Biosynthesis of Riboflavin. The Reaction Catalyzed by 6,7-Dimethyl-8-ribityllumazine Synthase Can Proceed without Enzymatic Catalysis under Physiological Conditions†

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6,7-Dimethyl-8-ribityllumazine is the biosynthetic precursor of the vitamin, riboflavin. The biosynthetic formation of the lumazine by condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione and 3,4-dihydroxy-2-butanone 4-phosphate is catalyzed by the enzyme, lumazine synthase. We show that the condensation reaction can proceed without enzyme catalysis in dilute aqueous solution at room temperature and neutral pH. The reaction rate is proportional to e^{pH}. The activation energy of the uncatalyzed reaction is $E_a = 46.3$ kJ mol^{-1}. The regioselectivity of the uncatalyzed reaction increases with pH and temperature (70% at 65 °C and pH 7.75). The data suggest partitioning of the uncatalyzed reaction via two different reaction pathways. The value of k_{cat}/k_{uncat} may be indicative for an entropy driven process for the enzyme-catalyzed reaction.

Introduction

It is generally assumed that constituent molecules of early organisms were generated by prebiotic chemical reactions occurring in aqueous solution. More than a century ago, Löw and Emil Fischer showed that carbohydrates could be obtained from formaldehyde.[1,2] These reactions have been studied in more detail by Eschenmoser and co-workers.[3] Miller, Urey, and their co-workers obtained evidence for the formation of amino acids by electric discharge in a gas phase which was considered to be similar to the early atmosphere, but more recent geophysical evidence suggests that the primitive atmosphere was different from that which was assumed by these authors.[4−6]

More recently, Wächtershäuser proposed that the formation of the first biomolecules could have occurred in hot submarine environments providing a supply of hydrogen sulfide, carbon monoxide, elemental hydrogen, and transition metal ions.[7] Recently, Huber and Wächtershäuser showed that amino acids and peptides can be obtained under these reaction conditions in relatively high yield.[8]

Flavins are a group of redox catalysts which are indispensable in all cellular organisms. Their biosynthesis has been studied in considerable detail (Figure 1; for review, see refs 9−12). Briefly, the heterocyclic part of the riboflavin molecule (**4**) is derived from GTP via the intermediate 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**). The pyrimidine is converted into 6,7-dimethyl-8-ribityllumazine (**3**) under the catalytic action of lumazine synthase by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (**2**) which is formed from ribulose 5-phosphate. An unusual dismutation of the lumazine **3** yields riboflavin. The second product of the dismutation reaction, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, is returned into the biosynthetic cycle.

Paterson and Wood found that riboflavin can be obtained by boiling a solution of its biosynthetic precursor, 6,7-dimethyl-8-ribityllumazine, in phosphate buffer, pH 7.3, without a catalyst.[13−15] The spontaneous and the enzyme-catalyzed reaction were shown to proceed with the same regiospecificity. Plaut and Beach subsequently showed that the nonenzymatic formation of riboflavin from **3** can proceed under neutral as well as acidic conditions.[16,17] More recently, Eschenmoser and Strupp obtained the lumazine **3** in 9.4% yield by boiling a neutral solution containing ribulose 1,5-bisphosphate and the pyrimidine **1**.[3,18]

We have found that the lumazine **3** can be formed from the biosynthetic precursors **1** and **2** in dilute, neutral aqueous solution at room temperature without enzyme catalysis, albeit with a low regioselectivity. This low regioselectivity may be a consequence of two reaction

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† Dedicated to Professor Albert Eschenmoser on the occasion of his 75th birthday.

