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Bromination of phenols in bromoperoxidase-catalyzed oxidations

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ABSTRACT

Phenol and *ortho*-substituted derivatives furnish products of selective *para*-bromination, if treated with sodium bromide, hydrogen peroxide, and the vanadate(V)-dependent bromoperoxidase I from the brown alga *Ascophyllum nodosum*. Relative rates of bromination in morpholine-4-ethane sulfonic acid (MES)-buffered aqueous *tert*-butanol (pH 6.2) increase by a factor 32, as the *ortho*-substituent in a phenol changes from F via Cl, OCH_3, $C(CH_3)_3$, and H to CH_3. The polar effect in phenol bromination by the enzymatic method, according to a Hammett-correlation ($\rho=-3$), compares to reactivity of molecular bromine under identical conditions ($\rho=-2$). Hypobromous acid is not able to electrophilically substitute bromine for hydrogen at pH 6.2 in aqueous *tert*-butanol. The tribromide anion behaves in MES-buffered aqueous *tert*-butanol as electrophile ($\rho\sim-3$), showing a similar polar effect in phenol bromination as molecular bromine.

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1. Introduction

Bromide, dissolved in ocean water ($c_{Br^-}\sim1$ mM) or deposited in minerals, is the major resource for production of molecular bromine, the most important reagent for synthesis of organobromines.[1–3] In an oxidative environment, such as the marine boundary layer or in hydrogen peroxide-producing compartments of living cells, bromide is rapidly oxidized into hypobromous acid, molecular bromine, and tribromide, to mention the major products.[4] All products of peroxidative bromide oxidation are able to convert hydrocarbons into organobromines, for example, as part of natural product synthesis, although with different functional group selectivity.[5]

Naturally occurring organobromines show considerable structural diversity regarding site of bromosubstitution and carbon skeleton the halogen atom is attached to.[6] Simple bromoalkanes, such as bromomethane or bromopentanes, are similarly found in the environment as more complex metabolites, for example, bromosubstituted fatty acids, phenylpropanes, or amino acids. Probably the most complex structures arise from highly substituted, chiral, cyclic terpenes, and acetogenins.[7,8] Functional groups that appear to be particularly receptive for bromination in putative biogenic progenitors, proposed on the basis of purely structural arguments, are carbon–carbon double bonds of donor-substituted alkenes or arenes, and the aromatic core of π-excess heteroarenes.[9,10]

Biosynthetic pathways for organobromine formation are largely unexplored.[11] From biochemical experiments it is known, that hydrogen peroxide oxidizes bromide at pH 6–7, if catalyzed by vanadate(V)-dependent bromoperoxidases (V_{Br}POs).[12–14] According to the general mechanism of V_{Br}PO-catalyzed bromide oxidation (Scheme 1),[12,14] hydrogen peroxide binds first to vanadate(V), in a reversible proton-assisted step. The peroxido-loaded cofactor is the active form of the enzyme and able react with bromide in a second reversible reaction, to furnish a structurally uncharacterized but kinetically relevant intermediate. Oxygen atom transfer from the peroxido complex to bromide follows, possibly resulting in a short-lived intermediate,[15] which rapidly hydrolyzes, to regenerate by the end of the catalytic cycle the resting state of the enzyme. Hydrolysis of the assumed intermediate furthermore provides one molecule of hypobromous acid, which is considered to be the primary product of enzymatic bromide oxidation (Scheme 1, top).[10,16] In an aqueous solution of bromide, such as ocean water, hypobromous acid, rapidly equilibrates to provide a mixture of molecular bromine, tribromide (Scheme 1, bottom), and possibly further products.[3,12,17]

The chemical behavior of hypobromous acid, bromine, and tribromide toward functional groups relevant for explaining organobromine formation in nature is distinctively different.

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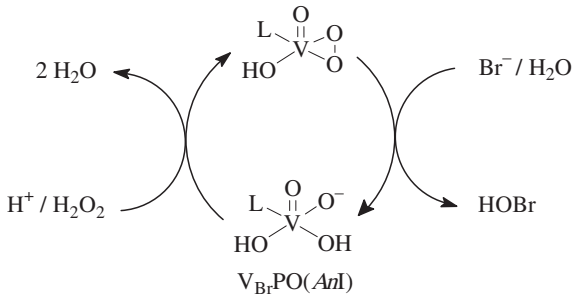
• *bromoperoxidase-catalyzed oxidation – primary product formation*



• *thermodynamic equilibration – secondary product formation*

$$HOBr + Br^- + H^+ \overset{K^1}{\rightleftharpoons} Br_2 + H_2O$$

$$Br_2 + Br^- \overset{K^2}{\rightleftharpoons} Br_3^-$$

Scheme 1. Proposed mechanism for bromide oxidation catalyzed by the vanadate(V)-dependent bromoperoxidase I ($V_{Br}PO$) from *Ascophyllum nodosum* (*An*) (top), and equilibria associated with secondary product formation in bromide-containing brines (bottom) [K^1=for example, 1.45×10^8 M^{-2} for H_2O at 20 °C (I=0.1 M, pH 2.6–3.8)[26] or 1.04×10^8 M^{-2} in H_2O at 25 °C (pH 1.5);[27] K^2=16.9 M^{-1} in H_2O at 25 °C[28]].[3]

Hypobromous acid bromohydroxylates alkenes to afford 2-bromoalcohols (bromohydrins). Unless activated by a strong Brønsted-acid, hypobromous acid is comparatively inert toward arenes and not able to electrophilically displace bromine for hydrogen. Tribromide, a weakly bound adduct between bromine and bromide, shows chemical reactivity at the borderline between nucleophilic and electrophilic,[18–20] and reacts with alkenes in polar aprotic solvents with different selectivity for vicinal dibromination than molecular bromine.[21–23] In protic solvents, such as water[24] or acetic acid,[25] tribromide seems to predominantly serve as safe-to handle substitute for bromine, liberating the halogen at a steady rate for converting, for example, phenol, 2-naphthol, aniline, and other strongly activated arenes into bromoderivatives with bromine-like selectivity.

