deactivated silica gel tlc technique

The basic strategy of our synthetic plan is to extend the chain from 3' toward 5'-ends by block addition. Since each of the intermediate fragments contained a 3'-benzoyl protecting group, its characterization after complete deblocking on PEI-tlc plate and by two-dimensional mobility shift fingerprinting method became easier. The general procedure followed in the synthesis of each deoxyribooligonucleotide was as follows: the 3'-O-benzoylated deoxyribooligonucleotide containing a 5'-hydroxyl group was reacted with an excess of 5'-dimethoxytrityldeoxyribooligonucleoside 3'-p-chlorophenyl phosphate (1.2-1.5 molar equivalent) in a concentrated pyridine solution using mesitylenesulfonyl tetrazole (3 molar equivalent) as a coupling reagent. The progress of the reaction was monitored by two tlc systems: the appearance of a slow moving trityl positive spot on reversed phase (RP-2 or RP-18) tlc plate in acetone-containing water (22.5-32.5%); the shifting of the trityl positive spot from the origin to R_f (0.2-0.7) on regular silica gel tlc plates in chloroform containing methanol. Generally the reaction was over within 2 hr. After work-up, the reaction mixture was fractionated by the

deactivated tlc technique using aqueous methylene chloride-acetone solvent systems on regular silica gel tlc plates. This technique was found to give better resolution of the product, in higher yields, than the conventional method of silica gel tlc in chloroform-methanol. It may be that fully protected compounds are more stable on deactivated silica gel absorbent. The main advantages were separation of a large amount of the reaction mixture (~300 mg) per tlc plate and excellent resolution of closely related compounds. The final yields and the reaction conditions are summarized in Table I.

The yield of each coupling reaction was dependent upon the individual sequence of the coupling fragment, with higher yield being obtained for pyrimidine-rich than for purine-rich sequence. All these fully protected compounds were quite stable in an anhydrous foam state at 4° except for those containing GG bases. These had to be freshly prepared before running the coupling reaction to obtain best yields.

Complete deblocking and isolation of product containing 3'-5'-phosphodiester linkages

Each of the synthesized fragments was completely deblocked by our published procedure[4]; that is, treatment with (2%) benzenesulfonic acid in chloroform solution to avoid any depurination, followed by treatment with concentrated ammonia[11] for 4-6 hr at 50°. The desired product was isolated on PEI-tlc plates using 0.5-0.7 M lithium chloride-7 M urea at 50° and its homogeneity was further verified on 20% gel electrophoresis[1]. The main advantage of this technique is that the UV-absorbing band containing the desired compound was easily identified by using the appropriate markers. In each case the major band corresponded to the desired product. The additional minor bands corresponded to the starting materials and minor degradation side products (~10-15%).

Characterization

(A) Deoxyribooligonucleotides containing phosphotriester linkages

The homogeneity of compounds containing phosphotriester linkages was checked by reversed phase tlc. Their purity as well as their base sequence was further confirmed by plasma desorption mass spectrometric techniques; these results are reported elsewhere[12].

