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Dibenzothiophene Catabolism Proceeds via a Flavin-N5-oxide Intermediate

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Supporting Information

ABSTRACT: The dibenzothiophene catabolic pathway converts dibenzothiophene to 2-hydroxybiphenyl and sulfite. The third step of the pathway, involving the conversion of dibenzothiophene sulfone to 2-(2-hydroxyphenyl)-benzenesulfinic acid, is catalyzed by a unique flavoenzyme DszA. Mechanistic studies on this reaction suggest that the C2 hydroperoxide of dibenzothiophene sulfone reacts with flavin to form a flavin-N5-oxide. The intermediacy of the flavin-N5-oxide was confirmed by LC-MS analysis, a co-elution experiment with chemically synthesized FMN-N5-oxide and $^{18}O_2$ labeling studies.

Acid rain is formed from sulfur dioxide generated during fossil fuel combustion and the removal of sulfur from these fuels is an important step in pollution control. The widely used metal-catalyzed hydrodesulfurization is only partially successful because it is inefficient with sulfur-containing heterocyclic compounds. Dibenzothiophene is the major sulfur-containing heterocycle in crude petroleum[1] and the possibility of removing this compound by microbial degradation has led to the discovery of its catabolic pathway in *Rhodococcus erythropolis* (Figure 1).[1,2] The first two steps[3] of

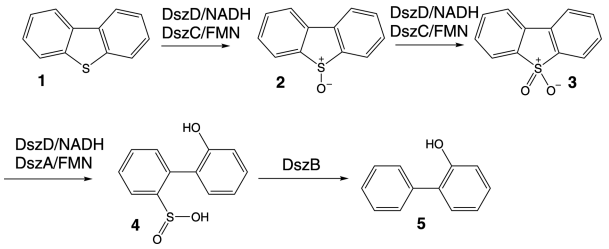

Figure 1. Four-step dibenzothiophene catabolic pathway in *Rhodococcus erythropolis*.

this pathway involve well-precedented flavin-hydroperoxide-mediated thioether oxidation[4] to sulfone **3** via sulfoxide **2**, and the last step[5] most likely involves bisulfite loss in an ipso substitution reaction. The mechanism of the third step, involving conversion of sulfone **3** to sulfinic acid **4**, is not obvious and does not conform to any of the well-established motifs in flavoenzymology. We describe studies to elucidate the mechanism of this reaction.

DszA was overexpressed in *E. coli* BL21(DE3) and purified by Ni-affinity chromatography. The protein overexpressed well (6 mg/Liter) and was purified to homogeneity (Figure S1). The DszA-catalyzed reaction was reconstituted by incubating sulfone (3), DszA, *E. coli* flavin reductase (to replace DszD), NADH, and FMN at 25 °C. The formation of sulfinic acid **4** was monitored by HPLC analysis (Figure S2).

The previously reported $S_{RN}1$ reaction of dibenzothiophene sulfone **3** with alkoxides to form the corresponding alkylated analog of **4** served as our first model for the enzymatic reaction,[6,7] and the corresponding $S_{RN}1$ proposal[7] for DszA is shown in Figure 2. In this proposal, electron transfer from FlH^-

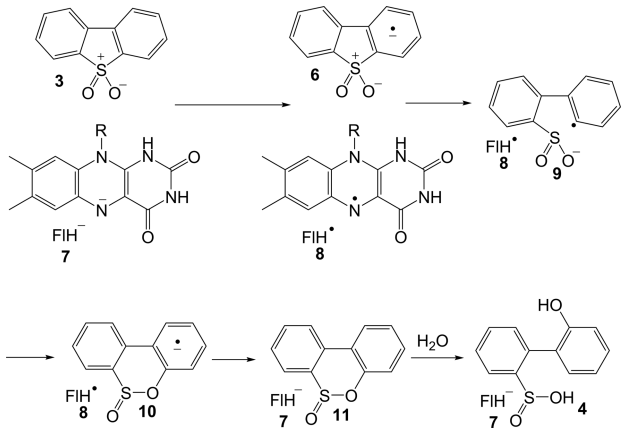

Figure 2. $S_{RN}1$ mechanistic proposal for the DszA-catalyzed reaction.

to the electron-deficient sulfone **3** gives the sulfone radical anion **6**. Fragmentation of the C−S bond gives **9**. Ring closure and electron transfer to the flavin semiquinone gives **11**. Hydrolysis of **11** completes the reaction. While the initial electron transfer to form **6** is likely to be unfavorable, rapid rearrangement of **6** to **10** could drive the reaction.

