Synthesis and Analysis of RNA Containing 6-Trifluoromethylpurine Ribonucleoside
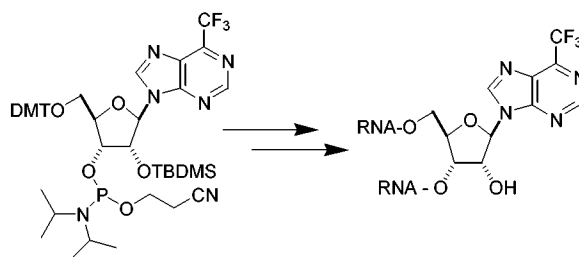
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ABSTRACT

We report the synthesis of a 5′-DMT-2′-TBDMS-protected phosphoramidite of 6-trifluoromethylpurine ribonucleoside (^{TFM}P) and its use in the site-specific incorporation of 6-trifluoromethylpurine into RNA. Properties of ^{TFM}P-substituted RNA suggest it will be valuable in the study of RNA structure and the binding of RNA-modifying enzymes, particularly the RNA-editing adenosine deaminases.

The enzymatic deamination of adenosine in mRNA results in inosine at that position. Since inosine is translated as guanosine, this reaction can lead to codon changes in mRNA, resulting in the synthesis of proteins that are structurally and functionally distinct from those encoded in the genome. These RNA-editing adenosine deamination reactions are catalyzed by the ADARs (adenosine deaminases that act on RNA) and require that the adenosine to be modified be present in duplex secondary structure.[1] Our current understanding of the mechanism of the ADAR-catalyzed deamination of adenosine in double helical RNA is limited. Indeed, given the lack of structural information on the enzyme, the nature of the active site can only be speculated upon based on sequence similarities to other enzymes.[2] We have used phosphoramidite chemistry to introduce adenosine analogues into model substrates as probes of the ADAR reaction mechanism.[3,4] These studies indicate that ADARs share mechanistic similarities with the well-characterized nucleoside-modifying enzyme adenosine deaminase (ADA). However, the hydrolytic deamination reaction catalyzed by ADA is an imperfect model for the steps occurring in the ADAR active site. In addition, fluorescence changes are observed when ADAR2 binds to a 2-aminopurine-modified RNA duplex that are consistent with a base-flipping mechanism wherein the nucleotide undergoing modification is extruded from the double helix.[5] This step is not required for the ADA reaction.

In this Letter, we report the synthesis and characterization of RNA containing an analogue of adenosine in which the C6 amine has been replaced with a trifluoromethyl group (6-trifluoromethylpurine ribonucleoside, ^{TFM}P). Trifluoromethylation of purine at C6 has been proposed to facilitate

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(2) Hough, R. F.; Bass, B. L. *RNA* **1997**, *3*, 356−370.

(3) Yi-Brunozzi, H.-Y.; Easterwood, L. M.; Kamilar, G. M.; Beal, P. A. *Nucleic Acids Res.* **1999**, *27*, 2912−2917.

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(5) Stephens, O. M.; Yi-Brunozzi, H.-Y.; Beal, P. A. *Biochemistry* **2000**, *39*, 12243−12251.

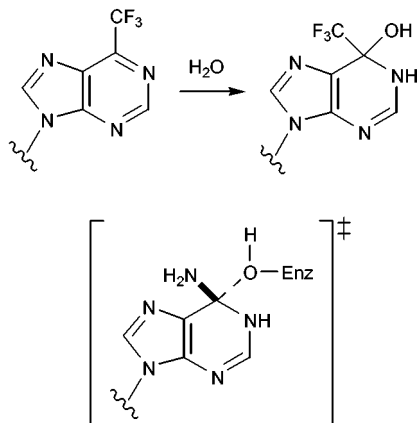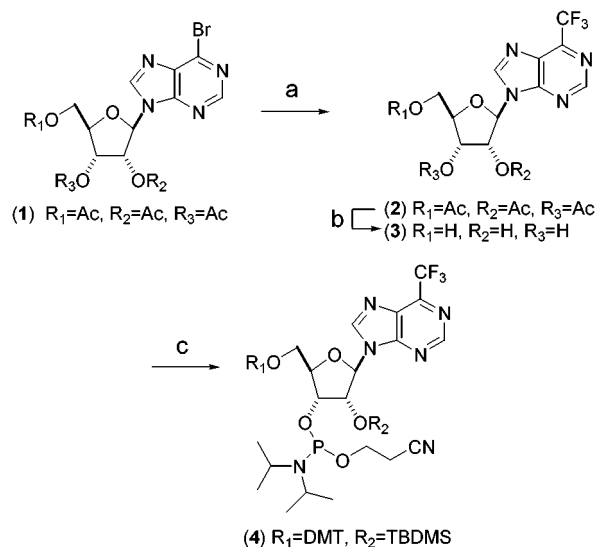hydration at this position, creating a mimic of the transition state for hydrolytic deamination of adenosine (Figure 1).[6]

Figure 1. Hydration of ^{TFM}P across the C6−N1 double bond generates a structure similar to the transition state proposed for the adenosine deamination reaction catalyzed by ADA.

