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A rapid deprotection procedure for phosphotriester DNA synthesis

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ABSTRACT

An equimolar solution of aldoxime and tetramethylguanidine at 70°C removes both the base and phosphate protection from oligonucleotides prepared by solid phase phosphate triester technology. The rate of cleavage from the succinyl linkage commonly used for solid phase synthesis is also increased. The method is simpler, faster and more easily automated than existing methods.

INTRODUCTION

The synthesis of DNA using the solid phase phosphotriester method has been extensively researched in recent years with a dramatic improvement in the rate of synthesis. However, relatively few studies have been carried out to investigate the rates of deprotection. The oximate method was introduced by Reese et al and has been widely used to remove phosphate protecting groups [1,2] and reverse base modifications [3,4]. Base protection is normally removed with concentrated ammonia followed by an acid step to remove the 5'-protecting group.

We have found that an equimolar solution of aldoxime and tetramethylguanidine at 70°C cleaves the chlorophenyl protecting group on phosphorous, removes base protection and also cleaves 3'-succinyl groups attaching the oligonucleotide to the support. After acid treatment and ion exchange HPLC, oligonucleotides produced in this way are identical in terms of purity and yield to those produced by standard methods.

MATERIALS AND METHODS

Reagents

1,1,3,3-Tetramethylguanidine (TMG), p-nitrobenzaldoxime and pyridine-2-carboxaldoxime were purchased from the Aldrich Chemical Co. Ltd. o-Nitrobenzaldoxime was obtained from Lancaster Synthesis Limited.

HPLC System

HPLC was carried out on a Varian 5060 Liquid chromatograph with a Vista 401 data system. Reverse phase separations were carried out on a Varian Micropak column (4.6 x 250mm, MCH-5-N-CAP) using a gradient elution (water:acetonitrile:3M triethylammonium formate, pH5.0 from 65:30:5 to 0:95:5 over 30 minutes). Peaks were detected at 254nm and their areas quantified and calibrated by reference to known standards.

Synthesis and Sequencing

All oligonucleotides were prepared by an automated DNA synthesiser [5], deprotected by the various methods described below and purified by ion exchange chromatography.

The purified oligonucleotides were labelled using [γ-^{32}P]ATP and T_4

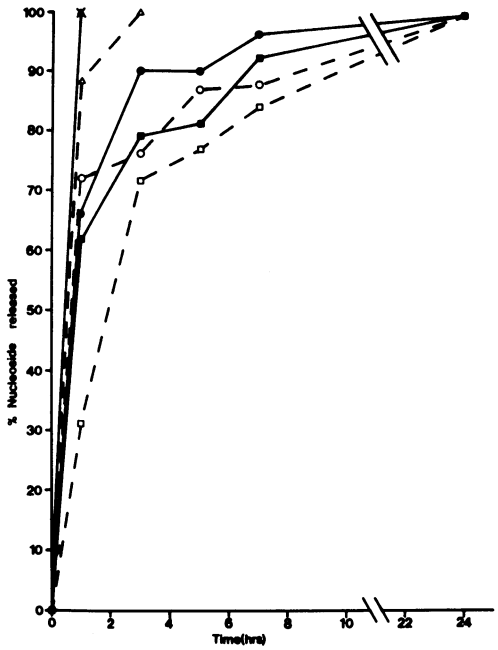

Figure 1.
Comparison of the rates of release of nucleoside from the support at 70°C.
$[(MeO)_2Tr]dbzAOCOCH_2CH_2CONH\sim$(P) + Oxime (+ TMG) ⟶
$[(MeO)_2Tr]dbzA_{OH}$ + $[(MeO)_2Tr]dA_{OH}$
✕——✕ p-nitrobenzaldoxime + TMG., ▲——▲ pyridine-2-carboxaldoxime + TMG.,
■——■ o-nitrobenzaldoxime + TMG., ○— — —○ TMG.,
□— — —□ p-nitrobenzaldoxime., ●——● o-nitrobenzaldoxime.,
△— — —△ pyridine-2-carboxaldoxime.

polynucleotide kinase and sequences confirmed by the mobility shift method of Jay et al [6].