This proposal predicts that the DszA-catalyzed reaction is oxygen independent. To test this, the DszA reaction was run under anaerobic conditions. No product was formed. The DszA reaction was also run in $[^{18}O]$-H_2O/$^{16}O_2$ buffer to look for the oxygen incorporation expected for the hydrolysis of **11** to **4**. No oxygen incorporation was detected (Figure 3A). In contrast, when the enzymatic assay was carried out in the presence of $^{18}O_2$, a 2 Da increase in the mass of the product was observed consistent with the incorporation of a single oxygen atom from molecular oxygen (Figure 3B). Oxygen incorporation into the phenol rather than the sulfinate was established by demonstrating retention of label when $[^{18}O_1]$-**4** was converted to **5** using

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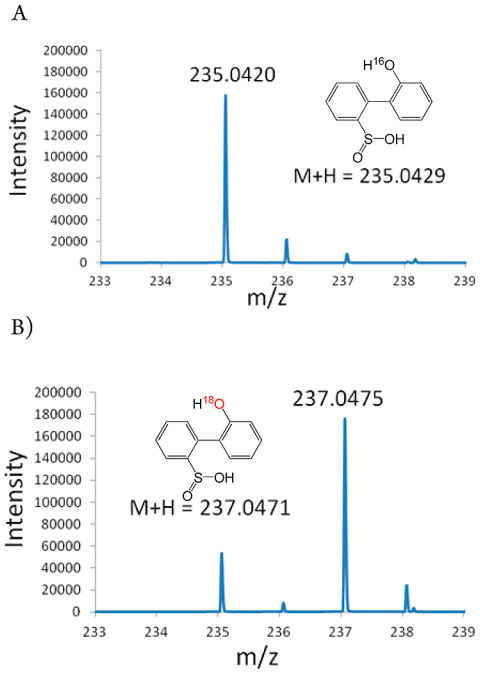

Figure 3. LC-MS analysis of the DszA reaction run in [^{18}O]-$H_2O/^{16}O_2$ buffer or $H_2O/^{18}O_2$. Panel A shows that oxygen from the buffer is not incorporated into the reaction product **4**. Panel B shows the incorporation of a single oxygen atom from molecular oxygen; *m*/*z* for **4** and [$^{18}O_1$]-**4** are 235 and 237 Da, respectively.

DszB (Figure S3). These experiments eliminate the possibility that the DszA reaction proceeds by an $S_{RN}1$ mechanism.

We next considered the possibility of a nucleophilic addition of flavin hydroperoxide **12** to **3**. The nucleophilicity of **12** is well established in the flavoenzyme-mediated Baeyer–Villiger oxidation of ketones[8] and the RutA-catalyzed uracil ring opening reaction.[9] In this proposal (Figure 4), addition of the flavin hydroperoxide to dibenzothiophene sulfone gives the sulfone-stabilized carbanion **13**. Protonation, followed by flavin elimination, gives hydroperoxide **14**. NADH-mediated reduction of this hydroperoxide, as previously observed for the RutA system, gives **16**, which is converted to product by a ring opening/tautomerization/bond rotation sequence (A Baeyer–Villiger-like mechanism, involving flavin peroxide attack on the sulfone sulfur, results in the formation of a sulfonic rather than a sulfinic acid and is therefore not possible). The normal mechanism of flavoenzyme-mediated peroxide cleavage[10] is also not possible because DszA is cysteine free.

