234. Simplified Liquid-Phase Preparation of Four Decadeoxyribonucleotides and their Preliminary Spectroscopic Characterization

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Summary

Four self-complementary decadeoxyribonucleotides, dApTpApTpApTpApTp-ApT, dApTpApTpCpGpApTpApT, dApTpApTpGpCpApTpApT and dApApAp-ApApTpTpTpTpT, were chemically synthesized by the phosphotriester procedure. Efforts were concentrated on keeping the procedure as simple as possible, concomitant with obtaining high-purity products at each step of the process. The decamers were elaborated from the 3′-end, starting with a 3′-*O*-benzoylated monomer according to the scheme: monomer + monomer → dimer + dimer → tetramer + dimer → hexamer + tetramer → decamer. Purity of intermediates and of the fully blocked decamers were monitored by chromatography and by 300-MHz ^{1}H-NMR. spectroscopy. The deblocked decadeoxyribonucleotides were characterized by their UV., CD., and ^{1}H-NMR. spectra.

Introduction. – The modified phosphotriester method is now well-established as the most efficient one for the synthesis of oligodeoxyribonucleotides [1–5]. Variants of the method have been used by different research groups to prepare synthetic genes [6] [7] and other long oligodeoxyribonucleotides for biological studies [8]. There is much current interest in the development of solid-phase synthesis to facilitate the preparation of sub-milligram quantities of long sequences for studies in these areas [9–11]. Defined-sequence oligodeoxyribonucleotides are also of considerable interest in ^{1}H-NMR. studies of nucleic acid structure [12–14], and the binding of both drugs [13] [14] and mutagens [15] to nucleic acids. For such studies fairly short oligonucleotides are needed because of the problems of resolution associated with large molecules. The relative insensitivity of the magnetic resonance technique in addition requires large (milligram) amounts of high-purity material. With the continual introduction of new coupling reagents [2] [4] [16] and more efficient procedures for deblocking the protected oligonucleotides [17], the liquid-phase synthesis of short oligonucleotides, up to 10 base pairs – one helical

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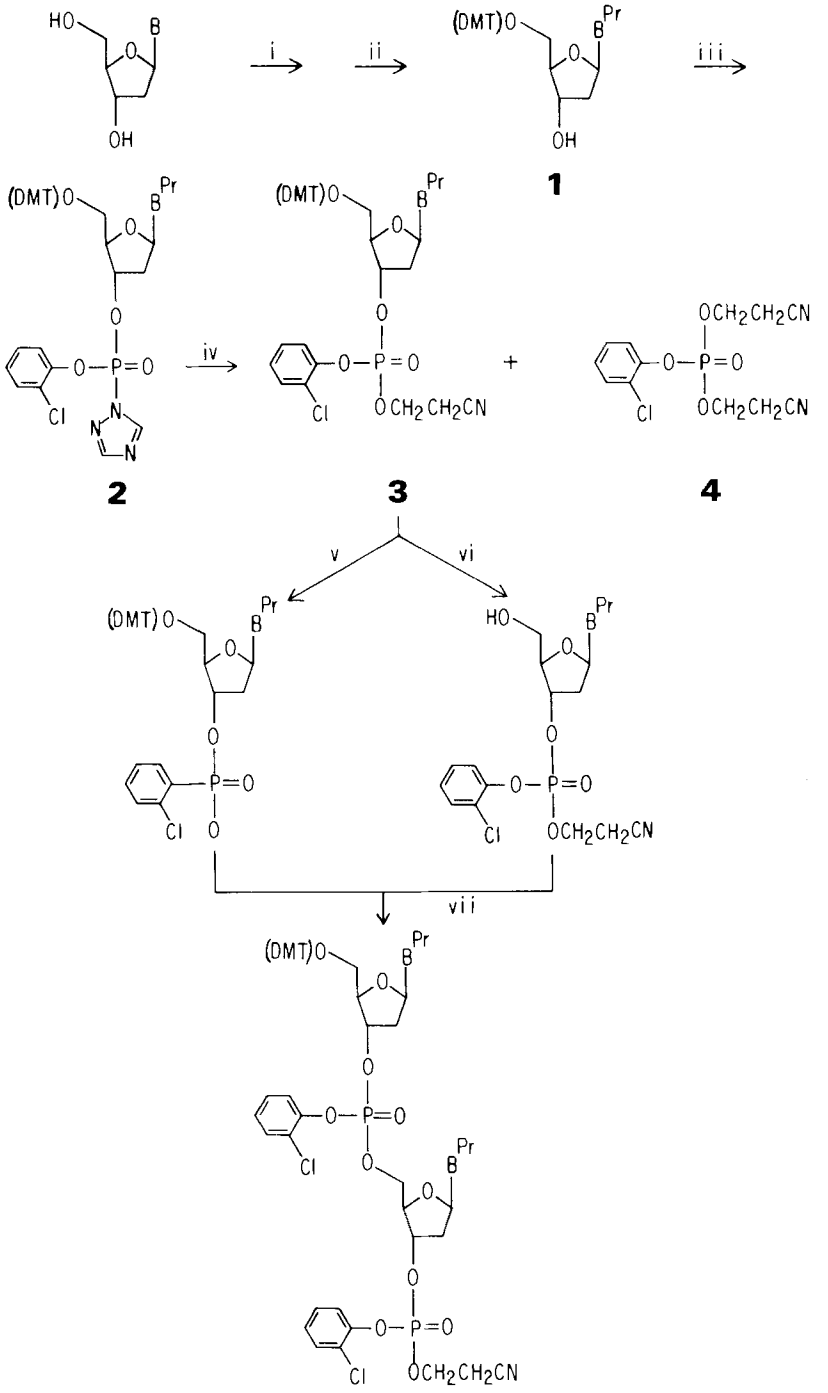
Scheme 1. *Synthesis of fully protected dinucleotides* (B = base (A, T, G or C); B^{Pr} = protected base; i) benzoyl chloride/pyridine (isobutyryl chloride/pyridine for B = G), followed by selective hydrolysis with NaOH; ii) 4,4′-dimethoxytrityl chloride/pyridine/10°/10 h; iii) 1,2,4-triazole-(*o*-chlorophenyl)-phosphodichloridate/dioxane/20°/2 h; iv) 3-hydroxypropionitrile, 1-methylimidazole/dioxane/20°/3 h; v) triethylamine/pyridine/20°/3 h; vi) benzenesulfonic acid/$CHCl_3$/CH_3OH/0°/15 min; vii) TPS-NT/pyridine/20°/3 h)



turn – from the fully protected mononucleotides has been simplified to the point where such molecules are accessible in suitable amounts and state of purity.

In this paper we present simplified procedures for the preparation of the fully protected starting mononucleotides together with a comparison of the efficiency of a variety of coupling reagents and strategies for oligodeoxynucleotide construction, determined during the synthesis of four decadeoxyribonucleotides. Preparation of approximately 0.1 millimol of each of these molecules from purchased 2′-deoxyribonucleosides required about two man-months, and was carried out using simple equipment.

Results and discussion. – *Fully protected monodeoxyribonucleotides* (**3**). These are the key starting materials for oligomer synthesis (see *Scheme 1*). We used *N*-benzoyl groups to protect base residues in dA and dC and *N*-isobutyryl groups for dG as introduced earlier [18] [19]. The protection was carried out according to published procedures by introducing simplifying modifications, which resulted in yields of at least 80% for all base-protected nucleosides. For selective protection of the 5′-hydroxy group, 4,4′-dimethoxytrityl (DMT) chloride [18] was the reagent. This reagent is very selective for the 5′-hydroxy groups, and is a suitable blocking group in itself (*e.g.* in contrast to *p*-chlorophenoxyacetyl chloride, which has to be replaced after phosphorylation of the 3′-position by the hydrazine-labile levulinyl moiety [4]), being quantitatively removed at 0° with benzenesulfonic acid [2]. Protection is typically carried out for 2–4 h at 20°, using a 1.2–1.4 mol excess of the trityl chloride [18] [20]. The non-crystalline (except for d-DMT-T) products are then separated from impurities, which include some 3′,5′-ditritylated material [18], by column chromatography. We have found that performance of this reaction at 0° for 16 h further improves the selectivity, ensuring virtually complete tritylation of the 5′-hydroxy group using a smaller excess of the trityl chloride (1.03–1.05 mol). The resulting crude products (in the case of d-DMT-bzA[3]), d-DMT-bzC[4]) and d-DMT-ibuG[5])) can then be used without further purification in the phosphorylation step.

The phosphorylation was carried out employing a recently described [22] two-step, one-pot reaction using a difunctional phosphorylating reagent, bis(triazolyl)-*o*-chlorophenylphosphate. Thus reaction of a C(5′)-protected nucleoside **1** with the reagent affords the monotriazolide **2**. This intermediate can then be reacted with a second hydroxyl moiety, 3-hydroxyproplionitrile in our case, in the presence of a catalyst such as 1-methylimidazole to force replacement of the second triazole group (which is a far worse leaving group than the first triazole moiety), allowing an efficient synthesis of the fully protected mononucleotide **3** [22]. Use of this method to prepare fully protected mononucleotides by the addition of 3-hydroxypropionitrile does not demand very stringent exclusion of moisture, since excess reagent can be used to prepare the triester **3** and destroy the excess of phosphorylating agent without complication of the chromatography by the consequent by-product **4**.

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[3]) d-BzA = *N*-6-Benzoyl-2′-deoxyadenosine.
[4]) d-BzC = *N*-4-Benzoyl-2′-deoxycytidine.
[5]) d-ibuG = *N*-2-Isobutyryl-2′-deoxyguanosine.

The reactions could be carried out in the open laboratory without the need for elaborate moisture-excluding equipment and the crude DMT-protected nucleosides could be used as starting materials without further purification. Details of the procedure are given in the *Experimental Part*. The reaction, including *in situ* formation of the phosphorylating agent and workup of the final crude product, occupied 8–10 h and was conveniently carried out in one day. The final reaction mixture was diluted with $CHCl_3$ and washed extensively with 0.1 N NaH_2PO_4 to remove all of the 1-methylimidazole, which is capable of slowly removing the β-cyanoethyl (CE) group from the triester [22]. After final workup, the products were co-evaporated with toluene to remove as much of the residual pyridine as possible. This minimized the quantities of silica gel needed for the chromatography, which was carried out with increasing percentages of CH_3OH in $CHCl_3$ until the product eluted. Overall yields of pure fully protected mononucleotides from the 2′-deoxynucleosides were about 55% (for five steps) in the case of A, C, and G, and about 85% (for three steps) for T with only one chromatographic purification.

