Volume 17 Number 20 1989 Nucleic Acids Research

Chemical synthesis of the 24 RNA fragments corresponding to hop stunt viroid

Hiroshi Tanimura, Masayuki Maeda, Tetsuya Fukazawa, Mitsuo Sekine and Tsujiaki Hata*

Department of Life Chemistry, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 227, Japan

Received July 11, 1989; Revised and Accepted September 8, 1989

ABSTRACT

A general and practical synthetic method of oligoribonucleotides (10−20 mers) by using the cyanoethyl phosphoramidite approach was described. In this experiment 9-phenylxanthen-9-yl (Pix) and 9-(4-methoxy)phenylxanthen-9-yl (Mox) groups were employed for the 5′-hydroxyls and tetrahydropyranyl (Thp) group was used for the 2′-hydroxyl protecting groups. In addition, suitable acyl groups were introduced for the protection of the lactam functions of guanine and uracil moieties.

INTRODUCTION

Although the chemical synthesis of DNA has already become routine by the phosphoramidite method, RNA synthesis has not yet been established because an ideal protecting group for the 2′-hydroxyl group of ribonucleosides is still missing. Recently, we have described (1−3) a practical method for the synthesis of oligoribonucleotides by means of an automatic DNA synthesizer where tetrahydropyranyl (Thp) group was employed for the 2′-hydroxyl protecting groups. More recently, Caruthers (4) has reported the RNA synthesis by the combined use of the 2′-Thp and 5′-dimethoxytrityl (DMTr) groups. Choice of the protecting group for the 2′-hydroxyls is of importance in solid phase synthesis. Reese (5) selected 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl group. Ohtsuka (6) and Gait (7) have described tetrahydrofuranyl and 4-methoxytetrahydropyran-4-yl groups, respectively. On the other hand, Ogilvie (8) has synthesized a 43 mer and a 77-nucleotide-long RNA by using the tert-butyldimethylsilyl group for the 2′-hydroxyl protecting groups. As an extension of our study, we were interested in the preparation of medium size RNAs (10−20 mers) which can not be transcribed correctly from the corresponding DNAs by use of T7 RNA polymerase and SP6 RNA polymerase.

From the synthetic point of view, 'viroid' is one of the attractive molecules as a proliferation potential molecule. Viroids (9) are the smallest pathogenic agent that induce serious disease of higher plants. They exist as single stranded and loop RNA molecules which have highly intramolecularly hybridized structures. Shikata (10) showed that hop stunt disease was caused by a viroid which consisted of 297 ribonucleotides. The nucleotide sequence of hop stunt viroid (HSV) was determined by Okada. (11)

As a strategy for the synthesis of HSV, we thought that the method in combination of chemical synthesis of the RNA fragments with enzymatic ligation of the fragments by means of T4 RNA ligase can be authorized. Thus, we designed to scrap HSV into 24 RNA fragments in consideration of the efficiency of ligation due to the nucleotide sequences. (12,13) Fragmentation of HSV is shown as bar in Fig. 1.

In this paper, we wish to report the synthesis of 24 oligoribonucleotides corresponding to HSV via the cyanoethyl phosphoramidite approach.

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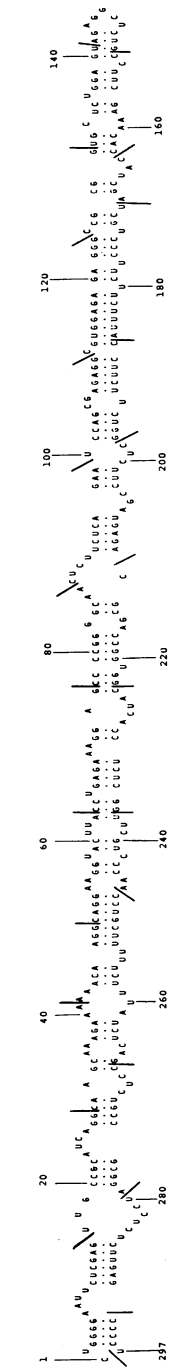


Fig 1. The structure of hop stunt viroid (HSV).

