Supporting Information

Catalytic Control of Spiroketal Formation in Rubromycin Polyketide Biosynthesis

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1. Materials and methods

1.1 Recombinant production and purification of RubL

In order to allow for the recombinant production of RubL, the respective gene was purchased, codon optimized for *Escherichia coli* and subcloned into a pET16b-vector (BioCat), in frame with the nucleotide sequence coding for an N-terminal histidine-tag. In addition, a tobacco etch virus (TEV)-cleavage site was inserted 5’ to the gene sequence, to enable the selective removal of the affinity tag prior to protein crystallization. Upon arrival, the dry plasmid DNA was dissolved in ddH₂O to a final concentration of 100 ng µL⁻¹ and transformed into *E. coli* BL21(DE3)-cells (Thermo Fisher Scientific) for subsequent gene expression.

For the recombinant production of the protein, TB-medium containing 100 µg mL⁻¹ ampicilllin was inoculated with a pre-culture grown in LB-medium (supplemented with the same amount of antibiotic) to an optical density at 600 nm (OD₆₀₀) of ~0.1. The cultures were incubated at 37 °C and 130 rpm, until OD₆₀₀ of ~0.5-0.6 was reached. Then, the temperature in the incubator was set to 18 °C and protein production was induced with 0.1-0.2 mM IPTG. After overnight incubation at 18 °C and 130 rpm, cells were harvested by centrifugation (4000 g for 15 min). For protein purification, the resulting pellets were resuspended in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4 (binding buffer) and cells were lysed by ultrasonication (3 s pulse, 2 s pause; 5 min total pulse time) with a Branson Digital Sonifier (Branson). Then, the lysate was cleared by centrifugation (18000 g for 40-50 min) and loaded onto two 5 mL Ni-NTA FF crude columns (GE healthcare, Cytiva) pre-equilibrated with binding buffer. Unspecifically bound proteins were removed by extensive washing with wash buffer (50 mM Tris, 300 mM NaCl, 30 mM imidazole, 10 % glycerol, pH 7.4) and RubL was eluted with elution buffer (50 mM Tris, 300 mM NaCl, 500 mM imidazole, 10 % glycerol, pH 7.4). Yellow fractions, containing pure RubL were pooled and buffer was exchanged to binding buffer using desalting columns (Hi-trap desalting, GE healthcare/Cytiva). RubL used in activity assays, was concentrated to 350 µM using centripreps (cut-off 30 kDa, Pall), flash frozen in liquid nitrogen and stored at -80 °C until further use.

RubL used for crystallization, after buffer-exchange was incubated with TEV-protease (10:1, m/m) at 4 °C overnight. After removal of the cleaved His-tag and the TEV-protease by a second round of affinity chromatography, the protein was applied to a Superdex 200 pg (HiLoad 16/600) column, pre-equilibrated with 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4, for further purification. Fractions containing pure RubL were combined, concentrated (to 15 mg mL⁻¹) and stored at 4 °C until further use.
1.2 Cloning and recombinant production of MBP-TEV-GrhO5

Since the initial MBP-GrhO5 construct generated by Frensch et al. \(^1\) did not contain a protease cleavage site allowing for the removal of the solubility tag (MBP-tag) prior to protein crystallization, grhO5 was recloned to the vector pETHis\(_{6}\)-MBP-TEV (between SspI and XhoI, site). Having confirmed the proper insertion of the gene by automated sequencing, the plasmid was transformed into *E. coli* BL21 (DE3)-cells for gene expression. Protein production and affinity purification (incl. removal of the affinity tag) were carried out as described for RubL above.

Like RubL, GrhO5 used for crystallization was applied to a Superdex 200 pg (10/600 HiLoad) column, in this case pre-equilibrated with 20 mM Tris, 50 mM NaCl, pH 7.4, for further purification. Again, yellow fractions corresponding to pure monomeric GrhO5 were combined, concentrated to 200 µM (11 mg mL\(^{-1}\)), flash frozen in liquid N\(_2\) and stored at -80°C until further use.

1.3 Determination of the molar extinction coefficient of GrhO5-/RubL-bound FAD

To allow for the determination of the molar extinction coefficients of the protein-bound FAD cofactors, a UV-visible absorption spectrum of both native and denatured GrhO5/RubL was recorded. Based on the assumption that the spectrum of denatured protein equals the one of free FAD (\(\varepsilon_{450} \approx 11300\ \text{M}^{-1}\ \text{cm}^{-1}\)), molar extinction coefficients of 10 250 M\(^{-1}\) cm\(^{-1}\) and 11 300 M\(^{-1}\) cm\(^{-1}\) were calculated for GrhO5 and RubL, respectively.

1.4 Anaerobic photoreduction of RubL-bound FAD

Photoreduction of RubL-bound FAD was carried out in a UV-1800PC photometer (Shimadzu) placed in an anaerobic chamber. A solution containing 11 µM RubL, 25 mM EDTA and 5 µM methylviologen (all components were rendered anaerobic prior to mixing) was prepared in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4 and a UV-visible absorption spectrum was recorded between 300 and 800 nm. Then, the sample was irradiated and changes in the UV-visible absorption characteristics were monitored until full reduction of the protein-bound FAD cofactor was observed (no further spectral changes). Finally, the solution was exposed to air for several minutes and a spectrum of “reoxidized” RubL was recorded.

