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Reengineering a Tryptophan Halogenase To Preferentially Chlorinate a Direct Alkaloid Precursor

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Supporting Information

ABSTRACT: Installing halogens onto natural products can generate compounds with novel or improved properties. Notably, enzymatic halogenation is now possible as a result of the discovery of several classes of halogenases; however, applications are limited because of the narrow substrate specificity of these enzymes. Here we demonstrate that the flavin-dependent halogenase RebH can be engineered to install chlorine preferentially onto tryptamine rather than the native substrate tryptophan. Tryptamine is a direct precursor to many alkaloid natural products, including approximately 3000 monoterpene indole alkaloids. To validate the function of this engineered enzyme in vivo, we transformed the tryptamine-specific RebH mutant (Y455W) into the alkaloid-producing plant Madagascar periwinkle (*Catharanthus roseus*) and observed the de novo production of the halogenated alkaloid 12-chloro-19, 20-dihydroakuammicine. While wild-type (WT) RebH has been integrated into periwinkle metabolism previously, the resulting tissue cultures accumulated substantial levels of 7-chlorotryptophan. Tryptophan decarboxylase, the enzyme that converts tryptophan to tryptamine, accepts 7-chlorotryptophan at only 3% of the efficiency of the native substrate tryptophan, thereby creating a bottleneck. The RebH Y455W mutant circumvents this bottleneck by installing chlorine onto tryptamine, a downstream substrate. In comparison with cultures harboring RebH and WT RebF, tissue cultures containing mutant RebH Y455W and RebF also accumulate microgram per gram fresh-weight quantities of 12-chloro-19,20-dihydroakuammicine but, in contrast, do not accumulate 7-chlorotryptophan, demonstrating the selectivity and potential utility of this mutant in metabolic engineering applications.

Natural product derivatization is a key strategy in the development of bioactive compounds with novel properties or improved activity.[1−4] Halogens, for example, often impact the pharmacological profile of a compound.[4,5] Notably, it has been demonstrated across different classes of natural products that incorporating simple precursor analogues into biosynthetic pathways is an effective strategy for producing complex natural products that are modified in a site-specific manner.[5−8] The biosynthesis of monoterpene indole alkaloids, a structurally diverse class of approximately 3000 natural products with a myriad of biological activities, commences with the decarboxylation of tryptophan (**1**) by tryptophan decarboxylase to yield tryptamine (**2**) (Figure 1A).[9] Here we describe how RebH, a flavin-dependent 7-L-tryptophan halogenase from an actinomycetes bacterial species, can be subjected to structure-based engineering to preferentially chlorinate **2**, a direct precursor in many biosynthetic pathways, including the monoterpene indole alkaloids. Furthermore, we introduced this RebH mutant into the metabolism of Madagascar periwinkle (*Catharanthus roseus*), a plant that produces approximately 130 monoterpene indole alkaloids.[10] We observed the de novo biosynthesis of the chlorinated alkaloid 12-chloro-19,20-dihydroakuammicine (**4b**) with no accumulation of halogenated tryptophan **1b**, clearly indicating that this reengineered enzyme functions in vivo as predicted. This study highlights the plasticity of these halogenases and the broad potential for halogenation of non-native substrates for metabolic engineering applications.

