Mycotoxin Biosynthesis

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Biosynthesis of the Halogenated Mycotoxin Aspirochlorine in Koji Mold Involves a Cryptic Amino Acid Conversion**

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Abstract: *Aspirochlorine (**1**) is an epidithiodiketopiperazine (ETP) toxin produced from koji mold (Aspergillus oryzae), which has been used in the oriental cuisine for over two millennia. Considering its potential risk for food safety, we have elucidated the molecular basis of aspirochlorine biosynthesis. By a combination of genetic and chemical analyses we found the acl gene locus and identified the key role of AclH as a chlorinase. Stable isotope labeling, biotransformation, and mutational experiments, analysis of intermediates and an in vitro adenylation domain assay gave totally unexpected insights into the acl pathway: Instead of one Phe and one Gly, two Phe units are assembled by an iterative non-ribosomal peptide synthetase (NRPS, AclP), followed by halogenation and an unprecedented Phe to Gly amino acid conversion. Biological assays showed that both amino acid transformations are required to confer cytotoxicity and antifungal activity to the mycotoxin.*

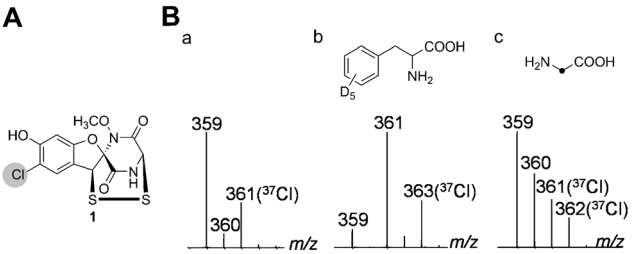

Figure 1. A) Structure of aspirochlorine (**1**) from the koji mold *Aspergillus oryzae*. B) Mass spectra of **1**. a) Natural isotope pattern showing ^{37}Cl signal; b) after incorporation of ring-D_5-labeled Phe (only two D are found in the final product due to further functionalization of the molecule); c) after incorporation of ^{13}C-labeled Gly (one ^{13}C incorporated).

Over two millennia ago the rites of the Zhou dynasty in China described the use of a fungus (*qu*) for food fermentation and brewing. This very fungus, nowadays known as the koji mold or *Aspergillus oryzae*, has led to the development of famous delicacies such as soy sauce (shoyu), miso, and sake, among many others. As in all microbial food fermentations, care must be taken to avoid the contamination with toxins, which are either produced by the fungus or by associated bacteria.[1] Considering its substantial use in Asian countries it is remarkable that *A. oryzae* is known to produce a variety of potentially harmful secondary metabolites including aspirochlorine (**1**, Figure 1).[2] Aspirochlorine (or antibiotic A30641) is an unusual halogenated spiro compound[3] with distinctive antifungal properties due to selective inhibition of protein biosynthesis.[4] In addition, the halogenated compound was shown to be active against bacteria, viruses, and murine tumor cells,[2a,4a] and even an immunosuppressant activity was reported.[5] Notably, **1** not only represents the major active constituent of the non-pathogenic *A. oryzae* but also of the taxonomically closely related human pathogen *Aspergillus flavus*.[3a,4b] Moreover, **1** belongs to the family of epidithiodiketopiperazine (ETP) toxins,[6] sharing the hallmark of a disulfide-bridged diketopiperazine (DKP). The prototypical ETP, gliotoxin, is a virulence factor of the human pathogenic fungus *Aspergillus fumigatus*.[7] It was shown that this toxin's epidithio bridge is crucial for inactivating proteins by thiol conjugation. In addition, sulfur-mediated redox cycling can generate reactive oxygen species (ROS), which usually lead to severe cell damage.[8] Thus, the capability of *A. oryzae* to produce toxic compounds such as aspirochlorine questions the safety of a large variety of traditional fermented Asian foods.[9] In light of the huge importance of koji in food biotechnology, it is surprising that the molecular basis of the *A. oryzae* secondary metabolism is underexplored and that nothing is known about the biosynthesis of **1**. Here we report the first insight into the aspirochlorine pathway in *A. oryzae* and show by a combination of genetic, biochemical, and chemical techniques that besides chlorination an unprecedented amino acid conversion takes place after assembly of the DKP core.