1.5 Determination of the reduction potential of RubL-bound FAD

The reduction potential of RubL-bound FAD was determined photometrically (UV-1800PC photometer, Shimadzu) in an anaerobic chamber using the xanthine-xanthine oxidase method first reported by Massey\(^2\). First, two solutions, one containing 35 µM RubL, 500 µM xanthine and 10 µM methylviologen and one containing ~40 µM anthraquinone-2,6-disulfonate were prepared in 50 mM Tris, 300 mM NaCl, pH 7.4. Then, these two solutions were mixed (1:1,
and a UV-visible absorption (starting) spectrum was recorded between 300 and 800 nm. Finally, catalytic amounts of xanthine oxidase (0.06 U, Roche) were added and changes of the UV-visible absorption characteristics were monitored until full reduction of the protein-bound FAD cofactor and the reporting dye was observed (~30 min). By plotting the log(RubL_{ox}/RubL_{red}) at 357 nm (isosbest of anthraquinone-2,6-disulfonate reduction) as a function of the log(dy_{ox}/dy_{red}) at 343 nm (isosbest of RubL-bound FAD reduction) a Nernst-plot was generated, allowing for the calculation of the reduction potential of RubL from the resulting y-axis intercept \[3\] (quadruplicate determination).

1.6 Steady state kinetics – reduction of \(3\) by RubL under anoxic conditions

Steady-state parameters for the reduction of collinone by RubL wild type were determined using a UV-1800PC photometer (Shimadzu) placed in an anaerobic tent. All assay components were rendered anaerobic and reaction mixtures containing 1 mM NADPH and 33 nM RubL were prepared in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4. Then, varying concentrations of collinone (25-350 µM) were added, and absorption changes at 650 nm (\(\epsilon_{650}\) of oxidized collinone in buffer pH 7.4: 3 660 M\(^{-1}\)cm\(^{-1}\)) were monitored for 180 s. By plotting the extracted reduction rates as a function of the respective substrate concentrations and by applying a hyperbolic fit, the enzyme characteristic parameters \(k_{cat}^{app}\) and \(K_{M}^{app}\) were obtained (rates at each substrate concentration were determined at least in triplicate).

1.7 Reduction of \(3\) by RubL-wild type and -variants under anoxic conditions

To assay the effect to several amino acid replacements in the active site of RubL on \(3\) reduction, steady-state assays were performed under anoxic conditions. Reaction mixtures containing 1 mM NADPH and 350 µM \(3\) were prepared in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4 and incubated at room temperature (ca. 23 °C) for 3 min. Then, RubL-WT or one of the variants was added (WT, 233 nM; R87K, 3.2 µM; E103Q, 360 nM; E103A, 390 nM; H225A, 420 nM; W289A, 4.3 µM; R374K, 2.3 µM; R374M, 1 µM) and absorption changes at 725 nm (\(\epsilon_{725}\) of oxidized collinone in buffer pH 7.4: 1 060 M\(^{-1}\)cm\(^{-1}\)) were monitored for 180 s. Reduction rates were extracted and normalized to the enzyme concentration to obtain apparent \(k_{cat}^{app}\) values for all variants (reduction rates were determined at least in triplicate for each variant).

For comparison, reaction rates were also determined in the presence of 50 µM \(3\). In this case, reaction mixtures containing 1 mM NADPH and 50 µM \(3\) were prepared in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4 and incubated at room temperature (ca. 23 °C) for 3 min. Then, RubL-WT or one of the variants was added (WT, 233 nM; R87K, 0.8 µM; E103Q, 120 nM; E103A, 130 nM; H225A, 140 nM; W289A, 530 nM; R374K, 2.3 µM; R374M, 1 µM) and absorption changes at 650 nm (\(\epsilon_{650}\) of oxidized collinone in buffer pH 7.4: 3 660 M\(^{-1}\)cm\(^{-1}\)) were monitored using a UV-1800PC photometer (Shimadzu) for 180 s. Reduction rates were again
extracted and normalized to the enzyme concentration to obtain apparent $k_{cat}$ values for all variants (reduction rates were determined at least in triplicate for each variant).

1.8 NADPH-concentration dependence of collinone reduction

To study the effect of the NADPH-concentration on the rate of collinone reduction, again a photometric steady-state assay was performed under anoxic conditions. Reaction mixtures containing 66 nM RubL (for the control no enzyme was used) and varying concentrations of NADPH (0.5-4 mM) were prepared in 50 mM Tris, 300 mM NaCl, 10 % glycerol pH 7.4, before adding collinone (250 µM final concentration) and monitoring the decrease in absorption at 725 nm ($\varepsilon_{725}$ of oxidized collinone in buffer pH 7.4: 1 060 M$^{-1}$cm$^{-1}$) for 180 s. Reduction rates were extracted and plotted as a function the respective NADPH-concentration, revealing a linear correlation between the two parameters (rates at each NADPH-concentration were determined in triplicate).

