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Regioselective Enzymatic Halogenation of Substituted Tryptophan Derivatives using the FAD-Dependent Halogenase RebH

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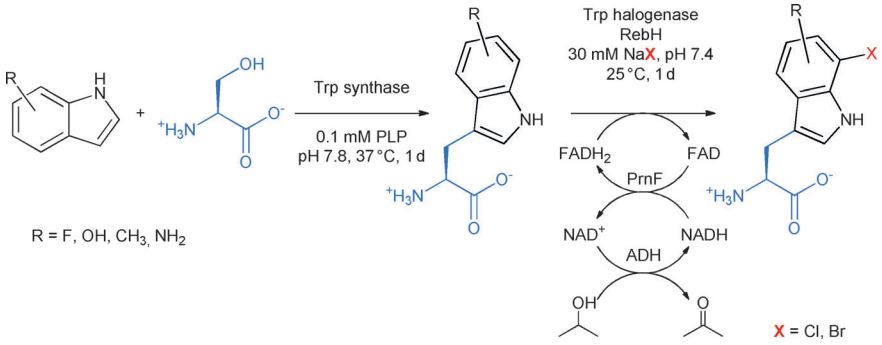
Regioselective methods to establish carbon–halide bonds are still rare, although halogenation is considered as a commonly used methodology for the functionalization of organic compounds. The incorporation of halogen substituents by organic synthesis usually requires hazardous conditions, shows poor regioselectivity and results in the formation of unwanted by-products. In addition, halogenation by electrophilic aromatic substitution (S_EAr) obeys distinct rules depending on electron-withdrawing or -donating groups already present in the aromatic ring. We employed the tryptophan-7-halogenase RebH for regioselective enzymatic halogenation to overcome these limitations. In combination with a tryptophan synthase, an array of C5- and C6-substituted tryptophan derivatives was synthesized and halogenated by RebH. The halogenase is able override these directing effects and halogenates at the electronically unfavored C7-*meta*-position, even in presence of *ortho*/*para*-directing groups.

Introduction

Halogenated compounds are important bioactive substances and essential intermediates in the chemical,[1] agrochemical,[2] and pharmaceutical industry,[3] owing to their metabolic stability and the ease of modification, e.g., by nucleophilic substitution or metal-catalyzed cross-coupling reactions. However, the introduction of halogen substituents, especially in mechanistically less-favored positions, remains a challenge. More than 5000 naturally occurring halogenated compounds are known,[4] including molecules with diverse biological activities like hormones (e.g. thyroxine[5]), antibiotics (e.g. chloramphenicol[6] and chlorotetracycline[7]), and cytostatics (e.g. cryptophycin[8]). Moreover, halogenation is in many cases essential for biological activity.[9] Although chemical halogenations mostly use molecular chlorine or bromine as hazardous reagents, often in a combination with Lewis acids, notably Nature employs for halogenation benign halide salts together with molecular oxygen and the reaction proceeds at pH 7 and 25 °C in aqueous media.[10,11] This reaction is catalyzed by FAD-dependent halogenases, one of the major enzyme families responsible for regioselective halogenation of aromatic or hetero-aromatic rings in natural metabolites.[12] The first member of this family, PrnA, was discovered in 2000.[11] Its close relative, the FAD-dependent tryptophan halogenase RebH belongs to the biosynthetic pathway of the chlorinated antitumor agent Rebeccamycin from *Lechevalieria aerocolonigenes*.[13] The enzyme is able to chlorinate and brominate L-tryptophan regioselectively at the electronically unfavored C7 position, while indole or tryptophan usually are halogenated at the C3 or C2 position respectively (Scheme 1).[14]



Scheme 1. Combination of tryptophan synthase from *Salmonella enterica* and tryptophan-7-halogenase RebH from *Lechevalieria aerocolonigenes* for the regioselective halogenation of substituted tryptophan derivatives.

Investigations by the groups of van Pée and Walsh, pioneers in the field of halogenases, have led to the elucidation of the enzymatic halogenation mechanism.[15,16] The proposed mechanism involves an additional flavin reductase, which supplies $FADH_2$. The latter is oxidized by molecular oxygen to form

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a flavin peroxide, which is commonly found in Baeyer–Villiger monooxygenases.[17–19] After nucleophilic attack of the halide ion (Cl^- or Br^-) on the flavin peroxide intermediate, hypohalous acid is formed and channeled along a 10 Å long tunnel from the $FADH_2$ binding site to the tryptophan binding site. Upon oxidation of the ε-amino function of a distinct lysine residue (K79) in the active site, a long-lived *N*-haloamine intermediate is formed that is responsible for the regioselective halogenation at the electronically unfavored C7 position of tryptophan.[15–17,20]

Results and Discussion

Establishment of an enzymatic halogenation system

As the regiochemistry of chemical halogenation of substituted arenes is restricted by electronic substituent effects, we became interested in the potential of enzymatic halogenation to overcome these limitations. This study focuses on enzymatic halogenation by the L-tryptophan-7-halogenase RebH that was amplified from genomic DNA of *L. aerocolonigenes* via PCR, cloned into the expression vector pET28a and overexpressed in *E. coli* BL21(DE3) pGro7[21] to establish an in vitro enzymatic halogenation system. After purification by means of immobilized metal ion affinity chromatography (IMAC), enzyme activity was determined on an analytical scale in the presence of the flavin reductase PrnF from *Pseudomonas fluorescens*[11] and an alcohol dehydrogenase from *Rhodococcus* sp.[22] for in situ cofactor regeneration (Figure 1 A). L-Tryptophan was converted almost quantitatively into 7-chloro-L-tryptophan (**1**), as identified both by a HPLC-standard and by mass spectrometry (Figure 1 B). Product formation was analyzed based on the consumption of L-tryptophan in comparison with a calibration curve (Figure 1 C). A catalytic constant of $k_{cat} = 1.0\ min^{-1}$ and a total turnover number (TTN) of 102 ± 3 could be extracted from these data.