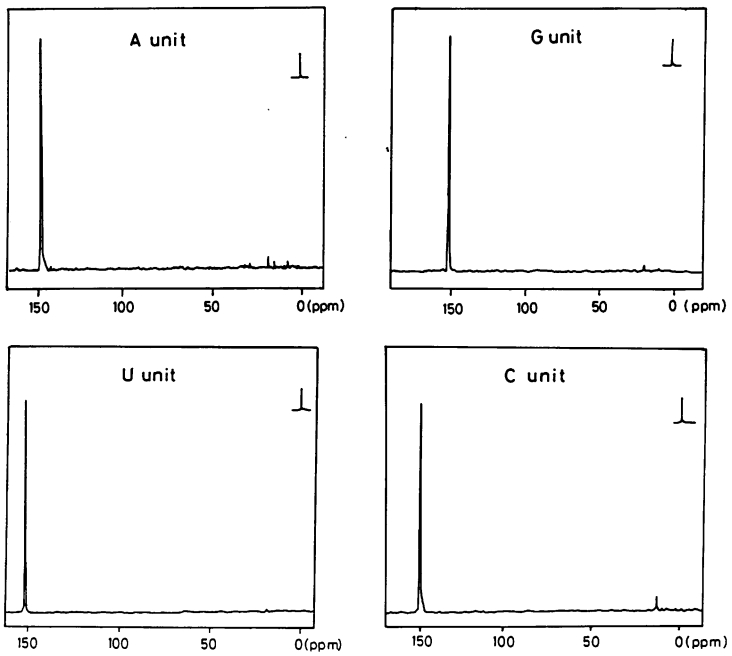
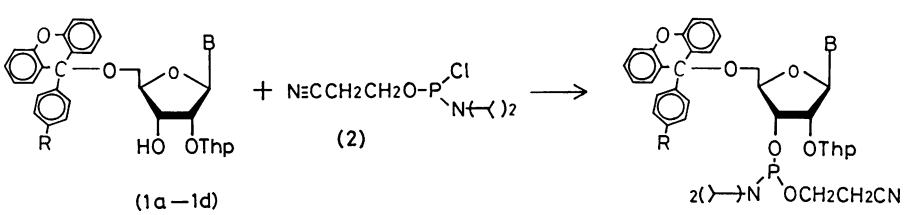


Fig 2. ^{31}P-NMR spectra of the ribonucleoside phosphoramidite units.

RESULTS AND DISCUSSION

Preparation of the protected ribonucleoside phosphoramidites (3a–3b).

Cyanoethoxydiisopropylaminochlorophosphine (**2**) was allowed to react with suitably protected ribonucleosides (**1a–1d**) according to a modification of the procedure of Köster. (14) As the 5′-hydroxyl protecting group, 9-phenylxanthen-9-yl (Pix) (15) was employed for adenosine and guanosine and the 9-(4-methoxy)-phenylxanthen-9-yl (Mox) group (16) was used for cytidine and uridine. The choice was based on the fact that trityl types of blocking groups attached to the 5′-hydroxyls of pyrimidine nucleosides were removed more slowly than those of purine derivatives by acid treatment. The purity of these nucleoside phosphoramidites was checked by means of ^{31}P-NMR after chromatography on a silica gel column. Guanosine and uridine phosphoramidites were satisfactorily obtained as



(a) B = N^6-benzoyladenine, R = H
(b) B = N^2-propionyl-O^6-diphenylcarbamoylguanine, R = H
(c) B = N^3-anisoyluracil, R = CH_3O
(d) B = N^4-anisoylcytosine, R = CH_3O

Table 1. Synthesis of the fully protected RNA fragments.

oligoribonucleotides	overall yield(%)	average yield(%)
(1) CUGGGGAAUUCUCGAG (16 mer)	63	97
(2) UUGCCGCAUCAGG (13 mer)	69	97
(3) CAAGCAAAGAAA (12 mer)	81	98
(4) AAACAAGG (8 mer)	86	97
(5) CAGGAAGGUACUUA (14 mer)	71	97
(6) CCUGAGAAAGGAG (13 mer)	66	96
(7) CCCCGGGGCAA (11 mer)	61	95
(8) CUCUUCUCAGAA (12 mer)	60	95
(9) UCCAGCGAGAGG (12 mer)	53	94
(10) CGUGGAGAGAGGG (13 mer)	76	98
(11) CCGCGG (6 mer)	76	93
(12) UGCUCUGGAG (10 mer) + pUmM	41	91
(13) AGAGGCUCUG (10 mer)	31	86
(14) CCUUCGAAA (9 mer)	65	94
(15) CACCAUCGA (9 mer)	36	86
(16) UCGUCCCUUCUUCUUUA (17 mer)	65	97
(17) CCUUCUUCUGG (11 mer)	71	96
(18) CUCUUCCGAUGAGA (14 mer)	38	92
(19) CGCGACCGGUGG (12 mer)	51	93
(20) CAUCACCUCUCGC (13 mer)	53	94
(21) UUCGUCCCAA (10 mer)	54	93
(22) CCUGCUUUUUGUCUAUCUGAG (21 mer)	72	98
(23) CCUCUGCCGCGGA (13 mer)	27	89
(24) UCCUCUCUUGAG (12 mer)	70	97
(25) CCCC (4 mer) + pUmM	71	84

chromatographically pure material (Fig. 2). However, adenosine and cytidine derivatives, especially, in the case of cytidine the hydrolyzed product (^{31}P NMR, 13 ppm) of **3** could not be removed completely by chromatography, these adenosine and cytidine

