



Cite this: *Chem. Commun.*, 2023, 59, 13587

Received 25th September 2023,  
Accepted 20th October 2023

DOI: 10.1039/d3cc04758j

rsc.li/chemcomm

## Total biosynthesis of fungal tetraketide pyrones†

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Fungal tetraketide pyrones possess important and potent bioactivities, but their detailed biosynthetic pathways are unknown and synthetic routes to their production are lengthy. Here we investigated the fungal pathways to the multiforins and compounds related to islandic acid. Heterologous expression experiments yield high titres of these compounds and pathway intermediates. The results both elucidate the pathway and offer a platform for the total biosynthesis of this class of metabolites.

The tetraketide pyrones form a large class of bioactive fungal specialized metabolites (SMs) that feature a wide variety of oxygenation and acylation patterns. For example, multiforin A **1A** and related compounds (Fig. 1) show significant *in vitro* immunosuppressive activity only 10-fold less potent than the standard drug cyclosporin A.<sup>1</sup> Related compounds such as islandic acid **2** from *Penicillium islandicum* possesses potent cytotoxicity vs. Yoshida sarcoma (IC<sub>50</sub> 1 µg ml<sup>-1</sup> for the methyl ester).<sup>2,3</sup> Similarly, allantopyrone **3** is also a potent cytotoxin (IC<sub>50</sub> 0.32 µM) *via* inhibition of the NF-κB signaling pathway; and rosellisin **4** has antibacterial properties (IC<sub>50</sub> 30 ppm vs. *S. aureus*).<sup>4</sup> Rosellisin **4** is known to be a polyketide derived from four acetate units<sup>5</sup> and it seems likely that **1A**, **2** and **3** arise from similar biosynthetic pathways. Production of islandic acid **2** and related compounds has been reported by multi-step total chemical synthesis,<sup>6</sup> but such methods are inefficient and production of these compounds by total biosynthesis would be advantageous. However, despite their potent and diverse bioactivities, and the presence of an intriguing *EZ* diene in **2** and **3**, this class of bioactive tetraketide pyrones has not been deeply investigated at the biogenetic level.

Fermentation of *Hypomontagnella monticulosa* during a series of experiments into the biosynthesis of the sporochartines,<sup>7</sup> revealed that this organism can produce the pyrone tetraketide multiforin H **1H**.<sup>1</sup> We screened the genome of *H. monticulosa* and two closely related species with the aim of discovering a biosynthetic gene cluster (BGC) which might be responsible for this pathway. We focussed on a BGC (*mfn*) encoding two highly-reducing polyketide synthases (*hrPKS*) *mfnPKS1* and *mfnPKS2*, together with an *O*-acyl transferase (*O*-AcT, *mfnL3*), two cytochrome P450 monooxygenases (*mfnL2* and *mfnL3*), an *O*-methyl transferase (*O*-MeT, *mfnL1*), a transcription factor (TF, *mfnR1*), an FAD-dependent oxidoreductase (FMO, *mfnR2*), and a short-chain dehydrogenase/reductase (SDR, *mfnR4*, Table 1).

The boundaries of this BGC were suggested by transcriptomic studies in *H. monticulosa* which showed this region to be transcriptionally co-regulated (see ESI,† Fig S1.1). Near identical BGCs were found in the related species *H. spongiphila* and *H. submonticulosa* (ESI,† Fig S1.2). Previous work had shown that selective gene knockout in *H. monticulosa* is inefficient.<sup>7</sup> We selected to use heterologous expression in the workhorse fungal host *Aspergillus oryzae* to probe the biosynthesis and provide a total biosynthesis platform.<sup>8,9</sup>

An intron-free construct of *mfnPKS1* was cloned into an *A. oryzae* expression vector. Transformation of *A. oryzae* yielded eight transformants, and PCR analysis confirmed six of these to

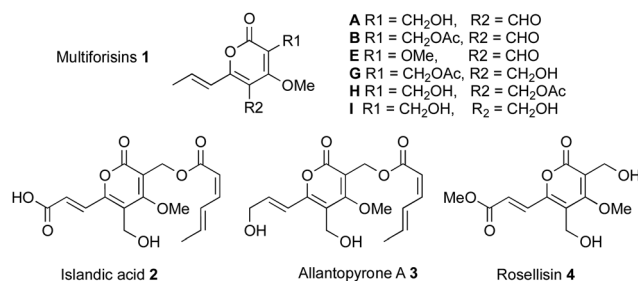


Fig. 1 Typical Fungal tetraketide Pyrones.

