^{19}F NMR Studies of the Mechanism of Riboflavin Synthase. Synthesis of 6-(Trifluoromethyl)-8-(D-ribityl)lumazine and Derivatives

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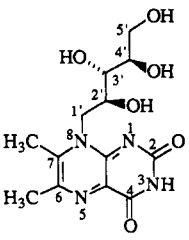
6-(Trifluoromethyl)-8-(D-ribityl)lumazine (**17**) was synthesized in order to study its reactivity at C-7 and its binding to riboflavin synthase of *Bacillus subtilis*. Compound **17** was prepared by reaction of 5-amino-4-[(D-ribityl)amino]-2,4-(1*H*,3*H*)-pyrimidinedione hydrochloride (**3**·HCl) with trifluoropyruvaldehyde hydrate (**18**). NMR studies revealed that under basic conditions, **17** forms only one major anionic species in which the oxygen of the 3′-hydroxyl group on the ribityl side chain binds covalently to C-7 of the lumazine, resulting in the formation of a pyran ring. As a model for possible addition of nucleophilic groups on the enzyme to C-7 of **17**, the reactions of **17** with a variety of sulfur nucleophiles were studied. Fluorolumazine **17** was found to form covalent adducts **27–31** with sulfite, sulfide, mercaptoethanol, D,L-1,4-dithiothreitol, and L-cysteine. Three molecules of **17** were found to bind per enzyme molecule (α subunit trimer). Equilibrium dialysis experiments and ^{19}F NMR spectroscopy provided dissociation constants K_D of 38 and 100 μM, respectively. The inhibition constant K_I was 58 μM. There was no evidence obtained for the formation of a covalent adduct between the fluorolumazine **17** and the enzyme, suggesting that the nucleophile adding to C-7 during the enzyme-catalyzed reaction is derived from water. The covalent adducts obtained from **17** were found to bind to the enzyme significantly more tightly than **17** itself. The covalent adducts **27–31** as well as **17** could be displaced from the enzyme by both riboflavin (**2**) and 5-nitroso-6-[(D-ribityl)amino]-2,4(1*H*,3*H*)-pyrimidinedione (**32**).

Introduction

Riboflavin synthase (EC 2.5.1.9) catalyzes an unusual dismutation reaction involving the transfer of a four-carbon unit from one molecule of 6,7-dimethyl-8-(D-ribityl)-lumazine (**1**) bound at the donor site of the enzyme to a second molecule of **1** bound at the acceptor site of the enzyme to form one molecule of riboflavin (**2**) and one molecule of the pyrimidinedione **3**.[1-3] A working hypothesis concerning the reaction mechanism has been advanced as shown in Scheme I.[4-6] Addition of an unidentified nucleophile to **1** yields **4** at the donor site, which then undergoes nucleophilic attack by an anion **5** formed by deprotonation of the 7-methyl group of lumazine **1** bound at the acceptor site. A 1,2-elimination in **6** followed by a 1,6-elimination in **7** yields a conjugated triene system in **8**, which undergoes a 3,3-sigmatropic rearrangement to afford intermediate **9**. Intermediate **9** then aromatizes by 1,2-elimination to yield the final products, riboflavin (**2**) and the pyrimidinedione **3**. Although the overall transformation is mechanistically complex, Wood and coworkers were able to demonstrate that riboflavin (**2**) forms in the absence of enzyme when the lumazine **1** was boiled in phosphate buffer at neutral pH.[7,8] Beach and Plaut showed that the nonenzymatic reaction also occurs under acidic conditions.[9] Both the catalyzed and uncatalyzed reaction are characterized by strict regiospecificity.



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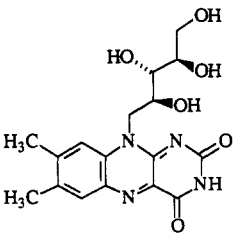

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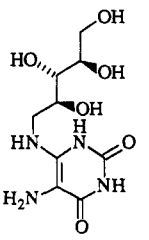

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Various possibilities for the identity of the nucleophile have been advanced, including a nucleophilic group from the enzyme, the 2′- or 3′-hydroxyl groups of the ribityl side chain, and water.[4,5,10] The pK_a of lumazine **1** is 8.4, and deprotonation yields a complex mixture of anions containing **10** (both diastereomers), **11** (both diastereomers), and **12**.[11-13] The equilibration among these species is slow

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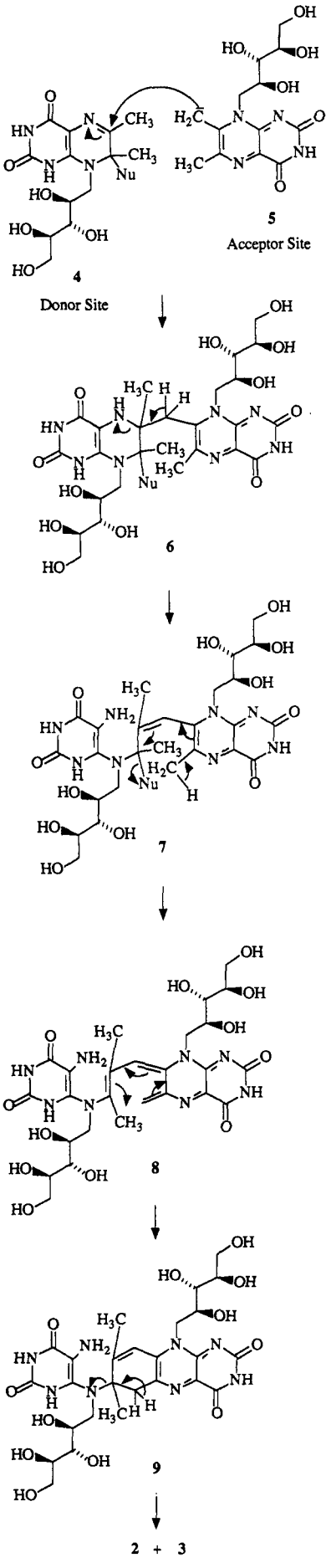
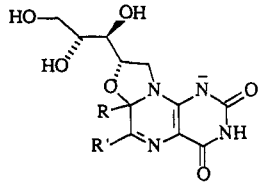
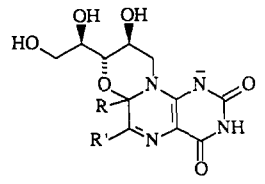
Scheme I



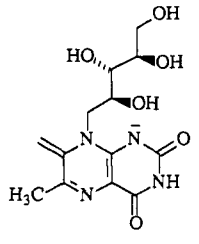
on the NMR time scale, and the equilibrium has been studied in some detail by NMR spectroscopy.



