Genetic, molecular, and biochemical basis of fungal tropolone biosynthesis

Jack Davison[a], Ahmed al Fahad[a], Menghao Cai[b], Zhongshu Song[a], Samar Y. Yehia[c], Colin M. Lazarus[d], Andrew M. Bailey[d], Thomas J. Simpson[a], and Russell J. Cox[a,1]

[a]University of Bristol, School of Chemistry, Cantock's Close, Bristol BS8 1TS, United Kingdom; [b]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Meilong Road 130, Shanghai 200237, China; [c]Future University in Egypt, Faculty of Pharmaceutical Sciences and Pharmaceutical Industries, New Cairo, Egypt 11477; and [d]University of Bristol, School of Biological Sciences, Woodland Road, Bristol BS8 1UG, United Kingdom

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A gene cluster encoding the biosynthesis of the fungal tropolone stipitatic acid was discovered in *Talaromyces stipitatus* (*Penicillium stipitatum*) and investigated by targeted gene knockout. A minimum of three genes are required to form the tropolone nucleus: tropA encodes a nonreducing polyketide synthase which releases 3-methylorcinaldehyde; tropB encodes a FAD-dependent monooxygenase which dearomatizes 3-methylorcinaldehyde via hydroxylation at C-3; and tropC encodes a non-heme Fe(II)-dependent dioxygenase which catalyzes the oxidative ring expansion to the tropolone nucleus via hydroxylation of the 3-methyl group. The tropA gene was characterized by heterologous expression in *Aspergillus oryzae*, whereas tropB and tropC were successfully expressed in *Escherichia coli* and the purified TropB and TropC proteins converted 3-methylorcinaldehyde to a tropolone in vitro. Finally, knockout of the tropD gene, encoding a cytochrome P450 monooxygenase, indicated its place as the next gene in the pathway, probably responsible for hydroxylation of the 6-methyl group. Comparison of the *T. stipitatus* tropolone biosynthetic cluster with other known gene clusters allows clarification of important steps during the biosynthesis of other fungal compounds including the xenovulenes, citrinin, sepedonin, sclerotiorin, and asperfuranone.

oxidative rearrangement | azaphilone | colchicine

In 1942 Harold Raistrick and coworkers working in London reported the isolation of the fungal metabolite stipitatic acid ($C_8H_6O_5$) **1** from *Penicillium stipitatum* (1). They wrote that "in spite of the large amount of experimental work which has been carried out on this substance, we have been unable up to the present to deduce an entirely satisfactory structural formula for it." In typically understated wartime style they continued, "the molecular constitution of stipitatic acid must therefore remain for the time being unsolved since, because of prevailing conditions, it has become necessary to postpone further work on the subject."

Regardless of the undoubtedly difficult prevailing conditions* (2), the structure of **1** was an inherently difficult problem. This problem was solved in 1945 by Michael Dewar who realized that the aromatic properties of **1** could be explained if an unprecedented type of nonbenzenoid aromatic system was invoked (3). Later Alexander Todd and coworkers provided the chemical proof for this hypothesis (4). Dewar named this seven-membered ring system tropolone and its discovery contributed strongly to developing ideas about aromaticity and bonding in organic chemistry (5). Indeed the study of tropolones and related nonbenzenoid aromatic systems contributed strongly to the development of the theoretical basis underpinning organic chemistry during the latter part of the 20th century. Stipitatic acid **1** and other fungal tropolones continue to stimulate interest—for example, puberulic acid **2** (5-hydroxy stipitatic acid, also discovered by Raistrick) (6) possesses potent antiplasmodial activity (IC_{50} 10 ng·mL^{-1}) and is currently a promising lead candidate as an antimalarial drug (7).

The biosynthesis of **1** and related compounds in fungi has also attracted much interest; as early as 1950, for example, Robert Robinson proposed that tropolones could be derived "by the condensation of polyhydric phenols with formaldehyde or its biological equivalent" (8). However, experimental support for this hypothesis did not come until 1963 when Ronald Bentley used ^{14}C labeling to show that the precursors of **1** are acetate, malonate, and a C_1 unit (9). Later studies showed that 3-methylorcinaldehyde **3** (and, less effectively, 3-methyl orsellinic acid) is a precursor of **1** (Scheme 1) (10). Feeding experiments using stable isotopic labels have shown that a single oxygen atom derived from atmospheric O_2 becomes incorporated into the tropolone skeleton during ring expansion. This observation is inconsistent with a mechanism in which the aromatic ring is first cleaved by a dioxygenase followed by C–C bond formation to form a tropolone because, if this were the case, then two atoms of oxygen would be retained in the product (route B, Scheme 1) (11). Thus the proposed model involves formation of the hydroxymethyl intermediate **4** by an unspecified mechanism coupled to a pinacol-type rearrangement (route A, Scheme 1). The later steps of stipitatic acid **1** biosynthesis were hypothesized to proceed via stipitalide **5** (12), stipitaldehydic acid **6**, and stipitatonic acid **7** (Scheme 1) (13). Our recent work has shown that 3-methylorcinaldehyde **3** is the direct product of a fungal nonreducing polyketide synthase (NR-PKS) which most likely appends the methyl group from *S*-adenosyl methionine during biosynthesis of the tetraketide (14), and which uses a reductive release mechanism to produce the observed aldehyde (15).

