2′-O-Trifluoromethylated RNA – a powerful modification for RNA chemistry and NMR spectroscopy†‡

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New RNA modifications are needed to advance our toolbox for targeted manipulation of RNA. In particular, the development of high-performance reporter groups facilitating spectroscopic analysis of RNA structure and dynamics, and of RNA–ligand interactions has attracted considerable interest. To this end, fluorine labeling in conjunction with 19F-NMR spectroscopy has emerged as a powerful strategy. Appropriate probes for RNA previously focused on single fluorine atoms attached to the 5-position of pyrimidine nucleobases or at the ribose 2′-position. To increase NMR sensitivity, trifluoromethyl labeling approaches have been developed, with the ribose 2′-SCF3 modification being the most prominent one. A major drawback of the 2′-SCF3 group, however, is its strong impact on RNA base pairing stability. Interestingly, RNA containing the structurally related 2′-OCF3 modification has not yet been reported. Therefore, we set out to overcome the synthetic challenges toward 2′-OCF3 labeled RNA and to investigate the impact of this modification. We present the syntheses of 2′-OCF3 adenosine and cytidine phosphoramidites and their incorporation into oligoribonucleotides by solid-phase synthesis. Importantly, it turns out that the 2′-OCF3 group has only a slight destabilizing effect when located in double helical regions which is consistent with the preferential C3′-endo conformation of the 2′-OCF3 ribose as reflected in the 3J (H1′–H2′) coupling constants. Furthermore, we demonstrate the exceptionally high sensitivity of the new label in 19F-NMR analysis of RNA structure equilibria and of RNA–small molecule interactions. The study is complemented by a crystal structure at 0.9 Å resolution of a 27 nt hairpin RNA containing a single 2′-OCF3 group that well integrates into the minor groove. The new label carries high potential to outcompete currently applied fluorine labels for nucleic acid NMR spectroscopy because of its significantly advanced performance.

Introduction

The attractiveness of fluorine labeling of biomolecules for 19F-NMR spectroscopic applications originates from its unique properties, namely a 100% natural abundance, high NMR sensitivity, and large chemical shift dispersion.1–9 Moreover, fluorine is bio-orthogonal, meaning that it is hardly encountered in native biomolecular systems.10–25 Appropriate probes for ribonucleic acids have mainly focused on single fluorine atoms attached to the 5-position of pyrimidine nucleobases26–29 or at the ribose 2′-position.21–25 To further increase sensitivity, trifluoromethyl labeling approaches have been sought after,26,27 one of them focuses on ribose 2′-trifluoromethylthio (2′-SCF3) modifications.28–30 A drawback of the 2′-SCF3 group, however, is its strong impact on RNA thermodynamic stability when located in base-paired regions.29 Interestingly, the structurally related 2′-O-trifluoromethyl (2′-OCF3) RNA has not been reported thus far. To the best of our knowledge, only one study is available that describes the thermodynamic stabilities of a short DNA containing a single 2′-OCF3 group that is paired to either a complementary DNA or RNA strand.23 We expected the 2′-OCF3 modification highly beneficial for RNA and 19F-NMR spectroscopic applications to analyze structural dynamics and ligand interactions, and therefore, we set out to overcome the underlying challenges in chemical synthesis. In this work, we present synthetic routes toward 2′-OCF3 nucleoside phosphoramidites.
and their incorporation into oligoribonucleotides by RNA solid-phase synthesis. Furthermore, we describe the impact of the 2'-OCF₃ group on thermodynamic stability of RNA double helices which is only slightly destabilizing when located in double helical regions. This finding is consistent with a preferential C3'-endo conformation of the 2'-OCF₃ ribose in short single stranded RNA as shown by measurements and interpretation of H1'-H2' coupling constants. Moreover, we demonstrate the exceptionally high sensitivity of the new label in ¹⁹F-NMR analysis of RNA structure equilibria and of RNA–small molecule interactions. The study is complemented by crystal structures of an RNA hairpin including 2'-OCF₃ modifications.

Results and discussion

The syntheses of 2'-OCF₃ nucleoside phosphoramidites followed the previously described route of a 2'-OCF₃ adenosine derivative and involved a method by Hiyama et al. who reported that methyl xanthates R-OC(S)SMe are converted into trifluoromethyl ethers R-OCF₃ by treatment with pyridinium trifluoromethane sulfonate (HF/pyridine) in the presence of 1,3-di-bromomethyl ether. The low yield of this transformation.

