On the Regiospecificity of Vanadium Bromoperoxidase

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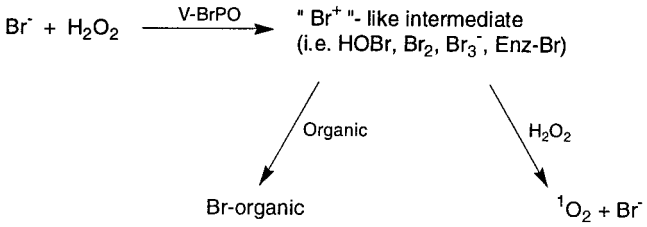
Abstract: Vanadium haloperoxidase enzymes catalyze the oxidation of halide ions by hydrogen peroxide, producing an oxidized intermediate, which can halogenate an organic substrate or react with a second equivalent of hydrogen peroxide to produce dioxygen. Haloperoxidases are thought to be involved in the biogenesis of halogenated natural products isolated from marine organisms, including indoles and terpenes, of which many are selectively oxidized or halogenated. Little has been shown concerning the ability of the marine haloperoxidases to catalyze regioselective reactions. Here we report the regiospecific bromoperoxidative oxidation of 1,3-di-*tert*-butylindole by V-BrPO from the marine algae *Ascophyllum nodosum* and *Corallina officinalis*. Both enzymes catalyze the regiospecific oxidation of 1,3-di-*tert*-butylindole in a reaction requiring both H_2O_2 and Br^- as substrates, but which produce the unbrominated 1,3-di-*tert*-butyl-2-indolinone product exclusively, in near quantitative yield (i.e. one H_2O_2 consumed per product). By contrast, reactions with the controlled addition of aqueous bromine solution (HOBr = Br_2 = Br_3^-) produce three monobromo and one dibromo-2-indolinone products, all of which differ from the V-BrPO-catalyzed product. Further, reactivities of 1,3-di-*tert*-butyl-2-indolinone with both aqueous bromine and V-BrPO differ significantly and shed light onto the possible nature of the oxidizing intermediate. This is the first example of a regiospecific bromination by a vanadium haloperoxidase and further extends their usefulness as catalysts.

Vanadium bromoperoxidase (V-BrPO), found primarily in marine algae, is thought to function in the biosynthesis of numerous halogenated marine natural products. These compounds span a wide range of structures, including halogenated phenols, terpenes, C-15 acetogenins, and indoles. Halogenated indoles are of particular interest as they are synthetic targets with potent antiinflammatory, anti-fungal, and anti-cancer activities.[1] Vanadium haloperoxidases catalyze halide oxidation by hydrogen peroxide.[2] This oxidized intermediate can halogenate an appropriate organic substrate or oxidize a second equivalent of hydrogen peroxide producing dioxygen, which in the case of bromide has been shown to be in the singlet excited state (Scheme 1).[3] It has also been established that bromination of organic substrates by vanadium haloperoxidases proceeds through an electrophilic (Br^+) rather than a radical ($Br^\bullet$) process.[4]

Recently the X-ray crystal structure of native V-BrPO from the brown alga *Ascophyllum nodosum* has been reported to 2.0 Å resolution.[5] This homodimeric protein is dominated by an α-helical topology with two four-helix bundles and three small β-sheet segments per monomer. The vanadium cofactor is bound at the bottom of a 15 Å deep substrate channel composed of residues contributed by both monomers with a high percentage of hydrophobic residues in the vicinity of the vanadium binding site. Vanadium(V), in the form of vanadate ion, is coordinated to the protein by one histidine ligand in a trigonal bipyramidal geometry. This histidine (His486) occupies one of the axial positions. The other axial position is occupied by hydroxide ion, which is hydrogen-bonded to what is thought to be a catalytically important histidine (His418).[5] Three oxygen atoms occupy the equatorial plane and are each hydrogen-bonded to multiple amino acid side-chain residues or the peptide backbone of the protein. The vanadate coordination environments of V-BrPO and the vanadium-dependent chloroperoxidase (V-ClPO) from the fungus *Curvularia inaequalis* are identical in terms of the vanadium ligation.[5] However, other residues in the vicinity of the vanadium site do differ between V-BrPO (*A. nodosum*) and V-ClPO (*C. inaequalis*), possibly leading to differences in the halide selectivity of these two enzymes.

Scheme 1



Vanadium-dependent haloperoxidases have been shown to be thermally stable with significant activity remaining at temperatures as high as 70 °C. Remarkably, they can recover any lost activity when reequilibrated at lower temperatures and are tolerant to organic solvents.[6] For V-BrPO from *A. nodosum*, this remarkable thermal stability is likely a result of the

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numerous inter- and intramolecular disulfide bonds present in the homodimer.[5] Whereas for V-BrPO from *C. officinalis*, this stability likely results from the binding of multiple divalent cations and from the dodecameric organization of the enzyme which would reduce solvent exposure, and not from disulfide bond stabilization.[7] Furthermore, vanadium-dependent haloperoxidases might well be preferable halogenating agents over heme-containing enzymes, which are subject to degradation as a result of heme oxidation. Therefore, the vanadium-dependent haloperoxidases with high thermal stability, tolerance to organic solvents, and ability to halogenate a wide range of substrates make them ideal candidates for use in organic synthesis. However, V-BrPO would be more attractive in organic synthetic schemes and industrial applications if it catalyzed enantio- or regioselective reactions.

