Vanadium Bromoperoxidase from *Delisea pulchra*: Enzyme-catalyzed formation of Bromofuranone and Disruption of Quorum Sensing

Moriah Sandy, Jayme N. Carter-Franklin, Jessica D. Martin, and Alison Butler*

Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA 93106-9510

SUPPORTING INFORMATION CONTENTS:

Figure S1. APCI mass spectrum of the sodium adduct of α , α -dibrominated-3-oxo-hexanoyl-homoserine lactone (4) formed at pH 6.

Figure S2. Effect of bromo-3-oxo-hexanoylhomoserine lactone on bioactivity with quorum sensing reporter strain *A. tumefaciens* NTL4.

Figure S3. Amino acid sequence alignment of DpDV-BrPO and DpEV-BrPO with V-BrPOs from red and brown algae.

Figure S4. GC-MS of 5*E*-bromomethylidenetetrahydro-2-furanone (2) synthesized enzymatically with V-BrPO

Figure S5. ¹H NMR spectrum of 5*E*-bromomethylidenetetrahydro-2-furanone (2) synthesized enzymatically with V-BrPO

Figure S6. GC-MS chromatogram and mass spectrum of bromofuranone 2 synthesized chemically.

Figure S7. ¹H NMR spectrum of bromofuranone 2 synthesized chemically.

Figure S8. ¹³C NMR spectrum of bromofuranone 2 synthesized chemically.

Table S1. Quorum sensing response of *A. tumefaciens* in the presence of varying amounts of bromofuranone **2**

Materials and Methods

Figure S1. APCI mass spectrum of the sodium adduct of α , α -dibrominated-3-oxohexanoylhomoserine lactone (4) formed at pH 6. Product 4 was generated by the bromination of 3-oxo-hexanoylhomoserine lactone (50 µM) by whole pieces of *D. pulchra* upon addition of 100 mM KBr in 0.15 sodium phosphate buffer (pH 6.0), and 1 – 2 mM H₂O₂. The products of this reaction inhibit quorum sensing in *A. tumifaciens*, consistent with a previous literature report (Fig. S2, below).¹



Figure S2. Effect of bromo-3-oxo-hexanoylhomoserine lactone on bioactivity with quorum sensing reporter strain *A. tumefaciens* NTL4. a) Growth of *A. tumefaciens* NTL4 in the presence of 20 μ L of the V-BrPO-catalyzed reaction mixture of bromo-3-oxo-hexanoylhomoserine lactone formed from reaction of 50 μ M 3-oxo-hexanoyhomoserine lactone, 40 mM KBr, and 1M H₂O₂, in 0.15M phosphate buffer at pH 6 with 2 % XGAL. The cream color of the bacterial streak is due to lack of β-galactosidase activity, and indicates that quorum sensing in *A. tumefaciens* NTL4 has been disrupted. b) Control reaction of growth of *A. tumefaciens* NTL4 in the presence of the reaction mixture of 50 μ M 3-oxo-hexanoyhomoserine lactone, 40 mM KBr, and 1M H₂O₂, 0.15M phosphate buffer at pH 6 with 2 % XGAL, without added V-BrPO. In b) the quorum-sensing response to AHL is indicated by the formation of the blue greed coloration, which results from expression of β-galactosidase activity *A. tumefaciens* NTL4 under quorum sensing has been disrupted and that β-galactosidase is not expressed.



Figure S3. Amino acid sequence alignment of DpDV-BrPO and DpEV-BrPO with V-BrPOs from red (*C. officinalis, C. pilulifera, P. cartilagineum, D. pulchra*) and brown algae (*A. nodosum, F. distichus*). The sequences obtained from GenBank were aligned using ClustalW Multiple Alignment program and displayed using GeneDoc. Sequence identity analysis was performed using GeneDoc. *C. officinalis* translated from nucleotide sequence, accession number AF218810, *C. pilulifera*-1, accession number BAA31261; *C. pilulifera*-2, accession number BAA31262; *A. nodosum* V-BrPO, accession number 1QI9A-B; *Fucus distichus* V-BrPO, accession number AAC35279; *P. cartilagineum, D. pulchra*-E (Clone E), *D. pulchra-D* (clone D). Shown in black are amino acids that are conserved 100% between all V-BrPOs. *D. pulchra* clones D and E differ in 3 amino acids: H553 and R553, respectively and at positions 302 and 545 indicated by the red arrows.

