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Chemoenzymatic Halogenation of Phenols by using the Haloperoxidase from *Curvularia inaequalis*

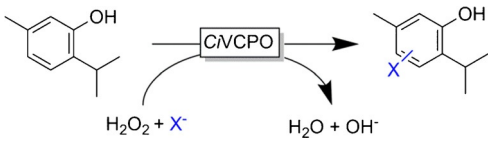
Elena Fernández-Fueyo,[a] Marco van Wingerden,[a] Rokus Renirie,[b] Ron Wever,[c] Yan Ni,[a] Dirk Holtmann,[d] and Frank Hollmann*[a]

The vanadium-dependent chloroperoxidase from *Curvularia inaequalis* is an efficient biocatalyst for the in situ generation of hypohalous acids and subsequent electrophilic oxidation/halogenation reactions. Especially, its superb activity and stability under operational conditions make it an attractive catalyst for organic synthesis. Herein, the efficient bromination of thymol was investigated, and turnover numbers of the enzyme were found to exceed 2 000 000. The major novelty of the work is that vanadium chloroperoxidase is more useful as a brominating enzyme than vanadium bromoperoxidase in terms of operational stability, besides being far more stable than heme-containing peroxidases.

The halogenation of phenols by using elementary hypervalent halogen species is a well-known and well-established reaction in organic synthesis. Hypochlorites and hypobromites are frequently used as reagents, but following an in situ generation strategy to generate the reactive hypohalogenites may be more advantageous. To attain this goal, vanadium-based catalysts[1] can be used to catalyze the formation of OBr^- or OCl^- ions from the corresponding halides by using H_2O_2 as the oxidant. However, owing to the relatively poor activity of these catalysts, high molar ratios of substrate to catalyst (S/C) are necessary to attain full conversion within a reasonable time frame. Alternatively, haloperoxidases enable significantly lower catalyst loadings owing to the intrinsically higher activity of these catalysts.[2] Particularly, so-called vanadium haloperoxidases are of interest here, as their stability towards the oxidant (H_2O_2) is significantly higher than that of the corresponding heme-dependent haloperoxidases, which suggests simpler and more robust reaction schemes.[3]

In the present study, we evaluated the vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (*Ci*VCPO) as a catalyst for the chemoenzymatic halogenation of phenols.[3c,4] In addition to its halogenating activity, the vanadium chloroperoxidase also catalyzes the sulfoxidation of sulfides to sulfoxides[4d] and is able to oxidize 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), a classical chromogenic heme peroxidase substrate, and the industrial sulfonated azo dye Chicago Sky Blue 6B.[4d]

As a model substrate for our studies we chose thymol (Scheme 1) because of the interesting antimicrobial activity of its halogenated derivatives.[5]



Scheme 1. Simplified reaction scheme for the *Ci*VCPO-mediated halogenation of thymol. *Ci*VCPO catalyzes the formation of hypohalites (XO^-), which then undergo chemical electrophilic substitution at the phenol moiety.

Production of *Ci*VCPO

*Ci*VCPO was obtained from heterologous expression in recombinant *Escherichia coli* by following a previously reported procedure.[4c] From a 2 L scale fermentation, 30 mg of essentially pure *Ci*VCPO was obtained by using a two-step purification procedure. The specific activity (A_{spec}) [monochlordimedon (MCD) assay] of the enzyme was 22.5 ± 0.1 U mg^{-1}.

Characterization of *Ci*VCPO

In a first set of experiments, we evaluated the basic biochemical properties of *Ci*VCPO. As shown in Figure 1, the pH range of *Ci*VCPO is fairly broad with an optimum at approximately pH 5.

