Enzymatic Synthesis of Riboflavin and FMN Specifically Labeled with ^{13}C in the Xylene Ring

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

[6α,7α-$^{13}C_2$]6,7-Dimethyl-8-ribityllumazine, [^{13}C]6,7-Dimethyl-8-ribityllumazine, [^{13}C]Riboflavin, [6,9,7α,8α-^{13}C]Riboflavin, [^{13}C]FMN

The condensation of 3-hydroximino-2-butanone (**1**) with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**2**) yields 6,7-dimethyl-8-ribityllumazine (**3**). At slightly alkaline pH, the carbonyl group of **1** reacts preferentially with the 5-amino group of **2** (regioselectivity, 4:1). Under acidic conditions, the reaction occurs with higher yield and marginal regioselectivity of opposite direction (1:1.4). Appropriately ^{13}C-labeled samples of **1** afford **3** labeled at C-6α, C-6, C-7 or C-7α. [6α,7α-$^{13}C_2$]-**3** was prepared by condensation of **2** with [1,4-$^{13}C_2$]diacetyl. The lumazines **3** were converted to riboflavin by the enzyme, riboflavin synthase, with almost quantitative yield. By this procedure, any C-atom of the carbocyclic moiety of riboflavin can be selectively labeled with ^{13}C at high abundance. Phosphorylation yields the respectively ^{13}C-labeled FMN samples.

Flavoenzymes have a central role in biochemical redox reactions involving a wide variety of different substrates [1]. The study of flavin-protein interactions by ^{13}C NMR observation has contributed significantly to the present knowledge on flavoprotein structure and mechanism [2–4]. These experiments require the reconstitution of an appropriate apoprotein with a ^{13}C-labeled flavocoenzyme. However, the studies have been limited to the pyrimidine C-atoms of the flavin chromophore which can be relatively easily labeled by a synthetic approach starting from ^{13}C-barbituric acid [2]. Flavins with ^{13}C-labels in the xylene moiety of the chromophore have not been available hitherto. We describe their preparation by an enzymatic approach, which permits the labeling of each C-atom of the xylene moiety from simple precursors.

Materials and Methods

Materials

Light riboflavin synthase was partially purified from the derepressed mutant *Bacillus subtilis* H 94 by published procedures [5]. Preparations used in this study had specific activities of approximately 1000 $nmol \cdot mg^{-1} \cdot h^{-1}$.

NMR measurements

75.4 MHz ^{13}C NMR spectra of riboflavin in DMSO-d_6 were recorded on a Bruker WM-300 NMR spectrometer. The spectra were acquired under conditions which gave equal intensities for the pairs of carbons of interest in natural abundance riboflavin. Absolute enrichments of protonated carbons were also checked by ^{1}H NMR.

*5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**2**)*

A suspension of palladium (10%) on charcoal (180 mg) and 5-nitro-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1.58 g, 5.2 mmol) [6] or 5-nitroso-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1.50 g, 5.2 mmol) [7] in 50 ml of water was hydrogenated at room temperature and atmospheric pressure until the absorption of hydrogen was terminated (about 20 to 40 h). The solution was used immediately.

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[^{13}C]Acetyl chloride

Freshly distilled $POCl_3$ (5.8 ml, 63 mmol) was added slowly to 5 g (60.2 mmol) of sodium [^{13}C]acetate at 0 °C with stirring [8]. Acetyl chloride was obtained by distillation (4.6 g, 57.8 mmol, 96.0%).

[1-^{13}C]2-Butanone

Methyl magnesium iodide was prepared under an atmosphere of nitrogen from 5 g (35 mmol) of [^{13}C]methyl iodide and 0.9 g of Mg in 15 ml of absolute ether. A solution of 3.6 ml (50.7 mmol) of propionitrile in 1.5 ml of ether was added. The mixture was stirred at 40 °C for 12 h. Icecold 5 M HCl (11 ml) was added together with 6 g of ice. The aqueous phase was extracted three times with ether (3 ml). Butanone was determined by gas chromatography. Yield: 8.75 mmol, 25.0%. The ether solution was used directly.

