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A new family of iron-dependent halogenases acts on freestanding substrates

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Regio- and stereospecific incorporation of a halogen atom to an unactivated *sp*³ carbon in a freestanding molecule is a challenging transformation that is currently missing in the inventory of enzyme-mediated reactions. Here we report what is to our knowledge the first example of a nonheme iron enzyme (WelO5) in the welwitindolinone biosynthetic pathway that can monochlorinate an aliphatic carbon in 12-*epi*-fischerindole U and 12-*epi*-hapalindole C, substrates that are free from peptidyl or acyl carrier protein.

Molecules containing carbon-halogen bonds are produced naturally across all kingdoms of life and constitute a large family of natural products with a broad range of biological activities[1]. The presence of halogen substituents in many bioactive compounds has a profound influence on their molecular properties, highlighted by the roles of chlorine substitutions on the conformational stability of vancomycin-type antibiotics, the antitumor activity of salinosporamide and the signaling capacity of differentiation-inducing factor 1 in *Dictyostelium* sp.[2–4]

A number of enzymatic strategies have been disclosed in the past two decades that use three distinct halogen species to incorporate halogen atoms into bioactive natural products[5]. They are the nucleophilic halide anion (X^-), the electrophilic hypohalite (^-OX) species (X^+ equivalent) generated by heme- or vanadium-dependent haloperoxidase or flavin-dependent halogenase and the halogen radical ($X\bullet$) equivalent provided by a halogen-bound high-valent Fe(IV)-oxo species, as in the case of non-heme iron (NHI)-dependent halogenases. Analysis of the timing of enzymatic halogenation for natural product biosynthesis readily reveals that all of the known enzymatic halogenation strategies are primarily used for the assembly of early biosynthetic intermediates from primary metabolites, whereas late-stage enzymatic halogenation on complex molecular scaffolds is restricted to enzymatic transformations involving electrophilic halogen species (X^+) on electron-rich sp^2 carbon centers. These examples include haloperoxidase-mediated cyclization events in the biosynthesis of brominated marine terpenoids and chlorinated prenylated polyketides (PKs) or flavin-dependent halogenase-mediated maturation of aromatic PK and nonribosomal peptide natural products[6–9]. Unlike the wide occurrence of cytochrome P450 enzymes capable of oxidatively tailoring freestanding terpenoids and/or PK scaffolds via enzymatic conversion of an unactivated sp^3 C-H bond to a C-O bond[10], a halogenation enzyme equipped with the same capacity remains to be discovered.

Hapalindole-type alkaloids, including hapalindoles, fischerindoles, ambiguines and welwitindolinones, are a large family of indole monoterpenoids produced by stigonematalean cyanobacteria *Fischerella* sp. and *Hapalosiphon* sp.[11] A key structural feature of hapalindole-type alkaloids is the wide occurrence of a chlorine substituent at the C13 of its indole-monoterpene backbone (**Fig. 1**). The biosynthetic origin of this chlorine atom was originally proposed to involve an enzymatic transformation using a chloronium ion–mediated cationic cascade to fuse two putative biosynthetic precursors, β-ocimene and 3-(2′-isocyanoethenyl) indole, into a tricyclic or tetracyclic framework[12,13]. However, careful analysis of the structural diversity of hapalindole-type alkaloids within each producing strain shows the frequent coexistence of chlorinated and deschlorinated structural pairs (**Fig. 1**). This led us to hypothesize the possible involvement of an unprecedented halogenase for the late-stage activation and conversion of an aliphatic C-H bond to a C-Cl bond in the hapalindole-type molecular scaffold.

