[Chem. Pharm. Bull. 34(10)4126—4132(1986)]

Synthesis of Oligoribonucleotides *via* the Phosphite-Triester Approach on a Polymer Support

TOSHIKI TANAKA,* KEN-ICHI FUJINO, SHIGERU TAMATSUKURI and MORIO IKEHARA

Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565, Japan

(Received April 22, 1986)

Four ribonucleoside phosphoramidites bearing 5′-*O*-dimethoxytrityl and 2′-*O*-tetrahydrofuranyl groups were synthesized with the use of bis(diisopropylamino)-methoxyphosphine in yields of 68—92%. By the same procedure, the phosphoramidite derivatives of dimers were also prepared for block condensation.

We measured the time course of the condensation reaction of ribonucleoside phosphoramidite and nucleoside linked to long-chain alkylamine-controlled pore glass (LCA-CPG) resin. It took 30 min for complete coupling if the nucleoside phosphoramidite was activated with 1-*H*-tetrazole. However, when 5-*p*-nitrophenyltetrazole was used for the activation, the condensation reaction was complete within 5 min. The behavior of the block condensation reaction using dimers was similar to that of monomers in the case of 5-*p*-nitrophenyltetrazole activation.

We synthesized oligoribonucleotides UUUU, CUCUCUU and GGCGCGCC in yields of 51, 33 and 3%, respectively, by the phosphite-triester approach by means of either stepwise or block condensation on the LCA-CPG resin.

Keywords——oligoribonucleotide; phosphite method; phosphoramidite; solid-phase synthesis; HPLC

The adaptation of phosphite-triester method[1] to solid phase synthesis[2] has allowed the rapid synthesis of oligodeoxyribonucleotides with defined sequences.[3] On the other hand, the synthesis of oligoribonucleotides with defined sequences is still difficult and time-consuming. One of the main reasons lies in the presence of the 2′-hydroxyl group, which does not exist in

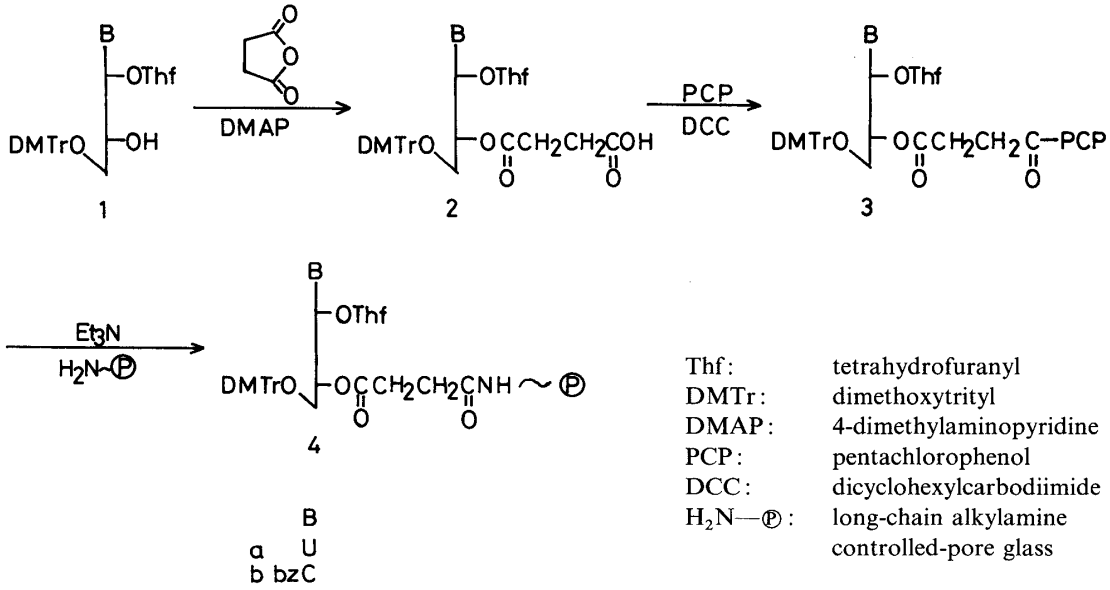

Chart 1

the 2′-deoxyribonucleosides. By using a tetrahydrofuranyl group, which can be removed by mild acid treatment, for 2′-hydroxyl protection, an oligoribonucleotide (chain length of 33) was synthesized in solution by the phosphotriester approach.[4] However, for rapid synthesis, the phosphite-triester approach is advantageous over the phosphotriester approach since the condensation reaction of the former is much faster than that of the latter. In this paper we described the synthesis of oligoribonucleotides by the phosphite-triester approach. Moreover, we applied this approach to block condensation.

