Novofumigatonin biosynthesis involves a non-heme iron-dependent endoperoxide isomerase for orthoester formation

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Novofumigatonin (1), isolated from the fungus Aspergillus novofumigatus, is a heavily oxygenated meroterpenoid containing a unique orthoester moiety. Despite the wide distribution of orthoesters in nature and their biological importance, little is known about the biogenesis of orthoesters. Here we show the elucidation of the biosynthetic pathway of 1 and the identification of key enzymes for the orthoester formation by a series of CRISPR-Cas9-based gene-deletion experiments and in vivo and in vitro reconstitutions of the biosynthesis. The novofumigatonin pathway involves endoperoxy compounds as key precursors for the orthoester synthesis, in which the Fe(II)/α-ketoglutarate-dependent enzyme NvfI performs the endoperoxidation. NvfE, the enzyme catalyzing the orthoester synthesis, is an Fe(II)-dependent, but cosubstrate-free, endoperoxide isomerase, despite the fact that NvfE shares sequence homology with the known Fe(II)/α-ketoglutarate-dependent dioxygenases. NvfE thus belongs to a class of enzymes that gained an isomerase activity by losing the α-ketoglutarate-binding ability.
Orthoesters are functional groups in which three alkoxy groups are bound to a single carbon atom. Orthoesters are widely distributed in plant metabolites, but are also found in biologically important bacterial natural products, such as the sodium channel blocker tetrodotoxin and the orthosomycin antibiotics. Despite their intriguing structures and biological activities, little is known about the molecular basis for orthoester biogenesis. To the best of our knowledge, the Fe(II)/α-ketoglutarate (αKG)-dependent dioxygenases involved in the biosynthesis of orthosomycins are the only examples of orthoester-producing enzymes known to date.

Novofumigatonin (1) is a fungal meroterpenoid discovered in Aspergillus novofumigatus IBT 16806 (CBS17520), and it possesses a congested skeleton with an unusual orthoester moiety. It has been proposed that 1 is biosynthesized via asnovolin A (2), which was also isolated from the same A. novofumigatus strain (Fig. 1a). Asnovolin A (2) is in turn derived from the polyketide precursor 3,5-dimethyloctanolic acid (DMOA, 3), which also serves as the starting material for diverse fungal meroterpenoids (Fig. 1b). We recently identified a putative biosynthetic gene cluster for 1 (the nfv cluster; Fig. 1b, Supplementary Figs. 1, 2 and Supplementary Table 1), but no biosynthetic study of 1 has been reported. Therefore, the biosynthetic pathway of 1, including the mechanism for the orthoester formation, has yet to be elucidated.

Here we show the complete biosynthetic pathway of novofumigatonin (1) and characterization of key enzymes for the orthoester synthesis. We initially create a series of gene-deletion strains of A. novofumigatus and isolated several metabolites, which we incorporated into a model for the biosynthetic route of 1 (Fig. 2a). The proposed pathway is further validated by heterologous expression experiments using Aspergillus oryzae and by in vitro enzymatic reactions with purified enzymes. In the course of our study, we achieve the total biosynthesis of 1 in the heterologous host A. oryzae, strongly indicating that the nfv cluster genes are sufficient to produce 1. We reveal the involvement of two key enzymes for the orthoester synthesis, namely NvfL and NvfE, which are responsible for the endoperoxidation and endoperoxide isomerization, respectively, to afford the orthoester. Most importantly, NvfE is characterized to be a non-heme iron-dependent endoperoxide isomerase despite its sequence similarity to known Fe(II)/αKG-dependent dioxygenases.

**Early-stage biosynthesis of 1.** Since the initial biosynthetic steps of DMOA-derived meroterpenoids adopted similar pathways to afford their first cyclized intermediates, four gene products, the prenyltransferase NvfB, the methyltransferase NvfJ, the flavin-dependent monoxygenase (FMO) NvfK, and the terpene cyclase NvfL, as well as the PKS NvfA, were expected to be required for the formation of a tetracyclic compound, by analogy to the biosyntheses of austinol, terretonin, and andrastin A (Supplementary Fig. 5). First, the involvement of NvfB in the biosyntheses and its function as a DMOA farnesyltransferase were confirmed by the deletion of nfvB (Fig. 2b, lane iii) and heterologous expression of nfvB with the known DMOA-synthase gene andMΔ16 in the Aspergillus oryzae NSARI strain17, which yielded farnesyl-DMOA (4) (Fig. 2d, lane ii and Supplementary Fig. 6).

