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Rapid Preparation of Isotopolog Libraries by in Vivo Transformation of ^{13}C-Glucose. Studies on 6,7-Dimethyl-8-ribityllumazine, a Biosynthetic Precursor of Vitamin B_2

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An *Escherichia coli* strain engineered for expression of the *ribABGH* genes of *Bacillus subtilis* was shown to produce 100 mg of the riboflavin precursor 6,7-dimethyl-8-ribityllumazine per liter of minimal medium. Growth of the recombinant strain in medium supplemented with [U-$^{13}C_6$]glucose and/or $^{15}NH_4Cl$ as single sources of carbon and/or nitrogen afforded 6,7-dimethyl-8-ribityllumazine universally labeled with ^{13}C and/or ^{15}N. The yield of [U-$^{13}C_{13}$]-6,7-dimethyl-8-ribityllumazine based on [U-$^{13}C_6$]glucose was 25 mg/g. Fermentation with [1-$^{13}C_1$]-, [2-$^{13}C_1$]-, or [3-$^{13}C_1$]glucose afforded mixtures of 6,7-dimethyl-8-ribityllumazine isotopologs, predominantly with ^{13}C enrichment of single carbon atoms. The isotope-labeled samples enabled a comprehensive NMR analysis of 6,7-dimethyl-8-ribityllumazine. Isotopolog libraries of a wide variety of microbial metabolites can be produced by the same experimental approach.

Introduction

Protein perturbation studies via NMR observation can afford information on the physical state of protein-bound ligands. They can also provide information on dynamic aspects of ligand exchange and on catalysis including the fate of cofactors, substrates, intermediates, and products during enzyme-catalyzed processes (for reviews, see refs 1−3). Frequently, however, these experiments require the labeling of the ligand under study with ^{13}C and/or ^{15}N for reasons of both sensitivity and selectivity.

This paper explores the potential of biotransformation methods for the preparation of isotopolog libraries of natural products designed for ligand perturbation experiments monitored by NMR. As an example, we used the biosynthetic precursor of vitamin B_2 (riboflavin), 6,7-dimethyl-8-ribityllumazine. This intermediate is the substrate of riboflavin synthase,[4,5] which catalyzes a mechanistically complex dismutation that is still poorly understood and deserves further study since the enzyme is a potential target for antiinfective chemotherapy.[6] Moreover, 6,7-dimethyl-8-ribityllumazine serves as the fluorophore of lumazine proteins acting as optical transponders for light emission by certain luminous marine bacteria.[7,8] Last but not least, it can be used as a flavocoenzyme model in mechanistic studies on flavoproteins.[9,10]

The carbon skeleton of riboflavin is biosynthesized from three pentose phosphate moieties and one molecule each of glycine, formate, and carbon dioxide (Figure 1) (for a review, see ref 11). The nitrogen atoms are contributed via the glycine building block and from glutamine and aspartate, respectively.

The first committed step in the biosynthetic pathway is the conversion of GTP into 2-amino-5-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (**3**) by GTP cyclohydrolase II.[12,13] The enzyme product is converted into 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**4**) by the action of a deaminase, a reductase, and a hitherto elusive phosphatase (for review, see refs 11 and 14). In eubacteria, the deamination and the reduction are catalyzed

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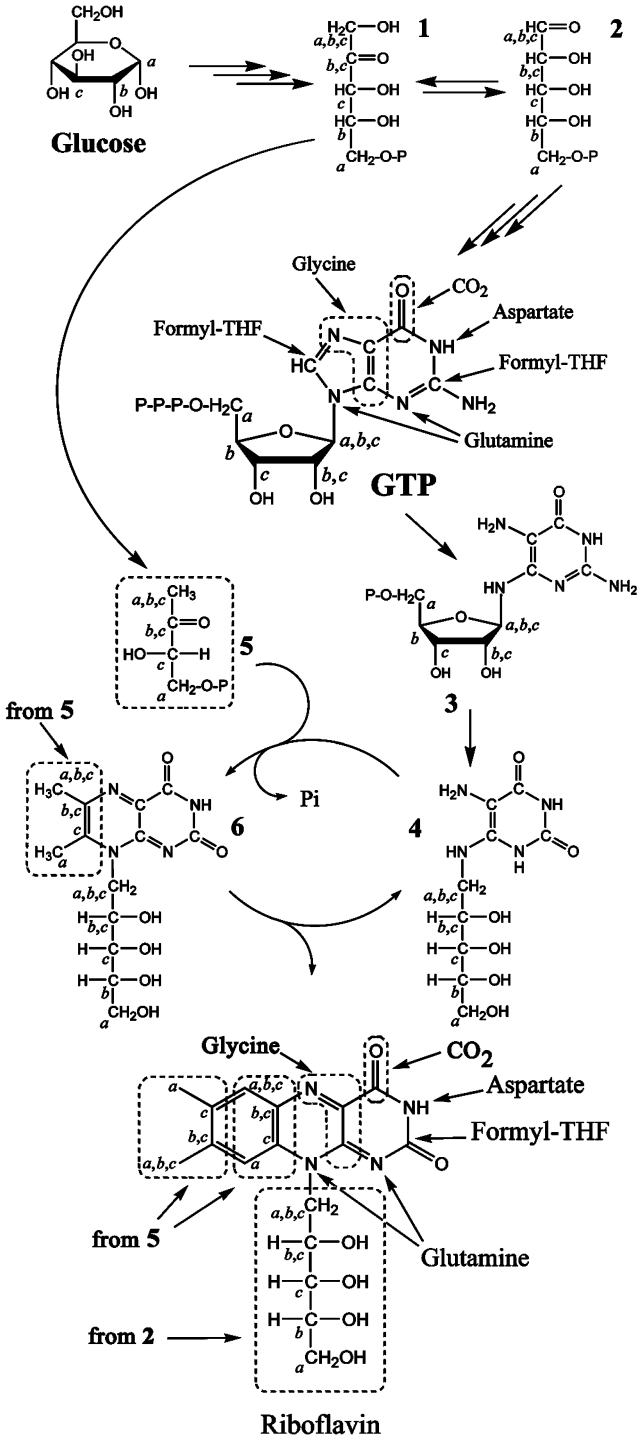