Long-chain alkylamine-controlled pore glass (LCA-CPG) was used as the support, since LCA-CPG resin is widely used in oligodeoxynucleotide synthesis by the phosphite-triester approach. As shown in Chart 1, 5′-*O*-dimethoxytrityl-*N*-acylated-2′-*O*-tetrahydrofuranylnucleosides (**1a**, **b**) were bound to the resin *via* a succinyl linkage by the reported procedure.[5] For the synthesis of oligoribonucleotides, the nucleoside phosphoramidite bearing a dimethoxytrityl group at 5′-OH and a tetrahydrofuranyl group at 2′-OH was used for the condensation and zinc bromide was used for removal of the 5′-*O*-dimethoxytrityl group. We also studied block condensation[6] by the phosphite-triester approach.

Results

Preparation of Nucleoside Phosphoramidites

5′-*O*-Dimethoxytrityl-*N*-acylated-2′-*O*-tetrahydrofuranyl nucleosides (**1a—d**)[7] were phosphitylated by treatment with bis(diisopropylamino)-methoxyphosphine in the presence of diisopropylamine hydrotetrazolide as the activating reagent.[8] The phosphoramidite derivatives (**5a—d**) were obtained in yields of 68—92% after purification from the mixture by silica gel column chromatography.

For the study of the block condensation, dinucleotides (**6a**, **b**) bearing an *o*-chlorophenyl group on the internal phosphate were prepared by the phosphotriester method[4] since dimer

