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OLIGODEOXYRIBONUCLEOTIDE SYNTHESIS BY USE OF S,S-DIPHENYL DEOXYRIBONUCLEOSIDE 3'-PHOSPHORODITHIOATES AND BIFUNCTIONAL CONDENSING REAGENTS IN THE PHOSPHOTRIESTER APPROACH

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Abstract—Four kinds of arenedisulphonyl chlorides as condensing reagents for oligodeoxyribonucleotide synthesis have been synthesised and their condensing abilities were examined in the synthesis of thymidylyl(3'-5')thymidine. Among them, mesitylenedisulphonyl chloride (MDS) and isodurenedisulphonyl chloride (DDS) proved to be effective for condensation and for separation of the product from the 5'-sulphonated by-product formed simultaneously. This paper describes detailed studies on the selective removal of one of two phenylthio groups by means of phosphinates from S,S-diphenyl deoxyribonucleoside 3'-phosphorodithioate derivatives, which were successfully used as the starting building units for the liquid phase synthesis of a dodecadeoxyribonucleotide, dCATTATTAATAC.

Over the last five years, the chemical synthesis of oligonucleotides has been extensively developed.[1] The manipulation has become increasingly practical by the introduction of the solid phase synthesis in both the phosphotriester and the phosphite–phosphoramidite approaches. However, the solid phase synthesis required large excess amounts of mono- or dinucleotide building blocks in each coupling step. Accordingly, when oligodeoxyribonucleotides are needed on a 100 mg scale for physicochemical studies, the liquid phase synthesis is still used since stoichiometric or small excess amounts of phosphodiester components are available for chain elongation.

We have described briefly a new method for the synthesis of oligodeoxyribonucleotides by the use of bifunctional condensing agents.[2] In this paper, we wish to report more detailed studies on oligodeoxyribonucleotide synthesis by the use of our own approach.

RESULTS AND DISCUSSION

Bifunctional condensing reagents

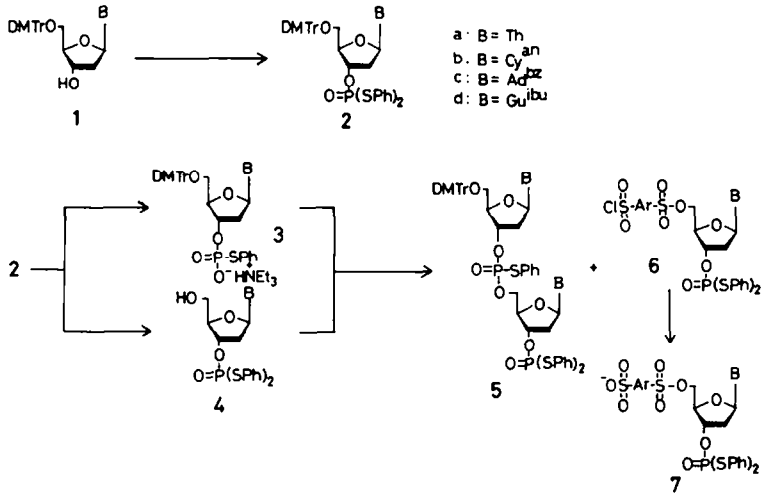
A number of condensing reagents have been explored for oligonucleotide synthesis.[1] Especially in the phosphotriester approach, monofunctional arenesulphonyl azoles such as mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT)[3] and triisopropylbenzenesulphonyltetrazole (TPSTe)[4] have been generally used with considerable success. However, these condensing reagents do not allow easy separation of fully protected oligodeoxyribonucleotides from the 5'-O-sulphonated by-products simultaneously formed.[5] In the hope of finding a solution to this problem, we prepared "bifunctional condensing reagents" that facilitated purification of the fully protected oligonucleotide building blocks. The bifunctional reagent is designed to have two chlorosulphonyl groups in the same molecule and one of them can work for the condensation reaction. When the reagent gives the 5'-O-sulphonated material, the other chlorosulphonyl group remaining in the by-product can lead to a polar material upon hydrolysis so that the isolated product is free from the sulphonated species as shown in Scheme 1.

Four kinds of bifunctional reagents were prepared and their condensing abilities were examined. 4,6 - Dimethoxybenzene - 1,3 - disulphonyl chloride (DMS) was prepared from 1,3-dimethoxybenzene as illustrated in Scheme 2. Direct chlorosulphonylation gave crude DMS in good yield. However, the crude material obtained by this route could not be purified by recrystallisation. 2,4,6 - Trimethylbenzene - 1,3 - disulphonyl chloride (mesitylenedisulphonyl chloride: MDS) and 2,4,5,6 - tetramethylbenzene - 1,3 - disulphonyl chloride (isodurenedisulphonyl chloride: DDS) were obtained by using a modification of the literature method.[6] 2,4,6 - Trimethoxybenzene - 1,3 - disulphonyl chloride and 2,4,6 - triisopropylbenzene - 1,3 - disulphonyl chloride could not be obtained by the same procedure. 2,3,5,6 - Tetramethylbenzene - 1,4 - disulphonyl chloride was obtained but was extremely unstable. Naphthalene-1,5-disulphonyl chloride (NDS) was prepared by chlorination of the corresponding disulphonic acid.[7]

At the start of this work, we tried to prepare arenedisulphonyl azolides. However, 2,4 - dimethoxybenzene - 1,3 - disulphonyltetrazole (DMSTe) was found to be extremely explosive on drying. On the basis of this observation, other arenedisulphonlytetrazole derivatives were not synthesised and their explosive properties are consequently unknown. To avoid the use of such dangerous derivatives, we tested combined reagents of arenesulphonyl chlorides and azoles. Seth and Jay[5d] reported that a mixture of TPS and tetrazole can be used in condensation in the same manner as TPSTe.

Condensing abilities of the bifunctional reagents

Triethylammonium S-phenyl 5' - O - (4,4' - dimethoxytrityl) - thymidine 3' - phosphorothioate (**3a**) was condensed with S,S-diphenyl thymidine 3'-phosphorodithioate (**4a**) by use of the bifunctional condensing reagents. The conditions and results are summarised in Table 1. The decreasing order of the



Scheme 1.

