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A General Approach to the Chemical Synthesis of Oligodeoxyribonucleotides

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A general procedure for the synthesis of oligodeoxynucleotides has been described using 9-phenylxanthen-9-yl as a 5'-protecting group. The building blocks *9* and *11*, all sixteen of them in each category (Tables 2 and 3), have been unambiguously synthesized and characterized. Their properties have been recorded. The application of these dimer blocks in deoxyoligonucleotide synthesis has been demonstrated by the preparation of three octamers, one undecamer and one tetradecamer using the phosphotriester approach.

Recent advances in genetic engineering has triggered the exploitation of microbiological methods for obtaining peptides and proteins that are of practical importance. In some cases this approach requires the unambiguous chemical synthesis of short deoxyribooligonucleotides with defined sequences of bases. It appears that the phosphotriester method is, indeed, the method of choice for the synthesis of short deoxyribooligonucleotides. Thus, the phosphotriester approach has resulted in the synthesis of various biologically important sequences such as *lac* operator DNA,[1] somatostatin[2] and insulin genes[3] and linker molecules containing restriction site of the endonucleases used for cloning.[4] Synthetic oligodeoxyribonucleotides of specific sequence have also been used to prime reverse transcription of a complementary mRNA present within a total mRNA population, thus directing the synthesis of double stranded cDNA corresponding to the coding region of the mRNA. Such an approach has been successfully used to isolate and characterize the hog gastrin mRNA by Noyes *et al.*[5] and human fibroblast interferon mRNA by Houghton *et al.*.[6,7] The present methodology[8] of synthesis of oligodeoxynucleotide fragments by the phosphotriester approach involves: (i) protection of 5'-OH group of thymidine or an appropriately *N*-protected deoxynucleoside; (ii) introduction of a phosphotriester or diester function specifically at 3'-OH of 5'-protected thymidine and other 5'- and *N*-protected deoxynucleoside blocks; (iii) coupling of the monomer phosphodiester salt *1A* to the 5'-OH function of an appropriately protected second building block with the help of an activating agent. The introduction of a 3'→5' linkage can be specifically performed with the second building block either with a free 3'-OH function[9,10] or protected by another group or a phosphotriester.[11] In the former example where a 3'→5' linkage is introduced by coupling *1B* with thymidine or an *N*-acyl-2'-deoxyribonucleoside derivative, a partially protected 3'→5' dinucleoside phosphate is formed as the major product (95 %) along with 3'→3' isomer. isomer. The undesirable 3'→3' isomer[12] could easily be separated from the 3'→5' isomer by short column chromatography. One could then block the 3'-OH function by a 9-phenylxanthen-9-yl (pixyl) group[13] and deprotect the 5'-OH function for a second condensation either with *1B* or with another dimer block with a phosphodiester function at 3' position. Thus a fully protected trinucleoside diphosphate or a tetranucleoside triphosphate could be easily synthesized in high overall yield. In the same approach, one also introduces a phosphodiester function, after removal of the pixyl group from the 3'-position, everytime one wishes to form an internucleotide linkage. This methodology[15] has led to the synthesis of a tridecanucleoside dodeca-

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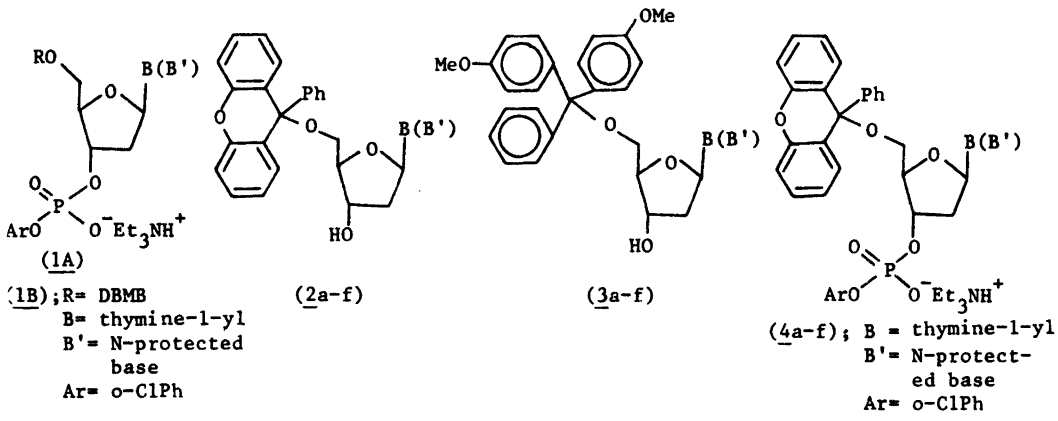


phosphate sequence of SV40 DNA. This paper reports a general methodology involving the use of the Pixyl group[13] for the protection of 5'-OH function which increases the overall lipophilicity of the fully protected oligodeoxynucleotide which in turn facilitates the purification process during extraction in the preparation of monomer phosphodiester salts *4a–f*. Another advantage of protecting the 5'-hydroxy function with the pixyl group is the sensitivty of its detection on thin layer chromatography (10^{-9} M). We also report the preparation of all 16 fully protected dimer diphosphates, where 5'-OH is being protected with the pixyl group and the 3'-terminal phosphate with a 2,2,2-tribromoethyl group and demonstrate their usefulness for the synthesis of various oligodeoxynucleotides of different chain lengths with a specific sequence.

RESULTS AND DISCUSSION

N-Acyl-2'-deoxyribonucleoside and mononucleotide building blocks. Chattopadhyaya and Reese have introduced the pixyl group for the protection of the 5'-OH function of *N*-acylated deoxynucleosides and showed that their introduction led to crystalline derivatives.[13] The stability of the pixyl hroup was comparable to the 4,4'-dimethoxytrityl group,[16] originally introduced by Khorana and his coworkers for the 5'-OH protection in the phosphodiester approach, which has been widely used in the phosphotriester approach by differentlaboratories. The pixyl group could be easily introduced at the 5'-OH function by reacting thymidine or other *N*-acyl-2'-deoxynucleosides in dry pyridine solution with a slight excess of 9-phenyl-9-xanthenyl (pixyl) chloride at room temperature to give the corresponding 5'-pixylated derivatives *2a–f*. Thus, the 5'-pixyl derivatives of (*a*) 6-*N*-(*m*-chlorobenzoyl)-2'-deoxyadenosine, (*b*) 6-*N*-(*p*-*t*-butylbenzoyl)-2'-deoxyadenosine, (*c*) 4-*N*-benzoyl-2'-deoxycytidine, (*d*) 2-*N*-phenylacetyl-2'-deoxyguanosine, (*e*) 2-*N*-(*p*-*t*-butylbenzoyl)-2'-deoxyguanosine and (*f*) thymidine were isolated in 74, 71, 87, 75, 83 and 83 % yields following a published procedure.[13]

