Regioselective halogenation of lavanducyanin by a site-selective vanadium-dependent chloroperoxidase

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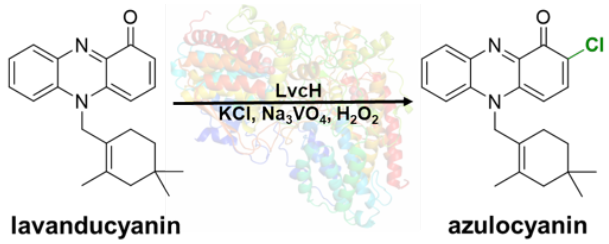

ABSTRACT: Halogenated phenazine meroterpenoids are a structurally unusual family of marine actinobacterial natural products that exhibit antibiotic, anti-biofilm, and cytotoxic bioactivities. Despite a lack of established phenazine halogenation biochemistry, genomic analysis of *Streptomyces* sp. CNZ-289, a prolific lavanducyanin and C2-halogenated derivative producer, suggested the involvement of vanadium-dependent haloperoxidases. We subsequently discovered lavanducyanin halogenase (LvcH), characterized it *in vitro* as a regioselective vanadium-dependent chloroperoxidase, and applied it in late-stage chemoenzymatic synthesis.

Biological halogenation is an important mechanism for the modulation of small molecule bioactivity and the expansion of chemical complexity in biosynthetic routes.[1–3] One family of halogenases are the vanadium-dependent haloperoxidases (VHPO) that use a vanadate co-factor, hydrogen peroxide, and aqueous halide ions to generate an electrophilic halogen species.[4] Classically, this family was understood to produce freely diffusible hypohalous acid that could spontaneously react with electron rich molecules.[5] However, site-selective VHPOs with specific roles in natural product biosynthesis or quorum sensor modification have been more recently characterized.[6–8] Despite catalyzing dramatic carbon skeleton rearrangements and the asymmetric installation of halogens,[9,10] the known pool of substrate scaffolds for these halogenases remains small. To discover novel VHPO substrates, we focused our efforts on the MAR4 clade of *Streptomyces* which are genomically enriched in VHPOs and abundantly produce halogenated meroterpenoid natural products,[11] including the majority of known halogenated phenazines. The biosynthesis of the phenazine scaffold has been well studied and the corresponding genes making up the *phz* operon are conserved across phenazine producers (Figure S1).[12] However, the large variety of strain-specific modifications and derivatizations has left questions for specific biosyntheses open.[13] Known naturally occurring halogenated phenazines appear to be restricted to derivatives of the phenazine-derived meroterpenoid, lavanducyanin (**1**), which display cytotoxic and antibiotic activities (Figure 1A).[14–16] Halogenated synthetic phenazines have been developed in parallel that display highly potent antibiotic, anti-biofilm, and cytotoxic effects (Figure 1B).[13,17–20] To date, the full biosynthetic route to **1** and its C2 chlorinated (WS-9659B, **2**) and brominated (marinocyanin A, **3**) derivatives, has not been elucidated. Given the general

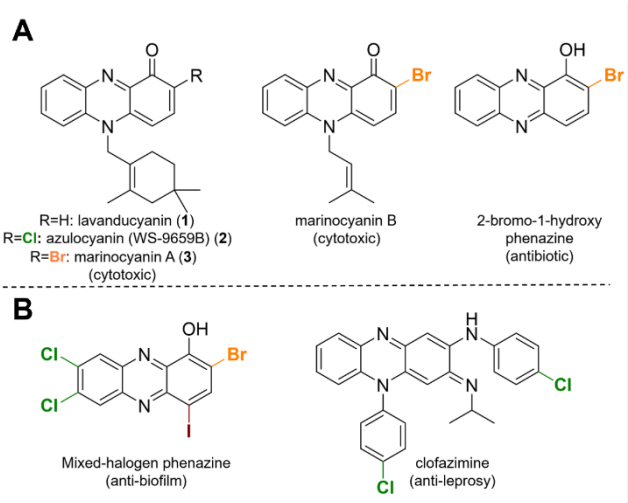

Figure 1. (**A**) Halogenated phenazines isolated from natural sources and their general bioactivities.[15] (**B**) Representative examples of synthetically prepared halogenated phenazines with notable bioactivities.[20]

