Siderophore Synthetase DesD Catalyzes N-to-C Condensation in Desferrioxamine Biosynthesis

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ABSTRACT: Desferrioxamine siderophores are assembled by the nonribosomal-peptide-synthetase-independent-siderophore (NIS) synthetase enzyme DesD via ATP-dependent iterative condensation of three N^1-hydroxy-N^1-succinyl-cadaverine (HSC) units. Current knowledge of NIS enzymology and the desferrioxamine biosynthetic pathway does not account for the existence of most known members of this natural product family which differ in substitution patterns of the *N*- and *C*-termini. The directionality of desferrioxamine biosynthetic assembly, N-to-C vs C-to-N, is a longstanding knowledge gap that is limiting further progress in understanding the origins of natural products in this structural family. Here, we establish the directionality of desferrioxamine biosynthesis using a chemoenzymatic approach with stable isotope incorporation and dimeric substrates. We propose a mechanism where DesD catalyzes the *N*-to-*C* condensation of HSC units to establish a unifying biosynthetic paradigm for desferrioxamine natural products in *Streptomyces*.

The desferrioxamines (DFOs) are a family of tri-hydroxamate siderophores produced by most actinomycetes.[1] The generic trimeric DFO scaffold is composed of repeating units of *N*-hydroxy-cadaverine joined via succinate groups resulting in either macrocyclic or linear variants. The linear structures vary by acylation patterns of the *N*- and *C*-termini.[2,3] Despite the widespread study of DFOs, there is still no unified biosynthetic paradigm accounting for the existence of all acylated DFO analogs.[4] Challis and coworkers identified the highly conserved *desABCD* biosynthetic operon responsible for DFO production.[5] The central biosynthetic intermediate N^1-hydroxy-N^1-succinyl-cadaverine (HSC) derives from L-Lys (some derive from L-Orn leading to incorporation of putrescine instead of cadaverine) via tandem action of DesA

(decarboxylase), DesB (*N*-monooxygenase), and DesC (*N*-acyltransferase).[6,7] DesD is a Nonribosomal-peptide-synthetase-Independent Siderophore (NIS) synthetase that catalyzes the ATP-dependent iterative condensation of HSC units where each HSC unit provides a *C*-terminal carboxyl and *N*-terminal amino group necessary for peptide bond formation.[8–11] Here, we sought to determine the inherent capacity of DesD to incorporate HSC derivatives with control of directionality in the biosynthetic assembly of DFO analogs.

A coupled enzyme assay was used by Challis and coworkers to demonstrate that DesD and AcsD NIS synthetases generate AMP as a byproduct from the adenylation of HSC and citrate, respectively.[8,12] Our group recently validated HSC acyl adenylate (HSC-AMP) as an intermediate in the DesD catalytic cycle using an HSC acyl sulfamoyl adenosine analog (HSC-AMS) which mimics the structure of HSC-AMP.[13] Presumably, the activated *C*-terminus of HSC-AMP is oriented favorably in the DesD active site for condensation with the free amino *N*-terminus of a second HSC molecule to form an HSC-HSC dimeric intermediate. Given that HSC serves as both the adenylation and condensation substrate for this dimerization it is essentially 'directionless'. To form a trimeric DFO product composed of HSC-HSC-HSC, also known as DFOG, DesD must perform an additional round of adenylation and condensation where there is inherent 'directionality' to the reaction sequence. Both HSC-HSC dimer and HSC monomer present free *C*- and *N*-termini as sites of possible adenylation and condensation, respectively. Adenylation of HSC-HSC dimer would result in so-called '*N*-to-*C*' directionality, while adenylation of HSC monomer as in the first round of catalysis would result in '*C*-to-*N*' biosynthetic assembly (**Figure 1**).

