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Solid phase synthesis of oligoribonucleotides using the o-nitrobenzyl group for 2'-hydroxyl protection and H-phosphonate chemistry

Toshiki Tanaka, Shigeru Tamatsukuri and Morio Ikehara

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan

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ABSTRACT

Oligoribonucleotides with chain length of 7, 11, 15, 17, 24 and 34 were synthesized on long chain alkylamine controlled pore glass beads (LCA-CPG) using o-nitrobenzyl protection of 2'-hydroxyls via a H-phosphonate approach either manually or by using an automatic synthesizer. The oligoribonucleotides were obtained in yields of 0.6 - 20 %, based on initial nucleoside bound to the LCA-CPG support.

INTRODUCTION

For the synthesis of oligonucleotides three types of synthetic approaches, i.e. phosphotriester[1,2], phosphite-triester[3-6] and H-phosphonate[7,8] approaches, are now available. These chemistries have been employed primarily for the synthesis of oligodeoxyribonucleotides. Even relatively long chain deoxyribooligonucleotides can be synthesized rapidly by the phosphite-triester[9] or H-phosphonate[10] approaches, and rapid synthesis by a modified phosphotriester approach has also been investigated[11-14]. On the contrary, few examples of rapid synthesis of oligoribonucleotide have been reported[15-17]. Recently we described the synthesis of hepta and undecaribonucleotides by the phosphite-triester method on a polymer support[18]. In this case, p-nitrophenyltetrazole was used as the activation reagent instead of the widely used tetrazole since the former reagent effected very rapid condensation reaction. However one drawback in the use of p-nitrophenyltetrazole is its low solubility in organic solvents such as CH_3CN.

We report here a study of the utility of the H-phosphonate method for synthesis of oligoribonucleotides. Advantages of the H-phosphonate method over the phosphite-triester method are: 1)

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the nucleoside-H-phosphonate is much more stable than the nucleoside phosphoamidite, 2) protection of the phosphorus is not required, 3) capping and oxidation reactions can be omitted during the course of the synthesis. That means the short time required to purse one elongation cycle (a single oxidation reaction is performed at the end of the synthetic sequence), and 4) all reagents are very soluble in the organic solvents used in the oligonucleotide synthesis.

In order to investigate the H-phosphonate method for ribo-oligonucleotide synthesis, ribooligonucleotides with chain length of 7, 11, 15, 17, 24 and 34 were synthesized manually or by using an antomatic synthesizer.

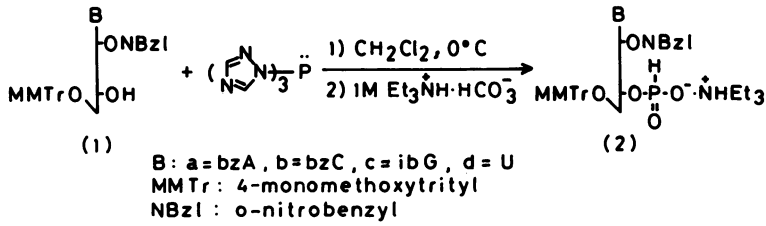
RESULTS AND DISCUSSION

Synthesis of nucleoside H-phosphonate derivatives

The synthesis of nucleoside H-phosphonates was accomplished according to Froehler et al[10]. 5'-O-Monomethoxytrityl-2'-O-o-nitrobenzoyl-N-acylated nucleosides (1a-d) were converted to their H-phosphonate derivatives (2a-d) by means of tris(1,2,4-triazol)phosphite in CH_2Cl_2 followed by the hydrolysis (Chart 1). The yields and ^{31}P NMR analysis are listed in Table 1. By analysis using ^{31}P NMR, all the H-phosphonate derivatives (2a-d) are more than 95 % pure.

