Mixing Up the Pieces of the Desferrioxamine B Jigsaw Defines the Biosynthetic Sequence Catalyzed by DesD

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Supporting Information

Abstract: Late-stage assembly of the trimeric linear siderophore desferrioxamine B (DFOB) native to Streptomyces pilosus involves two DesD-catalyzed condensation reactions between one N-acetyl-N-hydroxy-1,5-diaminopentane (AHDP) unit and two N-succinyl-N-hydroxy-1,5-diaminopentane (SHDP) units. AHDP and SHDP are products of DesBC-catalyzed reactions of the native diamine substrate 1,5-diaminopentane (DP). The sequence of DesD-catalyzed DFOB biosynthesis was delineated by analyzing the distribution of DFOB analogues and dimeric precursors assembled by S. pilosus in medium containing 1,4-diamino-2(E)-butene (E-DBE). Seven unsaturated DFOB analogues were produced that were partially resolved by liquid chromatography (LC). Mass spectrometry (MS) measurements reported on the combination of E-DBE- and DP-derived substrates in each trimer (uDFOA1 series, 1:2; uDFOA2 series, 2:1; uDFOA3, 3:0). MS/MS fragmentation patterns reported on the absolute position of the substrate derivative at the N-acetylated terminus, the internal region, or the amine terminus of the trimer. The uDFOA, and uDFOA, series each comprised three constitutional isomers (binary notation (DP-derived substrate "0," E-DBE-derived substrate "1"); direction, N-acetylated-internal-amine): uDFOA[001], uDFOA[010], uDFOA[100]; and uDFOA[011], uDFOA[110], and uDFOA[101]. E-DBE completely replaced DP in uDFOA[111]. Relative concentrations of the uDFOA, series were uDFOA[001] ≫ uDFOA[100] > uDFOA[010] and of the uDFOA, series, uDFOA[101] > uDFOA[011] ≫ uDFOA[110]. Dimeric compounds assembled from one N-acetylated and one N-succinylated substrate derivative were detected as trimer precursors: dDFX[00-] ≫ dDFX[10-] > uddfX[01-] (d = dimer, vacant position "-"). Relative concentrations of all species were consistent with the biosynthetic sequence: (i) SHDP activation, (ii) condensation with AHDP to form AHDP-SHDP, (iii) SHDP activation, and (iv) condensation with AHDP-SHDP to form DFOB.

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Desferrioxamine B (DFOB) is a linear trihydroxamic acid siderophore produced by several species of Streptomyces. This siderophore (Gk, "iron carrier") has been evolved as a high affinity chelator of Fe(III) and provides a mechanism for bacteria to acquire sparingly soluble environmental Fe(III) as essential for growth. DFOB has a long clinical history for the removal of excess iron that accumulates in patients who receive regular blood transfusions to treat genetic hemoglobin disorders, such as beta-thalassaemia, sickle cell anemia, and myelodysplastic syndromes. The chemical synthesis of DFOB is complex and multistep, and its production as a pharmaceutic relies on industrial-scale fermentation of an enzyme cluster DesABCD was subsequently shown by experiment to be functional via pathways independent of nonribosomal peptide synthetases in the biosynthesis of DFOG1 and DFOE, as siderophores native to S. coelicolor M145, and in DFOB biosynthesis. The current understanding of the biosynthesis of DFOB involves the decarboxylation of L-lysine to generate DP (DesA), mono-N-hydroxylation of DP to produce N-hydroxy-DP (DesB), succinylation or acetylation of N-hydroxy-DP to produce N-succinyl-N-hydroxy-DP (SHDP) or N-acetyl-N-hydroxy-DP (AHDP), respectively (DesC), and the condensation of two SHDP and one AHDP fragments (DesD) to form DFOB (Scheme 1a). The current work aimed to delineate the sequence of steps catalyzed by DesD in DFOB biosynthesis. The existing proposition was that DesD first catalyzed the condensation of two SHDP units and in the second step appended the AHDP unit to the SHDP homodimer. While this was a reasonable supposition based on the biosynthesis of DFOG1, and DFOE as...
SHDP homotrimers, the sequence was less clear as applied to heterotrimeric DFOB, since the dimer precursor could be either a SHDP−SHDP homodimer or an AHDP−SHDP heterodimer. To dissect the sequence of steps of DesD-catalyzed DFOB biosynthesis, a precursor-directed biosynthesis approach was used to measure the distribution of dimer and trimer products assembled in situ from combinations of fragments derived from native DP and non-native diamine precursors. Reconstituting the native assembly of DFOB analogues from different jigsaw pieces has established the directional preference of DFOB biosynthesis and identified the sequence of steps catalyzed by DesD.