Initial reports on the reactivity of V-BrPO failed to detect any regio or stereoselectivity in the oxidation of anisole and other prochiral aromatic compounds.[8−10] This lack of selectivity became, in part, the basis for the suggestion that the function of these enzymes is to produce a diffusible oxidized bromine intermediate such as hypobromite, bromine, or tribromide.[11] However, the kinetics of bromination and bromoperoxidative oxidation of certain indoles (e.g., 2-methylindole, 3-methylindole, 2-phenylindole, etc.) showed that the V-BrPO-catalyzed reactions were not consistent with halogenation by a released diffusible species (e.g., $HOBr = Br_2 = Br_3^-$).[12] In addition, fluorescence quenching of 2-phenylindole established that the indole binds to V-BrPO.[12] Given that organic substrates can bind to V-BrPO and that oxidized bromine species are not released from the enzyme active site in the presence of these substrates, it seemed likely that V-BrPO should be able to catalyze selective bromination or bromoperoxidative oxidation reactions, particularly given the stereo- and regio-defined structures of many halogenated marine natural products.

Recently it has been found that V-BrPO can catalyze stereospecific oxidation reactions of bicyclic sulfides to the corresponding sulfoxides, with a high degree of enantioselectivity (>95% ee).[13−15] These reactions, which were carried out in the absence of a halide, are a result of the direct oxidation of the sulfide by the peroxovanadium(V) active site of V-BrPO, and are the only known reactions involving the direct oxidation of an organic substrate in the absence of halide ion. Interestingly, it has been shown that the bromoperoxidases from *A. nodosum* and *Corallina pilulifera* direct the oxidation to differing enantiomers, while the similar recombinant V-ClPO from *C. inaequalis* catalyzes the production of the racemic sulfoxide mixture.[15] These results and recent X-ray crystallographic studies suggest subtle differences exist in the substrate channel of these enzymes.[5,7]

Although important advances have been made regarding the stereoselectivity of V-BrPO,[13−16] little in the way of this enzyme's ability to catalyze selective halogenation or halogen-dependent oxidation reactions has been reported. Herein we report the regioselective, halogen-dependent, oxidation of indoles catalyzed by V-BrPO (*A. nodosum* and *C. officinalis*) and compare them to reactions with aqueous bromine (i.e., $HOBr = Br_2 = Br_3^-$, etc).

Materials and Methods

General Methods. Vanadium bromoperoxidase from the brown algae, *A. nodosum* was purified as previously described.[17,18] The vanadium bromoperoxidase from the red alga, *C. officinalis* was purified as described with minor modifications.[19] Bromoperoxidase activity was determined by monitoring the bromination of 50 μM monochlorodimedone (MCD) spectrophotometrically at 290 nm under conditions of 0.1 M Br^- and 0.5 mM H_2O_2 in 0.1 M phosphate pH = 6.00. The extinction coefficient difference at 290 nm between MCD and brominated MCD is 19 900 cm^{-1} M^{-1}.[20] The specific activity of V-BrPO was 69 μmol brominated per min per mg of V-BrPO (units/mg).

^1H NMR and ^{13}C NMR spectra were recorded on a Varian 400 instrument using deuteriochloroform as the solvent ($CHCl_3$ standard, $\delta = 7.27$ ppm). Formation of 1,3-di-*tert*-butylindole (**1**) was monitored using a Hewlett-Packard 5890-series II gas chromatograph coupled with a Hewlett-Packard 592-A EI-mass selective detector and also with thin-layer chromatography (Silica Gel 60 F_{25} precoated plates). Exact mass determination of (**1**) was completed on a VG-70E instrument. Mass spectral analysis of the products (**2**, **3**, and **4**) were completed on the same instrument using 3-nitrobenzyl alcohol as matrix for FAB-MS.

Aqueous bromine was prepared by dilution of bromine vapors into 0.1 M NaOH and standardized by tri-iodide formation (I_3^-; λ_{max} 353 nm, $\Delta\epsilon = 26\,000$ cm^{-1} M^{-1} determined in 0.1 M citrate−phosphate pH 4.4 with 0.1 M KI). The dominant species in solution at pH 6−7 is HOBr; however, multiple oxidized bromine species are present in solution which is best represented as $OBr^- = HOBr = Br_2 = Br_3^-$.[21] This reaction mixture will be referred to as "aqueous bromine" throughout this article.

Reverse-phase HPLC was used to purify the products from the reactions of 1,3-di-*tert*-butylindole (**1**) or 1,3-di-*tert*-butyl-2-indolinone (**2**) with aqueous bromine or from the V-BrPO-catalyzed reactions, as well as for retention time analysis. A C_{18} column (semipreparative 250 × 10 mm or analytical 250 × 4.6 mm, ODS-AQ, YMC Inc., Wilmington, NC) was used on a Waters HPLC system equipped with two Waters 510 pumps. Products were eluted isocratically in 80% acetonitrile/20% water, and the UV absorbance (214 nm) of the eluate was measured with either a photodiode array or a dual wavelength detector.