1.9 Site-directed mutagenesis

In order to investigate the role of several active site residues in catalysis, different RubL-variants were generated using PCR-based mutagenesis. In all cases, the mutations were introduced to the pET16b-RubL wild type expression plasmid using both forward and reverse primers carrying the desired nucleotide replacements.

**Table 1: Forward and reverse primers used for site-directed mutagenesis, with the codon triplets carrying the desired nucleotide replacements shown in bold.**

| Variant | Primer | Primer sequence (5'-3') |
|---------|--------|------------------------|
| R87K    | fwd.   | CATCGTACCAAAAGCAGGCGTGACC |
|         | rev.   | CACGCCCTGCTTTGTTACGGTGTTG |
| E103Q   | fwd.   | GTAAGGAACAGCTGGAAGGTGACGATG |
|         | rev.   | CACCTTCCAGCTTCTTTAATAACAGAAC |
| E103A   | fwd.   | GTAAGGAAAGCTGTGAAGGTGACGATG |
|         | rev.   | CACCTTCCAGAGCTTCTTTAATAACAGAAC |
| H225A   | fwd.   | GCCGTGGTGCTGTAAAAATGGCGACG |
|         | rev.   | GGAATTTTACACAGCACAAGGCTATAACG |
| W289A   | fwd.   | GTGTTTCCGGCAGATATGGCCGG |
|         | rev.   | CGGCCATATCGGCGGAAAAACGACTAC |
| R374K   | fwd.   | CTGGCAGCTAAAAAGCGGACCGGACAAC |
|         | rev.   | GTGCCGCTTTTCAATGCCAGCTGCAG |
| R374M   | fwd.   | CTGGCAGCTATGAGCGGCAACGCAAC |
|         | rev.   | GTGCCGCTCATGCGCAGCTGCAG |
Having confirmed the successful introduction of the nucleotide replacements by automated sequencing, the constructs were transformed into *E. coli* BL21(DE3)-cells and protein production and purification were carried out as described for RubL wild type (see above).

1.10 **Short-time conversion assays (HPLC-analysis based)**

To study the effect of several amino acid replacements on the efficiency of collinone reduction, apart from the steady-state assay conducted under anoxic conditions, also a short turnover assay under aerobic conditions was performed. Generally, reaction mixtures (100 µL final volume) containing 1.5 mM NADPH and 10 µM RubL(-variant) were prepared in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4 and incubated at 30 °C for 2 min. Then, collinone (450 µM final concentration) was added and the samples were incubated at 30 °C and 750 rpm for 30 s. All reactions were stopped and extracted with 150 µL EtOAc:FA (9:1, v/v) and the organic layers were analyzed by HPLC-DAD immediately (min. three assays per variant).

1.11 **10 min conversion assays (HPLC-analysis based)**

To investigate the role of the key active site residues on the efficiency of collinone conversion/lenticulone formation, a 10 min turnover assay was performed. Reaction mixtures (100 µL final volume) containing 1.5 mM NADPH and 10 µM RubL(-variant), 5 mM MgCl$_2$, 10 mM glucose-6-phosphate (G6P) and 2 U of G6P-dehydrogenase were prepared in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4 and pre-incubated at 30°C for 2 min. Subsequently, turnover was started by the addition of collinone (450 µM final concentration) and the samples were incubated at 30 °C and 750 rpm for 10 min. The reactions were stopped and extracted with 150 µL EtOAc:FA (9:1, v/v) and the organic layers were analyzed by HPLC-DAD immediately (min. three assays per variant).

1.12 **Structure-based sequence alignment (group A monoxygenases)**

Sequences for the alignment were selected based on a blastp of the RubL sequence against the sequences/structures deposited in the protein database (PDB) – duplicates and sequences with no significant similarity to RubL were omitted. Instead, the sequences of the known RubL-homologs GrhO5, HlqO5, and HyalO5 were added. Then, a structure-based alignment was generated using the online program T-coffee$^4$ and visualized in Jalview$^5$. Upon comparison of the sequence alignment with the overlaid 3D-structures, errors in the alignment of phenol hydroxylase and *m*-hydroxybenzoate hydroxylase to the other sequences were observed, requiring manual editing of the alignment. Finally, the alignment was visualized using SeaView$^6$ and a bootstrapped distance tree (1000 repetitions) was generated in the same program. The rooted tree was exported in Newick format and visualized and color-edited using the online program iTOL$^7$. 

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1.13 Analytical gelfiltration
Analytical gelfiltration was carried out to determine the biologically active oligomer of RubL wild type and its variants as well as of GrhO5 in solution. Protein samples were applied to a Superdex 200 10/300 GL column, pre-equilibrated with 20 mM Tris, 50 mM NaCl, pH 7.4 and the molecular weight of the protein eluting in the main peak fraction was estimated based on a previously generated calibration curve.