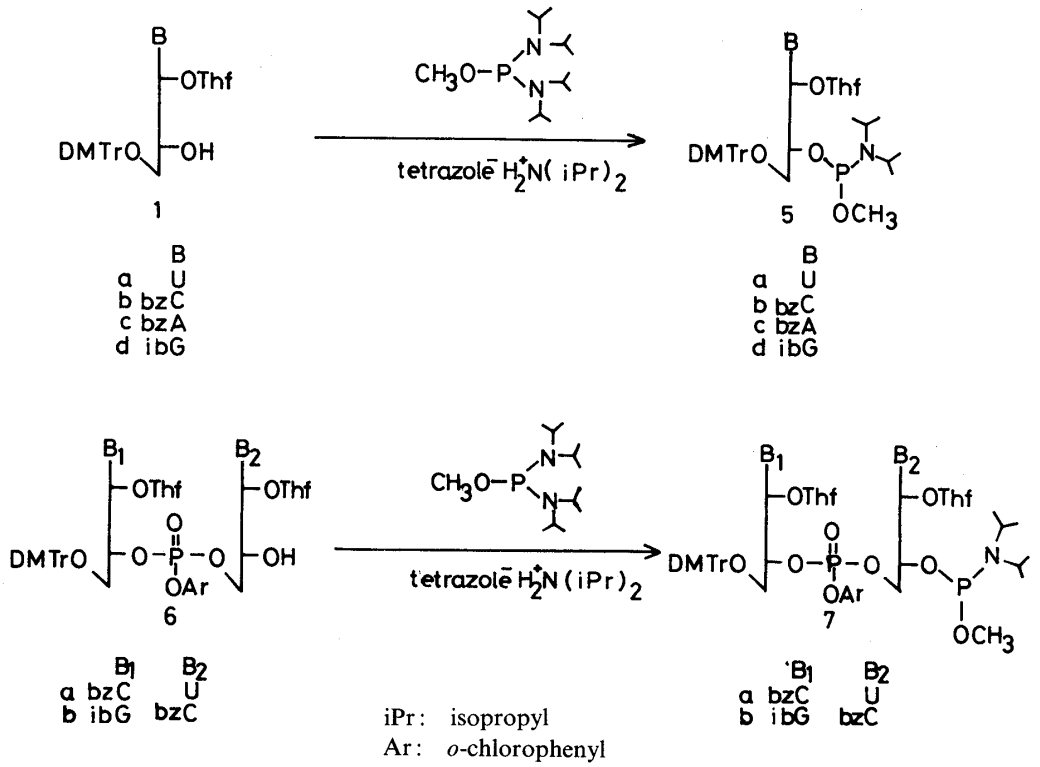

Chart 2

TABLE I. Yields and ^{31}P-NMR Data of **5a—d** and **7a, b**

Compound	Yield (%)	^{31}P-NMR chemical shifts
5a	68	147.37
5b	92	146.81, 147.02
5c	86	147.24, 147.75
5d	90	147.60
7a	67	135.40, 135.84, 136.31, 136.40, 136.83, −21.96, −22.01, −22.06, −22.17
7b	86	146.73, 146.95, 147.10, 147.68, 147.97, 148.26, 148.55, −7.92, −8.43, −8.79, −9.15, −10.32, −10.46, −10.61, −10.82

The chemical shift are downfield from trimethylphosphate in $CDCl_3$ as an external standard.

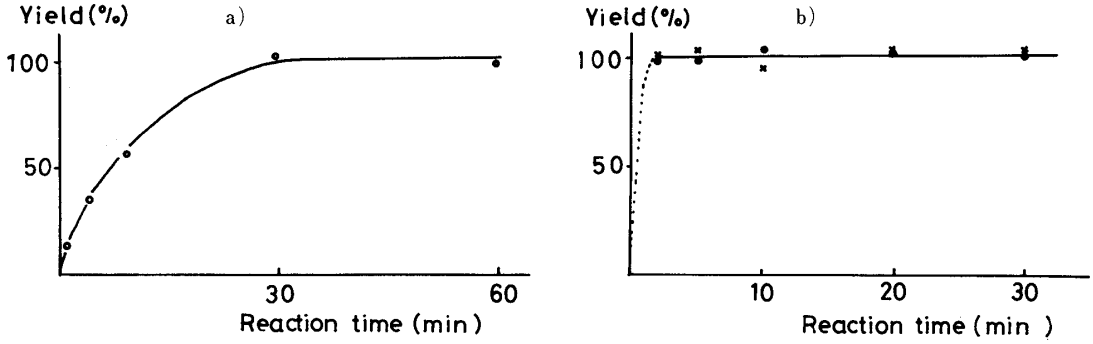

Fig. 1. Time Course of Coupling Reaction Using Monomer (**5a** or **5b**) (○—○) or Dimer (**7a**) (×—×) with Activation by Using a) 1-*H*-Tetrazole or b) 5-*p*-Nitrophenyltetrazole

synthesis in solution by the phosphotriester method is easily handled. The aryl protecting group is relatively stable during the purification step on silica gel and is removed easily by conc. ammonia treatment. The dimers bearing a free 3′-hydroxyl group were phosphitylated by the same procedure as described above. The yields of the phosphoramidite derivative of dimer (**7a, b**) were 67 and 86%, respectively, after purification on a silica gel short column.

The yields and the results of phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) analysis are summarized in Table I. The products (**5a—d**) contained two diastereoisomers as a result of phosphitylation since one of the diastereoisomers (**1a—d**) was used, and the ^{31}P-NMR analysis of **5b, c** showed double peaks. However, the double peaks of **5a, d** were not resolved. In the case of dimers (**7a, b**), multiple peaks in the ranges of 135—149 (phosphite) and −7——22 (phosphate) were seen since the dimers (**7a, b**) obtained contained mixtures of sixteen diastereoisomers derived from two tetrahydrofuranyl groups, internal phosphate and 3′-terminal phosphite. Not all the peaks were resolved. As shown in Table I, **7a** gave only five phosphite and four phosphate signals. In the case of **7b**, seven phosphite and eight phosphate signals were seen.

