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Synthesis of 2,6-Dioxo-(1*H*,3*H*)-9-*N*-ribitylpurine and 2,6-Dioxo-(1*H*,3*H*)-8-aza-9-*N*-ribitylpurine as Inhibitors of Lumazine Synthase and Riboflavin Synthase

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Abstract—2,6-Dioxo-(1*H*,3*H*)-9-*N*-ribitylpurine (**6**) and 2,6-dioxo-(1*H*,3*H*)-8-aza-9-*N*-ribitylpurine (**7**) have been synthesized and evaluated as inhibitors of lumazine synthase and riboflavin synthase. Reaction of 5-amino-6-ribitylaminouracil hydrochloride (**8**) with diethoxymethyl acetate (**9**) afforded the purine **6**, while diazotization of **8** afforded the 8-aza purine **7**. Compounds **6** and **7** were evaluated against lumazine synthase of *Bacillus subtilis* and riboflavin synthase of *Escherichia coli*. Both **6** and **7** were better inhibitors of lumazine synthase than riboflavin synthase. The 8-azapurine 7 had a lower K_I (0.33 and 0.39 mM) than the purine **6** (0.47 and 0.54 mM) when evaluated with lumazine synthase and riboflavin synthase, respectively.

Introduction

Riboflavin synthase has been observed in a variety of microorganisms and plants and the biosynthesis of riboflavin has been studied extensively.[1–7] The structure of the lumazine synthase-riboflavin synthase complex in *Bacillus subtilis* has been investigated in detail.[8–13] The reactions catalyzed by the enzyme complex are displayed in Scheme 1. The complex is comprised of an inner core consisting of 3 α subunits (riboflavin synthase) and an outer icosahedral capsid containing 60 β subunits (lumazine synthase).[14,15] The β-subunits catalyze the formation of 6,7-dimethyl-8-(D-ribityl)lumazine (**3**) from 5-amino-6-(D-ribitylamino)-2,4-(1*H*,3*H*)pyrimidinedione (**1**) and the novel four-carbon carbohydrate, L-3,4-dihydroxy-2-butanone-4-phosphate (**2**).[16–18] The α-subunits catalyze the dismutation of two molecules of 6,7-dimethyl-8-(D-ribityl)lumazine (**3**) to form one molecule of riboflavin (**4**) and one molecule of **1**. The pyrimidinedione **1** formed by the catalytic action of the α subunits can then be reutilized in the reaction catalyzed by the β subunits. The overall stoichiometry of the reaction sequence catalyzed by the enzyme complex involves the formation of one molecule of riboflavin (**4**) and two molecules of inorganic phosphate from one pyrimidinedione molecule **1** and two molecules of the carbohydrate phosphate precursor **2**.

Until relatively recently, the lack of information about the reaction catalyzed by lumazine synthase precluded the design and synthesis of potential inhibitors. However, the X-ray structure of reconstituted, icosahedral lumazine synthase capsids has now been obtained and information is available about the mechanism of the enzyme-catalyzed reaction.[5,10,13] This has provided a detailed description of the active site occupied by the substrate analog 5-nitro-6-(D-ribitylamino)-2,4-(1*H*,3*H*)pyrimidinedione (**5**). In addition, the elucidation of the structure of the four-carbon precursor **2** has made it feasible to assay potential lumazine synthase inhibitors. With the structures of both of the substrates **1** and **2** now in hand, detailed steady state enzyme kinetic parameters for recombinant β_{60} capsids devoid of α subunits have been measured,[6,7] and inhibition constants of potential lumazine synthase inhibitors can now be determined.

