Predicting the Substrate Scope of the Flavin-Dependent Halogenase BrvH

Pia R. Neubauer,[a] Silke Pienkny,[b] Ludger Wessjohann,[b] Wolfgang Brandt,[b] and Norbert Sewald*[a]

The recently described flavin-dependent halogenase BrvH is able to catalyse both the bromination and chlorination of indole, but shows significantly higher bromination activity. BrvH was annotated as a tryptophan halogenase, but does not accept tryptophan as a substrate. Its native substrate remains unknown. A predictive model with the data available for BrvH was analysed. A training set of compounds tested *in vitro* was docked into the active site of a complete protein model based on the X-ray structure of BrvH. The atoms not resolved experimentally were modelled by using molecular mechanics force fields to obtain this protein model. Furthermore, docking poses for the substrates and known non-substrates have been calculated. Parameters like distance, partial charge and hybridization state were analysed to derive rules for predicting activity. With this model for activity of the BrvH, a virtual screening suggested several structures for potential substrates. Some of the compounds preselected in this way were tested *in vitro*, and several could be verified as convertible substrates. Based on information on halogenated natural products, a new dataset was created to specifically search for natural products as substrates/products, and virtual screening in this database yielded further hits.

Introduction

Halogenated metabolites are found in nature in many different organisms. They form an important part of the natural products family, and are most often found in marine invertebrates, algae, and bacteria. Bioactive molecules like pharmaceuticals or plant protectants frequently contain halogens, too. Some of the halogenated natural products are biologically active. For example, thienodolin is produced by *Streptomyces albogriseulus* and promotes plant growth.[1] Balhimycin from an *Actinomycete* species shows antibiotic activity against *Staphylococcus aureus*, *S. epidermis*, *S. haemolyticus*, and *Streptococcus* species.[2] Another halogenated antibiotic is produced by *Pseudomonas aeruginosa* and was named pyoluteorin.[3] Cryptophycins isolated from cyanobacteria show anti-tumour activity.[4] A *Salinospora* strain, producing the proteasome inhibitor salinosporamide A, is another marine source for highly bioactive compounds.[5] New halogenated metabolites are being identified constantly from different sources, like 6-bromo-8-oxoconicamin A from the marine sponge *Oceanapia* sp. It shows micromolar activity against a human pancreatic cancer cell line.[6]

Chemical halogenation often requires harsh reaction conditions like Lewis acid, elevated temperature, and proceeds in organic solvents. The use of enzymes provides an elegant alternative since enzymes work in aqueous solution at room temperature. Flavin-dependent halogenases introduce halogen substituents in their substrates regioselectively and only require oxygen, a halide salt, and the reduced flavin $FADH_2$ as cofactor.[7] The most prominent members are the tryptophan halogenases, which catalyse the regioselective halogenation of tryptophan in different positions.[8] A flavin reductase is required for enzymatic cofactor regeneration with consumption of nicotinamide adenine dinucleotide (NADH). It was shown only recently that also NADH mimics can be employed instead of enzymatic cofactor regeneration in biocatalytic halogenation.[9] Furthermore, it is also possible to photochemically (455 nm) reduce bound FAD in the presence of EDTA.[10]

X-ray crystallographic structure analysis of PrnA helped to postulate a reaction mechanism for these enzymes.[11] $FADH_2$ reacts with oxygen to give a flavin hydroperoxide (FAD–OOH) which reacts with a halide nucleophile forming hypohalous acid (HOX). HOX then passes through a 10 Å tunnel to the substrate binding site. A highly conserved lysine residue (PrnA: K79) is important for the stabilization of HOX. It is still under debate whether HOX reacts with the lysine side chain giving an *N*-haloamine or whether there is an association by hydrogen bond formation.[11–13] In the substrate binding site, HOX or the haloamine react with the substrate in an electrophilic substitution reaction. A conserved glutamate residue (PrnA: E346) deprotonates the Wheland intermediate releasing the final halogenated product.[11,14] Recently, it was shown that substrate binding reduces the affinity for oxidized cofactor FAD to probably facilitate regeneration of $FADH_2$.[15]