Halogenation of substituted tryptophan derivatives

As the halogenation of the natural substrate L-tryptophan was successful with our in vitro system on an analytical scale, the influence of electron-withdrawing and -donating groups at the indole ring on the regioselectivity of halogenation by RebH was further studied. An array of C5- and C6-substituted L-tryptophan derivatives was envisaged to be synthesized enzymatically using a tryptophan synthase from *Salmonella enterica*.[23,24] Briefly, a lysate from a commercially available *E. coli* strain (ATCC strain 37845) producing recombinant tryptophan synthase was used to condense C5- or C6-substituted indoles with L-serine in the presence of catalytic amounts of pyridoxal phosphate to provide the corresponding array of L-tryptophan derivatives. These compounds were then used as substrates in RebH halogenation. All of the synthesized derivatives could be chlorinated, as well as brominated, although the TTN differ significantly among all substrates (Table 1). Electron-rich arenes like 5-hydroxy-L-tryptophan (**2**) show a TTN similar to the natural substrate L-tryptophan (TTN = 80 ± 3 and 102 ± 3 respectively), whereas arenes with electron-withdrawing groups are expectedly characterized by much lower TTN, for example, 5-fluoro-L-tryptophan (**3**) was converted with a TTN of 7 ± 4. The tendency of the TTN matches the substituent influence on S_EAr caused by the mesomeric and/or inductive properties. Upon

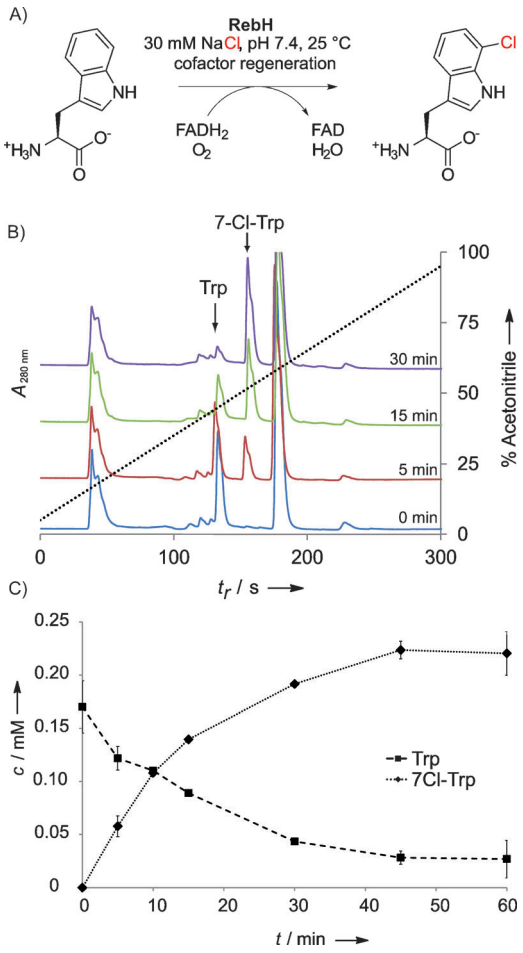

Figure 1. Halogenase activity assay of purified RebH. A) Enzymatic activity was measured in the presence of the flavin reductase PrnF and an alcohol dehydrogenase to promote cofactor regeneration. B) The activity of RebH was monitored by reversed-phase HPLC at $\lambda = 280$ nm. L-Tryptophan (0.25 mM) is eluted at a retention time of $t_r = 134$ s, whereas the signal at $t_r = 177$ s corresponds to 4-nitrophenol as an internal standard. During 30 min, a constant consumption of L-tryptophan was observed. In addition, a new signal at $t_r = 156$ s appeared, which was identified as 7-chloro-L-tryptophan (**1**) based on a standard, as well as by LC-MS. C) Product formation during the reaction process was calculated based on the decrease in peak area of the L-tryptophan signal in comparison with a calibration curve.

Table 1. Total turnover numbers [TTN] for the chlorination of selected Trp-derivatives.

Substrate	M/I effect	TTN
L-tryptophan	–	102 ± 3
5-hydroxy-L-tryptophan (**2**)	+M, -I	80 ± 3
5-methyl-L-tryptophan (**5**)	+I	55 ± 9
5-fluoro-L-tryptophan (**3**)	+M, -I	7 ± 4
5-bromo-L-tryptophan (**4**)	+M, -I	traces

halogenation of 5-bromo-L-tryptophan (**4**) only traces could be observed, probably due to the high van der Waals radius of the bromo substituent in combination with its highly negative inductive capacity. 5-Methyl-L-tryptophan (**5**) is converted at a moderate TTN of 55±9, which is in good agreement with the observed tendency due to its positive inductive effect.

