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Studies on Transfer Ribonucleic Acids and Related Compounds. XXV.[1] Synthesis of *E. coli* $tRNA_f^{Met}$ 5′-Terminal Tetranucleotide C-G-C-Gp and Its Sequence Analog U-G-C-Gp

Eiko Ohtsuka, Tetsuo Miyake, and Morio Ikehara

Faculty of Pharmaceutical Sciences, Osaka University[2]

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A tetranucleotide C–G–C–Gp (cytidylyl-(3′—5′)-guanylyl-(3′—5′)-cytidylyl-(3′—5′)-guanosine 3′-phosphate) was synthesized by condensation of (Bz)bzC(Bz)–ibG(Bz)p (**5**) with C(Bz)–ibG(Bz)*p*$NHC_6H_4S(O)CH_3$ (**6**) using 2,4,6-triisopropylbenzenesulfonyl chloride. This sequence corresponds to the *E. coli* $tRNA_f^{Met}$ 5′-terminus. A base substituted analog U–G–C–Gp was also synthesized by a similar procedure to that used for C–G–C–Gp. These tetranucleotides were characterized by enzymic hydrolysis.

Keywords——*p*-methylsulfinyl phosphoranilidate; hydrogen peroxide; ion-exchange chromatography; ribonuclease M; alkaline phosphatase; isoamyl nitrite

We have previously reported syntheses of fragments from *E. coli* $tRNA_f^{Met}$ termini by a number of approaches including stepwise condensation of mononucleotides with diesterified oligonucleotides,[3,4] and addition of phosphomonoesters to triesterified oligomers.[5,6] Phosphoranilidate and phosphoranisidate were used for protections of terminal phosphomonoesters in the synthesis of the yeast alanine tRNA fragments[7] and 5′-terminal tetranucleotide of yeast $tRNA^{Tyr}$.[8] In the present paper we report (a) synthesis of C–G–C–Gp by block condensation of dinucleotides using phosphoro-*p*-methylthioanilidate[9] as a protection of the 3′-phosphate. The tetranucleotide corresponds to the 5′-terminal sequence from *E. coli* $tRNA_f^{Met}$ [10] which has a unique unpaired 5′-cytidine, since the fifth base from the 3′-terminus is adenosine. Substitution of cytidine with uridine in the 5′-terminal tetranucleotide would provide a base paired 5′-end when U–G–C–G is joined to the rest of the molecule. Joining of chemically synthesized 3′-fragments from *E. coli* $tRNA_f^{Met}$ has been performed enzymically using bacteriophage T4 induced RNA ligase.[11] This approach is applicable to the 5′-quarter molecule of the tRNA. The chemically mutated 5′-quarter molecule can be joined to the naturally occurring three quarter molecule obtained by partial digestion with RNase T_1[12] to give a molecule with the paired 5′-end, which was shown to be the requirement for a specific interaction with bacterial protein elongation factor Tu.[13] The base substituted

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analog U–G–C–Gp was synthesized by condensation of protected U–Gp with the same 3′-terminal dinucleotide as used for the synthesis for C–G–C–Gp.

Synthesis of 3′-Phosphoro-*p*-methylsulfinylanilidate of N-Isobutyryl, 2′-O-Benzoylguanosine 3′-Phosphate (4)

The amidate (**4**) was synthesized from (MeOTr)ibG(Bz)p[4] (**1**) as shown in Chart 1 by the similar procedure to that described for Up.[9] The procedure included condensation of *p*-methylthioanilidate with **1** and oxidation of **2** with hydrogen peroxide followed by removal of the monomethoxytrityl function.

