58. Nucleosides and Nucleotides. Part 21. Synthesis of a Tridecanucleoside Dodecaphosphate Incorporating the Unnatural Base 2(1*H*)-Pyridone[1])

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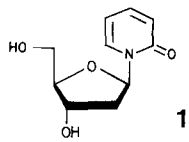
(19.XI.82)

Summary

The modified nucleoside Π_d (**1**) was used in the synthesis of the oligonucleotide d(TpTpΠpCpGpTpCpApApApApTpC). Diester methodology being unsatisfactory, the triester synthesis was investigated with the unnatural nucleoside. An improved method of nucleoside phosphorylation was developed for the synthesis of the fully-protected nucleotide **2e**. This molecule could be cleanly and selectively deprotected, and allowed the efficient synthesis of the desired oligonucleotide.

Introduction. – For our continuing studies on the specificity of DNA polymerase reactions [2] we required the oligonucleotide sequence d(TpTpΠpCpGpTpCpApApApApTpC)[3]), incorporating the unnatural nucleoside 1-(2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (Π_d, **1**) [3]. Although oligonucleotide synthesis in general has enjoyed explosive development during the last few years, relatively little work has been done on extending the techniques to such unnatural bases. In the present case, the synthetic problem is complicated by the relative instability of the glycosidic bond in **1** [4]. Solid-phase synthesis was in any case ruled out,



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[1]) Part 20: [1].

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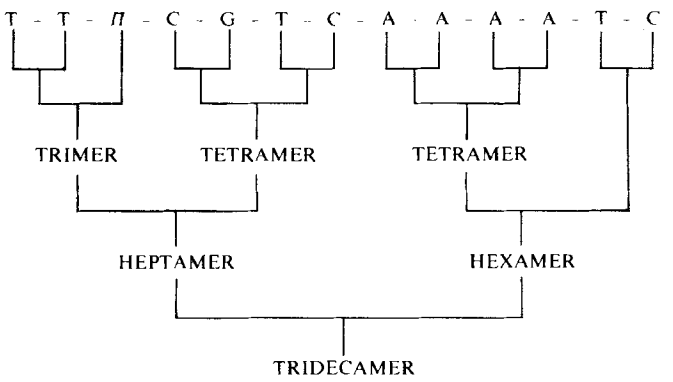
[3]) Abbreviations: d = 2′-deoxy, p = phosphate, C = cytidine, G = guanosine, A = adenosine, T = ribosylthymine, φ = *p*-chlorophenyl phosphate, (CNEt) = cyanoethyl, (MMTr) = 5′-*O*-methoxytrityl, (DMTr) = 5′-*O*-dimethoxytrityl, (bz) = 3′-*O*-benzoyl, bz = N^6-benzoyl, an = N^4-anisoyl, ib = N^2-isobutyryl, TEA = triethylamine, TEAB = triethylammonium hydrogencarbonate, BSA = benzenesulfonic acid, DCC = dicyclohexyl carbodiimide, TPS = 2,4,6-triisopropylbenzenesulfonyl chloride, *p*-NBSNI = *p*-nitrobenzenesulfonyl-4-nitroimidazole, *p*-NBSNT-*p*-nitrobenzenesulfonyl-3-nitro-1,2,4-triazole, TPSNT = 2,4,6-triisopropylbenzenesulfonyl-4-nitro-1,2,4-triazole, MSNT = 2,4,6-trimethylbenzenesulfonyl-4-nitro-1,2,4-triazole, MIZ = *N*-methylimidazole, tet. = 1*H*-tetrazole.

because of the difficulty of monitoring potentially delicate reactions on a solid support.

Diester methodology. – This has been shown [6] [7] to have disadvantages relative to triester or, more recently, phosphite chemistry. However, at the time this work was begun, it was felt that diester synthesis offered the mildest conditions for syntheses with unstable, unnatural nucleosides [8]. Π_d had already been used in the synthesis of a variety of di- and trinucleotides, including d(TpTpΠ) [9], and it was hoped to extend this to the desired sequence.

The synthetic strategy was well-precedented [10] [11]. The chain was built up from dinucleotide blocks, as shown in *Scheme 1;* this permitted multiple use of the d(pApA)-block, while simplifying purification by anion-exchange chromatography. Standard protecting groups were used [10].

Scheme 1



For each condensation the yield was optimized by varying the condensing agent, ratio of nucleotide starting materials, excess of condensing agent, and reaction time. The best results are summarized in *Table 1.*

Products were isolated by ion-exchange chromatography on *Sephadex A-25,* eluting with linear gradients of NH_4HCO_3 or TEAB. After removal of protecting

Table 1. *Condensation conditions and products*

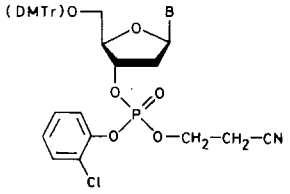
Product	Molar ratio of starting materials[a])	Condensing agent (equiv.)[b])	Reaction time	Yield
$d(pA^{bz}pA^{bz})$	1.2	DCC (3.0)	4.5 days	35%
$d(pA^{bz}pA^{bz}pA^{bz}pA^{bz})$	2.0	TPS (2.1)	4 h	35%
$d(pA^{bz}pA^{bz}pA^{bz}pA^{bz}pTpC^{an})$	5.0	TPS (0.95)	3.5 h	24%
$d(pTpC^{an})$	1.1	DCC (5.0)	5 days	58%
$d(pC^{an}pG^{ib})$	1.2	DCC (7.0)	6 days	55%
$d(pC^{an}pG^{ib}pTpC^{an})$	2.0	TPS (2.0)	6 h	28%

[a]) 3′-OH component used in excess.
[b]) Equiv. of condensing agent per dissociable phosphate.

