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1 **Biosynthesis of novel desferrioxamine derivatives requires unprecedented** 2 **crosstalk between separate NRPS-independent siderophore pathways**

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13 **Abstract**

14 Iron is essential to many biological processes, but its poor solubility in aerobic environments restricts its
15 bioavailability. To overcome this limitation, bacteria have evolved a variety of strategies, including the
16 production and secretion of iron-chelating siderophores. Here, we describe the discovery of four series of
17 siderophores from *Streptomyces ambofaciens* ATCC23877, three of which are unprecedented. MS/MS-
18 based molecular networking revealed that one of these series corresponds to acylated desferrioxamines
19 (acyl-DFOs) recently identified from *S. coelicolor*. The remaining sets include unprecedented tetra- and
20 penta-hydroxamate acyl-DFO derivatives, all of which incorporate a previously undescribed building
21 block. Stable isotope labeling and gene deletion experiments provide evidence that biosynthesis of the
22 acyl-DFO congeners requires unprecedented crosstalk between two separate non-ribosomal peptide
23 synthetase (NRPS) - independent siderophore (NIS) pathways in the producing organism. Although the
24 biological role(s) of these new derivatives remain to be elucidated, they may confer advantages in terms
25 of metal chelation in the competitive soil environment due to the additional bidentate hydroxamic
26 functional group(s). The metabolites may also find application in various fields including biotechnology,
27 bioremediation and immuno-PET imaging.

29 **Importance**

30 Iron-chelating siderophores play important roles for their bacterial producers in the environment, but they
31 have also found application in human medicine both in iron chelation therapy to prevent iron overload and
32 in diagnostic imaging, as well as in biotechnology, including as agents for biocontrol of pathogens and

33 bioremediation. In this study we report the discovery of three novel series of related siderophores, whose
34 biosynthesis depends on the interplay between two NRPS-independent (NIS) pathways in the producing
35 organism *S. ambofaciens* – the first example to our knowledge of such functional cross-talk. We further
36 reveal that two of these series correspond to acyl-desferrioxamines which incorporate four or five
37 hydroxamate units. Although the biological importance of these novel derivatives is unknown, the
38 increased chelating capacity of these metabolites may find utility in diagnostic imaging (for instance ⁸⁹Zr
39 based immuno-PET imaging) and other applications of metal chelators.

40

41 **Keywords**

42 NRPS-independent siderophore, biosynthetic pathway crosstalk, acyl-DFO, *Streptomyces*

43

44 **Introduction**

45 Although iron is abundant in the biosphere, its bioavailability is low due to the poor solubility of ferric
46 oxide and hydroxide complexes. To obtain this essential nutrient, many bacteria synthesize and excrete
47 specialized metabolites called siderophores which are capable of scavenging iron from their
48 surroundings. The resulting high-affinity Fe³⁺ complexes are then re-acquired by siderophore uptake
49 systems, followed by release of iron into the cytoplasm in Fe²⁺ form where it can play essential roles in
50 many biological processes (1). To date, substantial knowledge has been accumulated concerning the
51 diversity of siderophore chemical structures, as well as the molecular mechanisms underlying their
52 synthesis, export, uptake and regulation (2-5). Siderophores often function as virulence factors for
53 bacterial pathogens (e.g. aerobactin in enteric bacteria (6)) by sequestering iron from the animal or plant
54 host, but conversely, they can also play important roles in pathogen control (7). Siderophores have also
55 been implicated in mediating interspecies interactions (8). For example, siderophores produced by one
56 species can be expropriated by another species ('siderophore piracy'), or can induce the production of
57 endogenous siderophores under iron-limited conditions (9, 10). Together, these various modes of
58 siderophore action help to shape competition for iron in the environment, and facilitate various types of
59 social interactions in soil and marine bacterial communities (11).

60 Siderophores are selective for ferric iron, but can also bind a range of other metals including zinc,
61 manganese, copper, nickel, gallium, and aluminum (12). They are classified into four basic types
62 depending on the moiety involved in iron chelation: catecholate, hydroxamate, carboxylate and mixed-
63 type siderophores (13) (Fig. S1). These diverse structures are assembled via two principal means: NRPS
64 (14) and NRPS-independent siderophore (NIS) pathways (15). Notably, the siderophore products of NIS
65 pathways often contain diamine, citric acid, and dicarboxylic acid building blocks (Fig. S1).

66 The biosynthetic gene clusters (BGCs) encoding siderophores are widely distributed in

67 actinomycetes and *Streptomyces* in particular, allowing the bacteria to respond to resource competition
68 within the complex microbial community. In this context, *Streptomyces coelicolor* A3(2) has been well-
69 studied, and shown to produce coelichelin and desferrioxamines E (DFO-E) and B (DFO-B) under iron-
70 deficient conditions (16). Desferrioxamine B is of clinical importance, as it is approved by the US Food
71 and Drug Administration (FDA) for removal of excess iron from the body (chelation therapy). Coelichelin,
72 a tetrapeptide siderophore, is encoded by a NRPS gene cluster (*cch*), while the NIS pathway responsible
73 for DFOs-B and E comprises a locus of six genes (*desABCDEF*) (17) (Fig. 1A), with both clusters located
74 on the chromosome.