FIGURE 1. Biosynthesis of riboflavin. The fate of glucose carbon atoms is indicated by letters a−c. Partial scrambling in **1** is due to reactions of the pentose phosphate pathway.

by one bifunctional enzyme (specified in *Escherichia coli* by the *ribD* gene).[15]

The pyrimidine derivative **4** is condensed with 3,4-dihydroxy-2-butanone 4-phosphate (**5**) into 6,7-dimethyl-8-ribityllumazine (**6**) by the catalytic action of 6,7-dimethyl-8-ribityllumazine synthase.[16,17] The carbohydrate substrate **5** for that reaction is obtained from ribulose 5-phosphate (**1**) by an unusual rearrangement catalyzed by a single enzyme.[18,19] In the final reaction step, the pteridine type intermediate **6** undergoes a mechanistically unusual dismutation affording riboflavin and the riboflavin precursor **4** by the transfer of a 4-carbon unit between two substrate molecules, which is catalyzed by riboflavin synthase.[4,5,11] The transfer of carbon atoms from glucose into riboflavin under in vivo conditions is indicated by the letters a−c in Figure 1.

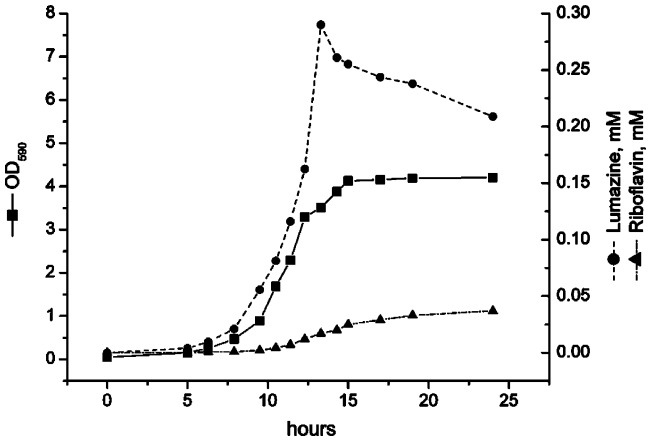

FIGURE 2. Production of metabolites during of fermentation of *E. coli* M15[pREP4, pRFN4]: 6,7-dimethyl-8-ribityllumazine (**6**), ●; riboflavin, ▲; cell density (OD_{590}), ■.

TABLE 1. ^{1}H^{13}C and ^{13}C^{13}C Correlation Pattern of 6,7-Dimethyl-8-ribityllumazine (6)

	NMR experiment		
position	HMQC	HMBC	INADEQUATE[a]
7α	7α		7
6α	6α		6
1′	1′, 1′ [b]	3′, 2′	2′
5′	5′, 5′ [b]	3′, 4′	4′
2′	2′	3′, 1′	1′, 3′
4′	4′	3′, 5′	5′, 3′
3′	3′	4′, 5′,1′ 2′	4′, 2′
4a		7α	8a
6		6α, 7α	6α, 7
8a		1′, 1[b]	4a
7		6α, 7α, 1′, 1′ [b]	7α, 6

[a] With the ^{13}C-enriched sample from the experiment with [U-$^{13}C_6$]glucose. [b] Indicates the downfield-shifted ^{1}H signal of a pair of hydrogen atoms at a diastereotopic center.

In *Bacillus subtilis*, all enzymes of the riboflavin pathway (with the exception of the elusive phosphatase) are specified by the *rib* operon comprising five open reading frames (*ribABGHT*).[20] Specifically, *ribA* specifies a bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-

Using an *E. coli* strain engineered for the production of 6,7-dimethyl-8-ribityllumazine (**6**), we show that libraries of isotopologs universally or selectively labeled with ^{13}C and/or ^{15}N can be prepared rapidly by in vivo biotransformation of ^{13}C-substituted glucose and/or ^{15}-NH_4Cl. Notably, we show that the use of glucose samples with ^{13}C in different positions can provide libraries of 6,7-dimethyl-8-ribityllumazine (**6**) with orthogonal label distribution.