An alternative approach to the preparation of protected mononucleotides has been described by *Gough et al.* [3] [8]. They obtained high yields of products by hydrolyzing the intermediate monotriazolide **2** and freeing this product from other by-products of the phosphorylation reaction by precipitation as the Ba-salts, which could be used directly as the phosphate components in coupling reactions. However, these compounds must be coupled with 3-hydroxypropionitrile to give the triesters if they are to be used as the 5′-hydroxy component in oligonucleotide synthesis [8]. We have found that it is also necessary to employ dimethoxytritylated nucleosides that have been purified by column chromatography, to ensure clear solutions on hydrolysis of the monotriazolide. For these reasons, this approach seems to offer no particular advantage over the one we have employed.

Formation of internucleotide bonds. Starting with the fully protected mononucleotides, the basic strategy for oligonucleotide synthesis is to selectively deprotect the 5′- and 3′-ends of the appropriate mononucleotides, and join the units together by the use of a coupling reagent. Repetition of this process affords products of longer chain length.

For deprotection of the 5′-hydroxy group, benzenesulfonic acid is the reagent of choice [2], effecting complete unmasking at 0° in 10 min or less, independent of the length of the oligonucleotide. The 5′-hydroxy compound can be freed from 4,4′-dimethoxytritanol by chromatography on silica gel [12], or more simply by precipitation of the product from ether/hexane 1:1. The latter method retains traces of 4,4′-dimethoxytritanol, but this does not interfere with the subsequent coupling [8], and was the method we employed. Yields in the reaction varied from 85–95%, but in the preparation of 5′-hydroxy-TpTpTpT-OBz and all higher oligomers, dioxane/chloroform 1:2 needed to be employed in the workup, due to insolubility of the products in pure $CHCl_3$.

Both aqueous [12] and anhydrous [20] triethylamine have been used for removal of the β-cyanoethyl group from the 3′-triester. The reaction with aqueous triethylamine is about six times as fast (30 min compared with 3 h under anhydrous conditions), but the extra time needed under anhydrous conditions is more than

Table 1. *Synthesis of protected oligodeoxyribonucleotides*

Starting material[a])	(mmol)	Condensing agent (mol-equiv.), reaction time[b])	Product[a])	Silica gel chromatography[c])		
				Adsorbent (ml)[d])	Eluting solvent	Yield[e])
Dimers						
DMT-Aṗ + 5′-OH-Tṗ-CE	(5.07) (3.90)	TPS-NT (1.15), 3.5 h	DMT-AṗTṗ-CE	150	97:3	62
DMT-Aṗ + 5′-OH-T-OBz	(4.50) (3.46)	TPS-NT (1.15), 3 h	DMT-AṗT-OBz	150	97:3	77
DMT-Aṗ + 5′-OH-Aṗ-CE	(4.11) (3.16)	TPS-NT (1.15), 3.5 h	DMT-AṗAṗ-CE	140	97:3	64
DMT-Tṗ + 5′-OH-Tṗ-CE	(3.75) (2.88)	TPS-NT (1.15), 2.5 h	DMT-TṗTṗ-CE	150	97:3	62
DMT-Tṗ + 5′-OH-Tṗ-OBz	(3.76) (2.89)	TPS-NT (1.15), 3 h	DMT-TṗT-OBz	100	98:2	88
DMT-Cṗ + 5′-OH-Gṗ-CE	(2.24) (1.72)	TPS-NT (1.15), 3 h	DMT-CṗGṗ-CE	100	95:5	71
DMT-Gṗ + 5′-OH-Cṗ-CE	(2.74) (2.28)	TPS-NT (1.15), 3.5 h	DMT-GṗCṗ-CE	100	95:5	71
Tetramers						
DMT-AṗTṗ + 5′-OH-AṗT-OBz	(2.16) (1.80)	TPS-NI (3), 18 h	DMT-AṗTṗAṗT-OBz	120	97:3	70
DMT-AṗTṗ + 5′-OH-AṗTṗ-CE	(1.60) (1.23)	TPS-NI (3), 18 h	DMT-AṗTṗAṗTṗ-CE	100	97:3	71
DMT-TṗTṗ + 5′-OH-TṗT-OBz	(1.70) (1.31)	TPS-NT (1.15), 2.5 h	DMT-TṗTṗTṗT-OBz	100	97:3	74
DMT-AṗAṗ + 5′-OH-AṗAṗ-CE	(0.95) (0.73)	TPS-NT (1.15), 3 h	DMT-AṗAṗAṗAṗ-CE	80	96:4, 95:5	66
Hexamers						
DMT-AṗTṗ + 5′-OH-AṗTṗAṗT-OBz	(0.60) (0.46)	TPS-NT (1.15), 3.5 h	DMT-AṗTṗAṗTṗAṗT-OBz	80	96:4	61
DMT-CṗGṗ + 5′-OH-AṗTṗAṗT-OBz	(1.17) (0.88)	TPS-NT (1.15), 2 h	DMT-CṗGṗAṗTṗAṗT-OBz	140	96:4	73
DMT-GṗCṗ + 5′-OH-AṗTṗAṗT-OBz	(1.31) (0.98)	TPS-NT (1.15), 2.5 h	DMT-GṗCṗAṗTṗAṗT-OBz	140	96:4	73
DMT-AṗTṗ + 5′-OH-AṗTṗTṗT-OBz	(0.61) (0.47)	TPS-NT (1.15), 2.5 h	DMT-AṗTṗTṗTṗT-OBz	60	96:4, 95:5	75
Decamers						
DMT-AṗTṗAṗTṗ + 5′-OH-AṗTṗAṗTṗAṗT-OBz	(0.29) (0.21)	TPS-NT (1.3), 2.5 h	DMT-AṗTṗAṗTṗAṗTṗAṗTṗAṗT-OBz	70	95:5	65
DMT-AṗTṗAṗTṗ + 5′-OH-CṗGṗAṗTṗAṗT-OBz	(0.59) (0.42)	TPS-NT (1.3), 3 h	DMT-AṗTṗAṗTṗCṗGṗAṗTṗAṗT-OBz	150	96:4	82
DMT-AṗTṗAṗTṗ + 5′-OH-GṗCṗAṗTṗAṗT-OBz	(0.21) (0.15)	TPS-NT (1.3), 3 h	DMT-AṗTṗAṗTṗGṗCṗAṗTṗAṗT-OBz	60	95:5	82
DMT-AṗAṗAṗAṗ + 5′-OH-AṗTṗTṗTṗTṗT-OBz	(0.25) (0.18)	TPS-NT (1.3), 3 h	DMT-AṗAṗAṗAṗAṗTṗTṗTṗTṗT-OBz	80	95:5	58

[a]) For the sake of clarity, the base-protecting groups have been omitted; A = d-BzA, C = d-BzC, G = d-ibuG, T = T. The symbol ṗ denotes an (*o*-chlorophenyl)phosphotriester linkage, after *Miller et al.* [12] DMT denotes the 5′-*O*-(4,4′-dimethoxytrityl) group, -OBz the 3′-*O*-benzoyl group. [b]) See detailed procedures in *Experimental Part.* [c]) Elution beginning with pure $CHCl_3$ and stepping up with 1% increments of CH_3OH. [d]) Approximate dry volume of adsorbent. [e]) Yield of TLC.-pure material, based on the 5′-hydroxy component of the reaction.

offset by the fact that rigorous redrying of the sample for the coupling reaction is then not necessary, and these conditions were employed in the present work.

Conditions for the deblocking of the CE-group vary with chain length, with increasingly basic conditions being needed for complete deblocking as the chain grows [2]. Since most strategies for oligonucleotide synthesis grow the chain from the 3′-end, this does not usually become a problem.

Table 2. *Rf-values of protected deoxynucleotide intermediates on silica gel TLC.*

Compound[a])	Rf ($CHCl_3$/CH_3OH 9:1)	
	Observed	Literature
DMT-Ap̀-CE	0.55	0.57 [12]
DMT-Cp̀-CE	0.54	0.55 [12]
DMT-Gp̀-CE	0.45/0.47	0.47/0.53 [12]
DMT-Tp̀-CE	0.52	0.52 [12]
5′-OH-Ap̀-CE	0.38	
5′-OH-Cp̀-CE	0.39	0.35
5′-OH-Gp̀-CE	0.30	0.32
5′-OH-Tp̀-CE	0.34	0.39
DMT-ApTp̀-CE	0.45	0.58 [22]
DMT-ApT-OBz	0.55	
DMT-ApAp̀-CE	0.49	0.60 [12], 0.58 [22]
DMT-TpTp̀-CE	0.39	0.34/0.37 [12], 0.58 [22]
DMT-TpT-OBz	0.50	
DMT-CpGp̀-CE	0.37/0.39	0.45 [22]
DMT-GpCp̀-CE	0.44/0.47	0.42/0.49 [12], 0.57 [22]
5′-OH-ApTp̀-CE	0.44	
5′-OH-ApT-OBz	0.31	
5′-OH-ApAp̀-CE	0.37	0.37 [12]
5′-OH-TpT-OBz	0.34	
DMT-ApTpApTp̀-CE	0.34	
DMT-ApTpApT-OBz	0.42	
DMT-TpTpTpT-OBz	0.33	
DMT-ApApApAp̀-CE	0.30	
5′-OH-ApTpApT-OBz	0.31	
5′-OH-TpTpTpT-OBz	0.28	
DMT-ApTpApTpApT-OBz	0.36	
DMT-CpGpApTpApT-OBz	0.32	
DMT-GpCpApTpApT-OBz	0.34	
DMT-ApTpTpTpTpT-OBz	0.32	
5′-OH-ApTpApTpApT-OBz	0.21	
5′-OH-CpGpApTpApT-OBz	0.25	
5′-OH-GpCpApTpApT-OBz	0.28	
5′-OH-ApTpTpTpTpT-OBz	0.26	
DMT-ApTpApTpApTpApTpApT-OBz	0.40	
DMT-ApTpApTpCpGpApTpApT-OBz	0.31	
DMT-ApTpApTpGpCpApTpApT-OBz	0.31	
DMT-ApApApApApTpTpTpTpT-OBz	0.38	

[a]) See footnote *a* in *Table 1.*

After the original introduction of arenesulfonyl chlorides as coupling reagents [23], a number of studies have suggested 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) as the most suitable one. Side reactions include competing sulfonylation of the 5′-hydroxy group and degradation of guanine bases [4]. To overcome these problems, a number of derivatives have been tried, and three have received wide use: 1-(2,4,6-triisopropylbenzenesulfonyl)tetrazole (TPST) [2], 1-(2,4,6-triisopropylbenzenesulfonyl)-4-nitroimidazole (TPS-NI) [16], and 1-(2,4,6-triisopropylbenzenesulfonyl)-3-nitro-1,2,4-triazole (TPS-NT) [4]. New coupling reagents continue to be reported, an example is *p*-nitrobenzenesulfonyl nitroimidazole (*p*-NBSNI) [39], which was reported after completion of our work. TPST and TPS-NT are much faster acting than TPS-NI, but TPST suffers the drawback of being somewhat unstable. In our hands, it also provided lower yields in coupling reactions than TPS-NT. Thus the present work was carried out using TPS-NI or TPS-NT as reagents for internucleotide bond formation. In synthesis up to the hexamer stage, no difference in yields could be detected using these two reagents under the appropriate conditions (a 3-fold mol excess of TPS-NI for 16 h, or a 1.15-fold mol excess of TPS-NT for 3 h). Thus the choice of the reagent is largely one of convenience; the use of TPS-NT permits the reaction (demasking of the phosphate followed by coupling) to be carried out in one day, whereas use of TPS-NI allows the reaction to be continued overnight. For preparation of the decamers, only the more efficient reagent TPS-NT was used.

