

Biosynthesis

Biosynthesis of 6-Hydroxymellein Requires a Collaborating Polyketide Synthase-like Enzyme

Lukas Kahlert, Miranda Villanueva, Russell J. Cox,* and Elizabeth J. Skellam*

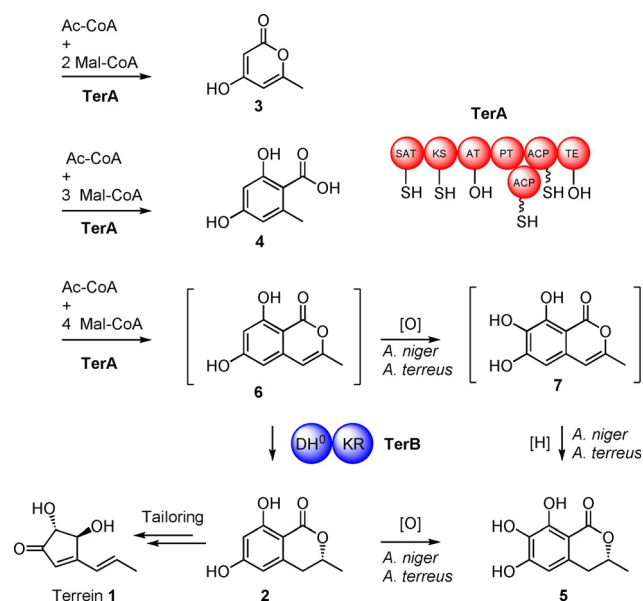
Abstract: The polyketide synthase (PKS)-like protein *TerB*, consisting of inactive dehydratase, inactive C-methyltransferase, and functional ketoreductase domains collaborates with the iterative non-reducing PKS *TerA* to produce 6-hydroxymellein, a key pathway intermediate during the biosynthesis of various fungal natural products. The catalytically inactive dehydratase domain of *TerB* appears to mediate productive interactions with *TerA*, demonstrating a new mode of trans-interaction between iterative PKS components.

Introduction

Fungal polyketide synthases (PKS) are iterative Type I systems in which catalytic domains form large programmed covalent complexes that can synthesise a very wide variety of structures.^[1,2] Fungal highly reducing (hr) PKS resemble mammalian fatty acid synthases^[3] (mFAS) in domain organisation, while fungal non-reducing (nr) PKS are simpler and lack ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) β -processing domains. In almost all known cases the catalytic domains are *cis*-acting: that is, they are part of the covalent complex. However, in some cases *trans*-acting enoyl reductase (ER)^[4] and (very rarely) *trans*-C-methyltransferase (C-MeT)^[5] and *trans*-KR domains^[6] are known. Other *trans*-acting interactions are also possible. For example nr-PKS usually have a starter-unit acyl transferase (SAT) domain which can accept an acyl chain priming unit directly from the acyl carrier protein (ACP) of an hr-PKS or FAS.^[7]

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Recently, Brock and co-workers described the biosynthetic gene cluster (BGC) of the fungal polyketide terrein **1** in *Aspergillus terreus*, which is derived from the polyketide precursor 6-hydroxymellein **2**.^[8] The *ter* BGC encodes *TerA* which is an apparently canonical nr-PKS consisting of SAT, ketosynthase (KS), acyl transferase (AT), product template (PT), twin ACP, and N-terminal thiolesterase (TE) domains (Scheme 1). Heterologous expression of *TerA* in *Aspergillus niger* results in the formation of three products: the triketide lactone **3**, the tetraketide orsellinic acid **4**, and the pentaketide 6,7-dihydroxymellein **5** (Scheme 1) that arises via shunt hydroxylation of proposed *TerA* product **6** to **7** followed by reduction.



Scheme 1. Summary of previous observations. Compounds in brackets were not isolated. See main text for abbreviations.

A second protein, *TerB*, was shown to be essential for biosynthesis of **2** in *A. terreus* by gene knock-out. In the absence of *TerB* **3**, **4**, and **5** were isolated. However, co-expression of *terAB* in *A. niger* did not lead to the expected production of **2**. Due to the observation of **2** in additional knock-out studies of later tailoring enzymes in *A. terreus*, the role of *TerB* was proposed as opening lactone **6** followed by subsequent reduction and relactonisation. We have previously used heterologous expression of fungal BGC in *Aspergillus oryzae* to successfully investigate the biosynthesis of other metabolites without the complications of shunt pathways and we therefore decided to use it to investigate this intriguing system *in vivo*.^[9,10]

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Results

Expression of *terA* and *terB*

Genes encoding TerA and TerB were cloned from *A. terreus* cDNA and/or gDNA (see SI for all experimental details). The fungal expression vector pTYGSarg-*terA* was assembled by yeast homologous recombination and expressed in *A. oryzae* NSAR1.^[11] Transformants expressing *terA* yield three compounds: the triketide pyrone **3** (71 mgL⁻¹), the tetraketide orsellinic acid **4** (153 mgL⁻¹), and the pentaketide acid **8** (91 mgL⁻¹, Figure 1 B, Figure 2), all confirmed by full structure elucidation by NMR and HRMS and comparison to literature values. Compound **8** is unshunted in *A. oryzae*, unlike in *A. niger* where it is evidently subjected to oxidative modifications by host enzymes. In contrast to previous reports by Oakley et al. the pentaketide lactone **6** was not observed (SI Figure S9).^[12]