RESULTS AND DISCUSSION

Rationale. Analysis of the structures and relative concentrations of DFOB analogues produced in cultures of S. pilosus supplemented with exogenous diamine substrates could provide new insight into the native DFOB biosynthetic pathway as a complement to knowledge built from genetics and molecular biology. Precursor-directed biosynthesis with Streptomyces olivaceus Tu2718 has been used to produce analogues of macrocyclic DFOE.20,21 This work examined analogues of DFOB produced in a medium inoculated with S. pilosus supplemented with 1,4-diamino-2(E)-butene (E-DBE) or 1,4-diaminobutane (DB), as the saturated analogue of E-DBE. The E-DBE substrate has been used in cultures of Shewanella putrefaciens to produce unsaturated macrocycles of the dihydroxamic acid putrebactin,22 which is one member of this small class of siderophores.23–26 The similarity of siderophore biosynthesis gene clusters between S. putrefaciens and S. coelicolor27–29 suggested that E-DBE would also be competent as a substrate in the biosynthesis of linear DFOB by closely related S. pilosus.

Unsaturated Analogues of DFOB from E-DBE. The LC trace from the semipurified extract of S. pilosus cultured in a medium optimized for DFOB production30 showed two peaks (Figure 1a). MS analysis of the major peak gave signals at m/z 561.4 and 281.2, consistent with DFOB (Chart 1, 1) as [M + H]+ and [M + 2H]2+ adducts, respectively. The minor peak analyzed as DFOA1 (m/z 547.3, 274.2), which is a known DFO-type siderophore7,31,32 assembled from two DP- and one DB-derived substrate, with the latter diamine available as an endogenous substrate at low levels.

Figure 1. LC-MS-Q trace with total ion current (TIC) detection mode from semipurified supernatant of S. pilosus cultured in (a) base medium or (b) medium supplemented with E-DBE, analyzed as isolated (column at left) or as Fe(III)-loaded (column at right) solutions. The gradient in a or c was the same in b or d, respectively. Peak numbering refers to the compounds in Charts 1 and 2 and Supporting Information Chart 1, or the equivalent 1:1 complexes formed with Fe(III) (number underlined).
The LC profile from the extract purified from the medium supplemented with E-DBE showed five well-resolved peaks (Figure 1b). The LC peak at tR 34.55 min was ascribed to DFOB (Figure 2a, lower x axis) and agreed well with calculated data (upper x axis). MS analysis of the peaks at tR 33.12 (Figure 2b) and 32.04 min (Figure 2c) each gave m/z values consistent with a trimer assembled from two DP- and one E-DBE-derived substrate (m/z 545.3, 273.2) attributable to isomers of uDFOA1, as unsaturated analogues of DFOA1. uDFOA1 could be formulated as three constitutional isomers, in which the E-DBE-derived substrate was inserted at the N-acetylated region, the internal region, or the amine region of the trimer, with the structural balance met by two units of the DP-derived substrate. A binary notation system (DP-derived substrate as "0," E-DBE-derived substrate as "1;" analogue direction from left to right: N-acetylated-internal-amine) would describe these constitutional isomers as uDFOA1[001] (2), uDFOA1[010] (3), and uDFOA1[100] (4). The LC results supported the presence of at least two of the three isomers, similar to previous results for DFOA1.32

The peak at tR 30.35 min gave m/z signals (m/z 529.3, 265.2; Figure 2d) consistent with uDFOA2. Similar to the uDFOA1 series, uDFOA2 could be formulated as three constitutional isomers: uDFOA2[001] (5), uDFOA2[010] (6), or uDFOA2[100] (7). The production of more than one uDFOA2 isomer was unclear, based on the observation of only a single peak. The complete exchange of the native DP-derived fragments for the non-native E-DBE-derived fragments was evident from the m/z signals from the peak at tR 27.37 min (m/z 513.2, 257.1), consistent with uDFOA2[111] (8; Figure 2e). Relative to DFOB ([M + H]+, 561.4), the m/z values decreased in a systematic fashion by 16 units for uDFOA1, uDFOA2, and uDFOA3 as a result of the incremental exchange of one DP-derived fragment for one E-DBE-derived fragment.

