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LARGE SCALE SYNTHESIS OF OLIGORIBONUCLEOTIDES ON A SOLID SUPPORT: SYNTHESIS OF A CATALYTIC RNA DUPLEX

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Abstract: Three oligoribonucleotides, octaadenosine heptaphosphate [$(Ap)_7A$] and two fragments of self-cleaving RNA, have been synthesized on a 10 μmol scale by the phosphoramidite approach using the tetrahydrofuranyl and levulinyl groups for protection of the 2'- and 5'-hydroxyl functions respectively.

With the progress of oligodeoxyribonucleotide synthesis by the phosphoramidite approach,[1] synthetic DNA fragments have been utilized not only as primers, probes, and gene fragments in biochemical experiments,[2] but also to study tertiary structures of DNA duplexes by X-ray crystallography[3,4] and NMR spectroscopy.[5] However, very few structural studies using synthetic oligoribonucleotides have been reported because of the difficulty of synthesizing RNA fragments on a scale sufficient for this purpose, although RNA structures may be very important in understanding its biological functions. In the last decade, some types of RNA sequences have been found to function as catalysts *in vitro*, without any protein component.[6] These catalytic RNAs, which are called ribozymes, include ribonuclease P RNA,[7] the intervening sequence of the ribosomal RNA precursor of *Tetrahymena*,[8] and the RNA transcripts which can form the hammerhead secondary structures found in certain viroids, virusoids, and satellite RNAs.[9,10] The properties of these ribozymes have been studied using RNA fragments prepared by *in vitro* transcription with bacteriophage SP6 and T7 RNA polymerases.[11,12] Chemical synthesis of oligoribonucleotides is potentially more efficient than these enzymatic methods for elucidating the mechanisms of the ribozymes when large scale synthesis for structural studies is achieved.

The most serious problem in the synthesis of oligoribonucleotides is the combination of protecting groups for the 2'- and 5'-hydroxyl functions. The *tert*-butyldimethylsilyl (TBDMS) group for 2'-protection has been studied intensively. It can be used for the machine assembly of oligonucleotides using the acid-labile 5'-monomethoxytrityl group and the

phosphoramidite approach in the same way as DNA synthesis, and long RNA fragments with chain lengths up to 77 have been synthesized.[13,14] An improved method using the labile phenoxyacetyl group for protection of the amino function has been developed to prevent removal of the 2'-alkylsilyl group and subsequent chain cleavage at the deprotection step with ammonia.[15,16] The H-phosphonate approach was employed for chain elongation using the TBDMS protection.[17] This protecting group was applied to the 10 μmol synthesis of two self-complementary dodecaribonucleotides which were purified by thin layer chromatography on silica gel and used for NMR studies.[18] The o-nitrobenzyl group is also compatible with the 5'-monomethoxytrityl group,[19,20] and two hexamers and a heptamer have been synthesized on a 10 μmol scale by the phosphotriester approach on a polymer support.[21]

On the other hand, acid-labile acetal groups for protection of the 2'-hydroxyl function are incompatible with the trityl derivatives during chain elongation on a polymer support. The 9-phenylxanthen-9-yl and 9-(4-methoxy)phenylxanthen-9-yl groups for 5'-protection, which are removed more readily than the dimethoxytrityl (DMTr) group, have been employed to synthesize RNA fragments with chain lengths up to 21 in combination with 2'-tetrahydropyranyl (Thp) protection.[22] We have previously reported the synthesis of oligoribonucleotides using the levulinyl group for protection of the 5'-hydoxyl function, which is removed with hydrazine and completely compatible with 2'-tetrahydrofuranyl (Thf) protection.[23] The 9-fluorenylmethoxycarbonyl group removed with 1,8-diazabicyclo[5.4.0]undec-7-ene has also been used for 5'-protection,[24] and the 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl group, which is stable under strongly acidic conditions, has been developed for 2'-protection.[25] These syntheses have been performed on a small scale to yield at most 30 A_{260} units of a pure RNA fragment.

In this paper we describe the 10 μmol synthesis of oligoribonucleotides on a solid support using 5'-levulinyl and 2'-Thf protection. The octamer, $(Ap)_7A$, has been synthesized to confirm the effectiveness of the methodology. Chain elongation was performed in good yields, but one Thf group remained within the octamer after deprotection at pH 2.0, this was removed completely at pH 1.0. Two RNA fragments with sequences corresponding to the naturally-occurring self-splicing RNA from the newt, one of which contains 2'-O-methylcytidine at the cleavage site, have been synthesized using 5'-DMTr derivatives at the last coupling step in order to facilitate purification of the product by reversed-phase chromatography. An NMR study of $(Ap)_7A$ will be published elsewhere,[26] and structural studies of the catalytic RNA duplex are in progress.

Results and Discussion

Synthesis of Octaadenosine Heptaphosphate. In order to examine the efficiency of the methodology for the synthesis of oligonucleotides, homooligomers such as oligo(thymidylic acid) or oligo(uridylic acid) are convenient because the products are eluted in accordance with their chain lengths by both reversed-phase and anion-exchange high-performance liquid chromatography (HPLC). $(Ap)_7A$ was selected for two reasons; a duplex containing oligo(adenylic acid) had been reported to form an interesting structure,[4] and an oligoadenylate-specific by-product observed in a previous experiment[23] needed to be investigated.