1.14 HPLC detection
Turnover assays requiring offline detection, were analyzed by HPLC using an Agilent 1100 chromatographic system (Technologies), equipped with a VP NUCLEODUR 100-5 C18ec (250 x 4.6 mm ID, 5 µM, Macherey-Nagel) coupled with a UNIVERSAL RP guard column (4 x 3 mm ID, Macherey-Nagel). The column was pre-equilibrated with ddH₂O + 0.1 % TFA (solution A) and acetonitrile + 0.1 % TFA (solution B) 90:10 (v/v) and samples were analyzed at a flow rate of 1.5 mL min⁻¹ using the following gradient: 10 % B (0-1 min), 10-100 % B (1-15 min), 100 % B (15-20 min), 10 % B (20-25 min). Considering the strong absorption of all analyzed compounds in the visible-light region, absorption changes at 254, 350, and 500 nm were selected to be monitored by the diode array detector connected to the chromatographic system.

1.15 Crystallization - RubL
RubL crystals were grown in sitting-drop vapor diffusion set-ups (MRC Maxi Plate – Polystyrene; Jena Biosciences). 250 µM RubL (15 mg mL⁻¹ presoaked with 1 mM 7) in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4, was mixed with an equal volume of a complex reservoir solution (20 mM DL-arginine hydrochloride, 20 mM DL-threonine, 20 mM DL-histidine monohydrochloride monohydrate, 20 mM DL-5-hydroxylysine hydrochloride, 20 mM trans-4-hydroxy-L-proline, 100 mM BES/TEA pH 7.5 buffer system, 12.5 % PEG 4000 and 2 0% 1,2,6-hexanetriol from Morpheus® II crystallization screen, Molecular Dimensions, Sheffield, UK) at 22 °C. Clusters of RubL needles appeared within 30 days and matured 60 days at 22 °C. Single crystals were dislodged from the cluster, dipped briefly into a drop of reservoir solution without any cryoprotectant, mounted into MiTeGen MicroMounts/MicroLoops, and instantly flash frozen in liquid nitrogen.

1.16 Crystallization - GrhO5
GrhO5 was crystallized using sitting-drop vapor diffusion (MRC Maxi Plate – Polystyrene; Jena Biosciences). 200 µM (~11 mg mL⁻¹) protein solution in 20 mM Tris, 50 mM NaCl, pH 7.4 was mixed with 20 % PEG 3350, 200 mM di-sodium malonate or with 20 % PEG 3350, 200 mM sodium formate (i.e. a crystallization screen condition from JBScreen PACT++, Jena
bioscience, jena, germany) in a 1:1 ratio (0.7 µl/0.7 µl) and plates were incubated at room temperature (23-24°C). after 4-6 months, intense yellow, rod-shaped crystals appeared, which were dipped briefly into a drop of reservoir solution supplemented with 20% (v/v) glycerol for cryoprotection, mounted into MITEGen MicroMounts/MicroLoops, and flash frozen in liquid nitrogen.

GrhO5 + 3 complex crystals were prepared by washing a dislodged single GrhO5 crystal in reservoir solution (2 times) and by subsequently soaking in reservoir solution supplemented with 4 mM 3 in 10% DMSO for 3 hours prior to cryoprotection and freezing.

1.17 X-ray Data Collection and Structure Determination

High-resolution X-ray diffraction datasets were collected at the PXI-X06SA beamline (SLS synchrotron, Villigen, Switzerland) at 12.4 KeV (λ=1.0 Å), using an Eiger 16M X (133 Hz, Dectris) detector. Intensity data were collected (φ scans of 0.1° over 180° or 360°) up to a resolution of 1.57 Å for RubL crystal and up to 1.75 Å for GrhO5 crystals. The datasets were processed by XDS[8] in I2 space group (a = 77.32, b = 57.51, c = 122.38; α = γ = 90°, β = 95.19°) for RubL crystal, P21 space group (a = 46.07, b = 104.44, c = 58.78; α = γ = 90°, β = 104.89°) for GrhO5 and P21 space group (a = 45.67, b = 103.75, c = 9°; α = γ = 90°, β = 95.19°) for GrhO5 in complex with collinone. The X-ray diffraction reflections were merged and scaled using SCALA[9]. Structure determination of RubL and GrhO5 was performed by molecular-replacement with PHASER[10] as implemented in CCP4 suite, using a RubL- or GrhO5-structure model generated by the FFAS server (https://ffas.godziklab.org/) or SWISS-model server[12], respectively, based on the crystal structure of Streptomyces purpurascens RdmE of rhodomyacin biosynthesis (PDB ID: 3ihg) as the search model[13]. The structures were modified manually with Coot[14] and refined with REFMAC[15] or PHENIX[16][10]. For omit map calculations Polder OMIT[17] maps tool was used. Restraints for collinone and 1,2,6-hexanetriol were generated using AM1 QM optimization method in eLBOW[18] as implemented in PHENIX[16]. All crystallographic figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 2.2.0 Schrödinger, LLC). The structures have been deposited in the Protein Data Bank with accession codes 7OUC for GrhO5, 7OUD for GrhO5 with collinone and 7OUJ for RubL. The data collection and refinement statistics are given in Table S1.
## 2. Supplementary Figures and Tables

