Regio- and Stereoselective Intermolecular Oxidative Phenol Coupling in *Streptomyces*

Andreas Präg,[†] Björn A. Grüning,[†] Matthias Häckh,[†] Steffen Lüdeke,[†] Marcel Wilde,[†] Andriy Luzhetskyy,[‡] Michael Richter,[#] Marta Luzhetska,[†] Stefan Günther,[†] and Michael Müller*[,†]

[†]Institute of Pharmaceutical Sciences, Albert-Ludwigs-Universität Freiburg, 79104 Freiburg, Germany
[‡]Helmholtz Institute for Pharmaceutical Research Saarland, 66123 Saarbrücken, Germany
[#]Laboratory for Biomaterials, Empa, Swiss Federal Laboratories for Materials Science and Technology, 9014 St. Gallen, Switzerland

Ⓢ *Supporting Information*

ABSTRACT: Intermolecular oxidative phenol coupling is the main process in nature for the formation of atroposelective biaryl compounds. Although well defined in plants and fungi, this type of dimerization reaction in bacteria is poorly understood. Therefore, the biosynthesis of julichromes, spectomycins, and setomimycin was investigated. The monomeric subunits of these biarylic pre-anthraquinones are derived from a common polyketidic precursor, yet the coupling reaction proceeds in a regioselective manner, with the position of attachment of the two subunits depending on the specific streptomycete strain. By using genome analysis and deletion experiments, the biosynthetic gene clusters were identified. Furthermore, it was established that cytochrome P450 enzymes are fundamentally involved during dimerization of the polyketide monomers.

A large diversity of natural products contain a C−C biaryl axis as a structural characteristic.[1] Barton and Cohen proposed that biaryl compounds such as biphenols and binaphthols are biosynthesized via intermolecular oxidative phenol coupling.[2] Based on experimental data obtained through feeding experiments, in vivo studies, and investigations of biosynthetic pathways in eukaryotes, several examples for the regioselective production of axially chiral biaryl compounds in fungi and plants have been described.[1,3] The enzymes involved in such biosyntheses could be identified in some cases. These include, for example, a cytochrome P450 enzyme that catalyzes the dimerization of chiral benzyltetrahydroisoquinolines to berbamunine;[4] furthermore, it has been demonstrated that another enzyme of this family is involved in the atroposelective coupling of P-(+)-kotanin in *Aspergillus niger*.[5] The enantioselective formation of (+)- and (−)-pinoresinol in plants is initiated via a laccase-catalyzed oxidation of (E)-coniferyl alcohol, whereas the stereochemical information for the free-radical coupling reaction is furnished by a noncatalytic dirigent protein.[6]

In contrast to what is known from plants and fungi, our mechanistic understanding of enzyme-catalyzed intermolecular oxidative phenol-coupling reactions in bacteria is limited. Recently, a flavoenzyme(s)-catalyzed atroposelective N,C-bipyrrole homocoupling in bacterial marinopyrrole biosynthesis

was described.[7] In this example, two different flavoenzymes, at least, are required to achieve atroposelective C−N coupling.[8]

For bacteria, enzymatic atroposelective intermolecular aryl coupling via C−C bond formation is as yet unknown. In the case of actinorhodin, one of the best-studied bacterial metabolites, the biocatalyst for the corresponding phenol-coupling step has not been identified.[9] Several examples of bacterial cytochrome P450 monooxygenase-catalyzed C−C cross-linking have been described; nevertheless, an unambiguous stereo- or regioselectivity could not be determined for any of the identified enzymes.[10−12]

Herein, we describe the identification and characterization of a bacterial biocatalytic system capable of performing a regio- and stereoselective intermolecular oxidative C−C phenol coupling. These results prove that the assumption of Barton and Cohen also applies to bacterial biotransformations, and might enable an application of such enzymes in synthesis by heterologous expression in other bacterial hosts.