lack of halogenated phenazines outside of the MAR4 clade, we speculated that the relatively unique VHPO family may be behind the biosynthesis of **2** and **3**. To maintain consistency with the '-cyanin' nomenclature used for this family of *N*-alkylated phenazinone molecules, including notable redox-active *Pseudomonas aeruginosa* metabolite pyocyanin,[21] we propose to rename **2** from WS-9659B to azulocyanin given its bright blue color. This is also precedented by renaming **1** from WS-9659A to lavanducyanin upon reisolation.[15]

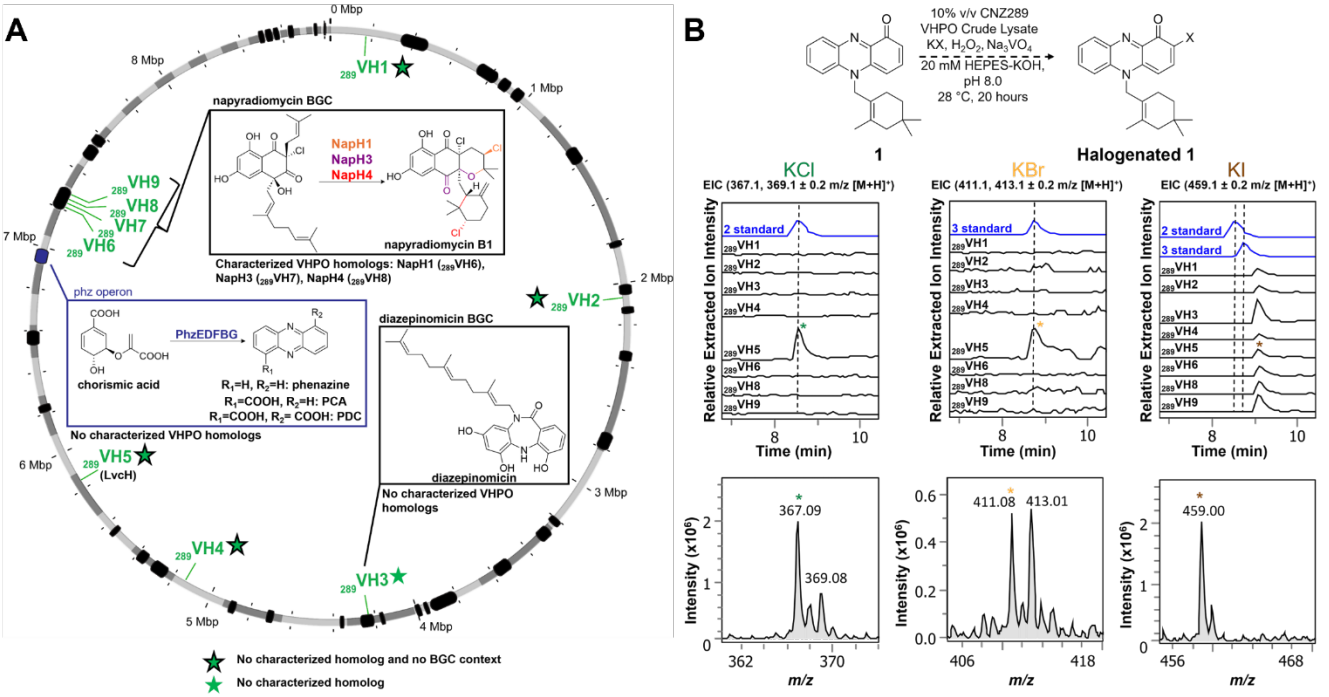

Figure 2. (**A**) Visualization of the genome of *Streptomyces* sp. CNZ-289 and its biosynthetic potential. BGCs predicted by antiSMASH are shown as black ovals. (**B**) *In vitro* assay with the crude lysates of each VHPO from *Streptomyces* sp. CNZ-289 incubated with **1** and each halide ion. The MS of the products peaks from $_{289}$VH5 display characteristic halogen isotope distributions.