1,3-Di-*tert*-butylindole (1) was prepared using a modification of Smith and Walters' procedure.[22] MeMgBr (5.5 mL of 3.0 M in Et_2O, 16.5 mmol) was added dropwise at room temperature over a 15 min period to a solution of indole (1.5 g, 12.8 mmol) in freshly distilled THF (10 mL). Neat *tert*-butyl bromide (2.0 mL, 16.6 mmol) was then added, and the resulting mixture was refluxed for 2 h. Subsequently, another 5.5 mL of MeMgBr (16.5 mmol) and 3.0 mL (26.0 mmol) of the *tert*-butyl bromide were added to the reaction vessel, and the mixture was refluxed for an additional 4 h after which time product formation was complete (monitored by TLC and GC/MS). The contents of the reaction vessel were then poured into saturated NH_4Cl at 0 °C, and the organic layer was separated and washed with brine. Upon removal of the solvent and purification by flash chromatography (3% Et_2O in pentane over 200−425 mesh SiO_2), 1.1 g (4.8 mmol) of 1,3-di-*tert*-

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butylindole was obtained (37%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (dt, 1H, J = 8.0, 0.8 Hz), 7.63 (dt, 1H, J = 8.4, 0.8 Hz), 7.14 (m, 1H), 7.06 (m, 1H), 6.99 (s, 1H) 1.73 (s, 9H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.00, 128.01, 125.51, 121.45, 121.45, 120.69, 119.95, 117.59, 113.39, 55.38, 31.55, 30.89, 29.82. HRMS calcd for $C_{16}H_{23}N$ 229.1831, found 229.1828

Bromination of 1,3-Di-*tert*-butylindole by V-BrPO or Aqueous Bromine. 1,3-Di-*tert*-butylindole (**1**) (0.5 mM) predissolved in ethanol was added to a 0.1 M phosphate (pH 6.00) solution containing 30% v/v ethanol and 0.1 M KBr. Enzymatic reactions containing 30 nM V-BrPO, were initiated by addition of 1 or 2 mol equiv of H_2O_2 in relation to the 1,3-di-*tert*-butylindole (**1**) concentration.

The nonenzymatic reactions were initiated by addition of 1 or 2 mol equiv of aqueous bromine to the indole compound as described above (without V-BrPO or H_2O_2). Aqueous bromine was added either in bulk or in a controlled manner by syringe pump at a rate similar to the bromination rate of MCD by V-BrPO ($-$dMCD/dt = +d[aqueous bromine]/dt = 63 nmol/min). All reaction mixtures were stirred. Similar reactions with V-BrPO and HOBr were completed using 1,3-di-*tert*-butyl-2-indolinone (**2**) as substrate.

HPLC analysis of reactions was completed by direct injection without prior extraction. All products were purified by HPLC, dried over $MgSO_4$, concentrated, diluted with ethyl ether, and dried in vacuo. (**2**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, 1H, J = 7.2 Hz), 7.21 (m, 2H), 6.95 (t, 1H J = 7.4 Hz), 2.93 (s, 1H), 1.71 (s, 9H), 1.08 (s, 9H) FAB-MS m/z 246 $(M + H)^+$ (**3**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (d,d 1H, J = 2.0, 0.8 Hz), 7.31 (ddd, 1H J = 8.8, 2.0, 0.4 Hz), 7.09 (d, 1H J = 8.8 Hz), 2.92 (broad s, peak width 2.1 Hz, 1H), 1.68 (s, 9H), 1.08 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.14, 144.30, 130.71, 129,-83, 128.96, 113.64, 113.45, 57.30, 55,68, 35.50, 28.99, 27.23. FAB-MS m/z 324 (M^+) The expected isotopic distributions of masses corresponding to one bromine was observed. NMR spectra given is for one of the monobromo-2-indolinone products obtained (see Figure 2b); however, NMR and FAB-MS spectra was obtained for the other two products establishing them as monobromo-2-indolinone derivatives. Two of these monobromo-2-indolinone products produced needlelike crystals; however, the general symmetry of the molecule precluded atom assignment of the crystallographic data. (**4**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (d, 1H, J = 2.4 Hz), 7.35 (dd, 1H J = 2.4, 8.8 Hz), 7.10 (d, 1H J = 8.8 Hz), 1.71 (s, 9H), 1.19 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.97, 141.95, 132.96, 131,59, 130.28, 114.45, 114.24, 66.72, 58,33, 39.24, 28.96, 25.35. FAB-MS m/z 404 $(M + H)^+$. The expected isotopic distribution of masses corresponding to two bromines was observed. The dibromo derivative did not produce diffracting crystals.

