Fluorinated Analogues of Desferrioxamine B From Precursor-Directed Biosynthesis Provide New Insight Into the Capacity of DesBCD

Thomas J. Telfer, Rachel Codd*

School of Medical Sciences (Pharmacology), The University of Sydney, New South Wales 2006, Australia

Corresponding author. Tel.: +61 2 9351 6738.

E-mail address: rachel.codd@sydney.edu.au (R. Codd).

Abstract: The siderophore desferrioxamine B (DFOB, **1**) native to *Streptomyces pilosus* is biosynthesized by the DesABCD enzyme cluster. DesA-mediated decarboxylation of L-lysine gives 1,5-diaminopentane (DP) for processing by DesBCD. *S. pilosus* culture medium was supplemented with *rac*-1,4-diamino-2-fluorobutane (*rac*-FDB) to compete against DP to generate fluorinated analogues of DFOB, as agents of potential clinical interest. LC-MS/MS analysis identified fluorinated analogues of DFOB with one, two or three DP units (binary notation: 0) exchanged for one (DFOA-F_1[001] (**2**), DFOA-F_1[010] (**3**), DFOA-F_1[100] (**4**)), two (DFOA-F_2[011] (**5**), DFOA-F_2[110] (**6**), DFOA-F_2[101] (**7**)), or three (DFOA-F_3[111] (**8**)) *rac*-FDB units (binary notation: 1). The two sets of constitutional isomers **2**–**4** and **5**–**7** arose from the position of the substrates in the *N*-acetyl, internal or amine-containing regions of the DFOB trimer. *N*-Acetylated fluorinated DFOB analogues were formed where the *rac*-FDB substrate was positioned in the amine region (*eg*, *N*-Ac-DFOA-F_1[001] (**2a**)). Other analogues contained two hydroxamic acid groups and three amide bonds. Experiments using *rac*-FDB, *R*-FDB or *S*-FDB showed a similar species profile between *rac*-FDB and *R*-FDB. These data are consistent with the following. (i) DesB can act on *rac*-FDB; (ii) DesC can act directly on *rac*-FDB; (iii) the products of DesBC or DesC catalysis of *rac*-FDB can undergo a second round of DesC catalysis at the free amine; (iv) DesD catalysis of these products gives *N*,*N*′-diacetylated compounds; (v) a minimum of two hydroxamic acid groups is required to form a viable DesD-substrate(s) pre-complex; (vi) one or more DesBCD-catalysed steps in DFOB biosynthesis is enantioselective. This work has provided a potential path to access fluorinated analogues of DFOB and new insight into its biosynthesis.

Introduction

Desferrioxamine B (DFOB) is a linear trimeric hydroxamic acid siderophore that forms a high affinity (logK_a 31) complex with Fe(III).[1,2] The soil bacterium *Streptomyces pilosus* produces DFOB as its native siderophore to acquire iron from the extracellular environment, as essential for growth.[3-5] DFOB is obtained from large-scale fermentation for clinical use to treat chronic iron overload that results from transfusion-dependent anaemias, such as β-thalassemia.[6,7] Together with the synthetic agents deferasirox and deferiprone,[8,9] DFOB has contributed to improved outcomes for patients with chronic iron overload, although its hydrophilicity (log$P$ –2.10), short plasma half-life ($t_{1/2}$ 20 min), and poor membrane permeability,[10-12] indicate potential for improvement. Strategies to increase the lipophilicity, cell permeability and circulation time of DFOB have involved grafting lipophilic low-molecular-weight ancillary units,[11,13,14] or biological polymers[15,16] onto the primary amine group.

An alternative strategy with potential to modulate the properties of DFOB would be to incorporate fluorine atoms into the backbone of the molecule. Hydrogen-for-fluorine atom exchange is being used increasingly in clinical agents, with fluorine analogues often showing superior performance than the hydrogen parent.[17-24] About 25% of current drugs contain fluorine, including the top sellers: 5-fluorouracil (anticancer), fluoxetine (Prozac, antidepressant) and atorvastatin (Lipitor, anti-cholesterol). Nine of the 33 new small molecules approved by the U.S. Food & Drug Administration in 2017 contain fluorine atoms, which shows the continuing impact of this element in drug design. The similarity in van der Waals radius between the C–F bond (1.47 Å) and the C–H bond (1.20 Å) makes fluorine a useful hydrogen atom replacement,[17-24] with its high electronegativity affecting pK_a values, lipophilicity, molecular conformation, intermolecular binding interactions, metabolic

stability, and membrane permeability.[17-27] The multistep total synthesis of DFOB[28-30] could limit access to fluorinated DFOB using chemical synthesis. Early studies that used precursor-directed biosynthesis (PDB) to generate new analogues of siderophores,[31-33] have been expanded recently to generate new macrocyclic siderophores,[34] and new analogues of DFOB.[35,36] This identified the possibility of using PDB as an approach to generate fluorinated analogues of DFOB. PDB has been successful in producing fluorinated analogues of several types of natural products,[37-41] with synthetic biology approaches also used in this endeavor.[42-45]

Scheme 1. Biosynthesis of (**a**) desferrioxamine B (DFOB, **1**) or (**b**) a representative regioisomer of the fluorinated analogue DFOA-F_3[111] (**8**) from the complete substitution of native DP with non-native *rac*-FDB.

The use of PDB is predicated on knowledge of the biosynthesis of the target natural product to allow judicious selection of exogenous substrates as competitors of native substrates. The biosynthesis of DFOB is governed by the DesABCD enzyme cluster (**Scheme 1a**).[35,46-49] The first step involves the generation of the diamine substrate 1,5-diaminopentane (DP) as the product of DesA-mediated decarboxylation of L-lysine.[50] Mono-*N*-hydroxylation of DP (DesB) gives *N*-hydroxy-DP (HDP),[51] which is *N*-acetylated (DesC) to give *N*-acetyl-*N*-hydroxy-DP (AHDP) or *N*-succinylated (DesC) to give *N*-succinyl-*N*-hydroxy-DP (SHDP).[46-49] One unit of SHDP is activated and condensed with AHDP (DesD, cycle 1 (C1)) to give AHDP-SHDP, followed by the activation and condensation of a second unit of SHDP (DesD, cycle 2 (C2)) with AHDP-SHDP to give AHDP-SHDP-SHDP (DFOB).[35] The fluorinated diamine *rac*-1,4-diamino-2-fluorobutane (*rac*-FDB) was examined in this work as

a competitor of DP in the biosynthesis of DFOB. This choice of substrate was guided by our previous work,[35] which showed that compared to 1,5-diamine-based substrates, 1,4-diamine-based substrates gave species that were sufficiently resolved by liquid chromatography from DFOB for analysis. The complete exchange of native DP for non-native *rac*-FDB would result in DFOA-F_3[111] (**8**), shown as one regioisomer (**Scheme 1b**).

This work describes the types of fluorinated analogues of DFOB produced by *S. pilosus* using PDB with *rac*-FDB. Experiments also undertook supplementation with *R*-FDB and *S*-FDB, to examine the possibility of the enantioselective activity of DesBCD. The types of species produced has provided new insight into DesBCD-mediated biosynthesis of DFOB, with implications for the biosynthesis of related siderophores. The identification of these species at an analytical level provides a basis for larger-scale production in future work to examine the properties of fluorinated analogues of DFOB.

