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COMMUNICATION

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RadH a Versatile Halogenase for Integration into Synthetic Pathways.

Binuraj R.K Menon,[‡] Eileen Brandenburger,[‡] Humera H. Sharif, Ulrike Klemstein, Sarah A. Shepherd, Michael F. Greaney and Jason Micklefield*

Abstract: *Flavin-dependent halogenases are useful enzymes providing halogenated molecules with improved biological activity or intermediates for synthetic derivatization. Here we demonstrate how the fungal halogenase RadH can be used to regioselectively halogenate a range of bioactive aromatic scaffolds. Site-directed mutagenesis of RadH was used to identify catalytic residues and provide insights into the mechanism of fungal halogenases. A high throughput fluorescence screen was also developed enabling a RadH mutant to be evolved with improved properties. Finally we demonstrate how biosynthetic genes from fungi, bacteria and plants can be combined, to encode a new pathway generating a novel chlorinated coumarin 'non-natural' product in* E. coli.

Flavin-dependent halogenase enzymes (Fl-Hal) halogenate aromatic precursors in the biosynthesis of a diverse range of halogenated natural products, including antibiotics, antitumor agents and other bioactive compounds.[1] The halogen substituents installed by these enzymes are often important for the bioactivity of the natural products and can also be used as an orthogonal handle for further synthetic derivatization.[2] Tryptophan halogenases (Trp-Hal), from bacteria, are the most well characterized Fl-Hal and have been subject to detailed structural and mechanistic investigations.[1,3] The regioselectivity and benign, aqueous operating condition of Trp-Hal, has also provoked considerable interest in the development of these enzymes for synthetic applications.[4] Many valuable materials, agrochemicals and *ca*. 30% of the leading pharmaceuticals possess halogens.[5] Haloaromatics are also widely used precursors and intermediates in synthesis. However, narrow substrate specificity, relatively low activity and poor stability of wild-type Trp-Hal limits their synthetic utility. Despite this, there has been progress in expanding the substrate scope, improving catalytic activity and altering the regioselectivity of Trp-Hal using targeted or random mutagenesis approaches.[4]

Genome mining reveals there are many other bacterial Fl-Hal.[6] However, the majority of these enzymes remain uncharacterized and many are likely to require carrier protein tethered substrates, which would limit their utility. On the other hand, there are a number of fungal Fl-Hal that halogenate phenolic intermediates, independent of carrier proteins, in the biosynthesis of natural products including radicicol,[7] griseofulvin,[8] aspirochlorine[9] and chaetoviridins[10] (Figure 1). To date the catalytic scope of these fungal halogenases has been little explored beyond the natural substrates.[11] In this paper, we explore the mechanism and catalytic scope of the fungal halogenase RadH. We introduce a high throughput fluorescence screening method to rapidly select RadH mutants with improved properties and demonstrate how an improved RadH variant can be integrated into an engineered pathway to deliver novel halogenated 'non-natural' products.

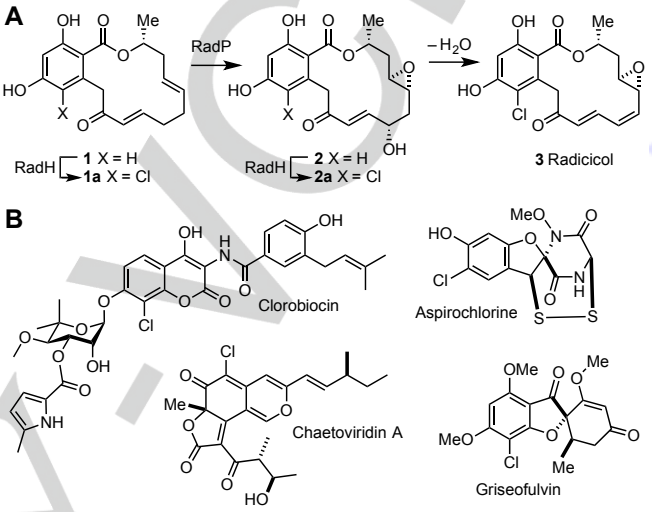

Figure 1. (A) Proposed final steps in the biosynthesis of radicicol. **(B)** Other natural products derived from Fl-Hal chlorination of phenolic precursors.

