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Early Oxidative Transformations During the Biosynthesis of Terrein and Related Natural Products

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Abstract: The mycotoxin terrein is derived from the C₁₀-precursor 6-hydroxymellein (6-HM) via an oxidative ring contraction. Although the corresponding biosynthetic gene cluster (BGC) has been identified, details of the enzymatic oxidative transformations are lacking. Combining heterologous expression and in vitro studies we show that the flavin-dependent monooxygenase (FMO) TerC catalyzes the initial oxidative decarboxylation of 6-HM. The reactive intermediate is further hydroxylated by the second FMO TerD to yield a

highly oxygenated aromatic species, but further reconstitution of the pathway was hampered. A related BGC was identified in the marine-derived *Rousoella* sp. DLM33 and confirmed by heterologous expression. These studies demonstrate that the biosynthetic pathways of terrein and related (polychlorinated) congeners diverge after oxidative decarboxylation of the lactone precursor that is catalyzed by a conserved FMO and further indicate that early dehydration of the side chain is an essential step.

Introduction

The mycotoxin terrein **1**, first isolated in 1935 from the ascomycete *Aspergillus terreus*,^[1] and related fungi such as *Aspergillus lentulus*,^[2] has been the focus of many studies due to its diverse biological activities. These include phytotoxic^[3] and anti-inflammatory properties,^[4] inhibition of tumour angiogenesis^[5] and melanogenesis^[6] as well as inhibition of biofilm formation in *Pseudomonas aeruginosa*.^[7] Isotope labelling^[8,9] and genetic experiments^[3] revealed that the oxygenated cyclopent-2-en-1-one moiety, present in **1**, is derived via ring contraction of the precursor *R*-6-hydroxymellein **2** (6-HM, Scheme 1A). Although the terrein biosynthetic gene cluster (BGC) was discovered in 2014, and the biosynthesis of **2** by collaboration of the non-reducing PKS (nr-PKS) TerA and the

PKS-like multidomain protein TerB has been investigated in detail,^[10] the later steps remain obscure.

By gene-knockout experiments in *A. terreus* Brock et al. identified that the late-stage genes *terCDEF* (and *terR* encoding a transcriptional activator) are essential for production of terrein **1** in vivo. However, no pathway intermediates following 6-HM **2** could be isolated from individual knockout strains.^[3] The group's alternative attempt to reconstitute the biosynthetic pathway by heterologous expression in *A. niger* also met with failure as native enzymes were thought to interfere with formation of **2**.

Structurally related (poly)chlorinated polyketides include the families of cyclohelminthols **3** (*Helminthosporium velutinum*),^[11] palmaenones **4** (*Lachnum palmae*),^[12] cryptosporiopsin **5** and cryptosporiopsinol **6** (*Cryptosporiopsis* sp., *Periconia macrospinosa* and *Phialophora asteris* f. sp. *Helianthi*)^[13–15] as well as the putative Diels-Alder adduct of **5**, roussoellatide **7** (*Rousoella* sp. DLM33, Scheme 1).^[16] These compounds are expected to utilize chlorinated congeners of **2**, such as the double chlorinated **8**, as biosynthetic precursors. All respective BGC share genes homologous to *terABC* and *terR*, but no homologs of *terDEF* of the terrein BGC are present in any of these systems (Scheme 1B).^[17]

Remarkably, labelling studies showed that the cyclopent-2-en-1-one moieties of terrein **1** and cryptosporiopsin **5** display opposite isotopic incorporation at the α -hydroxyl carbon indicating a different course of the crucial ring contraction in each case (Scheme 1A).^[16,18,19] It therefore appears that the biosynthetic pathways of **1** and its chlorinated congeners diverge at some point in the pathway, possibly after the action of TerC which is annotated as a flavin dependent monooxygenase (FMO). TerD and TerE are also likely oxidative enzymes (FMO and multi-copper oxidase, respectively), while TerF (a so-called Kelch protein) is of unknown function. The role of each

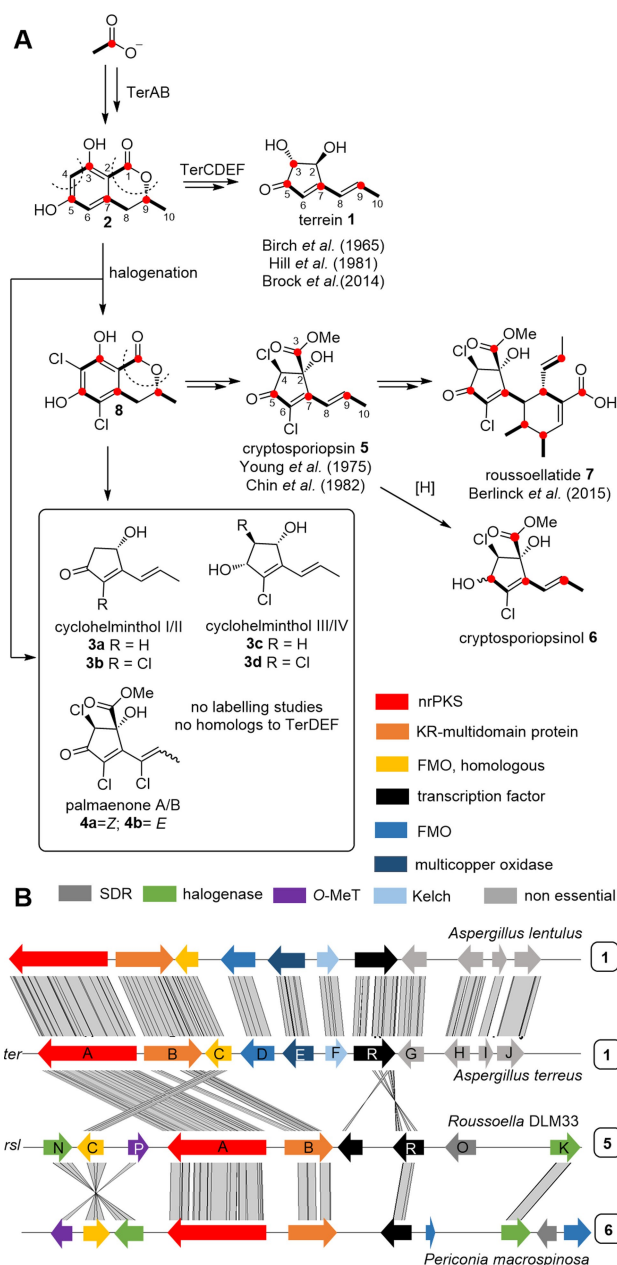
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Scheme 1. A, Labelling patterns in terrein 1 and cryptosporiopsin 5. Related compounds are boxed; B, Homology analysis^[23] between the terrein 1 BGC in *A. lentulus* / *A. terreus* and the putative cryptosporiopsin 5 /-ol 6 BGC in *Roussoella* sp. DLM33 and *P. macrospinosa*, respectively. Confirmed or expected pathway end-products are boxed. For more detailed analysis of indicated genes within the terrein 1 BGC see Table S1.

