

# Ergot alkaloid biosynthesis in *Aspergillus fumigatus*: Conversion of chanoclavine-I aldehyde to festuclavine by the festuclavine synthase FgaFS in the presence of the old yellow enzyme FgaOx3†

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Ergot alkaloids are toxins and important pharmaceuticals which are produced biotechnologically on an industrial scale. A putative gene *fgaFS* has been identified in the biosynthetic gene cluster of fumigaclavine C, an ergot alkaloid of the clavine-type. The deduced gene product FgaFS comprises 290 amino acids with a molecular mass of about 32.1 kDa. The coding region of *fgaFS* consisting of three exons was amplified by PCR from a cDNA library of *Aspergillus fumigatus*, cloned into pQE70 and overexpressed in *E. coli*. The soluble monomeric His<sub>6</sub>-FgaFS was purified by affinity chromatography and used for enzyme assays. It has been shown that FgaFS is responsible for the conversion of chanoclavine-I aldehyde to festuclavine in the presence of the old yellow enzyme FgaOx3. The structure of festuclavine including the stereochemistry was unequivocally elucidated by NMR and MS analyses. Festuclavine formation was only observed when chanoclavine-I aldehyde was incubated with FgaOx3 and FgaFS simultaneously or as a tandem-reaction with a sequence of FgaOx3 before FgaFS. In the absence of FgaFS, two shunt products were formed and did not serve as substrates for FgaFS reaction.

## Introduction

Ergot alkaloids are a complex family of indole derivatives with diverse structures and biological activities. The common structural feature of the most ergot alkaloids is the ergoline with a four fused ring scaffold.<sup>1,5</sup>

In previous studies, we have identified three genes, *fgaPT2*, *fgaMT* and *fgaDH*, from the biosynthetic gene cluster of fumigaclavine C in *Aspergillus fumigatus* (Fig. 1) which are involved in the steps towards the ergoline ring (Fig. 2). These genes were

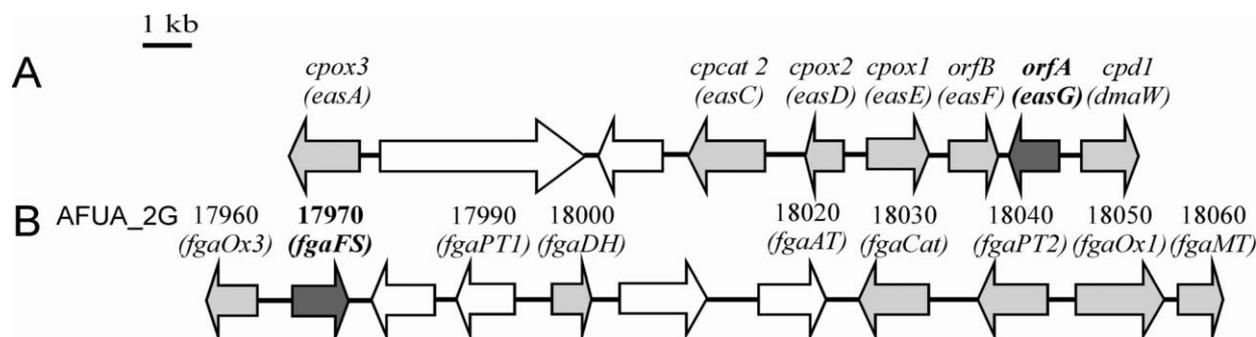
cloned, expressed and characterized biochemically.<sup>6–8</sup> FgaPT2 catalyzes the first pathway-specific step, *i.e.* the prenylation of L-tryptophan at position C-4 resulting in the formation of 4-dimethylallyltryptophan (4-DMAT),<sup>6</sup> which is then converted to N-methyl-4-dimethylallyltryptophan by the N-methyltransferase FgaMT.<sup>7</sup> FgaDH catalyzes the conversion of chanoclavine-I (**1**) to chanoclavine-I aldehyde (**2**) (Fig. 2).<sup>8</sup> Lorenz *et al.* has recently shown that the product of *ccsA* in *Claviceps purpurea* (former known as *cpox1*, a homologous gene of *fgaOx1* in *A. fumigatus*) is involved in the conversion of N-methyl-4-dimethylallyltryptophan, *i.e.* the product of FgaMT, to **1** (Fig. 2).<sup>9</sup>

This leaves three genes *fgaCat*, *fgaOx3* and *fgaOrfA*, which have been found in both *Claviceps purpurea*<sup>10</sup> and *A. fumigatus* (Fig. 2),<sup>8</sup> to be proven functionally. In view of the reaction steps, **2** should be converted to festuclavine (**4**) (Fig. 2) or its diastereomer pyroclavine in *Aspergillus* and *Penicillium*, which have been isolated from both genera.<sup>1,11</sup> However, an enzymatic formation of **4** or of pyroclavine in *A. fumigatus* has not yet been demonstrated by an overproduced and purified enzyme or by crude

