

Biosynthesis of a Tricyclo[6.2.2.0^{2,7}]dodecane System by a Berberine Bridge Enzyme-like Aldolase

Hang Li^[a], Jinyu Hu^[a], Haochen Wei^[b], Peter S. Solomon^[b], Keith A. Stubbs^[a], Yit-Heng Chooi^{*[a]}

Abstract: The aldol reaction is one of the most fundamental stereocontrolled carbon-carbon bond-forming reactions and is mainly catalyzed by aldolases in Nature. Despite aldol reaction has been widely proposed to be involved in fungal secondary metabolite biosynthesis, a dedicated aldolase that catalyze stereoselective aldol reaction has rarely been reported in fungi. Here, we activated a cryptic polyketide biosynthetic gene cluster that was upregulated in the fungal wheat pathogen *Parastagonospora nodorum* during plant infection and it resulted in the production of the phytotoxic stemphyloxin II (**1**). Via heterologous reconstruction of the biosynthetic pathway and *in vitro* assay using cell-free lysate from *Aspergillus nidulans* we demonstrated a berberine bridge enzyme (BBE)-like protein SthB catalyzes an intramolecular aldol reaction to establish the bridged tricyclo[6.2.2.0^{2,7}]dodecane skeleton in the post-assembly tailoring step. The characterization of SthB as an aldolase enriches the catalytic toolbox of classic reactions and the functional diversities of the BBE superfamily enzymes.

Aldol reactions involve nucleophilic attack by the α -carbon atom of an aldehydic or ketonic enolate upon the carbonyl carbon atom of another aldehyde or ketone to form a β -hydroxy carbonyl product (Figure 1A). The aldol reaction is one of the most powerful methods to generate carbon-carbon bonds in synthetic organic chemistry as it could simultaneously establish new chiral centers.^[1] Enzymes that are capable of catalyzing aldol reactions are known as aldolases which have shown synthetic utility.^[1a, 2] This reaction is also critical in the primary metabolism context, in which aldolases are found in biosynthetic pathways of carbohydrates, keto acids, and some amino acids.^[3] Some of these aldolases also have been successfully developed for intermolecular aldol reaction in organic synthesis. Considering that more and more aldolase-catalyzed asymmetric syntheses have been reported in organic synthesis for the construction of defined stereoselective centers,^[2, 4] the discovery of novel aldolases from Nature is thus of both fundamental mechanistic significance and practical importance.

Aldol reactions are also proposed to occur widely in fungal secondary metabolite biosynthetic pathways. For example, product template (PT) domains from fungal non-reducing polyketide synthases (NR-PKSs) are responsible for controlling the aldol cyclizations of poly- β -ketone intermediates during aromatic polyketide biosynthesis.^[5] Meanwhile, in

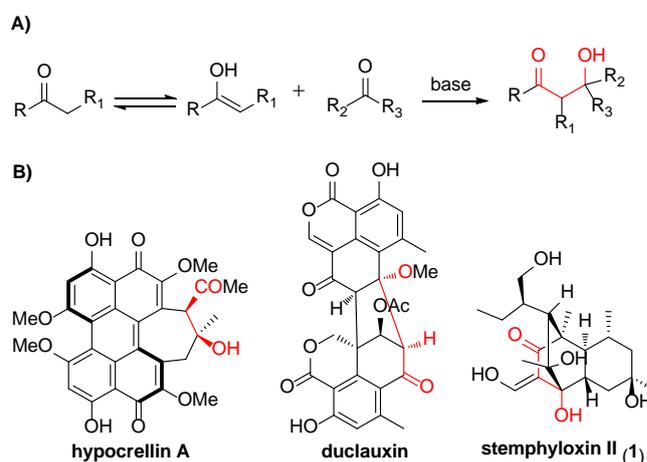


Figure 1. (A) The Aldol reaction and (B) Structures of fungal secondary metabolites containing β -hydroxy carbonyl moiety (highlighted in red) proposed to be biosynthesized via aldol reaction.