Such a structure, in the appropriate double helical RNA context, is a good candidate for high-affinity binding at the ADAR active site. Furthermore, the presence of the trifluoromethyl group creates a sensitive NMR probe of the electronic environment of this nucleotide in the RNA. ^{19}F NMR analysis of the binding of 5-fluorodeoxycytidine-substituted DNA to a cytosine DNA methyltransferase allowed for delineation of features of its base-flipping mechanism.[7] This approach would be valuable at further defining the mechanism by which ADARs base flip if the appropriately fluorine-labeled RNA were available.

6-Trifluoromethylpurine ribonucleoside had been previously synthesized from a sugar-protected 6-chloropurine ribonucleoside and trifluoromethyl copper (CF$_3$Cu).[8] However, the reaction time (60 h) and yield (29%) led us to investigate other methods to obtain this compound. Given the precedent of increased yields in copper-mediated coupling reactions with bromides over chlorides, we attempted copper-mediated trifluoromethylation with tri-*O*-acetyl-6-bromo-purine ribonucleoside (**1**) (Scheme 1).

Bromopurine derivative **1** is readily available from inosine via our recently reported procedure.[9] Two different trifluoromethylation reactions with **1** were investigated. Both FSO$_2$CF$_2$CO$_2$Me (MFSDA)/CuI/HMPA/DMF[10] (91%) and CF$_3$I/Zn/CuI/DMF[11] (96%) lead to a high yield of 6-trifluoromethylpurine ribonucleoside (**2**). However, the ease

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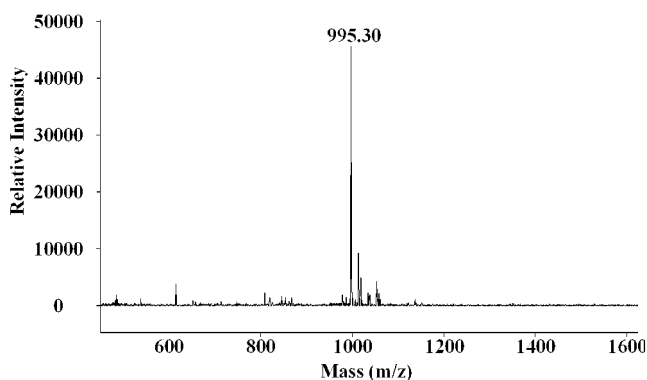
Scheme 1^a

^{a} (a) FSO$_2$CF$_2$CO$_2$Me, CuI, HMPA, DMF, 70 °C, 91% or CF$_3$I, Zn, CuI, DMF, 70 °C, 96%; (b) NH$_3$/MeOH, 97%; (c) (i) DMTCl, pyridine, AgNO$_3$, THF, 98%; (ii) TBDMSCl, TEA, AgNO$_3$, THF, 56%; (iii) β-cyanoethyldiisopropylphosphoramidochloridite, DIPEA, THF, 80%.

of manipulation of liquid MFSDA vs the gaseous CF$_3$I makes the former condition more desirable. To the best of our knowledge, this constitutes the first reported use of the MFSDA reagent in nucleoside synthesis. The acetyl protecting groups on ribose were removed in MeOH saturated with NH$_3$ to give ^{TFM}P (**3**) in greater than 80% yield in two steps from **1**. Recently, two alternative strategies for obtaining **3** were reported.[12,13] Although the trifluoromethylation step in each route is high yielding, the overall yield reported for each procedure is lower than that reported here.

Synthesis of a monomer useful for automated RNA synthesis proceeded initially by protection of the 5′-hydroxyl as the dimethoxytrityl (DMT) ether. Using the conditions of Ogilvie, the 2′-hydroxyl was silylated regioselectively with *tert*-butyldimethylsilyl chloride and AgNO$_3$.[14] Finally, the 3′-phosphoramidite (**4**) was generated via reaction of 2-cyanoethyl diisopropylphosphoramidochlorodite in the presence of Hünig's base. Using this phosphoramidite and standard automated RNA synthesis procedures, ^{TFM}P was incorporated into RNA. On-line trityl monitoring during automated RNA synthesis indicated quantitative coupling of the phosphoramidite (**4**).

The RNA was deprotected in a standard two-step sequence using NH$_4$OH/EtOH followed by TEA·3HF. After deprotection and purification of the oligonucleotides, ESI and MALDI mass spectrometric analysis confirmed that the ^{TFM}P nucleotide was incorporated into the RNA unaltered (Figure 2).

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Figure 2. The incorporation of [TFM]P into RNA in the sequence 5'-A[TFM]PG-3' was confirmed by ESI and MALDI MS (shown above).

