^{19}F NMR Studies on the Mechanism of Riboflavin Synthase. Synthesis of 6-(Trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine and 6-(Trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine

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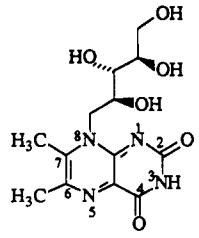
The reactions of hexafluoropropene oxide (**19**), methyl trifluoropyruvate (**21**), and 1,1,1-trifluorobutane-2,3-dione (**45**) with a series of ortho diamines were investigated as an approach to the synthesis of trifluoromethyl-substituted quinoxalinones and lumazines. 6-(Trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine (**11**) was synthesized by reaction of methyl trifluoropyruvate (**21**) with 5-amino-6-(D-ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione (**3**) hydrochloride and utilized as a ^{19}F NMR probe of the light riboflavin synthase of *Bacillus subtilis*. The fluorolumazine **11** was found to be an inhibitor of the enzyme with an inhibition constant K_I = 55 μM. Equilibrium dialysis experiments indicated the binding of six molecules of **11** per enzyme molecule, corresponding to one molecule bound at each of the three donor and three acceptor sites of the enzyme. The apparent dissociation constants K_D were approximately 4 and 112 μM. The enzyme-bound ligand gave rise to several broad ^{19}F NMR signals which were shifted to low field. The bound ligand **11** could be displaced from the enzyme by the enzyme product, riboflavin (**2**), and the product analog, 5-nitroso-6-(ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione (**56**). 6-(Trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (**13**) was synthesized by reaction of the hydrochloride salt of **3** with 1,1,1-trifluorobutane-2,3-dione (**45**). Three molecules of **13** can be bound relatively tightly per mole of riboflavin synthase, i.e., one ligand molecule per protein subunit. The inhibition constant K_I was determined to be 75 μM, while dissociation constants of 17 and 70 μM were determined by equilibrium dialysis and ^{19}F NMR, respectively. The bound ligand **13** could also be displaced by riboflavin and product analog **56**. A scheme for the catalytic cycle of riboflavin synthase is proposed.

Riboflavin synthase (E.C. 2.5.1.9.) catalyzes an interesting dismutation reaction in which two molecules of 6,7-dimethyl-8-(D-ribityl)lumazine (**1**) form one molecule of riboflavin (**2**) and one molecule of the pyrimidinedione **3**.[1-3] During this reaction, substrate bound at the donor



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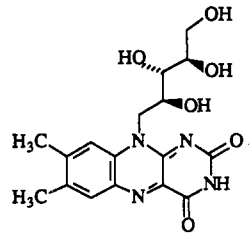

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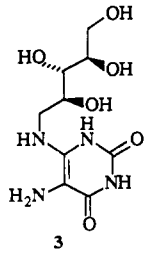

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site of the enzyme donates a four-carbon unit to substrate bound at the acceptor site. The mechanism of the reaction is thought to proceed as outlined in Scheme I.[4,5] In this process, the anion **5** formed from deprotonation of the C-7 methyl group of **1** at the acceptor site of the enzyme adds to the imine group of **4**, which is formed by addition of an unidentified nucleophile to C-7 of **1** bound at the donor site of the enzyme. Various suggestions for the identity of the nucleophile have been offered, including a nucleophilic group from the enzyme,[6] the 2′-hydroxyl or 3′-hydroxyl of the ribityl side chain,[7,8] and water.[9] A β-elimination involving cleavage of the N-5 to C-6 bond of the lumazine moiety of **6** at the donor site yields the alkene **7**. A 1,6-elimination involving loss of a proton from the C-6 methyl group of the lumazine moiety bound at the acceptor site and "Nu" then results in the formation of a conjugated triene **8**. A 3,3-sigmatropic rearrangement involving the triene system of **8** affords a six-membered ring of **9**. Aromatization of **9** by β-elimination of a methylene proton and final cleavage of a C–N bond then yields the final products of the reaction, riboflavin (**2**) and 5-amino-6-(D-ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione (**3**).

Recent interest in fluorinated ribityllumazines as potential inhibitors and ^{19}F NMR detecting shift probes[10] of riboflavin synthase has led us to an investigation of methodology for the preparation of fluorinated nitrogen heterocycles.[6,11,12] Both of the two epimeric 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrates **10** were recently synthesized, and only one of them was demonstrated by ^{19}F NMR spectroscopy to interact with the light riboflavin

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(1) Plaut, G. W. E.; Smith, C. M.; Alworth, W. L. *Ann. Rev. Biochem.* **1974**, *43*, 899.
(2) Brown, G. M.; Williamson, J. M. *Adv. Enzymol.* **1982**, *53*, 345.
(3) Bacher, A.; Le Van, Q.; Keller, P. J.; Floss, H. G. *J. Biol. Chem.* **1983**, *258*, 13431.
(4) Beach, R. L.; Plaut, G. W. E. *J. Am. Chem. Soc.* **1970**, *92*, 2913.
(5) Paterson, T.; Wood, H. C. S. *J. Chem. Soc., Chem. Commun.* **1969**, 290.
(6) Cushman, M.; Wong, W. C.; Bacher, A. *J. Chem. Soc., Perkin Trans. 1* **1986**, 1043.
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(8) Bown, D. H.; Keller, P. J.; Floss, H. G.; Sedlmaier, H.; Bacher, A. *J. Org. Chem.* **1986**, *51*, 2461.
(9) Otto, M.; Bacher, A. *Eur. J. Biochem.* **1981**, *115*, 511.
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(11) Cushman, M.; Wong, W. C.; Bacher, A. *J. Chem. Soc., Perkin Trans. 1* **1986**, 1051.
(12) Cushman, M.; Patrick, D. A.; Bacher, A.; Scheuring, J. *J. Org. Chem.* **1991**, *56*, 4603.

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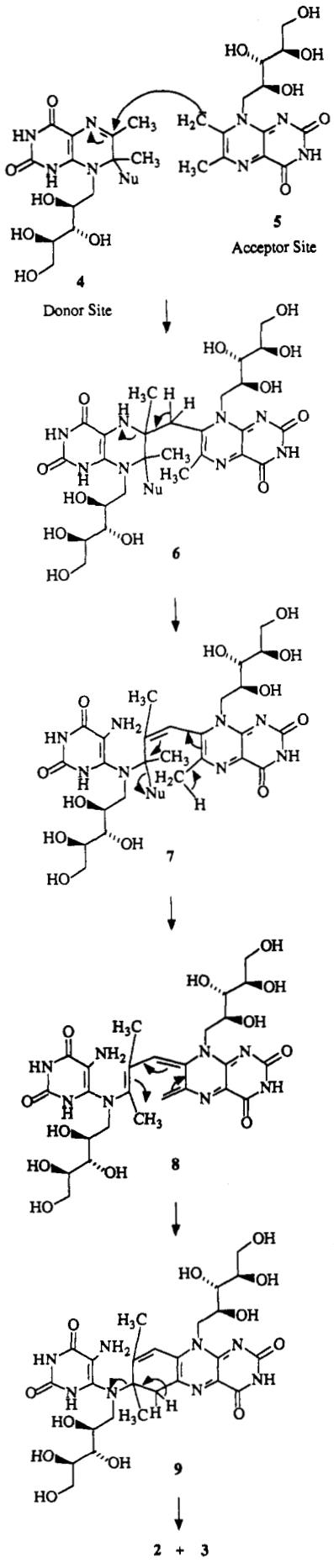
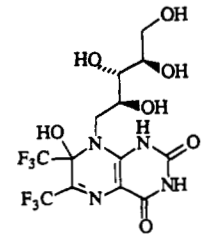
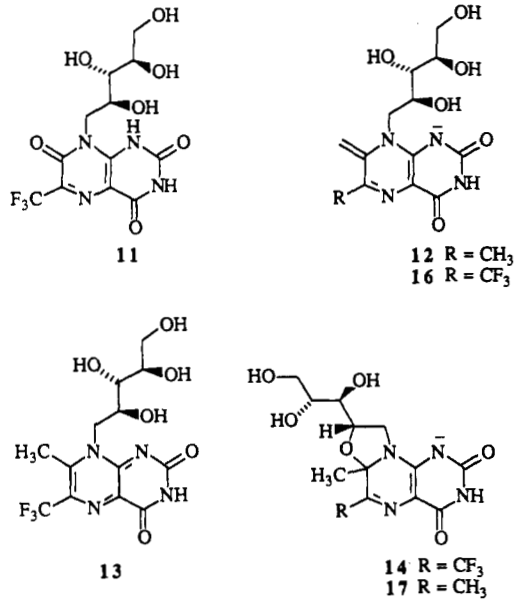
Scheme I





10

synthase of *Bacillus subtilis*.[12] This substance was also found to be a weak competitive inhibitor of the enzyme (K_i = 120 μM). As an extension of these studies, consideration has recently been directed to the preparation of 6-(trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine (**11**). The rationale for proposing this compound as an inhibitor of riboflavin synthase is that it might function as an analog of the anion **12** formed by the initial deprotonation of the substrate at the acceptor site of the enzyme.[4,5] The electronegativity of the 6-(trifluoromethyl) group as well as the presence of the keto group at C-7 would be expected to facilitate deprotonation at N-1 of **11** to form a stabilized anion resembling **12**. In fact, the corresponding 6-methyl



11

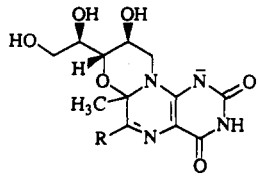
12 R = CH_3
16 R = CF_3

13

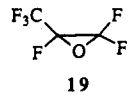
14 R = CF_3
17 R = CH_3

analog of **11** has been prepared and found to be a relatively good inhibitor of the *Ashbya gossypii* riboflavin synthase (K_i = 2 μM) as well as the riboflavin synthase from baker's yeast (K_i = 0.53 μM).[13,14] The simplified ^{19}F NMR spectrum of **11** relative to **10** might also prove useful during the employment of **11** as a ^{19}F NMR detecting shift probe[10] of the enzyme. In addition to the fluorolumazine **11**, 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (**13**) was also considered. It was anticipated that this compound, in contrast to the bis-trifluoromethylated diastereomers **10**, would have modulated reactivity at C-7 which would allow the reversible addition of nucleophiles to C-7. This would provide an opportunity to observe and study anions **14** (both diastereomers) and **15** (both diastereomers) resulting from addition of the 2′-hydroxyl and 3′-hydroxyl groups to C-7 of **13** under basic conditions, as well as the exomethylene anion **16**, also anticipated from deprotonation of **13** under basic conditions.[8] Anions **14** and **15** are

(13) Winestock, C. H.; Aogaichi, T.; Plaut, G. W. E. *J. Biol. Chem.* **1963**, *238*, 2866.
(14) Al-Hassan, S. S.; Kulick, R. J.; Livingston, D. B.; Suckling, C. J.; Wood, H. C. S.; Wrigglesworth, R.; Ferone, R. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2645.



15 R = CF_3
18 R = CH_3



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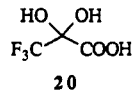

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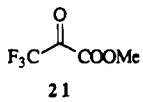

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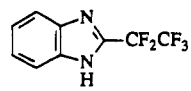

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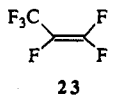

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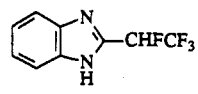

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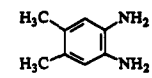
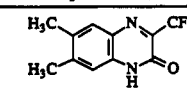
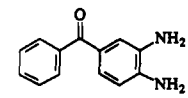
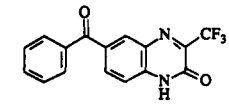
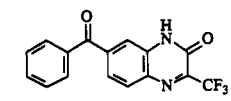
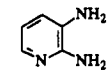
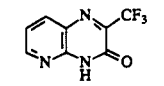
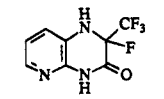
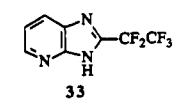
trifluoromethylated analogs of anions 17 and 18 that may be involved at the donor site of the enzyme, while anion 16 is an analog of the anion 12 (a resonance form of anion 5), which is thought to be involved at the acceptor site of the enzyme (Scheme I).[4,7,8] Methodology for the synthesis of 11 and 13, as well as related model heterocyclic systems, was therefore studied in order to investigate problems of reactivity and regiochemistry associated with the preparation of these types of trifluoromethylated nitrogen heterocycles.