The structure of **1** suggests that the ETP is assembled from phenylalanine and glycine. To verify this, we performed MS-based stable isotope labeling experiments using ^{13}C-labeled Gly and ring-D_5-labeled Phe. Indeed, we detected the incorporation of both amino acid-derived labels into

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1 (Figure 1). After formation of the DKP, sulfur would be incorporated into the DKP by conjugation with glutathione and its stepwise degradation in analogy to the classical gliotoxin ETP pathway.[10] Thus, for the discovery and functional investigation of the aspirochlorine (*acl*) biosynthesis genes, we initially screened the sequenced *A. oryzae* RIB40 genome[11] for characteristic ETP biosynthesis genes. We discovered a 42 kb gene locus harboring a non-ribosomal peptide synthetase (NRPS) gene (*aclP*) for the formation of the diketopiperazine core and a set of genes required for introducing the epidithio bridge. Specifically, enzymes for the formation of a glutathione adduct (AclG), its stepwise degradation to the thiol (AclIJK) and dithiol oxidation (AclT) were found encoded in the *acl* gene locus. In addition, we identified several genes for putative tailoring enzymes such as oxygenases (*aclBCLO*) and *O*-methyltransferases (*aclMU*). Furthermore, the presence of a putative flavin-dependent halogenase gene (*aclH*) in the *acl* gene locus suggested that this gene cluster could code for the biosynthesis of the halogenated ETP. Furthermore, genome database searches revealed that *acl*-like gene clusters are spread among several fungal orders, involving the aspirochlorine producer *A. flavus* and the dermatophytes *Trichophyton tonsurans* and *Trichophyton equinum* (Figure 2 A and Figure S4 in the Supporting Information, SI). To verify the identity of the tentative *acl* biosynthesis gene cluster and to investigate the function of the putative halogenase gene, we constructed an *aclH* deletion mutant of the *A. oryzae* RIB40 strain. Using a double crossover strategy, the target gene was successfully replaced by a pyrithiamine resistance gene cassette (Figure S1). HPLC-HRMS monitoring of the Δ*aclH* mutant culture showed that aspirochlorine biosynthesis was completely abolished (Figure 2 B). Complementation by ectopic integration of *aclH* in the Δ*aclH* mutant, however, restored aspirochlorine production (Figure S2). In contrast, no production of **1** was detected after addition of 3-chloro-L-tyrosine and 3-chloro-DL-phenylalanine to the *A. oryzae* Δ*aclH* mutant culture (SI), indicating that the amino acid is halogenated after formation of the peptide core of aspirochlorine. Consistent with this model, we noticed that the Δ*aclH* mutant culture accumulated a metabolite (**2**) with *m*/*z* 324 ($[M-H]^-$), which matches the expected mass of an aspirochlorine precursor lacking the chlorine atom. Indeed, the expected molecular formula of $C_{12}H_9N_2O_5S_2$ was deduced from HR-MS data. To confirm its structure, we isolated pure dechloroaspirochlorine (**2**, 880 μg) from an 80 L culture using preparative HPLC. ^{13}C NMR and DEPT135 spectra of **2** were

