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Methylamine Deprotection Provides Increased Yield of Oligoribonucleotides

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Abstract: Use of methylamine or methylamine/ammonium hydroxide as a cleavage and deprotection reagent for the solid phase synthesis of oligoribonucleotides has significantly increased the yield of the full length oligoribonucleotides as compared to the use of conventional ammonium hydroxide/ethanol.

Chemical synthesis of RNA is now practical with the development of phosphoramidite chemistry and the use of t-butyldimethylsilyl (t-BDMS) protecting group for the 2'-hydroxyl group.[1] However, synthesis of long sequences of RNA in reasonable yields has been a challenge partly due to the instability of the 2'-silyl protecting group towards basic deprotection conditions.[1-3] Concentrated aqueous ammonium hydroxide deprotection under heating causes some premature partial removal of the 2'-silyl group, thereby facilitating the attack of the liberated hydroxyl group on the adjacent phosphodiester group, ultimately resulting in the chain cleavage. Use of anhydrous ethanolic ammonia or a mixture of ethanol and ammonium hydroxide is reported to have significantly suppressed the undesirable desilylation.[1,2] Toward a similar goal, phenoxyacetyl,[2] dimethylformamidine[3] and t-butylphenoxyacetyl[4] protecting groups which are more labile toward aminolysis have been introduced to protect the exocyclic amino groups. However, the corresponding nucleoside phosphoramidites are associated with some drawbacks such as reduced stability[4] and increased cost.

We have reported earlier the development of methylamine/ammonium hydroxide[5] as a fast cleavage and deprotection reagent for the synthesis of oligodeoxyribonucleotides. Use of this reagent in the place of ammonium hydroxide reduced the cleavage time from 60 min to 5 min at room temperature and deprotection time from 3 h at 65°C to 5 min at 65°C or 75 min at room temperature. Methylamine alone gave very similar performance.[6] To avoid the transamination side product formed by methylamine with the commonly used dC^{bz} phosphoramidite, dC^{ac} phosphoramidite was developed. We have now reasoned that the use of methylamine or methylamine/ammonium hydroxide in the place of ammonium hydroxide for the cleavage and deprotection of oligoribonucleotides will avoid the exposure of oligoribonucleotides to elevated temperatures and also reduce the exposure time to the basic conditions, and consequently will result in reduced chain cleavage. In order to test this hypothesis, we have performed a comparative evaluation of methylamine, methylmine/ammonium hydroxide and ammonium hydroxide/ethanol as cleavage and deprotection reagents.[7]

Oligoribonucleotides of various lengths (Table I) were synthesized[8] using 5'-DMT-2'-TBDMS A^{bz}, G^{ibu}, T and C^{Ac} 3'-phosphoramidites. In order to avoid the transamination side product formation[5] with the use of methylamine or methylamine/ammonium hydroxide, it was necessary to use C^{Ac} phosphoramidite instead of the commonly used C^{bz} derivative. We synthesized it by a modification of

the literature procedure.[9] Cytidine was quantitatively converted to N^4-acetylcytidine by following the selective acetylation procedure.[10] It was tritylated with 1.2 equivalents of 4,4'-dimethoxytrityl chloride in pyridine at 5^0C for 20 h to yield 65% of the 5'-DMT product. Silylation of 2'-hydroxyl group with TBDMS chloride furnished 50% of the 2'-silyl cytidine derivative. Phosphorylation with 2 equivalents of β-cyanoethyl N,N-diisopropylchlorophosphoramidite in tetrahydrofuran in the presence of 4 equivalents of diisopropylethylamine afforded 65% of 5'-DMT-N^4-acetyl-2'-tBDMS-cytidine-3'-phosphoramidite. The overall yield starting from cytidine was 21% as compared to the reported yield of 4%.

After RNA synthesis, the solid support was divided into two or three portions. They were treated with ammonium hydroxide/ethanol (3:1) for 8 hrs at 55^0C or with methylamine/ammonium hydroxide (1:1) at room temperature for 90 minutes or with methylamine at room temperature for 90 minutes.[11] After evaporation of the reagents, the 2'-silyl protecting group was removed by treatment with 1.0 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran overnight. After quenching the excess of TBAF with 0.05 M ammonium acetate buffer (pH 7.0), desalting was performed on a SepPak (Millipore) column. Oligoribonucleotides were analyzed by capillary electrophoresis.[12] In our hands, capillary electrophoresis has been reliable in terms of resolution and reproducibility, and we also find it convenient and rapid to use. The relative percentages of the full length sequences in comparison to the short sequences, mostly arising from the combination of the failures during the sequence assembly and degradation during basic deprotection, are given in Table I. The capillary electropherograms are depicted in Figures 1 and 2. In order to confirm the resolution in the capillary electrophoresis, 'n-1' sequences were separately synthesized and coinjected with the 'n' sequences. They migrated distinctly different from each other.

Table I

% of full length oligoribonucleotides by absorbance at 260 nm

Sequence	Cleavage and deprotection reagent		
	NH_4OH/EtOH	CH_3NH_2/NH_4OH	CH_3NH_2
43 mer	21	38	43
Ribozyme 50 mer[3]	20	34	-
Ribozyme 59 mer[3]	21	34	-
70 mer	18 (Fig. 1A)	32 (Fig. 1B)	36 (Fig. 1C)
101 mer	a (Fig. 2A)	a (Fig. 2B)	-

a: Integration of peaks did not work satisfactorily.

