Natural Product Biosynthesis

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The Biosynthesis and Catabolism of the Maleic Anhydride Moiety of Stipitatonic Acid**

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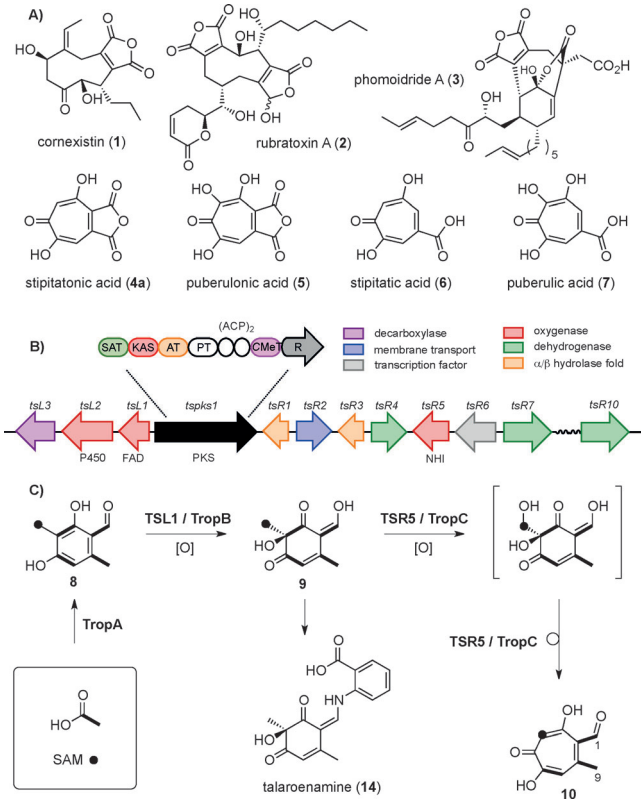
Abstract: *A series of directed knockout experiments, combined with an in vitro assay of pathway components, has elucidated for the first time the chemical steps involved in the biosynthesis of the tropolone class of fungal maleic anhydrides. The pathway involves the stepwise oxidation of aldehyde and methyl carbon atoms to form a 1,2-dicarboxylate. A hydrolase-catalyzed interconversion of this and the corresponding maleic anhydride, followed by decarboxylation of the diacid leads to the pathway's final product of stipitatic acid.*

Natural products containing a maleic anhydride moiety are recognized for their potent biological activities.[1] Examples include (Scheme 1 A) the selective herbicide cornexistin (**1**),[2] rubratoxin A (**2**)[3] (a potent inhibitor of phosphatase 2A),[4] and phomoidride B (**3**)[5] (a 20 μM Ras farnesylation inhibitor).[6] Maleic anhydrides are relatively common products of fungi and include the tropolones stipitatonic acid (**4a**) and puberulonic acid (**5**) which are precursors of stipitatic acid (**6**) and puberulic acid (**7**), respectively.[7] Puberulic acid (**7**) has recently been reported to be a potent antimalarial compound.[8] However, despite the interest in fungal compounds containing a maleic anhydride moiety, little is known about molecular aspects of their biosynthesis. We recently reported a putative biosynthetic gene cluster for the construction of tropolones in fungi and proved that the tropolone nucleus arises after the oxidative ring expansion of a polyketide-derived aromatic aldehyde **8**.[9] Here we report the determination of the function of enzymes encoded by other genes in the cluster and delineate the chemical steps leading to the formation and further metabolism of the maleic anhydride moiety of stipitatonic acid **4a**.