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3cc04758j>

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Table 1 Summary of BGCs, predicted protein function and heterologous expression experiments

Genomic map of the *mfn* locus in *H. monticulosa*, *P. islandicum*, and *Amphicorda felina*. The map shows genes *mfnPKS2*, *mfnL3*, *mfnL2*, *mfnL1*, *mfnPKS1*, *mfnR1*, *mfnR2*, *mfnR3*, *mfnR4*, *ilaPKS1*, *ilaR1*, *ilaR2*, *ilaR3*, *ilaR4*, *ilaR5*, *ilaR6*, *ilaR7*, *ilaPKS2*, *ilaR8*, and *ilaR9*. A 2.5kb scale bar and an identity percentage scale (0-100%) are shown. A legend identifies symbols for TF, hrPKS, OMeT, P450, SDR, FAD, P450, SDR, and Transporter.

Exp.	<i>mfnPKS2</i> hrPKS	<i>mfnL3</i> O-AcT	<i>mfnL2</i> P450	<i>mfnL1</i> O-MeT	<i>mfnPKS1</i> hrPKS	<i>mfnR2</i> FMO	<i>mfnR3</i> P450	<i>mfnR4</i> SDR	<i>ilaPKS2</i> hrPKS	<i>ilaR2</i> O-AcT	<i>ilaR8</i> SDR	Products
1					✓							5
2			✓		✓							5, 6
3					✓		✓					5, 6
4				✓	✓							5, 7, 8
5			✓	✓	✓							5, 9
6			✓	✓	✓		✓					11, 5, 9, 10
7			✓	✓	✓		✓					11, 5, 9
8			✓	✓	✓	✓	✓					11, 5, 9
9			✓	✓	✓		✓	✓				9, 11, 16
10		✓	✓	✓	✓		✓					11, 5, 9,
11	✓	✓	✓	✓	✓		✓					11, 1H, 9, 12
11 <sup>a</sup>	✓	✓	✓	✓	✓		✓					11, 1H, 13, 14, 15
12			✓	✓	✓		✓	✓				11, 5, 9
13	✓	✓	✓	✓	✓		✓	✓	✓	✓		11, 1H, 5, 9, 12
14			✓	✓	✓		✓	✓	✓	✓	✓	11, 5, 9

<sup>a</sup> Same as expt 11, with acid workup.

contain full-length *mfnPKS1*. These transformants produced a new compound **5** in high titre ( $123 \text{ mg L}^{-1}$ , Fig. 2) and traces of **6** (Fig S1.16, ESI<sup>†</sup>) that were not produced by untransformed *A. oryzae*. Larger scale fermentation (1L) allowed purification and structure elucidation of **5** that was identified by full NMR analysis as amphicoprone A **5**.<sup>10</sup>

Next, we undertook a systematic program of gene expression of the *mfn* genes (Table 1, experiments 2–11, see ESI<sup>†</sup> for vector details) followed by LCMS analysis (Fig. 2). Addition of P450 monooxygenase encoding genes *mfnL2* (exp. 2) or *mfnR3* (exp. 3) produced small but isolatable amounts of the 8-alcohol **6** (see ESI<sup>†</sup> for full NMR characterisation of all isolated compounds) with the majority of **5** unreacted. Alcohol **6** is not on

the pathway to the multiforins, and we assumed it to be an *A. oryzae* shunt.

