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# Neighbouring group participation in the unblocking of phosphotriesters of nucleic acids[1]

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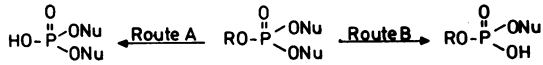
## ABSTRACT

Two examples of neighbouring group participation during the removal of protecting groups from phosphotriesters of partially or fully protected intermediates of nucleic acids are presented. The first example shows that ammonolysis of aryl groups from phosphotriesters of partially protected - 5'-hydroxy free - nucleic acids (e.g., 4b; Ar=2ClC$_6$H$_4$) gives rise to the formation of unnatural nucleic acids (e.g., 7 and 8). The second one illustrates that fluoride ion promoted hydrolysis of 2,2,2-trichloroethyl groups from phosphotriesters of fully protected nucleic acids (e.g., 18a), having t-butyldimethylsilyl groups at the 2'-positions, leads to the formation of a considerable amount of side-products (e.g., 20 and 21).

## INTRODUCTION

The final removal of all protecting groups from fully or partially-protected oligonucleotides to obtain RNA or DNA fragments, which contain solely 3'-5'-internucleotide phosphotriester linkages, is an essential part of a synthesis of nucleic acids *via* phosphotriester intermediates.

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Nu = Partially or fully protected nucleosides.

R = Aryl or alkyl. Scheme 1

Especially, the problems which may arise during the removal of protecting groups from phosphotriesters (*e.g.*, group R from 1), by a mechanism which entails a nucleophilic attack at phosphorus, are not fully appreciated. In the removal of protecting groups at phosphorus we may encounter, depending on the nature and the total number of blocking groups used for the protection of the nucleoside hydroxyl functions, two side reactions: internucleotide cleavage and neighbouring group participation (NGP). The first side-reaction leading to internucleotide cleavage (*e.g.*, route B instead of

route A in Scheme 1) has been well studied[2,3,4]. However, less attention has been paid to neighbouring group participation (NGP). The only studies so far reported[5] on this side-reaction clearly showed that hydrolysis of aryl-phosphotriesters (*e.g.*, 1; R=Aryl), containing partially (5'- or 3'- OH free) and properly protected nucleosides, by aqueous sodium hydroxide resulted in the formation of unwanted products.

In this paper we wish to report that NGP is not suppressed by hydrolysing aryl-phosphotriesters (*e.g.*, 4b; R=Aryl) with aqueous ammonia instead of sodium hydroxide and, furthermore, that NGP becomes the main process when alkyl-phosphotriesters (*e.g.*, 4b; R=Alkyl) are hydrolyzed under conditions which also remove protecting groups from nucleoside 2'-hydroxyl groups.

## RESULTS AND DISCUSSION

### Ammonia-promoted hydrolysis of aryl-phosphotriesters of oligonucleotides.

We previously reported[5] that the first step in the deblocking of partially-protected dinucleoside monophosphate triesters (*e.g.*, 4b; Ar=$2ClC_6H_4$), having a terminal 5'-hydroxy function, by a simple two-step process involving hydroxide ion-promoted hydrolysis (to remove the protecting groups from the internucleotide phosphotriester) followed by acid hydrolysis (to remove the methoxymethylene and methoxytetrahydropyranyl protecting groups) proceeded virtually exclusively *via* NGP. The latter was substantiated by the fast and exclusive formation of the intermediate 3',5'-cyclic dinucleoside phosphate (6), which was further hydrolyzed to give a mixture of oligomers containing not only 3'-5'-linkages, but also unnatural 5'-5'-internucleotide bonds (*e.g.*, 7).

However, the statement[6,7] of Narang *et al*, that ammonolysis of aryl-phosphotriesters (4b;Ar=$4ClC_6H_4$) of oligonucleotides with a terminal 5'-hydroxy function proceeded with a minimum formation of side-products, urged us to reinvestigate this result.

Thus treatment of the less polar diastereoisomer ($d_1$) of dimer (4b;Ar=$2ClC_6H_4$, *vide* Experimental) with aqueous ammonia for 16 h at 50°C, followed by acid treatment (pH=2;0.01 N NCl) and work-up of the reaction mixture gave a fluffy solid. Purification of the latter by anion-exchange (DEAE-Sephadex) chromatography revealed the presence of mainly two peaks (i and ii in Fig. 1). Analysis of the material in peak (i) by HPLC in combination with enzymatic digestion (RNase) indicated that it consisted of uridilyl-(3'-5')-uridine (5) and uridilyl-(5'-5')-uridine (7).

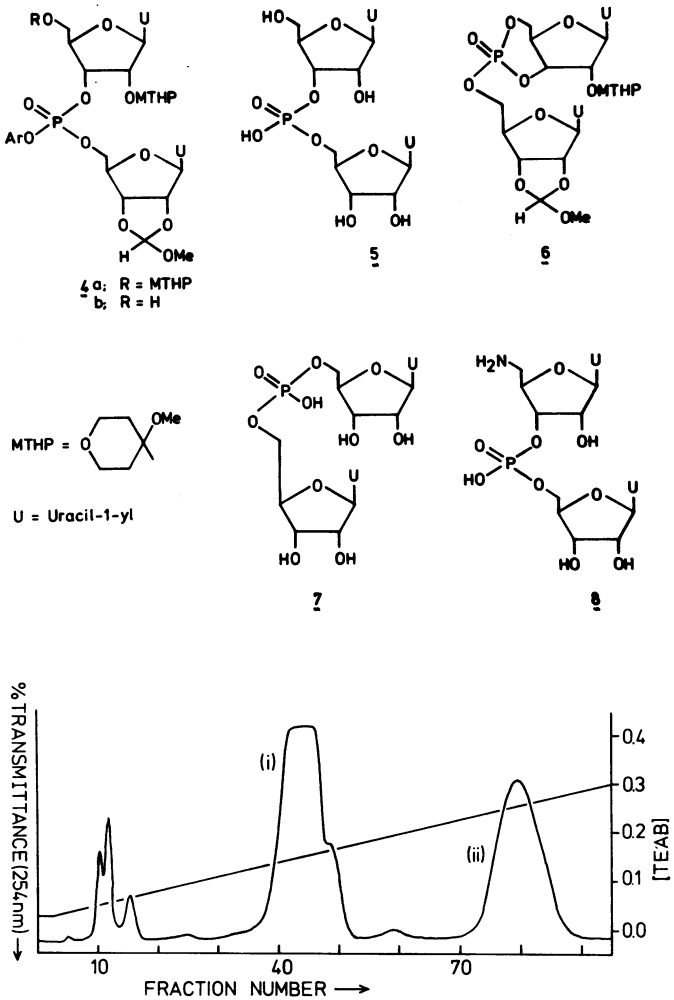
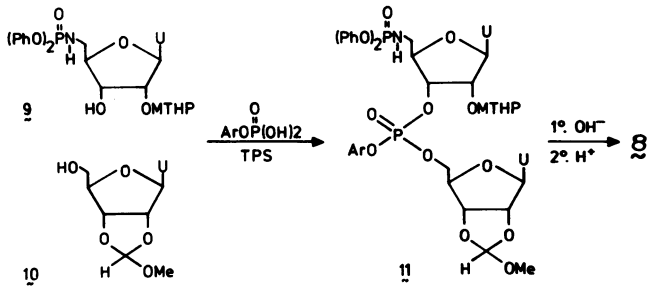



Fig.1 DEAE-Sephadex chromatography of the products obtained after ammonolysis (15 N aq. $NH_3$/dioxan, 4:1 v/v) followed by acid hydrolysis (0.01 N HCl) of 4b (Ar=2Cl$C_6H_4$).