The effect [TFM]P substitution has on duplex stability was investigated using thermal denaturation (T_m) studies of a 12 base pair RNA duplex (Figure 3). These experiments were

5'- C A U U A X G G U G G G -3'
3'- G U A G U Y C C A U C C -5'

X	Y	T_m, °C
A	U	52 ± 2
A	C	44 ± 2
A	G	42 ± 2
A	A	40 ± 1
[TFM]P	U	39 ± 1
[TFM]P	C	40 ± 2
[TFM]P	G	37 ± 1
[TFM]P	A	38 ± 2

Figure 3. The effect [TFM]P substitution has on duplex stability was tested in the context of the RNA shown above. [TFM]P was found to have a destabilizing effect on the same order as an A·A mismatch.

carried out to provide one measure of how effectively [TFM]P can mimic adenosine in the context of RNA. The nucleoside analogue was incorporated into the duplex opposite each of the four common bases (adenine (A), guanine (G), cytosine (C), and uracil (U)), and the T_m for each duplex was determined. The observed T_m values were compared to the T_m's of duplexes with A opposite each of the four common bases. Under the conditions of the denaturation experiment, the duplex containing an A·U base pair at the variable position had a $T_m = 52$ °C whereas an A·A mismatch caused the T_m to decrease to 40 °C. Each of the four duplexes containing [TFM]P had a T_m near 40 °C, with only 3 degrees separating the four T_m's measured. Therefore, the [TFM]P nucleotide destabilizes duplex RNA to a degree similar to that of a purine−purine mismatch with little pairing preference for any of the four common bases. A likely source to this destabilizing effect is the steric demand of the trifluoromethyl group, which has been suggested to be 2.5 times

greater than that of a methyl group.[15] The lack of any pairing preference for [TFM]P may indicate that this nucleotide does not base pair in duplex RNA and already exists in a flipped out conformation. If so, RNA containing [TFM]P may bind tightly to ADARs as it would not be necessary to overcome the energetic barrier to disrupt base-pairing interactions. However, this may limit the use of [TFM]P as a probe of the early steps in the flipping mechanism (see below).

^{19}F NMR analysis of fluorine-substituted nucleic acids has proven useful in the study of protein−nucleic acid interactions, including the binding of base-flipping enzymes.[7,16] To determine if ^{19}F NMR could be used to probe the electronic environment of the trifluoromethyl group of [TFM]P in oligoribonucleotides, we obtained ^{19}F NMR spectra for two different RNA trimers, 5'-A[TFM]PG-3' and 5'-C[TFM]PU-3'. Each trimer gave rise to a single peak near −67 ppm relative to CFCl₃ (Figures 4A and 4B). The spectrum for a 1:1 mixture
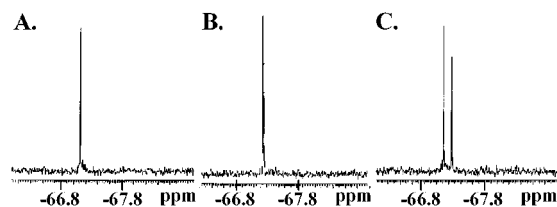

Figure 4. Analysis by ^{19}F NMR indicates that differences in the structural context of [TFM]P can be detected: (A) 400 μM 5'-C[TFM]PU-3' (−67.12 ppm); (B) 400 μM 5'-A[TFM]PG-3' (−67.25 ppm); (C) 1:1 mixture of 200 μM each 5'-C[TFM]PU-3' and 5'-A[TFM]PG-3' (−67.12 and −67.25 ppm).

of the two trimers clearly shows two well-resolved peaks corresponding to the two different [TFM]P nucleotides present (Figure 4C).

Thus, as has been observed with 5-fluorouridine-substituted RNA, the fluorine-substituted nucleotide gives rise to a unique ^{19}F NMR signal whose chemical shift is dependent on its structural context.[17] This result indicates that ^{19}F NMR could be used to monitor structural changes in [TFM]P-substituted RNA that significantly alter the electronic environment of the [TFM]P nucleotide, such as a base-flipping conformational change induced by an ADAR.

In summary, we have synthesized a phosphormadite of [TFM]P and incorporated this novel nucleoside into RNA. The [TFM]P modification is destabilizing to an RNA duplex equal to that of an A·A mismatch. The sensitivity of the ^{19}F chemical shift of [TFM]P to its structural context indicates it will be valuable in the study of RNA structure and the binding of RNA binding proteins, particularly the base-flipping RNA-editing adenosine deaminases. Furthermore, the electron-withdrawing properties of the trifluoromethyl group will increase the reactivity of the purine to covalent

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hydration. This latter property suggests that [TFM]P-containing RNA could inhibit RNA-editing adenosine deaminases by transition state mimicry. Experiments to test these hypotheses are currently underway in our laboratories.