75 DFO-B and E are biosynthesized from L-lysine, acetyl-CoA and succinyl-CoA as building blocks
76 (Fig. 1B). The first step involves generation of 1,5-diaminopentane (otherwise known as cadaverine) by
77 decarboxylation of L-lysine, which is catalyzed by DesA, a pyridoxal 5-phosphate-dependent
78 decarboxylase (16). This step is followed by mono-*N*-hydroxylation carried out by DesB, a FAD-
79 dependent amine monooxygenase, resulting in *N*-hydroxy-cadaverine. This compound is then *N*-
80 acetylated by DesC, an acyl CoA-dependent acyl transferase, to yield *N*-hydroxy-*N*-acetylcadaverine
81 (HAC). Alternatively, DesC catalyzes *N*-succinylation to give *N*-hydroxy-*N*-succinylcadaverine (HSC).
82 Three units of HSC are activated and condensed by DesD, an NRPS-independent peptide synthetase, to
83 produce desferrioxamine G1 (DFO-G1) (18, 19), followed by a ring-closing reaction which yields DFO-E
84 (Fig. 1B). Linear DFO-B is composed of two units of HSC and a unit of HAC (Fig. 1B). The two other
85 genes in the *des* cluster (Table S1), *desE* and *desF*, encode respectively a cell surface-associated
86 lipoprotein receptor component of an ABC transporter involved in uptake of the iron-chelated forms
87 (called ferrioxamines), and a ferrioxamine reductase, an enzyme that removes iron from hydroxamate
88 siderophore complexes.

89 Previous studies revealed that *S. ambofaciens* ATCC23877 harbors a *des* BGC and a *cch* BGC, both
90 of which show strong conservation with the corresponding clusters in *S. coelicolor* (20, 21). The six
91 enzymes encoded by the *S. ambofaciens desABCDEF* exhibit 83–95% amino acid sequence identity to
92 their homologs in *S. coelicolor* (Fig. 1A, Table S1), and are responsible for the biosynthesis of DFO-B and
93 E (21). In this work, we report four series of siderophores, in addition to the previously identified DFO-B
94 and E, produced by both *Streptomyces ambofaciens* and mutants obtained by genetic engineering of the
95 stambomycin gene cluster (22). One of these series corresponds to the acyl-DFOs, a class of recently-
96 identified siderophores produced by *S. coelicolor* upon interaction with other actinomycetes (23). The
97 additional three sets of DFOs, which we term acyl-DFOs+30, acyl-DFOs+200+30 and acyl-
98 DFOs+200+200+30, were observed exclusively in *S. ambofaciens*. The numbers (+30, +200+30 and
99 +200+200+30) in the named sets of acyl-DFOs represent the difference in mass units observed in
100 MS/MS analysis. Results obtained from stable isotope labeling and gene deletion experiments allow us to
101 propose a biosynthetic pathway for these novel acyl-DFO derivatives, which involves unprecedented
102 crosstalk between separate NIS pathways. We further provide evidence that the ‘acyl-DFOs+200+30’ and
103 ‘acyl-DFOs +200+200+30’ variants represent respectively tetra- and penta-hydroxamate structures

104 distinct from those of the common bis-hydroxamate (e.g. bisucaberin (24)) and tris-hydroxamate iron (e.g.
105 DFOs) chelators, and thus suggesting that NIS pathways are capable of extending oligomerization. Given
106 the expanded binding capacity offered by the tetra- and penta-hydroxamate structures, the discovered
107 metabolites may find utility in diverse applications as metal chelators (25).

108

109 **Results**

110 **Identification of novel forms of desferrioxamines from *Streptomyces ambofaciens***

111 In the course of HPLC-MS analysis of a *S. ambofaciens* ATCC23877 mutant (K7N1/OE484) obtained
112 by genetic engineering of the stambomycin gene cluster (22), four series of related, but previously
113 undetected metabolites were identified as monoprotonated ions ($[M+H]^+$) with $m/z = 673, 687, 701, 715,$
114 $729, 743, 757$ (first series); $703, 717, 731, 745, 759, 773, 787$ (second series); $903, 917, 931, 945, 959,$
115 $973, 987$ (third series); and $1103, 1117, 1131, 1145, 1159, 1173, 1187$ (fourth series) (Fig. S2 and Table
116 S2). The first two series of masses are separated from each other by 30 mass units and thus they were
117 initially named 'NMs' (Newly-detected Metabolites) and 'NMs+30', respectively. The last two clusters of
118 masses differ sequentially from NMs+30 by respectively +200 and +200+200, and as such were
119 annotated as 'NMs+200+30' and 'NMs+200+200+30'. Based on the observed masses, we originally
120 hypothesized that the NMs could correspond to shunt polyketide products released from module 13 of the
121 stambomycin polyketide synthase (PKS) (22), followed by the loss of a water molecule, either by
122 dehydration or cyclization (the predicted cyclic form is shown in Fig. S2C) (26). However, these
123 compounds were also identified in the wild type strain, albeit at lower levels, an observation which is
124 inconsistent with classical PKS function (27) (Fig. S2B). Taken together, these data implied that the
125 observed series of masses were unrelated to the stambomycin pathway.

