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SOLID PHASE SYNTHESIS OF OLIGORIBONUCLEOTIDES USING o-NITROBENZYL PROTECTION OF 2'-HYDROXYL via A PHOSPHOTRIESTER APPROACH

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Abstract - Oligoribonucleotides with chain length of 6-9 were synthesized on a polystyrene support using o-nitrobenzyl protection of 2'-hydroxyls via phosphotriester method. The condensation reactions between N-acyl-5'-O-monomethoxytrityl-2'-O-(o-nitrobenzyl) nucleoside 3'-(o-chlorophenyl)phosphate and nucleoside bound to the resin using 1-mesitylenesulfonyl-3-nitro-1H-1,2,4-triazole (MSNT) as a condensing agent were examined. It was found that the condensation reaction using MSNT was promoted in the presence of 1-methylimidazole as a catalyst. A mixture of MSNT and 1-methylimidazole was also found to be effective in the condensation reaction involving a dinucleotide with 3'-phosphodiester. The fully protected oligomer were deblocked and purified by chromatography in total yields of 3-21 % based on initial nucleoside bound to the support.

INTRODUCTION

For a synthesis of oligonucleotide, three synthetic approaches, i.e. phosphotriester[1,2], phosphite-triester[3-8] and H-phosphonate approaches[9-11], are available. One of the advantages of phosphite-triester and H-phosphonate method over phosphotriester method is that the condensation reaction proceeds very rapidly. In deoxyribooligonucleotide synthesis, some rapid synthetic approaches by the phosphotriester method were exploited. Efimov et al. showed the rapid condensation when 1-methylimidazole[12] or 4-alkoxypyridine-N-oxide[13] was used as a catalyst in the reaction with condensation agents such as 1,3,5-triisopropylbenzenesulfonyl chloride (TPSCl) or MSNT. Froeler and Matteucci[14] and Sproat et al.[15] employed o-(1-methylimidazoyl)phenyl phosphate protecting group in which the 1-methylimidazole moiety acted as the catalyst. The same type of activation was introduced by Sekine and Hata[16]. In this case, a 1-methylimidazole moiety attached to the meta position of one aromatic ring of dimethoxytrityl 5'-hydroxyl protecting group acted as the catalyst. By using these catalysts, the reaction rate was reported to be comparable to those of the phosphite-triester and H-phosphonate methods. In oligoribonucleotide synthesis, on the other hand, the catalyst such as 1-methylimidazole or 4-alkoxypyridine-N-oxide has never been used in the condensation reaction by phosphotriester method.

Here, we report the synthesis of oligoribonucleotides using 1-methylimidazole as a catalyst in a condensation reaction by the phosphotriester method on the polymer support.

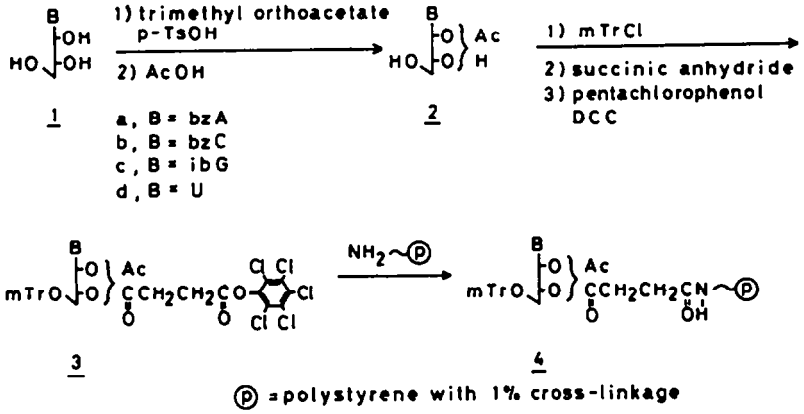

Chart 1

RESULTS AND DISCUSSION

We previously reported the preparation[7] of a nucleoside-bound resin where the nucleoside, protected by acetyl on the 2' or 3' hydroxyl, was bound to the resin via 3'- or 2'-O-succinyl linkages. On the synthesis, 2'- or 3'-O-acetyl nucleosides (2) were prepared by three step procedures. In order to prepare 2a easily, N-benzoyladenosine (1a) was treated with trimethyl orthoacetate (6 eq.)[17] in the presence of p-toluenesulfonic acid for 1 h. The mixture was then treated with aqueous acetic acid to afford a mixture of 2'-O- and 3'-O-acetyl derivatives (2a) in 80% yield after silica gel column chromatography. With the same procedure, 2b-d were prepared in 61-90% yields. The mixture 2 was converted to 5'-O-monomethoxytrityl-3'-O- and -2'-O-pentachlorophenylsuccinate derivatives (3) as described previously. The activated ester (3) was treated with aminomethylated polystyrene with 1 % cross-linkage in dimethylformamide overnight. The amount of loaded nucleosides was 134-200 μmol per gram, as determined from the amount of monomethoxytrityl cation released by 5% trichloroacetic acid in dichloromethane.

