Synthesis of Oligodeoxyribonucleotides Involving a Rapid Procedure for Removal of Base-Protecting Groups by Use of the 4,4′,4″-Tris(benzoyloxy)trityl (TBTr) Group

Mitsuo SEKINE,* Narihiro MASUDA, and Tsujiaki HATA*
Department of Life Chemistry, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 227
(Received January 18, 1986)

Fully protected building units for oligodeoxyribonucleotide synthesis in the phosphoramidite approach were prepared in high yields by the 5′-dimethoxytritylation of *N*-[4,4′,4″-tris(benzoyloxy)trityl]deoxyribonucleosides followed by the 3′-phosphitylation with methoxymorpholinochlorophosphine. The solid phase synthesis of oligodeoxyribonucleotides on a controlled pore glass gel using the amidite units was examined. The efficiency of condensation was discussed in detail. The 4,4′,4″-tris(benzoyloxy)trityl (TBTr) group was removed rapidly from a protected tetramer synthesized on the controlled pore glass gel to give dGpCpApT.

Recent developments of oligodeoxyribonucleotide synthesis have enabled us to synthesize relatively long DNA fragments in short time.[1] Reaction velocity of condensation has been dramatically accelerated by introduction of new technologies such as the tetrazole-catalyzed phosphoramidite approach[2] and the *N*-methylimidazole[3] or 4-substituted pyridine-*N*-oxide-promoted phosphotriester approach.[4] The use of sterically hindered diaryl phosphorochloridates also improved markedly the condensation time.[5] However, only little attention has been paid to the deprotection procedure of fully protected DNA fragments.[6] Especially, removal of *N*-acyl protecting groups from oligomers was the most sluggish in the whole synthetic procedures. Some improvements have been reported in this direction but they required rather drastic conditions such as elevated temperatures.[7]

In this paper, we report basic studies on improvement of the deprotection procedure for *N*-protecting groups by the use of 4,4′,4″-tris(benzoyloxy)trityl (TBTr) as a universal base-protecting group.

Results and Discussion

In a previous paper,[8] we showed that the TBTr group could be introduced into the four common base moieties by using the transient protection strategy. The glycosyl bond of *N*-TBTr-deoxyadenosine (**2c**) was also found to be 4 times more stable to 2% dichloroacetic acid in dichloromethane (CH_2Cl_2) than that of N^6-benzoyldeoxyadenosine. Since the TBTr group was rather resistant to acids owing to its electronic effect derived from the three benzoyloxy substituents, the 4,4′-dimethoxytrityl (DMTr) group could be used as the 5′-hydroxy protecting group in combination with the TBTr group. Therefore, we chose the latter as the *N*-protecting group in the phosphoramidite approach using the morpholino group as an activatable phosphite protecting group.

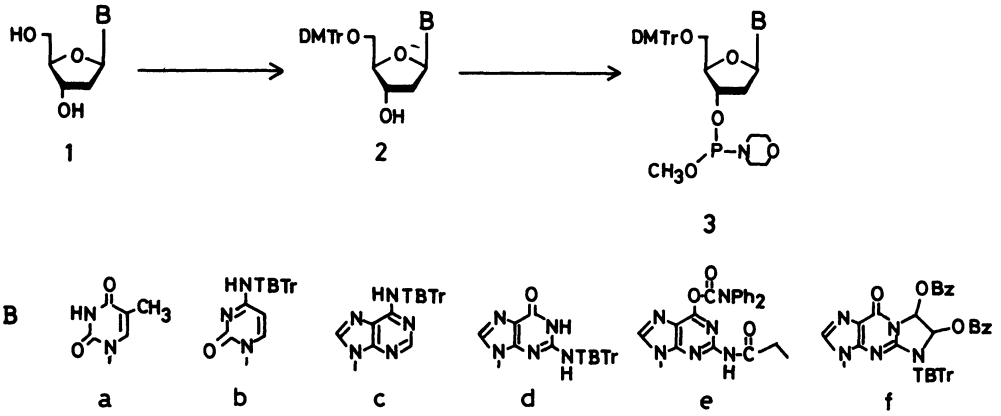
Preparation of Fully-Protected Deoxyribonucleoside Amidite Units (3b—d). *N*-[4,4′,4″-Tris(benzoyloxy)-trityl]deoxyribonucleosides (**1b—d**) were allowed to

Table 1. Synthesis of *N*-TBTr-5′-*O*-DMTr-deoxyribonucleosides (**2b—f**)

Compd	Yield %	^{1}H NMR ($CDCl_3$) ppm	Elemental analysis: Formula	Calcd (%) / Found (%) C	H	N
2b	98	1.90—2.95 (2, m, 2′-H), 3.34 (2, m, 5′-H), 3.63 (6, s, OMe), 4.03 (1, m, 4-H′),4.43 (1, m, 3′-H), 4.84 (1, d, *J*=7Hz, 5-H), 6.33 (1, t, *J*=5Hz, 1′-H), 6.65 (4, d, *J*=8Hz, ArH), 6.91—7.76 (31, m, ArH), 8.10 (6, m, ArH)	$C_{70}H_{57}O_{12}N_3$	74.26 73.26	5.07 5.36	3.71 3.63
2c	92	2.08—3.16 (2, m, 2′-H), 3.40 (1, m, 5′-Ha), 3.74, 3.87 (6, s, OMe), 3.56—3.88 (1, m, 5′-Hb), 4.14 (1, m, 4′-H), 4.66 (1, m, 3′-H), 6.33 (1, m, 1′-H), 6.81 (4, m, ArH), 7.08—8.32 (32, m, ArH)	$C_{71}H_{57}O_{11}N_5$	73.75 73.13	4.97 4.83	6.06 5.90
2d	93	2.10 (2, m, 2′-H), 3.63 (3, m, 4′-H and OMe), 4.09 (2, m, 3′-H), 5.72 (1, m, 1′-H), 6.60—6.70 (4, m, ArH), 6.92—7.63 (32, m, ArH and 8-H), 8.02—8.10 (6, m, ArH)	$C_{71}H_{57}O_{12}N_5$	72.75 72.65	4.90 4.99	6.00 5.87
2f	93	2.06 (2, m, 2′-H), 3.32 (2, m, 5′-H), 3.68, 3.73 (6, s, OMe), 4.01—4.38 (2, m, 3′,4′-H), 5.74 (1, m, 2′-H), 6.39, 6.43 (2, s, N-CH-O), 6.74 (6, m, ArH), 7.00—7.77 (31, m, ArH), 7.99 (4, m, ArH), 8.17 (6, br d, *J*=8Hz, ArH)	$C_{87}H_{67}O_{12}N_5$	72.64 72.27	4.70 4.80	4.87 4.83