Results and Discussion

Fluorinated Analogues of DFOB. The major species produced by *S. pilosus* when cultured in base medium optimised for siderophore production[52] was DFOB, as verified by mass spectrometry (MS) analysis of the major peak (t_R 34.8 min) in the liquid chromatogram (LC) (**Figure 1a**), giving ions characteristic for $[M+H]^+$ and $[M+2H]^{2+}$ adducts (m/z_{obs} 561.4, 281.2). The production of DFOB (**Chart 1**, **1**) and minor species, including AHDP-SHDP (**Chart 2**, **9**), as the dimeric precursor of DFOB; and $DFOA_1$ (t_R 33.9 min), which contains *N*-succinyl-*N*-hydroxy-1,4-diaminobutane (SHDB) as the terminal fragment, was consistent with previous work.[35,36] A minor signal at t_R 42.2 min characterised as $DFOD_1$ (**1a**), which is a variant of DFOB containing an *N*-acetyl group at the terminal amine.[4,53,54]

Chart 1. Desferrioxamine B (DFOB, **1**), and its fluorinated analogues containing one (**2**–**4**), two (**5**–**7**) or three (**8**) fluorine atoms in the main-chain region. Other modified fluorinated analogues of DFOB contained *N*-acetyl groups ('**a**' series), one additional amide bond and one less hydroxamic acid group ('**b**' series – with positional variation: '**i**', '**ii**', '**iii**'), or a combination of both modifications ('**c**' series – with positional variation: '**i**', '**ii**', '**iii**').

Chart 2. Dimeric precursors ('dDFO') of the AHDP-SHDP-type (**9**–**12**) or the SHDP-SHDP-type (**13**–**16**) and modified analogues containing *N*-acetyl groups ('**a**' series), one additional amide bond and one less hydroxamic acid group ('**b**' series – with positional variation: '**i**', '**ii**'), or a combination of both modifications ('**c**' series – with positional variation: '**i**', '**ii**').

The semi-purified (XAD, IMAC) supernatant from the culture supplemented with 10 mM *rac*-FDB showed reduced DFOB (t_R 34.8 min), coupled with the appearance of multiple new peaks (**Figure 1b**). MS/MS analysis (**Figure 2a**) showed a fragmentation pattern for the peak at t_R 34.8 min characteristic of DFOB (**Scheme 2, Supporting Information Table 1**), which based on the extracted ion chromatogram (EIC) peak integrals, was present in about one third of the concentration of the system with no supplementation. While the onset of the biosynthesis of Fe(III)-complexing species was delayed in the *rac*-FBD supplemented culture, the final concentration of Fe(III)-complexing species plateaued at a concentration almost twice that of the native culture (**Supporting Information Figure 1**). There was no significant difference in cell mass between the two systems: 352 mg dry weight

(supplemented), 348 mg dry weight (native) per 50-mL culture. These measurements showed that *rac*-FDB at 10 mM was not toxic to *S. pilosus*.

Figure 1 LC-MS traces (detection as total ion current (TIC): **a**, **b**, **i**; or EIC: **c**–**h**) from semi-purified supernatant of *S. pilosus* cultured in base medium with: (**a**) no supplementation; or (**b**) supplemented with *rac*-FDB (10 mM). The semi-purified sample from (**b**–**i**) was analysed following the addition of Fe(III) (**i**). Species as numbered (free ligands: non-italicized; Fe(III)-complexes: italicized) are identified in **Chart 1** and **Chart 2**. Peaks marked * were co-purified species that did not give MS/MS fragmentation patterns characteristic of siderophores.

Scheme 2. MS/MS fragmentation of DFOB (**1**) and fluorinated analogues of DFOB (**2**–**8**).

The peak at t_R 33.8 min in the *rac*-FDB-supplemented system gave *m*/*z* values (*m*/*z* 565.4, 283.2) consistent with the $[M+H]^+$ and $[M+2H]^{2+}$ adducts of an analogue of DFOB with one DP for *rac*-FDB exchange. MS/MS fragmentation showed that this species featured the *rac*-FDB substrate incorporated at the amine terminus, and was designated DFOA-F_1[001] (**2**). The analogues here are more correctly defined as DFOA, since *rac*-FDB is a fluorinated analogue of 1,4-diaminobutane (DB), which is a native substrate (produced from the DesA-mediated decarboxylation of L-ornithine) of the DFOA-type siderophores.[4,54] The assignment of DFOA-F_1[001] (**2**) was based on the presence of signals at *m*/*z* 361.2 and 443.2, and the absence of signals at *m*/*z* 365.2 and 447.2 (**Figure 2b**), which correlated with

its unique fragmentation pattern (**Scheme 2, Supporting Information Table 1**). The single DP-for-*rac*-FDB exchange gives rise to three possible constitutional isomers, including **2**, and those with *rac*-FDB incorporated in the internal region (DFOA-F_1[010], **3**) or at the *N*-acetyl region (DFOA-F_1[100], **4**) of DFOB. The binary naming system has been defined previously, with '0' or '1' representing the native or non-native substrate, respectively; and the substrate position as written from left-to-right mapped to the *N*-acetyl region, the internal region or the amine region of the asymmetric DFOB trimer.[35]

The peak at t_R 32.8 min contained a mixture of DFOA-F_1[010] (**3**) and DFOA-F_1[100] (**4**). Within the set of DFOA-F_1 isomers (**2**–**4**), the MS/MS signals at *m*/*z* 148.1, 247.1 and 319.1 were unique to DFOA-F_1[100] (**4**) (**Figure 2c**). Although the signals at *m*/*z* 143.9, 243.0 and 323.0 in the same MS/MS spectrum could be assigned to DFOA-F_1[001] (**2**) or DFOA-F_1[010] (**3**), the absence of signals at *m*/*z* 361.2 and 443.2 showed that DFOA-F_1[001] (**2**) was not present, and that the signals were due to DFOA-F_1[010] (**3**). The EIC peak integral was used as an estimate of concentration, together with the relative intensities of matched MS/MS fragment pairs for peaks that contained co-eluting isomers. This assumes the species had similar ionization properties, which was reasonable, based on the high structural similarity within a series and their like charge. The relative concentration of DFOA-F_1[001] (**2**) (16.2%) > DFOA-F_1[100] (**4**) (5.6%) > DFOA-F_1[010] (**3**) (1.9%) was consistent with the relative concentrations of matched constitutional isomers produced using PDB with different diamine substrates.[35,36] The presence of **9**, together with the concentration of DFOA-F_1[001] (**2**) > DFOA-F_1[100] (**4**), is consistent with the notion[35] of the first round of DesD catalysis involving the conjugation of activated SHDP to AHDP to produce the AHDP-SHDP heterodimer (**9**), with the second-round of DesD catalysis conjugating a second unit of activated SHDP to AHDP-SHDP to produce AHDP-SHDP-SHDP, as trimeric DFOB (**Scheme 1a**).

Figure 2. LC-MS trace (left column) and MS/MS fragmentation (right column) of: (**a**) DFOB (**1**), (**b**) DFOA-F_1[001] (**2**), (**c**) DFOA-F_1[010] (**3**) and DFOA-F_1[100] (**4**), (**d**) DFOA-F_2[011] (**5**), DFOA-F_2[110] (**6**) and DFOA-F_2[101] (**7**), (**e**) DFOA-F_3[111] (**8**), (**f**) aDFOA-F_1[001_a] (**2b**$_i$), (**g**) *N*-Ac-dDFO-F_1[01-] (**10a**), (**h**) *N*-Ac-dDFO-F_2[11-] (**12a**), and (**i**) *N*-Ac-DFOA-F_1[001] (**2a**).

The peak at t_R 31.4 min gave *m*/*z* values (*m*/*z* 569.3, 285.2) consistent with the $[M+H]^+$ and $[M+2H]^{2+}$ adducts of an analogue of DFOB with two DP for *rac*-FDB exchanges. MS/MS fragmentation analysis of this peak showed the presence of three constitutional isomers of the DFOA-F_2 set: DFOA-F_2[011] (**5**), DFOA-F_2[110] (**6**) and DFOA-F_2[101] (**7**) (**Figure 2d**). The relative concentrations were: DFOA-F_2[101] (**7**) (21.0%) > DFOA-F_2[011] (**5**) (7.3%) >> DFOA-F_2[110] (**6**) (trace), also consistent with previous work, and the proposition that assembly has a directional preference from the *N*-acetyl region through to the amine region.[35] The analogue showing the complete DP-for-*rac*-FDB exchange, DFOA-F_3[111] (**8**), was present in the peak at t_R 28.9 min (*m*/*z* 573.3, 287.1), with its assignment supported by its characteristic MS/MS fragmentation pattern. Each species had a different absolute retention time using LC-Q (**Figure 1**) or LC-QQQ (**Figure 2**) instrumentation, although the relative elution order of the series of analogues was maintained. The presence of **1**–**8** was evident using EIC as the detection mode, presented as a sum of the $[M+H]^+$ and $[M+2H]^{2+}$ adducts for each set of isomers (**Figure 1c**).