Key words: Lumazine synthase; riboflavin synthase; inhibition.
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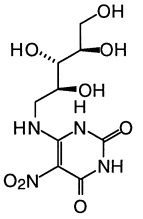
A rational approach to therapeutically useful antibiotics would be to selectively inhibit an enzyme present in a parasite but absent in the host. Inhibition of the biosynthesis of riboflavin provides such a strategy, since pathogenic microorganisms synthesize their own riboflavin, whereas mammals obtain this vitamin through dietary sources. Therefore, riboflavin synthase inhibitors might be useful as therapeutic agents for pathogenic bacterial, yeast and fungal infections, since effective treatment of these infections is often lacking when they are systemic. In particular, enterobacteria such as *Salmonella* and *Escherichia* species lack a riboflavin uptake system and are therefore required to biosynthesize riboflavin to remain viable.

Results and discussion

Taking advantage of the crystal structure of the active-site containing bound **5**,[13] 2,6-dioxo-(1*H*,3*H*)-9-*N*-ribityltpurine (**6**) and 2,6-dioxo-(1*H*,3*H*)-8-aza-9-*N*-ribityltpurine (**7**) were designed as potential inhibitors of lumazine synthase. Molecular modeling was performed using SCULPT version 2.0 (Interactive Simulations, Inc.). The compounds were docked into the active site of lumazine synthase (Figure 1). The pyrimidinedione rings of compounds **6** and **7** were superimposed on that of the substrate analog **5** within the hydrophobic region of the pocket. Structure **5** was then removed and the energy of either bound **6** or **7** was minimized. This placed the ribityl chain within the hydrophilic pocket of the enzyme. The hydrophobic region of the pocket is large and is able to accommodate the bicyclic rings of compounds **6** and **7** very well.



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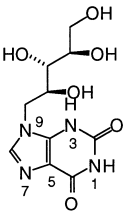

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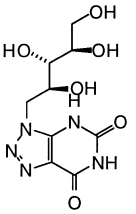
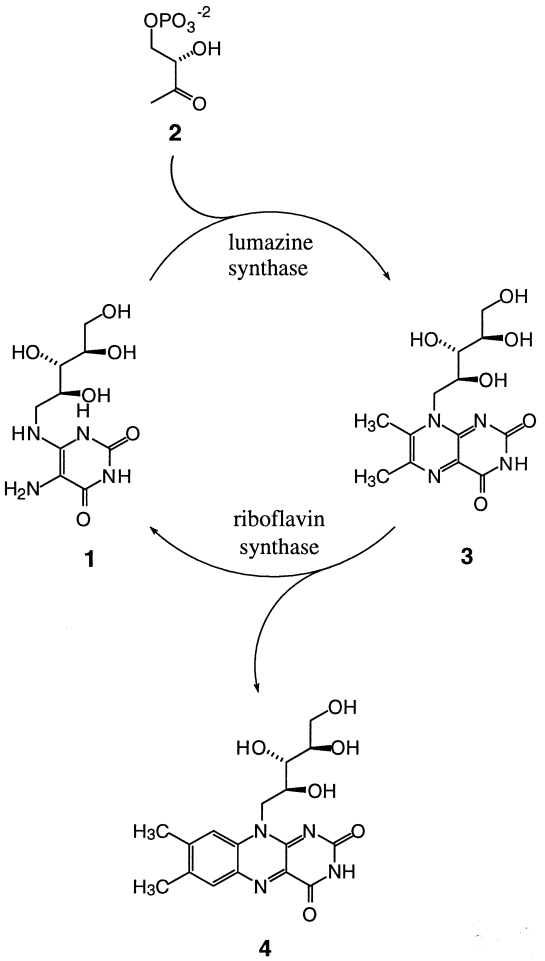


7

The β-capsid is a relatively dense structure except for the channels that run along the fivefold symmetry axes. It is assumed that substrate molecules are imported through these channels to access the inner surface of the capsid where the substrate binding sites are located. The substrate molecules are thought to be attracted and oriented by the positively charged 'windows' in the channels formed by protonated lysine side chains.[19] On the basis of published pK_a values of the corresponding 9-*N*-hydroxyethyl compounds, both the purine **6** and the azapurine **7** are expected to be anionic at physiological pH, with pK_a values close to 5.9 and 5.1, respectively.[20,21] The highly delocalized anions are formed in each case by deprotonation of the N-3 nitrogen atom.[20,21] Both compounds **6** and **7** should, therefore, be attracted by the positive 'windows' at the channel entrance. This would enable these inhibitors to access the substrate binding sites of the β-subunit on the inner surface of the β_{60} capsid.