126 Each mass series comprises 7 members, wherein each differs sequentially by +14 from its
127 predecessor. In addition, the retention time for each NM/NM+30/NM+200+30/NM+200+200+30 group is
128 quite close (Fig. S2). Both of these observations are consistent with compounds of related structure.
129 However, on the basis of the integrated EIC peaks, we observed that the yields of the various series of
130 metabolites differed both within and between the various strains, with the titers of NMs and NMs+30
131 superior to those of NMs+200+30 and NMs+200+200+30, and the high-molecular weight series present
132 at 22–170% relative yield compared to the NMs (Fig. S2 and Table S3).

133 Using the Global Natural Products Social Molecular Networking platform (GNPS) (28), a molecular
134 network of mutant K7N1/OE484 based on the fragmentation spectra was generated (Fig. S3). Via this
135 method, compounds represented by nodes in the network are sorted based on the similarity of their
136 fragmentation spectra. Nodes representing highly similar spectra are connected by edges and are likely to
137 be structurally similar. Notably, the molecular network revealed that the NM series of compounds (shown
138 with blue dots in Fig. 2 and Fig. S3) corresponds to various acyl-desferrioxamine siderophores (acyl-

139 DFOs). Based on the GNPS ID hits, we identified the blue dots as C9–C15 acyl-DFOs (Fig. 2); although
140 longer acyl-DFOs (C16 and C17) have been reported (23), they were not detected in this work. In
141 addition, the corresponding ferrioxamines (acyl-FOs, Fig. 2 and Fig. S3), were also observed and linked
142 to DFOs in the same subnetwork. Bisucaberin (24), DFO-E, DFO-D2 (29, 30), Desf-07 (30), and
143 aluminum (Al)-chelated DFO-B were also annotated within this subnetwork, as were certain members of
144 NMs+30, NMs+200+30 and NMs+200+200+30 (Fig. 2 and Fig. S3), consistent with their membership in
145 the same metabolic family. The NM nodes are connected to DFOs and acyl-DFOs but do not correspond
146 to compounds indexed within the GNPS database, identifying them as potentially novel acyl-DFO
147 derivatives. We therefore re-designated them as acyl-DFOs+30, acyl-DFOs+200+30 and acyl-
148 DFOs+200+200+30, respectively (Fig. 2 and Fig. S3).

149 **Structure analysis of novel acyl-DFO derivatives**

150 As the relatively low yields of the novel metabolites compounded with the difficulty of
151 chromatographic separation of closely-similar structures (Fig. S2) precluded structure elucidation by
152 NMR, we opted for an MS/MS-based analysis. As a starting point, inspection of the previously identified
153 MS/MS fragments for DFOs and acylated DFOs (23), revealed that certain fragments were common to all
154 7 acyl-DFOs (C9–C15) (e.g. m/z $[M+H]^+$ = 401, 319, 201) (Fig. S4). Thus, these fragments must arise
155 from the shared amine portion of the structures. We also observed fragments encompassing the acyl
156 regions, as these differed by +14 between the acyl-DFO family members consistent with the presence of
157 additional $-CH_2$ units (e.g. the series m/z $[M+H]^+$ = 397, 515 and 597 arising from C12 acyl-DFO vs. 411,
158 529, and 611 from C13 acyl-DFO) (Fig. S4). In the case of the novel derivatives, we observed the same
159 acyl-derived fragments as for the corresponding acyl-DFOs, allowing in each case identification of the
160 acyl group appended to the DFO+30, DFO+200+30 and DFO+200+200+30 series of compounds (Fig. 3
161 and Fig. S4–7). In contrast, a new set of fragments common to all of the novel metabolites was identified
162 with masses of +30 relative to those observed in DFOs and acyl-DFOs (m/z $[M+H]^+$ = 431, 349, 231; Fig.
163 3 and Fig. S4–7). Thus, the +30 region is located within the opposite region of the compounds to the acyl
164 terminus.

165 Detailed analysis of the fragmentation patterns of the acyl-DFOs+200+30 and acyl-
166 DFOs+200+200+30 also allowed us to propose that the +200 and +200+200 arise via incorporation of
167 one or two additional building blocks of HSC, respectively, into the metabolite backbones (Fig. 4). For
168 example, fragments encompassing the acyl group of C12 acyl DFO+30 (m/z $[M+H]^+$ = 515 and 597) are
169 additionally observed at +200 in C12 acyl-DFO+200+30 (m/z $[M+H]^+$ = 715 and 797) (Fig. 3), while these
170 same +200 fragments as well as +200+200 fragments (m/z $[M+H]^+$ = 915 and 997) were seen from C12
171 acyl-DFO+200+200+30 (Fig. 3 and Fig. S6, 7). Additional sets of fragments show this pattern: m/z $[M+H]^+$
172 = 349 and 431 common to all acyl-DFOs+30 can be correlated with +200 fragments in both the acyl-
173 DFO+200+30 and acyl-DFO+200+200+30 series (m/z $[M+H]^+$ = 549 and 631), while the corresponding
174 +200+200 fragments are observed with the acyl-DFOs+200+200+30 (m/z $[M+H]^+$ = 749 and 831) (Fig. 3

175 and Fig. S6, 7). The +200 and +200+200 versions of DFO-B, DFO-D2, DFO-E and DFO-G1 were not
176 seen.

