Substrate Specificity and Regioselectivity of Tryptophan 7-Halogenase from *Pseudomonas fluorescens* BL915

Manuela Hölzer,[a] Wassily Burd,[b] Hans-Ulrich Reißig,[c] Karl-Heinz van Pée[d,*]

[a] JEP GmbH, Rheingaustr. 190–196, 65203 Wiesbaden, Germany
[b] Kupala Grodno State University, 22 Ozkeshko Street, Grodno, 230023 Belarus
[c] Institut für Chemie – Organische Chemie, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany
[d] Institut für Biochemie, Technische Universität Dresden, 01062 Dresden, Germany
Phone: (+49) 351–463–4494; Fax: (+49) 351–463–5506; e-mail: karl-heinz.vanpee@chemie.tu-dresden.de

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Abstract: Tryptophan 7-halogenase which is involved in pyrrolnitrin biosynthesis is the first halogenating enzyme to be isolated that has substrate specificity and regioselectivity. This $FADH_2$-dependent halogenase catalyzes the chlorination of its natural substrate tryptophan exclusively at the 7-position, a position at which direct chemical chlorination is not possible. Other substrates such as *N*-Ω-methyltryptamine, 5-methyltryptamine, 5-methylindole, 3-methylindole, or indole-3-acetonitrile are also chlorinated by the enzyme, whereas compounds like 1-methyltryptophan, indole-3-carboxylic acid, indole-3-acetic acid, or indole are not accepted as substrates. In addition, phenylpyrrole derivatives are also chlorinated by the enzyme. However, in contrast to tryptophan, the tryptophan and indole derivatives are chlorinated at positions 2 or/and 3 of the indole ring system and not at the 7-position. Chlorination of the phenylpyrrole derivatives also proceeds without regioselectivity and a mixture of mono- and dichlorinated products is obtained.

Keywords: biohalogenation; chlorination; enzyme catalysis; enzymatic halogenation; oxidoreductases; regioselectivity

In contrast to other enzymes involved in secondary metabolism, halogenating enzymes seemed to lack substrate specificity and regioselectivity.[1] The halogenating enzymes that were known until 1997 were all haloperoxidases or perhydrolases. Elucidation of their three-dimensional structures and reaction mechanisms have shown that they produce hypohalous acids as the actual halogenating agent.[2–6] Thus, halogenation reactions catalyzed by haloperoxidases and perhydrolases proceed without substrate specificity and regioselectivity which is not in agreement with results obtained by investigations of the biosynthesis of halogenated metabolites. These investigations had shown that halogenating enzymes involved in halometabolite biosynthesis must have substrate specificity and regioselectivity.[6] The first two halogenating enzymes with these properties were detected in a pyrrolnitrin-producing *Pseudomonas fluorescens* strain. These two halogenases were originally believed to require NADH as a co-factor,[7] but more detailed investigations revealed that it was actually $FADH_2$, produced by non-specific flavin reductases from FAD and NADH, that was required by these halogenases.[8] One of these halogenases catalyzes the first step in pyrrolnitrin biosynthesis, the regioselective chlorination of tryptophan (**1**) to 7-chlorotryptophan (**2**).[9] In this paper, we describe the first investigations of the substrate specificity and regioselectivity of tryptophan 7-halogenase (Trp 7-hal) and their implication for pyrrolnitrin biosynthesis.

Since Trp 7-hal activity cannot be measured in cell-free extracts of the wild-type strains producing pyrrolnitrin, a recombinant mutant of *P. fluorescens* BL915, overexpressing Trp 7-hal from *P. fluorescens* BL915 was used.[9] The enzyme was partially purified which resulted in separation of most of the flavin reductase activity from the Trp 7-hal. Therefore, partially purified flavin reductase had to be added to the enzyme assays.[8]

To investigate which other tryptophan and indole derivatives in addition to the natural substrate **1** are chlorinated by Trp 7-hal, we tested a number of commercially available compounds. While 1-methyltryptophan and *N*-acetyltryptophan were not accepted as substrates, *N*-Ω-methyltryptamine (**3**) and 5-methyltryptamine (**5**) were chlorinated by the enzyme. However, whereas **1** is exclusively chlorinated in the 7-position, the tryptamine derivatives **3** and **5** were

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/home/asc/ or from the author.

