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FtmOx1, a non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase, catalyses the endoperoxide formation of verruculogen in *Aspergillus fumigatus*†

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Verruculogen is a tremorgenic mycotoxin and contains an endoperoxide bond. In this study, we describe the cloning, overexpression and purification of a non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase FtmOx1 from *Aspergillus fumigatus*, which catalyses the converstion of fumitremorgin B to verruculogen by inserting an endoperoxide bond between two prenyl moieties. Incubation with $^{18}O_2$-enriched atmosphere demonstrated that both oxygen atoms of the endoperoxide bond are derived from one molecule of O_2. FtmOx1 is the first endoperoxide-forming non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase reported so far. A mechanism of FtmOx1-catalysed verruculogen formation is postulated and discussed.

Introduction

Verruculogen (Scheme 1) is a tremorgenic mycotoxin from various *Aspergillus* and *Penicillium* strains.[1] Recently, it was reported that verruculogen associated with *Aspergillus fumigatus* hyphae and conidia modified the electrophysiological properties of human nasal epithelial cells.[2] Feeding experiments showed that L-tryptophan, L-proline, mevalonate and methionine are precursors in the biosynthesis of verruculogen.[3,4] In comparison to another tremorgenic mycotoxin fumitremorgin B (Scheme 1), verruculogen carries an endoperoxide bond linking the two prenyl moieties. Therefore, the former compound could be considered as a biosynthetic precursor of the latter. Co-existence of both compounds in different strains also suggests their biosynthetic relationship.[5–7] However, based on the results obtained from feeding experiments with TR-2, a minor metabolite from *Aspergillus fumigatus*, Willingale *et al.*[8] speculated that the conversion of fumitremorgin B to verruculogen would be a spontaneous reaction upon addition of oxygen rather than an enzymatic process. Molecular biological and biochemical investigations on the biosynthesis of verruculogen have not been reported yet.

The key step in the biosynthesis of verruculogen is the formation of the endoperoxide bond, which is also found in the structures of the antimalarial agent artemisinin from *Artemisia annua*[9] and prostaglandin G2 (PGG2) involved in inflammatory processes.[10] The well-investigated constitutively expressed cyclooxygenase (COX) I and the inducible isoform COX II are responsible for endoperoxide formation in PGG2 *via* a peroxide radical.[10] The active centres of these enzymes harbour an Fe(II)-containing heme group.[10] In contrast, little is known about the endoperoxide formation in the biosynthesis of artemisinin.[9] From the genome sequence of *Aspergillus fumigatus* Af293, a cluster containing nine putative biosynthetic genes was identified on chromosome 8 by using a bioinformatic approach.[11] Homologous gene clusters could also be identified in the genome sequences of *Aspergillus fumigatus* A1163 and *Neosartorya fischeri* NRRL181 with sequence identities of the gene products of 82–100% to those of Af293.[12] Our previous investigations showed that *ftmPS*, *ftmPT1* and *ftmPT2* from this cluster, encoding a non-ribosomal peptide synthetase and two prenyltransferases, respectively, are involved in the biosynthesis of fumitremorgin B.[11,13,14] We have speculated that the end product of this gene cluster could be fumitremorgin B,[11] but it could not be excluded that verruculogen or even fumitremorgin A (Scheme 1) is the true end product instead.[14] However, no homologue of a COX involved in the biosynthesis of PGG2 could be detected in this cluster.

Recently, Kato *et al.*[15] reported the involvement of three cytochrome P450s, *i.e.* FtmC, FtmE and FtmG (also termed FtmP450-1, FtmP450-2 and FtmP450-3[12]) from this cluster in the biosynthesis of fumitremorgin B. They catalysed the conversion of tryprostatin B to 6-hydroxytryprostatin B, tryprostatin A to fumitremorgin C, and fumitremorgin C to 12,13-dihydroxyfumitremorgin C, respectively (Scheme 1). This leaves three genes in the gene cluster with unknown function, namely *ftmOx1* (also called *ftmF*), *ftmO* (*ftmI*) and *ftmMT* (*ftmD*). *ftmMT* (AFUA_8G00200 according to genome annotation) showed sequence similarity to methyltransferases and could be responsible for the conversion of 6-hydroxytryprostatin B to tryprostatin A.[12,15] *ftmO* (AFUA_8G00260) is likely not involved in the biosynthesis.[15] *ftmOx1* (AFUA_8G00230) shows sequence homology to α-ketoglutarate-dependent dioxygenases (see below) and could be involved in an oxidoreduction process. As shown in Scheme 1, no additional genes/enzymes are required for the biosynthesis of fumitremorgin B, at least *in vitro*. Therefore,

