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# Rapid One-Pot Synthesis of Riboflavin Isotopomers

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Flavocoenzymes labeled with stable isotopes are important reagents for the study of flavoproteins using isotope-sensitive methods such as NMR, ENDOR, infrared, and Raman spectroscopy. We describe highly versatile one-pot methods for the preparation of riboflavin isotopomers labeled with $^{13}C$ in every desired position of the xylene moiety. The starting materials are commercially available $^{13}C$-labeled glucose samples, which are converted into riboflavin using enzymes of the oxidative pentose phosphate pathway in combination with recombinant enzymes of the riboflavin biosynthetic pathway. The overall reaction comprises six enzyme-catalyzed reaction steps for the synthesis of the vitamin and two auxiliary enzymes for in situ recycling of cofactors. The overall yields of riboflavin based on isotope-labeled glucose are 35−50%.

## Introduction

Flavocoenzymes derived from the vitamin riboflavin (**8**) are absolutely required in all organisms, and their central role in redox biochemistry has been investigated in considerable detail.[1−5] Flavins are also involved in a variety of nonredox processes such as blue light reception in plants,[6] photorepair of photodamaged DNA,[7] and in circadian time-keeping.[8]

The properties of the flavin chromophore can be modulated over a wide range by the protein environment in a specific flavoprotein. Monitoring the state of the flavin chromophore by physical techniques is therefore crucial for the mechanistic analysis of flavoproteins.

NMR spectroscopy has been used extensively for mechanistic studies of flavoproteins.[1,9−12] This approach requires stable isotope labeling of the flavin cofactor in order to enhance the selectivity and sensitivity of NMR detection. Stable isotope labeling of flavin cofactors can also serve as the basis for time-resolved infrared and Raman spectroscopy of flavoproteins and can contribute substantially to electron spin resonance experiments using nuclear spin sensitive methods such as ENDOR.[13−20]

Ideally, these spectroscopic techniques should utilize protein samples carrying a variety of differently isotope-substituted flavocoenzymes. Synthetic procedures for a number of riboflavin isotopomers have been reported in the literature,[10,21,22] but the preparation of a reasonably comprehensive library of isotopomers is laborious and time-consuming.

A more elegant approach to the preparation of isotopomer libraries by parallel synthesis is possible by enzyme-assisted procedures. The enzymes of the riboflavin biosynthetic pathway can be expressed in high yields in recombinant microbial hosts.[23−26] As shown in this

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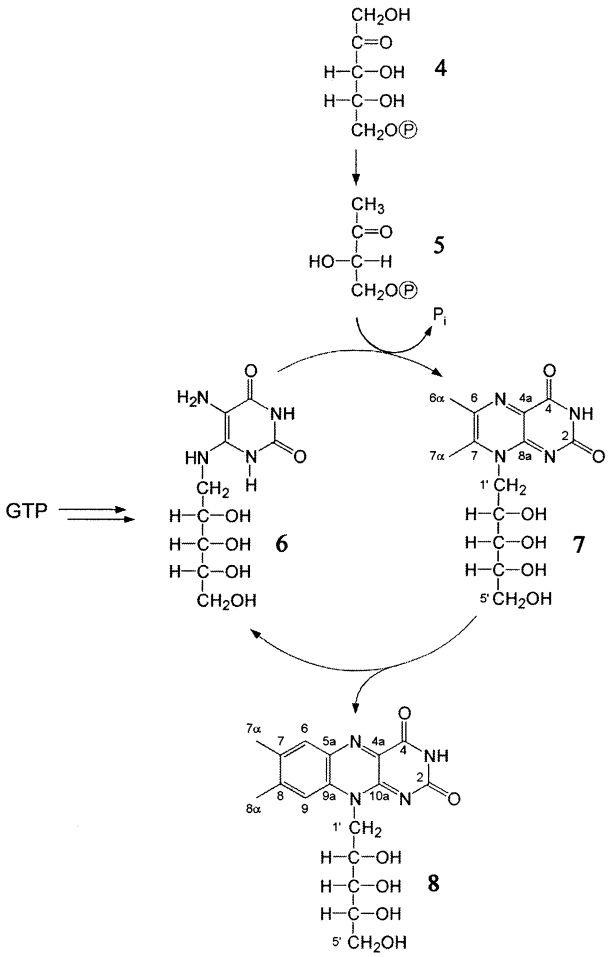


**FIGURE 1.** Biosynthesis of riboflavin (**8**).

paper, they can be used for the preparation of numerous different riboflavin isotopomers with overall yields of 35−50% by convenient one-pot procedures using $^{13}$C-labeled glucose as starting material.

