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Blockwise Mechanical Synthesis of Oligonucleotides by the Phosphoramidite Method[1)]

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For use in automated synthesis of oligodeoxyribonucleotides, a dimer containing the triesterified internucleotide linkage and the 3′-phosphoramidite has been prepared. 5′-*O*-Dimethoxytritylthymidylyl-(3′-5′)-(*o*-chlorophenyl)-thymidine was converted to the 3′-(methyl)*N*,*N*-diisopropylphosphoramidite, which has been used as a condensing unit for the synthesis of pentadecathymidylate on controlled pore glass by a deoxyribonucleic acid (DNA) synthesizer.

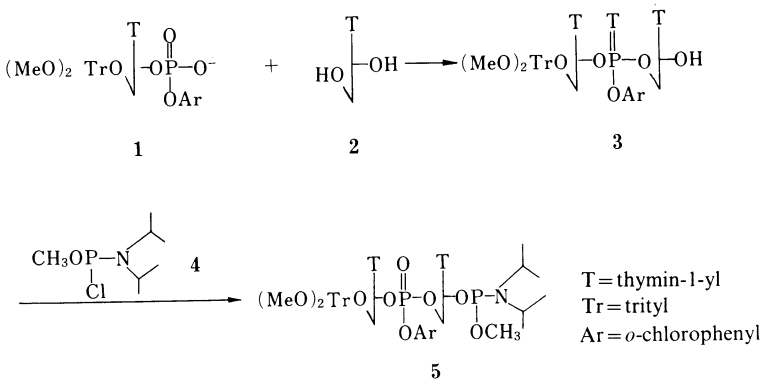
Keywords——solid-phase synthesis; *o*-chlorophenyl phosphotriester; 3′-terminal phosphite; mixed dinucleotide unit; DNA synthesizer

Solid-phase synthesis of polydeoxyribonucleotides has made it feasible to obtain a large number of gene fragments, as described in review articles.[2a,b)] The solid-phase phosphotriester synthesis using dinucleotide units has been applied to oligomers with 10—20 nucleotides,[2c,d)] and the use of the phosphoramidite synthesis on a machine can yield oligodeoxyribonucleotides with at least 20 nucleotides.[3)] Products obtained by the blockwise phosphotriester synthesis usually contain less by-products than those obtained by machine synthesis. Although high pressure liquid chromatography (HPLC) can be used to purify oligonucleotides, it is desirable to isolate oligonucleotides in quantity without extensive chromatography. In this paper, we describe a machine synthesis of pentadecathymidylate by condensation of dimers containing an internucleotidic triester using the phosphoramidite method to test the feasibility of automatic blockwise oligonucleotide syntheses. The purity of products is considered to be increased as a result of using fewer condensation steps, and this should be especially advantageous in large scale syntheses of oligonucleotides.

Preparation of a Dimer Unit

As a dimer unit, 5′-*O*-dimethoxytritylthymidylyl-(3′-5′)-(*o*-chlorophenyl)thymidine 3′-(methyl)*N*,*N*-diisopropylphosphoramidite (**5**) was prepared. For this preparation a 3′-unprotected dimer containing a triesterified internucleotide phosphate (**3**) was required. Compound **3** was synthesized by condensation of 5′-dimethoxytritylthymidine 3′-(*o*-chlorophenyl)phosphate (**1**)[4)] with an excess of thymidine (**2**) using 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT)[5)] as the condensing reagent (Chart 1). Although some papers have reported the absence of symmetrical 3′-3′-dinucleoside phosphates in the reaction of 3′,5′-unprotected nucleosides,[6)] formation of a few percent of 3′-3′-dinucleoside phosphates has been described.[7)] In the present experiment the desired product (**3**) was separated from the 3′-3′ by-product by chromatography on a column of silica gel in a yield of 71%.

The 3′-unprotected dimer (**3**) was converted to the phosphoramidite (**5**) by treatment with (methyl)*N*,*N*-isopropylchlorophosphoramidite (**4**) at room temperature for 30 min, and the phosphoramidite (**5**) was purified by silica gel chromatography in a yield of 85%.