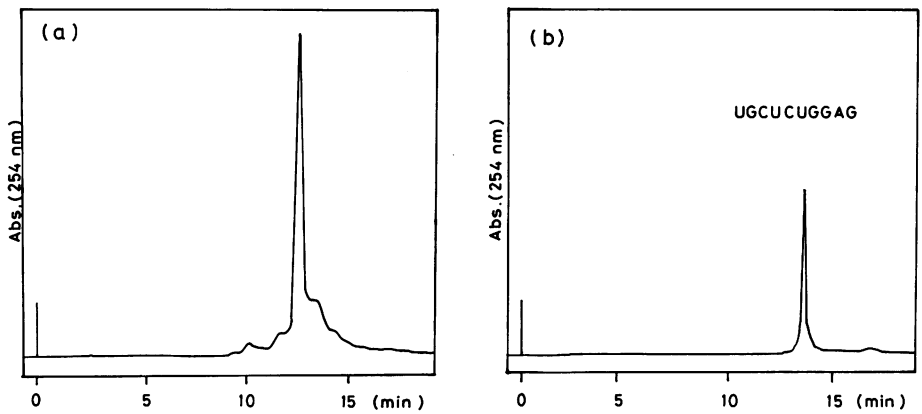


Fig 3. (a)Reversed phase HPLC profile of a decamer, UGCUCUGGAG, after prepurification by means of Seppak C_{18}. (b)Reversed phase HPLC profile of the purified decamer.

phosphoramidites were used sufficiently for the successive coupling reactions.

Synthesis of RNA fragments on controlled pore glass polymer supports.

The synthesis of RNA fragments was performed by the method reported previously. (1,3) In this experiment we have chosen so-called controlled pore glass (CPG)(Mean Pore Dia (A) 1000: Applied Biosystems Co. Ltd.) as a solid support and it was applied to manipulation of the solid phase procedure. Trifluoroacetic acid (TFA) (0.5%) in dichloromethane (5 sec×2) was used for removal of the Pix or Mox group. After washing, the support was dried for 5 min. Then, the coupling reaction was carried out for 10 min. The yields of the fully protected RNA fragments are listed in Table 1.

Deprotection from the fully protected oligoribonucleotides.

First, a fully protected RNA fragment on CPG was treated with ammonia (60°C. 6h and r.t. 12h). The crude mixture was washed with ether and passed through a Seppak C_{18} column. Then, acid treatment (pH 2.0, 24h) gave deprotected RNA fragments. These products were separated by reversed phase HPLC. Fig. 3(a) shows the elution profile of a decamer of UGCUCUGGAG after ammonia treatment. Fig. 3(b) shows the reversed phase HPLC profile of the purified decamer. The isolated amounts and yields of the RNA fragments are listed in Table 2. Fig. 4 shows gel electrophoresis in the presence of 7M urea after ^{32}P-labelling of the 5′-terminus of the RNA fragments.

Characterization of chemically synthesized RNA fragments.

The isolated products were completely hydrolyzed with nuclease P_1 to give the expected monomers in the correct ratios. The result of nuclease P_1 digestion for a decamer, UGCUCUGGAG, is shown in Fig. 5. The sequence analysis of the decamer and a tridecamer, CCUCUGCCGCGGA, was performed by the method of Donis-Keller. (17) These oligoribonucleotides were labelled by using [γ-^{32}P]ATP with polynucleotide kinase and analyzed by treatment with four types of ribonucleases, i.e., RNase T_1, RNAse U2, RNase Phy M and RNase B. Cer. The resulting autoradiograms are shown in Figs. 6a and 6b.

CONCLUSION

Several protecting groups for the 2′-hydroxyl function of ribonucleosides have been proposed. (5,6,7,18) Among them Thp group was found to be suitable and enough for

Table 2. Isolated amounts and yields of the deprotected RNA fragments.

oligoribonucleotides	isolated amount(OD)	isolated yield(%)
(1) CUGGGGAAUUCUCGAG (16 mer)	8.9	20
(2) UUGCCGCAUCAGG (13 mer)	8.4	22
(3) CAAGCAAAGAAA (12 mer)	16.9	37
(4) AAACAAGG (8 mer)	15.3	38
(5) CAGGAAGGUACUUA (14 mer)	12.8	24
(6) CCUGAGAAAGGAG (13 mer)	6.8	14
(7) CCCCGGGGCAA (11 mer)	8.0	27
(8) CUCUUCUCAGAA (12 mer)	10.4	29
(9) UCCAGCGAGAGG (12 mer)	15.1	44
(10) CGUGGAGAGAGGG (13 mer)	3.1	3
(11) CCGCGG (6 mer)	19.0	79
(12) UGCUCUGGAG (10 mer) + pUmM	30.0	31
(13) AGAGGCUCUG (10 mer)	7.7	44
(14) CCUUCGAAA (9 mer)	4.3	20
(15) CACCAUCGA (9 mer)	6.9	43
(16) UCGUCCCUUCUUCUUUA (17 mer)	13.9	24
(17) CCUUCUUCUGG (11 mer)	7.8	23
(18) CUCUUCCGAUGAGA (14 mer)	12.4	45
(19) CGCGACCGGUGG (12 mer)	10.6	37
(20) CAUCACCUCUCGC (13 mer)	3.6	14
(21) UUCGUCCCAA (10 mer)	10.9	37
(22) CCUGCUUUUUGUCUAUCUGAG (21 mer)	4.0	6
(23) CCUCUGCCGCGGA (13 mer)	15.0	26
(24) UCCUCUCUUGAG (12 mer)	10.0	36
(25) CCCC (4 mer) + pUmM	2.1	11