Manual synthesis of oligoribonucleotides

For comparison of the H-phosphonate method and phosphite-triester methods, we first synthesized the hepta (UACUAAC) and undecaribonucleotides (GUAUGUUAAUA) with the same sequence which we had prepared by the phosphite-triester method[18]. The synthesis was performed according to the elongation cycle shown in Table 2 in a small sintered glass filter as a reaction vessel. For the coupling step, 40 equiv. of nucleoside H-phosphonate (2a-d) and 200 equiv. of pivaloyl chloride as an activating reagent were allowed to react for 3 min with the 5'-hydroxyl group of an N-acylated-2' or 3'-O-acetyl nucleoside bound to LCA-CPG support *via* a succinyl linkage[18]. De-monomethoxytritylation was carried out by treatment with 5% trichloroacetic acid (TCA) in CH_2Cl_2 for 3 min. The time required for one elongation cycle was only 8 min. After the reaction was complete, the



B: a = bzA, b = bzC, c = ibG, d = U
MMTr: 4-monomethoxytrityl
NBzl: o-nitrobenzyl

Chart 1

resins were subjected to the oxidation and deblocking procedure described in Chart 2. Analysis of the reversed phase HPLC after partial or complete deblocking of the protecting groups showed that each oligonucleotide appeared as a main peak at the same retention time as that prepared by the phosphite-triester method (Fig. 1). Not much difference was seen in the yield or the purity of a given oligoribonucleotides prepared by the H-phosphonate method and phosphite-triester method.

Automatic synthesis of oligoribonucleotides

We next examined the use of an automatic synthesizer to prepare oligoribonucletides by the H-phosphate approach. The machine contains 8 bottles (four for the H-phosphonates; one each for pivaloyl chloride in pyridine, 5 % trichlorocetic acid in CH_2Cl_2, CH_2Cl_2 and CH_3CN) and one reaction column. Each reagent or solvent is moved by a slight high pressure of Ar gas. In order to find suitable conditions, r-A_{15} was first synthesized with changes in the amounts of A-phosphonate (2a) used for the condensation; 15, 30 or 40 equiv of A-phosphonate (2a) relative to the nucleoside bound to support was used, with pivaloyl chloride equal to 5 equiv of A-phosphonate. The reaction was

Table 1 Yields and ^{31}P-NMR Spectra Analysis of Compound (2,a-d)

compd.	yields (%)	^{31}P-NMR Chemical Shift (ppm)
2a	85.9	1.09
2b	85.5	1.64
2c	60.0	1.45
2d	92.0	1.34

The chemical shifts are reported relative to trimethylphosphate in CH_3CN:pyridine:Pyridine-d_6 (2:1:1) as an external standard.

Table 2 Steps involved in one elongation cycle

No.	Step	Solvent or Reagent	Time
1	Wash	CH_3CN × 2,	-
2	Wash	CH_2Cl_2 × 2	-
3	Detritylation	5% Trichloroacetic acid in CH_2Cl_2	3min
4	Wash	CH_2Cl_2	-
5	Wash	Pyridine	-
6	Wash	CH_2Cl_2 × 3	-
7	Coevaporation	Pyridine	1min
8	Coupling	Phosphonate (2a-d) 40equiv	3min
		Pivaloyl chloride 200equiv	
		CH_3CN-Pyridine (1:1) 200µl/1µmol-resin	

continued for 3 min. After oxidation by I_2-H_2O and treatment with NH_4OH described in Chart 2, partially protected pentadecamer was analyzed by reversed phase HPLC. The profils showed that the pentadecamers were synthesized with the same efficiency in each cases. Next, we synthesized oligoribonucleotides, with chain lengths of 17, 24 and 34, which were located at the 5'-exon-intron junction of Tetrahymena thermophila ribosomal RNA[19] (Fig. 2). The syntheses were performed by the same procedure employed

Fully protected oligomer bound to C.P.G.