Kinetic Profile Conditions. Time course experiments for the bromination of phenol red by aqueous bromine or catalyzed by V-BrPO, as a function of the concentration of 1,3-di-*tert*-butylindole (**1**), were carried out at 24 °C in the presence of 25.3 μM phenol red and 40 mM KBr in 0.1 M phosphate buffer pH 6.00 containing 30% v/v ethanol (total volume is 3 mL). Enzymatic experiments were initiated by addition of 0.416 mM H_2O_2 and 2.5 nM V-BrPO. The nonenzymatic competition experiments were initiated by addition of aqueous bromine (2.35 mM stock solution) in 10 μL aliquots at 30 s intervals. UV absorbance was measured 20 s after each addition of aqueous bromine. Production of bromophenol blue was monitored at 596 nm.

Results and Interpretation

V-BrPO-Catalyzed Reactions with 1,3-Di-*tert*-butylindole versus Reaction of Aqueous Bromine with 1,3-Di-*tert*-butylindole. When 1,3-di-*tert*-butylindole (**1**) (0.5 mM) is allowed to react with 1 equiv of H_2O_2, in the presence of V-BrPO (30 nM) and bromide (0.1 M), the sole product is 1,3-di-*tert*-butyl-2-indolinone (**2**) obtained in near quantitative conversion at pH 6.00. Identical results are obtained with addition of 2 equiv of H_2O_2 (Figure 1, Scheme 2; see Materials and Methods for product identification). No reaction was observed in the absence of bromide (data not shown), indicating that in the absence of halide, the peroxo V-BrPO complex could not directly oxidize 1,3-di-*tert*-butylindole (**1**). The formation of product (**2**) then likely results from electrophilic attack of the brominating species on the electron-rich C2−C3 double bond of the pyrrole ring yielding first a bromoindolinium species, followed by hydration at the C2 position (Scheme 2).[23] This mechanism is consistent with product analysis from numerous substituted indoles.[24,25] The regioselective oxidation at C2 was unaffected by a change in cosolvent from ethanol to methanol, tetrahydrofuran or dimethylformamide.

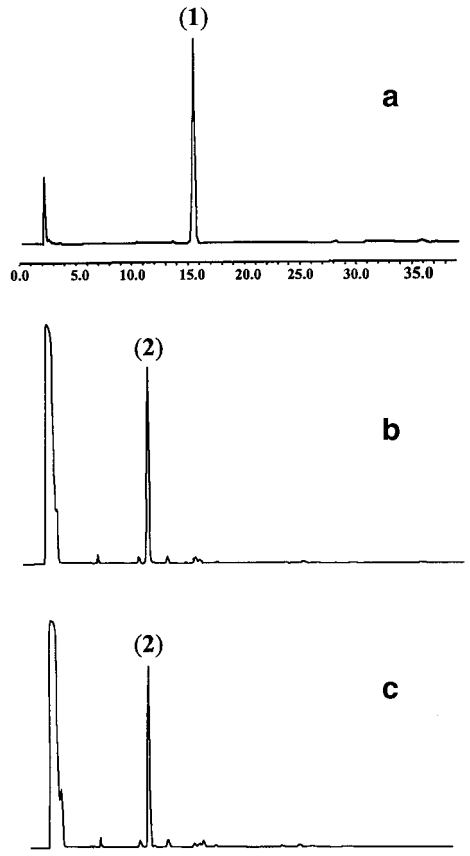
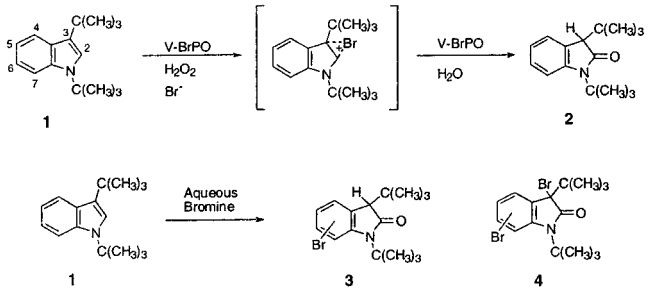


Figure 1. HPLC separation of products from the reaction of V-BrPO with 1,3-di-*tert*-butylindole. The reaction mixture contained 0.1 M KBr and 30 nM V-BrPO in 0.1 M phosphate (pH 6.00) with 30% v/v ethanol. The reactions were initiated by addition of H_2O_2 and allowed to react for 60 min at 24 °C. (a) 0.5 mM 1,3-di-*tert*-butylindole (**1**) (i.e. a control of the organic substrate alone without addition of H_2O_2); (b) 0.5 mM 1,3-di-*tert*-butylindole (**1**) and 0.5 mM H_2O_2; and (c) 0.5 mM 1,3-di-*tert*-butylindole (**1**) and 1.0 mM H_2O_2. The peak at 2 min is the solvent front. Peaks are denoted with numbers corresponding to their structures.

Scheme 2



(23) V-BrPO catalyzed reaction of indole with Br^- and $H_2{}^{18}O_2$ yielded unlabeled indigo, suggesting that the oxygen atom of 2-indolinone and 1,3-di-*tert*-butyl-2-indolinone originates from water and not hydrogen peroxide. Tschirret-Guth, R. A. Ph.D. Dissertation; University of California, Santa Barbara, 1996.