Results and Discussion

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TABLE 2. NMR Data of 6,7-Dimethyl-8-ribityllumazine (6)

	chemical shifts, ppm[a]			coupling constants, Hz[b]					
position	δ^{15}N	δ^{13}C	δ^{1}H	J_{CC}	J_{CH}	J_{HH}	J_{CN}	J_{HN}	J_{CD} [d]
7α		18.09	2.79	46.5 (7), 3.3 (6), 1.3 (6α)	131.8 (7α), 6.1 (6), 3.9 (7)		8.0	2.7	20.1 (7α)
6α		21.40	2.59	54.0 (6), 7.5 (7), 5.3 (4a), 1.3 (7α)	129.6 (6α), 6.8 (7), 3.1 (6)		8.5	3.1	
1′		51.21	4.92,[c] 4.67	39.4 (2′)	143.4 (1′)	14.0 (1′), 2.7 (2′) 13.8 (1′[c]), 9.8 (2′)	7.1, 2.0		
5′		62.60	3.77,[c] 3.62	40.9 (4′), 2, 2	142.0 (5′)	12.1 (5′), 3.0 (4′) 12.0 (5′[c]), 6.6 (4′)			
2′		69.16	4.32	40 (3′), 39.4 (1′)	147.2 (2′)	9.8 (1′), 4.9 (3′), 2.7 (1′[c])			
4′		71.98	3.85	41 (3′), 41 (5′)	143.1 (4′)	7.0 (3′), 7.0 (5′), 2.9 (5′[c])			
3′		73.40	3.79	41 (2′), 41 (4′)	143.0 (3′)	4.9 (2′), 7.2 (4′)			
4a		130.90		77.8 (4), 61.3 (8a), 9 (6), 9 (7), 5.1 (6α)			6, 6		
6		144.95		54.2 (6α), 59.5 (7), 3.3 (7α)					
8a		150.39		61.7 (4a), 9 (4), 9 (6)			17.2, 8.8		
7		152.30		46.7 (7α), 59.5 (6), 7.5 (6α), 7.5 (4a)			13.1, 4.9		4.7 (7α)
2		157.93		3 (4), 3 (4a), 3 (8a)			13.1, 8.6, 4.9		
4		163.12		77.8 (4a), 9.3 (8a), 6.4 (2), 1.3, 1.3			13.7, 8.0		
1	190.8								
3	176.7							5.0	
5	326.9								
8	154.0								

[a] ^{1}H and ^{13}C NMR chemical shifts were referenced to external trimethylsilylpropane sulfonate. ^{15}N NMR chemical shifts were referenced to external [5-^{15}N]-**6**.[24] [b] Coupling partners are given in parentheses. [c] Indicates the downfield shifted ^{1}H signal of a pair of hydrogen atoms at a diastereotopic center. [d] Observed with samples measured in 99% D_2O.

butanone 4-phosphate synthase, *ribB* specifies riboflavin synthase, *ribG* specifies a bifunctional deaminase/reductase, and *ribH* specifies 6,7-dimethyl-8-ribityllumazine synthase. The function, if any, of the putative open reading frame *ribT* is still unknown but is not required for the biosynthesis of the vitamin.[21]

We cloned the entire riboflavin operon of *B. subtilis* into the pNCO113 plasmid designed for efficient expression in *E. coli*. To stall the engineered oversynthesis of riboflavin at the level of the intermediate, 6,7-dimethyl-8-ribityllumazine (**6**), an F2A missense mutation was then introduced into the *ribB* gene specifying riboflavin synthase. That mutation had been shown previously to inactivate the enzyme[22] and should not result in polarity effects of any kind since the mutated gene can be translated in full length. The nucleotide sequence of the resulting plasmid pRFN4 is available under accession no. AY386222.

A recombinant *E. coli* strain carrying that plasmid produced 100 mg of 6,7-dimethyl-8-ribityllumazine (**6**) per liter of minimal medium in shaking cultures at 37 °C. The growth curve as well as the accumulation of 6,7-dimethyl-8-ribityllumazine (**6**) and riboflavin in the culture medium are shown in Figure 2. The lumazine derivative was accumulated in the culture medium in parallel to cell growth and reached a maximum level of 0.30 mM after 13 h of cultivation (late log phase). After 15 h, the glucose pool in the medium was exhausted, and the cells entered the stationary phase. The lumazine concentration began to decrease after 13 h of cultivation, whereas the concentration of riboflavin continued to increase even after 20 h of cultivation. This may be due to reuptake of **6** and intracellular transformation into riboflavin by wild type riboflavin synthase, which can be expressed, albeit in relatively small amounts, from the intact chromosomal *ribB* gene.

The maximum yield of **6** (2.5% based on glucose) was obtained with 5 μM IPTG in the culture medium. At IPTG concentrations above 25 μM, the production of **6** was negligible. Without IPTG added, the lumazine yield was about 1% based on glucose. The optimal glucose concentration was between 4 and 10 g/L. At concentrations below 1 g/L, the glucose pool was apparently exhausted before the cell suspension reached OD_{590} 0.8, and the yield of 6,7-dimethyl-8-ribityllumazine (**6**) was less than 1 mg/L. At concentrations above 10 g/L, the yield was less than 1% based on glucose.

To produce isotope-labeled **6**, the recombinant strain was grown in minimal medium supplied with [U-$^{13}C_6$]-, [1-$^{13}C_1$]-, [2-$^{13}C_1$]-, or [3-$^{13}C_1$]glucose (4 g/L) as carbon source. Nitrogen was supplied as $^{15}NH_4Cl$ when required. Under these conditions, **6** reached a maximum concentration of 100 mg/L after a culture period of 13 h at 37 °C. 6,7-Dimethyl-8-ribityllumazine (**6**) was isolated from the culture supernatants and was purified by column chromatography.