C.officinalis	1		1	-	
A.nodosum	:			-	
C.pilulifera-2	:		:	-	
C.pilulifera-1	:			-	
F.distichus	:	MLCHAADTTRGSPMPDTGVLRLLTSEQRAKGWRRQLEGEKSLGFHPSETPYIKYLEGSET		60	
P.cartilagineum	:			-	
D.pulchra-E	:			-	
D.pulchra-D	:		:	-	
C.officinalis	:	***************************************	1	-	
A.nodosum	1	***************************************	1	-	
C.pilulifera-2	:		:	-	
C.pilulifera-1	:	***************************************	:	-	
F.distichus	:	WKKVKLPTDGISASKILGKIMARVRIATALAVVLAAPCLAFDEVTASGVFPEEHKHTGEG		120	
P.cartilagineum	:		:	-	
D.pulchra-E	:			-	
D.pulchra-D	1			-	

C.officinalis	1	-MGIPADNLOSRAKASFDTRVSAAELALARGVVPSLANGEELLYRNPDPENGDPSFIVSF	:	59
A.nodosum	:	XTCSTSDDADDPAPPNERDDEAFASRVAAAKRELEGTGTVCQINNCETDLAAKF		54
C.pilulifera-2	:	-MGIPADNLQSRAKASFDTRVAAAELALARGVVPSFANGEELLYRNCETGDPSFIASF		57
C.pilulifera-1	:	-NGIPADNLQSRAKASFDTRVAAAELALNRGVVPSFANGEELLYRNPDPDNDDPSFIASF		59
F.distichus	:	RHLOTCT SDDALDPAAPN RDNVAFASR DAARRER CTGT COIT NCETDLATMS		177
P.cartilagineum	1	-MGIPADNLQSRAKASFDTRVSAAELALARGSVPSTANGEELLYRNPDPDNDDPSFIASF		59
D.pulchra-E	:	-NGIPADNLONRAKASFDTRVSAAELALARGVVPSTANGEELPYRDPDPENGDPSFIASF		59
D.pulchra-D	:	-MGIPADNLONRAKASFDTRV <mark>S</mark> AAELAL <mark>A</mark> RG <mark>VVPSF</mark> ANGEEL <mark>P</mark> YRD <mark>PDPE</mark> NGDPSFIASF	:	59

C.officinalis	:	TKGLPHDDNGAIIDPDDFLAFVRAINSGDEKEIADLTLC-PARDPDTGLPIWRSDLANSL		118
A.nodosum	:	HKSLPHDDLG-QVDADAFAALEDCILNGDLSICEDVPVGNSEGDPVG		100
C.pilulifera-2	:	TKGLPHDDNGATIDPDDFLAFVRAINSGDEKEIADLTLG-PARDPPTGLPIWRSDLANSL	:	116
C.pilulifera-1	:	TKGLPHDDNGATIDPDDFLAFVRAINSGDEKEIADLTLG <mark>-</mark> PARDP <mark>E</mark> TGLPIWRSDLANSL	:	118
F.distichus	:	BESHOHDELG-QVTADDBAILEDCILNGDFSICEDVPAGDPAG	:	219
P.cartilagineum	:	TKGLPHDDNGAIIDPDDFLAFVRAINSGDEKEIADLTLG-PARDPDTGLPIWRSDLANSL	:	118
D.pulchra-E	1	TKGLPHDDNGAIIDPDDFLAFVRAINSGDEKEIADLTLG <mark>-</mark> PARDP <mark>D</mark> TGLPIWRSDLANSL	:	118
D.pulchra-D	•	TKGLPHDDNGAIIDPDDFLAFVRAINSGDEKEIADLTLG <mark>-</mark> PARDP <mark>D</mark> TGLPIWRSDLANSL	:	118

C.officinalis	:	ELEVRGN NSSAGLTFDLEGPDAQSIAMPPAPVLTSPELTAEIAELYLMALCREIEFSEF	:	178
A.nodosum	:	RLVNPTAAFAIDISGPAFSATTIPPVPTLPSPELAAQLAEVYMMALARDVPFYQY	:	155
C.pilulifera-2	:	ELEVRGW NSSAGLTFDLEGPDAQSVAMPPAPVLISPELTAEMAELYLMALGRDIEFSEF	:	176
C.pilulifera-1	:	ELEVRGW NSSAGLTFDLEGPDAQSIAMPPAPVLTSPELVAEIAELYLMALOR <mark>B</mark> IEFSEF	:	178
F.distichus	:	RLVNPTAAPAIDISGPAPSATTIPPVPTLESPELAAQLAELYMALARDVFFYQY	:	274
P.cartilagineum	:	ELEVRGW-NESAGLTFDLEGPDAQSIAMPPAPVLTSPELIAEIAELYLMALGR-IEFSEF	:	178
D.pulchra-E	:	ELEVRGW <mark>ANSSAGLTFDLEGPDAQSIAMPPAPVLTSPELIAEIAELYLMALGR</mark> EIEFSEF		178
D.pulchra-D	:	elevrgw <mark>a</mark> nssagltfdlegpdaqsiamppapvltspeltaeiaelylmalgr <mark>e</mark> iefsef	:	178