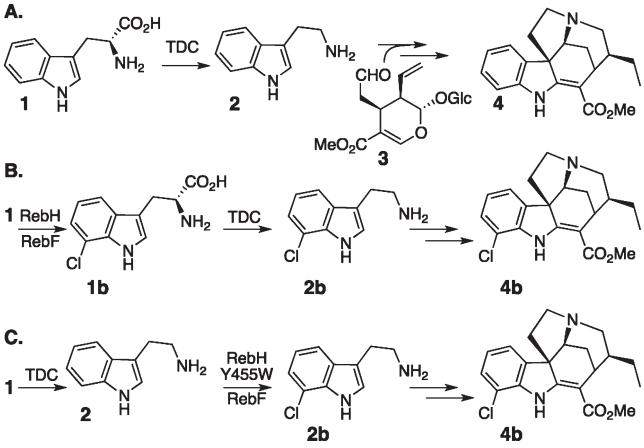

Figure 1. (A) Tryptophan (**1**) is decarboxylated by tryptophan decarboxylase (TDC) to form tryptamine (**2**), which, along with secologanin (**3**), is a precursor to all monoterpene indole alkaloids, including 19,20-dihydroakuammicine (**4**). (B) RebH and RebF can be expressed in periwinkle to yield 12-chloro-19,20-dihydroakuammicine (**4b**) in planta.[11] (C) Cultures transformed with RebH Y455W, which prefers **2**, also form **4b** but do not accumulate 7-chlorotryptophan (**1b**).

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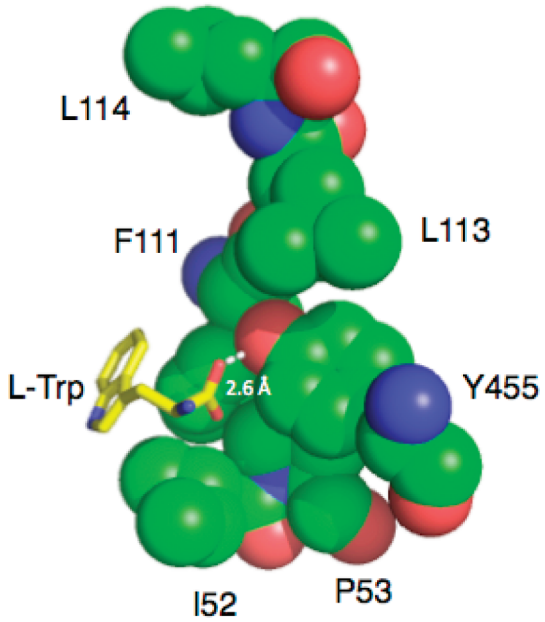

Figure 2. Tryptophan **1** complexed with RebH (PDB entry 2E4G). **1** is highlighted in yellow sticks. The residues proximal to the carboxylate moiety of **1** that were targeted to reengineer the substrate selectivity of RebH are shown in green space-filling models. The mutations made were I52F, I52Y, P53F, P53G, P53W, F111L, F111W, F111Y, L113F, L113G, L113W, L114F, L114G, L114W, Y455F, Y455L, and Y455W. The phenolic oxygen of Y455 is 2.6 Å from the nearest oxygen of the carboxylate moiety of **1**.

Previously, Runguphan et al.[11] transformed codon-optimized *rebH* along with its partner flavin reductase *rebF* into periwinkle root tissue and observed the production of halogenated alkaloid **4b** (Figure 1B). However, substantial amounts of 7-chlorotryptophan (**1b**) also accumulated in these tissues (50 ± 12 μg of **1b** per gram fresh weight, approximately double the amount of **4b**). The accumulation of **1b** suggests that tryptophan decarboxylase, which has a 30-fold preference for tryptophan **1** over analogue **1b**, acts as a bottleneck in vivo.[11] This bottleneck is undesirable because **1b** could be shuttled into the production of the more valuable halogenated alkaloid final product **4b**. Moreover, tryptophan is an essential metabolite that is involved in many central metabolic processes, including protein biosynthesis and, in the case of plants, auxin (growth hormone) biosynthesis. The accumulation of a halogenated primary metabolite seemingly has adverse effects on the growth rate of the tissues.

We hypothesized that overexpression of tryptophan decarboxylase in periwinkle would increase the turnover of **1b** and thereby alleviate the bottleneck. However, all efforts at constitutive overexpression of tryptophan decarboxylase, RebH, and RebF in periwinkle resulted in plant tissue that failed to survive selection. Additionally, tissues transformed with only tryptophan decarboxylase also failed to survive selection and could not be rescued through transfer to growth medium supplemented with 500 μM L-tryptophan [see the Supporting Information (SI)]. The apparent lethality of tryptophan decarboxylase overexpression suggests, perhaps unsurprisingly, that disrupting the flux of tryptophan is detrimental to plant survival.

