

Investigation of the Binding of Epimer A of the Covalent Hydrate of 6,7-Bis(trifluoromethyl)-8-D-ribityllumazine to a Recombinant F22W *Bacillus subtilis* Lumazine Synthase Mutant by $^{15}\text{N}\{^{19}\text{F}\}$ REDOR NMR

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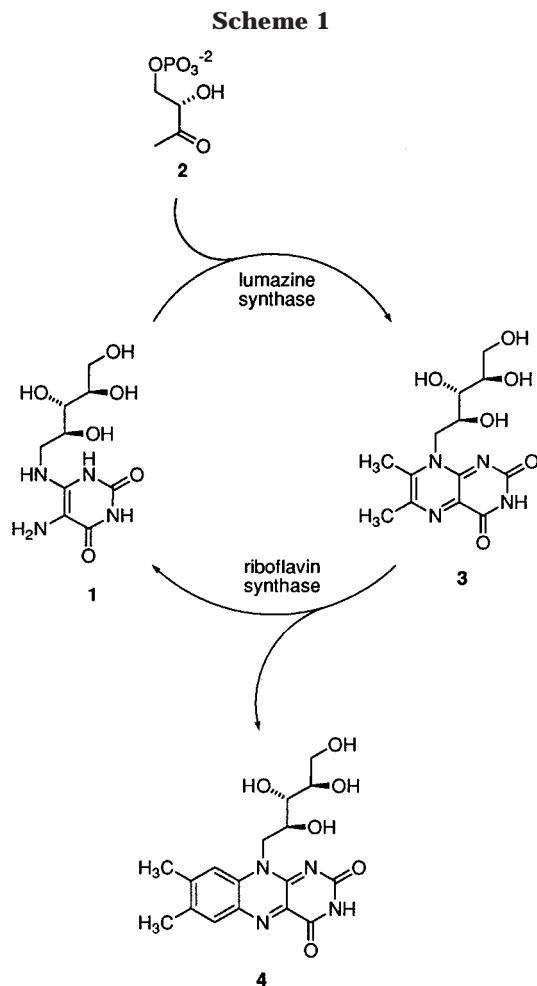
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The two epimeric covalent hydrates A and B of 6,7-bis(trifluoromethyl)-8-D-ribityllumazine are metabolically stable analogues of hypothetical intermediates proposed in the reactions catalyzed by riboflavin synthase and lumazine synthase. To confirm the stereochemical assignments previously based solely on results for epimer B, a $^{15}\text{N}\{^{19}\text{F}\}$ REDOR NMR study was performed on the complex formed from epimer A and a recombinant, uniformly ^{15}N -labeled F22W mutant of *Bacillus subtilis* lumazine synthase. The results indicate that the fluorines of the ligands are closer to the side chain nitrogens of Arg127 and farther away from the side chain nitrogens of Lys135 in epimer B than in epimer A. These results are consistent with the assignment of the earlier 7R configuration of epimer A and the 7S configuration of epimer B.

Riboflavin synthase catalyzes a mechanistically complex and incompletely understood dismutation reaction involving the transfer of a four-carbon unit from one molecule of 6,7-dimethyl-8-D-ribityllumazine (**3**) to another molecule of **3**, resulting in the formation of one molecule of riboflavin (**4**) and one molecule of the ribitylamino pyrimidine **1** (Scheme 1). Lumazine synthase catalyzes the formation of **3** from **1** and 3,4-dihydroxy-2-butanone 4-phosphate (**2**).^{1–3}

The lumazine synthase-catalyzed reaction is thought to proceed as outlined in Scheme 2. Schiff base formation between the ribitylamino pyrimidine **1** and the four-carbon unit **2** results in the formation of the imine **5**, which eliminates phosphate to form the enol **6**. Tautomerization of the enol **6** affords the ketone **7**, which reacts with the ribitylamino group to form the carbino-lumazine **8**. The final product **3** is formed by elimination of water from **8**.^{4,5}

