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5'-Levulinyl and 2'-tetrahydrofuranyl protection for the synthesis of oligoribonucleotides by the phosphoramidite approach

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ABSTRACT

The levulinyl group has been employed for protection of the 5'-hydroxyl group in the synthesis of oligoribonucleotides by the phosphoramidite approach, using the acid-labile 2'-tetrahydrofuranyl group. The hydrazine treatment was performed for 10 minutes in order to remove the levulinyl group on controlled pore glass. Four decaribonucleotides (AAAAAAAAAU, GGGGGGGGGU, CCCCCCCCCU and UUUUUUUUUU) and a heneicosamer (GCCUAGCUGAUGAAGGGUGAU) were prepared with an automatic synthesizer in good yields.

INTRODUCTION

In recent years, great progress has been made in the chemical synthesis of oligonucleotides by development of the phosphoramidite method on a polymer support. The synthesis of oligoribonucleotides, however, is still difficult compared with that of the deoxy-series because the 2'-hydroxyl function must be protected properly. Ogilvie and his coworkers have studied the use of the tert-butyldimethylsilyl group as the 2'-protecting group as well as the application of the phosphite approach to RNA synthesis, and the synthesis of a 43mer has been accomplished [1]. Tanaka et al. have synthesized oligoribonucleotides with chain lengths up to 34 using 2'-o-nitrobenzyl protection by the phosphoramidite or H-phosphonate approach [2,3].

Acid-labile acetal and ketal groups have also been used for protection of the 2'-hydroxyl group in the synthesis of oligoribonucleotides [4,5]. These protecting groups, however, are incompatible with the trityl derivatives, excellent 5'-protecting groups used commonly in oligonucleotide synthesis, during chain elongation on a polymer support [6,7]. Therefore the chain lengths have been limited to medium size, even though the phosphoramidite

approach has been applied [8,9]. There are two alternatives to solve this problem; one is to develop a new acetal or ketal group, for example the 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl group which is compatible with the 9-phenylxanthen-9-yl group [10], and the other is to develop a procedure without acid treatment during chain elongation. With regard to the latter approach, we have reported the synthesis of 9mers and a 18mer in the 3'-direction using p-anisidate protection of the phosphate residue and 2'-tetrahydrofuranyl protection [11]. The tetrahydrofuranyl group can be removed under milder conditions than the tetrahydropyranyl group [12]. In this paper we describe the synthesis of oligoribonucleotides by the phosphoramidite approach using 5'-levulinyl protection in combination with the 2'-tetrahydrofuranyl group. The 5'-levulinyl group has been used for the synthesis of oligoribonucleotides by the phosphotriester approach in combination with the 2'-methoxytetrahydropyranyl group [13,14]. Our aims are to develop a facile and high-yield synthesis of RNA fragments by the phosphoramidite approach, comparable to the DNA synthesis.

RESULTS AND DISCUSSION

Preparation of protected nucleosides and their 3'-phosphoramidite derivatives

The starting materials, N-acyl-5'-O-levulinyl-2'-O-tetrahydrofuranylnucleosides, were prepared from N-acyl-2'-O-tetrahydrofuranylnucleosides [15] with levulinic acid and 2-chloro-1-methylpyridinium iodide as described [13]. Although this procedure was reported to be 5'-selective, the occurrence of 3'-acylation was detected, which reduced the isolated yield. The 5',3'-dilevulinyl derivative could be separated by chromatography on silica gel. The 3'-monolevulinyl derivative was treated with 4,4'-dimethoxytrityl chloride and then removed by reverse-phase chromatography. The amount of the product was adequate to the solid-phase synthesis, although the 5'-selectivity should be improved. The purified 5'-levulinyl derivative was phosphitylated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite [16] followed by purification on silica gel.

