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ARTICLE TYPE

Enzymatic halogenation of the phenolic monoterpenes thymol and carvacrol with chloroperoxidase

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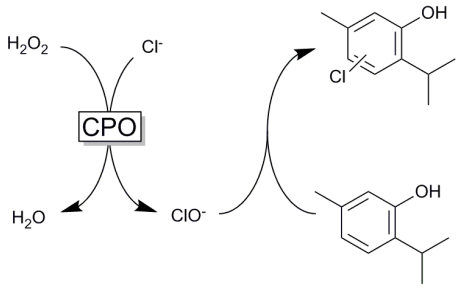
The conversion of the phenolic monoterpenes thymol and carvacrol into antimicrobials by (electro)enzymatic halogenation was investigated, using a chloroperoxidase (CPO) catalyzed process. The CPO catalysed process enables for the first time the biotechnological production of chlorothymol, chlorocarvacrol and bromothymol as well as a dichlorothymol with high conversion rates, total turnover numbers and space time yields of up to 90 %, 164,000 and 4.6 mM h^{-1}, respectively.

Introduction

Terpenes comprise a highly diverse class of natural products from which numerous commercial compounds are derived. Today, besides their use as flavor and fragrance compounds, the potential therapeutic properties of terpenes such as anti-cancer, anti-microbial and anti-inflammatory properties make them interesting and desirable molecules for the pharmaceutical, sanitary, cosmetic, agricultural and food industries.[1] Monoterpenes in particular represent a cheap and abundantly available source of chiral substances that can be transformed into valuable bioactive compounds.[2] Regio- or stereoselective functionalizations of terpenes is one of the main goals of synthetic organic chemistry, which are possible through radical reactions but are not selective enough to introduce the desired functions into those compounds. Beside oxidations[3], halogenations of the natural precursor allow the formation of more valuable products. The controlled introduction of halogen substituents into the structure of naturally occurring monoterpenes provides an entry into numerous important synthetic intermediates.[4] Halogenated secondary metabolites are common in marine plants (i.e. seaweeds), while rare in land plants, due to the abundance of chloride and bromide ions in seawater.[5] Of particular interest are the halogenated monoterpenes, a diverse class of organohalogen marine natural products found in only three genera of red macroalgae, *Plocamium*, *Portieria*, and *Ochtodes*.[6] Halogenated monoterpenes are pharmacologically active, and some acyclic structures exhibit potent and selective anti-tumor activity.[7, 8]

The essential oil of common thyme (*Thymus vulgaris*), contains 20-54% thymol.[9] Thymol, an antiseptic, is the main active ingredient in various mouthwashes and has also been shown to be effective against various fungi that commonly infect toenails. Chlorothymol is a chlorinated phenolic antiseptic used as an ingredient of preparations for hand and skin disinfection and topical treatment of fungal infections. It has also been used in preparations for anorectal disorders, cold symptoms, and mouth disorders. Chlorothymol has a 75 times higher bactericidal potency, while also possessing low human toxicity, compared to thymol. A comparison of larvicidal activity of different monoterpenoids shows that chlorothymol was found to be the most effective against the common house mosquito *Culex pipiens* followed by thymol, carvacrol and cinnamaldehyde.[10] Thymol, chlorothymol as well as the isomer carvacrol are also used as cosmetic preservatives.[11] Amongst the various halogenating agents established, *in situ* formed elementary halogens or hypohalogenites are the environmentally least harmful ones.[12] In this respect, enzymatic generation of the reactive hypohalogenites may be attractive due to the generally acknowledged mild reaction conditions and highly efficient catalysts.[13] Therefore we became interested in evaluating the peroxidase from *Caldriomyces fumago* (CPO)[14, 15] as biocatalyst for the in situ generation of hypochlorite to act as electrophilic chlorination agent for thymol (Scheme 1)



Scheme 1. Hypothesised chemoenzymatic halogenation of thymol.

