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Studies on Transfer Ribonucleic Acids and Related Compounds. XXXIV.[1] Stepwise Diester or Partial Triester Synthesis of Penta- to Octanucleotides Corresponding to Residues 41—46, 47—54, 61—65 and 66—71 of $tRNA_f^{Met}$ of *E. coli*

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A hexanucleotide and an octanucleotide corresponding to $tRNA_f^{Met}$ bases 41—46 and 47—54 were synthesized by stepwise addition of mononucleotides. The octanucleotide is the largest oligoribonucleotide synthesized by this method. A pentanucleotide (bases 61—65) and a hexanucleotide (bases 66—71) were synthesized *via* partially triesterified intermediates. The deblocked products were purified by ion-exchange chromatography and characterized by enzymatic hydrolysis. These oligonucleotides were used as substrates in joining reactions with RNA ligase.

Keywords——ribooligonucleotides; tRNA fragments; ion-exchange chromatography; reverse-phase chromatography; gel filtration

We have previously described the chemical synthesis of oligonucleotides corresponding to fragments of *E. coli* $tRNA_f^{Met}$[3] either by the phosphodiester method[4] or by the phosphotriester method.[5] A eukaryotic initiator loop IV sequence A–U–C–G–A–A–A has also been synthesized using triesterified oligonucleotide blocks.[6] In this paper we report a synthesis of *E. coli*. tRNA fragments corresponding to bases 41—46 (**1**), 47—54(**2**), 61—65 (**3**) and 66—71 (**4**). The locations of these oligonucleotides in the tRNA are shown in Fig. 1. The octanucleotide (**2**) is the largest ribooligonucleotide so far synthesized by the stepwise phosphodiester method.

Synthesis of the Hexanucleotide (1)

The hexanucleotide C–C–G–A–A–G[7] (**1**) was synthesized by stepwise addition of suitably protected nucleoside 3′-phosphates using essentially the same protecting groups described previously[4b] (Chart 1). The reaction conditions and results are summarized in Table I. Although the yield in the final step was not satisfactory, the product was isolated by ion-exchange chromatography on DEAE-cellulose in 7 M urea. The deblocked hexanucleotide

1) Paper XXXIII: E. Ohtsuka, T. Miyake, K. Nagao, H. Uemura, S. Nishikawa, M. Sugiura, and M. Ikehara, *Nucleic Acids Res.*, **8**, 611 (1980).
2) Location: *133-1 Yamadakami, Suita 565, Japan.*
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(**1**) was further purified by paper electrophoresis at pH 3.5 and characterized by enzymatic hydrolysis with RNase M;[8] the expected ratio of mononucleotides was obtained.

Synthesis of the Octanucleotide (2)

In order to investigate the limitation of the stepwise addition method, the octanucleotide G–U–C–G–U–C–G–G (**2**) was synthesized by the phosphodiester method. As shown in Chart 2, protected mononucleotides were condensed with the free hydroxyl group of the growing chain using dicyclohexylcarbodiimide (DCC) as the condensing reagent. Excesses of (MeOTr)-bzC(Bz)p (2.6 fold), (MeOTr)U(Bz)p (7.3 fold) and (MeOTr)ibG(Bz)p (11.1 fold) were used in the tri-, tetra- and pentanucleotide syntheses. The yields of the protected oligonucleotides isolated by ion-exchange chromatography on TEAE-cellulose were 27, 30 and 18% respectively. An aliquot of the pentanucleotide was deblocked by successive treatment with 80% acetic acid and methanolic ammonia. The pentanucleotide G–U–C–G–G was purified by ion-

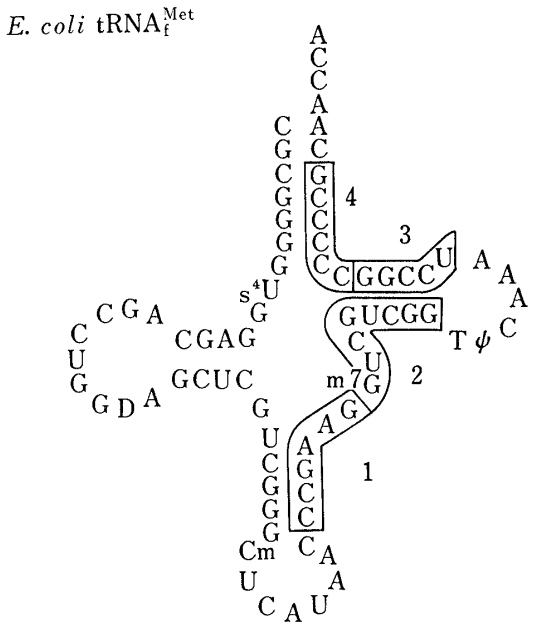

Fig. 1. Location of Compound **1** (bases 41—46), Compound **2** (bases 47—54), Compound **3** (bases 61—65) and Compound **4** (bases 66—71) in *E. coli* tRNA$_f^{Met}$