Table 2. Preparation of Phosphoramidite Units (**3b—f**)

Compd	Method for separation/ eluant	Isolated yield %	^{31}P NMR ($CDCl_3$) ppm	Elemental analysis: Formula	Calcd (%) Found (%) C	H	N
3b	Basic alumina/0.5% Et_3N/ CH_2Cl_2–benzene	68	−144.5, −144.2	$C_{75}H_{67}O_{14}N_4P$	70.41 69.38	5.28 5.25	4.38 4.40
3c	Basic alumina/1% Me-N□/ CH_2Cl_2–benzene	68	−144.3, −144.1	$C_{76}H_{67}O_{13}N_6P$	70.04 69.67	5.18 5.02	6.45 6.46
3d	Silica gel/1% Et_3N/ CH_2Cl_2–hexane	63	−144.4, −143.4	$C_{76}H_{67}O_{14}N_6P$	69.19 69.74	5.12 5.12	6.37 6.42
3e	Basic alumina/1% Me-N□ CH_2Cl_2–benzene	68	−144.5	$C_{52}H_{54}N_7O_{10}P$	64.52 64.33	5.62 5.52	10.13 10.33
3f	Basic alumina/0.3% Et_3N/ CH_2Cl_2	95	−144.5, −143.7	$C_{92}H_{77}O_{18}N_6P$	69.69 69.98	4.90 4.77	5.30 5.49



Scheme 1.

react with 4,4′-dimethoxytrityl chloride (DMTrCl) to give the corresponding 5′-tritylated products (**2b—d**) in 92—98% yields (Table 1). The amidite units (**3a—d**) were synthesized by a method similar to that described by Caruthers.[2b] Reaction of **2b—d** with methoxymorpholinochlorophosphine (MMCP) in the presence of *N,N*-diisopropylethylamine in CH_2Cl_2 gave the desired products (**3b—d**). The phosphoramidites **3b—d** were obtained in more than 90% yields by reprecipitation from CH_2Cl_2 into hexane. However, the purities of the precipitated materials were less than 70% from their NMR spectra. Several experiments were conducted to purify **3b—d** by using silica-gel or alumina column chromatography developed with various solvent systems. Because of the lability of the TBTr group to secondary amines such as morpholine the inert tertiary amines, triethylamine and *N*-methylpyrrolidine, were used with CH_2Cl_2–hexane or benzene. By the use of these solvent systems, **3b—d** were satisfactorily purified and their purities increased to more than 90% as shown in Chart 1. The impurities were found to be the hydrolyzed products (**4a—d**) of **3b—d** and the 3′-3′ linked dimer (**5b—d**). The former could be removed by chromatographic separation but the latter remained to a degree of 3—5% even after rechromatography. Nevertheless, the amidite units were used without further purification since the 3′-3′ by-products were inert during condensation and could be washed off from solid supports. The amidites appeared at −144 ppm in their ^{31}P NMR spectra as shown in Table 2.

Polymer Supported Synthesis of Oligodeoxyribonucleotides. Recent studies on the solid phase synthesis of oligodeoxyribonucleotides in the phosphoramidite approach pioneered by Caruthers[2] have shown that controlled pore glass (CPG)[9] was very effective as a nonswellable polymer support. Therefore, we employed two kinds of CPG gel supports, 3-aminopropyl CPG (P-CPG) and long-chain amino CPG (L-CPG) purchased from Funakoshi Co. Ltd. The latter has a hexamethylene chain in the spacer. Both the supports were bound to 4,4′-*O*-dimethoxytritylthymidine (DMTrT) via the succinate linker according to the well established procedure.[10] The loading amounts of DMTrT were 35.9 and 13.9 μmol g^{-1} for P-CPG and L-CPG, respectively.