Experimental

The production and purification of chloroperoxidase (CPO) from *C. fumago* is described elsewhere.[16] CPO activity was measured with the MCD assay under standard conditions (25 °C, 100 mM citric acid buffer, pH 2.75, 100 µM monochlorodimedone, 20 mM NaCl and 2 mM H_2O_2). CPO activity was photometrically detected by measuring absorption at 278 nm (ε_{MCD} = 12200 M^{-1} cm^{-1}). The concentration of CPO was photometrically determined at 400 nm (ε = 75300 M^{-1} cm^{-1}).[17]

The halogenation of the substrates was investigated in 5 mL glass reactors. Different concentrations and reaction conditions were evaluated. The reaction was started by adding the co-substrate H_2O_2. In general, the hydrogen peroxide is added in five equal doses after 0, 10, 20, 30 and 40 min of the reaction up to the final concentration. For each reaction time an independent experiment was setup and the reaction products were analyzed. Reaction products were determined with GC/MS (GC17A

(Shimadzu, Kyoto, Japan), Valcobond VB-5 column, l = 30 m; ID = 0.25 mm (VICI, Schenkon, Switzerland), temperature program 80 °C (5 min) then 10 °C min^{-1} to 200 °C, injector temperature: 250 °C, GC/MS interface temperature: 290 °C, scan range MS: 35 – 250 m/z. 950 µL of the samples were mixed with 50 µL of the internal standard (2 g · L^{-1} 1-octanol in ethanol) and subsequently extracted with 1 mL butyl acetate. The organic phase was dried with sodium sulfate and analyzed by GC/MS (Retention times: thymol 11.9 min, para-chlorothymol 12.7 min, ortho-chlorothymol 15.1 min). Several experiments were performed twice, the deviation between the experiments was less than 5.5%.

The reactor for the electroenzymatic conversion was described in detail previously.[18] Briefly, a gas diffusion electrode (GDE) with an apparent surface area of 5.5 cm^2 was used as cathode and platinum was used as anode. The reactors casing has three parts, the middle part forming the flow channel, with an internal volume of 8 mL. The platinum anode is placed to one side part, the gas diffusion cathode is placed to the second one. Oxygen from the air can diffuse through a notch of the casing to the reverse side of the GDE. A current generator was used to apply suitable currents for the electro generation of H_2O_2 by oxygen reduction. The reactor was applied in the bypass of a 50 mL reservoir and a flow of 30 mL min^{-1} was pumped from the reservoir through the reactor cell. Experiments were carried out with an untreated and a pretreated GDE, respectively. Pre-treatment of the GDE was done by saturation with 5 mM thymol solution for 24 h at room temperature to prevent adsorption of thymol to the PTFE layer of the electrode. The terminal voltage V depended linearly on the applied current I following V = 0.036 · I + 1.83 (R^2 = 0.975) between 5 and 45 mA for the saturated GDE.

Results and discussion

In a first set of experiments we evaluated the suitability of CPO as thymol halogenation catalyst. For this hydrogen peroxide was added at intervals to a mixture of thymol and CPO in citrate buffer. As shown in Figure 1, already under these arbitrarily chosen reaction conditions thymol was smoothly converted into chlorothymol at significant rates. Overall, a yield of more than 90% (based on hydrogen peroxide as the limiting component) was obtained. Interestingly, no indications for poly(thymol) formation (e.g. as a result from CPO-catalysed direct oxidation of the phenolic compound followed by radical chain polymerisation)[19] were found. It is worth mentioning here, that in the absence of the biocatalyst (CPO), no product formation was observed and the substrate was recovered completely.

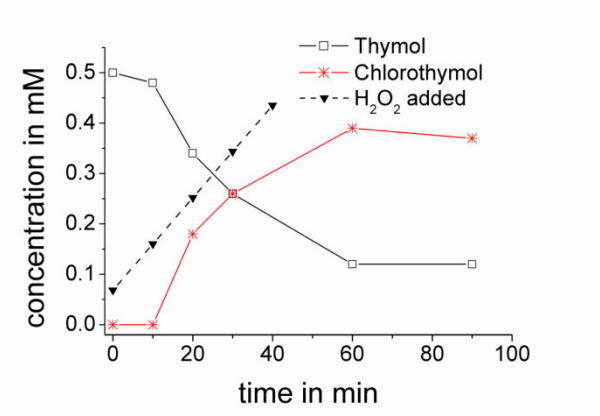


Figure 1. Representative time course of the CPO-initiated chlorinarion of thymol. Conditions: citrate buffer 0.1 M, pH 3.5, 10 nM CPO, 0.5 mM thymol, 10 mM sodium chloride, 0.088 mM H_2O_2 added every 10 min up to 0.44 mM, T = 25 °C. Each data point represents the endpoint of an independent experiment.

