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## Fungal siderophore biosynthesis catalysed by an iterative nonribosomal peptide synthetase<sup>†</sup>

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Siderophores play a vital role in the viability of fungi and are essential for the virulence of many pathogenic fungal species. Despite their importance in fungal physiology and pathogenesis, the programming rule of siderophore assembly by fungal nonribosomal peptide synthetases (NRPSs) remains unresolved. Here, we report the characterization of the bimodular fungal NRPS, SidD, responsible for construction of the extracellular siderophore fusarinine C. The use of intact protein mass spectrometry, together with *in vitro* biochemical assays of native and dissected enzymes, provided snapshots of individual biosynthetic steps during NRPS catalysis. The adenylation and condensation domain of SidD can iteratively load and condense the amino acid building block *cis*-AMHO, respectively, to synthesize fusarinine C. Our study showcases the iterative programming features of fungal siderophore-producing NRPSs.

## Introduction

Iron is an essential element to life, but its bioavailability is very limited owing to the poor solubility of ferric iron in aerobic environments. Hence, virtually all living organisms have evolved sophisticated mechanisms for iron acquisition and storage.<sup>1,2</sup> Siderophores are low-molecular-weight, high-affinity iron chelators and play a central role in maintaining iron homeostasis in bacteria, fungi, and plants.<sup>3</sup> Two major hydroxamate-based peptidyl siderophores are employed by fungi: the depsipeptides, exemplified by fusarinine C (FSC, 1) and triacetyl fusarinine C (TAFC, 2),<sup>4</sup> and the coprogen family of siderophores (Fig. 1A).<sup>5</sup> These compounds are excreted primarily to capture ferric iron, assisting iron uptake. In contrast, the intracellular siderophores, represented by the ferrichrome family, are primarily used for iron storage although some fungal species have been observed to secrete such siderophores (Fig. 1A).<sup>6</sup> Both extra- and intracellular siderophores are indispensable for the virulence of many notorious pathogenic fungal species, such as rice blast fungus *Magnaporthe*

*oryzae* and the human opportunistic pathogen *Aspergillus fumigatus*.<sup>7</sup>

Despite the preponderance of siderophores in fungal physiology and pathogenesis, the enzymes involved in fungal siderophore biosynthesis remain largely uncharacterized, in particular the core modular nonribosomal peptide synthetases (NRPS) that are essential in assembling peptidyl siderophores.<sup>8,9</sup> Representative examples of these NRPSs include the FSC synthetase, SidD, the coprogen synthetase, Nps6, and the ferricrocin synthetase, SidC (Fig. 1).<sup>10–12</sup>

Features unique to these NRPSs can be readily identified from their characteristic domain organizations. Firstly, they typically have incomplete C-terminal modules missing a functional adenylation (A) domain, which suggests the thiolation (T) domain in these modules must be aminoacylated by A domains from the upstream modules. Specifically, in both SidD and Nps6, module 2 harbours degenerate A (dA) domains which are truncated at their N-termini by nearly 290 amino acid residues and are predicted to be catalytically inactive.<sup>13</sup> These dA domains are likely vestiges of evolution and only play a structural role within the NRPS. In comparison, module 4 and 5 in SidC have evolved to completely lack A domains. A second defining feature is the number and order of modules in these siderophore-producing NRPSs do not match the length and sequence of their peptide siderophore products, indicating a high degree of non-colinearity.<sup>13,14</sup> These features distinguish them from the well-characterized fungal iterative cyclodepsipeptide (CDP) NRPSs,<sup>15,16</sup> all of which harbour complete modules and assemble depsipeptide antibiotics (e.g. enniatin) consisting of repeated dipeptide units.

