Tetrahedron Letters No. 30, pp 2727 - 2730. 0040-4039/78/0722-2727$02.00/0
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OXIMATE ION PROMOTED UNBLOCKING OF OLIGONUCLEOTIDE PHOSPHOTRIESTER INTERMEDIATES

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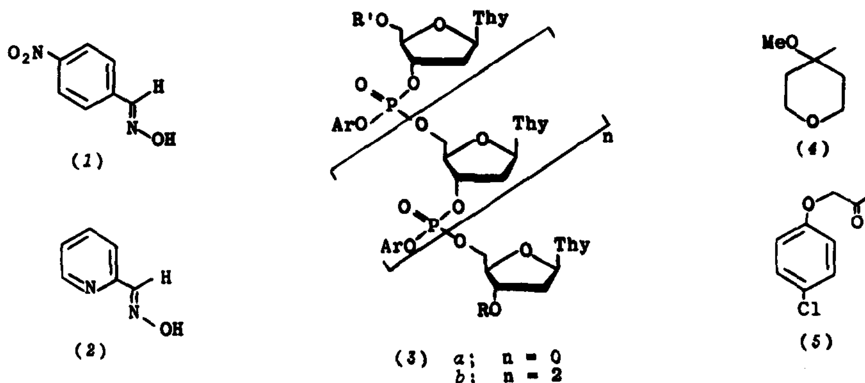
(Received in UK 12 May 1978; accepted for publication 25 May 1978)

Perhaps the most serious remaining problem in the synthesis of oligonucleotides by the phosphotriester approach[1] with aryl protecting groups has been the occurrence of internucleotide cleavage during the removal of the latter protecting groups. Thus, in a study on the synthesis of oligo- and poly-thymidylic acids with phenyl protecting groups, we found[2] that *ca.* 3% internucleotide cleavage per phosphotriester group occurred during unblocking under the most favourable conditions of alkaline hydrolysis which could be found.

There are two obvious approaches to the solution of this problem. The first approach involves the use of an aryl protecting group derived from a phenol which is more acidic than phenol itself. A recent study on the synthesis of oligothymidylic acids in which the internucleotide linkages were protected with 2-nitro-4-t-butylphenyl groups indicated[3] that the extent of internucleotide cleavage during unblocking by alkaline hydrolysis was only *ca.* 0.5% per phosphotriester group. However, when a protecting group derived from such an acidic phenol[4] is used, the phosphotriester functions are too susceptible to alkaline hydrolysis for the resulting intermediates to be suitable for the synthesis of high molecular weight oligo- and poly-nucleotides.

The other obvious approach to the solution of this problem involves the use of a nucleophile other than hydroxide ion in the unblocking step. Ammonia[5] [in aqueous solution] and tetra-alkylammonium fluorides [in tetrahydrofuran[6] or tetrahydrofuran-pyridine-water (8:1:1 v/v)[7]] have previously been used as alternative nucleophiles. Although ammonia, the use of which can lead both to phosphoramidate formation[3] and internucleotide cleavage[8], is perhaps marginally more satisfactory than hydroxide ion, it is, like hydroxide and fluoride ions (see Table and below), an unsuitable nucleophile for the unblocking of high molecular weight oligo- and polynucleotides. In this paper we show that the conjugate bases of *syn*-4-nitrobenzaldoxime[9] (*1*) and *syn*-pyridine-2-aldoxime[9] (*2*) are far superior nucleophiles for the present purpose and that their use leads to an acceptable level (not more than *ca.* 0.5 - 1% per phosphotriester group) of internucleotide cleavage when 2-chlorophenyl groups are used to protect the internucleotide linkages.

The substrate used in the unblocking experiments was the fully-protected tetranucleoside triphosphate (*3b*; R = R' = *4*). The fully-protected dinucleoside phosphate (*3a*; R = *4*, R' = *5*) was prepared by allowing the triethylammonium salt of 5'-*O*-*p*-chlorophenoxyacetylthymidine 3'-(2-chlorophenyl) phosphate[11], 3'-*O*-methoxytetrahydropyranylthymidine[3] and 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole[13] to react together in pyridine solution. The two partially-protected dinucleoside phosphates [*3a*; R = H, R' = *5* and *3a*; R = *4*, R' = H] were then prepared and linked together to give *3b* (R = *4*, R' = *5*). The latter compound was converted into *3b* (R = R' = *4*) by a previously reported procedure[2,3].