10 R = CH_3, R' = CH_3
20 R = H, R' = CF_3



11 R = CH_3, R' = CH_3
21 R = H, R' = CF_3



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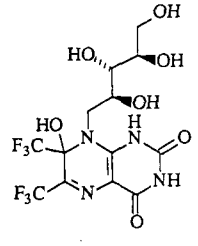

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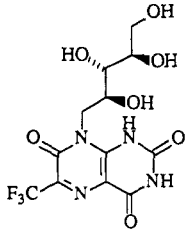

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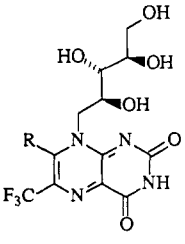

15 R = CH_3
16 R = CF_3
17 R = H

Our interest in fluorinated analogs of **1** as potential inhibitors and ^{19}F NMR-detecting shift probes of riboflavin synthase has recently resulted in the synthesis of the covalently hydrated, diastereomeric bis(trifluoromethyl)-lumazines **13**, as well as the 7-oxo compound **14** and the 6-(trifluoromethyl)-7-methyl-substituted lumazine **15**.[14-18] The diastereomeric lumazines **13** proved to be very stable analogs of intermediate **4** at the donor site in which "Nu" is a hydroxyl group, and only one of them was found to bind to the enzyme.[17] However, their stability toward dehydration, resulting from the highly electronegative CF_3 group at C-7, precluded exchange of the 7-hydroxyl group for other nucleophiles, which would have occurred through intermediate **16**. On the other hand, the 6-(trifluoromethyl)-7-methyl analog **15** proved to undergo reversible addition of the 2'- and 3'-hydroxyl groups at C-7 to form 5- and 6-membered rings, but no evidence was obtained for intermolecular addition of nucleophiles at C-7. Additional trifluoromethylated lumazines were therefore

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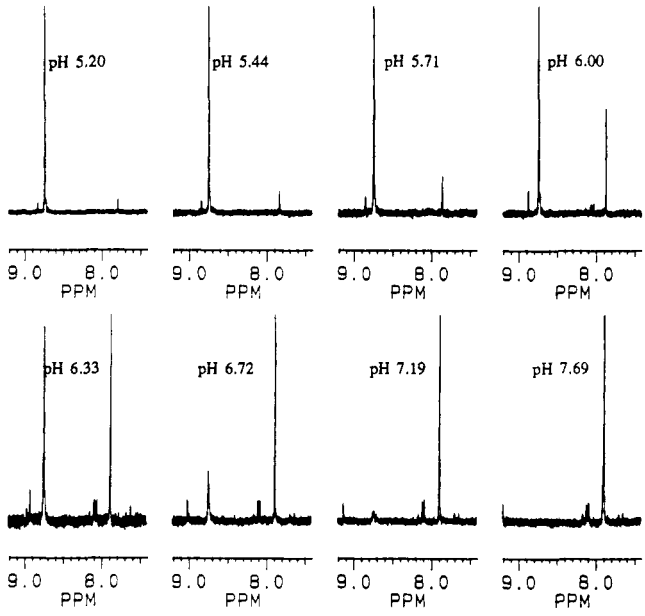
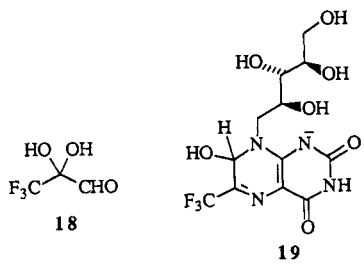


Figure 1. The ^{19}F NMR spectra of 6-(trifluoromethyl)-8-(D-ribityl)lumazine (17) recorded at different pH values.

sought which would have modulated reactivity allowing the study of intermolecular addition of small nucleophiles at C-7 as well as the possible attack at C-7 of nucleophilic groups located on the enzyme. If the nucleophile for the enzyme-catalyzed formation of riboflavin (2) is provided by an amino acid residue on the enzyme, it should be possible to form stable covalent adducts between the enzyme and appropriate substrate analogs. To test this hypothesis and to study possible reactivity at C-7, we prepared 6-(trifluoromethyl)-8-(D-ribityl)lumazine (17). It was anticipated that this compound would be more reactive at C-7 than the 6-(trifluoromethyl)-7-methyl analogs 15 due to decreased steric hindrance.

Chemistry. 6-(Trifluoromethyl)-8-(D-ribityl)lumazine (17) was synthesized by reaction of the hydrochloride salt of 3 with the covalent hydrate of trifluoropyruvaldehyde (18) in DMF.[16] The reaction mixture proved to be quite complex and the desired product 17 was isolated in 7% yield after a tedious purification procedure involving cation-exchange chromatography followed by anion-exchange chromatography.



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Spectrophotometric titration of 17 in phosphate buffer indicated that equal amounts of neutral molecule and anion exist at pH 6.3 (Figure 1). In phosphate buffer at pH 2.5, the ^{19}F NMR spectrum of 17 shows a singlet at δ 8.75 ppm downfield from trifluoroacetic acid, while in phosphate buffer of pH 7.7, the signal at δ 8.75 was replaced by one major signal at δ 7.85 (83%) and two minor signals at δ 8.04 and 8.06 (together 17%). Three other very minor singlets which probably correspond to anionic forms were also present (Figure 1). This suggests that the signal at

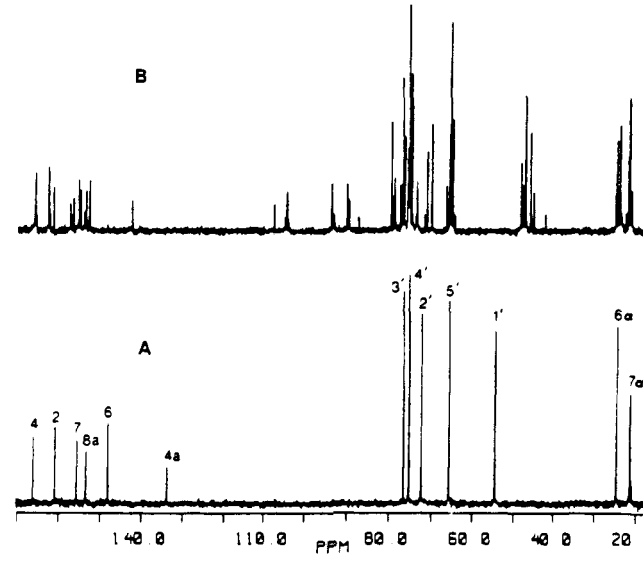


Figure 2. ^{13}C NMR spectra of 6,7-dimethyl-8-(D-ribityl)lumazine (1) in 0.5 M phosphate buffer in H_2O/D_2O at pH 7 (A) and pH 11 (B).[13]

δ 8.75 ppm corresponds to the neutral molecule 17, whereas the signals at δ 7.85, 8.04, and 8.06 correspond to the anions. It was interesting that only one major signal (δ 7.85, 83%) was observed in alkaline solution, since there are six possible structures for the anion (two diastereomers each of 19, 20, and 21).[13] The structure of the major anion was identified as one of the diastereomers of the 6-membered cyclic ring ether anion 21 on the basis of spectroscopic methods. The minor singlets which arise as the pH is increased likely represent the remaining five anionic structures.[13]

Negative ion FABMS of 17 (dissolved in water) using triethanolamine as a matrix showed a base peak at 365 ($M^- - 1$). It was determined by UV spectroscopy that in the presence of triethanolamine, 17 does exist as an anion. The mass spectral data suggest that the major anion was not one of the two diastereomers of 19. However, structure 19 is not rigorously excluded by the mass spectral data since 19 could conceivably undergo dehydration to 20 or 21 on the mass spectrometer probe.

Since the solution structures of the anions derived from 6,7-dimethyl-8-(D-ribityl)lumazine (1) have been published[13] and the ^{13}C signals are assigned, it seemed appropriate to use ^{13}C NMR to elucidate the structure of the major anion derived from 17. Proton-decoupled and ^{1}H-coupled ^{13}C NMR spectra of 17 were obtained in a phosphate buffer at pH 3.0, in which 17 exists as a neutral molecule, and at pH 8.0, in which it exists in an anionic form. Figures 2 and 3 show proton-decoupled ^{13}C NMR spectra of 1 and 17, respectively, in phosphate buffer pH 3 (A) and at pH 8 (B).