The LC traces from the native and the E-DBE supplemented extracts were reacquired following the addition of Fe(III) (Figure 1c and d, respectively). This provided an additional level of characterization and demonstrated the functional integrity of the analogues as siderophores. In the native system, observed MS signals were consistent with [M + H]+ and [M + 2H]2+ adducts (where [M] = [Fe(III)-DFOB-H3] or equivalent) of Fe(III)-DFOB and Fe(III)-DFOA1. MS signals from the LC peak at tR 28.96 min in the Fe(III)-loaded E-DBE supplemented system were similarly characteristic of Fe(III)-DFOB (m/z 614.3, 307.7; Figure 2f). Two LC peaks at tR 28.52 min (Figure 2g) and 26.62 min (Figure 2h) gave MS signals with identical m/z values (m/z 598.3, 299.6) consistent with the formation of Fe(III) complexes with the two constitutional isomers of uDFOA1. The single LC peak attributed to uDFOA2 was resolved in the Fe(III)-loaded extract into three peaks at tR 26.09 (Figure 2i), 25.35 min (Figure 2j), and 24.24 min (Figure 2k), which each gave MS signals (m/z 582.2, 291.6).
characteristic of Fe(III)-uDFOA$_1$ isomers. Signals for the peak at $t_k$ 24.24 min were of low intensity. Fe(III)-uDFOA$_1$ was present in the LC at $t_k$ 23.05 min ($m/z$ 566.2, 283.6), together with other species (Figure 2f).

**Analogs of DFOB from DB.** The LC profile from the extract from *S. pilosus* cultured in a medium supplemented with DB was similar to the E-DBE system (Supporting Information). Five major peaks were resolved in the LC (Supporting Information Figure 1a) that from the MS signals (Supporting Information Figure 2a–e) were characterized as DFOB (1), DFOA$_2$ (two LC peaks, with three possible isomers (DP/DBE derived substrate “0,” DB-derived substrate “1”): DFOA$_1[001]$ (9), DFOA$_1[010]$ (10), DFOA$_1[100]$ (11)), DFOA$_3$ (one LC peak, with three possible isomers: DFOA$_2[011]$ (12), DFOA$_2[101]$ (13), DFOA$_3[101]$ (14)), and DFOA$_4$ (15; Supporting Information Chart 1). The LC peaks from the Fe(III)-loaded extract were less well resolved than the E-DBE system (Supporting Information Figure 1b), with MS signals (Supporting Information Figure 2f–g) attributable to Fe(III)-DFOB, two isomers of Fe(III)-DFOA$_2$, and one isomer of Fe(III)-DFOA$_3$ and Fe(III)-DFOA$_4$.

**Constitutional Isomers of uDFOA$_1$ and uDFOA$_2$.** The E-DBE supplemented system was analyzed by LC-MS-QQQ using selected ion monitoring (SIM) as the detection mode, with values set for the relevant $[M + H]^+$ ion (Figure 3a–e, column at left). Well resolved peaks were consistent with DFOB (SIM 561), uDFOA$_1$ (SIM 545; major and minor peaks), uDFOA$_2$ (SIM 529), and uDFOA$_3$ (SIM 513). The peak areas ascribed to isomers of uDFOA$_1$ integrated in a ratio of 3:1. Overall relative concentrations were estimated from peak integration as DFOB (21.2%), uDFOA$_1$ (major; 34.0%), uDFOA$_1$ (minor; 11.3%), uDFOA$_2$ (29.1%), and uDFOA$_3$ (4.4%). This assumed that the extent of ionization was similar among the structural isomers, which was supported by the similarity in the distribution of signals for each species between different MS systems (LC-MS-Q, LC-MS-QQQ) using different detection modes (extracted ion chromatograms, EIC; SIM; Supporting Information Figure 3). The relative concentrations broadly reflected the yields of compounds isolated using semipreparative HPLC from one 50 mL culture: DFOB (4.1 mg, 17.0%), uDFOA$_1$ (major, 5.5 mg, 22.8%), uDFOA$_1$ (minor, 3.7 mg, 15.3%), uDFOA$_2$ (6.4 mg, 26.6%), and uDFOA$_3$ (4.4 mg, 18.3%). Peak integration showed the ratio of incorporation of DP/E-DBE into the library of analogues was 1:6:1.

