Biosynthesis of Riboflavin. Stereochemistry of the 3,4-Dihydroxy-2-butanone 4-Phosphate Synthase Reaction

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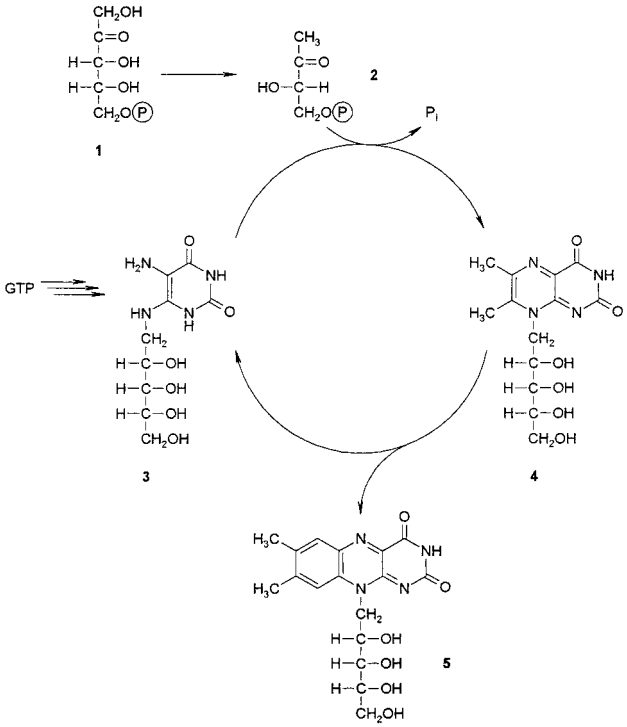
Flavocoenzymes are indispensable in all cells as cofactors of numerous enzyme-catalyzed redox reactions. A variety of pathogenic bacteria such as *Escherichia* and *Salmonella* lack the capacity to absorb riboflavin from the environment and are therefore absolutely dependent on endogenous synthesis of the vitamin.[1] Hence, the pathway of riboflavin biosynthesis is a potential target for chemotherapy of bacterial infections.

To develop a rational approach for the design of inhibitors directed against enzymes of riboflavin biosynthesis, the detailed study of the riboflavin pathway is in order. The early steps of the biosynthetic pathway[2] (Scheme 1) involve the formation of 5-amino-6-(ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione (**3**) from GTP. The pyrimidine intermediate **3** is then condensed with (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**) to form 6,7-dimethyl-8-ribityllumazine (**4**). Dismutation of the lumazine derivative yields riboflavin (**5**) and the pyrimidine intermediate **3**, which is subsequently reutilized in the biosynthetic pathway.

(3*S*)-3,4-Dihydroxy-2-butanone 4-phosphate (**2**), the second substrate for the formation of **4**, was identified relatively recently by studies with *Candida guilliermondii* and *Escherichia coli*.[3] The novel carbohydrate is formed from ribulose 5-phosphate (**1**) by a homodimeric 46 kDa enzyme requiring Mg^{2+} as cofactor. The enzyme-catalyzed reaction involves the elimination of C-4 of the substrate as formate and a rearrangement reaction forming a bond between C-3 and C-5 of the substrate. A hypothetical mechanism for this complex reaction has been proposed (Scheme 2).[3,4a,b] This paper describes the stereochemical course of the rearrangement reaction.

(5*R*)- and (5*S*)-[5-$^2H_1$]-ribulose 5-phosphate were synthesized in situ from (6*R*)- and (6*S*)-[6-$^2H_1$]glucose,[5] respectively, by published procedures.[4b,6a,b] The progress of the reactions was controlled using enzymatic assays (for further details see Supporting Information). Analogous experiments with [U-^{13}C]-labeled glucose were performed under the same conditions. ^{13}C NMR spectra clearly show that glucose is converted exclusively to ribulose 5-phosphate. The deuterium-labeled ribulose 5-phosphates could therefore be used without isolation as substrates for the enzyme, 3,4-dihydroxy-2-butanone 4-phosphate synthase. This enzyme was added when at least 65% of the glucose was converted to ribulose 5-phosphate (monitored by specific assays). The reaction mixtures contained 70 mM Tris buffer, pH 7.8, 8 mM

Scheme 1. Biosynthesis of Riboflavin



Scheme 2. Proposed Mechanism of the 3,4-Dihydroxy-2-butanone 4-Phosphate Synthase Reaction

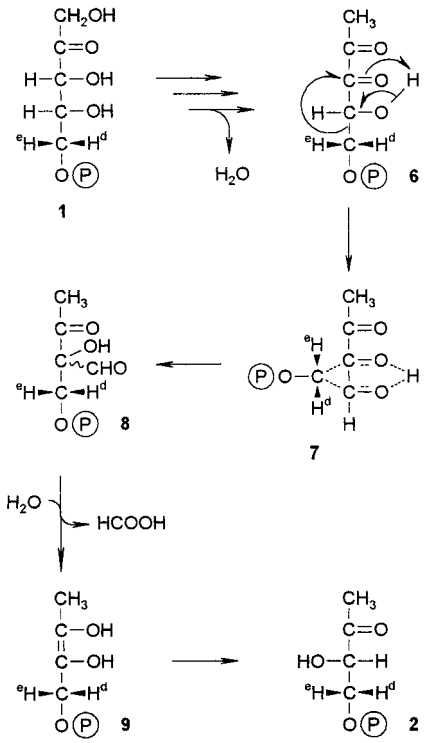


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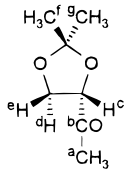
Table 1. NMR Parameters of 10

proton position[a]	chemical shift (ppm)	coupling constant (Hz)	NOE[b]
f	1.31, s, 3H		
g	1.42, s, 3H		
a	2.18, s, 3H		
d (*pro-S*)	3.92, dd, 1H	5.5 (d, c)	7.4 (a)
		8.6 (d, e)	6.1 (g)
			1.7 (f)
e (*pro-R*)	4.13, dd, 1H	7.7 (e, c)	14.2 (f)
		8.6 (e, d)	1.2 (a)
			1.0 (g)
c	4.34, dd, 1H	5.5 (c, d)	8.9 (f)
		7.7 (c, e)	8.7 (a)
			1.9 (g)

