Peptidic tryptophan halogenation by a promiscuous flavin-dependent enzyme

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ABSTRACT: Amino acids undergo numerous enzymatic modifications, including free amino acids and those incorporated into polypeptides. The extensive structural modifications observed in ribosomally synthesized and post-translationally modified peptides (RiPPs) provide compelling examples of natural modifications to amino acids. However, the broad applicability of amino acid-modifying enzymes for synthetic purposes is limited by narrow substrate scope and often unknown regulatory or accessory factor requirements. Here, we characterize ChlH, a flavin-dependent halogenase (FDH) from the chlorolassin biosynthetic gene cluster. Reconstitution of ChlH and the associated flavin reductase ChlR *in vitro* unexpectedly showed that ChlH acts on the linear precursor peptide ChlA rather than the threaded lasso peptide product. Unlike characterized peptide-modifying FDHs, which are limited to either heavily altered peptidic scaffolds or the terminus of a linear peptide, ChlH halogenates internal Trp residues of linear peptides, as well as N- and C-terminal Trp. Ala and Trp scanning mutagenesis of ChlA revealed a broad substrate tolerance and the capacity to modify Trp at nearly every position of the core region. Halogenation of disparate RiPP precursor peptides, pharmacologically relevant peptides, and an internal Trp of a protein was achieved using wild-type ChlH. A rapid cell-free biosynthetic assay was also used to assess a mutagenic panel of ChlA, which provided insight into ChlH's preferences. In contrast to characterized FDHs, ChlH halogenates diverse peptide sequences, and we predict this promiscuity may find utility in the modification of additional peptide and protein substrates of biotechnological value.

INTRODUCTION

Natural products have historically been a source of inspiration for drug development.[1] However, most natural products must be further derivatized to become suitable for clinical use. One common modification is halogenation. Many FDA-approved drugs contain at least a single halogen atom,[2] and numerous research groups have devised creative methods for robust halogen incorporation into small-molecule scaffolds.[3–9]

Natural product biosynthetic pathways similarly employ various strategies for bioactivity optimization. Many flavin-dependent halogenases (FDHs) act on the free amino acid Trp, and play a critical role in modifying bioactivity.[10–13] Unfortunately, FDHs frequently exhibit poor activity *in vitro* and require additional recognition motifs for robust enzymatic activity.[14–16] Recently, several approaches have been taken to obtain FDHs that halogenate peptidic Trp.[17–19] Several recently characterized FDHs are derived from ribosomally synthesized and post-translationally modified peptide (RiPP) biosynthetic gene clusters.

While relatively rare in RiPP biosynthesis, several examples of Trp halogenation have been reported. Microbisporicin (NAI-107) is a 24-residue lanthipeptide with Trp4 being chlorinated at the 5-position by the FDH MibH (WP_036325874.1).[20] However, MibH only accepts the lanthionine-containing, multicyclic peptide *deschloro*-NAI-107 as a substrate. This feature disqualifies wild-type MibH as a broadly applicable biocatalyst, as it does not tolerate linear substrates or lanthipeptides structurally similar to microbisporicin. Another RiPP-associated FDH, SrpI, catalyzes the 6-bromination of the C-terminal Trp of the SrpE precursor peptide.[21,22] Substrate tolerance studies showed that SrpI-catalyzed bromination is limited to the C-terminus and required an ~18-residue segment of the leader peptide for efficient product formation, narrowing its potential biocatalytic utility.[23] Separately, Thal (ABK79936.1), which modifies free *L*-Trp during the biosynthesis of thienodolin,[24] can also site-selectively chlorinate and brominate polypeptides, but is restricted to C-terminal Trp. An evolved Thal could also selectively brominate Trp when placed at the C-terminus of a protein.[25]

Chlorolassin is a lasso peptide recently isolated from *Lentzia jiangxiensis*.[26] The mature product features ChlH-dependent 5-chlorination of Trp8 and Trp12 (Figure 1). Here, we present the *in vitro* reconstitution of the chlorolassin halogenase ChlH (WP_090100303.1) and the cognate flavin reductase ChlR (WP_090100388.1). ChlH modifies the linear precursor peptide ChlA (WP_143022795.1) and does not modify threaded *des*-chlorolassin (chlorolassin lacking halogenation). In contrast to the stricter substrate requirements of MibH and SrpI, ChlH exhibited broad substrate tolerance and modified a wide range of ChlA variants. Furthermore, ChlH chlorinates Trp in unrelated peptides of various lengths, a macrocyclic peptide, and an internal residue of an unrelated, catalytically active enzyme. We glean insight into the substrate preferences of ChlH by extensively evaluating numerous substrates using either purified proteins or cell-free biosynthetic methods. These insights are then utilized to dramatically improve turnover of a poor substrate. This work demonstrates that wild-type ChlH is a versatile Trp chlorinating enzyme.

RESULTS

In vitro reconstitution of ChlHR.

We first sought to determine when Trp was halogenated during chlorolassin biosynthesis. With Trp14 being less accessible in the folded lasso peptide (PDB code: 8UKC),[26] and strict substrate recognition requirements for NAI-107 chlorination, we initially hypothesized that ChlH modified the folded lasso peptide product. However, given the observation of *des*-chlorolassin during the isolation of chlorolassin, lasso peptide cyclization was independent of the chlorination status of ChlA.[26] To address the timing of ChlH modification, we reconstituted the chlorination activity *in vitro*. *E. coli*-optimized genes encoding ChlH and ChlR (see Supporting Methods) were individually cloned with N-terminal His_6 tags. Following co-expression with the chaperones GroES/EL,[14] ChlH and ChlR were purified in high yield using immobilized metal-affinity chromatography (Figure S1). Reactions were initiated using purified ChlH/R and an excess quantity of NADH and FAD before analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). No chlorination of *des*-chlorolassin and *L*-Trp was observed after reaction with ChlH/R (Figure S2-3).

