PAPER

In vitro studies of maleidride-forming enzymes†

Sen Yin, Steffen Friedrich, Vjaceslavs Hrupins and Russell J. Cox [ID] *

In vitro assays of enzymes involved in the biosynthesis of maleidrides from polyketides in fungi were performed. The results show that the enzymes are closely related to primary metabolism enzymes of the citric acid cycle in terms of stereochemical preferences, but with an expanded substrate selectivity. A key citrate synthase can react both saturated and unsaturated acyl CoA substrates to give solely *anti* substituted citrates. This undergoes *anti*-dehydration to afford an unsaturated precursor which is cyclised *in vitro* by ketosteroid-isomerase-like enzymes to give byssochlamic acid.

Introduction

Alkyl citrates are a class of fungal secondary metabolites derived from a polyketide or fatty acid component and oxaloacetic acid. They include relatively simple monomeric compounds such as **1** and **2**, piliformic acid **3**,[1] oryzines A and B **4–5**,[2] sporothriolide **6**,[3] CJ-13,981 **7** (ref. 4 and 5) and hexylitaconic acids[6] known from *Aspergillus niger* (Fig. 1). More complex examples include compounds such as viridiofungin A **8** (ref. 7) and squalestatin S1 **9** (ref. 8 and 9) which are potent inhibitors of squalene synthase. In many cases dehydration of the alkyl citrate affords a maleic anhydride as observed in **1** and **2**, and dimerisation of these monomers leads to the formation of compounds with large alicyclic rings known as heptadrides, octadrides and nonadrides.[10] Collectively such compounds are known as maleidrides.[11] These include agnestadride A **10**,[11] zopfiellein **11** (ref. 12 and 13) byssochlamic acid **12** (ref. 14) and the selective herbicide cornexistin **13**.[15,16] These types of compounds are wide-spread in fungi (Fig. 1).[17]

To-date the main evidence for the biochemical reactions involved in the biosynthesis of alkyl citrates and maleidrides has come indirectly from *in vivo* genetic knockout or heterologous expression experiments. Thus, in the case of byssochlamic acid **12**, for example, we showed that co-expression in the host *Aspergillus oryzae* of genes encoding: a fungal highly-reducing polyketide synthase (hr-PKS); an αβ-hydrolase; a citrate synthase-like protein (CS); and a homolog of 2-methylcitrate dehydratase (2MCDH) results in production of **1** and **2**. Likewise, Oikawa and coworkers expressed homologous genes from the phomoidride BGC from unidentified fungus ATCC 74256 and related genes from *Talaromyces stipitatus* and showed production of anhydride monomers related to **1** and **2**.[18]

OCI, BMWZ, Leibniz University of Hannover, Schneiderberg 38, 30167, Hannover, Germany. E-mail: russell.cox@oci.uni-hannover.de

These experiments suggest a series of events in which a polyketide synthase produces a linear acyl group attached to its acyl carrier protein (ACP) **14**. This must be released (*e.g.* **15**) and reacted with oxaloacetate to form a vinyl citrate **16** by the citrate synthase enzyme (Scheme 1). Dehydration by the 2MCDH enzyme would then provide the observed substituted maleic
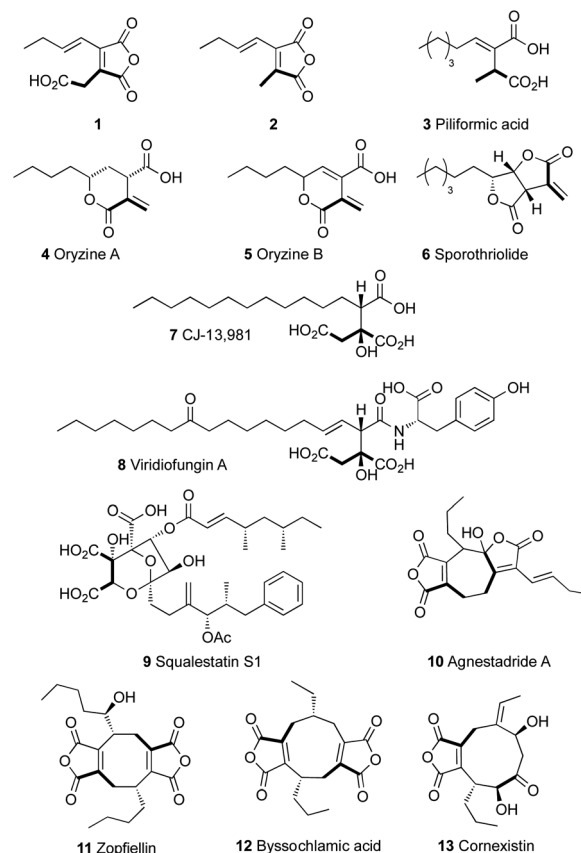

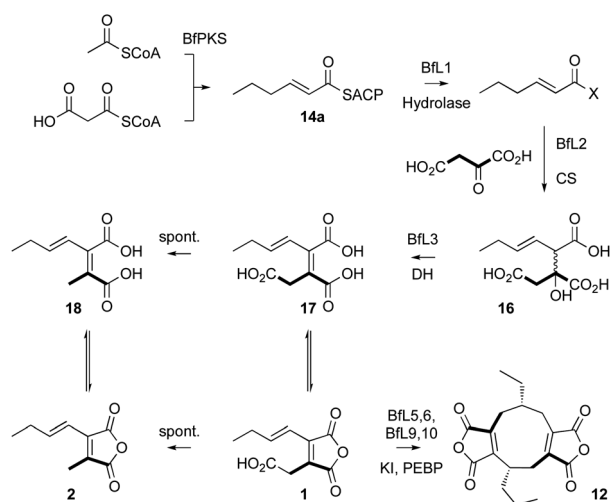Fig. 1 Structures mentioned in the text. Bold bonds indicate atoms derived from oxaloacetate.

