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**PAPER**

# New insights into ergot alkaloid biosynthesis in *Claviceps purpurea*: An agroclavine synthase EasG catalyses, *via* a non-enzymatic adduct with reduced glutathione, the conversion of chanoclavine-I aldehyde to agroclavine†

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Ergot alkaloids are indole derivatives with diverse structures and biological activities. They are produced by a wide range of fungi with *Claviceps purpurea* as the most important producer for medical use. Chanoclavine-I aldehyde is proposed as a branch point *via* festuclavine or pyroclavine to clavine-type alkaloids in Trichocomaceae and *via* agroclavine to ergoamides and ergopeptines in Clavicipitaceae. Here we report the conversion of chanoclavine-I aldehyde to agroclavine by EasG from *Claviceps purpurea*, a homologue of the festuclavine synthase FgaFS in *Aspergillus fumigatus*, in the presence of reduced glutathione and NADPH. EasG comprises 290 amino acids with a molecular mass of about 31.9 kDa. The soluble monomeric $His_6$-EasG was purified after overproduction in *E. coli* by affinity chromatography and used for enzyme assays. The structure of agroclavine was unequivocally elucidated by NMR and MS analyses.

## Introduction

Ergot alkaloids are a complex family of indole derivatives with diverse structures and biological activities.[1] They are toxins produced by fungi of the families Clavicipitaceae, *e.g. Claviceps* and *Neotyphodium*, and Trichocomaceae including *Aspergillus* and *Penicillium*.[2,3] On the other hand, some of these compounds and their semi-synthetic derivatives are important drugs, which are used in modern medicine, *e.g.* ergotamine and dihydroergotamine for the treatment of migraines[4,5] and dihydroergotoxine for dementia and age-related cognitive impairment.[6,7]

A characteristic structural feature of most ergot alkaloids is the tetracyclic ergoline ring system.[1,2,8] Fungi of the family Trichocomaceae produce mainly clavine-type alkaloids consisting of the ergoline system, whereas amides and peptides of lysergic acid, also termed ergoamides and ergopeptines respectively, are mainly found in fungi of the family Clavicipitaceae.[1]

The biosynthetic gene cluster for ergoamides and ergopeptines in *Claviceps purpurea* has been proposed to contain 14 genes, whereas the gene cluster of the clavine-type alkaloid fumigaclavine C in *Aspergillus fumigatus* likely comprises 11 genes (Fig. 1).[9–11] Seven orthologous/homologous genes with sequence identities of 46 to 66% on the amino acid level were found in both gene clusters, which are assumed to be involved in the early steps towards the ergoline ring (Fig. 2).[1,11]

The biosynthesis of the common ergoline ring begins with the prenylation of L-tryptophan by a dimethylallyltryptophan synthase, *e.g.* FgaPT2, resulting in the formation of 4-dimethylallyltryptophan (4-DMAT),[11] which is then converted to N-methyl-4-dimethylallyltryptophan (4-DMA-L-abrine) by an N-methyltransferase, *e.g.* FgaMT.[12] Lorenz *et al.*[13] have shown that the product of *ccsA* in *C. purpurea* (formerly known as *cpox1* or *easE*, a homologous gene of *fgaOx1* in *A. fumigatus*) is involved in the conversion of 4-DMA-L-abrine to chanoclavine-I (Fig. 2). An alcohol dehydrogenase, *e.g.* FgaDH, catalyses the conversion of chanoclavine-I to chanoclavine-I aldehyde (**1**).[14]

It has been demonstrated that **1** is the branch point for the biosynthetic pathways of ergot alkaloids in *Claviceps via* agroclavine (**2**) and in *Aspergillus via* festuclavine (**3**).[15] **2** and **3** were isolated from *C. purpurea* and *A. fumigatus*, respectively.[16–18]

**2** differs from **3** in the presence of a double bond between C8 and C9. For the formation of **2** from **1**, one reduction and an isomerisation step would be necessary. In contrast, for the conversion of **1** to **3**, two reduction steps and possibly an isomerisation process are required. It has been shown that **1** is converted to **3** by FgaFS in the presence of the old yellow enzyme FgaOx3 in *A. fumigatus*.[19] **3** serves then as a precursor of fumigaclavines A, B and C.[1] In *C. purpurea*, it is expected that **1** is converted to **2**, which is then converted *via* elymoclavine to lysergic acid, the acidic component of ergoamides and ergopeptines (Fig. 2).[20] During the preparation process of this manuscript, Cheng *et al.*[21] reported the conversion of **1** to **2** by FgaFS from *A. fumigatus* in

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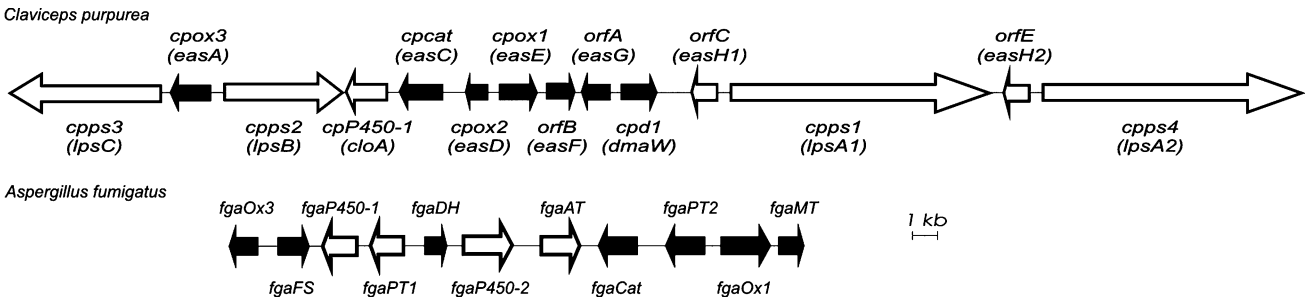