As an alternative to halogenation with purified enzyme, the reactions were also conducted with *E. coli* lysate containing overexpressed RebH. Surprisingly, in many cases the main product is still halogenated at the electronically unfavored C7 position, even in presence of deactivating and *ortho*/*para*-directing groups like the 5-fluoro-substituent. The position of halogenation in the case of 5-fluoro-L-tryptophan (**3**) can easily be identified as C7 by using ^{1}H NMR-spectroscopy (Figure 2).

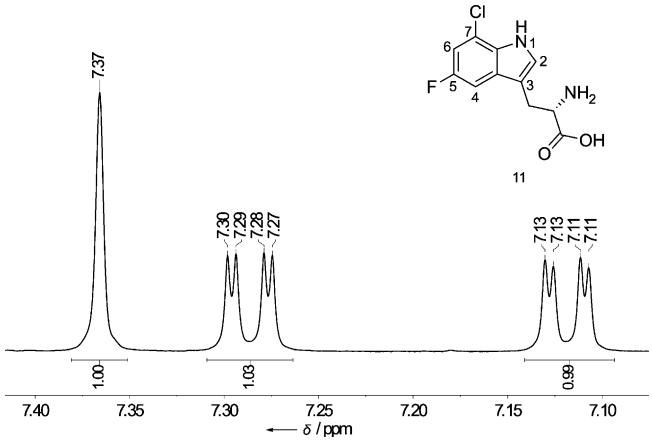

Figure 2. ^{1}H NMR spectrum of the regioselective halogenation product of 5-fluoro-L-tryptophan (**3**). Chlorination takes place in the *meta*-position of the fluoro-substituent, an deactivating *ortho*/*para*-directing group. This can be confirmed by the coupling constants of each proton to the C5-fluoro substituent, as well as by the ^4J coupling between both aromatic indole protons ($^3J_{H,F}$ = 9.5 Hz, $^4J_{H,H}$ = 2.2 Hz).

Two symmetric double doublet signals are observed for the two protons adjacent to the fluoro substituent in the ^{1}H NMR spectrum of 7-chloro-5-fluoro-L-tryptophan (**11**) with two coupling constants of $^3J_{H,F}$ = 9.5 Hz and $^4J_{H,H}$ = 2.2 Hz. Starting from 5-hydroxy-L-tryptophan (**2**), the position of chlorination in **9** can also be identified as C7, owing to the ^4J coupling of both aromatic protons in the indole ring. Interestingly, bromination takes place in the C6 position to give **10**, identified by a cross-signal of the C7 proton and the indole amine functionality in the ROESY-NMR spectrum. In addition, the C6 and C7 halogenated derivatives are formed together with dihalogenated product, if a low concentration of the reactant 5-methyl-L-tryptophan (**5**) and 5-amino-L-tryptophan (**6**), respectively, is used. In the case of 5-methyl-L-tryptophan (**5**), halogenation occurs at C6 and C7, respectively, to give **13** and **14**, as confirmed by ROESY-NMR cross-signals between the protons of the C5 methyl group and the adjacent protons. Upon chlorination of 5-methyl-L-tryptophan (**5**), a 1:1 ratio between tryptophan derivatives halogenated at C6 or C7 is observed, whereas bromination prevails at C6 to give **17**. 5-Amino-L-tryptophan (**6**) is mainly halogenated at the C6 position. In both cases, dihalogenation occurs in C6 and C7 position for 5-amino (**6**) and 5-methyl-L-tryptophan (**5**), as shown by ROESY-NMR cross-signals between Cβ and C2 and C4 protons at the indole ring. A possible explanation for the altered regioselectivity for 5-methyl-L-tryptophan (**5**) could be a change in the binding position of the substrate inside RebH in combination with the electronic preference of the *ortho*-position. Docking analysis hints toward a slight rotation of the 5-methyl-derivative in comparison to the natural substrate L-tryptophan (Figure 3 B), whereas no significant change was observable for 5-hydroxy-L-tryptophan (**2**, Figure 3 A) or 5-amino-L-tryptophan (**6**).

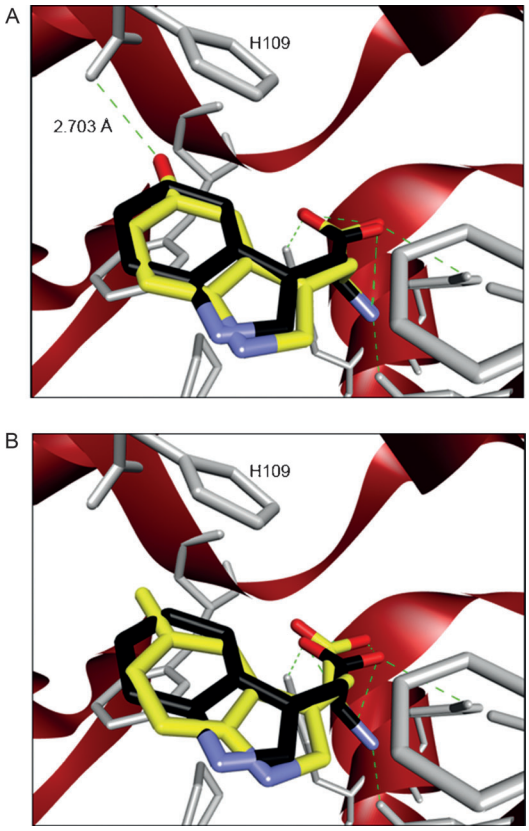

Figure 3. Docking analysis of L-tryptophan compared to 5-hydroxy-L-tryptophan (**A**) and 5-methyl-L-tryptophan (**B**). 5-hydroxy-L-tryptophan (yellow) is bound in a similar orientation compared to the natural substrate L-tryptophan (black), resulting in a halogenation at the C7 carbon atom, whereas 5-methyl-L-tryptophan is slightly shifted inside the binding pocket, leading to a mixture of both C6 and C7 halogenated product. (PDB ID: 2E4G)

