Novel RNA Synthesis Method Using 5′-*O*-Silyl-2′-*O*-orthoester Protecting Groups

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The ability to routinely synthesize RNA has become increasingly important as research reveals the multitude of RNA's biological functions.[1] Over the past 25 years, many chemical strategies have been explored for synthesizing RNA. Most approaches have focused on retaining the 5′-*O*-dimethoxytrityl (DMT) ether and adding a compatible 2′-hydroxyl protecting group such as fluoride-labile silyl ethers,[2] photolabile moieties,[3] or acid-labile acetals.[4] The acetals have exhibited many attractive features, but a delicate balance has been required to successfully utilize the 2′-*O*-acetals and the 5′-*O*-DMT ether in the same synthesis strategy.[5] Hence, other approaches have involved retaining the 2′-*O*-acetal while replacing the 5′-*O*-DMT.[6] Several reviews further document these strategies.[7] Of all of the RNA synthesis methods reported to date, the 5′-*O*-DMT-2′-*O*-*tert*-butyldimethylsilyl (TBDMS) and the 5′-*O*-DMT-2′-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl] (FPMP) chemistries are offered commercially. Unfortunately, neither allows RNA synthesis to be as routine and dependable as DNA. The current methods enable the synthesis of RNA in acceptable yields and quality, but a high level of skill appears to be required to deliver adequate results. The need and desire exists for more robust RNA synthesis methods which consistently produce higher quality RNA.

Whereas most previous approaches were adaptations of DNA methodologies, we focused on a *de novo* strategy and asked what would be optimal for RNA. According to the literature, the most desirable conditions for the final 2′-*O*-deprotection would be mildly acidic aqueous conditions. The obstacle to using mildly acid-labile 2′-*O*-groups has been the 5′-*O*-DMT group, which is removed under similar conditions. Our investigations led to the successful development of silyl ethers for protection of the 5′-hydroxyl.[8] These protecting groups can be removed with fluoride ions under neutral conditions which are compatible with an acid-labile 2′-hydroxyl moiety. However, it was subsequently discovered that 5′-*O*-silyl ether oligonucleotide synthesis chemistry in conjunction with 2′-*O*-acetals produced side products.[8] Acid-labile orthoester protecting groups[9] were investigated as alternatives and discovered to be suitable for the 2′-hydroxyl.

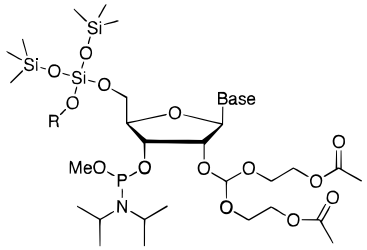

Figure 1. 5′-*O*-SIL-2′-*O*-ACE ribonucleoside phosphoramidites. (Base = *N*-benzoyladenine, *N*-acetylcytosine, *N*-isobutyrylguanine, or uracil. R = cyclooctyl for guanosine and uridine, R = cyclododecyl for adenosine and cytidine.)

We recently developed the 2′-*O*-bis(2-acetoxyethoxy)methyl (ACE) orthoester that is stable to nucleoside and oligonucleotide synthesis conditions but is modified via ester hydrolysis during base deprotection of the oligonucleotide.[10] The resulting 2′-*O*-bis(2-hydroxyethoxy)methyl orthoester is 10 times more acid-labile than the ACE orthoester. Complete cleavage of the 2′-*O*-protecting groups is effected using extremely mild conditions (pH 3, 10 min., 55 °C). The innovative features of this chemistry have enabled the synthesis of RNA oligonucleotides of unprecedented quality.

The structures of the four RNA nucleoside phosphoramidites are illustrated in Figure 1. The 3′-hydroxyl was functionalized as the methoxy *N*,*N*-diisopropylphosphoramidite. (We observed that the cyanoethyl-protected phosphoramidites were not compatible with fluoride reagents.[8]) Method optimization resulted in >99% coupling yields in <90 s.[11] These results are a clear leap over current yields and coupling times achieved with 2′-*O*-TBDMS phosphoramidites. The high yields observed with such a short coupling time are comparable with those routinely experienced in DNA synthesis.[12]

The following two oligonucleotides were synthesized for this study: **1** (UCU CCA UCU GAU GAG GCC GAA AGG CCG AAA AUC CCC) and **2** (GUU UUC CCU GAU GAG GCC GAA AGG CCG AAA UUC UCC X, where X = inverted abasic residue[13]). Following oligonucleotide synthesis,[11] the phosphate methyl protecting group was cleaved.[14] The oligonucleotides were then cleaved from the support concomitantly with removal of the acyl groups on the exocyclic amines and the acetyl groups on

