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FAST OLIGONUCLEOTIDE DEPROTECTION PHOSPHORAMIDITE CHEMISTRY FOR DNA SYNTHESIS

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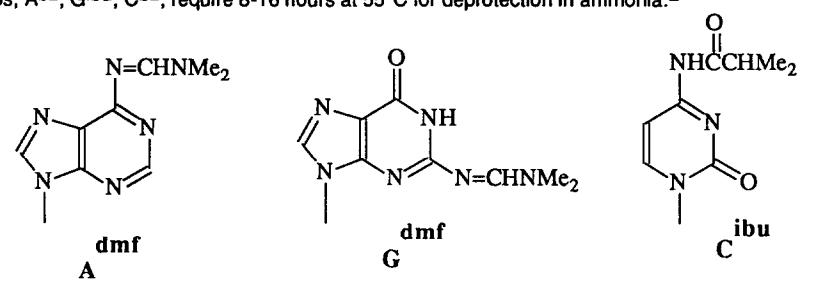
A new set of base protecting groups for cyanoethylphosphoramidite nucleosides and supports has been developed which decreases the post-synthesis time requirements. The traditional purine amide protecting groups, which require 8-16 hours at 55°C for deprotection in ammonia, have been replaced with the dimethylformamidine group. Oligonucleotides made with the new reagents require only 1 hour at 55°C or 8 hours at room temperature for complete deprotection. Dialkylformamidine phosphoramidites exhibit enhanced resistance to depurination compared to the traditional, or even the phenoxyacetyl, phosphoramidites.

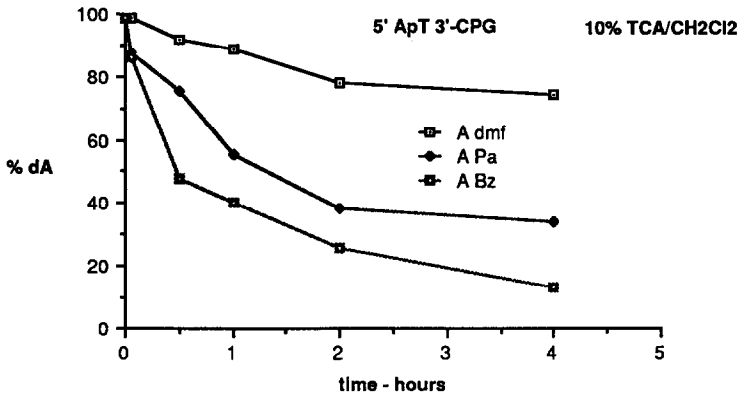
The optimal system for the rapid, efficient, convenient, and economical synthesis and purification of synthetic oligonucleotides has been advancing. In the interest of time savings, there remain many opportunities for improvements. By recognizing the very rapid reaction kinetics and taking advantage of an efficient, low volume delivery system, cycle times have decreased to about 5.5 minutes, without compromising performance.[1] Typical sequencing or PCR primers now require less than 2 hours synthesis time. After synthesis is complete, the traditional amide protecting groups, A^{bz}, G^{ibu}, C^{bz}, require 8-16 hours at 55°C for deprotection in ammonia.[2]



Dialkylformamidine (A,G) and isobutyryl (C) protection of the exocyclic amines of nucleosides has been shown effective in synthesis, and to impart depurination resistance.[3] For example, dialkylformamidine protected deoxyadenosine compounds were reported to be more resistant to depurination than N^6-benzoyl deoxyadenosine under the acidic detritylation conditions used for synthesizing DNA.[4] The set of A^{dmf}, G^{dmf}, and C^{ibu} cyanoethylphosphoramidites were synthesized and tested for optimum stability and rapid deprotection.

N^2-Dimethylformamidine-2'-deoxyguanosine(dG^{dmf})[5] was prepared in quantitative yield by reacting 2'-deoxyguanosine with N,N-dimethylformamide dimethyl acetal in methanol. No trace of N^1 or O^6 methylation was observed. The product dG^{dmf} was tritylated with 4,4'-dimethoxytrityl chloride in pyridine. After recrystallization, 5'-dimethoxytrityl-N^2-dimethylformamidine-2'-deoxyguanosine ($DMTdG^{dmf}$) resulted as a white powder (yield: 78%, purity by HPLC: 97%). Conversion to N^2-dimethylformamidine-2'-deoxyguanosine-3'-diisopropylaminocyanoethyl phosphoramidite was effected by treatment with bis(diisopropylamino)cyanoethyl phosphine and diisopropylammonium tetrazolide.[6] (^{31}P-NMR: 149.0 ppm; purity by HPLC: 98%). N^6-Dimethylformamidine-2'-deoxyadenosine-3'-diisopropylaminocyanoethyl phosphoramidite was prepared by similar procedures (dA^{dmf} - yield:

85%; DMTdA^{dmf} - yield: 80%, purity by HPLC: 92%; ^{31}P-NMR: 149.3; purity by HPLC: 96%). N^4-Isobutyryl-2'-deoxycytidine (dC^{iBu}) was prepared from 2'-deoxycytidine and isobutyric anhydride after transient silylation[7] (yield: 71%, purity by HPLC: 98%). DMTdC^{ibu} was obtained from tritylation with 4,4'-dimethoxytrityl chloride (yield:60%, purity by HPLC: 98%). N^4-Isobutyryl-2'-deoxycytidine-3'-diisopropylaminocyanoethyl phosphoramidite was prepared by the usual procedure (^{31}P-NMR: 149.1, 149.6 ppm, purity by HPLC: 98%). The supports were prepared by succinylation of the protected nucleosides (DMTdA^{dmf}, DMTdG^{dmf}, DMTdC^{ibu}) and coupling to aminopropyl CPG by the conventional method.[8]

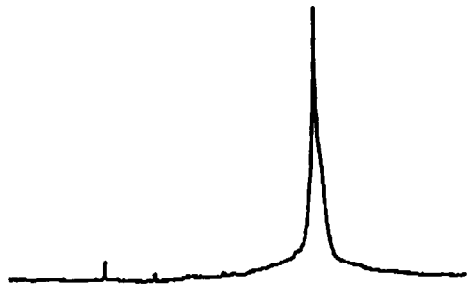


The depurination rates of N^6-protected 2'-deoxyadenosines; benzoyl (Bz), phenoxyacetyl (Pa)[9], and dimethylformamidine (dmf) were examined by treatment of dimer 5' ApT 3'-CPG with 10% TCA/CH_2Cl_2.[10] Depurination was terminated by washing thoroughly with 5% triethylamine/methanol and dichloromethane at 0.05, 0.5, 1, 2, and 4 hours. The dimers were cleaved from the support and deprotected with concentrated aqueous ammonia at room temperature (Pa and dmf) or at 55 C (Bz) for 8 hours. Depurination was assessed by HPLC analysis of the products obtained from the enzymatic digestion of the dimers. Measurement of the remaining deoxyadenosine was determined by the dA/T ratio of the dimer. The results show that the dimethylformamidine group conferred significantly more stability than benzoyl or phenoxyacetyl. It can be expected that dimethylformamidine protected bases will undergo less depurinative degradation during the course of DNA synthesis.

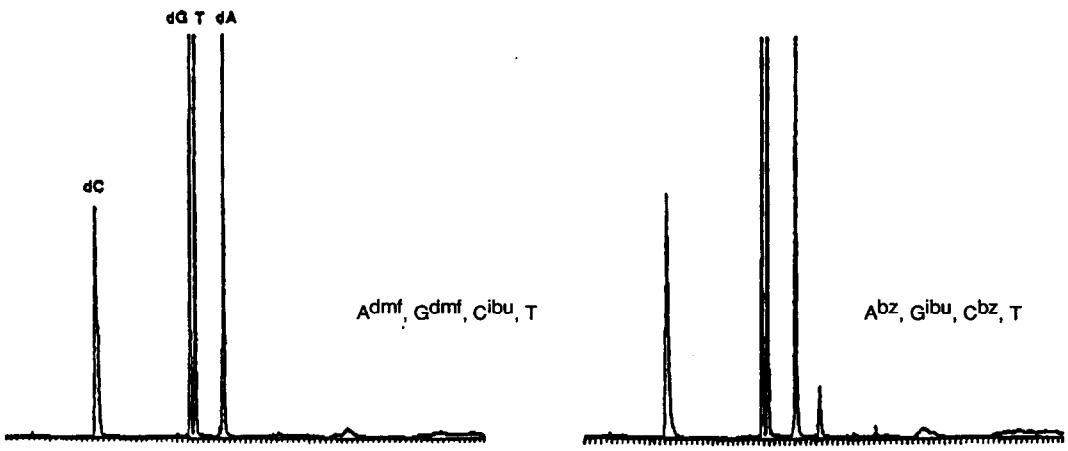
A large array of oligonucleotides (18 to 72mers) were synthesized with the new phosphoramidite reagents. Deprotection was complete after 1 hour at 55 C, or 8 hours at room temperature in concentrated aqueous ammonia. Dimethoxytrityl cation assay routinely gave 98% stepwise coupling yields. During the course of these investigations, it became apparent that the trityl assay does not always accurately indicate synthesis efficiency and predict high purity, authentic product. While the DMT assay is convenient and gives immediate data, it is indirect, measuring a by-product generated by impurities, as well as from the correct sequence oligonucleotide. More sensitive and direct analytical methods, especially capillary electrophoresis, give more definitive guidance to optimize oligonucleotide synthesis. HPLC and gel capillary electrophoresis[11] (Applied Biosystems Model 270A) analyses of the crude oligonucleotides showed high purity.