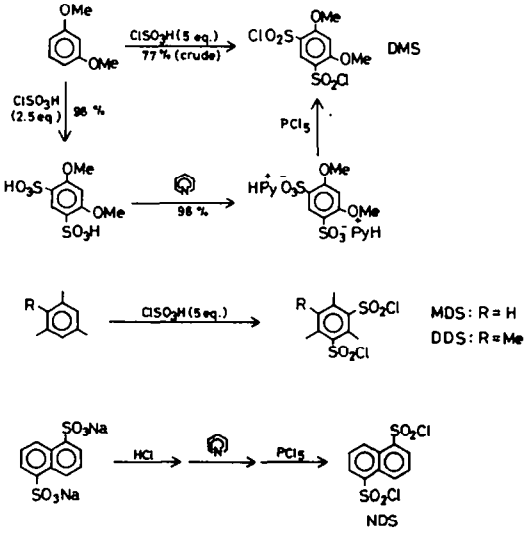
reactivity of the condensing reagents was NDS, MDS, DDS, DMS as expected from the electron-donating effect of the substituents. Although NDS was the most reactive, the yield of the dimer (**5**) was much lower than those from the others because of the significant 5'-sulphonation. The reaction by use of DMS was considerably slower. The hindered bifunctional reagents, MDS and DDS, were found to have similar condensing abilities to mesitylene-sulphonyl chloride (MS). The latter gave an optimum yield of **5**. In these experiments, all the 5'-sulphonated by-products (**6**) were converted to base-line materials (**7**) as evidenced by TLC analysis.

We know that MS severely decomposed when stored at room temperature for several months. Compared with the lability of MS on storage, MDS and DDS can be stored as stable crystalline materials at room temperature for at least a year.

The azoles, 3-nitro-1,2,4-triazole (NT) and tetrazole (Te) gave good results whereas triazole took longer periods of time and 4-(dimethylamino)pyridine (DMAP) which is known to be an effective catalyst in general acylation reactions, gave a complex mixture.

Mononucleotide units

Fully protected mononucleotide units (**2a–d**) are the key starting compounds for the modified phosphotriester approach to oligonucleotide synthesis. We used the isobutyryl, anisoyl and benzoyl groups for protecting the amino groups of deoxyguanosine, deoxycytidine and deoxyadenosine, respectively. The 5'-hydroxyls were protected by the 4,4'-dimethoxytrityl (DMTr) group in the usual manner. The 3'-phosphoryl residue was protected with the bis(phenylthio)phosphoryl (BPTP) group. Phosphorylation on N-protected 5'-O-(4,4'-dimethoxytrityl) deoxyribonu-



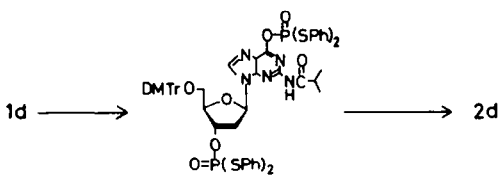
Scheme 2.

Table 1. Conditions and results of condensation of **3a** with **4a**[a]

Condensing reagent[b] (equiv. to **4a**)	Time (min)	Yield of **5a** (%)
DMS (3.0) Te (6.0)	40	74
MDS (3.0) Te (6.0)	25	86
DDS (3.0) Te (6.0)	30	78
NDS (3.0) Te (6.0)	20	48
MDS (2.0) Te (4.0)	60	71
MDS (2.0) Tri (4.0)	300	70
MDS (2.0) NT (4.0)	60	71
MDS (2.0) DMAP (4.0)	60	44
MDS (1.5) Te (3.0)	180	51
DMS (3.0) Te (3.0)	60	78
DDS (2.0) Te (3.0)	120	92
DDS (3.0) NT (3.0)	40	96
DDS (2.0) NT (3.0)	40	92

[a] The ratio of **3a** to **4a** is 1.2 in all the reactions.

[b] Te, Tri, NT, and DMAP refer to tetrazole, 1,2,4-triazole, 3-nitro-1,2,4-triazole, and 4-(dimethylamino)pyridine, respectively. For abbreviations of the condensing agents see Scheme 2.



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Scheme 3.

Table 2. Phosphorylation of **1a–d** with PSS by use of DMS

Compound	PSS/1	DMS/1	Time (hr)	Yield (%)
1a	1.1	1.2	18	87
1b	1.2	1.3	21	89
1c	1.2	1.3	23	86
1d	1.2	1.3	29	60
1d	4.0	2.0	4	87

cleotides (**1a–d**) was carried out by use of cyclohexylammonium S,S-diphenyl phosphorodithioate (PSS)[9,10] and DMS. Compounds **1a–c** underwent the phosphorylation smoothly with the use of small excess amounts of the reagents. In the case of **1d**, an O^6-phosphorylated compound (**8**) was observed (Scheme 3) and the reaction was not completed under the same conditions. Therefore, in this case, the nucleoside **1d** was converted to **8** by treatment with large excess amounts of PSS and DMS and then the final product was hydrolysed to **2d** by the addition of sodium bicarbonate. These results are lised in Table 2. Elemental analysis and some physical properties of **2a–d** are listed in Tables 3 and 4.

Table 3. Melting points and elemental analysis of compounds **1a–d**

Compound	M.p.°[a]	Formula	C	H	N	S
2a	126–127	$C_{43}H_{41}N_2O_8PS_2$	63.84[b]	5.11	3.46	7.93
			63.59[c]	5.24	3.40	8.00
2b	96–99	$C_{50}H_{44}N_5O_7PS_2 \cdot 1/2H_2O$	64.50	4.87	7.52	6.89
			64.39	4.87	7.42	7.19
2c	95–105	$C_{50}H_{46}N_3O_3PS_2$	64.71	5.00	4.53	6.91
			64.89	5.09	4.36	6.99
2d	106–112	$C_{47}H_{46}N_5O_8PS_2$	62.45	5.13	7.75	7.09
			62.58	5.31	7.73	7.20

[a] Decomposition.
[b] Upper figure % calc.
[c] Lower figure % found.

Table 4. ^{1}H-NMR spectra of compounds **2a–d**[a]

Compound	1'-H	2'-H	3'-H	4'-H	5'-H	Others
2a	6.33 t J = 7 Hz	2.36 m	5.21 m	3.07 m	3.39 m	1.71 (s, 3, CH_3), 3.78 (s, 3, CH_3O), 6.80 (d, J = 9 Hz, 4, ArH), 7.02–7.65 (m, 21, ArH), 8.77 (s, 1, NH)
2b	5.34 t J = 6 Hz	2.30 m	2.81 m	5.37 m	4.29 m	3.50 m 3.82 (s, 3, CH_3O), 3.93 (s, 3, CH_3O (an)), 6.90 (d, J = 9 Hz, 4, ArH), 7.05 (d, J = 9 Hz, 2, ArH), 7.19–7.77 (m, 19, ArH), 7.95 (d, J = 9 Hz, 2, ArH (an)), 8.17 (d, J = 8 Hz, 1, 5-H)
2c	6.42 m	2.55 m	5.49 m	4.33 m	3.38 m	3.78 (s, 3, CH_3O), 6.84 (d, J = 9 Hz, ArH), 7.10–7.82 (m, 19, ArH), 8.76 (s, 1, 2-H), 8.76 (s, 1, 8-H), 9.29 (br s, NH)
2d	6.13 t J = 7 Hz	2.166 m	5.86 m	4.26 m	3.48 m	1.20 (m, 6, CH_3), 2.53 (m, 1, CH), 3.83 (s, 3, CH_3O), 6.80 (d, J = 9 Hz, 4, ArH), 7.08–7.77 (m, 20, ArH), 7.80 (s, 1, 8-H), 9.21 (br s, 1, NH)

[a] The solvent was $CDCl_3$. Chemical shifts are given in ppm (δ).

