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Studies on Transfer Ribonucleic Acids and Related Compounds. XLI.[1] Synthesis of tRNA Fragments containing Modified Nucleosides

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tRNA fragments containing modified nucleosides have been synthesized either by condensation of nucleotides containing a modified base or by modification of oligonucleotides. Ribothymidine 3′-phosphate was prepared by condensation of the silylated base with protected D-ribose followed by phosphorylation. Ribothymidine-containing trimer, T-ψ-C, was synthesized by the phosphodiester method. A ribothymidine-containing hexamer T-U-C-A-A-A and a 2′-*O*-methylcytidine containing hexamer C-G-G-G-Cm-Up were synthesized by the phosphotriester method. A dihydrouridine-containing trimer D-A-G and a 7-methylguanosine-containing trimer m^7G-U-C were obtained by modification of the trimers by reduction and methylation, respectively.

Keywords——ribooligonucleotide; modified base; tRNA fragments; phosphodiester method; phosphotriester method

We have previously reported the synthesis of oligoribonucleotides with sequences identical to that of the nascent strand of *E. coli* $tRNA_f^{Met}$, and these oligonucleotides have been joined with RNA ligase to construct the tRNA molecule.[2] For studies on the structure-function relationship of tRNAs and on the role of modified nucleosides, it is necessary to synthesize tRNAs containing some modified bases. In the present paper, we describe the synthesis of oligonucleotides containing ribothymidine (T), pseudouridine (ψ), 2′-*O*-methylcytidine (Cm), 7-methylguanosine (m^7G) and dihydrouridine (D). A trimer T-ψ-C[3] were synthesized by the phosphodiester method using ribothymidine obtained by the silyl method.[4] T-U-C-A-A-A and C-G-G-G-Cm-Up were synthesized by the phosphotriester method involving 2′-*O*-(*o*-nitrobenzyl) and phosphoranilidate protection.[5] m^7G-U-C and D-A-G were synthesized by methylation of G-U-C and reduction of U-A-G.

Synthesis of Ribothymidine and T-Ψ-C

Ribothymidine was prepared by condensation of bis (trimethylsilyl)-thymine with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose[6] in the presence of stannic chloride,[4] followed by removal of the protecting groups. The 5′-hydroxyl group of ribothymidine was protected with a monomethoxytrityl group and phosphorylation was carried out with morpholino-phosphorodichloridate[7] as shown in Chart 1. The 2′-hydroxyl group was protected by benzoylation after hydrolysis with RNase A and the protected nucleotide (**4**) was obtained. T-ψ-C (**9**) was synthesized by the phosphodiester method using essentially the same procedure as described previously.[8] Pseudouridine 3′-phosphate was monomethoxytritylated and benzoylated to give 5. N-1 was benzoylated together with the 2′-hydroxyl group with benzoic anhydridein tetraethylammonium benzoate under the same condition. This may be advantageous for preventing isomerizaion[9] of ψ during acid treatment. Compound **7** was obtained in a yield of 64% and was condensed with **4** to yield fully protected T-ψ-C (**8**). Deblocking with 80% acetic acid and with ammonia gave the trimer T-ψ-C (**9**). The product (**9**) was purified by chromatography on DEAE-Sephadex A-25 and characterized by digestion with RNase M[10] to give Tp, ψp and C in the expected ratio. The timer T-ψ-C is difficult to