In contrast to 1, which exists as five major anionic species (two diastereomers each of 10 and 11, in addition to 12) showing a complex ^{13}C NMR spectrum (Figure 2), compound 17 shows a simple spectrum under basic conditions (Figure 3). The spectrum contains two quartets at δ 120.23 and 128.35 corresponding to C-6α (CF_3) and C-6, respectively. Ten other carbons are seen as singlets. This indicates the presence of one major species of the anion derived from 17. The proximity of the C-7 and C-3' signals under basic conditions initially prevented an unambiguous assignment. However, the ^{13}C NMR assignment was

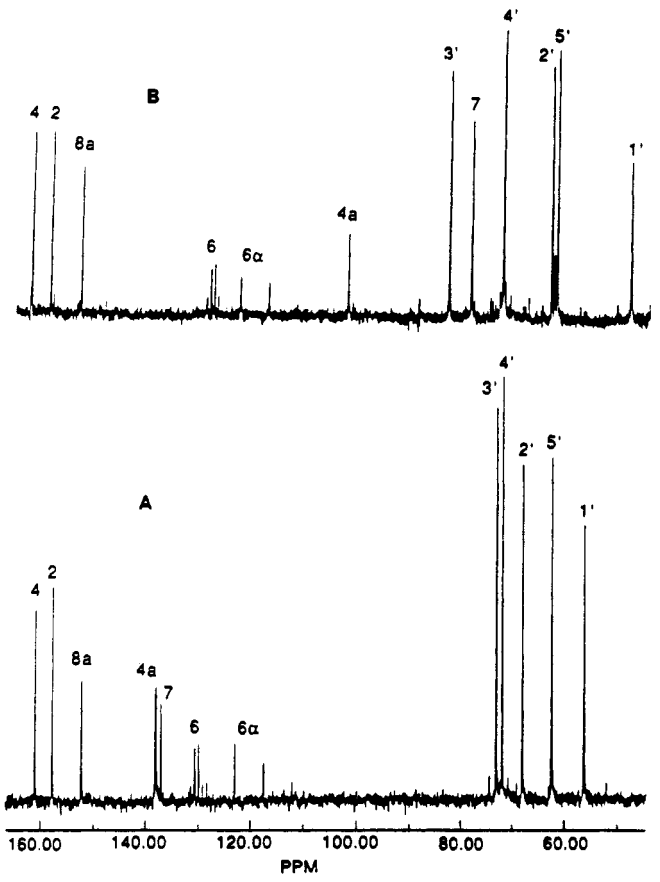
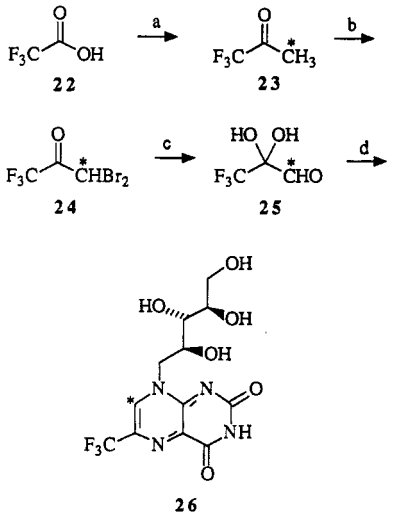


Figure 3. ^{13}C NMR spectra of 6-(trifluoromethyl)-8-(D-ribityl)-lumazine (17) in phosphate buffer in H_2O at pH 3 (A) and pH 8 (B).

Scheme II[a]



[a] (a) $^{13}CH_3MgI$, n-Bu_2O, −10 °C (90 min); (b) Br_2, H_2SO_4, 23 °C; (c) H_2O, NaOAc, 98 °C (30 min); (d) 3·HCl, DMF, 23 °C (18 h).

eventually made by preparing compound **17** enriched in ^{13}C at C-7, starting with ^{13}C enriched trifluoropyruvaldehyde. The synthesis of the labeled compound **26** is described in Scheme II.[19]

There are some differences in the chemical shifts of the corresponding carbons of **1** and **17**, which are obviously due to different substitutions at positions 6 and 7. The

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Table I. ^{13}C NMR Chemical Shifts (ppm) of 8-(D-Ribityl)-6-(trifluoromethyl)lumazine (17) and Anion 21

carbon assignment	neutral molecule 17	anion 21
2	157.64	159.59
4	161.00	163.24
4a	138.04	102.30
8a	152.19	153.76
6a	120.17	120.23
6	130.22	128.35
7	137.02	78.50
1′	56.31	47.56
2′	67.97	62.92
3′	72.99	82.85
4′	71.83	72.35
5′	62.38	61.85

Table II. ^{13}C NMR Chemical Shifts (ppm) of Anion 21, Derived from 17, and the Major Diastereomeric Forms of Anions 10 and 11, Derived from 1

carbon assignment	21	10	11
4a	102.3	105.0	104.7
7	78.5	93.7	89.7
1′	47.7	47.1	45.9
2′	62.9	76.8	70.1
3′	82.8	74.6	79.5

chemical shift assignments of lumazine **17** and the corresponding carbons of its major anion **21** are listed in Table I.

Five signals corresponding to C-4a, C-7, C-1′, C-2′, and C-3′ are significant for the structural assignment of the anion **21**. The chemical shifts of the other carbons are not affected much by formation of anion. It can be seen from the spectrum that at pH 8 two signals at δ 138.04 (C-4a) and 137.02 (C-7) disappear and two new signals at δ 102.30 (C-4a) and 78.50 (C-7) are formed. The upfield shift of C-4a and of C-7, which is associated with a change in hybridization (sp^2 in the neutral molecule and sp^3 in the anion), are well in line with the published data.[13] In the case of **1** the chemical shifts of the anions ranges for C-4a from δ 104–105 and for C-7 from δ 93.7–87.3. The upfield shift of C-1′ in **17** versus of corresponding anion (δ 56.31 in neutral molecule versus 47.56 in anion) is also in agreement with the published data for C-1′ of **1** (δ 54.3 for neutral molecule versus δ 48–45 for anions).

Cyclization involving the bonding of one of the oxygens on the side chain and C-7 in **1** during anion formation results in the downfield shift of the attached carbon on the side chain as compared to the uncyclized alcohol.[13] The chemical shift of C-3′ shifted downfield when **17** was converted to the anionic form (δ 82.85 in the anion compared to δ 67.97 in the neutral molecule). This indicates that the anion derived from **17** exists in the form **21** which has a pyran ring derived from an intramolecular attack of the 3′-hydroxyl group on C-7. In addition, an NOE was observed at pH 8 between the signal of the 7-H proton at δ 5.08 ppm and the signal of the 3′-H at δ 3.82 ppm in the ^1H–^1H NOESY spectrum, which confirms the presence of the six-membered ring in the anion. For comparison, important ^{13}C chemical shifts of anions of **17** and **1** are shown in Table II. In the case of **1**, only the major diastereomeric forms of 5-membered and 6-membered ring anions are shown. In contrast to **17**, which exists in only one major anionic species **21** (at least 83% of the mixture) having a 6-membered ring, the natural substrate **1** exists as the 7α *exo*-methylene anion **12** (17%), the diastereomeric 5-membered ring anions **10** (40 and 22%), and the diastereomeric 6-membered ring anions **11**

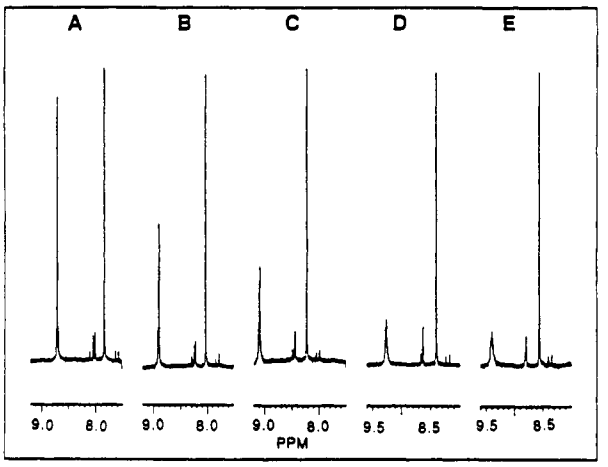


Figure 4. Temperature dependence of the ^{19}F NMR spectrum of 6-(trifluoromethyl)-8-(D-ribityl)lumazine (17) at a concentration of 1.8 mM in H_2O/D_2O (80/20) at pH 6.3. A, 20 °C; B, 30 °C; C, 40 °C; D, 50 °C; E, 60 °C.

