VIP Bacterial Alkaloids

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Regiodivergent N–C and N–N Aryl Coupling Reactions of Indoloterpenes and Cycloether Formation Mediated by a Single Bacterial Flavoenzyme**

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Indoloterpenoid natural products play an eminent role as drugs, and their significance for medicine has propelled a plethora of synthetic and biosynthetic studies. Interestingly, these alkaloids are a hallmark of plant and fungal metabolism, but virtually nothing was known about the corresponding bacterial pathways. Only recently, in the context of profiling the bacterial endophytes of widespread mangrove trees,[1] we discovered the unprecedented bacterial indolosesquiterpenes xiamycin (**1**), indosespene (**2**), and sespenine (**3**; Figure 1).[2] Together with oridamycin (**4**)[3] and oxiamycin (**5**)[4] these multicyclic alkaloids constitute a new family of bacterial indoloterpenes. The co-occurrence of these diverse hybrid metabolites in a single organism implies a biogenetic relationship. Thus, we and another research group have independently analyzed xiamycin (*xia*) biosynthesis gene clusters.[5,6] Through mutational analyses and heterologous expression, we found that the pathway involves an unparalleled cyclization sequence to yield diverse ring systems. The heterologous expression of the entire gene cluster also led to the discovery of N–C and N–N coupled xiamycin dimers,[5] which had been overlooked in the wild-type strain owing to their low production.[2] These structurally intriguing bixiamycins represent the first examples of bacterial bisindolosesquiterpenes (BIST), and their biosynthesis has remained enigmatic. Herein, we report the discovery and full characterization of a series of highly regiodivergent, N–C and N–N aryl-coupled xiamycin dimers, and reveal their potent antibacterial activities. Furthermore, we show that a single flavoprotein not only mediates diverse aryl couplings, but also ether formation. Finally, we support a radical-based mechanism by a biomimetic synthesis of the xiamycin derivatives.

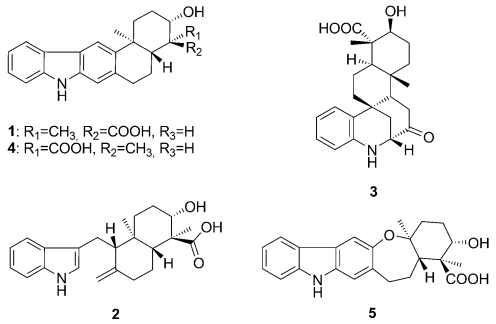

Figure 1. Structures of bacterial indolosesquiterpenes, xiamycin (**1**), indosespene (**2**), sespenine (**3**), oridamycin (**4**), and oxiamycin (**5**).

To reveal the range of bixiamycins produced, we inspected the metabolic profile of *Streptomyces albus* carrying the entire *xia* biosynthesis gene cluster. HPLC-HRMS analyses indicated that the strain produces a number of compounds that likely result from the dimerization of xiamycin (**1**), as they have the same molecular formula ($C_{46}H_{48}N_2O_6$), but differ in retention times. To obtain sufficient amounts of these new compounds for a full structural characterization, the culture was scaled up. Both mycelia and culture filtrate of a scaled-up fermentation (50 L) were extracted with ethyl acetate, and the combined extracts were subjected to fractionation by flash chromatography, first through silica, then through a Sephadex LH-20 column. Final purification by preparative HPLC yielded various dimers, including the atroposiomeric pair of N–N coupled bixiamycins (**6a**/**6b**)[4,5] and other types of dimers (Figure 2) as pure compounds: **6a** (10 mg), **6b** (5 mg), **7a** (3 mg), **7b** (1.0 mg), and **8** (23.1 mg). NMR analysis of the new dimers revealed that they all possess the xiamycin backbone. Compounds **7a** and **7b** were obtained as a pair of atropodiastereomers. Their ^{1}H NMR spectra show two sets of signals, thus indicating that the coupling sites of the two moieties of the molecule are not identical. As only a signal for H21 is visible, whereas the counterpart (H21′) is missing in the ^{1}H NMR spectrum, a N–C coupling between N1 and C21′ was proposed. COSY and HMBC correlations confirmed the proposed structure for the two halves of the dimer. Comparison of the experimental CD spectra with the TDCAM-B3LYP/6-31G*//B97D/TZVP calculated ones revealed that **7a** has a *P* configuration, whereas the minor atropodiastereomer **7b** has an *M* configuration at the N–C axis (Figure 3; see the Supporting Information for details).