[2-^{13}C]2-Butanone

Copper zinc alloy (17.0 g) was suspended under an atmosphere of nitrogen in a mixture containing 6.0 ml of anhydrous diethyleneglycol diethyl ether, 3.0 g of 2-pentylbutyrate, and a trace of iodine. Ethyl iodide (8.4 ml, 105 mmol) was added with stirring [9]. The suspension was heated to 90 °C within 75 min. The mixture was allowed to cool to about 35 °C and was filtered through a pad of glass wool under an atmosphere of nitrogen. [1-^{13}C]Acetyl chloride (4.6 g, 57.8 mmol) in 10 ml of diethyleneglycol diethylether was added at 0 °C during a period of 40 min. The solution was allowed to warm to room temperature and was stirred for 25 min. The product (33.0 mmol, 56.9%) was obtained by distillation.

[4-^{13}C]2-Butanone

Potassium hydride (55 mmol) was suspended in 35 ml of anhydrous diethyl ether under an atmosphere of nitrogen. A solution of acetone (2.9 ml) in 10 ml of ether was added slowly at room temperature with stirring. After 40 min, the suspension was cooled to 0 °C. TEMED (5.5 ml) and 2.5 M butyl lithium (14.2 ml, 35.5 mmol in *n*-hexan) were dissolved in ether (15 ml) and the solution was added to the reaction mixture over a period of 2 min [10]. The yellow suspension was stirred at 0 °C for 20 min and subsequently cooled to −78 °C. [^{13}C]Methyl iodide (5 g, 35 mmol) in 5 ml of diethyl ether was slowly added with vigorous stirring. The flask was connected to a condensor cooled by liquid nitrogen, and the solvent was removed by lyophilization at a pressure of 0.03 Torr. The residue was hydrolyzed at 0 °C by the addition of 10 ml of 7 M hydrochloric acid and 12 ml of ether. The flask was sealed to a condensor cooled with liquid nitrogen. The condensate was obtained by distillation at room temperature under reduced pressure in a closed system. The organic phase was separated from water at −18 °C. Yield: 14.7 mmol, 42%.

[^{13}C]3-Hydroximino-2-butanone [11]

[^{13}C]2-Butanone (8 mmol) and 30 µl of conc. HCl were dissolved in 6 ml of dry ether. Ethylnitrite (6.5 ml of a 15% solution in ethanol) was added over a period of 30 min at room temperature. The flask was sealed to a condensor cooled in liquid nitrogen, and the solvent was removed by lyophilization at a pressure of 0.02 Torr. The product was sublimated from the residue at 60 °C and 2×10^{-2} Torr to a flask cooled by liquid nitrogen. Yield: 698 mg, 6.1 mmol, 76.3%.

[2-^{13}C]Acetic acid ethyl ester

[2-^{13}C]Acetyl-chloride (1.8 ml, 25.0 mmol) was stirred in a 25 ml flask equipped with a reflux condensor and 1.5 ml of dry ethanol was slowly added at 0 °C. After the addition of β-picoline (2.5 ml, 25.5 mmol) the flask was sealed to a condensor cooled in liquid nitrogen. The product was obtained by distillation at room temperature and under reduced pressure (1.97 ml, 20.1 mmol, 80.7%).

[1,4-$^{13}C_2$]2,3-Bistrimethylsiloxy-2-butene [12]

Powdered sodium (0.95 g, 41.3 mmol) was suspended in a mixture of 15 ml of anhydrous diethyl ether and 5.2 ml (41.3 mmol) trimethylchlorosilane under an atmosphere of nitrogen. The mixture was stirred at 40 °C, and a solution of [2-^{13}C]acetic acid ethyl ester (19.8 mmol) in 10 ml of diethyl ether was added dropwise over a period of 45 min. The mixture was boiled under reflux for 20 h. The liquid was removed, and the residue was extracted 4 times with 10 ml aliquots of diethyl ether under an atmosphere of nitrogen. Subsequent to the evaporation of ether, the product was distilled (69 °C, 13 Torr). Yield: 1.35 ml (5.01 mmol, 50.6%).