**Fig. 1** Biosynthetic gene clusters of ergot alkaloids from *Claviceps purpurea* and of fumigaclavine C from *Aspergillus fumigatus Af293*. Homologous genes are indicated as black arrows.

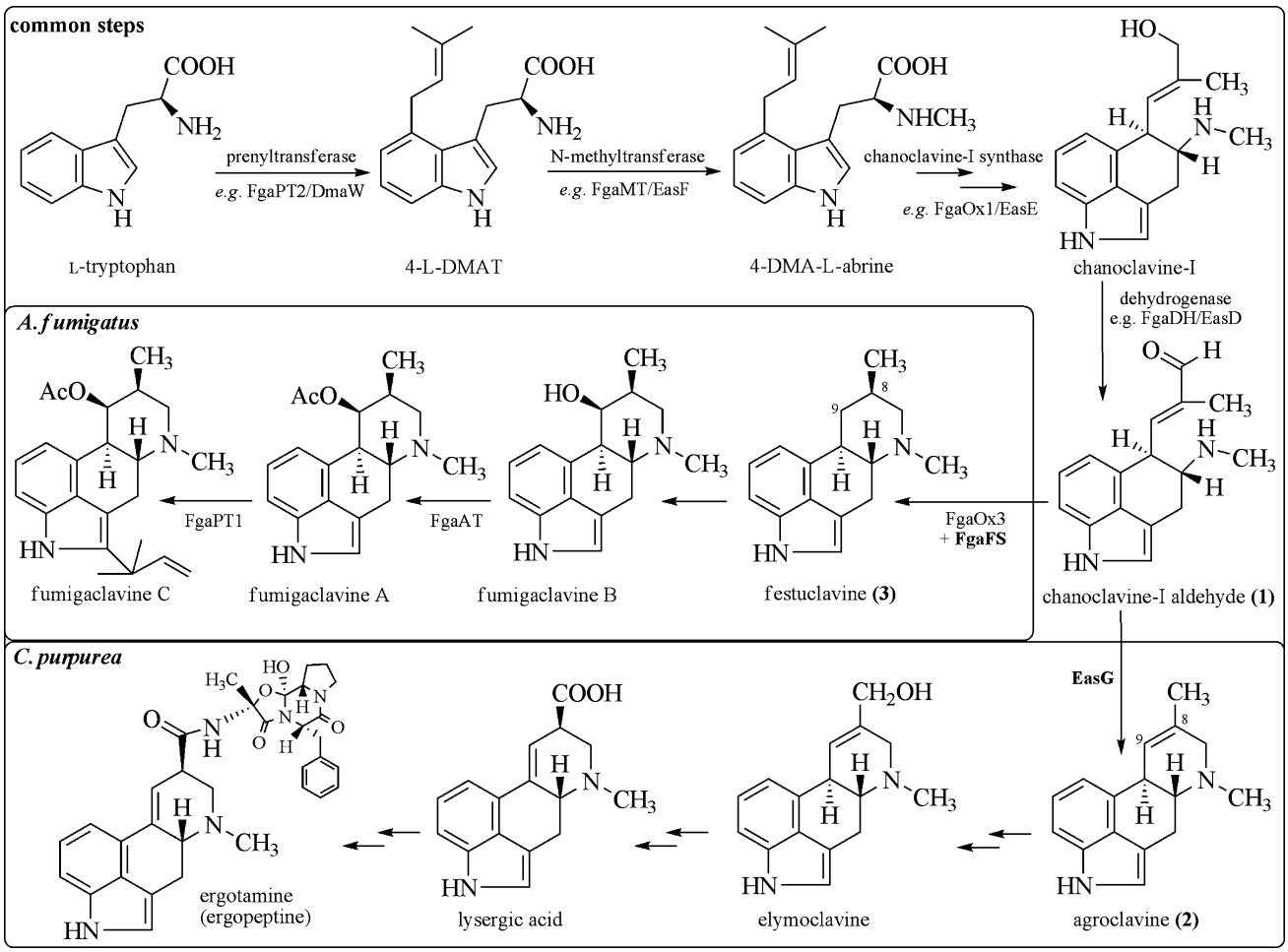


**Fig. 2** Putative biosynthetic pathways of ergot alkaloids in *Aspergillus fumigatus* and *Claviceps purpurea*.

the presence of EasA from *Neotyphodium lolii*, a homologue of FgaOx3 from *A. fumigatus*. These authors mentioned that EasA from *C. purpurea* was catalytically inactive after heterologous expression in *E. coli* due to a lack of the expected flavin cofactor.[21]

Here we report that EasA is not necessary for the conversion of **1** to **2**, at least in our *in vitro* experiments. EasG of *C. purpurea*, a homologue of FgaFS from *A. fumigatus*, is sufficient for the formation of **2** from **1** in the presence of NADPH, *via* a non-enzymatic adduct with reduced glutathione (GSH). The chemical structure of the enzyme product **2** was unequivocally proven by NMR and MS analyses including HSQC, HMBC and ROESY spectra.