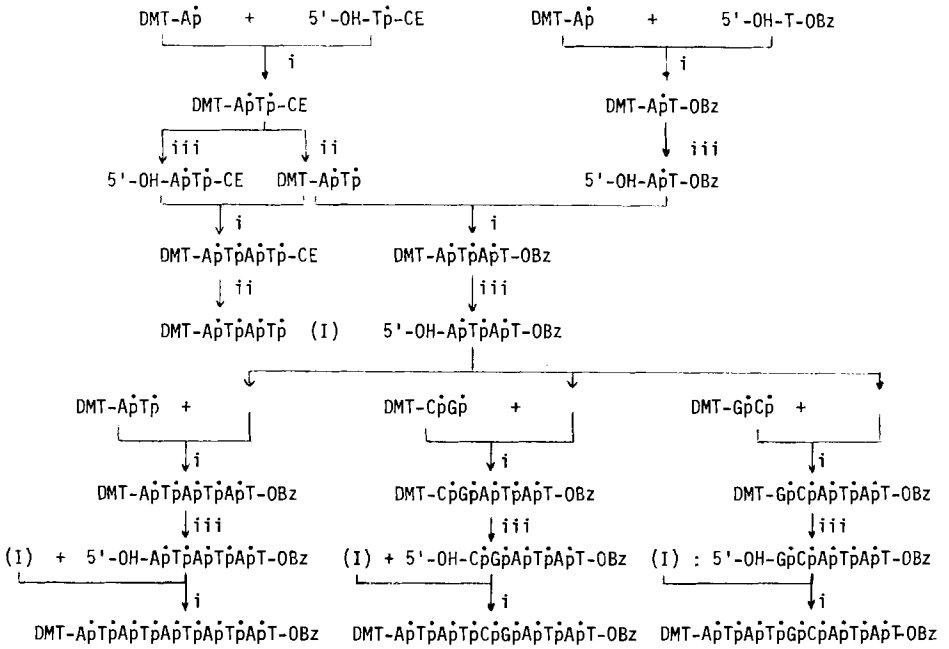
General procedures for the coupling reaction are given in the *Experimental Part*, and the results obtained are listed in *Table 1*. The yields quoted are for TLC.-homogeneous material recovered from the silica gel column. It can be seen that yields in the coupling reaction are essentially independent of chain length up to the decamer stage, as previously reported [24]. Purity of the products was judged by TLC. on silica gel and by ^{1}H-NMR. spectroscopy. The excess of the phosphate component employed in the reactions was always retained on the column. Although it was difficult to judge the purity of crude products before chromatography (due to traces of pyridine that degraded the resolving power of the plate), it proved quite possible by monitoring of the fractions off the column to resolve the product from any unreacted 5′-hydroxy starting material contaminant. These materials always ran slightly slower than the corresponding coupled product (see *Table 2*), and of course did not give a positive trityl-group test. Most of the coupling reactions appeared to go to completion, and little or no 5′-hydroxy component was detected in any of the column fractions. In all four reactions to form the decamers, the later column fractions did contain decamer contaminated with the 5′-hydroxy hexamer starting material, but these fractions were generally less than 10% of the material recovered from the column. In all cases the yields quoted in *Table 1* are based on the 5′-hydroxy component, and the decamers are the combined fractions in which no 5′-hydroxy hexamer contaminant was detected by TLC. It should be noted that careful ion-exchange chromatography after deblocking enabled even the hexamer-contaminated fractions to be utilized for the preparation of pure decadeoxyribonucleotides. TLC. of the fully protected intermediates proved adequate to monitor the purity of products and the extent of completion of reactions. In spite of the formation of diastereoisomers with each phosphotriester coupling, TLC. on

silica gel in $CHCl_3/CH_3OH$ 9:1 provided tight spots at low loadings; only for the GC. dimers was there evidence of resolution of the diastereoisomers. The Rf-values obtained are better used in a comparative sense rather than as absolute values, although they are in good agreement with those given in [1]. It proved possible to distinguish between the different classes of fully protected monomers, dimers, tetramers and hexamers, and between each class and its 5′-deblocked analogs *(Table 2)*. Even the fully protected decamers were sufficiently well-resolved from the 5′-hydroxy hexamer components used in their synthesis to allow rapid monitoring of column fractions by TLC. Those fractions apparently free of hexamer contamination were pooled for deblocking (later ion-exchange chromatography of deblocked material using more sensitive detection methods showed that the compounds contained less than 5% of this impurity).

Strategy of oligonucleotide synthesis. The syntheses described here were undertaken to explore the phosphotriester approach to synthesize large quantities of oligonucleotides, and to provide model compounds for studies of nucleic-acid dynamics and structure, and studies of ligand binding to small defined-sequence nucleic acids. General requirements were thus that the sequences chosen be self-complementary, to avoid the synthesis of both strands separately, and that the chain length be sufficiently long to enable duplex formation readily at ambient temperatures and at concentrations suitable for UV. measurements. A length of 10 base-pairs was judged appropriate, as this represents one turn of the DNA-helix, and it has been reported [12] that the self-complementary duplex formed by d(CCAAGGTTGG) has, at a strand concentration of 1.3×10^{-5} M, a melting temperature T_M of 47° in 0.11 M salt, and the duplex formed by d(AATTGCAATT) at the same strand and salt concentration, has a melting temperature T_M of 33° [31]. To improve the stability of the short DNA-duplexes, it was decided to prepare the oligonucleotides without terminal phosphate groups. Although it is possible to selectively remove such groups by the use of alkalinephosphatase [8], we considered it advantageous to utilize the flexibility of the synthetic approach and use an appropriately protected nucleotide to form the 3′-end of the oligonucleotides. While both acetyl and benzoyl groups have been used to mask the 3′-hydroxy function, the benzoyl group is preferred because it provides intermediates of greater solubility in organic solvents, and with a greater tendency to solidify [2].

The general strategy of the syntheses was: dimer + dimer → tetramer + dimer → hexamer + tetramer → decamer, and is outlined in detail for the four different decamers in *Schemes 2* and *3*. The chains were extended from the 3′-end, and the size of the phosphate-bearing component was kept as small as possible to facilitate complete coupling. This reaction appears to be dependent on the size of the phosphate-bearing component, and it is sluggish with the larger molecules. Thus in the present work, no 5′-hydroxy component was detected at the end of coupling reactions where the phosphate component was a monomer or dimer. When this component was a tetramer, as in the final coupling reactions, small amounts of 5′-hydroxy component were detected in the products, even though the mole ratio of phosphate to 5′-hydroxy component was increased to 1.4:1. In a similar reaction involving the use of a hexameric phosphate component [12], the yield in the coupling reaction was only 65%. In the latter case, the hexameric phosphate was

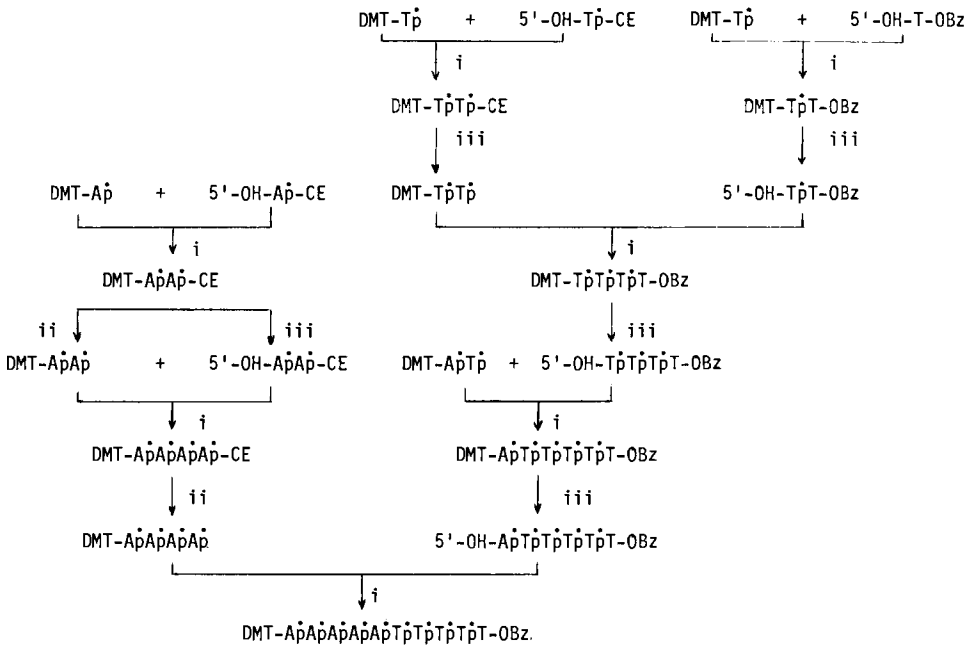
Scheme 2. *Synthesis of three fully protected decadeoxyribonucleotides* (For the sake of clarity the base-protecting groups have been omitted; A = d-BzA, C = d-BzC, G = d-ibuG, T = T. The symbol ṗ denotes an (*o*-chlorophenyl)phosphotriester linkage, after *Miller et al.* [12] DMT denotes the 5′-*O*-(4,4′-dimethoxytrityl) group, -OBz the 3′-*O*-benzoyl group. i) Triethylamine; ii) 1-(2,4,6-triisopropylbenzenesulfonyl)-3-nitro-1,2,4-triazole; iii) benzenesulfonic acid)



also lipophilic enough for some of it to be eluted off the silica gel column, further contaminating the product decamer; no similar contamination of products by the tetrameric phosphate components was detected in the current work.