Close examination of the coding region of *terB* indicated that the NCBI sequence was likely truncated. Reanalysis identified an additional 193 amino acid residues at the C-terminus that contribute to a KR domain (SI Figures S1–S8). Conserved domain database (CDD)^[13] analysis of the longer ORF identifies an N-terminal DH domain lacking key active site residues (DH⁰) as well as the C-terminal KR. In addition, InterPro^[14,15] and Phyre2^[16] further identify an inactive C-methyltransferase (C-MeT⁰) located centrally. Alignment with a number of characterised hr-PKS also indicates sequence homology with C-MeT domains. TerB thus appears to be a truncated hr-PKS that has lost KS, AT, ER, and ACP domains. Since reduction of the 9-ketone of **8** is required during biosynthesis of **2**, and TerA does not contain a KR domain, it seemed possible that TerB fulfils this role.

Coexpression of the longer *terB* with *terA* in *A. oryzae* results in production of *R*-6-hydroxymellein **2** (Figure 1 C, Figure 2, see SI for full characterisation) in very high titres (> 500 mgL⁻¹). Prolonged incubation times (> 4 days) result in hydroxylation of **2** by *A. oryzae* to give 5,6-dihydroxymellein as reported previously (SI Figure S11).^[17] In addition to **2**, **3**, **4**, and **8**, a new minor compound was observed and 2D-NMR analysis revealed it to be the tetraketide lactone **9**, showing a secondary alcohol function in the aliphatic side chain (SI Figs S63–S67). The *R*-configuration was assigned by Mosher's esterification (SI Figure S68).^[18,19]

Coexpression of *terAB* gives **2** at five-fold higher titre (530 mgL⁻¹) than production of pentaketide **8** by expression of *terA* alone. Based on this observation we envisaged two potential biosynthetic routes to **2** (Scheme 2). First, early release would provide free ketone **8** which could be reduced by the KR of TerB to alcohol **10**, followed by lactonisation. In a second possibility, ketoreduction could occur first while the polyketide, for example, **8A**, is still tethered to TerA. In this case final release could be spontaneous by intramolecular lactonisation, or release could be catalysed by the TE, and conceivably these possibilities could occur while the intermediate is bound to either ACP (**8A/10A**) or TE (**8T/10T**).

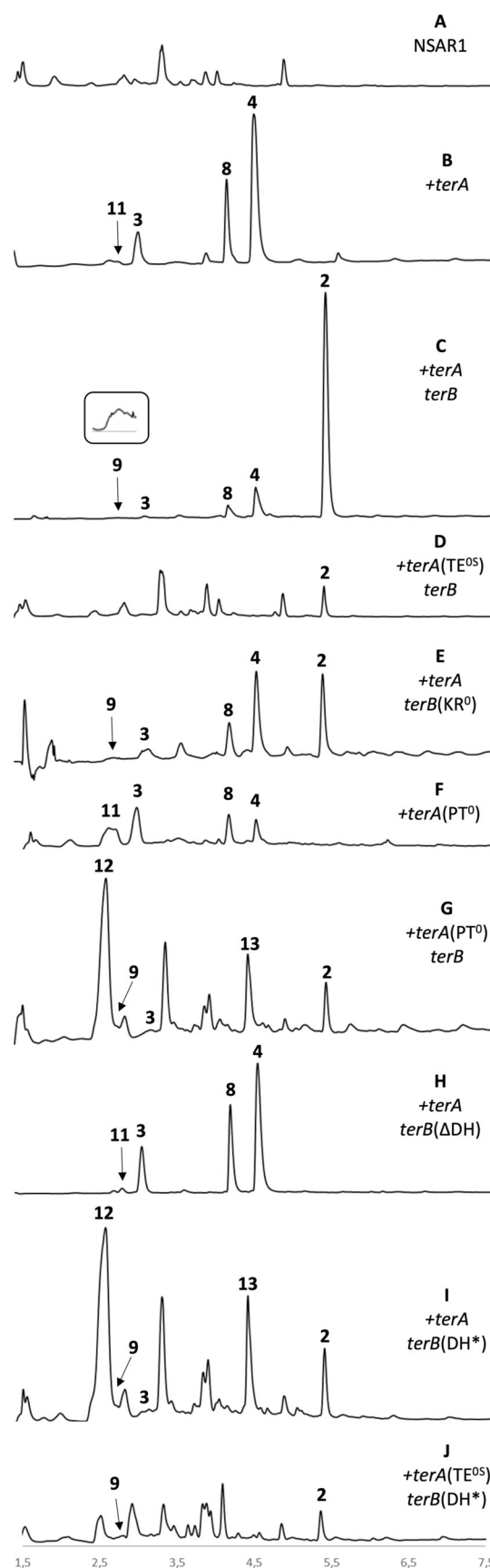


Figure 1. Selected DAD (210–600 nm) chromatograms of the indicated *A. oryzae* expression experiments. Unlabelled peaks are unrelated.