High resolution mass spectrometry (HRMS) analysis of samples of DFOA-F_1[001] (**2**) (found $[M+H]^+$ 565.33557, $[C_{24}H_{46}FN_6O_8]^+$ requires 565.33615) and DFOA-F_3[111] (**8**)

(found $[M+H]^+$ 573.28567, $[C_{22}H_{40}F_3N_6O_8]^+$ requires 573.28600), as purified by semi-preparative HPLC, supported the structure assignment.

Dimeric Precursors of Fluorinated Analogues of DFOB. The production of **1**–**8** depends upon the availability of the cognate dimeric AHDP-SHDP-like precursors **9**–**12** (**Chart 2**), which were detected by EIC (**Figure 1g**). Dimeric precursor **9** (dDFO[00-], where 'd' denotes 'dimer' and '-' denotes the vacant position) would be produced and consumed for the synthesis of DFOB (**1**) and DFOA-F_1[001] (**2**). Dimeric precursor **10** (dDFO-F_1[01-]) would be produced and consumed for DFOA-F_1[010] (**3**) and DFOA-F_2[011] (**5**); dimeric precursor **11** (dDFO-F_1[10-]) for DFOA-F_1[100] (**4**) and DFOA-F_2[101] (**7**); and dimeric precursor **12** (dDFO-F_2[11-]) for DFOA-F_2[110] (**6**) and DFOA-F_3[111] (**8**). The dimeric precursors assembled from SHDP-SHDP-like fragments **13**–**16** were also detected, with low intensity signals for **13**–**15** and the signal for **16** (dDFO-F_2[-11]) dominant within this sub-set. The SHDP-SHDP-like dimeric precursors have been proposed as less viable substrates in trimer assembly, due to a reduced propensity towards DesD activation than the SHDP monomer,[35] and could be produced in relatively low amounts and/or as terminal products.

The semi-purified extract from the *rac*-FDB supplemented system yielded a species distribution that was more complex than other systems using minimally modified diamine substrates,[35,36] and showed the presence of other modified fluorinated analogues of DFOB (**Figure 1d–f**, **Table 1**) and modified dimers (**Figure 1h**). An additional set of fluorinated analogues of DFOB were detected comprising combinations of three substrates: DP, *rac*-FDB and DB. Further discussion of this complex tri-substrate system has been omitted.

Table 1. LC-MS Characteristics of Trimeric DFOB and Fluorinated Analogues of DFOB, As Assemblies of Three Monomers: **A**–**F**, **I**(**H**),[a] or **M**(**L**)[b] (Refer to **Scheme 3** for Structures).

No[c]	**Name**	t_R **(min)**	$[M+H]^+_{calc}$	$[M+H]^+_{obs}$	**Assembly**[c]
1	DFOB	34.8	561.4	561.4	ABB
2	DFOA-F_1[001]	33.8	565.3	565.3	ABD
2a	*N*-Ac-DFOA-F_1[001]	40.5	607.3	604.4	ABI(H)[a]
3	DFOA-F_1[010]	32.8	565.3	565.3	ADB
4	DFOA-F_1[100]	32.8	565.3	565.3	CBB
5	DFOA-F_2[011]	31.4	569.3	569.3	ADD
5a	*N*-Ac-DFOA-F_2[011]	38.5	611.3	611.1	ADI(H)[a]
6	DFOA-F_2[110]	31.4	569.3	569.3	CDB
7	DFOA-F_2[101]	31.4	569.3	569.3	CBD
7a	*N*-Ac-DFOA-F_2[101]	38.5	611.3	611.1	CBI(H)[a]
8	DFOA-F_3[111]	29.0	573.3	573.3	CDD
8a	*N*-Ac-DFOA-F_3[111]	36.3	615.3	615.3	CDI(H)[a]
2b$_i$	aDFOA-F_1[001$_a$]	33.1	549.3	549.3	ABF
2c$_i$	*N*-Ac-aDFOA-F_1[001$_a$]	37.7	591.4	591.3	ABM(L)[b]
3b$_i$	aDFOA-F_1[01$_a$0]	31.7	549.3	549.3	AFB
4b$_i$	aDFOA-F_1[1$_a$00]	31.7	549.3	549.3	EBB
5b$_i$	aDFOA-F_2[011$_a$]	30.7	553.3	553.3	ADF
5c$_i$	*N*-Ac-aDFOA-F_2[011$_a$]	37.4	595.3	595.3	ADM(L)[b]
5b$_{ii}$	aDFOA-F_2[01$_a$1]	30.7	553.3	553.3	AFD
5c$_{ii}$	*N*-Ac-aDFOA-F_2[01$_a$1]	37.4	595.3	595.3	AFI(H)[a]
6b$_i$	aDFOA-F_2[11$_a$0]	ND[d]	553.3	ND[d]	CFB
6b$_{ii}$	aDFOA-F_2[1$_a$10]	ND[d]	553.3	ND[d]	EDB
7b$_i$	aDFOA-F_2[101$_a$]	30.7	553.3	553.3	CBF
7c$_i$	*N*-Ac-aDFOA-F_2[101$_a$]	37.4	595.3	595.3	CBM(L)[b]
7b$_{ii}$	aDFOA-F_2[1$_a$01]	30.7	553.3	553.3	EBD
7c$_{ii}$	*N*-Ac-aDFOA-F_2[1$_a$01]	37.4	595.3	595.3	EBI(H)[a]
8b$_i$	aDFOA-F_1[111$_a$]	27.9	557.3	557.3	CDF
8c$_i$	*N*-Ac-aDFOA-F_1[111$_a$]	35.2	599.3	599.3	CDM(L)[b]
8b$_{ii}$	aDFOA-F_1[11$_a$1]	27.9	557.3	557.3	CFD
8c$_{ii}$	*N*-Ac-aDFOA-F_1[11$_a$1]	35.2	599.3	599.3	CFI(H)[a]
8b$_{iii}$	aDFOA-F_1[1$_a$11]	27.9	557.3	557.3	EDD
8c$_{iii}$	*N*-Ac-aDFOA-F_1[1$_a$11]	35.2	599.3	599.3	EDI(H)[a]

[a] Monomers **H** and **I** would be indistinguishable by MS/MS. [b] Monomers **M** and **L** would be indistinguishable by MS/MS. [c] Refer to **Chart 1** for structures of trimers.[d] ND = not detected.

***rac*-FDB is a Viable Substrate for DesBCD.** The fluorinated analogues of DFOB **2**–**8**, and modified trimeric analogues to be discussed in subsequent sections, can be considered an assembly of three monomers **A**–**N** (**Scheme 3**), which themselves are products of different pathways of DesBC catalysis (**Table 2**). In the native system (**Path I**), DesB-catalysed mono-*N*-hydroxylation of DP produces HDP, which is subject to DesC-catalysed *N*-acetylation to produce AHDP (**A**) or *N*-succinylation to produce SHDP (**B**). The two cycles of DesD catalysis ultimately produce trimeric DFOB, coded as **ABB** (**Table 1**). The production of **2**–**8** shows that *rac*-FDB is a viable substrate for DesB (**Path II**), with the product HFDB a viable substrate for DesC-mediated *N*-acetylation to give *N*-acetyl-*N*-hydroxy-1,4-diamino-2-fluorobutane (AHFDB) (**C**) or *N*-succinylation to give *N*-succinyl-*N*-hydroxy-1,4-diamino-2-fluorobutane (SHFDB) (**D**). Two DesD-catalysed cycles to condense different combinations of the monomers **A**, **B**, **C** and **D** would give DFOA-F_1[001] (**2**) (**ABD**), DFOA-F_1[010] (**3**) (**ADB**) or DFOA-F_1[100] (**4**) (**CBB**), DFOA-F_2[011] (**5**) (**ADD**), DFOA-F_2[110] (**6**) (**CDB**), DFOA-F_2[101] (**7**) (**CBD**) or DFOA-F_3[111] (**8**) (**CDD**). This pathway generated the highest fidelity fluorinated analogues of DFOB in this study, and reflects understanding from other studies of DFOB biosynthesis using PDB[35,36] and other approaches.[47-51]

Scheme 3. Action of DesB and/or DesC on DP or *rac*-FDB to yield monomeric precursors **A**–**N** as potential substrates for DesD-mediated assembly of dimers or trimers.

