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Studies on transfer ribonucleic acids and related compounds. XL.[1] Synthesis of an eicosaribonucleotide corresponding to residues 35-54 of $tRNA_f^{Met}$ from E. coli

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ABSTRACT

An E.coli $tRNA_f^{Met}$ fragment [C-A-U-A-A-C-C-C-G-A-A-G-G-U-C-G-U-C-G-G (bases 35-54)] containing the anticodon triplet has been synthesized by the phosphotriester method involving protected oligonucleotide blocks. Di- or tri-nucleotide blocks were prepared by condensation of 2'-O-(o-nitrobenzyl)nucleotide derivatives and used for the synthesis of pentanucleotide blocks. The 5'-hydroxy, heterocyclic amino and internucleotide linkage were protected with monomethoxytrityl, acyl and p-chlorophenyl groups, respectively. The 3'-phosphates of the pentanucleotides, except for the GUCGG block where 2'-O-benzoyl 3'-O-(o-nitrobenzyl) N-isobutyrylguanosine was used, were protected with p-chlorophenyl and anilido groups. The anilido groups were removed by treatment with isoamyl nitrite and the 3'-phosphodiesters of resulting pentamers were activated with mesitylenesulfonyl nitrotriazolide to give protected decanucleotides in yields of 61-89%. The two decanucleotides were condensed similarly to yield the protected eicosanucleotide in a yield of 59%. The product was deblocked and purified by ion-exchange chromatography on DEAE-Sephadex A-25 and characterized by enzymatic hydrolysis after labelling the 5'-end by phosphorylation using polynucleotide kinase and [γ-^{32}P]ATP.

INTRODUCTION

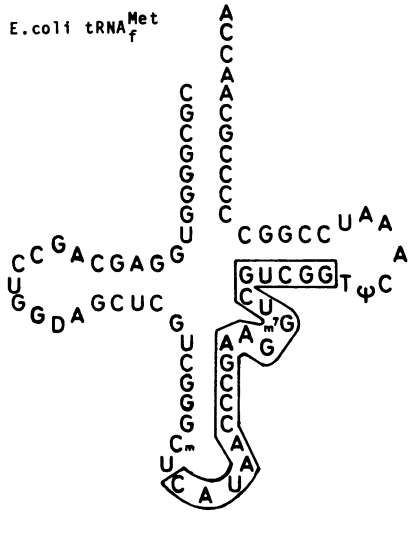
We have previously reported the synthesis of a ribodecanucleotide AGCAGCCUGG which corresponds to the bases 11-20 of the E.coli $tRNA_f^{Met}$ by condensation of di- and tri-oligonucleotide blocks.[2] Penta-[3] and hexa-[4] nucleotides having sequences from the same tRNA were also synthesized by similar triester methods. These ribo-oligonucleotides were used for enzymatic joining with RNA ligase to construct the total $tRNA_f^{Met}$ nascent strand.[5] For the synthesis of tRNAs or their analogs in quantity useful for biochemical studies, it is desirable to prepare larger oligonucleotide fragments chemically and to reduce the number of enzymatic ligation steps. To find the size limit in the conden-

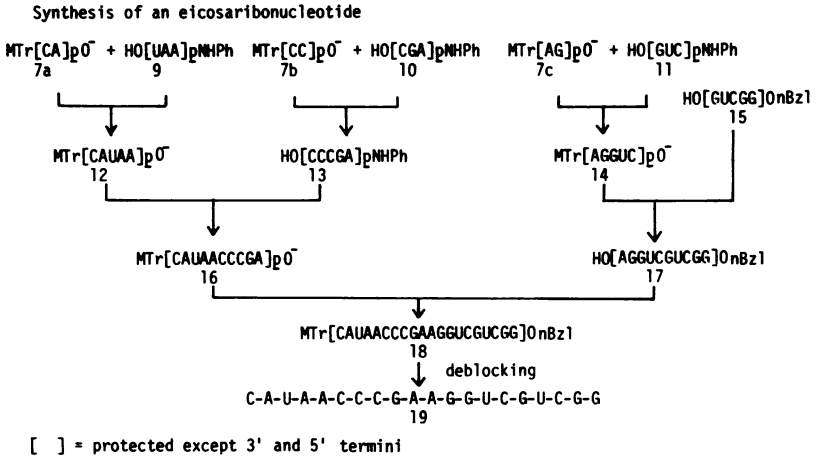
sation of protected ribo-oligonucleotides, hexadeca-, and heptadeca-nucleotides corresponding to the 3'-end of the $tRNA_f^{Met}$ were synthesized using various sizes of blocks, and we found that larger blocks such as penta- or hexa-nucleotides could be activated to almost the same extent as smaller blocks.[1] To extent this approach further we describe in this paper the condensation of penta- and deca-nucleotide blocks to yield an eicosanucleotide having the sequence of the E.coli $tRNA_f^{Met}$ bases 35-54 (Fig. 1). The abbrebiated scheme for the synthesis is shown in Chart 1.

RESULTS

Synthesis of the dinucleotides (7)

2'-O-(o-Nitrobenzyl) derivatives of N-protected ribonucleosides (1) were prepared either by using o-nitrobenzyl bromide[6] or o-nitrophenyldiazomethane[7] and the 5'-monomethoxytrityl group was introduced as described previously.[2] Using these nucleosides (2) as starting materials, the 3'-O-(p-chlorophenyl) phosphate derivatives (5) were synthesized either by treatment with p-chlorophenyl phosphoroditriazolide[8] (3) or with p-chlorophenyl phosphate (4) plus DCC[9] as shown in Chart 2. Dinucleotides having the 3'-phosphodiester (7) were prepared by a rapid method[10] by





condensing (5) with N,2'-protected nucleosides (1) using mesitylenesulfonyl nitroimidazolide[11] (MSNI) as the activating reagent. The intermediates 6 were isolated by chromatography on silica gel and silanized silica gel. 6 were phosphorylated by a similar method as described for the synthesis of 5. The conditions for condensation reactions to yield the dimers (6) are summarized in Table I and those for phosphorylation of (6) to give (7) are lis-