Owing to this rotation, the C6 carbon atom of the 5-methyl derivative is in close proximity to the original halogenation position of the C7 atom of L-tryptophan, resulting in a mixture of C6 and C7 halogenated product. Although *ortho*/*para*-directing groups in C5 position usually dominate the regioselectivity of further S_EAr reactions, the halogenase is able to override these directing effects for deactivating and activating substituents like hydroxyl functionalities, whereas strongly activating groups like amines force the enzyme to halogenate the substrate at the electronically preferred position. Nevertheless,

a combination of steric and electronic properties of the substrate, as well as the nature of electrophile (*N*-chloroamine or *N*-bromoamine at K79) seems to influence the actual position of the second S_EAr.

Conclusions

Tryptophan is a precursor molecule e.g., of the mammalian hormones serotonin and melatonin that regulate several important physiological processes in the human body like blood pressure, mood, as well as the day and night rhythm.[25,26] Regioselective halogenation of tryptophan and its derivatives might lead to a direct access to intermediates for the synthesis of potential serotonin and melatonin agonists and antagonists. In addition, halogenated amino acids are of great interest for the synthesis of peptides, as they can easily be modified by well-developed processes, for example, nucleophilic substitution, or Pd-catalyzed cross coupling reactions.[1] In parallel to our studies, an enzymatic halogenation system also based on RebH was established independently by Payne et al., showing the potential of RebH for preparative applications.[21] In combination with the tryptophan synthase from *Salmonella enterica*,[23,24] we established an artificial enzymatic halogenation system based on the halogenase RebH for its natural substrate L-tryptophan, as well as for C5- and C6-substituted tryptophan derivatives as non-natural substrates (Figure 4). Halogenation by RebH clearly indicates that the orientation of substrates inside the binding pocket of enzymes has a high impact on the resulting product.

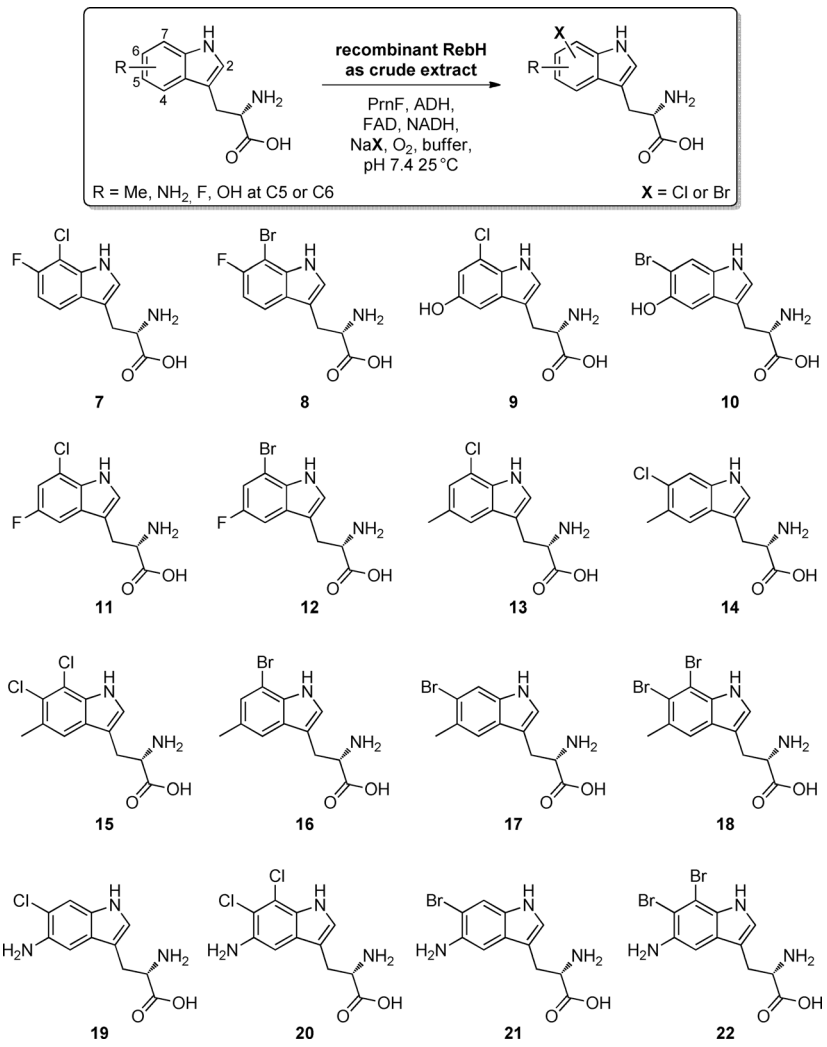

Figure 4. Halogenation products of tryptophan derivatives by RebH. Halogenation occurs in many cases preferred in the electronically unfavored C7 position, even in presence of deactivating *ortho/para*-directing groups like 5-fluoro.

