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Studies on Deoxyribonucleic Acids and Related Compounds. III.[1] Synthesis of Oligodeoxyribonucleotides Complementary to the Anticodon Loop of *Escherichia coli* $tRNA_f^{Met}$ by an Improved Triester Method

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p-Chlorophenyl N-(*p*-methoxyphenyl)chlorophosphoramidate was used to phosphorylate the 3′-hydroxyl groups of N,5′-O-protected deoxyribonucleosides. These nucleotides served as the 3′-terminal unit in the synthesis of fully protected oligonucleotides, and the *p*-anisidine was readily removed by treatment with isoamyl nitrite to generate the 3′-phosphodiester for condensations. This approach was suitable for complete purification of fully protected oligonucleotides by chromatography, which was essential to obtain products. Deoxyoligonucleotides complementary to the anticodon loop of *E.coli* $tRNA_f^{Met}$ were synthesized by condensation of di-, tri- and tetranucleotide blocks (dATp, dTATp and dTTATp) with protected dGAG. The products (dA–T–G–A–G, d–T–A–T–G–A–G and d–T–T–A–T–G–A–G) were isolated by anion-exchange or reverse phase high pressure liquid chromatography.

Keywords——phosphotriester method; phosphoro-*p*-anisidate; phosphorylation; complementary oligodeoxyribonucleotide; high pressure liquid chromatography

Recently, several investigators have described improved triester approaches for the synthesis of deoxyribooligonucleotides using a variety of combinations of protecting groups for phosphates and hydroxyl groups.[2] We have reported[3] the synthesis of deoxyribooligonucleotides by the use of anilidates of phosphodiesters, which had been proved to be a suitable protecting group in the synthesis of ribooligonucleotides with chain lengths of ten[4] to twenty.[5] In the present paper we wish to report that substitution of the anilidate with *p*-anisidate can facilitate the synthesis by shortening the time required for removal; adequate stability is retained during the purification of intermediates. In the present paper, the use of the new phosphorylating reagent containing *p*-anisidine in the synthesis of penta- (**19**), hexa- (**20**) and hepta-deoxynucleotide (**21**) which are complementary to the anticodon loop of the *E. coli* $tRNA_f^{Met}$ (Fig. 1) is described. Complementary deoxyribooligonucleotides might become important substances for site-specific cleavage of RNA by the use of RNase H as reported by several investigators.[6] This would allow fragmentation of RNA, which is otherwise difficult because of the lack of restriction endonuclease type RNases such as those used in DNA fragmentation.[7] Since an enzyme able to join single-strand RNA, RNA ligase,[8] is available, joining of two pieces of RNA, insertion of oligoribonucleotides between two parts, and modification of RNA structures

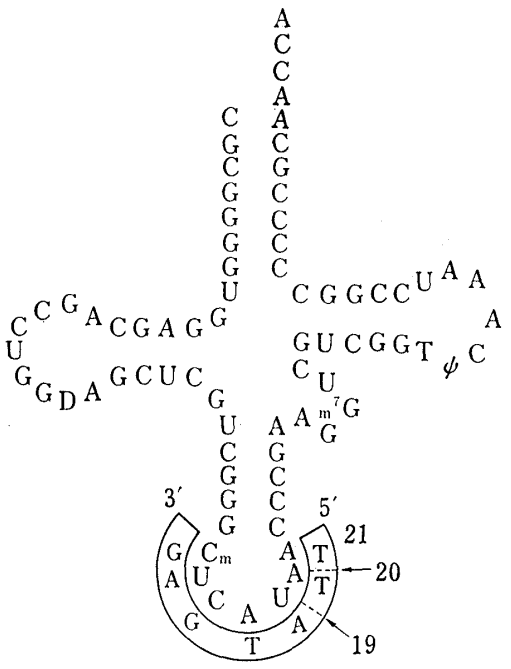


Fig. 1. *E. coli* $tRNA_f^{Met}$ and Complementary Deoxyribooligonucleotides

are feasible for studies on structure-function relationships of RNA. The availability of larger quantit ies of oligodeoxyribonucleotides with defined sequences makes it possible to carry out structural studies of DNA by X-ray diffraction analysis[9] or nuclear magnetic resonance spectroscopy.[10] Improving the purity of synthetic DNA fragments by introducing new protecting groups would facilitate those studies as well as biological studies involving DNA.