To distinguish between '*N*-to-*C*' and '*C*-to-*N*' directions in DesD catalysis, we synthesized an HSC-HSC dimer (**Figure S1**). We performed *in vitro* enzymatic reactions with four DesD orthologs using the HSC-HSC dimer and a ^{13}C-labeled HSC monomer as substrates with inclusion of excess ATP and Mg^{2+}. The four DesD orthologs were cloned from *Streptomyces coelicolor* A3(2) (ScDesD), *Streptomyces griseoflavus* DSM 40698 (SgDesD), *Streptomyces pilosus* JCM 4403 (SpDesD), and *Streptomyces violaceus* DSM 8286 (SvDesD) which all produce unique DFO natural products.[13] The ^{13}C-labeled HSC was derived *in situ* from L-Lys-$^{13}C_6$ (Cambridge Isotopes) by inclusion of DesABC along with necessary cofactors (PLP, FAD) and cosubstrates (succinyl-CoA, O_2, NADPH) (**Figure S2**). We analyzed the complete DesABCD-catalyzed reactions by ESI LC-MS/MS to leverage mass fragmentation as a method to distinguish between the two possible trimeric products – HSC-HSC-($^{13}C_5$-HSC) (*N*-to-*C* direction) or ($^{13}C_5$-HSC)-HSC-HSC (*C*-to-*N* direction) (**Figure 2ab**). The MS^2 spectra for all DesD-catalyzed reactions produced base peaks with apparent *m/z* of either 319 or 306 which corresponds exclusively to fragmentation of the HSC-HSC-($^{13}C_5$-HSC) product via N–C bond cleavage at the central hydroxamate amide bond (**Figure 2bc**). These data support that DesD catalyzes adenylation of HSC-HSC to

produce HSC-HSC-AMP followed by condensation with an HSC monomer in the *N*-to-*C* direction in the biosynthetic assembly of DFOG.

Next, we designed a series of experiments to determine if the *N*-to-*C* biosynthetic directionality holds for DFOB (primary product from *S. pilosus*) which is composed of two units of HSC and one unit of N^1-hydroxy-N^1-acetyl-cadaverine (HAC).[14] Similar to HSC, HAC is derived from L-Lys via DesABC-catalyzed reactions where DesC employs acetyl-CoA instead of succinyl-CoA for acyl group transfer.[7] We synthesized the HAC monomer (**Figure S1**) and used this as a substrate in a series of DesD-catalyzed reactions. First, we combined HSC and HAC monomers in a variety of ratios ranging from 10:1 to 1:10 (HSC:HAC) in the presence of ScDesD, SgDesD, SpDesD (native DFOB producer), and SvDesD (only data for 1:1 ratio is shown in **Figure S3**). All combinations of HSC and HAC resulted in exclusive production of DFOG (HSC-HSC-HSC) and DFOE (macrolactam product derived from DFOG) with no appearance of DFOB (HSC-HSC-HAC) as judged by LC-MS analysis of the reaction mixtures.

Given this surprising result, we synthesized the HSC-HAC dimer (**Figure S1**) and attempted to produce DFOB by the DesD-catalyzed reaction with HSC monomer (**Figure 3a**). The lack of a carboxy terminus in HSC-HAC forces exclusive adenylation of HSC by DesD. The combination of HSC-HAC and HSC failed to produce DFOB detectable by LC-MS and once again produced DFOG and DFOE as the only detectable products. We next attempted to form DFOB using HSC-HSC dimer and HAC monomer as substrates for DesD (**Figure 3b**). Under these substrate conditions, we were finally able to observe DesD-catalyzed formation of DFOB (HSC-HSC-HAC) by LC-MS. To our surprise, we also observed the formation of a tetrameric product DFOG-HSC (HSC-HSC-HSC-HSC, ESI HR-MS calculated *m/z* for $C_{36}H_{67}N_8O_{13}^+$ $[M+H]^+$ 819.4822, observed 819.4829) and its corresponding macrolactam cyclic derivative, c-DFOG-HSC (ESI HR-MS calculated *m/z* for $C_{36}H_{65}N_8O_{12}^+$ $[M+H]^+$ 801.4716, observed 801.4722). Interestingly, we did not observe these tetrameric products when combinations of HSC-HSC dimer and HSC monomer were used as substrates for DesD (**Figure S4**). However, a combination of HSC-HSC and HSC-HAC dimers produced all possible tetrameric products including DFOG-HSC, c-DFOG-HSC, and DFOG-HAC (ESI HR-MS calculated *m/z* for $C_{34}H_{65}N_8O_{11}^+$ $[M+H]^+$ 761.4767, observed 761.4773) (**Figure S5**). Additionally, treatment of SgDesD with HSC-HSC as the only substrate initially produced DFOG-HSC followed by the time-dependent cyclization to c-DFOG-HSC (**Figure S6**). The formation of DFOB and various tetrameric products from substrate combinations of HSC-HSC with HAC and HSC-HSC with HSC-HAC is consistent with a preference for DesD-catalyzed adenylation of HSC-HSC followed by condensation with amine nucleophiles in the *N*-to-*C* biosynthetic direction.

