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Regioselective Halogenation of Lavanducyanin by a Site-Selective Vanadium-Dependent Chloroperoxidase

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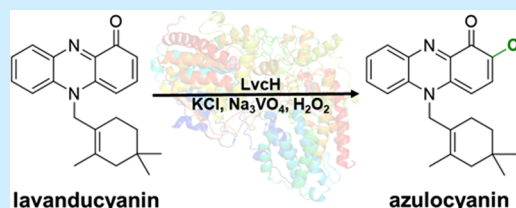


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ABSTRACT: Halogenated phenazine meroterpenoids are a structurally unusual family of marine actinobacterial natural products that exhibit antibiotic, antibiofilm, and cytotoxic bioactivities. Despite a lack of established phenazine halogenation biochemistry, 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 cofactor, 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 In contrast, site-selective VHPOs with specific roles in natural product biosynthesis or quorum sensor modification have been more recently characterized and control the delivery of oxidized halides to their respective targets.^{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, antibiofilm, 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 lack of halogenated phenazines outside of the MAR4 clade, we speculated that the

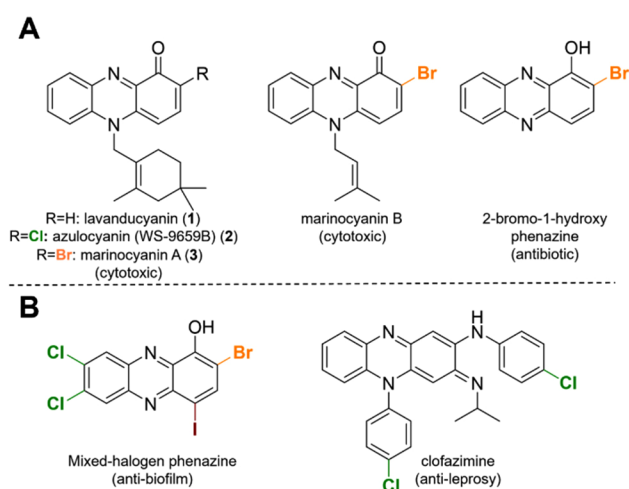


Figure 1. (A) Halogenated phenazines isolated from natural sources and their general bioactivities.¹⁵ (B) Representative examples of synthetically prepared halogenated phenazines with notable bioactivities.²⁰

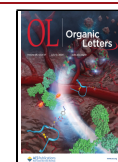
relatively unique VHPO family may be behind the biosynthesis of compounds 2 and 3. To maintain consistency with the “-cyanin” nomenclature used for this family of *N*-alkylated phenazinone molecules, including notable redox-active *Pseudomonas aeruginosa* metabolite pyocyanin,²¹ we propose to rename