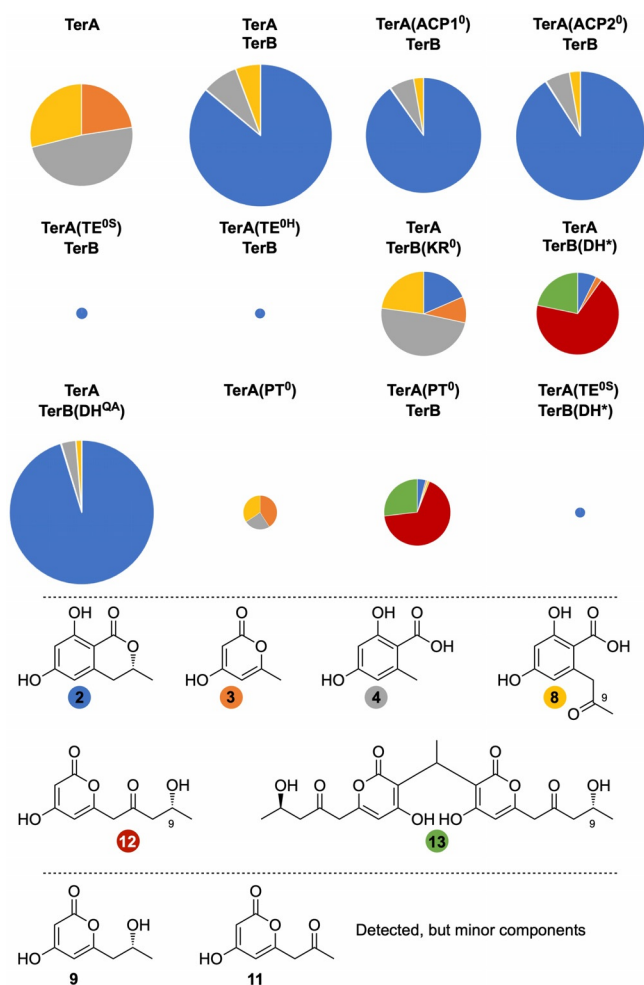
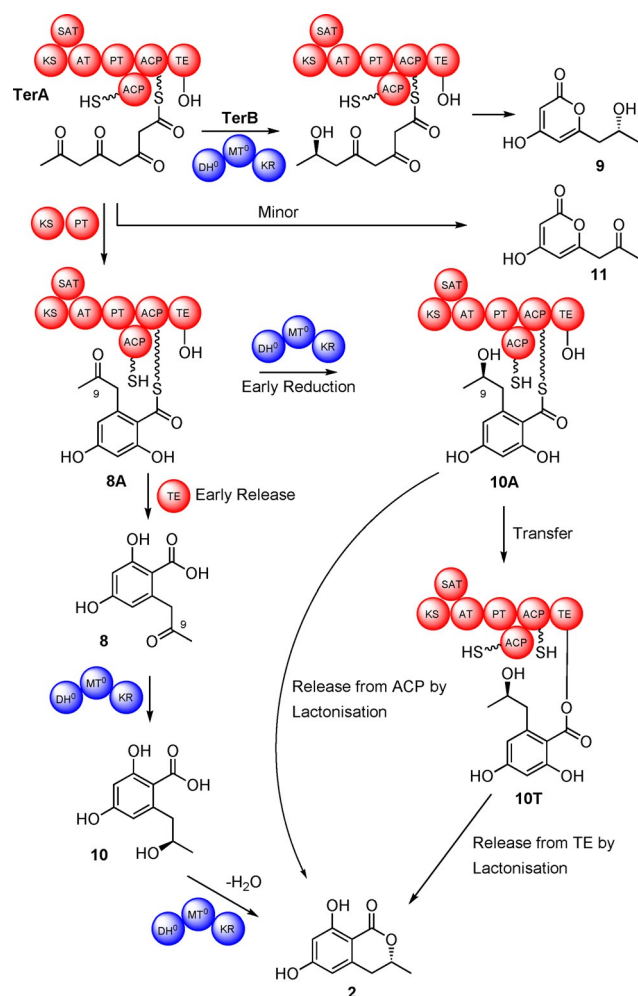


Figure 2. Titres of isolated compounds from the indicated heterologous expression experiments in *A. oryzae*. Area of pie charts is proportional to total titre in each case. Results of lower production experiments not shown. All titres are also summarised in Table S6.

In Vitro Study of Reduction and Lactonisation

In order to probe these release possibilities we generated *A. oryzae* transformants solely expressing *terB* and prepared a cell-free extract (CFE). The CFE was incubated with pentaketide **8** in the presence of NADPH for 3 h, followed by LCMS. Ketone **8** was converted to **2** only in the presence of TerB and NADPH, but not in the untransformed *A. oryzae* NSAR1 control (SI Figure S12). The intermediate secondary alcohol **10** was not detected suggesting that TerB also catalyses lactonisation. This is further supported by studies of the chemical properties of alcohol **10** generated by reduction of **8** with NaBH_4 .

Alcohol **10** is stable in water and in the *A. oryzae* CFE (SI Figure S15), but upon acidification immediately converts to **2** (SI Figures S13, S14). These results show that the TerB KR can reduce free **8**, but do not rule out the possibility that TerB can also reduce enzyme-bound intermediates such as **8A** (Scheme 2).



Scheme 2. Possible reduction and release pathways during the formation of **2**.

