FULL PAPER

DOI:10.1002/ejic.201500086

Thymol Bromination – A Comparison between Enzymatic and Chemical Catalysis

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Keywords: Vanadium / Enzyme catalysis / V-bromoperoxidases / Sustainable chemistry / Bromination / Thymol

The catalytic activity of the vanadium-dependent bromoperoxidase isolated from the brown alga *Ascophyllum nodosum* is compared with the activity of a cheap, commercially available V-catalyst precursor in the bromination of thymol. Organic solvents have been avoided to make the system appealing from a sustainable chemistry point of view. It is noteworthy that, notwithstanding the low solubility of the substrate, the thymol bromination reactions were performed in water, with a safe brominating source, under mild conditions, and with relatively inexpensive reagents. In this regard, the *greenness* of the systems was evaluated by the estimation of the *E*-factor value; the result is that the chemical reaction has a lower environmental impact than the enzymatic process, with an *E*-factor in the range of eco-friendly processes.

Introduction

Brominated compounds are the most abundant organohalides in nature.[1] This class of molecules finds applications in different fields such as agrochemicals and pharmaceuticals, mainly because of their interesting antifungal, antibacterial, antiviral, and anti-inflammatory activities. Furthermore, brominated derivatives are considered important synthetic intermediates for several selective and efficient transformations. This applies likewise for brominated thymol derivatives.

For these reasons, bromination of organic compounds is a very important reaction in organic synthesis. Classical methods usually involve the use of molecular bromine, which is corrosive and toxic; therefore, the development of safer and *greener* protocols for halogenation reactions is mandatory. Haloperoxidase enzymes (HalPOs), isolated mainly from marine algae and fungi, have received much attention, because they are known to play a major role in the biosynthesis of brominated compounds, such as halogenated indoles, terpenes, acetogenins, phenols, and hydrocarbons.[2–4]

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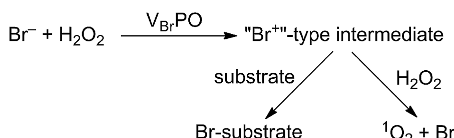
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejic.201500086.

Haloperoxidases are able to oxidize halide ions to halogenating species, whose nature may vary as a function of the reaction conditions, in the presence of hydrogen peroxide. A synthetically useful HalPO for bromo-functionalization of various substrates is the vanadium-dependent bromoperoxidase ($V_{Br}PO$) isolated from the brown alga *Ascophyllum nodosum*. $V_{Br}PO$-catalyzed oxidation of bromide in the presence of hydrogen peroxide has proved to be a sustainable methodology to synthesize various bromo derivatives. Edge and X-ray absorption near-edge structures of this bromoperoxidase were obtained, and they substantiate its mechanism of action.[5]

The catalytic effect of $V_{Br}PO$ is related to the formation of a vanadium–peroxido complex, in the active site, in the presence of hydrogen peroxide.[6] This species, which is a stronger oxidant than H_2O_2, is able to catalyze the oxidation of bromide ion to a brominating intermediate, which may then react with an appropriate organic substrate to form products or with another oxidant molecule to cause its decomposition with formation of singlet dioxygen[7] (Scheme 1).



Scheme 1. $V_{Br}PO$-catalyzed bromide oxidation by hydrogen peroxide.

These processes likely occur in different regions of the enzyme. In particular, the first one may take place in a

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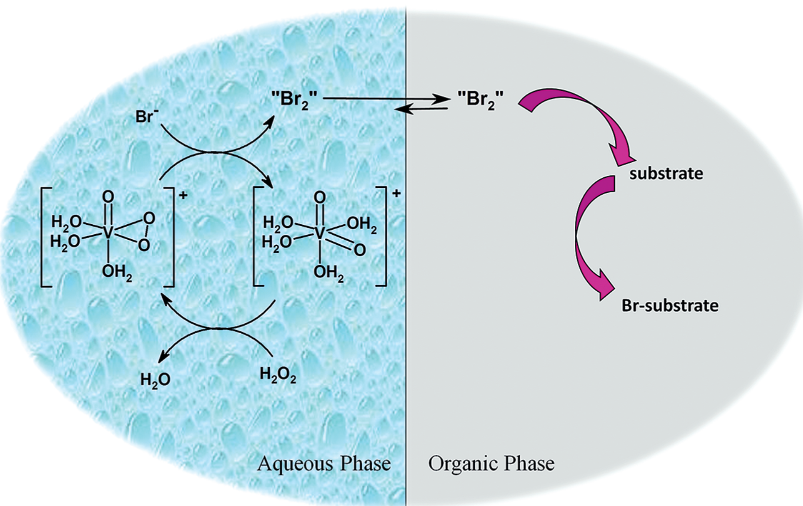

Scheme 2. V^V-based two-phase system for bromination reactions.

hydrophilic portion close to the active site, considering that at least two molecules of water and a proton are required to oxidize bromide,[8] while the functionalization of the substrate may occur in a more hydrophobic location.

