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Communication

Enzymatic Halogenation of Terminal Alkynes

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ABSTRACT: The biosynthetic installation of halogen atoms is largely performed by oxidative halogenases that target a wide array of electron-rich substrates, including aromatic compounds and conjugated systems. Halogenated alkyne-containing molecules are known to occur in Nature; however, halogen atom installation on the terminus of an alkyne has not been demonstrated in enzyme catalysis. Herein, we report the discovery and characterization of an alkynyl halogenase in natural product biosynthesis. We show that the flavin-dependent halogenase from the jamaicamide biosynthetic pathway, JamD, is not only capable of terminal alkyne halogenation on a late-stage intermediate en route to the final natural product but also has broad substrate tolerance for simple to complex alkynes. Furthermore, JamD is specific for terminal alkynes over other electron-rich aromatic substrates and belongs to a newly identified family of halogenases from marine cyanobacteria, indicating its potential as a chemoselective biocatalyst for the formation of haloalkynes.

Halogenated natural products are found in a variety of environments and are especially abundant in marine ecosystems. Enzymes responsible for installing halogen atoms are defined as halogenases and are categorized by the cofactor used, type of halogenation performed (electrophilic, nucleophilic, or radical), and the nature of the carbon atom to be functionalized.[1,2] Among the halogenases, one of the most versatile groups is the flavin-dependent halogenases (FDHs), which are capable of electrophilic halogenation of aromatic rings, conjugated systems, and 1,3-diketones.[3] Due to the inherent substrate promiscuity and high levels of site-, regio-, and chemoselectivity exhibited by FDHs, these enzymes are of significant interest for biocatalytic applications.[3] However, major challenges remain in expanding the structural diversity of substrates accepted and in improving the catalytic efficiency of FDHs overall.[4−6] We sought to address these limitations by searching for homologues of one of the most efficient FDHs discovered to date, AetF.

AetF is an FDH from the biosynthetic pathway for the cyanobacterial toxin aetokthonotoxin (AETX, **3**), where it is responsible for the sequential dibromination of L-tryptophan (**1**) to yield 5,7-dibromo-L-tryptophan (**2**) (Figure 1a).[7,8] AetF is distinct from known FDHs in amino acid sequence, exhibits remarkable catalytic efficiency and substrate promiscuity, and operates as a standalone "single-component" enzyme (Figure 1b).[7−13] This rare configuration improves ease of use for biocatalysis, as the vast majority of FDHs are known as "two-component" halogenases and require an exogenous flavin reductase to initiate the catalytic cycle (Figure 1b).[5,6,14,15] We anticipated that AetF homologues in publicly available databases might harbor similarly impressive properties with diverse substrate preference or reactivity.

In the search for novel AetF homologues, we investigated the evolutionary context of AetF in an unbiased manner by constructing a maximum likelihood (ML) phylogenetic tree of the FAD-NAD(P)-binding enzyme family. Approximately 150,000 unique representative sequences were used for initial

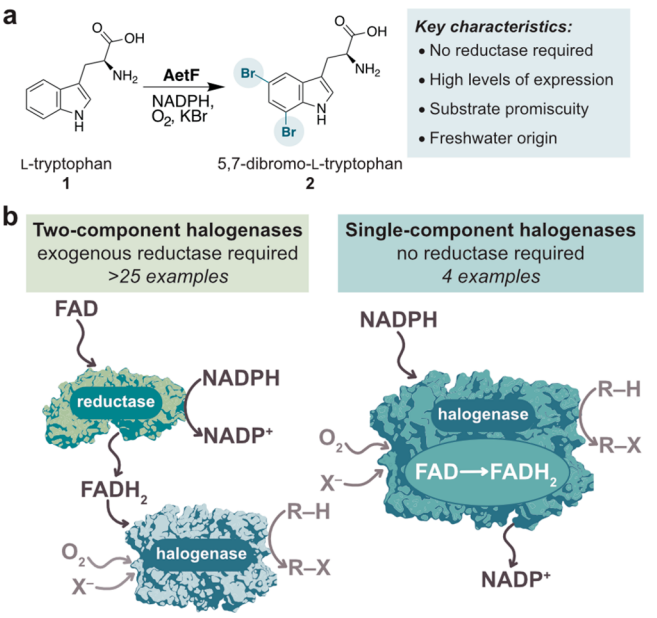

Figure 1. (a) Dibromination reaction performed by the single-component halogenase AetF. (b) Modes of reactivity for two-component and single-component FDHs.