post-assembly tailoring steps, aldol reactions could increase the complexity of natural product scaffolds by introducing additional C-C linkages and stereocenters. Notable examples include the light-sensitive phytotoxin hypocrellin A,^[6] the RNA synthesis inhibitor duclauxin^[7] and the phytotoxic stemphyloxin II **1**^[8] (Figure 1B). However, a dedicated enzyme that can catalyze such intramolecular stereoselective aldol reactions has not been reported in post-modification steps, which is surprising given the prevalence of aldol reaction-derived complex structures found in Nature. In this study, we report the identification of the biosynthetic gene cluster of **1** using a chemical ecogenomics approach, and its biosynthetic pathway was unveiled by successful heterologous expression in *A. nidulans*. Further biochemical analysis uncovered an aldolase responsible for the construction of the unusual bridged tricyclo[6.2.2.0^{2,7}]dodecane scaffold.

As part of our ongoing efforts to discover new bioactive metabolites and small molecule virulence mediators from fungi using chemical ecogenomics,^[9] we identified a PKS gene cluster *sth* in the wheat pathogen *Parastagonospora nodorum* is highly upregulated in expression during plant infection (Figure S2).^[10] Detailed bioinformatic analysis showed that the *sth* cluster shares high homology to the previously identified *bet* cluster responsible for the production of the phytotoxin betaenone **6** and **C 4** in the sugar beets pathogen *Phoma betae* Fr.^[11] with the only difference being the presence of an additional cytochrome P450 gene *sthD* (Figure 2A and Table S2). The extensive overlaps between the *sth* and *bet* gene clusters suggested that the *sth* cluster may also encode for the production of betaenone-like compound(s). Interestingly, the presence of such betaenone-like metabolites has never been reported in *P. nodorum*. Homologous gene clusters are also

[a] H. Li, J. Hu, S. Alsop, Dr. K. A. Stubbs, Dr. Y.-H. Chooi
School of Molecular Sciences
The University of Western Australia
Perth, WA 6009, Australia
E-mail: yitheng.chooi@uwa.edu.au

[b] H. Wei, Dr. P. S. Solomon
Division of Plant Science, Research School of Biology
The Australian National University
Canberra, ACT 2601, Australia

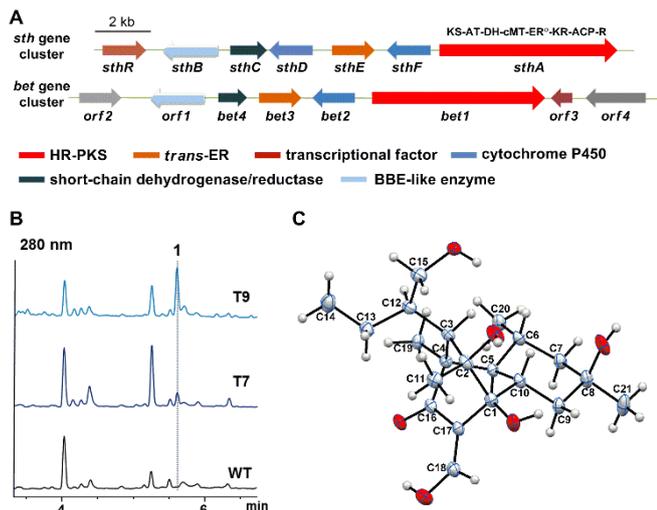


Figure 2. The *sth* cluster encodes the production of stemphyloxin II (**1**). (A) The *sth* gene cluster in comparison to the *bet* cluster. KS, ketosynthase; AT, malonyl-CoA transferase; DH, dehydratase; cMT, methyltransferase; ER, enoyl reductase; KR, ketoreductase; ACP, acyl carrier protein; R, reductase; HR-PKS, highly reducing polyketide synthase. (B) HPLC metabolite profile (280 nm) of *sthR*-OE containing strains in comparison to WT. (C) The crystal structure of **1** refined as an inversion twin.

