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Identification and Biosynthetic Characterization of Natural Aromatic Azoxy Products from *Streptomyces chattanoogensis* L10

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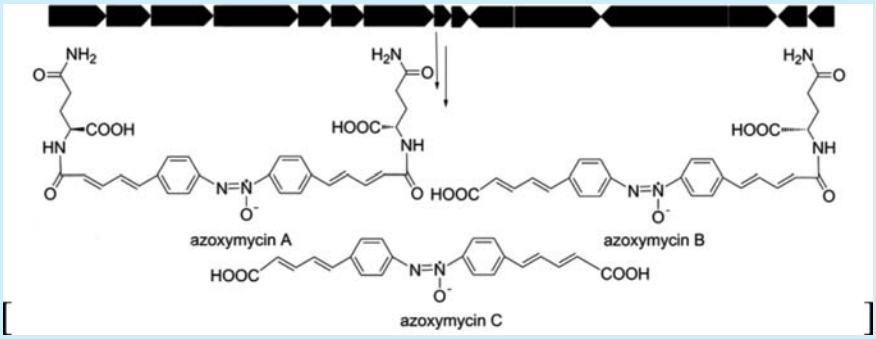
Supporting Information



ABSTRACT: Aromatic azoxy compounds recently attracted wide interest for their unique liquid crystalline properties. However, biosynthetic pathways of natural azoxy products have rarely been reported. Three novel aromatic azoxy compounds, azoxymycins A, B, and C, have been isolated and identified from *Streptomyces chattanoogensis* L10, and their biosynthetic pathways have been reported.

Azoxy compounds are widely used as dyes, reducing agents, chemical stabilizers, polymerization inhibitors, therapeutic agents, and energetic materials.[1−3] More recently, the liquid crystalline properties of aromatic azoxy compounds have gained considerable attention.[4] These light-controllable materials have novel applications, are used in liquid crystal laser systems,[5,6] and are potentially capable of converting light energy into macroscopic motion,[7,8] where a light-induced switch from the *trans* to *cis* configuration of the azoxy bond between their two aromatic rings is utilized.[9] To date, a few natural azoxy compounds have been reported,[10−15] and a few biosynthetic studies have been published.[10,16,17] Thus, the biosynthetic mechanism of these compounds remains a mystery. Unmasking the biosynthesis of these important natural products could facilitate their application as liquid crystalline materials.

In this research, three novel aromatic azoxy compounds, azoxymycins A, B, and C (Figure 1), were isolated and identified from *Streptomyces chattanoogensis* L10. Their biosynthetic pathways were also characterized.

Azoxymycins A, B, and C were separated by semipreparative HPLC from the culture broth of *S. chattanoogensis* L10, which had been cultivated in an optimized supplemented minimal medium (SMM culture). All three compounds appeared yellow. Their molecular formulas, $C_{32}H_{34}N_6O_9$ (*m*/*z* 645.2306 [M − H]$^-$), $C_{27}H_{26}N_4O_7$ (*m*/*z* 517.1719 [M − H]$^-$), and $C_{22}H_{18}N_2O_5$ (*m*/*z* 389.1139 [M − H]$^-$), were calculated with high-resolution time-of-flight mass (HR-TOF-MS) data. To confirm the calculated molecular formulas and analyze their elemental composition, stable isotope replacement experiments were performed by replacing the glucose and NH_4NO_3 with ^{13}C-labeled glucose and ^{15}N-labeled NH_4NO_3 in the culture medium, respectively. By comparing the MS data of the ^{13}C- and ^{15}N-labeled azoxymycins with their unlabeled analogues, the calculated molecular formulas were confirmed (Figures S1−S9).