Rate of Cleavage of Nucleoside from a Succinyl Support

Kieselguhr composite [7], (10mg, 0.8-1μmole of 2'-deoxyadenosine) was treated with a 0.3M solution of aldoxime and tetramethylguanidine in dioxan/water (1:1, v/v, 1ml) at 70°C with shaking. The supernatant was analysed by HPLC at 1,3,5,7 and 24 hour intervals.

Removal of Base Protecting Groups

5'-Dimethoxytrityl protected nucleosides (^{bz}C, ^{ibu}G, ^{bz}A, 17 μmoles) were treated with a 0.3M solution of aldoxime and tetramethylguanidine in

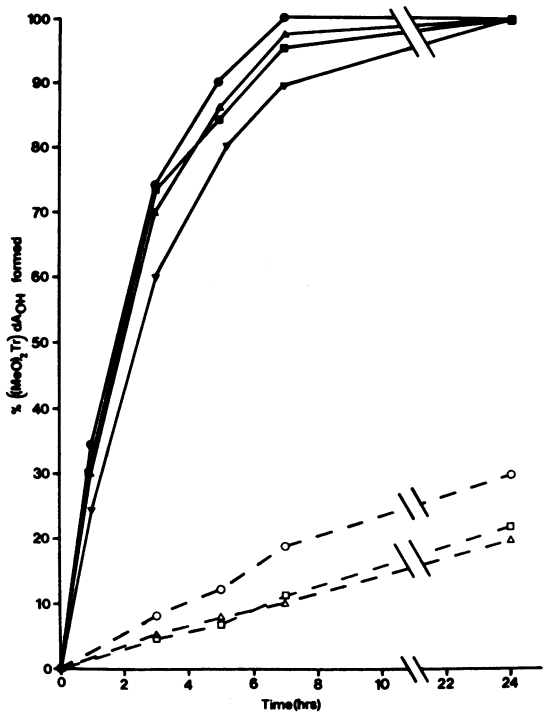

Figure 2.
Comparison of the rates of removal of the N-benzoyl group from $[(MeO)_2Tr]dbzA_{OH}$ at 70°C.

$[(MeO)_2Tr]dbzA_{OH}$ + Oxime (+ TMG) ⟶ $[(MeO)_2Tr]dA_{OH}$

17 μmoles 300μmoles 300μmoles

▲——▲ p-nitrobenzaldoxime + TMG., ■——■ o-nitrobenzaldoxime + TMG.,
▼——▼ pyridine-2-carboxaldoxime + TMG., ●——● TMG.,
□- - -□ pyridine-2-carboxaldoxime-, O- - -O p-nitrobenzaldoxime
△- - -△ o-nitrobenzaldoxime

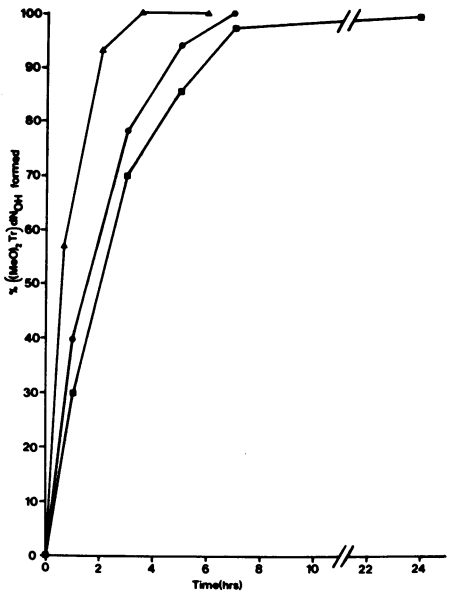

Figure 3.

Comparison of the rates of removal of the N-protecting group of $[(MeO)_2Tr]dbzA_{OH}$, $[(MeO)_2Tr]dbzC_{OH}$ and $[(MeO)_2Tr]dibuG_{OH}$ at 70°C with a 0.3M solution p-nitrobenzaldoxime and 1,1,3,3-tetramethylguanidine in dioxan/water (1:1).