The identity of the material in peak (ii) was corroborated by the specific action of enzymes which hydrolyze 3'-5'-internucleotide phosphodiester bonds, as well as by comparison with synthetic model compounds. Enzymatic digestion of material in peak (ii) with (a) spleen phosphodiesterase (SPD) was negative; (b) venom phosphodiesterase (VPD) gave uridine-5'-phosphate and

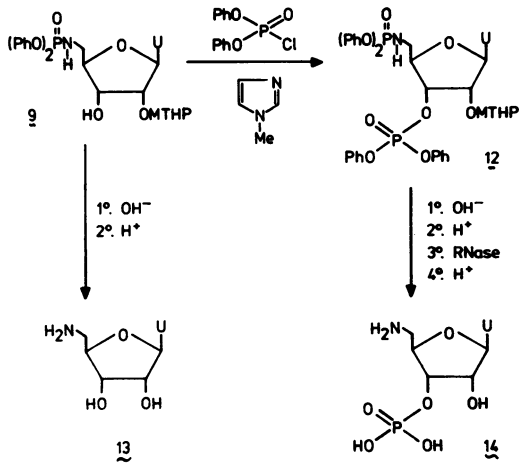
a nucleoside; (c) pancreatic ribonuclease (RNase) afforded uridine and a nucleoside-3'-phosphate. These enzymatic studies indicate that the material in peak (ii) should possess a 3'-5'-internucleotide linkage and, taking into account the negative result obtained with SPD, another than hydroxy function at the 5'-position. The most likely proposition[8] of the nature of the group at the 5'-terminus of material in peak (ii) is an amino group. The formation of this amino function can be visualized by ringopening, at the 5'-carbon, of the intermediate 3',5'-cyclic phosphotriester (6) with ammonia. Acidic hydrolysis of the resulting 5'-amino-deoxy-derivative should give 5'-amino-deoxy-uridilyl-(3'-5')-uridine (8). Model compound (8) was prepared (Scheme 2) by treating the fully protected derivative 11, obtained by a two-step phosphorylation procedure of nucleoside 9 and nucleoside 10 with aryl dihydrogen phosphate[9] (Ar=2ClC$_6$H$_4$) under the influence of TPS[10], with base followed by acid.




SCHEME 2

Dimer 8 thus obtained was in every aspect identical with material in peak (ii) in Fig. 1. Thus dimer 8 failed, as expected, to react with SPD. RNase digestion of 8 should give uridine and 5'-amino-deoxy-uridine-3'-phosphate (14). The identity of the latter compound was independently confirmed by comparison with synthetically-prepared 14 (Scheme 3).
Digestion of dimer 8 with VPD should give exclusively uridine-5'-phosphate and 5'-amino-deoxy-uridine (13). In this case too the digestion products were identical, in every aspect, with uridine-5'-phosphate and synthetically-prepared 5'-amino-deoxy-uridine (13). The above findings clearly indicate that the compound in peak (ii) of Fig. 1 should be 5'-amino-deoxy uridilyl-(3'-5')-uridine (8).

Having established the identity of the side products formed during the NGP process, we turned our attention on the quantitation of these products in relation to the diastereoisomeric forms ($d_1$ and $d_2$) of the starting product 4b and the intermediate 3',5'-cyclic-phosphate 6.



SCHEME 3

It can be seen from the Table (experiments 1 and 2) that different proportions of 7 and 8 were obtained from each diastereoisomer of 4b (Ar= $2ClC_6H_4$).

Table Ammonolysis[a] followed by acid hydrolysis[b] of partially protected dinucleoside phosphates 4b and fully protected 3',5'-cyclic dinucleoside phosphates 6.

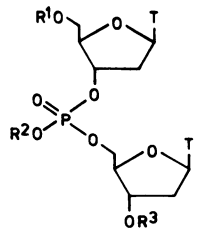
| Experiment | Substrate | Diastereo-isomer[c] | Distribution[d] of products | | |
|---|---|---|---|---|---|
| | | | % 5 | % 7 | % 8 |
| 1 | 4b; Ar = $2ClC_6H_4$ | $d_1$ | 75 | 15 | 10 |
| 2 | 4b; Ar = $2ClC_6H_4$ | $d_2$ | 85 | 6 | 9 |
| 3 | 6 | $d_1$ | 49 | 30 | 21 |
| 4 | 6 | $d_2$ | 71 | 11 | 18 |
| 5 | 4b; Ar = $C_6H_5$ | $d_1 + d_2$ | 83 | 9 | 8 |

[a] With 15 N aq. $NH_3$ in dioxan (4:1, v/v) for 24 hr at 50° C. [b] With 0.01 N HCl (pH=2) for 24 hr at 20° C. [c] The less polar and more polar diastereoisomers are indicated by $d_1$ and $d_2$, respectively, mixtures are indicated by $d_1 + d_2$. [d] As estimated by HPLC after removal of the acid-labile protecting groups.

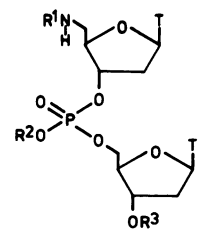
A comparison of the results of experiments 1 and 3 reveals that the proportion of 7 and 8 obtained from the less polar diastereoisomer ($d_1$) of 4b

(Ar=2ClC$_6$H$_4$) was two times less as for the corresponding diastereoisomer ($d_1$) of 6. It can also be seen that the same holds for the diastereoisomers ($d_2$) of 4b (Ar=2ClC$_6$H$_4$) and 6. These results indicate that ammonolysis of 4b (Ar=2ClC$_6$H$_4$) proceeds, contrary to the hydroxide-ion promoted hydrolysis of 4b, only for half of it *via* 6. Furthermore, the results of experiment 5 suggest that ammonolysis of 4b (Ar=C$_6$H$_4$) leads to the formation of 7 and 8.

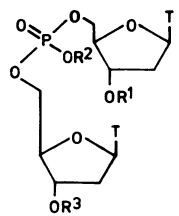
We also demonstrated that the same type of unwanted side-products are formed in the ammonolysis of aryl-phosphotriesters of deoxyoligomers with a terminal 5'-hydroxy function.



15 a; $R^1 = R^3 =$ MTHP, $R^2 =$ Ar
b; $R^1 =$ H, $R^2 =$ Ar, $R^3 =$ MTHP
c; $R^1 = R^2 =$ H, $R^3 =$ MTHP
d; $R^1 = R^2 = R^3 =$ H



16 a; $R^1 = (PhO)_2P(O)-$, $R^2 =$ Ar, $R^3 =$ MTHP
b; $R^1 = R^2 =$ H, $R^3 =$ MTHP
c; $R^1 = R^2 = R^3 =$ H



17 a; $R^1 =$ Bz, $R^2 =$ Ar, $R^3 =$ MTHP
b; $R^1 = R^2 =$ H, $R^3 =$ MTHP
c; $R^1 = R^2 = R^3 =$ H

T = Thymin-1-yl

Thus, HPLC analysis of the mixture obtained after ammonolysis of dimer 15b (Ar=2ClC$_6$H$_4$) revealed the presence of three peaks (Fig. 2a). The identity of the products in these peaks was corroborated by comparison with synthetically-prepared dimers 16b, 17b and 15c (*vide*, Experimental). These studies indicated that the material in peaks (i), (ii) and (iii) was identical with the dimers 16b, 17b and 15c, respectively. Further proof of their identity was obtained as follows. Removal of the acid-labile methoxytetrahydropyranyl groups from the products in the three peaks in Fig. 2a should give 16c, 17c and 15d which, in turn, should be identical with the corresponding synthetically-prepared dimers. Indeed, the retention times of the hydrolysis products

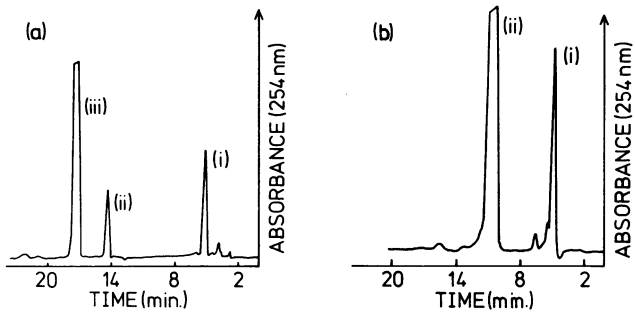



Fig. 2 (a) HPLC analysis of the products obtained after ammonolysis (15 N aq. $NH_3$/dioxan, 4:1, v/v) of 15b (Ar=$2ClC_6H_4$).
(b) HPLC analysis of the products obtained after ammonolysis followed by acid hydrolysis (0.01 N HCl; pH=2) of 15b (Ar=$2ClC_6H_4$).