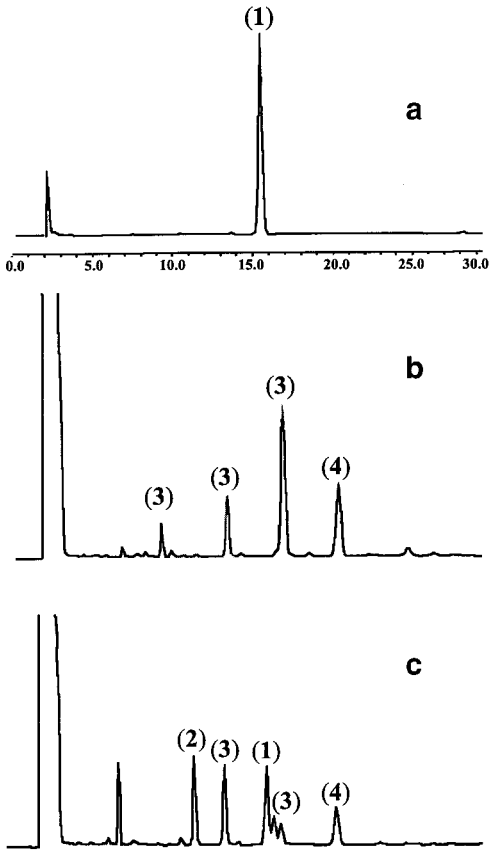


Figure 2. HPLC separations of products from the reaction of aqueous bromine with 1,3-di-*tert*-butylindole. Comparison of bulk versus controlled addition of aqueous bromine. The reaction mixture contained 0.1 M KBr and 0.5 mM 1,3-di-*tert*-butylindole (**1**) in 0.1 M phosphate (pH 6.00) with 30% v/v ethanol. The reactions were initiated by addition of NaOBr and allowed to react at 24 °C for 60 min. (a) 0.5 mM 1,3-di-*tert*-butylindole (**1**) (i.e. a control of the organic substrate alone without addition of NaOBr); (b) 1.0 mM NaOBr by syringe pump at a rate of 63 nmol/min; and (c) 1.0 mM NaOBr by bulk addition. Peaks are denoted with numbers corresponding to their structures. All monobrominated-2-indolinone derivatives have differing bromine substitution; however, each is denoted as (**3**) for clarity.

In contrast to the selectivity exhibited by reaction of 1,3-di-*tert*-butylindole (**1**) with V-BrPO, when 2 equiv of aqueous bromine are added by syringe pump to 1,3-di-*tert*-butylindole (**1**) at pH 6.00, at a rate equal to enzymatic turnover by V-BrPO, a mixture of products is obtained (Figure 2b, Scheme 2). These products include one dibromo- and three monobromo-2-indolinone derivatives of 1,3-di-*tert*-butylindole (**1**). For two of the monobromo products (**3**) the bromine resides at either C5 or C6 of the benzene ring. The third monobromo product was identified by mass spectrometry; however, because of limited amounts of material, the position of the bromine substitution could not be accurately determined. In the dibromo-derivative (**4**), the bromine is at the C3 position as well as at either C5 or C6 (see Materials and Methods for characterization of these products).[26] In contrast to the controlled addition of aqueous bromine by syringe pump, when 2 equiv of aqueous bromine are added at once, many more derivatives are formed, resulting in incomplete consumption of starting material (Figure 2c). Addition of 1 equiv of aqueous bromine in either manner gives similar product profiles as seen in Figure 2c with overall lower starting material consumption (data not shown).

Competitive Kinetics for Reactions of 1,3-Di-*tert*-butylindole versus Phenol Red. In a mixture of 1,3-di-*tert*-butylindole (**1**) and phenol red, V-BrPO preferentially catalyzes the bromoperoxidative oxidation of 1,3-di-*tert*-butylindole (**1**) as seen by a lag phase in the appearance of bromophenol blue (Figure 3), which is also consistent with the reactivity of other indoles.[12] The lag phase increases with increasing concentration of 1,3-di-*tert*-butylindole (**1**). After all of the 1,3-di-*tert*-butylindole (**1**) is consumed, the rate of bromination of phenol red occurs at the same rate as in the absence of added indole. In addition, the lag phase is not observed in the competitive reactions of aqueous bromine with 1,3-di-*tert*-butylindole (**1**) and phenol red (Figure 3), which is also consistent with previous observations with other indoles.[12] Thus, the difference in reactivity between the V-BrPO/H_2O_2/Br^- system and aqueous bromine suggests that the sterically bulky 1,3-di-*tert*-butylindole binds to V-BrPO, resulting in the observed specificity.

