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Deoxyribonucleic Acids and Related Compounds. VIII.[1] Solid-Phase Synthesis of Deoxyribooligonucleotides with 3′-Modification by Elongation in the 3′-Direction

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Four dodecanucleotides dATGTTTCCCTCTpOAr (**12a**), dATGATGTGGTATpOAr (**12b**), dATGTTGCCCGTTpOAr (**12c**) and dATGTGTCTGCGGpOAr (**12d**) having the 3′-(*o*-chlorophenyl)phosphate moiety have been synthesized on a polymer support by elongation in the 3′-direction. The 5′-succinyl group of N-protected deoxynucleoside 3′-(*o*-chlorophenyl)-phosphoro-*p*-anisidates was reacted with the amino group of the aminomethylene derivative of 1% cross linked polystyrene and the 3′-phosphoro-*p*-anisidate was converted to the phosphodiester by treatment with isoamyl nitrite. The chain was elongated by condensation of the 3′-phosphodiester with 5′-deblocked dinucleotides or trinucleotides having the 3′-(*o*-chlrophenyl)phosphoro-*p*-anisidate moiety by using 1-(mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole as the activating reagent. The reaction was repeated and products were deblocked except for the *o*-chlorophenyl group on the 3′-phosphate. The overall yields of the dodecamers were 9—16%. The method can be applied to the synthesis of oligonucleotides having nucleoside derivatives at the 3′-end.

Keywords——phosphotriester method; polystyrene support; deoxyribopolynucleotide 3′-(*o*-chlorophenyl)phosphate; HPLC; reversed-phase chromatography; ion-exchange chromatography

Deoxyribooligonucleotide syntheses on polymer supports have been shown to be very useful for the rapid synthesis of gene fragments.[2] Primers for the synthesis of complementary deoxyribonucleic acid (DNA) by reverse transcription can also be obtained by polymer-supported synthesis.[3] These syntheses have involved activation of incoming nucleotides or nucleotide blocks and elongation of the chain in the 5′-direction, since 3′-succinylated deoxynucleosides have successfully been used in linking to aminomethylene derivatives of polystyrene.[4] Products synthesized by this approach have the 3′-hydroxyl end and can serve as substrates for DNA ligase or polymerases.[5] In the present paper we describe a new approach for the solid phase synthesis of deoxyribooligonucleotides by elongation in the 3′-direction. By using the present method, the 3′-(*o*-chlorophenyl)phosphate moiety is introduced as a terminal modification. Complementary deoxyribooligonucleotides bearing blocked 3′-ends would stop replication or reverse transcription by forming hydrogen bonds with DNA or messenger ribonucleic acid (mRNA) at the appropriate position and would not start new chains. This type of oligomer in turn can be converted to a primer if the 3′-hydroxyl group is generated. In this respect, elongation in the 3′-direction dictates a versatile method for the synthesis of deoxyribooligonucleotides. However, activation of the 3′-phosphodiester has to be carried out on elongating molecules as shown in Chart 1. A recent report on a polymer-supported synthesis by addition of 5′-deblocked deoxyribonucleoside 3′-(*p*-chlorophenyl)-*β*-cyanoethyl phosphate gave rather efficient couplings to the elongating chain, and it was pointed out that the 5′-sulfonylation did not interfere with the chain.[6] In the present study, dinucleotide 3′-(*o*-chlorophenyl)phosphoro-*p*-anisidates were used as key

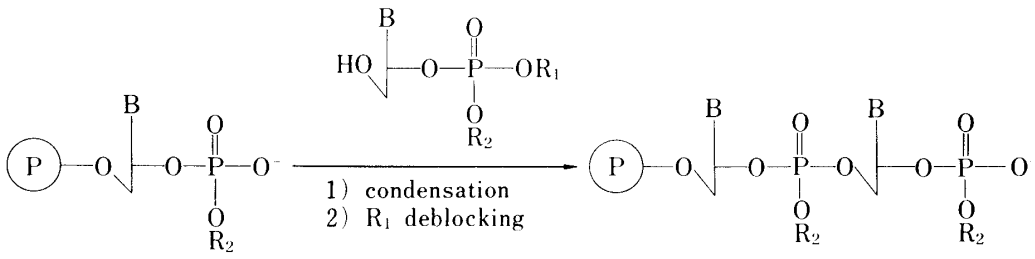


Chart 1

intermediates and the phosphoro-*p*-anisidate was converted to the phosphodiester by treatment with isoamyl nitrite[7] in each step. The phosphoro-*p*-anisidate derivatives of deoxyribonucleosides have previously been prepared and used for the synthesis of 15,[7] 17[8] and 22 mers.[8] An improved synthetic procedure for deoxyribodinucleotides having the 3′-phosphoro-*p*-anisidate moiety is also described in this report.