Chemistry. Reactions of Hexafluoropropene Oxide and Methyl Trifluoropyruvate with Ortho Diamines. Synthesis of 6-(Trifluoromethyl)-7-oxo-8-(D-ribityl)-lumazine (11). Reagents that were considered for the preparation of 11 from 3 included hexafluoropropene oxide (19), trifluoropyruvic acid hydrate (20), and methyl trifluoropyruvate (21). Although these substances might reasonably be expected to react in the desired fashion, there are potential difficulties associated with each reagent. Depending on the reaction conditions, hexafluoropropene oxide (19) may isomerize to pentafluoropropionyl fluoride, which reacts with *o*-phenylenediamine to afford product 22.[15] Reaction of trifluoropyruvic acid hydrate (20) with nucleophiles requires high temperatures.[16] Reactions of methyl trifluoropyruvate (21) with unsymmetrical ortho diamines have not been investigated previously and therefore pose problems in regiochemistry. Since both trifluoropyruvic acid hydrate (20) and methyl trifluoropyruvate (21) are prepared from hexafluoropropene oxide (19), initial studies were performed with 19.

All of the known methods for synthesis of hexafluoropropene oxide (19) are based on the reaction of commercially available hexafluoropropene (23) with an oxygen donor.[17] Two general methods involve reaction of 23 with alkaline hydrogen peroxide[18,19] or with hypohalites in alkaline solution.[20-23] The hexafluoropropene oxide used in the present study was prepared by reaction of 23 with hydrogen peroxide in KOH.[18] The crude product was used without separation of unreacted hexafluoropropene (23). It was assumed that hexafluoropropene oxide would react faster than 23 with the ortho diamine. In fact, no byproducts related to 24 resulting from reaction of 23 with ortho diamines were detected,[23-25] and the results with the crude product were identical to those obtained with commercially available 19. The results of these studies are listed in Table I.

Table I. Reaction of Hexafluoropropene Oxide with Ortho Diamines

reactant	solvent (time, temp)	product(s)	% yield
 25	CH_2Cl_2 (12 h, 23 °C)	 26	96
 27	$(C_2H_5)_2O$ (12 h, 23 °C)	 28	71
		 29	13
 30	CH_2Cl_2 (12 h, 23 °C)	 31	3
		 32	31
		 33	6

Ishikawa and Sasaki found in their studies of the reaction of hexafluoropropene oxide (19) with ortho diamines that use of polar solvents like acetonitrile accelerates isomerization of 19 to pentafluoropropionyl fluoride, which would be expected to give products related to 22.[15] The use of less polar solvents like diethyl ether, together with sodium bicarbonate to remove the hydrogen fluoride formed, increased the yield of the desired products.[15] This was found to be true in the present case with diamines 25 and 27, which afforded products in good yield. Two products, 28 and 29, were formed from the reaction involving 3,4-diaminobenzophenone (27). The structures of the two isomers 28 and 29 were assigned on the basis of the known chemistry of hexafluoropropene oxide.[26] It is reported that nucleophiles readily attack the central carbon. This may be attributed to the powerful electron-withdrawing properties of the trifluoromethyl group.[26]

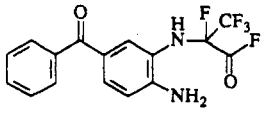
(15) Ishikawa, N.; Sasaki, S. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2164.
(16) Mustafa, M. E.; Takaoka, A. *Bull. Soc. Chim. Fr.* **1986**, 944.
(17) Millauer, H.; Schwertfeger, W.; Siegmund, G. *Angew. Chem., Int. Ed. Engl.* **1985**, 161.
(18) Knunyants, I. L.; Shokina, V. V.; Galakhov, I. V. *Khim. Geterotsiki Soedin.* **1966**, 873.
(19) Soshin, V. A.; Vilenchik, Y. M.; Kharchenko, A. P. *Chem. Abstr.* **1977**, *86*, 139820.
(20) Kolenko, I. P.; Filyakova, T. I.; Zapevalov, A. Y.; Lure, E. P. *Izv. Akad. Nauk. SSSR* **1979**, 2509.
(21) Filyakova, T. I.; Zapevalov, A. Y.; Kolenko, I. P. *Chem. Abstr.* **1979**, *91*, 140335s.
(22) *Chem. Abstr.* **1983**, *99*, 22293u.
(23) Ishikawa, N.; Muramtsu, T. *Nippon Kagaku Kaishi* **1973**, 563.
(24) Nakai, T.; Hassan, N. M.; Ishikawa, N. *Bull. Chem. Soc. Jpn.* **1977**, 3014.
(25) Harada, H.; Mitzutaki, S.; Hayashi, S.; Ishikawa, N. *J. Fluorine Chem.* **1978**, 211.
(26) Sianesi, D.; Pasetti, A.; Tarli, F. *J. Org. Chem.* **1966**, *31*, 2312.

Hence, it was assumed that the more reactive amino group of **27** attacks the central carbon atom to give intermediate **34**, which then cyclizes and dehydrohalogenates to give isomer **28** as the major product.

The reaction of **30** gave three products **31**, **32**, and **33**, with the expected product **31** obtained in the lowest yield. The structure of **31** was assigned on the basis of comparison of its spectral data with the authentic compound made from methyl trifluoropyruvate and diamine **30** (vide infra). The structure of **32** was assigned on the basis of spectral data: CIMS showed an $M^+ + 1$ ion m/z 236, IR showed the presence of a carbonyl group at 1715 cm^{-1}, ^{1}H NMR showed the presence of two exchangeable protons at δ 6.05 and 6.04 ppm, and ^{19}F NMR showed a doublet at δ −4.73 ppm (3 F) and a quartet at δ −77.30 ppm (1 F), upfield from trifluoroacetic acid. High-resolution CIMS gave an $M^+ + 1$ value at m/z 236.0416, which agrees with the chemical formula $C_8H_6F_4N_3O$. All attempts to dehydrohalogenate **32** using triethylamine, DBU, or KOtBu in DMSO were unsuccessful. Either starting material was recovered or extensive decomposition occurred. The structure of **32** was assigned on the assumption that the more reactive 3-amino group of **30** attacks the central carbon of hexafluoropropene oxide. However, since the yield of the overall reaction is quite low, the isomeric structure **35** cannot be rigorously excluded. The structure of the other byproduct **33** was also determined on the basis of its spectral data. The IR showed the absence of a carbonyl, ^{1}H NMR showed in addition to the three aromatic protons one proton at δ 14.93–14.55 that was exchangeable with D_2O, and the ^{19}F NMR displayed a triplet (3 F) at δ −7.28 ppm and a quartet (2 F) at δ −39.49 ppm upfield from trifluoroacetic acid.

In view of the complexity of the reaction of **19** with **30**, it was thought that reaction of **19** with 5-amino-6-(D-ribitylamino)pyrimidinedione (**3**) to obtain **11** might be difficult. It may also require protection of the side-chain hydroxyl groups. To avoid the extra protection–deprotection steps, attention was focused on the other reagent, methyl trifluoropyruvate (**21**).

Methyl trifluoropyruvate (**21**) was prepared from the ester, methyl 2-methoxy-2,3,3,3-tetrafluoropropionate, by hydrolysis in the presence of sulfuric acid.[27] This ester was obtained by alcoholysis of hexafluoropropene oxide (**19**) with methanol.[27] Methyl trifluoropyruvate (**21**) has been reported to yield 3-(trifluoromethyl)quinoxalone (**36**) on reaction with *o*-phenylenediamine, and it reacts with ethylenediamine to afford 3-hydroxy-3-(trifluoromethyl)-piperazin-2-one (**37**).[28]



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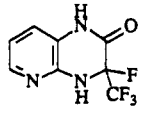

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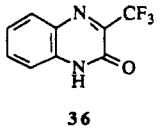

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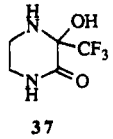

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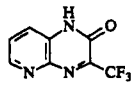
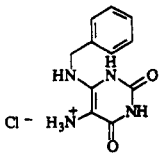
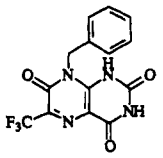
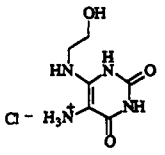
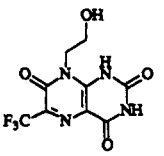
The products **26**, **28**, and **29** obtained from reaction of methyl trifluoropyruvate (**21**) with the corresponding diamines **25** and **27** (Table II) were identified by comparing spectral data with compounds obtained from the corresponding hexafluoropropene oxide reactions. Two products, **31** and **38**, were obtained from 2,3-diaminopyridine (**30**). The ratio of the two products was determined to be 55:45 by ^{19}F NMR. The major product **31** formed through the initial attack of the more reactive amino group of **30** on the ketone group of methyl trifluoropyruvate and then intramolecular cyclization involving attack of the less reactive amino group on the ester group. The structure of the more polar, minor product **38** has been confirmed by X-ray analysis. This also establishes the structure of **31**. Reactions involving diamine hydrochlorides **39** and **41** gave one product in each case. The structure of each product was assigned on the basis of ^{19}F NMR data. The ^{19}F NMR spectrum of **40** in DMSO-d_6 showed one signal at δ 9.67 ppm downfield from trifluoroacetic acid. If the trifluoromethyl group were located on the C-7 position, then it would be located on an sp^3-hybridized carbon atom and would appear upfield from trifluoroacetic acid, as in both diastereomers of **10**.[6,11,12] Similar arguments also apply for compound **42**.

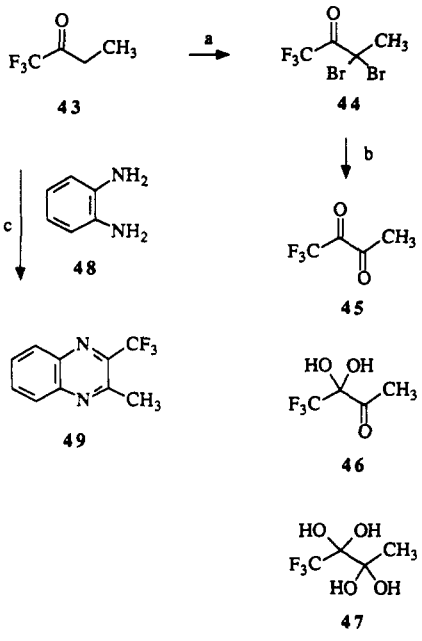
Table II. Reaction of Methyl Trifluoropyruvate with Ortho Diamines

reactant	solvent (time, temp)	product(s)	% yield
25	DMF (3 h, 80 °C)	**26**	98
27	DMF (3 h, 80 °C)	**28** **29**	54 12
30	DMF (5 h, 80 °C)	**31**	33
		 **38**	26
 **39**	DMF (3 h, 80 °C)	 **40**	32
 **41**	DMF (5 h, 80 °C)	 **42**	24

The synthesis of **11** was achieved by reacting 5 equiv of methyl trifluoropyruvate (**21**) with the hydrochloride salt of **3** in DMF at 80 °C for 7 h. All operations were performed in the dark. The ^{19}F NMR spectrum of **11** in phosphate buffer showed a singlet which shifted as a function of pH. The signal appeared at δ 6.88 ppm downfield from trifluoroacetic acid at pH 1 and gradually shifted to δ 7.80 as the pH was titrated to 4.9. Further increase in basicity did not result in a further downfield shift of the signal. This change in the chemical shift of the ^{19}F NMR signal is due to deprotonation of N-1, and the pK_a value of 2.5 indicated by titration monitored by ^{19}F NMR was in agreement with the value of 2.6 when the titration was monitored spectrophotometrically. For

(27) Knunyants, I. L.; Shokina, V. V.; Tyuleneva, V. V. *Dokl. Akad. Nauk. SSR* **1966**, *169*, 549.
(28) Saloutin, V. I.; Piterskih, I. A.; Pashkevich, K. I.; Kodess, M. I. *Izv. Akad. Nauk. SSSR, Ser. Khim.* **1983**, 2568.

Scheme II[a]



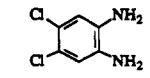
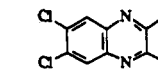
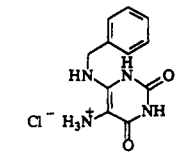
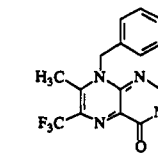
[a] Key: (a) Br_2, NaOAc, AcOH, $C_6H_5COOOCOC_6H_5$, CCl_4, hν, 23 °C (36 h); (b) Aq NaOAc, reflux (3 h); (c) SeO_2, water, heat (15 h).

comparison, the pK_a of the natural substrate 1 (deprotonation of the 7-methyl group to form 12) is about 8.5. It is therefore clear that in contrast to the natural substrate 1, we are dealing totally with the anion of 11 at physiological pH. This anion of 11 is a close structural analog of the anion 12 formed from 1 at the acceptor site of the enzyme.