Ar = 2-ClC$_6$H$_4$-; Thy = thymin-1-yl

The phosphotriester linkages of the substrate (*3b*; R = R' = *4*) were unblocked in six different ways by treatment with (i) 0.1 *M*-sodium hydroxide in aqueous dioxan (1:1 v/v), (ii) concentrated aqueous ammonia, (iii) 0.05 *M*-tetra-n-butylammonium fluoride (TBAF) in anhydrous tetrahydrofuran, (iv) 0.05 *M*-TBAF in tetrahydrofuran-pyridine-water (8:1:1 v/v), (v) 0.3 *M*-N^1,N^1,N^2,N^2-tetramethylguanidinium(TMG) 4-nitrobenzaldoximate in aqueous dioxan (1:1 v/v), and (vi) 0.3 *M*-TMG pyridine-2-carboxaldoximate in aqueous dioxan (1:1 v/v). When the unblocking of the internucleotide linkages was complete, the 3'- and 5'-terminal methoxytetrahydropyranyl protecting groups were removed by acidic hydrolysis. Some details relating to these experiments are given in Table 1 and the results obtained are illustrated in Figure 1.

TABLE 1. Unblocking of Fully-Protected (Tp)$_3$T (*3b*; R = R' = *4*) at 20°

Experiment No.	Substrate Concentration ($M \times 10^3$)	Unblocking Reagent[a]	Solvent	Reaction Time (hr)	Yield of Unprotected (Tp)$_3$T (%)
(i)	8.5	0.1 *M*-NaOH	H_2O-dioxan (1:1 v/v)	16	93
(ii)	2.5	NH_3 (*d* 0.88)	H_2O	16	93.5
(iii)	8.5	0.05 *M*-TBAF	THF	0.5	51
(iv)	8.5	0.05 *M*-TBAF	THF-C_5H_5N-H_2O (8:1:1 v/v)	4	89
(v)	10.0	0.3 *M*-NBO[b,c]	H_2O-dioxan (1:1 v/v)	16	>98[d]
(vi)	10.0	0.3 *M*-PAO[b]	H_2O-dioxan (1:1 v/v)	16	97

[a] After the internucleotide linkages had been unblocked, the 3'- and 5'-terminal methoxytetrahydropyranyl protecting groups were removed by acidic hydrolysis (0.01 *M*-hydrochloric acid, 16 hr, 20°).

[b] N^1,N^1,N^2,N^2-tetramethylguanidinium salts of *syn*-4-nitrobenzaldoxime (*1*) and *syn*-pyridine-2-carboxaldoxime (*2*) are abbreviated to NBO and PAO, respectively. After 4 hr, an additional quantity (10 molecular equivalents with respect to substrate) of N^1,N^1,N^2,N^2-tetramethylguanidine was added.

[c] Following treatment with NBO, the products were allowed to react with aqueous ammonia (*d* 0.88) for 16 hr. at 20° before the final acidic hydrolysis step.

[d] This material underwent complete digestion in the presence of spleen phosphodiesterase to give thymidine (1 part) and thymidine 3'-phosphate (3 parts).

The fully-unblocked products obtained in each experiment were chromatographed on DEAE Sephadex A25 with triethylammonium bicarbonate (pH 7.5, linear gradient from 0.001 - 0.6 *M*) as the eluting buffer. The major product obtained in experiment (i) [Table 1 and Figure 1 (i), peak (d)], which was eluted from the column with the buffer concentration expected for $(Tp)_3T$, accounted for 93% of the total number of absorbance units (at 267 nm) of nucleoside and nucleotide products eluted. Peaks (a), (b) and (c) similarly corresponded to nucleoside/nucleotide cleavage products bearing charges of 0, -1 and -2, respectively, at pH 7.5. When ammonia was used in the first unblocking step [Table 1 and Figure 1 (ii)], the result was marginally better but internucleotide cleavage still exceeded 2% per phosphotriester group. By far the worst result was obtained when the conditions recently recommended by Ogilvie and his co-workers[6] were used [see Table and Figure 1 (iii)]. In this case, the yield of $(Tp)_3T$ was almost certainly less than 51% as it was not certain how much of the material eluted in peak (d) was the desired product. Thymidine [peak (a)] accounted for over 4% of the absorbance units eluted and cleavage products bearing charges of -1 and -2 [peaks (b) and (c)] for 17 and 28%, respectively. Although the alternative conditions involving fluoride ion [Table 1, Figure 1 (iv)], which were first used by Narang and his co-workers[7] and very recently recommended by van Boom and Burgers[14] led to less internucleotide cleavage (*ca.* 4% per phosphotriester group), they are clearly quite unsatisfactory for the unblocking of high molecular weight oligo- and poly-nucleotides.