[a] See Scheme 3 for designation of atoms in **10**. [b] NOE buildup rates (dNOE/dt_{mix}; arbitrary units); coupled ^{1}H atom in parentheses.

deuterium-labeled ribulose 5-phosphate, 12 mM $MgCl_2$, and approximately 1 mg of recombinant 3,4-dihydroxy-2-butanone 4-phosphate synthase[7] in a total volume of 10 mL. The reaction mixtures were incubated for 3 h at 25 °C. The enzyme product, (3*S*)-[4-$^2H_1$]-3,4-dihydroxy-2-butanone 4-phosphate, was purified as its barium salt and was subsequently dephosphorylated by treatment with alkaline phosphatase. The resulting (3*S*)-[4-$^2H_1$]-3,4-dihydroxy-2-butanone was isolated and was converted to (4*S*)-[5-$^2H_1$]-2,2-dimethyl-4-acetyl-1,3-dioxolane[8] (**10**) by treatment with dry acetone in the presence of $ZnCl_2$/ether complex solution.[9]



10

It is obvious from the course of the chemical reactions that the configuration of the relevant group is changed neither during the formation of [5-$^2H_1$]ribulose 5-phosphate from [6-$^2H_1$]glucose nor during the synthesis of **10** from (3*S*)-[4-$^2H_1$]-3,4-dihydroxy-2-butanone 4-phosphate. So the configuration of **10** shows directly whether the rearrangement that is investigated proceeds with inversion or retention of the configuration at the phosphomethylene group of ribulose 5-phosphate.

^{1}H and ^{2}H NMR spectra of **10** were recorded in C_6F_6 using a Bruker 500 MHz NMR spectrometer equipped with fluorine lock. Stereospecific assignments of the hydrogen atoms at C-5 of **10** were obtained by two-dimensional NOESY spectroscopy. NOE buildup rates were obtained from NOESY experiments conducted with various mixing times (Table 1). Specifically, the protons of the methyl group f show NOEs with the H atoms c and e. On the other hand,

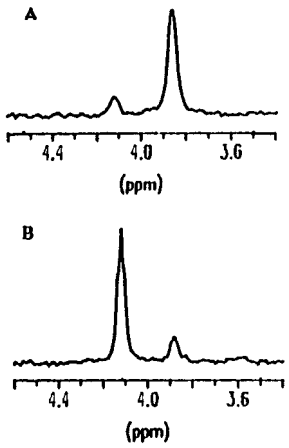


Figure 1. Deuterium NMR (^{1}H-decoupled) spectra of **10** in C_6F_6 (A, from (5*S*)-[5-$^2H_1$]-ribulose 5-phosphate; B, from (5*R*)-[5-$^2H_1$]-ribulose 5-phosphate).

H atom d shows strong NOEs to the methyl protons of g and the methyl protons a of the acetyl unit.

On the basis of these data, we assign the resonance at 3.92 ppm to H-d (*pro-S*) and the resonance at 4.13 ppm to H-e (*pro-R*) in the ^{1}H NMR spectrum of **10**. ^{2}H NMR (^{1}H-decoupled) spectra of **10** obtained from (5*R*)- and (5*S*)-[5-$^2H_1$]ribulose 5-phosphate are shown in Figure 1. The (5*R*)-substrate yields a strong ^{2}H signal at 4.1 ppm and a relatively weak signal at 3.9 ppm. Conversely, the (5*S*)-substrate yields a strong ^{2}H signal at 3.9 ppm and a weak signal at 4.1 ppm. Independently, we could show by NMR spectroscopy that the [6-$^2H_1$]glucose samples that had been used for preparation of enzyme substrates had a diastereomer ratio of approximately 85:15 with regard to the chirally labeled methylene group at C-6 of glucose. The diastereomer ratio of the resulting **10** has a similar value of 85:15.

On the basis of these data, we conclude that 3,4-dihydroxy-2-butanone 4-phosphate synthase generates (3*S*,4*S*)-[4-$^2H_1$]dihydroxy-2-butanone 4-phosphate from (5*S*)-[5-$^2H_1$]-ribulose phosphate and (3*S*,4*R*)-[4-$^2H_1$]dihydroxy-2-butanone 4-phosphate from (5*R*)-[5-$^2H_1$]ribulose phosphate. Thus, the enzyme-catalyzed rearrangement reaction shown in Scheme 2 proceeds with retention of configuration at C-5 of the substrate.

The hypothetical reaction mechanism proposed by Volk and Bacher[4] implicates the migration of C-5 of the enzyme substrate **1** as a carbanion. A carbanion mechanism should proceed with retention of configuration. The observed stereochemical course of the enzyme-catalyzed reaction is thus consistent with the proposed enzyme mechanism.

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Supporting Information Available: Experimental details for the synthesis and characterization of D-ribulose 5-phosphate (**1**), (3*S*)-3,4-dihydroxy-2-butanone, and 2,2-dimethyl-4-acetyl-1,3-dioxolane (**10**) (11 pages).

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