Table 2. Characterisation and Biosynthesis of Monomers **A**–**N**

Label	Substrate	Code	Region of trimer	T I[a]	T II[b]	HXA[c]	DesB[d]	$DesC_{C1}$[e]	$DesC_{C2}$[f]
A	DP	AHDP	*N*-Acetyl	*N*-Ac	NH_2	+	+	+	–
B	DP	SHDP	Internal or Amine	*N*-Suc	NH_2	+	+	+	–
C	*rac*-FDB	AHFDB	*N*-Acetyl	*N*-Ac	NH_2	+	+	+	–
D	*rac*-FDB	SHFDB	Internal or Amine	*N*-Suc	NH_2	+	+	+	–

E	*rac*-FDB	AFDB	*N*-Acetyl	*N*-Ac	NH_2	–	–	+	–
F	*rac*-FDB	SFDB	Internal or Amine	*N*-Suc	NH_2	–	–	+	–
G	*rac*-FDB	AHAFDB	Unviable	*N*-Ac	*N′*-Ac	+	+	+	+
H	*rac*-FDB	AHSFDB	Amine	*N*-Ac	*N′*-Suc	+	+	+	+
I	*rac*-FDB	SHAFDB	Amine	*N*-Suc	*N′*-Ac	+	+	+	+
J	*rac*-FDB	SHSFDB	Unviable	*N*-Suc	*N′*-Suc	+	+	+	+
K	*rac*-FDB	AAFDB	Unviable	*N*-Ac	*N′*-Ac	–	–	+	+
L	*rac*-FDB	ASFDB	Amine	*N*-Ac	*N′*-Suc	–	–	+	+
M	*rac*-FDB	SAFDB	Amine	*N*-Suc	*N′*-Ac	–	–	+	+
N	*rac*-FDB	SSFDB	Unviable	*N*-Suc	*N′*-Suc	–	–	+	+

[a] *N*-terminal chemical motif. [b] *N′*-terminal chemical motif. [c] HXA, containing an internal hydroxamic acid. [d] Substrate of DesB. [e] First-cycle substrate of DesC. [f] Second-cycle substrate of DesC.

Modified Fluorinated Analogues of DFOB: *N*-Acetylation. In order to examine the ability of **1**–**8** as Fe(III) chelators, an aliquot of Fe(III) was added to the mixture and the solution was re-analysed using LC-MS (**Figure 1i**). Signals for the Fe(III) complexes of **1**–**8** were detected with *m*/*z* values consistent with the corresponding $[M+H]^+$ adducts (where M is defined as the neutral complex between Fe(III) and the triple-deprotonated hydroxamate ligand). Two dominant signals at t_R 26.0 and 25.1 min gave *m*/*z* values at 460.1 and 464.2, respectively, which were closer to values representing $[M]^+$ adducts of Fe(III) complexes of double-deprotonated dimeric ligands rather than trimeric ligands. With this assumption, back-calculation gave *m*/*z* values of the $[M+H]^+$ adducts of the free ligands of 407.2 and 411.2, respectively, which correlated with dimers **10a** and **12a** as *N*-acetylated products of **10** and **12** (**Chart 2**). Signals for free ligands **10a** and **12a** were detected using EIC (**Figure 1h**) and gave MS/MS fragmentation patterns consistent with the proposed structures (**Figure 2g**–**h**). The dimeric precursors **10** (dDFO-F_1[01-]) and **12** (dDFO-F_2[11-]) both contained a fluorine atom in the region proximal to the terminal amine group, which suggested this perturbation to electronic structure could promote the generation of the *N*-acetyl derivatives. As consistent with this hypothesis, *N*-acetylated variants of **14** (dDFO-F_1[-01]) or **16** (dDFO-F_2[-11]) were

detected as **14a** or **16a**, respectively. *N*-Acetylated variants of **9** (dDFO[00-]) or **11** (dDFO-F_1[10-]) were not detected.

Further analysis of the data for the trimeric fluorinated DFOB analogues **1**–**8** showed the presence of *N*-acetylated variants of **2** (**2a**), **5** (**5a**), **7** (**7a**), and **8** (**8a**) that were evident in the LC using EIC detection. Analogues **2a** and **8a** were well resolved, with **5a** and **7a** co-eluting (**Figure 1d**). MS/MS fragmentation analysis confirmed the assignment of each of **2a**, **5a**, **7a**, and **8a** (data for **2a** shown in **Figure 2i**, **Supporting Information Table 2**, **Supporting Information Figure 2**). Each of **2**, **5**, **7**, and **8** contained a *rac*-FDB unit in the amine region of DFOB. Similar to the native system, *N*-Acetyl-DFOB ($DFOD_1$, **1a**) was detected in the *rac*-FDB system. The absence of characteristic MS/MS fragments at *m*/*z* 405.2 (**3a** and **4a**) and *m*/*z* 409.2 (**6a**) indicated the *N*-acetyl variants of DFOA-F_1[010] (**3**), DFOA-F_1[100] (**4**) or DFOA-F_2[110] (**6**), which contained a DP-based substrate in the amine-containing region, were below detection levels. The relative increase in hydrophobicity of the *N*-acetyl variants compared to the amine parent compounds was evident from the systematic increase in LC retention time for both the trimeric (**Figure 1c**–**d**) and the dimeric compounds (**Figure 1g**–**h**).

***rac*-FDB Promotes Two Cycles of DesC Catalysis With Products Viable DesD Substrates.** The production of the *N*-acetyl fluorinated analogues of DFOB indicated the possibility that the first-round products of DesC catalysis, AHFDB (**C**) or SHFDB (**D**), remained competent as substrates for a second round of DesC catalysis (**Path II**, C2), which in the instance of the use of acetyl-CoA as co-substrate, would produce *N*-acetyl-*N*-hydroxy-*N*′-acetyl-1,4-diamino-2-fluorobutane (AHAFDB) (**G**) or *N*-succinyl-*N*-hydroxy-*N*′-acetyl-1,4-diamino-2-fluorobutane (SHAFDB) (**I**), respectively. Monomer **G** would be predicted to be a terminal product, due to the flanking *N*,*N*′-diacetyl groups resulting in a loss of

reactivity. The second-round DesC processing of AHFDB (**C**) or SHFDB (**D**) with succinyl-CoA as co-substrate, would give *N*-acetyl-*N*-hydroxy-*N*′-succinyl-1,4-diamino-2-fluorobutane (AHSFDB) (**H**) or *N*-succinyl-*N*-hydroxy-*N*′-succinyl-1,4-diamino-2-fluorobutane (SHSFDB) (**J**), respectively. Although monomers **H** and **I** have distinct chemical structures, these monomers would be indistinguishable by LC-MS/MS. Similar to monomer **G**, the dicarboxylic acid-containing monomer **J** would likely be a terminating product. The viability of monomer **I** (or **H**) as a substrate for DesD was consistent with the production of *N*-Ac-DFOB-F_1[001] (**2a**), coded as trimer **ABI**(**H**). The viability of monomer **I** (or **H**) as a DesD substrate also correlated with the production of *N*-Ac-DFOB-F_2[011] (**5a**) (**ADI**(**H**)), *N*-Ac-DFOB-F_2[101] (**7a**) (**CBI**(**H**)) and *N*-Ac-DFOB-F_3[111] (**8a**) (**CDI**(**H**)). The DesD-mediated installation of the *N*-acetyl capped fluorinated monomer **I** (or **H**) was viable only in the terminal position (**2a**, **5a**, **7a**, **8a**).