Chart 2

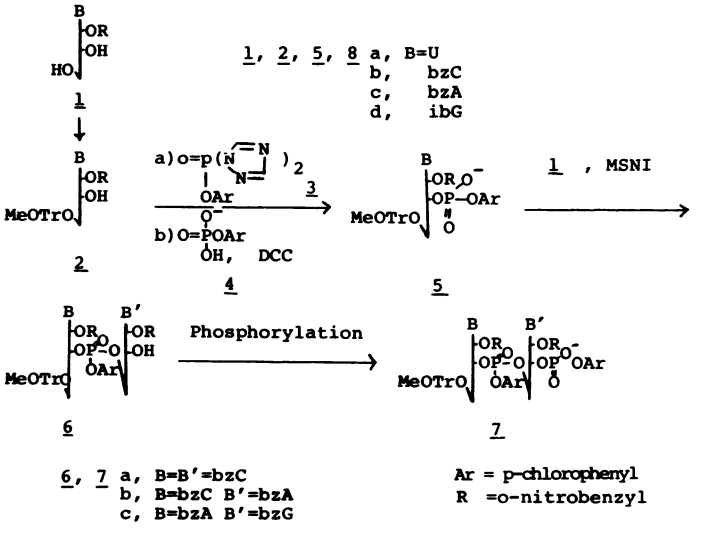


Table I Reaction Conditions for the Synthesis of Dimers (6)

5'-OH Component (mmol)	3'-Phospho-diester Component	MSNI (mmol)	Pyridine (ml)	Time (hr)	Product	Yield (%)
1b (3.25)	5b (2.5)	5.23	12	17	6a	60
1c (2.86)	5b (2.2)	3.30	10	16	6b	72
1d (2.10)	5c (1.5)	2.35	14	19	6c	74

ted in Table II.

Synthesis of the trinucleotides (9, 10, 11)

The trinucleotides (9, 10, 11) which had the fully blocked 3'-phosphate were prepared by stepwise condensations of phosphodiesters (5) to the 3'-blocked mononucleotide (e,g, 2'-O-(o-nitrobenzyl)-N-benzoyladenosine 3'-p-chlorophenyl phosphoroanilidate ,[2] (8c) followed by repeated addition of 5 after removal of the 5'-monomethoxytrityl group. The scheme for the synthesis of 9 is shown in Chart 3. The reaction conditions for the 1st and 2nd condensations are summarized in Table III. After the first condensation, the monomethoxytritylated dimer was isolated by chromatography on silica gel and treated with 1:1 formic acid-chloroform[12] to yield the free 5'-hydroxyl group. The product was purified by chromatography as above then subjected to the 2nd condensation with phosphodiesters (5). The trimers were isolated using silica gel, and the 5'-monomethoxytrityl group of trimers was removed in the same way. In the case of 11, the demonomethoxytritylated dimer and trimer (11) were further purified by reverse phase chromatography on silanized silica gel

Table II Conditions for the Phosphorylation od Dimers (6)

Compound (mmol)	Phosphorylation reagent	Product (mmol)	Yield %
6a (1.18)	4 (4.72)	(nBzl)MeOTrbzCpbzCp (0.855) (7a)	73
6b (1.05)	4 (4.20)	(nBzl)MeOTrbzApibGp (1.01) (7b)	96
6c (1.50)	3 (4.50)	(nBzl)MeOTrbzCpbzAp (1.2) (7c)	80

Table III Conditions for the Synthesis of Trimers

5'-OH(mmol) Component (mmol)	3'-Phospho- diester Component (mmol)	MSNI (mmol)	2nd 3'-Phospho- diester Component (mmol)	Product	Overall Yield %
8c (1.6)	5c (1.92)	2.88	5a (1.9)	9	57
8c (1.0)	5d (1.30)	1.95	5c (1.15)	10	42
8b (1.00)	5a (1.2)	2.0	5d (1.1)	11	48

using 60-80% aqueous acetone to remove contaminating starting 5'-hydroxyl components. It seems essential to purify protected oligonucleotides completely for the synthesis of larger oligonucleotides by block condensations. Extensive purification of deblocked products is required if starting materials contain impurities. These trinucleotide blocks were checked for their purity by thin layer chromatography (TLC) on silica gel and reverse phase thin layer chromatography (RTLC).

Synthesis of pentanucleotide blocks 12, 13, 14 or 15

As shown in Chart 1 pentanucleotides 12, 13 and 14 were prepared from trinucleotides obtained above by condensation with protected dinucleotides (7). The 3'-terminal block (15) was synthesized by using the 3'-O-(o-nitrobenzyl)guanosine derivative as the terminal unit, and stepwise addition of mononucleotide yielded the pentanucleotide in an overall yield of 10%. The 3'-O-(o-nitrobenzyl) group would be stable during deblocking of the p-chlorophenyl group on internucleotidic phosphates in alkaline conditions and prevents attack by the 3'-hydroxyl group at internucleotidic phosphates. The 2'-hydroxyl of the terminus was protected with benzoyl group, since the 2'-position was considered to be less able to react at the internucleotide phosphate.

The conditions for the synthesis of fully protected pentamers are suumarized in Table IV. The 3'-diesterified pentanucleotides 12 and 14 were obtained from fully protected pentamers by removal of the anilido group with isoamyl nitrite. The 5'-

Table IV Reaction Conditions for the Synthesis of Fully Protected Pentanucleotides

5'-OH Component (mmol)		3'-Phosphodiester Component (mmol)		MSNI (mmol)	Product (mmol)	Yield (%)
[UAA] (9)	0.93	[CAp]	(7a)	1.18	2.09	0.43	46
[CGA](10)	0.414	[CCp]	(7b)	0.559	0.983	0.22	54
[GUC](11)	0.47	[ACp]	(7c)	0.658	0.987	0.186	39
(ibG*Bz)(nBzl)	0.7	5d		0.91	1.365		72
		5b		0.654	1.31		
		5a		0.42	1.06		
		5d		0.204	0.408	0.017	10(overall)

deblocked pentamers (13, 15) were prepared by treatment with 1:1 formic acid-chloroform. These pentanucleotide blocks were purified by chromatography either on silica gel or on silanized silica gel.