4

C.officinalis A.nodosum C.pilulifera-2 C.pilulifera-1 F.distichus P.cartilagineum D.pulchra-E D.pulchra-D	: DSPKNAEYIQFAIDQLNGLEWFNTPAMLGDPPAEIRRRGEVTVGNLFRGILPGSEVGPY : GTDDITVTAAANLAGMEGFPNLDAVSIGSDGTVDPLSQLFRATFVGVETGPF : ESPKNAEYIQFAIDQLNGLEWFNTPAKLGDPPAEIRRRGEVTVGNLFRGILPGSEVGPY : DSPKNAEYIQFAIDQLNGLEWFNTPAKLGDPPAEIRRRGEVTVGNLFRGILPGSEVGPY : DSPKNAEYIQFAIDQLNGLEWFNTPAKLGDPPAEIRRRGEVTVGNLFRGILPGSEVGPY : DSPKNAEYIQFAIDQLNGLEWFNTPAKLGDPPAEIRRRGEVTVGNLFRGILPGSEVGPY : DSPKNAEYIQFAIDQLNGLEWFNTPAKLGDPPAEIRRRGEVTVGNLFRGILPGSEVGPY	: : : : : : : : : : : : : : : : : : : :	238 207 236 238 326 238 238 238
C.officinalis A.nodosum C.pilulifera-2 C.pilulifera-1 F.distichus P.cartilagineum D.pulchra-E D.pulchra-D	: LSQYIIVGSKQIGSAT <mark>CON</mark> KTLVSPNAADEFDGEIAYGSITISQRVRIATPGRDFMTDLK : ISQLVNSFTIDSITVEPKQETFAPDVNYMVDFD : LSQYIIVGSKQIGSATVCNKTPVSPNAADEFDGEIAYGSITISQRVRIATPGRDFMTDLK : VSQLIVGSKQIGSATVCNKTPVSPNAADEFDGEIAYGSITISQRVRIATPGRDFMTDLK : VSQLIVNSFTIDATTVEPKQETFAPDLNYMVDFD : LSQYIIVGSKQIGSATVCSKTLVSPNAADEFDGEIAYGSITISQRVRIATPGRDFMTDLK : LSQYIIVGSKQIGSATVCSKTLVSPNAADEFDGEIAYGSITISQRVRIATPGRDFMTDLK : LSQYIIVGSKQIGSATVCSKTLVSPNAADEFDGEIAYGSITISQRVRIATPGRDFMTDLK	:::::::::::::::::::::::::::::::::::::::	298 241 296 298 360 298 298 298
C.officinalis A.nodosum C.pilulifera-2 C.pilulifera-1 F.distichus P.cartilagineum D.pulchra-E D.pulchra-D	: VFLDVQDAADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN : EWLNIQNGGPPAGPELLDDELRFVRNARDLARVTFTDNINTEAYRGALILLGLDAFNRAG : VFLDVQDGADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANGVPFDPN : VFLDVQDAADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANGVPFDPN : EWLNIQNGGPPAGPEELDEELRFIRNARDLARVSFVDNINTEAYRGSLILLELGAFSRFG : VFLDVQDAADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN : VFLDVQDADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN : VFLDVQDADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN : VFLDVQDADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN : VFLDVQDADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN : VFLDVQDADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN : VFLDVQDADFRGFESVEPGARLIRTIRDLATWVHFDALVEAYLNACLILLANRVPFDPN	: : : : : : :	358 301 356 358 420 358 358 358
C.officinalis A.nodosum C.pilulifera-2 C.pilulifera-1 F.distichus P.cartilagineum D.pulchra-E D.pulchra-D	 IPFQQEDKLDNQDVFVNFGDAHVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISV VNGPFID-IDROAGFVNFGISHYFRLIG-AAELAORSSWYQKWQVHRFARPEALGGTLHL LPFQQEDKLDNHDVFVNFGSAHVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISV LPFQQEDKLDNQDVFVNFGSAHVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISV INGPFID-SDROAGFVNFGTSHYFRLIG-AAELAORASCYQKWQVHRFARPEALGGTLHN IPFQQECKLDNQDVFVNFGDAHVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISV IPFQQEDKLDNQDVFVNFGDAHVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISV IPFQQEDKLDNQDVFVNFGDAHVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISV 		418 359 416 418 478 418 418 418
C.officinalis A.nodosum C.pilulifera-2 C.pilulifera-1 F.distichus P.cartilagineum D.pulchra-E D.pulchra-D	 NKIAAEKG-ESVFPEVDLAVEELEDILEKAEISNRKONIADGDPDPDPSFLLPOAFAEGS TIKGE-LNADFDLSLENAELLKRVAAINAAONPNNEVTYLLPOAIQEGS NKKSFLAGSDIIFPEVSELVEELSSILDDVAESNEKONRADGIVSPDKSFLLPMAFAEGS NKIAPQKG-ESIFPEVDLAVEELGDILEKAEISNRKONIADGDPDPDPSFLLPMAFAEGS TIAGD-LDADFDISLENDELLKRVAEINAAONPNNEVTYLLPOAIQVGS NKIAAEKG-ESVFPEVDLAVEELGDILEKAEISNRKONIADGDPDPDPSFLLPOAFAEGS NKIAAEKG-ESVFPEVDLAVEELGDILEKAEISNRKONIADGDPDPDPSFLLPOAFAEGS NKIAAEKG-ESVFPEVDLAVEELGDILEKAEISNRKONIADGDPDPDPSFLLPOAFAEGS NKIAAEKG-ESVFPEVDLAVEELGDILEKAEISNRKONIADGDPDPDPSFLLPOAFAEGS NKIAAEKG-ESVFPEVDLAVEELGDILEKAEISNRKONIADGDPDPDPSFLLPOAFAEGS 		477 408 476 477 527 477 477