Condensation Time

It is well known that in deoxyribonucleotide synthesis *via* the phosphite approach, the phosphoramidite activated by 1-*H*-tetrazole reacts very rapidly with the 5′-OH group of nucleoside linked to the resin.[9] Usually the condensation reaction is completed within a few min. This is one of the most advantageous features of the phosphite-triester approach over the phosphotriester approach. At first we measured the condensation time course in ribonucleotide synthesis with 1-*H*-tetrazole activation.

After removal of the dimethoxytrityl group of the resin (**4a**) by treatment with $ZnBr_2$ (1 M $ZnBr_2$ in CH_2Cl_2–isopropanol, 85 : 15, v/v), the phosphoramidite derivative (**5a**) (20 eq) was added with 1-*H*-tetrazole (50 eq) as an activating reagent in CH_3CN. After 2, 5, 10, 30, and 60 min, the resin was treated with I_2 (0.1 M I_2 in THF–lutidine–H_2O, 40 : 10 : 1, v/v/v) then $ZnBr_2$ to remove the 5′-*O*-dimethoxytrityl group. The condensation yields were calculated from the amounts of released dimethoxytrityl cation. As shown in Fig. 1a, the condensation reaction was much slower (30 min) than that obtained in deoxyribonucleotide synthesis. Froehler and Matteucci reported[10] that the condensation reaction proceeded much faster if the nucleoside phosphoramidite was activated with 5-*p*-nitrophenyltetrazole instead of 1-*H*-tetrazole. Next, we studied the effect of condensation time by using 5-*p*-nitrophenyltetrazole. The resin (**4a**), after removal of the dimethoxytrityl group, was treated with a phosphoramidite derivative (**5b**, 20 eq) and 5-*p*-nitrophenyltetrazole (50 eq) in CH_3CN for periods of 2, 5, 10, 20, and 30 min followed by oxidation with iodine. To calculate the condensation yield, the dimethoxytrityl group was removed by $ZnBr_2$ and the amount of liberated dimethoxytrityl cation was measured. As shown in Fig. 1b, the condensation reaction was complete within 5 min. Finally we studied block condensation by the same approach. Using the phosphoramidite of the dimer (**7a**) with 5-*p*-nitrophenyltetrazole as the activating reagent, we obtained the same coupling yield as in the case of monomer coupling (Fig. 1b).

Synthesis of Ribooligonucleotides on the Polymer Support

One cycle for elongation of the chain is summarized in Table II. De-dimethoxytritylation was carried out with 1 M $ZnBr_2$ in CH_2Cl_2–isopropanol (85 : 15, v/v). This treatment was continued until the color of the dimethoxytrityl cation was no longer seen on adding the $ZnBr_2$ solution at 10 min intervals. For the coupling reaction, the resin was dried by coevaporation with pyridine and toluene, then nucleoside phosphoramidite and the activating reagent dissolved in CH_3CN were added. The reaction was continued for 60 min in the case of 1-*H*-tetrazole activation or 10 min in the case of 5-*p*-nitrophenyltetrazole activation. After oxidation with I_2–H_2O, unreacted 5′-hydroxyl group was capped by acetylation. One cycle required about 2 h for 1-*H*-tetrazole activation or 1 h for 5-*p*-nitrophenyltetrazole activation. We synthesized the tetramer UUUU by using **5b**, heptamer CUCUCUU by using the dimer

TABLE II. One Cycle of Operation

Step	Reagent or solvent	Reaction time (min)	No. of repetitions
1	1 M $ZnBr_2$[a]	10	
2	CH_2Cl_2–iso-PrOH (85 : 15)	0.2	3
	repeat above steps		
3	*n*-BuOH–pyridine (1 : 1)	0.2	3
4	Pyridine	0.2	3
5	Toluene	Co-evaporation	1
6	Amidite[b]		
7	Coupling reagent	60[c] or 10[d]	
8	CH_3CN	0.2	3
9	Oxidation[e]	2	1
10	CH_3CN	0.2	3
11	Capping reagent[f]	5	1
12	Pyridine	0.2	3
13	Toluene	Co-evaporation	1

a) CH_2Cl_2–iso-PrOH (85 : 15) solution. *b*) 20 eq. *c*) 1-*H*-Tetrazole (50 eq) in CH_3CN. *d*) 5-*p*-Nitrophenyltetrazole (50 eq) in CH_3CN. *e*) 0.1 M I_2 in tetrahydrofuran–2,6-lutidine–H_2O (40 : 10 : 1). *f*) Ac_2O: 0.1 M dimethylaminopyridine in pyridine (1 : 9).

