Supporting Information

A Water-Soluble Anthraquinone Photocatalyst Enables Methanol Driven Enzymatic Halogenation and Hydroxylation Reactions

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Materials

All chemicals were purchased from Alfa-Aesar, Acros, Sigma-Aldrich or Fluka with the highest purity available and used without further purification. The peroxyzymes were produced in house as described below.

Preparation of *Ci*VCPO

The heterologous expression and purification of vanadium chloroperoxidase from *Curvularia inaequalis* (*Ci*VCPO) were performed according to the reported procedures.[1] A 2 litre culture of *Escherichia coli* transformant (*E. coli* TOP10 (Invitrogen) with the construct *pBAD-VCPO*) was grown at 37 °C in LB medium supplemented with 100 µg/mL ampicillin to an OD 600 nm between 0.6 and 0.8. Protein expression was induced after the fermentation broth was cooled to 20 °C and of 0.02 % L arabinose was added. The incubation continued for another 24 hours at 25 °C.

Purification of *Ci*VCPO

Cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C (4.3 xg). The cells were re-suspended to 1 g mL^{-1} in 50 mM Tris/H_2SO_4, pH 8.1 fortified with protease inhibitors, lysozyme (2 mg mL^{-1}) and DNaseI. Cells were lysed using a cell disruptor and debris was removed by centrifugation at 15000 rpm for 1 h at 4 °C. Then an equal volume of isopropyl alcohol was added to the supernatant to precipitate nucleic acids and unstable proteins. After centrifugation (30 min at 15000 rpm), the clear supernatant was applied to a DEAE Sephacel column (Amersham Pharmacia Biotech) (5mL min^{-1}) equilibrated with 50 mM Tris/H_2SO_4 pH 8.1. After washing of the column with 2 volumes of 50 mM Tris/ H_2SO_4, pH 8.1, and 2 volumes of 0.1 M NaCl in 50 mM Tris/H_2SO_4, pH 8.1, the enzyme was eluted with 1 M NaCl in 50 mM Tris/HCl, pH 8.1. Finally, the pure apoenzyme was dialyzed against 100 µM orthovanadate in 50 mM Tris H_2SO_4, pH 8.1 to obtain the reconstituted holoenzyme. The protein concentration was estimated by the BSA assay.

Preparation of r*Aae*UPO

The unspecific peroxygenase from *Agrocybe aegerita* (*Aae*UPO, main isoform II) was produced and purified according to previous methods.[2] The culture broth with *P. pastoris* cells containing r*Aae*UPO was clarified by centrifugation at 8000 rpm for 2 hours at 4 °C. The supernatant was filtered through a 20 µm filter and kept at -80°C. The r*Aae*UPO activity was determined to be 652 ± 5 U mg^{-1} (pH 5.0 in NaPi buffer). One unit of the enzyme activity was defined as the amount of the enzyme catalysing the oxidation of 1 µmol of ABTS per minute.

Purification of r*Aae*UPO

The supernatant was concentrated (Amicon 10-kDa-cut-off) and dialyzed against 100 mM sodium phosphate, pH 7.0. r*Aae*UPO was purified using an NGC Chromatography system (Biorad), in one single step. The separation was performed on a Q Sepharose FF 30-mL cartridge with a flow rate of 5 mL min^{-1}. After 90 mL, the retained protein was eluted with a 0–50 % NaCl gradient in 450

mL, followed by 50–100 % gradient in 50 mL and 100 % NaCl in 75 mL. The peroxidase activity was followed by ABTS oxidation in the presence of H_2O_2, and the appropriate fractions were pooled, concentrated and dialyzed against 100 mM sodium phosphate buffer (pH 7). The purification of the UPO was confirmed by sodium dodecyl sulfate (SDS)–PAGE in 12% gels stained with Coomassie brilliant blue R-250 (Sigma).

