

Synthesis of Nonadeca- and Octadecaribonucleotides Using the Solid-Phase Phosphotriester with Tetrahydropyranyl Groups as the 2'-Hydroxyl-Protecting Group

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Nonadeca- and octadecaribonucleotides corresponding to the D-loop of tRNA^{Phe} from yeast and the leader sequence of phage f1 coat protein mRNA were synthesized by the activated phosphotriester method. Coupling yield in the synthesis of oligoribonucleotides depended on the extent of nucleosides loaded on controlled pore glass beads (CPG). *N*-Acyl-5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranyl derivatives were used as fully protected ribonucleotide monomer units. The 18mer and 19mer corresponding to the D-loop did not serve as substrates for tRNA (guanosine-2'-)methyltransferase from *T. thermophilus*, but inhibited methylation of the 5'-half fragment of tRNA^{Phe}. This indicates that both fragments possess some affinity with the enzyme.

The synthesis of long-chain oligoribonucleotides up to about the icosamer (20mer) has recently been achieved by the solid phase approach: 19mer¹⁾ was synthesized by the chlorophosphate method, 15mer,²⁾ 11mer,³⁾ 43mer,⁴⁾ 19mer,⁵⁾ and 13mer,⁶⁾ 77mer⁷⁾ by the phosphoramidite method, 21mer⁸⁾ and 24mer⁹⁾ by the H-phosphonate method, 13mer¹⁰⁾ by the triester method and 9mer¹¹⁾ and 8mer¹²⁾ by the phosphoro-*p*-anisidate method. However, the phosphotriester approach though easy to conduct, is attended by various problems such as the long time required for the coupling reaction, its low yield and the occurrence of side reactions on the uracil and guanine moieties.¹³⁾ The synthesis of long-chain oligoribonucleotides (about 20mer) by the phosphotriester solid-phase method has not been reported so far.

The starting materials, 5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranyl-*N*-acylribonucleosides¹⁴⁾ were prepared from 3',5'-*O*-(tetraisopropylidisiloxane-1,3-diyl) derivatives¹⁵⁾ on a large scale. We previously reported the selective deprotection of the 5'-*O*-dimethoxytrityl group of oligoribonucleotides by treatment with 1% dichloroacetic acid in dichloromethane at room temperature without removal of the 2'-*O*-tetrahydropyranyl (THP) group,¹⁶⁾ followed by the synthesis of undecaribonucleotides (11mers)¹⁷⁾ on a polystyrene support using 2-chlorophenyl-*O,O*-bis(1-benzotriazolyl) phosphate, initially reported by van Boom et al.¹⁸⁾ Recently, Kierzek et al. have reported the synthesis of oligoribonucleotides by the phosphoramidite approach, using our method in combination with the THP group.¹⁹⁾

However, we were unable to synthesize nonadecaribonucleotides (19mers) by this method, possibly since long-chain oligoribonucleotides were easily adsorbed on the polystyrene resin, used for the solid support, during the synthesis. We thus attempted the solid-phase synthesis using controlled pore glass (CPG), a well-known support for nucleic acids synthesis,²⁰⁾

instead of a polystyrene resin. When the coupling reaction was carried out on the long-chain ω -aminoalkyl CPG resin using acetonitrile as the solvent, with a loading amount less than 20 $\mu\text{mol g}^{-1}$ the average coupling yields exceeded 95% during a 15 min reaction period.

In the present study, the simple solid-phase synthesis of nonadeca- and octadecaribonucleotides corresponding to the D-loop of yeast tRNA^{Phe} and the leader sequence of phage f1 coat protein mRNA were carried out by the phosphotriester approach.