Several reactions with different substrates have been performed by using $V_{Br}PO$ and, under appropriate conditions, bromocyclization reactions, indole functionalizations, and bromohydrin synthesis have been achieved.[3] Also oxidative bromination reactions of phenol and substituted phenols have been performed, and these reactions have usually led to selective *para*-bromination with respect to the hydroxy group together with formation of the corresponding *ortho,para*-diBr derivatives, both resulting from the classical electrophilic aromatic substitution.[8,9] No products of side-chain bromination were detected. The role of vanadium haloperoxidases in the formation of volatile brominated compounds from natural sources has been recently discussed in a Perspective Article, evidencing their impact on the environment.[10] Bromoperoxidases as catalysts for oxidative bromination have been reviewed.[4]

A wide variety of vanadium complexes have been synthesized and used as functional models for V-haloperoxidases in order to have a better understanding of the mechanism of action of such enzymes, and these complexes are effective catalyst precursors in various oxidation reactions such as epoxidation of alkenes, oxidation of sulfides, alkanes, arenes and alcohols, as well as in oxidative bromination reactions in the presence of hydrogen peroxide[11–25] or alkyl hydroperoxides.[26]

Inspired by the mechanism of action of $V_{Br}PO$, a biphasic procedure for bromination reactions was proposed in 1994.[27] According to this procedure, in order to mimic the hydrophobic and hydrophilic regions of the enzyme, a double-phase system is required (Scheme 2).

With this system, good results in terms of yields and selectivity have been achieved for the bromination of aromatic substrates, alkenes and alkynes[15] and, according to these results, the system seems to be a simple and inexpensive method to mimic the mode of action of $V_{Br}PO$, providing the opportunity to study the chemistry of such enzymes. Additionally, the oxidative bromination of toluene has been performed with the system described above, achieving the functionalization at the benzylic position.[28] The reaction has also been carried out without solvent, thus avoiding the use of chlorinated co-solvents to obtain products derived from the electrophilic aromatic substitution.

The aim of the present work is to directly compare the oxidative bromination reaction catalyzed by a cheap, commercially available vanadium catalyst precursor, such as NH_4VO_3, with that of the $V_{Br}PO$ from *Ascophyllum nodosum* by using thymol, a monoterpenoid phenolic compound, as model substrate. To the best of our knowledge, only few examples of thymol bromination reaction are reported in the literature.[4,29,30] To be able to demonstrate the sustainability of our procedures, the chemical and the enzyme-catalyzed bromination of thymol were performed without solvent.

Results and Discussion

The V^V catalyst was used to perform reactions on a preparative scale in order to isolate and characterize the reaction products (Scheme 3). The reactions proceeded heterogeneously, yielding high amounts of product mixtures.

V-cat, KBr, H_2O_2, pH = 1, 30 °C

Scheme 3. Product analysis of the V-catalyzed oxidative thymol bromination.

Thymol bromination accomplished with an equimolar amount of bromide with respect to the substrate occurred on the activated position for electrophilic aromatic substitution in the phenol ring. Specifically, the bromination reac-

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tion led to the formation of two isomers, 4-bromothymol being, as expected, the main product because of steric hindrance at the *ortho* position. A further bromination of the *para* isomer gave 2,4-dibromothymol in good yield.

Subsequently, the reaction was explored by changing parameters that may influence the rate and the overall yield of the chemical bromination of thymol. In particular, as shown in Table 1, KBr and H_2O_2 concentrations, together with reaction temperature and pH, were changed systematically.

Table 1. Oxidative bromination of thymol: chemical catalysis. Reaction conditions: 100 mM thymol, 100 mM KBr, 200 mM H_2O_2, unless otherwise stated.

Entry	NH_4VO_3 (mM)	Conv. (%)	OH, Br	OH, Br	OH, Br, Br
1[a]	4.8	89 %	86 %[b]	14 %[b]	–
2[a,c]	4.8	82 %	85 %[b]	15 %[b]	–
3[a,d]	4.8	100 %	82 %[b]	13 %[b]	5 %[b]
4[a]	0.5	46 %	86 %[b]	14 %[b]	–
5[a]	50	72 %	45 %[b]	11 %[b]	44 %[b]
6[a]	4.8	7 %	79 %[b]	21 %[b]	–
7[f]	4.8	100 %[g]	70 %[b]	14 %[b]	9 %[b]

[a] Reaction volume: 1 mL, at 30 °C, pH = 1 for 24 h. [b] Calculated with respect to substrate conversion. [c] 100 mM H_2O_2. [d] 200 mM KBr. [e] Reaction volume: 1 mL, at 30 °C, pH = 2.5 for 24 h. [f] Reaction volume: 1 mL, at 45 °C, pH = 1 for 24 h. [g] 2-Isopropyl-5-methyl-1,4-benzoquinone detected as further product (7%).

Reactions performed with about 5% of the catalyst precursor (Entries 1–3) showed high yields and good selectivity toward the formation of 4-bromothymol. Conversions and product distributions did not vary significantly when either the initial bromide or H_2O_2 concentrations were changed.

With lower precatalyst concentration, lower substrate conversion with similar product distribution was observed (Entry 4). On the other hand, in the presence of 0.5 equiv. of NH_4VO_3 (Entry 5), a dibromination reaction occurred: under these conditions the relatively high catalyst concentration likely accelerates successive bromination.