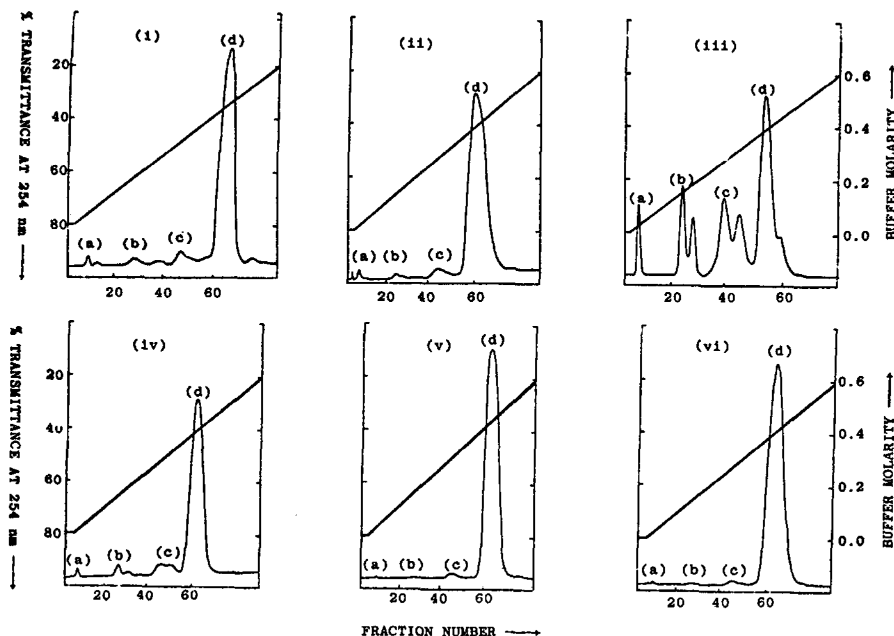


Figure 1. DEAE-Sephadex chromatography of the completely unblocked products obtained from the fully-protected tetranucleoside triphosphate (*3b*; R = R' = *4*). The 2-chlorophenyl protecting groups were removed (i) by treatment with 0.1 *M*-sodium hydroxide in dioxan-water (1:1 v/v), (ii) by treatment with aqueous ammonia (*d* 0.88), (iii) by treatment with 0.05 *M*-TBAF in anhydrous tetrahydrofuran, (iv) by treatment with 0.05 *M*-TBAF in tetrahydrofuran-pyridine-water (8:1:1 v/v), (v) by treatment with 0.3 *M*-TMG 4-nitrobenzaldoximate in dioxan-water (1:1 v/v), and (vi) by treatment with 0.3 *M*-TMG pyridine 2-carboxaldoximate in dioxan-water (1:1 v/v).

The N^1,N^1,N^2,N^2-tetramethylguanidinium salts of *syn*-4-nitrobenzaldoxime (*1*) and *syn*-pyridine-2-carboxaldoxime (*2*) are much more suitable unblocking reagents [Table 1, Figure 1 (v and vi)] than any of the others previously used. Indeed, it is not yet clear that the use of either of these reagents leads to any detectable internucleotide cleavage as it is not certain that the purity of the substrate (*3b*; R = R' = *4*) used in the experiments was greater than 98%. In experiment (v), the products were treated with aqueous ammonia before they were subjected to acidic hydrolysis but this additional step appeared to make virtually no difference. The unblocking of *o*-chlorophenyl esters of protected oligonucleotides occurs quite rapidly at 20° when a tenfold excess (per phosphotriester group) of the conjugate base of *1* or *2* in aqueous dioxan solution is used. Thus the fully-protected dinucleoside phosphate (*3a*; R = R' = *4*) was completely unblocked in under 3 hr. Phenyl-protected oligonucleotides undergo hydrolysis more slowly under the same conditions. It seems likely that other oximes with pK_a's of *ca.* 10, such as pyridine-3-aldoxime, pyridine-4-aldoxime and quinoline-2-aldoxime[15], will prove to be equally suitable for the present purpose.

We propose to discuss the results[13] of a mechanistic study of the reaction between oximate ions and phosphotriesters in a later publication. In conclusion, we believe that the work described in this paper constitutes a solution to what has perhaps been the most serious remaining problem in the synthesis of oligo- and poly-nucleotides by the phosphotriester approach.

Acknowledgements

We thank the Science Research Council and I.C.I. Corporate Laboratory (C.A.S.E. Studentship to R.C.T.) for generous support of this work.

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