ACP, TE, and KR Active Site Mutations

TerA contains tandem ACP domains and we were curious whether one ACP domain specifically interacts with TerB. In other PKS or polyunsaturated fatty acid synthase (PUFAS) systems individual ACP domains within a tandem repeat are often functionally equivalent.^[20] The conserved serine in each TerA ACP was mutated. TerA(ACP1⁰) carries an S1674A mutation; TerA(ACP2⁰) has S1793A, while TerA(ACP12⁰) has both mutations. The respective *terA* mutants were co-expressed with *terB* and production of **2**, **3**, **4**, and **8** was monitored and quantified (Figure 2). Production of **2** falls to approximately 65–80% in TerA(ACP1⁰) and TerA(ACP2⁰), however it is still 3.5–4.5 times higher than production of pentaketide **8** by native TerA alone (Figure 2). Production of all polyketides was abolished in TerA(ACP12⁰). It appears that possession of two ACP domains increases the efficiency of TerA, but both ACP appear to be functionally identical in these experiments.

Next, we attempted to determine whether the TE domain in TerA is required for the formation of **2**, or whether TerB directs the release of the pentaketide intermediate. S1968 was identified as the active site nucleophile and H2117 as the

active site base of the TE domain (SI Figure S7) and we created TerA(TE^{OS}) and TerA(TE^{OH}), respectively, by mutation of these residues to alanine.^[21] Expression of either *terA*(TE^{OS}) or *terA*(TE^{OH}) in *A. oryzae* almost completely abolishes production of TerA-derived polyketides: only orsellinic acid **4** was observed in extracted ion chromatograms (EIC) at barely detectable levels indicating that catalysis by the TE domain is required for release of *any* product, including pyrone **3**, confirming the previous observation of Brock and co-workers.^[8]

Co-expression of *terA*(TE⁰) with *terB* similarly significantly affects production of TerA-derived polyketides. These mutants produce mostly **2**, but overall titres drop 100-fold to below 5 mg L⁻¹ (Figure 1D, Figure 2). The tetraketide lactone **9** was also detected in minor amounts, however low overall titres prevented quantification. The striking observation is that once again co-expression of *terB* with *terA* is more productive than TerA alone: even with a non-functional TE domain, TerA(TE⁰)/TerB leads to **2** while TerA(TE⁰) alone cannot produce the corresponding pentaketide **8**. These results suggest that **8A** accumulates on the TerA ACP and is reduced by the TerB KR to **10A**. Release by lactonisation must be slow, blocking the productivity of TerA. This shows that the TerB KR is unlikely to be able to catalyse significant lactonisation and product release for ACP-bound intermediate **10A** and that the TE must catalyse the intramolecular release.

We next turned our attention to the KR domain of TerB. The conserved acid likely to activate the substrate carbonyl for reduction by protonation was identified as Y991 (SI Figure S5).^[22] TerB(KR⁰) was created by forming mutant Y991F. Co-expression of *terA* with *terB*(KR⁰) results in reduction of the titre of **2** by 90% confirming that the KR domain in TerB is active and essential for biosynthesis of **2** (Figure 1E, Figure 2).

PT and DH⁰ Mutations

To confirm the role of the TerA PT domain, its catalytic histidine, required for C–C bond formation, was identified by sequence alignment^[23] as H1320 (SI Figure S8). The H1320A mutant was created and is referred to as TerA(PT⁰). Expression of *terA*(PT⁰) alone results in a significant decrease in production of **3**, **4**, and **8** as expected (Figure 1F). Careful examination of the culture extract revealed formation of tetraketide ketone **11** (3 mg L⁻¹, Figure 1F, Scheme 2) and also allowed it to be identified in the expression of *terA* alone (Figure 1B) as a minor component.

The lack of any pentaketide pyrone products formed by the PT⁰ mutant, together with the low titres of **3** and **11** in particular, suggests that TerA(PT⁰) has stalled at the tetraketide stage and shows that the TE is unable to efficiently remove the linear tetraketide and pentaketide intermediates via catalysed pyrone formation or by intermolecular hydrolysis with H₂O.

When *terA*(PT⁰) is co-expressed with *terB*, two new major compounds **12** and **13** appear in high titre (Figure 1G, Figure 2). Purification and full structure elucidation (SI Fi-

gures S81–92) revealed that **12** is a pentaketide pyrone, while **13** is a dimer of **12** formally fused to acetaldehyde and both are reduced at C-9. Dimeric compounds related to **13** have been observed before,^[24] but not previously in *A. oryzae*, indicating the presence of a highly unusual shunt pathway.

In order to probe the role of the TerB DH⁰ domain, *terA* was co-expressed with *terB* lacking its initial 320 codons (*terB*ΔDH). In this experiment no TerB-related compounds were produced (Figure 1H, SI Figure S16) and the chemotype is identical to expression of *terA* alone (Figure 1B), suggesting that the DH⁰ domain is required for structural integrity of TerB.

Alignment of the TerB DH⁰ with well-studied PKS DH domains such as that from the squalestatin tetraketide synthase SQTKS^[25] reveals that the TerB DH⁰ lacks the catalytic histidine within the highly conserved 46-H(X₃)G-(X₄)P-55 motif.^[26] A glutamine residue replaces the histidine. In contrast, the second conserved 215-D(X₃)Q-219 motif is present. As no catalytic role for the DH⁰ domain of TerB was immediately obvious, we attempted to re-activate the domain by introducing the active-site H46 and observe any effect on polyketide production. TerB(DH*) was created by introducing mutation Q46H. Co-expression of *terA* with *terB*(DH*) results in a 95% reduction in production of **2**, and **3**, **4**, and **8** are almost undetectable (Figure 1I, Figure 2). No unsaturated congeners were observed, showing that TerB(DH*) remains deficient in dehydratase activity.