$DFOD_1$ as the *N*-acetylated variant of DFOB was first identified as a minor product from a large-scale culture of *S. pilosus* (Stamm ETH 21 748),[53] and was identified in this work as a minor species. This indicates that at least monomer **B**, as one of the native first-round products of DesC catalysis, has some competence as a substrate for second-round DesC catalysis. In the presence of its first-round product SHDP and acetyl-CoA as co-substrate, DesC could generate the second-round product *N*-succinyl-*N*-hydroxy-*N*′-acetyl-1,5-diaminopentane (SHADP). DesD-mediated condensation of SHADP to the dimeric precursor **AB** would form $DFOD_1$. Other bacteria, including *Streptomyces chartreusis*,[55] *Salinispora tropica* CNB-440,[54] *Streptomyces avermitilis*,[56] and *Micrococcus luteus* KLE1011,[57] have been shown to produce *N*-acetyl variants of DFOB, which supports the broader substrate specificity for DesCD, and reflects a chemical motif commonly present in marine siderophores.[58,59] Others have speculated on an iterative function of the acylation

enzyme involved in the biosynthesis of symmetric rhizoferrin,[60] in support of the posit for DesC made here.

The current work has demonstrated that medium supplementation with *rac*-FDB under PDB conditions increased the propensity of *S. pilosus* to biosynthesize *N*-acetyl fluorinated analogues of DFOB, likely due to the fluorine atom proximal to the amine group of AHFDB (**C**) or SHFDB (**D**) enhancing a second-round of DesC-catalyzed *N*′-acetylation of the monomer. The *N*-acetyl fluorinated DFOB analogues *N*-Ac-DFOA-F_1[001] (**2a**), *N*-Ac-DFOA-F_2[011] (**5a**), *N*-Ac-DFOA-F_2[101] (**7a**) or *N*-Ac-DFOA-F_3[111] (**8a**) are likely assembled from the DesD-mediated second-round condensation of the *N*-acetyl monomer **I** (or **H**) with the respective dimeric precursors **AB**, **AD**, **CB** or **CD**. For example, the DesD-mediated condensation of the *N*-acetyl monomer **I** (SHAFDB) to the native heterodimer AHDP-SHDP (**9**) would generate *N*-Ac-$DFOA_1$[001] (**2a**) (**Scheme 4a**, solid lines).

Scheme 4. Biosynthesis of *N*-Ac-DFOA-F_1[001] (**2a**), *N*-Ac-dDFO-F_1[01-] (**10a**) or *N*-Ac-dDFO-F_2[11-] (**12a**) from: (**a**) the concerted use by DesD of *N*-acetyl monomer **I** (SHAFDB) as the second-round product of DesC-mediated catalysis of SHFDB; or (**b**) the use by DesD of SHFDB in discrete reactions, with dimeric and trimeric products subject to *N*-acetylation in the final step. The dotted or solid lines refer to the biosynthesis of the dimeric or trimeric species, respectively.

An alternative explanation could involve the assembly of the cognate hydroxamic acid-containing trimer **ABD** (**2**), **ADD**, (**5**), **CBD** (**7**), or **CDD** (**8**), with these products subject to *N*-acetylation, as mediated by DesC catalysis, or by an alternative enzyme (**Scheme 4b**, solid lines). This latter proposition, as intimated by others,[56] would seem less likely than the

first, for several reasons. The most compelling reason relates to the gain in biosynthetic efficiency relating to the production of the *N*-acetyl dimers **10a** and **12a**. In the first scenario, the *N*-acetyl monomer **I**, as the second-round product of DesC catalysis on substrate SHFDB, could be used in a concerted fashion by DesD to produce in two separate reactions **10a** and **12a** (**Scheme 4a**, dotted lines), requiring a total of three reactions from SHFDB to produce **10a** and **12a**. The production of **10a** and **12a** under the alternative scenario would require two separate DesD-catalysed reactions to generate **10** and **12** and two separate DesC-catalysed reactions to install the *N*-acetyl group (**Scheme 4b**, dotted lines), giving a total of four reactions. This would also require DesC to accept variable sized dimeric and trimeric hydroxamic acid substrates, rather than optimising its function to transform minimally modified mono-hydroxamic acids as its principal substrate type. Further, given the energetic costs of siderophore biosynthesis,[61,62] it would seem redundant for biology to evolve an enzyme distinct from DesC that catalyses the *N*-acetylation of dimeric and trimeric hydroxamic acids.

Modified Fluorinated Analogues of DFOB: Distribution of Hydroxamic Acid and Amide Groups. One other type of analogue of DFOB was observed from the *rac*-FDB supplemented system. A signal at t_R 33.1 min (**Figure 1b**) gave an *m*/*z* value 549.3, which was 16 units less than the *m*/*z* value calculated for the fluorinated DFOB analogues **2**–**4**. Analysis of the MS/MS fragmentation pattern was consistent with a trimer with one DP-for-*rac*-FDB exchange and that contained two hydroxamic acid units (one less than DFOB) and three amide bonds (one greater than DFOB). The fragmentation pattern of the signal at t_R 33.10 was consistent with aDFOA-F_1[001_a] (**2b**$_i$) (**Figure 2f**, **Supporting Information Table 3**, **Supporting Information Figure 3**), where the 'a' prefix in the compound notation denotes the presence of an additional amide bond, with its position marked as the subscript in

square brackets. Of this class of species, **2b$_i$** was detected as the major species (**Figure 1e**) as the amide modified **2**, and was present in sufficient concentration to allow the detection of the corresponding Fe(III) complex (**Figure 1i**). HRMS analysis of isolable aDFOA-F$_1$[001$_a$] (**2b$_i$**) (found $[M+H]^+$ 549.34065, $[C_{24}H_{46}FN_6O_7]^+$ requires 549.34123) supported the structure assignment. Tri-amide-containing **3b$_i$** and **4b$_i$** as the modified analogues of **3** and **4**, respectively, co-eluted with lower intensity signals.

The fluorinated analogues of DFOB that contained two DP-for-*rac*-FDB exchanges **5** and **7** were also detected as the tri-amide variants. Each of **5** and **7** was detected as two different isomers, with positional differences in the amide bond. DFOA-F$_2$[011] (**5**) was present with aDFOA-F$_2$[011$_a$] (**5b$_i$**) and aDFOA-F$_2$[01$_a$1] (**5b$_{ii}$**); and DFOA-F$_2$[101] (**7**) was present with aDFOA-F$_2$[101$_a$] (**7b$_i$**) and aDFOA-F$_2$[1$_a$01] (**7b$_{ii}$**). aDFOA-F$_2$[11$_a$0] (**6b$_i$**) and aDFOA-F$_2$[1$_a$10] (**6b$_{ii}$**) were not detected, similar to the detection of DFOA-F$_2$[110] (**6**) in only trace amounts. DFOA-F$_3$[111] (**8**) was present with three isomers: aDFOA-F$_3$[111$_a$] (**8b$_i$**), aDFOA-F$_3$[11$_a$1] (**8b$_{ii}$**) and aDFOA-F$_3$[1$_a$11] (**8b$_{iii}$**). *N*-Acetylated variants of the tri-amide compounds **2b$_i$**, **5b$_i$**, **5b$_{ii}$**, **7b$_i$**, **7b$_{ii}$**, **8b$_i$**, **8b$_{ii}$**, **8b$_{iii}$** were detected as **2c$_i$**, **5c$_i$**, **5c$_{ii}$**, **7c$_i$**, **7c$_{ii}$**, **8c$_i$**, **8c$_{ii}$**, **8c$_{iii}$** in low signal-to-noise peaks, in cases where the fluorinated substrate was present in the amine region of DFOB (**Figure 1f**, **Supporting Information Table 4**, **Supporting Information Figure 4**).