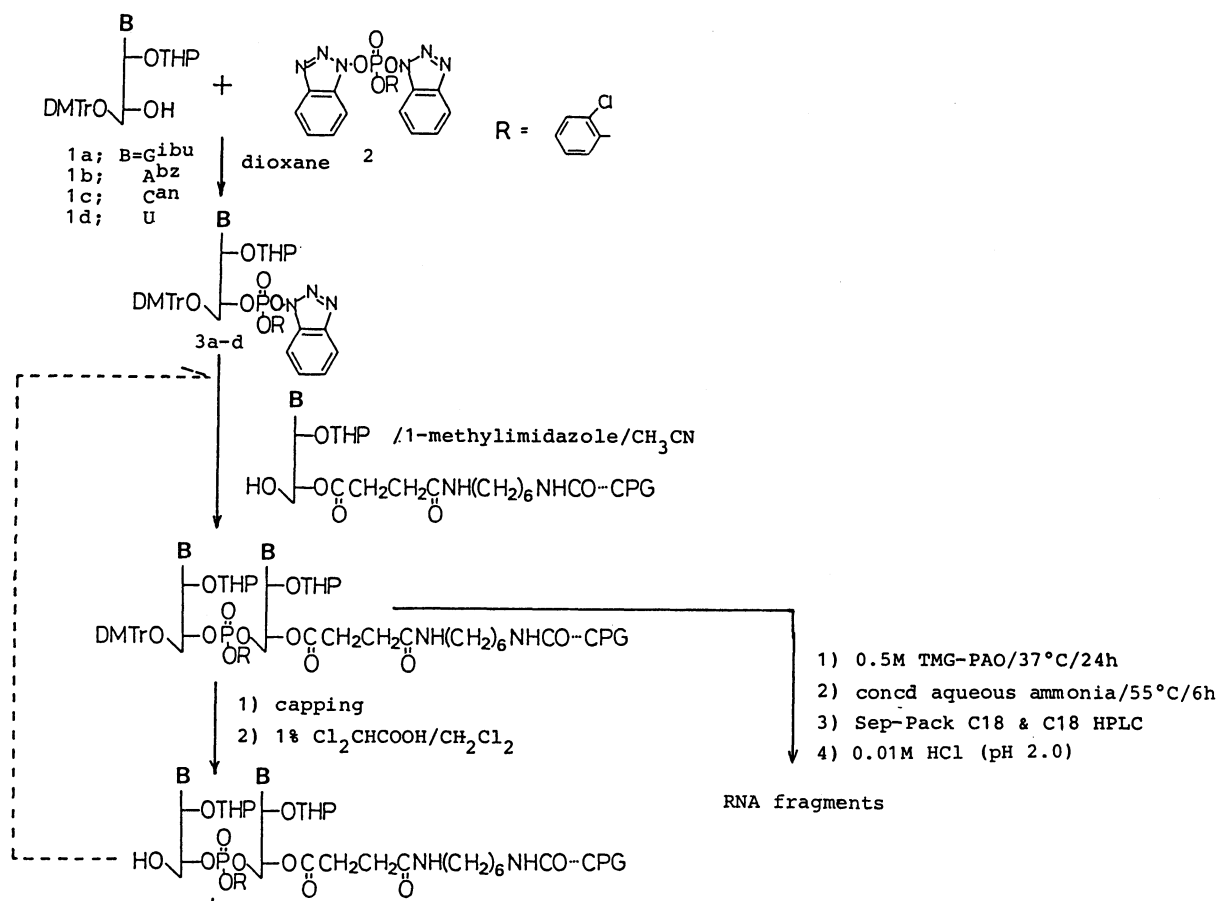
Results and Discussion

For preparation of guanosine or adenosine bound to the CPG resin, 5'-*O*-dimethoxytrityl-*N*²-isobutyryl-2'-*O*-tetrahydropyranylguanosine **1a** or *N*⁶-benzoyl-5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranyladenosine **1b** was succinylated at the 3'-hydroxyl group, followed by its activation with 1-hydroxybenzotriazole and dicyclohexylcarbodiimide (DCC) applying the same procedure as that for preparing, the 2'-*O*-deoxyribonucleoside resin.²¹⁾ The activated ester of guanosine derivative was subsequently allowed to react with various CPG resins such as 3-aminopropyl (AP)-, aminoaryl (AA)-, and long-chain aminoalkyl (LCA)-CPG. Unreacted amino groups on the CPG resin were capped with a mixture of 5% acetic anhydride, 3.5% 4-dimethylaminopyridine, and 5% 2,6-lutidine in dry tetrahydrofuran or pyridine.

We first tested the effects of solvents on the condensation reaction of a AA-CPG resin covering a guanosine derivative with 3'-*O*[(1-benzotriazolylloxy)(2-chlorophenoxy)phosphinyl]-5'-*O*-dimethoxytrityl-*N*²-isobutyryl-2'-*O*-tetrahydropyranylguanosine **3a**: When 1,4-dioxane, benzene, dichloromethane, or acetonitrile were used as the solvents for promoting the condensation reaction on the polystyrene resin. 1,4-Dioxane was found to be effective solvent. While in case of the CPG resin, a reaction conducted in acetonitrile, provided the highest coupling yield (data not shown).