[1,4-$^{13}C_2$]Diacetyl

The product from the previous step was dissolved in 1.5 ml of diethyl ether, and 0.5 ml of 1 M hydrochloric acid was added [12]. The mixture was boiled under reflux with vigorous stirring for 1 h. A 65% aqueous solution of $FeCl_3$ (9 ml) was added, and the mixture was heated under reflux for 2.5 h under vigorous stirring. The flask was sealed to a condensor cooled in liquid nitrogen, and the product was obtained by distillation at room temperature under reduced pressure (0.02 Torr). The residual slurry was dissolved in 6 ml of 1 N HCl, stirred at 60 °C for 1 h and distilled as described above. The distilled fractions were combined. The diacetyl content of the solutions was determined by gas chromatography. Yield: 1.57 mmol, 31.3%.

*[^{13}C]6,7-Dimethyl-8-ribityllumazine (**3**) from [^{13}C]3-hydroximino-2-butanone (**1**)*

Method A – All operations were performed in darkness or dim light to avoid photodecomposition of **3**. A freshly prepared aqueous solution of **2** (50 ml, 0.1 M) was adjusted to pH 7.65 by the addition of 4 M NaOH. [^{13}C]3-Hydroximino-2-butanone (**1**) (500 mg, 4.90 mmol) was added, the pH was again adjusted to 7.65 and the mixture was heated at 89 °C for 3.5 h under an atmosphere of nitrogen. Remaining **1** (about 200 mg) was extracted with diethyl ether (6 × 15 ml). The palladium catalyst was removed by filtration, and the solution was placed on a column of Dowex 50 WX8 (H^+ form, 2.2 × 20 cm) [13]. The green-fluorescent **3** was eluted with water and concentrated to dryness. Crystallization from water yielded 173 mg (17.6%) of yellow crystals.

Method B – [^{13}C]3-Hydroximino-2-butanone (300 mg, 2.94 mmol) was added to a freshly prepared aqueous solution of 0.1 M **2** (50 ml). The pH was adjusted to 5.0. The mixture was heated in the dark at 80 °C for 20 h under an atmosphere of nitrogen. The product was isolated and crystallized as described above. Yield: 1.46 mmol, 49.2%.

[6α,7α-$^{13}C_2$]6,7-Dimethyl-8-ribityllumazine from [1,4-$^{13}C_2$]diacetyl

An aqueous solution of [1,4-$^{13}C_2$]diacetyl (1 mmol) was added to a freshly prepared solution of 3.3 mmol of **2** under an atmosphere of nitrogen [13]. The pH was adjusted to 5, and the mixture was kept in the dark at room temperature overnight. The product was purified as described above. Yield: 306 mg, 0.93 mmol, 93.3%.

*[^{13}C]Riboflavin (**4**)*

All operations were performed in darkness or under dim light. [^{13}C]6,7-Dimethyl-8-ribityllumazine (**3**) (66 mg, 0.2 mmol) was dissolved in 400 ml of 0.1 M phosphate buffer pH 7.0 containing 10 mM sodium sulfite, 10 mM EDTA, and 0.02% sodium azide. Riboflavin synthase (42,000 units) was added, and the mixture was incubated at 42 °C for 12 h. The solution was passed through a column of Florisil (2.8 × 10 cm). The column was washed with 600 ml of water. Fluorescent material was eluted with a mixture of acetone/2 M ammonia (1:1, v/v; 600 ml). The solution was evaporated to dryness under reduced pressure. The residue was dissolved in 100 ml of H_2O at 60 °C, and the solution was placed on a column of Dowex 50 WX8 (H^+ form, 1.5 × 15 cm). The column was developed with 0.1 M HCl. Fractions were pooled and concentrated to dryness under reduced pressure. The residue was crystallized from 2 M acetic acid. Yield: 0.96 mmol, 96%.

[^{13}C]Riboflavin 5'-phosphate (FMN) [14, 15]

Freshly prepared monochlorophosphoric acid (1.2 ml) was added to 35 mg (0.093 mmol) of [^{13}C]-riboflavin. The mixture was kept in the dark at room temperature for 2 days, and 3 g of ice were added at 0 °C. The pH was adjusted to 5.0 by the addition of 25% ammonia. The product was isolated by preparative HPLC (Lichrosorb RP 18, 10 μ, 16 × 250 mm). The eluent contained 35 mM ammonium formate, 35 mM formic acid, and 25% methanol. Fractions were collected and lyophilized. The product contained approximately 80% riboflavin 5'-phosphate and 20% 4'-phosphate. Yield: 31 μmol, 34%. Approximately 0.02 mmol (21.5%) of riboflavin were recovered.