177 Nonetheless, at this stage, the precise structural differences giving rise to the +30 mass discrepancy
178 were unclear. One possible explanation we considered was transformation of the terminal amine group
179 ($-NH_2$) into a nitro group ($-NO_2$). This chemistry has been reported to occur during LC-ESI-MS analysis,
180 as electrochemical reactions can take place at the liquid-metal interface on the capillary, leading to
181 reactive oxygen species (e.g. $\bullet OOH$ and H_2O_2) capable of amine oxidation (31, 32). Alternatively, the NO_2
182 could derive from spontaneous enzymatic oxidation of NH_2 . To assess this latter possibility, we carried
183 out a BLASTP search with *S. ambofaciens* using the sequence of PrnD from *Pseudomonas fluorescens*,
184 which encodes a [2Fe-2S] Rieske non-heme *N*-oxygenase involved in the biosynthesis of the antifungal
185 antibiotic pyrrolnitrin (33, 34). This analysis identified SAM23877_0811, with 39% amino acid sequence
186 identity to PrnD (Fig. S8). The second overall explanation is that the +30 compounds incorporate an
187 alternative 1,3-diaminopropane-derived building block (*vide infra*).

188 **Incorporation *in vivo* of isotopically-labeled lysine**

189 In order to differentiate between the potential origins of the +30 moiety (an enzymatic or chemical
190 derivative of lysine vs. integration of an alternative monomer) as well as to obtain more direct evidence
191 for the number of HSC building blocks present in each series of molecules, we supplemented cultures of
192 strain K7N1/OE484 with isotopically-labeled lysine ($^{13}C_6$, $^{15}N_2$ -lysine, 1 mM). If lysine were the origin of
193 the +30 moiety, we would have expected to observe as many as three Lys molecules incorporated into
194 the acyl-DFOs+30, four into the acyl-DFOs+30+200, and five into the acyl-DFOs+200+200+30. Instead,
195 using the C12 acyl-DFO series as an example, analysis of the isotopic distribution of the parent ions in
196 fed vs. unfed samples, showed that while the C12 acyl-DFO incorporated up to three units of labeled
197 lysine (mass shifts of +7, +14 and +21 Da) (Fig. 5B), only a maximum of two lysines were integrated into
198 the +30 derivative, three into the +200+30 compound and four into the +200+200+30 analogue (Fig. 5D,
199 F, H). These data are fully consistent with the +200 masses arising from incorporation of additional HSC
200 units, but not with *N*-oxidized lysine as the origin of the +30 mass difference. Furthermore, integration of
201 labeled lysine did not increase the mass of a characteristic fragment of C12 acyl-DFO+30 containing the
202 +30 region ($m/z [M+H]^+ = 149$), while a deletion mutant in the putative *N*-oxygenase SAM23877_0811 still
203 produced the +30/+200+30/+200+200+30 congeners of the acyl-DFOs (Table S3).

204 **A putative 1,3-diaminopropane-derived building block involved in the biosynthesis of novel DFO** 205 **derivatives**

206 In general, the structures of NIS-mediated siderophores, and in particular the hydroxamate-type,
207 incorporate a diamine moiety, such as 1,3-diaminopropane, 1,4-diaminobutane (putrescine) or 1,5-
208 diaminopentane (cadaverine) (Fig. S11). The origins of the backbones of 1,3-diaminopropane, putrescine
209 and cadaverine are (*S*)-2-amino-4-oxobutanoic acid, L-ornithine and L-lysine, respectively. More
210 specifically, in the biosynthetic pathway of schizokenin in *Nostoc sp.* PCC 7120 (also known as

211 *Anabaena* sp. PCC 7120), (S)-2-amino-4-oxobutanoic acid is transformed into 1,3-diaminopropane by
212 sequential action of an L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase (DABA AT, *all0396*)
213 and a L-2,4-diaminobutyrate decarboxylase (DABA DC, *all0395*) (Fig. 6A, B) (35-37). Genes encoding the
214 DABA AT and DABA DC pair are also present in the BGCs of the 1,3-diaminopropane-based
215 siderophores synechobactins (38), rhizobactin 1021 (39), and acinetoferrin (40).