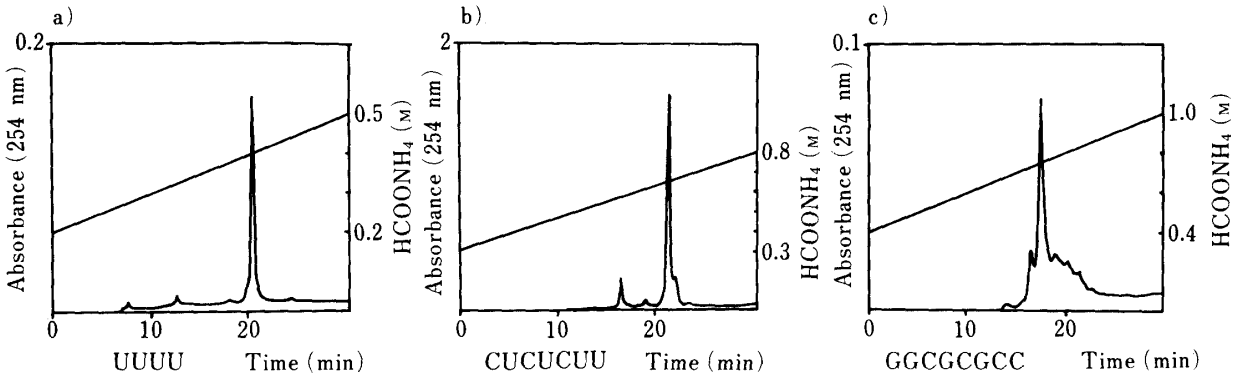

Fig. 2. HPLC Analysis of a) UUUU, b) CUCUCUU and c) GGCGCGCC by Ion Exchange Column Chromatography (TSK-GEL DEAE 2 SW, 0.46 i.d. × 25 cm)
Elution was performed with a linear gradient of $HCOONH_4$ in 20% acetonitrile.

