Sulfa Drugs

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Bacterial Synthesis of Unusual Sulfonamide and Sulfone Antibiotics by Flavoenzyme-Mediated Sulfur Dioxide Capture

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Abstract: *Sulfa drugs, such as sulfonilamide and dapsone, are classical antibiotics that have been in clinical use worldwide. Despite the relatively simple architectures, practically no natural products are known to feature such aromatic sulfonamide or diarylsulfone substructures. We report the unexpected discovery of three fully unprecedented, sulfonyl-bridged alkaloid dimers (sulfadixiamycins A–C) from recombinant Streptomyces species harboring the entire xiamycin biosynthesis gene cluster. Sulfadixiamycins exhibit moderate antimycobacterial activities and potent antibiotic activities even against multidrug-resistant bacteria. Gene inactivation, complementation, and biotransformation experiments revealed that a flavin-dependent enzyme (XiaH) plays a key role in sulfadixiamycin biosynthesis. XiaH mediates a radical-based, three-component reaction involving two equivalents of xiamycin and sulfur dioxide, which is reminiscent of radical styrene/SO_2 copolymerization.*

The discovery of sulfa drugs is regarded as a landmark for the treatment of bacterial infections and started a new era of modern medicine in the early 20th century.[1] Inspired by the remarkable antibacterial properties of an azo dye (prontosil red), which is actually a prodrug of sulfanilamide (**1**, Figure 1), numerous sulfonamide-based chemotherapeutics were developed and brought into the clinics.[2] The success of the sulfa drugs, which culminated in the 1939 Nobel Prize in Medicine for Gerhard Domagk,[3] is grounded in their potent inhibition of the folate pathway. Specifically, sulfonamides block dihydropteroate synthetase, which is essential for bacterial growth and development.[4] The same crucial pathway is targeted by numerous structural variants of sulfonamides, including a diarylsulfone named dapsone (diaminodiphenylsulfone, **2**). Despite its age, dapsone is still used in combination therapies to cure leprosy and infections in highly immunocompromized patients.[5] Considering the outstanding antibacterial efficacy of the sulfonamides and diarylsulfones, it is surprising that such structural moieties are practically absent in naturally occurring molecules. Of the 265 000 known natural products listed in comprehensive compound databases (Chapman & Hall/CRC chemical database), the only aromatic sulfonamide is the synthetic plasticizer *N*-butylbenzenesulfonamide (**3**), which was isolated (likely as a contaminant) from various biological samples.[6] As to natural diarylsulfones, the single known representative is echinosulfone A (**4**), a bromoindole derivative from a marine sponge (*Echinodictyum* sp.).[7] It appears that sulfonyl-bridged aromatic natural products are clearly an exception, and to date nothing has been known about their biogenesis. Herein, we report the discovery of novel antibacterial natural products with sulfanilamide and dapsone substructures from a single bacterial strain. Based on extensive in vivo experiments and retrobiosynthetic analysis, we propose a biosynthetic pathway for sulfonamide and diarylsulfone formation involving an unprecedented enzyme-mediated sulfur dioxide capture.

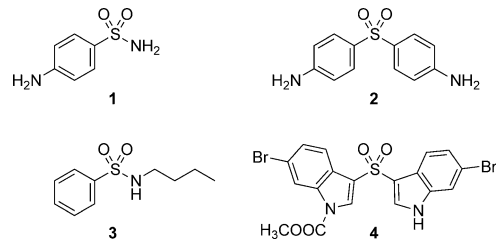

Figure 1. Structures of synthetic sulfa drugs sulfanilamide (**1**) and dapsone (**2**), plasticizer *N*-butylbenzenesulfonamide (**3**), and sponge metabolite echinosulfone A (**4**).

