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Rapid Synthesis of Oligodeoxynucleotides by using *N*-Methylimidazole as a Condensation Catalyst. Syntheses of Dodecanucleotides corresponding to Complementary Deoxyribonucleic Acid of the Tetrapeptide Fragments of Cholecystokinin-Pancreozymin and Vasoactive Intestinal Peptide

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It has been found that *N*-methylimidazole (MeIm) activates stable condensing reagents such as 2,4,6-triisopropylbenzenesulfonyl-4-nitroimidazolide (TPSNI) and mesitylene-sulfonyl-4-nitroimidazolide (MSNI). Fully protected trinucleotides which could serve as key intermediates were synthesized by using MeIm and TPSNI or MSNI. These trinucleotides were utilized in the syntheses of the dodecamers, dCATCCACCCCAT and dAGCCATCTGCTT, which correspond to the specific tetrapeptides of cholecystokinin-pancreozymin and vasoactive intestinal peptide.

Keywords—DNA synthesis; *N*-methylimidazole; nucleotide condensation; CCK-PZ; VIP; HPLC

Introduction

Synthetic oligonucleotides of certain defined sequences have been proven to be useful tools for studies in the field of molecular biology.¹⁾ Although synthetic methods for deoxy-oligonucleotides have been greatly improved by the introduction of the triester procedure,²⁾ some problems still remain to be solved.³⁾ For instance, a better condensing reagent is desirable. Bulky condensing reagents such as 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPSTe),⁴⁾ mesitylenesulfonyl 3-nitro 1,2,4-triazolide (MSNT)⁵⁾ and 2,4,6-triisopropylbenzenesulfonyl 3-nitro 1,2,4-triazolide (TPSNT)⁶⁾ lead to rapid formation of internucleotide bonds with high yields, and are generally recognized as suitable reagents for use in deoxyoligonucleotide synthesis. However, some side reactions such as sulfonylation of hydroxy groups and certain base modifications have been observed in the course of the triester condensation³⁾ and these reagents are so labile that they must be freshly prepared when required. Van Boom's and Gilham's groups developed some stable reagents, such as 2,4,6-triisopropylbenzenesulfonyl 4-nitroimidazolide (TPSNI)^{7,8)} and mesitylenesulfonyl 4-nitroimidazolide (MSNI),⁹⁾ which promoted condensation without any significant side reactions but a longer time was required to complete the condensation reaction.

We investigated reagents which could activate such stable condensing reagents, TPSNI and MSNI, and found a satisfactory catalyst, *N*-methylimidazole (MeIm), which has already been used for acylation⁶⁾ and phosphorylation reactions.¹⁰⁾ This condensation method was successfully applied to the syntheses of the two dodecanucleotides corresponding to the genes of the tetrapeptide sequences of cholecystokinin-pancreozymin (CCK-PZ) and vasoactive intestinal peptide (VIP). After the completion of our work,¹¹⁾ a similar method was reported recently as a short letter.¹²⁾ We present here the details of this novel condensation method, which is useful for oligonucleotide synthesis.

MeIm as a Catalyst in Condensation

MeIm is not only a good catalyst for acylation but is also used for phosphorylation of hydroxy groups and some condensation reactions. As we expected that stable condensing

reagents might be activated by MeIm, the effects of MeIm were investigated under various conditions. As shown in Table I, by addition of a 6-fold excess of MeIm and a 3-fold excess of MSNI to suitably protected mononucleotides, coupled dinucleotides were obtained in less than 2 h with high yields. It is likely that MeIm activates the condensation reaction by formation of an intermediate similar to that proposed in acylation, because the same catalytic activity was observed when *N*-dimethylaminopyridine (DAP)¹³⁾ was used instead of MeIm. DAP is known to be a good catalyst for acylation. Since the active intermediate complex of the condensation is thought to be a mixed anhydride between a phosphate and a sulfonic acid, or to be a phosphoronitroimidazolide, the formation of this intermediate may be accelerated by MeIm or DAP. MeIm could be used in the synthesis of nucleotides longer than dinucleotides because it gave a homogeneous solution under usual reaction conditions. On the other hand, the insolubility of DAP in the reaction solvents made it difficult to purify the coupled nucleotides. Therefore, DAP seems to be a less suitable catalyst than MeIm.