Experimental Section

Chemical analysis and compound isolation

Reactions were monitored by using RP-HPLC (Thermo Scientific Accela 600 equipped with a Thermo Scientific Hypersil GOLD 3 μm column (C_{18}, 150×2.1 mm, eluent A $H_2O/CH_3CN/TFA = 95:5:0.1$, eluent B $H_2O/CH_3CN/TFA = 5:95:0.1$, flowrate 0.7 mL min^{-1} using a gradient from 0–100% B over 5 min)). LC-MS analysis was accomplished by using a Waters Alliance HT equipped with a Waters Symmetry 3.5 μm column (C_8, 100×2.1 mm, eluent A $H_2O/HCOOH = 100:0.1$, eluent B $CH_3CN/HCOOH = 100:0.1$, flowrate 0.4 mL·min^{-1} using a gradient from 5–95% B over 10 min) coupled with a Waters micromass ZQ2000 ESI-MS. For preparative HPLC purification, a LaChrom System (Merck Hitachi) equipped with a Phenomenex Jupiter column (10 μm, C_{18}, 300 Å, 250×21.1 mm, eluent A $H_2O/CH_3CN/TFA = 95:5:0.1$, eluent B $H_2O/CH_3CN/TFA = 5:95:0.1$, flowrate 10 mL·min^{-1} using a gradient from 0–35% B over 65 min). For very polar analysts (e.g. 5-amino-L-tryptophan) an acetonitrile free eluent A (H_2O:TFA 100:0.1) was used. NMR spectra were recorded by using a Bruker DRX-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) or Avance 600 (^{1}H: 600 MHz, ^{13}C: 150 MHz) spectrometer at 298 K and chemical shifts are reported relative to residual solvent peaks (D_2O: ^{1}H: 4.79 ppm, [D_6]DMSO: ^{1}H: 2.50 ppm). High resolution mass spectrometry experiments were performed using a Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer APEX III (Bruker Daltonik GmbH, Bremen, Germany) equipped with a 7.0 T, 160 mm bore superconducting magnet (Bruker Analytik GmbH - Magnetics, Karlsruhe, Germany), infinity cell, and interfaced to an external (nano)ESI or MALDI ion source. Nitrogen served both as the nebulizer gas and the dry gas for ESI. Nitrogen was generated by a Bruker nitrogen generator NGM 11. Argon served as cooling gas in the infinity cell and collision gas for MS^n experiments. Scan accumulation and Fourier transformation were performed with

XMASS NT (7.08) on a PC Workstation, for further data processing DataAnalysis 3.4 was used. Docking experiments were conducted using AutoDockVina[27] and AutoDockTools[28] with the crystal structure of RebH.[18]

Organisms and growth conditions

The *Escherichia coli* strains DH5α and BL21(DE3) (Novagene) were grown at 37 °C using Luria-Bertani (LB) agar or broth with shaking (150 rpm), supplemented with 100 μg mL^{-1} ampicillin, 60 μg mL^{-1} kanamycin and 50 μg mL^{-1} chloramphenicol where appropriate. *Pseudomonas fluorescens* strain BL915 ΔORF1-4 was grown at 30 °C in LB agar or broth with shaking (150 rpm), supplemented with 30 μg mL^{-1} tetracyclin where appropriate. The bacterial strain *Lechevalieria aerocolonigenes* (strain C-38383-RK2, DSMZ-No. 44217) was grown on GYM Streptomyces agar (4 g L^{-1} glucose, 4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 2 g L^{-1} $CaCO_3$, 12 g L^{-1} agar, pH 7.2) for 4 days at 28 °C.

Cloning and expression

Standard methods were used for agarose gel electrophoresis, DNA restriction, ligation and transformation of *E. coli*. Restriction enzymes, T4 DNA ligase and Phusion Polymerase were supplied by New England Biolabs (Ipswich, MA). Agarose was obtained from Peqlab (Erlangen, Germany). Plasmid DNA was extracted employing a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). dNTP mixture was supplied by Fermentas (Vilnius, Lithuania).

The kanamycin-resistant plasmid vector pET28a+ was used to clone the *rebH* gene and overexpress its product prior to its subsequent purification. To amplify the *rebH* gene of the bacterial strain *Lechevalieria aerocolonigenes*, two primers, forward (5′-GTACGTCATATGTCCGGCAAGATTGACAAG-3′) and reverse (5′-GTCAGCAAGCTTTCAGCGGCCGTGCTGTGCC-3′), with *Nde*I and *Hin*dIII restriction sites, respectively included as underlined were designed based on the sequence of the 5′ and 3′ ends of the *rebH* gene (GenBank accession number AAN01216.1). A bacterial colony of *L. aerocolonigenes* was picked, resuspended in 200 μL PBS Puffer, heated for 20 min at 100 °C and immediately used for colony-PCR. A PCR product of approximately 1.6 kb was obtained and directly purified to prevent degradation by bacterial endonucleases. The amplificate was digested with *Nde*I and *Hin*dIII, purified again and ligated into the expression vector pET28a+ that had been digested with the same restriction enzymes. The ligation product pMF-rebH was transformed into *E. coli* strain BL21(DE3) pGro7 and selected utilizing the kanamycin resistance of the pET28a+ vector and the chloramphenicol resistance of the pGro7 vector, resulting in the strain *E. coli* BL21(DE3)-rebH. The correct base sequence of the inserted gene was confirmed by DNA sequencing (GATC Biotech, Köln, Germany).