Because of similarities in the sequences of three of the decamers, only seven dimers were needed. Two of these contained a 3′-*O*-benzoyl group; DMT-ApT-OBz for the 3′-end of the alternating A-T oligonucleotides, and DMT-TpT-OBz for the end of the block co-polymer. These two dimers were elaborated successively to tetramers and hexamers by addition of dinucleotide blocks; conditions and yields for these reactions are given in *Table 1*. The two phosphate-bearing tetranucleotides DMT-ApApApAp and DMT-ApTpApTp were similarly prepared by dimer coupling, and in the last step these tetramers and the appropriate 5′-hydroxy hexamers were coupled together to provide the desired fully protected decadeoxyribonucleotides. The purity of the products was checked by TLC. and ^{1}H-NMR. spectroscopy. All DMT-containing compounds showed a characteristic resonance at $\delta = 3.8$ ppm from the CH_3-protons of the methoxy groups. Furthermore, the fully blocked nucleotides exhibit two multiplets at $\delta = 2.75$ and 4.4 ppm which we attribute to the CH_2-protons of the cyanoethyl substituent. In the case of the mononucleotides, these spectra can be assigned by comparison with deblocked mononucleotides in the region between 6.5 and 1 ppm. For the blocked dinucleotides,

Scheme 3. *Synthesis of the fully protected decamer d(AAAAATTTTT)* (For the sake of clarity the base-protecting groups have been omitted; A = d-BzA, C = d-BzC, G = d-ibuG, T = T. The symbol ṗ denotes an (*o*-chlorophenyl)phosphotriester linkage, after *Miller et al.* [12] DMT denotes the 5′-*O*-(4,4′-dimethoxytrityl) group, -OBz the 3′-*O*-benzoyl group. i) Triethylamine; ii) 1-(2,4,6-triisopropylbenzenesulfonyl)-3-nitro-1,2,4-triazole; iii) benzenesulfonic acid)



the spectra are too complicated to allow a straightforward assignment. In those cases, the purity was checked by comparing the integrated intensities of the resonance at 3.8 ppm with all aromatic proton resonances.

Deblocking of fully protected deoxyribonucleotides. There are three distinct types of blocking groups to be considered in deblocking of the fully protected oligonucleotides; the alkali-labile base-protecting *N*-acyl and 3′-*O*-acyl groups, the acid-labile 5′-*O*-DMT group, and the aryl groups protecting the internucleotide phosphates. The characteristics of these three classes of blocking groups determine the nature and sequence of the deblocking steps, and the sensitivity of the final products towards the deblocking reagents make it advisable to remove these immediately after each step. The most difficult problem has been the avoidance of internucleotide cleavage during deblocking of the phosphodiester linkage [1]. Employment of relatively acidic phenols assists by increasing the alkali lability of the blocking group, but if the phenol is too acidic then the group becomes too labile to act as an adequate blocking group during the synthesis [17]. The use of *o*- and *p*-chlorophenols seems to be an adequate compromise in this regard. Ammonia, hydroxide, and fluoride have all been employed as nucleophiles, but all result in small but detectable and (for the deblocking of long sequences) unacceptable amounts of internucleotide cleavage (1–2% per phosphotriester). Recently the use of the con-

jugate bases of 4-nitrobenzaldehyde oxime and pyridine-2-carbaldehyde oxime has been reported [12] [17]. Slow nucleophilic attack by the conjugate base on phosphorus is followed by fast, base-promoted cleavage of the (O,N)-bond to give the aryl cyanide and the phosphodiester, with less than 0.5% of concomitant internucleotide cleavage [25]. In the present case, a 50- to 80-fold excess of tetramethylguanidinium pyridine-2-aldoximate in dry dioxane at 20° for 20 h efficiently removed the internucleotide protecting groups. The DNA was recovered from the excess reagent by diluting the reaction mixture with 60% ethanol and passing it through a short *DEAE-Sephacel* column. After washing out the reagents, the DNA was eluted with a solution of 2 M triethylammonium bicarbonate (TEB) in 60% ethanol. This buffer can be removed under vacuum at 30° to give the crude DNA. The base-protecting *N*-acyl groups were then easily removed with further alkali treatment, using pyridine/aqueous ammonia 1:2 for 100 h at 20°. This treatment also efficiently removes the 3′-*O*-benzoyl group from the 3′-terminal nucleotide. After removal of all traces of base under high vacuum, treatment for 30 min with acetic acid (to deblock the 5′-*O*-(dimethoxytrityl) group of the 5′-terminal nucleotide) completes the process. The acid hydrolysis is carried out last because the *N*-acylated purines, especially adenosine, have a much greater tendency to undergo acid-catalysed hydrolysis of the (N,C(1′))-glycosidic bond than do the unacylated derivatives [1].

Final purification of the DNA was effected by chromatography on *DEAE-Sephacel*, using a linear gradient of aqueous TEAB from 0.02 to 0.50 M. Elution

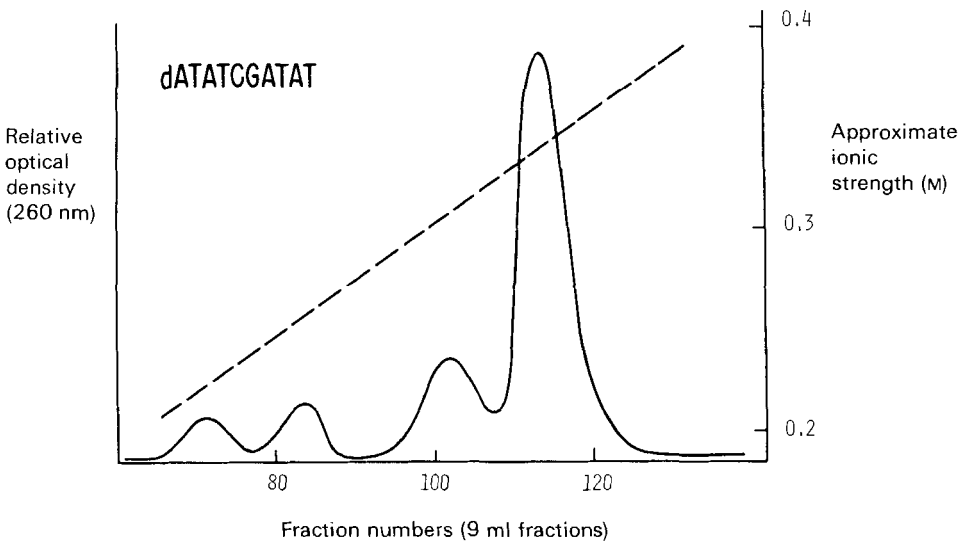


Fig. 1. *Elution profile of a decadeoxyribonucleotide on* DEAE-Sephacel *ion-exchange column* (Elution was achieved with a gradient of aqueous triethylammonium bicarbonate from 0.02 to 0.5 M, collecting fractions of 9 ml and following the absorbance at 260 nm. The sloping line gives the approximate ionic strength of the eluant. The highest peak is the decadeoxyribonucleotide; the preceeding peak is due to unreacted hexamer component from the final coupling reaction)

of the decamers occurred at an ionic strength of about 0.35–0.40 M, with shorter fragments beginning to be eluted from about 0.25 M. A typical elution profile is given in *Figure 1*. This profile is from approximately 1800 OD. units (optical density at 260 nm) of crude DNA, which in turn resulted from deblocking of 175 mg (0.035 mmol) of fully protected decamer. Although the profile is different for each of the four decamers, deblocking of different samples of the same decamer under slightly different conditions gave essentially identical profiles. This suggests that most of the short material in the samples, eluting between 0.25–0.35 M TEAB and containing between 10–15% of the total OD., results from impurities in the fully protected decamers, rather than from those introduced during the deblocking procedure. Further evidence for this is shown by the profiles obtained by deblocking the less pure samples of fully protected decamers. These were obtained as the later fractions off the silica gel columns during purification of the fully protected decamers, and could be shown by TLC. to contain some 5′-hydroxy hexamer component (see above). When these less pure samples were deblocked, the gradient elution profile showed an increased amount of OD. associated in particular with the shorter fragments, allowing identification of these as the corresponding 5′-hydroxy hexamer. Thus, even the samples of fully protected decamer, considered to be pure by TLC., probably contained up to 5% of the hexamer component.

Conversion of the decanucleotides from the triethylammonium form to the Na-form (more suitable for NMR. studies) was effected either by filtration of a water solution through a Na-form ion-exchange column, or by ethanol or acetone precipitation from 0.3 M NaCl-solution (see *Experimental Part*). The precipitation method is very efficient for DNA-fragments as small as 20–30 base pairs, but with the decanucleotides the efficiency was only 60–70%, even using three volumes of acetone. Thus the ion-exchange method is to be prefered for DNA of this length; recovery in this case was quantitative.

Recently it was reported [37] that TPS-NT reacts slowly with the 6-carbonyl group of guanosine to yield a nitrotriazole derivative; it is thus preferable to also protect the 6-carbonyl group of guanosine. We did not do so, but after deblocking we were not able to detect the characteristic resonance of the nitrotriazole proton at $\delta \approx 9.5$ ppm [38] in the ^{1}H-NMR. spectra of the guanosine-containing decanucleotides. We, therefore, conclude that the modification of guanosine by TPS-NT does not occur to a large extent under our experimental conditions (small excess of TPS-NT relative to the 5′-hydroxy component in the coupling reaction; short coupling time). Very recently it was demonstrated [38] that the oximate treatment in the deblocking process reverses the modification of guanosine by TPS-NT, thus giving a further explanation for our non-detection of the modified guanosine.

^{1}H-NMR. Results. All four decanucleotides formed duplexes readily on dissolution in buffer (0.1 M NaCl, 0.01 M Na-cacodylate or Na-phosphate at pH 7.0), as evidenced by the low-field (10–14 ppm) ^{1}H-NMR. spectrum at 300 MHz *(Fig. 2a–d)* in H_2O. In aqueous solution, the only signals appearing in this region are those from the exchangeable guanine-N(1)- and thymidine-N(3)-protons, which form N–H · · · N-imino H-bonds to the N(3) of cytosine and N(1) of adenine in the *Watson-Crick* base pairs [26]. These protons are then protected from rapid exchange with the solvent, and their observation is a sensitive monitor of duplex formation.