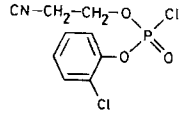
groups, enzymatic degradation and subsequent HPLC. analysis showed correct base ratios for the tetramers and hexamer.

Although it was possible to carry out a substantial part of the synthesis in this way, we had to abandon diester chemistry. Condensations were consistently inefficient, requiring a substantial excess of one nucleotide component (which could rarely be recovered), and giving complex product mixtures with unsatisfactory yields of the desired compound. This was particularly true of adenosine-containing sequences, which readily underwent depurination, but in all cases sulfonation, self-condensation of nucleotides and formation of symmetric pyrophosphates all contributed to the problem. Workup and purification of the crude products were difficult and time-consuming, resulted in further substantial losses (particularly through loss of protecting groups during *Sephadex* chromatography), and rarely allowed the isolation of pure products. The overall yield of d(ApApApApTpC) was only 1%. Thus it became clear that, despite years of optimization and the appearance of encouraging results in the literature (*e.g.* [10–13]), diester methodology contains too many insoluble problems to be practical, even in special cases such as that presented here. Such a view is, of course, reinforced by the contrast with alternative strategies, which have recently undergone enormous improvements in the relatively short period since we began these studies.

Synthesis of triester mononucleotides. – The triester synthesis was based on standard fully-protected mononucleotides of the type **2**. The natural nucleotides **2a–d** can be prepared by methods analogous to those previously published [14], but problems were experienced in the case of Π_d. Phosphorylation of the corresponding nucleoside (DMTr)Π_d with **3** under standard conditions (methylimidazole, dioxane, r.t.) resulted in significant decomposition, probably due to attack of **3** on the pyridone ring, with subsequent cleavage of the glycosidic bond. In addition, only a low recovery of **2e** was obtained; normally, the nucleotide is precipitated in ether/petroleum ether 2:3 in order to remove the methylimidazole, but **2e** was abnormally soluble in this solvent. Use of pure petroleum ether failed, as this is immiscible with methylimidazole.



2a B = A^{bz}
2b B = C^{an}
2c B = G^{ib}
2d B = T
2e B = Π



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4a R = butyl
4b R = hexyl
4c R = octyl
4d R = hexadecyl

A range of more lipophilic alkylimidazoles **4a–d** were tested as catalysts in the phosphorylation of (DMTr)A_d^{bz}. The hexadecyl derivative **4d**, a crystalline solid, was not adequately soluble in the reaction mixture, and gave only low yields of nucleotide. In contrast, **4a**, **4b** and **4c** worked very well, and were readily removed at the end of the reaction by precipitation of the nucleotide in pure petroleum ether. The butyl derivative **4a** is commercially available, and appears to be the reagent of choice for reactions of this type.

Attempts were then made to optimize the reaction with Π_d to minimize decomposition of the product. Working in pyridine at 0° was an improvement, but it was not possible to achieve complete phosphorylation without significant decomposition. The best yields were obtained by using two equivalents of **3** with short reaction times; a small amount of starting material remained, but was easily removed from the product by silica gel chromatography. Compound **2e** was isolated as a pale honey in 54% yield. It failed to produce the usual foam when evaporated from $CHCl_3$, and was found unstable at room temperature.

Preliminary investigations with Π_d nucleotides in triester synthesis. – Before attempting to build the unnatural nucleotide into a long chain, it was necessary to test its stability under the various conditions of triester synthesis.

Decyanoethylation of **2e** (TEA, pyridine, r.t., 6 h) proceeded cleanly to give a single product with the expected TLC. characteristics. Detritylation of **2e** was carried out with BSA (2% in $CHCl_3$/MeOH 7:3), giving a 71% isolated yield of the desired product. Comparable results were obtained using $ZnBr_2$ [15] [16].

With the partially-deprotected monomers in hand, condensations were then carried out to synthesize (MMTr)$T_d\varphi T_d\varphi\Pi_d\varphi$ (CNEt) and (DMTr)$\Pi_d\varphi C_d^{an}\varphi$ (CNEt), using a TPS/tetrazole mixture as condensing agent [17]. The reactions, monitored by TLC., proceeded smoothly. The products were deprotected by treatment with 2-nitrobenzaldoximate [18] (6 h, r.t.), followed by aqueous ammonia (15 h, 50°) and 50% acetic acid (1 h, r.t.). Analysis by anion-exchange HPLC. and UV., and comparison with authentic material [9] [19], confirmed that the correct products had been obtained.

Thus modern methodology is mild enough to allow the 2-pyridone nucleotide to survive largely intact during oligonucleotide synthesis, and during deblocking of the resulting chain.