Photoenzymatic halogenation reactions

The photochemical enzymatic halogenation reactions using *Ci*VCPO were performed at 30 °C in 1.0 mL of sodium phosphate buffer (NaPi, pH 6.0, 60 mM). Specifically, a stock solution (5 mM) of sodium anthraquinone sulfonate (SAS) in above NaPi buffer and thymol (100 mM) in methanol were firstly prepared. 100 µL of each stock solution was added to 795 µL of a pre-mixed solution (300 µL methanol and 495 µL NaPi buffer) in a 4 mL glass vial. Afterwards the *Ci*VCPO was added (5 ul). In the final solution the reaction condition was: [substrate] = 10 mM, [*Ci*VCPO] = 50 nM, [NaBr] = 25 mM, [SAS] = 0.5 mM, pH 6.0 (NaPi buffer, 60 mM) and 40% of methanol in 1.0 mL. For those reactions to investigate the influence of SAS and *Ci*VCPO concentration, the adjustment of the reaction mixture was necessary so that the reaction conditions were identical. As the final step, the reaction vial was closed, and exposed to visible light (Philips 7748XHP 150 W, white light bulb) under gentle magnetic stirring (200 rpm).[3,4] At intervals, aliquots were withdrawn, extracted with ethyl acetate (containing 5 mM of dodecane as internal reference, extraction ratio 1 : 2) and analyzed by Gas Chromatography (SHIMADZU). All above reactions were performed in independent duplicates.

Photoenzymatic hydroxylation reactions

The photochemical enzymatic hydroxylation reactions using r*Aae*UPO were performed in a very similar approach as described for halogenation reactions.

In a typical monophase reaction, a stock solution (5 mM) of sodium anthraquinone sulfonate (SAS) in NaPi buffer (pH 6.0, 60 mM) was firstly prepared. 100 µL of the stock solution was added to 895 µL of a pre-mixed solution (400 µL methanol and 495 µL NaPi buffer) in a 4 mL glass vial. Afterwards the substrate (ethyl benzene or cyclohexane) r*Aae*UPO was added. In the final solution the reaction conditions were: [substrate] = 50 mM, [r*Aae*UPO] = 100 nM, [SAS] = 0.5 mM, pH 6.0 (NaPi buffer, 60 mM) and 40% of methanol in 1.0 mL at 30 °C. As the final step, the reaction vial was closed, and exposed to visible light (Philips 7748XHP 150 W, white light bulb) under gentle magnetic stirring (200 rpm). At intervals, aliquots were withdrawn, extracted with ethyl acetate (containing 5 mM of dodecane as internal reference, extraction ratio 1 : 2) and analyzed by Gas Chromatography.

In a typical two-phase reaction, approximately 500 µL of a pre-mixed solution (200 µL methanol and 300 µL NaPi buffer) was added in a 4 mL glass vial. r*Aae*UPO was first added to this mixture, followed by addition of 500 µL substrate (ethyl benzene or cyclohexane) as the organic phase. In the final solution the reaction conditions were: substrate/ NaPi buffer (pH 6.0, 60 mM) phase ratio

= 1:1 (v/v). In the aqueous phase: [r*Aae*UPO] = 100 nM, [SAS] = 0.5 mM, 40% of methanol, 30 °C. The reaction was then irradiated under visible light. At intervals, aliquots were taken, 5 μL of organic phase was diluted by 195 μL of ethyl acetate (containing 5mM of dodecane as internal standard) and analyzed by Gas Chromatography. All above reactions were performed in independent duplicates.

Measurement of the concentration of H_2O_2

The in situ concentration of H_2O_2 in the absence of enzymes was measured by ABTS oxidation assay. A calibration curve was first drawn based on the oxidation of ABTS (1 mM) in the presence of varied concentration of H_2O_2 (0 to 0.5 mM) and r*Aae*UPO (50 nM) in NaPi buffer (pH 5.5, 100 mM) at 30 ºC. The absorbance at 420 nm was recorded. To measure the concentration of H_2O_2 in real samples, the reaction mixture with SAS (0.5mM) and 40% methanol in NaPi buffer was irradiated under visible light. Then, at intervals 25 ul of the mixture was taken and diluted to 100 ul, which was then added to 900 ul of ABTS solution. The final condition was identical to the approach used for the calibration curve. The H_2O_2 concentration was read based on the calibration curve.