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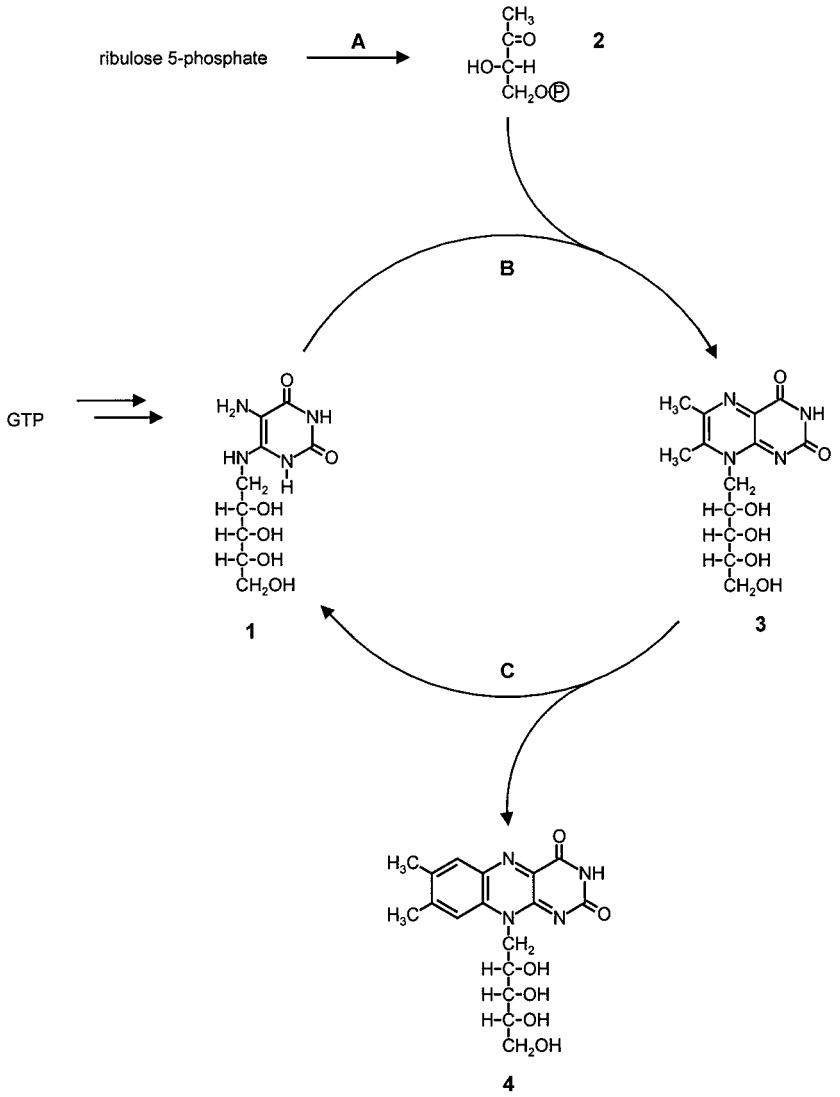

Figure 1. Biosynthesis of riboflavin. A, 3,4-dihydroxy-2-butanone 4-phosphate synthase; B, lumazine synthase; C, riboflavin synthase.

pathways with different regioselectivities. These observations support the hypothesis that flavin type molecules could have been formed naturally in prebiotic environments without enzyme catalysis.

Results

In the course of kinetic studies on lumazine synthase, we observed that 6,7-dimethyl-8-ribityllumazine (**3**) was formed from 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**) and 3,4-dihydroxy-2-butanone 4-phosphate (**2**) in blank samples devoid of lumazine synthase. This observation prompted a more detailed study of the uncatalyzed condensation reaction.

Kinetic analysis of the spontaneous condensation reaction was performed in aqueous solution containing the biosynthetic intermediates **1** and **2** in the mM concentration range. At pH 7.0 and 37 °C, the reaction was found to be pseudo first order for the substrate **1**. A logarithmic plot of the reaction velocity versus pH gave a straight line indicating a pH dependence of $v = 0.0185\ \mu\text{M min}^{-1} \times e^{\text{pH}}$. The rate law is $v = k \times [\mathbf{1}] \times [\mathbf{2}] \times e^{\text{pH}}$. At a temperature of 37 °C, the rate constant k has a value of $0.0022\ \text{M}^{-1}\ \text{min}^{-1}$.

The temperature dependence of the uncatalyzed reaction in the range from 20 °C to 80 °C is shown in Figure 2. The Arrhenius-plot of $\log(v)$ versus $1/T$ gave a straight line indicating an overall activation energy of 46.3 kJ mol^{-1} with a preexponential term P of 180 M min^{-1}. Using the overall rate law $v = k \times [\mathbf{1}] \times [\mathbf{2}] \times e^{\text{pH}} = A \times e^{(-Ea/RT)} \times [\mathbf{1}] \times [\mathbf{2}] \times e^{\text{pH}}$ the frequency factor A can be calculated from the preexponential factor P by the equation $P = A \times [\mathbf{1}] \times [\mathbf{2}] \times e^{\text{pH}}$. At pH 7, A had a calculated value of $122 \times 10^3\ \text{M}^{-1}\ \text{min}^{-1}$, and thus the value of k at pH 7 and 37 °C was $0.0020\ \text{M}^{-1}\ \text{min}^{-1}$.