Our findings in this letter are consistent with the biosynthetic directionality originally proposed (but not supported by any experiments such as those reported here) by Challis and coworkers,[8] but seem to contradict some of the conclusions from previous studies reported by Codd and coworkers.[14,15,16] However, we do believe our findings are in fact consistent with the data reported in all of these published works. For example, Codd and coworkers used precursor directed biosynthesis in cultures of *S. pilosus* supplemented with substituted diamines such as 1,4-diamino-2(*E*)-butene to track the sites of incorporation in DFO products and biosynthetic intermediates including dimers.[15] The analysis of labeling patterns in resulting DFOB analogs revealed a preference for incorporation of the 1,4-diamino-2(*E*)-butene group at the terminus of the product with minimal product incorporating this as the central unit. An analysis of the relative concentrations of biosynthetic intermediates revealed a significant accumulation of HSC-HAC dimer leading the authors to the conclusion that this dimer is on pathway towards DFOB in a *C*-to-*N* direction of adenylation and condensation by DesD. Here, we show that HSC-HAC is not a preferred substrate for DesD and hence its accumulation in *S. pilosus* cultures could be attributed to this lack of flux through the biosynthetic pathway towards the primary product, DFOB in this case. The cell-based precursor-directed biosynthetic feeding studies with 1,4-diamino-2(*E*)-butene resulting in labeling of the DFOB terminus is fully consistent with a requirement for *N*-to-*C* biosynthetic assembly (**Figure S7**). Further, Böttcher and coworkers found that the distance of the hydroxamate from the carboxyl terminus is important for substrate adenylation while the distance of the terminal amino group from the hydroxamate was important for substrate condensation. Such analysis helps to rationalize how the 1,4-diamino-2(*E*)-butene derivative might be a substrate for adenylation and subsequent condensation with HSC or HAC, but may not serve as the nucleophilic amine in the condensation step (**Figure S8**).[17]

DesD is unique compared to other NIS enzymes in the ability to iteratively condense HSC units and incorporate variable monomers including HSC and HAC. Additionally, the ability of DesD to produce tetrameric DFOG-HSC and c-DFOG-HSC products supports a high degree of enzyme plasticity. In the presence of HSC monomer under all conditions explored in this study, DesD favors trimerization to linear DFOG and macrocyclic DFOE (apparent thermodynamic end product). This product preference implies a model of 'volume control' for DesD that is supported by calculating the apparent 'closed' active site volume of DesD (7TGM) when bound to an HSC acyl adenylate mimic, 620 $Å^3$ (CAVER web v1.1), which matches and slightly outsizes the apparent ligand volume of DFOE, 586 $Å^3$ (MoloVol v1.0.0) (**Figure S9**).[18,19] Applying this method to other NISs appears to further support a 'volume control' model for catalysis where AcsD (2W03), active site volume 240 $Å^3$ (CAVER web v1.1), is responsible for condensing a citrate acyl adenylate with the hydroxyl group side chain of L-Ser to form an *O*-citryl-Ser ester with 200 $Å^3$ (MoloVol v1.0.0) ligand volume.[12]