↓ Oxidation

0.1M I_2 in THF/Py/H_2O=44/3/3, r.t. 15-60min

↓ conc.NH_4OH treatment

27% Ammonia water, 55-60°C 6-12hrs

↓ Purification by RP-HPLC

↓ Acetic acid treatment

80% aq.CH_3COOH, 30°C 2-3hrs

↓ UV irradiation

0.1M $HCOONH_4$ (pH3.5), 60min

↓ Gel filtration

Sephadex G-25

↓ Purification by RP-HPLC

Ribo-oligonucleotide

Chart 2

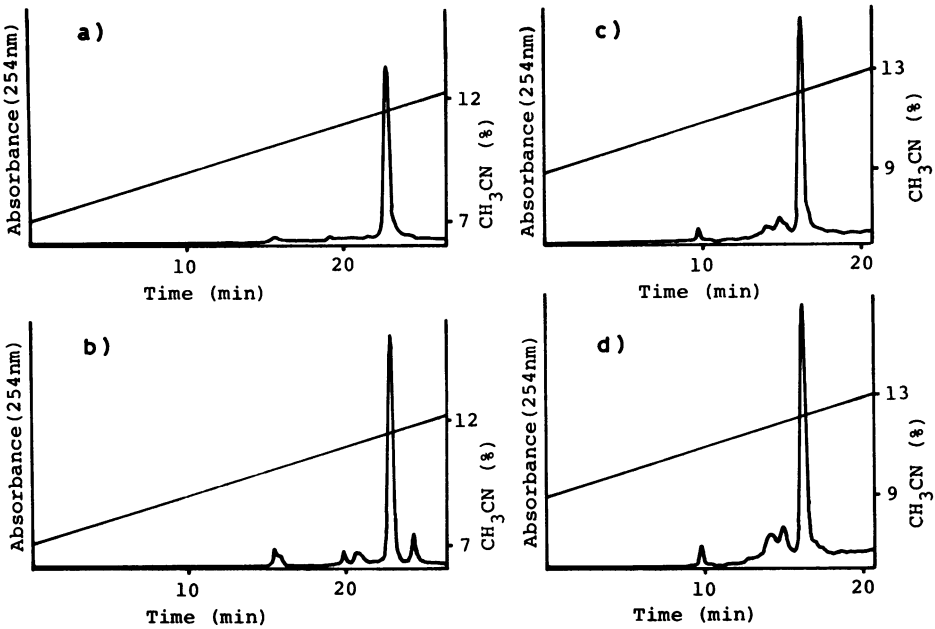


Fig 1. The analysis of the hepta and undecaribonucleotides by the reversed phase HPLC. a) r-7mer synthesized via a phosphite method, b) r-7mer synthesized via a H-phosphonate method, c) r-11mer synthesized via a phosphite method, d) r-11mer synthesized via a H-phosphonate method.

for r-A_{15} except that 20 equiv. of nucleoside H-phosphonate (2a-d) was used. After the synthesis, the resin was oxidized (30 min for 17 and 24 mer, 60 min for 34 mer) and subjected to the deblocking procedure as described in Chart 2. After the

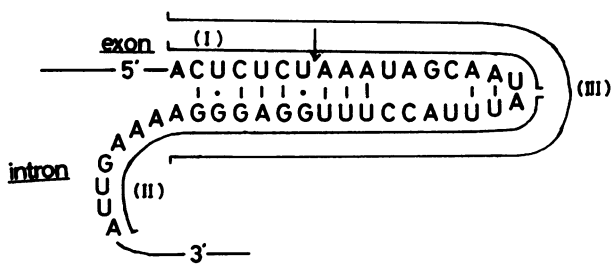


Fig 2. The nucleotide sequence at the exon-intro junction of Tetrahymena ribosomal RNA. The synthesized oligoribonucleotides were designated as (I),(II) and (III). The arrow indicates the splice site.