## Results and discussion

### Sequence analysis and cloning of *easG* and *easA*

The deduced gene product of *easG* from *C. purpurea* (AY836771 in GenBank) comprises 290 amino acids and has a calculated molecular mass of 31.9 kDa. In the case of *easA* (AJ703809 in GenBank), the deduced gene product consists of 369 amino acids

and has a calculated molecular mass of 41.5 kDa. By using the program BLAST 2 SEQUENCES (http://blast.ncbi.nlm.nih.gov), EasG and EasA from *C. purpurea* share sequence identities of 47% and 58% with their homologues from *A. fumigatus*, *i.e.* FgaFS (CAG28312.1) and FgaOx3 (AAW57089.1), respectively. A structurally informed sequence alignment for EasG and FgaFS as well as for EasA and FgaOx3 is provided as Fig. S1 (ESI†).

For the overexpression in *E. coli*, the sequences of *easG* and *easA* from *C. purpurea* had been synthesised chemically by MWG Biotech AG and cloned into the NcoI and BamHI sites of the expression vector pQE60, resulting in the expression vectors pMM044 containing *easG* and pMM046 containing *easA*, respectively. The sequences in both vectors were also confirmed by sequencing.

### Overproduction and purification of $His_6$-EasG and $His_6$-EasA

For the expression of *easG* and *easA*, *E. coli* cells harbouring pMM044 and pMM046 were cultivated at 26 °C and 22 °C respectively, and were induced by addition of IPTG to a final concentration of 0.1 mM (see Experimental Section). The $His_6$-tagged EasG and EasA proteins were purified on Ni-NTA agarose. With pMM044, a major protein band with a migration above the 30 kDa size marker was detected (Fig. 3), as expected for the $His_6$-EasG with a calculated mass of 33.3 kDa. For EasA, a major protein band with a migration below the 45 kDa size marker was observed (Fig. 3), as expected for the $His_6$-EasA with a calculated mass of 41.5 kDa. The protein yield was 3.4 mg per litre of culture for purified $His_6$-EasG and 0.5 mg per litre of culture for purified $His_6$-EasA.

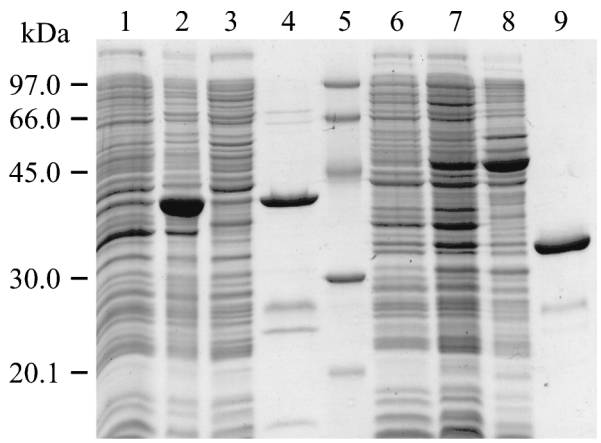


**Fig. 3** Analysis of the overproduction and purification of $His_6$-EasA (lanes 1-4) and $His_6$-EasG (lanes 6-9). The proteins were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1: Total proteins before induction; 2: Total proteins after induction; 3: Soluble proteins after induction; 4: Purified $His_6$-EasA; 5: Molecular weight standard; 6: Total proteins before induction; 7: Total proteins after induction; 8: Soluble proteins after induction; 9: Purified $His_6$-EasG.