The accumulation of HSC-HSC dimer with depleted concentrations of HSC appears to be essential in creating a window of opportunity for DesD to utilize HAC as a substrate. This consideration of 'cellular substrate pool' availability has been proposed by Böttcher and coworkers.[17,20] The control of metabolic flux and relative concentrations of biosynthetic precursors/intermediates could play a role in the incorporation of unique termini into DFO scaffolds in the microbial cell. Central to this paradigm is the formation of an HSC-HSC acyl adenylate intermediate, HSC-HSC-AMP, that is captured by either HSC or HAC for *N*-to-*C* type condensation producing DFOG or DFOB, respectively (**Figure 4**). While both HSC and HSC-HSC are presumably adenylated by DesD, it is clear from LC-MS (**Figures 2**, **3**) and AMP-release assays (**Figure S10**) that HSC is the preferred nucleophile over HSC-HSC. The increased dwell time of the HSC-HSC-AMP intermediate could allow for the intercepting of alternate nucleophilic substrates like HAC during formation of DFOB. Unanswered questions remain including how strains like *S. pilosus* control the metabolic pool to favor condensation of HSC-HSC dimer and HAC monomer to form DFOB when it seems the favored product of SpDesD *in vitro* is trimerization of HSC providing DFOG/DFOE.[21,22] Additionally, there are likely secondary genes/operons present in microbial producers that contribute to post-NIS tailoring of DFO analogs, including acylation of termini. One such gene, *desG*, encodes for an amidase and has a high rate of co-appearance with *desABCD* in DFO producing microbes.[1] There are likely additional operons involved in regulation and biosynthetic tailoring of DFO analogs in *Streptomyces*,[23] including genes in *S. violaceus* and *S. griseoflavus* that are associated with biosynthesis and conjugation of the structural complex antibiotic moieties found in the salmycin and ferrimycin DFO natural products.[13] The establishment of a unified biosynthetic directionality for DFO assembly in *Streptomyces* settles a longstanding knowledge gap in natural product biosynthesis. The characterization of more NIS synthetases from diverse genus and strains is needed to determine if biosynthetic directionality is a conserved evolutionary feature of this enzyme family. This study and future studies of this type will inform genome mining efforts and mechanistic study of NIS-associated siderophore natural products.[24]

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. The following items are included in the Supporting Information document:

general experimental procedures for compound synthesis and enzyme assays; synthetic schemes, LC-MS purity analysis, and NMR data for compounds **2**-**15**; tables of strains, plasmids, gene sequences, and protein sequences used in this study; LC-MS chromatograms for product formation assays, AMP formation assays, and molecular modeling of ligands in DesD active site.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; BGC, biosynthetic gene cluster; DFO, desferrioxamine; DFOB, desferrioxamine B; DFOE, desferrioxamine E; DFOG, desferrioxamine G; DFOG-AMP, acyl adenylate of DFOG; DFOG-AMS, DFOG acyl sulfamoyl adenosine; DFOG-HAC, tetramer composed of DFOG and HAC monomer; DFOG-HSC, tetramer of HSC; c-DFOG-HSC, cyclic tetramer of HSC; EIC, extracted ion chromatogram; ESI, electrospray ionization; FAD, flavin adenine dinucleotide; HAC, N^l-hydroxy-N^l-acetyl-cadaverine; HSC, N^l-hydroxy-

N^1-succinyl-cadaverine; HSC-AMP, acyl adenylation of HSC; HSC-AMS, HSC acyl sulfamoyl adenosine; HSC-HAC, dimer of HSC and HAC; HSC-HSC, dimer of HSC; HSC-HSC-AMP, acyl adenylate of HSC-HSC; HSC-HSC-AMS, HSC-HSC acyl sulfamoyl adenosine; HSC-HSC-HAC, trimer composed of HSC-HSC dimer and HAC monomer also known as DFOB; HSC-HSC-HSC, trimer of HSC also known as DFOG; LC-MS, liquid chromatography mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NIS, nonribosomal peptide independent siderophore; PDB, protein data bank; PLP, pyridoxal phosphate; P_i, phosphate; PP_i, pyrophosphate

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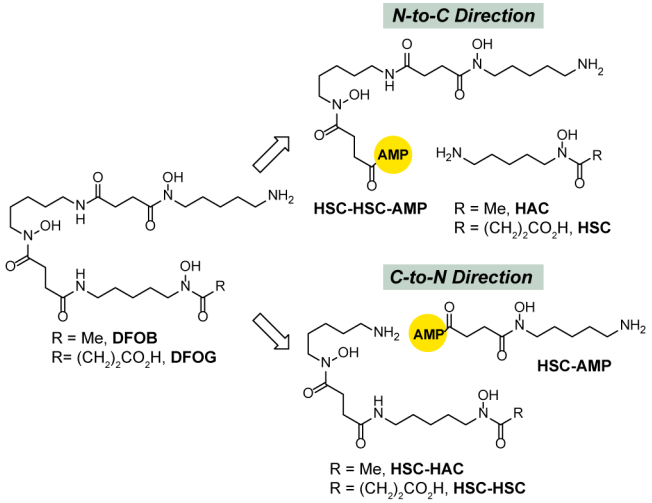

Figure 1. Chemical structures of representative DFO natural products, DFOB and DFOG, with retrosynthetic analysis highlighting nomenclature and acyl adenylates for possible DesD-mediated condensation directions, *N*-to-*C* vs *C*-to-*N*.