We chose two dimeric pre-anthraquinones, julichrome $Q_{3−3}$ (**1**) from *Streptomyces afghaniensis* NC 5228 and setomimycin (**2**) from *S. aurantiacus* JA 4570, and their derivatives for our studies.[13,14] It can be assumed that some of these compounds are formed atroposelectively, and different regioisomers are produced selectively by different *Streptomyces* strains. The monomers of these compounds are most likely derived from a nonaketidic precursor which is regioselectively coupled to provide the corresponding biaryls via oxidative phenol coupling within each organism (Scheme 1). Hence, we hypothesized that the respective polyketide synthases are probably highly homologous. Moreover, the biosynthetic clusters to be identified might also contain the enzymes responsible for the phenol coupling.

To test our hypothesis, the structures of compounds **1** and **2** isolated from two *Streptomyces* species were reexamined. We paid particular attention to the existence of potential regio- and stereoisomers of **1** and **2** from each bacterial strain. Moreover, it was not known whether setomimycin (**2**) is formed atroposelectively at all.

Julichrome $Q_{3−3}$ (**1**) and setomimycin (**2**) were isolated and then fully characterized by 1D and 2D NMR measurements. Furthermore, julichrome $Q_{3−5}$ (**3**, Scheme 1), a nonsymmetrically coupled member of the julimycin B-complex, was isolated

Scheme 1. Bacterial System for Investigation of Intermolecular Oxidative Phenol Coupling
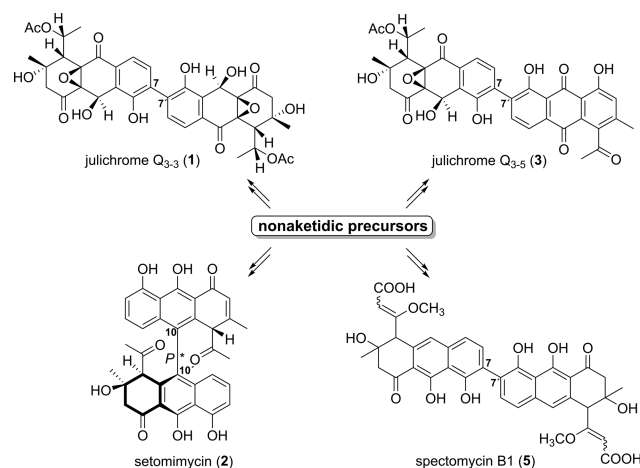
from *S. afghaniensis*. The analytical data obtained for this compound definitively demonstrated that the C−C bond between the two subunits is located at position C7−C7′. Similarly, it was confirmed that the setomimycin moieties are connected at position C10−C10′ (Figures S1−S8 and S15−S21). A HPLC-MS method was established which enabled a quick identification of the compounds. The total ion chromatogram of the metabolites from the wild-type strain of *S. afghaniensis* showed several peaks with a mass range (m/z) of the molecular ion peak from 669 to 749 $[M − 1]^-$. Two peaks could be assigned to julichrome Q_{3-3} (**1**) and julichrome Q_{3-5} (**3**), respectively (see Figures 2A and S25). The remaining peaks were not further investigated; however, the similar fragmentation pattern suggests that they are likely due to other compounds derived from the julimycin B-complex (Figure S26). This HPLC-MS assay was also used to monitor the occurrence of possible regioisomers. Even though it cannot be entirely ruled out, there was no indication that any other regioisomer exists in extracts from either *S. afghaniensis* or *S. aurantiacus*.

The absolute stereochemistry of both the biaryl axis of setomimycin (**2**) and the asymmetric carbons in the subunits was determined by combining NOESY/ROESY NMR spectroscopy with vibrational circular dichroism (VCD, see Supporting Information). Theoretical VCD spectra were calculated on the basis of the relative stereochemistry of **2** obtained from the 2D NMR experiments. A comparison of observed to calculated VCD spectra led to the assignment $P,4S,3'R,4'S$ (Figure S22 and Scheme 1). The P configuration is also suggested from the electronic circular dichroism spectrum of **2**, which shows a distinct exciton coupling effect (Figure S24). This is a clear proof of axial chirality and indicates an atroposelective biosynthesis for this compound.

In addition, VCD analysis of the julichrome Q_6 monomer (**4**, see Scheme 2 and Figure S23) indicated that the tricyclic subunits of the julichromes and setomimycin (**2**) do not have the same absolute configuration, although their relative configuration is identical.