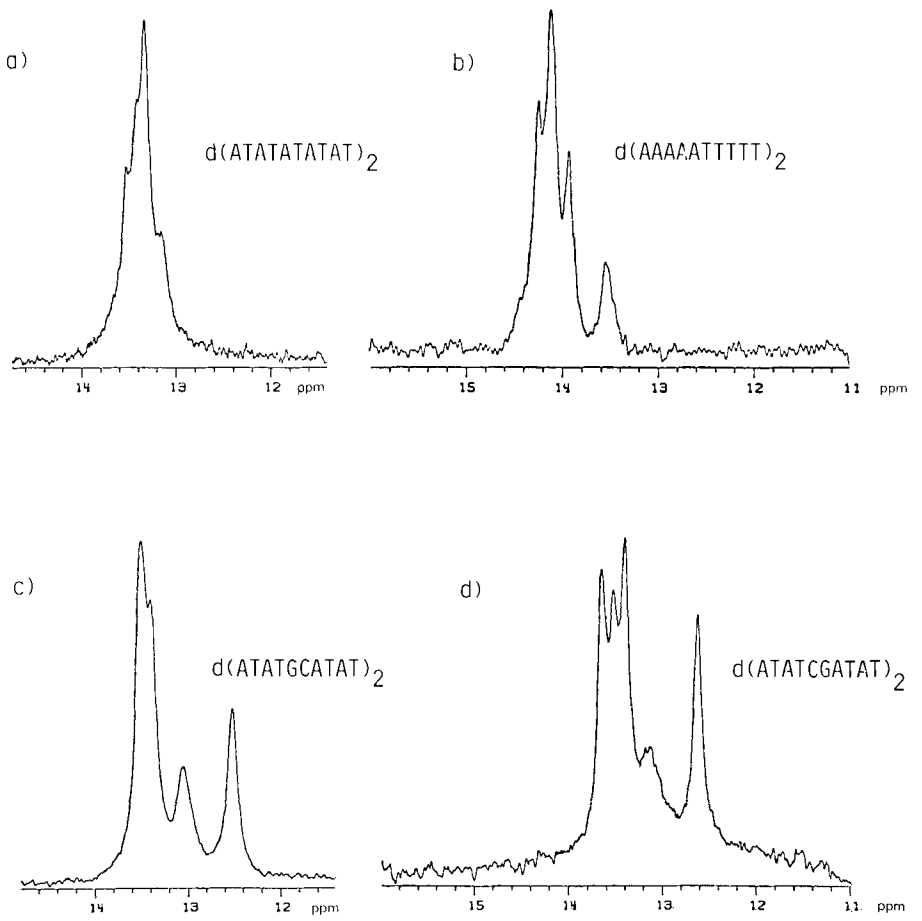


Fig. 2. *Low-field 300-MHz ^{1}H-NMR. spectrum of decadeoxyribonucleotides at 5° in aq. solution* (0.1N NaCl; 0.01M Na-cacodylate; pH 7).

The imino protons from AT base pairs resonate at about 13.0–14.0 ppm, while those from GC. base pairs appear upfield at 12.1–13.2 ppm. These resonances are shifted upfield from the position expected from an isolated N–H · · · N-imino proton, due to shielding by the ring currents of the nearest-neighbor and next-nearest-neighbor base pairs in the duplex [27]. Thus, the exact position of a particular resonance line is dependent on the environment of the base pairs, and some resolution of the low-field resonances can be expected, even for molecules of repeating sequence such as d(ATATATATAT)$_2$ *(Fig. 2a)*. The decamers are all self-complementary, palindromic, thus possessing only five non-identical base-pair imino protons. Although the protons of the terminal AT base pair experience the least ring-current shielding, being adjacent to only one other base pair, the resonance assigned to the terminal AT in each molecule is the low-intensity line slightly upfield of the main AT peak. Both the low-intensity and the upfield shift of this

resonance from the expected position are due to a substantial degree of transient fraying of the ends of the short duplex, even at 6°. The spectrum of the block co-polymer d(AAAAATTTTT)$_2$ *(Fig. 2b)* is broadly similar to that one of d(ATATATATAT)$_2$ except that the individual resonances are better resolved in the former molecule. The two guanine-cytosine containing molecules show the expected additional resonance from the GC. base pairs with that for d(ATATGCATAT)$_2$ at 12.52 ppm *(Fig. 2c)* and for d(ATATCGATAT)$_2$ at 12.62 ppm *(Fig. 2d)*. For the latter two molecules, the intensity ratio of the GC- and AT-resonances is about 1 : 3.6 which, after allowing for some degree of exchange for the terminal AT-protons indicates that the molecules have the expected number and type of base pairs and this provides additional support that the molecules have the correct sequence. By comparison, the imino protons of d(AATTGCAATT)$_2$ resonate at 12.50 ppm (two GC-imino protons) and 14 ppm (eight AT-imino protons) [31], thus showing a similar shift for the GC-imino protons as in d(ATATGCATAT)$_2$ and similar shifts for the AT-imino protons as in d(AAAAATTTTT)$_2$. Thus, high-resolution NMR. in this region is a powerful analytical technique for monitoring sequence, structure and duplex formation in small oligodeoxyribonucleotides.

The ^{1}H-NMR. spectra of the non-exchangeable protons of oligonucleotides is much more complicated than the low-field spectrum of the exchangeable protons, and an assignment of the resonances to specific protons in the oligonucleotide is much more difficult. As an example, *Figure 3* shows the non-exchangeable ^{1}H-NMR. spectrum of d(AAAAATTTTT)$_2$ in D_2O at 20° and 80°. The spectrum at 20° is representative of the duplex form, while the one at 80° is for the random coil. The changes in the spectrum upon going from 20° to 80° demonstrate again the ability of the decamer to form a duplex, as seen in many other oligonucleotides [36]. The base protons can be easily assigned by comparison with NMR. spectra of similar oligonucleotides: H–C(8) and H–C(2) of A and H–C(6) of T between 7.0 and 8.2 ppm, H_3C–C(5) of T between 1.3 and 2 ppm. The assignment of the resonances to the individual base protons in the sequence is only straightforward for the CH_3-resonances, based on their chemical shift: the thymines at the center of the block polymer are the only ones which have adenine as a nearest neighbor, which induces a much larger ring-current shift than a neighboring thymine. Thus, in the spectrum at 20° we assign the resonance at 1.28 ppm to the CH_3-protons of the thymines in the center of the duplex and the resonance at 1.58 ppm to the CH_3-protons of the adjacent thymines. As seen in the spectra (shown in *Fig. 3*) the two CH_3-resonances at high-field shift down-field upon melting of the duplex due to loss of the induced ring-current shifts. The spectrum at higher temperatures demonstrates also that even at 80° there occurs some residual base-pair stacking in the single coil state, because the CH_3-resonances should be chemically identical in an unstacked oligomer.

The ratio of the integral over the base protons at lower field to the CH_3-protons, experimentally observed to be 1.0, provides additional proof for the decanucleotide structure. The assignment of the remaining resonances in the spectrum to the individual base and sugar protons in the sequence is still under investigation for all four synthesized decanucleotides.

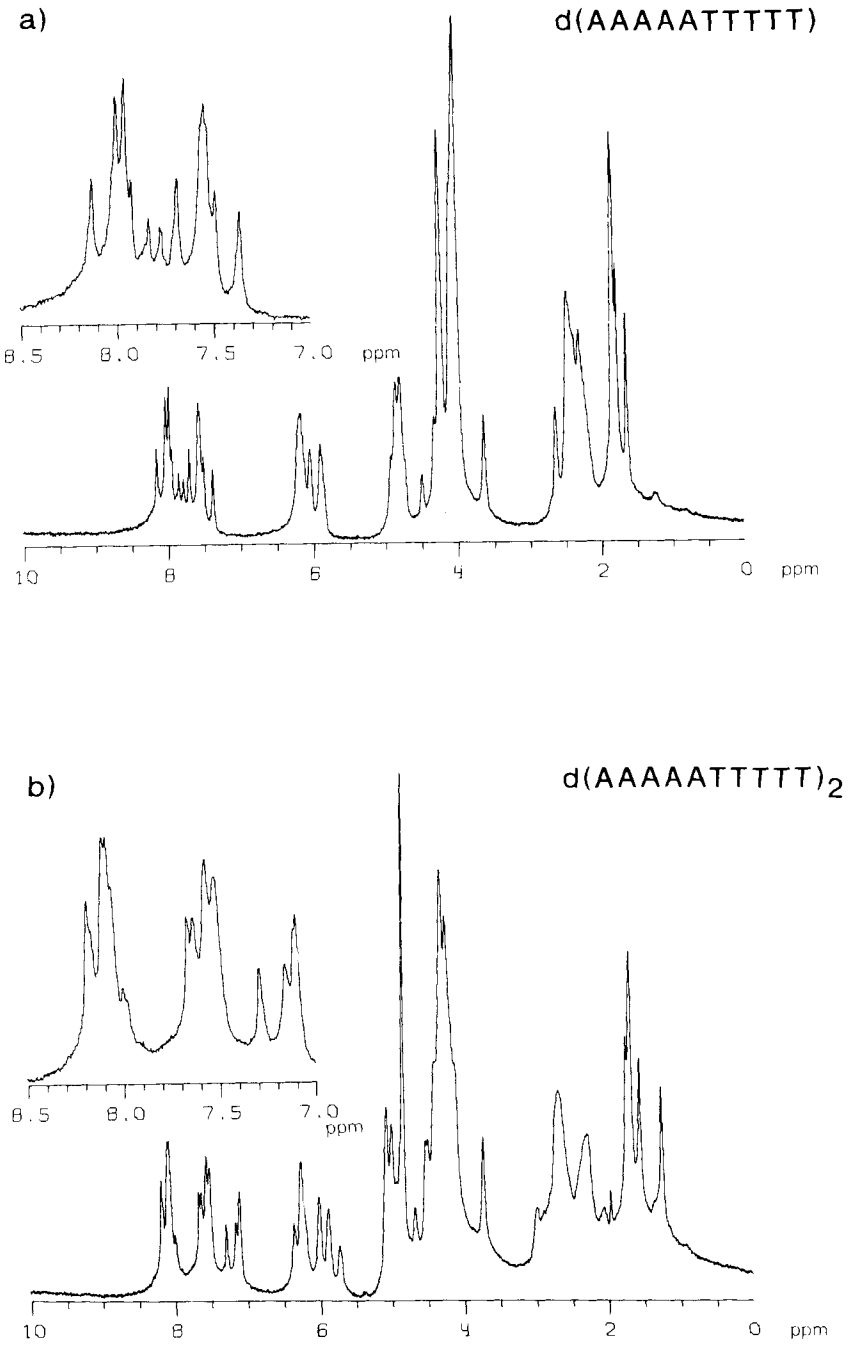


Fig. 3. *360-MHz ^{1}H-NMR. spectrum of d(AAAAATTTTT)$_2$ at 20° (a) and 80° (b) in D_2O* (0.1N NaCl, 0.01M NaH_2PO_4; pH = 7)

Table 3. *Spectroscopic properties of the fully deblocked decanucleotides*

	d(ATATATATAT)$_2$	d(AAAAATTTTT)$_2$	d(ATATGCATAT)$_2$	d(ATATCGATAT)$_2$
UV.[a])	λ_{max} = 263 nm ε (263 nm) = 13200 M^{-1} cm^{-1}	261 nm ε (261 nm) = 12000 M^{-1} cm^{-1}	263 nm ε (263 nm) = 13000 M^{-1} cm^{-1}	263 nm ε (263 nm) = 13000 M^{-1} cm^{-1}
CD.[b])	λ_{max} (nm) 227, 270 λ_{min} (nm) 251	219, 262, 283 249, 266	215, 270, 291 (*S*) 249	224, 270, 290 (*S*) 248
^{1}H-NMR.[c]) δ (ppm)	13.09 (2H) 13.29 (4 H) 13.39 (2 H) 13.48 (2 H)	13.54 (2 H) 13.93 (2 H) 14.15 (4 h) 14.27 (2 H)	12.52 (2 H) 13.09 (2 H) 13.41 (2 H) 13.51 (4 H)	12.62 (2 H) 13.21 (2 H) 13.42 (2 H) 13.55 (2 H) 13.66 (2 H)

[a]) Aqueous solutions in 1-mm cell (7.9×10^{-4} M in base pair, 1 M NaCl, 0.01 M Na-cacodylate, pH 7, T = 19°). Values of ε are ± 15%. [b]) Same as in (a) except T = 8°. [c]) At 300 MHz; in aqueous solution (2.3×10^{-2} M in base pairs, 0.1 M NaCl, 0.01 M Na-cacodylate; pH 7; T = 5°), δ (ppm) relative to δ (DSS) = 0).