MS/MS fragmentation patterns were analyzed to identify and establish the distribution of constitutional isomers within the peak(s) that corresponded with the uDFOA$_1$ or the uDFOA$_2$ series (Figure 3a–e, column at right). The fragmentation of DFOB as the parent compound was similar to previous studies of DFOB or related siderophores.33–36

MS/MS data showed that in the uDFOA$_1$ series, the major signal corresponded with uDFOA$_1[001]$ (2). The presence of MS/MS signals at $m/z$ 361.1 and 443.2 specific to fragments of uDFOA$_1[001]$ (2), together with the absence of signals at $m/z$ 345.2 and 427.2 for the analogous fragments common to uDFOA$_2[011]$ (3) and uDFOA$_3[101]$ (4), made this assignment unambiguous (Table 1). The LC peak intensity showed that uDFOA$_1[001]$ (2) was produced from the uDFOA$_2$ series as the major isomer, which correlated with its isolation in higher yield. The minor signal in the uDFOA$_1$ series gave a fragmentation pattern consistent with the presence of a mixture of uDFOA$_1[010]$ (3) and uDFOA$_1[100]$ (4). Signals at $m/z$ 128.1, 227.1, and 319.2 were attributable to fragments unique to uDFOA$_1[001]$ (3). These latter signals would also arise from fragments of uDFOA$_1[001]$ (2); however, this isomer was not present in the minor peak, based on the absence of characteristic signals at $m/z$ 361.2 and 443.2. The relative intensity of the signals due to pairs of fragment analogues of uDFOA$_1[011]$ (3) at $m/z$ 243.2 and uDFOA$_1[100]$ (4) at $m/z$ 227.1 or $m/z$ 303.3 (3) and $m/z$ 319.2 (4) suggested a higher relative concentration of uDFOA$_1[100]$ (4) than uDFOA$_1[011]$ (3), in a ratio of about 2:1.

In the uDFOA$_2$ series, MS/MS data showed signals that correlated with the presence of a mixture of isomers. Signals of fragments characteristic of both uDFOA$_2[011]$ (5) and uDFOA$_2[101]$ (7) were present at $m/z$ 345.2 and 427.2,
confirming the presence of at least one of these isomers.

Fragment signals at m/z 391.1 and 411.1, which within the uDFOA2 series were unique to uDFOA2[110] (6), were detected at very low levels indicating that this isomer was present in trace amounts. MS analysis across the peak indicated low levels of uDFOA2[110] in the peak front. The minor peak at tR 24.66 min did not characterize as a DFOB analogue. The relative intensity of the signals due to pairs of fragment analogues of uDFOA2[011] (5) at m/z 243.1 and uDFOA2[101] (7) at m/z 227.2 (5) and m/z 287.2 (5) and m/z 303.2 (7) suggested a higher relative concentration of uDFOA2[101] (7) than uDFOA2[011] (5) in a ratio of about 2:1. A signal at m/z 369.2 from the MS/MS analysis of uDFOA1[001] (2) suggested a higher relative intensity of MS/MS signals resolved for isomers of the uDFOA1 or uDFOA2 series. The term 8.9C describes breaking the bond between atoms 8 and 9 of the main chain of DFOB (refer Scheme 2 for numbering) in which the relevant fragment contains the terminal acetyl group (C terminus). Where the subscript C is replaced with N, the relevant fragment contains the terminal amine group (N terminus). The subscript I describes an internal fragment. Atom numbers are referenced to DFOB and may differ from the absolute atom number in a given analogue.
fragmentation of the LC peak at \( t_R \) 23.11 min was characteristic of uDFOA\(_{111}\).