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

5'-Protected component[a] (μmole)	5'-Hydroxyl component (μmole)	Condensing reagent (μmole)	Reaction time (hr)	Product isolated (yield %)	Deblocked product
[(MeO)$_2$Tr]dT-T-T-IsobG-ClPh (35)	dT-bzC-bzA-bzA-T-bzC-bzA+IsobG-OBz (20)	180	1.5	[(MeO)$_2$Tr]dT-T-T-IsobG-T-bzC-bzA-bzA-T+bzC-bzA+IsobG-OBz (50)	dT-T-T-G-T-C-A-A-T-C-A-G (I)
[(MeO)$_2$Tr]dbzC-bzA-bzC-bzC-ClPh (30)	dT-T-T-IsobG-T-IsobG+IsobG-T-OBz (15)	120	1.5	[(MeO)$_2$Tr]dbzC-bzA-bzC-bzC-T-T-T+IsobG-T+IsobG-IsobG+T-OBz (55)	dC-A-C-C-T-T-T-G-T-G-G-T (II)
[(MeO)$_2$Tr]dT-bzC-T-bzC-ClPh (25)	dbzA-bzC-bzC-T-IsobG+IsobG-T-IsobG-OBz (11)	100	1.5	[(MeO)$_2$Tr]dT-bzC-T-bzC-bzA-bzC-bzC+T+IsobG+IsobG+T-IsobG-OBz (50)	dT-C-T-C-A-C-C-T-G-G-T-G (III)
[(MeO)$_2$Tr]dIsobG-bzA+IsobG-IsobG+bzC-ClPh (15)	dT-bzC-T-IsobG-T-bzA+bzC-OBz (10)	110	1.5	[(MeO)$_2$Tr]dIsobG-bzA-IsobG-IsobG+bzC-T+bzC-T+IsobG+T+bzA+bzC-OBz (25)	dG-A-G-G-C-T-C-T-G-T-A-C (IV)
[(MeO)$_2$Tr]dbzC-T-IsobG+IsobG-ClPh (20)	dT-IsobG-T-IsobG-T-IsobG+IsobG-OBz (12)	115	1.5	[(MeO)$_2$Tr]dbzC-T-IsobG-IsobG+T-IsobG+T+IsobG+T+IsobG+IsobG-OBz (20)	dC-T-G-G-T-G-T-G-T-G-G (V)
[(MeO)$_2$Tr]dIsobG-IsobG+bzA+bzA-ClPh (28)	dbzC-IsobG-T-IsobG+IsobG+T+T-OBz (13)	120	1.5	[(MeO)$_2$Tr]dIsobG-IsobG+bzA-bzA-bzC+IsobG+T+IsobG+IsobG+T+T+T-OBz (45)	dG-G-A-A-C-G-T-G-G-T-T-T (VI)
[(MeO)$_2$Tr]dbzC-T-T-bzC-ClPh (35)	dT-bzA-bzC-bzA-bzC-bzA+bzC-bzC-OBz (20)	150	1.5	[(MeO)$_2$Tr]dbzC-T-T-bzC-T-bzA+bzC-bzA+bzC+bzA+bzC-OBz (50)	dC-T-T-C-T-A-C-A-C-A-C-C (VII)
[(MeO)$_2$Tr]dbzC-bzA+bzA+IsobG-ClPh (30)	dbzA-bzC-bzC-bzC+IsobG+T+bzC+IsobG+T-OBz (14)	140	1.5	[(MeO)$_2$Tr]dbzC-bzA+bzA+IsobG+bzA+bzC+bzC+bzC+IsobG+T+bzC+T+bzC+IsobG+T-OBz (55)	dC-A-A-G-A-C-C-C-G-T-C-G-T (VIII)
[(MeO)$_2$Tr]dbzA-bzA+IsobG+bzC-ClPh (30)	dT-T-bzA-bzA+IsobG+bzC+IsobG+T-OBz (13)	180	1.5	[(MeO)$_2$Tr]dbzA-A+IsobG-bzC+T-T-bzA+bzA+IsobG+bzC+IsobG+T-OBz (35)	dA-A-G-C-T-T-A-A-G-C-G-T (IX)
[(MeO)$_2$Tr]dbzA-bzA+IsobG+IsobG+T-ClPh (16)	dIsobG-bzC+T+IsobG+bzA+T-OBz (12)	80	3	[(MeO)$_2$Tr]dbzA-bzA+IsobG+IsobG+T+IsobG+bzC+T+IsobG+bzA+T-OBz (45)	dA-A-G-G-T-G-C-T-G-A-T (X)