Using the tetrahydrofuranyl and levulinyl groups for protection of the 2'- and 5'-hydroxyl functions respectively, four decamers composed of a single kind of nucleoside and one heneicosamer containing four kinds of nucleosides had been synthesized on a 1 μmol scale by the phosphoramidite approach in good yields.[23] This procedure was applied to the 10 μmol synthesis of $(Ap)_7A$ (Scheme I and II).

The starting material, 6-N-benzoyl-2'-O-tetrahydrofuranyladenosine (1a), contained a chiral carbon atom in the Thf group, and two diastereomers were separated by chromatography on silica gel. Each isomer was separately subjected to levulinylation as described.[27] The 5'-levulinyl compound (2a-h) derived from the higher isomer (1a-h), which moved faster on a silica gel plate, was attached to a controlled-pore glass (CPG) support covalently via a succinyl linkage, and 2a-l from the lower isomer (1a-l) was phosphitylated with 2-cyanoethyl N, N-diisopropyl-

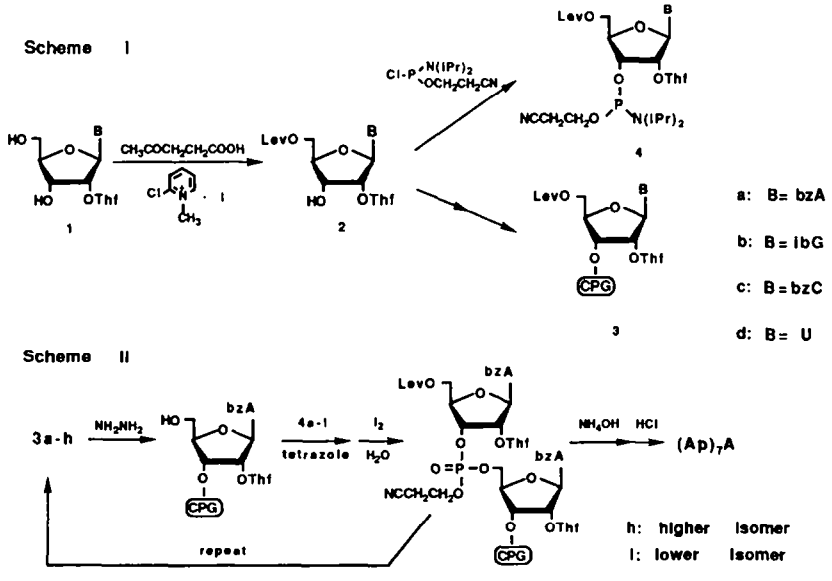


Table. Reaction Cycle for Chain Elongation

step	reagent	time
1.	0.5 M hydrazine monohydrate in pyridine/acetic acid (3:2, v/v)	15 min
2.	70 mM nucleoside 3'-phosphoramidite and 0.25 M tetrazole in acetonitrile	20 min
3.	0.44 M acetic anhydride and 0.88 M 1-methylimidazole in 2,6-lutidine/tetrahydrofuran (1:17, v/v)	3 min
4.	0.11 M iodine in pyridine/tetrahydrofuran/water (18:80:2, v/v/v)	3 min

chlorophosphoramidite.[28] The phosphoramidite derivative (4a) was purified by chromatography on silica gel and obtained as a foam by evaporation.

Chain elongation was performed on a synthesizer (Applied Biosystems 381A) using a synthetic program modified from the procedure for the 1 μmol synthesis. Starting from 0.26 g (10 μmol) of adenosine-CPG (3a) packed in a column (6 mm I.D. x 50 mm L.), removal of the 5'-levulinyl group with hydrazine, coupling of 4a using tetrazole as an activator, capping of the remaining 5'-hydroxyl function by acetylation, and oxidation to the phosphotriester linkage with iodine were repeated seven times (Table). For removal of the solution of hydrazine or iodine, a continuous flow of acetonitrile (90 sec) and a reverse flush of argon (60 sec) were repeated four times after each reaction.

The oligonucleotide was cleaved from the CPG support with ammonia water at room temperature and deprotected by heating the solution in a sealed vessel at 55 ^{o}C for 5 h. After evaporation of ammonia water, the Thf group was removed with hydrochloric acid at pH 2.0. This treatment was prolonged to 36 h because the oligoadenylate-specific by-product had been found to change to the desired product under acidic conditions,[23] and then the solution was neutralized with diluted ammonia water.

The deprotected product was analyzed by reversed-phase HPLC (Figure 1A). Peak I corresponded to benzamide originating from the benzoyl group, and peak III was the by-product discussed below. The amount of shorter oligomers was very small, which showed good coupling yields and the effectiveness of this method. Purification was performed first by reversed-phase chromatography using a column of alkylated silica gel. The fractions containing the desired product (peak II) were collected and purified further by HPLC to remove slight contaminants of shorter oligomers (Figure 1B). The overall yield from 3'-terminal adenosine on the CPG support was 31%, and 311 A_{260} units of purified $(Ap)_7A$ were obtained. The purity of the final product was examined by both reversed-phase and