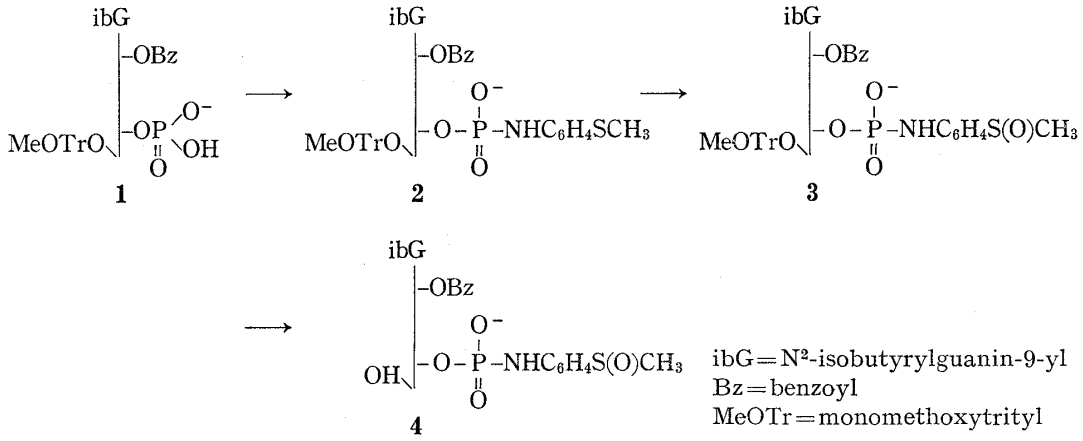

Chart 1

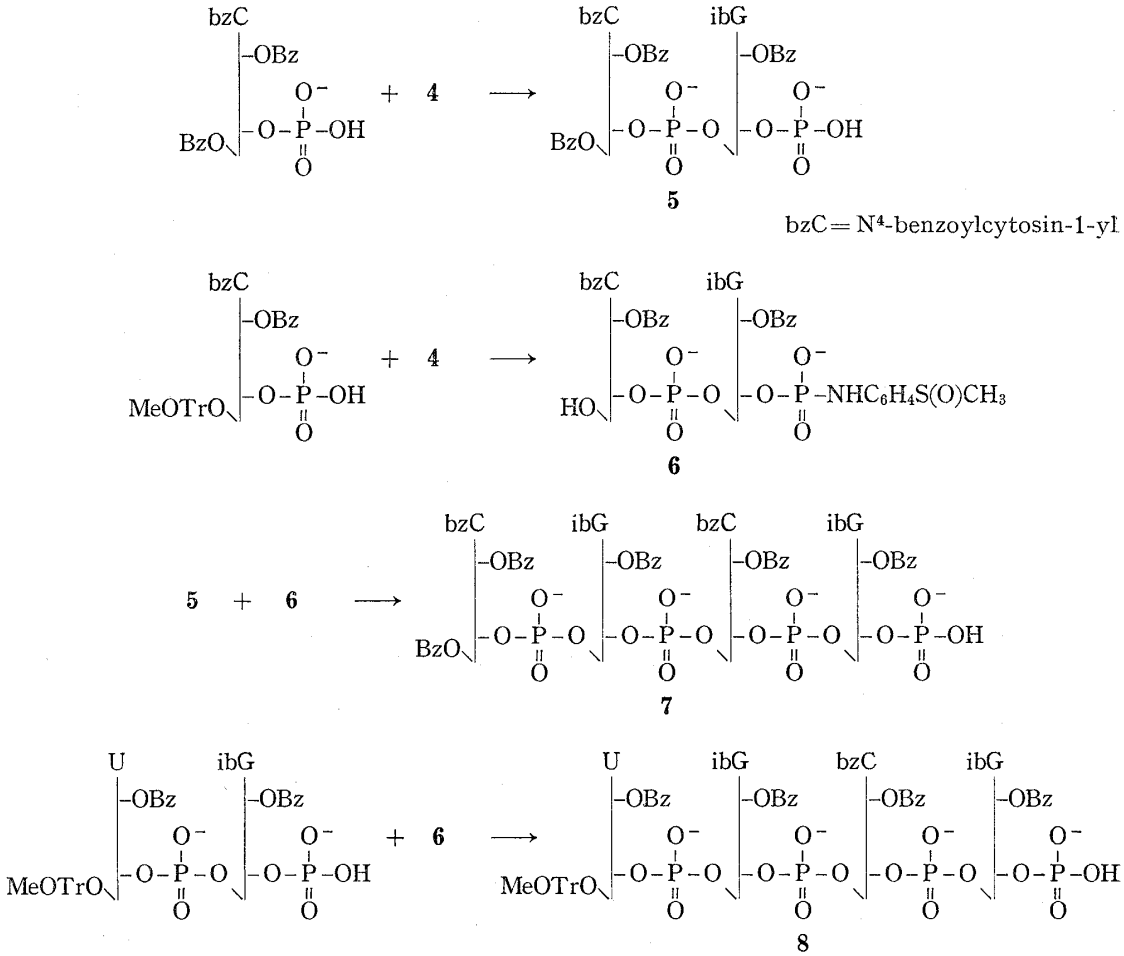

Chart 2

Synthesis of Dinucleotides and Their Condensation to Yield the Tetranucleotides (7, 8)