The condensation reaction was thus carried out in

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Scheme 1.

Table 1. Yields of Coupling Reactions on Various CPG-Resins

Entry	2c in CH_3CN		CPG ^{a)} mg	Time min	Base ^{b)}	Coupling yield/%
	Volume/ml	Concn/mol dm^{-3}				
1	0.5	0.1	AP (21.2)	20	1-MeIm	66
2	0.5	0.1	AA (20.0)	20	1-MeIm	51
3	0.5	0.1	LCA (80.9)	20	1-MeIm	91
4	0.4	0.125	AA (20.0)	20	1-MeIm	67
5	0.4	0.125	AA (20.0)	20	1-MeMo	52
6	0.4	0.125	LCA (50.0)	15	1-MeIm	101

a) AA, aminoaryl-; LCA, long-chain ω -aminoalkyl-; AP, 3-aminopropyl-. b) 1-MeIm, 1-methylimidazole; 1-MeMo, 1-methylmorpholine.

acetonitrile on three different CPG resins (AA, AP, and LCA) each having a guanosine derivative. To obtain the 5'-free hydroxyl group, the CPG resins were first treated with 1% dichloroacetic acid in CH_2Cl_2 for 1.5 min. *N*⁴-Anisoyl-5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranylcytidine **1c** (1.1 mole equiv) was phosphorylated with *O,O*-bis(benzotriazolyl)-phosphate **2** (1.0 mole equiv) according to the procedure reported by van Boom.¹⁸⁾ The condensation reaction was conducted with a ZEON Genet manual DNA synthesizer (NIPPON ZEON Co., Ltd.). The resulting *N*⁴-anisoyl-3'-*O*-[(1-benzotriazolyl-2'-chlorophenoxy)phosphinyl]-5'-*O*-dimethoxytrityl-2'-

O-tetrahydropyranylcytidine **3c** and a base catalyst were added to the CPG resin in the reaction vessel. The condensation yields were estimated on the basis of the colorimetric determination of the released dimethoxytrityl cation (Scheme 1 and Table 1).

The LCA-CPG resin (Table 1, Entries 3 and 6) was found to give a dimer in satisfactory yield and lessen the reaction time (15 min). The AA-CPG resin with the highest content of the guanosine derivative provided only a low yield of 50–70% (Table 1, Entries 2 and 5). Next, clarification was made of the relationship between coupling yield and amount of nucleoside loaded on the CPG resin. Coupling efficiency

Table 2. Yield of Each Fragment

Sequence no.	Sequence (5'—3')	Average coupling yields/%	Yields of pure oligomers(OD ₂₆₀ U)
RNA 1	UAGCUCAGUUGGGAGAGCG	93	0.76
RNA 2	AGCUCAGUUGGGAGAGCG	97	0.50
RNA 3	ACUGGAAACUCCUCAUG	95	2.33
RNA 4	ACUAAAAACUCCUCAUG	97	1.81
RNA 5	ACUCCAAACUCCUCAUG	94	0.96
RNA 6	UAAGGAAACUCCUCAUG	93	0.64

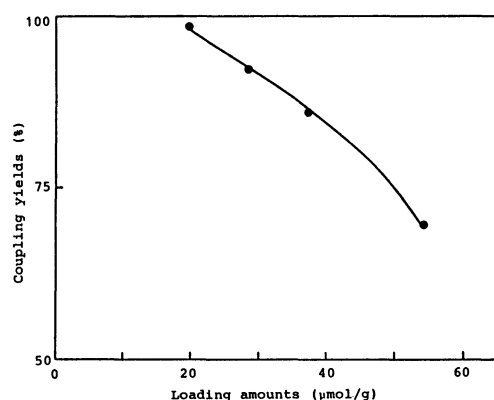


Fig. 1. Coupling yields plotted against loading amounts of the nucleoside on the LCA-CPG resin.

was assessed using activated cytidine 3'-phosphotriester unit **3c** (0.27 M^{††}, 0.27 ml) and LCA-CPG resins bound to the protected adenosin derivative at various loading amounts, [Weight of the LCA-CPG resin (g)=0.66 (μmol)/loading amount (μmol g⁻¹)], with 1-methylimidazole (0.027 ml) at 30 °C for 10 min. In Fig. 1, the coupling yield has increased with decrease in the amount of nucleoside loaded on the resin. This is possible due to steric hindrance of a nucleoside present on the CPG resin. Therefore the LCA-CPG resin derivatized with a 5'-O-dimethoxytrityl ribonucleoside, at a loading amount less than 20 μmol g⁻¹ used.