The stability of protecting groups other than the levulinyl

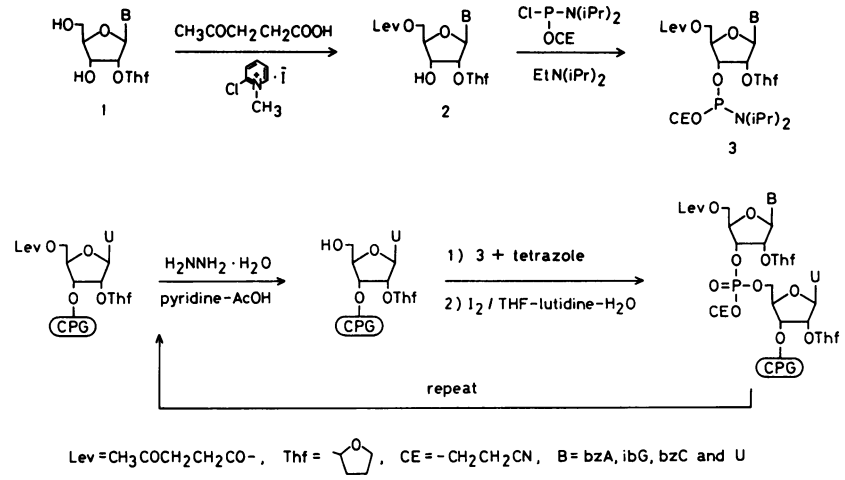

Fig. 1 Procedure for the synthesis of oligoribonucleotides.

group to hydrazine was tested using a fully-protected dinucleoside monophosphate, d(DMTr)$T_{P(CE)}$bzC(Bz), and N-acyl-2'-O-tetrahydrofuranyl nucleosides. Whilst no removal of the cyanoethyl group protecting the phosphate residue was detected after treatment with 0.5M hydrazine monohydrate in pyridine-acetic acid (3:2, v/v) for 5hr, about half of the benzoyl group protecting adenine and cytosine was removed in 3hr and 5hr respectively. The effect of this debenzoylation on the synthesis of oligonucleotides is discussed below.

Coupling reaction using a 2'-tetrahydrofuranylnucleoside 3'-phosphoramidite

In order to study the reaction conditions required for high coupling yield, we measured the time course of the reaction using a 2'-tetrahydrofuranylnucleoside 3'-phosphoramidite (Fig. 2). Controlled pore glass (CPG) containing 1 μmol of 5'-O-dimethoxytritylthymidine was treated with trichloroacetic acid in dichloromethane to remove the dimethoxytrityl group, coupled with 5'-O-dimethoxytrityl-2'-O-tetrahydrofuranyluridine 3'-β-cyanoethyl N,N-diisopropylphosphoramidite using tetrazole as an activator, followed by oxidation, and detritylated again. The coupling yield was calculated by spectrophotometric determination of the trityl cation. It was necessary to prolong the coupling

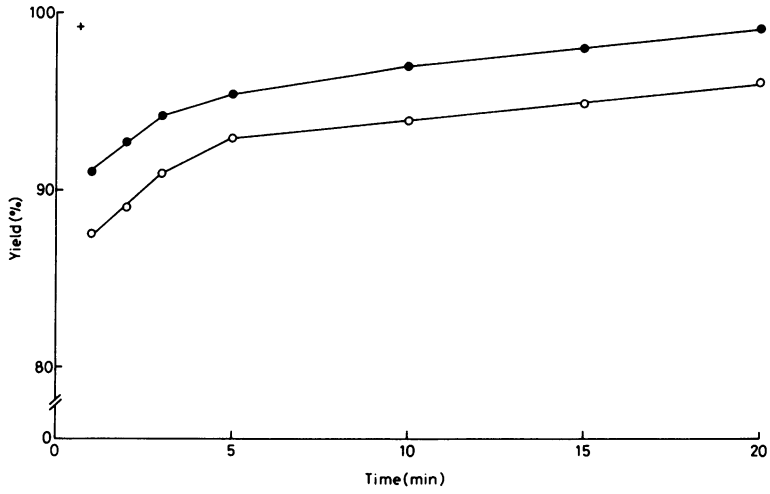

Fig. 2 Time course of the coupling reaction.
The concentration of the phosphoramidite derivative is 42.0mM (○) and 67.2mM (●). + is the coupling yield of 5'-O-dimethoxytrityl-thymidine 3'-phosphoramidite.

reaction to 20min, using a higher concentration of the phosphoramidite derivative, to obtain a yield of 99%, whilst a deoxynucleoside 3'-phosphoramidite could react within 40sec at the lower concentration to give the same yield.