enzyme beyond the formation of **2** is therefore speculative or unknown.

We,^[20,21] and others,^[22] have shown that reconstruction of cryptic fungal biosynthetic pathways in the host organism *Aspergillus oryzae* NSAR1 is an effective way to understand complex transformations. Here, we deploy those methods and in vitro studies of isolated enzymes aiming to overcome the hurdles that have previously hampered characterization of

pathway intermediates during the biosynthesis of these intriguing metabolites.^[3]

Results

Characterization of the FMOs TerC and TerD

The BGC of terrein 1 and related natural products each harbor one homologous FMO encoding gene (*terC*). Bioinformatic analysis suggests that the sequence of TerC deposited at NCBI is truncated at the N-terminus, with the true *terC* sequence featuring an unusual intron starting after the first two 5' codons (Figure S2). We therefore generated *A. oryzae* transformants co-expressing either the truncated (*terC*^t) or the extended sequence of *terC* with *terAB* following an established protocol.^[24] TerA and TerB produce 6-HM **2**^[25] that is the putative substrate for TerC.^[3,10] Expression of the truncated *terC*^t with *terAB* led to no change over expression of *terAB* alone. However, transformants coexpressing the extended sequence of *terC* with *terAB* exhibited a distinct red colouration of the fermentation media that intensified with prolonged incubation (Figure 1), similar to that of $\Delta terD$ - $\Delta terF$ strains of *A. terreus* SBUG844.^[3] In

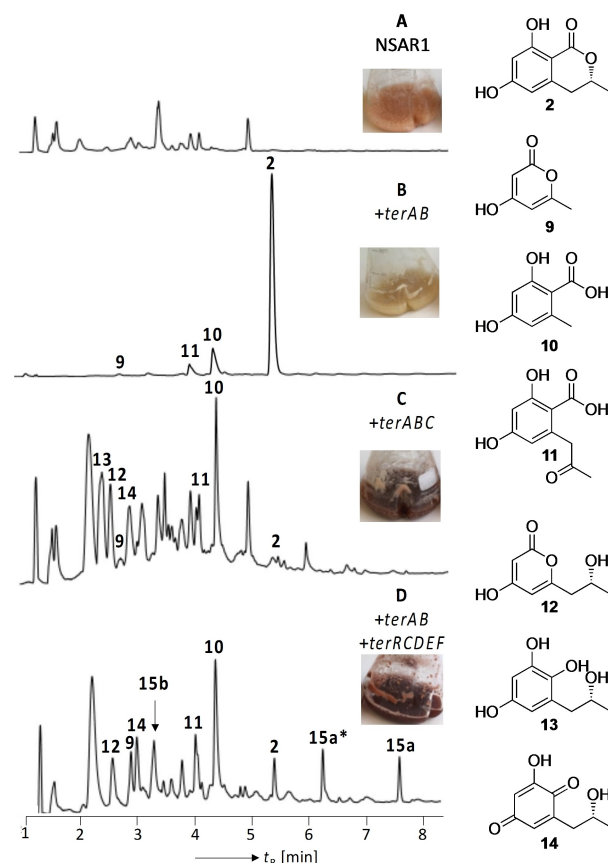


Figure 1. LCMS chromatograms (DAD 210–600 nm) of *A. oryzae* transformants expressing the indicated genes with example images of fungal liquid cultures. Fungal strains depicted in lane A–C were grown for 3 days; lane D was grown for 5 days. Note that the chromatogram in C is expanded 4-fold vs. A and unlabelled peaks in C are also present in the WT A.

addition to TerA-derived polyketide products 9–11 and the tetraketide pyrone 12, LCMS analysis revealed that 2 was present in much lower quantities compared to transformants solely expressing *terAB* (Figures 1B and 1C).

We initially identified two new peaks during early stages of cultivation of *terABC* transformants corresponding to the very polar compounds 13 ($C_9H_{12}O_4$; $[M+H]^+$ calculated 185.0814, found 185.0802) and 14 ($C_9H_{10}O_4$). Based on their molecular weights, both 13 and 14 could be possible pathway intermediates. Compound 13 was purified to homogeneity (~12 mg from 1 L fungal culture) and its structure was revealed by full NMR analysis (Figures S34–S38). The *R*-configuration of 13 was assumed from the known configurations of the precursor 6-HM 2 and the off-pathway lactone 12. Compound 14 proved even more unstable than 13 and could not be purified. Based on its mass, retention time and distinctive UV spectrum we propose 14 to be a quinone derived from 13.

