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REVIEW ARTICLE

Genome mining and biosynthesis of fumitremorgin-type alkaloids in ascomycetes

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This review summarizes the recent progress on the biosynthesis of fumitremorgin-type alkaloids; that is, the identification of the biosynthetic gene clusters from genome sequences by genome mining and proof of gene function by molecular biological and biochemical investigations.
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INTRODUCTION

Indole alkaloids of the fumitremorgin-type contain tryprostatins, cyclotryprostatins, spirotryprostatins, fumitremorgins and verruculogen as well as derivatives thereof. These compounds have been isolated from various fungal sources, mainly from *Aspergillus* and *Penicillium* strains. They carry not only intriguing chemical structures, but also interesting biological and pharmacological activities. Early-feeding experiments showed that the members of this group of natural products are derived from tryptophan, proline and mevalonic acid. Recently, biosynthetic gene clusters of the fumitremorgin-type alkaloids consisting very likely of nine genes each has been identified in genomes of one *Neosartorya fischeri* and three *Aspergillus fumigatus* strains. The functions of seven genes and their roles in the biosynthesis were proven by molecular biological and biochemical approaches. The experimentally identified end product of the cluster is until now verruculogen.

STRUCTURES OF FUMITREMORGIN-TYPE ALKALOIDS AND THEIR PRODUCERS AS WELL AS BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES

Prenylated indole alkaloids are hybrid natural products containing indole/indoline and isoprenoid moieties or structures derived thereof.[1,2] Fungal secondary metabolites derived from the diketopiperazine brevianamide F (**1**) consisting of L-tryptophan and L-proline (Figure 1) represent a large group within the prenylated indole alkaloids.[1,2] The enormous structure diversity is only possible by prenylation and other modification reactions of the cyclic dipeptide skeleton. Derivatives of the regularly C2-prenylated brevianamide F; that is, tryprostatin B (**2**), are classified as fumitremorgin-type alkaloids, which include tryprostatins (**2** and **3**), spirotryprostatins (**4** and **5**), cyclotryprostatins (for example **6**), fumitremorgins (**7–10**) and verruculogen (**11**).[1,2] Finding and proof of the genetic information for the biosynthesis of these compounds are focus of this review. Reversely C2-prenylated brevianamide F; that is, deoxybrevianamide E (**12**), is the biosynthetic precursor of brevianamides; for example, brevianamide A (**13**), austamide (**14**) and notoamides like notoamide A (**15**).[2,3] During the reviewing process of this manuscript, a biosynthetic gene cluster for notoamides was identified in an *Aspergillus* sp. and two prenyltransferases from this cluster including NotF, a brevianamide F C2 reverse prenyltransferase, have been characterized biochemically.[4]

Simple fumitremorgin-type alkaloids with one prenyl moiety at position C2 are tryprostatins (**2** and **3**) or derivatives thereof. Spirotryprostatins (**4** and **5**) and fumitremorgin C (**8**) as well as its demethoxylated derivative **7** can be considered as derivatives of **2** and **3** after cyclization and modification. All of these compounds were isolated from several fungal strains.[5–7] Some of these substances have been found to show interesting biological and pharmacological activities. For example, **2** and **3** as well as their diastereomeres were found to exhibit cytotoxicity toward various cancer cell lines, even more potent than etoposide.[8,9] Compounds **8** and **3** are specific inhibitors of the breast cancer resistance protein, an ATP-binding cassette (ABC) transporter (ABCG2) and can reverse the resistance of some tumor cell lines against antitumor agents.[10–12] Therefore, **2**, **3** and **8** as well as their analogues could serve as interesting candidates for development of anticancer agents.