Synthesis of deca- and eicosanucleotides

Pentanucleotides 12 and 13 were condensed with mesitylene-sulfonyl nitro-triazolide (MSNT)[13] to yield the fully protected decanucleotide, which was isolated by silica gel chromatography and then converted to 16 (Chart 1) by treatment with isoamyl nitrite. 16 was purified by reverse phase chromatography. The 3'-terminal decamer (17) was obtained by similar condensation of two pentamers (14, 15) followed by 5'-demonomethoxytritylation. Reaction conditions for these condensations together with those for the synthesis of the protected eicosanucleotide (18) are listed in Table V. Since unreacted 15 contaminates the fully protected decamer during silica gel chromatography, the mixture was acetylated to block the 5'-hydroxyl group of 15 before demonomethoxytritylation to yield 17. It was observed that the two fully protected decamers and the eicosamer (18) were not observed on silica gel completely. Absorption had to be repeated using a newly packed silica gel column. Resolutions were

Table V Conditions for Condensation of Penta- and Deca-nucleotide

5'-OH Component (mmol)		3'-Phospho-diester Component (mmol)		MSNI (mmol)	Product (mmol)		Yield (%)
13	0.16	12	0.24	0.6	fully protected decamer	0.142	89
15	0.06	14	0.09	0.225	fully protected decamer	0.037	61
17	0.0158	16	0.0237	0.0948	18	0.0093	59

not complete. The product (18) was deprotected by treatment with N^1,N^1,N^2,N^2-tetramethylguanidinium syn-pyridine-2-carboxaldoximate to remove the internucleotidic protection.[13] Treatments with ammonia and 80% acetic acid were given successively to remove N,2'-O-acyl groups and the 5'-O-monomethoxytrityl group. The 2'-O-(o-nitrobenzyl) groups and 3'-terminal o-nitrobenzyl group were cleaved with UV light.[6] The eicosamer (19) was roughly isolated by gel filtration on Sephadex G-50 and purified by ion-exchange chromatography on DEAE-Sephadex A-25 in the presence of 7 M urea. (Fig. 2) The main fraction was further purified by reverse-phase high pressure chromatography on alkyl(octadodecyl, ODS) silica gel. Fig. 3 shows the elution profile. The eicosaribonucleotide thus purified, C-A-U-A-A-C-C-C-G-A-A-G-G-U-C-G-U-C-G-G was characterized by base composition and mobility shift analysis[14] of the labeled product with nuclease P1 (Fig. 4). For this relatively larger oligonucleotides, labeling at the 3',5'-termini was performed by using RNA ligase plus [5'-^{32}P]pCp[15] (for 3') and polynucleotide kinase[16] plus [γ-^{32}P]ATP (for 5'). This allowed to read spots from both ends. The chain length of the eicosamer and decamers was confirmed by electrophoresis on 10% polyacrylamide gel (Fig. 5). Thus the riboeicosanucleotide (19) was obtained by condensation of purified relatively larger blocks. The crude yield of the protected product (18) was 59% and the practical yield of 19 from 18 after purifi-

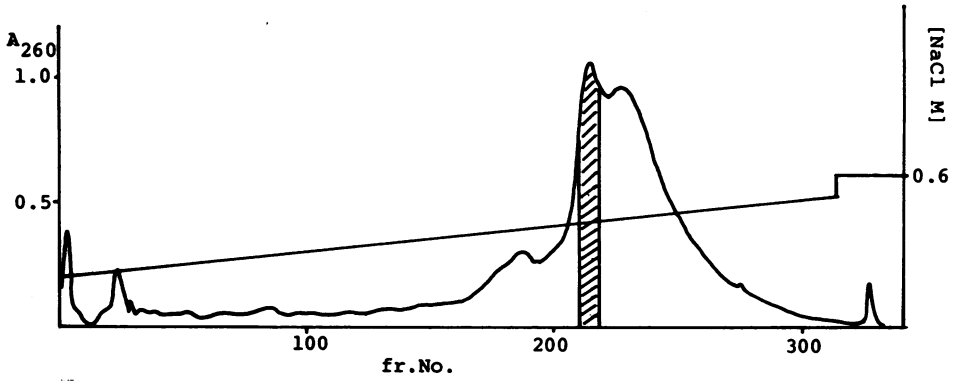


Fig. 2 Chromatography of the eicosamer (19) on a column (0.9 x 64 cm) of DEAE-Sephadex A25 (chloride) in 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The elution was performed with a linear gradient of NaCl (0.2-0.55 M, total 600 ml).

