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The Products of 5-Fluorouridine by the Action of the Pseudouridine Synthase TruB Disfavor One Mechanism and Suggest Another

Edward J. Miracco[†] and Eugene G. Mueller[*,‡]

[†]Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, United States

[‡]Department of Chemistry, University of Louisville, Louisville, Kentucky 40292, United States

Supporting Information

ABSTRACT: The pseudouridine synthase TruB handles 5-fluorouridine in RNA as a substrate, converting it into two isomeric hydrated products. Unexpectedly, the two products differ not in the hydrated pyrimidine ring but in the pentose ring, which is epimerized to arabinose in the minor product. This inversion of stereochemistry at C2′ suggests that pseudouridine generation may proceed by a mechanism involving a glycal intermediate or that the previously proposed mechanism involving an acylal intermediate operates but with an added reaction manifold for 5-fluorouridine versus uridine. The arabino product strongly disfavors a mechanism involving a Michael addition to the pyrimidine ring.

The pseudouridine synthases (Ψ synthases) isomerize uridine to pseudouridine (Ψ) in RNA and fall into six families. Despite their statistically insignificant global sequence similarity, the families share a common protein fold and constellation of active-site residues, so they also likely share a common mechanism.[1] The only absolutely conserved amino acid residue is an esssential Asp that has been proposed as a nucleophile that forms either a Michael adduct by attacking C6 of the uracil ring or an acylal intermediate by attacking C1′ of the ribose ring.[2,3] Pioneering mechanistic studies using RNA containing 5-fluorouridine, [F^5U]-RNA, and the Ψ synthase TruA demonstrated the formation of a protein−RNA adduct that was stable in denaturing gels unless the sample was heated, in which case a hydrated product of F^5U was isolated. These results were reasonably interpreted in favor of the mechanism involving a Michael adduct, with hydration resulting from the hydrolysis of the ester linkage between the active-site Asp and the pyrimidine ring.[2,3] Subsequent ^{18}O-labeling studies, however, showed that the direct hydration of F^5U rather than ester hydrolysis gives rise to the products.[4] Similar studies had already shown the same result for the Ψ synthases TruB[5] and RluA,[6] and the cocrystal structures[7,8] of both enzymes with [F^5U]-RNA showed that a covalent adduct was not present and that the F^5U was rearranged to a *C*-glycoside (like Ψ) as well as becoming hydrated. Furthermore, analysis of the products after digestion of the RNA with S1 nuclease and alkaline phosphatase revealed that both TruB and RluA generate *two* hydrated products of F^5U (in a ratio of ∼3:1), both of which are *C*-glycosides and recovered as dinucleotides with the residue following F^5U in the RNA.[4] To seek any mechanistic insight that the products might offer, their full structural elucidation by NMR methods was undertaken. TruB was examined first, for it does not make a tight adduct with [F^5U]RNA (as TruA and RluA do) but instead handles it as a simple substrate,[5] which eases the technical demands of generating appropriately large quantities of the products, the characterization of which is reported here.

The isolation of a mixture of the major and minor dinucleotide products after incubation of [F^5U]RNA with TruB was reported previously.[4] Overlap of the ribose protons in the one-dimensional ^1H NMR spectrum necessitated their assignment by a suite of two-dimensional methods. For both dinucleotide products, all of the protons and carbon atoms in the product of F^5U (for convenience denoted as F^5U^*) and the cytidine (Cyd) that follows it in the RNA sequence were unequivocally assigned [see the Supporting Information (SI)]. The major product is, unsurprisingly, the one observed in the cocrystal of TruB and [F^5U^*]-RNA, but what is the minor product?

The two products share the same skeletal structure (see the SI), leaving conformational or stereochemical isomerism as the difference between them. The dogged maintenance of the ratio of the two products through digestion, isolation, and storage argued against conformational isomerism unless the conformers were remarkably strongly locked. Degradation of the 3′-Cyd by treatment with periodate and base yielded a mononucleotide F^5U^*, the spectrum of which matched that of the major product. The yield, however, was less than that of the major product originally present, and a commensurate loss of the minor product would take it below the threshold of NMR detection, so no definitive conclusion could be drawn concerning the conversion of the minor product to the major product by this treatment.