On the basis of the results obtained above, the 19mer including the D-stem and loop of yeast tRNA^{Phe} and the 18mer lacking the 5'-terminal uridine in the 19mer were synthesized. Two dihydrouridine residues in the D-loop were replaced with uridine in both the 18mer and 19mer (RNA 1 and 2 in Table 2). Synthesis was made also of the sequence of the phage f1 coat protein mRNA (18mer) as well as the AUG initiation codon (RNA 3 in Table 2) and several 18mers in each of which two guanosine residues in the Shine-Dalgarno sequence²²⁾ were replaced by different nucleosides (RNA 4—6 in Table 2).

As protected ribonucleoside monomers, *N*²-isobutyryl-guanosine **1a**, *N*⁶-benzoyladenine **1b**, *N*⁴-anisoyl-cytidine **1c**, and uridine **1d**, each protected at both the 5'- and 2'-hydroxyl groups with the 4,4'-dimethoxy-

Table 3. Synthetic Cycle

Step	Reagent or solvent	Time/min
1	1% Cl ₂ CHCOOH-CH ₂ Cl ₂	1.5(A,U,C) 1.0(G)
2	CH ₃ CN×4	3.0
3	0.26M Nucleotide-CH ₃ CN (0.3 ml), 1-methylimidazole (0.03 ml), 30 °C	15.0
4	CH ₃ CN×3	2.0
5	5% (CH ₃ CO) ₂ O-3.5% DMAP -5% 2,6-lutidine-THF	1.0
6	CH ₃ CN×3	2.0

Synthesizer: ZEON Genet H-6 DNA manual synthesizer. Total time for cycle: 25—25.5 min.

trityl and tetrahydropyranyl groups, respectively¹⁴⁾ were used. These protected ribonucleosides **1** (0.088 mmol/coupling reaction) were converted to *O*-benzotriazolyl phosphate derivatives **3** by treatment with *O,O*-bis(benzotriazolyl) 2-chlorophenyl phosphate **2**¹⁸⁾ (0.08 mmol/coupling reaction). In order to perform the reaction of **2** with **1**, a slight excess of **2** was used. By TLC-analysis of the reaction mixture, the resulting **3** was detected along with small amounts of 3',3'-dinucleotide and remaining **1**. The trityl group of **1a** was removed by treatment with 1-hydroxybenzotriazole in acetonitrile for few hours. Following preparation of the activated ribonucleotide units as the key intermediates **3**, a small amount of pyridine was added to neutralize the resulting slightly acidic species 1-hydroxybenzotriazole and excess pyridine was evaporated off. However, this treatment caused considerable decomposition resulted from hydrolysis of **3** catalyzed by a trace amount of pyridine still remaining. Thus, for the synthesis of long-chain oligoribonucleotides, fresh ribonucleotide units **3** must be prepared within five hours.

Guanosine-bound-LCA-CPG (20—50 mg, 0.3—0.8 μmol) was detritylated by 1% dichloroacetic acid in dichloromethane at 30 °C. To the LCA-CPG resin in the reaction vessel were added **3** in acetonitrile (0.250 ml) and 1-methylimidazole (0.025 ml). The condensation was carried out for 15 min at 30 °C. Fully protected oligoribonucleotides were prepared in the same way. One cycle of the synthesis required 25 min for a coupling yield more than 90% (see Scheme 1 and Table 2). The synthesis cycle is shown in Table 3.

†† 1 M=1 mol dm⁻³.

Following their synthesis, deprotection of the fully protected oligoribonucleotides was carried out according to the general procedure (see experimental section). 5'-O-DMTr-2'-O-THP oligomers were purified first by Sep-Pak C-18 (Waters) and then by high-performance liquid chromatography (HPLC) with a reversed-phase C-18 column (M&S Co.) (Fig 2). The eluted oligomers were treated with 0.01 M HCl (pH 2.0) to remove the 2'-O-tetrahydropyranyl and 5'-O-dimethoxytrityl groups. These crude oligomers were purified by HPLC at 55 °C or room temperature (Fig.