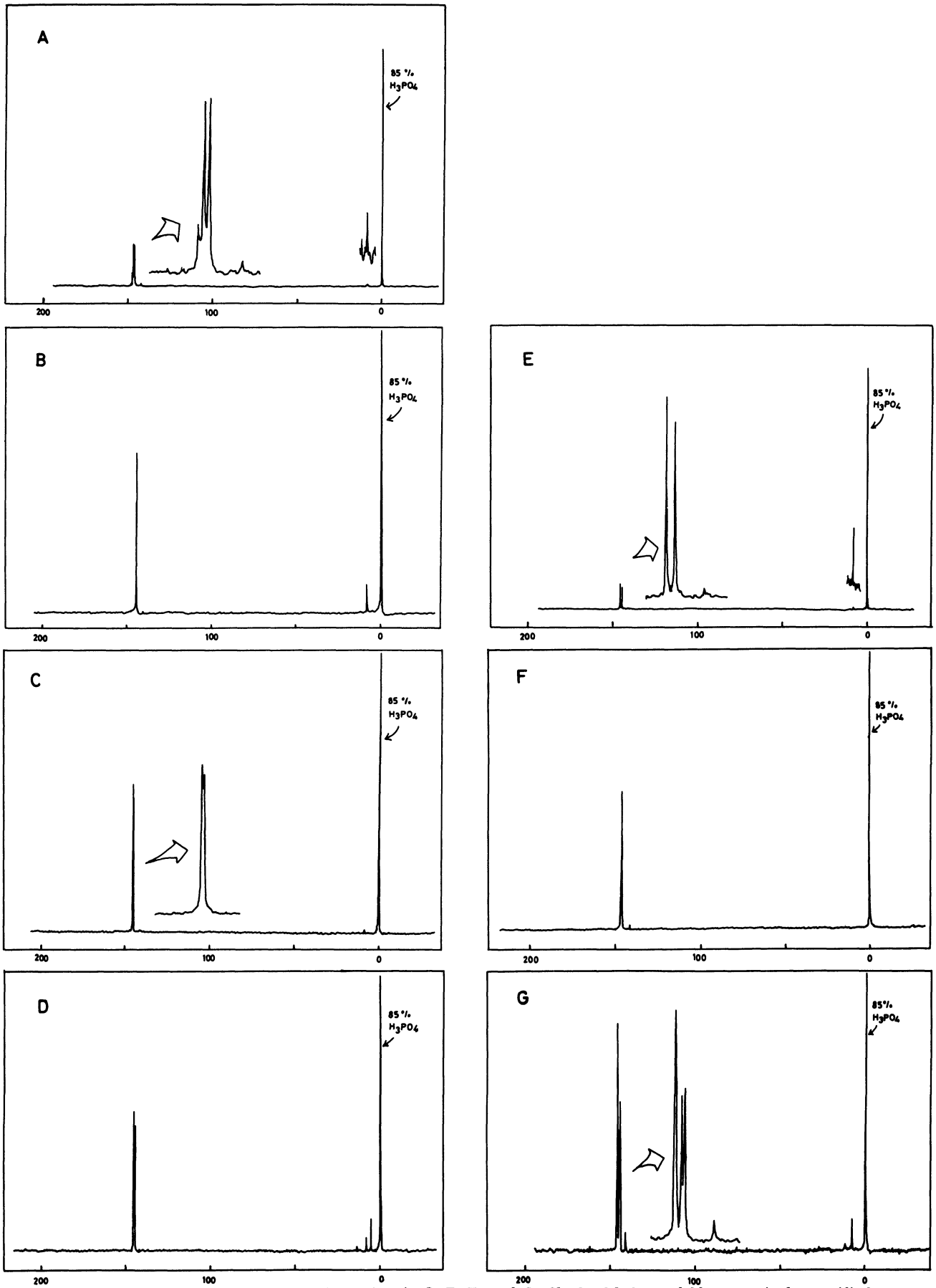


Chart 1. The ^{31}P NMR spectra of **3b**—**f**. A, C, E, F, and G: **3b**, **3c**, **3d**, **3e**, and **3f**, respectively, purified by silica-gel or alumina column chromatography; B and D: **3c** and **3d**, respectively, obtained by reprecipitation from CH_2Cl_2 into hexane after the extractive workup.

Table 3. Elongation Cycle for Polymer Support Synthesis of Oligoribonucleotides

Step	Manipulation	Time/min	Volume/ml
1	1% TFA/CH_2Cl_2	2	3
2	Wash with CH_2Cl_2, MeCN	1	5
3	0.2M phosphoramidite (20 equiv)/MeCN (0.18 ml)+0.4M tetrazole (40 equiv)/MeCN (0.18 ml)	5 or 10	0.36
4	Wash with MeCN, THF		
5	0.1M I_2/2,6-lutidine–THF–H_2O (1:2:2, v/v/v)	1	1
6	Wash with MeCN, THF	1	5
7	0.07M DMAP/Ac_2O–THF–lutidine (2:6:1, v/v/v)	2	3
8	Wash with THF, CH_2Cl_2	1	5

DMTrT-CPG (35.9 μmol/g, 50 mg, 1.8 μmol) was used in each experiment.

Prior to condensation of **3b—d** on these supports, we reconfirmed suitable conditions of coupling cycle by using a modification of the procedure reported by Adams.[2c] The modified cycle for the polymer-supported synthesis is shown in Table 3.

Condensation of **3a** with T-L-CPG was repeated three times under the different conditions using 0.05 and 0.1 M† solutions of **3a**. However, the average coupling yields, which were estimated by the DMTr cation assay, were only 83 and 86% in the case of 0.05 and 0.1 M solutions of **3a**, respectively.

amidite(M)	time	solvent	T—T—T—T——	[L-CPG]
3a (0.05)	5 min	MeCN	87 87 74 (%)	av. 83 %
3a (0.1)	5 min	MeCN	95 89 73	86

Although a relatively long spacer was involved in L-CPG like a long alkylated CPG (LA-CPG) used by Gilham,[9a] Adams,[2c] and Köster,[9d] the unexpected results were obtained. This is due probably to lack of affinity of the activated phosphorylated species of **3a** for the aliphatic hexamethylene chain. The presence of somewhat polar bonds like LA-CPG seems to be important for smooth coupling. However, the coupling of T-P-CPG with **3a** was found to proceed more effectively than that of T-L-CPG under the same conditions. Under the conditions of 10 min and 0.1 M solution of **3a**, the average coupling yield increased to 98% satisfactorily as reported in the original paper.[2b]

amidite(M)	time	solvent		
3a (0.1)	5 min	MeCN		
3a (0.1)	10 min	MeCN		
	T—T—T—T—T—T—T——			[P-CPG]
	80 84 83 79 84 87	(%)		av. 83 %
	94 98 97 99 99 104			98

We feel that the coupling time requires at least 10 min when the morpholino group is used. A relatively long reaction period has already been employed by Köster.[2d,8d]