The compounds **6** and **7** were synthesized as detailed in Scheme 2 from the common precursor amine hydrochloride **8**, which was prepared freshly by reduction of



Scheme 1.

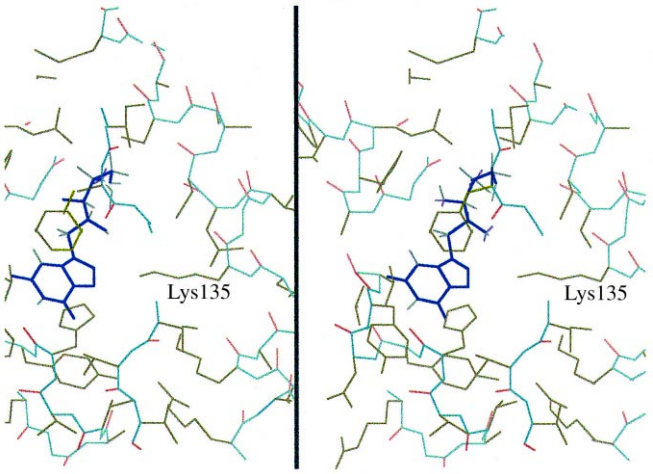
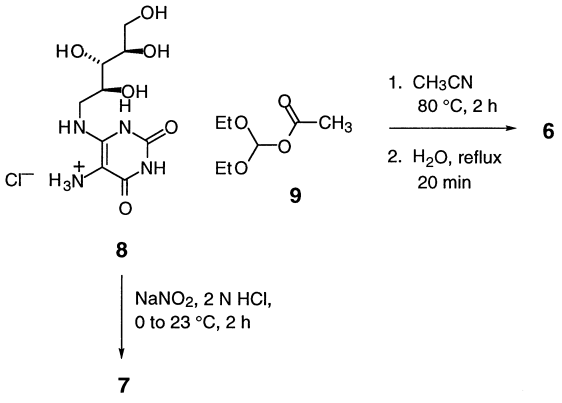


Figure 1. Stereoview (crosseye) of 8-azapurine **7** docked in the active site of lumazine synthase.

the corresponding nitro compound **5**.[22,23] Attempts to synthesize the purine **6** using formamide resulted in a complex mixture of products. A number of xanthines have been synthesized from *o*-diaminopyrimidines using diethoxymethyl acetate as the one-carbon source in the ring closure step.[24] The purine **6** was obtained in 56% yield from 5-amino-6-(ribitylamino)pyrimidine-2,4-(1*H*,3*H*)-dione hydrochloride (**8**)[23] via modification of the procedure reported for the xanthines, utilizing diethoxymethyl acetate (**9**). The ^{1}H NMR spectrum showed the singlet of the aromatic 8-CH at δ 7.34.

o-Diaminopyrimidines have also been utilized in the synthesis of various 8-azapurines in the presence of nitrous acid.[25,26] Hence, utilizing diazotization conditions ($NaNO_2$ in 2 N HCl), the precursor amine hydrochloride **8** was converted to the 8-azapurine **7**. The straw-colored solution of the amine hydrochloride changed to dark red upon addition of the $NaNO_2$. The IR spectrum (CaF_2) of the dark red reaction mixture indicated the N≡N stretch at 2135 cm^{-1}. As the unstable diazonium species cyclized to the stable 8-azapurine **7**, the color of the reaction mixture changed from red to fluorescent yellow. This change in color corresponded to the disappearance of the N≡N stretch at 2135 cm^{-1} in the IR spectrum of the reaction mixture. The UV spectrum of the reaction mixture now showed a λ_{max} at 230 nm, which was absent in the spectrum of the starting material and of the reaction mixture at the diazonium intermediate stage. The product **7** was isolated from the reaction mixture in 28% yield after anion exchange chromatography.