This LC-MS/MS analysis allowed the identification of isomers and an estimate of the relative concentrations within the uDFOA\(_{1}\) and the uDFOA\(_{2}\) series as uDFOA\(_{100}\) \( \gg \) uDFOA\(_{101}\) \( \gg \) uDFOA\(_{102}\) \( \gg \) uDFOA\(_{110}\). The MS/MS fragmentation patterns from the species produced in the DB-supplemented system (Supporting Information Figure 4 and Scheme 1) showed the same trend in isomer distribution within the DFOA\(_{1}\) and DFOA\(_{2}\) series: DFOA\(_{100}\) \( \gg \) DFOA\(_{101}\) \( \gg \) DFOA\(_{102}\) \( \gg \) DFOA\(_{110}\) (Supporting Information Table 1).

**NMR Spectroscopy.** The two peaks that were resolved for the uDFOA\(_{1}\) series (major and minor), the single peak of the uDFOA\(_{2}\) series and the single peak containing uDFOA\(_{1}\) were isolated from the LC, and these purified fractions were analyzed by HRMS and by 1D and 2D (COSY) \(^1\)H NMR spectroscopy. The \(^1\)H--\(^1\)H COSY experiment confirmed that the major peak from the uDFOA\(_{1}\) series contained uDFOA\(_{101}\) (Supporting Information Figures 5 and 6). The methine protons in this sample gave a signal at \( \delta \) 5.66 ppm, with \(^1\)H--\(^1\)H correlations to \( \delta \) 4.06 ppm and \( \delta \) 3.30 ppm. The latter correlation was evidence for the incorporation of the E-DBE-derived substrate in the amine region of the trimer. The \(^1\)H--\(^1\)H COSY data were in agreement with the conclusions based on MS/MS fragmentation that the minor peak from the uDFOA\(_{1}\) series contained a mixture of uDFOA\(_{101}\) and uDFOA\(_{100}\), while these isomers were unable to be distinguished. The \(^1\)H--\(^1\)H COSY correlation data were consistent with the incorporation of two or three E-DBE-derived substrates in the uDFOA\(_{1}\) or uDFOA\(_{2}\) samples, respectively (Supporting Information Figures 7–12).

**Dimeric Precursors of DFOB and Analogues.** A well-resolved signal in the extract from the DB-supplemented system at \( t_R \) 28.79 min (Supporting Information Figure 1a) was analyzed as \( m/z \) 361.2 (Supporting Information Figure 2f) and in the presence of Fe(III) as \( m/z \) 414.1 (Supporting Information Figure 2a). These data were consistent with the assignment of this species as the dimer formed from one AHDP and one SHDP fragment: dDFX\([00\text{-}]\), where "d" = dimer and "-" = vacant position at the amine region (Chart 2, 16). This species was also observed at low levels in the extracts from the native and the E-DBE-supplemented systems (Figure 1). LC-MS-QQQ analysis using SIM detection from the E-DBE extract showed the presence of dDFX\([00\text{-}]\) (16) and signals in accord with the presence of the other dimers that could be assembled from the N-acetylated region: udDFX\([01\text{-}]\) (17), udDFX\([10\text{-}]\) (18), and udDFX\([11\text{-}]\) (19) (Figure 4a–c). The identity of each dimer was supported from characteristic MS/MS fragmentation patterns (Table 2, Supporting Information Figure 13 and Scheme 2). The relative concentration of the dimers from SIM peak integration was dDFX\([00\text{-}]\) (16) \( \gg \) udDFX\([10\text{-}]\) (18) \( \gg \) udDFX\([01\text{-}]\) (17) \( \gg \) udDFX\([11\text{-}]\) (19).