Acidic pH values were rather detrimental to the activity of the enzyme with half-life times of a few hours. Notably, the apparent inactivation is not due to enzyme denaturation but rather due to dissociation of the catalytically active vanadate from the active site of the enzyme. Preliminary experiments

[a] Dr. E. Fernández-Fueyo, M. van Wingerden, Y. Ni, Dr. F. Hollmann
Department of Biotechnology
Delft University of Technology
Julianalaan 136
2628BL Delft (The Netherlands)
E-mail: f.hollmann@tudelft.nl

[b] Dr. R. Renirie
IVAM UvA BV
Plantage Muidergracht 24
1018TV Amsterdam (The Netherlands)

[c] Prof. Dr. R. Wever
Van't Hoff Institute for Molecular Sciences (HIMS), Faculty of Science
University of Amsterdam
Science Park 904
1098 XH Amsterdam (The Netherlands)

[d] Dr. D. Holtmann
DECHEMA Research Institute
Theodor-Heuss-Allee 25
60486 Frankfurt am Main (Germany)

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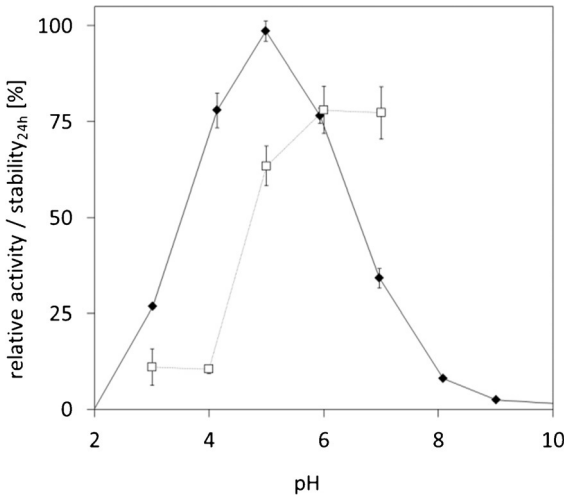


Figure 1. pH range of *Ci*VCPO. Activity (◆) and stability (□, residual activity after 24 h incubation) at different pH values. Conditions: 100 mM B&R buffer, 50 μM MCD, 5 mM KBr, 5 mM H_2O_2, 10 nM *Ci*VCPO, $T = 30\,^{\circ}C$; 100% corresponds to a A_{spec}(*Ci*VCPO) of 118 U mg^{-1} (TOF = 131 s^{-1}).

adding additional vanadate indeed suggested that even at low pH values the full activity can be reconstituted.

Next, we investigated factors influencing the robustness of the enzyme. In contrast to heme-dependent chloroperoxidases, *Ci*VCPO exhibited superb stability in the presence of H_2O_2 (Figure 2): upon exposure to even 100 mM H_2O_2 for more than 1 day, the enzyme activity decreased by only approximately 14%. Compared to the very high sensitivity of heme-dependent peroxidases towards even micromolar concentrations of H_2O_2, but also compared to the vanadium-dependent peroxidase from *Ascophyllum nodosum*,[6] the stability of *Ci*VCPO is impressive. Furthermore, the stability of *Ci*VCPO against polar water miscible and immiscible solvents such as methanol and ethyl acetate was excellent and showed no significant decrease even in the presence of 50 vol% after 3 days of incubation. The unexpected apparent increase in the activity of *Ci*VCPO over time in the presence of ethyl acetate is most likely due to slow hydrolysis of ethyl acetate and concomitant acidification of the incubation medium (see also Figure 1).

Overall, we concluded that *Ci*VCPO is a very robust biocatalyst that may potentially be useful for chemical halogenation reactions.

*Ci*VCPO-mediated thymol halogenation

Encouraged by these promising catalytic and stability properties, we set off to evaluate the *Ci*VCPO-mediated halogenation of thymol (Scheme 1). We were pleased to find that under the (arbitrarily chosen) reaction conditions significant chlorination and bromination of thymol occurred. Notably, no conversion of thymol was observed if *Ci*VCPO, H_2O_2, or the halide was omitted from the reaction mixture. Interestingly, the bromination reaction was complete within 6 h, whereas the corresponding chlorination reaction (under otherwise identical reaction conditions) gave approximately 50% conversion