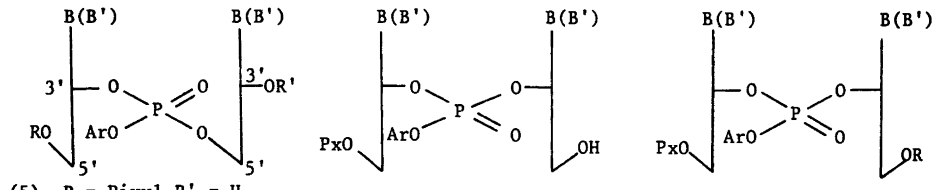
The pixyl groups from these 5'-pixylated derivatives could be completely removed[13] within 9 – 14 min in 80 % acetic acid at 20 °C. The stability of the pixyl group is, thus comparable to the dimethoxytrityl group of noncrystalline 5'-O-dimethoxytrityl thymidine or other *N*-acyl derivatives *3a–f*.[13] Initially we used 6-*N*(*p*-*t*-butylbenzoyl)2'-deoxyadenosine and 2-*N*-phenylacetyl-2'-deoxyguanosine for our work for obvious reasons of lipophilicity[8] and ease of removal of the phenylacetyl group under mild conditions[13] but their preparations and purifications were cumbersome and the yields were not reproducible in different hands. Therefore we have chosen to use 6-*N*-(*m*-chlorobenzoyl)-2'-deoxyadenosine and 2-*N*-(*t*-butylbenzoyl)-2'-deoxyguanosine since both are crystalline compounds and are obtainable in over 92 and 82 % yields, respectively. The acyl groups could be completely removed in aqueous NH_3 (*d* 0.88) under 75 h at 20 °C.

Recently[12,15] it has been shown that the first step of phosphorylation in the phosphotriester approach can be specifically performed by reacting 5'-O-(2-dibromomethylbenzoyl)-(DBMB) derivatives of an appropriately *N*-protected nucleoside or oligodeoxynucleotide with a free 3'-hydroxy group with an excess of *o*-chlorophenylphosphorodi-

(1,2,4-triazolide) for 40 min at 20 °C followed by hydrolysis with a mixture of triethylamine, water and pyridine. The 5'-O-DBMB nucleoside or oligonucleotide phosphodiester triethylammonium salt produced could then be separated from the bis-triethylammonium salt of *o*-chlorophenylphosphate by partitioning between a saturated solution of $NaHCO_3$ and $CHCl_3$ to generate pure phosphodiester triethylammonium salt in the $CHCl_3$ phase. In the present work we have successfully applied this procedure to introduce the phosphodiester function at the 3' position of all the 5'-pixylated building blocks *2a–f*. The lipophilic nature of the pixyl group assisted in clear partioning of the phosphodiester salts from bis-triethylammonium salts of *o*-chlorophenylphosphate without forming an emulsion. Thus, we obtained pure 3'-phosphodiester salts *4a–f* of all deoxynucleoside building blocks *2a–f* as white powders in 92, 85, 95, 98, 96 and 94 % yields. These phosphodiester salts could be stored for over six months at −20 °C without any decomposition. Any decomposition of these salts due to the removal of the pixyl group could be easily detected under a 366 nm UV lamp after spraying the TLC plate with 10 % solution of H_2SO_4 and then warming the plate slightly. This allowed us to detect any minute decomposition at the level of 10^{-9} M concentration.

Preparation of dimer blocks. The dry and clear pyridine solution of each of the phosphodiester salts *4a–f* and thymidine or an *N*-acyl-2'-deoxyribomucleoside derivative is treated with an excess of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MS–NT)[12,14] for 25 min at 20 °C to obtain a mixture of 3'→5' linked partially protected dinucleoside monophosphate (>95 % of the mixture) *5* and 3'→3' isomer *6*. 3'→3' isomers in all sixteen cases have an higher R_f on TLC than 3'→5' isomers and they were easily separated by short column chromatography on silica gel in satisfactory yields (Table 1). Each of the 3'→3' isomers which formed in small quantity (not exceeding 3 – 5 % of the total product) was synthesized in an unambiguous way by reacting 5'-*O*-pixylphosphodiester *4a–f* and 5'-*O*-DBMB derivative of thymidine or *N*-acylnucleoside in anhydrous pyridine solution in the presence of an excess of MS–NT to obtain fully protected dimer *7*, followed by removal of the DBMB-group to *8* (see Table 1).

The partially protected, dideoxynucleoside monophosphate with a free 3'-hydroxy group, thus obtained, was again phosphorylated using *o*-chlorophenylphosphorodi-(1,2,4,-triazolide),[12] generated from *o*-chlorophenylphosphorodichloridate, 1,2,4-triazole and triethylamine. Following a standard procedure (experimental section), the corresponding dideoxynucleoside diphosphate *9* of all 16-dimer blocks could be obtained in high yield (Table 2) and they were pure on TLC. Their purities were further demonstrated to be over 95 %, after the removal of all protecting groups, by both high pressure liquid chromatography and TLC in two solvent systems (Table 2). These dideoxynucleoside diphosphate bearing a 3'-terminal phosphodiester salt seemed to us an ideal intermediate for the preparation of fully protected dinucleoside diphosphate bearing a 3'-terminal phosphotriester *10* which would allow us to prepare 5'-hydroxy components *11* for extending the chain at the 5'-end after removal of the pixyl group. A proper combination of partially protected dinucleoside diphosphate salt *9* either with 5'-hydroxy component *11* or 3'-acylated-5'-hydroxy dimer component *5b* or in conjuntion with the trimer blocks *13*, produced from the condensation of *4a–f* with *5b* followed by depixylation of *12*, would be able to produce any deoxyoligonucleotide of any odd or even chain length. Thus we chose 2,2,2-tribromoethyl group



(5), R = Pixyl, R' = H
(5a), R = Pixyl, R' = m-ClBz
(5b), R = H, R' = m-ClBz

(6), Px = Pixyl

(7), R=DBMB, Px=Pixyl
(8), R=H, Px=Pixyl

Ar = o-ClPh

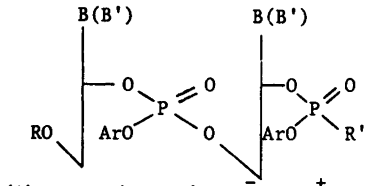
Table 1. Preparation of 16 partially protected dimers *5* using MS-NT.