TABLE I. Effect of MeIm and DAP in Oligonucleotide Synthesis

3' Aryl phosphate compound (mmol)	5' Hydroxyl compound (mmol)	MSNI mmol	MeIm mmol	DAP mmol	Reaction time (h)	Yield % (mmol)
DMT T° (1.5)	dibGbz (1.2)	2.3			14	78(0.9)
DMT T° (0.12)	Tbz (0.1)	0.36			3	30(0.03)
DMTdbzA° (1.5)	dbzC (2.0)	2.3		3.0	4	50(0.75)
DMTdbzA° (1.3)	dbzC°-T°-(CE) (1.0)	2.0	2.6		14	80(0.8)
DMT T° (1.8)	Tbz (1.5)	5.4	10.8		2	80(1.2)
DMTdbzA°-dbzA°-dbzA° (0.3)	dibG°-T°-dbzCbz (0.2)	0.9	1.8		2	67(0.13)

DMT: dimethoxy trityl, bz: benzoyl, ib: isobutyryl, o: *o*-chlorophenyl phosphotriester linkage, CE: β -cyanoethyl.

We further studied whether unfavorable sulfonylation might occur in our condensing reaction. 3'-Benzoylthymidine was treated with MSNI and MeIm in an anhydrous pyridine-dioxane mixture at room temperature, and the rate of sulfonylation of the primary 5'-hydroxy group was determined periodically. A 1.5-fold excess of MSNI and a 3-fold excess of MeIm gave 7% (3 h) and 13% (17 h) yields of sulfonylation product while a 3-fold excess of MSNI and a 6-fold excess of MeIm gave 17% (3 h) and 40% (17 h) yields, respectively. Although we could not exclude this typical side reaction, these rates are slower than those in the case of arylsulfonyltetrazolide,¹⁴⁾ which is a common condensing reagent in the nucleotide synthesis.

Syntheses of Trinucleotide Blocks

Suitably protected deoxyoligonucleotide blocks, especially trimers, are useful intermediates for the synthesis of polynucleotides. In order to save time in carrying out syntheses, Itakura *et al.* have reported a rapid synthesis of trinucleotide blocks.²⁾ However, it was difficult to obtain a pure trinucleotide block simply by extraction with an organic solvent from the reaction mixture. In these reactions the condensed dinucleotide blocks were purified by chromatography on silica gel and subsequently condensed with other nucleotides without further precipitation processes. The condensed trinucleotides were similarly purified by chromatography. Each condensation could be completed within 2 h with a high yield by using MSNI and MeIm. The overall yields of fully protected trimers based on the 5'-hydroxy mononucleotides are listed in Table II.

Synthesis of Dodecanucleotide

The trinucleotide blocks obtained by the above-mentioned procedures served as key intermediates in the syntheses of the dodecanucleotides corresponding to the genes for the amino acid sequences Met-Gly-Trp-Met of CCK-PZ and Lys-Gln-Met-Ala of VIP. The

TABLE II. Synthesis of Trimer Blocks

Protected trinucleotide	<i>Rf</i> ^{a)}	Yield (%) ^{d)}
A A A	0.39	40
A C T	0.40	49
A C C bz ^{b)}	0.61	69
A G G bz ^{b)}	0.54	31
C A T	0.49	69
C C A	0.38	63
C C C	0.37	62
C T G	0.34	35
C A T bz ^{b)}	0.61	57
G A T	0.39 ^{c)} 0.46	51
G T T	0.44	32
G C T bz ^{b)}	0.51	51
G A A bz ^{b)}	0.52 ^{c)} 0.59	52

- a) Chloroform-methanol 10:1 v/v.
b) 3'-Benzoyl fully protected trinucleotide block.
c) Two isomers were separated.
d) Overall yield from 3'-end mononucleoside.

