Tetrahedron Letters No. 17, pp 1431 - 1434, 1977. Pergamon Press. Printed in Great Britain.

ATTEMPTS TO SUPPRESS INTERNUCLEOTIDE CLEAVAGE DURING UNBLOCKING OF OLIGONUCLEOTIDE PHOSPHOTRIESTER INTERMEDIATES

Ryszard W. Adamiak, René Arentzen and Colin B. Reese*

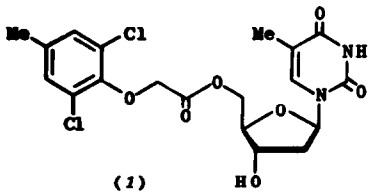
Department of Chemistry, King's College, Strand, London WC2R 2LS, England.

(Received in UK 31 January 1977; accepted for publication 14 March 1977)

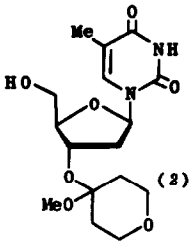
Perhaps the most serious problem which we have encountered in the synthesis of oligonucleotides by the phosphotriester approach with phenyl and other aryl protecting groups[1], is the internucleotide cleavage which accompanies the removal of the latter protecting groups. For example, in a recent study on the block synthesis of oligo- and poly-thymidylic acids in which phenyl protecting groups were used, we found[2] that the extent of internucleotide cleavage was *ca.* 3% per phosphotriester function even under the most favourable conditions of alkaline hydrolysis. Thus loss of product during unblocking was considerable except for oligonucleotides of comparatively low molecular weight.

An obvious approach to the solution of this problem is to use an aryl protecting group derived from a phenol which is more acidic than phenol itself. However, it was clear from earlier studies[3] that, if internucleotide cleavage was to be restricted to an acceptable amount, say, to *ca.* 0.5% per phosphotriester function, it would be necessary to use an aryl protecting group derived from a phenol with a $pK_a \sim 7.5$. It was therefore decided to undertake the block synthesis of oligothymidylic acids with the 2-nitro-4-t-butylphenyl protecting group. While the reason for the introduction of the 2-nitro group[4] is clear in the context of the present discussion, the 4-t-butyl group was introduced to increase the lipophilicity of the phosphotriester intermediates[5].

The 2-nitro-4-t-butylphenyl protected oligothymidylic acids were prepared by the procedure previously described[2] for the corresponding phenyl-protected intermediates. The fully-protected dinucleoside phosphate (*5a*, n = 0) was prepared in 66% isolated yield (Table) from stoicheiometric quantities of the two monomeric building blocks [(*1*) and (*2*)] and 2-nitro-4-t-butylphenyl dihydrogen phosphate (*3*)[7] in the presence of an excess of 2,4,6-tri-isopropylbenzenesulphonyl chloride (*4*, TPS) in anhydrous pyridine solution. It soon became apparent that 2-nitro-4-t-butylphenyl protected intermediates were sensitive even to mildly basic conditions. Indeed, it was necessary to acidify the eluting solvent during the chromatographic purification of these intermediates on neutral silica gel[8]. Treatment of (*5a*, n = 0) with 0.5 *M*-anhydrous hydrazine/acetonitrile and 0.086 *M*-HCl/aqueous dioxan (3:4 v/v) gave (*5b*, n = 0) and (*5c*, n = 0), respectively, in high yields (Table). Stoicheiometric quantities of the latter two intermediates were linked together[9] to give the fully-protected tetramer (*5a*, n = 2) which was then selectively unblocked at the 5'- and 3'-ends by treatment with hydrazine and acid to give (*5b*, n = 2) and (*5c*, n = 2), respectively. Finally, the fully-protected octamer (*5a*, n = 6) was obtained in modest yield (Table) by linking together (*5b*, n = 2) and (*5c*, n = 2); treatment of (*5a*, n = 6) with hydrazine gave (*5b*, n = 6). In



(1)



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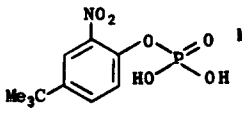

