JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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A Stereoselective Vanadium-Dependent Chloroperoxidase in Bacterial Antibiotic Biosynthesis

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Supporting Information

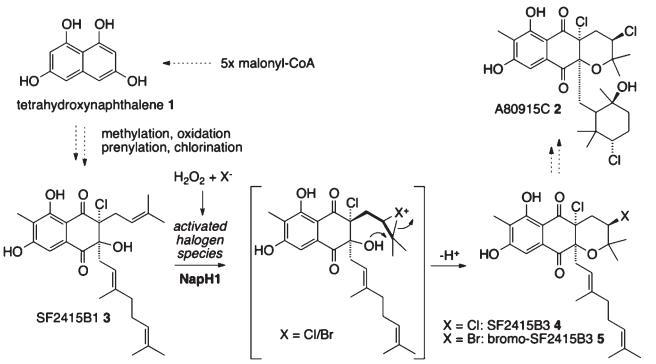
ABSTRACT: Halogenases catalyze reactions that introduce halogen atoms into electron-rich organic molecules. Vanadium-dependent haloperoxidases are generally considered to be promiscuous halogenating enzymes that have thus far been derived exclusively from eukaryotes, where their cellular function is often disputed. We now report the first biochemical characterization of a bacterial vanadium-dependent chloroperoxidase, NapH1 from *Streptomyces* sp. CNQ-525, which catalyzes a highly stereoselective chlorination−cyclization reaction in napyradiomycin antibiotic biosynthesis. This finding biochemically links a vanadium chloroperoxidase to microbial natural product biosynthesis.

Many halogenated natural products have therapeutic potential for human health, and there is great interest in discovering and exploiting enzymes that trigger halogenation reactions. Nature has developed an extensive repertoire of halogenation strategies to incorporate fluorine, chlorine, bromine, and iodine into organic molecules,[1,2] Although many studies in recent years have increased our mechanistic understanding of halogenase biochemistry, vanadium-dependent haloperoxidases (V-HPOs) remain poorly understood in the context of natural product biosynthesis.[3] Herein, we describe the first functional characterization of a bacterial V-HPO and show that the enzyme stereoselectively converts SF2415B1 into SF2415B3, two napyradiomycin antibiotics isolated from actinomycetes (Scheme 1).[4−6]

V-HPO utilizes hydrogen peroxide and a covalently bound vanadate cofactor to oxidize halides (X^-) into a mixture of activated halogenating agents, often represented by hypohalous acid (*HOX*), which can then react with electron-rich organic substrates. All currently characterized V-HPOs are eukaryotic in origin and isolated as vanadium bromoperoxidase (V-BPO) or chloroperoxidase (V-CPO) in red and brown algae and dematiaceous hyphomycete fungi.[7−12]

The cellular function of (eukaryotic) V-HPOs is often speculative as it is challenging to establish authentic substrates for the reactions they catalyze. In some cases, the producing organism does not produce any known halogenated metabolites.[3] There is indirect evidence, however, that electron-rich organic substrates bind to V-BPOs isolated from the marine red algae *Corallina*, *Laurencia*, and *Plocamium*. For example, V-BPOs from *Corallina* convert the commercially available terpenoid (*E*)-(+)-nerolidol into single stereoisomers of known halogenated metabolites α-, β-, and γ-snyderol and (+)-3β-bromo-8-epicaparrapi oxide.[13−18]

Scheme 1. Napyradiomycin Biosynthesis Starting from Malonyl-CoA and Leading to A80915C 2 Proceeds via a V-CPO-Catalyzed Chlorination−Cyclization of SF2415B1 3 To Form SF2415B3 4



We recently identified three genes (*napH1*, *H3*, and *H4*), homologous to eukaryotic V-HPOs, in a bacterial gene cluster encoding the formation of chlorinated polyketide-terpenoid antibiotics, called napyradiomycins.[19] The presence of these genes in a bacterial gene cluster presented us with a unique opportunity to characterize a V-HPO in its biosynthetic context.