## Results

The xylene ring of riboflavin is biosynthetically assembled from two molecules of 3,4-dihydroxy-2-butanone 4-phosphate that become connected in an antiparallel orientation by a unique reaction sequence (Figure 1). Briefly, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**6**) is condensed with 3,4-dihydroxy-2-butanone 4-phosphate (**5**), affording 6,7-dimethyl-8-ribityllumazine (**7**) under the catalytic action of 6,7-dimethyl-8-ribityllumazine synthase. The lumazine is subsequently dismutated by riboflavin synthase under formation of riboflavin and **6**.[27−29] In this bisubstrate reaction, one lumazine type substrate serves as the donor of a four-carbon unit and the second lumazine molecule serves as acceptor, whereby it is converted to riboflavin. The pyrimidine type product **6** of riboflavin synthase can be recycled by lumazine synthase.[30]

3,4-Dihydroxy-2-butanone 4-phosphate **(5)** is formed enzymatically from ribulose 5-phosphate (**4**) by the catalytic activity of 3,4-dihydroxy-2-butanone 4-phosphate synthase.[31] The enzyme substrate, ribulose 5-phosphate, can be obtained enzymatically from glucose by published procedures.[32−34] A variety of $^{13}$C-substituted glucose isotopomers are commercially available. It is therefore possible to generate a variety of riboflavin isotopomers with $^{13}$C substitution of the xylene ring from appropriate glucose substrates.

The reaction sequence for enzymatic conversion of glucose to riboflavin is summarized in Figure 2. Glucose (**1**) is converted to ribulose 5-phosphate (**4**) in three enzymatic steps requiring 1 equiv of ATP and 2 equiv of $NADP^+$. ATP can be regenerated in situ by pyruvate kinase using phosphoenolpyruvate (PEP) as phosphate donor, and $NADP^+$ can be regenerated in situ by glutamate dehydrogenase using 2-ketoglutarate as oxidant.

The enzyme-catalyzed conversion of ribulose 5-phosphate to 3,4-dihydroxy-2-butanone 4-phosphate involves the release of the C-4 of ribulose phosphate as formate and is thermodynamically irreversible.[31] The reaction catalyzed by lumazine synthase affords **7**, water, and inorganic phosphate and is also thermodynamically irreversible.[35] The dismutation reaction catalyzed by riboflavin synthase results in the formation of an aromatic ring and is again thermodynamically irreversible.[27] Thus, the overall reaction sequence in Figure 2 is characterized by a significant negative Gibbs free energy increment. It involves six forward reaction steps, two cofactor regeneration cycles, and a total of eight enzymes. The only products generated in stoichiometric amount are riboflavin, glutamate, pyruvate, formate, carbon dioxide, and inorganic phosphate. ATP and $NADP^+$ are only required in catalytic amounts. Most importantly, the entire reaction sequence can be performed as a one-pot reaction. Incubation of the reaction mixture for 3 days at 37 °C is followed by workup, affording yields of 35−50% based on the proffered glucose. Riboflavin crystallizes spontaneously from the one-pot reaction mixture and can be harvested by centrifugation followed by recrystallization. The yield can be improved by workup of the mother liquors via absorption of riboflavin on magnesium silicate.

The transfer of carbon atoms from glucose into riboflavin is indicated by the letters a−f in Figure 2. Carbon atoms 2−4 and 6 of glucose become part of the xylene moiety of riboflavin. As a consequence of the dismutation reaction catalyzed by riboflavin synthase, a single-labeled glucose precursor diverts $^{13}$C to two positions in the xylene ring of the vitamin.

Using this approach, a variety of [$^{13}C_2$]- and [$^{13}C_8$]-isotopomers of riboflavin can be obtained with a minimum of experimental effort. In fact, it is convenient to synthesize all desired isotopomers in parallel one-pot reactions, since the reactant mixtures are all identical except for the $^{13}$C-labeled glucose.

