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A new solid-phase synthesis of oligoribonucleotides by the phosphoro-*p*-anisidate method using tetrahydrofuranyl protection of 2'-hydroxyl groups

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ABSTRACT

Six nonaribonucleotides containing the 5'-splice site, one complementary nonamer and an octadecamer containing the 3'-splice site have been synthesized on a polymer support using the phosphoro-p-anisidate method. A 5'-linked 2'-O-tetrahydrofuranyl-N-protected nucleoside 3'-(o-chlorophenyl)phosphoro-p-anisidate was used as the starting nucleotide, and the chain elongated in the 3'-direction by removing the p-anisidate protecting group with isoamyl nitrite under neutral conditions. The octadecamer has been synthesized using dinucleotide blocks and a 3'-terminal trinucleotide.

INTRODUCTION

The solid-phase synthesis of oligodeoxyribonucleotides has been improved and has provided not only a large number of gene fragments but also probes for gene analysis.[1] The solid-phase method for oligoribonucleotide synthesis, on the other hand, has provided a relatively small number of products, although several approaches have been investigated either using the tert-butyldimethylsilyl,[2,3] o-nitrobenzyl,[4] or tetrahydropyranyl[5] groups for protecting the 2'-hydroxyl groups or using 2'-methoxytetrahydropyranyl protection in combination with the 5'-levulinyl group.[6]

We have previously reported the synthesis in solution of the 5'-half of a tRNA molecule using 5'-dimethoxytrityl-2'-tetrahydrofuranyl nucleosides where the dimethoxytrityl group was removed by chelation with zinc bromide.[7] The tetrahydrofuranyl group is useful for protection because it is rapidly and completely removed at the deprotection stage. In a polymer support synthesis, however, detritylation by zinc

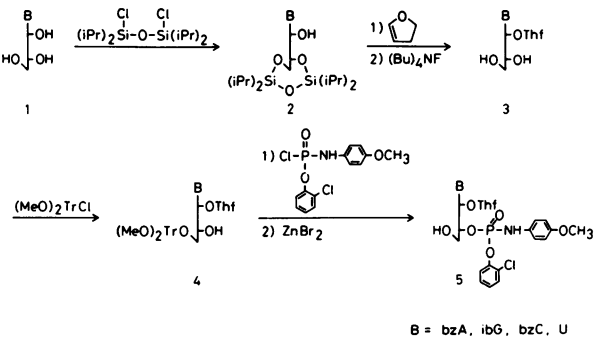

Fig. 1 Preparation of the mononucleotide unit.

bromide requires a longer period of time, and the tetrahydrofuranyl group may be cleaved by water contamination.[8,9]

For a facile and reliable synthesis of oligoribonucleotides using the tetrahydrofuranyl group, we have employed phosphoro-p-anisidate protection on the phosphate group, and these derivatives have been used for elongating the chain in the 3'-direction, as described in a preliminary report.[10] In the present paper we describe the synthesis, by stepwise elongation, of seven nonaribonucleotides containing sequences related to the 5'-splice site, and of one octadecanucleotide related to the 3'-splice site by block condensation using di- and trinucleotide units. The use of these oligonucleotides in biochemical experiments on splice reactions will be reported elsewhere.

RESULTS AND DISCUSSION

Synthesis of nonanucleotides

To synthesize oligoribonucleotides by the phosphoro-p-anisidate method in the 3'-direction, 2'-O-tetrahydrofuranyl-N-acyl nucleoside 3'-(o-chlorophenyl)phosphoro-p-anisidate units (5), were prepared as illustrated in Fig. 1. The tetrahydrofuranyl group produced two diastereomers of the protected nucleoside and each isomer was separately used in order to simplify the succeeding reactions; however, the two diastereomers of the nucleotide unit (5) caused by the chirality of the phosphoramidate were not separated.