Remarkably, pyrone **12** and its related dimer **13** again constitute the major compounds produced. The combined titre of the pentaketides **12** (approx. 150 mg L⁻¹) and **13** (47 mg L⁻¹) is twice as high as the pentaketide product **8** naturally produced by TerA alone, and almost no tetraketide product is observed. Not only does the TerB(DH*) mutant change the product profile, it also increases the efficiency of TerA towards the formation of pentaketide products. The structures of **12** and the tetraketide **9** indicate that the TerA PT domain has failed to function, since neither of them arise via C-2/C-7 cyclisation evident in the TerA-derived aromatic products **4** and **8**.

The result of the DH* mutation is almost identical to the TerA(PT⁰)/TerB experiment and shows that the effect of the DH* mutation is to inactivate the TerA PT. This is a significant finding and can only be explained by the collaborative action of TerB that enables the release of linear intermediates by pyrone formation when the PT domain of TerA is inactive. Additionally, since almost no **2** is produced it can be excluded that the DH domain in TerB functions as a PT domain that aids C-2/C-7 cyclisation. This result also suggests that reduction of C-7 at the tetraketide stage represses early C-2/C-7 cyclisation—all systems with defective or missing KR make considerably more tetraketide orsellinic acid **4**.

To determine whether the PT domain programming in TerA is disrupted by TerB(DH*) specifically due to the presence of H46, as opposed to the lack of the native Q46, we generated the Q46A mutation [TerB(DH^{QA})]. Co-expression of *terA* with *terB*(DH^{QA}) leads to slightly increased titres of **2** (600 mg L⁻¹, Figure 2), confirming that specifically the presence of H46 in TerB inactivates the PT domain of TerA.

To establish whether it is TerB that releases the linear tetraketide and pentaketide intermediates from the ACP or the TE domain of TerA, we co-expressed *terA*(TE^{OS}) with *terB*(DH*) that also effectively inactivates the TerA PT domain. Similarly to co-expression of *terA*(TE^{OS}) with the native *terB*, production of **2** is significantly affected and decreased by almost 95% (<3 mgL⁻¹) while tetraketide lactone **9** is still found in minor amounts (Figure 1J, Figure 2). However, no pentaketide pyrone **12** (or its dimer **13**) is observed. This result demonstrates that TerB does not catalyse release of polyketide intermediates while they are attached to the ACP, and therefore shows that release occurs for intermediates bound to the TE.

Discussion and Conclusion

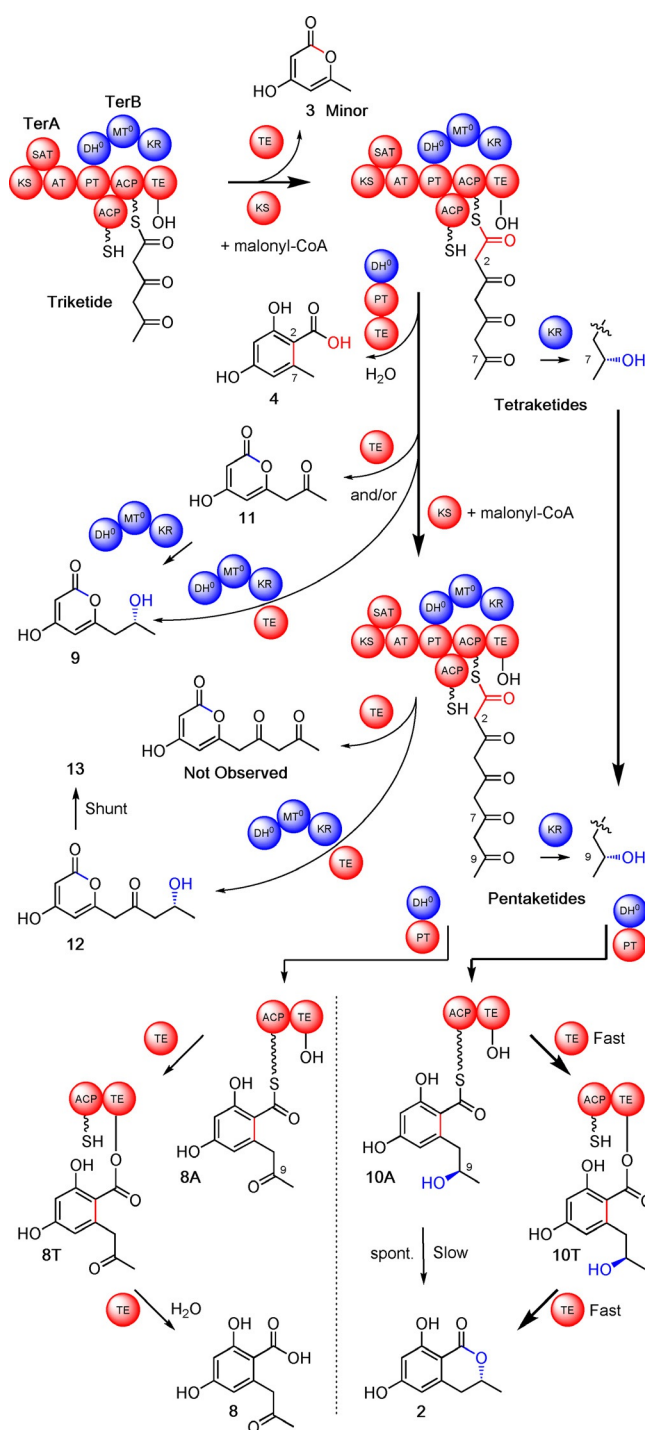
Taken together our results strongly support a collaborative model for TerA and TerB during the biosynthesis of **2** (Scheme 3). In all cases TerA is more productive in the presence of TerB suggesting a close interaction. The fact that mutation in TerB (e.g. DH*) affects chemistry catalysed by TerA (e.g. PT) strongly supports this conclusion.