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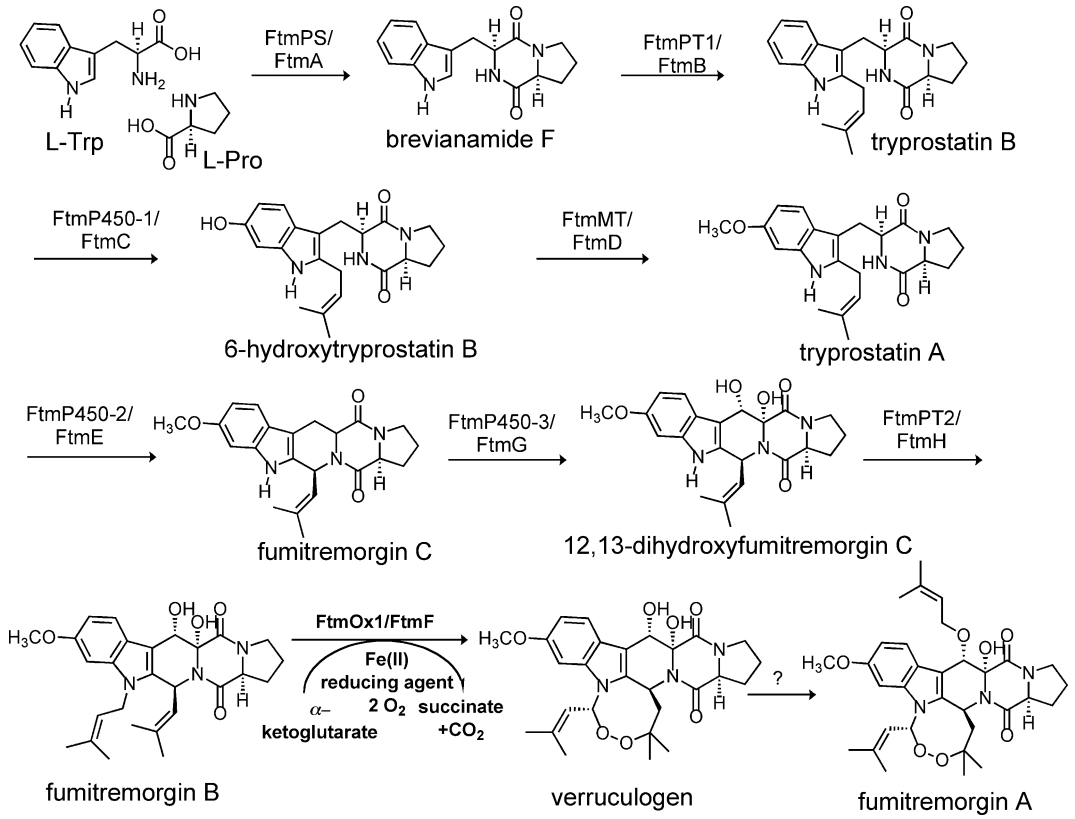


Scheme 1 Putative biosynthetic pathway of verruculogen and fumitremorgin A in *Aspergillus fumigatus* Af293. FtmPS (FtmA, Accession No. EAL85149.1): nonribosomal peptide synthetase; FtmPT1 (FtmB, EAL85145.1): brevianamide F prenyltransferase; FtmP450-1 (FtmC, EAL85147.1): Cytochrome P450 enzyme; FtmMT (FtmD, EAL85146.1): putative methyltransferase; FtmP450-2 (FtmE, EAL85144.1): Cytochrome P450 enzyme; FtmP450-3 (FtmG, EAL85142.1): Cytochrome P450 enzyme; FtmPT2 (FtmH, EAL85141.1): 12,13-dihydroxyfumitremorgin C prenyltransferase; FtmOx1 (FtmF): α-ketoglutarate and Fe(II) dependent dioxygenase.

ftmOx1 should be involved in the metabolism of fumitremorgin B, if it is a structure gene belonging to the cluster. In this study, we report the function of FtmOx1 as a non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase catalysing the conversion of fumitremorgin B to verruculogen.