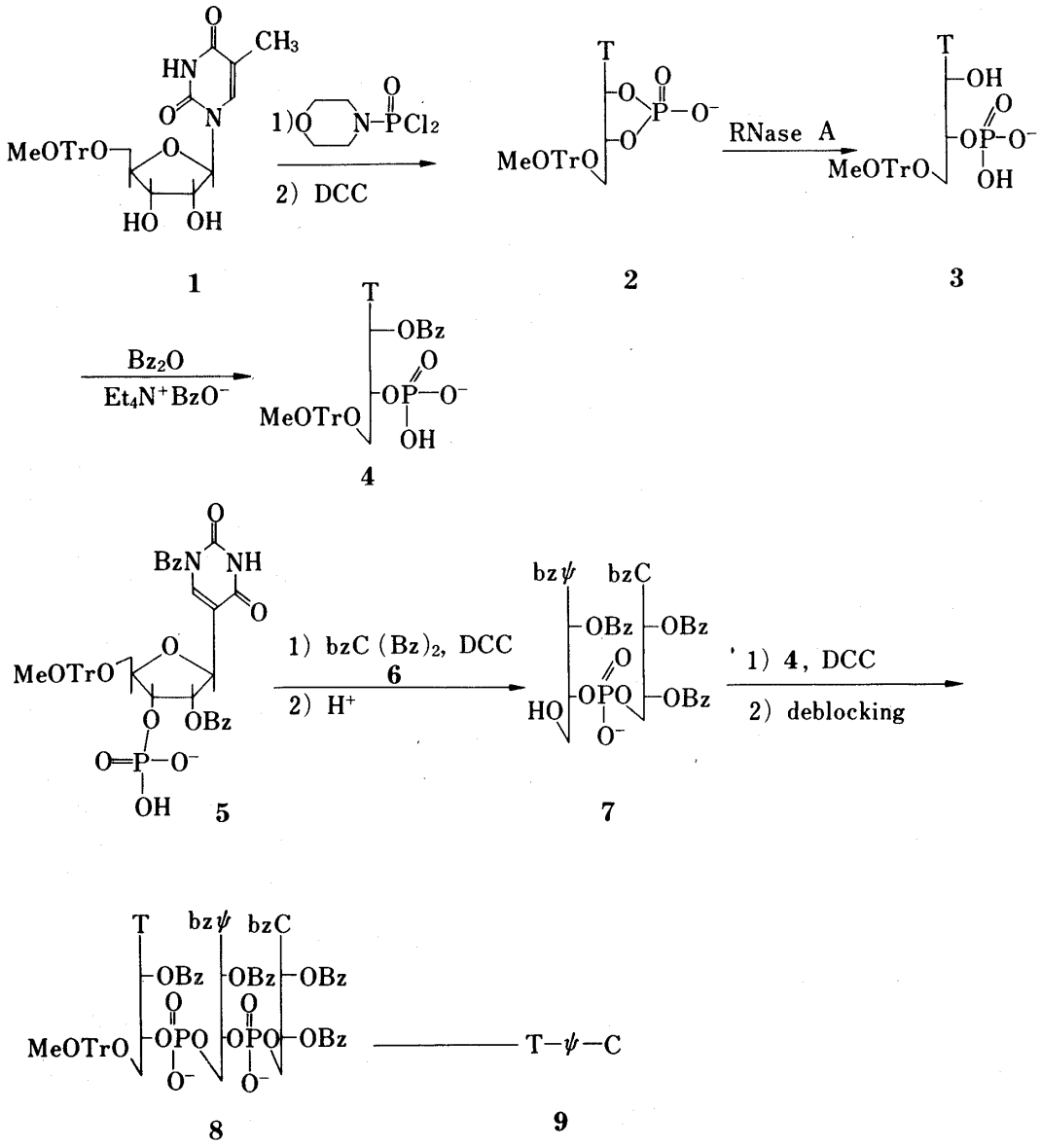

Chart 1

obtain by enzymatic digestion of tRNA. The chemically synthesized trimer can be tested as a substrate for RNA ligase.

Synthesis of T-U-C-A-A-A (17) and C-G-G-G-Cm-Up (29)

The hexanucleotides were synthesized by the phosphotriester method involving 2′-*O*-(*o*-nitrobenzyl) and phosphoranilidate protection.[5] Ribothymidine was converted to the corresponding 2′-*O*-(*o*-nitrobenzyl) derivative (**10**) by treatment with *o*-nitrophenyldiazomethane[11] followed by separation from the 3′-isomer by chromatography on silica gel. The yields of the 2′- and 3′-isomers were 24 and 30%, respectively. These *o*-nitrobenzylribothymidines were characterized by elemental analysis, nuclear magnetic resonance spectroscopy (NMR) and ultraviolt (UV) absorption spectroscopy. The 5′-hydroxyl group of 2′-*O*-(*o*-nitrobenzyl) ribothymidine was protected with a monomethoxytrityl group to give **11** (Chart 2). The nucleoside (**11**) was phosphorylated with *p*-chlorophenyl dihydrogen phosphate plus dicyclohexylcarbodiimide (DCC) to yield **12**. Condensation of **11** with protected UpCp (**13**) using mesitylenesulfonyl triazolide (MST, 1-(2,4,6-trimethylbenzenesulfonyl)-1*H*-1,2,4-triazole) gave the trimer (**14**), which was then treated with isoamyl nitrite to convert it to the