Previous gene deletion experiments indicate that RadH is required for the production of radicicol in the fungus *Chaetomium chiversii*.[12] Sequence similarity (Figure S1) suggests that RadH is likely to fulfill the same function as Rdc2, which was shown to catalyze the chlorination of monocillin II **1** to give **1a**, in the biosynthesis of radicicol **3** in a different fungal strain (Figure 1).[7] We overproduced RadH in *E. coli,* and as expected RadH does accept monocillin II as a substrate. However, the halogenation of **1** to **1a** was slow, reaching only 84 % conversion after 18 hours. In addition, unlike Rdc2,[7] RadH does not halogenate related natural products zearalenone **4** or curvularin **5**, suggesting that despite close sequence similarity (87%) RadH and Rdc2 exhibit different active site architecture.

To further explore the biocatalytic potential of RadH a number of aromatic compounds were tested as potential substrates (Figure 2, S2 & S3). This showed that RadH is more promiscuous than other Fl-Hal. RadH halogenates a range of natural and synthetic phenolic compounds (Figure 2 & S3). Interestingly, RadH halogenates several of these compounds with higher efficiency than the proposed natural substrate **1**.[7,12] Of the compounds tested 6-hydroxyisoquinoline **6**, and the plant natural product 7-hydroxycoumarin **8**, are the best substrates for RadH (Figure 2). In addition, RadH also halogenated plant derived flavonoids (*e.g.* **12**, **13** & **14**). In all cases, we observe regioselective halogenation *ortho* to the phenolic hydroxyl group and for the majority of bicyclic or tricyclic substrates where there are two possible *ortho*-positions that could be halogenated, RadH regioselectively halogenates proximal to the bridging position. To explore whether the phenolic moiety is required for RadH activity, we tested a range of electron rich aromatic compounds that lack hydroxyl substituents and found that none

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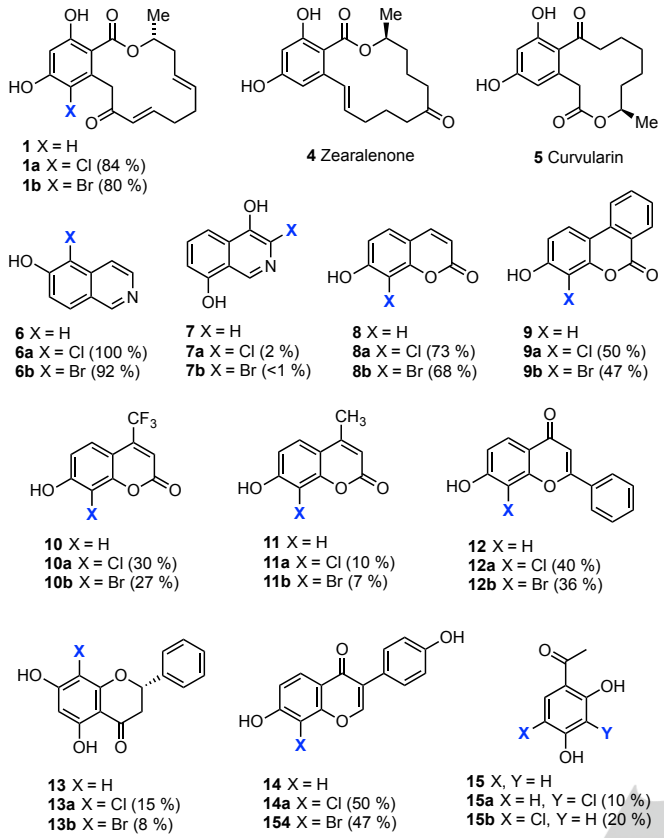

Figure 2. RadH substrate scope: RadH (15 μM), Fre (2.5 μM), FAD (1 μM), NADH (2.5 mM), $MgCl_2$ (or KBr for bromination reactions) (10 mM) and substrate (0.5 mM) in 10 mM potassium phosphate buffer at pH 7.4, Reactions were analyzed by HPLC after 2 hour incubation at 30 °C and 200 rpm. For reactions with monocillin II higher enzyme concentration, RadH (100 μM) and Fre (20 μM), was used and the conversions were measured after 18 hours.

of these were substrates (Figure S2). In addition, compounds with methoxy instead of hydroxyl groups proximal to the halogenation site, including the methoxy derivatives of substrates **6, 8** & **12**, were not halogenated by RadH despite having similar overall structure and electronic properties (Figure S2). This suggests that deprotonation of the phenol hydroxyl group is essential in the mechanism of RadH, and contributes to the regioselectivity of halogenation (Figure 3). In contrast to the highly regioselective RadH, conventional (non-enzymatic) halogenation of phenols usually gives rise to mixtures of *ortho*, *para* and di-substituted products. The kinetic parameters for RadH with selected substrates were determined (Table 1 & Figure S4-S10). Notably, the k_{cat} values for RadH with iso-quinoline **6** and coumarin **8** were significantly higher than those reported for Trp-Hals with the natural substrate.[3a,13] Whilst kinetic analysis shows monocillin II (**1**) is turned over more slowly than the other substrates, k_{cat}/K_m was not determined for **1** as saturation of the Michaelis-Menten curve was not attained.