(14 and 7%). No explanation for this unexpected behavior of 17 under basic conditions can be offered at this time. For that, detailed kinetic analyses and further studies with different side chain substituents in the 8-position would be necessary. The analogous 6-methyl-8-(D-ribityl)lumazine exhibits the same behavior, as it seems to exist in only one anionic species under basic conditions as indicated by proton NMR and NOESY experiments. This behavior occurs when C-7 of the lumazine is unsubstituted.

The effect of temperature on the ^{19}F NMR spectrum of 6-(trifluoromethyl)-8-(D-ribityl)lumazine (17) was also investigated. The ^{19}F NMR spectra were recorded at 20 °C, 30 °C, 40 °C, 50 °C, and 60 °C and the results are shown in Figure 4. As the temperature increased, the signals shifted about 0.7 ppm to lower field relative to the external standard of potassium–sodium trifluoroacetate, pH 7.0. During the temperature increase, the peak corresponding to the major anion remained sharp, while that of the neutral molecule broadened (Figure 4). This may reflect the greater conformational flexibility of the side chain in the neutral molecule as compared to the anion. This may imply that a greater number of conformers of the side chain become accessible at higher temperature. These temperature effects were reversible.

Derivatives of 17 with quaternary C-7 can be formed at neutral pH by nucleophilic addition of appropriate nucleophiles to C-7 as shown by ^{19}F NMR spectroscopy. Thus, 17 has been shown to react with a variety of thio compounds such as sulfite, sulfide, and organic thiols to form products 27–31. The titration of 17 with sulfite at pH 6.8 is shown in Figure 5. The NMR signals of the neutral molecule (8.7 ppm) and anion (7.9 ppm) are progressively replaced by two closely spaced signals at 9.45 and 9.50 ppm. The intensities of the new signals have a ratio of about 2:1 and represent the two possible epimers with inverted configurations at C-7. A dissociation constant for the dissociation of the sulfite adduct 27 (150 μM) can be estimated from titration experiments monitored by ^{19}F NMR. The formation of the adducts required several seconds at room temperature, thus allowing the detection of the equilibrium between 17 and the adducts on the NMR time scale.

The ^{19}F NMR spectra of adducts 27–31 formed by reaction of 17 with sulfite, sulfide, mercaptoethanol, D,L-1,4-dithiothreitol and L-cysteine are shown in Figure 6. The ^{19}F NMR chemical shifts are summarized in Table

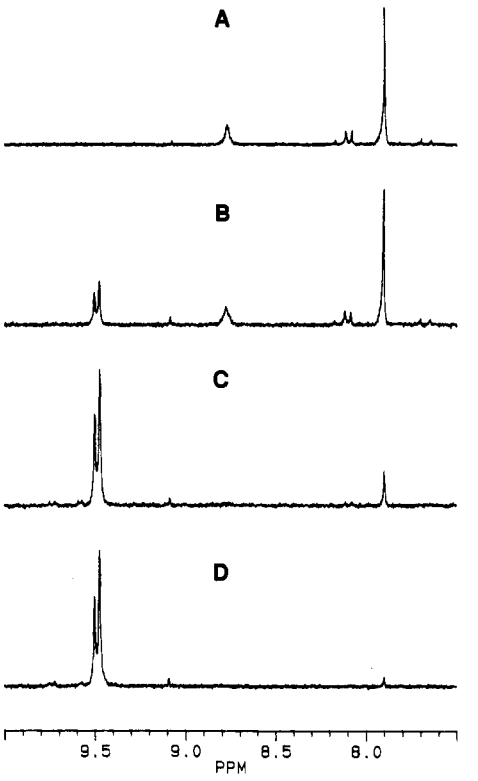
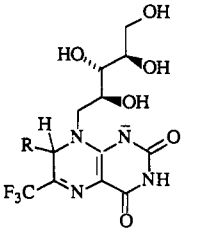


Figure 5. Titration of 2.4 mM 6-trifluoromethyl-8-(D-ribityl)-lumazine (17) with sulfite. A) No sulfite. B) 1.2 mM Na_2SO_3. C) 2.3 mM Na_2SO_3. D) 3.4 mM Na_2SO_3. The ^{19}F NMR spectra were recorded at 24 °C in 100 mM phosphate, pH 7.0.



27 R = SO_3^-
28 R = S^-
29 R = SCH_2CH_2OH
30 R = $SCH_2CHOHCHOHCH_2S^-$
31 R = $SCH_2CH(NH_2)COO^-$

III. The adducts derived from organic thiols have ^{19}F NMR chemical shift values close to the value of 17 and their formation can only be diagnosed by the loss of the signal of the anion at δ 7.9 ppm. The sulfite and sulfide adducts are characterized by considerable low field and high field shifts, respectively. Complete formation of the adduct required the presence of an excess of the sulfur reactant.

The proton at C-7 has chemical shift values of δ 8.9 and 5.8 ppm in the neutral molecule and anion of 17. In the sulfite adduct 27 this proton is characterized by two signals with chemical shift values of δ 5.6 and 5.55 ppm. The ratio between the signal integrals is about 1:2, thus suggesting that the signals represent the different configurations at C-7. This confirms the addition of the nucleophile occurs at C-7.

Enzyme Studies. If an amino acid side chain (serine, threonine, or cysteine) of the enzyme serves as a nucleophile for the formation of a covalent adduct as an early step in the reaction mechanism of riboflavin synthase, the lumazine 17 should be an ideal probe for its detection and analysis. Kinetic experiments, equilibrium dialysis, and

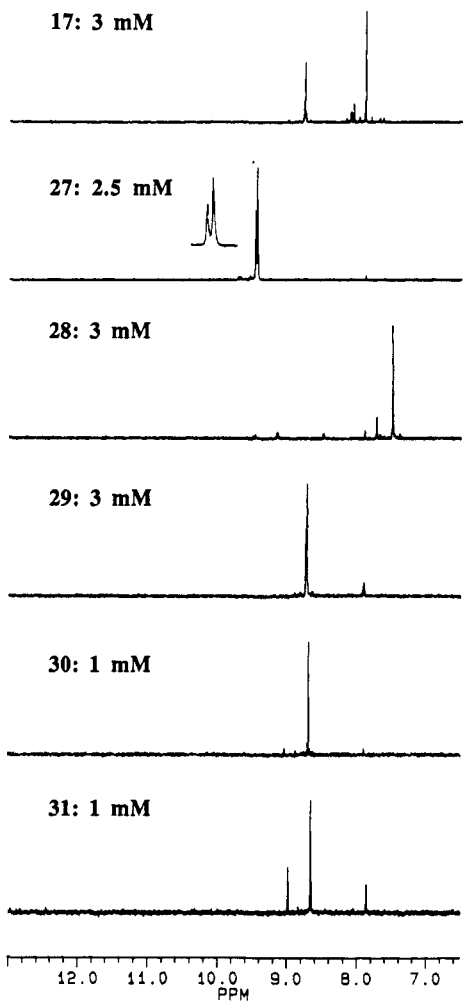


Figure 6. ^{19}F NMR spectra of 17 and products 27–31, pH = 6.8 at 24 °C. Reactant concentrations: Na_2SO_3 and Na_2S, 20 mM; $HOCH_2CH_2SH$ and dithiothreitol, 50 mM; cysteine, 100 mM.

Table III. ^{19}F NMR Chemical Shifts of 17 and Products 17–31

compound	free ligand	enzyme-bound ligand
17	8.7 (neutral)	9.3
	7.9 (anion)	10.3
27	9.5	10.7
		11.7
28	7.9	10–12 (max 10.7)
29	8.7	9.4
		9.8
30	8.7	9.2
		9.8
31	8.7	9.4
		9.7
		10.2

^{19}F NMR spectroscopy studies were initiated in order to test this hypothesis.