The reaction performed at higher pH value (Entry 6) has a very low yield, probably due to the formation of a diperoxido–metal complex. In fact, it is known that vanadate dissolved in aqueous solutions in the presence of hydrogen peroxide forms several species in equilibrium. The prevalence of the different peroxido complexes is strongly influenced by pH.[31,32] In particular, at pH = 2.5 species with more than one peroxido group bound to the metal are favored, while at pH = 1 the monoperoxido complex is the most abundant species in solution (Scheme 4).

H_2O_2 H_2O
$(H_2O)_x$ $(H_2O)_y$

Scheme 4. Mono- and diperoxido vanadium(V) complexes in equilibrium at pH = 2.5.

Furthermore, the monoperoxido vanadium complex was more reactive than the diperoxido complex toward the oxybromination of alkenes and alkynes;[27,32] recently good results have also been obtained in the benzylic bromination of toluene.[28] At higher reaction temperature (Entry 7), quantitative conversion of the substrate was observed, and the bromination reaction proceeded faster. However, it was accompanied by the oxidation of the substrate with hydrogen peroxide, which also led to the formation of 2-isopropyl-5-methyl-1,4-benzoquinone as byproduct.

The kinetic behavior of the model bromination is shown in Figure 1. It can be seen that the initial part of the process is quite fast; in fact, substrate conversion and product formation are almost complete only after 3 h, while the catalyst remains active even after 24 h, indicating that, under these experimental conditions, the decomposition of hydrogen peroxide is not predominant.

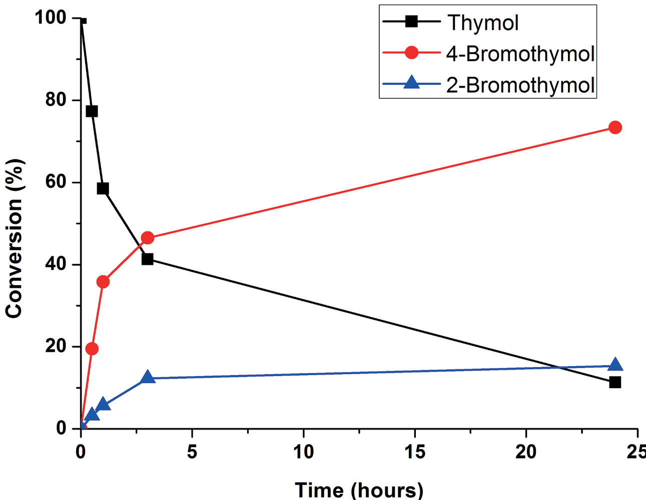

Figure 1. V^V-catalyzed oxidative thymol bromination as a function of time. Reaction conditions: volume 1 mL, 100 mM thymol, 100 mM KBr, 200 mM H_2O_2, 5 mM NH_4VO_3, pH = 1, 30 °C.

The results obtained with the $V_{Br}PO$ enzyme are collected in Table 2. Interestingly, under experimental conditions similar to those used for the chemical model, a lower conversion of the substrate together with a different product distribution were observed (Table 2, Entry 1). In this case, the amounts of both 2- and 4-bromothymol were similar, and formation of dibrominated 2,4-dibromothymol was also observed. Possible explanations of this behavior are either a permanence of the monobrominated derivatives near the active site of the enzyme, which makes a second functionalization possible, or different reactivity due to the higher pH.[6]

As far as the enzymatic process is concerned, the kinetic plot (Figure 2) shows that the reaction does not proceed any further after 3 h. The low substrate conversion (27%) might be due to inactivation of the enzyme by hydrogen peroxide. To substantiate this hypothesis, the stability of the enzyme in the presence of high concentrations of H_2O_2 was investigated, and indeed after 5 h of incubation, the enzyme lost 85% of its initial activity, in agreement with the work by Soedjak and Butler,[33] who showed inactivation and inhibition at high peroxide concentrations (Figure 3).

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Table 2. Oxidative bromination of thymol: enzymatic catalysis. Reaction conditions: 100 mM thymol, 100 mM KBr.

Entry	H_2O_2 (mM)	Conv. (%)	OH / Br (4-bromothymol)	Br / OH (2-bromothymol)	Br / OH / Br (2,4-dibromothymol)
1[a]	100	27 %[b]	38 %[b]	50 %[b]	12 %[b]
2[a]	10 × 10[c]	42 %[b]	41 %[b]	43 %[b]	16 %[b]

[a] Reactions were carried out in 50 mM citrate buffer pH = 6.3 (final volume 1 mL), at 30 °C for 24 h using $V_{Br}PO$ 1 mg/mL. [b] Calculated with respect to the substrate conversion. [c] 10 µL aliquots of 1 M H_2O_2 were added every 30 min for 10 times (final concentration 100 mM).