Reactions of 1,1,1-Trifluoro-2,3-butanedione with Ortho Diamines. Synthesis of 6-(Trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (13). 1,1,1-Trifluoro-2,3-butanedione (45) was required for the synthesis of 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (13). Compound 45 can be prepared by hydrolysis of 3,3-dibromo-1,1,1-trifluoro-2-butanone (44) in aqueous sodium acetate. Intermediate 44 can in turn be prepared by bromination of 1,1,1-trifluoro-2-butanone (43) in sodium acetate–acetic acid.[29] Compound 45 can also be prepared by oxidation of 43 with selenium dioxide; however, the product has not been isolated in pure form. Its presence was implied by reaction of crude material with *o*-phenylenediamine (48) to give 2-methyl-3-(trifluoromethyl)quinoxaline (49).[29]

During the present study, an improved method was developed for the preparation of 44, which involved irradiation of 1,1,1-trifluoro-2-butanone (43) in the presence of *N*-bromosuccinimide.[30] Like trifluoropyruvaldehyde, 1,1,1-trifluoro-2,3-butanedione exists in aqueous solution principally as hydrates 46 and 47. The ^{19}F NMR spectrum of a solution obtained after aqueous sodium acetate hydrolysis of 3,3-dibromo-1,1,1-trifluoro-2-butanone showed one singlet at δ −2.7 ppm upfield from trifluoroacetic acid. Since the ^{19}F NMR chemical shift of the dihydrate of trifluoropyruvaldehyde lies at δ −2.5 ppm, it was assumed that 1,1,1-trifluoro-2,3-butanedione also exists principally as dihydrate 47 in aqueous solution.[31] Because the scope and regiochemistry of the reaction of trifluoropyruvaldehyde with ortho diamines have already been established and 1,1,1-trifluoro-2,3-butanedione was expected to give similar results, an extensive study of its reactions with ortho diamines did not seem necessary.[31] Only two diamines, one symmetrical (50) and one unsymmetrical (51), were used in the model study (Table III).

Table III. Reaction of a Solution of 1,1,1-Trifluorobutane-2,3-dione (39) and 1,1,1-Trifluorobutane-2,3-dione Hydrates (40 and 41) with Ortho Diamines

reactant	solvent (time, temp)	product(s)	% yield
 50	aq MeOH (1.5 h, 23 °C)	 51	83
 52	DMF (5 h, 80 °C)	 53	35

The reaction of 52 with 47 gave only one product. The structure 53 was determined on the basis of UV spectroscopy and ^{19}F NMR data. In aqueous HCl (pH 1), compound 53 shows absorbance at λ_{max} 400 (log ϵ 3.80) and 264 nm (4.00), while in aqueous NaOH (pH 13), it displays λ_{max} values at 390 (log ϵ 3.51), 299 (4.14), and 240 nm (4.16). These data are in agreement with structure 53 under acidic conditions and suggest that 53 undergoes some deprotonation to form a 7-exomethylene anion 54 related to the anion 12 formed at the acceptor site of the enzyme.[8] In deuterated methanol containing HCl, the ^{19}F NMR spectrum of 53 shows a singlet at δ 13.08 downfield from trifluoroacetic acid, while in the presence of sodium hydroxide the signal at δ 13.08 is replaced by one at 6.53 ppm, indicating conversion to the anion. The spectral data indicate that the trifluoromethyl group is located at C-6, since if it were located at C-7, the substance would be expected to exist as a covalent hydrate related to 10 and the trifluoromethyl group should therefore be expected to appear upfield from trifluoroacetic acid near δ −4.7, as the C-7 trifluoromethyl group does in the covalently hydrated lumazine 10.[12] Compound 53 was found to be unstable in solutions upon heating, and decomposition products were obtained when recrystallization was attempted.

One product isolated from the reaction of the hydrochloride of 3 with 45–47 was characterized as 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (13) on the basis of its ^{19}F NMR spectrum. In D_2O containing HCl (pH 1), the ^{19}F NMR spectrum of 13 showed a singlet at δ 12.86 downfield from trifluoroacetic acid (Figure 6A). In the pH range 6.9–11.0, three additional signals appeared at δ 10.97, 10.22, and 9.70 ppm, which may be assigned to monoanionic forms of the lumazine, corresponding to diastereomers of 14 or 15, or to the anion 16.[8] The two very minor singlets observed at δ 10.22 and 9.70 ppm under acidic pH (Figure 6A) may also represent cyclized forms in which the oxygens of the 2′- and/or 3′-hydroxyl groups of the side chain are bound covalently to C-7. The ^{19}F NMR data of 13 (Figure 6) confirm the location of the trifluoromethyl group at the 6-position. Otherwise, the trifluoromethyl group would be located on an sp^3-hybridized carbon in the anionic forms and would therefore appear upfield from trifluoroacetic acid near δ −4.7, as the C-7 trifluoromethyl group does in the covalently hydrated lumazine 10.[12] The results demonstrate that ^{19}F NMR is

(29) Belcher, R.; Sykes, A.; Tatlow, J. C. *J. Chem. Soc.* **1957**, 2393.
(30) Modari, B.; Kshoshdel, E. *J. Org. Chem.* **1977**, *42*, 3527.
(31) Cushman, M.; Patel, H.; McKenzie, A. *J. Org. Chem.* **1988**, *53*, 5088.

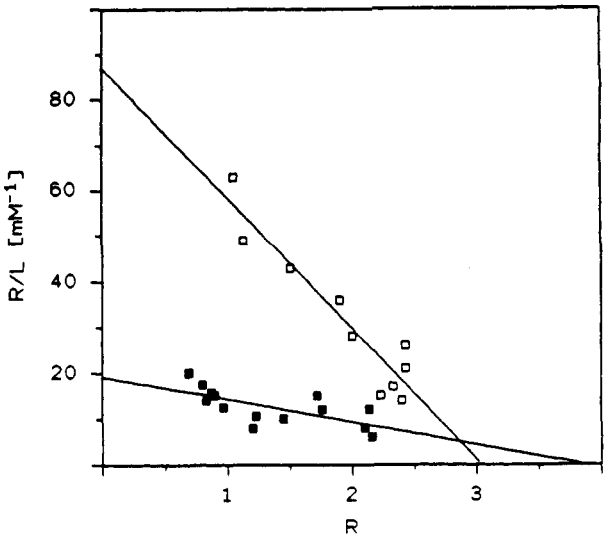


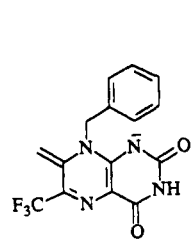
Figure 1. Binding of riboflavin to riboflavin synthase (Scatchard plot) at 24 °C. The protein concentration was 3 mg/mL: □, binding of riboflavin; ■, binding of riboflavin in the presence of 0.2 mM **56**.

indeed very sensitive to the local magnetic enviornment. An additional piece of evidence in support of structure **13** is that rapid exchange of the protons of the 7-methyl group was observed in D_2O. At pH 6.0, complete exchange was observed within 45 min. At higher pH, the exchange was so rapid that no signal for the C-7 methyl group was observed after about 5 min. This rapid exchange would only be expected if the methyl group were at C-7.[8] The deuterium exchange of the 7-methyl group also indicates that the anionic species **16** must be present to some extent. A careful titration in phosphate buffer, monitored spectrophotometrically, indicated that at pH 6.57 the ratio of neutral molecule to anions is 1:1.

A pK_a of 6.6 was determined by titration as monitored by ^{19}F NMR spectroscopy. In the pH range >11, a second equilibrium could be observed as two additional signals at δ 12.06 and 11.89 ppm appeared (Figure 6D). This may represent an equilibrium between the monoanion and the dianion with a pK_a of about 13. It is obvious that these anionic forms do not exchange rapidly on the NMR time scale.

Enzymatic Studies. Riboflavin synthase of *Bacillus subtilis* is a trimer of identical 24 kD_a subunits.[32,33] The protein has been sequenced and is characterized by extensive sequence homology between the N-terminal and the C-terminal parts.[33] The enzyme catalyzes a dismutation reaction involving the regiospecific transfer of a four-carbon unit between two molecules of **1**. This would require adjacent donor and acceptor sites. In line with this expectation, Otto and Bacher found that each enzyme subunit can bind two molecules of several lumazine derivatives studied.[9] Moreover, it has been shown that each subunit can bind one molecule of riboflavin or of the nitrosopyrimidine **56**, which is a close structural analog of the second enzyme product **3**.

However, it was unknown whether the flavin and the pyrimidine **56** bind to the same site or to different binding sites on the enzyme. We have addressed this question by equilibrium dialysis experiments in mixtures containing enzyme, riboflavin, and the pyrimidine **56**. Scatchard plots (Figure 1) indicated that the two different ligands can indeed bind simultaneously, each at a 1:1 stoichiometry (ligand/protein). The dissociation constant for riboflavin was 35 μM in the absence of the pyrimidine ligand, and 3.0 flavin molecules were bound per protein trimer. In the presence of 200 μM **56**, the enzyme bound one molecule of the pyrimidine per α subunit, irrespective of the flavin concentration. Moreover, the enzyme bound riboflavin with a dissociation constant of about 200 μM in the presence of 200 μM **56**. The ratio was 1.3 flavin molecules per subunit.



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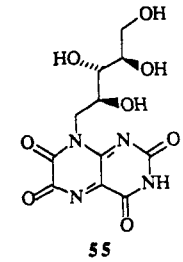

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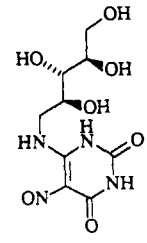


56

We conclude that each subunit can bind one molecule each of riboflavin and the pyrimidine. However, each of the ligands reduces the affinity of the protein for the second type of ligand. In agreement with Plaut's conventions, we will subsequently designate the binding site for riboflavin as the acceptor site and the site binding the pyrimidine as the donor site. This designation implies that a substrate molecule bound at the donor site can donate a 4C-moiety to a substrate molecule located at the acceptor site in the course of the enzyme-catalyzed dismutation.

6-(Trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine. The fluorolumazine **11** was found to be an inhibitor of the enzyme from *B. subtilis* with an inhibition constant K_I = 55 μM. Equilibrium dialysis experiments performed in 170 mM phosphate buffer, pH 6.8, at 4 °C gave a nonlinear Scatchard plot shown in Figure 2. The data indicate the binding of about six molecules of **11** per enzyme molecule. Since the enzyme contains three subunits, each with an acceptor site and a donor site, it follows that the fluorolumazine **11** binds at both the donor sites and the acceptor sites. The dissociation constants K_D were found to be approximately 4 μM for the first molecule bound per subunit and 112 μM for the second molecule bound per subunit.

^{19}F NMR spectra recorded during a titration of the enzyme with the fluorolumazine **11** are shown in Figure 3. Besides the signal for the free ligand at δ 7.8 ppm, there is a group of three signals extending from about 8 to 11 ppm downfield from sodium trifluoroacetate. An additional signal is displayed in the lower-field region at δ 16.0 ppm. These signals are very broad (line widths of 150–250 Hz). The signal of the free ligand is broadened to 130 Hz. This indicates an exchange of the enzyme bound ligand with the free ligand on the NMR time scale.

Quantitative evaluation of the NMR spectra obtained in titration experiments was performed by integration of the NMR signals. T_1 relaxation was determined for the

(32) Bacher, A.; Baur, R.; Eggers, U.; Harders, H.-D.; Otto, M. K.; Schnepple, H. *J. Biol. Chem.* **1980**, *255*, 632.
(33) Schott, K.; Kellerman, J.; Lottspeich, F.; Bacher, A. *J. Biol. Chem.* **1990**, *265*, 4204.

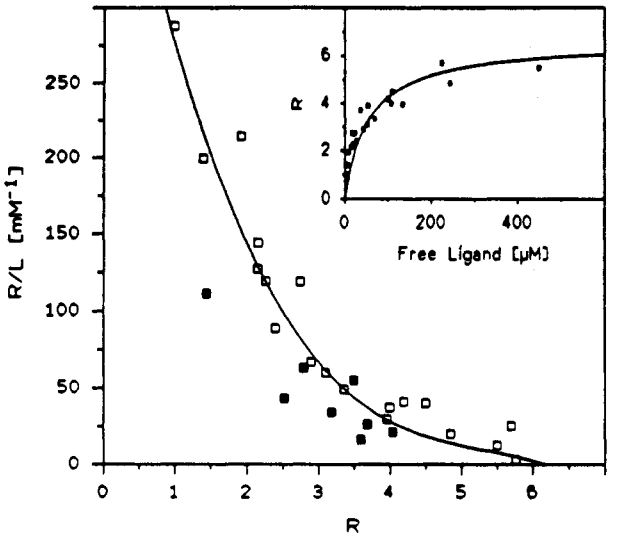


Figure 2. Scatchard plot of the binding of **11** by the light riboflavin synthase of *B. subtilis*. Data were obtained by equilibrium dialysis (□) (pH 6.8, 4 °C) and ^{19}F NMR spectroscopy (■) (pH 6.8, 24 °C). Inset: hyperbolic plot of the equilibrium dialysis binding data before transformation by Scatchard analysis. R is the number of ligand molecules bound per molecule of enzyme at equilibrium, and L is the free ligand concentration at equilibrium. The ^{19}F NMR contribution to the Scatchard analysis is based on NMR integrations of the signals attributed to bound and free ligand determined at different concentrations of the ligand.

signals of the bound and the free ligand by inversion recovery and was found in the range of 0.5 s. Spectral acquisition using 30° pulse with a pulse interval of 1 s should therefore give spectra without significant intensity distortions. Test experiments with longer pulse intervals gave the same quantitative results. The reproducibility of the NMR experiments with different enzyme batches was excellent.