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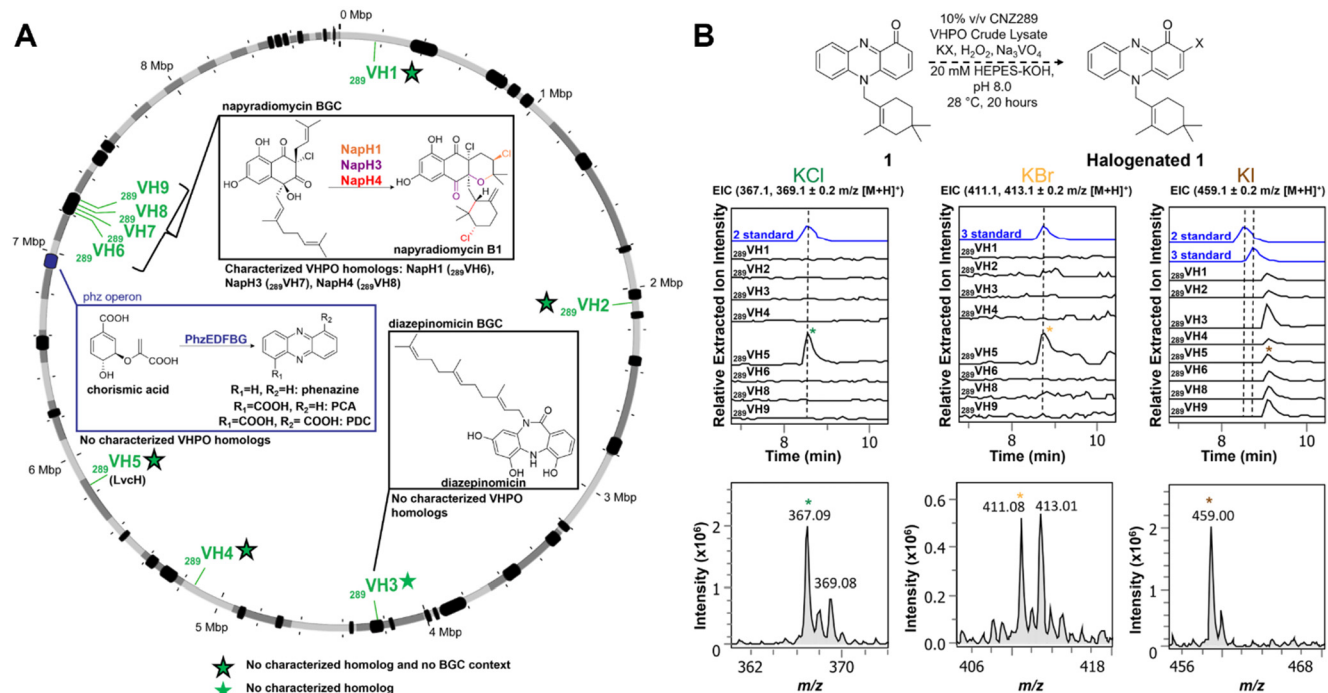


Figure 2. (A) Visualization of the genome of *Streptomyces* sp. CNZ-289 and its biosynthetic potential. BGCs predicted by antiSMASH are shown as black ovals. (B) *In vitro* assay with the crude lysates of each VHPO from *Streptomyces* sp. CNZ-289 incubated with **1** and each halide ion. The MS of the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

2 from WS-9659B to azulocyanin given its bright blue color. This is also preceded by renaming **1** from WS-9659A to lavanducyanin upon reisolation.¹⁵

We focused on a high biosynthetic potential MAR4 strain, *Streptomyces* sp. CNZ-289 (generously provided by Prof. Paul Jensen). After culturing this bacterium in 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 ¹H 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} antiSMASH 7.0²³ genomic analysis 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 nonredundant VHPO genes (289VH1–289VH9) within the genome (Figure 2A, Table S4). Each of these genes was annotated as VHPOs and displayed high % similarities and identities with characterized VHPOs (Table S5). Four VHPOs (289VH6–289VH9) colocalized to the napyradiomycin BGC and demonstrated high sequence similarity and genomic synteny with the previously characterized homologues *NapH1* (289VH6), *NapH3* (289VH7), and *NapH4* (289VH8).⁹ The auxiliary 289VH9 in the cluster was most similar to those of *NapH1* and *NapH4* but has no characterized function. Putative VHPO 289VH3, was located within the predicted diazepinomicin BGC; however, no direct biosynthetic role has been proposed.^{25,26} The final four VHPOs (289VH1,

289VH2, 289VH4, and 289VH5) were distributed throughout the genome with no obvious BGC localization or secondary metabolite contexts (Table S4).

There is precedent for the delocalization of site-selective VHPOs from the BGCs of their substrates,⁸ so we hypothesized one of these uncharacterized and genomically distant homologues 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 an 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 and then analyzed by UPLC-MS for the presence of monohalogenated **1** (Figure 2B). Excitingly, we observed that 289VH5 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 ³⁵Cl/³⁷Cl, 1:1 ⁷⁹Br/⁸¹Br ratios) provided additional evidence of the expected halogenation chemistry. Interestingly, there were no other *Streptomyces* sp. CNZ-289 VHPOs that catalyzed the monohalogenation of **1** under lysate conditions. Since *NapH1* and *NapH4* are site-selective napyradiomycin chloroperoxidases capable of nonselectively releasing HOBr,^{6,7,9} the homologous 289VH6 and 289VH8 could serve as controls for nonselective haloperoxidase activity. 289VH7 was omitted from this analysis since its *NapH3* homologue 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} Despite halogenating a phenazine-derived substrate, 289VH5 displays 53–59% sequence identity