found in many plant pathogens in the Dothideomycete and Sordariomycete classes (Figure S3), however, none of the orthologous gene clusters in these fungi have been associated with any metabolite product. Given no betaenone-like metabolites could be detected in various culture conditions tested, as expected from the lack of expression of *sth* gene cluster in *in vitro* cultures (Figure S2), we overexpressed a putative Zn₂Cys₆ transcriptional regulator encoded by *sthR* in the cluster using the constitutive *A. nidulans* *gpdA* promoter.^[12] LC-DAD-MS analysis of the acetone extracts from the resulting mutants T7 and T9 on minimal media liquid culture revealed the accumulation of a new peak **1** (λ_{\max} 276 nm, m/z [M+H]⁺ 383) not found in the wild-type (WT) strain (Figure 2B). Large scale culture of T9 mutants and subsequent stepwise purification afforded enough of **1** (1.1 mg/L) for structural elucidation. NMR analyses suggested that the structure of **1** was identical to stemphyloxin II, previously isolated from *Stemphylium botryosum* f. sp. *Lycopersici*.^[8] The compound was successfully crystallized, which allowed the absolute configuration to be determined for the first time using X-ray single crystal diffraction (Figure 2C). Given that stemphyloxins were previously reported to be phytotoxic,^[8] the PKS gene *sthA* was deleted in *P. nodorum* to examine the role of *sth* cluster in virulence against wheat. However, the Δ *sthA* mutant showed no significant difference compared to WT in our assays (Figure S8). This could be due to redundancy and the presence of other virulence factors.

Notably stemphyloxin II **1** contains a β -hydroxy carbonyl bridge, presumably formed from an aldol reaction, in the tricyclo[6.2.2.0]dodecane scaffold. Previous heterologous expression of *bet* genes (*bet1-4*) from *P. betae* in *A. oryzae* by Oikawa and coworkers resulted in the production of the decalin **6**.^[11] However, the enzyme responsible for the aldol reaction to

construct the unique tricyclo[6.2.2.0]dodecane scaffold in **1** and the related betaenone **5** from *P. betae* remained enigmatic. Here, to further shed light into the biosynthesis of **1**, we employed a similar heterologous pathway reconstruction approach in *A. nidulans* LO7890^[13] using a hybrid yeast-fungal artificial chromosome (pYFAC) expression system.^[6b, 14] Coexpression of the polyketide synthase gene *sthA* and the partnering *trans*-ER gene *sthE* in *A. nidulans* led to the production of dehydroprobetaenone I **2** (Figure 3), which corresponds to coexpression of *bet1* and *bet3* (homologous to *sthA* and *E*, respectively) in *A. oryzae*.^[11] Likewise, when coexpressing *sthC* (homologous to *bet4*) encoding a reductase with *sthA/E*, the *A. nidulans* strain expectedly yielded probetaenone I **8**, which confirmed that *SthC* acts as an aldehyde reductase akin to *Bet4*.

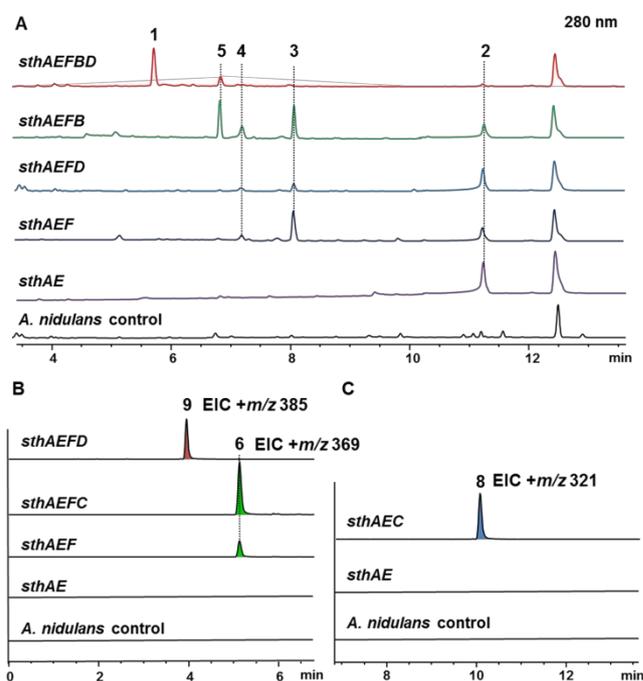


Figure 3. Chromatographic profiles of metabolites produced by *A. nidulans* transformants. (A) HPLC traces at 280 nm (B) Extracted ion chromatography (EIC) of LC-MS traces showing production of metabolites **6** ($+m/z$ 369) and **9** ($+m/z$ 385) (C) EIC ($+m/z$ 321) of LC-MS showing production of **8**.