1 2 3 5

Chart 1

TABLE I. DNA Synthetic Cycle and Cleavage

Step manipulation	Solvent or reagent	Time[a]
(1) Synthetic cycle		
1 Wash resin (×1)	Acetonitrile	39 s
2 Detritylation	Trichloroacetic acid	50 s
3 Wash resin (×2)	Acetonitrile	232 s
4 Condensation	Amidite+tetrazole	60 s
5 Filtration		20 s
6 Capping	Acetic anhydride+dimethyl-aminopyridine	140 s
7 Filtration		26 s
8 Oxidation	Iodine–water	53 s
9 Wash resin (×3)	Acetonitrile	165 s
		(13 min/cycle)
(2) Cleavage		
1 Demethylation	Thiophenol	600 s×3+43 s
2 Wash resin (×3)	Methanol, acetonitrile	157 s
3 Cleavage and collection	Conc. aqueous ammonia	900 s×8+74 s
4 Wash resin (×1)	Acetonitrile	50 s

a) At room temperature (*ca.* 25 C).

Condensation of the Dimer on a Support by a Deoxyribonucleic Acid (DNA) Synthesizer

The phosphoramidite dimer (**5**) was used to synthesize pentadecathymidylic acid. The condensation to the 3′-succinylthymidine on controlled-pore glass was performed at room temperature for 50 s using a DNA synthesizer and repeated 6 times. Manipulations for a cycle of chain elongation and the cleavage procedure of the synthesized oligonucleotide from the polymer support are summarized in Table I. The overall yield was estimated as 95.7% by means of the dimethoxytrityl color reaction.[8] The product cleaved from the polymer support was treated with ammonia to remove *o*-chlorophenyl protecting groups on phosphate. Then, the dimethoxytritylated pentadecamer was isolated by reversed phase chromatography and the completely deblocked product was analyzed by reversed phase HPLC (Fig. 1a). Impurities were removed by fractionation by the same chromatography, and the product was analyzed by reversed phase and anion-exchange HPLC (Fig. 1b). Purity and the chain length were confirmed by mobility shift analysis.[9]

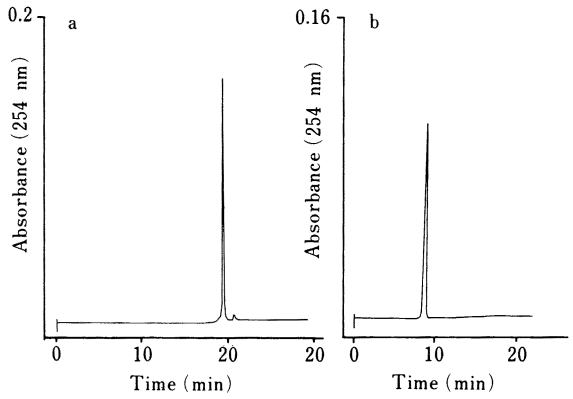


Fig. 1. a) Reversed-Phase HPLC of Pentadecathymidylate

Column: 10 × 300 mm. Solvent: A, 5% CH_3CN–0.1 M triethylammonium acetate; B, 25% CH_3CN–0.1 M triethylammonium acetate. Gradient: (B/A), 30—60%, 1.5%/min. Flow rate: 2 ml/min.

b) Anion-Exchange HPLC of Pentadecathymidylate

Column: 4.6 × 250 mm. Solvent: A, 20% CH_3CN; B, 1 M $HCOONH_4$–20% CH_3CN. Gradient: (B/A), 30—50%, 1%/min. Flow rate: 1 ml/min.