The structures of azoxymycins A and B were elucidated using 1D and 2D NMR (Figures S10−S13). Due to low solubility, the clear NMR spectra of azoxymycin C were not determined. Thus, the structure of azoxymycin C was elucidated by MS, UV absorption, and acid hydrolysis experiments. There were two sets of similar moieties in azoxymycin A according to the 1D NMR data, which were assigned as ^{1}H and ^{13}C NMR signals in Table S1. The ^{1}H NMR spectrum showed the presence of two aromatic rings (2, 6, 3, 5 and 2′, 6′, 3′, 5′), two sets of double conjugated alkene groups (7, 8, 9, 10 and 7′, 8′, 9′, 10′), four alkane methylene groups (15, 16, 15′, 16′), two imino groups, and four other hydrogen atoms. The *J*-values of H7/H8, H9/H10, H7′/H8′, H9′/H10′ indicated an *E* configuration for these double bonds. The ^{13}C and DEPT 135 spectra supported

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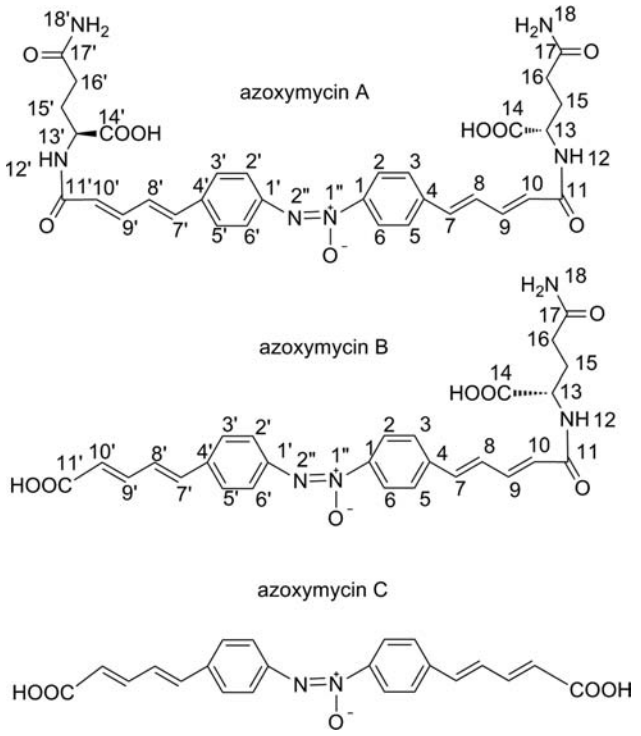

Figure 1. Structures of azoxymycins A, B, and C.

the above moieties. The chemical shifts of C1 (δ_C = 143.15) and C1′ (δ_C = 146.75) suggested that they were most probably linked to the nitrogen atom. According to the ^{15}N NMR spectrum of ^{15}N-labeled azoxymycin A (Figure S24), two nitrogen atoms were coupled to each other. Based on the chemical shift and ^{15}N NMR spectra, the C1 and C1′ should be linked by an azoxy bond. The structure of azoxymycin A is shown in Figure 1. The HMBC and COSY correlations of azoxymycin A supported this structure (Figure S25). The ^{1}H and ^{13}C NMR spectra of azoxymycin B were similar to those of azoxymycin A, except that azoxymycin B lacked one glutamine group. The assignment structure of azoxymycin B is shown in Figure 1 (HMBC and COSY correlation in Figure S25). To determine the stereochemistry of the glutamine moiety, azoxymycins A and B were hydrolyzed with 6 mol L^{-1} HCl at 90 °C for 48 h. According to HPLC and MS data, both azoxymycins A and B had been totally converted to azoxymycin C and glutamate (Figure S26). The specific rotation of hydrolyzed glutamate was measured as +18.4°. This indicates that the glutamine moieties of azoxymycins A and B were in the L configuration. The identical UV absorption of azoxymycins A, B, and C indicated that they had the same main conjugated systems. Based on the molecular formula of azoxymycin C and the hydrolysis experiments of azoxymycins A and B, the structure of azoxymycin C was proposed (Figure 1).

