

# 3',5'-Cyclic Dinucleoside Phosphates: Undesirable Intermediates in the Phosphotriester Approach to Oligonucleotide Synthesis

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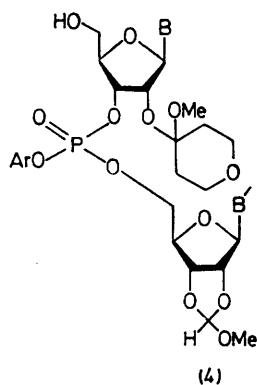
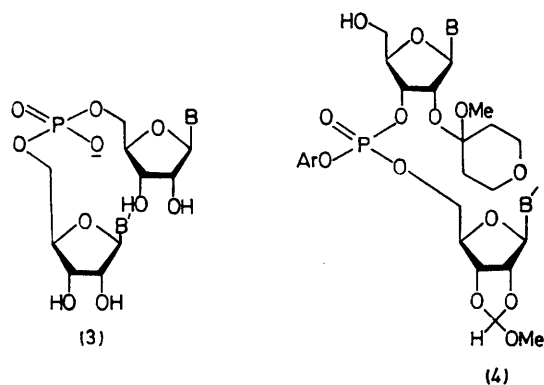
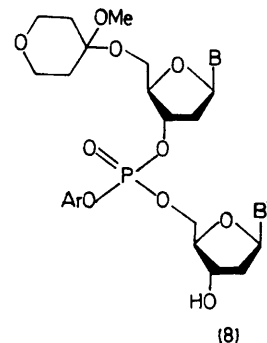
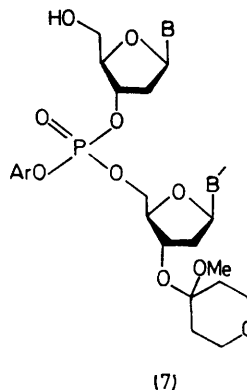
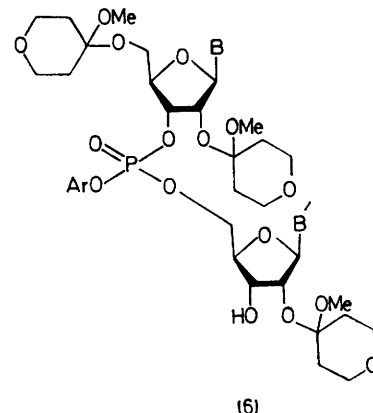
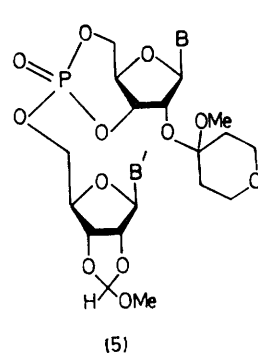
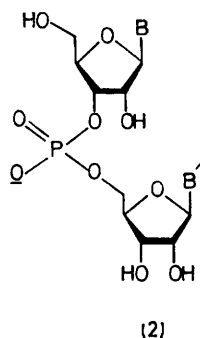
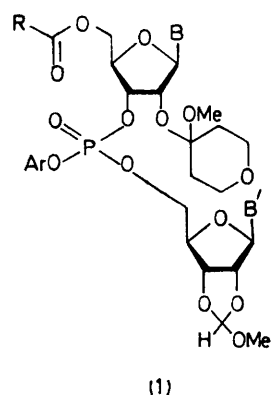
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**Summary** Mild alkaline hydrolysis of (4a) gives the 3',5'-cyclic dinucleoside phosphate (5a); more drastic alkaline hydrolysis of phosphotriester intermediates with free vicinal 5'- or 3'-hydroxy functions [(4a) and (7b), or (6a) and (8b)] leads to products with, respectively, 5' → 5'- or 3' → 3'- in addition to 3' → 5'- internucleotide linkages.

We have previously reported<sup>1,2</sup> a stepwise synthesis, by the phosphotriester approach, of oligoribonucleotides involving extension from the 5'-ends of the growing chains and leading to fully protected intermediates such as (1). It was at first envisaged that it might be possible to unblock (1) and corresponding longer chain protected oligoribonucleotides by a simple two-step process involving alkaline hydrolysis (to remove the protecting groups from the 5'-hydroxy functions and the internucleotide linkages) followed by acidic hydrolysis (to remove the methoxymethylene and methoxytetrahydropyranyl protecting groups) to give (2) and longer chain unprotected oligomers with exclusively 3' → 5'- internucleotide linkages. However, several years ago, we observed<sup>3</sup> that if fully-protected diribonucleoside phosphates

[e.g. (1)] were unblocked in this way, significant amounts of the 5' → 5'-isomers (3) were obtained in addition to the desired products (2). We concluded<sup>3</sup> that this result was most probably due to the cyclization of the intermediate 5'-hydroxy derivatives (4) and showed<sup>2,3</sup> that the formation



(a); B = B' = uracil-1-yl

(b); B = B' = thymine-1-yl

(a); B = B' = uracil-1-yl

of 5' → 5'-isomers could be completely suppressed by tetrahydropyranylation of the 5'-hydroxy functions prior to the unblocking of the internucleotide linkages. We now report the isolation for the first time of the putative intermediate 3',5'-cyclic dinucleoside phosphates (5) and provide evidence for the hydroxide ion-promoted hydrolysis of (4) [and hence of (1) unless the 5'-ester protecting group is unusually stable to alkali] proceeding virtually exclusively via (5).

