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Identification of the Key Enzyme of Roseoflavin Biosynthesis

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Abstract: *The bacteria Streptomyces davawensis and Streptomyces cinnabarinus produce roseoflavin, the only known natural riboflavin (vitamin B_2) analogue with antibiotic activity. Roseoflavin can be considered a natural antimetabolite and has been postulated to be biosynthesized from riboflavin via the key intermediate 8-demethyl-8-aminoriboflavin (AF). The required site-specific substitution of one of the methyl groups on the dimethylbenzene ring of riboflavin by an amino group (to give AF) is challenging. The pathway from riboflavin to AF has remained elusive, and the corresponding enzyme/s was/were unknown. Herein, we show by systematic gene deletion, heterologous gene expression, and biochemical studies that the enzyme specified by the gene BN159_7989 from S. davawensis is able to carry out a whole set of chemical reactions starting from riboflavin-5′-phosphate to give the final product 8-demethyl-8-aminoriboflavin-5′-phosphate (AFP).*

Vitamin analogues can serve as lead structures for the development of novel antibiotics, and they could help to replenish the arsenal of antimicrobials, which is urgently needed to fight multidrug-resistant bacterial pathogens.[1] Three naturally occurring vitamin analogues with antibiotic functions have been reported: the vitamin B_1 analogue bacimethrin,[2] the vitamin B_6 analogue ginkgotoxin,[3] and the vitamin B_2 (riboflavin) analogue roseoflavin (RoF).[4]

The Gram-positive soil bacteria *Streptomyces davawensis* and *Streptomyces cinnabarinus* are the only organisms known to produce RoF.[5] RoF is efficiently taken up by target cells via riboflavin transporters, widespread proteins[6] that seem to also accept RoF as a substrate.[6,7] Following uptake, RoF is converted into the flavin cofactor analogues roseoflavin-5′-phosphate (RoFP) and roseoflavin adenine dinucleotide (RoFAD) by flavokinases and FAD synthetases, respectively.[1,8] These enzymes are present in all organisms. Obvious cellular targets for RoFP and RoFAD are flavoproteins, which make up 0.1% to 3.5% of predicted proteins in organisms sequenced so far.[9] Flavoproteins depend on the cofactors riboflavin-5′-phosphate and flavin adenine dinucleotide (FAD) and were found to be less active or completely inactive when RoFP or RoFAD were bound instead.[8b,10] Riboflavin-5′-phosphate riboswitches, genetic elements that regulate riboflavin biosynthesis and transport, have been reported to be turned off in the presence of RoFP, thereby causing riboflavin deficiency,[11] and to represent additional targets for RoF.

Earlier publications on the biosynthesis of RoF suggest that RoF is synthesized from riboflavin via 8-demethyl-8-aminoriboflavin (AF) and 8-demethyl-8-methylaminoriboflavin (MAF).[12] The intermediates AF and MAF were confirmed by the discovery of the first enzyme of RoF biosynthesis, the *S*-adenosyl methionine (SAM)-dependent dimethyltransferase RosA.[13] This enzyme synthesizes RoF from AF (via MAF; Figure 1, conversion of compound **6** into compound **8**). The corresponding gene *rosA* is present in a cluster comprising nine genes (gene cluster 7, Figure 2). The remaining genes of this cluster were found not to be involved in RoF synthesis.[13] The pathway from riboflavin to AF remained unknown and several enzymes were postulated to be involved in the formation of this key intermediate of RoF biosynthesis.[5] An important step with regard to the identification of the as yet unknown AF biosynthesis genes was the result of the following expression experiment. A cosmid (pESAC13-SD106kbp)[5] containing a specific 106 kbp subgenomic fragment from *S. davawensis* with 95 coding sequences (Figure 2) was inserted into the chromosome of the expression host *S. coelicolor*. This led to the production of RoF by the corresponding recombinant *S. coelicolor* strain.[5] *S. coelicolor* naturally produces neither RoF nor AF (nor any other flavin analogue). The result of this experiment thus suggests that all of the genes necessary for RoF synthesis (and consequently all of the genes necessary for the synthesis of the intermediate AF) are present on this 106 kbp subgenomic fragment from *S. davawensis*. The 106 kbp subgenomic fragment contains the gene *rosA*, which is responsible for the formation of RoF from AF (see above). Most of the experiments described in the present work are aimed at identifying and characterizing the genes/enzymes responsible for the synthesis of AF. Prior to that, however, we set out to confirm the previous finding that riboflavin is a precursor of RoF biosynthesis.[12a]