Synthesis of Protected Mononucleotides (4) by Phosphorylation with *p*-Chlorophenyl N-(*p*-Methoxyphenyl)Chlorophosphoramidate (1)

The phosphorylating reagent (**1**) was prepared by a method similar to that described for the corresponding anilidate[11] from *p*-chlorophenyl phosphorodichloridate[12] (Chart 1). The phosphorylation of 5'-O-dimethoxytritylthymidine,[13] d[$(MeO)_2$Tr]T[14] (**2a**), 5'-O-dimethoxy-

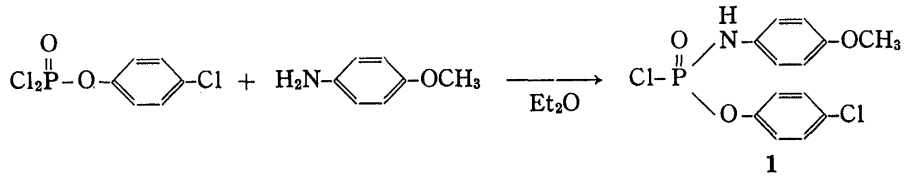
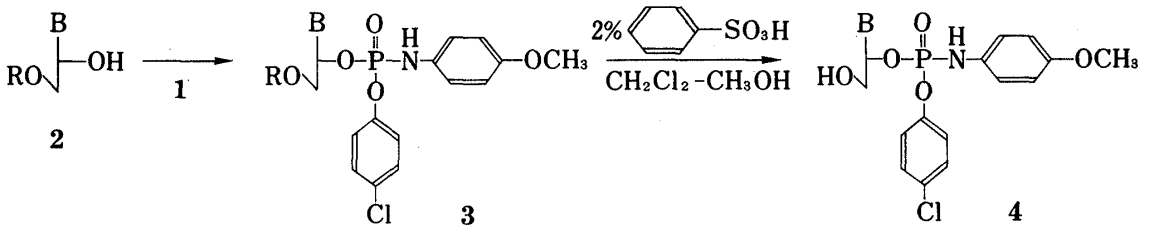
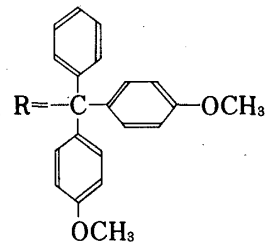


Chart 1





a: B=T
b: B=bzC
c: B=bzA
d: B=ibC

Chart 2

TABLE I. Reaction Conditions for Preparation of 3

Compound	Solvent[a]	Reagent (1) eq.	Time (h)	Isolated yield (%)
2a	Pyridine	2.0	16	93
	Dioxane[b]	2.2	2	98
2b	Pyridine	2.2	43[c]	56
	Acetonitrile[b]	2.2	6	83
2c	Pyridine	2.2	24	86
	Dioxane[b]	2.2	8	88
2d	Pyridine	3.0	24	59
	Acetonitrile[b]	3.0	4.5	88

a) Concentration: 0.25 M.
b) In the presence of 1-methyl imidazole (2.2 equivalents to 1).
c) At −20°C.

trityl-N-benzoyldeoxycytidine[13] (**2b**), 5′-O-dimethoxytrityl-N-benzoyldeoxyadenosine[13] (**2c**) and 5′-O-dimethoxytrityl-N-isobutyryl-deoxyguanosine[15] (**2d**) was performed by using the monofunctional phosphorylating reagent (**1**) in the presence of either pyridine or N-methylimidazole (Chart 2). Table I summarizes the synthesis of **3** under these codnitions. The phosphorylation in pyridine gave a high yield in the case of thymidine (**3a**) and deoxyadenosine (**3c**) derivatives. However, fully protected deoxycytidine 3′-phosphate (**3b**) was obtained as a minor product in the reaction at room temperature; this may be due to a side reaction caused by elimination of the 3-phosphate, and lowering the temperature to −20°C improved the yield to 56%. The phosphorylation of the deoxyguanosine derivative suffered from ring phosphorylation, although the extraphosphate could be removed during dedimethoxytritylation with benzenesulfonic acid (BSA).[16] N-Methylimidazole-catalyzed phosphorylation,[17] on the other hand, gave satisfactory yields (83—98%) in all four cases. The 5′-O-dimethoxytritylated nucleotides (**3**) thus obtained were treated with 2% BSA to yield the 5′-deblocked nucleotides (**4**). These nucleotides were isolated by chromatography on silica gel. Resolution