[a] Dr. P. R. Neubauer, Prof. Dr. N. Sewald
Organic and Bioorganic Chemistry, Department of Chemistry
Bielefeld University
Universitätsstrasse 25, 33501 Bielefeld (Germany)
E-mail: norbert.sewald@uni-bielefeld.de
[b] Dr. S. Pienkny, Prof. Dr. L. Wessjohann, Dr. W. Brandt
Leibniz Institute for Plant Biochemistry (IPB)
Weinberg 3, 06120 Halle (Germany)

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In recent years, many new flavin-dependent halogenases have been identified and strategies to overcome their drawbacks of low catalytic activity and low stability have been explored by directed evolution and structure-based mutagenesis.[16] Frese et al. employed the immobilization technique of crosslinked enzyme aggregates (CLEAs) to achieve higher stability of flavin-dependent halogenases and were able to finally obtain preparative amounts of halogenated products.[17] A combination of different strategies including directed evolution, rational design, as well as site-saturation mutagenesis led to a more thermostable and stronger elevated activity of a Thal variant.[18] Moreover, Moritzer et al. switched the regioselectivity of the tryptophan-6-halogenase Thal almost completely to tryptophan halogenation in position 7 by only changing five amino acid residues.[19]

Altogether flavin-dependent halogenases show a fascinating mechanism, are involved in the biosynthesis of natural products with interesting bioactivities and might help to develop greener methods for the synthesis of halogenated compounds. Such halogenated aromatic amino acids have been further derivatized, for example, in Suzuki-Miyaura cross-coupling reactions.[20]

Most known flavin-dependent halogenases prefer chloride over bromide.[21] In 2014, two brominases were identified from a marine habitat; they only catalyse bromination, but not chlorination.[22] In 2018, we described the new flavin-dependent halogenase BrvH, which was retrieved from a metagenome by Hidden-Markov-Model (HMM) based bioinformatic analysis. BrvH is able to catalyse both the bromination and chlorination of indole, but shows significantly higher bromination activity.[23] In 2019 other novel flavin-dependent halogenases from *Xanthomonas campestris pv. campestris* B100 were identified, which prefer bromination.[24] Fisher et al. generated a sequence-similarity network for the identification of novel flavin-dependent halogenases and identified in total 39 novel halogenases, which all show brominase activity and only 16% chlorinase activity.[25] Recently, a marine viral flavin-dependent halogenase that prefers iodination was identified in a cyanophage. It has a wider active site than, say, BrvH and accepts sterically demanding substrates.[26]

Results and Discussion

Although BrvH had been bioinformatically annotated as a tryptophan halogenase, it does not convert tryptophan. The native substrate of BrvH is yet unknown. For this study we investigated the substrate scope of BrvH in a virtual screening approach. We further investigated the substrate scope of BrvH by using structure–activity-relationship analyses to identify features of the compounds brominated by BrvH. Furthermore, we intended to suggest further substrates accepted by BrvH.

Indole-derived substrates as a test set for virtual screening

BrvH had previously been identified from a metagenome data set originating from Botany Bay, Australia. The enzyme was produced recombinantly and shown to catalyse bromination of indole in C3 position.[23] Because of lacking knowledge on the natural substrate, we attempted to employ virtual screening methodology frequently employed in medicinal chemistry. An array of 26 different compounds (**1**–**26**) served as a training set. Conversion of the individual members of the training set was analysed by LC-MS. Twelve substrates were shown to be brominated by BrvH under standardized conditions (substrates) within 48 h in yields between 12–100%, whereas 14 compounds were not halogenated (non-substrates) by BrvH under these conditions (Table 1). The model was validated by the leave-one-out cross-validation. Despite the small set of starting compounds, the approach was successful due to the high quality of the lab data and a sensible selection of potential substrates tested. With the structures of the different tested compounds (Table 1) and the specifications *yes* (substrate) and *no* (non-substrate) a binary model could be created using the following descriptors: H-bond donor capacity at −4.0 (vsurf_HB6), differences of bonded atom polarizabilies (bpol), number of aromatic atoms (a-aro), molecular flexibility (KierFlex) and the log octanol/water partition coefficient (log P_{OW}). A neutral pH was assumed and the structures were protonated or deprotonated according to the pK_a values of the functional groups. With these descriptors the experimental data can be described well. The leave-one-out cross-validation correctly predicted the activities for all but two substances. The two outliers are 5-cyanoindole and 2-indolylmethanol. The cross-validated accu-

Table 1. Compounds of the training set.