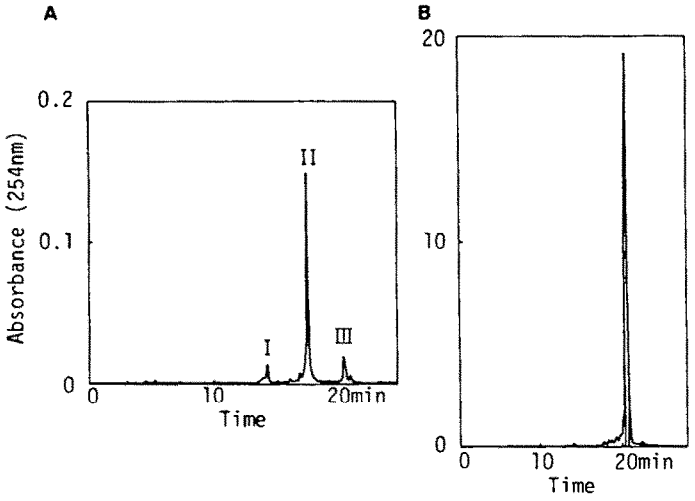


Figure 1. Elution profiles of crude $(Ap)_7A$: (A) HPLC analysis before the first purification; (B) purification after elution from a reversed-phase column. For analysis a YMC A-303 column (4.6 mm I.D. x 250 mm L.) was used at a flow rate of 1.0 mL/min with a linear gradient of acetonitrile (from 7 to 15% during 20 min) in 0.1 M TEAA. Purification was performed by injecting 1/25 of the total volume for each run using a YMC A-324 column (10 mm I.D. x 300 mm L.) at a flow rate of 2.0 mL/min with the same gradient.

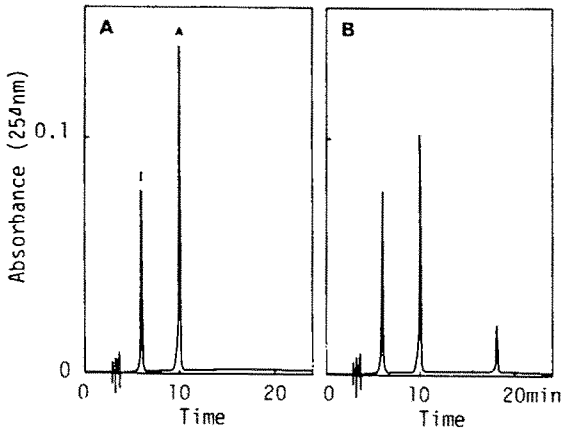


Figure 2. HPLC analysis of the digests with snake venom phosphodiesterase and bacterial alkaline phosphatase: (A) $(Ap)_7A$; (B) the by-product. A TSKgel ODS-80T_M column (4.6 mm I.D. x 250 mm L.) was used at a flow rate of 1.0 mL/min with a linear gradient of acetonitrile (from 5 to 15% during 20 min) in 0.1 M TEAA.

anion-exchange HPLC, and the chain length was confirmed by limited alkaline hydrolysis followed by homochromatography (Figure 3).

By-product Specific to Oligo(adenylic acid). In our previous report[23] an extra peak, which diminished after prolonged acid treatment, was detected behind the main peak in the 1 μmol synthesis of $(Ap)_9U$ by the

same procedure. This peak was not detected in the syntheses of the other homooligomers, $(Gp)_9U$, $(Cp)_9U$, and $(Up)_9U$. A similar by-product (peak III in Figure 1) was also obtained in the 10 μmol synthesis of $(Ap)_7A$, which was separated by reversed-phase chromatography, further purified by HPLC, and digested with snake venom phosphodiesterase and bacterial alkaline phosphatase.[29] The resulting nucleosides were separated by reversed-phase HPLC, and a modified nucleoside which was equivalent to one nucleoside in the octamer was eluted besides inosine produced by contamination with adenosine deaminase (Figure 2). This nucleoside showed the same UV spectrum as that of adenosine, and its retention time coincided with that of the lower isomer of 2'-O-tetrahydrofuranyladenosine. Therefore the by-product was attributed to $(Ap)_7A$ containing one Thf group which could not be removed by acid treatment, although the Thf group could be removed more readily than the Thp group.[30] Moreover it was reported that the presence of a vicinal phosphodiester linkage facilitates hydrolysis of a 2'-acetal protecting group,[31] but the nucleoside with the remaining Thf group was the lower isomer, which was used as the phosphoramidite derivative, while the more stable Thf group at the 3'-end (the higher isomer) was completely removed. In order to specify the residue where the Thf group remained, the by-product (peak III) was phosphorylated with [γ-^{32}P]ATP and T4 polynucleotide kinase, subjected to limited alkaline hydrolysis,[32] and separated by homochromatography (Figure 3). It was found that the Thf group remained not at one specific site, but at several residues within the octamer. The structure of oligo(adenylic acid) in an acidic solution may have some influence on the stability of the Thf group against acid catalyzed hydrolysis.

The conditions for complete deprotection of oligo(adenylic acid) were investigated using decaadenosine nonaphosphate [$(Ap)_9A$] synthesized on a 1

Figure 3. Autoradiograms of the separated products after limited alkaline hydrolysis: (A) $(Ap)_7A$; (B) the by-product.