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chlorinated in the 2-position (Table 1). Indole derivatives with only a carboxy group in the side chain such as indole-3-carboxylic acid and indole-3-acetic acid were not accepted as substrates. This was also the case for indole and some indole derivatives substituted in position 3 such as indole-3-carbonitrile, indole-3-carboxaldehyde, and gramine. Surprisingly, 5-methylindole (**7**) and 3-methylindole (**9**) were chlorinated by Trp 7-hal. As in the case of the tryptamine derivatives **3** and **5**, chlorination did not occur at position 7, but at the 2-position (Table 1). All the above-mentioned substrates **3, 5, 7,** and **9** were monochlorinated. A substrate from which a dichlorinated product is obtained is indole-3-acetonitrile (**11**). The main product was 2-chloroindole-3-acetonitrile (**12**) and as minor products 2,3-dichloroindole-3-acetonitrile (**13**) and 3-chloroindole-3-acetonitrile (**14**) were obtained (Table 1).

Chlorination of 3-(2'-aminophenyl)pyrrole (**19**) and monodechloroaminopyrrolnitrin [4-(3'-chloro-2'-

Table 1. Chlorination of tryptophan and several tryptophan and indole derivatives by tryptophan 7-halogenase in the presence of an FADH2-producing flavin reductase, chloride, and oxygen.

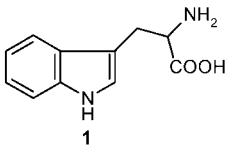
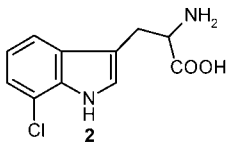
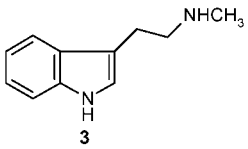
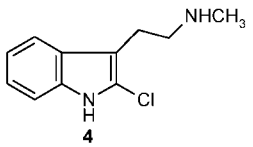
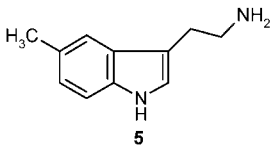
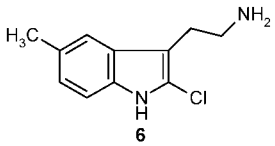
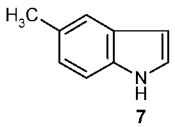
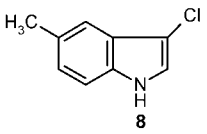
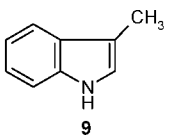
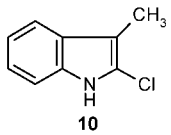
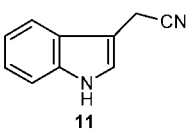
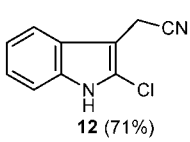
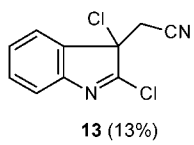
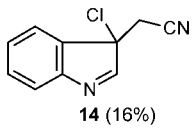
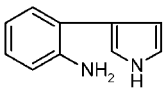
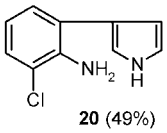
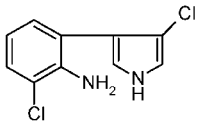
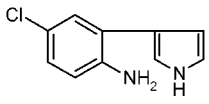
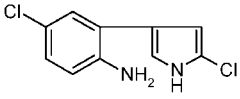
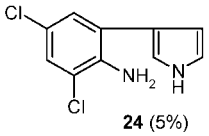
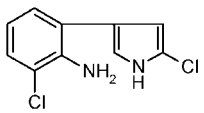
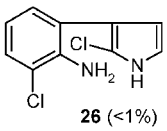
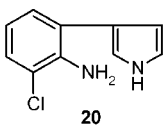
Substrate	Product (ratios of different products in % according to peak areas)
 **1**	 **2**
 **3**	 **4**
 **5**	 **6**
 **7**	 **8**
 **9**	 **10**
 **11**	 **12** (71%)  **13** (13%)  **14** (16%)

Table 2. Chlorination of 3-(2'-aminophenyl)pyrrole (**19**) and monodechloroaminopyrrolnitrin (**20**) by tryptophan 7-halogenase in the presence of an $FADH_2$-producing flavin reductase, chloride and oxygen.