Expt. No.	Partially protected dimer[a]	% ethanol $CHCl_3$[b]	R_f[c]		Yield/%[d]
			3′→3′	3′→5′	
1	d[Px-ApA-OH	5.5	0.48, 0.43	0.42, 0.30	72.6
1a	d[Px-ApA$^+$-OH	4.5	0.51	0.47	74.3
1b	d[Px-A$^+$pA$^+$-OH]	4.5	0.52	0.49	65.0
2	d[Px-ApG-OH]	6.0	0.38	0.33	63.6
3	d[Px-ApC-OH]	7.0	0.50	0.46	65.4
4	d[Px-ApT-OH]	6.0	0.42	0.39	70.0
5	d[Px-GpA-OH]	5.0	0.49, 0.45	0.43, 0.34	61.3
6	d[Px-G^{x}pG^x-OH]	8.0	0.47, 0.41	0.39, 0.29	66.0
6a	d[Px-GpG-OH]	10.0	0.67, 0.58	0.55, 0.63[e]	68.1
7	d[Px-GpC-OH]	5.0	0.56, 0.49	0.47, 0.42	64.0
8	d[Px-GpT-OH]	6.0	0.48, 0.42	0.40, 0.34	65.0
9	d[Px-CpA-OH]	6.0	0.54, 0.49	0.45, 0.42	61.0
10	d[Px-CpG-OH]	8.0	0.45, 0.40	0.36, 0.32	76.0
11	d[Px-CpC-OH]	5.0	0.49	0.45	65.2
12	d[Px-CpT-OH]	6.5	0.45	0.42	69.6
13	d[Px-TpA-OH]	6.0	0.41	0.37	72.0
14	d[Px-TpG-OH]	7.0	0.33	0.29	70.0
15	d[Px-TpC-OH]	5.0	0.40	0.38	65.5
16	d[Px-TpT-OH]	6.0	0.37	0.33	57.0

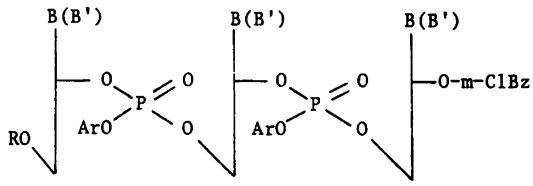
[a] 9-Phenylxanthen-9-yl is abbreviated to Px. 6-*N*-(*m*-Chlorobenzoyl)-2′-deoxyadenosine, 2-*N*-(*p*-*t*-butylbenzoyl)-2′-deoxyguanosine, 4-*N*-benzoyl-2′-deoxycytidine are represented by A, G and C, respectively. 6-*N*-(*p*-*t*-Butylbenzoyl)-2′-deoxyadenosine and 2-*N*-(phenylacetyl)-2′-deoxyguanosine are indicated by A^+ and G^x, respectively. Internucleotide phosphotriester is protected with *o*-chlorophenyl group. Abbreviations adopted here following the suggestion of Chattopadhyaya and Reese.[15] [b] Short column chromatography [18] (column size: 4 cm long, 3 cm diameter) has been used for purification. [c] Solvent system (E) has been used for TLC on silica gel plates. [d] Isolated as powder starting from 1 mmol of the 5′-protected component (see experimental section for details). [e] Solvent system (A) has been used for TLC on silica gel plates.

(TBE) to mask the 3′-phosphodiester function of *9* to convert it to a triester level *10*. This could be easily performed by reacting the diester triethyl ammonium salt *9* and 2,2,2-tribromoethanol with two molar equivalence in anhydrous pyridine solution in presence of excess of MS – NT for 45 min at 20 °C. A standard work up and purification through a column of silica gel gave pure *10* of all 16-dimer blocks in high yield (Table 3 and experimental).

5′-Hydroxy dideoxynucleoside diphosphate component *11* of 16-dimers could be easily obtained from *10* in good yields by treating a 2 % ethanol – $CHCl_3$ solution of each of these dimers with a stock solution of 4-toluenesulfonic acid, H_2O (3 molar equivalents) in the same solvent mixture for 90 s at 20 °C. The 5′-hydroxy components, thus obtained, were purified by precipitation of a $CHCl_3$ solution (2 – 3 ml volume) in cyclohexane as white powders,



(9), R = Pixyl, R' = $O^-Et_3NH^+$
(10), R = Pixyl, R' = $O-CH_2CBr_3$
(11), R = H, R' = $O-CH_2CBr_3$



(12), R = Pixyl
(13), R = H

Ar = o-ClPh

Table 2. Preparation and properties of 16-dideoxynucleoside diphosphates *9*, bearing 3′-terminal phosphodiester triethylammonium salt.

Expt. No.	Partially protected dideoxynucleoside diphosphate salt *9*[a]			Fully deprotected dideoxy nucleoside diphosphate		
		Yield/%[b]	R_f[c]	R_f[d]	R_f[e]	Retention time[f] (min) in HPLC
1	d[Px-ApAp]	92.0	0.65	0.47	0.40	6.30
1a	d[Px-ApA$^+$p]	95.2	0.68	–	–	–
2	d[Px-ApGp]	99.4	0.61	0.42	0.23	6.56
3	d[Px-ApCp]	98.7	0.66	0.49	0.40	5.76
4	d[Px-ApTp]	87.0	0.57	0.58	0.40	6.23
5	d[Px-GpAp]	98.0	0.53	0.42	0.24	6.43
6	d[Px-GpGp]	97.0	0.57	0.36	0.09	6.53
6a	d[Px-G^{x}pG^{x}p]	93.4	0.43	–	–	–
7	d[Px-GpCp]	95.0	0.57	0.42	0.20	6.87
8	d[Px-GpTp]	90.0	0.49	0.53	0.18	6.32
9	d[Px-CpAp]	85.2	0.63	0.53	0.44	6.10
10	d[Px-CpGp]	98.5	0.59	0.44	0.17	6.40
11	d[Px-CpCp]	87.6	0.64	0.51	0.34	4.70
12	d[Px-Cp-Tp]	92.1	0.53	0.62	0.28	6.10
13	d[Px-TpAp]	98.3	0.52	0.60	0.41	6.44
14	d[Px-TpGp]	92.8	0.78	0.44	0.15	6.95
15	d[Px-TpCp]	84.4	0.55	0.60	0.30	5.46
16	d[Px-TpTp]	94.9	0.52	0.67	0.40	6.72

[a] Abbreviations[15] are same as adopted in Table 1. [b] Isolated as powder. [c] 30% methanol-$CHCl_3$ has been used for TLC on silica gel plates. [d] Solvent system (C) for TLC on cellulose plates. [e] Solvent system (B) for TLC on cellulose plates. [f] Permaphase AAX column, linear gradient: 0.01 M KH_2PO_4. 0.0 M KCl to 0.05 M KH_2PO_4. 0.3 M KCl (pH 4.45) at 60 °C.

and they were free of pixyl ethyl ether, 9-hydroxy-9-phenylxanthen (pixanol) or any other side product. Earlier Gough *et al.*[19] have prepared similar fully protected dinucleoside diphosphate blocks by condensation of the monomer tetraethylammonium phosphate with a 5′-hydroxy component. They obtained 5′-hydroxy component after the removal of dimethoxytrityl group from the monomer phosphotriester block which was synthesized by reacting 5′-protected monomer component with an excess of cyanoethanol in presence of 1-(*p*-toluenesulfonyl)-4-nitroimidazole.