Selective deprotection from the mononucleotide units and oligodeoxyribonucleotide building blocks

Properties of the BPTP group have been reported previously.[9] One of the two phenylthio groups can be removed from the BPTP group by mild alkaline treatment[9] or by the action of pyridinium phosphinate (PSA) under neutral conditions.[2a] Oligonucleotide synthesis requires selectivity in partial deprotection between the internucleotidic and 3'-terminal phenylthio groups for chain elongation of oligomers. In order to satisfy this situation, various conditions for the selective phosphinate treatment were examined in detail as shown in Table 5. The 3'-terminal dephenylthiolation was partially dependent on temperature and the concentration of phosphinic acid. In the case of higher phosphinate concentrations (greater than 1.5 M) elimination of the DMTr group was often observed. To prevent the detritylation, triethylamine was added and the phosphinate concentration could then be increased to 5 M.[11] Use of such a high concentration shortened the time of the phosphinate treatment. Under these conditions the internucleotidic phenylthio groups were stable while one of the two phenylthio groups was easily removed from the 3'-terminal BPTP group. Interestingly, dephenylthiolation was independent of the type of counter ions of phosphinate as suggested in Table 5. Dephenylthiolation was more conveniently done from the fully protected mononucleotide units. The units **2a–d** were treated with triethylamine–water–pyridine (2:1:2, v/v/v) at room temperature for 20–60 min. After the reaction was completed, the mixture was evaporated to dryness and the residue was used as a phosphodiester component in the next condensation without further purification. These results are summarised in Table 6. This method was available only for the mononucleotide units, since the internucleotidic phenylthio groups might be lost under these conditions. In conclusion, we used the following two methods for removal of the 3'-terminal phenylthio group: 5 M pyridinium phosphinate (pyridine solution)–triethylamine (2:1, v/v) at 40° (method A); triethylamine–water–pyridine (2:1:2, v/v/v) at room temperature (method B).

Table 6. Removal of one of the two phenylthio groups from **2a–d** by treatment with triethylamine–water–pyridine (2:1:2, v/v/v)[a]

Compound	Time (min)	Yield of 3 (%)
2a	60	98
2b	60	94
2c	25	95
2d	40	96

[a] The reaction was carried out at room temp.

To prepare 5'-terminal hydroxyl components, trifluoroacetic acid (TFA) was the reagent used for the removal of the DMTr group. Complete deblocking was achieved at 0° in less than 5 min, being independent of the chain length. Under these conditions, depurination of the deoxyadenosine was not observed over a period of 15 min.

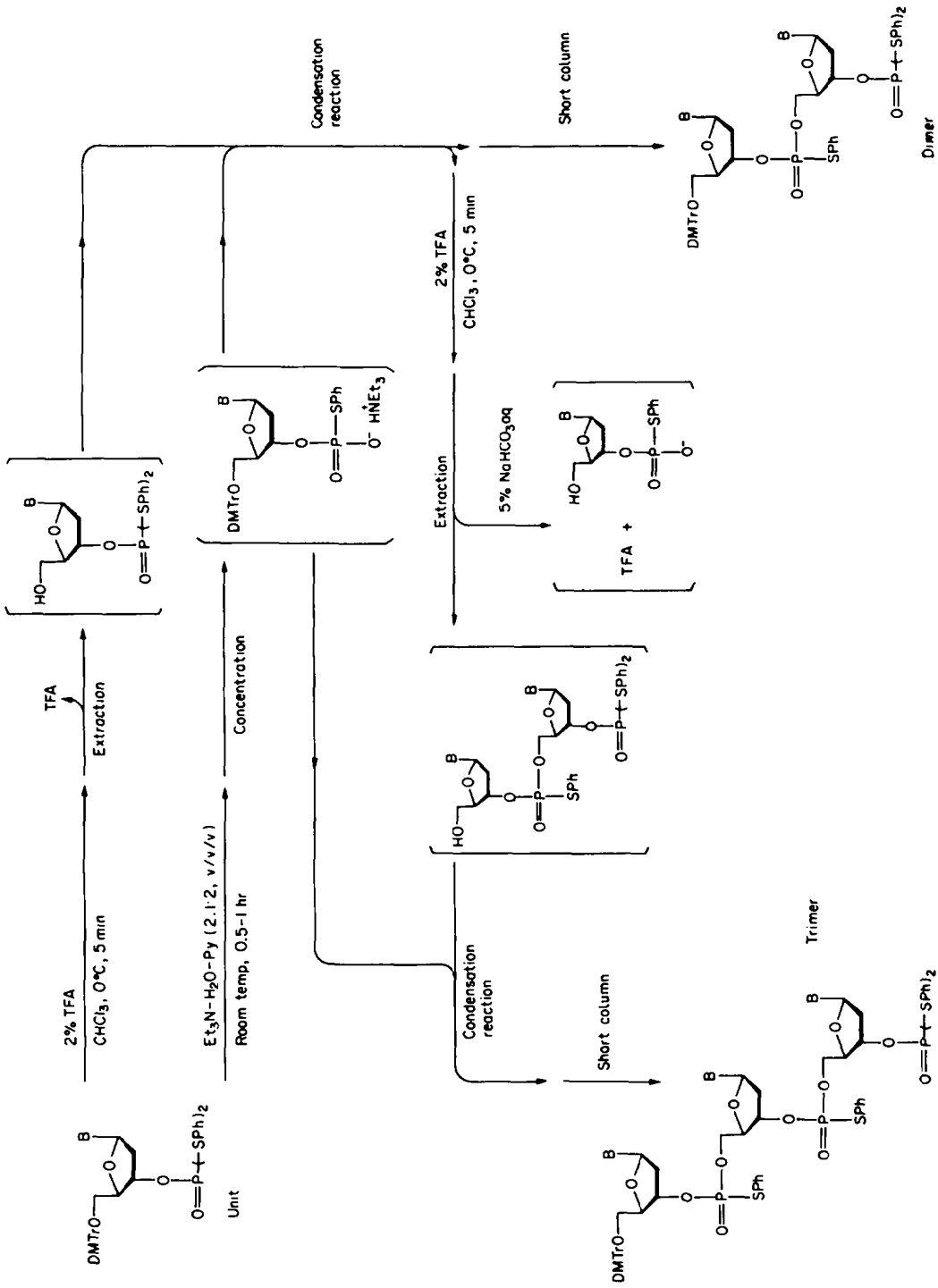
Formation of internucleotidic bonds

Compared with the solid phase synthesis, the liquid phase synthesis involved time-consuming procedures for the purification of synthetic intermediates. However, we could omit the tedious purification of the phosphodiester and hydroxyl components because both the thiol and the tritanol released by the selective partial deprotection were entirely inert in the condensation.