Table S1: Data collection and refinement statistics.

|                         | GrhO5•FAD | GrhO5•FAD•3 | RubL•FAD |
|-------------------------|-----------|-------------|----------|
| **PDB code**            | 7OUC      | 7OUD        | 7OUJ     |
| **Wavelength (nm)**     | 1.0       | 1.0         | 1.0      |
| **Resolution range (Å)**| 44.52 - 1.75 (1.84 - 1.75) | 44.19 - 2.3 (2.42 - 2.30) | 46.08 - 1.57 (1.66 - 1.57) |
| **Space group**         | P 1 2 1   | P 1 2 1     | I 1 2 1  |
| **Unit cell (Å), (°)**  | 46.07 104.44 69.23 | 45.67 103.75 69.00, 90 104.44 90 | 77.32 57.51 122.38, 90 95.19 90 |
| **Total reflections**   | 60464 (8879) | 87229 (12544) | 168341 (24765) |
| **Unique reflections**  | 62285 (8665) | 26914 (3911) | 70014 (10481) |
| **Completeness (%)**    | 97.8 (92.7) | 97.4 (97.5) | 94.4 (96.9) |
| **Mean I/σ(I)**         | 5.0 (1.2) | 5.8 (2.8) | 9.4 (2.1) |
| **R-merge (%)**         | 14.6 (63.5) | 25.1 (48.6) | 4.6 (54.9) |
| **CC1/2**               | 0.99 (0.76) | 0.92 (0.83) | 0.99 (0.69) |
| **R-work (%)**          | 19.43 | 22.50 | 18.30 |
| **R-free (%)**          | 23.27 | 26.29 | 20.94 |
| **Number of non-hydrogen atoms** | 4429 | 4255 | 4264 |
| **Macromolecules**      | 3885 | 3885 | 3983 |
| **Ligands**             | 53 | 92 | 87 |
| **Solvent**             | 491 | 278 | 194 |
| **Protein residues**    | 527 | 533 | 541 |
| **RMS (bonds)**         | 0.010 | 0.009 | 0.012 |
| **RMS (angles)**        | 1.594 | 1.543 | 1.706 |
| **Ramachandran favored (%)** | 98.09 | 96.18 | 97.40 |
| **Ramachandran allowed (%)** | 1.87 | 3.06 | 2.60 |
| **Ramachandran outliers (%)** | 0.38 | 0.76 | 0.00 |
| **Rotamer outliers (%)** | 2.35 | 4.44 | 1.48 |
| **Clashscore**          | 4.75 | 7.02 | 2.98 |
| **Average B-factor (Å²)** | 25.35 | 10.11 | 29.35 |
| **Macromolecules (Å²)** | 24.31 | 10.06 | 29.15 |
| **Ligands (Å²)**        | 15.17 | 12.50 | 26.78 |
| **Solvent (Å²)**        | 34.68 | 9.96 | 34.59 |
Table S2: Overview of all group A FPMOs and their origin that are displayed in the phylogenetic tree of Figure 3. A. The active sites feature unique conserved tryptophan residues for GrhO5-like type I enzymes. While the FAD cofactor in GrhO5 is held in the "OUT"-position by sandwich π-π stacking with W281, this particular tryptophan is missing in the type II enzymes such as MtmOIV and other group A FPMOs such as PHHY. These enzymes, however, exhibit another conserved tryptophan residue, which is missing in the type I enzymes. Note that GrhO6 and the functional homologs of GrhO5 (HlgO5 and HyalO5) required for formation of other rubromycin polyketides have not been structurally characterized and that GrhO6 clusters with type II enzymes rather than the GrhO5-like type I. All shown enzymes are involved in the biosynthesis or modification of natural products apart from phenol hydroxylase (PHHY)\textsuperscript{(19)}, meta-hydroxybenzoate hydroxylase (MHBH)\textsuperscript{(20)} and 2-hydroxybiphenyl 3-monooxygenase (HbpA)\textsuperscript{(21)} from bacterial and fungal primary metabolism. For a comprehensive tree including enzymes from primary metabolism and fungal group A FPMOs, see ref. \textsuperscript{(22)}.