In the course of biosynthetic studies on rare aromatic alkaloid natural products from bacteria associated with mangrove plants, we observed the formation of unusual, sulfur-containing congeners. We have previously investigated these endophytes for their potential to produce bioactive compounds and discovered a family of polycyclic indolosesquiterpenes (IST), which have been unprecedented for prokaryotes.[8] Biosynthetic studies at the genetic and chemical levels revealed that the pentacyclic ring system of the major component of the complex, xiamycin (**5**, Figure 2), results from unusual terpenoid cyclizations.[9] To obtain larger quantities of the rare IST compounds, the genes encoding the entire biosynthetic pathway were expressed in a heterologous host.[10] Using this recombinant strain, it became possible to isolate and characterize minor congeners of the complex such as *N,C*- and *N,N*-fused dimers (named bixiamycins or dixiamycins, **6**–**8**).[10] Because various dixiamycins exhibit potent antibacterial activities,[9b,10] we sought to isolate and characterize more congeners for structure–activity relationships. Therefore, the metabolic profile of the recombinant

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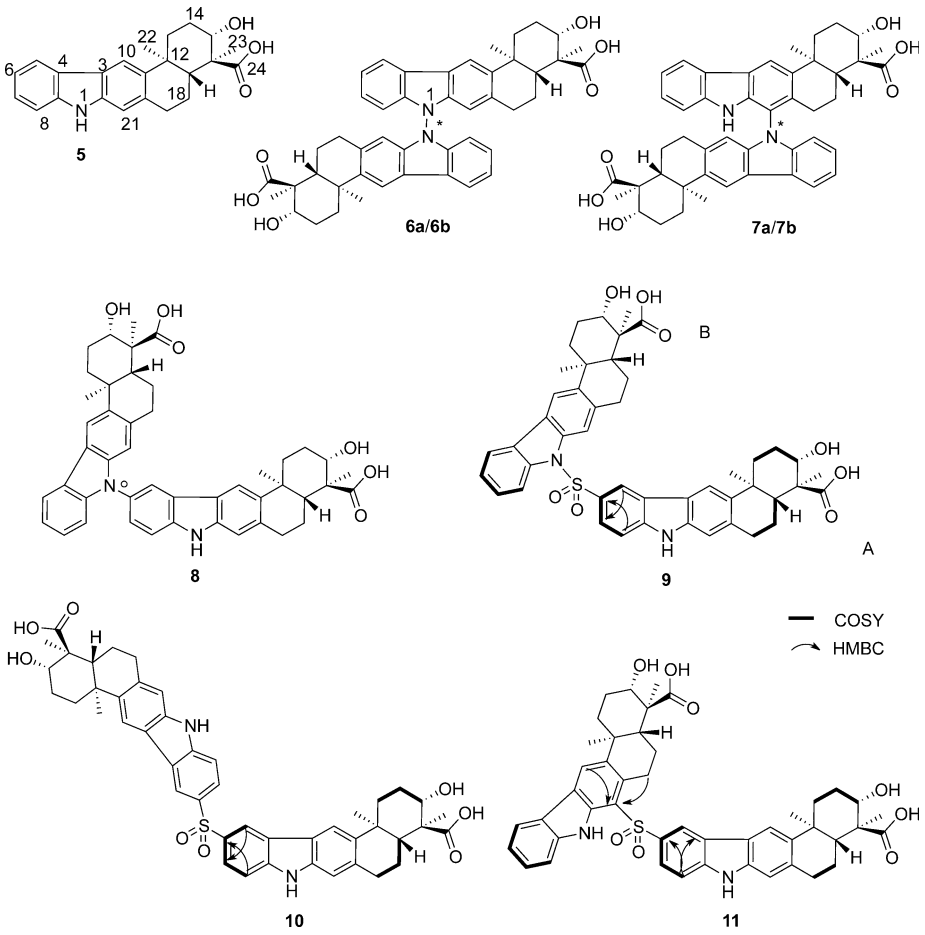