A hypothetical mechanism for the formation of riboflavin is outlined in Scheme 3. The early steps in this proposal involve the addition of a nucleophile to the lumazine **3** that will function as the donor of the four-carbon unit to form **9** and the deprotonation of the C-7 methyl group of the lumazine **3** that will function as the acceptor of the four-carbon unit to form the anion **10**.^{6–9}



The nucleophile "Nu" in structure **9** could be a hydroxyl group derived from water,⁷ the primary hydroxyl group

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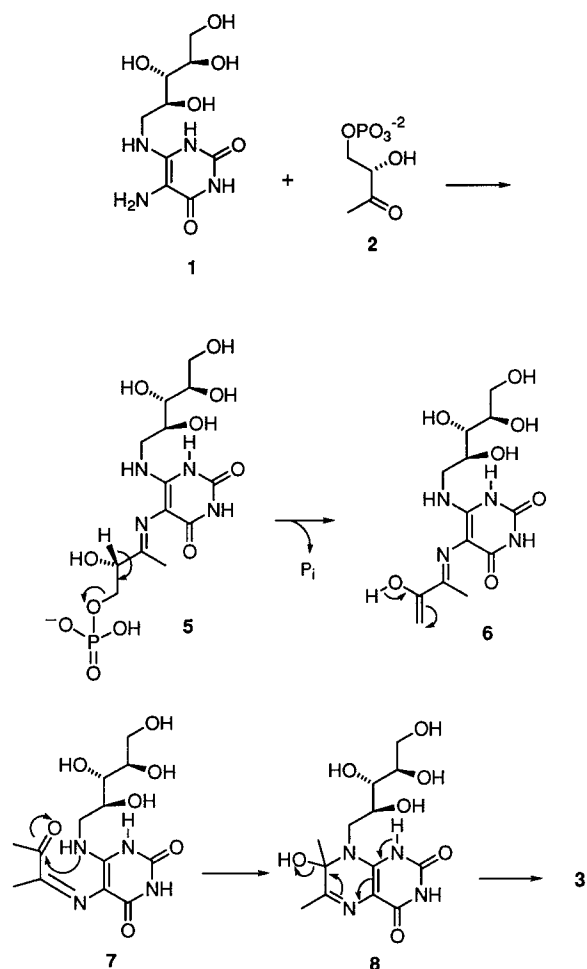
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Scheme 2



of Ser41 provided by the enzyme,^{6,10} or the 2'- or 3'-hydroxyl group of the ribityl side chain.^{11,12} Nucleophilic attack of the anion **10** on C-6 of **9** affords intermediate **11**, which could tautomerize to form **12**. Tautomerization of the imine moiety of **12** to the enamine **13**, followed by nucleophilic displacement of "Nu", could afford the pentacyclic intermediate **14**. Two sequential amine elimination reactions involving two C–N bond cleavages, which could proceed, for example, through intermediate **15**, would result in the two products **2** and **3**. This pathway is supported by the recent isolation and structure elucidation of the proposed pentacyclic intermediate **14** from reaction mixtures containing the S41A mutant of *Escherichia coli* riboflavin synthase, as well as by its conversion to **2** and **3** in the presence of wild-type riboflavin synthase.¹³ However, other reasonable mechanistic pathways have been proposed that are not inconsistent with the isolation of the pentacyclic compound **14**.⁶