Rather than the expected early intermediate, the nfvΔA strain accumulated the tetracyclic molecule 5, which was named asnovolin I (Fig. 2b, lane xi). This result surprisingly revealed that NvfJ acts after the cyclization event and therefore differs from the methyltransferases in the known DMOA-derived pathways, which require the methyl esterification of 4 for the activity of the terpene cyclases6. Consistent with this observation, the nfvKΔA and nfvLΔA mutants both yielded compounds 4 and 6 with a free carboxyl group, respectively (Fig. 2b, lanes xii and xiii). Compound 6 has a diol moiety at the terminus of the farnesyl group, which is likely to be a consequence of the hydrolysis of the expected epoxide 7. Collectively, the early-stage biosynthesis is proposed as follows. The PKS NvfA forms DMOA (3), followed by the farnesylation by NvfB, the epoxidation by NvfK, and the protonation-initiated cyclization of 7 by NvfL, to afford 8 via the cationic intermediate 9 (Fig. 2a).

**Mid-stage biosynthesis of 1.** In addition to the above-mentioned mutants, three more strains, nfvCΔA, nfvHΔA, and nfvMΔA, were unable to produce both 1 and 2 (Fig. 2b, lanes iv, ix, and xiv), and therefore these genes are likely to be engaged in the pathway to 2. Among the three mutants, the nfvCΔA strain did not accumulate any detectable unknown metabolites, and therefore the function of nfvC was investigated using the A. oryzae heterologous expression system; the transformant harboring andMΔ, nfvBΔ, nfvKΔ, nfvLΔA, and nfvC yielded both 8 and chernesin D (10), while the transformant lacking nfvC only produced the alcohol form 8 (Fig. 2d, lanes iii and iv and Supplementary Fig. 6), establishing the function of NvfC as a 3-OH dehydrogenase of 8. Compound 8 was hereby designated as asnovolin H.

The nfvHΔA strain accumulated the product 11, which was named asnovolin J. The structure of 11 is nearly identical to that of 2, and only lacks the oxygen atom between C-3 and C-4, which established the FMO NvfH as a Baeyer–Villiger monooxygenase (BVMO). Another close analog of 2, 12, with a double bond between C-5’ and C-6’, was obtained from the nfvMΔ strain and designated as asnovolin K. This indicates that NvfM is an enoylreductase that reduces this double bond. Taken together, our results suggest that there are two pathways to synthesize 2 from 10. In one branch, 10 undergoes Baeyer–Villiger oxidation, methylation, and enoyl reduction to form 5, 12, and 2 in this order, while in the other branch, the methylation precedes the Baeyer–Villiger oxidation and the enoyl reduction to give 13, 11, and 2, respectively (Fig. 2a). Consistently, the introduction of the above-mentioned eight genes into A. oryzae successfully yielded 2 (Fig. 2d, lane v and Supplementary Fig. 6). In this proposed pathway, the methyl esterification by NvfJ occurs just before the NvfM-catalyzed reduction. Without the methylation, a β-keto acid that spontaneously undergoes decarboxylation would be generated upon the enoyl reduction. Thus, the methyl group could serve as a protecting group of carboxylic acid, which
explains the different timing of the methylation in the biosyntheses of 1 and the other known DMOA-derived meroterpenoids (Fig. 2a and Supplementary Fig. 5).