Thus, it was confirmed that in both bacterial strains just one regioisomer is formed and that this process, at least in the case of setomimycin (**2**), proceeds in an atroposelective manner.

Feeding experiments with ^{13}C-labeled sodium acetate have revealed the polyketide origin of setomimycin (**2**).[14] We set out to elucidate the biogenic origin of **2** and the julichromes at the

genetic level. The aim was to identify type II polyketide synthase biosynthetic gene clusters, which should enable the identification of potential "coupling enzymes".

The genomes of both *Streptomyces* strains were sequenced and screened for type II PKS genes.[15,16] Additionally, the up- and downstream regions of the putative minimal PKS genes were analyzed. We identified two potential type II PKS gene clusters in the genome of *S. afghaniensis* and one in the genome of *S. aurantiacus*. Comparison of the three putative biosynthetic gene clusters by sequence revealed two clusters showing high similarity. Ten genes within these clusters have a distinctive homology at the protein level, up to 94% in the case of the putative β-ketosynthase, which argues for the formation of the same polyketidic precursor (Figure 1 and Table 1). Consequently, it was possible to identify one putative type II PKS cluster of particular interest in each strain.
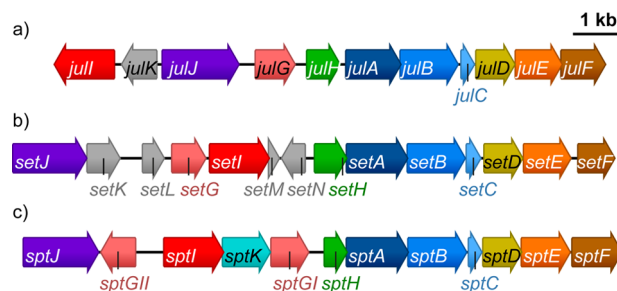

Figure 1. Predicted gene organization of the essential parts of (a) the julichrome cluster (*jul*), (b) the setomimycin cluster (*set*), and (c) the spectomycin cluster (*spt*); same color indicates same predicted function (see Table 1).

Table 1. Alignment of the Homologous Proteins from the Julichrome (Jul) and the Setomimycin (Set) Biosynthetic Gene Cluster

designation of proteins *S. afghaniensis* / *S. aurantiacus*	deduced role	aa sequence homology
JulI / SetI	cytochrome P450	63%
JulJ / SetJ	resistance transporter	38%
JulG / SetG	transcriptional regulator	61%
JulH / SetH	cyclase	59%
JulA / SetA	β-ketosynthase/KSα	94%
JulB / SetB	chain length factor/KSβ	90%
JulC / SetC	acyl carrier protein	82%
JulD / SetD	polyketide ketoreductase	93%
JulE / SetE	cyclase/dehydratase	85%
JulF / SetF	hydrolase/thioesterase	63%

To confirm this assumption, deletion experiments in the genome of julichrome producer *S. afghaniensis* were carried out. The minimal PKS genes (*julA*, *julB*, *julC*) were disrupted through an inactivation construct, in which the three genes of interest were replaced by a spectinomycin resistance gene using a modified version of Redirect technology (Scheme S1).[17] The extract of the resulting double-crossover mutant of *S. afghaniensis*, Δ*julA,B,C*, was analyzed for the presence of julichromes using the previously developed HPLC-MS assay.

An obvious change of the phenotype occurred during cultivation of the *S. afghaniensis* Δ*julA,B,C* mutant. In contrast to the extract of the wild-type strain, no peaks corresponding to julichromes were observed in the HPLC-MS chromatogram of
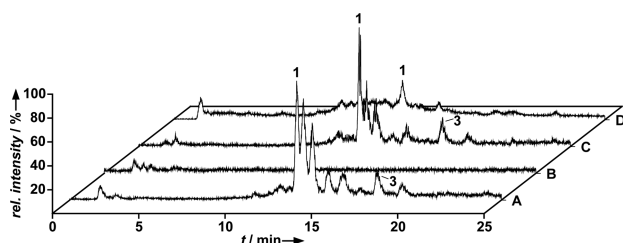
Figure 2. Total ion chromatogram of *S. afghaniensis* metabolites: (A) wild-type strain; (B) *S. afghaniensis* Δ*julA,B,C* mutant; (C) complemented *S. afghaniensis* Δ*julA,B,C* mutant with the integrative plasmid pΔ*julABC*-comp; and (D) *S. afghaniensis* Δ*julA,B,C* mutant cultivated with the addition of julichrome Q$_6$ (**4**). The relative intensities of the chromatograms were scaled to the peak at 1.6 min (for complete chromatograms, see Figures S25, S27−S29).
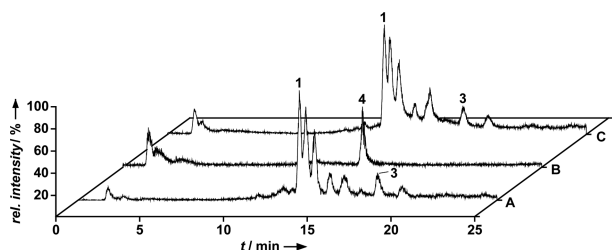

