Electronic Supplementary Information

Vanadate(V)-dependent Bromoperoxidase immobilized on Magnetic Beads as Reusable Catalyst for Oxidative Bromination

Diana Wischang[a], Jens Hartung*[a], Thomas Hahn[b], Roland Ulber[b], Tobias Stumpf[c], and Claudia Fecher-Trost[c].

[a] *Fachbereich Chemie, Organische Chemie, Technische Universität Kaiserslautern, Erwin-Schrödinger-Straße, D-67663 Kaiserslautern, Germany*

[b] *Fachbereich Maschinenbau und Verfahrenstechnik, Technische Universität Kaiserslautern, Gottlieb-Daimler-Straße, D-67663 Kaiserslautern, Germany*

[c] *Fachbereich Biologie, Proteinfunktion/Proteomics, Technische Universität Kaiserslautern, Paul-Ehrlich-Straße, D-67663 Kaiserslautern, Germany*

Contents

1 General Remarks

(*i*) The compound numbering in the Electronic Supplementary Information is consistent with that of the accompanying publication. (*ii*) References refer exclusively to the Electronic Supplementary Information. (*iii*) The following substances were used in *pro analysi* quality: NaBr (*Merck*) and *t*BuOH (*Sigma-Aldrich*). (*iv*) Silica gel (60, 40–63 µm, *Merck*) was used for column chromatography.

2 Instrumentation

Melting points [°C] were determined on a melting point apparatus 9100 (*Electrothermal*) and were not corrected.

^{1}H and ^{13}C spectra were recorded with FT-NMR DPX 200, DPX 400 and DMX 600 instruments (*Bruker*). Chemical shifts refer to the δ-scale. The resonances of residual protons and those of carbon of deuterated solvents $CDCl_3$ (δ_H 7.26, δ_C 77.2) were used as internal standards. Overlap of two ^{13}C resonances in NMR spectra, as indicated by 2 C, was established with the aid of supplementing HMQC-measurements.

Mass spectra (EI, 70 eV) were recorded with a Mass Selective Detector HP 6890 (*Hewlett Packard*).

GC analyses were performed on a HP 6890 Series (*Hewlett Packard*) with a ZB5 column (*Phenomenex*, 30 m x 0.25 mm, 0.25 µm). Temperature program: 40 °C (3 min), linear temperature gradient (10 °C min^{-1}) to 280 °C, final temperature 280 °C (10 min).

IR spectra were measured from pelletized samples in KBr using a Perkin Elmer FT/IR 1600 spectrometer.

UV/Vis enzyme kinetics and UV/vis spectra were recorded with a Varian Cary 100 Conc double beam spectrophotometer.

Microplate Reader Bio Tek EL 808 was used for photometric measurement of Bradford assays.

MALDI-TOF measurements (Ultraflex MALDI-TOF-TOF, *Bruker Daltonics*) were performed by accumulating one thousand spectra per sample processing them with Flex-

Analysis 2.2 (*Bruker Daltonics*). Peptide mass fingerprints spectra were analyzed with Biotools 2.2 (*Bruker Daltonics*).

Denatured polyacrylamide gel electrophoresis (PAGE) was performed with a NUPAGE®-Novex gelelectrophoresis system (Invitrogen) in the presence of a 3-(*N*-morpholino) propanesulfonic acid (MOPS) system.

Reaction progress was monitored via thin layer chromatography (TLC) on aluminium sheets coated with silica gel (60 F_{254}, *Merck*). Compounds were detected on the basis of UV absorption (254 nm) or colored compound formation upon spraying Ekkert's reagent on developed TLC sheets followed by gentle warming.