No intermediates between 3-methylorcinaldehyde **3** and stipitalide **5** have been observed, and the molecular mechanisms and enzymes responsible for the ring-expansion step remain obscure. The mechanism of the oxidative ring expansion which forms the fungal tropolone nucleus has thus remained one of the longest-standing puzzles in the study of natural product biosynthesis.

Results

In initial work, we grew *Talaromyces stipitatus* (*Penicillium stipitatum*) under tropolone-producing conditions and observed the production of **1**, **5**, and **6** as well as methyl stipitate **8** (Fig. 1*A*).† In previous work, we showed that the *Acremonium strictum* gene,

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The authors declare no conflict of interest.

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*The London School of Hygiene and Tropical Medicine, where Raistrick was based, was bombed on the night of May 10, 1941, destroying much of the building.

†The structure of **8** was confirmed by full high-resolution MS, NMR, and X-ray crystal structure determination. This compound has not previously been reported as a metabolite of *T. stipitatus*. It arises by reaction of stipitatonic acid **7** with methanol during the extraction procedure.

[1]To whom correspondence should be addressed. Email: r.j.cox@bris.ac.uk.

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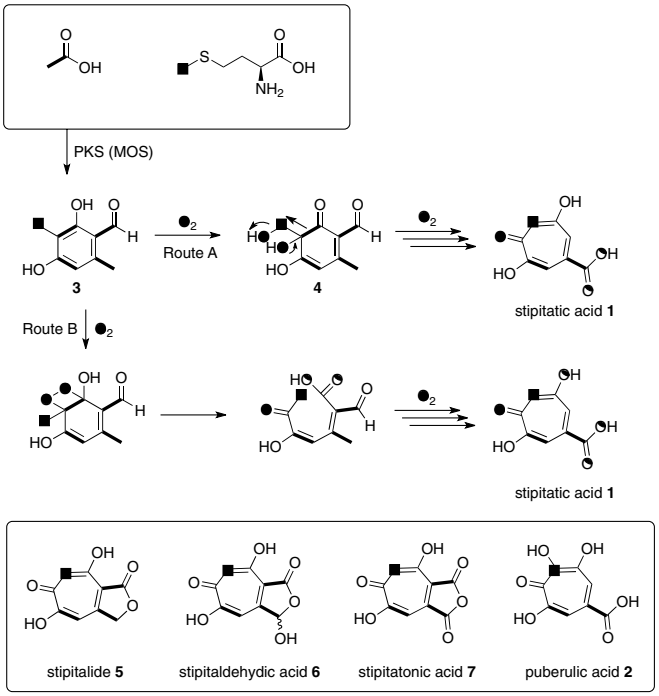


Scheme 1. Isotopic labeling patterns observed in stipitatic acid biosynthesis.

aspks1, encodes an NR-PKS known as methylorcinaldehyde synthase (MOS), which synthesizes 3-methylorcinaldehyde **3** using a reductive release domain (R) (14, 15). 3-Methylorcinaldehyde **3** is a known precursor of tropolones in *T. stipitatus* (10), so we began by searching the publicly available genome sequence of *T. stipitatus* for biosynthetic gene clusters featuring an NR-PKS gene homologous to aspks1. BLAST searching revealed four such clusters (see *SI Appendix*). One of these consisted of 11 open reading frames centered on a fungal NR-PKS gene (tspks1, TSTA_117750) encoding a protein of 294 kD. Protein domain analysis revealed a domain structure consistent with the production of methylorcinaldehyde—i.e., starter unit acyl transferase, ketoacylsynthase, acyl transferase, product template, acyl carrier protein, *C*-methyl transferase, and acyl CoA thiolester reductase domains (Fig. 1) (16) with overall identity of 38.7% (51.9% similarity) to MOS from *A. strictum*. To confirm its role in tropolone biosynthesis, and thus the likely role of the gene cluster, we performed a knockout experiment using the duplex-KO method of Nielsen and coworkers (17) with a bleomycin selection marker from *Streptomyces verticillus* (18). Of nine selected transformants, four were shown to be deficient in tropolone biosynthesis by liquid chromatography mass spectrometry (LCMS) (Fig. 1*B*)—further genetic experiments showed that in all cases the bleomycin resistance cassette had successfully integrated into the tspks1 gene.