The addition of riboflavin (100 μM) to a *S. davawensis* culture stimulated RoF production, leading to 43 μM RoF in the culture supernatant instead of 21 μM (untreated control). Feeding experiments in *S. davawensis* with [U-^{15}N]riboflavin (50 μM) and liquid chromatography–mass spectrometry (LC/MS) analyses of the culture supernatants revealed that all four nitrogen atoms of [U-^{15}N]riboflavin were conserved in RoF (see Figure S1 in the Supporting Information). This indicates that riboflavin is used directly (without breakdown) as a starting point to synthesize RoF.[12a]

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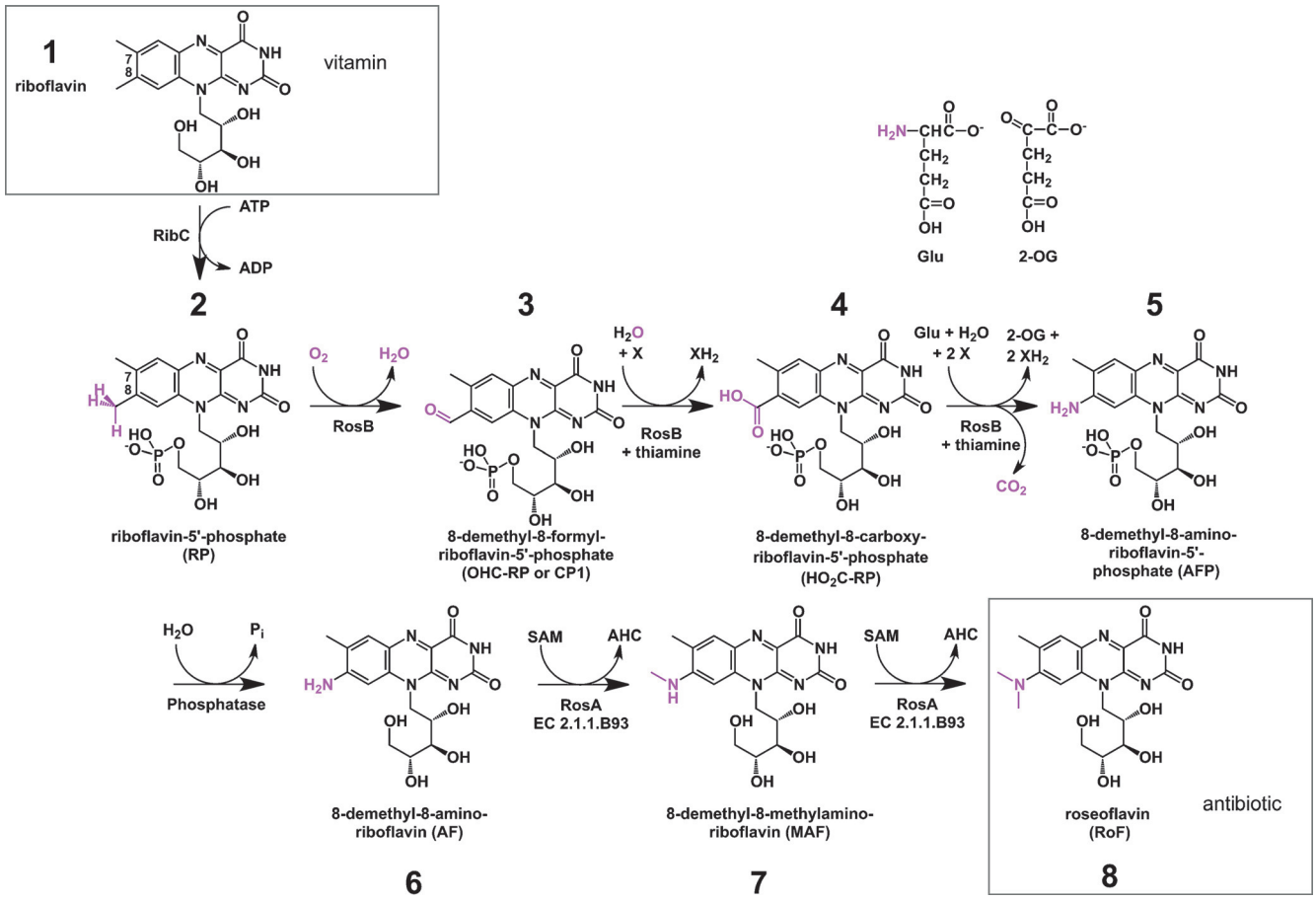