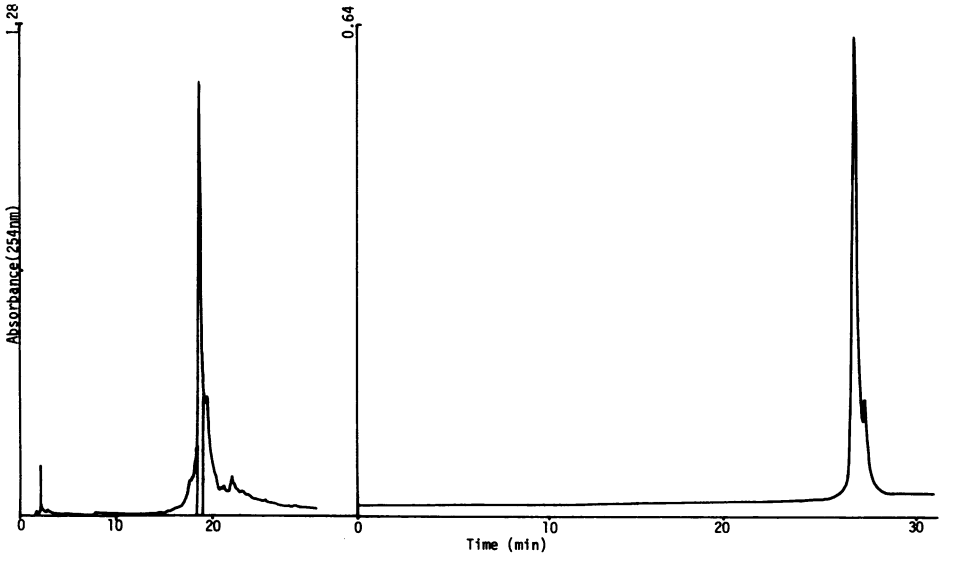


Fig. 3 Reversed phase HPLC of the eicosamer (19) on a column (4.6 x 250 mm) of Hypersil ODS 5 µ. The elution was performed with a linear gradient of acetonitrile: a, 5-15 % (30 min); b, 0-25 % (45 min). The product in the main peak in (a) was desalted by liophylization and re-chromatographed as shown in (b).

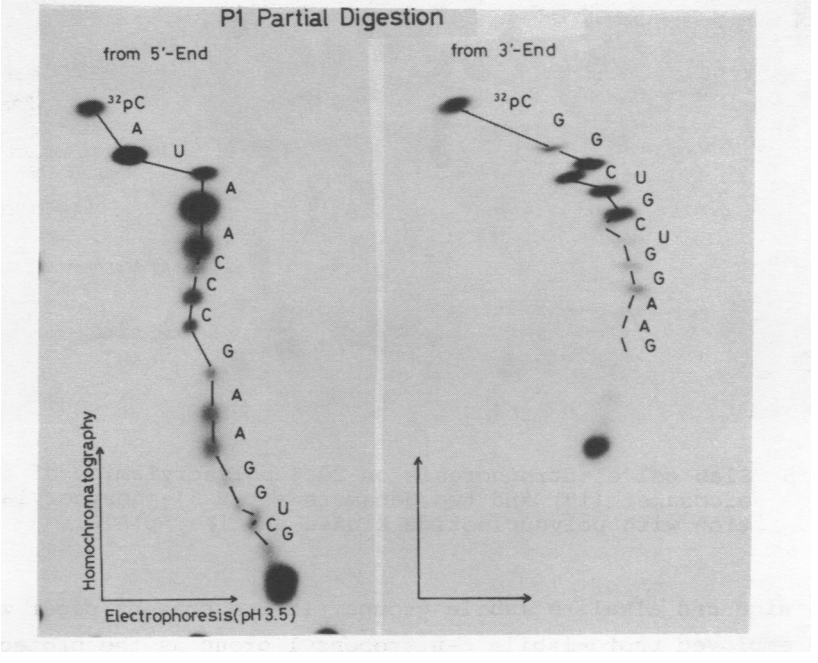


Fig. 4 Mobility shift analysis of the eicosamer (19). (a) nuclease P1 partial digestion of the 5'-labeled eicosamer; (b) nuclease P1 partial digestion of the 3'-labeled product obtained by addition of [5'-^{32}P]pCp to the eicosamer with RNA ligase followed by phosphatase treatment.

cation was ca. 5%.

DISCUSSION

The eicosanucleotide has been synthesized by the phosphotriester method involving protections for internucleotidic phosphates with p-chlorophenyl, 2'-hydroxyl functions with o-nitrobenzyl, 5'-hydroxyl groups with monomethoxytrityl and heterocyclic amino groups with acyl group. Apparently the phosphotriester approach was prefered to the phosphodiester method for the synthesis of larger oligonucleotides by condensation of protected oligonucleotide blocks. In the ribo-series an acid and alkaline stable protecting group for the 2'-hydroxyl group is nescessary if the 5'-hydroxyl and internucleotidic phosphate are protected

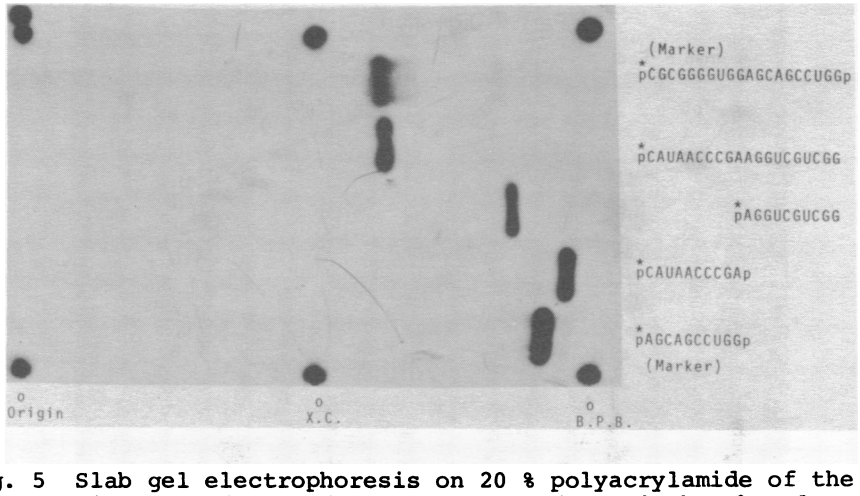


Fig. 5 Slab gel electrophoresis on 20 % polyacrylamide of the eicosamer (19) and two decamers after 5'-phosphorylation with polynucleotide kinase and [γ-^{32}p]ATP.

with aicd and alkaline labile groups. In the present paper we have employed photo-labile o-nitrobenzyl group as the protecting group for the 2'-hydroxyl function. We have examined conditions for removal of the o-nitrobenzyl group with UV light in various concentrations and rection periods. The present conditions were relatively better than the previous ones. Trapping of o-nitrobenzaldehyde with resin containing carbonyl reagents[18] would improve yields of the deblocking. The p-chlorophenyl groups on internucleotidic phosphates were removed either with oxamate[13] or ammonia. In both cases the yields of deblocking seemed lower than those obtained in corresponding deoxyribooligonucleotides. More acidic phenols may be desirable especially in the ribo-series.