Scaling-up the synthesis of 4-Br-thymol

The reaction conditions were: [substrate] = 15 mM, [*Ci*VCPO] = 75 nM, [NaBr] = 25 mM, [SAS] = 0.5 mM, pH 6.0 (NaPi buffer, 60 mM), 40% of methanol, 30 °C. 100 mL of the mixture was placed in a 120 mL glass bottle and stirred gently. To guarantee the light transmission, 6 batches were used. The mixture was irradiated for 36 hours at 30 °C under visible light. At the end of the reaction, the organic compounds were extracted by using ethyl acetate (3×). The organic phases were combined and dried over anhydrous Na_2SO_4. The organic phases were then evaporated under reduced pressure. The obtained yellowish oil was purified by silica column using petroleum ether/ethyl acetate (40 : 2, v/v) as eluent. 810 mg of 4-Br-thymol (5.9 mM) and 157 mg of 2,4-Br-thymol (0.84 mM) were obtained, corresponding to 45% isolated yield.

GC analysis

The Gas Chromatography equipped with a CP Wax 52CB column (25 m × 0.25 mm × 1.2 μm), FID, and N_2 as carrier gas was used. Dodecane (5 mM in ethyl acetate) was used as the internal standard.

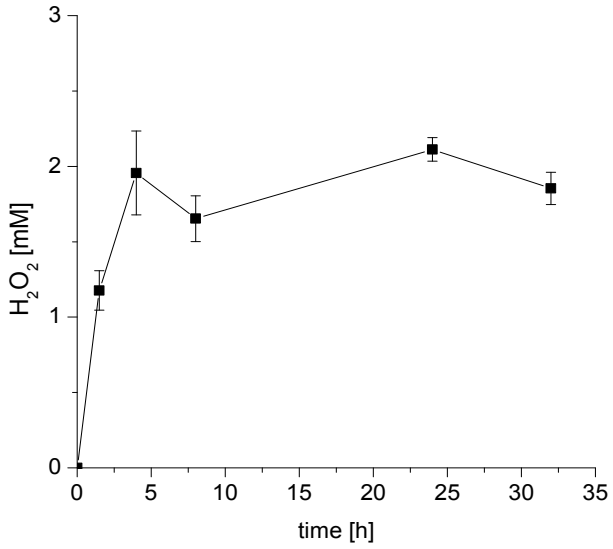


Figure S1. In situ H_2O_2 concentrations of SAS-catalyzed methanol oxidation. Reaction conditions: SAS] = 0.5 mM, pH 6.0 (NaPi buffer, 60 mM), 40% of methanol, and visible light irradiation (λ > 400 nm) at 30 ºC. No enzyme and substrate were added. Error bars represent the standard deviation of duplicate experiments.