Interestingly, there was a significant lag time for product formation observed in all experiments. Published K_M-values of CPO to H_2O_2 are in the range of 0.03 – 1.14 mM.[20, 21] Therefore, it is possible that the hydrogen peroxide concentration after the first dose (0.088 mM) is too low to enable full CPO activity. More probably the lag phase may be caused by a comparably low rate of the uncatalysed electrophilic halogenation of thymol by hypochloride. GC analysis of the reaction product revealed the formation of the ortho- and para-chlorination product in an approximate 70:30 ratio. This product distribution was expected for the chemical halogenation reaction. Next, we further explored the reaction parameters influencing rate and overall yield of the chemoenzymatic thymol halogenation. As shown in Table 1, the reaction temperature, pH, and [NaCl] were varied systematically.

Table 1. Influence of reaction parameters on the product formation of chlorothymol. Conditions: 100 mM citrate buffer, 10 nM CPO; 0.5 mM thymol; 0.44 mM H_2O_2; 25 °C, enzyme inactivation was prevented by stepwise addition of H_2O_2, reaction was stopped until no further substrate conversion takes place (reaction time 90-150 min)

pH / -	Temperature / °C	[NaCl] / mM	Product formation rate / mM h^{-1}	Conversion / %
Variation of pH				
2.8[a]	25	10	0.33	83
3.5[b]	25	10	0.29	86
4	25	10	0.27	73
6	25	10	0.02	15
Variation of temperature				
3.5	25	10	0.29	86
3.5	30	10	0.30	80
3.5	37	10	0.29	75
Variation of NaCl concentration				
3.5	25	1	0.19(1)	56
3.5	25	5	0.39	77
3.5	25	10	0.29	86
3.5	25	25	0.35	77
3.5	25	50	0.24	79

[a] experiment was performed twice, the standard deviation was 5.5 % [b] experiment was performed twice, the standard deviation was 5.1 %

In regard to substrate conversion and product formation pH 3.5 was identified as the best condition in the range of performed experiments. Between 25 °C and 37 °C and a pH range of 2.8 to 4 there was only a low impact of temperature and pH, respectively, on product formation. However, the conversion was improved at low temperatures. In general, the CPO catalyzed chlorination of thymol is less affected by changes of the temperature and pH in the ranges mentioned before. For a technical process no laborious control of these parameters is needed, the reaction can be performed at the given room temperature. Sodium chloride had a notable influence on the product formation. The highest product formation rate was measured at 5 mM NaCl and the highest product concentration at 25 mM NaCl. The rate of chloride incorporation decreased with increased halogen concentration. At 1 mM 28 % of the chloride was incorporated in chlorothymol. At

higher concentrations an increase of the product concentration occurred, in parallel the yield on chloride decreased. The halogenation process was optimized by testing different concentration ratios between the monoterpene and hydrogen peroxide (Table 2).

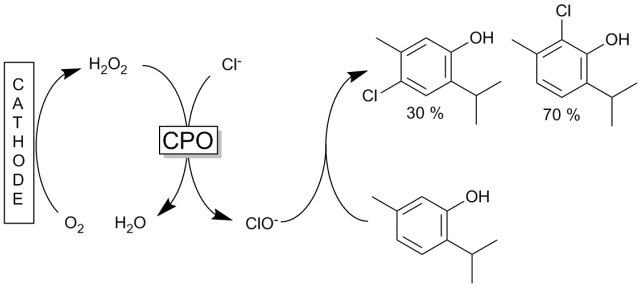
Table 2. Influence of varying thymol/H_2O_2 ratios on the chemoenzymatic halogenation**.** Conditions: 100 mM citrate buffer pH 3.5, 25 mM sodium chloride, 10 nM CPO, T = 30 °C, reaction was stopped until no further substrate conversion takes place (reaction time 90-150 min)