The tspks1 gene was then cloned into a vector allowing heterologous expression in the fungal host *Aspergillus oryzae* using the inducible amyB promoter (P_{amyB}). Two variants were constructed: The first contained the genomic sequence of tspks1; the second contained tspks1 lacking the 74 bp intron and with egfp fused in-frame at the 3′ terminus. *A. oryzae* transformants containing each of these vectors were grown in the presence of amylose which induces P_{amyB}. The transformants containing tspks1 with its intron produced no new compounds vs. untransformed *A. oryzae*. However, LCMS analysis showed that *A. oryzae* clones that contained the intronless tspks1-egfp construct produced a compound eluting with the same retention time as 3-methylorcinaldehyde **3** (7.7 min, Fig. 1*E*), as well as a compound at 4.3 min (Fig. 1*E*). Microscopic examination of the mycelia from these fermentations also confirmed the presence of green fluorescence

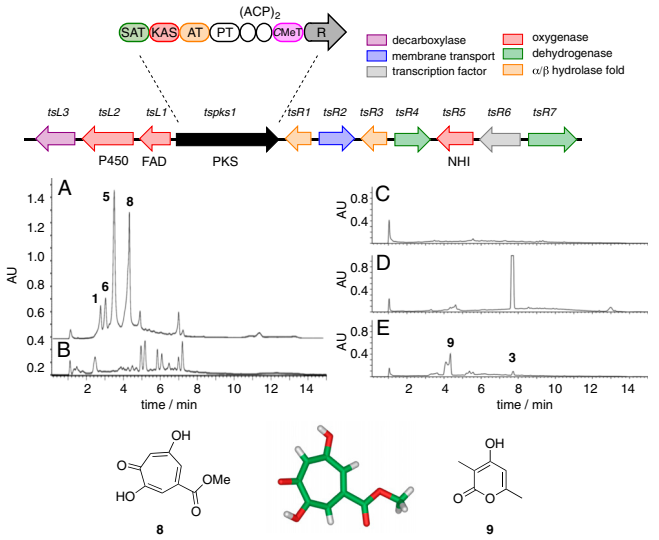


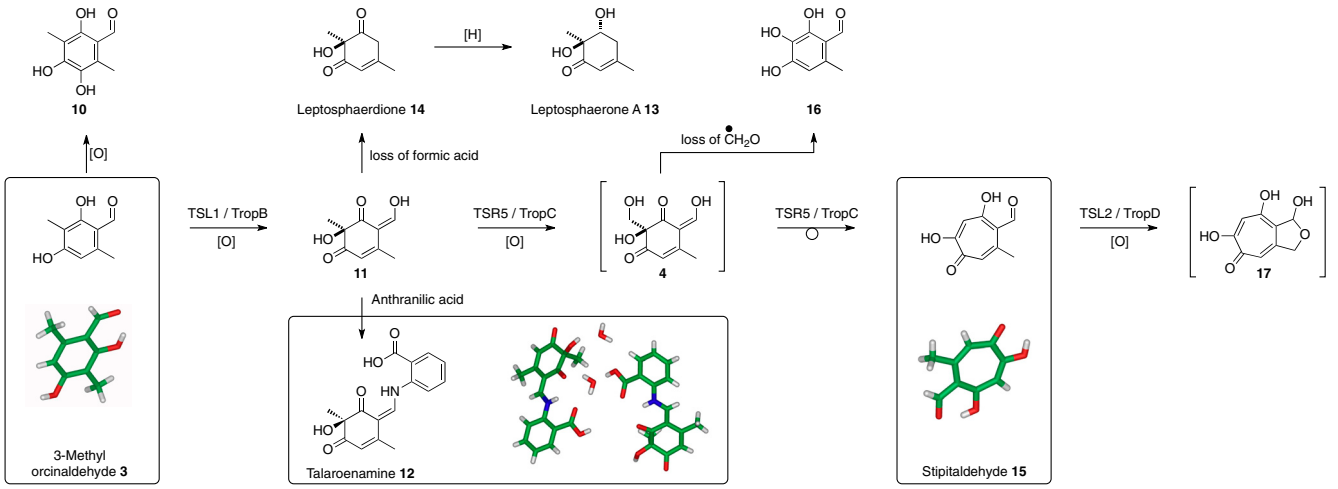
Fig. 1. Involvement of tspks1 (tropA) in the biosynthesis of methylorcinaldehyde and tropolones in *T. stipitatus*. PKS domains: SAT, starter unit acyl transferase; KAS, ketosynthase; AT, acyl transferase; PT, product template; ACP, acyl carrier protein; *C*MeT, *C*-methyl transferase; R, acyl CoA thiolester reductase. HPLC analysis of tspks1 KO: (*A*) UV chromatogram at 260 nm for WT *T. stipitatus*; (*B*) UV chromatogram at 260 nm for *T. stipitatus tspks1* KO. HPLC analysis of tspks1 expression in *A. oryzae*: (*C*) UV chromatogram at 293 nm for untransformed *A. oryzae*; (*D*) UV chromatogram at 293 nm for *A. oryzae* expressing aspks1; (*E*) UV chromatogram at 293 nm for *A. oryzae* expressing tspks1.

