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SOME OBSERVATIONS RELATING TO PHOSPHORYLATION METHODS IN OLIGONUCLEOTIDE SYNTHESIS

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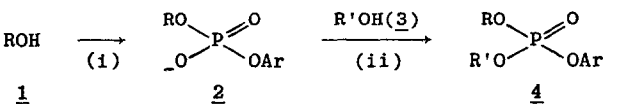
Summary. A very convenient procedure for the conversion of (N),O-5'-protected deoxyribonucleosides (8) into the triethylammonium salts of the corresponding 3'-(o-chlorophenyl) phosphates (9) is described. Good yields of partially-protected 3'→5'-dinucleoside phosphates (10) are obtained from the latter (9) and 3'-unprotected nucleoside building blocks (12a).

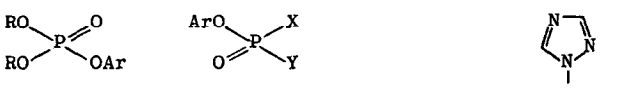
Two separate phosphorylation steps [(i) and (ii), Scheme 1] are required in the synthesis of oligonucleotides by the phosphotriester approach[1]. The first phosphorylation step (i), involves the conversion of a partially-protected nucleoside or oligonucleotide [ROH(1)] with a free (usually 3'-) hydroxy group into the corresponding aryl phosphate (2) or into an activated form of 2. In our original studies[2] on the synthesis of oligonucleotides by the phosphotriester approach, we used phenyl phosphorodichloridate (6; Ar = Ph, X = Y = Cl) as the phosphorylating agent. As might be expected, the formation of symmetrical by-products (such as 5) could not then be avoided. We later found that 4-nitrophenyl phenyl[3] and 2-chlorophenyl 4-nitrophenyl[4] phosphorochloridates (6a; Ar = C_6H_5 and 2-ClC_6H_4, respectively) were suitable phosphorylating agents for the first step [(i), Scheme 1] of oligonucleotide synthesis. However, further studies[5] have revealed that the latter reagents may only be used effectively in stepwise synthesis. Other monofunctional phosphorylating agents have also been suggested[6,7].

Scheme 1



1 2 4

Ar = aryl



5 6 a; X = Cl, Y = 4-$O_2NC_6H_4$ 7

Narang and his coworkers[8] have developed a method for converting 5'-protected 2'-deoxyribonucleosides and their N-acyl derivatives into the corresponding p-chlorophenyl 2-cyanoethyl phosphates in good yields by reaction first with p-chlorophenyl phosphorodi-(1,2,4-triazolide) [6; Ar = 4-ClC_6H_4, X = Y = 7] and then with 2-cyanoethanol. Agarwal and Riftina have reported[9] that the reaction of 5'-protected 2'-deoxyribonucleosides (corresponding to 1) with p-chlorophenyl phosphorodi-(1,2,4-triazolide) is not accompanied by the formation of symmetrical

3'→3'-dinucleoside phosphates (corresponding to 5). This result suggests that, although p-chlorophenyl phosphorodi-(1,2,4-triazolide) [6; Ar = 4-ClC_6H_4, X = Y = 7] is apparently a bifunctional phosphorylating agent and has been used as such[8], it is effectively monofunctional when it is used in excess. We now report that when 5'-protected 2'-deoxyribonucleoside derivatives (such as 8)[10] are treated with an excess of o-chlorophenyl phosphorodi-(1,2,4-triazolide) [6; Ar = 2-ClC_6H_4, X = Y = 7] and the products subjected tc hydrolysis, the corresponding 3'-(o-chlorophenyl) phosphates (9) are obtained and may readily be isolated[11] as their triethylammonium salts in very high yields (Table 1). Thus additional steps[8] involving the preparation and selective unblocking of phosphotriester intermediates may be avoided[12]. The phosphodiester products (9, corresponding to 2) may generally be isolated as stable colourless solids, uncontaminated (as indicated by ^{31}P n.m.r. spectroscopy and t.l.c.) with triethylammonium o-chlorophenyl phosphate or other impurities[13].