C.officinalis A.nodosum C.pilulifera-2 C.pilulifera-1 F.distichus P.cartilagineum D.pulchra-E D.pulchra-D		PFHPSYGSGHAVVAGACVTILKAFFDSN-FQIDQVFEVDKDEDKLVKSSFKG-TLTVAGE PTHPSYPSGHATQNGAFATVLKALIGLDRGGDCYPDPVYPDDDGLKLIDFRGSCLTFEGE PFHPSYGSGHAVVAGACVTILKAFFDAN-FQIDKVFEVDTDEDKLVKSSFKG-TLTVAGE PFHPSYGSGHAVVAGACVTILKAFFDSG-IEIDQVFEVDKDEDKLVKSSFKG-TLTVAGE PTHPSYGSGHATQNGAFATVLKALIGLDRGGECFPNPVFPSDDGLELINFEGACLTYEGE PFHPSYGSGHAVVAGACVTILKAFFDSN-FQIDQVFEVDKDEDKLVKSSFKG-TLTVAGE PFHPSYGSGHAVVAGACVTILKAFFDSN-FQIDQVFEVDKDEDKLVKSSFKG-TLTVAGE PFHPSYGSGHAVVAGACVTILKAFFDSN-FQIDQVFEVDKDEDKLVKSSFKG-TLTVAGE	:::::::::::::::::::::::::::::::::::::::	535 468 534 535 587 535 535 535
C.officinalis	:	LNKLADNIAIGRNMAGVHYFSDOFESILLGEOVAIGILEEOSLTYGENFFFNLPKFDGTT	:	595
A.nodosum		INKLAWNVAFGROMLGIHYRFDGIOGLULGETITWRTLHOELMTFAFESTFEFRLFTGEV		528
C.pilulifera-2		LNKLADNVALGRNMAGVHYFSDOFFSLLLGEOTATGTLEEOSLTYGENEFFNLBKFDCTT		594
C nilulifera-1		LNKLADNTAT CONVACUEVESDOFESTILL CEQUATOTI FEOST TVCENEFENT DZEDOFT	:	505
E distichus	:		:	535
F.disticnus	•	INKIANNVAFGROUIGITTIKFDGIQGHIHGETTIVKTIHQEDWUTABEATHEFRIHTGEV	•	547
P.cartilagineum	•		:	595
D.pulchra-E	:	LNKLADNIA TORNWAGVRYFSDQFESILLGEQVAIGILEEQSLTYGENFFFNLPKFDGTT	:	595
D.pulchra-D	:	LNKLADNIAICRNAAGVHYF5DQFESILLGEQVAIGILEEQSLTYGENFFFNLPKFDGTT	:	595
		545 553		
C.officinalis	:	IQI : 598		
A.nodosum	:	IKLFQDGTFTIDGFKCPGLVYTGVENCV- : 556		
C.pilulifera-2	:	101 : 597		
C.pilulifera-1	:			

 F.distichus
 : IKLFQDGTFSIDGDMCSGLVYTGVADCQA : 676

 P.cartilagineum
 : ICIXX------ : 600

 D.pulchra-E
 : ICI----- : 598

 D.pulchra-D
 : ICI

Figure S4. GC-MS Mass spectrum of 5*E*-bromomethylidenetetrahydro-2-furanone synthesized enzymatically with V-BrPO (parent ion m/z 176/178 M+H⁺). 4-Pentynoic acid (1) (10 mM) was pre-dissolved in a small volume of ethanol and added to 0.15 M sodium phosphate buffer (pH 6.0), 100 mM KBr. Enzymatic reactions containing 40 nM partially purified V-BrPO from *D*. *pulchra* were initiated by addition of 2 mol equivalents of H₂O₂ in relation to 4-pentynoic acid (1) concentration via syringe pump (1 equivalent per hour).



Figure S5. ¹H NMR spectrum of 5*E*-bromomethylidenetetrahydro-2-furanone (2) synthesized enzymatically. In addition to the product, bromofuranone 2, marked as resonances 2, 3 and 5, the starting material, 4-pentynoic acid, is also present in the crude reaction mixture and marked as resonances 1a, 2a, 3a and 4a.



Figure S6. GC-MS chromatogram (top) and mass spectrum (bottom) of bromofuranone 2 synthesized chemically following the procedure of Krafft et al.²



Electronic Supplementary Material (ESI) for Chemical Communications This journal is The Royal Society of Chemistry 2011

Figure S7. ¹H NMR spectrum of bromofuranone **2** synthesized chemically following the procedure of Krafft et al.²



10

Figure S8. ¹³C NMR spectrum of bromofuranone 2 synthesized chemically, following the procedure of Krafft et al.² Impurities are marked with an X.