Scheme 2.

Compounds **6** and **7** were tested for their inhibition of lumazine synthase from *Bacillus subtilis* and riboflavin synthase from *Escherichia coli* (Table 1). Both **6** and **7** were better inhibitors of lumazine synthase than riboflavin synthase. The **8**-azapurine **7** had a lower K_I than the purine **6** with both enzymes. The lower K_I of **7** versus **6**

Table 1. Inhibition constants of **5**, **6**, and **7** versus lumazine synthase and riboflavin synthase

Compund	Lumazine synthase (K_I)[a] (μM)	Riboflavin synthase[b] (K_I) (μM)
5	—	120
6	470	540
7	330	390

[a]Recombinant β_{60} capsids from *B. subtilis*.
[b]Recombinant riboflavin synthase from *E. coli*.

observed with lumazine synthase may reflect a possible favorable electrostatic interaction between the N-8 of **7** with the adjacent positively charged Lys135 (N8-Lys135 δNH_2, distance 3.05 Å) residue of the enzyme, which would not be possible with the 8-CH moiety of compound **6** (Figure 1). The N-8 nitrogen of the azapurine **7** would be expected to be more negative than the C-8 of **6** because of the greater electronegativity of the nitrogen as well as the nonbonded pair of electrons on the nitrogen atom. In order to investigate this in more detail, the negative electrostatic isopotential surfaces of both **6** and **7** were calculated. Geometry optimization and energetics calculations for semiemperical AM1 quantum chemical levels were performed with MacSpartan Plus version 1.0 (Wavefunction, Inc.). On the basis of the expected pK_a values of **6** and **7**, it can be expected that both molecules would be anionic when bound in the active site of the enzyme, but in order to cover both possibilities, calculations were performed on both neutral and anionic **6** and **7**. The negative electrostatic isopotential surfaces were calculated at −30 kcal/mol for neutral **6** and **7**, and at −110 kcal/mol for the corresponding anions. The resulting isopotential surfaces are shown in Figure 2. In both the neutral molecules and their corresponding anions, the 8-azapurine **7** has significantly more negative charge concentrated near the 8-position, which could possibly bind ionically to the positively charged Lys135 residue in the active site of the enzyme (Figure 1). This is also reflected in the calculated atomic charges (AM1 fits to electrostatic potentials), which are listed in Table 2. In addition to electrostatic attraction, the N-8 of the 8-azapurine could form a hydrogen bond with the protonated Lys135 of lumazine synthase, which would not be possible with the purine **6**.

Conclusion

Although a variety of riboflavin synthase inhibitors have previously been reported,[15,23,27–32] compounds **6** and **7** are the first documented inhibitors of lumazine