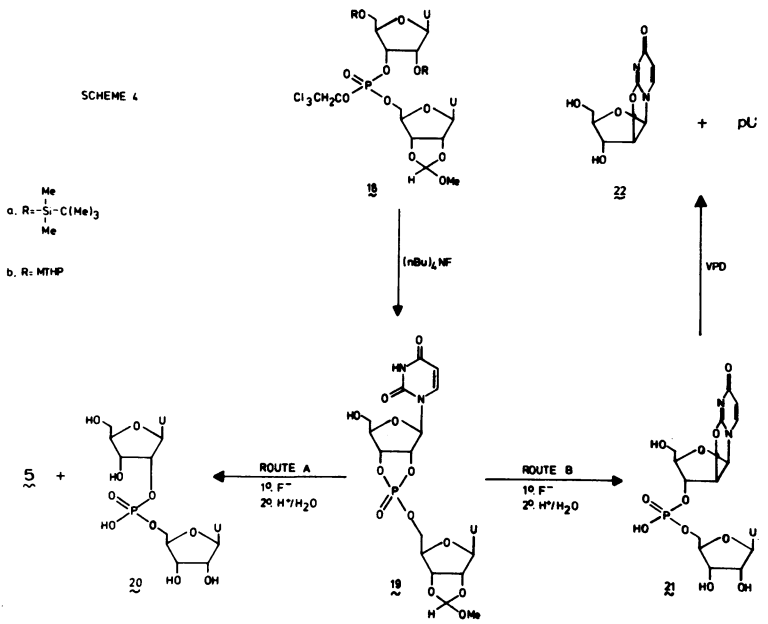
were the same as for the synthetic dimers (see Fig. 2b).

The proportion of the three products formed after ammonolysis of 15b (Ar=$2ClC_6H_4$) could be estimated directly by HPLC-analysis and showed to be: 12% (16b), 7% (17b) and 81% (15c).

In conclusion the data presented above clearly show that NGP will be suppressed completely if the terminal hydroxy groups of oligonucleotides are protected with base-stable protecting groups prior to the unblocking of the internucleotide linkages with ammonia.

Fluoride-ion promoted hydrolysis of alkyl-phosphotriesters of oligonucleotides.

Recently, Ogilvie *et al.* reported[11,12] that alkyl-phosphotriesters of fully protected oligoribonucleotides (*e.g.*, 18a), having 2,2,2-trichloroethyl (TCE) as a protecting group at phosphorus and t-butyldimethylsilyl (TBDMS) groups for the protection of the 2'- and 5'-hydroxyl functions of the nucleosides, could be deblocked completely and rapidly with tetrabutylammonium fluoride ($Bu^4_nNF$) in dry DMF to give oligoribonucleotides with solely 3'-5'-internucleotide linkages. However, we found that the removal, under the same conditions, of TCE groups from phosphotriesters of oligomers (*e.g.*, 18b) with methoxytetrahydropyranyl (MTHP) instead of TBDMS groups was extremely slow. In order to explain this difference in hydrolysis rate,we postulated[13] that the increase in hydrolysis rate of Ogilvie's phosphotriesters could be explained by NGP and that, as a consequence, the deblocked oligomers would contain a




considerable amount of unwanted products.

To test our hypothesis we prepared the fully protected dimer 18a (see Scheme 4). Deblocking of the TCE groups with the reagent zinc/2,3,4-triisopropulbenzenesulphonic acid/pyridine followed by acid, to remove the methoxymethylene and TBDMS groups, and neutralization gave dimer (5), which was completely digested by RNase to give solely uridine and uridine-3'-phosphate. This experiment shows that model compound (18a) contains solely 3'-5'-internucleotide linkages. Compound (18a) was now treated with dry $Bu^n_4NF$ in THF followed by acid. The deblocked product thus obtained was purified by DEAE-ion exchange chromatography (Fig. 3a). The product in peak (i) was collected and treated with RNase. Analysis of the digestion mixture by HPLC showed the presence of mainly four peaks, two of which were identical with uridine and uridine-3'-phosphate. This finding indicates that only part of the deblocked product consists of natural uridilyl-(3'-5')-uridine (5). Purification of the crude digest revealed (Fig. 3b) that it contained four products (*i.e.*, peaks (i)-(iiii)). The products in peak (i) and (iiii) were, as proved before, identical with uridine and uridine-3'-phosphate, respectively. The structure of the product in peak (iii) was determined by $^1$H-NMR-spectroscopy and showed to be uridilyl-(2'-5')-uridine (20). The identity of the pro-

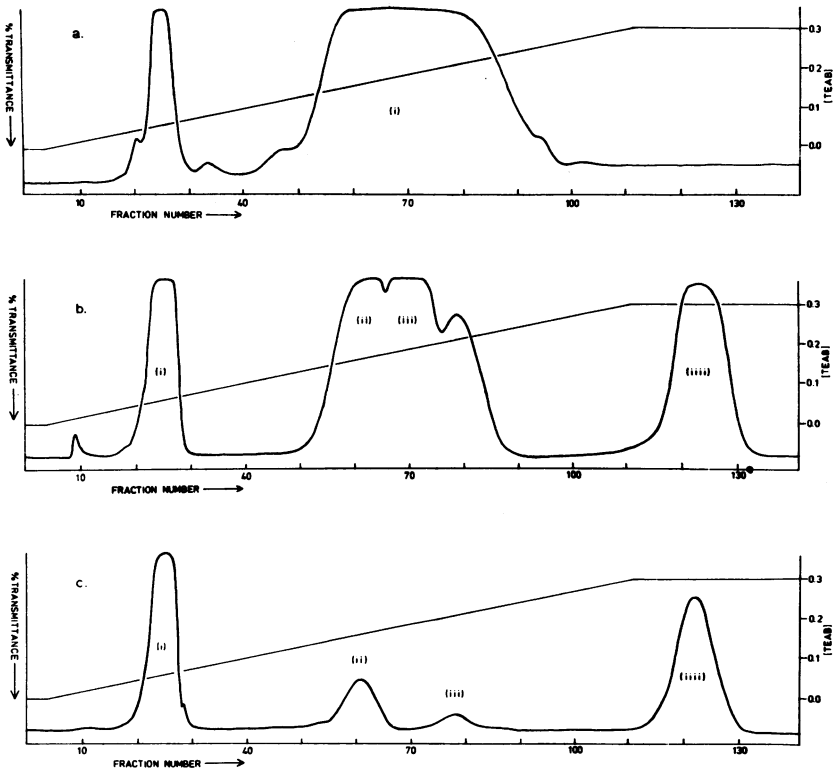



Fig. 3 (a) DEAE-Sephadex chromatography of the products obtained after complete deblocking (fluoride followed by acid treatment) of dimer 18a.
(b) DEAE-Sephadex chromatography of the products obtained after RNase digestion of the product in peak (i) in Fig. 3a.
(c) DEAE-Sephadex chromatography of the products obtained after VPD digestion of the product in peak (ii) in Fig. 3b.

duct in peak (ii) was corroborated as follows. Exhaustive treatment of the product in peak (ii) with VPD, followed by purification of the digestion mixture, gave, apart from starting product, mainly two peaks (Fig. 3c). The product in peak (iiii) was, as expected, identical with uridine-5'-phosphate. Furthermore, the product in peak (i) was in every aspect -TLC behaviour, $^{1}$H-NMR-spectroscopy - identical with commercially available 2,2'-O-anhydrouridine (22 in Scheme 4). These findings clearly show that the product in peak (ii) of Fig. 3b must be 2,2'-O-anhydrouridilyl-(3'-5')-uridine (21).

