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Studies on Transfer Ribonucleic Acids (tRNA) and Related Compounds. XLIX.[1] Solid-Phase Synthesis of Nonadecaribonucleotide Corresponding to the 5′-Half of the Formylmethionine tRNA Deficient in D-Loop and Stem Region

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Ribooligonucleotides (nonamer, decamer and nonadecamer) were synthesized by the solid phase phosphotriester method using 2′-*O*-tetrahydrofuranyl nucleosides. These oligomers were designed in order to obtain *E. coli* $tRNA_f^{Met}$ deficient in D-loop and D-stem and its analogs. Condensation was performed by using dimer blocks and the dimethoxytrityl group on the 5′-hydroxyl group was removed selectively by 1 M $ZnBr_2$ without affecting the 2′-protection.

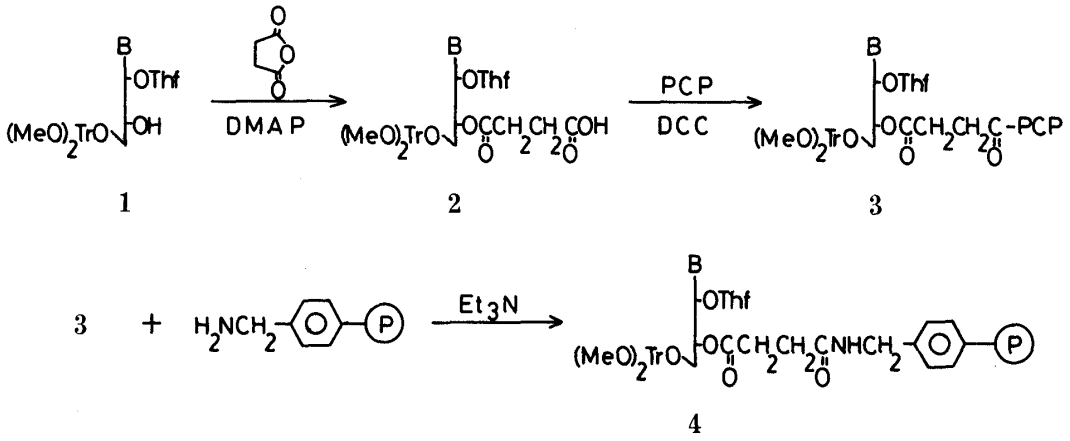
Keywords——solid-phase synthesis; ribooligonucleotide; D-loop deficient tRNA; high pressure liquid chromatography; mobility shift analysis

Chemical synthesis of ribooligonucleotides is an important subject in organic chemistry and is essential for studies on the biological and physico-chemical properties of ribonucleic acids (RNAs). The synthesis of deoxyoligonucleotides has been facilitated by the development of the solid-phase method, which has the following advantages over solution syntheses. 1) The desired product, attached to the resin, can be easily separated from reagents by filtration, so the synthesis is rapid. 2) Since no chromatographic separation is involved, heterogeneous products can be used during synthesis of oligonucleotides. We have previously reported the syntheses of ribooligonucleotides using the tetrahydrofuranyl group[2] for protection of the 2′-hydroxyl function,[3] and the 5′-half molecule of *E. coli* $tRNA_2^{Gly}$ has been synthesized by using this method.[4] In this paper, the usefulness of the 2′-*O*-tetrahydrofuranyl protection in combination with zinc bromide deprotection of the 5′-dimethoxytrityl group is described. We report here the solid phase synthesis of three ribooligonucleotides (C–G–C–G–G–G–G–U–G–G, C–G–U–C–G–G–G–G–C–U and C–G–C–G–G–G–G–A–U–U–C–C–U–C–G–G–G–C–U) which can be used to construct truncated analogs of the $tRNA_f^{Met}$ of *E. coli*.