However, the guanosine unit **3d** was found to form a gel in acetonitrile. The other new amidite units **3b,c** were soluble in this solvent. Therefore, a mixed solvent of 1,2-dichloroethane(DCE)–acetonitrile (1:1, v/v) was chosen during the condensation step to compare the reactivities of the three amidite units **3b—d** under the same conditions. In the case of the deoxycytidine unit **3b**, the coupling yields increased gradually with an increase in chain length and finally up to more than 95% after the five consecutive couplings. Compared with this result, the coupling yields in the case of **3c** were constant with an average yield of 79%. The average coupling yield was improved by the use of only acetonitrile to 89%.

amidite(M)	time	solvent		
3b (0.1)	10 min	DCE–MeCN	(1:1, v/v)	
	C—C—C—C—C—C—T——			[P-CPG]
	96 95 87 78 83 73	(%)		av. 85 %

amidite(M)	time	solvent		
3c (0.1)	10 min	DCE–MeCN	(1:1, v/v)	
3c (0.1)	10 min	MeCN		
	A—A—A—A—A—A—T——			[P-CPG]
	77 79 83 78 88 69	(%)		79 %
	— 90 87 87 94 87	(%)		89 %

A mixed solvent with 1,2-dichloroethane was somewhat inferior to acetonitrile in our coupling system. In an attempt to improve the coupling yield, a little more acidic catalyst, 5-(4-nitrophenyl)tetrazole (NPT), reported by Matteucci,[11] was employed. How-

† 1 M=1 mol dm^{-3}.

ever, this catalyst was ca. one-forth less soluble in acetonitrile than tetrazole so that a relatively dilute solution of NPT (0.1 M) had to be used. As a consequence, high yield couplings were attained at the second and third condensations although the first coupling was ineffective.

amidite(M)	NPT(M)	time	solvent
3c (0.1)	0.1	10 min	DCE-MeCN (1:1, v/v)

A—A—A—T—— [P-CPG]
97 89 35 (%) av. 74 %

However, a large amount of precipitate appeared 1 min after the tetrazole was added at every coupling stage. This precipitate, probably, morpholinium salt of NPT, caused difficult shaking during the reaction.

In the case of deoxyguanosine, the average coupling yield was very low in 1,2-dichloromethane-acetonitrile. It was observed that the coupling yields after the second condensation were considerably lower than that obtained in the first coupling. This low reactivity of **3d** led us to examine if the reagent was stable in 1,2-dichloroethane-acetonitrile. The same coupling cycle was repeated after **3d** was left in the mixed solvent in the presence of tetrazole for 5 h. However, a similar result was obtained.

amidite(M)	time	solvent
3d (0.1)	10 min	DCE-MeCN (1:1, v/v)
3d (0.1)*	10 min	DCE-MeCN (1:1, v/v)

G—G—G—T—— [P-CPG]
32 37 65 (%) av. 45 %
25 26 57 36

*In this case, **3d** was used 5h after tetrazole was added.

Next, in order to see if the low yield is due to side reactions at the guanine moiety, several experiments were conducted. First, the four consecutive couplings of T-P-CPG with a newly-synthesized amidite unit (**3e**) of deoxyguanosine were carried out. The compound **3e** was obtained in 67% yield from the deoxyguanosine derivative (**2e**) as the substrate which could eliminate any side reactions on the guanine moiety since the reactive amidite site was blocked completely with the diphenylcarbamoyl (DPC) group.[12]

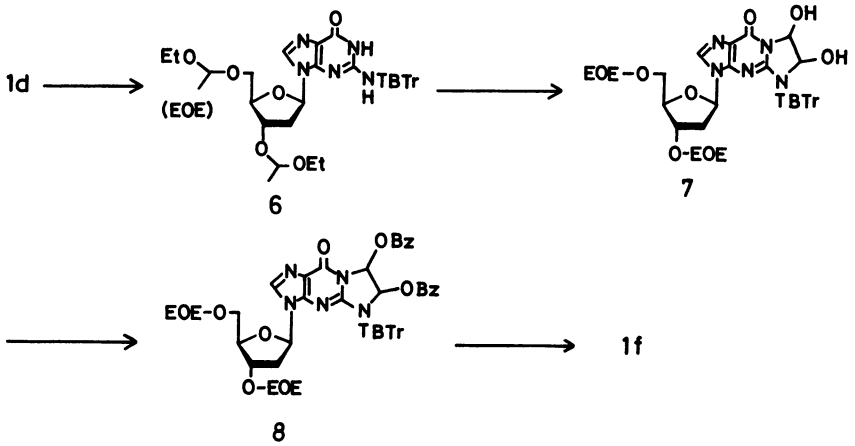
amidite(M)	time	solvent
3c (0.1)	10 min	DCE-MeCN (1:1, v/v)

G*—G*—G*—G*—G*—G*—T—— [P-CPG]
83 85 83 79 81 89 (%) av. 83 %

G* refers to 2-*N*-propionyl-*O*^{6}-diphenylcarbamoyldeoxyguanosine.

Consequently, a nearly constant average yield of 83% was obtained. Since we aimed to develop rapid deprotection procedures through this study and removal of the DPC group required somewhat long periods of time, the double protection mode using glyoxal was employed. As expected from our previous paper, 1,2-bis(acyloxy)ethylene groups such as the 1,2-bis(isobutyryloxy)ethylene group[13] would be removed readily under mild basic conditions. The compound **1d** was treated with ethyl vinyl ether in the presence of trifluoroacetic acid in CH_2Cl_2 to give a bis(1-ethoxyethyl)ated derivative (**6**), which was in situ converted to a glyoxal adduct (**7**) by a method similar to that reported previously.[13] The two hydroxyl groups of **7** were further acylated with benzoic anhydride. The resulting product (**8**) was partially deblocked with 1 M zinc bromide in CH_2Cl_2-2-propanol[14] to give the key intermediate (**1f**) in an overall yield of 55% from **1d**.

