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Fast Cleavage and Deprotection of Oligonucleotides

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Abstract: We have developed methylamine/ammonia as a fast cleavage and deprotection reagent which effects complete cleavage of oligonucleotides from the solid support in 5 min at room temperature and complete deprotection in 5 min at 65°C. The problem of transamination side product formation, faced with the commonly used dC^{bz} (10.0% side product) upon treatment with methylamine/ammonia, has been successfully solved by the use of dC^{ac} (0.0% side product). DMT dC^{ac} phosphoramidite –methylamine/ammonia system furnished oligonucleotides in equal or superior quality as compared to the other systems.

Present state-of-the-art of the DNA synthesis primarily involves (a) sequential assembling of nucleotides on an insoluble solid support (b) cleavage of the synthesized oligonucleotide from the solid support and (c) deprotection of the oligonucleotide to produce biologically active material. The past decade has witnessed revolutionary improvements in the coupling chemistry as well as automation which enables one to assemble a typical oligonucleotide sequence (~20 mer) on the solid support in a matter of 1-2 h. However, cleavage of the synthesized oligonucleotide from the solid support using ammonia still takes one hour and no improvements have been made in this area. Complete deprotection of the oligonucleotide requires further treatment at 65°C for 3h when the conventional and well established nucleotide derivatives .. dA^{bz}, dC^{bz}, dG^{ibu}, and T .. are employed.

In order to reduce the deprotection time, several protecting groups like phenoxyacetyl,[1] dimethylformamidine[2] and tert-butylphenoxyacetyl[3] groups which are more labile towards aminolysis have been developed in recent years. While the use of these protecting groups reduced the deprotection time to anywhere between 15 min and 60 min at 55 °C, they have been reported to have inherent drawbacks.[3-5]

In this study, we have taken an alternative approach which uses the well established nucleotide derivatives, but uses a reagent which is more nucleophilic than ammonia for cleavage and deprotection. We have tested the following amines: methylamine, dimethylamine, ethylamine, diethylamine, propylamine, isopropylamine, n-butylamine, n-pentylamine and n-hexylamine and their mixtures with ammonia, and found that methylamine/ammonia[6] gave the fastest cleavage and deprotection kinetics. This reagent, which incidentally is more volatile than ammonia and hence can be conveniently evaporated after the use, facilitates cleavage of oligonucleotides from the solid support in 5 min at room temperature as shown in the kinetic plot in Figure 1 and it deprotects oligonucleotides significantly faster as shown in Table I.

Figure 1. Cleavage of oligonucleotides from the CPG support

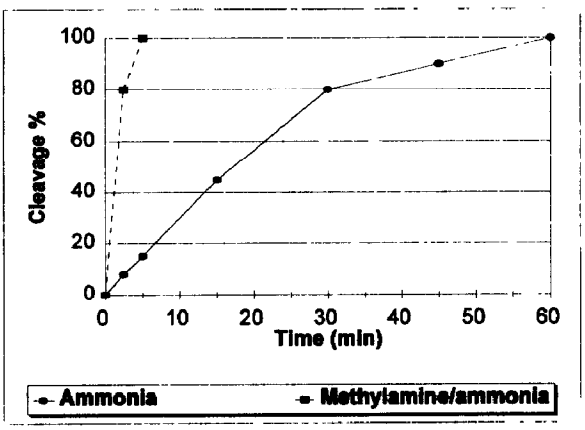


Table I. Deprotection Time[7]

Temperature	Methylamine/ Ammonia	Ammonia
25°C	75 min	72 h
37°C	30 min	20 h
55°C	10 min	4 h
65°C	5 min	3 h
80°C	2 min	1 h

Although methylamine/ammonia gave faster cleavage and deprotection kinetics, it was associated with a significant problem, namely, the generation of transamination side product during the deprotection of dC^{bz}. This is consistent with the observations made by Khorana et al,[8] Miller et al,[9] Polushin et al[10] and Hogrefe et al[11] when they used alkylamines for deprotection. It is formed in a competitive reaction as depicted in Figure 2 in which alkylamine attacks the pyrimidine ring of deoxycytidine instead of the carbonyl carbon of the deoxycytidine protecting group. We have rationalized that an increase in the electropositivity of the carbonyl carbon will result in the acceleration of the rate of deprotection, thereby reducing or eliminating the chances of an attack on the pyrimidine ring. As the data in Table II shows, protection of deoxycytidine with the acetyl group has virtually eliminated the side product formation.

Figure 2.