A decamer, UUUUUUUUUU (U_{10}), was synthesized with an Applied Biosystems 381A synthesizer using 1 μmol of uridine-CPG (Fig. 1 and Table 1). The hydrazine treatment was performed with a continuous flow for 5min per cycle. After nine cycles of chain

Table 1 Reaction cycle for chain elongation

step	reagent	time
1.	0.5M hydrazine monohydrate / pyridine-acetic acid (3:2, v/v)	10min (or 5min)
2.	70mM nucleoside 3'-phosphoramidite & 0.25M tetrazole / acetonitrile	20min
3.	0.53M acetic anhydride & 0.27M 4-dimethylaminopyridine / 2,6-lutidine-tetrahydrofuran (1:18, v/v)	0.5min
4.	0.1M I_2 / 2,6-lutidine-tetrahydrofuran-water (10:40:1, v/v/v)	1min

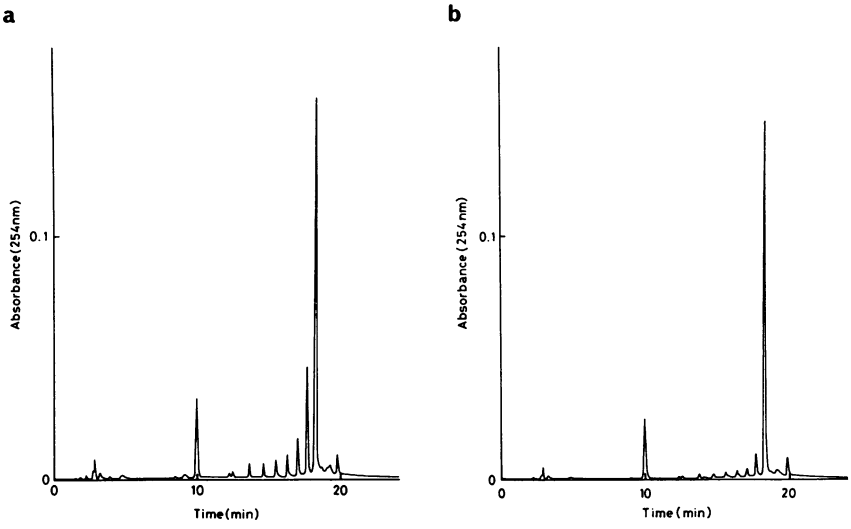

Fig. 3 HPLC analysis of crude C_9U.
A μBONDASPHERE 5μ C18-300Å column (3.9mmI.D.x15cmL.) was used with a linear gradient of acetonitrile (from 0 to 7.5% during 20min) in 0.1M triethylammonium acetate (pH7.0). The hydrazine treatment was performed for 5min (a) and 10min (b).

elongation, the decamer was cleaved from the CPG with ammonia water, deprotected with 0.01N hydrochloric acid for 3hr, and analyzed by high-pressure liquid chromatography (HPLC).

Treatment with hydrazine

A decamer, CCCCCCCCCU (C_9U), was synthesized with hydrazine treatment for 5min or 10min per cycle to ensure complete removal of the levulinyl group and to examine the influence of the debenzoylation described above. After chain elongation, the oligonucleotide was cleaved from the CPG, deprotected first with ammonia water at 55°C for 5hr and then with 0.01N hydrochloric acid at room temperature for 5hr, and analyzed by HPLC (Fig. 3). It appears that the hydrazine treatment must be performed for 10min to remove the levulinyl group completely and that removal of the benzoyl group with hydrazine has no influence upon the synthesis of C_9U.