The enzyme-catalyzed formation of the lumazine **3** has been shown to be regiospecific.[19,20] This suggested that the initial step in the enzyme-catalyzed reaction is a nucleophilic attack of the position 5 amino group of **1** on the carbonyl group of **2** yielding the Schiff's base **5** (Figure 3).

To study the regiospecificity of the uncatalyzed reaction, we prepared [1-$^{13}C_1$]-**2** by a modification of the procedure reported earlier.[19] Reaction with the pyrimi-

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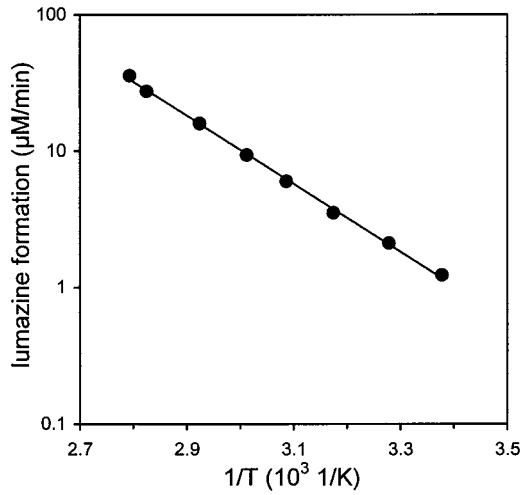

Figure 2. Arrhenius plot of uncatalyzed formation of **3** from **1** and **2**. The fit of the data afforded the relationship $\ln(v) = 18.99 - 5567.3 \times 1/T$ with $R^2 = 0.998$ corresponding to an activation energy of $E_a = 46.3$ kJ mol^{-1}; 1.0 mM **1**, 1.2 mM **2**, pH 7.0.

dine **1** afforded **3** which was analyzed by NMR spectroscopy.

The ^{1}H NMR signal of the position-6 methyl group of **3** obtained from [1-$^{13}C_1$]-**2** shows a central line for the $^{12}CH_3$ isotopomer and two satellite lines for the $^{13}CH_3$ isotopomer. The isotopomer composition can be obtained by integration of these different signal components. It is relevant for the calculation that the ^{13}C enrichment of [1-$^{13}C_1$]-**2** was about 99%. Data from experiments performed with [1-$^{13}C_1$]-**2** samples obtained by chemical synthesis or by enzymatic conversion from [2-$^{13}C_1$]-D-glucose gave closely similar results. It follows that the presence of small amounts of the 3,4-dihydroxy-2-butanone 3-phosphate (an impurity from the chemical synthesis) did not influence the observed regioselectivity of the condensation reaction.

The regioselectivity of lumazine formation as a function of temperature and pH is shown in Figures 4 and 5. The regioselectivity of the reaction was found to increase with temperature in the range of 27 °C to 65 °C. It was also found to increase with pH in the range of pH 5.25 to 8.25. The highest value of regioselectivity observed in this sequence of experiments was 70% at 65 °C and pH 7.75.

Discussion

The formation of 6,7-dimethyl-8-ribityllumazine (**3**) from **1** and **2** catalyzed by lumazine synthase is strictly regiospecific (at least 98%).[19,20] The mechanism of the enzyme-catalyzed reaction has been studied in some detail, and it has been proposed that the initial step of this reaction is the nucleophilic attack of the carbonyl group of **2** by the 5-amino group of **1** (Figure 3).[19,20] It was further proposed that the Schiff's base intermediate **5** could eliminate phosphate with subsequent formation of the pyrazine ring of the lumazine chromophore.