Napyradiomycins constitute a class of over 30 natural products produced by terrestrial and marine streptomycetes.[4,5,20,21] Comparing metabolites and gene clusters isolated from *Streptomyces* sp. CNQ-525 and *S. aculeolatus* led us to propose a pathway starting from the aromatic polyketide tetrahydroxynaphthalene **1** leading to the trichlorinated meroterpenoid A80915C **2** via two intermediate meroterpenoids, SF2415B1 **3** and SF2415B3 **4** (Scheme 1).[19] We hypothesized that at least one of the three V-HPO-encoding genes catalyzes the conversion of **3** into **4**, through a C−C and C−Cl bond-formation reaction. This type of reaction occurs in the V-BPO-catalyzed bromonium-ion-mediated cyclization of (*E*)-(+)-nerolidol by red algal V-BPOs.[15]

We cloned the genes coding for NapH1, H3, and H4 into the *Escherichia coli* expression vector pHis8.[22] After heterologous expression and purification using Ni-NTA affinity chromatography, we analyzed the purification steps and found that while all

Received: February 3, 2011
Published: March 08, 2011

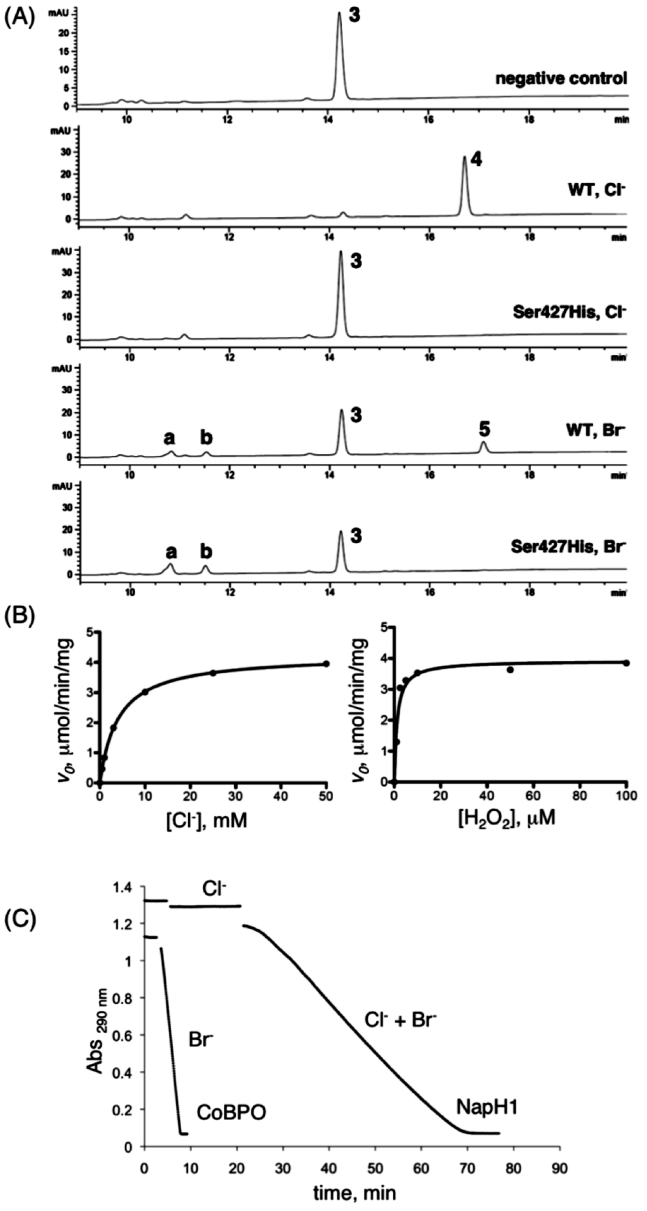


Figure 1. HPLC and spectrophotometric assay of heterologously expressed NapH1. (A) HPLC assay that monitors the chlorination and bromination of SF2415B1 **3** catalyzed by wild-type (WT) or Ser427His NapH1. The negative control is heat-inactivated WT NapH1 incubated in a complete assay mixture containing chloride. Bromohydrins **a** and **b** are of unknown structure. (B) Pseudo-first-order approximation steady-state kinetic analysis of NapH1 catalysis in the chlorination or SF2415B1 **3**. Three independent measurements at each substrate concentration gave the displayed nonlinear regression fit (line) to the averaged data (points). (C) Spectrophotometric assay that monitors the bromination and chlorination of monochlorodimedone (MCD), which results in a decrease in absorbance at 290 nm. The assay mixture is equilibrated at 30 °C (initial minutes) and then started by addition of either NapH1 (above) or *Corallina officinalis* V-BPO (CoBPO) (below). After no activity was observed in the presence of chloride (0.2 M), bromide (0.2 M) was added, and the reaction started and continued until all MCD was depleted.

three enzymes expressed well, only NapH1 (Figure S1, Supporting Information [SI]) and NapH3 were expressed in the soluble fraction as octahistidyl-tagged proteins. Since all attempts to reconstitute insoluble NapH4 failed, we focused our attention on NapH1 and H3.