^{19}F NMR spectra of enzyme-ligand complexes were recorded at 338 MHz using 30° pulses with a pulse interval of 1 s, and T_1 was estimated by inversion recovery to be in the range of 0.5 s. Control measurements using longer pulse intervals gave no evidence of saturation effects. Figure 7 shows a series of NMR spectra from a titration experiment. The lumazine 17 was added to a solution of riboflavin synthase in phosphate buffer at pH 6.8. The signals representing the neutral molecule and anion of nonbound 17 are broadened to a line width of about 20 Hz. At higher concentrations of the ligand (trace D),

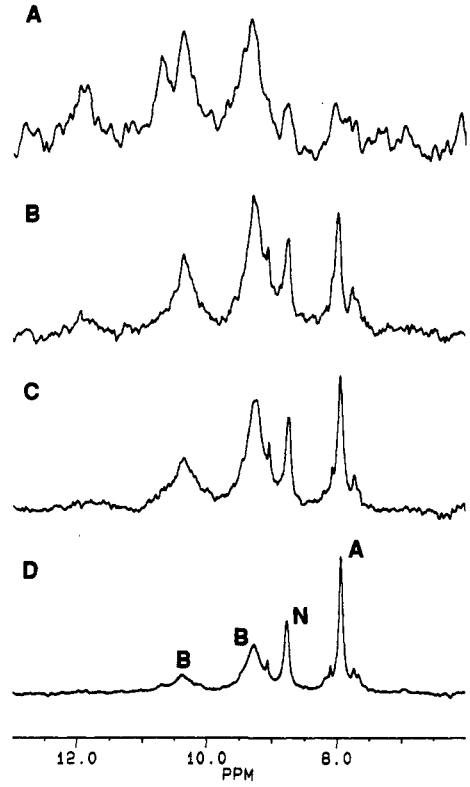


Figure 7. Titration of riboflavin synthase of *Bacillus subtilis* with 6-(trifluoromethyl)-8-(D-ribityl)lumazine (17) in phosphate buffer (no sulfite), pH 6.8 at 24 °C. (A) 182 μM fluorolumazine, 187 μM riboflavin synthase. (B) 525 μM fluorolumazine, 180 μM riboflavin synthase. (C) 687 μM fluorolumazine, 177 μM riboflavin synthase. (D) 993 μM fluorolumazine, 170 μM riboflavin synthase. B, bound ligand; N, neutral free ligand; A, anionic free ligand.

enzyme-bound 17 gives rise to two signals at δ 9.3 and 10.4 ppm which are shifted to lower field relative to the signals of the free ligand. These signals are very broad with line widths of about 150 Hz. At lower concentrations of the ligand (trace A), two additional signals at approximately δ 10.7 and 11.7 ppm are apparent. These additional signals may be due to the formation and binding of the sulfite adduct 27 (Figure 9 and Table III). It is possible that the enzyme solution contained trace amounts of sulfite, remaining from the purification procedure, which react with 17 to form 27. At increased concentration of 17, these signals become too weak for detection. The line broadening to about 150 Hz observed with the enzyme-bound lumazines appears to be partly due to exchange broadening. Studies to determine the exchange rates are currently in progress.

Multiple (two or three) ^{19}F NMR signals of enzyme-bound species have also been observed in binding experiments with other lumazine derivatives. The present case is further complicated by the coexistence of neutral and anionic forms of 17 at the pH of investigation. It remains unknown whether the two signals of the enzyme-bound species represent neutral molecule, anion, or both.

A ^{19}F NMR-monitored titration similar to that shown in Figure 7 gave the Scatchard plot shown in Figure 8. It is apparent that each enzyme molecule can bind three ligand molecules (i.e. one per subunit) with a dissociation constant of about 100 μM. This value was similar to K_D = 38 μM obtained by equilibrium dialysis experiments (data not shown). Kinetic experiments showed an inhi-

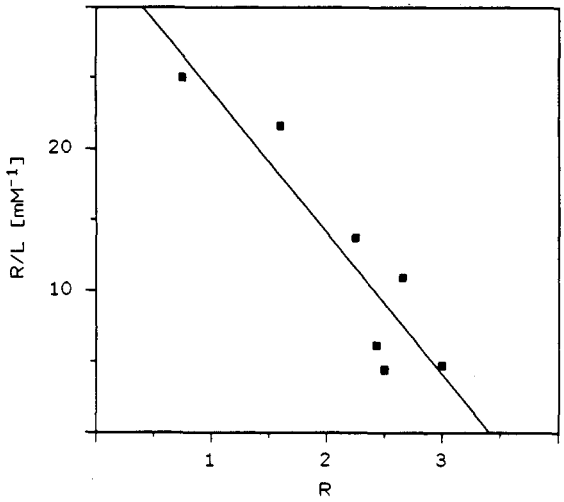


Figure 8. Scatchard plot of the ^{19}F NMR spectra of 6-(trifluoromethyl)-8-(D-ribityl)lumazine and riboflavin synthase of *Bacillus subtilis* in 170 mM phosphate buffer (pH 6.8, 24 °C). R is the number of ligand molecules bound per enzyme molecule at equilibrium, and L is the free ligand concentration at equilibrium. The values of R and L were calculated from the ^{19}F NMR integrations of the signals attributed to bound and free ligand determined at different concentrations of the ligand (Figure 7).

bition constant of 58 μM. These data show that **17** is only a weak inhibitor of the enzyme.

The same techniques were then used to study the interaction of the enzyme with the derivatives **27–31**. Enzyme solutions used in titration experiments contained 20 mM sulfide or sulfite, or 50 mM of the respective organic thiol. The NMR spectra shown in Figure 9 indicate that all the derivatives were bound to the enzyme. The enzyme-bound species always appear as broad signals shifted to lower field by about 1–3 ppm. The sulfite adduct showed two enzyme-bound signals separated by about 1 ppm. The sulfide adduct gave a single, very broad signal (line width about 200 Hz). The enzyme-bound adducts with organic thiols gave signals with a low-field shift of about 0.5–1.5 ppm and a line width of about 150 Hz.

Quantitative evaluation of NMR-monitored titrations gave the Scatchard plots shown in Figures 10–13. Data from equilibrium dialyses of the sulfite adduct are also included in Figure 10 and document the agreement between the two experimental approaches.

Surprisingly, all adducts studied bind more tightly to the enzyme than the lumazine **17** (Table IV). The cysteine adduct was characterized by a $K_D < 3$ μM, which could not be determined accurately by the method used. In all cases, about one molecule of the respective derivative was bound per enzyme subunit. The K_I values of the products formed with sulfide organic thiols were in the range of 15–20 μM. They are more potent inhibitors of the enzyme than **17** (K_I 58 μM, Table IV).