Next, we cloned the precursor peptide, ChlA, from *Lentzia jiangxiensis* with an N-terminal maltose-binding protein (MBP) tag. Removal of MBP via tobacco etch virus (TEV) protease resulted in linear ChlA peptide. Upon reaction with ChlH/R, NADH, FAD, and Cl^-, two chlorinations were observed on ChlA within 2 h (Figure 1). Although ChlH alone partially chlorinated ChlA, the addition of FAD, NADH, and ChlR yielded full dichlorination. All subsequent reactions therefore included ChlR, FAD, and NADH. MALDI-LIFT-TOF/TOF-MS (hereafter, MALDI-LIFT-MS) analysis established Trp8 and Trp12 as the chlorination sites, in agreement with the site-selectivity observed in native chlorolassin (Figures 1, S4). Analysis of monochlorinated ChlA from earlier time points revealed that Trp12 was modified before Trp8 (Figure S5).

After extended reaction times (24 h), a low intensity ion indicative of three ChlA chlorinations was detected (Figure S6). Localization by MALDI-LIFT-MS revealed Trp14 as the third chlorination site (Figure S7).[27] Previous work on the FDH RebH (CAC93722.1) identified Lys139 of ChlH as a potential catalytic residue (Figure S8). Characterized FDHs use chloride, O_2, and $FADH_2$ to generate hypochlorous acid. A conserved catalytic Lys then interacts with hypochlorous acid either through chloramine formation or by serving as a proton donor to chlorinate Trp.[10,15] A previous report showed that hypohalous acid can also spontaneously react with Trp.[28] To determine if the conserved Lys139 of ChlA was necessary for Trp chlorination, we prepared the K139A variant (Figure S1). Upon identical reactions with ChlA, the K139A variant gave no masses indicative of chlorination, even after extended reaction times (Figure S9). This confirms that Trp14 chlorination, while only observed *in vitro*, still requires catalytically active ChlH.

ChlH can brominate Trp residues of ChlA.

To evaluate if ChlH could utilize other halide sources, a series of reactions were supplemented with 100 mM NaF, NaBr, or NaI (Figure S10). Initially, no attempts were made to remove chloride, which remained at ~50 mM to allow for halide competition. NaF supplementation did not result in ChlA fluorination but rather exclusive chlorination. NaBr supplementation gave a mixture of chlorination and bromination. The stock solutions of ChlA, TEV protease, MBP-ChlA, ChlH, and ChlR were then extensively buffer exchanged to reduce chloride to trace levels. Upon NaBr supplementation, exclusive bromination of ChlA was observed (Figure S11). Unfortunately, ChlH precipitated in the NaI-supplemented buffer and iodination was not investigated further.

ChlA halogenation does not require the leader peptide.

To assess the role of the leader peptide in ChlH activity, we cleaved MBP-ChlA at the native leader peptide-core peptide junction using the leader peptidase FusB (WP_011291590.1) and associated RiPP-recognition element FusE (WP_011291591.1) from *Thermobifida fusca*. Despite this non-cognate usage, FusB/E readily yielded $ChlA_{core}$. The relative chlorination efficiency of full-length ChlA and $ChlA_{core}$ was then compared (Figure 2). $ChlA_{core}$ was fully dichlorinated at 2 h, at the same approximate rate as the full-length (40-residue) ChlA precursor peptide. This demonstrated that ChlH activity was not contingent on the leader peptide, unlike other characterized RiPP-associated Trp-halogenases.[29] Given that $ChlA_{core}$ is poorly soluble in water, we opted to use full-length ChlA for most of the *in vitro* experiments hereafter.

Ala variants of ChlA highlight substrate promiscuity.

To explore the site-selective dichlorination of ChlA, we performed an Ala scan of the core region of ChlA (Figure 3). Halogenation activity was assessed *in vitro* at 2 h and 24 h by MALDI-TOF-MS (Figures S12-S13).

Most Ala substitutions were well-tolerated, with minimal differences observed in processing compared to wild-type ChlA. Single chlorination of the W8A and W12A variants at 2 h demonstrated that chlorination of Trp8 was independent of Trp12 chlorination and *vice versa*. However, we note that neither W8A nor W12A was fully monochlorinated at 2 h. To determine if aromatic amino acids at position 8 or 12 enhanced chlorination efficiency at the non-varied site, we generated and analyzed the W8F, W8Y, W12F, and W12Y variants (Figure 3). After 2 h of reaction with ChlH, each variant was fully monochlorinated (Figure S14).

A modest extent of L13A trichlorination was observed at 2 h and full trichlorination was achieved within 24 h. This result implied Leu13 hindered Trp14 chlorination and could aid in understanding the observed chlorination pattern on chlorolassin. To further probe this idea, we generated ChlA variants P7L and A11L, which place Leu immediately before Trp8 and Trp12, respectively (Figure 3). These variants were poorly modified compared to wild-type ChlA and P7A at 2 h (Figure S14), supporting the inhibitory role of Leu at the (-1) position for Trp chlorination. Similarly, ChlH processing of variant W14A gave a mixture of 0-2 chlorinations at 2 h but was fully dichlorinated at 24 h. Predicting that Trp14 contributed to efficient substrate recognition, we evaluated W14F, which was fully dichlorinated at 2 h (Figure 3, S14-15). This result underscores a role for aromatic amino acids near the C-terminus of ChlA but demonstrates they are dispensable at longer reaction times.

The characterized FDH SrpI recognizes the C-terminal region of SrpE, the cognate precursor peptide.[29] To assess the importance of the C-terminus of ChlA for ChlH activity, we evaluated a truncated ChlA peptide lacking Leu13, Trp14 and Asp15 (ChlA-L13*, Figure 3). A low-intensity monochlorination product was observed at 2 h while fully dichlorinated product was observed at 24 h (Figures S14-15). Accordingly, a 19-residue addition to the C-terminus of ChlA that appended a hexahistidine tag ($ChlA_{ext,}$ Figures 3, S16) resulted in complete monochlorination at 2 h and complete dichlorination at 24 h after ChlH processing (Figures S14-S15). These data

demonstrate that the identity of the most C-terminal residues of the substrate peptide can influence the processing rate of ChlH but are otherwise dispensable.

ChlH chlorinates non-native Trp residues in ChlA.