Figure 1. Roseoflavin (RoF) biosynthesis in *S. davawensis* starting from riboflavin. RP (**2**) is formed from riboflavin and ATP by the flavokinase RibC.[8b] The first RosB reaction, the conversion of RP into OHC-RP (CP1; **3**), occurs in the presence of oxygen only. The conversion of OHC-RP into HO_2C-RP (**4**) depends on thiamine, with oxygen not being required. HO_2C-RP is decarboxylated and transaminated to AFP (**5**) in the presence of thiamine and glutamate with the concomitant synthesis of 2-oxoglutarate (2-OG). This reaction also occurrs in the absence of oxygen. The SAM dependent dimethyltransferase RosA is responsible for the conversion of AF to RoF (via MAF).[13] As a side product, *S*-adenosylhomocysteine (AHC) is formed. Notably, AFP is not a substrate for RosA;[13] the dephosphorylation of AFP is carried out by an as yet unknown enzyme.

The following expression experiments were carried out to reduce the number of candidate genes for AF biosynthesis present on the cosmid pESAC13-SD106 kbp. 5′- and 3′-trimmed versions containing 84 and 76 genes, respectively, were generated (Figure 2) and transferred to *S. coelicolor* by conjugation. Supernatants from stationary-phase cultures of the resulting recombinant *S. coelicolor* strains were found to contain RoF, thus strongly suggesting that the gene(s) responsible for the formation of the intermediate AF were still present on these modified cosmids. Since cluster 7 was shown not to contain candidate genes for AF synthesis,[13] the gene/s had to be present in clusters 1, 2, 3, 4, 5, 6, or 8 (Figure 2). These gene clusters were removed (one at a time) from the chromosome of *S. davawensis*, and supernatants from the resulting deletion strains were analyzed for the presence of RoF. Only deletion of gene cluster 2 led to a strain that did not produce RoF (Figure 2, chromatogram b). This suggests that the gene/s responsible for the synthesis of AF is/are present in gene cluster 2. The five genes present in gene cluster 2 were removed (one at a time) from the chromosome of *S. davawensis*, and supernatants from the resulting deletion strains were analyzed with regard to the presence of RoF. Only deletion of the gene *BN159_7989* in cluster 2 generated a strain that did not produce RoF (Figure 2, chromatogram c), thus indicating that a single enzyme (the gene product of *BN159_7989*) is responsible for the synthesis of AF. This idea was supported by the results of the following experiments. Overexpression of *S. davawensis BN159_7989* in *S. coelicolor* led to the synthesis of AF (Figure 2, chromatogram d), and coexpression of *S. davawensis rosA* and *BN159_7989* in *S. coelicolor* and in the Gram-negative bacterium *Escherichia coli* resulted in the synthesis of RoF. Most interestingly, the results of the expression experiments in *E. coli* indicate that the reaction product of BN159_7989 may be AFP (the phosphorylated form of AF) and not AF (Figure S2 in the Supporting Information). The following analyses show that the protein BN159_7989 indeed forms AFP and that an additional enzyme, a phosphatase, is required to generate AF from AFP.

BN159_7989 was compared to other proteins listed in public databases and was found to contain a putative RP binding site (Figure S3 in the Supporting Information). This