Interestingly, 13 is not red and no obvious new peaks in the respective LCMS chromatograms of *terABC*-transformants were observed concomitantly with 13. We therefore increased the cultivation time to five days and separated the organic extract into 20 fractions by preparative HPLC. Although almost all fractions displayed a light red-brownish hue, three fractions in particular exhibited a deep red colouration. Two isomers 15a and 15a* were identified in this extract that show the same UV-absorption and molecular weight ($C_{18}H_{16}O_7$; $[M+H]^+$ calculated 345.0974, found 345.0977). A third peak 15b ($C_{18}H_{18}O_8$; $[M-H]^-$ calculated 361.0923, found 361.0904) is more highly oxidised. However, despite many attempts the structure of these dimeric compounds could not be unambiguously assigned.

In order to corroborate that 2 is the precursor of 13 and 15ab, three individual *A. oryzae* transformants solely expressing *terC* were pulse fed with 2. Each 50 ml fermentation was fed with 9 mg of 2 each day for 3 days. At the end of the experiment extracts were examined by LCMS. Very low levels of 13 and 15a were detected, but only in extracted ion chromatograms. Meanwhile, the peak for 2 completely disappeared in the supplemented *terC*-strains, but not in the supplemented control strain lacking *terC* (Figure S3). This observation emphasizes the *in vivo* instability of the new intermediate 13. In addition, only the fermentation media of the *terC*-strain supplemented with 2 displayed a red colouration, but not the supplemented control strain (Figure S4).

In order to monitor the conversion of 6-HM 2 into 13 in detail, TerC was produced as soluble protein in *Escherichia coli* (Figure S5). Incubation of 2 with TerC *in vitro*, initially leads to production of a more polar compound 16 with 16 additional mass units ($C_{10}H_{10}O_5$; $[M-H]^-$ calculated 209.0450, found 209.0457) that we propose to be the C-2 hydroxyl-congener of 2 (Figure 2B). Lactone 16, in turn, readily converts to 13. Upon prolonged incubation, the peak for 16 is completely absent and 14 and 15b are formed at the expense of 13 (Figure 2C). In contrast to previous *in vivo* experiments neither 15a nor 15a* are formed *in vitro*.

TerD is also an FMO, but is specific to the terrein BGC. We prepared TerD as recombinant protein in *E. coli* (Figures S6–S7). Incubation of TerD with 6-HM 2 yields a single new product 17

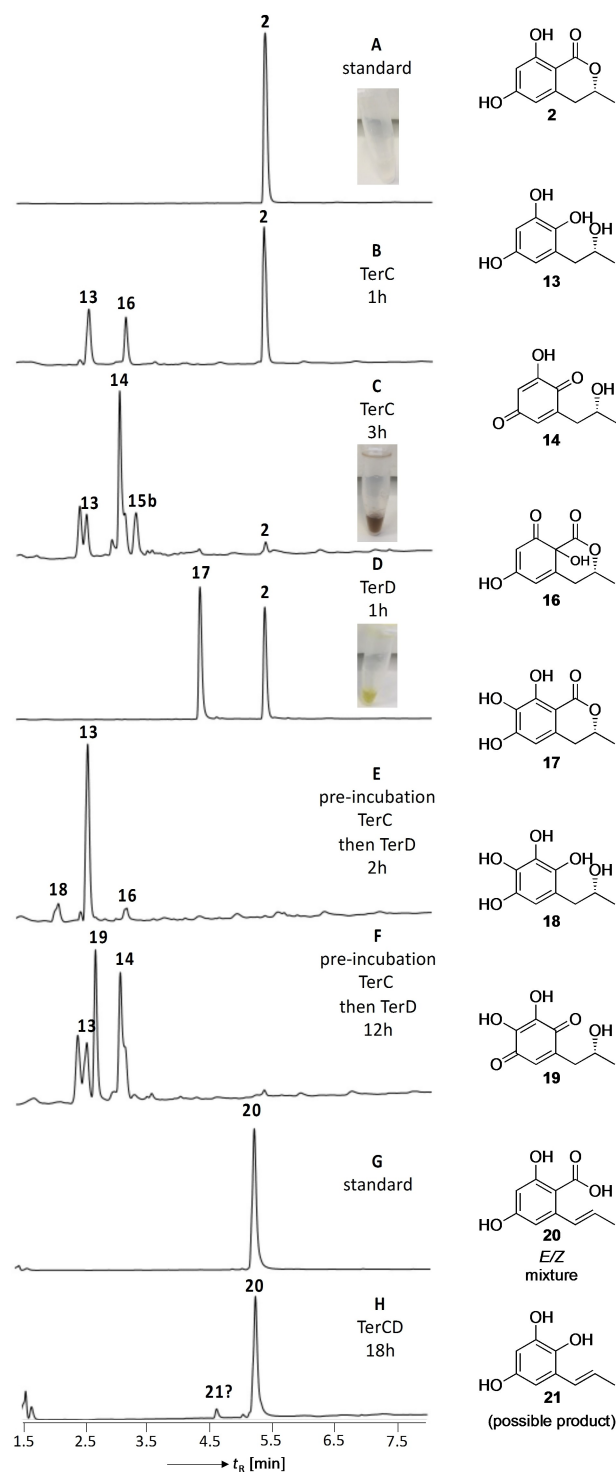


Figure 2. LCMS-chromatograms (DAD 210–600 nm) of *in vitro* assays with TerC/D using substrate 6-HM 2 or 20 under conditions as indicated. Colour changes of selected enzymatic conversions are given next to the chromatograms.

(Figure 2D) that was identified as the C-4-hydroxyl-congener of 2 by 2D NMR analysis (Figures S54–S58).

Co-incubation of TerC and TerD with 2, as well as pre-incubation of 6-HM 2 with TerC followed by addition of TerD,

yields another very polar new minor product **18** (Figure 2E) in addition to the major product **13**. Compared to the TerC-derived tetra-ol **13**, compound **18** shows additional 16 mass units ($C_9H_{12}O_5$; $[M-H]^-$ calculated 199.0606, found 199.0605). Based on UV, mass spectra, retention time and comparison to previously observed compounds, **18** appears to constitute the C-4 hydroxyl-congener of **13**. Compound **18** slowly oxidises to **19** (Figure 2F) similar to the oxidation of **13** to **14**. UV analysis of **19** is consistent with the quinone structure.^[26]

Late steps during the biosynthesis of terrein 1

In order to reconstitute and decipher the late-stage biosynthesis of terrein **1** we individually co-expressed all of the proposed essential genes *terA-terF* in *A. oryzae*. In this experiment each structural gene was expressed from a promoter known to be functional in *A. oryzae*.^[24] However, none of the transformants showed production of **1** and no other putative pathway intermediates other than **13/14** and the corresponding dimeric forms including **15a** could be identified (Figure S8). Unexpectedly, the TerD-derived compounds **17–19** were not observed in vivo, indicating an unknown incompatibility with our host organism.