Table 1 Summary of protein production. *bf*, genes from *B. fulva*; *pv*, genes from *P. variotii*; I, insoluble protein; ✓, soluble protein observed; ✗, no protein observed; –, experiment not tried; † cell-free extract used; green shading, functional protein

Gene	Putative function	E. coli			Yeast
		Complete ORF	Δ signal peptide	Δ signal peptide + chaperones	
bfL1	Hydrolase	✓	–	–	–
bfL2	Cit. synthase	✓	–	–	–
bfL3	Dehydratase	I	–	I	✓
pvL2	Dehydratase	✗	–	✗	✓
bfL5	PEBP-1	✗	I	✓	†
bfL6	KI-1	✗	I	✓	†
bfL9	PEBP-2	✓	–	–	†
bfL10	KI-2	✗	I	✓	†
bfACP	ACP	✓	–	–	–

Scheme 1 Proposed pathway to the maleidride byssochlamic acid **12**.

acids such as **17** and its equilibraiting anhydride from **1**. Anhydride **1** decarboxylates spontaneously to give **2**, which is also in equilibrium with a diacid form **18**.[19] We also showed that **1** appears to be a substrate for a pair of enzymes with homologies to ketosteroid isomerase-like (KI) and phosphatidylethanolamine-binding protein (PEBP) which then produce the alicyclic rings of dimeric maleidrides **10**, **12** and **13** by an as-yet unknown mechanism. In order to probe these reactions and enzymes in more detail we chose to study them *in vitro* with the aim of determining their selectivities. Since the byssochlamic acid **12** and cornexistin **13** pathways were previously described in our hands, we focussed our efforts on the study of enzymes from these systems.

Results

Gene cloning, expression and protein purification

Initial attempts to clone codon optimised fungal whole-length ORFs in *E. coli* expression systems (pRSETA, Rosetta, IPTG

induction) with an N-terminal his$_6$-tag were successful for BfL1 (hydrolase), BfL2 (citrate synthase) and BfL9 (PEBP) only (Table 1), giving soluble protein. In other cases either insoluble protein was detected, or no significant expressed protein was detected. In the cases of BfL5, BfL6 and BfL10 (PEBP and KI) putative signal peptides sequences were detected at their N-termini (SignalP 5.0 server).[20] These were removed by cloning N-truncated versions into pET28a and expression trials were set up in *E. coli* BL21(DE3). This resulted observable production of protein, but in the insoluble fraction in each case. The truncated *bfL5*, *bfL6* and *BfL10* genes were then individually co expressed in the presence of the pG-KJE8 chaperone system in *E. coli*, resulting in observation of soluble protein in each case. The final genes, *bfL3* and *pvL2*, encoding putative dehydratases could not be expressed in *E. coli* under any of the previously tried conditions. These genes were cloned into pESC-URA vectors and expressed in *Saccharomyces cerevisiae* strain W303-1b, induced by the addition of galactose. Soluble protein was detected in each case by SDS-PAGE analysis.

The ACP region of the *B. fulva* PKS was synthesised in *E. coli* optimised form (S2511 – E2618) in pET28a by a commercial supplier. The fragment was expressed in *E. coli* BL21 DE3 and soluble *apo*-protein was purified by nickel affinity chromatography and converted to *holo*-ACP using the Sfp phosphopantetheine transferase *in vitro*.[21] Both *apo* and *holo*-proteins were confirmed by mass spectrometry (see ESI† for details).
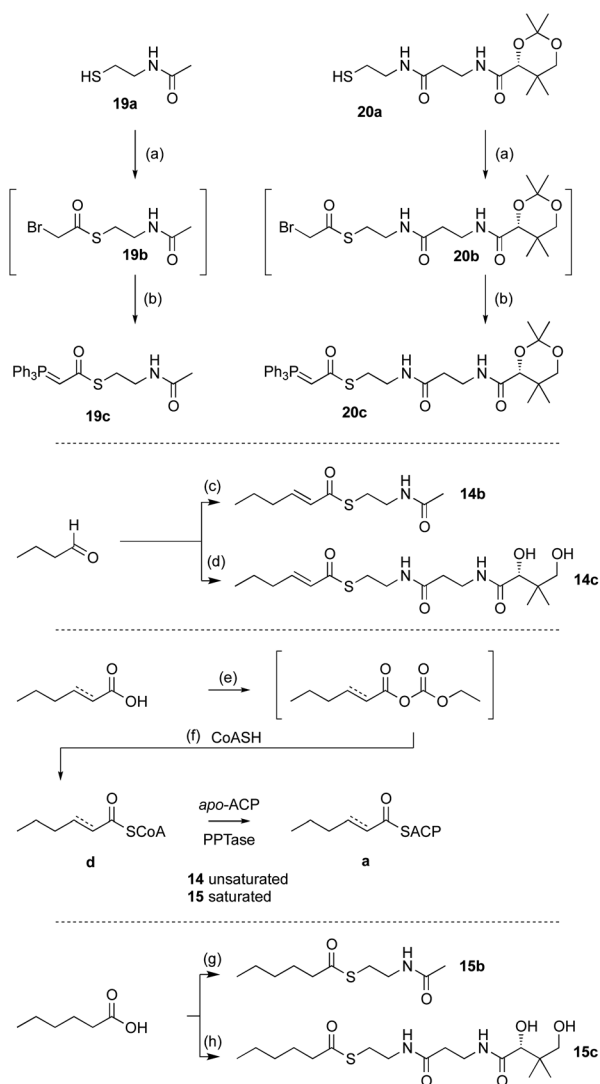
Hydrolase

Previous *in vivo* results indicate that BfPKS builds the hex-2-enoyl intermediate required for construction of monomeric maleic anhydrides (Scheme 1). We assumed that release of the hexenoyl unit from the PKS is achieved by the BfL1 hydrolase, and observations from coexpression studies were in agreement with this hypothesis.