Substrate	Conversion after 48 h
indole **1**	100%
2-methylindole **2**	100%
3-methylindole **3**	98%
5-methylindole **4**	97%
5-nitroindole **5**	89%
7-azaindole **6**	12%
5-cyanoindole **7**	99%
5-bromoindole **8**	52%
5-fluoroindole **9**	97%
2-indolylmethanol **10**	99%
indole-2-carboxylic acid **11**	35%
5-hydroxyindole **12**	96%
L-tryptophan **13**	0%
L-tyrosine **14**	0%
L-phenylalanine **15**	0%
L-5-hydroxytryptophan **16**	0%
tryptophol **17**	0%
3-indolylacetonitrile **18**	0%
indole-3-carbaldehyde **19**	0%
indole-5-carbaldehyde **20**	0%
5-indolylmethanol **21**	0%
3-(3-indolyl)propionic acid **22**	0%
benzoxazole **23**	0%
indazole **24**	0%
quinoxaline **25**	0%
4-hydroxybenzoic-acid **26**	0%

racy resulted for substrate in 86%, and for non-substrate in 93%.

Binary activity model development

A structure-activity relationship model was developed to evaluate whether a given compound is likely to be halogenated. A set of compounds, tested for conversion in the lab as substrates, was used and the 3D structures, their properties and their predicted activity values were compared.

Analysis of crystal structure, addition of hydrogen atoms and addition of the cofactor

The crystal structure of the BrvH (6FRL) was superimposed with the Trp-7 halogenase PrnA (2ARD).[18] The deviation of the C^{α} atoms between the two monomers is only 3.1 Å. In the area of the cofactor binding site there are no major deviations between the backbone atoms, therefore the superposition was used to insert FAD into the structure of BrvH. The positions of the oxygen atoms of the crystallized water partly correspond to the positions of the heteroatoms of the cofactor, the chloride ion and water molecules.

Preparation of pharmacophore

For indole, different docking poses were generated with the program GOLD and evaluated according to the position of C3 (Figure 1). It is known from the X-ray structures of RebH,[27] PrnA, and PyrH[12] that the N^{ε} of the lysine involved in catalysis has to be about 4 Å from the positions to be halogenated (C7 or C5). This distance was also assumed for BrvH. Further limitations are due to the position of the carboxyl group of Glu349, which was also considered to be involved in catalysis. The carboxylate is the proton acceptor from the Wheland intermediate during halogenation.

After the calculation of the docking poses and the analysis of the relevant amino acids of the binding pocket of BrvH, several features (usually called pharmacophores in a medicinal chemistry context) describing the properties of the binding site (Figure 2) were created using MOE. They were tested with the substrate list as follows: The 3D coordinates of the structures were generated, and their conformations calculated. This substrate conformation database was searched with the respective pharmacophore, with the aim of achieving the best possible recovery rate of the converted substrates and a low recovery rate of the unreacted compounds.

The best pharmacophore finds 12 out of 13 substrates and only five out of 13 non-substrates. A complete separation is not to be expected, since the numbers are small, substrates and non-substrates are very similar, and probably also non-substrates can bind to the protein, but are not being converted. The best pharmacophore tested in this way contains five so-called features that describe the interactions of a ligand with the protein (Figure 2). One of these features describes the position of the carbon atom to be halogenated (F1, derived from docking) and must be present in all hits. The others describe two possible hydrogen bridge acceptors (F2, F3-carbonyl group of Glu349 and F4 of Pro101) and hydrophobic properties (F1 and F5). In addition, the space occupied by the structure of the BrvH was defined as excluded volume. I.e.,

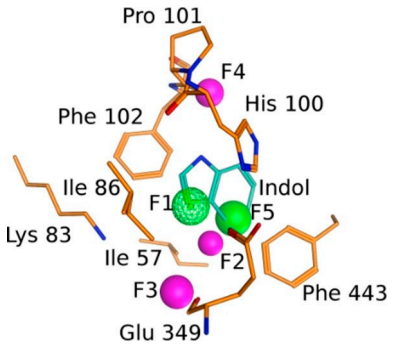

Figure 2. Selected amino acids of BrvH (carbons in orange) and the docked substrate indole (carbons in cyan) with the pharmacophore features F1 to F5 (as spheres in green and pink).