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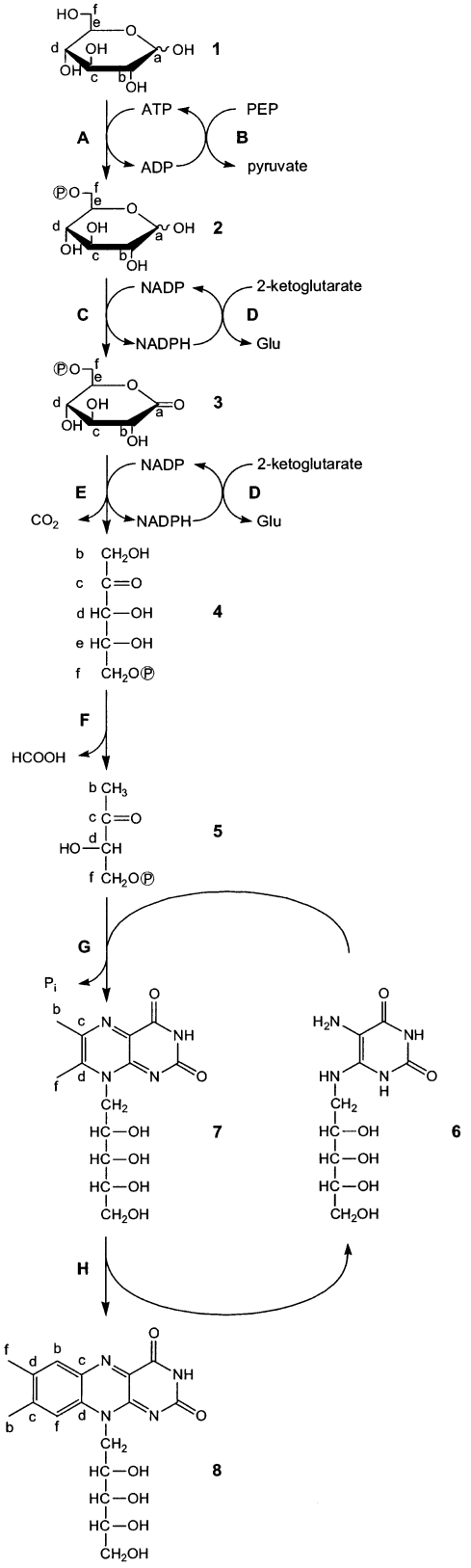


**FIGURE 2.** Enzymatic synthesis of riboflavin (**8**): A, hexokinase; B, pyruvate kinase; C, glucose 6-phosphate dehydrogenase; D, glutamate dehydrogenase; E, 6-phosphogluconate dehydrogenase; F, 3,4-dihydroxy-2-butanone 4-phosphate synthase; G, 6,7-dimethyl-8-ribityllumazine synthase; H, riboflavin synthase.

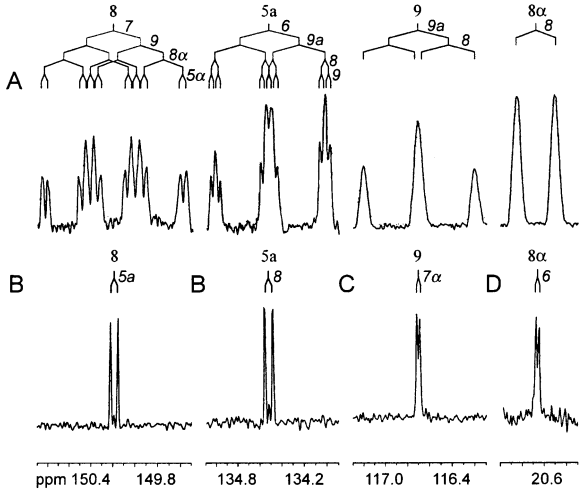


**FIGURE 3.** $^{13}C$ NMR signals of (A) [5a,6,7,7α,8,8α,9,9a-$^{13}C_8$]-riboflavin, (B) [5a,8-$^{13}C_2$]riboflavin, (C) [7α,9-$^{13}C_2$]riboflavin, and (D) [6,8α-$^{13}C_2$]riboflavin.