To understand the molecular mechanism of this unique family of NRPSs, we focused on the elucidation of SidD-catalysed biosynthesis of FSC. Here, employing

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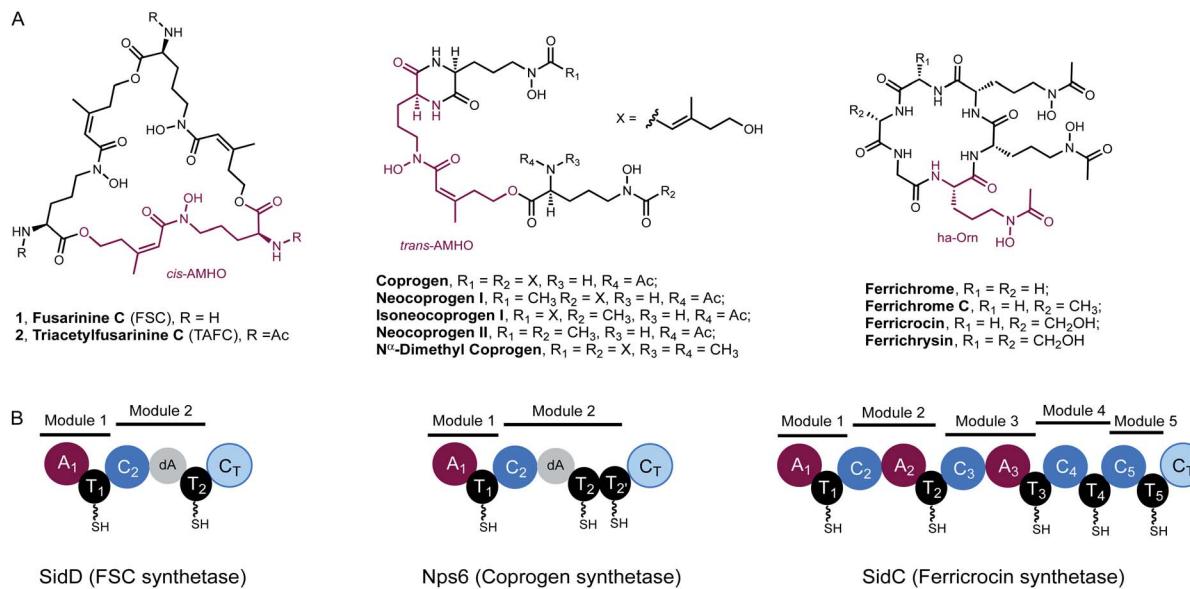
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**Fig. 1** Fungal hydroxamate siderophores and the domain organisation of NRPSs responsible for their biosynthesis. (A) Structures of extra- and intra-cellular siderophores shown in the desferri-form. In each case, a repeating hydroxamate unit has been highlighted in purple. TAFC and ferricrocin possess  $Fe^{3+}$  binding constants of  $10^{32}$  and  $10^{27}$ , respectively. (B) NRPS domain organisation of SidD, Nps6 and SidC (left to right). In SidD and Nps6, the dA notation represents a degenerated A domain. In SidC, module 4 and module 5 completely lack A domains.

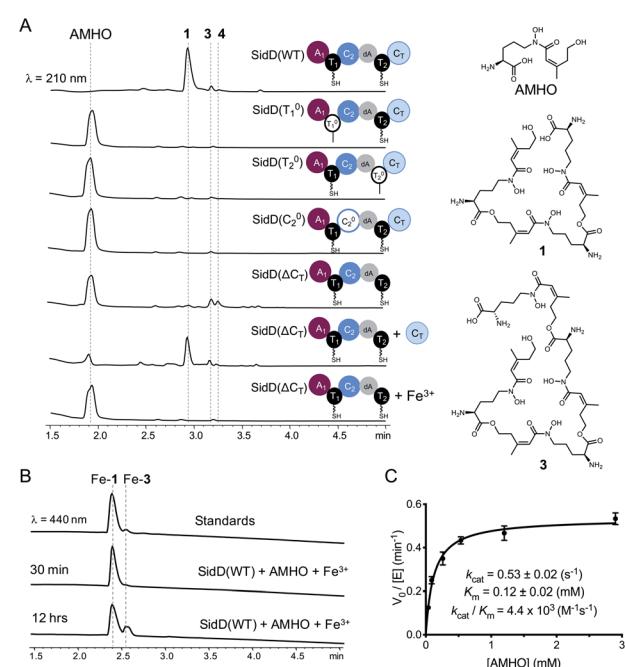
a combination of *in vitro* biochemical assays and intact protein mass spectrometry (MS), we show how the bimodular NRPS, SidD, is precisely programmed to iteratively assemble the trimeric siderophore FSC.