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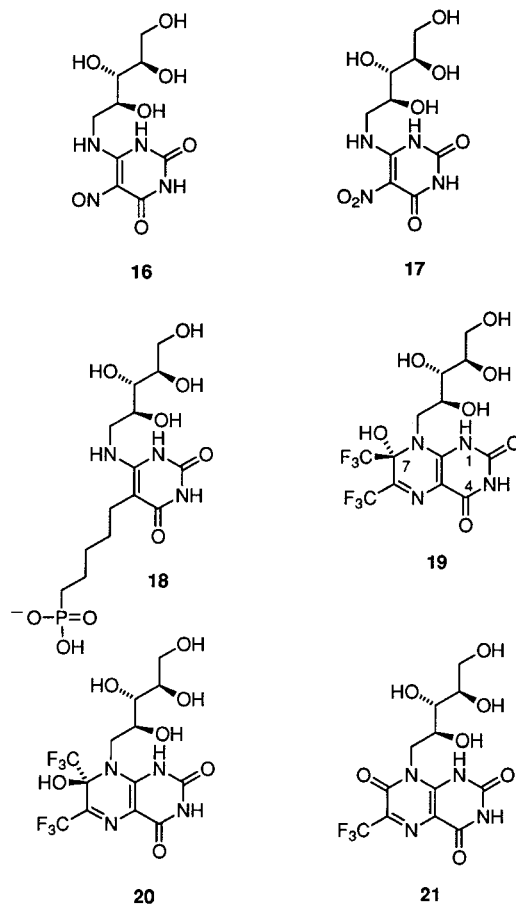
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The pathways proposed in Schemes 2 and 3 must be regarded as hypothetical and speculative given the present state of experimental evidence. In addition, the catalytic roles of the active site residues are unclear. On the other hand, substantial progress has been made on the structures of both lumazine synthase and riboflavin synthase. The X-ray structures of lumazine synthase complexed with the substrate analogues **16** and **17** provide information concerning the locations of active site residues with respect to the substrate **1**,^{14,15} and the X-ray structure of **18**¹⁶ in complex with *Saccharomyces cerevisiae* lumazine synthase indicates the orientation of the hypothetical intermediate **5** in the active site.¹⁷ In addition, the X-ray structure of riboflavin synthase has been determined and should yield mechanistically useful information when the coordinates become available and the enzyme is crystallized with bound ligands.¹⁸



Our approach to the study of the mechanisms of the reactions catalyzed by riboflavin synthase and lumazine synthase has been to design and synthesize metabolically stable analogues of the hypothetical reaction intermediates and to investigate the structures of their complexes with lumazine synthase and riboflavin synthase by X-ray crystallography,¹⁹ ^{19}F NMR, and

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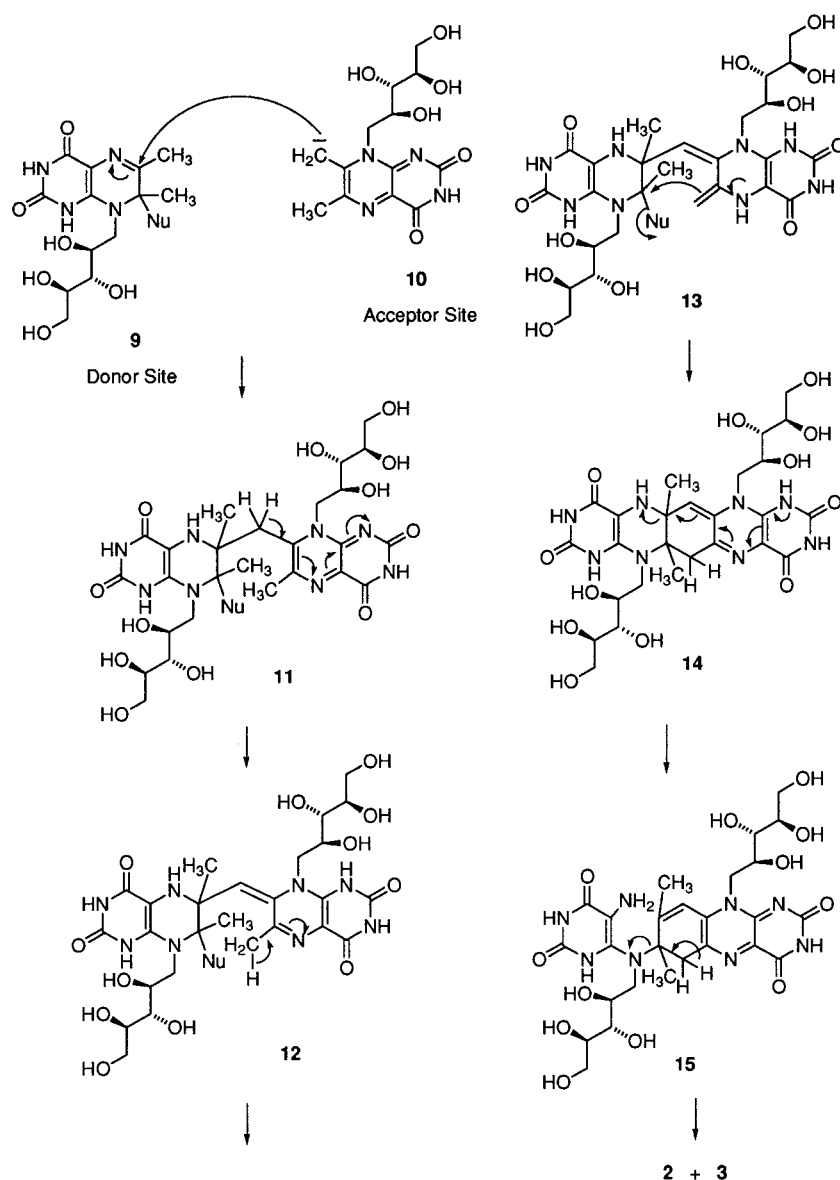
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Scheme 3