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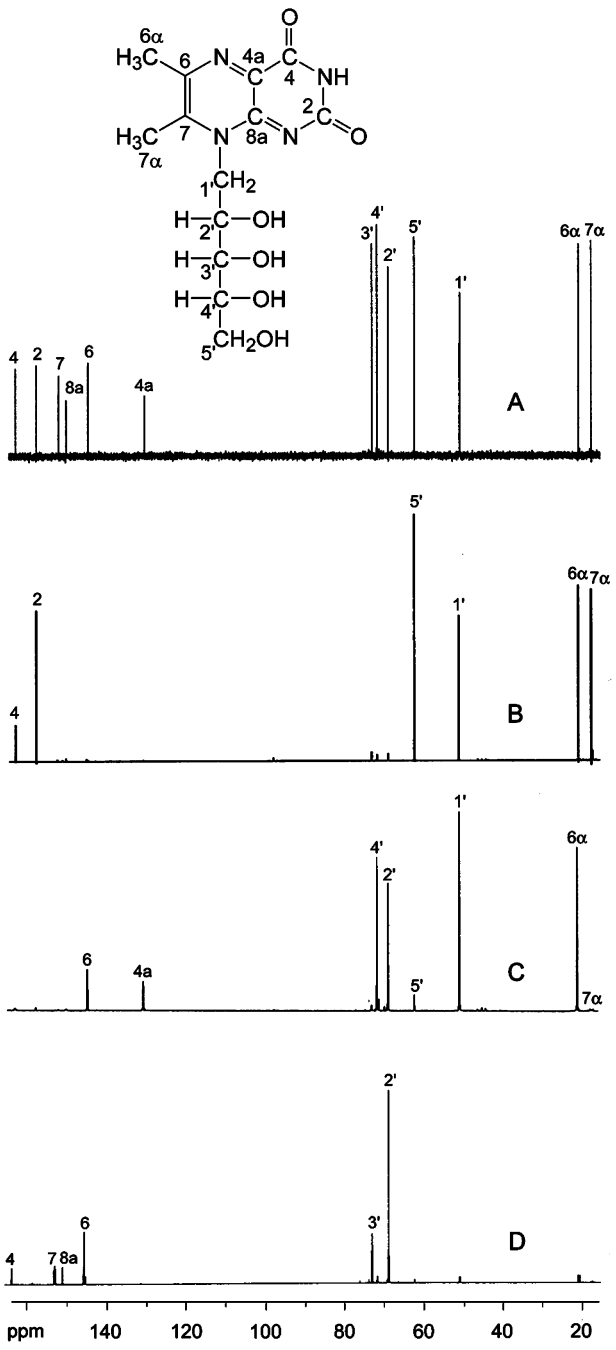

FIGURE 3. ^{13}C NMR spectra of 6,7-dimethyl-8-ribityllumazine (**6**): (A) of a sample with natural ^{13}C abundance; (B) of a sample ^{13}C-enriched from [1-^{13}C]glucose; (C) of a sample ^{13}C-enriched from [2-^{13}C]glucose; and (D) of a sample ^{13}C-enriched from [3-^{13}C]glucose.

Due to ^{13}C labeling, the sensitivity and selectivity of two-dimensional homonuclear and heteronuclear ^{13}C experiments (e.g., INADEQUATE and HMBC) was substantially increased. The detected correlations (Table 1) confirmed the published ^{13}C NMR signal assignments.[23−25] ^{1}H and ^{13}C NMR data of **6** are summarized in Table 2.

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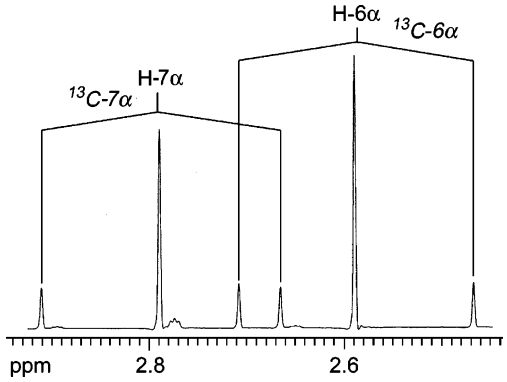

FIGURE 4. ^{1}H NMR signals of 6,7-dimethyl-8-ribityllumazine (**6**) from the experiment with [1-^{13}C]glucose. ^{13}C-coupled satellite signals are indicated.