All solvents were purified according to standard procedures.[1]

3 Analysis of Bromoperoxidase I (*A. nodosum*)

3.1 Denatured polyacrylamide gel electrophoresis (PAGE)

7.5 µg of purified protein[2] elute were denatured with loading buffer (NUPAGE®-Novex, Invitrogen) at 70 °C for 10 min, separated in 4 %–12 % precast [Bis-(2-hydroxyethyl) imino-tris-(hydroxymethyl)-methane-HCl] (Bis-Tris) buffered gels in the presence of a 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer system (NUPAGE®-Novex, Invitrogen). Protein bands were visualized with a colloidal CoomassieG250 ($C_{47}H_{48}N_3NaO_7S_2$, sodium salt) stain [34 % (*v*/*v*) CH_3OH, 2 % (*v*/*v*) H_3PO_4, 17 % (*w*/*v*) NH_4SO_4, 0.066 % (*w*/*v*) Coomassie G250] for 14 h. The gel was destained in deionized water. Protein bands were cut and prepared for MALDI mass spectrometry (section 3.2).

3.2 MALDI mass spectrometry and database search

Excized bands were alternately washed twice with 50 mM aq. NH_4HCO_3 (Solution A) and 50 % (*v*/*v*) aq. NH_4HCO_3 (50 mM), 50 % (*v*/*v*) CH_3CN (Solution B). Reductive cleavage of disulfide bridges was performed by incubation for 30 min at 56 °C in 50 mM aq. NH_4HCO_3 solution, 10 mM dithiothreitol DTT, followed by carbamidomethylation for 30 min at 56 °C in darkness in 50 mM aq. solution of NH_4HCO_3 with 5 mM iodoacetamide. Gel pieces were washed twice using solutions A and B in an alternating manner and vacuum dried (eppendorf concentrator 5301). In-gel digestion was performed by incubating the gel pieces with 4.5 µL of a 25 µg/mL solution of porcine trypsin (*Promega*, Madison, MA, USA) at 37 °C overnight. Resulting peptides were extracted by shaking the gel in 0.1 % CF_3CO_2H. Extracted peptides were concentrated with PerfectPure C-18 tips (*Eppendorf*, Hamburg, Germany) and applied on a steel target (*Bruker Daltonics*, Bremen, Germany) using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix (*Bruker Daltonics*). To prepare a thin peptide/matrix layer the bound peptides were eluted from C18 tips with 3–5 µL of a saturated CHCA matrix solution [1 % (*w*/*v*) CHCA, 50 % CH_3CN, 0.1 % CF_3CO_2H] and applied on the steel target manually, the matrix peptide layer was formed upon solvent evaporation. MALDI-TOF measurements (Ultraflex MALDI-TOF-TOF, *Bruker Daltonics*) were performed by accumulating one thousand spectra per sample processing with settings of Flex-Analysis 2.2 (*Bruker Daltonics*). MASCOT searches were performed with settings of carbamidomethylation as fixed modification and oxidation of methionine as variable

modification. Peptide mass tolerance was set to 0.15 Da. For database search the NCBI database was used. Database searches were conducted using the Mascot™ search algorithm (www.matrixscience.com, MatrixScience) against the whole NCBI non-redundant database (13th Nov 2007, 5633163 sequences). By this approach V_{Br}PO(*An*I) (P81701, PDB 1QI9, gi|13124466 and gi|9257121) was identified in two bands by peptide mass fingerprint analysis (Figure 2, black arrows). The amino acid coverage of the protein was 20.3 %. The amino acid sequence of the prominent protein band, which migrates above 66 kDa could not be identified.