Results and Discussion

In order to optimize the experimental effort in the ^{13}C NMR analysis of flavoproteins reconstituted with ^{13}C labeled flavocoenzymes, it is desirable to incorporate several ^{13}C atoms into a given flavin molecule in such a way that no ^{13}C atom has a direct ^{13}C neighbour (any two contiguous ^{13}C atoms would result in

Table I. Preparation of ^{13}C labeled 6,7-dimethyl-8-ribityllumazine (**3**) and riboflavin (**4**).

Starting material	pH	Position of ^{13}C label 6,7-Dimethyl-8-ribityllumazine	Riboflavin
[1-^{13}C]-**1**	7.65	[6α,(7α)-$^{13}C_1$][a]	[6,8α,(9),(7α)-$^{13}C_2$][a]
[2-^{13}C]-**1**	7.65	[6,(7)-$^{13}C_1$][a]	[5a,8,(9a),(7)-$^{13}C_2$][a]
[2-^{13}C]-**1**	5.0	[6,7-$^{13}C_1$][b]	[5a,9a,7,8-$^{13}C_2$][b]
[4-^{13}C]-**1**	7.65	[7α,(6α)-$^{13}C_1$][a]	[9,7α,(6),(8α)-$^{13}C_2$][a]
[1,4-$^{13}C_2$]Diacetyl	5.0	[6α,7α-$^{13}C_2$]	[6,9,7α,8α-$^{13}C_4$]

[a] Carbon atoms in brackets have only about 18% ^{13}C enrichment.
[b] All labeled atoms in product have ^{13}C enrichments close to 45%.

^{13}C-^{13}C coupling with splitting of NMR signals and a consequent loss of sensitivity). This can be achieved by the strategy described below which allows the introduction of ^{13}C atoms from simple and cost-efficient precursors ($^{13}CH_3I$ and [^{13}C]acetate) in good yield.

Various ^{13}C labeled 2-butanones have been prepared as described under Methods. Briefly, [1-^{13}C]2-butanone was obtained from propionitrile and $^{13}CH_3MgI$, [2-^{13}C]2-butanone from [1-^{13}C]acetyl chloride and C_2H_5ZnI [9], and [4-^{13}C]2-butanone from acetone and $^{13}CH_3I$ [10]. Reaction of a labeled 2-butanone with ethyl nitrite [15] yielded the corresponding 3-hydroximino-2-butanone (**1**) labeled with ^{13}C in position 1, 2, or 4.

Reaction of **1** with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**2**) yields the lumazine **3** (Fig. 1). This reaction was studied under a variety of pH conditions. ^{13}C Enrichments were determined by ^{13}C NMR in each experiment. The ^{13}C signals of **3** have been unequivocally assigned on the basis of ^{13}C-^{13}C coupling patterns as well as spontaneous proton exchange at the 7α methyl groups with D_2O [16–18].

Optimum regioselectivity of the condensation reaction was obtained at pH 7.65 at 89 °C. Under these conditions, the carbonyl group of **1** reacts preferentially with the 5-amino group of **3** (selectivity 4:1). Thus, [4-^{13}C]3-hydroximino-2-butanone yielded **3** with absolute ^{13}C enrichments of 72 and 18% at positions 7α and 6α, respectively. These experimental conditions (Method A) were also used to prepare samples of **3** with predominant labeling at carbons 6α or 6 from [1-^{13}C]-**1** or [2-^{13}C]-**1** (Table I). The preparation of [7-^{13}C]-**3** is feasible by the same approach from [1-^{13}C]ethyliodide *via* [3-^{13}C]-**1**, but the compound has not been prepared in the present study.