## Results and discussions

To decipher the mechanism of SidD, we purified recombinant SidD and its mutants (designed to dissect the domain function, *vide infra*) from *Saccharomyces cerevisiae* JHY686 strain (Fig. S1†).<sup>17</sup> To ensure protein samples were all in the *holo*-form, purified SidD variants were enzymatically phosphopantetheinylated using the phosphopantetheinyl transferases NpgA (*A. nidulans*) or Sfp (*B. subtilis*), as described previously.<sup>18–21</sup> The substrate, *N*<sup>5</sup>-*cis*-anhydromevalonyl-*N*<sup>5</sup>-hydroxy-L-ornithine (*cis*-AMHO) was prepared by alkaline hydrolysis of **1**.<sup>22</sup> We isolated **1** from an *A. nidulans* mutant in which the acetyltransferase gene *sidG* was knocked out to prevent acetylation of **1** to give **2** *in vivo* (ESI methods†).<sup>12</sup> In the presence of ATP and  $Mg^{2+}$ , wild-type SidD could synthesize desferri-**1** utilizing *cis*-AMHO as building blocks (Fig. 2A), while ferri-**1** was observed when  $Fe^{3+}$  was present in the substrate solution (prepared from hydrolysis of ferri-FSC without removing  $Fe^{3+}$  ions). The product identity was confirmed by comparison to a chemical standard of ferri-**1** on LC-MS (Fig. 2B). The reaction has an apparent  $K_M$  of  $120\ \mu M$  for *cis*-AMHO and an overall  $k_{cat}$  of  $0.5\ s^{-1}$  (Fig. 2C). Trace amounts of linear-FSC (**3**) were observed in both the authentic standard and the reaction mixture. This is likely the result of slow non-enzymatic hydrolysis of the ester bond in **1** under the assay conditions (phosphate buffer, pH 7.8), or in the reaction mixture, where additional linear-FSC (**3**) could arise from both

ester hydrolysis or non-enzymatic thioester hydrolysis of the *cis*-AMHO trimer appended to the SidD protein.

Successful reconstitution of SidD activity *in vitro* allowed us to further dissect its domain function. Both thiolation (T)



**Fig. 2** Comparison of *in vitro* enzymatic assays for wild-type SidD and mutational variants. (A) HPLC traces of the enzymatic reactions for different SidD variants. The domain architectures of the variants are displayed. Mass spectral data of each peak are shown in Fig. S2–S9.† (B) Comparison of HPLC traces for the enzymatic products (in the ferri-form) with chemical standards. (C) Steady-state kinetics of wild-type SidD.

domains are essential for SidD activity, as disabling either T domain by mutating the key serine that serves as the phosphopantetheinyl group attachment site (S801A and S1594A, designated as  $T_1^0$  and  $T_2^0$ , respectively), completely abolished FSC formation. Inactivating the condensation (C) domain from module 2 by mutating the catalytic histidine residue (H999A, designated as  $C_2^0$ ) also resulted in no product formation. Interestingly, deletion of the C-terminal condensation domain ( $\Delta C_T$ ) yielded two shunt products: linear-FSC (3) and a new compound, 4, whose molecular weight, retention time, and ability to chelate  $Fe^{3+}$  suggested a linear tetrameric derivative of FSC (Fig. 2A and S2–S9†). Addition of equimolar standalone  $C_T$  domain restored FSC synthetase activity of SidD( $\Delta C_T$ ) mutant, albeit with lower efficiency compared to wild-type. These results together indicate that cyclization, but not the chain-elongation reaction, is compromised by the  $\Delta C_T$  truncation mutant, whereas chain-elongation is prevented by the  $C_2^0$  mutant. This result is consistent with the general programming rule that,  $C_T$  domains found in fungal NRPS usually offload the peptidyl products through cyclization.<sup>23</sup> This is in contrast to the role played by  $C_T$  domains in CDP NRPSs, which have been shown to catalyse chain elongations in addition to cyclizations.<sup>15,16</sup>