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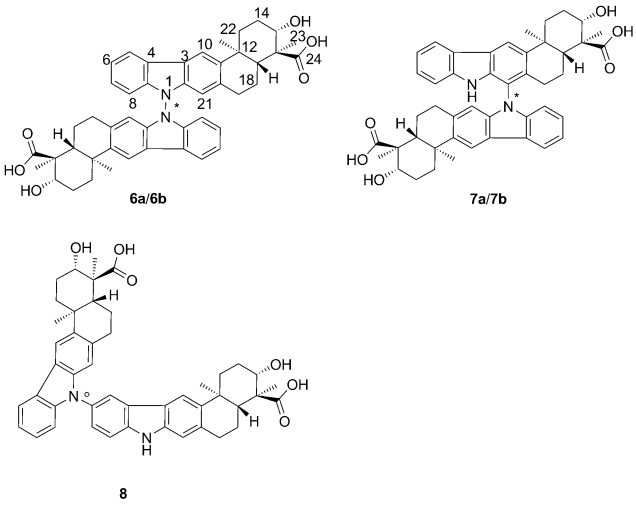


Figure 2. Structures of bixiamycins. *: Configurationally stable, °: configurationally unstable.

The ^{1}H NMR spectrum of **8** shows two sets of signals, which indicates that the coupling sites of the molecular portions are different. Eleven aromatic signals are visible. COSY and HMBC spectra established the structures for both halves of the isomer. A N–C linkage between N2′ and C6 was proposed, and fully established (Figure 2).

All bixiamycins were assayed in a panel of representative bacteria, yeasts, and filamentous fungi. Selective antibacterial activities against Gram-positive bacteria were observed (see the Supporting Information). N–N coupled dimers were most active, with MIC values as low as 0.2 μg mL^{-1}. The monomer xiamycin (**1**) exhibited only weak antimicrobial activities, whereas the N–C dimers showed moderate to strong activities. Moreover, in a test against three tumor cell lines (HUVEC, K-562, and HeLa), it is remarkable that neither cytotoxicity nor antiproliferative effects (GI_{50}, CC_{50} > 50 μg mL^{-1}) were observed for any of the tested dimers.

The structural variety of the bixiamycins suggests that one or more enzymes catalyze regiodivergent aryl couplings of the monomers. First, using a medium control, we excluded the spontaneous formation of dimers during the time of fermentation and concluded that the reaction is enzyme catalyzed. To unveil the biochemical basis of the coupling reactions, we performed mutational analyses and biotransformation experiments. For this purpose, we employed mutants lacking tentative oxygenase genes (*xiaF-J* and *xiaL*),[5] supplemented them with xiamycin, and monitored product formation by HPLC-MS (Figure 4a).

To exclude the involvement of genes of the heterologous host *S. albus*, we also performed a control experiment with the wild type lacking the *xia* genes. Initially, the cytochrome P450 monooxygenase XiaJ appeared to be the best candidate, as various related enzymes are capable of promoting aryl–aryl

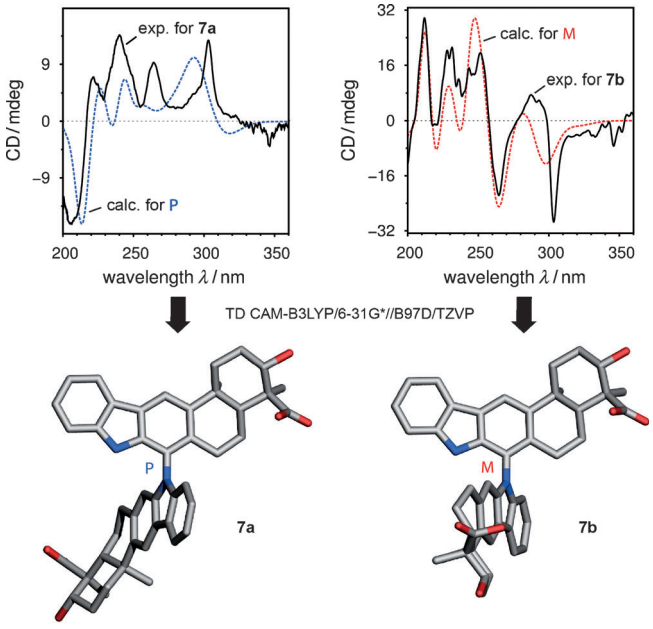


Figure 3. Determination of the absolute configuration of **7a** and **7b** by comparison of the experimental CD spectra with the ones calculated of the two possible *P* and *M* atropodiastereomers of **7**.