For the overexpression of the halogenase RebH, 1.5 L LB medium was inoculated with an overnight culture of *E. coli* BL21(DE3)-rebH with an OD_{600nm} = 0.01 and cultivated at 37 °C with shaking at 150 rpm. Protein expression was induced at an OD_{600nm} = 0.6 by the addition of 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and 2 mg mL^{-1} L-arabinose. After induction, temperature was decreased to 25 °C and the cells were harvested after 20 h of incubation by centrifugation (4000 × g, 30 min, 4 °C) and stored at −20 °C.

For the overexpression of the flavin reductase PrnF, the expression vector pCIBhis-prnF was electroporated into *P. fluorescens* BL915 ΔORF1-4. The resulting strain *P. fluorescens* BL915 pCIB-his-prnF was cultivated overnight at 30 °C in LB medium containing 30 μg mL^{-1} tetracycline. 1 L of LB medium, containing the appropriate antibiotic, was then inoculated with 10 mL of overnight culture and cultivated for 3 d at 30 °C. The cells were spun down and stored at −20 °C.

For the overexpression of the alcohol dehydrogenase from *Rhodococcus* sp., chemically competent *E. coli* BL21(DE3) were simultaneously cotransformed with the expression vector pET21-ADH and pGro7 (Takara), resulting in the strain *E. coli* BL21(DE3)-ADH. For the expression, 30 mL overnight culture containing 50 μg mL^{-1} chloramphenicol and 100 μg mL^{-1} ampicillin were used to inoculate 1.5 L of LB medium containing the same antibiotics. After an optical density of OD_{600nm} = 0.3 had been reached, the temperature was decreased to 25 °C. After 30 min, $ZnCl_2$ and L-arabinose were added to final concentrations of 0.5 mM and 2 mg mL^{-1} respectively. Expression of the alcohol dehydrogenase was induced by adding 0.1 mM IPTG at an OD_{600nm} = 0.6. After 16 h, the cells were harvested and stored at −20 °C.

For the expression of the tryptophan synthase, 1.5 L LB medium containing 100 μg mL^{-1} ampicillin was inoculated with 15 mL of an *E. coli* pSTB7 (ATCC strain 37845) overnight culture and grown for 12–16 h at 37 °C. The cells were harvested by centrifugation, washed once with 100 mM K_2HPO_4 pH 7.4 and stored at −20 °C.

Tryptophan synthase bioconversion

Bioconversion of indoles with L-serine for the production of the C5- or C6-substituted tryptophan derivatives were performed according to Goss et al.[23] in a modified protocol. Briefly, 12.1 mM indole, 12.1 mM L-serine and 100 μM pyridoxal phosphate in 15 mM NH_4HCO_3 (pH 7.8) were converted with 2 mL of *E. coli* lysate (1.5 L pellet lysed twice by FrenchPress in 20 mL 500 mM Tris-HCl, 5 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, and 0.1 mM pyridoxal phosphate), trapped in a sealed dialysis bag, within two days at 37 °C into the corresponding tryptophan derivative. The progress of the reaction was monitored by reversed-phase HPLC at λ = 280 nm. For analytical data of the substituted tryptophan derivatives, refer to the Supporting Information.

Enzyme purification

All steps of the enzyme purification process were performed at 4 °C. To purify the His_6-tagged halogenase RebH, *E. coli* cells from 1.5 L culture were resuspended in 30 mL equilibration

buffer (50 mM NaH_2PO_4, 300 mM NaCl and 50 μM PMSF pH 8.0 adjusted with NaOH) and lysed twice by FrenchPress. The crude lysate was centrifuged (10000×g, 30 min, 4 °C) and filtered through a 0.2 μm Whatman filter. The supernatant was loaded on a HisTALON agarose affinity column (0.5 mL min^{-1}) with a bed volume of 1 mL (Clontech, Mountain View, CA) that had been equilibrated with equilibration buffer before. The column was washed with 20 mL equilibration buffer (1 mL min^{-1}) and subsequently washed with 20 mL washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0, 1 mL min^{-1}). The protein was eluted with elution buffer containing 300 mM imidazole. Protein fractions were analyzed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The respective protein band was excised from the gel, de-stained twice with 200 μL 30% acetonitrile 0.1 M NH_4HCO_3 and dried completely for 30 min in a speedvac (SpeedVac Concentrator 5301 Eppendorf, Hamburg, Germany). The proteins were overnight digested in-gel at 37 °C with 15 μL of 6.7 μg mL^{-1} trypsin (Promega, Madison, WI) in 10 mM NH_4HCO_3 and subsequently identified by MALDI-ToF mass spectrometry employing a Bruker Ultraflextreme instrument (Bruker Daltonics, Bremen, Germany) with peptide mass fingerprint. The flavin-reductase PrnF was purified analogously. For the purification of the alcohol dehydrogenase, a heat precipitation step was used. *E. coli* cells from 1.5 L of culture were resuspended in 20 mL 100 mM K_2HPO_4 buffer at pH 7.4, lysed twice by FrenchPress and centrifuged (10000×g, 30 min, 4 °C). The crude lysate was heated to 60 °C in 1.5 mL aliquots for 20 min in a thermomixer (Eppendorf, Hamburg, Germany). Denaturated protein was removed by centrifugation and the crude extract was stored at −20 °C. Protein concentrations were determined in Bradford assays (Applichem, Darmstadt, Germany) with bovine serum albumin (BSA) as standard.