Many isoquinolines, coumarins and flavonoids have been found to possess bioactivity for pharmaceutical and other applications (Figure S11). For example, the antibiotics clorobiocin and simocyclinone (Figure 1 & S11), both possess the 8-chloro-7-hydroxycoumarin moiety (**8a**) with the 8-chloro substituent contributing to antimicrobial activity.[14] Given that RadH, can halogenate common pharmacophores, we sought to

Table 1. Kinetic parameters for wild-type and D456E/T501S RadH.

RadH	Sub.	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [min^{-1}.μM] x10^{-3}
WT	**6**	3.2 ± 0.2	277 ± 3	12 ± 0.7
	8	2.9 ± 0.4	379 ± 5	7.7 ± 1.1
	10	0.65 ± 0.04	309 ± 19	2.1 ± 0.2
	11	0.56 ± 0.01	364 ± 18	1.5 ± 0.08
	12	0.032 ± 0.003	347 ± 2	0.092 ± 0.009
	14	0.0054 ± 0.002	281 ± 2	0.019 ± 0.007
D456E /T501S	**6**	5.8 ± 0.6	268 ± 10	22 ± 2.4
	8	5.3 ± 0.4	465 ± 4	11 ± 0.9
	10	ND	ND	ND
	11	ND	ND	ND
	12	0.031 ± 0.002	383 ± 4	0.081 ± 0.005
	14	0.009 ± 0.001	315 ± 1	0.029 ± 0.003

engineer RadH variants with improved properties that might be used to generate new halogenated products with altered bioactivity, or intermediates for further synthetic elaboration.

To enable screening of a larger number of RadH mutants, a high throughput assay for RadH activity was developed that exploits the fluorescence of 7-hydroxycoumarin derivatives.[15] The UV absorbance and fluorescence properties of coumarin substrates and halogenation products from RadH reactions were determined (Figures S12 & S13). Chlorination of **8** to give 8-chloro-7-hydroxycoumarin **8a**, resulted in the most significant change, with enhanced fluorescence emission at 456 nm upon excitation at 386 nm, which could easily be monitored in a 96 well plate format. To demonstrate that this assay can be used to select for improved RadH variants, a library of genes encoding RadH, with an average of 2-3 amino acid mutations per clone, was generated by epPCR and 960 colonies were picked then grown in 96 deep well plates. Crude cell lysates were then assayed for chlorination of **8**, and the 24 mutants with highest activity were further analysed using UPLC. The most promising mutant D465E/T501S showed a 15-fold improvement in activity with **8** over wild-type RadH in lysate assays (Figure S14). D465E/T501S was purified and shown to have significantly improved relative activity for a range of substrates (Table S1) as well as enhanced k_{cat} values with **6**, **8** and **14** compared with the wild-type RadH (Table 1). To explain the more significant 15-fold increase in activity observed in lysate assays, with substrate **8**, protein production levels and the stability of D465E/T501S were compared with the wild type RadH. Whilst SDS-PAGE analysis of cell lysates indicate that the double mutant is produced to a significantly higher level in *E. coli* cells than the wild type (Figure S15), variable temperature CD indicates that thermal stability of the wild type and mutant enzymes are similar (Figure S16). Thus in addition to possessing improved catalytic activity (Tables 1 & S1), the mutant RadH is more efficiently overproduced in *E. coli*.

In order to gain insights into the mechanism of RadH, and to rationalise how the D465E/T501S mutations may effect RadH structure and activity, an homology model for RadH was generated based on the X-ray structure of a related flavoprotein (PDB 3ATQ, Figure S17).[16] The model shows RadH with three separate domains, the FAD-binding, catalytic and C-terminal domains. The RadH catalytic domain has a large cavity (Figures 3 & S18) near to the isoalloxazine ring of FAD, which is likely to

be the substrate binding site. To verify the model, individual lysine residues predicted to be close to (K73 & K74) or more distant (K80 & K84) to the putative substrate binding site were mutated. Whilst K73A, K80A and K84A all showed activity similar to the wild type, the K74A mutation completely abolishes enzyme activity (Figure S19). The model predicts that K74 is *ca.* 4 Å away from the halogenation site (C6) of radicicol docked in the putative active site (Figures 3 & S18). It is likely that the halide ion attacks C4a-hydroperoxyflavin generating hypohalous acid, which reacts with K74 to provide the key chloroamine or bromoamine electrophile.[3] Interestingly, D465E and T501S are distant from the putative active site in the C-terminal region of RadH which may influence protein dynamic and interactions between the FAD binding and substrate binding domains.