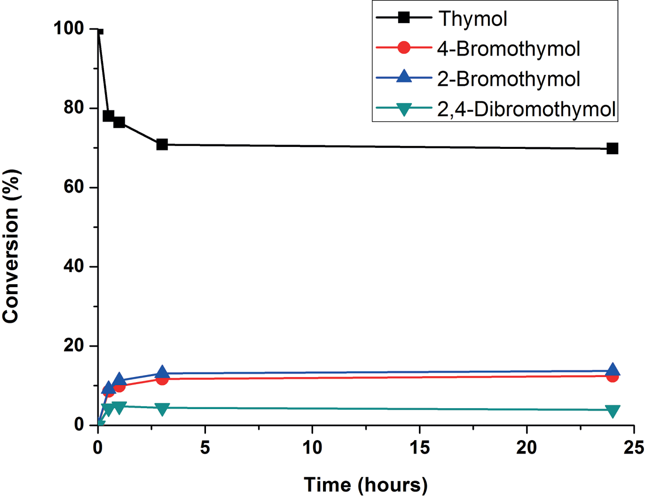


Figure 2. $V_{Br}PO$-catalyzed oxidative thymol bromination as function of time. Reaction conditions: volume 1 mL, 100 mM thymol, 100 mM KBr, 100 mM H_2O_2, $V_{Br}PO$ 1 mg/mL, 50 mM citrate buffer pH = 6.3, 30 °C.

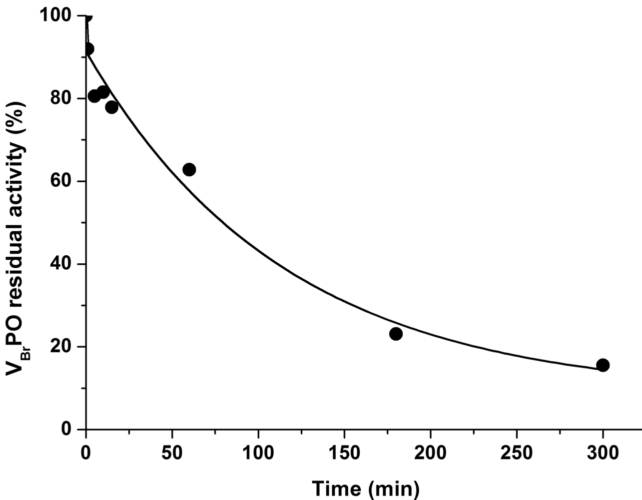


Figure 3. $V_{Br}PO$ remaining activity after incubation in 100 mM of H_2O_2.[34] Incubation conditions: [$V_{Br}PO$] = 1 mg/mL; 100 mM H_2O_2; 50 mM citrate buffer pH = 6.3, 30 °C; activity assay: 50 mM phosphate buffer, pH = 8; 100 mM KBr, 1 mM H_2O_2, 100 µM TB; 0.01 mg/mL $V_{Br}PO$.

Moreover, in order to evaluate $V_{Br}PO$ activity in presence of KBr, the initial rate of thymol blue (TB) bromination was measured as a function of KBr concentration with 1 mM H_2O_2 (Figure 4).

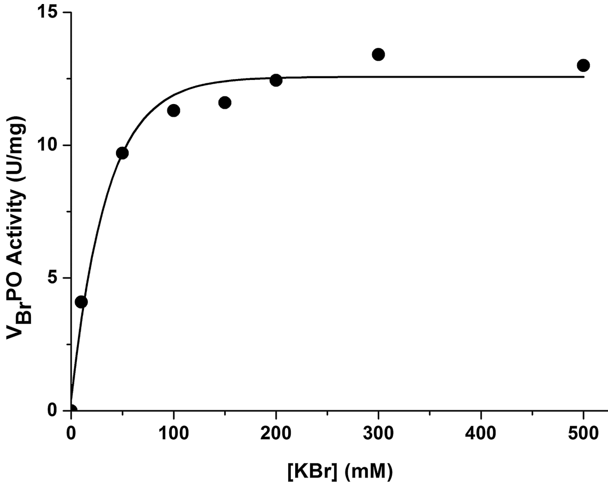
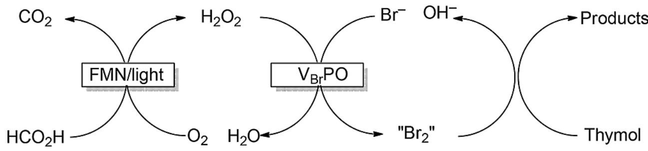


Figure 4. $V_{Br}PO$ activity at different KBr concentrations.[34] Reaction conditions: phosphate buffer 50 mM, pH = 8, 1 mM H_2O_2, 100 µM TB, $V_{Br}PO$ 0.002 mg/mL, 25 °C.

A saturation kinetic behavior was observed. In addition, with bromide concentrations equal to those used to perform chemical bromination (between 100 and 200 mM), the enzyme showed the highest activity while bromide inhibition was not detected.

In an attempt to reduce the inactivation o the enzyme due to the presence of high hydrogen peroxide concentrations particularly at the beginning of the reaction, the bromination was performed by adding the oxidant stepwise. As expected, a higher yield was observed (Table 2, Entry 2), accompanied by a different product distribution.

To further improve the enzyme performance, it is advisable to generate H_2O_2 in situ.[35–37] Preliminary experiments using light-driven H_2O_2 generation with flavin mononucleotide (FMN) as photocatalyst and sodium formate as sacrificial electron donor have been performed (Scheme 5).



Scheme 5. Light-driven in situ H_2O_2 generation.