Our results support a biosynthetic scheme in which triketide intermediates can only be extended, or released as the pyrone **3**, and that the TE is required for this step. Tetraketides are rarely released as a pyrone **11** for the WT system, but more often when the PT domain is disabled. Formation of **9** could come from release of a tetraketide 7-alcohol, or by reduction of **11**. In the presence of an active PT, tetraketides are cyclised to orsellinic acid **4** and then released by TE. This pathway is enhanced in systems lacking KR.

However, in the presence of KR, tetraketide intermediates prefer to be extended, suggesting that reduction occurs at the tetraketide stage which suppresses formation of **4**.

At the pentaketide stage the WT system prefers PT-catalysed C-2/C-7 Claisen cyclisation to form **10A**. Final transfer to the TE then leads to fast intramolecular cyclisation and release of **2**. Disruption of the Claisen cyclisation, either by mutation of PT, or by formation of the TerB DH* mutation, leads to effective off-loading of pentaketide 9-alcohol **12** as a pyrone. Importantly, pentaketide 9-keto pyrones are not observed, again suggesting that reduction occurs before release, either of the tetraketide or pentaketide intermediates.

This observation that mutation of PT and DH leads to almost identical results suggests that the TerA PT and TerB DH domains are likely to interact. This is an appealing hypothesis since, based on their structural (double hotdog folds) and catalytic features (His-Asp dyads), PT domains in nr-PKS are described as evolutionary ancestors of DH domains from hr-PKS.^[27,28] Interestingly, it is known that while the overall fold of DH and PT domains is very similar, DH domains dimerise at their N-termini, while PT domains have a different dimerisation surface which leaves the N-termini free.^[29] It is tempting to speculate that the N-terminus of the TerB DH may interact with the N-termini of the PT domains in TerA. Interaction via a PT–DH interaction is also supported by our observation that the TerB lacking the DH



Scheme 3. Overall description of the interaction of TerA and TerB during the biosynthesis of **2**. Bold arrows show main pathway flux of the WT system.

domain is inactive in the presence of TerA. However, structural work, which is currently not possible due to non-availability of soluble proteins, will be required to confirm this hypothesis.

It is also noteworthy that TerB(DH*) is unable to catalyse dehydration as no unsaturated products were observed. This is perhaps unsurprising since PKS and FAS DH domains normally dehydrate β -hydroxy thioesters^[25] rather than β -

hydroxy ketones, consistent with TerAB reduction occurring at the tetraketide stage or later.

Mutation of the TE domain shows that it is required for all release modes, including intermolecular hydrolysis in the formation of **4** and **8**, and intramolecular reactions involved in the formation of pyrones (**3**, **9**, **11**, **12**) as well as release of **2** from **10T**. In the TE^{OS} mutant, transfer of intermediates to the TE is not possible, and formation of **2** in this case must come from slow spontaneous intramolecular release from ACP-bound intermediate **10A**. While pyrone formation is often regarded as a default release mechanism when other catalysed routes are absent, some other nr-PKS TE domains, such as that of the cercosporin PKS described by Townsend and co-workers,^[30] are also known to catalyse this type of release.

Other examples of collaborating and *trans*-acting enzymes have been reported in fungi, including: nr-PKS SAT domains,^[31] *trans*-TE domains that are necessary for chain length determination and product release,^[32] *trans*-ER domains that perform specific enoyl reduction prior to release from the PKS,^[33,34] and an unusual ψ ACP-C-MeT protein that binds to the KS domain of an nr-PKS enabling methylation of the polyketide intermediate.^[35] Tan and co-workers recently reported a short-chain dehydrogenase/reductase (SDR) ChrB which interacts with an iterative hr-PKS ChrA during the biosynthesis of precursors of dalmanol,^[6] but ChrB is unrelated to the KR of TerB (<15% identity) which forms part of a truncated hr-PKS itself. Tang and co-workers also reported an interesting interaction between a bacterial Type II KR (act III) and the fungal nr-PKS4 from *G. fujikuroi*. Interestingly, this KR can also reduce at C-9, but it appears to reduce full-length octa- and nona-ketides which then prevents the function of PT,^[36] unlike the case of the TerAB system where immature tetraketides are reduced and PT activity enhanced. Thus the proposed DH-PT interaction in TerAB appears unique. Although some of these *trans*-acting systems have been studied in detail, the precise interaction points have not yet been determined due to a lack of structural data. Future investigations will also be necessary to probe the proposed interactions in the TerAB system using a combination of in vitro domain assays with synthetic intermediates to determine the precise timing of reduction.^[37]