Results and discussion

FtmOx1 shows clear sequence homology to α-ketoglutarate-dependent dioxygenases. For example, FtmOx1 (= EAL85143.1) shares a sequence identity of 29% on the amino acid level with Fum3p, an α-ketoglutarate-dependent non-heme Fe(II) dioxygenase in the biosynthesis of fumonisin in *Fusarium verticilloides*. Fum3p was shown to catalyse the hydroxylation of fumonisin B_3 to fumonisin B_1.[16,17] In addition, FtmOx1 showed significant sequence similarity to eukaryotic phytanoyl-CoA-hydroxylases (PhyH) (ESI, Fig. S1†), which catalyse the hydroxylation of phytanoyl-CoA, derived from the side chain of chlorophyll, in the α-position to a keto group.[18] Members of the PhyH superfamily also belong to the α-ketoglutarate-dependent dioxygenases. Furthermore, the typical binding motif for Fe(II) and α-ketoglutarate, His(129)-X-Asp(131)-X_{73}-His(205)-X_{12}-Arg(218),[19,20] could be found in the amino acid sequence of FtmOx1 (ESI, Fig. S1). Therefore, we speculate that the gene product FtmOx1 could be an Fe(II)- and α-ketoglutarate-dependent dioxygenase and responsible for the endoperoxide formation of verruculogen by two hydroxylation steps at the two prenyl moieties of fumitremorgin B and subsequent oxidative linking of these two hydroxy groups or by direct incorporation of both oxygen atoms from a molecular oxygen (O_2) between the two prenyl moieties.

To prove its function, *ftmOx1* consisting of only one exon of 876 bp was amplified by PCR from genomic DNA of the genome reference strain *A. fumigatus* Af293 in form of BAC plasmid AfB8B11. The PCR fragment was then ligated into the cloning vector pGEM-T. After confirming of the sequence, the gene was then cloned into the expression vector pQE9 *via* BamHI and HindIII. *E. coli* XL1 blue MRF′ cells harbouring the final expression construct pAG025 were induced by isopropyl-β-thiogalactoside (1 mM) at 37 °C for 16 hours. SDS-PAGE analysis showed that the desired N-terminal His_6-FtmOx1 could be successfully overproduced and purified to apparent homogeneity (Fig. 1). The observed size of the purified protein was 38 kDa, which corresponded very well to the calculated molecular mass of 35.1 kDa. A protein yield of 10 mg of pure His_6-FtmOx1 per litre culture could be obtained. Using gel permeation chromatography, the native molecular mass was determined as 81 kDa, suggesting that the native protein was a homodimer.

Incubation of the purified FtmOx1 with fumitremorgin B in the presence of α-ketoglutarate and ascorbate resulted clearly in

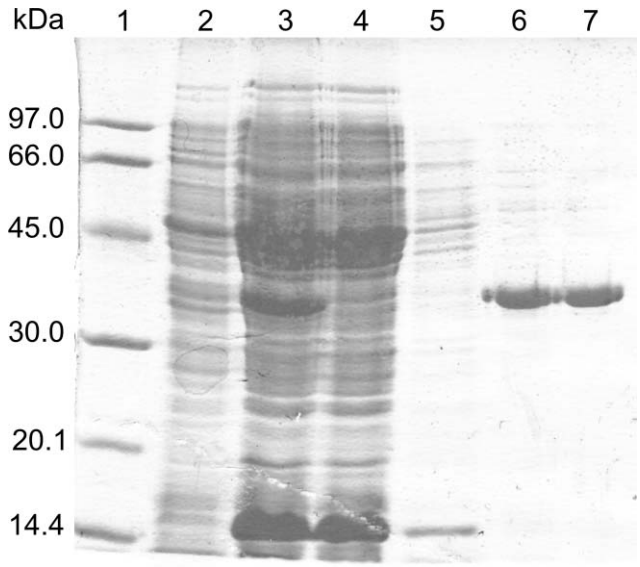


Fig. 1 Purification of His_6-FtmOx1 after gene expression. The SDS polyacrylamide gel (12% *w*/*v*) was stained with Coomassie brilliant blue R-250. Lane 1: molecular mass standard; lane 2: soluble protein before induction; lane 3: soluble protein after induction for 16 hours; lane 4: flow through; lane 5: wash fraction; lanes 6 and 7: purified His_6-FtmOx1.