Table II. Analysis of Nucleotides **4**

		Calcd (Found)				UV in 99% EtOH	
		C	H	N	Cl	λ_{max} (10^{-4})	λ_{min} ($\times 10^{-4}$)
4a	$C_{23}H_{25}ClN_3O_8P$	51.36	4.68	7.81	6.59	266 (0.99)	248 (0.96)
	(537.89)	51.44	4.64	7.79	6.58	232 (sh)	
4b	$C_{29}H_{28}ClN_4O_8P$	55.55	4.50	8.94	5.66	302 (1.17)	285 (1.04)
	(626.99)	55.26	4.41	8.67	5.71	261 (2.56)	247.5 (1.96)
						231 (sh)	
4c	$C_{30}H_{28}ClN_6O_7P \cdot 1/2H_2O$	54.60	4.43	12.73	5.37	279 (2.28)	251 (1.17)
	(660.02)	54.35	4.20	12.51	5.56	231 (sh)	
4d	$C_{27}H_{30}ClN_6O_8P$	51.23	4.78	13.28	5.60	279 (1.39)	271 (1.30)
	(632.99)	50.98	4.69	13.08	5.46	254 (1.76)	246.5 (1.59)
						232 (sh)	

Table III. NMR Spectral Data for Compounds **4**

Compound	5-H	6-H	2-H	8-H	Ar–H	NH	ArNH	OCH_3
4a-I	1.80	7.69			6.77—7.48	11.47	8.31	3.71
	s 3	s 1			m8	bs1	d 1	s 3
4a-II	1.78	7.68			6.76—7.48	11.33	8.32	3.70
	s 3	s 1			m8	bs1	d 1	s 3
4b-I		8.34			6.78—8.04	11.26	8.34	3.70
		d 1			m14	bs1	d 1	s 3
4b-II		8.33			6.78—8.07	11.26	8.34	3.70
		d 1			m14	bs1	d 1	s 3
4c-I			8.74	8.69	6.78—8.09	11.15	8.37	3.71
			s 1	s 1	m13	bs1	d 1	s 3
4c-II			8.73	8.66	6.78—8.09	11.11	8.36	3.71
			s 1	s 1	m13	bs1	d 1	s 3
4d-I			8.26		6.80—7.51	11.73 12.13	8.34	3.71
			s 1		m8	bs1 (each)	d 1	s 3
4d-II			8.24		6.80—7.51	11.70 12.13	8.36	3.70
			s 1		m8	bs1 (each)	d 1	s 3

of diastereoisomers was observed in the dimethoxytritylated nucleotides (**3**): *e.g.*, **3b** showed two spots in thin layer chromatography (TLC) (20: 1, 0.40 and 0.49). Detritylated diastereoisomers (**4**) were not resolved under the same conditions. The two diastereoisomers of **3** were separately detritylated and **4** was identified by elemental analysis and ultraviolet (UV) spectroscopy as shown in Table II. Nuclear magnetic resonance (NMR) data of **4** detritylated separately from diastereoisomers of **3** (I, high *Rf*; II, low *Rf*) are summarized in Table III.

Preparation of Protected Oligonucleotide Blocks

Chart 3 illustrates the preparation of oligonucleotide blocks. Since the 3′-terminal fragment (**8**) did not require the 3′-phosphate, 3′-O-benzoyl-N-isobutyryldeoxyguanosine (**6**) was used as the 3′-terminal unit and condensed with diesterified internal unit (**5c**). **5c** was synthesized from 5′-dimethoxytrityldeoxyadenosine (**2c**) by phosphorylation with *p*-chlorophenyl

