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A Safe and Practical Procedure for Global Deprotection of Oligoribonucleotides

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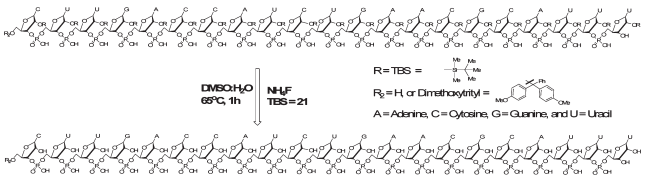
Received February 10, 2009



We report a practical global deprotection of RNA 2′-*O*-*tert*-butyldimethylsilyl (TBS) ethers using commercially available aqueous NH_4F. The procedure is applicable to both 96-well plate format and large-scale production of RNA. This improved procedure provides a safe, mild, and cost-effective alternative to highly hazardous $Et_3N\cdot 3HF$, a reagent commonly used in the routine synthesis of RNA.

Modern methods for the assembly of RNA via the phosphoramidite approach rely heavily on suitable protection of the reactive 2′-hydroxyl group of ribonucleoside building blocks.[1] The ideal protecting group must be completely stable to automated solid-phase RNA assembly and basic conditions used in the subsequent cleavage−deprotection steps that release crude product from solid support and reveal the free nucleobases and phosphate groups. Significant effort has been made to develop alternative 2′-OH protecting groups.[2] The most notable is the use of the 2′-*O*-triisopropylsilyloxymethyl group (TOM),[3a] which requires prolonged aging (1−50 h) for deprotection using Bu_4NF and the use of the 2′-*O*-bis(2-acetoxyethoxy) methyl orthoester (ACE) protecting group.[3b] Despite significant advances made toward developing alternative protecting groups, the *tert*-butyldimethylsilyl (TBS) ether has emerged as the most frequently used in the routine synthesis of RNA.[4] This can be attributed to the relative ease of installation at the 2′-OH position of ribonucleosides and its excellent stability under automated solid-phase synthesis of RNA. After initial cleavage−-deprotection with either ammonium hydroxide[4a,c] or aqueous methylamine,[5] a global deprotection of RNA 2′-*O*-TBS ethers is routinely carried out using a large excess of highly hazardous triethylamine trihydrogen fluoride ($Et_3N\cdot 3HF$).[6] Aside from safety concerns during handling, $Et_3N\cdot 3HF$ is also known to cause detritylation and decomposition of oligoribonucleotides.[7] Unfortunately, alternative reagents such as tetrabutylammonium fluoride (TBAF),[8] pyridine·HF, and other HF complexes[9] offer little improvement in terms of handling safety and have associated drawbacks such as sluggish reactions and challenging workup conditions. Safe, efficient, and user-friendly global desilylation procedures are thus needed for routine RNA synthesis.

After a screen of various potential substitutes[10] for $Et_3N\cdot 3HF$ in RNA deprotections, we identified potassium fluoride and ammonium fluoride as the reagents of choice for desilylation of RNA. These fluoride salts are readily available, inexpensive, easy to handle, and less toxic relative to $Et_3N\cdot 3HF$. Preliminary experiments indicated that modified oligoribonucleotides containing < 10 2′-*O*-TBS ethers could be deprotected using aqueous KF buffered with malonic acid (see the Supporting Information). However, application of these conditions to 21-mer RNA gave erratic results such as sluggish and incomplete deprotection.

In comparison, deprotection of RNA using aqueous NH_4F showed excellent performance with either mixed or full 21-mer RNA. While KF desilylation remains useful for mixed RNA sequences (2′-*O*-TBS < 10), aqueous NH_4F was selected for further optimization and application to high-throughput (96-well format) and large scale (800 μmol) deprotections. The reaction can be performed in either DMSO/H_2O (∼3:1) or dimethylacetamide (DMAc)/H_2O (∼1:1). The solvent ratios are critical to maintain both the fluoride salt and RNA in solution during the reaction and reproducibly achieve complete deprotection. The reactions can be performed under a wide range of pH. Although the reaction proceeds readily at pH 5−12, optimal operating conditions were determined to be pH 7.5−9.5 for RNA product integrity. Mild organic acids such as citric acid, glycolic acid, malonic acid, and tartaric acid can be used for pH

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(10) (a) Reagents that were evaluated for desilylation of RNA include KF, KF/alumina, TMSCl/KF, $CuCl_2$, $KHSO_4$, LiF, NaF, NH_4F, CsF, CaF_2, MgF_2, KHF_2, and $ZrCl_4$. (b) Zhang, W.; Robins, M. J. *Tetrahedron Lett.* **1992**, *33*, 1177–1180. (c) Serafinowski, P. J.; Barnes, C. L. *Tetrahedron* **1996**, *52*, 7929–7938.