Finally, quantitative analysis of the crude reaction product obtained by deblocking 18a, under exactly the same conditions as used by Ogilvie, gave the following proportions of products: 61%(5), 30%(20) and 9%(21).

The formation of the products discussed before may be explained by taking into account NGP during the hydrolysis of triester 18a with $Bu_n^4NF$ (Scheme 4). The first step in this process will be the removal of the TBDMS groups from the 2'- and 5'-hydroxyl groups to give the corresponding 2'- and 5'-alkoxide ions. The 5'- or 2'-nucleoside hydroxide ions may attack the phosphotriester function with simultaneous removal of the TCE group from phosphorus to afford a 3',5'- or 2',3'-cyclic phosphotriester intermediate, respectively. Due to the close proximity of the nucleoside 2'-hydroxide ion, in comparison with the 5'-hydroxide ion, to the 3'-phosphotriester function we may assume that intermediate 19 will be formed predominantly. This intermediate may react in two ways with excess fluoride ions. Firstly (Route A), nucleophilic attack by fluoride ion at phosphorus, which results in a non-specific ringopening of the 2',3'-cyclic phosphotriester, should give, after hydrolysis of the intermediate P-F bonds, the dimers 5 and 20. Secondly (Route B), fluoride ion may react as a base[14] by removing the amino-proton ($-N_3H$) from the uracil moiety. The negative charge thus created in the uracil ring may lead, *via* oxygen at carbon at the 2'-position, to a nucleophilic attack at the 2'-carbon of the ribose moiety to afford, after acidic hydrolysis (0.01 N HCl; pH=2), the stable 2,2'-O-anhydrouridine-derivative 21.

In conclusion the data presented clearly show that (a) 2'-hydroxy functions should not be protected with TBDMS groups in oligoribonucleotide synthesis unless the internucleotide linkages are unblocked before the removal of the silyl protecting groups and (b) that NGP provides a plausible explanation for the fast hydrolysis of TCE groups from phosphotriesters[15] (*e.g.*, 18a) with dry $Bu_n^4NF$.

## EXPERIMENTAL

All solvents were dried as described previously[16]. UV absorption spectra were measured with a Cary C15 recording spectrophotometer. $^1$H-NMR spectra were measured at 100 MHz with a JEOL JNM PS 100 spectrometer; chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS). Compounds 20 and 22 were measured relative to tetramethylammonium chloride (TMA) with a JEOL JNM PS 100 spectrometer, equipped with a EC-100 computer, operating in the Fourier Transform mode. This system was also used for $^{31}$P-NMR with 85% $H_3PO_4$ as an external standard.

Schleicher & Schüll DC Fertigfolien F 1500 LS 254 were developed in chloroform-methanol (92:8 v/v, system A). Merck DC Alufolien Cellulose F 254 plates were developed in aqueous 1 M $NH_4OAc$/EtOH (3:7 v/v, system B). The high-performance liquid chromatography system used in this study has been described elsewhere[17]. High-performance anion-exchange chromatography was performed on a column (25 cm x 4.6 mm) packed with Nucleosil 10 SB (Chrompack). Elution was effected, starting with buffer A (0.005 M $KH_2PO_4$, pH 4.5) and applying 2% buffer B (0.05 M $KH_2PO_4$, 0.5 M KCl, pH 4.5) per min (system C) or starting with buffer A and applying, at t=6 min, 3% of buffer B per min (system D). A flow rate of 1 ml per min at a pressure of 50 kp/cm$^2$ at 20° was standard. In system E high-performance anion-exchange chromatography was performed on a column (25 cm x 4.6 mm) packed with Partisil PXS 10/25 SAX (Whatman). The column was eluted with buffer A at a flow rate of 1 ml per min and a pressure of 45 kp/cm$^2$. Retention times (Rt) in systems C, D and E were measured relative to the injection peak. Merck Kieselgel H was used for short column adsorption chromatography. Preparative anion-exchange chromatography was performed on a column (24 cm x 7 cm$^2$) of DEAE-Sephadex A25 (Pharmacia) ($HCO_3^-$ form) suspended in 0.05 M triethylammonium bicarbonate (TEAB, pH 7.5). The column was eluted with a linear gradient of 0.05 M - 0.3 M TEAB for 16 h at a flow rate of 40 ml per hour. Fractions of 6 ml were collected.

2',5'-Bis-O-methoxytetrahydropyranyluridilyl-(3'-5')-2',3'-O-methoxymethyleneuridine 2-chlorophenyl ester (4a)

Anhydrous pyridine (5 ml) was added to a dry mixture of 2',5'-bis-O-methoxytetrahydropyranyluridine[18] (0.47 g, 1.0 mmole), 2-chlorophenyl dihydrogen phosphate[19] (0.22 g, 1.05 mmole) and 2,4,6-triisopropylbenzenesulphonyl chloride[10] (TPS, 0.76 g, 2.5 mmole) and the solution was stored at 20°. After 4 h, 2',3'-O-methoxymethyleneuridine[20] (0.32 g, 1.1 mmole) was added and, after another 15 h, the products were concentrated under reduced pressure to an oil. A solution of the oil in chloroform (50 ml) was washed with 10% aqueous $NaHCO_3$ (25 ml) and water (25 ml). The organic layer was dried ($MgSO_4$) and concentrated to a glass. This material, dissolved in chloroform-methanol (96:4 v/v, 5 ml), was applied to a column (11 cm x 9 cm$^2$) of Kieselgel H (40 g), suspended in the same solvent. Elution with chloroform-methanol (95.5:4.5 v/v) gave pure 4a. The appropriate fractions were concentrated to a small volume and 4a was precipitated from petroleum ether (40-60°, 150 ml), filtered off and dried in vacuo (KOH). Yield 0.64 g (69%), Rf 0.41 (system A).

2'-O-Methoxytetrahydropyranyluridilyl-(3'- 5')-2',3'-O-methoxymethyleneuridine 2-chlorophenyl ester (4b)

The fully protected dinucleotide 5'-O-p-chlorophenoxyacetyl-2'-O-methoxytetrahydropyranyluridilyl-(3'- 5')-2',3'-O-methoxymethyleneuridine 2-chlorophenyl ester was prepared and purified according to the same procedure as described for 4a and using 5'-O-p-chlorophenoxyacetyl-2'-O-methoxytetrahydropyranyluridine[21] (0.53 g, 1.0 mmole) as the 3'-hydroxy component. Yield 0.69 g (70%), Rf 0.40 (system A). The fully protected dinucleotide (0.69 g, 0.7 mmole) was dissolved in dry dioxan (14 ml) and dry methanol (49 ml). Methanolic $K_2CO_3$ (0.1 M, 7 ml) was added to the solution and after 8 min at 20° the reaction mixture was neutralized with aqueous $KH_2PO_4$ (2 M, 2ml). The solvents were evaporated and the residue was partitioned between chloroform (50 ml) and water (25 ml). The organic layer was dried ($MgSO_4$) and concentrated to give a glass. This material, dissolved in chloroform-methanol (95:5 v/v, 5 ml), was applied to a column (11 cm x 9 $cm^2$) of Kieselgel H (40 g), suspended in the same solvent. Elution with chloroform-methanol (94.5:5.5 v/v) afforded the two pure diastereoisomers of 4b.

a. Less polar diastereoisomer ($d_1$). Yield 0.22 g (39%), Rf 0.29 (system A). UV (MeOH-$H_2O$, 9:1 v/v) $\lambda_{max}$ 257 (ε 19,400) $\lambda_{min}$ 228 nm (ε 5,400). $^{31}$P-NMR ($(CD_3)_2CO$) - 8.16 (s).

b. More polar diastereoisomer ($d_2$). Yield 0.17 g (30%), Rf 0.27 (system A). UV (MeOH-$H_2O$, 9:1 v/v) $\lambda_{max}$ 257 (ε 19,400) $\lambda_{min}$ 228 nm (ε 5,200). $^{31}$P-NMR ($(CCD_3)_2CO$)-8.21 (s).