Finally, these results also highlight the importance of melleins as fungal secondary metabolite intermediates. The pentaketide mellein skeleton is known to be produced by at least four independent systems in fungi. As shown here TerA and TerB form an unusual collaborative PKS system combining nr-PKS and hr-PKS components. CdmE is an hr-PKS from the chrodriamanin B pathway that directly synthesises **2**.^[38] Men2 is a nr-PKS, involved in ascotrichalactone A biosynthesis, that synthesises **2** as a derailment product.^[39] Finally, mellein itself is synthesised by a partially reducing PKS (pr-PKS)^[1,40] with a programmed *cis*-acting KR. All four systems contain enzymes with differently constructed and programmed KR domains. The TerAB-type architecture appears relatively common, being also known to be involved in the biosynthesis of other 6-HM-derived compounds such as roussoellatide,^[41] cyclohelminthols, and palmaenones.^[17] It remains to be seen whether nature also uses this architecture in other systems.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: iterative polyketide synthase · ketoreductase · mellein · terrein · *trans*-acting domain

- [1] R. J. Cox, *Org. Biomol. Chem.* **2007**, *5*, 2010–2026.
- [2] Y.-H. Chooi, Y. Tang, *J. Org. Chem.* **2012**, *77*, 9933–9953.
- [3] O. Piech, R. J. Cox, *RSC Adv.* **2020**, *10*, 18469–18476.
- [4] J. Kennedy, K. Auclair, S. G. Kendrew, C. Park, J. C. Vederas, C. R. Hutchinson, *Science* **1999**, *284*, 1368–1372.
- [5] S. Kishimoto, Y. Tsunematsu, T. Matsushita, K. Hara, H. Hashimoto, Y. Tang, K. Watanabe, *Biochemistry* **2019**, *58*, 3933–3937.
- [6] Z. Z. Zhou, H. J. Zhu, L. P. Lin, X. Zhang, H. M. Ge, R. H. Jiao, R. X. Tan, *Chem. Sci.* **2019**, *10*, 73–82.
- [7] J. M. Crawford, B. C. Dancy, E. A. Hill, D. W. Udway, C. A. Townsend, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 16728–16733.
- [8] C. Zaehle, M. Gressler, E. Shelest, E. Geib, C. Hertweck, M. Brock, *Chem. Biol.* **2014**, *21*, 719–731.
- [9] R. Nofiani, K. de Mattos-Shipley, K. E. Lebe, L.-C. Han, Z. Iqbal, A. M. Bailey, C. L. Willis, T. J. Simpson, R. J. Cox, *Nat. Commun.* **2018**, *9*, 3940.
- [10] L. Kahlert, E. F. Bassiony, R. J. Cox, E. J. Skellam, *Angew. Chem. Int. Ed.* **2020**, *59*, 5816–5822; *Angew. Chem.* **2020**, *132*, 5865–5871.
- [11] K. A. K. Pahirulzaman, K. Williams, C. M. Lazarus, *Methods Enzymol.* **2012**, *517*, 241–260.
- [12] Y.-M. Chiang, E. C. Oakley, M. Ahuja, R. Entwistle, A. Schultz, S.-L. Chang, C. T. Sung, C. C. Wang, B. R. Oakley, *J. Am. Chem. Soc.* **2013**, *135*, 7720–7731.
- [13] A. Marchler-Bauer, Y. Bo, L. Han, J. He, C. J. Lanczycki, S. Lu, F. Chitsaz, M. K. Derbyshire, R. C. Geer, N. R. Gonzales, et al., *Nucleic Acids Res.* **2017**, *45*, D200–D203.
- [14] M. Blum, et al., *Nucleic Acids Res.* **2021**, *49*, D344–D354.
- [15] P. Jones, D. Binns, H.-Y. Chang, M. Fraser, W. Li, C. McAnulla, H. McWilliam, J. Maslen, A. Mitchell, G. Nuka, S. Pesseat, A. F. Quinn, A. Sangrador-Vegas, M. Scheremetjew, S.-Y. Yong, R. Lopez, S. Hunter, *Bioinformatics* **2014**, *30*, 1236–1240.
- [16] L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, M. J. E. Sternberg, *Nat. Protoc.* **2015**, *10*, 845–858.
- [17] T. Ugai, A. Minami, S. Tanaka, T. Ozaki, C. Liu, H. Shigemori, M. Hashimoto, H. Oikawa, *ChemBioChem* **2020**, *21*, 360–367.
- [18] T. R. Hoye, C. S. Jeffrey, F. Shao, *Nat. Protoc.* **2007**, *2*, 2451–2458.
- [19] J. M. Seco, E. Quiñoá, R. Riguera, *Chem. Rev.* **2004**, *104*, 17–117.
- [20] A. Watanabe, I. Fujii, U. Sankawa, M. E. Mayorga, W. E. Timberlake, Y. Ebizuka, *Tetrahedron Lett.* **1999**, *40*, 91–94; T. Thongkawphueak, A. J. Winter, C. Williams, H. J. Maple, S.