The *Talaromyces stipitatus* gene cluster (Scheme 1 B) responsible for the biosynthesis of stipitatonic acid (**4a**) contains 12 putative open reading frames clustered around the gene *tspks1* (=*tropA*) which encodes a non-reducing polyketide synthase (nrPKS) known as methylorcinaldehyde synthase (MOS).[9,10] Previously reported genetic and in vitro experiments have proven that the non-reducing polyketide synthase[11] TropA synthesizes 3-methylorcinaldehyde (**8**) which is the substrate (Scheme 1 C) for TropB to give the dearomatized hydroxycyclohexadione **9**. TropC then performs the oxidative ring expansion to provide the first tropolone metabolite **10**. However, the detailed functions of the remaining cluster genes are unknown.[9]

Previous experiments have shown that the maleic anhydride moiety of stipitatic acid is derived from the two terminal carbon atoms of the polyketide chain, that is, positions C-1 and C-9 of **10**.[12] It is thus clear that both of these positions require further oxidation. The cluster encodes four oxidases which might be involved in these steps, but also two putative hydrolases with no predicted biosynthetic roles. We therefore



Scheme 1. A) Fungal metabolic products containing maleic anhydride units. B) *T. stipitatus* gene cluster responsible for the biosynthesis of stipitatonic acid. C) Biosynthesis of the tropolone nucleus in *T. stipitatus*. SAM = *S*-adenosyl methionine.

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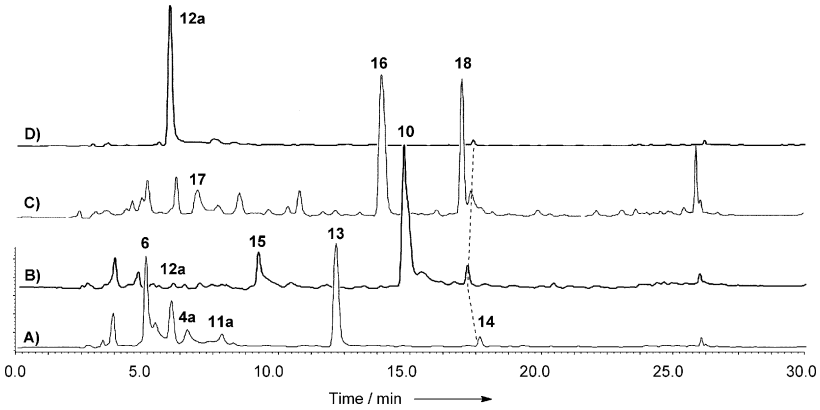


Figure 1. HPLC chromatograms of WT and KO strains of *T. stipitatus*. A) WT; B) Δ*tsL2*/*tropD*; C) Δ*tsR7*/*tropE*; D) Δ*tsR4*/*tropI*.

set out to probe the activity of these genes using a combination of knockout (KO) and in vitro enzymology.

We first analyzed tropolones and related compounds produced by the wild type (WT) *T. stipitatus* under standardized conditions using LCMS. All compounds of interest were purified and fully characterized by NMR spectroscopy and mass spectrometry. These proved to be: stipitatonic acid (**4a**), stipitatic acid (**6**), stipitalide (**11a**), stipitaldehydic acid (**12a**), methyl stipitate (**13**), and talaroenamine (**14**)[9] (Figure 1 and Scheme 2). In the cases of **4a**, **11a**, **12a**, and **13**, crystals were obtained and the structures also solved by X-ray crystallography. Methyl stipitate (**13**) has not been reported previously, although ethyl stipitate has been purified from *T. stipitatus* and is known to arise from ethanolic treatment of stipitatonic acid (**4a**).[13] Our standard isolation procedure involved the use of methanol. Under conditions where methanol was rigorously excluded **13** was not observed and increased titers of stipitatonic acid (**4a**) and stipitatic acid (**6**) were detected. Thus **13** is likely to arise from methanolysis of **4a** and is not a true metabolite of *T. stipitatus*.