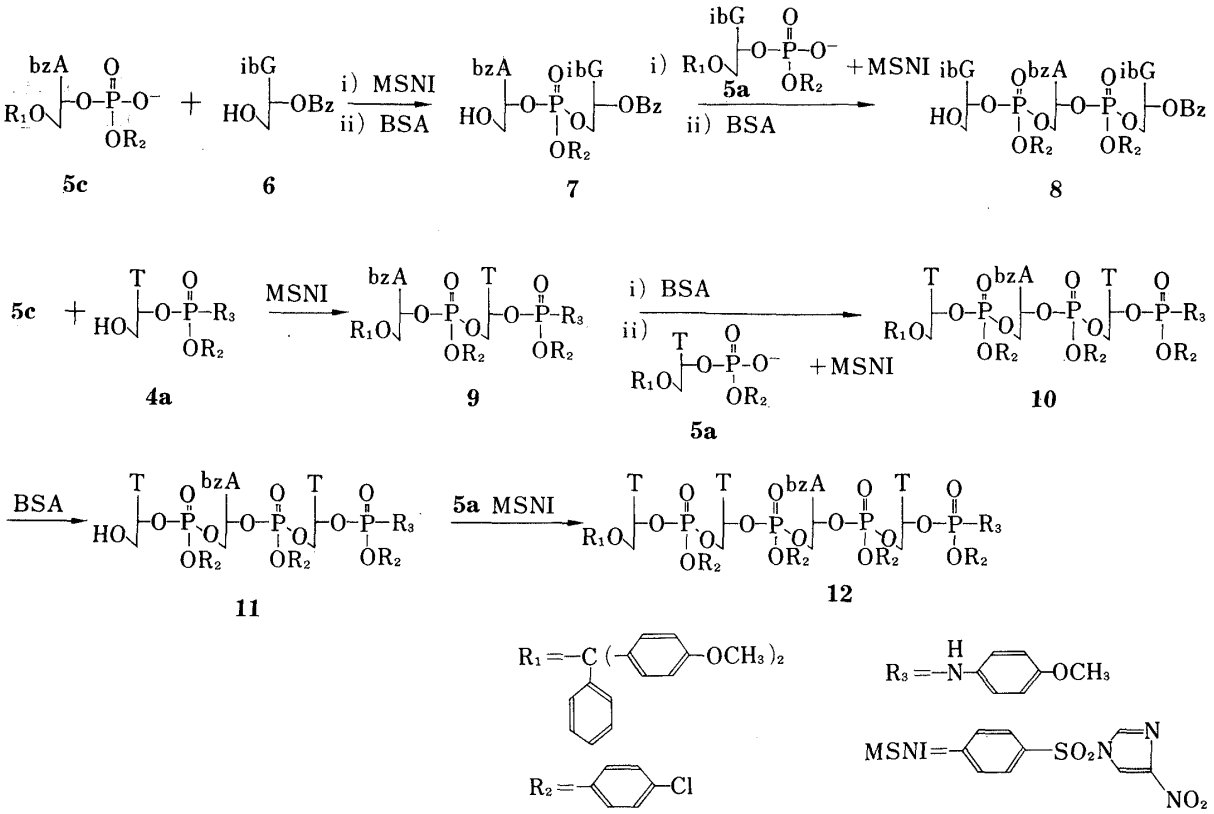


Chart 3

TABLE IV. Reaction Conditions for Condensation

3′-Phospho diester component (mmol)		5′-Hydroxyl component (mmol)		MSNI (mmol)	Reaction time (h)	Product (mmol)		Yield (%)
5c	1.2	**6**	0.7	1.8	17	**7**	0.57	81
5d	1.0	**7**	0.57	1.5	14	**8**	0.38	66
5c	1.8	**4a**	1.0	2.7	16	**9**	0.92	92
5a	1.3	**9**	0.6	1.56	15	**10**	0.58	96
5a	0.55	**10**	0.32	0.99	22	**11**	0.28	88
13	0.18	**8**	0.12	0.27	14	**16**	0.096	80
14	0.17	**8**	0.10	0.31	16	**17**	0.084	84
15	0.27	**8**	0.15	0.49	16	**18**	0.096	64

phosphoroditriazolide.[17] Mesitylenesulfonyl-4-nitroimidazolide (MSNI)[18] was used as the condensing reagent as shown in Table IV, since the nitroimidazolide was found to be quite stable and gave less by-products during condensation. However, more active condensing reagents such as the corresponding 3-nitro-1,2,4-triazolide[19] or tetrazolide[20] could have been used with a shorter reaction time, as side reactions can be minimized under certain conditions.[21]

The condensed dimer was isolated by chromatography on silica gel after removal of the dimethoxytrityl group, and the 5′-deblocked product (**7**) was then condensed with diesterified Gp (**5d**). The trimer **8** was also isolated after detritylation. All yields shown in Table IV are based on products purified by column chromatography and they thus depend on the recovery from silica gel. Stepwise increase of polarity of the eluent was important to obtain good recoveries. The purity of the oligonucleotide was checked by TLC and reversed phase TLC (RTLC). *Rf* values are listed in Table V.

Three oligonucleotide blocks **9**, **10** and **12** were prepared from the 3′-phosphorylated terminal unit (**4a**) as shown in Chart 3. Conditions for these reactions are also summarized in Table IV. These oligonucleotides were converted to 3′-phosphodiesters by decomposition of the *p*-anisidate with isoamyl nitrite as described below and condensed with **8**.

Conversion of Phosphoro-*p*-anisidate to Phosphate for Condensations and Synthesis of Penta-. Hexa- and Hepta-Nucleotides

For the next condensation the phosphoro-*p*-anisidate on **9**, **10** and **12** was converted to the phosphate to give **13**, **14** and **15**, respectively, by treatment with isoamyl nitrite for 2—2.5 h in pyridine–acetic acid (Chart 4). Decomposition of the anilidate on phosphodiesters[4] with isoamy nitrite is known to be slower than to that on phosphomonoesters[22] and prolonged treatment[1] in this slightly acidic medium was not desirable in deoxyoligonucleotide syntheses, especially when N-benzoyldeoxyadenosine was involved. Substitution of aniline with *p*-anisidine on the phosphate in **3c** shortened the reaction time markedly (7 h to 1 h). This could be due to acceleration of nitrosation on the phosphoramidate by substitution of the electron-donating group on the aniline. The times required for the removal of *p*-anisidine