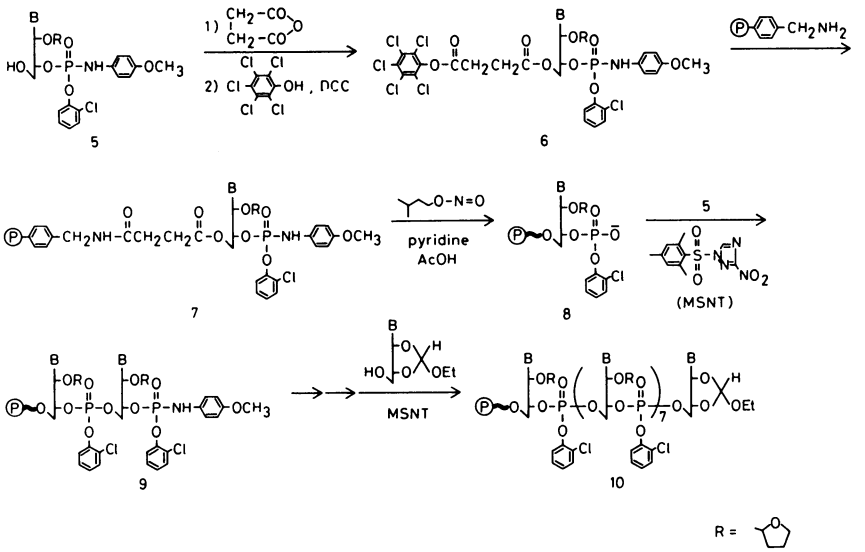

Fig. 2 Synthesis of a nonanucleotide.

Using these mononucleotide units, seven nonanucleotides were synthesized whose sequences were CAGGUAAGU, ACUUACCUG, CAGGUUGGU, CACUGACUC, CAGCUAAGU, GUAAGUAUC and GUUGGUAUC. Polystyrene with a 1% cross-link was used for the preparation of the nucleotide resins (7), and the chain was elongated by deprotecting the anisidate group using isoamyl nitrite in the presence of pyridine and acetic acid under nearly neutral conditions and by subsequent condensation of the mononucleotide unit using 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT)(Fig. 2). Aromatic amidates of protected nucleotides have been used in the phosphodiester method[11] and in the phosphotriester method,[7] and the conditions used for deprotection of phosphoro-p-anisidate are known to be tolerated by the acid-labile 5'-dimethoxytrityl and 2'-tetrahydrofuranyl groups.

The reaction conditions for the operations performed are summarized in Table 1. The washing steps after the nitrite treatment (steps 3-7) had been used previously after the zinc bromide treatment.[12] After the condensation steps, the unreacted phosphate group was capped by condensation with

Table 1 Reaction cycle for chain elongation

STEP	SOLVENT or REAGENT	AMOUNT	REACTION TIME	NUMBER OF OPERATIONS
1	pyridine-AcOH (1:1,v/v)	2 ml	0.1min	2
2	isoamyl nitrite pyridine-AcOH (1:1,v/v)	0.3ml 1.5ml	2.5hr	1
3	pyridine	2 ml	0.1min	3
4	0.5M TEAA in DMF	2 ml	0.1min	3
5	CH_2Cl_2	2 ml	0.1min	3
6	ether	2 ml	0.1min	3
7	tetrahydrofuran	2 ml	0.1min	3
8	pyridine	2 ml	0.1min	3
9	pyridine	0.3ml	co-evaporation	1
10	nucleotide in pyridine	15mg/0.3ml	co-evaporation	1
11	MSNT in pyridine	22mg/0.3ml	30 min (40°C)	1
12	MSNT 10% MeOH in pyridine	22mg 0.3ml	10 min	1
13	pyridine	2 ml	0.1min	3

methanol at step 12. For synthesizing the nonanucleotides, eight such reaction cycles were repeated, and 2',3'-ethoxymethylidene nucleoside[13] was used for the last condensation. The oligonucleotide was then cleaved from the support and deblocked by successive treatments with $\underline{N}^1$, $\underline{N}^1$, $\underline{N}^3$, $\underline{N}^3$-tetramethylguanidinium syn-pyridine-2-aldoximate, concentrated ammonia water, and 0.01N hydrochloric acid. After gel filtration, the product was purified by high pressure liquid chromatography (HPLC) using an anion exchange column, as shown in Fig. 3a. An aliquot of the product obtained in the main peak was analyzed by reversed phase HPLC (Fig. 3b) and purified further whenever impurities were detected. The yields of the purified products from the nucleotide resins (7) were 1-3%. Their chain lengths and sequences were confirmed by mobility-shift analysis[14] (Fig. 6a).

