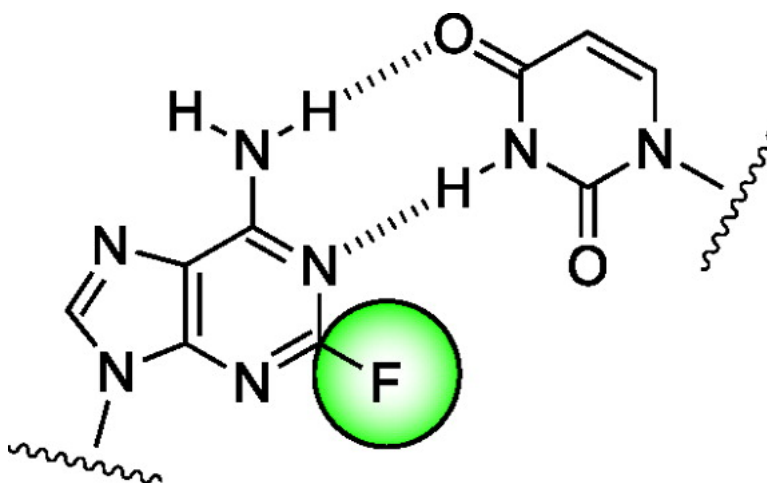## Communication

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Enzymatic Synthesis and ¹⁹F NMR Studies of 2-Fluoroadenine-Substituted RNA

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The production of isotopically labeled nucleotides remains critical for structural studies of RNA oligonucleotides using nuclear magnetic resonance (NMR) spectroscopy.[1] Through the use of ¹³C and ¹⁵N isotopic labeling and multidimensional heteronuclear NMR experiments, studies of 15-kDa RNAs are commonplace.[2,3] However, as the size of the RNA increases, the spectral crowding and resonance line widths also increase, making heteronuclear NMR experiments less sensitive and assignments more difficult. Site-specific deuteration has been successfully used to simplify spectra and to improve sensitivity.[4] An alternative approach is to label with a nonnatural nucleus such as fluorine-19 (¹⁹F) that affords high natural abundance and sensitivity. Furthermore, ¹⁹F chemical shift dispersion is about 100-fold that of ¹H, making it an ideal site-specific probe for monitoring functionally important conformational transitions.[5] Here we show that 2-fluoroadenosine-5′-triphosphate (2F-ATP) can readily be incorporated into the HIV-2 transactivation response element (TAR) RNA without affecting its structural integrity and thermodynamic stability. The approach may therefore be generally applicable to structural studies of RNA molecules.

Previously, we had demonstrated the efficient in vitro enzymatic synthesis of ribonucleotide-5′-triphosphates (NTPs).[4,6,7] The nucleotide analogue 2F-ATP can be produced metabolically in *Escherichia coli* supplied with 9-β-D-arabinofuranosyl-2-fluoro-adenine, which proceeds first by phosphorolysis to 2-fluoroadenine (2F-Ade), followed by conversion via the purine salvaging pathway to 2F-ATP.[8] We anticipated that 2-fluoroadenine (2F-Ade)-substituted RNA could be prepared via in vitro enzymatic synthesis of 2F-ATP,[9] followed by in vitro transcription.
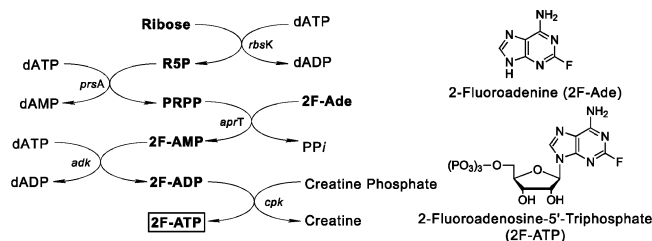
The efficient in vitro enzymatic synthesis of 2F-ATP (Scheme 1) began with the C-5-phosphorylation of ribose by ribokinase