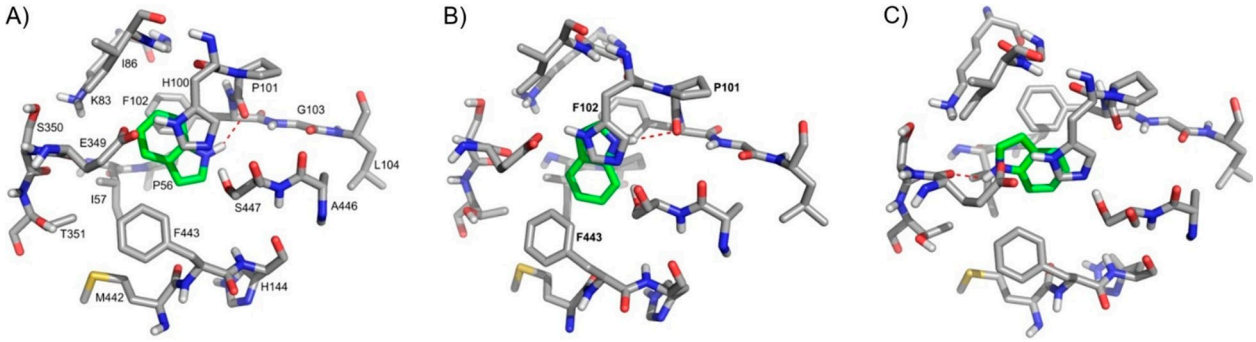

Figure 1. Docking of indole in diverse poses (cluster analysis based on RMSD) resulted in three reasonable poses: hydrogen bond of the indole NH to the carbonyl oxygen (red dashed lines) of A) and B) Pro101, and C) Glu349. The orientation of the indole in (A) and (B) differs due to hydrophobic interactions with A) Phe102, and B) additionally with Phe443.

these regions cannot be occupied by atoms of the ligands to be searched for.

Screening of the IPB in-house database

The IPB in-house database contains nearly 16.000 entries of compounds available in substance. This database was searched with the pharmacophore. As a result, 581 hits were found. These hits were selected for compounds where electrophilic aromatic halogenation seems possible based on chemical reasons. Hits which already contain a halogen at the carbon in position F5, a sulfur, or a tertiary carbon were excluded. An already existing halogen would likely prevent further halogenation and sulfur was excluded as unlikely. Furthermore, structures were excluded which allow a hydrogen bridge to the amino group of the side chain of Lys83 by functional groups such as carboxyl or hydroxyl groups and thus would make halogenation unlikely. In addition, only structures meeting at least three of the four non-essential pharmacophore features, were selected. Thus, 95 compounds remained as potential substrates for BrvH (SI1 in the Supporting Information).

Screening in the Plant Natural Product Database

A database with almost 12.000 entries, compiled from structures of the Beilstein database (identified in 2010 with the keywords "Plant" and "Natural Product"), was also searched with the pharmacophore and gave 118 hits. The same selection restrictions as for the InHouseDB hit list, were applied and resulted in a shortlist of 13 structures and their Beilstein IDE.RXN numbers (SI2 in the Supporting Information).

Screening in the MOE lead-like database

The so-called lead-like database of the MOE software package from 2012 contains over 600.000 entries of structures of potentially commercially available compounds. The search with pharmacophore revealed 322 hits with the manufacturers and identification numbers. Non-aromatic compounds were again discarded based on mechanistic reasons and the availability and price were further parameters for further consideration of a compound in the assay. Thus 103 compounds remained for further analysis (SI3 in the Supporting Information).