CCK-PZ								
		²⁸	²⁹	³⁰	³¹	³²	³³	
		-Met	-Gly	-Trp	-Met	-Asp	-PheNH ₂	
mRNA	5'	AUG	GGG U C A	UGG	AUG	GAC U	UUU C	3'
DNA	3'	TAC	CCC A G T	ACC	TAC	CTG A	AAA G	5'
synthetic DNA	3'	TAC	CCC	ACC	TAC			
VIP								
		¹⁵	¹⁶	¹⁷	¹⁸			
		-Lys	-Gln	-Met	-Ala-			
mRNA	5'	AAG A	CAG A	AUG	GCU A C G	3'		
DNA	3'	TTC T	GTC T	TAC	CGA T G C	5'		
synthetic DNA	3'	TTC	GTC	TAC	CGA	5'		

Fig. 1. Possible DNA Sequences corresponding to Amino Acids of CCK-PZ and VIP

sequences of these deoxyoligonucleotides complementary to mRNA were chosen mainly on the basis of the frequency of codon usage in porcine gastrin as shown in Fig. 1.^{1a)} As described by Gilham *et al.*,⁹⁾ we did not isolate the detritylated oligonucleotides; free dimethoxytritanol did not interfere with internucleotide bond formation. Dodecanucleotides were easily isolated by chromatography on silica gel plates and prepared in good yields by using a 6-fold excess of MeIm as shown in Table III. Both nucleotides, dCATCCACCCAT and dAGCCATCTGCTT,