The basis for the discovery of fumitremorgin-type alkaloids in the 1970s was their ability to induce tremors in vertebrates.[13,14] As results, fumitremorgins A (**10**) and B (**9**) as well as verruculogen (**11**) (Figure 1) were identified as tremorgenic mycotoxins.[13–15] Verruculogen (**11**) containing an endoperoxide bridge between the two prenyl moieties of fumitremorgin B (**9**) was found in several strains of ascomycota[16,17] including *A. fumigatus*[5,18,19] and *N. fischeri*[20] as well as *Penicillium* strains.[21,22] Fumitremorgin A (**10**) carries, in

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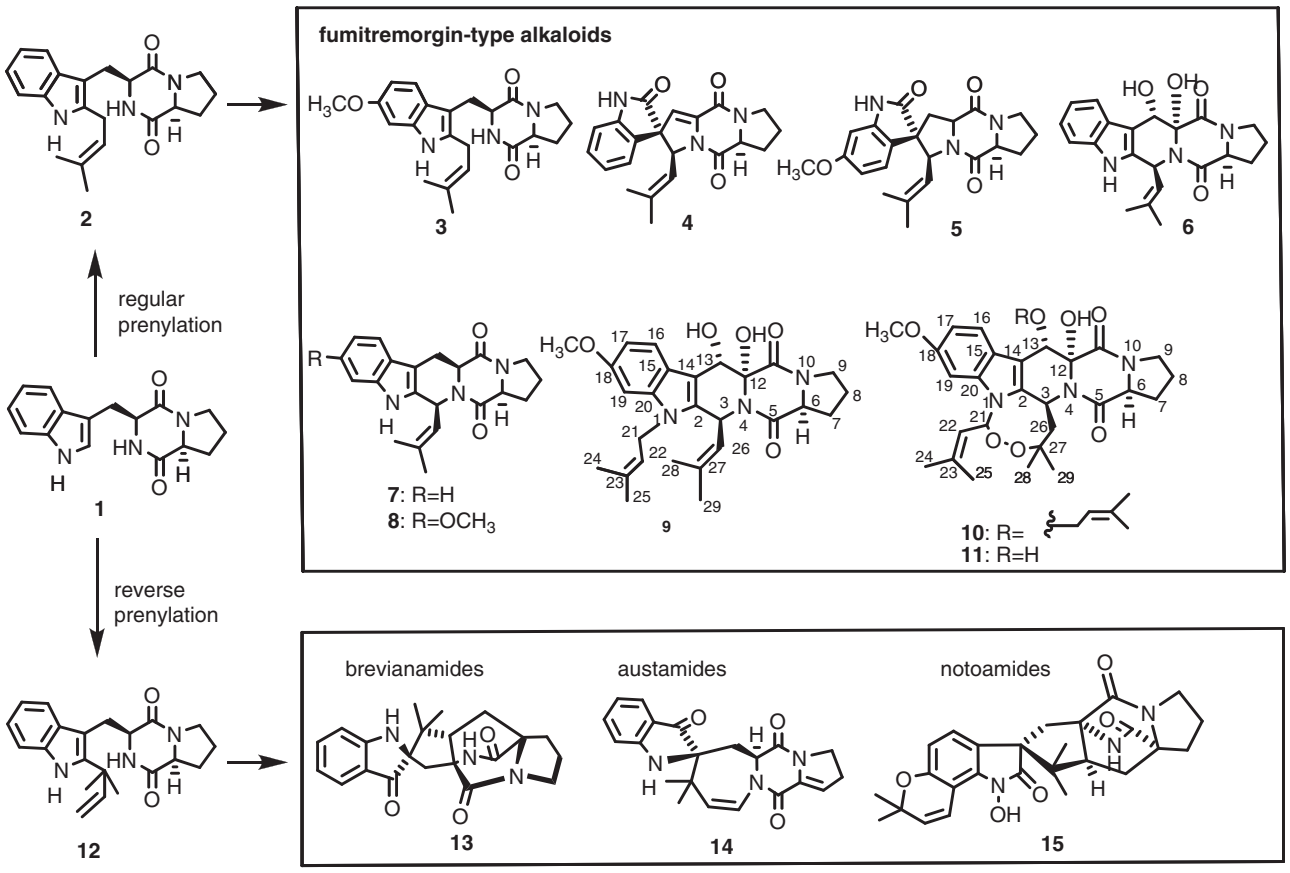