Enzymatic digestion of the oligonucleotides by snake venom phosphodiesterase and bacterial alkaline phosphatase gave the expected deoxynucleoside composition, with no detectable protected nucleosides or base

GEL CAPILLARY ELECTROPHORESIS
65mer, 98.1% trityl yield, OPC purified[12]
A^{dmf}, G^{dmf}, C^{ibu}, T phosphoramidites
Model 380B DNA synthesizer,
5.5 minute/cycle ssceaf3, version 2.00

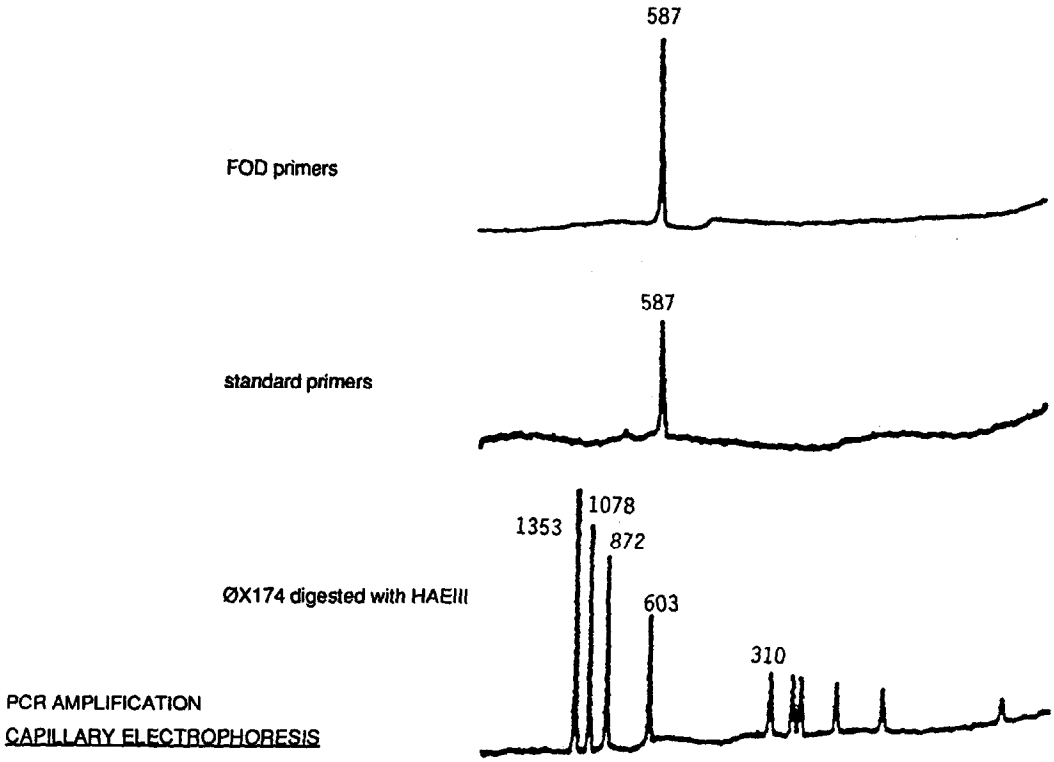


modifications.[13] HPLC analysis, shown below, of the digest products from crude 72mers made with the two sets of phosphoramidites showed no essential differences, other than benzamide from deprotection of A^{bz} and C^{bz}.



Oligonucleotide primers were tested in the polymerase chain reaction (PCR).[14] Crude 21mer primers were made with formamidine and standard phosphoramidites and compared in the amplification of a 587bp template within the 603bp fragment of ØX174 DNA digested with HAEIII restriction enzyme. The reactions were analyzed by polyacrylamide gel electrophoresis with ethidium bromide staining and by capillary electrophoresis (shown below). In both analyses, the primers made by each set of phosphoramidites yielded efficient amplification of the correct length product, with no spurious products.

The new set of fast deprotecting phosphoramidites, A^{dmf}, G^{dmf}, and C^{ibu}, fulfill the performance and applications criteria for the production of quality oligonucleotides in high yield. The dimethylformamidine phosphoramidites exhibit enhanced resistance to depurination compared to the traditional, or even the phenoxyacetyl, phosphoramidites. Oligonucleotides made with the new reagents require only 1 hour at 55 C or 8 hours at room temperature for complete deprotection. DNA synthesizers with the automated cleavage feature, started in the evening, yield fully deprotected oligonucleotides the next morning. As an illustration, typical primers can be ready (designed, synthesized, cleaved, deprotected, and quantitated) in just half of a working day.



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