$^{15}\text{N}\{^{19}\text{F}\}$ REDOR NMR.^{10,16,17,19–37} The covalent hydrate **8** in Scheme 2 is a late intermediate in the lumazine synthase-catalyzed reaction and may also be the early intermediate **9** in the riboflavin synthase-catalyzed reaction, assuming that Nu = OH.³⁶ The two epimeric covalent hydrates **19** and **20** of 6,7-bis(trifluoromethyl)-8-D-ribityllumazine were synthesized as metabolically

stable analogues of the proposed intermediates **8** and **9**.²¹ The electronegativity of the trifluoromethyl groups at C-7

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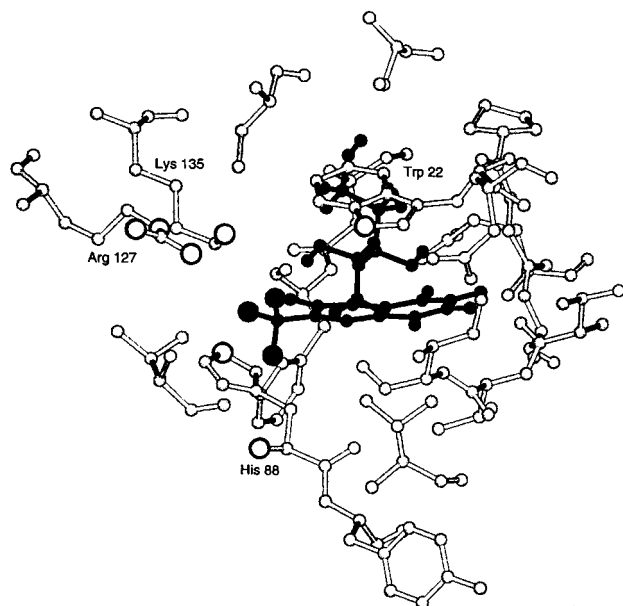


Figure 1. Arrangement of the ligand **21** and residues observed in the active site of lumazine synthase observed after a REDOR distance-restrained molecular dynamics simulation. Nitrogens whose distances to the CF_3 group were used as restraints in the simulation are highlighted by size and shading.

of **19** and **20** stabilize the covalent hydrates to the extent that these two epimers can be separated and do not interconvert. Only one epimer (either **19** or **20**), designated as “epimer A”, binds to the light riboflavin synthase of *Bacillus subtilis*,²¹ the N- and C-terminal domains of *E. coli* riboflavin synthase,¹⁰ and the bioluminescence transducer, lumazine protein, of *Photobacterium phosphoreum*.³² On the other hand, both epimers bind to lumazine synthase of *B. subtilis*, but the binding of epimer A results in the stereoselective catalysis of its conversion to the 7-oxo compound **21** via the haloform reaction.³³ These results emphasize the importance of determining the stereochemistry of epimer A, since it is likely to be relevant to the stereochemistry of the hypothetical intermediates **8** and **9** in Schemes 2 and 3, respectively.