V-BrPO-Catalyzed Reactions with 1,3-Di-*tert*-butyl-2-indolinone versus Reaction of Aqueous Bromine with 1,3-Di-*tert*-butyl-2-indolinone. Comparison of the reactivity of 1,3-di-*tert*-butyl-2-indolinone (**2**) in the V-BrPO/H_2O_2/Br^- system to that of aqueous bromine shows that no additional bromination or oxidation of 1,3-di-*tert*-butyl-2-indolinone is catalyzed by V-BrPO (Figure 4b); however, reaction with aqueous bromine does produce one monobromo-2-indolinone product exclusively (Figure 4c). This product, also seen in Figure 2b and 2c, was determined to be brominated at either C5 or C6 (see Materials and Methods). Although the exact placement of the bromine is not clear, it is likely brominated at the C5 position of the benzene ring due to the tendency of the amide to direct toward the para position. This attenuated reactivity toward bromination is very similar to what is seen with the acetylated aniline derivative, acetanilide. Aniline, by itself, is highly reactive toward ortho and para bromination producing 2,4,6-tribromoaniline, making it difficult to achieve selective halogenation; however, following conversion of the amino group to an amide, its reactivity is moderated producing only *p*-bromoacetanilide.[27]

Although the reaction of 1,3-di-*tert*-butyl-2-indolinone (**2**) with V-BrPO does not yield any products, this result does not rule out a direct oxidation of H_2O_2 by an enzyme-produced brominating species, if released by the enzyme, as competition for substrate bromination in solution. However, when 1 equiv of aqueous bromine, added by syringe pump at a rate equal to that of enzymatic turnover, is reacted with a solution containing 1 equiv of 1,3-di-*tert*-butyl-2-indolinone (**2**) and H_2O_2, the substrate is brominated (Figure 4d).[28] Furthermore, in a mixture of 1,3-di-*tert*-butyl-2-indolinone (**2**) and phenol red, V-BrPO preferentially catalyzed the bromination of phenol red to phenol blue (Figure 5). This experiment suggests that this modified indole does not bind V-BrPO. Clearly, this substrate is not an inhibitor of V-BrPO, as quantitative conversion of 1,3-di-*tert*-butylindole (**1**) to 1,3-di-*tert*-butyl-2-indolinone (**2**) occurs under catalytic conditions of excess substrate over enzyme. These results suggest that reaction with V-BrPO (*A. nodosum*) are not consistent with the formation and release of hypobromous acid.

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(28) 1,3-Di-*tert*-butylindole (**1**) also underwent bromination in the presence of H_2O_2.

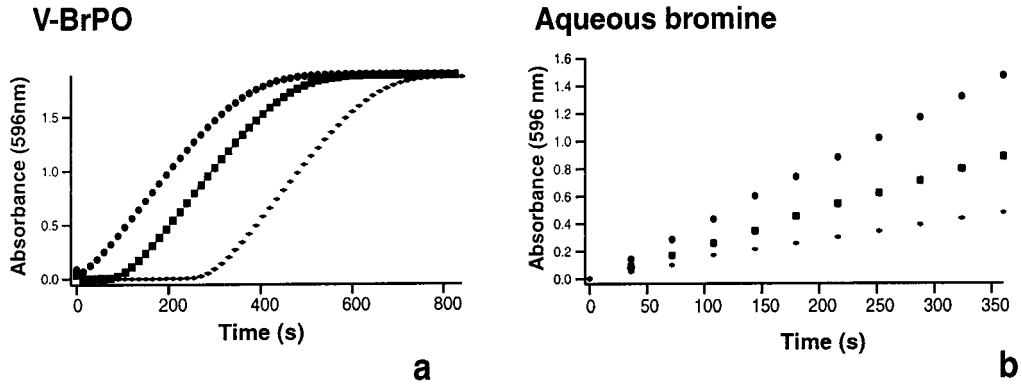

Figure 3. Time course for the bromination of phenol red by (a) V-BrPO catalysis or (b) by NaOBr as a function of the 1,3-di-tert-butylindole concentration. The reactions were carried out at 24 °C in the presence of 25.3 μM phenol red and 40 mM KBr in 0.1 M phosphate buffer (pH 6.00) with 30% v/v ethanol. Enzymatic reactions were initiated by addition of 0.416 mM H_2O_2 and 2.5 nM V-BrPO. Nonenzymatic reactions were initiated by addition of NaOBr (stock solution of 2.35 mM in 0.02 M NaOH) in 10 μL aliquots at 30 s intervals. Production of bromophenol blue was monitored at 596 nm. Concentration of 1,3-di-*tert*-butylindole (**1**): ●, 0 μM; ■, 28 μM; ▲, 87 μM.

Selectivity Comparison of V-BrPO from *Ascophyllum nodosum* and *Corallina officinalis*. V-BrPO isolated from *C. officinalis* was reacted with 1,3-di-*tert*-butylindole (**1**) and 1,3-di-*tert*-butyl-2-indolinone (**2**) under similar conditions and specific activity as reactions with V-BrPO from *A. nodosum*. The product from reaction with 1,3-di-*tert*-butylindole (**1**) was 1,3-di-*tert*-butyl-2-indolinone (**2**), and there was no further reactivity of this substrate with V-BrPO (*C. officinalis*). These results are identical to results from reaction with V-BrPO from *A. nodosum*.