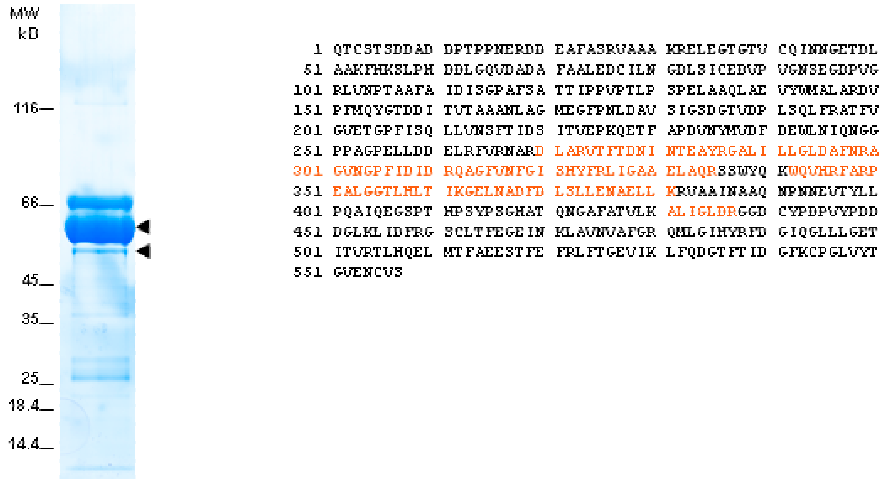


Figure S1. V_{Br}PO(*An*I) analysis showing a Coomassie-stained polyacrylamide gel of purified proteins [black arrowheads represent protein bands, where V_{Br}PO(*An*I) was found via MALDI-MS and the coverage of the amino acid sequence of V_{Br}PO(*An*I) by the peptides identified by peptide mass fingerprint (PMF) analysis using MALDI-MS analysis (letters printed in red).

4 Determination of Bromoperoxidase Activity and Concentration

4.1 Triiodide assay.[3,4]

Reference and sample cuvettes (10.0 mm) were charged with aq. phosphate buffer (3.00 mL, 132 mM K_2HPO_4, 33.9 mM citric acid monohydrate, pH 6.2), stock solutions of aq. KI (100 µL, 200 mM), and aq. H_2O_2 (100 µL, 26.6 mM). The cuvettes were closed and thermostated to 20 ± 0.5 °C. H_2O (100 µL) was pipetted into the reference cuvette. The contents were mixed. Enzyme solution (100 µL) was pipetted into to the stirred solution of the sample solution followed by immediate monitoring of triiodide absorption (λ = 350 nm) as a function of time.

4.2 Bradford assay.[5,6]

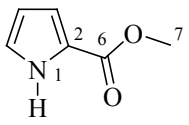
Preparation of protein reagent. Coomassie Brilliant Blue G-250 (100 mg) was dissolved in ethanol (95%, 50 mL). To this solution phosphoric acid (85%, 100 mL) was added. The resulting solution was diluted with distilled H_2O to a final volume of 1 L. Before utilization the protein reagent was filtrated.

Calibration. Bovine serum albumin (*Sigma*) served as reference. The concentrations ranged between 0–0.5 mg/mL in aq. MES-buffer (50 mM, pH 6.2) for **1a** and **1b** or in aq. Tris(HCl)-buffer (100 mM, pH 8.8) for **2a** and **2b**.

Microprotein assay. Samples as well as standards (10 µL, each protein solutions containing between 0 to 5 µg protein) were pipetted into test tubes. Protein reagent (290 µL) was added to each tube and the contents were mixed. After 5 min the absorption (λ = 595 nm) of the samples and standards against the reagent blank [MES-buffer (50 mM, pH 6.2 or Tris(HCl)-buffer (100 mM, pH 8.8)] were measured at 30 °C.

5 Preparation of Methyl 1*H*-pyrrole-2-carboxylate (3)

To a freshly prepared solution of $NaOCH_3$ from sodium (0.25 g, 10.9 mmol) in anhydrous CH_3OH (20 mL) was added in batches 2,2,2-trichloro-1-(1*H*-pyrrol-2-yl)-ethanone[7] (4.70 g, 22.0 mmol) over a period of 10 min in an atmosphere of N_2. After stirring for 30 min at 23 °C the solvent was removed under reduced pressure (11 mbar, 40 °C). The dark residue was partitioned between Et_2O (50 mL) and 2 M aq. HCl (15 mL). The organic layer was separated and kept. The aqueous layer was extracted with Et_2O (2 × 10 mL). Combined organic layer and extracts were washed with satd. aq. $NaHCO_3$ (15 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure (10 mbar, 40 °C) to afford a purple solid which was purified by column chromatography (Et_2O). Yield: 2.56 g (20.0 mmol, 92 %), colourless solid, mp 74–77 °C (from Et_2O). IR (KBr) $\tilde{\nu}$ /cm^{-1} 3290, 3122, 2953, 1685, 1559, 1446, 1406, 1128. δ_H (600 MHz; $CDCl_3$) 3.86 (3 H, s, OCH_3), 6.26–6.28 (1 H, m, H-4), 6.91–6.93 (1 H, m, H-3), 6.95–6.96 (1 H, m, H-5), 9.22 (1 H, br.s, -NH-). δ_C (150 MHz; $CDCl_3$) 51.6 (C-7), 110.6 (C-4), 115.4 (C-3), 122.8 (C-2), 123.0 (C-5), 161.7 (C-6). MS (EI) *m*/*z* 125 (66), 94 (100), 66 (36).