Polystyrene containing 1% divinylbenzene was used as the support, since cleavage of oligonucleotides from the 1% cross-linked resin was found to be successful[5] and acrylamide resin can not be used in conjunction with the zinc bromide treatment[6] due to possible complex formation of amide groups with zinc ions. 5′-*O*-Dimethoxytrityl-*N*-acylated-2′-*O*-tetrahydrofuranylnucleosides (**1**) were converted to the 3′-succinyl derivatives (**2**) as shown in Chart 1 by treatment with succinic anhydride in the presence of 4-dimethylaminopyridine (DMAP). The succinylated group of **2** was activated by esterification with pentachlorophenol and linked to aminomethylated polystyrene containing 1% divinylbenzene to yield the nucleoside resins (**4**). Unreacted amino groups on the resin were capped by treatment with acetic anhydride and DMAP in pyridine.

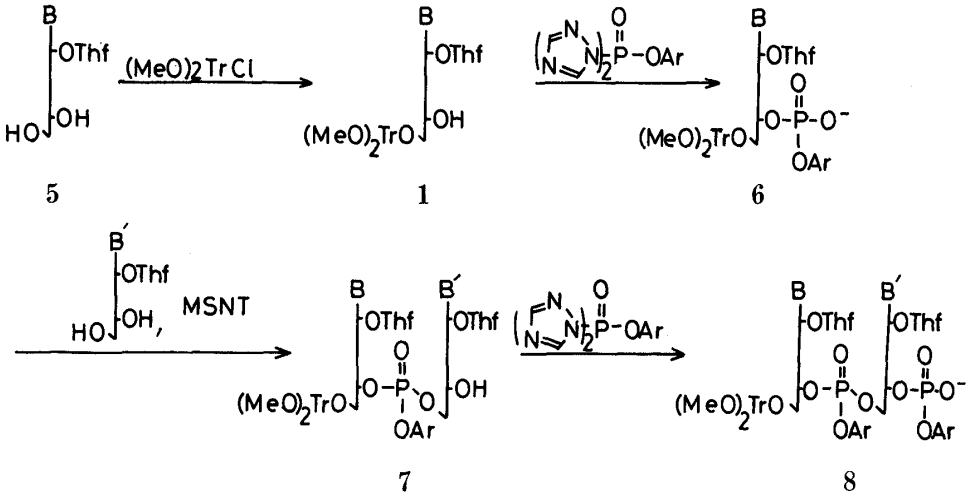
Preparation of Dimer Blocks

We selected dimer blocks as the chain elongation unit. Dimer blocks are considered to be



B = U or ibG
Thf = 2-tetrahydrofuranyl
DMAP = 4-dimethylaminopyridine
PCP = pentachlorophenol
DCC = 1,3-dicyclohexylcarbodiimide
P = polystyrene co-polymer with 1% divinylbenzene

Chart 1



B, B′ = bzA, bzC, ibG, U
Ar = *o*-chlorophenyl
MSNT = 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole

Chart 2

the most suitable size for the solid-phase synthesis, as seen in the deoxyribo series.[7] The method of preparation of the blocks is shown in Chart 2. 3′-*O*-Chlorophenylphosphoryl derivatives (**6**) were synthesized by phosphorylation of 5′-*O*-dimethoxytrityl-2′-*O*-tetrahydrofuranyl nucleosides with *o*-chlorophenylphosphoroditriazolide. Dimer units (**7**) were prepared by condensation of 3′,5′-unprotected nucleosides (**5**) with 5′-*O*-dimethoxytrityl-2′-*O*-tetrahydrofuranyl nucleoside-3′-(*o*-chlorophenyl)phosphate (**6**) using 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT). The reaction conditions for the preparation of dimers are summarized in Table I. The products (**7**) were purified by reversed phase chromatography on octadecylated (C-18) silica gel. Dinucleotides (**8**) with a 3′-phosphodiester moiety were prepared by phosphorylation of dimers (**7**) with *o*-chlorophenylphosphoroditriazolide and purified by C-18 column chromatography. The purity of prepared

dimer blocks (**8**) was checked by reversed phase thin layer chromatography (TLC) in conjunction with a TLC-scanner, and dimer blocks which had a purity of over 95% were used for the synthesis.