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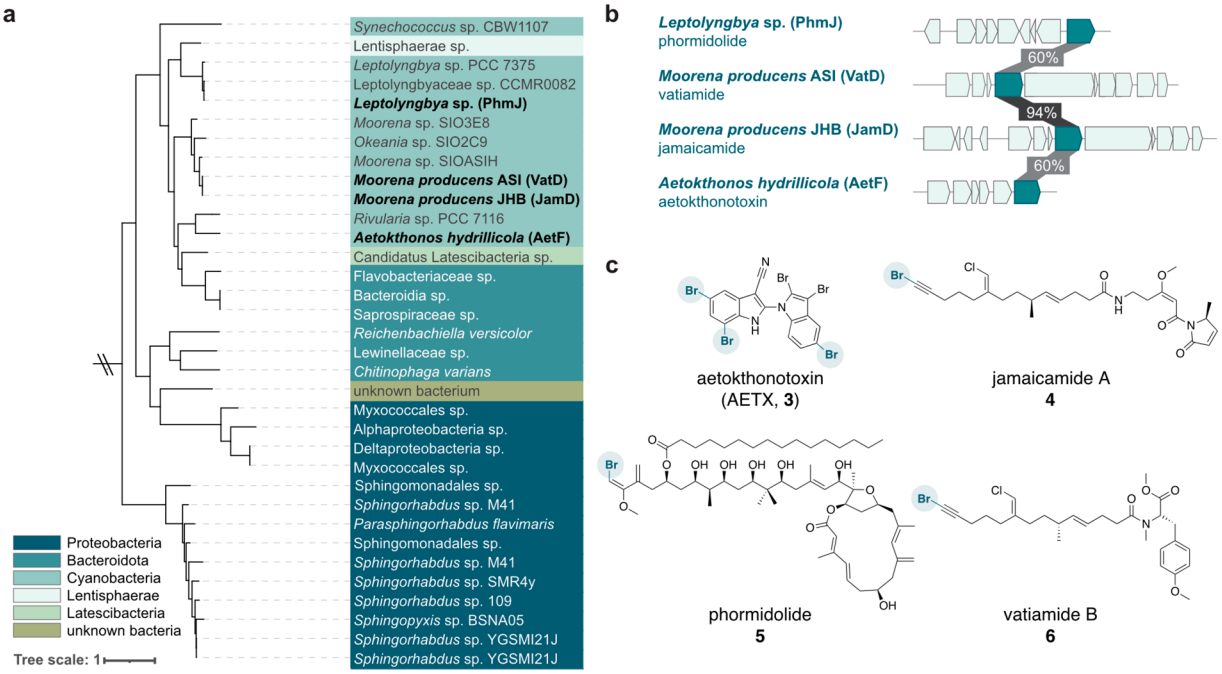

Figure 2. Phylogenetic analysis reveals AetF relatives from known BGCs. (a) AetF-containing clade from a flavin-dependent enzyme phylogenetic tree. (b) Synteny plots of known BGCs highlighting AetF relatives in dark blue; percent identity indicated between relatives in gray. Partial regions of BGCs containing AetF relatives are shown due to cluster length. (c) Structures of natural products associated with highlighted BGCs with bromine atoms proposed to be installed by the corresponding AetF-like halogenases highlighted in blue.

phylogenetic tree construction followed by iterations of clustering and manual tree trimming to yield a curated set of 269 sequences that is representative of the large variety of known flavin-dependent enzyme sequences (Table S1). AetF does not clade with the other known single-component halogenases, which also appear to be distinct from one another and emerge on three separate occasions (Figure S3). Instead, AetF and its homologues appear to be more closely related to pyridine nucleotide-disulfide oxidoreductases and glucose-methanol-choline (GMC) family oxidoreductases, neither of which have been shown to catalyze a halogenation reaction.