The cytochrome P450 *Bet2* (homolog of *SthF*) was previously proposed to catalyze the multistep oxidation of **2** to betaenone **4**.^[11] However, **4** was completely reduced to **6** in *A. oryzae*, likely by an endogenous host reductase. Here, to further confirm the function of *SthF*, which shares 80% identity to *Bet2*, *sthF* was coexpressed with *sthA/E*. This led to the production of three compounds, including a new major metabolite **3** (m/z [M+H]⁺ 335), and small amount of the expected **4** and **6** (Table S8). Compound **3** was characterized to contain an α,β -unsaturated aldehyde and an epoxide. NMR analysis of **3** showed the appearance of a coupled aldehyde

Scheme 1. Proposed Biosynthetic Pathway for 1.

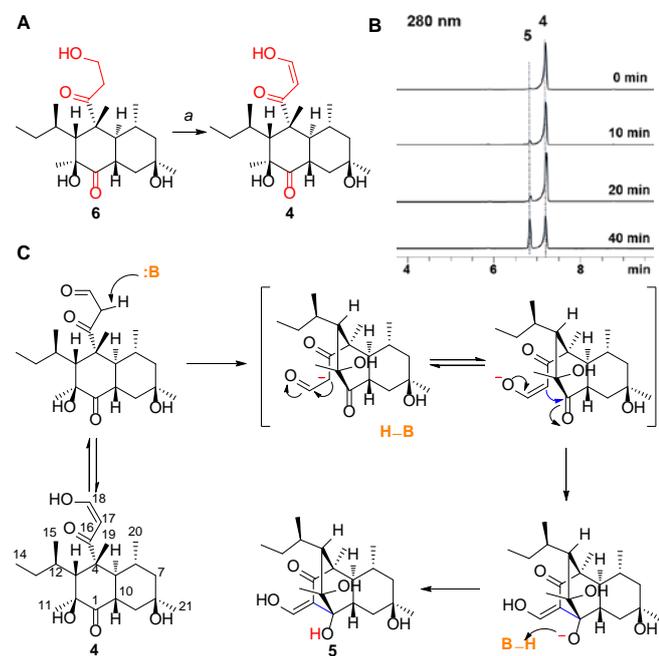
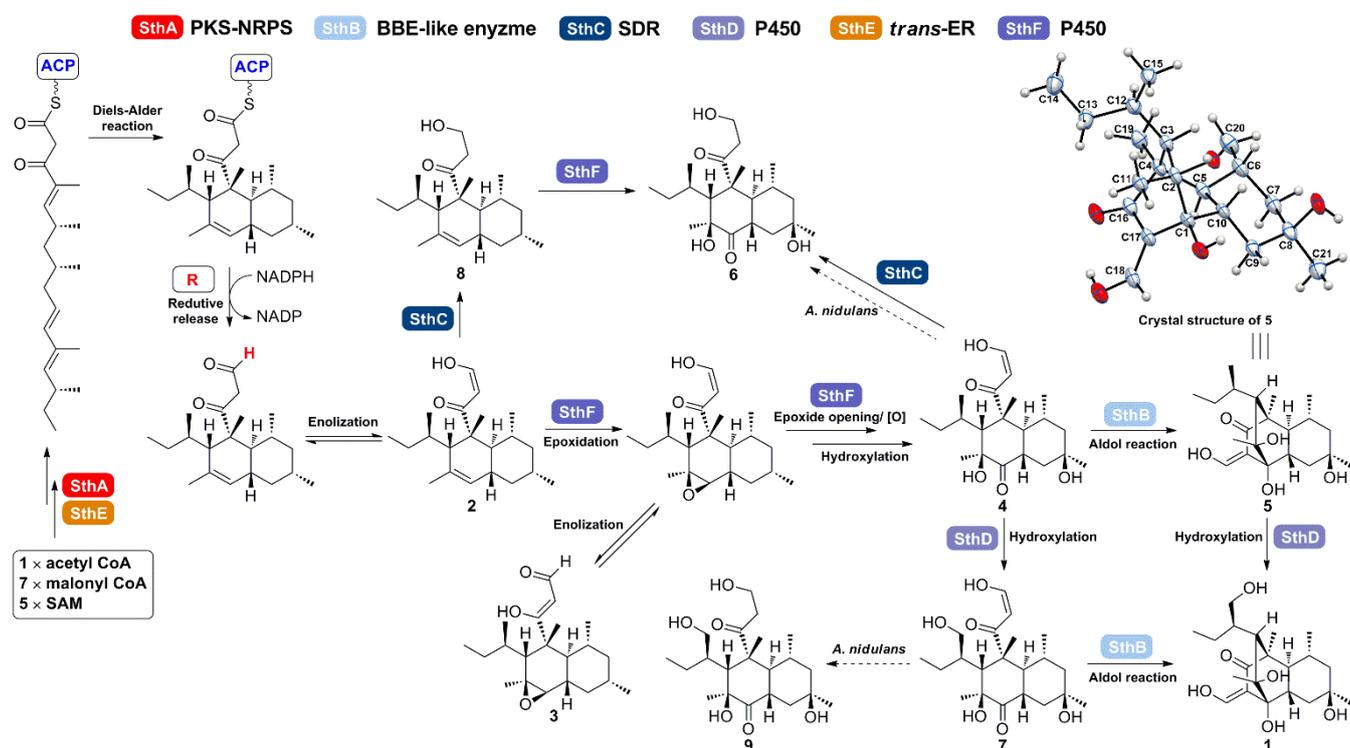