We then performed a Trp scan of the core region of ChlA to assess chlorination at non-native positions (Figures 3, S17-18). ChlA variants S4W and G5W were fully trichlorinated at 2 h while the remaining variants exhibited an array of modification states. The P7W, D9W, A11W, and L13W variants were modified less extensively than wild-type ChlA and all other Trp variants at 2 h. Notably, either partial or complete modification was observed for all Trp variants at 24 h.

ChlA variants L13W and D15W were fully tetrachlorinated at 24 h, supporting a role of these positions in delaying Trp14 chlorination in wild-type ChlA. To better gauge the pattern of Trp chlorination, several ChlA Trp variants were reacted with ChlH/R, then FusB/E, purified by high-performance liquid chromatography (HPLC), and analyzed by MALDI-LIFT-MS (Figures S19-S23). This revealed a general trend in which Trp8 and Trp12 are modified more effectively than Trp14 or the non-native Trp residues introduced into ChlA. Overall, these data collectively show that certain positions of the core peptide, when changed to Trp, are preferentially chlorinated compared to other positions. Still, these preferences can be overridden with longer reaction times.

ChlH modifies peptides unrelated to ChlA.

Given that ChlH exhibited broad, leader peptide-independent substrate tolerance, we next asked whether ChlH could halogenate FusA, an unrelated lasso precursor peptide. After enzymatic processing, FusA is converted into fusilassin (also known as fuscanodin),[30–33] which contains two Trp. Linear $FusA_{core}$ was reacted with ChlH for 24 h and a combination of unmodified, mono- and dichlorinated $FusA_{core}$ was observed (Figure 4). Analogous to the lack of activity on *des*-chlorolassin, ChlH did not accept the threaded lasso peptide fusilassin as a substrate (Figure S24). Although ChlA and FusA are both lasso precursor peptides, they show no core region similarity (Figure S25). We next assessed if a linear variant of the darobactin precursor peptide was a substrate for ChlH (i.e., DarA-Q8*).[34,35] We observed monochlorination of DarA-Q8* after reaction with ChlH (Figure 4). As with cyclic lasso peptides, we did not detect chlorination of a cyclic darobactin intermediate (Figure S26). In contrast, pyritide A1, a macrocyclic RiPP featuring a central pyridine and two Trp, was predominantly monochlorinated and partially dichlorinated, by ChlH (Figure 4).[36,37] HR-MS confirmed the molecular formula of the $[M+H]^+$ for the dichlorinated pyritide A1 ($C_{49}H_{59}N_{10}O_9Cl_2$; theoretical, 1001.3838 Da; observed, 1001.3835 Da, 0.3 ppm error) (Figure S27). Attempts to localize the chlorination site(s) were inconclusive given the limited fragmentation observed. For the dichlorinated product, we hypothesize each Trp was singly chlorinated, as there has been no evidence that ChlH performs dichlorination of a single Trp. Collectively, these results show that ChlH can halogenate certain macrocyclic peptides, although the extent of tolerance will require additional investigation.

We next evaluated if ChlH could chlorinate a pharmacologically relevant peptide, specifically growth hormone-releasing peptide 6. GHRP-6 is a hexapeptide containing both *L*-Trp and *D*-Trp that stimulates growth hormone secretion and displays cardioprotective properties.[38–40] Upon reaction with ChlH, we observed monochlorinated GHRP-6 and a new mass deviating by +1 Da that we hypothesized resulted from deamidation of the C-terminus (Figure 4).[41] Tandem MS localized the chlorination event to *D*-Trp2 of GHRP-6 (Figure S28). This result was reminiscent of a recent report on Thal that showed properly placed *L*- and *D*-Trp could be halogenated.[17] To evaluate if an all-*L*-configured isomer of GHRP-6 was a ChlH substrate, a synthetic DNA template was prepared and subjected to *in vitro* transcription/translation. The resulting peptide was treated with ChlH, then analyzed by MALDI-TOF-MS. The highest intensity peak corresponded to monochlorination while a lower intensity ion was consistent with dichlorination (Figure 4). MALDI-LIFT-MS localized the major chlorination site to *L*-Trp2 (Figure S29).

Cell-free insights into ChlH substrate tolerance.

Emboldened by the variety of substrates ChlH modified *in vitro*, we designed several additional ChlA variants to further probe substrate tolerance. These substrates were prepared by *in vitro* transcription/translation. Given the leader peptide independence of ChlH, this region was replaced with a single N-terminal Met (installed as formyl-Met, ^{f}Met) to allow translation initiation.[42] These experiments employed a I2K variant of ChlA to provide a cationic site and enhance analytical sensitivity. We refer to the *in vitro* transcription/translation prepared ChlA core peptide bearing N(-1)^{f}M and I2K substitutions as $ChlA_{PE}$.

Analogous to ChlA and $ChlA_{core}$, $ChlA_{PE}$ was dichlorinated at Trp8 and Trp12 after a 1 h reaction with ChlH (Figure 5). A $ChlA_{PE}$ variant with residues 8-15 duplicated and appended to the C-terminus was next tested (Figure S30). This variant contains six Trp, two being preceded by Leu and thus likely to undergo delayed chlorination. After reaction with ChlH for 1 h, a mixture of masses indicative of 3-4 chlorinations was observed. MALDI-LIFT-MS tentatively localized chlorination to Trp6, 10, 14, and 18 (Figure S31), highlighting that motif duplication doubled the number of chlorinations observed. An inverted $ChlA_{PE}$ sequence was also tested, which yielded low levels of chlorination after treatment with ChlH (Figure S32). The position corresponding to Trp14 of ChlA, was preferentially chlorinated, while the position corresponding to Trp12 of ChlA was likely unmodified (Figure S33). This highlights the role of both flanking residues in modification by ChlH.

To again address the potential inhibitory role of preceding Leu residues, we generated $ChlA_{PE}$ variant P7L/A11L, which placed Leu directly before all three Trp residues. Upon reaction with ChlH for 1 h, monochlorination was observed (Figure S34). MALDI-LIFT-MS localized chlorination to Trp8, indicating that Leu11 effectively blocked Trp12 chlorination yet Leu7 did not block Trp8 chlorination (Figure S35).