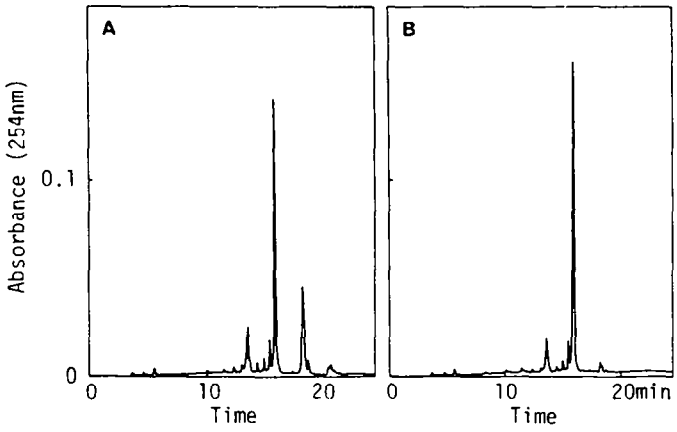


Figure 4. Elution profiles of crude $(Ap)_9A$. Deprotection was performed: (A) at pH 2.0 for 5 h; (B) at pH 1.0 for 3 h. The conditions for analysis are mentioned in the legend to Figure 1.

μmol scale. After cleavage and deprotection with ammonia water, the solution was divided into three portions, and ammonia water was removed by evaporation separately. The first decamer was treated with diluted hydrochloric acid at pH 2.0 for 5 h, the second at pH 1.0 for 3 h, and the third at pH 1.0 for 15 h, followed by neutralization and HPLC analysis (Figure 4). A relatively large peak of the by-product discussed above was detected for the mixture treated at pH 2.0. This peak was only a trace in the case of the pH 1.0 sample. The product treated at pH 1.0 for 15 h showed the same elution profile as that of the 3 h sample, and the main peak was partitioned by HPLC followed by digestion with RNase T_2 after 5'-^{32}P-labeling.[33] Formation of the 2'-5' phosphodiester linkage was not observed, also no chain cleavage was observed in the elution profile, although these side reactions have been reported for prolonged acid treatment at pH 1.0.[34] Therefore the yield in the synthesis of oligo(adenylic acid) using the Thf group for protection of the 2'-hydroxyl function can be improved by deprotection at pH 1.0 for a short period of time.

Large Scale Synthesis of Catalytic RNA. Some types of RNA sequences have been found to function as a catalyst in vitro, and the components which can form the hammerhead structures containing the consensus nucleotides are the smallest catalytic RNA. These sequences have been prepared by in vitro transcription with T7 RNA polymerase,[9,10] and we have studied these kinds of ribozymes using oligoribonucleotides synthesized chemically.[35-37] Because structural studies seem to be essential to elucidate the mechanism of the catalytic function, we have synthesized two oligoribonucleotides which can form the hammerhead structure derived from

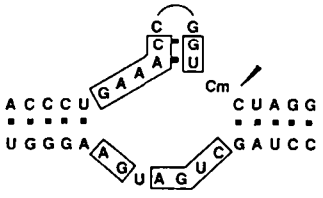
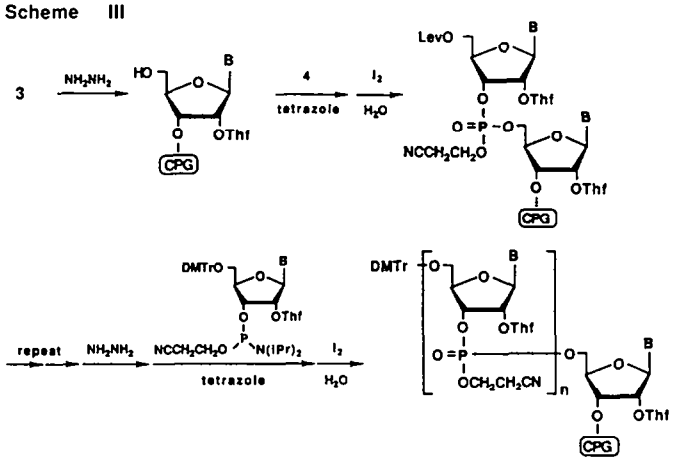


Figure 5. The hammerhead structure formed by the synthetic oligoribonucleotides. The consensus nucleotides are boxed, and the cleavage site is indicated by an arrowhead. Cm is 2'-O-methylcytidine.

Scheme III



the self-cleaving transcripts of satellite DNA from the newt[38] (Figure 5).

In the solid-phase synthesis of oligonucleotides, purification of the deprotected product is a crucial problem because shorter oligomers to which coupling reactions have not occurred are cleaved from the support together with the desired product. In the synthesis of oligodeoxyribonucleotides, the hydrophobicity of the DMTr group which is left at the 5'-terminus at the end of the deprotection steps is generally used to make purification by reversed-phase chromatography easier.[2] For this purpose, phosphoramidite derivatives using the DMTr group for protection of the 5'-hydroxyl function were prepared by phosphitylation of 5'-DMTr and 2'-Thf protected nucleosides because the levulinyl group in our method is removed simultaneously with cleavage from the support.

Another problem in the synthesis of catalytic RNA sequences is the cleavage reaction during purification, and especially in structural studies. 2'-O-Methylcytidine (Cm) was employed to avoid this reaction because hammerhead-type ribozymes were reported to require the 2'-hydroxyl function at the cleavage site.[37] 4-N-Benzoyl-2'-O-methylcytidine prepared as described[39] was levulinylated and phosphitylated in the same manner.