Synthesis of 2'-OCF₃ cytidine

For building block C7 (Scheme 1), we started the synthesis from cytidine C1, which was simultaneously protected at the 3' and 5' oxygen atoms with the tetraisopropyldisiloxane (TIPDS) group (Scheme 1). Compound C2 was then treated with tert-BuLi, carbon disulfide and methyl iodide in tetrahydrofuran at −78 °C to yield the 2'-O-[(methylthio)-thiocarbonyl]cytidine derivative C3. After acetylation of the exocyclic NH₂ group to furnish C4a, the desired 2'-O-trifluoromethyl derivative C5a was obtained in low yields by treatment with pyridinium poly(hydrogen fluoride) (HF/pyridine) in the presence of N-bromosuccinimide (NBS) instead of DBH as mentioned above. Finally, C5a was transformed into the dimethoxytritylated compound C6a, and conversion into the corresponding phosphoramidite C7a was accomplished in good yields by reaction with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. Starting with cytidine C1, our route provides C7a in 4% overall yield in six steps with six chromatographic purifications; in total, 0.5 g of C7a was obtained in the course of this study. We mention that the yields of trifluoromethylation can be increased by switching from N²-acetyl to N²-benzoyl protection (Scheme 1). The higher stability of the latter (compound 4b) against hydrosis increased the yields by 50% comparing the transformation of 4a into 5a with 4b into 5b. However, one has to be aware that N²-benzoyl cytidine in synthetic RNA can be transaminated to some extent (<10%) when deprotection reagents containing methylamine are applied.

Synthesis of 2'-OCF₃ adenosine

For building block A7 (Scheme 2), we started the synthesis from adenosine A1, which was simultaneously protected at the 3’ and 5’ oxygen atoms with the TIPDS group (Scheme 2). Compound A2 was treated with tert-BuLi, carbon disulfide and methyl
iodide in tetrahydrofuran at −78 °C to yield the 2′-O-[[methylothio]-thiocarbonyl]cytidine derivative A3. After benzylation of the exocyclic NH2 group to furnish A4, the desired 2′-O-trifluoromethyl derivative A5 was obtained by treatment with HF/pyridine in the presence of NBS. Yields were significantly higher compared to the same transformation on cytidine (C4 into C5). Finally, A5 was transformed into the dimethoxytritylated compound A6, and conversion into the corresponding phosphoramidite A7 was accomplished in good yields by reaction with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. Starting with adenosine A1, our route provides A7 in 12% overall yield in six steps with six chromatographic purifications; in total, 1.2 g of A7 was obtained in the course of this study.

We mention that in an attempt to increase the yields for the 2′-O trifluoromethylation step, we tested silver-mediated oxidative O-trifluoromethylation of a 3′,5′-O protected adenosine derivative using TMSCF3, following a published protocol, but unfortunately failed. Currently, we are planning to elaborate routes for 2′-OCF3 uridine and guanosine building blocks via the here described methyl xanthate intermediates. This should be feasible provided proper protection concepts for N3-H and N1-H, respectively, can be identified.

RNA solid-phase synthesis

The solid-phase synthesis of RNA with site-specific 2′-OCF3 modifications was performed following the 2′-O-[(triisopro|pylsilyl)oxy]methyl (TOM) approach. Coupling yields of the novel building blocks were higher than 98% according to the triyl assay. Cleavage of the oligonucleotides from the solid support and their deprotection were performed using methylamine in water/ethanol or methylamine/ammonia in water (AMA), followed by treatment with tetra-n-butylammonium fluoride (TBAF) in tetrahydrofuran. Salts were removed by size-exclusion chromatography, and RNAs were purified by anion-exchange chromatography under denaturing conditions (6 M urea, 80 °C; Fig. 1 and ESI Table 1†). The molecular weights of the purified RNAs were confirmed by liquid-chromatography (LC) electrospray-ionization (ESI) mass spectrometry (MS). The sequences of 2′-OCF3 containing RNAs synthesized in the course of this study are listed in ESI Table 1†.