The dimeric fragments assembled from the amine region from the E-DBE extract were identified using SIM detection (Figure 4d–f) and MS/MS fragmentation patterns (Table 2, Supporting Information Figure 14 and Scheme 2). The concentration of the dimer species dDFX\([-00\text{-}]\) (20), where "-" = vacant position at the N-acetylated region, was about 70-fold less than dDFX\([00\text{-}]\) (16; Figure 4a,d). It was possible that this difference in concentration was a result of distinct ionization properties of the differentially terminated fragments.

**Figure 4.** LC-MS-QQQ signals detected from the E-DBE supplemented system (a–f) at SIM values (a) 361, (b) 345, (c) 329, (d) 419, (e) 403, or (f) 387 or from the DB supplemented system (g–l) at SIM values (g) 361, (h) 347, (i) 333, (j) 419, (k) 405, or (l) 391.

(N-acetyl/amine, carboxylic acid/amine). MS analysis of a mixture of two standard compounds that modeled each fragment type (N-(5-aminopentyl)-N-hydroxyacetamide and 4-(5-aminopentyl) (hydroxy)amino]-4-oxobutanoic acid) gave similar signal intensities (Supporting Information Figure 15), which supported that the concentration differences were...
real and not an artifact of the analysis. Dimers assembled from the amine region from one DP and one E-DBE-derived fragment (SIM 403) were present at similarly low levels as dDFX[-00] (20), with udDFX[-01] (21) > udDFX[-10] (22). From this dimer series udDFX[-11] (23), assembled from two E-DBE-derived fragments, was present in the highest relative concentration.

Trends in the distribution of the dimeric precursors of DFOB and analogues were similar between the E-DBE- and the DB-supplemented systems (Supporting Information Table 2). Dimer dDFX[00-] (16) was the dominant dimer, with dDFX[-00] (20) present in about 30-fold less concentration (Figure 4g, j). The concentration of dDFX[10-] (25) ≫ dDFX[01-] (24) (Figure 4h), with the identity of the dimers confirmed from

### Table 2. MS/MS Fragmentation Patterns from Dimeric Precursors of DFOB and Analogues Assembled from the N-Acetylated or the Amine Region from Substrates DP and E-DBE

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<th>F18</th>
<th>F19</th>
<th>F20</th>
<th>F21</th>
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**a** Determined from peak integration. **b** The term 13.14N describes breaking the bond between atoms 13 and 14 of the main chain of dDFX[00-] or dDFX[-00] in which the relevant fragment contains the terminal amine group (N terminus). Where the subscript N is replaced with C, the relevant fragment contains the terminal acetyl group (C terminus). The subscript I describes an internal fragment. Atom numbers are referenced to dDFX[00-] or dDFX[-00] and may differ from the absolute atom number in a given analogue. **c** NR, not relevant for this compound.

### Scheme 3. Biosynthesis of (a) DFOB (1) and (b–h) Analogues (2–8) Shown in the Direction from C Amide to N Amide (Pathway at Left) or N Amide to C Amide (Pathway at Right)ad

ad Refer to Charts 1 and 2 for full structures. Fragments derived from native DP or non-native E-DBE are depicted as solid lines in black or gray, respectively. Amide bonds are represented as narrow lines perpendicular to the solid lines.
Identification of a preference in the biosynthetic direction of DFOB and analogues was only possible by virtue of its assembly from two different N-acetylated substrates. Linear desferrioxamine G\(_1\) (DFOG\(_1\)) and its macromycic product desferrioxamine E (DFOE) are assembled from SHDP as the unique N-acetylated substrate.\(^{25,38}\) In these cases, it would not be possible to use a precursor-directed biosynthesis approach to determine a preference in the biosynthetic direction of the final-stage condensation steps, since the dimer precursors would be identical.