Figure 3. Total ion chromatogram of *S. afghaniensis* metabolites: (A) wild-type strain; (B) *S. afghaniensis* Δ*julI* mutant; and (C) complemented *S. afghaniensis* Δ*julI* mutant with the integrative plasmid pΔ*julI*-comp. The relative intensities of the chromatograms were scaled to the peak at 1.6 min (for complete chromatograms, see Figures S29−S31).

the mutant extract (Figure 2B), which implies a complete breakdown of the production of these secondary metabolites in the mutant strain. To confirm this result, the biosynthetic cluster was complemented by insertion of the disrupted genes (*julA, julB, julC*) into the mutant strain. The genes were integrated at the original position of the genome; thereby, production of the julichrome compounds was completely restored (Figure 2C).

The neighboring genes of the potential type II PKS clusters were analyzed for genes which might catalyze an oxidative phenol coupling. In both cases, a gene coding for a putative cytochrome P450 enzyme (JulI, SetI) was detected (Table 1 and Figure 1). The *julI* gene was deleted and subsequently complemented using a procedure analogous to the previous experiment (see Supporting Information). The total ion chromatogram of the metabolites from the *S. afghaniensis* Δ*julI* mutant showed only one major peak, with $m/z = 343$ $[M -1]^-$ (Figure 3B). Disregarding minor impurities obtained from the media, the produced compound represented the main component of the crude extract. Based on ^1H, ^{13}C, and 2D NMR spectra of the isolated material, plus the information derived from MS measurements, this compound is the julichrome Q$_6$ monomer (**4**, Figures S9−S14, S30).

In summary, the julichrome biosynthetic gene cluster was identified by the deletion experiments, as was a putative cytochrome P450 enzyme that plays an important role in the phenol-coupling step (Scheme 2). Furthermore, it can be assumed that the highly homologous type II PKS gene cluster (*set*) from the genome of *S. aurantiacus* is responsible for the formation of setomimycin (**2**).

Spectomycin B1 (**5**), a closely related dimeric dihydroan-thracene antibiotic, has been isolated from *S. spectabilis* UC 2294.

Scheme 2. Postulated Biosynthesis of Julichrome Compounds
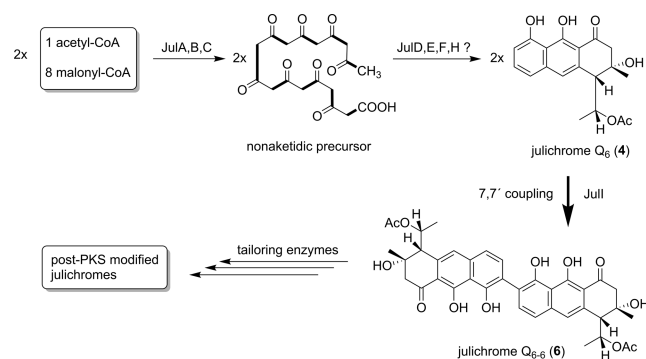
The spectomycin monomer is assumed to be derived from a nonaketidic precursor similar to that for the julichromes and setomimycin (**2**) (Scheme 1).[18] The considerable structural difference is the presence of a terminal carboxyl group, which has been removed in the julichromes and setomimycin (**2**). A further variation is the methylated hydroxyl group at positions 11 and 11′ of spectomycin B1 (**5**). The coupling reaction of the spectomycin monomeric subunits takes place at the same relative position as the julichromes.