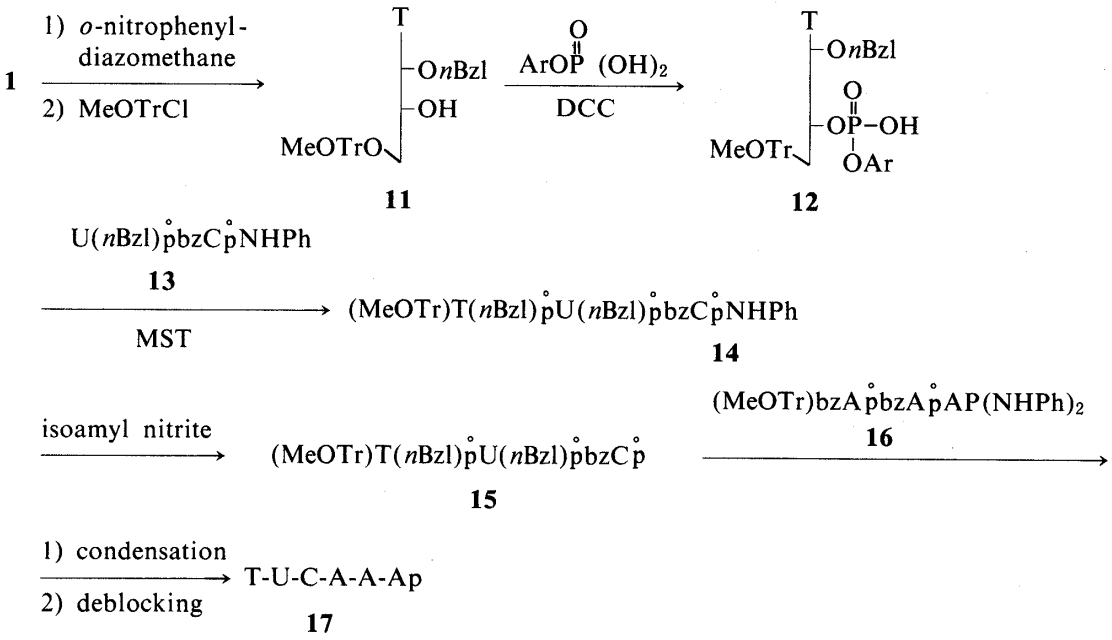

Chart 2

diester (**15**). Condensaton of **15** with protected ApApAp (**16**) were performed by a procedure similar to that used for the synthesisof other tRNA fragments.[5] The product T-U-C-A-A-Ap (**17**) was purified by chromatography on DEAE-cellulose in the presence of 7M urea and characterized by base ratio and mobility shift analysis.[12]

The 2′-*O*-methylcytidine containing hexanucleotide (**29**) was synthesized as shown in Chart 3. 2′-*O*-Methylcytidine[13] was protected to give 5′-*O*-monomethoxytrityl 2′-*O*-methyl-*N*-benzoylcytidine (**18**), which was phosphorylated with *p*-chlorophenyl phosphoroditriazolide (*p*-chlorophenyl bis(1*H*-1,2,4-triazol-1-yl)phosphate). The nucleotide (**19**) was condensed with 2′-*O*-(*o*-nitrobenzyl)uridine 3′-phosphate (**20**) using mesitylenesulfonyl nitroimidazolide (MSNI, 4-nitro-1-(2,4,6-trimethylbenzenesulfonyl)-1*H*-imidazole) to yield the fully protected dinucleotide (**21**). The dimer (**21**) was deblocked at the 5′-position to give **22** for elongation in the 5′-direction. Two protected dimers dGpGp and dCpGp (**26**) were prepared as described for the synthesis of **26** using the method reported previously.[1] The tetramer (**27**) was prepared by condensation of **22** and protected dGpGp, followed by 5′-deblocking with acid. The protected hexanucleotide (**28**) was obtained by condensation of **27** and **26** with mesitylenesulfonyl tetrazolide (1-(2,4,6-trimethylbenzenesulfonyl)-1*H*-tetrazole and the protecting groups were removed as described previously.[1] The fully deblocked hexamer C-G-G-G-Cm-Up (**29**) was characterized as above. Cm-Up was identified as shown in Fig. 1.

Modification of Oligonucleotides to Yield D-A-G and m^7G-U-C

The dihydrouridine containing trimer D-A-G was synthesized by reductionof U-A-G with platinum oxide in 10% acetic acid with slight modifications as described elsewhere[15] and isolated in a yield of 47% by ion-exchange chromatography on Sephadex A-25. The structural integrity was confirmed by mobility shift analysis (Fig. 2) and complete digestion followed by chromatography on polyethyleneimine cellulose plates together with authentic Dp, which was obtained by hydrolysis of tRNAs (Fig. 3).

The trimer containing 7-methylguanosine, m^7G-U-C, was prepared by methylation of G-U-C with dimethyl sulfate at pH 4, under which condition cytidine would not be methylated because of by protonation. Methylation of guanosine and cytidine in neutral aqueous solution with dimethyl sulfate was reported earlier.[16] m^7G-U-C was isolated by ion-exchange chromatography and the selective methylation of guanosine was confirmed by complete