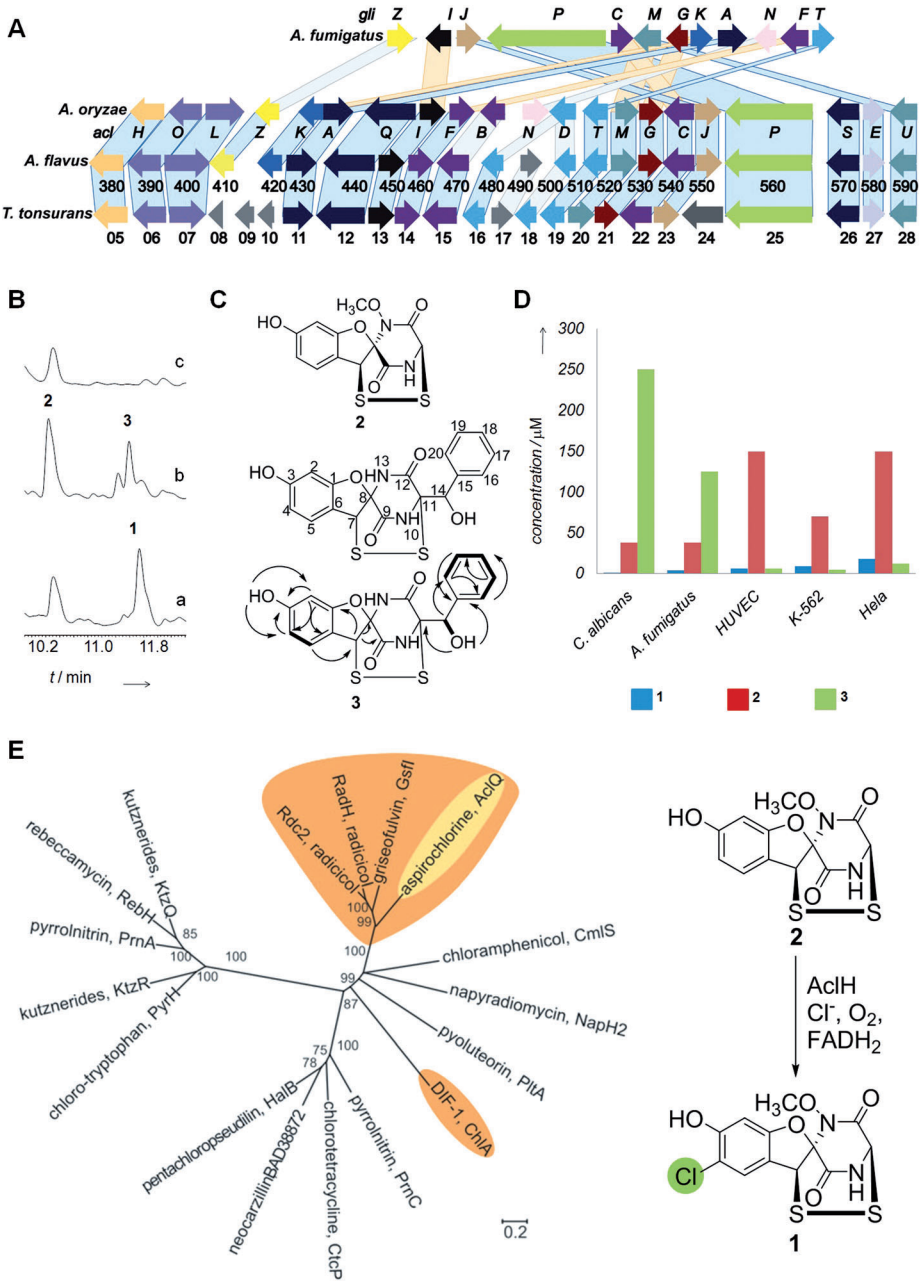


Figure 2. A) Comparison of the gliotoxin biosynthesis (*gli*) gene cluster and verified and putative aspirochlorine (*acl*) biosynthesis gene clusters. B) HPLC profiles of *A. oryzae* wild type (a), Δ*aclH* mutant (b), and Δ*aclP* mutant (c). C) Structures of dechloroaspirochlorine (**2**) and diaryl congener **3** and key HMBC and COSY correlations. D) Antifungal, cytotoxic, and antiproliferative effects of **1**–**3** in cell-based assays. E) Phylogenetic analysis of AclH and related enzymes.

almost identical with those of **1**, except for the presence of a methine carbon signal at 109.3 ppm in lieu of the halogenated quaternary carbon signal. The absolute configuration of **2** was found to be identical with **1** by comparison of CD spectra (SI). To corroborate the timing of chlorination, we added dechloroaspirochlorine to a mutant lacking the NRPS gene (see below), and HPLC-HRMS analysis (see SI) revealed that aspirochlorine production was restored. Thus, chlorination is the last step in the aspirochlorine pathway. These experiments not only confirmed that the *acl* gene locus codes for aspirochlorine biosynthesis but also revealed that AclH is a chlorinase. It should be highlighted that besides radicicol[12] and griseofulvin[13] biosynthesis, this is only the third fungal pathway involving a characterized halogenase, and AclH represents the first fungal chlorinase that modifies an amino acid.