At pH values around 5, the condensation of **1** with **3** occurs almost without regiospecificity. More specifically, the carbonyl group of **1** shows a slight preference (1.4:1) for the reaction with the ribitylamino group at position 6 of **2**, whereas the opposite selectivity has been found at alkaline pH (see above). Thus, [2-^{13}C]-**1** yields an almost equal mixture of lumazine molecules labeled at C6 and C7. The yield is substantially better at pH 5 than at pH 7.65.

The reaction of **2** with [1,4-$^{13}C_2$]diacetyl (prepared from [2-^{13}C]acetate as described under Methods) yields [6α,7α-$^{13}C_2$]-**3**. It should be noted that each molecule contains two ^{13}C atoms in contrast to the samples described above where each individual molecule contained only one labeled atom.

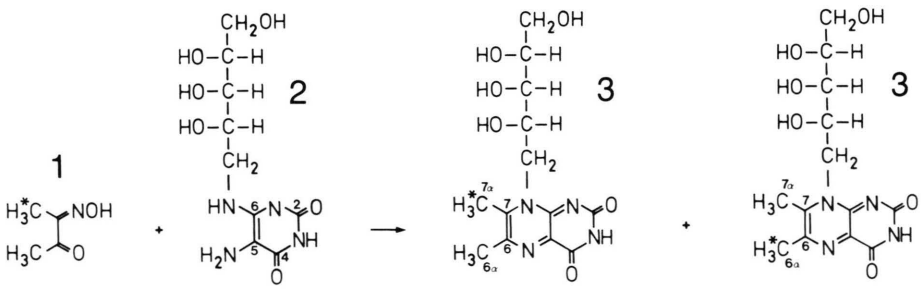

Fig. 1. Preparation of 6,7-dimethyl-8-ribityllumazine (**3**) from [4-^{13}C]3-hydroximino-2-butanone (**1**). Asterisks indicate ^{13}C atoms. The 6α methyl group is predominantly labeled at pH 7.65 (selectivity, 4:1).

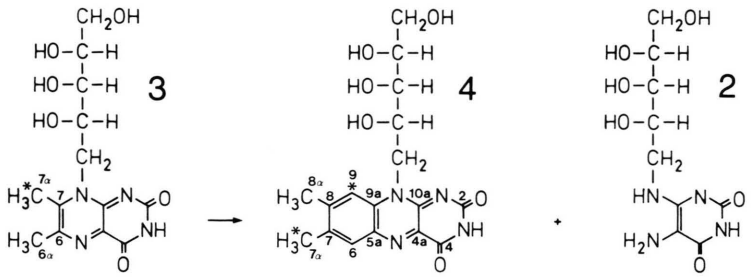

Fig. 2. Enzymatic synthesis of riboflavin (**4**) from 6,7-dimethyl-8-ribityllumazine (**3**). Asterisks indicate the regiospecificity of the enzyme, riboflavin synthase.

Riboflavin (**4**) is obtained from the lumazine **3** by treatment with the enzyme, riboflavin synthase. This enzyme catalyzes the dismutation of two molecules of **3** to one molecule each of riboflavin and the pyrimidine **2** (for review see [19]). The regiospecificity of the enzyme-catalyzed reaction as shown in Fig. 2 has been deduced from NMR studies utilizing ^{2}H- and ^{13}C labeled precursors [20, 21]. The net result is the formation of the xylene ring of **4** from two identical 4C units. Hence, the presence of one ^{13}C atom in the 4C-precursor results in the presence of two labeled atoms in **4**. The flavins thus obtained are summarized in Table I. Riboflavin obtained from [7α-^{13}C]6,7-dimethyl-8-ribityllumazine showed the following ^{13}C enrichments: C-7α and C-9, 72%; C-6 and C-8α, 18%.

The various riboflavin samples were phosphorylated by the chlorophosphoric acid method of Scola-Nagelschneider and Hemmerich [14]. This procedure yields a mixture of several isomeric monophosphates and bisphosphates. Pure 5′-monophosphate can be obtained by preparative HPLC using reverse phase columns [15]. However, the rigorous isomer purification is not required if the apoenzyme has a high selectivity for the 5′-isomer. Subsequent to the reconstitution of the holoenzyme, the undesired isomers which do not bind to the apoenzyme can be conveniently removed by dialysis prior to NMR experimentation [22].

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