**Late-stage biosynthesis of 1.** Asnovolin A (2), but not 1, was detected in extracts from five mutants, *nvfΔA, nvfΔE, nvfΔS, nvfGΔ*, and *nvfΔA* (Fig. 2b, lanes v to vii and x), suggesting that all five genes are involved in the synthesis of 1 from 2. Except for the *nvfΔA* mutant, all produced 2 in combination with another compound. The mutants without *nvfΔD* and *nvfΔE* accumulated the metabolites 14 and 15, respectively (Fig. 2b, lanes v and vi). Compound 14 was also produced by the *nvfΔA, nvfΔE, nvfΔS, nvfGΔ* quadruple-mutant strain (Fig. 2b, lane xv), indicating that 14 is the product of NvfI. To facilitate the isolation of 14, we created another mutant in which a non-ribosomal peptide synthase gene named *c-anaPS* was deleted. The mutants without *nvfΔG* and *nvfΔE* accumulated the metabolites 14 and 15, respectively (Fig. 2b, lanes vi and vii). Compound 14 was also produced by the *nvfΔD, nvfΔE, nvfΔG*, and *nvfΔG* double-mutant strain (Fig. 2b, lane xv), indicating that 14 is the product of NvfI. To facilitate the isolation of 14, we created another mutant in which a non-ribosomal peptide synthase gene named *c-anaPS* was deleted. The mutants without *nvfΔG* and *nvfΔE* accumulated the metabolites 14 and 15, respectively (Fig. 2b, lanes vi and vii). Compound 14 was also produced by the *nvfΔD, nvfΔE, nvfΔG*, and *nvfΔG* double-mutant strain (Fig. 2b, lane xv), indicating that 14 is the product of NvfI. To facilitate the isolation of 14, we created another mutant in which a non-ribosomal peptide synthase gene named *c-anaPS* was deleted. The mutants without *nvfΔG* and *nvfΔE* accumulated the metabolites 14 and 15, respectively (Fig. 2b, lanes vi and vii).

**Characterization of NvfI as an endoperoxidase.** The gene-deletion experiments indicated involvement of endoperoxide precursors for orthoester generation, but the endoperoxidation mechanism remained to be clarified. To obtain deeper insight into the endoperoxide synthesis, the predicted Fe(II)/αKG-dependent dioxygenase NvfI was purified as a His6-tagged protein for in vitro enzymatic reactions (Supplementary Fig. 10). Based on the predicted function, the recombinant NvfI protein was reacted with asnovolin A (2) under conditions similar to those used for other αKG-dependent enzymes in our previous studies. Interestingly, the reaction did not provide the expected product 14, but yielded its isomer 21 (Fig. 4a, lane iv). Compound 21 was isolated from a large-scale enzymatic reaction and determined to possess a hemiacetal linkage between C-13 and C-2', instead of the endoperoxide of 14, which seems to be derived from a rearrangement of the endoperoxide by the iron

![Fig. 1 Novofumigatonin and its biosynthetic gene cluster.](image_url)

**Fig. 1** Novofumigatonin and its biosynthetic gene cluster. a Structures of novofumigatonin (1) and its predicted biosynthetic precursors. b Schematic representation of the *nvf* cluster and predicted function of each gene based on BLASTP comparison with characterized proteins. PKS polyketide synthase, FMO flavin-dependent monoxygenase, SDR short-chain dehydrogenase/reductase.
added to the reaction mixture (Fig. 4d). Therefore, we performed the reaction in the absence of exogenous iron, in which iron ions copurified with NvfI can be used for the catalysis. As a result, the reaction successfully generated 14 as the major product (Fig. 4a, lane v), demonstrating that NvfI is solely responsible for the endoperoxidation reaction. The reaction also yielded the mono-hydroxylated asnovolin B\(^6\) (22) as a minor product, which was also detected from \textit{A. oryzae} transformants harboring \textit{nvfI} (Figs. 2c, 4d). Furthermore, metal- and \textit{aKG}-dependences of NvfI were confirmed by observations that the reaction was abolished in the presence of EDTA or in the absence of \textit{aKG} (Fig. 4a, lanes ii and iii).

As an \textit{aKG}-dependent enzyme, the catalysis by NvfI should follow the common mechanism seen in this class of oxidative enzymes\(^23\): an Fe(IV)-oxo species is generated by the oxidative decarboxylation of \textit{aKG} to abstract a hydrogen atom from 2 to
initiate the reaction. Thus, the mechanism for the endoperoxidation by NvfI is as proposed (Fig. 4e): the hydrogen atom at C-13 is abstracted to give the radical species 23; a molecular oxygen is then incorporated to generate the peroxy radical 24, which undergoes C-O bond formation at C-2′ to yield 25; and an oxygen rebound at C-3′ completes the reaction to provide 14. This proposed mechanism for the endoperoxidation is similar to that proposed for the fumitremorgin B endoperoxidase (FtmOx1)24,25, which is another αKG-dependent enzyme. However, the mechanisms for the radical quenching differ in these two enzymes; FtmOx1 requires a reducing agent as a hydrogen donor at the end of the reaction, while Nvf completes the reaction by the oxygen rebound, altogether introducing three oxygen atoms to the substrate. For the formation of the minor product 22, the hydrogen abstraction would occur on the C-7′ methyl group, which is immediately followed by the rebound of the hydroxyl radical (Fig. 4d).