The reactions catalyzed by lumazine synthase and riboflavin synthase are regiospecific. $^1H$ and $^{13}C$ NMR analysis showed that the $^{13}C$ enrichment in the various $^{13}C_2$-labeled riboflavin samples is about 95%. The formation of undesired regioisomers can be explained by spontaneous decay of **5**, affording butanedione. Butanedione can react spontaneously with the pyrimidine **6** under formation of **7**, which can subsequently serve as a donor or acceptor substrate for riboflavin synthase. For example, starting from [3-$^{13}C_1$]glucose, [2-$^{13}C_1$]**5** is generated by the consecutive action of four enzymes (cf. Figure 2). The spontaneous decay of [2-$^{13}C_1$]**5** leads to [2-$^{13}C_1$]-butanedione. Due to the symmetry of butanedione, the reaction with **6** results in a 1:1 mixture of [6-$^{13}C_1$]**7** and [7-$^{13}C_1$]**7**, which reacts to give a mixture of [5a,8-$^{13}C_2$]**8**, [5a,7-$^{13}C_2$]**8**, [7,9a-$^{13}C_2$]**8**, and [8,9a-$^{13}C_2$]**8**. Indeed, the $^{13}C$ NMR spectrum of riboflavin obtained from [3-$^{13}C_1$]glucose indicated the presence of small amounts of [5a,7-$^{13}C_2$] and [8,9a-$^{13}C_2$] isotopomers (note the small signals in Figure 3B located approximately at the center of the doublets arising by coupling between the labeled C-5a and C-8). The slight contamination of the products by these impurities appears tolerable in the biophysical study of flavoenzymes reconstituted with the synthetic flavins. Anyhow, it should be noted that all nonlabeled positions contain about 1.1% $^{13}C$, representing the natural $^{13}C$ abundance.

Using the synthetic riboflavin samples, we could determine 17 $^{13}C-^{13}C$ coupling constants for the xylene ring of riboflavin which are summarized in Table 1. Using these parameters, the complex multiplets of [5a,6,7,7α,8,8α,9,9a-$^{13}C_8$]riboflavin can be simulated in excellent agreement with the experimental data (Figure 4). Notably, certain lines of [5a,6,7,7α,8,8α,9,9a-$^{13}C_8$]riboflavin are significantly broadened due to long-range coupling via coupling constants that are too small to be resolved but jointly result in appreciable line broadening.

## Discussion

Chemical synthesis of complex organic molecules typically requires the isolation and purification of intermediates after each reaction step. The process is laborious,

**TABLE 1. NMR Data of $^{13}$C-Labeled Riboflavin Isotopomers in $D_2O$**

| | chemical shifts (ppm) | | coupling constants (Hz)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| position | $^{13}$C | $^{1}$H | 5a | 6 | 7 | 7α | 8 | 8α | 9 |
| 5a | 134.5 | | | | | | | | |
| 6 | 130.2 | 7.53 | 67 | (164) | | (4) | | | |
| 7 | 139.2 | | | 51 | | | | | |
| 7α | 18.5 | 2.26 | | (5) | 44 | (128) | | | |
| 8 | 150.2 | | 7 | | 59 | 2 | | | |
| 8α | 20.7 | 2.37 | | 3 | 2 | | 43 | (128) | (6) |
| 9 | 116.7 | 7.62 | 5 | | | 2 | 51 | (5) | (162) |
| 9a | 131.7 | | 58 | 5 | 5 | | | 6 | 65 |

[a] $^{13}$C−$^{13}$C and $^{1}$H−$^{13}$C coupling constants; $^{1}$H−$^{13}$C coupling constants are shown in parentheses.

and the overall yield depends on the completeness of each reaction step. The disposal of undesired side products can be a major cost factor.

On the other hand, the biosynthesis of complex natural products in cells and organisms proceeds virtually without the formation of undesired side products, and a very large number of reactions (on the order of hundreds to thousands depending on the genetic complexity of the organism under study) can proceed simultaneously and without undesired interference. These simultaneous procedures occur in "reactors" (i.e. cells and organelles) with volumes in the femtoliter to picoliter range (cells are quintessential "nanoreactors").