Taking a closer look at the AetF-containing clade (Figure 2a), interesting patterns emerge. First, every member in the clade is of marine origin, which is unexpected given the freshwater source of the AetF-producing cyanobacterium, *Aetokthonos hydrillicola*.[8] Second, AetF homologues appear in separate taxonomic clades. Most strikingly, three uncharacterized enzymes from annotated assembly line biosynthetic gene clusters (BGCs) in marine cyanobacteria appear as AetF homologues: JamD, VatD, and PhmJ (Figure 2b,c).[16−18] Each of these enzymes is hypothesized to be a halogenase in their respective biosynthetic pathways; however, activity has not been previously demonstrated. JamD and VatD are both localized in mixed polyketide synthase/nonribosomal peptide synthetase (PKS-NRPS) pathways that produce the molecules jamaicamide A (**4**) and vatiamide B (**6**), respectively (Figure 2c).[16,17] PhmJ is encoded in a PKS pathway, and its corresponding natural product, phormidolide (**5**), is structurally distinct from jamaicamide A (**4**), vatiamide B (**6**), and AETX (**3**) (Figure 2c).[18,19] Although the halogenases from this clade share moderate to high sequence identity with AetF (Figure 2b), their associated biosynthetic gene clusters generate vastly different structures compared with AETX (**3**). Furthermore, analysis with the Enzyme Function Initiative-Genome Neighborhood Tool (EFI-GNT) revealed that many of the remaining uncharacterized homologues are also localized in PKS and NRPS-like assembly line pathways (Figure S4), bringing into question the evolution of the unusual AETX (**3**) BGC in a freshwater cyanobacterium.[20]

We hypothesized that JamD and VatD may be capable of halogenating terminal alkyne substrates since nonbrominated jamaicamide and vatiamide analogs have been isolated previously with the terminal alkyne intact.[16,17] Furthermore, temporal studies on jamaicamide biosynthesis using ^{15}N-feeding and mass spectrometry also indicated jamaicamide B (**7**) was the substrate for a late-stage bromination reaction.[21] To test this hypothesis for jamaicamide biosynthesis *in vitro*, we extracted both jamaicamide B (**7**) and jamaicamide A (**4**) from *Moorena producens* cultures for assays and heterologously expressed and purified JamD from *Escherichia coli* (Figures S1 and S2).[16] We tested the activity of JamD with jamaicamide B (**7**), supplementary FAD, potassium bromide, and NADPH generated *in situ* using the PtdH phosphite dehydrogenase NADP$^+$ recycling system.[22,23] To our delight, we observed bromination of jamaicamide B (**7**) by LC-MS and confirmed the identity of a single product to be jamaicamide A (**4**) by direct comparison with a verified standard (Figure 3). These results demonstrated that JamD not only directly brominates a terminal alkyne but also performs the reaction without the addition of an external redox partner. Significantly, this represents the first example of the enzyme-mediated halogenation of a terminal alkyne to yield a haloalkyne.