6 O_2/D-Glucose/D-Glucose Oxidase as Oxidant

To a solution of methyl 1*H*-pyrrole-2-carboxylate (**3**) (22.5 mg, 0.18 mmol), NaBr (18.5 mg, 0.18 mmol), and D-(+)-glucose (541 mg, 3.00 mmol) in MES-buffer (50 mM, pH 5.9, 18.8 ml) and *t*BuOH (6.2 ml) were added glucose oxidase (11.4 µg, 1.7 *U*) and $V_{Br}PO(AnI)$ (37.5 µl, 6.2 U_T, 0.05 mmol%). The reaction mixture was stirred at 23 °C for 1 d in an open flask having contact to laboratory atmosphere. The aqueous layer was extracted with Et_2O (3 × 10 ml). The combined organic extracts were dried with $MgSO_4$. The solvent was removed under reduced pressure (12 mbar, 40 °C) to afford a product mixture which was analyzed by ^{1}H-NMR. Yields: 59 % of **4**$_{\mathbf{4\text{-}Br}}$, 11 % of **4**$_{\mathbf{5\text{-}Br}}$ and 7 % of **7**. 526 U_T^0 mg^{-1}, 269 U_T^{final} mg^{-1}, pH^{final} 5.80.

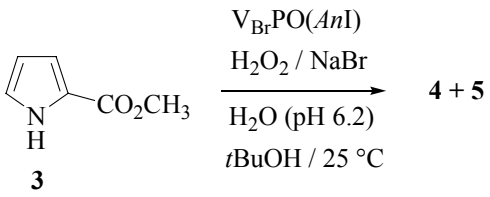
Methyl 4,5-dibromo-1*H*-pyrrole-2-carboxylate (5).[8] Yield: 3.56 mg (12.6 µmol, 7 %). δ_H (600 MHz; $CDCl_3$) 3.90 (3 H, s, -OCH_3), 6.87 (1 H, d, J = 2.9, H-3), 10.06 (1 H, br.s, -NH-). MS (EI) *m*/*z* 285 (27), 283 (58), 281 (32), 253 (48), 251 (100), 249 (52), 226 (6), 224 (14), 222 (6), 199 (6), 197 (15), 195 (8), 144 (14), 142 (14).

7 Multiple Use of $V_{Br}PO(AnI)$ in Sequential Pyrrole Brominations

To a solution of methyl 1*H*-pyrrole-2-carboxylate (**3**) (18.0 mg, 0.14 mmol), NaBr (14.8 mg, 0.14 mmol), and H_2O_2 [4 mL, 36 mM, prepared in MES-buffer (50 mM, pH 6.2)] in MES-buffer (50 mM, pH 6.2, 15.0 mL) and *t*BuOH (5.0 mL), $V_{Br}PO(AnI)$ (30.0 µL, 5.0 U_T, 0.05 mmol%) was added. The reaction mixture was stirred at 23 °C for 1 d. The aqueous layer was extracted with Et_2O (3 × 5 mL). Combined organic extracts were dried ($MgSO_4$). The solvent was removed under reduced pressure (13 mbar, 40 °C) to afford a product mixture which was analyzed by ^{1}H NMR ($CDCl_3$).

Additional methyl 1*H*-pyrrole-2-carboxylate (**3**) (18.0 mg, 0.14 mmol), NaBr (14.8 mg, 0.14 mmol), H_2O_2 [4 mL, 36 mM, prepared in MES-buffer (50 mM, pH 6.2)] and *t*BuOH (5.0 mL) was added to the aqueous layer. The resulting homogeneous solution that was left from the ethereal extraction of the reaction mixture was stirred for additional 1 d at 23 °C and worked up as described in the first iteration. This procedure was repeated until no further bromopyrrole formation was detectable (^{1}H NMR).