- Soontaranon, C. Kaewhan, D. J. Campopiano, M. P. Crump, P. Wattana-Amorn, *Biochemistry* **2021**, *60*, 219–230; T. A. M. Gulder, M. F. Freeman, J. Piel, *Top. Curr. Chem.* **2011**, *298*; L. Gu, E. B. Eisman, S. Dutta, T. M. Franzmann, S. Walter, W. H. Gerwick, G. Skinnotis, D. H. Sherman, *Angew. Chem. Int. Ed.* **2011**, *50*, 2795–2798; *Angew. Chem.* **2011**, *123*, 2847–2850; H. Jiang, R. Zirkle, J. G. Metz, L. Braun, L. Richter, S. G. Van Lanen, B. Shen, *J. Am. Chem. Soc.* **2008**, *130*, 6336–6337.
- [21] I. Fujii, A. Watanabe, U. Sankawa, Y. Ebizuka, *Chem. Biol.* **2001**, *8*, 189–197.
- [22] A. T. Keatinge-Clay, *Chem. Biol.* **2007**, *14*, 898–908.
- [23] Y. Xu, T. Zhou, Z. Zhou, S. Su, S. A. Roberts, W. R. Montfort, J. Zeng, M. Chen, W. Zhang, M. Lin, J. Zhan, I. Molnár, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 5398–5403.
- [24] H. V. Kemami Wangun, C. Hertweck, *Eur. J. Org. Chem.* **2007**, 3292–3295.
- [25] E. Liddle, A. Scott, L.-C. Han, D. Ivison, T. J. Simpson, C. L. Willis, R. J. Cox, *Chem. Commun.* **2017**, *53*, 1727–1730.
- [26] L. Tang, Y. J. Yoon, C. Y. Choi, C. R. Hutchinson, *Gene* **1998**, *216*, 255–265; A. Keatinge-Clay, *J. Mol. Biol.* **2008**, *384*, 941–953; D. Gay, Y. O. You, A. Keatinge-Clay, D. E. Cane, *Biochemistry* **2013**, *52*, 8916–8928.
- [27] J. M. Crawford, T. P. Korman, J. W. Labonte, A. L. Vagstad, E. A. Hill, O. Kamari-Bidkorpoh, S. C. Tsai, C. A. Townsend, *Nature* **2009**, *461*, 1139–1143.
- [28] J. F. Barajas, K. Finzel, T. R. Valentic, G. Shakya, N. Gamarra, D. Martinez, J. L. Meier, A. L. Vagstad, A. G. Newman, C. A. Townsend, M. D. Burkart, S. C. Tsai, *Angew. Chem. Int. Ed.* **2016**, *55*, 13005–13009; *Angew. Chem.* **2016**, *128*, 13199–13203.
- [29] D. A. Herbst, C. A. Townsend, T. Maier, *Nat. Prod. Rep.* **2018**, *35*, 1046–1069.
- [30] A. G. Newman, A. L. Vagstad, K. Belecki, J. R. Scheerer, C. A. Townsend, *Chem. Commun.* **2012**, *48*, 11772–11774.
- [31] J. M. Winter, D. Cascio, D. Dietrich, M. Sato, K. Watanabe, M. R. Sawaya, J. C. Vederas, Y. Tang, *J. Am. Chem. Soc.* **2015**, *137*, 9885–9893.
- [32] A. O. Zabala, Y. H. Chooi, M. S. Choi, H. C. Lin, Y. Tang, *ACS Chem. Biol.* **2014**, *9*, 1576–1586.
- [33] A. A. Yakasai, J. Davison, Z. Wasil, L. M. Halo, C. P. Butts, C. M. Lazarus, A. M. Bailey, T. J. Simpson, R. J. Cox, *J. Am. Chem. Soc.* **2011**, *133*, 10990–10998.
- [34] B. D. Ames, C. Nguyen, J. Bruegger, P. Smith, W. Xu, S. Ma, E. Wong, S. Wong, X. Xie, J. W. H. Li, J. C. Vederas, Y. Tang, S. C. Tsai, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 11144–11149.
- [35] M. Chen, Q. Liu, S. S. Gao, A. E. Young, S. E. Jacobsen, Y. Tang, *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 5499–5504.
- [36] S. M. Ma, J. Zhan, X. Xie, K. Watanabe, Y. Tang, W. Zhang, *J. Am. Chem. Soc.* **2008**, *130*, 38–39.
- [37] J. S. Parascandolo, J. Havemann, H. K. Potter, F. Huang, E. Riva, J. Connolly, I. Wilkening, L. Song, P. F. Leadlay, M. Tosin, *Angew. Chem. Int. Ed.* **2016**, *55*, 3463–3467; *Angew. Chem.* **2016**, *128*, 3524–3528.
- [38] T. Bai, Z. Quan, R. Zhai, T. Awakawa, Y. Matsuda, I. Abe, *Org. Lett.* **2018**, *20*, 7504–7508.
- [39] W. Bunnak, P. Wonnapijit, A. Sriboonlert, C. M. Lazarus, P. Wattana-Amorn, *Org. Biomol. Chem.* **2019**, *17*, 374–379.
- [40] Y. H. Chooi, C. Krill, R. A. Barrow, S. Chen, R. Trengove, R. P. Oliver, P. S. Solomon, *Appl. Environ. Microbiol.* **2015**, *81*, 177–186.
- [41] E. L. Ferreira, D. E. Williams, L. P. Ióca, R. P. Morais-Urano, M. F. Santos, B. O. Patrick, L. M. Elias, S. P. Lira, A. G. Ferreira, M. R. Passarini, et al., *Org. Lett.* **2015**, *17*, 5152–5155.

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