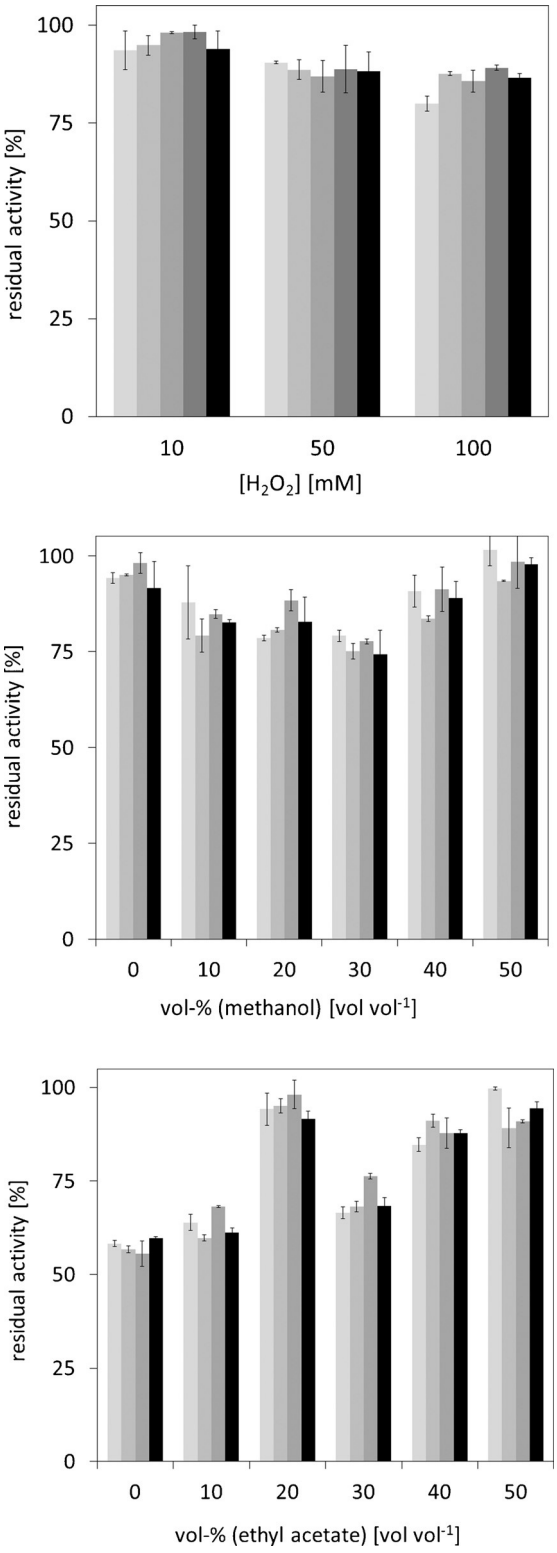


Figure 2. Stability of *Ci*VCPO in the presence of H_2O_2 (top), methanol (middle), and ethyl acetate (bottom). The *x* axes indicate the concentrations of H_2O_2 and the solvents. From left to right (light gray to black), the relative residual activity determined after 0, 1, 2, 3, and 27 h of incubation for H_2O_2 and after 0, 19, 45, and 66 h for methanol and ethyl acetate.

(Figure 3). Most likely, this can be attributed to the different kinetic properties of *Ci*VCPO towards Cl^-, which necessitates