this purpose. Reese (19) and Gait (20), however, suggested that the 3′−5′ linked oligomers were contaminated by the 2′−5′ linked isomers when the 2′-Thp and 5′-trityl type protecting groups were used. We have recently demonstrated that the 3′−5′ linked oligomers could be obtained selectively without contamination of the 2′−5′ regioisomers on polymer support synthesis (3). On the other hand, it was shown that the side reactions of guanine and uracil

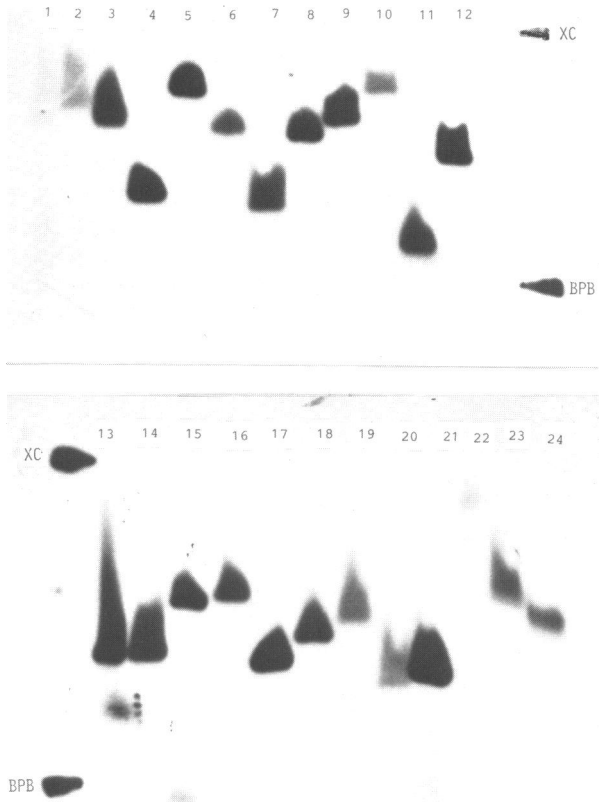


Fig 4. 20% Polyacrylamide gel electrophoresis of the deprotected RNA fragments.

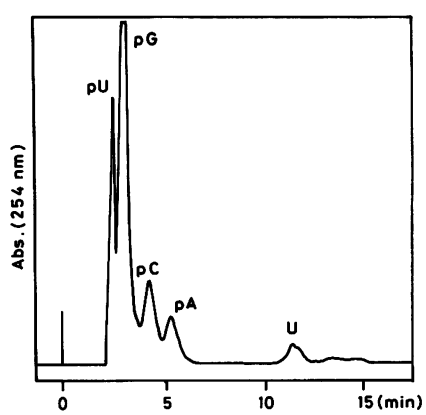


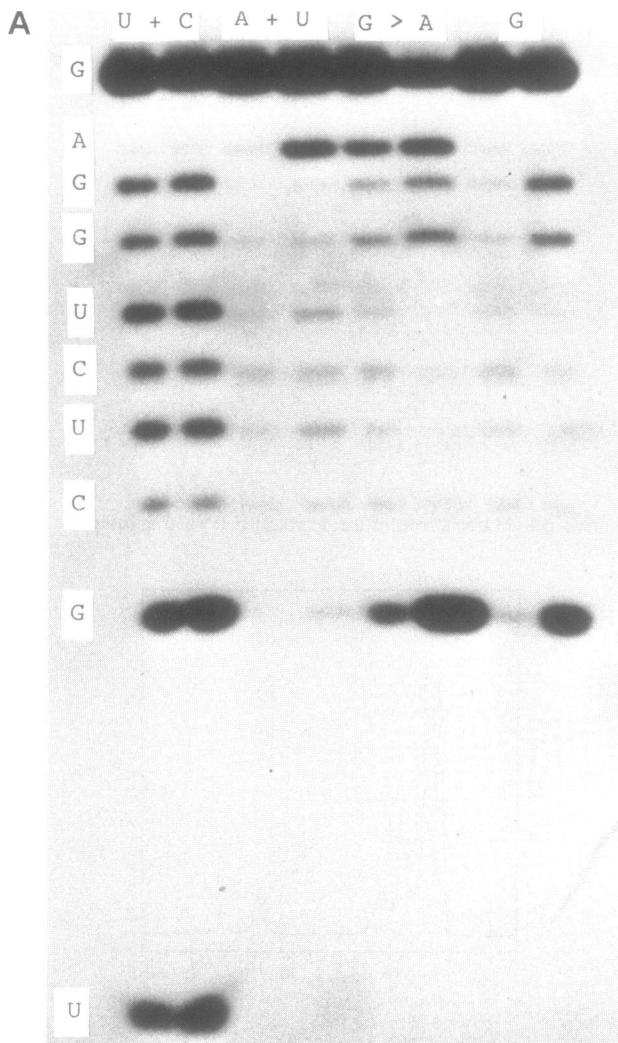
Fig 5. Nuclease P_1 digestion of a decamer, UGCUCUGGAG.

moieties as pointed out by Ogilvie (21) could be avoided by introduction of the suitable acyl protecting groups (22) to the lactam functions of these bases.

