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Vanadium bromoperoxidase from *Delisea pulchra*: enzyme-catalyzed formation of bromofuranone and attendant disruption of quorum sensing†

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Vanadium bromoperoxidase was isolated and cloned from the marine red alga *Delisea pulchra*. This enzyme catalyzes the bromolactonization of 4-pentynoic acid forming 5*E*-bromomethylidenetetrahydro-2-furanone, a compound which is shown herein to inhibit quorum sensing in the engineered reporter strain, *Agrobacterium tumefaciens* NTL4.

All surfaces in aquatic environments are susceptible to biofouling. Marine algae, which rely on photosynthesis to survive and thrive in the ocean, therefore must have evolved molecular mechanisms to limit microbial fouling of their surfaces to ensure adequate light exposure, as well as access to essential nutrients. The lack of significant microbial fouling of the marine red alga *Delisea pulchra* has intrigued marine ecologists for decades.[1,2] Of particular interest are several brominated furanones (Fig. 1A) isolated from the surface of *D. pulchra*,[3,4] which have been shown to inhibit bacterial quorum sensing.[5]

Quorum sensing is the process by which bacteria monitor cell density and regulate such phenotypic responses as biofilm formation, virulence, swarming motility and bioluminescence, among other responses.[6,7] Many bacteria secrete small signaling molecules, called autoinducers (AI) as their quorum sensing compounds.[8] The AI-1 system in Gram-negative bacteria is regulated by acyl homoserine lactones (HSL; Fig. 1B), variations of which are specific to distinct bacterial species. The AI-2 system is species nonspecific and is regulated by furanosyl compounds (Fig. 1B). The bromofuranones from *D. pulchra* have been shown to disrupt both the AI-1 and the AI-2 systems of quorum sensing by either displacing the AI-1 signaling molecules from their corresponding receptor (*e.g.* LuxR), or by covalently modifying and thus inactivating LuxS, the AI-2 producing enyme.[9–11]

Intriguingly, 3-oxo acyl homoserine lactones (Fig. 1B) are readily susceptible to electrophilic halogenation at the C2 position, as demonstrated not only by reaction with aqueous bromine (*i.e.*, $HOBr/OBr^-/Br_2$), but also by a putative haloperoxidase of *Laminaria digitata* (Scheme 1).[12,13] The bromoacyl HSL products have been shown to disrupt quorum sensing in engineered reporter strains of *Chromobacterium violaceum* and *Agrobacterium tumefaciens*.[13]

Haloperoxidase enzymes have been identified in many different marine algae.[14] We have previously shown that vanadium bromoperoxidase (V-BrPO) present in a variety of red algae catalyzes the biosynthesis of natural products.[15–17] V-BrPO functions as a Lewis acid catalyst of bromide oxidation by H_2O_2, effecting electrophilic halogenation reactions. Given that *D. pulchra* produces biologically active bromofuranones, we have been interested in whether this alga has a haloperoxidase and if so, whether this enzyme is involved in the biosynthesis of bromofuranones, as well as in the bromination of 3-oxo-acylhomoserine lactones.

We report herein the isolation, cloning and expression of vanadium bromoperoxidase (V-BrPO) from *D. pulchra*. We demonstrate, for the first time, the V-BrPO-catalyzed synthesis of a bromofuranone. This bromofuranone also disrupts quorum sensing in the reporter strain *A. tumefaciens* NTL4. In addition, the *D. pulchra* V-BrPO catalyzes the bromination of 3-oxo acyl HSL signaling molecules.

Addition of whole pieces of the alga *D. pulchra* (1–2 mg) to a solution of phenol red (100 μM) and bromide (100 mM) in phosphate buffer (0.15 M, pH 6) results in the bromination of phenol red to produce bromophenol blue upon addition of H_2O_2 (1–2 mM) (Fig. 2). In control experiments in the absence of the alga, bromophenol blue is not formed. Thus this haloperoxidase is present at or near the surface of the alga. In addition, and consistent with previous observations for the

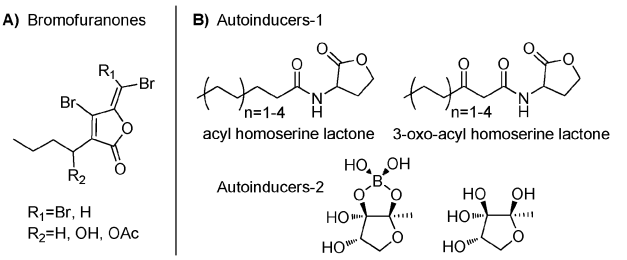

Fig. 1 (A) Brominated furanone natural products isolated from *D. pulchra*. (B) Selected examples of autoinducers, AI-1 and AI-2.