DOI: 10.1021/jo100648e Published on Web 07/02/2010

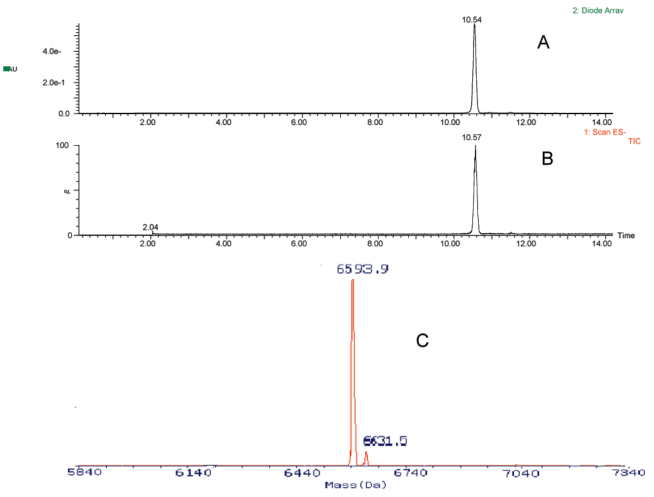

FIGURE 1. UPLC-MS analysis for SAX-HPLC purified 21-mer RNA (Table 1, entry 1). MW calcd for $C_{197}H_{246}N_{72}O_{147}P_{20}$ = 6594, found = 6594; calcd [M + K] = 6633, obtained [M + K] = 6632. (A) UV detector λ = 260 nm, (B) MS ESI, (C) deconvoluted mass spectrum.

TABLE 1. Oligoribonucleotides Desilylated with NH_4F

		UPLC/MS	
entry	sequence 5′ → 3′[a]	calcd	found
1	CUU GAC CAU CUG AAC GCA UUU	6594[c]	6594
2	CCC GAC CAU CUU AAC GCA AAU	6902[b]	6904
3	CUC GAC CAU AAA AAC GUA AAU	6671[c]	6672
4	CGC GAC CAU UUU AAC GCG AAU	6959[b]	6958
5	UGC GAU GUC UAU UCG AGU GUC AU	7302[c]	7304
6	UAC GGA CUA CGU CGA UGA UCU GCA G	8300[b]	8299

[a] A = adenosine, U = uridine, C = cytidine, G = guanosine. [b] 5′-Dimethoxytrityl-on. [c] 5′-Dimethoxytrityl-off.

adjustment with minimal loss of the 5′-DMT protecting group (< 2%, by RP HPLC). We excluded acidic conditions to avoid formation of hazardous hydrogen fluoride. Using optimized NH_4F desilylation conditions, a representative 21-mer RNA was fully deprotected in 1 h at 65 °C (Figure 1), giving similar UPLC and reversed-phase HPLC profiles to reactions performed using standard $Et_3N \cdot 3HF$ desilylation conditions. Optimized NH_4F desilylation conditions were successfully applied to various RNA sequences (Table 1, Figure 2).

A valid concern during RNA synthesis and deprotection is control over a side reaction involving phosphoryl group migration. This reaction has been shown to be dependent on various factors, including RNA sequence and reaction conditions such as pH and temperature.[11] Although deprotection with NH_4F proceeds under controlled pH conditions, we wanted to ascertain if phosphoryl group migration was indeed operative under the reaction conditions. Authentic samples of model 3′-5′-dinucleotide **M1** and 2′-5′-dinucleotide **M2** were synthesized. Deprotection of **M1** using either $Et_3N \cdot 3HF$ or aqueous NH_4F were carried out, and the products were analyzed by ^1H, ^{13}C and ^{31}P NMR spectroscopy in D_2O. As illustrated in Figure 3, deprotections using either $Et_3N \cdot 3HF$ or NH_4F provide indistinguishable ^1H NMR traces and do not lead to phosphoryl group migration. Deprotection of **M2** was done using TEA·3HF.

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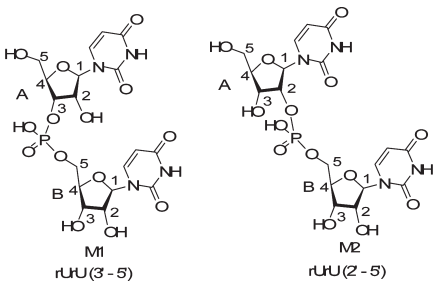


FIGURE 2. Structure of synthetic dinucleotides rUrU with 3′−5′ (M1) and 2′−5′ (M2) internucleotide linkages.