It was found that the 1,2-bis(benzoyloxy)ethylene (BBE) group was rapidly eliminated from **1f** by treatment with 0.5 M NaOH-pyridine (1:1,v/v) at room temperature for 20 min. Under these conditions, the TBTr group was simultaneously removed to afford deoxyguanosine. It was also found that compound **1f** was stable to iodine in THF-lutidine-water like **1a**—



Scheme 2.

e. On the TFA treatment, **1f** has a relatively long half time of 24 h toward depurination. These results led us to synthesize a new deoxyguanosine amidite unit (**3f**). The compound **1f** was tritylated with DMTrCl to give **2f** in 93% yield. The subsequent phosphitylation of **2f** with MMCP gave **3f** in 95% yield. A high yield synthesis of **2f** was achieved with the aid of the highly lipophilic protecting groups contained in the molecule, which facilitated elution of the product from an alumina column.

The coupling reaction of the unit **3f** with T-P-CPG was tested under various conditions. However, unsatisfactory results were obtained. In 1,2-dichloroethane–acetonitrile, the coupling yields at the first and second condensations were 56 and 27% yields, respectively.

amidite(M)	time	solvent	
3f (0.1)	10 min	DCE–MeCN	(1:1, v/v)
3f (0.1)	60 min	DCE–MeCN	(1:1, v/v)
3f (0.1)	10 min	MeCN	

G′—G′—G′—G′—G′—T—— [P-CPG]					
		27	56	(%)	av. 47 %
	23	32	66		40
60	62	47	49	74	58

G′ refers to 2-*N*-TBTr-N^1,N^2-bis(benzoyloxy)ethylene-deoxyguanosine.

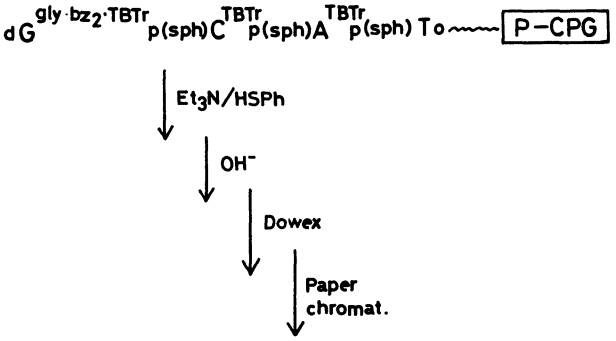
When the time for the condensation was prolonged to 60 min, remarkable improvements were not made. When acetonitrile was used in place of the mixed solvent, somewhat better results were obtained. These results indicate that the low coupling yield in the case of **3d** may be due to the steric hindrance of the TBTr group attached to the exo amino group. In fact, the CPK model suggests that the TBTr residue can approach to the 3′-position. An alternative way to solve this problem should be studied.

In spite of the problem on the deoxyguanosine unit, we demonstrated rapid deprotection procedure after coupling using the four phosphoramidite units, **3a—c** and **3f**, on CPG gel. In order to see if the TBTr group can be smoothly removed from CPG gel, we synthesized a sequence of dGpCpApT on P-CPG. The results are shown below.

amidite(M)	time	solvent
3b, 3c, 3f (0.1 each)	10 min	MeCN

G′—C—A—T—— [P-CPG]				
45	84	86	(%)	av. 72 %

The CPG gel loading this sequence was first treated with benzenethiol–Et_3N–dioxane (1:1:1, v/v)[2a] and then with 0.5 M NaOH–pyridine (1:1, v/v) at room temperature for 30 min. It was confirmed that under the latter conditions the succinate linker and the TBTr and BBE groups were rapidly removed as expected.[8,15] The reaction could be easily monitored by the strength of color of rosolic acid, which was generated as the deprotection proceeded. Since the sensitivity of this color test was as good as that of the DMTr cation assay under acidic conditions, the completion of deprotection can be confirmed directly by eyes.



Scheme 3.

These properties will be advantageous when applied to automated synthesis of oligodeoxyribonucleotides. Rosolic acid and benzoic acid generated were easily removed by extraction with ether. The mixture obtained from the aqueous layer was chromatographed on Whatman 3 MM papers with 1-propanol–concentrated aqueous ammonia–H_2O (55:10:35, v/v/v) to afford dGpCpApT in 70% yield. The tetramer was characterized by enzymatic digestion with snake venom phosphodiesterase which gave G, pC, pA, and pT in the ratio of 1.08:0.85:1.00:0.88.

Conclusion

As shown in the present study, the TBTr and BBE groups could be removed promptly from the oligomer chain within 30 min. Since the phosphodiester linkage is rather stable under alkaline conditions[7] compared with phosphotriester linkage, relatively strong alkali like NaOH is available for removal of base protecting groups after elimination of the methyl group with benzenethiol. The conventional protecting groups of acyl-type were, however, resistant to alkali such as dilute NaOH except for labile acyl groups like the acetyl group on N^4-acetyldeoxycytidine[16] which can not be utilized in oligodeoxyribonucleotide synthesis. Therefore they have been removed mostly by treatment with concentrated aqueous ammonia. It is noteworthy that the TBTr and BBE groups are removed simply under mildly alkaline conditions that can be applied to the "full" automated-synthesis of DNA fragments which involves the deprotection procedure. The color