Figure 1 Structures of prenylated indole alkaloids derived from cyclo-L-tryptophan–L-proline and their biosynthetic relationship: brevianamide F (**1**), tryprostatins B (**2**) and A (**3**), spirotryprostatins B (**4**) and A (**5**), cyclotryprostatin C (**6**), demethoxyfumitremorgin C (**7**), fumitremorgin C (**8**), fumitremorgins B (**9**) and A (**10**), verruculogen (**11**), deoxybrevianamide E (**12**), brevianamide A (**13**), austamide (**14**) and notoamide A (**15**).

comparison to verruculogen (**11**), an additional O-prenyl moiety at C13 and is produced by *A. fumigatus* and *N. fischeri*,[19,20,23] but not by the genus of *Penicillium*. Verruculogen (**11**), associated with *A. fumigatus* hyphae and conidia, was reported to be able to modify the electrophysiological properties of human nasal epithelial cells and could be involved in the pathogenesis of this fungus.[24]

Recently, a number of modified derivatives of **2**–**11** have been isolated from *Aspergillus* strains including *A. fumigatus*.[18,25] Some of them differ from **2**–**11** just in their stereochemistry at C12 and C13; that is, β-hydroxyl instead of α-hydroxyl groups. In some cases, the 13-hydroxyl was oxidized to keto group or methylated to methoxy group. Compounds with modifications at one or both prenyl moieties, such as hydroxylation, oxidation and peroxidation, have also been isolated.[18,25]

BIOSYNTHETIC STUDIES ON FUMITREMORGIN-TYPE ALKALOIDS BY FEEDING EXPERIMENTS

Feeding experiments with ^{13}C or ^{14}C-labeled acetate, proline, mevalonic acid and methionine in *Aspergillus* and *Penicillium* strains, and characterization of the isolated secondary metabolites such as fumitremorgin B and verruculogen revealed clearly that the common precursor of these compounds is the cyclic dipeptide brevianamide F consisting of L-tryptophan and L-proline. The prenyl moieties are derived from mevalonic acid. Cultivation of *Penicillium verruculosum* under an atmosphere of $^{18}O_2$ and characterization of verruculogen indicated the formation of the endoperoxide bond by incorporation of an oxygen molecule.[26] For details on the results of the feeding experiments, the review by Professor Williams[2] and literature cited there are recommended.

IDENTIFICATION OF THE BIOSYNTHETIC GENE CLUSTERS OF FUMITREMORGIN-TYPE ALKALOIDS BY GENOME MINING

Significant progress on the biosynthesis of fumitremorgin-type alkaloids was only achieved after availability of genome sequences. A gene cluster for the biosynthesis of fumitremorgin-type alkaloids was initially identified by our group in the genome of *A. fumigatus* Af293.[27] This strain was the first sequenced *Aspergillus* species and its genome sequence was released to the public in 2003. By blasting the genome sequence with *cpd1*, a prenyltransferase gene coding for a 4-dimethylallyltryptophan synthase in the biosynthesis of ergot alkaloids in *Claviceps purpurea*,[28] a segment of 25 kbp on chromosome 8 was identified as a putative biosynthetic gene cluster for fumitremorgin B.[27] The identified cluster was proposed to comprise 10 genes.[27] Later on, orthologous clusters were also identified in *A. fumigatus* A1163 and *N. fischeri* NRRL181.[29,30] Comparison of these clusters revealed the presence of all of the 10 genes in both strains. Remarkably, the orthologous gene of *orf17*[27] in NRRL181, termed *ftmP* in the PhD thesis of A Grundmann,[30] is separated from *ftmO* by a segment of 24 kbp and is likely not a member of the gene cluster of fumitremorgin-type alkaloids.[30] Therefore, we speculate that the mentioned clusters consist of nine genes each as illustrated in Figure 2, which was also identified in *A. fumigatus* BM939 (Figure 2).[31] However, it cannot be excluded that different strains carry similar, but not identical clusters