Several lines of evidence indicate that epimer A is **19** and epimer B is **20**. An overlap of the structure of **21** with that of **17** in the X-ray structure of the complex of **17** and lumazine synthase, followed by removal of the structure of **17** and a series of distance-restrained molecular dynamics simulations, led to the $^{15}\text{N}\{^{19}\text{F}\}$ REDOR NMR structure of **21** in the active site (Figure 1).³⁶ The $^{15}\text{N}\{^{19}\text{F}\}$ REDOR spectrum of epimer B in complex with lumazine synthase indicated that the fluorine was relatively closer to the side chain nitrogens of Arg127 and farther away from the side chain nitrogen of Lys135 than in the 7-oxo compound **21**. An overlap of the structure of **20** with that of **21**, followed by a similar distance-restrained molecular dynamics simulation, led to the prediction that the trifluoromethyl groups of structure **20** would in fact be closer to the nitrogens of Arg127 than they would be in **19**, and therefore epimer B was assigned structure **20**.³⁶ It was also calculated that the 7-hydroxyl group of **19**, as opposed to **20**, would be closer to the basic residues Lys135 and His88 that would be available to catalyze the haloform reaction, which would be consistent with epimer A being assigned structure **19**.³⁶ Finally, ^{19}F

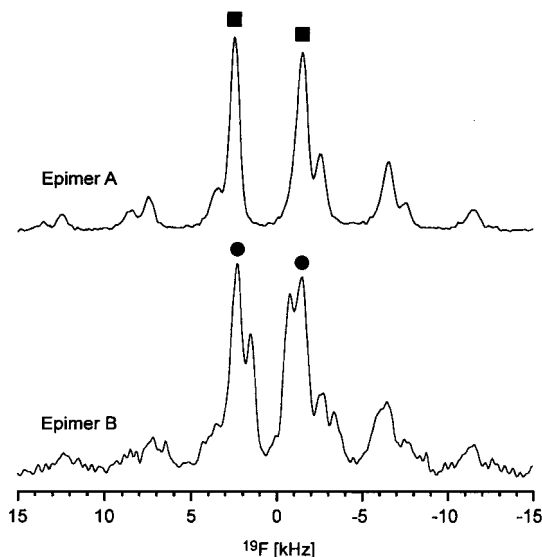


Figure 2. ^{19}F cross-polarization magic-angle spinning NMR spectra (188 MHz) of epimer A complexed to [uniform- ^{15}N]-F22W–lumazine synthase at -30°C and epimer B complexed at -2°C . Only two major isotopic shifts are observed in the top spectrum of epimer A (solid squares), indicating that complex formation by low-temperature trapping, followed by low-temperature lyophilization, avoided degradation; the minor lines arise from the 5 kHz magic-angle spinning. Additional lines in the bottom spectrum of epimer B (solid circles) indicate chemical modification of the epimer.

NMR measurements of complexes of both epimers A and B with *B. subtilis* mutants led to the discovery of a remarkable downfield shift of 7.8 ppm of the signal assigned to the C-7 trifluoromethyl group in epimer B, relative to that of the free ligand, caused by deshielding by the imidazole ring of His88.³⁷ This would also be consistent with the assignment of structure **20** to epimer B.

Because of the importance of the assignment of the absolute configurations at C-7 in epimers A and B, a further confirmation of the validity of the $^{15}\text{N}\{^{19}\text{F}\}$ REDOR arguments has now been completed using epimer A as the ligand. The reason epimer B had originally been investigated instead of epimer A was that epimer A undergoes an enzyme-catalyzed haloform reaction and is therefore less stable than epimer B in the presence of the enzyme. More specifically, the haloform elimination rate constant for epimer A in the presence of the enzyme is $15.9\text{ nmol}^{-1}\text{ mg}^{-1}\text{ h}^{-1}$ at 37°C and pH 7, whereas that of epimer B is $0.4\text{ nmol}^{-1}\text{ mg}^{-1}\text{ h}^{-1}$.³⁷ However, despite this known instability issue, according to the arguments presented above, it seemed logical to conclude that if a sufficiently stable complex of epimer A could in fact be obtained in the solid state, the REDOR difference signals corresponding to the Arg127 residues should be smaller in the $^{15}\text{N}\{^{19}\text{F}\}$ REDOR difference spectrum of epimer A than those of epimer B and the Lys135 difference peak should be larger.