Thymol / mM	H_2O_2 / mM	ttn / mol product mol enzyme^{-1}	Product formation rate / mM h^{-1}	Product yield / %
0.5	0.44	43,000	0.29	86
1.0	0.44	45,000	0.30	47
1.0	0.7	69,000	0.28	66
1.0	1	88,000	0.59	90
2.5	0.44	50,000	0.33	22
2.5	2.5	145,000	0.78	73
2.5[a]	2.5	164,000	0.68	78

[a] in the presence of 0.25 mM caffeic acid[22]

Total turnover number and space time yield can be optimized by high substrate concentrations and equimolar concentrations of H_2O_2. A ttn of 145,000 mol product per mol enzyme was measured at equimolar concentrations of thymol and H_2O_2. The addition of caffeic acid as an antioxidant[22] led to an improved ttn of 164,000. On the other hand the production rate decreased to 0.68 mM h^{-1}. The measured product yield is in the range of the theoretical product yield.

Having set the stage with external addition of hydrogen peroxide we advanced to systems enabling its *in situ* generation based on catalytic reduction of molecular oxygen. Electrochemical methods[14,23] appear attractive compared to established systems[17, 24] as the cathode serves as reagent-free source of reducing equivalents and accumulation of co-products in the reaction mixture can be avoided. Recently, we have demonstrated that an electrochemical generation of H_2O_2 at a gas diffusion electrode (GDE) can be used to develop efficient CPO catalyzed processes.[18] Therefore, we used this reactor system to improve the chlorination of thymol (Scheme 2).



Scheme 2. Electro-chemoenzymatic halogenation of thymol to para- and ortho-chlorothymol with a ratio of 30 % to 70 %.

In a first set of experiments we investigated the correlation between H_2O_2 generation rate and the applied current (citrate buffer 1 mM, pH 3.5). Using untreated electrodes a linear increase of the H_2O_2-generation rate with increasing current was observed until 20 mA (0.53 µmol H_2O_2 min^{-1} cm^{-2}). Beyond that value, the H_2O_2 generation rate did not increase any more, most probably due to O_2 diffusion to the cathode surface becoming overall rate limiting. Using thymol-equilibrated electrodes (*vide infra*) H_2O_2-production rates were reduced by roughly 30% but showed linear dependency on the current applied up to 60 mA (0.7 µmol H_2O_2 min^{-1} cm^{-2}). Overall, we concluded that the GDE-mediated reduction may be a viable approach to provide CPO with appropriate amounts of H_2O_2 to sustain high hypochloride generation rates while minimising H_2O_2-related inactivation of the biocatalyst. Next, we coupled the electrogeneration of H_2O_2 to the CPO-initiated halogenation of thymol. However, using untreated electrodes, apparent productivities (chlorothymol accumulation rates) were approximately 2.3 times lower than expected from the H_2O_2 generation rate. At the same time, the mass-balance of the reaction was not closed and only 40% of the substrate was recovered (either as unreacted thymol of product). We suspected adsorption of the reactants to the hydrophobic PTFE membrane of the gas diffusion electrode to account for both observations. Indeed, repeating the electroenzymatic experiments in reactor setups pre-equilibrated with thymol, improved mass-balances with 66 % substrate recovery were obtained. Nevertheless, a further improvement of the GDE for hydrophobic substrates is necessary to obtain a closed mass-balance. Again, the productivity of the overall reaction system depended on the current applied (Figure 2). Compared to the productivity of the initial system using externally added H_2O_2 (up to 0.78 mM h^{-1}) the productivity of this electro enzymatic process was up to 4.6 mM h^{-1}.

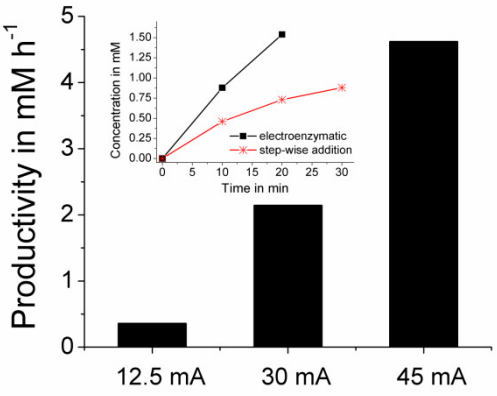


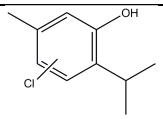
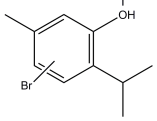
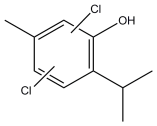
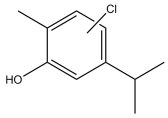
Figure 2. Formation of chlorothymol in an electroenzymatic reactor at a thymol-saturated electrode (0.1 M citrate buffer, pH 3.5, 10 nM CPO, 2.5 mM thymol, 50 mM sodium chloride, continuous production H_2O_2, T = 30 °C). The insert shows the product formation over time of a step-wise addition of hydrogen peroxide and the electro-enzymatic conversion at 45 mA. Kinetics of the reactions are shown in the supplementary information.