Synthesis of the tridecanucleoside dodecaphosphate. – The synthetic strategy is designed, for economic reasons, to combine building blocks of same or similar size. However, we tried to minimize the number of synthetic steps involving Π_d. The buildup of the tridecamer *via* dinucleotide blocks is outlined in *Scheme 2*. Standard protecting groups were used [7]: benzoyl, anisoyl and isobutyryl on the bases, monomethoxytrityl on the terminal 5′-OH, dimethoxytrityl on other 5′-OH groups, benzoyl on the terminal 3′-OH, and cyanoethyl and 2-chlorophenyl [18] on the phosphates. Detritylation of mono- and dinucleotides was carried out with BSA, while for larger chains $ZnBr_2$ was used [16]. Decyanoethylations were performed with TEA in pyridine; the resulting triethylammonium salts were evaporated twice from pyridine, then used immediately in the coupling reaction. During the course of the synthesis a variety of condensing agents were applied, but in the later stages TPSNT [20] [21] was used exclusively[4]). The results of the various reactions are

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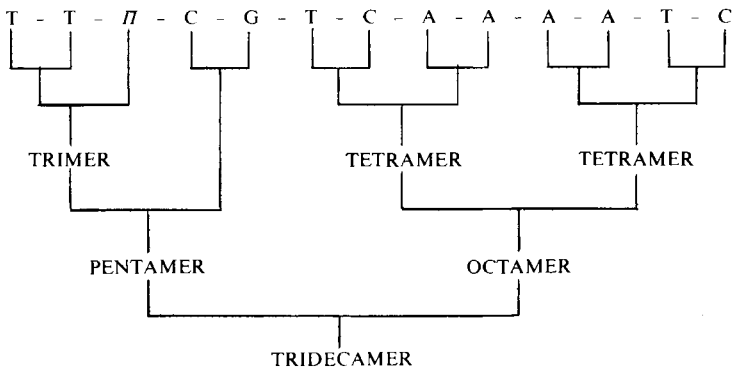
[4]) In a separate experiment, we carried out the condensation to give (DMTr)$C_d^{an}\varphi G_d^{ib}\varphi$ (CNEt) using a range of different condensing agents, and analyzed the crude products by HPLC. TPSNT and TPS/tetrazole [17] both gave very clean products, the former somewhat more rapidly. *p*-NBSNI [5] gave a small amount of by-product, and was rather slow; *p*-NBSNT (kindly supplied by *Fluka AG*, Buchs) was extremely fast, but gave *ca.* 20% sulfonated by-product.

summarized in *Table 2*. In the case of dinucleotides, the purity of the products were checked by HPLC. and shown to be at least 95% in all cases.

Scheme 2



All coupling reactions were monitored by TLC. However, in some cases the product and unreacted 5′-OH starting material were not resolved: (MMTr)$T_d\varphi T_d\varphi \Pi_d\varphi$ (CNEt) and $\Pi_d\varphi$ (CNEt) eluted at the same Rf, as did (MMTr)$T_d\varphi T_d\varphi \Pi_d\varphi C_d^{an}\varphi G_d^{ib}\varphi$ (CNEt)/ $C_d^{an}\varphi G_d^{ib}\varphi$ (CNEt) and tridecamer/$T_d\varphi C_d^{an}\varphi A_d^{bz}\varphi A_d^{bz}\varphi A_d^{bz}\varphi A_d^{bz}\varphi T_d\varphi C_d^{an}$(bz). In these cases it was difficult to assess when the reaction was complete. Some indication could be obtained by observing the disappearance of diester component, and the coloration of the product/5′-OH component spot after spraying the TLC. plate with $HClO_4$. More reliably, the absence of starting material could be proved by detritylating small aliquots of the reaction mixture, as the detritylated product was well-resolved from starting material on TLC. Nevertheless, incomplete reaction was a common problem in coupling large nucleotide blocks, as demonstrated by anion-exchange HPLC. analysis of deprotected samples. In an attempt to compensate for this, we used the diester component in larger excess as the chains grew longer *(cf. Table 2)*.

At the end of each reaction, excess condensing agent was hydrolyzed with aqueous pyridine, the solution evaporated, and (except in the case of tridecamer) the product partitioned between CH_2Cl_2 and aqueous $NaHCO_3$. After evaporation of the organic solvent, the crude product was in most cases purified by flash chromatography [22] or short-column chromatography on silica gel. However, it was found that this gave poor recovery of longer chains (hexamer/octamer), especially after detritylation. In such cases the purification was most conveniently carried out by preparative layer chromatography (PLC.); surprisingly, this gave much better recovery of nucleotide material.

Deblocking, purification and analysis. – After initial purification by PLC., the tridecanucleotide was fully deprotected by the methods used earlier for d(TpTpΠ) and d(ΠpC). HPLC. analysis of the crude product on a *Partisil-10 SAX* column showed mainly the desired product *(Fig. 1)*, plus a substantial peak for d(TpCpApApApApTpC) owing to incomplete reaction in the final step. The