The *tsL2* gene, which encodes a putative cytochrome P450 monooxygenase, was targeted for KO. Using the bipartite KO strategy pioneered by Nielsen and co-workers[14] we rapidly generated a number of potential deletion mutants using a bleomycin selection marker. Bleomycin-resistant transformants were grown in shake culture under standard growth conditions. Analysis of organic extracts by HPLC (Figure 1B) clearly showed that the mutants were incapable of synthesizing **4a**, **6**, **11a**, **12a**, or **13**. However, two tropolones were formed. Purification and full structure determination identified them to be stipitaldehyde (**10**), the known product of TropC[9] and its known deformylated product **15**.[15]

The next gene targeted for knockout was *tsR7*, which encodes a putative NAD-dependent dehydrogenase. Compounds **4a**, **6**, **11a**, **12a**, and **13** were again absent from the metabolic profile of the KO mutants but several new peaks were apparent in the chromatogram (Figure 1C). The new compounds were purified and identified by full spectroscopic structure determination. They were identified as the new compound stipitafuran (**16**), the previously known antimalarial compound cordytropolone (**17**)[16], and the novel dimeric tropolone talaroditropolone (**18**). The structure of stipitafuran (**16**) was confirmed by X-ray crystallography. All isolated compounds are oxidized at C-9 indicating that TsL2 is the C-9 hydroxylase. However, the direct product of C-9 oxidation, stipitacetal **19a/b**, was not observed. Stipitafuran (**16**) presumably arises by a shunt dehydration of **19a**, and adventitious reduction of **16** would give **17**. Talaroditropolone (**18**) is formed by dimerization of **19a** (see the Supporting Information for a possible mechanism).

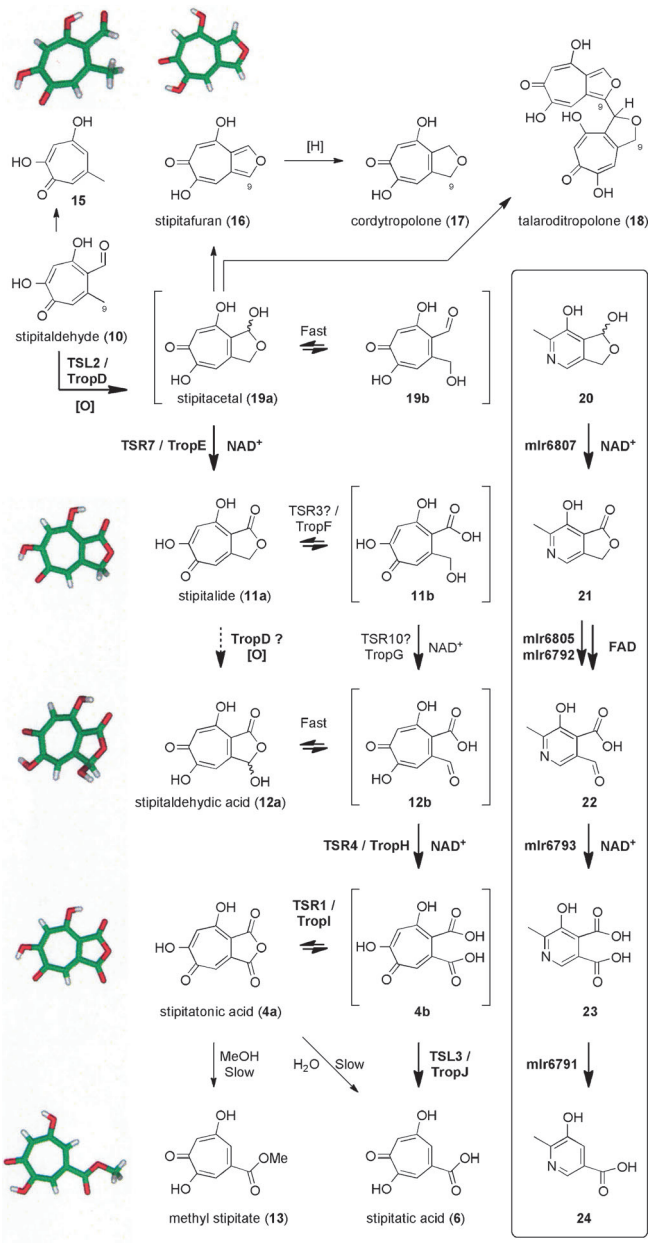
The gene *tsR4* encodes another putative NAD-dependent dehydrogenase and its KO again led to the creation of mutants incapable of stipitatonic acid formation. Stipitaldehydic acid (**12a**) was produced instead (Figure 1D). Further elucidation of the function of TsR4 and its substrate was obtained by an in vitro assay. The dehydrogenase (cloned from cDNA with an N-terminal his_6 tag) was heterologously expressed in *E. coli* BL21-codon plus. The enzyme was obtained in soluble form and the purified enzyme was incubated with **12a** and NAD^+. This readily oxidized the aldehyde to the diacid form **4b** of stipitatonic acid (see the Supporting Information).