We focused on a MAR4 strain, *Streptomyces* sp. CNZ-289 (generously provided by Prof. Paul Jensen), with high biosynthetic potential. After culturing this bacterium in an artificial seawater media for 14 days, extracting metabolites using XAD-7-HP resin, and performing a liquid-liquid extraction with ethyl acetate, a vibrant green colored organic extract was generated. Subsequent color-guided fractionation and UPLC-MS analyses suggested that the deep-blue mixed phenazine-terpene natural product **1** was the major secondary metabolite responsible for this pigmentation. Meroterpenoid **1** was generated in high titers (~30 mg/L) and matched previous ^{1}H NMR characterization of isolated and synthetic product (Table S1).[14,22] Both chlorinated and brominated **1** derivatives were detected within the crude extract, putatively representing the previously isolated meroterpenoids **2** and **3** respectively (Figure S2).[14,15] Initial genomic analysis using antiSMASH 7.0[23] predicted 36 biosynthetic gene clusters (BGC) including the characterized napyradiomycin meroterpenoid pathway.[9] Using the established NapH1 as a bioinformatic hook,[24] we identified 9 non-redundant VHPO genes ($_{289}$VH1-$_{289}$VH9) within the genome (Figure 2A). Four VHPOs ($_{289}$VH6-$_{289}$VH9) co-localized to the napyradiomycin BGC and demonstrated high sequence similarity and genomic synteny with the previously characterized homologs NapH1 ($_{289}$VH6), NapH3 ($_{289}$VH7), and NapH4 ($_{289}$VH8).[9] The auxiliary $_{289}$VH9 in the cluster was most similar to NapH1 and NapH4 but has no characterized function. Putative VHPO $_{289}$VH3, was located within the predicted diazepinomicin BGC, however no direct biosynthetic role has been proposed.[25,26] The final four VHPOs ($_{289}$VH1, $_{289}$VH2, $_{289}$VH4, and $_{289}$VH5) were distributed throughout the genome with no obvious BGC localization or secondary metabolism contexts.

There is precedent for the de-localization of site-selective VHPOs from the BGCs of their substrates,[8] so we hypothesized one of these uncharacterized and genomically distant homologs played a role in **1** halogenation. Each VHPO gene was cloned from *Streptomyces* sp. CNZ-289 genomic DNA for recombinant expression in *Escherichia coli* BL21(DE3) as a N-terminal hexahistidine tagged construct. Crude VHPO lysates (10% v/v) were incubated with **1** in the presence of buffered aqueous sodium orthovanadate and hydrogen peroxide alongside either KCl, KBr, or KI. Lysate assays were incubated overnight at room temperature, then analyzed by UPLC-MS for the presence of monohalogenated **1** (Figure 2B). Excitingly, we observed that $_{289}$VH5 exhibited mass ion peaks that aligned with chlorinated **2** and brominated **3** standards. The presence of diagnostic mass isotope patterns for the halide of interest (3:1 ^{35}Cl:^{37}Cl, 1:1 ^{79}Br:^{81}Br ratios) provided additional evidence for the expected halogenation chemistry. Interestingly, no other *Streptomyces* sp. CNZ-289 VHPOs catalyzed the monohalogenation of **1** under lysate conditions. Since NapH1 and NapH4 are site-selective napyradiomycin chloroperoxidases capable of non-selectively releasing HOBr,[6,7,9] the homologous $_{289}$VH6 and $_{289}$VH8 could serve as controls for non-selective haloperoxidase activity. $_{289}$VH7 was omitted from this analysis since its NapH3 homolog catalyzes a thermodynamically favorable α-hydroxyketone rearrangement instead of halogenation biochemistry.[10,24] A mass ion peak for iodinated **1** was observed in each crude VHPO lysate, however iodide can spontaneously oxidize to HOI in the presence of hydrogen peroxide and vanadium so this is not indicative of site specific halide installation.[27,28] Given the chlorination and bromination activity on **1** by $_{289}$VH5, we renamed it LvcH