Another decamer, AAAAAAAAAU (A_9U), synthesized by the same procedure gave an extra peak behind the main peak which was identified as a decamer by anion-exchange HPLC (Fig. 4a). This

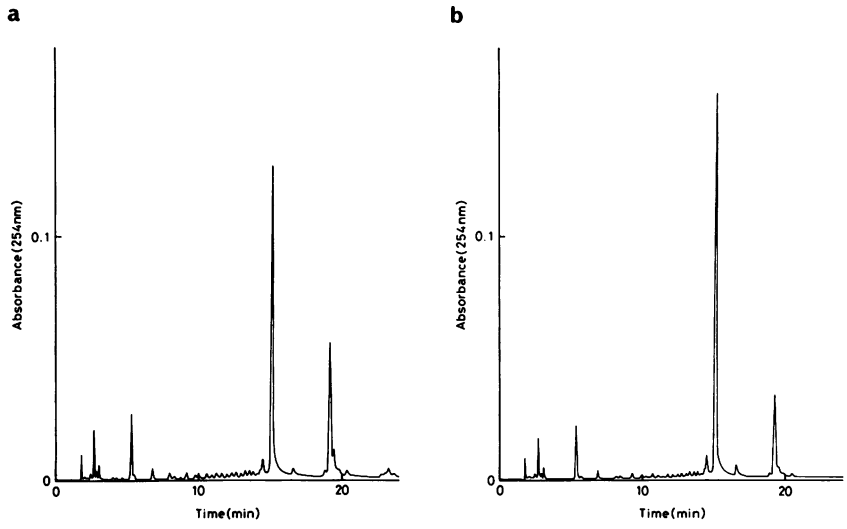

Fig. 4 HPLC analysis of crude A_9U.
A μBONDASPHERE 5μ C18-300Å column was used with a linear gradient of acetonitrile (from 5 to 11% during 20min). The acid treatment (pH2.0) to remove the tetrahydrofuranyl group was performed for 5hr (a) and 15hr (b).

peak diminished after prolonged acid treatment, probably due to hydrolysis of the phosphoramidate formed by reaction with the amino group of adenine (Fig. 4b).

Purification of the decamers

The remaining decamer, GGGGGGGGGU (G_9U), was also synthesized, and the four decamers were purified by reverse-phase HPLC. The main peak was partitioned and 0.30-0.65 A_{260} units were obtained by injecting one-sixtieth of each crude product. The

Table 2 Isolated yields of the decamers

sequence	yield*1	
AAAAAAAAAU (A_9U)	0.65 A_{260} unit	(33%*2)
GGGGGGGGGU (G_9U)	0.45	(27%)
CCCCCCCCCU (C_9U)	0.32	(27%)
UUUUUUUUUU (U_{10})*3	0.30	(19%)

*1 One-sixtieth of the crude product was purified.
*2 The overall yield from the 3'-terminal uridine on CPG.
*3 The hydrazine treatment was performed for 5min per cycle.

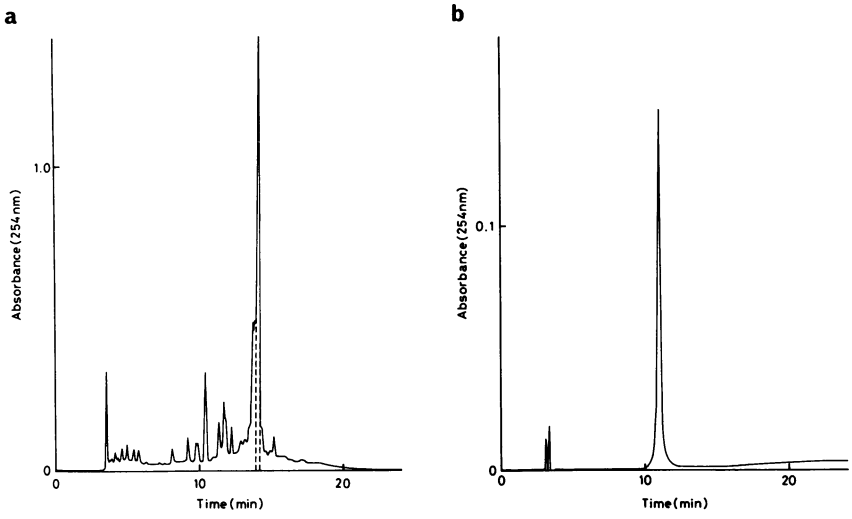

Fig. 5 Purification and analysis of the 21mer.
(a) The 21mer was purified by reverse-phase HPLC using a YMC A-303 column (4.6mmI.D.x25cmL.) with a linear gradient of acetonitrile (from 7 to 13% during 20min). (b) The purified 21mer was analyzed by anion-exchange HPLC using a TSK gel DEAE-2SW column (4.6mmI.D.x25cmL.) with a linear gradient of ammonium formate (from 0.6 to 1.0M during 20min) in 20% aqueous acetonitrile.

overall yields from the 3'-terminal uridine on CPG (1 μmol), using calculated molar extinction coefficients [17], are listed in Table 2. The purified decamers were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and analyzed by 10% polyacrylamide gel electrophoresis (PAGE) containing 8M urea.