(see *SI Appendix*). Both compounds were purified and their structures determined by full NMR analysis (see *SI Appendix*). The 7.7 min was 3-methylorcinaldehyde **3** as expected, whereas the 4.3-min compound was proven to be the 3,5-dimethyl pyrone **9**. These experiments prove that tspks1 also encodes a 3-methylorcinaldehyde synthase and that **3** is a precursor of the tropolones. We thus propose to name the tspks1 gene tropA.

The tropA gene is flanked by three oxygenase-encoding genes (Fig. 1)—to the left a putative FAD-dependent monooxygenase (tsL1, TSTA_117740) and a cytochrome P450 monooxygenase (tsL2, TSTA_117730), and to the right a non-heme iron dioxygenase (tsR5, TSTA_117800). Because the steps required to convert **3** to a tropolone are oxidative, we began by constructing knockouts of each of these genes in turn. Knockout strains were analyzed by LCMS of organic extracts after fermentation in production medium and compared carefully with extracts from the WT organism.

Knockout of tsL1 led to the production of strains that were incapable of tropolone biosynthesis (see *SI Appendix* for chromatograms). A major product was observed in the LCMS traces of these mutants, and this was shown to be 3-methylorcinaldehyde **3** (Scheme 2) by chromatographic comparison and after purification by NMR. A minor component was also isolated, which had the molecular formula $C_9H_{10}O_4$ by high-resolution MS (calculated for $C_9H_9O_4$ $[M\text{-}H]^-$ 181.0501, measured 181.0506) corresponding to hydroxylation of **3**. This prediction proved to be the case when the minor component was identified as 5-hydroxy-3-methyl-orcinaldehyde (19) **10** by full NMR analysis (see *SI Appendix* for details).

Knockout of tsR5 using the same procedure also led to strains incapable of tropolone biosynthesis (see *SI Appendix* for chromatograms). In this case, LCMS analysis showed that a different $C_9H_{10}O_4$ isomer was produced. Isolation and full NMR analysis proved this compound to be dione **11**, the 3-hydroxylation product of 3-methylorcinaldehyde **3**. Additionally, a compound arising from the condensation of this compound with anthranilic acid (probably formed by degradation of tryptophan) was isolated



Scheme 2. Overview of the results from KO experiments. Compounds in square brackets were not observed directly. Note that the absolute configurations of **11**, **13**, and **14** are unknown.

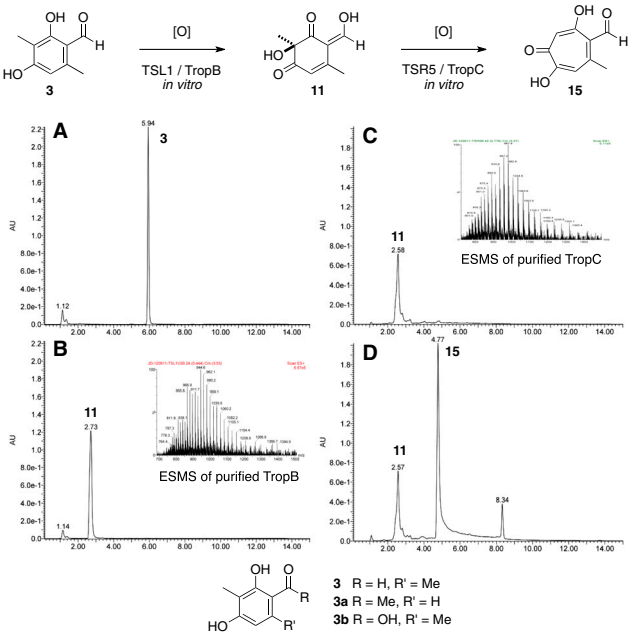
and named talaroenamine **12**. Its structure was proven by full NMR analysis and X-ray crystallography. The Flack parameter, which is the usual measure of whether the assigned stereochemistry is correct, is in this case not reliable. However, Bijvoet pair analysis gives the Hooft parameter for this structure as −0.01(6). The probability (P2) that the handedness has been correctly assigned is 1.000 and "P3 (false)" is 0.2 e-09 (20), indicating that the absolute configuration of **12** is as shown in Scheme 2—i.e., *R* at C-3. Two other minor compounds were isolated: These proved to be the known fungal metabolite leptosphaerone A **13** (21, 22) and its oxidized precursor **14** (which we name leptosphaerdione). Dione **14** probably arises by loss of formic acid via a retro-Claisen reaction from **11** itself. These compounds were not observed in WT *T. stipitatus* extracts grown under the same conditions and appear to be shunt metabolites.

Knockout of tsL2 gave a chromatographically complex mixture, a major component of which was the tropolone **15** ($C_9H_8O_4$), which we name stipitaldehyde. As previously, this compound was isolated and fully characterized by NMR and X-ray crystallography. A second, minor, component had the molecular formula $C_8H_8O_4$ and was shown by NMR analysis to be 3-hydroxyorcinaldehyde **16**.