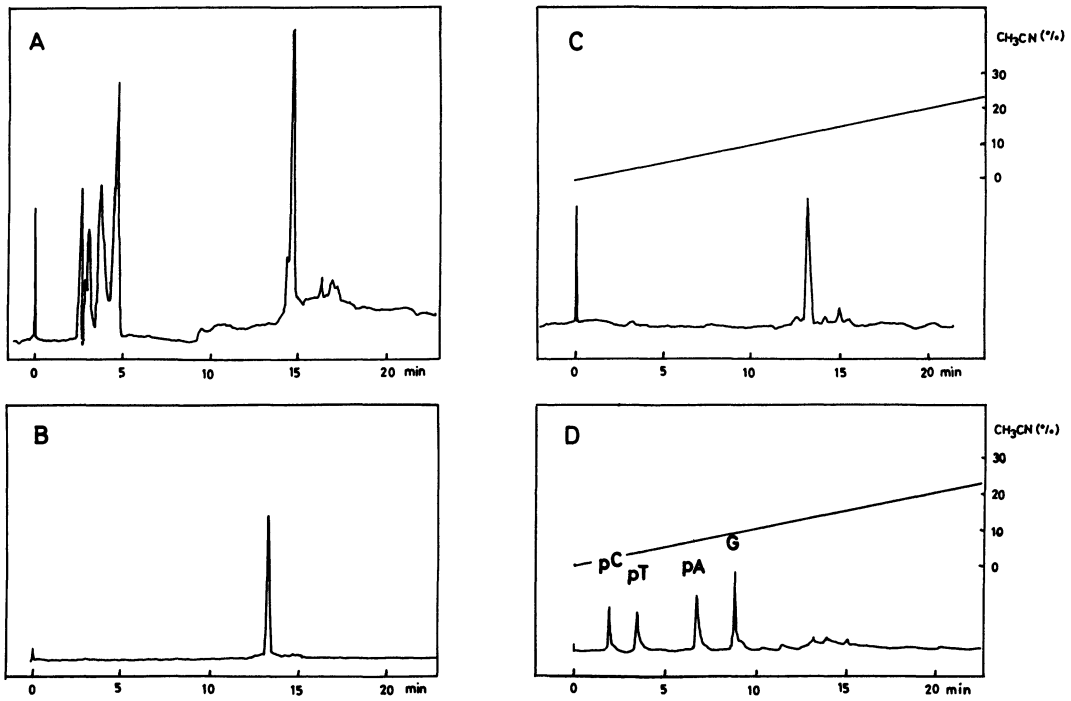


Chart 2. A: The ion-exchange HPLC profile of the mixture obtained after the protecting groups and linker were removed; B: The reverse-phase HPLC profile of dGpCpApT separated by paper chromatography; D: The reverse-phase HPLC profile of the mixture obtained by enzyme digestion of dGpCpApT with snake venom phosphodiesterase.

marker of rosolic acid enabled us to observe visibly the degree of the complete deprotection since the succinate linker and the BBE group were eliminated under the conditions where the TBTr group was removed. However, some improvements are needed for obtaining higher coupling yields. Especially, more suitable alkali-labile protecting groups should be explored for our purpose. We are now challenging to solve these problems in this derection.

Experimental

^{1}H NMR spectra were recorded at 100 MHz on a JEOL UNM PS-100 spectrometer using tetramethylsilane as an internal standard. UV spectra were obtained on a Hitachi 220A spectrophotometer. Paper chromatography was performed by use of a descending technique with Whatman 3 MM papers using the following solvent system: Solvent I (1-propanol–concentrated aqueous ammonia–water, 55:10:35, v/v/v). Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd. or alumina Woelm B-Supper I from Kanto Co. Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. TLC was performed on precoated TLC plates of silica gel 60 F-254 (Merck) or aluminium oxide 60 F-254 (Merck).

Ion-exchange HPLC was performed on a JASCO TRI ROTAR type II apparatus equipped with a Whatman Partisil 10 SAX column (3.9×250 mm), a GP-A30 solvent programmer, a UVIDEC 100-II UV detecter and an RC-225 recorder. A linear gradient (0→99%) starting with buffer A (0.005 M KH_2PO_4, 20% CH_3CN, pH 6.54) and applying buffer B (0.5 M KH_2PO_4, 20% CH_3CN, pH 6.54) was used at a flow rate of 2 ml min^{-1} for 32 min for analysis and purification of dGpCpApT. Reverse phase HPLC was performed on a Waters Model A25 using a μ-Bondapack C-18 column using a linear gradient starting from buffer C (0.1 M NH_4OAc, pH 7.0) and applying CH_3CN at a flow rate of 2 ml min^{-1} for 30 min.

Deoxyribonucleosides were purchased from Yoshitomi Co. Ltd. Compounds **1b—d**,[8] **2a**,[17] and **2e**[12] were prepared to the published procedures. Pyridine was distilled two times from *p*-toluenesulfonyl chloride and from calcium hydride and then stored over molecular sieves 3A. Acetonitrile was distilled over calcium hydride and stored over molecular sieves 3A. 1,2-Dichloroethane was dried over P_4O_{10}, decanted, distilled over K_2CO_3, and stored over molecular sieves 3A.

Venom phosphodiesterase was purchased from Boehringer Co. Ltd. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

General Procedure for Synthesis of 2b—f. An appropriate *N*-TBTr deoxyribonucleoside[7] (**1b—f**, 5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (50 ml). To the solution was added DMTrCl (1.05—1.20 equiv), and the mixture was kept for 3—5 h. Then ice-water was added and the mixture was extracted with CH_2Cl_2. The organic layer was washed with satd $NaHCO_3$ solution, dried over Na_2SO_4, filtered, and evaporated to a gum. The residue was evaporated several times with toluene and chromatographed on silica gel (70 g) with CH_2Cl_2→CH_2Cl_2–MeOH in the presence of 0.3% pyridine to give **2b—f** as listed in Table 1.

General Procedure for Synthesis of 3b—f. An appropriate *N*-TBTr-5′-*O*-DMTr-deoxyribonucleoside (**2b—f**, 1 mmol) was

rendered anhydrous by coevaporation with dry pyridine and then with dry toluene two times each and finally dissolved in dry CH_2Cl_2 (10 ml). To the solution were added successively diisopropylethylamine (609 μl, 3.5 mmol) and MMCP (224 μl, 1.5 mmol). After being stirred at room temperature for 10 min, the mixture was dissolved with ethyl acetate (20 ml) which had been washed with satd $NaHCO_3$ solution. The organic phase was again washed with satd $NaHCO_3$ solution (20 ml). After drying over Na_2SO_4, the extract was filtered and evaporated to a foam. The foamy material was chromatographed on a silica-gel or alumina column as shown in Table 2. The elemental analyses and ^{31}P NMR spectra of **3b—f** are also given in Table 2.