A 17mer (CCUAGCUGAUGAAGGGU) and a 20mer (ACCCUGAAACCGGUCmCUAGG) were synthesized as shown in Scheme III starting from 10 μmol of uridine- and

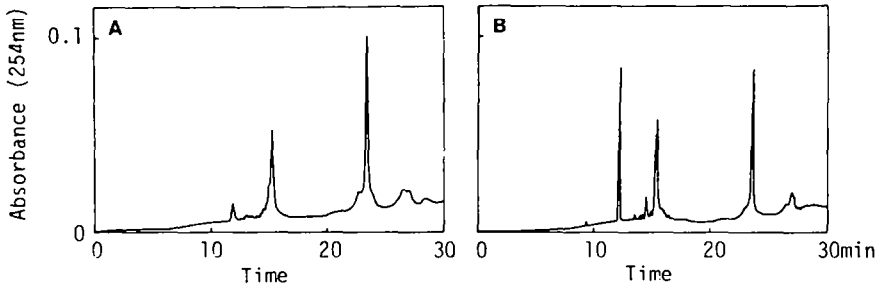


Figure 6. Elution profiles of the oligonucleotides having the 5'-DMTr and 2'-Thf groups: (A) the 17mer; (B) the 20mer. A YMC A-303 column was used with a linear gradient of acetonitrile (from 5 to 50% during 30 min) in 0.1 M TEAA.

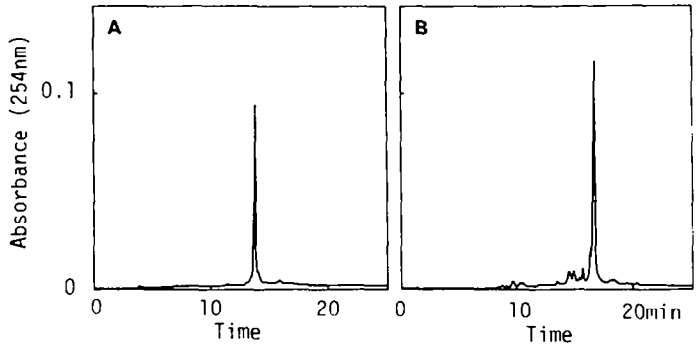


Figure 7. Elution profiles of the fully deprotected oligomers: (A) the 17mer; (B) the 20mer. A YMC A-303 column was used with a linear gradient of acetonitrile (from 7 to 13% during 20 min) in 0.1 M TEAA. Further purification was performed using a YMC A-324 column with the same gradient.

guanosine-CPG respectively. 2'-O-Methylcytidine was introduced at the proper position, and the 5'-DMTr derivatives were used only at the last coupling step. After chain elongation, oligonucleotides were cleaved from the CPG support and deprotected with ammonia water, and the products were analyzed by reversed-phase HPLC (Figure 6). Each main peak eluted later seemed to contain the desired oligomer having the 5'-DMTr and 2'-Thf groups, which was purified by reversed-phase chromatography using a column of alkylated silica gel. After treatment with hydrochloric acid at pH 2.0 for 5 h, the fully deprotected product was analyzed and purified by HPLC (Figure 7). The isolated yields of the 17mer and the 20mer were 250 A_{260} units (15% from the 3'-terminal nucleoside on the CPG support) and 127 A_{260} units (7%) respectively. The purity of each oligomer was examined by both reversed-phase and anion-exchange HPLC, and the sequences were confirmed by partial digestion with snake venom phosphodiesterase followed by homochromatography (Figure 8) along with determination of the 5'-terminal nucleoside by complete digestion of the 5'-^{32}P-labeled oligomer.

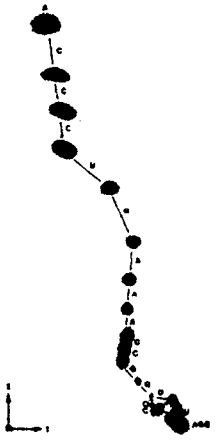


Figure 8. Sequence analysis of the 20mer.

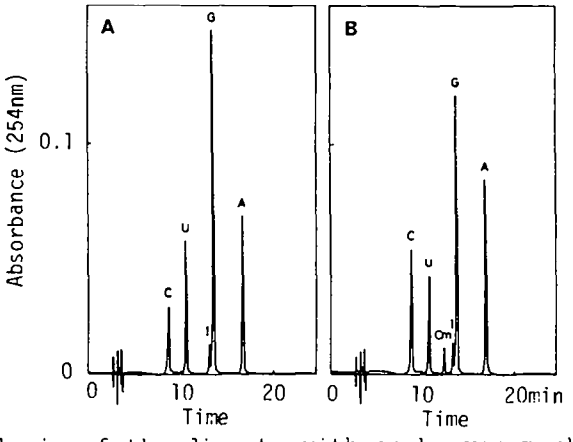


Figure 9. HPLC analysis of the digests with snake venom phosphodiesterase and bacterial alkaline phosphatase: (A) the 17mer; (B) the 20mer. A YMC A-303 column was used with a linear gradient of acetonitrile (from 0 to 15% during 20 min) in 0.1 M TEAA.

The base composition was also confirmed by digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase followed by HPLC analysis (Figure 9, A:G:C:U = 3.9:6.3:2.9:3.9 for the 17mer, and A:G:C:U:Cm = 5.0:5.3:5.8:2.9:1.0 for the 20mer), and no peaks resulting from base modification or incomplete deprotection were detected.

Experimental Section

General. N-Acyl-2'-O-tetrahydrofuranyl nucleosides[40] and 4-N-benzoyl-2'-O-methylcytidine[39] were prepared via the 3', 5'-(1, 1, 3, 3-tetra-isopropyldisiloxan-1, 3-diyl) derivatives as described.