CD. and UV. Spectra. All four decanucleotides exhibit a structureless UV. spectrum with a maxima near 260 nm (see *Table 3*) characteristic for DNA. These spectra demonstrate that the UV. spectroscopy is not a useful analytical tool for the characterization of oligonucleotides. By contrast, the CD. spectra of the four decanucleotides exhibit distinctive characteristic features for each decamer (see *Fig. 4* and *Table 3*). The spectrum of d(ATATATATAT)$_2$ shows maxima at 270 and 227 nm and a minimum at 251 nm and resembles very much the spectrum of poly(dAdT) [33]. The CD. spectra of d(ATATGCATAT)$_2$ and d(ATATCGATAT)$_2$ are very similar to each other, exhibiting slightly different wavelengths for the maxima and minima and a more pronounced shoulder at large wavelength for the former duplex. This shoulder at long wavelength for the GC-containing decamers occurs in the spectral region where poly(dGdC) shows a maximum in its CD. spectrum [40], thus proving the GC-content in these decamers. Whereas the overall shape of the spectra of d(ATATATATAT)$_2$, d(ATATGCATAT)$_2$, and d(ATATCGATAT)$_2$ are similar, the CD. spectrum of d(AAAAATTTTT)$_2$ is quite different, and it is interesting to note that it closely resembles that of poly dA · poly dT [34] in every aspect. This indicates that the geometry of this block co-polymer contains the same features as already found in solution studies for poly dA · poly dT [33] but different ones than the other decanucleotides (these findings are consistent with the results of a X-ray study of crystalline d(CGCGAATTCGCG)$_2$ [35], which also showed that the fragment -AATT- possesses different stacking geometry than the fragment -CGCG-). These four spectra demonstrate that the CD. spectrum is much more sensitive to the nature and sequence of the oligonucleotides than is the UV. spectrum.

A note of caution should be added: many authors state extinction coefficients at the wavelength of the maximum of the UV. spectrum for short oligonucleotides. To be meaningful, these values must be measured under conditions where the oligonucleotides exist either completely in the duplex or completely in the random coil state.

Conclusions. – In the work described here we show that it is possible to synthesize short oligonucleotides in large amounts, high purity and short time using the phosphotriester approach in liquid phase. Since this work was completed, we have succeeded in synthesizing four more decadeoxynucleotides in even shorter time [30], thus demonstrating that the proposed procedures do provide a real simplification for the synthesis of synthetic DNA-fragments. In addition we have also shown that ^{1}H-NMR. spectroscopy of the exchangeable proton is a powerful analytical tool for the characterization of short oligonucleotides in duplex form.

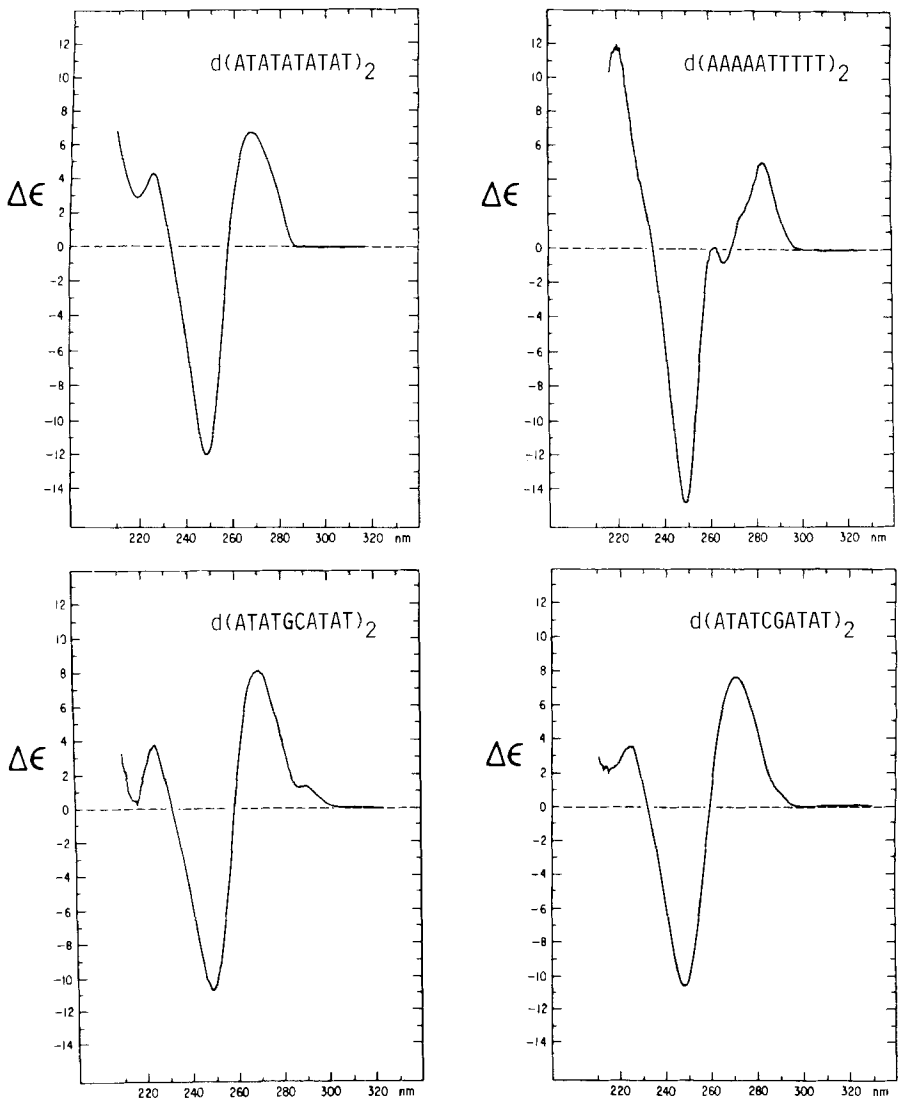


Fig. 4. *CD. Spectra of the decadeoxyribonucleotides at 8° in aq. solution* (7.9×10^{-4} M in base pairs, 1 N NaCl, 0.01 M Na-cacodylate; pH 7)

The support of the *American Cancer Society* (CH-32) and the *National Science Foundation* (PCM 7911571) to *D.R. Kearns* and the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung* to *W. Leupin* is most gratefully acknowledged. We also thank Dr. *Peter Mirau*, Ms. *N. Assa-Munt*, and Ms. *J. Feigon* for providing some NMR. spectra, and Dr. *R. Ogden* and Mr. *C. Broka* for helpful discussions.

Experimental Part

General remarks. Deoxynucleosides *(Sigma)* were used as received after checking their purity by TLC. 2'-Deoxycytidine was used as the hydrochloride without liberation of the free base. 1,2,4-Triazole *(Sigma)*, 3-amino-1,2,4-triazole *(Aldrich)*, 2,4,6-triisopropylbenzenesulfonyl chloride *(Aldrich)* and 4,4'-dimethoxytrityl chloride *(Alfa)* were used as received. 3-Hydroxypropionitrile *(Aldrich)*, 1-methylimidazole *(Aldrich)* and pyridine *(Aldrich Gold Label)* were dried over 4-Å molecular sieve. Reagent-grade dioxane and triethylamine were refluxed for 7 h over calcium hydride and distilled onto 4-Å molecular sieve. $CHCl_3$ and CH_3OH *(Mallinkrodt Analytical Reagents)* for column chromatography were used as received. (*o*-Chlorophenyl)phosphodichloridate was prepared by the method of *Owen & Reese* [28]. 1-(2,4,6-Triisopropylbenzenesulfonyl)-4-nitroimidazole (TPS-NI) and 1-(2,4,6-triisopropylbenzenesulfonyl)-3-nitro-1,2,4-triazole (TPS-NT) were prepared by the methods of *van Boom et al.* [16], *Kroger & Mietcher* [29] and *de Rooij et al.* [4], respectively, except for anh. dioxane was used as solvent. Triethylammonium bicarbonate (TEAB) solutions were freshly prepared by passing CO_2 through a cooled solution or emulsion of triethylamine in the appropriate solvent until the pH was 7.4–7.6. Silica gel chromatography was carried out at ambient temperature and pressure using *Merck* silica gel *60* (70–230 mesh). Thin layer chromatography (TLC.) was performed on *Merck* silica gel F_{254} plastic-backed TLC. sheets; chromatograms were checked under UV. and developed with HCl gas to visualize 4,4-dimethoxytrityl-containing products. *DEAE-Sephacel* (Swollen Bead Size 40–150 μ) was purchased from *Sigma*. UV. spectra were recorded on a *Beckman Acta CIII* UV./VIS. spectrophotometer. The CD. spectra were recorded on a *Joubin-Yvon Dichrograph III*, using 1-mm cells. ^{1}H-NMR. spectra were recorded on a *Varian Associates HR 300* NMR. spectrometer operated in the FT- or CW-mode, or on a 360-MHz spectrometer constructed in these laboratories around an *Oxford Instrument* narrow-bore 8.45 T magnet and a modified *Varian HR-220* electronics console [32].

Synthesis of base-protected 2'-deoxyribonucleosides. – N-*6-benzoyl-2'-deoxyadenosine (d-bzA).* 2'-Deoxyadenosine monohydrate (10 g, 37.1 mmol) was dissolved in dry pyridine (50 ml) and evaporated to dryness. The gummy residue was stirred rapidly with dry pyridine (120 ml) to give a crystalline suspension. Benzoyl chloride (20 ml, 220 mmol, 2 mol excess) was added dropwise to this stirred suspension below 10°. The mixture was allowed to warm to 20°, kept at 20° for 1 h, and poured into ice-water (1 l). Extraction with $CHCl_3$ (3 × 400 ml) gave the crude tribenzoyl compound. This was dissolved in pyridine (100 ml) and prechilled C_2H_5OH (120 ml) was added, followed as quickly as possible by a prechilled solution of NaOH (12.8 g, 320 mmol) in water (80 ml) and C_2H_5OH (160 ml), keeping the reaction temperature below 20°. The mixture was stirred until it became homogeneous (10–15 min), neutralized to pH 7 with acetic acid, and diluted to 1 l with water. The solution was filtered, if necessary, from traces of starting material, and the filtrate evaporated to 500 ml and clarified with charcoal. On standing, *d-bzA* separated, m.p. 112–114° ([18]: m.p. 113–115°). Yield from two crops was 10.3 g (78%).