Figure 2. Structures of xiamycin (**5**), dixiamycins (**6**–**8**), and sulfadixiamycins A–C (**9**–**11**). Sulfanilamide and dapsone substructures are found in **9** and **10**.

strain was reevaluated by HPLC/MS analyses of culture extracts. Several new compounds with a mass of 788 Da were detected that differ from the dixiamycins by 64 Da. Yet, these metabolites were only produced in minuscule amounts. To obtain sufficient quantities for a full characterization, *S. albus* harboring the xiamycin biosynthesis gene cluster was grown at a large scale (50 L). Both mycelia and culture filtrate were extracted with ethyl acetate to afford a crude extract. The extract was subjected to flash chromatography on silica gel and open-column chromatography on Sephadex LH-20. Final purification was achieved by preparative HPLC (RP-C_{18}) yielding pure **9** (0.5 mg), **10** (0.8 mg), and **11** (1.5 mg). In contrast to the dixiamycins, all of the new compounds showed an isotope pattern diagnostic for sulfur content (^{34}S peak) (Supporting Information, Figures S2, S3). Furthermore, HRESIMS data indicated that all of the new compounds share the same molecular formula ($C_{46}H_{48}N_2SO_8$).

The deduced molecular formula of compounds **9**, **10**, and **11** indicated that the novel molecules differ from the dixiamycins by one sulfur atom and two oxygen atoms. We also noted marked differences in the UV spectra for **9**, **10**, and **11** in comparison to other dixiamycins. However, the NMR data of the new compounds were similar to the dixiamycin congeners. Thus, we speculated that sulfonyl groups are attached to the aromatic chromophore. The positions of the substituents were unequivocally elucidated by 1D and 2D NMR analyses. The ^1H NMR spectrum of compound **9** exhibited signals for eleven aromatic protons. Further analysis of the COSY spectrum indicated a substitution at C-6. Thus, we concluded that the sulfonyl group is located between N-1′ and C-6. Consequently, **9** represents an unusual type of natural sulfonamide. In the IR spectrum we observed the characteristic absorption for an aromatic sulfonamide group at 1244 and 1167 cm^{-1} (S=O stretching), which confirmed the structure proposal for sulfadixiamycin A (**9**). Both ^1H NMR and ^{13}C NMR spectra of compound **10** exhibit a similar signal pattern as xiamycin (**5**). However, only five sets of aromatic protons ($\delta = 8.82$, 7.97, 7.93, 7.53, 7.05 ppm) were detected. Based on the coupling constant and COSY spectrum, we confirmed the connection between H-5 ($\delta = 8.82$ ppm (d, 1.5 Hz)), H-7 ($\delta = 7.97$ ppm (dd, $J = 8.6$, 1.8 Hz)), and H-8 ($\delta =$ 7.53 ppm (d, 8.6 Hz)). Two other singlets ($\delta = 7.93$, 7.05 ppm) result from the resonances of H-10 and H-21 of the carbazole ring, respectively. Since the H6 and H6′ were absent, the most plausible explanation would be that the sulfonyl group is located between C6 and C6′. Consequently, **10** is a symmetrical sulfonyl-bridged dimeric xiamycin. HMBC correlations rigorously confirmed the structure (Figure 2). The structure proposal for sulfadixiamycin B (**10**) is also supported by the IR spectrum, which showed the characteristic absorption for an aromatic sulfonyl group (1286 and 1126 cm^{-1}). Compound **11** is the third sulfonyl-bridged dimer of xiamycin. Ten aromatic protons were observed in the ^1H NMR spectrum. Compared to xiamycin, H-6 and H-21′ were obviously substituted. COSY and ^1H NMR spectra confirmed that the sulfonyl bridge is located between C-6 and C-21′. The structure of the sulfadixiamycin C (**11**) was corroborated by HMBC correlations and the IR spectrum, which showed absorptions (1286 and 1121 cm^{-1}) diagnostic of diarylsulfones.