### Enzyme activity and identification of the enzyme product

In a previous study, we have demonstrated the conversion of **1** to **3** by the festuclavine synthase FgaFS in the presence of the old yellow enzyme FgaOx3, both from *A. fumigatus*.[19] Initially, we speculated that the conversion of **1** to **2** in *C. purpurea* would be catalysed by their homologues EasG and EasA. Therefore, **1** was incubated with the purified EasG and EasA in the presence of NADH and FMN, which were successfully used in the enzyme assays for the conversion of **1** to **3**.[19] **1** is commercially not available and was therefore prepared enzymatically from chanoclavine-I with FgaDH according to Wallwey *et al.*[14] (See experimental section). Surprisingly, no product peak was observed in the HPLC chromatogram of the incubation mixture of **1** with EasG and EasA (Fig. 4B). Replacing NADH by NADPH did not improve the result (Fig. 4C). One explanation for this unexpected result could be that the recombinant proteins EasG and/or EasA were catalytically inactive, as speculated by Cheng *et al.*[22] However, this would be in contrast to the successful conversion of **1** by FgaFS and FgaOx3.[19] Another reason could be that the reaction conditions are not correct, *e.g.* a required cofactor is missing. Therefore, different cofactors were tested for the incubation mixtures of **1** with both EasG and EasA. The HPLC chromatogram of **1** with EasG and EasA, in the presence of 1 mM reduced GSH, showed a minor peak with the same retention time as that of **2** (Fig. 4D). A low conversion of 6% was observed under this condition. Interestingly, the incubation mixture without EasA also showed a similar profile in the HPLC chromatogram (Fig. 4E). However, the consumption of **1** was much higher than the product peak in Fig. 4D and 4E. This discrepancy is caused by the chemical reaction of GSH with **1**, which is clearly observed in the absence of the enzymes (see below and Fig. S1, ESI†). Addition of NADPH to the reaction mixture of EasG and GSH enhanced the product formation significantly (Fig. 4F). A conversion rate of 59% was detected in the reaction mixture. However, incubation of **1** with EasG and NADPH, lacking GSH, did not result in the formation of any product (Fig. 4G). Product formation was only detected in assays with active (Fig. 4F), but not with heat-inactivated EasG (Fig. 4H). In the presence of 1 mM GSH, product formation showed a linear dependence on the protein amount up to 0.1 μg (Fig. S2, ESI†) and on the incubation time up to 10 min (Fig. S3, ESI†). This proved that EasA was not necessary for the conversion of **1** to (very likely) **2**. EasG was sufficient for this conversion in the presence of GSH and NADPH. The low conversion without NADPH (Fig. 4E) can be explained by traces of NADPH in the purified EasG. A conversion of **2** to **3** was not observed in incubation mixtures with EasG and EasA under various reaction conditions (data not shown), indicating that agroclavine is not likely to be an intermediate to festuclavine.

To determine the structure, 1.5 mg of **2** were isolated on HPLC after incubation of **1** with EasG in the presence of GSH and NADPH, and subjected to NMR and MS analyses. The $^1$H-NMR spectrum of **2** in its protonated form corresponded perfectly to that of an authentic agroclavine sample after treatment with HPLC solvents containing trifluoroacetic acid (Fig. S13 and S14, ESI†). The identity of the agroclavine standard had been confirmed by comparison of the $^1$H-NMR and $^{13}$C-NMR data of its free base form (Table S1, Fig. S7–S11, ESI†) with the data published by Flieger *et al.*[23,24] and Ninomiya *et al.*[25] Assignments of $^1$H and $^{13}$C signals of the authentic agroclavine in its deprotonated form have been confirmed by DQF-COSY, HSQC, HMBC and ROESY and are given in Table S1 and the spectra are supplied as Fig. S9–12 (ESI†). These data proved unambiguously that **2** was agroclavine with a chemical formula of $C_{16}H_{18}N_2$, which was also confirmed

 