Next, we isolated SF2415B1 **3**, the putative substrate (Scheme 1), from *S. aculeolatus* NRRL 18422, and verified its structure by comparing its spectral properties with literature values.[6] Gratifyingly, when this molecule was incubated with NapH1 in the presence of hydrogen peroxide and chloride in aqueous buffered (pH 6) solution, a single new peak appeared in the chromatogram (Figure 1A). Isolation and spectral analysis (HRMS and NMR) of the enzymatic product showed that it was indeed a single stereoisomer, SF2415B3 **4** (SI). NapH1 catalyzed this reaction with an optimum pH of 6 (Figure S2, SI) and following Michaelis−Menten kinetics with chloride and hydrogen peroxide (Figure 1B). The maximum rate of the reaction (V_{max}) at pH 6 was 3.9 ± 0.2 and 4.3 ± 0.1 μmol/min/mg for hydrogen peroxide and chloride, respectively. The Michaelis constant (K_m) was 1.2 ± 0.4 μM and 4.0 ± 0.1 mM for hydrogen peroxide and chloride, respectively. We were unable to determine the K_m for SF2415B1 **3**, but estimate the value to below the detection limit of our assay system (K_m < 1 μM).

NapH3 and *C. officinalis* V-BPO (CoBPO) failed to catalyze the formation of SF2415B3 **4**, in the presence of chloride, or bromo-SF2415B3 **5**, in the presence of bromide. Furthermore, NapH1 did not accept (±)-nerolidol or lapachol (4-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,2-dione) as alternate substrates (Figure S3, SI). Since lapachol contains the hydroxy and isoprene units in the analogous positions to substrate SF2415B1 **3**, and since NapH1 did not convert lapachol, we conclude that **3** likely interacts intimately with NapH1 during catalysis. In contrast, CoBPO catalyzed the complete conversion of lapachol into numerous products (Figure S3, SI). The high *in vitro* selectivity of NapH1 distinguishes this enzyme from other characterized vanadium haloperoxidases, and strongly supports a link between the *napH1*-containing gene cluster and production of napyradiomycins in *S. aculeolatus* and *Streptomyces* sp. CNQ-525. It also provides the first evidence that V-CPOs can catalyze stereoselective and substrate-specific chlorination−cyclization reactions.

When chloride and bromide were added in an equimolar ratio, NapH1 catalyzed the preferential bromination of SF2415B1 **3** to form bromo-SF2415B3 **5** (Figure S4, SI). However, while the chlorination reaction rapidly proceeded to completion, the reaction with bromide, in comparison, resulted in only a partial conversion into bromo-SF2415B3 **5** (Figure 1A). This partial conversion is likely due to a modification to the enzyme as further conversion occurred once additional NapH1 supplemented the reaction (Figure S5, SI). In addition to bromo-SF2415B3 **5**, NapH1 triggered the formation of two new compounds with identical molecular formulas $C_{26}H_{34}O_6BrCl$ based on HRMS analysis. This observation is consistent with the formation of two bromohydrin stereoisomer derivatives of SF2415B1, presently of unknown structure, that form upon hydrolysis of a common intermediate bromonium ion (Figure 1A, and SI).

