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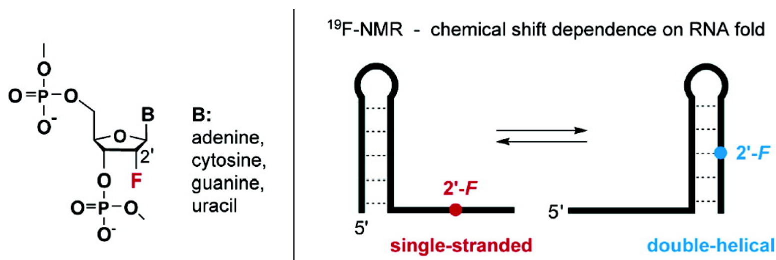

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Ribose 2′-*F* Labeling: A Simple Tool for the Characterization of RNA Secondary Structure Equilibria by ^{19}F NMR Spectroscopy

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The regulatory functions of RNA often rely on base sequences with the propensity to adopt different defined secondary structures, most impressively documented by a number of naturally occurring, metabolite-sensing riboswitches that have been discovered in recent years.[1,2] Secondary structure ambivalence is also encountered for very small RNAs comprising only 20−40 nucleotides.[3,4] Although the characterization of such monomolecular structural equilibria can be performed on nonlabeled probes by comparative imino proton ^{1}H NMR spectroscopy,[5] a principal drawback of this approach is the reliance on *exchangeable* N*H*−N nuclei. This is disadvantageous for the precise determination of the equilibrium position, for investigations at elevated temperatures, or for investigations at different buffer conditions that affect H/D exchange rates. Moreover, for larger RNAs, the imino proton chemical shift region becomes significantly crowded with severe signal overlaps.[5] Here we report on a basic concept that employs ^{19}F NMR spectroscopy and its advantages for the characterization of bistable RNAs.

The ^{19}F nucleus has 100% natural abundance, possesses an intrinsic NMR sensitivity almost as high as proton sensitivity, and most importantly, offers a chemical shift dispersion that is about 100-fold that of ^{1}H. These properties make fluorine an ideal candidate for monitoring functionally important conformational transitions. With respect to nucleic acids, these properties have been exploited to some extent for studies on metal ion binding,[6] hairpin−duplex transitions,[7] hammerhead ribozyme folding,[8] DNA methylation,[9] oligonucleotide hybridization,[10] and on a very basic level, on tRNA and 5S rRNA characterization.[11,12] Remarkably, most of the ^{19}F NMR studies on nucleic acids utilize nucleosides that have been modified with fluorine at the nucleobase, such as 5-fluorouracil,[6−8,11,12] 5-fluorocytosine,[9] 5-(trifluoromethyl)uracil,[13] and very recently, 2-fluoroadenine.[14] Incorporation of these labels has been performed by either chemical or enzymatic synthesis.

The concept presented here relies on site-specific labeling of RNA with 2′-deoxy-2′-fluoro (2′-*F*) nucleosides, thereby mimicking the 2′-hydroxyl group by a 2′-*F* atom. Our aim to distinguish alternative RNA secondary structures of the same sequence by ^{19}F NMR spectroscopy should be feasible if a distinct 2′-*F* labeled nucleoside resides within an intact double helix of one fold where it is part of a single-stranded region within the alternative fold (Figure 1). Because the chemical environment of a 2′-*F* atom is significantly different for the two cases, we can expect different chemical shift values for the corresponding resonances. In this sense, the key feature of the approach relies on the strategically “correct” positioning of single, site-specific 2′-*F* labels within the RNA sequence of interest. Such a strategy implies that the replacement of a 2′-OH group by a 2′-*F* atom only slightly alters the overall RNA structure and, in particular, the thermodynamic stability of an RNA double helix. This assumption is reasonable and has been verified for the particular case as discussed below.