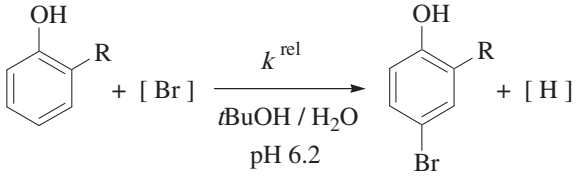
In bromoperoxidase chemistry, the chemical nature of the reagent that controls reactivity and selectivity for carbon–bromine bond formation, so far has not been determined. Endogeneous substrates that bind to the active site in vanadate(V)-dependent bromoperoxidases are bromide and hydrogen peroxide. A binding site for an organic substrate close to the vanadate co-factor has not yet been identified. This observation correlates with the lack in hydrocarbon specificity and regio- or stereoselectivity for carbon–bromine bond formation in bromoperoxidase-catalyzed oxidations.[3,10]

In a project dealing with biomimetic synthesis of brominated natural products, we encountered the problem to predict selectivity for preparing the correct starting material via bromoperoxidase-catalyzed oxidation. We therefore specified the chemical nature of the bromoelectrophile that is directly involved in carbon–bromine bond formation in enzymatic reactions. The major results from a combined synthetic and competition kinetic study on phenol bromination show that bromination in oxidations catalyzed by the vanadate(V)-dependent bromoperoxidase I from the brown alga *Ascophyllum nodosum* [$V_{Br}PO$(*An*I)] shows considerable parallels to the chemistry of molecular bromine in water. This information allowed us to derive a reaction model for explaining a striking difference between phenol- and anisol bromination.

2. Results and discussion

2.1. Concept

The strategy used in this study to reference reactivity and selectivity in bromoperoxidase-catalyzed oxidation combines a product study for determining individual reactivity of *ortho*-substituted phenols in enzymatic reactions with competition kinetics for determining polar substituent effects on rates in phenol bromination. The results from bromoperoxidase-catalyzed oxidations subsequently were compared to controls, using hypobromous acid, molecular bromine, and tetrabutylammonium tribromide as bromination reagents (Scheme 2).



[Br] = Br_2, HOBr, NBu_4Br_3, $NaBr / H_2O_2 / V_{Br}PO$(*An*I)

1, **2**	R
a	H
b	CH_3
c	$C(CH_3)_3$
d	OCH_3
e	Cl
f	F

Scheme 2. Summary of bromination reagents and indexing of phenols used in this study ([H]=e.g., H^+).

The approach in chemistry to quantify polar substituent effects on chemical reactivity is correlation analysis.[29,30] In the present study, we used the phenol competition system to determine relative rates of bromination in bromoperoxidase-catalyzed oxidations, and in non-enzymatic references (Scheme 2). Correlation of decadic logarithms of relative rate constants $\lg k^{rel}=\lg(k^R/k^H)$ with substituent constants[31] σ_m, according to equation 1, provides the reaction parameter ρ. Sign and magnitude of ρ characterize responsivity of polar substituent effects for accelerating or retarding the rate determining step, and helps to characterize the chemical nature of the reagent that is directly involved in the reactivity determining step.

$$\lg k^{rel} = \lg \frac{k^R}{k^H} = \rho \cdot \sigma_m \quad (1)$$

2.2. Bromoperoxidase isolation and preparation of bromination reagents

The bromoperoxidase used in this study is the isoenzyme I from the brown alga *A. nodosum* [$V_{Br}PO$(*An*I), EC 1.11.1.18, PDB-code 1QI9].[32,33] The enzyme was isolated from specimen collected in Roscoff, France, following an established freeze–drying, milling, and liquid–liquid partitioning process.[34] The crude bromoperoxidase fraction, which precipitated upon addition of acetone from the final extract of the extraction process, was dialyzed against sodium metavanadate for restoring bromoperoxidase activity of the

protein. From the mixture of active bromoperoxidases, $V_{Br}PO(AnI)$ was separated by hydrophobic interaction chromatography and subsequent size exclusion chromatography, providing samples of specific activity between 558±11 $U_T^0\,mg^{-1}$ to 611±6 $U_T^0\,mg^{-1}$, based on the triiodide assay[35,36] (pH 6.2; phosphate buffer).

For synthetic and kinetic experiments on phenol bromination with the $V_{Br}PO(AnI)$, we used a solvent/buffer-combination composed of 25 volume percent of *tert*-butanol for dissolving the organic components, 75 volume percent of water as major reaction medium, and morpholine-4-ethane sulfonic acid (MES) to maintain pH of the solutions close to 6.2. In reference reactions for phenol bromination starting from hypobromous acid, molecular bromine, and tetrabutylammonium tribromide, we tried as close as possible to adhere to these conditions.

The applied solution of hypobromous acid was prepared from bromine and silver nitrate in aqueous MES-buffer, containing 25vol% of *tert*-butanol. From the solubility product of silver bromide in water [$4.948\cdot10^{-3}$ (mol/L),[2] 25 °C],[37] we estimated a residual bromide concentration in this solution of 7×10^{-7} M.

Since bromine in the required concentration of 0.75 M did not quantitatively dissolve in the solvent of interest, we added the halogen for synthesis and for competition kinetics as solution in *tert*-butanol via syringe pump to solutions containing a phenol **1** in aqueous MES-buffered *tert*-butanol.

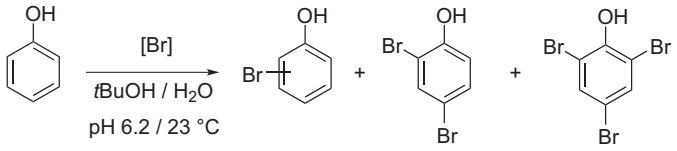
Tribromide salts having tetrabutylammonium or cesium as countercation, are sparingly soluble in *tert*-butanol and aqueous solutions of this alcohol. We therefore added tetrabutylammonium tribromide as single batch to MES-buffered solutions of a phenol **1** in aqueous *tert*-butanol for bromophenol syntheses, and as homogeneous solution in acetonitrile via syringe pump for competition kinetic experiments.

2.3. Phenol bromination in aqueous *tert*-butanol

2.3.1. Bromophenol synthesis. For phenol bromination via enzymatic oxidation, we used 17.3 units (U_T) of $V_{Br}PO(AnI)$, referenced versus the triiodide assay, and the solvent/buffer-system outlined in the previous section. To minimize bromoperoxidase-activity loss, we used sodium bromide and the organic substrate in an equimolar ratio and added not more than 1.1 equiv of hydrogen peroxide as 0.825 M solution in aqueous *tert*-butanol (pH 6.2) with a syringe pump to the solution containing the enzyme, the phenol **1**, and sodium bromide in the same solvent.[14] Under such conditions, 90% of phenol (**1a**) (0.75 mmol) were converted into bromophenols **2a**–**4a** with a total yield of 79% (Table 1, entry 1). By the end of the reaction, the original pH of the mixture had increased to 6.8. A noteworthy feature of the enzymatic reaction is the selectivity for providing monobromophenol **2a** as major product (vide infra).