Arensulfonyl triazolides or tetrazolides[19] facilitated the activation of phosphodiesters. Relatively powerful condensing reagents such as MSNT[13] seemed to be suitable for activation of larger oligonucleotide blocks and weaker reagents such as MSNI[11] may be prefered for activation of mononucleotides or dinucleotides, since the nitroimidazolide gave less side reactions which were found with the nitrotriazolide.[20]

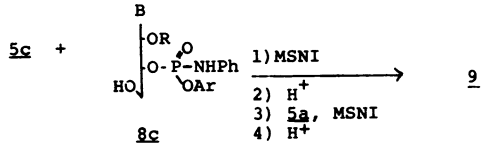
To obtain oligonucleotide blocks we employed two approaches.

One approach involved condensation of 3'-phosphodiester components with 3',5'-unsubstituted nucleosides followed by phosphorylation either by treatment with p-chlorophenyl phosphoroditriazolide or by using p-chlorophenyl phosphate plus DCC which was found to give byproducts in the case of pyrimidine nucleosides. The other method to synthesize oligonucleotide blocks via fully protected dimer (Chart 3) can be used conveniently to elongate the chain in the 5'-direction. Protected oligonucleotides containing phosphoranilidates were found to be stable during chromatography and suitable for purification. It seems that purification of intermediates is important to obtain pure final products. High performance TLC and RTLC facilitated detection of impurities. Preparative reversed phase chromatography was found to be a useful tool for purification of oligonucleotides. Overall yields of deblocking may be improved further by introducing different protecting groups. However, base sequences of oligonucleotides seem to affect yields of isolation. For example, cytidine rich oligomers are relatively labile and decompose during deblocking and purification. Not many papers mentioned absolute amounts of products in HPLC. Yields are often reported as percentages in eluted materials. It is important to compare yields of deblocking from known amounts of protected oligonucleotides and to calculate them from isolated amounts.

EXPERIMENTS

Paper chromatography was performed using solvent systems: A, isopropanol-c. ammonia-water (7:1:2, v/v); B, n-propanol-c. ammonia-water (55:10:35, v/v). Paper electrophoresis was performed using 0.05 M triethylammonium bicarbonate (pH 7.5) or 0.2 M morpholinium acetate (pH 3.5) at 900 V/40 cm. Gel electrophoresis on 10 or 20 % acrylamide was described previously.[17]

Chart 3



Thin layer chromatography (TLC) was performed on plates of silica gel (Kieselgel HF_{254}, Merck) using a mixture of chloroform and methanol. For reversed phase thin layer chromatography (RTLC), silanized silica gel, HPTLC RP-2 or RP-8 F_{254} (Merck) were used with a mixture of acetone-water. For columns, silica gel (Merck, type 60) was packed with chloroform and compounds were applied as a concentrated solution in chloroform. Elution was performed with a mixture of chloroform-methanol, unless otherwise specified. For reversed phase column chromatography, silanized silica gel (Merck, 70-230 mesh) was equilibrated with 60-70 % acetone and compounds in acetone were applied with addition of water until slight turbidity. Elution was performed with 60-80 % aqueous acetone. For oligomers having phosphodiester ends, ca. 0.5 % pyridine was added to eluants. High pressure liquid chromatography (HPLC) was carried out by using an Altex 332 MP apparatus.

Other general methods for removal of the o-nitrobenzyl group and for the characterization of oligonucleotides were as described previously.[9]

General procedure for preparation of dinucleoside monophosphates (6) (Table I)

For example, 6a was prepared by condensation of triethylammonium 5'-O-monomethoxytrityl-2'-O-(o-nitrobenzyl)-N-benzoylcytidine 3'-p-chlorophenyl phosphate (5b) with 2'-O-(o-nitrobenzyl)-N-benzoylcytidine (1b) under the conditions shown in Table I. The starting materials (1b, 5b) were dissolved in pyridine (20 ml) and added to condensing reagent (3.75 mmol). The mixture was concentrated to ca. 12 ml and the extent of the reaction was checked by TLC. Additional amounts of the reagent were used when starting material was detected. The reaction was terminated by addition of water (0.5 ml) under cooling and insoluble materials were removed by filtration. The product was dissolved in chloroform (50 ml), washed 3 times with 0.1 M triethylammonium bicarbonate (50 ml), and the anhydrous concentrated solution in chloroform was applied to a column of silica gel G (140 g). The product (Rf 0.31 in RP-2) was eluted with 50:1 chloroform-methanol and an impurity (Rf 0.14 in Rp-2) was

removed by reverse phase silica gel chromatography on a column (2.4 x 25 cm) of silanized silica gel. The product was precipitated with n-pentane from its solution in chloroform. The yield was 2.12 g, 1.50 mmol, 60 %.

General procedure for phosphorylation of 6 to yield 7 (Table II)

a) The dimer (6c) (2.15 g, 1.5 mmol) was phosphorylated with p-chlorophenyl phosphoditriazolide (4.5 mmol), which was prepared by reacting p-chlorophenyl phosphodichloridate (4.5 mmol), with 1,2,4-triazole (9.4 mmol) at 0° by addition of triethylamine (9 mmol) in dioxane (15 ml) followed by stirring at room temperature for 1 hr. The filtered solution of the reagent was added to the dimer 6 and stirred until TLC showed completion of the reaction. Triethylammonium bicarbonate (1 M, 12 ml) was added with cooling and the product was extracted with chloroform (50 ml). The organic layer was washed 3 times with 0.1 M triethylammonium bicarbonate (50 ml), concentrated, and the residue was applied to a column (2.4 x 25 cm) of silanized silica gel. The product was checked by RTLC and precipitated with n-pentane-ether (1:1, 20 times in volume) from its solution in chloroform. The yield was 2.06 g, 1.2 mmol, 80 %.

b) The dimer (6b) (1.52 g, 1.0 mmol) was treated with p-chlorophenyl phosphate (0.88 g, 4.2 mmol) plus DCC (1.51 g, 7.35 mmol) in pyridine (8 ml) at room temperature overnight. The extent of reaction was checked by TLC, and the mixture was treated with water (2 ml). The filtered solution was diluted with 50 % aqueous pyridine (100 ml) and DCC was removed by extraction with ether. The product was extracted 3 times with ethyl acetate (50 ml). The organic layer was washed 3 times with 0.1 M triethylammonium bicarbonate (100 ml). The organic solvents were evaporated and the product was precipitated with ether-n-pentane (1:1) from its solution in chloroform.