EXPERIMENTAL

General methods

^{1}H-NMR spectra were recorded at 100 MHz on a JNM PS-100 spectrometer, and the chemical shifts (Hz) are shown relative to an internal standard of 2% TMS. ^{31}P-NMR spectra were recorded on a JEOL PS-100FT (40.50 MHz), and the chemical shifts (Hz) are reported relative to an external capillary standard of 85% H_3PO_4. UV spectra were obtained on a Hitachi 220A spectrometer. Column chromatography was carried out by using silica gel C-200 purchased from Wako Co. Ltd. HPLC was performed on a μ



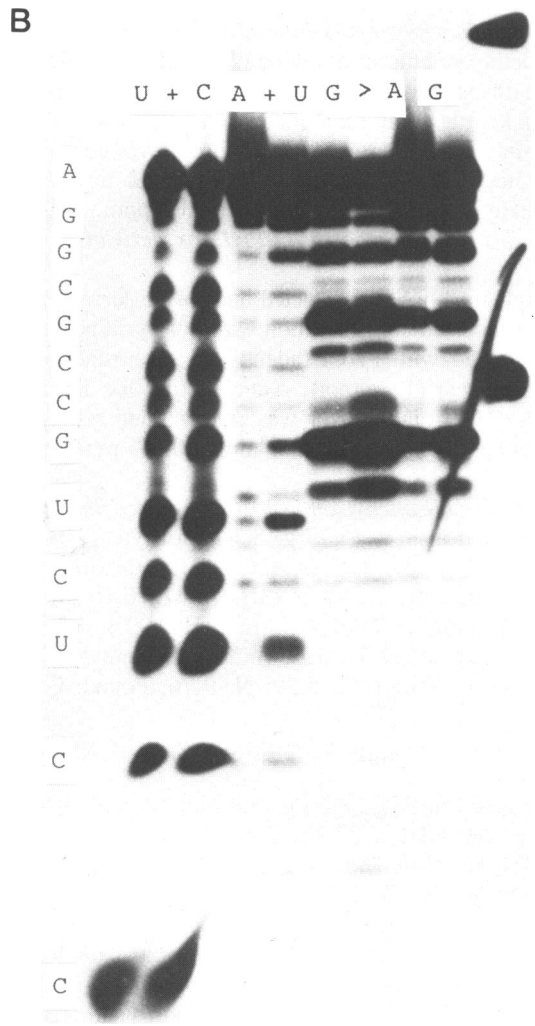


Fig 6A. Sequence analysis of a decamer, UGCUCUGGAG, by Donis-Keller's method.
Fig 6B. Sequence analysis of a tridecamer, CCUCUGCCGCGGA, by Donis-Keller's method.

Bondapak C_{18} column using 0.1M ammonium acetate (pH 7.0) at the flow rate of 1.5 ml/min. Polyacrylamide gel electrophoresis was performed using a normal vertical slab gel apparatus. Dichloromethane and acetonitrile were purified by repeated distillation first from phosphorus pentoxide and then from calcium hydride and stored over molecular sieves 4A. Elemental analysis has been done by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

Preparation of the protected ribonucleoside phosphoramidites.

Cyanoethoxydichlorophosphine.

This compound was prepared in 85% yield (b.p. 92−104°C/1.5 mmHg) (11) by a modification of the procedure of Fujii. (23)