3), and the fractions corresponding to the main peak were collected. The HPLC profiles of the purified oligomers are shown in Fig. 3.

The purity of the oligomers was assessed by electrophoresis on a 20% polyacrylamide gel containing 7M urea, and when a single band could not be detected, purification was conducted again by polyacrylamide gel electrophoresis (Fig. 4). Yields of pure fragments are shown in Table 2. One reason for the low yields is that each fragment was partially lost through the purification process used for increasing the purity.

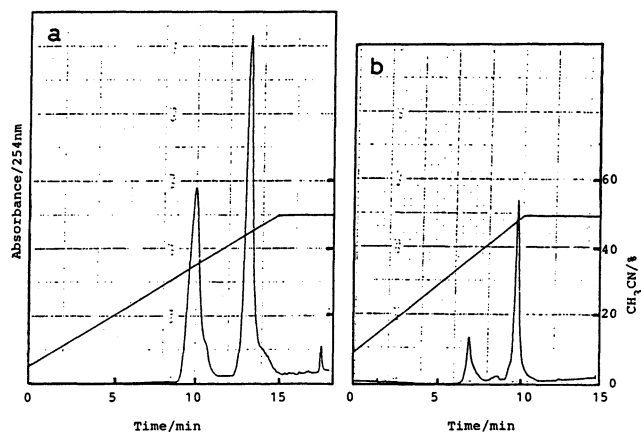


Fig. 2. Separation of 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl r-18mer (RNA 3) (a) and r-19mer (RNA 1) (b) by reversed-phase C-18 HPLC.

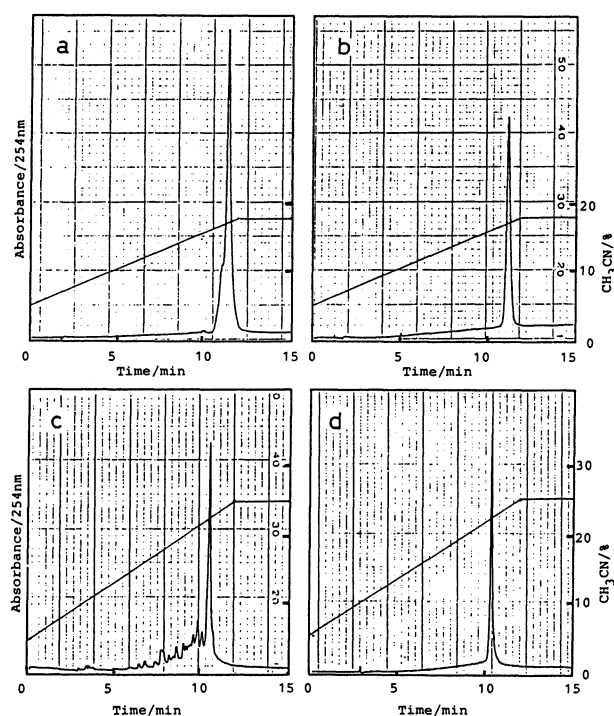


Fig. 3. Reversed-phase HPLC profiles for purification and analysis; the crude product mixture containing r-18mer (RNA 3) (a) and r-19mer (RNA 1) (c), and the elution profiles of the main peaks of RNA 3 (b) and RNA 2 (d).

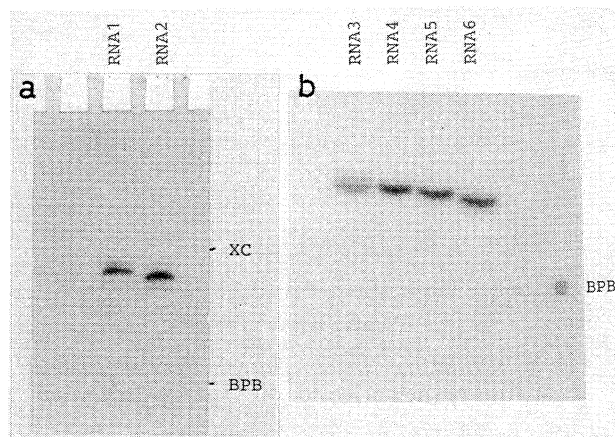


Fig. 4. 20% Polyacrylamide-7 M urea gel electrophoresis of RNA 1 and RNA 2, followed by dyeing with 0.02% Methylene Blue (a); 32 P-labeled RNA 3-6 (b).