To obtain further insights into the function of  $C_2$  and  $C_T$  in SidD, we prepared *cis*-AMHO-derived aminoacyl-*N*-acetylcysteamine thioester substrates and assayed individual C domain activity *in vitro*.<sup>24</sup> However, this approach was impeded by the instability of *cis*-AMHO and its derivatives: the *cis*-AMHO moiety is prone to degradation and yields *N*<sup>5</sup>-hydroxy-L-ornithine and  $\Delta^2$ -anhydro-mevalonate lactone under acidic conditions (Fig. S10†).<sup>25,26</sup> We therefore turned to intact protein MS to provide snapshots of SidD during the assembly of FSC. This approach has been used successfully to study the enzymology of simplified polyketide synthases (PKS) and NRPS model systems.<sup>27–29</sup> Here we applied this technique to capture the steps of an iterative biosynthetic assembly line.

In order to visualize enzyme-bound intermediates using intact protein MS, we chose the SidD( $\Delta C_T$ ) mutant instead of wild-type SidD. Based on our previous biochemical assays (Fig. 2), this mutant assembly line is stalled without the releasing domain ( $C_T$ ). This leads to accumulation of intermediates that can be captured by intact protein MS. Accordingly, *in vitro* reactions of SidD( $\Delta C_T$ ) were subject to UHPLC-ESI-Q-TOF-MS analyses (Methods see ESI†). Taking *holo*-SidD( $\Delta C_T$ ) as a starting point, after providing *cis*-AMHO and ATP as substrates for 15 min, we observed a new species from the deconvoluted mass spectrum (Fig. 3A). This new species corresponded to one *cis*-AMHO unit tethered to the assembly line (designated as intermediate I), as indicated by the mass shift of +242 Da relative to the mass of SidD( $\Delta C_T$ ) in the *holo*-form. Rather than building up more intermediates, prolonged incubation (1 h) resulted in complete disappearance of intermediate I (Fig. 3A).

When we repeated this analysis using  $Fe^{3+}$ -containing *cis*-AMHO substrates, we observed a new species with a +1021 Da mass shift relative to *holo*-SidD( $\Delta C_T$ ), corresponding to loading of four *cis*-AMHO units onto the assembly line plus a chelated ferric ion (designated as intermediate IV). We interpreted this