The contribution of core residues was next evaluated by creating an extensive mutagenic panel that probed positions 7-15 of $ChlA_{PE}$. Additionally, all three Trp were individually replaced with Ala, Glu, Phe, Pro, Arg, and Tyr for a total of 120 variants tested. After *in vitro* transcription/translation and reaction with ChlH, the samples were subjected to MS analysis to determine chlorination stoichiometry and location (Figures 5, S36-44, and Supplementary Data Set 1).

We first analyzed monochlorinated products. Most variants tested displayed at least one chlorination, highlighting the general substrate tolerance of ChlH. A total of 43 variants were fully modified at Trp8 while 50 variants were fully modified at Trp12. While ChlH displayed versatility in accepting many

substrate sequences, the residues flanking the Trp modification site influenced the reaction outcome. For example, Trp8 was only modified when Asp9 was changed to Gly; however, Trp12 was chlorinated in every tested Asp9 variant. Additionally, many Ala11 variants displayed reduced or no chlorination at Trp12, but over 50% of the tested variants were fully chlorinated at Trp8. Generally, small residues were preferred in the (-1) position (i.e., core positions 7 and 11), while tolerance at the (+1) positions (i.e., core positions 9 and 13) differed substantially between Trp8 and Trp12.

We then analyzed dichlorinated products from the 120-membered panel of $ChlA_{PE}$ variants (Figure 5). Several variants (29, 24%) resulted in full dichlorination. Position 15 was the most permissive of those tested, as every substitution except Pro yielded a dichlorinated product. Positions 10 and 13 exhibited similar preferences for hydrophobic residues. Positively charged residues at position 10 prevented modification and broadly inhibited chlorination at all positions except 15.

ChlH modifies a GLP-1 variant.

Building on the mutagenic panel, we next tested whether ChlH could halogenate glucagon-like peptide 1 ($GLP\text{-}1_{7\text{-}36}$). This peptide serves as the basis for a series of type 2 diabetes- and obesity-related drugs.[43–45] $GLP\text{-}1_{7\text{-}36}$ contains a single Trp that received only trace chlorination after reaction with ChlH (Figure 5). While Trp25 is flanked by small hydrophobic residues, the nearby Lys28 and Arg30 residues may be inhibitory, as evidenced from the variant library where Lys/Arg residues prevented chlorination of nearby Trp. ChlH processing of the K28A/R30A variant of $GLP\text{-}1_{7\text{-}36}$ was substantially improved (Figure 5).

ChlH modifies an internal Trp of an active enzyme.

Lastly, we asked if ChlH could act upon a full-length protein. Thus, we tested a set of readily available, purified proteins that are responsible for thiomuracin biosynthesis.[37,46] MBP-TbtA, -TbtD, -TbtE, -TbtF, and -TbtG and His_6-TbtB and -TbtC were individually treated with ChlH, trypsinized, and analyzed by MS. Out of 97 unique Trp across seven proteins, we observed near-complete modification of MBP-TbtG Trp471 (WP_013130815.1, Figure 6). As no experimentally determined structure is available for MBP-TbtG, we generated an AlphaFold3 model of TbtG. Trp74 of TbtG (corresponding to Trp471 in MBP-TbtG), is not solvent exposed in the predicted structure. MBP-TbtG was confirmed to be catalytically active, confirming proper folding of the protein (Figure S45). [47] We speculate that TbtG may be flexible, such that ChlH primarily modified an alternate conformation that was solvent exposed. Alternatively, it is possible that the AlphaFold model does not accurately represent the solution structure of TbtG. Potential complexation with the precursor peptide TbtA, TbtE, or TbtF may alter the conformation. Notably, the local sequence of TbtG that was chlorinated is partially analogous to ChlA (Figure S46).

DISCUSSION

The data reported here show that ChlH acts on the linear precursor peptide ChlA and not the threaded lasso peptide *des*-chlorolassin. ChlH shares 58% sequence identity with the lanthipeptide-modifying MibH. An AlphaFold 3-enabled structural alignment of ChlH with the crystal structure of MibH (PDB: 5UAO) shows high predicted structural similarity with a global RMSD of 0.5 Å (Figure S47).[20] Despite this similarity, these proteins modify their respective substrates at distinct biosynthetic stages. This may be rationalized by the size differences in the active site cavities of each enzyme. CASTpFold calculated a 442 $Å^3$ volume for ChlH while the active site cavity for MibH was nearly 50% larger (652 $Å^3$, Figure S48).[48] Caution should be applied in interpreting these values, however, as they are derived from static structures. Additionally, while FDHs acting on free *L*-Trp are often sequence-similar, known FDHs acting on peptide substrates can be highly sequence-diverse (Figure S49).

Using ChlA as the substrate led us to wonder why Trp14 was unmodified *in vivo* but minimally modified *in vitro*. Our results indicated that rather than utilizing the leader peptide of ChlA for substrate recognition, ChlH seems to recognize the C-terminal region of the ChlA core peptide (i.e., Ala11-Trp14). While the C-terminal motif expedited modification *in vitro*, it was dispensable, as numerous substrates lacking the motif were successfully modified. Despite contributing to processing elsewhere, Trp14 is slowly modified *in vitro*. Our results show that ChlH is broadly tolerant and capable of modifying disparate Trp-containing peptides. Because of this, ChlH activity may need to be tightly regulated in *Lentzea jiangxiensis*. Indeed, both *des*-chlorolassin and monochlorolassin were observed during the purification of chlorolassin,[26] demonstrating that during native production, ChlA is incompletely modified prior to lasso cyclization.

Nature often utilizes halogenation to tailor the physical properties of natural products. For example, the FDH DarH naturally brominates the 5-position of Trp1 in darobactin.[49] While capable of iodination, DarH could not chlorinate darobactin. ChlH, in contrast, can chlorinate and brominate substrates but only acts on the linear precursor of darobactin. The complementary activities of DarH and ChlH could be envisioned to provide access to new-to-nature halogenated darobactin variants.