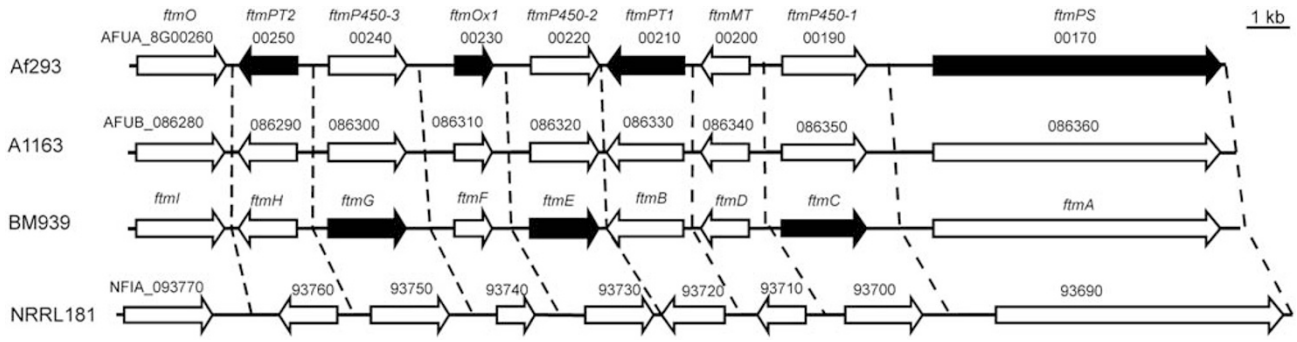

Figure 2 Biosynthetic gene clusters of fumitremorgin-type alkaloids in *Aspergillus fumigatus* Af293, A1163, BM939 and *Neosartorya fischeri* NRRL181. Orthologous genes are indicated between dotted lines. Functions of genes with black arrows have been proven experimentally.

and produce different end products. The nine genes from the four strains share high homology to each other with sequence identities of 85–99% on the amino acid level. Furthermore, they have same order and orientation in these clusters. The end product of this cluster was proven to be verruculogen (**11**) (see below),[32] but it could not be excluded that fumitremorgin A (**10**) is the true end product instead. The fumitremorgin/verruculogen cluster was very likely not expressed in *A. fumigatus* Af293; therefore, no secondary metabolites of this cluster could be detected in this strain.[33] In contrast, a number of intermediates including tryprostatins A (**3**) and B (**2**), fumitremorgin C (**8**), 12,13-dihydroxyfumitremorgin C, fumitremorgin B (**9**) as well as verruculogen (**11**) were isolated from *A. fumigatus* BM939.[5] No data on the expression of the gene clusters in *A. fumigatus* A1163 and *N. fischeri* NRRL181 are available.

MOLECULAR BIOLOGICAL AND BIOCHEMICAL INVESTIGATIONS ON THE BIOSYNTHESIS OF FUMITREMORGIN-TYPE ALKALOIDS

Functional proof of the biosynthetic genes and gene cluster of fumitremorgin-type alkaloids was carried out by gene inactivation experiments in the producing strain BM939,[31] by identification of the accumulated secondary metabolites after heterologous expression in *Aspergillus* strains[33,34] or by biochemical investigation of the recombinant enzymes from *Escherichia coli* or *Saccharomyces cerevisiae*.[27,31,32,35] In total, functions of seven genes have been proven experimentally. On the basis of the obtained bioinformatic, molecular biological and biochemical results, the following biosynthetic pathway has been postulated (Figure 3).[1]