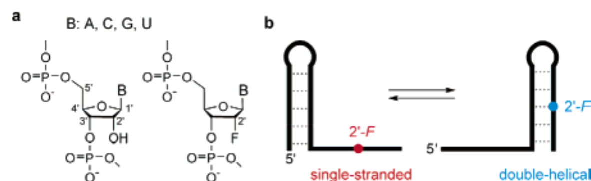

Figure 1. (a) RNA nucleotide unit and the 2′-*F* labeled counterpart. (b) Concept for the determination of different RNA secondary structures by a single 2′-*F* label: two ^{19}F resonances of different chemical shifts are expected according to single-stranded vs double-helical environment.

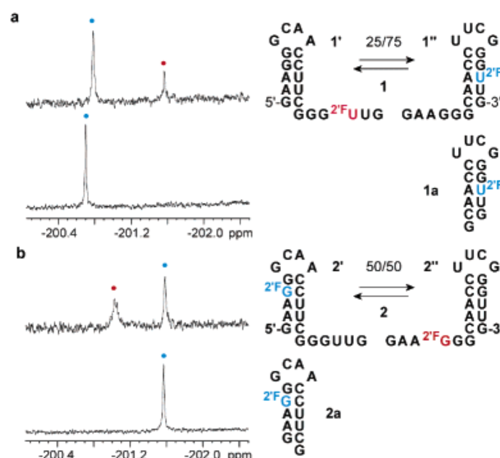

Figure 2. Proton-decoupled ^{19}F NMR spectra. (a) 2′-*F* uridine labeled RNA **1** existing in two conformations **1′** and **1″**, reference sequence **1a** (below). (b) Same as (a) but 2′-*F* guanosine labeled sequence, **2** (conformers **2′** and **2″**; reference **2a**). Conditions: 0.3 mM RNA, 25 mM sodium phosphate buffer pH 7, 298 K, recorded on a Bruker Avance DRX 400 (^{1}H/^{13}C/^{31}P/^{19}F QNP probe). ^{19}F resonances relative to external CCl_3F.

Figure 2 illustrates the realization of our concept displaying the proton-decoupled ^{19}F NMR spectra of a typical bistable 20 nt RNA labeled with a single fluorine either at the 2′-carbon of an uridine in the 3′-part of the sequence (**1**) or at the 2′-carbon of a guanosine in the 5′-part of the sequence (**2**), respectively. The positioning of the labels within the individual sequence (e.g., **1**) fulfills the criteria of participating in either the single-stranded or the double-helical region of the two proposed secondary structures. Accordingly, we observed two signals in a defined ratio for sequence **1** (Figure 2a). The assignment of the signals was performed by comparison with reference **1a**. This hairpin adopted only a single conformation, and its ^{19}F resonance matched well with the one arising from the double helix in conformer **1″** of sequence **1**. Likewise, the chemical shifts of the ^{19}F resonances of reference hairpin **2a** and of conformer **2′**

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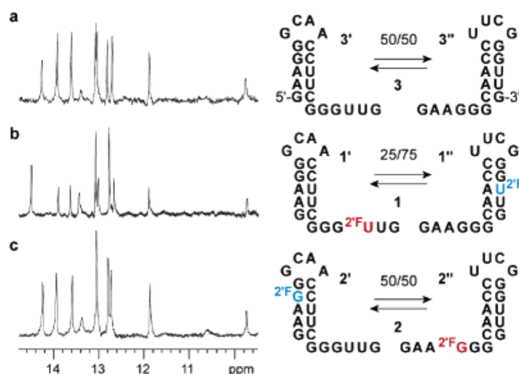

Figure 3. Influence of the 2′-OH/2′-F replacement on the equilibrium position analyzed by ^{1}H NMR spectroscopy. (a) Nonlabeled bistable RNA, **3**. (b) 2′-*F* uridine labeled RNA, **1**. (c) 2′-*F* guanosine labeled RNA, **2**. Conditions as above. Recorded on a Varian Inova 500.

for sequence **2** were in excellent agreement. The ^{19}F NMR spectra therefore unequivocally confirmed the existence of two conformers for the bistable RNA. The rate constant k_1 of the interconversion of conformers for a closely related bistable RNA has been determined recently and is on the order of 0.1 s^{-1} at 298 K.[15]

