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IMPROVEMENTS IN THE PHOSPHOTRIESTER SYNTHESIS OF DEOXYRIBOOLIGONUCLEOTIDES - THE USE OF HINDERED PRIMARY AMINES AND A NEW ISOLATION PROCEDURE

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Two improvements greatly enhancing the rate of phosphotriester oligonucleotide systhesis were developed 1) the use of hindered primary amines, e.g. t-butyl amine for decyanoethylation of oligonucleotide triester intermediates, 2) a simplified isolation procedure that eliminates the tedious bicarbonate extraction after each condensing reaction.

The improved phosphotriester method[1] is by far the most convenient and popular method for the synthesis of deoxyriboligonucleotides in solution. The new phosphite method developed by Caruthers[2] and Ogilvie[3] is suitable only for solid state synthesis of deoxyribooligonucleotides. Although the phosphite reagent is reactive, the phosphite method has the disadvantage that the reagents may be extremely sensitive to moisture and air.

This report describes two new improvements in the phosphotriester method that will increase the speed of solution phosphotriester synthesis greatly. The improved procedure can be used to make any oligonucleotide 12 to 18 units long in one day (comparable to the speed of the phosphite solid phase synthesis). In addition, since the phosphotriester is rather stable, the condensing reaction can be performed in an open atmosphere with consistent results.

In the phosphotriester synthesis, the key intermediate is a fully protected mononucleotide (1).

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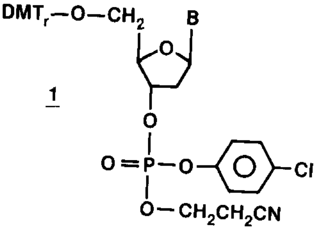


B = bases(a, N-benzoyladenine; b, N-benzoylcytosine, c, N-isobutyrylguanine and d, thymine)

DMT$_r$ = dimethoxytrityl

The standard condensing reactions involved first decyanoethylation of this protected nucleotide with either triethylamine[4,5] or diisopropyl amine[6]. Decyanoethylation reaction rates were investigated with various amines in order to find a better reagent for this reaction.

Two criteria were considered in choosing good decyanoethylating reagents. First, the amines should be volatile to be removed rapidly by evaporation. Secondly, the reagent should remove the cyanoethyl group preferentially. Since all the amines tested are volatile amines, the first criterion was met without further experimentation. Therefore, the selective removal of the cyanoethyl group was investigated.

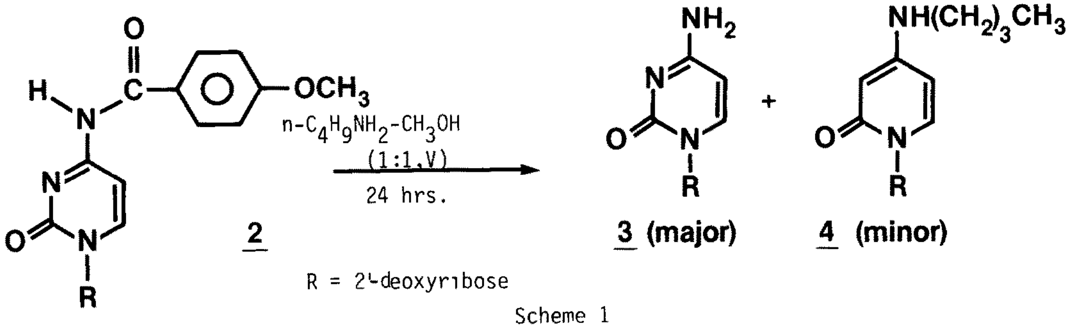
Fully protected mononucleotide 5'-O-dimethoxytritylthymidine -3'-β-cyanoethyl -p-chlorophenylphosphate (1 d) was used as a model compound and the results are shown in Table 1. The primary amines are generally faster decyanothylating agents than secondary amines, which, in turn, are faster than tertiary amines (Table 1).