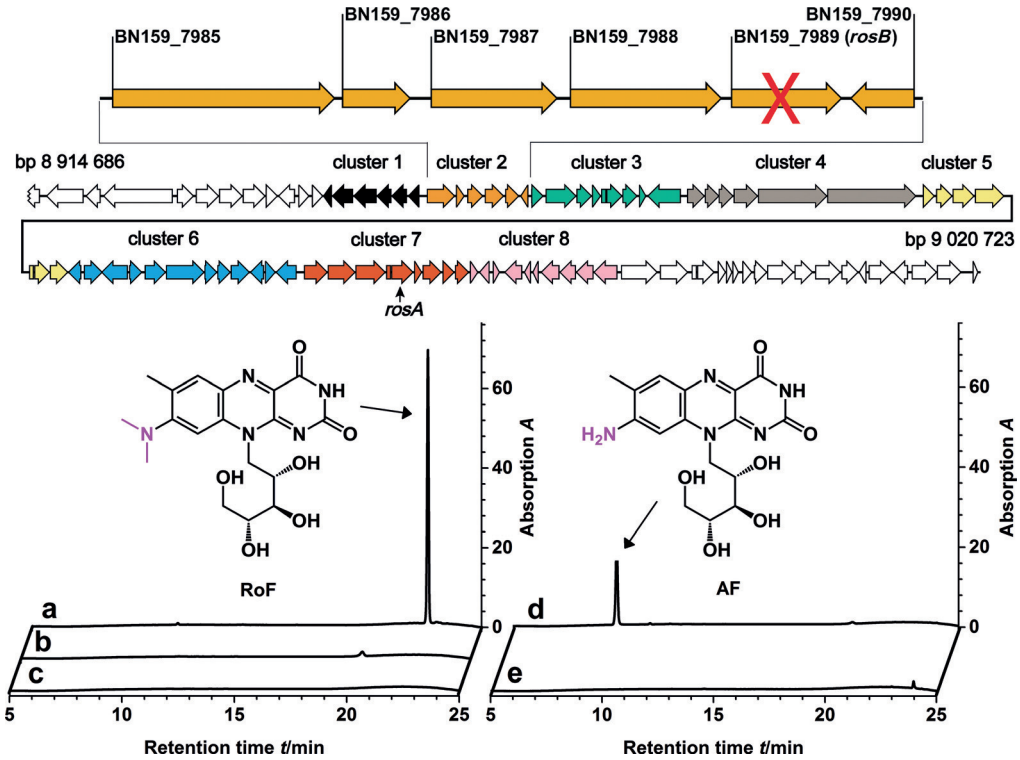

Figure 2. Identification of the key gene responsible for the formation of AF through systematic gene deletion and expression experiments. Middle: schematic representation of the 95 coding sequences present on a 106 kbp subgenomic fragment of *S. davawensis* (from bp 8 914 686 to bp 9 020 723).[5] The white genes at the 5′- and 3′-ends were removed ("cosmid trimming") and were found not to be relevant for AF synthesis. The gene clusters 1, 2, 3, 4, 5, 6 and 8 were sequentially removed from the chromosome of *S. davawensis*. Bottom: analysis of cultures of *S. davawensis* deletion strains for synthesis of RoF and AF by HPLC (absorption wavelength $\lambda = 480$ nm). *S. davawensis* wild-type (control) produces RoF (a). Deletion of cluster 2 (systematic names of the coding sequences are shown)[5] abrogated synthesis of both RoF and AF (b). Upon deletion of the gene *BN159_7989* (*rosB*) (red cross in top panel), neither RoF nor AF wre produced (c). Expression of *BN159_7989* in *S. coelicolor* led to the accumulation of AF in the supernatant (d). *S. coelicolor* wild-type (control) produces neither AF nor RoF (e).

suggests that RP (and not riboflavin) is the starting point for RoF synthesis. A recombinant version of BN159_7989, BN159_7989-His_6, was overproduced in *E. coli* and purified to apparent homogeneity (Figure S4 in the Supporting Information). This procedure yielded a yellow tetrameric protein. Denaturation of the yellow BN159_7989-His_6 preparation led to the release of a yellow compound, which was identified as AFP (Figure S5 in the Supporting Information). Upon modification of the initial purification procedure BN159_7989-His_6 preparations were obtained that were largely free (< 5%) of AFP and were used for biochemical analysis of this protein.

BN159_7989-His_6 (< 5% AFMN) was added to an enzyme assay containing RP as the sole substrate. Following incubation, the reaction mixture was analyzed by ultra-performance liquid chromatography coupled with electrospray-ionization tandem mass spectrometry (UPLC/ESI/MS2). An unknown compound (CP1; **3**) with an m/z value of 471 was synthesized from RP (but not from riboflavin) with a specific activity of 0.44 nmol min^{-1} (mg protein)$^{-1}$ (± 0.01; $n = 6$; K_m for RP < 20 μM). This reaction occurred in the presence of oxygen only (Figure S6b in the Supporting Information), with no other substrate or cofactor being added to the assay. The UV/Vis spectrum of CP1 was similar to that of RP (Figure S7 in the Supporting Information), thus indicating that CP1 is a flavin. CP1 was further analyzed by UPLC/ESI/MS2, and comparison of its product ion spectra with those of RP, AFP (Figure S6a,h), [U-^{15}N]riboflavin, [U-^{13}C]riboflavin, and [U-^{13}C,U-^{15}N]riboflavin (Figure S8 in the Supporting Information) indicated that CP1 is a flavin with a modified dimethylbenzene ring. The m/z value of CP1 and the mass differences in the product ion spectra were consistent with oxidation of one of the methyl groups on the dimethylbenzene ring of RP to a formyl group in CP1 (see Figure 1, conversion of compound **2** into compound **3**). The identification of CP1 as 8-demethyl-8-formylriboflavin-5′-phosphate (OHC-RP) is in line with the need for oxygen in the reaction and the broad chromatographic peak observed for the aldehyde CP1, which indicates the reversible on-column formation of a hemiacetal with methanol (Figure S6b). The rapid reaction of CP1 with phenylhydrazine (which is a detection reagent for primary aldehydes) to give the predicted sharp-eluting hydrazone (Figure S6c), as well as the oxidation of CP1 to 8-demethyl-8-carboxyriboflavin-5′-phosphate (HO_2C-RP) with hydrogen peroxide (Figure S6d,e), confirmed the presence of an aldehyde group in CP1 (see also Figure S9).