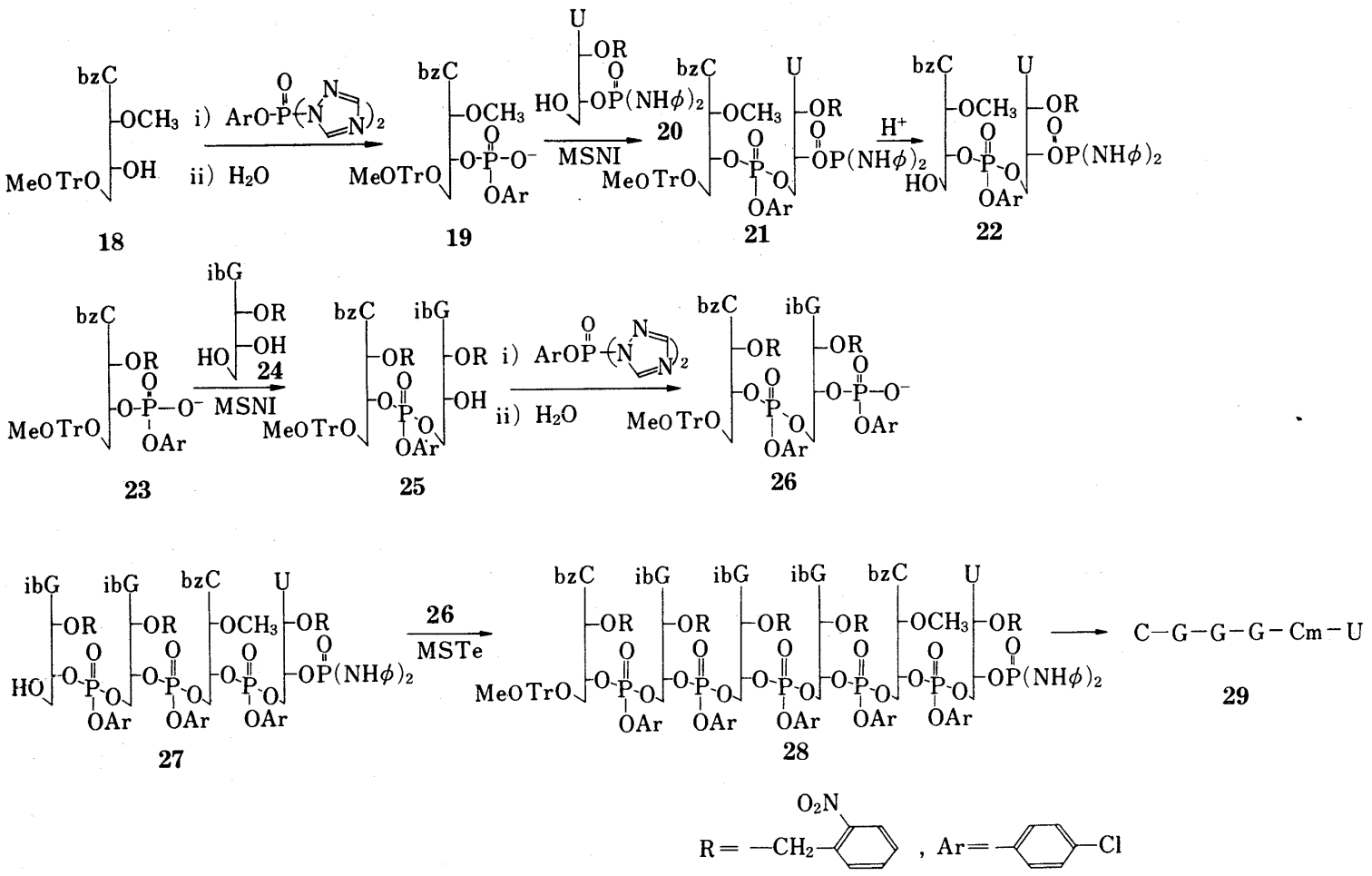

Chart 3

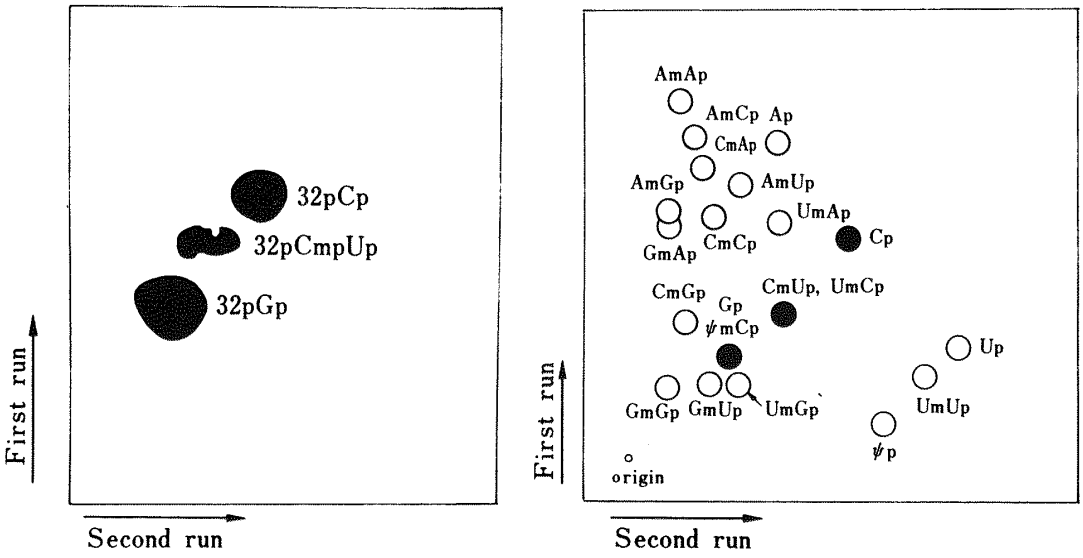

Fig. 1. Two-dimensional TLC of the Products obtained by Digestion of C-G-G-G-Cm-U with RNase T_2 followed by 5′-Phosphorylation with [γ-^{32}p] ATP and Polynucleotide Kinase

Two solvent systems were used on cellulose plates, as shown in the authentic map (right).[20] First run, isobutyric acid–0.5M ammonium hydoxide (5:3, v/v); second run, isopropanol–conc. hydrochloric acid–water (70:15:15, v/v).

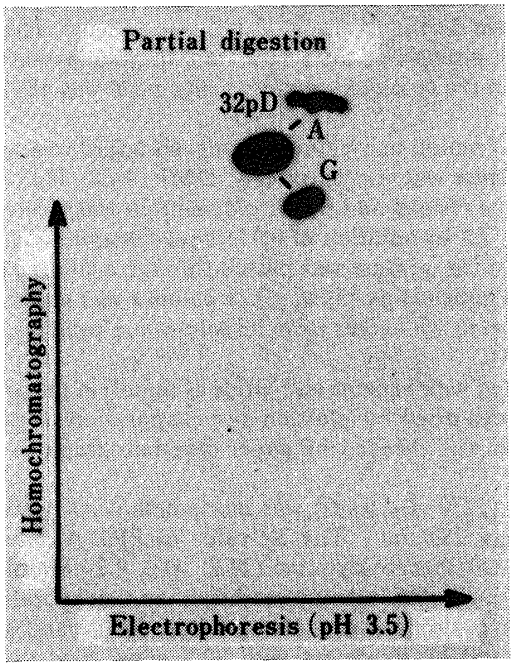

Fig. 2. Two-dimensional Homochromatography of ^{32}pD-A-G partially digested with Nuclease P1