product formation, as demonstrated by HPLC analysis (Fig. 2). HPLC chromatogram of the complete assay (Fig. 2B) showed the presence of two product peaks with retention times of 7.8 and 12.5 min, respectively, which were absent in the reaction mixture with heat-inactivated enzyme (Fig. 2C). The peak at 12.5 min had the same retention time as that of an authentic verruculogen sample (Fig. 2A), which was also confirmed by LC-MS analysis (ESI, Fig. S2†). In the negative ESI mode, $[M - 1]^-$ at *m*/*z* 510 could be clearly detected in both verruculogen standard and enzymatic product. In the positive ESI mode, $[M + 1]^+$ at *m*/*z* 512 was poorly detected in both samples, being consistent with the results reported by other groups.[2,21] However, $[M + Na]^+$ at *m*/*z* 534 and $[M + H - H_2O]^+$ at *m*/*z* 494 could clearly be detected in both samples. In addition, the dissociation MS-MS spectrum of the ion at *m*/*z* 494 of the enzymatic product showed an identical fragmentation pattern to that of authentic verruculogen (Fig. S2). Therefore, the enzymatic product at 12.5 min was unequivocally identified as verruculogen. An additional peak at 11.7 min could be found in all of the reaction mixtures (Figs. 2B–F). Its peak area was significantly smaller in the reaction mixture with native enzyme than in the assays with inactivated protein, as well as those with EDTA or without α-ketoglutarate or ascorbate, indicating that this substance was formed non-enzymatically from fumitremorgin B and might also function as substrate of FtmOx1. To prove this hypothesis, the peak at 11.7 min was isolated by HPLC and incubated with active and heat-inactivated FtmOx1 in the presence of α-ketoglutarate and ascorbate. As shown in Fig. 2G, almost no change was observed in the reaction mixture with heat-inactivated enzyme. In the presence of active FtmOx1 (Fig. 2H), however, the peak area was significantly reduced and new peaks, including one at 7.8 min, were detected. This peak was also observed in the enzyme assay with fumitremorgin B (Fig. 2B). This demonstrated that the peak at 7.8 min was an enzymatic product of a degradation product of fumitremorgin B, but is very likely not involved in the

