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Identification of the Product of Toxoflavin Lyase: Degradation via a Baeyer–Villiger Oxidation

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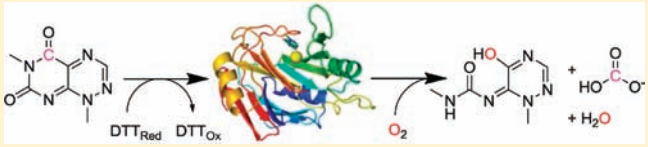
Supporting Information

ABSTRACT: Toxoflavin (an azapteridine) is degraded to a single product by toxoflavin lyase (TflA) in a reaction dependent on reductant, Mn(II), and oxygen. The isolated product was fully characterized by NMR and MS and was identified as a triazine in which the pyrimidine ring was oxidatively degraded. A mechanism for toxoflavin degradation based on the identification of the enzymatic product and the recently determined crystal structure of toxoflavin lyase is proposed.



INTRODUCTION

Toxoflavin (**1**) was originally isolated from *Pseudomonas cocovenenans* as one of two agents that caused food poisoning in Java.[1−3] Isolation of the structurally similar compounds fervenulin (**2**) and reumycin (**3**) (Figure 1) has been reported

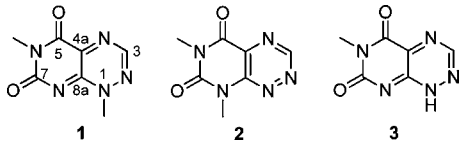


Figure 1. Chemical structures of toxoflavin (**1**), fervenulin (**2**), and reumycin (**3**).

from *Streptomyces fervens*[4] and *Burkholderia glumae*, respectively.[5] Toxoflavin has been identified as a factor necessary for the infection of rice seedlings by *B. glumae*[6] and studies on its biosynthesis and degradation are important for the control of this plant pathogen.

Early investigations into the biosynthesis of toxoflavin revealed that the pyrimidine ring was derived from a purine base (most likely guanine) and that glycine was most likely incorporated as an intact unit to complete the bicyclic core structure.[7] More recently, investigations of infected rice seedling biomes yielded a bacterium capable of surviving in the presence of toxoflavin and it was identified as *Paenibacillus polymyxa* strain JH2. Creation of a genomic library and screening in *Escherichia coli* revealed that a gene, subsequently named *tflA*, encoded a protein responsible for the detoxification of toxoflavin.[8] Initial studies using heterologously overexpressed and purified protein (TflA) showed that the degradation of toxoflavin was Mn(II), dithiothreitol (DTT), and oxygen dependent.[8−10] In addition, the recent structure of the toxoflavin/Mn(II)/TflA complex revealed a novel 1-His-2-carboxylate triad coordinating the Mn(II) center in the active site.[9]

Here, we determine the product of the enzymatic degradation reaction of toxoflavin by TflA and propose a mechanism for its formation.

METHODS AND MATERIALS

General Methods. All chemicals except those noted below were from Sigma Aldrich (St. Louis, MO) and used without further purification. *E. coli* BL21(DE3) cells were from Invitrogen (Carlsbad, CA). All micobiological and molecular biology protocols were performed according to standard procedures.[11] Pall protein concentrators (10 000 MWCO) were centrifuged at 14 000*g* for 45 min to remove protein prior to high-performance liquid chromatography (HPLC) analysis.

TflA Overexpression and Purification. Heterologous expression in and purification from *E. coli* BL21(DE3) was performed as previously described[9] except that cultures were induced with a final concentration of 0.1 mM isopropyl *β*-D-1-thiogalactopyranoside. Purified protein was buffer exchanged into Buffer A (50 mM Tris (pH 7.5), 50 mM NaCl, 10% (w/v) glycerol, and 5 mM *β*-mercaptoethanol (BME)) using an Econo-Pac 10DG column according to the manufacturer's instructions (BioRad, Hercules, CA). Protease inhibitor cocktail from Sigma Aldrich was added to the lysis buffer during sonication, according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO) as a modification of the previously described purification procedure.[9]

Toxoflavin Synthesis. Toxoflavin was synthesized as previously described.[12,13] Labeled toxoflavin was synthesized using $^{13}C_3$-diethyl malonate and ^{13}C-formaldehyde (Cambridge Isotope Labs, Andover, MA).