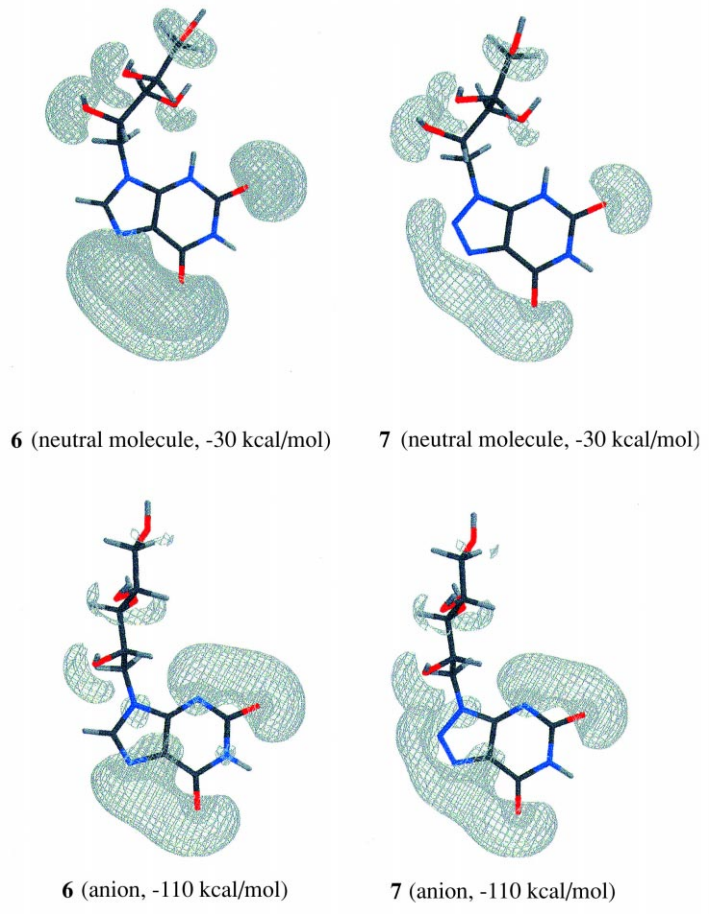


Figure 2. Negative electrostatic isopotential surfaces for **6** and **7**.

Table 2. Calculated atomic charges for **6** and **7** (AM1 fits to electrostatic potentials)[a]

	6		7	
Atom	Neutral	Anion	Neutral	Anion
1	−0.62	−0.73	−0.54	−0.66
2	0.78	0.90	0.76	0.89
3	−0.58	−0.86	−0.62	−0.87
4	0.23	0.56	0.21	0.54
5	−0.09	−0.30	−0.05	−0.23
6	0.66	0.73	0.55	0.60
7	−0.43	−0.44	−0.20	−0.21
8	0.17	0.16	−0.18	−0.20
9	−0.12	−0.39	0.24	−0.90

[a]Calculations were performed using MacSpartan Plus, Wavefunction, Inc. Charges are in electrostatic units.

synthase to be studied in detail. Although these compounds have proven to be weak inhibitors of lumazine synthase, they provide a starting point for the design of additional compounds with enhanced inhibitory activity.

Experimental

Melting points are uncorrected. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were recorded on a 300 MHz spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; s = singlet, d = doublet, m = multiplet, bs = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. The mass spectra were determined on a plasma desorption mass spectrometer (PDMS) which utilizes a 252Cf ionizing source which produces MeV fission fragments. The interaction of the fission fragments with the sample produces ions which are mass analyzed with a time-of-flight mass spectrometer.[33] The compounds were applied to a nitrocellulose-coated mylar target and allowed to dry prior to being put into the mass spectrometer. The accelerated potential was set at 17 000 kV with data being collected for 15 min. Proportions of solvents used for TLC are by volume. Analytical samples were dried in vacuo (0.2 mm Hg) in an Abderhalden drying apparatus over P_2O_5 and refluxing acetone. Elemental analyses were performed by the Purdue Microanalytical Laboratory. Fractional moles of water and solvents frequently found in some analytical samples were not removed in spite of 24–48 h of drying in vacuo and were confirmed by their presence in the ^{1}H NMR spectrum. All solvents and chemicals were purchased from Aldrich Chemical Co. and were used as received.