TABLE V. Chromatography and Electrophoresis

	TLC $CHCl_3$–CH_3OH (10: 1)	RTLC Acetone–H_2O (7: 3)	Paper chromatography solvent A (*Rf*)	Electrophoresis pH 7.5 (Rm, dpA-dA)
[AG](Bz) (**7**)	0.33			
[GAG](Bz) (**8**)	0.11 & 0.20	0.49		
DTr[AT]p̊NHPh	0.40	0.36		
[AT]p̊NHPh	0.19	0.62		
DTr[TAT]p̊NHPh (**9**)	0.24	0.40		
[TAT]p̊NHPh	0.20	0.51		
DTr[TTAT]p̊NHPh (**10**)	0.18	0.39		
DTr[AT]p̊ (**13**)		0.81		
DTr[TAT]p̊ (**14**)		0.73		
DTr[TTAT]p̊ (**15**)		0.76		
DTr[ATGAG]p̊NHPh (**16**)	0.10	0.42		
DTr[TATGAG]p̊NHPh (**17**)	0.23	0.27		
DTr[TTATGAG]p̊NHPh (**18**)	0.09	0.66		
dA–T–G–A–G (**19**)			0.34	0.93
dT–A–T–G–A–G (**20**)			0.25	0.97
dT–T–A–T–G–A–G (**21**)			0.12	0.99

[ ]: Protected except for termini.
p: *p*-Chlorophenyl phosphoryl.

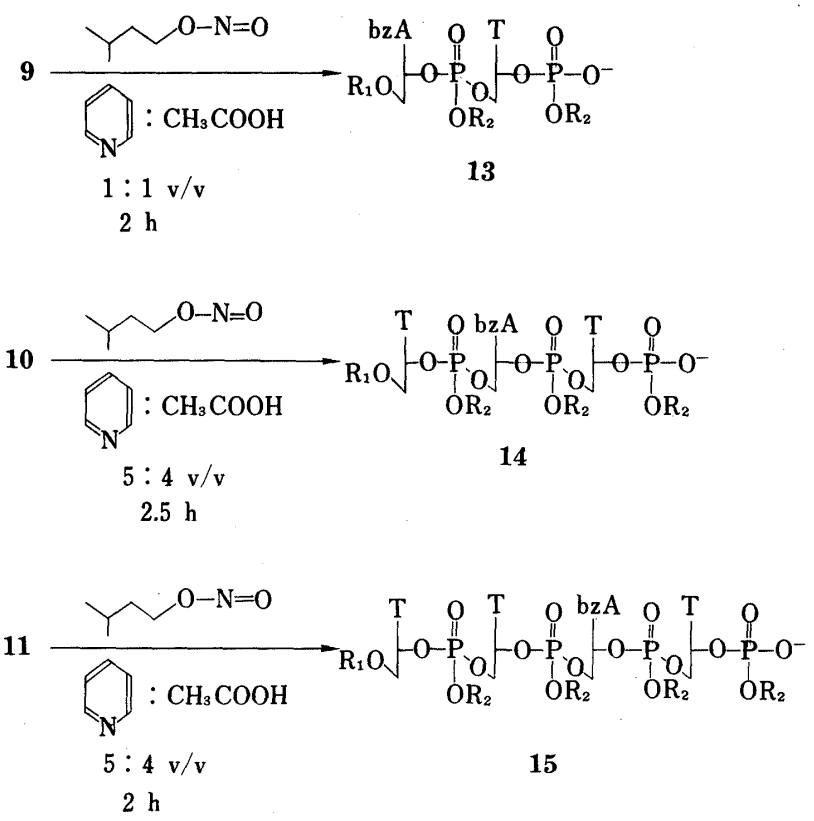


Chart 4

from the corresponding oligonucleotides were longer than for the monomer (**3c**) . Decrease of the amount of acetic acid in the medium retarded the reaction with **3c** (data are not shown), and this effect was also observed with the oligomers (Chart 4).