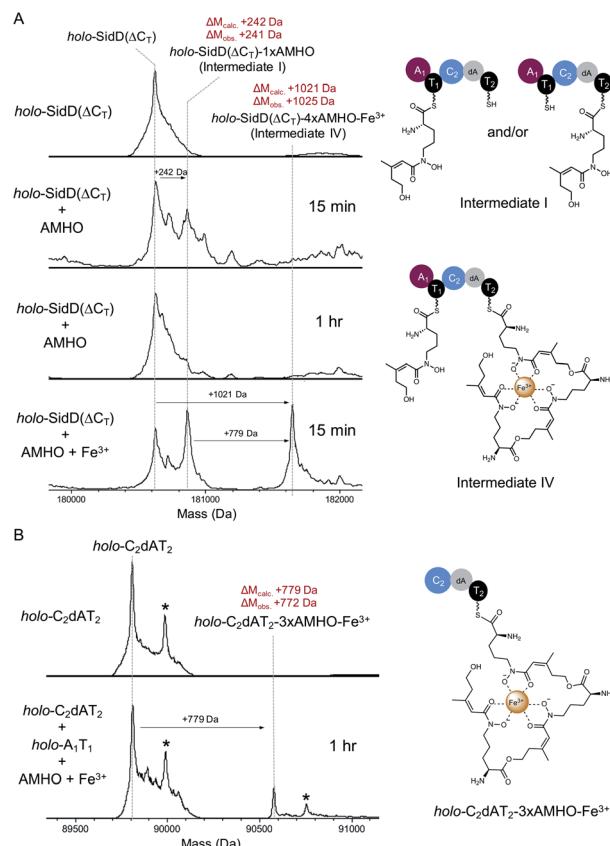


Fig. 3 Snapshots of late-stage biosynthetic intermediates. Deconvoluted intact protein mass spectra of (A) SidD( $\Delta C_T$ ) mutant acting *in cis* (B) split SidD( $\Delta C_T$ ) mutant ( $C_2$ dAT<sub>2</sub> + A<sub>1</sub>T<sub>1</sub>) acting *in trans*. Mass shifts corresponding to biosynthetic steps are highlighted with arrows, and proposed intermediates are displayed. Peaks labeled with asterisks indicate N-terminal gluconylation, a known post-translational modification of recombinant heterologous proteins in *E. coli*.<sup>11</sup> Exact measured and observed masses are detailed in Table S5.†

intermediate as most likely T<sub>1</sub> charged with one *cis*-AMHO while T<sub>2</sub> tethered with a linear trimeric depsipeptide coordinating to a  $Fe^{3+}$  ion (Fig. 3A). However, because SidD has two phosphopantetheinyl (Ppant) arms, it is uncertain as to which T domain each proposed peptidyl intermediate is tethered and different combinations of intermediates can be envisaged (e.g. dimeric depsipeptides appended to both T<sub>1</sub> and T<sub>2</sub>, with only one of these dimeric depsipeptide chelating the  $Fe^{3+}$  ion). To resolve this ambiguity, we performed a similar assay, using a split version of the SidD construct (A<sub>1</sub>T<sub>1</sub> + C<sub>2</sub>dAT<sub>2</sub>) in which module 1 and module 2 must interact *in trans*, such that the mass changes on individual Ppant arm can be tracked unambiguously. As shown in Fig. 3B, a +779 Da mass shift relative to the mass of C<sub>2</sub>dAT<sub>2</sub> in the *holo*-form was clearly observed for a new species corresponding to three *cis*-AMHO units attached to C<sub>2</sub>dAT<sub>2</sub> plus a coordinated  $Fe^{3+}$  ion. The low population of this new species may be attributed to less efficient crosstalk between two modules acting *in trans* as compared to the intact bimodule construct. Nevertheless, this result confirmed our interpretation of intermediate IV, and these snapshots together strongly support our proposal that  $C_T$  domain is solely responsible for



cyclization and product release, and is not involved in chain elongation steps as seen in fungal CDP NRPSs.<sup>15,16</sup>

Successful trapping of the trimeric intermediate **IV** achieved only in the presence of  $\text{Fe}^{3+}$  ions indicates that shielding of the hydroxamate moieties from the oligomeric peptidyl intermediate through coordination to  $\text{Fe}^{3+}$  ion can suppress the nonenzymatic thioester cleavage leading to **3** (and **4**). Indeed, when  $\text{Fe}^{3+}$ -containing *cis*-AMHO was supplied as substrates, no product was observed in our LC-MS analysis (Fig. 2A), which is consistent with the assembly line stalled at the stage of intermediate **IV**. It is likely that in the absence of  $C_T$  domain, a nearby free hydroxamate group may act as a general base to assist direct hydrolytic cleavage of the scissile thioester bond leading to spontaneous release of the peptidyl intermediates **3** and **4**; or help cleave the thioester bond through an intramolecular displacement mechanism driven by its nucleophilicity (Fig. S11†).