As shown in Figure 5, by using in situ H_2O_2 generation, thymol consumption was observed for 70 h, and a conversion of about 80% was obtained. This shows that the enzyme activity is preserved over many hours, and conversions similar to those with the chemical system can be achieved. Further optimization is currently ongoing in our laboratories to turn this system into a practical and synthetically relevant one.

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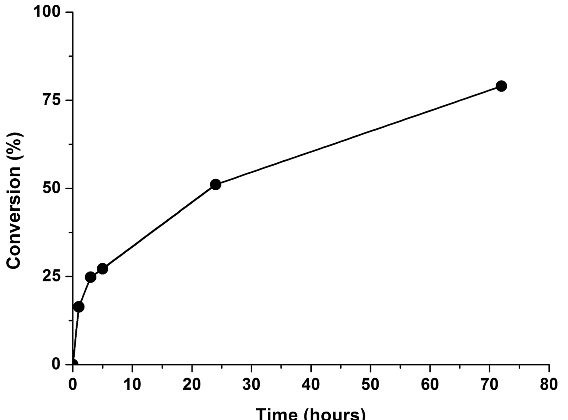

Figure 5. $V_{Br}PO$-catalyzed oxidative thymol bromination in a light-driven reaction as a function of time. Reaction conditions: volume 1 mL, 100 mM thymol, 200 mM KBr, 0.5 mg/mL $V_{Br}PO$, 200 mM sodium formate, 5 mM FMN, 50 mM citrate buffer, pH = 6.3, 30 °C.

Enzymatic reactions are often claimed as environmentally friendly in comparison to conventional catalytic reactions. For this reason, the environmental impact of the enzymatic bromination of thymol was evaluated and compared to that of the catalytic process. To this end, the environmental factor (*E*-factor = $kg_{waste} \times kg_{product}^{-1}$) can be used to firstly evaluate the *greenness* of a reaction.[37–40] Obviously, the ideal *E*-factor is zero.

In the present work, the *E*-factor value for the optimized chemical reaction is 280, while for the non-optimized enzymatic reaction it is around 1000. It should be noted that the two main contributions for both systems derive from the solvent used for the reaction (i.e. water) and from the solvent (i.e. diethyl ether) used to extract the products (see Figure 6).

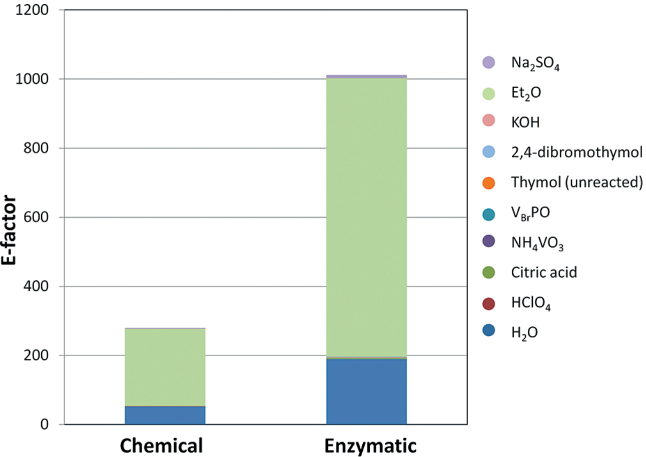

Figure 6. *E*-factor values for chemical and enzymatic thymol bromination.

Nevertheless, water is usually considered a green solvent and it is generally excluded from the *E*-factor calculation. However, this assumption is valid only for pure water, whereas when the water is contaminated it needs treatment and purification, which constitutes a negative contribution to the eco-compatibility of the system. As for diethyl ether, in principle, solvents can be recycled, and this should significantly decrease the *E*-factor.

For these reasons, when solvent contributions are not taken into account, *E*-factor values decrease drastically for both reactions. In particular, a value of 3.7 $kg_{waste} \times kg_{product}^{-1}$ has been obtained for the chemical reaction and 16 $kg_{waste} \times kg_{product}^{-1}$ for the enzymatic one (Figure 7). Hence, these estimations indicate that at present V^{V}-catalyzed thymol bromination is more sustainable than the enzymatic reaction in terms of produced wastes, having an *E*-factor in the range of eco-friendly processes.

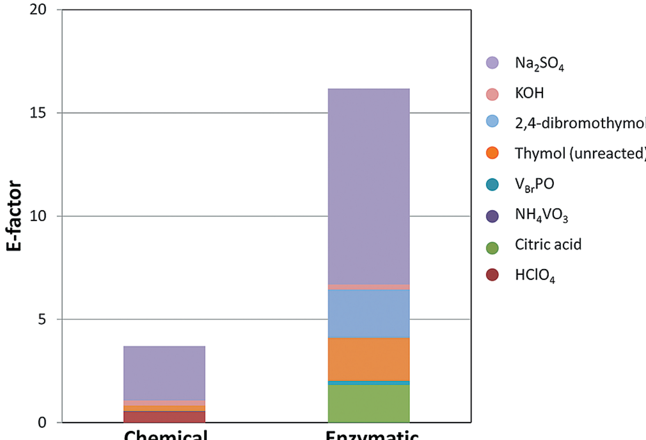

Figure 7. *E*-factor values for chemical and enzymatic thymol bromination (solvents contributions not considered).