Application of the dimer blocks in longer oligodeoxynucleotide synthesis. The usefulness of the dimer blocks *9* and *11*, the latter is obtained through *10*, have been adequately demonstrated by the successful synthesis of three octamers, one undecamer and one tetradecamer (I) AAAAAAAA: (II) TTTTAAAA; (III) TTTTTTTT; (IV) GCTTGTTTCAT; (V) GCCCATTTTTGGAA. These oligonucleotides were assembled from five tetramers, one pentamer and two hexamers. The deoxyoligomers (I,II,IV and V) had an *m*-chlorobenzoyl group as anchoring group at the 3′ position and (III) had a 3′-terminal phosphotriester group. The partially protected dinucleotides (d[Px-ApA-OH]) and (d[Px-ApT-OH]) (Table 1) were reacted with an excess of *m*-chlorobenzoyl (*m*-ClBz) chloride in pyridine solutions to obtain the corresponding N^6,$N^{6'}$,$O^{3'}$-tri-*m*-chlorobenzoyl derivative and N^6, $O^{3'}$-di-*m*-chlorobenzoyl derivative in high yields which were then depixylated to obtain 5′-hydroxy dimer components in 73 and 68% overall yield, respectively. Five tetramers were synthesized (Table 4 and experimental) by reacting a slight excess of building block of the type *9* with *11* (Tables 2, 3, 4 and experimental) in anhydrous pyridine solutions in the presence of an excess of MS – NT for 90 min at 20 °C. A prolonged reaction time has been found to contribute to the formation of side products especially involving guanine residue which appear as fluorescent compound. Reese and Ubasawa[20] have

Table 3. Preparation and properties of fully protected 16-dideoxynucleoside diphosphates *10* and 5′-hydroxy dimer components *11*.

Expt. No.	Fully protected dideoxynucleoside diphosphate *10*[a]				5′-Hydroxy dimer component *11*	
		Yield/%[b]	% ethanol-$CHCl_3$ to elute[c]	R_f[d]	Yield/%[b]	R_f[d]
1	d[Px-ApApTBE]	79.0	4.0	0.63	94.5	0.49
1a	d[Px-ApA$^+$pTBE]	80.0	4.0	0.69	93.0	0.57
2	d[Px-ApGpTBE]	70.1	5.0	0.50	95.0	0.42
3	d[Px-ApCpTBE]	93.7	4.0	0.65	80.0	0.58
4	d[Px-ApTpTBE	80.6	80.6	6.0	0.63	0.49
5	d[Px-GpApTBE]	75.4	5.0	0.45	87.4	0.40
6	d[Px-GpGpTBE]	93.2	5.0	0.52	90.8	0.34
7	d[Px-GpCpTBE	85.0	6.0	0.57	99.0	0.34
8	d[Px-GpTpTBE]	82.7	5.0	0.51	92.1	0.38
9	d[Px-CpApTBE]	79.5	6.0	0.58	90.8	0.50
10	d[Px-CpGpTBE]	77.0	4.5	0.65	95.0	0.51
11	d[Px-CpCpTBE]	84.5	6.0	0.66	83.7	0.48
12	d[Px-CpTpTBE]	84.0	5.0	0.57	91.3	0.45
13	d[Px-TpApTBE]	86.2	5.0	0.56	89.7	0.47
14	d[Px-TpGpTBE]	77.0	6.0	0.38	86.0	0.22
15	d[Px-TpCpTBE]	70.1	4.0	0.60	91.8	0.45
16	d[Px-TpTpTBE	84.0	6.0	0.53	84.0	0.35

[a] Abbreviations [15] are same as Table 1; 3′-terminal phosphotriester is designated by pTBE. [b] Isolated as powder. [c] Short column chromatography (column size: 4 cm long, 3 cm diameter) has been used for purification. [d] Solvent system (E) for TLC on silica gel plates. [e] Obtained by depixylation of (*10*) using 4-toluenesulfonic acid, H_2O in 2% ethanol-$CHCl_3$ solution (experimental section).

characterized the modified guanine residue to be an adduct of 3-nitro-1,2,4-triazole at C−6 position of guanine. This, as it has been pointed out by these workers, does not seem to cause a serious problem since any such modification can be reversed to original guanine structure at the deprotection stage using N^1N^1,N^3,N^3-tetramethylguanidinium-*syn*-4-nitrobenzaldoximate ion in aqueous dioxan solution at 20 °C.

The most serious problem we have encountered in our study is the depurination of both adenine and guanine residues on prolonged condensation time. Narang and his coworkers [21] have observed similar depurination. A detailed study of this aspect is under investigation in this laboratory. The pixyl group from the tetramers, (d[Px-CpCpApTpTBE]), (d[Px-TpCpApT-*O*-*m*-ClBz]), (d[Px-TpTpTpTpTBE]), (d[Px-ApA$^+$pApA-*O*-*m*-ClBz]), (d[G^{x}pG^{x}pApA-*O*-*m*ClBz]) were removed within 90 s at 20 °C to give 5′-hydroxy components in 89.5, 82, 75.5, 88 and 92 % yield as powder, which were free of pixanol or pixyl ethylether, after precipitation from cyclohexane. These could be directly used for condensation with the desired diester block (Table 4). The diester triethylammonium salts (5′-protected components of Table 4) were prepared by the removal of 2,2,2-tribromoethyl group from the following fully protected phosphotriesters, (d[Px-TpTpTpTpTBE]), (d[Px-ApA$^+$pApA$^+$pTBE]) by stirring their pyridine solutions with zinc and acetylacetone at 20 °C for min in 67.8 and 70.7 % isolated yield (experimental). Earlier, Adamiak *et al.* [22] have claimed complete removal of 2,2,2-trichloroethyl group from internucleotidic phosphotriester within 5−10 min under the above reaction condition. We have failed to reproduce this efficiency with fully protected deoxyoligomer longer than tetranucleoside tetraphosphate, And, in fact, this has led us to choose the tribromoethyl group for 3′-terminal phosphotriester protection, for the above reductive elimination reaction condition would be severalfold more effective. However, we confirm that there is no detectable side reaction with pyrimidine residues in the above deprotection condition. The pentamer block for the undecamer synthesis was assembled by the block condensation of (d[Px-GpCp]) and (d[HO-TpTpGpTBE]). The latter trimer block

Table 4. Condensation reactions leading to higher deoxyoligonucleotides with MS-NT in pyridine solution.