Table S1. Quorum sensing response of *A. tumefaciens* in the presence of varying amounts of bromofuranone 2.

Assay Plate	Concentration of bromofuranone 2 added to the <i>A. tumefaciens</i> NTL4 streak.	Quorum Sensing Response after 48 (Indicated as + for formation of blue color or – for inhibition of formation of the blue color)
1	20 μ L of 0.025 μ M of (2) in CH ₂ Cl ₂	(+)
2	20 μ L of 0.25 μ M of (2) in CH ₂ Cl ₂	(+)
3	20 μ L of 2.5 μ M of (2) in CH ₂ Cl ₂	(+)
4	20 μ L of 25 μ M of (2) in CH ₂ Cl ₂	(+)
5	20 μ L of 50 μ M of (2) in CH ₂ Cl ₂	(-)
6	20 μ L of 60 μ M of (2) in CH ₂ Cl ₂	(-)
7	20 μ L of 70 μ M of (2) in CH ₂ Cl ₂	(-)
8	20 μ L of 80 μ M of (2) in CH ₂ Cl ₂	(-)
9	20 μL of 90 μM of (2) in CH ₂ Cl ₂	(-)
10	20 μ L of 1 mM of (2) in CH ₂ Cl ₂	(-) no growth

In each of these plates, the 20 μ L was added as 2 μ L spots around the *A. tumefaciens* NTL4 streak.

The threshold is at 20 μ L of 50 μ M **2** above which, inhibition of quorum sensing is observed.

Materials and Methods

General Experimental Procedures

¹H NMR spectra were recorded on a Varian 400 MHz instrument in *d*-chloroform (CDCl₃, Cambridge Isotope Laboratories). GC-MS spectra were obtained using a Hewlett Packard 5890-Series II gas chromatograph coupled with a Hewlett Packard 592-A EI-mass selective detector. Atmospheric pressure chemical ionization (APCI) mass spectrometry was performed using a VG-Fisons Platform II (Micromass) quadrupole mass spectrometer. The APCI probe was operated at 500 °C and the ion source temperature was maintained at 150 °C. The cone voltage was set at 50V.

The temperate marine red alga *Delisea pulchra*, was collected off the coast of Antarctica. Partial purification of V-BrPO from the alga was performed as previously described for other V-BrPOs isolated from marine red algae.³ General bromoperoxidase activities of whole algal samples or purified enzyme were detected by bromination of phenol red forming bromophenol blue. Assays consisted of 100 μ M phenol red, 100 mM KBr in 0.15 M sodium phosphate buffer (pH 6.0) and 1 – 2 mg of washed *D. pulchra* or 40 nM partially purified V-BrPO in 1 – 2 mL. Reactions were initiated with 1 – 2 mM H₂O₂ (final concentration). Production of bromophenol blue was monitored at 596 nm or by visible detection.

Chemical reagents 4-pentynoic acid, NBS, Bu_4NOH , and $KHCO_3$ were purchased from Aldrich and used as received. Consumption and formation of reaction substrates and products were monitored using thin layer chromatography on Whatman SiO₂ pre-coated plates (60 F₂₅₄, 250 µm thick) with mixtures of hexanes/ethyl acetated as mobile phases. Flash column chromatography was performed using 230-400mesh silica gel (EM Science) with mixtures of hexanes/ethyl acetate as eluent.

Quorum sensing reporter strains, *A. tumefaciens* NTL4 was kindly provided by Professor Clay Fuqua, Indiana University, and was maintained as reported in the literature.⁴

Reaction of acyl-homoserine lactones with the marine macroalga *D. pulchra*: Reaction conditions with whole algal samples

Small pieces (approx. 2.5 mm x 2 mm) of *D. pulchra* were repeatedly washed with doubly deionized water. Cleaned algal pieces (1 - 2 mg) were placed in 1 mL of either 0.15 M phosphate (pH 6.0), 100 mM KBr or natural seawater (NSW), 100 mM KBr containing 50 μ M 3-oxo-hexanoylhomoserine lactone in a sterile Petri dish. Reactions were initiated by the addition of hydrogen peroxide over ten minutes to a final concentration of 2 mM. Reactions were gently agitated for 1 hour on a shaking platform at 25 rpm at ambient room temperatures (22 – 27 °C). At the completion of an hour the algal pieces were removed using ethanol-sterilized tweezers and the aqueous mixture extracted with 3 volume methylene chloride. The organic phases were combined and dried under a gentle stream of argon. The colorless crude residue was resolubilized in 20 μ L of acetonitrile, and injected directly for APCI mass spectrometry.⁵

Reaction of isolated V-BrPO from D pulchra with acyl-homoserine lactones.