Synthesis of Fully Protected dATGAG (16), dTATGAG (17) and dTTATGAG (18) and Their Deblocking

To synthesize oligodeoxynucleotides, dA–T–G–A–G (**19**), dT–A–T–G–A–G (**20**) and dT–T–A–T–G–A–G (**21**), 3′-phosphodiesterified oligonucleotides (**13**, **14** and **15**) were condensed with the 3′-terminal dGAG block (**8**) and deblocked completely. Conditions for condensation are listed in Table IV and the protected penta- (**16**), hexa- (**17**) and hepta- (**18**) nucleotides were isolated by chromatography on silica gel. As shown in Table IV, the yields were satisfactory despite the high contents of purine nucleosides. *Rf* values of the protected oligonucleotides shown in Table V. The deblocking was performed by the following procedures. The *p*-chlorophenyl groups were removed by treatment with tetramethyl guanidium pyridine-2-carboxaldoximate.[19] N- and 3′-O-benzoyl groups were also removed partially by this treatment. Subsequent treatment with concentrated ammonia deblocked the acyl groups completely. Finally the dimethoxytrityl groups were removed with 80% acetic acid. Since these fully deprotected oligomers (**19**, **20** and **21**) had rather short chain length, they could be isolated by paper chromatography (Table V) and were also subjected to paper electrophoresis. Relative mobilities of the products are also shown in Table V. The products thus obtained were checked by high pressure liquid chromatography (HPLC) on anion-exchange support and a reversed phase support as shown in Figs. 2 and 3, respectively. The products isolated by reverse phase HPLC were characterized by mobility shift analysis,[23] base composition and 5′-terminal analysis.[1] These analyses were carried out essentially as described for the ribooligonucleotides.[4,24]

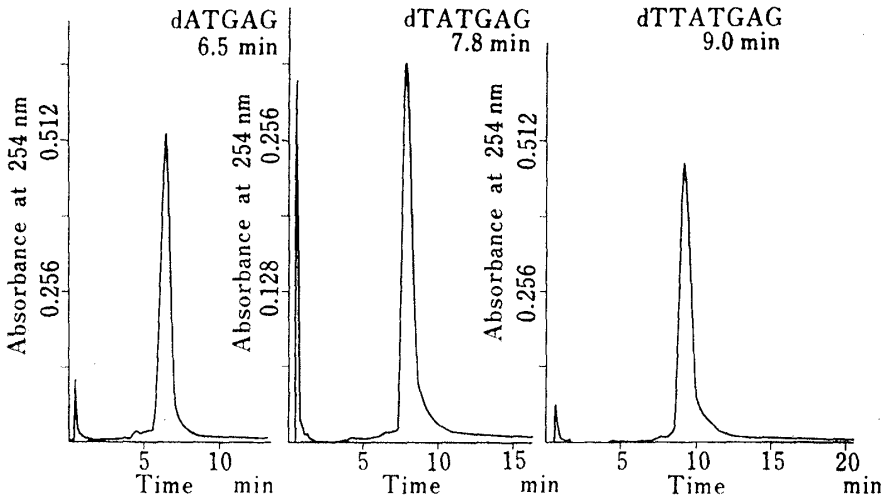


Fig. 2. Anion-exchange HPLC of the Deoxyribooligonucleotides on Permaphase AAX (2.1×500 mm)

Elution was performed with a linear gradient formed from 0.01 M KH_2PO_4 (pH 4.5) and 0.05 M KH_2PO_4 (pH 4.5) containing 1 M KCl in 30 min with a flow rate of 2 ml/min.

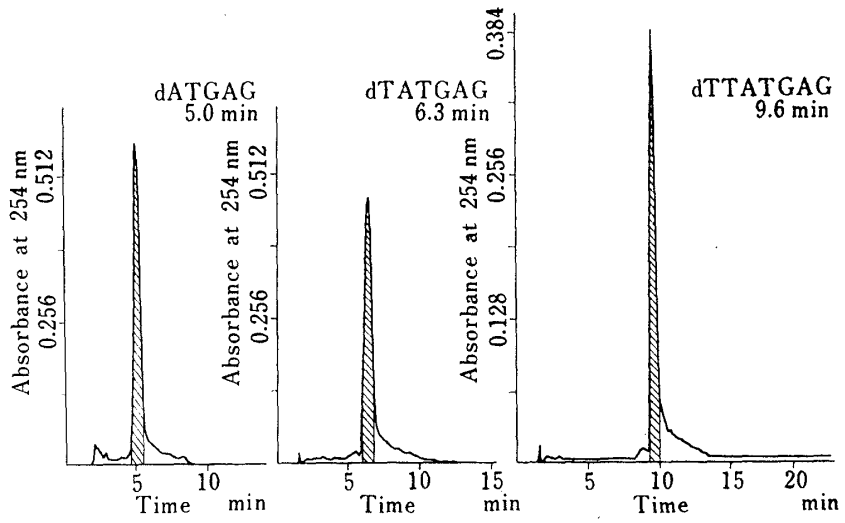


Fig. 3. Reverse-phase HPLC of the Deoxyribooligonucleotides on HypersilODS (5 μ, 4.6×250 mm)

Elution was performed with a linear gradient of acetonitrile (10—15%) in 0.1 M triethylammonium acetate (pH 7.0) in 30 min.

