Experimental section

Strains, plasmids and culture conditions

Eschericha coli XL1 Blue MRF' (Agilent Technologies, Waldbronn, Germany) was used for cloning and expression experiments and grown in liquid or on solid Luria Bertani medium [1] at 37 °C. Carbenicillin (50 µg/ml) was used for selection of recombinant *E. coli* strains. pGEM-T and pQE9 were obtained from Promega (Mannheim, Germany) and Qiagen (Hilden, Germany), respectively.

BAC AfB8B11, containing the fumitremorgin/verruculogen cluster of *A. fumigatus* Af293, was kindly provided by David Harris from the Wellcome Trust Sanger Institute (Cambridge, UK) and used as genomic DNA for PCR amplification.

Chemicals

Fumitremorgin B was obtained by conversion of 12, 13-dihydroxyfumitremorgin C by using FtmPT2 as described in a previous study.[2] Verruculogen was purchased from Alexis Biochemicals (Lörrach, Germany). $^{18}O_2$ (99 %) was obtained from icon services Inc. (Summit, NY, USA) and succinate kit was from megazyme (Wicklow, Ireland).

Cloning of *ftmOx1*

For construction of the expression construct pAG025, *ftmOx1* (AfuA_8G00230) was amplified by PCR from genomic DNA on BAC AfB8B11 by using the primers ftmOx1_for (TT**GGAT**C**C**ATGACCGTCGAC) and ftmOx1_rev (GA**A**AG**CT**TCTAAGCCGGCGA). The bold letters represent mutations instead of the original sequence to give the underlined restriction sites BamHI and HindIII, respectively. The PCR fragment was ligated into pGEM-T to give pAG020. The resulting plasmid was sequenced by MWG (Martinsried, Germany) to confirm the sequence integrity. *ftmOX1* was then released from pAG020 with

BamHI and HindIII and ligated into pQE9 to give pAG024. In order to have the possibility to remove the His-tag, a thrombin cleavage site was inserted as following to give the expression plasmid pAG025: Two oligo nucleotides ThrombinBam_for (GATCTGGTTCCGCGTGGTTCC) and ThrombinBam_rev (ACCAAGGCGCACCAAGG CTAG), encoding a thrombin-cleavage site, were ligated into pAG024, which had been restricted by BamHI previously.

Expression of *ftmOx1* and purification of the gene product

For gene expression, *E. coli* cells harbouring the expression plasmid pAG025 were cultivated in 300 ml Erlenmeyer flasks containing 100 ml liquid medium and grown for 4 h at 200 rpm. For induction, IPTG (1 mM) was added and the bacteria were cultivated for further 16 hours before harvest. The cell pellet was resuspended in 2 ml lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). After addition of 1 mg/ml lysozyme and incubation on ice for 30 minutes, the cells were sonicated six times for 10 s at 200 W. To separate the cellular debris from the soluble protein, the lysate was centrifugated 30 min at 14 000 x *g*, 4 °C. One-step purification was carried out according to the manufacturer's instruction by affinity chromatography with Ni-NTA agarose resin (Qiagen, Hilden, Germany). The protein was eluted with imidazole (250 mM). To exchange the buffer, the protein fractions were passed through a NAP-5 column (GE Healthcare, Munich, Germany), which had been equilibrated with Tris-HCl (50 mM, pH 7.5) containing 15 % glycerol.

For removal of metal ions from recombinant protein, FtmOx1 was stirred on ice in the presence of 1 mM EDTA for 1 hr and then passed twice through a NAP-5 column, which had been equilibrated with Tris-HCl (50 mM, pH 7.5) containing 15 % glycerol. The protein was stored at -20 °C until use.

Protein analysis

Standard protein techniques were used as described elsewhere.[3;4] The subunits of FtmOx1 were analysed by SDS-PAGE, carried out according to the method of Laemmli [4], and protein bands were stained with Coomassie brilliant blue R-250. The native molecular mass of active recombinant FtmOx1 was determined by gel filtration on a HiLoad 16/60 Sephadex^{TM} 200 column that had been equilibrated with Tris-HCl (20 mM, pH 7.5) containing NaCl (200 mM). The column was calibrated with conalbumin (75 kDa), ovalbumin (43 kDa), carboanhydrase (29 kDa), ribonuclase A (13.7 kDa) and aprotinin (6.5 kDa). The proteins were eluated with the same buffer as used for column equilibration.