Encouraged by the successful visualization of intermediate **IV**, we next applied this methodology to intercept intermediates at early stages by further inactivating  $T_1$  and  $C_2$  domains from the  $\text{SidD}(\Delta C_T)$  mutant, respectively. When the reaction mixture of  $\text{SidD}(\Delta C_T, C_2^0)$  mutant was subject to MS analysis, two species were observed (Fig. 4A). One species represents intermediate **I** while the other one has an additional mass shift of +242 Da relative to intermediate **I**, which indicates addition of totally two *cis*-AMHO units to  $\text{SidD}$  (designated as intermediate **II**).

Since no dipeptidyl-shunt products were observed in our *in vitro* assays, intermediate **II** was best interpreted as one *cis*-

AMHO unit tethered on each  $T$  domain. This interpretation is also consistent with the proposed role of  $C_2$ ; namely that it  $C_2$  carries out chain-elongation reactions. When the assay mixture of  $\text{SidD}(\Delta C_T, T_1^0)$  mutant was analysed, only one major species corresponding to one *cis*-AMHO unit tethered was observed (Fig. 4B). Since now only  $T_2$  has the obligatory  $\text{P}_{\text{Pant}}$  arm for substrate tethering, this covalently-bound monomeric *cis*-AMHO unit must be attached to  $T_2$  (intermediate **I'**). This result also unambiguously proves that direct intermodular substrate loading between  $A_1$  and  $T_2$  has occurred and bypassed  $T_1$ . Thus, the queuing model hypothesis, in which loading of  $T_2$  is  $T_1$ -dependent (activated *cis*-AMHO substrate is transferred from  $T_1$  to  $T_2$  through transthioesterification), can be ruled out (Fig. S12†).

Taken together, we propose a working model for  $\text{SidD}$  (Fig. 5). The only functional  $A$  domain from module 1 ( $A_1$ ) must aminoacylate  $T$  domains from both modules. However, whether loading of each module is in an ordered or random sequence remains unresolved. Once both  $T$  domains are charged with *cis*-AMHO, chain elongation will be catalysed by  $C_2$  to give a dimeric *cis*-AMHO intermediate (tethered to  $T_2$ ). Meanwhile, the vacant  $T_1$  will be charged with another *cis*-AMHO unit by  $A_1$ . The  $C_T$  domain is likely to dictate the chain-length by favouring a 36-membered ring closure cyclization reaction on a trimeric intermediate bound on  $T_2$ . Similar chain-length determining role of  $C_T$  has been proposed in CDP NRPSs.<sup>15,16</sup> Hence, if the dimer intermediate reaches  $C_T$ , it will be rejected and return to  $C_2$  to condense another *cis*-AMHO unit from  $T_1$ , yielding the desired on-pathway trimer intermediate that can be offloaded by  $C_T$  through cyclization to yield FSC (1). It is noteworthy that in this biosynthetic model, the  $C_2$  domain must accommodate substrates of different chain lengths appended to  $T_2$  for each of the condensation events (*i.e.* monomeric *cis*-AMHO vs. dimeric *cis*-AMHO). Although this scenario would require the  $C_2$  domain to catalyse chain elongation using different acyl-acceptors, *i.e.* the terminal hydroxyl moiety from substrates of vastly different chain lengths (12-membered vs. 24-membered), similar iterative use of  $C$  domain has been proposed for asperlicin biosynthesis.<sup>30</sup>

Our proposed working model could also explain the formation of shunt products **3** and **4**. In the absence of  $C_T$ , the  $\text{SidD}$  assembly line will progress and stall when the trimer intermediate is reached. Hydroxamate-assisted, slow non-enzymatic thioester cleavage will occur, eventually releasing the stalled intermediate as shunt product **3**. Furthermore, the long-lived trimer intermediate could now have a chance to complete an aberrant chain-elongation reaction presumably at  $C_2$  with a rate comparable to that of the hydrolytic releasing of **3**, which could explain the formation of derailed product **4** (Fig. S13†). Formation of the longer-than-desired product **4** in the absence of  $C_T$  further reinforced the idea that  $C_T$ , rather than  $C_2$  is the chain-length determining domain. Offloading either **3** or **4** will free up  $T_2$  and enable multiple turnovers of the substrate.