DesC Can Act Directly on *rac*-FDB as a Substrate. The identification of fluorinated analogues containing one additional amide bond (total = 3) and one less hydroxamic acid group (total = 2) than DFOB (**2b$_i$**, **3b$_i$**, **4b$_i$**, **5b$_i$**, **5b$_{ii}$**, **7b$_i$**, **7b$_{ii}$**, **8b$_i$**, **8b$_{ii}$**, **8b$_{iii}$**) was consistent with the notion that DesC could act directly on *rac*-FDB as a substrate (**Scheme 3**, **Path III**). Other DFOB or DFOE analogues with partial *N*-hydroxylation have been reported, as assembled from exogenous 1,6-diaminohexane[32] or endogenous DP.[56,63] DesC-mediated *N*-

acetylation or *N*-succinylation of *rac*-FDB would give *N*-acetyl-1,4-diamino-2-fluorobutane (AFDB) (**E**) or *N*-succinyl-1,4-diamino-2-fluorobutane (SFDB) (**F**), respectively. Second-round DesC catalysis of monomers **E** or **F** with acetyl-CoA as co-substrate, would give *N*,*N*′-diacetyl-1,4-diamino-2-fluorobutane (AAFDB) (**K**) or *N*-succinyl-*N*′-acetyl-1,4-diamino-2-fluorobutane (SAFDB) (**M**). DesC processing of **E** or **F** with co-substrate succinyl-CoA would give *N*-acetyl-*N*′-succinyl-1,4-diamino-2-fluorobutane (ASFDB) (**L**) (indistinguishable from monomer **M** by LC-MS/MS) or *N*,*N*′-disuccinyl-1,4-diamino-2-fluorobutane (SSFDB) (**N**), respectively. Similar to **G** and **J** (**Path II**), *N*,*N*′-diacetylated **K** and *N*,*N*′-disuccinylated **N** would be predicted as terminal products.

The production of trimeric **2b$_i$**, **3b$_i$**, **4b$_i$**, **5b$_i$**, **5b$_{ii}$**, **7b$_i$**, **7b$_{ii}$**, **8b$_i$**, **8b$_{ii}$** or **8b$_{iii}$** would result following two cycles of DesD-mediated assembly of different combinations of the non-hydroxamic acid containing monomers **E** and **F**, and the hydroxamic acid-containing monomers **A**–**D** (**Table 1**). Trimer **2c$_i$**, as the *N*-acetyl variant of **2b$_i$**, would be assembled from **AB** as the code of the dimeric precursor common to **2b$_i$**, with the terminal monomer **F** replaced by monomer **M** (or **L**). The analogous replacement of terminal monomer **F** (**5b$_i$**, **7b$_i$**, **8b$_i$**) with monomer **M** (or **L**) would give **5c$_i$**, **7c$_i$**, **8c$_i$**; or terminal monomer **D** (**5b$_{ii}$**, **7b$_{ii}$**, **8b$_{ii}$**, **8b$_{iii}$**) with monomer **I** (or **H**) giving **5c$_{ii}$**, **7c$_{ii}$**, **8c$_{ii}$**, **8c$_{iii}$**.

Substrate Specificity of DesD. A noteworthy feature of these tri-amide-containing variants of fluorinated analogues of DFOB was the increase in the number of amide bonds and corresponding decrease in the number of hydroxamic acid groups remained constant at one, even in compounds that contained two or three *rac*-FDB-containing substrates. This was an unusual phenomenon, since it might be envisaged that the number of additional amide bonds was correlated with the number of *rac*-FDB substrates incorporated.

One conceivable explanation for this relates to the substrate selectivity of DesD. In the second-round of DesD-mediated catalysis, the enzyme-substrate complex must comprise a dimer and a monomer, with condensation forming the ultimate trimer. The results here indicate that a viable DesD enzyme-substrate complex must have an overall configuration of molecules containing at least two hydroxamic acid groups. The dimeric precursor may contain between 1-2 hydroxamic acid units, and the terminal monomer between 0-1 hydroxamic acid units, with the sum across the dimer and monomer necessarily meeting the minimum threshold of 2 to ensure a viable condensation reaction. This notion suggests that the hydroxamic acid units are likely to play an important role in intermolecular interactions in the DesD active site, with a high likelihood of participating in hydrogen bonding networks. X-ray crystallographic data from DFO-type siderophore biosynthetic enzymes with bound substrates and/or products,[64,65] will provide further insight into the nature of these intermolecular interactions. The broad substrate specificity of DesBCD is in agreement with related enzymes used in the biosynthesis of macrocyclic dihydroxamic acid siderophores.[66,67]

It was considered that any mono-hydroxamic acid-containing trimers (4 amide bonds, 1 hydroxamic acid group) that could form a counterpoint to this argument may have been lost during the purification protocol. LC-MS analysis from the crude supernatant showed no detectable compounds of this type. The presence of a minimum of two hydroxamic acid units in the DesD-dimer-monomer assembly for second-round catalysis appears a reasonable supposition from a functional and evolutionary vantage,[68] since the assembly of a dihydroxamic acid trimer would retain some Fe(III) chelating ability, while a mono-hydroxamic acid trimer would have a modest affinity and poor selectivity towards Fe(III).

Fluorinated Analogues of DFOB: Enantioselective Assembly. The majority of the work, including repeat experiments, was undertaken using *S. pilosus* ATCC 19797 (**Figure 1** and

Figure 2). During the course of the study, reduced levels of DFOB production in *S. pilosus* ATCC 19797 prompted the use of the alternative DFOB-producing species *S. pilosus* AS5745 ISP2 for some work (**Figure 3**). Experiments were undertaken for a series of cultures of *S. pilosus* AS5745 ISP2 supplemented with *rac*-FDB, *R*-FDB or *S*-FDB, to examine any enantioselective effects of DesBCD catalysis. These effects would not be observable with the symmetric diamine substrates used in the biosynthesis of achiral DFOB, but might be discernible using chiral substrates. The enantiomeric excess (e.e.) of *R*-FDB or *S*-FDB was estimated as 42% or 40%, respectively, based upon ^{1}H NMR spectra acquired in the absence and presence of (18-crown-6)-2*R*,3*R*,11*R*,12*R*-tetracarboxylic acid (Supporting Information Figure 5), which has been shown to be an effective chiral shift reagent for several substituted diamines.[69] These spectra and additional LC-MS measurements verified the chemical purity of *rac*-FDB, *R*-FDB and *S*-FDB (Supporting Information Figure 6).

The semi-purified extract from a *rac*-FDB supplemented culture was analysed by LC-MS with EIC signals added for the $[M+H]^+$ and $[M+2H]^{2+}$ adducts of DFOB (**1**), the set of DFOA-F_1 isomers (**2**–**4**), the set of DFOA-F_2 isomers (**5**–**7**), and DFOA-F3[111] (**8**), shown in **Figure 3** in panels **a**, **d**, **g** and **j**, respectively. In this system, the peak integrals increased in a systematic fashion from DFOB (**1**) (5.7%) to **2**–**4** (10.5%), **5**–**7** (35.5%) to **8** (48.3%). The distribution of these species closely matched that from the *R*-FDB supplemented culture (**Figure 3b**,**e**,**h**,**k**). In the case of the *S*-FDB supplemented culture, the major species was DFOB, with a systematic attenuation in the peak integrals for **2**–**4**, **5**–**7** and **8** (**Figure 3c**,**f**,**i**,**l**). These results suggest the preferential use of *R*-DFB as a substrate, compared to *S*-FDB, and indicated that one or more enzymes from the DesBCD cluster was acting in an enantioselective fashion. These results suggest that DesBCD could be useful in chiral syntheses of low-molecular weight compounds, with potential applications in pharmaceutics development.

Figure 3. EICs for the $[M+H]^+$ and $[M+2H]^{2+}$ adducts of DFOB (**1**) (**a**–**c**), the cumulative set of DFOA-F_1 isomers (**2**–**4**) (**d**–**f**), the cumulative set of DFOA-F_2 isomers (**5**–**7**) (**g**–**i**), or DFOA-F_3 (**8**) (**j**–**l**) from semi-purified supernatant of *S. pilosus* AS5745 ISP2 cultures supplemented with *rac*-FDB (**a**, **d**, **g**, **j**), *R*-FDB (**b**, **e**, **h**, **k**) or *S*-FDB (**c**, **f**, **i**, **l**). Peak areas are given relative to the total area for **1**–**8** within each of the *rac*-FDB, *R*-FDB or *S*-FDB systems.