The ^{19}F resonance for conformer **1′** of sequence **1** (and for conformer **2″** of sequence **2)** originated from the 2′-*F* label within the single-stranded 6 nt overhang. Interestingly, the reference sequence GGG(2′-*F*-U)UG **1b** (GAA(2′-*F*-G)GG **2b**) mimicking this overhang displayed an ^{19}F resonance that was shifted downfield compared to the one of the complete sequence, with an increased line width (Supporting Information). In both cases of **1b** and **2b**, the formation of higher order structures (such as tetraplexes) was experimentally supported by their hysteresis UV-melting profiles (Supporting Information). This individual behavior of the 6 nt reference RNA may explain the slightly decreased matching of signal form and shift with the corresponding ^{19}F resonance obtained from the complete RNA. In general, the ^{19}F signals arising from single-stranded regions displayed increased line widths, what may also reflect the interconversion among local conformational minima of the 2′-*F* riboses. In this context, we mention that in a detailed study on ^{1}H−^{19}F dipolar couplings a ribose 2′-*F* substitution within the loop of an RNA hairpin was found to perturb the equilibrium population distribution of the C2′- versus C3′-endo sugar pucker.[16]

We also investigated the influence of a single 2′-OH replacement by fluorine on the equilibrium position of the bistable sequences used in this study. The nonlabeled RNA **3** existed in a 50:50 ratio of conformers **3′** and **3″** as judged by ^{1}H NMR spectroscopy (Figure 3).[5] While the 2′-*F* guanosine label left this ratio unchanged (sequence **2**), the 2′-*F* uridine slightly shifted the ratio in favor of conformer **1″**. These observations were in accordance with a detailed thermodynamic analysis of the individual references via their UV-melting profiles. Hairpin **2a** had the same thermodynamic stability compared with its nonlabeled counterpart; however, the stability of hairpin **1a** was slightly increased by the fluorine substitution (Supporting Information). In general, we observed equilibrium shifts of about 20−25% for the replacements with 2′-*F* pyrimidine nucleosides to favor the structure with a 2′-*F* label in a stem helix. This was also the case for the 34 nt RNA **4**, which represents another sequence example for a bistable secondary structure (Figure 4). Two distinct ^{19}F signals were detected in favor of conformer **4′** (**4′**/**4″** = 75:25; the corresponding non-fluorine

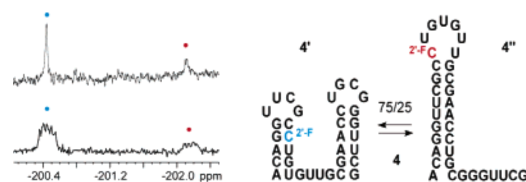

Figure 4. Bistable 34 nt RNA, **4**. ^{19}F NMR spectra: proton-decoupled (top) and non-proton decoupled (bottom). Conditions as above.

sequence was equally populated; see ref 5). Remarkably, in the case of purine nucleosides, we have never observed any RNA secondary structure equilibrium shifts upon the replacement of 2′-OH by 2′-*F*. More data will be needed to understand why 2′-*F* purine nucleosides do not influence the equilibrium position. In any case, they represent the ideal candidates for 2′-*F* labeling as these RNA derivatives can be directly compared to their natural counterparts.

Thus far, we have not explored fluorinated nucleobases for ^{19}F NMR spectroscopy of bistable RNAs. We suppose that their base pairing strengths are influenced to a high and less predictable extent which in turn affects the equilibrium position.

The approach presented here shows that the simple discrimination of double helical versus single-stranded regions in RNA is feasible via ribose 2′-*F* labeling by ^{19}F NMR spectroscopy. This is most promising for the investigation of folding pathways of large RNAs. Detecting a single NMR spin label, like fluorine, may facilitate interpretation of complex RNA structural rearrangements involving even more than two conformational states. Moreover, all four standard nucleosides are commercially available as 2′-*F* phosphoramidites, and this may contribute to a fast dissemination of ^{19}F NMR spectroscopy as a general approach for RNA folding studies.

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Supporting Information Available: ^{1}H NMR and ^{19}F NMR spectra, 2′-*F*-adenosine, and thermodynamic analysis of 2′-*F* RNAs. This material is available free of charge via the Internet at http://pubs.acs.org.

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