According to the same procedure we prepared the two diastereoisomers ($d_1$ and $d_2$) of dimer 4b (Ar= 2,4-dichlorophenyl).

a. Less polar diastereoisomer ($d_1$).

Pure 4b($d_1$, Ar= 2,4-dichlorophenyl, 30 mg) was crystallized from ethylacetate/methanol/5-chloro-1-methylimidazole (6:1:1 v/v). M.p. 158°, Rf 0.31 (system A). (Anal.: $C_{32}H_{37}O_{17}N_4PCl_2$ (851.54); calcd. C 45.14 H 4.38 N 6.58; found C 45.23 H 5.12 N 6.35.)

b. More polar diastereoisomer($d_2$).

Pure 4b ($d_2$, Ar= 2,4-dichlorophenyl, 420 mg) was crystallized from acetonitrile (16 ml) to which was added 5-chloro-1-methylimidazole (0.05 ml). M.p. 184°, Rf 0.26 (system A). (Anal.: $C_{32}H_{37}O_{17}N_4PCl_2$ (851.54; calcd. C 45.14 H 4.38 N 6.58; found C 45.16 H 4.80 N 6.74.)

Uridilyl-(3'- 5')-uridine (5)

Aqueous ammonia (15N, 5ml) was added to a solution of 4a (47 mg, 0.05 mmole) in dioxan (1 ml), the vessel was sealed and kept at 50° for 16 h. After evaporation of the solvents, the residue was dissolved in 0.01 N HCl (10

ml) and the pH was adjusted to 2.0 by addition of 0.1 N HCl. After 16 h at 20° the solution was carefully neutralized (pH 8) with 0.5 M ammonia and lyophilized to give the ammonium salt of 5. Rf 0.38 (system B). HPLC analysis (system C) revealed one peak (Rt 2.2 min), which accounted for over 95% of the UV absorption. Enzymatic digestion with RNase, VPD and SPD gave the expected products in the correct ratios.

Preparation of fully protected 3',5'-cyclic phosphate dinucleoside (6)

a. Less polar diastereoisomer ($d_1$).

To a solution of the less polar diastereoisomer ($d_1$) of dimer 4b (163 mg, 0.2 mmole) in dioxan (2.5 ml) was added 0.125 N NaOH (10 ml). After 6 min at 20° the solution was neutralized by the addition of aqueous $NaH_2PO_4$ (1 M, 10 ml). The solution was concentrated under reduced pressure to a small volume (7 ml) and extracted several times with chloroform (3 x 20 ml). The combined organic layer was dried ($MgSO_4$), evaporated down to a small volume (1 ml) and added dropwise to stirred n-hexane (40 ml). The colourless precipitate was collected by filtration and showed to be homogeneous on TLC (system A). Yield 76 mg (55%), Rf 0.31 (system A). (Anal.: $C_{26}H_{33}N_4O_{16}P$ (688,55); calcd. C 45.35 H 4.83 P 4.50; found C 44.70 H 5.25 P 4.45.) UV (MeOH-$H_2O$, 9:1 v/v) $\lambda_{max}$ 256 (ε 18,900) $\lambda_{min}$ 227 nm (ε 5,500). $^{31}$P-NMR ($(CD_3)_2CO$)-6.48 (s).

b. More polar diastereoisomer ($d_2$).

According to the same procedure as described for 6 ($d_1$) the more polar diastereoisomer 4b ($d_2$) (163 mg, 0.2 mmole) afforded chromatographically (TLC, system A) pure 6 ($d_2$). Yield 37 mg (26%), Rf 0.27 (system A). (Anal.: $C_{26}H_{33}N_4O_{16}P$ (688.55); calcd. C 45.35 H 4.83 P 4.50; found C 45.20 H 4.60 P 4.40.) UV (MeOH-$H_2O$, 9:1 v/v) $\lambda_{max}$ 258 (ε 19,300) $\lambda_{min}$ 228 nm (ε 5,100). $^{31}$P-NMR ($(CD_3)_2CO$)-5.16 (s).

Uridilyl-(5'- 5')-uridine (7)

A mixture of 2',3'-O-methoxymethyleneuridine (0.29 g, 1 mmole), 2-chlorophenyl dihydrogen phosphate (0.114 g, 0.55 mmole) and TPS (0.39 g, 1.3 mmole) was dissolved in anhydrous pyridine (5 ml). After 20 h TLC (system A) showed the reaction to be complete. Work-up of the reaction mixture and purification of fully protected 7 was according to the same procedure as described for 4a. Yield 0.28 g (74%), Rf 0.38 (system A). The 2',3'-O-methoxymethyleneuridilyl-(5'- 5')-2',3'-O-methoxymethyleneuridine 2-chlorophenyl ester thus obtained was deblocked in the same way as described for 5, affording 7 in the ammonium form. Rf 0.30 (system B). HPLC analysis (system C) revealed one peak (Rt 2,2 min), which accounted for over 95% of the UV absorption. Dimer 7 was completely digested by VPD to uridine and uridine 5'-phosphate, but was resistant

to spleen phosphodiesterase and RNase.

2'-O-Methoxytetrahydropyranyl-N-5'-deoxy-uridine-5'-diphenyl phosphoramidate (9)

a. 2'-O-Methoxytetrahydropyranyl-5'-azido-deoxy-uridine[22]

A solution of triphenylphosphine (3.41 g, 13 mmole) in dry DMF (20 ml) was added dropwise to a mixture of 2'-O-methoxytetrahydropyranyluridine[23] (3.58 g, 10 mmole), tetrabromomethane (4.98 g, 15 mmole) and lithiumazide (1.47 g, 30 mmole) in dry DMF (40 ml) over a period of 10 min at 20°. After stirring another 10 min, TLC (system A) indicated that the reaction was complete, and the mixture was concentrated to an oil, which was triturated with ether (2 x 200 ml). The precipitate was dissolved in chloroform-methanol (96.5:3.5 v/v, 10 ml) and applied to a column (13 cm x 9 $cm^2$) of Kieselgel H (45 g) suspended in the same solvent. The appropriate fractions were concentrated to a small volume (5 ml) and upon addition of chloroform-petroleum ether (40-60°) (3:7 v/v, 100 ml), pure 2'-O-methoxytetrahydropyranyl-5'-azido-deoxy-uridine crystallized. The crystals were filtered off and concentration of the filtrate, followed by addition of the same solvent mixture (50 ml) afforded an additional amount of product. Yield 3.55 g (92%), m.p. 120-121°, Rf 0.29 (system A.) UV 95% EtOH) $\lambda_{max}$ 259 (ε 9,700) $\lambda_{min}$ 229 nm (ε 2,600). NMR ($(CD_3)_2SO$) 7.79 ($H_6$, d, J 8Hz); 5.97 ($H_1$, d, J 7.5Hz); 5.73 ($H_5$, d, J 8Hz); 4.44 ($H_{3'}$, m); 3.00 ($OCH_3$, s).

b. 2'-O-Methoxytetrahydropyranyl-N-5'-deoxy-uridine-5'-diphenyl phosphoramidate (9)