If 1 equiv of hypobromous acid is added to a solution of phenol (**1a**) in MES-buffered (500 mM) aqueous *tert*-butanol, the pH of the solution immediately changes from 6.2 to 5.7. This solution, however, does not afford within a standard reaction time of 72 h any of the bromophenols **2a**–**4a** (Table 1, entry 2). By successively increasing the amount of hypobromous acid from one via two to three aliquots, the pH gradually declines from 5.7 to 1, still without any sign of bromophenol formation (Table 1, entries 2–4). As we reduced the buffer capacity to 50 mM, the pH of the reaction mixture decreased to 1, directly after adding the first equivalent of hypobromous acid. From this solution we isolated 21% of bromophenol (**2a**) with an *ortho*/*para*-selectivity of 24:76. The yield of bromophenol **2a** further increased as we doubled (47%) and tripled (57%) the amount of hypobromous acid, without finding experimental evidence for dibromophenol- and tribromophenol formation (Supplementary data). From the results we concluded that hypobromous acid under mildly acidic conditions, used for conducting bromoperoxidase-catalyzed oxidations, is not able to brominate phenol (**1a**).

Table 1
Products of phenol bromination formed in bromoperoxidase-catalyzed oxidation and non-enzymatic reactions



Entry	[Br]	$[Br]_{eq.}$	Conv. **1a**/%[a]	**2a**/% (*o*/*p*)[b]	**3a**/%	**4a**/%	pH[c]
1	$NaBr/H_2O_2/V_{Br}PO(AnI)$	1	90	69 (9/91)	4	6	6.8
2	HOBr	1	6	—[d]	—[d]	—[d]	5.7
3	HOBr	2	—[d]	—[d]	—[d]	—[d]	3.1
4	HOBr	3	6	—[d]	—[d]	—[d]	1.0
5	Br_2	1	53	17 (18/82)	3	29	6.2
6	Br_2	2	80	33 (18/82)	7	38	5.9
7	Br_2	3	*quant.*	13(<2/98)	8	77	5.3
8	NBu_4Br_3	1	53	14 (<2/98)	13	23	6.1
9	NBu_4Br_3	2	83	10 (<2/98)	14	59	6.0
10	NBu_4Br_3	3	*quant.*	—[d]	—[d]	99	5.8

[a] Continuous addition (syringe pump, 8 h) of a solution of H_2O_2 (0.825 M, 1.1 equiv) and NaBr (0.75 mmol, 1.0 equiv) in aq MES buffer (500 mM, pH 6.2) {$V_{Br}PO(AnI)$ [55.2 μL, 0.5626 mg/mL, 558 U_T/mg, 17.3 U_T, 0.036 mmol%} (entry 1); batch addition of a solution of HOBr in aq MES buffer (500 mM) (0.2–0.3 M, 0.75–2.25 mmol, 1.0–3.0 equiv, pH 1) (entries 2–4); continuous addition (syringe pump, 8 h) of a solution of Br_2 (0.75–2.25 M, 1.0–3.0 equiv) (entries 5–7); batch addition of $TABABr_3$ (0.75–2.25 mmol, 1.0–3.0 equiv) (entries 8–10); 0.75 mmol of **1a** were used as starting material for all entries; the final reaction medium was aq MES buffer (pH 6.2)/^{t}BuOH=75/25% (*v*/*v*) for all entries.

[b] *ortho* and *para* relative to OH; unless otherwise stated ^{1}H NMR yields.

[c] pH-electrode (for pH 3.1–6.8) or pH-indicator paper (for pH<3), pH-value after reaction of the aqueous layer.

[d] Not detectable.

Molecular bromine rapidly converts phenol (**1a**) in solutions of aqueous *tert*-butanol (pH 6.2) into bromophenols **2a**–**4a**, with the substrate conversion and product formation improving as the amount of bromine increases from one to three aliquots (Table 1, entries 5–7). The main product from the synthesis is 2,4,6-tribromophenol (**4a**), which correlates with information from a standard laboratory procedure.[38] The yields of bromophenols remained slightly below the values obtained from bromoperoxidase-catalyzed oxidations.

Tetrabutylammonium tribromide, if added as neat solid to solution of phenol (**1a**) in aqueous *tert*-butanol (pH 6.2), furnishes a slurry that turns into a homogeneous clear solution by the end of the reaction. From this solution, we isolated tribromophenol **4a** in yields that gradually increases as the amount of tribromide is raised from one (23%) via two (59%) to three aliquots (99%; Table 1, entries 8–10). Singly and doubly brominated products **2a** and **3a** appeared as minor components in experiments performed with one and two equiv of the bromination reagent. In a control on the solvent effect in tetrabutylammonium tribromide-mediated phenol bromination, ratios of mono- versus di- and tribromide, and the yield of products **2a**–**4a** remained unchanged, as we replaced *tert*-butanol by acetonitrile, or administered the bromination reagent either as neat material batchwise or as homogeneous solution in acetonitrile with a syringe pump. From this information we concluded that acetonitrile and *tert*-butanol have a similar solvent effect in tetrabutylammonium tribromide-mediated phenol bromination and are able to replace one another without changing selectivity.

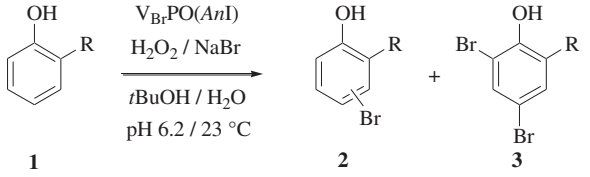
To sum up, molecular bromine, tetrabutylammonium tribromide, and the combination of sodium bromide, hydrogen peroxide, and $V_{Br}PO(AnI)$ are able to brominate phenol (**1a**) in aqueous *tert*-butanol (pH 6.2). The enzymatic oxidation selectively furnishes

monobromophenol **2a**, whereas bromine- and tribromide-mediated reactions give tribromide **4a** as major product.