Synthesis of an octadecanucleotide

For synthesizing longer oligonucleotides by the phosphotriester approach, block condensation using di- or trinucleo-

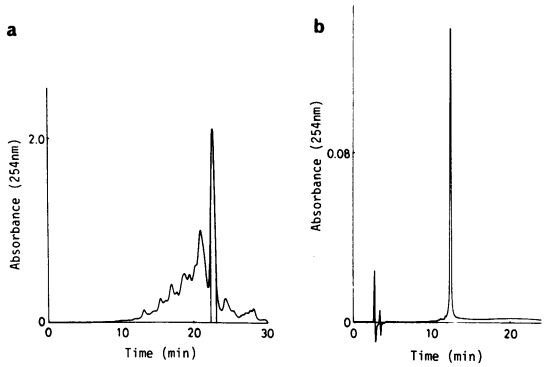

Fig. 3 Purification and analysis of the nonamer, GUAAGUAUC.
a) Anion exchange HPLC using a TSK gel DEAE-2SW column (7.8 mmI.D. x 300 mmL.) with a linear gradient of ammonium formate (from 0.4 to 0.7 M during 20 min) in 20% aqueous acetonitrile.
b) Reversed phase HPLC using a TSK gel ODS-80T_M column (4.6 mmI.D.x 250 mmL.) with a linear gradient of acetonitrile (from 7 to 15 % during 20 min) in 0.1M triethylammonium acetate (pH 7.0).

tide units is useful. To synthesize an octadecanucleotide, UCUUUCUUCUUCCAGGAU, five dinucleotide units, CU, UU, UC, CC and AG, carrying a 3'-(o-chlorophenyl)phosphoro-p-anisidate group (1~3) and a trimer GAU containing the terminal 2',3'-ethoxymethylidene group (1~5) were prepared as shown in Fig. 4. The dinucleotides (1~3) were separated from by-products by reversed phase chromatography, and the trimer (1~5) was purified by chromatography on silica gel.

The octadecanucleotide was synthesized by the same procedure as that described for the nonamers using 5 μmol (24 mg) of the uridine resin as the starting material and 20 μmol of the di- or trimer unit at each condensation step. The cleaved and deprotected product was first purified by anion exchange HPLC, as shown in Fig. 5a. Impurities in the fractions containing the main peak were separated by reversed phase HPLC (Fig. 5b). The yield was 1.5 %, based on the uridine resin, and the sequence was confirmed by mobility-shift analysis (Fig. 6b). The chain length was also confirmed by 20% polyacrylamide gel electrophoresis containing 7M urea since the

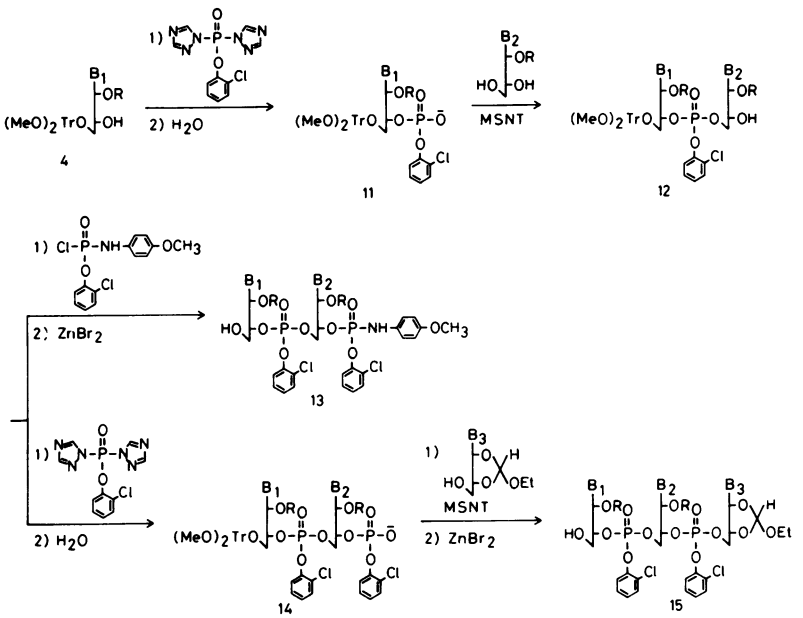

Fig. 4 Preparation of the di- and trinucleotide units.

seventeenth and the eighteenth spots in Fig. 6b were unresolved.