Discussion and Conclusions

V-BrPO catalyzes the regiospecific oxidation of 1,3-di-*tert*-butylindole (**1**) leading to formation of one product, 1,3-di-*tert*-butyl-2-indolinone (**2**); whereas, the reaction with aqueous bromine is not selective producing three monobromo- and one dibromo-2-indolinone products. The difference between the enzyme-catalyzed reaction and the molecular reaction with aqueous bromine solution is striking, especially when one considers that electrophilic bromination of 1,3-di-*tert*-butylindole could result in bromination at nearly all of the unsubstituted benzene ring carbons as well as C2−C3 of the pyrrole ring. Interestingly, under conditions of controlled addition of HOBr (Figure 2b) neither 1,3-di-*tert*-butyl-2-indolinone (**2**) nor 5- or 6- bromo-di-*tert*-butylindole is detectable, suggesting that attack of the benzene ring of this bulky substrate might occur at roughly the same rate as attack at the C2−C3 position.

It is not surprising that for both enzyme-catalyzed and molecular reactions the primary modification is indolinone formation, as the electron-rich C2−C3 double bond predisposes the heterocyclic ring toward electrophilic attack by the oxidized halogen species.[29,30] What is surprising is that no alternative or additional products form in the reaction with V-BrPO, given that the oxidized intermediate (e.g., "Br^+"-like) of V-BrPO is at the same oxidation level as NaOBr. The brominating intermediate of V-BrPO has not been detected under conditions of optimal pH (e.g., pH ≈ 6.5), because it reacts rapidly with organic substrates or with excess H_2O_2 to produce O_2.[12] At a more acidic pH, HOBr-type species (e.g., HOBr = Br_2 = Br_3^-) have been detected in solution with V-BrPO (*A. nodosum*) under specific conditions of low pH, high bromide, high enzyme, and low peroxide concentrations (e.g. pH 5.0; 100 mM Br^-; 130

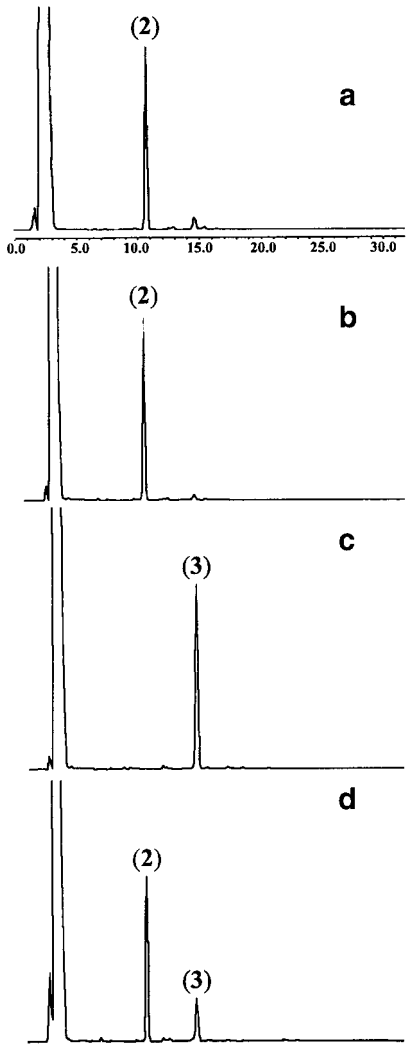

Figure 4. HPLC chromatograms of the V-BrPO-catalyzed and aqueous bromine reactions with 1,3-di-*tert*-butyl-2-indolinone. The reaction mixture contained 0.1 M KBr and 0.5 mM 1,3-di-*tert*-butyl-2-indolinone (**2**) in 0.1 M phosphate (pH 6.00) with 30% v/v ethanol. (a) 1,3-di-*tert*-butyl-2-indolinone (**2**) (i.e. the control in the absence of addition of H_2O_2/V-BrPO or NaOBr; (b) 30 nM V-BrPO and 0.5 mM H_2O_2; (c) 0.5 mM NaOBr added by syringe pump at a rate of 63 nmol/min; and (d) 0.5 mM NaOBr added by syringe pump in the presence of 0.5 mM H_2O_2. All reactions were carried out at 24 °C for 60 min. Peaks are denoted with numbers corresponding to their structures.

(29) Franssen, M. C. R. *Catal. Today* **1994**, *22*, 441−457.
(30) Gilchrist, T. L. *Heterocyclic Chemistry*, 2nd ed.; Wiley and Sons: New York, 1992.

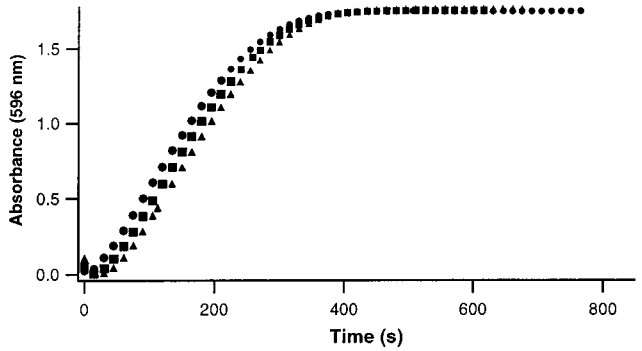

Figure 5. Time course for the bromination of phenol red catalyzed by V-BrPO as a function of the 1,3-di-tert-butyl-2-indolinone concentration. The reactions were carried out at 24 °C in the presence of 25.3 μM phenol red and 40 mM KBr in 0.1 M phosphate buffer (pH 6.00) with 30% v/v ethanol. Reactions were initiated by addition of 0.416 mM H_2O_2 and 2.5 nM V-BrPO. Production of bromophenol blue was monitored at 596 nm. Concentration of 1,3-di-*tert*-butyl-2-indolinone (**2**): ●, 0 μM; ■, 28 μM; ▲, 87 μM.