Short oligodeoxyribonucleotide blocks (dimer and trimer) could be conveniently prepared by a modified method of Broka *et al.*[13] The outline of the simplified preparation of dimers and trimers is illustrated in Scheme 4. The mononucleotide units were converted to the phosphodiester and hydroxyl components, which *in situ* were condensed by treatment with the bifunctional condensing reagents. Dimers were easily obtained by flash column chromatography. For the trimer synthesis, crude fully protected dimers obtained by the extractive workup were detritylated without further purification. At this stage the excess

Table 5. Times required for complete removal of one of the two phenylthio groups from **2a** under various conditions

Concentration **2a** (M)	PSA (M)	Additive (vol %)	Temperature (°)	Time (hr)
0.1	0.12		room temp	3 days
0.1	0.2		room temp	1 day
0.1	0.4		room temp	1 day
0.1	1.0		room temp	16
0.05	0.2		40	5
0.1	1.0		40	2
0.1	1.5		40	1.5
0.1	1.0	Bu_4NOH (cat.)	room temp	20
0.1	1.0	Bu_4NOH (0.1 M)	room temp	3
0.1	1.0	H_2O (10%)	40	3
0.1	2.0	H_2O (10%)	40	3
0.1	1.0	MeOH (10%)	room temp	6
0.1	1.0	MeOH (10%)	40	3
0.1	5.0	Et_3N (33%)	40	0.4


2% TFA
CHCl3, 0°C, 5 min
TFA
Extraction
Unit
Et3N-H2O-Py (2:1:2, v/v/v)
Room temp, 0.5-1 hr
Concentration
Condensation reaction
Short column
Trimer
Condensation reaction
Extraction
5% NaHCO3aq
2% TFA
CHCl3, 0°C, 5 min
Short column
Dimer
TFA +

Scheme 4.

Table 7. Simplified synthesis of fully protected dimers and trimers

Unit (equiv. to terminal unit)[a]					Condensing reagent			Time (hr)		Yield (%)
		Tp	(1.2)	Tp	MDS	(3)	Te (6)	23		80
		Tp	(1.3)	Tp	MDS	(3)	NT (3)	20		80
		Tp	(1.3)	Tp	DDS	(3)	NT (3)	40		94
		dCp	(1.2)	dAp	DDS	(3)	NT (3)	40		80
		dCp	(1.3)	dAp	MDS	(3)	Te (6)	25		80
		dCp	(1.3)	dAp	MDS	(3)	NT (3)	45		95
		dAp	(1.2)	Tp	MDS	(3)	NT (3)	30		84
		dAp	(1.3)	Tp	DDS	(3)	NT (3)	50		96
		dAp	(1.3)	dCp	MDS	(3)	NT (3)	45		76
		dAp	(1.3)	dCbz	DDS	(3)	NT (3)	40		89
Tp	(1.2)	Tp	(1.2)	Tp	MDS	(3)	NT (3)	30	45	69
Tp	(1.2)	Tp	(1.2)	Tp	DDS	(3)	NT (3)	40	60	78
Tp	(1.3)	Tp	(1.3)	Tp	MDS	(3)	NT (3)	50	60	88
Tp	(1.2)	Tp	(1.2)	dAp	MDS	(3)	NT (3)	50	90	83
Tp	(1.3)	Tp	(1.3)	dAp	MDS	(3)	NT (3)	40	50	88
Tp	(1.2)	Tp	(1.2)	dAp	DDS	(2)	NT (3)	40	60	82
dAp	(1.2)	Tp	(1.2)	dAp	DDS	(3)	NT (3)	50	60	77
dCp	(1.2)	dAp	(1.2)	Cp	DDS	(2)	NT (3)	25	35	91
Tp	(1.2)	dAp	(1.2)	Cp	DDS	(2)	NT (3)	40	60	78
Tp	(1.2)	dAp	(1.2)	dAp	DDS	(2)	NT (3)	40	30	65

[a] Tp, dCp, dAp and dCbz refer to **2a**, **2b**, **2c** and N^4-anisoyl-3'-O-benzoyl deoxycytidine, respectively.

phosphodiesters used in the first coupling reaction could be separated from the desired 5'-hydroxyl dimer components by extraction with 5% sodium bicarbonate soln. Finally, the fully protected trimers were synthesised by condensation with the crude dimers and the 3'-terminal phosphodiesters prepared in the same way. The trimers could also be purified rapidly by short column chromatography. In the simplified method, the bifunctional condensing reagents were extracted into the aqueous layer as well as the hydrolysed 5'-sulphonated by-products. According to this method, protected dimers and trimers could be prepared in 1 and 1.5 days, respectively, from the fully protected units which could be stored for long periods of time on a large scale. These results are summarised in Table 7.

Strategy for the synthesis of a dodecamer

The phosphinate approach was applied to the synthesis of a dodecamer which was complementary to the 5'-leader sequence of brome mosaic virus mRNA filament No. 4 as depicted in Fig. 1. We chose two synthetic strategies as shown in Fig. 1. Several dimer and trimer building blocks were synthesised by the simplified method described above. Block condensation was carried out without purification of the phosphodiester and 5'-hydroxyl components. These conditions and results are summarized in Table 8. As the oligomer chain was elongated, elution of the product from a silica gel column became increasingly difficult and the yield became lower. Nonetheless, TLC analysis showed that all the condensations were

Table 8. Block condensation for the synthesis of the fully protected dodecadeoxyribonucleotide[a]

Diester component (mmol)	Hydroxyl component (mmol)	Phosphinate treatment[b] (min)	Time taken for removal of DMTr group (min)	Ratio of diester/ hydroxyl	Condensing agent (equiv. to hydroxyl) $Ar(SO_2Cl_2)_2$		Azole		Time (min)	Yield of oligomer (%)
dATp	dAC_{bz}	25	8	1.2	MDS	(3)	NT	(3)	30	66
				1.3	DDS	(2)	NT	(3)	40	91
dCAp	dTTAp	20	5	1.2	MDS	(3)	NT	(3)	50	79
				1.2	DDS	(2)	NT	(3)	60	78
dTTAp	$dATAC_{bz}$	60	5	1.5	MDS	(3)	NT	(3)	40	66
				1.3	DDS	(3)	NT	(3)	80	68
dTTAp	$dTTAATAC_{bz}$	60	7	1.3	MDS	(3)	NT	(3)	40	68
				1.3	DDS	(2)	NT	(3)	60	68
dCATTAp	$dTTAATAC_{bz}$	40	5	1.4	MDS	(3)	NT	(3)	90	54
				1.3	DDS	(3)	NT	(3)	60	52
dCAp	$dTTATTAATAC_{bz}$	50	7	1.3	DDS	(2)	NT	(3)	60	28

[a] $dN^1N^2 \ldots N^m p$ and $dN^1N^2 \ldots N^m{}_{bz}$ refer to the fully protected deoxyribonucleotide building blocks having the 3'-terminal BPTP and benzoyl groups, respectively.