While several residues of ChlA (Ala11-Trp14) were important for robust processing by ChlH, an atomic-resolution co-crystal structure was not obtained and thus the precise substrate-binding mode remains to be investigated. Furthermore, mutagenic scanning revealed that modification of Trp8 and Trp12 are not entirely independent. For example, most Pro7 variants experienced hindered processing not only at the adjacent Trp8 but also at the more distant Trp12. Further work is needed to shed light on the precise details of the ChlA-ChlH interaction.

Several general observations about the substrate preferences of ChlH can be made from our extensive screening effort. When modifying wild-type ChlA, an N-terminal (-1) Leu is inhibitory. Small hydrophobic residues are often preferred. Positively charged residues are broadly inhibitory. We suspect that this could be due to electrostatic repulsion against positively charged residues within the ChlH active site, although this remains to be experimentally assessed.

CONCLUSION

In conclusion, we have reconstituted a versatile Trp halogenase enzyme ChlH and its cognate flavin reductase ChlR. Unexpectedly, ChlH modifies the linear precursor peptide ChlA rather than the threaded lasso peptide *des*-chlorolassin. We further assessed the substrate scope of ChlH and achieved

chlorination in a broad array of Trp-containing peptidic scaffolds. Linear and macrocyclic peptides, as well as a larger protein, were effectively chlorinated without the need for additional recognition motifs. While several residues of ChlA do contribute to more efficient processing by ChlH, they are not strictly required for modification. The broad applicability of ChlH makes it an attractive candidate for the biocatalytic halogenation of peptidic Trp residues.

ASSOCIATED CONTENT

The supporting information is available free of charge at *hyperlink here*
Experimental methods and Supporting Figures S1-S49 (PDF)

Supplementary Data Set 1: Assigned ions associated with MS/MS data (.xlsl)

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Author Contributions

A.J.R. conceived of the project. A.J.R., M.G.G., and D.A.M. designed research. A.J.R. and M.G.G. designed and performed experiments. All authors analyzed data. D.A.M. supervised the study. A.J.R. and M.G.G. wrote the manuscript with extensive input from D.A.M. A.J.R. and M.G.G. contributed equally.

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Notes

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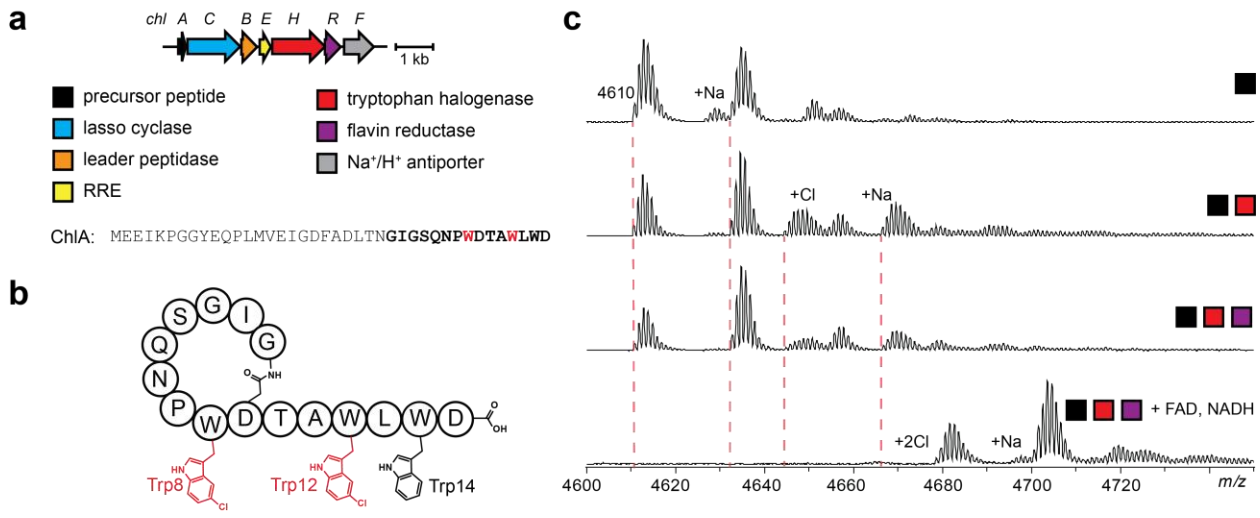


Figure 1. Chlorolassin biosynthesis, structure, and reconstitution. (a) Biosynthetic gene cluster and precursor peptide sequence for ChlA. (b) The structure of chlorolassin shown in an unthreaded conformation for clarity. Trp8 and Trp12 are each chlorinated at position 5 of the indole, while Trp14 is unmodified. (c) *In vitro* reconstitution of ChlH/R activity. Expected *m/z* values for unmodified, mono-, and dichlorinated ChlA are 4610, 4644, and 4678 respectively. Colored squares represent reaction components as defined in panel a.

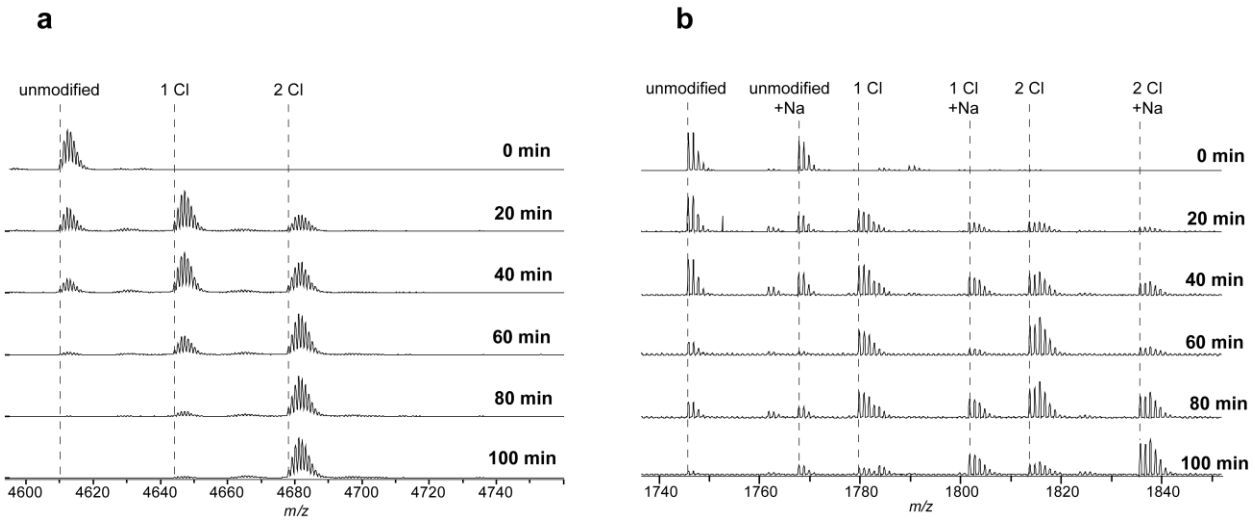


Figure 2. Relative rates of ChlH processing. (a) MALDI-TOF-MS monitoring of ChlA precursor peptide and (b) $ChlA_{core}$. The reactions were identical except for the tested substrate.