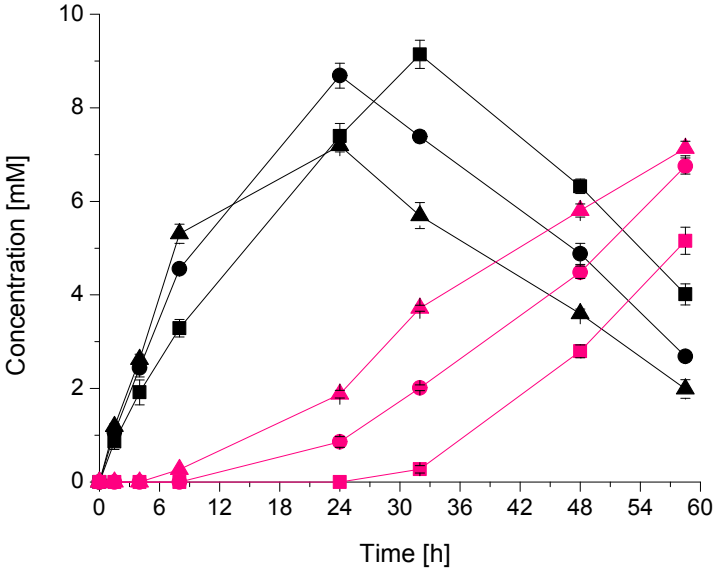


Figure S2. Halogenation of thymol by combining *Ci*VCPO and visible light-driven in situ generation of H_2O_2 using SAS. The formation of 4-bromo-thymol (**1b**, black line) and 2,4-dibromo-thymol (**1c**, red line) was recorded with varied concentrations of SAS (■=0.25 mM, ●=1 mM and ▲ = 2 mM). Reaction conditions: [substrate] = 10 mM, [*Ci*VCPO] = 100 nM, [NaBr] = 25 mM, [SAS] = 0.5-2 mM, pH 6.0 (NaPi buffer, 60 mM), 40% of co-solvent, and visible light irradiation (λ > 400 nm). Error bars represent the standard deviation of duplicate experiments.

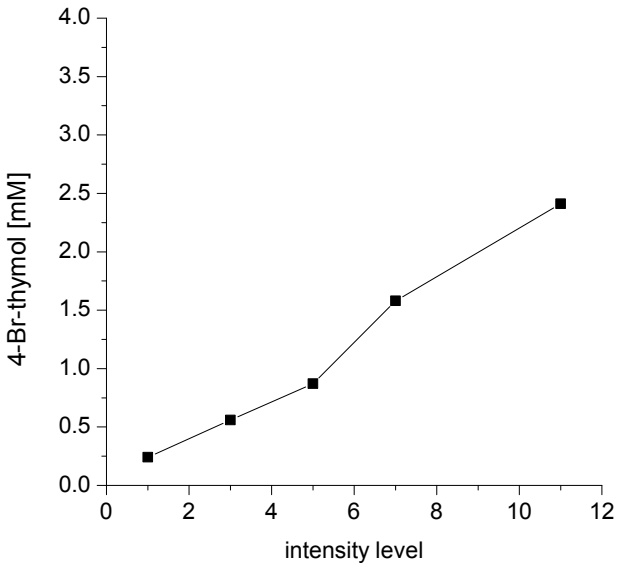


Figure S3. Influence of light intensity on the 4-br-thymol formation (**1b**). Reaction conditions: [substrate] = 10 mM, [*Ci*VCPO] = 50 nM, [SAS] = 0.5 mM, [NaBr] = 25 mM, pH 6.0 (NaPi buffer, 60 mM), 40% of co-solvent. The reactions continued for 8 h. For a blue LED light stripe in our lab, there are 11 levels of light intensity available. The level 1, 3, 5, 7 and 11 were used, respectively.

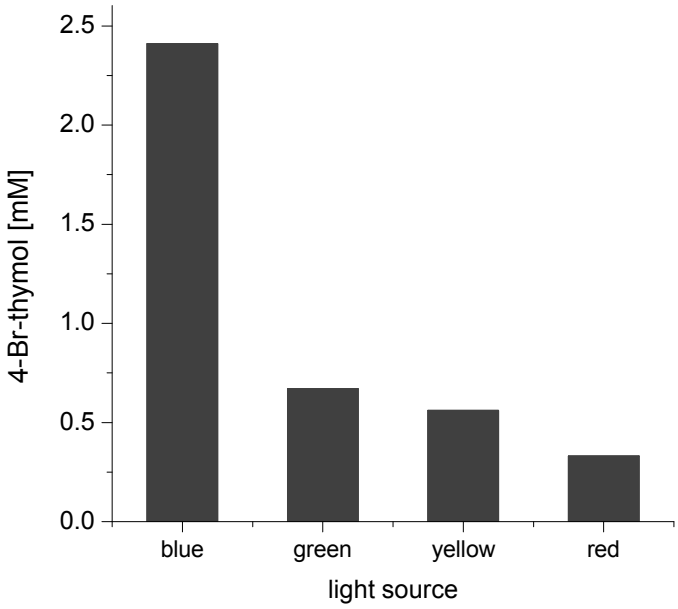


Figure S4. Influence of light sources on the 4-br-thymol formation (**1b**). Reaction conditions: [substrate] = 10 mM, [*Ci*VCPO] = 50 nM, [SAS] = 0.5 mM, [NaBr] = 25 mM, pH 6.0 (NaPi buffer, 60 mM), 40% of co-solvent. The reactions continued for 8 h. LED stripes were used as the light source.