N-*4-Benzoyl-2'-deoxycytidine (d-bzC).* Crude tribenzoyl-2'-deoxycytidine was prepared as described above for adenosine. The crude product (from 10 g, 37,9 mmol) of 2'-deoxycytidine hydrochloride was dissolved in a mixture of THF (500 ml), CH_3OH (400 ml) and water (100 ml), and cooled to below 3°. A prechilled solution of NaOH (8 g) in water (100 ml) was added as fast as possible while keeping the reaction temperature below 5°. The mixture was stirred an additional 10 min at 0–3°, and brought to pH 7 with acetic acid. Water was added to a volume of 2 l, the mixture was evaporated to half volume, clarified with charcoal while warm and filtered. On cooling, *d*-bzC crystallized, m.p. 175–177° ([18]: m.p. 174–175°). Yield from two crops was 10.03 g (80%).

N-*2-Isobutyryl-2'-deoxyguanosine (d-ibuG).* 2'-Deoxyguanosine (Grade II, 95% assay) (10 g, 37.4 mmol) was suspended in dry pyridine (50 ml) and evaporated to dryness. The residue was stirred with dry pyridine (100 ml) for 15 min to give a fine suspension. This was cooled to below 10° and kept at

this temperature while isobutyryl chloride (20 ml, 191 mmol; 1.7 mol excess) was added dropwise. After stirring 3 h at 20°, the mixture was cooled to 10° and dry CH_3OH (2 ml) was added. After a further 2 h at 20°, the mixture was diluted with $CHCl_3$ (1 l) and washed with ice-cold 1% aq. $NaHCO_3$ and water. Evaporation of the solvent gave the crude triisobutyrylated compound as a gum. This was dissolved in C_2H_5OH (100 ml), cooled to 3°, and kept at this temperature while a prechilled solution of NaOH (6.4 g) in water (80 ml) was added. The mixture was kept at 0–3° until hydrolysis was complete (30–40 min), as monitored by the TLC. The solution was adjusted to pH 7 with ice-cold 3N H_2SO_4 and the resulting suspension evaporated to dryness at low temperature and azeotroped once with C_2H_5OH. The residue was extracted with warm C_2H_5OH (2× 150 ml) and filtered from Na_2SO_4. The filtrate was evaporated, dissolved in water at 50° (200 ml), and clarified with charcoal. On standing, *d-ibuG* separated. Yield from two crops was 9.35 g (78% based on starting material of 95% purity).

Synthesis of fully protected 2'-deoxyribonucleotides. – *5'-O-(Dimethoxytrityl)thymidine 3'-(o-chlorophenyl β-cyanoethyl phosphate) (DMT-Tp-CE).* Thymidine (10 g, 41.3 mmol) was suspended in dry pyridine (50 ml) and evaporated to dryness. Solid 4,4'-dimethoxytrityl chloride (14.4 g, 42.5 mmol) was added to the residue, followed by dry pyridine (40 ml). The mixture was held in an ice bath for 5 min, then gently swirled until all solids were dissolved. The solution was kept for 16 h at 0°, dry CH_3OH (2 ml) was added and the solution kept a further 1 h at 20°. Solvent was evaporated at low temperature, and the residue was dissolved in $CHCl_3$ (200 ml) and washed with water (2× 100 ml). The solvent was evaporated and the residue dissolved in warm benzene (200 ml) and clarified with charcoal. Hexane was added to the boiling solution until turbidity (about 100 ml needed). On cooling 5'-*O*-(dimethoxytrityl)thymidine separated, m.p. 115–118° ([6]: m.p. 116–118°), 21.2 g, 94%.

A solution of (*o*-chlorophenyl)phosphoroditriazolide was prepared using the method of *Broka et al.* [22] 1,2,4-Triazole (3.17 g, 46 mmol) was dissolved in dry pyridine (20 ml) and evaporated to dryness. The residue was suspended in dry dioxane (70 ml) and (*o*-chlorophenyl)phosphodichloridate (3.43 g, 14 mmol) was added. The mixture was stirred vigorously at 0–3° during the dropwise addition of dry triethylamine (4.18 ml, 30 mmol). The mixture was stirred for 1 h at 20°, rapidly filtered and the collected triethylamine hydrochloride washed with 20 ml of dry dioxane. The combined filtrates were cooled to 0–3° with rigorous exclusion of moisture.

5'-*O*-(Dimethoxytrityl)thymidine (5.44 g, 10 mmol) was co-evaporated twice with dry pyridine (20 ml) and dissolved in dry dioxane (70 ml). This solution was concentrated to half volume and added dropwise with rigorous exclusion of moisture to the above ice-cold well-stirred solution of (*o*-chlorophenyl)phosphoroditriazolide. After stirring for 2 h at 20°, 3-hydroxypropionitrile (1.73 ml, 25 mmol) was added, followed by 1-methylimidazole (2.33 ml, 30 mmol). After stirring for 3 h at 20°, solvent was removed at low temperature and the residue was dissolved in $CHCl_3$ (200 ml) and washed with 0.1N NaH_2PO_4 (3× 75 ml) and water. Removal of solvent gave a gum which was chromatographed on silica gel (200 ml). $CHCl_3$ and $CHCl_3/CH_3OH$ 99:1 eluted impurities, whereas $CHCl_3/CH_3OH$ 97:3 eluted the protected nucleotide together with some 3-hydroxypropionitrile. The appropriate combined fractions were dissolved in toluene (200 ml) and washed with water (3× 100 ml). Evaporation of the solvent (finally using the oil pump) gave the pure *DMT-Tp-CE* as a brittle foam (12.9 g, 89%). – ^{1}H-NMR. ($CDCl_3$): 1.35 (*s*, 3 H, CH_3); 3.8 (*s*, 6 H, 2 OCH_3); 5.35 (*m*, 1 H, H–C(1')); 6.5 (*m*, 1 H, H–C(1')).

N-6-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxyadenosine 3'-(o-chlorophenyl β-cyanoethyl phosphate) (d-DMT-bzAp-CE). *N*-6-Benzoyl-2'-deoxyadenosine (5 g, 14.08 mmol) was treated with 4,4'-dimethoxytrityl chloride (5.18 g, 15.3 mmol) as described above. The crude non-crystalline product was phosphorylated as described above without further purification. The product from the reaction was azeotroped with toluene to remove traces of pyridine and chromatographed on silica gel (300 ml). $CHCl_3$ and $CHCl_3/CH_3OH$ 99:1 eluted impurities. $CHCl_3/CH_3OH$ 98:2 eluted the protected nucleotide, which was dissolved in toluene (200 ml) and washed with water (3× 75 ml) to remove traces of 3-hydroxypropionitrile. Removal of solvent (finally at oil pump pressure) gave the *d-DMT-bzAp-CE* as a pale yellow foam (9.3 g, 73% overall). – ^{1}H-NMR. ($CDCl_3$): 3.8 (*s*, 6 H, 2 OCH_3); 5.5 (*m*, 1 H, H–C(3')); 6.55 (*m*, 1 H, H–C(1')).

N-4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxycytidine 3'-(o-chlorophenyl β-cyanoethyl phosphate) (d-DMT-bzCp-CE). *N*-4-Benzoyl-2'-deoxycytidine (5 g, 15.1 mmol) was treated as above for the adenosine derivative. Chromatography of the final product on silica gel gave the protected nucleotide in the $CHCl_3/CH_3OH$ 98:2 fraction. Removal of traces of 3-hydroxypropionitrile by toluene water

partitioning gave pure *d-DMT-Cp-CE* as a brittle foam (9.3 g, 70% overall). – ^{1}H-NMR. ($CDCl_3$): 3.8 (*s*, 6 H, 2 OCH_3); 5.35 (*m*, 1 H, H–C(3′)); 6.35 (*m*, 1 H, H–C(1′)).

N-2-Isobutyryl-5′-O-(dimethoxytrityl)-2′-deoxyguanosine 3′-(o-chlorophenyl β-cyanoethyl phosphate) (d-DMT-ibuGp-CE). *N*-2-Isobutyryl-2′-deoxyguanosine (5 g, 14.84 mmol) was tritylated as above, and the crude product chromatographed on silica gel (300 ml). $CHCl_3$ and $CHCl_3/CH_3OH$ 98:2 eluted impurities. $CHCl_3/CH_3OH$ 95:5 eluted the 5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine (8.0 g, 84%). This pure material was then phosphorylated as described above, and the crude product chromatographed on silica gel (300 ml). $CHCl_3$ and $CHCl_3/CH_3OH$ 98:2 eluted impurities. $CHCl_3/CH_3OH$ 95:5 eluted *d-DMT-ibuGp-CE* (7.4 g, 69%) which was isolated as a brittle white foam (58% overall yield from the base-protected nucleoside). – ^{1}H-NMR. ($CDCl_3$): 1.0 (*m*, 3 H, CH_3); 1.1 (*m*, 3 H, CH_3); 3.8 (*s*, 6 H, 2 OCH_3); 6.25 (*m*, 1 H, H–C(1′)).

3′-O-Benzoylthymidine. 5′-*O*-(Dimethoxytrityl)thymidine (15 g, 27.6 mmol) was dissolved in dry pyridine (60 ml) and benzoyl chloride (4.05 ml, 44 mmol) was added dropwise to the stirred solution at below 10°. After 1 h at 20°, the mixture was poured into ice-water (600 ml) and extracted with $CHCl_3$. The solvent was removed and the residue azeotroped with toluene to remove all traces of pyridine and dissolved in a ice-cold 2% solution of benzenesulfonic acid in $CHCl_3/CH_3OH$ 7:3 *v/v* (1 l). After 10 min at 0°, the solution was washed with 5% $NaHCO_3$-solution (2× 300 ml) and with water. Removal of the solvent and crystallization of the residue from C_2H_5OH gave pure product, m.p. 215–217° ([4]: m.p. 215°). The yield was 8.5 g (89%) in three crops.

Preparation of fully protected oligodeoxyribonucleotides. – The fully protected mono- or oligodeoxyribonucleotide component destined to form the 3′-end of a longer molecule was detritylated as described above using approximately 100 ml of benzenesulfonic acid solution per mmol of oligonucleotide. In the case of d-DMT-TpTpTpT-OBz and all other molecules longer than a tetramer, the 5% $NaHCO_3$-solution and water-washed solutions were backwashed with half their volume of $CHCl_3$/dioxane 2:1, and the combined organic layers were dried using the smallest possible amount of drying agent. After evaporation of the solvent, the residue was dissolved in $CHCl_3$ or $CHCl_3$/dioxan 3:1 (approximately 2 ml per mmol) and precipitated by dropwise addition to a well-stirred mixture of ether/hexane 1:1 (200 ml per mmol). The product was checked for purity by TLC. Yields in all cases were 85–95%; chromatographic characteristics are given in *Table 2*.