TABLE I. Reaction Conditions for the Synthesis of Dimers (7)

3′-Phosphodiester component (mmol) (**1**)[a]	5′-OH component (mmol) (**5**)	MSNT (mmol)	Time (min 30 °C)	Product (**7**)	Yield (%)
DT[G]OH (1.50)	HO[G]OH (1.95)	2.7	30	DT[GG]OH	64
DT[G]OH (1.50)	HO[U]OH (1.95)	2.7	20	DT[GU]OH	62
DT[G]OH (1.50)	HO[C]OH (1.95)	2.7	35	DT[GC]OH	62
DT[G]OH (1.50)	HO[A]OH (1.95)	3.0	30	DT[GA]OH	66
DT[U]OH (1.30)	HO[U]OH (1.82)	2.6	30	DT[UU]OH	73
DT[U]OH (1.00)	HO[C]OH (1.68)	2.0	25	DT[UC]OH	62
DT[C]OH (1.00)	HO[G]OH (1.30)	2.0	30	DT[CG]OH	80
DT[C]OH (1.30)	HO[C]OH (1.69)	2.6	30	DT[CC]OH	53

DT=$(MeO)_2$Tr, [ ]=protected except for termini. *a*) DT[N]OH was converted to 3′-phosphodiester component by phosphorylation with *o*-chlorophenylphosphoroditriazolide followed by treatment with triethylammonium bicarbonate buffer.

TABLE II. One Cycle of Operation

Step	Reagent or solvent	Reaction time (min)	No. of operation
1	1 M $ZnBr_2/CH_2Cl_2$–iso-PrOH	[a]	3
2	CH_2Cl_2–iso-PrOH	0.1	2
3	0.5 M AcOH–Et_3N/DMF	0.2	2
4	Pyridine	0.1	3
5	Pyridine–Dimer[b]	Coevaporation	1
6	Pyridine	Coevaporation	1
7	Pyridine–MSNT[c]	40 (35 °C)	1
8	Pyridine	0.1	3
9	Capping mixture[d]	5	1
10	Pyridine	0.1	3
11	CH_2Cl_2–iso-PrOH	0.1	3

a) Steps 1 and 2 were repeated until the color of dimethoxytrityl cation was not observed. *b*) Dimer block (40 mg) was dissolved in 0.3 ml pyridine. *c*) MSNT (50 mg) was dissolved in 0.4 ml pyridine. *d*) Ac_2O : 0.1 M DMAP in pyridine = 9 : 1.

TABLE III. Summary of Yields

Step	Sequence of linked oligonucleotide	Yield (%)	Step	Sequence of linked oligonucleotide	Yield (%)
1	U-P		1	U-P	
2	GCU-P	80	2	GCU-P	94
3	GGGCU-P	110	3	GGGCU-P	90
4	UCGGGCU-P	93	4	UCGGGCU-P	104
	(CGUCGGGCU)		5	CCUCGGGCU-P	[a]
1	G-P		6	UUCCUCGGGCU-P	[a]
2	GG-P	94	7	GAUUCCUCGGGCU-P	97
3	GUGG-P	82	8	GGGAUUCCUCGGGCU-P	76
4	GGGUGG-P	78	9	CGGGGAUUCCUCGGGCU-P	61
5	CGGGGUGG-P	82		(CGCGGGGAUUCCUCGGGCU)	
	(CGCGGGGUGG)				

a) Yield could not be calculated. P: 1% cross-linked polystyrene.

Synthesis of Ribooligonucleotides on the Support

Procedures for one cycle of elongation of the chain are shown in Table II. Detritylation was performed by treatment with a solution of $ZnBr_2$ (1 M) in dichloromethane–isopropyl alcohol (85 : 15, v/v) for 30—60 min. After the detritylation, zinc cations were removed by washing with 0.5 M triethylammonium acetate in dimethylformamide (DMF). For the condensation reaction, slightly larger excesses of dimer blocks and MSNT were used compared to the synthesis of deoxyribonucleotide, because ribonucleotides are less reactive than deoxyribonucleotides. Unreacted 5′-hydroxyl groups were capped by acetylation at the final step. One cycle took 1—2 h. The condensation yields are shown in Table III.