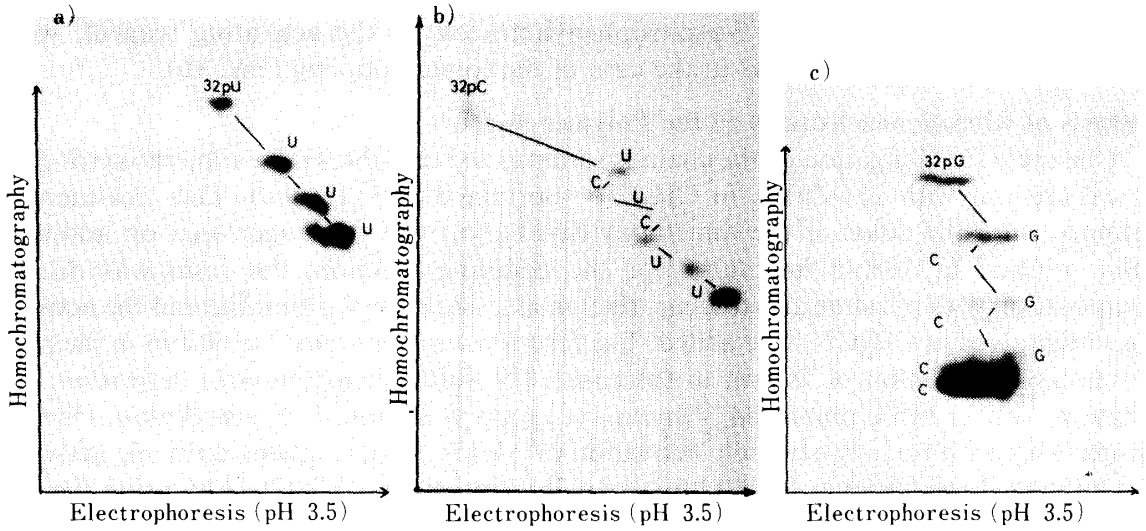

Fig. 3. Mobility Shift Analysis of a) UUUU, b) CUCUCUU and c) GGCGCGCC

(**7a**) with 1-*H*-tetrazole activation and octamer GGCGCGCC by using **7b** and **5c** with 5-*p*-nitrophenyltetrazole activation. The average coupling yields using **5b** or **7a** were more than 98%. In the case of **7b**, the average coupling yield was somewhat lower (88%).

The synthesized oligoribonucleotides bound to the support were treated first with thiophenol–triethylamine to remove the methyl group on the phosphate. Then the resin was treated with conc. NH_4OH at 55 °C overnight to remove the *o*-chlorophenyl phosphate protecting group and heterocyclic amino protecting group, and to release the nucleotidic compounds from the resin. The oligonucleotides bearing a dimethoxytrityl group at the 5′-hydroxyl and a tetrahydrofuranyl group at the 2′-hydroxyl function were subjected to separation by reversed-phase C_{18} column chromatography. The fractions containing the oligomer with a dimethoxytrityl group, which corresponded to the major peak, were collected and treated with 80% acetic acid to remove the 5′-*O*-dimethoxytrityl and 2′-*O*-tetrahydrofuranyl groups. The fully unprotected oligomer was purified by anion exchange column chromatography (Fig. 2). From 1.5 μmol of the resin, 25 A units of UUUU (total yield of 51%), 25 A units of CUCUCUU (total yield of 33%) and 3 A units of GGCGCGCC (total yield of 3%) were obtained. Finally the nucleotide sequences of all the oligomers were confirmed by mobility shift analysis[11] (Fig. 3).

Discussion

The use of phosphite-triester synthesis on a polymer support enabled us to synthesize deoxyoligonucleotide very rapidly, and we applied this technique to the synthesis of ribooligonucleotides. We prepared the nucleoside phosphoramidite bearing 5′-*O*-dimethoxytrityl and 2′-*O*-tetrahydrofuranyl groups. Activation of the ribonucleoside phosphoramidite with 1-*H*-tetrazole, which is commonly used for the activation of deoxyribonucleoside phosphoramidite, was rather slow (30 min). However, 5-*p*-nitrophenyltetrazole was able to activate the ribonucleoside phosphoramidite very rapidly. The condensation reaction was complete within 5 min. Since selective de-dimethoxytritylation from the 5′-hydroxyl function by $ZnBr_2$ was relatively slow (usually 30—60 min), we aimed to minimize the number of times that this step was used during the synthesis. Therefore, we prepared the phosphoramidite of dinucleotides. The activation of dinucleotide phosphoramidite by 5-*p*-nitrophenyltetrazole was found to be equivalent to that of the monomer.

We synthesized tetra-(UUUU), hepta-(CUCUCUU) and octaribonucleotide (GGCGCGCC) by the phosphite-triester approach on an LCA-CPG polymer support by either monomer or dimer coupling. The oligomer bearing dimethoxytrityl and tetrahydrofuranyl group after partial deblocking was easily obtained by reversed-phase C_{18} silica gel column chromatography. After the removal of the dimethoxytrityl and tetrahydrofuranyl groups by acid treatment, fully unprotected oligomers were isolated by anion exchange high-pressure liquid chromatography (HPLC). The HPLC profiles showed the desired oligomers as the major peak. In the case of the octamer, the yields was low and HPLC analysis showed some by-products since the octamer was guanosine-rich and self-complementary. The procedure used in this paper allowed us to synthesize oligoribonucleotides rapidly. The synthesis of oligomers with longer chain lengths is under investigation.

Materials and Methods

Thin-layer chromatography (TLC) was performed on plates of Kieselgel $60F_{254}$ (Merck). Column chromatography was performed on Kieselgel 60 (Merck). LCA-CPG (pore size 500 A, particle size 125—177 μm) were purchased from Pierce Chemical Company.