Introduction of DMTrT to CPG. To a suspension of P-CPG (pore size 569 Å, NH_2 97 μmol g^{-1}) or L-CPG (pore size 569 Å, NH_2 123 μmol g^{-1}) were added triethylamine (3 equiv), 4-(dimethylamino)pyridine (0.75 equiv), and 5′-*O*-dimethoxytrityl-3′-*O*-[3-(pentachlorophenoxycarbonyl)propionyl]thymidine (1.5 equiv). After the mixture was rotated slowly in a sealed flask by using a rotary evaporator for 20 h, the gel was washed with CH_2Cl_2 and dried over P_4O_{10} and NaOH in vacuo. The resulting gel was used for condensation with **3**.

***N*²-[4,4′,4″-Tris(benzoyloxy)trityl]-*N*¹,*N*²-bis(benzoyloxy)-ethylenedeoxyguanosine (8).** To a mixture of **1d** (2.31 g, 2.61 mmol) and ethyl vinyl ether (5.07 ml, 53 mmol) was added trifluoroacetic acid (408 μl, 5.3 mmol). The solution was stirred at room temperature for 100 min and the solvent and the ether was removed under reduced pressure. The residue was coevaporated successively with toluene and dry pyridine two times each and then dissolved in 2.5 M solution of glyoxal in dry pyridine (10 ml). The mixture was evaporated ten times with dry pyridine (20 ml) and finally dissolved in CH_2Cl_2 (100 ml). The CH_2Cl_2 solution was washed five times with water (100 ml), dried over Na_2SO_4, filtered, and evaporated to a gum. The gum was rendered anhydrous by repeated coevaporation with dry pyridine and then evaporated several times with dry toluene to remove the last traces of pyridine. The residue was dissolved in dry CH_2Cl_2 (25 ml) and treated with benzoic anhydride (4.72 g, 20.9 mmol), triethylamine (2.9 ml, 20.9 mmol), 4-(dimethylamino)pyridine (32 mg, 0.26 mmol). After the resulting mixture was kept at room temperature for 17 h, water was added. The dibenzoate was extracted three times with CH_2Cl_2 and the extract was washed with satd $NaHCO_3$ solution. The extracts were combined and evaporated to dryness under reduced pressure. To the residue was added 1 M solution of zinc bromide in CH_2Cl_2–2-propanol (85:15, v/v, 26 ml), and the mixture was stirred for 1 h. Then pyridine was added and the resulting precipitate was removed by filtration. The filtrate was partitioned between CH_2Cl_2 and satd $NaHCO_3$ solution. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated to a foam under reduced pressure. The residue was chromatographed on silica gel with CH_2Cl_2–MeOH to give **8** (1.63 g, 55%); ^1H NMR ($CDCl_3$) δ=2.02 (2H, m, 2′-H), 3.52 (2H, m, 5′-H), 3.87 (1H, m, 4′-H), 4.29 (1H, m, 3′-H), 5.71 (1H, m, 1′-H), 6.24, 6.50 (2H, br s, N-CH-O), 7.04—7.80 (22H, m, ArH), 7.96 (4H, m, ArH), 8.17 (6H, br d, *J*=8 Hz, ArH). Calcd for $C_{66}H_{49}N_5O_{14}$: C, 69.77; H, 4.35; N, 6.16%. Found: C, 68.75; H, 4.10; N, 6.03%.

Solid Phase Synthesis of dGpCpApT on P-CPG. This synthesis was done by using DMTrT-P-CPG (50 mg, 1.8 μmol). The final DMTr cation assay showed that 0.575 μmol of the tetramer was formed.

Isolation of dGpCpApT. The P-CPG gel containing fully-protected tetramer was treated with benzenethiol–triethylamine–dioxane (1:1:2, v/v/v, 4 ml) at room temperature for 45 min with gentle rotation using a rotary evaporator. The gel was washed successively with MeOH and ether and then allowed to react with 0.5 M NaOH in pyridine–water (1:1, v/v, 1 ml) for 30 min using a rotary evaporator to keep constant rotation. The gel was removed by filtration and washed with pyridine–water (1:1, v/v, 5 ml). The eluant and washings were combined and passed through Dowex 50 W×2 (100—200 mesh, pyridinium form, 5 ml). The resin was washed with pyridine–water (1:1, v/v, 5 ml). The eluant and washing were combined and evaporated and then the residue was evaporated three times with a small amount of concentrated aqueous ammonia to remove the last traces of pyridine. Part (71%) of the residue was applied to Toyo Roshi No 51 Å papers. Chromatography developed with Solvent I afforded dGpCpApT (10.2 OD, 70% based on the assumption of 15% hypochromicity using ε 3.55×10^4 at 258 nm).

Enzymic Assay of dGpCpApT. The tetramer (2.4 OD unit at 258 nm) was dissolved in Tris-HCl buffer (0.05 M, pH 8.0, 50 μl) and snake venom phosphodiesterase (10 μl) was added. The mixture was incubated at 37 °C for 1.5 h. After being heated at 100 °C for 3 min, the mixture was analyzed by reverse phase HPLC. The calculated ratio of dG, pC, pA, and pT was given in the text. The following ε values at 254 nm were used for calculation: 5630, 6080, 13400, and 14100 for pC, pT, pA, and dG, respectively.