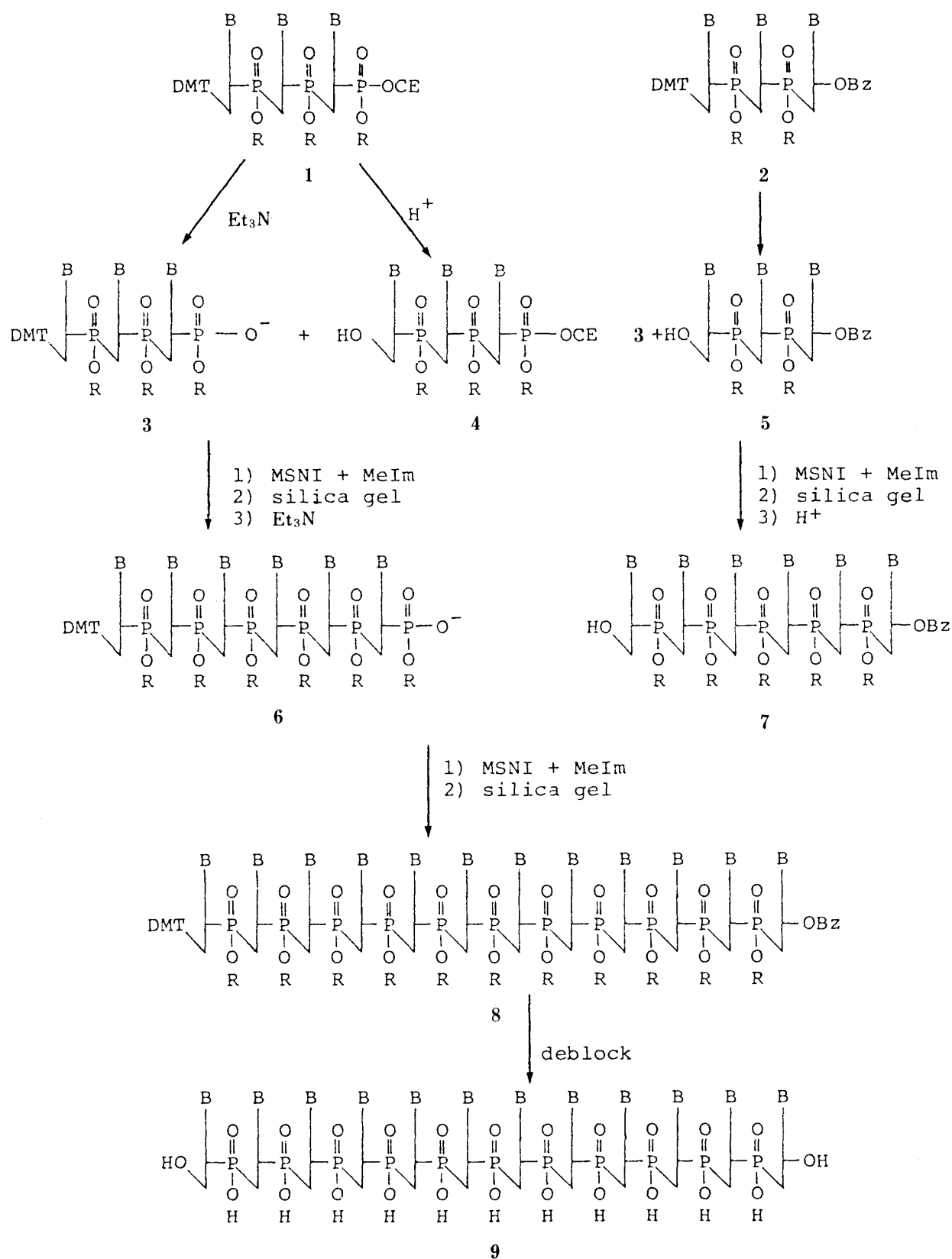


Fig. 2. Synthesis of fully Protected Dodecanucleotide by the Improved Triester Approach

DMT: dimethoxytrityl. CE: β -cyanoethyl. R: *p*-chlorophenyl. Bz: benzoyl.

were synthesized by condensation between the corresponding hexanucleotides for a few hours and then characterized by silica gel and reverse-phase thin layer chromatography (Fig. 2).¹⁵⁾ It is an important finding that MeIm is able to work as an effective catalyst in condensation between oligonucleotides.

Deprotection and Analyses of Dodecanucleotides

The first reaction for deprotection usually removes aryl groups from the internucleotide phosphates and this is followed by detritylation by treatment with acetic acid. On the other hand, Narang *et al.* obtained good results by deprotecting the acid-labile trityl group first.¹⁷⁾ We investigated suitable conditions for deprotection of the fully protected trimer (dACC) by means of the following two procedures.

Procedure A: 1) 0.3 M pyridinealdoximate (PAO) in dioxane-water (1:1); 2) aqueous ammonia; 3) 80% acetic acid.

Procedure B: 1) 2% benzenesulfonic acid (BSA);⁴⁾ 2) 0.3 M PAO; 3) aqueous ammonia. Although there have been several reports suggesting that oximate is the most suitable reagent

TABLE III. Reaction Conditions for Synthesis of Dodecanucleotides

3' Aryl phosphate ^{a)} compound (mmol)	5' Hydroxyl ^{b)} compound (mmol)	MSNI (mmol)	MeIm (mmol)	Reaction time (h)	Product (Yield)
DMTdbzC ^o -dbzA ^o - T ^o -(CE) (0.3)	DMTdbzC ^o -dbzC ^o - dbzA ^o -(CE) (0.2)	0.9	1.8	2	DMTdbzC ^o -dbzA ^o -T ^o -dbzC ^o - dbzC ^o -dbzA ^o -(CE) (62)
DMTdbzC ^o -dbzC ^o - dbzC ^o -(CE) (0.15)	DMTdbzC ^o -dbzA ^o - Tbz (0.1)	0.45	0.9	4	DMTdbzC ^o -dbzC ^o -dbzC ^o - dbzC ^o -dbzA ^o -Tbz (98)
DMTdbzC ^o -dbzA ^o -T ^o - dbzC ^o -dbzC ^o -dbzA ^o - (CE) (0.04)	DMTdbzC ^o -dbzC ^o - dbzC ^o -dbzC ^o -dbzA ^o - Tbz (0.02)	0.12	0.24	3	DMTdbzC ^o -dbzA ^o -T ^o -dbzC ^o - dbzC ^o -dbzA ^o -dbzC ^o -dbzC ^o - dbzC ^o -dbzC ^o -dbzA ^o -Tbz (87)
DMTdbzA ^o -dibG ^o - dbzC ^o -(CE) (0.3)	DMTdbzC ^o -dbzA ^o - T ^o -(CE) (0.2)	0.9	1.8	2.5	DMTdbzA ^o -dibG ^o -dbzC ^o - dbzC ^o -dbzA ^o -T ^o -(CE) (54)
DMTdbzC ^o -T ^o -dibG ^o - (CE) (0.3)	DMTdbzC ^o -T ^o -Tbz (0.2)	0.9	1.8	2	DMTdbzC ^o -T ^o -dibG ^o -dbzC ^o - T ^o -Tbz (87)
DMTdbzA ^o -dibG ^o - dbzC ^o -dbzC ^o -dbzA ^o - T ^o -(CE) (0.04)	DMTdbzC ^o -T ^o - dibG ^o -dbzC ^o -T ^o -Tbz (0.02)	0.12	0.24	4	DMTdbzA ^o -dibG ^o -dbzC ^o - dbzC ^o -dbzA ^o -T ^o -dbzC ^o -T ^o - dibG ^o -dbzC ^o -T ^o -Tbz (55)