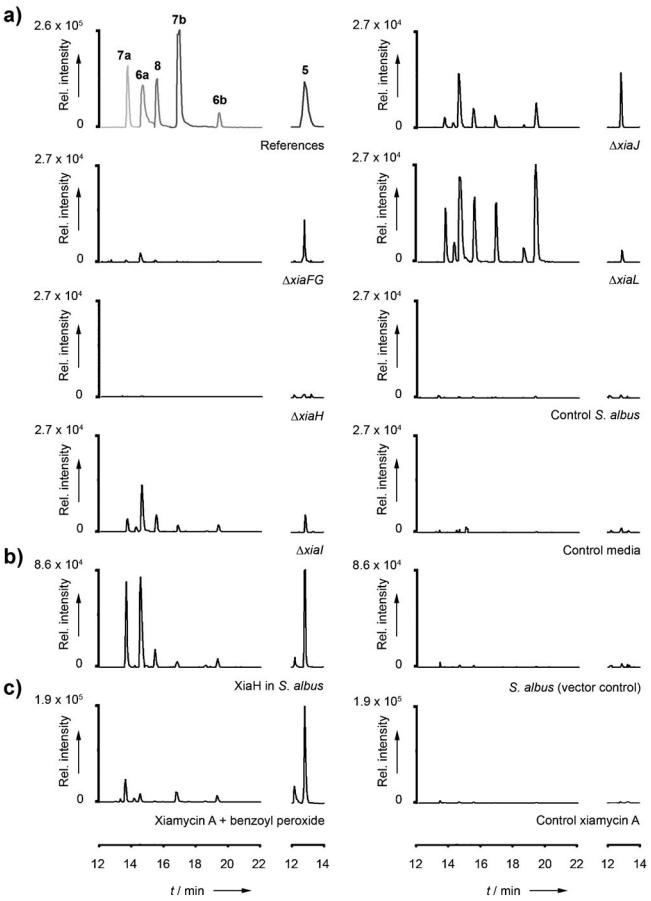


Figure 4. HPLC profiles (HRMS detection; extracted ion chromatograms) showing the results of a) feeding of xiamycin to mutants lacking tentative oxygenase genes to identify the gene involved in aryl–aryl coupling; b) biotransformation experiments of **1** using heterologously expressed *xiaH*; c) synthetic conversion of **1** into bixiamycins (**6a**/**b**, **7a**/**b**) and oxiamycin (**5**) using a radical starter. The profile showing the references is composed of different measurements of pure compounds.

couplings in alkaloids,[7] glycopeptides,[8] and polyketides.[9] However, the *ΔxiaJ* deletion mutant still produced the aryl-coupled dimers. Likewise, the *ΔxiaF, G, I*, and *L* mutants retained aryl–aryl coupling activity. We found that the dimers were only absent in the *xiaH* deletion strain and in the negative controls. Furthermore, in the course of the metabolite analyses, we made a surprising observation: oxiamycin could not be detected in the *ΔxiaH* deletion mutant, whereas all other mutants produced the xiamycin-derived cycloether.

A bioinformatic analysis indicated that XiaH belongs to the family of flavoproteins. By a phylogenetic analysis (Figure 5) we further specified that XiaH is related to the flavin adenine dinucleotide (FAD) dependent aromatic ring hydroxylases that are mainly involved in the degradation of