Further evidence for these processes was obtained by heterologous expression of the oxidative enzymes. The tsL1, tsL2, and tsR5 genes were cloned by standard procedures using RT-PCR from *T. stipitatus* total RNA (so that introns were removed) into pET28a, and then transformed into *Escherichia coli* BL21 CodonPlus RP cells. Both TsL1 and TsR5 proteins were obtained in soluble form and electrospray mass spectrometry analysis of the His-tag-purified proteins indicated that they had been expressed as expected without any mutations or deleterious modifications (Scheme 3). However, TsL2 could not be obtained as soluble protein from *E. coli* expression. Calibrated gel filtration chromatography indicated that TsL1 exists as a trimer, whereas TsR5 appears to be monomeric.

Bioinformatic analysis indicated that TsL1 is likely to require FAD as a cofactor and this was confirmed by precipitation of the protein and LCMS analysis of the supernatant (see *SI Appendix*). Incubation of purified TsL1 protein with 3-methylorcinaldehyde **3** and NAD(P)H under aerobic conditions rapidly led to complete conversion to the hydroxylated dearomatized product **11** demonstrated by LCMS comparison with authentic material (Scheme 3*B*). Kinetic analyses showed that NADPH was the marginally preferred cofactor ($k_{cat} = 3.4\ s^{-1}$; $K_M = 65.3\ \mu M$) over NADH ($k_{cat} = 3.1\ s^{-1}$, $K_M = 158.2\ \mu M$). A number of analogous substrates were also tested in vitro with TsL1 (see *SI Appendix*). The only compound observed to serve as a substrate in these reactions was methyl ketone **3a**. All other tested substrates, including 3-methylorsellinic acid **3b**, were not hydroxylated.

Incubation of purified TsR5 protein with intermediate **11**, Fe(II), α-ketoglutarate, and ascorbate led to the formation of stipitaldehyde **15**—again detected by LCMS in comparison with authentic material (Scheme 3*D*). Small quantities of 3-hydroxyorcinaldehyde **16** were observed, consistent with the intermediacy of **4** (see *SI Appendix*). The in vitro assays were also used to show that Fe(II) and α-ketoglutarate are essential for turnover in vitro, but that lack of ascorbate leads only to a reduction in production of **15** (see *SI Appendix*).



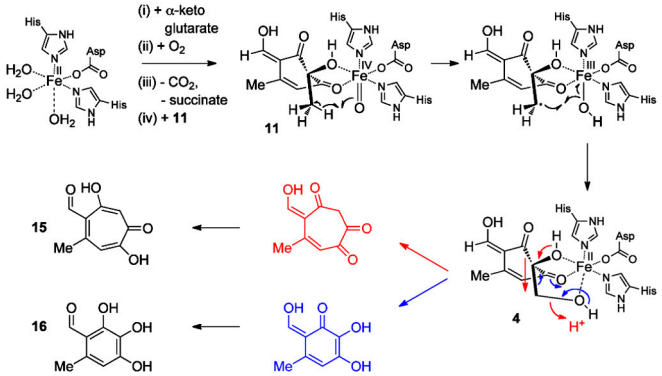
Scheme 3. In vitro assay of TsL1 (TropB) and TsR5 (TropC) using purified proteins: (*A*) HPLC trace (300 nm) of boiled TropB incubated with **3** (2 mM), potassium phosphate (20 mM, pH 7.6), EDTA (1 mM), and NADPH (3 mM); (*B*) TropB incubated with **3** (2 mM) under the same conditions (ESMS, electrospray mass spectrometry); (*C*) HPLC trace (375 nm) of boiled TropC incubated with **11** (2.5 mM), Tris (50 mM, pH 7.5), ascorbate (4 mM), α-ketoglutarate (2.5 mM), and $FeSO_4$ (0.1 mM); (*D*) TropC incubated with **11** (2.5 mM) under the same conditions. See *SI Appendix* for experimental details and further controls.

Thus TsL1 and TsR5 catalyze the two key oxidations and the rearrangement required during the formation of the tropolone nucleus in fungi. We therefore propose to name the encoding genes tropB and tropC. The gene tsL2 must encode an enzyme which catalyzes the next step in the pathway. It seems likely that this step is an oxidation of the unactivated methyl group of **15** to form putative intermediate **17** (Scheme 2), which requires further oxidation to form the lactone **5**. We therefore rename tsL2 as tropD.