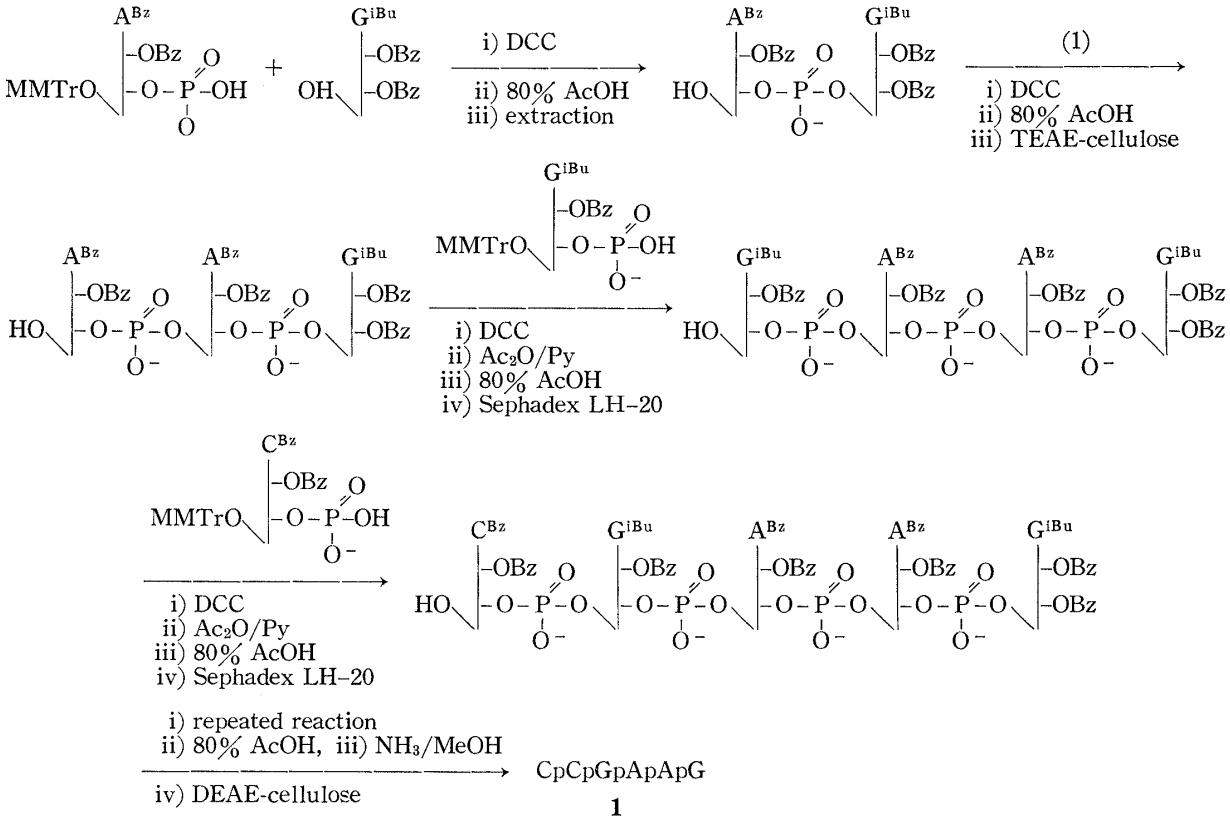

Chart 1

8) M. Imazawa, M. Irie, and T. Ukita, *J. Biochem.*, **64**, 595 (1968).

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Table I. Reaction Conditions for the Synthesis of **1**

Protected oligomer	T.O.D. A_{280}	mmol	Protected monomer	mmol	Pyridine ml	DCC mmol	Product	T.O.P. A_{280}	mmol	Yield %
G	15300	1.3	Ap	1.5	10	15	ApG	27000	0.82	63
ApG	27000	0.82	Ap	1.18	10	11.8	ApApG	14940	0.27	35
ApApG	14940	0.27	Gp	1.11	5	12	GpApApG	2526	0.038	14
GpApApG	2526	0.038	Cp	2.26	10	11.3	CpGpApApG	650	0.0085	22
GpGpApApG	650	0.0085	Cp	0.184	2	1.84	deblocked CpCpGpApApG	10	0.1113	3.9

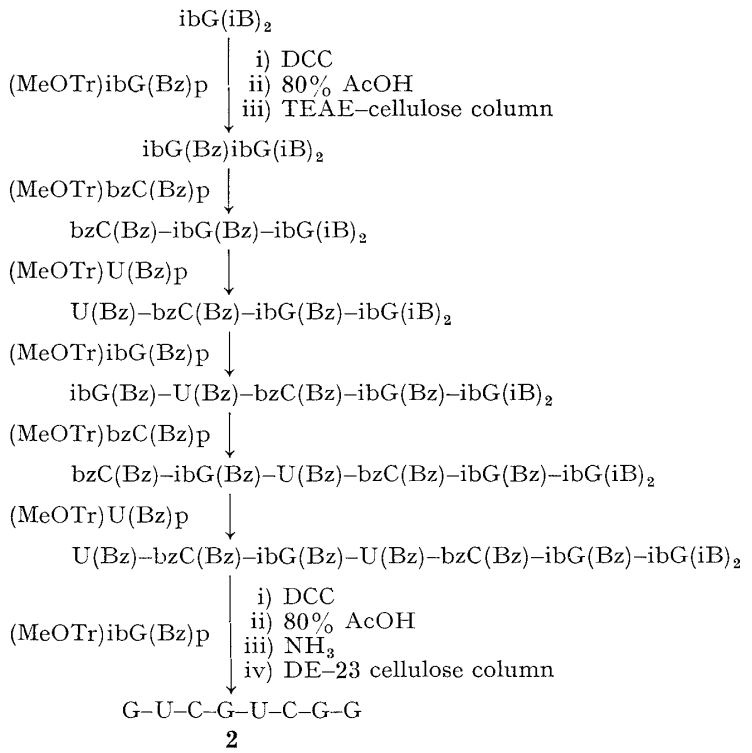

Chart 2

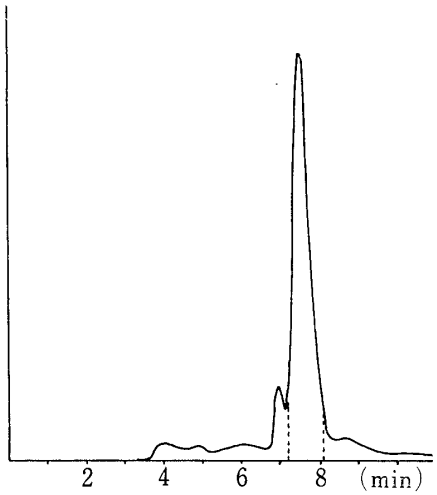

Fig. 2. High-pressure Liquid Chromatography of the Deblocked Pentamer G–U–C–G–G (3) on a Column (0.4×25 cm) of C-18 Alkylsilica

Elution was performed with 4% acetonitrile containing 0.1 M ammonium acetate (pH 7.5) at a flow rate of 0.5 ml/min, 25 kg/cm².