Fluorinated Analogues of DFOB: Additional Isomerisation. The asymmetry of *rac*-FDB gives rise to the possibility of the formation of two different types of isomers following substrate conversion, featuring variable positions of the fluorine atom and/or stereochemistry (*R*-FDB, *S*-FDB). In terms of the regioisomers, DesB-catalysed mono-*N*-hydroxylation of *rac*-FDB could produce either *N*-hydroxy-1,4-diamino-2-fluorobutane (HFDB) (**Scheme 1b**) or *N*-hydroxy-1,4-diamino-3-fluorobutane (not shown). The amine group in *rac*-FDB distal to the fluorinated region might be thought as the preferred site of *N*-hydroxylation, based on the structure of alcaligin, which contains the *C*-hydroxyl groups distal to the hydroxamic acid motifs,[70-72] correlating with *N*-hydroxy-1,4-diamino-2-hydroxybutane as substrate. However, this remains speculation, since no information on regioisomerism was discernible from ^{1}H or ^{19}F NMR spectra from DFOA-F_1[001] (**2**) or DFOA-F_3[111] (**8**) (Supporting Information Figure 7). It is possible that DesB-mediated catalysis of *rac*-FDB could give HFDB and *N*-hydroxy-1,4-diamino-3-fluorobutane, leading to mixed regioisomers of fluorinated analogues of DFOB, which could potentially be resolved by LC. This is a possible explanation for the presence of at least three signals resolved in the LC at the SIM value *m*/*z* 573, correlating with DFOA-F_3[111] (**8**) (**Figure 1d**, **Figure 2e**). Each of the peaks gave MS/MS

fragmentation patterns consistent with DFOA-F_3[111] (**8**), although the 2- and/or 3-fluoro regioisomers would be indistinguishable by MS/MS. The multiple signals for DFOA-F_3[111] (**8**) could also be due to LC-resolved diastereoisomers produced from enantioselective DesBCD-mediated biosynthesis.

Conclusions. Fluorinated analogues of DFOB were produced using a precursor-directed biosynthesis (PDB) approach involving supplementing *S. pilous* medium with the fluorinated diamine *rac*-FDB to compete against the native diamine DP during assembly. Fluorinated analogues were characterised by LC-MS and LC-MS/MS that contained one DP-for-*rac*-FDB exchange: DFOA-F_1[001] (**2**), DFOA-F_1[010] (**3**), DFOA-F_1[100] (**4**); two DP-for-*rac*-FDB exchanges: DFOA-F_2[011] (**5**), DFOA-F_2[110] (**6**), DFOA-F_2[101] (**7**); and exhaustive exchange: DFOA-F_3[111] (**2**). The two sets of constitutional isomers **2**–**4** and **5**–**7** have been similarly observed in recent PDB studies of *S. pilosus*.

Other types of species were observed, which provided new insight into the biosynthetic capacity of DesBCD. These species comprised different combinations of monomers (**A**–**N**) containing variable *N*-terminal motifs, arising from the iterative action of DesC in a first cycle (C1) or second cycle (C2) reaction; and the presence or absence of a hydroxamic acid unit, based on the action of DesC on HFDB or directly on *rac*-FDB, respectively. *N*-Acetylated trimers were observed of the type *N*-Ac-DFOA-F_1[001] (**2a**), with this modification present in analogues that contained the *rac*-FDB substrate in the amine-containing region of DFOB. Analogues containing one additional amide bond (total = 3) than DFOB, and one less hydroxamic acid group (total = 2) than DFOB, were observed. The additional amide bond was associated with the region containing the *rac*-FDB substrate, such as aDFOA-F_1[001_a] (**2b$_i$**). The composition of this class of species was constant in its containing 3 amide bonds and 2 hydroxamic acid groups, even in cases where two or three

rac-FDB substrates were incorporated into the trimer, which gave rise to regioisomers aDFOA-F_2[011_a] (**5b$_i$**) and aDFOA-F_2[01_a1] (**5b$_{ii}$**). These two phenomena (*N′*-acetylation, amide bond) could occur simultaneously in the same compound: *N′*-Ac-DFOA-F_1[001_a] (**2c$_i$**). Supplementation experiments using *rac*-FDB, *R*-FDB or *S*-FDB showed a similar species profile between *rac*-FDB and *R*-FDB. These data are consistent with the following. (i) DesB is competent in the *N*-hydroxylation of *rac*-FDB, with the products viable substrates for DesCD; (ii) DesC can act directly on *rac*-FDB, with the non-*N*-hydroxylated products viable substrates for DesD; (iii) the products of DesBC or DesC-mediated catalysis of *rac*-FDB can undergo a second round of DesC-mediated catalysis at the free amine; (iv) second-round DesC catalysis provides selected monomers that remain viable substrates for DesD, resulting in the production of *N*,*N′*-diacetylated dimeric and trimeric compounds; (v) the second-round DesD-mediated assembly of trimeric fluorinated DFOB analogues requires a minimum of two hydroxamic acid groups to form a viable enzyme-substrate(s) pre-complex; (vi) one or more DesBCD-catalysed steps in DFOB biosynthesis is enantioselective.

This work has provided a potential path to access fluorinated analogues of DFOB, which could have modified pharmacokinetic properties with clinical utility. The work has provided greater insight into the biosynthesis of this clinical metabolite, and has proposed that DesC acts in an iterative fashion *via* two in-sequence rounds of catalysis. This is being further explored in our laboratory using an *in vitro* approach with recombinant DesC, substrates and co-factors. The work highlights the merit of using a PDB approach to better understand the nuances of the biosynthesis of secondary metabolites, as a prelude to undertaking more targeted molecular biology approaches.

Materials and Methods

Reagents. Difco yeast mold (YM) broth was sourced from BD Biosciences. Potassium phosphate monobasic (≥99%), sodium phosphate dibasic (≥99%), trizma base (≥99%), zinc sulfate heptahydrate (≥99%), L-threonine (≥99.5%), chelex 100 sodium form, hydrochloric acid (37%), sodium hydroxide (≥98%), iron(III) perchlorate hydrate (≥99%), perchloric acid (70%), Amberlite XAD-2, chrome azurol S (CAS; 65%), piperazine (99%), 5-sulfosalicyclic acid dihydrate (≥99%), imidazole (99%), sodium chloride (≥99%), dimethyl sulfoxide (DMSO, ≥99%), formic acid (≥95%), and nitrilotriacetic acid (NTA; 99%) were sourced from Sigma-Aldrich. Calcium chloride (≥94%), magnesium sulfate heptahydrate (≥98%), methanol (≥99%), ethanol (≥99.5%), acetonitrile (ACN; 190 grade), and iron(III) chloride hexahydrate (≥98%) were sourced from Ajax Finechem. Ni(II) Sepharose 6 Fast Flow was from GE Healthcare, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acetate, disodium (EDTA; 99%) from AMRESCO, and *rac*-1,4-diamino-2-fluorobutane (*rac*-DFB), *R*-1,4-diamino-2-fluorobutane (*R*-DFB), and *S*-1,4-diamino-2-fluorobutane (*S*-DFB) from Innovapharm Ltd. (18-Crown-6)-2*R*,3*R*,11*R*,12*R*-tetracarboxylic acid was from Toronto Research Chemicals. All chemicals and solvents were used as received. MilliQ water was used in all experiments requiring water.