Table 2. *Synthesis of the*

Product	Diester component (equiv.)
(MMTr)$T_{d}\varphi T_{d}$(CNEt)	(MMTr)$T_{d}\varphi^{\ominus}$ (1.2)
(DMTr)$C_{d}^{an}\varphi G_{d}^{ib}\varphi$(CNEt)	(DMTr)$C_{d}^{an}\varphi^{\ominus}$ (1.3)
(DMTr)$T_{d}\varphi C_{d}^{an}\varphi$(CNEt)	(DMTr)$T_{d}\varphi^{\ominus}$ (1.3)
(DMTr)$A_{d}^{bz}\varphi A_{d}^{bz}\varphi$(CNEt)	(DMTr)$A_{d}^{bz}\varphi^{\ominus}$ (1.2)
(DMTr)$A_{d}^{bz}\varphi A_{d}^{bz}\varphi$(CNEt)	(DMTr)$A_{d}^{bz}\varphi^{\ominus}$ (1.25)
(DMTr)$T_{d}\varphi C_{d}^{an}$(bz)	(DMTr)$T_{d}\varphi^{\ominus}$ (1.3)
(MMTr)$T_{d}\varphi T_{d}\varphi\Pi_{d}\varphi$(CNEt)	(MMTr)$T_{d}\varphi T_{d}\varphi^{\ominus}$ (1.3)
(MMTr)$T_{d}\varphi T_{d}\varphi\Pi_{d}\varphi$(CNEt)	(MMTr)$T_{d}\varphi T_{d}\varphi^{\ominus}$ (1.4)
(MMTr)$T_{d}\varphi T_{d}\varphi\Pi_{d}\varphi C_{d}^{an}\varphi G_{d}^{ib}\varphi$(CNEt)	(MMTr)$T_{d}\varphi T_{d}\varphi\Pi_{d}\varphi^{\ominus}$ (1.4)
(DMTr)$T_{d}\varphi C_{d}^{an}\varphi A_{d}^{bz}\varphi A_{d}^{bz}\varphi$(CNEt)	(DMTr)$T_{d}\varphi C_{d}^{an}\varphi^{\ominus}$ (1.3)
(DMTr)$A_{d}^{bz}\varphi A_{d}^{bz}\varphi T_{d}\varphi C_{d}^{an}\varphi$(bz)	(DMTr)$A_{d}^{bz}\varphi A_{d}^{bz}\varphi^{\ominus}$ (1.4)
(DMTr)$T_{d}\varphi C_{d}^{an}\varphi A_{d}^{bz}\varphi A_{d}^{bz}\varphi A_{d}^{bz}\varphi T_{d}\varphi C_{d}^{an}$(bz)	(DMTr)$T_{d}\varphi C_{d}^{an}\varphi A_{d}^{bz}\varphi A_{d}^{bz}\varphi^{\ominus}$ (1.4)
(MMTr)$T_{d}\varphi T_{d}\varphi\Pi_{d}\varphi C_{d}^{an}\varphi G_{d}^{ib}\varphi T_{d}\varphi C_{d}^{an}\varphi A_{d}^{bz}\varphi A_{d}^{bz}\varphi A_{d}^{bz}\varphi A_{d}^{bz}\varphi T_{d}\varphi C_{d}^{an}\varphi$(bz)	(MMTr)$T_{d}\varphi T_{d}\varphi\Pi_{d}\varphi C_{d}^{an}\varphi G_{d}^{ib}\varphi^{\ominus}$ (1.5)

[a]) Yield after detritylation. [b]) When attempting this procedure [23] we experienced considerable difficulties with suitable for solution chemistry. [c]) See the *Exper. Part.* [d]) Yield from PLC., including octanucleotide impurity.

product was isolated by anion-exchange chromatography on *Sephadex A-25;* evaporation of the TEAB-buffer gave *ca.* 300 OD_{254}-units of nucleotide, mixed with some water-soluble *Sephadex* residues. A small fraction of this nucleotide was purified by reverse-phase HPLC. on *μ-Bondapack C_{18} (Fig. 2),* giving apparently homogeneous product. This was completely digested by snake venom phosphodiesterase, and HPLC. analysis of the resulting mononucleotides showed the correct base ratios.

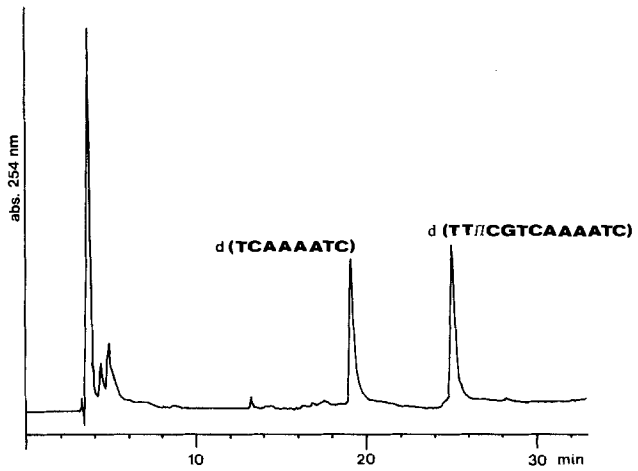


Fig. 1. *HPLC. analysis of crude tridecamer* (*Partisil SAX,* system A)

tridecanucleoside dodecaphosphate

5'-OH component (1 equiv.)	Condensing agent (equiv.)	Reaction time (min)	Yield (%)
$T_d\varphi$(CNEt)	TPS (3)/tet. (9)	90	83
$G^{ib}_d\varphi$(CNEt)	MSNT (3)	45	62[a])
$C^{an}_d\varphi$ (CNEt)	TPS (3)/tet. (9)	50	70
$A^{bz}_d\varphi$ (CNEt)	TPS (3)/tet. (9)	30	52
$A^{bz}_d\varphi$ (CNEt)	TPSNT (3)	45	76
C^{an}_d(bz)	TPS (3)/MIZ (9)[b])	45	86
$\Pi_d\varphi$ (CNEt)	TPS (3)/tet.(9)	90	59
$\Pi_d\varphi$ (CNEt)	TPSNT (4.2)	90	61
$C^{an}_d\varphi G^{ib}_d\varphi$ (CNEt)	TPSNT (3)	75	72
$A^{bz}_d\varphi A^{bz}_d\varphi$ (CNEt)	TPSNT (3)	60	64
$T_d\varphi C^{an}_d$(bz)	TPSNT (3)	60	50
$A^{bz}_d\varphi A^{bz}_d\varphi T_d\varphi C^{an}_d$(bz)	TPSNT (3)	120	63
$T_d\varphi C^{an}_d\varphi A^{bz}_d\varphi A^{bz}_d\varphi A^{bz}_d\varphi A^{bz}_d\varphi T_d\varphi C^{an}_d$(bz)	TPSNT (15)[c])	340	71[d])

the workup (emulsions, contamination of the product with MIZ), and felt that this reagent was not very

Final remarks. – With a few modifications, established triester methodology was adequate for the synthesis of an oligonucleotide which contains an unnatural nucleoside much less stable than those normally used. The desired sequence was obtained within a reasonable time, in substantial quantities, and in a high state of purity. Work is now in progress to extend these results to syntheses using other unnatural nucleosides.