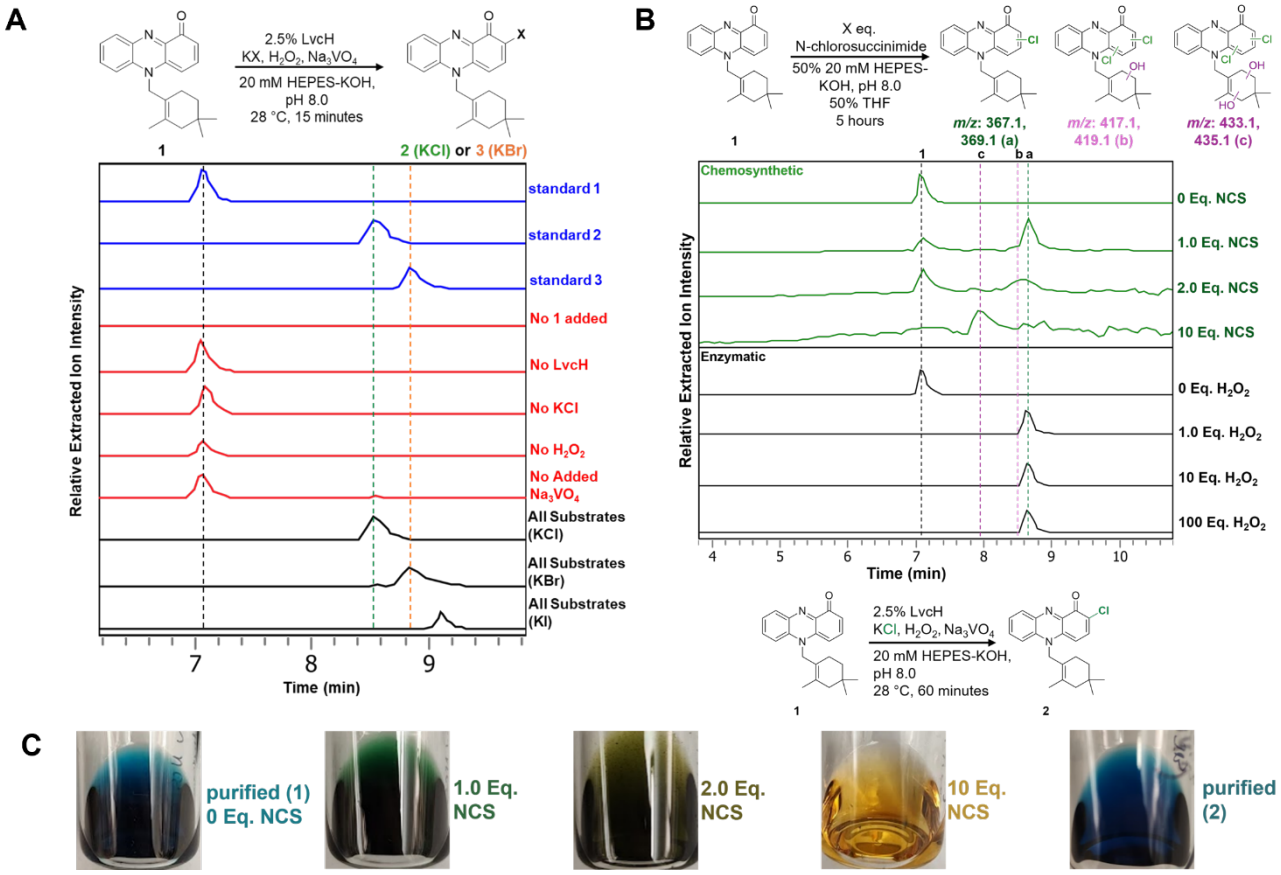

Figure 3. (**A**) *In vitro* LvcH assays in comparison to **1-3** standards (blue), omitting individual assay components (red) or introducing alternative halides (black). Chromatograms are extracted for mass ions corresponding to substrate and expected product (**B**) (top) EIC traces of incubation of **1** at different NCS stoichiometric equivalents. (bottom) EIC trace of LvcH-catalyzed **1** chlorination in the presence of increasing H_2O_2 stoichiometric equivalents. (**C**) Images of each chemosynthetic NCS reaction on **1** and purified **2**.

(lavanducyanin halogenase) and interrogated its enzymatic activity *in vitro*.