exchange chromatography on DEAE-cellulose in 7 M urea and further purified by reverse phase chromatography on C-18 alkylsilica (Fig. 2). The product was analyzed by determination of the base ratio and by the mobility shift method[9] after labelling the 5′-hydroxyl group with ^{32}P using [γ-^{32}P]ATP and polynucleotide kinase.[10] The pentanucleotide should be a useful substrate for RNA ligase[11] in reactions with m^{7}G–U–C or A–U–C for the preparation of an N-7 methylguanosine (m^{7}G) containing octanucleotide m^{7}G–U–C–G–U–C–G–G or an octanucleotide A–U–C–G–U–C–G–G found in minor species of $tRNA_f^{Met}$. Chemical condensation of nucleotides containing m^{7}G seems to be difficult because of the lability of m^{7}G under alkaline conditions. Methylation of G–U–C gave m^{7}G–U–C in a satisfactory yield.[12] Enzymatic joining of m^{7}G–U–C to the pentamer should yield the modified base-containing octanucleotide rather easily.

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The octanucleotide (2) was synthesized from the protected pentanucleotide by the stepwise method used in the above reaction except that acetic anhydride treatment and a rapid stepwise elution of nucleotides were employed in these last three steps. Acetic anhydride treatment was used to decompose pyrophosphates of internuleotide phosphates by acetolysis. An example of such a reaction is shown in Chart 3. Tri- or tetrasubstituted pyrophosphates were assumed to be hydrolyzed in aqueous pyridine. However, these pyrophosphates of higher oligonucleotides seemed to become more stable as the chain length increased. Thus, acetolysis followed by hydrolysis of acetyl phosphates might be necesary. Another advantage of acetic anhydride treatment was the blocking of the 3′-hydroxyl group of the unchanged starting oliognucleotides by acetylation. In the synthesis of the protected hexanucleotides, the pentamer was condensed with (MeOTr)bzC(Bz)p using DCC and the reaction mixture was treated with acetic anhydride before removal of the monomethoxytrityl group with 80% acetic acid. The demonomethoxytritylated mixture was applied to a column of TEAE-cellulose and oligomers were isolated by stepwise elution as shown in Fig. 3a. The fractions which contained hexamer were used for to the next reaction and worked up under conditions similar to those described above. The elution profile of the heptanucleotide is shown in Fig. 3b. The octanucleotide was synthesized by condensation of (MeOTr)ibG(Bz)p with the

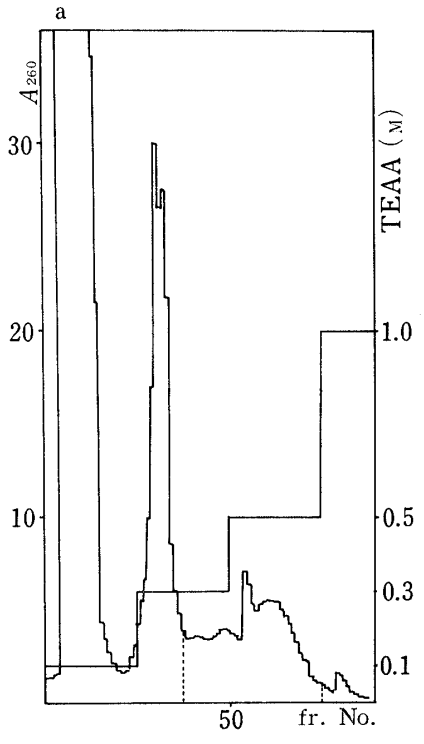

Chart 3

Fig. 3–a. Chromatography of the Reaction Mixture for the Synthesis of the Hexamer on a Column (2.5×14 cm) of TEAE-cellulose (Acetate)

Elution was performed by stepwise increases of triethyl ammonium acetate concentration in 80% ethanol as shown. Fractions shown by dotted lnies were pooled and used for the next reaction.

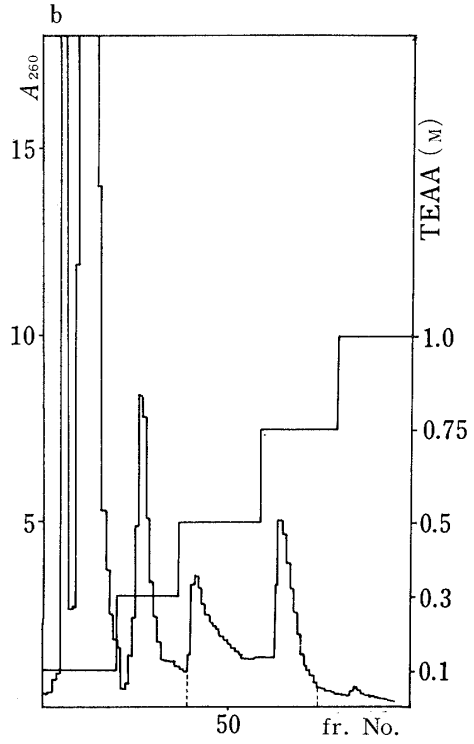

Fig. 3–b. Chromatography of the Reaction Mixture for the Synthesis of the Heptamer on a Column (2.5×12 cm) of TEAE-cellulose

Conditions for elution were the the same as in (a).

