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Effect of excess water on the desilylation of oligoribonucleotides using tetrabutylammonium fluoride

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ABSTRACT

The most commonly available 2′ hydroxyl protecting group used in the synthesis of oligoribonucleotides is the *tert*-butyldimethylsilyl moiety. This protecting group is generally cleaved with 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF). The efficiency of this reaction was tested on ribonucleotidyldeoxythymidine dinucleotides (AT, CT, GT, and UT). We have found that the efficiency of desilylation of uridine and cytidine is greatly dependent on the water content of the TBAF reagent. Conversely, the water content of the TBAF reagent [up to 17% (w/w)] had no detectable effect on the rate of desilylation of adenosine and guanosine. It was concluded that for effective desilylation of pyrimidine nucleosides the water content of the TBAF reagent must be 5% or less, which is readily achieved using molecular sieves. TBAF dried in such a manner was shown to be effective in deprotecting an oligoribonucleotide containing both purine and pyrimidine residues.

INTRODUCTION

RNA phosphoramidites protected at the 2′ position with the *tert*-butyldimethylsilyl group first introduced by Ogilvie (1,2) are the most common RNA synthesis reagents available commercially. These amidites have been used to successfully prepare many oligoribonucleotides. However, we, and others (Paul Miller, personal communication), noted a number of heterogeneous product mixtures after deprotection apparently due to incomplete desilylation using TBAF. These failures occurred even with freshly opened bottles. Furthermore, it was observed that purine rich sequences deprotected more rapidly and completely than pyrimidine rich sequences did.

Studies of the initial ammonium hydroxide deprotection step were well described (3,4), but there are few reports in the literature about the efficiency of desilylation. Although the same TBAF deprotection is common to all reports using 2′ silyl protection, the length of the desilylation step ranges from 4 hours (3) to 24 hours (5), with reaction times of 4–6 hours being the most common.

We propose that the efficiency of desilylation by 1 M TBAF in THF is adversely effected by the presence of excess water. Disturbingly, it was found that the water concentration present in some freshly opened bottles is often sufficient to cause decreased efficiency. We also verified that significant differences exist in the desilylation rates of the ribosyl purine and the ribosyl pyrimidine nucleosides.

RESULTS

The rates of desilylation of the various bases were determined on ribosyldeoxythymidine dinucleotides which contain a single silyl group, yet still maintain a realistic environment by having a 3′ neighboring nucleoside. The dinucleotides were synthesized, cleaved from support with the silyl group left intact, and isolated by reverse phase HPLC.

Each dinucleotide was treated with 1 M TBAF. A freshly opened bottle of TBAF was used for the experiment. The extent of desilylation was determined by reverse phase HPLC at one hour intervals for six hours. The ribosyl purine containing dinucleotides were completely deprotected in one hour. Conversely, desilylation of the UT dimer required greater than 3 hours to complete and the CT dinucleotide was not yet complete in 6 hours. All the dimers were fully deprotected in 24 hours with no apparent cleavage or modifications.

The 1 M TBAF used in the above experiment was found to contain 11% (w/w) water (+/− 0.5%) when assayed by Karl-Fisher titration (K-F). (Note: all subsequent percent water readings are defined as w/w.) A series of previously unopened 5 ml bottles of TBAF was assayed for water content. The results indicated that a range of 5 to 8% water was normal for this product. We chose a bottle that contained approximately 5% water, removed aliquots, and added water to increase the water content in 5% increments. The absolute percentages were determined by K-F. Aliquots of each dinucleotide were treated with the reagents, quenched after 4 hours at room temperature, and the crude reaction mixtures analyzed by reverse phase HPLC. The results are shown in figure 1. Both of the ribosyl purine containing dinucleotides were completely deprotected in 4 hours by 1 M TBAF containing as much as 17% water, whereas the ribosyl pyrimidine containing dinucleotides were incompletely deprotected after 4 hours when the fluoride reagent contained more than 5% water. Since we found that some of the fresh bottles of 1 M TBAF contained 8% water, it is not surprising that desilylation of oligoribonucleotides often failed. A similar experiment was done with CT and UT for a 24 hour period at

room temperature. The results are also shown in figure 1. The dinucleotides were incompletely deprotected when the fluoride reagent contained more than 7% water.