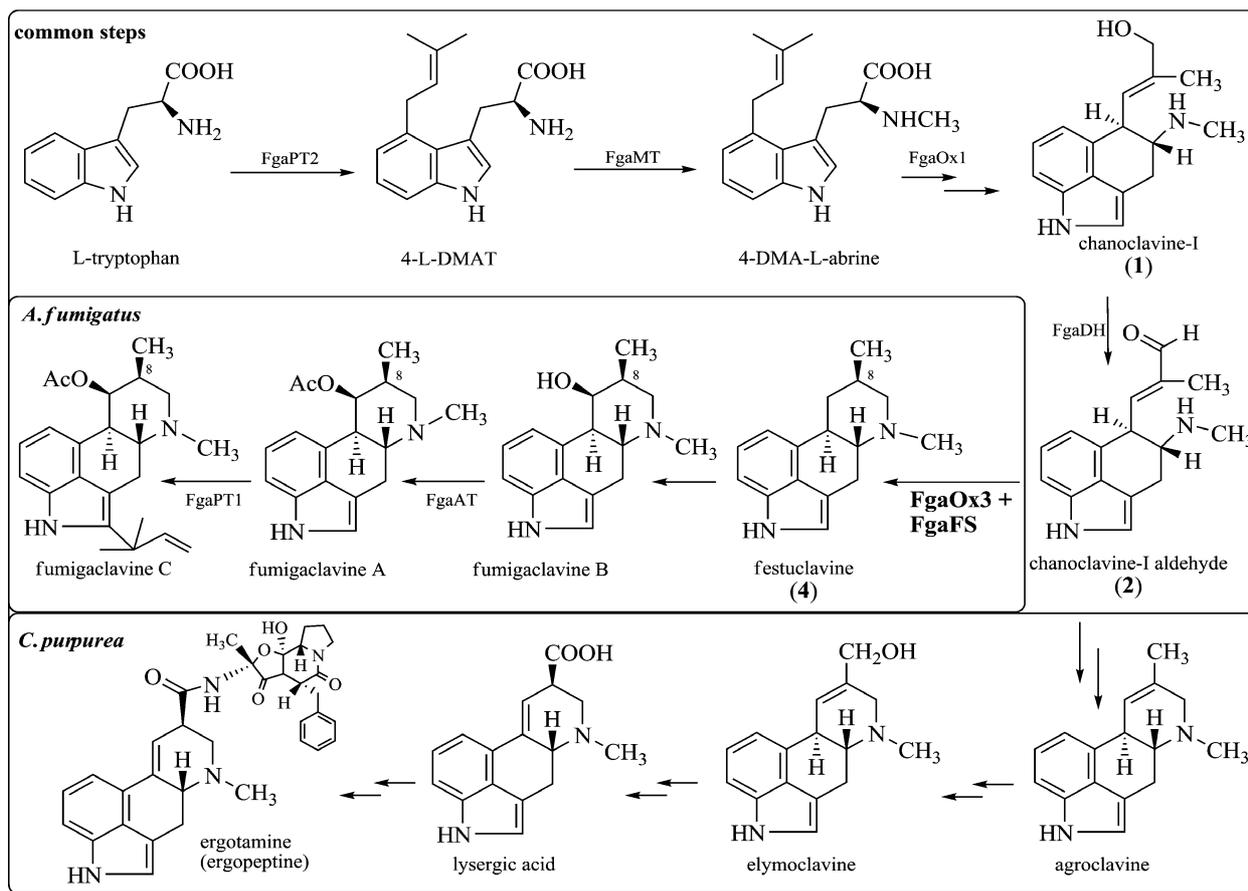
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**Fig. 1** Biosynthetic gene clusters of ergot alkaloids from different sources. Homologous genes are marked grey. A: part of the ergot alkaloid gene cluster from *Claviceps purpurea*; B: fumigaclavine C gene cluster from *Aspergillus fumigatus* Af293.



**Fig. 2** Putative biosynthetic pathway of ergot alkaloids in fungi. Stereochemistry of the methyl group at C-8 in fumigaclavines A, B and C from *Aspergillus fumigatus* was given in the literature either as  $\alpha$ -<sup>12</sup> or as  $\beta$ -configuration<sup>3,4</sup> as illustrated here.

enzyme extracts from a producer. When we started this work, there were no reports on structural genes which could be involved in the conversion of **2** to **4** or to its precursors. *fgaCat* shares high sequence homology with known catalases, whose role could not be speculated in the biosynthesis of ergot alkaloids. Therefore, FgaOrfA (renamed FgaFS in this study) and FgaOx3 remain as candidates for the conversion of **2** to **4** or to pyroclavine. During preparing process on this manuscript, Cheng *et al.*<sup>12</sup> reported that the old yellow enzyme EasA, an orthologue of FgaOx3, catalyzed likely the formation of an intermediate in the conversion of **2** to **4**. They proposed that **2** was converted *via* dihydrochanoclavine-I aldehyde to a cyclized iminium intermediate, which was verified by detection of the molecular ion in the mass spectrum. The incubation mixture of **2** and EasA was reduced by NaCNBH<sub>3</sub> and subjected to <sup>1</sup>H-NMR analysis. The obtained spectrum was then compared with that of the reduction products of agroclavine with Pt-black. The authors mentioned that the products of agroclavine reduction by this method should be a mixture with festuclavine as a major product. However, assignments for protons were given neither in the cited publication by Nakahara *et al.* from 1977<sup>13</sup> nor by Cheng *et al.*<sup>12</sup> Furthermore, a series of additional signals were found in the spectrum of the obtained sample with **2** and EasA, especially in the region between 4.0 and 2.5 ppm. This region is critical for determination of the ergoline structure including stereochemistry. In addition, no <sup>1</sup>H-NMR data were provided for pyroclavine by Nakahara *et al.*<sup>13</sup> Therefore, it is

difficult to follow the conclusion by Cheng *et al.*<sup>12</sup> that festuclavine was the product of the chemical reduction. Here we report the conversion of **2** to **4** by two overproduced enzymes FgaOx3 and FgaFS from *A. fumigatus* B5233. The chemical structure including the stereochemistry of the enzyme product **4** was unequivocally proven by NMR analysis including HSQC, HMBC and NOESY spectra.

## Results and Discussion

### Sequence analysis, cloning of *fgaOx3* and *fgaFS*

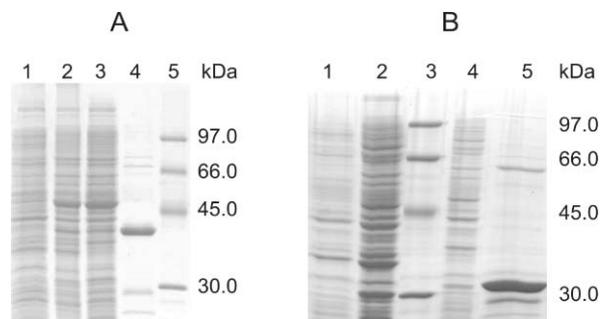
*fgaFS* from the genome reference strain *A. fumigatus* Af293, termed *AFUA\_2G17970*, consists probably of three exons of 418, 200 and 255 bp, interrupted by two introns of 52 and 59 bp, respectively. The deduced gene product FgaFS (=EAL94096.1 in GenBank) comprises 290 amino acids and has a calculated molecular mass of 32.1 kDa. Sequencing of pMM002 (see Experimental Section) revealed that the mRNA sequence of *fgaFS* from *A. fumigatus* B5233 differed from that of the predicted sequence of *AFUA\_2G17970* just by four base pairs, resulting in an amino acid exchange at position 21 from Asp in Af293 to His in B5233. In the case of *fgaOx3* which was sequenced in plasmid pCW02, changes at eight positions were detected. In addition, three bases were inserted between positions 369 and 370 of the sequence *AFUA\_2G17960* from Af293. In total, a difference of six

amino acids was detected between FgaOx3 from B5233 and its orthologue from Af293.