We performed liter-scale *E. coli* BL21(DE3) expressions of an N-terminally hexahistidine tagged LvcH and purified it using immobilized metal affinity chromatography (IMAC). Purified His_6-LvcH was obtained in a yield of about 2 mg/L and sufficient purity for downstream enzymatic reactions with **1** (Figure S3). We observe a duplication in the SDS-PAGE gel for LvcH consistent with the presence (58 kDa) and absence (54 kDa) of the N-terminal His_6-tag and signal peptide; we have observed this phenomenon with other recombinantly expressed actinobacterial VHPOs.[7] Subsequent size exclusion chromatography purification revealed that LvcH exists as a homodimer in solution, rationalizing how the smaller construct lacking the N-terminal hexahistidine tag could be purified using IMAC. A significant proportion of purified LvcH was soluble aggregate and excluded from further biochemical characterization (Figure S4). We first tested the ability for LvcH to release diffusible hypohalous acid with the monochlorodimedone (MCD) assay (Figure S5). This enzyme was able to non-selectively release HOBr, but not HOCl, a trait consistent with other actinobacterial site-selective vanadium chloroperoxidases (VCPO).[6] We next tested LvcH activity by individually omitting each enzyme component to assess dependence for each substrate (Figure 3A). All substrates and co-factors are strictly required to observe efficient **1** halogenation by LvcH. There is residual activity when exogenous sodium orthovanadate is omitted, most likely due to co-purification of the enzyme with trace vanadate from the culture media. Additionally, LvcH is able to oxidize chloride, bromide, and iodide by substituting the halide salt, which is consistent with the halide specificities of selective and non-selective VCPOs. The chlorination activity of LvcH is robust and displays quantitative conversion of **1** to **2** in 15 minutes at 2.5 mol% at analytical scale (Figure S6). LvcH bromination activity is comparatively more rapid, however **3** decreases even over short time periods, indicative of non-selective bromination *in vitro* (Figure S7).

To investigate the extent of halogenation by LvcH, we compared its *in vitro* biochemistry to chemosynthetic one-step halogenation reactions with *N*-halosuccinimides (Figures 3B and S8). Phenazinone **1** was incubated with *N*-chlorosuccinimide (NCS) in 1:1 aqueous enzyme assay buffer conditions:THF (for solubility) for 5 hours before analysis by UPLC-MS. With only 1 equivalent of NCS added to the reaction, dichlorinated and oxidized *m/z* features were detected, albeit at lower abundance compared to the monochlorinated product. Increasing the NCS equivalents under the same timescale reduced the abundance of monochlorinated **1**, in favor of dichlorinated and oxidized products. By qualitative observation, the chromophore of **1** was dramatically altered by the addition of just 1 equivalent of NCS (Figure 3C). Comparatively, the enzymatic reaction exhibited strict control over the extent of chlorination, even in the presence of saturating halide concentrations, high concentrations of oxidant hydrogen peroxide, and a prolonged

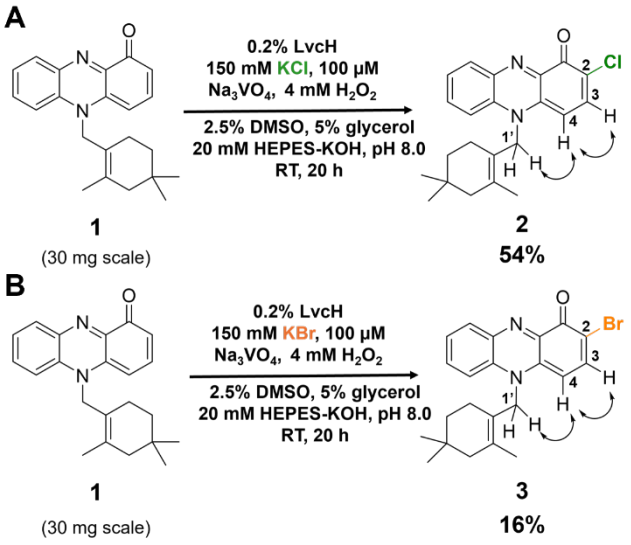

Figure 3. (**A**) Preparative scale reaction conditions and yield for the production of **2** by LvcH. (**B**) Preparative scale reaction conditions and yield for the production of **3** by LvcH. Key NOE interactions for **2** and **3** LvcH halogenation regioselectivity are shown as double headed arrows.