Conclusions

It is fairly difficult to tell which brominating method is “more sustainable”. Table 3 compares some important features of both methods.

Table 3. Key parameters to compare chemical and enzymatic bromination of thymol.

Parameter	NH_4VO_3	$V_{Br}PO$
Conversion (%)	100	42
TON ($mol_{prod} \times mol_{cat}^{-1}$)	92	1804
TF_{max} ($TON \times h^{-1}$)	15	1481
Catalyst consumption ($g_{cat} \times g_{prod}^{-1}$)	0.25	0.0031

The chemical method yields full conversion, whereas the enzyme catalyst enables only 42%. The major reason for this discrepancy was shown to be the rather poor robustness of the biocatalyst towards the oxidant (H_2O_2). Already portionwise addition of H_2O_2 resulted in significantly higher product concentrations. Further improvements can be expected from in situ generation of H_2O_2 to supply the biocatalyst with sufficient but not detrimental amounts of H_2O_2 for generation of the brominating species.[30,35–37]

A simple comparison of the environmental impact of the two methods by using the *E*-factor[39–41] demonstrates that biocatalysis is not per se environmentally more benign than chemical catalysis. In both cases, the relatively high solvent contribution (due to the low product concentrations used)

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again demonstrates the importance of high reactant concentrations to improve not only the economic attractiveness but also the environmental impact.

Experimental Section

Instrumentation: Gas chromatographic analyses were carried out with a Varian 3900 instrument equipped with an FID 1770 detector and a 30 m Supelco SPB-5 column (0.25 mm diameter and 0.25 µm internal film). GC–MS spectra were recorded with a Shimadzu GC–MS-QP2010 Ultra spectrometer. ^{1}H NMR spectra were recorded with a Bruker Avance 300 MHz instrument, with $CDCl_3$ as solvent. UV assays were performed with use of a Shimadzu UV/Vis spectrophotometer UV-2401 PC, with disposable plastic cuvettes of 1.5 mL volume.

Materials: All commercial reagents and solvents were used as received, without further purification. Thymol bromination products were synthesized according to the catalytic procedure described below and purified chromatographically (SiO_2; eluent: hexane/dichloromethane 3:1).

4-Bromo-2-isopropyl-5-methylphenol (4-Bromothymol): The compound was isolated as a white powder. MS (EI): *m/z* (%) = 228 (30) $[M]^+$, 230 (30) $[M + 2]^+$, 213 (85) $[M – CH_3]^+$, 134 (100) $[M – CH_3Br]^+$. ^{1}H NMR ($CDCl_3$): δ = 7.31 (s, 1 H), 6.66 (s, 1 H), 4.65 (s, 1 H), 3.19–3.07 (m, 1 H), 2.32 (s, 3 H), 1.26–1.23 (d, J = 7 Hz, 6 H) ppm.

2-Bromo-6-isopropyl-3-methylphenol (2-Bromothymol): The compound was isolated as a colorless oil. MS (EI): *m/z* (%) = 228 (30) $[M]^+$, 230 (30) $[M + 2]^+$, 213 (85) $[M – CH_3]^+$, 134 (100) $[M – CH_3Br]^+$. ^{1}H NMR ($CDCl_3$): δ = 7.08–7.05 (d, J = 7 Hz, 1 H), 6.83–6.79 (d, J = 8 Hz, 1 H), 5.72 (s, 1 H), 3.39–3.25 (m, 1 H), 2.39 (s, 3 H), 1.28–1.23 (d, J = 7 Hz, 6 H) ppm.

2,4-Dibromo-6-isopropyl-3-methylphenol (2,4-Dibromothymol): The compound was synthesized with the same procedure but with the previously synthesized 4-bromothymol as substrate. MS (EI): *m/z* = 306 (20) $[M]^+$, 308 (40) $[M + 2]^+$, 310 (20) $[M + 4]^+$, 291 (55) $[M – CH_3]^+$, 293 (100) $[M + 2 – CH_3]^+$, 295 (55) $[M + 4 – CH_3]^+$, 212 (50) $[M – CH_3Br]^+$, 214 (50) $[M + 2 – CH_3Br]^+$. ^{1}H NMR ($CDCl_3$): δ = 7.33 (s, 1 H), 5.70 (s, 1 H), 3.35–3.21 (m, 1 H), 2.54 (s, 3 H), 1.26–1.22 (d, J = 7 Hz, 6 H) ppm.

Catalytic Procedure: Thymol (15 mg ,0.1 mmol) and KBr (11.9 mg ,0.1 mmol) were dissolved in an aqueous solution of NH_4VO_3 (960 µL, 5 mM). Then, H_2O_2 (20 µL, 10.4 M) and perchloric acid (20 µL of 5 M) were added to obtain a pH value of 1. The mixture was stirred at 30 °C for 24 h. Afterwards, the reaction products were extracted with three portions of diethyl ether, dried with anhydrous Na_2SO_4, and filtered. The solvent was evaporated. The reaction products were dissolved in $CDCl_3$ (500 µL). Then, an aliquot (50 µL) of this solution was diluted with $CDCl_3$ (450 µL) containing CH_3NO_2 as internal standard and analyzed by ^{1}H NMR spectroscopy.