The evidence for stereochemical isomerism, on the other hand, is overwhelming. Surprisingly, the difference between the major and minor products is not at C6 of F^5U^* (the site of hydration in the pyrimidine ring) as previously proposed[5,9] but instead at C2′ of its pentose ring, which means that the minor product is the *arabino* isomer of the product seen in the cocrystal (Figure 1). This outcome was first indicated by a comparison of the chemical shifts of the pentose protons in F^5U and F^5U^*, which match the differences between (ribo)uridine and arabinouridine with a diagnostic deshielding of $H^{2'}$ and C2′ (Figure 2; also see Figure S25 in the SI). Confirming a difference in the configurations at C2′ in the major and minor products, both $^1H-^1H$ and $^1H-^{19}F$ nuclear Overhauser effect (NOE) experiments (Figure 2 and Figures S19−S21) showed the expected differences for the approach of $H^{2'}$ to other pentose protons and F^5 in a ribose ring for the major

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product and an arabinose ring for the minor product. Saliently, the major product evinces strong NOEs between $H^{2'}$ and $H^{5'}/H^{5''}$, but these interactions are absent in the minor product, in which $H^{2'}$ lies on the opposite side of the pentose ring as $H^{5'}/H^{5''}$ in arabinose. Even though strong NOEs between F^5 and $H^{3'}$ in both the major and minor products indicate that the fluoro group lies over the pentose ring, only the major product shows a strong interaction between F^5 and $H^{2'}$. Similarly, the minor product lacks a detectable NOE between $H^{2'}$ and H^6 (an interaction evident in the major product) but does show an NOE between $H^{2'}$ and $H^{4'}$ (Figure 3), although it is somewhat weak, as expected since the energy-minimized structures reveal that these two protons are splayed out away from each other (Figure 1B) and lie 3.99 Å apart; the intensity is also approximately equal to that of the NOE between $H^{1'}$ and $H^{4'}$, which

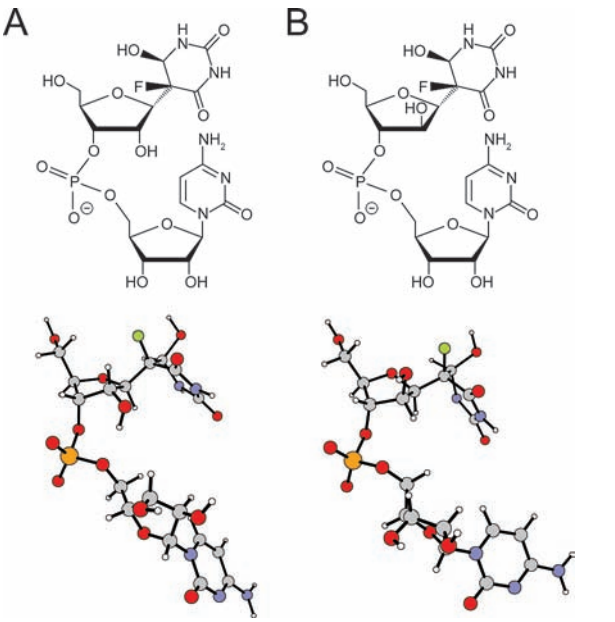

Figure 1. Structures of the (A) major and (B) minor products, which were isolated as dinucleotides. The models were energy-minimized using DFT (see the SI).