The spontaneous isomerization of azoxymycins was noted during the HPLC-DAD analysis. Azoxymycin A could split into two peaks with different retention times and UV/vis spectra (Figures S27 and S30). Azoxymycin C displayed the same split during HPLC analysis (Figures S29 and S32). The split of azoxymycin B was even more complex, as it had a main M-sized peak which could then split to another M-sized peak (Figures S28 and S31). These data indicate that both azoxymycins A and C are mixtures of two constantly exchanging isomers, whereas azoxymycin B is a mixture of four constantly exchanging isomers. Usually, the double bond between two nitrogen atoms within the azoxy group has either a *trans* or *cis* conformation. The *trans* conformation has been found to be more stable, but light and thermal fluctuations (even at room temperature) can cause a mutual isomerization between these two conformations.[18,19] Moreover, the oxygen atom can migrate from one nitrogen atom to another within the azoxy bond.[20] These splits align with the characteristics of azoxy compounds, further validating the deduced structures of these azoxymycins. To measure the photoactivity of azoxymycins, they were treated with UV and heat, followed by fast HPLC analysis. Results showed that the proportions of the *cis* azoxymycins were increased after UV exposure for 2 h. The ratio then returned to the original level within 10 min after the removal of UV stimulus (Figures S33−S35). Heat also caused changes between the *trans* and *cis* configurations (Figures S36−S38).

According to the structures of azoxymycins A, B, and C, at least one keto-synthase is expected to be involved in their biosynthesis. The sequenced genome of *S. chattanoogensis* L10 was screened for the potential ketosynthase encoding gene(s) for azoxymycins using the native Basic Local Alignment Search Tool (BLAST). Among all screened genes, azoF and azoG were the most focused, as their encoding protein exhibited 41% and 34% identity to AsuC14 and AsuC13,[21] respectively. AsuC14 and AsuC13 are a pair of keto-synthases participating in the formation of the benzene ring linked polyene moiety.[23] The structures of azoxymycins contain two similar benzene ring linked polyene moieties. While AsuC14 and AsuC13 are proposed to catalyze three cycle of polyketide elongation, the AzoF and AzoG are supposed to catalyze two cycles of elongation. The gene cluster harboring azo F and G is illustrated in Figure 2. BLAST results of the genes within the

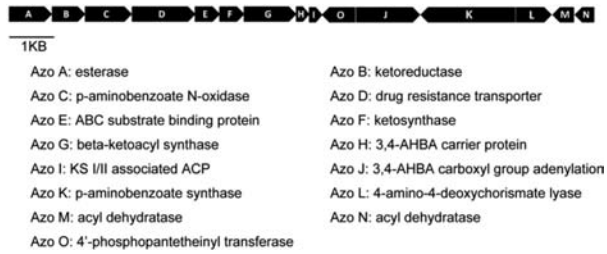

Figure 2. Proposed genetic organization of azoxymycin biosynthetic gene cluster.

cluster (Table S2) implied that they had a reasonable biosynthesis logic to azoxymycins (Figure 3). Following the proposed biosynthesis pathway, AzoJ is supposed to anticipate the load of *p*-aminobenzoic acid to ACP, while AzoF and G together are supposed to elongate the side alkene chain of *p*-aminobenzene; AzoC is expected to catalyze the azoxy conjugation of the precursor(s).

To validate this gene cluster and the proposed biosynthetic pathway, three deletion mutant strains, Δ*azoFG*, Δ*azoJ*, and Δ*azoC*, were constructed by replacing *azoFG*, *azoJ*, *azoC* with an apramycin resistant gene in *S. chattanoogensis* L10 (Figures S39−S41), using the polymerase chain reaction (PCR)-targeting method.[22] Inactivation of either *azoFG* or *azoJ* caused a loss of the yellow color (Figure 4C). HPLC analysis indicated the abolishment of azoxymycin production in both the Δ*azoFG* mutant and the Δ*azoJ* mutant (Figure 4A). Interestingly, the culture of the ΔazoC mutant still showed a light yellow color despite HPLC analysis indicating no azoxymycins production.