The distinguishing features of cellular biosynthesis are (i) the use of catalysts of high (although not absolute) specificity with regard to substrate selectivity, regiocontrol, and stereocontrol and (ii) the control of Gibbs free energy gradients. Notably, the rate of enzyme catalysis is not always exceedingly high. Although the rates of certain fast enzymes are diffusion-controlled, the enzymes for anabolic processes typically operate with turnover numbers in the range of milliseconds to minutes. As an example for low rates, enzymes involved in the biosynthesis of riboflavin have turnover numbers in the range of 2 per minute and per subunit.[23−25]

Some of the characteristics of cellular metabolism can be emulated for the purpose of natural product synthesis in vitro. (i) The availability of enzymes catalyzing a wide variety of reactions is rapidly increasing. This is due, among other factors, to the unfolding sequencing projects for a progressively larger variety of microorganisms, plants, and animals. Using recombinant gene technology in combination with affinity purification of, for example, histidine-tagged recombinant proteins on metal chelate columns, the cognate proteins can be obtained with a limited and fairly predictable effort. (ii) A sufficiently large Gibbs free energy gradient in order to drive reactions to completion can be maintained easily when specific enzyme steps of the biosynthetic pathway used are inherently thermodynamically irreversible. In the present example, 3,4-dihydroxy-2-butanone 4-phosphate synthase, 6,7-dimethyl-8-ribityllumazine synthase, and riboflavin synthase are all characterized by large negative $\Delta G$ values (due to elimination of formate in the case of 3,4-dihydroxy-2-butanone 4-phosphate, a condensation reaction accompanied by release of phosphate and water in the case of 6,7-dimethyl-8-ribityllumazine synthase, and of aromatic ring formation in case of riboflavin synthase). Moreover, negative $\Delta G$ values can be enforced by coupling of biosynthetic reaction steps to exergonic reactions using auxiliary enzyme systems in conjunction with auxiliary substrates (pyruvate kinase and glutamate dehydrogenase in the case of riboflavin production described in this study).

A potent method used by cells to modulate $\Delta G$ consists of the transport of metabolites across compartmental boundaries. Thus, metabolites can be sequestered by deposition in plant vacuoles or by excretion into the surrounding medium (intercellular fluid or fermentation medium). Control of free enthalpy gradients via sequestration of the final metabolite is not easily implemented in biomimetic in vitro methods. The situation is particularly favorable in the specific case of riboflavin, which precipitates from the reaction mixture due to its poor solubility in water. However, the thermodynamic aspects of the overall reaction would have been sufficiently favorable, even without that additional free energy sink.

The reactions described in this paper proceed with an overall yield of 35−50%. Apart from losses during workup, the relatively long incubation times may have resulted in the loss of phosphate residues from intermediates by residual phosphatase activities present in the commercial enzymes and/or the recombinant enzymes used with a consecutive decrease of yield.

Generally, enzyme purity is an important factor in multienzyme one-pot reaction. A detrimental contaminating enzyme can be introduced with any of the enzymes in the mixture.

Recombinant enzymes can frequently be designed for efficient purification by affinity chromatography via fusion with metal-chelating, carbohydrate-binding, or protein-binding fusion domains. An important factor limiting the overall efficacy of enzyme-assisted synthesis may then be the limited purity of commercially obtained auxiliary enzymes.

Biomimetic procedures are particularly useful for the preparation of isotope-labeled molecules. In case of flavocoenzymes, a wide variety of single-labeled as well as multiply labeled isotopomers is required for biophysical studies on flavoproteins using NMR, EPR, infrared, and Raman spectroscopy techniques.[1,9−21]

Methods for the synthesis of certain riboflavin isotopomers have been reported, but they are laborious by comparison with the present method. $^{13}$C-Labeled flavins have been reported earlier (e.g. [2-$^{13}C_1$]-, [4,10a-$^{13}C_2$]-, and [4a-$^{13}C_1$]riboflavin).[10,21,22] An earlier method for the introduction of $^{13}$C into the xylene ring had only limited regiospecificity.

The enzyme-assisted method described in this paper can be scaled down to very small volumes. Thus, it could be easily adapted for the production of radiolabeled riboflavin at the highest possible specific activity from a readily available starting material, glucose. Generally, down-scaling chemical synthesis methods for the purpose of preparing radiolabeled compounds of high specific activity can pose very difficult problems. By comparison, down-scaling as well as upscaling is simple with enzyme-catalyzed processes.