N-Acyl-5'-O-monomethoxytrityl-2'-O-(o-nitrobenzyl)nucleoside 3'-(o-chlorophenyl)phosphate (5a) and 3'-diesterified dinucleotide units (5b) were prepared according to the published procedure[18,19]

Synthetic protocol of oligoribonucleotide is shown in Chart 2 and Table 1. One cycle consists of three reactions, five washing and one co-evaporation steps. The resin (4) was first treated with 5% trichloroacetic acid in CH_2Cl_2 for 3 min to remove monomethoxytrityl group. A mixture of de-monomethoxytritylated resin (6) and properly protected 3'-(o-chlorophenyl)phosphate derivative (5 eq.) were treated with MSNT (50 eq.) for 30 min in a condensation mixture, i.e. pyridine only or pyridine containing 4-methoxypyridine-N-oxide (4 eq. of MSNT) or 1-methylimidazole (2 or 4 eq. of MSNT). After unreacted 5'-hydroxyl group of 6 was acetylated with acetic anhydride in pyridine in the presence of 4-dimethylaminopyridine (DMAP) for 1 min, the resin was subjected to acid treatment. The

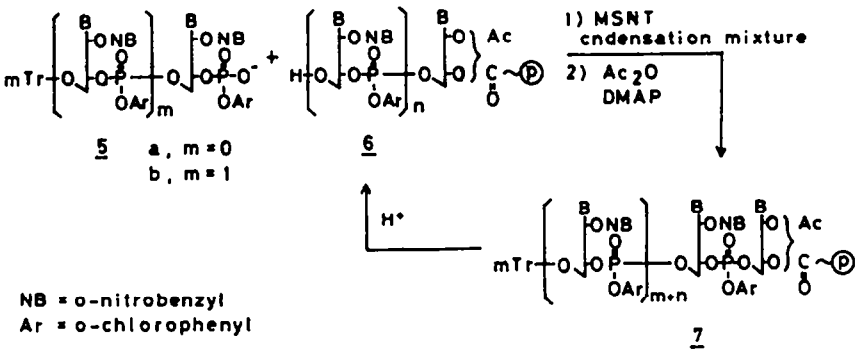

Chart 2

Table 1 Step involved in one elongation cycle

No.	Step	Solvent or Reagent	Amount*	Number of operation	Time
1	Wash	Pyridine	2 ml	2	---
2	Capping	AC_2O(1M), DMAP(50mM) in pyridine	1.2 ml	1	1 min
3	Wash	Pyridine	2 ml	3	---
4	Wash	CH_2Cl_2	2 ml	5	---
5	Detritylation	5% Trichloroacetic acid in CH_2Cl_2	5 ml	1	3 min
6	Wash	CH_2Cl_2	2 ml	3	---
7	Wash	Pyridine	2 ml	2	---
8	Coevaporation	Nucleotide(5eq) in pyridine	0.3 ml	1	5 min
9	Condensation	MSNT(50eq), condensation mixture	0.4 ml	1	30 min

* The amount corresponds to a 3 μmol scale

time required for one elongation cycle was about 45 min. Condensation yields, determined from the amount of the released monomethoxytrityl cation, were compared for the different condensation mixtures and summarized in Table 2. When the condensation solvent was pyridine only, the average condensation yield was low at 66%. By using 4-methoxypyridine-N-oxide as a catalyst, the condensation yield was somewhat improved. However, by adding 1-methylimidazole in the reaction mixture, the best coupling yields were obtained so far tested. Even for dimer condensation, 1-methylimidazole was still effective in the condensation reaction.