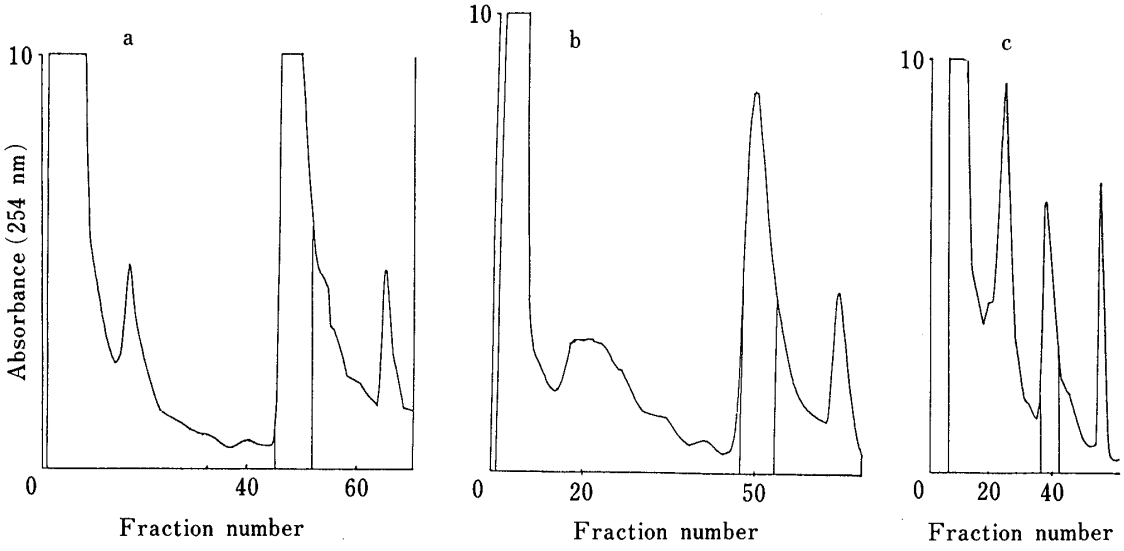


Fig. 1. Chromatography of the Nonamer (a), Decamer (b) and Nonadecamer (c) Having the $(MeO)_2Tr$ Group on a Column (0.7 × 10 cm) of Reversed Phase C-18 Silica Gel

Elution was performed with a linear gradient of acetonitrile (5%—40%) in 50 mM triethylammonium acetate (pH 7.0).

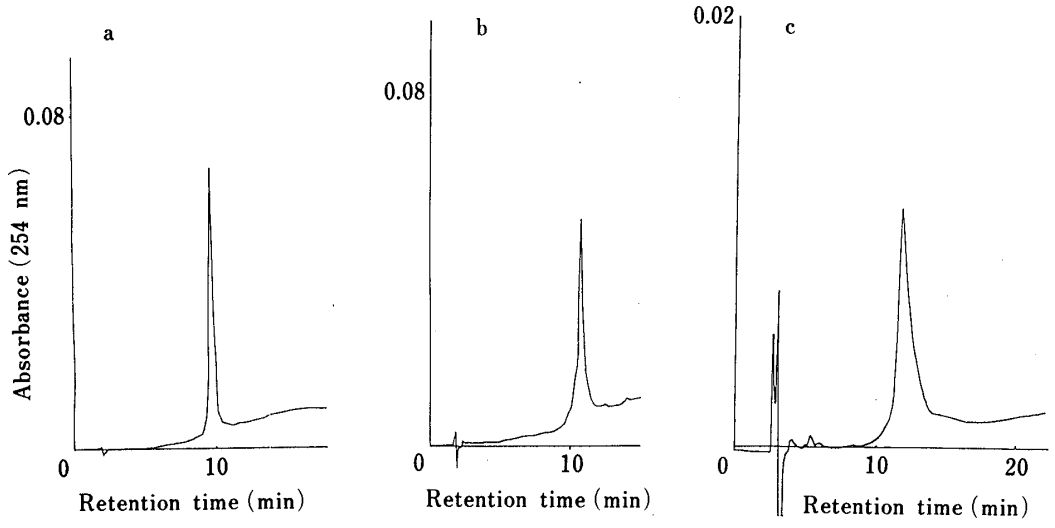


Fig. 2. HPLC of the Nonamer (a), Decamer (b) and Nonadecamer (c)

The nonamer (a) and decamer (b) were analyzed by reversed-phase HPLC (TSK-GEL LS410AK, 0.46 × 25 cm). Elution was performed with a gradient of acetonitrile (5%—25%) in 0.1 M triethylammonium acetate (pH 7.0) during 30 min (a) or 37.5 min (b). The nonadecamer (c) was analyzed by ion exchange HPLC (TSK-GEL DEAE 2SW, 0.46 × 25 cm). Elution was performed with $HCOONH_4$ (0.5—0.8 M) in 20% acetonitrile during 25 min.