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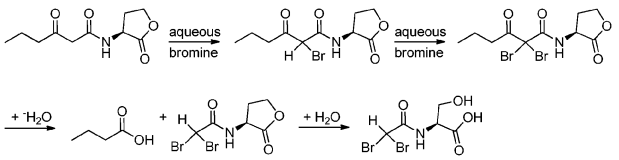


Scheme 1 Bromination of 3-oxo-hexanoyl homoserine lactone and subsequent hydrolysis of the dibromo product.[12] The corresponding chlorinated products also disrupt quorum sensing.[13]

Fig. 2 Bromination of phenol red to bromophenol blue by whole algal pieces of *D. pulchra*. 3 min after addition of hydrogen peroxide. At pH 6 phenol red appears yellow.

brown alga *L. digitata*,[13] whole pieces of *D. pulchra* catalyze the bromination of 3-oxo-hexanoylhomoserine lactone, forming the dibromo-3-oxo-hexanoylhomoserine lactone (ESI,† Fig. S1).

We next cloned and sequenced the *D. pulchra* haloperoxidase. Two different clones from *D. pulchra* were obtained, labelled DpDV-BrPO and DpEV-BrPO, designating clones D and E. The DpDV-BrPO and DpEV-BrPO clones, as well as the *Corallina officinalis* and *Plocamium cartilagineum* V-BrPO clones,[18] are all 1794 nucleotides in length, and the open reading frame of each gene encodes a protein of 598 amino acids. The calculated molecular mass for each *D. pulchra* V-BrPO is 65,458 Da, consistent with the subunit molecular mass of other red algal V-BrPOs.[18]

Amino acid sequence alignment of DpDV-BrPO and DpEV-BrPO with the sequences of V-BrPO from red and brown algae[18] establish DpDV-BrPO as a vanadium bromoperoxidase (ESI,† Fig. S3), along with its reactivity (see below). The translated amino acid sequences for DpDV-BrPO, DpEV-BrPO, and the V-BrPO from red algae *C. officinalis*, *C. pilulifera* and *P. cartilagineum* show greater than 90% sequence identity and 100% conservation of all active site residues,[18] with one exception: in clone E from *D. pulchra*, DpEV-BrPO, the residue at position 553 is an arginine, whereas in clone D, DpDV-BrPO, residue 553 is a histidine. In fact, in *all* other vanadium haloperoxidases, the amino acid at this position is a histidine. (ESI,† Fig. S3). Moreover, it is the sole ligand coordinating to the vanadate ion, although hydrogen bonding from other side chains helps to stabilize the vanadate-protein complex.[14]

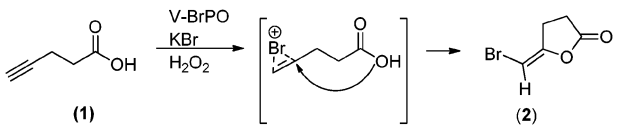
Following from our initial discovery that V-BrPOs from red algae catalyze the asymmetric bromination and cyclization of certain terpenes, including nerolidol, from which known marine natural products are formed,[16,17] we sought to investigate whether V-BrPO could also catalyze formation of bromofuranone compounds. We reasoned that 4-pentynoic acid could be a simple substrate that could undergo electrophilic bromolactonization, forming a bromofuranone. Reaction of 0.4 mM 4-pentynoic acid with purified V-BrPO (*D. pulchra* and *C. officinalis*) in the presence of bromide ion and hydrogen peroxide results in the production of 5*E*-bromomethylidenetetrahydro-2-furanone **2** (Scheme 2), as identified by mass spectrometry, *m*/*z* 176/178 M + H^+ and ^{1}H NMR (ESI,† Fig. S4 and S5).

Bromofuranone **2** was also synthesized chemically for comparison purposes following a previously reported procedure.[19] The reaction between 4-pentynoic acid and the Br^+-generating species NBS in dichloromethane resulted in a 25% yield of **2** after chromatographic purification. The ^{1}H NMR spectrum of **2** is consistent with the reported values (ESI,† Fig. S7).[19,20] The ^{13}C NMR spectrum of **2**, which was not reported previously, has 5 distinct carbon resonances corresponding to 1 carbonyl carbon (δ 174.3), 2 alkenyl carbons (δ 152. 6 and δ 85.0) and 2 methylene carbons (δ 29.4 and δ 24.7). The downfield position of the alkenyl methine carbon (δ 85.0) indicates that the bromine is bound to the β-carbon of the αβ-unsaturated carbonyl system (ESI,† Fig. S8). The chemical shift of the vinylic proton (δ 5.84) establishes the *E*-configuration of the double bond.[19,20]