After the synthetic cycles were completed, a fully protected oligoribonucleotides bound to a polystyrene resin (7), which were prepared by activation with MSNT and 1-methylimidazole, were treated with 1M N^1,N^1,N^3,N^3-tetramethylguanidinium-syn-pyridine-2-aldoximate (TMG-PAO) in a mixture of dioxane, pyridine

Table 2 Comparison of coupling yields in oligoribonucleotide synthesis

Sequence	Solvent	Addition	Coupling times	Total yield (%)	Average yields (%)
CUCUCUAAA	A	monomer	8	3.6	66
UCGUAA	B	monomer	5	13	67
UUUGGAGGG	B	monomer	8	6.0	71
CUCUCUAAA	C	monomer	8	22	83
CUCUCUAAA	D	monomer	8	37	89
UCGUAA	D	monomer	5	59	90
UUUGGA	D	monomer	5	29	80
CUCUCUAAA	D	dimer	4	24	71
UCUAAA	D	dimer	3	52	80
CUCUAAA	D	dimer	3	45	77
UUUGGA	D	dimer	3	61	85

A, pyridine; B, 4-methoxypyridine-N-oxide (4eq.) in pyridine;
C, 1-methylimidazole (2eq.) in pyridine; D, 1-methylimidazole (4eq.) in pyridine.

and water at room temperature for 12 h then with concentrated ammonia at 60° for 5 h to remove the o-chlorophenyl phosphate protecting groups and acyl exocyclic amino protecting groups and to release the nucleotidic compounds from the resin. The mixture was separated by a reversed-phase silica gel column chromatography which was performed by a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (pH 7). The desired product was then treated with 80% aqueous acetic acid for 4 h to remove monomethoxytrityl group from 5'-hydroxyl group. Finally, o-nitrobenzyl protecting groups were removed by irradiation of UV light[7] in pH 3.5 for 1 h. After gel filtration on a Sephadex G25 column to remove non-nucleotidic compounds, the mixture eluted at the void volume was analyzed and separated by reversed-phase chromatography. The yields obtained were summerized in Table 3. After the gel filtration, except for one example, the amounts of A_{260} unit obtained decreased to about 60%. This is explained by taking account of the moler absorption coefficient (ε), 5400 at 265 nm, for o-nitrobenzyl alcohol. The ε of oligonucleotides should be diminished to 60-65 % after the removal of o-nitrobenzyl group. The separation patterns of the nonanucleotide (CUCUCUAAA) synthesized by dimer addition were shown in Fig. 1. The 5'- and 2'-hydroxyl protected nonanucleotide (63 A_{260} units) were obtained by reversed phase column chromatography (Fig. 1a). After removal of the 5'- and 2'-hydroxyl protecting groups and separation on a Sephadex G25 column, 39 A_{260} units of the crude nonanucleotide were obtained. Only 12 A_{260} units of the pure nonanucleotide, however, were obtained after isolation by reversed phase HPLC, though the crude mixture is considered to contain predominantly the desired nonanucleotide from the profile of HPLC (Fig. 1b). Probably some of the nonanucleotide was clinged to the reversed-phase resin and not eluted. The same phenomena were also seen in the case of CUCUCUAAA (monomer addition). In other cases, a regular reversed-phase column was used instead of the reversed-phase HPLC at the last isolation step. The isolation of UCUAAA was shown in Fig. 2. The 5'- and 2'-hydroxyl protected hexamer was separated by reversed phase column chromatography. Then the hexamer was isolated with same column after removal of

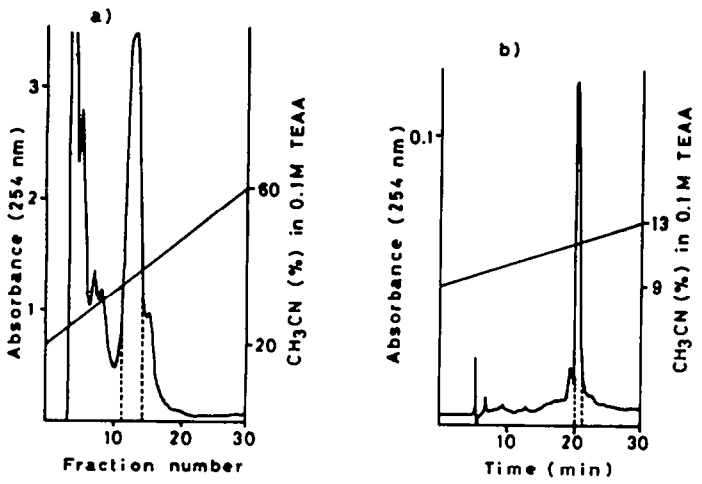

Fig 1. a) Separation of mTrC(NB)U(NB)C(NB)U(NB)C(NB)U(NB)A(NB)A(NB)A on a reversed-phase column. b) Isolation of CUCUCUAAA by reversed-phase HPLC. The fractions between the dotted lines were collected.