In contrast, coexpression of *mfnL1* that encodes an O-MeT (exp. 4) substantially converted **5** to known methyl ether **7** ( $140 \text{ mg L}^{-1}$ ) that does appear to be a pathway intermediate.<sup>10</sup> Minor amounts of presumed shunt 8-carboxylate **8** were also observed. Compound **7** is fully converted to the known C-9 alcohol **9** ( $134 \text{ mg L}^{-1}$ ) when the *mfnL2* P450 monooxygenase is added (exp. 5). On the other hand, **7** is converted to **10** (majority,  $31 \text{ mg L}^{-1}$ ), **9**, and some diol **11** (minority) by *mfnR3* that encodes the other P450 monooxygenase (exp. 6). Expression of both P450 monooxygenases at this stage (exp. 7) leads to the expected diol multiforin **11**

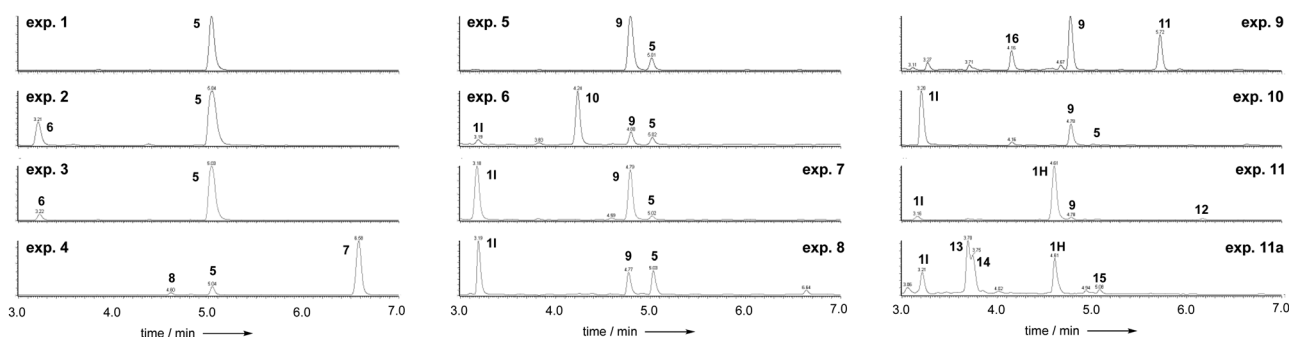


Fig. 2 LCMS results for experiments 1–11a. Diode array detector (200–600 nm) data shown. Absorbance units are arbitrary.



(36 mg L<sup>-1</sup>). At this point, expression of *mfnR2* that encodes the FMO (exp. 8) did not appear to give any further new compounds. However, coexpression of *mfnR4* that encodes the SDR, oxidises intermediate **9** to the aldehyde **11** (exp. 9, 10 mg L<sup>-1</sup>). Addition of the *mfnL3*-encoded *O*-AcT alone at this juncture gave no new compounds (exp. 10), but coexpression of *mfnL3* with *mfnPKS2* led to direct synthesis of multiforisin H **1H** (exp. 11, 28 mg L<sup>-1</sup>). Finally, a series of thioether shunts (**13**–**16**) were isolated after acidification of the media during extraction (exp. 11a). These compounds likely arise by formation of quinomethide-type structures that are quenched by 2-hydroxyl-3-thiopropionate or 2-thioacetate.

We next searched the publically available genome of *Penicillium islandicum*, the producing organism of islandic acid **2**, using the *mfn* genes as bait. This revealed a near identical BGC (*ila*, Table 1) encoding homologs of: *mfnPKS1* (*ilaPKS1*), two parallel cytochrome P450s (*ilaR4* & *ilaR6*); the *O*-methyltransferase (*ilaR1*); the transcriptional regulator (*ilaR3*), the SDR (*ilaR7*), and FMO (*ilaR5*). However the second PKS (*ilaPKS2*) and the acyl transferase (*ilaR2*) do not show significant similarity with the cognate proteins encoded by the *mfn* BGC, consistent with the formation and attachment of different PKS-derived acyl groups. Additionally the *ila* BGC encodes a second SDR (*ilaR8*) that shows low similarity to *mfnR4*. We hypothesised that *ilaPKS2* from the proposed *P. islandicum* BGC might synthesise the unusual *EZ* triketide which may be transferred to the C-2 hydroxymethyl group by the *ilaAT* in analogy to the multiforisin chemistry.