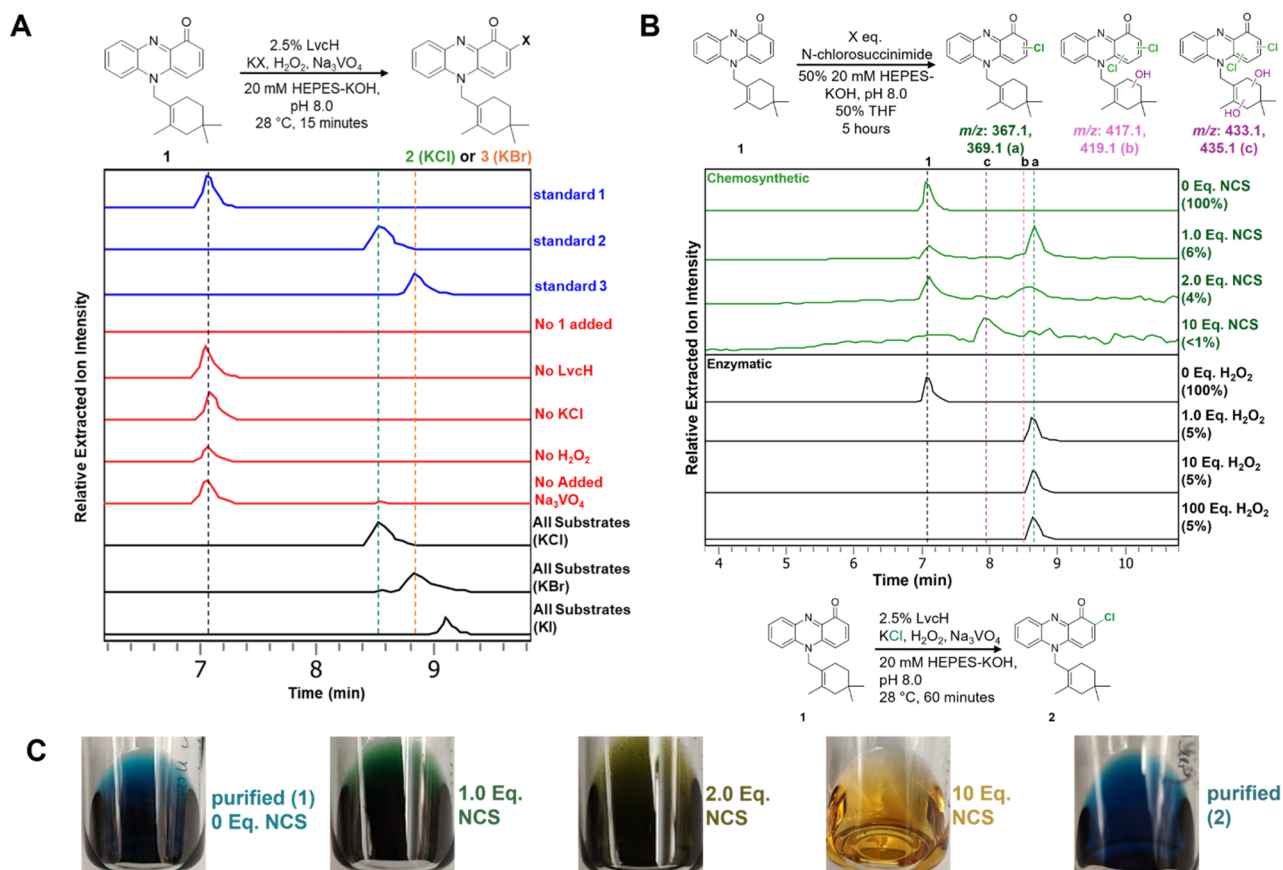


Figure 3. (A) *In vitro* LvcH assays in comparison to 1–3 standards (blue), omitting individual assay components (red) or introducing alternative halides (black). Chromatograms are extracted for mass ions corresponding to the substrate and expected product. (B) (Top) EIC traces of incubation of **1** at different NCS stoichiometric equivalents. (Bottom) EIC trace of LvcH-catalyzed **1** chlorination in the presence of increasing H₂O₂ stoichiometric equivalents. The remaining **1** for each reaction condition is shown in parentheses. (C) Images of each chemosynthetic NCS reaction on **1** and purified **2**.