Cyanoethoxydiisopropylaminochlorophosphine (2).
To a solution of cyanoethoxydichlorophosphine (22.9 ml, 0.2 mol) in dry ether (300 ml) was kept at 0°C, and diisopropylamine (56.1 ml, 0.4 mol) was added dropwise for 30 min. The mixture was kept at room temperature for 12 h. The precipitate was removed by using a glass filter in a closed system and the filtrate was concentrated in vacuo. The residue was distilled under reduced pressure to obtain **2** in 73% (33.1g) yield; b.p. 110−126°C/0.15 mmHg: ^{31}P-NMR ($CDCl_3$); δ 180.03 ppm. (14)
General method for the preparation of 5'-O-Pix/Mox derivatives of suitably protected nucleosides (1a−1d).
2'-O-Tetrahydropyranyl-suitably base-protected nucleoside derivative (10 mmol) (22) was rendered anhydrous by repeated coevaporation with dry pyridine. It was dissolved in dry pyridine (50 ml) and mixed with PixCl (adenosine, guanosine derivatives) or MoxCl (uridine, cytidine derivatives) (12 mmol). After stirring for 10 min, the mixture was extracted with CH_2Cl_2. The organic layer was concentrated and chromatographed on a silica gel column by using CH_2Cl_2-hexane containing 0.5% pyridine to afford **1a−1d** as white powder.
2'-O-Tetrahydropyranyl-5'-O-9-phenylxanthen-9-yl-N^6-benzoyladenosine (1a)
Compound **1a** was obtained in 81% (5.77 g, 8.1 mmol) yield. ^{1}H NMR (ppm); δ 9.25 (bs, 1H, NH), 8.78 (s, 1H, H-8), 8.23 (s, 1H, H-2), 8.08 (m, o-H of benzoyl), 7.56 (m, 3H, m and p-H of benzoyl), 7.24 (m, 13H, Ar-H), 6.16 (d, 1H, H-1'), 5.00 (m, 1H, H-3'), 4.70 (m, 1H, acetal of Thp), 4.55 (m, 1H, H-2'), 4.24 (m, 1H, H-4'), 3.46 (m, 4H, O-methylene of Thp and H-5'), 1.60 (m, 6H, C-methylene of Thp). Anal. Calcd for $C_{41}H_{37}N_5O_7 \cdot 1/3H_2O$: C, 68.60; H, 5.29; N, 9.76. Found: C, 68.62; H, 5.53; N, 9.61%.
2'-O-Tetrahydropyranyl-5'-O-9-phenylxanthen-9-yl-N^2-propionyl-O^6-diphenylcarbamoylguanosine (1b)
Compound **1b** was obtained in 98% (8.60 g, 9.8 mmol) yield. ^{1}H-NMR (ppm); δ 8.19 (s, 1H, H-8), 7.99 (bs, 1H, NH), 7.27 (m. 23H, Ar-H), 6.14 (d, 1H, H-1'), 4.90 (m, 1H, H-3'), 4.70 (m, 1H, acetal of Thp), 4.62 (m, 1H, H-2'), 4.28 (1H, H-4'), 3.35 (m, 4H. O-methylene of Thp and H-5'), 2.75 (q.2H, methylene of propionyl), 1.55 (m. 6H, C-methylene of Thp), 1.20 (t, 3H, methyl of propionyl). Anal. Calcd for $C_{50}H_{46}N_6O_9 \cdot 1/4H_20$: C, 68.29; H, 5.33; N, 9.56. Found: C, 68.46; H, 5.64; N, 8.95%.
2'-O-Tetrahydropyranyl-5'-O-9-(4-methoxy)phenylxanthen-9-yl-N^3-anisoyluridine (1c)
Compound **1c** was obtained in 80% (5.40 g, 7.21 mmol) yield. ^{1}H-NMR (ppm); δ 7.95 (d, 1H, H-6), 7.24 (m, 12H, Ar-H), 7.02 (m, 2H, 3', 5'-H of Mox), 6.94 (m, 2H, 3, 5-H of anisoyl), 6.84 (d, 1H, H-5), 6.19 (d, 1H, H-1'), 4.85 (m, 1H, H-3'), 4.70 (m, 1H, acetal of Thp), 4.50 (m, 1H, H-2'), 4.35 (m, 1H, H-4'), 3.87 (s, 3H, OCH_3 of Mox), 3.77 (s, 3H, OCH_3 of anisoyl), 3.48 (m, 4H, O-methylene of Thp and H-5'), 1.63 (m, 6H, C-methylene of Thp). Anal. Calcd for $C_{42}H_{40}N_2O_{11} \cdot 1/5C_6H_{14}$: C, 67.74; H, 5.63; N, 3.66. Found: C, 67.80; H, 5.40; N, 3.46%.
2'-O-Tetrahydropyranyl-5'-O-9-(4-methoxy)phenylxanthen-9-yl-N^4-anisoylcytidine (1d)
Compound **1d** was obtained in 84% (3.13 g, 4.19 mmol) yield. ^{1}H-NMR (ppm); δ 8.80 (bs, 1H, NH), 7.82 (d, 1H, H-6), 7.20 (m, 12H, Ar-H), 7.00 (m, 2H, 3', 5'-H of Mox), 6.90 (m, 2H, 3, 5-H of anisoyl), 6.82 (d, 1H, H-5), 6.22 (d, 1H, H-1'), 5.04 (m, 1H, H-3'), 4.90 (m, 1H, acetal H of Thp), 4.40 (m, 1H, H-2'), 4.16 (m, 1H, H-4'), 3.92 (s, 3H, OCH_3 of Mox), 3.87 (s, 3H, $0CH_3$ of anisoyl), 3.50 (m, 4H, O-methylene of

Thp and H-5′), 1.70 (m, 6H, C-methylene of Thp). Anal. Calcd. for $C_{42}H_{41}N_3O_9$: C, 67.46; H, 5.53; N, 5.62. Found: C, 67.42; H, 5.61; N, 5.45%.

General method for the preparation of nucleoside phosphoramidite units (3a–d).