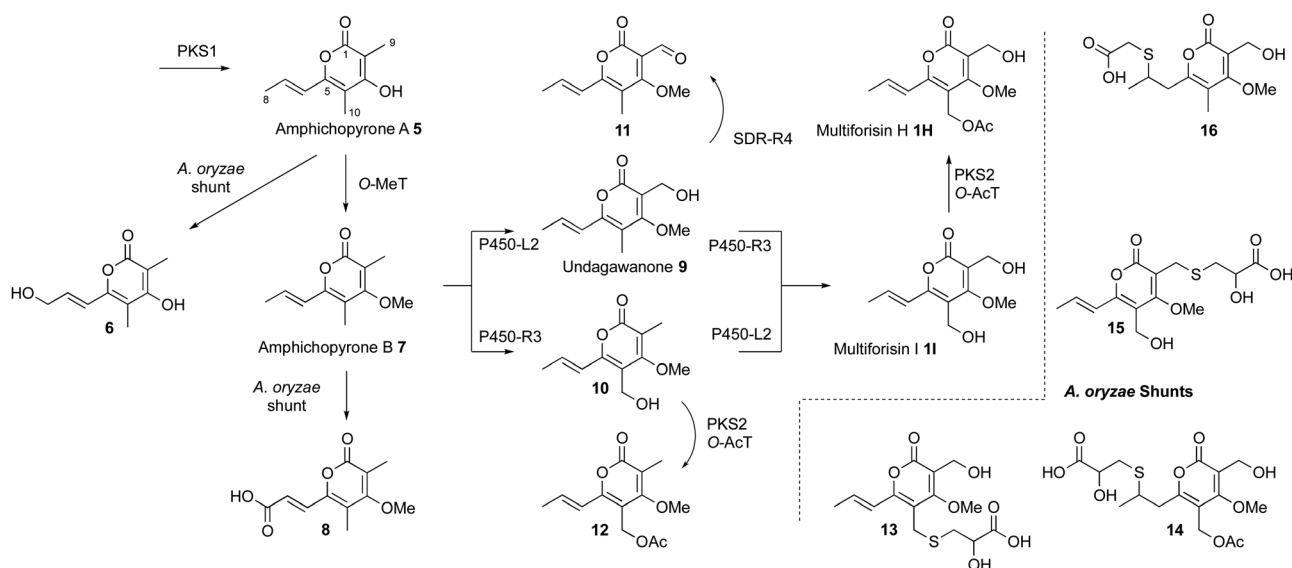
In the *H. monticulosus* case it appears that the role of PKS2 is simply to supply an acetyl group for attachment at the 10-OH. This is consistent with the observed mutation of the active site cysteine of the MfnPKS2 KS domain to serine (ESI,† Section 1.1.5): presumably the PKS can load an acetyl starter unit but not extend it. Tang and coworkers recently described another Type I PKS component with similar activity.<sup>11</sup>

We next attempted to synthesise **2** or **3** by expressing *ilaPKS2* in *A. oryzae* that produces multiforisin pathway intermediates. Three experiments were set up. In the first (exp. 12), *A. oryzae* from expt. 7 that produces **1I**, **5**, and **9** was cotransformed with *ilaPKS2* alone. Second (exp. 13), *A. oryzae* from exp. 11 that produces **1I**, **1H**, **9**, and **12** was cotransformed with *ilaPKS2* and *ilaR2* that encodes a putative acyl transferase. Finally (exp. 14), *A. oryzae* from exp. 7 that produces **1I**, **5**, **9** and was cotransformed with *ilaPKS2*, *ilaR2* and *ilaR7* that encodes an SDR. However, none of these transformants produced any compounds in addition to the those already produced by the *A. oryzae* hosts.