The partially-protected uridylyl-(3' → 5')-uridine derivative (4a; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>), obtained<sup>4</sup> in good yield from its 5'-O-*p*-chlorophenoxyacetate ester (1a; R = 4-ClC<sub>6</sub>H<sub>4</sub>-OCH<sub>2</sub>, Ar = 2-ClC<sub>6</sub>H<sub>4</sub>), was separated by chromatography on silica gel into two pure diastereoisomers† which could be

† The presence of diastereoisomers of (4a; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) is due to the chiral phosphotriester group. A pure diastereoisomer of 2',3'-O-methoxymethylneuridine, (see B. E. Griffin, M. Jarman, C. B. Reese, and J. E. Sulston, *Tetrahedron*, 1967, 23, 2301) was used in the preparation of (1; B = B' = uracil-1-yl, R = 4-ClC<sub>6</sub>H<sub>4</sub>-OCH<sub>2</sub>, Ar = 2-ClC<sub>6</sub>H<sub>4</sub>).

distinguished by t.l.c.<sup>‡</sup> and <sup>1</sup>H n.m.r. spectroscopy. Treatment of the less polar (*R<sub>F</sub>* 0.29) diastereoisomer ( $\delta_1$ ) of (**4a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) with an excess of 0.1 M sodium hydroxide in aqueous dioxan (4:1 v/v) for 6 min at 20 °C gave the less polar (*R<sub>F</sub>* 0.31) diastereoisomer ( $\delta_1$ ) of (**5a**) which was isolated as a colourless solid in 55% yield. Under similar conditions, the more polar (*R<sub>F</sub>* 0.27) diastereoisomer ( $\delta_2$ ) of (**4a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) was converted somewhat more rapidly into the more polar (*R<sub>F</sub>* 0.27) diastereoisomer ( $\delta_2$ ) of (**5a**).§ The latter compound was isolated in 26% yield. It is thus apparent that the cyclization reaction is stereospecific and it is reasonable to assume<sup>5</sup> that it proceeds with inversion of configuration at phosphorus. The absolute configuration at phosphorus has not yet been established for either of the diastereoisomers of (**4a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) or (**5a**).

TABLE. Alkaline hydrolysis\* of partially-protected dinucleoside phosphates (**4**) and fully-protected 3',5'-cyclic dinucleoside phosphates (**5**)

Experiment	Substrate	Diastereoisomer <sup>b</sup>	Distribution of products <sup>c</sup>	
			% ( <b>2a</b> )	% ( <b>3a</b> )
1	( <b>4a</b> ; Ar = 2-ClC <sub>6</sub> H <sub>4</sub> )	$\delta_1$	61	39
2	( <b>4a</b> ; Ar = 2-ClC <sub>6</sub> H <sub>4</sub> )	$\delta_2$	80	20
3	( <b>4a</b> ; Ar = 2-ClC <sub>6</sub> H <sub>4</sub> )	$\delta_1 + \delta_2$	72	28
4	( <b>5a</b> )	$\delta_1$	58	42
5	( <b>5a</b> )	$\delta_2$	79	21
6	( <b>4a</b> ; Ar = Ph)	$\delta_1 + \delta_2$	78	22
7	( <b>4a</b> ; Ar = 2,4-Cl <sub>2</sub> C <sub>6</sub> H <sub>3</sub> )	$\delta_1 + \delta_2$	70	30

\* With 0.1 M sodium hydroxide in dioxan-water (1:4 v/v) for 3 h at 20 °C. <sup>b</sup> The less polar and more polar (higher and lower *R<sub>F</sub>*) diastereoisomers are indicated by  $\delta_1$  and  $\delta_2$ , respectively. Mixtures of diastereoisomers of (**4**), obtained directly by deacylation of the corresponding fully-protected dinucleoside phosphates (**1**) are indicated by  $\delta_1 + \delta_2$ . <sup>c</sup> As estimated by h.p.l.c. after removal of the methoxymethylene and methoxytetrahydropyranyl protecting groups.

It can be seen from the Table (experiments 1 and 2) that alkaline hydrolysis of either diastereoisomer of (**4a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) and subsequent removal of the acid-labile protecting groups led to a mixture of uridylyl-(3' → 5')-uridine (**2a**) and uridylyl-(5' → 5')-uridine (**3a**). It can also be seen that different proportions of (**2a**) and (**3a**) were obtained from each diastereoisomer. A comparison of the results of experiments 1 and 4 reveals that the proportions of (**2a**) and (**3a**) obtained from the less polar ( $\delta_1$ ) diastereoisomers of (**4a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) and (**5a**) were, within the limits of experimental error, virtually identical. The proportions of (**2a**) and (**3a**) obtained from the more polar ( $\delta_2$ ) diastereoisomers of (**4a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) and (**5a**) (experiments 2 and

5) were also virtually identical. These results indicate that alkaline hydrolysis of (**4a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) proceeds almost exclusively *via* (**5a**) and that direct attack of hydroxide ion on the phosphotriester group occurs, if at all, only to a minor extent. A comparison of the results of experiments 3, 6 and 7 suggests that a similar situation obtains in the alkaline hydrolysis of the corresponding phenyl and 2,4-dichlorophenyl esters (**4a**; Ar = Ph and 2,4-Cl<sub>2</sub>C<sub>6</sub>H<sub>3</sub>, respectively).