Isolation of Vanadium Bromoperoxidase: Isolation of the bromoperoxidase was performed by a literature procedure.[42] Seaweed (*Ascophyllum nodosum*) was collected along the Afsluitdijk, near Kornwerderzand, The Netherlands, towards the end of November. The seaweed was washed and stored at –20 °C. A portion (500 g) of this seaweed was chopped in a meat grinder for 30 min, suspended in TRIS–H_2SO_4 buffer (1 L, 0.2 M, pH 8.3), and centrifuged (2800 rpm, 15 min). $CaCl_2$ solid was added in portions to the supernatant to obtain a final concentration of 100 mM, followed by another centrifugation step (2800 rpm, 15 min). $(NH_4)_2SO_4$ was added to the supernatant to a final saturation of 80 %. The precipitated bromoperoxidase was separated from the supernatant by centrifugation (13000 rpm, 30 min). The precipitate was homogenized in a medium containing ethanol (60 %) and TRIS–H_2SO_4 (0.2 M, pH 8.3). After centrifugation at 10000 rpm for 30 min, the pellet obtained was resuspended in TRIS–H_2SO_4 buffer (0.2 M, pH 8.3) and loaded onto a DEAE–Sephacel column. The protein was eluted with TRIS–H_2SO_4 buffer (1 M, pH 8.3) containing NaCl (1 M). Fractions containing bromoperoxidase activity were pooled, concentrated on an Amicon ultrafiltration cell (Filter, PM 30), and dialyzed against sodium acetate (10 mM, pH 5). Portions of this enzyme preparation were stored at –20 °C.

V_{Br}PO Activity Assay: V_{Br}PO activity was evaluated by using a colorimetric assay based on thymolsulfonphthalein (Thymol Blue, TB) bromination at 25 °C.[34] A stock solution of TB (1 mM, 100 µL) dissolved in H_2O/dimethyl sulfoxide (4:1) was added to phosphate buffer (880 µL, 100 mM, pH 8) containing KBr (100 mM). Then, H_2O_2 (10 µL, 100 mM) and V_{Br}PO (10 µL) from the 50 × diluted stock solution were added (final V_{Br}PO concentration 2 µg/mL). The absorbance change of the resulting mixture at 620 nm was recorded every minute, and it was converted to dibromothymolsulfonphthalein ($TBBr_2$) formed per minute (in mM), by using ε = 37.2 mM^{-1} cm^{-1}. The specific activity in thymol blue bromination was (11.2 ± 0.6) U/mg.

General Procedure for V_{Br}PO Catalyzed Reactions: Thymol (15 mg, 0.1 mmol) and KBr (11.9 mg, 0.1 mmol) were dissolved in citrate buffer (890 µL, 50 mM, pH 6.3). Then, H_2O_2 (10 µL, 10.4 M) and the stock solution of V_{Br}PO (100 µL, 10.8 mg/mL) were added, and the mixture was stirred at 30 °C. Afterwards, the reaction products were extracted with three portions of diethyl ether, dried with anhydrous Na_2SO_4, and filtered. The solvent was evaporated. The reaction products were dissolved in $CDCl_3$ (500 µL). Then, this solution (50 µL) was diluted with $CDCl_3$ (450 µL) containing a known amount of CH_3NO_2 and analyzed by ^{1}H NMR spectroscopy.

Acknowledgments

COST Action CM1003 "Biological Oxidation Reactions – Mechanisms and Design of New Catalysts" is acknowledged for having allowed a stimulating European Research Environment and a Short Term Scientific Mission for F. S. Dr. Heiko Lange is gratefully acknowledged for GC–MS analysis and Dr. Martina Tiravia for helpful discussions.

[1] See, for example: a) G. W. Gribble, *Chem. Soc. Rev.* **1999**, *28*, 335–346; b) M. Liu, P. E. Hansen, X. Lin, *Mar. Drugs* **2011**, *9*, 1273–1292; c) B. G. Wang, J. B. Gloer, N. Y. Ji, J. C. Zhao, *Chem. Rev.* **2013**, *113*, 3632–3685.
[2] A. Butler, *Coord. Chem. Rev.* **1999**, *187*, 17–35.
[3] A. Butler, J. N. Carter-Franklin, *Nat. Prod. Rep.* **2004**, *21*, 180–188.
[4] D. Wischang, O. Brücher, J. Hartung, *Coord. Chem. Rev.* **2011**, *255*, 2204–2217.
[5] J. M. Arber, E. de Boer, C. D. Garner, S. S. Hasnain, R. Wever, *Biochemistry* **1989**, *28*, 7968–7973.
[6] E. de Boer, R. Wever, *J. Biol. Chem.* **1988**, *263*, 12326–12332.
[7] R. R. Everett, H. S. Soedjak, A. Butler, *J. Biol. Chem.* **1990**, *265*, 15671–15679.
[8] D. Wischang, M. Radlow, J. Hartung, *Dalton Trans.* **2013**, *42*, 11926–11940.
[9] D. Wischang, J. Hartung, *Tetrahedron* **2012**, *68*, 9456–9463.