Attempts to use alternative start sites for *terD-terF*, cloning individual genes from gDNA instead of cDNA, using different fermentation media and cultivation protocols, also met with failure to generate new pathway intermediates or the end product terrein **1**. Additional introduction of the four genes *terG-terJ* (previously shown to be non-essential for production of **1** in *A. terreus*),^[3] also with known strong *A. oryzae* promoters, did not lead to any change in the metabolic output (Figure S8). Since **1** shows antifungal activity,^[27] and its production could conceivably kill transformants successfully expressing the complete pathway, we tested its toxicity against *A. oryzae*. No inhibition of growth at a concentration up to 10 mg·mL⁻¹ was observed on solid agar plates.

Earlier studies showed that the transcriptional regulator TerR induces high-level transcription of all essential terrein pathway genes *terA-terF* and is a useful tool for the construction of new fungal expression systems.^[28] To exclude the possibility that cloning mistakes or uneven transcription levels between *terC-terF* caused by the use of different *Aspergillus* promoters (P_{amyB} , P_{enor} , P_{adhV} , P_{gdpA}) hampered production of **1**, we constructed a *terR*-based expression vector. In contrast to the previously applied modular expression system, this construct (pTYGSade-*terRCDEF*) features *terR* cloned downstream of the starch-inducible P_{amyB} , followed by its native terminator and a contiguous *A. terreus* genomic fragment covering the promoter region of *terC* up to the terminator region of *terF* (Figure S9). This approach should ensure that *terCDEF* are expressed as closely as possible to the native situation.

In former *A. oryzae* transformants *terA-C* are equally functional when cloned from either gDNA or cDNA, showing that *A. terreus* introns are generally spliced correctly in *A. oryzae*. The vector pTYGSade-*terRCDEF* was co-transformed with pTYGSarg-*terAB* and positive transformants that integrated the desired

fragments were confirmed by PCR (Figure S10). However, the *terABRCDEF* transformants only produced the dimeric compounds related to **15** in elevated titres compared to previous transformants. Once again neither **1** nor any new pathway intermediates were observed (Figure 1D).

Unsuccessful reconstitution of the terrein pathway in vivo prompted us to revisit our in vitro strategy. However, attempts to obtain the multicopper oxidase TerE and the Kelch protein TerF as soluble proteins in *E. coli* met with failure (Figures S11–S12). We tried to simulate the general oxidative ability of TerE by addition of Cu^{2+} ions to the TerCD+**2** in vitro assay. In the absence of copper this reaction gives a faint red reaction mixture, but upon presence of copper ($CuCl_2$, 5 mM; Figure S13) it immediately adopts an intense red colouration. Yet, no new products were observed by LCMS.

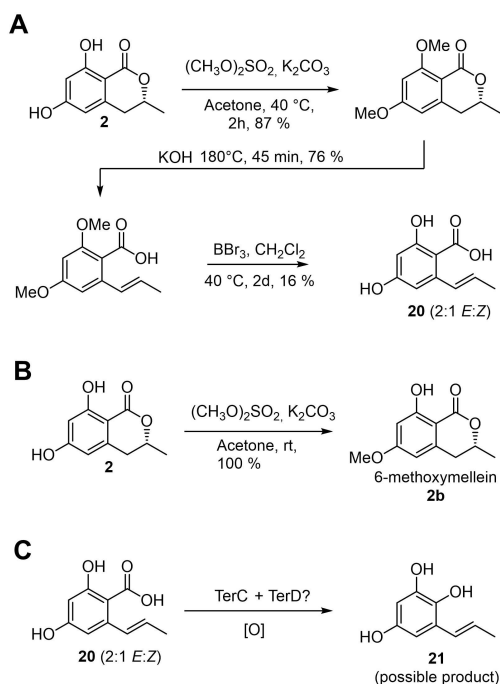
Unlike the final pathway product terrein **1**, the intermediates **13/14** and **18/19** possess a secondary hydroxyl-group at C-9 that has to be eliminated at some stage. The only candidate enzyme encoded in the terrein BGC that could conceivably catalyse this reaction is the SDR TerH. Yet, TerH has been shown non-essential for production of **1** in *A. terreus*.^[3] TerH was obtained as soluble protein from *E. coli* and added to either TerC+**2** or TerCD+**2** in vitro. No new compounds were observed. Addition of the N-terminal catalytically inactive DH-domain of TerB also yielded no new intermediates (data not shown, Figures S14–S15).

We considered it possible that dehydration of any intermediate might have to occur before the oxidative steps, and that the dehydration might be catalysed by proteins encoded outside the known terrein BGC. We therefore prepared **20** in a three-step synthesis from **2** as an alternative substrate for in vitro studies (Scheme 2A). *O*-methylation of **2** was followed by base catalysed elimination, and then BBr_3 -mediated demethylation. This afforded a ca. 2:1 mixture of inseparable *E:Z* isomers of **20**.

Neither TerC nor TerD alone show any conversion of **20**, but simultaneous incubation with both enzymes results in a new small peak in LCMS-chromatograms (Figures 2G and 2H) of assay components. HRMS confirmed a composition of $C_9H_{10}O_3$ ($[M-H]^-$ calculated 165.0552, found 165.0549) that would match the structure of tri-ol **21** that has been proposed as a potential biosynthetic intermediate.^[17,19] However, very low production of **21** thwarted structural elucidation by NMR. In addition, the methoxy-derivative 6-methoxymellein **2b** (Scheme 2B) was probed as an alternative substrate, but neither TerC nor TerD exhibited any conversion (Figure S16).