We showed earlier that each subunit of riboflavin synthase can simultaneously bind one molecule each of riboflavin (**2**) and 5-nitroso-6-(D-ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione (**32**).[18] It appears plausible that these two ligands were bound, respectively, at the acceptor and donor site. It was also shown earlier that various bound fluorolumazine derivatives could be displaced from the enzyme by both riboflavin and the pyrimidine **32**.[17,18] Similarly, ^{19}F NMR studies showed clearly that all enzyme-bound lumazine derivatives used in this study could also

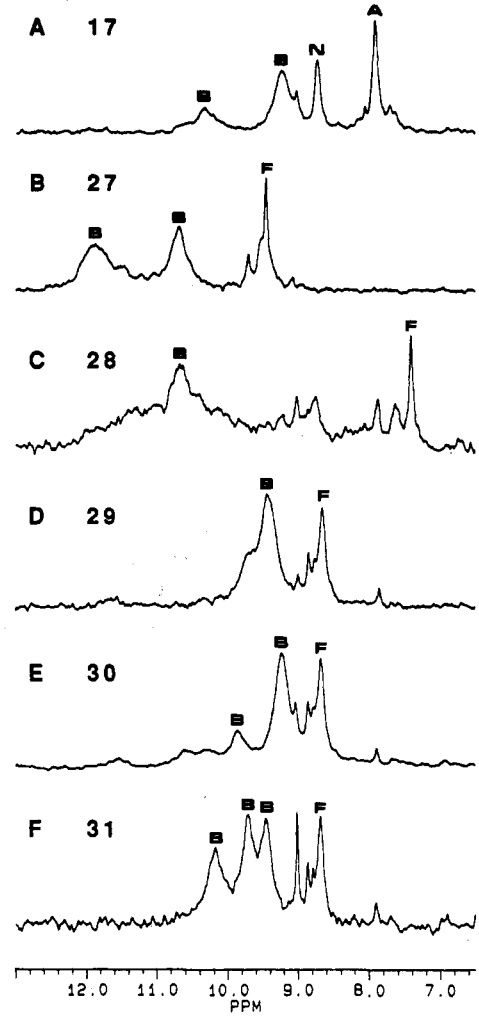
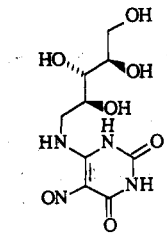


Figure 9. ^{19}F NMR spectra obtained from mixtures containing light riboflavin synthase of *Bacillus subtilis* and fluorolumazines **17** and **27–31**, pH 6.8 at 24 °C. (A) 170 μM enzyme, 993 μM lumazine, (B) 150 μM enzyme, 400 μM lumazine, 20 mM Na_2SO_3. (C) 200 μM enzyme, 616 μM lumazine, 20 mM Na_2S. (D) 190 μM enzyme, 352 μM lumazine, 50 mM mercaptoethanol. (E) 170 μM enzyme, 720 μM lumazine, 50 mM D,L-1,4-dithiothreitol. (F) 143 μM enzyme, 605 μM lumazine, 100 mM L-cysteine. B, bound; N, free neutral form; A, free anionic form; F, free.

be completely displaced from the enzyme by the addition of riboflavin or **32** (data not shown).



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Discussion. The lumazine **17** can reversibly form covalent adducts with a variety of thio compounds. Both the free lumazine and the adducts can bind to riboflavin synthase as shown by kinetic experiments, equilibrium dialysis, and ^{19}F NMR spectroscopy. Some of the adducts bind significantly more tightly to the enzyme than the free lumazine. However, in each case the protein-bound lumazines could be displaced by the enzyme product,

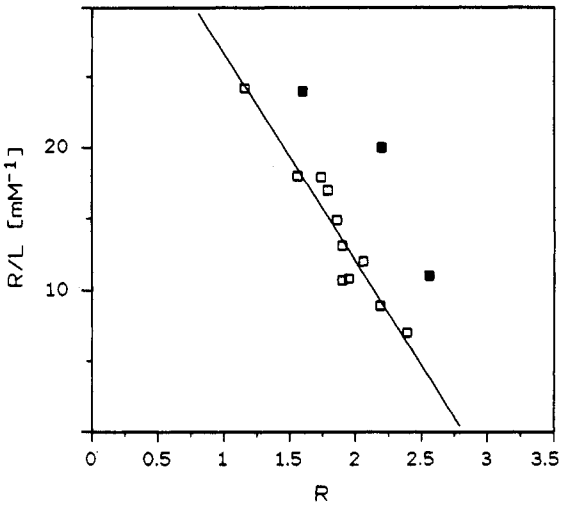


Figure 10. Scatchard plot of the ^{19}F NMR spectra (■) and equilibrium dialysis (□) of the binding of **27** to riboflavin synthase of *Bacillus subtilis*. For details see Figure 8.

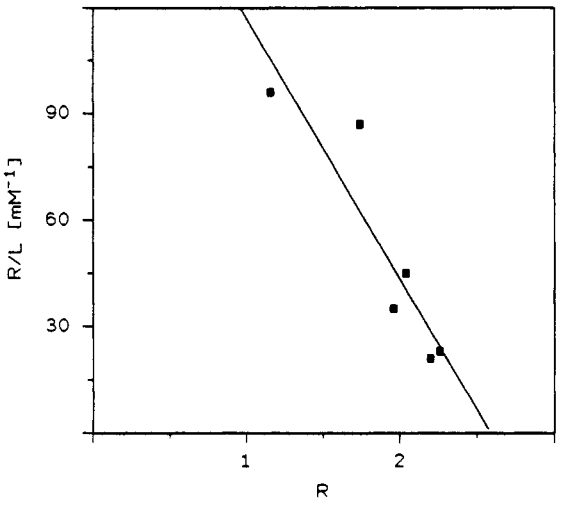


Figure 11. Scatchard plot of the ^{19}F NMR spectra of **28** and riboflavin synthase of *Bacillus subtilis* in 170 mM phosphate buffer (pH 6.8). For details see Figure 8.

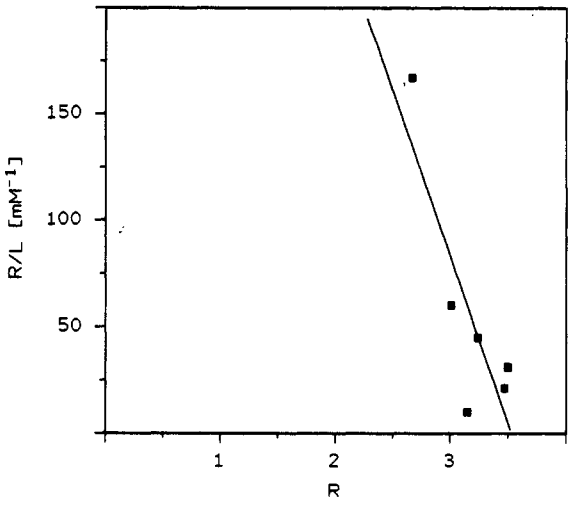


Figure 12. Scatchard plot of the ^{19}F NMR spectra of **29** and riboflavin synthase of *Bacillus subtilis* in 170 mM phosphate buffer (pH 6.8). For details see Figure 8.

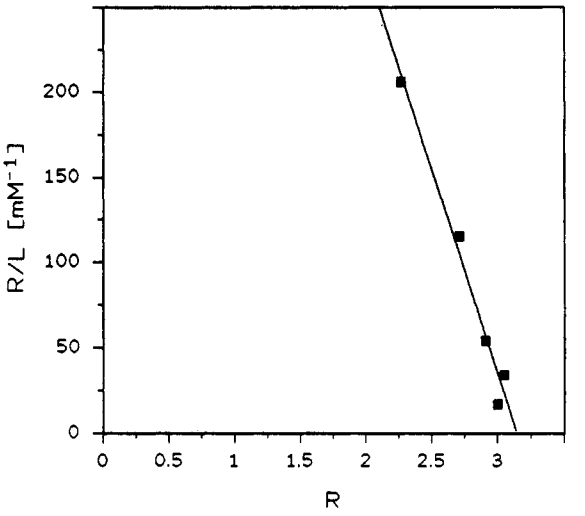


Figure 13. Scatchard plot of the ^{19}F NMR spectra of **30** and riboflavin synthase of *Bacillus subtilis* in 170 mM phosphate buffer (pH 6.8). For details see Figure 8.

riboflavin, or the product analog, 5-nitroso-6-(D-ribityl-amino)-2,4(1*H*,3*H*)-pyrimidinedione (**32**).

