Elimination of the 7-Trifluoromethyl Group from 6,7-Bis(trifluoromethyl)-8-ribityllumazines. Stereoselective Catalysis by the Lumazine Synthase of *Bacillus subtilis*

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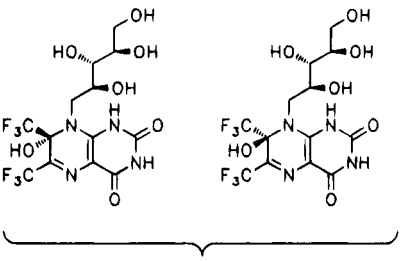
Both diastereomers of 6,7-bis(trifluoromethyl)-7-hydroxy-8-ribityllumazine decompose in neutral or mild basic aqueous solution by elimination of the 7-CF_3 group yielding 6-(trifluoromethyl)-7-oxo-8-ribityllumazine. It is suggested that the reaction starts with deprotonation of the 7-OH group, followed by release of the CF_3 carbanion and formation of a carbonyl oxygen. The reaction was catalyzed diastereoselectively by the lumazine synthase/riboflavin synthase complex from *Bacillus subtilis*, which resulted from a decrease in the pK_a of the 7-OH group by the enzyme.

Bis(trifluoromethyl)-7-hydroxy-8-ribityllumazine (**1**) has been synthesized as a potential inhibitor of the enzyme riboflavin synthase.[1] This lumazine exists as two stable C-7 epimers. The absolute configurations of the diastereomers are unknown and were designated "epimer A" and "epimer B" according to their elution times on HPLC-C18 columns. As shown by ^{19}F NMR spectroscopy, the epimers interact highly stereoselectively with the enzyme riboflavin synthase from *Bacillus subtilis*[1] and the phototransducer protein, designated lumazine protein, from *Photobacterium phosphoreum*.[2] Whereas epimer A was bound to both proteins, no interaction was observed in the case of epimer B. In contrast to NMR results, other experimental techniques such as enzyme inhibition kinetics[1] and fluorescence anisotropy decay measurements[3] seemed to indicate an interaction between epimer B and these proteins. While looking for a reason for this apparent paradox, we found an interesting decomposition reaction of **1** which is described in the present communication.

Using ^{19}F NMR spectroscopy and analytical reverse phase HPLC, we found that samples of both nonfluorescent epimers contained an additional, fluorescent compound. This substance was identified as 6-(trifluoromethyl)-7-oxo-8-ribityllumazine (**2**), which had been synthesized earlier by condensation of (trifluoromethyl)-pyruvaldehyde and 5-amino-6-(ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione.[4]

Samples of both epimers of **1** in aqueous solution, pH 7.2, were incubated for several hours at different temperatures. Samples were retrieved and analyzed by HPLC. An increasing amount of **2** and decreasing amounts of the diastereomers of **1** were observed at temperatures above 37 °C. The half-lives of **1** under these conditions were determined as about 90 min at 50 °C and 15 min at 60 °C. At 24 °C and 4 °C, no formation of **2** was detectable. Monitoring the reaction by ^{19}F NMR spectroscopy showed that the signals of the trifluoromethyl groups of **1** disappeared and the trifluoromethyl group signal of **2** increased (Figure 1). These observations indicate an unusual elimination reaction of the 7-trifluoromethyl group of the 6,7-bis(trifluoromethyl)-8-ribityllumazine **1**.

Scheme 1



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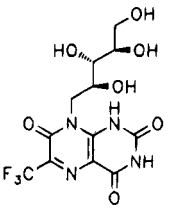

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To study the mechanism of the reaction, we determined the reaction velocity at different pH values at 50 °C. The results show that the elimination occurs with first order kinetics. Plotting the k-values against pH yielded the titration curve shown in Figure 2. The curve showed clearly that the reaction velocity depended on pH and indicated a pK_a of about 7.5. Below pH 5.5, no formation of **2** was observed, and pH values above 8.5 caused no further acceleration. As described earlier, **1** has two pK_a values of 4.3 and about 7.5.[1] As to the second pK_a values of the two epimers of **1**, the ^{19}F NMR signals of the 7-CF_3 groups of **1** shifted upfield in the pH range 6 to 9 by 0.5 ppm (epimer A) and 1.5 ppm (epimer B). This is most likely due to the deprotonation of the 7-hydroxyl groups of the lumazine hydrates **1**. On the basis of this agreement of the kinetic and NMR data, we can propose a