Figure 3 shows ^{13}C signals of 6,7-dimethyl-8-ribityllumazine samples (**6**) obtained from experiments with single ^{13}C-labeled glucose supplements. Relative ^{13}C abundances of biolabeled **6** were obtained by comparison of the ^{13}C NMR intensities with the signal intensities of **6** at natural ^{13}C abundance (cf. Figure 3A). Absolute ^{13}C abundances for selected carbon atoms were obtained from the ^{1}H^{13}C coupling satellites in the ^{1}H NMR spectra. As an example, the relative fraction of the ^{13}C coupled satellites (J_{CH} = 129.6 Hz, cf. Table 2) in the global intensity of the ^{1}H NMR signal for the H-6α methyl group of **6** (δ = 2.59 ppm; Figure 4) from the experiment with [1-$^{13}C_1$]glucose accounted for 26.4% (cf. Table 3). In other words, 26.4% of 6,7-dimethyl-8-ribityllumazine (**6**) isolated from the experiment with [1-$^{13}C_1$]glucose contained ^{13}C at position 6α. Similarly, the absolute ^{13}C abundance of 7α was determined as 29.4% from the relative intensity of ^{13}C coupled satellites in the ^{1}H NMR signal of H-7α (J_{CH} = 131.8 Hz). With these data in hand, relative ^{13}C abundances extracted from the ^{13}C NMR spectra (see above) were normalized, and absolute abundances for all carbon atoms in **6** were obtained (Table 3).

Some of the ^{13}C signals showed coupling satellites of significant intensity reflecting the formation of multiply labeled biochemical precursors by stochastic recombination of labeled intermediary metabolites. As an example, the signals of C2′, C-3′, C-6, and C-7 of **6** from the experiment with [3-^{13}C]glucose displayed intense coupling satellites indicating the presence of [2′,3′-$^{13}C_2$]- and [6,7-$^{13}C_2$]-**6** at molar abundances of 16%. The formation of these molecular species is due to glycolytic cycling of glucose prior to its use as precursor of lumazine biosynthesis.

More specifically, glycolysis of [3-^{13}C]glucose 6-phosphate is conducive to the formation of [1-^{13}C]glyceraldehyde phosphate (**8**), which is in equilibrium with [1-^{13}C]-dihydroxyacetone 3-phosphate (**9**) (Figure 5). Regeneration of hexose phosphate by glucogenesis affords [3,4-$^{13}C_2$]-glucose 6-phosphate, which is further converted into [2,3-$^{13}C_2$]ribulose 5-phosphate (**1**) by the pentose phosphate pathway. Catalytic transformation of [2,3-$^{13}C_2$]ribulose 5-phosphate by 3,4-dihydroxy-2-butanone 4-phosphate

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TABLE 3. ^{13}C Abundance of 6,7-Dimethyl-8-ribityllumazine Samples (6) Obtained by Biotransformation of ^{13}C-Labeled Glucose with an *E. coli* Strain Harboring *ribABG* Genes of *Bacillus subtilis*[a]

		^{13}C abundance (%)		
position[b]	biosynthetic origin[c]	[1-^{13}C]glucose	[2-^{13}C]glucose	[3-^{13}C]glucose
7α	C-5 of ribulose 5-phosphate	**29.4**	2.1	1.3
6α	C-1 of ribulose 5-phosphate	**26.4**	**73.6**	5.6
1′	C-1 of ribose 5-phosphate	**23.8**	**71.7**	6.0
5′	C-5 of ribose 5-phosphate	**28.9**	3.8	1.1
2′	C-2 of ribose 5-phosphate	1.4	**30.0**	**61.6**
4′	C-4 of ribose 5-phosphate	1.1	**34.3**	2.8
3′	C-3 of ribose 5-phosphate	1.3	2.5	**24.9**
4a	C-2 of glycine	1.9	**48.7**	4.4
6	C-2 of ribulose 5-phosphate	1.7	**31.9**	**73.1**
8a	C-1 of glycine	2.5	4.3	**43.1**
7	C-3 of ribulose 5-phosphate	1.8	2.3	**31.7**
2	formyl tetrahydrofolate	**72.0**	6.4	1.6
4	CO_2	**20.8**	10.7	**30.9**

[a] Values larger than 20% are shown in bold. [b] The numbering of carbon atoms is shown in Figure 3. [c] On the basis of in vivo and in vitro studies with *B. subtilis*[26−28,16,29,30] (cf. also Figure 1).

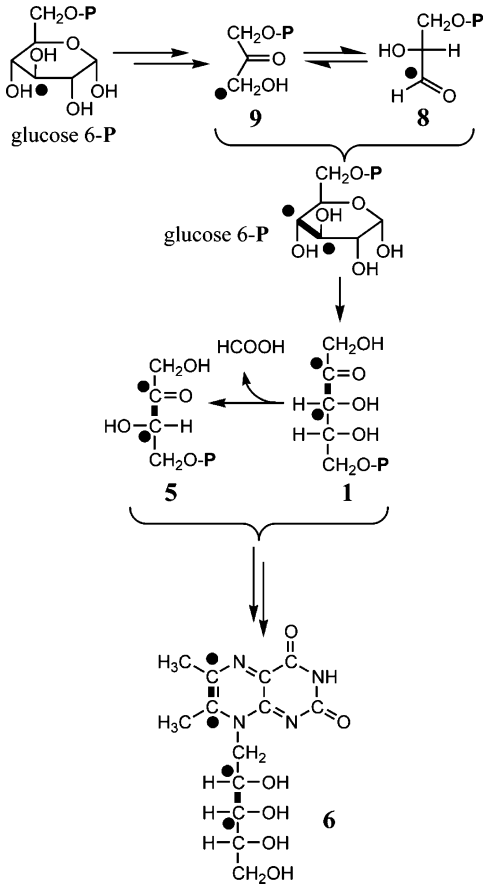

FIGURE 5. Biosynthetic pathways conducive to the formation of [2′,3′-$^{13}C_2$]- and [6,7-$^{13}C_2$]-6,7-dimethyl-8-ribityllumazine (**6**) from [3-$^{13}C_1$]glucose. Filled circles indicate the ^{13}C labels. Bars connect ^{13}C atoms in a given molecule.