Homo-mix IV[21] was used for the second run.

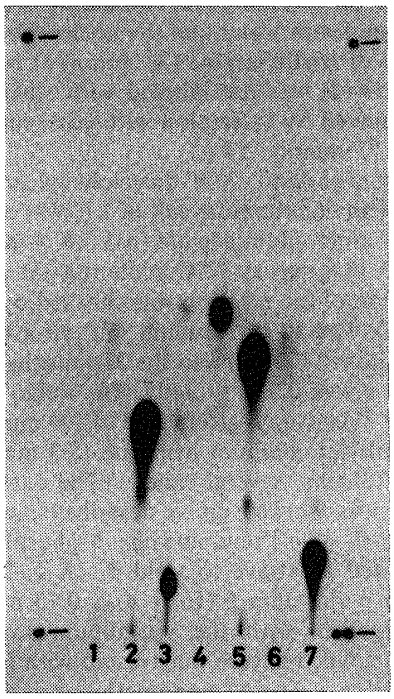

Fig. 3. Chromatography of the Products obtained by Digestin of D-A-G followed by 5′-Labelling (4, ^{32}pDp) on Polyethyleneimine Cellulose with 0.55 M Ammonium sulfate at 4°C[2,12]

Markers; 1 (Pi), 2 (^{32}pCp), 3 (^{32}pDp, ^{32}pCp and [γ-^{32}p]ATP: ^{32}pOp obtained by RNase T2 hydrolysis of the 5′-labeled three quarter of $tRNA_f^{Met}$), 5 (^{32}pUp), 6 (^{32}pp), 7 (^{32}pGp).

product with venom phosphodiesterase. m^7G-U-C was homogeneous in paper chromatography and paper electrophoresis.