Synthesis of a heneicosamer

Using the phosphoramidite derivatives of four nucleosides, a 21mer, GCCUAGCUGAUGAAGGGUGAU, was synthesized. This sequence is a part of satellite DNA transcripts of the newt [18,19]. After chain elongation using a synthesizer, the 21mer was cleaved from the CPG, deprotected first with ammonia water as described above, and then with 0.01N hydrochloric acid for 24hr, and purified by reverse-phase HPLC (Fig.5a). The main peak was partitioned and further purified by 10% PAGE to remove a small amount of shorter oligonucleotide contaminants. One-thirtieth of the crude product was purified, and 0.55 A_{260} units were obtained. The overall yield from uridine-CPG was ca. 8%.

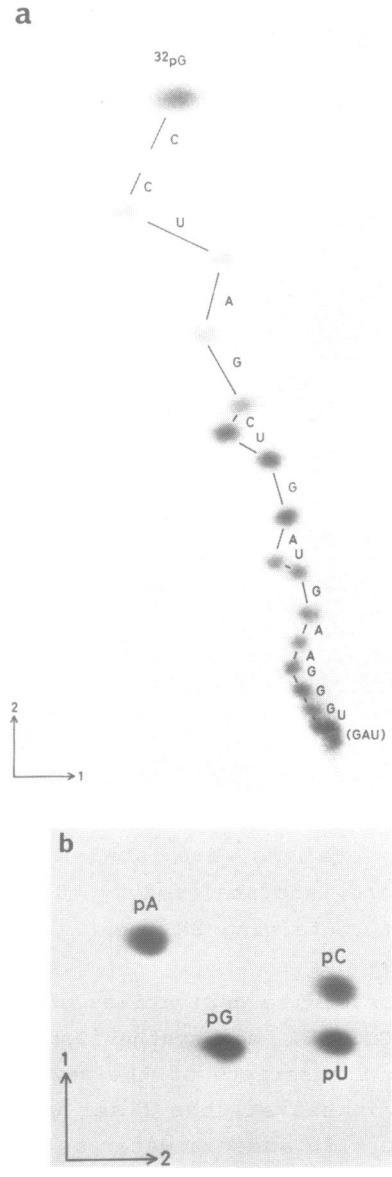

Fig. 6 Sequence analysis and RNase T_2 digestion of the 21mer.

The purified 21mer was phosphorylated with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the sequence was confirmed by two-dimensional homochromatography [20] (Fig. 6a, the three spots at

the 3'-end are not separated from each other.) and identification of 5'-terminal guanosine by paper electrophoresis after digestion with nuclease P1. The 21mer was also digested completely with RNase T_2 to give pA, pG, pC and pU by two-dimensional thin layer chromatography on a cellulose plate after kination and nuclease P1 digestion [2,21] (Fig. 6b).

These results demonstrate that 5'-levulinyl protection is compatible with the 2'-tetrahydrofuranyl group and the phosphoramidite procedure. This method is facile and effective for the synthesis of oligoribonucleotides with an automatic synthesizer.

EXPERIMENTAL

General methods

N-Acyl-2'-O-tetrahydrofuranylnucleosides were prepared as described previously [15].

Thin layer chromatography (TLC) was performed on Kieselgel $60F_{254}$ plates (Merck) with chloroform-methanol (10:1, v/v). For reverse-phase TLC, Kieselgel $60F_{254}$ silanisiert plates (Merck) were used with a solvent system of acetone-20mM triethylammonium acetate (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 100MHz with a JEOL JNM-FX100 spectrometer. ^{31}P-NMR spectra of the phosphoramidite derivatives were measured at 36.25MHz with a JEOL JNM-FX90Q spectrometer using trimethyl phosphate as an internal standard.