In order to probe the selectivity of the BfL1 hydrolase we synthesised SNAC, pantetheine, CoA and ACP-bound potential substrates (Scheme 2).[22,23] In our previous work we assumed that the acyl group should be unsaturated (*e.g.* an *E*-hex-2-enoyl substrate) but this was unproven,[10] so we made both *E*-hex-2-enoyl **14a–d** and hexanoyl **15a–d** substrates. Initial experiments showed that direct formation of thiolesters of α,β-unsaturated species (*e.g.* in carbodiimide type couplings) was accompanied by unwanted Michael addition, the products of which could not be easily removed. This was overcome by formation of the ylides **19c** and **20c** from the corresponding thiols **19a** and **20a**. These reacted smoothly with aldehydes to give the desired **14b** and **14c** after deprotection (Scheme 2). CoA thiolesters were formed more easily by direct coupling of mixed anhydrides with CoASH itself, and in turn these were loaded onto the isolated *apo*-ACP of the *B. fulva* PKS using the phosphopantetheinyl transferase (PPTase) enzyme Sfp.[24,25] Finally, saturated SNACs and panthetheins **15b** and **15c** were made by standard EDCI coupling reactions.

In order to assess the substrate selectivity of the BfL1 hydrolase we incubated it with hex-2-enoyl and hexanoyl
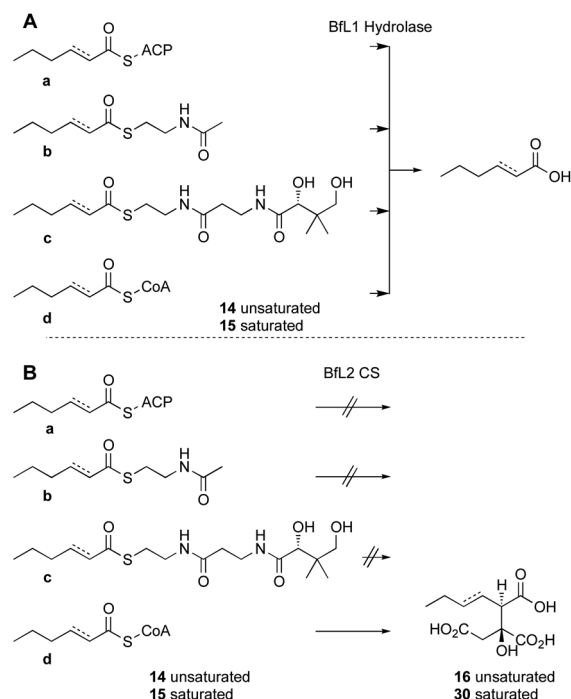
Scheme 2 Synthesis of hex(en/an)oyl substrates. Reagents and conditions: (a) BrCH₂CO₂H, EDCI, DMAP, CH₂Cl₂, RT; (b) PPh₃, toluene, 80 °C, then aq. NaOH; (c) **19c**, CH₂Cl₂, RT, 72 h; (d) **20c**, CH₂Cl₂, 40 °C, 3 d then THF/CF₃CO₂H, RT, 60 min; (e) ClCO₂Et, CH₂Cl₂, Et₃N, 0 °C; (f) CoASH, aq. NaHCO₃; (g) **19a**, EDCI, DMAP, CH₂Cl₂; (h) **20a**, EDCI, DMAP, CH₂Cl₂, then THF/CF₃CO₂H, RT, 60 min.

Scheme 3 *In vitro* assays of substrate analogs. A, results of incubation with the BfL1 hydrolase; B, results of incubation with the BfL2 citrate synthase and oxaloacetic acid.

Citrate synthase

Citrate synthase [EC 2.3.3.1] is a key enzyme of primary metabolism and is known to react acetyl CoA with oxaloacetic acid to produce citric acid. The enzyme performs both the condensation reaction and the hydrolytic-release of CoA. The gene *bfL2* from the byssochlamic acid BGC in *B. fulva* encodes a citrate synthase homolog which is 20% identical to the mitochondrial (presumed primary metabolism) CS of *B. fulva*.[10] *In vitro* assays were set up containing purified BfL2, oxaloacetic

thiolester species **14b–d** and **15b–d** and acyl-BfACP **14a** and **15a**. Assays were set up *in vitro* and contained substrate and purified BfL1 in buffer only. Reactions were incubated at 30 °C in Tris buffer containing MgCl₂, NaCl and the non-nucleophilic reducing agent tris(2-carboxyethyl)phosphine (TCEP, 1 mM), then quenched by addition of 1 volume of CH₃CN. Protein was precipitated by centrifugation and the residue analysed directly by LCMS.

In the case of the ACP species **14a** and **15a**, the acyl ACP was directly monitored by ESIMS. In all cases **14a–d** and **15a–d** we observed very rapid hydrolysis to the respective carboxylic acids, and by direct observation of the formation of *holo*-BfACP (Scheme 3A, see ESI† for bfACP mass data).
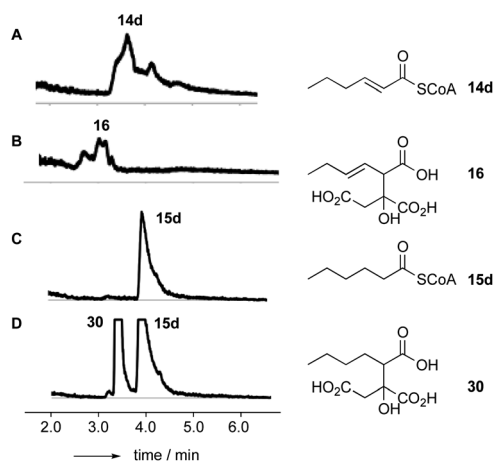

Fig. 2 LCMS results for incubation of saturated and unsaturated CoAs with oxaloacetic acid and BfL2 (CS), ELSD traces. A, hex-2-enoyl CoA **14d**; B, **14d** + BfL2 + oxaloacetic acid; C, hexanoyl CoA **15d**; D, **15d** + BfL2 + oxaloacetic acid.

acid and substrates **14a–d** and **15a–d**. The reactions were followed by LCMS. ACPs, SNACs and pantetheines were not substrates for BfL3, but both CoA thiolesters **14d** and **15d** were converted to the corresponding citrates **16** and **30** (Scheme 3, and Fig. 2).