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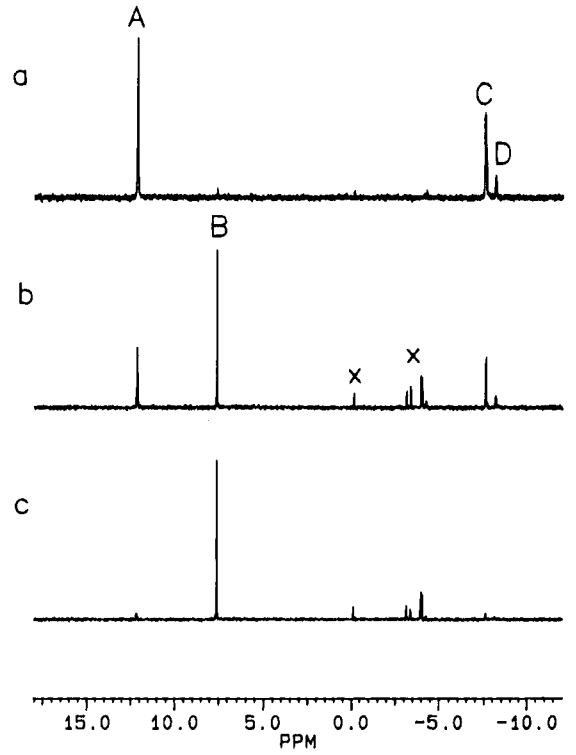


Figure 1. 338 MHz ^{19}F NMR spectra of **1** at different time intervals. The sample, containing 1.5 mM **1**, a mixture of 85% epimer B and 15% epimer A, in 150 mM phosphate buffer, pH 7.5, was incubated at 50 °C. The spectra were calibrated using an external standard containing sodium trifluoroacetate, pH 7.0. (a) Spectrum before incubation, (b) spectrum after 1 h, (c) spectrum after 3 h. A, signal of the 6-CF_3 group of **1**, both epimers; B, signal of the 6-CF_3 group of **2**; C, signal of the 7-CF_3 group of **1**, epimer B; D, signal of the 7-CF_3 group of **1**, epimer A; x, impurities.

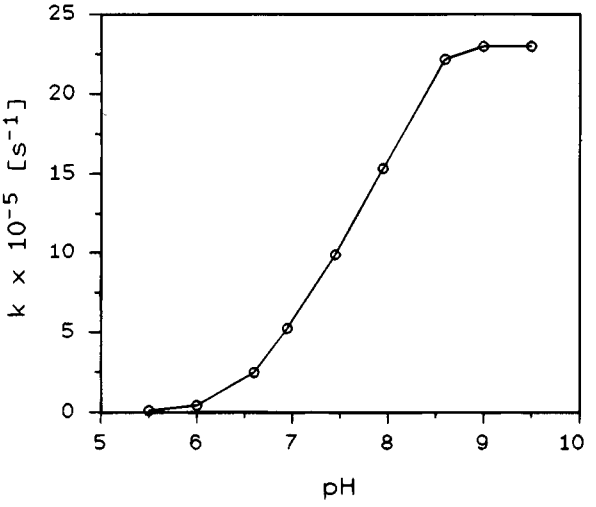
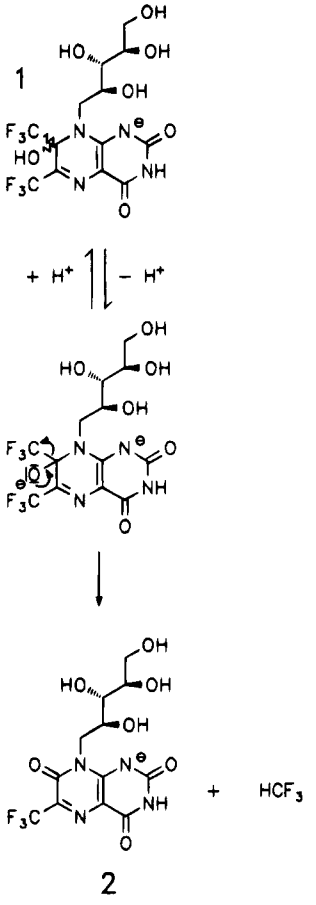