To test a simple solution to this problem, we added molecular sieves (3 Å) to a 1 M TBAF solution that contained 15% water. After about 4 days the water content was lowered to 3% water. The ability of TBAF reagents dried with molecular sieves to cleanly deprotect an oligoribonucleotide was examined. Water was added to a fresh bottle of TBAF to a final concentration of 21% water. One half of the reagent was transferred to another vessel and dried with molecular sieves. After 5 days, the water content in the dried reagent dropped to 2.3%. Both the wet and molecular sieve dried reagents were used to deprotect a model oligoribonucleotide with the sequence 5′-rACGUACGU. The oligonucleotide was treated with ammonium hydroxide/ethanol to cleave it from support and remove the base protecting groups. The oligoribonucleotide was then aliquoted into 6 separate glass vials and treated with fluoride reagents containing 21% water or 2.3% water for 3, 6, and 24 hours. The oligoribonucleotides were analyzed by reverse phase HPLC and PAGE. Deprotection with the TBAF reagent dried with molecular sieves was complete within 3 hours, whereas the deprotection with the reagent containing 21% water was not complete within 6 hours.

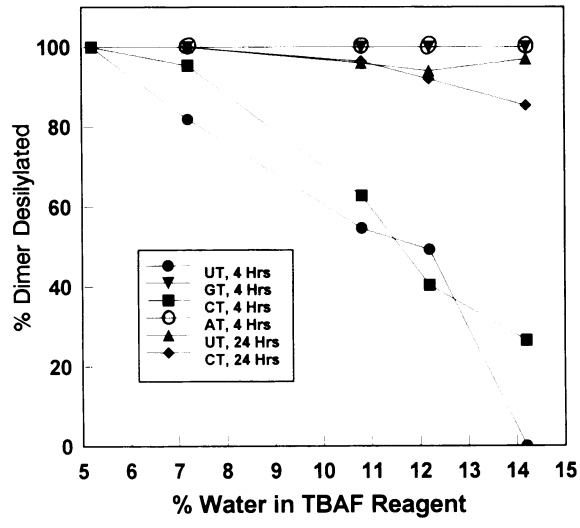

Figure 1. Graph representing the effect of increasing water concentration in 1 M TBAF on the efficiency of desilylation of ribosyldeoxyribose dinucleotides. The graph shows data from the 4 hour treatment of all four dinucleotides (AT, CT, GT, UT) with TBAF containing increasing amounts of water, as well as 24 hour treatments of the pyrimidine dinucleotides, CT and UT, with similarly contaminated TBAF. Note: The plots of the percent desilylation of both AT and GT dinucleotides lie on the same line, 100% desilylated at every water concentration.

DISCUSSION

It is apparent that many of our oligoribonucleotide deprotection failures in the past were due to high water content in the TBAF reagent. Concentrations of water as low as 7% can cause incomplete deprotection when times shorter than 24 hours are used. Furthermore, it appears that some fresh, unopened bottles of 1 M TBAF may already be too wet to be effective (>5%).

The deprotection rate difference we noted with oligoribonucleotide strands of all purine or pyrimidine base composition was borne out by the dimer study. The effect of slight increases in water content in the TBAF reagent was immediate and severe on the rates of desilylation of the ribosyl pyrimidine nucleosides; whereas the rates of desilylation of the ribosyl purine nucleosides were impervious to water contents of up to 17%, and deprotected rapidly. Perhaps this is due to inductive effects of the purine ring on the 2′ position, although the extent is unexpected.

The effect of water is significant enough to warrant attention to the quality of the TBAF reagent in use when deprotecting oligoribonucleotides containing pyrimidine nucleosides. We now routinely add sieves to freshly opened bottles of TBAF. The addition of sieves to the reagent will ensure a dry reagent for some time, although we still recommend checking the water content by Karl-Fisher titration. We do not recommend use of a reagent that assays for more than 5% water content. Since we began this practice we have not noted any incomplete deprotections in over 100 oligomers.