Figure 4. SthB catalyzes the intramolecular aldol reaction from 4 to form 5. (A) Chemical conversion of 6 to 4. ^aDess–Martin periodinane, CH₂Cl₂, rt, 2 h (B) *In vitro* conversion of 4 to 5 using cell-free lysate from *A. nidulans* expressing *sthB* (C) Proposed mechanism for the SthB-catalyzed aldol reaction.

proton at δ_{H} 9.86 (1H, d, 8.5 Hz) and a conjugated trisubstituted alkene at δ_{H} 4.99 (1H, d, 8.5 Hz) (Table S7). Based on the extensive NMR and MS information, the structure of 3 was assigned to be epoxybetaenone (Scheme 1). The production of 3 as an intermediate from *sthA/E/F* transformants indicated that the epoxidation is likely to be the first step catalyzed by SthF to convert 2 to 4, and the appearance of 4 clearly demonstrated that the P450 SthC can catalyze successive epoxidation, oxidation (resulting from epoxide opening) and hydroxylation to install a tertiary alcohol. Like previous heterologous expression studies in *A. oryzae*, the small amount of 6 produced is likely due to an endogenous reductase in *A. nidulans* that mediates the same reduction catalyzed by SthC. However, it appears that the *A. nidulans* endogeneous reductase is less active against 4 (compared to that found in *A. oryzae*) as it allowed for isolation of 4. The coexpression of *sthA/E/F/C* (adding the reductase gene *sthC*) resulted in exclusive production of 6 as expected and further supported the function of SthF as a multifunctional P450 oxygenase. Even though multifunctional P450 oxygenases are now well-known, e.g., in fumagillin^[15] and communesin^[16] biosynthesis in fungi, they continue to be a rich source of new catalytic abilities.

Conserved domain prediction based on NCBI's conserved domain database (CCD)^[17] showed that the enzyme encoded by *sthB* comprises a typical FAD-binding domain^[18] and a berberine bridge enzyme (BBE) domain^[19] (Figure S10). Homology modeling by Phyre2^[20] further showed that SthB is a putative flavoprotein belonging to the BBE family, which has a bi-covalent attachment of the FAD cofactor (Figure S11 and

S12).^[24] To examine the function of *sthB* further, we coexpressed it together with *sthA/E/F*. The resulting transformants accumulated the tricyclo[6.2.2.0]dodecane **5**, which indicated that SthB is responsible for the conversion of **4** to **5**. This is in stark contrast to the previous studies,^[11] where coexpression of the *P. betae sthB* homolog (*orf1*) with other genes in the *bet* cluster resulted in no new product. This is likely due to the substrate **4** being rapidly reduced by the endogenous *A. oryzae* aldehyde reductase to **6** *in vivo*.