The 5′-terminal dinucleotide (**5**) was synthesized by condensation of (Bz)bzC(Bz)p with the anilidate (**4**) using dicyclohexylcarbodiimide (DCC) as the condensing reagent as shown in Chart 2. The hydroxyl component (**6**) was synthesized from (MeOTr)bzC(Bz)p with **4** by condensation with DCC followed by acidic treatment (Chart 2). Block condensation of these two dinucleotides **5** and **6** was performed with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS).[14] The 3′-phosphoro-*p*-methylsulfinylanilidate was removed by treatment with isoamyl nitrite in 1:1 pyridine–acetic acid.[7] The tetranucleotide (**7**) was isolated by ion-exchange chromatography using TEAE-cellulose. The isolated yield of **7** was 12%. Treatment of **7** with methanolic ammonia gave the completely deblocked tetranucleotide C–G–C–Gp, which was characterized by enzymic dephosphorylation with alkaline phosphatase and hydrolysis with RNase M.

The base substituted analog (**8**, Chart 2) was synthesized by treatment of (MeOTr)U(Bz)-ibG(Bz)p with **6** and TPS using the same conditions as above.

Conclusion

Two teranucleotides were synthesized by block condensation of dimers. The terminal 3′-phosphoranilidate was substituted with *p*-methylsulfinyl group to stabilize P–N linkage during condensation in pyridine.[15] Although stepwise condensation of mononucleotides may be more straightforward method for synthesis of short oligonucleotides, the block condensation is useful in syntheses of partially substituted analogs. The 3′-terminal dinucleotide block (**6**) was used repeatedly in synthesis of C–G–C–Gp and its base substituted analog U–G–C–Gp. The yield of this type of condensation is being improved by introduction of the 3′-terminal phosphorodianilidate and protection of the internucleotide linkages.[6,16]

Experimental

General Methods——Paper chromatography was performed by the descending technique using solvent systems: A, 2-propanol–concentrated ammonia–water (7:1:2, v/v); B, ethanol–1 M ammonium acetate (pH 7.5) (7:3, v/v); C, 1-propanol–concentrated ammonia–H_2O (55:15:35, v/v). Paper electrophoresis was performed using 0.05 M triethylammonium bicarbonate (pH 7.5) at 900 V/40 cm.

Other general methods and conditions for enzymic hydrolyses were performed as described previously.[7]

ibG(Bz)*p*-NHC$_6$H$_4$S(O)CH$_3$ (4)——(MeOTr)ibG(Bz)p (**1**) (0.5 mmol) and *p*-aminophenyl methyl sulfide (970 mg, 7 mmol) were treared with DCC (1.03 g, 5 mmol) in 10:2 *t*-BuOH–H_2O at 27° for 2 days. The extent of the reaction was monitored by paper electrophoresis. The reaction was ceased by addition of water and the mixture was applied to a column (2×10 cm) of Dowex 50 W×2 (pyridinium form) after removal of dicyclohexylurea and DCC by extraction of the filtered solution with hexane. The column was washed with 50% aqueous pyridine and the product (**2**) was precipitated with 3:2 ether–hexane from its solution in anhydrous pyridine. The precipitate (**2**) was treated with 30% hydrogen peroxide (1.17 ml) in DMF (16.5 ml) at 27° for 2 days. An aliquot was deblocked for checking in paper chromatography (see Table I). Manganese oxide was added until no bubbling occurred by shaking and the filtered solution was applied to a column (2×10 cm) of Dowex 50 W×2 (pyridinium form). The column was washed with 50% pyridine and the product (**3**) was precipitated with 3:2 ether–hexane from its solution in pyridine. **3** was treated with 80% acetic acid at 30° for 1.5 hr and acetic acid was removed by evaporation of added aqueous 1-butanol. The demonomethoxytritylated product (**4**) was dissolved in 70% ethanol and applied to a column (3.5×56 cm). The column was washed with 70% ethanol and eluted with a linear gradient of triethylammonium acetate (0.30—0.09 M, 31) in 70% ethanol. Fractions of 18 ml were collected every 16 min. The appropriate fractions (61—75) were identified by measurement of UV spectra ($A^{EtOH}_{230/260}$ and $A^{OH^-}_{260/310}$) and concentrated *in vacuo*. The residue was dissolved in butanol, washed with water. Evaporate with added aqueous pyridine and **4** was precipitated with 3:2 ether–hexane from its solution in anhydrous pyridine. The yield was 9000 A_{260} units, 0.28 mmol (56%). An aliquot was deacylated for identification (Table I).