Discussion

Knockout and expression of tropA (tspks1) shows that it encodes a 3-MOS, identical in function to MOS from *A. strictum* (15). The expression of tspks1 in *A. oryzae* led to the production of two compounds—3-methylorcinaldehyde **3** itself and the methylated pyrone **9**, which was previously observed as a metabolite of *T. stipitatus* (23) and which is also produced by *A. strictum* MOS as a by-product (14). The results from knockout and expression of tropB and tropC confirm the general route shown in Scheme 1, but also unexpectedly show the oxidative rearrangement to be a two-step process involving an initial oxidation catalyzed by a FAD-dependent monooxygenase (TropB), followed by a second oxidation catalyzed by a non-heme Fe(II)-dependent dioxygenase (TropC). The in vitro results strongly support the observation from feeding experiments that 3-methylorcinaldehyde **3** is a much better starting material than 3-methylorsellinic acid **3b**. Although **3b** is not a substrate for TropB in vitro, it may be reduced to **3** in vivo and thus serve as a surrogate substrate at low level. The absolute configuration of **11** was inferred from the absolute configuration of **12**, determined by X-ray crystallography, which is opposite to that reported in some other compounds such as sclerotiorin **19** and asperfuranone **20**. However, there are numerous examples where both antipodes of such compounds are observed (e.g., leptosphaerones A and C, refs. 21 and 22, and in many azaphilones, ref. 24), so it seems likely that relatively minor mutations in the active sites of TropB enzymes could lead to an exchange in facial selectivity for the delivery of oxygen.

The second step of the process is hydroxylation of **11** to give **4** catalyzed by TropC, which is a non-heme Fe(II) and α-ketoglutarate-dependent oxidase. Sequence comparison shows it to have a conserved 2-His-1-carboxylate iron-binding facial triad (H210, D212, H269; see *SI Appendix*). The oxidative mechanisms of such enzymes are well understood (25): Initial binding of α-ketoglutarate and O_2 leads to oxidative decarboxylation of α-ketoglutarate and the formation of the active Fe(IV) oxo species (Scheme 4). This species would bind **11** and hydroxylate it, leading to **4** bound to an Fe(II) species. Xin and Bugg have shown that compounds similar to **4** can be rearranged to tropolones in vitro by liganded Fe(II) species and by the *E. coli* extradiol dioxygenase MhpB which is itself a non-heme Fe(II) enzyme (26)—albeit with low overall homology to TropC (23.1% similarity, 11.9% identity). Thus TropC must also catalyze the ring expansion to form the first tropolone stipitaldehyde **15**. Our results also suggest that bound **4** could undergo a complementary reaction involving deformylation of the same enzyme-bound intermediate to give 3-hydroxyorcinaldehyde **16**. Thus the observation of **16** (both in vivo and in vitro) is good evidence for the existence of **4**. It is also conceivable that **16** arises by a second oxidation of **4**, which would lead to a product that could lose formic acid, again forming the observed **16**. The homologous enzyme CtnA may catalyze a similar double oxidation (see below).



Scheme 4. Proposed mechanisms for the production of **15** and **16** by TropC.

Finally, cytochrome P450 monooxygenation of the methyl group of **15** begins the process of formation of the maleic anhydride moiety of stipitatonic acid **7**, presumably via the unobserved hemiacetal **17** (Scheme 2), which is then further oxidized via **5**, **6**, and **7** and decarboxylated to form **1**. However, further detailed in vitro investigation of TropD could not be performed because of its insolubility.

Thus these results show that fungal tropolone biosynthesis requires a minimum of three proteins: a nonreducing polyketide synthase with a *C*-methyltransferase, a FAD-dependent monooxygenase, and a non-heme Fe(II)-dependent oxidase. With this knowledge in hand, we searched the genomes of several other fungi for the presence of putative tropolone biosynthetic gene clusters (Table 1).

Potential tropolone biosynthetic clusters were detected in at least 10 organisms. For example a tropolone cluster is found surrounding aspks1 (which is a tropA homologue) in *A. strictum*, featuring homologues of tropB–D (Table 1). It appears highly likely that these genes form part of the xenovulene **21** biosynthetic cluster (35); however, further proof will have to await the development of gene knockout tools that are effective in this organism. Similar chemistry is likely to lead to the erythropoietin stimulators epolones A and B **22** (36) and other complex ditropolones such as eupenifeldin and pycnidione (37). *Metarhizium anisopliae* also contains a similar cluster, although this organism is not yet known to produce tropolones. Similarly the genomes of sequenced *Aspergillus* species contain clusters spanning tropA–C, although again none are yet known to produce tropolones.

Our results also shed light on the biosynthesis of other important fungal compounds, including model compounds such as citrinin **18**, sclerotiorin **19**, and asperfuranone **20** (Scheme 5). Citrinin **18** is a mycotoxin produced by *Monascus purpureus*. Its biosynthetic gene cluster has been sequenced, but the function of the encoded proteins has not been fully elucidated (38). The core of the cluster consists of an NR-PKS (encoded by *pksCT*) homologous to TropA (56.0% similarity, 41.4% identity). Citrinin does not undergo a ring expansion during its biosynthesis and the gene cluster lacks a tropB homologue consistent with this observation. However, there is a TropC homologue (CtnA, 49.0% similarity, 35.6% identity) which is likely to catalyze the required oxidation at the 3-methyl position. Double oxidation would give an aldehyde, which could be the substrate for the *ctnC*-encoded aldehyde dehydrogenase that would form the observed carboxylic acid functionality of citrinin **18**. Sepedonin **23** (*Sepedonium chrysospermum*) is a tropolone closely related to citrinin **18** (39). It appears likely that **23** is biosynthesized by a similar route, although the pathway would have to include a TropB homologue.