This hypothesis correlates well with the genetic information encoded in the ambiguine (*amb*) and welwitindolinone (*wel*) biosynthetic gene clusters, recently disclosed by our group[14,15]. Both the *amb* and *wel* pathway, identified from *Fischerella ambigua* UTEX1903 and *Hapalosiphon welwitschii* UTEX B1830, lack the genes coding for a previously proposed class I terpene synthase required for β-ocimene biosynthesis and the putative halogenase that can provide the electrophilic Cl^+ species. Instead, they contain an identical list of genes for the assembly of the early pathway biosynthetic precursors geranyl pyrophosphate and 3-((*Z*)-2′-isocyanoethenyl) indole and a varied set of genes coding tailoring enzymes for the maturation of the indole monoterpenoid scaffold in hapalindole-type alkaloids[14,15]. Although most of the tailoring enzymes encoded in the *wel* and *amb* pathways are bioinformatically predicted to be NHI-dependent oxygenases[14,15], none are homologous to the previously characterized

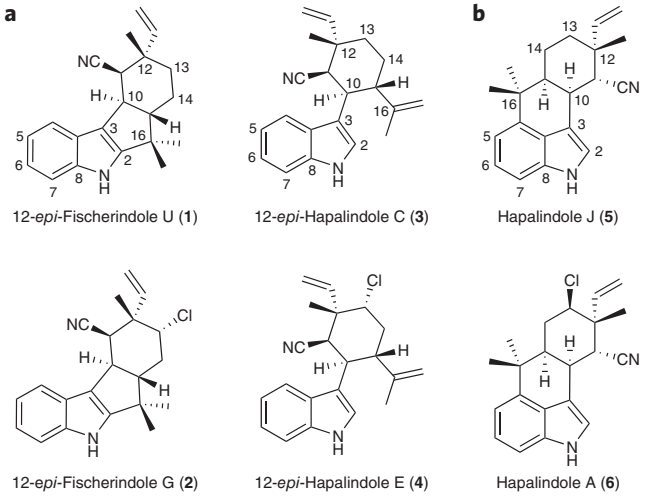

Figure 1 | Selected chlorinated and deschlorinated pairs of hapalindole-type molecules from two distinct stigonematalean cyanobacteria. (**a**) The 12-*epi*-fischerindole U (**1**)–12-*epi*-fischerindole G (**2**) pair and the 12-*epi*-hapalindole C (**3**)–12-*epi*-hapalindole E (**4**) pair from *H. welwitschii* UTEX B1830 and IC-52-3 (ref. 13). (**b**) The hapalindole J (**5**)–hapalindole A (**6**) pair from *F. muscicola* UTEX LB1829 (ref. 12).

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NHI-dependent halogenases that are active only on amino acyl or acyl substrates delivered via a prosthetic phosphopantetheine arm from a carrier protein[16–20].

The *wel* gene cluster from *H. welwitschii* UTEX B1830 contains six genes (*wel*M and *wel*O1–5) encoding tailoring enzymes for welwitindolinone biogenesis[15]. We have previously characterized WelM as a pathway-specific methyltransferase for the generation of *N*-methylwelwitindolinone C from welwitindolinone C and proposed that the sequential oxidative actions of WelO1–5 would be responsible for the maturation of welwitindolinone C from 12-*epi*-fischerindole U (**1**) (**Supplementary Results**, **Supplementary Fig. 1**)[15]. WelO5 shows C-terminal sequence similarity to a family of Fe(II)/α-ketoglutarate (α-KG)–dependent oxygenases and is distinct from WelO1–4, which have a conserved Rieske oxygenase domain (**Supplementary Fig. 2**). As NHI-dependent oxygenases and halogenases share strong mechanistic homologies[21], we proposed that WelO5 would be the halogenase to act on freestanding **1** and stereoselectively chlorinate its C13 to give 12-*epi*-fischerindole G (**2**)[15]. This chlorination event is most likely to be the first committed step of a series of late-stage oxidative maturation events in welwitindolinone biosynthesis. This hypothesis also calls for the promiscuity of WelO5, which needs to act on 12-*epi*-hapalindole C (**3**) to give 12-*epi*-hapalindole E (**4**), both of which are found in minor quantities in the welwitindolinone producer *H. welwitschii* UTEX B1830 (ref. 15).