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Table I. Paper Chromatography and Electrophoresis

Compound	*Rf* Solvent A	*Rf* Solvent B	*Rf* Solvent C	Relative mobility pH 7.5
G	0.32	0.55	0.55	0.00
G>p	0.45	0.62	0.58	0.53
Gp	0.05	0.19	0.32	0.86
C	0.49	0.66	0.62	0.00
C>p	0.37	0.51	0.62	0.61
Cp	0.10	0.26	0.43	1.00
C–Gp	0.02	0.05	0.22	1.00
C–G–C–Gp		0.01	0.06	0.92
C–GpNHC$_6$H$_4$CH$_3$	0.23	0.47	0.68	0.66
C–GpNHC$_6$H$_4$S(O)CH$_3$	0.08	0.29	0.56	0.66
bzC(Bz)-ibG(Bz)*p*NHC$_6$H$_4$S(O)CH$_3$				0.42
(Bz)bzC(Bz)-ibG(Bz)p				0.71
(Bz)bzC(Bz)-ibG(Bz)-bzC(Bz)ibG(Bz)p				0.65
U	0.55	0.68	0.70	0.00
U>p	0.46			
Up	0.19	0.28	0.58	1.00
U–Gp	0.09	0.11	0.39	1.05
U–G–C–Gp			0.18	1.00

bzC(Bz)-ibG(Bz)p (5)——(Bz)bzC(Bz)p[17] (7300 A_{305}, 0.73 mmol) and ibG(Bz)*p*NHC$_6$H$_4$SOCH$_3$ (14000 A_{260}, 0.44 mmol) were applied to a column (2×10 cm) of Dowex 50 W×2 (pyridinium form) separately. The nucleotide were precipitated with 3:2 ether–hexane from its solution in pyridine and combined with dried Dowex 50 W×2 (pyridinium salt). The mixture was rendered anhydrous by evaporation of added pyridine 3 times and treated with DCC (1.51 g, 7.3 mmol) in pyridine (1—2 ml) at 27° for 7 days. Aqueous pyridine (50%, 10 ml) was added and DCC was removed by extraction with hexane (6 ml). After 24 hr the filtered solution was concentrated and the nucleotides were precipitated with 3:2 ether–hexane from its solution in pyridine. The phosphoramidate linkage was cleaved by treatment with isoamyl nitrite (1 ml) in 1:1 pyridine–acetic acid (5 ml) at 27° for 14 hr. The volatile materials were removed by coevaporation of water and the residue (in 200 ml of 70% ethanol) was applied to a column (3.5×56 cm) of TEAE-cellulose (acetate form). The elution was performed with a linear gradient of triethylammonium acetate in 70% ethanol (0—0.3 M, 6 l). The dinucleotide (5) was eluted at 0.21 M salt. The yield was 7800A_{260}, 0.118 mmol, 42%. The product was desalted by extraction with water (5 ml) 3 times from its solution in 1-butanol (20 ml) and precipitated with 3:2 ether–hexane. An aliquot was deacylated and C–Gp was hydrolyzed with RNase A to yield Cp and Gp in a ratio of 1.00 to 1.03.