Instrumentation. Liquid chromatography-mass spectrometry (LC-MS) was conducted using an Agilent system consisting of a 1260 series quaternary pump with an inbuilt degasser, a 1200 series autosampler, thermostated column compartment, diode array detector, and fraction collector, and a 6120 series single quadrupole mass spectrometer. The drying gas flow, temperature, and pressure were set to 12 L min^{-1}, 350 °C, and 35 psi. Agilent OpenLAB Chromatography Data System (CDS) ChemStation Edition (B.04.02) was used for

data acquisition and processing. LC-MS/MS was conducted using an Agilent system consisting of a 1290 series quaternary pump with inbuilt degasser, autosampler, thermostated column compartment, and diode array detector, and a 6460 series triple quadrupole mass spectrometer with jet stream technology. Product ion mode was used for fragmentation using collision energy voltages optimised for individual precursor ions, ranging from 14–42 V. The fragmentor voltage was set to 150 V and the drying gas flow, temperature, and pressure to 10 L min^{-1}, 300 °C, and 25 psi. Agilent MassHunter Workstation (B.07.01) was used for data acquisition and processing. An Agilent Zorbax Eclipse XDB-C18 column (5 µm particle size, 150 mm length, 4.6 mm internal diameter) was used for analytical runs (10 µL injections) on both machines at a 0.2 mL min^{-1} flow rate with a gradient of 0–30% ACN (0.1% formic acid) in water (0.1% formic acid) over 40 min at 30 °C. An Agilent Zorbax Eclipse XDB-C18 column (5 µm particle size, 250 mm length, 9.4 mm internal diameter) was used for semi-preparative runs (100 µL injections) under the same conditions with a 1 mL min^{-1} flow rate on the same system used for LC-MS. Electrospray ionization was used in positive ion mode on both machines with a 4000 V capillary voltage. The systems were flushed with an NTA solution (20 mM phosphate, 2 mM NTA, pH 7.0) to remove residual Fe(III) prior to analysis.[73] High-resolution MS (HRMS) was conducted using a Bruker Apex qE 7T fourier transform ion cyclotron resonance mass spectrometer with an Apollo(II) ESI/MALDI dual source. The instrument was externally calibrated using PEG 600 and PEG 1500 before analysis. ^{1}H-NMR spectra were recorded in 5 mm Bruker SampleJet tubes, using a Bruker Avance III 600 MHz NMR spectrometer at a frequency of 600.13 MHz at 25 °C operated with Topspin 3.5pl.7 software. The spectral data are reported in ppm (δ) and referenced to residual solvent (methanol-d_4 4.97 and 3.42 ppm). ^{19}F-NMR spectra were recorded in 5 mm Pyrex tubes, using a Bruker Avance III 400 MHz wide-bore NMR spectrometer at a

frequency of 376.42 MHz at 25 °C operated with Topspin 3.5pl.7 software. The spectral data are reported in ppm (δ) and referenced to residual solvent (DMSO-d_6 2.60 ppm).

Bacterial culturing. Frozen stocks of *Streptomyces pilosus* ATCC 19797 or *S. pilosus* AS5745 ISP2, the latter strain which was kindly provided by Dr Ernest Lacey (Microbial Screening Technologies), were stored at –80 °C in DMSO (10% v/v) in YM broth (2.1% w/v). Precultures were grown in YM broth (2.1% w/v, pH 6.00 ± 0.05) for 4 d at 28 °C and 160 rpm in an Eppendorf New Brunswick Innova 42 Shaking Incubator. The base medium was made by combining a phosphate buffer with solutions of YM broth and other components. The final composition was YM broth (2.1%), potassium phosphate monobasic (235 mM), trizma base (35.0 mM), calcium chloride (13.6 mM), sodium phosphate dibasic (11.2 mM), magnesium sulfate heptahydrate (2.43 mM), threonine (0.84 mM), and zinc sulfate heptahydrate (13.9 μM).[52] All YM broth solutions and the phosphate buffer were stirred with chelex 100 for 3 h to remove residual Fe(III), were pH adjusted to 6.00 ± 0.05 using hydrochloric acid or sodium hydroxide, and were autoclaved (121 °C, 100 kPa, 20 min) for sterilisation. All work was completed in a biosafety cabinet using sterilised consumables and Fe(III)-depleted glassware or plastic-ware. Individual conditions were established by the addition of *rac*-DFB (10 mM), *R*-DFB (10 mM), or *S*-DFB (10 mM) solutions (pH 6.00 ± 0.05) to the required concentration. The other component mixture and substrate solutions were sterilised with Minisart 0.2 μm syringe filters before use. The bacterial cultures were grown for 8–10 d at 28 °C and 160 rpm. 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 after 10 min at 465 nm using a BMG Labtech FLUOstar Omega microplate reader. The supernatant was collected for purification by centrifugation (5000

rpm) when siderophore production had plateaued, and the quantity of bacterial cells in each culture was measured following lyophilisation with a Labconco FreeZone freeze-dryer.

Purification. XAD-2 purification was modified from previous methodology.[74,75] Amberlite XAD-2 resin (100 mL) was activated in methanol, suspended in a column with water, backwashed 3 times to give a homogenous resin distribution, and washed with 2 column volumes (CV) of water. The supernatant was introduced, column washed with 2 CV of water, and sample eluted with 2-4 CV of aqueous methanol (50% v/v). A flow rate of 5 mL min^{-1} was used. Siderophore presence was tracked using a CAS assay.[34,76] The CAS positive fractions (40 mL) from the elution step were taken to dryness *in vacuo* using a Buchi Rotavapor R-300. Ni(II)-based immobilised metal ion affinity chromatography (IMAC) was adapted from previous methodology.[34] Ni(II) sepharose (40 mL) was washed with water (5 CV) and charged with binding buffer (5 CV; 0.2 M sodium chloride, 10 mM HEPES, pH 9.0). The sample was dissolved in binding buffer (2 mL) and adsorbed onto the resin, which was then washed with binding buffer (5 CV), and the sample eluted with elution buffer (5 CV; 0.2 M sodium chloride, 10 mM HEPES, pH 5.5). The CAS positive fractions (40 mL) from the elution step were taken to dryness *in vacuo* and were desalted by dissolution in methanol. Individual peaks were isolated after XAD-2 and IMAC purification using semi-preparative HPLC, and were lyophilised. About 8 mg of fluorinated DFOB analogues were obtained per 50-mL culture. As averaged across five repeat experiments, the yields were DFOA-F_1[001] (**2**) (1.2 mg), aDFOA-F_1[001_a] (**2b$_i$**) (1.6 mg), DFOA-F_1[010] (**3**) and DFOA-F_1[100] (**4**) (1.4 mg as a mixture), DFOA-F_2[011] (**5**), DFOA-F_2[110] (**6**) and DFOA-F_2[101] (**7**) (1.6 mg as a mixture), and DFOA-F_3[111] (**8**) (2.2 mg).

High Resolution Mass Spectrometry (HRMS). Five aliquots were analysed by HRMS. DFOA-F_1[001] (**2**): found $[M+H]^+$ 565.33557, $[C_{24}H_{46}FN_6O_8]^+$ requires 565.33615. aDFOA-F_1[001_a] (**2b$_i$**): found $[M+H]^+$ 549.34065, $[C_{24}H_{46}FN_6O_7]^+$ requires 549.34123. DFOA-F_1[010] (**3**)/DFOA-F_1[100] (**4**): found $[M+Al^{3+}–2H]^+$ 589.29360, $[C_{24}H_{43}AlFN_6O_8]^+$ requires 589.29363. DFOA-F_2[011] (**5**)/DFOA-F_2[110] (**6**)/DFOA-F_2[101] (**7**): found $[M+H]^+$ 569.31050, $[C_{23}H_{43}F_2N_6O_8]^+$ requires 569.31108. DFOA-F_3[111] (**8**): found $[M+H]^+$ 573.28567, $[C_{22}H_{40}F_3N_6O_8]^+$ requires 573.28600.

LC-MS and LC-MS/MS analysis. Samples (0.1 mg) were dissolved in HPLC grade methanol (1 mL) for analysis by LC-MS and LC-MS/MS. Fe(III)-loaded samples were produced by adding 100 µL of an Fe(III) chloride solution (350 µM) to 100 µL of the analyte and incubating for 1 h.

Associated Content

Supporting Information (theoretical and experimental MS/MS fragmentation data, production of Fe(III)-complexing species in bacterial supernatant, LC-MS traces, ^{1}H NMR spectra) is available free of charge on the ACS Publications website: https://pubs.acs.org.

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