To validate the postulated roles of WelO5, we isolated its candidate substrates **1** and **3** to homogeneity from *H. welwitschii* UTEX B1830. Recombinant WelO5 with an N-terminal His_7 tag was overexpressed in *Escherichia coli* and readily purified (25 mg l^{-1}) using immobilized metal affinity chromatography. The initial preparation of WelO5 purified under aerobic conditions did not provide active proteins. Upon a series of optimizations (data not shown), we found that apo-WelO5 depleted of divalent metal ions can be rapidly activated aerobically with freshly prepared solutions of $(NH_4)_2Fe(SO_4)_2$, α-KG and NaCl in a nitrogen-purged HEPES buffer. Subsequent incubation of WelO5 (20 μM) with **1** (1 mM) aerobically at 30 °C in the presence of Fe^{2+}, α-KG and NaCl rapidly turned **1** into a new product, as observed by HPLC and LC/MS analysis. Its retention time and high-resolution (HR) mass spectrum matched those of authentic **2** (**Fig. 2a** and **Supplementary Fig. 3**). To further ascertain the identity of the WelO5 product from **1**, the enzymatic reaction was scaled up to 3 ml, and the newly generated compound was isolated. Its ^1H NMR spectrum matched that reported for **2** (**Supplementary Fig. 4**). The enzymatic activity of WelO5 strictly depends on the presence of Fe^{2+} and α-KG in the reaction mixture, as the absence of either component or substitution of Fe^{2+} with other redox-active divalent metals including Co^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+} completely abolished the reactivity of WelO5 toward **1** (**Fig. 2a** and **Supplementary Fig. 5**). A detailed kinetic analysis is complicated by the auto-oxidative inactivation of WelO5 and limited substrate availability. However, at a 20-μM concentration in a 100-μl scale assay, 2 nmol of WelO5 converted up to 150 nmol of **1** to **2**, which translates to ~75 turnovers (**Supplementary Fig. 6**). This number is 5–10 times greater than that for CmaB and SyrB2 (refs. 16,17), two previously characterized NHI-dependent halogenases that act on amino acyl substrates tethered to a peptidyl carrier protein. This greater robustness for multiple turnovers highlights the distinct catalytic ability of WelO5, a unique feature of this new family of NHI-dependent halogenases.

Having established the chlorination capacity of WelO5 on freestanding **1**, we next assessed its activity toward a second potential substrate **3**. Upon mixing WelO5 with **3** under the same assay conditions, the formation of a new product was detected by HPLC (**Fig. 2b**). Subsequent LC/HRMS indicated that the enzymatic product is a monochlorinated derivative of **3** (**Supplementary Fig. 7**). As the authentic **4** was not readily obtainable from *H. welwitschii*