Experimental

Paper chromatography was performed by the descending technique using *n*-propanol–c. ammonia–water (55 : 10 : 35, v/v) Paper electrophoresis was performed using 0.05 M triethylammonium bicarbonate (pH 7.5) at 900 V/40 cm.

TLC was performed on plates of silica gel (Kieselgel 60 F_{254}, Merck) using a mixture of chloroform and methanol. For RTLC, silanized silica gel (Kieselgel 60 F_{254} silanisiert, Merck) was used with a mixture of acetone and water. For columns, silica gel (60 H, Merck) was used unless otherwise specified. HPLC was performed by using an Altex 332 MP apparatus with an anion-exchange column (Permaphase AAX, 2.1×500 mm, Dupont) or a reverse phase column (alkylated silica, Hypersil ODS 5 μ, 4.6×250 mm, Chandon).

***p*-Chlorophenyl N-(*p*-Methoxyphenyl)chlorophosphoramidate (1)**——*p*-Anisidine (37 g, 300 mmol) was dissolved in ether–benzene (2 : 1) and added to *p*-chlorophenyl phosphorodichloridate (25 ml, 150 mmol) in ether (75 ml) at 0°C with stirring. The mixture was stirred at room temperature for 6 h and the hydrochloride was removed by filtration. The precipitate was washed with ether (100 ml). The filtrate and

washings were concentrated to *ca.* 100 ml. The crystallized product was washed with pentane and recrystallized from benzene–pentane. The yield was 36.5 g, 110 mmol, 73%; mp 96°C; TLC, chloroform–acetone (5: 1) 0.52. *Anal.* Calcd for $C_{13}H_{12}$ NOP: C, 47.01; H, 3.64; N, 4.22. Found: C, 47.18; H, 3.53; N, 4.42. NMR ($CDCl_3$, δ): 6.78—7.36 (8H, m, Ar–H), 6.55 (1H, d, $J=12$ Hz), 3.78 (3H, s, OCH_3).

Preparation of 3′-*p*-Chlorophenyl Phosphoranisidate of N-Protected Deoxynucleosides (4) (Table I)——a) Thymidine-3′-O-*p*-chlorophenyl Phosphoranisidate (**4a**) prepared in the Presence of Pyridine: **2a** (1 mmol) in pyridine (4 ml) was cooled in an ice bath and the phosphorylating reagent **1** (2 mmol) was added with stirring. The mixture was stirred at 25°C for 16 h and checked by TLC. Aqueous pyridine (50%, 1 ml) and 5% sodium bicarbonate were added with cooling. The product was extracted 3 times with methylene chloride (20 ml), and the extract was washed twice with 5% sodium bicarbonate and water (15 ml). The organic layer was concentrated and the residue was coevaporated 3 times with toluene. The residue was dissolved in methylene chloride and the solution was applied to a column of silica gel (23 g). The product (**3a**) was eluted with 2% ethanol in methylene chloride and precipitated with pentane from its solution in methylene chloride. **3a** (0.3 mmol) was dissolved in methylene chloride (18 ml) and treated with benzenesulfonic acid (264 mg) in methanol (8 ml) at 0°C for 15 min. The reaction was terminated by stirring with 5% sodium bicarbonate (15 ml). The aqueous layer was extracted twice with methylene chloride (15 ml) and the combined organic solutions were washed with sodium bicarbonate and water (10 ml each). The organic layer was dried with sodium–magnesium, concentrated. The residue was purified on a small column of silica gel.

b) N-Isobutyryldeoxyguanosine 3′-O-*p*-Chlorophenyl Phosphoranisidate, 1-Methylimidazole Catalyzed Synthesis: **2d** (6.4 g, 10 mmol) and 1-methylimidazole (4.78 g, 60 mmol) were dissolved in acetonitrile (40 ml) and **1** (9.96 g, 30 mmol) was added with cooling. The mixture was stirred at 25°C for 4.5 h and the reaction was checked by TLC (chloroform–methanol, 15: 1). The bisphosphorylated isomers (*Rf*, 0.71, 0.62) and **3d** (0.42, 0.32) were detected. Water (5 ml) and methylene chloride (100 ml) were added. The organic layer was washed with 5% sodium bicarbonate (40 ml) and back-extracted twice with methylene chloride (50 ml). The combined organic solutions were washed with 5% sodium bicarbonate (40 ml) and twice with water (40 ml). The solution was concentrated, and the residue was coevaporated twice with toluene then applied to a column of silica gel as a methylene chloride solution. Elution was performed with a linear gradient of ethanol (0.5—5%) in methylene chloride containing 1% pyridine. The bisphosphorylated compounds disappeared during chromatography. **3d** was collected, concentrated, coevaporated with toluene and treated with 2% benzenesulfonic acid as above. **4d** was purified by chromatography on silica gel. The yield was 4.54 g, 7.17 mmol, 72%.