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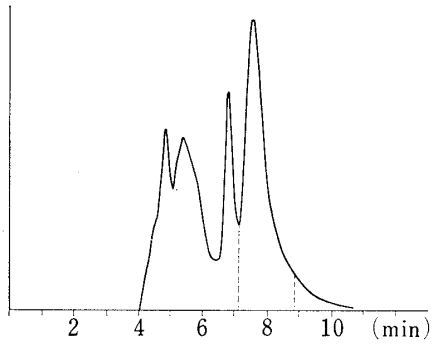

Fig. 4. High-pressure Liquid Chromatography of the Octamer G–U–C–G–U–C–G–G

The column and elution conditions were the same as in Fig. 2.

heptamer-containing fraction and was isolated by ion-exchange chromatography on DEAE-cellulose in 7 M urea after deblocking. The product was further purified by reverse phase chromatography (Fig. 4) and characterized by mobility shift analysis.[9]

Synthesis of the Pentanucleotide (3) and Hexanucleotide (4)

These intermediate-sized oligonucleotides were synthesized by a mixed phosphodiester-phosphotriester stepwise addition method with separation of oligomers longer than trimers from the incoming mononucleotide and its pyrophosphate by gel filtration. Chart 4 illustrates the condensation of mononucleotides to β-cyanoethylated oligonucleotides. As discussed in a preliminary communication,[8] the yields of condensations were considerably higher compared to those involving phosphodiester oligomers. The triesterified trimers were prepared by TPS-

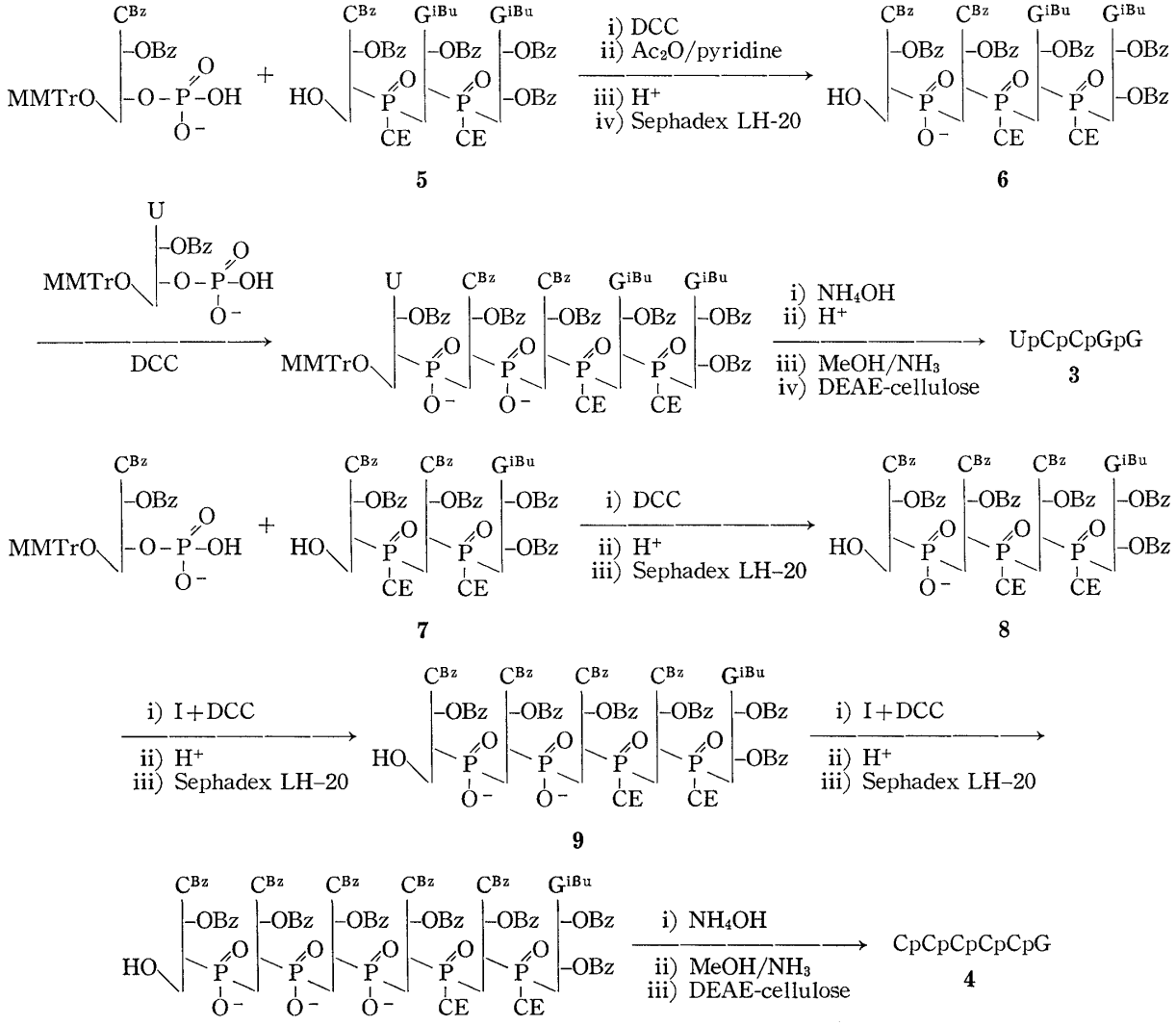

Chart 4

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catalyzed β-cyanoethylation of the 5′-monomethoxytritylated trinucleoside diphosphates and purified by silica gel chromatography. The 5′-deblocked trimer was condensed with (MeOTr)bzC(Bz)p and the tetramer was separated from the monomer by gel filtration on Sephadex LH-20. Although this gel filtration could not resolve the trimer and tetramer, the mixture of oligomers was used for the next condensation, since the same cytidine nucleotide was added in the synthesis of **4**. The protected pentanucleotide C–C–C–C–G was worked up by the same method and the hexamer C–C–C–C–C–G was purified by ion-exchange chromatography on DEAE-cellulose in 7 M urea.

In the case of **3** the reaction mixture after each condensation was treated with acetic anhydride to cleave pyrophosphates before demonomethoxytritylation and gel filtration. This treatment also blocked the 3′-hydroxyl group of unchanged starting oligonucleotides. The deblocked product U–C–C–G–G was isolated by ion-exchange chromatography on DEAE-cellulose (carbonate form) and further purified by chromatography under denaturing conditions. The structural integrity of these oligonucleotides was confirmed by base ratio analysis and by mobility shift analysis[9] using electrophoresis and homochromatography.