■——■ $[(MeO)_2Tr]dA_{OH}$, ●——● $[(MeO)_2Tr]dG_{OH}$,
▲——▲ $[(MeO)_2Tr]dC_{OH}$

dioxan/water (1:1, v/v, 1ml) at 70°C. Aliquots were removed at 1,3,5,7 and 24 hour intervals and analysed by HPLC.

Rapid Method of Deprotection of Oligonucleotides

A 1.0M solution[8] of p-nitrobenzaldoxime and tetramethylguanidine in dioxan/water (1:1, v/v, 5ml) was added to the resin (150mg) and heated at 70°C for 17 hours. The resin was filtered off, washed with dioxan/water (3x5ml) and the combined filtrate adjusted to pH7.0 with 80% acetic acid and extracted with diethyl ether (3x5ml). The aqueous layer was evaporated in vacuo and treated with 80% acetic acid (5ml) for 15-30 minutes at room temperature. Water (5ml) was added and the mixture was extracted with diethyl ether. The aqueous layer was evaporated in vacuo, dissolved in water (2ml) and filtered prior to ion exchange purification on partisil 10 SAX column (25cmx0.7cm) at 60°C using a gradient elution (water: acetonitrile:3M triethylammonium formate, pH5.0 from 57:25:18 to 39:25:36 over 60 minutes).

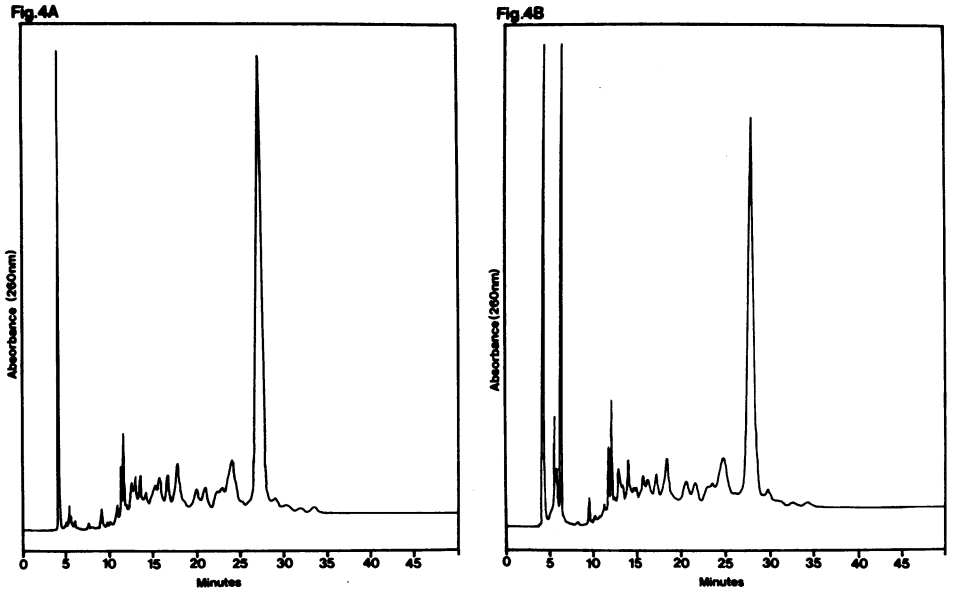

Figure 4A
An ion-exchange chromatogram of dAGAGATCTCT deprotected with
1) 0.3M 1,1,3,3-tetramethylguanidine salt of p-nitrobenzaldoxime for 18 hr at 37°C.
2) 0.880 ammonia for 3 hr at 70°C.
3) 80% acetic acid for 20 min.

Figure 4B.
An ion-exchange chromatogram of dAGAGATCTCT deprotected with
1) 1.0M 1,1,3,3-tetramethylguanidium salt of p-nitrobenzaldoxime for 18 hours at 70°C.
2) 80% acetic acid for 20 mins.