A synthesis was developed for the production of the proposed substituted-citrate products of the CS (Scheme 4). The synthesis involves a Reformatsky reaction with an oxalate diester. Diethy oxaloacetate is commercially available, and it reacts satisfactorily to give citrate products, however the ethyl citrates could not be hydrolysed in our hands. We therefore required dimethyl oxalate **24**. This innocuous compound is not commercially available and attempts to produce it by esterification of oxaloacetic acid, or transesterification of diethyloxalate were unsuccessful. However, starting from dimethylacetylene dicarboxylate (DMAD) **21**, selective hydration of the alkyne was achieved in two steps by the controlled addition of morpholine **22** to give the intermediate **23**, followed by hydrolysis in the presence of oxalic acid. This yielded **24** as yellow crystals.

Although this material did not give satisfactory spectroscopic analysis, it did react smoothly and as expected in the following step in which it was reacted with either methyl-α-

bromohexanoate **25** or its 3-unsaturated congener **27** (itself derived from **26**) in a Reformatsky reaction in DMF.[26] This afforded a 2 : 1 mixture of diastereomers of the required trimethyl esters **28** (unsaturated) and **29** (saturated). These were hydrolysed by extended reflux in aqueous HCl (3 days) to afford good yields of the required triacids **16** (unsaturated) and **30** (saturated) – again as 2 : 1 mixtures of diastereomers.

Previous work by Barrett and coworkers showed that similar reactions are *anti*-selective[27,28] and this was confirmed by ^{1}H NMR analysis of the mixture of diastereomers of **16** which showed that the major compound is the 2,3-*anti* (3S*, 4R*) diastereomer **16a**. The ^{1}H chemical shifts of H-4 are particularly diagnostic, with H-4$_{syn}$ consistently resonating at lower field than H-4$_{anti}$ (see ESI† for details). HPLC analysis showed that the major *anti* diastereomer **16a** elutes slightly before the minor *syn* diastereomer **16b**.
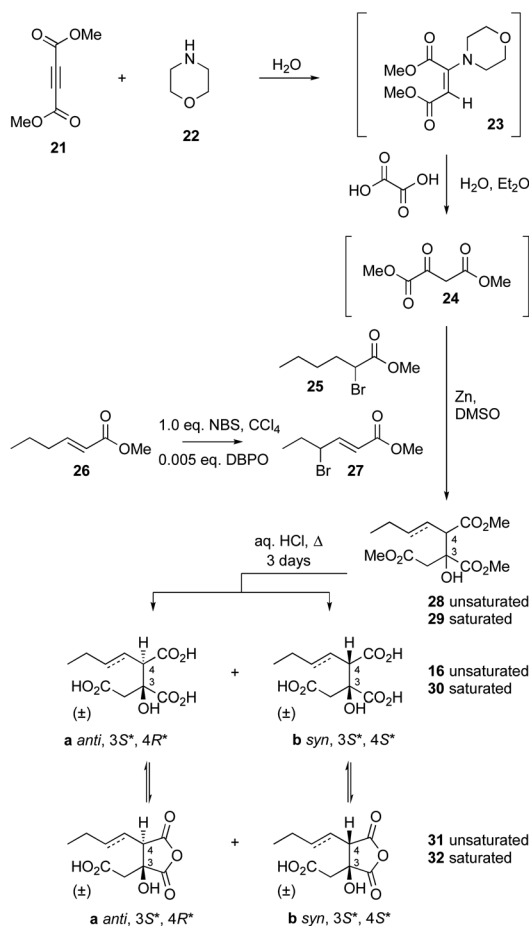
The individual diastereomers of synthetic **16** were separated by HPLC to yield the pure racemic stereoisomers. NMR and LCMS analysis also revealed the presence of equilibrating mixtures of the corresponding cyclic anhydrides **31**, again with a prevalence of the 3,4 *anti* diastereomer (3S*, 4R*, ESI Fig S5.1†). Chromatographic comparison of the pure synthetic diastereomers with the products of the *in vitro* assay of BfL2 showed that the enzyme product is exclusively the *anti* diastereomer **16a** (ESI Fig S5.3†).

Structures of many primary metabolism citrate synthases have been determined at high resolution. In particular the structures of CS from *Escherichia coli*,[29] *Thermus thermophilus*,[30] *Pyrococcus furiosus*[31] and *Sus scrofa*[32] are understood in significant detail. Multiple sequence alignment between BfL2 and CS from these organisms showed that the known active site residues D377, H284 and R332 are fully conserved (BfL2 numbering, see ESI†).
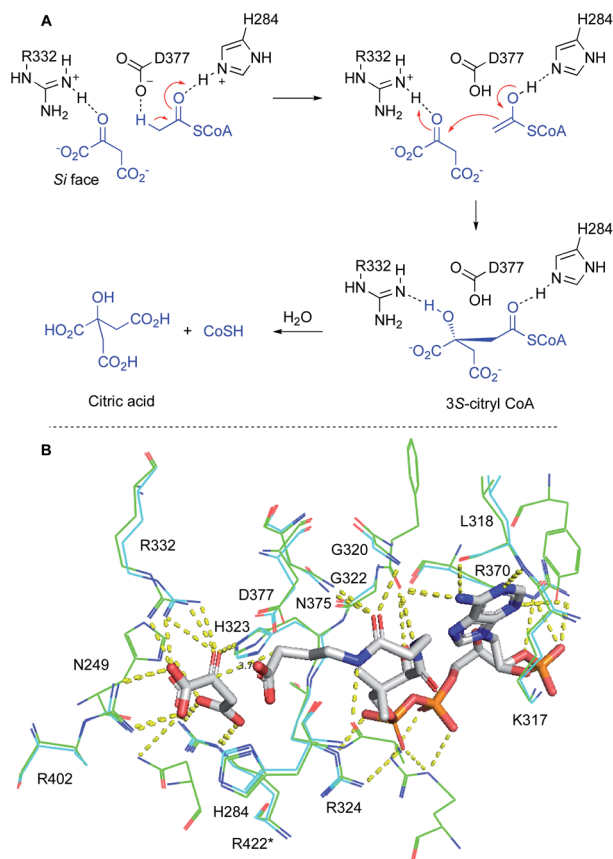
Other residues known to be involved in binding oxaloacetate (*e.g.* G322, H323, R402 and R422*) and acyl CoA (*e.g.* K/R317, L/I318, G320, R324, R/K370 and N375) are also conserved. N249 is replaced by histidine in the other organisms, but modelling (*vide infra*) shows that this residue forms an auxiliary hydrogen bond to the C-4 carboxylate of oxaloacetate which can be achieved by either histidine or asparagine.