[b] Phosphodiester components were prepared by method A.

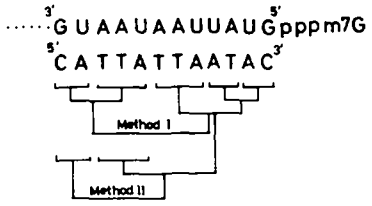


Fig. 1. Strategy for the synthesis of the fully protected dodecadeoxyribonucleotide which is complementary to the 5′-terminal leader sequence of brome mosaic virus mRNA filament No. 4.

completed under the conditions employed and independent of the chain length.

Deprotection of fully protected oligomers

The following four kinds of deprotection modes were examined: A (i) oximate,[14] (ii) conc ammonia, (iii) 80% acetic acid; B (i) conc ammonia, (ii) 80% AcOH; C (i) 0.1 M NaOH, (ii) conc ammonia, (iii) 80% AcOH; D (i) silver acetate in aqueous pyridine, (ii) concentrated ammonia, (iii) 80% AcOH. A fully protected tetramer of dApTpApC was treated by use of the above four methods. The crude unprotected tetramer was separated by paper chromatography and then analysed by ion exchange HPLC. The yields of dApTpApC were estimated to be 78, 43, 67 and 71% yields based on the HPLC chromatograms derived from methods A–D, respectively. The tetramers obtained by methods A–D were very pure, whereas the one from method B was accompanied with some degradation products. Method C was especially useful as the operation was the simplest and gave a result similar to that obtained by method A, which took a much longer time at the first step. Therefore, the fully protected dodecamers obtained using methods I and II shown in Fig. 1 were deprotected using method C.

Purification was performed by column chromatography using Toyopearl 650 M with a linear gradient of NaCl in the presence of 7 M urea (Fig. 2) followed by desalting with Bio-Gel P-2. Thus, the unprotected dodecamers were isolated in *ca* 20% yields from both methods I and II. The HPLC (Fig. 4) and

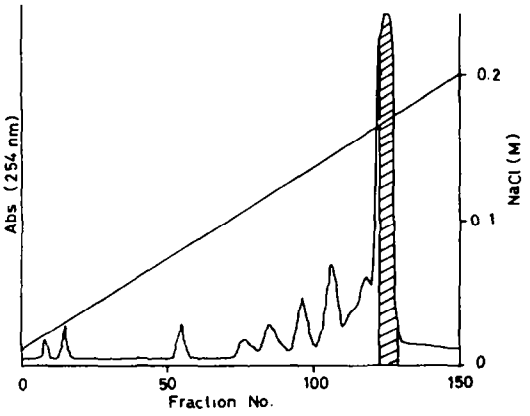


Fig. 2. Separation of the dodecamer on a DEAE Toyopearl 650 M column.

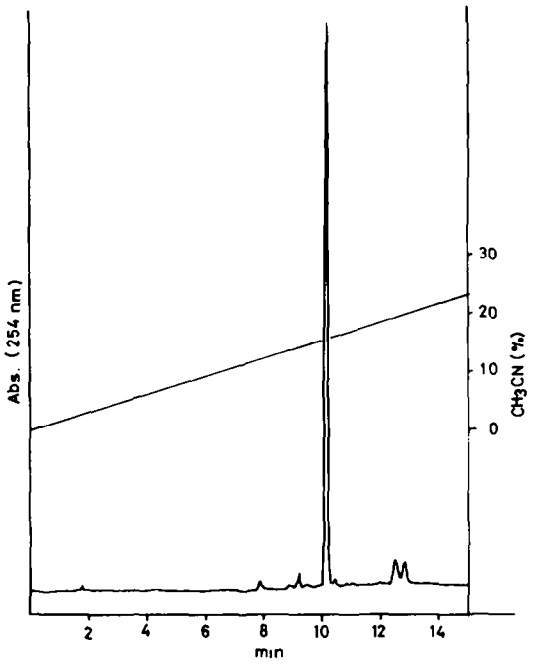


Fig. 3. HPLC profile of the purified tetramer, dApTpApC.

polyacrylamide gel electrophoresis analyses showed that the desired product was homogeneous. (Only data derived from method II are shown in Figs 2 and 4.) The structure of the dodecamers was also confirmed by the enzymatic degradation with snake venom phosphodiesterase and the two-dimensional base sequencing method.[8]

EXPERIMENTAL

^{1}H-NMR spectra were recorded at 100 MHz on a JEOL JNM-PS-100 spectrometer. Chemical shifts are given in ppm

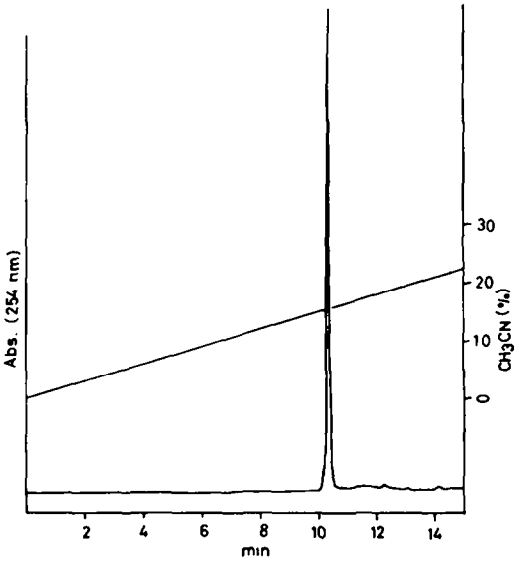


Fig. 4. HPLC profile of the purified dodecamer dCpApTpTpApTpTpApApTpApC.