It is worth mentioning here that throughout the experiments reported here, no dihalogenation product was observed. Only upon using chlorothymol as starting material, gradual accumulation of a further product was observed, which we identified as dichlorothymol via GC/MS. Due to the lack of authentic standards, an exact quantification of this dihalogenation reaction is not possible at present, but – as very roughly estimated

from the GC area – the second halogenation step proceeded very significantly slower than the first one. This may also explain the very high chemoselectivity in the reactions observed.

Finally, the product scope of the proposed enzymatic halogenation was explored to some extent. As shown in Table 3, also thymol bromination was feasible. Quite expectedly, no fluorination was observed. Likewise, carvacrol was also converted smoothly into bromocarvacrol.

Table 3. Halogenation of thymol, chlorothymol and carvacrol were basically performed like the experiments shown in Figure 1 (0.1 mM citrate buffer (pH 3,5, T = 30 °C), substrate concentration 1 mM, 25 mM sodium salt of halide, 10 nM CPO, H_2O_2 was added five times every 10 min to a final concentration of 1 mM). The products were identified by m/s spectra without quantification.

Substrate	Halide	Product
Thymol	Cl^-	
Thymol	Br^-	
Thymol	F^-	-
Chlorothymol	Cl^-	
Carvacrol	Cl^-	

Conclusions

Halogenated natural products are widely distributed in nature, some of them being potent biologically active substances. Incorporation of halogen atoms into drugs is a common strategy to modify molecules in order to vary their bioactivities and specificities[25]. Chemical halogenation, however, often requires harsh reaction conditions and results in unwanted byproduct formation. Therefore it is of great interest to investigate the biosynthesis of halogenated natural products and the biotechnological potential of halogenating enzymes[25]. Here, we demonstrate for the first time the chemo-enzymatic production of halogenated phenolic monoterpenes thymol and carvacrol.

H_2O_2 is considered to be an environmentally friendly chemical, since it leaves no hazardous residues, such as other oxidants. Electrogeneration of H_2O_2 is an attractive approach since it does not require additional chemicals, and electricity is readily available.

Compared to the current state of the art using Cu^{II}-catalysed *in situ* generation of hypochlorite from chloride and molecular oxygen,[26] our method excels especially by the catalyst performance of CPO compared to simple transition metal salts. Hence, the catalyst loading (0-0004 - 0.002 mol-%) is orders of magnitude lower than in the chemical counterpart (12.5 mol-%) while achieving comparable product yields. Additionally, the milder reaction conditions (RT and aqueous reaction media vs. 80 °C and acetic acid as solvent) presumably contribute to higher product qualities (and reduced downstream processing and purification efforts).

Admittedly, at present, the low substrate loadings used impair preparative applicability. However, further improvements such as the use of cosolvents, two-liquid-phase systems or slurry-to-slurry reaction setups are currently being implemented in our laboratory. We are confident that these improvements will result in a truly environmentally more benign alternative to existing chemical technologies.

Notes and references

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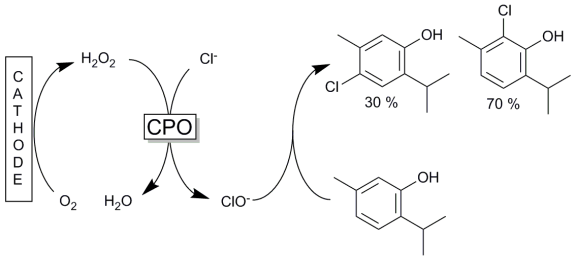

Enzymatic halogenation of thymol and its derivatives has been shown using a bioelectrochemical approach with chloroperoxidase.