Selecting for a low electronegativity value

Because active site binding does not mean conversion of a substrate, we wanted to include knowledge on the reaction mechanism to preselect likely substrates from binders only. Therefore, all the resulting 211 (95 inHouseDB, 13 Beilstein DB, 103 MOE) final hits from virtual screening were docked to the active site of BrvH. From 100 poses for each ligand, the best ones according to score and cluster size were selected. Using Amber10EHT as force field, the best poses were energetically optimized. The partial charges calculated for the carbon atoms in a range of max. 4 Å to the N^{ε} of Lys83 were used to rank the results. A more negative value (higher electron density) was regarded as more likely to enhance conversion, referring to the proposed mechanism for halogenases.[14] Altogether from the 211 docked compounds 137 were proposed to be substrates of BrvH for halogenation.

Experimental evaluation of calculated substrates

Six proposed substrates were selected for more detailed experimental analysis: five from the MOE lead-like database and one from the IPB in-house library (Figure 3).

Compounds **28**, **31** and **32** were not identical, but only similar to the proposed substrates, however, more readily available. For five (**28**–**32**) of the six chosen substrates, halogenation activity by BrvH could be shown (Figure 3).

Alternative six-membered ring aromatic substrates were chosen because BrvH halogenated **32** and the virtual screening proposed several further aromatic substrates for BrvH. In the

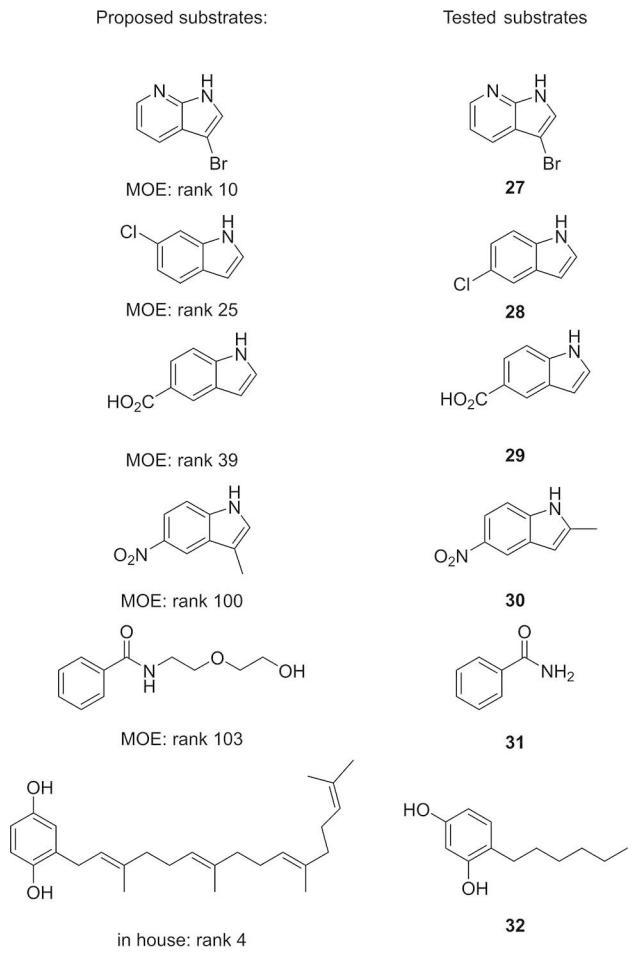

Figure 3. Structures selected from the MOE lead-like database and the IPB in-house database as well as the closest compounds available for experimental verification.