Materials

5-Nitro-6-(ribitylamino)pyrimidine-2,4-(1*H*,3*H*)-dione (**5**),[22] 5-amino-6-(ribitylamino)pyrimidine-2,4-(1*H*,3*H*)-dione hydrochloride (**8**),[23] and L-3,4-dihydroxy-2-butanone-4-phosphate (**2**)[34] were prepared by published procedures. Recombinant riboflavin synthase of *Escherichia coli* and lumazine synthase of *Bacillus subtilis* were also prepared by published procedures.[35,36]

2,6-Dioxo-(1*H*,3*H*)-9-*N*-ribitylpurine (6). A mixture of the amine hydrochloride **8** (200 mg, 1.23 mmol), acetonitrile (15 mL) and diethoxymethylacetate (**9**) (0.4 mL, 2.46 mmol) was heated at 80 °C for 2 h. The solvent was evaporated under reduced pressure and the residue triturated with water (5 mL) at 90 °C for 20 min. The solid obtained upon lyophilization of this mixture was dissolved in $HCOONH_4$ buffer at pH 10 and loaded onto an anion-exchange column (2.5 cm × 58.4 cm, Dowex-1×2–400, 20 g) and eluted, sequentially, with $HCOONH_4$ buffers at pH 10 (100 mL), pH 7 (100 mL) and pH 4 (200 mL). Fractions corresponding to the product were pooled, concentrated and loaded onto a cation-exchange column (2.5 cm × 58.4 cm, Dowex-50W×2–400, 20 g) and eluted with water (200 mL). The product fractions were pooled and lyophilized to yield **6** (115 mg, 56%): mp 100–105 °C dec; PDMS *m*/*z* 287 (MH^+); ^{1}H NMR (DMSO-d_6) δ 7.34 (s, 1 H, 8-CH), 4.05 (m, 3 H), 3.55 (m, 2 H), 3.38 (m, 1 H), 3.21 (m, 1 H); Anal. calcd for $C_{10}H_{14}N_4O_6 \cdot 1.7\ HCOONH_4 \cdot 0.8\ H_2O$: C, 34.46; H, 5.96; N, 19.58. Found: C, 34.12; H, 5.61; N, 19.27.

2,6-Dioxo-(1*H*,3*H*)-8-aza-9-*N*-ribitylpurine (7). The amine hydrochloride **8** (0.13 g, 0.42 mmol) was dissolved in 6 N HCl (10 mL) and the light yellow solution was cooled to 0 °C in an ice bath. A solution of $NaNO_2$ (0.029 g, 0.42 mmol) in water (5 mL), cooled to 0 °C, was added dropwise to the solution of the amine over a period of 0.5 h. The solution was stirred at 0 °C for 1 h, then allowed to warm to room temperature and was stirred for an additional hour. The solution was then basified with 1 N NaOH (pH 10) and loaded onto an anion-exchange column (2.5 cm × 58.4 cm, Dowex-1×2–400, 10 g) and eluted, sequentially, with water and 10% HCOOH. The bright greenish-yellow fluorescent fractions corresponding to the product were pooled and evaporated to dryness under reduced pressure. The residue was suspended in ethanol (2 mL) and Et_2O (15 mL), filtered and dried to yield **7** (33 mg, 28%): mp 125–130 °C; PDMS *m*/*z* 288 (MH^+); ^{1}H NMR (DMSO-d_6) δ 12.09 (s, exchangeable, 1 H), 11.21 (s, exchangeable, 1 H), 4.50–5.02 (bs, exchangeable, 4 H), 4.54 (m, 1 H), 4.41 (m, 1 H), 4.01 (m, 1 H), 3.41–3.61 (m, 4 H); IR (KBr) 3653, 3645, 3626, 3591, 2350, 2310, 1842, 1515, 1494, 1418, 1194 cm^{-1}; UV (aqueous HCl, pH 1) λ_{max}

270, 230 nm; Anal. calcd for $C_9H_{13}N_5O_6 \cdot 1.7\ HCl \cdot 0.6\ HCOOH$: C, 30.60; H, 4.25; N, 18.58. Found C, 30.65; H, 4.23; N, 18.54.