Experimental

Paper chromatography (PPC) was performed by the descending technique using the following solvent systems: A, ethyl alcohol–0.1 M ammonium acetate (pH 7.5) (7:3, v/v); B, *n*-propanol–conc.ammonia–water (50:10:35, v/v). Paper electrophoresis (PEP) was performed at 900V/40 cm with 0.05 M trimethylammonium bicarbonate (pH 7.5) and 0.2 M morpholinium acetate (pH 3.5). Thin layer chromatography (TLC) and reversed phase thin layer chromatography (RTLC) were performed on plates of silica gel (Kieselgel HF_{254}, Merck, in chloroform–methanol) and silanized silica gel (Merck, in acetone–water), respectively. For column chromatography, silica gel and silanized silica gel were used under the conditions described previously.[5d]

U-A-G and G-U-C were prepared by the phosphotriester[5] and phosphodiester[8] methods, respectively. Other general methods for removal of the *o*-nitrobenzyl group and for the characterization of oligonucleotides were as described previously.[17]

5'-*O*-Monomethoxytritylribothymidine (1)———Ribothymidine was prepared by condensation of bis-(trimethylsilyloxy) thymine[4] and 1-*O*-methyl-2,3,5-*O*-benzoyl-β-D-ribofuranose[6] using stannic chloride in ethylene dichloride, followed by deblocking with concentrated ammonia under the reported conditions.[4]

Ribothymidine (1.29 g, 5 mmol) was dried by addition and evaporation of pyridine and treated with monomethoxytrityl chloride (1.92 g, 6 mmol) for 16 h. Ammonia (7N, 5 ml) was added to the mixture. The product was extracted with chloroform, washed with water and precipitated with hexane from its solution in pyridin. Recrystallization from benzene–ethyl acetate (2:1) gave 1.77 g (67%), mp 114—116°C.

(MeOTr)T(Bz)p (4)———(MeOTr)T (**1**) (1.59 g, 3 mmol) was dissolved in dioxane (30 ml) and DMF (5 ml), then treated dropwise with morpholinophosphorodichloridate (1.28 g, 6 mmol) in dioxane (1 ml) in the presence of 2,6-lutidine (12 mmol) at room temperature. The reaction was checked after 2 d and stopped by addition of dilute ammonia (0.05N, 120 ml) and pyridine (40 ml). Morpholine was removed by extraction with ether and the nucleotide (**2**) containing ring opened phosphates was extracted with butanol. The organic phase was washed with water and the nucleotides were treated with DCC (6.18 g, 30 mmol) in pyridine (75 ml) in the presence of triethylamine (30 mmol) at room temperature for 22 h. Water (120 ml) was added. The cyclic phosphate (**2**) was precipitated with ether from its solution in pyridine after DCC and dicyclohexylurea had been removed by extraction with ether and after filtration of the aqueous solution. The yield was 18078 A_{265} units (1.57 mmol), 52%.

(4-Morpholino)-*N,N'*-dicyclohexylcarboxamidine (0.494 mmol) in pyridine (10 ml) was added to a solution of the triethylammonium salt of **2** (0.494 mmol) and the mixture was concentrated. DMF (4 ml), H_2O (10 ml) and 0.1N ammonium acetate (pH 7.8, 5 ml) were added to the residue and the turbid solution was treated with RNase A (0.4 mg) at 37°C while the pH was maintained at 7.8 by addition of 0.01N aqueous ammonia. The completion of the reaction was checked by PEP (pH 7.5) and the mixture was passed through a column (2×10 cm) of Dowex 50 (pyridinium salt) after being diluted with aqueous pyridine. The column was washed with 10% pyridine (150 ml) and the 3'-phosphate (**3**) was precipitated with ether from its solution in pyridine. The yield of **3** was 0.162 g (2405 A_{260} units, 0.209 mmol), 85%.

The 2'-hydroxyl group of **3** (0.209 mmol) was benzoylated with benzoic anhydride (4 mmol) in the presence of tetraethylammonium benzoate (2.3 mmol) after **3** had been dried by addition and evaporation of pyridine then of toluene, several times. The extent of the reaction was checked by paper chromatography and the mixture was worked up as described previously.[8]

T-Ψ-C———Pseudouridine 3'-phosphate (Sigma. Co.) (2270 A_{260} units), was monomethoxytritylated and benzoylated as above to yield **5**. λ_{max} 223, 260 nm, λ_{max} 282 nm. The *Rf* value in PPC(A) was 0.76.

The pyridinium **5** (1630 A_{260}, 0.13 mmol) and *N*,2',3'-*O*-tribenzoylcytidine (2420 A_{305}, 0.24 mmol) were dried together with the pyridinium form of Dowex 50×2 (0.5 ml) by addition and evaporation of pyridine. The mixture was treated with DCC (1.3 mmol) in pyridine at room temperature for 3 d and worked up as above. The 5'-monomethoxytrityl group was removed by treatment with 80% acetic acid for 1.5 h (checked by TLC). The yield of **7** was 2860 A_{260}, 0.083 mmol, 64%.