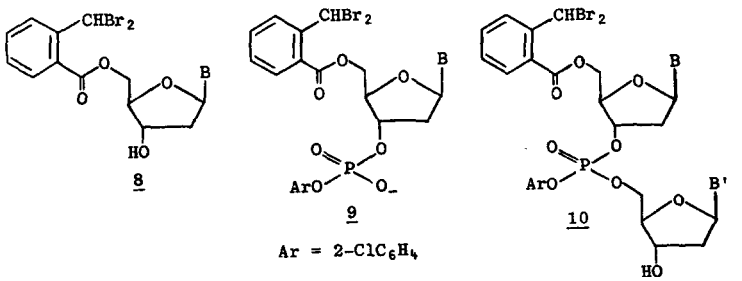
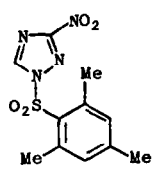


TABLE 1. Preparation of 3'-(o-Chlorophenyl) Phosphates

Substrate	Yield of Product (%)[a]	δ(p.p.m.)[b]	$R_F(A)$[c]	$R_F(B)$[c]
8 [B = 6-N-(p-t-butylbenzoyl)-adenin-9-yl]	93	- 5.89	0.79	0.45
8 [B = 4-N-benzoylcytosin-1-yl]	95	- 5.81	0.80	0.53
8 [B = 2-N-(p-t-butylphenylacetyl)-guanin-9-yl]	94	- 5.93	0.70	0.41
8 [B = thymin-1-yl]	95	- 6.43	0.89	0.58
10 [B = B' = thymin-1-yl]	94	-	-	-

[a] Isolated by precipitation as solid triethylammonium salts.
[b] These data relate to ^{31}P n.m.r. spectra in pyridine solution. Negative chemical shifts indicate resonance signals upfield from that of orthophosphoric acid which was used as an external standard. The chemical shift of the resonance signal of triethylammonium o-chlorophenyl phosphate is -4.62 p.p.m. in pyridine solution.
[c] T.l.c. was carried out on Merck Alufolien Cellulose GF_{254} in solvent systems (A) [ethanol-M-ammonium acetate (5:2 v/v)] and (B) [isobutyric acid - ammonia (d 0.88) - water (66:1:33 v/v)].



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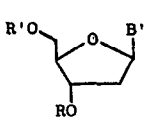
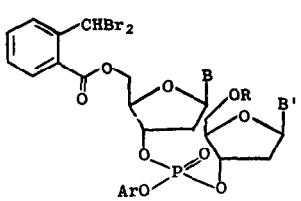


12 a; R = R' = H
b; R = Px, R' = H
c; R = H, R' = Px



13 a; R = H
b; R = Px

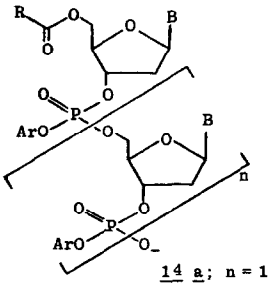
Ar = 2-ClC_6H_4; Px = 9-phenylxanthen-9-yl (pixyl)

We have found[4,14] that 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT, 11) is a very effective condensing agent in the second step [(ii), Scheme 1] of the phosphotriester approach. If a phosphodiester intermediate (9, corresponding to 2) is allowed to react with a 3'-protected 2'-deoxyribonucleoside building block [e.g. 12b, corresponding to 3] in the presence of MSNT (11), the internucleotide linkage in the product (corresponding to 4) will be unambiguously 3'→5'. However, the present study clearly demonstrates that it is not necessary to protect the 3'-hydroxy group in 12. Thus when a solution of 9 (B = thymin-1-yl, Et_3NH^+ salt; 2.0 mmol) and unprotected thymidine (12a; B' = thymin-1-yl; 2.5 mmol) in pyridine (40 ml) was treated with MSNT (11; 12.5 mmol) at room temperature and the reaction quenched after 20 min, 10 (B = B' = thymin-1-yl) was obtained as by far the major product. While the latter compound (R_F 0.28, Table 2) was obtained in 79% isolated yield, the yield of its 3'→3'-isomer (13a; B = B' = thymin-1-yl; R_F 0.38) was not greater than ca. 1-2%. Good yields of 3'→5'-isomers and only small quantities of 3'→3'-isomers were obtained in all five other examples of this regiospecific reaction so far examined (Table 2). Each of the 3'→3'-isomers (13a) was prepared in an unambiguous way from the appropriate phosphodiesters (9) and 5'-O-pixyl derivatives (12c)[15]. As, in all cases examined, the 3'→3'-isomers (13a) had higher R_F's (Table 2) than the 3'→5'-isomers (10), the latter could be isolated in a pure state following chromatography on silica gel[16].

TABLE 2. Preparation of Partially-Protected Dinucleoside Phosphates (10)

B (in 9)	B' (in 12a)	Yield[a] of 10 (%)	R_F[b] of 10	R_F[b] of 13a
6-N-(p-t-butylbenzoyl)-adenin-9-yl	2-N-(p-t-butylphenylacetyl)-guanin-9-yl	78	0.32	0.46
6-N-(p-t-butylbenzoyl)-adenin-9-yl	thymin-1-yl	78.5	0.32	0.35
4-N-benzoylcytosin-1-yl	4-N-benzoylcytosin-1-yl	81	0.36	0.46
4-N-benzoylcytosin-1-yl	thymin-1-yl	84	0.31	0.35
thymin-1-yl	4-N-benzoylcytosin-1-yl	76	0.33	0.38
thymin-1-yl	thymin-1-yl	79	0.28	0.38

[a] Isolated by precipitation, following purification by chromatography on silica gel.
[b] T.l.c. was carried out on Merck pre-coated silica gel F_{254} plates which were developed in the solvent system: $CHCl_3$-MeOH (9:1 v/v).