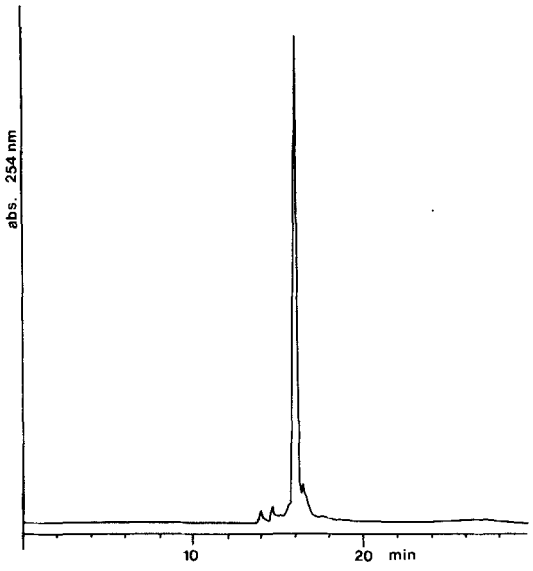


Fig. 2. *HPLC. purification of tridecamer* (μ-*Bondapack* C_{18}, system D)

Significant improvements were achieved in the phosphorylation of nucleosides to produce fully-protected mononucleotide starting material. This was of particular importance for the unnatural base, but the results can usefully be applied to all other nucleosides.

One of us *(C. L.)* gratefully acknowledges the award of a *Royal Society European Science Exchange Fellowship.* We thank the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung* for financial support.

Experimental Part

General. See [14], except for the following changes and additions: triisopropylbenzenesulfonyl chloride, tetrazole, *N*-butylimidazole and acetonitrile *(puriss. p.a.)* were purchased from *Fluka AG*, Buchs, 2-chlorophenol, formamide (zur Analyse) and phosphodiesterase *I* from *E. Merck*, Darmstadt, potassium dihydrogen phosphate *(A.R.)* from *Riedel de Haën AG*, Hannover, and ammonium acetate (AnalaR) from *BDH Chemicals Ltd.*, Poole, England. TPSNT [21] and 2-nitrobenzaldoxime [18] were prepared by published procedures. HPLC. was carried out using a *Pye Unicam LC-XPD* pump and *LC-XP* gradient programmer, an *ISCO UA-5* absorbance monitor and *Hewlett Packard 3380A* integrator. Columns used were 7 µm *LiChrosorb Si60* (250×4.6 mm) from *Knauer, Partisil PXS 10/25 SAX* (4.6×250 mm) from *Whatman*, and *µ-Bondapack* C_{18} (3.9×300 mm) from *Waters*. The following buffers were used in HPLC. separations: system A, 30 min linear gradient from 10^{-2}M KH_2PO_4, pH 6.6, 10% $HCONH_2$ to $4 \cdot 10^{-1}$M KH_2PO_4, pH 6.6, 10% $HCONH_2$; system B, 30 min linear gradient from $3 \cdot 10^{-2}$M KH_2PO_4, pH 6.6, 20% EtOH to $4 \cdot 10^{-1}$M KH_2PO_4, pH 6.6, 20% EtOH; system C, isocratic elution with $5 \cdot 10^{-2}$M KH_2PO_4, pH 3.3; system D, 15 min linear gradient from acetonitrile/0.1M NH_4OAc, pH 7.0 5:95 to acetonitrile/0.1M NH_4OAc, pH 7.0 20:80. Buffers were filtered through *Whatman GF/F* glass microfibre before use, and the pH adjusted within ±0.02. All glassware containing aqueous solutions of nucleotide was treated with dichlorodimethylsilane before use. 1-(2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (**1**) was prepared by the method of *Séquin & Tamm* [3]. Fully-protected mononucleotides with natural bases were prepared as previously described [14].

o-*Chlorophenyl(2-cyanoethyl)phosphochloridate* (**3**). The synthesis was carried out as for the *p*-chloro-derivative [14], and similar results were obtained; $d = 1.39$ (crude product).

(DMTr)Π$_{dφ}$(CNEt) (**2e**). (DMTr)Π$_d$ (1 equiv.) was twice evaporated from dry pyridine at 20°/1 Torr, and the rotary evaporator filled with Ar after each evaporation. The residue was dissoved in dry pyridine (6 ml/mmol), the solution cooled in ice, and phosphochloridate **3** (2 equiv., 0.45 ml/mmol nucleoside) and butylimidazole (2 equiv., 0.26 ml/mmol nucleoside) added. The mixture was stirred under Ar at 0° for 5 min, then hydrolyzed with 50% aq. pyridine (4 ml/mmol) for 10 min. The solution was evaporated, 4% aq. $NaHCO_3$ added, and the product extracted 4 times with CH_2Cl_2. The combined org. layers were dried and evaporated, the residue redissolved in EtOAc (50 ml/mmol), and added dropwise (*ca.* 2–3 ml/min) to vigorously-stirred petroleum ether. The resulting white precipitate was filtered off, dissolved in CH_2Cl_2, and the solution evaporated. TLC. showed a major product spot, a minor starting material spot, and decomposition products of low Rf. The crude product was purified on a silica gel column using a stepwise gradient of MeOH in CH_2Cl_2 (for 2 mmol: 150 g silica gel, 4 cm diameter, flow *ca.* 25 ml/min). The desired product eluted with 1.5–2% MeOH, and was isolated as a pale honey in 54% yield.