Assay for FtmOx1 activity

The standard reaction mixture (100 µl) contained Tris-HCl (50 mM, pH 7.5), FtmOx1 (110 µM), fumitremorgin B (0.5mM) or degradation product, ascorbate (1 mM), *α*-ketoglutarate (1 mM), catalase (160 U). For investigation of the dependency of enzymatic activity on metal ions, Ca(II), Co(II), Cu(II), Fe(II), Mg(II), Mn(II) or Zn(II) at a final concentration of 1 mM were added to the reaction mixture containing FtmOx1 after treatment with EDTA.

After incubation for 16 hours at 37 °C, the enzymatic reaction was stopped by addition of 100 µl methanol, the precipitated protein was removed by centrifugation at 13 000 x *g* for 10 minutes and the supernatant was subjected to HLPC analysis.

Assay in the presence of $^{18}O_2$ enriched atmosphere

For enzymatic reaction in the presence of $^{18}O_2$, FtmOx1 assay (500 µl) contained same components as in the standard reaction mixture. $^{16}O_2$ in the reaction mixture was partially removed by application of vacuum followed by flushing with argon for three times. The argon was then removed by vacuum and finally $^{18}O_2$ was allowed to enter the flask. After

incubation for 16 hours, the reaction was stopped by addition of 500 µl methanol and subjected to LC-MS analysis.

Assay for succinate formation

Succinate was detected in analogy to that of TauD [5] by using a test kit from Megazyme, Ireland, according to the manufacturer's instructions. The assays were carried out in a volume of 2 ml at 25 °C and succinate formation was followed with a photometer at 340 nm for 12 hours.

HPLC analysis

Enzymatic products were analysed on a HPLC by using a Multosphere 120 RP-18 column (250 mm x 4 mm, 5 µm; C&S Chromatographie; Langerwehe, Germany). A linear gradient of 35 – 70 % (*v*/*v*) acetonitrile in water was run for 15 minutes, followed by 100 % (*v*/*v*) acetonitrile for 5 minutes. Before the next injection, the column was equilibrated with 35 % (*v*/*v*) acetonitrile for 5 minutes. The substances were detected with a Photo Diode Array detector and illustrated at 296 nm.

LC-MS-analysis

For identification of verruculogen as enymatic product, the assays were analysed by positive and negative electrospray ionization (ESI) mass spectrometry with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled with an Agilent HPLC series 1100 equipped with a RP18-column (2 x 250 mm, 5 µm). For separation, the column was run with 10 % (*v*/*v*) solvent B (CH_3OH) in solvent A (H_2O, each containing 0.1 % (*v*/*v*) HCOOH) for 5 min, followed by a gradient from 10 % to 100 % (*v*/*v*) B over 30 min. After washing with 100 % (*v*/*v*) B, the column was equilibrated with 10 % (*v*/*v*) B for 10 min. The flow rate was at 0.2 ml min^{-1}.

For detection of ^{18}O-verruculogen, the assays were analysed by secondary ion mass (SIM) spectrometry with an Acquity UPLC BEH C18-column (2.1 x 50 mm, 1.7 µm). For separation, the column was run with 5% (*v*/*v*) solvent B (acetonitrile) in solvent A (H_2O, each containing 0.1 % (*v*/*v*) HCOOH) for 1 min, followed by a gradient from 5 % to 95 % (*v*/*v*) B over 9 min. Afterwards the column was washed with 95 % (*v*/*v*) B for 1 minute. The flow rate was at 0.6 ml min^{-1}.