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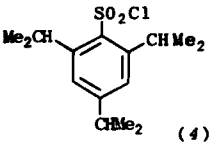

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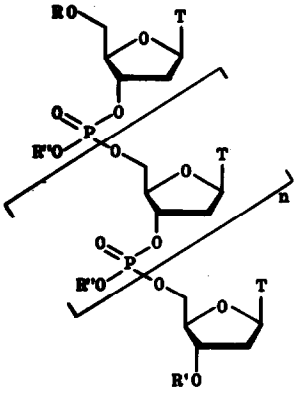
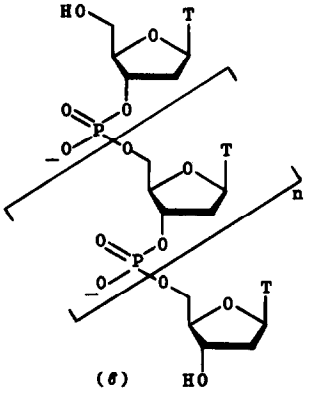
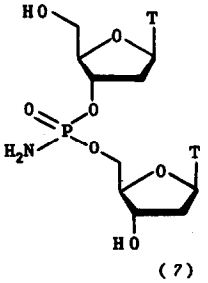


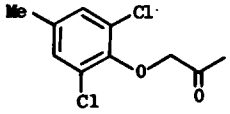


(6)

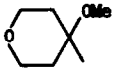


(7)

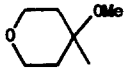
(5) *a*; R =



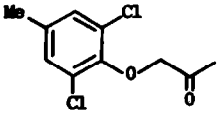
R' =



b; R = H , R' =



c; R =

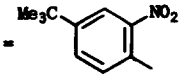


R' = H

d; R = R' =

T = thymin-1-yl

R' =



order to prevent terminal phosphoryl migration[10], (*5b*, n = 0), (*5b*, n = 2) and (*5b*, n = 6) were converted into their 5'-ketals [(*5d*, n = 0), (*5d*, n = 2) and (*5d*, n = 6), respectively] before they were further unblocked.

Table. Yields of Phosphotriester Intermediates

	% Yields[a]		
	n = 0	n = 2	n = 6
(*5a*)	66[b]	58[b]	37[b]
(*5b*)	93[c]	74[c]	57[c]
(*5c*)	90[c]	80[c]	
(*5d*)	53[de]	71[d]	57[d]

[a] All products were isolated as solids by precipitation.

[b] Based on (*1*) and (*2*), (*5b*, n = 0) and (*5c*, n = 0) or (*5b*, n = 2) and (*5c*, n = 2) as starting materials.

[c] Based on (*5a*, n = 0), (*5a*, n = 2) or (*5a*, n = 6) as starting material.

[d] Based on (*5b*, n = 0), (*5b*, n = 2) or (*5b*, n = 6) as starting material.

[e] A comparatively low yield of (*5d*, n = 0) was obtained as no acid stabilizer was added to the eluting solvent during the chromatographic purification step.

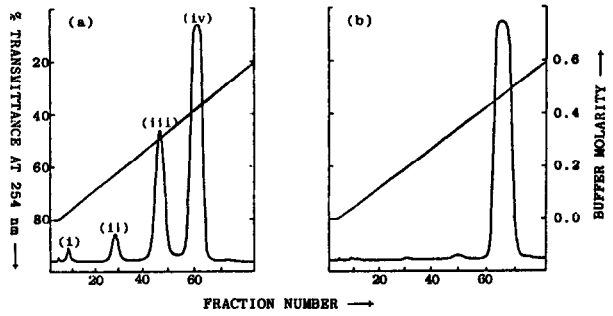


Figure 1. DEAE-Sephadex chromatography of products obtained by treating the fully-protected tetramer (*5d*, n = 2) (a) first with 10 *M*-NH_3/aqueous acetonitrile (1:1 v/v) followed by 0.01 *M*-hydrochloric acid and (b) first with 0.2 *M*-NaOH/aqueous dioxan (1:1 v/v) followed by 0.01 *M*-hydrochloric acid.