This approach offered the attractive feature of the separation of oligomers from mononucleotides. Protection of the internucleotide phosphate increased the yield of condensations with mononucleotides. However, the yield of β-cyanoethylation for preparation of the blocked trimer was not satisfactoty, presumably due to the instability of the β-cyanoethyl group during silica gel chromatography. Further triesterification of larger oligonucleotides seemed to be difficult, since the overall yield would decrease if losses of material during purifications on silica gel accumulated. The use of a more stable protecting group such as aryl derivatives in combination with a different 2′-hydroxyl protection method might be more suitable for longer oligonucleotide syntheses.[5,6]

Table II. Paper Chromatography and Paper Electrophoresis Results

Compound	Paper chromatography			Paper electrophoresis
	Solvent A	Solvent B	Solvent B′	pH 7.5
C	0.52	0.65		0.00
Cp cyclic	0.42	0.59		0.63
Cp	0.14	0.47		1.00
ApG		0.38		0.42
ApApG		0.27		0.69
GpApApG		0.11		0.85
CpGpApApG		0.06		
CpCpGpApApG (**1**)		0.04		
GpG	0.05	0.33		0.52
GpGpG	0.02	0.27		0.65
CpCpGpG		0.12		0.76
UpCpGpG		0.22		0.70
UpCpCpGpG (**3**)		0.11		0.83
GpUpCpGpG		0.07		0.80
CpG			0.36	0.46
CpCpG			0.26	0.65
CpCpCpG			0.10	0.78
CpCpCpCpG			0.08	0.87
CpCpCpCpCpG (**4**)			0.06	0.90

Experimental

Paper chromatography was performed by the descending technique using the following solvent systems: A, propan-1-ol-concentrated ammonia–water (7: 1: 2, v/v), and B, propan-1-ol-concentrated ammonia–water (55: 10: 35, v/v), B′, (propan-1-ol-concentrated ammonia–water) (6: 1: 3, v/v). Paper electrophoresis

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was performed using 0.05 M triethylammonium bicarbonate (pH 7.5) or 0.2 M morpholinium acetate (pH 3.5) at 900 V/cm. *Rf* values and mobilities in paper chromatography and electrophoresis are shown in Table II. High-pressure liquid chromatography was performed on a Hitachi liquid chromatograph, model 635, using a Lichrosorb C-18 (0.4×25 cm) column. The elution conditions were essentially the same as those used for deoxyoligonucleotides.[13] Conditions for partial nuclease P1[14] digestion and other general methods were as described previously.[6] Enzymatic hydrolyses were also described in ref. 15. Preparation procedures for mononucleotides were according to the cited references: (MeOTr)ibG(Bz)p,[4b] (MeOTr)bzA(Bz)p,[4f] MeOTrBzC(Bz)p,[4f] (MeOTr)U(Bz)p.[16] Abbreviations used here include: TEAB, triethylammonium bicarbonate, TEAA, triethylammonium acetate; TLC, thin-layer chromatography: PPC, paper chromatography: PEP, paper electrophoresis.

C-C-G-A-A-G (1)

The protected trimer bzA(Bz)–bzA(Bz)–ibG(Bz)$_2$ was synthesized under the conditions shown in Table I and isolated by ion-exchange chromatography on a column (2.8×88 cm) of TEAE-cellulose (acetate) in 80% ethanol with a linear gradient of TEAA (0—0.22 M, total 6 l). The product was eluted at a salt concentration of *ca.* 0.15 M and desalted as described previously.[4b] The protected tetranucleotide was separated from the mononucleotide by gel filtration on a column (2.5×80 cm) of Sephadex LH-20 in 95% ethanol. The reaction mixture in 95% ethanol (1 ml) was applied to the column and elated at a flow rate of 3 ml in 23 min. The protected pentanucleotide was purified by chromatography on a column (2.8×88 cm) of TEAE-cellulose in 80% ethanol with a linear gradient of TEAA (0—0.23 M, total 6 l plus 0.23—0.4 M, total 7 l). The reaction mixture for synthesis of the hexanucleotide was subjected to gel filtration using a column (2.4×74 cm) of Sephadex LH-20. Fractions containing larger oligomers were combined, deblocked, and applied to a column (1.0×100 cm) of DEAE-cellulose (chloride) in 7 M urea–0.02 M Tris-HCl. Elution was performed with a linear gradient of NaCl (0.05—0.3 M, total 1 l). Fractions of 3 ml were collected every 20 min and fractions 144—152 (20 A_{260}) were desalted.[4b] The oligonucleotide (0.2 A_{260}) was characterized by base analysis after digestion with RNase M. The ratio of Cp: Ap: Gp was estimated with a Varian LCS-1000 nucleic acid analyzer and found to be 2.08: 2.00: 1.00.

Synthesis of the Octanucleotide GU-C-G-U-C-G-G (2)

i) **Protected Pentanucleotide G-U-C-G-G**——The protected tetranucleotide U(Bz)–bzC(Bz)–ibG(Bz)–ibG(Bz)$_2$ (5080 A_{260}, 0.074 mmol) was obtained by stepwise condensation using the method described for the synthesis of **1** and was reacted with (MeOTr)ibG(Bz)p (16200 A_{260}, 0.82 mmol) using DCC (8.2 mmol) in pyridine (5 ml) for 6 days. The pentanucleotide was isolated by ion-exchange chromatography on a column (3.5×58 cm) of TEAE-cellulose (acetate form) with a linear gradient of TEAA in 80% ethanol (0.2—0.5 M, total 6 l). The yield was 1093 A_{260}, 0.013 mmol, 18%.

ii) **Deprotection of the Pentanucleotide**——An aliquot (800 A_{260}) of the pentamer was treated with acetic anhydride (5 ml) in pyridine (5 ml) at room temperature overnight, then aqueous pyridine (50%, 20 ml) was added to the mixture. The solution was kept overnight, concentrated and the residue was treated with methanolic ammonia at 30° for 2 days. The deblocked pentanucleotide was isolated by ion-exchange chromatography on DEAE-cellulose (chloride). A column (1.0×130 cm) was equilibrated with 0.05 M NaCl, 0.02 M Tris-HCl (pH 7.5) and 7 M urea. Elution was performed with a linear gradient of NaCl (0.05—0.25 M, total 1.6 l) and the product was desalted by absorption on DEAE-cellulose (bicarbonate) followed by elution of chloride ions with 0.05 M TEAB and of the oligonucleotide with 1 M TEAB. The pentanucleotide G–U–C–G–G was further purified by reverse-phase chromatography on C-18 alkylsilica as described in Fig. 2 and characterized by complete hydrolysis with RNase M to give Up: Cp: Gp: G=1.00: 0.91: 1.92: 1.02. The sequence was confirmed by two-dimensional homochromatography of the partially digested pentamer with nuclease P1.