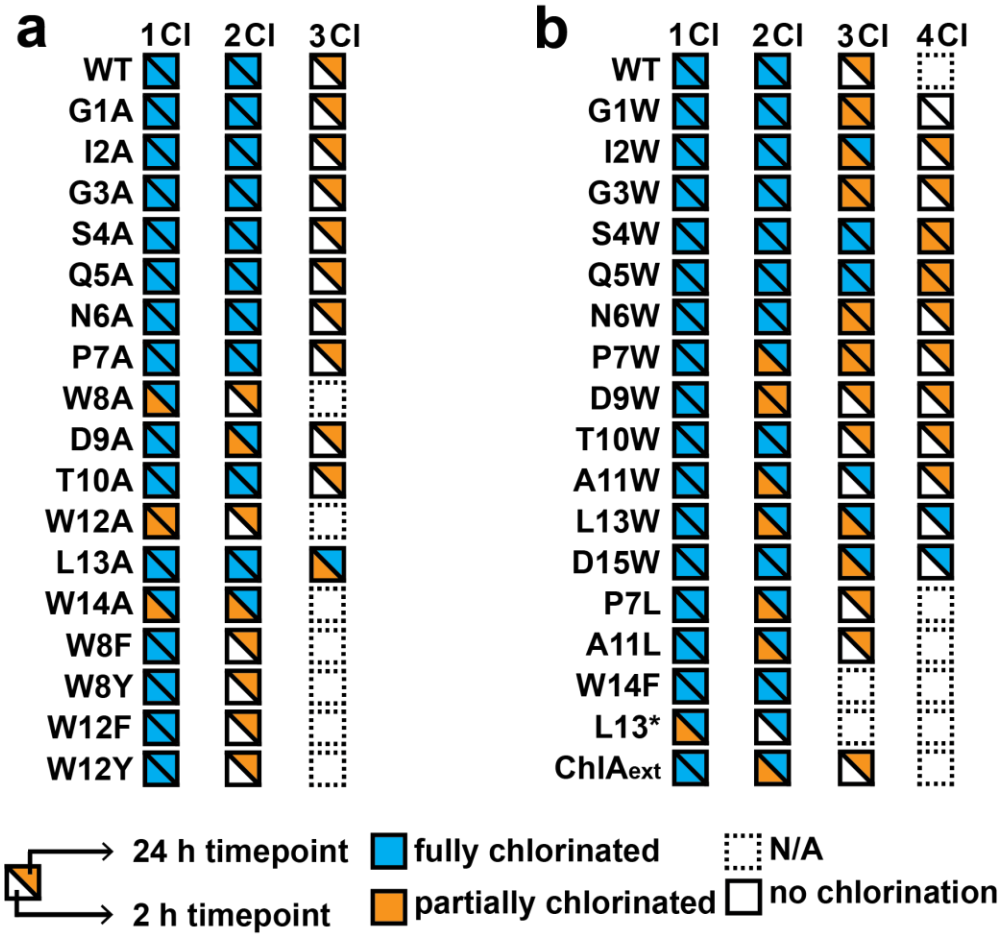


Figure 3. *In vitro* chlorination of ChlA variants. (a) Compiled results from the Ala scan of ChlA and other variants. The lower left half of each square represents chlorination status after 2 h of reaction with ChlH/R. The upper right half represents the same after 24 h. WT = wild-type ChlA. (b) Compiled results from the Trp scan of ChlA with other variants. L13* represents a stop codon at position 13 of ChlA. $ChlA_{ext}$ represents a C-terminally extended variant of ChlA, the sequence of which can be found in Figure S16.