An alternative strategy for bypassing this bottleneck is to reengineer enzyme substrate specificity. Since the three-dimensional structure of RebH is known,[12] we envisioned reengineering the

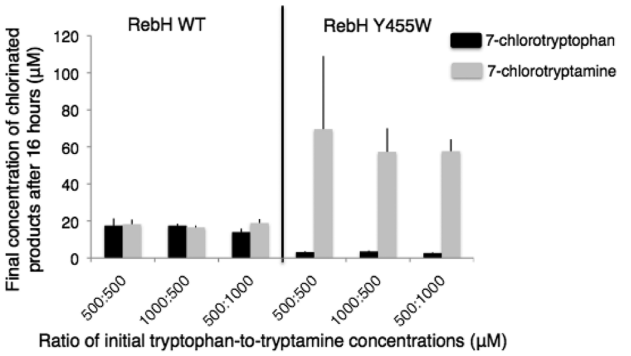

Figure 3. WT RebH and RebH Y455W competition assays with tryptophan **1** and tryptamine **2**. Both WT RebH (left) and RebH Y455W (right) were incubated with three different **1**:**2** ratios. RebH Y455W produces a 30-fold higher accumulation of 7-chlorotryptamine (60 μM) over 7-chlorotryptophan (2 μM), indicating that this mutant is highly selective for **2**.

halogenase to preferentially chlorinate a downstream biosynthetic precursor removed from primary metabolism. Specifically, we envisioned that RebH could be readily reengineered to chlorinate **2** directly (Figure 1C).

We examined the crystal structure of RebH complexed with **1** (PDB entry 2E4G) to propose a series of 17 mutations to the active site, specifically targeting residues proximal to the carboxylate moiety of the natural substrate **1** (Figure 2).[12,13] Gratifyingly, one RebH mutant, RebH Y455W, preferentially accepts **2**. Only one other mutant, RebH L113G, retained activity for either **1** or **2**. However, RebH L113G did not possess the desired selectivity (see the SI). Slow conversion to product in vitro prevented accurate measurement of the steady-state enzyme kinetics parameters for both wild-type (WT) RebH and RebH Y455W. Thus, to assess the substrate selectivity rigorously, we utilized competition assays where either WT RebH or the Y455W mutant was incubated with a mixture of **1** and **2**. We tested three different **1**:**2** ratios (500 μM:500 μM, 1000 μM:500 μM, and 500 μM:1000 μM).

WT RebH chlorinated both **1** and **2** under these assay conditions (Figure 3). However, we observed an approximately 30-fold higher accumulation of 7-chlorotryptamine (**2b**) than **1b** in RebH Y455W assays across all three substrate ratios (Figure 3). Relative to WT RebH in these competition assays, the production of **1b** was diminished 10-fold while the production of **2b** was augmented approximately 3-fold with RebH Y455W. The crystal structure suggests that RebH Y455W partially occludes **1** from the redesigned active site while not impeding access for tryptamine. This mutational analysis (see the SI) is congruent with the observation that the RebH Y455W mutant shows a clear preference for **2** as a substrate, even when **1** is present at initial concentrations twice as high as **2** (Figure 3). These results demonstrate that we successfully altered the substrate specificity of RebH in vitro to make it highly specific for **2**, a direct indole alkaloid precursor.

Notably, Hölzer and co-workers demonstrated that PrnA, a tryptophan 7 halogenase involved in pyrollnitrin biosynthesis (55% sequence identity to RebH) does accept analogues of **2**.[12a,14] However, PrnA was shown to install chlorine atoms at the more nucleophilic 2-position of various analogues of **2**, not at the 7-position, suggesting that the non-native substrates have altered binding to PrnA.[14] To ensure that the regioselectivity of RebH Y455W was unaltered, and also to determine whether