The gene *tsR1* encodes a putative hydrolase. Knockout of *tsR1* and LCMS analysis showed that only the usual range of WT compounds was produced, although the productivity of these strains was diminished. An intronless clone of *tsR1* was obtained by RT-PCR and subcloned into pET28a. Expression in *E. coli* BL21 codon-plus led to the production of significant amounts of soluble TsR1, which was purified by nickel-affinity chromatography. This protein rapidly hydrolyzed the bright yellow stipitatonic acid (**4a**) to give colorless diacid **4b**, which was identified by LCMS analysis (see the Supporting Information).

Under normal fermentation conditions *T. stipitatus* accumulates stipitatic acid (**6**) as the final product of the pathway. The gene cluster contains *tsL3* which encodes a putative decarboxylase and it seemed reasonable to assume that this gene is responsible for the final chemical step. Knockout of *tsL3* could not be achieved. To probe this step further, *tsL3* was cloned from cDNA and expressed in *E. coli* with an N-terminal his_6 tag. This afforded good yields of soluble TsL3 which was purified by nickel-affinity chromatography. The pure protein converted **4a** to **6** in vitro. However, much faster reaction was observed when the hydrolase TsR1 was included in the mixture, showing that the decarboxylase TsL3 most likely reacts with the dicarboxylate **4b**.

Further in vitro experiments supported the role of TsR4, TsR1, and TsL3 as the final tailoring enzymes in the stipitatic acid biosynthetic pathway. The three enzymes were mixed together in an in vitro assay with **12a** and NAD^+ at pH 7.0. The reaction mixture was incubated at room temperature and monitored by LCMS. The HPLC chromatograms of the time course showed rapid conversion of **12a** to **6** (Figure 2A). In an identical reaction mixture lacking TsR1 the reaction proceeded more slowly and did not reach completion (Figure 2B).

The *tsR6* gene, encoding a pathway regulator, was subjected to KO. In this case abolition of all tropolone



Scheme 2. Elucidated pathway for the formation and degradation of stipitatonic acid (**4a**) in *T. stipitatus*. Compounds in square brackets were not isolated. The panel shows the known pathway for the degradation of pyridoxal **20**.