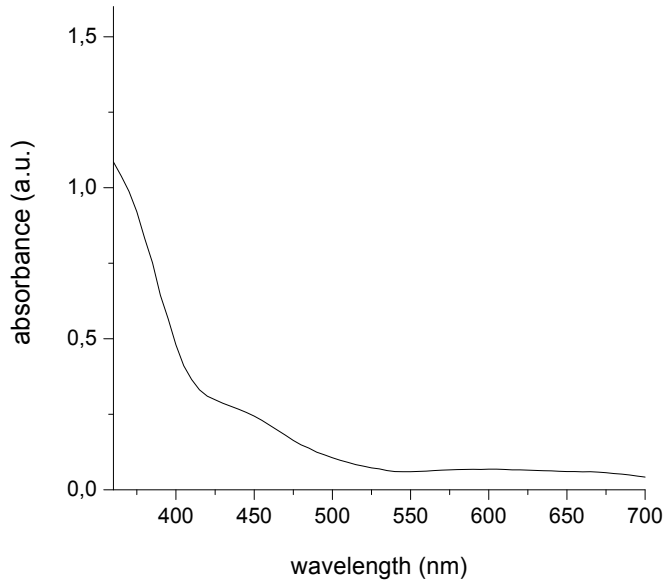


Figure S5. UV-Vis spectrum of SAS in water.

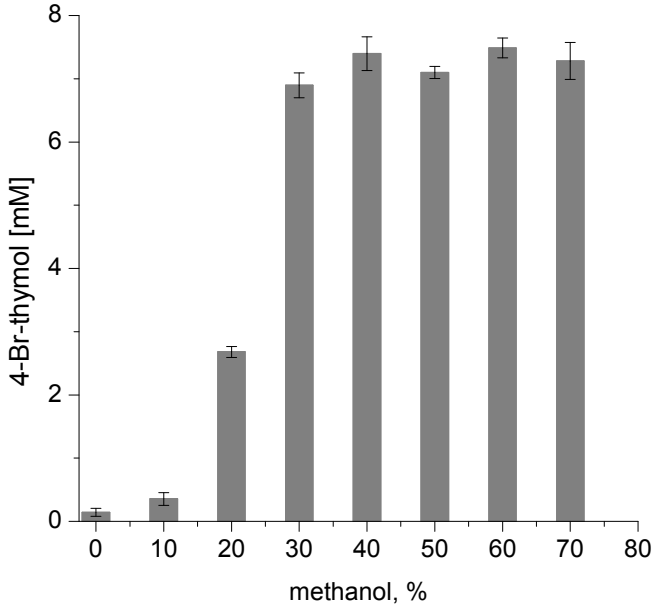


Figure S6. Influence of methanol concentrations on the formation of product **1b**. Error bars represent the standard deviation of duplicate experiments.