We built a 3D model of BfL2 using the SwissModel server[33] and CS from *Acetobacter aceti*[34] as a template (pdb 2H12, Scheme 5B, see ESI† for details). The *A. aceti* structure was obtained in complex with oxaloacetate and an acetyl CoA mimic which allows the determination of residues which bind these substrates.[33] Overlay of the BfL2 model and the *A. aceti* structure shows that all of the residues involved in catalysis and binding oxaloacetate and acyl CoA are structurally highly conserved (Scheme 5B). The model shows that the expected *Si* face of the oxaloacetate ketone is within 3.7 Å of the nucleophilic carbon, consistent with the formation of an *S*-configured tertiary alcohol upon carbon–carbon bond formation.

Since the oxaloacetate-binding and catalytic residues of the BfL2 model and the structure of 2H12 are identical, and overlay to within 1.15 Å RMSD over 13 residues, it is highly likely that the BfL2 alkyl citrate synthase also creates a 2S-stereocentre. Since we already know that BfL2 synthesises exclusively the *anti*

Scheme 4 Synthesis of citrates. Abbreviations: DBPO, dibenzoylperoxide; NBS, N-bromosuccinimide; DMSO, dimethylsulfoxide.

Scheme 5 Conserved residues in citrate synthase: A, mechanism of citrate synthase; B, model of BfL2 (cyan) overlayed with crystal structure data from *A. aceti* citrate synthase 2H12 (green). Residue numbering from BfL2 throughout. Oxaloacetate and an acetyl CoA mimic bound to 2H12 are shown in grey. * = residue from other dimer subunit.

diastereomer, then we conclude that the product of BfL2 possesses 2S,3R configuration.

Dehydratase

Dehydratase assays were also set up *in vitro* and monitored by analytical LCMS. In initial reactions, the synthetic mixture of *E*-1-butenyl-citrate diastereomers **16ab** was incubated with the BfL3 and PvL2 dehydratase enzymes in separate reactions. When observed by diode array detector (200–300 nm), an increase in the area of the later-running peak corresponding to the *syn* citrate was noted. The citrates themselves have no significant UV absorbtion above 220 nm, but the new peak has a maximum UV absorbtion at 270 nm consistent with the formation of the expected unsaturated product **17** which co-elutes with **16b** (Fig. 3).

Observation of the reaction at 270 nm more clearly showed the increase in this species. Mass analysis also indicated a loss of 18 mass units, consistent with the expected dehydration. PvL2 was more active that BfL3 and was therefore used for all further assays.

We next tested the *anti* **16a** and *syn* **16b** diastereomers of the citrate substrate separately. The *anti* diastereomer **16a** was converted to the new peak, while the *syn* diastereomer **16b** was unreactive. The new compound **17** elutes with the same retention time as the *syn* diastereomer of the citrate **16b**. Extended reaction never reduced the amount of (±) starting material below 50% suggesting that the enzyme is selective for a single enantiomer, presumably the 2S,3R stereoisomer. Although the UV and MS analysis of the product of the reaction were consistent with formation of the expected 2,3-unsaturated system, the *in vitro* assays did not yield enough material for full NMR characterisation (Fig. 4).

In order to fully characterise **1** it was re-isolated from a fermentation of *A. oryzae* expressing *bfPKS*, *bfL1* (hydrolase), *bfL2* (CS) and *bfl3* (dehydratase). A time course experiment
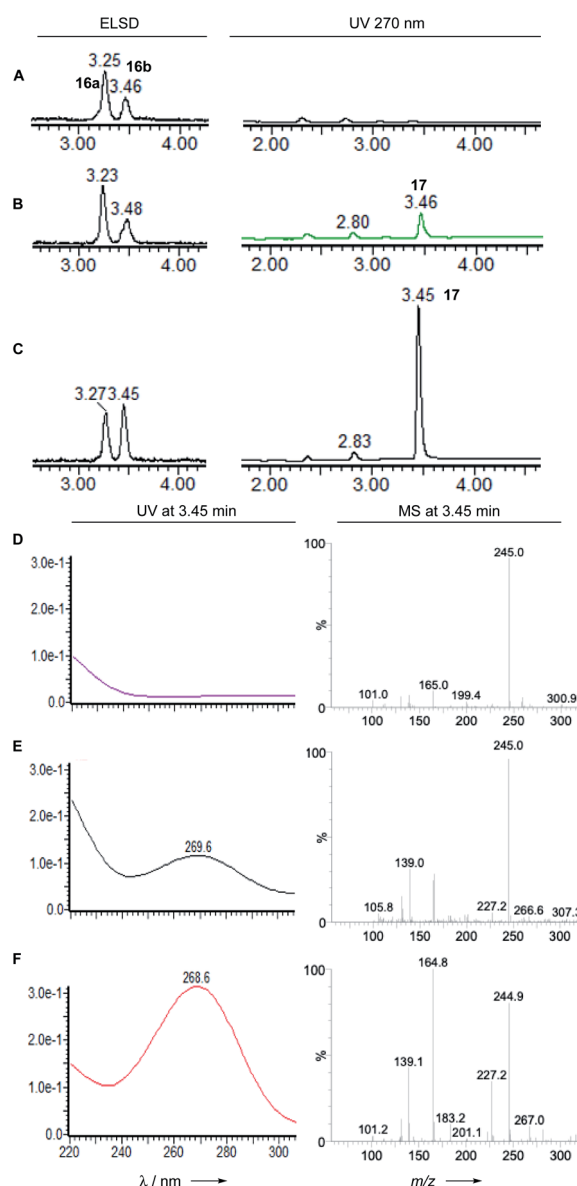