A subtle variation on this pathway could also be envisioned, in which the dimeric *cis*-AMHO intermediate bound to  $T_2$  following the first condensation event is “back transferred” onto  $T_1$  via a *trans*-thioesterification step, followed by loading of

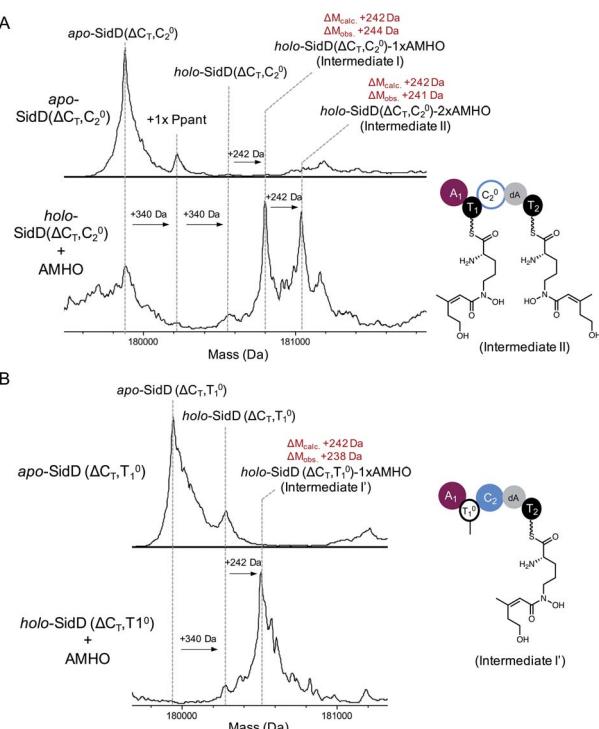
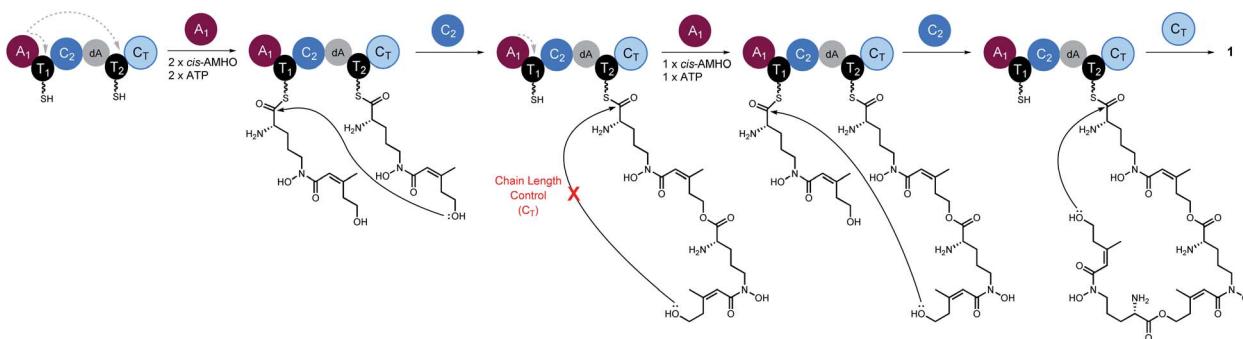


Fig. 4 Snapshots of early-stage biosynthetic intermediates. Deconvoluted intact protein mass spectra of (A)  $\text{SidD}(\Delta C_T, C_2^0)$  mutant and (B)  $\text{SidD}(\Delta C_T, T_1^0)$  mutant. Exact measured and observed masses are detailed in Table S5.†