Mechanistic investigation of the orthoesterification. To unveil how the endoperoxide precursors are transformed into novofumigatin (1) with its unique orthoester moiety, two other putative Fe(II)/αKG-dependent dioxygenases, NvfE and NvfF, were also purified as His6-tagged proteins (Supplementary Fig. 10). Initially, the reaction by NvfE was investigated using similar reaction conditions to those for NvfI. The reaction with 15 as a substrate gave two isomeric products (Fig. 4b, lane vi), one of which was identified as 18 obtained from the A. novofumigatus nvyFA strain. The other product 26 was isolated from a large-scale reaction for the structural characterization, and quite surprisingly, it was elucidated that 26 has a different orthoester moiety from those seen in 1 and 18 (Figs. 2a, 4f), in which the hydroxyl group at C-5′ is not involved in the orthoester formation. We also found that 26 is easily transformed into 18 when formic acid was added after the completion of the reaction (Fig. 4b, lane vi). Thus, we reasoned that 18 is not an enzymatic product, and that 26 undergoes an acid-catalyzed rearrangement to yield 18 (Supplementary Fig. 11).

To evaluate the intermediacies of 18 and 26 in the novofumigatin pathway, these compounds were individually incubated with NvfF, which seems to be responsible for the very last steps of the biosynthesis. As a result, 26 was efficiently converted to 1 in a metal- and αKG-dependent manner (Fig. 4c, lanes iii to vi), but 18 was not utilized as a substrate of NvfF (Fig. 4c, lanes i and ii), indicating that 26 is the genuine product and substrate of NvfE and NvfF, respectively, and that 18 is a shunt pathway product. Collectively, NvfF catalyzes two rounds of oxidation to transform 26 into the end product, novofumigatin (1).

Despite the sequence similarity between NvfE and the known Fe(II)/αKG-dependent dioxygenases, the reaction catalyzed by NvfE is not an oxidation, but an isomerization event, which led us to further investigate this intriguing enzyme. Surprisingly, the reaction was not inhibited in the absence of αKG or in the presence of 20 mM EDTA, and only the purified enzyme and substrate were required for the complete reaction (Fig. 4b, lanes ii to iv). This observation suggested that NvfE is a cofactor/cosubstrate-free enzyme. We thus carefully analyzed the sequence alignment between NvfE and other αKG-dependent enzymes involved in fungal secondary metabolism (Supplementary Fig. 12). As a result, we found that one highly conserved glutamine residue important for the αKG binding24,26–28 is substituted with glutamate (E149) in NvfE, while all of the residues consisting of the iron-binding facial triad29 (H152, D154, and H234) are still conserved. To reveal the importance of these residues, four genes containing each of the following codon changes, E149Q, H152A, D154A, and H234A, were constructed and the resulting mutant enzymes were subjected to in vitro enzymatic reactions. When incubated only with substrate 15, each of the mutant enzymes exhibited reduced activity but did not completely lose activity (Supplementary Fig. 13). Quite interestingly, the reactions by all four of the mutants were enhanced in the presence of Fe2+ and/or ascorbate and inhibited in the presence of EDTA (Supplementary Fig. 13). Altogether, these results show that the orthoester formation proceeds in a metal-dependent manner. The differences between the reactions catalyzed by wild-type NvfE and the mutants could be attributed to the fact that E149 participates in the iron binding in the active site via its carboxyl group together with the other three residues, resulting in stronger iron-binding affinity. However, this hypothesis should be confirmed by a future X-ray crystallographic study on NvfE complexed with iron.

It is well known that Fe(II) causes the cleavage and rearrangement of endoperoxide linkages in a variety of compounds, such as the antimalarial agent artemisinin30. Thus, we reasoned that the NvfE-catalyzed orthoesterification is also initiated by the iron-mediated cleavage of the endoperoxide and proposed the following reaction mechanism (Fig. 4f). First, the ferrous iron in the active site would bind to the oxygen atom at C-13, to cleave the endoperoxide. The oxygen-centered radical 27 generated herein then undergoes β-scission to give the carbon-centered radical 28, which is oxidized by Fe(III) to form the carbocatic species 29. Finally, the carbocation is quenched by the two rounds of heterocyclization, to afford the orthoester 26.