5'-Protected component[a] (μmole)	5'-Hydroxyl component (μmole)	Condensing reagent (μmole)	Reaction time (hr)	Product isolated (yield %)	Deblocked product
[$(MeO)_2$Tr]dIsobG-T-IsobG-bzA-ClPh (35)	dIsobG-bzA-bzA-bzC-bzC-bzA-bzC-bzA-OAC (30)	200	3	[$(MeO)_2$Tr]dIsobG-T-IsobG-bzA-IsobG-bzA-bzA-bzC-bzC-bzA-bzC-A-OAC (48)	dG-T-G-A-G-A-A-C-C-A-C-A (XI)
[$(MeO)_2$Tr]dbzA-IsobG-bzC-bzC-ClPh (40)	dT-bzC-bzC-bzA-bzC-bzC-bzA-IsobG-OBz (25)	250	3	[$(MeO)_2$Tr]dbzA-IsobG-bzC-bzC-T-bzC-bzC-bzA-bzC-bzC-bzA-IsobG-OBz (44)	dA-G-C-C-T-C-C-A-C-C-A-G (XII)
[$(MeO)_2$Tr]dbzC-bzA-bzC-bzC-ClPh (17)	dbzA-IsobG-IsobG-T-bzA-bzC-bzA-IsobG-OBz (10)	100	3	[$(MeO)_2$Tr]dbzC-bzA-bzC-bzC-bzA-IsobG-IsobG-T-bzA-bzC-bzA-IsobG-OBz (40)	dC-A-C-C-A-G-G-T-A-C-A-G (XIII)
[$(MeO)_2$Tr]dbzA-bzC-IsobG-T-ClPh (34)	dT-bzC-bzC-bzC-bzC-bzA-bzC-bzA-OAC (21)	200	3	[$(MeO)_2$Tr]dbzA-bzC-IsobG-T-T-bzC-bzC-bzC-bzC-bzA-bzC-bzA-OAC (52)	dA-C-G-T-T-C-C-C-C-A-C-A (XIV)
[$(MeO)_2$Tr]dIsobG-T-bzA-IsobG-bzA-ClPh (15)	dbzA-IsobG-bzA-bzA-bzA-bzC-bzC-OBz (15)	60	2.5	[$(MeO)_2$Tr]dbzG-T-bzA-IsobG-bzA-bzA-IsobG-bzA-bzA-bzA-bzC-bzC-OBz (32)	dG-T-A-G-A-A-G-A-A-A-C-C (XV)
[$(MeO)_2$Tr]dIsobG-IsobG-T-ClPh (10)	dbzC-T-T-IsobG-IsobG-IsobG-T-IsobG-T-OBz (2.5)	10	2.5	[$(MeO)_2$Tr]dIsobG-IsobG-T-bzC-T-T-IsobG-IsobG-IsobG-T-IsobG-T-OBz (40)	dG-G-T-C-T-T-G-G-G-T-G-T (XVI)
[$(MeO)_2$Tr]dbzA-bzC-IsobG-bzC-T-T-ClPh (16)	dbzA-bzA-IsobG-bzC-T-T-bzA-bzC-IsobG-bzA-bzC-IsobG-OBz (12)	100	3	[$(MeO)_2$Tr]dbzA-bzC-IsobG-bzC-T-T-bzA-bzA-IsobG-bzC-T-T-bzA-bzC-IsobG-bzA-bzC-IsobG-OBz (36)	dA-C-G-C-T-T-A-A-G-C-T-T-A-C-G-A-C-G (XVII)

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

(B) Deoxyribooligonucleotides containing 3'→5' phosphodiester linkages

Each of the unprotected oligomers was labelled with ATP-[γP^{32}] and polynucleotide kinase and the labelled compound was sequenced by the mobility shift method[13]. The fingerprinting patterns of the seventeen oligomers synthesized in the present studies are presented in Figures 2 and 3.