To investigate the impact of the chloride substituent on the biological activity, we compared **1** with **2** in various assays. The dechloro variant proved to be substantially less active against *Candida albicans* and *Aspergillus fumigatus* than aspirochlorine. Furthermore, the lack of the chlorine resulted in radically reduced antiproliferative and cytotoxic activities (Figure 2D). Consequently, the presence of chlorine contributes significantly to the biological activity of aspirochlorine.

In this respect it is interesting to note that bioactive aspirochlorine derivatives are also produced under conditions applied in food industry, for example, in sake production (see SI, Figure S1) which may represent a potential risk for food safety.

In the search for additional pathway intermediates in the block mutant we made an unexpected discovery. By HPLC-MS we detected small amounts of another aspirochlorine derivative (**3**) with *m/z* 401 ($[M-H]^-$; Figure 2C and SI) and a molecular formula of $C_{18}H_{13}N_2O_5S_2$, as deduced from HRESI-MS data. HPLC-MS also revealed traces of **3** in the wild-type culture, but its peak was covered by the dominant aspirochlorine peak. To fully elucidate its structure we isolated **3** from a 100 L culture of Δ*aclH* by silica gel chromatography, repeated size exclusion chromatography, and preparative HPLC, eventually yielding 1.2 mg of pure compound. The 1D-NMR and 2D-NMR spectra were similar to those of **1** and **2** indicating the presence of a DKP moiety. However, **3** differs from its congeners by an additional phenyl substituent and the absence of the methoxy group. The phenyl substitution was verified by marked HMBC couplings of the aromatic ring protons to the adjacent C–H carbon (C14). Moreover, H,H-COSY analyses indicated that 14-OH is adjacent to H14, and this was perfectly supported by HMBC couplings of 14-OH to C11, C14, and C15 (SI, Table S2).

The finding of compound **3** is most surprising because its structure is derived from a DKP composed of two Phe moieties. To prove that the diketopiperazines **2** and **3** are produced by the same NRPS (AclP) we engineered a Δ*aclP* mutant by double crossover replacing *aclP* with a pyrithiamine resistance cassette (Figure S2). HPLC-MS monitoring of the Δ*aclP* mutant culture showed that the biosynthesis of both **2** and **3** was completely abrogated (Figure 2B and SI, Figure S2). In this context it should be noted that the activation of two different amino acids generally requires the presence of two A domains with differing amino acid specificity. For example, the NRPS (GliP) involved in the gliotoxin pathway consists of two full NRPS modules with A domains, A_1 and A_2, that activate phenylalanine and serine, respectively (Figure 3A). The structures of aspirochlorine and its congeners suggest that, analogously to GliP, AclP would assemble a Phe (or Tyr) and a Gly residue.

To gain insight into the specificity of the NRPS we first constructed a phylogenetic tree on the basis of A domain sequences from AclP and other characterized ETP synthetases, NRPSs, and NRPS–PKS hybrids. ETP synthetases (clade II) are clearly distinct from other enzymes (Figure 3A and SI). Notably, the AclP A domain is most closely related to the AtaP A domain, and both share closest phylogenetic relationship to the serine-specific domains of SirP and GliP (marked in dark purple). We also found that AclP possesses only a single A domain with a predicted specificity for Phe. It shares an identical nonribosomal code (DGHIYVMCGK) with the AtaP NRPS from *A. terreus*[14] (Figure 3A), which fuses two Phe units. Nonetheless, one may conceive that AclP has a degree of flexibility in DKP assembly, utilizing both Phe and Gly.