The present work adds emphasis to our earlier conclusion:<sup>3</sup> it is essential in the phosphotriester approach to oligoribonucleotide synthesis to protect terminal 5'-hydroxy functions with base-stable (*e.g.* tetrahydropyranyl) groups before unblocking the internucleotide linkages. Similar precautions are also necessary in the case of terminal 3'-hydroxy functions: thus treatment of (**6a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) first with alkali and then with aqueous acid gave (**2a**) (88%) and uridylyl-(3' → 3')-uridine (12%). Protection of terminal 5'- and 3'-hydroxy functions before unblocking is equally necessary in the deoxyribose series: thus when (**7b**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) was treated first with alkali and then with acid, a mixture of thymidylyl-(3' → 5')-thymidine (81%) and thymidylyl-(5' → 5')-thymidine (19%) was obtained and when (**8b**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) was unblocked in the same way, a mixture of thymidylyl-(3' → 5')-thymidine (70%) and thymidylyl-(3' → 3')-thymidine (30%) was obtained.¶ It is reasonable to conclude that alkaline hydrolysis of partially-protected dinucleoside phosphates of the types (**6**), (**7**) and (**8**) proceeds mainly *via* the corresponding 3',5'-cyclic dinucleoside phosphates but, so far, no attempt has been made to isolate such intermediates. When (**6a**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>), (**7b**, Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) and (**8b**; Ar = 2-ClC<sub>6</sub>H<sub>4</sub>) were tetrahydropyranylated before unblocking, the dinucleoside phosphates obtained contained solely 3' → 5'-internucleotide linkages.

It should finally be observed that, in the case of longer chain oligonucleotides corresponding to (**4**), (**6**), (**7**) and (**8**), partial migration of the terminal internucleotide linkages will occur unless the 3'- and 5'-terminal hydroxy functions are both protected with base-stable groups during the alkaline hydrolysis step. This conclusion should be emphasized as it appears from recent reports in the literature<sup>8</sup> that its importance is not fully appreciated in several laboratories in which the synthesis of oligonucleotides by the phosphotriester approach is being undertaken.

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‡ T.l.c. was carried out on glass plates coated with Merck Kieselgel GF<sub>254</sub> in the solvent system CHCl<sub>3</sub>-MeOH (92:8 v/v).

§ The two diastereoisomers ( $\delta_1$  and  $\delta_2$ ) of (**5a**) could easily be distinguished from each other and from the corresponding diastereoisomers of (**4a**) by <sup>1</sup>H and <sup>31</sup>P n.m.r. spectroscopy.

¶ Alkaline hydrolysis of the phenyl ester of thymidylyl-(3' → 5')-thymidine has recently been reported (H. Rokos, A. Myles, W. Hutzenlaub, and W. Pfeleiderer, *Chem. Ber.*, 1975, **108**, 2872) to give 74% of thymidylyl-(3' → 5')-thymidine together with 13% each of its (3' → 3')- and (5' → 5')-isomers.

<sup>1</sup> R. Saffhill, Ph.D. Thesis, Cambridge University, 1968, p. 89; C. B. Reese, *Colloques Internationaux du C.N.R.S.*, 1970, No. 182, 319.

<sup>2</sup> J. H. van Boom, P. M. J. Burgers, G. R. Owen, C. B. Reese, and R. Saffhill, *Chem. Comm.*, 1971, 869.

<sup>3</sup> G. R. Owen, Ph.D. Thesis, Cambridge University, 1971, p. 41.

<sup>4</sup> See ref. 2 for method of preparation.

<sup>5</sup> M. J. Gallagher and I. D. Jenkins, 'Topics in Stereochemistry,' vol. 3, eds. E. L. Eliel and N. L. Allinger, Interscience, New York, 1968, p. 31.

<sup>6</sup> K. Itakura, C. P. Bahl, N. Katagiri, J. J. Michniewicz, R. H. Wightman, and S. A. Narang; *Canad. J. Chem.*, 1973, **51**, 3649; K. Itakura, N. Katagiri, and S. A. Narang, *ibid.*, 1974, **52**, 3689; K. Itakura, N. Katagiri, S. A. Narang, C. P. Bahl, K. J. Marians, and R. Wu, *J. Biol. Chem.*, 1975, **250**, 4592; M. Sekine and T. Hata, *Tetrahedron Letters*, 1975, 1711; R. L. Letsinger, J. L. Finman, G. A. Heavner, and W. B. Lunsford, *J. Amer. Chem. Soc.*, 1975, **97**, 3278.