Preparation of Deoxyribodinucleoside 3′-(*o*-Chlorophenyl)-phosphoro-*p*-anisidates

Condensation of nucleotides using phosphoromonotriazolide derivatives (**4**, Chart 2) has

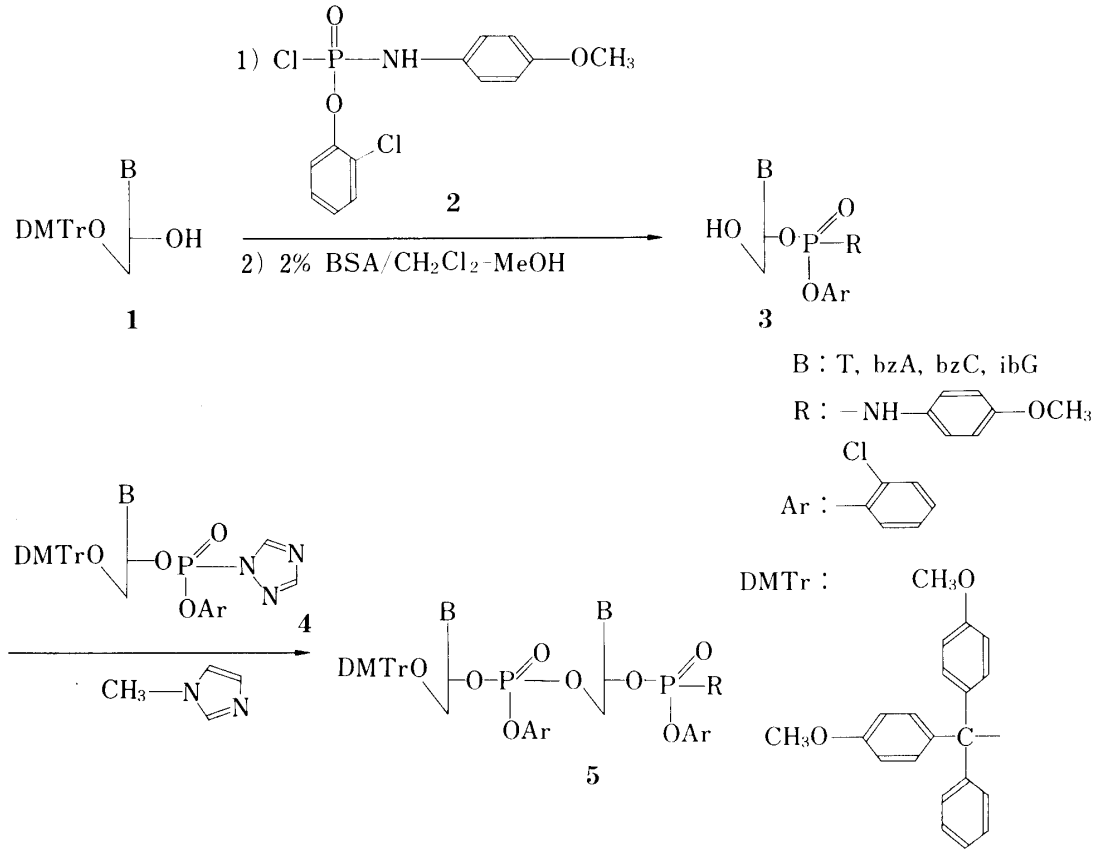


Chart 2

been used for preparation of fully protected dinucleotides having 3′-*β*-cyanoethyl phosphates.[9] This approach was shown to be suitable for condensation involving deoxyribonucleoside 3′-(*o*-chlorophenyl) phosphoro-*p*-anisidates (**3**), since the anisidate is more stable than the *β*-cyanoethyl ester in the presence of 1-methylimidazole, which is a catalyst for activation of the phosphoromonotriazolide. The yield and analytical data of **3** are summarized in Table I. Dichloromethane was found to give high yields in the phosphorylation of **1**

TABLE I. Data for the Mononucleotides (3)