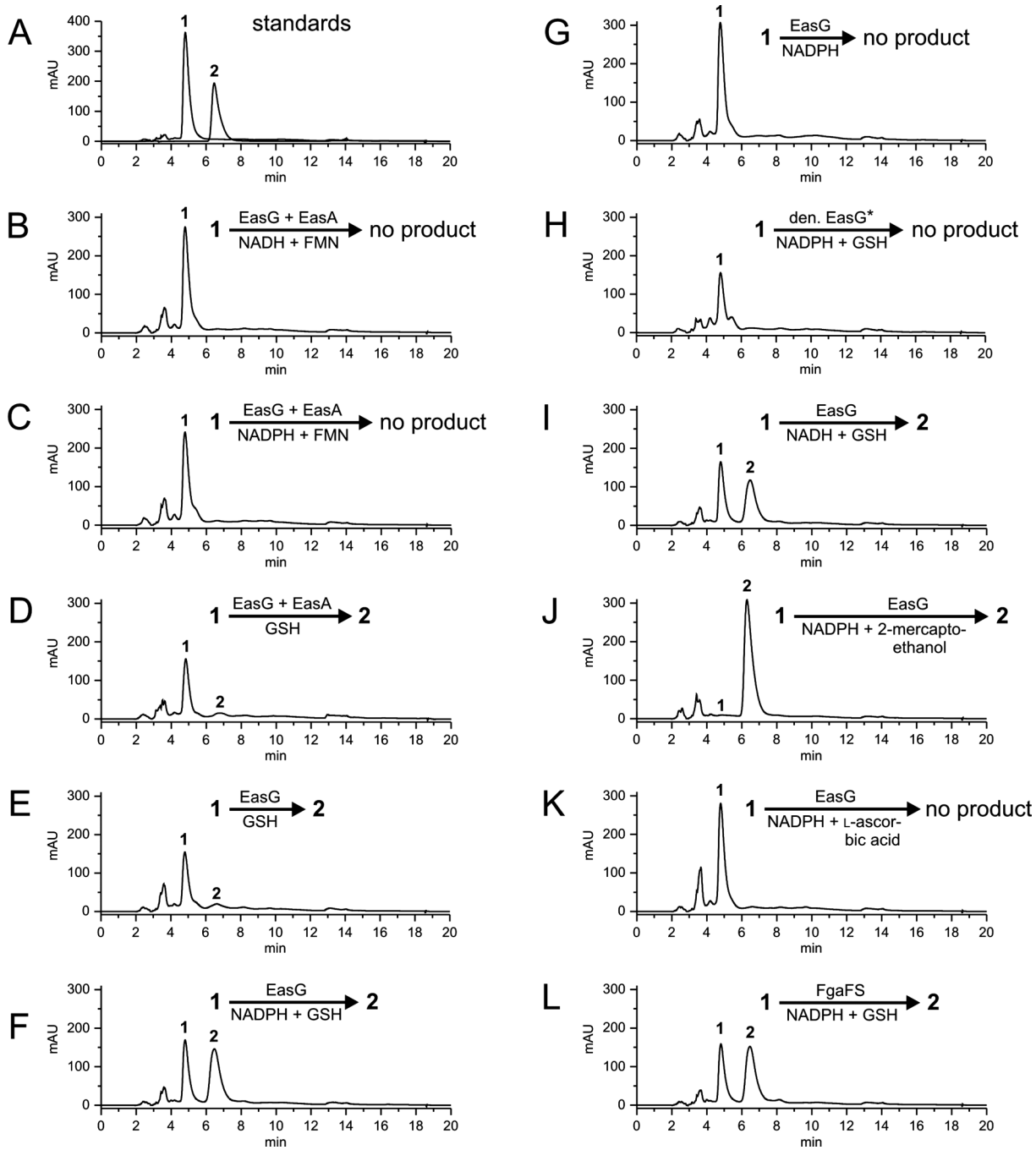


**Fig. 4** HPLC chromatograms of incubation mixtures under different conditions. The reaction mixtures contained 1.5 mM of the cofactors FMN, NADH or NADPH, 1 mM of GSH, 2-mercaptoethanol or L-ascorbic acid, 1 mM chanoclavine-I aldehyde and 2.5 μg of the recombinant enzymes and were incubated at 30 °C for 2 h. The substances were detected with a Photo Diode Array detector and illustrated for absorption at 282 nm. (*: heat-inactivated EasG by boiling for 60 min). **1**: chanoclavine-I aldehyde; **2**: agroclavine.

by detection of the $[M+1]^+$ ion at $m/z$ = 238.8 in the ESI-MS spectrum.

In summary, EasG from *C. purpurea*, together with GSH, catalyses the conversion of **1** to **2** in the presence of NADPH *in vitro* and therefore functions as an agroclavine synthase. These results also raised the question about the role of EasA *in vivo* in the biosynthesis of ergot alkaloids in *C. purpurea*. Inactivation of *easA* or its orthologues in a *Claviceps* strain would provide more information about its involvement in the biosynthesis of agroclavine.

NADPH and GSH can be replaced by NADH and other thiol compounds, respectively. An incubation mixture of **1** and GSH, with NADH, showed a comparable conversion rate of 54% to that with NADPH (Fig. 4I). Even higher conversion rates of 99 and 67% were obtained in the incubation mixtures with 2-mercaptoethanol (Fig. 4J) and dithiothreitol (data not

shown), respectively. GSH, 2-mercaptoethanol and dithiothreitol are reducing agents. GSH and its oxidised form, GSSG, play an important role as redox systems in living organisms.[26–28] On the other hand, GSH also serves as a thiolation agent, *e.g.* in the detoxification of formaldehyde.[29] No product formation was detected in the incubation mixture with L-ascorbic acid instead of GSH (Fig. 4K), indicating that GSH, 2-mercaptoethanol and DTT act very likely as thiolation agents in the conversion of **1**.

The results obtained from this study promoted us to re-investigate the enzyme reaction catalysed by FgaFS and FgaOx3 from *A. fumigatus* in the presence and absence of GSH. When we incubated FgaFS with NADPH and GSH in the absence of FgaOx3, formation of product **2** with a conversion rate of 60% was observed (Fig. 4L). ESI-MS analysis of the isolated product from this incubation mixture (Fig. 4L) confirmed the presence of **2** and the absence of **3** by detection of an ion at $m/z$ for [M+1] of **2**. This indicates that, together with GSH, FgaFS also catalyses the conversion of **1** to **2** in the absence of FgaOx3. However, the production of **2** by *A. fumigatus* has not been reported in the literature.

To further investigate these contradictory results, we carried out additional incubations. An incubation of **1** with FgaFS and FgaOx3, in the presence of NADPH and FMN, resulted in the formation of **3** (Fig. 5B), as reported previously.[19] Addition of 1 mM GSH to the reaction mixture did not change the result (Fig. 5C). The formation of a small amount of **2** was observed, when 25 mM GSH was added to the reaction mixture (Fig. 5D). The presence of **2** and **3** in the reaction mixture was confirmed by detection of their $[M+1]^+$ ions at $m/z$ = 238.9 and 240.9, respectively. These results clearly demonstrated that in the presence of FgaOx3 from *A. fumigatus*, the reaction of **1** with an involvement of GSH was not preferred and **3** was the main enzyme product. This explains why **3** instead of **2** has been identified in *A. fumigatus*. In *C. purpurea*, it is plausible that EasG converts **1** to **2** with the help of cellular GSH, which is usually found in a concentration range of 0.1–10 mM in living cells.[27]

Incubation of **1** with EasA from *C. purpurea* and FgaFS from *A. fumigatus*, in the presence of NADH and FMN, resulted in the formation of neither **3** nor any other products (Fig. 5E). This indicates that EasA was catalytically inactive or the role of FgaOx3 in the conversion of **1** to **3** could not be replaced by EasA. Incubation of **1** with EasA and FgaFS in the presence of additional GSH resulted in the formation of **2** (Fig. 5F), being identical to the results with FgaFS, GSH and NADPH (see also Fig. 4L).