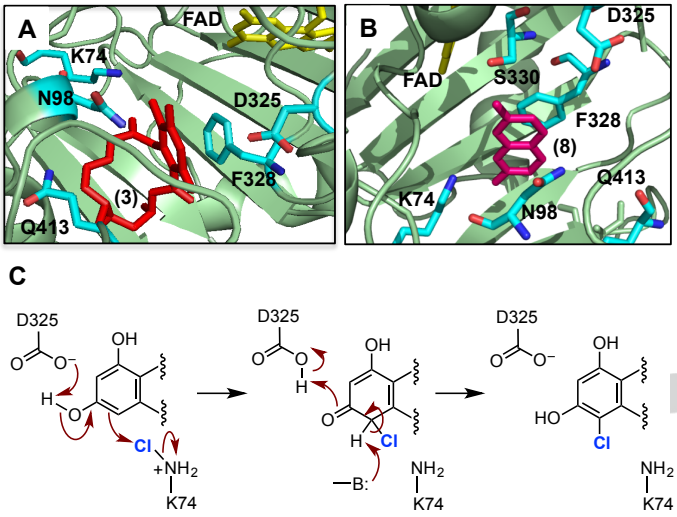

Figure 3. Active site model of RadH **(A)** with radicicol and **(B)** with 7-hydroxycoumarin (**8**) bound. **(C)** Proposed mechanism for RadH halogenation.

The RadH structural model also showed the presence of aromatic residues F327 and F328 close to the substrate binding site, and indicates that F328 may be involved in π-π stacking interactions with the aryl group of the substrates. Accordingly, a F328A mutant was prepared (Figure S20) and shown to have significantly reduced activity, consistent with F328 π-stacking with the substrate. The model also suggests that there are several residues such as N98, D325, S329, S330 and Q413 that are within H-bonding distance to the substrate. To establish if any of these residues may be involved in catalysis the corresponding alanine mutants were created. Whilst all the alanine mutants showed significantly reduced RadH activity, only the D325A completely abolished the catalytic activity (Figure S20). This suggests that both D325 and K74 are essential for catalysis. Given that the model indicates D325 would be in close proximity to the hydroxyl group which is directly adjacent to the substrate halogenation site, it is possible that D325 may function as a general base to deprotonate the phenol group during or after attack of the electrophile (Figure 3C). Presumably, another general base or possibly a water molecule is required to deprotonate the sp^3-hybridised centre of the halogenated intermediate facilitating rearomatization. This proposed mechanism (Figure 3C) is also consistent with the observation that substrates which have been methylated at the hydroxyl group, proximal to the halogention site, are not turned over by RadH (Figure S2B). Moreover, sequence alignments reveal that K74, D325 and F328 residues are conserved across known and putative fungal Fl-Hal, which also recognize phenolic substrates (Figure S21).

Previously, we showed how RadH can be integrated into synthetic pathways with chemocatalysts.[2] To further demonstrate the versatility of RadH, we sought to integrate this enzyme into an engineered biosynthetic pathway to generate novel halogenated 'non-natural' products *in vivo*. The *de novo* biosynthesis of 8-chloro-7-hydroxycoumarin **8a** was selected as a target (Figure 4) as RadH can efficiently halogenate 7-hydroxycoumarin **8** (Figure 2), also known as umbelliferone, a central intermediate in the biosynthesis of coumarins produced by plants. Natural coumarins and synthetic derivatives have been developed as commercial fluorescent dyes and as therapeutic agents (e.g. methoxsalen used to treat psoriasis, the antibiotic novabiocin and anticoagulants including warfarin).[17] To establish microbial production of 8-chloro-7-hydroxycoumarin **8a**, a plasmid (RSFDuet-1) with genes encoding 4-coumaryl-CoA ligase (4CL) from *Streptomyces coelicolor*[18] and feruloyl CoA 6´-hydroxylase (F6´H) from the plant *Ipomoea batatas*,[19] was generated and used to transform *E. coli* cells. Incubation, of the transformant, with precursor *p*-coumaric acid **16** resulted in production of 7-hydroxycoumarin **8** (Figure 4 & S22). A second plasmid pCDF, with a gene encoding the RadH variant (D465E/T501S) was next introduced and co-expression of this halogenase, along with 4CL and F6´H, led to the production of 6.5 mgL^{-1} of **8** and 0.8 mgL^{-1} of 8-chloro-7-hydroxycoumarin **8a**. Finally to demonstrate how **8a** can produced directly from glucose *via* fermentation, a third strain of *E. coli* was engineered with an additional plasmid (pACYC) encoding a tyrosine ammonia-lyase (TAL) from *Saccharothrix espanaensis*.[19] The TAL enzyme catalyzes the elimination of ammonia from the cellular pool of L-tyrosine to generate *p*-coumaric acid *in vivo*, which led to production of 1.4 mgL^{-1} **8** and 1.1 mgL^{-1} **8a** under fermentation conditions (Figure 4 & S22). These titres could be increased by feeding L-tyrosine. However, significantly higher levels of *p*-coumaric acid were produced by the engineered *E. coli* strain than **8** or **8a,** which may be due to higher production or activity of TAL compared with 4CL and F6´H.