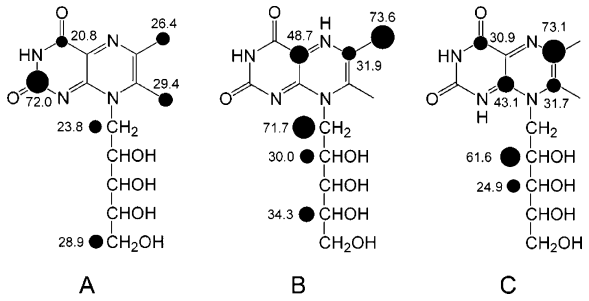

FIGURE 6. Labeling patterns of 6,7-dimethyl-8-ribityllumazine (**6**) obtained by biotransformation of ^{13}C-labeled glucose samples with an *E. coli* strain M15[pREP4, pRFN4]: (A) from the experiment with [1-^{13}C]glucose; (B) from the experiment with [2-^{13}C]glucose; (C) from the experiment with [3-^{13}C]-glucose. ^{13}C enrichments are indicated by scaled dots. The numbers indicate ^{13}C abundances (cf. also Table 3).

synthase yields [2,3-$^{13}C_2$]3,4-dihydroxy-2-butanone 4-phosphate (**5**). Processing of these molecular species in the riboflavin biosynthetic pathway leads to the detected [2′,3′-$^{13}C_2$]- and [6,7-$^{13}C_2$]6,7-dimethyl-8-ribityllumazine specimens (**6**) (Figure 5).

Fermentations using only three differently labeled glucose isotopologs (i.e., [1-$^{13}C_1$]-, [2-$^{13}C_1$]-, and [3-$^{13}C_1$]-glucose) were sufficient to obtain significant labeling (ranging from about 20−70% ^{13}C in our samples) of each of the 13 carbon atoms of **6** in at least one of the samples (Table 3, Figure 6). As a sideline, it should be noted that the different degrees of ^{13}C abundance in the more highly labeled carbon atoms of a given isotopolog mixture provide an important advantage for ligand/protein interaction studies because the intensities of the signals differ accordingly and the different intensities of individual carbon signals as well as their coupling signatures in protein perturbation experiments can be useful to cross-check signal assignments.

The fractional ^{13}C enrichment of **6** from the specifically labeled glucose samples used as carbon source in this study is in line with known reactions involved in glucose metabolism (e.g., glycolysis and pentose phosphate pathway) and riboflavin biosynthesis (cf. Figure 1). The detailed analysis of the contributive roles of different routes in the carbohydrate metabolism of the bacterium is outside the scope of this study and will be discussed elsewhere.

All ^{13}C NMR signals of **6** obtained from the experiment with [U-$^{13}C_6$]glucose were characterized by $^{13}C^{13}C$ coupling extending over one to three bonds. Some of the signals are shown in Figure 7A. Coupling constants via one bond were in the range of 39−78 Hz (Table 2). A network of long-range couplings was found with coupling constants in the range of 1−10 Hz. By comparing the sizes of the coupling constants, most of the long-range

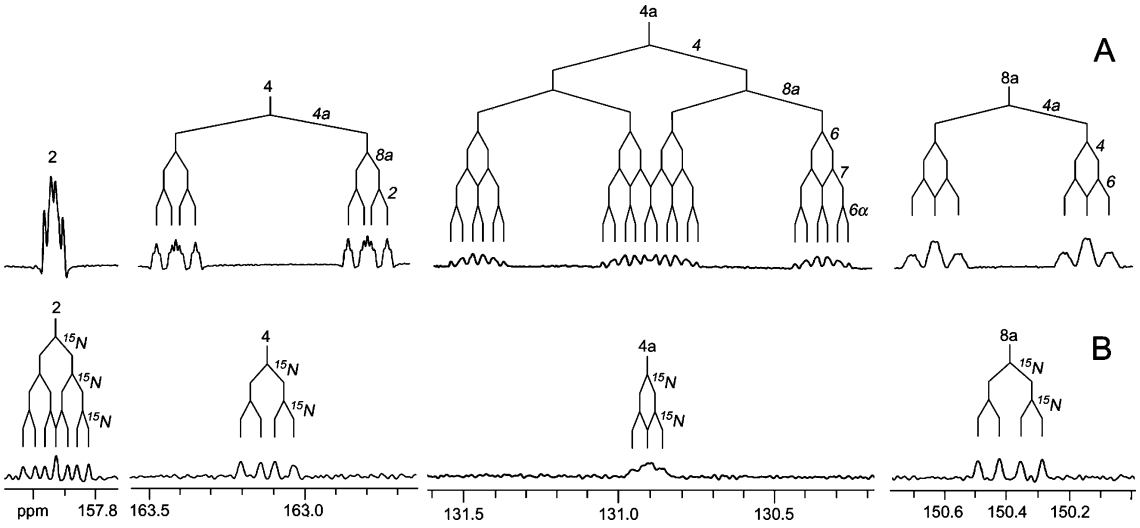

FIGURE 7. ^{13}C NMR signals of 6,7-dimethyl-8-ribityllumazine (**6**): (A) from the experiment with [U-$^{13}C_6$]glucose; (B) from the experiment with $^{15}NH_4Cl$. $^{13}C^{13}C$ and $^{13}C^{15}N$ couplings are indicated in A and B, respectively.