The phosphoro-p-anisidate method, described above, is useful for synthesizing oligoribonucleotides with chain lengths of up to 20. Although a relatively long period of time is required for deprotecting the anisidate group, the nucleotide units used for chain elongation are stable during storage. The methoxy group on the aromatic amidate makes deprotection easy since the 4-methoxyanilide group can be removed three times as fast as the anilide group, and the 3,4-dimethoxyanilide twice as fast as the 4-methoxyanilide although, probably due to steric hindrance, the 2,4-dimethoxy derivative reduces the rate of deprotection (data not shown). The phosphoro-3,4-dimethoxyanilidate group may, therfore, be suitable for rapid synthesis. The overall yield was lower than that in the deoxy series[15]; however, the present work provides a reproducible method for obtaining sufficient oligoribonucleotides for biochemical experiments.

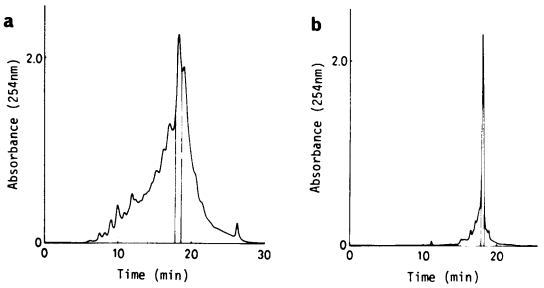

Fig. 5 Purification of the octadecamer, UCUUUCUUCUUCCAGGAU. a) Anion exchange HPLC using a TSK gel DEAE-2SW column (7.8 mmI.D. x 300 mmL.) with a linear gradient of ammonium formate (from 0.5 to 0.8 M during 20 min) in 20% aqueous acetonitrile.
b) Reversed phase HPLC using a M&S PACK C18 column (10 mmI.D. x 300 mmL.) with a linear gradient of acetonitrile (from 9 to 13 % during 20 min) in 0.1 M triethylammonium acetate (pH 7.0).

MATERIALS AND METHODS

General Methods

5'-O-Dimethoxytrityl-2'-O-tetrahydrofuranyl-N-acyl nucleosides were prepared as described previously.[7]

TLC was performed on Kieselgel 60 F_{254} plates (Merck) with chloroform-methanol (10:1, v/v). For reversed phase TLC, Kieselgel 60 F_{254} silanisiert (Merck) was used with the acetone-20 mM triethylammonium acetate (7:3 or 6:4, v/v) solvent system.

For column chromatography, Wakogel C-300 (Wako Pure Chemicals) and Preparative C18 (Waters) were used. HPLC was performed using a Gilson apparatus and the columns are described in the legends to Figs. 3 and 5.

For analyzing chain lengths and sequences, the 5'-labeled oligoribonucleotide was partially digested by venom phosphodiesterase (Boehringer-Mannheim), and homochromatography was performed on POLYGRAM CEL 300 DEAE (Macherey-Nagel) using Homomix VI for the nonamers and Homo-mix III for the octadecamer.[16]

Preparation of the mononucleotide unit (5)

5'-O-Dimethoxytrityl-2'-O-tetrahydrofuranyl-N-acyl nucleoside (4)(1 mmol) was dissolved in dichloromethane (5 ml)