The NMR spectra shown in Figure 3 gave a Scatchard plot (Figure 2) which was in close agreement with the results of the equilibrium dialysis study. The ^{19}F NMR data confirm the binding of two ligand molecules per protein subunit. We propose tentatively that the signals at higher and lower field reflect the binding of the ligand at the donor and the acceptor site. The three signals of the bound fluorolumazine **11** in the 8–11 ppm region resemble the low-field region in the ^{19}F NMR spectrum of bound **10**, where it corresponds to the 6-(trifluoromethyl) group.[12] The binding diastereomer of **10** binds to only one of the two binding sites per enzyme subunit, which is presumably the donor site.[12] This implies that the three higher field signals of the bound fluorolumazine **11** in Figure 3 correspond to ligand bound at the donor site.

The Scatchard data indicate that three ligand molecules are bound with K_D approximately 10 μM and another three ligand molecules are bound with K_D approximately 80 μM. However, the buildup of both signal groups for the bound ligand occurs in parallel, and there is no evidence indicating a higher affinity for either the high-field or low-field species. This will be discussed in more detail below.

All ^{19}F NMR signals attributed to protein-bound **11** gradually disappear during the titration of the enzyme ligand complex with 5-nitroso-6-(ribitylamino)-2,4-(1*H*,3*H*)-pyrimidinedione (**56**, Figure 4) or with riboflavin (Figure 5). The experiments described above indicated that riboflavin binds at the acceptor site and **56** binds at the donor site. Apparently, the fluorolumazine **11** can be displaced from both binding sites by ligands which can only bind to one of the respective sites.

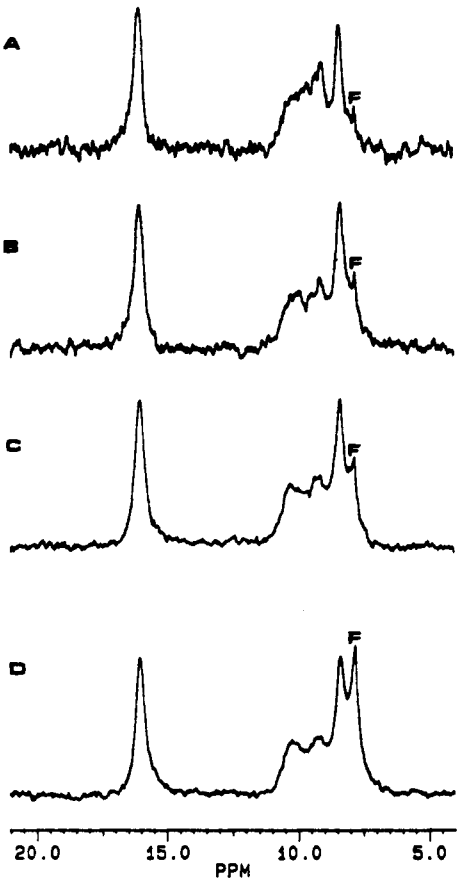


Figure 3. 338-MHz ^{19}F NMR spectra of the light riboflavin synthase of *B. subtilis* plus various concentrations of fluorolumazine **11** in 170 mM phosphate buffer pH 6.8 containing 10 mM sodium sulfite and 5% D_2O. The peak designated F is free ligand, and the remaining signals correspond to bound ligand: A, 410 μM fluorolumazine and 275 μM light riboflavin synthase; B, 790 μM fluorolumazine and 265 μM light riboflavin synthase; C, 970 μM fluorolumazine and 260 μM light riboflavin synthase; D, 1140 μM fluorolumazine and 255 μM light riboflavin synthase.

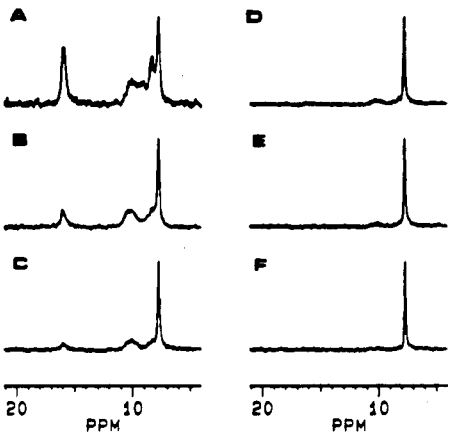


Figure 4. Displacement of bound fluorolumazine **11** from the enzyme by 5-nitroso-6-(ribitylamino)-2,4(1*H*,3*H*)pyrimidinedione (**56**). The experiment was performed in 170 mM phosphate buffer pH 6.8 containing 10 mM sodium sulfite and 5% D_2O at 24 °C. The complex was made using 150 μM light riboflavin synthase and 630 μM fluorolumazine: A, no **56** added; B, 135 μM **56**; C, 270 μM **56**; D, 530 μM **56**; E, 1 mM **56**; F, 4.5 mM **56**.

6-(Trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine. The fluorolumazine **13** inhibited the light riboflavin synthase of *B. subtilis*. The inhibition constant was determined to be 75 μM. Equilibrium dialysis experiments performed at 4 °C in phosphate buffer at pH 6.8 gave a nonlinear Scatchard plot as shown in Figure 7a. The data

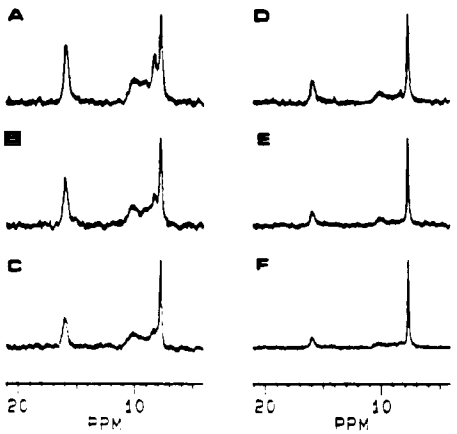


Figure 5. Displacement of bound fluorolumazine 11 from the enzyme by riboflavin (2). The experiment was performed in 170 mM phosphate buffer pH 6.8 containing 10 mM sodium sulfite and 5% D_2O at 24 °C. The complex was made using 150 μM light riboflavin synthase and 630 μM fluorolumazine: A, no riboflavin added; B, 20 μM riboflavin; C, 60 μM riboflavin; D, 90 μM riboflavin; E, 160 μM riboflavin; F, saturated with riboflavin (approximately 500 μM).

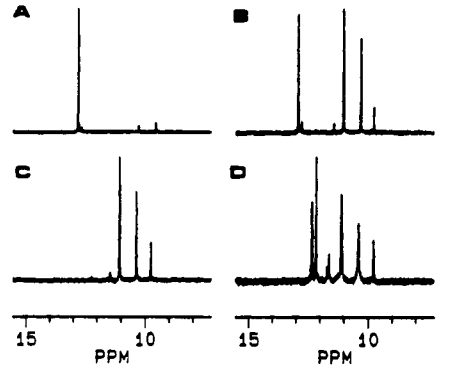


Figure 6. ^{19}F NMR spectrum of 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (13) at pH 1 (A), pH 6.9 (B), pH 11.0 (C), and pH 13 (D).

suggest that three ligand molecules per enzyme molecule can be bound relatively tightly with a K_D of about 17 μM. Additional ligand molecules can be bound weakly, but their number could not be determined unequivocally.

A series of ^{19}F NMR spectra was recorded during the titration of the light riboflavin synthase with 13 at 24 °C (Figure 8). The spectra show the signals of free neutral fluorolumazine at 12.9 ppm and the signals of three free anionic species at 10.9, 10.2, and 9.7 ppm. Additionally, two broad signals corresponding to bound ligand are displayed with maxima at 14.5 and 15.0 ppm. Small, broad signals at 7.3 and 8.5 ppm may also represent enzyme bound anionic forms of the fluorolumazine. The signals of the bound fluorolumazine 13 are broadened to a line width of about 130 Hz. The signals of the free anionic species have line widths between 10 and 14 Hz, whereas the signal corresponding to the free neutral molecule has a line width of 25 Hz. This suggests that the neutral free molecule is exchanging with the protein-bound form on the NMR time scale.

The Scatchard plot of the ^{19}F NMR monitored titration (Figure 7b) indicates a K_D of 70 μM and three bound molecules of 13 per enzyme molecule. By comparison, the equilibrium dialysis experiment indicated the tight binding of three ligand molecules and the additional weak binding of an undetermined number of additional molecules. If the weakly bound ligand molecules have the same chemical shift as the free ligand or if they are in rapid exchange with

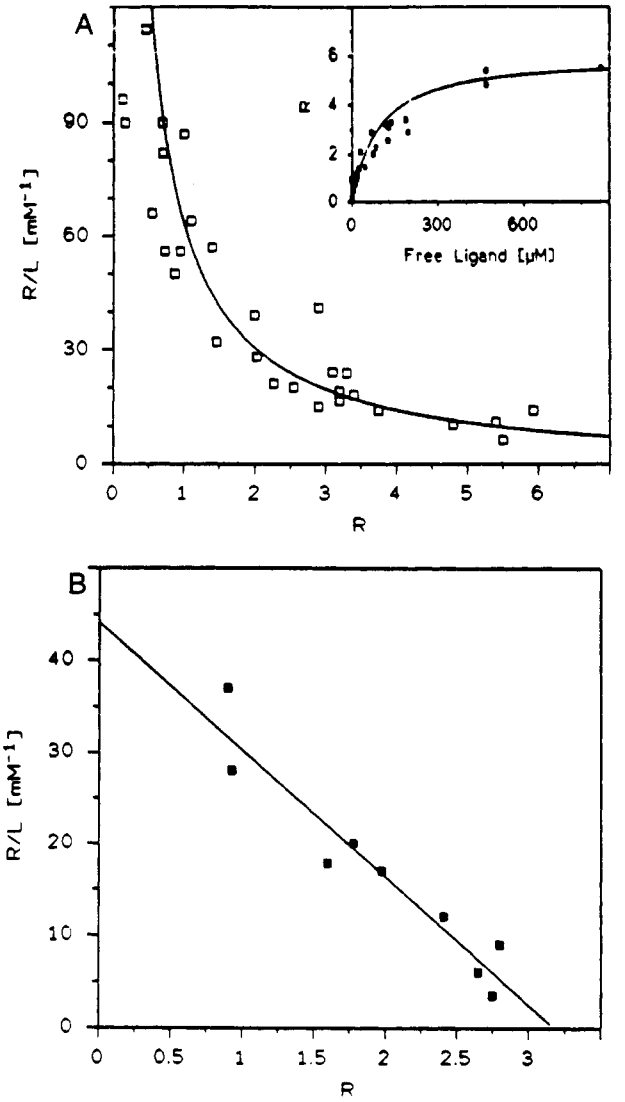


Figure 7. (A) Scatchard plot of the equilibrium dialysis of 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (13) and the light riboflavin synthase of *B. subtilis* in 170 mM phosphate buffer (pH 6.8) containing 10 mM sodium sulfite at 4 °C. Inset: hyperbolic plot of the equilibrium dialysis data before transformation by Scatchard analysis. (B) Scatchard plot of the ^{19}F NMR spectra of 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (13) and the light riboflavin synthase of *B. subtilis* in 170 mM phosphate buffer (pH 6.8) containing 10 mM sodium sulfite at 24 °C. R is the number of ligand molecules bound per 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.

the free ligand in solution, they would remain undetected in the ^{19}F NMR spectra.

Titration of the fluorolumazine-enzyme complex with riboflavin resulted in displacement of the bound lumazine (Figure 9). The failure of riboflavin to completely displace bound 13 from the enzyme may be due to the poor solubility of riboflavin.