The details of the reactions catalyzed by NvfF to finalize the biosynthesis are still not very clear, as we were not able to obtain the product from the first-round reaction. Nevertheless, it is evident that NvfF is engaged in two sequential oxidative reactions, the dehydrogenation to introduce the C-C double bond between C-1 and C-2 and the aldehyde formation at C-13. Homologs of NvfF are often found in the DMOA-derived meroterpenoid pathways, and most of them catalyze multistep reactions as NvfF16,22,31,32. Interestingly, contrary to the commonly seen desaturation event, the aldehyde forming reaction by NvfF is relatively rare for reactions catalyzed by αKG-dependent enzymes. Based on the reaction mechanism proposed for aldehyde formation by this class of dioxygenases33,34, the mechanism for the NvfF-catalyzed reaction to yield the aldehyde with the rearranged orthoester could be
proposed as follows (Fig. 4g). Initially, the ferryl-oxo species generated in the active site of the enzyme abstracts a hydrogen atom from C-13 of 26, or its dehydrogenated form 30, and then hydroxylates this position, which would be the only role of NvfF in this transformation. The resultant hemiacetal 31 is spontaneously converted to an aldehyde, by which a hemiothoester group is generated as well. The resulting hemiothoester 32 would then be transformed to the orthoester via the cationic intermediate 33 by the participation of the hydroxyl group at C-5′, thus completing the reaction. These proposed mechanisms also explain the formation of 19 in the absence of the ketoreductase gene nvfG (Supplementary Fig. 11). In this case,
In this study, we elucidated the biosynthetic pathway of novel-fumigatin (1) by CRISPR-Cas9-guided gene-deletion experiments, heterologous fungal expression, and in vitro enzymatic reactions. We believe that we have demonstrated the powerfulness of combining these three different approaches to efficiently elucidate a fungal natural product pathway and to identify key enzymes for the biosynthesis.

We have identified and characterized the endoperoxidase NvfI and the endoperoxide isomerase NvfE for the orthoester synthesis. The reactions catalyzed by NvfI and NvfE somewhat resemble those by prostaglandin endoperoxide H synthases (PGHSs) and prostacyclin or thromboxane A synthases, respectively, but differ in that these enzymes involved in prostanoid biogenesis are heme proteins, while NvfI and NvfE utilize non-heme iron to perform a similar set of reactions. Enzymatic endoperoxidation has been well studied in PGHSs, but except that the resultant orthoester is different from that of known endoperoxidases, and NvfI thus represents a rare example of an endoperoxide-producing enzyme.

It is remarkable that the orthoester synthesis is achieved by a non-heme iron-dependent isomerase, NvfE. Non-heme iron-utilizing enzymes are widespread in nature and perform versatile reactions, but are mostly involved in oxidative reactions. Enzymatic orthoesterification can be performed by the non-heme iron-dependent enzymes for the orthosomycins pathways as well, but their orthoester synthesis is an oxidative reaction without any structural rearrangement, thus completely differing from the NvfE-catalyzed reaction. Nevertheless, there are some examples in which isomerization reactions are catalyzed by NvfE homologs, such as CarC, SnoN, and AndA, in the carbanion, nogalamycin, and anditomin pathways, respectively. The reactions by these enzymes, however, are all αKG-dependent, and they retain their functions as oxidative enzymes as well. In contrast, NvfE appears to have completely lost the dioxygenase activity, and to the best of our knowledge, NvfE is the first reported enzyme that gained the isomerase activity by losing the αKG-binding ability.

Other intriguing findings regarding the NvfE-catalyzed reaction are that the resultant orthoester is different from that of 1 and that the NvfE product 26 completely possesses the same backbone structure as that of another orthoester-containing natural product, fumigatoin (Fig. 5). We previously speculated that the two different orthoesters in 1 and fumigatoin are respectively biosynthesized in somewhat different manners, but our present study revealed that the novel-fumigatin-type orthoester actually originates from the fumigatoin-type orthoester. Overall, 26 would be the common precursor for the biosyntheses of both 1 and fumigatoin, and the biosynthetic gene cluster for fumigatoin should contain a few more genes as compared with those of the nvf cluster, to allow for the C-6 hydroxylation and the two acetylations (Fig. 5).