The identification of OHC-RP (CP1) as an intermediate of AFP synthesis by BN159_7989-His_6 suggested that a decarboxylation reaction (Figure 1, conversion of compound **4** into compound **5**) paves the way for the introduction of an amino group. Such a reaction would require the prior oxidation of OHC-RP to HO_2C-RP, which was not observed in the initial enzyme assay containing RP and BN159_7989-His_6. Different components were added to this initial assay mixture (one at a time; see Table S1 in the Supporting Information) to stimulate the anticipated formation of HO_2C-RP by BN159_7989-His_6. In the presence of thiamine, the oxidation of OHC-RP to HO_2C-RP indeed was observed. HO_2C-RP

was formed by BN159_7989-His_6 with a specific activity of 0.61 nmol min^{-1} (mg protein)$^{-1}$ ($\pm$0.04; $n=6$; Figure 6e,f). Thiamine pyrophosphate was not as effective in stimulating the formation of HO_2C-RP from OHC-RP (Table S1) when compared to thiamine. Neither oxygen nor any other substrate or cofactor was required for this reaction step. The role of thiamine in the oxidation of OHC-RP to HO_2C-RP remains elusive. Clearly, synthesis of the final product AFP, which requires the transfer of an amino group from an as yet unknown donor, did not occur. A modified enzyme assay, now containing RP, thiamine, and BN159 7989-His_6, was thus used to screen putative amino group donors (Table S1). As a result of this screening, glutamate was identified as the amino group donor for AFP synthesis (Figure 6g,h and Figure S9). In the presence of RP, thiamine, and glutamate, AFP was formed by BN159_7989-His_6 with a specific activity of 0.33 nmol min^{-1} (mg protein)$^{-1}$ ($\pm$0.06; $n=6$; Figure S6e,f). Transfer of the amino group from glutamate to HO_2C-RP was verified using [^{15}N]glutamate (instead of ^{14}N-glutamate) as a cosubstrate: the expected +1 u mass shift from 8-demethyl-8-aminoriboflavin-5′-phosphate (^{14}N-AFP) to [8-^{15}N]8-demethyl-8-aminoriboflavin-5′-phosphate (8-[^{15}N]AFP) was detected (Figure S6i and Figure S9). The expected concomitant synthesis of 2-oxoglutarate was validated using a coupled enzyme assay with (*R*)-2-hydroxyglutarate dehydrogenase.[14] The addition of NAD^+ (but not of $NADP^+$) to this final BN159_7989 assay enhanced AFP formation by a factor of 1.7 (see Table S1).

We conclude that the key reactions of RoF biosynthesis are carried out by BN159_7989, which converts the riboflavin-derived cofactor RP into AFP. An as yet unknown phosphatase hydrolyses AFP to give AF, which is converted into RoF by RosA. Hence, it takes four enzymes to convert the vitamin into a potent antibiotic (Figure 1). Being the second enzyme of RoF biosynthesis to be discovered, BN159_7989 was renamed RosB. RosB is an 8-demethyl-8-aminoriboflavin-5′-phosphate synthase and the first member of a novel class of enzymes (with a novel reaction mechanism) that are oxidoreductases, decarboxylases, and aminotransferases in one.

Genome sequencing of *S. cinnabarinus*, the second known RoF producer, revealed that the corresponding homologous genes (*rosA* and *rosB*) are present. Notably, the primary structure of RosB from *S. cinnabarinus* is 100% identical to that of RosB from *S. davawensis* (Figure S3).

RosB could be extremely useful for chemical synthesis. Careful engineering of RosB may allow the site-specific replacement of methyl groups by amino groups in polyaromatic compounds of commercial interest. Structural studies are underway that will hopefully shed light on the reaction mechanism. A working hypothesis is shown in Figure S10 in the Supporting Information.

Keywords: riboflavin · riboflavin-5′-phosphate · roseoflavin · *streptomyces davawensis*

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