Preparation of the trimer (9) (Table III)

Triethylammonium salt of 5c (2.05 g, 1.92 mmol) was condensed with 2'-O-(o-nitrobenzyl)-N-benzoyladenosine 3'-p-chlorophenyl phosphoranilidate[2] (1.24 g, 1.6 mmol) by treatment with MSNI (0.85 g, 288 mmol) in pyridine (10 ml) for 16 hr. Trie-

thylammonium bicarbonate (1 M, 3 ml) was added to the mixture and the solution was concentrated with pyridine then with toluene. The residue was applied to a column of silica gel (28 g) and the monomethoxytrityl group of the dimer was removed by treatment with formic acid (20 ml) in chloroform (20 ml) at room temperature for 15 min. The demonomethoxytritylated dimer was extracted 4 times with chloroform (20 ml), washed with water 3 times then 0.1 M triethylammonium bicarbonate (50 ml), concentrated and purified by chromatography on silica gel (20 g). The dimer was then condensed with 5a (1.79 g, 1.9 mmol) by treatment with MSNI (0.67 g, 2.28 mmol) for 15 hr, then with additional reagent (0.61 mmol) for 5 hr. The reaction was stopped by addition of 1 M triethylammonium bicarbonate (2.9 ml) and the mixture was concentrated by evaporation with pyridine then with toluene. The product (9) was isolated by chromatography on silica gel (35 g) and impure fractions were rechromatographed to remove the dimer. The combined product was precipitated with n-pentane from its solution in chloroform. The overall yield from 8c was 59%, 2.16 g, 0.95 ml. The monomethoxytrityl group was removed as above and 9 was isolated by chromatography on silica gel (20 g).

Preparation of pentanucleotides (12 and 14) (Table IV)

For example, 12 was synthesized by condensation of 9 (prepared from 0.93 mmol of the monomethoxytritylated trimer) and 7a (2.04 g, 1.18 mmol) by treatment with MSNI (1.89 mmol) in pyridine (8 ml) overnight. Additional reagent was reacted for 4 hr. Water (1 ml) was added and the mixture was diluted with ethylacetate-ether (2:1, 90 ml). The organic layer was washed with 0.1 M triethylammonium bicarbonate containing 25% pyridine, concentrated with added pyridine and then with toluene. The product was isolated by chromatography on silica gel G (70 g) with chloroform-methanol (50:1) as an eluent. The yield was 1.54 g, 0.43 mmol (46%). The anilidate was removed by treatment of the fully protected pentanucleotide (1.54 g, 0.43 mmol) with isoamyl nitrite (3 ml) in pyridine-acetic acid (5:4) at room temperature for 1.5 days. Aqueous pyridine (40%, 50 ml) was added to the mixture, and the product was extracted with chloro-

form (50 ml, 30 ml, 20 ml). The organic layer was washed 4 times with 0.1 M triethylammonium bicarbonate and concentrated. The product (12) was isolated by chromatography on a silanized silica gel column (2 x 25 xm). Impure fractions were rechromatographed using a column (1.6 x 45 cm) of the same support. The combined product was precipitated with n-pentane-ether (1:1) . The yield was 1.10 g, 0.302 mmol, 70%.

The pentanucleotide (13)

The triethylammonium salt of protected C-Cp (7b) and protected C-G-Ap (10) were condensed under the conditions shown in Table IV. The procedure for isolation of the fully protected form was the same as described for the synthesis of (12). The fully protected pentanucleotide (789 mg, 0.215 mmol) was demonomethoxytritylated by treatment with formic acid (10 ml) in chloroform (10 ml) at room temperature for 30 min. The mixture was neutralized with pyridine (20 ml) in an ice bath, diluted with water (50 ml), and the product was extracted 3 times with chloroform (10 ml). The organic solution was washed with water (80 ml), 0.1 M triethylammonium bicarbonate (60 ml), concentrated with added pyridine then with toluene, and the residue was applied to a column (2.6 x 28 cm) of silanized silica gel (15 g). The product (13) was precipitated with n-pentane from its solution in chloroform. The yield was 0.569 g, 0.168 mmol, 78%.

Stepwise synthesis for the pentamer (15)

The 3'-terminal nucleoside, 2'-O-benzoyl-3'-O-(o-nitrobenzyl)-N-isobutyrylguanosine (0.593 g, 1 mmol) was reacted with the triethylammonium salt of 5d (1.37 g, 1.30 mmol) using MSNI (0.575 g, 1.95) in pyridine (8 ml) for 13 hr. The mixture was treated with 1 M triethylammonium bicarbonate (2 ml) and extracted with ethylacetate (60 ml). The organic phase was washed twice with 0.1 M triethylammonium bicarbonate (50 ml), concentrated, coevaporated with pyridine then with toluene, and the residue was chromatographed on silica gel (30 g). The dimer was further purified by chromatography on a column (1.6 x 50 cm) of silanized silica gel. An aliquot (30 %) of the fully protected dimer was precipitated with n-pentane (0.328 mg, 0.215 mmol, 72