The sulfonyl-bridged bixiamycins **9**–**11** were tested for their cytotoxic and antimicrobial activitities in cell-based assays. We found that both diarylsulfonyl congeners (**10**, **11**) have selective, yet moderate antimycobacterial properties (minimum inhibitory concentration (MIC) = 25 $\mu g\,mL^{-1}$). In contrast, sulfonamide **9** exhibited the highest potency against several bacterial test strains including *Bacillus subtilis* (MIC 6.25 $\mu g\,mL^{-1}$) and pathogenic strains like *Staphylococus*

aureus (MIC 3.12 μg mL^{-1}) as well as methicillin-resistant *S. aureus* (MRSA) (MIC 6.25 μg mL^{-1}; Supporting Information, Table S4). Notably, an assay comprising a panel of three human cell lines revealed that none of the three dimers exerts cytotoxic or antiproliferative effects. Considering the negligible cytotoxicity and the marked antibiotic properties, sufonamide **9**, in particular, is a potential anti-infective agent. However, considering the bulky substitution pattern, it is likely that the sulfonamide and the diarylsulfones have a different mode of action and molecular target than the classical sulfa drugs.

The discovery of aromatic sulfonyl and sulfonamide alkaloids is highly surprising because their structures are fully unprecedented and most unusual from a biosynthetic point of view. To date, practically nothing is known about the biosynthesis of sulfones and sulfonamides in nature. The few reported aliphatic sulfonamide- or sulfone-bearing natural products likely result from incorporation of taurine-derived building blocks and enzymatic oxygenation of thioethers, respectively.[11] However, these biosynthetic schemes are not credible for the formation of aromatic sulfonamides or diarylsulfones.

To elucidate the involvement of specific *xia* pathway genes in the formation of sulfadixiamycins, we interrogated mutants of the natural IST producer that lack tentative oxygenase genes (*xiaF-J* and *xiaL*).[9a] Each mutant culture was supplemented with xiamycin, and product formation was monitored by HPLC-MS. Whereas the *ΔxiaF*, *G*, *I*, *J*, and *L* mutants were unaffected, the mutant with a gene deletion of *xiaH* proved to be incapable of producing any sulfadixiamycins (Supporting Information, Figure S4). We have previously demonstrated that *xiaH* codes for a flavoprotein (XiaH) that promotes an aryl coupling to yield dixiamycins.[10] To corroborate the involvement of XiaH in the biosynthesis of sulfadixiamycins, we deleted *xiaH* on the heterologous expression plasmid and compared the metabolic profile of the corresponding *S. albus* mutant with the one that heterologously expressed the intact *xia* pathway gene cluster (Figure 3a). Indeed, the lack of XiaH results in a complete shutdown of sulfadixiamycin and dixiamycin production (Figure 3b). Complementation with *xiaH* restored the initial phenotype (Figure 3c). These results unequivocally confirmed the involvement of XiaH in sulfadixiamycin formation and its close connection to dixiamycin biosynthesis. Next, to determine whether XiaH alone is sufficient to convert **5** into the sulfa compounds, we employed a *S. albus* mutant that heterologously expressed *xiaH*.[10] Indeed, **5** was transformed into the dimers as well as sulfadixiamycins (Figure 3d), corroborating that no other *xia* pathway gene is required for their biosynthesis. The same host–vector system without *xiaH* served as negative control (Figure 3e). The crucial role of XiaH in the biosynthesis of dixiamycins and sulfadixiamycins was supported by monitoring xiamycin production. The precursor of both kinds of dimers (**5**) accumulates only in the absence of XiaH (Figure 3b,e).