Yuan and coworkers recently reported a fungal BGC (*amp*) from *Amphichorda felina* that also encodes the biosynthesis of tetraketide pyrones. In comparison to the *mfn* and *ile* BGCs, the genes encoding PKS1, *O*-MeT and the two P450 monooxygenases are conserved. However, in their work only expression of the first catalysts from the pathway led to the production of metabolites (**5** and **7**). In our hands the pathway expression is more productive, showing that hydroxylation of **7** can occur in parallel, to form either or both of **9** and **10** as intermediates, before formation of multiforisin **I 1I** (Scheme 1).

Final *O*-acetylation is achieved by using an apparently partially active PKS and AT pair. Similar *O*-acylation systems are known in the cases of lovastatin<sup>12</sup> where a diketide is added and in the case of squalestatin S1<sup>13</sup> where a tetraketide is added. The multiforins often feature a 9-aldehyde and this is installed by the SDR encoded by *mfnR4* (e.g. **11** from exp. 9).

Thus systematic heterologous expression of the *mfn* genes provides a convenient total biosynthesis platform for the multiforins in good overall titres. However, attempts to redirect the pathway towards islandic acid **2** and allantopyrone **3** intermediates by adding the islandic acid triketide were unsuccessful. The *ilaPKS2* has a carnitine acyl transferase (cAT) C-terminal domain. Such domains are very rare in fungal PKS, but in one



**Scheme 1** Deduced pathway for multiforisin H biosynthesis in *H. spongiphila* showing shunts observed in *A. oryzae*.



case where a cAT domain has been investigated, Tang and coworkers showed that it was responsible for the reversible transfer of the completed polyketide to a polyol.<sup>14</sup> Here we reasoned that a compound such as **1I** could serve as the acceptor, but coexpression of the *ilaPKS2* in cultures that produce **1I** did not afford **2**. Further addition of the putative AT encoded by *ilaR2* did not help. We reasoned that the terminal carboxylate of **2** might be required as a recognition feature, but the *ila* BGC does not encode any additional monooxygenases that could oxidise position 8. The oxygenation of C-8 in *A. oryzae* was unreliably performed by a presumably *A. oryzae* native enzyme forming shunt metabolites **6** and **8**. It is possible that 8-oxygenation during islandic acid biosynthesis may also be achieved by an oxygenase not encoded by the *ila* BGC, and that the *ilaSDR-R8* may oxidise this to a carboxylic acid. However, inclusion of *ilaSDR-R8* did not allow this chemistry.

Overall, we have achieved rapid total biosynthesis of a number of known and new fungal tetraketide pyrones and elucidated the pathway to the multiformisins. It should be possible to rationally engineer future members of this family using sustainable synthetic biology rather than multi-step synthetic chemistry methods.<sup>6</sup> However, further work is required to understand the biosynthesis and attachment of the *EZ* triketide side chain of **2** and **3**. Experiments in this direction are ongoing in our laboratories.

RJC, EK, DT and YS designed the study. DT and YS performed all bioinformatic analysis. JO, GLG and GA isolated and characterised compounds from *H. spongiphila*. YS and DT did all other experimental work. The MS was drafted by RJC, and all authors were involved in final editing.

The China Scholarship Council is thanked for scholarships to Y. S. (201908360165) and D. T. (201706670001). This work was also funded by the Deutsche Forschungsgemeinschaft (DFG) priority program "Taxon-Omics: New Approaches for Discovering and Naming Biodiversity" (SPP 1991), specifically

CO 1328/4-2 and CO 1328/4-1. Professor Thomas Ostenfeld Larsen (Danish Technical University) is thanked for the gift of *Penicillium islandicum*. The publication of this article was partially funded by the Open Access Fund of the Leibniz University of Hannover.

## Conflicts of interest

There are no conflicts to declare.

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