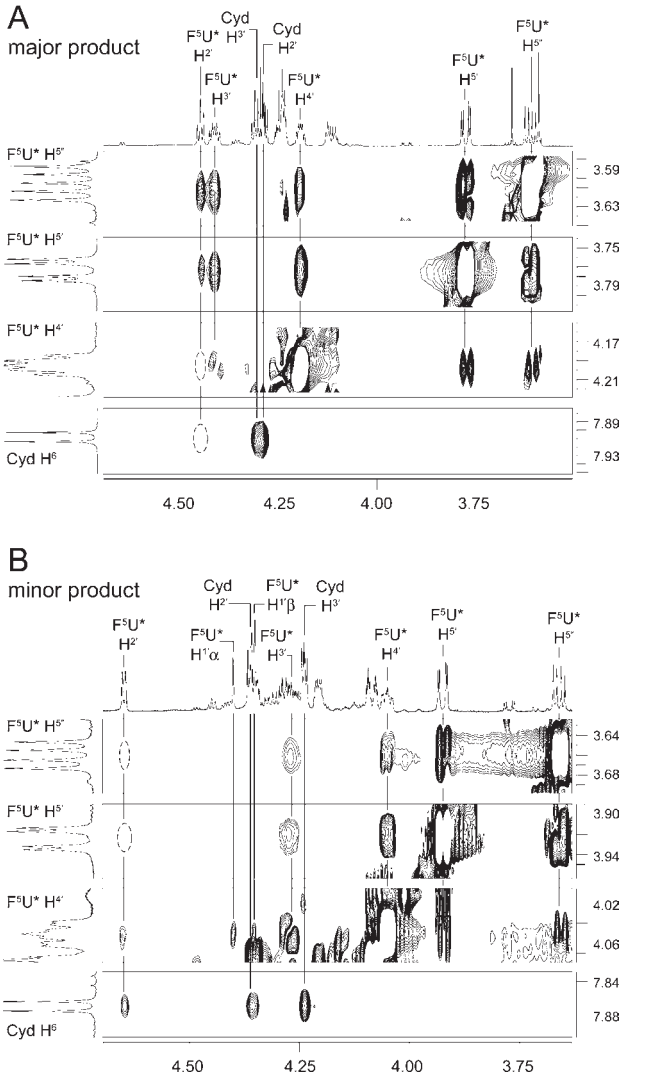

Figure 3. $^1H-^1H$ NOE spectra showing the correlations involving $H^{5'}$, $H^{5''}$, and $H^{4'}$ of F^5U^* and H^6 of Cyd in the major (A) and minor (B) products. Dotted ovals indicate NOEs whose absence is stereochemically informative.

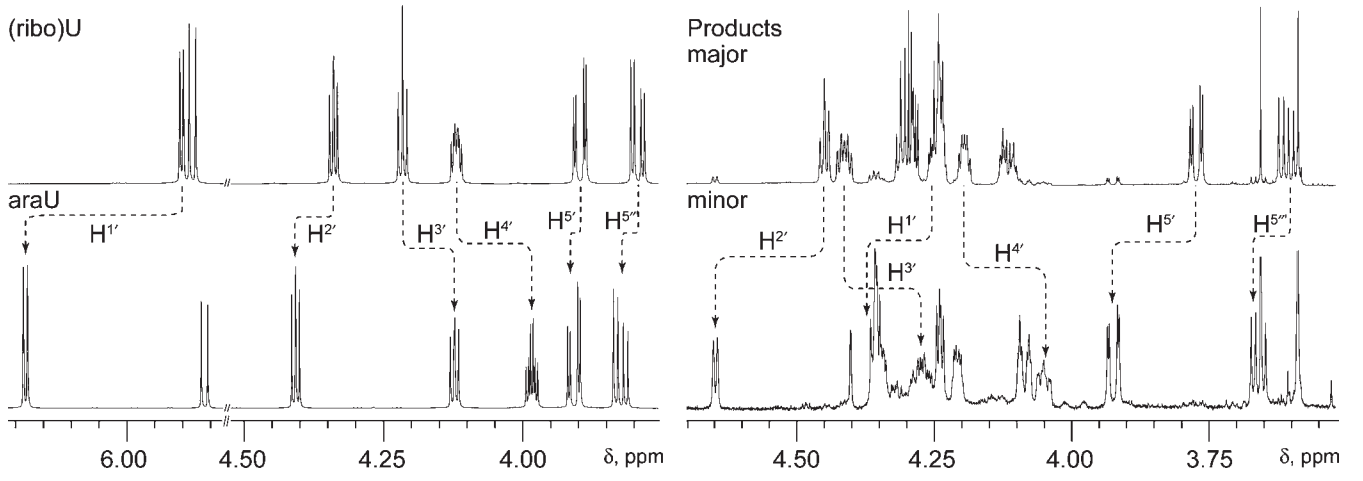

Figure 2. Differences in the chemical shifts of the F^5U^* pentose protons in the major and minor products, which qualitatively match those between uridine (riboU) and arabinouridine (araU).