The displacement of bound 13 by 5-nitroso-6-(ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione (56), which binds preferentially to the donor site of the enzyme, was also studied (Figure 10). At low concentrations of this product analog, the signals of bound 13 at 14.5 and 15.0 ppm were progressively broadened and shifted to lower field. Increasing the nitrosopyrimidine concentration further resulted in the disappearance of the ^{19}F NMR signals of the bound fluorolumazine. This behavior is similar to that

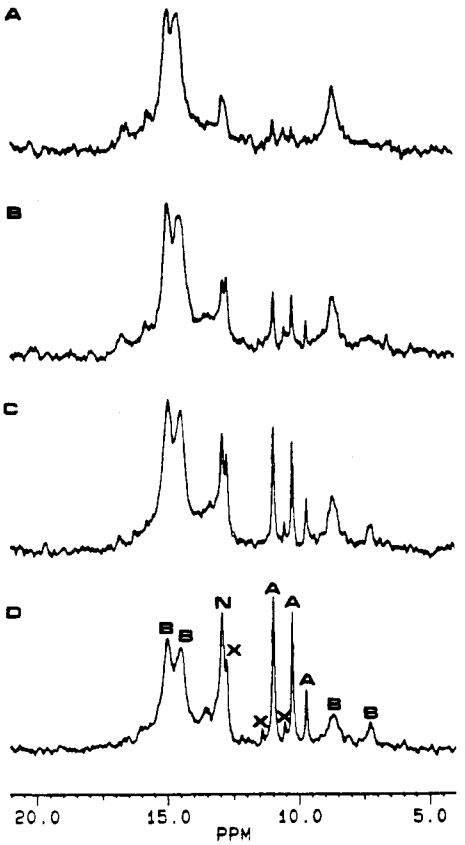


Figure 8. 338-MHz ^{19}F NMR spectra of the light riboflavin synthase of *B. subtilis* and various concentrations of the fluorolumazine **13** in 170 mM phosphate buffer, pH 6.8, containing 10 mM sodium sulfite at 24 °C. The spectra were measured with 16 K data points. The line broadening was 20 Hz. The peaks designated B are bound ligand, N is the free neutral, and A is the free anionic species. The signals designated X were minor impurities: (A) 330 μM fluorolumazine, 320 μM enzyme; (B) 640 μM fluorolumazine, 310 μM enzyme; (C) 935 μM fluorolumazine, 300 μM enzyme; (D) 1210 μM fluorolumazine, 293 μM enzyme.

Table IV. Interaction of Lumazine Derivatives with the Light Riboflavin Synthase of *Bacillus subtilis*

compd	K_I (μM)	K_D (μM)	mol bound/ enzyme
10 (epimer A)	120	13.0[a]	2.9[a]
		16.2[b]	3.1[b]
10 (epimer B)	500[c]	nbo[d]	0
11	55	4, 112[a]	6.2[a]
		10, 80[b]	5.5[b]
13	75	17[a]	>3[a]
		70[b]	3.2[b]
riboflavin	100[9]	35[e]	3.0[e]
riboflavin (in presence of 200 μM **56**)		200[e]	3.8[e]

[a] From equilibrium dialysis. [b] From ^{19}F NMR at 24 °C. [c] This inhibition constant is an artifact due to the presence of a minor impurity. Since **10** (epimer B) does not bind to the enzyme, it is unlikely that it inhibits the enzyme. [d] nbo: no binding observed. [e] From equilibrium dialysis at 24 °C.

observed with the fluorolumazine **11**.

For comparison, the dissociation constants, inhibition constants, and stoichiometries of binding of both diastereomers of **10**,[12] the 7-oxofluorolumazine **11**, and the 7-methylfluorolumazine **13** are listed together in Table IV.

Discussion

The xylene ring of riboflavin is biosynthesized in an unusual way by a dismutation of **1** which involves the transfer of a 4-carbon moiety. The byproduct of the reaction, the pyrimidine **3**, is the biosynthetic precursor of **1** and can be returned to the biosynthetic pathway. The reaction catalyzed by riboflavin synthase appears mechanistically complex, and it is surprising that the reaction can proceed nonenzymatically. However, as shown by Wood and his co-workers, riboflavin can be obtained by boiling **1** in phosphate buffer at neutral pH.[34,35] Later, it was shown by Plaut and Beach that the nonenzymatic reaction can also proceed under acidic conditions.[36]

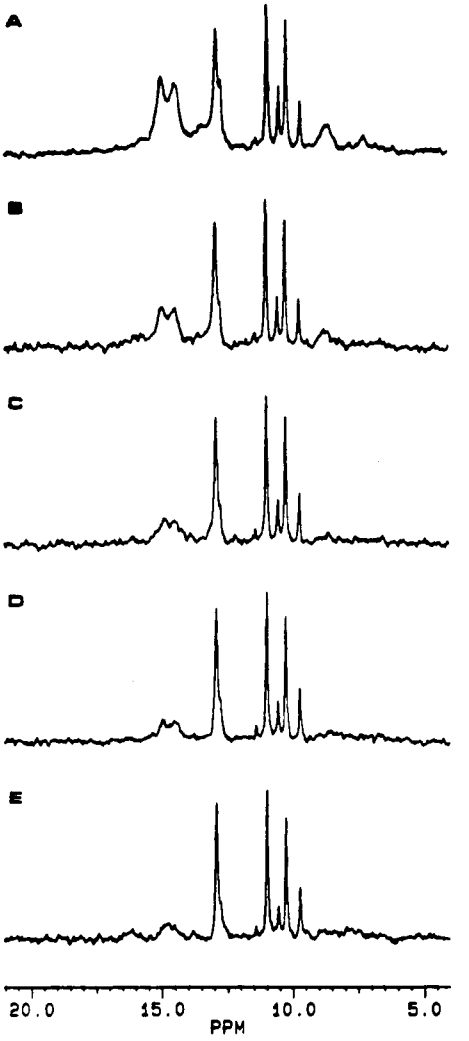


Figure 9. Titration of the complex between fluorolumazine **13** and the light riboflavin synthase of *B. subtilis* with riboflavin (**2**). The concentration of the enzyme was 180 μM, and the concentration of **13** was 800 μM: (A) no riboflavin; (B) 175 μM riboflavin; (C) 285 μM riboflavin; (D) 380 μM riboflavin; (E) saturated with riboflavin (about 570 μM).

Plaut and his co-workers studied riboflavin synthase from baker's yeast in considerable detail.[37] A wide variety of substrate analogs was tested for substrate or inhibitor activity. These authors suggested that the proposed dismutation mechanism required the binding of two substrate molecules in close proximity, but a qualitative determination of the binding isotherms was not performed.

Later, Otto and Bacher found that the enzyme from *B. subtilis* is a trimer and can bind one molecule of riboflavin or of the pyrimidine **56** per protomer.[9] It remained unknown whether these compounds were bound at the same or different sites. Moreover, it was shown that several

(34) Rowan, T.; Wood, H. S. C. *Proc. Chem. Soc.* **1963**, 21.
(35) Rowan, T.; Wood, H. S. C. *J. Chem. Soc.* **1968**, 452.
(36) Beach, R.; Plaut, G. W. E. *Tetrahedron Lett.* **1969**, 3489.
(37) Harvey, R. A.; Plaut, G. W. E. *J. Biol. Chem.* **1966**, *241*, 2120.

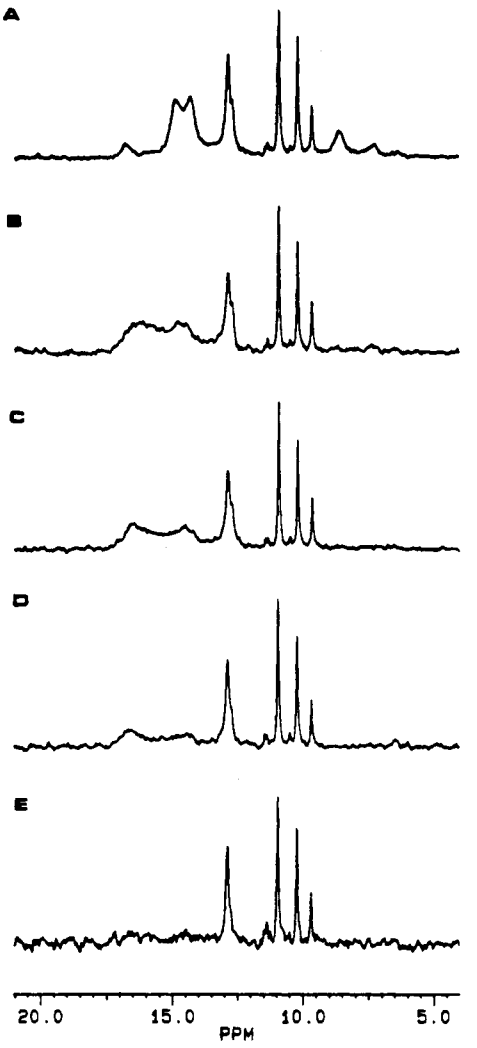


Figure 10. Titration of the complex between fluorolumazine **13** and the light riboflavin synthase of *B. subtilis* with 5-nitroso-6-(D-ribitylamino)pyrimidine-2,4(1*H*,3*H*)-dione (**56**). The concentration of enzyme was 123–95 μM, and the concentration of **13** was 500–400 μM: (A) no **56**; (B) 120 μM **56**; (C) 239 μM **56**; (D) 700 μM **56**; (E) 1300 μM **56**.

lumazine analogs could bind with a stoichiometry of 2:1 (ligand per protomer). Nonlinear Scatchard plots were found in these cases.

We have now shown that riboflavin and the pyrimidine **56** can bind simultaneously at each protein subunit. These findings suggest that riboflavin can bind to the acceptor site but not to the donor site. Vice versa, the pyrimidine can bind to the donor site but not to the acceptor site. Moreover, the affinity of the donor site for the pyrimidine is reduced by the presence of the riboflavin at the acceptor site, and the affinity of the acceptor for riboflavin is reduced by the presence of the pyrimidine. This may indicate some steric hindrance if the riboflavin and the pyrimidine are bound in close proximity at their respective sites.

Earlier studies had shown that several substrate analogs including 7-methyl-8-ribityllumazine and 6,7-dioxo-8-ribityllumazine (**55**) bind at a stoichiometry of 2:1 (ligand/subunit).[9] Generally, lumazine analogs with oxo groups in position 7 bind tightly to the enzyme, and it has been proposed that they represent transition state analogs mimicking the structure of the proposed intermediate **12**.[14]

The present data show that the affinity of the enzyme for 7-oxo type compounds is significantly reduced by the introduction of the trifluoromethyl group in the 6-position. A relatively low affinity (K_D = 13 μM) for riboflavin synthase has also been observed earlier with the binding diastereomer of 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrate (**10**).[12] Thus, it appears that trifluoromethyl substitution of the pteridine ring may be generally unfavorable for binding to riboflavin synthase.

Similar to the results with **55**, the trifluoromethyl derivative **11** binds at a stoichiometry of 2:1 (ligand/protomer). The Scatchard plot (Figure 2) for binding of the compound is significantly curved and suggests dissociation constants of 4 and 112 μM for the binding of the first and second ligand molecules bound per subunit whereas the K_D of **55** was 1.6 μM.[9] The NMR spectrum of the protein-bound ligand is complex and consists of a group of three signals with a downfield shift of about 8–11 ppm and an additional signal with a downfield shift of about 16 ppm. The group of signals at higher field resembles the NMR signature of the 6-(trifluoromethyl) group of **10**. In light of the 2:1 binding stoichiometry of **11** it appears plausible that the high- and low-field signals could stem from molecules bound at the donor and acceptor sites, respectively.

On the basis of the Scatchard plot one might conclude that the donor and the acceptor site of the protein have different affinities for the ligand **11**. Contrary to this expectation, the titration experiment in Figure 2 indicates that the signals at 8–11 and 16 ppm increase in parallel upon addition of the ligand. This finding can be explained if we assume that the affinities of the donor and acceptor sites have approximately the same value in the ligand-free protein, thus permitting the binding of **11** to the donor or acceptor site with similar probability. However, if one site is already occupied, the affinity of the second site is reduced by steric interference. This behavior is in parallel with the binding of the products where each product, when bound, was shown to reduce the affinity of the protein for the other product.

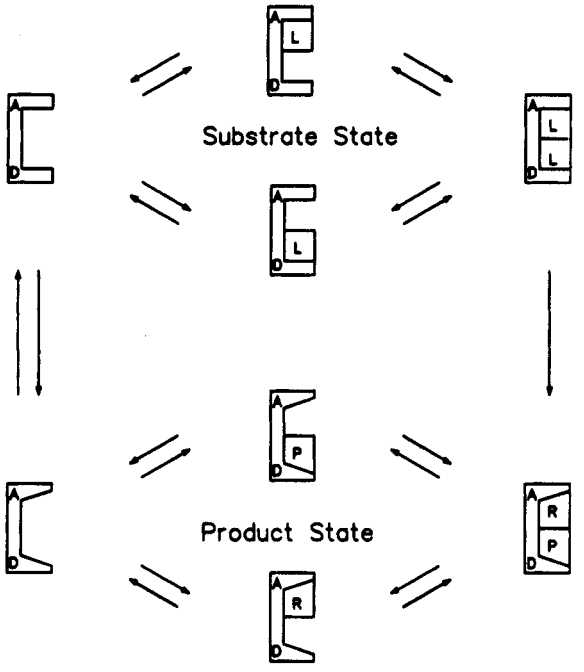
All bound substrate analogs studied by ^{19}F NMR spectroscopy can be displaced from the enzyme either by riboflavin or by an analog of the pyrimidine product. In other words, the product-type compounds can displace substrate analogs from the donor and the acceptor site although they bind only to one respective site.