^{31}P-NMR spectra were recorded with a JEOL GX500 spectrometer operating at 202.42 MHz using trimethylphosphate as an external standard. Ultraviolet (UV) spectra were measured on a Hitachi model 200-10 spectrophotometer.

HPLC was performed on an Altex 322MP chromatography system. Anion exchange chromatography was performed using anion exchange resin from Toyo Soda Co. Reversed phase HPLC was carried out on Nucleosil (5 μ) packed into a stainless steel column (0.6 i.d. × 20 cm) under a pressure of 500 kg/cm^2.

Synthesis of Phosphoramidite Derivatives (5a—d, 7a, b)——Bis(diisorpropylamino)-methoxyphosphine (240 μl, 1.3 mmol) was added to a solution of the protected nucleoside (**1a—d**, **6a**, **b**) (1 mmol) (U, A, G; low *Rf* isomer on TLC, C; high *Rf* isomer on TLC) and diisopropylhydrotetrazole (86 mg, 0.5 mmol) in dichloromethane (5 ml) with stirring for 4 h. Completion of the reaction was confirmed by TLC (AcOEt) (*Rf* 0.57—0.87), and the mixture was evaporated to a small volume. The residue was dissolved in dichloromethane (50 ml) and washed with sat. $NaHCO_3$ twice (10 ml each), then with water (10 ml × 3). The organic solvent was evaporated off, then the product was applied to a column (3 i.d. × 3 cm) of silica gel and eluted with ethyl acetate. The fraction which contained the desired product was collected and evaporated to a foam. The yields and the results of analysis by ^{31}P-NMR are shown in Table I.

Preparation of the Ribonucleoside Resin (4a, b)——5′-*O*-Dimethoxytrityl-2′-*O*-tetrahydrofuranyl uridine (**1a**) and 5′-*O*-dimethoxytrityl-*N*-benzoyl-2′-*O*-tetrahydrofuranyl cytidine (**1b**) was derivatized to nucleoside-3′-pentachlorophenyl succinate according to the literature.[5] Each succinate derivative (0.1 mmol) was dissolved in dimethyl formamide (DMF) (2 ml), and LCA-CPG (500 mg) and triethylamine (0.11 mmol) were added. The mixture was shaken at room temperature for 20 h, then the resin was washed with DMF (5 ml) and dichloromethane (5 ml), and mixed with capping reagent (0.1 M 4-dimethylaminopyridine (DMAP) in pyridine : Ac_2O = 9 : 1, v/v, 1 ml). The reaction mixture was allowed to stand at room temperature for 10 min, then washed with DMF (5 ml), methanol (5 ml), dichloromethane (5 ml) and ether (5 ml) and dried in a vacuum. The amount of ribonucleoside loaded on the resin was 30 μmol per gram.

The Measurement of Condensation Time——The resin (**4a**) (20 mg, 0.6 μmol) was first treated with $ZnBr_2$ to remove the dimethoxytrityl group. The ribonucleoside resin was evaporated with pyridine and toluene, and a phosphoramidite derivative (**5a**, **b** or **7a**) (20 eq) was added. To this mixture, a solution of 1-*H*-tetrazole (50 eq) dissolved in acetonitrile (80 μl) or 5-*p*-nitrophenyltetrazole (50 eq) dissolved in acetonitrile (400 μl) was added. The resin was washed with acetonitrile and treated with 1 ml of 0.1 M I_2 solution in a mixture of tetrahydrofuran–lutidine–H_2O (40 : 10 : 1, v/v/v) for 2 min. The resin was washed well with pyridine and coevaporated with toluene. Then the dimethoxytrityl group from incoming nucleoside was removed by treatment with 1 M $ZnBr_2$ solution in dichloromethane–isopropanol (85 : 15, v/v). The amount of dimethoxytrityl cation liberated by $ZnBr_2$ treatment was measured at 500 nm to calculate the coupling yield. The result are shown in Fig. 1.