The successful biotransformation experiment indicates that the biogenesis of **9**–**11** involves the fusion of two equivalents of xiamycin with insertion of an equivalent of SO_2. Such a reaction would be reminiscent of the classical

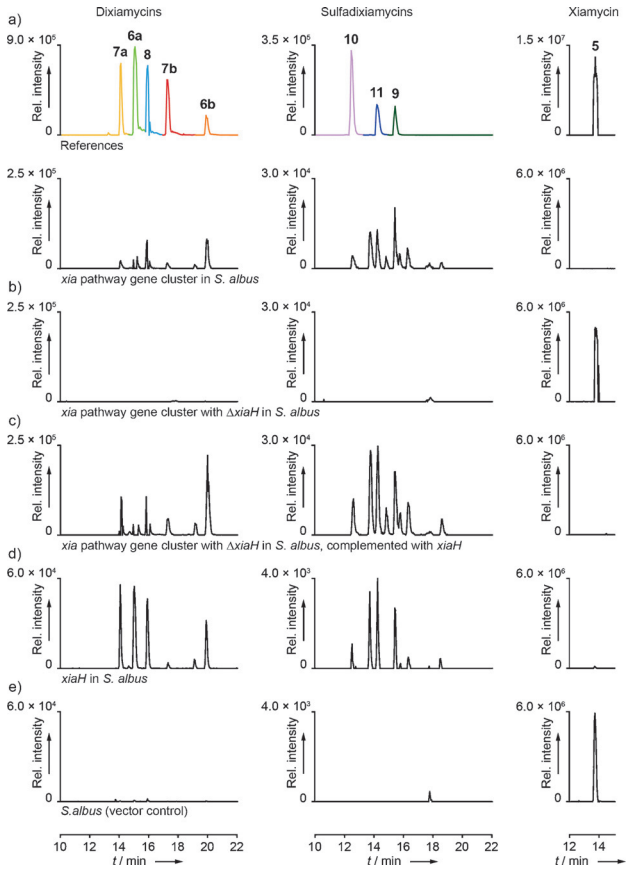
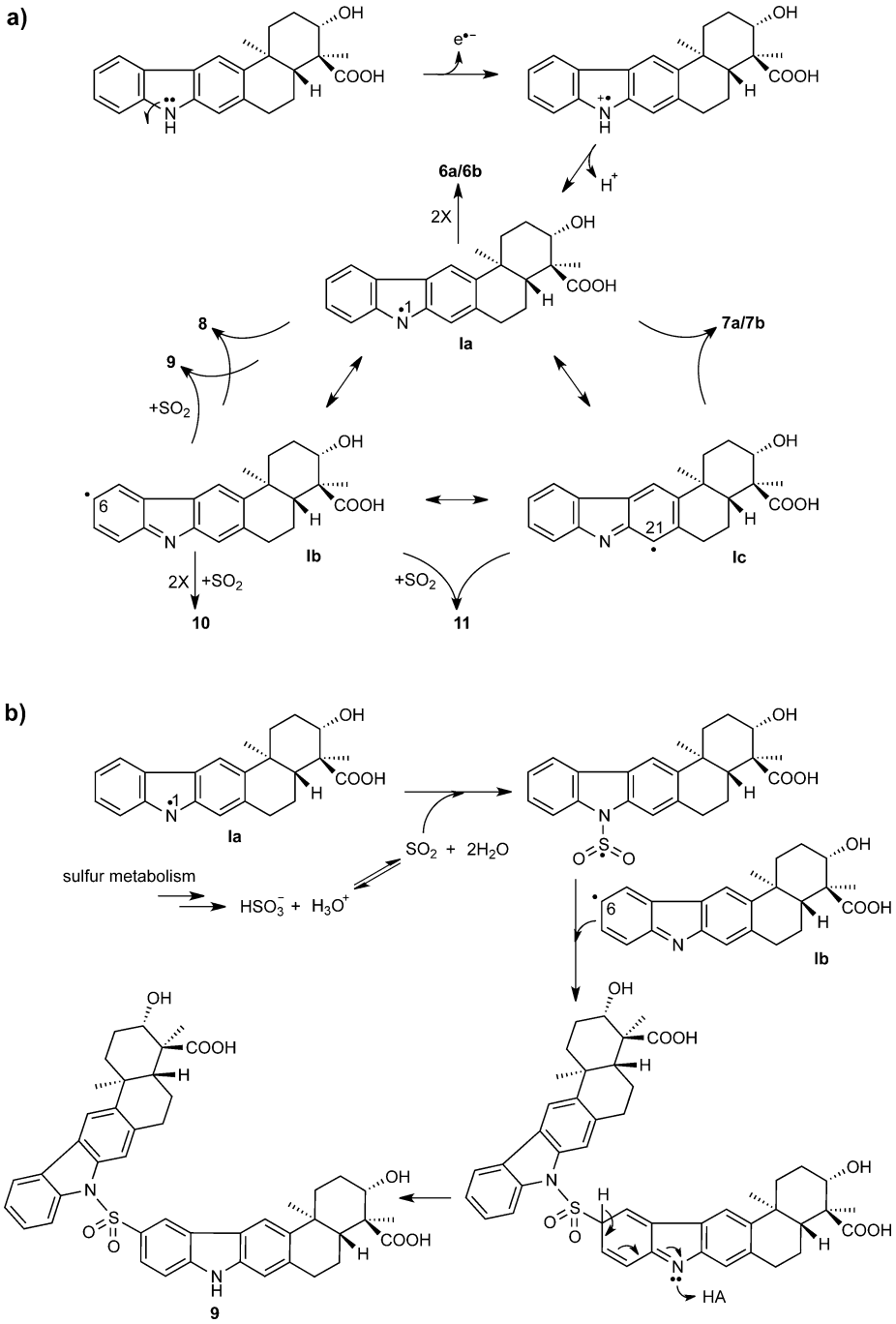