Di-, tetra- and octanucleotides. Details of the individual reactions are given in *Table 2*. General procedures were as used previously [5] [16], except that column chromatography was accelerated by applying N_2-pressure. All compounds were eluted with a stepwise gradient of MeOH in CH_2Cl_2. *Table 3* shows the MeOH-content required in each case to elute the desired product.

Particular care was needed in the detritylation of adenosine-rich sequences, especially AA and AATC, which were prone to decomposition and gave below-average yields of detritylated product. Detritylation of the octanucleotide was relatively clean, but most of the desired compound remained

Table 3. *Required MeOH-content*

Sequence	Fully-protected	Detritylated
TT	3–3.5%	–
CG	4%	4–5%
TC	3–4%	–
AA	3–4%	4%
TC(bz)	2–2.5%	4%
TCAA	4%	–
AATC(bz)	4–5%	10%
TCAAAATC(bz)	6–8%	Failed

on the column (elution with CH_2Cl_2/MeOH 3:1). The crude product was instead purified by PLC., eluting with CH_2Cl_2/MeOH 88:12, and was isolated in 63% overall yield. However, this product was contaminated with several percent of tritylated material (octamer starting material).

All sequences were analyzed by HPLC.: dinucleotides in the fully-protected form on silica gel *(LiChrosorb)*, eluting with water-saturated MeOH/CH_2Cl_2 mixtures, and longer chains on *Partisil-10 SAX* after complete deprotection. In the latter case various buffer systems were applied. Some early work was done using EtOH-containing phosphate buffer [24], but more recently we have used formamide-containing buffers with excellent results (*Figures 3–5*, system A).

(MMTr)$T_{d\varphi}T_{d\varphi}\Pi_{d\varphi}$*(CNEt)*. Reaction conditions: *Table 2*. The product and $\Pi_{d\varphi}$(CNEt) had the same Rf on TLC., and could not be resolved by HPLC. After hydrolysis and extraction the product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 97:3) and isolated as a white foam. A small sample of the trinucleotide was deprotected (2-nitrobenzaldoximate/acetic acid) and analyzed by ion-exchange HPLC. *(Fig. 6)*. The major peak was collected. – UV.: max. 268, 302 nm; $\varepsilon_{268}/\varepsilon_{302} = 3.39$ (*cf.* d(TpTpΠ), $\varepsilon_{268}/\varepsilon_{302} = 3.38$ [9]).

(MMTr)$T_{d\varphi}T_{d\varphi}\Pi_{d\varphi}C^{an}_{d}\varphi G^{ib}_{d}\varphi$*(CNEt)*. Reaction conditions: *Table 2*. Starting material and product had the same Rf. Detritylation of a sample of the product after workup showed the absence of starting material. After complete deprotection (2-nitrobenzaldoximate/NH_3/AcOH) a sample of the product gave the HPLC. trace of *Figure 7*.

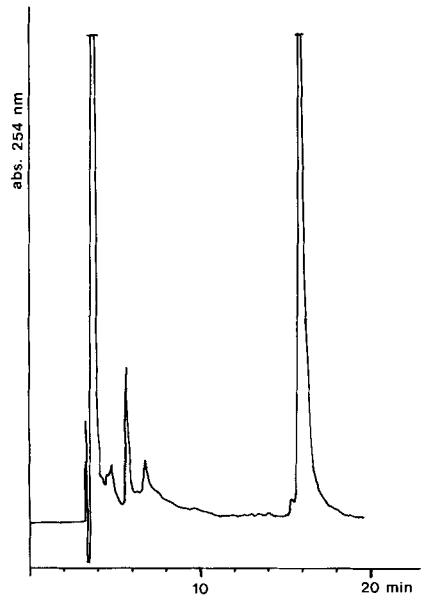


Fig. 3. *Crude d(TpCpApAp(C₆H₄Cl))* (*Partisil SAX*, system A)

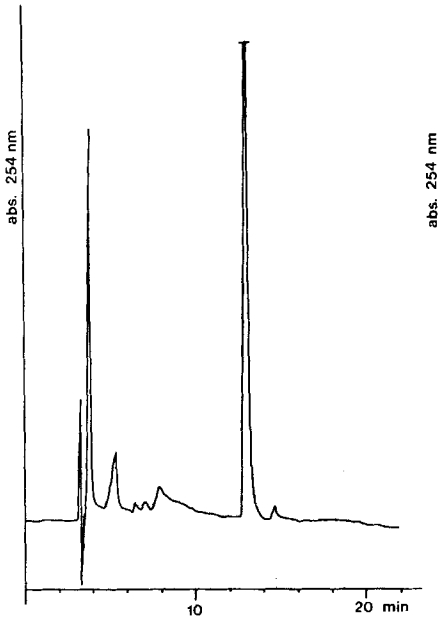


Fig. 4. *Crude d(ApApTpC)* (*Partisil SAX*, system A)