The biosynthesis of fumitremorgin-type alkaloids begins by condensation of the two amino acids L-tryptophan and L-proline to brevianamide F, catalyzed by the non-ribosomal peptide synthetase FtmPS (also termed FtmA), which was proven by heterologous overexpression of the NRPS gene *ftmPS* in *Aspergillus* and identification of the accumulated product brevianamide F.[33] Brevianamide F is then prenylated by the prenyltransferase FtmPT1/FtmB in the presence of dimethylallyl diphosphate, resulting in the formation of tryprostatin B, as demonstrated by using the recombinant FtmPT1.[27] By gene inactivation in *A. fumigatus* BM939 and heterologous expression in *S. cerevisiae*, Kato *et al.*[31] showed that the three cytochrome P450 enzymes, FtmP450-1/FtmC, FtmP450-2/FtmE and FtmP450-3/FtmG, are responsible for the conversion of tryprostatin B (**2**) to 6-hydroxytryprostatin B, tryprostatin A (**3**) to fumitremorgin C (**8**) and fumitremorgin C (**8**) to 12,13-dihydroxyfumitremorgin C, respectively. Using recombinant enzymes, we have demonstrated that FtmPT2/FtmH catalyzes the prenylation of 12,13-dihydroxyfumitremorgin C in the presence of dimethylallyl diphosphate, resulting in the formation of fumitremorgin B,[35] which is then converted to verruculogen by FtmOx1/FtmF by inserting an endoperoxide bond between the two prenyl moieties.[32] We have shown that FtmOx1 reaction was absolutely dependent on the presence of Fe(II) and α-ketoglutarate, and therefore functions as a non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase. Known α-ketoglutarate-dependent dioxygenases usually transfer one oxygen atom to the substrate and another to α-ketoglutarate resulting in the formation of succinate.[36] In contrast, both oxygen atoms from a single O_2 molecule were incorporated into the structure of verruculogen, which was proven by incubation of fumitremorgin B with FtmOx1 in $^{18}O_2$ enriched atmosphere.[32] Therefore, FtmOx1 represents the first endoperoxide forming non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase reported so far. Until now, functions of two genes *ftmMT/ftmD* and *ftmO/ftmI* have to be proven experimentally. The putative methyltransferase FtmMT/FtmD is expected for the conversion of 6-hydroxytryprostatin B to tryprostatin A.[27] Kato *et al.*[31] reported that inactivation of *ftmO/ftmI* in *A. fumigatus* BM939 did not abolish the fumitremorgin B accumulation. Using overproduced and purified His_6-FtmO, we could not provide any evidence on its role in the biosynthesis of fumitremorgin-type alkaloids (unpublished results).

Until now, a conversion of verruculogen to fumitremorgin A was demonstrated neither by an overproduced protein nor by a crude protein extract from a producer. Furthermore, no candidate gene could be identified in the clusters mentioned above. Verruculogen was found in much more strains including *A. fumigatus*,[5,18,19] *N. fischeri*[20,37] and *Penicillium* species[21,22] than fumitremorgin A, which was not identified in *Penicillium* strains. This indicates a genetic difference of these strains or non-expression of the prenyltransferase being responsible for the transfer of a prenyl moiety to the hydroxyl group of verruculogen in *Penicillium* strains.

As mentioned previously, a series of derivatives of the intermediates in the biosynthesis illustrated in Figure 3, including stereoisomers and oxidation products, have been isolated from different *Aspergillus* strains.[7,18,25,38] Obviously, the nine genes of the clusters mentioned above could not account for the synthesis of all of these compounds. It should be questioned whether some of these compounds are enzymatic products in living organisms or results of chemical reactions; for example, oxidation by atmospheric oxygen. It cannot be excluded that unspecific genes/enzymes out of the clusters convert the biosynthetic intermediates to the respective products. It is of course also possible that the biosynthetic enzymes in the cluster catalyze similar reactions, resulting in the formation of side products of the pathway. The last possibility is that there are more than nine genes in the related clusters in such strains.