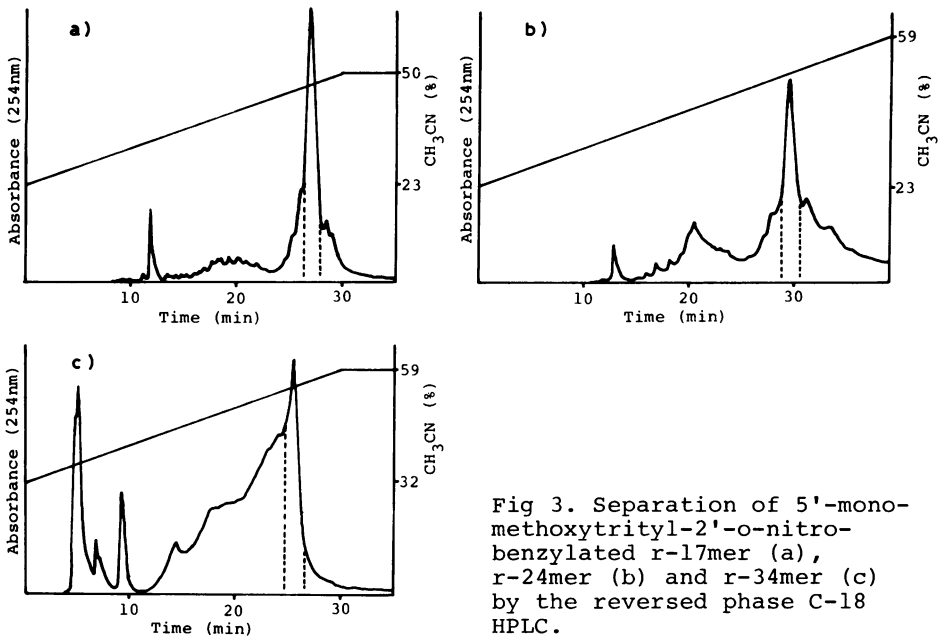


Fig 3. Separation of 5'-monomethoxytrityl-2'-o-nitrobenzylated r-17mer (a), r-24mer (b) and r-34mer (c) by the reversed phase C-18 HPLC.

treatment with NH_4OH, the partially protected 17, 24 and 34-mers were analyzed by reversed phase HPLC, as shown in Fig. 3. The fractions between the dotted lines were pooled and treated with 80 % AcOH to remove the monomethoxytrityl group. Finally, o-nitrobenzyl groups were removed by UV irradiation in pH 3.5 buffer and the mixtures were evaporated and separated on a

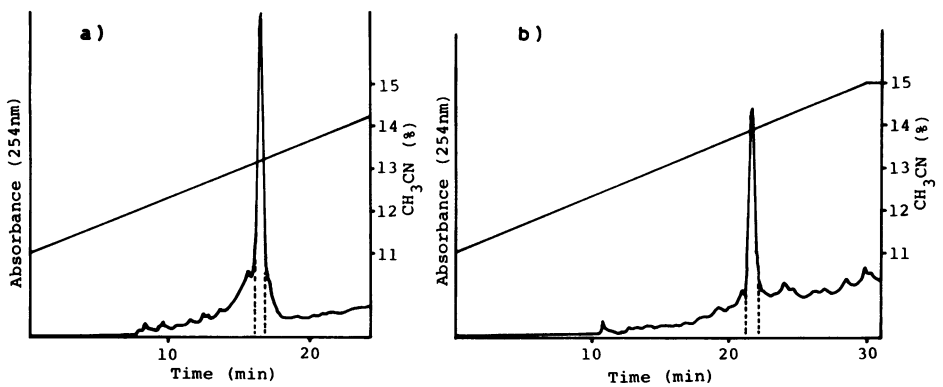


Fig 4. Purification of r-17mer (a) and r-24mer (b) by the reversed phase C-18 HPLC.

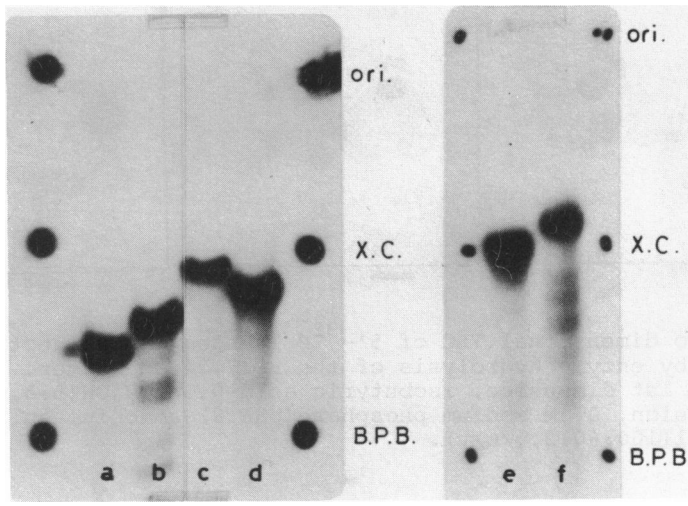


Fig 5. Analysis by the 20% polyacrylamide gel electrophoresis. lane a, d-17mer; lane b, synthesized r-17mer; lane c, synthesized r-24mer; lane d, d-24mer; lane e, d-34mer; lane f, synthesized r-34mer.