A mixture of triphenylphosphite (10.85 g, 35 mmole) and 2'-O-methoxytetrahydropyranyl-5'-azido-deoxy-uridine (3.45 g, 9 mmole) in dry dioxan[24] (75 ml) was heated under reflux for 4 h. After cooling to 30°, dioxan was evaporated under reduced pressure. The remaining oil was dissolved in chloroform and washed with 5% aqueous $NaHCO_3$ (50 ml) and water (50 ml). The organic layer was dried ($MgSO_4$), concentrated to a small volume and triturated with petroleum ether (40-60°, 200 ml). The precipitate was dissolved in chloroform-methanol (96.5:3.5 v/v, 10 ml) and applied to a column (14 cm x 20 $cm^2$) of Kieselgel H (100 g), suspended in the same solvent. The appropriate fractions were concentrated to a small volume and pure 9 was precipitated from petroleum ether (40-60°, 200 ml). Yield 2.6 g (49%), Rf 0.28 (system A). Anal.: $C_{27}H_{32}N_3O_{10}P$ (589.54); calcd. C 55.01 H 5.47 N 7.12 P 5.25; found C 54.74 H 6.10 N 7.21 P 5.10.) UV (95% EtOH) $\lambda_{max}$ 261 (ε 10,500) $\lambda_{min}$ 228 nm (ε 10,500) $\lambda_{min}$ 228 nm (ε 3,200). NMR ($(CD_3)_2SO$) 7.80 ($H_6$, d, J 8Hz); 7.29 ($C_6H_5$, m); 5.93 ($H_{1'}$, d, J 7.5Hz); 5.66 ($H_5$, d, J 8Hz); 2.96 ($OCH_3$, s).

Preparation of the fully protected dimer 11.

A mixture of 9 (353 mg, 0.6 mmole), 2-chlorophenyl dihydrogen phosphate (156 mg, 0.75 mmole) and TPS (453 mg, 1.5 mmole) was dissolved in anhydrous pyridine (3 ml). After 20 h at 20°, TLC (system A) indicated that the reaction was complete. The mixture was diluted with chloroform (100 ml) and washed with aqueous 1 M triethylammonium bicarbonate (TEAB, pH 7.5, 9 ml) and 0.1 M TEAB (9 ml). The organic layer was concentrated to an oil and dried, after addition of 2',3'-O-methoxymethyleneuridine (143 mg, 0.5 mmole), by repeated coevaporation with anhydrous pyridine (3 x 10 ml). The mixture was concentrated to a small volume (3 ml) and TPS (212 mg, 0.7 mmole) was added. TLC (system A), after 20 h at 20°, demonstrated that the reaction was complete. Work-up of the reaction mixture and purification of 11 was according to the same procedure as described for 4a. Yield 325 mg (62%), Rf 0.31 (system A).

5'-Amino-deoxy-uridilyl-(3'- 5')-uridine (8)

Aqueous 0.125 N NaOH (16 ml) was added to a solution of 11 (105 mg, 0.1 mmole) in dioxan (4 ml). After 36 h at 20°, Dowex 50W cation-exchange resin (20 g, 100-200 mesh, ammonium form) was added. The resin was filtered off after 5 min, and washed with water (3 x 10 ml). The combined filtrates were concentrated to a small volume (1 ml), diluted with aqueous 0.01 N HCl (15 ml) and washed with ether (3 x 15 ml). The aqueous layer was adjusted to pH 2.0 by addition of 0.1 N HCl and left for 20 h at 20°. Addition of aqueous 0.5 M ammonia up to pH 8 and lyophilization of the solution afforded 8. Rf 0.21 (system B). HPLC analysis (system C) revealed one peak (Rt 0.9 min)., which accounted for over 90% of the UV absorption. Dimer 8 was resistant to SPD. Digestion with VPD gave uridine 5'-phosphate and 5'-amino-deoxy-uridine (identical with chemically synthesized 13). Dimer 8 was completely digested by RNase, affording uridine and 5'-amino-deoxy-uridine 3'-phosphate (identical with chemically synthesized 14).

5'-Amino-deoxy-uridine (13).

Aqueous 0.125 N NaOH (4 ml) was added to a solution of 9 (59 mg, 0.1 mmole) in dioxan (1 ml). After 36 h at 20°, Dowex 50W cation-exchange resin (5 g, 100-200 mesh, ammonium form) was added. Work-up of the reaction mixture and acid treatment were according to the same procedure as described for 8. Rf 0.50 (system B). HPLC analysis (system B) revealed one peak, which coincided with the injection peak (Rt = 0.0 min). All analytical data of 13 were in accordance with those published before[25].

Preparation of the fully protected mononucleotide 12.

Diphenyl phosphorochloridate (1.14 g, 5 mmole) was added to a solution

of 9 (1.77 g, 3 mmole) and 1-methylimidazole (0.5 g, 6 mmole) in dry acetonitrile (15 ml). After 1 h at 20°, TLC (system A) revealed that the reaction was complete. The mixture was concentrated under reduced pressure to an oil, which was dissolved in chloroform (100 ml) and washed with 5% aqueous $NaHCO_3$ (50 ml) and water (50 ml). The organic layer was dried ($MgSO_4$), evaporated down to a small volume and triturated with petroleum ether (40-60°, 2 x 200 ml). The precipitate was dissolved in chloroform-methanol (96.5:3.5 v/v, 7 ml) and applied to a column (11 cm x 9 $cm^2$) of Kieselgel H (40 g), suspended in the same solvent. Concentration of the appropriate fractions to a small volume (10 ml) followed by addition of petroleum ether (40-60°, 200 ml) afforded pure 12 as a white precipitate. Yield 0.87 g (36%), Rf 0.70 (system A).

5'-Amino-deoxy-uridine 3'-phosphate (14)

Aqueous 0.125 N NaOH (16 ml) was added to a solution of 12 (81 mg, 0.1 mmole) in dioxan (4 ml). After 36 h at 20°, Dowex 50W cation-exchange resin (20 g, 100-200 mesh, ammonium form) was added. The resin was filtered off and washed with water (3 x 10 ml). The combined filtrates were concentrated to a small volume (1 ml), diluted with 0.01 N HCl (10 ml) and washed with ether (3 x 10 ml). The aqueous layer was adjusted to pH 2.0 with 0.1 N HCl and left for 2 h. The solution was neutralized with aqueous 0.5 M ammonia and concentrated to dryness. The residue was dissolved in 0.2 M Tris-HCl buffer (pH 8.0, 1 ml) and incubated with 1 mg Pancreatic Ribonuclease (Merck) at 37° for 2 h. The enzymatic digest was transferred to a centrifuge tube and a mixture of chloroform-isopentylalcohol (24:1 v/v, 2 ml) was added. The two layer system was mixed thoroughly and centrifuged. The aqueous layer was removed and applied to a column (8 cm x 2 $cm^2$) of Dowex 50W cation-exchange resin (10 g, 100-200 mesh, ammonium form) which was eluted with water. The aqueous solution was concentrated to a small volume (1 ml), diluted with 0.01 N HCl (20 ml) and adjusted to pH 2.0 with 0.1 N HCl. After 16 h, the solution was neutralized with aqueous 0.5 M ammonia and lyophilized. Rf 0.07 (system B). HPLC analysis (system C) revealed one main peak (Rt 3.1 min).

Deblocking of dimers 4b and 6.

Ammonia-promoted hydrolysis of the aryl-phosphotriesters of 4b ($d_1$ and $d_2$) and 6 followed by acid treatment to remove the remaining protecting groups was performed as described for 5. The crude reaction mixtures were purified on DEAE-Sephadex. Peaks, eluted from this column were analysed by TLC (system B) and HPLC (system C) in combination with enzymatic digestion by RNase, VPD and SPD.