(δ) relative to TMS as internal standard. UV spectra were obtained on a Hitachi 124 spectrophotometer. M.ps were measured on a Fisher–Jones melting point block and are uncorrected. Ion exchange HPLC was carried out on a JASCO TRIROTOR system with a Whatman Partisil 10/25 SAX column (250 × 4 mm). Linear gradient elution was performed by starting with buffer A (0.005 M KH_2PO_4 (pH 4.5)–CH_3CN, 4 : 1, v/v) and applying with 3% buffer B (0.05 M KH_2PO_4 plus 0.5 M KCl (pH 4.5)–CH_3CN, 4 : 1, v/v) per min (system I). Reverse-phase HPLC was performed with a Waters μBondapack C-18 column (300 × 4 mm) with a linear gradient of 0–50% CH_3CN in 0.1 M ammonium acetate (pH 7.0) (system II). TLC was carried out on plates of silica gel 69 F-254 (Merck) using the following solvent systems: solvent A: CH_2Cl_2–MeOH (12 : 1, v/v); solvent B: CH_2Cl_2–MeOH (9 : 1, v/v); solvent C: 1-propanol–conc ammonia–water (55 : 10 : 35, v/v/v); solvent D: 2-propanol–conc ammonia–water (7 : 1 : 2, v/v/v); solvent E: 2-propanol–conc ammonia–water (6 : 1 : 3, v/v/v). Paper chromatography was performed on Whatman 3 MM papers using the descending technique with solvents C and D.

Reagent grade pyridine was distilled by addition of *p*-toluenesulfonyl chloride, redistilled over calcium hydride after reflux for several hours, and stored in the presence of 4 Å molecular sieves for several weeks.

Compounds **1a–d** were prepared by the lit. method.[15] Isolation of appropriately protected nucleotide derivatives of the triester type was performed by using silica gel C-200 (Wako).

Purification of the completely deprotected dodecamer was carried out on a Gilson LC system using the anion exchange resin DEAE Toyopearl 650 M (Toyo Soda) eluted with a linear gradient of 0.02–0.2 M NaCl in buffer D (7 M urea, 20 mM Tris–HCl, pH 8.0). Desalting chromatography was performed with Bio-Gel P-2 (Bio Rad) impregnated with 0.05 M TEAB (pH 8.0).

Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, Nagatsuta.

4,6-Dimethoxybenzene-1,3-disulphonic acid

A soln of chlorosulphonic acid (35 ml, 0.5 mol) in CH_2Cl_2 (250 ml) was added dropwise over a period of 1 hr to a stirred soln of 1,3-dimethoxybenzene (27.6 g, 0.2mol) in CH_2Cl_2 (300 ml) at −5°. The mixture was allowed to reach room temp and stirred for 2 hr. A white ppt was filtered off with a glass filter and washed with CH_2Cl_2 (2 × 200 ml) and then ether (2 × 200 ml). The white powder was dried in a desiccator to give the required compound (58.6 g, 98%). This compound was used without further purification for the preparation of dipyridinium 4,6-dimethoxybenzene-1,3-disulphonate.

Dipyridinium 4,6-dimethoxybenzene-1,3-disulphonate

The disulphonic acid (58.6 g, 0.2 mol), obtained in the above experiment, was suspended in pyridine (300 ml) and stirred at room temp for 1 hr. Then, a white ppt was filtered off, washed with ether (2 × 100 ml), and dried *in vacuo* to give the dipyridinium salt (83.0 g, 92%). (Calc for $C_{18}H_{20}N_2O_8S_2$: C, 47.36; H, 4.42; N, 6.14; S, 14.05. Found: C, 46.71; H, 4.23; N, 5.83%.)

4,6-Dimethoxybenzene-1,3-disulphonyl chloride (DMS)

The pyridinium salt (22.9 g, 50 mmol) and PCl_5 (25.0 g, 120 mmol) were mixed and heated at 60° in an oil bath. After 4 hr crushed ice was carefully added and the mixture was diluted with water (200 ml). The resulting white ppt was collected by filtration, washed with cold water (100 ml) and ether (2 × 100 ml), and dried in a desiccator to give the analytically pure disulphonyl chloride (16.4 g, 98%): m.p. 175–178° (dec); NMR ($CDCl_3$) 4.13 (s, 6H, CH_3O), 6.61 (s, 1H, 3-H), 8.46 (s, 1H, 6-H). (Calc for $C_8H_8Cl_2S_2$: C, 28.67; H, 2.40; Cl, 21.16; S, 19.13. Found: C, 28.59; H, 2.43; Cl, 21.09%.)

Mesitylenedisulphonyl chloride (MDS)

Chlorosulphonic acid (33 ml, 0.49 mol) was added while stirring dropwise over a period of 30 min to mesitylene (12 g, 0.1 mol) at −5°. The mixture was stirred at −5° for an additional 1 hr, warmed to room temp, and then stirred under reduced pressure for 3 hr. A white ppt was collected by filtration with a glass filter and washed carefully with 2 M H_2SO_4 (3 × 70 ml) and then with cold water (2 × 100 ml). The solid was washed further with hexane (2 × 100 ml) and dried *in vacuo* over P_4O_{10} to give crude MDS. Recrystallisation from hexane–benzene (5 : 2, v/v, 280 ml) gave the pure material (22.1 g, 79%): m.p. 124°; NMR ($CDCl_3$) 2.77 (s, 6H, 4, 6-CH_3), 3.313 (s, 3H, 2-CH_3), 7.15 (s, 1H, 5-H). (Calc for $C_9H_{10}Cl_2O_4S_2$: C, 34.16; H, 3.19; Cl, 22.15; S, 20.27. Found: C, 34.05; H, 3.18; S, 20.30%.)

2,4,5,6-Tetramethylbenzenedisulphonyl chloride (DDS)

This compound was obtained from 1,3,4,5-tetramethylbenzene (13.4 g, 0.1 mol) by the same procedure as described in the case of MDS except for the reaction time (9 hr). Yield 15 g, 48% (after recrystallisation): m.p. 135°; NMR ($CDCl_3$) 2.35 (s, 3H, 5-CH_3), 2.72 (s, 6H, 4, 6-CH_3), 2.98 (s, 3H, 2-CH_3). (Calc for $C_{10}H_{12}Cl_2S_2$: C, 36.35; H, 3.66; Cl, 21.21; S, 19.41. Found: C, 36.26; H, 3.60; S, 19.47%.)

Naphthalene-1,5-disulphonyl chloride (NDS)

Disodium salts of naphthalene-1,5-disulphonic acid (2.72 g, 10 mmol) were suspended in pyridine (40 ml). To the suspension was added 0.5 M HCl (40 ml) and the resulting mixture was stirred until a clear soln was obtained. The solvent was removed under reduced pressure. The solid residue and PCl_5 (6.25 g, 30 mmol) were mixed and heated at 80° for 5.5 hr. The mixture was then cooled to room temp and cold water was carefully added. The white solid was collected by filtration and washed with cold water (3 × 50 ml). After the crude product (2.3 g, 88%) was dried *in vacuo*, it was recrystallised from hexane–benzene (2 : 3, v/v, 50 ml) giving pure white crystals (1.0 g, 38%).