TflA Activity Assays. Reaction mixtures were set up as previously described.[9] Briefly, the mixtures consisted of 50 *μ*L solutions of 20 mM Tris (pH 6.5), 10 mM $MnCl_2$, 0.5 mM toxoflavin, and 9 *μ*M TflA. The reaction was initiated by the addition of DTT to a final

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concentration of 10 mM. At the specified time points (1, 5, 10, 15, and 30 min), the reaction was quenched by addition of 100 μL of 10 M urea followed by filtration through a PALL protein concentrator. A portion of the reaction mixture (100 μL) was analyzed by HPLC as described below. Alternatively, after a set time (30−45 min), TflA was removed from the reaction mixture using a PALL protein concentrator and a portion of the filtrate was analyzed by liquid chromatography−mass spectrometry (LC−MS) as described below.

Large-Scale Purification of the Enzymatic Product. The purification of the enzymatic product was performed as previously described.[9] Briefly, a master mix of 495 μL of 50 mM potassium phosphate (pH 6.8), 80 μL of toxoflavin (5 mM), 80 μL of $MnCl_2$ (0.1 M), and 80 μL of TflA (∼200 μM) was mixed in a microcentrifuge tube. The mixture was aliquoted to a total of 4 tubes containing 190 μL of master mix, to which 10 μL of DTT (0.1 M) was added to initiate the reaction. A total of 14 reactions were performed in this manner because small volumes were required to allow the reaction to proceed to completion. The reactions were incubated at 30 °C for 45 min followed by centrifugation to eliminate the precipitate (14 000*g*, 15 min, 4 °C) and then filtered through a PALL protein concentrator. The product was then purified by HPLC using an SPLC-18DB column (10 × 250 mm, 5 μm, Supelco) at a flow rate of 2 mL/min using an Agilent 1100 HPLC system with a quaternary pump and manual injector where Line A was water and Line B was methanol. The column was pre-equilibrated in 100% A and upon injection of the sample immediately switched to 95% A/5% B. This composition was held for 2 min and then changed to 35% A/65% B over the next 18 min using a linear gradient. This composition was held for 5 min after which the mobile phase was recycled to 100% A in 1 min and the column was equilibrated in 100% A for 9 min prior to the injection of the next sample. Typical injection volumes were 25−50 μL. The product eluting at approximately 12 min was collected, fractions were pooled, and solvent was removed under reduced pressure. The residue was redissolved in 450 μL of water and 50 μL of D_2O and analyzed by NMR spectroscopy as described below. The product derived from [3,4a,5,8a-$^{13}C_4$]-toxoflavin was prepared and isolated in an identical fashion.

Mass Spectrometric Analysis of the Enzymatic Product. HPLC purified product (preceding section) was analyzed using a Bruker MicroToF-Q II mass spectrometer with the following settings; End plate offset, −500 V; Capillary, −4500 V; Nebulizer, 0.4 bar; Dry gas, 4 L/min; Dry temp, 180 °C; Funnel 1 RF, 200 Vpp; Funnel 2 RF, 200 Vpp, ISCIP energy, 0.0 eV; Hexapole RF, 100 Vpp; Quadrapole ion energy, 3.0 eV; Low mass, 55.0 *m*/*z*; Collision cell collision energy, 8.0 eV; Collision RF, 150.0 Vpp; Transfer time, 121.7 μs, Prepulse storage, 1.0 μs The sample was infused at 3 μL/min. Data were analyzed using DataAnalysis 4.0 software (Bruker Daltonics, Billerica, MA).

LC−MS Analysis of TflA Reaction Mixtures. Reaction mixtures were set up and prepared as described in TflA Activity Assays above. After removal of the protein using a PALL concentrator, an aliquot (10 μL) was analyzed by LC−LCQMS. Fractionation of the reaction mixture (10 μL) was accomplished using a Surveyer HPLC system consisting of a quad pump and an autosampler coupled to a DECA-LCQ mass spectrometer operated in ESI positive mode. The reaction mixtures were separated using an Aquasil HPLC column (2.1 × 150 mm, 3 μm, Thermo Scientific) at a flow rate of 0.2 mL/min. The column was pre-equilibrated with 95% H_2O/5% MeOH prior to sample injection. The mobile phase was held constant for 2 min and then increased to 40% H_2O/60% MeOH over the next 13 min, followed by holding at this concentration for 5 min. The mobile phase was then recycled to 5% aq MeOH over 1 min and the column was equilibrated in this mobile phase for 6 min before the next injection. The ESIMS was set to positive mode with the following settings: 250 °C capillary temperature, 60 arb units sheath gas, 20 arb units auxiliary sheath gas, 4.5 V ESI voltage.