216 Interestingly, we identified two loci on the chromosome of *S. ambofaciens*, one of which contains
217 homologs of *all0392* (43% identity at the AA sequence level) and *all0395* (39%) involved in the
218 biosynthesis of schizokenin, while the other comprises homologs of *all0396* (39%), *all0393* (37%), and
219 *all0394* (40%) (Fig. 6A). It thus appears that a portion of an ancestral *szk* cluster was relocated to an
220 alternative region within the *S. ambofaciens* genome. Nonetheless, a homolog of gene *all0390* encoding
221 the second NIS enzyme required for schizokenin biosynthesis is not present in either locus, and thus the
222 pathway appears incomplete. In accord with this observation, no schizokenin was detected in cultures of
223 *S. ambofaciens* wild type and its mutants (data not shown). Nonetheless, the availability of additional
224 genes capable of diamine synthesis prompted us to consider a new pathway to a 1,3-diaminopropane-
225 derived building block in *S. ambofaciens* (Fig. 6C), based on collaboration between enzymes encoded by
226 the desferrioxamine and schizokenin-like (*szk*-like) gene clusters.

227 In this scheme, DesABC would be responsible for generation of the cadaverine-based units HSC and
228 *N*-hydroxy-*N*-acyl-cadaverine, while either of the enzyme pairs SAM23877_5502 (DABA
229 AT)/SAM23877_2103 (DABA DC) and/or SAM23877_5502 (DABA AT)/DesA_Sa (DABA DC) (36) would
230 produce 1,3-diaminopropane. Subsequently, 1,3-diaminopropane would be *N*-hydroxylated by DesB and
231 SAM23877_2102 (or *N*-hydroxylated twice by one of the two enzymes) on its two amino termini, followed
232 by *N*-acetylation and *N*-succinylation by DesC and SAM23877_5504, respectively (or via bifunctional
233 catalysis by one of the two enzymes). The final step would involve concatenation of the monomers via
234 amide bonds, catalyzed by the two NIS synthetases DesD and SAM23877_5503 (or alternatively by one
235 of the two), resulting in the synthesis of acyl-DFOs+30, acyl-DFOs+200+30 and acyl-DFOs+200+200+30.

236 Given that the +30 building block could in principle be incorporated into other desferrioxamines
237 produced by the strain, we also scrutinized the extracts for the corresponding versions of DFO-B, DFO-E
238 and DFO-G1. This analysis revealed the +30 forms of both linear DFO-B and DFO-G1 (Fig. S5), but not
239 of cyclic DFO-D2 and DFO-E.

240 **Testing the biosynthetic hypothesis by gene deletion within the *des* and *szk*-like clusters**

241 To evaluate whether the biosynthesis of novel DFO derivatives requires crosstalk between the *des*
242 and *szk*-like clusters, several mutants were created using the stambomycin engineering mutant K7N1 as
243 the base strain. We first deleted genes *desC* (*N*-acetylase/*N*-succinylase) and *desD* (NIS synthetase) in
244 the *des* cluster using CRISPR-Cas9 (Fig. S12), followed by the introduction of plasmid pOE484 (in order
245 to make it equivalent to the control strain K7N1/OE484), resulting in the mutant K7N1/ Δ *desCetD*/OE484.
246 Analysis by HPLC-MS of the mutant relative to the parental strain revealed that all DFO production was

247 completely abolished (Fig. S15B). Thus, the *des* cluster is essential for biosynthesis of all types of DFOs
248 in *S. ambofaciens*. The split *szk*-like cluster was also inactivated by CRIPSR-Cas9-mediated deletion by
249 generation of a mutant in each locus, K7N1/ Δ *sam2102et03*/OE484 (*N*-hydroxylase and DABA DC
250 mutant) and K7N1/ Δ *sam5502et03et04*/OE484 (DABA AT, NIS synthetase and *N*-acetylase/*N*-succinylase
251 mutant) (Fig. S13, 14). Although neither *szk* deletion mutant completely abrogated DFO production (Fig.
252 S15C, D), the yields of the various series of DFOs were all reduced by 10–100 fold relative to
253 K7N1/OE484 (Table S3), strongly implicating the *szk*-like cluster in their biosynthesis (Fig. 6C).

254 Indeed, the continued production of these novel acyl-DFOs by K7N1/ Δ *sam2102et03*/OE484 is fully
255 consistent with the proposed functional redundancy between the *N*-hydroxylases SAM23877_2102 and
256 DesB, and the DABA DCs SAM23877_2103 and DesA_Sa. Production of +30 containing acyl-DFOs by
257 mutant K7N1/ Δ *sam5502et03et04*/OE484 was less readily explained, as no enzyme encoded by the *des*
258 cluster could functionally complement the missing SAM23877_5502 for DABA synthesis. However,
259 reinspection of the *S. ambofaciens* genome sequence revealed multiple SAM23877_5502 homologs
260 which could rescue the missing activity, including SAM23877_1053 (32% identity and 41% similarity at
261 the aa level), SAM23877_6419 (29% identity, 42% similarity), and SAM23877_1939 (28% identity, 41%
262 similarity). SAM23877_1939 is especially interesting in this context, as it functions as a DABA AT (EctB)
263 during biosynthesis of DABA in the pathway to ectoine and hydroxyectoine (Fig. S16) (41).