4,6-Dimethoxybenzene-1,3-disulphonyl tetrazole

DMS (1.006 g, 3 mmol) and 1H-tetrazole (506 mg, 7.2 mmol) were dissolved in CH_2Cl_2–dioxan (2 : 1, v/v, 45 ml) and cooled at −5°. Et_3N (1.01 ml, 7.2 mmol) was added dropwise and the mixture was stirred at −5° for 10 min. The resulting mixture was then warmed to room temp and stirred for 3 hr. A white powder was collected by filtration and washed with CH_2Cl_2 (3 × 10 ml). Drying *in vacuo* gave the bis-tetrazolide (904 mg, 75%). *Caution*: purification and drying of this compound often led to explosion.

Reaction of MDS with the 3′-hydroxyl group of 5′-O-monomethoxytritylthymidine (MMTrT)

MMTrT (11 mg, 0.02 mmol) was dissolved in dry pyridine (0.3 ml). MDS (33 mg, 0.1 mmol) was added and the soln was stirred at room temp. The reaction was monitored by TLC (solvent B). As the reaction proceeded, MMTrT (R_f : 0.47) and two new spots (R_f : 0.05 and 0.51) appeared. After 1 day, water (0.1 ml) was added and the soln was stirred for 3 hr. TLC showed that the spot at R_f 0.51 disappeared while the starting material and a base-line product which was 3′-sulphonated MMTrT were detected.

Coupling reaction by use of bifunctional condensing reagents

Typical procedure. A mixture of **3a** (98 mg, 0.12 mmol), **4a** (51 mg, 0.1 mmol), and 1 H-tetrazole (42 mg, 0.6 mmol) was rendered anhydrous by repeated coevaporations with dry pyridine (3 × 2 ml). MDS (84 mg, 0.3 mmol) was added and the reaction was monitored by TLC (solvent A). After 25 min, **4a** disappeared on TLC. Then, the mixture was diluted with CH_2Cl_2 (10 ml) and washed with cold water (20 ml). The aqueous layer was extracted with CH_2Cl_2 (2 × 5 ml), and the organic extracts were combined and dried over Na_2SO_4. The solvent was removed *in vacuo* and coevaporated with toluene several times. The residue was chromatographed on a column of silica gel (3 g) and eluted with CH_2Cl_2 containing methanol (0–1.5%) to give fully protected thymidine dimer (96 mg, 86%).

Preparation of mononucleotide units

Typical procedure for the thymidine unit. Cyclohexylammonium S,S-diphenyl phosphorodithioate (PSS) (4.20 g, 11 mmol) was rendered anhydrous by repeated coevaporations with dry pyridine (3 × 10 ml) and dissolved in pyridine (60 ml). DMS (4.20 g, 12 mmol) was added and the soln was stirred for 1 hr. To the mixture was added 5'-O-dimethoxytritylthymidine (5.55 g, 10 mmol), pre-dried by coevaporations with dry pyridine (3 × 5 ml), and then the soln was stirred at room temp for 7 hr. Then ice cubes (10 g) were added and the mixture further stirred for 20 min. The mixture was diluted with CH_2Cl_2 (100 ml) and transferred into a separatory funnel. Water (100 ml) was added and the organic layer was separated. Extraction was performed with CH_2Cl_2 (2 × 100 ml) and the combined extracts were dried over Na_2SO_4. The solvent was removed *in vacuo* and the last traces of pyridine were completely removed by repeated coevaporations with toluene. The residue was charged on a column of silica gel (200 g) and eluted with CH_2Cl_2 containing MeOH (0–0.5%) to give the thymidine unit (7.04 g, 87%).

Preparation of deoxyguanosine unit. PSS (1.53 g, 4 mmol) was rendered anhydrous by coevaporations with dry pyridine (3 × 5 ml) and dissolved in pyridine (10 ml). DMS (0.67 g, 2 mmol) was added and the mixture was stirred at room temp for 1 hr. To 5' - O - dimethoxytrityl - N^2 - isobutyryldeoxyguanosine (0.64 g, 1 mmol), pre-dried by coevaporations with dry pyridine (3 × 3 ml), was added with pyridine (2 + 2 + 1 ml), and the resulting soln was stirred for 4 hr. Then cold 5% $NaHCO_3$ aq (5 ml) was added and the clear soln was stirred for 1 hr. The mixture was transferred into a separatory funnel with CH_2Cl_2 (10 + 5 + 5 ml) and washed with water (30 ml). The organic layer was separated and further extraction from the aqueous layer was performed with CH_2Cl_2 (2 × 20 ml). The organic layer was concentrated and coevaporated several times with toluene to give a foam. The foam was dissolved in CH_2Cl_2–hexane and applied to a column of silica gel (30 g) and eluted with CH_2Cl_2 containing MeOH (0–1%) to give the deoxyguanosine unit (778 mg, 87%).

Data for the physical properties and elemental analysis of all units are described in Tables 3 and 4.

Removal of the 3'-terminal phenylthio group

A pyridine soln of PSA was prepared as follows: commercially available phosphinic acid (5 M aqueous soln) was coevaporated with dry pyridine repeatedly and diluted with dry pyridine to the desired concentration of 1–5 M.

Method A—general procedure. A fully protected oligomer (0.1 mmol) was treated with 5 MPAS–Et_3N (2:1, v/v, 2.3 ml) at 40°. The reaction was monitored by TLC (solvent A or B). When the starting compound was converted to a base-line compound on TLC, the mixture was transferred into a separatory funnel with $CHCl_3$ (10 + 3 + 3 ml) and washed with water (3 × 10 ml). The organic phase was collected and the combined aqueous layers were extracted with $CHCl_3$ (10 ml). The latter organic layer was washed with water (10 ml). All the extracts were combined and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the residue was coevaporated with toluene repeatedly and used without further purification in the next condensation as the phosphodiester component. Purification of the above component was carried out as follows: The residue was dissolved in $CHCl_3$ (0.5 ml) and added by stirring dropwise to hexane (100 ml). A white precipitate was collected by filtration and washed with hexane (3 × 30 ml) to give the pure triethylammonium salt of phosphodiester component.

Method B—typical procedure. Thymidine unit (809 mg, 1 mmol) was dissolved in pyridine (10 ml). To the solution was added triethylamine (10 ml) and water (5 ml). The mixture was stirred for 1 hr at room temp. The solvent was removed under reduced pressure and coevaporated with toluene to remove the last traces of pyridine completely. The residue was dissolved in CH_2Cl_2 (2 ml) and dropped with stirring into hexane (200 ml). A white ppt was collected by filtration, washed with hexane (3 × 40 ml), and dried *in vacuo* to give the phosphodiester derivative (800 mg, 98%).