Scheme 1

(*rbs*K) to give ribose-5-phosphate (R5P) that was further phosphorylated at the C-1 position by 5-phospho-D-ribosyl-α-1-pyrophosphate synthase (*prs*A), forming 5-phospho-D-ribosyl-α-1-pyrophosphate (PRPP). Next, adenine phosphoribosyltransferase (*apr*T) coupled 2F-Ade to PRPP, forming 2-fluoroadenosine-5′-monophosphate (2F-AMP). The monophosphate 2F-AMP was subse-

quently converted to 2F-ATP by sequential action of adenylate kinase (*adk*) and creatine phosphokinase (*cpk*). A key feature of the synthesis is the use of a dATP as the phosphate donor that was regenerated using *adk*, *cpk*, and an excess of creatine phosphate. The nucleotide analogue 2F-ATP was easily separated in 90% isolated yield from the dATP and incorporated into RNA as readily as ATP by DNA template-directed transcription using phage T7 RNA polymerase.[10] A single 40-mL in vitro transcription reaction yielded 860 nM of 2F-Ade-substituted HIV-2 TAR RNA (see Supporting Information) that was used for further characterization by UV and NMR spectroscopy.

To determine the effect of 2F-Ade substitutions on the thermodynamic stability of HIV-2 TAR RNA, optical melting profiles were performed for 2F-Ade-substituted as well as unmodified RNA samples (Supporting Information Figure 1). Both RNAs exhibited essentially identical hyperchromicity in a single transition, with the T_m for 2F-Ade-substituted RNA being only 2° lower than unmodified RNA (348.0 ± 0.2 and 350.4 ± 0.7 K, respectively). The enthalpy values of denaturation for 2F-Ade-substituted and unmodified RNAs were also quite similar ($\Delta H° = -92.9 \pm 0.7$ and -94.1 ± 1.4 kcal·mol⁻¹, respectively), clearly demonstrating that the ¹⁹F substitutions do not substantially change overall RNA stability. Any destabilizing electrostatic interactions in a 2F-A−U base pair involving positions Ade ¹⁹F-2 and Ura O-2 are compensated for via favorable base stacking[11] and ¹⁹F-N-1 secondary electrostatic hydrogen-bond donor and acceptor arrangements.[12] Apparently, 2F-Ade substitutions are very similar in character to 2,6-diaminopurine substitutions.[12,13]

The ¹⁹F and imino proton resonance assignments for the 2F-Ade-substituted RNA were straightforward to obtain with homo- and heteronuclear NOE experiments shown in Figure 1. Two out of four ¹⁹F nuclei, A20 and A27, could be identified on the basis of intense heteronuclear NOE correlations[14,15] to exchangeable imino protons on the complementary base-paired U42 and U38, respectively. The exchangeable U42 and U38 imino protons experience very pronounced upfield chemical shift changes compared to the unmodified RNA (-2.63 and -2.60 ppm, respectively) because of the altered shielding in the presence of the ¹⁹F nuclei on the complementary base involved in the Watson−Crick base pair (Supporting Information Figure 2). However, chemical shifts of guanine imino protons involved in canonical G−C base pairs are relatively unperturbed ($+0.04$ ppm on average, Supporting Figure 2), greatly facilitating the assignments of imino protons in the 2F-Ade-substituted RNA (Figure 1C). The U40 imino proton resonance is not detectable at 283 K because of unfavorable exchange properties with water, and consequently, heteronuclear NOE correlations to the base-paired ¹⁹F nuclei of A22 were not observed. The ¹⁹F resonance of A22 could be unambiguously assigned using a sequential heteronuclear NOE correlation to the
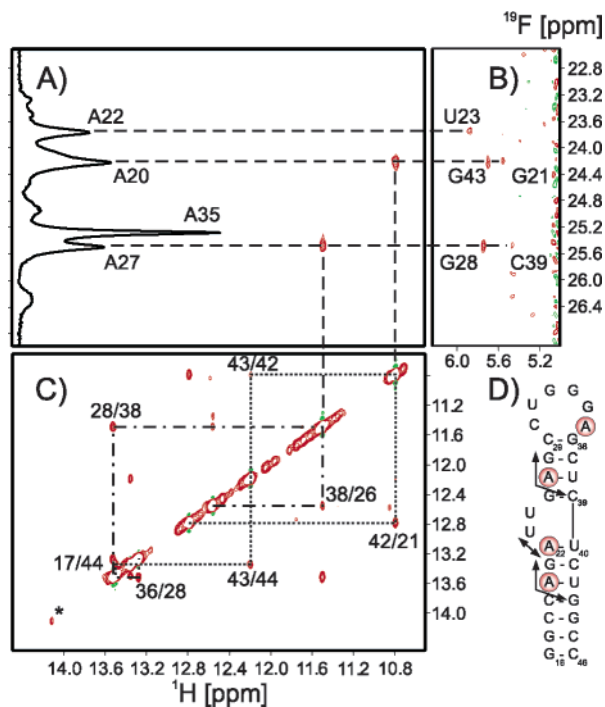
Figure 1. (A) and (B) Proton-detected ^{1}H, ^{19}F-HOESY (optimized for detecting exchangeable protons; for details see Supporting Information Figure 3) showing cross-peaks between ^{19}F nuclei and (A) imino and (B) anomeric H-1′ protons. The inset in (A) shows a 1-D ^{19}F spectrum with residue-specific assignments for the four 2-^{19}F adenine resonances. (C) ^{1}H, [1]H-NOESY experiment showing imino−imino proton connectivities for the lower stem (dotted line) and corresponding connectivities for the upper stem (dash−dotted line). Assignments of individual homonuclear imino−imino NOESY cross-peaks are given. The diagonal peak marked with an asterisk represents a minor impurity corresponding to U38 imino proton resonance frequency in the absence of a 2F-Ade substitution at position A27. (D) Sequence and secondary structural representation of HIV-2 TAR RNA. The 2F-Ade substitutions are highlighted. Observable [19]F−[1]H-1′ HOESY correlations (B) are indicated by arrows. [19]F spectra (A and B) were recorded on a three-channel Bruker Avance 400 MHz spectrometer equipped with a z-gradient ^{1}H/^{13}C/^{31}P/^{19}F QNP probe at a temperature of 283 K. The ^{1}H, [1]H-NOESY experiment (C) was recorded on a four-channel Bruker DRX 800 MHz spectrometer equipped with a z-gradient ^{1}H/^{13}C/^{15}N triple-resonance probe, again at a temperature of 283 K.