Deblocking and Purification

Synthesized oligonucleotides were cleaved from the support together with *o*-chlorophenyl groups by treatment with N^1,N^1,N^3,N^3-tetramethylguanidinium pyridine-2-

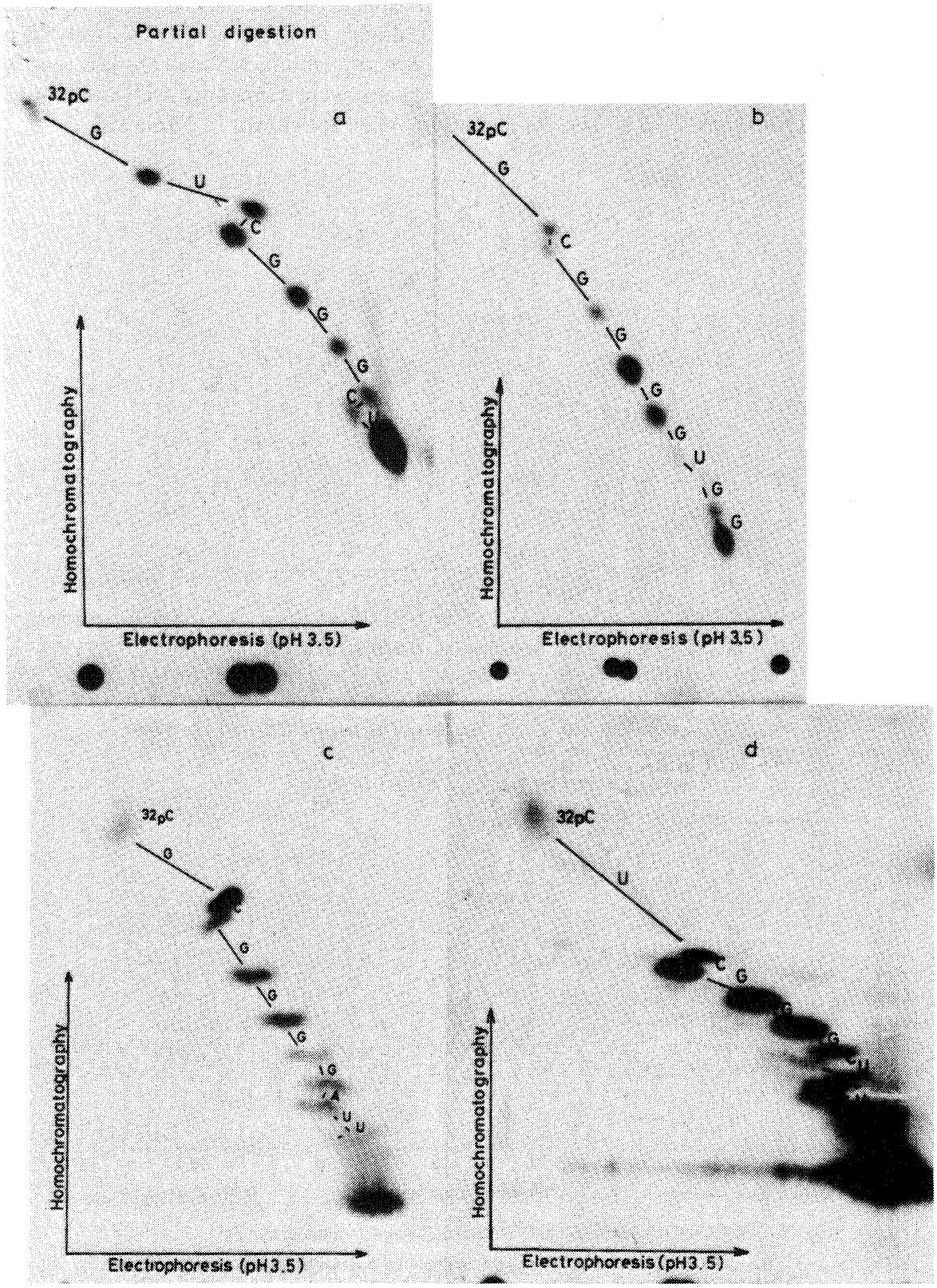


Fig. 3. Mobility Shift Analysis of the Nonamer (a), Decamer (b) and Nonadecamer (c) using Homo-mix I