iii) **The Octanucleotide G-U-C-G-U-C-G-G** (2)——The protected pentanucleotide (4000 A_{260}, 0.061 mmol) and (MeOTr)bzC(Bz)p (0.93 mmol) were treated with DCC (9.5 mmol) in the presence of Dowex 50×2 (pyridinium form, 2 ml) in pyridine (5 ml) at room temperature for 4 days. The reaction mixture was treated with acetic acid (10 ml) in pyridine (10 ml) after treatment with aqueous pyridine and worked up to remove solid materials. The oligonucleotide mixture was applied to a column of TEAE-cellulose under the conditions given in Fig. 3a and separated from the mononucleotides. The mixture containing the protected hexamer (2690 A_{260}) was subjected to condensation with (MeOTr)U(Bz)p (1.11 mmol) using DCC (10 mmol) in the presence of Dowex 50×2 (1 ml) in pyridine (5 ml). After 3 days the mixture was worked up as above and the heptamer was separated from the mononucleotide as shown in Fig. 3b. The fractions containing the heptamer (1260 A_{260}) were condensed with (MeOTr) ibG(Bz)p (0.55 mmol) using DCC (5.5 mmol) in the presence of Dowex 50×2 (1 ml) in pyridine (5 ml) at room temperature for 5 days. The mixture was worked

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up as described for the hexamer and the demonomethoxytritylated octanucleotide was deacylated by treatment with methanolic ammonia (10 ml) at 30° for 2 days. The deblocked octanucleotide was fractionated by ion-exchange chromatography on a column (1.2×23 cm) of DEAE-Sephadex A-25 (bicarbonate). The column was washed with 0.1 M TEAB and the oligomers were eluted with 1 M TEAB. The octanucleotide was isolated by ion-exchange chromatography on a column (1.0×95 cm) of DEAE-Sephadex A-25 (chloride) with a linear gradient of sodium chloride (0.1—0.3 M, total 1.3 l) in 20 mM Tris-HCl (pH 7.5), 7 M urea. The fractions eluted at 0.22 M sodium chloride (13.5 A_{260}) were further purified by a reverse-phase chromatography as shown in Fig. 4. The product was characterized by partial nuclease P1 digestion after labeling at the 5′-end followed by acidic electrophoresis and homochromatography.[9]

Synthesis of the Pentanucleotide U-C-C-G-G (3)

i) **bzC(Bz)p(CE)ibG(Bz)pibG(Bz)$_2$ (5)**——ibG(Bz)p(CE)ibG(Bz)$_2$ was prepared from the diester dimer ibG(Bz)–ibG(Bz)$_3$[4b] (6500 A_{260}, 0.17 mmol) by treatment with TPS (205 mg) in pyridine (0.5 ml) for 30 min followed by addition of β-cyanoethyl (1.7 mmol) at 4°. After reactions for 24 hr at 4° formation of the triester dimer was confirmed by TLC (chloroform–methanol, 10: 1) and the dimer was extracted with chloroform. The organic layer was concentrated, coevaporated with toluene and the residue was applied to a column (3.5×55 cm) of silica G type 60. Elution was performed with 15: 1 chloroform–methanol. The yield of the triester dimer was 3720 A_{260}, 0.102 mmol, 61%. For the synthesis of the trimer, the triester dimer (0.274 mmol) and (MeOTr)bzC (0.301 mmol) were treated with DCC (3 mmol) in the presence of Dowex 50×2 (pyridinium form, 2 ml) in pyridine (4 ml) at room temperature for 6 days. The diester trimer was isolated by ion exchange chromatography on a column (2.8×7 cm) of TEAE-cellulose (acetate). Elution was performed by stepwise increase of TEAA concentration. The monomer was removed with 0.04 M TEAA and the trimer was eluted with 0.5 M TEAA. The trimer was triesterified with β-cyanoethanol (0.18 ml) and TPS (185.7 mg) as described for the dimer. The triester trimer was isolated by preparative TLC (3 sheets) and the demonomethoxytritylated trimer (**5**) was obtained by treatment with 80% acetic acid followed by preparative TLC. The yield was 4600 A_{260}, 0.078 mmol, 29%.

ii) **The Tetramer (16)**——The trimer (**5**) (4600 A_{260}, 0.078 mmol) and (MeOTr)bzC(Bz)p (0.1 mmol) were treated with DCC (1 mmol), Dowex 50×2 (pyridinium form, 1 ml) and pyridine (2 ml) at room temperature for 6 days. The mixture was treated with aqueous pyridine and then with acetic anhydride (8 ml) in pyridine (8 ml) at 10° for 14 hr. After aqueous pyridine treatment the monomethoxytrityl group was removed with 80% acetic acid and the mixture was applied to a column (2.1×75 cm) of Sephadex LH-20 in 95% ethanol. Fractions of 1.3 ml were collected every 10 min. Fractions 90—115 (2400 A_{260}, 0.031 mmol) were combined and used for the next reaction.