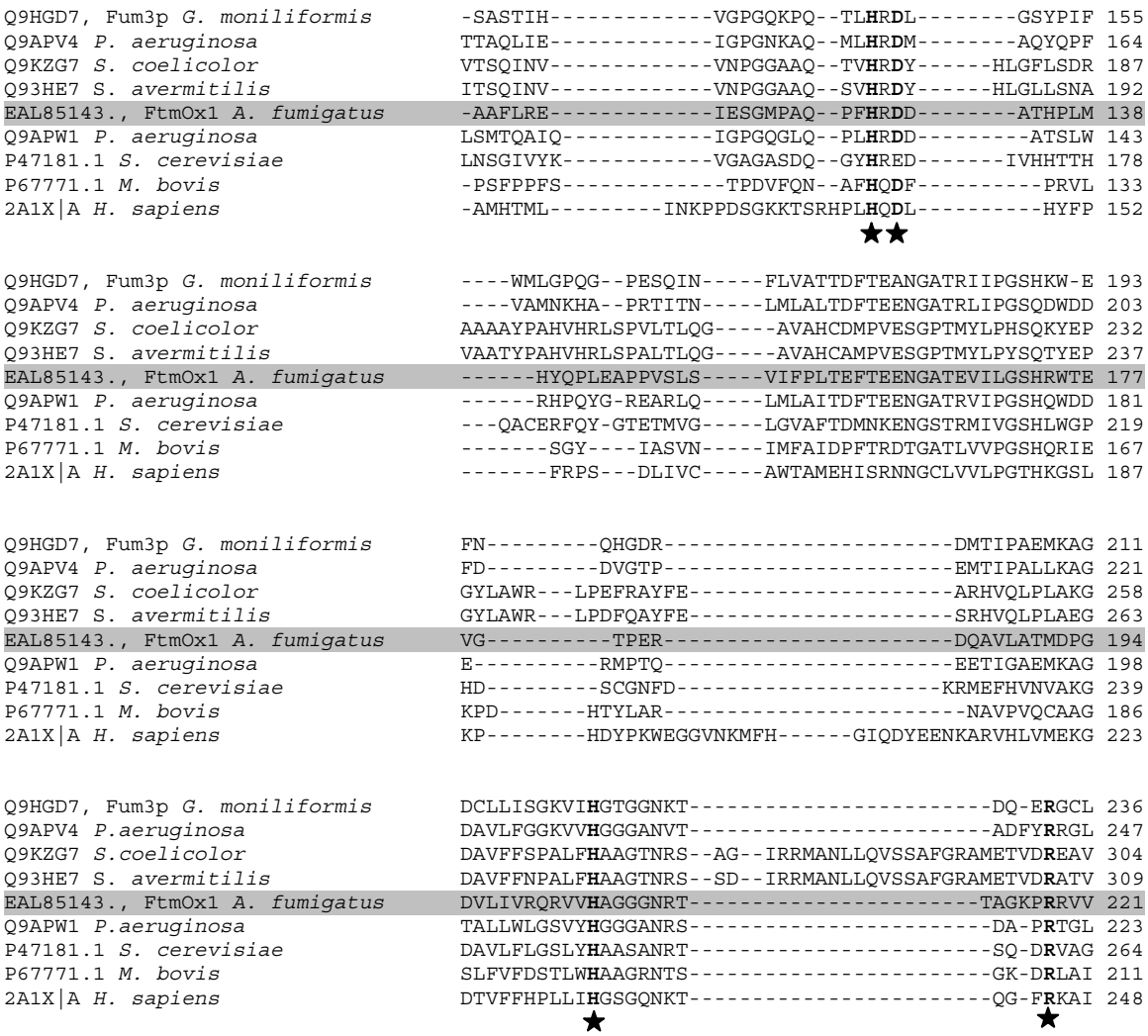

Figure S1: Alignments of FtmOx1 with Fum3p and enzymes of the PhyH superfamily (NCBI blast pfam05721). Amino acids of the binding motif HXD…H…R are indicated in bold letters and are labelled with an asterisk. Q9HGD7: hydroxylase from *Gibberella moniliformis* involved in fumonisin biosynthesis; Q9APV4: putative α-ketoglutarate dependent dioxygenase from *Pseudomonas aeruginosa*, Q9KZG7: putative α-ketoglutarate dependent dioxygenase from *Streptomyces coelicolor*, Q93HE7: putative α-ketoglutarate dependent hydroxylase from *Streptomyces avermitilis*, FtmOx1: α-ketoglutarate dependent endoperoxidase from *Aspergillus fumigatus*, P47181.1: putative Phytanoyl-CoA dioxygenase from *Saccharomyces cerevisiae,* P67771.1*:* putative Phytanoyl-CoA dioxygenase from *Mycobacterium bovis*, 2A1X|A: phytanoyl-CoA-2-hydroxylase from *Homo sapiens*.