Determination of PrnF activity

The volumetric activity of the elution fractions of the Flavin reductase PrnF was determined as triplicates by monitoring the decrease of absorption at $\lambda = 340$ nm due to the oxidation of $NADH + H^+$ to NAD^+ ($\varepsilon = 6.3$ mL μmol^{-1} cm^{-1}) in a final volume of 1 mL containing 20 μL of the diluted elution fractions (dilution 1:100, usually ~30 μg mL^{-1}), 10 mM K_2HPO_4 pH 7.4, 20 μM FAD and 240 μM NADH. The stock solutions were preequilibrated to 25 °C and pH 7.4 and temperature was held constant at 25 °C in a tempered Jasco V-630 photometer. The conversion rate of the substrate was determined by regression of the linear range (usually 15 s after addition of the enzyme, less than 10% substrate conversion).

Determination of ADH activity

The volumetric activity of the diluted crude extract of the alcohol dehydrogenase after heat precipitation (20 μL, 1:100 dilution) was determined as triplicates in a final volume of 1 mL containing 250 μM NAD^+, 10 mM K_2HPO_4 pH 7.4 and 20% (v/v) *iso*-propanol. The conversion rate of the substrate was determined by regression of the linear range (usually 15 s after addition of the enzyme, less than 10% substrate conversion).

Determination of RebH activity

Reactions were assembled in 96-well plates as triplicates in a final volume of 75 μL from a 3 mL master mix containing 30 mM NaCl, 10 mM K_2HPO_4 pH 7.4, 0.1 mM FAD, 0.1 mM NADH, 0.25 mM L-tryptophan, 5% (v/v) *iso*-propanol, 2.5 U ml^{-1} PrnF, 1 U ml^{-1} ADH and 10 μM RebH with 0.5 mM 4-nitrophenol as an internal standard. The reactions were left in an incubator at 25 °C and stopped at distinct times by the addition of 75 μL methanol to each well. The stopped solution was directly transferred to Eppendorf tubes for removal of precipitated proteins by centrifugation. The resulting supernatant was analyzed by reversed-phase HPLC. Product formation was calculated based on the peak area in comparison to a calibration curve of L-tryptophan and 4-nitrophenol as an internal standard.

Determination of Total Turnover Numbers (TTN) for RebH

Reactions were assembled as triplicates in a final volume of 200 μL containing 1.25 mM substrate, 30 mM NaCl, 10 mM K_2HPO_4 pH 7.4, 0.1 mM FAD, 0.1 mM NADH, 5% (v/v) *iso*-propanol, 2.5 U ml^{-1} PrnF, 1 U ml^{-1} ADH and 5 μM RebH. The reactions were left in an incubator shaker at 25 °C and 600 rpm for 16 h. At the end of the reaction, proteins were precipitated by the addition of 200 μL methanol. After centrifugation, the supernatant was analyzed by reversed-phase HPLC. Product formation was calculated based on the decrease in peak area of the reactant in comparison to a calibration curve of each substrate.

General procedure for enzymatic halogenation reactions using *E. coli* lysate

For the halogenation of L-tryptophan and its derivatives, *E. coli* lysate from 1.5 L of culture, containing the overexpressed halogenase RebH, was used. The cells were lysed by FrenchPress in a final volume of 30 mL lysis buffer containing 30 mM NaCl or NaBr, 10 mM K_2HPO_4 pH 7.4, 50 μM PMSF, 100 μg mL^{-1} ampicillin, 10 μM FAD, 20 mM DTT, 1 mM NADH, 5% (v/v) *iso*-propanol, 2.5 U ml^{-1} PrnF, 1 U ml^{-1} ADH and an excess of substrate (2 to 6 mM), whereas DTT, NADH, *iso*-propanol, PrnF, ADH and the substrate were added after lysis and centrifugation (30 min, 10000 x g, 4 °C) of the crude lysate. The supernatant was transferred into an Erlenmeyer flask (50 mL) and incubated at 25 °C in an orbital shaker at 100 rpm. The reaction was monitored by reversed-phase HPLC and stopped at 16–24 h, depending on the reaction process. The reaction solution was transferred into a sealed dialysis bag and the product was removed by step-wise dialysis against Millipore water containing 0.1% of TFA (2x 500 mL for 4 h, 1x 500 mL overnight). The combined dialysis fractions were pooled, the solvent was removed at reduced pressure and the remaining liquid containing the halogenated tryptophan derivative was purified by

preparative HPLC. For the analytical data of the halogenated tryptophan derivatives, refer to the Supporting Information.