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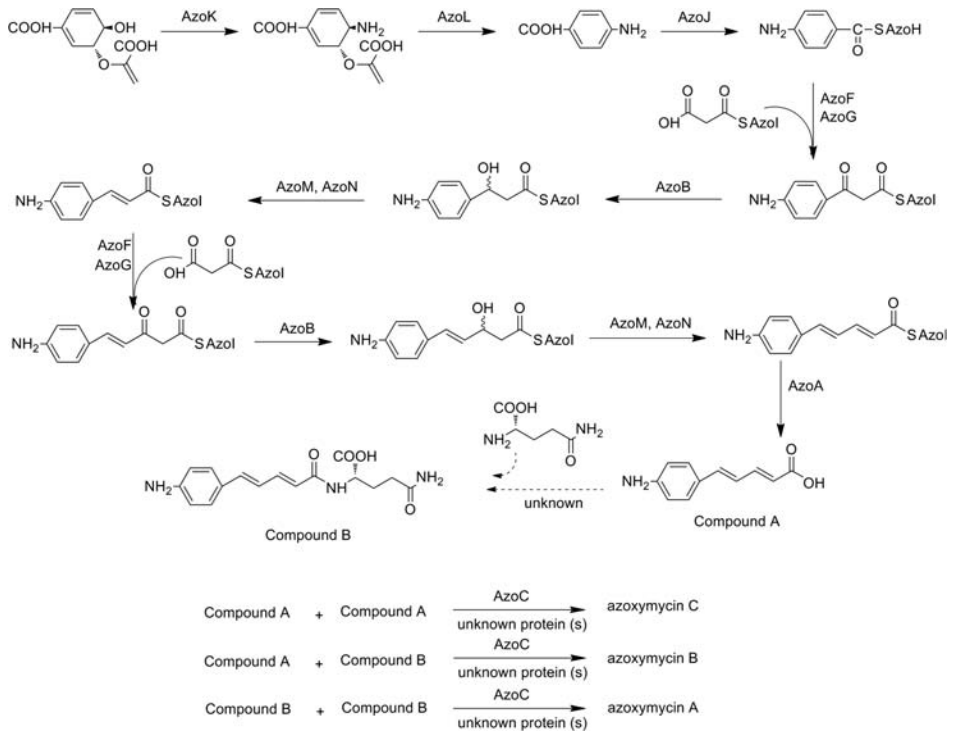

Figure 3. Proposed biosynthetic pathway of azoxymycins in *S. chattanoogensis* L10.