This can be explained if we assume that riboflavin synthase can exist in two conformational states. In one state, the protein can accept one or two substrate molecules per monomer, but interacts poorly with the products. We will subsequently designate this state as substrate state. In the other conformational state, subsequently designated as product state, the enzyme can bind one flavin and/or one pyrimidine type ligand but interacts poorly with the substrate.

In the framework of this hypothesis, the catalytic cycle of riboflavin can be described as follows (Scheme III). The unliganded enzyme can adopt two different conformations, the substrate state and the product state, which are indicated by symbols. An approaching substrate molecule can either bind to the donor or acceptor site, which have similar affinities. Although the affinity of the other site is somewhat lowered, a second lumazine substrate can bind to the enzyme which is now ready for catalytic formation of products. The flavin or the pyrimidine product can be released, and the remaining product is released subsequently. Binding of new substrates is only possible after both product molecules have been released.

Up to now, we have only discussed the interaction of one protein subunit with substrate and products. However, the enzyme is a trimer of identical subunits, and this may

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.

complicate the overall picture. Thus, we have seen that the NMR signal of the bound **13** is shifted to lower field upon titration of the complex with **56** (Figure 10). This could imply that one of the subunits has adopted the product state by binding **56**. The other subunits may still be in the substrate state with bound lumazine, but the bound lumazine is influenced by the conformational state of the subunit which binds **56**.

Edman sequencing of riboflavin synthase indicated a C-terminal sequence which was shorter by 13 amino acids than the corresponding gene sequence.[33] This may indicate that the C-terminus of the protein has been modified by proteolytic processing. Attempts to check the protein for microheterogeneity by ion spray mass spectrometry were unsuccessful. However, the NMR results were highly reproducible with different enzyme batches, and it appears unlikely that the multiplicity of signals observed is a consequence of variable proteolytic processing of subunits.

The three-dimensional structure of riboflavin synthase is not known. In particular, it is unknown whether the protein has C_3 symmetry. If the structure is nonsymmetric, the multiplicity of ^{19}F NMR signals could be a result of positional nonequivalence of the protein subunits.

Experimental Section

All reactions were performed under nitrogen atmosphere. Analytical thin-layer chromatography was performed on Baker-flex silica gel 1B2-F sheets or EM Science silica gel $60F_{254}$ glass plates. Microanalyses were obtained from the Purdue Microanalytical Laboratory. The mass spectra were determined using an ionization potential of 70 eV. The chemical ionization mass spectra (CIMS) were obtained by using methane, 2-methylpropane, or ammonia as the reagent gas, as noted. *N,N*-Dimethylformamide was distilled from calcium hydride. For analytical and preparative HPLC work, distilled water and HPLC-grade acetonitrile and methanol were used.

6,7-Dimethyl-3-(trifluoromethyl)-2(1*H*)-quinoxalinone (26). A mixture of 4,5-dimethyl-*o*-phenylenediamine (**25**, 0.96 g, 7.1 mmol) and sodium bicarbonate (1.8 g, 21.4 mmol) in dichloromethane (30 mL) was allowed to react with hexafluoropropene oxide (**19**, 1.792 g, 10.79 mmol) in diethyl ether (5 mL) for 12 h at room temperature in a pressure bottle. The crude product (1.961 g) was filtered and washed with water (20 mL). Recrystallization of the crude product from ethyl acetate–hexane afforded compound **26** (1.326 g, 78%) as white crystals: mp 273–274 °C; UV (methanol) λ_{max} 373 (log ϵ 3.80), 301 (3.88), 233 nm (4.30); IR (KBr) 3600–3250, 3200–2700, 1660, 1620, 1540, 1480, 1435, 1385, 1350, 1290, 1210, 1175, 1155, 1130, 1040, 900, 870, 840, 730, 680 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 13.06–12.82 (br s, exchangeable with D_2O), 7.70 (s, 1 H), 7.16 (s, 1 H), 2.36 (s, 3 H), 2.31 (s, 3 H); ^{19}F NMR (DMSO-d_6) δ 10.6 (s, 3 F); CIMS (NH_3 ionizing gas) m/z (relative intensity) 260 (M^+ + 18, 100), 243 (M^+ + 1, 28). Anal. Calcd for $C_{11}H_9F_3N_2O$: C, 54.53; H, 3.75; F, 23.55; N, 11.57. Found: C, 54.60; H, 3.55; F, 23.43; N, 11.26.

6-Benzoyl-3-(trifluoromethyl)-2(1*H*)-quinoxalinone (28). A mixture of 3,4-diaminobenzophenone (**27**, 2.123 g, 10 mmol) and sodium bicarbonate (2.54 g, 30 mmol) in diethyl ether (50 mL) was allowed to react with hexafluoropropene oxide (**19**, 1.8 g, 10.8 mmol) in diethyl ether (10 mL) for 12 h at room temperature in a pressure bottle. The white solid product (3.1 g) containing **28** and **29** was filtered, washed with water (20 mL), and dried. The ratio of products was estimated to be 86:14 by ^{19}F NMR. The mixture was then chromatographed on a column of silica gel (150 g, 60–200 mesh, 4.1 × 30 cm), eluting with ethyl acetate–hexane (3:17) to afford **29** (392 mg, 13%). Elution of this column with ethyl acetate–hexane (1:3) then afforded **28** (2.139 g, 71%). The analytical sample of **28** was recrystallized from ethyl acetate–hexane: mp 253–255 °C; UV (methanol) λ_{max} 359 (log ϵ 3.86), 269 nm (4.60); IR (KBr) 3200, 3140, 1700, 1650, 1600, 1485, 1465, 1435, 1345, 1310, 1300, 1290, 1270, 1245, 1215, 1175, 1160, 1140, 1120, 1040, 980, 915, 895, 845, 810, 770, 730, 710, 685, 620 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 13.35 (s, 1 H, exchangeable with D_2O), 8.11–8.09 (m, 2 H), 7.78–7.75 (m, 2 H), 7.72–7.68 (m, 1 H), 7.61–7.57 (m, 2 H), 7.52 (d, 1 H, J = 9 Hz); ^{19}F NMR ($CDCl_3$–DMSO-d_6) δ 6.15 (s, 3 F); CIMS (NH_3 ionizing gas) m/z (relative intensity) 336 (M^+ + 18, 2), 319 (M^+ + 1, 100), 301 (M^+ − 17, 5). Anal. Calcd for $C_{16}H_9F_3N_2O_2$: C, 60.37; H, 2.85; F, 17.92; N, 8.80. Found: C, 60.15; H, 2.58; F, 17.95; N, 8.54.

7-Benzoyl-3-(trifluoromethyl)-2(1*H*)-quinoxalinone (29). Compound **29** was obtained as described above, and the analytical sample was recrystallized from ethyl acetate–hexane: mp 280–282 °C; UV (methanol) λ_{max} 374 (log ϵ 3.74), 282 (4.18), 230 nm (4.51); IR (KBr) 3220, 3200, 3040, 1705, 1650, 1620, 1600, 1570, 1450, 1390, 1350, 1320, 1310, 1290, 1270, 1240, 1220, 1190, 1160, 1130, 1050, 995, 985, 905, 880, 840, 825, 800, 740, 710, 690, 680, 630 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 13.13 (s, 1 H, exchangeable with D_2O), 8.07 (d, 1 H, J = 8 Hz), 7.80–7.77 (m, 2 H), 7.75–7.71 (m, 1 H), 7.69 (d, 1 H, J = 2 Hz), 7.67 (dd, 1 H, J = 8, 2 Hz), 7.62–7.58 (m, 2 H); ^{19}F NMR ($CDCl_3$–DMSO-d_6) δ 6.02 (s, 3 F); CIMS (i-C_4H_{10} ionizing gas) m/z (relative intensity) 319 (M^+ + 1, 100), 301 (M^+ − 17, 11). Anal. Calcd for $C_{16}H_9F_3N_2O$: C, 60.37; H, 2.85; F, 17.92; N, 8.80. Found: C, 60.34; H, 2.69; F, 17.55; N, 8.73.

Reaction of Hexafluoropropene Oxide (19) with Pyridine-2,3-diamine (30) To Afford 31, 32, and 33. Hexafluoropropene oxide (**19**, 1.79 g, 10.8 mmol) in diethyl ether (5 mL) was added to a mixture of pyridine-2,3-diamine (**30**, 1.09 g, 10 mmol) and sodium bicarbonate (2.54 g, 30 mmol) in dichloromethane (50 mL). The mixture was stirred at room temperature for 12 h in a pressure bottle. The brown solid product (450 mg) which contained **31**, **32**, and **33** was filtered, washed with water (5 mL), and dried. Removal of the solvent from the filtrate under vacuum afforded **32** (616 mg, 26%) as the only product. The solid containing **31**, **32**, and **33** was chromatographed on a column of silica gel (20 g, 60–200 mesh, 2 × 16 cm). Elution with hexane–ethyl acetate (5:1) afforded the least polar component **33** (143 mg, 6%). Further elution with hexane–ethyl acetate (4:1) gave **31** (47 mg, 3%) and with hexane-ethyl acetate (3:1) afforded **32** (119 mg, 5%, total yield 31%).

2-(Trifluoromethyl)pyrido[2,3-*b*]pyrazin-3-one (31). This compound was found to be identical to product **31** prepared by reaction of pyridine-2,3-diamine (**30**) and methyl trifluoropyruvate (**21**).

2-Fluoro-2-(trifluoromethyl)-2,3-dihydropyrido[2,3-*b*]-pyrazin-3-one (32). The analytical sample of **32** was obtained by recrystallizing the crude material obtained above from dichloromethane to give yellow needles: mp 191–193 °C; UV

(methanol) λ_{max} 419 (log ϵ 3.94), 276 nm (3.81); IR (KBr) 3410, 3300, 1715, 1625, 1610, 1560, 1495, 1430, 1340, 1355, 1270, 1200, 1180, 1165, 1110, 1070, 1010, 980, 890, 725, 690 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.64 (dd, 1 H, J = 1 and 7 Hz), 7.00 (dd, 1 H, J = 1 and 8 Hz), 6.86 (dd, 1 H, J = 7 and 8 Hz), 6.05 (s, 1 H, exchangeable with D_2O), 6.04 (s, 1 H, exchangeable with D_2O); ^{19}F NMR ($CDCl_3$) δ -4.73 (d, 3 F, J = 9 Hz), -77.30 (q, 1 F, J = 9 Hz); CIMS (i-C_4H_{10} ionizing gas) m/z (relative intensity) 237 (M^+ + 2, 8), 236 (M^+ + 1, 100), 218 (M^+ - 17, 4); high-resolution CIMS (i-C_4H_{10} ionizing gas) calcd for $C_8H_6F_4N_3O$ m/z 236.0447 (M^+ + 1), found 236.0416.

2-(Pentafluoroethyl)pyrido[2,3-*b*]imidazole (33). The analytical sample of **33** was obtained by recrystallizing the crude material obtained above from ethyl acetate–hexane: mp 178–180 °C; UV (methanol) λ_{max} 290 (log ϵ 3.93), 284 (3.96), 246 nm (3.51); IR (KBr) 3000–2600, 1585, 1490, 1450, 1410, 1380, 1335, 1315, 1290, 1270, 1210, 1175, 1150, 1045, 1035, 1025, 920, 800, 790, 780, 735, 700 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 14.93–14.55 (br s, 1 H, exchangeable with D_2O), 8.56 (d, 1 H, J = 5 Hz), 8.30 (d, 1 H, J = 8 Hz), 7.45 (dd, 1 H, J = 5 and 8 Hz); ^{19}F NMR (DMSO-d_6) δ -7.28 (t, 3 F, J = 3 Hz), -39.49 (q, 2 F, J = 3 Hz); CIMS (i-C_4H_{10} ionizing gas) m/z (relative intensity) 239 (M^+ + 2, 9), 238 (M^+ + 1, 100); high-resolution CIMS (i-C_4H_{10} ionizing gas) calcd for $C_8H_5F_5N_3$ m/z 238.0404 (M^+ + 1), found 238.0400.