All chain elongation reactions were performed on an Applied Biosystems 381A synthesizer using a CPG column containing 1 µmol of uridine.

Reverse-phase HPLC was performed on a Shimadzu LC-6A system using a µBONDASPHERE 5µ C18-300Å column (Waters) for analysis and a YMC A-303 column (Yamamura Chemical Laboratories) for purification with a linear gradient of acetonitrile in 0.1M triethylammonium acetate (pH7.0). For anion-exchange HPLC, a TSK gel DEAE-2SW column (Tosoh Corporation) was used with a linear gradient of ammonium formate in 20% aqueous acetonitrile.

Enzymes for analysis of oligonucleotides were purchased from the following companies: T4 polynucleotide kinase (E. coli A19)

from Takara Shuzo, snake venom phosphodiesterase from Boehringer Mannheim, nuclease P1 from Yamasa Shoyu, and RNase T_2 from Sankyo.

N-Acyl-5'-O-levulinyl-2'-O-tetrahydrofuranylnucleoside

A solution of N-acyl-2'-O-tetrahydrofuranylnucleoside (3mmol) in dioxane (24ml) was added to a suspension of 2-chloro-1-methylpyridinium iodide (1.53g, 6mmol) in acetonitrile (6ml). To the resulting suspension a solution of levulinic acid (1.23ml, 12mmol) and 1,4-diazabicyclo[2.2.2]octane (1.62g, 14.4mmol) in dioxane (12ml) was added, and the mixture was stirred for 30min. Chloroform (200ml) and 2% aqueous $NaHCO_3$ (200ml) were added, and the aqueous layer was extracted with chloroform (50ml x3). The combined organic layer was dried with Na_2SO_4 and concentrated. After coevaporation with pyridine, the residue was dissolved in pyridine (5ml), and 4,4'-dimethoxytrityl chloride (0.51g, 1.5mmol) was added. After 1hr, methanol (1ml) and then chloroform (50ml) were added, and the solution was washed with water. The organic layer was concentrated and applied to a column (3cmI.D.x6cmL.) of Preparative C18 eluted with acetone-0.1% aqueous pyridine (25:75, v/v). The eluted product was further purified using a column (3cmI.D.x 5.5cmL.) of Wakogel C-300 (20g) eluted with 1% methanol in chloroform. The 5'-O-levulinyl-2'-O-tetrahydrofuranyl derivatives of 6-N-benzoyladenosine, 2-N-isobutyrylguanosine and 4-N-benzoylcytidine were precipitated with hexane-ethyl ether (1:1, v/v). 5'-O-levulinyl-2'-O-tetrahydrofuranyluridine was obtained as a foam by evaporation.

6-N-Benzoyl-5'-O-levulinyl-2'-O-tetrahydrofuranyladenosine

Yield: 0.61g (38%). R_f value ($CHCl_3$:MeOH=10:1): 0.45. ^{1}H-NMR ($CDCl_3$/TMS): δ[ppm]=9.06(s,1H,-NH-), 8.80(s,1H,H-8), 8.29(s,1H,H-2), 8.1-7.5(m,5H,arom), 6.15(d,1H,H-1'), 5.34(br,1H,Thf), 4.92(t,1H,H-2'), 4.5-4.3(m,4H,H-3',4',5'), 4.1-3.8(m,2H,Thf), 3.43(d,1H,3'-OH), 2.9-2.5(m,4H,Lev), 2.19(s,3H,Lev), 2.1-1.9(m,4H,Thf).

2-N-Isobutyryl-5'-O-levulinyl-2'-O-tetrahydrofuranylguanosine

Yield: 0.98g (63%). R_f value ($CHCl_3$:MeOH=10:1): 0.41. ^{1}H-NMR ($CDCl_3$/TMS): δ[ppm]=9.34(s,1H,-NH-), 7.82(s,1H,H-8), 5.85(d,1H,H-1'), 5.19(br,1H,Thf), 4.77(t,1H,H-2'), 4.6-4.3(m,4H,H-3',4',5'),

4.1-3.8(m,2H,Thf), 3.34(d,1H,3'-OH), 2.9-2.5(m,5H,Lev,ib), 2.16(s,3H,Lev), 2.1-1.8(m,4H,Thf), 1.27(d,6H,ib).