Analysis by using the program BLAST 2 SEQUENCES showed that the deduced gene product FgaOx3 from B5233 shares an identity of 56% with EasA from *C. purpurea* (CAG28312.1)<sup>5</sup> and 56% with EasA from *C. fusiformis* (ABV57819.1), respectively. The deduced gene product FgaFS shares somewhat lower identity of 46% with EasG from *C. purpurea* (AAW57089.1)<sup>5</sup> and 48% with EasG from *C. fusiformis* (ABV57825.1), respectively. FgaFS shows no sequence homology to known proteins.

### Overproduction and purification of His<sub>6</sub>-FgaOx3 and His<sub>6</sub>-FgaFS

The His<sub>6</sub>-tagged proteins FgaOx3 and FgaFS were overproduced in *E. coli* and purified on Ni-NTA agarose. A major protein band with migration below the 45 kDa size marker was observed on SDS-PAGE for overproduced FgaOx3 (Fig. 3A), as expected for the His<sub>6</sub>-FgaOx3 with a calculated mass of 43.5 kDa. In the case of FgaFS, a major protein band with migration above the 30 kDa size marker was detected (Fig. 3B), as expected for the His<sub>6</sub>-FgaFS with a calculated mass of 33.3 kDa. The protein yield was 5 mg per litre of culture for purified His<sub>6</sub>-FgaOx3 and 0.4 mg for purified His<sub>6</sub>-FgaFS.



**Fig. 3** Analysis of the overproduction and purification of His<sub>6</sub>-FgaOx3 (A) and His<sub>6</sub>-FgaFS (B). The proteins were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. (A) Lane 1: Total protein before induction; 2: Total protein after induction; 3: Soluble protein after induction; 4: Purified His<sub>6</sub>-FgaOx3; 5: Molecular weight standard (B) Lane 1: Total protein before induction; 2: Total protein after induction; 3: Molecular weight standard; 4: Soluble protein after induction; 5: Purified His<sub>6</sub>-FgaFS.

### Enzymatic activity and identification of the enzyme product

To investigate the conversion of **2** to **4**, we tested different enzyme combinations. **2** is commercially not available and was therefore prepared enzymatically from **1** with FgaDH. As shown in Fig. 4A, about 28% of **1** was converted in the presence of FgaDH and NAD<sup>+</sup> to its aldehyde **2**, as reported previously.<sup>8</sup> In the incubation mixture of **1** with FgaDH and FgaOx3 in the presence of the cofactors NAD<sup>+</sup>, NADH and FMN, higher conversion of **1** was detected (Fig. 4B) and a new peak **3** at 18.9 min instead of **2** was detected by HPLC. This peak is broad and asymmetrical, indicating the presence of a substance mixture. The observed ion of M<sup>+</sup> at *m/z* 239 for EasA (= FgaOx3) product reported by Cheng *et al.*<sup>12</sup> was also detected in a sample prepared from peak **3**

(data not shown), indicating that peak **3** contained the proposed intermediate and therefore confirming the function of FgaOx3.

Incubation of **1** with the three enzymes FgaDH, FgaOx3 and FgaFS in the presence of the cofactors NAD<sup>+</sup>, NADH and FMN resulted in formation of a new predominant peak **4** at 20.5 min (Fig. 4C). The observed peak **3** at 18.9 min (Fig. 4B) in the assay with FgaDH and FgaOx3 was strongly reduced. In the presence of 5 μg FgaDH and 5 μg FgaOx3, the FgaFS reaction showed a linear dependence on the protein amount up to 0.5 μg (see ESI†) and on the incubation time up to 90 min (see ESI†). Interestingly, the conversion of **1** was much higher in the presence of FgaOx3 or FgaOx3 and FgaFS than FgaDH alone. In the first two cases, **1** was converted almost completely to **3** and **4**, respectively. These results could be explained by a possible product inhibition of the FgaDH reaction which was almost abrogated by further conversion of **2** in the presence of FgaOx3. However, it is more plausible that the equilibrium between **1** and **2** was shifted from substrate to product by removing **2** in the presence of FgaOx3. Incubation of the isolated FgaDH product, *i.e.* **2**, with FgaOx3 and FgaFS resulted also clearly in formation of **4** (data not shown).