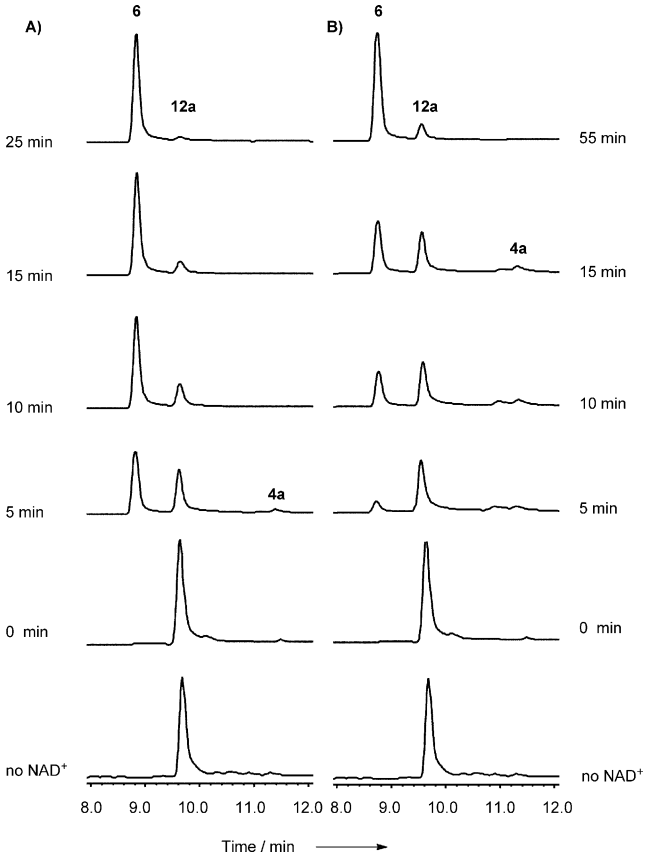
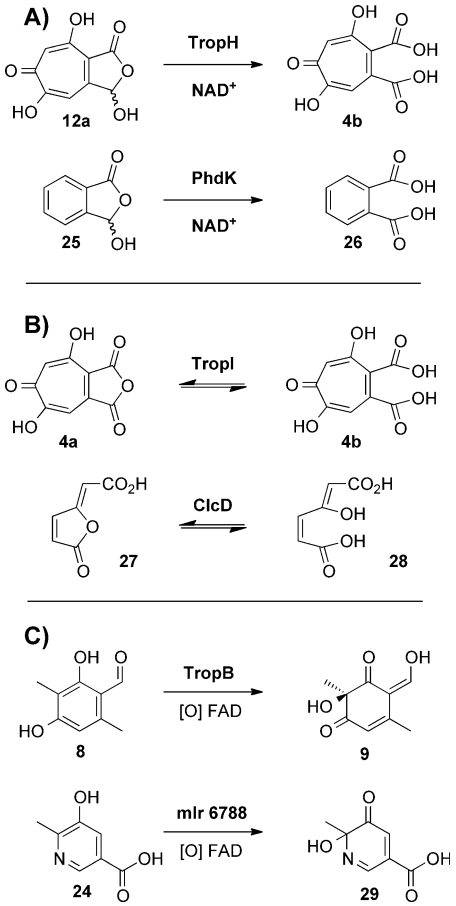


Figure 2. HPLC time course analyses of in vitro reactions catalyzed by TsR4, TsR1, and TsL3. A) Analysis of a reaction containing **12a**, NAD^+ and TsR4, TsR1, and TsL3. B) The conditions are identical to the previous ones, but lacking TsR1.

biosynthesis was observed and no new compounds were formed. In the case of the final two genes in the *trop* cluster, *tsR3* and *tsR10*, satisfactory disruptants could not be generated. All transformants tested displayed the same chemotype as the WT strain, and genetic analysis did not confirm successful insertion of the bleomycin selection cassette.

The combined information from knockout and in vitro assay experiments supports the pathway shown in Scheme 2. The cytochrome P450 monooxygenase TsL2 (=TropD) appears to hydroxylate stipitaldehyde (**10**) at C-9 to form the primary alcohol **19b** which would be expected to be in equilibrium with its hemiacetal form **19a**. In the absence of TsR7 these metabolites are not directly observed, but shunted to **16**–**18**. Thus TsR7 encodes the next step in the pathway and is renamed TropE. TropE is homologous (21% identity, 37% similarity) to the bacterial enzyme mlr6807 (pyridoxal-4-dehydrogenase), which catalyzes the oxidation of pyridoxal (**20**) to the lactone **21** during the early steps of pyridoxal degradation (Scheme 2, panel) and TropE is therefore responsible for the formation of **11a**.[17]