Lumazine synthase assay[7]. Reaction mixtures contained 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM dithiothreitol, and recombinant β_{60} lumazine synthase capsids (800 μg, specific activity 12.5 μmol $mg^{-1}\,h^{-1}$). The mixtures, also containing various concentrations of 5-amino-6-ribitylaminouracil **1** (4–400 μM) and inhibitors **6** or **7** (30 to 1000 μM), were preincubated at 37 °C. The reactions were started adding 400 μM of L-3,4-dihydroxy-2-butanone 4-phosphate (**2**) to a total volume of 100 μL. At intervals, aliquots were taken, and the reactions were quenched by the addition of trichloroacetic acid to a final concentration of 0.4M. Concentrations of lumazine were determined by HPLC analysis using a reversed phase column Nucleosil 10 C_{18} (4×250 mm). The effluent was monitored fluorimetrically (excitation, 408 nm; emission, 487 nm). With an eluent contanining 7% methanol and 30 mM formic acid, lumazine was eluted with a retention volume of 8.4 mL. A Lineweaver Burk plot of the initial ratios gave strictly competitive inhibition. The K_I values result from a secondary plot (1/v versus $c_{inhibitor}$).

Riboflavin synthase assay[35]. Reaction mixtures contained buffer (100 mM potassium phosphate, 10 mM EDTA, 10 mM sodium sulfite), inhibitor **6** or **7** (50 to 1000 μM), and riboflavin synthase (10 μg, specific activity 50 μmol $mg^{-1}\,h^{-1}$). After preincubation, the reactions were started by the addition of various amounts of 6,7-dimethyl-8-ribityllumazine (**3**) (10 to 500 μM) to a total volume of 500 μL. The formation of riboflavin (**4**) was measured online with a computer controlled photometer at 470 nm ($e_{Riboflavin} = 9100\ M^{-1}\,cm^{-1}$). The K_I evaluation was performed in the same manner as described above.

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References

1. Plaut, G. W. E.; Smith, C. M.; Alworth, W. L. *Ann. Rev. Biochem.* **1974**, *43*, 899.

2. Plaut, G. W. E. In *Comprehensive Biochemistry*; Florkin, M.; Stotz, E. H., Eds.; Elsevier: Amsterdam, 1971; Vol. 21, p 11.

3. Brown, G. M.; Williamson, J. M. In *Escherichia coli and Salmonella typhimurium*; Neidhardt, F. C.; Ingraham, J. L.; Low, K. B.; Magasanik, B.; Schaechter, M.; Umbarger, H. E., Eds.; American Society for Microbiology: Washington, DC, 1987; p 521.

4. Bacher, A.; Ladenstein, R. In *Chemistry and Biochemistry of Flavoenzymes*; Müller, F., Ed.; Chemical Rubber Co.: Boca Raton, FL, 1991; Vol. II; pp 293–316.

5. Bacher, A.; Fischer, M.; Kis, K.; Kugelbrey, K.; Mörtl, S.; Scheuring, J.; Weinkauf, S.; Eberhardt, S.; Schmidt-Bäse, K.; Huber, R.; Ritsert, K.; Cushman, M.; Ladenstein, R. *Biochem. Soc. Trans.* **1996**, *24*, 89.

6. Kis, K.; Volk, R.; Bacher, A. *Biochemistry* **1995**, *34*, 2883.

7. Kis, K.; Bacher, A. *J. Biol. Chem.* **1995**, *270*, 16788.

8. Ladenstein, R.; Schneider, M.; Huber, R.; Bartunik, H. D.; Wilson, K.; Schott, K.; Bacher, A. *J. Mol. Biol.* **1988**, *203*, 1045.

9. Bacher, A.; Eisenreich, W.; Kis, K.; Ladenstein, R.; Richter, G.; Scheuring, J.; Weinkauf, S. In *Bioorganic Chemistry Frontiers*; Dugas, H.; Schmidtchen, F. P., Eds.; Springer–Verlag: Berlin, 1993; Vol. 3, p 147.