Removal of the DMTr group

General procedure. A fully protected oligomer (0.1 mmol) was dissolved in $CHCl_3$ (4 ml) and cooled to 0°. Trifluoroacetic acid (TFA) (0.08 ml) was added at once and the mixture was stirred at 0° continuously. The reaction was monitored by TLC (solvent B) until the starting material had disappeared. It usually took 3–8 min. The reaction was then quenched by the addition of pyridine (0.4 ml). The resulting soln was transferred into a separatory funnel with $CHCl_3$ (2 × 3 ml) and washed with water (3 × 10 ml). The organic phase was collected, and the aqueous layers were combined and extracted with $CHCl_3$ (10 ml). The latter organic layer was washed with water (10 ml) and combined with the former. The soln was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The resulting residue could be used as the hydroxyl component directly in the next condensation or was purified, if necessary, by silica gel column chromatography.

Simplified procedure for the preparation of fully protected dimer blocks

Typical procedure for dCpAp block. The fully protected deoxycytidine unit (334 mg, 0.36 mmol) was treated with Et_3N–H_2O–pyridine (2:1:2, v/v/v, 9 ml) at room temp for 30 min. The solvent was removed *in vacuo* to give the crude phosphodiester component. The adenosine unit (277 mg, 0.3 mmol) was treated with 2% TFA in $CHCl_3$ (12 ml) at 0° for 4 min and pyridine (0.5 ml) was added. TFA was removed by extraction as described previously. The soln was dried over Na_2SO_4 and concentrated to an oil. The oil was mixed with 3-nitro-1,2,4-triazole (103 mg, 0.9 mmol) and the crude phosphodiester prepared as above. The mixture was rendered anhydrous by repeated coevaporations with dry pyridine and dissolved in dry pyridine (3 ml), and then DDS (297 mg, 0.9 mmol) was added. The condensation was performed with stirring for 40 min. When the hydroxyl deoxyadenosine unit disappeared on TLC, the mixture was purified in the usual manner (extraction, drying over Na_2SO_4, concentration and column chromatography on silica gel (20 g)) to give the fully protected dCpAp (489 mg, 95%).

Simplified procedure for preparation of trimer blocks

General procedure. A crude fully protected dimer block (0.3 mmol) was prepared by the same procedure as described previously except for column chromatographic purification. The crude material was treated with 2% TFA in $CHCl_3$ (12 ml) at 0° for 5–7 min. The reaction was stopped by the addition of pyridine (1 ml). The resulting soln was transferred into a separatory funnel with $CHCl_3$ (2 × 5 ml) and washed with 5% $NaHCO_3$ aq (3 × 15 ml). The organic phase was collected. The combined aqueous layers were extracted again with $CHCl_3$ (15 ml) and the latter organic layer was washed with the bicarbonate soln. The combined organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo* to prepare the OH component of the dimer. The above residue was mixed with 3-nitro-1,2,4-triazole (114 mg, 0.9 mmol) and the crude phosphodiester monomer (prepared from 0.36 mmol of the mononucleotide unti by method C). The mixture was rendered anhydrous by repeated coevaporations with pyridine and dissolved in pyridine (3 ml). A condensing reagent (0.9 mmol) was added followed by stirring at room temp. The reaction was monitored by TLC (solvent A) until the OH component had disappeared. The condensation usually took 30–60 min. After the usual workup (extraction, drying over Na_2SO_4, evaporation and coevaporation with toluene), the resulting residue was applied to a column of silica gel (30 g) and eluted with CH_2Cl_2 containing MeOH (0–1.5%) to give the fully protected trimer block.

General procedure of coupling reaction. A phosphodiester, an OH component, and an azole were mixed (for the molar ratio see Table 6) and rendered anhydrous by repeated coevaporations with dry pyridine. The mixture was dissolved

in pyridine (1 ml/0.1 mmol of the OH component) and finally a condensing reagent (ordinarily 2 to 3 equiv.) was added. The mixture was stirred at room temp and the reaction was monitored by TLC (solvent A or B). A spot of the OH component decreased gradually on TLC as the reaction proceeded. When the spot could not be observed on TLC, the reaction was quenched as follows. The mixture was transferred into a separatory funnel with $CHCl_3$ and washed with cold water. The aqueous layer was extracted again with $CHCl_3$ twice and the combined extracts were dried over Na_2SO_4. The solvent was removed under reduced pressure and the last traces of pyridine were completely removed by repeated coevaporations with toluene. The residue was purified by a column of silica gel to give the fully protected oligomer.

Full deprotection of the dodecamer. The fully protected dodecamer (13 mg) was dissolved in pyridine (1 ml) and cooled at 0°. To the soln, 0.2 M NaOH (1 ml) was added dropwise at 0°. The mixture was kept at 0° for 35 min. TLC analysis (solvent E) showed the phenylthio group and the benzoyl group were completely removed under the above conditions. Then the soln was applied on a column of Dowex 50W × 8 (pyridinium form, 1.8 ml) and eluted with pyridine–water (1 : 1, v/v, 10 ml). The eluent was concentrated *in vacuo* and conc ammonia (10 ml) was added. The mixture was kept with stirring at room temp. After 48 hr ammonia and the solvent were carefully removed under reduced pressure and the resulting oil was coevaporated three times with water. The residue was further treated with 80% AcOH for 15 min at room temp and the AcOH was evaporated and coevaporated with water completely. Then the residue was dissolved in 0.05 M triethylammonium bicarbonate (TEAB) buffer (3 ml) and washed several times with ether. The aqueous layer was concentrated and dissolved in separation buffer (7 M urea, 20 mM Tris–HCl, pH 8.0), and finally chromatographed on an ion exchange column of Toyopearl 650 M column (Cl^- form, 2.5 cm × 45 cm) by a linear gradient of 0.01–0.2 M NaCl in 20 mM Tris–HCl pH 8.0 in the presence of 7 M urea. The column was eluted at the flow rate of 47 ml/hr and each 5.4 ml of fraction was collected. Finally fractions containing the desired product were desalted by a column of Bio-Gel P-2 (0.05 M TEAB). The eluent was collected and lyophilised twice. The dodecamer (35 OD units at 260 nm) was analysed by HPLC (Fig. 4) and 20% acrylamide gel electrophoresis.

Incubation of the dodecamer (1 OD) with snake venom phosphodiesterase in 0.05 M Tris–HCl buffer (100 μl, pH 8.0) at 37° for 15 hr gave a digestion mixture of dC, pdC, pdA and pdT in the correct ratios, which were analysed by HPLC.

The sequencing analysis was performed by the standard method described by Jay *et al.*[8]

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