3-Oxo-hexanoyl homoserine lactone (50 μ M) pre-dissolved in ethanol was added to 0.15 M phosphate (pH 6.0), 40 mM KBr, or natural seawater (NSW) containing 40 mM KBr.

Purification of V-BrPO from the alga was performed as described below. Vanadium bromoperoxidase (40 nM) was added, and the reaction initiated by the bulk addition of H_2O_2 to a final concentration of 1 mM. The final reaction volume was 1 mL. The reaction progress was monitored on a Varian Cary spectrophotometer between 190 – 350 nm. Background and baseline correction modes were used. Scans were completed every 30 seconds for 10 minutes. Non-enzymatic control reactions were performed as described above without added V-BrPO.

Effect of dibrominated products from reactions of 3-oxo hexanoyl homoserine lactone with the whole alga *D. pulchra* and with *D. pulchra* V-BrPO on quorum sensing of *A. tumefaciens* NTL4

A. tumefaciens NTL4 (pCF218)(pCF372) is an acyl homoserine lactone (HSL) reporter strain. Products from the reaction between partially purified V-BrPO from *D. pulchra* with 3-oxo-hexanoyl homoserine lactone were tested for their ability to elicit a quorum sensing response in *A. tumefaciens* NTL4. Single colonies of *A. tumefaciens* NTL4 were streaked on six L.B. agar plates containing 4.5 μ g/mL tetracycline and 2% X-gal. For each plate 20 μ L of the corresponding reaction mixture was placed in 2 μ L aliquots around the bacterial streak.^{1, 6}

D. pulchra	v-BrPO	reactions	used	in	the	bioactivity	quorum	sensing	reporter	assay	with	A.
tumefacien	s NTL4 w	vere:										

Plate number	Sample						
1	pH 6.0, V-BrPO reaction mixture without added 3-oxo- hexanoylhomoserine lactone						
2	oH 6.0, Positive control reaction with 3-oxo-hexanoylhomoserine actone without added V-BrPO						
3	oH 6.0, V-BrPO reaction with 3-oxo-hexanoylhomoserine lactone						
4	NSW, V-BrPO reaction without added 3-oxo-hexanoylhomoserine actone						
5	NSW, Positive control reaction with 3-oxo-hexanoylhomoserine lactone without added V-BrPO						
6	NSW, V-BrPO reaction with 3-oxo-hexanoylhomoserine lactone						

L.B. agar plates were dried face up for 30 minute at room temperature, followed by incubation at 30 °C for 48 hours. A quorum sensing response by *A. tumefaciens* NTL4 was detected by the appearance of blue color in the bacteria due to the conversion of X-gal by β -galactosidase.

Biomimetic synthesis of halogenated lactones by D. pulchra V-BrPO

4-Pentynoic acid (1) (10 mM) was pre-dissolved in a small volume of ethanol and added to 0.15 M sodium phosphate buffer (pH 6.0), 100 mM KBr. Enzymatic reactions containing 40 nM partially purified V-BrPO from *D. pulchra* were initiated by addition of 1 - 4 mol equivalents of H_2O_2 in relation to 4-pentynoic acid (1) concentration via syringe pump (1)

equivalent per hour). Reactions were carried out in the dark and the disappearance of the starting material 4-pentynoic acid was monitored by thin layer chromatography (TLC). After completion of the reaction, the aqueous mixture was extracted with four volumes of dichloromethane. The organic layer was dried with magnesium sulfate. After concentrating the organic layer in vacuo, flash chromatography was used with increasing gradient mixtures of ethyl acetate in hexanes to purify compound (2). Bromofuranone (2): 25% yield, colorless oil; MS (EI): m/z 176,178 [M⁺]; ¹H NMR δ 5.84 (t, 1H, J = 2 Hz), δ 2.66 (m, 4H) The ¹H NMR characterization of enzymatically produced 2 agrees with the literature values. ^{2,7}

Chemical synthesis of brominated furanone: 5E-bromomethylidenetetrahydro-2-furanone

Experimental procedures for the synthesis of 5*E*-bromomethylidenetetrahydro-2-furanone (2) were adapted from Kraft et al.² Dichloromethane (25 mL), 4-pentynoic acid (1) (397 mg, 4.05mmol), NBS (728 mg, 4.05mmol), KHCO₃ (420 mg, 4.05mmol) were added to an ovendried 100 mL round bottom flask equipped with a stir bar. 0.5mL of a 1.0M solution Bu₄NOH was added 10 minutes later. The reaction was stirred vigorously for 35 minutes, at which point the reaction was quenched by adding 20 mL of a 5% solution of sodium thiosulfate to the round bottom flask. The product was extracted in dichloromethane (4 x 20mL CH₂Cl₂). The dichloromethane fraction was first concentrated via rotovap. After concentrating in vacuo, bromofuranone (2) was purified by flash chromatography using increasing mixtures of hexanes and ethyl acetate. Bromofuranone (2): 25% yield, colorless oil; MS (EI): m/z 176,178 [M⁺]. ¹H NMR δ 5.84 (t, 1H, J = 2Hz), 2.66 (m, 4H); ¹³C NMR δ 174.3, δ 152.6, δ 85.0, δ 29.4, δ 24.7. The ¹H NMR of **2** is in agreement with the literature values.^{2,7}

Effect of 5E-bromomethylidenetetrahydro-2-furanone on quorum sensing in *A. tumefaciens* NTL4

Purified bromofuranone (2) was tested for its ability to disrupt quorum sensing in *A*. *tumefaciens* NTL4 following the procedure outlined by Borchart et al. and Stickler et al.^{6, 8} Agar plates (LB or AT media) with 40 μ L of 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal) (20 mg/mL stock solution in dimethyl formamide) were used for the bioassay experiments.