Sclerotiorin **19** and the closely related asperfuranone **20** are representatives of the large class of bioactive fungal azaphilones (40). The asperfuranone gene cluster in *Aspergillus nidulans* was recently discovered by Wang and coworkers, although without detailed evidence of gene function they could not accurately predict the roles of individual genes (32). The NR-PKS encoded by *afoE* accepts a dimethylated tetraketide as starter unit (41). As expected, AfoE is homologous to TropA (51.3% similarity, 36.9% identity). The cluster also encodes a protein homologous to TropB (AfoD; 52.0% similarity, 35.6% identity) which was previously hypothesized to hydroxylate C-7. However, of the two FAD-dependent monooxygenases in the cluster, AfoD is the best

Table 1. Proteins encoded in gene clusters homologous to the *T. stipitatus* tropolone cluster

Organism	TropA, NR-PKS	TropB, FAD monooxygenase	TropC, non-heme iron II	TropD, cytochrome P450	Source
Acremonium strictum	MOS 51.9/38.7	AsL1 59.9/46.4	AsL3 41.6/41.6	AsR2 50.9/39.3	14, 15
Metarhizium anisopliae	MAA_07925 57.2/43.5	MAA_07924 53.6/39.7	MAA_07923 70.0/58.1	MAA_07921 67.4/55.1	27
Aspergillus nidulans	AN7903 65.4/48.7	AN7902 74.2/57.1	AN7893 72.3/58.2	—	28
Aspergillus oryzae (conserved in *A. flavus* and *A. nidulans*)	AOR_1_694164 56.1/42.0 [AFL2G$_0$9051.2/AN0523]	AOR_1_682164 62.2/47.6 [AFL2G$_0$9047.2/AN0530]	AOR_1_688164 67.8/55.1 [AFL2G$_0$9051.2/AN0526]	—	29, 30
Leptosphaeria maculans	LEMA_P086730.1 57.6/42.1	LEMA_P086690.1 51.1/35.6	LEMA_P086640.1 64.2/51.6	—	31
Aspergillus nidulans	AfoE 51.3/36.9	AfoD 52.0/35.6	—	—	32
Monascus purpureus	PksCT 56.0/41.4	—	CtnA 49.0/35.6	—	33
Coccidioides imitis (conserved in *C. posadasii*)	CIMG_07081 63.8/47.7	CIMG_07079 69.6/54.0	CIMG_07085 71.5/56.4	—	34
Colletotrichum higginsianum accession no. PRJNA47061	CH063_02139.1 38.7/28.0	CH063_02138.1 67.0/49.5	CH063_02136.1 72.0/57.2	—	NCBI