Identification a homologous BGC in *Rousoella* sp. DLM33

Inability to progress understanding of the terrein system beyond the activity of TerC led us to consider the related biosynthetic systems which lack TerD and the later proteins. We hypothesized that such pathways may use more tractable rearrangement systems and that results from such studies might help to draw conclusions about the functions of TerE and



Scheme 2. Synthesis of alternative substrates. **A**, Synthesis of **20**; **B**, synthesis of 6-methoxymellein **2b**; **C**, possible product of enzymatic conversion of substrate **20**.

TerF that were previously shown to be essential for formation of **1**.^[3]

FungiSMASH analysis^[29] identified a candidate BGC in the genome of *Rousoella* sp. DLM33, a producer of the double chlorinated lactone **8** and the cryptosporiopsin **5** derived roussoellatide **7** (for details of genome sequencing see Supporting Information).^[16] Refinement using the gene prediction tools AUGUSTUS^[30] and FGENSESH^[31] associated nine genes with the proposed *rsI* BGC (~36 kb). The central genes encode RslA and RslB which are homologs of TerA and TerB and are expected to produce 6-HM **2**. Two additional genes encode an FMO (RslC, homologous to TerC) and a transcriptional activator (RslR, homologous to TerR) and are also conserved among related BGC (Scheme 1B). Further genes in the *rsI* BGC encode: two flavin-dependent halogenases (RslK and RslN); a short-chain dehydrogenase/reductase (SDR, RslO); an *O*-methyltransferase (RslP); and another putative incomplete transcription factor. Seven of these genes are also conserved in the putative cryptosporiopsinol **6** BGC of *Periconia macrospinoso* (Scheme 1B).

The putative *rsI* BGC was confirmed by heterologous expression of *rsIA* in *A. oryzae*. This yields the same three polyketides **9–11** as previously reported for expression of *terA*, in a combined titre of 125 mg L⁻¹ (Figure 3B).^[10] Addition of *rsIB* results in formation of the key intermediate 6-HM **2** in high titres of approx. 500 mg L⁻¹ (Figure 3B).

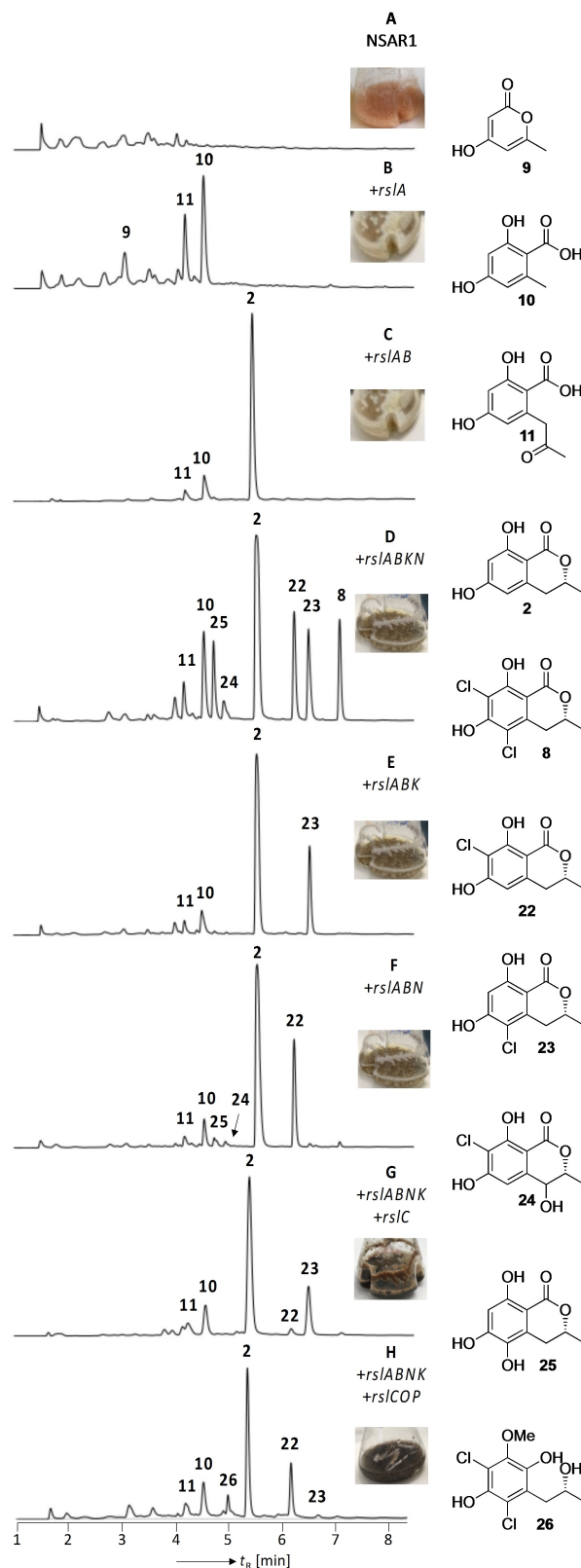


Figure 3. LCMS chromatograms (DAD 210–600 nm) of *A. oryzae* transformants expressing the indicated genes from the *rsI* BGC of *Rousoella* sp. DLM33. Example images of fungal liquid cultures are given next to the chromatograms. Fungal strains depicted in lane A–G were grown for 4 days. The fungal strain depicted in lane H was grown for 2 days to show a visible peak for **26**.