### Thermodynamic stability of 2′-OCF3 modified RNA

A single 2′-OCF3 adenosine exhibited only moderate attenuation of RNA double helix stability if the modification was located in the Watson–Crick base-pairing region (Table 1). For instance, UV melting profile analysis of the hairpin-forming RNA 5′-GA(2′-OCF3-A)GG-GCAA-CCUUGG (Fig. 2A) revealed a decrease of the $T_m$ value by 3 °C determined at micromolar RNA concentrations ($T_m$ 70.3 °C), compared to the unmodified counterpart ($T_m$ 73.3 °C). We remind that the same RNA but with a 2′-SCF3 modification at the same nucleotide position caused a much stronger drop in the $T_m$ value, namely by 14 °C (Table 1). As a second example, the palindromic RNA 5′-GGUUGG(2′-OCF3-A)CC (Fig. 2B) also suffered from an average $T_m$ value decrease of 3 °C per 2′-OCF3-A modification (micromolar RNA concentrations), compared to the unmodified counterpart.

Next, the same hairpin was analyzed with 2′-OCF3 at cytidine instead of adenosine, 5′-GAAGGGGCAAC(2′-OCF3-C)UUCG (Fig. 2C). The destabilization was reflected by a 6.5 °C lower $T_m$
The inherent preference of a modified nucleoside to adopt either C2'-endo or C3'-endo conformation is crucial for its impact on thermodynamic base pairing stability. To analyze the ribose conformation of a 2'-OCF₃ nucleoside in detail, we synthesized a short, single-stranded RNA, 5'-GGCAG(2'-OCF₃)-A)GGC, and determined the 1H (H1'-H2') coupling constant from a 2D 1H,1H double quantum filtered COSY spectrum (DQFCOSY). The coupling constant amounted to 5.9 Hz, which translates into a population of about 60% of C2'-endo ribose conformation in the single strand (Fig. 3A and B; for 1H/1H DQFCOSY (B) NMR spectra of single-stranded RNA 5'-GGCAG(2'-OCF₃)-A)GGC. For the 2'-OCF₃ adenosine moiety, the 3-bond scalar coupling constant of H1' and H2' (3JH1'-H2') was extracted from the corresponding crosspeak and amounted to 5.9 Hz. Assuming a pure C2'/C3'-endo equilibrium, this value is correlated to a C2'-endo (South) population of 58%, 38,40 (C) 1H NMR spectrum of the self-complementary 8 nt RNA with 2'-OCF₃-A group (red); 3JH1'-H2' is smaller than 1 Hz, consistent with prevalent C3'-endo conformation in the double helix. Conditions: c(RNA) = 0.3 mM; 15 mM Na[AsO2(-CH3)]·3H2O, 25 mM NaCl, 3 mM NaNO3 in D2O, pH 6.5, 296 K.

### 2'-OCF₃ ribose conformation

The inherent preference of a modified nucleoside to adopt either C2'-endo or C3'-endo conformation is crucial for its impact on thermodynamic base pairing stability. To analyze the ribose conformation of a 2'-OCF₃ nucleoside in detail, we synthesized a short, single-stranded RNA, 5'-GGAAGG-GC(2'-OCF₃)-A)A-CCUUG (Fig. 2D and Table 1). The stabilization is rationalized by the fact that unmodified adenosine in GCA loops preferentially adopts the C3'-endo ribose pucker (70%). The C3'-endo conformation in this loop becomes locked when the 2'-OCF₃ label compared to a 2'-SCF₃ label of the same hairpin sequence with the modification in the GNRA loop motif (D). Conditions: c(RNA) = 12 μM; 10 mM Na2HPO4, 150 mM NaCl, pH 7.0. Nucleotides in red color indicate the positions for 2'-OCF₃ modification.

**Fig. 2** Thermal stabilities of unmodified versus 2'-OCF₃ modified oligoribonucleotides. UV-melting profiles of hairpin and self-complementary duplex RNAs with the modification (either at adenosine or cytidine) located in the base-pairing region (A–C), and UV-melting profile of the same hairpin sequence with the modification in the GNRA loop motif (D). Conditions: c(RNA) = 12 μM; 10 mM Na2HPO4, 150 mM NaCl, pH 7.0. Nucleotides in red color indicate the positions for 2'-OCF₃ modification.

value compared to the unmodified hairpin, still significantly less compared to the 2'-SCF₃-C modification that caused a Tₘ reduction by 20 °C of this hairpin (Table 1). We point out that the significantly larger destabilization of 2'-SCF₃ compared to the 2'-OCF₃ modified double helices is of entropic origin (Table 1).