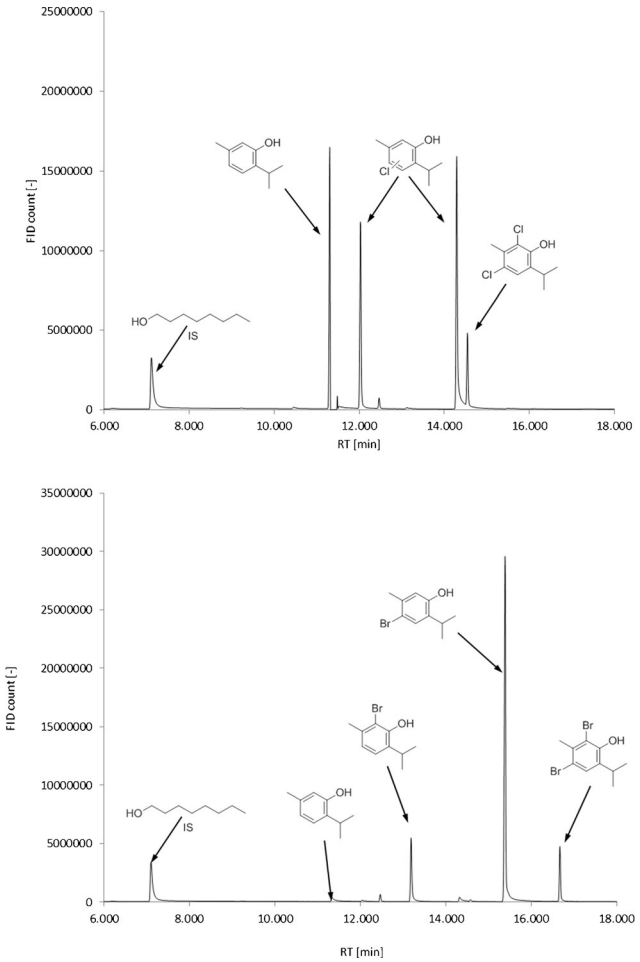


Figure 3. Chromatograms of the chlorination (top) and bromination (bottom) reactions. In the case of bromination, the chemical structures were confirmed with authentic standards. In the case of chlorination, the products were identified by GC–MS. Reaction conditions: 5 mM thymol in 100 mM citrate buffer (pH 5), 10 mM KBr or KCl, 18 nM VCPO, and 1 mM H_2O_2 every 30 min for 6 h.

either lower pH values or significantly increased chloride concentrations compared to Br^-.[4f]

Notably, the product distributions of both the bromination and the chlorination reactions corresponded to the pattern obtained through chemical halogenation. Therefore, we assume that the role of *Ci*VCPO is limited to the formation of freely diffusing XO^-.

In further experiments we focused on bromination. As shown in Table 1, the starting concentration of H_2O_2 and KBr (using equimolar concentrations of H_2O_2 and KBr) had no significant effect on the product distribution. The apparent product formation rate was almost linearly dependent on the starting concentration of both reagents. Hence, the apparent turnover frequency (TOF) of *Ci*VCPO (determined as an average rate over the entire reaction) increased from 2.2 to over 55 s^{-1}.

Higher concentrations of Br^- led to very significant inhibition of *Ci*VCPO (data not shown), which is why we decided to opt for a continuous provision of both substrates, H_2O_2 and Br^-, for some larger-scale reactions. Trials aiming at a slurry-to-slurry reaction setup (i.e., by using no co-solvents to dissolve the thymol) were feasible but suffered from severe mass-balance issues. Therefore, we used ethanol as a co-solvent, which enabled homogeneous solutions of up to 200 mM (30 g L^{-1}) of thymol under the reaction conditions. A typical time course of such a reaction is shown in Figure 4.

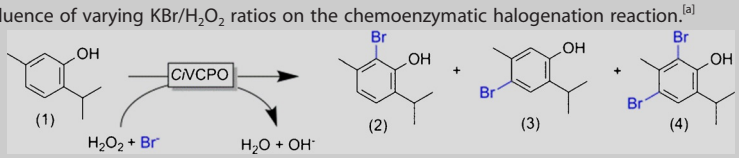
Within less than 24 h thymol was converted into the corresponding 2,4-dibromothymol. Interestingly, under these reaction conditions hardly any formation of 2-bromothymol was observed (less than 1 mM), whereas the 4-bromo isomer appeared to be the primary product of the first halogenation step. Currently, we are lacking a plausible explanation for this apparent discrepancy. This may possibly be due to the continuously low bromide concentration relative to that for the reactions reported in Table 1. Apparently, the overall product formation rate in this experiment (Figure 4) was limited by the addition rate of H_2O_2/Br^- (8 $mM_{final}\,h^{-1}$). Further investigations will be necessary to understand this unexpected selectivity of the chemical bromination step.

Finally, this setup also allowed for the semipreparative bromination of thymol (1.5 g), which yielded practical amounts of pure products after column chromatography (94, 690, and 138 mg of 2-bromo-, 4-bromo-, and 2,4-dibromothymol, respectively; refer to the Supporting Information for further details).