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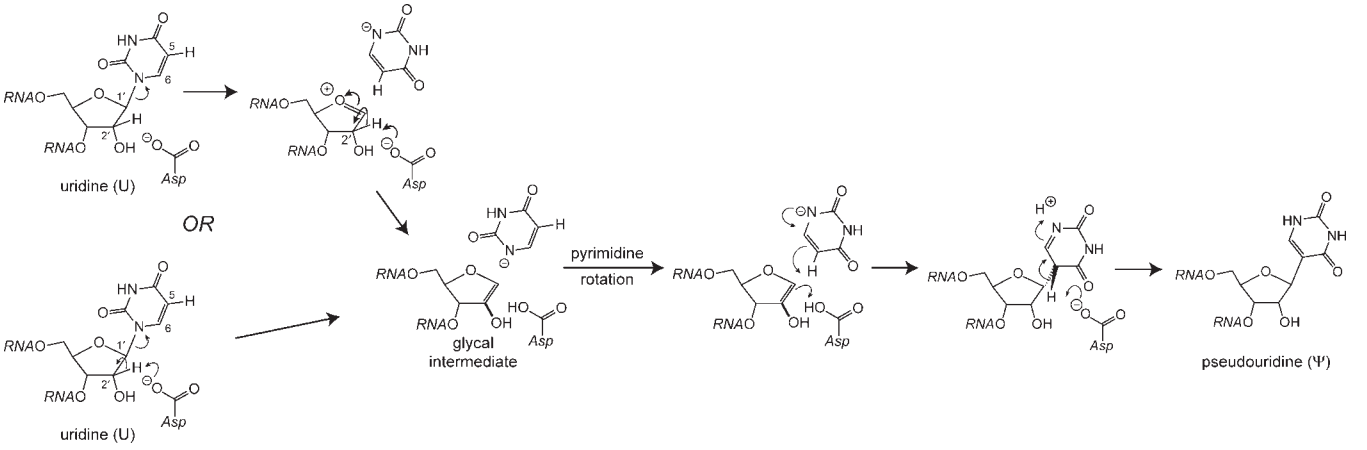
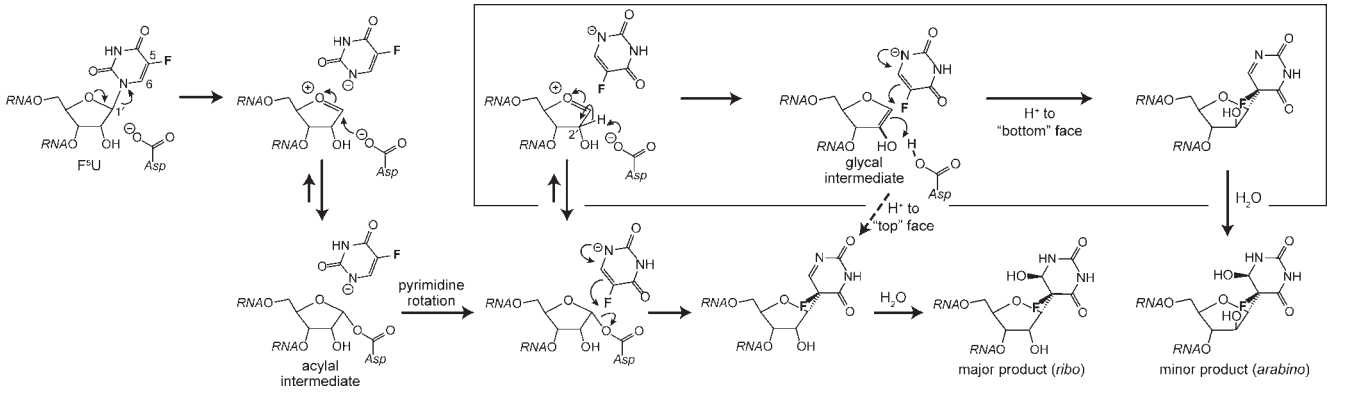
Scheme 1. "Glycal Mechanism" for Ψ Generation, in Which the Elimination of Uracil May Be Stepwise (Upper Path) or Concerted (Lower Path)



Scheme 2. "Acylal Mechanism" for Ψ Generation Modified To Account for Epimerization at C2′ via the Boxed Reaction Manifold[a]



[a] The stereospecific hydration across C=N could occur at the active site or in solution with a directing hydrogen bond between the fluoro group and the incoming water molecule. The reaction manifold made accessible by the longer lifetime of the acylal/oxocarbenium intermediates is boxed.

clearly lie on the same side of the pentose ring. Together, these data show the opposite disposition of $H^{2'}$ on the pentose ring in the two products. That conclusion is bolstered (Figure 3) by the NOE between $H^{2'}$ of F^5U^* and H^6 of Cyd that is detectable in the minor product (with reference to the standard Haworth projection, $H^{2'}$ points "down" toward Cyd and H^6 points "up" toward F^5U^*) but not in the major product (in which $H^{2'}$ points "up" and away from Cyd).