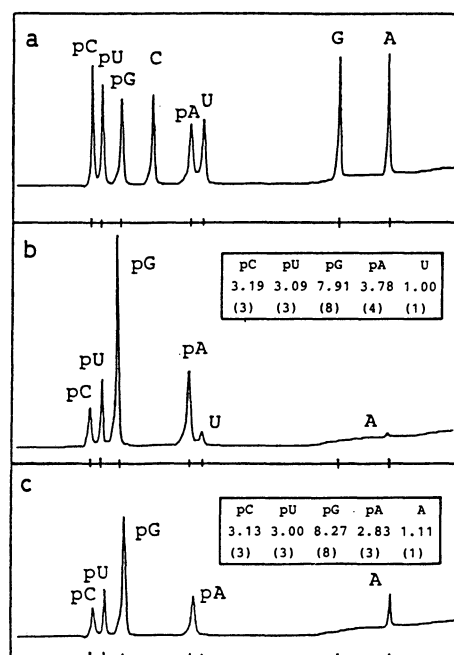


Fig. 5. Reversed-phase HPLC profiles for composition analysis of RNA 1 (b) and RNA 2 (c) both digested with nuclease P1, and authentic ribonucleosides and ribonucleotides (a). The insets show the ratios of their nucleoside and nucleotide compositions, as determined from peak area.

Base compositions were determined by enzymatic digestion using nuclease P1 (Fig. 5). It was observed that the initial digestion product, adenosine 5'-phosphate, underwent simultaneous hydrolysis to adenosine with nuclease P1 but only to a slight degree. The base sequences of the synthesized oligomers were confirmed by partial enzymatic degradation of the 5'-end-labeled oligomers in polyacrylamide gel electrophoresis (Fig. 6).

A convenient method has been presented above for the synthesis of oligoribonucleotides. Compared with the phosphoramidite or H-phosphonate method, our synthetic cycle (see Table 3) is simple owing to elimination of an oxidation step. Side reactions due to the use of 1-hydroxybenzotriazole have been reported,²³⁾ but no other products was detected in the purified oligomers. However, the by-products shown in Figs. 3a and 3c may possibly resulted from base modification or release of the tetrahydropyranyl group; the latter was not observed in our previous paper on the synthesis of the 11mer.¹⁷⁾

The synthesized 18mer and 19mer corresponding to the D-loop of yeast tRNA^{Phe} (Table 2, RNA 1 and RNA 2) were examined for their ability to be methylated with tRNA (guanosine-2')-methyltransferase (Gm-methylase) isolated from an extreme thermo-

Table 4. Inhibitory Effect of the Synthetic 18mer and 19mer to the Methylation of the 5'-Half Fragment of tRNA^{Phe} with Gm-methylase (a) and of tRNA^{Met} with m¹-methylase (b)

	Conditions	Methylation activity/%
(a)	5'-Half fragment	100
	Plus 18mer	31±3
	Plus 19mer	43±5
(b)	tRNA ^{Met}	100
	Plus 18mer	95
	Plus 19mer	97

19mer, 18mer, 5'-half fragment and tRNA^{Met}; 0.05 OD₂₆₀ unit.

phile, *Thermus thermophilus* HB 27.²⁴⁻²⁶⁾ It has been already elucidated that the D-loop-and-stem structure of tRNA is a minimal prerequisite for recognition with Gm-methylase (Matsumoto et al., unpublished), and that s⁴U8 of *E. coli* tRNA^{Met} is involved in the recognition with Gm-methylase.²⁷⁾ Therefore, regarding on the substrate ability for Gm-methylase, comparison of the 18mer (RNA 2) having no 5'-terminal U corresponding to U8 of tRNA, with the 19mer (RNA 1) having the U, would be useful to confirm importance of the U residue.

However, the result was negative, showing that neither 19mer nor 18mer was methylated with the enzyme, instead both fragments inhibited to a great extent methylation of the 5'-half fragment of yeast tRNA^{Phe} which is known as a good substrate for the enzyme (Matsumoto et al., unpublished) as shown in Table 4. A possibility that the inhibition was caused by an impurity which may have been contaminated in the fragment preparations was avoided by the fact that both fragments did not inhibit the methylation of *E. coli* tRNA^{Met} with tRNA(1-methyladenosine)-methyltransferase (m¹A-methylase)²⁷⁾ (Table 4). Therefore, it is concluded that the minimal fragment for the substrate with Gm-methylase is longer than the 19mer (RNA 1) synthesized here and it has to take a special tertiary structure which may be supported by other parts in a complete tRNA molecule. The 18mer corresponding to the leader sequence of phage f1 coat protein mRNA is now being examined for its binding efficiency to ribosomes.