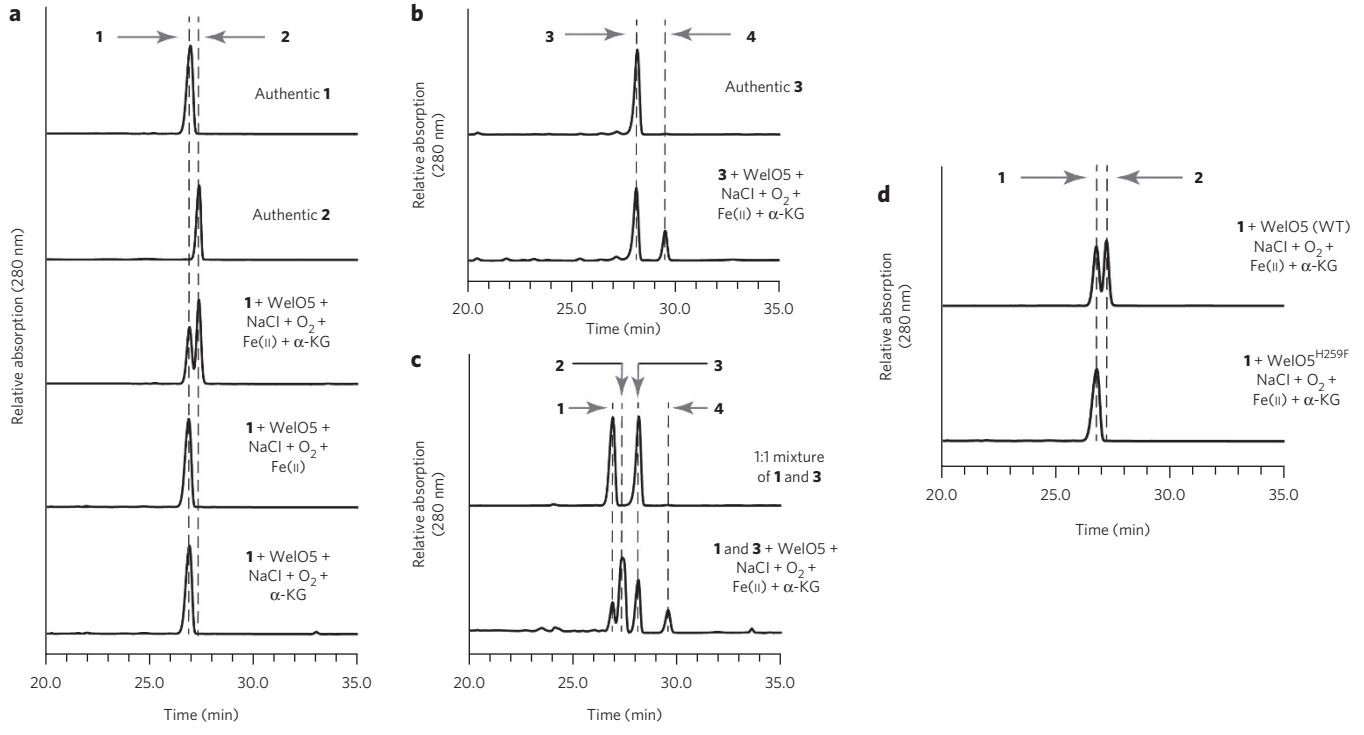

Figure 2 | *In vitro* characterization of WelO5 and its mutant. (**a**–**d**) HPLC chromatographs with a UV detector at 280 nm showing that WelO5 is a Fe(II)/α-KG–dependent halogenase for the formation of **2** from **1** (**a**); WelO5 also takes **3** as a substrate to generate **4** (**b**); **1** is a preferred substrate for WelO5, as confirmed by an *in vitro* competition assay with **2** (**c**); and the mutant $WelO5^{H259F}$ lacks chlorination activity on **1** (**d**). All of the HPLC data shown are *in vitro* assays conducted with WelO5 or its mutant (20 μM) with 1 mM of substrate (or substrates; **1** and/or **3**) for 1 h at 30 °C. Assays were conducted in triplicate, and representative results are shown.

UTEX B1830 owing to its low production yield, the reaction of **3** with WelO5 was scaled up, and the enzymatic product was isolated. Its ^{1}H NMR spectrum is in excellent agreement with that originally reported for **4** (**Supplementary Fig. 8**), thus providing clear evidence that WelO5 is also capable of chlorinating **3** at its C13 site, as observed for **1**.

We next examined the substrate specificity of WelO5. We observed that the rate of conversion of **3** to **4** by WelO5 is less than that for conversion of **1** to **2** (**Fig. 2a**,**b**). To explain this trend, a competition experiment with equimolar **1** and **3** (0.5 mM each) was carried out with WelO5 (20 μM). Subsequent HPLC analysis revealed that the conversion of **1** to **2** under these experimental conditions is approximately eight times greater than conversion of **3** to **4** (**Fig. 2c**), clearly demonstrating that **1** is the preferred substrate for WelO5. These data corroborate our initial hypothesis that the conversion of **1** to **2** by WelO5 constitutes the major pathway to provide the efflux of chlorinated intermediate required for welwitindolinone biogenesis. It also agrees with the observation that the production ratio of **4** to **3** in the *wel* cluster containing *H. welwitschii* UTEX B1830 is considerably less than the ratio of **2** to **1** (**Supplementary Fig. 9**).