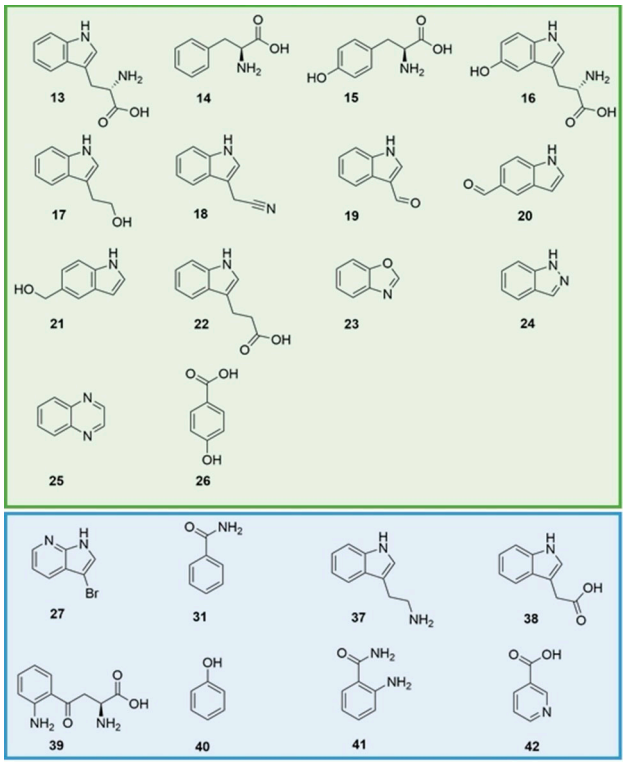

Figure 4. Substrates not accepted by BrvH. green: previously identified non-substrates in the training set; blue: additionally tested compounds not halogenated by BrvH.

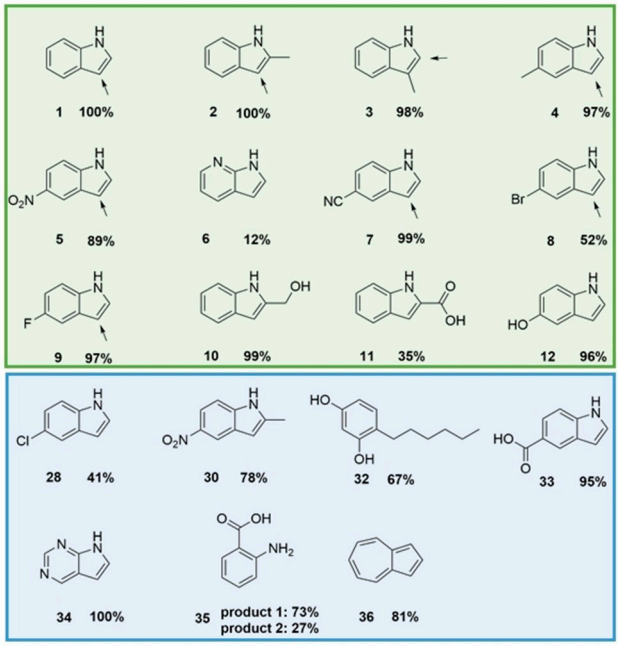

Figure 5. Substrates accepted by BrvH. Conversion was determined by RP-HPLC after a reaction time of 48 h. The arrows show the position of halogenation, which was identified by NMR analysis. green: previously identified substrates (training set); blue: newly identified substrates suggested by virtual screening.

literature it was shown that brominases like Bmp5 and the Xcc halogenases are able to brominate phenol.[27,29] BrvH does not catalyse bromination of phenol (**40**), benzamide (**31**), 2-aminobenzamide (**41**), and nicotinic acid (**42**; Figure 4). Interestingly, for anthranilic acid (**35**), BrvH shows bromination activity, but no regioselectivity.

In total 40 compounds were tested for enzymatic halogenation by BrvH. Nineteen substrates were brominated with different conversion rates (Figure 5). The position of halogenation was determined for the substrates indole (**1**), 2-methylindole (**2**), 3-methylindole (**3**), 5-methylindole (**4**), 5-nitroindole (**5**), 5-cyanoindole (**7**), 5-bromoindole (**8**), and 5-fluoroindole (**9**). In all cases the halogenation took place in the most activated (electron rich) C3 position. Only 3-methylindole was halogenated in C2 because the C3 position was already blocked with the methyl group. Interestingly, indole derivatives with larger substituents in C3 position were not accepted by BrvH.

In 2018, we elucidated the reason for the non-conversion of tryptophan based on the crystal structure of BrvH.[23] We suspected that the lack of side chains which keep the amino and carboxyl group of tryptophan in place in tryptophan halogenases are also the reason for the non-acceptance of the other tryptophan-like substrates (**16**, **17, 22**, **37, 38, 39**) as well as of tyrosine (**14**) and phenylalanine (**15**). We also postulated that larger substrates with longer side chains could be accepted by BrvH because of the wider and more open active side in comparison to tryptophan halogenases.[23] The screened and calculated substrates also predicted many substrates with longer side chains. In this study we were able to show that also 4-*n*-hexylresorcinol (**32**) with a hexyl side chain can be brominated by BrvH. All accepted substrates give exclusively one monobrominated product, except anthranilic acid (**35**). In this case two monobrominated products occur, which are halogenated in different positions.