2.3.2. Bromination of ortho-substituted phenols in bromoperoxidase-catalyzed oxidation. Prior to conducting competition kinetic experiments, we verified that *ortho*-substituted phenols **1b**–**f** provide in bromoperoxidase-catalyzed oxidations comparable yields of bromophenols **2b**–**f**. The substrates chosen for relative rate analysis, and thus the synthetic part of the study, bear a methyl-, *tert*-butyl-, methoxy-, chloro-, and a fluoro- substituent in *ortho*-position to the phenolic hydroxyl group. The hydroxyl group is a strong *para*-directing group in electrophilic aromatic substitution, which we hypothesized also to operate as underlying mechanism in enzymatic reactions. Decisive selectivity for aromatic substitution *para* to the hydroxyl group, being *meta* to R, is a prerequisite for obtaining meaningful information from Hammett-correlations. For the synthetic study, we restricted ourselves to bromoperoxidase-catalyzed oxidations, since bromine and tetrabutylammonium tribromide have been successfully used before for this purpose.[39–43]

For attaining maximum yields of bromophenols **2b**–**f**, and minimizing bromoperoxidase activity loss, we added 1.1 equiv of hydrogen peroxide as a 0.825 M, MES-buffered solution of *tert*-butanol via syringe pump to a reactant solution composed of one equivalent of sodium bromide, 17.3 U_T of $V_{Br}PO(AnI)$, and an *ortho*-substituted phenol (compounds **1b**–**f**; 0.75 mmol) in aqueous *tert*-butanol (pH 6.2, MES-buffer). From likewise prepared reaction mixtures, we isolated bromophenols **2b**–**f** as major compounds (Table 2). The yields of target products **2b**–**f** depended on the nature of the *ortho*-substituent in **1b**–**f**, and gradually decreased along the series of *ortho*-substituents R=CH_3 (68%), via OCH_3 (56%), $C(CH_3)_3$ (42%), F (19%) to Cl (13%). In the latter two instances, we isolated 18–26% of dibromides **3e** and **3f** (Table 2, entries 5–6), leading to a satisfactory mass balance for sodium bromide utilization between 46% (for **1c**) and 95% (**1a**; cf. Table 2).

Table 2
Products of phenol bromination in bromoperoxidase-catalyzed oxidation



Entry	**1**–**3**	R	Conv. **1**/%[a]	**2**/% (*o*/*p*)[b]	**3**/%	Mass balance/%[c]	pH^{final} [d]	Activity U_T^{final} mg^{-1}[e]
1	**a**	H	90[f]	69 (9/91)	4	89	6.8	133
2	**b**	CH_3	86	68 (16/84)	3	85	6.5	138
3	**c**	$C(CH_3)_3$	53	42 (36/64)	2	91	6.4	—[g]
4	**d**	OCH_3	68	56 (21/79)	10	98	6.4	—[g]
5	**e**	Cl	43	13 (<2/98)	26	96	6.3	129
6	**f**	F	51	19 (<2/98)	18	86	6.4	160

[a] Continuous addition (syringe pump, 8 h) of a solution of H_2O_2 (0.825 M, 1.1 equiv) and NaBr (0.75 mmol, 1.0 equiv) in aq MES buffer (pH 6.2)/^{t}BuOH=75/25% (*v*/*v*); 0.75 mmol of **1** were used as starting material.
[b] *ortho* and *para* relative to OH; unless otherwise stated ^{1}H NMR yields.
[c] Sum of recovered **1** and bromides **2**–**4**.
[d] pH electrode.
[e] Initial enzyme activity 558 U_T^0 mg^{-1} (entries 1 and 3) and 611 U_T^0 mg^{-1} (entries 2 and 4–6).
[f] Additional product: 6% of 2,4,6-tribromophenol (**4a**).
[g] No final bromoperoxidase activity detected.

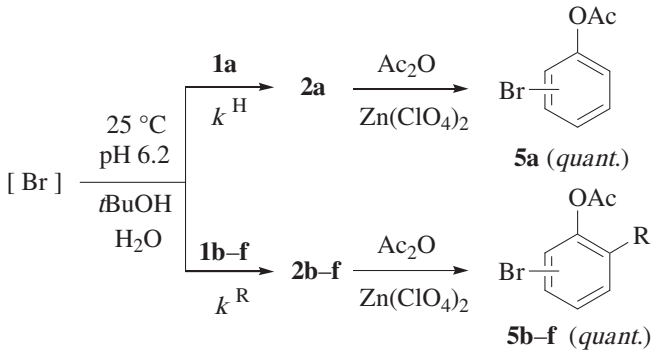
By the end of the enzymatic oxidation, the pH had slightly increased to 6.4–6.8. Final bromoperoxidase activity had declined to 18–27% of the initial value, except of experiments on guaiacol- and *tert*-butyl phenol bromination, which consistently led to quantitative $V_{Br}PO(AnI)$-activity loss (Table 2, entries 3 and 4).

2.4. Competition kinetics and correlation analysis

With the knowledge about the performance of $V_{Br}PO(AnI)$ in synthetic scale oxidative brominations, we turned our attention to competition kinetics for determining relative reactivity of phenols under such conditions. The underlying reaction model thereby considers, for reasons given above, no organic substrate binding to the vanadate active site. Phenol bromination in this model occurs at some distance from the active site, and it was the aim of the kinetic part of the study to clarify whether the reactivity determining bromoelectrophile compares to a reagent familiar from the chemistry of bromine in water.

In the general experimental set-up for competition kinetics under pseudo-first order conditions, bromoperoxidase-catalyzed oxidations furnished exclusively products of monobromination. The fractions of *ortho*-substituted products and the yields of bromophenols **2a**–**f** obtained from the diluted solutions used for competition kinetics [62±4% for **2a**+**2b**, 81±17% for **2a**+**2c**, 34±3% for **2a**+**2d**, 92±4% for **2a**+**2e**, and 78±11% for **2a**+**2f**] in most instances were higher than those obtained from the more concentrated solutions applied for synthesis (Supplementary data). Competition kinetics for phenol bromination by tetrabutylammonium tribromide surprisingly provided substantial amounts of dibrominated halophenols **3e**–**f**. All attempts to circumvent this problem by lowering phenol concentrations, or using phenols in larger excess, failed to solve this problem. We therefore excluded substrates **1e** and **1f** from correlation analysis for tribromide-mediated reactions.