%). The rest of material was demonomethoxytritylated with formic acid (20 ml) as described for the synthesis of (13) and the dimer was condensed with 5b (0.654 g, 1.3 mmol) by the same procedure as described above (Table V). The fully protected trimer was isolated by chromatography on silica gel and silanized silica gel. The trimer was 5'-deblocked as for the dimer, and the product was used for condensation with 5a (0.396 g, 0.42 mmol). The fully protected tetramer was isolated as described for the trimer and the 5'-deblocked tetramer was reacted with 5d (0.215 g, 0.204 mmol)(Table IV). The pentamer was extracted with ethyl acetate (50 ml) from the reaction mixture after addition of 1 M triethylammonium bicarbonate (1 ml) and pyridine (6 ml). The organic layer was washed with 0.1 M triethylammonium bicarbonate (80, 70, 50 ml), concentrated, and the residue was applied to a column (2.6 x 28 cm) of silanized gel. Impure fractions were rechromatographed on a column (1.6 x 50 cm) of the same support. The fully protected pentamer was precipitated with pentane (0.243 g, 0.071 mmol, 10% from the protected guanosine). The monomethoxytrityl group of the product was removed by using formic acid (5 ml) as described for 13 and the pentamer 15 was precipitated with n-pentane. The yield was 0.189 g, 0.06 mmol, 89%.

The decanucleotide (16)

The triethylammonium salt of the pentanucleotide (12)(0.87 2 g) and the 5'-deblocked pentanucleotide (13)(0.544 g) were treated with MSNT (0.178 g) in pyridine (1.5 ml) as described in Table V. After 30 min RTLC (RP-8) showed no starting material. The mixture was treated with 1 M triethylammonium bicarbonate (1 ml) and pyridine (3 ml) for 30 min, and the product was extracted with ethyl acetate (50 ml). The organic phase was washed twice with 0.1 M triethylammonium bicarbonate, concentrated with pyridine then with toluene, and the residue was applied to a column of silica gel. A part of the product was washed off with 1% methanol in chloroform. The rest of the product was eluted with increasing methanol content. Fractions were checked by RTLC and the fully protected decanucleotide which was contaminated with a trace of starting materials was precipitated with

n-pentane in a crude yield of 86 %, 0.979 g, 0.142 mmol. The product (0.692 g, ca. 0.1 mmol) was treated with isoamyl nitrite (0.9 ml) in pyridine-acetic acid (5:4, 4.5 ml) for 24 hr. The removal of the anilido group was confirmed by TLC. Aqueous pyridine (50%, 50 ml) and ether-pentane (1:1, 50 ml) were added to the mixture. The product 16 was extracted with ethyl acetate, washed 4 times with 0.1 M triethylammonium bicarbonate concentrated, dissolved in a small amount of pyridine, and water was added until slightly turbid. The turbid solution was applied to a column (2.6 x 26 cm) of silanized silica gel. The decanucleotide (16) was precipitated with ether-n-pentane (1:1) from its solution in chloroform (0.389 g, 0.056 mmol, 56 %).

The decamer (17) (Table V)

The triethylammonium salt of the pentanucleotide (14) (324 mg) and the pentamer (15) (187 mg) were dissolved in pyridine (1 ml) and MSNT (70 mg) added, then concentrated to ca. 0.5 ml. After 30 min, the reaction was checked by TLC (10:1, the decamer , Rf 0.39) and RTLC (8:2, the decamer, Rf 0.19). The reaction was stopped (reaction time, 50 min) by addition of 1 M triethylammonium bicarbonate (0.5 ml) and the pyridine (6 min). The mixture was stirred for 40 min and the product was extracted with ethyl acetate (50 ml). The organic layer was washed twice with 0.1 M triethylammonium bicarbonate (50 ml), concentrated with pyridine then with toluene, and the residue was applied to a column of silica gel (15 g). Most of the fully protected decamer was not adsorbed to the column. Aplication was repeated twice to the same kind of silica gel. The product was eluted with chloroform-methanol (28:1). Fractions containing the product (Rf 0.39 in TLC, 10:1; 0.19 in RP-8, 8:2) were collected and precipitated with n-pentane from solution in chloroform (243 mg, 36.8 μmol, crude yield 61%, contaminated with the starting material (15)). The product was acetylated with acetic anhydride (1 ml) in pyridine (5 ml) to block the 5'-hydroxyl group of 15 before demonomethoxytritylation. The acetylated product was chromatographed on silica gel (G) and demonomethoxytritylated by treatment with formic acid (4 ml) in chloroform (4 ml) at room temperature for 30 min. The mixture was worked up as described

for 13 and purified by chromatography on silica gel (5 g). The yield of 17 was 100 mg, 15.8 μmol. Additional material was eluted with 10% methanol in chloroform from the column of silica gel (G), which was demonomethoxytritylated to give 17 (68.3 mg, 10.7 μmol).

The eicosamer (19)

The triethylammonium salt of the decanucleotide (16) (165 mg , 23.7 μmol) and the decamer (17) (100 mg, 15.8 μmol) were dissolved in pyridine (1 ml) and MSNT (28 mg, 94.8 umol) added. The mixture was concentrated to ca. 0.5 ml and checked by TLC after 30 min. A new trityl positive spot was observed (Rf, 0.5 in 10: 1 TLC, 0.33 in 8:2 RP-8). The reaction was terminated after 1 hr by treatment with 1 M triethylammonium bicarbonate (0.1 ml) and pyridine (5 ml) for 40 min. The product was extracted with ethyl acetate (40 ml) washed twice with 0.1 M triethylammonium bicarbonate (50 ml), concentrated with pyridine then with toluene, and applied to silica gel H (5 g) packed with suction in a sintered glass funnel. Fractions containing products were rechromatographed similarly on silica gel H (6 g). The product (18) was precipitated with n-pentane (124.7 mg, 9.3 μmol, 59 %, crude).