Thin layer chromatography (TLC) was performed on Kieselgel $60F_{254}$

plates (Merck) with chloroform-methanol (10:1, v/v). For reversed-phase TLC, Kieselgel $60F_{254}$ silanisiert plates (Merck) were used with a solvent system of acetone-20 mM triethylammonium acetate (TEAA, pH 7.0) (6:4, v/v). For column chromatography, Wakogel C-300 (Wako Pure Chemical Industries) and PREPARATIVE C18 (Waters Associates) were used.

^{1}H-NMR spectra were measured at 100 MHz with a JEOL JNM-FX100 spectrometer. ^{31}P-NMR spectra of the phosphoramidite derivatives were measured at 36.25 MHz with a JEOL JNM-FX90Q spectrometer using trimethyl phosphate as an internal standard.

Reversed-phase HPLC analysis was performed on a Shimadzu LC-6A system using a YMC A-303 column (4.6 mm I.D. x 250 mm L., Yamamura Chemical Laboratories) at a flow rate of 1.0 mL/min with a linear gradient of acetonitrile in 0.1 M TEAA. For purification, a YMC A-324 column (10 mm I.D. x 300 mm L., Yamamura Chemical Laboratories) was used on a GILSON's system at a flow rate of 2.0 mL/min with the same gradient. Anion-exchange HPLC was performed on a Shimadzu LC-3A system using a TSKgel DEAE-2SW column (4.6 mm I.D. x 250 mm L., Tosoh Corporation) at a flow rate of 1.0 mL/min with a linear gradient of ammonium formate in 20% aqueous acetonitrile.

Purified oligonucleotides were quantified by UV absorption at 260 nm measured with a Beckman DU-65 spectrophotometer, and overall yields from the 3'-terminal nucleoside on the CPG support were determined using calculated extinction coefficients.[41]

N-Acyl-5'-O-levulinyl-2'-O-tetrahydrofuranyl Nucleosides (2). A solution of N-acyl-2'-O-tetrahydrofuranyl nucleoside (1, the lower isomer, 20 mmol) in dioxane (160 mL) was added to a suspension of 2-chloro-1-methylpyridinium iodide (10.22 g, 40 mmol) in acetonitrile (40 mL). To the resulting suspension a solution of levulinic acid (8.19 mL, 80 mmol) and 1,4-diazabicyclo[2.2.2]octane (10.77 g, 96 mmol) in dioxane (80 mL) was added, and the mixture was stirred for 30 min. Chloroform (1L) and 2% aqueous $NaHCO_3$ (1L) were added, and the aqueous layer was extracted with chloroform (200mL x 3). The combined organic layer was dried with Na_2SO_4 and concentrated. The product was partially purified using a column of Wakogel C-300 (150 g) with a solvent system of chloroform-methanol. After concentration and coevaporation with pyridine, the residue was dissolved in pyridine (20 mL), and 4, 4'-dimethoxytrityl chloride (ca. 0.5 equivalent to the obtained product) was added. After 1 h, methanol (3 mL) and then chloroform (300 mL) were added, and the solution was washed with water. The organic layer was dried with Na_2SO_4, concentrated, and applied to a silica gel column again. The 5'-O-levulinyl-2'-O-tetrahydrofuranyl derivatives of 6-N-benzoyladenosine (2a) and 2-N-isobutyrylguanosine (2b)

eluted with 2-3% methanol in chloroform were precipitated with hexane-ethyl ether (1:1, v/v) after concentration. Those of 4-N-benzoylcytidine (2c) and uridine (2d) were obtained as a foam by evaporation. Compound 2a-1; yield 5.00 g, 9.27 mmol, 46%. ^{1}H-NMR ($CDCl_3$) 9.18 (br s, 1H, NH), 8.80 (s, 1H, H-8), 8.22 (s, 1H, H-2), 8.1-7.4 (m, 5H, arom), 6.18 (d, 1H, H-1'), 5.28 (br s, 1H, Thf), 4.86 (t, 1H, H-2'), 4.60 (t, 1H, H-3'), 4.44 (d, 2H, H-5'), 4.4-4.2 (m, 1H, H-4'), 3.8-3.5 (m, 3H, 3'-OH and Thf), 2.9-2.5 (m, 4H, Lev), 2.18 (s, 3H, Lev), 2.1-1.7 (m, 4H, Thf). Compound 2b; yield 7.05 g, 13.52 mmol, 68%. ^{1}H-NMR ($CDCl_3$) 9.88 (br s, 1H, NH), 7.80 (s, 1H, H-8), 5.87 (d, 1H, H-1'), 5.24 (br s, 1H, Thf), 4.8-4.2 (m, 5H, H-2', 3', 4', and 5'), 3.8-3.5 (m, 3H, 3'-OH and Thf), 2.9-2.5 (m, 4H, Lev), 2.17 (s, 3H, Lev), 2.07 (s, 1H, isobutyryl), 2.1-1.8 (m, 4H, Thf), 1.26 (d, 6H, isobutyryl). Compound 2c; yield 4.20 g, 8.14 mmol, 41%. ^{1}H-NMR ($CDCl_3$) 8.1-7.5 (m, 7H, H-5, 6, and arom), 6.06 (d, 1H, H-1'), 5.54 (br s, 1H, Thf), 4.5-4.2 (m, 5H, H-2', 3', 4', and 5'), 4.1-3.8 (m, 3H, 3'-OH and Thf), 3.0-2.6 (m, 4H, Lev), 2.21 (s, 3H, Lev), 2.1-1.9 (m, 4H, Thf). Compound 2d; yield 2.48 g, 6.01 mmol, 30%. ^{1}H-NMR ($CDCl_3$) 8.90 (br s, 1H, NH), 7.45 (d, 1H, H-6), 5.86 (d, 1H, H-1'), 5.78 (d, 1H, H-5), 5.31 (br s, 1H, Thf), 4.4-4.1 (m, 5H, H-2', 3', 4', and 5'), 4.0-3.8 (m, 3H, 3'-OH and Thf), 2.9-2.5 (m, 4H, Lev), 2.19 (s, 3H, Lev), 2.1-1.9 (m, 4H, Thf).