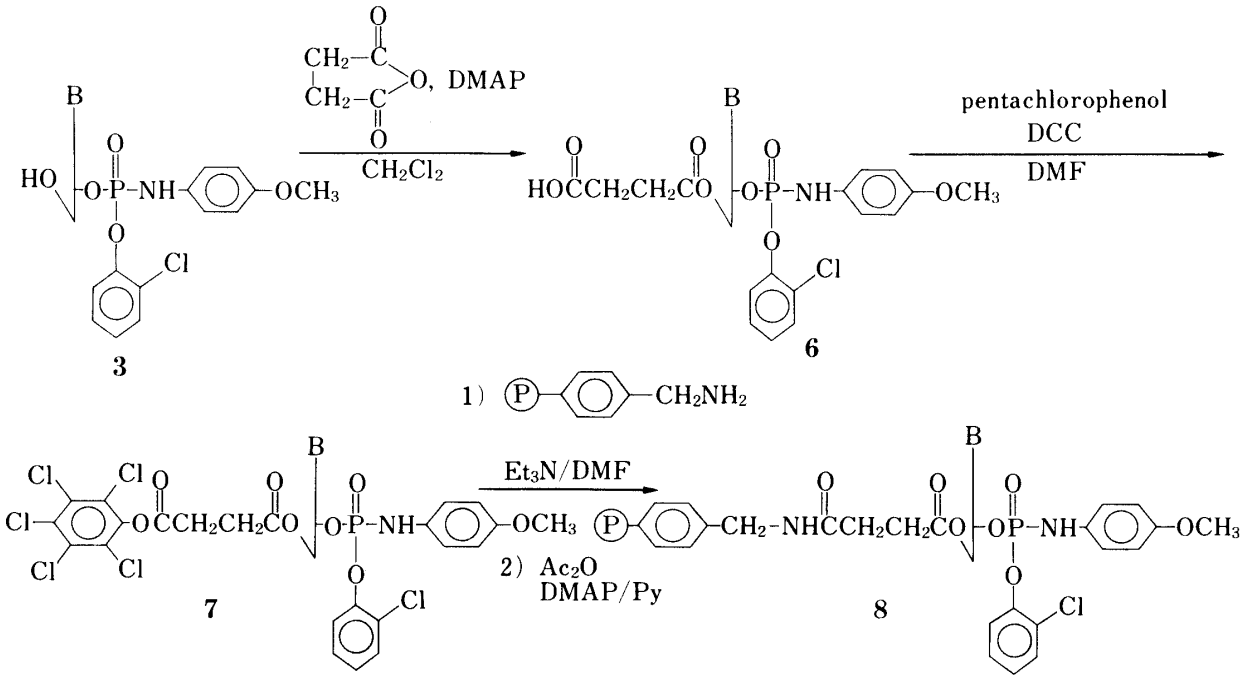
Compound	Yield (%)		Elemental analysis (%) Calcd (Found) C	H	Cl	N	TLC (*Rf* value[a])	RTLC (*Rf* value[b])
3a[c]	91	$C_{23}H_{25}ClN_3O_8P$ (537.89)	51.36 (51.14	4.68 4.63	6.59 6.69	7.81 7.65)	0.29	0.72
3b	83	$C_{29}H_{28}ClN_4O_8P$ (626.99)	55.55 (55.58	4.50 4.61	5.66	8.94 8.90)	0.38	0.66
3c	85	$C_{30}H_{28}ClN_6O_7P \cdot H_2O$ (669.03)	53.86 (53.64	4.22 4.44	5.30 5.18	12.56 12.56)	0.39	0.62
3d	85	$C_{27}H_{30}ClN_6O_8P \cdot 2/3H_2O$ (645.01)	50.28 (50.23	4.69 4.58	5.50 5.54	13.03 13.13)	0.24	0.68

a) Solvent system: $CHCl_3$–MeOH (10 : 1).
b) Solvent system: acetone–H_2O (7 : 3).
c) **3a**, B = T; **3b**, B = bzC; **3c**, B = bzA; **3d**, B = ibG.

with *o*-chlorophenyl phosphoro-*p*-anisidochloridate[7] (**2**). The same solvent was also used to prepare phosphoromonotriazolides (**4**) and a 10 fold excess of pyridine with respect to the phosphorylated monomer (**4**) was used to prevent dedimethoxytritylation; it also accelerated the coupling with the 5′-hydroxyl components (**3**). The yields of fully protected dinucleotides (**5**) were between 84—95% using a two fold excess of the 3′-phosphodiester component. Fully protected trinucleotides were prepared by elongation in the 5′-direction by dedimethoxytritylation of **5** with benzenesulfonic acid (BSA)[10] followed by condensation with **4** using a procedure similar to that described for **5**.

Preparation of 5′-Linked Deoxynucleotide Resin

The 5′-deblocked nucleosides (**3**, B = Thymin-1-yl and *N*-benzoyladenin-9-yl) were converted to the 5′-succinyl derivatives (**6**) as shown in Chart 3 by treatment with succinic



DCC: dicyclohexylcarbodiimide.

Chart 3

anhydride in the presence of 4-dimethylaminopyridine using a procedure similar to that described for preparation of the 3′-succinyl-5′-dimethoxytrityl-*N*-protected deoxynucleosides.[4a] The carboxyl group of **6** was activated by esterification with pentachlorophenol and linked to aminomethylated polystyrene containing 1 or 2% divinylbenzene to yield the nucleotide resin (**8**). Unreacted amino groups on the resin were blocked by acetylation.