Figure 2. Rate constants for the elimination of the 7-CF_3 group of 6,7-bis(trifluoromethyl)-8-ribityllumazine (**1**, epimer A) at 50 °C. The samples contained 250 μM **1** in 100 mM phosphate. Samples were taken at intervals and analyzed by HPLC by published procedures.[1,4] k-Values were determined from the decreasing concentration of **1**.

mechanism for the elimination of the 7-CF_3 group (Scheme 2). The initial step is the deprotonation of the 7-hydroxyl groups to form the dianions of **1**. The resulting dianions will stabilize by release of the 7-CF_3 group as CF_3^- and formation of a carbonyl group at C-7 yielding the anion

Scheme 2



of **2**. The CF_3 anion will be protonated to form HCF_3. This reaction takes place under mild basic or neutral conditions in aqueous solution at relatively low temperature.

The conversion of **1** into **2** can be considered as a nitrogen analog of the later stages of the well-known haloform reaction, which has been documented for a variety of trifluoromethylated carbonyl compounds, including trifluoroacetaldehyde,[5] trifluoromethyl alkyl ketones,[6,7] trifluoromethyl benzyl ketones,[6] and trifluoromethyl aryl ketones.[8-10] These transformations are typically carried out in strongly alkaline aqueous sodium or potassium hydroxide solutions, often at elevated temperatures.[6-8] In one case, the reaction was performed in DMF at 60 °C with sodium hydride as the base.[10] In contrast, the present elimination of the trifluoromethyl carbanion from **1** to form **2** occurs under comparatively mild conditions. This may reflect the formation of the relatively stable, conjugated amide carbonyl present in the product **2**.

Both epimers of **1** were used in ligand studies with the lumazine synthase/riboflavin synthase complex[11] and both could be shown to bind to β-subunits, the lumazine

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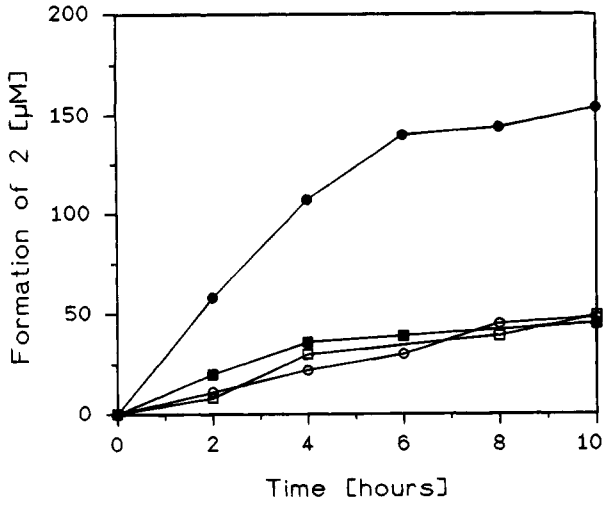

Figure 3. Formation of **2** in the presence and absence of the lumazine synthase/riboflavin synthase complex at 37 °C, pH 7.0 in 100 mM phosphate buffer. The samples contained 100 μM of the respective epimer. The enzyme sample additionally contained 2.5 mg/mL riboflavin synthase/lumazine synthase complex from *B. subtilis* (corresponding to 150 μM β-subunits). Formation of **2** was determined by HPLC by published procedures.[1,4] (●), epimer A and enzyme, (○), epimer A alone, (■) epimer B and enzyme, (□) epimer B alone.