| Enzyme | PDB-ID | Species | Kingdom of life |
|--------|--------|---------|-----------------|
| PHHY  | 1pn0   | *Trichosporon cutaneum* | fungus          |
| MHBH  | 2dkh  | *Comamonas testosterone* | bacterium       |
| RebC  | 2r0c  | *Lentzea aerocolonigenes* | bacterium       |
| PieE  | 6u0p  | *Streptomyces sp. SCSIO 3032* | bacterium       |
| HbpA  | 4z2t  | *Pseudomonas nitroreducens* | bacterium       |
| RdmE  | 3lhg  | *Streptomyces purpurascens* | bacterium       |
| GrhO5 | 7ouc  | *Streptomyces JP95* | bacterium       |
| RubL  | 7ouj  | *Streptomyces collinus* | bacterium       |
| Tmn9  | 6ui5  | *Streptomyces sp. NRRL 11266* | bacterium       |
| PyrE3 | 5xgv  | *Streptomyces rugosporus* | bacterium       |
| AlpK  | 6j0z  | *Streptomyces ambofaciens* | bacterium       |
| BexE  | 4xj4  | *Amycolatopsis orientalis subsp. vinearia* | bacterium       |
| CabE  | 2qa2  | *Streptomyces* | bacterium       |
| PgaE  | 2qa1  | *Streptomyces sp. PGA64* | bacterium       |
| MtmOIV| 4k5r  | *Streptomyces argillaceus* | bacterium       |
| GrhO6 | n.a.  | *Streptomyces JP95* | bacterium       |
| RIFMO | 5vqb  | *Streptomyces venezuelae ATCC 10712* | bacterium       |
| OxyS  | 4k2x  | *Streptomyces rimosus subsp. rimosus ATCC 10970* | bacterium       |
Figure S1: Uncropped structure-based multiple sequence alignment (cf. Figure 3 main text) showing the spiroketal synthases RubL and GrhO5, as well as previously crystallized group A monooxygenases. In addition, GrhO5/RubL homologs from heliquinomycin (HlqO5) and hyaluromycin (HyalO5) biosynthesis and the second group A monooxygenase involved in griseorhodin A biosynthesis (GrhO6) were included. The structure-based sequence alignment was generated using the online tool T-coffee[4] and visualized in SeaView[6]. Final color editing to highlight important amino acid residues (FAD-binding and solvent gate keeping) and structurally conserved regions (blue boxes) was carried out in PowerPoint. For PDB-IDs of the proteins included in the alignment, see Table S2.
Figure S2: Surface representation of PHHY indicating the putative gate-keeping role of Trp336. While Trp336 (indicated in blue) shields the active site in the wild type enzyme (A), the replacement of the same residue by an Ala would lead to a strongly increased solvent accessibility of the cofactor-binding cleft (B).
Figure S3: GrhO5 ternary complex structure with FAD and collinone (3). Polder omit electron density maps (blue mesh) for collinone (3) was contoured at 3σ above the mean (collinone was positioned as stick model in the extra densities, but not included in the refinement).
Figure S4: LigPlot showing the interactions of GrhO5 (3-soaked)-bound FAD ("OUT"-conformation) with the amino acid backbones and side-chains in the active site.
Figure S5: LigPlot showing the interactions of GrhO5-bound collinone (3) with the active site residues Arg79, His217 and Arg366. See main text for details.
Figure S6: Putative O2-reaction site in RubL shown in face-on (A) and side-view (B). The Cl-ion observed in the crystal structure of RubL may well indicate the superoxide binding site in the spiroketal synthase as it is found in about 4 Å distance to the C4a-position of the FAD cofactor, which is within the optimal range for covalent oxygen adduct formation between the superoxide and the flavin (in its semiquinonic form).
Figure S7: LigPlot showing the interactions of RubL-bound FAD ("IN"-conformation) with the amino acid backbones and side-chains in the active site. The backbones of V55 and G56 as well as of N323, A322 and G319 (not visible from the LigPlot) interact with the C4=O and C2=O of FAD, respectively. Similarly, interactions of the backbones of A322 as well as of G56 and G319 (not visible from the LigPlot) with the N1 and N3, respectively, are observed.
Figure S8: Overlay of the overall structures of GrhO5 (grey) and RubL (teal) with the FAD-cofactor in “OUT”- (yellow) and “IN”- (orange) conformation, respectively. A. The overlay of the overall structures of GrhO5 and RubL indicates that movement of the FAD-cofactor from the “OUT” to the “IN” position goes along with a shift of domain II and the ordering of the loop region between residues 90-110 when co-crystallized with substrate analog 7.
Figure S9: Comparison of the spectral properties of RubL wild type and the W289A variant. While the UV-visible absorption spectrum of RubL wild type (black line), apart from the characteristic flavin absorption maxima at 370 and 450 nm, exhibits a long-wavelength absorption between 500 and 650 nm, the latter is no longer observed in the W289A variant (red line). Therefore, W289 very likely determines the spectral characteristics of the wild-type enzyme, which is in line with the observed sandwiched π-π interaction between the isoalloxazine ring and the indole moiety of W289 in the crystal structure.
Figure S10: UV-visible absorption spectra of native (black) and denatured (red) GrhO6. The spectrum of native GrhO6 was recorded between 300 and 800 nm in 50 mM Tris, 300 mM NaCl, 10 % glycerol, pH 7.4 prior to the addition of 0.5 % SDS to release the FAD-cofactor for determination of the extinction coefficient of GrhO6-bound FAD.
Figure S11: FPLC-chromatograms recorded in the course of the gel-filtration of RubL wild type (A) and the W289A-variant (B). While only a clear peak corresponding to monomeric enzyme (~15 mL; ~60 kDa) is observed in the gel-filtration chromatogram of the RubL wild type, additionally, both a substantial aggregate peak at ~10 mL and a peak corresponding to free FAD (~20 mL) are found in the elution profile of RubL-W289A.
Figure S12: Determination of the reduction potential of RubL wild type. A, Selected UV-visible absorption spectra recorded in the course of the xanthine oxidase catalyzed reduction of the RubL-bound FAD cofactor and the reporting dye anthraquinone-2,6-disulfonate (E°: -184 mV).[23] B, Nernst-plot generated by plotting the log(ox/red) of the protein-bound FAD (at 357 nm) as a function of the log(ox/red) of the dye (at 343 nm). Using the value of the y-axis intercept, subsequently the midpoint potential of RubL was calculated (-177 ± 1 mV; from 4 determinations). Due to the overlapping spectra, the reduction potential was calculated based on the spectral changes at the isobestic points observed in the course of the separate reduction of RubL (343 nm) and anthraquinone-2,6-disulfonate (357 nm).
Figure S13: Estimation of the upper and lower boundary of the reduction potential of RubL-bound FAD. A. Selected UV-visible absorption spectra recorded in the course of the xanthine oxidase catalyzed reduction of the RubL-bound FAD cofactor and the reporting dyes indigo carmine (A; $E^\circ$, -125 mV\textsuperscript{[24]}), and anthraquinone-2-sulfonate (B; $E^\circ$, -225 mV\textsuperscript{[25]}). A. The spectral changes indicate that only the reporting dye, but not the RubL-bound FAD became reduced, suggesting a more negative reduction potential (than -125 mV) for the enzyme-bound cofactor. B. The spectral changes indicate that first only the protein-bound cofactor and later the reporting dye became reduced, suggesting a more positive reduction potential (than -225 mV) for the enzyme-bound cofactor.
Figure S14: UV-visible absorption spectra of RubL WT (A) and RubL-W289A (B) in the absence and presence of 5 and NADP⁺. The black spectrum corresponds to native RubL-WT (A) and RubL-W289A (B) in 50 mM Tris, 300 mM NaCl, pH 7.4, while the purple spectra were recorded in the presence of 5 (free 5 was used as blank). Upon 5 addition, the original charge-transfer interaction (between FAD and W289) remains intact for the RubL-WT while an additional charge-transfer interaction is established. The further addition of 1 mM NADP⁺ to the enzyme-5 mixture (orange lines) resulted in a significant loss of long-wavelength absorption (particularly for the WT-enzyme), suggesting that NADP⁺ binding affects the interaction of W289 with the FAD cofactor.
Upon reduction of 3 in buffer pH 7.4 (under anoxic conditions), a rapid decrease of the absorption between 550-800 nm and an strong increase of the absorption between 400 and 550 nm are observed.
Figure S16: Basic kinetic characterization of the first step of collinone (3) turnover (reduction to dihydrocollinone (5)). A, Michaelis-Menten plot generated from the initial rates determined at six different 3 concentrations between 25 and 350 µM (rates at each substrate concentration were determined at least in triplicate; standard deviations are shown as error bars). By applying a hyperbolic fit, the enzyme typical kinetic parameters $k_{\text{cat app}}$ (4.6 ± 0.2 s$^{-1}$) and $K_{M \text{app}}$ (22 ± 4 s$^{-1}$) were obtained. B, Effect of the NADPH-concentration on 3 reduction. Interestingly, the rate of 5 formation linearly increased with the NADPH concentration (0.5 - 4 mM), both in the absence (purple squares) and in the presence (black spheres) of 1 µM RubL, indicating weak or only transient binding of NADPH to the RubL-3 complex (the rates at each NADPH-concentration were determined at least in triplicate; standard deviations are displayed as error bars).
Figure S17: HPLC-analysis of the conversion of 3 (10.3 min, pink line) into 5 (12.4 min, yellow line) catalyzed by RubL wild type or several active site variants. To investigate the influence of several amino acid replacements on the rate of 3 reduction, RubL wild type and seven variants (each 10 µM) were separately mixed with a 350 µM 3 solution (in the presence of 1.5 mM NADPH) and incubated at 30°C and 750 rpm for 30 s under aerobic conditions. Then, the reactions were stopped with EtOAc:FA (9:1) and the organic layers immediately analyzed by HPLC-DAD. While almost full conversion of 3 into 5 was observed for the wild type enzyme, particularly the arginine-variants (R87K, R374K, and R374M) and the tryptophan-variant (W289A) showed a strongly reduced reduction activity. Note that in the shown WT trace, 3 was almost completely converted to 5 without detectable amounts of the final products 4 and 7 with retention times of 14.6 min and 10.1 min, respectively (compare Figure S18). This suggests that enzyme-bound 5 is most likely displaced by remaining 3 before hydroxylation can occur.
Table S3: Summary of the relative collinone (3) reduction rates determined under anoxic (column 2 and 3) and aerobic conditions (column 4). Both in the photometric, as well as in the HPLC-based assays, very similar effects of the amino acid replacements on 3 reduction were observed. While the substitution of E103 or H225 resulted in residual activities of 20-30 %, the replacement of R87, R374 or W289 diminished the reduction activity to 3-10 %. Interestingly though, for some of the variants the relative reduction rates at the lower 3 concentration of 50 µM were slightly higher rather than lower, despite the anticipated role of both arginine residues in substrate binding (however, evaluation of the data at a substrate concentration of 50 µM is challenging, as the absorption at 650 nm ($\lambda_{max}$) is close to the detection limit).