The uncatalyzed reaction has only a relatively low regioselectivity (at pH 7.5, 37 °C, 60%). However, the velocity of the uncatalyzed reaction and its degree of regioselectivity increase with pH and with temperature. Our regioselectivity data suggest that the uncatalyzed reaction can proceed via two different pathways, one of which is similar to the pathway of the enzyme-catalyzed reaction (pathway A, Figure 3). The alternative pathway (B) could involve the formation of diacetyl from **2** by β-elimination of phosphate. The subsequent condensation of diacetyl with the pyrimidine **1** would be characterized by the absence of regiospecificity due to its symmetric structure.

The velocity of the uncatalyzed lumazine formation increases with increasing pH. At high pH, the rate of the β-elimination of **2** should be increased because the removal of the acidic proton at C-3 of **2** is facilitated. On the other hand, the 5-amino group of **1** obviously is deprotonated in basic solution, and therefore the nucleophilic attack of this amino group to the carbonyl group of **2** proceeds faster. The enhanced regioselectivity of the lumazine formation shows that pathway A is favored at higher pH and that the reactivity of the deprotonated 5-amino group of **1** exceeds the β-elimination of **2**. An additional point of evidence may arise directly from Figure 5. The point of inflection of the pH dependent regioselectivity at pH 6.8 may reflect the pK_a of a certain species in the proposed reaction sequence.

Studies on the velocity of the spontaneous lumazine formation showed an exponential dependence on temperature. The data gave rise to a linear Arrhenius plot, indicating an activation energy of about 46 kJ mol^{-1} for the overall reaction. As shown recently, the abstraction of the proton at C-3 of **2** irreversibly leads to the elimination of the phosphate group under formation of diacetyl.[19] This reaction should proceed faster at elevated temperature. However, the enhanced regioselectivity at elevated temperature shows that pathway A is preferred. This could imply that the elimination of phosphate is facilitated by Schiff's base formation which is supported by experiments of Bender and Williams[21] and Hine et al.[22] who studied the deuterium exchange of the α-protons of carbonyl compounds in the presence of primary amines. They observed a dramatic increase in the exchange velocity and explained their observation by the formation of a Schiff's base.

The biomimetic formation of lumazine seems to be attributed to a combination of the two pathways A and B. In basic solution and at elevated temperature the contribution of pathway A increases due to an enhanced reactivity of **1** (at high pH) and a faster Schiff's base formation. The Schiff's base may be easily protonated, and the enhancement of the velocity is a consequence of the basicity of the Schiff's base. A high pH is necessary for the formation of a Schiff's base from the free amino group of **1**. However, if the pH was too high, a protonation of the Schiff's base would be no longer possible. Therefore, the inflection point of the regioselectivity at pH 6.8 may reflect the pK_a of the Schiff's base **5**. pK_a Values for Schiff's bases typically are in the range of 5.8 to 7.5.[23,24]

The rate constant k_{uncat} of the spontaneous lumazine formation was calculated from the pH-dependence and temperature-dependence experiments and had a value of about 0.002 M^{-1} min^{-1}. The corresponding rate constant k_{cat} of the enzyme-catalyzed reaction is 0.074 s^{-1}.[19]

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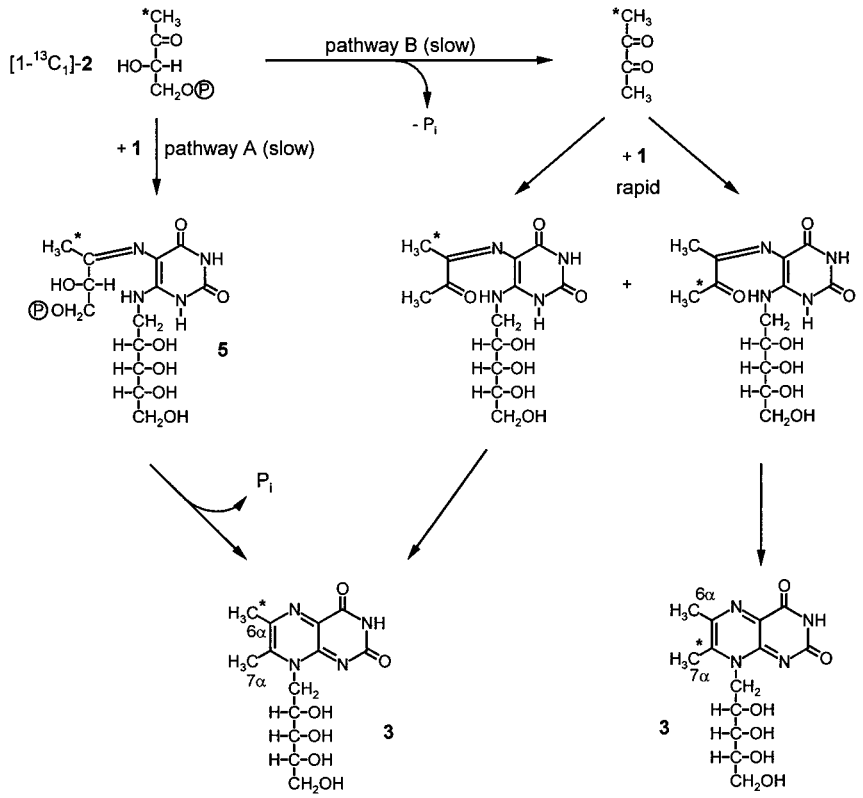