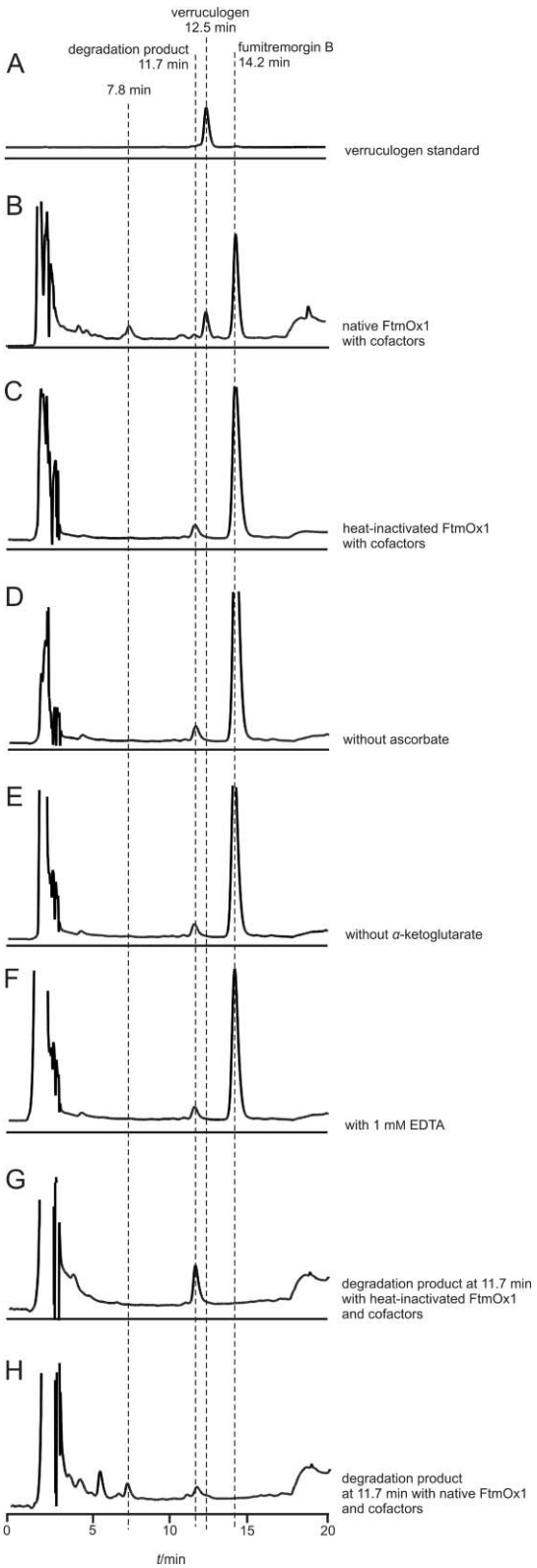


Fig. 2 HPLC chromatograms of the FtmOx1 assays.

conversion of the latter compound to verruculogen. Due to the low amount, the structures of the peaks at 7.8 and 11.7 min could not be identified in this study. As a consequence of the low stability of fumitremorgin B in the enzyme assay and involvement of FtmOx1

Table 1 Dependency of FtmOx1 activity on divalent metal ions

Enzyme treatment[a]	Metal ion added[b]	Relative activity [%]
No treatment	None	100.0
EDTA	None	≤1.0
EDTA	Ca(II)	4.8
EDTA	Co(II)	≤1.0
EDTA	Cu(II)	≤1.0
EDTA	Fe(II)	30.0
EDTA	Mg(II)	2.0
EDTA	Mn(II)	≤1.0
EDTA	Zn(II)	≤1.0

[a] FtmOx1 was treated with 1 mM EDTA for 1 h. Afterwards, the chelating agent was removed by gel filtration. [b] Metal ions were added to achieve a final concentration of 1 mM.

in the conversion of the degradation product, it was not possible to determine the kinetic parameters of FtmOx1 for the formation of verruculogen.

The formation of verruculogen was strictly dependent on the presence of α-ketoglutarate and ascorbate. Succinate formation by FtmOx1 could be clearly detected by using a succinic acid test kit (see ESI†) (data not shown). Incubation of fumitremorgin B with FtmOx1 in the absence of α-ketoglutarate or ascorbate did not result in formation of any enzymatic product (Figs. 2D and 2E). Addition of EDTA (1 mM) to the reaction mixture abolished the enzymatic formation of verruculogen too (Fig. 2F), indicating the involvement of metal ions in the enzymatic reaction.

To provide evidence that Fe(II) is required for the enzymatic reaction, assays were carried out with FtmOx1, which had been previously treated with EDTA, and divalent metal ions at a final concentration of 1 mM (Table 1). Incubations with untreated FtmOx1 and treated FtmOx1 without addition of metal ions were used as positive and negative controls, respectively. It could be shown that incubation without addition of metal ions to the treated FtmOx1 assay did not result in formation of verruculogen. After addition of Fe(II), 30.0% of the initial enzymatic activity before treatment with EDTA could be restored. 4.8 and 2.0% of the initial activity could be recovered after addition of Ca(II) and Mg(II), respectively. No enzymatic activity was detected after addition of other ions such as Mn(II), Co(II), Cu(II) or Zn(II). These results confirmed that the formation of verruculogen was dependent on the presence of Fe(II) ions and that FtmOx1 belongs to the group of Fe(II) and α-ketoglutarate-dependent enzymes.

Members of the α-ketoglutarate-dependent dioxygenases have been identified in a large range of organisms, including bacteria, fungi, plants and mammals.[20,22–26] Some of these enzymes are involved in the biosynthesis of pharmaceutically important compounds *e.g.* cephalosporins, scopolamine, vinblastine and vincristine.[26–28] They catalyse a variety of two-electron oxidations, including hydroxylation, desaturation, epoxydation, ring expansions and ring-closure reactions.[19,26,29] But until now, no endoperoxide formation had been described for this enzyme group. Therefore, FtmOx1 is the first endoperoxide-forming α-ketoglutarate-dependent dioxygenase reported so far.