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(8) The 5′-*O*-silyl ether (Figure 1) was arrived at through screening approximately 30 derivatives (Scaringe, S. A.; Caruthers, M. H., in preparation). Lability to fluoride and stability toward RNA synthesis conditions were the key criteria for selecting this protecting group. The phosphoramidites (Figure 1) were synthesized ($200/gram) in four steps from the 5′-*O*-3′-*O*-tetraisopropyldisiloxane-*N*-acyl-protected nucleosides in overall yields of 45−55%. Synthesis methods will be published separately.

(9) Hata, T.; Azizian, J. *Tetrahedron Lett.* **1969**, 4443−4446.

(10) A protected protecting group strategy in RNA synthesis was first elaborated by Sandstrom, A.; Kwiatkowski, M.; Chattopadhyaya, J. *Acta Chem. Scand. B* **1985**, *39*, 273−290.

(11) Coupling yields were determined via integration of HPLC analyses of various homopolymers 5−10 nucleotides long. For oligonucleotide synthesis, a modified 380B (PE-ABI) was used. Cycle consisted of the following reactions and appropriate washes: 35 s 5′-deprotection with 1.1 M HF, 1.6 M triethylamine in DMF, 90 s coupling with 15 equiv amidite (0.05 M) and 30 equiv *S*-ethyl tetrazole (0.25 M), 30 s 10% acetic anhydride/10% *N*-methylimidazole, 40 s oxidation with 3 M *tert*-butylhydroperoxide in toluene. The synthesis cycle required 11.5 min for 3 parallel syntheses. Syntheses were performed on polystyrene supports loaded with appropriate nucleoside via a succinate linkage (5−7 μmol/g).

(12) Methyl-protected phosphoramidites were investigated with 5′-DMT-2′-TBDMS chemistry. No improvement in RNA quality was observed.

(13) Beigelman, L.; Karpeisky, A.; Usman, N. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1715−1720.

(14) 1 M Disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate (S_2Na_2) in DMF in 30 min. Dahl, B. J.; Bjergarde, K.; Henriksen, L.; Dahl, O. *Acta Chem. Scand.* **1990**, *44*, 639−641.

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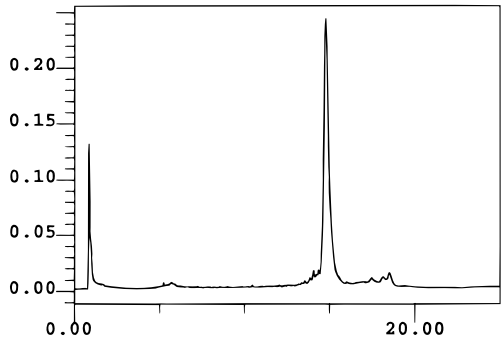

Figure 2. Anion-exchange HPLC chromatograph of unpurified 2′-*O*-protected **1** (5′-*O*-SIL-2′-*O*-ACE chemistry) (*Y*-axis units AU_{260}).

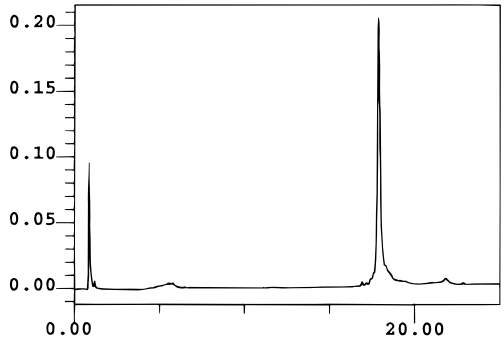

Figure 3. Anion-exchange HPLC chromatograph of fully deprotected **1** (5′-*O*-SIL-2′-*O*-ACE chemistry) (*Y*-axis units AU_{260}).

the 2′-*O*-orthoester.[15] In the 2′-*O*-protected state, the RNA was analyzed by HPLC chromatography (Figure 2).[16]

To effect complete 2′-*O*-deprotection, the RNA was incubated in aqueous buffers.[17] HPLC analysis showed **1** in 71% yield (Figure 3)[16] and **2** in 68% yield (data not shown). The HPLC profiles of the synthesis with and without 2′-*O*-protecting groups are comparable. (Identical gradient conditions were used in Figures 2−4.) These profiles represent the entire crude reaction mixtures. No purification nor additional workups were performed prior to analysis.