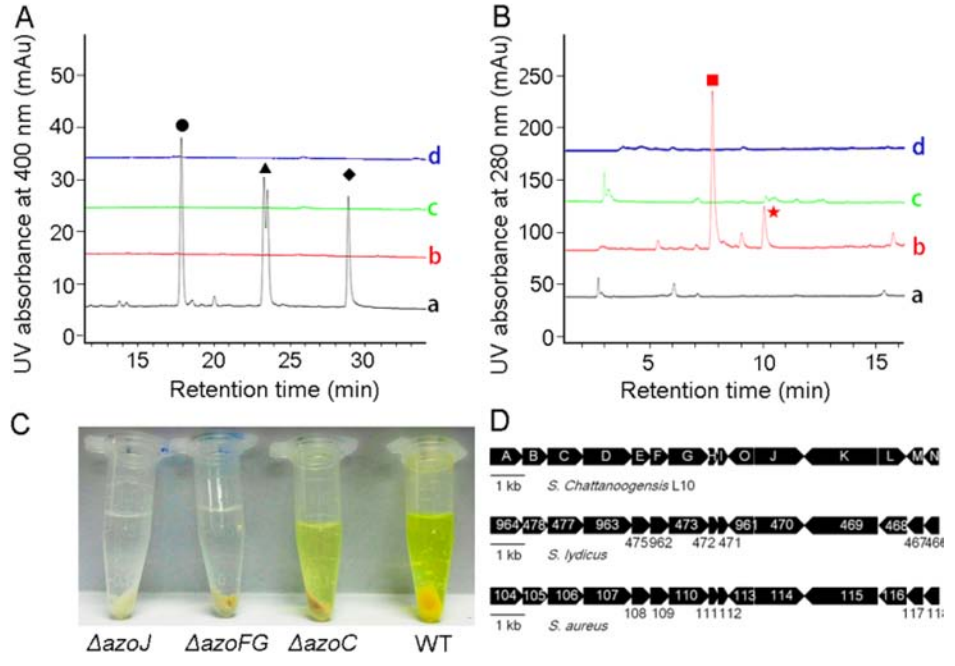

Figure 4. (A) HPLC spectra of azoxymycins A, B, and C in *S. chattanoogensis* L10, (a) wild type, (b) Δ*azoC* mutant, (c) Δ*azoFG* mutant, (d) Δ*azoJ* mutant. (B) HPLC spectra of compound **A** and compound **B** in *S. chattanoogensis* L10, (a) wild type, (b) Δ*azoC* mutant, (c) Δ*azoFG* mutant, (d) Δ*azoJ* mutant. (C) Phenotypic appearance of culture broth of different mutant strains and wild type. (●) Azoxymycin A, (▲) azoxymycin B, (◆) azoxymycin C, (■) compound **B**, (★) compound A. (D) Comparison of the azoxymycins biosynthesis gene clusters in *S. chattanoogensis*, *S. lydicus*, and *S. aureus*.

Meanwhile, two new components were detected at the 280 nm peak (Figure 4B), which were denoted as compounds **A** and **B** (Figures S42 and S43). The MW of compounds **A** and **B** were 189 and 317 respectively (Figures S44−45). The HPLC splits, UV/vis absorptions, and MS of compounds **A** and **B** were identical to those for the two compounds (compounds **1** and **3**) reported by Potterat et al.[23] The abolishment of azoxymycin production in these three deletion mutants complied with the biosynthetic pathway and further confirmed the proposed biosynthetic pathway of azoxymycin; the accumulation of compounds **A** and **B** in Δ*azoC* mutant indicated that AzoC could probably be a key enzyme in the biosynthesis of the azoxy bond.

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The structures of azoxymycins A and C indicate that they contain two sets of identical subunits which are linked by an azoxy bond. Moreover, the structure of azoxymycin B implies that it contains two azoxy bond-linked different subunits. Their biosynthetic pathway suggests that azoxymycins A and C are synthesized from two identical precursors, and azoxymycin B is synthesized from two different precursors. While the structure of azoxymycin C is deduced without NMR spectra, the perfect match for the azoxymycins' biosynthetic pathway and structures suggests that the deduced structure of azoxymycin C is correct.

Furthermore, the National Center for Biotechnology Information (NCBI) genome database search revealed that azoxymycin biosynthesis gene clusters are spread among several other *Streptomyces* species, such as *Streptomyces lydicus* (99% identity) and *Streptomyces aureus*[24] (88% identity). Their gene cluster arrangement was almost the same as that of the azoxymycin biosynthesis cluster in *S. chattanoogensis* L10 (Figure 4D and Table S2). Interestingly, *S. lydicus and S. aureus* both show yellow color,[25,26] which might be caused by the azoxymycin-like compounds synthesized by these clusters. The existence of the azoxymycin biosynthesis gene cluster in other *Streptomyces* species implies that these aromatic azoxy natural products might have some kind of bioactivity similar to that of these *Streptomyces*.

To summarize, three novel aromatic azoxy natural products, azoxymycins A, B, and C, were isolated and identified from *S. chattanoogensis* L10. All three azoxymycins showed light inducible properties. Their biosynthetic pathway was characterized and strongly supported by gene deletion experiments. The discovery and biosynthesis of azoxymycins suggest that these promising azoxy liquid crystalline compounds could be biologically synthesized in replacement of traditional chemical methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03137.

Experiment details, biological assays, NMR, MS, HPLC, UV/vis spectra of compounds described in the text (PDF)

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Notes

The authors declare no competing financial interest.

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