d is a sequence analysis from the 3′-end by single addition of 5′-labeled pCp.

aldoximate[8] for 2—3 d at room temperature under shaking. These guanine-rich oligonucleotides were considered to require a longer time for deblocking as compared with deoxyribonucleotides. The base protecting acyl groups were then removed by treatment with concentrated ammonia for 5 h at 60 °C. In this step, the oligonucleotides were subjected to chromatography on a C-18 silica gel column and fractions containing the oligomer with the dimethoxytrityl group were collected. The elution profiles are shown in Fig. 1. The protecting groups, dimethoxytrityl and tetrahydrofuranyl groups, were deblocked by treatment with 0.01 N HCl for 6 h at room temperature. Further purification was performed by reversed phase high-pressure liquid chromatography (HPLC) and ion-exchange HPLC (Fig. 2). Purified oligonucleotides were analyzed by the mobility shift analysis[9] after labelling with [γ-^{32}P] adenosine triphosphate (ATP) followed by partial digestion with nuclease P1 (Fig. 3a, 3b, 3c). In the case of the nonadecamer, sequencing from the 3′-end was also performed by single addition of 5′-labelled pCp as shown in Fig. 3d.

Discussion

The solid phase method has been used successfully in deoxyoligonucleotide syntheses, and it has been applied to the construction of synthetic genes[10] and synthesizing probes for gene manipulation.[11] In the ribo-series, short oligonucleotides have been synthesized by the phosphodiester[12] or phosphotriester[13] method. We have shown in the present work that the solid phase method is also applicable to the synthesis of relatively large ribooligonucleotides. For the synthesis of ribooligonucleotide, we have used dimer blocks because of their availability and the easy purification of deblocked products, especially in obtaining longer oligonucleotides. In the synthesis of three ribooligonucleotides, we prepared the solvents and reagents carefully to exclude moisture. The synthesis was performed under a nitrogen atmosphere, since moisture could cause deprotection of the tetrahydrofuranyl groups leading to subsequent cleavage of internucleotide linkages. In the purification step, reversed phase chromatography of oligonucleotides having the dimethoxytrityl group[12] was employed. These oligonucleotides had guanosine-rich sequences, so aggregation was observed in HPLC purification, but aggregation could be prevented by warming the sample to 60 °C, instead of warming the column. The nonamer and decamer were purified until they became homogeneous on reversed-phase HPLC. However, the nonadecamer was difficult to purify by reversed phase HPLC because of its aggregation, so in this case ion-exchange HPLC was used. The oligomers were analyzed by mobility shift analysis and used to construct D-loop and stem deficient tRNAs.

Experimental

TLC was performed on plates of silica gel (Kieselgel 60 HF_{254}, Merck) with a mixture of chloroform and methanol. For reversed phase TLC (RTLC), silanized silica gel was used with a mixture of acetone and water. For column chromatography, Silica gel 60H (Merck) was used with a mixture of chloroform and methanol, and reversed-phase chromatography was performed on octadecylated silica gel (35—105 μm, Waters) with a mixture of acetone–0.2% aqueous pyridine. For preparative anion-exchange chromatography, HPLC was performed on an anion-exchange column (TSK-GEL IEX-540K or DEAE 2SW, Toyo Soda Co.) or a reversed phase column (TSK-GEL LS410AK, Toyo Soda Co.) on an Altex 332 MP apparatus. Two-dimensional homochromatography (mobility shift analysis) was performed as described previously.[8] Nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi R-22 spectrometer (90 MHz) with tetramethylsilane (TMS) as an internal standard.