incubation time scale. The treatment of **1** with *N*-bromosuccinimide (NBS) resulted in more pronounced polybrominated molecules; in the presence of 1 equivalent of NBS the major product mass ion corresponded to dibrominated-phenazinol, suggesting dibromination and a loss of the cyclolavandulyl moiety (Figure S8). A minor mass ion peak corresponding to this metabolite was observed in the higher hydrogen peroxide equivalents with LvcH and KBr, which corresponds with observed diffusible HOBr activity. It was previously reported that while NBS treatment of **1** results in C2 bromination, treatment with NCS results in C4 chlorination (Figure S9).[16] While only C2-halogenated products involving the lavanducyanin scaffold have been naturally isolated, we wanted to experimentally confirm LvcH regioselectivity on **1** and assess its scalability.

Enzymatic reactions on 30 mg scale **1** and 0.2 mol% LvcH were individually performed in the presence of either chloride or bromide ions (Figure 4A and 4B). After work-up and purification of the enzymatic reaction by semi-preparative HPLC, the chlorination reaction resulted in a 54% yield of the monochlorinated product. Comparison of the ^{1}HNMR spectrum showed alignment between enzymatically produced and isolated/synthesized **2** (Table S2).[14,16] The C2-hydrogen signal had completely disappeared and key NOE correlations between the C3, C4 and C1' hydrogens validated the regioselectivity of chlorination at the C2 position. The chemoenzymatic yield of **2** by LvcH shows improvement compared to the current best synthetic route to **2**.[16] The regioselectivity of the major LvcH monobrominated product was determined in a similar method (Table S3),[15,22] however the yield of **3** was considerably lower compared to chemoenzymatically produced **2** and chemosynthetic preparation by NBS.[22] This lower yield is likely due to reduced control of bromination, evidenced by the MCD assay results. During **3** HPLC purification, a second minor product was able to be collected and had a mass ion and isotopic distribution pattern that corresponded to dibrominated-phenazinol. 2-bromo-1-hydroxyphenazine has been previously isolated from a MAR4 *Streptomyces* species for its antibiotic activity and likely represents a natural catabolite of this pathway.[15]

In summary, we have bioinformatically identified, biochemically characterized, and chemoenzymatically applied novel regioselective VHPO, LvcH. Despite lack of co-localization with the *phz* operon, we have shown its involvement in the biosynthesis of halogenated phenazine-derived meroterpenoids, **2** and **3**. To the best of our knowledge, LvcH represents the first phenazinone halogenase in the literature. Assuming that halogenation is the final biosynthetic step, we are actively investigating the construction of **1** from primary metabolic precursors. We have confirmed LvcH as a VCPO that displays regioselective **1** monochlorination with an isolated yield of 54%. Intriguingly, the chlorination regioselectivity of **1** differs in the presence of NCS, highlighting the site-selective enzymatic control (Figure S9).[16] Conversely, LvcH produces diffusible HOBr that reacts with substrate **1** based on its inherent nucleophilicity, mirroring the reaction with electrophilic bromine source NBS. Despite the thousand-fold difference in chloride vs. bromide ion concentrations in the marine environment, this difference in LvcH halide reactivity rationalizes the multiple naturally isolated brominated marinocyanins, while **2** remains the only isolated chlorinated **1** derivative. Halogenated phenazines, both natural and synthetic, have been shown to display significant bioactivities and intriguing non-bactericidal antibiotic effects,[16,18] such as the disruption of microbial biofilms.[20] This bioactivity opens interesting questions into how and when LvcH is expressed to modulate **1** and the biological and ecological reasons for why this meroterpenoid is utilized over other phenazine scaffolds. Finally, the robust phenazinone halogenase activity and regiocontrol makes LvcH an exciting candidate to develop as a novel halogenation biocatalyst.

ASSOCIATED CONTENT

Data Availability

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

- FAIR data, including the primary NMR FID files for compounds **1**, **2**, and **3** (ZIP)
- Experimental procedures, Supplementary figures, NMR spectroscopic data, and supplementary tables (PDF)

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Notes

The authors declare no competing financial interest.

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