Expt. No.	5'-Protected component/(mmol)[a]	5'-Hydroxy component/(mmol)[a]	Pyridine/ ml	MS-NT/ mmol	Product[a]	Yield[a] %	R_f[b]	%EtOH-$CHCl_3$[c]
1	d[Px-TpTp](0.73)	d[HO-TpTpTBE](0.58)	3.0	5.8	d[Px-TpTpTpTpTBE	76.1	0.45	6.0
2	d[Px-ApA$^+$p](0.57)	d[HO-ApA$^+$pTBE](0.42)	6.0	4.2	d[Px-ApA$^+$pApA$^+$pTBE	96.0	0.59	4.0
2a	d[Px-ApA$^+$p](0.33)	d[HO-ApA-*O*-*m*-ClBz](0.28)	4.0	3.4	d[Px-ApA$^+$pA-*O*-*m*-ClBz]	85.0	0.68	5.0
3	d[Px-TpCp](0.18)	d[HO-ApT-*O*-*m*-ClBz](0.16)	4.0	2.0	d[Px-TpCpApT-*O*-*m*-ClBz]	65.0	0.48	5.0
4	d[Px-G^{i}pG^{i}p](0.35)	d[HO-ApA-*O*-*m*-ClBz](0.35)	3.0	3.5	d[Px-G^{i}pG^{i}pApA-*O*-*m*-ClBz]	56.0	0.40	4.0
5	d[Px-CpCp](0.08)	d[HO-ApTpTBE](0.076)	1.0	1.2	d[Px-CpCpApTpTBE]	78.4	0.50	7.0
6	d[Px-GpCp](0.1)	d[HO-TpTpGpTBE](0.07)	2.0	1.5	d[Px-GpCpTpGpTBE]	67.5	0.27	6.0
7	d[Px-GpCp](0.067)	d[HO-CpCpApTpTBE](0.053)	0.7	1.35	d[Px-GpCpCpCpApTpTBE]	73.0	0.39	7.5
8	d[Px-TpTp](0.12)	d[HO-TpCpApT-*O*-*m*-ClBz](0.09)	2.0	1.2	d[Px-TpTpTpCpApT-*O*-*m*-ClBz]	77.7	0.44	5.0
9	d[Px-TpTpTpTp](0.13)	d[HO-TpTpTpTpTBE](0.11)	1.4	1.70	d[Px-TpTpTpTpTpTpTpTpTBE]	39.4	0.45	4.5
10	d[Px-TpTpTpTp](0.15)	d[HO-ApA$^+$ApA-*O*-*m*-ClBz](0.12)	1.5	2.3	d[Px-TpTpTpTpApA$^+$pApA-*O*-*m*-ClBz]	80.0	0.53	5.0
11	d[Px-TpTpTpTp](0.26)	d[HO-G^{i}pG^{i}pApA-*O*-*m*-ClBz](0.19)	1.0	2.0	d[Px-TpTpTpTpG^{i}pG^{i}pApA-*O*-*m*-ClBz]	63.0	0.41	6.0
12	d[Px-ApA$^+$pApA$^+$p](0.09)	d[HO-ApA$^+$ApA-*O*-*m*-ClBz](0.08)	1.0	1.0	d[Px-ApA$^+$pApA$^+$pApA$^+$pApA-*O*-*m*-ClBz]	80.0	0.40	6.0
13	d[Px-GpCpTpTpGp](0.06	d[HO-TpTpTpCpApT-*O*-*m*-ClBz](0.056)	1.0	0.9	d[Px-GpCpTpTpGpTpTpTpCpApT-*O*-*m*-ClBz	57.0	0.21	6.0
14	d[Px-GpCpCpCpApTp] (0.014)	d[HO-TpTpTpTpG^{i}pG^{i}pApA-*O*-*m*-ClBz] (0.014)	0.3	0.3	d[Px-GpCpCpCpApTpTpTpTpTpG^{i}pG^{i}pApA-*O*-*m*-ClBz]	49.8	0.31	9.0

[a] Isolated as powder. [b] Solvent system E on silica gel plates. [c] Solvent mixture used for silica gel column chromatography.

was obtained in 68 % yield by condensing (d[Px-Tp]) with d(HO-TpGpTBE]). The hexamer blocks (d[Px-TpTpTpCpApT-*O*-*m*-ClBz]) and (d[Px-GpCpCpCpApTBE]) were synthesized by condensation of (d[Px-TpTp]), (d[Px-GpCp]) with (d[HO-TpCpApT-*O*-*m*-ClBz]) and (d[HO-CpCpApTp-TBE]), respectively, in 77.7 and 73.0 % yield. As indicated in Table 4 the undecamer and tetradecamer were assembled by the appropriate condensation of 5'-protected component with 5'-hydroxy component (experiments 13 and 14 in Table 4) in 57 and 49.8 % isolated yield. The 5'-protected components for the above condensations were prepared in 73.5 and 72.0 % yield by the removal of tribromethyl group (experimental) from fully protected phosphotriesters, (d[Px-GpCpTpTpGpTBE]) and (d[Px-GpCpCpCpApTpTBE]) and the 5'-hydroxy components were prepared from (d[Px-TpTpTpCpApT-*O*-*m*-ClBz]) and (d[Px-TpTpTpTpG^{x}pG^{x}pApA-*O*-*m*-ClBz]) in 96 and 94 % yield by depixylation with 4-toluenesulfonic acid, H_2O in 2 % EtOH – $CHCl_3$ solution at 20 °C for 90 s.

Deblocking of the fully protected oligonucleotides [obtained from experiments No. 9, 10, 12, 13 and 14, Table 4]. The protecting groups on these oligooxynucleotides were removed following the published procedure[14,15] after the replacement of pixyl group at 5'-position by benzoyl group, by the treatment of 4-toluenesulfonic acid in 2 % EtOH – $CHCl_3$ for 90 s followed by benzoylation in pyridine solution (earlier study[23] has shown that an oligonucleotide with 5'-O-2-dibromomethylbenzoyl group undergoes less than 0.5 % terminal phosphoryl migration in 4-nitrobenzaldoximate ion

Fig. 1. HPLC elution pattern of AAAAAAAA (Permaphase AAX at 65 °C, linear gradient, 0.01 M KH_2PO_4, 0.0 M KCl to 0.05 KH_2PO_4 and 0.7 M KCl, pH 4.45) and a partial enzyme digest (A).

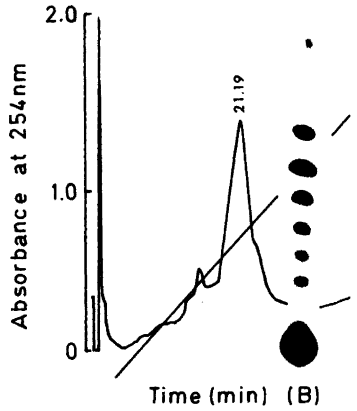


Fig. 2. HPLC elution pattern of TTTTAAAA (Permaphase AAX at 65 °C, linear gradient, 0.01 M KH_2PO_4 and 0.7 M KCl, pH 4.45) and a partial enzyme digest (B).