This mechanism predicts that it should be possible to trap hydroperoxide **14** if the DszA reaction is run in the absence of excess reducing agent. These reaction conditions were achieved by initial photoreduction of the flavin using EDTA and by running the reaction in phosphate buffer using stoichiometric amounts of photoreduced FMN, DszA, and dibenzothiophene sulfone. The reaction mixture did not contain DTT, protein thiol, free FMN, or NADH, and the photogenerated glyoxylic acid was not consumed (Figure S5). It was prepared under anaerobic conditions in a glovebox and then exposed to air to allow formation of the enzyme bound flavin hydroperoxide. Under these reaction conditions, sulfinic acid **4** was the major reaction product, and peroxide **14** was not detected raising the question as to how peroxide **14** is reduced.

In the absence of any obvious reducing agent in the reaction buffer, the possibility of a flavin-N5-oxide was considered (mechanism 3, Figure 4). This new flavin oxidation state was recently identified in the EncM-catalyzed Favorskii rearrangement involved in enterocin biosynthesis.[11−13] HPLC analysis of the DszA reaction mixture (containing stoichiometric amounts of photoreduced FMN, DszA, and dibenzothiophene sulfone) demonstrated the consumption of FMN and the formation of a new species eluting after 20.3 min (Figure 5A). This species comigrates with and has an identical UV–vis spectrum to a synthetic sample of FMN-N5-oxide (Figure 5B,C). MS analysis of $I_{20.3}$ is also consistent with the FMN-N5-oxide structure (Figure 5D,E), and running the DszA reaction in the presence of $^{18}O_2$ showed the expected 2 Da mass increase (Figure 5F). Finally, the ratio of FMN consumed to sulfinic acid formed in a reaction containing stoichiometric amounts of photoreduced FMN, DszA, and dibenzothiophene sulfone was 1:0.9 consistent with the stoichiometric conversion of FMN to FMN-N5-oxide during the course of the DszA-catalyzed reaction (Figure S4).

Our studies on DszA suggest a mechanism involving initial formation of flavin hydroperoxide from reduced flavin. This adds to **3** to form the sulfone-stabilized carbanion **13**. Protonation followed by flavin elimination gives **14** and **15**. Flavin-mediated peroxide cleavage[14,15] would form **16** and **17**. The catalytic cycle is then completed by the conversion of **16** to **4** and **17** to **15** (Figure 4).

Decades ago flavin-N5-oxide **17** was considered as an intermediate in flavin-dependent oxygenase chemistry but was almost entirely replaced by the flavin hydroperoxide mecha-

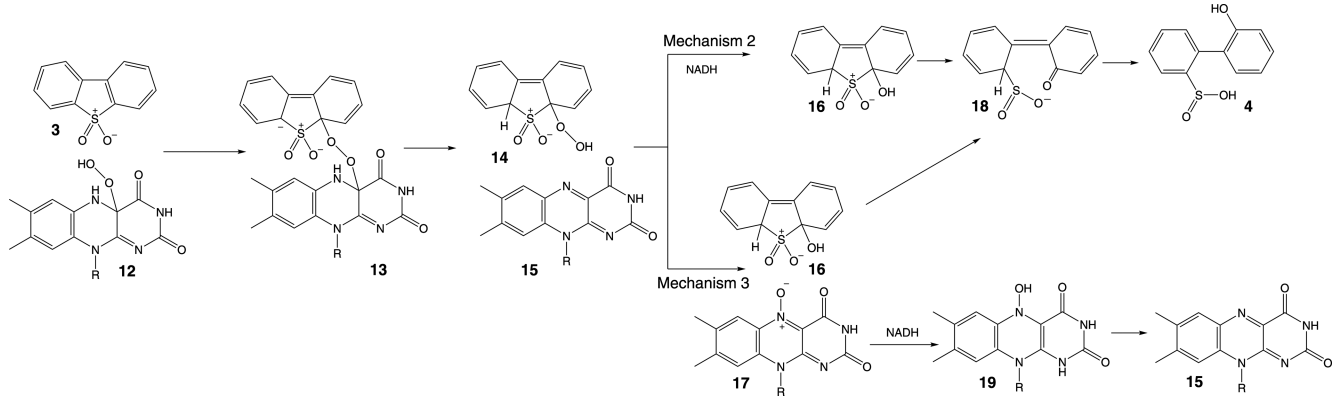

Figure 4. Proposed DszA-catalyzed dibenzothiophene sulfone ring opening via a flavin hydroperoxide intermediate.