To challenge our results, the genome of *S. spectabilis* DSM 40779, a strain which is equivalent to the original spectomycin producer, was sequenced. The sequence data revealed a type II PKS gene cluster highly homologous to the genes already known, up to 92% in the case of the β-ketosynthase SptA. The genes from the core area (*sptA* to *sptF*) are arranged in the same way as the corresponding genes in the biosynthetic clusters for setomimycin (**2**) and the julichromes (Figure 1). Furthermore, all other homologous genes could be identified in the cluster, including a gene that codes for a putative cytochrome P450 enzyme.

To clarify whether julichrome Q$_6$ (**4**) is indeed utilized as a substrate for the coupling reaction, *S. afghaniensis* Δ*julA,B,C* was cultivated with the addition of **4**. The extract resulting from this feeding experiment showed a composition comparable to that of the extract of the wild-type strain, thus confirming our original assumption (Figure 2D).

To establish conclusively that the putative cytochrome P450 monooxygenase JulI is able to catalyze an intermolecular C−C coupling reaction, the enzyme was heterologously expressed in *E. coli*, together with the electron-transfer partners putidaredoxin reductase (PdR) and putidaredoxin (Pdx) from *Pseudomonas putida* DSM 50198.[19] A set of in vitro experiments was performed using different cell-free extracts (CFE), NADH, and julichrome Q$_6$ (**4**). HPLC-MS analysis of the bioconversions indicated formation of a compound with $m/z = 685$ $[M − 1]^-$ in preparations containing JulI (Figure 4C). According to the ion fragmentation pattern, which is similar to those of known dimeric julichromes, it can be assumed that this compound is julichrome Q$_{6-6}$ (**6**, Scheme 2 and Figure S37). Furthermore, preparations without JulI did not show this conversion of julichrome Q$_6$ (**4**, Figure 4A,B). Thus, it has been demonstrated that the identified cytochrome P450 monooxygenase is responsible for the intermolecular oxidative phenol-coupling reaction during the biosynthesis of bacterial dimeric pre-anthraquinones.

In conclusion, we have demonstrated that not only plants and fungi but also bacteria, in particular streptomycetes, are able to form axially chiral biaryl compounds by regio- and stereoselective intermolecular oxidative phenol coupling (Scheme 1). By using genome analysis and deletion experiments, we have identified
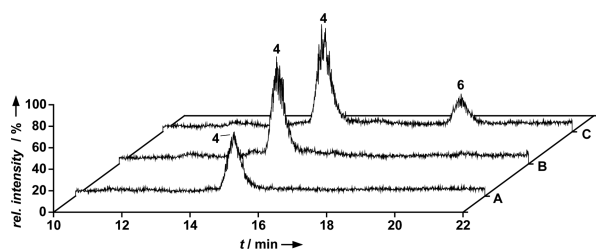
Figure 4. Total ion chromatogram of in vitro experiments using julichrome Q$_6$ (**4**), NADH, and CFE of BL21-Gold(DE3): (A) blank; (B) with PdR and Pdx; and (C) with PdR, Pdx, and JulI (for complete chromatograms, see Figures S35–S37).

three highly homologous gene clusters responsible for biosynthesis of the julichromes and most probably of setomimycin (**2**) and spectomycin B1 (**5**). In addition to genes which are essential for the biosynthesis of bacterial aromatic polyketides, a cytochrome P450 enzyme is located within each gene cluster. The disruption of the corresponding gene *julI* resulted in the production of the monomeric precursor. In the subsequent in vitro experiments it could be verified that these bacterial cytochrome P450 monooxygenases are able to catalyze regioselective oxidative phenol-coupling reactions. Heterologous expression and in vitro C−C bond formation represent a promising application of these enzymes. Due to the bacterial origin of the proteins, this process is expected to be facilitated relative to those of analogous fungal and plant proteins. Mechanistic studies and experiments concerning substrate specificity and selectivity of these enzymes are currently in progress.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

michael.mueller@pharmazie.uni-freiburg.de

Notes

The authors declare no competing financial interest.

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