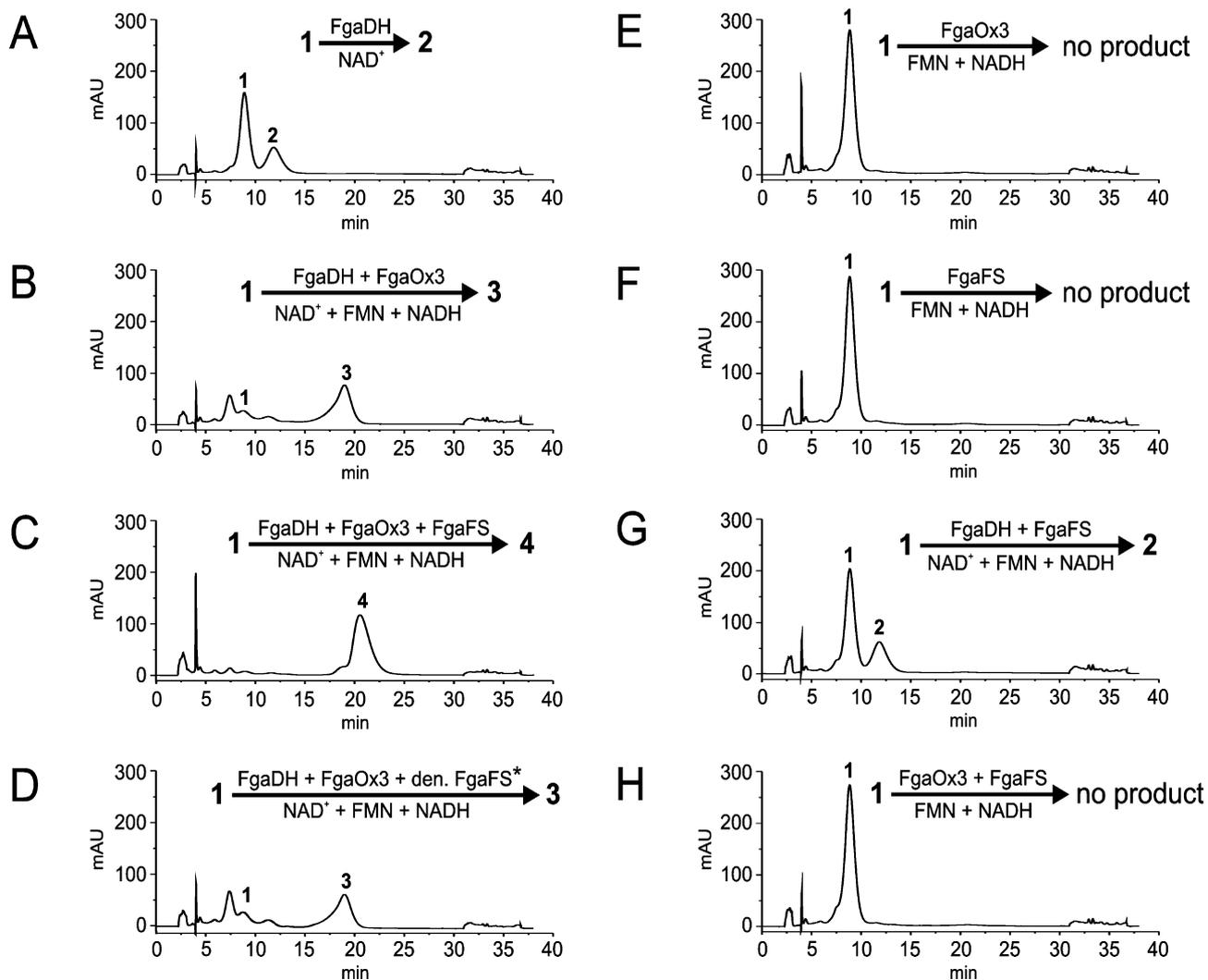
Peak **4** was only detected in the incubation mixture with active, but not in the assay with heat-denatured FgaFS (Fig. 4D). The formation of this peak was observed neither in the incubations of **1** with FgaOx3 (Fig. 4E) or FgaFS (Fig. 4F) alone, nor in combinations of FgaDH with FgaFS (Fig. 4G) or FgaOx3 with FgaFS (Fig. 4H). This proved that **2**, but not **1**, was converted to **3** by FgaOx3 and to **4** by FgaOx3 and FgaFS together.

To prove the roles of FgaOx3 and FgaFS in the conversion of **2** to **4**, we carried out another series of experiments. In these assays, **1** was tandem-incubated with the three enzymes mentioned above. The low molecular weight fraction of the first assay was extracted with ethyl acetate and used as substrate for the second assay, and from the second for the third assay. Due to the different HPLC equipments, all of the substances were eluted slightly earlier than in the first series of experiments (Fig. 5).

As expected, incubation of **1** with FgaDH resulted in the formation of its aldehyde **2** (Fig. 5A), which is then converted to **3** by FgaOx3 (Fig. 5B) and to **4** by FgaOx3 and FgaFS together (Fig. 5C). Because FgaDH was removed by extraction with ethyl acetate before its low molecular weight products were further incubated with FgaOx3 or FgaOx3 and FgaFS, only already produced **2** but not **1** was converted to **3** or **4**. Therefore, the conversion of **1** to **3** or **4** were much lower than those in Fig. 4B and Fig. 4C. **1** was still detected as a predominant peak in these assays. This was confirmed by almost complete conversion of **1** to **4** in the incubation with all three enzymes together (Fig. 5D), as demonstrated above (Fig. 4C).

Interestingly, when we incubated the low molecular weight products of the FgaOx3 reaction with FgaFS, which were obtained by co- or tandem-incubation of **1** with FgaDH and FgaOx3, no formation of **4** was observed (Fig. 5E and F). Even in the presence of FgaOx3 and FgaFS, the FgaOx3 product was not converted to **4** (Fig. 5G). This proved clearly that the major substance under peak **3** was not the substrate for FgaFS. **3** cannot be activated or converted by FgaOx3 and served then as substrate for FgaFS (Fig. 5G).

To determine the structure, 4 mg of **4** were isolated on HPLC after incubation of **1** with FgaDH, FgaOx3 and FgaFS and subjected to NMR and MS analyses. <sup>1</sup>H and <sup>13</sup>C signal assignments



**Fig. 4** HPLC chromatograms of incubation mixtures with different enzyme combinations. The reaction mixtures contained 5 mM of  $\text{NAD}^+$  and/or FMN and NADH, 1 mM chanoclavine-I and 5  $\mu\text{g}$  of the recombinant enzymes and were incubated at 30 °C for 16 h. The substances were detected with a Photo Diode Array detector and illustrated for absorption at 282 nm. (\*: heat-denatured FgaFS by boiling for 30 min).