a) Used after decyanoethylation.

b) Used after detritylation.

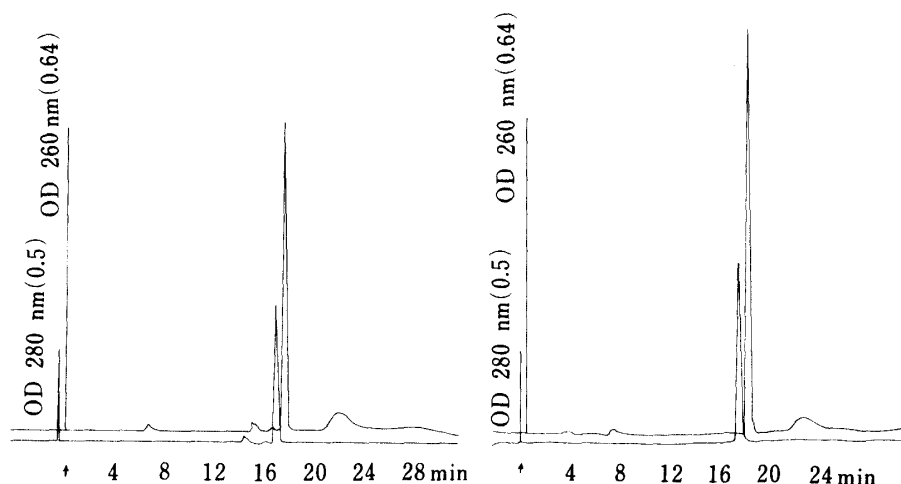


Fig. 3. HPLC Patterns of the Trinucleotide d(ACC) obtained by Deprotection Procedures A (Right) and B (Left)

Column: μ Bondapak C18, 3 \times 300 mm.

Elution: A 30-min linear gradient of CH₃CN (0 \rightarrow 30%) in 0.1 M AcONH₄ (pH 7.0).

Flow rate: 1 ml/min.

for cleavage of aryl groups from internucleotide linkages,¹⁶⁾ so far no report has dealt with the preferred order of removal of the protective groups.

After being desalted on a DEAE-cellulose column, each reaction mixture was analyzed by high performance liquid chromatography (HPLC). As can be seen from Fig. 3, procedure A is superior to procedure B in terms of the purity of the product. The major product obtained from procedure A accounted for 99% of the absorbance. When 2% BSA was used in the first step to avoid any depurination¹⁷⁾ (procedure B), the major product accounted for 95%. It seems likely that 80% acetic acid does not cause depurination when amino groups are free. On the other hand, alkaline treatments (0.3 M PAO and aqueous ammonia) certainly produce some side products when primary hydroxy groups are present. Dodecanucleotides were completely deprotected by procedure A and purified by reverse-phase HPLC¹⁸⁾ (Fig. 4). The final products were confirmed to possess the expected sequences by enzymatic digestion with venom phosphodiesterase and subsequent analyses of the hydrolysates by means of HPLC.