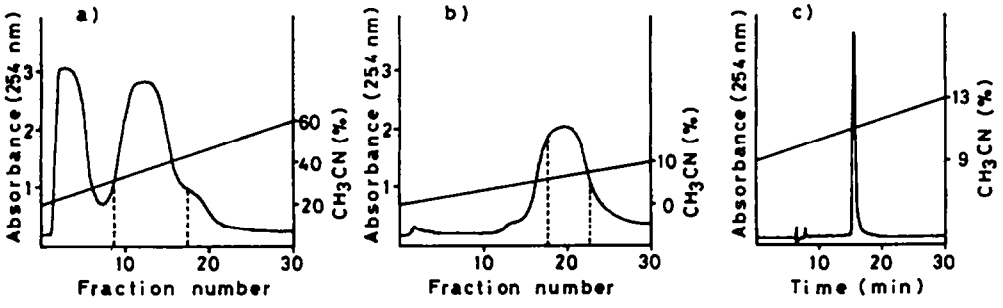

Fig 2. Separation of a) mTrU(NB)C(NB)U(NB)A(NB)A(NB)A and b) UCUAAA on a reversed-phase column. The fractions between the dotted lines were collected. c) Analysis of UCUAAA by reversed-phase HPLC.

Table 3. Yields in the synthesis of oligoribonucleotides via phosphotriester method

Sequence	Addition	Scale (μmol)	Total coupling yield, %[a)]	A_{260} unit obtained after reversed phase column	A_{260} unit obtained after Sephadex G-25 column	Isolated Yield A_{260} unit (%)[b)]	
CUCUCUAAA	monomer	3	37	70	21	8	(3%)
CUCUCUAAA	dimer	3	24	63	39	12	(4%)
UUUGGA	monomer	10	29	126	80	38	(6%)
UCUAAA	dimer	10	52	364	234	126	(21%)
UCGUAA	monomer	3	59	50	32	17	(9%)
CUCUAAA	dimer	10	45	490	282	121	(17%)
UUUGGA	dimer	3	61	99	53	37	(21%)

a) Yields were determined by the mTr cation assay.
b) Yields were calculated from the amount of initial nucleoside bound to the support.

all protecting groups. The isolation yield was somewhat improved though only the fractions which were to contain the pure hexamer by reversed-phase HPLC analysis were collected. Structures of the oligoribonucleotides were confirmed by enzymatic procedures[20,21].

In conclusion, it was found that 1-methylimidazole was effective as a catalyst in the condensation reaction using MSNT for oligoribonucleotide synthesis by phosphotriester method. The condensation reaction was over in 30 min. Even when dimers were used, the coupling yields did not decrease. After the synthesis, the procedures for the removal of 5'-hydroxyl and base protecting groups, excision from the support and the separation were almost the same as in the case of oligodeoxyribonucleotide synthesis. Finally, o-nitrobenzyl groups were removed by UV irradiation and oligoribonucleotides were efficiently recovered by gel filtration column chromatography. This process was also performed easily. Therefore this method is comparable to that for oligodeoxyribonucleotide synthesis in ease and efficiency. By using this method, we could obtain several oligoribonucleotides, which were located at the 5' or 3'-exon-intron junction of Tetrahymena thermophila ribosomal RNA[22], in relatively large amount, which is sufficient for NMR study. NMR study using these oligoribonucleotides will be published elswhere.

EXPERIMENTAL

Thin-layer chromatography (TLC) was performed on a plate of Kieselgel $60F_{254}$ (Merck). For column chromatography, Kieselgel 60 (Merck) was used. Reversed-phase chromatography was performed on an alkylated silica gel (C-18, 55-105μ, Waters Associates Inc.)column. High pressure liquid chromatopraphy (HPLC) was performed on an Altex 322Mp chromatography system. For HPLC work, Nucleosil (C-18, 5 μ, Macherey-Nagel) was packed under 500 Kg/cm^2 in the stainless steel column (φ0.6 × 20 cm). UV spectra were measured on Hitachi model 200-10 spectrophotometer. Conditions for removal of the o-nitrobenzyl group was described previously[7].