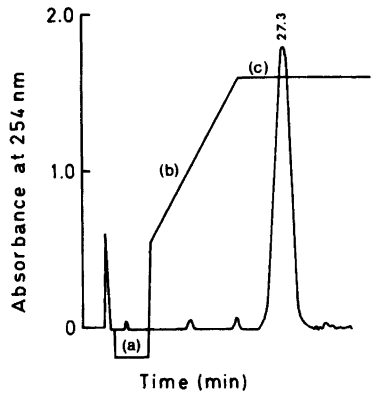


Fig. 4. HPLC elution pattern of GCTTGTTTCAT (Permaphase AAX at 65 °C, (a) isocratically with 0.05 M KH_2PO_4 and 0.15 M KCl, pH 4.45; (b) linear gradient with 0.05 M KH_2PO_4, 0.15 M KCl to 0,05 M KH_2PO_4 and 0.7 M KCl, pH 4.45; (c) isocratically with the solvent system (b), PH 4.45.

promoted deblocking condition, despite the fact that 2-dibromomethylbenzoyl group is three times less stable to base than benzoyl group and has approximately the same stability as of 5'-O-acetyl group). This is important due to the fact that considerable depurination[8] takes place if 80 % acetic acid reaction condition for the removal of pixyl group is employed following the removal of internucleotide and exocyclic amino protecting group. This loss can be completely avoided if pixyl group is removed using 4-toluenesulfonic acid-EtOH-$CHCl_3$ reaction condition before any other deblocking treatments. The blocking of 5'-hydroxy function, thus regenerated, is highly desirable before subjecting the protected oligomer to oximate ion promoted deblocking condition[14] to minimize any 5'-terminal phosphoryl migration.[17] The occurrence of such a terminal phosphoryl migration in alkaline deblocking condition has been adequately demonstrated [17,25]. Recent work by Gough[19,26], Narang[11,21] and more recently by Itakura[27] and their coworkers do not seem to take account of occurrence of such a terminal phosphoryl migration.

The deblocked octamers (experimentals) were directly examined by HPLC (Permaphase AAX column) and the deblocked undecamer and tetra-

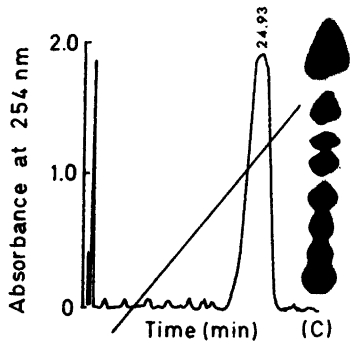


Fig. 3. HPLC elution pattern of TTTTTTTT (Permaphase AAX at 65 °C, linear gradient, 0.01 M KH_2PO_4 and 0.7 M KCl, pH 4.45) and a partial enzyme digest (C).

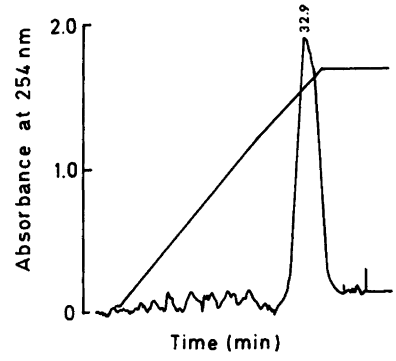


Fig. 5. HPLC elution pattern of GCCCATTTTT-GGAA (Permaphase AAX at 65 °C, linear gradient, 0.01 M KH_2PO_4, 0.0 M KCl to 0.05 M KH_2PO_4 and 0.7 M KCl, pH 4.45.

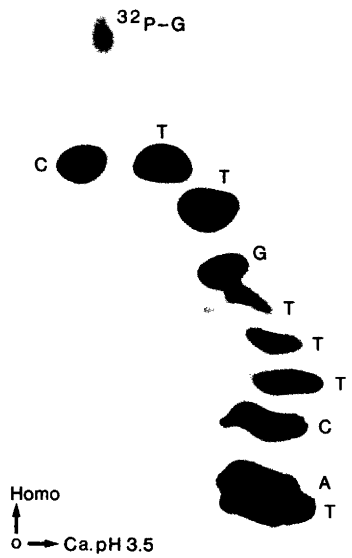


Fig. 6. Two dimensional chromatographic finger print of an undeca deoxyribooligonucleotide fragment: GCTTGTTTCAT.

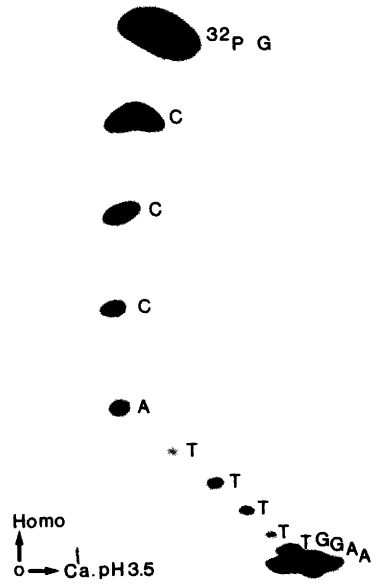


Fig. 7. Two dimensional chromatographic finger print of a tetradeca deoxyribooligonucleotide fragment L: GCCCATTTTTGGAA.

decamer were purified by chromatography through a G-25 Sephadex (fine) column using 0.01 M $Et_3NH^+HCO_3^-$ (pH 7.5) buffer for isocratic elution and then their purities were examined by HPLC. The elution patterns are shown in Figs. 1–5. The desired oligonucleotide under each of the main peaks was found to contain 75, 71, 87, 85 and 82 % of the total number of A_{260} units eluted from the column. The oligonucleotides (I, II, IV and V) were completely digested by snake venom and calf spleen phosphodiesterases and (III) was fully digested by nuclease P_1 and spleen phosphodiesterase as examined in different partition systems on cellulose. The structure of TTTTTTTT, TTTTAAAA and AAAAAAAA were confirmed by partial enzymatic digestion (TTTTTTTT was partially digested by nuclease P_1 from *Penicillium citrium*. TTTTAAAA and AAAAAAAA were partially digested by *Crotalus adamanteus* snake venom phosphodiesterase) of respective 5′-^{32}P labeled material followed by one dimensional homochromatography on DEAE-cellulose plates and then they were visualized by autoradiography as shown in Figs. 1–3. The structure of undecamer and tetradecamer was confirmed by sequence analysis of the siolated material eluted under the main peak as shown in Figs. 6 and 7, respectively.

EXPERIMENTAL

UV absorption spectra were measured with a Cecil CE 545 double beam scanning spectrophotometer, ^{1}H NMR spectra were measured at 60 MHz with a Perkin-Elmer R12B and 100 MHz with a Jeol FX 100 spectrophotometer. IR spectra were measured with a Perkin-Elmer 298 spectrometer.