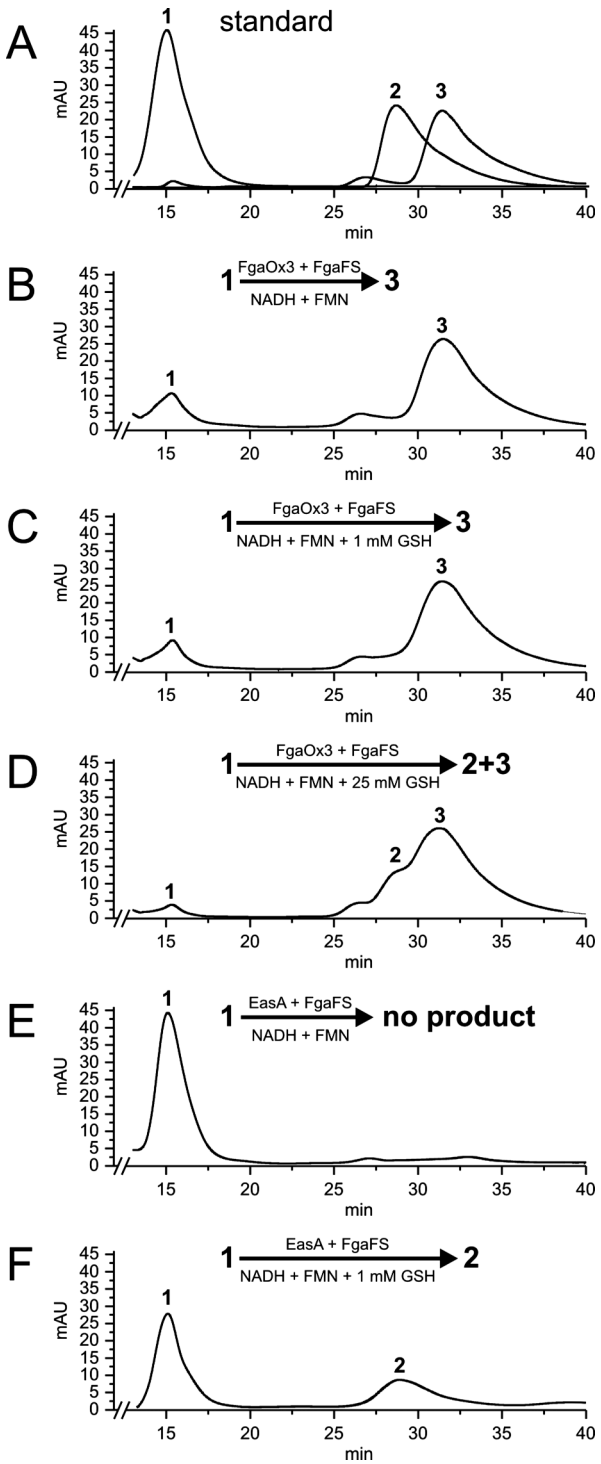


**Fig. 5** HPLC chromatograms of the product formation with different enzyme and cofactor combinations. The reaction mixtures contained 5 mM of the cofactors FMN and NADH, 1 mM or 25 mM of GSH, 1 mM chanoclavine-I aldehyde and 5 μg of the recombinant enzymes and were incubated at 30 °C for 2 h. The substances were detected with a Photo Diode Array detector and illustrated for absorption at 282 nm. **1**: chanoclavine-I aldehyde; **2**: agroclavine; **3**: festuclavine.

### Non-enzymatic reaction of GSH with chanoclavine-I aldehyde and proposed mechanism of the EasG reaction

In the enzyme assays with low yields for **2** (Fig. 4D and 4E), consumption of **1** was much higher than the product formation, indicating the involvement of a non-enzymatic reaction between **1** and GSH. To investigate this reaction, we incubated **1** only with GSH at 30 °C. Dependencies of the decreasing of **1** on GSH concentrations up to 10 mM and on time with GSH at 5 mM and **1** at 1 mM are given in Fig. S4 and S5 (ESI†). About 22, 60 and 80% of **1** were consumed in the incubation mixtures with 1, 3 and 5 mM GSH for 80 min, respectively. With 5 mM GSH, 40% of **1** was consumed after 15 min. This demonstrated clearly that chemical reactions between **1** and GSH had taken place. It could be speculated that GSH attacks C9 of **1**, as observed for thiols with other α,β-unsaturated aldehydes,[30,31] resulting in the formation of the enolate of a thiolated saturated aldehyde **4a** (Fig. 6). Different

 