An aliquot of 18 (53.4 mg, 4 μmol) was stirred with 0.5 M N^1,N^1,N^2,N^2-tetramethylguanidinium syn-pyridine-2-carboxaldloximate (in 50 % aqueous dioxane 9.2 ml) at room temperature 2.5 days, diluted with 50 % aqueous pyridine (200 ml), washed with ethyl acetate (200 ml) and the organic phase was back extracted with water (50 ml). The aqueous layer was concentrated, heated with conc. ammonia (50 ml) at 60° for 6 hr, concentrated and the residue was dissolved in 50 % pyridine. The solution was passed through a column (25 ml) of Dowex 50 x 2 (pyridinium) and the column was washed with 50 % pyridine (100 ml). The combined solution was concentrated to make 30 % pyridine (50 ml) and washed with ethyl acetate (50 ml). The aqueous phase was concentrated with added toluene, treated with 80 % acetic acid (50 ml) at 30° for 16 hr, evaporated with added toluene and the residue was dissolved in 10 % pyridine (50 ml). The solution was washed twice with ethyl acetate (50 ml), concentrated with water, and the residue was dissolved in water (240 ml), irradiated with

UV light for 20 min, washed with ethyl acetate (100 ml), evaporated to remove ethyl acetate, diluted with water (120 ml), and irradiated for 20 min. The solution was washed similarly and irradiated for 2 hr to yield 924 A_{260} units of material. The solution was concentrated to ca. 50 ml, washed with ethyl acetate, concentrated, and an aliquot (234 A_{260}) was applied to a column (0.9 x 64 cm) of DEAE-Sephadex A 25 (chloride) equilibrated with 0.05 M NaCl containing 0.02 M Tris-HCl, pH 7.5 and 7 M urea. Elution was performed with a linear gradient of NaCl (0.2 -0.55 M, total 600 ml). The main peak (Fig. 2) contained the deblocked eicosamer (19) (28.63 A_{260}) which was further purified by HPLC on alkylated silica (Hypersil ODS 5 μ, ϕ 4.6 x 250 cm). The crude yield of deblocking was 13 %. Fig. 3a shows the elution profile in preparative conditions. Several runs yielded 8.1 A_{260} of the product. The main peak was analyzed in different elution conditions (Fig. 3b) to give a purity of 81 %. The eicosamer thus obtained was subjected to two dimensional homochromatography (Fig. 4) and slab gel electrophosresis (Fig. 5). The base composition of the eicosamer was measured by digestion of an aliquot (0.58 A_{260}) with either RNase T2 or nuclease P, followed by analysis on high-pressure anion-exchange chromatography (Varian nucleic acid analyzer). The ratios were Cp:Up: Ap:Gp = 6.00:3.09:4.77:4.95 and pC:pU:pA:pG = 5.00:3.19:4.95: 6.29.

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REFERENCES

1) Ohtsuka, E., Fujiyama, K., Tanaka, T. and Ikehara, M. Chem. Pharm. Bull., in press.
2) Ohtsuka, E., Tanaka, T. and Ikehara, M. (1979) J. Am. Chem. Soc., 101, 6409-6414.
3) Ohtsuka, E., Tanaka, T. and Ikehara, M. (1979) Nucleic Acids Res., 7, 1283-1296.
4) Ohtsuka, E., Tanaka, T. and Ikehara M. (1980) Chem. Pharm. Bull., 28, 120-125.
5) Ohtsuka, E., Markham, A.F., Tanaka, S., Tanaka, T., Miyake, T., Nakagawa, E., Wakabayashi, T., Taniyama, Y., Fujiyama,

K., Nishikawa, S., Fukumoto, R., Uemura, H., Doi, T., Tokunaga, T. and Ikehara, M. (1980) Nucleic Acids Res. Symp. Ser. 7, 335-343.
6) Ohtsuka, E., Tanaka, S. and Ikehara, M. (1974) Nucleic Acids Res., 1, 1351-1358, Idem., (1977) Chem. Pharm. Bull., 25, 949-959; Idem., (1977) Synthesis, 453-454.
7) Ohtsuka, E., Wakabayashi, T., Tanaka, S., Tanaka, T., Oshie, K., Hasegawa, A. and Ikehara, M. (1981) Chem. Pharm. Bull., 29, 318-324.
8) Broka, C., Hozumi, T., Arenzen, R. and Itakura, K. (1980) Nucleic Acids Res., 8, 5461-5471.
9) Ohtsuka, E., Tanaka, S. amd Ikehara, M. (1978) J. Am. Chem. Soc., 100, 8210-8213.
10) Ohtsuka, E., Wakabayashi, T. and Ikehara, M. (1981) Chem. Pharm. Bull., 29, 759-765.
11) van Boom, J.H. and Burgers, P.M.J. (1977) Tetrahedron Lett., 4875-4878. Gough, G.R., Singleton, C.K., Weith, H.L. and Gilham, P.T. (1979) Nucleic Acids Res., 6, 1557-1581.
12) Bleaney, R.C., Jones, A.S. and Walker, R.T. (1975) Nucleic Acids Res., 2, 699-706.
13) Reese, C.B., Titmas, R.C. and Yau, L. (1978) Tetrahedron Lett., 2727-2730.
14) Sanger, F., Donelson, J.E., Coulson, A.R., Kössel, H. and Fischer, D. (1973) Proc. Natl. Acad. Sci. U.S.A., 70, 1209-1213: Silberklang, M., Gillum, A.M. and RajBhandary, (1977) Nucleic Acids Res., 4, 4091-4108.
15) Richardson, C. (1965) Proc. Nat. Acad. Sci.U.S. 54, 158-165.
16) England, T.E. and Uhlenbeck, O.C. (1978) Nature, 275, 561-562.
17) Ohtsuka, E., Nishikawa,S., Fukumoto, R., Uemura, H., Tanaka, T., Nakagawa, E., Miyake, T. and Ikehara, M. (1980) Eur. J. Biochem., 105, 481-488.
18) Rubinstein, M., Amit, B. and Pachornik, A. (1975) Tetrahedron Lett., 1445-1448.
19) Stawinski, J., Hozumi, T., Narang, S.A., Bahl, C.P. and Wu, R. (1977) Nucleic Acids Res., 4, 353-372.
20) Reese, C. and Ubasawa, A. (1980) Tetrahedron Lett., 21, 2265-2268.