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[10] R. Wever, M. A. van der Horst, *Dalton Trans.* **2013**, *42*, 11778–11786.
[11] V. Conte, B. Floris, *Dalton Trans.* **2011**, *40*, 1419–1436 and references cited therein.
[12] J. A. L. da Silva, J. J. R. Fraústo da Silva, A. J. L. Pombeiro, *Coord. Chem. Rev.* **2011**, *255*, 2232–2248 and references cited therein.
[13] G. Licini, V. Conte, A. Coletti, M. Mba, C. Zonta, *Coord. Chem. Rev.* **2011**, *255*, 2345–2357 and references cited therein.
[14] V. Conte, A. Coletti, B. Floris, G. Licini, C. Zonta, *Coord. Chem. Rev.* **2011**, *255*, 2165–2177 and references cited therein.
[15] V. Conte, B. Floris, *Inorg. Chim. Acta* **2010**, *363*, 1935–1946 and references cited therein.
[16] M. R. Maurya, A. A. Khan, A. Azam, S. Ranjan, N. Mondal, A. Kumar, F. Avecilla, J. Costa Pessoa, *Dalton Trans.* **2010**, *39*, 1345–1360.
[17] T. K. Si, M. G. B. Drew, K. K. Mukherjea, *Polyhedron* **2011**, *30*, 2286–2293.
[18] M. R. Maurya, C. Haldar, A. Kumar, M. L. Kuznetsov, F. Avecilla, J. Costa Pessoa, *Dalton Trans.* **2013**, *42*, 11941–11962.
[19] U. Saha, T. Kr. Si, P. Kr. Nandi, K. K. Mukherjea, *Inorg. Chem. Commun.* **2013**, *38*, 43–46.
[20] C. Chen, Q. Sun, D.-X. Ren, R. Zhang, F.-Y. Bai, Y.-H. Xing, Z. Shi, *CrystEngComm* **2013**, *15*, 5561–5573.
[21] C. Das, P. Adak, S. Mondal, R. Sekiya, R. Kuroda, S. I. Gorelsky, S. K. Chattopadhyay, *Inorg. Chem.* **2014**, *53*, 11426–11437.
[22] G. Grivani, V. Tahmasebi, A. D. Khalaji, *Polyhedron* **2014**, *68*, 144–150.
[23] G. Grivani, V. Tahmasebi, A. D. Khalaji, V. Eigner, M. Dusek, *J. Coord. Chem.* **2014**, *67*, 3664–3677.
[24] M. R. Maurya, N. Chaudhary, A. Kumar, F. Avecilla, J. Costa Pessoa, *Inorg. Chim. Acta* **2014**, *420*, 24–38.
[25] M. R. Maurya, N. Chaudhary, F. Avecilla, *Polyhedron* **2014**, *67*, 436–448.
[26] O. Brücher, J. Hartung, *ACS Catal.* **2011**, *1*, 1448–1454.
[27] V. Conte, F. Di Furia, S. Moro, *Tetrahedron Lett.* **1994**, *35*, 7429–7432.
[28] P. Galloni, M. Mancini, B. Floris, V. Conte, *Dalton Trans.* **2013**, *42*, 11963–11970.
[29] R. Kaur, M. P. Darokar, S. K. Chattopadhyay, V. Krishna, A. Ahmad, *Med. Chem. Res.* **2014**, *23*, 2212–2217.
[30] L. Getrey, T. Krieg, F. Hollmann, J. Schrader, D. Holtmann, *Green Chem.* **2014**, *16*, 1104–1108.
[31] V. Conte, O. Bortolini, M. Carraro, S. Moro, *J. Inorg. Biochem.* **2000**, *80*, 41–49.
[32] O. Bortolini, V. Conte, *J. Inorg. Biochem.* **2005**, *99*, 1549–1557.
[33] H. S. Soedjak, J. V. Walker, A. Butler, *Biochemistry* **1995**, *34*, 12689–12696.
[34] E. Verhaeghe, D. Buisson, E. Zekri, C. Leblanc, P. Potin, Y. Ambroise, *Anal. Biochem.* **2008**, *379*, 60–65.
[35] D. I. Perez, M. Mifsud Grau, I. W. C. E. Arends, F. Hollmann, *Chem. Commun.* **2009**, *44*, 6848–6850.
[36] E. Churakova, M. Kluge, R. Ullrich, I. W. C. E. Arends, M. Hofrichter, F. Hollmann, *Angew. Chem. Int. Ed.* **2011**, *50*, 10716–10719.
[37] C. E. Paul, E. Churakova, E. Maurits, M. Girhard, V. B. Urlacher, F. Hollmann, *Bioorg. Med. Chem.* **2014**, *22*, 5692–5696.
[38] R. A. Sheldon, *J. Chem. Technol. Biotechnol.* **1997**, *68*, 381–388.
[39] R. A. Sheldon, *Chem. Commun.* **2008**, *29*, 3352–3365.
[40] Y. Ni, D. Holtmann, F. Hollmann, *ChemCatChem* **2014**, *6*, 930–943.
[41] R. A. Sheldon, *Green Chem.* **2007**, *9*, 1273–1283.
[42] R. Wever, H. Plat, E. de Boer, *Biochim. Biophys. Acta* **1985**, *830*, 181–186.

Received: January 27, 2015
Published Online: March 20, 2015

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