**Sequence of DesD-Catalyzed DFOB Biosynthesis.** Within each of the uDFOA\(_1\) and the uDFOA\(_2\) series, two trimers were built following the second condensation step from a dimer precursor comprised of one native DP-derived substrate (AHDP or SHDP) and one E-DBE-derived substrate (AH-E-DBE or SH-E-DBE). For the uDFOA\(_4\) isomer subset, udDFX\(_{10}\) (4) > udDFX\(_{101}\) (3), and for the uDFOA\(_2\) isomer subset, udDFX\(_{101}\) (7) > udDFX\(_{111}\) (5). As paired by relative concentration, the high concentration isomers udDFX\(_{10}\) (4) and udDFX\(_{111}\) (7) used 18 as the common heterodimer precursor, and the low concentration isomers udDFX\(_{101}\) (3) and udDFX\(_{111}\) (5) used heterodimer 17 (Scheme 3). This suggested that the dimer precursor udDFX\(_{10}\) (18) was available at higher concentration than udDFX\(_{111}\) (17). This was supported by the SIM measurements (Figure 4b), with the dimers identified from characteristic MS/MS fragmentation patterns (Supporting Information Figure 13).

The dominance of udDFX\(_{10}\) (18) above udDFX\(_{111}\) (17) allowed for subtle insight into the sequence of steps catalyzed by DesD in DFOB biosynthesis. Since the relative concentration of products from reactions between combinations of native AHDP and SHDP would be expected to be higher than those involving non-native AH-E-DBE and SH-E-DBE, the trend of udDFX\(_{10}\) (18) > udDFX\(_{111}\) (17) was consistent with the notion that the activation of native SHDP occurred more readily than the activation of SH-E-DBE as the first step of the biosynthesis, and that the availability of activated SHDP was the major determinant of the final concentration of a given dimer precursor. It has been established that the activation of the SHDP unit occurs as part of the DesD-catalytic cycle via the formation of an acyl adenylate intermediate.\(^{19}\) Levels of activated SHDP were sufficient to allow the condensation of non-native AH-E-DBE to form udDFX\(_{10}\) (18). Levels of activated SH-E-DBE were relatively low, which reduced the concentration of udDFX\(_{111}\) (17) formed from the condensation with native AHDP. This notion was also in accord with dDFX\(_{10}\) (16) > dDFX\(_{111}\) (18), whereby activated SHDP was condensed in the first step with either native AHDP (high concentration) or non-native AH-E-DBE (low concentration). The trimer products would ultimately be formed following the second condensation step between the available dimer precursors and the second unit of activated SHDP or activated non-native SH-E-DBE.

Consideration of the relative concentration of the trimeric DFOB analogues and the dimer precursors has allowed the sequence of steps in the two DesD-catalyzed condensation reactions in DFOB biosynthesis to be defined (Scheme 4). The first step involves the activation of SHDP, which is then condensed with AHDP to form an AHDP-SHDP heterodimer. A second aliquot of activated SHDP would next be formed which would undergo condensation with the heterodimer to
form the final DFOB trimer. The condensation between AHDP and activated SHDP would effectively cap one end of the dimer product, thereby reducing the propensity of side reactions. It would be likely that the activation of SHDP would occur as two discrete steps (step i and step iii), since a pool of activated SHDP produced in a single step in excess of AHDP could promote the formation of SHDP oligomers.

**Sequence of DesD-Catalyzed Biosynthesis:** DFOB, DFOG, DFOE. The biosynthetic sequence for DFOB can be considered in terms of the effectiveness of monomeric SHDP or dimeric SHDP−SHDP as a substrate in the DesD-catalyzed activation step. Results from this work suggest that SHDP is the optimal substrate for activation, with SHDP−SHDP significantly less effective. DFOB biosynthesis from the N-acetylated region to the amine region would only ever require the activation of a SHDP monomer. In the reverse direction, the biosynthesis would require activation of the SHDP−SHDP dimer as the step prior to AHDP condensation. In the case of DFOB biosynthesis, this necessarily prescribes a preference in the biosynthetic direction in appending activated SHDP to AHDP−SHDP rather than AHDP to poorly activated SHDP−SHDP. In the case of siderophores DFOG and DFOE assembled only from SHDP, a directional preference in the biosynthesis would not be prescribed, since the second-step condensation could occur between an activated SHDP monomer and SHDP−SHDP. This rationale is consistent with a study that in DB-supplemented cultures of *S. pilosus* with a study that in DB-supplemented cultures of *S. pilosus* with a study that in DB-supplemented cultures of *S. pilosus*.