Pre-kinetic evaluations furthermore clarified that bromophenols **2a**–**f** had to be derivatized for obtaining reproducible yields from gas chromatographic analysis. We found that the combination of acetic anhydride and catalytic amounts of zinc perchlorate-hexahydrate[44] quantitatively copies ratios of **2a** versus **2b**–**f** into ratios of *O*-acetyl derivatives **5a** versus **5b**–**f** (Scheme 3, Supplementary data), which solved the analytical problem.



$$\frac{[\,\mathbf{5b–f}\,]}{[\,\mathbf{5a}\,]} = \frac{k^R}{k^H}\frac{[\,\mathbf{1b–f}\,]}{[\,\mathbf{1a}\,]} \qquad \text{(eq. 2)}$$

Scheme 3. Reaction scheme for kinetic experiments in the phenol-competition system {for data analysis, see equations 1–2; [Br]=Br_2, NBu_4Br_3, or $NaBr/H_2O_2/V_{Br}PO(AnI)$}.

From competition experiments performed at three different ratios of phenol **1a** versus one of the reporter substrates **1b**–**f** at fixed bromide-, hydrogen peroxide-, and bromoperoxidase concentration we derived from linear correlations according to equation 2 relative rate constants $k^{rel}=k^R/k^H$ (Supplementary data). Correlations considering exclusively products of *para*-bromination hereafter are abbreviated as $k_p^{rel} = k_p^R/k_p^H$, and those taking

products of *ortho*- and *para*-bromination into account as $k_{op}^{rel} = k_{op}^{R}/k_{op}^{H}$. The correlation coefficients ($R2$) of the fits for calculating k^{rel}-values were satisfactory ($R2$ for k_p^{rel}=0.995 for **1b**, 0.909 for **1c**, 0.963 for **1d**, 0.855 for **1e**, and 0.999 for **1f**).

The kinetic data show that the rate of phenol bromination in bromoperoxidase-catalyzed oxidation, as expressed in k_p^{rel} *and* k_{op}^{rel}, gradually increases, along the sequence of *ortho*-substituents in **1** from R=CH_3 via C(CH_3)$_3$, OCH_3, and Cl to F (Table 3). Relative rates for the most reactive phenol (**1b**, R=CH_3) and the least reactive substrate (**1f**, R=F) differed by a factor 32 for k_p^{rel} and 16 for k_{op}^{rel}. The difference between the two values arises from a change in *ortho*/*para*-selectivity from 26:74 for phenols **1b**–**d** [R=CH_3, C(CH_3)$_3$, and OCH_3] to 47:53 for **1e**–**f** (R=Cl, F; Supplementary data).

Table 3
Relative rate constants of phenol bromination[a] in bromoperoxidase-catalyzed oxidation and non-enzymatic methods, and σ_m parameters[31] used for correlation analysis

Entry	R/**1**	σ_m	$V_{Br}PO(AnI)$		Br_2		NBu_4Br_3	
			k_p^R/k_p^H	k_{op}^R/k_{op}^H	k_p^R/k_p^H	k_{op}^R/k_{op}^H	k_p^R/k_p^H	k_{op}^R/k_{op}^H
1	CH_3/**1b**	−0.11	2.25	1.89	1.35	1.17	3.15	2.96
2	C(CH_3)$_3$/**1c**	−0.02	0.98	0.67	—[b]	—[b]	—[b]	—[b]
3	OCH_3/**1d**	0.05	0.48	0.43	0.97	1.18	0.99	1.37
4	Cl/**1e**	0.31	0.10	0.15	0.22	0.21	—[c]	—[c]
5	F/**1f**	0.36	0.07	0.12	0.19	0.18	—[c]	—[c]

[a] *o*(*rtho*) and *p*(*ara*) with respect to the hydroxyl group in **1**; *op* refers to values considering products of *ortho*- and *para*-substitution.
[b] Not considered due to a poor quality in correlation analysis.
[c] Not considered for correlation analysis due to dibromide formation.

The reactivity of phenols **1b**–**f** toward molecular bromine in aqueous *tert*-butanol (pH 6.2) follows the same trend as described for $V_{Br}PO(AnI)$-catalyzed oxidations. The fastest (**1b**, R=CH_3) and the slowest reaction (**1f**, R=F) differed by a factor 7.1 for k_p^{rel} and 6.5 for k_{op}^{rel}. The *ortho*/*para*-ratio in all phenol brominations by bromine under pseudo-first order conditions was close to 25:75.

The data available for classifying the polar effect in phenol bromination by tetrabutylammonium tribromide show that *ortho*-cresol **1b** reacts by a factor 3.2 for *para*-bromination and 2.2 for *ortho*- and *para*-bromination faster than phenol (**1a**), whereas reference **1a** is approximately similar reactive as guaiacol **1d**. The *ortho*/*para*-ratio in the competition experiment is about 10:90 for **2b**, and 30:70 for **2d**.

From correlation of decadic logarithms of relative rate constants k^{rel} and substituent constants σ_m, we obtained reaction parameters ρ for the three bromination methods (Table 3). The σ_m-parameter selected for correlation analysis refers to fluorine-19 NMR-chemical shift-changes caused by substituent R in *meta*-substituted fluorobenzenes,[31] and is almost independent from solvent effects. This substituent parameter provided excellent correlations (Fig. 1),

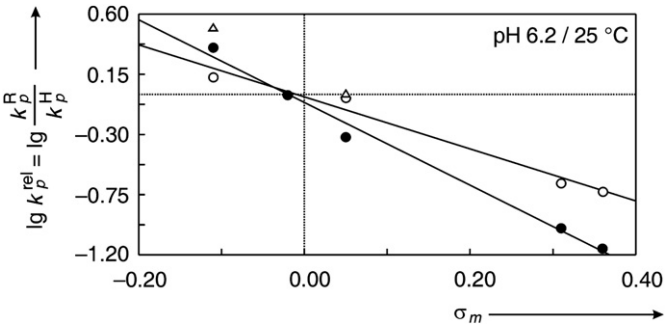


Fig. 1. Correlation of log k^{rel} from phenol bromination by bromine (○) (correlation coefficient $R2$=0.958), tetrabutylammonium tribromide (△), and the combination of sodium bromide, hydrogen peroxide, and $V_{Br}PO(AnI)$ (●) ($R2$=0.995) versus σ_m according to equation 1.

whereas others, such as the original values proposed by Hammett,[29] and alternative values put forward by Taft, resulted in a scatter of data.[30,31]

The ρ-value for describing *para*-substitution of **1** (ρ_p=−3.1) in $V_{Br}PO(AnI)$-catalyzed oxidations is slightly more negative than the value referring to *ortho*- and *para*-bromination (ρ_{op}=−2.3; Table 4, entry 1). Since *ortho*-substitution adds a steric component to chemical reactivity that is not covered by σ_m, and k_{op}^{rel} summarizes effects from two elementary reactions, which not necessarily have to respond similarly to polar effects, we restricted ourselves for the general mechanistic discussion to compare of ρ_p-values rather than the ρ_{op}-values.