To gain insight into the chemoselectivity of JamD, we compared its reactivity to that of AetF and a well-studied vanadium-dependent bromoperoxidase from the red alga *Corallina officinalis* (Co-VBPO).[24−26] By comparing substrates typically accepted by each enzyme, L-tryptophan (**1**) for AetF,[8] phenol (**8**) for Co-VBPO,[27,28] and jamaicamide B (**7**) for JamD,[16] we anticipated that patterns in reactivity and substrate preference would emerge (Figure 4a). Vanadium-dependent

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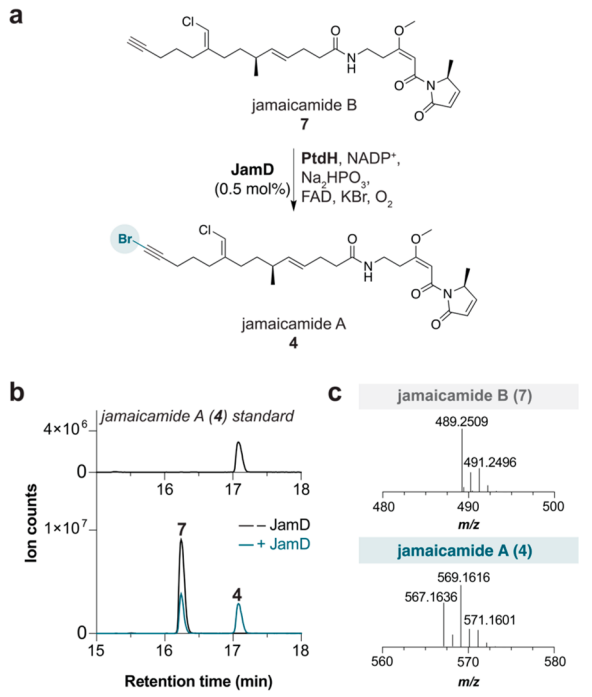


Figure 3. Demonstration of JamD reactivity. (a) Scheme of JamD reaction with jamaicamide B (**7**) to yield jamaicamide A (**4**). (b) LC-MS trace showing the extracted ion chromatograms (EICs) of jamaicamide B (**7**, $[M + H]^+$ = 489.2515) and jamaicamide A (**4**, $[M + H]^+$ = 567.1620) compared to an authentic standard of jamaicamide A (**4**). (c) MS isotope patterns of jamaicamide B (**7**) and jamaicamide A (**4**).

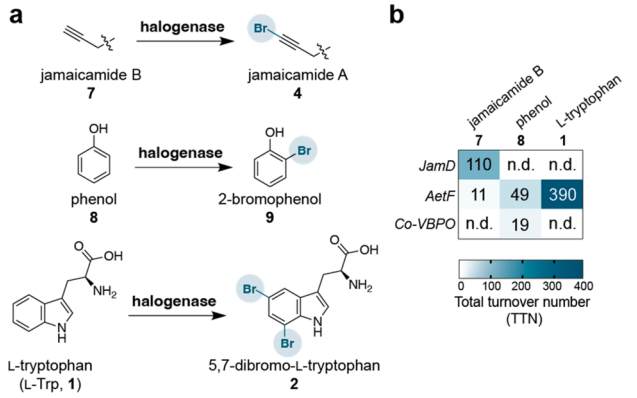
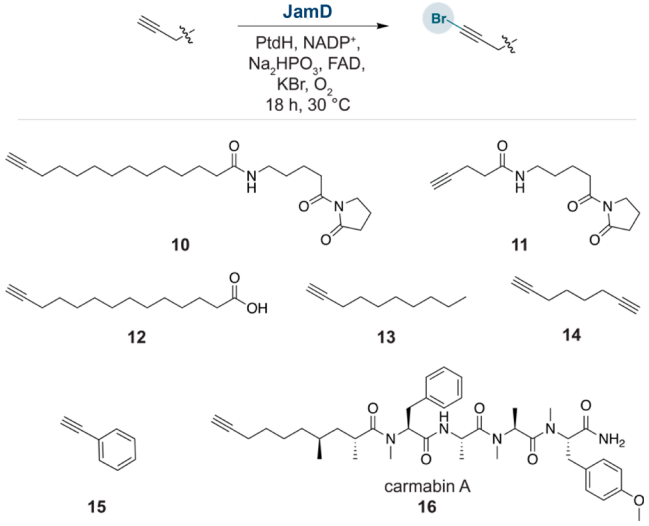