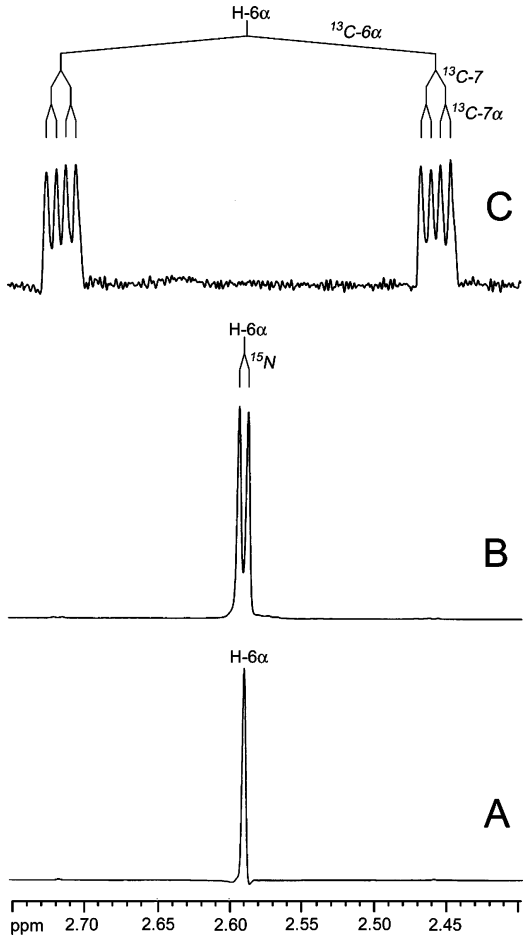

FIGURE 8. ^{1}H NMR signals for H-6α of 6,7-dimethyl-8-ribityllumazine (**6**): (A) with natural ^{13}C and ^{15}N abundance; (B) from the experiment with $^{15}NH_4Cl$; (C) from the experiment with [U-$^{13}C_6$]glucose. Coupling patterns are indicated.

^{13}C couplings could be assigned (Table 3). Signal patterns indicative of isotopologs with ^{12}C were not observed. The high abundance of totally ^{13}C labeled **6** was also reflected in the ^{13}C-coupled ^{1}H NMR signals. Central signals (reflecting hydrogen atoms connected to ^{12}C) were not observed (cf. Figure 8C). Thus, the ^{13}C label of [U-$^{13}C_6$]-glucose was transferred efficiently to all carbon atoms of **6** under the fermentation conditions using glucose as single carbon source in the medium.

TABLE 4. Bacterial Strains and Plasmids Used in This Work

strain or plasmid	source	properties
bacterial strains		
XL1-blue	stratagene	Tc^R
M15[pREP4]	Zamenhof and Villarejo, 1972[31]	Km^R
plasmids		
pNCO113	Stüber et al., 1990[32]	Ap^R
pRF2	Perkins et al., 1991[21]	Cm^R
p602/-CAT	Mörtl et al., 1996[33]	Km^R
pRFN4	this study	Ap^R

^{15}N-Labeling of **6** was achieved by fermentation of the recombinant *E. coli* strain with $^{15}NH_4Cl$. The ^{15}N NMR spectrum displayed four signals at chemical shifts (Table 2) reported earlier for [U-$^{15}N_4$]-**6**.[24,25] With the exception of the signals of C-2′, C-3′, C-4′, C-5′, and C-6, the ^{13}C NMR signals of this sample appeared as multiplets due to $^{15}N^{13}C$ couplings with coupling constants in the range of 2−20 Hz (Table 2) (Figure 7B). The presence of ^{15}N could also be gleaned from $^{15}N^{1}H$ couplings via three bonds in the ^{1}H NMR signals of the methyl protons (Figure 8B) (Table 2).

In summary, our data show that isotopolog libraries of **6** can be prepared by in vivo biotransformation with limited effort. By comparison, the preparation of the same number of different chemical entities by chemical synthesis would have been prohibitive because it would have involved a variety of different synthetic strategies tailored to label different positions, whereas the present approach used a single experimental strategy with ^{13}C-glucose specimens and/or $^{15}NH_4Cl$ as starting materials, which are all commercially available.

There can be no doubt that the general strategy for the preparation of orthogonally labeled isotopolog mixtures by in vivo biotransformation of different isotopologs

TABLE 5. Oligonucleotides Used in This Study

primer	restriction site	codon replacement	primer sequence[a]
RO 1	*Bam*HI	no	5′-GAGGATCCGGGCTTTTTTGACGGTAAATAACAAAAG
RO 2	*Hind*III, *Spe*I, *Bst*BI	no	5′-GAGAAGCTT ACTAGT ATTTCGAACCGTGAACAGCTGAACCGCC
BS-L	no	no	5′-TTGGCACAGTGAAAGCCGACAATCC
BS-R	no	no	5′-CTTTTCTCTTCAATTCGTGTGATTTCCGCA
BS-M	*Bcl*I	F2 → A	5′-GGATGGTGATCATG**GCT**ACAGGAATTATCGAAGAA

[a] Codon specifying modified amino acid residue is shown in bold. Synthetic restriction sites are underlined.

of glucose or other carbon sources can be similarly applied to a wide variety of other natural products.