Fig. 3 LCMS analysis of PvL2 and BfL3 reactions *in vitro*. A, **16** alone; B, **16** + BfL3; C, **16** + PvL2; D, **16** alone; E, **16** + BfL3; F, **16** + PvL2.
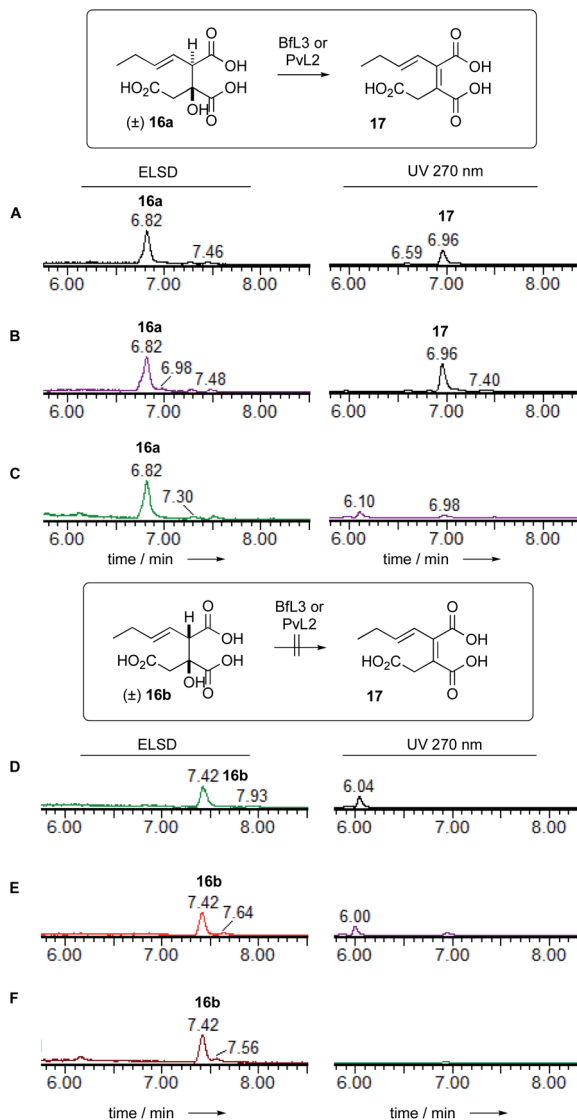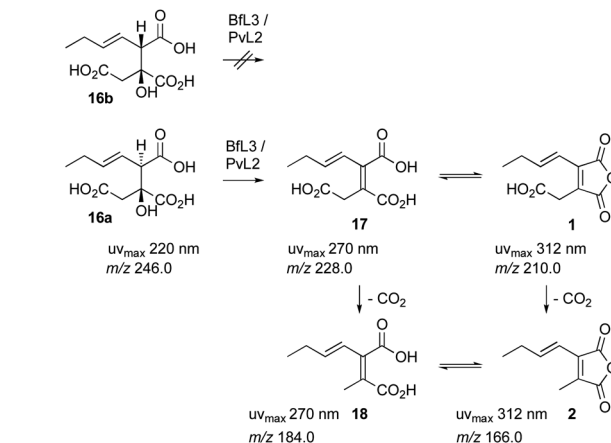
Fig. 4 LCMS analysis of the indicated DH reactions. Top (2R,3S/2S,3R) *anti* substrate **16a** reacted with enzyme: A, BfL3; B, PvL2; and C, empty vector control respectively. Bottom (2R,3R/2S,3S) *syn* substrate **16b** reacted with enzyme: D, BfL3; E, PvL2; and F, empty vector control.

determined that **1** reaches maximum concentration after 4 days of fermentation. After this time cells were collected, and extracted with EtOAc. Extended contact with organic solvents such as $CHCl_3$ and DMSO causes **1** to decarboxylate to give **2**. Similarly, freeze-drying procedures also decarboxylated **1**. However, rapid purification by preparative HPLC in CH_3CN/H_2O mixtures, followed by removal of CH_3CN under mild vacuum led to stable aqueous solutions of **1** which could be analysed by NMR and used for further *in vitro* assays (*vide infra*). The product of the *in vitro* reaction of PvL2 and BfL3 was thus proven to be identical to **1** isolated from fermentation (Scheme 6).

LCMS analysis also showed that the maleic acid **17** (UV max 268 nm) and maleic anhydride **1** (UV max 313 nm) forms are in equilibrium. The diacid **17b** elutes significantly earlier than the

Scheme 6 Reactions catalysed by BfL3 and Pvl2 dehydratases, and facile decarboxylation.

anhydride **1**. Attempts to purify either compound always led to recovery of mixtures.

KI and PEBP

Our previous *in vitro* work showed that expression of KI genes with the earlier pathway genes led to dimerisation of **1** and formation of byssochlamic acid, while addition of the PEBP genes increased the titre. However, we were unable to conclude whether the PEBP proteins played a role in the catalysis or, alternatively, provided an uncharacterised resistance mechanism to byssochlamic acid **12** which is known to be toxic to fungi.[35] Here we tested the effects of the isolated proteins with the proposed substrate **1** *in vitro*, once again following the reactions by analytical LCMS.