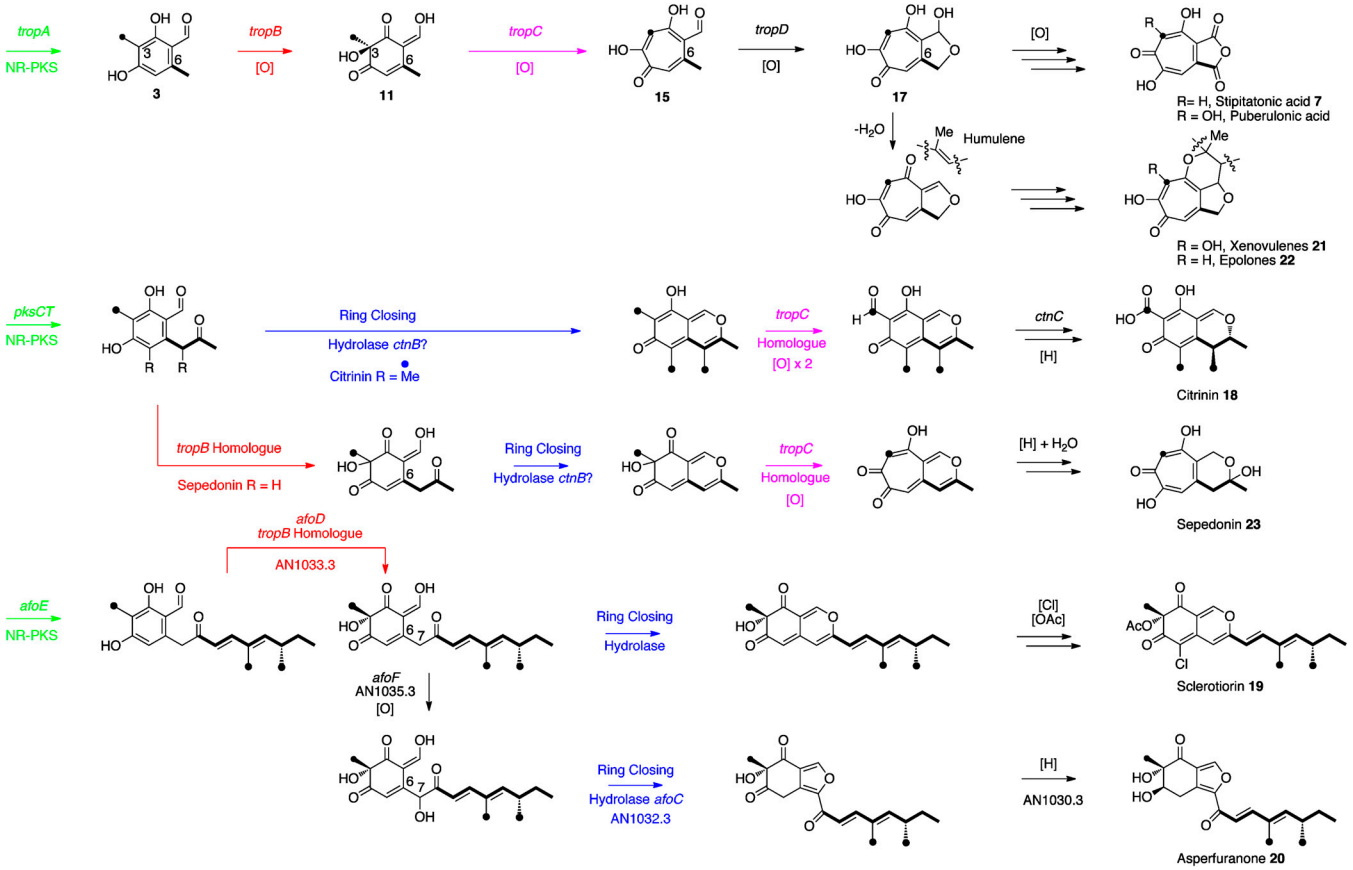
Figures show % similarity, % identity for the translated proteins.

match for TropB, whereas AfoF, which was previously given the role of C-3 hydroxylation, shows low overall homology to TropB (21.1% similarity, 11.0% identity). We suggest that AfoF hydroxylates C-7 and represents a branchpoint between the sclerotiorin and asperfuranone pathways.

The citrinin cluster contains another gene, *ctnB*, which has previously been annotated as encoding an oxidoreductase, but this assignment of function is incorrect (see *SI Appendix* for details). In fact, CtnB is a member of the hydrolase superfamily. The asperfuranone cluster contains a gene which encodes a CtnB homologue (AfoC, 53.4% similarity, 42.9% identity), and we suggest that these hydrolases are involved in forming the pyran rings of **18**, **19**, and **23** and the furan ring of **20**. These two hydrolases are similar to TsR3, which plays no role in the early steps of tropolone biosynthesis but may be involved in the formation of the maleic anhydride moiety of **7**.

Previously AfoC was ascribed a redox role in the final reduction required for asperfuranone biosynthesis, but this is an unlikely reaction for a hydrolase. We suggest that the previously unannotated *A. nidulans* gene AN1030 catalyzes the required reductive step as it encodes an oxidoreductase.

Thus our experiments have solved the 70-y-old mystery of how tropolones are formed by fungi. We have demonstrated that TropB and TropC are active in vitro, setting the scene for detailed



Scheme 5. Biosynthetic relationships between tropolones and azaphilones: Bold lines indicate likely PKS starter units; black circles denote carbons derived from *S*-adenosyl methionine.

investigations of their mechanisms and selectivities and future use in engineered biosynthetic pathways. The results have also illuminated the biosynthesis of a wide range of other important fungal compounds and this will aid in the elucidation of many silent biosynthetic gene clusters in fungi. It may not be coincidental that the biosynthesis of bioactive tropolones such as colchicine in plants also requires an oxidative rearrangement (42) and it seems possible that this too may be catalyzed by a non-heme Fe(II) dioxygenase. The later steps of conversion of stipitaldehyde **15** to stipitatic acid **1** remain to be elucidated.

Materials and Methods

Transformation of *A. oryzae* and *T. stipitatus*, knockout of *T. stipitatus* genes, and heterologous expression in *A. oryzae* were conducted using literature procedures (17, 43). Strains were fermented in standard production media and extracted using EtOAc and extracts were analyzed by LCMS using a Waters 2767 HPLC linked to a Waters ZQ mass spectrometer. Standard chromatographic methods were run using Kinetex columns (Phenomenex), and peaks were detected using either a Waters 996 diode array detector between 210 and 400 nm or the mass spectrometer scanning an m/z range between 80 and 600 Da. Full experimental details for all procedures, compound characterization, and supporting chromatographic and bioinformatic information is contained in the *SI Appendix*.

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