Further insight into the role of WelO5 in welwitindolinone biosynthesis was obtained by examining the activity of WelO5 against hapalindole J (**5**), a deschlorinated analog of hapalindole A (**6**). Both **5** and **6** have been isolated from *Fischerella muscicola* UTEX LB1829, which naturally produces a different set of hapalindole-type alkaloids from *H. welwitschii* UTEX B1830 (ref. 12). They also exhibit a different fused 6-6-5-6 tetracyclic framework with stereoconfiguration at C10 and C12 that is opposite from that in **1** and **3**. HPLC and LC/MS analysis of the enzymatic reaction mixture of **5** with WelO5 revealed no detectable formation of **6** after 6-h incubation at 30 °C (**Supplementary Fig. 10**), providing additional evidence that WelO5 is a *wel* pathway–specific halogenase for tailoring **1** and **3** in the context of welwitindolinone biosynthesis.

As the activity of WelO5 strictly depends on the presence of Fe^{2+} and α-KG as cofactor and co-substrate, respectively, the mechanism for its diastereoselective chlorination at C13 of **1** and **3** is likely to be analogous to those of other NHI-dependent halogenases that are active only on thioester substrates tethered to carrier proteins (**Supplementary Fig. 11**)[22,23]. A chlorine-bound high-valent Fe(IV)-oxo species, generated by oxygen-dependent decarboxylation of α-KG in an Fe(II) complex, is most likely the key catalytic intermediate responsible for the diastereotopic abstraction of the *pro*-R hydrogen at C13 of **1** or **3**. The resulting secondary carbon radical would undergo a stereoselective chlorine rebound by accepting Cl• from a Fe(III) complex to furnish **2** or **4**. Close comparison of the WelO5 sequence with other members in the Fe(II)/α-KG–dependent oxygenase superfamily revealed that WelO5 has two histidine residues (H164 and H259) in the characteristic HX(D/E)X_nH motif for iron binding, but, as in the carrier protein–dependent aliphatic halogenases, the carboxylate-containing D/E residue is absent, replaced in this case by a glycine residue (G166) (**Supplementary Fig. 12**). It is therefore plausible that WelO5 adopts two histidines for iron binding and leaves an open coordination site for chloride binding in the resting Fe(II) state, as observed for other NHI-dependent halogenases[24,25]. The critical importance of H259 in WelO5 was confirmed by site-directed mutagenesis. The substitution of the histidine residue by phenylalanine completely abolished the activity of WelO5 toward **1** (**Fig. 2d**).

In summary, by successfully reconstituting the activity of WelO5 *in vitro*, we provided conclusive evidence on a long-standing question regarding the enzymatic origin of chlorine substitution in the biogenesis of hapalindole-type alkaloids. WelO5 is a Fe(II)/α-KG–dependent halogenase that performs the first committed oxidative maturation transformation in welwitindolinone biosynthesis by stereospecifically replacing a *pro*-R hydrogen at the C13 of hapalindole-type molecules **1** and **3** with a chlorine. WelO5 is, to our knowledge, the first example of an enzyme with the capacity to mediate this reaction type on a freestanding small molecule. The pathway-specific nature of WelO5 also implies that other individual hapalindole-type alkaloid producers will most likely encode an analogous halogenase responsible for a dedicated chlorination event in each pathway, such as AmbO5 in ambiguine biosynthesis[14]. We expect that this discovery, followed by further structural and biochemical studies of WelO5 and related halogenases, will present new opportunities to evolve new catalysts for selective late-stage halogenations on unactivated carbons in complex molecular scaffolds.