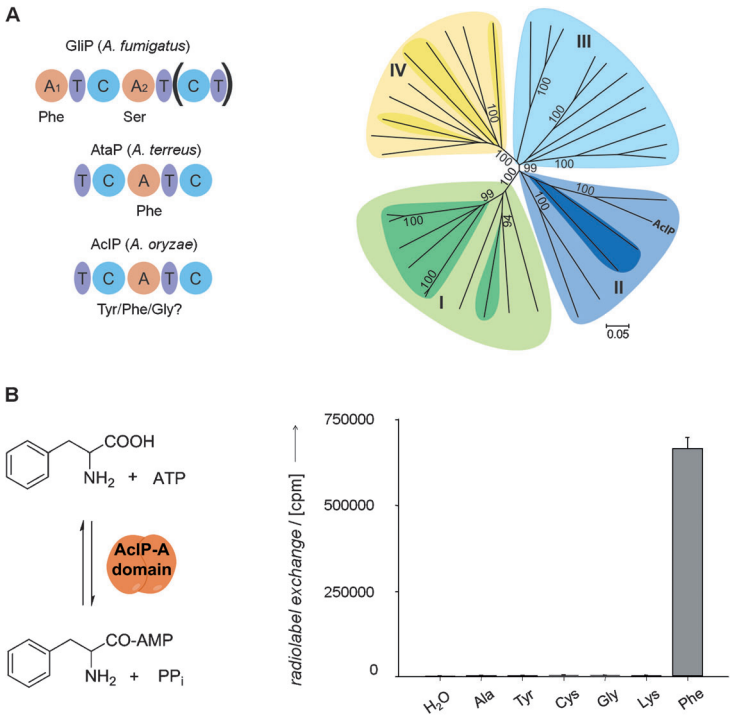
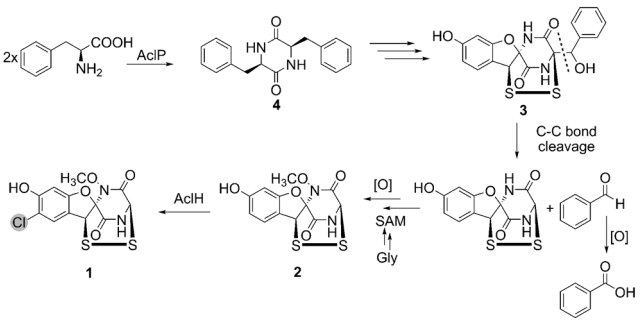


Figure 3. A) Architectures of the NRPSs involved in the biosynthesis of gliotoxin, aspirochlorine, and acetylaranotin in *Aspergillus spp.* and phylogenetic tree (neighbor joining algorithm) of characterized A domains from ETP synthetases and selected other NRPSs. NRPS–PKS hybrids, II: ETP synthetases, III: siderophore (all prefer aliphatic amino acid), IV: Other NRPS products (I, II, IV marked in dark colors prefer aliphatic amino acids). B) Substrate preference of the AclP A domain in the aspirochlorine gene cluster determined in vitro.

To unequivocally determine the substrate specificity of the single A domain of AclP we performed an in vitro ATP-[^{32}P] pyrophosphate exchange assay.[15] Therefore, the synthetic A domain-encoding portion of *aclP* was cloned into an expression vector, and the functional domain was heterologously produced in *E. coli*. Using a selection of representative amino acids, the assay showed a pronounced substrate preference for L-phenylalanine. In stark contrast, glycine incorporation was negligible (Figure 3B).

To exclude the possibility that the A domain can select both Phe and Gly in vivo we performed an additional stable isotope labeling experiment with U-$^{13}C_2$-^{15}N-Gly. Notably, HPLC-HRMS analysis of the culture broth showed the incorporation of only one ^{13}C carbon label in the DKP core of **1** and **2** (Figure S4, SI). To elucidate the exact position of the ^{13}C carbon label we isolated ^{13}C-**1** from an up-scaled culture supplemented with 1,2-$^{13}C_2$-Gly. The ^{13}C NMR spectrum of ^{13}C-labeled **1** showed that the carbon resonating at 65.8 ppm (OCH_3) is enriched in ^{13}C (Figure S6, SI). In contrast, the Gly residue was not labeled, which is in perfect agreement with the lack of Gly activation observed in the A domain assay. The finding of the ^{13}C label in the methoxy group could be rationalized by the glycine cleavage system.[16] In this major amino acid catabolic pathway, Gly is degraded into CO_2 and NH_4^+, whereas the C-2 is loaded onto tetrahydrofolate to yield N^5,N^{10}-methylenetetrahydrofolate, the C1 donor in methionine biosynthesis.[17] Met-derived adenosylmethionine (SAM) represents the most plausible methyl donor for the enzymatic *O*-methylation in the aspirochlorine pathway (Figure S7, SI).[18]