Sephadex G-25 column to remove the non-nucleotidic materials. The fractions eluted at the void volume were analyzed by reversed phase HPLC, shown in Fig. 4. The main peaks corresponded to the desired 17 and 24-mer. These oligoribonucleotides were analyzed by 20 % polyacrylamide gel electrophoresis (PAGE) containing 7M urea after labeling at 5'end by [γ-^{32}P]ATP and T_4-polynucleotide kinase (Fig. 5). In the case of the 34-mer, a clean peak was not seen by reversed phase HPLC. This difficulty probably was due to the self-complementary sequence of the 34-mer. However, the 34-mer showed up as a clear band on the 20 % PAGE (7M urea). It was isolated by 20 % PAGE; then analyzed after labeling by [γ-^{32}P] at 5'end (Fig. 5).

The 5'-terminal nucleosides of the purified 17, 24 and 34 mers were determined as followed. The oligonucleotides were treated with [γ-^{32}P]ATP and polynucleotide kinase to give 5'-labeled oligonucleotides, which were hydrolyzed by nuclease P1. The labeled nucleotide was then analyzed by paper electrophoresis at pH 3.5 and found to be A in every cases.

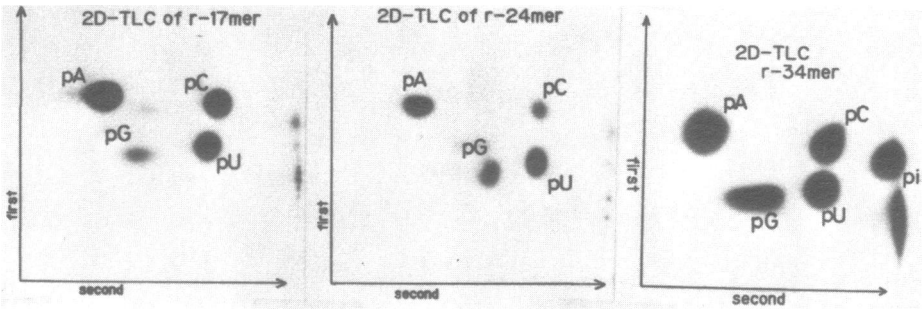


Fig 6. Two dimensional TLC of 5'-^{32}P labeled mononucleotides obtained by enzyme hydrolysis of the r-17,24 and 34mer. Solvents: 1st dimension, isobutyric acid-0.5M NH_4OH(5:3,v/v); 2nd dimension, 0.1M sodium phosphate(pH6.8)-ammonium sulfate-2-propanol(100:60:2,v/w/v).

The purified 17, 24 and 34 mers were treated with RNase T_2; then the 5'ends were labeled by [γ-^{32}P]ATP and T_4-polynucleotide kinase to give ^{32}pNp's, which was treated with nuclease P1 to remove the 3'-phosphate. The resultant ^{32}pN's were analyzed by two dimensional thin layer chromatography on cellulose (Fig. 6). This test showed complete digestion of the 17, 24 and 34 mer by RNase T_2.