References

1) a) V. Amarnath and A. D. Broom, *Chem. Rev.*, **77**, 183 (1977); b) C. B. Reese, *Tetrahedron*, **34**, 3143 (1978); c) M. Ikehara, E. Ohtsuka, and A. F. Markham, *Adv. Carbohy. Chem. Biochem.*, **36**, 135 (1978); d) G. A. Urbina, G. M. Sathe, W. C. Liu, M. F. Gillen, P. D. Duck, R. Bender, and K. K. Ogilvie, *Science*, **214**, 270 (1981); e) E. Ohtsuka, M. Ikehara, and D. Söll, *Nucleic Acids Res.*, **10**, 6553 (1982); f) K. Itakura, *Trends Biochem. Sci.*, **7**, 442 (1982); g) S. A. Narang, *Tetrahedron*, **39**, 3 (1983); h) W. Pfleiderer, *KONTAKTE* (Merck), 3 (1983); i) J. E. Davies and H. G. Gassen, *Angew. Chem. Int. Ed. Engl.*, **22**, 13 (1983); j) K. Itakura, J. J. Rossi, and R. B. Wallace, *Annu. Rev. Biochem.*, **53**, 323 (1984); k) "Oligonucleotide Synthesis, a Practical Approach," ed by M. J. Gait, IRL Press, Oxford (1984); l) M. H. Caruthers, *Science*, **230**, 281 (1985).

2) a) S. L. Beaucage and M. H. Caruthers, *Tetrahedron Lett.*, **23**, 1859 (1982); b) L. J. McBride and M. H. Caruthers, *ibid.*, **24**, 245 (1983); c) S. P. Adams, K. S. Kavka, E. J. Wykes, S. B. Holder, and G. R. Galluppi, *J. Am. Chem. Soc.*, **105**, 661 (1983); d) N. D. Sinha, J. Biernat, and H. Köster, *Tetrahedron Lett.*, **24**, 5843 (1983).

3) a) V. A. Efimov, S. V. Reverdatto, and O. G. Chakhmakhcheva, *Nucleic Acids Res.*, **10**, 6675 (1982); b) V. A. Efimov, S. V. Reverdatto, and O. G. Chakhmakhcheva, *Tetrahedron Lett.*, **23**, 961 (1982); c) V. A. Efimov, A. A. Buryakova, S. V. Reverdatto, O. G. Chakhmakhcheva, and Yu. A. Ovchinnikov, *Nucleic Acids Res.*, **11**, 8369 (1983).

4) V. A. Efimov, O. G. Chakhmakhcheva, and Yu. A. Ovchinnikov, *Nucleic Acids Res.*, **13**, 3651 (1985).

5) J. Matsuzaki, H. Hotoda, M. Sekine, and T. Hata,

Tetrahedron Lett., **25**, 4019 (1984).

6) K. K. Ogilvie, M. J. Nemer, G. H. Hakimelahi, Z. A. Proba, and M. Lucas, *Tetrahedron Lett.*, **23**, 2615 (1982).

7) T. P. Patel, M. A. Chauncey, T. A. Millican, C. C. Bose, and M. A. W. Eaton, *Nucleic Acids Res.*, **12**, 6853 (1984).

8) M. Sekine, N. Masuda, and T. Hata, *Tetrahedron*, **41**, 5445 (1985).

9) a) G. R. Gough, M. J. Brunden, and P. T. Gilham, *Tetrahedron Lett.*, **22**, 4177 (1981); b) H. Köster, A. Stumpe, and A. Wolter, *ibid.*, **24**, 747 (1983); c) B. S. Sproat and W. Bannwarth, *ibid.*, **24**, 5771 (1983); d) N. D. Sinha, J. Biernat, and H. Köster, *ibid.*, **24**, 5843 (1983); e) S. L. Beaucage, *ibid.*, **25**, 375 (1984); f) C. Claesen, G. I. Tesser, C. E. Dreef, J. E. Marugg, G. A. van der Marel, and J. H. van Boom, *ibid.*, **25**, 1307 (1984); g) W. J. Stec and G. Zon, *ibid.*, **25**, 5275 (1984); h) **25**, 5279 (1984); i) N. D. Sinha, J. Biernat, J. McManus, and H. Köster, *Nucleic Acids Res.*, **12**, 4539 (1984); j) R. T. Pon and K. K. Ogilvie, *Nucleosides & Nucleotides*, **3**, 485 (1984); k) C. Minganti, K. N. Ganesh, B. S. Sproat, M. J. Gait, *Anal. Biochem.*, **147**, 63 (1985); l) R. T. Pon, M. J. Damha, and K. K. Ogilvie, *Tetrahedron Lett.*, **26**, 2525 (1985); m) P. T. Pon, M. J. Damha, and K. K. Ogilvie, *Nucleic Acids Res.*, **13**, 6447 (1985).

10) K. Miyoshi, T. Miyake, T. Hozumi, and K. Itakura, *Nucleic Acids Res.*, **8**, 5473 (1980).

11) a) B. C. Froehler and M. D. Matteucci, *Tetrahedron Lett.*, **24**, 3171 (1984); b) W. G. Finnegan, R. A. Henry, and R. Lofguist, *J. Am. Chem. Soc.*, **80**, 3908 (1958).

12) a) T. Kamimura, M. Tsuchiya, K. Koura, M. Sekine, and T. Hata, *Tetrahedron Lett.*, **24**, 2775 (1983); b) T. Kamimura, K. Urakami, K. Koura, M. Sekine, K. Shinozaki, K. Miura, and T. Hata, *J. Am. Chem. Soc.*, **106**, 4552 (1984).

13) M. Sekine, J. Matsuzaki, and T. Hata, *Tetrahedron Lett.*, **23**, 5287 (1982).

14) R. Kierzek, H. Ito, R. Bhatt, and K. Itakura, *Tetrahedron Lett.*, **22**, 3761 (1981).

15) M. Sekine and T. Hata, *J. Org. Chem.*, **48**, 3011 (1983).

16) H. Köster, K. Kolikowski, T. Liese, W. Heikens, and V. Kohli, *Tetrahedron*, **37**, 363 (1981).

17) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3821 (1965).