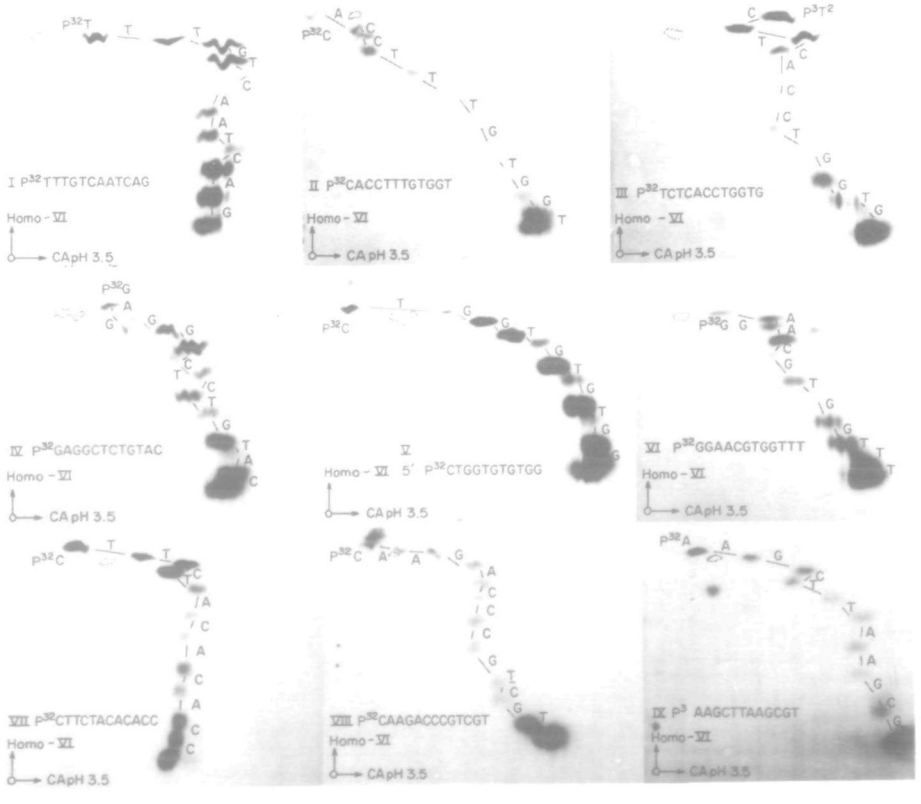

Figure 2. Two dimensional chromatographic fingerprint of synthetic deoxyoligonucleotides (corresponding to upper strand) after partial snake venom phosphodiesterase digestion of (I) d^{32}PT-T-T-G-T-C-A-A-T-C-A-G; (II) d^{32}PC-A-C-C-T-T-T-G-T-G-G-T; (III) d^{32}PT-C-T-C-A-C-C-T-G-G-T-G; (IV) d^{32}PG-A-G-G-C-T-C-T-G-T-A-C; (V) d^{32}PC-T-G-G-T-G-T-G-T-G-G; (VI) d^{32}PG-G-A-A-C-G-T-G-G-T-T-T; (VII) d^{32}PC-T-T-C-T-A-C-A-C-A-C-C; (VIII) d^{32}PC-A-A-G-A-C-C-C-G-T-C-G-T; (IX) d^{32}PA-A-G-C-T-T-A-A-G-C-G-T. 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 tlc plate.