Single colonies of *A. tumefaciens* NTL4 were streaked onto separate assay plates. 20 μ L of different concentrations (0.025 μ M, 0.25 μ M, 2.5 μ M, 25 μ M, 50 μ M, 60 μ M, 70 μ M, 80 μ M, 90 μ M and 1 mM) of (2) dissolved in dichloromethane was added in 2 μ L aliquots around the bacterial streak. The assay plates were dried face up for 30 minutes at room temperature followed by incubation at 30°C for 48 hours. Plates were monitored for a color change over 48 hours. A positive quorum sensing response by *A. tumefaciens* NTL4 was detected by the appearance of blue color in the bacterial streak due to the conversion of X-gal by β -galactosidase.

Control plates were prepared using the same method described above. Positive control 1 was prepared by streaking a single colony of *A. tumefaciens* NTL4 on an agar plate. Positive control 2 was prepared by streaking a single colony of *A. tumefaciens* NTL4 on an assay plate, and then adding 20 μ L of 50 μ M 3-oxo-hexanoylhomoserine lactone in 2 μ L aliquots around the streak. Positive control 3 was prepared by streaking a single colony of *A.tumefaciens* NTL4 on an assay plate and then adding 20 μ L of dichloromethane in 2 μ L aliquots around the streak. Positive control 4 was prepared by streaking a single colony of *A. tumefaciens* NTL4 on an assay plate, and then adding 20 μ L of 0.1 M 4-pentynoic acid in 2 μ L aliquots around the streak. All positive control reactions showed the expected positive quorum sensing response in the *A*.

tumefaciens NTL4 bioassay, as indicated by the appearance of blue color in the bacterial streak due to the conversion of X-gal by β -galactosidase.

Isolation and purification of V-BrPO from D. pulchra.

Approximately 300 grams of algae were ground in a cooled mortar and pestle with liquid nitrogen to obtain a fine red powder. Two volume equivalents of 0.1 M Tris-buffer pH 8.0 was added to the powdered algae and blended at high speed at 4 °C for 10 minutes (commercial Waring Blender). Crude extracts were centrifuged at 6000 rpm for 30 minutes, and the supernatants retained. Remaining algal pellets were rehomogenized two more times followed by centrifugation. The pooled supernatants were concentrated on an Amicon stir-cell with a 30 kDa cut-off filter membrane. The concentrated supernatants were batch loaded onto 200 mL of DEAE FF resin (Pharmacia). Protein was eluted by step-gradient using 1M NaCl in 0.1 M Tris-SO₄ pH 8.0. Haloperoxidase activity eluted within the 0.3 - 0.5 M NaCl fraction. Fractions showing haloperoxidase activity were further purified by Superose-6 gel filtration in 0.1 M Tris-HCl pH 8.0, and by Mono Q anion exchange chromatography (same buffers as was used for DEAE FF).

RT-PCR cloning of V-BrPO from *D. pulchra*

<u>Small-scale isolation of total RNA.</u> Frozen alga was rinsed in doubly deionized water (ddH₂O, Nanopure) followed by air drying at room temperature for 1 hour. Dried alga was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Six hundred milligrams of powdered algae was used with the Qiagen Plant RNeasy Kit (Qiagen) according to the manufacturer's protocols. Isolated total RNA was resuspended in 50 μ L of DEPC-treated ddH₂O.

<u>Reverse-Transcriptase Polymerase Chain Reaction</u>. RT-PCR was performed using the One Step RT-PCR kit (Qiagen) according to the manufacturer's protocols. Primers used in the reactions were BPO1, BPO2, For3Full, For02, and FullRev:

Primer Name	Sequence
For3Full	5'ATGGGTATTCCAGCTGACAACCT3'
For02	5'CATGACGACAATGGCGCTATTAT3'

Products from RT-PCR reactions were visualized on 0.7% - 1.5% agarose/TAE gels with ethidium bromide. DNA of interest was excised from the gel and purified using the GIBCO Concert Gel Extraction Kit (GIBCO-BRL) according to the manufacturer's protocol. Purified products were subcloned into the PCR-Script-Amp^r cloning vector (Stratagene) and transformed into Ultracompetent XL-1 Gold *E. coli* cells, all according to manufacturer's protocols. Transformed *E.coli* (at least 10-20 colonies) were grown to confluence, and plasmids isolated using the Plasmid Isolation Kit (BioRad). The presence of the insert of interest was confirmed by restriction digest analysis using PST1. Plasmids harboring the DNA of interest were sequenced by the Iowa State Sequencing Facility. The cloned inserts were sequenced using T3/T7 primers from opposite directions by an automated DNA sequencer.