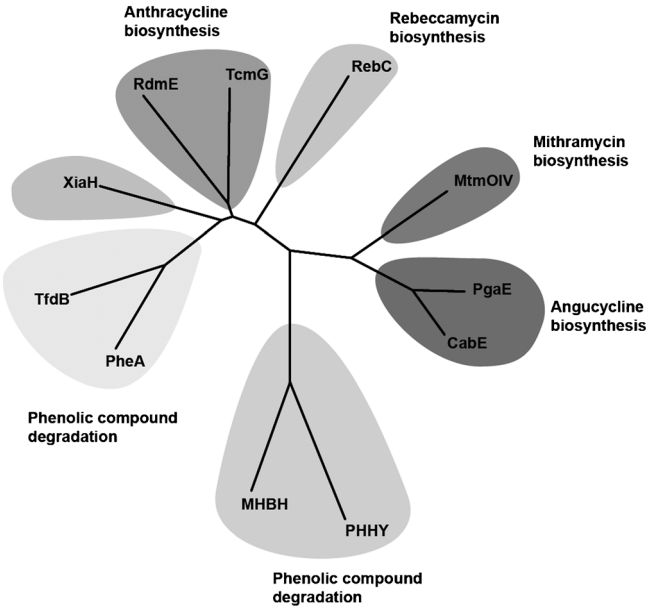
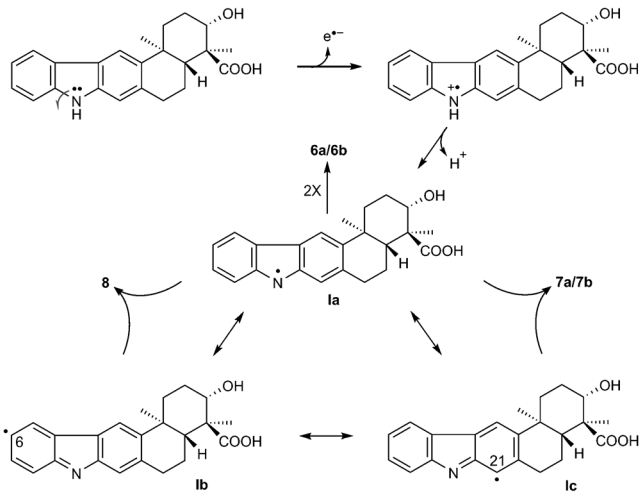


Figure 5. Phylogenetic tree of XiaH and related flavoenzymes (for detailed cladograms, see the Supporting Information).

phenolic compounds or the tailoring of aromatic polyketides such as aklavinone[10] and alkaloids such as rebeccamycin.[11]

To test whether the flavoprotein is indeed responsible for the aryl couplings and for transforming xiamycin into oxiamycin, *xiaH* was cloned into the integrative *E. coli–Streptomyces* shuttle vector pKJ55[12] and heterologously expressed in *S. albus*. We then added xiamycin to the strain overexpressing *xiaH* and monitored the metabolic profile by HPLC-HRMS. Whereas no conversion took place in the control, the xiamycin dimers and oxiamycin were produced in the presence of XiaH (Figure 4b), thus supporting the assumption that the flavoprotein catalyzes both aryl couplings and ether formation.

These results, however, posed the riddle of how a single enzyme can catalyze such a broad range of N–C, N–N, and C–O bond formation reactions. From a chemical perspective, the positions of the dimer links are suggestive of a radical mechanism, as the resulting postulated radical **I** not only has spin density on the nitrogen (mesomeric structure **Ia**), but

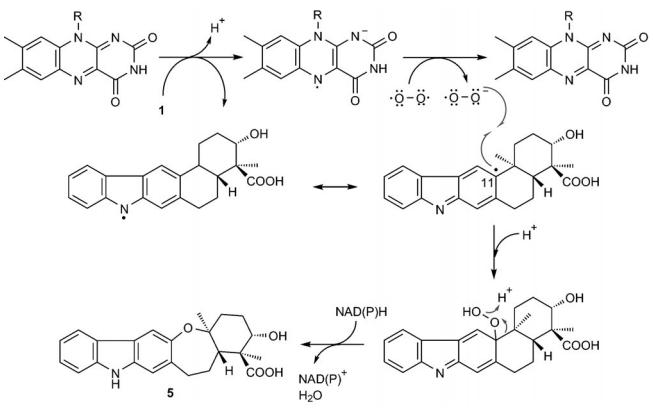


Scheme 1. Proposed radical mechanism for xiamycin dimer formation.

also on different carbon atoms, among them C6 (**Ib**) and C21 (**Ic**) (Scheme 1).