The formation of bromofuranone **2** likely results from an initial V-BrPO-catalyzed bromination reaction at the terminal alkyne, followed by cyclization from intermolecular nucleophilic attack by the terminal hydroxyl group (Scheme 2), in accord with previous V-BrPO-catalyzed bromination and cyclization of terpenes.[17,21]

Bromofuranone **2** was tested for its ability to disrupt quorum sensing in the reporter strain *A. tumefaciens* NTL4 (pCF218)(pCF372).[22] In this engineered strain, β-galatosidase, expressed under quorum sensing conditions, catalyzes the hydrolysis of X-gal (5-bromo-4-chloro-3-indoyl-β-D-*galacto*-pyranoside), which leads to the formation of the blue-green compound, 5,5′-dibromo-4,4′-dichloro-indigo. Bromofuranone **2** was found to inhibit quorum sensing in *A. tumefaciens* NTL4 when 20 μL of 50 μM **2** was applied in 2 μL aliquots around the bacterial streak, as indicated by the off-white colour of the bacterial streak (Fig. 3a) and thus, the lack of β-galactosidase activity. Positive control reactions with the addition of 20 μL of 50 μM 3-oxo-hexanoyl homoserine lactone to the *A. tumefaciens* NTL4 streak showed the expected quorum sensing response as indicated by the blue-green colour (Fig. 3b).

Given that histidine is coordinated to vanadate in all V-BrPOs, we have begun to investigate the reactivity of the clones D and E to determine the importance of His at position 553. Recombinant DpDV-BrPO and DpEV-BrPO, as well as the site specific mutant R553H of DpEV-BrPO were expressed in *E. coli*. Bromoperoxidase activity of the DEAE purified recombinant proteins was monitored by the phenol red assay (Fig. 4). DpDV-BrPO and the site specific mutant R553H catalyze the bromination of phenol red to bromophenol blue, however the DpEV-BrPO (*i.e.*, R553) protein was not active,



Scheme 2 Proposed reaction sequence for the V-BrPO-catalyzed bromolactonization of 4-pentynoic acid.

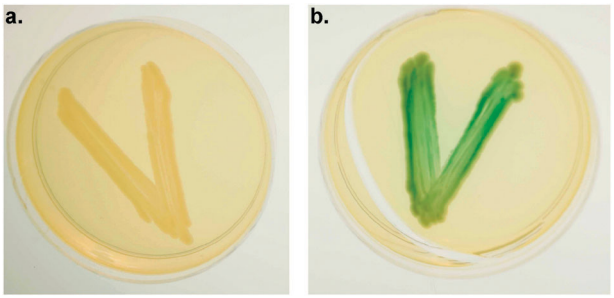


Fig. 3 Effect of bromofuranone **2** on quorum sensing in reporter strain *A. tumefaciens* NTL4. (a) 20 μL of 50 μM bromofuranone **2** in CH_2Cl_2 was added around the bacterial streak in 2 μL aliquots. (b) Control assay of *A. tumefaciens* NTL4 in which 20 μL of 50 μM 3-oxo-hexanoylhomoserine lactone dissolved in CH_2Cl_2 was added around the streak in 2 μL aliquots. Neither 4-pentynoic acid nor CH_2Cl_2 affect quorum sensing. Agar plates contained X-gal (ESI,† Materials and Methods).

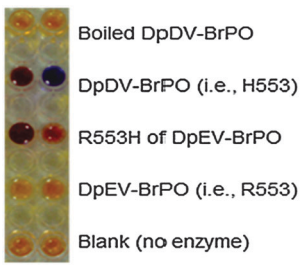


Fig. 4 Bromoperoxidase assay of recombinant DpDV-BrPO, DpEV-BrPO and the site specific construct, R553H of DpEV-BrPO. From top: boiled sample of DpDV-BrPO, recombinant DpDV-BrPO, site specific R553H mutant of DpEV-BrPO, recombinant DpEV-BrPO and the control without added enzyme. The assay consisted of 250 μL 0.1 M phosphate, pH 5.7 in a microtiter plate, with 60 μL 1 M KBr, 10 μL 5 mM phenol red, 50 μL recombinant protein sample, and 6.6 μL 0.2 M H_2O_2. Each well corresponds to a fraction collected from FPLC purification using a fresh DEAE cartridge in the purification of each recombinant protein sample to avoid possible contamination.

suggesting that the vanadium-ligated histidine is important for activity.

Further experiments are in progress to determine if vanadium is bound in the active site of the DpEV-BrPO (*i.e.*, R553) isoform, and thus whether the lack of haloperoxidase activity is a result of the absence of coordinated vanadium, or the absence of histidine-ligated vanadium.