The dismutation reaction to form riboflavin (**2**) requires the binding of two substrate molecules **1** in close proximity at the active site of the enzyme. The sequence of the riboflavin synthase subunit shows strong homology between and *N*-terminal and *C*-terminal half, and it has been proposed that the subunit can form homologous domains, each of which provides for binding of one substrate molecule.[20] Indeed, it has been shown that each subunit can bind two molecules of appropriate substrate analogs such as **14**.[18,21]

Earlier studies have shown that the bis(trifluoromethyl)-lumazine **13** forms stable covalent hydrates characterized by a quaternary carbon at C-7.[17] Both diastereomers have been isolated. Only one of the epimers (epimer A) bound to riboflavin synthase, and the binding stoichiometry was 1:1 (ligand subunit).

The lumazine derivatives **27–31** and epimer A of **13** are structural analogs of **4**, which has been proposed as an intermediate bound at the donor site in the enzyme mechanism.[4–6,10] In light of this similarity, it seems likely that the lumazine derivatives under study bind specifically at the donor site but not the acceptor site of the protein.

The hypothetical mechanism of the enzyme-catalyzed reaction requires a nucleophile for the formation of a covalent adduct at C-7 of the donor molecule (Scheme I). In the uncatalyzed reaction, a hydroxyl ion from water or a hydroxyl group of the ribityl side chain could serve this function. In the enzyme-catalyzed reaction, an amino acid side chain from serine, threonine, or cysteine could also serve this purpose. In this case, it should be possible to form covalent adducts between the enzyme and appropriate substrate analogs. The lumazine **17** should be an excellent candidate for covalent adduct formation with the enzyme in light of the facile formation of a variety of covalent adducts with small molecules. However, we have shown that **17** binds significantly less tightly to the enzyme than the adducts **27–31** characterized by quaternary C-7. No evidence was obtained which would indicate the formation of a covalent adduct between **17** and the enzyme.

(20) Schott, K.; Kellerman, J.; Lottspeich, F.; Bacher, A. *J. Biol. Chem.* **1990**, *265*, 4204.
(21) Otto, M.; Bacher, A. *Eur. J. Biochem.* **1981**, *115*, 511.

Table IV. Summary of the Kinetic and Thermodynamic Parameters of the Interactions of Fluorolumazines with the Light Riboflavin Synthase of *Bacillus subtilis*

compound	K_I (μM)	K_D (μM)	stoichiometry[a]
13 (epimer A)[15]	120 (pH 7.4)	13.0[b]	2.9[b]
	38 (pH 6.8)	16.2[c]	3.1[c]
13 (epimer B)[15]	500[d]	nbo[f]	0
14[16]	55	4, 112[b]	6.2[b]
		80[c]	5.5[c]
15[16]	75	17[b]	>3[b]
		70[c]	3.2
17	58	38[b]	3.9
		100[c]	3.4
27	70	70[c]	2.8
		77[b]	3.4
28	17	14[c]	2.6
29	17	6[c]	3.5
30	15	4[c]	3.1[c]
31	20	<3[c]	ca. 3.0
2	100[12]	35[e]	3.0

[a] Moles bound per enzyme molecule (α-subunit trimer). [b] From equilibrium dialysis at 4 °C. [c] From ^{19}F NMR at 24 °C. [d] This equilibrium constant is an artifact due to the presence of a minor impurity. Since epimer B does not bind to the enzyme, it is unlikely that it inhibits the enzyme. [e] From equilibrium dialysis at 24 °C. All measurements were performed at pH 6.8. [f] nbo = no binding observed.

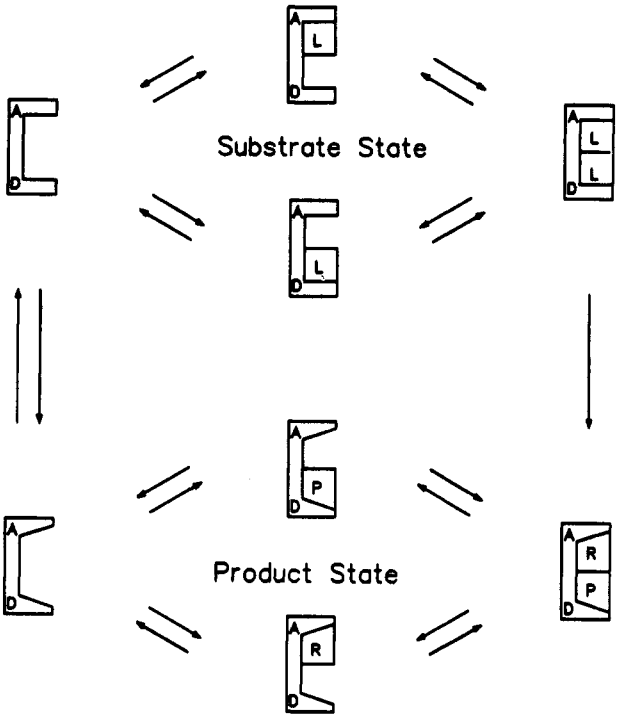
On the basis of these findings, we propose that the nucleophile involved in enzyme catalysis is neither an amino acid nor a hydroxyl group of the side chain. On the contrary, the data suggest that water or a hydroxyl ion serves as a nucleophile for the enzyme-catalyzed reaction as well as the uncatalyzed reaction. This would imply that the enzyme does not interact covalently with its substrate, and that its catalytic role is exclusively based on providing for an optimum spatial arrangement of the substrate molecules. The increased affinity of the derivatives **27–31** could be due to the quaternary C-7. The K_D values are similar to that of the binding diastereomer of **13**. Additionally, new contacts can be formed between the substituents at C-7 and the enzyme.

It has been known for some time that each subunit of riboflavin synthase can bind one molecule of riboflavin (**2**) or of the product analog **32**. More recently, we could show that riboflavin and the pyrimidine **32** can bind simultaneously at the active site of a given protein subunit, and it appears plausible that riboflavin should bind at the acceptor site and the pyrimidine **32** at the donor site.[18]

Only one molecule of the substrate analogs **27–31** can bind per enzyme subunit, most probably at the donor site as discussed above. The bound compounds can be displaced from the enzyme by both riboflavin and the pyrimidine **32**. The same is true for epimer A of **13** as shown earlier. Moreover, it has been shown that the lumazine **14** which binds both to the donor and acceptor site is also completely displaced by either riboflavin or the pyrimidine **32**. Since riboflavin is supposed to bind at the acceptor site, it is not immediately obvious that it should displace any ligands from the donor site.

However, the findings can be explained if we assume that the enzyme can adopt two different conformational states as proposed earlier (Scheme III).[18] Specifically, we propose that the enzyme can adopt a conformation designated the substrate state which can interact with one or two lumazine molecules. Alternatively, the enzyme can adopt a conformation designated the product state, which can interact with a flavin and a pyrimidine ligand. The simultaneous binding of one substrate and one product type molecule, however, is unfavorable in such a system.

Scheme III[a]



[a] A hypothetical model for the catalytic cycle of riboflavin synthase. A, acceptor site; D, donor site; L, lumazine; R, riboflavin; P, pyrimidine.

Experimental Section

All reactions were performed under nitrogen atmosphere. ^{19}F NMR chemical shifts are reported relative to trifluoroacetic acid as external standard. Analytical thin-layer chromatography was performed on Baker-flex silica gel 1B2-F sheets or EM Science Silica gel 60F_{254} glass plates.