In conclusion, we have revealed the complex biosynthetic route to novel-fumigatin (1) and discovered the intriguing endoperoxide isomerase, NvfE, for the orthoester formation, which was accomplished by establishing genetic engineering in A. novofumigatus. Further biochemical and biophysical characterizations of NvfE will clarify this unusual chemistry and could even provide opportunities to engineer known αKG-dependent enzymes into αKG-independent biocatalysts to expand nature’s catalytic versatility.

**Methods**

**General.** Solvents and chemicals were purchased from Sigma-Aldrich, VWR International, Fisher Scientific International, Inc., Wako Chemicals Ltd., or Kanto Chemical Co., Inc., unless noted otherwise. Oligonucleotide primers (Supplementary Data 1) were purchased from Integrated DNA Technologies Inc., Eurofins Genetics, or Sigma-Aldrich. PCR was performed using a T100 Thermal Cycler (BIO-RAD) or a TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa), with Phusion DNA polymerase, iProof DNA-RAD, or Phire Plant Direct PCR Master Mix (Thermo Scientific). Sequence analyses were performed by Eurofins Genetics. Flash chromatography was performed using an Isolera flash purification system (Biotage). Preparative HPLC was performed using a Waters 600 controller with a 996 photodiode array detector (Waters) or a Shimadzu Prominance LC-20AD system. NMR spectra were obtained with Bruker AVANCE 400 MHz, 600 MHz, or 800 MHz spectrometers at DTU NMR Center • DTU, or JEOL ECX-500 or ECZ-500 spectrometers, and chemical shifts were recorded with reference to solvent signals (1H NMR: CD3I 7.26 ppm, CD3OD 3.34 ppm, DMSo-d6 2.49 ppm, acetome-d2 2.05 ppm; 13C NMR: CD3I 77 ppm, CD3OD 49 ppm, DMSo-d6 39.5 ppm, acetome-d2 29.9 ppm). LC–MS samples from the gene-deletion experiments were injected into a Dionex Ultimate 3000 UHPLC system (Thermo Scientific)—a maXi 3Q TOF orthogonal mass spectrometer (Bruker Daltonics), using electrospray ionization with a Kinetex C18 column (2.1 i.d. x 100 mm; Phenomenex). LC–MS samples from the heterologous expression and in vitro experiments were injected into a Shimadzu Prominance LC-20AD system with a compact mass spectrometer (Bruker Daltonics), using electrospray ionization with a COSMOsil 2.5C8–MS-II column (2.1 i.d. x 75 mm; Nacalai Tesque, Inc.).

**Strains.** Aspergillus novofumigatus IBT 16806 (IFO 55215) was obtained by the IBT Culture Collection at the Department of Biotechnology and Biomedicine, Technical University of Denmark, Denmark, and from the Medical Mycology Research Center, Chiba University, Japan. Aspergillus oryzae NSAR1 (nis·c and pgl−) was used as the host for fungal heterologous expression. Standard DNA engineering experiments were performed using Escherichia coli DH5α. E. coli Rosetta®(DE3)PlsS (Novagen) was used for the expression of the NvfI, NvfE, NvfF, and NvfE mutants.

**Construction of plasmids for fungal transformations.** For the construction of plasmids for the CRISPR-Cas9 system, two fragments for the expression cassette of the targeted gene (the target) were amplified by PCR with pFC334 as the template, and ligated into the pFC332 vector, which had been digested with PacI and subsequently treated with Nt.BbvCI, by the Uracil-Specific Excision Reagent (USER) fusion method. For the construction of plasmids for gene-deletion experiments, approximately 1-kb of the 5′ and 3′ flanking regions of the targeted gene were amplified by PCR from the genomic DNA of A. novofumigatus IBT 16806, and introduced into the pAC/Nt.BbvCI USER cassette sites of pU2002c. The plasmids constructed in this study and the primers used for the construction of each plasmid are listed in Supplementary Data 2.