Attachment of Nucleosides to the CPG Support. 5'-O-Levulinyl-2'-O-tetrahydrofuranyluridine (2d, 0.21 g, 0.5 mmol) was dissolved in dichloromethane (4 mL), and succinic anhydride (75 mg, 0.75 mmol) and 4-dimethylaminopyridine (DMAP, 92 mg, 0.75 mmol) were added. After 2 h, the solution was diluted with dichloromethane (50 mL) and washed with 0.5 M potassium phosphate buffer (pH 5.0). The organic layer was dried with Na_2SO_4, concentrated, and applied to a column of Wakogel C-300 (12 g). The 3'-succinyl derivative was eluted with 3% methanol in chloroform and obtained as a foam (169 mg, 0.33 mmol) by evaporation. This intermediate was dissolved in N, N-dimethylformamide (DMF, 3 mL), and pentachlorophenol (97 mg, 0.363 mmol) and 1, 3-dicyclohexylcarbodiimide (102 mg, 0.495 mmol) were added. After stirring for 16 h, the mixture was filtered and concentrated. 1, 3-Dicyclohexylurea was removed by filtration of the suspension in benzene followed by concentration. The activated ester was precipitated with hexane-ethyl ether (1:1, v/v) from a chloroform solution and shaken with CPG / long chain alkylamine (purchased from PIERCE, 1.0 g) and triethylamine (26 μL, 0.19 mmol) in DMF (4.5 mL) for 9 h. After filtration, CPG containing uridine (3d) was washed with DMF and pyridine, shaken with 0.1 M DMAP in pyridine (9 mL) and acetic anhydride (1 mL) for 10 min, and then washed with pyridine and dichloromethane in turn. Adenosine and guanosine derivatives (3a and 3b respectively) were attached

to the CPG support in the same manner. The nucleoside content was determined by UV absorption after cleavage and deprotection with ammonia water using 10 mg of each product; 3a 38 μmol/g. 3b 36 μmol/g. 3d 38 μmol/g.

Synthesis of the Phosphoramidite Derivatives (4). After coevaporation with pyridine, N-acyl-5'-O-levulinyl-2'-O-tetrahydrofuranyl nucleoside (2, 2 mmol) was dissolved in tetrahydrofuran (20 mL), and N, N-diisopropylethylamine (1.39 mL, 8 mmol) was added. For the derivative of guanosine (2b), dichloromethane was used as a solvent due to low solubility in tetrahydrofuran. To this solution 2-cyanoethyl N, N-diisopropylchlorophosphoramidite (0.89 mL, 4 mmol) was added dropwise. After 1 h, ethyl acetate (200 mL) was added and washed with saturated aqueous $NaHCO_3$ (80 mL x 2). The organic layer was dried with Na_2SO_4, concentrated, and applied to a column of Wakogel C-300 (30 g) eluted with 2% methanol in chloroform containing 0.1% pyridine. The purified phosphoramidite derivative was precipitated with n-pentane (150 mL) from a chloroform solution (10 mL), dissolved in chloroform again, and obtained as a foam by evaporation. The 3'-phosphoramidite derivatives of 5'-DMTr-protected nucleosides and 4-N-benzoyl-5'-O-levulinyl-2'-O-methylcytidine were synthesized in the same way starting from 0.6 mmol of each nucleoside. Compound 4a; yield 1.18 g, 1.60 mmol, 80%. ^{31}P-NMR ($CDCl_3$) 148.10, 147.54. Compound 4b; yield 1.18 g, 1.64 mmol, 82%. ^{31}P-NMR ($CDCl_3$) 147.81, 147.20. Compound 4c; yield 1.15 g, 1.61 mmol, 81%. ^{31}P-NMR ($CDCl_3$) 148.15, 147.67. Compound 4d; yield 1.04 g, 1.70 mmol, 85%. ^{31}P-NMR ($CDCl_3$) 148.08, 147.81. 6-N-Benzoyl-5'-O-dimethoxytrityl-2'-O-tetrahydrofuranyl-adenosine 3'-phosphoramidite; yield 0.42 g, 0.45 mmol, 75%. ^{31}P-NMR ($CDCl_3$) 148.21, 147.54. 4-N-Benzoyl-5'-O-dimethoxytrityl-2'-O-tetrahydrofuranylcytidine 3'-phosphoramidite; yield 0.42 g, 0.45 mmol, 75%. ^{31}P-NMR ($CDCl_3$) 147.94, 147.65. 4-N-Benzoyl-5'-O-levulinyl-2'-O-methylcytidine 3'-phosphoramidite; yield 0.34 g, 0.51 mmol, 85%. ^{31}P-NMR ($CDCl_3$) 148.08.