Synthesis of Ribooligonucleotides——For removal of the dimethoxytrityl group, the resin was suspended in 2 ml of $ZnBr_2$ (1 M) solution in dichloromethane–isopropanol (85 : 15, v/v) for 10 min. This step was repeated until dimethoxytrityl cation was no longer detectable. The resin was washed successively with a mixture of *n*-butanol and pyridine (1 : 1, v/v) (2 ml × 3) and pyridine (2 ml × 3). After co-evaporation of the resin with toluene, phosphoramidite (20 eq) and 1-*H*-tetrazole (50 eq) in acetonitrile (200 μl) or 5-*p*-nitrophenyltetrazole (50 eq) in acetonitrile (400 μl) was added. The reaction mixture was allowed to stand at room temperature for 60 min or 10 min, respectively. After the reaction, the resin was washed with acetonitrile (2 ml × 3) and treated with capping reagent (1 ml) (0.1 M DMAP in pyridine : Ac_2O = 9 : 1, v/v) for 5 min. After the reaction, the resin was washed with pyridine (2 ml × 3).

Deblocking and Purification——The resin was mixed with thiophenol–triethylamine–dioxane (1 : 2 : 2, v/v/v, 2 ml) at room temperature for 30 min. After washing with pyridine, conc. NH_4OH (3 ml) was added, and the reaction mixture was allowed to stand at 55 °C for 12 h. The resin was removed and the solution was evaporated. The residue was chromatographed over C_{18} silica gel (0.7 i.d. × 7 cm). Elution was performed with a linear gradient of acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0) (5—40%, total 200 ml). The fractions eluted at 20—30% acetonitrile concentration were collected and evaporated. The residue was dissolved in 80% acetic acid (10 ml) for 2 h at room temperature and then evaporated. An aliquot was purified by anion-exchange HPLC as shown in Fig. 2. The sequences of the purified oligoribonucleotides were confirmed by mobility-shift analysis (Fig. 3).

References

1) R. L. Letsinger and W. B. Lunsford, *J. Am. Chem. Soc.*, **98**, 3655 (1975).
2) M. D. Matteucci and M. H. Caruthers, *Tetrahedron Lett.*, **21**, 719 (1980).
3) *a*) T. Tanaka and R. L. Letsinger, *Nucleic Acids Res.*, **10**, 3249 (1982); *b*) S. P. Adams, K. S. Kavka, E. J. Wykes, S. B. Holder and G. R. Galluppi, *J. Am. Chem. Soc.*, **105**, 661 (1983); *c*) M. A. Dorman, S. A. Noble, L. J. McBride and M. H. Caruthers, *Tetrahedron*, **40**, 95 (1984); *d*) H. Koster, J. Biernat, J. McManus, A. Wolter, A. Stumpe, C. K. Narang and N. D. Sinha, *ibid.*, **40**, 103 (1984).
4) E. Ohtsuka, A. Yamane, T. Doi and M. Ikehara, *Tetrahedron*, **40**, 47 (1984).
5) E. Ohtsuka, J. Matsugi, A. Yamane, H. Morioka and M. Ikehara, *Chem. Pharm. Bull.*, **33**, 4152 (1985).
6) *a*) R. L. Letsinger, E. P. Groody, N. Lander and T. Tanaka, *Tetrahedron*, **40**, 137 (1984); *b*) G. Kumer and M. S. Poonian, *J. Org. Chem.*, **49**, 4905 (1984).
7) E. Ohtsuka, A. Yamane and M. Ikehara, *Nucleic Acids Res.*, **11**, 1325 (1983).
8) A. D. Barone, J. Y. Tang and M. H. Caruthers, *Nucleic Acids Res.*, **12**, 4051 (1984).
9) S. L. Beaucage and M. H. Caruthers, *Tetrahedron Lett.*, **22**, 1859 (1981).
10) B. C. Froehler and M. D. Matteucci, *Tetrahedron Lett.*, **24**, 3171 (1983).
11) *a*) E. Jay, R. Bambora, R. Padmanabhan and R. Wu, *Nucleic Acids Res.*, **1**, 331 (1974); *b*) E. Jay, C. P. Bahl and R. Wu, *Anal. Biochem.*, **74**, 73 (1976).