The fully protected mono- or oligodeoxyribonucleotide component destined to provide the phosphate group was azeotroped twice with dry pyridine and treated with a 25-fold excess of dry triethylamine for 3 h at 20°. The solvents were evaporated and the residue azeotroped with dry pyridine (finally at the oil pump). The solid 5′-hydroxy component was then added, permitting a 1.3 mol excess of the phosphate component (in the case of the final coupling reactions to form the decamer, a 1.4 mol excess of the phosphate component was used). The mixture was co-evaporated twice with dry pyridine (finally at the oil pump) and dissolved in dry pyridine (10 ml per mmol of phosphate component). Solid TPS-NT (usually 1.15 mol excess over the phosphate component; 1.33 mol excess for preparation of decamers) or TSP-NI (3 mol excess over the phosphate component) was added, and the mixture kept at 20° for 3 h (TPS-NT) or 18 h (TPS-NI). For reactions on less than 1 mmol of material, an additional 10% of coupling reagent was added after 1 h. Prior to workup, reactions involving a phosphorylated G-residue in the coupling were treated with water (5 ml per mmol) to avoid the formation of branched trinucleotide by-products [3]. The mixture was diluted with $CHCl_3$ (100 ml per mmol). In the case of the decamers, the same amount of a 2:1 mixture of $CHCl_3$/dioxane was used. The organic layer was washed once with half its own volume of 5% $NaHCO_3$-solution and once with water, dried over the smallest amount of Na_2SO_4 and the solvents evaporated at low temperature (finally at the oil pump). The residue was dissolved in $CHCl_3$ (2 ml per mmol) and added dropwise to well-stirred hexane (200 ml per mmol). The crude precipitated product was collected and purified by chromatography on silica gel; details and yields are given in *Table 1*.

Deblocking of fully protected decadeoxyribonucleotides. – A sample of a fully protected decadeoxyribonucleotide (175 mg, 0.035 mmol) was dissolved in dry dioxane (2 ml) and treated with a 1M solution of tetramethylguanidinium pyridine-2-aldoximate (25 ml). The clear solution which resulted turned cloudy within 1 min, and was kept at 20° for 4 h. Additional tetramethylguanidine (3 ml) was added, and the mixture kept for 16 h at 20°. Dilution with 60% aq. C_2H_5OH (500 ml) gave

a clear solution which was passed through a column of *DEAE-Sephacel* (2.7×12 cm; bed volume 70 ml). The column was washed with 60% aq. C_2H_5OH (150 ml) and then eluted with 0.05M TEAB in 60% aq. C_2H_5OH until fractions possessed no OD. at 240 nm (maximum for pyridine-2-aldoxime). Elution with 2M TEAB in 60% aq. C_2H_5OH (100 ml) recovered the DNA. After removal of the buffer under vacuum at 30°, the crude sample was treated with a 1:2 mixture of pyridine conc. aq. NH_4OH-solution (50 ml) for 100 h at 20°. Evaporation and co-evaporation with water to remove all of the base was followed by treatment with 80% acetic acid (10 ml) for 30 min at 20°. Evaporation and co-evaporation with water gave a semicrystalline residue which was suspended in water (25 ml) and extracted three times with 10 ml of EtOAc to remove the benzoic acid and tritanol. The aqueous phase was evaporated to dryness, and the total optical density at 260 nm checked (usually this was between 1600–1800 OD. units).

The total crude sample was applied to a column of *DEAE-Sephacel* (2.7×28 cm, bed volume 160 ml) packed in 0.02M aq. TEAB, and eluted with a linear gradient of aq. TEAB from 0.02M to 0.50M (total eluate 1.5 liters). Fractions of 7–8 ml were collected, and the DNA was usually eluted between fractions 80–160, at a buffer concentration of 0.25–0.40M. The decamer comprised the main bulk of the samples and was eluted in a narrow band at a buffer concentration of 0.35–0.38M. Appropriate fractions were pooled and evaporated and co-evaporated with C_2H_5OH to remove the buffer. A representative elution profile for one of the four decamers is given in *Figure 1*.

Samples of the decanucleotides in the triethylamine form (500–800 OD. units) were dissolved in water (1 ml) and passed through a cation exchange column (2.5×15 cm; *Dowex 50W-X8*, 25–50 mesh, Na-form). Elution with water (20–40 ml) gave the decanucleotide in Na-form (virtually complete recovery of the OD.). Alternatively, the decanucleotide in triethylammonium form (500–800 OD. units) was dissolved in 0.3M NaCl (1 ml), diluted with 3 ml of cold C_2H_5OH or acetone and kept at −10° for 12 h. The resulting suspension was centrifuged and the pellet redissolved in 1 ml 0.3M NaCl and reprecipitated. The resulting pellet was dried under high vacuum to give the pure Na-form (60–70% recovery of OD.). Both techniques removed all of the triethylammonium cations, as evidenced by loss of the associated C_2H_5-proton resonances in the ^{1}H-NMR. spectrum.

REFERENCES

[1] *C.B. Reese*, Tetrahedron *34*, 3134 (1978).
[2] *J. Stawinski, T. Hozumi, S.A. Narang, C.P. Bahl & R. Wu*, Nucleic Acids Res. *4*, 353 (1977).
[3] *G.R. Gough, K.J. Collier, H.L. Weith & P.T. Gilham*, Nucleic Acids Res. *7*, 1955 (1979).
[4] *J.F.M. de Rooij, G. Wille-Hazeleger, P.H. van Deursen, J. Serdijn & J.H. van Boom*, Recl. Trav. Chim. Pays-Bas *98*, 537 (1979).
[5] *T. Hirose, R. Crea & K. Itakura*, Tetrahedron Lett. *1979*, 2449.
[6] *K. Itakura, T. Hirose, R. Crea, A.D. Riggs, H.L. Heyneker, F. Bolivar & H.W. Boyer*, Science *198*, 1056 (1977).
[7] *W.L. Sung, H.M. Hsiung, R. Brousseau, J. Michniewicz, R. Wu & S.A. Narang*, Nucleic Acids Res. *7*, 2199 (1979).
[8] *G.R. Gough, C.K. Singleton, H.L. Weith & P.T. Gilham*, Nucleic Acids Res. *6*, 1557 (1979).
[9] *A.F. Markham, M.D. Edge, T.C. Atkinson, A.R. Greene, G.R. Heathcliffe, C.R. Newton & D. Scanlon*, Nucleic Acids Res. *8*, 5193 (1980).
[10] *K.E. Norris, F. Norris & K. Brunfeldt*, Nucleic Acids Res. Symposium Series *7*, 233 (1980).
[11] *K. Myoshi, T. Huang & K. Itakura*, Nucleic Acids Res. *8*, 5491 (1980).
[12] *P.S. Miller, D.M. Cheng, N. Dreon, K. Jayaraman, L.-S. Kan, E.E. Leutzinger, S.M. Pulford & P.O.P. Ts'o*, Biochemistry *19*, 4688 (1980).
[13] *D.J. Patel & C.W. Hilbers*, Biochemistry *14*, 2651 (1975).
[14] *D.R. Phillips & G.C.K. Roberts*, Biochemistry *19*, 4795 (1980).
[15] *R.M. Santella, E. Kriek & D. Grunberger*, Carcinogenesis *1*, 897 (1980).
[16] *J.H. van Boom, P.M.J. Burgers, G. van der Marel, C.H.M. Verdegal & G. Wille*, Nucleic Acids Res. *4*, 1047 (1977).
[17] a) *C.B. Reese, R.C. Titmas & L. Yau*, Tetrahedron Lett. *1978*, 2727; b) *C.B. Reese & L. Zard*, Nucleic Acids Res. *9*, 4611 (1981).

[18] *H. Schaller, G. Weinmann, B. Lerch & H. G. Khorana*, J. Am. Chem. Soc. *85*, 3821 (1963).
[19] We are indebted to *C. Broka* and *R. Ogden* for this suggestion.
[20] *S. A. Narang, R. Brousseau, H. M. Hsiung & J. J. Michniewicz*, Methods in Enzymology *65*, 610 (1980).
[21] *K. Itakura, N. Katagiri, C. P. Bahl, R. H. Wightman & S. A. Narang*, J. Am. Chem. Soc. *97*, 7327 (1975).
[22] *C. Broka, T. Hozumi, R. Arentzen & K. Itakura*, Nucleic Acids Res. *8*, 5461 (1980).
[23] *R. L. Letsinger & K. K. Ogilvie*, J. Am. Chem. Soc. *89*, 4801 (1967).
[24] *R. Arentzen, C. A. A. van Boeckel, G. van der Marel & J. H. van Boom*, Synthesis, *1979*, 123.
[25] *C. B. Reese & L. Yau*, Tetrahedron Lett. *1978*, 4443.
[26] *D. R. Kearns, D. J. Patel & R. G. Shulman*, Nature *229*, 338 (1971).
[27] *D. R. Kearns*, Ann. Rev. Biophys. Bioeng. *6*, 477 (1977).
[28] *G. R. Owens & C. B. Reese*, Synthesis *1974*, 704.
[29] *C.-F. Kroger & R. Mietcher*, Z. Chem. *9*, 378 (1969).
[30] *W. Leupin, W. A. Denny & D. R. Kearns*, in preparation.
[31] *N. T. Thuong, M. Chassignol, G. Lancelot, R. Mayer, B. Hartmann, M. Leng & C. Helene*, Biochimie *83*, 775 (1981).
[32] *J. M. Wright, J. Feigon, W. A. Denny, W. Leupin & D. R. Kearns*, J. Magn. Reson. *45*, 514 (1981).
[33] *G. Bernardi & S. N. Timasheff*, J. Mol. Biol. *48*, 43 (1970).
[34] *M.-Th. Sarocchi & W. Guschlbauer*, Eur. J. Biochem. *34*, 232 (1973).
[35] *R. M. Wing, H. R. Drew, T. Takano, C. Broka, S. Tanaka, K. Itakura & R. E. Dickerson*, Nature *287*, 755 (1980).
[36] *D. J. Patel*, Acc. Chem. Res. *12*, 118 (1979).
[37] *C. B. Reese & A. Ubasawa*, Tetrahedron Lett. *1980*, 2265.
[38] *J. A. J. den Hartog, G. Wille, R. A. Scheublin & J. H. van Boom*, Biochemistry *21*, 1009 (1982).
[39] *C. A. Leach, F. Waldmeier & Ch. Tamm*, Helv. Chim. Acta *64*, 2515 (1981).
[40] *F. M. Pohl & T. M. Jovin*, J. Mol. Biol. *67*, 375 (1972).