For the construction of fungal expression plasmids for A. oryzae, each gene in the nvf cluster, except for nvfK and nvfL, was amplified from A. novofumigatus IBT 16806 genomic DNA using the published genome sequence, with the primers listed in Supplementary Data 1 and 2, and initially ligated into the pTAex3 vector, while nvfK and nvfL were introduced into the pUSA44 and pUNA45 vectors, respectively, using an In-Fusion™HD Cloning Kit (TaKaRa Clontech). To construct the gene-containing vectors, fragments containing the amylB terminator (PamB) and the amyB terminator (TamyB) were amplified from the pTAex3-based plasmids, and ligated into the previously constructed single gene-containing

**Fig. 5** Biosynthesis of fumigatoin. Structure of fumigatoin and reactions required to synthesize fumigatoin from 26 are shown. 20, the 4′-keto analog of 15, would be accepted by NvfE and NvfF to undergo similar rearrangement and oxidation reactions as shown in Fig. 4f, g but a water molecule is used for the neutralization at the last step of the reaction to give the orthoester of 19.
the nvfD-based plasmids (Supplementary Data2) were used as well, since transformations by TF solution 2 (10 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 50 mM CaCl2, 5 mM homologous recombination (Supplementary Fig.3), and were further selected with 5-FOA. The selected transformants were sensitive to 5-FOA. The selected transformants in which the pyrG gene was eliminated from the strain was transformed with the pU2002c-based plasmids (ΔpyrG). To obtain an intron-free ΔpyrG strain was then transformed to delete the pyrG marker gene (AfpfgG:pyrG from Aspergillus flavus) via intramolecular homologous recombination (Supplementary Fig. 3), and were further selected with 5-FOA, hereby creating the pyrG−, ligDA strain. The pyrG−, ligDA strain was transformed with the pu2002c-based plasmids corresponding to the targeted gene(s) (Supplementary Data 2), and transformants were selected on minimal media. When deleting pyrG, mfM, and pyrD-F, pFC332-Asnpov_pyrG was used for transformation of A. novofumigatus IBT 16806. Transformants were selected on transformation media supplemented with 1.12 g L−1 NaNO3, 0.52 g L−1 KCl, 0.52 g L−1 MgSO4·7H2O, 1.52 g L−1 KH2PO4, 10 g L−1 d-glucose, 10 mg L−1 thiamine, and 0.5 mL L−1 of a trace element solution26, which was poured on the top of the pre-made transformation media plate. The plate was further incubated for about a week at 25 °C until mutant colonies appear.

For the deactivation of the pyrG gene (Protein ID in JGI database: 399623), pFC332-Asnpov_pyrG was used for transformation of A. novofumigatus IBT 16806. Transformants were selected on transformation media supplemented with 1.12 g L−1 NaNO3, 0.52 g L−1 KCl, 0.52 g L−1 MgSO4·7H2O, 1.52 g L−1 KH2PO4, 10 g L−1 d-glucose, 10 mg L−1 thiamine, 20 g L−1 agar, supplemented with 1 mL L−1 of a trace element solution26, containing 1.30 g L−1 of a trace element solution48), which was poured on the top of the pre-made transformation media plate. The plate was further incubated for about a week at 25 °C until mutant colonies appear. To construct the strain lacking pyrG, A. novofumigatus, wild type and mutants were inoculated on 100 to 200 YES agar plates (ca. 2–4 L), respectively, and cultivated for ~10 days at 25 °C. The resulting fungal cultures were extracted with ethyl acetate, fractionated by flash chromatography, and purified by preparative HPLC. For the isolation of each metabolite from A. oryzae transformants, media from 1 to 4 L of the culture were extracted with ethyl acetate at room temperature, concentrated, and reextracted with ethyl acetate. Both extracts were combined and subjected to open column chromatography and further purification by preparative HPLC. The detailed purification procedures and structural characterizations of the compounds are described in Supplementary Notes 1 to 18, and the final product data are provided in Supplementary Figs. 14 to 100 and Supplementary Tables 5 to 22.

**Hydrogenolysis of fumigatobon A (15).** To a stirred solution of 14.4 mg (7.3 μmol) of fumigatobon A in 5% MeOH (14 mL), was added 10% palladium on activated charcoal (~5 mg), and the mixture was stirred for 5 h under hydrogen atmosphere. The suspension was then filtered through a PTFE syringe filter (0.45μm), and the eluent was removed in vacuo. The residue was dissolved in 1 mL 20% DMF-DMSO, lyophilized by reverse-phase preparative HPLC (40% aqueous acetonitrile, 5 μmol mL−1) on a Luna C18 column (250×10 mm, 5 μm, Phenomenex), to yield 3.6 mg (82%, 7.5 μmol) of a white amorphous solid. HR-ESI-MS found m/z 489.2463 [M+N]+ (calcd. 489.2459 for C25H26O11).