Merck silica gel 60 F_{254} pre-coated plates and DC-plasticfolien cellulose F_{254} sheets were used for TLC in the following solvent systems: (A) $CHCl_3$–EtOH (8:2 v/v); (B) *i*-PrOH–aq.NH_3 (d0.88)–water (7:1:2 v/v); (C) EtOH–*M*-aq.NH_4OAc (6:3 v/v); (D) $CHCl_3$–MeOH (7:3 v/v); (E) $CHCl_3$–MeOH (9:1 v/v). Gel. electrophoresis was carried out in a home made apparatus on 20 % polyacrylamide gel at 1400 V, 100 mA for 4 h (buffer pH 7.9). HPLC was carried out with a Waters apparatus on a Permaphase AAX column at 60 °C using linear gradients. Merck kieselgel H was used for short column chromatography. DEAE-cellulose (MN 300 DEAE) plates were purchased from Macherey-Nagel Co., West Germany.

Dry dioxane, acetonitrile and pyridine and dimethylformamide were prepared and stored in the laboratory following published procedure.[14]

9-Hydroxy-9-phenylxanthene, 9-chloro-9-phenylxanthene, 5'-*O*-(9-phenylxanthene-9-yl) (pixyl)thymidine, 4-*N*-benzoyl-5'-*O*-pixyl-2'-deoxycytidine and 6-*N*-(*p*-*t*-butylbenzoyl)-5'-*O*-pixyl-2'-deoxyadenosine have been prepared following the procedure originally developed in Professor Reese's laboratory.[13]

*6-N-(*m*-chlorobenzoyl)-5'-*O*-pixyl-2'-deoxyadenosine.* This compound was prepared in an identical way[13] and it was isolated in 74 % yield after purification through a silica gel column followed by recrystallization from ethanol, m.p. 171–174 °C [Found: C, 66.71; H, 4.18; N, 10.71; $C_{36}H_{28}N_5O_5$ requires C, 66.92; H, 4.36 and N, 10.84 %].

2-N-Phenylacetyl-5'-O-pixyl-2'-deoxyguanosine. This compound was prepared in the same way[13] from 2-*N*-phenylacetyl-2'-deoxyguanosine and was isolated in 75 %. Yield after recrystallization from acetone m.p. 198–204 °C. [Found: C, 68.89; H, 4.71; N, 10.74. $C_{37}H_{31}N_5O_6$ requires: C, 69.2; H, 4.87; N, 10.91 %].

*2-N-(*p*-t-Butylbenzoyl)-5'-O-pixyl-2'-deoxyguanosine.* This compound was prepared in the same way[13] from 2-*N*-(*p*-*t*-butylbenozyl)-2'-deoxyguanosine after purification by column chromatography. This was isolated in 83 % yield after recrystallization from ethanol m.p. 191–193 °C. [Found: C, 69.92; H, 5.31; N, 9.97. $C_{40}H_{37}N_5O_6$ requires: C, 70.2; H, 5.45; N, 10.24 %].

General procedure for the preparation of triethylammonium salt of 5'-O-pixyl-2'-deoxyribonucleoside-3'-(o-chlorophenyl) phosphates. 5'-O-pixyl-2'-deoxyribonucleoside (2.5 mmol) in dry pyridine (20 ml) solution was treated with a phosphorylating mixture containing *o*-chlorophenylphosphorodichloridate (5 mmol), 1,2,4-triazole (11 mmol) and triethylamine (10 mmol) in dry acetonitrile (20 ml) for 30 min at 20 °C, when a product with $R_f = 0$ in solvent system: (E) was formed. The monotriazolide formed could then be easily hydrolyzed to the diester salt by the addition of a mixture of Et_3N (11 mmol) and water (30 mmol) within 10 min at 20 °C. The desired phosphodiester triethylammonium salt and *o*-chlorophenylphosphate-bis-triethylammonium salt could be easily separated by partioning between $CHCl_3$ (200 ml) a saturated $NaHCO_3$ solution (200 ml). The $CHCl_3$ phase was then washed with more $NaHCO_3$ solution (3 × 60 ml), dried ($MgSO_4$) and concentrated under reduced pressure. A solution of the residue in $CHCl_3$ (2–5 ml) is precipitated from petroleum ether (b.p. 30–50 °C, 250 ml). The precipitate was collected and dried in a desiccator to obtain pure phosphodiesters over 95 % yield. The purity of all six phosphodiesters was checked by TLC on silica gel plates in solvent system D. The R_f values for *4a–f* were 0.46, 0.56, 0.63, 0.42, 0.46 and 0.69, respectively. No hydrolysis of pixyl group could be detected during the preparation of these phosphodiester salts.

Preparation of partially protected dinucleoside phosphates with free 3'-hydroxy functions 5. 1-Mesitylenesulfonyl-3-nitro-1,2,4-triazole (MS–NT) (5 mmol) was added at room temperature to a magnetically-stirred pyridine (15 ml) solution of appropriate triethylammonium 5'-*O*-pixyl-2'-deoxyribonucleoside-3'(*o*-chlorophenyl) phosphate (1 mmol) and thumidine or the *N*-acyl-2'-deoxyribonucleoside (1.15 mmol). After 23 min the reaction was quenched by adding aqueous saturated $NaHCO_3$ solution (2 ml). The stirring was continued for another 10 min and then the reaction mixture was pured into a separating funnel containing saturated $NaHCO_3$ solution (100 ml). The mixture was then extracted in $CHCl_3$ (5 × 60 ml). The $CHCl_3$ extracts were pooled, dried ($MgSO_4$) and concentrated to a glass (coevaporations with toluene) under reduced pressure. This was purified by column chromatography (Table 1 for solvent mixture for elution, R_f of 3'→5' and 3'→3' products and the yields).

Preparation of dinucleoside diphosphates 9 *with 3'-terminal phosphodiester triethylammonium salt (Table 2).* These were made essentially following the general method of preparation of triethylammonium salt of 5'-*O*-Pixyl-2'-deoxyribonucleoside-3'-(*o*-chlorophenyl) phosphates.

Preparation of fully protected dinucleoside diphosphates 10 *with 3'-terminal phosphotriester.* MS–NT (5 mmol) was added at room temperature to a magnetically-stirred pyridine (10 ml) solution of appropriate dinucleoside diphosphates *9* (1 mmol) and 2,2,2-tribromoethnaol (2 mmol). The reaction mixture was stirred for 45 min, then it was quenched by adding aqueous saturated $NaHCO_3$ solution (2 ml). After a period of 10 min, the reaction mixture was poured into a separating funnel containing aq.satd. $NaHCO_3$ (100 ml). The resulting mixture was extracted with $CHCl_3$ (5 × 60 ml). The $CHCl_3$ extracts were pooled together and concentrated to a glass (coevaporations with toluene). This glass was purified through a short column of silica gel (Table 3 for solvent mixture for purification by column chromatography and yields).