The versatility of enzyme-assisted long-shot one-pot synthesis using cascades of recombinant enzymes is growing rapidly with the availability of an increasingly larger number of genes and recombinant enzymes from a large variety of organisms. The set of isotope-labeled

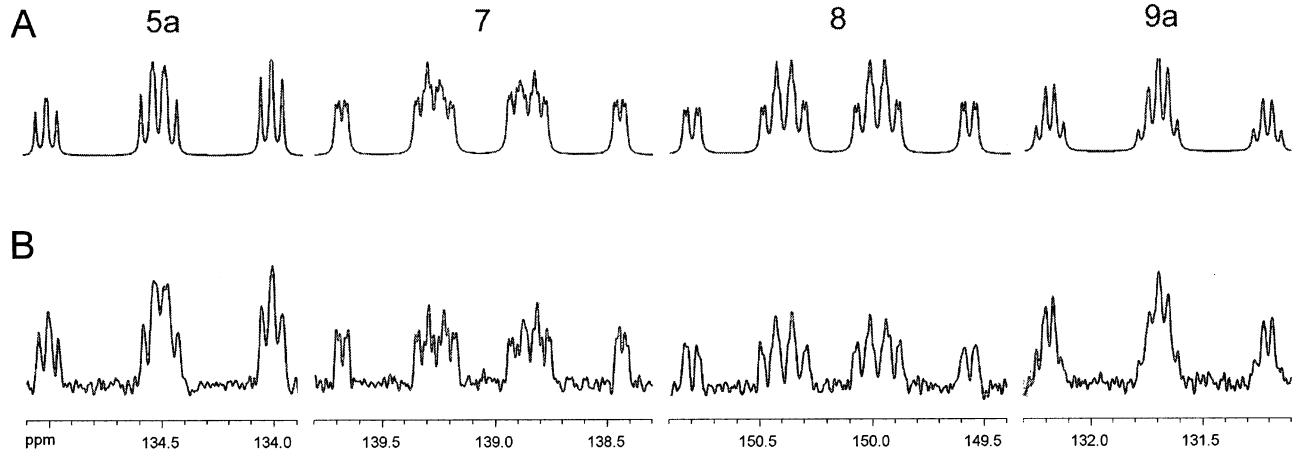


**FIGURE 4.** $^{13}C$ NMR signals of [5a,6,7,7α,8,8α,9,9a-$^{13}C_8$]riboflavin: (A) simulated and (B) experimental data.

substrates required as starting materials is quite limited, because all natural products are assembled from a small number of intermediary metabolites. Thus, processes similar to those used in this paper may become practical even for preparation of unlabeled natural products.

## Experimental Section

**Materials.** [2-$^{13}C_1$]-, [3-$^{13}C_1$]-, [4-$^{13}C_1$]-, [6-$^{13}C_1$]-, and [U-$^{13}C_6$]-glucose were obtained from Omicron, South Bend, IN. 5-Nitroso-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione was prepeared by a published procedure.[22,36]

**Proteins.** Recombinant 3,4-dihydroxy-2-butanone 4-phosphate synthase from *Escherichia coli*, 6,7-dimethyl-8-ribityllumazine synthase from *Bacillus subtilis*, and riboflavin synthase from *E. coli* were prepared by published procedures.[23−26]

**General Procedures.** $^{13}C$ NMR spectra were recorded at 125.6 MHz. The chemical shifts were referenced to external trimethylsilylpropane sulfonate. Riboflavin spectra were recorded in 90% $H_2O$/10% $D_2O$ (pH 7.0, uncorrected glass electrode reading).

**[5a,6,7,7α,8,8α,9,9a-$^{13}C_8$]Riboflavin.** A solution containing 100 mM Tris hydrochloride (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.02% sodium azide, 500 mg (2.69 mmol) of [U-$^{13}C_6$]-glucose, 30 mg (0.05 mmol) of ATP, 610 mg (2.96 mmol) of phosphoenolpyruvate, 40 mg (0.05 mmol) of $NADP^+$, 995 mg (5.92 mmol) of 2-ketoglutarate, 317 mg (5.92 mmol) of $NH_4Cl$, 380 mg (1.38 mmol) of **6** [freshly prepared by hydrogenation of 5-nitroso-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione over Pd/charcoal], 100 U of hexokinase, 100 U of pyruvate kinase, 50 U of glucose 6-phosphate dehydrogenase, 50 U of 6-phosphogluconate dehydrogenase, 100 U of glutamate dehydrogenase, 10 U of phosphoriboisomerase, 5 mg (1.4 U) of 3,4-dihydroxy-2-butanone 4-phosphate synthase from *E. coli*, 75 mg (15 U) of 6,7-dimethyl-8-ribityllumazine synthase from *B. subtilis*, and 50 mg (1.1 U) of riboflavin synthase from *E. coli* in a total volume of 200 mL was incubated for 72 h under an atmosphere of argon in the dark at room temperature. During the first hours of incubation, the pH was repeatedly adjusted to 7.8 by the addition of 3 M NaOH. The orange precipitate was harvested by centrifugation and crystallized from 3 M acetic acid.