The in vitro assay strongly suggested that AclP fuses two Phe units, one of which is later converted into Gly. To unequivocally prove this scenario in vivo, we added 1-^{13}C-L-Phe to cultures of wild-type *A. oryzae* and the Δ*aclH* mutant. Indeed, by HPLC-HRMS we observed incorporation of two ^{13}C-enriched carbons, not only for the DKP core of **3**, but also in **1** and **2** (Figure 3E). Consequently, the presumed glycine unit in the DKP core of aspirochlorine actually results from an unprecedented C–C bond cleavage of a phenylalanine moiety after DKP assembly. This unusual reaction has only been described in primary metabolic pathways.[19] To clarify the fate of the lacking benzyl unit we administered the ^{13}C-labeled compound **3** (obtained from the 1-^{13}C-L-Phe feeding experiment) to the Δ*aclP* mutant culture. By LC-HRMS monitoring we found that ^{13}C-aspirochlorine is formed, clearly showing that **3** is a precursor of **1** (Figure S5A). In addition, we supplemented D_5-labeled Phe to the wild-type *A. oryzae* culture, and by HPLC-HRMS analysis we detected the incorporation of D atoms into the phenyl ring of **1** and **2** and into both phenyl rings of **3** (Figure S5B). Moreover, by HPLC-HRMS analysis of the culture extract and comparison with a benzoic acid reference, we detected a peak corresponding to D_5-benzoic acid. HRESI-MS data revealed a formula of C_7D_5O with *m*/*z* 126.0607 ([M−H]$^-$, calcd. 126.0609; Figure S5C). The finding of the cleavage products is strongly suggestive of an unusual oxidative C–C cleavage reaction, which could be mediated by one or more oxygenases encoded in the *acl* gene locus.



Scheme 1. Model for aspirochlorine biosynthesis via **3** and **2**, involving an unusual Phe to Gly conversion.

Taken together, in the *acl* pathway phenylalanine enters two different reaction corridors after formation of the DKP (**4**, Scheme 1): one Phe residue is tailored into chlorotyrosine by hydroxylation and chlorination, whereas the second Phe undergoes a fully unexpected conversion into glycine. We have provided numerous lines of evidence for this hidden—or cryptic—post-NRPS amino acid transformation. Although amino acid residues of non-ribosomal peptides are often modified, for example, by oxygenation, aryl coupling or glycosylation,[20] to the best of our knowledge the formal conversion of two proteinogenic amino acids in an NRPS-derived peptide is unprecedented.

In conclusion, we have elucidated the molecular basis for the biosynthesis of the mycotoxin aspirochlorine in *A. oryzae*, which is of utmost importance for the safety of Asian food biotechnology. We report the formation of this toxin under sake producing conditions. Moreover, by genome mining we also identified identical gene loci in the genomes of the aspirochlorine-producing human pathogen *A. flavus*, in the human pathogenic dermatophyte *T. tonsurans* and the horse pathogen *T. equinum*. In addition to providing a genetic marker to identify toxin producers, the finding that the pathogenic *Trichophyton* species are genetically equipped to produce the toxin sheds light on the underinvestigated metabolic potential of pathogens and has implications for infection biology. Our study also revealed several biosynthetic novelties. We identified the first fungal halogenase that modifies an amino acid, and found that chlorination has a massive impact on the biological activity of the toxin. The most unexpected finding was that the aspirochlorine peptide core is assembled from two Phe units. One Phe undergoes chlorination and oxygenation, whereas the other Phe moiety is cleaved to yield a Gly unit, which remains in the *acl* backbone, and benzoic acid. Notably, this unusual dephenylation reaction is a prerequisite for the strong antifungal activity of aspirochlorine. The discovery of the cryptic amino acid conversion in aspirochlorine biosynthesis not only grants first insight into a novel peptide modification sequence, but also highlights once more the potential of merging chemical and biochemical methods in biosynthetic studies.

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