General procedure for the preparation of partially protected dinucleoside diphosphate with free 5'-hydroxy function 11 *and other 5'-hydroxy components.* A solution of 4-toluenesulfonic acid (5 mmol) in 2 % ethanol-$CHCl_3$ (20 ml) was added to a solution of substrate (1 mmol) in the same solvent mixture (30 ml) in a separating funnel at room temperature. The reaction mixture was swirled for 90 s and then saturated aqueous $NaHCO_3$ (100 ml) was added to quench the reaction by vigorous shaking. The resulting mixture was extracted with $CHCl_3$ (5 × 60 ml). The $CHCl_3$ extracts were combined and concentrated to a glass. This glass was then dissolved in $CHCl_3$ (2–4 ml) and was precipitated from

cyclohexane (150 ml) to obtain *11* (Table 3) or other 5'-hydroxy components (Table 4) which were free of any 9-hydroxy-9-phenylxanthen and/or its derivatives.

A general method of preparation of 5' protected components: removal of tribromoethyl group by reductive elimination of 3'-terminal phosphotriesters to generate 3'-terminal triethylammonium salt of phosphodiester. To the magnetically-stirred pyridine (10 ml) solution of fully protected oligomer (1 mmol), zinc[22] (5 mmol) and acetylacetone (5 mmol) were added at room temperature. The reaction was stirred vigorously for 20 min when TLC (solvent system: (E)) revealed a compound with $R_f = 0$. Then triethylamine (5 mmol) was added. After 2 min $CHCl_3$ (40 ml) was added to this reaction mixture and was filtered through a cotton plug directly into a separating funnel containing aqueous saturated $NaHCO_3$ solution (50 ml). The resulting mixture was extracted by $CHCl_3$ (5 × 60 ml). The $CHCl_3$ extracts were combined and were re-extracted with satd. $NaHCO_3$ solution (3 × 50 ml). This operation removed all zinc cations. The $CHCl_3$ extracts were concentrated, after coevaporations with toluene, to a glass. This glass was then dissolved in $CHCl_3$ (2 – 5 ml) and the solution was precipitated from light petroleum (30 – 50 °C) and diethyl ether mixture (200 ml, 1:1 v/v).

Following this general experimental procedure, the 5'-protected components of tetramers (experiments 9 to 12 of Table 4), pentamer (experiment 13, Table 4) and the hexamer (experiment 14, Table 4) were obtained in 74, 70, 68, 71, 73.5 and 72 % yield, respectively and they were also found to be homogeneous on TLC (R_f: 0.47, 0.49, 0.40, 0.67, 0.45 and 0.64, respectively, in solvent system D).

A general method of condensation of 5'-protected component with 5'-hydroxy component Table 4). An excess of the phosphodiester component to the 5'-hydroxy component, both in powder form, in 1.1 – 1.36 to 1.0 mol equivalence, respectively, were dissolved in dry pyridine solution. To this well-stirred clear solution, MS – NT (5 – 15 molar equivalent to the phosphodiester component) was added and stirred for 90 min at room temperature. The reaction was quenched by the addition of an excess of saturated $NaHCO_3$ solution (0.2 – 0.5 ml). After stirring for an additional period of 10 min the reaction mixture was poured in a separating funnel containing saturated $NaHCO_3$ (Ca. 100 ml). the mixture was then partitioned with $CHCl_3$ (6 × 75 ml). The $CHCl_3$ extracts were combined and evaporated under reduced pressure (coevaporations with toluene) to obtain a glass which was purified by column chromatography (Table 4 for isolated yields as powder, solvent mixture used for chromatography, stoichiometries of the reactants, MS – NT and volume of pyridine used for each condensation).

Deblocking of the fully protected oligodeoxynucleotides [obtained from the experiments 9, 10, 12, 13 and 14 of Table 4]. Pixyl group from these fully protected oligomers (20 mg) was removed by the action of 4-toluenesulfonic acid, H_2O in 2 % EtOH – $CHCl_3$ as described above (see the general procedure for the preparation of 5'hydroxy components) and then the 5'-hydroxy function was protected by treating with excess of benzoylchloride (10 molar equivalent) in the their pyridine solution was magnetically stirred for 2 h at room temperature. A solution of saturated $NaHCO_3$ (0.3 ml) was added to it. After stirring for 15 min, the reaction mixture. A solution of saturated $NaHCO_3$ (0.3 ml) was added to it. After stirring for 15 min, the reaction mixture was poured in a separating funnel containing saturated $NaHCO_3$ solution (50 ml). This was extracted with $CHCl_3$ (8 × 20 ml). The $CHCl_3$ extracts were pooled and evaporated (with toluene) to obtain a residue [the tribromoethyl group was removed from 3'-terminal phosphotriester of TTTTTTTT by Zn-acetylacetone treatment following standard reaction condition before subjecting it to the next treatment] which was then treated with N^1,N^1,N^3,N^3-tetramethylguanidinium salt of *syn-p*-nitrobenzaldoximate ion in aqueous dioxan solution following the published procedure.[14,15] After 24 h, aqueous NH_3 (*d* 0.88, 3 ml) was added and the reactants were stirred at room temperature for 80 h. The products were concentrated, dissolved in 50 % aqueous acetic acid (15 ml) and the solution was immediately extracted with $CHCl_3$ (15 × 15 ml). The aqueous layer was then concentrated under reduced pressure. The residue (from experiments 9, 10 and 12) were then directly examined by HPLC on Permaphase AAX column using a linear gradient of 0.01 M KH_2PO_4, 0.0 KCl to 0.05 M KH_2PO_4 and 0.7 M KCl (pH 4.45) at 60 °C (see elution profiles, Figs. 1 – 3). The residue from experiments 13 and 14 were purified through a G-50 (medium) column (0.01 $Et_3NH^+HCO_3^-$ buffer, pH 7.5 was used for isocratic elution) and then were examined by HPLC on permaphase AAX column at 60 °C with buffers (pH 4.45) [*undecamer:* linear gradients; (a) isocratically with 0.05 M KH_2PO_4 and 0.15 M KCl; (b) 0.05 M KH_2PO_4 and 0.15 M KCl to 0.05 M KH_2PO_4 and 0.7 KCl; (c) isocratically with solvent system (b); *tetradecamer:* linear gradient; 0.01 M KH_2PO_4, 0.0 M KCl to 0.05 M KH_2PO_4 and 0.7 KCl] (Figs. 4 and 5). Large scale purifications of these oligomers for different physical studies were carried out through a DEAE-Sephadex A25[15] column using $Et_3NH^+HCO_3^-$ buffer as linear gradient (0.001 M to 1.3 M, pH 7.5).

5'-^{32}P-labeled oligodeoxynucleotides have been prepared and identified by partial enzyme digest (Figs. 1, 2 and 3 for octamers) and by two dimensional mobility shift (Figs. 6 and 7 for undecamer

and tetradecamer, respectively) methods following a literature procedure.[28]

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