| Protein | relative activity (%) 50 µM 3 | relative activity (%) 350 µM 3 | HPLC-assay collinone red (%) |
|---------|-------------------------------|-------------------------------|-------------------------------|
| WT      | 100 ± 3                       | 100 ± 2                       | 85 ± 2                        |
| R87K    | 7 ± 0                         | 3 ± 0                         | 4 ± 0                         |
| E103Q   | 19 ± 1                        | 22 ± 3                        | 21 ± 2                        |
| E103A   | 25 ± 1                        | 26 ± 2                        | 25 ± 1                        |
| H225A   | 37 ± 1                        | 26 ± 1                        | 32 ± 2                        |
| R374K   | 10 ± 1                        | 7 ± 0                         | 7 ± 1                         |
| R374M   | 3 ± 0                         | 3 ± 0                         | 7 ± 1                         |
| W289A   | 6 ± 0                         | 5 ± 0                         | 5 ± 1                         |
Figure S18: HPLC-analysis of the conversion of 3 (10.3 min, pink line) via 5 (12.4 min, yellow line) into 4 (14.6 min, light orange line) and 7 (10.1 min, light blue line) catalyzed by RubL wild type or several active site variants. To investigate the influence of several amino acid replacements on the efficiency of 3 turnover and 4/7 formation, RubL wild type and seven variants (10 µM) were separately mixed with a 350 µM 3 solution (in the presence of 1.5 mM NADPH) and incubated at 30°C and 750 rpm for 10 min. Then, the reactions were stopped with EtOAc:FA (9:1) and the organic layers immediately analyzed by HPLC-DAD. While substantial conversion of 3 into 4 and 7 was observed in assays with the wild type protein as well as the glutamine and the histidine variants, turnover was significantly lower in the reactions containing the R374K variant. No reaction products were found in the assay mixtures containing the W289A-, the R87K- or the R374M variant, suggesting a crucial role of these residues in catalysis.
Table S4: Summary of the 3 turnover-efficiency observed for RubL wild type and several active site variants.
Reactions were quenched before full turnover occurred to allow for comparison of the reaction rates. Shown are relative amounts of residual 3 in the assay mixture (column 1) and of produced compounds 5, 7 and 4 (columns 2, 3 and 4, respectively). n.d. = not detectable.