Finally, the introduction of the second P450 gene *sthD* (not present in the *bet* cluster) successfully transformed **5** into the end product stemphyloxin II **1**, thus achieving the total biosynthesis of **1** in *A. nidulans* and confirming SthD catalyzed a hydroxylation at the C-15 methyl group on the side chain. Given that stemphyloxin I **7**, a decalin with a hydroxyl group at the same position, was previously reported as a natural product from *S. botryosum*,^[8] we examined whether the *sth* cluster can synthesize **7** in *A. nidulans*. Coexpression of *sthA/E/F/D* (omitting *sthB*) resulted in the production of a novel metabolite **9**. The structure of **9** was characterised to contain an additional hydroxyl group at C-15 compared to **6**, which was confirmed by the crucial ¹H–¹H COSY correlations of H₂-15 (δ_{H} 3.43 and 3.49)/H-12 (δ_{H} 1.89) (Table S9), and is herein named as stemphyloxin III. The production of **9** instead of **7** is likely due to an endogenous host aldehyde reductase, as mentioned above, converting **7** to **9**. Combining the above pathway reconstruction results, it is reasonable to conclude that the order of SthB and SthC in the pathway is exchangeable in the biosynthetic pathway and SthB could directly catalyze the conversion of **7** to **1** via the same intramolecular aldol reaction.

Although the biosynthesis of **1** was successfully reconstituted heterologously, the function of the BBE-like oxidase SthB remained ambiguous. Heterologous pathway reconstruction revealed that SthB likely catalyzes an intramolecular aldol reaction from **4** to **5**. To obtain a more in-depth insight into the reaction catalyzed by SthB, the intron-free *sthB* from *P. nodorum* was cloned under the *ADH2* promoter and transformed into *Saccharomyces cerevisiae* strain BJ5464.^[21] However, Western blotting analysis of the soluble and membrane fractions showed no expression of the SthB, and no new product could be detected when **4** was supplemented to the yeast culture (Figure S5). We then attempted to achieve the *in vitro* conversion of **4** to **5** using a cell-free extracts preparation from *A. nidulans* expressing *sthB* only. As the production of the precursor **4** is low in the heterologous host, in order to obtain enough substrate for subsequent feeding assays, we performed a Dess-Martin oxidation^[22] of **6** to afford **4** (Figure 4A), which was purified and confirmed by MS and NMR. In DMSO, the purified **4** was relatively stable and did not convert to any new compound after 28 days at room temperature (Figure S6). Using cell-free lysate of *A. nidulans* expressing SthB, we observed consumption of **4** and the production of **5** *in vitro* (Figure 4B). Meanwhile, **4** was not converted to **5** in parallel controls (Figure S7). These results clearly established that SthB catalyzes the intramolecular aldol reaction between C-1 and C-17 to form the bridged tricyclic system in **5** and **1**.

BBE-like oxidases have been widely proposed to be involved in oxidation reactions via nucleophilic attack with hydride transfer.^[23] However, in this case, the annulation reaction likely

proceeds via a standard base-catalyzed aldol reaction, which is non-oxidative, facilitated by SthB and involves proton abstraction to generate an enolate followed by a nucleophilic attack at the C1-carbonyl on the decalin (Figure 4C). Though BBE-like oxidases have been discovered to be able to catalyze an astonishing diversity of chemical reactions,^[24] the namesake of this family is the (S)-reticuline oxidase from *California poppy* that catalyzes the conversion of (S)-reticuline to (S)-scoulerine by mediating an oxidative cyclization;^[19, 25] no enzyme in this family has been reported to be an aldolase. Thus the discovery of SthB as an intramolecular aldolase in the biosynthesis of **1** expands the catalytic diversity of this family of enzymes. Further mutagenesis, biochemical and structural characterization is required to understand the role (if any) of the FAD cofactor in the intramolecular aldol reaction.