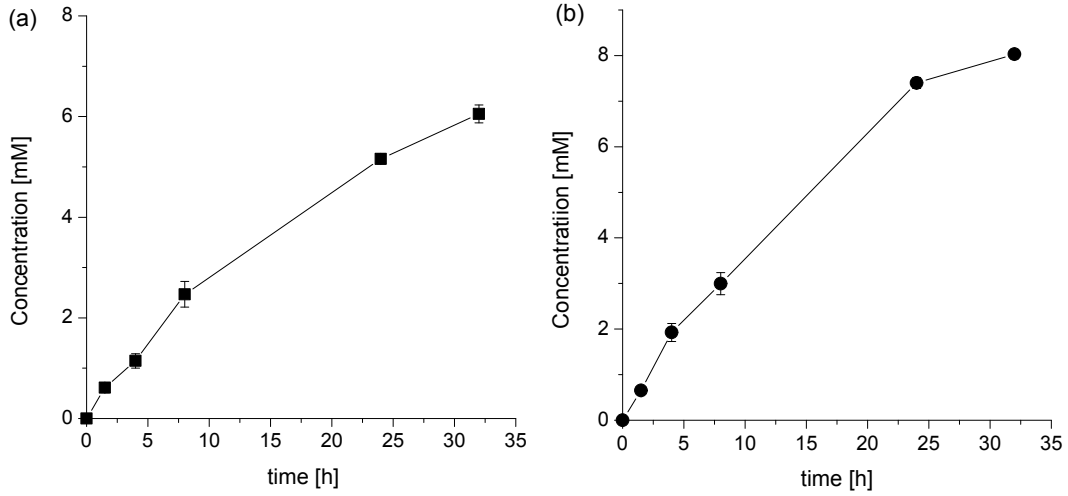


Figure S7. Hydroxybromination (a) and bomocyclization (b) reactions by combining *Ci*VCPO and visible light-driven in situ generation of H_2O_2 using SAS. Reaction conditions: [substrate] = 10 mM, [*Ci*VCPO] = 50 nM, [SAS] = 0.5 mM, pH 6.0 (NaPi buffer, 60 mM), 40% of methanol, 32h, and visible light irradiation ($\lambda > 400$ nm). Error bars represent the standard deviation of duplicate experiments.

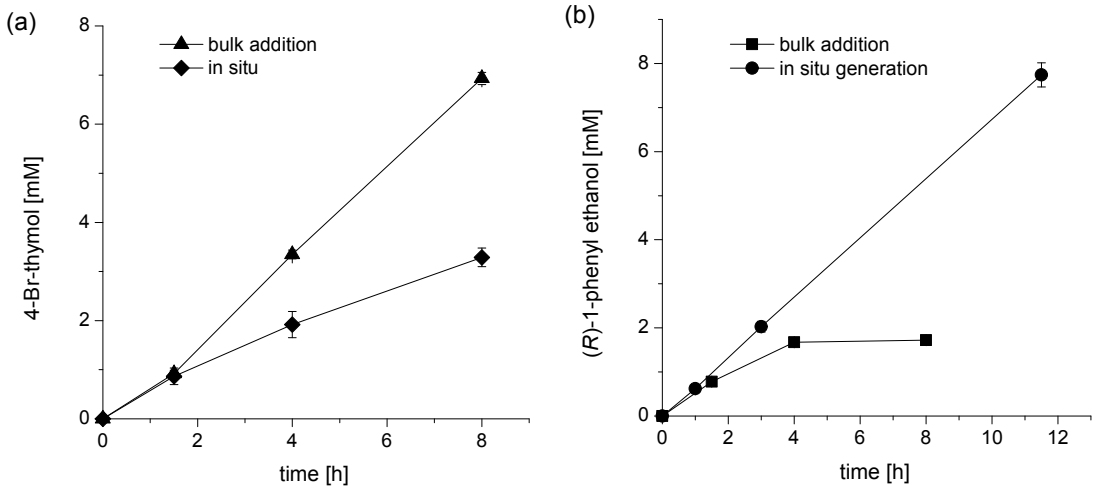


Figure S8. Comparison of bulk addition of H_2O_2 and in situ generation of H_2O_2 to drive enzymatic halogenation (a) and hydroxylation reactions (b). For bulk addition H_2O_2, the dose rate of 1.0 mM h^{-1} was used. For the in situ generation of H_2O_2, data of Figure 1a and Figure 3 were used respectively.

Figure S9. Image of the house-made light setup for photobiocatalytic reactions. Photograph taken by Wuyuan Zhang.