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Methods

Methods and any associated references are available in the online version of the paper.

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Author contributions

M.L.H. and X.L. designed the study. M.L.H. performed all experiments. M.L.H. and X.L. analyzed the data. X.L. wrote the manuscript with input from M.L.H.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to X.L.

ONLINE METHODS

General methods. All PCRs were carried out on a C1000 thermal cycler (Bio-Rad). DNA sequencing was performed by Elim BioPharm Inc. Preparative-scale reverse-phase HPLC was performed using a Dionex instrument equipped with a 21 × 250 mm Luna C18 column (Phenomenex). Analytical reverse-phase HPLC was performed using a Dionex UHPLC with a photo-diode array UV/Vis detector (Thermo Fisher Scientific) and a 4.6 × 250 mm Luna C18 column (Phenomenex). HRMS analysis was conducted using a Q Exactive Benchtop Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a Dionex RSLC (Thermo Fisher Scientific). The NMR spectrum was recorded on a Bruker Avance III 700 MHz spectrometer equipped with a $^{1}H/^{13}C/^{15}N$ triple-resonance inverse probe (1.7 mm 'microprobe').

Materials. Synthetic oligonucleotides for gene amplification by PCR were purchased from Life Technologies or Integrated DNA Technology. Kappa HiFi DNA polymerase was obtained from Kappa Biosystems. Restriction endonucleases, T4 DNA ligase and Antarctic phosphatase were purchased from New England BioLabs. LB broth and agar used for culturing *E. coli* were obtained from Teknova. All other reagents, including inorganic salts and cofactors, were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise stated.

Strains and plasmids. *H. welwitschii* UTEX B1830 and *F. muscicola* UTEX LB1829 were obtained from the UTEX Culture Collection of Algae, the University of Texas at Austin. *E. coli* TOP10 cell (Life Technologies) was used for routine cloning and plasmid propagation. *E. coli* C43(DE3) cell (Lucigen) was used for protein expression. pQTEV cloning plasmid was obtained from Addgene.

Gene cloning. The 873-bp *wel*O5 gene was PCR amplified from *H. welwitschii* UTEX B1830 genomic DNA, using the following primers: forward, GATCGGATCCATGTCCAATAACACCGTCTCTAC; reverse, GATC GCGGCCGCTTAACTCCAGTAATAAATCTTA. After digestion with BamHI and NotI, the *wel*O5 gene was ligated into digested and dephosphorylated pQTEV vector (BamHI and NotI sites) using T4 ligase. The ligation reaction was drop-dialyzed against water and transformed to electrocompetent Top10 *E. coli* cell. Colonies were selected on ampicillin-containing (100 μg/ml) LB agar plates. Plasmid DNA from selected clones were digestion-verified with BamHI and NotI restriction enzymes for correct insert. Selected plasmid with the target insert was further verified by Sanger sequencing.

Bioinformatic analysis of WelO5. To identify protein homologs of WelO5, protein BLAST was performed using BLAST-P online at NCBI with default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome/). Homologs with high similarity (query cover > 85%, identity > 30%, and positivity > 50%) were summarized in **Supplementary Figure 2a**. Comparative conserved domain analysis of WelO5 with other known NHI-dependent halogenases (SyrB2 and CurA_Hal) was performed using CD-search provided online at NCBI with default parameters (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and summarized in **Supplementary Figure 2b**. Further homology search of WelO5 using HHpred (http://toolkit.tuebingen.mpg.de/hhpred/), an open-source online program for protein homology detection and structure prediction, was performed using the default parameters and allowed for the identification of the putative HX(D/E)XnH motif for iron binding (**Supplementary Fig. 12**).

