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

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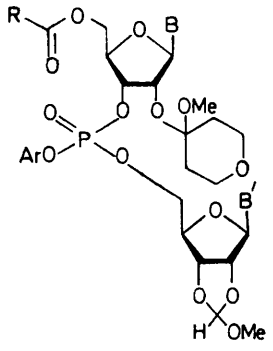
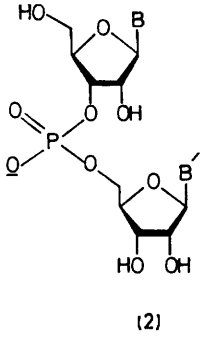
Summary Mild alkaline hydrolysis of (**4a**) gives the 3′,5′-cyclic dinucleoside phosphate (**5a**); more drastic alkaline hydrolysis of phosphotriester intermediates with free vicinal 5′- or 3′-hydroxy functions [(**4a**) and (**7b**), or (**6a**) and (**8b**)] leads to products with, respectively,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

WE have previously reported[1,2] 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[3] 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[3] that this result was most probably due to the cyclization of the intermediate 5′-hydroxy derivatives (**4**) and showed[2,3] that the formation



(1)



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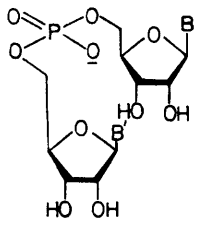
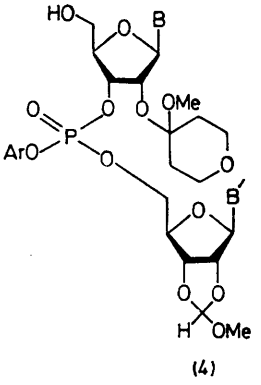
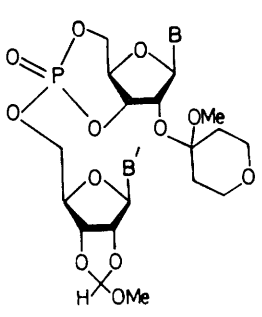


(3)

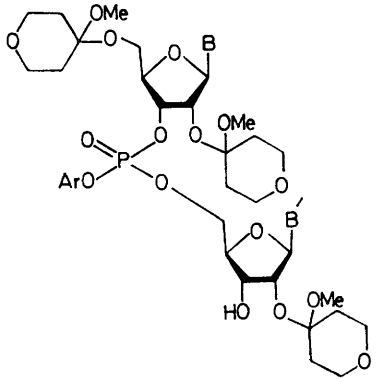


(4)

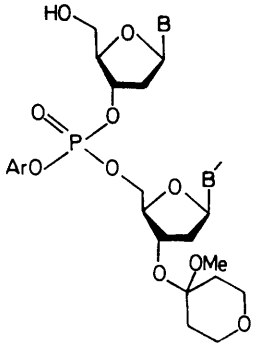
(a); B = B′ = uracil-1-yl



(5)



(6)



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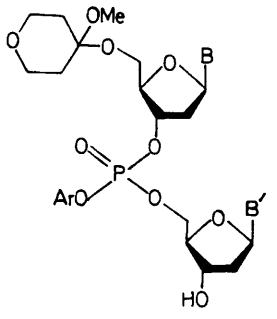

(8)

(a); B = B′ = uracil-1-yl
(b); B = B′ = thymin-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_6H_4), obtained[4] in good yield from its 5′-*O*-*p*-chlorophenoxyacetate ester (**1a**; R = 4-ClC_6H_4-OCH_2, Ar = 2-ClC_6H_4), was separated by chromatography on silica gel into two pure diastereoisomers† which could be

† The presence of diastereoisomers of (**4a**; Ar = 2-ClC_6H_4) is due to the chiral phosphotriester group. A pure diastereoisomer of 2′,3′-*O*-methoxymethyleneuridine, (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-$ClC_6H_4OCH_2$, Ar = 2-ClC_6H_4).

distinguished by t.l.c.‡ and ^{1}H n.m.r. spectroscopy. Treatment of the less polar (R_F 0·29) diastereoisomer (δ_1) of (**4a**; Ar = 2-ClC_6H_4) 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_F 0·31) diastereoisomer (δ_1) of (**5a**) which was isolated as a colourless solid in 55% yield. Under similar conditions, the more polar (R_F 0·27) diastereoisomer (δ_2) of (**4a**; Ar = 2-ClC_6H_4) was converted somewhat more rapidly into the more polar (R_F 0·27) diastereoisomer (δ_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[5] 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_6H_4) or (**5a**).

TABLE. Alkaline hydrolysis[a] of partially-protected dinucleoside phosphates (**4**) and fully-protected 3′,5′-cyclic dinucleoside phosphates (**5**)

Experiment	Substrate	Diastereoisomer[b]	Distribution of products[c] % (**2a**)	% (**3a**)
1	(**4a**; Ar = 2-ClC_6H_4)	δ_1	61	39
2	(**4a**; Ar = 2-ClC_6H_4)	δ_2	80	20
3	(**4a**; Ar = 2-ClC_6H_4)	$\delta_1 + \delta_2$	72	28
4	(**5a**)	δ_1	58	42
5	(**5a**)	δ_2	79	21
6	(**4a**; Ar = Ph)	$\delta_1 + \delta_2$	78	22
7	(**4a**; Ar = 2,4-$Cl_2C_6H_3$)	$\delta_1 + \delta_2$	70	30

[a] With 0·1 M sodium hydroxide in dioxan–water (1:4 v/v) for 3 h at 20 °C. [b] The less polar and more polar (higher and lower R_F) diastereoisomers are indicated by δ_1 and δ_2, respectively Mixtures of diastereoisomers of (**4**), obtained directly by deacylation of the corresponding fully-protected dinucleoside phosphates (**1**) are indicated by $\delta_1 + \delta_2$. [c] 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_6H_4) 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 (δ_1) diastereoisomers of (**4a**; Ar = 2-ClC_6H_4) and (**5a**) were, within the limits of experimental error, virtually identical. The proportions of (**2a**) and (**3a**) obtained from the more polar (δ_2) diastereoisomers of (**4a**; Ar = 2-ClC_6H_4) and (**5a**) (experiments 2 and 5) were also virtually identical. These results indicate that alkaline hydrolysis of (**4a**; Ar = 2-ClC_6H_4) 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-$Cl_2C_6H_3$, respectively).

The present work adds emphasis to our earlier conclusion:[3] 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_6H_4) 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_6H_4) 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_6H_4) 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_6H_4), (**7b**, Ar = 2-ClC_6H_4) and (**8b**; Ar = 2-ClC_6H_4) 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[6] 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_{254} in the solvent system $CHCl_3$—MeOH (92:8 v/v).

§ The two diastereoisomers (δ_1 and δ_2) of (**5a**) could easily be distinguished from each other and from the corresponding diastereoisomers of (**4a**) by ^{1}H and ^{31}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. Pfleiderer, *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.

[1] R. Saffhill, Ph.D. Thesis, Cambridge University, 1968, p. 89; C. B. Reese, *Colloques Internationaux du C.N.R.S.*, 1970, No. 182, 319.
[2] J. H. van Boom, P. M. J. Burgers, G. R. Owen, C. B. Reese, and R. Saffhill, *Chem. Comm.*, 1971, 869.
[3] G. R. Owen, Ph.D. Thesis, Cambridge University, 1971, p. 41.
[4] See ref. 2 for method of preparation.
[5] 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.
[6] 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.