Figure 4. JamD reaction profiling. (a) Halogenase reactions for chemoselectivity assessment. (b) Heatmap of total turnover numbers (TTNs) of Co-VBPO, AetF, and JamD with phenol (**8**), L-tryptophan (**1**), and jamaicamide B (**7**). TTN is calculated based on the number of catalytic cycles (i.e., bromination events) to generate the indicated product relative to the molar quantity of enzyme used. n.d. = not detected.

haloperoxidases like Co-VBPO are capable of generating the same reactive brominating species as in FDHs, hypobromous acid (HOBr); however, the species is released into solution to react with nucleophilic organic molecules rather than engaging in active-site-controlled halogenation.[29] We hypothesized that free HOBr should not be solely responsible for terminal alkyne bromination in jamaicamide B (**7**), and therefore, alkynyl bromination by Co-VBPO should not be observed. Similarly, we anticipated that phenol (**8**) could be used to detect the uncontrolled reactivity of HOBr by monitoring the generation of 2-bromophenol (**9**) as a favored product of electrophilic aromatic substitution.

We heterologously expressed and purified Co-VBPO from *E. coli* and verified the HOBr-generating activity using the standard monochlorodimedone (MCD) assay for VBPO activity (Figure S5).[30] We then subjected jamaicamide B (**7**) to Co-VBPO and observed no halogenation of the substrate (Figure 4b, Figure S6). AetF was able to brominate jamaicamide B (**7**) to yield jamaicamide A (**4**), albeit with a 10-fold reduction in turnovers compared with JamD (Figure 4b, Figure S6). Conversely, JamD and Co-VBPO were unable to halogenate L-tryptophan (**1**) (Figure 4b, Figure S8). Phenol (**8**) was brominated effectively by Co-VBPO and AetF to produce 2-bromophenol (**9**), but no product was detectable in the reaction with JamD (Figure 4b, Figure S7). This assessment revealed that there is a unique property of JamD that enables chemoselective alkyne halogenation that is not permitted by enzymes such as Co-VBPO and AetF.

We next probed the minimal substrate requirements for the JamD reaction. First, we synthesized a simplified version of jamaicamide B (**7**) with many chemical complexities of the natural product removed (**10**, Figure 5). In the reaction with **10**, we observed that JamD could brominate the simplified scaffold (Figure 5, Figures S9 and S10). We also synthesized a shorter alkynyl substrate, **11**, and observed complete



Substrate	Enzyme Loading (mol%)	% Conversion (LC- or GC-MS)	Product Isolated Yield
10	5	n.a.*	33%
11	5	>99%	19%
12	5	>99%	88%
13	0.5	>99%	n.a.†
14	0.5	>99%‡	n.a.†
15	0.5	>99%	n.a.†
16	5	>99%	36%

Figure 5. Simple and complex alkynes accepted by JamD. Reactions with substrates **10**–**12** and **16** were analyzed by LC-MS, products were isolated and characterized by NMR, and the reactions with substrates **13**–**15** were analyzed by GC-MS compared to synthetic product standards. *Percent conversion n.a. (not available) due to irregular precipitation. †Product isolated yield n.a. (not available) due to volatility. ‡Dibromination observed exclusively.

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consumption of the substrate by LC-MS, motivating us to test even simpler substrates to find the minimum substrate requirements (Figure 5, Figures S11 and S12). JamD also readily accepted the long chain alkyne precursor in our synthesis of **10**, 13-tetradecynoic acid (**12**), and to our surprise efficiently accepted simple hydrocarbons like 1-decyne (**13**), 1,7-octadiyne (**14**), and phenylacetylene (**15**) (Figure 5, Figures S13, S14, and S16−S18). The structures of each of the bromoalkyne products of these reactions were verified by characterizing the isolated biocatalytic products by NMR (substrates **10**−**12** and **16**) or by comparison to a synthesized product standard by GC-MS (substrates **13**−**15**). Limitations associated with the volatility of **13**−**15** and their corresponding brominated products under aqueous reaction conditions prevented efficient scale-up and isolation, despite the apparent complete conversion of substrate to product.