Enzymatic digestion of the deblocked dinucleotides

a. Venom phosphodiesterase (VPD): a solution of the dinucleotide (1 mg) in a buffer (0.1 ml) containing 25 mM Tris-HCl (pH 9.0), 5 mM $MgCl_2$ and 10 µg snake venom phosphodiesterase (Crotalus terr.terr., Boehringer) was incubated at 37° for 3 h.

b. Spleen phosphodiesterase (SPD): a solution of the dinucleotide (1 mg) in a buffer (0.1 ml) containing 0.1 M $NH_4OAc$ (pH 5.7) and 20 µg spleen phosphodiesterase (Boehringer) was incubated at 37° for 3 h.

c. RNase: the dinucleotide (1 mg) was incubated with 100 µg Pancreatic Ribonuclease (Merck) in 0.2 M Tris-HCl buffer (pH 8.0, 0.1 ml) at 37° for 2 h.

5'-O-Methoxytetrahydropyranyldeoxythymidilyl-(3'- 5')-3'-O-methoxytetrahydropyranyldeoxythymidine 2-chlorophenyl ester (15a)

A mixture of 5'-O-methoxytetrahydropyranyldeoxythymidine[23] (0.39 g, 1.1 mmole), 2-chlorophenyl dihydrogen phosphate (0.25 g, 1.2 mmole) and 2,4,6-triisopropylbenzenesulphonyl-4-nitroimidazole[26] (TPS-NI, 0.95 g, 2.5 mmole) was dissolved in anhydrous pyridine (5 ml) and stored for 16 h at 20°. The reaction mixture was diluted with chloroform (100 ml) and washed with aqueous 2 M triethyl ammonium bicarbonate (TEAB, pH 7.5, 15 ml) and 1 M TEAB. The organic layer was concentrated to an oil and dried, after addition of 3'-O-methoxytetrahydropyranyldeoxythymidine[27] (0.36 g, 1.0 mmole), by repeated coevaporation with anhydrous pyridine (3 x 10 ml). The mixture was concentrated to a small volume (3 ml) and TPS-NI (0.46 g, 1.2 mmole) was added. After 18 h at 20° TLC (system A) showed that the reaction was complete and the reaction mixture was concentrated to an oil. A solution of this oil in chloroform (50 ml) was washed with 5% aqueous $NaHCO_3$ (25 ml) and water (50 ml). The organic layer was dried ($MgSO_4$), evaporated down to a small volume (5 ml) and triturated with petroleum ether (40-60°, 2 x 150 ml). The precipitate was dissolved in chloroform-methanol (96:4 v/v, 5 ml) and applied to a column (10 cm x 7 $cm^2$) of Kieselgel H (25 g), suspended in the same solvent. The column was eluted with the same solvent mixture and after evaporation of the appropriate fractions pure 15a was precipitated from petroleum ether (40-60°, 150 ml). Yield 0.76 g (86%), Rf 0.45 (system A).

Deoxythymidilyl-(3'- 5')-3'-O-methoxytetrahydropyranyldeoxythymidine 2-chlorophenyl ester (15b)

5'-O-p-Chlorophenoxyacetyldeoxythymidilyl-(3'- 5')-3'-O-methoxytetrahydropyranyldeoxythymidine 2-chlorophenyl ester was prepared, starting from 5'-O-p-chlorophenoxyacetyldeoxythymidine[28] (0.27 g, 1.1 mmole), according to the same procedure as described for 15a. The crude, fully protected dimer was

dissolved in dry dioxan (10 ml) and dry methanol (20 ml) and methanolic $K_2CO_3$ (0.1 M, 5 ml) was added. After 5 min at 20°, the reaction was quenched by the addition of aqueous $KH_2PO_4$ (2 M, 2.5 ml). The solvents were evaporated and the residue was partitioned between chloroform (50 ml) and water (25 ml). The organic layer was concentrated to a small volume and precipitated from petroleum ether (40-60°, 150 ml). Purification by short column chromatography was performed as described for 15a. Yield 0.60 g (77%), Rf 0.28 (system A).

Preparation of the fully protected dinucleotide (16a)

a. N-5'-Deoxy-deoxythymidine-5'-diphenyl phosphoramidate[24].

A mixture of triphenylphosphite (6.2 g, 20 mmole) and 5'-azido-5'-deoxy-deoxythymidine[22] (1.34 g, 5 mmole) in dry dioxan (40 ml) was heated, under reflux, for 2.5 h. The solution was allowed to cool and diluted with ether (500 ml). The precipitate was filtered off and the filtrate was extracted with water (4 x 100 ml). The combined aqueous layers were evaporated down to 50 ml and upon cooling to 5° pure product crystallized. Yield 1.12 g (47%), m.p. 164-165°, Rf 0.20 (system A). (Anal.: $C_{22}H_{24}N_3O_7P$ (473.42); calcd. C 55.81 H 5.11 P 6.54; found C 55.81 H 5.66 P 6.11.) UV (95% EtOH) $\lambda_{max}$ 265 (ε 10,100) $\lambda_{min}$ 233 nm (ε 3,200). NMR ($(CD_3)_2SO$) 7.55 ($H_6$, s); 7.32 ($C_6H_5$, m); 6.16 ($H_{1'}$, t, J 7Hz); 6.00 (NH-P, m); 4.20 ($H_{3'}$, m); 3.16 ($H_{5''}$, m); 1.74 ($CH_3$, s).

b. Synthesis of dimer 16a

Dimer 16a was prepared and purified according to the same procedure as described for 15a, starting from N-5'-deoxy-deoxythymidine-5'-diphenyl phosphoramidate (0.52 g, 1.1 mmole) Yield 0.79 g (79%), Rf 0.37 (system A).

3'-O-Benzoyldeoxythymidilyl-(5' -5')-3'-O-methoxytetrahydropyranyldeoxythymidine (17a).

The fully protected dimer 17a was prepared and purified as described for 15a. Yield 0.71 g (90%), Rf 0.47 (System A).

Deblocking of the fully protected dimers d-TpT (15a, 16a and 17a)

a. Dimer 15a (89 mg, 0.1 mmole) was deblocked in the same way as described for the preparation of 5. Product 15d was analysed by HPLC (system D) and accounted for 97% of the total UV absorption (Rt = 8.0 min). Digestion of 15d with VPD and SPD gave the expected products in the correct ratio.

b. Dinucleotide 16a (100 mg, 0.1 mmole) was deblocked according to the same procedure as applied for the preparation of 8, to afford 16c (HPLC, system D, Rt 2.2 min). Dimer 16c was resistant to SPD, but was completely digested by VPD to give deoxythymidine 5'-phosphate and 5'-amino-5'-deoxy-deoxythymidine in the correct ratio. To a solution of 16c (16 mg, 0.03 mmole)

and mesitylenesulphonic acid (1 mg) in dry DMF (0.5 ml) was added 4-methoxy-5,6-dihydro-2H-pyran (0.1, 0.8 mmole). After 1 h at 20° the reaction mixture was poured into stirred ether (50 ml). Filtration afforded 16b as the main product. Rt 2.4 min (system D).

c. To a solution of 17a (79 mg, 0.1 mmole) in tetrahydrofuran (6.4 ml) was added 0.25 M tetrabutylammonium fluoride in pyridine-water[29] (1:1 v/v, 1.6 ml). After 30 min at 20° Dowex 50W cation-exchange resin (4 g, 100-200 mesh, ammonium form) was added. The resin was filtered off and washed with water (2 x 20 ml). The combined filtrates were evaporated to dryness, the residue was dissolved in 25% aqueous ammonia (20 ml) and left for 16 h at 20°. The solution which now contained 17b was concentrated to a small volume (2 ml) and 0.1 ml was withheld for HPLC analysis. The remaining solution was acidified (20 ml 0.01 N HCl) and adjusted to pH 2.0. After 3 h at 20° the mixture was neutralized by the addition of aqueous ammonia and lyophilized to give 17c. HPLC analysis (system D) of 17b showed the presence of one peak (Rt = 11.5 min) HPLC analysis (system D) of 17c gave also one peak (Rt 7.9 min). Digestion of 17c with VPD afforded deoxythymidine 5'-phosphate and deoxythymidine. Dimer 17c was resistant to SPD.