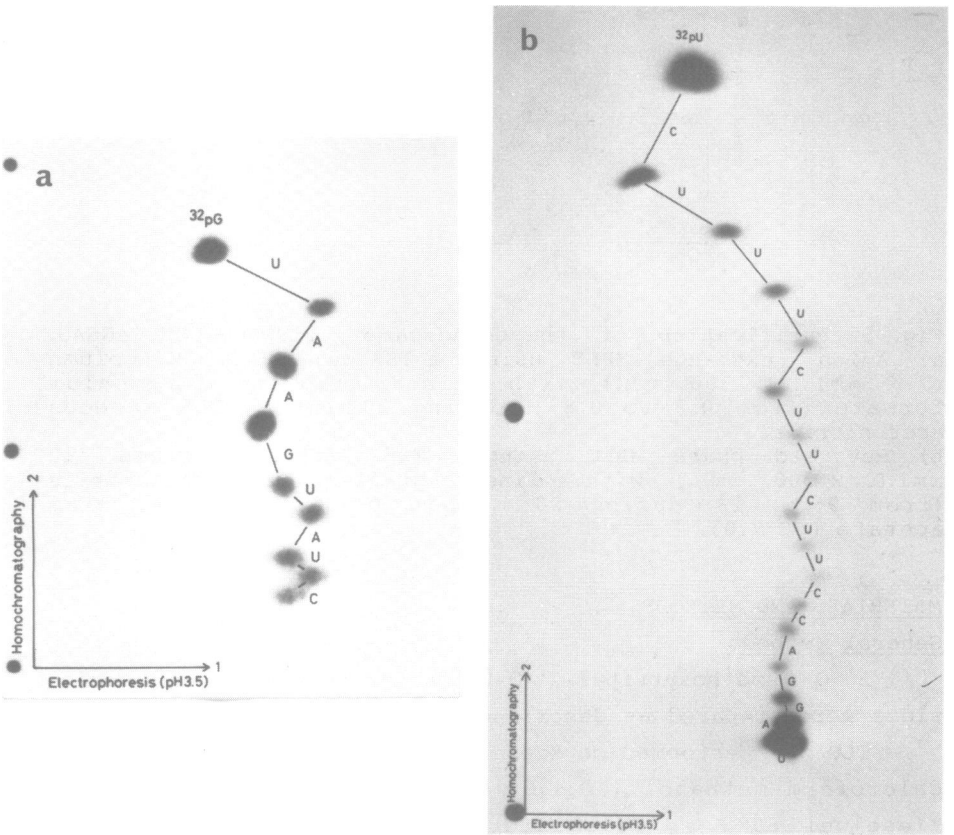

Fig. 6 Sequence analysis of the nonamer GUAAGUAUC (a) and the octadecamer UCUUUCUUCUUCCAGGAU (b).

containing 1-methylimidazole (0.16 ml, 2 mmol). This solution was cooled in an ice bath, and o-chlorophenyl phosphoro-p-anisidochloridate (0.66 g, 2 mmol) added in three portions at 15 min intervals. An hour after the last addition, dichloromethane (10 ml) was added, and the mixture washed first with saturated sodium bicarbonate and then with water. After purification by chromatography on silica gel, the fully protected nucleotide was treated with 1 M zinc bromide in dichloromethane-isopropanol (85:15, v/v)(40 ml) for 1 hr, and the mixture washed by the successive use of 1M ammonium acetate, saturated sodium bicarbonate and water. Again after

purification by chromatography on silica gel, the 2'-O-tetrahydrofuranyl-N-acyl nucleoside 3'-(o-chlorophenyl)phosphoro-p-anisidate (5) was obtained by precipitation with hexane (100 ml). When the nucleoside was uridine, the yield was 0.37 g (0.60 mmol, 60 %).

Preparation of the dinucleotide block (13, B_1=U, B_2=bzC)

5'-O-Dimethoxytrityl-2'-O-tetrahydrofuranyluridine (4, B_1= uracil-1-yl)(0.49 g, 0.8 mmol) was dried by co-evaporation with pyridine, and a dioxane solution of o-chlorophenyl phosphoro-ditriazolide (3.36 ml, 1.12 mmol) added. After stirring for 30 min, 30% aqueous pyridine was added and the product extracted with chloroform. The organic layer was washed with 0.1 M triethylammonium bicarbonate, dried with sodium sulfate, and the solvent evaporated. To this residue 2'-O-tetrahydrofuranyl-N-benzoylcytidine (0.42 g, 1 mmol) in pyridine (2 ml) was added, dried previously by co-evaporation with pyridine. Following this, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT)(0.47 g, 1.6 mmol) was added and the mixture stirred for 40 min. Aqueous pyridine (30%) was then added, the product extracted with chloroform and the organic layer washed with water. The dinucleotide (12) was purified by chromatography on C18 silica gel and phosphorylated with o-chlorophenyl phosphoro-p-anisidochloridate (0.39 g, 1.2 mmol) in dichloromethane (5 ml) in the presence of 1-methylimidazole (0.096 ml, 1.2 mmol) at 0°C for 2 hr. The solution was first washed with saturated sodium bicarbonate and then with water, and the product purified by chromatography on C18 silica gel. The phosphorylated dinucleotide was treated with a 1M solution of zinc bromide in dichloromethane-isopropanol (85:15, v/v)(30 ml) for 1 hr and worked up in the same way as described above. The dinucleotide block (13) was purified by chromatography on C18 silica gel and precipitated with hexane (50 ml). The yield was 0.40 g (0.33 mmol, 42 %).