In this study we demonstrated that vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (*Ci*VCPO) is indeed a potentially very useful catalyst for the in situ generation of hypohalous acids (especially HOBr) for subsequent chemical oxidation/halogenation reactions. As a model reaction, the electrophilic halogenation of thymol was chosen.

Under semioptimized reaction conditions, full conversion of thymol (30 g L^{-1}) into 2,4-dibromothymol was achieved. Principally, under kinetic control, selective monobromination is also

Table 1. Influence of varying KBr/H_2O_2 ratios on the chemoenzymatic halogenation reaction.[a]



Entry	$[H_2O_2]_0=[KBr]_0$ [mM]	[**2**] [mM]	[**3**] [mM]	[**4**] [mM]	Product formation rate [mM h^{-1}]
1	1	0.29 ± 0.24	0.51 ± 0.07	0	0.81
2	4	0.69 ± 0.028	3.00 ± 0.39	0.935 ± 0.33	4.63
3	8	0.58 ± 0.04	7.34 ± 0.42	1.00 ± 0.049	8.93
4	16	1.50 ± 0.395	10.55 ± 3.58	2.68 ± 0.43	14.74
5	20	1.96 ± 0.09	15.24 ± 0.806	2.65 ± 0.77	19.8

[a] Conditions: 100 mM citrate buffer (pH 5), 20 mM thymol, 100 nM VCPO, $T=30\,^{\circ}C$. The reactions were stopped after 1 h.

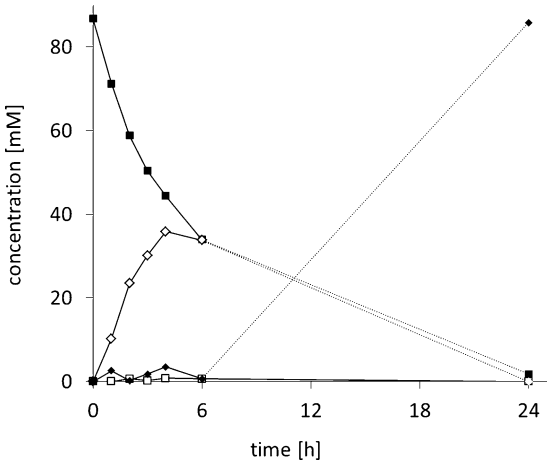


Figure 4. Representative time course of the chemoenzymatic bromination of thymol (thymol: ■; 2-bromothymol (**2**): □; 4-bromothymol (**3**): ◇; 2,4-dibromothymol (**4**): ◆. Conditions: 100 mM thymol, 100 nM *Ci*VCPO in 100 mM citrate buffer (pH 5.0) and 50% (v/v) ethanol, $T = 30\,^{\circ}C$. H_2O_2 and KBr were added continuously at 8 mM h^{-1} from a 1.5 M stock solution.

feasible. Noteworthy in these experiments is that the overall yield with respect to H_2O_2 was always above 95%, which confirmed the lack of any catalase activity of the vanadium haloperoxidases, a major asset compared to heme-containing haloperoxidases. Most interesting is the superb catalytic performance of *Ci*VCPO. For example, from Figure 4 an average turnover frequency of more 25 s^{-1} can be calculated for *Ci*VCPO within the first 4 h of the reaction. This underlines the very high robustness of this enzyme under the strongly oxidizing reaction conditions. Another indication for this robustness is the exceptionally high total turnover number of more than 2000000 (i.e., 0.00005 mol% of catalyst) achieved in these experiments.

In this respect, *Ci*VCPO clearly excels over other chloroperoxidases reported such as the heme-dependent chloroperoxidase from *Caldariomyces fumago*[2f] or even the vanadium-dependent bromoperoxidase from *Ascophyllum nodosum*.[6] Both enzymes, as a result of their sensitivity to H_2O_2, require careful control over the in situ concentration of H_2O_2. Consequently, also for bromination reactions, a vanadium chloroperoxidase can actually be superior to a vanadium bromoperoxidase. Furthermore, the high robustness of *Ci*VCPO against water (im)-miscible solvents and its facile preparation at scale make it a very promising catalyst for further applications.

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