Synthesis of $(Ap)_7A$. Chain elongation was performed on an Applied Biosystems 381A synthesizer using a column of adenosine-CPG (3a, 0.26 g, 10 μmol) and a synthetic program outlined in Table. The synthetic cycle was repeated seven times using the phosphoramidite derivative of adenosine (4a) at the coupling steps, and then the oligomer was cleaved by treatment with concentrated ammonia water (2 mL x 6) for 1.5 h. The resulting solution was heated in a sealed vial at 55 °C for 5 h. After evaporation of ammonia water, 0.01 N hydrochloric acid (10 mL) was added, and the pH was adjusted to 2.0 by addition of 0.1 N hydrochloric acid. The solution was stirred for 36 h, neutralized with diluted ammonia water, and applied to a column (1.7 cm I.D. x 14 cm L.) of PREPARATIVE C18 equilibrated with

50 mM TEAA. Elution was performed with a gradient of acetonitrile (from 0 to 15%) in 50 mM TEAA, and the fractions in the main peak were collected and concentrated. The residue was dissolved in 2.5 mL of water and purified by HPLC as shown in Figure 1; yield 311 A_{260} units, 3.13 μmol, 31%.

Enzymatic Degradation. The octamer [$(Ap)_7A$] or the by-product (peak III in Figure 1) (4.0 A_{260} units) was dissolved in 0.1 M Tris-HCl (pH 8.2) containing 2 mM $MgCl_2$ (953 μL), and snake venom phosphodiesterase (2 mg/mL, 40 μL) and alkaline phosphatase (from E. coli A19, 0.44 unit/μL, 7 μL) were added. The mixture was incubated at 37 °C for 20 h, and ethanol (2.5 mL) was added. After 1 h at -20 °C, the proteins were pelleted by centrifugation, and the supernatant was concentrated in vacuo. The residue was dissolved in water (0.5 mL), filtered through a membrane filter, and analyzed by HPLC as shown in Figure 2. The peak of the modified nucleoside was partitioned, and its UV spectrum was compared with that of adenosine. Authentic nucleosides were also eluted under the same conditions.

Limited Alkaline Hydrolysis. The octamer or the by-product (0.04 A_{260} unit) was dissolved in water (5.8 μL) and a buffer (2 μL) containing 250 mM Tris-HCl (pH 9.6), 50 mM $MgCl_2$, 10 mM spermine, 50 mM dithiothreitol, and 0.5 M KCl. To this solution, [γ-^{32}P]ATP (2 μL) and T4 polynucleotide kinase (from E.coli A19, 10 units/μL, 0.2 μL) were added, and the mixture was incubated at 37 °C for 1 h. The 5'-^{32}P-labeled oligomer was separated from [γ-^{32}P]ATP by TLC on a DEAE-cellulose plate using Homo-mix III[42] and eluted with 2 M triethylammonium bicarbonate after washing with ethanol. The resulting solution was concentrated in vacuo and coevaporated with water. The residue was dissolved in water (50 μL), and one-fiftieth of the solution was treated with 50 mM Na_2CO_3/$NaHCO_3$ (pH 11.0, 20 μL) at 90 °C for 20 min. After cooling in an ice bath, 2 N hydrochloric acid (2 μL) was added, and the mixture was kept at 4 °C overnight. Then 2 N aqueous NaOH (1 μL) was added, and the product was analyzed by homochromatography (Figure 3).

Synthesis of the RNA Fragments. Chain elongation was performed by the same procedure for the synthesis of $(Ap)_7A$ except that 5'-DMTr-protected phosphoramidite derivatives were used at the last coupling step. After cleavage from the CPG support and deprotection by heating, ammonia water was removed by evaporation, and the residue (HPLC analysis is shown in Figure 6) was applied to a column (1.7 cm I.D. x 14 cm L.) of PREPARATIVE C18 equilibrated with 5% acetonitrile in 0.1 M TEAA. Elution was performed with a gradient of acetonitrile (from 5 to 50%) in 0.1 M TEAA, and the fractions in the main peak eluted later were collected and desalted using a column (1.7 cm I.D. x 36 cm L.) of Sephadex G-25 eluted with 50 mM

triethylammonium bicarbonate. After coevaporation with water, the excluded substance was treated with 0.01 N hydrochloric acid adjusted to pH 2.0 by addition of 0.1 N hydrochloric acid for 5 h, and then neutralized with diluted ammonia water. The resulting solution was desalted by gel filtration and concentrated. The products were analyzed and purified by HPLC as shown in Figure 7. The isolated yields of the 17mer and the 20mer were 250 A_{260} units (1.47 μmol) and 127 A_{260} units (0.67 μmol) respectively. The purity of each final product was examined by both reversed-phase and anion-exchange HPLC. The sequences were analyzed by partial digestion with snake venom phosphodiesterase followed by homochromatography,[23,42] and the 3'-5' phosphodiester linkages were confirmed by RNase T_2 digestion.[23]

Conclusions

The results described above demonstrate that the synthetic method using the 2'-Thf group in combination with the 5'-levulinyl group is effective for the synthesis of oligoribonucleotides on a scale sufficient for structural studies of RNA. The purification procedure can be simplified by using a 5'-DMTr-protected phosphoramidite derivative for the last coupling, and it was found that acid treatment at pH 1.0 was needed for complete removal of the Thf group uniquely in the case of oligo-(adenylic acid).

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