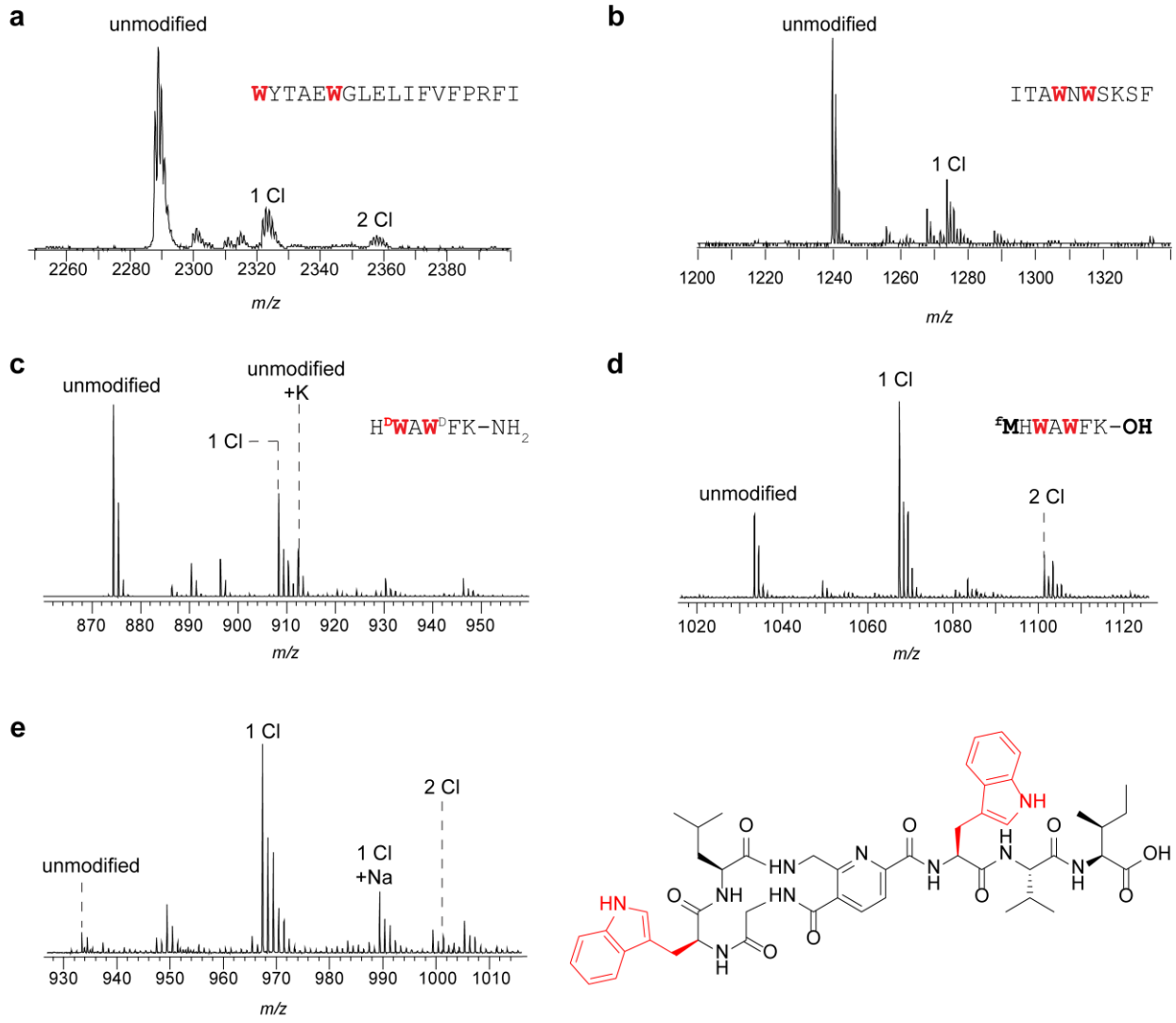


Figure 4. ChlH reactivity towards alternative substrates. (a) MALDI-TOF-MS of $FusA_{core}$ (expected *m/z*: unmodified, 2287; 1 Cl, 2321; 2 Cl, 2355) and (b) DarA-Q8* (shown: GluC fragment, expected *m/z*: unmodified, 1240; 1 Cl, 1274) after treatment with ChlH/R. (c) MALDI-TOF-MS of GHRP-6 (expected *m/z*: unmodified, 873; 1 Cl, 907) and (d) an in vitro-prepared analog (expected *m/z*: unmodified, 1034; 1 Cl, 1067; 2 Cl, 1101) after treatment with ChlH/R. (e) MALDI-TOF-MS of pyritide A1 (expected *m/z*: unmodified, 934; 1 Cl, 967; 2 Cl, 1001). Note *m/z* 1001 overlaps with a 1Cl+32 species, presumably arising from Trp oxidation.