$^{18}O_2$ Incorporation Experiment. Reagents were preincubated in an anaerobic chamber (≤10 ppm O_2, COY Laboratories, Grass Lake, MI) for 30 min prior to mixing. Reaction mixtures containing 35 mM potassium phosphate buffer (pH 6.8), 0.25 mM toxoflavin, 15 μM TflA, and 10 mM DTT were then prepared. The total volume of the reaction was 500 μL and was split into 2 × 250 μL aliquots in 2 mL microcentrifuge tubes. After assembly, the $^{18}O_2$ reaction mixture was sealed in a round-bottom flask with a rubber septum and removed from the anaerobic chamber. The atmosphere of the flask was evacuated and then refilled with $^{18}O_2$ (Cambridge Isotope Laboratories, ≥98% ^{18}O) and the reaction was allowed to proceed for 1 h at 22 °C. The reaction mixture was filtered though a PALL protein concentrator (10 000 MWCO, 14 000*g*, 30 min, 4 °C). The filtrate (450 μL) was combined with 50 μL of D_2O and then analyzed by ^{13}C NMR as described below. The $^{16}O_2$ control experiment was handled identically except that upon removal from the anaerobic chamber the reaction mixture was simply exposed to the atmosphere. Each reaction was first analyzed by ^{13}C NMR to ensure product purity and then each NMR sample (250 μL) was combined, mixed, and reanalyzed. A portion (10 μL) of the individual reaction mixtures was also analyzed by LC−LCQMS as described above.

Detection of Bicarbonate As a Reaction Product. Reaction mixtures were set up as quickly as possible and all vessels were flushed with either argon or oxygen to prevent contamination with atmospheric carbon dioxide. Phosphate buffer and DTT solutions were made using degassed water (ddH_2O acidified with concentrated HCl, ∼5−6 drops per 300 mL, followed by boiling for 20 min and then cooling and storing under an argon atmosphere). Phosphate buffer (57 mM, pH 8.0) was made by mixing 96 mg K_2HPO_4 and 5 mg K_3PO_4 in 10 mL degassed water and was stored under an argon atmosphere until use. Reactions were made by mixing 350 μL of phosphate buffer, 25 μL of toxoflavin (5 mM initial concentration), and 75 μL of TflA (21.8 mg/mL) or Buffer A for the enzymatic control reaction. The reactions were mixed and split into 2 × 225 μL portions in 2 mL microcentrifuge tubes to which was added 25 μL of DTT (0.1 M). The solutions were then mixed and quickly placed in a round-bottom flask sealed with a septum and the atmosphere was purged with oxygen for 30 s to remove traces of carbon dioxide. The reactions were then incubated for 1 h at room temperature to complete the reaction. The two 250 μL reaction aliquots were combined and a portion of the reaction mixture (450 μL) was diluted with 50 μL of D_2O and mixed in an NMR tube by inversion. The tubes were then sealed with septa and purged with argon gas and finally sealed with parafilm. The reactions were analyzed by NMR as described below.

NMR Analysis. All NMR analyses were performed using a Bruker Avance III 500 MHz instrument with H−C−N Cryoprobe (^1H 500 MHz, ^{13}C 125 MHz) in 5 mm Wilmad labglass 535-PP-7 high precision NMR tubes at room temperature (22 °C) using standard instrument settings and processing parameters.