WelO5 protein expression. Positive pQTEV∷*wel*O5 plasmid was transformed to electrocompetent C43 (DE3) *E. coli* cells. Individual colonies were picked and inoculated to a 5-ml culture overnight, which was used to inoculate a 2.8-l Fernbach flask containing 1 l of LB medium with 100 μg/ml ampicillin. The cultures were grown at 37 °C to an optical density of 0.6, and then the temperature was dropped to 16 °C, and isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM) was added to induce protein expression. After 16 h, the cells were centrifuged for 20 min at 8,800*g*. Cell pellets were resuspended in 10 ml of protein lysis buffer (50 mM Tris, pH = 7.4, 500 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol (β-ME) and 0.1% Tween-20) and sonicated for 10-s intervals for 3 min on ice and then was centrifuged for 30 min at 20,000*g* at 4 °C to pellet cell debris. The cleared lysate was transferred to 2 ml of pre-washed Ni-NTA bead (Qiagen) slurry and incubated for 1 h at 4 °C. Thereafter, the Ni-NTA beads were loaded on to a column and washed with 60 ml of binding buffer (50 mM Tris, pH = 7.4, 500 mM NaCl, 0.1% Tween-20, 20 mM imidazole, 10 mM β-ME). The His-tagged protein was then eluted by addition of 5 ml of elution buffer (50 mM Tris, pH = 7.4, 500 mM NaCl, 100 mM and then 250 mM imidazole, 10 mM β-ME) to the bead bed. The eluted protein was subjected to two rounds of dialysis using a 14-kDa molecular weight cutoff membrane (Spectrum Laboratory Products). First, a buffer containing 50 mM HEPES, pH = 7.4, 10 mM EDTA, 10 mM NaCl, 10% glycerol, 0.5 mM DTT was used. Second, a buffer containing 50 mM HEPES, pH = 7.4, 10 mM NaCl, 10% glycerol, 0.5 mM DTT to remove EDTA was used. The purified protein was analyzed by SDS-PAGE to ensure its homogeneity (**Supplementary Fig. 13**), concentrated and then flash-frozen using liquid nitrogen and stored at −80 °C for activity assays. The WelO5 protein concentration was determined using a NanoDrop spectrometer (Thermo Fisher Scientific) at 280 nm using the estimated extinction coefficient of 33,350 M^{-1} cm^{-1}.

Isolation of fischerindole and hapalindole authentic standards. Deschlorinated substrates (**1**, **3**, **5**) used for testing the activity of WelO5 as well as their chlorinated counterpart molecules (**2**, **6**), which were used as HPLC standards, were isolated from *H. welwitschii* B1830 and *F. muscicola* LB1829, respectively. In brief, *H. welwitschii* B1830 and *F. muscicola* LB1829 were routinely cultured at a 20-l scale at room temperature in BG-11 medium with a 12 h–12 h light-dark cycle. After ~5 weeks, the algal cells were harvested by filtration and lyophilized to give 5–6 g of freeze-dried tissue, which was further extracted with CH_2Cl_2/MeOH (v/v = 1:1) overnight with stirring. The crude extract was dried under reduced pressure, and the methanol soluble portion was filtered and injected on a Phenomenex Luna C18 21.5 × 20 mm reverse phase preparative HPLC column for initial separation of hapalindole-related compounds using a gradient of 50–100% methanol over 100 min. For further purification of targeted molecules, a gradient of 50–80% acetonitrile over 35 min was used for the mobile phase separation on a C18 Luna Phenomenex 250 × 4.6 mm column and detected by UV absorbance at a wavelength of 280 nm. This procedure typically results in 0.2-1 mg of targeted authentic standards (**1**–**3**, **5** and **6**) from a 20-l cyanobacterial culture. 12-*epi*-Hapalindole E (**4**), the C13 chlorinated version of **3**, was not isolatable from *H. welwitschii* B1830 (5 g lyophilized tissue) owing to its low production yield. The authenticity of each standard was ensured by ^{1}H NMR analysis in comparison with literature data[13,26]. For 12-*epi*-fischerindole U (**1**), see **Supplementary Figure 14**. For 12-*epi*-fischerindole G (**2**), ^{1}H-NMR data is in accordance with that shown in **Supplementary Figure 4**. For 12-*epi*-hapalindole C (**3**), see **Supplementary Figure 15**. For hapalindole J (**5**), see **Supplementary Figure 16**. For hapalindole A (**6**), ^{1}H-NMR data are as reported in our previous work[14].