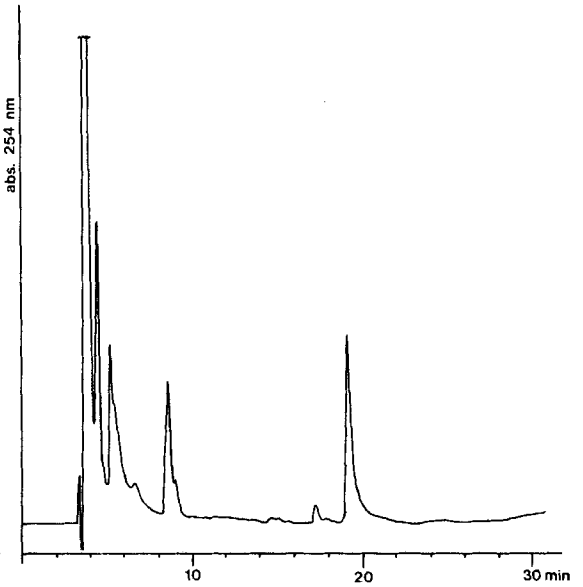


Fig. 5. *Crude d(TpCpApApApApTpC)* (*Partisil SAX*, system A)

(MMTr)$T_d\varphi T_d\varphi\Pi_d\varphi C_d^{an}\varphi G_d^{ib}\varphi T_d\varphi C_d^{an}\varphi A_d^{bz}\varphi A_d^{bz}\varphi A_d^{bz}\varphi A_d^{bz}\varphi T_d\varphi C_d^{an}$(bz). (MMTr)$T_d\varphi T_d\varphi\Pi_d\varphi C_d^{an}\varphi G_d^{ib}\varphi$-(CNEt) (29.7 mg, 11.4 μmol, 1.5 equiv.) was decyanoethylated in the usual way, then 30.6 mg (7.8 μmol, 1 equiv.) $T_d\varphi C_d^{an}\varphi A_d^{bz}\varphi A_d^{bz}\varphi A_d^{bz}\varphi A_d^{bz}\varphi T_d\varphi C_d^{an}$(bz), 0.5 ml pyridine and 14.8 mg (39 μmol, 5 equiv.) TPSNT were added. After 1.5 h no detectable reaction had occurred. A further 5 equiv. TPSNT was added to the reaction mixture[5]). After 1 h more, the reaction was proceeding slowly, and a third batch of 5 equiv. TPSNT was added. After a further 2 h, the TTΠCG component had completely disappeared. Excess condensing agent was hydrolyzed with 0.5 ml 50% aq. pyridine, then the solution evaporated (0.5 Torr). The crude product was dried by evaporation from pyridine, residual pyridine removed by repeated evaporation from $CHCl_3$, then the sample purified by PLC., eluting with CH_2Cl_2/MeOH 85:15. The nucleotide band was scraped off and the product removed with CH_2Cl_2/MeOH 75:25. Crude yield 35.5 mg (nominally 71%).

Deprotection, purification and analysis of the tridecamer. The product of the previous reaction was dissolved in 1 ml of a 0.5 M solution of 2-nitrobenzaldoximate in 50% aq. dioxane [18] and stirred for 1 h at r.t., 5 ml conc. aq. ammonia were then added, the reaction vessel sealed, and the solution stirred for 17 h at 50°. The solvent was evaporated, 1.5 ml 50% aq. acetic acid added, and stirred 1 h at r.t. The solution was diluted with 10 ml water, washed with 3×25 ml $CHCl_3$, then lyophilized. The crude deprotected sample was analyzed by HPLC. on *Partisil-10 SAX*, giving the results in

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[5]) The sample of TPSNT used was later shown to be substantially decomposed (after 3 months at r.t.); hence the actual concentration of condensing agent was lower than the calculated values. Figures shown here are nominal amounts, assuming pure condensing agent. It was at first supposed that moisture in the pyridine was causing difficulties, as this condensation was carried out at a lower concentration than all others. Analysis of freshly-dried pyridine showed a water content of only 0.006%, equiv. to 3.4 μmol/ml, or 0.2 equiv. in the above reaction.

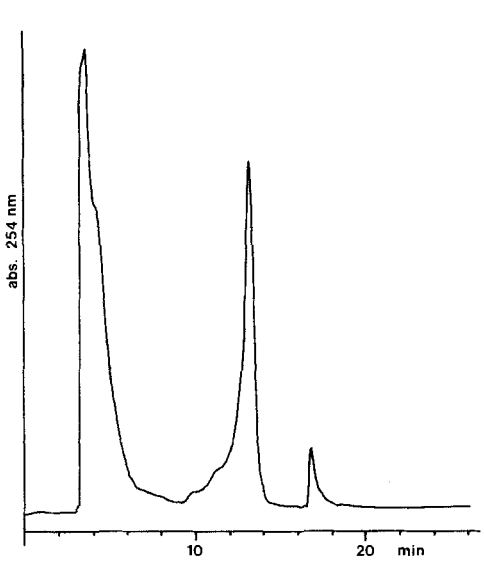

Fig. 6. *Crude d(TpTpΠp(C_6H_4Cl))* (*Partisil SAX*, system B)