Table I

Time needed for complete decyanoethylation of fully protected mononucleotide (1 d)

triethylamine	180 min.
diisopropylamine	60 min.
diethylamine	30 min.
sec-butyl amine	20 min.
t-butyl amine	10 min.
n-propyl amine	2 min.

In 1972, Weber and Khorana[7] reported that a primary amine (n-butyl amine) could react with protected nucleoside p-anisoyldeoxycytidine (scheme 1). The product are deoxycytidine (3, major) and n-butyl derivative of deoxycytidine (4, minor).



R = 2'-deoxyribose

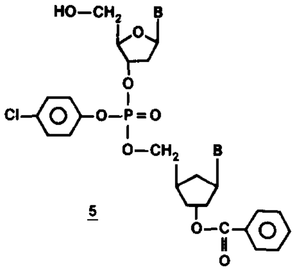
Scheme 1

The extent of the reactions of all four protected nucleosides with various amines[8] was investigated. The products (3 and 4) as suggested by Scheme 1 are more polar than starting protected nucleosides and therefore, could be separated from starting material (2) easily by silica gel tlc. The results (Table II) showed that most protected nucleosides were stable under these reaction conditions with all the amines tested, except that protected deoxycytidine and protected deoxyadenosine were somewhat sensitive to n-propyl amine. Thus, n-propyl amine is not suitable, but hindered primary amines such as t-butyl amine or sec-butyl amine should be ideal for decyanoethylation of triester.

Table II

Protected Nucleosides Amines	5'O-Dimethoxy tritylthymidine	5'O-Dimethoxy-trityl-N-benzoyl deoxycytidine	5'O-Dimethoxy-trityl-N-benzoyl deoxyadenosine	5'O-Dimethoxy trityl-N-Isobutyryl-deoxyguanosine
triethylamine	N.R.	N.R.	N.R.	N.R.
diethylamine	N.R.	N.R.	N.R.	N.R.
diisopropyl amine	N.R.	N.R.	N.R.	N.R.
N-Propyl amine	N.R.	trace polar products	trace polar products	N.R.
t-butyl amine	N.R.	N.R.	N.R.	N R.

Another possible side reaction is cleavage of the protecting chlorophenyl group on the oligonucleotide phosphotriester compound. To test this possibility, I have treated various protected dinucleotide triester compounds of general structure (5) with t-butylamine pyridine (1 9,V) for 10 min.[9]



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B = bases (N-benzoyladenine, N-benzoylcytosine, N-isobutyryl guanine and thymine)

The products from the cleavage of the chlorophenyl group are ionic phosphodiester species, which are easily separable from non ionic protected phosphotriester starting materials by reversed phase hplc (μ-Bondpak C-18, Waters - with acetonitrile H_2O (3 1,V). HPLC results indicated that no polar side products (detection limit 0.2 percent) were present.

Another time saving improvement was achieved by using anhydrous ether or ether/petroleum ether mixture to precipitate the oligonucleotide triester components from the condensing reaction mixture. The triester product can be isolated with greater than 90 percent purity in one step. The old procedure required several tedius steps of washing with saturated $NaHCO_3$, and H_2O (to decompose condensing agents), drying in vacuo and finally precipitating the products by ether. The new procedure requires one precipitation step with ether, and the condensing agents can be removed by decanting the supernatant.

Using these improvements, oligonucleotides can be prepared in an average of one per day, the major work load for oligonucleotide synthesis is now purfication and sequencing rather than synthesis. More than 100 oligonucleotides with defined sequences have been prepared by this new modified procedure

Acknowledgements

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Reference and Footnotes

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8. Each of the protected nucleosides (100 mg) was treated with 1 ml of amine/pyridine (1 10,V) for 1 hour.
9. Since the decyanoethylation reactions of all nucleotide triester compounds are finished in less than 10 minutes, I chose this time period to investigate the side reactions.

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