Reaction of Methyl Trifluoropyruvate (21) with 4,5-Dimethyl-*o*-phenylenediamine (25) To Afford 26. Methyl trifluoropyruvate (**21**, 393 mg, 2.5 mmol) was added to a solution of 4,5-dimethyl-*o*-phenylenediamine (**25**, 272 mg, 2 mmol) in DMF (5 mL). The mixture was heated at 80 °C for 3 h before the solvent was removed under vacuum on a rotary evaporator (bath 60 °C). The brown solid product was then chromatographed on a silica gel column (20 g, 60–200 mesh, 2 × 17 cm), eluting with ethyl acetate–hexane (1:4). Evaporation of the fractions containing **26** gave this substance as a light brown solid (473 mg, 98%). This compound was identical to compound **26** prepared by reaction of 4,5-dimethyl-*o*-phenylenediamine and hexafluoropropene oxide.

Reaction of Methyl Trifluoropyruvate (21) with 3,4-Diaminobenzophenone (27). Methyl trifluoropyruvate (**21**, 638 mg, 4 mmol) was added to a solution of 3,4-diaminobenzophenone (**27**, 636 mg, 3 mmol) in DMF (4 mL). The mixture was heated at 80 °C for 4 h before the solvent was removed under vacuum on a rotary evaporator (bath 70 °C). The ratio of the two products was estimated by ^{19}F NMR to be 83:17. The crude product was chromatographed on a column of silica gel (50 g, 60–200 mesh, 3 × 25 cm). Elution with ethyl acetate–hexane (3:17) afforded the less polar minor component (**29**, 12%) and with ethyl acetate–hexane (1:3), the more polar component (**28**, 467 mg, 54%). The compounds were found to be identical with the corresponding compounds prepared by reaction of hexafluoropropene oxide and **27**.

Reaction of Methyl Trifluoropyruvate (21) with 2,3-Diaminopyridine (30) To Afford 2-(Trifluoromethyl)pyrido[2,3-*b*]pyrazin-3-one (31). Methyl trifluoropyruvate (**21**, 1.03 g, 6.6 mmol) in DMF (2.5 mL) was added to a solution of 2,3-diaminopyridine (**30**, 545 mg, 5 mmol) in DMF (2 mL). The mixture was heated at 80 °C for 5 h before the solvent was removed under vacuum on a rotary evaporator (bath 60 °C). The ratio of products was estimated to be 55:45 by ^{19}F NMR. The crude product was chromatographed on a column of silica gel (20 g, 60–200 mesh, 2 × 23 cm), eluting with ethyl acetate–hexane (3:7), to afford the less polar compound **31** (351 mg, 33%). Further elution of the column with ethyl acetate–hexane (1:1) gave the more polar compound **38** (278 mg, 26%). The analytical sample of **31** was recrystallized from ethyl acetate–hexane: mp 250–251.5 °C; UV (methanol) λ_{max} 351 (log ϵ 3.91), 223 nm (4.40); IR (KBr) 3000, 2940, 2880, 2800, 1695, 1600, 1570, 1500, 1425, 1350, 1300, 1220, 1210, 1175, 1150, 1115, 1060, 1030, 900, 830, 800, 775, 750, 720 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.76–13.28 (br s, 1 H, exchangeable with D_2O), 8.70 (dd, 1 H, J = 2 and 5 Hz), 8.38 (dd, 1 H, J = 2 and 8 Hz), 7.48 (dd, J = 5 and 8 Hz); ^{19}F NMR ($CDCl_3$–DMSO-d_6) δ 6.34 (s, 3 F); CIMS (i-C_4H_{10} ionizing gas) m/z (relative intensity) 216 (M^+ + 1, 100). Anal. Calcd for $C_8H_4F_3N_3O$: C, 44.65; H, 1.88; F, 26.51; N, 19.54. Found: C, 44.46; H, 1.68; F, 26.14; N, 19.75.

3-(Trifluoromethyl)pyrido[2,3-*b*]pyrazin-2-one (38). The more polar compound **38** from above was recrystallized from ethyl acetate–methanol: mp 280–282 °C; UV (methanol) λ_{max} 359 (log ϵ 3.84), 227 nm (4.33); IR (KBr) 3000, 2930–2700, 1695, 1580, 1490, 1455, 1350, 1300, 1240, 1200, 1170, 1150, 1050, 1030, 920, 890, 820, 800, 740, 730, 630 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.18 (s, 1 H, exchangeable with D_2O), 8.64 (dd, 1 H, J = 2 and 4 Hz), 7.80 (dd, 1 H, J = 2 and 8 Hz), 7.71 (dd, J = 4 and 8 Hz); ^{19}F NMR ($CDCl_3$–DMSO-d_6) δ 5.82 (s, 3 F); CIMS (i-C_4H_{10} ionizing gas) m/z (relative intensity) 217 (M^+ + 2, 10), 216 (M^+ + 1, 100). Anal. Calcd for $C_8H_4F_3N_3O$: C, 44.65; H, 1.88; F, 26.51; N, 19.54. Found: C, 44.47; H, 1.62; F, 26.28; N, 19.68.

8-Benzyl-7-oxo-6-(trifluoromethyl)lumazine (40). 5-Amino-4-(benzylamino)uracil hydrochloride (**39**, 340 mg, 2.2 mmol) was suspended in DMF (7 mL) in a 25-mL two-necked flask fitted with a reflux condenser. Upon addition of triethylamine (202 mg, 2 mmol), the solid went into the solution. Methyl trifluoropyruvate (**21**, 340 mg, 2.2 mmol) was added, and the mixture was heated at 80 °C for 3 h. The reaction mixture was concentrated to a small volume (1 mL) under vacuum (0.5 mm Hg) and triturated with cold water (10 mL). The yellow product was filtered and washed with water (5 mL). Recrystallization of the crude product from benzene–methanol afforded compound **40** (260 mg, 32%): mp 247–249 °C dec; UV (aqueous HCl, pH 1) λ_{max} 343 (log ϵ 4.01), 297 (3.50), 272 nm (3.99); UV (aqueous NaOH, pH 13) λ_{max} 374 (log ϵ 4.15), 264 nm (4.10); IR (KBr) 3600–2800, 1710, 1680, 1500, 1440, 1170, 1130, 1020, 850, 690 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 11.62 (s, 1 H, exchangeable with D_2O), 7.44–7.27 (m, 5 H), 5.34 (s, 2 H); ^{19}F NMR (DMSO-d_6) δ 9.68 (s, 3 F); CIMS (i-C_4H_{10} ionizing gas) m/z (relative intensity) 340 (M^+ + 2, 16), 339 (M^+ + 1, 100); high-resolution CIMS (i-C_4H_{10} ionizing gas) calcd for $C_{14}H_{10}F_3N_4O_3$ m/z 339.0705 (M^+ + 1), found 339.0702.

8-(2-Hydroxyethyl)-7-oxo-6-(trifluoromethyl)lumazine (42). 5-Amino-4-[(2-hydroxyethyl)amino]uracil hydrochloride (**41**, 444 mg, 2 mmol) was suspended in DMF (6 mL) in a 25-mL two-necked flask connected with a condenser, and triethylamine (202 mg, 2 mmol) was added. Methyl trifluoropyruvate (**21**, 340 mg, 2.2 mmol) was added, and the mixture was heated at 80 °C for 5 h. The solvent was removed under vacuum (0.5 mmHg). The brown semisolid was dissolved in water (15 mL) and applied to a column of alumina (Sigma, neutral, type WN-6, activity-grade super 1, 100 g, 3.7 × 10.5 cm), eluting with water. Evaporation of the solvent from fractions containing **42** gave light yellow solid (141 mg, 24%). The analytical sample was recrystallized from water: mp >300 °C; UV (aqueous HCl, pH 1) λ_{max} 335 (log ϵ 4.14), 270 (3.98), 248 nm (3.90); UV (aqueous NaOH, pH 13) λ_{max} 347 (log ϵ 3.98), 254 nm (3.91); IR (KBr) 3600–2800, 1730, 1700, 1650, 1575, 1520, 1420, 1340, 1320, 1180, 1130, 1110, 1030, 980, 800, 690 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 11.69 (s, 1 H, exchangeable with D_2O), 4.40–4.36 (m, 2 H), 4.23–4.19 (m, 2 H); ^{19}F NMR (DMSO-d_6) δ 9.67 (s, 3 F); CIMS (i-C_4H_{10} ionizing gas) m/z (relative intensity) 293 (M^+ + 1, 18), 275 (M^+ - 17, 100); high-resolution CIMS (i-C_4H_{10} ionizing gas) calcd for $C_9H_8F_3N_4O_4$ m/z 293.0498 (M^+ + 1), found 293.0503.

6-(Trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine (11). Triethylamine (404 mg, 4 mmol) and methyl trifluoropyruvate (**21**, 2.34 g, 15 mmol) in DMF (7 mL) were added to a flask containing 5-amino-4-(D-ribitylamino)-2,4-(1*H*,3*H*)-pyrimidinedione hydrochloride (**3**•**HCl**, 936 mg, 3 mmol). The reaction mixture was heated at 80 °C in the dark for 7 h before the solvent was removed under vacuum (0.5 mmHg) at room temperature. The semisolid, dark red material was dissolved in water (7 mL), ethanol (70 mL) was added, and the mixture was refrigerated for 7 h before the solid impurities were filtered off. The filtrate was concentrated on a rotary evaporator (bath 40 °C) to afford a semisolid mass. It was dissolved in an ammonium acetate buffer (10 mL, 0.1 M, pH 8.34) and applied to a column of anion exchange resin (Biorad, AG-1-X2, acetate form, 4 g, 2 × 5 cm). The column was eluted with ammonium acetate buffer (0.1 M, pH 8.34). Fractions (6 mL) were collected and analyzed by analytical HPLC (μ-Bondapak C-18, 10 μM, 3.9 × 300 mm, detecting at 270 nm), eluting with 0.1% trifluoroacetic acid in 7% acetonitrile. Fractions 23–60 were combined and lyophilized to give **11** (217 mg, 19%) as a very light yellow solid. The analytical sample was purified by preparative HPLC (Dynamax-300A, C-18 83-213-C, 10 × 350 mm; pump A = 0.1% trifluoroacetic acid in water, pump B = 0.1% trifluoroacetic acid in acetonitrile, gradient 0%–17% B in 32 min,

flow rate 3 mL/min, detecting at 295 nm) to give a white solid (133 mg, 12%): mp 168–170 °C; UV (aqueous HCl, pH 1) λ_{max} 341 (log ϵ 4.09), 274 (4.00), 243 nm (3.80); UV (aqueous NaOH, pH 13) λ_{max} 373 (log ϵ 4.25), 263 nm (4.10); IR (KBr) 3620–2600, 1730, 1700, 1600, 1550, 1535, 1460, 1430, 1320, 1260, 1180, 1140, 1030, 780, 680 cm^{-1}; ^1H NMR (D_2O, 500 MHz) δ 4.55 (dd, 1 H, J = 9 and 14 Hz), 4.42 (dd, 1 H, J = 3 and 14 Hz), 4.20 (m, 1 H), 3.89 (dt, 1 H, J = 3 and 7 Hz), 3.83–3.77 (m, 2 H), 3.68 (dd, 1 H, J = 7 and 12 Hz); ^{19}F NMR (D_2O) δ 7.12 (s, 3 F); FABMS (glycerol + HCl) m/z (relative intensity) 383 (M^+ + 1, 20); high-resolution FABMS (glycerol + HCl) calcd for $C_{12}H_{14}F_3N_4O_7$ m/z 383.0815 (M^+ + 1), found 383.0812.

3,3-Dibromo-1,1,1-trifluoro-2-butanone (44). 1,1,1-Trifluoro-2-butanone (**43**, 24.865 g, 0.197 mol), *N*-bromosuccinimide (91.2 g, 0.512 mol), and benzoyl peroxide (25 mg) in CCl_4 (200 mL) were heated at reflux with illumination from a 100-W tungsten bulb for 36 h. The solid succinimide was filtered off and washed with CCl_4 (2 × 30 mL). Fractional distillation of the filtrate gave **44** (51.987 g, 93%) as a colorless liquid: bp 124 °C (lit.[29] by 124 °C); ^1H NMR ($CDCl_3$, 200 MHz) δ 2.67 (s, 3 H).