anomeric H-1′ ribose proton of the bulged U23 (Figure 1B). In A-form helical structure, cross-strand distances ($d < 6$ Å) are observed from the H-2 base proton of adenine to the H-1′ proton on ribose. The pattern of observable ^{19}F, ^{1}H heteronuclear NOEs resembles the corresponding homonuclear ^{1}H, ^{1}H NOEs in unmodified RNA; thus, cross-strand ^{19}F, ^{1}H connectivities are readily observed between A20 and the anomeric H-1′ ribose proton of G43 as well as between A27 and C39 H-1′. The remaining ^{19}F resonance of A35 was assigned by exclusion and its unique properties among the four adenines. Adenine-35 is part of the largely unstructured apical loop (C30−A35), and therefore it exhibits greater flexibility with respect to the ordered helical stem parts of the RNA.[16,17] The absence of any observable heteronuclear NOE correlations of A35 to other protons (Figure 1) is consistent with the observed homonuclear NOE pattern in unmodified RNA and indicates that this base is flipped out and accessible to the solvent. The considerably reduced ^{19}F line width of A35 with respect to the other three adenines further supports the reported assignments (Figure 1A).

A closer inspection of the ^{19}F spectrum of 2F-Ade-substituted RNA reveals the presence of minor populations of RNA in addition to the assigned four major resonances, which can be attributed to both incomplete ^{19}F labeling and conformational heterogeneity. The NOESY diagonal peak integrals of the U38 imino proton in the presence and absence of the ^{19}F nuclei on the complementary base A27 (Figure 1C) can be attributed to a contamination of 1.3% unmodified adenine present in the synthesis of 2F-ATP, which was subsequently incorporated during transcription. Conformational heterogeneity of fully 2F-Ade-substituted RNA could be due to a combination of base-pair opening dynamics and equilibria between interconverting 2F-A−U base-pairing geometries.[18]

Here we introduced an efficient in vitro enzymatic synthesis of the fluorinated nucleotide analogue 2F-ATP. We demonstrate the stable base-pairing interaction of 2F-Ade with uracil in a helical RNA structure. Both thermal denaturation studies and NMR analysis suggest that there is only a mild perturbation of the helical RNA structure by incorporation of multiple 2F-Ade residues. The introduction of ^{19}F substitution into the adenine bases provides a uniquely positioned, sensitive NMR reporter to monitor structural changes in RNA molecules due to conformational changes or ligand binding.

Supporting Information Available: Detailed experimental and supporting figures for the synthesis, thermal melts, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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