Notably, radical intermediates of the organic substrate have not been reported for any relatives of XiaH. However, a catalytic mechanism involving organic radicals was proposed for FAD-dependent enzymes such as monoamine oxidases, where the amino group is believed to be the target of a one-electron extraction by FAD. As a consequence, an aminyl radical cation is formed to initiate the oxidation reaction.[13] Although other mechanisms have been discussed, the single-electron-transfer mechanism gained importance after tyrosyl radicals from the protein backbone were shown to be in close proximity to the FAD.[14] In analogy, we suggest that the XiaH-mediated aryl–aryl coupling is initiated by the formation of a *N*-radical cation. After deprotonation, two xiamycin radicals could be stabilized by a homocoupling reaction (Scheme 1).

Interestingly, the xiamycin *N*-radical generated by XiaH could also form the basis for an alternative reaction pathway towards the tetrahydrooxepine ring of oxiamycin. A plausible reaction mechanism would be analogous to what has been proposed for the "flavin destructase" BluB.[15] In vitamin B_{12} biosynthesis, BluB promotes the oxygenation of flavin mononucleotide (FMN) to flavin hydroperoxide. In contrast to canonical FAD-dependent enzymes where flavin hydroperoxide acts as a cofactor, the fate of this molecule is to become the substrate, and the peroxo group decomposes with ring expansion of FMN. In analogy, the C11-radical of xiamycin could react with a superoxide, generated by a transfer of one electron from the flavin semiquinone to oxygen, to yield xiamycin peroxide. Alternatively, reaction of the C11-radical of xiamycin with molecular oxygen followed by reduction of the xiamycin peroxide radical would also be conceivable. The resulting hydroperoxide intermediate would then rearrange by ring expansion with concomitant release of water. This step would require the reduction of a positively charged oxiamycin intermediate. Flavoproteins related to XiaH use NAD(P)H to regenerate its cofactor, FAD. As this reduction step is not required in the proposed reaction cycle



Scheme 2. Proposed mechanism for the conversion of xiamycin (**1**) into oxiamycin (**5**).

of oxiamycin biosynthesis, NAD(P)H could be used to reduce the oxiamycin intermediate (Scheme 2).

To test the radical-based mechanism, we sought to mimic the proposed enzymatic reaction conditions in the active site of XiaH by using a radical forming agent. Therefore, we treated xiamycin dissolved in benzene with benzoyl peroxide and monitored the reaction by HPLC-HRMS. Under these radical-forming conditions, bixiamycins and, most surprisingly, even oxiamycin were formed (Figure 4c). These results strongly support the proposed radical-mediated mechanism for biaryl and ether formation. Although other mechanisms are conceivable for the enzyme-mediated transformation, the sites of aryl fusions, the lack of marked *P*/*M* selectivity, and the results from the synthetic study all provide strong support for a radical reaction promoted by the flavoprotein. Although the proposed mechanism and the range of reactions initiated are highly unusual for FAD-dependent enzymes, we believe that this scenario is actually more widespread in nature. Only recently, the two FAD-dependent halogenases Mpy10 and Mpy11 were implicated in the aryl coupling of marinopyrrole monomers. The authors speculated on a cryptic halogenation, but also considered a radical-based mechanism as an alternative.[16]

In summary, through heterologous expression of the entire *xia* gene cluster, we have discovered three types of N–C and N–N fused bixiamycins, which are a new class of non-cytotoxic, potent antibiotics. The high structural diversity of N–C and N–N aryl-coupled indolosesquiterpenes in one strain is unprecedented. By targeted gene deletions, heterologous expression, and biotransformations we pinpointed *xiaH* to be involved in the aryl couplings. Furthermore, we found that XiaH also mediates C–C bond cleavage of the xiamycin D-ring and oxygen insertion to yield the tetrahydrooxepine of oxiamycin. Moreover, we succeeded in a biomimetic synthesis of the bixiamycins and oxiamycin by employing a radical starter.[17] Thus, a plausible, radical-based mechanism was proposed for N–C and N–N coupling, and cyclic-ether formation, which is unusual for a single flavoprotein.[18] Considering the exceptional structural diversity generated, and the potent antibiotic activities of the bixiamycins, XiaH may be used as a novel biocatalyst to generate bixiamycin analogues.

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