Table S1. Efficiency of product formation in $V_{Br}PO(AnI)$-catalyzed reactions[a] in sequential runs



3

entry / cycle	conv. **3** [b] / %	pH^{final} [c]	**4** / % (*4*/*5*) [b]	**5** / % [b]
1	79	6.3	56 (79/21)	10
2	39	6.5	34 (79/21)	2
3	33	6.5	10 (80/20)	– [d]
4	15	6.4	8 (75/25)	– [d]

[a] 4.96 U_T corresponding to 0.05 mmol% $V_{Br}PO(AnI)$; 144 µmol of **3**, 1.0 equiv. of H_2O_2 and NaBr, H_2O/*t*BuOH = 75/25 (*v*/*v*). [b] ^{1}H NMR yields. [c] pH electrode. [d] not detected.

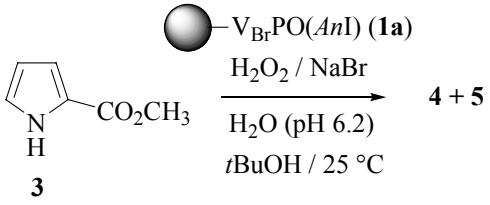
8 Multiple use of immobilized $V_{Br}PO(AnI)$ in sequential pyrrole brominations

8.1 Optical density measurement

For the optical density study the colloidal solutions of M PVA E01, M PVA E02, M PVA N11 and M PVA N12 (0.2 mL, 50 mg/mL) were diluted in distilled H_2O (2.8 mL, 3.3 mg/mL) and pipetted into the sample cuvette. Distilled H_2O (3 mL) was pipetted into the reference cuvette. The contents were mixed. Nd/Fe bar magnet (20 × 10 × 5 mm, N42 magnetization) was justified directly to sample cuvette, followed by immediate monitoring the absorption at 600 nm as a function of time (up to 5 minutes).

8.2 Immobilized onto magnetite particles having epoxide endgroups (M PVA E01) (1a)

Table S2. Efficiency of product formation in $V_{Br}PO(AnI)$-catalyzed reactions[a] in sequential runs if immobilized onto M PVA E01

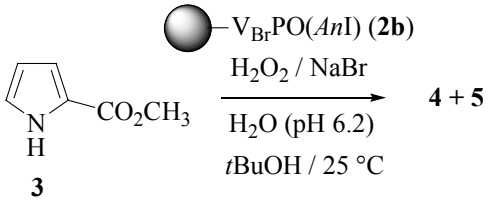


entry / cycle	conv. **3** [b] / %	pH^{final}	**4** / % (*4*/*5*) [b,c]	**5** / % [b]
1	54	7.2	40 (90/10)	2
2	55	7.2	40 (88/12)	2
3	56	7.0	40 (85/15)	2
4	63	7.0	39 (85/15)	2
5	56	7.0	35 (83/17)	2
6	58	6.8	33 (82/18)	2
7	57	6.8	34 (82/18)	– [d]
8	56	6.8	29 (83/17)	– [d]
9	62	6.8	25 (88/12)	– [d]
10	31	6.8	26 (85/15)	– [d]
11	30	6.8	23 (83/17)	– [d]
12	34	6.6	14 (86/14)	– [d]
13	32	6.4	14 (86/14)	– [d]
14	38	6.4	16 (63/37)	– [d]
15	25	6.4	9 (89/11)	– [d]

[a] 6.9 U_T corresponding to 0.37 mmol% $V_{Br}PO(AnI)$; 0.20 mmol of **3**, 1.0 equiv. of H_2O_2 and NaBr, H_2O/*t*BuOH = 75/25 (*v*/*v*). [b] Yields determined via ^1H NMR versus pentachlorobenzene as internal standard. [c] Ratio of 4/5-regioisomers of bromopyrrole **4**. [d] not detected.