Synthesis of Dodecamers Having the 3′-*o*-Chlorophenyl Phosphate Moiety

Four dodecamers having the 3′-*o*-chlorophenyl phosphate moiety dATGTTTCCCT-CTpOAr (**12a**), dATGATGTGGTATpOAr (**12b**), dATGTTGCCCGTTpOAr (**12c**) and dATGTGTCTGCGGpOAr (**12d**) were synthesized as illustrated in Chart 4. Dimers (**9**) or

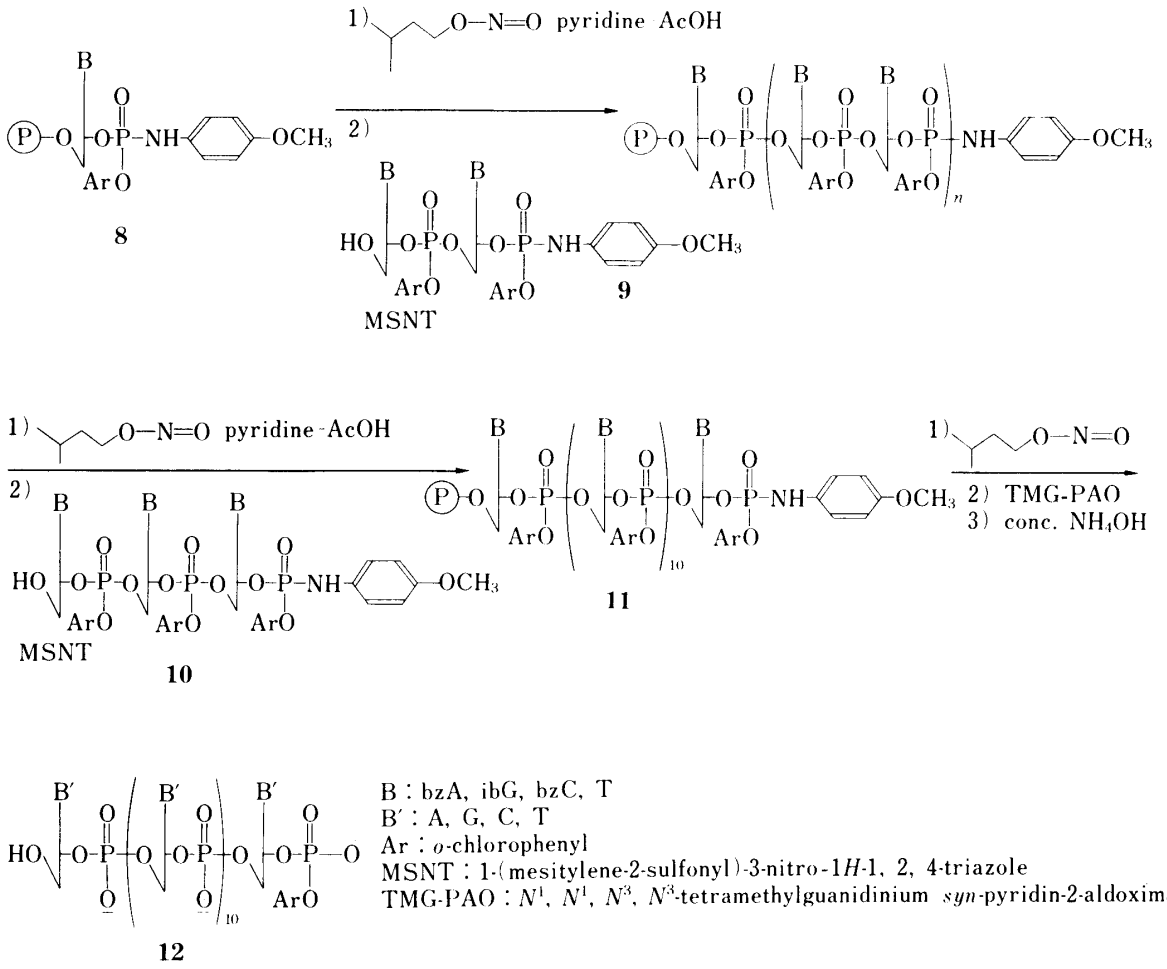


Chart 4

trimers (**10**) were condensed to the 3′-phosphodiester on the growing chain which had been generated by treatment of the phosphoro-*p*-anisidate with isoamyl nitrite. Oligonucleotide blocks used in each condensation are shown in Table II. 1-(Mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT)[11] was used as the condensing reagent. Procedures for condensation at each step are summarized in Table III. After condensation, unchanged phosphodiester ends were blocked by treatment with methanol and the same condensing reagent. The dodecamers (**11**) thus synthesized were deblocked first with isoamyl nitrite[7] to remove the anisidate and with N^1, N^1, N^3, N^3-tetramethylguanidinium *syn*-pyridin-2-aldoximate (TMG-PAO)[11] to cleave the succinyl group as well as the *o*-chlorophenyl ester. The oligonucleotides cleaved from the support were *N*-deacylated by treatment with concentrated ammonia and separated

TABLE II. Oligonucleotide Blocks (**9**, **10**) Used for Dodecamers (**12**)