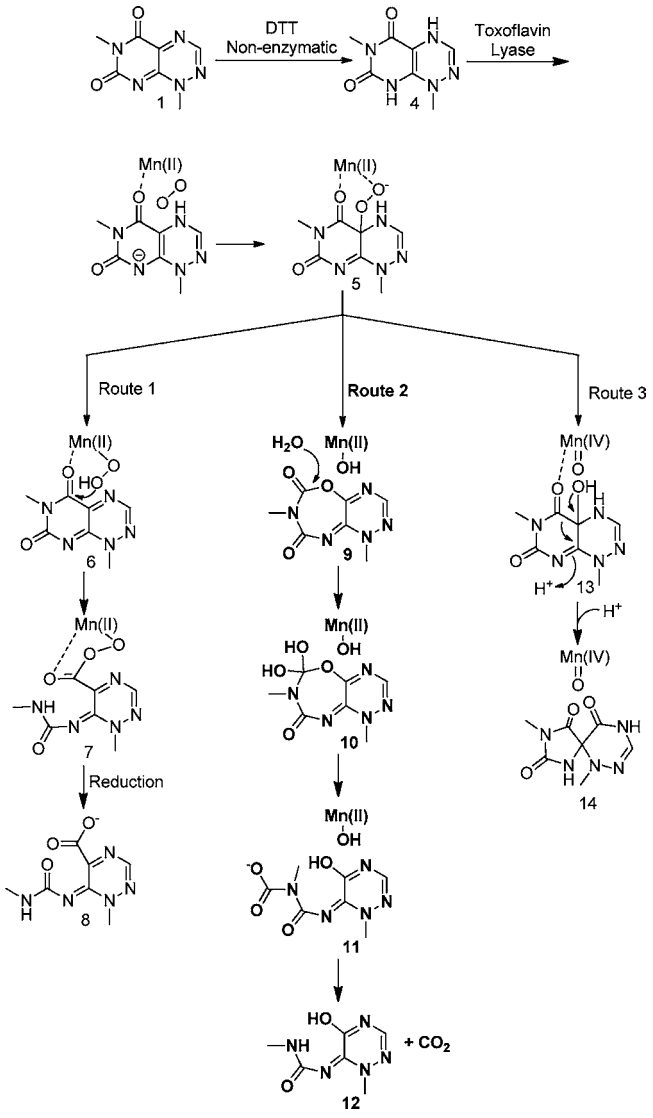
Molecular Modeling. Molecular models of some of the proposed reaction intermediates were prepared using the program Desmond[14] within the Schrödinger software package (Schrödinger, Inc.). These calculations included energy minimizations (with an energy gradient convergence threshold of 0.001 kcal/(mol/Å)) and molecular dynamics using the OPLS 2005 force field.[15] The simulated system was an adaptation of the crystal structure of TflA bound to Mn(II) and toxoflavin[9] (PDB ID 3PKX). All crystallographic waters except those located near the reaction center were removed. TIP3P waters[16] were then added to ensure a margin of 10.0 Å between the protein and the three sides of an orthorhombic periodic simulation volume. Na^+ ions were included to achieve overall electrical neutrality.

For modeling ligand−Mn(II) interactions, restraints ("zero-order bonds" within Schrödinger) were applied to maintain the crystal structure coordination geometry for the Mn(II) chelating side chain atoms of His60, Glu113, and Glu138. These restraints were imposed in all modeling steps. In contrast, the remaining Mn(II) ligands, which included toxoflavin-derived atoms, water molecules, and hydroxide ions, were unrestrained during energy minimization and molecular dynamics trajectories, except during molecular dynamics simulation of the carbamate intermediate. In this case, restraints were also applied for modeling the carbamate carbonyl oxygen−Mn(II) interaction.

RESULTS AND DISCUSSION

We have previously demonstrated that degradation of toxoflavin by TflA is oxygen, Mn(II), and DTT dependent.[9] We also observed the facile reduction of toxoflavin by DTT to yield dihydrotoxoflavin (**4**), which is readily reoxidized by air. This chemistry is analogous to well-characterized flavin redox chemistry[17] and suggests that dihydrotoxoflavin **4** is the substrate for the lyase and reacts at the active site with molecular oxygen to form peroxytoxoflavin (**5**).[9] Three possible routes for the degradation of this intermediate were considered (Scheme 1). In the first route, elimination of metal bound peroxide gives toxoflavin (**6**). Addition of peroxide to the C5 carbonyl followed by ring-opening gives (**7**), by analogy to the RutA catalyzed ring-opening of uracil.[18] This intermediate would then be reduced to **8** by excess DTT in the buffer. In the second route, a Baeyer–Villiger oxidation to yield **9** followed by hydrolysis and decarboxylation would yield **12**. The final route involves O–O bond cleavage to generate Mn(IV)=O and hemiaminal **13**, which could rearrange to (**14**) as reported for oxo-guanosine[19] and hydroxyflavin.[20]