8.3 Immobilized onto magnetite particles having amino endgroups (M PVA N12) (2b)

Table S2. Efficiency of product formation in $V_{Br}PO(AnI)$-catalyzed reactions[a] in sequential runs if immobilized onto M PVA N12



entry / cycle	conv. **3** [b] / %	pH^{final}	**4** / % (*4*/*5*) [b]	**5** / % [b]
1	75	7.8	32 (94/6)	2
2	60	7.8	37 (89/11)	2
3	57	7.8	40 (90/10)	2
4	64	7.8	43 (86/14)	– [c]
5	74	7.6	39 (85/15)	– [c]
6	55	7.6	40 (85/15)	– [c]
7	60	7.4	40 (85/15)	– [c]
8	43	7.2	31 (87/13)	– [c]
9	66	6.8	22 (86/14)	– [c]
10	47	6.8	16 (81/19)	– [c]
11	41	6.4	17 (88/12)	– [c]
12	15	6.2	7 (86/14)	– [c]
13	18	6.2	5 (80/20)	– [c]
14	16	6.2	2 (>99/– [c])	– [c]

[a] 6.9 U_T corresponding to 0.17 mmol% $V_{Br}PO(AnI)$; 0.20 mmol of **3**, 1.0 equiv. of H_2O_2 and NaBr, H_2O/*t*BuOH = 75/25 (*v*/*v*). [b] Yields determined via ^1H NMR versus pentachlorobenzene as internal standard. [c] Ratio of 4/5-regioisomers of bromopyrrole **4**. [d] not detected.

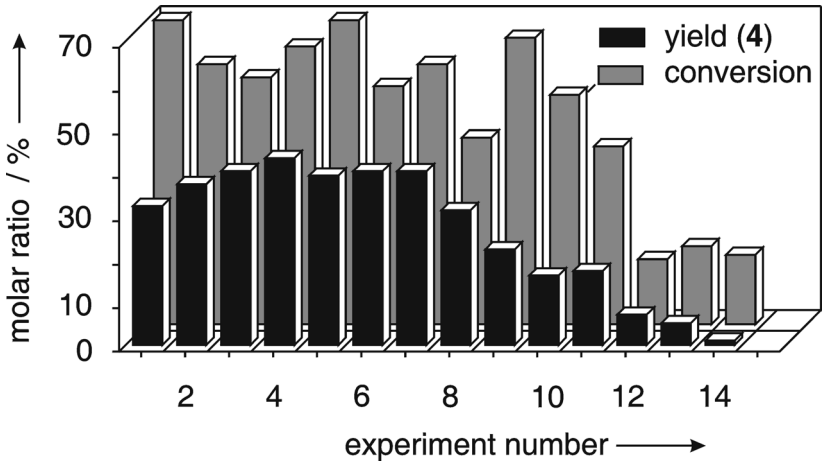


Figure S2. Conversion of substrate **3** (c^0 = 35 mM; gray columns) and efficiency of methyl pyrrole-2-carboxylate bromination (black columns) upon multiple use of $V_{Br}PO(AnI)$-preparation **2b** (6.9 U_T) for catalyzing oxidative bromination in aqueous *t*BuOH (pH 6.2, 23 °C) using 1.0 equiv. each of H_2O_2 and NaBr (3 h, see text).

9 References

1 D.D. Perrin, W.L.F. Armarego and D.R. Perin, in *Purification of Laboratory Chemicals*, Pergamon Press, Oxford, 2nd edn., 1980.

1 J. Hartung, O. Brücher, D. Hach, H. Schulz, H. Vilter and G. Ruick, *Phytochem.*, 2008, **69**, 2826–2830.

3 F. Björkstén, *European J. Biochem.*, 1968, **5**, 133–142.

4 H. Vilter, K.-W. Glombitza and A. Grawe, *Bot. Marina*, 1983, **26**, 331–340.

5 M.M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.

6 S.J. Compton and C.G. Jones, *Anal. Biochem.*, 1976, **151**, 369–374.

7 D.M. Bailey, R.E. Johnson and N.F. Albertson, *Org. Synth.*, 1971, **51**, 100.

8 C. Schroif-Gregoire, N. Travert, A. Zaparucha and A. Al-Mourabit, *Org. Lett.*, 2006, **8**, 2961–2964.