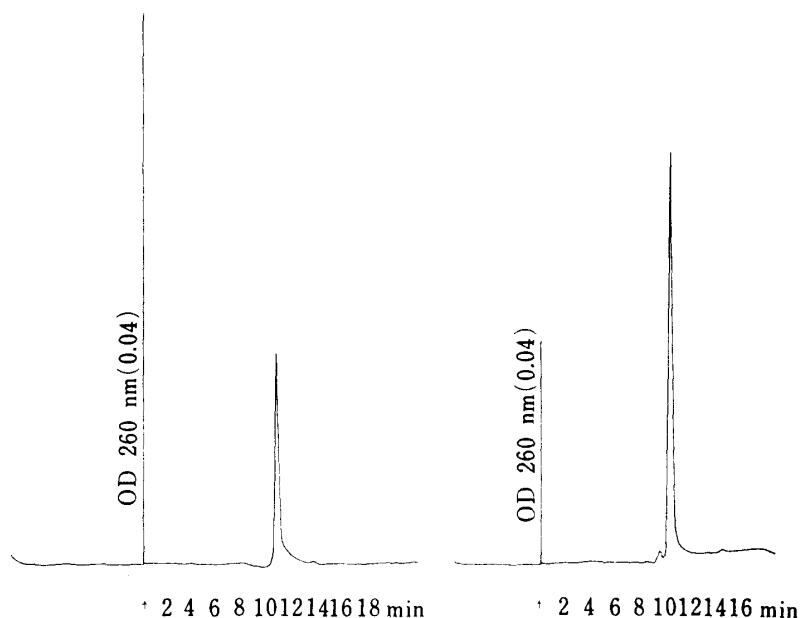


Fig. 4. HPLC Patterns of Dodecanucleotides dCATCCACCCCAT (Left) and dAGCCATCTGCTT (Right)

Column: Nucleosil C18, 4.6 × 250 mm.
Elution: A 20-min linear gradient of CH₃CN (10→30%) in 0.1 M AcONH₄ (pH 7.0).
Flow rate: 1 ml/min.

Experimental

Materials—Thymidine, deoxyadenosine, deoxycytidine, and deoxyguanosine (Yamasa), mesitylenesulfonyl chloride, benzenesulfonic acid, pyridinealdoxime, *N*¹,*N*¹,*N*²,*N*²-tetramethylguanidine, β-cyanoethanol, and *N*-methylimidazole (Tokyo Kasei), triazole (Wako), dimethoxytrityl chloride (Aldrich), silica gel HF₂₅₄ thin-layer chromatographic (TLC) plates, silica gel 60HF₂₅₄ silanized TLC plates, silica gel 60H grade for column chromatography (Merck), and venom phosphodiesterase (Sigma) were purchased commercially. HPLC was carried out by using a Waters compact type unit (6000/U/440) and column effluents were monitored with an optical absorbance detector, UVIDEC-100-2 (JASCO). Fully protected deoxymononucleotides were prepared by the method described elsewhere.^{4,19)}

General Method for the Synthesis of fully Protected Trinucleotide Blocks—5'-Dimethoxytrityl-*N*-acyl nucleoside (1.2 m eq to 5'-free hydroxyl protected nucleoside) was dissolved in anhydrous pyridine (2 ml per mmol of the nucleoside component). A mixture of *p*-chlorophenyl phosphorodichloridate (2.4 m eq), triazole (5.3 m eq) and triethylamine (5.3 m eq) was stirred in anhydrous dioxane (6 ml per mmol), first at 0°C and then at room temperature for 1 h. The precipitates were filtered off and the filtrate was added to the pyridine solution mentioned above. The mixture was concentrated to about half the initial volume and kept for 1 h