nM V-BrPO; and 300 μM H_2O_2).[11] However, at optimal pH, a released brominating species cannot be detected with similar H_2O_2 and enzyme concentrations (*C. pilulifera*).[8]

What likely accounts for the regiospecific modification of 1,3-di-*tert*-butylindole is a specific orientation within the enzyme's active site. The sequences and crystal structures for bromoperoxidase from *C. officinalis* and *A. nodosum* show that each has a relatively hydrophobic substrate channel with the exception of three hydrophilic amino acids which differ between the enzymes from these two species.[5,7] Within each species these three amino acids are the only hydrophilic residues within 7.5 Å of the vanadate O^4 oxygen atom, except those directly involved in coordination or hydrogen-bonding to the vanadate ion.[5,7] In *A. nodosum* the three amino acids are Ser337, Gln334, and Gln421.[5] In *C. officinalis* they are Glu124, Arg395, and Asp292.[7] These amino acid differences have been suggested to lead to differences in substrate selectivity and specificity for the direct oxidation of sulfides catalyzed by V-BrPO (in the absence of bromide).[5] For example, it has been found that the V-BrPO from *A. nodosum* converts methyl phenyl sulfide to the (*R*)-enantiomer of the sulfoxide, whereas V-BrPO from *C. pilulifera* (highly homologous to *C. officinalis*) produces the (*S*)-enantiomer.[15] Furthermore, V-ClPO (*C. inaequalis*) whose active site differs somewhat compared to that of V-BrPO (*A nodosum* and *C. officinalis*) produces a racemic mixture of sulfoxides.[15] Thus, differences in discrete active-site residues may well account for differences in reactivity. However, in this study, no difference in reactivity or substrate selectivity was detected for V-BrPO from either *A. nodosum* or *C. officinalis*.

Earlier work has shown that the reactivity of V-BrPO with substrates is not consistent with a freely diffusible brominating species.[8,12] From those initial experiments, the nature of this brominating species as enzyme-bound or enzyme-trapped could not be distinguished. If a brominating species is trapped by a docked substrate, one might expect occasional bromination of the indole as a consequence of substrate release and therefore release of the brominating species. However, our current results show that no further bromination of 1,3-di-*tert*-butylindole (**1**) past indolinone formation occurred. Yet, when 1,3-di-*tert*-butyl-2-indolinone (**2**) (or 1,3-di-*tert*-butylindole (**1**)) is reacted with 1 equiv of aqueous bromine added by syringe pump into a solution containing 1 equiv of H_2O_2, a significant level of bromination does occur, even in the presence of the competing reaction of reduction of aqueous bromine by H_2O_2. Similar results were seen by Itoh and colleagues for the substrate anisole (*C. pilulifera*); anisole bromination by NaOBr occurs in the presence of H_2O_2 (pH 6.0), showing that the OBr^- reaction with anisole is competing with the oxidation of H_2O_2 by OBr^-.[9] Furthermore, the quantitative conversion of 1,3-di-*tert*-butylindole to 1,3-di-*tert*-butyl-2-indolinone, under catalytic conditions (i.e., [substrate] $\gg$ [V-BrPO]), suggests that 1,3-di-*tert*-butyl-2-indolinone is not a binding inhibitor of V-BrPO nor acting to potentially block the release of HOBr by V-BrPO. Furthermore, the competitive kinetic profile seen for 1,3-di-*tert*-butyl-2-indolinone demonstrates that it is not an inhibitor of the V-BrPO bromination of phenol red. Thus, under our reaction conditions, the reactivity of V-BrPO from *A. nodosum* is not consistent with the formation and release of hypobromous acid in agreement with Itoh and colleagues (V-BrPO, *C. pilulifera*).[8]

In summary, V-BrPO catalyzes the regiospecific oxidation of 1,3-di-*tert*-butylindole (**1**) in a reaction that requires both H_2O_2 and Br^- as substrates, but which produces the unbrominated 1,3-di-*tert*-butyl-2-indolinone product exclusively. By contrast many products are produced in the reaction with aqueous bromine solution, all of which differ from the V-BrPO-catalyzed product.[31] This is the first example of regiospecific bromination by V-BrPO. Site-directed mutagenesis experiments on V-BrPO are in progress to probe the role of selected active-site amino acid residues in the regio- and stereospecific enzyme-catalyzed reactions.

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(31) The product ratios of the aqueous bromine reactions depend on its rate of addition (Figure 2b and 2b). The molecular basis of this difference is under investigation.