Figure 3. LC-MS profiles (HRMS detection; extracted ion chromatograms) of extracts from cultures of *S. albus* heterologously expressing the entire *xia* pathway gene cluster (a), *ΔxiaH* mutant (b), and complemented mutant (c), as well as biotransformation experiments of **5** using heterologously expressed *xiaH* (d). Except for xiamycin, the profiles showing the reference compounds are composed of different measurements of pure compounds (color-coded).

synthetic copolymerization of styrene with sulfur dioxide.[12] In bacteria, low quantities of SO_2 are typically formed to counterbalance high concentrations of cysteine or sulfate, and *Streptomyces* spp. are known to generate sulfite from sulfate in inorganic sulfur metabolism.[13] These genes for the assimilation of sulfate are well conserved in *S. albus* and in the IST producer,[8] generating sulfite, which reaches equilibrium with bisulfite and molecular SO_2 in aqueous solution.

Thus, the most plausible biosynthetic route to the sulfadixiamycins involves a three-component reaction of SO_2 and two equivalents of **5**. The substitution pattern of the sulfadixiamycins is highly suggestive for a radical-based mechanism as proposed for the XiaH-mediated homocoupling. Experimental evidence for carbazolium radical cations as the prerequisite for dimer formation has been obtained in the biomimetic synthesis of dixiamycins using radical starter[10] or electrochemical oxidation.[14] Likewise, the synthesis of *N*–*N*-, *N*–*C*-, and *C*–*C*-coupled carbazole dimers can be achieved via electrochemically produced radical cations.[15] Further support for a proposed radical mechanism comes from recent synthetic studies towards aryl *N*-aminosulfonamides, where sulfur dioxide was inserted into small molecules by a radical process.[16]