Conclusion

The dimer unit for a machine synthesis of pentadecathymidylate has been prepared by using unprotected thymidine. *o*-Chlorophenyl and methyl groups were incorporated alternatively during the synthesis as protecting group for internucleotide phosphates. The condensation yield of this relatively bulky dimer was comparable to those reported in condensations involving 3′-phosphitylated dimers,[10] despite the presence of the bulky phenyl group. The yield of the isolated product after reversed phase HPLC was about 30%. In the present particular case, anion-exchange chromatography was not required. It seems likely that a combination of a conventional dimer synthesis using 3′,5′-unprotected nucleosides and the phosphoramidite condensation of preformed protected oligonucleotides has a practical value.

Experimental

General Method——Preparation of protected nucleosides and the 3′-phosphodiester were described previously.[11] Characterization of nucleotides by thin layer chromatography (TLC) and HPLC was performed as described. Preparative silica gel chromatography was performed by using Wakogel C-300 (Wako Pure Chemical Industries) in chloroform–methanol. The phosphitylating reagent was obtained from American BioNuclear Inc. A mechanical synthesis of oligonucleotides was performed by using an Applied BioSystems synthesizer, model 380 A. Mobility shift analysis was performed after partial digestion of the 5′-phosphorylated oligonucleotide with venom phosphodiesterase.[9b] HPLC was performed by using columns of C-18 silica gel (M & S Pack, M & S Co.) and diethylaminoethyl (DEAE)-silica gel (TSK Gel DEAE-2SW, Toyo Soda Co.).

Preparation of the Dimer Unit (5)——5′-*O*-Dimethoxytritylthymidine 3′-(*o*-chlorophenyl)phosphate (**1**, 2 mmol) and thymidine (**2**, 10 mmol) were dried by evaporation of pyridine twice and dissolved in pyridine (4.8 ml). The solution was cooled in an ice bath, and MSNT (4 mmol) was added. The mixture was kept at room temperature for 1 h and treated with aqueous pyridine (30%). The product was extracted with chloroform, washed with water and applied to a column of silica gel. The 3′-5′ linked dimer (**3**) was eluted with 1.5% methanol in chloroform and analyzed by TLC (*Rf* 0.7 in 10 : 1 chloroform–methanol). The 3′-3′ linked by-product, which had a higher *Rf* value in TLC, was separated. The product (**3**) was precipitated with ether–hexane (1 : 1). The yield was 71% (1.36 g).

The dimer (**3**) (0.48 g, 0.5 mmol) was dried with benzene azeotropically and dissolved in dichloromethane (5 ml). *N*,*N*-diisopropylethylamine (0.35 ml, 2.0 mmol) was added to the mixture and the phosphitylating reagent (**4**) (0.196 ml, 1 mmol) was added through a septum by using a syringe. The reaction mixture was kept at room temperature for 30 min. The solvent was evaporated off, and the residue was dissolved in ethyl acetate. The solution was washed twice with sat. sodium bicarbonate and then with water. The product was isolated by silica gel column chromatography using a mixture of dichloromethane–ethyl acetate–triethylamine (1 : 1 : 0.05) and analyzed by TLC in ethyl acetate–triethylamine (1 : 0.05). The yield was 85% (0.475 g).

Synthesis of Pentadecathymidylic Acid——Dimethoxytritylthymidine (1 μmol) linked to controlled pore glass through a succinyl group (Applied BioSystems) in the machine was used as the support. The dimer (**5**) (0.25 g) was dissolved in acetonitrile (2 ml) and the solution was passed to the synthesizer. The condensation was repeated seven times and subsequently, the oligonucleotide was partially deblocked with thiophenol and cleaved from the support as

shown in Table I. Then, the partially deprotected pentadecathymidylate was treated with aq. ammonia (2 ml) at 50°C for 5 h. The dimethoxytritylated oligonucleotide was separated by reversed phase chromatography on a column of C-18 Silica gel using a gradient of acetonitrile (5—35%) in 0.01 M triethylammonium bicarbonate. The product was completely deblocked by treatment with 80% acetic acid and analyzed by reversed phase HPLC. The product was fractionated by similar chromatography (39.3 A_{260} units). The pentadecathymidylate thus obtained was homogeneous in reversed phase and ion-exchange chromatography.

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