To elucidate the mechanism of this fascinating novel reaction, FtmOx1 assays were carried out in an $^{18}O_2$-enriched atmosphere and the enzymatic product was analysed by LC-MS using a verruculogen standard as control. As observed in the other experiment mentioned above, ions corresponding to $[M + H - H_2O]^+$, $[M + H]^+$ and $[M + Na]^+$ could be detected at *m/z* 494, 512 and 534 for the verruculogen standard, respectively (Fig. 3A). These ions could also be clearly detected for verruculogen obtained by incubation in

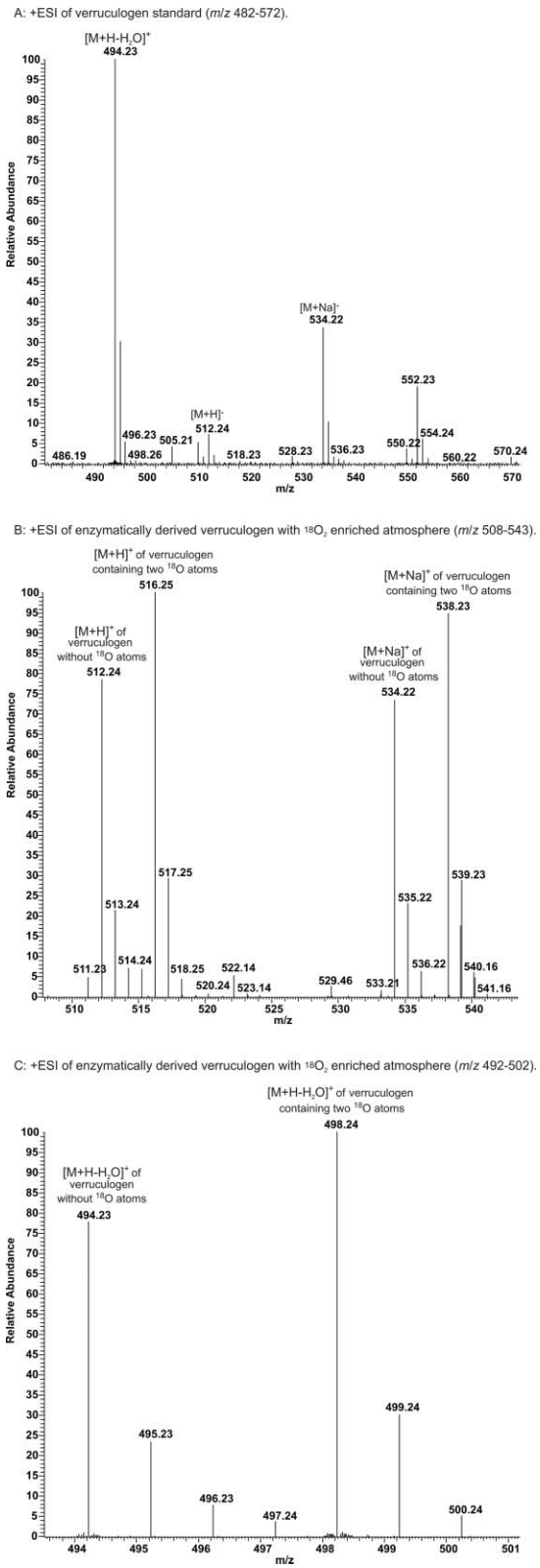
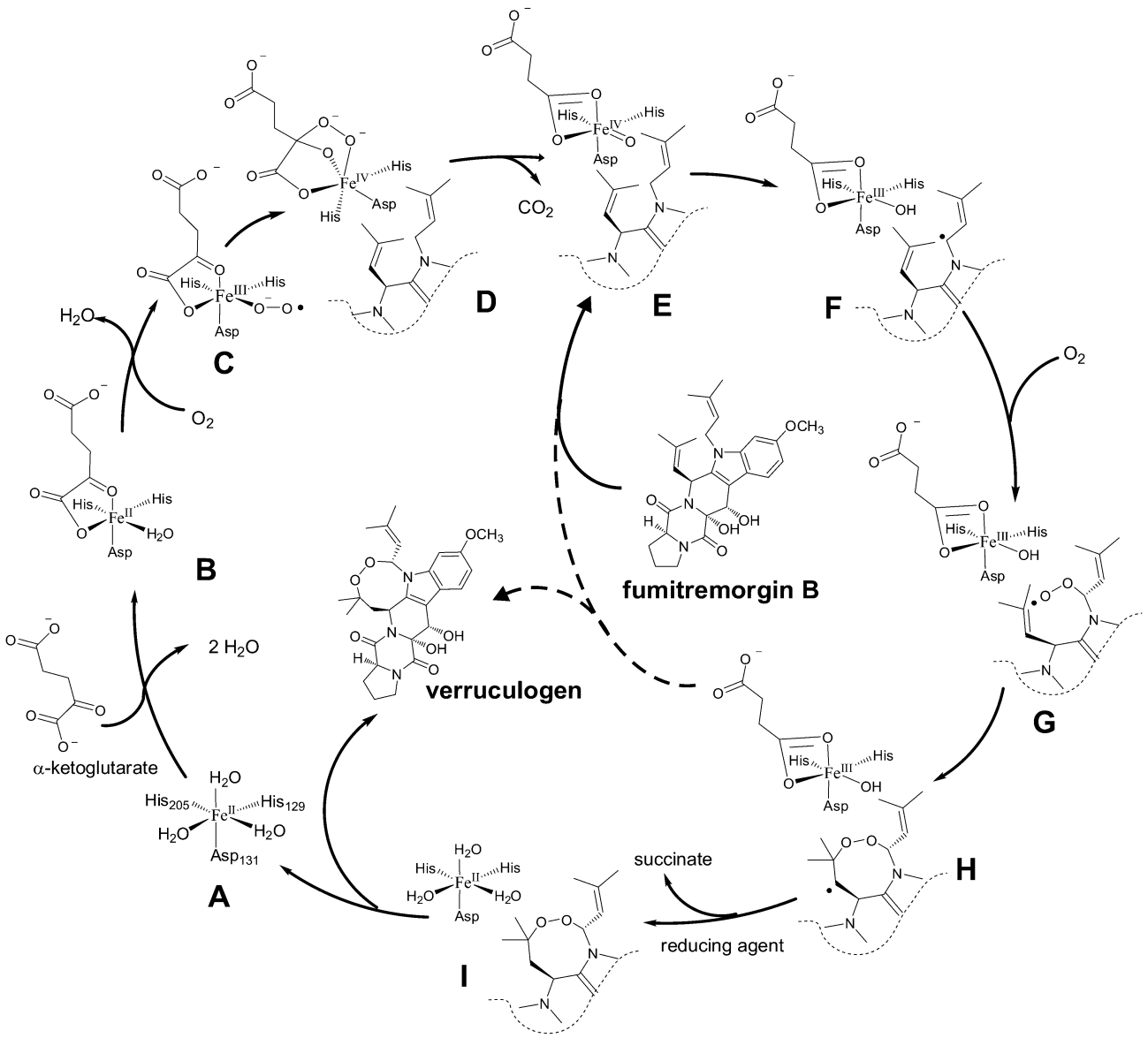