14 a; n = 1
b; n = 2

R = 2-$Br_2CHC_6H_4$; Ar = 2-ClC_6H_4

As indicated in Table 1 (final entry), partially-protected dinucleoside phosphates (10) may also be converted into their 3'-(o-chlorophenyl) phosphates (14a) in high yields by reaction with o-chlorophenyl phosphorodi-(1,2,4-triazolide) [6; Ar = 2-ClC_6H_4, X = Y = 7]. The partially-

protected dinucleotides (14a) thereby obtained may then be condensed with nucleoside building blocks (12a or 12b) to give partially- or fully-protected trinucleoside diphosphates. The latter may then readily be converted in the same way into the corresponding trinucleotide blocks (14b). We believe that the methods outlined in this paper suggest a very convenient approach to the synthesis of oligodeoxyribonucleotides. Indeed, preliminary results confirm that this approach is capable of leading to oligonucleotides of high quality.

Acknowledgement. We thank the Science Research Council for generous support of this work.

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[10] The 2-dibromomethylbenzoyl (DBMB) protecting group [as in 8; see J.B. Chattopadhyaya, C.B. Reese and A.H. Todd, J.C.S. Chem. Comm., in the press (1979)] has been especially designed for use in oligonucleotide synthesis; it may be removed from a protected nucleoside or oligonucleotide under very mild conditions indeed, by treatment first with silver perchlorate and 2,4,6-collidine in acetone-water (98:2 v/v) and then briefly with morpholine.

[11] The following procedure is recommended for the first step of the phosphotriester approach: To a solution of o-chlorophenyl phosphorodichloridate (0.60g, 2.44 mmol) in acetonitrile (2.5 ml) is added 1,2,4-triazole (0.44g, 6.36 mmol), followed by more acetonitrile (2.5 ml). Triethylamine (0.50g, 0.68 ml, 4.9 mmol) is then added and the reactants are stirred at room temperature. After 15 min, the nucleoside building block (8, 1.0 mmol) is added, followed by pyridine (5 ml). After 40 min, a solution of triethylamine (0.64g, 0.9 ml, 6.3 mmol) and water (0.3 ml) in pyridine (2 ml) is added and, after a further period of 10 min, the products are poured into a separating funnel containing saturated aqueous $NaHCO_3$ (100 ml). After thorough shaking, the resulting cloudy solution is extracted with chloroform (2-5 x 35 ml). The combined chloroform extracts are washed with saturated aqueous $NaHCO_3$ (2-5 x 80 ml), dried ($MgSO_4$) and concentrated under reduced pressure. A solution of the residual glass in chloroform (2.5 - 4 ml) is added dropwise to stirred petroleum ether (b.p. 30 - 40°C, 200 ml). The resulting colourless precipitate is collected by filtration and dried in a desiccator.

[12] Recently, Gough et al. [G.R. Gough, C.K. Singleton, H.L. Weith and P.T. Gilham, Nucleic Acids Res. 6, 1557 (1979)] have converted (N),O-5'-protected deoxyribonucleoside derivatives into the tetraethylammonium salts of the corresponding 3'-O-p-chlorophenyl phosphates by treatment with an excess of p-chlorophenyl phosphorodi-(1,2,4-triazolide). However, the products were isolated in a much more laborious manner which did not involve the removal of the p-chlorophenyl phosphate by extraction.

[13] The presence of 2-N-acylguanine residues does not appear to present a problem. Thus 2-N-benzoyl-2',3',5'-tri-O-benzoylguanosine was recovered in 94% yield after it had been treated with 1.5 molecular equivalents of o-chlorophenyl phosphorodi-(1,2,4-triazolide) [6; Ar = 2-ClC_6H_4, X = Y = 7] for 45 min at room temperature.

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[15] J.B. Chattopadhyaya and C.B. Reese, J.C.S. Chem. Comm. 639 (1978).

[16] Cashion et al. [P. Cashion, K. Porter, T. Cadger, G. Satha, T. Tranquilla, H. Notman and E. Jay, Tetrahedron Letters 3769 (1976)] have also concluded that it is unnecessary to protect the 3'-hydroxy groups of the nucleoside building blocks (15). However, these workers implied that the partially-protected dinucleoside phosphates which they obtained were uncontaminated with 3'→3'-isomers and, in any case, did not discuss the possibility of removing the latter impurities by chromatography.

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