Figure 3. Proposed mechanisms for the uncatalyzed formation of **3**. ^{13}C labels are denoted by asterisks.

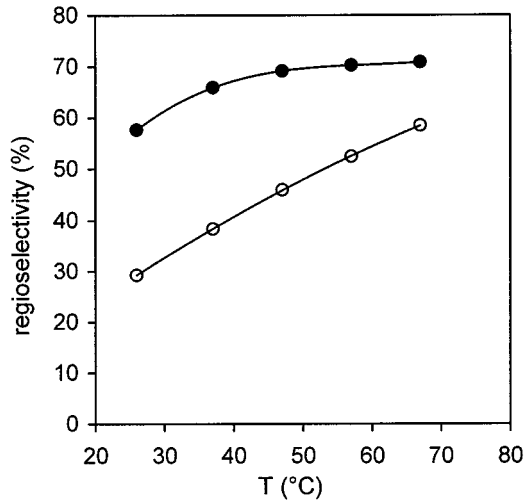

Figure 4. Regioselectivity of the biomimetic lumazine formation at different temperatures. 40 mM **1**, 1.8 mM [1-$^{13}C_1$]-**2**; ○, pH 6.70; ●, pH 7.75 (pH was not corrected for temperatures).

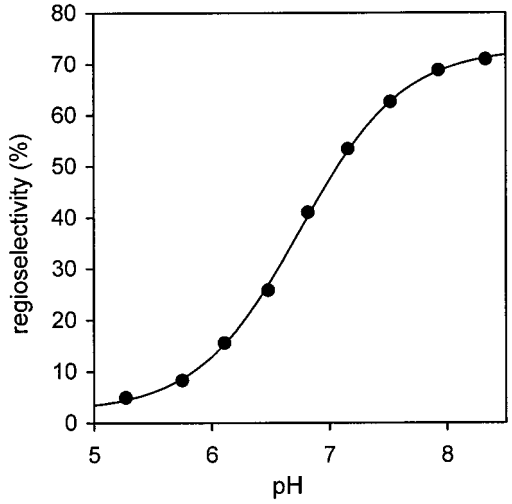

Figure 5. Regioselectivity of the biomimetic lumazine formation at different pH. 40 mM **1**, 1.8 mM [1-$^{13}C_1$]-**2**, 37 °C. Data were fitted to the function $r = (\text{low} + \text{high} \times 10^{(\text{pH} - w)})/(10^{(\text{pH} - w)} + 1)$ according to Leatherbarrow;[31] values for low, high, and w were solved by the Newton method implemented in Microsoft Excel 8.0 Solver; low = 2.2, high = 70.1, w = 6.8, sum of square errors = 2.14; the point of inflection at pH 6.8 corresponds to a regioselectivity of 39.5%

The value of k_{cat}/k_{uncat} is therefore 2220 M. It is difficult to interpret this value. However, a possible explanation may be that the nonenzymatic reaction could be as fast as the enzymatic reaction when the substrate concentrations are in the order of 10^3 M. In light of the concentration of pure water (56 M) a concentration of 10^3 M is not possible. The large value of k_{cat}/k_{uncat} may show that the enzymatic reaction is an entropy driven process.