The 24-mer was hydrolyzed by RNase T_1. Then the mixture was labeled at the 5'-hydroxyl group by [γ-^{32}P]ATP and polynucleotide kinase and the labeled products were analyzed by homochromatography. As expected, five spots corresponding to the products (pGp, pUUA, pAGp, pAAAAGp and pAUUUACCUUUUGp) resulting from cleavage 3' to guanosine were seen, through the spot 1 contained two products (Fig. 7). The 5'-nucleotides obtained from these spots after nuclease P_1 digestion were analyzed by paper electrophoresis at pH 3.5. They were found to be pG, pU, pA, pA and pA, respectively, as expected. This test was also tried with the 34-mer. However, we couldn't see all the spots expected from cleavage by RNase T_1. This result is probably due to the self complementary sequence of the 34-mer. However, the results of the analysis by polyacrylamide gel electrophoresis, observation of the correct nucleoside at 5'-end, and the ratio of products from degradation by RNase T_2 show that the 34-mer was obtained.

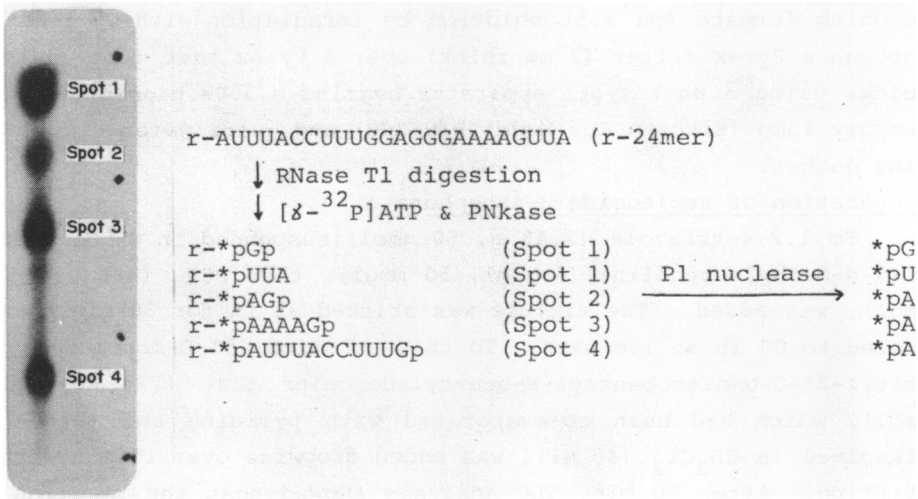


Fig 7. Analysis of the 24mer digested by RNase T1 on the homochlomatography.

Conclusion

By using the o-nitrobenzyl group for protection of 2'-hydroxyl groups, we achieved rapid synthesis of oligoribonucleotides with chain length up to 34 by the H-phosphonate method, both manually and with an automatic synthesizer. Starting with the synthesis, it took only three days to obtain these oligonucleotides. Accordingly, this method opens the way for biological studies in the field of RNA as well as DNA.

MATERIALS AND METHODS

High pressure liquid chromatography (HPLC) was performed on an Altex 322MP chromatography system. For the reversed-phase HPLC, Nucneosil C-18 (5 μ) was packed under 500 Kg/cm^2 in a stainless steel column (φ 0.6 × 20 cm). The elution was performed with a linear gradient of CH_3CN in 0.1M triethylammonium acetate (pH7) at a flow rate of 0.7 ml per min.

^{31}P NMR spectra were recorded with a JEOL GX500 Spectrometer operating at 202.42 MHz using trimethyl phosphate as an external standard.

Removal of the o-nitrobenzyl groups was performed in

ammonium formate (pH 3.5) solution by irradiation with UV light through a Pyrex filter (2 mm thick) over a Pyrex test tube (1 mm thick) using a photolysis apparatus bearing a 300W high-pressure mercury lamp (Eikosha Co. Model PIH 300) and quarz water-circulating jachet.