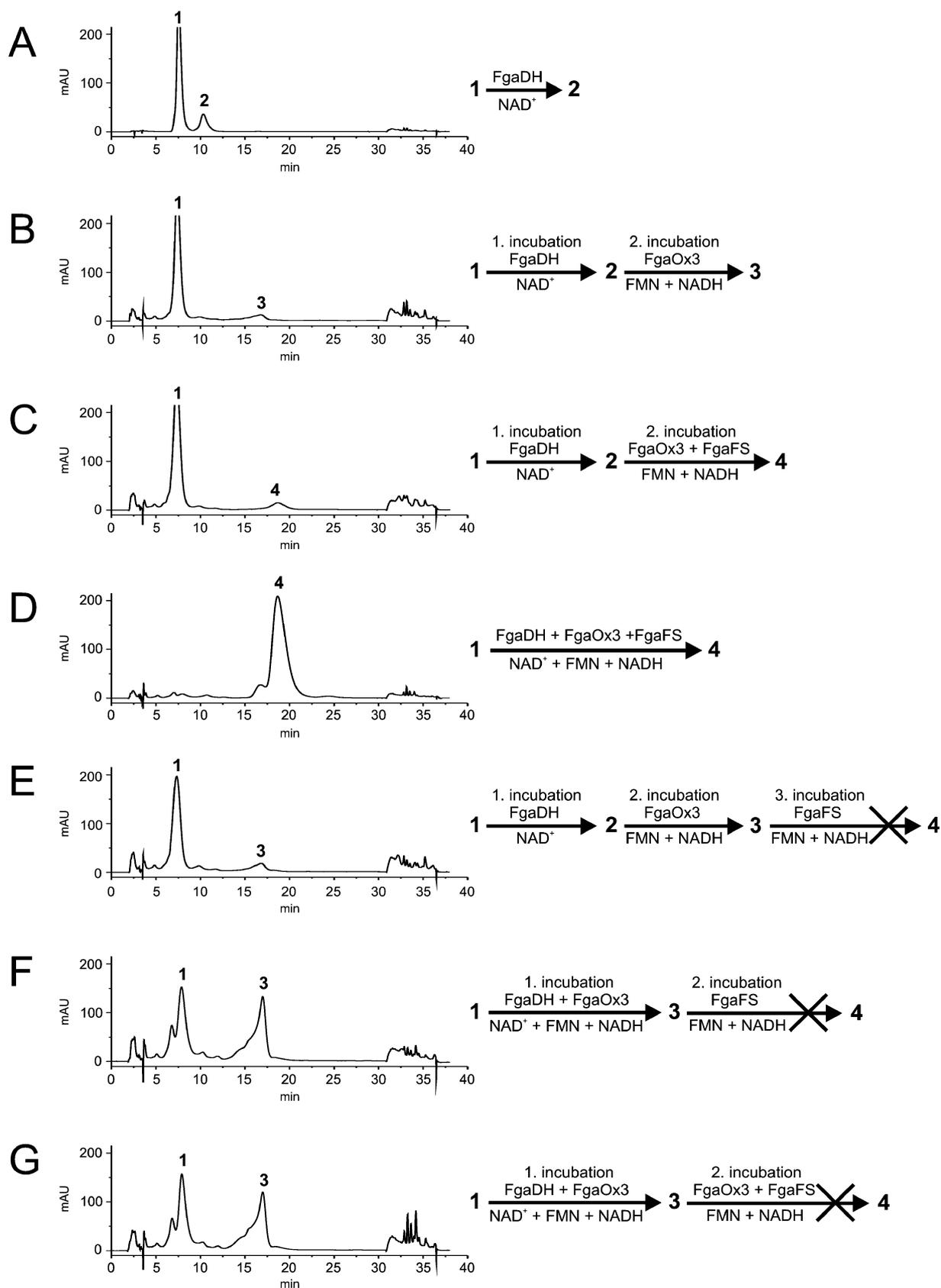
of **4** in its protonated form after HPLC separation have been proven by DQF-COSY, HSQC and HMBC and are given in Table 1. The spectra are supplied in the ESI<sup>†</sup>.

The signals of aldehyde and olefinic protons in **2** (see Wallwey *et al.*<sup>8</sup>) had disappeared in the <sup>1</sup>H-NMR spectrum of **4**. Signals for aromatic and olefinic protons were only found for protons at the indole ring, but not for those at the C- and D-rings (Table 1, Fig. 6). HMBC between H-18 and C-7 as well as H-7<sub>α</sub> and C-5 and H-7<sub>β</sub> and C-5 (Table 1) proved unequivocally the closure of the ring D (Fig. 2).

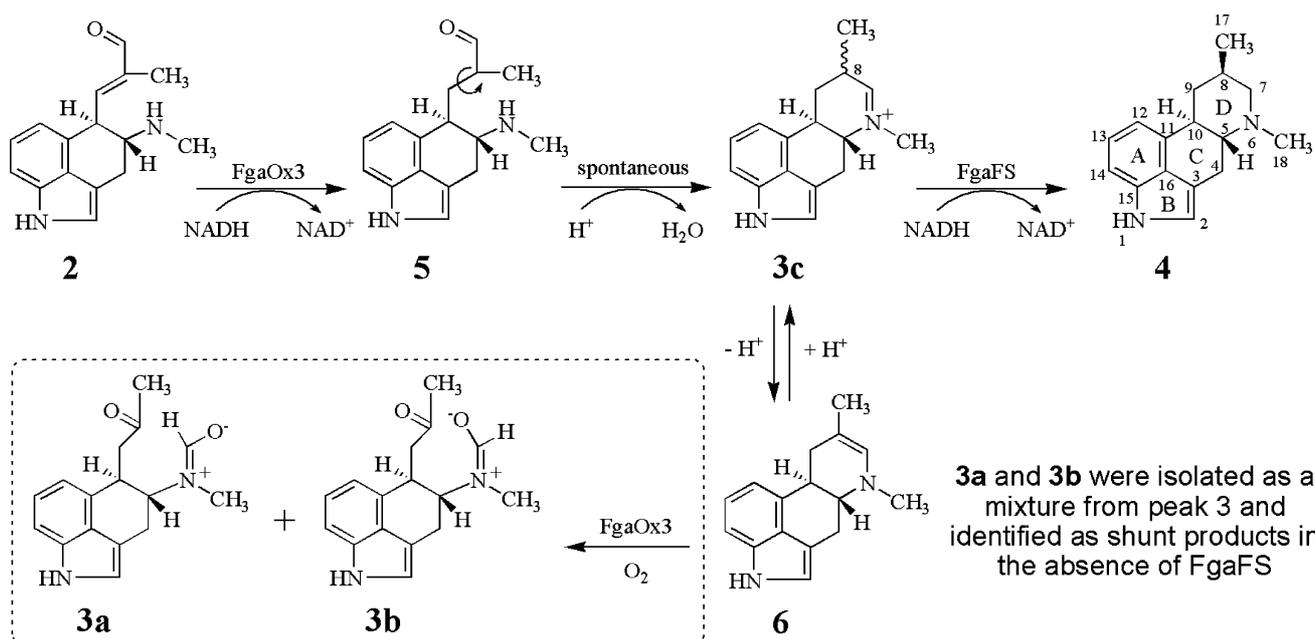
To prove the stereochemistry, we determined the NOE contacts of the aliphatic protons within rings C and D, with reference to the known configuration at C-5 and C-10, *i.e.* β-configured H-5 (H-5<sub>β</sub>) and α-configured H-10 (H-10<sub>α</sub>) (Table 2). Medium NOE correlation was observed between H-8 and H-10<sub>α</sub>, proving that H-8 is α-substituted. This was supported by the medium NOE contacts between H-8<sub>α</sub> and H-7<sub>α</sub> as well as H-8<sub>α</sub> and H-9<sub>α</sub>. Both H-7<sub>α</sub> and H-9<sub>α</sub> showed no NOEs with H-5<sub>β</sub>, which showed medium NOEs with H-7<sub>β</sub> and H-9<sub>β</sub>.