Notably, when the 2'-OCF₃ group resides in a single-stranded region, the impact on thermodynamic stability is minor, and in the case of the extra-stable GNRA loop motif even modestly stabilizing, reflected by an increase of the Tₘ value and a favorable ΔG value for 5'-GAAAGG-GC(2'-OCF₃)-A)A-CCUUG (Fig. 2D and Table 1). The stabilization is rationalized by the fact that unmodified adenosine in GCA loops preferentially adopts the C3'-endo ribose pucker (70%).

The C3'-endo conformation in this loop becomes locked when the 2'-OCF₃ label of the same hairpin sequence with the modification in the GNRA loop motif (D). Conditions: c(RNA) = 12 μM; 10 mM Na2HPO4, 150 mM NaCl, pH 7.0. Nucleotides in red color indicate the positions for 2'-OCF₃ modification.

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By examining the 19F NMR spectroscopic sensitivity of the 2'-OCF₃ group (Fig. 2,‡), the very same hairpin was also used to demonstrate the impressive19F NMR spectroscopic sensitivity of the 2'-OCF₃ group (red); 3JH1'-H2' is smaller than 1 Hz, consistent with prevalent C3'-endo conformation in the double helix. Conditions: c(RNA) = 0.3 mM; 15 mM Na[AsO2(-CH₃)]·3H₂O, 25 mM NaCl, 3 mM NaNO₃ in D₂O, pH 6.5, 296 K.

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Fig. 3 19F (A) and 1H/1H DQFCOSY (B) NMR spectra of single-stranded RNA 5'-GGCAG(2'-OCF₃)-A)GGC. For the 2'-OCF₃ adenosine moiety, the 3-bond scalar coupling constant of H1' and H2' (3JH1'-H2') was extracted from the corresponding crosspeak and amounted to 5.9 Hz. Assuming a pure C2'/C3'-endo equilibrium, this value is correlated to a C2'-endo (South) population of 58%, 38,40 (C) 1H NMR spectrum of the self-complementary 8 nt RNA with 2'-OCF₃-A group (red); 3JH1'-H2' is smaller than 1 Hz, consistent with prevalent C3'-endo conformation in the double helix. Conditions: c(RNA) = 0.3 mM; 15 mM Na[AsO2(-CH₃)]·3H₂O, 25 mM NaCl, 3 mM NaNO₃ in D₂O, pH 6.5, 296 K.

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X-ray structures of 2'-OCF₃ containing RNA

We set out for the X-ray analysis of a 2'-OCF₃ modified RNA (Fig. 4). To this end, we used the 27 nt fragment of E. coli 23S rRNA sarcin-ricin loop (SRL) region. This sequence is known to be a robust and well-behaved crystallization scaffold that can accommodate small modifications.⁴⁴ For the installation of 2'-OCF₃, we deemed nucleotide A2670 appropriate which forms a Watson–Crick base pair with U2650 in the regular A-form double helical region of this RNA, as well as nucleotide C2667 which forms a water-mediated mismatch with U2653. The latter RNA crystallized in the same tetragonal crystal form as the unmodified RNA⁴⁴ and also diffracted to atomic resolution (0.9 Å). A new monoclinic crystal form was obtained with 2'-OCF₃ A2670-modified SRL RNA (relative to former SRL RNA crystal forms), containing three molecules per asymmetric unit and diffracting to 2.4 Å resolution (ESI Table 2†). The 2'-OCF₃ modification did not affect the overall structure (r.m.s.d. ~0.4 Å for 2'-OCF₃–C2667 compared to the unmodified structure; PDB ID 3DVZ), including sugar puckers of modified positions (ESI Fig. 4†). As previously observed for the 2'-SCF₃–C2667 modification,²⁹ a fluorine atom of the 2'-OCF₃ group closely approached the oxygen atom of its cytosine nucleobase (O2). It is tempting to assume that the short distance observed (2.9 Å) is indicative of a halogen bond, however, fluorine (as opposed to chlorine, bromine, or iodine) usually retains a strongly electronegative electrostatic potential in biomolecules.⁶³,⁶⁴ Nevertheless, we note that the trifluoromethyl modification additionally comes also close the O4’ of the next G2668 residue (O–F distance is 3.2 Å, ESI Fig. S1†) which further fuels speculations on possible stabilizing interactions.