Scheme 1. Mechanistic Proposals for the Degradation of Toxoflavin by TflA



Product characterization should differentiate between these mechanistic proposals. This was a non trivial task because of the paucity of NMR signals (2 methyl, 1 methine, and 1 NH signal, Supporting Information Figures 1 and 2) and the large number of nitrogen atoms present in toxoflavin prevented long-range correlations from being obtained. Isotopically labeled toxoflavin was therefore essential to determine the product structure and we synthesized [3,4a,5,8a-$^{13}C_4$]-toxoflavin using the previously published method for the synthesis of the nonlabeled compound.[13] ^{1}H NMR analysis of the labeled TflA product revealed four product signals (2 methyl, 1 methine, and 1 NH, Supporting Information Figure 3) as previously observed for the unlabeled product. The ^{13}C NMR spectrum was very informative as only three signals could be seen (2 doublets, 1 singlet, Supporting Information Figure 4). This indicated that one of the labeled carbon atoms from toxoflavin was lost during conversion to the product. This information immediately ruled out compounds **8** and **14** and was consistent with compound **12**. Full 2D NMR analysis (^{13}C–^{13}C COSY, ^{1}H–^{13}C HSQC, and HMBC, Supporting Information Figures 5–7) further supported this structural assignment. Analysis of the isolated product by ESI-MS revealed a base peak at m/z 209.0754 which was assigned as the $[M + Na^+]^+$ ion with an error value of 2.4 ppm (calc. 209.0749 m/z). In addition the $[2M + Na^+]^+$, $[M + K^+]^+$, and $[M + H^+]^+$ ions were also observed at m/z 395.1594, 225.0891, and 187.0921, respectively. MS/MS analysis of the $[M + Na^+]^+$ ion yielded two prominent daughter ions that were consistent with the assigned structure (Supporting Information Figures 8 and 9).

The identification of the TflA product structure suggested that the C5 oxygen of triazine **12** is derived from molecular oxygen. NMR analysis of the reaction product obtained using $^{16}O_2$ and $^{18}O_2$ was performed to test this. When the spectra were observed separately, the most downfield carbon signal (155 ppm) was offset by 7 Hz between the spectra (Supporting Information Figure 10a,b). Mixing of the two products transformed the doublet at 155 ppm into two offset doublets while all other signals were unchanged (Supporting Information Figure 10c). ^{1}H–^{13}C HMBC analysis of the mixture showed a correlation from the methine proton (7.3 ppm) to the signal at 155 ppm indicating that this carbon was derived from C4a of toxoflavin and LC–MS analysis of the individual reactions gave the expected masses and showed that ^{18}O was incorporated into the product (Supporting Information Figure 11).

To test for bicarbonate production, the TflA reaction was run at pH 8.0, all solutions were carefully degassed, and great care was taken to purge all vessels with argon. Under these conditions, the TflA free sample showed the presence of starting material and a small bicarbonate peak (singlet overlapping with the upfield portion of the doublet at 160.5 ppm). In contrast, the full reaction showed a large bicarbonate peak of approximately equal intensity to the methine carbon signal. This suggests that bicarbonate is also a product of the degradation reaction as expected for the formation of **12** (Supporting Information Figure 12).

Toxoflavin lyase (TflA) is a monooxygenase, catalyzing the degradation of toxoflavin to give the triazine **12**. A mechanistic proposal for its formation is outlined in Scheme 1 (route 2). In this proposal, toxoflavin is first reduced nonenzymatically to dihydrotoxoflavin **4**, which is the substrate for the lyase. This reduction has been previously observed as an NADH-dependent process using cell free extracts of *Saccharomyces*

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cerevisiae, suggesting that dihydrotoxoflavin is readily available *in vivo* for detoxification by TflA.[21] Enzymatic oxidation to give peroxide **5**, which then undergoes a Baeyer–Villiger rearrangement to give **9**. Hydrolytic ring-opening through the tetrahedral intermediate **10**, followed by decarboxylation of compound **11**, yields triazine **12** to complete the degradation reaction.

Molecular modeling was used to gain insight into the plausibility of this mechanism starting from the previously proposed peroxytoxoflavin intermediate shown in Figure 2A.[9]