It had been noted earlier that significant decomposition of even the more stable epimer B occurred during complex formation at -2°C , as evidenced by two “extra” peaks in the ^{19}F NMR spectrum (see Figure 2, bottom).³⁶ To avoid decomposition of the less stable epimer A during formation of the solid complex with a uniformly ^{15}N -labeled F22W mutant of *B. subtilis* lumazine synthase, the ligand was complexed and trapped in a lyophilized matrix.³⁸ As evidenced by the ^{19}F NMR spectrum dis-

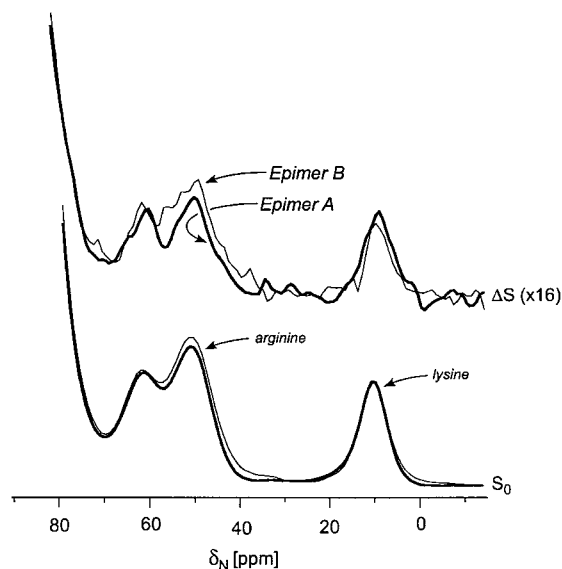


Figure 3. Top: 20.3 MHz $^{15}\text{N}\{^{19}\text{F}\}$ REDOR difference spectra of epimers A and B complexed to [uniform- ^{15}N]-F22W-lumazine synthase after 96 rotor cycles of dipolar evolution with magic-angle spinning at 5.000 kHz. Bottom: the corresponding full-echo REDOR spectra of epimers A and B.

played in Figure 2 (top), no decomposition was detected provided all of the operations performed during complex formation were carried out at $-30\text{ }^{\circ}\text{C}$.

The $^{15}\text{N}\{^{19}\text{F}\}$ REDOR difference spectrum of the complex of epimer A with a ^{15}N -labeled F22W recombinant *B. subtilis* lumazine synthase mutant is shown in Figure 3 (top), along with that previously determined for epimer B. Comparisons of absolute peak intensities for different samples are generally difficult, so these spectra have been normalized for equal integrated intensities for the lysine full-echo peaks at 10 ppm (Figure 3, bottom). The REDOR difference spectra (Figure 3, top) have arginine signal amplitudes significantly greater for the complex of epimer B (light line) than for the complex of epimer A (bold line) for each value of the frequency between about 40 and 60 ppm. Thus, the total integrated intensity of the REDOR difference spectrum corresponding to the complexed side chain nitrogens of Arg127 is greater for epimer B than for epimer A, indicating that these nitrogens are closer to the fluorines of epimer B than they are to the fluorines of epimer A. On the other hand, the integrated intensity of the difference peak corresponding to the Lys135 side chain nitrogen of the complex of epimer B is comparable to, or perhaps slightly smaller than, the difference peak for the complex formed from epimer A, indicating that this nitrogen is a little farther away from the fluorines of epimer B than the fluorines of epimer A. The results presented in Figure 3 are therefore in complete agreement with the predictions discussed above from the previous $^{15}\text{N}\{^{19}\text{F}\}$ REDOR experiments.³⁶ In that study, the differences expected between complexes of epimers A and B were clear for Arg127 (the fluorines of epimer B were significantly closer) but less clear for Lys135 (the fluorines of epimer B were only slightly farther away). The results of Figure 3 are also in harmony with the prior solution-state ^{19}F NMR investigation.³⁷ Taken as a whole,

the NMR results provide strong evidence that structure **19** is epimer A and structure **20** is epimer B.