7 (0.083 mmol) and **4** (0.050 mmol) were condensed with DCC (160 mg) in pyridine (1 ml) in the presence of Dowex 50×2 (pyridinium) under the conditions described for the synthesis of **7**. After 5 d, the reaction was stopped and the mixture was worked up as above. The protected trimer (**8**) was treated with acetic anhydride in pyridine for 4 h and then with aqueous pyridine for 24 h. **8** was deblocked first by treatment with 80% acetic acid for 1 h and then with 15N methanolic ammonia for 16 h. The deblocked trimer (**9**) was applied to a column (1.3×21 cm) of DEAE-Sephadex A-25 (bicarbonate) equilibrated with 0.01 M triethylammonium bicarbonate. Elution was performed with a linear gradient of the buffer (0.05—0.25 M, total 2 l). The trimer (**9**) was isolated in a yield of 8% (99 A_{260} unit, 4 μmol). An aliquot was digested with RNase A to give Tp (0.372 A_{267} in 50 mM triethylammonium bicarbonate), ψp (0.489 A_{280} in 0.01N HCl) and C (0.489 A_{280} in 0.01N HCl) in a ratio of 1.00:0.92:0.96.

2′-*O*-(*o*-Nitrobenzyl)ribothymidine———Ribothymidine (10 mmol) obtained as described for the synthesis of **1** was treated with *o*-nitrophenyldiazomethane[11] (40 mmol) in triethyleneglycol (40 ml) in the presence of $SnCl_2$ (200 mg) and DMF (80 mmol) at 30°C overnight. TLC (10:1) showed two spots (*Rf* 0.63 and 0.65). Volatile materials were removed by evaporation and the residue was dissolved in pyridine (10 ml), then precipitated with pentane (200 ml). The precipitated syrup was washed with pentane, dried by addition and evaporation of pyridine, and dissolved in chloroform (15 ml). The solution was applied to a column (3.6×46 cm) of silica gel G (200 g) and 2′(3′)-*O*-[(*o*-nitrobenzyl)ribothymidine was eluted with chloroform–methanol (50:1). The mixture was precipitated with pentane (300 ml) from its solution in chloroform (20 ml) in a yield of 74% (2.92 g, 7.42 mmol). The isomers were separated by rechromatography under the same conditions. The 3′-isomer was eluted first (1.14 g, 2.89 mmol, not crystallized) and the 2′-isomer (479 mg, 1.21 mmol) was obtained as a powder.

The same amount of the 2′-isomer was collected by recrystallization of the crude material eluted after the pure 2′-isomer from pyridine–chloroform. *Anal.* Calcd for $C_{17}H_{17}N_3O_8$: C, 51.91; H, 4.87; N, 10.68. Found: C, 51.73; H, 4.77; N, 10.70. ^{1}H-NMR ($CDCl_3$) δ: 5.98 (d, 1H, J=5.6 Hz, H_1), 4.22 (1H, H_3), 4.07 (1H, $H_{2'}$Y), 3.93 (iH, H_4), 3.67 (2H, H_5) 5.0—5.5 (br, 2H, 3′ and 5′ OH), 1.28 (br s, 1H, 3 NH), 1.77 (s, 3H, $5CH_3$), 7.4—8.1 (m, 4H, aromatic), 4.95 and 5.07 (q, 2H, J_{gen}=14.4 Hz, benzyl CH_2), UV: λ_{max} 266 (H_2O, ϵ, 12700), 266 (0.1N HCl, ϵ, 12700) 265 (0.1N NaOH, ϵ, 11100), 266 (EtOH, ϵ, 14000).

5′-*O*-monomethoxytrityl-2′-*O*-(*o*-nitrobenzyl)ribothymidine (11)———2′-*O*-(*o*-Nitrobenzyl)ribothymidine (479 mg, 1.22 mmol) was treated with monomethoxytrityl chloride (1.59 mmol) in pyridine (7 ml) at 30°C for 40 h, then water (5 ml) was added to stop the reaction. The product was extracted with chloroform, washed with water and precipitated with pentane (100 ml) from its solution in chloroform (7 mmol). The yield was quantitative.

(MeOTr)T(*n*Bzl)p (12)———**11** (1.25 mmol) was treated with *p*-chlorophenyl phosphate (3.75 mmol) and DCC (9 mmol) in pyridine at 27°C for 30 h, and the mixture was worked up as described for the synthesis of the guanosine derivative.[17] The yield was 91% (1.08 g, 1.11 mmol).