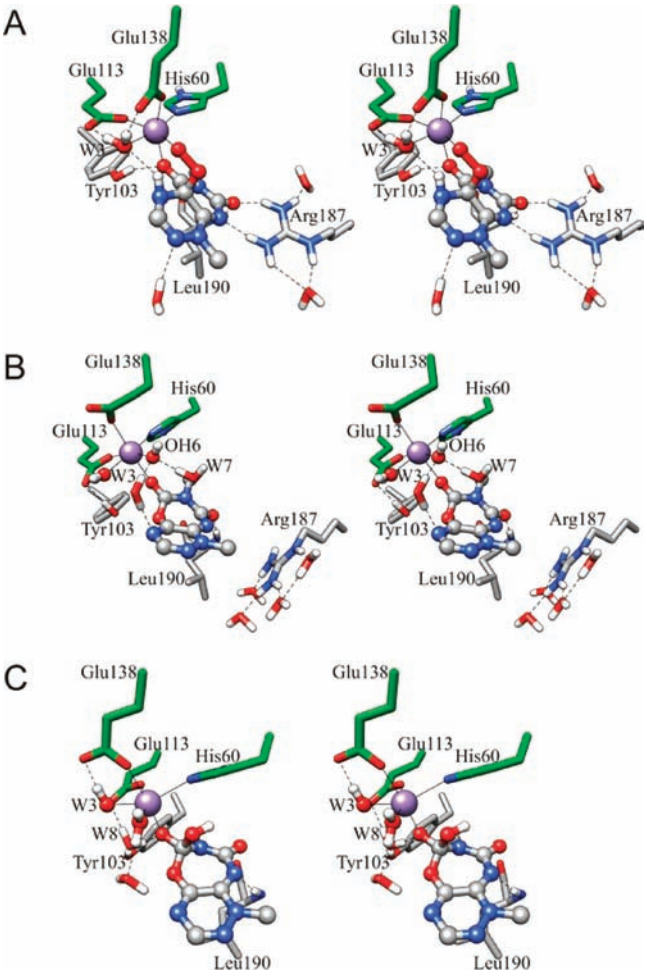

Figure 2. Molecular model of toxoflavin degradation by TflA. (A) Peroxytoxoflavin **5** bound to TflA; (B) the carbamate intermediate **9**; (C) the tetrahedral intermediate **10** generated by the addition of W7 to the C5 carbonyl of **9**.

In this structure, the C5–C4a–O–O dihedral angle (90°) does not satisfy the stereoelectronic requirements for the ring expansion. A 90° rotation about the C4a–O bond is therefore required to generate **9** (Figure 2B). For the carbamate hydrolysis, a water molecule (W7) is suitably positioned to add to the carbonyl group to give the tetrahedral intermediate (**10**) shown in Figure 2C. Collapse of this tetrahedral intermediate (**10**) followed by decarboxylation generates the product **12**. It is interesting to note that no protein side chains are directly involved in catalyzing the conversion of **5** to **12**.

The degradation of toxoflavin may have relevance to some poorly understood aspects of flavin hydroperoxide reactivity. For example, the enzyme involved in the catabolic conversion of riboflavin to quinoxaline **17** has not been identified but could proceed via a Baeyer–Villiger oxidation of a flavin hydroperoxide.[22] The enzyme involved in the conversion of flavin hydroperoxide to dimethylbenzimidazole **18**, the axial ligand of Vitamin B_{12}, has been structurally characterized,[23,24] but the mechanism of this reaction has not yet been determined. A pathway initiated by a Baeyer–Villiger oxidation is one possibility (Figure 3). Sequence analysis of TflA shows no

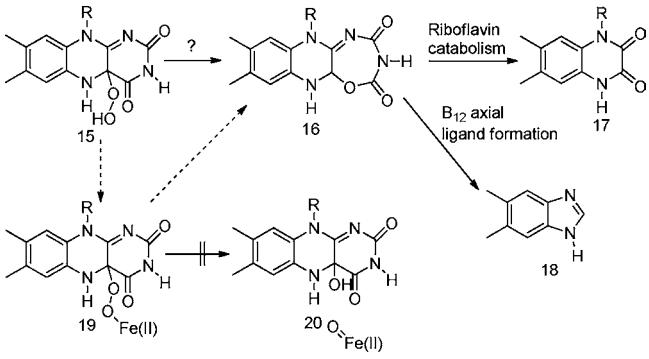

Figure 3. Proposed role for the Baeyer–Villiger oxidation of flavin hydroperoxide **15** in riboflavin catabolism, dimethylbenzimidazole biosynthesis, and in the apparent absence of flavin mediated Fe(IV)=O formation.

homology to BluB. In addition, the formation of dimethylbenzamidazole was not metal-dependent. Lastly, in contrast to biopterin hydroperoxide,[25] flavin hydroperoxide has never been identified as a source of an iron oxo-intermediate (**19** to **20**). Since flavin mediated redox chemistry has been extensively studied, it is likely that there are chemical reasons for the absence of this reaction motif in flavoenzymology. Our studies on toxoflavin degradation suggest the possibility that evolution may have selected against this chemistry because a metal-activated flavin hydroperoxide **19** may be too reactive to form an iron oxo-intermediate, undergoing instead degradation by a Baeyer–Villiger oxidation (**19** to **16**).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR and MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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