10. Ritsert, K.; Huber, R.; Turk, D.; Ladenstein, R.; Schmidt-Bäse, K.; Bacher, A. *J. Mol. Biol.* **1995**, *253*, 151.

11. Bacher, A.; Ludwig, H. C.; Schnepple, H.; Ben-Schaul, Y. *J. Mol. Biol.* **1986**, *187*, 75.

12. Ladenstein, R.; Meyer, B.; Huber, R.; Labischinski, H.; Bartels, K.; Bartunik, H.-D.; Bachman, L.; Ludwig, H. C.; Bacher, A. *J. Mol. Biol.* **1986**, *187*, 87.

13. Ladenstein, R.; Ritsert, K.; Richter, G.; Bacher, A. *Eur. J. Biochem.* **1994**, *223*, 1007.

14. Bacher, A.; Baur, R.; Eggers, U.; Harders, H.-D.; Otto, M. K.; Schnepple, H. *J. Biol. Chem.* **1980**, *255*, 632.

15. Cushman, M.; Patel, H.; Scheuring, J.; Bacher, A. *J. Org. Chem.* **1992**, *57*, 5630.

16. Neuberger, G.; Bacher, A. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 1111.

17. Volk, R.; Bacher, A. *J. Am. Chem. Soc.* **1988**, *110*, 3651.

18. Volk, R.; Bacher, A. *J. Biol. Chem.* **1990**, *265*, 19479.

19. Karshikoff, A.; Ladenstein, R. *Proteins* **1989**, *5*, 248.

20. Pfleiderer, W.; Nübel, G. *Justus Liebigs Ann. Chem.* **1960**, *631*, 168.

21. Nübel, G.; Pfleiderer, W. *Chem. Ber.* **1965**, *98*, 1060.

22. Plaut, G. W. E.; Harvey, R. A. *Methods Enzymol.* **1971**, *XVIIIB*, 515.

23. Cushman, M.; Patrick, D. A.; Bacher, A.; Scheuring, J. *J. Org. Chem.* **1991**, *56*, 4603.

24. Cottam, H. B.; Shih, H.; Tehrani, L. R.; Wasson, D. B.; Carson, D. A. *J. Med. Chem.* **1996**, *39*, 2.

25. Franchetti, P.; Sheikha, G. A.; Cappellacci, L.; Grifantini, M.; De Montis, A.; Piras, G.; Loi, A. G.; La Colla, P. *J. Med. Chem.* **1995**, *38*, 4007.

26. Pfleiderer, W.; Bühler, *E. Chem. Ber.* **1966**, *99*, 3022.

27. Winestock, C. H.; Aogaichi, T.; Plaut, G. W. E. *J. Biol. Chem.* **1963**, *238*, 2866.

28. Plaut, G. W. E.; Beach, R. In *Flavins and Flavoproteins*; Singer, T. P., Ed.; Elsevier: Amsterdam, 1976; p 737.

29. 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.

30. Wood, H. C. S.; Wrigglesworth, R.; Yeowell, D.; Gurney, F. W.; Hurlbert, B. S. *J. Chem. Soc., Perkin Trans. 1* **1974**, 1225.

31. Otto, M.; Bacher, A. *Eur. J. Biochem.* **1981**, *115*, 511.

32. Cushman, M.; Patel, H. H.; Scheuring, J.; Bacher, A. *J. Org. Chem.* **1993**, *58*, 4033.

33. Wood, K. V.; Bonham, C. C.; Hipskind, J.; Nicholson, R. L. *Phytochemistry* **1994**, *37*, 557.

34. Kis, K.; Volk, R.; Bacher, A. Manuscript in preparation.

35. Eberhardt, S.; Richter, G.; Gimbel, W.; Werner, T.; Bacher, A. *Eur. J. Biochem.* **1996**, *242*, 712.

36. Bacher, A.; Eberhardt, S.; Fischer, M.; Mörtl, S.; Kis, K.; Kugelbrey, K.; Scheuring, J. *Methods Enzymol.* **1997**, in press.