The <sup>1</sup>H-NMR spectrum of **4** in its protonated form corresponded perfectly to that of an authentic festuclavine sample (see ESI<sup>†</sup>) after treatment with HPLC solvents containing trifluoroacetic acid. The identity of the festuclavine standard had been confirmed by comparison of the <sup>1</sup>H-NMR spectrum of its free base form (see ESI<sup>†</sup>) with the data published by Bach *et al.*<sup>14</sup> These data proved unambiguously **4** as festuclavine with a chemical formula of C<sub>16</sub>H<sub>20</sub>N<sub>2</sub>, which was also confirmed by the HR-ESI-MS data of the [M+1]<sup>+</sup> ion at *m/z* 241.1673 (calculated value 241.1705). These results proved that FgaFS together with FgaOx3 catalyzes the conversion of **2** to **4** in the presence of FMN and NADH and therefore functions as a festuclavine synthase.

As speculated above, peak **3** was indeed a mixture consisting of at least three compounds. Isolation and structure elucidation revealed that the two isomers **3a** and **3b** (Fig. 6) with a ratio of 3 : 1 were determined as predominant components of peak **3** and made up at least 99%. The proposed iminium intermediate **3c** (Fig. 6)<sup>12</sup> existed in trace and was only detected by MS but not by NMR spectroscopy. The structures of **3a** and **3b** have been determined



**Fig. 5** HPLC chromatograms of the product formation with different enzyme and cofactor combinations. Between two incubations the low molecular weight fraction of the first reaction was extracted with ethyl acetate and used as substrate for the next reaction. The reaction mixtures contained 5 mM of each cofactor, 1 mM chano clavine-I and 5  $\mu\text{g}$  of the recombinant enzymes and were incubated at 30  $^{\circ}\text{C}$  for 16 h. The substances were detected with a Photo Diode Array detector and illustrated for absorption at 282 nm.



**Fig. 6** Proposed mechanisms of the FgaOx3 and FgaFS reactions in *Aspergillus fumigatus*.

**Table 1**  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  data of festuclavine **4** in protonated form ( $\text{CD}_3\text{OD}$ )

Position	$\delta_{\text{C}}$	$\delta_{\text{H}}$ , multi., $J/\text{Hz}$	HMBC correlation
2	120.15	7.02, d, 1.4	C-2 to H-4 $_{\alpha}$
3	108.22	—	C-3 to H-2, H-4 $_{\alpha}$
4 $_{\alpha}$	25.62	2.98, ddd, 13.9, 11.7, 1.6	C-4 to H-2, H-18
4 $_{\beta}$	3.64, dd, 12.7, 5.0	—	—
5 $_{\beta}$	68.35	3.20, dt, 4.1, 11.3	C-5 to H-4 $_{\alpha}$ , H-7 $_{\alpha}$ , H-7 $_{\beta}$ , H-9 $_{\beta}$ , H-10 $_{\alpha}$ , H-18
7 $_{\alpha}$	63.13	3.55, ddd, 12.1, 3.8, 1.7	C-7 to H-9 $_{\beta}$ , H-17, H-18
7 $_{\beta}$	2.86, t, 12.4	—	—
8 $_{\alpha}$	30.01	2.25, m	C-8 to H-9 $_{\beta}$ , H-17
9 $_{\alpha}$	35.55	2.79, ddt, 13.3, 1.9, 3.6	C-9 to H-7 $_{\alpha}$ , H-7 $_{\beta}$ , H-10 $_{\alpha}$ , H-17
9 $_{\beta}$	1.33, dt, 12.2, 12.7	—	—
10 $_{\alpha}$	40.13	3.29, dt, 3.2, 11.4	C-10 to H-9 $_{\beta}$ , H-12
11	130.23	—	C-11 to H-9 $_{\beta}$ , H-10 $_{\alpha}$ , H-13
12	113.77	6.90, d, 7.6	C-12 to H-10 $_{\alpha}$ , H-14
13	123.63	7.11, t, 7.7	C-13 to H-12
14	110.45	7.21, d, 8.1	C-14 to H-12
15	134.99	—	C-15 to H-2, H-13
16	126.71	—	C-16 to H-2, H-12, H-14
17	18.56	1.13, d, 6.6	C-17 to H-7 $_{\beta}$ , H-8 $_{\alpha}$ , H-9 $_{\beta}$
18	41.31	3.06, s	—

by HR-ESI-MS,  $^1\text{H-NMR}$ , NOESY, COSY, HSQC and HMBC (data not shown). Due to the complexity of their structures and especially the interpretation of the NMR spectra, the structure elucidation of **3a** and **3b** will be published elsewhere.

Based on the data obtained from this and the previous study,<sup>12</sup> a mechanism as shown in Fig. 6 was postulated for the FgaOx3 and FgaFS reactions. As proposed by Cheng *et al.*,<sup>12</sup> chanoclavine-I

**Table 2** NOE contacts for proving the stereochemistry of **4**

Protons	Strength
H-8 $_{\alpha}$ to H-10 $_{\alpha}$	Medium
H-8 $_{\alpha}$ to H-7 $_{\alpha}$	Medium
H-8 $_{\alpha}$ to H-9 $_{\alpha}$	Medium
H-5 $_{\beta}$ to H-7 $_{\beta}$	Medium
H-5 $_{\beta}$ to H-9 $_{\beta}$	Medium
H-17 to H-7 $_{\beta}$	Medium
H-17 to H-9 $_{\beta}$	Medium