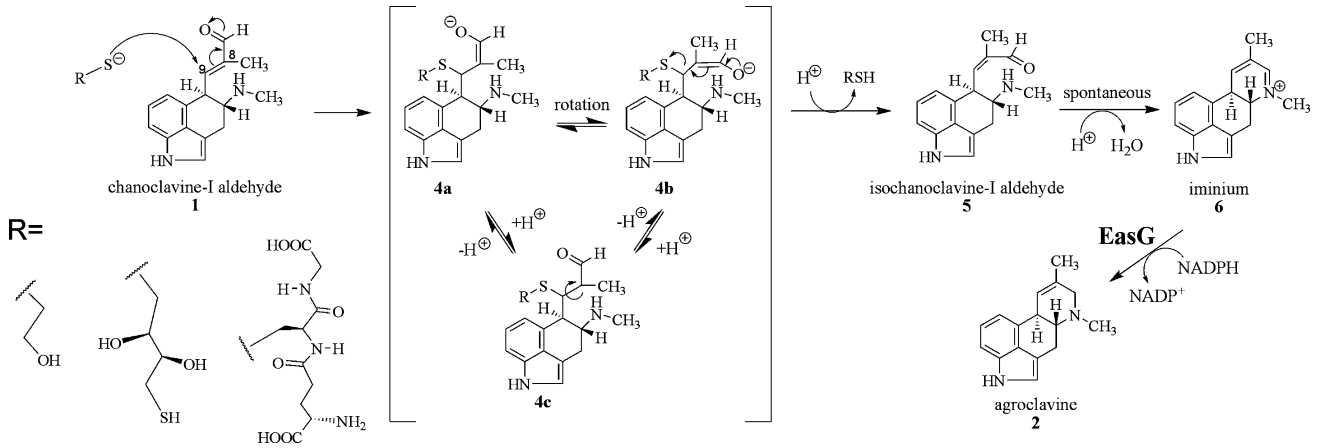


**Fig. 6** Proposed mechanism of the conversion of chanoclavine-I aldehyde (**1**) to agroclavine (**2**) by non-enzymatic isomerisation with the help of GSH and reduction of an iminium by EasG.

forms of **4a**, *e.g.* **4b** and **4c**, can be expected after protonation and single bond rotation. Elimination of the thiol group from **4b** would lead to the formation of isochanoclavine-I aldehyde, **5**, which would undergo spontaneous cyclisation to the iminium, **6**, by elimination of one molecule of water. Reduction of **6** by EasG in the presence of NADPH would result in the formation of agroclavine, **2** (Fig. 6). According to this hypothesis, the reaction catalysed by EasG from *C. purpurea* is very similar to that by its homologue FgaFS from *A. fumigatus*, *i.e.* a reduction reaction on a double bond of an iminium ion.[19]

Attempts to identify reaction products of **1** with GSH, such as **4a**, **4b** or **4c**, failed. No noticeable peaks were observed in the HPLC chromatograms of **1** with GSH, GSH and NADPH or GSH with NADPH and EasG (Fig. S6, ESI†). As demonstrated above, **2** was detected in the last case. In the reaction mixtures of **1** with GSH in the presence and absence of NADPH, insoluble black precipitates were observed. Different solvents, *e.g.* acetone, methanol or DMSO, were tested to dissolve the precipitate for MS and NMR analysis, but without any success.

We therefore decided to investigate the reactions of **1** with 2-mercaptoethanol and dithiothreitol, under the same conditions as for GSH (Fig. S6, ESI†). In the chromatograms of the incubation mixtures of **1** with 2-mercaptoethanol and DTT without EasG, an additional peak was detected at 6.7 and 8.1 min, respectively (Fig. S6A and S6B, ESI†). These peaks were also found in the incubation mixtures containing NADPH and EasG (Fig. S6C, ESI†). Both peaks were isolated by HPLC and incubated with EasG in the presence of NADPH. None of them were converted by EasG (Fig. S7, ESI†). MS analysis provided no indication for the presence of intermediates mentioned in Fig. 6.

Detailed inspection of the HPLC chromatogram of the incubation mixture of **1** with DTT revealed the presence of an additional minor peak at 6.5 min (Fig. S6 and S7, ESI†). This peak was also isolated on HPLC and subjected to MS analysis. ESI-MS showed an ion at $m/z$ = 409.1, corresponding to the $[M+1]^+$ of **4c**. More importantly, incubation of the isolated product with EasG, in the presence of NADPH, resulted in the formation of a product with the same retention time as that of **2** (Fig. S7A, ESI†). Due to the low amount, a structure elucidation by NMR was not possible in the present study. Furthermore, the isolated substance was unstable at room temperature and the peak at 8.1 min was detected as the dominant one in the isolated sample (Fig. S7B, ESI†).

## Conclusions

In this study, we demonstrated clearly that the conversion of chanoclavine-I aldehyde to agroclavine was catalysed by EasG of *C. purpurea* in the presence of NADPH, *via* a non-enzymatic adduct with reduced glutathione. Its homologue FgaFS of *Aspergillus fumigatus* catalyses this conversion under the same conditions as well. However, in the presence of FgaOx3, FgaFS converted chanoclavine-I aldehyde to festuclavine, even in the presence of 1 mM GSH. It can therefore be concluded that chanoclavine-I aldehyde could be converted to agroclavine in *C. purpurea* by EasG after double bond isomerisation by GSH and to festuclavine in *A. fumigatus* by the two enzymes FgaFS and FgaOx3 together.

## 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). A codon optimisation was done for the expression of the genes *easG* and *easA* in *E. coli* XL1 Blue MRF′ cells using the program GENEius (www.genius.de).

### Chemicals

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

### Bacterial strains, plasmids and cultural conditions

The pQE60 vector was obtained from Qiagen (Hilden, Germany.

*Escherichia coli* XL1 Blue MRF′ (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 or 22 °C.[32] Carbenicillin (50 μg ml$^{-1}$) was used for selection of recombinant *E. coli* strains.