Substrate	Product (ratios of the different products in % according to peak areas)
 **19**	 **20** (49%)  **21** (<1%)  **22** (30%)  **23** (5%)  **24** (5%)  **25** (10%)  **26** (<1%)
 **20**	**21, 24, 25, 26**

aminophenyl)pyrrole] (**20**) resulted in a mixture of products, chlorinated at different positions of the phenyl and the pyrrole rings. The main products obtained with **19** as the substrate are **20** and 3-(5'-chloro-2'-aminophenyl)pyrrole (**22**); the other products are formed by further chlorination of these two compounds (Table 2). Chlorination of the corresponding nitro compound [3-(2'-nitrophenyl)pyrrole] could not be observed. Although Trp 7-hal can chlorinate **19** to **20**, further chlorination of **20** to aminopyrrolnitrin (**21**) hardly occurs. This could explain why chlorination of **20** to **21** by tryptophan 7-halogenase obviously does not occur *in vivo*, since a mutant with an inactive monodechloroaminopyrrolnitrin 3-halogenase (Mcap 3-hal) cannot produce pyrrolnitrin, showing that Trp 7-hal cannot substitute the Mcap 3-hal.[9]

The formation of the obtained products is consistent with the proposed mechanism for $FADH_2$-dependent halogenases (Figure 1). In this hypothetical mechanism, $FADH_2$ is formed by the reduction of FAD with NADH catalyzed by a non-specific flavin reductase. $FADH_2$ is suggested to be bound by the tryptophan 7-halogenase where it reacts with oxygen. The formed tryptophan 7-halogenase-bound flavin hydroperoxide activates the organic substrate by a monooxygenase-like reaction leading to the formation of an epoxide which would not leave the enzyme. The epoxide would be opened by the nucleophilic attack of a chloride ion resulting in formation of a chlorohydrin. Specific removal of water would result in the formation of the chlorinated product.[6] However, so far none of the postulated intermediates has been isolated.

Trp 7-hal can accept a number of different tryptophan, indole, and phenylpyrrole derivatives, but only **1** is regioselectively chlorinated in the 7-position of the indole ring. With all the other compounds, the reaction proceeds with a relaxed regioselectivity. Obviously, only **1** is positioned at the active site of the enzyme in a way that allows the chlorination to take place at a position at which direct chemical halogenation is not possible. This shows that halogenating enzymes can have substrate specificity and that they can catalyze regioselective halogenation reactions, the latter being strongly dependent on the chemical structure of the substrate, and that in some cases they are even superior to chemical halogenation.