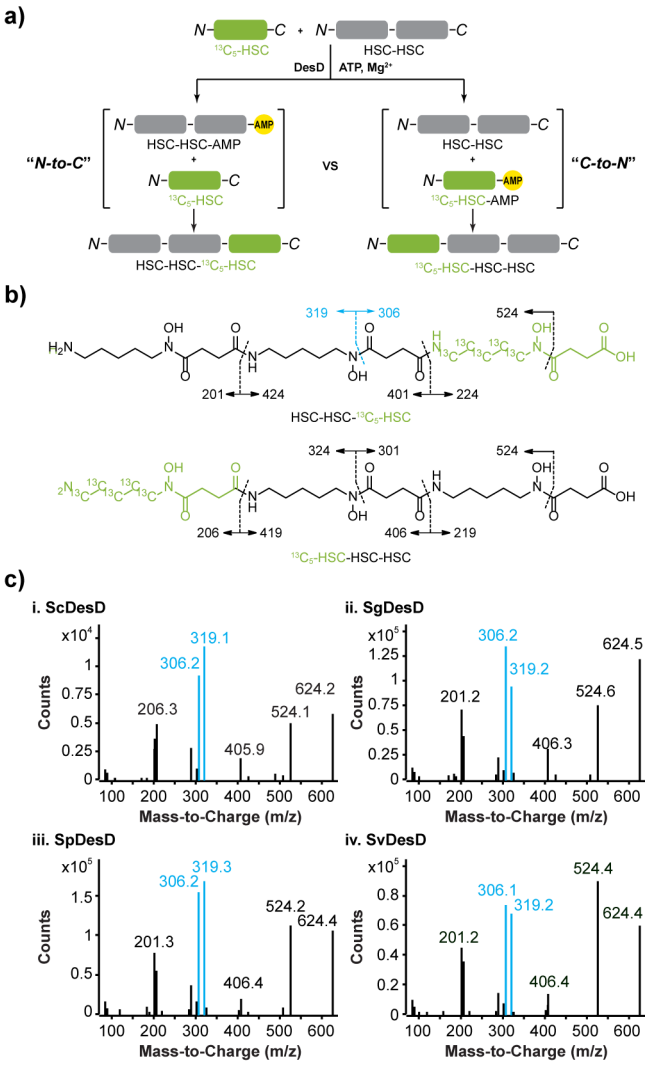

Figure 2. DesD-catalyzes *N*-to-*C* condensation of HSC-HSC and HSC in DFOG biosynthetic assembly. (a) Schematic depicting the directionality for *N*-to-*C* (left) and *C*-to-*N* (right) condensation. (b) Expected MS/MS fragmentation patterns of $^{13}C_5$-DFOG (*m/z* 624 for $[M+H]^+$ parent ion) for isomers resulting from *N*-to-*C* (top) or *C*-to-*N* (bottom) condensation. (c) MS/MS spectra of $^{13}C_5$-DFOG from reactions catalyzed by ScDesD (i), SgDesD (ii), SpDesD (iii), and SvDesD (iv) showing base peaks consistent with *N*-to-*C* condensation direction (*m/z* values 306 and 319).