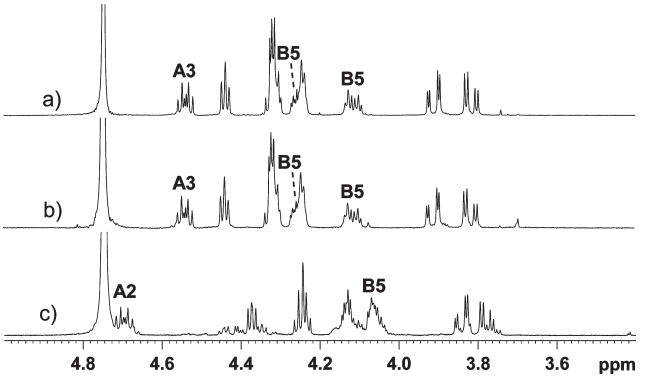


FIGURE 3. ^1H NMR spectra (D_2O): (a) dinucleotide M1 deprotected with aqueous NH_4F; (b) dinucleotide M1, deprotected with $Et_3N \cdot 3HF$; (c) dinucleotide **M2**, deprotected with $Et_3N \cdot 3HF$.

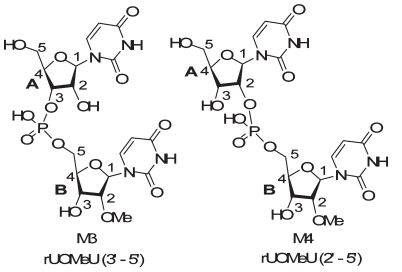


FIGURE 4. Structure of synthetic dinucleotides rUOMeU with 3′−5′ (**M3**) and 2′−5′ (**M4**) internucleotide linkages.

An authentic sample of model 3′−5′-dinucleotide **M3** (Figure 4) deprotected using either $Et_3N \cdot 3HF$ or aqueous KF and analyzed by ^1H, ^{13}C, and ^{31}P NMR spectroscopy in D_2O resulted in similar observations. 2′−5′-Dinucleotide **M4** was deprotected with TEA·3HF (Figure 5).

In summary, we have developed a practical procedure for global deprotection of RNA 2′-*O*-TBS ethers. This procedure obviates the use of hazardous $Et_3N \cdot 3HF$ in the routine synthesis of RNA in 96-well plate format (500 nmol) and larger scales (800 μmol).

Experimental Section

General Methods. All reagents were obtained from commercial sources and used without further purification. All oligoribonucleotides were synthesized on controlled-porosity glass (CPG) using the phosphoramidite approach on GE automated oligo synthesizers (50−800 μmol scale) or on a MerMade 12 (Bioautomation) instrument (10−50 μmol) and MerMade 192 (500 nmol, 96-well format plate synthesis). Isolated yields are

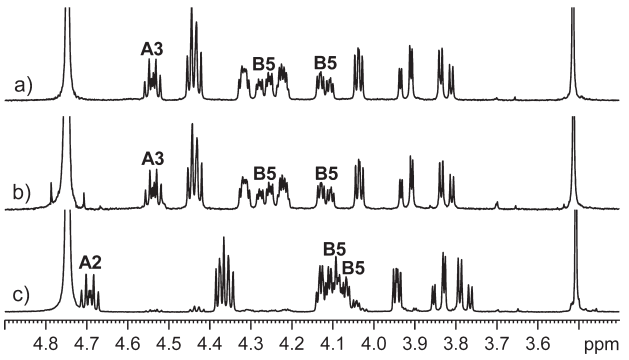


FIGURE 5. ^{1}H NMR spectra (D_2O): (a) dinucleotide **M3** deprotected with $Et_3N\cdot HF$; (b) dinucleotide M3, deprotected with aqueous KF; (c) dinucleotide **M4**, deprotected with $Et_3N\cdot HF$.

calculated based on CPG-bound leader nucleoside starting material. pH measurements were carried out using a UB-10, UltraBasic pH/mV meter.