The protected nucleoside derivative (**1a–1d**) (3 mmol) was rendered anhydrous by repeated coevaporation with pyridine, toluene, and CH_2Cl_2 (10 ml). After addition of diisopropylethylamine (2.6 ml, 15 mmol), the mixture was kept at 0°C, then the phosphitylating reagent **2** (0.6 ml, 3.3 mmol) was added. It was stirred at room temperature for 1 h. The reaction mixture was extracted three times with CH_2Cl_2/sat. aq. $NaHCO_3$ (1:1, v/v). The CH_2Cl_2 extracts were combined and dried over Na_2SO_4 for 15 min. The solvent was removed under reduced pressure and coevaporated with toluene for removal of diisopropylethylamine. After chromatographical purification on a column of silica gel, compound (**3a–3d**) was obtained as white powder.

Adenosine phosphoramidite unit (3a).

Compound **3a** was obtained in 92% (2.51 g) yield. ^{31}P-NMR (ppm); δ 150.01, 150.55, 150.69, 150.84; ^{1}H NMR (ppm); δ 8.69 (s, 1H, H-8), 8.21 (s, 1H, H-2), 8.00 (m, O-H of benzoyl), 7.54 (m, 3H, m and p-H of benzoyl), 7.20 (m, 13H, Ar-H), 6.20 (d, 1H, H-1′), 5.16 (m, 1H, H-3′), 4.72 (m, 1H, acetal of Thp), 4.52 (m, 1H, H-2′), 4.24 (m, 1H, H-4′), 3.58 (m, 4H, O-methylene of Thp and H-5′), 3.22 (m, 2H, methine of isopropyl), 2.48 (m, 4H, methylene of cyanoethyl), 1.58 (m, 6H, C-methylene of Thp), 1.15 (m, 12H, methyl of isopropyl). Anal. Calcd for $C_{50}H_{54}N_7O_8 \cdot 1/8\ C_6H_{14}$: C,66.06; H,6.09; N,10.63. Found: C,65.55; H,6.52; N,10.72%.

Guanosine phosphoramidite unit (3b).

Compound **3b** was obtained in 90% (2.90 g) yield. ^{31}P-NMR (ppm); δ 149.92, 150.55, 151.88; ^{1}H NMR (ppm); δ 8.13 (s, 1H, H-8), 7.89 (bs, 1H, NH), 7.24 (m, 23H, Ar-H), 6.09 (d, 1H, H-1′), 4.92 (m, 1H, H-3′), 4.74 (m, 1H, acetal of Thp), 4.58 (m, 1H, H-2′), 4.23 (m, 1H, H-4′), 3.50 (m, 4H, O-methylene of Thp and H-5′), 3.22 (m, 2H, methine of isopropyl), 2.70 (m, 4H, C-methylene of Thp), 1.18 (m, 12H, methyl of isopropyl). Anal. Calcd for $C_{59}H_{63}N_8O_{10}P \cdot 1/3C_6H_{14}$: C,66.37; H,6.18; N,10.15.
Found: C,66.71; H,6.35; N,9.94%.

Uridine phosphoramidite unit (3c).

Compound **3c** was obtained in 91% (2.99 g) yield. ^{31}P-NMR (ppm); δ 150.21, 150.64; ^{1}H NMR (ppm); δ 7.90 (d, 1H, H-6), 7.22 (m, 12H, Ar-H), 6.98 (m, 2H, Mox), 6.88 (m, 2H, anisoyl), 6.78 (d, 1H, H-5), 6.18 (d, 1H, H-1′), 4.88 (m, 1H, H-3′), 4.70 (m, 1H, acetal of Thp), 4.52 (m, 1H, H-2′), 4.33 (m, 1H, H-4′), 3.82 (s, 3H, OCH_3 of Mox), 3.79 (s, 3H, OCH_3 of anisoyl), 3.58 (m, 4H, O-methylene of Thp and H-5′), 2.60 (m, 4H, methylene of cyanoethyl), 1.62 (m, 6H, C-methylene of Thp), 1.16 (m, 12H, methyl of isopropyl). Anal. Calcd for $C_{51}H_{57}N_4O_{12}P \cdot 1/3\ C_6H_{14}$: C, 65.11; H,6.36; N,5.73. Found: C,65.40; H,6.45; N,5.84%.

Cytidine phosphoramidite unit (3d).

Compound **3d** was obtaind in 90% (2.56 g) yield. ^{31}P-NMR (ppm); δ 149.82, 150.55, 150.74; ^{1}H NMR (ppm); δ 8.32 (bs, 1H, NH), 7.83 (d, 1H, H-6), 7.20 (m, 12H, Ar-H), 6.98 (m, 2H, ArH of Mox), 6.91 (m, 2H, ArH of anisoyl), 6.84 (d, 1H, H-5), 6.23 (d, 1H, H-1′), 5.06 (m, 1H, H-3′), 4.46 (m, 1H, H-2′), 4.20 (m, 1H, H-4′), 3.89 (s, 3H, OCH_3 of Mox), 3.82 (s, 3H, OCH_3 of anisoyl), 3.56 (m, 4H, O-methylene of Thp and H-5′), 3.22 (m, 2H, methine of isopropyl), 2.70 (m, 4H, methylene of cyanoethyl), 1.60 (m, 6H, C-methylene of Thp), 1.19 (m, 12H, methyl of isopropyl). Anal. Calcd for $C_{51}H_{58}N_5O_{11}P \cdot 1/6C_6H_{14}$: C,64.90; H,6.32; N,7.28. Found: C,64.53; H,6.76; N,7.38%.