Resin–dA cross link	Fully protected oligonucleotides in each step (μmol)					Product
	1	2	3	4	5	
1%	TG	TTG	CCC	TCT		**12a**
5 μmol	20	20	20	20		
2%	TG	ATG	TG	GT	AT	**12b**
20 μmol	60	60	60	60	60	
2%	TG	TTG	CCC	CTT		**12c**
24 μmol	60	60	60	60		
1%	TG	TG	TCT	GC	GG	**12d**
5 μmol	20	20	20	20	20	

TABLE III. Procedure for the Synthesis

Step	Solvent or reagent	Amount (ml)	Shaking time (min)	Number of operations
1	Pyridine	2	0.5	2
2	Isoamyl nitrite	0.5	2.5 h	1
	in pyridine–AcOH	2.5		
3	Pyridine–AcOH	2	0.5	2
4	0.5 M TEAA in DMF	2	0.5	3
5	CH_2Cl_2	2	0.5	3
6	Ether	2	0.5	3
7	THF	2	0.5	3
8	Pyridine	2	0.5	2
9	Dimer or trimer in pyridine		Co-evaporation	
10	MSNT in pyridine		30	2
11	Pyridine	2	0.5	1
12	MeOH in pyridine	0.3	10	1
	MSNT			

TEAA; triethylammonium acetate: THF; tetrahydrofuran.

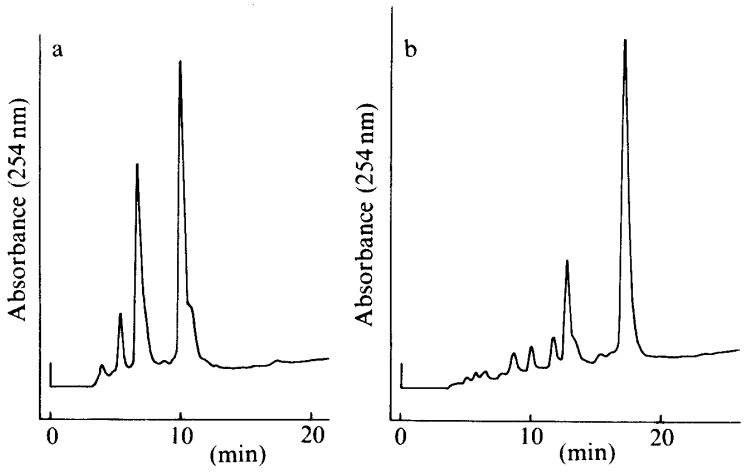


Fig. 1. Anion-Exchange HPLC

a, dATGTTTCCCTCTp(OC_6H_4Cl) (**12a**); b, dATGTGTCTGCGGp(OC_6H_4Cl) (**12d**). A column (4.6 × 250 nm) of Partisil-10 SAX was used with a gradient system of the following buffers: A, 0.005 M KH_2PO_4, pH 6.3; B, 0.3 M KH_2PO_4 (pH 6.3) in 30% ethanol. The content of buffer B was increased from 50 to 90% in 30 min with a flow rate of 1 ml/min.

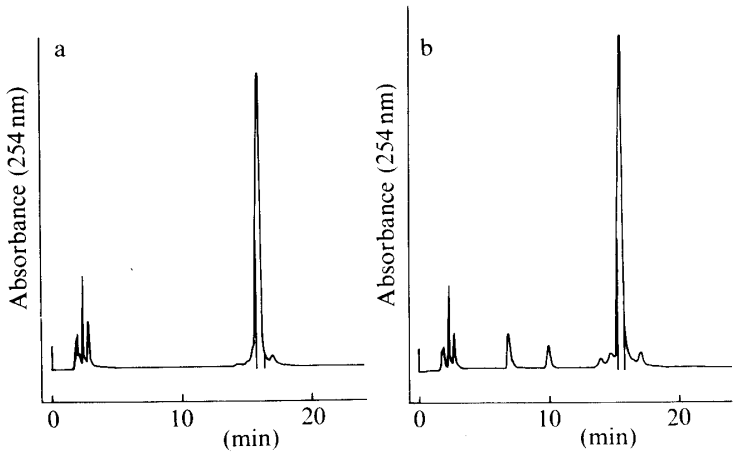


Fig. 2. Reversed-Phase HPLC

a, **12a**; b, **12d** on a column of μ-Bondapak C-18 silica gel. Elution was carried out with a gradient system of acetonitrile solutions (A, 5%; B, 25%) in 0.1 M triethylammonium acetate. The content of buffer B was increased from 25 to 75% in 20 min.