43 mer: ^{5}CAUAACCCGAAGAUCGUCGGUUCAAAUCCGGCCCCCGCAACCA^3

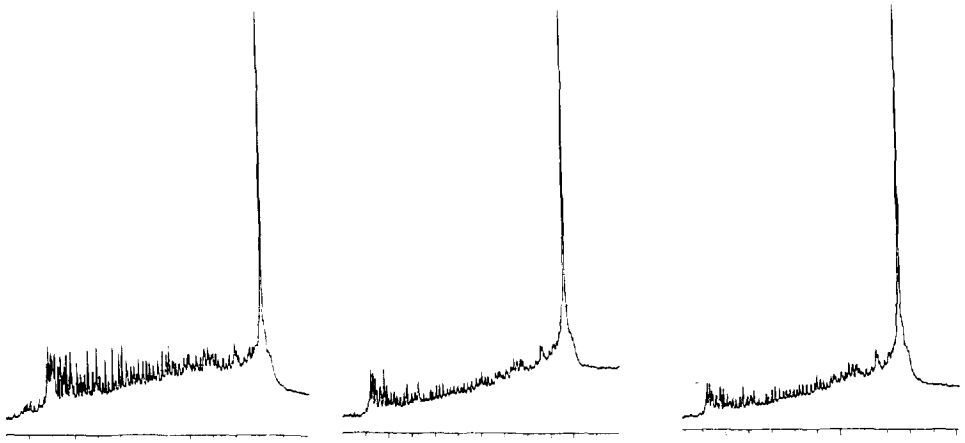
Ribozyme R50: ^{5}AAACAGAGAAGUCAACCAGAGAAACACACGUUGUGGUAUAUUACCUGGUA^3

Ribozyme R59: ^{5}AAACAGAGAAGTCAACCAGAGAAACACACGGACUUCGGUCCGUGGUAUAUUACCUGGUA^3

70 mer: ^{5}GAUGCCAGUUCGGUCAUCCGAUGCUCGGUCACGGAACUGUCAACGGUACCUACUUGUC-GUAACGUAGGAU^3

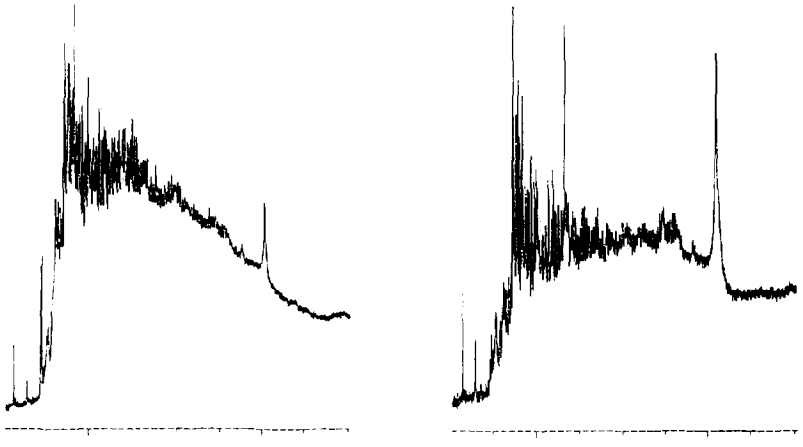
101 mer: ^{5}GCUGCCAGUUCGGUCAUCCGAUCCUCGGUCACGCAACUGUCAACGGCACCUACUCCUCGU-AACGUAGGACAGUCCGAUUCGCACGUGCAAAGCCCAUUCAU^3

Figure 1. Capillary Electrophoretic scan of a 70 mer oligoribonucleotide



A: NH_4OH/EtOH B: CH_3NH_2/NH_4OH C: CH_3NH_2

Figure 2. Capillary Electrophoretic scan of a 101 mer oligoribonucleotide



A: NH_4OH/EtOH B: CH_3NH_2/NH_4OH

It is evident from the data that the use of methylamine or its 1:1 mixture with ammonium hydroxide improved the yield of the full length sequences as compared to the use of ammonium hydroxide/ethanol (3:1). Methylamine appears to have performed slightly better than methylamine / ammonium hydroxide. The improvement in the yield obtained with the use of methylamine or methylamine/ammonium hydroxide is presumably be due to the relatively milder room temperature deprotection instead of harsher elevated temperature conditions required for the ammonium hydroxide deprotection. Further detailed studies need to be performed to better understand the reasons. Experiments are planned to evaluate the biological activity of ribozymes synthesized using the improved method.

In conclusion, the use of methylamine or methylamine/ammonium hydroxide for the cleavage and deprotection has significantly improved the yield of full length oligoribonucleotides in addition to providing rapid cleavage and deprotection.

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6. Although methylamine by itself gave very similar performance, we slightly preferred a mixture of methylamine/ammonium hydroxide, due to its compatibility with certain plastic 'O' ring materials used for the vials.
7. While our investigation was in progress, a publication appeared on the use of methylamine/ammonium hydroxide in RNA synthesis. Strobel, S. A.; Cech, T. R.; Usman, N.; Beigelman, I. *Biochemistry* **1994**, 33, 13824-13835.
8. The oligoribonucleotides were synthesized using phosphoramidite chemistry on Gene Assembler Plus (Pharmacia) synthesizer using standard protocols with a coupling time of 12 minutes.[1]
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11. The deprotection times were determined by digesting the oligoribonucleotides with phosphodiesterase I and alkaline phosphatase, followed by reverse phase HPLC analysis.
12. Capillary electrophoresis (CE) was run on Beckman P/ACE 2000. The capillary gel column was obtained from Beckman Instruments, U100P urea Gel column, Cat. # 338480, and was loaded and cut to 37 cm long. The Tris-borate, 7M urea buffer was also from Beckman, Gel buffer kit (cat. # 338481) and was used according to directions. The absorbances of the oligoribonucleotides were in the range of 1 to 5 $OD/_{260\ nm}$/ml, depending upon the quality and length of oligonucleotide. Injection was at 10 kV for 3 sec, while separation was at 11kV for 30-60 min, depending upon the length.

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