Oligonucleotides **1** and **2** were also synthesized with 5′-*O*-DMT-2′-*O*-TBDMS chemistry using published methodologies.[15a] Anion-exchange HPLC analysis showed **1** in 45% yield (Figure 4) and **2** in 43% yield (data not shown). The results illustrated in Figure 4 are representative of the best yields reported using TBDMS chemistry. When Figure 4 is compared directly to Figure 3, the improvements in quality and yields are remarkable.

To further assess the authenticity of the RNA produced by our novel chemistry, a number of assays were performed. When

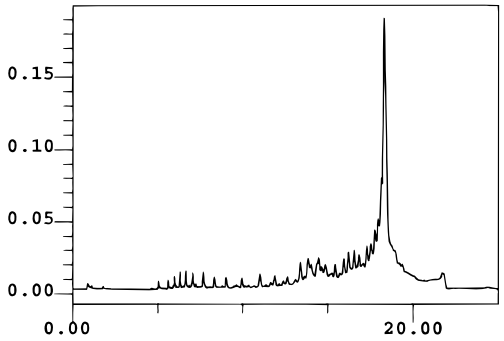

Figure 4. Anion-exchange HPLC chromatograph of fully deprotected **1** (5′-*O*-DMT-2′-*O*-TBDMS chemistry) (*Y*-axis units AU_{260}).

oligonucleotides **1** and **2** were assayed for base composition, the expected nucleoside ratio was observed, and no modifications were detected.[18] The identity of oligonucleotide **2** was further confirmed when its mass was measured at 11663.0 (predicted mass of 11665.86).[19] The biochemical reactivity of ribozyme **1** was assessed via an enzymatic cleavage assay. The initial rates and extents of cleavage were comparable for **1** synthesized by both methods. Finally, the extent, if any, of 3′- to 2′-migration of the internucleotidic phosphate bonds was assayed.[20] No contaminating 5′−2′ linkages were observed.[21]

5′-*O*-SIL-2′-*O*-ACE oligonucleotide chemistry is a definitive advance in RNA synthesis technology. Nucleoside coupling yields are comparable to DNA and require only 90 s. The final acid deprotection is mild and fast with minimal handling. Several assays confirmed the authenticity of 5′-*O*-SIL-2′-*O*-ACE synthesized RNA. Furthermore, this chemistry enables HPLC analysis and purification of stable 2′-*O*-protected RNA. This innovation has tremendous value. The opportunity to degrade the RNA is minimized. In addition, it may be possible to analyze troublesome sequences, which, when fully deprotected, do not easily resolve into one major conformation due to strong secondary structure. 5′-*O*-SIL-2′-*O*-ACE chemistry enables the routine synthesis of high quality RNA oligonucleotides. Studies involving the incorporation of modifications, e.g., phosphorothioates and halogenated bases, are in progress.

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Supporting Information Available: Experimental details (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(16) HPLC conditions: buffer A, 85% 10 mM $NaClO_4$, 15% acetonitrile; buffer B, 85% 300 mM $NaClO_4$, 15% acetonitrile; column, Dionex Pa-100; temperature: 55 °C.

(17) 10 min at 55 °C in 150 mM sodium acetate buffer at pH 3. An equal volume of 300 mM Tris-HCl buffer at pH 8.7 was added to raise the pH to 7.7−8.0. The sample was then incubated for 10 min at 55 °C. This cleaved any 2′-formyl groups resulting from the orthoester deprotection. (a) Griffin, B. E.; Jarman, M.; Reese, C. B.; Sulston, J. E. *Tetrahedron* **1967**, *23*, 2301−2313. (b) Fromageot, H. P. M.; Griffin, B. E.; Reese, C. B.; Sulston, J. E. *Tetrahedron* **1967**, *23*, 2315−2331.

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(19) MALDI-TOF on Voyager-DE (Perseptive Biosystems) in negative ion mode.

(20) Morgan, M. A.; Kazakov, S. A.; Hecht, S. M. *Nucleic Acids Res.* **1995**, *23*, 3949−3953. A 5′−3′ dimer control (Sigma) was completely digested, whereas no digestion of a 5′−2′ dimer (Sigma) was observed.

(21) In Morgan et al. the best result for a U21-mer synthesized by 5′-DMT-2′-FPMP chemistry was 0.45% contaminating 5′−2′ linkages and 1.2% 5′−2′ linkages for 5′-DMT-2′-TBDMS chemistry. However, it was reported that no attempt was made to optimize 2′-TBDMS oligonucleotide synthesis or deprotection conditions.