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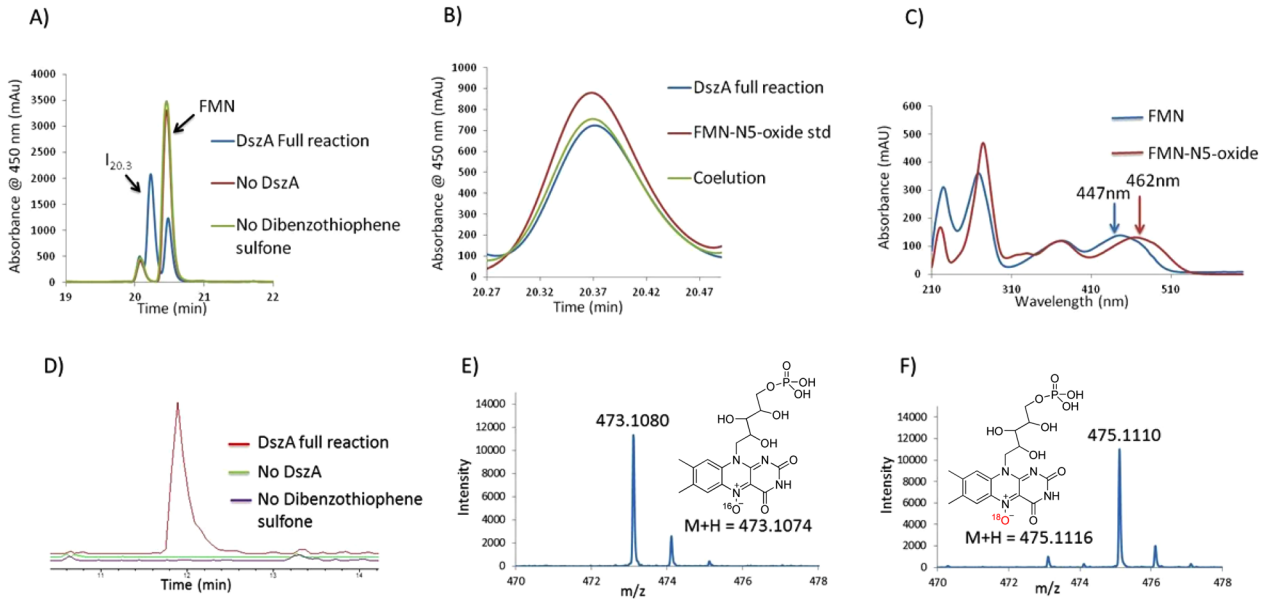

Figure 5. Characterization of a new intermediate in the DszA-catalyzed reaction. (A) Partial HPLC chromatogram of the DszA/photoreduced FMN reaction mixture showing the new intermediate ($I_{20.3}$) eluting at 20.3 min. The peak eluting at 20.1 min corresponds to an impurity present in commercial FMN. (B) Co-elution of $I_{20.3}$ with synthesized FMN-N5-oxide. (C) UV−vis spectra of FMN and FMN-N5-oxide. The $I_{20.3}$ spectrum was identical to that of FMN-N5-oxide. (D) Extracted ion chromatograms for [M + H] = 473.1 Da demonstrate that $I_{20.3}$ is present only in the full reaction mixture. (E) Exact mass of $I_{20.3}$ consistent with the mass expected for FMN-N5-oxide ([M + H] = 473.11 Da). (F) Exact mass of $I_{20.3}$ generated using $^{18}O_2$ consistent with the mass expected for [$^{18}O_1$]-FMN-N5-oxide ([M + H] = 475.11 Da).

nistic paradigm.[11,12] Since FMN-N5-oxide and organic peroxides are easily reduced by widely used biochemical reducing agents, (DTT, TCEP, NADH etc.),[16] it is easy to miss this intermediate, and a systematic search for other FMN-N5-oxide-mediated reactions is merited.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b00583.

Experimental details and data (PDF)

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Notes

The authors declare no competing financial interest.

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