2'-OCF₃ NMR analysis of structure-ambivalent RNA

The biological function of RNA is determined by the secondary and tertiary structure, defining the RNA fold.⁴⁶ The folding path usually proceeds via intermediates that represent local minima in the RNA folding free energy landscape. When these intermediates are separated by large energy barriers they constitute folding traps and, therefore, the timescale of the folding process may take up to minutes and longer. One of the most prominent examples is a single RNA sequence of 150 nucleotides in length that co-exists in two stable folds harboring distinct ribozyme examples is a single RNA sequence of 150 nucleotides in length that can accommodate small modifications.⁴⁶ For the installation of 2'-OCF₃, we deemed nucleotide A2670 appropriate which forms a Watson–Crick base pair with U2650 in the regular A-form double helical region of this RNA, as well as nucleotide C2667 which forms a water-mediated mismatch with U2653. The latter RNA crystallized in the same tetragonal crystal form as the unmodified RNA⁴⁴ and also diffracted to atomic resolution (0.9 Å). A new monoclinic crystal form was obtained with 2'-OCF₃ A2670-modified SRL RNA (relative to former SRL RNA crystal forms), containing three molecules per asymmetric unit and diffracting to 2.4 Å resolution (ESI Table 2†). The 2'-OCF₃ modification did not affect the overall structure (r.m.s.d. ~0.4 Å for 2'-OCF₃–C2667 compared to the unmodified structure; PDB ID 3DVZ), including sugar puckers of modified positions (ESI Fig. 4†). As previously observed for the 2'-SCF₃–C2667 modification,²⁹ a fluorine atom of the 2'-OCF₃ group closely approached the oxygen atom of its cytosine nucleobase (O2). It is tempting to assume that the short distance observed (2.9 Å) is indicative of a halogen bond, however, fluorine (as opposed to chlorine, bromine, or iodine) usually retains a strongly electronegative electrostatic potential in biomolecules.⁶³,⁶⁴ Nevertheless, we note that the trifluoromethyl modification additionally comes also close the O4’ of the next G2668 residue (O–F distance is 3.2 Å, ESI Fig. S1†) which further fuels speculations on possible stabilizing interactions.

Fig. 4 X-ray structure of 2'-OCF₃ modified RNA at atomic resolution. (A) Secondary structure of the E. coli Sarcin-ricin stem-loop (SRL) RNA used for crystallization. The 2'-OCF₃ modified cytidine is labeled in red. Adenosine in green highlights the second 2'-OCF₃ modified RNA solved in this study (see ESI Fig. 4 and S5†) and uridine in blue refers to a previously solved 2'-SCF₃ modified SRL RNA for reason of comparison.²⁹ (B) Superimposition of the 2'-OCF₃–C2667 modified RNA (PDB ID 6ZYB red) and 2'-SCF₃–U2650 modified RNA (PDB ID 4NHX, blue). Trifluoromethyl modifications are visible outside the RNA stem loop (on the center-right for OCF₃ and in the bottom-left for SCF₃). (C) 2Fₐ – Fcalc electron density map contoured at 1.5 o level showing the U2653/2'-OCF₃–C2667 base pair. Water molecules are shown as red spheres (PDB ID 6ZYB).

to the NMR time scale. Two distinct ¹⁹F resonances are obtained (Fig. 5A and B) and assigned by comparison to a short reference hairpin that matches one substructure and adopts a single fold only (Fig. 5A). Interestingly, the line-widths of the two ¹⁹F resonances arising from the 2'-OCF₃ group in the two folding states exhibit a large difference. The half-height line-width is 7 Hz for the ‘single strand’ ¹⁹F resonance while it is increased by a factor of almost three for the ‘double helix’ ¹⁹F resonance (20 Hz). Likely, the rotation of the 2'-OCF₃ group in the base-paired stem is sterically hindered leading to signal broadening due to exchange on the µs to ms time scale.