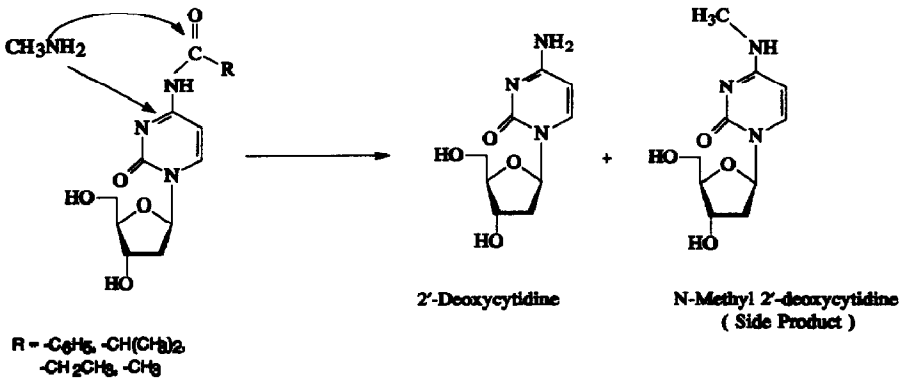
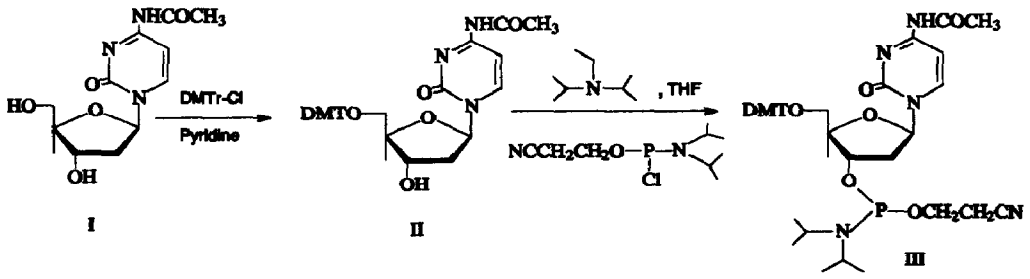


Table II.

Protected deoxycytidine	Deprotection time by methylamine/ammonia[12]	Side product formed[13] (N-methyldeoxycytidine)
$dC^{benzoyl}$	1 hour	10.0%
$dC^{isobutyryl}$	2 min	0.7%
$dC^{propionyl}$	1 min	0.05%
dC^{acetyl}	< 1 min	0.0%

In order to use the acetyl protected deoxycytidine in the DNA synthesis, 5'-DMT- N^4-acetyl-2'-deoxycytidine 3'-phosphoramidite (III) was synthesized starting from N^4-acetyl- 2'-deoxycytidine[14] (I) as shown in Figure 3. It was tritylated to give the compound II in 80% yield with a HPLC purity of 99% which was then converted to the phosphoramidite (III) using standard procedures[15] in 85% yield (99.66% pure by HPLC, ^{31}P NMR: 149.99 ppm, 149.43 ppm corresponding to the two diastereomers).

Figure 3.



Several oligonucleotides with the lengths ranging from 10 mer to 120 mer were synthesized by using dC^{ac}, dA^{bz}, dG^{ibu} and T phosphoramidites for the synthesis and methylamine/ammonia for the cleavage and deprotection. The quality of the oligonucleotides was at least as good as those synthesized using dC^{bz}, dA^{bz}, dG^{ibu} and T phosphoramidites---ammonia system as monitored by capillary electrophoresis, slab gel electrophoresis and reverse phase HPLC. Figure 4 shows the capillary electrophoresis of a 101 mer synthesized using the Beckman Oligo -1000. Also, there was no discernible difference in the performance of oligonucleotides in the following applications: PCR amplification, DNA sequencing by dideoxy termination method, 5'- kinasing, 3'-terminal transferase extension, hybridization probes and Tm analysis.

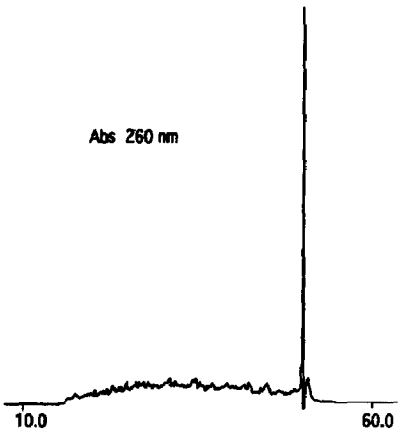


Figure 4. Capillary electrophoretic scan of a 101 mer run on the Beckman P/ACE 2000 using a gel filled capillary.

In conclusion, we have developed methylamine/ammonia used in conjunction with DMT dC^{ac} phosphoramidite as a fast cleavage and deprotection system[16] which facilitates cleavage of oligonucleotides from the solid support in 5 min at room temperature and deprotection in 5 min at 65°C. The chemical and biological integrity of the oligonucleotides synthesized using this fast system has been established.

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6. The reagent is prepared by mixing in equal volumes 40% aqueous methylamine and 29% aqueous ammonia.
7. The extent of deprotection of oligonucleotides was monitored by taking aliquots at various times, digesting them into the individual nucleosides, using a mixture of phosphodiesterase I and alkaline phosphatase, and analysing by reverse phase HPLC.
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12. Deprotection time was determined by both reverse phase HPLC analysis and TLC analysis.
13. The percentage of N-methyldeoxycytidine was determined from the reverse phase HPLC.
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16. a) Reddy, M. P.; Hanna, N. B.; Farooqui, F. **1992**, Patents pending. b) Reddy, M. P.; Hanna, N. B.; Farooqui, F. **1993**, Presented at the San Diego conference: Beyond DNA probes, Abstract # 43. c) It is available from Beckman Instruments under the name "UltraFast Cleavage and Deprotection kit".

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