Preparation of the nucleotide resin (7)

As an example, 2'-O-tetrahydrofuranyl-N-benzoylcytidine 3'-(o-chlorophenyl)phosphoro-p-anisidate (Fig. 1, structure 5, B=N-benzoylcytosin-1-yl)(1.64 g, 2.3 mmol) was dried by co-evaporation with pyridine and treated with succinic anhydride

(0.35 g, 3.45 mmol) in dichloromethane (9 ml) in the presence of 4-dimethylaminopyridine (0.42 g, 3.45 mmol) for 2.5 hr. Potassium phosphate buffer (pH 5.0) was added to the mixture, and the organic layer washed with water. The succinyl derivative was applied to a column of C18 silica gel equilibrated with 40% aqueous acetone containing 0.1 % pyridine, and the product eluted with 60% aqueous acetone and precipitated with hexane from a chloroform solution. The yield was 1.12 g (1.38 mmol, 60 %). The 5'-succinyl nucleotide was converted to the pentachlorophenyl ester as described.[17]

The pentachlorophenyl derivative (6)(0.70 g, 0.66 mmol) was reacted with 1 % cross-linked aminomethylated polystyrene (0.21 g, 0.2 mmol) in N,N-dimethylformamide (DMF) (6 ml) in the presence of triethylamine (0.03 ml, 0.22 mmol) at 30°C for 16 hr. The resin was washed three times each with DMF, dichloromethane, and methanol. The amount of nucleotide covalently linked to the support was estimated by measuring the amino group as its picrate salt (0.09 mmol/g); the amino group which had not reacted was then capped by acetylation.

Synthesis of GUAAGUAUC

Guanosine resin (7, B=N-isobutyrylguanin-9-yl)(24 mg, 5 μ mol) was used as the starting material. The chain elongation operations are shown in Table 1. After eight cycles, the product was cleaved from the support by treatment with 0.5 M N^1, N^1, N^3, N^3-tetramethylguanidinium syn-pyridine-2-aldoximate in dioxane-water (9:1, v/v)(1 ml) at 30°C for 16 hr. The filtrate and washings (50 % aqueous pyridne) were concentrated and passed through a column (0.7 x 4 cm) of Dowex 50W x 2 (pyridinium form). The column was washed with 30% aqueous pyridine (20 ml), and the concentrated residue treated with concentrated ammonia water (10 ml) at 55°C for 5 hr. The mixture was concentrated and washed with ethyl acetate, after which the aqueous layer containing the oligonucleotide was concentrated, mixed with 0.01 N HCl (10 ml), and the pH of the solution adjusted to 2.0 by adding 0.1 N HCl. The mixture was stirred for 3 hr and neutralized with 0.1 N aqueous ammonia.

The solution was applied to a column (1.4 x 40 cm) of Sephadex G-25 equilibrated with 0.1 M triethylammonium bicarbo-

nate. The excluded fractions (140 A_{254} units) were concentrated and co-evaporated with water. An aliquot (one fourteenth) was subjected to anion exchange HPLC as shown in Fig. 3a. Fractions containing the nonanucleotide were combined (from four runs) and desalted by gel filtration on Sephadex G-25. The product (3.56 A_{260} units) was analyzed by reversed phase HPLC as shown in Fig. 3b. The overall yield was 3%, based on the nucleotide on the support. The other oligoribonucleotides were synthesized using an analogous procedure.

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