264 ***In vivo* incorporation of labeled acetate**

265 In our proposed pathway to the alternative building block (Fig. 6), a 1,3-diaminopropane-derived unit
266 bearing both an *N*-succinyl and an *N*-acetyl is generated via *szk*-like chemistry. As acetate is the
267 distinguishing feature of this monomer relative to *N*-hydroxy-*N*-acyl-cadaverine and HSC, we reasoned
268 that feeding of 2-¹³C-acetate would result in more pronounced labeling of all the +30 compounds relative
269 to the acyl-DFOs, while MS/MS analysis would help us to localize the sites of increased incorporation. It
270 is important to note that we did not expect exclusive labeling of the +30 metabolites, as 2-¹³C-acetate
271 could be converted to succinate by the TCA cycle as well as into long-chain fatty acids, and thus
272 incorporated into the acyl-DFOs.

273 Compared to the K7N1/OE484 strain cultivated in unsupplemented medium, the +1 peak for C12
274 acyl-DFO following growth in the presence of 1 mM sodium acetate-2-¹³C, increased in relative intensity
275 from 41.9 ± 0.4% (Fig. 7A) to 52.0 ± 0.2% (Fig. 7B). Likewise, the +1 peaks for DFO-B and G1 increased
276 in relative intensity from 28.8 ± 0.4% to 35.2 ± 0.3%, and from 31.1 ± 0.3% to 38.1 ± 0.2%, respectively
277 (Table S7). These observations are in accord with the conversion of the fed acetate into more elaborate
278 precursors followed by incorporation. However, the intensity of the +1 peak for the C12 acyl-DFO+30
279 increased by an additional 3.6% compared to that of C12 acyl-DFO (from 52.0 ± 0.2% in Fig. 7B to 55.6 ±
280 0.3% in Fig. 7D-) while the +2-peak showed similarly higher enrichment, consistent with greater
281 incorporation of acetate-2-¹³C into C12 acyl-DFO+30 relative to C12 acyl-DFO (Fig. 7 and Table S7).

282 In order to track the site of incorporation, we sought to compare the MS/MS fragmentation patterns
283 between C12 acyl-DFO and C12 acyl-DFO+30. For this, we relied on fragments of the molecules which
284 are diagnostic for their structures, as they exhibit a mass difference of +30 and so encompass the
285 divergent regions: m/z $[M+H]^+$ = 319 and 119 for acyl-DFOs, versus m/z $[M+H]^+$ = 349 and 149 for acyl-
286 DFOs+30 (Fig. 3). This analysis showed that the +1 peaks of 319 and 119 were observed at $55.0 \pm 0.5\%$
287 and $6.0 \pm 0.0\%$ relative intensity, respectively, in the presence of $2\text{-}^{13}\text{C}$ -acetate. As a +1 peak of 5.4%
288 intensity would be expected for the 119-fragment based on natural ^{13}C abundance ($5\text{C} \times 1.07\%$), these
289 data show that intact acetate incorporation into this portion of C12 acyl-DFO was minimal (Fig. 8A). In
290 contrast, the +1 peaks of the 349 and 149 fragments were of substantially higher intensity ($60.0 \pm 0.6\%$
291 and $10.2 \pm 0.5\%$, respectively) (Fig. 8B and Table S8), providing evidence that they both incorporate an
292 intact acetate unit. Thus overall, the acetate feeding experiments are in line with our proposed
293 biosynthetic pathway (Fig. 6).

294

295 Discussion

296 Acyl-DFOs are desferrioxamines bearing a saturated fatty acid of variable length (C9–C17) on the
297 terminal hydroxamate group (22). Amphiphilic siderophores are commonly produced by marine bacteria
298 (e.g. marinobactins (42), aquachelins (42), amphibactins (43), ochrobactins (44), and synechobactins
299 (38)). The hydrophobic acyl appendages of these metabolites modify their physical properties, allowing
300 them to embed in the cell membrane (for acyl chain lengths from C14–C21 (45)), and to form micelles
301 and vesicles (C12–C16 (43)). These features are thought to minimize loss of these metabolically
302 expensive compounds into the open ocean, an unstructured and highly diffuse environment, while
303 facilitating iron acquisition. Nevertheless, hydrophilic siderophores have been also identified in sea water
304 such as in iron-replete ocean regions, where ferrioxamines predominate (5, 46). In contrast, terrestrial
305 bacteria usually produce highly diffusible siderophores, as the soil is a structured habitat that intrinsically
306 limits the diffusion of molecules (47). Nonetheless, amphiphilic siderophores have been also
307 characterized in several soil bacteria such as *Burkholderia* (ornibactins (48)), *Pseudomonas*
308 (corrugatin,(49)), *Sinorhizobium* (rhizobactin (39)) and *Streptomyces* (23) (now including *S.*
309 *ambofaciens*). This observation raises questions about the role(s) of these compounds in structured
310 habitats, and argues for the commonality of acyl-DFO composition between terrestrial and marine
311 bacteria.