Preparation of 2——5′-*O*-Dimethoxytrithyl-*N*-protected-2′-*O*-tetrahydrofuranylnucleoside (**1**) (0.3 mmol), succinic anhydride (60 mg, 0.60 mmol) and 4-dimethylaminopyridine (73 mg, 0.60 mmol) were dissolved in dichloromethane (1.2 ml) and the mixture was stirred at room temperature. After 1.5 h, the reaction was monitored by TLC. Finally 0.1 M triethylammonium bicarbonate buffer (pH 7.5, 3 ml) was added, and the product was extracted three times with chloroform. The organic layer was washed three times each with 0.1 M tetraethylammoniumbromide (TEAB) buffer (10 ml), 0.5 M potassium dihydrogen phosphate buffer (pH 4.3, 10 ml), and water (10 ml), then dried

with Na_2SO_4, filtered, and evaporated. The residue was dissolved in a small portion of chloroform and chromatographed on silica gel. The fractions containing the desired product were collected and concentrated. The product was precipitated with pentane. The yield was 94% (0.28 mmol) for uridine and 60% (0.18 mmol) for guanosine.

Preparation of the Active Ester (3)——5′-*O*-Dimethoxytrityl-*N*-protected-2′-*O*-tetrahydrofuranyl-3′-*O*-succinatenucleoside (**2**) (0.2 mmol), dicyclohexylcarbodiimide (62 mg, 0.30 mmol), and pentachlorophenol (59 mg, 0.22 mmol) were dissolved in DMF (1.6 ml) and stirred at room temperature. After 16 h, TLC showed that the reaction was completed. The reaction mixture was concentrated and the residue to dissolved in benzene. Insoluble material was removed by filtration and the solution was concentrated. The product was precipitated with pentane. The yield was 0.2 mmol (100%) for uridine, and 0.19 mmol (98%) for guanosine.

U Succinate (**3a**): ^{1}H-NMR ($CDCl_3$) δ ppm: 9.82 (br s, 1H, NH), 7.60 (d, 1H, H-6, J=8 Hz), 7.20 (m, 9H, H-arom), 6.77 (m, 4H, H-arom), 6.01 (d, 1H, H-5, J=8 Hz), 5.33 (m, 2H, H-1′, H-1″), 5.13 (br s, 1H, H-2′), 4.60 (m, 1H, H-3′), 4.20 (m, 1H, H-4′), 3.80 (br s, 8H, $-OCH_3 \times 2$, H-5′), 3.43 (br s, 2H, H-5″), 2.68 (br s, 4H, $-CO(CH_2)_2CO-$), 1.83 (br s, 4H, H-3″, H-4″).

G Succinate (**3b**): ^{1}H-NMR ($CDCl_3$) δ ppm: 8.79 (br s, 1H, NH), 7.81 (s, 1H, H-8), 7.5—7.1 (m, 9H, H-arom), 6.8—6.6 (m, 4H, H-arom), 5.72 (d, 1H, H-1′, J=10 Hz), 5.52 (m, 1H, H-1″), 5.3—5.15 (m, 2H, H-2′, H-3′), 4.25 (br s, 1H, H-4′), 3.70 (s, 6H, $-OCH_3 \times 2$), 3.4—3.2 (m, 4H, H-5′, H-5″), 2.68 (br s, 4H, $-CO(CH_2)_2CO-$), 2.10—1.5 (m, 5H, H-2″, H-3″, $-CH(CH_3)_2$), 1.0—0.8 (m, 6H, $-CH_3 \times 2$).

Preparation of Nucleoside Resin (4)——Active ester (**3**) (0.16 mmol) was dissolved in DMF (5 ml), and 1% cross-linked aminomethylated polystyrene (500 mg, amino group 0.065 mmol) and triethylamine (0.18 mmol) were added. The mixture was shaken at room temperature for 70 h. After the reaction, the resin was washed three times with DMF (5 ml) and dichloromethane (5 ml), and mixed with capping reagent (0.1 M DMAP in pyridine: Ac_2O=9 : 1, 2 ml). The reaction mixture was allowed to stand at room temperature for 5 min, then washed with pyridine (5 ml), dichloromethane (5 ml) and ether (5 ml) and dried *in vacuo*. The loading yield was 92% (**4a**) or 63% (**4b**) as determined by measuring dimethoxytrityl cation in $HClO_4$–EtOH (3 : 2, v/v) at 499 nm.