Table 4
Reaction parameter ρ for phenol bromination in aqueous *tert*-butanol (pH 6.2, 25 °C)

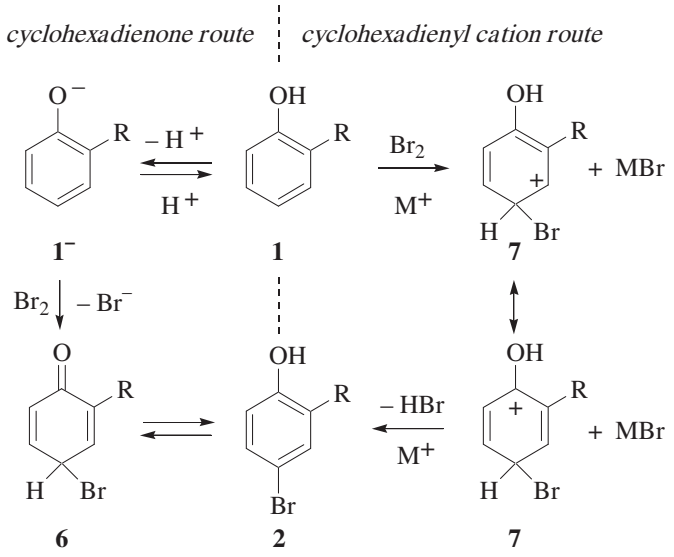
Entry	[Br]	ρ_p	ρ_{op}
1	$NaBr/H_2O_2/V_{Br}PO(AnI)$	−3.1	−2.3
2	Br_2	−1.9	−1.9
3	NBu_4Br_3	(−3.1)[a]	(−3.0)[a]

[a] Estimated on the basis of k^{rel} values for **1b** and **1d** in MES-buffered aqueous acetonitrile (see Table 3).

The reaction parameters for phenol bromination in aqueous *tert*-butanol (pH 6.2) by molecular bromine (ρ_p=−1.9), via $V_{Br}PO(AnI)$-catalyzed oxidations, (ρ_p=−3.1), and the approximated value for tetrabutylammonium tribromide-mediated brominations (Table 4) are within a reasonable confidence level for correlation analysis similar. Since electron releasing substituents enhance relative rates of phenol bromination, the sign of ρ_p is negative and shows that the attacking bromination reagent is an electrophile.

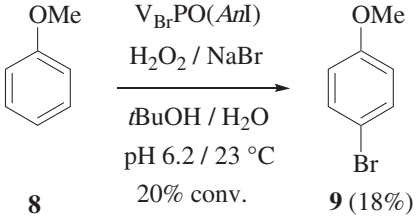
According to the data summarized in the previous paragraphs, reactivity and selectivity of phenol bromination in bromoperoxidase-catalyzed oxidation is best described by the chemistry of molecular bromine in water.[45,46] Alternative reagents, such as hypobromous acid or the tribromide-anion, are expected to co-exist with bromine in typical bromoperoxidase reaction mixtures, but are not expected to be directly involved in the carbon–bromine bond forming step.

2.4.1. Mechanistic implications. The noteworthy propensity of molecular bromine for electrophilically displacing hydrogen in phenols under physiological conditions arises from a mechanism for aromatic substitution, which proceeds via cyclohexadienone intermediates (e.g., **6**) instead of cyclohexadienyl cations (e.g., **7**; Scheme 4).[45,47,48] 4-Bromocyclohexadienones **6** or the 6-bromoisomers (not shown in Scheme 4), according to this mechanism, are formed in the reaction between a phenolate $\mathbf{1}^-$ and molecular bromine. Although the phenolate fraction in aqueous solution at pH 6.3, the average value in bromoperoxidase-catalyzed oxidations, is very small (2×10^{-4} for $\mathbf{1a}^-$/**1a**; for other $\mathbf{1}^-$/**1**-ratios,[49–54] see the Supplementary data), this mechanism is the kinetically favored pathway for phenol bromination. The rate constant, for example, for the reaction between $\mathbf{1a}^-$ and bromine (k=2.4×10^{10} M^{-1} s^{-1}; pH 7, 25 °C) is close to the diffusion limit,[56] whereas bromophenol formation from **1a** and bromine is by five orders of magnitude slower (4.1×10^5 M^{-1} s^{-1}; pH 7, 25 °C). The alternative mechanism furthermore helps to explain the magnitude of experimentally determined ρ-values for phenol bromination in water, being less negative for reactions proceeding via uncharged cyclohexadienone intermediates [−3.1 (Table 4, entry 1), −2.9[55] or −5.2 under slightly different conditions[56]] than for electrophilic halogenations via cyclohexadienyl cations ($-8>\rho>-12$).[57,58]



Scheme 4. Mechanistic pathways for phenol bromination in aqueous solution[45,56] [R=for example, CH_3, $C(CH_3)_3$, H, OCH_3, Cl, F; M^+=Lewis- or a Brønsted-acid].

To test the hypothesis that phenol bromination in bromoperoxidase-catalyzed oxidation follows the cyclohexadienone route, we investigated the chemistry of anisole bromination in enzymatic reactions (Scheme 5). Bromination of the phenol ether has to follow the cyclohexadienyl cation route and should therefore occur with a slower rate than phenol bromination.



Scheme 5. Anisole bromination in bromoperoxidase-catalyzed oxidation.

Under standard conditions of bromoperoxidase-catalyzed oxidations used in this study, anisole bromination consistently stopped at about 22% conversion to furnish 18% of *para*-bromoanisole **9**. Attempts to raise the yield of bromoether **9** by increasing the amount of enzyme to 34.6 U_T, and adding further sodium bromide- and hydrogen peroxide aliquots, until the reaction mixture contained a twofold excess of the reagents, failed to improve rate and efficiency of anisole turnover. A competition kinetic experiment performed with a 100/10-anisole/phenol-ratio referenced versus one equivalent of sodium bromide and hydrogen peroxide, provided 14% of bromoanisole **9** (*ortho*:*para*=43:57) and 86% of bromophenol **2a** (*ortho*:*para*=24:76). From these data we estimated that anisole bromination occurs by a factor of 60 slower than phenol bromination, which is in line with a mechanistic interpretation outlined above.