Preparation of nucleoside H-phosphonate

To 1,2,4-triazole (3.45 g, 50 mmol) suspended in CH_2Cl_2 (50 ml), N-methylmorpholine (5.5 ml, 50 mmol), then PCl_3 (436 µl, 5 mmol), was added. The mixture was stirred at rt for 30 min then cooled to 0° in an ice-bath. To this solution, 5'-O-monomethoxytrityl-2'-O-o-nitrobenzoyl-N-benzoyladenosine (1a) (779 mg, 1 mmol), which had been co-evaporated with pyridine and then dissolved in CH_2Cl_2 (40 ml), was added dropwise over 15 min with stirring. After 10 min, TLC analysis showed that the reaction was complete (TLC, CH_2Cl_2:MeOH, 20:1, Rf 0.83 → 0.18). A mixture of 1M TEAB (100 ml) and triethylamine (2 ml) was added to the mixture. The product was extracted with CH_2Cl_2 (50 ml), washed with 1M TEAB (50 mlx2) and dried over $MgSO_4$ for 10 min. $MgSO_4$ was filtered off and washed with CH_2Cl_2 (30 ml). The combined CH_2Cl_2 layer was evaporated to an oil which was separated on a silica gel column (ϕ 3x3 cm). The elution was performed by increasing the EtOH concentration (0 → 10 %) in CH_2Cl_2 containing NEt_3 (2 %). The fractions containing (2a) were pooled, washed twice with a mixture of 1M TEAB (100 ml) and NEt_3 (2 ml), and dried over $MgSO_4$. $MgSO_4$ was filtered off and washed with CH_2Cl_2 (30 ml). The combined CH_2Cl_2 layer was evaporated to afford a solid; yield, 810 mg (0.86 mmol, 86 %).

Other nucleoside H-phosphonates (2b-d) were prepared by the same procedure.

Synthesis of oligoribonucleotide

For the manual synthesis, a small sintered glass filter[18] was used as a reaction vessel. The condensation reaction was performed as follows. To a nucleoside resin (0.2 µmol) (6.9 mg for C-resin, 6.4 mg for A-resin), which had been co-evaporated with pyridine, the nucleoside H-phosphonate derivatives (2a-d) (7.4 mg, 20 µmol) and then pivaloyl chloride (34.4 µl, 100µmol) in CH_3CN:pyridine (1:1) (1.2 ml) was added. The reaction was continued for 3 min. After the resin was washed with CH_3CN and

CH_2Cl_2, it was treated with 5 % trichloroacetic acid in CH_2Cl_2 for 3 min followed by washing with CH_2Cl_2, pyridine and CH_2Cl_2. This cycle was repeated until the desired oligonucleotide was formed.

For the automatic machine synthesis, an Applied Biosystems model 380-A DNA synthesizer was used. The conditions were changed so that the condensation was carried out with 20 equiv of nucleoside H-phosphonate and 100 equiv of pivaloyl chloride for 3 min. De-tritylation was carrried out by treatment with 5% trichloroacetic acid in CH_2Cl_2 for 3 min.

After the synthesis, the resin was treated with 0.1 M I_2 in a mixture of THF-pyridine-H_2O (44:3:3) (1 ml) at rt for 15 min (7 and 11 mer) or 30 min (15, 17 and 24 mer) or 60 min (34 mer). The resin was washed with pyridine, CH_2Cl_2, ether and dried.

Deblocking and Purification

The nucleotide resin was heated in a mixture of pyridine-c.NH_4OH (1:9, v/v) (5 ml) at 55°-60° for 6-12 hr. The resin was filterd off and washed with H_2O (1 ml). The combined filtrate was evaporated and the residue was applied to a reversed phase C-18 HPLC. The fraction containing nucleotides bearing a monomethoxytrityl group was pooled and evaporated to a small volume and then treated with 80 % AcOH (10 ml) at rt for 2-3 hr. The solvent was evaporated and the residue was dissolved in 0.1 M ammonium formate solution (pH 3.5) to adjust to a concentration of 10 A_{260}/ml. Then the mixture was irradiated with UV for 60 min, evaporated to a small volume, and applied to Sephadex G-25 column (ϕ1 x 45 cm). Elution was performed with 50 mM TEAB and monitored at 254 nm. The fraction eluted at the void volume was evaporated and the residue was separated by reversed phase C-18 HPLC (Fig. 1, 4). For isolution of the 34-mer, half of the sample was loaded onto 20 % PAGE containing 7 M urea (1 cm width, 20 cm length, 2 mm thickness) and run at 300V until bromophenol migrated about 15 cm. The portion of the gel which absorbed UV on irradiation was cut and suppended in a 1 ml solution of 0.5 M ammonium acetate, 10 mM magnesium acetate and 0.1 mM EDTA at 37° for 12 hr. The solution was applied to Sep Pak C-18 (Waters). The column was washed with H_2O (6 ml) and then the 34-mer was eluted with 30% CH_3CN (5 ml).