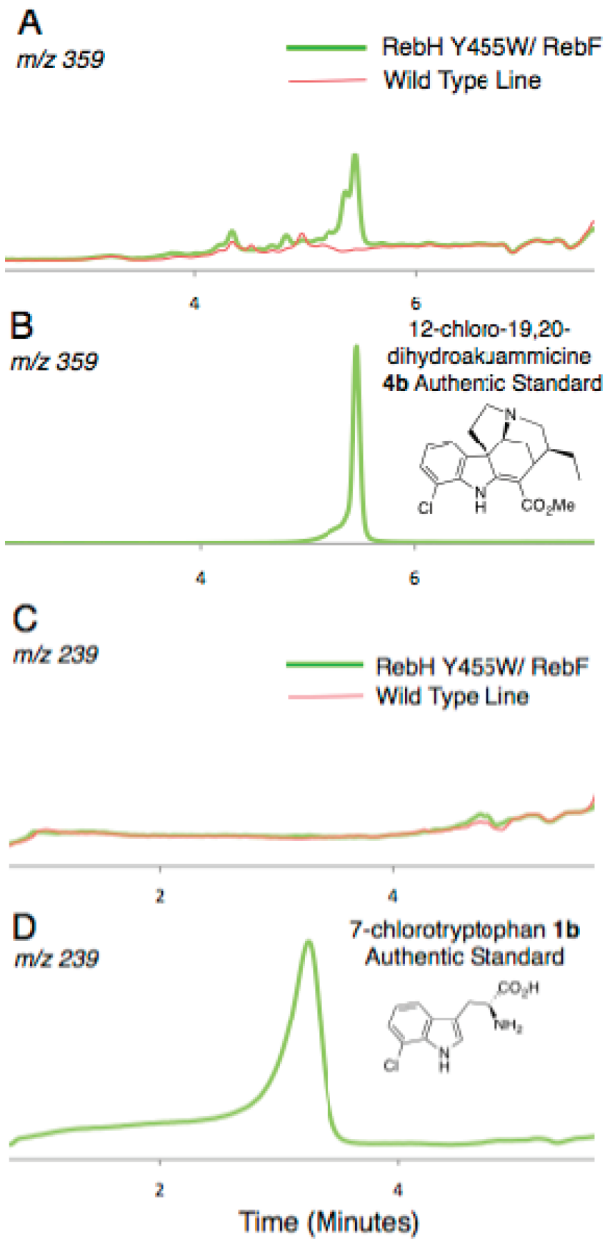

Figure 4. Metabolic analysis of lines harboring RebH Y455W and RebF. (A) Selected ion monitoring for 12-chloro-19,20-dihydroakuammicine **4b** (m/z 359) in transgenic root lines. This line accumulates 2.65 ± 1.08 μg per gram fresh weight of **4b**. (B) Authentic standard of **4b**.[11] (C) Selected ion monitoring of 7-chlorotryptophan **1b** (m/z 239). (D) Authentic standard of **1b**.[11] Lines expressing RebH Y455W and RebF do not display a peak corresponding to **1b** but do produce alkaloid **4b**.

WT RebH possesses the same regioselectivity for the non-native substrate **2**, we compared the tryptamine enzymatic products of WT RebH and RebH Y455W with authentic standards of all possible monochlorinated tryptamine isomers, namely, **2** chlorinated at the 2-, 4-, 5-, 6-, or 7-position of the indole ring. Using LC−MS to monitor the retention times of the various chlorotryptamine isomers (m/z 195), we noted that the WT RebH and RebH Y455W tryptamine enzymatic products coeluted exclusively with the 7-chlorotryptamine authentic standard (see the SI). Thus, WT RebH and RebH Y455W retained regioselectivity for the 7-position of the indole ring with substrate **2**.