Fig. 3 ESI+ of verruculogen standard (A) and enzymatically derived verruculogen (B and C) from assays in an $^{18}O_2$-enriched atmosphere.



Scheme 2 Putative mechanism of verruculogen formation by FtmOx1. The key step is the formation of a radical at the α-position to C=C of the *N*-prenyl moiety by reduction of the Fe(IV)=O species to Fe(III)–OH. *In vitro*, ascorbate was used to regenerate of Fe(III) to Fe(II). The *in vivo* reducing agent/system is as yet unknown. The direct conversion of the radical **H** to **E** with release of verruculogen and involvement of fumitremorgin B (dashed line) is possible, but less plausible.

an $^{18}O_2$-enriched atmosphere (Figs. 3B and C), demonstrating the presence of $^{16}O_2$ in the $^{18}O_2$-enriched atmosphere and the presence of verruculogen with an endoperoxide bridge containing two ^{16}O atoms. In addition, isotopic ions at *m/z* 516, 538 and 498, four mass units larger than those of the corresponding fragments at *m/z* 512, 534 and 494, were detected in enzymatically derived verruculogen with an intensity ratio of 55:45 to those of the ions at *m/z* 512, 534 and 494. This provided evidence for the presence of verruculogen containing two atoms of ^{18}O in the endoperoxide. In contrast, the signals of the isotopic ions at *m/z* 496, 514 and 536, two mass units larger than those of the corresponding parent ions, showed an intensity of about 5% of those at *m/z* 494, 512 and 534 in both standard and enzymatic product. This indicated that the presence of the ions at *m/z* 496, 514 and 536 was a consequence of natural isotopic abundance rather than an enrichment of one ^{18}O atom in the molecule. In the last case, the intensities of these peaks would be increased significantly. In summary, the endoperoxide bond of verruculogen obtained from incubation in an $^{18}O_2$-enriched atmosphere contains either two ^{16}O or two ^{18}O, but not one of each isotope, which is consistent with the results from feeding experiments by Horak and Vleggaar.[5] Therefore, the possibility of verruculogen formation by two hydroxylation steps and subsequent oxidative linking can be excluded, as this would result in formation of verruculogen containing both ^{16}O and ^{18}O isotopes in the $^{18}O_2$-enriched atmosphere used in this study.

The incorporation of both oxygen atoms from a single O_2 molecule into the structure of verruculogen is in contrast to reactions catalysed by other α-ketoglutarate-dependent dioxygenases, which usually transfer one oxygen atom to the substrate and another to α-ketoglutarate, resulting in formation of

succinate. [30] As discussed above, the presence of α-ketoglutarate as well as ascorbate was essential for the enzymatic conversion of fumitremorgin B to verruculogen by FtmOx1 (Fig. 2), which excludes the possibility that formation of verruculogen and oxidative decarboxylation of α-ketoglutarate to succinate would be two independent activities of FtmOx1. Therefore, we postulate a reaction mechanism of verruculogen formation including Fe(II), α-ketoglutarate and ascorbate as well as two O_2 molecules (Scheme 2).

According to this hypothesis, formation of the Fe(IV)=O species (**E**) from Fe(II) (**A**) *via* Fe(III) (**C**) and a trioxo Fe(IV)-complex (**D**) is in analogy to other known α-ketoglutarate-dependent dioxygenases.[20,30,31] The Fe(IV)=O species (**E**) could then generate a radical at the α-position to the C=C bond of the *N*-prenyl moiety of fumitremorgin B by its own reduction to an Fe(III)–OH species (**F**) as proposed for TauD from *E. coli*, where Fe(IV)=O attacks the substrate by hydrogen abstraction.[20] This is also plausible, if the sequence homologues of FtmOx1 from the PhyH family, which hydroxylate the α-position of a C=O bond,[18] are taken into consideration. In addition, radical formation in an allylic position has also been proposed for COX-catalysed peroxide formation.[10] Similar to that proposed for COX, [10] the molecular oxygen would then attack the radical at the *N*-prenyl moiety, resulting in formation of the radical **G**, which would undergo cyclization to enzyme-bound verruculogen radical (**H**). To complete the reaction, the verruculogen radical (**H**) has to abstract a hydrogen atom to form free or enzyme-bound verruculogen. This could take place, as indicated by the dashed line in Scheme 2, by abstraction of one hydrogen atom from the Fe(III)–OH species in **H** with involvement of a new molecule of fumitremorgin B and formation of a Fe(IV)=O species **E**, which would then undergo another reaction cycle. However, this hypothesis is in contrast to our finding that the enzymatic activity was strictly dependent on the presence of ascorbate. Therefore, reduction of **H** to enzyme-bound verruculogen and Fe(II) complex (**I**) would be more plausible. The reducing agent/system *in vivo* for this reaction is as yet unknown, which could explain the low conversion rate of fumitremorgin B to verruculogen by FtmOx1 *in vitro* by using ascorbate as a reducing agent.

Conclusions

The results reported in this study have provided evidence for the biosynthetic relationship of fumitremorgins to verruculogen. Furthermore, this study has described the first α-ketoglutarate-dependent dioxygenase, which functions as an endoperoxide-forming enzyme. Both oxygen atoms of the endoperoxide bond are derived from a single molecule of oxygen (O_2), which was clearly demonstrated by incubation of FtmOx1 in an $^{18}O_2$-enriched atmosphere in the presence of fumitremorgin B, Fe(II), ascorbate and α-ketoglutarate. Further investigations with different reducing partners such as ferredoxin might give new insights into this novel enzyme group.

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