4-N-Benzoyl-5'-O-levulinyl-2'-O-tetrahydrofuranylcytidine

Yield: 0.45g (29%). R_f value ($CHCl_3$:MeOH=10:1): 0.51. ^{1}H-NMR ($CDCl_3$/TMS): δ[ppm]=8.75(br,1H,-NH-), 8.26(d,1H,H-5), 8.0-7.4(m,6H,arom,H-6), 5.91(s,1H,H-1'), 5.58(br,1H,Thf), 4.6-3.8(m,7H,H-2',3',4',5',Thf), 3.62(d,1H,3'-OH), 2.9-2.5(m,4H,Lev), 2.22(s,3H,Lev), 2.2-1.9(m,4H,Thf).

5'-O-Levulinyl-2'-O-tetrahydrofuranyluridine

Yield: 0.40g (32%). R_f value ($CHCl_3$:MeOH=10:1): 0.41. ^{1}H-NMR ($CDCl_3$/TMS): δ[ppm]=8.99(br,1H,-NH-), 7.68(d,1H,H-6), 5.90(d,1H,H-1'), 5.80(d,1H,H-5), 5.41(br,1H,Thf), 4.5-3.8(m,7H,H-2',3',4',5',Thf), 3.36(d,1H,3'-OH), 2.9-2.5(m,4H,Lev), 2.20(s,3H,Lev), 2.1-1.9(m,4H,Thf).

Nucleoside 3'-β-cyanoethyl N,N-diisopropylphosphoramidite

After coevaporation with pyridine, N-acyl-5'-O-levulinyl-2'-O-tetrahydrofuranylnucleoside (0.5mmol) was dissolved in tetrahydrofuran (5ml), and N,N-diisopropylethylamine (0.35ml, 2mmol) was added. To this solution 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.24ml, 1mmol, purchased from American Bionuclear) was added dropwise. After 1hr, ethyl acetate (50ml) was added and washed with saturated aqueous $NaHCO_3$ (20ml x2). The organic layer was dried with Na_2SO_4, concentrated, and applied to a column (3cmI.D.x3cmL.) of Wakogel C-300 (10g) eluted with 1% methanol in chloroform containing 0.1% pyridine. The purified phosphoramidite derivative was precipitated with n-pentane (50ml) from a chloroform solution (3ml), dissolved in chloroform again, and obtained as a foam by evaporation.

Yield: B=bzA, 0.34g (77%); B=ibG, 0.38g (81%); B=bzC, 0.25g (70%); B=U, 0.22g (90%). R_f value ($CHCl_3$:MeOH=10:1): B=bzA, 0.55; B=ibG, 0.48; B=bzC, 0.57; B=U, 0.46. ^{31}P-NMR ($CDCl_3$/trimethyl phosphate): B=bzA, δ=148.62, 147.74ppm; B=ibG, δ=148.62, 148.01ppm; B=bzC, δ=148.15, 147.47ppm; B=U, δ=148.01, 147.74ppm.

Synthesis of oligoribonucleotides

5'-O-levulinyl-2'-O-tetrahydrofuranyluridine was linked to controlled pore grass (Aminopropyl-CPG-550Å 120-200mesh, Fluka) as described [22]. The nucleoside content was 30μmol/g by spectrophotometric determination after cleavage with ammonia water.