6-(Trifluoromethyl)-8-(D-ribityl)lumazine (17). 3,3-Dibromo-1,1,1-trifluoroacetone (1.08 g, 4 mmol) was added to a solution of sodium acetate (1.2 g, 14.8 mmol) in water (10 mL) and heated at 90 °C for 30 min. The reaction mixture was cooled to room temperature, diluted with water (10 mL), and extracted with diethyl ether (4 × 50 mL). The ether extracts were dried ($MgSO_4$) and evaporated under vacuum to obtain a yellow liquid. DMF (2 mL) was added, and the resulting solution was added to a solution of 5-amino-4-(D-ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione hydrochloride (**3**·HCl, 340 mg, 1.13 mmol) in DMF (4 mL). The reaction mixture was stirred at room temperature in the dark for 24 h before the solvent was removed under vacuum (0.5 mmHg). The dark red, semisolid residue was triturated with water (2 mL) at 98 °C for 3 min. The solution was cooled to room temperature and applied to a column of cation-exchange resin (Dowex 50 × 8–200, H^+ form, 2 × 66 cm). The column was eluted with water, and fractions (4 mL) were collected and analyzed by analytical HPLC (μ-Bondapak C-18, 10 mm 3.9 × 300 mm, detecting at 270 nm) eluting with 10% acetonitrile. Fractions 15–24 containing **17** and some impurities were combined and lyophilized to give yellow solid (91 mg). This solid was dissolved in an ammonium acetate buffer (0.1 M, pH 8.01, 2 mL) and applied to a column of anion-exchange resin (Biorad AG-1-X2, acetate form, 1 g, 1 × 3.5 cm). The column was eluted with ammonium acetate buffer (0.1 M, pH 8.01, 30 mL) followed by ammonium acetate buffer (0.1 M, pH 4.8, 40 mL). Fractions (2 mL) were collected and analyzed by analytical HPLC as described above. Fractions containing the desired compound were combined and lyophilized to give yellow solid product (27 mg, 7%): mp 134–136 °C; UV (aqueous HCl, pH 1) λ_{max} 397 (log ϵ 3.84), 264 nm (4.18); UV (aqueous NaOH, pH 13) λ_{max} 327 (log ϵ 3.74), 253 nm (3.95); IR (KBr) 3630–2600, 1720, 1700, 1630, 1565, 1440, 1400, 1365, 1275, 1200, 1165, 1140, 1075, 1050, 1000, 900, 800, 685 cm^{-1}; ^{1}H NMR (D_2O, DMSO-d_6, 500 MHz) δ 8.91 (s, 1 H), 5.09 (m, 1 H), 3.90 (dt, 1 H, J = 3 and 7 Hz), 3.82 (m, 2 H), 3.69 (dd, 1 H,

J = 7 and 12 Hz); ^{19}F NMR (phosphate buffer pH 5.2) δ 8.7 (s, 3 F); ^{19}F NMR (phosphate buffer pH 7.7) δ 7.9 (s, 3 F); ^{13}C NMR (phosphate buffer pH 3.0) δ 161.00 (s), 157.64 (s), 152.19 (s), 138.04 (s), 137.02 (s), 130.22 (q, J = 38 Hz), 120.17 (q, J = 272 Hz), 72.99 (s), 71.83 (s), 67.97 (s), 62.38 (s), 56.31 (s); ^{13}C NMR (phosphate buffer pH 7.9) δ 163.24 (s), 159.59 (s), 153.76 (s), 128.35 (q, J = 36 Hz), 120.28 (q, J = 274 Hz), 102.30 (s), 82.85 (s), 78.50 (s), 72.35 (s), 62.92 (s), 61.85 (s), 47.56 (s); FABMS (glycerol + HCl) m/e (rel inten) 367 (M^+ + 1, 39); FABMS negative ion mode (triethanolamine) m/e (rel inten) 365 (M^- − 1); high resolution FABMS (glycerol + HCl) calcd for $C_{12}H_{14}F_3N_4O_6$ m/e 367.0865 (M^+ + 1), found 367.0873.

[3-^{13}C]-1,1,1-Trifluoroacetone (23). A 100-mL two-necked flask fitted with a dry ice–acetone condenser and a dropping funnel was charged with magnesium turnings (2.4 g, 0.1 mol) and dry di-*n*-butyl ether (25 mL). A solution of methyl iodide (1.0 g ^{13}C-labeled and 13.2 g unlabeled, total 14.2 g, 0.1 mol) and dry di-*n*-butyl ether (10 mL) was added dropwise. The flask was immersed in a water bath and the temperature was maintained between 23 and 28 °C. The addition was completed in 30 min. After an additional 20 min, the flask was cooled to −10 °C and trifluoroacetic acid (**22**, 3.8 g, 0.03 mol) in di-*n*-butyl ether was added dropwise with continuous stirring. The stirring was continued for a further 90 min at −10 °C before water (6 mL) was added, followed by 7 N hydrochloric acid (12 mL). Fractional distillation of the mixture, without prior separation of organic and aqueous layers, gave [3-^{13}C]-1,1,1-trifluoroacetone (2.8 g, 75%): bp 25 °C. The compound was used immediately in the next step.

[3-^{13}C]-3,3-Dibromo-1,1,1-trifluoroacetone (24). Ice-cold sulfuric acid (8 mL) was added to a flask containing [3-^{13}C]-1,1,1-trifluoroacetone (**23**, 2.8 g, 25 mmol) cooled in an ice bath (5 °C). Bromine (9.6 g, 60 mmol) was added to this solution dropwise and the mixture was stirred at 23 °C for 72 h. The lower layer was separated and distilled to give **24** (1.8 g, 27%): bp 87–95 °C. The compound was used in the next step without purification.

[7-^{13}C]-6-(Trifluoromethyl)-8-(D-ribityl)lumazine (26). [7-^{13}C]-3,3-Dibromo-1,1,1-trifluoroacetone (**24**, 1.08 g, 4 mmol) was added to a solution of sodium acetate (1.3 g, 16 mmol) in water (10 mL) and heated at 98 °C for 30 min. The reaction mixture was cooled to room temperature, diluted with water (10 mL), and extracted with diethyl ether (4 × 50 mL). The ether extracts were dried ($MgSO_4$) and evaporated under vacuum to obtain a yellow liquid. DMF (2 mL) was added to this liquid, and the resulting solution was added to a solution of 5-amino-4(D-ribityl)amino-2,4(1*H*,3*H*)pyridinedione hydrochloride (**3**·HCl, 395 mg, 1.3 mmol) in DMF (4 mL). The reaction mixture was stirred at room temperature in the dark for 18 h before the solvent was removed under vacuum (0.5 mmHg). The dark red semisolid residue was triturated with water (2 mL) at 98 °C for 3 min. The solution was cooled to room temperature and the product was purified by cation-exchange chromatography followed by anion-exchange chromatography as described for unlabeled **17** to give **26** as yellow solid (12 mg, 3%).

Protein. Light riboflavin synthase was purified to a specific activity of 50000 nmol mg^{-1} h^{-1} from the derepressed *B. subtilis* H94 mutant as described earlier.[20] The purified enzyme gave a single band in sodium dodecyl sulfate polyacrylamide gel electrophoresis. Enzyme activity was measured as described. One unit of enzyme catalyzes the formation of one nmol of riboflavin per hour at 37 °C.

NMR Spectroscopy. The ^{19}F NMR spectra of the enzyme–ligand complex were measured at 9.6 Tesla using a Bruker AM360 NMR spectrometer. The samples contained 10–15 mg of protein and ligand as indicated. The experiments were performed in 170 mM phosphate, pH 6.8, and 10% D_2O. The samples of the experiments with the adducts **27–31** contained additionally an excess of sulfite, sulfide, or the organic thiol as indicated. The ^{19}F NMR measurements were performed at 24 °C using a pulse angle of 30° (2 μs) and a repetition rate of 1 s. Chemical shifts were calibrated using an external standard containing sodium trifluoroacetate at pH 7.0. Equilibrium dialysis and enzyme kinetics were performed by published procedures.[17,18]

Adducts of Compound 17. The adduct compounds of **17** were formed by the addition of Na_2SO_3, Na_2S, mercaptoethanol, dithiothreitol, or cysteine to solutions of **17** in 170 mM phosphate, pH 6.8, at 24 °C. The formation of the adducts was monitored by NMR spectroscopy.

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