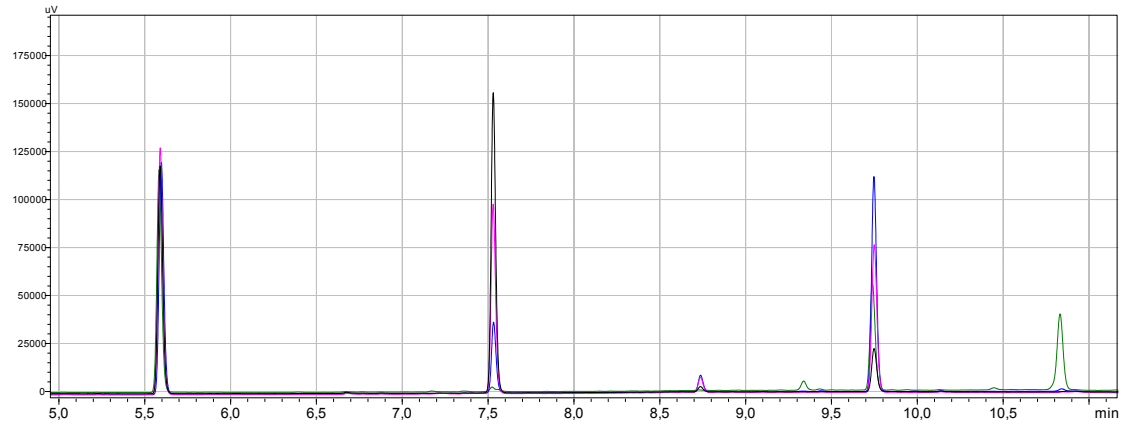


Figure S10. Representative GC chromatograms of the halogenation of thymol for 1.5 h (black), 8 h (pink), 24 h (blue) and 48 h (green). The retention time of the compounds: dodecane (internal standard) 5.57 min; thymol 7.07 min; 4-bromo-thymol 9.74 min; 2-bromo-thymol 8.63 min; 2,4-dibromo-thymol 10.82 min.

Table S1. Different buffers examined[a]

Buffer	Sodium phosphate buffer	Tris-HCl buffer	Sodium citrate buffer
1b [mM]	9.14	8.82	0.33

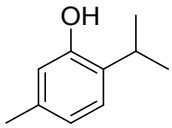
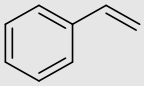
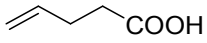
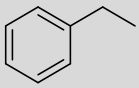
[a] The buffer conditions: pH 6.0, 60 mM.

Table S2. Comparison between the bulk addition of H_2O_2 and the in situ generation of H_2O_2[a]

	TON of the peroxyzymes	
Methods of H_2O_2 supply	r*Aae*UPO	*Ci*VCPO
Bulk addition of H_2O_2	17240	69300
In situ generation of H_2O_2	77400	32900

[a]The TON was calculated by using the last time point and compared based on the information from Figure S8.

Table S3. Details for GC analysis[a]

Substrate	T_R [min]	Temperature profile
 2-Isopropyl-5-methylphenol (Thymol)	Thymol 7.07 2-bromothymol 8.63 4-bromothymol 9.74 2,4-dibromothymol 10.82 IS 5.57	120 °C hold 3 min, 30 °C /min to 150 °C hold 1 min, 30 °C /min to 210 °C hold 2 min, 15 °C /min to 240 °C hold 4.5 min.
 Styrene	styrene 3.21 styrene epoxide 6.09 2-bromo-1-phenylethan-1-ol 11.21	130 °C hold 4 min, 30 °C /min to 200 °C hold 4.5 min, 30 °C /min to 240 °C hold 3 min.
 4-Pentenoic acid	4-pentenoic acid 9.17 5-(bromomethyl)dihydrofuran-2(3*H*)-one 11.95	130 °C hold 3 min, 30 °C /min to 200 °C hold 4.5 min, 30 °C /min to 240 °C hold 3 min.
 Ethyl benzene	ethylbenzene 2.91 1-phenylethanol 8.89 acetophenone 7.34	130 °C hold 3.5 min, 30 °C /min to 200 °C hold 4.5 min, 30 °C /min to 250 °C hold 1.5 min.
 Cyclohexane	cyclohexanol 8.81 cyclohexanone 7.67	90 °C hold 3 min, 10 °C /min to 180 °C hold 1 min, 30 °C /min to 230 °C hold 1 min.

[a]: CP Wax 52CB column (25 m × 0.25 mm × 1.2 µm), FID, N_2 as the carrier gas.

References

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