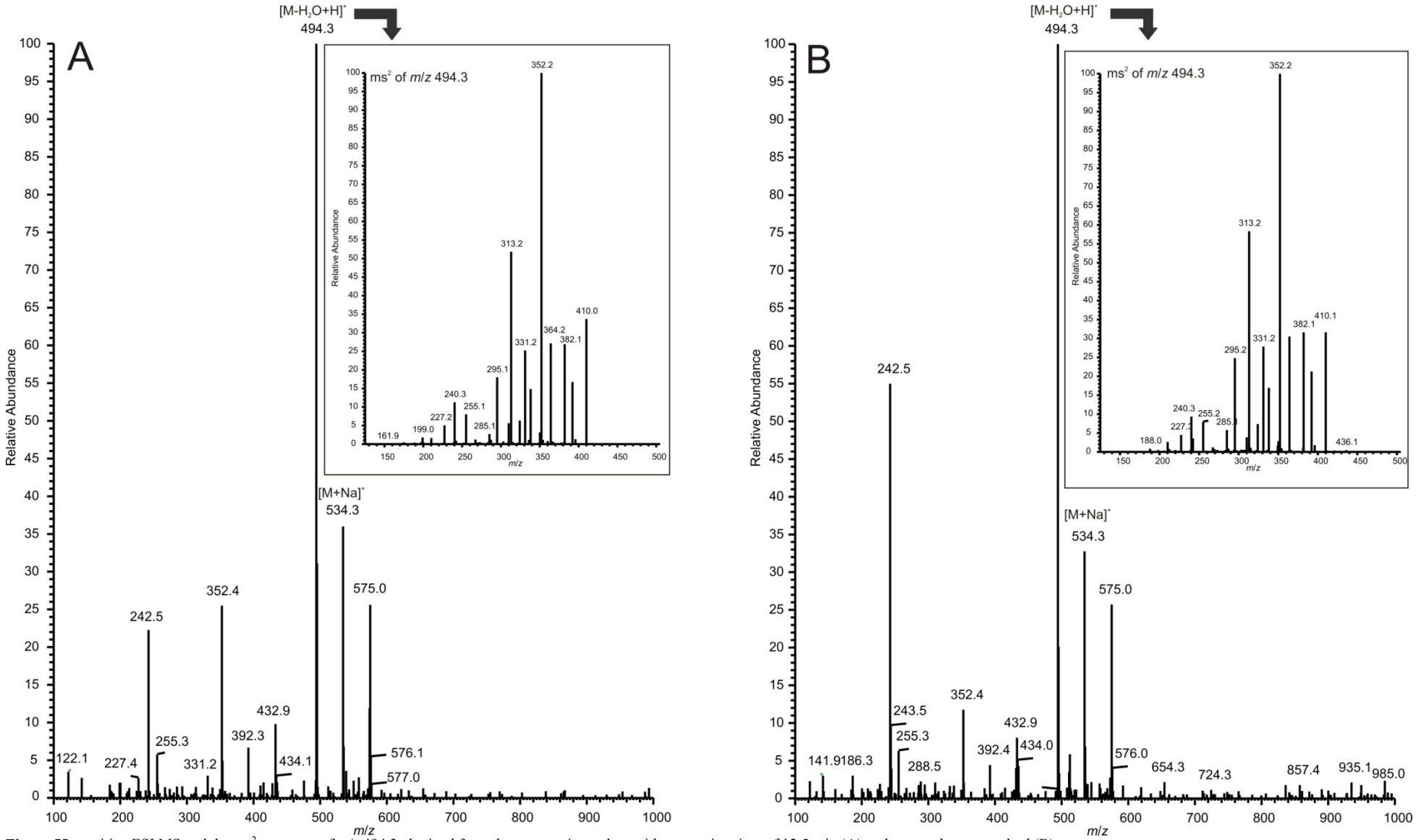

Figure S2: positive ESI MS and the ms^2 spectrum of *m/z* 494.3 obtained from the enzymatic product with a retention time of 12.5 min (A) and verruculogen standard (B).

Reference List

[1.] J. Sambrook, D. W. Russell, *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, New York **2001**.

[2.] A. Grundmann, T. Kuznetsova, S. S. Afiyatullov, S.-M. Li, *Chembiochem* **2008,** *9*, 2059-2063.

[3.] M. M. Bradford, *Anal.Biochem.* **1976,** *72*, 248-254.

[4.] U. K. Laemmli, *Nature* **1970,** *227*, 680-685.

[5.] E. Eichhorn, J. R. vanderPloeg, M. A. Kertesz, T. Leisinger, *J.Biol.Chem.* **1997,** *272*, 23031-23036.