**Conclusion.** The DesBCD enzyme cluster in *S. pilosus* was competent in the use of E-DBE as a competitive n-native substrate against native DP to produce a library of unsaturated DFOB analogues that retained function as Fe(III) chelators *in vitro*. The distribution of dimer precursors and trimer products assembled from a mixture of DP- and E-DBE-derived substrates has enabled the steps in DesD-catalyzed DFOB biosynthesis to be defined. DFOB biosynthesis involves (i) the activation of SHDP, (ii) condensation with AHDP to form AHDP−SHDP, (iii) SHDP activation, and (iv) condensation with AHDP−SHDP to form DFOB. This directional preference for DFOB biosynthesis is prescribed by the substrate selectivity of DesD toward activating monomeric SHDP but not dimeric SHDP−SHDP.
plasticware. Cells were withdrawn from the precultures, centrifuged (5000 rpm), resuspended in fresh medium, and used to inoculate 50 mL cultures of the base medium. Individual conditions were established by the addition of DB or E-DBE solutions (pH 6.00 ± 0.05) to a concentration of 20 mM with respect to the dihydrochloride salt. The other component mixtures and substrate solutions were filtered with Minisart 0.2 µm syringe filters before use. The bacteria was grown at 28 °C and 160 rpm for 8 days. Siderophore production was tracked daily by adding supernatant (200 µL) to ferric perchlorate (10 mM) in perchloric acid (200 mM; 100 µL) and measuring absorbance at 465 nm using a BMG Labtech FLUOstar Omega microplate reader. The supernatant was collected for purification by centrifugation (5000 rpm), and the quantity of bacterial cells in each culture was measured following lyophilization, using a Labconco FreeZone freeze-dryer.

**Purification.** XAD-2 purification was modified from previous methodology.23,39 Amberlite XAD-2 resin (100 mL) was activated in methanol, suspended in a column in water, and washed with two column volumes (CVs) of water. The supernatant was adsorbed on the resin which was then washed with 2 CVs of water. The sample was eluted using 2–4 CVs of aqueous methanol (50% v/v). Siderophore presence was tracked using a CAS assay.40,41 The CAS positive fractions from the elution step were taken to dryness in vacuo and were analyzed in positive ion mode.

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**Sample Preparation:** LC-MS-Q and LC-MS/MS/QQQ. Samples (0.1 mg) were dissolved in HPLC grade methanol (1 mL) for analysis by LC-MS-Q and LC-MS/MS/QQQ. Fe(III)-loaded samples were generated by adding 100 µL of an iron(III) chloride solution (350 µM) to 100 µL of analyte and incubating for 1 h. Aliquots (10 µL) were analyzed in positive ion mode.

## ASSOCIATED CONTENT

© Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00056.

Structures of DFOB analogues assembled from DB-supplementation (Supporting Information Chart 1) or of dimers from DB-supplementation (Supporting Information Chart 2). LC-MS-Q (Supporting Information Figure 1, LC), MS (Supporting Information Figure 2, MS), and LC-MS/MS/QQQ data (Supporting Information Figure 4, Supporting Information Scheme 1, Supporting Information Table 1) from DB-supplementation. LC-MS/QQQ (SIM: [M + H]+) and LC-MS-Q (Sum EIC: [M + H]+ + [M+2H]+) from E-DBE-supplementation (Supporting Information Figure 3). NMR spectra from uDFOA1 major peak (Supporting Information Figures 5 and 6), uDFOA1 minor peak (Supporting Information Figures 7 and 8), uDFOA2 (Supporting Information Figures 9 and 10), and uDFOA3 (Supporting Information Figures 11 and 12). LC-MS/MS/QQQ data from dimers from E-DBE. (Supporting Information Figures 13 and 14, Supporting Information Scheme 2) or DB- (Supporting Information Figures 16 and 17, Supporting Information Scheme 3, Supporting Information Table 2) supplementation. LC-MS/QQQ from standards (Supporting Information Figure 15) (PDF)

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Notes

The authors declare no competing financial interest.

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## REFERENCES

iron(III), molybdenum(VI) or chromium(V), through the upstream inhibition of ornithine decarboxylase. *FEMS Microbiol. Lett.* **42**, 135–139.