General procedure for the synthesis of RNA fragments on polymer supports
A CPG gel (Mean Pore Dia l000 A) which was loaded with a 3′-terminal nucleoside unit (capacity of 50 mg, 1.65 μmol) was packed in a column of a manual synthesizer. First, the CPG gel was treated twice with TFA (0.5%) in CH_2Cl_2 for 5 sec, and it was immediately washed with CH_2Cl_2 for l min. These solutions were combined and estimated the coupling yield. The CPG gel was successively dried in vacuo for 5 min. Then an appropriately protected nucleoside phosphoramidite (50 μmol, 30 equiv.) and tetrazole (500 μmol), predried in vacuo, was added into the column, and dissolved in acetonitrile (300 μl). The manual synthesizer was placed in a desiccator for coupling reaction. After l0 min, the gel was washed with acetonitrile and oxidized with 0.1 M I_2 in THF−lutidine−H_2O (7:2:1, v/v/v) for l min. This support was washed with acetonitrile followed by addition with THF−acetic anhydride (9:1, v/v) in the presence of a catalytic amount of DMAP for l0 sec. Then the CPG gel was washed with acetonitrile and CH_2Cl_2, and dried over P_4O_{10} in vacuo for 5 min. Repetition of the above chain elongation cycle gave the desired RNA fragment on the solid supports. The results of the average and overall yields of the coupling reactions are listed in Table 1.
Deprotection of the fully protected RNA fragments from the solid supports
To the protected RNA fragment of CPG (50 mg) was added pyridine (2 ml) and concentrated ammonia (20 ml). The suspension was sealed and kept at 60°C for 6 h and then at room temperature for l2 h. The mixture was filtered, the filtrate was evaporated under reduced pressure. During the evaporation, pyridine was added to avoid partial loss of the Pix or Mox and Thp groups. The residue was dissolved in sterilized water (50 ml). The aqueous solution was washed with ether (50 ml ×3) and concentrated under reduced pressure. The residue was passed slowly through a mini column of Seppak C_{18} and the column was first washed with sterilized water (l00 ml) and eluted with 30% aqueous MeOH. The eluate was evaporated under reduced pressure. The residue containing the RNA fragment protected with the Pix or Mox and Thp groups were further dissolved in a 0.0l M solution of HCl in sterilized water (l0 ml) and the solution was adjusted to pH 2.0 and readjusted after 30 min. Then the mixture was kept at room temperature for 24 h. The solution was neutralized by addition of diluted ammonia and it was concentrated under reduced pressure. The residue was passed slowly through a mini column of Seppak C_{18} and the mini column was washed with sterilized water (l00 ml) and eluted with 30% aqueous MeOH. The eluate was evaporated under reduced pressure. Final purification was performed by HPLC (μ Bondapak C_{18} column). The isolated amounts and yields of the RNA fragments are listed in Table 2.
5′-Labelling of RNA fragments.
To a solution of a RNA sample (0.00l OD/μl, 8 μl) in a kination solution [5× kination buffer (24), l0μl; H_2O, 20 μl; [γ-^{32}P]ATP, 3 μl (l0 pci/μl); polynucleotide kinase, 2 μl (l0 U/μl)] was incubated at 37°C for l h. The mixture was heated at 90°C for 30 sec and added to 0.1 M dye solution of formamide (l0 μl). Then the mixture was subjected to 20% polyacrylamide gel.(17) Electrophoresis was performed at room temperature by using the usual apparatus. Autoradiography was performed for the proof of the chain length (Fig. 4)
RNA sequencing analysis
The gel-sequencing was performed by Donis-Keller's method. (17) The above 5′-labelled RNA fragments were treated with RNase T_1 (G specific), RNase U_2 (G and A specific), RNase Phy M (U and A specific) and RNase B. Cereus (U and C specific), under partial

digestion conditions, and the products were analyzed by 20% polyacrylamide gel electrophoresis. Autoradiograms are shown in Figs. 6A and 6B.

ACKNOWLEDGEMENTS

We heartily thank Mrs. Chieko Sugimura for typewriting of the manuscript. This work was supported by Takeda Chemical Industries Ltd. and a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

*To whom correspondence should be addressed

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24. 5× Kination buffer; 0.25 M Tris-HCl (pH 7.6), 0.05 M $MgCl_2$. 25mM 1,4-dithiothreitol, 0.5 mM spermidine, 0.5 mM EDTA.

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