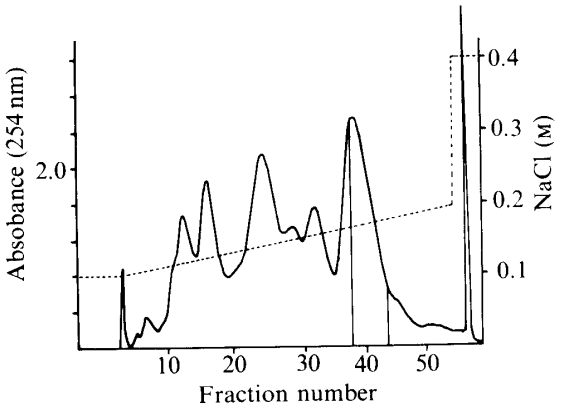


Fig. 3. Anion-Exchange Chromatography of dATGTTGCCCGTTp(CoH_4Cl) (**12c**) on DEAE-Toyopearl 650S

A column (0.7 × 22 cm) was used with a linear gradient of sodium chloride (0.1—0.2 M, total 200 ml) in 7 M urea containing 0.02 M Tris-HCl (pH 7.5). Fractions of 3.7 ml were collected every 7 min.

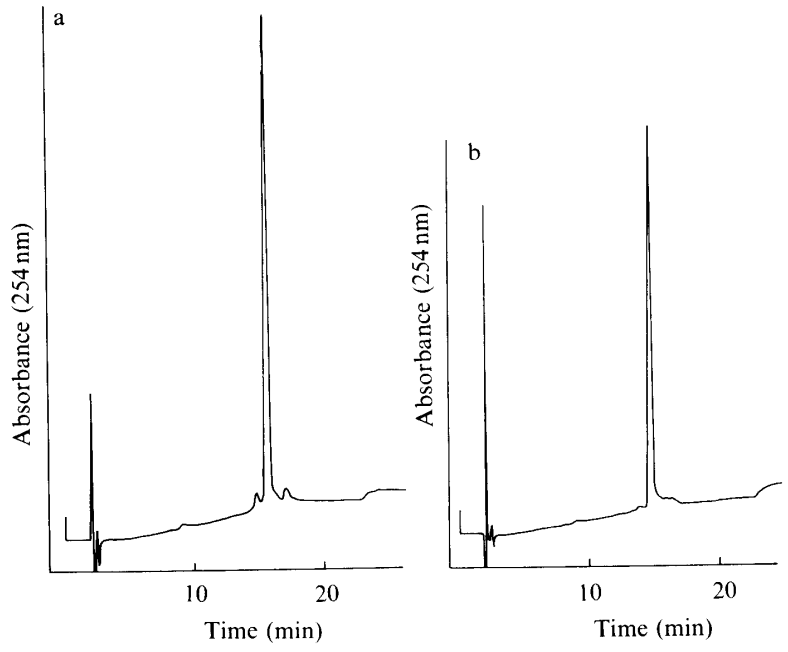


Fig. 4. Reversed-Phase HPLC

a, dATGTTGCCCGTTp(OC_6H_4Cl) (**12c**); b, dATGTTGCCCGTTp($NHC_6H_4OCH_3$). Elution conditions were the same as in Fig. 2.

by gel filtration on Sephadex G-25. Aliquots of the main peaks were analyzed by high pressure liquid chromatography (HPLC) on an anion-exchange column. The elution profiles of **12a** and **12d** are shown in Fig. 1. The products were fractionated on the same column. The purity of the dodecamers (**12a**, **d**) was checked by reversed-phase HPLC. The products were fractionated as shown in Fig. 2. The overall yield of **12d** was estimated to be 16%. Base composition was determined by complete digestion with nuclease P1[12] followed by anion-exchange HPLC. The sequence of the products (**12**) was confirmed by mobility shift analysis[13] of partially digested labeled oligomers using nuclease P1. The 3′-terminal *o*-chlorophenyl phosphate was found to be stable under the conditions used in the partial digestion. Even with five times more of the enzyme than was required to generate nucleoside 5′-phosphate, 53% of the starting material remained when thymidine 3′-(*o*-chlorophenyl)phosphate was incubated with nuclease P1 at 37 °C for 4 h. Yields of other dodecamers were almost the same as above. The products (**12b**, **12c**) obtained by using 2% cross-linked polystyrene were subjected to preparative anion-exchange chromatography on diethylaminoethyl (DEAE)-Toyopearl 650S after gel filtration. The elution profile of **12c** is shown in Fig. 3. The last peak in Fig. 3 was found to contain the dodecamer, which was further purified by reversed-phase HPLC. The HPLC profile of the purified dodecamer (**12c**) is shown in Fig. 4, together with that of the same dodecamer having the 3′-phosphoro-*p*-anisidate moiety, obtained by deblocking without the isoamyl nitrite treatment. These results indicated that the two derivatives could be distinguished by HPLC. The retention times of **12c** and the anisidate were 15.6 and 14.6 min, respectively. The stability of the anisidate at the 3′-end was tested under acidic or alkaline conditions and the moiety was found to survive under conditions such that the corresponding monomers were decomposed.[14] Oligomers having the 3′-phosphoroanisidate moiety should be stable under the conditions used for enzymatic reactions.