Preparation of dimer 15c

5'-O-p-Chlorophenoxyacetyldeoxythymidilyl-(3'- 5')-3'-O-methoxytetrahydropyranyldeoxythymidine (see preparation of 15b) (94 mg, 0.1 mmole) was treated with TBAF and $NH_4OH$ as described for the deblocking of 17a, to afford 15a (Rt 15.0 min, system D).

Ammonolysis of dimer 15b

Aqueous ammonia (25%, 8 ml) was added to a solution of 15b (78 mg, 0.1 mmole) in dioxan (2 ml) and kept at 50° for 16 h. The solution was concentrated untill neutral and analysed by HPLC (system D) which revealed three peaks (Fig. 2a). In this system peak (i) was identical with 16b (Rt 2.4 min), peak (ii) had the same retention time (15.0 min) as 15c and peak (iii) coincided with 17b (Rt 11.5 min). The solution was diluted with 0.01 N HCl (10 ml) and adjusted to pH 2.0. After 2 h at 20°, aqueous ammonia was added to neutralize the reaction mixture which was analysed by HPLC (system D, Fig.2b). The material in peak (ii) (Rt 2,2 min, identical with synthetical 16c) was resistant to SPD, but was completely digested by VPD to give deoxythymidine 5'-phosphate and 5'-amino-5'-deoxy-deoxythymidine (identical with synthesized material[24], ninhydrine positive). The retention time of the product in peak (ii) in Fig. 2b was the same as for 15d and 17c (7.9 min). The material in peak (ii) was completely digested by VPD to give deoxythymidine 5'-phos-

phate and deoxythymidine. Treatment with SPD gave, as expected, only partial hydrolysis.

2',5'-Bis-O-tert-butyldimethylsilyluridilyl-(3'- 5')-2',3'-O-methoxymethyleneuridine 2,2,2-trichloroethyl ester (18a)

The synthesis and purification of 18a, starting from 2',5'-bis-O-tert-butyldimethylsilyluridine[11] (0.57 g, 1,2 mmole) and 2,2,2-trichloroethyl dihydrogen phosphate[9] (0.34 g, 1.5 mmole) was performed as described for 11. Yield 0.26 g (27%), Rf 0.52 (system A).

2',5'-Bis-O-methoxytetrahydropyranyluridilyl-(3'- 5')-2',3'-O-methoxymethyleneuridine 2,2,2-trichloroethyl ester (18b)

The preparation and purification of 18b, starting with 2',5'-bis-O-methoxytetrahydropyranyluridine[18] (0.56 g, 1.2 mmole) and 2,2,2-trichloroethyl dihydrogen phosphate[9] (0.34 g, 1.5 mmole) were according to the same procedure as described for 11. Yield 0.53 g (56%), Rf 0.47 (system A).

Deblocking of dimer 18a

a. Reductive cleavage of the 2,2,2-trichloroethyl group.

Activated zinc[26] (ca 1 mmole) was added to a stirred solution of 18a (47.5 mg, 0.05 mmole) and 2,4,6-trisopropylbenzenesulphonic acid (3 mg, 0.01 mmole) in anhydrous pyridine (0.5 ml). After 3 min the reaction mixture was filtered to remove excess zinc and diluted with chloroform (100 ml). The solution was washed with 1 M triethylammonium bicarbonate (TEAB, pH 7.5, 3 ml) and 0.1 M TEAB. The organic layer was concentrated under reduced pressure to an oil and coevaporated with water (2 x 50 ml). The residue was dissolved in aqueous 0.01 N HCL (15 ml) and the pH was adjusted to 2.0 with 0.1 N HCL. After 18 h at 20° the solution was adjusted to pH 8 by addition of aqueous 0.5 M ammonia and lyophilized. TLC (system B) and HPLC (system C) revealed the presence of solely UpU (5). The homogeneity of 5 thus obtained was also confirmed by its complete digestion with RNase, VPD and SPD to give the expected products in the correct ratios.

b. Removal of the 2,2,2-trichloroethyl group by fluoride ion treatment with dry TBAF.

Dimer 18a (152 mg, 0.16 mmole) was dissolved in dry tetrahydrofuran (3.2 ml) containing 0.5 M tetrabutylammonium fluoride[12] (TBAF). TLC (system A) showed that both TBDMS groups as well as the 2,2,2-trichloroethyl group were removed within 1 min at 20°. Dowex 50W cation exchange resin (16g, 100-200 mesh, ammonium form) was added. The resin was filtered off and washed with water. The combined filtrates were evaporated down to dryness and the residue was dissolved in 0.01 N HCl (20 ml). The solution was adjusted to pH 2.0 by the

addition of 0.1 N HCl, neutralized, after 16 h at 20°, with 0.5 M aqueous ammonia and concentrated to a small volume. DEAE-ion exchange chromatography gave one main peak (Fig. 3a). The appropriate fractions were collected and lyophilized. This material was dissolved in 0.2 M Tris-HCl buffer (pH 8.0, 16.5 ml) containing RNase (8 mg). After 16 h at 37° the mixture was analysed by HPLC (system E) and purified on a column of DEAE-Sephadex (Fig. 3b). Both systems showed the presence of four peaks (*i.e.*, peaks i-iiii in Fig. 3b). The product in peak (i) was identical with uridine, Rf 0.32 (system B) and Rt 1.2 min (system E). The product in peak (ii) was identified as 2.2'-anhydrouridilyl-(3'- 5')-uridine (21, Rt 12.9 min, system E) as based on the following evidence. The combined fractions of peak (ii) (in Fig. 3b) were lyophilized and the solid was dissolved in 25 mM Tris-HCl buffer (pH 9.0, 2ml) containing 20 µg VPD. Separation on DEAE-Sephadex (Fig. 3c) gave mainly two peaks, one of which was identical with uridine 5'-phosphate. The compound in the other peak was in every aspect, $^1$H-NMR[30,31] and TLC (Rf 0.38, system B), identical with commercially (Sigma) obtained 2.2'-O-anhydrouridine (22, in Scheme 4). The identity of the product in peak (iii) in Fig. 3b was corroborated as follows. The product in peak (iii) was lyophilized, converted into the sodium form (Dowex 50W cation-exchange resin, 100-200 mesh, $Na^+$ form) and lyophilized with $D_2O$ (3 x 2 ml). $^1$H-NMR ($D_2O$, 24°) 2.88 ($H_{1'}$, Up, d, J 5.4Hz); 2.69 ($H_{1'}$, pU, d, J 3.2Hz). $^1$H-NMR ($D_2O$, 75°) 2.84 ($H_{1'}$, Up, d, J 5.4Hz); 2.71 ($H_{1'}$, pU, d, J 4.6Hz); 1.56 ($H_{2'}$, Up, $J_{P2'}$ 8.7Hz). These values show a distinct difference with the chemical shift and coupling constants of the corresponding protons in UpU (3'- 5') as reported in literature[32], but are very similar to the values in UpT (2'- 5')[33]. The NMR data, together with the finding that the product in peak (iii) was not digested by RNase indicate that the product in peak (iii) must be uridilyl-(2'- 5')-uridine (20, in Scheme 4, Rt 10.5 min in system E).

The product in peak (iiii) in Fig. 3b) was identical (Rf 0.13, system B and Rt 55.8 min, system E) with uridine 3'-phosphate.

Deblocking of dimer 18b with dry TBAF.

Dimer 18b (53 mg, 0.05 mmole) was treated with 0.5 M TBAF in dry tetrahydrofuran (1 ml). Removal of the 2,2,2-trichloroethyl group was complete within 50 h (t1/2,5 h, estimated on TLC, system A). Work-up and acid treatment were the same as described for 18a. HPLC-analysis of the product showed, apart from internucleotide cleavage products, the presence of UpU (5; Rt 10.5 system E).

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