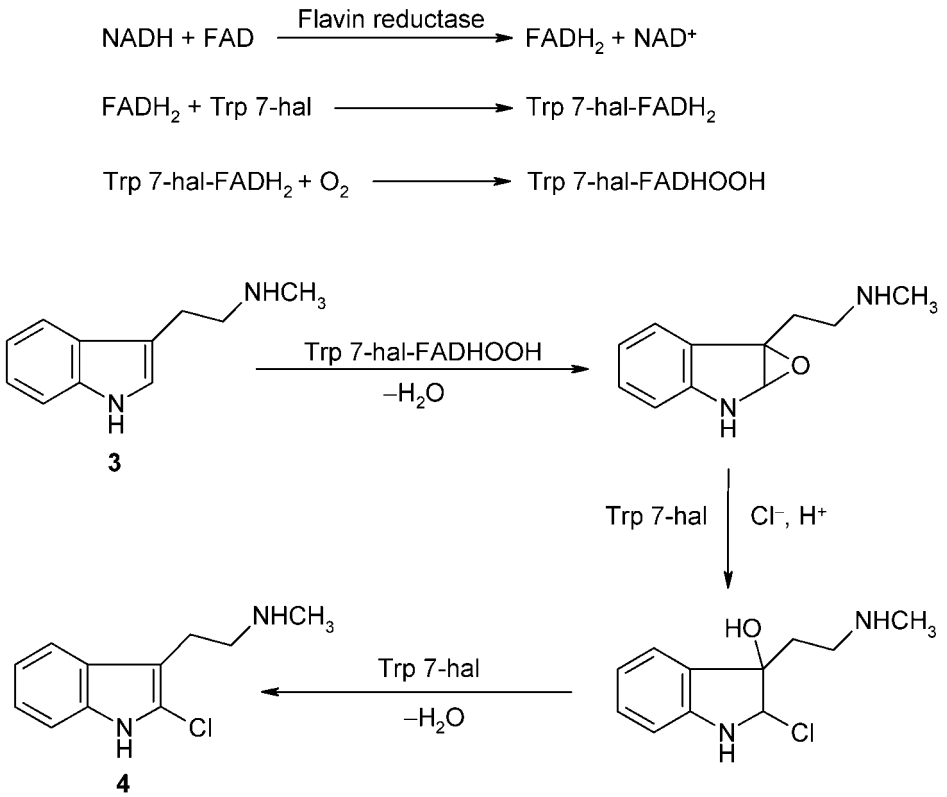


Figure 1. Hypothetical reaction mechanism for chlorination catalyzed by the $FADH_2$-dependent tryptophan 7-halogenase with *N*-Ω-methyltryptamine (**3**) as the substrate.

The genes of similar halogenases have recently been detected in a number of biosynthetic gene clusters for halogenated antibiotics such as the vancomycin-group antibiotics chloroeremomycin,[10] balhimycin,[11] and the antifungal compound pyoluteorin.[12] However, nothing is known about the corresponding enzymes and their properties, but according to what is known about the biosynthetic pathways for these halometabolites it has to be expected that these halogenases also have substrate specificity and regioselectivity.

Experimental Section

General Procedure

Trp 7-hal and flavin reductase from a recombinant *P. fluorescens* BL915 mutant were partially purified by ion exchange and hydrophobic interaction chromatography as described previously.[8] The phenylpyrrole derivatives **19** – **21** were synthesized as described in the literature.[13] The enzyme assays contained 4.45 mU Trp 7-hal, 50 mM NADH, 100 mM NaCl, 550 μU flavin reductase, 10 μM FAD, 0.6 mM **1** or indole derivative or 0.1 mM phenylpyrrole derivative in 10 mM potassium phosphate buffer, pH 7.2, in a total volume of 64 mL in the case of the tryptophan and indole derivatives and a total volume of 200 mL in the case of the phenylpyrrole derivatives. A blank containing all components of the assay mixture except tryptophan 7-halogenase was incubated in parallel and analyzed in the same way as the assay mixture. After incubation at 30 °C for 16 h the reactions were stopped by extraction with *tert*-butyl methyl ether. After extraction the samples were evaporated to dryness under vacuum and redissolved in 1 – 2 mL of methanol. The conditions used for the purification of the products for ^{1}H NMR analysis are presented in Table 1 of the supporting information. Chlorination of the products was analyzed by HPLC-electrospray MS [substrate **5**: LichroCRAT 250–4 column, CH_3OH/H_2O, 60:40 + 1% HCO_2H, 0.6 mL min^{-1}] and GC-MS (substrates **3**, **7**, **9**, **11**, **19**, and **20**: capillary column DB-5, 25 m × 0.32 mm; temperature program 100 – 240 °C at 10 °C min^{-1}, injection port and detector 240 °C). The structures of the purified products were elucidated by ^{1}H NMR analysis and comparison of these data with data from the literature.[13] The MS and ^{1}H NMR data are presented as supporting information.

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