The trinucleotide (14)———The dinucleotide (**13**) (0.49 mmol) was prepared as described previously[5a] and condensed with **12** (0.59 mmol) in pyridine using MST (1.77 mmol) at room temperature. After 2 d, water was added with cooling. The product was extracted with chloroform, washed with 0.1M triethylammonium bicarbonate, and dried by addition and evaporation of pyridine. The residue in chloroform (1 ml) was applied to a column (2.5×12 cm) of silica gel G (30 g). The trinucleotide (**14**) was eluted with 50:1 chloroform–methanol and precipitated with pentane (50 ml) from its solution in chloroform (5 ml). The yield was 71% (907 mg, 0.42 mmol). An aliquot of **14** was deblocked with isoamyl nitrite, concentrated ammonia and 80% acetic acid. T(*n*Bzl)pU(*n*Bzl)C(*n*Bzl)p was separated by PPC (solvent B) and checked by PEP (pH 7.5). The trinucleotide was deblocked by irradiation with UV light (λ 280 nm), treated with 0.05N HCl at 30°C for 30 min and subjected to PPC. T-U-Cp was characterized by hydrolysis with RNase A to yield Tp (0.575 A_{266}), Up (0.605 A_{260}) and Cp (0.841 A_{279}) in 0.01N HCl. The ratio was 0.991:1.00:1.04.

T-U-C-A-A-Ap (17)———The trinucleotide (**14**) (0.42 mmol) was treated with isoamyl nitrite (6 ml) in 2:1 pyridine–acetic acid (30 ml) at 30°C for 16 h. The extent of reaction was checked by TLC (30:1). The same amount of isoamyl nitrite was added to the mixture. After 72 h, 0.1Mtriethylammonium bicarbonae was added and the diester **15** was extracted with chloroform. The organic phase was washed 3 times with the same buffer and **15** was precipitated with pentane, yield 868 mg (0.40 mmol, 74%).

The trimer (**16**) (0.1 mmol) prepared as described previously[17,18] was condensed with **15** (0.15 mmol) using MSNI (0.3 ml) in pyridine (1 ml) at 30°C for 14 h. TLC (20:1) and RTLC (acetone–water, 75:25) showed completion of the reaction. The fully protected hexanucleotide was isolated by silica gel G (12 g) with 50:1 chloroform-methanol. The yield was 231 mg (0.055 mmol, 55%). An aliquot (21 mg, 5 μmol) of the protected hexamer was deblocked as described elesewhere[5e] and isolated by PPC (solvent B). **17** (133 1A_{260}) was purified by ion-exchange chromatography on a column (1.0×51 cm) of DEAE-celllose in 7M urea, 0.02M Tris–HCl (pH 8.0). Elution was performed with a linear gradient of sodium chloride (0.1—0.25M, total 800 ml). The yield was 71 A_{260} units. T-U-C-A-A-Ap was analyzed by base ratio analysis[5a] and two dimensional chromatography as described previously.[17]

D-A-G———U-A-G (720 A_{260}) in 10% acetic acid (0.4 ml) was hydrogenated at atmospheric pressure in the presence of platinum oxide (40 mg) for 5 h with shaking. The mixture was subjected to PEP (pH 7.5) using 7 sheets of Whatman 3 MM paper. The nucleotides (340 A_{260}, *Rm*, 0.60) were eluted and applied to a column (1.6×34 cm) of DEAE Sephadex A-25 (bicarbonate). Elution was performed with a gradient of triethylammonium bicarbonate (0—0.3M, total 3 l). D-A-G was eluted with 0.162M salt, and the starting material U-A-G was recovered at 0.178M salt. Fractions containing D-A-G were checked by homochromatography and high pressure liquid chromatography on a reversed-phase support (Hypersil ODS, 5μ). The yield was 34 A_{260}, 5%. The result of two dimensional homochromatography of partially digested D-A-G is shown in Fig. 2. Dihydrouridylate was characterized on a polyethyleneimine plate as shown in Fig. 3.

m^{7}G-U-C———G-U-C (59.5 A_{260}, 2.06 μmol) in 0.2M sodium acetate (pH 4.0, 1 ml) was stirred with dimethyl sulfate (10 μl) at room temperature for 3 h. Water (3 ml) was added and the mixture was washed 3 times with ether. The aqueous solution was made up to 50% pyridine and applied to a column (1×5 cm) of

Dowex 50×2 (pyridinium). The combined washings were applied to a column (1.0×15 ml) of DEAE Sephadex A-25 (bicarbonate). Elution was performed with a linear gradient of triethylammonium bicarbonate (0—0.25 M, total 300 ml). m^7G-U-C was eluted at 0.1 M salt. Fractions containing the product were combined and treated with Dowex 50×2 (pyridinium form, 9.5 ml) to remove triethylammonium ions. The washings were concentrated with added pyridine, and m^7G-U-C (17.8 A_{260}, 0.62 μmol) was stored at −20°C in 50% aqueous pyridine. The structure was confirmed by hydrolysis with venom phosphodiesterase under the reported condition[19] to give m^7G (0.153 A_{258}) and pU+pC (0.289 A_{260}), which were measured in 50 mM Tris-HCl (pH 7.5)(2ml) after PEP (pH 7.5). pC and pU were separated by PEP (pH 3.5) and measured similarly: pU (0.156 A_{260}) and pC (0.193 A_{260}) in 0.03N HCl (1.5 ml).

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