Our mutational analyses and successful biotransformation experiments unequivocally show that the flavoenzyme XiaH is sufficient to mediate the three-component coupling. Flavin coenzymes are very versatile heterocycles, the catalytic repertoire of which includes both two- and one-electron transfers that allow flavoenzymes to facilitate an enormous range of redox transformations in biosynthetic pathways. Radical mechanisms are part of the standard repertoire of one-electron pathways catalyzed by flavoproteins, such as the one-electron reduction of dioxygen that yields a superoxide anion and a flavin radical.[17] These radicals then react to yield 4a-flavin hydroperoxide, which is the active species in various flavoproteins like monooxygenases, halogenases, and Baeyer–Villigerases.[17] The reverse one-electron transfer reaction, comprising the one-electron oxidation of a substrate and the reduction to the flavin radical as proposed for xiamycin coupling is not frequently observed. However, such a mechanism has been implicated for flavin-dependent monoamine oxidases, where the amino group is the target of a one-electron oxidation by flavin adenine dinucleotide (FAD) to yield an aminyl radical cation.[18] Futhermore, other flavoproteins might well parallel the proposed scenario of bixiamycin formation, such as the enzymatic *N*–*C*-bipyrrole homocoupling in marinopyrrole biosynthesis, for which a flavoenzyme-catalyzed single electron transfer mechanism to give an aminyl radical cation was suggested as one plausible route.[19] Taking these biochemical and synthetic data together, XiaH-mediated sulfadixiamycin formation is most likely initiated by the formal abstraction of a hydrogen radical or one-electron oxidation to the radical cation, followed by deprotonation. Via both routes, reactive xiamycin intermediates would be formed, in which resonance stabilization leads to unpaired electrons positioned at N-1 (**Ia**), C-6 (**Ib**), and C-21 (**Ic**), among others (Scheme 1a). These radicals may pair to yield dixiamycins, or, alternatively, they could react with sulfur dioxide, yielding a sulfonyl radical that could pair with another xiamycin radical (Scheme 1b). It is remarkable that the positions for bond formation in dixiamycin and sulfadixiamycin biosynthesis (N-1, C-6, and C-21) are equal, although other positions might also be possible on the basis of radical resonance stabilization. This observation further supports the hypothesis that both kinds of dimers share the same biogenetic origin.



Scheme 1. Model for dixiamycin and sulfadixiamycin biosynthesis. a) Formation of the resonance-stabilized xiamycin radicals **Ia**, **Ib**, and **Ic**, after a proposed one-electron oxidation to the aminyl radical cation, followed by deprotonation. These radicals may pair to yield dixiamycins or alternatively, could react with sulfur dioxide to the sulfadixiamycins. b) Exemplary reaction to yield **9**: **Ia** could react with sulfur dioxide, which is in equilibrium with sulfite from sulfur metabolism, yielding a sulfonyl radical that could pair with **Ib**, followed by rearomatization.

The discovery of the highly unusual sulfonyl-bridged alkaloids from a mangrove endophyte and the elucidation of their bacterial synthesis have several implications. Sulfadixiamycins are the first natural products featuring sulfanilamide and dapsone substructures, and are a new class of antibiotics. Their marked antibacterial potency and low cytotoxicity make them promising leads for therapeutics. From a biosynthetic point of view, we elucidated an unprecedented biosynthetic route for sulfadixiamycins involving a three-component reaction with two equivalents of carbazole derivatives and one equivalent sulfur dioxide. The successful biotransformation revealed a new role for a radical-forming flavoprotein that sets the stage for *C–S* and *N–S* bond formation. Flavin-dependent enzymes have been the subject of countless biochemical studies and it was found that they catalyze a vast array of reactions.[17] However, this is the first report on a flavoprotein-mediated SO_2 incorporation. Furthermore, the XiaH-catalyzed reaction is a mechanistically unprecedented route for carbon–sulfur bond formation in nature. This unusual reaction, which mimics a synthetic copolymerization procedure, is an important contribution to the currently available enzymatic repertoire for the generation of sulfur-organyls.[20] Considering the frequently observed enzyme-mediated aryl couplings in metabolic pathways,[19,21] sulfonyl-bridged aromatic compounds are likely to be found as congeners of known natural products. Thus, our findings may inspire exploring these new scaffolds by biomimetic synthesis and stimulate the search for related bioactive natural products.

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