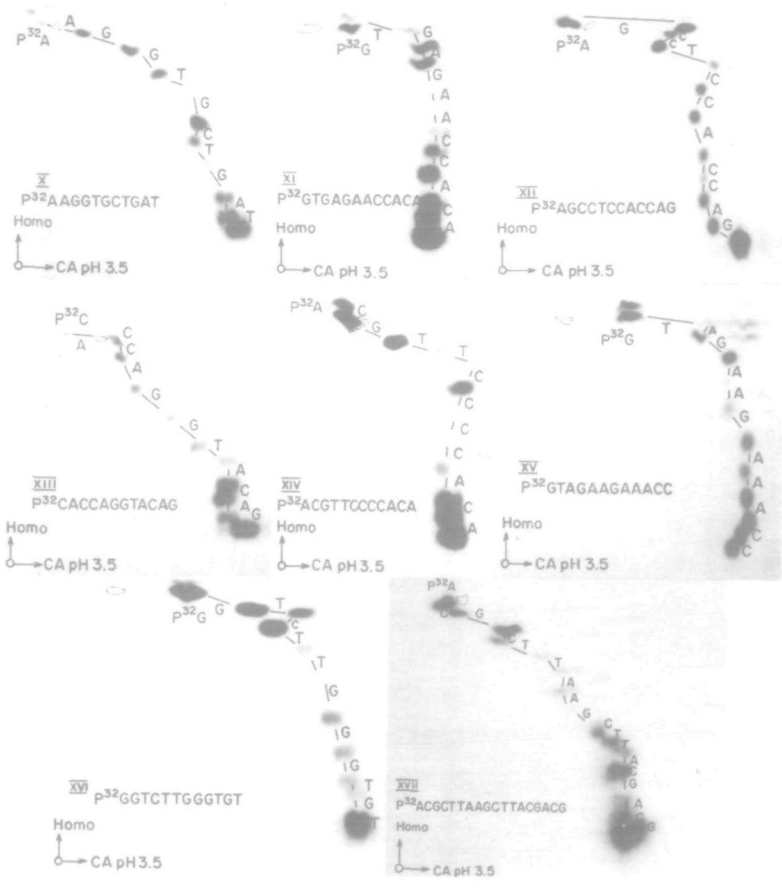

Figure 3. Two dimensional chromatographic fingerprint of synthetic deoxyribo-oligonucleotides (corresponding to the lower strand) after partial snake venom phosphodiesterase digestion of (X) d^{32}PA-A-G-G-T-G-C-T-G-A-T; (XI) d^{32}PG-T-G-A-G-A-A-C-C-A-C-A; (XII) d^{32}PA-G-C-C-T-C-C-A-C-C-A-G; (XIII) d^{32}P-C-A-C-C-A-G-G-T-A-C-A-G; (XIV) d^{32}PA-C-G-T-T-C-C-C-C-A-C-A; (XV) d^{32}PG-T-A-G-A-A-G-A-A-A-C-C; (XVI) d^{32}PG-G-T-C-T-T-G-G-G-T-G-T; (XVII) d^{32}PA-C-G-C-T-T-A-A-G-C-T-T-A-C-G-A-C-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 tlc plate.

Concluding remarks

The results reported in this paper clearly establish the modified phos-

photriester as a simple and efficient method of oligonucleotide synthesis. Combined with recent advancement in DNA recombinant and rapid DNA sequencing techniques[14], the synthesis of a well defined DNA of biological interest has been rendered practical.

EXPERIMENTAL SECTION

General methods and materials

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

Solvent systems

(A) Reversed phase tlc chromatography on (RP-2 or RP-18) plates for fractionating deoxyribooligonucleotides containing phosphotriester linkages

(i) Acetone containing 27.5%-30% water for dimers;

(ii) Acetone containing 25%-27.5% water for trimer-pentamer;

(iii) 0.5 M NaCl[15] in acetone containing 27.5%-30% water for pentamer-nonamer;

(iv) 0.5 M NaCl[15] in acetone containing 25%-27.5% water for nonamer-dodecamer;

(B) Deactivated silica gel tlc chromatography

(v) Methylene chloride-water-acetone (69.5:0.5:30 v/v) for dinucleotides containing 3'-O-benzoate;

(vi) Methylene chloride-water-acetone (59:1:40 v/v) for dinucleotides;

(vi) Methylene chloride-water-acetone (43:2:55 v/v) for trimer and tetramer;

(viii) Methylene chloride-water-acetone (32:3:65 v/v) for pentamer-nonamer;

(ix) Methylene chloride-water-acetone (20:5:75 v/v) for octa-tridecamer.

Modified procedure for the isolation of pure fully protected mononucleotides

(A) 5'-Dimethoxytrityl N-acyl mononucleoside

These compounds were prepared according to the earlier procedure[15]. After work-up, the crude product (~70 g) was dissolved in methylene dichloride (70 ml) and precipitated from petroleum ether (30-60°) (600 ml) by slow addition with vigorous stirring. The clear supernatant was decanted and the precipita-

tion procedure was repeated. The precipitate thus obtained was easily purified by fast column chromatography on tightly packed silica gel using methylene dichloride containing 5% methanol.

(B) 5'-Dimethoxytrityl 5'-acyl mononucleoside 3'-p-chlorophenyl-β-cyanoethyl phosphate

The phosphorylation of monomer was carried out as described earlier[15]. After work-up, residual pyridine and β-cyanoethanol was removed by precipitation with ethyl ether as described above. Final purification was achieved by fast column chromatography on tightly packed silica gel using methylene dichloride containing 5% methanol.

The presence of residual cyanoethanol was detected by two triplets in nmr at chemical shifts of δ 2.21 and 3.48, partially overlapped by signals from the fully protected monomer.