Conclusion

The present synthesis of the dodecamers on polymer support gave satisfactory overall yields and indicated that activation of phosphodiesters on oligomers linked to polystyrene occurred to an extent comparable with that on mononucleotides in solution. Phosphoro-*p*-anisidate protection for the 3′-end of di- or trinucleotide blocks was found to be convenient, since the group was stable during storage and reaction. However, the time required for removal of the anisidate was 2 h at 40 °C and the total time required for one cycle was greater than in conventional solid-phase syntheses in the 5′-direction, including removal of the 5′-dimethoxytrityl group. Despite this problem, the present approach has several valuable features in synthesizing oligonucleotides. Neither acid treatment nor zinc bromide treatment was required on the support. Further, by-products formed by sulfonylation at the 5′-hydroxyl group can be removed by filtration; this may be the most advantageous feature of the synthesis in the 3′-direction. The present method can be applied to any kind of oligomer for modification of the 3′-end. As regards the support, 1% cross-linked polystyrene was found to be more suitable for the synthesis of oligonucleotides than 2% cross-linked polystyrene, as reported independently during the course of this study.[15] Easier swelling and better recovery of products from 1% cross-linked resin were confirmed in the present syntheses.

Experimental

Thin layer chromatography (TLC) was performed on plates of silica gel (Kieselgel 60 F_{250}, Merck) or high performance TLC (HPTLC) plates (Merck) with a mixture of chloroform and methanol. For reversed phase TLC (RTLC), silanized silica gel or HPTLC RP-8 and RP-18 (Merck) were used with a mixture of acetone–water or

acetone–0.02 M triethylammonium acetate. For column chromatography, Silica gel 60 or 60H (Merck) or Wakogel C-200 was used with a mixture of chloroform–methanol. Reversed-phase chromatography was performed on alkylated silica gel (C-18 35—105 μ, Waters Co.) with a mixture of acetone–0.1—1% pyridine. For preparative anion-exchange chromatography, DEAE-Toyopearl 650 S (Toyo Soda Co.) was used under medium pressure. HPLC was performed using reversed-phase columns (μBondapak C-18 silica gel, Waters Co.; TSK-gel LS410AK C-18 silica gel, Toyo Soda Co.) and an anion-exchange column (Partisil-10 SAX, Whatman Co.).

***N*-Acyldeoxynucleoside 3′-(*o*-Chlorophenyl)phosphoro-*p*-anisidates (3)**——5′-*O*-Dimethoxytrityl-*N*-benzoyldeoxycytidine (**1c**) (0.6337 g, 1 mmol) and 1-methylimidazole (0.16 ml, 2 mmol) were dissolved in dichloromethane (5 ml). The mixture was cooled to −10 °C and *o*-chlorophenyl phosphoro-*p*-anisidochloridate (**2**) (0.1642 g, 2 mmol) was added in 3 portions at 15 min intervals. At 15 min after the last addition, completion of the reaction was confirmed by TLC and 5% sodium bicarbonate solution (20 ml) was added to the mixture. The product was extracted 3 times with dichloromethane (20 ml) and the solution was washed with 5% sodium bicarbonate then with water. The dimethoxytrityl group was removed by treatment of the product in chloroform (30 ml) with benzenesulfonic acid in methanol (0.38 M, 13 ml, 5 mmol) at 0 °C for 15 min. The solution was neutralized by addition of 5% sodium bicarbonate (15 ml) and the organic layer was washed with water. The aqueous layer was back extracted with dichloromethane and the extract was washed with 5% sodium bicarbonate. The organic layers were combined, and the product (**3c**) was isolated by silica gel chromatography as described previously.[7] Nucleotides **3a**, **b**, **d** were also prepared by the same procedure except that 3 eq of the phosphorylating reagent (**2**) was used for the deoxyguanosine derivative (**1d**). *Rf* values of **3a**, **b**, **c**, **d** in TLC and RTLC are shown in Table I.

Preparation of Dinucleotides (5)——5′-*O*-Dimethoxytrityl-*N*-protected deoxynucleoside 3′-(*o*-chlorophenyl)phosphorotriazolides (**4**) were prepared by phosphorylation of **1** (2 mmol) with *o*-chlorophenyl phosphoroditriazolide[9] in dichloromethane (0.326 M, 8.6 ml, 2.8 mmol) in the presence of pyridine (0.82 ml, 10.2 mmol) at room temperature for 1 h. After a check by TLC, excess phosphorylating reagent was decomposed with 1 M aqueous pyridine (0.7 mmol) at 0 °C for 10 min. The nucleotide (**3**) (1 mmol) was added at room temperature, followed by 1-methylimidazole (0.48 ml, 6.0 mmol), and the mixture was stirred for 3—6 h until TLC showed no starting material (**3**). The reaction was stopped by addition of aqueous pyridine (30%, 10 ml) and the product was extracted twice with chloroform (10 ml). The organic layer was washed once with 0.5 M KH_2PO_4 (15 ml) and twice with 0.1 M triethylammonium bicarbonate (15 ml). The dinucleotide (**5**) was purified by chromatography on silica gel (50—100 g) as described[7] or on C-18 silica gel. For example, fully protected dGAp was applied to a column (3 × 12 cm) of C-18 silica gel and eluted with a gradient of acetone (60—70%) in 0.1% pyridine. The yield was 92%. This compound was a mixture of 4 diastereoisomers which showed 4 spots (*Rf*=0.13, 0.15, 0.17 and 0.19 in 20 : 1 $CHCl_3$–MeOH) in HPTLC and migrated as a single spot in RTLC (*Rf*=0.57 in 7 : 3 acetone–water).