with characterized meroterpenoid VHPOs (Table S6). Given the chlorination and bromination activity on **1** by ₂₈₉VHS, we renamed it LvcH (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 His₆-LvcH (2 mg/L) was obtained at 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 His₆-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 by using IMAC. A significant proportion of purified LvcH was soluble aggregate and was excluded from further biochemical characterization (Figure S4). We first tested the ability of 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 the dependence for each substrate (Figure 3A). All substrates and cofactors are strictly

required to observe efficient **1** halogenation by LvcH. There is residual activity when exogenous sodium orthovanadate is omitted, most likely due to copurification 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 nonselective VCPOs. The chlorination activity of LvcH displays quantitative conversion of **1** to **2** in 15 min at 2.5 mol % at analytical scale (Figure S6). LvcH bromination activity is more rapid; however, **3** decreases even over short time periods, indicative of nonselective 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 (Figure 3B and Figure S8). Phenazinone **1** was incubated with *N*-chlorosuccinimide (NCS) in 1:1 aqueous enzyme assay buffer conditions: THF (for solubility) for 5 h before analysis by UPLC-MS. With only 1 equiv 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 time scale 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 equiv 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 incubation time scale. The treatment of **1** with *N*-bromosuccinimide (NBS) resulted in more pronounced polybrominated molecules; in the presence of 1 equiv 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 the 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 the LvcH regioselectivity on **1** and assess its scalability.

Enzymatic reactions on a 30 mg scale for **1** and 0.2 mol % LvcH were individually performed in the presence of either chloride or bromide ions (Figure 4A,B). After workup and

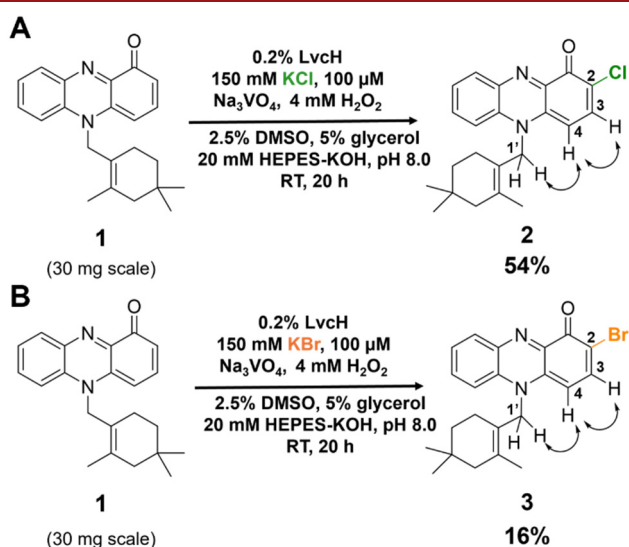


Figure 4. (A) Preparative scale reaction conditions and yield for the production of **2** by LvcH. (B) Preparative scale reaction conditions and yield for the production of **3** by LvcH. Key NOE interactions for **2** and **3** LvcH halogenation regioselectivity are shown as double headed arrows.

purification of the enzymatic reaction by semipreparative HPLC, the chlorination reaction resulted in a 54% yield of the monochlorinated product. Comparison of the ^1H NMR 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**; however, due to the different synthetic approaches, the yields are not directly comparable (Figure S9).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, as 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 colocalization 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 in 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 nonbactericidal 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 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 Statement

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 at <https://pubs.acs.org/doi/10.1021/acs.orglett.4c01869>.

Experimental procedures, structures, ion chromatogram, SDS-PAGE gel, SEC, monochlorodimedone and *in vitro* assays, EIC traces and optical images, synthetic methods, amino acid sequence, NMR spectra, and tables of NMR data, genomic contexts, cloning primers, and sequence percent similarity/identity comparisons (PDF)

FAIR data, including the primary NMR FID files, for compounds **1**–**3** (ZIP)

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Notes

The authors declare no competing financial interest. A preprint was previously posted on ChemRxiv.²⁹

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