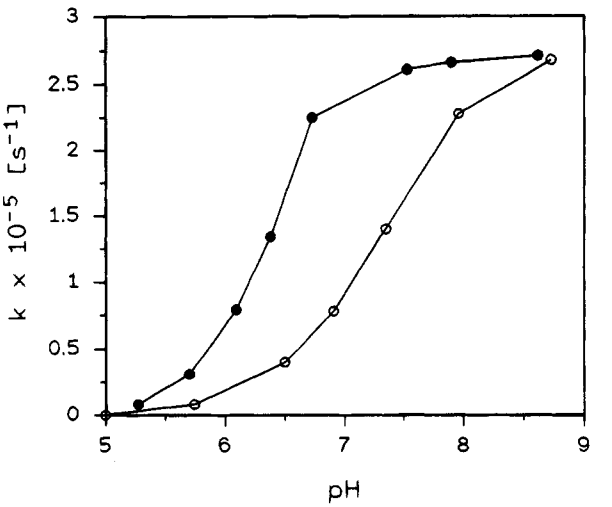

Figure 4. Kinetic constants of the enzyme-catalyzed and spontaneous elimination of the 7-CF_3 group of **1** (epimer A), at 37 °C. The samples contained 200 mM phosphate, 500 μM epimer A, and lumazine synthase/riboflavin synthase complex *from B. subtilis* (1.7 mg/mL, 100 μM β-subunits) as indicated. *k*-Values were determined as described in Figure 2. (○), epimer A alone; (●), epimer A and enzyme.

synthase active subunits, of this bifunctional enzyme (to be published elsewhere). In the absence of enzyme, aqueous solutions, pH 6.8, of both epimers were stable for several months during storage at 4 °C. Surprisingly, in the presence of the lumazine synthase/riboflavin synthase complex it was observed that epimer A, but not epimer B reacts to form oxolumazine **2** under these conditiones. By incubation of samples of the epimers with and without the enzyme in phosphate buffer, pH 7.0, at 37 °C it could be shown that in the sample containing epimer A and the enzyme, the elimination velocity was about 3 times faster than in the other samples (Figure 3). In these experiments, the samples contained nearly stoichiometric amounts of epimer A and β-subunit binding sites. The results indicate that the enzyme has a stereoselective catalytic influence on this reaction. Samples of epimer A and the enzyme and epimer A alone were incubated at different pH values at 37 °C and the kinetic constants were determined. The data were plotted against pH as described above. The titration curve in presence of the enzyme yielded a pK_a of about 6.5 in contrast to the value for the spontaneous reaction which was 7.5 (Figure 4), i.e., the enzyme decreased the pK_a by about 1 unit. However, the maximal reaction velocity of both the enzymatic and the spontaneous reaction were in the same range. A possible explanation of this behavior could be that the enzyme causes a decrease of the pK_a value of the 7-OH group of epimer A of **1**. A basic amino acid residue on the enzyme could act as the proton acceptor. This would also explain the stereoselectivity of the catalysis. The interaction between the 7-OH group of the ligand and a specific proton acceptor is evidently only possible for one diastereomer of **1**. After deprotonation to form the alkoxide anion, the following elimination of the CF_3 group and the resulting carbonyl formation are not influenced by the enzyme and happen at the same rate for both the reaction in the presence and absence of the enzyme. It seems that at increased pH only the spontaneous deprotonation occurs and the enzyme has no further influence.

Experimental Section

6,7-Bis(trifluoromethyl)-7-hydroxy-8-ribityllumazine (**1**) and 6-(trifluoromethyl)-7-oxo-8-ribityllumazine (**2**) were synthesized as described earlier.[1,4] The riboflavin synthase/lumazine synthase complex from *B. subtilis* was purified by published procedures.[12]

NMR spectroscopy. ^{19}F NMR spectra were measured at 338 MHz using a pulse angle of 30° (2 μs) and a repetition time of 1 s.

Kinetic Measurements. Samples of 200–500 μM **1** in 200 mM phosphate, pH and temperature as indicated, were incubated for 4–6 h. In enzymatic experiments the samples contained additionally 2–3 mg lumazine synthase/riboflavin synthase complex from *B. subtilis* per milliliter. Samples were taken at intervals, and 15% trichloroacetic acid was added to stop the reaction and precipitate the enzyme. Concentrations of **1** and **2** were determined by analytical reverse phase HPLC as described earlier.[1,4]

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