Determination of specific activities of BrvH towards indole derivatives

For eight substrates (**1**–**5, 7**–**9**) the specific activities were determined. BrvH shows the highest specific activity towards 5-fluoroindole (**9**). The specific activity of the known tryptophan halogenases to their natural substrates is almost five times higher in comparison to the specific activity of BrvH to 5-fluoroindole. Therefore, we calculated the specific activities of RebH (23.2 mU/mg) and PrnA (18.3 mU/mg) by using the known turnover numbers (RebH: 1.4 min^{-1}; PrnA: 1.1 min^{-1})[28,29] and the enzyme mass (RebH: 60297 g/mol; PrnA: 60000 g/mol; Figure 6).

We showed that BrvH accepts different indole derivatives as well as phenol-like substrates even with longer side chains like the 4-*n*-hexylresorcinol. Determination of specific activities revealed around five times lower activities in comparison to RebH and PrnA towards their natural substrate L-tryptophan.

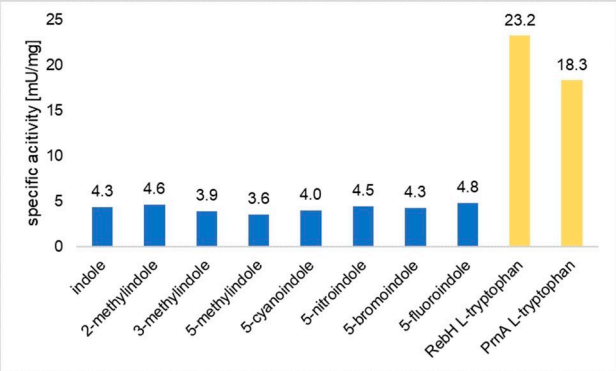

Figure 6. Specific activities of BrvH for the substrates 5-, 3-, 2-methylindole, indole, 5-fluoro-, 5-bromo-, 5-cyano- and 5-nitroindole in comparison to the calculated specific activities of RebH and PrnA based on the turnover numbers (RebH: 1.4 min^{-1}, 60297 g/mol; PrnA: 1.1 min^{-1}, 60000 g/mol).[28,29]

Conclusion

Metagenomic analysis turned out to be a powerful tool for the identification of novel flavin-dependent halogenases. A bottleneck of this identification technique is the lack of knowledge of the natural substrates. In this study we tried to overcome this issue by employing a virtual screening process, docking a plethora of potential substrates including natural products into the active site of BrvH generating a preliminary SAR based on first conversion results. 137 structures were predicted for correct BrvH active site binding. Six very promising compounds with regard to predicted high binding energy and correct positioning were chosen from the MOE lead-like database and one from the IPB in-house database, of which five identical or very similar compounds were brominated by BrvH. Interestingly, with compounds **31** and **32**, 6-membered ring aromatic substrates different from indole were accepted. This is important as aromatic compounds, especially phenolics, are ubiquitous natural products and halogenated derivatives are common moieties in bioactive compounds. Moreover, the 3D structure of BrvH proposed the acceptance of a bulkier substrate[23], and indeed even 4-*n*-hexylresorcinol (**32**) with its lipophilic side chain can be brominated, which underlines our hypothesis that BrvH is able to halogenate substrates with longer alkyl chains.[23]

The flavin-dependent halogenase BrvH accepts indole derivatives with electron-withdrawing as well as electron-donating groups in the six-membered ring of the indole moiety. In a comparison of different halogenated indole substrates, it shows the highest activity for 5-fluoroindole. Fluoride, bromide and chloride show all a +M and −I effect, but the fluoro substituent has the smallest van-der-Waals radius, which might be the reason for preference of 5-fluoroindole over 5-bromo- and 5-chloroindole.

The virtual screening process not only enabled a deeper insight into the substrate binding and SAR, but allowed the identification of novel substrates for BrvH, including an indole-free phenol as representative of a compound class very abundant in natural environments.