**Fig. 5** Proposed biosynthetic model for SidD-catalysed formation of FSC (1). Grey dashed arrows indicate productive interactions between the  $A_1$  domains and the  $T_1/T_2$  domains. Our data suggest that the  $C_T$  domain, not the central  $C_2$  domain, determines the chain-length of the nascent peptidyl chain permitted for cyclisation. A subtle variation of this model involving a chain back-transfer step cannot be ruled out and is outlined in Fig. S13.†

$T_2$  with another *cis*-AMHO unit. This would mean that  $C_2$  always utilises monomeric *cis*-AMHO from the  $T_2$  domain (Fig. S14†). Although such “chain back-transfer” step cannot be ruled out by our current data, it is unprecedented for NRPS enzymology. It is worth noting, that although “pass-back chain extension” has been observed in fungal CDP NRPSs and bacterial PKS–NRPS hybrids,<sup>15,31</sup> it is different from the “chain back-transfer” proposed here. The aforementioned, “chain back-transfer” does not involve chain elongation, whilst “pass-back chain extension” is essentially a chain elongation step except the direction is not forward. This difference also renders SidD distinct from the closely related CDP NRPSs. The  $C_T$  domains from CDP NRPSs not only catalyze chain cyclization to offload the final cyclicdepsipeptides, but also catalyses “pass-back chain extension” to enable iterative use of the whole assembly line; whereas the  $C_T$  domain from SidD is solely involved in cyclization since elongated products are still observed in the absence of  $C_T$ .

Our mechanistic study of SidD also provides valuable insights into the biosynthesis of other fungal siderophores, in particular, the coprogen-type siderophores. Nps6 shares several striking similarities with SidD: (1) they adopt almost identical domain architectures (except in Nps6 tandem repeated T domains are present in module 2; and (2) they utilize very similar building blocks (SidD activates *cis*-AMHO while Nps6 activates *trans*-AMHO), yet they make distinct products: coprogen is a partially linear depsipeptide while FSC is a *bona fide* macrocycle. Based on our study of SidD, we expect that Nps6 should iteratively use  $A_1$  to aminoacylate T domains in both of its modules (*i.e.* three T domains in total). The tandem T domains in Nps6 module 2 are likely employed to increase overall flux.<sup>32</sup> Assuming the  $C_T$  domain in Nps6 also catalyses cyclization and chain release to forge the diketopiperazine scaffold, then the  $C_2$  domain must be iteratively used to account for the extra chain elongation in this bimodular NRPS. Intriguingly, if this proposal is correct, the  $C_2$  domain from Nps6 would be a C domain with dual-function; catalysing both ester bond and amide bond formation. We hypothesise that when the Nps6  $C_2$  domain catalyses a second chain elongation event, it attaches  $T_1$ -tethered *trans*-AMHO to the amino group of  $T_2$ -tethered *trans*-AMHO dimers instead of the hydroxy group at

the end of the growing chain as proposed for the SidD  $C_2$  domain. Then a  $C_T$ -catalysed intra-molecular cyclisation would simultaneously yield the diketopiperazine moiety and release the coprogen product from the NRPS (Fig. S15†). Although this biosynthetic proposal is consistent with our working model for SidD, the exact molecular mechanism of Nps6 undoubtedly warrants more in-depth biochemical investigation in the future.

## Conclusions

We have provided evidence supporting an iterative NRPS mechanism for SidD, including an iterative A domain, iterative chain-elongation C domain, and a chain-releasing  $C_T$  domain catalysing cyclization and determining product chain-length, respectively. The iterative use of A domains to incorporate repeat units of hydroxamate amino acids is a hallmark for fungal siderophore-producing NRPS and a remarkable strategy to improve atom economy for biosynthetic assembly-lines. Understanding the molecular mechanism underpinning such programmed and highly efficient iterative domain usage may guide future design and engineering of novel and smaller NRPS assembly-lines.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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