3. Concluding remarks

The chemistry of phenol bromination that follows bromide oxidation by hydrogen peroxide in reactions catalyzed by the vanadate(V)-dependent bromoperoxidase I from the brown alga *A. nodosum* [$V_{Br}PO(AnI)$] shows considerable parallels to the chemistry of molecular bromine in water. This information closes the gap between the mechanistic details for bromide oxidation derived from steady-state kinetics (Scheme 1), and reactivity/selectivity-guidelines required to apply the enyzme in synthesis. $V_{Br}PO(AnI)$ is readily available from renewable resources, retains full bromoperoxidase activity if stored in Tris/HCl-buffer, is comparatively thermostable, and tolerates significant concentrations of organic co-solvents, and substrates. On the basis of the findings summarized in this article, we think that synthesis of organobromines via bromoperoxidase-catalyzed oxidation using hydrogen peroxide as terminal oxidant and ocean water as bromide source is an attractive perspective, worth-while to pursue for further developing sustainable synthesis.

Alternative bromine compounds for mechanistically explaining phenol bromination, such as hypobromous acid and tribromide, are for thermochemical reasons expected to co-exist with bromine in typical bromoperoxidase reaction mixtures, but seem not to be directly involved in carbon-bromine bond formation. Hypobromous acid, for example, does not react with phenols at pH 6.2 in aqueous morpholine-4-ethane sulfonic acid-buffered solution. The tribromide ion dissociates in protic solvents providing reactivity and selectivity for arene bromination that is largely governed by molecular bromine.[24,25]

As the mechanistic picture for organic substrate bromination in oxidations catalyzed by $V_{Br}PO(AnI)$ becomes clearer, the role of the bromoperoxidase protein on selectivity deserves closer attention. Once the primary product hypobromous acid diffuses from the active site (cf. Scheme 1) and secondary products, such as bromine and tribromide have been formed, hydrocarbon bromination still can take place at the outer rim of the bromoperoxidase protein. The fact that phenol (**1a**) furnishes tribromophenol **4a** from the reaction between molecular bromine in aqueous solution, but monobromophenol **2a** in the bromoperoxidase-catalyzed reaction points to such a selectivity effect. Competition kinetic data show, that this selectivity effect is not relevant for describing chemical reactivity, as expressed in relative rate constants and the Hammett-parameter ρ. A selectivity effect originating from the bromoperoxidase protein or possibly from an external co-factor is an attractive feature for attaining new means of stereocontrol in this chemistry and therefore is being addressed at the moment in our laboratory.

4. Experimental

4.1. General remarks

Standard instrumentation and general remarks have been disclosed previously (see also ESI). All solvents and reagents were purified following recommended standard procedures.[59] $V_{Br}PO(AnI)$ was isolated from *A. nodosum* collected in April 2009 (France, 48° 43′ N, 3° 58′ W) as described previously.[34]

4.2. Phenol bromination

4.2.1. Phenol bromination with HOBr—general procedure A. A solution of HOBr[60,61] (0.2–0.3 M, 0.75–2.25 mmol, 1.0–3.0 equiv, pH^{final} 1 for all reactions, 3.0–9.0 mL) in aq MES buffer (500 mM) was added to a solution of substrate **1a** (0.75 mmol) in MES-buffer (500 mM, pH^{final} 6.2, 2–8 mL) and ^{t}BuOH (3.3 mL). The reaction mixture was stirred at 23 °C for 3 d. The aqueous layer was extracted with Et_2O (3×20 mL). Combined organic extracts were dried ($MgSO_4$). The solvent was removed under reduced pressure (40 mbar, 40 °C) to afford a product mixture, which was analyzed by ^{1}H NMR and GC, using pentachlorobenzene (^{1}H NMR) as an internal standard in comparison to spectroscopic data from authentic references. The pH-value of the aqueous layer was determined at the end of reaction. For spectroscopic data of **2a$_{4\text{-Br}}$**,

$2a_{2\text{-Br}}$, **$3a_{2,4\text{-Br}_2}$**, and **$4a_{2,4,6\text{-Br}_3}$** refer to Section 4.3.2 of the experimental part. For yields refer to Table 1.

4.2.2. Phenol bromination with Br_2—general procedure B. A solution of Br_2 (120–360 mg, 0.75–2.25 mmol) in ^{t}BuOH (1.0 mL) was added with a syringe pump (8 h, 2.08 μL/min) to a solution of substrate **1a** (0.75 mmol) in MES-buffer (500 mM, pH^{final} 6.2, 11.0 mL) and ^{t}BuOH (2.3 mL). The reaction mixture was stirred at 23 °C for 3 d. The aqueous layer was extracted with Et_2O (3×20 mL). Combined organic extracts were washed with aq $Na_2S_2O_3$ solution (0.1 M, 25 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure (40 mbar, 40 °C) to afford a product mixture, which was analyzed by ^{1}H NMR and GC, using pentachlorobenzene (^{1}H NMR) as an internal standard in comparison to spectroscopic data from authentic references. The pH-value of the aqueous layer was determined at the end of reaction. For spectroscopic data of **$2a_{4\text{-Br}}$**, **$2a_{2\text{-Br}}$**, **$3a_{2,4\text{-Br}_2}$**, and **$4a_{2,4,6\text{-Br}_3}$** refer to section 4.3.2. of the experimental part. For yields refer to Table 1.

4.2.3. Phenol bromination with NBu_4Br_3—general procedure C. NBu_4Br_3 (362–723 mg, 0.75–2.25 mmol) was added to a solution of substrate **1a** (0.75 mmol) in MES-buffer (500 mM, pH^{final} 6.2, 11.0 mL) and ^{t}BuOH (3.3 mL). The suspension was stirred at 23 °C for 3 d. The aqueous layer was extracted with Et_2O (3×20 mL). Combined organic extracts were dried ($MgSO_4$). The solvent was removed under reduced pressure (40 mbar, 40 °C) to afford a product mixture, which was analyzed by ^{1}H NMR and GC, using pentachlorobenzene (^{1}H NMR) as an internal standard in comparison to spectral data from authentic references. The pH-value of the aqueous layer was determined at the end of reaction. For spectroscopic data of **$2a_{4\text{-Br}}$**, **$2a_{2\text{-Br}}$**, **$3a_{2,4\text{-Br}_2}$**, and **$4a_{2,4,6\text{-Br}_3}$** refer to Section 4.3.2 of the experimental part. For yields refer to Table 1.