Residual dipolar coupling methods can determine the relative orientation of bond vectors within a molecule,[10] and the data for the F^5U^* moieties fit an arabino minor product (Q = 0.251) better than a ribo one (Q = 0.571) and also indicate a ribo (Q = 0.290) rather than an arabino (Q = 0.442) major product (Q values represent the extent of deviation of the data from that calculated for a candidate structure, with a lower value indicating a better fit). The interatomic distances from the energy-minimized proposed structures and the density functional theory (DFT) chemical shift predictions for F^5 match the NMR data very well (see the SI). For the major product, the conformations seen in molecular dynamics runs and the predicted ring puckering from the coupling constants of the ribose protons match well, but they diverge notably for the minor product. However, the correlation between the puckering and the coupling constants was derived for ribo- and deoxyribonucleotides, and the inadequacy of the correlation for α-arabinofuranosides has been reported,[11] with a β-arabinofuranoside posing even greater difficulties for accurate theoretical treatment.[12] Although the matter is under further examination, the unusual nature of this particular β-arabinonucleoside (a *C*-glycoside with both carbons fully saturated) makes it a test case for method development rather than a candidate for description by "canned" protocols based on ribonucleoside parameters.

The arabino minor product indicated by the preponderance of the data offers mechanistic insight. Epimerization at C2′ requires deprotonation to form a glycal intermediate followed by reprotonation on the opposite face. Uridine phosphorylase offers a precedent for such an intermediate in an enzyme-catalyzed reaction,[13] and the Ψ synthase reaction with the natural substrate (U) may proceed by a similar mechanism (Scheme 1), with deprotonation effected either by the active-site Asp (as shown) or O^2 of uridine. The process can be either a stepwise elimination with deprotonation of an oxocarbenium intermediate or a concerted

syn elimination, a process that is generally disfavored but is catalyzed by α-1,4-glucan lyase.[14] No evidence for arabino-Ψ has ever appeared in our numerous experiments with TruB, RluA, and TruA, so they must be rigidly stereospecific with their natural substrates, which is not surprising.

A variation of the mechanism involving attack by the active-site Asp at C1′ can also accommodate the arabino minor product of F^5U (Scheme 2). The decreased nucleophilicity of the anion of 5-fluorouracil versus uracil increases the lifetime of the acylal intermediate, which is in equilibrium with an oxocarbenium species and free Asp, effectively giving both of these intermediates more time to access an alternate reaction pathway. The acidity of C2′ is accentuated in the oxocarbenium intermediate, which facilitates deprotonation to generate the glycal intermediate. The glycal intermediate can be reprotonated on either face during pyrimidine reattachment (as shown in Scheme 2) or beforehand to regenerate oxocarbenium intermediates of each configuration at C2′ that subsequently undergo pyrimidine reattachment.

Whether O^2 or O^4 of the 5-fluorouracilate anion or the Asp effects the deprotonation (from the "top"), the proton must be transferred to Asp to afford the arabino product, as no other active-site group in the cocrystal[7] is positioned to protonate the glycal from the "bottom", at least not without major structural reorganization. The alternative protonation of the glycal directly from solution to generate the arabino product is ruled out by the lack of deuterium incorporation when the reaction (with either U or F^5U in RNA) was run in buffered D_2O with protein and RNA pre-equilibrated in the same buffer; this result also indicates that the protonated Asp is sequestered from exchange with solvent. The need for participation by the active-site Asp as an acid precludes its involvement in a Michael addition to the pyrimidine ring as a part of the mechanism, for the Asp would be tied up in the resulting ester linkage throughout the subsequent course of events and therefore unable to protonate C2′ of the glycal intermediate.

A major caveat of experiments with substrate analogues is the chance that the alteration of the substrate significantly perturbs its chemistry. In this instance, however, the fluoro group does not appear to alter the fundamental chemistry but merely enhances the lifetime of an intermediate (glycal or acylal/oxocarbenium), thereby allowing it to access a secondary and mechanistically informative reaction manifold. Hydrogen bonding to the fluoro group may also help depress the pK_a of C2′ in the intermediates, but this effect seems likely to be minor in comparison with the increased lifetime of the intermediate with a detached pyrimidine.

The arabino minor product of F^5U from the action of TruB thus suggests another plausible mechanism for the Ψ synthases and strongly disfavors one of the two mechanisms that have dominated discussion. In view of the similarity of the NMR spectra of the products of F^5U from the action of RluA to those reported here, the conclusions will likely prove general for all Ψ synthases, but the full characterization of the "RluA products" is underway to confirm that supposition, as are experiments to test for the formation of a glycal intermediate during the conversion of U to Ψ.

■ ASSOCIATED CONTENT

Supporting Information. NMR spectra and assignments, detailed structural analysis, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
eugene.mueller@louisville.edu

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