Stipitalide (**11a**) (or its hydrolyzed product **11b**) is then a substrate for another oxidation. There are two possibilities: either a second oxidation by the cytochrome P450 TropD, or an oxidation by the NAD-dependent dehydrogenase encoded by *tsR10*. Attempts to knockout *tsR10* failed, and knockout of *tropD* already blocks the pathway at an earlier step. Attempts to express *tropD* and *tsR10* have not yet been successful; however oxidation of stipitalide (**11a**) would give stipitaldehydic acid (**12a**), and this was shown to be the substrate for TsR4 in vitro, while knockout of *tsR4* resulted in accumulation of **12a** itself. TsR4 is thus renamed TropH. The product of the TropH-catalyzed reaction is dicarboxylate **4b**. TropH shows homology to 2-carboxybenzaldehyde dehydrogenase (PhdK)[18] involved in the degradation of polyaromatic hydrocarbons: the enzymes share 61% similarity and 42% identity (Scheme 3A). TropH also catalyzes a reaction chemically analogous to the conversion of **22** to **23** during pyridoxal



Scheme 3. Reactions catalyzed by enzymes homologous to those on the Trop pathway.

degradation, although it shares little sequence homology to mlr6793 (Scheme 2, panel).

The hydrolase encoded by *tsR3* could not be knocked out, but a plausible role for TsR3 is the interconversion of **11a** and **11b** prior to oxidation to **12a**/**12b**, and so TsR3 is tentatively renamed TropF. Once again a chemically similar hydrolysis/oxidation sequence (conversion of **21** to **22**) occurs in the pyridoxal degradation pathway. The other hydrolase, TsR1, catalyzes the rapid hydrolysis of **4a** to **4b**. TsR1 is homologous to the class of dienelactone hydrolases, exemplified by ClcD (Scheme 3B). ClcD shares 28% identity of its amino acid sequence with TsR1 and has a known catalytic function during the hydrolysis of *cis*-dienelactone (4-carboxymethylenebut-2-en-4-olide) **27**, which is an intermediate in the degradation pathway of 3-chlorobenzoic acid, to maleylacetate **27** (Scheme 3).[19] TsR1 is thus renamed TropI.

The pathway features substrates bearing carbon atoms at the hemiacetal (**19a**), lactone (**11a**), lactol (**12a**), and anhydride (**4a**) oxidation levels and chemical interconversion between the open and closed forms is required. In the case of the lactone/anhydride pairs this reaction is known to be relatively slow and hydrolase enzymes (TropF and TropI) are present to increase the rate. However, interconversion of the lactol/hemiacetal pairs is expected to be much faster and no enzyme catalysis is required. Evidence for this hypothesis comes from the work of McClelland and co-workers who showed that closely related intramolecular reactions of 2-hydroxymethyl benzaldehydes are around 10^3 times faster[20] than the corresponding reactions of 2-hydroxymethyl benzoic acids.[21]

TropI is not essential to the pathway: its knockout does not prevent the formation of **6**, and in vitro assays show that TropH and TropJ are sufficient for the formation of **6** from **12a/b**. However the in vitro assays showed that the presence of TropI increases the rate of the decarboxylation reaction both in the presence of TropH and its absence. The role of TropI may therefore be to scavenge stipitatonic acid (**4a**), or simply to increase pathway throughput.

The last step in the pathway is catalyzed by TsL3 (=TropJ) and involves decarboxylation of **4b**. TropJ is homologous to another pyridoxal degradation enzyme, mlr6791 (3-hydroxy-2-methylpyridine-4,5-dicarboxylate decarboxylase) which converts **23** to **24**,[22] and with which it shares 32% identity and 49% similarity. Finally, it is also interesting to note that the last enzyme of the pyridoxal degradation pathway, mlr6788, catalyzes a very similar hydroxylation (**24** to **29**) to that catalyzed by TropB at the start of the tropolone pathway (**8** to **9**, Scheme 3C), although these FAD-dependent enzymes share only 18% identity and 32% similarity.

Overall, we have shown for the first time the molecular steps operating during the synthesis and degradation of the tropolone class of fungal maleic anhydrides. Further work is underway to investigate the biosynthesis of other classes of fungal maleic anhydrides.

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