We also tested the activity of JamD with a complex natural product from our compound repository containing both a terminal alkyne and phenylic residues, carmabin A (**16**),[31] which does not have a natural brominated counterpart (Figure 5). Satisfyingly, JamD readily accepted carmabin A (**16**), emphasizing the chemoselective preference and broad substrate tolerance of JamD (Table S2 and Figure S15). To determine whether the terminal alkyne moiety was essential for reactivity, we tested the terminal alkene 1-dodecene (**S10**) and the internal alkyne 5-decyne (**S12**), observing no reaction with either substrate, indicating the exclusive preference of JamD for terminal alkynes (Figures S19 and S20).

Since natural jamaicamide analogs containing iodine instead of bromine, such as jamaicamide F (**S14**), have been reported previously, we anticipated that JamD may also be able to iodinate jamaicamide B (**7**).[32] Such halide flexibility was also recently reported for AetF in performing iodination as well as bromination reactions.[13] Substituting potassium iodide for potassium bromide, we performed the JamD reaction in the presence of jamaicamide B (**7**) and did indeed observe a single iodinated product with the corresponding exact mass of jamaicamide F (**S14**) and retention time similar to that of jamaicamide A (Figure S21). Furthermore, JamD appeared to prefer bromination over iodination, and no reaction was observed with the enzyme in the presence of sodium chloride (Figure S21).

This initial substrate and enzyme analysis indicates that the reactivity of JamD on terminal alkynes is specific and that the mechanism is likely distinct from that of other known FDHs that operate on aromatic substrates. Since PhmJ shows significant amino acid sequence similarity with JamD, we tested its potential for alkynyl bromination as well.[18] To our surprise, PhmJ was able to produce jamaicamide A (**4**) under similar reaction conditions, even though the associated natural product, phormidolide (**5**), does not contain a terminal alkyne (Figure S22).[18] Phormidolide (**5**) instead contains a terminal bromoalkene with an appended vinylic methoxyl group, and perhaps this structure provides a hint of its mechanism of formation, although no terminal alkyne version of phormidolide (**5**) has yet been identified.

To summarize, we discovered a new reaction in enzyme chemistry that is mediated by alkynyl halogenase JamD, which is a member of a new and emerging class of FDHs. We revealed the activity of JamD in its associated biosynthetic pathway using the natural product jamaicamide B (**7**). We found that JamD is capable of directly halogenating alkyne substrates of varying complexity with a distinct chemoselective preference for terminal alkynes, suggesting its promise as a biocatalyst. We anticipate that JamD and its homologues have the potential to be powerful additions to the biocatalytic repertoire for halogenation, enabling the specific activation of alkynes under environmentally benign conditions. Further studies will be focused on characterizing the breadth of reactivity among homologues and applying these enzymes in biocatalysis as well as gaining structural and mechanistic insights into the nature of enzymatic alkyne halogenation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c05750.

General procedures, experimental details, supplemental figures, and NMR spectra (PDF)

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The manuscript was written through contributions of all authors.

Notes

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■ ABBREVIATIONS

BGC, biosynthetic gene cluster; EFI-GNT, Enzyme Function Initiative-Genome Neighborhood Tool; EIC, extracted ion chromatogram; FAD, flavin adenine dinucleotide; FDH, flavin-dependent halogenase; GC-MS, gas chromatography mass spectrometry; GMC, glucose-methanol-choline; HOBr, hypobromous acid; LC-MS, liquid chromatography mass spectrometry; MCD, monochlorodimedone; ML, maximum likelihood; NADPH, nicotinamide adenine dinucleotide phosphate hydride; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; SSN, sequence similarity network; TTN, total turnover number; VBPO, vanadium-dependent bromoperoxidase

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