RESULTS AND DISCUSSION

The results of the succinyl cleavage (figure 1) show that a solution of tetramethylguanidine at 70°C cleaves the ester linkage completely within 24 hours. The addition of an equimolar concentration of p-nitrobenzaldoxime or pyridine-2-carboxaldoxime to the tetramethylguanidine solution increased the rate of hydrolysis so that the reaction was essentially complete after one hour. Interestingly, the addition of o-nitrobenzaldoxime did not appear to increase the rate of cleavage.

The results of the base deprotection studies for N^6-benzoyl-2'-deoxy-adenosine (the slowest case) using a basic solution of tetramethylguanidine are shown in figure 2. In this case the hydrolysis is only dependent on the concentration of tetramethylguanidine, the addition of aldoximes having

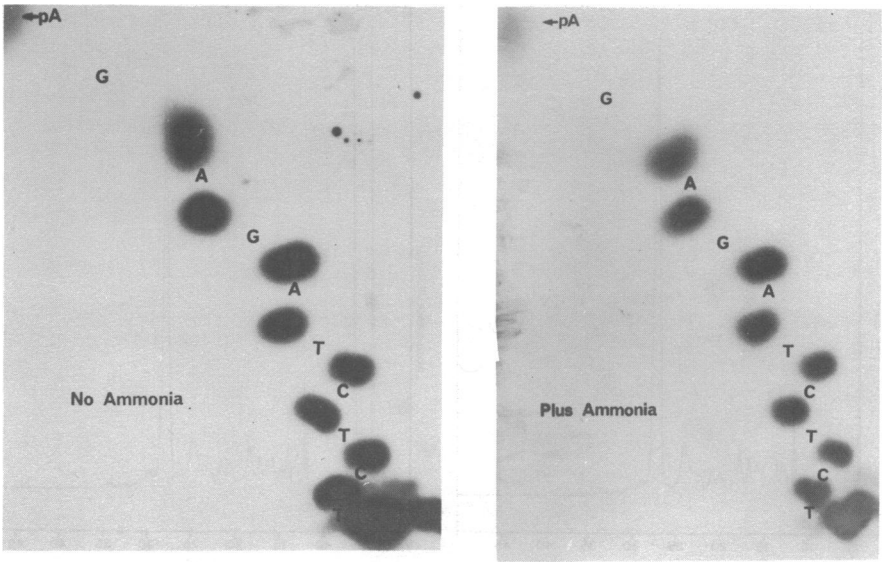

Figure 5
Mobility shift analysis of an oligonucleotide deprotected with and without ammonia as in legend to Figure 4.

no significant effect. A comparison of the rates of deprotection of N^6-benzoyl-2'-deoxyadenosine, N^4-benzoyl-2'-deoxycytidine and N^2-isobutyryl-2'-deoxyguanosine with tetramethylguanidine (+ p-nitrobenzaldoxime) is shown in figure 3. The rates of hydrolysis were in the order C^{bz} ($t_{\frac{1}{2}}$ ~54 mins)>G^{ibu}($t_{\frac{1}{2}}$ ~80 mins)>A^{bz}($t_{\frac{1}{2}}$ ~126 mins). The reverse phase HPLC traces of all four deprotected nucleosides showed no evidence of modification of the bases nor was any deamination of cytidine apparent after 24 hours reaction.

The ion exchange HPLC profiles of an oligonucleotide deprotected by the rapid method and by a standard method using ammonia are shown in figures 4A and 4B. As can be seen there are no significant differences detected by this analytical method, neither can any differences be seen (figure 5) using the mobility shift method of analysis. We have examined the reversal of base modifications under these conditions and find them to be essentially complete[9]. Furthermore the oligomers produced by this method have been found to be active in a range of biochemical experiments.

In conclusion therefore this deprotection procedure offers a simpler, safer and faster method which can be incorporated easily into the cycle of an automated DNA synthesiser.

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8. It has been found experimentally that some large oligonucleotides may be deprotected incompletely by 0.3M oximate and the concentration has therefore been increased to 1.0M to overcome this problem.
9. Unpublished observations.