The sensitive 2'-OCF₃ label was also used to analyze the underlying kinetics of refolding for the bistable RNA. For that purpose, we used ¹⁹F longitudinal exchange NMR spectroscopy.⁵¹–⁵⁵ As expected we found two ¹⁹F correlation peaks (fold A and fold B) in the ¹⁹F NOESY exchange spectrum of the RNA (with a single 2'-OCF₃ labeled adenosine) for which exchange
peaks could be identified (Fig. 5C). The forward and backward folding rates of the secondary structure equilibrium were determined at 30 °C (Fig. 5D). We found a forward rate constant $k_{BA}$ of $0.36 \pm 0.01$ s$^{-1}$ and a rate constant $k_{AB}$ for the folding process from state A to state B of $0.21 \pm 0.01$ s$^{-1}$, giving a good agreement with the equilibrium constant obtained from peak integration [$k_{AB}^{30^\circ C} = 1.9$ and $k_{AB}^{30^\circ C} = k_{BA}/k_{AB} = 1.7$]. Using our $^{19}$F labeling approach, we were thus able to characterize the refolding kinetics of the bistable RNA under near physiological conditions.

Furthermore, to analyze the impact of the labeling position we synthesized the same bistable RNA but with $2'$-OCF$_3$ at C10. As expected, we observed two distinct $^{19}$F NMR resonances for the two folds in slow exchange, albeit the chemical shift difference was smaller, consistent with the label located in double helical regions in both folds (Fig. 5F). The equilibrium position (50:50) (Fig. 5G and ESI Fig. 7) was only slightly shifted compared to the $2'$-OCF$_3$ A3 labeled counterpart (40:60) and confirmed the flexibility for $2'$-OCF$_3$ positioning despite of being a non-isosteric label. At this point, we also mention that the native (unmodified) RNA exists in a 50:50 equilibrium as was determined earlier.$^{21}$

With respect to increasing temperatures, the $^{19}$F NMR resonances shifted to lower magnetic field. This shift was more pronounced for the $2'$-OCF$_3$-C labeled RNA (Fig. 5G) compared to the $2'$-OCF$_3$-A labeled RNA (Fig. 5B), which might be due to fraying of the loop closing base pair of which $2'$-OCF$_3$-C is part of (Fig. 5F, fold B).

Finally, we characterized both RNAs by recording $^{19}$F-$^{13}$C HMBC spectra (Fig. 5E and H). We were able to obtain high quality natural abundance $^{19}$F-$^{13}$C correlation spectra for rather dilute samples (ca. 150 µM each fold) overnight. By adding the carbon dimension, better spectral dispersion is obtained, which in principle would allow to apply $^{13}$C ZZ exchange spectroscopy in $2'$-O-$^{13}$CF$_3$ labeled systems. It will be further highly interesting to explore the TROSY properties of the $2'$-O-$^{13}$CF$_3$ methyl group in RNA by combining $^{19}$F and $^{13}$C stable isotope labeling. In a recent work by Sykes and co-workers no favorable TROSY effect in proteins was found using 3-bromo-1,1,1-trifluoroacetone as labeling reagents. The absence of the TROSY effect was attributed to the dominating CSA relaxation mechanism in the alpha CF$_3$ group suggesting that gains from CF$_3$-HMBC experiments should be only observable at low magnetic fields.$^{68}$ Nevertheless, it remains to be clarified if the $2'$-O-$^{13}$CF$_3$ shows a more beneficial TROSY effect due to the different chemical environment.

$2'$-OCF$_3$ NMR analysis of RNA-ligand binding

The new $2'$-OCF$_3$-labeled system is highly practical for NMR studies of RNA-ligand interactions. In Fig. 6, a comparison of the 7-aminomethyl-7-deazaguanine (preQ$_1$) sensing class-I riboswitches from _Thermoanaerobacter tengcongensis_ (Tte) [Fig. 6A] and _Fusobacterium nucleatum_ (Fn) is illustrated (Fig. 6B).$^{56-58}$ These RNAs adopt the same overall fold but differ in sequence and ligand affinity, the latter by about an order of magnitude ($K_d$(Tte) ~ 20 nM versus $K_d$(Fn) ~ 260 nmol).$^{57,58}$ Moreover, for the _Tte_ riboswitch, crystal structures at high resolution exist of both, the free and the preQ$_1$-bound RNA,$^{59}$ and therefore a solid foundation to explore the underlying conformational adaptions during ligand-induced folding is available.