Experimental Section

(7*R*)-6,7-Bis(trifluoromethyl)-7-hydroxy-8-D-ribityllumazine (19, Epimer A). This compound was prepared as described previously.²¹

Uniformly ^{15}N -Labeled F22W Mutant of *B. subtilis* Lumazine Synthase. The construction of the plasmid p602-ribH-F22W directing the hyperexpression of the gene specifying the F22W mutant of *B. subtilis* will be described elsewhere.³⁹ The plasmid was transduced into the *E. coli* strain M15-[pRep4], yielding strain M15-[pRep4]-p602-ribH-F22W. The recombinant strain was grown in M9 medium⁴⁰ containing 0.7 g of $^{15}\text{NH}_4\text{Cl}$ per liter as the only source of nitrogen. IPTG (final concentration, 2 mM) was added to shaking cultures when they had reached an optical density of 0.7 (600 nm). Incubation was continued for 5 h. The cells were harvested by centrifugation. Lumazine synthase was purified as described earlier.⁴¹

Preparation of the Complex of ^{15}N -Labeled Lumazine Synthase-F22W Mutant with (7*R*)-6,7-Bis(trifluoromethyl)-7-hydroxy-8-ribityllumazine (19, Epimer A). Use of a method developed in our laboratory for trapping and stabilizing enzyme substrates in a lyophilized matrix³⁸ allowed the unstable epimer A to be successfully complexed to lumazine synthase without evidence of significant degradation. Uniformly ^{15}N -labeled lumazine synthase-F22W (100 mg) was exchanged into 50 mM triethanolamine-formate, pH 6.0, containing 30% methanol as an antifreeze reagent, 30 mM trehalose as a lyoprotectant, and 0.4% PEG 8000 (w/v) as a cryoprotectant. The solution, in a lyophilization flask, was then placed in a $-25\text{ }^{\circ}\text{C}$ bath until equilibrated. An equimolar amount of epimer A, dissolved in methanol, was added to this fluid $-25\text{ }^{\circ}\text{C}$ mixture. After mixing by swirling, the solution was incubated for 10 min at $-25\text{ }^{\circ}\text{C}$ and then rapidly frozen in liquid nitrogen. The flask was attached to a 5 mTorr vacuum and placed in a $-80\text{ }^{\circ}\text{C}$ bath. Lyophilization was accomplished by keeping the external flask temperature at subzero temperatures throughout the primary drying phase, thereby stabilizing the unstable substrate in a solid matrix. When the rate of sublimation decreased, as measured by a vacuum sensor mounted near the flask, the bath temperature was gradually raised to remove any residual solvent.

$^{15}\text{N}\{^{19}\text{F}\}$ REDOR NMR. $^{15}\text{N}\{^{19}\text{F}\}$ REDOR was performed using a four-frequency transmission-line probe having an analytical coil with a length of 14 mm and an inside diameter of 9 mm and a Chemagnetics/Varian Kel-F stator. Powdered samples were contained in thin-wall Chemagnetics/Varian zirconia rotors with outside diameters of 7.5 mm. The rotors were spun at 5000 Hz with the speed under active control to within ± 2 Hz. The spectrometer was controlled by a Tecmag pulse programmer. ^{15}N radio frequency pulses (20.3 MHz) were produced by a 1 kW Electron Navigation Industries LPI-10 power amplifier. ^1H (200 MHz) and ^{19}F (188 MHz) radio frequency pulses were generated by 1 kW Kalmus 166 HP power amplifiers. The π pulse lengths were 10 μs for both ^{15}N and ^{19}F . Distance measurements using ^{19}F dephasing were calibrated using the two-bond coupling of [^{19}F]polycarbonate. Standard XY-8 phase cycling was used for $^{15}\text{N}\{^{19}\text{F}\}$ REDOR. A 4.7 T static magnetic field was provided by an 89 mm bore Magnex superconducting solenoid. Proton-nitrogen cross-polarization transfers were made in 2 ms at 50 kHz. Proton dipolar decoupling was 100 kHz during REDOR evolution and data acquisition.

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