Since NapH1 appeared to perform a *nonspecific* bromination of **3**, we tested the activity of NapH1 in the general haloperoxidase assay that relies on detecting the chlorination or bromination of an electron-rich indicator substrate, monochlorodimedone (MCD), using spectrophotometry (Scheme 1C).[23] Our assay results clearly demonstrated that NapH1 is *inactive* when chloride is the only halide source and only *active* after addition of bromide. This brominating activity is dramatically lower compared to the nonspecific brominating activity of *C. officinalis* V-BPO (CoBPO) (Figure 1C, Figure S6, SI). The nonspecificity in bromination

may be a result of production of diffusible hypobromous acid. Our results suggest that although the MCD assay is a benchmark assay used to quantify and isolate new general-activity haloperoxidases,[23] it may not be suitable for isolating new V-CPOs in bacterial natural product biosynthesis.

To obtain insight into the substrate binding of NapH1, we compared amino acid sequence alignments with available crystal structures of the more promiscuous V-HPOs, *C. inaequalis* V-CPO (CiCPO) and CoBPO (SI).[24,25] From this, we selected two residues in NapH1, His420 and Ser427, that had previously been proposed to be involved in determining the specificity of V-HPOs.[3,19] His420 is a histidine and phenylalanine in CoBPO and CiCPO, respectively, while Ser427 is a histidine in CoBPO *and* CiCPO. After constructing the His420Phe and Ser427His mutants of NapH1 using site-directed mutagenesis, we expressed and purified the resulting enzyme variants. Purified His420Phe NapH1 was inactive in the MCD assay and in the chlorination and bromination of SF2415B1 **3** (Figure S7, SI). Interestingly, however, while Ser427His NapH1 showed near wild type activity at approximately 70% in the MCD assay, this mutant enzyme was unable to catalyze the conversion of substrate **3** into SF2415B3 **4** or bromo-SF2415B3 **5**. Instead, Ser427His NapH1 exclusively catalyzed the formation of SF2415B1-based bromohydrins (Figure 1A). Hence the activity with SF2415B1 **3** can be completely abolished by a single point mutation to NapH1, Ser427His, without compromising the ability to produce activated brominating species.

V-BPO is known to endogenously contain bromine atoms on side-chains,[26] presumably acquired in post-translation through self-halogenation. It is possible that the activated brominating species produced by NapH1 reacts with an amino acid side-chain, and thus creates an alternative means for the activated brominating species to brominate MCD, or initiate the formation of bromohydrins. Future experiments will seek to further understand the interplay of specific and nonspecific halogenation events.

In summary, we have described the first highly substrate-selective vanadium haloperoxidase in natural product biosynthesis, where the chloroperoxidase NapH1 carries out a stereoselective chlorination−cyclization reaction with a known polyketide-terpenoid natural product.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1−S7, experimental methods, sequence alignment, HRMS data, NMR spectra of SF2415B3. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

S. aculeolatus NRRL 18422 was provided by Drs. McAlpine and Zhao (Thallion Pharmaceuticals, Canada), while the SF2415B3 standard was a kind gift from Dr. Ohyama (Meiji Seika Kaisha, Ltd., Japan). We gratefully acknowledge Drs. Su and Mrse at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility and NMR Facility, respectively, for instrument expertise. This work was generously supported by research grants from the NIH (AI047818) and the California Sea Grant Program (R/NMP-100) to B.S.M., postdoctoral fellowships from the NIH (GM096711) to P.B. and JSPS to A.M., a Ruth L. Kirschstein National Research Service Award to J.M.W. from the NIH Training Program in Marine Biotechnology (GM067550), and the Naito Foundation for sabbatical support to T.O.

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