WelO5 *in vitro* assay. Individual assays of WelO5 with a single or mixed substrate (or substrates) (**1** and/or **3** or **5**) was carried out at a 100-μl scale, containing WelO5 (20 μM), selected substrate (or substrates; total concentration 1 mM), α–KG (2 mM), NaCl (10 mM), HEPES (20 mM) and $(NH_4)_2Fe(SO_4)_2$ (500 μM). In a typical procedure, all of the components except the metal salt solution and WelO5 are mixed under ambient conditions and then purged with nitrogen gas. To this mixture was added the WelO5 protein and a freshly prepared Fe(II) salt solution that was prepurged with nitrogen. The resulted solution was incubated briefly (~10 min) under a nitrogen atmosphere before being purged with air and incubated aerobically at 30 °C for 1 h. The assay was stopped by extracting with ethyl acetate and centrifuged. Ethyl acetate extracts were combined, dried under a stream of nitrogen gas and redissolved in methanol (50 μl). A portion of the methanol solution was injected in to a Dionex HPLC over a C18 Luna Phenomenex 250 × 4.6 mm column. Analysis was conducted using a mobile phase gradient of 50–80% acetonitrile in water over 35 min and was monitored by UV absorption at 280 nm. For accurate mass determination of WelO5–derived enzymatic product, the same methanol extract was injected to a Dionex RSLC paired with a Thermo Scientific Q Exactive Benchtop Quadrupole-Orbitrap mass spectrometer. The conversion of **1** to **2** by WelO5 was used as a standard reaction to assess the influence of pH, cofactors including α-KG and metal ions (Fe^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+}) to WelO5 activity and stability. To estimate the maximal turnover number of WelO5 at 20 μM, a series of 100-μl scale assays with **1** (1.5 mM and 2.0 mM) were conducted, and the reaction was analyzed by HPLC at four different time points (1 h, 3 h, 6 h and 24 h). All analytical-scale assays (100 μl) with WelO5 were performed in triplicate, and representative results are shown.

WelO5 enzymatic product structural determination. To determine the structure of enzymatic products derived from WelO5-mediated chlorination of **1**

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and **3**, the reactions were scaled proportionally to 3 ml from a 100-µl scale reaction described above and incubated at 30 °C for 4 h. The reaction mixture was extracted with ethyl acetate (2 ml × 3), and combined ethyl acetate extracts were dried under nitrogen gas. The crude material was dissolved in methanol and purified by a Dionex HPLC equipped with a C18 Luna Phenomenex 250 × 4.6 mm column. The target peaks were collected and dried. The ^{1}H NMR of isolated materials were analyzed on a Bruker Avance III 700 MHz spectrometer using capillary NMR tubes.

Site-directed mutagenesis. The single amino acid mutant (H259F) of WelO5 was generated according to the protocol described in the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) using plasmid pQTev::*wel*O5 as the template and the following primers: forward, AATGGTGGTCGATACTACTTTCGAGTTAGTGAAGTAATT; reverse, AATTACTTCA CTAACTCGAAAGTAGTATCGACCACCATT. The correct mutant plasmids were verified by Sanger sequencing. The mutant protein was overproduced and purified in the same manner as that described for the wild-type WelO5. Its homogeneity was assessed by SDS-PAGE analysis (**Supplementary Fig. 13**), and its activity was assayed using **1** as a substrate, as described for wild-type WelO5.

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