METHODS

General methods and materials

Tetrabutylammonium fluoride (TBAF), 1 M in THF, in 5 ml bottles, was purchased from Aldrich Chemicals Company, Inc., Milwaukee, WI. Molecular sieves (3 Å) and all solvents were purchased from Fisher Scientific, Inc., Pittsburgh, PA. The TBAF solutions were treated with enough sieves to form an approximately 1/2 inch bed on the bottom of the 5 ml bottle.

RNA 2′ silylated phosphoramidites and support bound nucleosides were purchased from Peninsula Laboratories, Inc., Belmont, CA. All other synthesis reagents were purchased from Glen Research Corporation, Sterling, VA.

Karl-Fisher titrations were obtained on a Model 447 coulomatic titrator from Fisher Scientific Inc. UV absorbances were obtained on a Model 3000 Photodiode Array Spectrophotometer from Milton Roy Company, Rochester, NY. HPLC was done on a Beckman, Fullerton, CA, System Gold Model 126. Columns and running conditions are described below.

All water was treated with 0.05% diethylpyrocarbonate and autoclaved prior to use. All buffers were prepared and used under sterile conditions. Baked glassware was used throughout.

Oligoribonucleotide synthesis

Syntheses were done on a Milligen (Milford, MA) 8750 using a modified method that allows for the longer RNA amidite coupling times. Each dinucleotide and the octomer were synthesized on a 4×1 μmole scale. The detritylated oligomers were cleaved from the support and partially deprotected by treatment with 2 ml of ammounium hydroxide/ethanol (3/1) overnight at 55°C (3), yielding crude, 2′ silylated dinucleotides and octomer. The silylated dinucleotides were isolated on HPLC using a Whatman (Clifton, NJ) RAC II reverse phase column. The gradient was 0 to 60% B over 15 min. with a flow of 1 ml/min. Solvent A was 0.05 M triethylammonium actetate (TEAA) and solvent B was acetonitrile. Retention times of the 2′-O-*tert*-butyldimethylsilyl-dinucleotides were: AT, 11.0 min.; CT, 10.5 min.; GT, 10.2 min.; and UT, 11.4 min. The UV λ maximums in water, pH 7, were: AT, 262.0 nm; CT, 268.8 nm; GT, 257.4 nm and 268.8 nm (shoulder); and UT, 264.3 nm.

Dinucleotide desilylations

All of the deprotection studies were done as described in the following example. Time of reaction and percentage of water in the TBAF reagent varied as described in the results section.

A dried aliquot (0.5 O.D.$_{260}$ unit) of HPLC purified 2'-O-*tert*-butyldimethylsilyladenosinyldeoxythymidine was treated with 200 μl of 1 M TBAF. The reaction was quenched by the addition of 200 μl of 2 M TEAA. The reaction mixture was analyzed by HPLC using the column and gradient described above. An aliquot (40 μl) of the reaction mixture was injected for analysis. Retention times of the desilylated dinucleotides were: AT, 6.7 min.; CT, 5.6 min.; GT, 6.2 min.; and UT, 6.2 min.

Oligoribonucleotide desilylations

Aliquots (0.3 O.D.$_{260}$ unit) of the crude silylated octomer were treated with 40 μl of 1 M TBAF which contained 21% or 2.3% water for 3, 6, and 24 hours. The reactions were quenched by adding 100 μl of 2 M TEAA. The reaction mixtures were analyzed on a C-4 analytical HPLC column from Vydac, Inc., Hesperia, CA, using the following gradient: 0% B for 10 min., 0–45% B over 15 min., 45 to 100% B over 10 min. at a flow rate of 1 ml/min. The retention time of the silylated oligoribonucleotide was 35.2 min., while the retention time of the desilylated oligoribonucleotide was 23.2 min. Solvents A and B are the same as described above for use with the Whatman column. Samples were also analyzed by 20% denaturing PAGE using standard kinasing and running conditions (6). Bands were visualized by autoradiography.

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