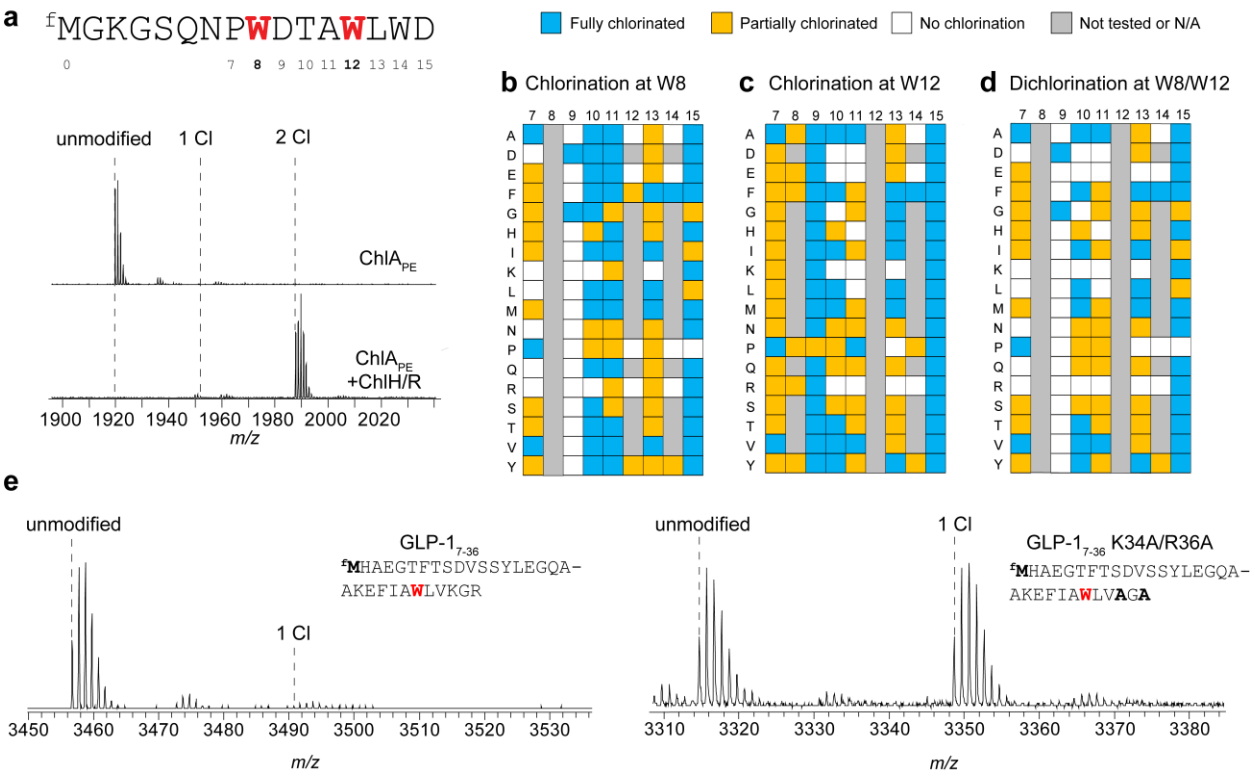


Figure 5. Cell-free mutagenic scanning informs substrate engineering. (a) Sequence and MALDI-TOF-MS of ChlA$_{PE}$ after reaction with ChlH/R. Expected *m/z*: unmodified, 1920; 2 Cl, 1988. (b) Assessment of Trp8 chlorination of the 120-membered mutagenic panel after reaction with ChlH/R. (c) Same as b, except the status of Trp12 chlorination is indicated. (d) Assessment of Trp8 and Trp12 dichlorination. (e) Left spectrum, MALDI-TOF-MS of GLP-1$_{7-36}$ after reaction with ChlH/R. Expected *m/z*: unmodified, 3457; 1 Cl, 3491. Right spectrum, same except with the K34A/R36A variant of GLP-1$_{7-36}$. Expected *m/z*: unmodified, 3315; 1 Cl, 3349.

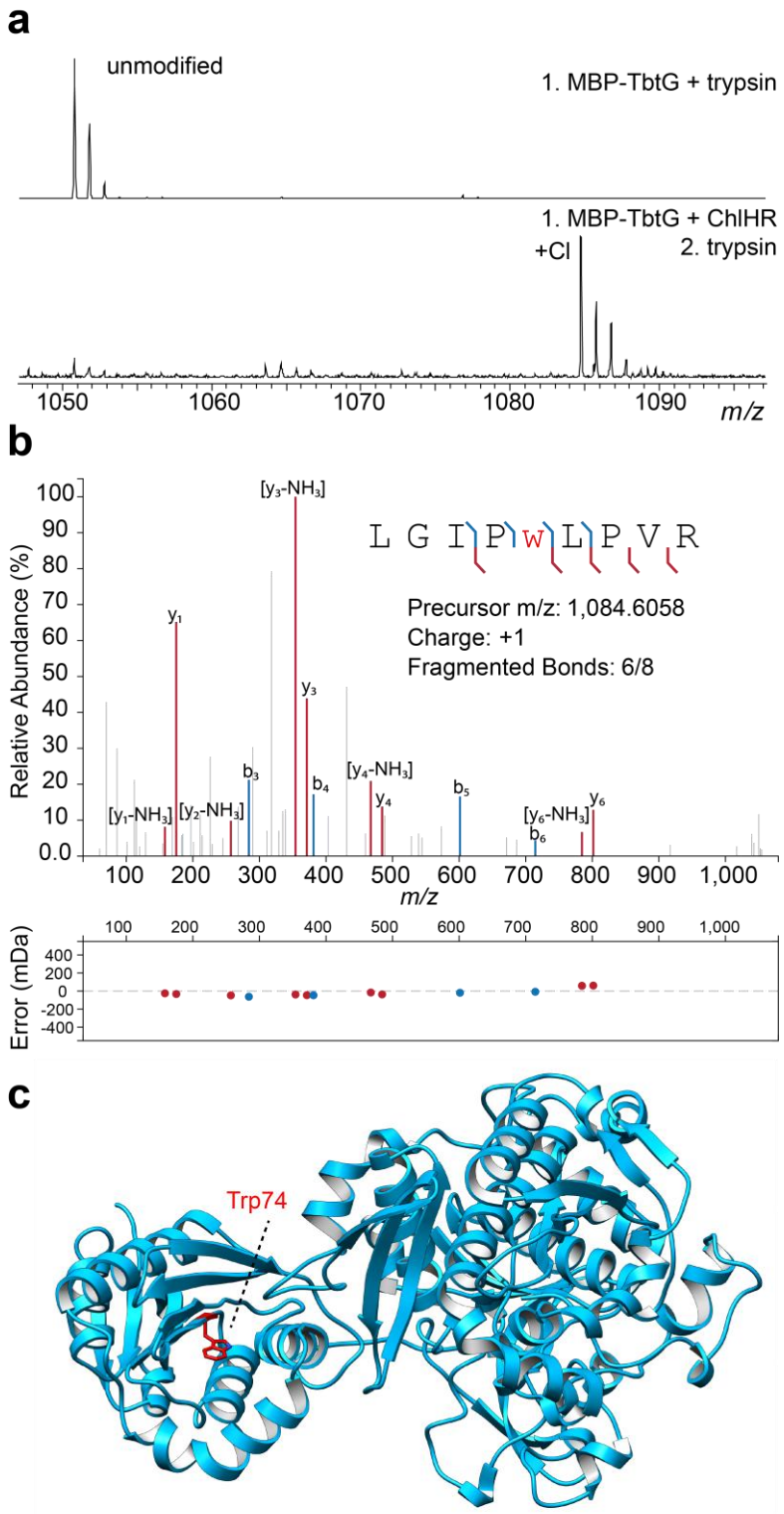
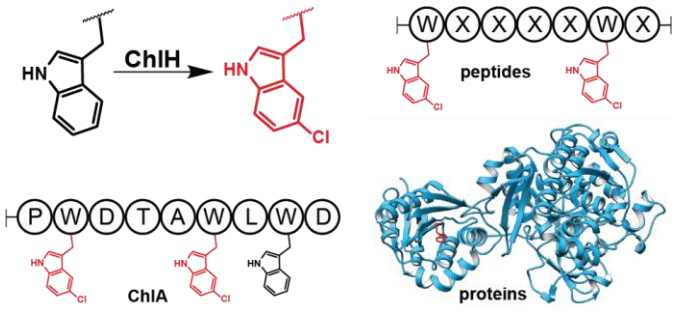


Figure 6. Evaluation of ChlH protein substrate halogenation. (a) MALDI-TOF-MS of MBP-TbtG reacted with ChlH/R with subsequent trypsin digestion. The expected *m/z* values are 1050.6 for the unmodified fragment and 1084.7 for the chlorinated fragment. (b) MALDI-LIFT-MS analysis of the tryptic peptide containing Cl-Trp (lowercase, red w). (c) AlphaFold 3 structural prediction of TbtG (modified Trp is red).



TOC Figure