Table 3 Yields in the synthesis of oligoribonucleotides via H-phosphonate method

Sequence	Length	Scale	Yields
$UACUA_2C$	7	0.2μmol	$2.5A_{260}$ (20%)
$UACUA_2C$ (phosphite method, ref. 18)	7	1.0μmol	$18.4A_{260}$ (28%)
$GUAUGU_2A_2UA$	11	0.2μmol	$3.0A_{260}$ (14%)
$GUAUGU_2A_2UA$ (phosphite method, ref. 18)	11	1.0μmol	$18.7A_{260}$ (17%)
A_{15}	15	0.2μmol	$1.3A_{260}$ (3.5%)
$ACUCUCUA_3UAGCA_2U$	17	0.5μmol	$1.4A_{260}$ (1.8%)
$AU_3AC_2U_3G_2AG_3A_4GU_2A$	24	1.0μmol	$1.3A_{260}$ (0.6%)
$ACUCUCUA_3UAGCA_2UAU_3AC_2U_3G_2AG_3A$	34	0.3μmol	$1.9A_{260}$ (2.0%)

The yields of the oligoribonucleotides obtained are listed in Table 3.

Analysis of the 5'-terminal nucleoside

A sample of thr oligoribonucleotide (0.02 A_{260} unit) was reacted with [γ-^{32}P]ATP (4000 cpm/pmol, 10 μl, 1 μl) and T_4-polynucleotide kinase (1 unit/μl) (1 μl) in 5 μl of 50 mM Tris-HCl (pH 9.6), 1 mM $MgCl_2$, 2 mM spermine, 10 mM dithiothreitol and 0.1 M KCl at 37° for 1 hr. The mixture was heated at 90° for 3 min and separated on DEAE cellulose by development by Homo mixI to remove the unreacted [γ-^{32}P]ATP. The part which had radioactivity was scraped off and the oligonucleotide was eluted from the DEAE cellulose by 1M TEAB. The eluant was evaporated. The residue was dissolved in H_2O (40 μl) and evaporated again. The residue was treated with nuclease P1 (mg/ml) (1 μl) in 40 mM $AcONH_4$ (pH 5) (5 μl) at 37° for 2 hr. The whole was analyzed by paper electrophoresis in 0.2 M morpholine acetate buffer (pH 3.5) at 900V.

Enzyme digestion[18]

This analysis consists of RNase T_2 digestion, labeling at 5'end by [γ-^{32}P]ATP, and Nuclease P1 digestion. The resultant ^{32}pN's were analyzed by two dimensional thin layer chromatography on cellulose. By measuring the amount of incorporated [γ-^{32}P] relative to nucleotides with a 5'-hydroxyl group, the ratio of pA, pG, pC, pU obtained were 7.0:0.9:4.2:3.8 (theoretical 7:1:4:4) for 17mer, 7.5:6.0:2.3:7.3 (theoretical, 7:6:2:8) for 24mer and 10.0:5.3:5.1:11.1 (theoretical, 10:6:6:11) for 34mer (Fig. 6). In this reaction, the 3'-terminal nucleoside was not

labeled by the polynucleotide kinase due to the lack of 3'-phosphate.

RNase T_1 digestion of 24 mer

The 24 mer (0.02 A_{260} unit) was treated with RNase T_1 (1.25 unit/μl) (1 μl) in 4 μl of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM spermine at 37° for 1 hr; then it was treated directly with T_4-polynucleotide kinase (10 unit/μl) (0.1 μl) and [γ-^{32}P]ATP (100 μM) (1 μl) at 37° for another 1 hr. The whole was applied to DEAE cellulose, which was developed in Homo mix I^{20} (Fig. 7). Spots 1-4 were cut and eluted with 1M TEAB. These products were subjected to the analysis of 5'-terminal nucleoside as indicated previously.

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