Initial work focussed on attempts to detect conversion of maleic anhydride monomer **1** to bysochlamic acid **12** using BfL5, BfL6, BfL9 and BfL10 obtained by *E. coli* expression of the genes (lacking signal peptides and containing N-terminal his6 tags) either singly or in combination. However despite numerous attempts and varied conditions we could never observe any activity. We then attempted to express the full-length genes in yeast using the pESC system already described. In these cases we were not able to observe significant amounts of new soluble proteins by SDS-PAGE, or purify the proteins because they were cloned without his6 tags. However, we tested cell-free extracts of the induced yeast strains and compared the effects *vs.* control strains lacking the expression plasmids. In these cases we could clearly observe the conversion of **1** to byssochlamic acid **12** by a combination of all 4 protein extracts. The same results were observed for the combination of the two KI components, and for the KI components tested singly. The PEBP proteins appeared to be inactive in all conditions tested. They did not form **12** either alone or in combination, and their addition to the KI proteins did not seem to significantly increase the amount of **12** formed (Scheme 7).

Finally, decarboxylated maleic anhydride **2** was also tested as a substrate in the dimerisation assay. However no conversion to byssochlamic acid **12** was observed, and **2** and its hydrolysed

congener **18** were the only compounds observed by LCMS analysis (see ESI† for details).

Discussion

Alkyl citrates are a class of fungal secondary metabolites which are closely related to primary metabolites. In the case of the maleidrides byssochlamic acid **12** and cornexistin **13** the pathway starts with a highly-reducing polyketide synthase (hr-PKS),[36] but in many other cases such as the sporothriolides[3] the pathway begins with a typical fungal fatty acid synthase (FAS). The function of these synthases at the start of the pathways is subtly different.
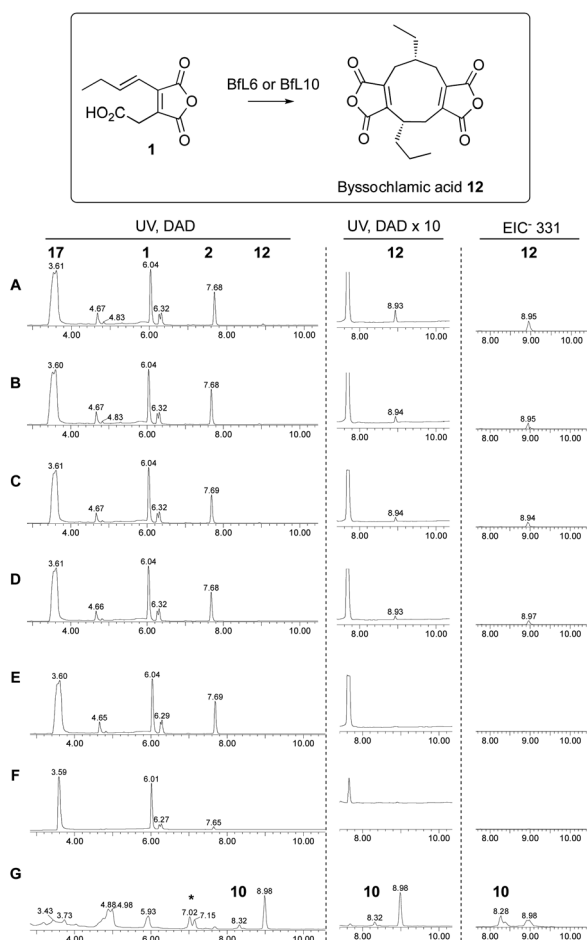
hr-PKS such as BfPKS1 can control chain-length and functionalisation of the acyl chain, but these hr-PKS usually lack an integrated release mechanism. Thus BfPKS1 appears to synthesise a hex-2-enoyl product which is released by a dedicated *trans*-acting hydrolase BfL1 as a carboxylic acid. This is demonstrated *in vitro* here where we showed that BfL1 can hydrolyse acyl-BfPKS-ACP species. Similar hydrolytic release

mechanisms are known in other fungal PKS systems such as phaeospelide A.[37] However, BfL1 also appears to rapidly hydrolyse other thiolesters, so how its selectivity is controlled *in vivo* is not yet known. This release mechanism differs from that used by fungal FAS (*e.g.* SpoFas)[3] where the malonyl-palmitoyl transferase (MPT) domain is known to both load malonyl CoA extenders, and release products directly as CoA thiolesters.[38]
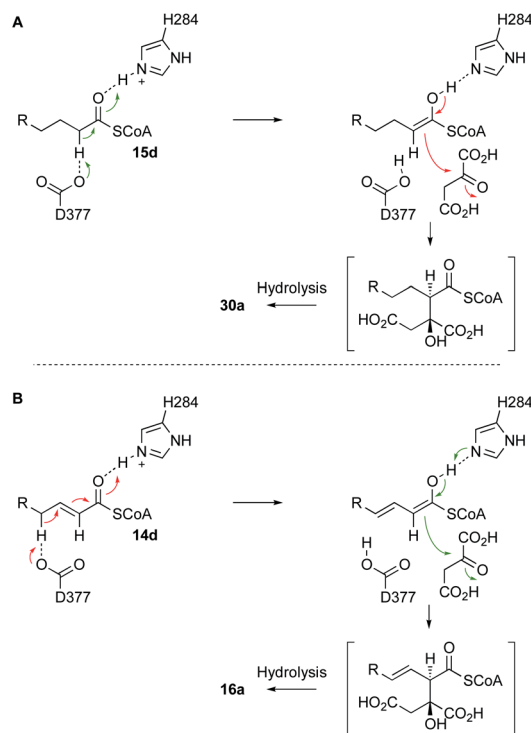
The next step of the pathway is also closely related to primary metabolism. We showed that the citrate synthase BfL2 selectively reacts acyl CoAs **14d** and **15d** with oxaloacetate. This differs from the suggestion by Tang and coworkers that similar CS enzymes react directly with ACP-bound acyl groups, for example during the biosynthesis of squalestatins.[39] This reaction does not occur in the case of the byssochlamic acid pathway where we showed that acyl-ACPs (*i.e.* **14a** and **15a**) are not substrates for BfL2.

The requirement for CoA substrates by the CS raises an interesting question. In the case of FAS-based pathways acyl CoAs are released directly by the FAS, but for the PKS + hydrolase pathways the released carboxylic acids must be activated to CoA thiolesters, however the required CoA synthetase does not appear to be encoded within the Bf BGC. Presumably such short-chain thiolesters could be synthesised by endogenous primary metabolism enzymes.