Experimental

Reagents and solvents were purified and dried according to the procedure reported by van Boom et al.⁸⁾ 1-Hydroxybenzotriazole was purchased from DOJINDO and coevaporated with pyridine just before use.

HPLC was conducted by the Gilson gradient chromatography system with a C-18 column (M&S Co.) for reversed-phase HPLC and the elution buffer was a mixture of CH₃CN and 0.1 M triethylammonium acetate (pH 7.0).

RNase P1 was purchased from Seikagaku Kogyo (a product of Yamasa Co.), RNase U₂ from Sankyo Co., and

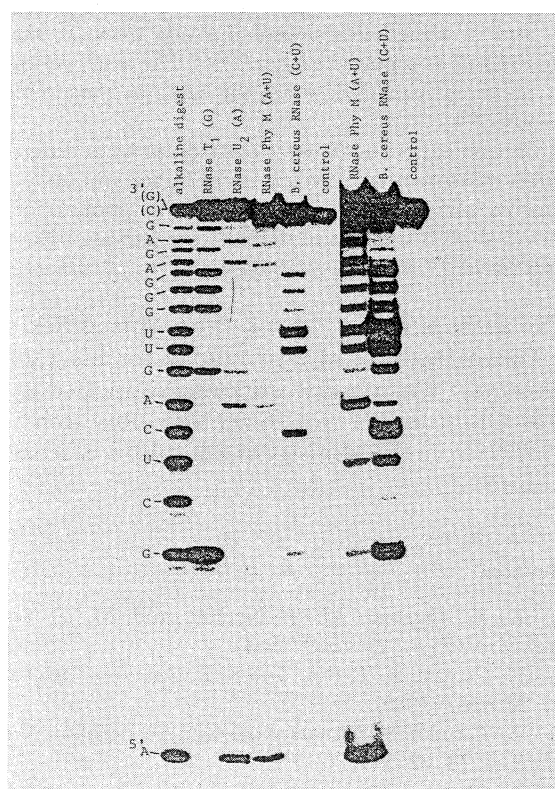


Fig. 6. Autoradiogram of electrophoresis on a 23% polyacrylamide gel containing 7 M urea for RNA sequencing. The 5'-end-³²P-labeled RNA 1 was partially digested with ribonucleases according to the method of Donis-Keller.³⁰⁾

RNase T1, RNase Phy M and *B. cereus* RNase, from Pharmacia Co.

Preparation of Fully Protected Nucleosides: The 5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranyl derivatives of *N*²-isobutyrylguanosine **1a**, benzoyladosine **1b**, *N*⁴-anisoylcytidine **1c**, and uridine **1d** were prepared from 3',5'-*O*-(tetraisopropylidisiloxane-1,3-diyl) derivatives according to the methods described previously.¹⁴⁾

The starting material, *N*²-isobutyryl-3',5'-*O*-(tetraisopropylidisiloxane-1,3-diyl)guanosine **1a**, was synthesized as follows. To a solution of guanosine (14.2 g, 50 mmol) in an anhydrous mixture of *N,N*-dimethylformamide (250 ml) and pyridine (100 ml) was added 1,3-dichloro-1,1,3,3-tetra-isopropylidisiloxane (18 ml). The mixture was stirred overnight at room temperature, followed by the addition of trimethylchlorosilane²⁸⁾ (25 ml) and stirring for 1 h. Isobutyric anhydride (45 ml) was then added and stirring was continued for another 3 h at room temperature. The mixture thus obtained was hydrolyzed with water (60 ml) for 15 min at 0°C, followed by treatment with aqueous ammonia (65 ml) for 15 min at 0°C. It was concentrated under reduced pressure and the resulting residual oil was dissolved in CH₂Cl₂ (500 ml). The CH₂Cl₂ layer was followed by washing with 1 M sodium hydrogencarbonate, drying with magnesium sulfate and concentrating under reduced pressure. The residue was dissolved in a small amount of CH₂Cl₂ and precipitated into a rapidly stirred mixture of hexane (750 ml) and ethyl acetate (250 ml). The precipitate was crystallized from ethyl acetate, to give **1a** (18.4 g, 60% yield from guanosine).