4.3. $V_{Br}PO(AnI)$-catalyzed reactions

4.3.1. General procedure D. A solution of H_2O_2 (1.0 mL, 0.825 M) and NaBr (77.2 mg, 0.75 mmol) in MES-buffer (500 mM, pH 6.2) was added with a syringe pump (8 h, 2.08 μL/min) to a solution of substrate **1**, or **8** (0.75 mmol) and $V_{Br}PO(AnI)$ [55.2 μL (0.5626 mg/mL; 558 U_T/mg), 17.3 U_T, 0.036 mmol% for **1a**, **1c**, and **8**; 62.8 μL (0.4505 mg/mL; 611 U_T/mg), 17.3 U_T, 0.031 mmol% for **1b** and **1d**–**f**] in MES-buffer (500 mM, pH 6.2, 10.0 mL) and *t*BuOH (3.3 mL). The reaction mixture was stirred at 23 °C for 3 d. The aqueous layer was extracted with Et_2O (4×10 mL). Combined organic extracts were dried ($MgSO_4$). The solvent was removed under reduced pressure (14 mbar, 40 °C) to afford a product mixture, which was analyzed by ^{1}H NMR or GC, using pentachlorobenzene (^{1}H NMR, **1a**–**d** and), 1-bromodecane (^{1}H NMR, for **1e**), or *n*-decane (GC, for **8**) as an internal standard in comparison to spectroscopic data from authentic references. pH and bromoperoxidase activity (triiodide assay[35]) were determined from the aqueous layer.

*4.3.2. Bromination of phenol (**1a**).* Reactants: **1a** (70.6 mg, 0.75 mmol), NaBr (77.2 mg, 0.75 mmol), H_2O_2 (1.0 mL, 0.825 M), and $V_{Br}PO(AnI)$ (17.3 U_T, 0.036 mmol%) in MES-buffer (500 mM, pH 6.2, 10.0 mL) and ^{t}BuOH (3.3 mL) were converted as described in general procedure D. Yield: 114 mg, yellowish oil; 11/71/7/4/7-mixture of **1a**/**$2a_{4\text{-Br}}$**/**$2a_{2\text{-Br}}$**/**$3a_{2,4\text{-Br}_2}$**/**$4a_{2,4,6\text{-Br}_3}$**-isomers. **4-Bromophenol** (**$2a_{4\text{-Br}}$**).[62] δ_H (600 MHz; $CDCl_3$) 4.83 (1H, s, –OH), 6.72 (2H, d, *J* 8.7, 2-H, and 6-H), 7.33 (2H, d, *J* 8.9, 3-H, and 5-H). *m*/*z* (EI, 70 eV) 174 (100), 172 (100), 93 (29). **2-Bromophenol** (**$2a_{2\text{-Br}}$**).[62] δ_H (600 MHz; $CDCl_3$) 5.51 (1H, s, –OH), 6.82–6.83 (1H, m, 6-H), 7.02 (1H, dd, *J* 7.0 and 1.5, 4-H), 7.23–7.24 (1H, m, 5-H), 7.46 (1H, dd, *J* 7.9 and 1.5, 3-H). *m*/*z* (EI, 70 eV) 174 (100), 172 (91), 93 (18). 2,4-**Dibromophenol** (**$3a_{2,4\text{-Br}_2}$**).[62] δ_H (600 MHz; $CDCl_3$) 5.50 (1H, s, –OH), 6.90 (1H, d, *J* 8.7, 6-H), 7.32–7.35 (1H, m, 5-H), 7.60 (1H, d, *J* 2.3, 3-H). *m*/*z* (EI, 70 eV) 254 (39), 252 (100), 250 (43), 174 (22), 172 (22), 93 (9). 2,4,6-**Tribromophenol** (**$4a_{2,4,6\text{-Br}_3}$**).[62] δ_H (600 MHz; $CDCl_3$) 5.88 (1H, s, –OH), 7.59 (2H, s, 3-H, and 5-H). *m*/*z* (EI, 70 eV) 334 (39), 332 (94), 330 (100), 328 (38), 172 (14), 170 (9). 558 U_T^0 mg^{-1}, 133 U_T^{final} mg^{-1}, pH^{final} 6.80.

4.4. Competition kinetics

4.4.1. Competition kinetics for $V_{Br}PO(AnI)$—general procedure E. In three separate reactions phenol (**1a**) and 2-substituted phenol derivative **1b**–**1f** in the proportions 1:1, 1:2, and 2:1 (200 μmol, 1.0 equiv or 400 μmol, 2.0 equiv) were added to a stock solution A [3.75 mL, corresponding to 0.1 equiv NaBr (20 μmol)], consisting of MES-buffer (50 mM, pH 6.2, 225 mL), and NaBr (124 mg, 1.20 mmol). MES-buffer (50 mM, pH 6.2, 3.75 mL), ^{t}BuOH (2.5 mL) and $V_{Br}PO(AnI)$ [14.5 μL (0.4505 mg/mL; 611 U_T/mg), 4.0 U_T, 0.27 mmol%] were added. After the addition of H_2O_2 (2 mL, 10 mM, 0.1 equiv) in a dropwise manner within 2 min, the reaction mixture was stirred at 25 °C for 24 h, acidified with 2 M HCl (pH 1) and extracted with Et_2O (2×15 mL, 1×10 mL). Combined organic extracts were dried ($MgSO_4$). The volatiles were removed under reduced pressure (13 mbar, 40 °C). To the crude product mixture a stock solution B (0.3 mL), consisting of $Zn(ClO_4)_2$×$6H_2O$ (30.0 mg, 80.0 μmol) in Ac_2O (551 mg, 5.40 mmol) and Et_2O (0.9 mL), was added. The homogenous reaction mixture was stirred at 25 °C for 18 h. The purified product mixture by adsorption chromatography (SiO_2, EtOAc) was analyzed by GC, using pentachlorobenzene (**1c**) or *n*-decane (**1a**, **1b**, **1d**–**1f**) as an internal standard in comparison to spectroscopic data from authentic references.

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Supplementary data

Instrumentation, reagent specification, details about competition kinetics, experimental procedures, spectral, and analytical data of compounds. Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2012.08.081.

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[†] Notation for enzymatic activity: 1 unit (U) refers to the amount of enzyme required for turning over 1 μmol of substrate per minute. Bromoperoxidase activity determined with the aid of the triiodide assay (T) was abbreviated as U_T. U_T mg^{-1} refers to the specific bromoperoxidase activity in units per mg of enzyme.

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