Compatibility between TerA/RsIA and TerB/RsIB

TerB is a tridomain protein with an N-terminal catalytically inactive dehydratase (ψ DH), a central catalytically inactive C-methyltransferase- (ψ C-MeT) and a functional C-terminal keto-reductase (KR).^[10] Interestingly, RsIB, that is ca. 300 amino acid residues shorter than TerB, does not contain the central ψ C-MeT domain (InterPro^[32] analysis). Since TerA and TerB interact during the biosynthesis of 6-HM **2** in vivo we were interested whether any of these enzymes could be exchanged with its homologous protein RsIA or RsIB, respectively.^[10] We therefore generated two transformant strains of *A. oryzae* expressing either *terA + rsIB* or *rsIA + terB*. Both transformant strains produce **2** very efficiently (ca. 400 mg L⁻¹ and 450 mg L⁻¹ vs. 500 mg L⁻¹ for *terA + terB*; Figure S17).

Characterization of the flavin-dependent halogenases RsIK and RsIN

Addition of the two annotated halogenase genes (*rsIK* and *rsIN*) to the *A. oryzae* transformants leads to production of the dichlorinated **8** in vivo, that was also isolated from cultures of *Rousoella* sp. DLM33.^[16] The mono-chlorinated congeners **22** and **23** are also produced (Figure 3D; NMR in Supporting Information). Individual expression revealed regioselectivity for RsIK at the C-6 and for RsIN at the C-4 position, respectively (Figures 3E and 3F). These results are in agreement with recent in vitro characterization of the homologous halogenases ChmKN (cyclohelminthols) and PloKN (palmaeonones).^[17]

Both **22** and **23**, but not **8**, are slowly converted into their C-8 hydroxyl-derivatives including **24** by endogenous enzymes in *A. oryzae*, supported by feeding studies (Figure S18, S81-S85). 6-HM **2** in turn is hydroxylated at C-6 to give **25** upon prolonged incubation which is also consistent with previous observations.^[10,17] Supplementation of the fermentation medium with sodium bromide (50 μ g mL⁻¹) results in the successful formation of mono- and dibrominated derivatives of **2** as well as mixed chloro-bromo species based on distinct isotope ratios in the respective mass spectra (Figure S19). However, iodinated derivatives were not formed in the presence of NaI.

Characterization of the flavin-dependent monooxygenase RsIC and the O-methyltransferase RsIP

The TerC/RsIC-type FMO are encoded by all related BGC (Scheme 1B). In fact, *rsIC* appears to exhibit the same unusual intron pattern located directly at the 5-end of the corresponding mRNA analogous to *terC* (Figure S2).

Similar to previous in vivo expression of *terABC*, *rsIABCKN*-transformants display a distinct reddish-brown colouration of the fermentation medium. However, no new compounds could be detected by LCMS analysis (Figure 3G). Shortened or prolonged cultivation also revealed no new products by LCMS.

All attempts to obtain RsIC as soluble and active protein in *E. coli* met with failure. Alternatively, TerC was incubated with **8**

in vitro and although no turnover was visible by LCMS analysis the samples displayed a reddish-brown colouration as observed in vivo (data not shown), indicative of oxidation.

Since potential RsIC-derived products may also be chemically unstable or prone to degradation in *A. oryzae* (e.g. as observed for **13**), we proceeded to add the two ancillary genes *rsIP* (O-methyltransferase) and *rsIO* (SDR) to generate “full cluster” transformants. A new pale brownish di-chlorinated compound **26** was found in the fermentation media of respective transformants exclusively on the third day of cultivation (before the culture medium adopted a dark colouration, Figure 3H).

Compound **26** is two mass units heavier than the expected pathway end product cryptosporiopsin **5** (C₁₀H₁₂O₄Cl₂; ([M-H]⁻ calculated 265.0034, found 265.0037). 2D NMR analysis revealed that **26** is a di-chlorinated derivative of the aromatic TerC product **13** with an additional O-methylation at the C-3 hydroxyl group (isolated 6 mg L⁻¹; Figures S87-S91). To exclude spontaneous methylation all analytical steps were repeated in the absence of MeOH whereby **26** was still produced. Interestingly, **26** has been previously isolated from *Periconia macrospinosa*, a producer of cryptosporiopsin **6**.^[33] This suggests that O-methylation is introduced by RsIP. No obvious function could be attributed to the SDR RsIO.

In analogy to the TerC-derived **13**, the RsIC-derived **26** features a secondary hydroxyl-group at C-9 that is not present in the proposed pathway end-product cryptosporiopsin **5**. Since no gene encoding an enzyme that would catalyze dehydration at this position is conserved among homologous BGC (Scheme 1B) this step remains obscure. Despite heterologous expression of seven genes (*rsIABCKNOP*) in vivo, no compounds were identified that feature a re-arranged cyclopentenone/-ol skeleton.

Discussion

6-HM **2** is an intermediate during the biosynthesis of terrein **1** and related (poly-chlorinated) natural products such as **5**.^[3,17] In the terrein pathway lactone **2** is formed by collaboration between an nrPKS and a multidomain protein with catalytically active KR-domain in vivo.^[10] Bioinformatic analysis allowed the identification of a related BGC in the marine-derived *Rousoella* sp. DLM33 that shares the two corresponding genes *rsIAB*, amongst others. Heterologous expression of either *rsIA* alone or in combination with *rsIB* revealed the same qualitative and quantitative product manifold reported for the homologous proteins of the terrein **1** BGC.^[10] In contrast to TerB, RsIB does not feature a central ψ C-MeT domain. Despite this altered domain architecture combining homologous genes from two different species (*terA + rsIB* and *rsIA + terB*) yielded the product 6-HM **2** in titres nearly as high as obtained for the native system (Figure S17). These results show that collaboration is also facilitated between homologous proteins originating from different fungal species. Furthermore, since RsIB physically lacks ψ C-MeT it appears that this domain is neither involved in

protein–protein interactions, or fulfils a catalytic role, in line with previous findings.^[10]

It is likely that collaboration during the biosynthesis of 6-HM 2 is a general feature of such homologous systems (e.g. also ChmAB from *H. velutinum*),^[17] but further in-depth investigations are required to characterize the interacting elements in greater detail.