iii) **The Pentanucleotide (3)**——The tetranucleotide (**6**) (1690 A_{260}) and (MeOTr)U(Bz)p (1640 A_{260}) were treated with DCC (1 mmol) in the presence of Dowex 50×2 (pyridinium form, 1 ml) in pyridine (1 ml) at room temperature for 4 days. The mixture was treated with aqueous pyridine and then with concentrated ammonia (1 ml) at 0° for 5 min. Volatile materials were removed and the residue was treated with 80% acetic acid. Acyl groups were then removed by treatment with methanolic ammonia at 30° for 1 day. One-half of the materials was applied to a column (1.8×40 cm) of DEAE-cellulose (bicarbonate) and eluted with a linear gradient of TEAE (0—0.6 M, total 5 l) then with 0.6 M TEAB. Oligomers were then applied to a column (1.3×73 cm) of DEAE-cellulose (chloride) and eluted with a linear gradient of sodium chloride (0—0.25 M, total 2 l) in 7 M urea, 0.02 M Tris-HCl (pH 7.5). Fractions of 4.2 ml were collected every 15 min and fractions 305—335 were desalted.[5] The yield of the deblocked pentamer (**3**) was 529 A_{260}. An aliquot was hydrolyzed with RNase M, and subjected to PPC (solvent A): each spot was eluted with water (1.5 ml). The ratio of G: Gp: (2Cp+Up)=1.00: 1.05: 0.92 was obtained by absorbance measurements: G (0.201 A_{252}), Gp (0.212 A_{252}) and CP+UP (0.36 A_{270}). Cp and Up were separated by acidic PEP and the ratio of Cp to Up was found to be 1.93 to 1.00. Partial nuclease P1 digestion of the 5′-labeled product was performed for mobility shift analysis.

Synthesis of the Hexanucleotide C-C-C-C-C-G (4)

i) **bzC(Bz)-ibG(Bz)$_2$**——Pyridinium (MeOTr)bzC(Bz)p (730 mg, 0.9 mmol), ib G (Bz)$_2$ (550 mg, 1.0 mmol) and Dowex 50 (Pyridinium form) (*ca.* 1 ml) were dried by coevaporation with pyridine (5 times). Pyridine (8 ml) and DCC (1.03 g, 5 mmol) were added and after shaking for 10 min the solution was evaporated to an immobile gum. After 5 days at 26°, 50% aqueous pyridine (20 ml) was added and the solution was extracted with hexane (20 ml×4). After 12 hr at 4° and 6 hr at room temperature, DCU and Dowex resin were filtered off, then the filtrate was concentrated and coevaporated with ethanol. 80% acetic acid was added and removed after 2 hr by evaporation followed by coevaporation twice with aqueous butanol. The residue was dissolved in ethanol (200 ml) then applied to a column (3.4×50 cm, 54 ml) of TEAE-cellulose (acetate form). After washing with ethanol (1 l) elution was performed with a linear gradient of ethanol and 0.2 M triethylammonium acetate (TEAA) in ethanol (total volume 4 l), followed by 1.0 M TEAA in ethanol (1 l). 21.5 ml fractions were collected every 15 min. Fractions 161—192 contained pure-protected CpG (37820 A_{260}, 0.83 mmol) a 91% conversion with respect to the starting (MeOTr)bzC(Bz)p. (UV: 50% ethanol λ_{max} 261 nm, 274 nm(s), 282 nm(s)). Protected nucleotide in the pyridinium salt form was precipitated from ether–hexane (3:2) as described previously. Deblocking of an aliquot with methanolic ammonia afforded CpG (Table II) (UV: pH 7.0, λ_{max} 253 nm, 270 nm (s), λ_{min} 224 nm; pH 1, λ_{max} 261 nm (s), 276 nm,

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λ_{min} 232 nm, pH 11, λ_{max} 266 nm, λ_{min} 233 nm). Digestion of material purified by PPC and PEP with ribonuclease A (standard conditions) gave Cp and G in a ratio of 1.16 to 1. Digestion of $3A_{260}$ with venom phosphodiesterase (30 μg of enzyme, in 50 μl of 50 mM TEAB at 37° for 5 hr) gave C+pG in a molar ratio of 1 to 1.06.

ii) **(MeOTr)bzC(Bz)-bzC(Bz)-ibG(Bz)$_2$**——(MeOTr)bzC(Bz)p, (855 mg, 30160 A_{260} 1.04 mmol) and bzC(Bz)–ibG(Bz)$_2$ (570 mg, 22165 A_{260} 0.485 mmol) as their pyridinium salts plus Dowex 50×2 (pyridinium form) (*ca.* 20 mg) were dried and treated with DCC (2.06 g, 10 mmol) as described above. After 5 days at 28° the reaction mixture was worked up as above and, without removal of the monomethoxytrityl group, the residue was applied to the same column of TEAE-cellulose (acetate form) in ethanol (150 ml) and pyridine (100 ml). After washing with ethanol elution was performed with a linear gradient of ethanol and 0.4 M TEAA in ethanol (6 l, total volume) and fractions of 21.5 ml were collected every 20 mn. Fractions 181—219 contained the pure protected trimer (18040 A_{260}, 0.25 mmol; total recovery from the column, 36625 A_{260}) (UV properties in ethanol, λ_{max} 230 nm, 260 nm, 305 nm (s), λ_{min} 248 nm). Complete deblocking of this material, after recovery as the pyridinium salt and precipitation from ether–hexane (3: 2), afforded CpCpG, and an aliquot of the fully protected trimer after treatment with methanolic ammonia alone afforded (MeTr)-CpCpG which gave positive color reactions on spraying with 30% H_2SO_4 and warming. Digestion of CpCpG, purified by PPC followed by PEP, with ribonuclease A gave only Cp and G in a molar ratio of 1.95 to 1. (CpCpG UV properties: pH 7.0, λ_{max} 257 nm, 267 nm (s), λ_{min} 227 nm, pH 1.0, λ_{max} 277 nm, λ_{min} 234 nm: pH 11.0, λ_{max} 267 nm, λ_{min} 235 nm).