aldehyde **2** was converted by FgaOx3 in the presence of NADH to the intermediate **5**, which was then cyclized spontaneously to the iminium **3c**. The addition of FMN to the FgaOx3 reaction was necessary to convert the enzyme from its apo- to its holo-form. Reduction of **3c** by FgaFS resulted in formation of festuclavine **4**. It can be expected that the formation of **3c** is a stereochemically unspecific reaction and two isomers with a  $\beta$ - or an  $\alpha$ -methyl group at position 8 are possible (Fig. 6). It is plausible that FgaFS from *Aspergillus fumigatus* accepted predominantly **3c** with a  $\beta$ -methyl group at position 8, and not or only small amount of its stereoisomer with an  $\alpha$ -methyl group. In the later case, pyroclavine rather than festuclavine should be formed. We speculate that equilibration between **3c** and its tautomer **6** ensures the conversion of **3c** with an  $\alpha$ -methyl group to that with a  $\beta$ -methyl group, so that festuclavine was identified as predominant enzyme product. It could be speculated that the iminium **3c** was immediately passed from FgaOx3 to FgaFS in a manner of substrate channeling.<sup>15</sup> Association of FgaOx3 and FgaFS was however not observed by size exclusion chromatography after incubation of the two enzymes with or without **2** and cofactors. In the absence of FgaFS, the intermediate **6** was oxidized by FgaOx3 to **3a** and **3b**, which were not accepted by FgaFS. The signals of **3a** and **3b**, especially those for the formyl group at 8.5 ppm, have also been detected in the  $^1\text{H-NMR}$  spectrum of the chemically reduced product of EasA

and chanoclavine-I aldehyde by Cheng *et al.*<sup>12</sup> Detailed studies on the proposed reaction mechanism are under investigation.

### Biochemical properties of the festuclavine synthase FgaFS

By using size exclusion chromatography, the native molecular mass of the recombinant His<sub>6</sub>-FgaOx3 was determined to be 46 kDa, suggesting that the native FgaOx3 is a monomer. The native molecular mass of the recombinant His<sub>6</sub>-FgaFS was determined to be 39 kDa, suggesting that the native FgaFS is also a monomer.

The FgaOx3 reaction was dependent on the presence of FMN or FAD and NADH or NADPH. FgaOx3 was more active in the presence of FMN and NADH than other combinations, *e.g.* FMN and NADPH, FAD and NADH or FAD and NADPH (see ESI†). The FgaOx3 reaction was absolutely essential for the FgaFS reaction and already contained FMN and NADH as cofactors. Therefore, it is difficult to determine whether or which cofactor is necessary for the FgaFS reaction. Because FgaOx3 was essential for the conversion of **2** to **4** the  $K_M$  value for FgaFS could not be determined in this study. The second problem is the unknown concentration of **2** which was also dependent on the following reactions.

### Conclusions

In this study, we demonstrated clearly that the conversion of chanoclavine-I aldehyde **2** to festuclavine **4** was catalyzed by the two enzymes FgaOx3 and FgaFS. In the absence of FgaFS, **3a** and **3b** was accumulated as shunt products of the FgaOx3 reaction.

### Experimental Section

#### Computer-assisted sequence analysis

Sequence similarities were obtained by alignments of amino acid sequences using the BLAST program “BLAST 2 SEQUENCES” (www.ncbi.nlm.nih.gov).

#### Chemicals

The cofactors were obtained from Sigma-Aldrich (Munich, Germany). Chanoclavine-I and authentic festuclavine were kindly provided by Prof. Leistner (Bonn, Germany).

#### Bacterial strains, plasmids and cultural conditions

pGEM-T Easy and pQE70 vectors were obtained from Promega (Mannheim, Germany) and Qiagen (Hilden, Germany), respectively. A Uni-ZAP<sup>®</sup> XR Premade Library of *A. fumigatus* strain B5233 (= ATCC13073) was purchased from Stratagene and used to obtain phagemids as cDNA template for PCR amplification.

*Escherichia coli* XL1 Blue MRF<sup>+</sup> (Stratagene, Amsterdam, The Netherlands) was used for cloning and expression experiments and grown in liquid or on solid Luria-Bertani or Terrific-Broth medium with 1.5% (w/v) agar at 37 °C, 30 °C or 22 °C.<sup>16</sup> Carbenicillin (50 µg ml<sup>-1</sup>) was used for selection of recombinant *E. coli* strains.

#### DNA isolation, PCR amplification and cloning

Standard procedures for DNA isolation and manipulation were performed as described.<sup>16</sup> PCR amplification was carried out

on a MiniCycler from BioRad (Munich, Germany). A PCR fragment of 1147 bp containing the entire coding sequence of *fgaOx3* was amplified using Expand High Fidelity Kit (Roche Diagnostics GmbH, Mannheim, Germany) from the cDNA library of *A. fumigatus* B5233 by using the primers fgaOx3-for (5'-GGCATGCGAGAAGAACCGTCC-3') at the 5'-end and fgaOx3\_rev2 (5'-TTCTCGGGATCCGACGGGGA-3') at the 3'-end of the gene. A PCR fragment of 888 bp containing the entire coding sequence of *fgaFS* was amplified using Expand High Fidelity Kit (Roche Diagnostics GmbH, Mannheim, Germany) from the cDNA library of *A. fumigatus* B5233 by using the primers fgaFS\_for (5'-CGCATGCCTATCCTCGTGCTG-3') at the 5'-end and fgaFS\_rev2 (5'-CCAAAATAGGATCCATGCCGCAT-3') at the 3'-end of the gene. Bold letters represent mutations inserted in comparison to the original genome sequence of Af293 to give the underlined restriction site SphI at the start codon in fgaOx3\_for and in fgaFS\_for and to give the underlined restriction site BamHI located at the predicted stop codon in fgaOx3\_rev2 and in fgaFS\_rev2. The PCR fragment of *fgaOx3* was cloned into pGEM-T Easy resulting in plasmid pCW02 and the PCR fragment of *fgaFS* was cloned into pGEM-T Easy resulting in plasmid pMM002, which were subsequently sequenced (MWG Biotech AG, Ebersberg, Germany). To create the expression vectors pCW04 for *fgaOx3* and pMM003 for *fgaFS*, pCW02 and pMM002 were partially digested with SphI and BamHI. The resulted SphI - BamHI fragments of 1129 bp and 868 bp, respectively, were isolated from the agarose gel and ligated into pQE70, which had also been digested with SphI and BamHI, previously.