Collectively, our results combined with earlier studies show that the multifunctional P450 SthF is responsible for electrophilic ketone formation through a combination of epoxidation and oxidation of the double bond. Significantly, a BBE-like enzyme SthB can catalyze an intramolecular aldol reaction between the nucleophilic aldehyde enolate and the electrophilic ketone group to form the complex bridged tricyclo[6.2.2.0^{2,7}]dodecane scaffold. The cytochrome P450 SthC then catalyzes the hydroxylation of C-15 to afford the final metabolite **1**. The biosynthetic pathway of **1** is highly concise given its structural complexity. To our knowledge, the discovery of SthB as an aldolase represents a first example of a dedicated aldolase in fungal secondary metabolite biosynthesis. This further illustrates the remarkably functional diversity of the BBE-like oxidases in fungi and the prospects of using these enzymes as beacons for new natural product discovery.

Acknowledgements

CCDC 1940177 and 1940179 contain the supplementary crystallographic data for this paper. The authors thank Dr. Alexandre N. Sobolev at the UWA Centre for Microscopy, Characterisation & Analysis (CMCA) for assistance with single crystal X-ray crystallography data acquisition and interpretation. Samuel Alsop is thanked for his assistance as an undergraduate research student. This study was supported by an Australian Research Council (ARC) Discovery Project grant (DP170100228). Y-HC is supported by an ARC Future Fellowship (FT160100233). HL and JH are supported by an Australian Government International Postgraduate Research Scholarship. NMR and X-ray diffraction data were collected at the CMCA.

Keywords: fungal polyketide • biosynthesis • aldol reaction • Berberine Bridge enzyme-like • aldolase

- [1] a) M. Brovotto, D. Gamenara, P. Saenz Méndez, G. A. Seoane, *Chem. Rev.* **2011**, *111*, 4346-4403; b) C. Palomo, M. Oiarbide, J. M. García, *Chem. Soc. Rev.* **2004**, *33*, 65-75; c) R. Mahrwald, *Springer: Dordrecht, The Netherlands* **2009**.
- [2] S. M. Dean, W. A. Greenberg, C.-H. Wong, *Adv. Synth. Catal.* **2007**, *349*, 1308-1320.
- [3] J. Mlynarski, J. Paradowska, *Chem. Soc. Rev.* **2008**, *37*, 1502-1511.
- [4] a) T. D. Machajewski, C.-H. Wong, *Angew. Chem. Int. Ed.* **2000**, *39*, 1352-