iii) **bzC(Bz)pCEbzC(Bz)pCEibG(Bz)$_2$ (7)**——Pyridinium (MeOTr)bzC(Bz)–bzC(Bz)–ibG(Bz)$_2$ (493 mg, 0.24 mmol, 18000 A_{260}), dried 5 times with pyridine, was activated with TPS (290 mg, 0.96 mmol) in pyridine (3 ml) for 30 min. β-Cyanoethanol (4.8 mmol, 0.35 ml) was added and TLC (silica, $CHCl_3$ iEtOH, 20: 1) after 40 hr at room temperature indicated *ca.* 33% formation of an MeOTr-containing component (*Rf*, 0.42). Attempted removal of remaining phosphodiesters and triisopropyl benzene sulfonic acid by passage through a column of TEAE-cellulose (acetate form) in ethanol was unsuccessful, as significant losses of the β-cyanoethyl group occurred. A portion of the reaction mixture was dissolved in $CHCl_3$ (20 μl) and extracted with 5% aqueous pyridine (20 ml×6). After removal of chloroform by evaporation the residue was treated with 80% acetic acid for 2 hr at room temperature, and a major band (*Rf* 0.47) was isolated by preparative layer chromatography on silica eluting with $CHCl_3$/EtOH (10: 1). The yield of material eluted with $CHCl_3$/MeOH (10: 1) and precipitated from hexane was 74.1 mg. Treatment of an aliquot with methanolic ammonia afforded CpCpG alone as determined by PPC and PEP (Table II).

iv) **bzC(Bz)-bzC(Bz)pCEbzC(Bz)pCEibG(Bz)$_2$**——bzC(Bz)pCEbzC(Bz)pCEibG(Bz)$_2$ (74 mg, 0.043 mmol 3112 A_{260}) was treated with pyridinium (MeOTr)bzC(Bz)p (74.1 mg, 0.090 mmol, 2610 A_{260}) and DCC (185.4 mg, 0.9 mmol) in pyridine in the presence of Dowex 50×2 (pyridinium form) as described above. After 3 days at 27°, the reaction mixture was worked up as before and treated with 80% acetic acid for 1 hr at room temperature. Examination by PPC and PEP of a completely deprotected aliquot indicated extensive (*ca.* 65%) conversion to CpCpCpCpG (Table II). Complete digestion with ribonuclease A gave Cp and G in a ratio of 3.12: 1.00. UV properties: pH 7.0, λ_{max} 258 (s), 273, λ_{min} 237 nm; pH 1.0, λ_{max} 277, λ_{min} 238 nm, pH 11.0, λ_{max} 267, λ_{min} 248 nm. The product was dissolved in pyridine (1 ml) and applied to a column (2 cm× 80 cm, 250 ml) of Sephadex LH-20 packed in ethanol. Elution was performed with ethanol and fractions of 1 ml were collected every 15 min. Fractions 105—285 contained bzC(Bz)p, and were pooled and concentrated. The crude yield without further purification was 120.0 mg (0.051 mmol with respect to the tetramer, 118% yield). The only nucleotide contaminant detected after complete deprotection was CpCpG, so this material was used for the next coupling reaction.

v) **The Pentamer (9)**——The above protected tetramer (*ca.* 0.051 mmol) was the pyridinium salt was treated with pyridinium (MeOTr)bzC(Bz)p (0.15 mmol), 132.4 mg) and DCC (1.5 mmol, 309 mg) in pyridine in the presence of Dowex 50×2 (pyridinium form) as described above for 4 days at 27°. Again, examination of a completely deprotected aliquot by PPC and PEP after the usual work-up indicated extensive conversion to CpCpCpCpG (Table II). (UV Properties: pH 7.0, λ_{max} 270, λ_{min} 236 nm; pH 1.0, λ_{max} 278, λ_{min} 238 nm; pH 11.0, λ_{max} 267, λ_{min} 250 nm). The compound was completely degraded with ribonuclease A to yield only Cp and G in a ration of 3.86: 1.00. After removal of the 5′-protecting group, the reaction mixture was applied to the same column of Sephadex LH-20 as described above. Elution under identical conditions afforded bzC(Bz)p free material in fractions 55—125 (95.5 mg), which contained traces of unprotected tetramer as judged by PPC after complete deprotection. This material (*ca.* 0.05 mmol as the pentamer) was used without further purification for the subsequent coupling reaction.

vi) **The Protected Hexamer and Deblocking to Yield C-C-C-C-C-G**——The pyridinium salt of the protected pentamer (*ca.* 0.05 mmol, 95 mg) was treated with pyridinium (MeOTr)bzC(Bz)p (0.15 mmol, 132 mg) and DCC (1.5 mmol, 310 mg) as described above. After 3 days at 27°, deprotection of an aliquot and examination by PPC and PEP showed conversion to the hexamer. (UV properties: pH 7.0, λ_{max} 270, λ_{min} 255 nm; pH 1.0, λ_{max} 278, λ_{min} 242 nm; pH 11.0, λ_{max} 270, λ_{min} 265 nm). The compound was completely digested with ribonuclease A to yield only Cp and G in a riato of 4.95: 1.00. After the usual work-up, half the material was treated with 80% acetic acid and applied (78 mg) in the manner described previously to the same column of Sephadex LH-20. Fractions 55-107 contained no bzC(Bz)p, as judged by PPC and PEP after deprotection

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of an aliquot, and were pooled and concentrated. The yield was 954 A_{260} (0.0062 mmol with respect to CpCpCpCpCpG, neglecting hypochromicity). Deprotection of this material with methonolic ammonia afforded 425 A_{260}, which was applied to a column (1.3 × 85 cm, 113 ml) of DEAE-cellulose (chloride form) pre-equilibrated with 7 M urea containing 0.02 M Tris-HCl, pH 7.5. Five ml fractions were collected every 20 min. After washing with the starting buffer (22 fractoins) elution was performed with a linear gradient formed from 7 M urea, 0.02 M Tris-HCl, pH 7.5, and 7 M urea, 0.02 M Tris-HCl, pH 7.5, 0.3 M NaCl (1 l, total volume). Fractions 150—160 contained the hexamer (8.00 A_{260}), which was characterized by 5′-terminal labelling with ^{32}P (polynucleotide kinase), partial digestion with snake venom phosphodiesterase and 2-dimensional analysis to confirm its structure (first dimension electrophoresis on cellulose acetate, pH 3.5; second dimension homochromatography-homomix III).

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