After chain elongation with a synthesizer using a column of uridine-CPG (33mg, 1μmol) and a synthetic program outlined in Table 1, the oligonucleotide was cleaved by treatment with concentrated ammonia water (0.5ml x4) for 1hr. The resulting solution was heated in a sealed vial at 55°C for 5hr, except U_{10}. After evaporation of ammonia water, 0.01N hydrochloric acid (5ml) was added, and the pH was adjusted to 2.0 by addition of 0.1N hydrochloric acid. The solution was stirred for 3-24hr (as described in the text), neutralized with 0.1M aqueous ammonia, and passed through a membrane filter (EKICRODISC 13, Gelman Sciences Japan). The deprotected oligonucleotide was analyzed and purified by reverse-phase HPLC as shown in Fig. 3-5. For purification, 100μl of the oligonucleotide solution was injected to a YMC A-303 column (4.6mmI.D.x25cmL.), and the main peak was partitioned. The 21mer was further purified by electrophoresis on 10% polyacrylamide containing 8M urea and desalted on a column (1.7cmI.D.x40cmL.) of Sephadex G-25 eluted with 0.1M triethylammonium bicarbonate followed by coevaporation with water. The overall yields of the 10mers are listed in Table 2, and that of the 21mer was ca. 8%. The purity and the chain length were analyzed by anion-exchange HPLC and polyacrylamide gel electrophoresis.

Kination of the 21mer

The 21mer (0.04 A_{260} unit) was dissolved in water (2μl) and a buffer (1μl) containing 250mM Tris-HCl (pH9.6), 50mM $MgCl_2$, 10mM spermine, 50mM dithiothreitol and 0.5M KCl. To this solution 100μM ATP (0.5μl), [γ-^{32}P]ATP (1μl, 1μCi) and T4 polynucleotide kinase (E.coli A19, 0.5μl, 1unit) were added, and the mixture was incubated at 37°C for 1hr. The 5'-^{32}P-labeled 21mer was separated from [γ-^{32}P]ATP by DEAE-cellulose TLC using Homo-mix III and eluted with 2M triethylammonium bicarbonate followed by coevaporation with water. The residue was dissolved in water (50μl) and used for the following experiments.

Partial digestion of the ^{32}P-labeled 21mer

The ^{32}P-labeled 21mer (7μl) was digested with snake venom phosphodiesterase (2μg) at 37°C in 10μl containing 50mM Tris-HCl (pH8.0) and 10mM $MgCl_2$. After 2, 5, 10, 20 and 30min, 2μl samples were taken and added to 5mM EDTA (5μl) followed by heating at

100°C for 2min. An aliquot (1µl) of the combined digest was analyzed by DEAE-cellulose TLC using Homo-mix III.

Sequence analysis of the 21mer

The partially-digested 21mer (2µl) was applied to a cellulose acetate strip (2.5cmx40cm) and electrophoresis was carried out in a pyridinium acetate buffer (pH3.5) containing 7M urea at 5,000V for 15min. After transfer onto a DEAE-cellulose plate (20cmx40cm), TLC in the second dimension was performed using Homo-mix III. An autoradiogram was obtained after overnight exposure at -80°C.

Analysis of the 5'-terminal nucleoside

The ^{32}P-labeled 21mer (2µl) was digested with nuclease P1 (1µg) in 10µl of 40mM ammonium acetate (pH5.0) at 37°C for 1hr. An aliquot (5µl) was applied to a filter paper (Toyo No.51A), and electrophoresis was carried out with four nucleoside 5'-monophosphate as markers in 0.2M morpholinium acetate (pH3.5) at 700V for 2hr.

Digestion with RNase T_2

The 21mer (0.025 A_{260} unit) was digested with RNase T_2 (1unit) in 2µl of 50mM ammonium acetate (pH4.5) at 37°C for 2hr. The mixture was heated at 100°C for 2min and then concentrated. To this residue H_2O (5.5µl), kination buffer (2µl), 100µM ATP (1µl), [γ-^{32}P]ATP (1µl, 0.16µCi) and T4 polynucleotide kinase (0.5µl, 2.5units) were added. After incubation at 37°C for 1hr, the mixture was heated at 100°C for 2min, and an aliquot (1µl) was treated with nuclease P1 (1µg) in 5µl of 40mM ammonium acetate (pH5.0) at 37°C for 1.5hr. The digest was heated at 100°C for 2min, and subjected to two-dimensional TLC on a cellulose plate (10cmx10cm) using the following solvent systems; isobutyric acid-ammonia water-water (66:1:33, v/v/v) in the first dimension and 0.1M sodium phosphate (pH6.8)-ammonium sulfate-n-propanol (100:60:2, v/w/v) in the second dimension.

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