General method of large-scale rapid synthesis of di- and trinucleotides

5'-Dimethoxytrityl N-protected deoxymononucleoside 3'-p-chlorophenyl phosphate (4 mmoles) was coupled in anhydrous pyridine solution (30 ml) with 5'-hydroxyl deoxymono- or dinucleoside 3'-p-chlorophenyl β-cyanoethyl phosphate (3 mmoles) in the presence of mesitylenesulfonyl tetrazole (8 moles) at room temperature for 2 hr. After checking the completion of the reaction by silica gel and reversed phase tlc, the mixture was then decomposed with cold distilled 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 water (1 x 100 ml). The organic layer was dried over sodium sulfate and analyzed by tlc on (a) reversed phase, (b) regular silica gel plates using aqueous organic solvent systems (v)-(vii). The desired product was isolated by fast column chromatography on silica gel type 60 (230-400 mesh) tightly packed in methylene chloride (wt. ratio between silica gel to the material applied is 5 to 1) and eluted with solvent system (v) for dimer or (vi) for trimer. The pure product was isolated in (60-75%) yield in less than 2 hr.

General method of deoxyribooligonucleotide synthesis

Before coupling, each of the intermediate fragments was critically analyzed for its homogeneity on PEI-tlc after complete deblocking (as described below). The sequence of the longer fragments between octamer to dodecamer

are built by growing the chain from the 3'-benzoylated fragment. At each step of condensation, the 5'-dimethoxytrityl oligomer containing 3'-p-chlorophenyl-phosphate component was used in excess 20-30% and condensed with the 5'-hydroxyl oligonucleotide containing a 3'-benzoylated protecting group in the presence of mesitylenesulfonyl tetrazole (3-5 fold excess) for 2 to 4 hr. After the usual work-up as described above, the crude reaction (300 mg/tlc plate) was fractionated by preparative tlc on silica gel plates using solvent systems (viii) and (ix) for octamer-tridecamers. The mixture was resolved into well defined bands. The desired band was eluted with methylene chloride-methanol (20% v/v) and checked for its purity, after complete deblocking step on PEI-tlc plate.

Alternatively, these compounds up to 30 mg per plate could also be fractionated on RP-2 or RP-18 tlc plates using solvent (iii) or (iv) containing 0.5 M NaCl. The reaction conditions and yields for the coupling reactions in the synthesis of seventeen fragments are given in Table I.

Complete deblocking, isolation and characterization of deoxyribooligonucleotides containing 3'→5' phosphodiester linkages

(A) Deblocking procedure

The 5'-dimethoxytrityl group was removed by treating the fully protected compound (5 mg) with 2% benzenesulfonic acid solution in chloroform containing methanol (30% by volume) (1 ml) at 0° for 30 min. After washing the organic layer with 5% sodium bicarbonate (0.5 ml x 2) and water (0.5 ml x 2), and drying, it was next evaporated to dryness under reduced pressure. The residual material was treated with concentrated ammonia (2 ml) containing pyridine (0.2 ml) for 4-6 hr at 50°. After the careful removal of ammonia under vacuo, the residue was redissolved in water (1 ml) and washed with ether (2 x 1 ml) and evaporated to dryness.

(B) Isolation of the desired product

The above aqueous solution was fractionated on PEI-tlc plates (2) using (i) 0.6 M lithium chloride-7 M urea-0.025 tris-HCl pH 7.4 for the sequence of dodecamer size, and 0.7 M lithium chloride-7 M urea-0.025 tris-HCl pH 7.4, for pentadecamer to octadecamer. The compound from the expected band was isolated by eluting with 2 M triethylammonium bicarbonate

pH 9.6 and verified its purity on (20 cm x 40 cm) slab for preparative gel electrophoresis in 20% gel at pH 7.5 as described elsewhere[1].

(C) Characterization

The base sequence of each chemically synthesized oligomer was conformed by two-dimensional mobility shift method and results of each radioaudiography are shown in Figures 2 and 3. The expected fingerprinting was obtained in each case.

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11. J.H. van Boom and his coworker (Nuclear Acid Res. (1979) 6, 2237) used drastic ammonolysis conditions such as concentrated ammonia in dioxane for 16 hr at 50°, and reported the formation of side product (19%). Under our conditions, i.e. concentrated ammonia for 4-6 hr at 50°, we detected side product up to 15%. Reese and his coworker (Tetrahedron Lett. (1978) 2727) has reported the use of oximate ion in removing aryl protecting groups from the internal phosphotriester function without significant cleavage of internucleotide bonds. This observation has been further supported by Gough *et al.* (Nucleic Acid Res. (1979) 6, 1557) and also by us (manuscript in preparation).
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