#### Overproduction and purification of His<sub>6</sub>-FgaOx3 and His<sub>6</sub>-FgaFS

For expression of the gene *fgaOx3*, *E. coli* XL1 Blue MRF<sup>+</sup> cells harbouring the plasmid pCW04 were cultivated in 2000 ml Erlenmeyer flasks containing liquid Luria-Bertani medium (1000 ml) supplemented with carbenicillin (50 µg ml<sup>-1</sup>) and grown at 37 °C to an absorption at 600 nm of 0.6. For induction, isopropyl thiogalactoside (IPTG) was added to a final concentration of 1.0 mM and the cells were cultivated for further 4 h at 30 °C before harvest.

For expression of the gene *fgaFS*, *E. coli* XL1 Blue MRF<sup>+</sup> cells harbouring the plasmid pMM003 were cultivated in 2000 ml Erlenmeyer flasks containing liquid Terrific-Broth medium (1000 ml) supplemented with carbenicillin (50 µg ml<sup>-1</sup>) and grown at 22 °C to an absorption at 600 nm of 0.6. For induction, isopropyl thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and the cells were cultivated for further 16 h at 22 °C before harvest.

The bacterial cultures were centrifuged and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, pH 8.0) at 3 ml per gram wet weight. After addition of 1 mg ml<sup>-1</sup> lysozyme and incubation on ice for 30 min, the cells were sonicated 6 times for 10 s each at 200 W. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 14,000  $\times$  g for 30 min at 4 °C. One-step purification of the recombinant His<sub>6</sub>-tagged fusion protein by affinity chromatography with Ni-NTA agarose resin (Qiagen, Hilden, Germany) was carried out according to the manufacturer's instructions. The protein was eluted with 250 mM imidazole in

50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0. In order to change the buffer, the protein fraction was passed through a PD-10 column (GE Healthcare, Freiburg, Germany), which had been equilibrated with 50 mM Tris-HCl, 15% (v/v) of glycerol, pH 7.5, previously. The proteins were eluted with the same buffer and stored frozen at – 80 °C for enzyme assays.

### Protein analysis

Proteins were analyzed by SDS-PAGE according to the method of Laemmli<sup>17</sup> and stained with Coomassie Brilliant Blue R-250.

### Determination of the molecular mass of active His<sub>6</sub>-FgaOx3 and His<sub>6</sub>-FgaFS

The molecular mass of the recombinant His<sub>6</sub>-FgaOx3 and His<sub>6</sub>-FgaFS was determined by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Health Care, Freiburg, Germany), with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl as eluent. The column was calibrated with blue dextran 2000 (2000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa) (GE Healthcare, Freiburg, Germany).

### Enzyme assays with His<sub>6</sub>-FgaOx3 and His<sub>6</sub>-FgaFS

All of the enzyme assays contained 50 mM Tris-HCl, pH 7.5 and 0.5–6.0% (v/v) of glycerol. The reaction mixtures were incubated at 30 °C for different incubation times and the reactions were then extracted twice with 2 volumes of ethyl acetate after adjusting to pH 9.0 with 1 M NaOH. The combined organic phase was evaporated to dryness and dissolved in 2 µl DMSO and 98 µl methanol. The enzyme products were analyzed on a HPLC system described below. Assays for determination of the enzyme activity and the product formation (100 µl) contained 1 mM chanoclavine-I, 5 mM of the respective cofactors and optionally 5 µg (0.42 µM) of FgaDH, 5 µg (1.2 µM) of FgaOx3 and/or 5 µg (1.5 µM) of FgaFS. The incubation time was 16 h.

The reaction mixture for isolation of the enzyme product for structural elucidation (20 ml) contained chanoclavine-I (1 mM), 1.5 mg (0.63 µM) of purified FgaDH, 1.5 mg (1.7 µM) of purified FgaOx3 and 1.5 mg (2.3 µM) of purified FgaFS. After incubation for 16 h, the pH value of the reaction mixture was adjusted to 9 with aqueous ammonium hydroxide and extracted twice with ethyl acetate. The combined organic phase was evaporated on a rotation evaporator at 32 °C to dryness. The residue was dissolved in 250 µl methanol and centrifuged at 14,000 *x g* for 30 min at 4 °C before injection onto HPLC for isolation of festuclavine.

### HPLC analysis and isolation of festuclavine

Reaction mixtures were analyzed on an Agilent HPLC Series 1200 by using a Multospher 120 RP18 column (4 × 250 mm, 5 µm, Agilent) at a flow rate of 1 ml min<sup>-1</sup>. Water (solvent A) and acetonitrile (solvent B), each containing 0.5% (v/v) trifluoroacetic acid, were used as solvents. The substances were detected with a Photo Diode Array detector. The assays were analyzed by beginning with 25% B for 8 min and then with a gradient from 25 to 35% B over 20 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 25% solvent B for 5 min.

For isolation of the enzyme product, a gradient from 36 to 45% B over 28 min was used. After washing with 100% solvent B for 5 min, the column was equilibrated with 36% solvent B for 5 min.

The collected fractions containing the enzyme product after HPLC separation were evaporated to dryness and subjected to NMR and MS analyses.

### NMR experiments

Spectra were recorded at room temperature on a Bruker Avance 600 MHz spectrometer equipped with an inverse probe with z-gradient. The DQF-COSY, HSQC, and HMBC spectra were recorded with standard methods.<sup>18</sup> Gradient-selected NOESY experiment<sup>19</sup> was performed in phase-sensitive mode. For all two-dimensional spectra, 16 transients were used. For NOESY spectra, a mixing time of 1.5 s and a relaxation delay of 2.5 s were used. <sup>1</sup>H spectra were acquired with 65 536 data points, while 2D spectra were collected using 4096 points in the F<sub>2</sub> dimension and 512 increments in the F<sub>1</sub> dimension. Chemical shifts were referenced to CD<sub>3</sub>OD. All spectra were processed with Bruker TOPSPIN 2.1.

### ESI-MS of the enzyme product

The positive electrospray ionization (ESI) mass spectrometry was carried out on a Q-Trap Quantum (Applied Biosystem).

### Nucleotide sequence accession numbers

The nucleotide sequence of the genomic DNA from *A. fumigatus* Af293 reported in this study is available at GenBank under the accession number AAHF01000001.1. The coding sequence of *fgaFS* from *A. fumigatus* B5233 was deposited at GenBank under the accession number GU929210. The coding sequence of *fgaOx3* from *A. fumigatus* B5233 was deposited at GenBank under the accession number GU929211.

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