The terminal two steps in the pathway of riboflavin biosynthesis can proceed under relatively mild conditions in the absence of catalyst. The spontaneous dismutation of the lumazine[13,15,16] and the formation of the lumazine from ribulose 1,5-bisphosphate[3] require a temperature around the boiling point of water. The uncatalyzed reaction discussed in this paper proceeds under even milder conditions and has an appreciable velocity at room temperature. Although biomimetic reaction conditions for the formation of the riboflavin precursor **1** have not been found so far, these observations address the question whether riboflavin or a related isoalloxazine could have been formed prior to the evolution of enzyme catalysis.

Experimental Section

Materials. [1-$^{13}C_1$]Methyl iodide (99% ^{13}C) was purchased from Campro, Emmerich, Germany. [2-$^{13}C_1$]-D-glucose (99% ^{13}C) was purchased from Omicron Biochemicals Inc., South Bend, IN. 5-Nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione,[25] 6,7-dimethyl-8-ribityllumazine,[10] and (2*S*)-2,3-*O*-isopropylideneglyceraldehyde[19] were prepared by published procedures. Solvents were distilled before use.

Preparation of 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (1). 5-Nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (36.7 mg, 0.1 mmol) was suspended in 10 mL of water. The suspension was hydrogenated over Pd/charcoal at room temperature and atmospheric pressure for 2 days. The solution was then quickly passed through a 0.2 μm membrane filter under an atmosphere of inert gas. A 10 μL aliquot was removed and diluted with 1 mL of hydrochloric acid (0.1 M) for the determination of the product concentration (ϵ_{268} = 24500 M^{-1} cm^{-1}).[25] The main solution was stabilized by the addition of dithiothreitol (123 mg, 0.8 mmol) and stored at −70 °C.

Preparation of [4-$^{13}C_1$]-(2*S*,3*RS*)-1,2-*O*-Isopropylidene-1,2,3-butanetriol. [1-$^{13}C_1$]Methylmagnesium iodide was prepared from magnesium (35 mmol, 0.85 g) and [1-$^{13}C_1$]methyl iodide (35 mmol, 5.0 g) in absolute ether. A solution of (2*S*)-2,3-*O*-isopropylideneglyceraldehyde in absolute ether (prepared from 17.5 mmol (5*S*)-5,6-*O*-isopropylidene-L-gulono-1,4-lactone[19]) was added. After 17 h, the reaction mixture was poured on ice and treated with a saturated solution of ammonium chloride to solubilize the precipitate. The phases were separated, and the water phase was extracted with ether. The combined organic layers were washed with 10 mL of water and dried over $MgSO_4$. The solvent was removed under reduced pressure.

Preparation of [1-$^{13}C_1$]-(3*S*)-3,4-Dihydroxy-2-butanone 4-Phosphate ([1-$^{13}C_1$]-2). The reaction steps for the preparation of [1-$^{13}C_1$]-(3*S*)-3,4-dihydroxy-2-butanone 4-phosphate ([1-$^{13}C_1$]-**2**) were carried out according to Kis et al.[19] starting with [4-$^{13}C_1$]-(2*S*,3*RS*)-1,2-*O*-isopropylidene-1,2,3-butanetriol.

Enzymatic Synthesis of [1-$^{13}C_1$]-(3*S*)-3,4-Dihydroxy-2-butanone 4-Phosphate ([1-$^{13}C_1$]-2). Small amounts of [1-$^{13}C_1$]-**2** were prepared enzymatically from [2-$^{13}C_1$]-D-glucose as described elsewhere.[26]

Proteins. The lumazine synthase/riboflavin synthase complex (formerly designated heavy riboflavin synthase) was purified from cell extracts of the derepressed mutant H94 of *Bacillus subtilis* by published procedures.[27,28]

NMR Spectroscopy. ^1H and ^{13}C NMR spectra were recorded at room temperature with AM 360, AC 250, and AC 200 spectrometers from Bruker Instruments, Karlsruhe, Germany.