Preparation of Deoxyadenylic Acid Resin (8)——The mononucleotide (**3c**) (0.46 mmol) was stirred with succinic anhydride (0.92 mmol) in dichloromethane (2 ml) in the presence of 4-dimethylaminopyridine (90 mg, 0.75 mmol) at 30 °C for 2 h; conversion of **3** (*Rf*, 0.55) to the 5′-succinyl nucleotide (**6**) (*Rf*, 0.38) was confirmed by TLC (10 : 1). The solution was washed with 0.5 M KH_2PO_4 and water. The product was separated by chromatography on silica gel (type 60, 3 × 1.5 cm) using 95 : 5 chloroform–methanol and then precipitated with pentane from its solution in chloroform. The yield of **6** was 85%, 290 mg, 0.39 mmol). Compound **6** (0.39 mmol) was treated with dicyclohexylcarbodiimide (DCC, 0.6 mmol) and pentachlorophenol (0.43 mmol) in dimethylformamide (DMF, 3 ml) at 30 °C overnight and the product was isolated by filtration followed by precipitation with pentane from its solution in chloroform. This active ester (**7**) (0.28 g, 0.28 mmol) was allowed to react with 1% cross-linked aminomethylene polystyrene (1 g, containing 0.13 mmol amino group) in DMF (6 ml) in the presence of triethylamine (0.31 mmol) with shaking for 26 h at room temperature. The resin was washed 3 times each with DMF (5 ml) and pyridine (5 ml), and then treated with acetic anhydride (1 ml) in 0.1 M 4-dimethylaminopyridine in pyridine (9 ml) for 30 min. The resin (**8**) was then washed 3 times each with pyridine (5 ml), dichloromethane (5 ml) and ether (5 ml). The product (827 mg) was dried *in vacuo*, and an aliquot (10.4 mg) was treated with 0.5 M TMG-PAO (0.2 ml) for 48 h then heated with conc. ammonia (2 ml) at 55 °C for 6 h. The filtrate and washings were passed through a small column of Dowex 50 (pyridinium form), and PAO was removed by washing with ethyl acetate. Deoxyadenosine 3′-phosphoro-*p*-anisidate cleaved from the resin was characterized and the amount was estimated by subjecting an aliquot (10%) to paper electrophoresis (0.05 M triethylammonium bicarbonate, pH 7.5). The content of nucleotide was found to be 0.12 mmol/g of the resin (**8**).

Synthesis of Dodecamers (12)——The 5′-deblocked dimers (**9**) and trimers (**10**) were prepared by removal of the dimethoxytrityl group of the fully protected nucleotides. For example, **5** (protected dTGp) (0.24 mmol) was treated with 2% benzenesulfonic acid in 7 : 3 dichloromethane–methanol (11 ml) at 0 °C for 20 min then neutralized with 5% sodium bicarbonate. The product (**9**) was extracted with chloroform and precipitated. The starting nucleoside resin (**8**, B = *N*-benzoyladenin-9-yl) (5—24 μmol) was allowed to react with a 2.5—4 fold excess of oligonucleotides as shown in Table II. About 60—100 μmol of MSNT was used in each step. Operations in each cycle are shown in Table III. When 2% cross-linked resin was used for the synthesis of **12b**, **c**, **8** was soaked in pyridine overnight. Deblocking was performed first with isoamyl nitrite (1 ml) in pyridine–acetic acid (1 : 1, 5 ml) at 37 °C for 3 h followed by washing as shown in Table III. About 10 μmol of the resin was treated with 0.5 M TMG-PAO (90% aqueous dioxane, 3 ml) at 25 °C for 48 h. The resin was washed with 70% aqueous pyridine (10 ml) and 50% aqueous pyridine (6 ml), and the

filtrate and washings were treated with conc. ammonia. The products **12a, d** thus obtained were roughly purified by application to a column of Sephadex G-25 (1.8 × 36 cm) and elution with 0.05 M triethylammonium bicarbonate. Aliquots were purified by anion-exchange HPLC as shown in Fig. 1 and the products in the main peaks were further purified by reversed-phase HPLC as shown in Fig. 2. Compound **12c** was subjected to ion-exchange chromatography on DEAE-Toyopearl as shown in Fig. 3 and analyzed by reversed-phase HPLC as shown in Fig. 4 together with the dodecanucleotide with the 3′-phosphoro-*p*-anisidate moiety, obtained by deblocking without isoamyl nitrite treatment.

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