at room temperature. Aqueous pyridine (50%) was added to the mixture and the product was extracted with chloroform. The chloroform layer was washed with 0.1 M triethylammonium bicarbonate (TEAB) and then concentrated under reduced pressure. To this syrup, 5'-hydroxy-*N*,3'-protected nucleoside (1 m eq) was added and the product was coevaporated with anhydrous pyridine. The dried residue was treated with MSNI (3 m eq to 3'-phosphodiester component) and MeIm (6 m eq) in pyridine for 2 to 3 h at room temperature. Aqueous pyridine was added to the reaction mixture and the product was extracted with chloroform. The extract was washed with 0.1 M TEAB and concentrated to dryness. After coevaporation with pyridine and then with toluene, the residue was dissolved in chloroform-methanol (7:3 v/v) containing 2% BSA and kept at 0°C. After 15 min, the reaction mixture was washed with 5% sodium bicarbonate solution and then with water. The chloroform layer was concentrated under reduced pressure and the residue was purified by silica gel column chromatography. 5'-Dimethoxy *N*-acyl 3'-*p*-chlorophenyl phosphonucleoside (1.1 m eq) synthesized by the previously described method¹⁹ was added to the product and the mixture was coevaporated in order to eliminate moisture completely. The dried residue was first treated with MSNT and MeIm for 2 to 3 h and then the product was isolated by using a silica gel column. The reaction conditions and isolated yields are given in Table I.

Detritylation and Decyanoethylation of fully Protected Phosphotriester Intermediates—Detritylation was effectively performed by treatment with 2% BSA in chloroform (7:3 v/v) at 0°C for 10 min; 44 ml of this solution was used for each mmol of protected intermediates. When trityl cleavage was completed, the reaction mixture was neutralized with 5% NaHCO₃ (20 ml) and the chloroform layer was separated. The aqueous layer was extracted again with chloroform and the extract was combined with the first extract. This organic layer was washed with 5% NaHCO₃ and then twice with water. The residue after the removal of the chloroform was used directly as the 5'-hydroxyl component in the subsequent condensation reaction without further purification. Selective decyanoethylation was carried out by treatment with anhydrous pyridine-triethylamine (1:1) solution (12 ml) containing the fully protected triester intermediate (0.1 mmol) at 30 to 35°C for 2 h. Most of the solvents were evaporated off *in vacuo*. Traces of water were removed by repeated evaporations with dry pyridine, and the residue was employed as the 3'-arylphosphodiester component in the subsequent reaction.

Syntheses of the Dodecanucleotides—a) DMTdbzC°dbzA°T°CE (546.3 mg, 0.3 mmol) was decyanoethylated and DMTdbzC°dbzC°dbzA°CE (384.5 mg, 0.2 mmol) was detritylated as described above. The resulting intermediates were mixed and coevaporated with pyridine, and then treated with 4 ml of anhydrous pyridine solution containing MSNI (296.4 mg, 0.9 mmol) and MeIm (0.18 ml, 1.8 mmol) for 2 h. The product was extracted with chloroform after 1 ml of aqueous pyridine (50%) had been added to the reaction mixture. The extract was washed with 0.1 M TEAB and concentrated to dryness. After coevaporation with pyridine and then with toluene in order to remove traces of moisture, the residue was dissolved in a small amount of chloroform and applied to a small column of silica gel (15 g) for purification. The product was eluted with a mixture of chloroform:methanol (40:1) and the yield was 416 mg (62%).

b) DMTdbzC°dbzA°TBz (168.3 mg, 0.1 mmol) was detritylated and combined with decyanoethylated DMTdbzC°dbzC°dbzC°CE (284.8 mg, 0.15 mmol) in anhydrous pyridine (1 ml). MSNI (133 mg, 0.45 mmol) and MeIm (0.09 ml, 0.9 mmol) were added and the reaction mixture was allowed to stand for 4 h. The product was isolated as described above and purified to yield 314 mg (98%).

c) DMTdbzC°dbzA°T°dbzC°dbzC°dbzA°CE (133.6 mg, 0.04 mmol) was decyanoethylated and combined with detritylated DMTdbzC°dbzC°dbzC°dbzC°dbzA°TBz (60.9 mg, 0.02 mmol) in anhydrous pyridine (1 ml). MSNI (37.7 mg, 0.12 mmol) and MeIm (24 μl, 0.24 mmol) were added and the reaction mixture was allowed to stand for 3 h. The reaction product was isolated and purified by preparative TLC on silica gel as described previously to yield 102 mg (87%).

d) Fully protected dodecamer dAGCCATCTGCTT was synthesized by the same procedure as described above in a yield of 66 mg from the hexamers (55%).