|     | substrate 3 | intermediate 5 | shunt product 7 | product 4 |
|-----|-------------|----------------|-----------------|-----------|
| WT  | 23 ± 0      | 53 ± 1         | 13 ± 1          | 12 ± 1    |
| R87K| 36 ± 3      | 61 ± 2         | n.d.            | < 0.5     |
| E103Q| 23 ± 1     | 56 ± 1         | 10 ± 1          | 12 ± 0    |
| E103A| 20 ± 1     | 57 ± 2         | 11 ± 1          | 14 ± 2    |
| H225A| 20 ± 1     | 56 ± 2         | 13 ± 1          | 11 ± 1    |
| R374K| 31 ± 3     | 59 ± 1         | 6 ± 1           | 4 ± 1     |
| R374M| 62 ± 2     | 38 ± 2         | n.d.            | n.d.      |
| W289A| 23 ± 3     | 77 ± 2         | n.d.            | n.d.      |
Figure S19: SDS-PAGE analysis of the different fractions collected in the course of the purification of a selected RubL-variant. Shown are: lane 1, cell pellet after lysis (P); lane 2, cleared lysate (S); lane 3, column flow-through (FT); lane 4, wash fraction (W); lane 5, selected elution fraction; lane 6, prestained protein ladder (M, Thermo Fisher Scientific®).
Figure S20: Analytical size exclusion chromatography of different RubL-variants. Shown are the chromatograms for all analyzed RubL-variants (A, R87K; B, E103Q; C, E103A; D, H225A; E, R374K; F, R374M) using a Superdex200 GL10/300 column pre-equilibrated with 20 mM Tris, 50 mM NaCl, pH 7.4. The sharp peak at 15 mL (~60 kDa) indicates that the major fraction of all variants is present as monomeric protein in solution (like the wild type) and that the single amino acid replacements introduced in the active site of RubL did not have any adverse effects on protein folding. For wild type enzyme and the W289A variant, see Figure S12.
Figure S21: Surface representation of RubL, showing the NADPH-entry to the active site and the FAD-cofactor modeled in OUT-position. The purple sphere indicates the putative NADPH-reaction site.
Figure S22: Surface representation of 3-soaked GrhO5 with the FAD-cofactor (yellow), 3 (purple) and the amino acid side chains coordinating 3/5 (grey) shown as sticks. Both views demonstrate the rather open active site of GrhO5, even in the presence of 3/5 and indicate that ring A of 3 can easily be accessed by NADPH, allowing for direct hydride transfer and formation of 5 independent of the FAD cofactor.
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