In summary *Delisea pulchra* has evolved a double-pronged molecular approach to defend against microbial colonization using V-BrPO, which is active at the alga's surface, to brominate 3-oxo-acylhomoserine lactones, producing compounds that disrupt bacterial quorum sensing, as well as to biosynthesize bromofuranone compounds that also disrupt microbial quorum sensing. V-BrPO catalyzes the bromolactonization of 4-pentynoic acid **1** forming 5*E*-bromomethylidenetetrahydro-2-furanone **2**, a bromofuranone that disrupts quorum sensing in *A. tumefaciens* NTL4. Quorum sensing inhibition is not affected by the lack of unsaturation within the furanone ring of **2**, a structural feature which differentiates **2** from the naturally occurring *D. pulchra* bromofuranones (Fig. 1A), as the synthetic bromofuranone **2** is effective at disrupting quorum sensing in *A. tumefaciens* NTL4. While the precursors for the naturally occurring *D. pulchra* bromofuranones are still unknown, the ability of V-BrPO to direct the bromolactonization of 4-pentynoic acid in the synthesis of **2** expands the arsenal of chemical analogues known to act as antagonists of bacterial quorum sensing.

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Notes and references

1 R. de Nys, P. D. Steinberg, P. Willemsen, S. A. Dworjanyn, C. L. Gabelish and R. J. King, *Biofouling*, 1995, **8**, 259–271.
2 R. Maximilien, R. de Nys, C. Holmstrom, L. Gram, M. Givskov, K. Crass, S. Kjelleberg and P. D. Steinberg, *Aquat. Microb. Ecol.*, 1998, **15**, 233–246.
3 R. de Nys, S. A. Dworjanyn and P. D. Steinberg, *Mar. Ecol.: Prog. Ser.*, 1998, **162**, 79–87.
4 S. A. Dworjanyn, R. De Nys and P. D. Steinberg, *Mar. Biol.*, 1999, **133**, 727–736.
5 P. D. Steinberg, R. de Nys and S. Kjelleberg, in *Marine Chemical Ecology*, ed. J. B. McCLintock and B. J. Baker, CRC Press LLC, Boca Raton, Editon edn, 2001, pp. 355–387.
6 A. Camilli and B. L. Bassler, *Science*, 2006, **311**, 1113–1116.
7 W. R. J. D. Galloway, J. T. Hodgkinson, S. D. Bowden, M. Welch and D. R. Spring, *Chem. Rev.*, 2011, **111**, 28–67.
8 Y. J. Wang, J. J. Huang and J. R. Leadbetter, *Adv. Appl. Microbiol.*, 2007, **61**, 27–58.
9 M. Manefield, R. de Nys, N. Kumar, R. Read, M. Givskov, P. Steinberg and S. A. Kjelleberg, *Microbiology*, 1999, **145**, 283–291.
10 D. C. Ren, L. A. Bedzyk, R. W. Ye, S. M. Thomas and T. K. Wood, *Biotechnol. Bioeng.*, 2004, **88**, 630–642.
11 T. Zang, B. W. K. Lee, L. M. Cannon, K. A. Ritter, S. Dai, D. Ren, T. K. Wood and Z. S. Zhou, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 6200–6204.
12 J. J. Michels, E. J. Allain, S. A. Borchardt, P. Hu and W. F. McCoy, *J. Chromatogr., A*, 2000, **898**, 153–165.
13 S. A. Borchardt, *Appl. Environ. Microbiol.*, 2001, **67**, 3174–3179.
14 A. Butler and M. Sandy, *Nature*, 2009, **460**, 848–854.
15 A. Butler and J. N. Carter-Franklin, *Nat. Prod. Rep.*, 2004, **21**, 180–188.
16 J. N. Carter-Franklin and A. Butler, *J. Am. Chem. Soc.*, 2004, **126**, 15060–15066.
17 J. N. Carter-Franklin, J. D. Parrish, R. A. Tschirret-Guth, R. D. Little and A. Butler, *J. Am. Chem. Soc.*, 2003, **125**, 3688–3689.
18 J. N. Carter, K. E. Beatty, M. T. Simpson and A. Butler, *J. Inorg. Biochem.*, 2002, **91**, 59–69.
19 G. A. Krafft and J. A. Katzenellenbogen, *J. Am. Chem. Soc.*, 1981, **103**, 5459–5466.
20 W. Dai and J. A. Katzenellenbogen, *J. Org. Chem.*, 1991, **56**, 6893–6896.
21 J. M. Winter and B. S. Moore, *J. Biol. Chem.*, 2009, **284**, 18577–18581.
22 J. Zhu, J. W. Beaber, M. I. More, C. Fuqua, A. Eberhard and S. C. Winans, *J. Bacteriology*, 1998, **180**, 5398–5405.