Preparation of 2'- or 3'-O-acetylnucleoside (2)

N-Acylated nucleoside (1) (4 mmol) was mixed with p-toluenesulfonic acid monohydrate (200 mg) and trimethyl orthoacetate (3 ml, 23.6 mmol) with stirring at room temperature for 1 h. In the case of 1a,b, the mixture was dissolved in dichloromethane (30 ml) and washed with water (10 ml × 2). In the case of 1c, dichloromethane (20 ml) and methanol (20 ml) was added to the mixture. Then the resultant precipitates were filtered off and washed with methanol (10 ml). The eluant and washing were combined, diluted with dichloromethane (50 ml) and washed with water (10 ml × 2). The organic solvent was evaporated. In the case of 1d, the reaction mixture was neutralized by NH_4OH and directly evaporated. The residue was dissolved in a mixture of acetic acid (4 ml) and water (20 ml). After 10 min, the mixture was evaporated to an oil which was applied onto a column of silica gel (40 g). Elution was performed with a stepwise gradient of methanol in dichloromethane. Fractions containing the 2'- and 3'-O-acetylated product (2) were collected and evaporated to give a solid. Yield was 61-90 %.

Preparation of ribonucleoside polystyrene (4)

To a suspension of aminomethylated polystyrene (1 g) in dimethylformide (10 ml), the pentachlorophenyl ester derivative (3) (476 mg) and triethylamine (133 μl) were added. After the mixture was shaken at room temperature overnight, the

resin was collected by suction filtration, washed successively with dichloromethane, methanol, dichloromethane, ether and dried under vacuum. Then to acetylate the unreacted amino group of the resin, a mixture of pyridine (8 ml) and acetic anhydride (2 ml) was added to the resin, which was kept at room temperature for 2 h. The resin was collected by suction filtration, washed successively with pyridine, dichloromethane, methanol, dichloromethane, ether and dried under vacuum. The polystyrene was loaded with the ribonucleoside to the extent of 134-200 μmol per gram.

Synthesis of oligoribonucleotide

A small sintered glass filter (φ1.5 × 7 cm) was used as a reaction vessel[7]. The ribonucleoside resin (4) was placed on the sintered glass filter. For removal of monomethoxytrityl group, the resin was suspended in 5% trichloroacetic acid in dichloromethane for 3 min. After the acidic solvent was drained, the resin was washed successively with dichloromethane and pyridine. Nucleotide (5) (5 eq.) dissolved in pyridine was added to the resin and co-evaporated. To the mixture, condensation mixture that is MSNT (50 eq.) in pyridine or MSNT (50 eq.) and 4-methoxypyridine-N-oxide (200 eq.) in pyridine or MSNT (50 eq.) and 1-methylimidazole (100 or 200 eq.) in pyridine, was added and the mixture was kept at room temperature for 30 min. The resin was washed with pyridine, then treated with 1M acetic anhydride in pyridine in the presence of 50 mM DMAP for 1 min. The resin was washed successively with pyridine and dichloromethane.

Deblocking and Purification

The resin (7) was mixed with 1M TMG-PAO in a mixture of dioxane-pyridine-water (4:2:1, 1 ml) at room temperature for 15 h. After volatile materials were evaporated, concentrated ammonia (5 ml) and pyridine (1 ml) were added. The mixture was heated at 50° for 5 h. The resin was removed and the solution was evaporated. The residue was chromatopraphed on a reversed-phase C-18 silica gel column (φ0.7 × 9 cm for 3 μmol scale, φ0.9 × 9 cm for 10 μmol scale). Elution was performed with a linear gradient of acetonitrile in 50 mM triethylammonium acetate (TEAA) (pH 7) (20 → 60 %, total 100 ml for 3 μmol scale, total 200 ml for 10 μmol scale). The fractions eluted at 35 - 40 % acetonitrile concentration were collected and evaporated. The residue was treated in 80% aqueous acetic acid (10 ml) for 4 h at room temperature and then evaporated. The residue was dissolved in 0.1 M ammonium formate solution (pH 3.5) to adjust the oligonucleotide concentration to 10 A_{260}/ml. Then the mixture was irradiated with UV light for 60 min, evaporated to a small volume and separated by gel filtration on a Sephadex G-25 column (φ1 × 45 cm). Elution was performed with 50 mM triethylammonium bicarbonate (pH 7.5). The fraction eluted at the void volume was evaporated and the residue was separated by reversed phase C-18 HPLC or reversed phase C-18 silica gel column chromatography. HPLC was performed with a linear gradient of acetonitrile (9 → 13%) in 50 mM TEAA for 30 min at a flow rate of 0.7 ml/min. Reversed phase column chromatography (φ0.9 × 9 cm) was performed with a linear gradient of acetonitrile (0 → 10%) in 50 mM TEAA (total 200 ml).

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