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[1] F. Diederich, P. J. Stang, *Metal-Catalyzed Cross-Coupling Reactions*, Wiley, **2008**.
[2] P. Jeschke, *ChemBioChem* **2004**, *5*, 570 – 589.
[3] R. Wilcken, M. O. Zimmermann, A. Lange, A. C. Joerger, F. M. Boeckler, *J. Med. Chem.* **2013**, *56*, 1363 – 1388.
[4] G. W. Gribble, *Alkaloids Chem. Biol.* **2012**, *71*, 1 – 165.
[5] A. R. Knaggs, *Nat. Prod. Rep.* **2001**, *18*, 334 – 355.
[6] K. Podzelinska, R. Latimer, A. Bhattacharya, L. C. Vining, D. L. Zechel, Z. Jia, *J. Mol. Biol.* **2010**, *397*, 316 – 331.
[7] T. Zhu, X. Cheng, Y. Liu, Z. Deng, D. You, *Metab. Eng.* **2013**, *19*, 69 – 78.
[8] C. Weiss, B. Sammet, N. Sewald, *Nat. Prod. Rep.* **2013**, *30*, 924 – 940.
[9] E. Rodrigues Pereira, L. Belin, M. Sancelme, M. Prudhomme, M. Ollier, M. Rapp, D. Sevère, J. F. Riou, D. Fabbro, T. Meyer, *J. Med. Chem.* **1996**, *39*, 4471 – 4477.
[10] E. Yeh, S. Garneau, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3960 – 3965.
[11] S. Keller, T. Wage, K. Hohaus, M. Hölzer, E. Eichhorn, K.-H. van Pée, *Angew. Chem. Int. Ed.* **2000**, *39*, 2300 – 2302; *Angew. Chem.* **2000**, *112*, 2380 – 2382.
[12] X. Zhu, W. De Laurentis, K. Leang, J. Herrmann, K. Ihlefeld, K.-H. van Pée, J. H. Naismith, *J. Mol. Biol.* **2009**, *391*, 74 – 85.
[13] C. Sánchez, I. A. Butovich, A. F. Braña, J. Rohr, C. Méndez, J. A. Salas, *Chem. Biol.* **2002**, *9*, 519 – 531.
[14] M. Tani, H. Ikegami, M. Tashiro, T. Hiura, H. Tsukioka, C. Kaneko, T. Notoya, M. Shimizu, M. Uchida, Y. Aida et al., *Heterocycles* **1992**, *34*, 2349 – 2362.
[15] S. Flecks, E. P. Patallo, X. Zhu, A. J. Ernyei, G. Seifert, A. Schneider, C. Dong, J. H. Naismith, K. van Pée, *Angew. Chem. Int. Ed.* **2008**, *47*, 9533 – 9536; *Angew. Chem.* **2008**, *120*, 9676 – 9679.
[16] E. Yeh, L. C. Blasiak, A. Koglin, C. L. Drennan, C. T. Walsh, *Biochemistry* **2007**, *46*, 1284 – 1292.
[17] E. Yeh, L. J. Cole, E. W. Barr, M. Bollinger, D. P. Ballou, C. T. Walsh, *Biochemistry* **2006**, *45*, 7904 – 7912.
[18] E. Bitto, Y. Huang, C. A. Bingman, S. Singh, J. S. Thorson, G. N. Phillips Jr., *Proteins Struct. Funct. Bioinf.* **2008**, *70*, 289 – 293.
[19] C. Dong, S. Flecks, S. Unversucht, C. Haupt, K.-H. van Pée, J. H. Naismith, *Science* **2005**, *309*, 2216 – 2219.
[20] K.-H. Pée, E. P. Patallo, *Appl. Microbiol. Biotechnol.* **2006**, *70*, 631 – 641.
[21] J. T. Payne, M. C. Andorfer, J. C. Lewis, *Angew. Chem. Int. Ed.* **2013**, *52*, 5271 – 5274; *Angew. Chem.* **2013**, *125*, 5379 – 5382.
[22] W. Hummel in *New Enzym. Org. Synth.*, Springer, Heidelberg, **1997**, pp. 145 – 184.
[23] R. J. M. Goss, P. L. A. Newill, *Chem. Commun.* **2006**, 4924 – 4925.
[24] M. Winn, A. D. Roy, S. Grüschow, R. S. Parameswaran, R. J. M. Goss, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4508 – 4510.
[25] L. F. Mohammad-Zadeh, L. Moses, S. M. Gwaltney-Brant, *J. Vet. Pharmacol. Ther.* **2008**, *31*, 187 – 199.
[26] P. Pevet, E. Challet, *J. Physiol.* **2011**, *105*, 170 – 182.
[27] O. Trott, A. J. Olson, *J. Comput. Chem.* **2010**, *31*, 455 – 461.
[28] G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, *J. Comput. Chem.* **2009**, *30*, 2785 – 2791.

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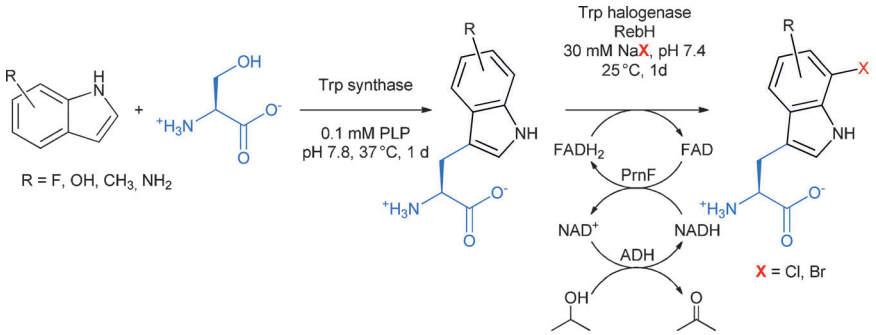
Regioselective Enzymatic Halogenation of Substituted Tryptophan Derivatives using the FAD-Dependent Halogenase RebH



No business as usual: The tryptophan halogenase RebH from *Lechevalieria aerocolonigenes* is able to halogenate at the electronically unfavored C7-*meta*-position of C5-substituted tryptophan derivatives, even in presence of deactivating *ortho*/*para*-directing groups.