Metabolic engineering approaches have previously been developed for production of valuable natural plant coumarins in *E. coli*.[19-21] However, as far as we are aware, this is the first example of production of a 'non-natural' halogenated coumarin from glucose in *E. coli*. Notably, the engineered pathway to **8a** is comprised of enzymes from plants, bacteria and fungi. Production of **8a** directly by fermentation also offers advantages over traditional chemistry used to synthesize **8a** that requires deleterious and toxic reagents.[22] Further optimization of this pathway and addition of other enzymes from coumarin biosynthesis may also lead to additional halogenated derivatives with altered bioactivities. The C8 chloro group also provides an orthogonal handle for further synthetic derivatization.[2]

In summary, we have explored the biocatalytic and biosynthetic scope of the fungal halogenase, RadH, showing that this enzyme is more promiscuous than other halogenases described to date. A phenolic hydroxyl is prerequisite for RadH activity, and halogenation is highly regioselective occurring *ortho*

to phenol hydroxyl group. Two active site residues, K74 and D325, were shown to be essential for RadH activity and from this a mechanism is proposed (Figure 3) that is supported by the observation that compounds possessing methoxy groups, in place of the substrate phenolic hydroxyl, are not halogenated by RadH. In addition, we have developed a high throughput fluorescence assay, which was used to screen for a RadH mutant that exhibited higher activity. Deployment of the improved RadH variant into a coumarin biosynthetic pathway assembled in an *E. coli* host strain, demonstrates that biosynthetic enzymes from diverse plant, bacterial and fungal origins can be combined to create new pathways to novel halogenated 'non-natural' products directly *via* fermentation.

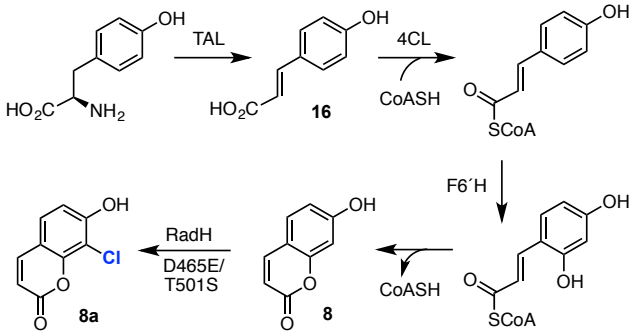

Figure 4. Engineered pathway to 8-chloro-7-hydroxycoumarin **8a** in *E. coli*.

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Keywords: Halogenase • Enzyme Mechanism • Directed Evolution• Natural Products • Pathway Engineering

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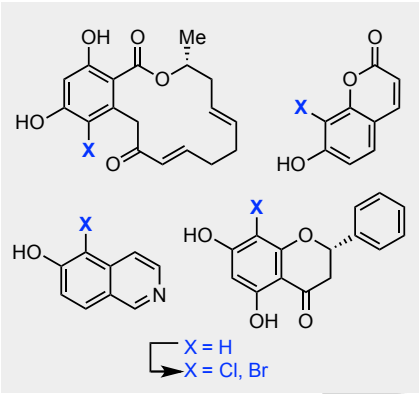
COMMUNICATION

The fungal halogenase enzyme RadH regioselectively halogenates a range of bioactive scaffolds. Structure-guided mutagenesis provides insights into the selectivity and mechanism of RadH and related halogenases. A high throughput fluorescence screen was also developed to evolve a RadH mutant with improved activity. The improved RadH and other enzymes, from diverse origins, were assembled in *E. coli*, to create a novel chlorinated 'non-natural' product by fermentation.



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