-
- 1375; b) W. A. Greenberg, A. Varvak, S. R. Hanson, K. Wong, H. Huang, P. Chen, M. J. Burk, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 5788-5793; c) J. K. Howard, M. Müller, A. Berry, A. Nelson, *Angew. Chem. Int. Ed.* **2016**, *55*, 6767-6770; d) J. Fang, D. Hait, M. Head-Gordon, M. Chang, *Angew. Chem. Int. Ed.*, **2019**, *58*, 11841-11845.
- [5] a) Y.-H. Chooi, Y. Tang, *J. Org. Chem.* **2012**, *77*, 9933-9953; b) J. F. Barajas, G. Shakya, G. Moreno, H. Rivera, D. R. Jackson, C. L. Topper, A. L. Vagstad, J. J. La Clair, C. A. Townsend, M. D. Burkart, S.-C. Tsai, *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, E4142-E4148.
- [6] a) A. G. Newman, C. A. Townsend, *J. Am. Chem. Soc.* **2016**, *138*, 4219-4228; b) J. Hu, F. Sarrami, H. Li, G. Zhang, K. A. Stubbs, E. Lacey, S. G. Stewart, A. Karton, Andrew M. Piggott, Y.-H. Chooi, *Chem. Sci.* **2019**, *10*, 1457-1465.
- [7] S.-S. Gao, T. Zhang, M. Garcia-Borràs, Y.-S. Hung, J. M. Billingsley, K. N. Houk, Y. Hu, Y. Tang, *J. Am. Chem. Soc.* **2018**, *140*, 6991-6997.
- [8] S. Manulis, Y. Kashman, D. Netzert, I. Barash, *Phytochemistry* **1984**, *23*, 2193-2198.
- [9] Y.-H. Chooi, P. S. Solomon, *Front. Microbiol.* **2014**, *5*, 640.
- [10] S. V. S. Ipcho, J. K. Hane, E. A. Antoni, D. A. G. Ahren, B. Henrissat, T. L. Friesen, P. S. Solomon, R. P. Oliver, *Mol. Plant Pathol.* **2012**, *13*, 531-545.
- [11] T. Ugai, A. Minami, R. Fujii, M. Tanaka, H. Oguri, K. Gomi, H. Oikawa, *Chem. Commun.* **2015**, *51*, 1878-1881.
- [12] Y.-H. Chooi, J. Fang, H. Liu, S. G. Filler, P. Wang, Y. Tang, *Org. Lett.* **2013**, *15*, 780-783.
- [13] Y.-M. Chiang, M. Ahuja, C. E. Oakley, R. Entwistle, A. Asokan, C. Zutz, C. C. Wang, B. R. Oakley, *Angew. Chem. Int. Ed.* **2016**, *55*, 1662-1665.
- [14] a) H. Li, J. Hu, H. Wei, P. S. Solomon, D. Vuong, E. Lacey, K. A. Stubbs, A. M. Piggott, Y.-H. Chooi, *Org. Lett.* **2018**, *20*, 6148-6152; b) Y. Tsunematsu, N. Ishikawa, D. Wakana, Y. Goda, H. Noguchi, H. Moriya, K. Hotta, K. Watanabe, *Nat. Chem. Biol.* **2013**, *9*, 818-825.
- [15] H.-C. Lin, Y. Tsunematsu, S. Dhingra, W. Xu, M. Fukutomi, Y.-H. Chooi, D. E. Cane, A. M. Calvo, K. Watanabe, Y. Tang, *J. Am. Chem. Soc.* **2014**, *136*, 4426-4436.
- [16] H.-C. Lin, T. C. McMahon, A. Patel, M. Corsello, A. Simon, W. Xu, M. Zhao, K. N. Houk, N. K. Garg, Y. Tang, *J. Am. Chem. Soc.* **2016**, *138*, 4002-4005.
- [17] A. Marchler-Bauer, S. Lu, J. B. Anderson, F. Chitsaz, M. K. Derbyshire, C. DeWeese-Scott, J. H. Fong, L. Y. Geer, R. C. Geer, N. R. Gonzales, M. Gwadz, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, F. Lu, G. H. Marchler, M. Mullokandov, M. V. Omelchenko, C. L. Robertson, J. S. Song, N. Thanki, R. A. Yamashita, D. Zhang, N. Zhang, C. Zheng, S. H. Bryant, *Nucleic Acids Res.* **2010**, *39*, D225-D229.
- [18] O. Dym, D. Eisenberg, *Protein Sci.* **2001**, *10*, 1712-1728.
- [19] T. M. Kutchan, H. Dittrich, *J. Biol. Chem.* **1995**, *270*, 24475-24481.
- [20] L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, M. J. E. Sternberg, *Nat. Protoc.* **2015**, *10*, 845.
- [21] S. M. Ma, J. W.-H. Li, J. W. Choi, H. Zhou, K. K. M. Lee, V. A. Moorthie, X. Xie, J. T. Kealey, N. A. Da Silva, J. C. Vederas, Y. Tang, *Science* **2009**, *326*, 589-592.
- [22] Y. Wu, C. Du, C. Hu, Y. Li, Z. Xie, *J. Org. Chem.* **2011**, *76*, 4075-4081.
- [23] M.-C. Tang, Y. Zou, K. Watanabe, C. T. Walsh, Y. Tang, *Chem. Rev.* **2017**, *117*, 5226-5333.
- [24] B. Daniel, B. Konrad, M. Toplak, M. Lahham, J. Messenlehner, A. Winkler, P. Macheroux, *Arch. Biochem. Biophys.* **2017**, *632*, 88-103.
- [25] a) H. Dittrich, T. M. Kutchan, *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 9969-9973; b) A. Winkler, F. Hartner, T. M. Kutchan, A. Glieder, P. Macheroux, *J. Biol. Chem.* **2006**, *281*, 21276-21285.
- ...
-