<u>Nucleotide and protein sequence alignment analyses</u>. Nucleotide sequences of V-BrPO genes were aligned with CLUSTAL W without manual adjustment using the BIOEDIT sequence alignment editor.⁹ The amino acid sequences for cloned V-BrPOs were also aligned using

CLUSTAL W. Sequence identity analysis was performed using the GENEDOC program, v.2.5.¹⁰ The recombinant *D. pulchra* V-BrPO was expressed and purified following the procedures outlined by Carter et al.³

Mutagenesis to generate the R553H mutant

Site-directed mutagenesis of DelpulE.A using the Stratagene QuickChange II kit successfully produced the R553H mutant (regenerating the original active site). Plasmid containing the gene for the arginine isoform (pTrc.DelpulE.A) was isolated from a small overnight culture of pTrc.DelpulE.A.XL10 in LB ampicillin using the BioRad Quantum Prep plasmid isolation kit. Plasmids were eluted in 100 μ L of DEPC-treated nanopure water and stored at -20 °C. Mutagenesis was accomplished using primers synthesized by Invitrogen: DP R553H FWD: (DNA)-5'-CGG GCG GAA CTG GCA GGT GTT CAC TAC TTC TCT GAC CAG TTC GAG-3' and DP R553H REV: (DNA)-5'-CTC GAA CTG GTC AGA GAA GTA GTG AAC ACC TGC CAG TTC CGC CCG-3'.

The mutation was generated by PCR using the following reaction conditions: 1 μ L dNTP mix, 2 μ L plasmid template, 1.25 μ L DP R553H FWD (100 ng/ μ L), 1.25 μ L DP R553H REV (100 ng/ μ L), 38.5 μ L DEPC treated nanopure water, 1 μ L PfuUltra HF DNA polymerase in 5 μ L 10x reaction buffer. All solutions, except the plasmid template, were obtained from the Stratagene QuickChange II site-directed mutagenesis kit and used as received. The thermal cycling program was 95 °C for 30 s, a three step cycle of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 6 min, followed by a hold at 37 °C. The parental DNA was digested for 1 hour at 37 °C with 1 μ L of DpnI restriction enzyme (10 U/ μ L). The supplied XL1-Blue supercompetent cells were transformed with the resulting solution (which contains the mutated plasmid). Colonies were picked from the LB ampicillin plates, inoculated into 4 mL of LB ampicillin, and grown overnight at 37 °C, shaking at 225 rpm. Plasmids were isolated using the BioRad Quantum Prep MiniPrep kit and submitted for sequencing. Isolates were stored as glycerol stocks at -80 °C. Plasmids from a successful mutation were used to transform BL21-Gold for expression of the enzyme.

A small amount of protein from each strain was isolated and evaluated by the phenol red assay over a period of days. The assay consisted of 250 μ L 0.1 M phosphate buffer pH 5.7, 60 μ L 1 M KBr, 10 μ L 5 mM phenol red, 50 μ L sample (from DEAE purifications using a prepacked column), and 6.6 μ L 0.2 M H₂O₂ and were incubated at room temperature.

- 1. S. A. Borchardt, Appl. Environ. Microbiol., 2001, 67, 3174-3179.
- 2. G. A. Krafft and J. A. Katzenellenbogen, *Journal of the American Chemical Society*, 1981, **103**, 5459-5466.
- 3. J. N. Carter, K. E. Beatty, M. T. Simpson and A. Butler, J. Inorg. Biochem., 2002, 91, 59-69.
- 4. J. Zhu, J. W. Beaber, M. I. More, C. Fuqua, A. Eberhard and S. C. Winans, *Journal of Bacteriology*, 1998, 180, 5398-5405.
- 5. J. J. Michels, E. J. Allain, S. A. Borchardt, P. Hu and W. F. McCoy, J. Chromatogr. A, 2000, 898, 153-165.
- 6. D. J. Stickler, N. S. Morris, R. J. C. McLean and C. Fuqua, *Applied and Environmental Microbiology*, 1998, **64**, 3486-3490.
- 7. W. Dai and J. A. Katzenellenbogen, *Journal of Organic Chemistry*, 1991, **56**, 6893-6896.
- 8. S. A. Borchardt, E. J. Allain, J. J. Michels, G. W. Stearns, R. F. Kelly and W. F. McCoy, *Applied and Environmental Microbiology*, 2001, **67**, 3174-3179.
- 9. T. A. Hall, Nucl. Acids Symp. Ser., 1999, 41, 95-98.
- 10. K. B. Nicholas, H. B. Nicholas Jr. and D. W. Deerfield, *EMBnet News*, 1997, 4, 14.