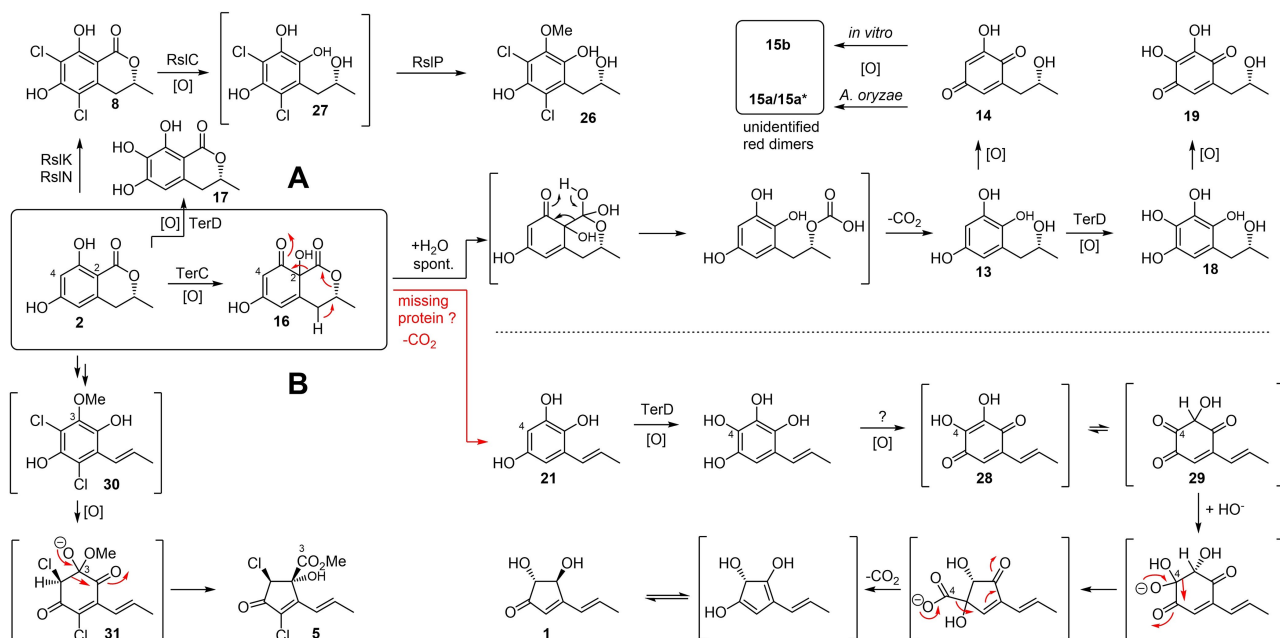
The *rsI* BGC encodes two flavin-dependent halogenases RslK and RslN that halogenate 6-HM 2 at the C-4 (RslN) and C-6 (RslK) positions (Scheme 3). The observed regioselectivity corresponds with previous reports on homologous enzymes.^[17] The formation of brominated congeners in the presence of NaBr reveals that both halogenases show halide promiscuity. This is a potentially useful approach to generate novel brominated species.

Of primary interest was the elucidation of the function of the flavin-dependent monooxygenases that are common to all the related pathways (e.g. TerC and RslC, Scheme 1B) and that are likely to catalyse the final common step before divergence of the terrein and other pathways. Characterization of TerC shows that this enzyme initially hydroxylates 6-HM 2 adjacent to the carbonyl-position at C-2 to yield intermediate 16. TerC thus matches the function expected for a classical flavin-dependent monooxygenase.^[34,35] Intermediate 16 decarboxylates spontaneously to the tetra-ol 13 in vitro (Scheme 3A). An analogous mechanism was proposed by Townsend et al. for the FMO-dependent oxidative lactone opening during the biosynthesis of cercosporin and related perylenequinones.^[36,37] Intermediate 13 is unstable both in vivo and in vitro and it is converted into the corresponding quinone 14 by autoxidation^[38] that readily dimerises to 15b that exhibits a

strong red colouration (Figure S13). Tetra-ol 13 appears to be a substrate for TerD which hydroxylates at C-4. But the product, 18, does not proceed to ring-contracted species, giving only dimers by autoxidation (Scheme 3A). TerD appears inactive in *A. oryzae* expression experiments for unknown reasons. However, TerD is a potential tool for the in vitro stereoselective hydroxylation of isocoumarin/ phenol-derivated compounds. Further investigations and screening of a substrate library will be necessary to evaluate its substrate promiscuity.

A significant difficulty in this study has been the instability of compounds beyond 13 and our inability to satisfactorily identify, isolate or characterise them. Dimers such as 15b were identified by HRMS in vitro and in vivo, but do not appear to be on the pathway to 1. Formation of 15a exclusively in vivo suggests that a native enzyme in *A. oryzae* is involved in its formation, adding another layer of complexity to efforts to detect and identify pathway intermediates. Oxidative coupling of phenol-related compounds in fungi is often mediated by laccases or cytochrome P450-enzymes.^[39,40] A recently described laccase catalyses the coupling of two subunits structurally very similar to 14, yielding the bibenzoquinone oosporein that also exhibits a strong red colour.^[41]

As anticipated, the homologous FMO RslC catalyses the same fundamental reaction, i.e. oxidative lactone opening of the di-chlorinated 8, that we could only observe in vivo. However, the direct product 27 defied detection. Similar to previous transformants expressing *terABC*, *A. oryzae* *rslABC* transformants adopted a distinct dark colouration (cf. Figures 3G and 3H) indicative of further oxidation. Alternatively, production of 27 might have induced production of fungal



Scheme 3. Summary of observed and hypothetical reactions on the pathway to terrein 1 and cryptosporiopsinol 5. **A**, Reactions and intermediates observed in vivo and in vitro. All compounds feature the C-9 hydroxyl which is not observed in 1 and 5; **B**, hypothetical pathways to 1 and 5 involving early dehydration and late benzilic acid rearrangements. See Supporting Information for DFT calculations of the respective rearrangements in each case.

melanins that function as a defence mechanism against chemical environmental stressors.^[42]

The *O*-methylated congener **26** was identified only in the presence of the *O*-methyltransferase RslP. Based on labelling studies *O*-methylation within **26** and cryptosporiopsin **5** shares the same position at C-3 (Scheme 1A). It remains elusive whether **26** constitutes a true pathway intermediate or an off-pathway shunt. Despite elevated titres of 6-HM **2** in the respective transformants (Figure 3G), the non-chlorinated **13** was not observed. This indicates that RslC requires chlorination at C-4 and C-6 for substrate recognition, and rules out the activity of RslC before chlorination.