Experimental Section

Materials: All chemicals and solvents were purchased from commercial suppliers in highest purity (*p.a.*). Prof. Dr. Karl-Heinz van Pée kindly provided the plasmid pClBhis-PrnF encoding the flavin reductase from *Pseudomonas fluorescens* and Prof. Dr. Werner Hummel donated the plasmid vector pET-21_ADH encoding for alcohol dehydrogenase. The plasmid pGro7 for the chaperone system GroEL-GroES was obtained from TaKaRa Bio Inc. Competent cells *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were obtained from Novagen.

Analytical reversed-phase high performance liquid chromatography: For analytical RP-HPLC a Shimadzu Nexera XR Luna 13 µm C_{18}(2) 100 Å, Column from Phenomenex was used (100×2 mm, eluent A: H_2O/TFA (100:0.1), eluent B: CH_3CN/TFA (100:0.1), flow-rate 650 µL/min). A linear gradient was applied starting with 95% eluent A and 5% eluent B over 5 min to 95% eluent B and 5% eluent A, staying for 0.5 min, then back to 95% eluent A and 5% eluent B for 3 min.

Liquid chromatography–mass spectrometry: For LC-MS analysis a Agilent Technologies 1200 with Hypersil Gold C_{18} (3 µm, 150x2.1 mm; eluent A: H_2O/CH_3CN/formic acid (95:5:0.1), eluent B: H_2O/CH_3CN/formic acid (5:95:0.1); flow rate: 300 µL/min; eluent A) from Thermo Fisher Scientific and a mass spectra Agilent Technologies 6220 Accurate Mass TOF/LC-MS (gas temperature: 325 °C, capillary voltage: 2500 V, fragmentor voltage: 175 V) with a Dual-ESI (spray voltage: 2.5 kV) was used. A linear gradient starting with 100% eluent A changes over 10 min to 98% eluent B and 2% eluent A, stays for 1 min, then over 0.5 min back to 100% eluent A for 3.5 min.

NMR spectroscopy: For determination of regioselectivity 1D (^{1}H NMR, ^{13}C NMR) and 2D NMR (^{1}H,^{1}H COSY, ^{1}H,^{1}H ROESY, HMQC) in deuterated D_6-DMSO) at 25 °C was carried out using DRX-500 and AV500, Bruker (^{1}H: 500 MHz; ^{13}C: 126 MHz).

Vector preparation, heterologous protein expression and protein purification: Vector expression, heterologous protein expression and purification was carried out as described in our previous publication.[23]

Substrate conversion assays: Substrate assays with BrvH were carried out with PrnF and ADH for cofactor regeneration as described by Frese et al.[17] 1.25 mg/mL enzyme was incubated with 1 mM substrate for 48 h, 500 rpm, at 25 °C in 100 mM Na_2HPO_4 buffer and 1 µM FAD, 100 µM NAD, 50 mM NaBr, 2.5 U/mL PrnF, 2 U/mL RR-ADH and 5% (*v*/*v*) *iso*-propanol. Methanol (1:1) was added to stop enzyme activity. Next, the samples were purified with C_{18} silica (0.035–0.07 mm) for RP-HPLC and LC-MS measurements.

Determination of specific activity: For determination of the specific acitivity tests were carried out in triplicates with 50 µM substrate and 10 µM enzyme incubated for 20 min. Every 5 min a sample was taken, stopped with 1:1 methanol, purified on C_{18} silica (0.035–0.07 mm) and analysed with RP-HPLC. With the percentage product yield and the molecular weight of BrvH the specific activity (mU mg^{-1}) was calculated.

Computational chemistry: The crystal structures of BrvH (PDB ID: 6FRL)[23] and of Trp-7 halogenase PrnA (PDB ID: 2ARD)[11] were downloaded from the protein data bank (www.rcsb.org)[30] and subsequently prepared for the modelling studies by adding the

hydrogen atoms using the 3d-protonate module implemented in MOE 2019.0101 [www.chemcomp.com Molecular Operating Environment, Chemical Computing Group Inc., Montreal, QC, Canada]. The analysis of structure-activity relationships (binary activity model), pharmacophore definition and *in silico* screening were also performed in MOE using the corresponding tools. Docking studies were carried out with the GOLD Suite v5.2.2 and GOLD-score as fitness function. For all other parameters standard settings were applied.[31]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bromination · docking · halogenation · structure-activity · relationships · virtual screening

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