Assay of (3*S*)-3,4-Dihydroxy-2-butanone 4-Phosphate. The concentration of (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**) was determined enzymatically using the lumazine/riboflavin synthase complex (heavy riboflavin synthase).[29] Assay mixtures contained 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 0.7 mM **1**, 2 μg of heavy riboflavin synthase, and **2** in a total volume of 100 μL. The concentration of **2** should be in the range of 20−50 μM for optimum accuracy. Assay mixtures were incubated at 37 °C for 1 h. Protein was precipitated by the addition of 15% trichloroacetic acid (TCA, 100 μL). The precipitate was removed by centrifugation (14000 rpm, 5 min). The samples were analyzed by reversed phase HPLC using a column of Nucleosil $10C_{18}$ (4 × 250 mm) (Macherey & Nagel, Düren, Germany). An eluent containing 10% methanol and 30 mM formic acid was used for determination of 6,7-dimethyl-8-ribityllumazine (**3**); the effluent was monitored fluorimetrically (excitation, 408 nm; emission, 487 nm). An eluent containing 40% methanol and 0.1 M ammonium formate was used for the determination of riboflavin (**4**); the effluent was monitored fluorimetrically (excitation, 445 nm; emission, 516 nm). The molar amount of **2** consumed by lumazine synthase corresponds to the amount of lumazine **3** plus two times the amount of riboflavin (**4**).

Influence of the Concentration of 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione on the Nonenzymatic Formation of Lumazine. Assay mixtures (100 μL) contained 22.5 mM potassium phosphate (pH 7.0), 5 mM **2**, and **1** (from 11 to 75 mM) at 37 °C. Aliquots (15 μL) were retrieved at intervals, quenched with 50 μL of TCA (15%), and analyzed by HPLC as described above.

pH Dependence of Nonenzymatic Formation of 6,7-Dimethyl-8-ribityllumazine. Assay mixtures containing 2 mM EDTA, 22.5 mM **1**, and 90 mM potassium phosphate buffer at pH values ranging from 5.8 to 7.3 were preincubated for 3 min at 37 °C. The reaction was started by the addition of **2** to a final concentration of 3.3 mM in a total assay volume of 75 μL. Aliquots (10 μL) were retrieved and acidified with 50 μL of TCA (15%). 6,7-Dimethyl-8-ribityllumazine was analyzed by HPLC as described above.

Temperature Dependence of Nonenzymatic Formation of 6,7-Dimethyl-8-ribityllumazine. A solution containing 112 mM potassium phosphate, pH 7.0, 2.2 mM EDTA, and 1.1 mM **1** in a total volume of 420 μL was preincubated at the indicated temperatures for 3 min. The reaction was started by the addition of 5 μL of 12.1 mM **2**. Aliquots (10 μL) were retrieved and acidified with 50 μL of TCA (15%). 6,7-Dimethyl-8-ribityllumazine was analyzed by HPLC as described above.

Regiochemistry of the Nonenzymatic Formation of 6,7-Dimethyl-8-ribityllumazine. To avoid oxidative decomposition of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**), these experiments were carried out under an atmosphere of N_2/H_2 (9:1).

Solutions containing 6.2 mM [1-$^{13}C_1$]-**2** or 85.3 mM **1** or 2 M potassium phosphate were preincubated at the desired temperature. Aliquots of these solutions (buffer, 120 μL; **2**, 220 μL; and **1**, 300 μL) were combined and incubated at the respective temperature. pH was controlled at the end of the incubation period, when all of [1-$^{13}C_1$]-**2** was consumed. Hydrochloric acid (1 M, 400 μL) was added, and precipitate was removed by centrifugation. 6,7-Dimethyl-8-ribityllumazine (**3**) was purified by HPLC using a column of Nucleosil $10C_{18}$ (20 × 250 mm). The eluent contained 10% methanol and 50 mM formic acid. The flow rate was 40 mL/min. The effluent was monitored fluorimetrically (excitation, 408 nm; emission, 487 nm). Fractions were pooled and freeze-dried. The residue was dissolved in 540 μL of 50 mM formic acid and 60 μL of D_2O were added. ^{13}C abundance at the methyl groups of the lumazine **3** was analyzed by NMR spectroscopy.[30]

Definition of Regioselectivity. The regioselectivity of the lumazine formation is defined by $((L^*_{6\alpha} - L_{6\alpha})(a + i))/(L^*_{6\alpha} + L_{6\alpha})(a - i))$ where $L^*_{6\alpha}$ and $L_{6\alpha}$ denote the amount of lumazine with or without the ^{13}C label in the 6α-methyl position, respectively; a is the ^{13}C enrichment of the starting material (99%), and i is the natural abundance of ^{13}C (1.1%).

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