Though the mutant enzyme was sluggish in vitro, we rationalized that a steady supply of fresh enzyme in the plant cell may allow the mutant enzyme to function adequately over extended periods to yield isolable quantities of chlorinated alkaloids. To test this reengineered enzyme in the context of a biosynthetic pathway in vivo, we introduced RebH Y455W and RebF into periwinkle (*C. roseus*) via *Agrobacterium rhizogenes* to yield stably transformed root cultures. To streamline the engineering process, neither gene was codon-optimized for expression in periwinkle. Each gene was placed into a commercially available plant vector (pCAMBIA1305.1) and under the control of the constitutive promoter CaMV 35S. Precursor-directed biosynthesis studies in periwinkle with **2b**, as well as the prior studies in which periwinkle was transformed with WT RebH and RebF, indicated that analogues of **4** are the major alkaloid analogue products when the 7-position of the indole ring is modified.[7,11,15] Notably, when the tryptamine precursor is chlorinated at other positions of the indole ring and integrated into periwinkle metabolism, the resulting chlorinated alkaloid profiles are drastically different, and chlorinated dihydroakuammicine is not a major product.[11,15] Methanolic extracts of the transformed roots were analyzed with selected ion monitoring for 12-chloro-19,20-dihydroakuammicine **4b** (m/z 359) and 7-chlorotryptophan **1b** (m/z 239). We observed several root lines harboring RebH Y455W and RebF that produced **4b**. RebH Y455W/RebF (line 13), for example, accumulated 2.65 ± 1.08 μg per gram fresh weight of the product **4b** (averaged across three biological replicates) with no measured accumulation of **1b**, indicating that RebH Y455W displays the desired substrate selectivity in planta as well as in vitro (Figure 4). The accumulation of **4b** (characterized by coelution with an authentic standard), the major metabolite expected from **2b**, provides further confirmation that RebH Y455W installs chlorine regioselectively at the 7-position of the indole ring of **2**. Chlorinated alkaloids aside from **4b** were not observed in this study, as evidenced by selected ion monitoring. Moreover, chlorination at the 2-position of the indole ring of tryptamine, as was observed in the study with Hölzer and co-workers, would preclude the formation of the tetrahydro-β-carboline via a Pictet−Spengler mechanism, a necessary step in the biosynthesis of the monoterpene indole alkaloids.[16]

Notably, no **2b** accumulated in these transformed hairy root lines, suggesting that **2b** is readily shuttled into the alkaloid metabolism of periwinkle. Moreover, the lines harboring RebH Y455W and RebF survived selection and grew more rapidly than lines overexpressing tryptophan decarboxylase, RebH WT, and RebF (see the SI), demonstrating that reengineering the halogenase was the superior method of alleviating the tryptophan decarboxylase bottleneck. However, the yield of unnatural alkaloid **4b** in this study remained low (approximately 1% of the total alkaloid content),[11] indicating that this system is not yet at a stage where large-scale production of **4b** is practical. We hypothesize that as tractable heterologous hosts are developed to produce plant-derived alkaloid pathways in high yields, incorporation of this redesigned biosynthetic enzyme (along with selected downstream biosynthetic enzymes that have also been engineered to favor chlorinated substrates) may play a crucial role in improving the production of chlorinated alkaloids.

Halogen moieties in natural products have been shown to confer potency and modulate molecular bioactivity and pharmacokinetics.[1,2,4,5,13] Additionally, halogens offer unique, site-specific handles that can be utilized in cross-coupling methodology

for further derivatization.[17] Notably, halogens appear in 25% of pharmaceutical compounds.[2,4] We have demonstrated the de novo biosynthesis of a halogenated "unnatural" plant natural product by redesigning a halogenase to preferentially install a chlorine atom onto a direct alkaloid precursor, tryptamine **2**, and subsequently integrating this redesigned enzyme into the alkaloid biosynthesis of periwinkle. Interestingly, RebH can brominate the 7-position of tryptophan **1** in the presence of bromide ions.[11,13a] However, bromination does not occur selectively in the presence of chloride sources (such as sodium chloride), which to date has prevented selective formation of brominated products using chlorinase enzymes in whole-cell systems.[11]

This mutant allows us to circumvent a metabolic bottleneck involving tryptophan decarboxylase. Moreover, this work, along with other recently reported studies,[18] highlights the potential use of halogenases for more widespread applications.

■ ASSOCIATED CONTENT

Supporting Information. Protein purification and mutant end point screening assays (noncompetitive and competitive), enzymatic product characterization, PyMol mutational analyses, plant transformations, genomic and metabolic characterization, and full methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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