At the left side of Fig. 6A, a secondary structure folding model for the preQ$_1$ riboswitch is shown. The two RNAs with...
a previous NMR study where the formation of individual (15N-labeled) base pairs of the same riboswitch system was tracked.29 We furthermore believe that two of the three folding states likely correspond to the conformational distinction seen in high-resolution crystal structures of free versus preQ1-bound Tte RNA. In the ligand-free pseudoknot form, A14 takes the position of the preQ1 ligand and is stacked between the two base pairs of G11–C30 and G5–C16.27,29 This observation can be rationalized by a conformational rearrangement from a solvent-exposed base C15 to a flipped-in conformation of C15 that becomes the Watson–Crick pairing partner of preQ1 in the ligand-bound pseudoknot RNA.60

Fig. 6B depicts NMR spectra of the titration of the Fn preQ1 riboswitch which binds preQ1 13-fold weaker compared to the Tte counterpart. Furthermore, the loop size of the Fn RNA contains two additional nucleotides and the pseudoknot interaction allows formation of a continuous 4 bp double helix.64 We placed the 2′-OCF3 labels in different positions, one in the tail to sense pseudoknot formation, and the other one in the loop next to the cytosine that can form a Watson–Crick base pair with preQ1. Both labels allow to monitor the binding process. By increasing the concentration of preQ1 stepwise, an increasing fraction of the preQ1-RNA complex is observed, reflected in a second 19F resonance that is shifted to lower field. In contrast to the Tte RNA, the Fn riboswitch shows a significant population of unbound RNA, even at a ten-fold excess of ligand over RNA. Furthermore, a folding intermediate is also indicated by the appearance of a third resonance for the 2′-OCF3 A16 labeled RNA, although existing in a minor population only. Another interesting feature is that the line width of the 19F resonance of the label next to the pseudoknot double helix (A30) is rather small and the OCF3 group seems to be hardly hindered in rotational freedom compared to the other three labeling positions in the two riboswitch systems.

Conclusions

Over the last decades, several 19F labels for nucleic acids NMR spectroscopy have been introduced, among them are single fluorine labels (such as pyrimidine 5-F,16–20 ribose 2′-F,21–23 and 4′-F),21,23 4′-C-[(4-trifluoromethyl-1H-1,2,3-triazol-1-yl)methyl] ribose,27 2′-SCF3,28–30 and very recently 8-SCF3 guanine,44), and a nine-fluorine-atom label (in form of 5-[4,4,4-trifluoro-3,3-bis(trifluoromethyl)but-1-ynyl] 2′-deoxyuridine).4 Comparing them, the first subgroup is superior with respect to steric demands but suffers from low sensitivity and the need for proton decoupling;23–25 the opposite is true for the nine-fluorine-atom label which is highly sensitive but sterically demanding.4 Therefore, trifluoromethyl labels appear to be a good compromise.23–25 The pyrimidine 5-SCF3 group, however, has been described to be chemically unstable during deprotection of synthetic nucleic acids, resulting in low yields and length limitation.28 Differently, the 2′-SCF3 group – although synthetically well accessible – strongly affects thermodynamic base pairing strength which is a drawback for applications that aim at the elucidation of folding pathways.28–30 All critical aspects
and requirements are very well satisfied by the new 2′-OCF₃ label as shown in this study; it therefore has potential to advance to the most broadly applicable fluorine label in NMR spectroscopy of nucleic acids. The 2′-OCF₃ group possesses pronounced sensitivity and exhibits large chemical shift dispersion so that it is possible to distinguish distinct base sequences in double helical regions. Moreover, the ribose 2′ position guarantees principal synthetic accessibility to all of the four nucleosides. Additionally, the size of the OCF₃ label matches the naturally occurring 2′-OCH₃ group. For the investigation of naturally occurring 2′-OCH₃ modification patterns in biologically meaningful settings, the 2′-OCF₃ group will be an ideal candidate.

Conflicts of interest

There are no conflicts to declare.

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