Preparation of CPG Resins Carrying Nucleosides: Nucleoside-containing CPG resins were prepared according to the literatures.²¹⁾ The condensation reaction of fully protected nucleoside 3'-succinate with the CPG resin was carried out at 37°C for 10–20 min.

Preparation of *N*-Acyl-3'-*O*-(1-benzotriazolyl-2-chlorophenoxy)phosphinyl-5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranylnucleoside (3**):** The method of van Boom¹⁸⁾ was applied as follows with slight modification.

To dried *N*-acyl-5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranylnucleoside **1a**, **1b**, **1c**, or **1d** (0.088 mmol/one coupling reaction) was added a 0.2 M solution (0.4 ml) of **2** in dioxane followed by stirring at room temperature for 30 min. The solution was then evaporated, the residue dissolved in pyridine (0.1 ml) and evaporation was continued to dryness. The residue thus obtained was dissolved in acetonitrile (0.3 ml) which was used within 5 h following the synthesis of oligoribonucleotides.

Synthesis of Oligonucleotides: The fully protected nucleoside derived LCA-CPG resin (50 mg, 0.76 μmol) was introduced into a reaction vessel of the ZEON Genet DNA manual synthesizer (NIPPON ZEON Co.). The cycle for synthesis is shown in Table 3. At the time of the condensation reaction, a solution (0.4 ml) of **3** and 1-methylimidazole (0.04 ml) was added followed by shaking at 30°C for 15 min.

Following completion of the above cycle, the resin was treated with 0.5 M 2-pyridinecarbaldehyde oxime-tetramethylguanidinium in a mixture of pyridine and water (9:1 v/v) (0.2 ml) at 37°C for 24 h, filtered and then washed with a mixture of pyridine and water (9:1 v/v) (1 ml×3). The filtrate was evaporated, and the residue was applied on a Sep Pak C18 (Waters Associates) after treatment with concd aqueous ammonia (55°C, 1 h) and washed successively with

10 ml of 0.1 M triethylammonium hydrogencarbonate (TEAB) and 20 ml of 15% acetonitrile in 0.1 M TEAB. The 5'-*O*-dimethoxytrityl-2'-*O*-tetrahydropyranyl oligoribonucleotide was finally eluted with 10 ml of 35% acetonitrile in 0.1 M TEAB and then this oligomer was applied on a reversed-phase C18 column (M&S Pack C18, M&S Co.). Elution was conducted with a linear gradient from 5% or 9.5% CH₃CN in 0.1 M triethylammonium acetate (TEAA, pH 7.0) to 50% CH₃CN in 0.1 M TEAA (Fig. 2). The desired product fraction was pooled and evaporated to dryness. The residue was treated with 0.01 M HCl (pH 2.0, 5 ml) at room temperature for 48 h. The solution was neutralized with dil. aqueous ammonia, washed with ethyl acetate (10 ml×5), and evaporated.

Crude oligomers were purified by a reversed-phase C-18 column. Elution was performed with a linear gradient of 5%–25% CH₃CN in 0.1 M TEAA (Fig. 3). Slightly crude oligomers were purified by 20% polyacrylamide gel electrophoresis in the usual manner.²⁹⁾

Enzyme Digestion and Nucleotide Sequencing: The above oligomers (0.1 A₂₆₀ units) were treated with Nuclease P1 (1 μg) in 0.1 M ammonium acetate (pH 5.3) at 37°C for 20 min. The mixtures thus produced were analyzed by HPLC on a C-18 column (elution buffer: CH₃CN–50 mM NH₄H₂PO₄, pH 5.2) (Fig 5).

The labeled oligomers were partially digested with RNases or alkali and sequenced by the 20–23% polyacrylamide-7M urea gel electrophoresis developed by Donis-Keller³⁰⁾ for distinguishing G, A, A+U, and C+U from each other.

Preparation of Methyltransferases and the Enzyme Assay: Gm- and m¹A-methylases were purified from *T. thermophilus* HB 27 as reported earlier.^{24,25)} The assay conditions for Gm- or m¹A-methylase are the same as those reported previously.²⁵⁾

Inhibition experiments were carried out by adding to the reaction mixture several to ten times larger amounts of the fragments (the 18mer or 19mer) than the amounts of substrates (5'-half fragments of tRNA^{Phe} for Gm-methylase and *E. coli* tRNA^{Met} for m¹A-methylase).

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