The combined mother liquors were applied to a column of Florisil (2.8 × 10 cm) which was washed with 400 mL of water and developed with 200 mL of acetone/water (1:4 v/v) followed by 600 mL of acetone/water (1:1, v/v). Fractions were analyzed by thin-layer chromatography using cellulose plates that were developed in 3% $NH_4Cl$. Fractions were combined. Acetone was evaporated under reduced pressure. Riboflavin was crystallized from the concentrated aqueous solution for a total yield of 204 mg (0.53 mmol, 39%): $^{13}C$ NMR ($D_2O$, 126 MHz) $\delta$ 18.5 (7α, d, $J_{CC}$ = 44 Hz), 20.7 (8α, d, $J_{CC}$ = 43 Hz), 116.7 (9, dd, $J_{CC}$ = 65 Hz, 51 Hz), 130.2 (6, dd, $J_{CC}$ = 67 Hz, 51 Hz), 131.7 (9a, ddddd, $J_{CC}$ = 65 Hz, 58 Hz, 6 Hz, 5 Hz, 5 Hz), 134.5 (5a, dddd, $J_{CC}$ = 67 Hz, 58 Hz, 7 Hz, 5 Hz), 139.2 (7, ddddd, $J_{CC}$ = 59 Hz, 51 Hz, 44 Hz, 5 Hz, 2 Hz), 150.2 (8, dddd, $J_{CC}$ = 59 Hz, 51 Hz, 43 Hz, 7 Hz); MS (ESI, $CH_3CN$) *m*/*z* 385.3 $[M + 1]^+$.

**[6,8α-$^{13}C_2$]Riboflavin.** The isotopomer was prepared as described above from 500 mg of [2-$^{13}C_1$]**1** as labeled starting material to yield 189 mg (0.50 mmol, 36%): $^{13}C$ NMR ($D_2O$, 126 MHz) $\delta$ 20.7 (8α, d, $J_{CC}$ = 3 Hz), 130.2 (6, d, $J_{CC}$ = 3 Hz); MS (ESI, $CH_3CN$) *m*/*z* 379.3 $[M + 1]^+$.

**[5a,8-$^{13}C_2$]Riboflavin.** [5a,8-$^{13}C_2$]**8** was prepared as described above from 350 mg of [3-$^{13}C_1$]**1** as labeled starting material to yield 178 mg (0.47 mmol, 48%): $^{13}C$ NMR ($D_2O$, 126 MHz) $\delta$ 134.5 (5a, d, $J_{CC}$ = 7 Hz), 150.2 (8, d, $J_{CC}$ = 7 Hz); MS (ESI, $CH_3CN$) *m*/*z* 379.3 $[M + 1]^+$.

**[7,9a-$^{13}C_2$]Riboflavin.** [7,9a-$^{13}C_2$]**8** was prepared as described above from 55 mg of [4-$^{13}C_1$]**1** as labeled starting material to yield 28 mg (74 μmol, 49%): $^{13}C$ NMR ($D_2O$, 126 MHz) $\delta$ 131.7 (9a, d, $J_{CC}$ = 5 Hz), 139.2 (7, d, $J_{CC}$ = 5 Hz); MS (ESI, $CH_3CN$) *m*/*z* 379.3 $[M + 1]^+$.

**[7α,9-$^{13}C_2$]Riboflavin.** [7α,9-$^{13}C_2$]**8** was prepared as described above with 500 mg of [6-$^{13}C_1$]**1** as labeled starting material to yield 177 mg (0.47 mmol, 34%): $^{13}C$ NMR ($D_2O$, 126 MHz) $\delta$ 18.5 (7α, d, $J_{CC}$ = 2 Hz), 116.7 (9, d, $J_{CC}$ = 2 Hz); MS (ESI, $CH_3CN$) *m*/*z* 379.3 $[M + 1]^+$.

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