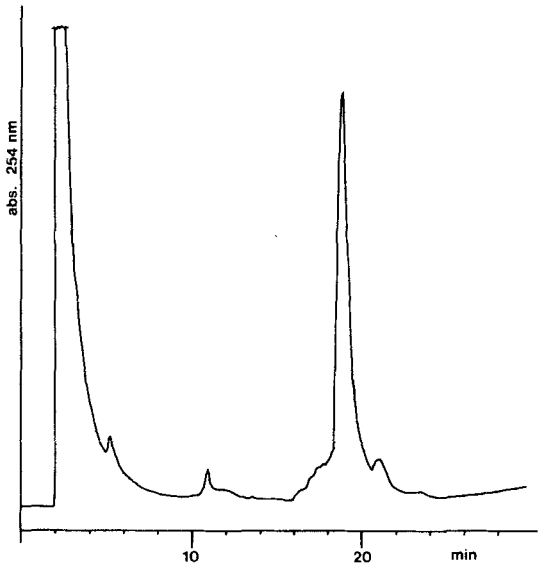

Fig. 7. *Crude d(TpTpΠpCpGp(C_6H_4Cl))* (*Partisil SAX*, system A)

Figure 1. As expected, there was significant contamination by d(TpCpApApApApTpC). This arose partly as a result of incomplete coupling in the last step, and partly from the tritylated impurity in the starting material for this step.

Initial purification of the product was carried out on a column of *DEAE Sephadex A-25* (45 × 2 cm) eluted with a linear gradient of triethylammonium bicarbonate (0.3 to 1.2 M, 2.5 l total volume). In contrast to the HPLC. result, this column separated the crude product into three major peaks *(Fig. 8)*. Analysis of these by HPLC. confirmed that peak I was octanucleotide, while peaks II and III were not separated on *Partisil-10 SAX*, co-eluting as the 'product' peak. The total weight of the three isolated components was well above the expected value; the extra mass was presumed to be due to contamination by UV.-inactive *Sephadex* residues.

The *Sephadex* fractions were further analyzed by reverse-phase HPLC. on *μ-Bondapack C_{18}* (system D). Peak I was a very pure sample of the octanucleotide *(Fig. 9)*, confirming the efficiency of *Sephadex* columns in separating such mixtures. Peak II was purified by preparative HPLC. with the RP-column to obtain high-purity samples for further analysis; this was later shown to be the desired product, and was also the major component in the original mixture. Peak III was not eluted from the HPLC. column by buffer system D (max. 20% MeCN); it was eluted with 26% MeCN, compared with 12% for peak II. Spotting the solution from peak III onto a TLC. plate and spraying with $HClO_4$ confirmed the presence of a monomethoxytrityl group. Further treatment with 50% AcOH-solution gave a product identical to peak II. Thus it appears that the detritylation step of the deprotection was incomplete; tritylated and detritylated nucleotides are not resolved on *Partisil-10 SAX* (relatively pure ion-exchange separation), but are well separated by *Sephadex* (hydrophobic interactions with the gel). The total yield in peaks II and III was 304 OD_{254} units, purity *ca.* 85% by RP-HPLC.

The pure tridecanucleotide isolated from reverse-phase HPLC. was digested with phosphodiesterase I, and the absence of residual oligonucleotides confirmed by HPLC. (system A). The resulting mixture of monomers was analysed by HPLC. (system C), giving the expected base ratios:

T_d		pC_d		pA_d		pG_d		$pΠ_d$
1.23	:	3.11	:	3.82	:	0.99	:	1.00

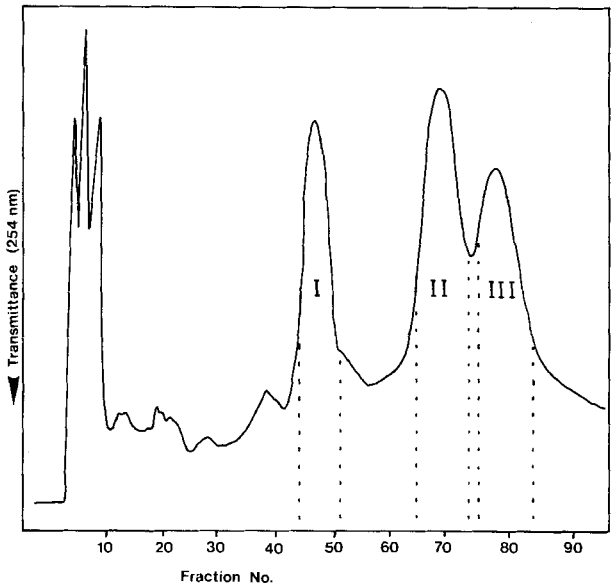

Fig. 8. *Purification of crude tridecanucleotide on* DEAE-Sephadex (Peak I contains d(TpCpApApApA-pTpC), peak II detritylated d(TpTp*Π*pCpGpTpCpApApApApTpC), and peak III tritylated tridecamer)

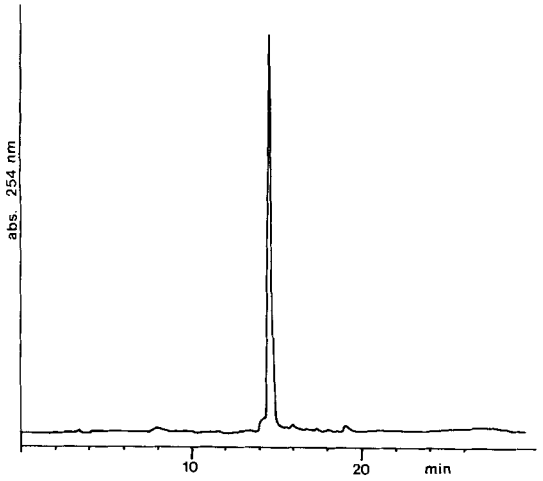

Fig. 9. *d(TpCpApApApApTpC) from* DEAE-Sephadex *chromatography* (peak I) (*μ-Bondapack* C_{18}, system D)

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