### Chemical synthesis, DNA isolation and cloning

Standard procedures for DNA isolation and manipulation were performed as described.[32] The genes *easG* and *easA* were synthesized chemically and cloned into pCR2.1 and pBluescript II SK (+) (MWG Biotech AG, Ebersberg, Germany), resulting in plasmid pMM043 and pMM045, respectively. For cloning in the expression vector pQE60, a NcoI restriction site was added to the start codon. The original stop codon was removed by insertion of a BamHI restriction site.

To create the expression vector pMM044 for *easG* and pMM046 for *easA*, the plasmids pMM043 and pMM045 were digested with NcoI and BamHI. The resulted NcoI–BamHI fragments of 872 bp and 1109 bp, respectively, were isolated from the agarose gel and ligated into pQE60, which had also been digested with NcoI and BamHI, previously.

### Overproduction and purification of $His_6$-EasG and $His_6$-EasA

For expression of the genes *easG* and *easA*, *E. coli* XL1 Blue MRF′ cells harbouring the plasmids pMM044 and pMM046 were cultivated in 2000 ml Erlenmeyer flasks containing liquid Terrific–Broth medium (1000 ml) supplemented with carbenicillin (50 μg ml$^{-1}$) 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 0.1 mM and the cells containing the plasmid pMM044 were cultivated for 15 h at 26 °C before harvest. The cells containing the plasmid pMM046 were cultivated for 15 h at 22 °C before harvest.

The bacterial cultures were centrifuged and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.0) at 1.4 ml per gram wet weight. After addition of 1 mg ml$^{-1}$ lysozyme, 10 μg ml$^{-1}$ RNaseI, 5 μg ml$^{-1}$ DNaseI 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 × *g* for 30 min at 4 °C. One-step purification of the recombinant $His_6$-tagged fusion proteins by affinity chromatography with Ni-NTA agarose resin (Qiagen, Hilden, Germany) was carried out according to the manufacturer's instructions. The proteins were eluted with 250 mM imidazole in 50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.0. In order to change the buffer, the protein fractions were 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 analysed by SDS-PAGE according to the method of Laemmli[33] and stained with Coomassie Brilliant Blue R-250.

### Determination of the molecular mass of active $His_6$-EasG and $His_6$-EasA

The molecular mass of the recombinant $His_6$-EasG and $His_6$-EasA 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). The molecular mass of the recombinant $His_6$-EasG was determined to be 32.6 kDa. This suggests that the native EasG is a monomer. For the recombinant $His_6$-EasA, the molecular mass was determined to be 114 kDa. This suggests that the native EasA is likely a trimer.

### Enzyme assays with $His_6$-EasG and $His_6$-EasA

All of the enzyme assays contained 50 mM Tris-HCl, pH 7.5 and 1.3–3.0% (*v*/*v*) of glycerol. The reaction mixtures were incubated at 30 °C for 2 h and 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 100 μl methanol. The enzyme products were analysed on a HPLC system described below.

The reaction mixture for isolation of **1** (120 ml) contained chanoclavine-I (1 mM), 12 mg (0.86 μM) of purified FgaDH and 5 mM NAD. The reaction mixture was incubated for 4 h at 30 °C.

The reaction mixture for isolation of the enzyme product, **2**, for structural elucidation (30 ml) contained chanoclavine-I (1 mM), 3 mg (0.86 μM) of purified FgaDH, 3 mg (3 μM) of purified EasG, 5 mM NAD, 5mM NADPH and 10 mM GSH. After incubation for 15 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 30 °C to dryness. The residue was dissolved in 350 μl methanol and centrifuged at 14 000 × *g* for 30 min at 4 °C before injection onto HPLC for isolation of **2**.

### HPLC analysis and isolation of agroclavine

Reaction mixtures were analysed 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$^{-1}$. 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 analysed with a gradient from 38 to 54% B over 10 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 38% solvent B for 5 min. For better separation of **2** and **3** (Fig. 5), the substances were eluted with 21% B for 38 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 21% solvent B for 5 min. For isolation of the enzyme product **2**, a gradient from 30 to 37% B over 28 min was used. After washing with 100% solvent B for 5 min, the column was equilibrated with 30% 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.

Non-enzymatic products of **1** with thiols were analysed, after incubation at 30 °C for 5 or 16 h, on the same equipment and with the same solvents aforementioned. After running with 10% B for 14 min, a gradient from 10 to 74% B over 17 min was used. The column was then washed with 100% solvent B for 5 min, and equilibrated with 10% solvent B for 5 min.

 

### 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.[34] ROESY experiment was performed in phase-sensitive mode.[35]

For all two-dimensional spectra, 16 transients were used. For ROESY spectra, a mixing time of 300 ms and a relaxation delay of 3 s were used. $^1$H spectra were acquired with 65 536 data points, while 2D spectra were collected using 4096 points in the $F_2$ dimension and 512 increments in the $F_1$ dimension. Chemical shifts were referenced to $CD_3OD$. 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 with an AutoSpec instrument (Micromass Co. UK Ltd).

### Nucleotide sequence accession numbers

The coding sequences of *easG* and *easA* from *C. purpurea* are available at GenBank under the accession numbers AY836771 and AJ703809.

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