Deprotection of Trinucleotide Block—Procedure A: DMTdbzA°dbzC°dbzCBz (10 mg) was treated with 2 ml of 0.3 M *N*¹,*N*¹,*N*²,*N*²-tetramethylguanidium pyridine 2-carboxaldoximate (PAO) solution (dioxane:water 1:1) at room temperature. After 19 h the reaction mixture was concentrated to dryness and dissolved in 4 ml of conc. ammonia at 50°C for 3 h. The residual material obtained by evaporating off the ammonia was dissolved in 5 ml of 80% aqueous acetic acid. The product was obtained by evaporation of acetic acid and desalted on a DEAE-cellulose column using TEAB buffer. This eluate was directly analyzed for purity by HPLC.

Procedure B: DMTdbzA°dbzC°dbzCBz (10 mg) was treated with 2% BSA in chloroform-methanol (7:3) at 0°C for 5 min. The mixture was neutralized with 5% NaHCO₃, and the chloroform layer was separated from the aqueous layer and washed successively with 5% NaHCO₃ and water. The chloroform was evaporated off and the residual materials were completely deblocked with 2 ml of 0.3 M PAO solution at room temperature for 19 h. The reaction mixture was evaporated to dryness and the residue was dissolved in 4 ml of concentrated ammonia. After 3 h at 50°C, the ammonia was evaporated off and the product was dissolved in a small amount of water for application to a DEAE-cellulose column as described above. The eluate was analyzed by HPLC.

Deprotection of Dodecanucleotide—Fully protected dodecanucleotides, dCATCCACCCCAT (2.5 μ mol) and dAGCCATCTGCTT (2.7 μ mol), were treated with 0.3 M dioxane–water solution (1: 1) of PAO (1.6 ml and 1.3 ml, respectively) at room temperature for 19 h. Each reaction mixture was concentrated by evaporation of the solvents, and the residue was treated with 10 ml of concentrated ammonia at 50°C for 3 h. The residues obtained by evaporation of the ammonia were each dissolved in 10 ml of 80% aqueous acetic acid and kept at room temperature for 30 min. After evaporation of the aqueous acetic acid, each residue was dissolved in 15 ml of water and purified on a DEAE-cellulose column (1 \times 10 cm). The column was washed first with 0.05 M TEAB and then with 1 M TEAB. The eluate containing the reaction product was concentrated and chromatographed by reverse-phase HPLC on a Nucleosil C₁₈ column (4.6 \times 500 mm). The column was eluted with a linear gradient of acetonitrile from 10 to 30% in 0.1 M ammonium acetate buffer, pH 7. The products thus obtained were each desalted on a DEAE-cellulose column (1 \times 10 cm) and shown to have a purity greater than 95% by HPLC on a Nucleosil column.

Enzymatic Hydrolysis of dCATCCACCCCAT and dAGCCATCTGCTT—A solution of dCATCCACCCCAT (3 OD units) in 80 μ l of 0.05 M TEAB (pH 7.5) was incubated with 10 μ l of venom phosphodiesterase solution (1 μ g/ml) at 37°C for 6 h. The HPLC pattern showed complete digestion of the dodecanucleotide to C, pA, pT, and pC in ratios of 1.19: 3.00: 2.16: 6.21 (Calcd 1: 3: 2: 6). dAGCCATCTGCTT (1.5 OD units) was also digested with venom phosphodiesterase in a similar manner and yielded pC, pT, pG, pA, and A in ratios of 4.09: 4.08: 2.11: 0.94: 1.00 (Calcd 4: 4: 2: 1: 1).

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