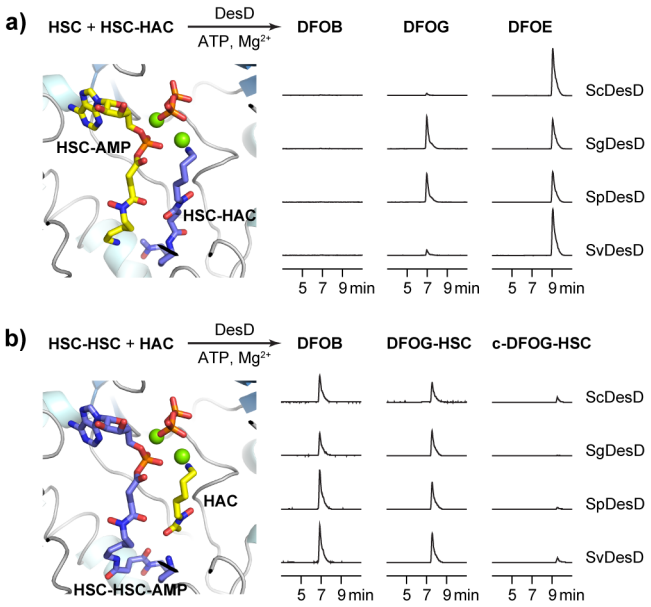

Figure 3. DesD-catalyzes *N*-to-*C* condensation of HSC-HSC and HAC in DFOB biosynthetic assembly. (a) DesD-catalyzed reaction between HSC monomer and HSC-HAC dimer (1:1 ratio) produces only DFOG and DFOE (not detectable DFOB). (b) DesD-catalyzed reaction between HSC-HSC dimer and HAC monomer (1:1 ratio) produces DFOB along with tetrameric products DFOG-HSC and c-DFOG-HSC. For both panels, the insets show a molecular model of the putative substrate acyl adenylate, HSC-AMP or HSC-HSC-AMP, and putative condensation substrate, HSC-HAC or HAC, along with the di-Mg^{2+} catalytic center and bound PPi based on the SvDesD/HSC-AMS co-crystal structure (7TGM). The LC-MS traces represent extracted $[M+H]^+$ ion chromatograms for DFOB (*m/z* 561), DFOG (*m/z* 619), DFOE (*m/z* 601), DFOG-HSC (*m/z* 819), c-DFOG-HSC (*m/z* 801) at the 2 h timepoint of reactions catalyzed by ScDesD, SgDesD, SpDesD, and SvDesD as indicated. Each experiment was performed in at least duplicate as independent trials.

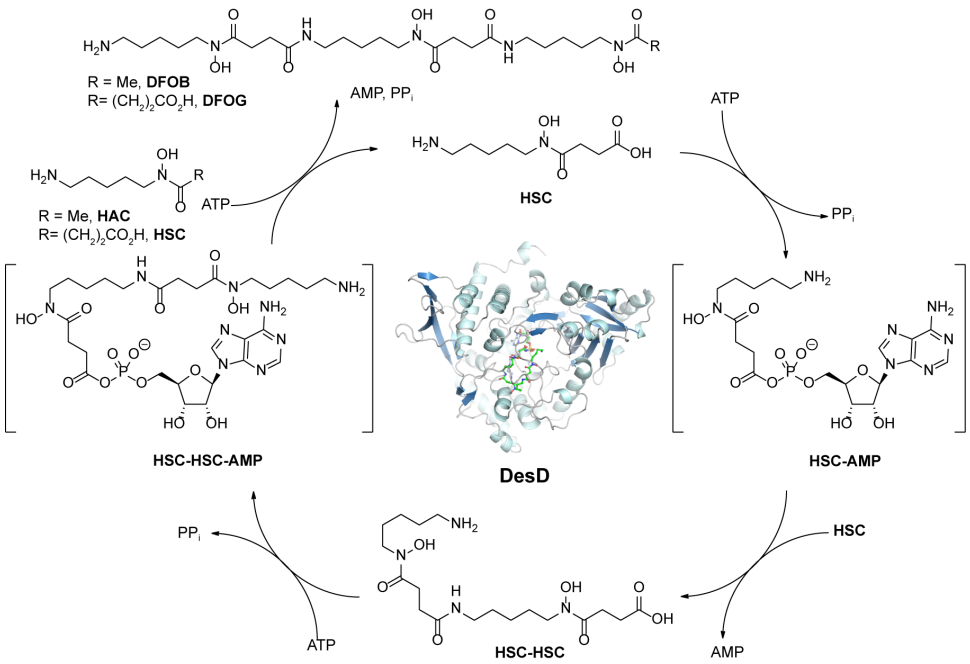

Figure 4. Proposed catalytic cycle for iterative ATP-dependent *N*-to-*C* condensation of HSC and/or HAC precursors by DesD during DFOG and DFOB biosynthesis in *Streptomyces*.

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