312 Interestingly, in *S. coelicolor*, acyl-DFOs were shown to diffuse away from the colonies (23).
313 Therefore, the ability to produce amphiphilic siderophores might be beneficial in some specific micro-
314 environments within the soil which, although considered to be a structured habitat, is not homogenous.
315 For example, all soils harbor microaggregates that, with the physiochemical heterogeneity of their
316 surfaces and their three-dimensional structure, provide a wide variety of ecological niches (50). Such
317 metabolites could also be advantageous not only when the producer interacts with other species as

318 shown for *S. coelicolor* (23), but additionally in response to the perturbation of growth conditions. For
319 example, acyl-DFO production was observed from *S. coelicolor* cultured using an OSMAC approach, in
320 which the growth conditions were systematically varied in order to induce specialized metabolite
321 production (51). In addition, although acyl-DFOs diffuse away from the producing bacteria (23), they may
322 occupy a distinct spatial location in the environment relative to DFOs such as foroxymithine. Indeed, the
323 latter has been suggested to sequester iron surrounding a *Streptomyces* colony growing within
324 polymicrobial communities, while DFOs have been proposed to contribute to the common good (52).

325 In this work, we have enlarged the known family of acyl-DFOs by identifying three novel sets of
326 related metabolites from *S. ambofaciens*, acyl-DFOs+30, acyl-DFOs+200+30, and acyl-
327 DFOs+200+200+30, and provided evidence for their synthesis via an unprecedented interplay between
328 two distinct NRPS-independent siderophore pathways. An incomplete *szk*-like pathway is also present in
329 *S. coelicolor* suggesting that it should also have the ability to produce these novel acyl-DFO derivatives,
330 at least under certain growth conditions. Indeed, acyl-DFO production from *S. ambofaciens* is
331 environment-dependent, with yields substantially higher when the strain is grown on liquid M5 medium
332 (22) instead of 26A agar medium ((9), data not shown), reflecting the fact that nutrients and precursors
333 present under different cultivation conditions can influence the product spectrum within a particular
334 metabolite family. Of note, we also observed the +30 forms for DFO-B and DFO-G1 (Fig. S5), which
335 demonstrates that the interplay between the *des* and the *szk*-like pathways is not restricted to the acyl-
336 DFOs, but encompasses all linear desferrioxamine derivatives.

337 It is interesting to consider the potential biological and/or ecological benefits of these octa- and
338 docadentate acyl-DFO derivatives. DFOs, including DFOB, are known to form complexes with several
339 metal ions and metalloids (including toxic metals) although the binding strength of these compounds is
340 lower relative to iron (53). It can be speculated that increased coordination potential (8 or 10 vs. 6 sites)
341 may improve the efficiency of chelating metal(loid)s, and/or the stability of the resulting complexes, which
342 could confer competitive benefit in terms of internalizing biologically-relevant metals, or external
343 sequestration of toxic compounds. Microbial siderophores including DFOs are also known to reduce the
344 growth of plant pathogens by locking away iron, providing plants both with protection against
345 phytopathogens and nutrition to stimulate their growth (12, 54). In view of this, it would be interesting to
346 directly investigate if the newly discovered multidentate acyl-DFOs also play a role in plant defense and
347 growth promotion.

348 DFO siderophores find application in medicine as imaging agents, sensors and therapeutics (55, 56),
349 and in various domains of biotechnology, including pathogen biocontrol and pollutant bioremediation (54),
350 and in eco-friendly processes such as the recovery of rare earth elements and other elements critical for
351 technology from electrical and electronic end-of-life products (57, 58). Nevertheless, DFOs exhibit certain
352 practical limitations due to their physical properties and distribution/metabolism in the body. For example,
353 their strongly hydrophilic character and short plasma half-life necessitate regular subcutaneous

354 administration during chelation therapy for iron overload, while their poor ability to penetrate cell
355 membranes restricts their utility in cancer treatment (59). In this context, the novel derivatives produced
356 by *S. ambofaciens* could be of interest due to their appended acyl chains (and therefore increased
357 hydrophobicity), and/or their octa- or dodecadentate structures that should improve their ability to chelate
358 certain metals. Indeed, while capacity of DFO-B to coordinate [⁸⁹Zr]Zr⁴⁺ has led to its clinical use in
359 immune-PET imaging, the resulting complex exhibits suboptimal stability *in vivo*, as DFO-B only offers six
360 coordination sites for Zr⁴⁺ via its three hydroxamate moieties (60). This observation has inspired the
361 chemical synthesis of various octadentate versions of DFO-B capable of saturating the Zr⁴⁺ coordination
362 sphere (61, 62). Our results show that nature also assembles a range of extended DFOs, the
363 acylDFO+200+30 and +200+200+30, which may now find utility in this and other medical and
364 biotechnological applications (25). Given the evident difficulty of purifying the metabolites from the native
365 host, however, it would be worthwhile to chemically synthesize the compounds (63) for further evaluation.