Experimental Section

Materials. All materials were purchased from commercial suppliers.

Bacterial Strains and Plasmids. Bacterial strains and plasmids used in this work are shown in Table 4. A 885 bp DNA segment was amplified by PCR using the oligonucleotides RO1 and RO2 as primers (Table 5) and the plasmid pRF2 as template (Table 4). The amplificate was digested with *Bam*HI and *Hind*III and was ligated into plasmid pNCO113, which had been digested with the same restriction enzymes. The resulting plasmid pRFN1 was transformed into *E. coli* XL1 blue.

Treatment of plasmid pRF2 with *Bsi*WI and *Xba*I afforded a 4.5 kbp DNA fragment, which was ligated into the pRFN1 plasmid that had been digested with *Bsi*WI and *Nhe*I. The resulting plasmid pRFN2 was transformed into *E. coli* strain XL1-blue affording the recombinant strain *E. coli* XL1-blue [pRFN2].

Treatment of the plasmid p602/-CAT with *Xho*I and *Eco*RI afforded a 190 bp fragment that was isolated and was then ligated into the plasmid pRFN2, which had been treated with the same restriction enzymes. The resulting plasmid pRFN3 was transformed into *E. coli* strain XL1-blue affording the recombinant strain *E. coli* XL1-blue [pRFN3].

A DNA segment of 360 bp was amplified using the oligonucleotides BS-R and BS-M as primers (Table 5) and the plasmid pRFN3 as template. A second PCR round was then performed with the oligonucleotides BS-R and BS-L as primers. The amplificate was treated with *Sal*I and *Pst*I and was then ligated into the plasmid pRFN3, which had been treated with the same restriction enzymes. The resulting plasmid pRFN4 was transformed into *E. coli* strain M15[pREP4] affording strain M15[pREP4, pRFN4].

Bacterial Culture. Recombinant *E. coli* strains were grown overnight at 37 °C in shaking flasks containing 100 mL of LB medium[34] supplemented with 50 mg of ampicillin and 15 mg of kanamycin per liter. Cells were collected by centrifugation (5000*g*, 4 °C, 10 min) and were resuspended in 0.8 L of M9 medium[34] supplemented with vitamins, trace elements, and ampicillin (50 mg per liter), as well as ^{13}C-labeled glucose (4 g/L) and/or $^{15}NH_4Cl$ (2 g/L). The suspensions were incubated at 37 °C with shaking. Aliquots of bacterial cultures were retrieved at intervals for HPLC analysis. After incubation for 13 h, cell suspensions were centrifuged; the cells were discarded.

Isolation of 6,7-Dimethyl-8-ribityllumazine (6). Supernatants of bacterial cultures (see above) were passed through columns of Florisil (1 × 1.5 cm), which were then washed with 10 mL of water and developed with 5 mL of ammonium hydroxide/acetone/water (1:250:250, v/v). Eluates were evaporated to a small volume under reduced pressure and were then lyophilized. The residue was dissolved in 2 mL of 50 mM hydrochloric acid and applied to a column of Hypersil RP 18 (20 × 250 mm), which was developed with a mixture of methanol/formic acid/water (25:1:288, v/v). The retention volume of 6,7-dimethyl-8-ribityllumazine (**6**) was 160 mL.

HPLC. Analytical high performance liquid chromatography was performed with an RP18 column (5 μm, 4 × 250 mm), which was developed with a mixture of methanol/formic acid/water (25:1:288, v/v). The flow rate was 1.5 mL min^{-1}. The effluent was monitored photometrically at 408 and 470 nm.

NMR Spectroscopy. ^{1}H, ^{13}C, and ^{15}N NMR spectra were recorded at 25 °C using a spectrometer equipped with four channels and a pulsed gradient unit. The transmitter frequencies were 500.1, 125.6 and 50.68 MHz for ^{1}H, ^{13}C, and ^{15}N, respectively. Samples were dissolved in a H_2O/D_2O mixture (9:1; v/v). Two-dimensional COSY, HMQC, HMBC, and INADEQUATE experiments were performed according to standard Bruker software (XWINNMR).

Determination of ^{13}C Enrichments. ^{13}C-Enrichments were determined by quantitative NMR spectroscopy.[35] For this purpose, ^{13}C NMR spectra of **6** from the experiments with the ^{13}C-labeled glucose specimens and of **6** with natural ^{13}C abundance (i.e., with 1.1% ^{13}C abundance) were measured under the same experimental conditions. The ratios of the signal integrals of the biolabeled compound and of the compound at natural abundance were then calculated for each respective carbon atom. Absolute ^{13}C abundances for certain carbon atoms (i.e., for carbon atoms with at least one attached hydrogen atom displaying a ^{1}H NMR signal in a noncrowded region of the spectrum) were then determined from the ^{13}C coupling satellites in the ^{1}H NMR spectra. The relative ^{13}C abundances determined for all other positions in **6** were then referenced to this value, thus affording absolute ^{13}C abundances for every single carbon atom (% ^{13}C in Table 3).

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