bzC(Bz)-ibG(Bz)pNHC$_6$H$_4$S(O)CH$_3$ (6)——The pyridinium salts of (MeOTr)bzC(Bz)p (6600 A_{305}, 0.66 mmol) and ibG(Bz)pNHC$_6$H$_4$S(O)CH$_3$ (4) (16000 A_{260}, 0.50 mmol) were combined with Dowex 50 W×2 (pyridinium form) (2 ml). The mixture was rendered anhydrous by evaporation of added pyridine 3 times. It was treated with DCC (1.44 g, 7 mmol) in pyridine (1—2 ml) at 27° for 6 days and the nucleotides were precipitated with 3:2 ether–hexane after removal of dicyclohexylurea and Dowex 50 W×2. The precipitate was dissolved in 70% ethanol (100 ml) and applied to a column (3.5×56 cm) of TEAE-cellulose (acetate form). The elution was performed with a linear gradient of triethylammonium acetate (0—0.3 M, total 4 l) in 70% ethanol. The dinucleotide was eluted with 0.14 M of the salt and desalted by extraction with butanol (20 ml) from the concentrated appropriate fractions. The layer was washed with water (5 ml) 3 times and the concentrated residue was precipitated with 3:2 ether–hexane from its solution in pyridine. The precipitate was treated with 80% acetic acid for 1.5 hr and 6 was precipitated with ether–hexane as above. The yield was 4700 A_{260} units, 0.084 mmol, 17%. Pancreatic RNase digestion of the deblocked product gave Cp and Gp in a ratio of 1.00 to 0.99.

(Bz)bzC(Bz)-ibG(Bz)-bzC(Bz)-ibG(Bz)p (7)——**5** (3700 A_{260}, 0.098 mmol) and **6** (4500 A_{260}, 0.083 mmol) were rendered anhydrous with evaporation of pyridine 3 times and TPS (172 mg, 0.57 mmol) was added to the solution in pyridine (10 ml). The solution was concentrated to *ca.* 1 ml and kept at 15° for 7 hr. Aqueous pyridine (50%, 8 ml) and N,N-diiscpropyl ethylamine (0.15 ml) were added with cooling. After 14 hr at 15° the nucleotides were extracted with butanol (50 ml), washed with water (10 ml) 3 times and precipitated with 3:2 ether–hexane from its solution in pyridine. The precipitate was treated with isoamyl nitrite (0.21 ml) in 1:1 pyridine–acetic acid (3 ml) at room temperature for 17 hr and applied to a column (3.6×56 cm) of TEAE-cellulose after removal of the volatile materials. The elution was performed first with 0.05

(800 ml) and then with a linear gradient of triethylammonium acetate (0.08—0.6 M, 8 l) in 75% ethanol. The tetranucleotide (7) was eluted with 0.4 M salt, desalted by extraction with butanol and precipitated with ether–hexane. The yield was 794 A_{260} (9.7 μmol), 12%. An aliquot was deblocked by treatment with methanolic ammonia and C–G–C–Gp was hydrolyzed with RNase M or pancrearic RNase. Cp, Gp and G–Cp were separated by paper chromatography in solvent A in ratios of Cp: Gp: C=1: 1, 13: 1.01.

(MeOTr)U(Bz)-ibG(Bz)p——ibG(Bz)*p*$NHC_6H_4SOCH_3$ (**4**) (7300 A_{260}, 0.23 mmol) and (MeOTr)U(Bz)p (5000 A_{260}, 0.25 mmol) were condensed with DCC (515 mg, 2.5 mmol) using conditions described for **5**. The yield after chromatography on TEAE-cellulose was 3200 A_{260}, 0.075 mmol, 34%. An aliquot was deblocked for identification and U–Gp was completely hydrolyzed with RNase M to yield Up and Gp in the correct ratio. Chromatographic mobilities are shown in Table I.

MeOTrU(Bz)-ibG(Bz)-bzC(Bz)-ibG(Bz)p (**8**)——MeOTrU(Bz)-ibG(Bz)p (1620 A_{260}, 0.053 mmol) and **4** (2650 A_{260}, 0.052 mmol) were treated with TPS (97 mg, 0.32 mmol) in pyridine (1.5 ml) for 3.5 hr. The reaction was stopped and worked up as described for **2**. The product was isolated by chromatography on TEAE-cellulose as above and the completely deblocked product (7.7 A_{260}) was isolated by paper chromatography in solvent C. *Rf* values and relative mobility of U–G–C–G are shown in Table I.

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