5′-O-Dimethoxytrityl-N-protected Deoxynucleoside-3′-O-*p*-chlorophenyl Phosphate (5)——1,2,4-Triazole (287 mg, 4.16 mmol) and triethylamine (0.56 ml, 4.0 mmol) were dissolved in dioxane (10 ml). *p*-Chlorophenyl phosphorodichloridate (0.333 ml, 2.0 mmol) was added with cooling and the mixture was stirred at 25°C for 70 min. The filtered solution was added to **2** (1 mmol) in pyridine (3 ml). The mixture was kept at 30°C for 40 min and the progress of the reaction was checked by TLC. Triethylammonium bicarbonate (0.1 M, pH 7.5, 20 ml) was added to the mixture. The product (**5**) was extracted 3 times with methylene chloride (20 ml). The extract was washed 3 times with 0.1 M triethylammonium bicarbonate (20 ml) and concentrated, and the residue was dried by evaporation with pyridine. The yield was quantitative.

General Procedure for Condensation——3′-Phosphodiester component (1.5—2.1 mmol) and 5′-hydroxyl component (1 mmol) were dried by coevaporation with pyridine. The mixture was dissolved in pyridine (8 ml) and treated with MSNI (2.2—3.2 mmol) at 30°C for 14—20 h. The completion of the reaction was checked by TLC (chloroform–methanol, 10: 1) and RTLC (acetone–water, 7: 3). The reaction was terminated by addition of 50% pyridine (1 ml) and 0.1 M triethylammonium bicarbonate (20 ml). The product was extracted 3 times with methylene chloride (20 ml), washed 3 times with 0.1 M triethylammonium bicarbonate (20 ml) and concentrated by coevaporation with pyridine then with toluene. The residue was dissolved in methylene chloride (5 ml) and applied to a column of silica gel (15 g). Elution was performed with a stepwise increase of ethanol concentration. Dedimethoxytritylation of protected deoxyoligonucleotides was performed as described for the synthesis of **4a**.

Deamidation of the fully Protected Oligomer (10) to give 15——**10** (612 mg, 0.28 mmol) was treated with isoamyl nitrite (1.49 ml, 11 mmol) in pyridine–acetic acid (5: 4, 7.5 ml) at 25°C for 2 h. RTLC (acetone–water, 7: 3) showed the disappearance of **10** (*Rf* 0.36), and a new spot (*Rf* 0.76) was detected as a dimethoxytrityl-positive product. The reaction was terminated by addition of pyridine (15 ml) and 0.2 M triethylammonium bicarbonate (30 ml). The mixture was washed 5 times with ether–pentane (1: 1). The product was extracted 3 times with methylene chloride (20 ml), then the extract was washed 3 times with 0.2 M triethylammonium bicarbonate (20 ml) and concentrated. The residue was dried by evaporation with pyridine and precipitated with ether–pentane (1: 1, 50 ml) from solution in pyridine (3 ml). The yield was 602 mg, 0.273 mmol, 98%.

Deprotection of the Heptanucleotide (18) to give 21 by a General Procedure——**18** (10 μmol) was dissolved in dioxane (2 ml) and treated with a solution of tetramethylguanidium pyridine 2-carboxaldoximate (0.6 M 2 ml) at 25°C for 24 h. The mixture was concentrated and dissolved in pyridine (10 ml). Concentrated ammonia (28%, 15 ml) was added and the mixture was kept at 25°C overnight then at 60°C for 6 h. Ammonia

was removed by evaporation and the solution was passed through a column (20 ml) of Dowex 50×2 (pyridinium form). The resin was washed with 30% aqueous pyridine and the combined solution was concentrated to dryness. A trace of pyridine was removed by coevaporation with toluene and the dimethoxytrityl group was removed by treatment with 80% acetic acid for 20 min; its removal was checked by TLC (chloroform–methanol, 10: 1). Acetic acid was removed by coevaporation with water and the residue was dissolved in water. The solution was washed with methylene chloride, then concentrated and the residue was dissolved in 20% pyridine (1 ml). An aliquot (0.16 ml) was subjected to paper chromatography and the product was eluted from sheets of paper with 0.1 M triethylammonium bicarbonate (61 A_{260} units).

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