Synthesis of Dimer Blocks (8)——5′-*O*-Dimethoxytrithyl-*N*-protected-2′-*O*-tetrahydrofuranylnucleoside (**1**) (1 mmol) which had been coevaporated twice with a small amount of pyridine was allowed to react with *o*-chlorophenylphosphoditriazolide (1.5 mmol) at room temperature for 20 min. After analysis by RTLC (6 : 4), 0.1 M TEAB buffer (10 ml) was added. The product was extracted twice with chloroform and evaporated in the presence of pyridine. The residue was mixed with *N*-protected-2′-*O*-tetrahydrofuranylnucleoside (1.3 mmol). The mixture was dried by coevaporation with pyridine three times and treated with MSNT (593 mg, 2 mmol) in pyridine (4 ml) at 30 °C. After 30 min, the reaction was checked by RTLC (7 : 3) and 0.1 M TEAB buffer (10 ml) was added. The reaction mixture was extracted three times with chloroform (20 ml), and the organic layer was concentrated. The residue was dissolved in a mixture of acetone–0.2% aqueous pyridine, and chromatographed on C-18 silica gel. The fractions containing the desired product (**7**) were collected and concentrated, and the product was precipitated with pentane. The dimer (0.5 mmol) was phosphorylated with *o*-chlorophenylphosphoroditriazolide (0.75 mmol) by the procedure described above. Phosphorylated dimer (**8**) was purified by reversed-phase silica gel chromatography and precipitated with pentane containing 1% triethylamine. The yields are shown in Table I.

Synthesis of Ribooligonucleotides——For removal of the dimethoxytrityl group, a mixture of the resin (50 mg, *ca.* 5 μmol) and a 1 M solution (2 ml) of $ZnBr_2$ in dichloromethane–isopropyl alcohol (85 : 15, v/v) was allowed to stand at room temperature for 10 min, and this procedure was repeated until the color of the dimethoxytrityl cation had disappeared. After the reaction, the mixture was filtered and washed with dichloromethane–isopropyl alcohol (85 : 15, v/v) (2 ml), triethylammonium acetate in DMF (0.5 M, pH 7—8) (2 ml), and pyridine (2 ml). The resin was mixed with the dimer block (40 mg) and the mixture was coevaporated twice with pyridine. Then MSNT (50 mg) was added, and the reaction mixture was allowed to stand at 35 °C for 40 min. After the reaction, the resin and capping reagent (2 ml) (0.1 M DMAP in pyridine : Ac_2O=9 : 1) were allowed to react at room temperature for 5 min.

Deblocking and Purification——The resin was shaken with 0.5 M N^1,N^1,N^3,N^3-tetramethylguanidinium-*syn*-pyridine-2-carboxaldoximate (dioxane : H_2O=9 : 1, 2 ml) at room temperature for 2—3 d and washed with 50% aqueous pyridine (10 ml). The filtrate was evaporated and conc. NH_4OH (10 ml) was added. The solution was allowed to stand at 60 °C for 5 h, then evaporated. The residue was chromatographed over C-18 silica gel. Elution was performed with a linear gradient of acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0) (5%—40%, total 200 ml) (Fig. 1). The fractions containing the desired oligomer were collected and coevaporated with water. The residue was taken up with 0.01 N HCl (10 ml), then the solution was adjusted to pH 2 by addition of 0.1 N HCl and allowed to stand at room temperature for 6 h. The solution was neutralized with dilute NH_4OH, concentrated, and applied to a Sephadex G-25 column (i.d. 1.7 cm × 40 cm) for desalting. The product was eluted with 0.1 M TEAB buffer and purified by reversed-phase HPLC (elution was performed with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate buffer). Further purification of the nonadecamer was performed by HPLC on an anion-exchange column (Fig. 2c). The two-dimensional homochromatograms of partially digested products are shown in Fig. 3. Sequencing from the 3′-end was performed by a single addition of 5′-labeled pCp using T4 RNA ligase.

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