The BfL2 CS can react both saturated and *E*-unsaturated substrates. In the case of *E*-unsaturated substrates the alkene migrates during the reaction. Since it is known that the active site base of CS is a highly conserved aspartate (D377, Scheme 8),

Scheme 7 LCMS analysis of reactions catalysed by KI enzymes BfL6 and BfL10, and PEBP proteins BfL5 and BfL9: A, two KSI and two PEBP; B, two KI; C, BfL6; D, BfL10; E, empty vector control; F, only substrate; G, producing *A. oryzae* transformant. * mixture of diacid forms of **12**. Note that data is qualitative rather than quantitative.

Scheme 8 A, Likely mechanism of BfL2 during the synthesis of alkyl citrates; B, adapted mechanism for the synthesis of vinyl citrates *via* a dienol intermediate.

we speculate that this residue could be involved in both processes, forming a dienol intermediate during the synthesis of **16a** (Scheme 8B). Thus-far we have not experimented with *Z*-alkenes as substrates, but this would be an interesting future goal.

We showed that the BfL2 CS produces exclusively 3,4-*anti* products, and structural considerations strongly suggest this is the 3*S*,4*R* stereoisomer. In the cases of other known citrate-derived metabolites (and in primary metabolism) the 3*S* configuration is also observed, but in the known cases of squalestatin S1 **9**, viridiofungin **8** and CJ-13,981 **7**, for example, the CS products are 3*S*,4*S*.[7,40,41] Differences in configuration at the 4-position must be controlled by the geometry of the enoyl CoA intermediate, and in-turn this may be related to the ability of the BfL2 CS to react 2,3-unsaturated substrates. Detailed structural work will be required to probe this question further. The family of secondary metabolite alkyl citrate synthases resemble methylcitrate synthases involved in propionate metabolism. Recent work by Reddick and coworkers has reassigned the product of these enzymes as 3*S**,4*R** in agreement with our stereochemical assignment.[42]

In the next step the dehydratase BfL3 removes a water molecule. This step is closely related to the primary metabolism enzyme 2-methylcitrate dehydratase (2MCDH). Again the stereoselectivity of this step by the primary metabolism 2-methylcitrate dehydratase has been debated, but Reddick's results[41] conclusively show that the enzyme takes 3*S**,4*R** isomer to give *Z*-methyl aconitate – *i.e. anti* dehydration.[43] Again, the BfL3 and PvL2 dehydratases follow exactly the same stereochemical course, emphasising the similarity of the primary and secondary metabolism pathways. The dehydrated products spontaneously equilibrate between diacid **17** and anhydride **1** forms. Reddick showed that the primary metabolism 2MCDH enzymes can dehydrate both the *anti* and *syn* diastereomers of 2-methylcitrate, but the BfL3 enzyme cannot dehydrate the *syn* substrate diastereomer **16b**.

Finally, we showed that ketosteroid-isomerase-like (KI-like) enzymes BfL6 and BfL10 catalyse the dimerisation of **1** to the nonadride byssochlamic acid **12**. They appear to act alone and both are individually active. Interestingly, decarboxylated congener **2** is not a substrate for the enzyme catalysed reaction. In previous *in vitro* work Baldwin[44] and others[45] have shown that related decarboxylated compounds can give low yields of dimeric products in organic solvents in the presence of medium to strong bases which are likely to be able to form low concentrations of anionic intermediates. However our results suggest that while such intermediates could be involved in dimerisation, they are likely to be generated by enzyme-catalysed decarboxylation of **1**.

We could not obtain high yields of the KI-like enzymes in soluble form, and only cell-free extracts produced in yeast showed any tangible activity. We were therefore unable to perform more detailed studies of the mechanisms of these intriguing enzymes, but never-the-less this is the first report of their activity *in vitro* and sets the scene for future more detailed studies. Also unexpected is the observation that the KI proteins appear to make a single nonadride product *in vitro*: formation of

the heptadride **10** was not observed *in vitro*, raising the question of how this is formed *in vivo*.

The PEBP proteins studied here appeared catalytically inactive. The *B. fulva* **12** BGC encodes two pairs of KI and PEBP enzymes, but most other maleidride clusters encode only a single pair, so it seems likely that the *B. fulva* system has undergone gene duplication. Lack of catalytic activity of PEBP proteins supports previous suggestions that these may be part of resistance systems as they are encoded by most maleidride BGC, however another function cannot be ruled out.

Thus the *in vitro* studies of the function and stereoselectivity of enzymes involved in fungal alkyl citrate biosynthesis show that the pathway is very closely related to primary metabolism. Indeed simple compounds like CJ-13981 **7** should require only a fatty acid synthase and an alkylcitrate synthase, both presumably duplicated from primary metabolism, to produce a compound with useful activity as an inhibitor of squalene synthase.[4,5] Exchange of the FAS for an hr-PKS (and hydrolase) allows more complex chains to be built – for example squalestatin precursors which are also squalene synthase inhibitors.[46–48] Finally, gain of a dehydratase (again almost directly from primary metabolism) and a KI-like protein then allows the pathway to make maleidrides. Other pathways like those involved in squalestatin or sporothriolide diversify by gain of more obviously secondary metabolism steps involving C–H activation by oxidation. The close relationship of such secondary metabolism pathways to key primary metabolic enzymes shows how complex secondary metabolic pathways have likely evolved. However, for the same reasons, this makes it difficult for bioinformatic systems trained to find secondary metabolism genes and pathways to recognise these pathways.

Experimental details

All experimental details and characterisations of individual compounds and proteins is contained in the ESI.†

Author contributions

VH cloned the BfPKS ACP, prepared acylated ACPs and conducted assays with these species, and worked with Sfp. SY cloned all other genes, expressed all other proteins and performed all other *in vitro* assays. SF synthesised all small molecules and performed hydrolase assays. RJC devised the study, gained funding and wrote the paper.

Conflicts of interest

There are no conflicts to declare.

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