Both aqueous ammonia[11] and hydroxide ion[1,2,3] have been used to unblock aryl-protected internucleotide linkages. The danger of using aqueous ammonia became clear when treatment of (*5d*, n = 0) with 10 *M*-NH_3/aqueous acetonitrile followed by 0.01 *M*-hydrochloric acid, gave thymidylyl-(3' → 5')-thymidine (*6*, n = 0; *ca.* 90%) and the corresponding phosphoramidate (*7*; *ca.* 10%). Unblocking of (*5d*, n = 2) in the same way gave (*6a*, n = 2; *ca.* 69%) [Figure 1a, peak (iv)], di-anionic material (*ca.* 26%) [peak (iii)], mono-anionic material (*ca.* 4%) [peak (ii)] and a small amount (*ca.* 1%) of uncharged material [peak (i)]. The material in peaks (iii) and (ii) probably consisted of isomeric tetramer mono- and di-phosphoramidates, respectively, and the material in peak (i) probably contained tri-phosphoramidate.

In contrast, treatment of (*5d*, n = 0) with 0.2 *M*-NaOH/aqueous dioxan (1:1 v/v) proceeded in a straightforward manner and was virtually complete after 1 hr at 20°. No trace of internucleotide cleavage products such as (*2*) or its 5'-isomer could be detected by t.l.c. on silica gel. The corresponding tetramer (*5d*, n = 2) was similarly treated with alkali and then with 0.01 *M*-hydrochloric acid to remove all of its protecting groups. Chromatography of the resulting fully-unblocked material on DEAE-Sephadex revealed (Figure 1b) that (*6*, n = 2) accounted for *ca.* 98.5% of the total nucleotide products. Thus internucleotide cleavage had

been restricted to *ca.* 0.5% per phosphotriester function by the use of the 2-nitro-4-t-butylphenyl protecting group. Unfortunately the results obtained by unblocking the fully-protected octamer (*5d*, n = 6) were complex and it seems virtually certain that the latter material was impure. Even in the presence of an acid stabilizer, the chromatographic purification of 2-nitro-4-t-butylphenyl protected oligonucleotides on silica gel is difficult and becomes much more so with increasing molecular weight. We have therefore reluctantly come to the conclusion that the problem of internucleotide cleavage during unblocking cannot be solved solely by the introduction of electron-withdrawing substituents into the aryl groups.

It has been known for several years[12] that phosphotriesters are readily susceptible to nucleophilic attack by fluoride ion and, recently, three groups have reported[13] the use of the latter nucleophile in the unblocking of aryl-protected oligonucleotides. We are at present investigating the use of nucleophiles other than hydroxide ion and ammonia for this purpose and, in our opinion, this now seems to be the most promising approach to the problem.

Acknowledgements

We thank the Science Research Council and the Nuffield Foundation for generous support of this work.

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[3] J.H. van Boom, P.M.J. Burgers, P.H. van Deursen, R. Arentzen, and C.B. Reese, Tetrahedron Letters 3785 (1974).

[4] The pK_a of 2-nitrophenol is 7.23. It is anticipated that the introduction of the 4-t-butyl group would increase the pK_a by *ca.* 0.3 unit.

[5] We have observed that the solubility of phenyl-protected oligonucleotides in chloroform decreases markedly with increasing molecular weight. This leads to difficulties in the working-up of reactions and in the purification of products by short column chromatography[6] on silica gel.

[6] B.J. Hunt and W. Rigby, Chem. & Ind. 1868 (1967).

[7] This compound was obtained as a pure crystalline solid by Mr. Y.T. Yan Kui.

[8] Short column chromatography[6] was carried out on Reeve Angel CT silica gel with chloroform-ethanol mixtures containing 0.2 - 0.3% dimethylacrylic acid as eluting solvents. The acid was removed by extraction of the concentrated fractions with aqueous sodium hydrogen carbonate.

[9] As in the preparation of (*5a*, n = 0), a stoicheiometric quantity of (*3*) and an excess of TPS (*4*) were used.

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