6,7-Dichloro-2-(trifluoromethyl)-3-methylquinoxaline (51). 3,3-Dibromo-1,1,1-trifluoro-2-butanone (**44**, 595 mg, 2.1 mmol) was added to a solution of sodium acetate (720 mg, 8.8 mmol) in water (4 mL). The solution was heated at 98 °C for 1.5 h. After the solution was cooled to room temperature, a suspension of 4,5-dichloro-*o*-phenylenediamine (**50**, 177 mg, 1 mmol) in methanol (4 mL) was added, and the mixture was stirred at room temperature for 1.5 h. The crude product (268 mg) was filtered and washed with water (10 mL). The filtrate was extracted with CH_2Cl_2 (3 × 15 mL). The CH_2Cl_2 extracts were dried ($MgSO_4$), filtered, and evaporated to give light brown residue. The combined crude product was purified by sublimation at 60–70 °C (0.1 mmHg) to give a white crystalline compound **51** (233 mg, 83%). The analytical sample was recrystallized from CH_2Cl_2–hexane: mp 113–114 °C; UV (methanol) λ_{max} 330 (log ϵ, 3.81), 241 nm (4.51); IR (KBr) 3080, 1590, 1560, 1450, 1415, 1395, 1335, 1265, 1245, 1185, 1160, 1140, 1100, 1040, 955, 885, 870, 835, 750, 730 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 8.28 (s, 1 H), 8.19 (s, 1 H), 2.90 (s, 3 H); ^{19}F NMR ($CDCl_3$–DMSO-d_6) δ 9.00 (s, 3 F); CIMS (*i*-C_4H_{10} ionizing gas) m/e (relative intensity) 283 (M^+ + 3, 63), 281 (M^+ + 1, 100). Anal. Calcd for $C_{10}H_5Cl_2F_3N_2$: C, 42.71; H, 1.79; Cl, 25.24; F, 20.29; N, 9.97. Found: C, 42.52; H, 1.46; Cl, 25.31; F, 20.56; N, 9.96.

8-Benzyl-6-(trifluoromethyl)-7-methyllumazine (53). 3,3-Dibromo-1,1,1-trifluoro-2-butanone (**44**, 568 mg, 2 mmol) was added to a solution of sodium acetate (600 mg, 7.3 mmol) in water (4 mL). The solution was heated at 98 °C for 4 h. The solution was cooled to room temperature, diluted with water (10 mL), and extracted with ether (3 × 40 mL). The ether extracts were dried ($MgSO_4$), filtered, and evaporated under vacuum to obtain a colorless liquid. To this liquid, was added DMF (2 mL), and the resulting solution was added to a suspension of 5-amino-4-(benzylamino)uracil hydrochloride (**52**, 268 mg, 1.0 mmol) in DMF (3 mL). The reaction mixture was then heated at 84 °C for 5 h. The solid impurities were filtered off before the solvent was removed under vacuum (0.5 mmHg). The yellow, semisolid residue was purified by flash column chromatography using silica gel (40 g, 230–400 mesh, 2 × 12.5 cm). Elution was carried out first with benzene–methanol (2:23, 400 mL) followed by benzene–methanol (1:9, 400 mL). Evaporation of solvent from fractions containing **53** gave this substance as a green solid (119 mg, 35.4%). The analytical sample was purified by preparative HPLC (Dynamax-300A, C-18 83-213-C column, 10 × 250 mm, detecting at 270 nm) eluting with 30% acetonitrile: mp 172–174 °C; UV (aqueous HCl, pH 1) λ_{max} 400 (log ϵ 3.81), 264 nm (4.00); UV (aqueous NaOH, pH 13) λ_{max} 299 (log ϵ 4.14), 240 nm (4.16); IR (KBr) 3600–2780, 1715, 1700, 1600, 1530, 1450, 1375, 1270, 1240, 1200, 1150, 1130, 1030, 830, 690 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 7.43–7.15 (m, 5 H), 5.92 (s, 2 H), 2.67 (s, 3 H); ^{19}F NMR (DMSO-d_6) δ 9.45 (s, 3 F); CIMS (*i*-C_4H_{10} ionizing gas) m/e (relative intensity) 338 (M^+ + 2, 19), 337 (M^+ + 1, 100), 247 (M^+ – 89, 69); high-resolution CIMS (*i*-C_4H_{10} ionizing gas) calcd for $C_{15}H_{12}F_3N_4O_2$ m/e 337.0912 (M^+ + 1), found 337.0902.

6-(Trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (13). 3,3-Dibromo-1,1,1-trifluoro-2-butanone (**44**, 4.26 g, 15 mmol) was added to a solution of sodium acetate (5 g, 60.8 mmol) in water (30 mL), and the mixture was heated at reflux for 3 h. The reaction mixture was cooled to room temperature, diluted with water (10 mL), and extracted with diethyl ether (3 × 50 mL). The ether extracts were dried ($MgSO_4$) and evaporated under vacuum to obtain a colorless liquid. To this liquid was added DMF (5 mL), 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**, 940 mg, 3 mmol) in DMF (10 mL). The reaction mixture was heated at 80 °C in the dark for 5 h before the solvent was removed under vaccum (0.5 mmHg) at room temperature. The dark red, semisolid residue was dissolved in hot water (5 mL), hot ethanol (70 mL) was added, and the mixture was refrigerated for 24 h before the solid impurities were filtered off. The filtrate was concentrated on a rotary evaporator (bath 40 °C) to yield a semisolid mass. It was dissolved in 0.1 N NaOH (5 mL) and applied to a column of anion-exchange resin (Biorad AG-1-X2, acetate form, 2 g, 2 × 2 cm). The column was eluted in the following order: water (120 mL), 0.1 N NH_4OH (pH 12.00, 180 mL), ammonium acetate buffer (0.1 M, pH 9.9, 240 mL), and 0.1 N acetic acid (pH 2.5, 180 mL). Fractions (~6 mL) were collected and analyzed by analytical HPLC (μ-Bondapak C-18, 10 mm 3.9 × 300 mm, detecting at 270 nm), eluting with 7% acetonitrile. Fractions 75–124 containing **13** and some impurities were combined and lyophilized to give yellow solid. This was dissolved in water (7 mL) 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 (5 mL/3 min) were collected and analyzed by analytical HPLC as described above. Fractions (29–48) containing the desired compound **13** were combined and lyophilized to give yellow solid product (148 mg, 13%): mp 204–206 °C; UV (aqueous HCl, pH 1) λ_{max} 397 (log ϵ 3.88), 262 nm (4.13); UV (aqueous NaOH, pH 13) λ_{max} 333 (log ϵ 3.91), 246 nm (4.16); IR (KBr) 3600–3000, 1700, 1660, 1590, 1520, 1370, 1260, 1180, 1130, 1020, 800 cm^{-1}; ^1H NMR (D_2O, 500 MHz) δ 4.95 (m, 1 H), 4.44 (m, 1 H), 3.95–3.59 (m, 5 H); ^{19}F NMR (D_2O–HCl pH 2) δ 12.86 (s, 3 F), ^{19}F NMR (D_2O–NaOH, pH 13) δ 12.06 (s, 0.28 F), 11.89 (s, 0.34 F), 10.80 (s, 1.17 F), 10.09 (s, 0.83 F), 9.46 (s, 0.38 F); FABMS (glycerol + HCl) m/e (relative intensity) 381 (M^+ + 1, 5); high-resolution FABMS (glycerol + HCl) calcd for $C_{13}H_{16}F_3N_4O_6$ m/e 381.1022 (M^+ + 1), found 381.1017.

Table V. HPLC Conditions for the Analysis of Ligand Concentrations[a]

compd	eluent	detection	wavelength[b]	retn vol. (min)
pyrimidine **56**	10 mM AmFo/Fo	UV/vis	254	4
riboflavin	35% MeOH 100 mM AmFo	fluorescence	470/530	4
11	0.1% TFA 15% MeOH	fluorescence	340/385	4
13	0.1% TFA	UV/vis	411	5.5

[a] A column of Nucleosil RP18 (4 × 250 mm) was used. The flow rate was 2 mL/min. [b] UV/vis absorbance or fluorescence (excitation/emission).

Protein. Light riboflavin synthase was purified to a specific activity of 50 000 nmol mg^{-1} h^{-1} from the derepressed *B. subtilis* H94 mutant as described earlier.[33] 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 1 nmol of riboflavin per hour at 37 °C.

NMR Spectroscopy. ^{19}F NMR spectra of the enzyme–ligand complex were measured at 9.6 T using a Bruker AM360 NMR spectrometer. The samples contained 10–25 mg of protein and ligand as indicated in buffer containing 170 mM phosphate, pH 6.8, 10 mM sodium sulfite, and 10% D_2O. ^{19}F NMR measurements were performed at 4 °C and 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. Equilibrium dialysis experiments were performed at 4 °C using microdialysis cells from Dianorm and Visking dialysis tubes. The buffer contained 170 mM phosphate,

pH 6.8, and 10 mM sodium sulfite. The protein concentration was 2-4 mg/mL. The total ligand concentration was between 0.05 and 4 mM. The dialysis cells were allowed to equilibrate 5 h under slow rotation. The protein was precipitated by the addition of trichloroacetic acid to a final concentration of 5%. The ligand concentrations were determined by HPLC analysis. HPLC conditions for the analysis of ligand concentrations are given in Table V. Scatchard plots were evaluated using the program LIGAND (J. P. Munson).

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Supplementary Material Available: ^1H and ^{19}F NMR spectra of compounds **32**, **33**, and **40**, a ^1H NMR spectrum of **42**, ^1H and ^{19}F spectra and reversed-phase HPLC trace of **11**, ^1H NMR and ^{19}F NMR spectra and HPLC data for compound **13**, and ^1H and ^{19}F NMR data for compound **53** (41 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and may be ordered from the ACS; see any current masthead page for ordering information.

One-Pot Synthesis of Optically Active Cyanohydrin Acetates from Aldehydes via Lipase-Catalyzed Kinetic Resolution Coupled with in Situ Formation and Racemization of Cyanohydrins

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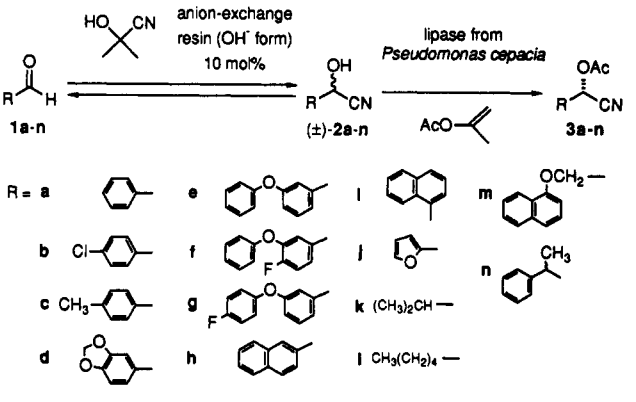
A novel one-pot synthesis of optically active cyanohydrin acetates from aldehydes has been accomplished by lipase-catalyzed kinetic resolution coupled with in situ formation and racemization of cyanohydrins in an organic solvent. Racemic cyanohydrins **2**, generated from aldehydes **1** and acetone cyanohydrin in diisopropyl ether under the catalysis of basic anion-exchange resin (OH^- form), were acetylated stereoselectively by a lipase from *Pseudomonas cepacia* (Amano) with isopropenyl acetate as an acylating reagent. The (*S*)-isomer of **2** was preferentially acetylated by the lipase, while the unreacted (*R*)-isomer was continuously racemized through reversible transhydrocyanation catalyzed by the resin. These processes thus enabled one-stage conversion of various aldehydes **1a-n** into the corresponding (*S*)-cyanohydrin acetates **3a-n** with up to 94% ee in 63-100% conversion yields. The racemization of the optically active cyanohydrin **2e** by Amberlite IRA-904 (OH^- form) was found to be much faster than the enzymatic acetylation, confirming the effective second-order asymmetric transformation. Enzymatic deacetylation of (*S*)-cyanohydrin acetates in an organic solvent and the synthesis of optically active pyrethroids are also described.

Optically active cyanohydrins are important starting materials for the synthesis of a number of chiral pharmaceuticals and agricultural chemicals because cyanohydrins are easily transformed into multifunctional chiral synthons such as β-hydroxy amines,[1] α-hydroxy carboxylic acids,[1c,2] and α-hydroxy ketones.[3] Among several chemical[4,5] and biochemical approaches[1-3,6-8] for the synthesis of optically active cyanohydrins, kinetic resolution by lipases[7] or microorganisms[8] has been extensively studied; optically active cyanohydrin esters were conveniently prepared by stereoselective hydrolysis or transesterification catalyzed by these biocatalysts. However, the recovery of optically active cyanohydrins from the reaction mixture has failed in many cases[7a,b,8b,d,e] because cyanohydrins are unstable and susceptible to decomposition or racemization in aqueous media. The unstable nature of cyanohydrins has thus hampered the enzymatic approach to the kinetic resolution of cyanohydrins. In addition, these approaches were all based on conventional kinetic resolution where the maximum obtainable yield of one enantiomer cannot exceed 50%, and the product ee is dependent on the conversion.[9] It is therefore highly desirable to develop a new method for the enzymatic kinetic resolution of cyanohydrins.

Scheme I



Introducing in situ racemization of substrate is a promising approach to this because it would allow for quanti-

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