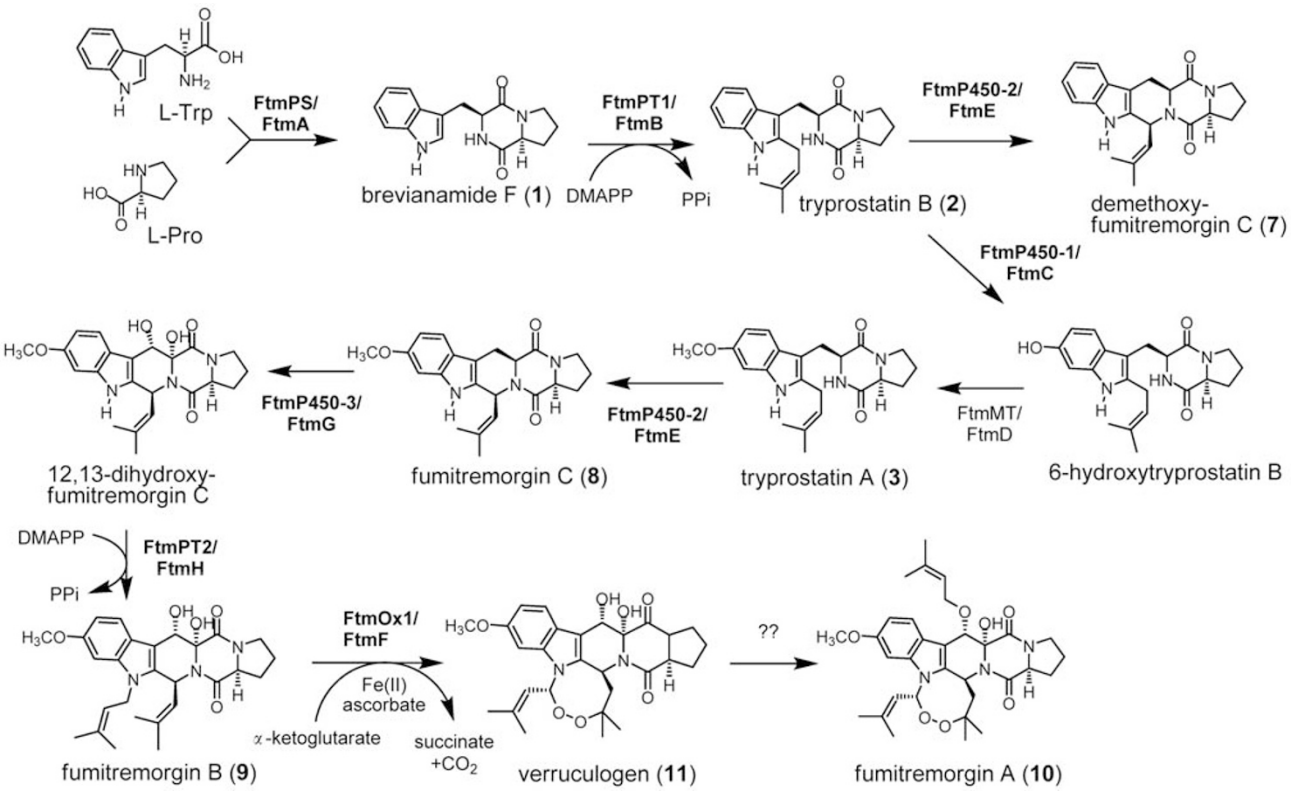

Figure 3 Proposed biosynthetic pathway for verruculogen and fumitremorgin A in *Aspergillus fumigatus*. Abbreviation: DMAPP, dimethylallyl diphosphate; PPi, pyrophosphate.

CONCLUSION AND DISCUSSION

Since 1970's, a number of fumitremorgin-type alkaloids and related substances have been identified in various fungal strains.[2] Significant progress on the biosynthesis of these compounds was only achieved after sequence release of genome sequencing projects, especially for *Aspergillus* strains in 2003/2004. With an exception for the methyltransferase, the genes/enzymes involved in the reaction steps from L-tryptophan and L-proline to verruculogen have been characterized experimentally. This provides an excellent example for identification of genetic information of natural products by genome mining. The availability of more and more genome sequences provides an enormous chance for the study of secondary metabolites. Functional proof of these genes and identification of the natural products coded by the gene clusters would be the main challenges for natural product chemists and biologists in the next years or even decades.

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