Characterization of both FMOs TerC and RslC provides experimental evidence that the biosynthetic pathways towards terrein **1** and related (poly-chlorinated) congeners branch after oxidative lactone cleavage. The subsequent ring contractions follow a different pathway in each case as indicated by distinct labelling patterns (Scheme 1A). However the pathway beyond this decarboxylation remains unclear in both cases.

A key difficulty revealed by our results is the timing of the dehydration required to provide the *E*-propenyl side chain of terrein **1**, cryptosporiopsin **5** and the majority of other compounds in this class. The fact that this moiety is widely present suggests that the dehydration should occur early in the pathway. Since **13** is also produced by TerC *in vitro*, we can exclude that the secondary hydroxyl-group at C-9 is (re) introduced by native enzymes of our *in vivo* host *A. oryzae*.^[43–46] Likewise **18**, that is derived from sequential catalysis of TerC and TerD *in vitro*, does not feature the *E*-propenyl side chain.

Furthermore, previous studies^[3] imply that the TerD-derived lactone **17** is not an intermediate but a shunt metabolite: Feeding of **17** to a $\Delta terB$ strain of *A. terreus* did not restore production of terrein **1**. Similarly, the $\Delta terD$ strain of *A. terreus* did not show production of **1** in the presence of **17**.

However, neither TerAB nor TerC (or the respective homologs) appear capable of catalysing the required dehydration. An attractive possibility would be coupling the oxidative decarboxylation catalysed by TerC/RslC with the dehydration (Scheme 3B). The fact that we did not observe this *in vitro* or *in vivo* may indicate that TerC/RslC requires another protein to achieve such a transformation. However, since there are no more homologous genes in the two BGC a candidate is hard to identify. In addition, full expression of both clusters in *A. oryzae* could not progress either pathway further, suggesting that if such a protein exists, it is not encoded within the *ter* or *rsI* BGC. In other systems, such as those responsible for azaphilone biosynthesis in *Monascus* species, a specific *O*-acetylation and elimination sequence (MrPigM and MrPigO) provides the prop-2-enyl moiety.^[47] However homologs of these proteins are not encoded within the terrein or related BGC.

For this reason, it appears unlikely that C-9-hydroxylated compounds such as **18** and **19** are true intermediates. Although we did not observe compounds such as **28** (the C-8/C-9 dehydrated homolog of **19**), this compound could be envisaged as a potential direct precursor of **1** *via* tautomerisation to **29** and then an α -ketol, or related benzilic acid rearrangement (Scheme 3B).^[48] Such a mechanism is supported as feasible by

DFT calculations (see Supporting Information for details). Ring contractions in natural product biosynthesis have been previously proposed to proceed following such rearrangements. Examples include the biosynthetic pathways of xenovulene A,^[49] preuisolactone A^[50] (both C₇ to C₆) and fredericamycin A (C₆ to C₅).^[51] In synthetic chemistry such reactions are often performed in the presence of metal-ion catalysts, pointing out a potential function of the predicted copper-dependent TerE.^[52,53] Decarboxylation after rearrangement would give terrein **1** after tautomerisation.

A similar rearrangement mechanism could be envisaged during the biosynthesis of cryptosporiopsin **5** (Scheme 3B). Chlorination of C-4, however, in this case, prevents an identical mechanism. Here, 3-methoxy hydroquinone **30** could oxidise to benzoquinone **31**. Rearrangement of **31** by a benzilic acid reaction would also lead to ring-contraction, but *via* extrusion of C-3. This is consistent with the observed labelling pattern. Early methylation of O-3 would create a methyl ester after rearrangement and thereby prevent the decarboxylation which must occur during the rearrangement on the route to **1**. Again, DFT calculations (Supporting Information section 2.14) suggest this type of mechanism would be feasible.

Conclusion

The biosynthesis of terrein **1** and related compounds such as **5** and **6** has remained mysterious for more than 85 years. Despite the recent discovery of the BGC and molecular studies involving gene knockouts that confirmed the intermediacy of 6-hydroxymellein **2**, no more details on their biosynthesis could be found. Here we have advanced knowledge in this area by another small step. The FMO TerC (and its homologs) are involved in the oxidative decarboxylation which removes C-1 early in the biosynthesis, and this seems to be the last step in common between the terrein and (poly)chlorinated pentenone pathways. However we have not identified the origin of the required dehydration, but our studies suggest this must also occur early in the pathways. The highly oxygenated intermediates produced by TerC and TerD are unstable and difficult to characterise, but if **1** or **5** had been formed they would have been detected. The lack of these compounds suggests the likely importance of early dehydration. It is noteworthy that while our approach to sequentially reconstitute fungal secondary metabolite biosynthetic pathways by heterologous expression in *A. oryzae* NSAR1 is usually very expedient and reliable, it was not able to decipher the final transformation during the biosynthesis of **1** and **5**.^[20,43,49,54,55] Inexplicably TerD seems inactive in heterologous expression experiments in *A. oryzae*, although it is active *in vitro*.

We propose that polyhydroxylated intermediates probably form quinones and then benzilic acid type rearrangements could connect the biosynthesis of terrein **1** and the related compounds such as **5**. However, we have so-far failed to discover the catalysts involved in the key dehydration and rearrangement steps and it is clear that significantly more work will have to be done to finally elucidate these steps.

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Conflict of Interest

The authors declare no conflict of interest.

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