**Expression and purification of the Nvf enzymes.** To express nvfI, nvfE, and nvfF in E. coli, the plasmid was introduced into the plasmid vector pET-28a(+) vector (Novagen), using an In-Fusion® HD Cloning Kit (Supplementary Data2). To obtain an intron-free nvf gene, total RNA was extracted from an A. oryzae transformant expressing nvf using ISOGEN (NipponGene Co., Ltd.), and CDNA was synthesized with SuperScript™ III Reverse Transcriptase (Invitrogen) from the extracted RNA. The intron-free nvf gene was generated by amplifying and ligating the two predicted exons. The nvfF gene has no intron, and therefore it was directly amplified from A. novofumigatus genomic DNA. NvFe mutants were constructed by PCR methods, using pairs of the mutation primers. For the expression of NvFe, NvF, and NvE, mutants, E. coli Rosetta™2 (DE3)pLysS was transformed with the pET-28a(+) based plasmid for each gene. Transformants of A. oryzae were grown in shaking cultures in YM-G medium59 supplemented with 50 mg L−1 kanamycin sulfate and 12.5 mg L−1 chloramphenicol. When the cultures had grown to an OD600 of 0.6, gene expression was induced by the addition of 0.5 mM IPTG. Then, the incubation was continued for 14 h at 30°C. Cells were centrifuged (4500×g, 15 min) and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM imidazole, 5% glycerol). After lysis on ice by sonication, the cell debris was removed by centrifugation. The supernatant was loaded onto a Ni-NTA affinity column. Bound proteins were removed with 30 column volumes of wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM imidazole, 5% glycerol). His-tagged proteins were eluted with 5 column volumes of elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 500 mM imidazole, 5% glycerol). The purity of the enzymes was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Supplementary Fig. 10). The protein concentrations were determined using a SimpliNano Spectrophotometer (GE Healthcare Life Sciences).

**Enzymatic reaction assay of purified proteins.** All of the enzymatic reactions were performed in 50 μL reaction mixtures at 30 °C, and terminated by adding 50 μL of methanol and vortex mixing, and then the supernatant obtained after centrifugation was analyzed by LC-MS.

The standard enzymatic reaction of NvF with viceninol A (2) was performed in reaction mixtures containing 0.5 mL Tris-HCl buffer (pH 7.5), 230 μM of 2, 2′-diaminobenzaldehyde, 5 mM FeSO4 or 1 mM EDTA was added to the reaction.

The standard enzymatic reaction of NvF with fumigatobon B (15) was performed in reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.5), 50 μM of fumigatobon B, 20 mM ferric ammonium citrate, and 20 mM FeSO4 was required. 20 mM EDTA was added to the reaction. To investigate the stability of 26, formic acid (final conc. 0.5%) was added after the completion of the reaction.
The standard enzymatic reaction of NvfE with axonovolin G (18) or fumigatoid B (15) was performed in reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.5), 50 μM of 18 or 26, 2.5 mM α-ketoglutarate, 4 mM ascorbate, and 11.1 μM NvfE, for 1 h. When required, 1 mM EDTA was added to the reaction. The standard enzymatic reaction of NvfE or its mutants with fumigatoid B (15) was performed in reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.5), 50 μM of 15, and 1 μM NvfE or its mutants, for 1.5 h. When required, 0.1 mM FeSO₄, 4 mM ascorbate, or 1 mM EDTA was added to the reaction.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information and from the corresponding authors upon reasonable request.

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Author contributions
Y.M., I.A., and T.O.L designed the research. Y.M. performed the gene-deletion experiments, heterologous fungal expression, in vitro enzymatic reactions, and structural characterization of new compounds. T.B. constructed the heterologous fungal expression systems. C.B.W.P. synthetically derivatized compound 15 for the structural characterization. C.S.N. and U.H.M. developed the methodology for the gene-deletion experiments in A. novofumigatus. Y.M., I.K., T.C.V., and M.R.A. discovered and analyzed the novofumigatonin biosynthetic gene cluster. Y.M., C.H.G., I.A., and T.O.L. analyzed the data. Y.M., C.B.W.P., I.A., and T.O.L. wrote the paper. All the authors reviewed the manuscript.

Additional information
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