Representative Procedure with NH_4F. Global desilylation of 21-mer RNA (CUU GAC CAU CUG AAC GCA UUU) Table 1, example 1: Protected RNA (11.1 g, 72 μmol/g, ~ 800 μmol, CPG-bound) was treated with methylamine (50 mL, 40% aqueous). The resulting suspension was aged for 45 min in a shaker (200 rpm) at 35 °C. The suspension was filtered and washed with DMSO (200 mL). The filtrate (pH 13−14) was cooled to 0 °C, and glycolic acid (~40 mL, 70% aqueous) was added dropwise to the reaction mixture until pH 8.6. Ammonium fluoride (40 mL, 40% aqueous) was then added, and the cloudy reaction mixture was aged for 60 min at 65 °C in a shaker (200 rpm, pH 8.5 end of reaction). The resulting solution was cooled to 0 °C, diluted to 1:2 with water, and purified by preparative SAX-HPLC. Lyophilization afforded the desired RNA as a white solid (1.5 g, ~28% yield, 98 A% SAX-HPLC); UPLC analysis t_R = 10.5 min; UPLC-MS analysis calcd for $C_{197}H_{246}N_{72}O_{147}P_{20}$ = 6594, found = 6594 residual NH_4^+ ion < 0.3 wt % as ascertained by capillary gel electrophoresis.

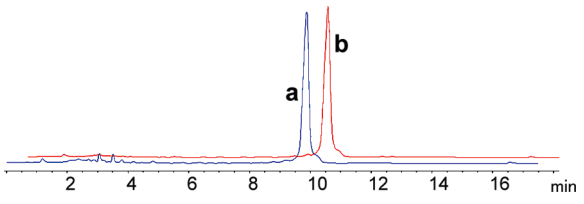
Representative Procedure for Global Desilylation of a 21-mer Mixed RNA/Non-RNA Sequence with KF. AUC FUFCOMEG OMeGOMeAOMeA OMeAFCFU FUFUFC OMeGOMeGOMeA OMeGOMeUOMeU (three 2′-*O*-TBDMS groups): Protected RNA (6.4 g, ~200 μmol, CPG-bound) was treated with methylamine (20 mL, 40% aqueous). The resulting suspension was aged for 45 min in a shaker (200 rpm) at 35 °C. The suspension was filtered and washed with DMSO (24 mL). The filtrate (pH 13−14) was treated with malonic acid (14 mL, 4.8 M aqueous) until pH 8.5. Potassium fluoride (1.6 mL, 9.2 M aqueous) was then added, and the reaction mixture was aged 60 min at 65 °C (200 rpm). The resulting solution was cooled to 0 °C, diluted 1:2 with water, and purified by preparative SAX-HPLC. Lyophilization afforded the desired RNA as a white powder (310 mg, 21.6% isolated yield, SAX HPLC, t_R = 9.3 min, 98A%). ESI-MS calcd for $C_{210}H_{261}F_7N_{76}O_{140}P_{20}$ 6842, obtained 6842. KF desilylation of the same sequence was compared with TEA·3HF desilylation.[12] FU = 2′-fluorouridine, FC = 2′-fluorocytidine, OMeC = 2′-*O*-methylcytidine, OMeG = 2′-*O*-methylguanosine, OMeA = 2′-*O*-methyladenosine, OmeU = 2′-*O*-methyluridine.

Representative Procedure for Global Desilylation of Oligoribonucleotides on a 96-Well Plate with NH_4F (500 nmol Scale). Parallel synthesis of multiple 21-mer oligoribonucleotides was carried out on a CPG solid support in a 96 well-plate format (see the Supporting Information). After the synthesis was complete, synthesis tips were treated with $MeNH_2$ (2 × 100 μL) and aged at rt (2 × 4 min). The CPG support was then washed with DMSO (2 × 300 μL). The deep-well plate with TBS protected oligomers in DMSO/methylamine was aged at 37 °C (200 rpm) over 45 min for complete deprotection of phosphate and nucleobases. The reaction plate was cooled to rt and treated with 100 μL of glycolic acid (70% aq)[13] followed by 200 μL of NH_4F solution (40% aq). The plate was then aged at 65 °C over 1 h. The plate with desilylated oligomers was cooled to 0 °C, and individual wells were diluted with NaCl (1 M, 600 μL). The crude reaction mixture was then loaded onto a 96-well C-18 column for purification. The identity of individual oligoribonucleotides was confirmed with LC/MS, and purity was evaluated via SAX HPLC.

Acknowledgment. We thank Sarah Young and Dr. Yong Liu for analytical support.

Supporting Information Available: HPLC, LC−MS, and ^{1}H, ^{13}C and ^{31}P NMR of dinucleotides **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

(12) RP and SAX HPLC conditions are described in the Supporting Information. A waterfall chromatogram (SAX HPLC) of purified 21-mer (trityl-off) with three 2′-*O*-TBS groups is shown (AUC FUFCOMEG OMeGOMeAOMeA OMeAFCFU FUFUFC OMeGOMeGOMeA OMeGOMeUOMeU (a) desilylation with TEA·3HF, pH = 11, (b) desilylation with KF, pH = 9:



(13) Alternatively, the excess $MeNH_2$ can be removed under vacuum and NH_4F added without a buffering acid.