366

367 **Materials and Methods**

368 **Materials.** All reagents and chemicals were obtained from Sigma-Aldrich, except the following: BD
369 (tryptone, yeast extract, TSB powder), Thermo Fisher Scientific (Tris), VWR (glycerol, NaCl, NaNO₃),
370 ADM France (NutriSoy flour), and New England Biolabs (T4 DNA ligase, restriction enzymes).
371 Oligonucleotide primers were synthesized by Sigma-Aldrich. DNA sequencing of PCR products was
372 performed by Sigma-Aldrich. PCR reactions were carried out with Taq DNA polymerase (Thermo Fisher
373 Scientific), or Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) when higher fidelity was
374 required. Isolation of DNA fragments from agarose gel, purification of PCR products and extraction of
375 plasmids were carried out using the NucleoSpin® Gel and PCR Clean-up or NucleoSpin® Plasmid DNA
376 kits (Macherey Nagel, Hoerd, France).

377 **Strains and media.** Unless otherwise specified, all *Escherichia coli* strains were cultured in LB medium
378 (yeast extract 10 g, tryptone 5 g, NaCl 10 g, distilled water up to 1 L, pH 7.0) or on LB agar plates (LB
379 medium supplemented with 20 g/L agar) at 37 °C. *Streptomyces ambofaciens* ATCC23877 and the
380 mutants thereof were grown in TSB (TSB powder 30 g (tryptone 17 g, soy 3 g, NaCl 5 g, K₂HPO₄ 2.5 g,
381 glucose 2.5 g), distilled water up to 1 L, pH 7.3) or on TSA plates (TSB medium supplemented with 20 g/L
382 agar), and sporulated on SFM agar plates (NutriSoy flour 20 g, D-mannitol 20 g, agar 20 g, tap water up
383 to 1 L) at 30 °C. All strains were maintained in 20% (v/v) glycerol in 2 mL Eppendorf tubes and stored at
384 -80 °C. For fermentation of *S. ambofaciens* ATCC23877 and its mutants, spores were streaked on TSA
385 with appropriate antibiotics (apramycin, etc..) and after incubation for 48 h at 30 °C, a loop of mycelium
386 was used to inoculate 7 ml of MP5 medium (yeast extract 7 g, NaCl 5 g, NaNO₃ 1 g, glycerol 36 mL,
387 MOPS 20.9 g, distilled water up to 1 L, pH 7.4) supplemented with selective antibiotics and sterile glass
388 beads, followed by incubation at 200 rpm and 30 °C for 24–48 h. Finally, the seed culture was centrifuged
389 and resuspended into 2 mL fresh MP5 before being inoculated into 50 mL MP5 medium in a 250 mL

390 Erlenmeyer flask, and cultivated at 200 rpm and 30 °C for 4 days. Labeled molecules were fed to the
391 media at a final concentration of 1 mM after inoculation of seed culture at 24h incubation.

392 **CRISPR-Cas9 mediated genetic engineering.** Plasmid pCRISPomyces-2 was used for construction of
393 all mutants generated in this study (Tables S4, 5). For each gene or locus to be deleted, the crRNA
394 sequence was selected to match the DNA segment which contains NGG on its 3' end (N is any
395 nucleotide, and the NGG corresponds to the protospacer-adjacent motif (PAM)). The annealed crRNA
396 fragment and two homologous arms (HAL and HAR, flanking the target region) were sequentially inserted
397 into the delivery plasmid pCRISPomyces-2 using the restriction sites *BbsI* and *XbaI*, respectively, to
398 afford the specific recombinant plasmid for each mutant (Fig. S12–14).

399 **LC-ESI-HRMS analysis of fermentation metabolites.** The fermentation broth was centrifuged at 4,000
400 x *g* for 10 min. The acyl-DFOs and their derivatives were then extracted from the mycelia by first
401 resuspending the cells in 40 mL distilled water, followed by centrifugation (4,000 x *g*, 10 min, repeated
402 3x) to remove the water-soluble components. After decanting the water, the cell pellets were weighed
403 and extracted with MeOH by shaking at 150 rpm for 2 h at room temperature. Thereafter, the MeOH
404 extracts were filtered to remove the cell debris, followed by rotary evaporation to dryness. The obtained
405 extracts were then dissolved in MeOH, whose volume was determined according to the initial weight of
406 the mycelia (70 µL MeOH to 1 g of cell pellet). The resulting mycelium extracts were then centrifuged at
407 16,000 x *g* at 4 °C for 20 min and analyzed in heated positive electrospray mode (HESI+) by HPLC-
408 HRMS on a Thermo Scientific Orbitrap ID-X Tribrid Mass Spectrometer using an Alltima™ C18 column
409 (2.1 x 150 mm, 5 µm particle size, Grace-Alltech) or an Acclaim™ C18 column (2.1 x 100 mm, 2.2 µm
410 particle size, ThermoScientific). Separation was carried out with ultrapure water (Purelab Flex system,
411 Veolia) containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B), using the
412 following elution profile: 0–50 min, linear gradient 5–95% solvent B; 50–54 min, constant 95% solvent B;
413 54–60 min, constant 5% solvent B. Mass analysis was carried out in ESI positive ion mode (ESI⁺) and
414 mass spectrometry conditions were as follows: spray voltage was set at 3.5 kV; source gases were set (in
415 arbitrary units/min) for sheath gas, auxiliary gas and sweep gas at 40, 8, and 1, respectively; vaporizer
416 temperature and ion transfer tube temperature were set at 320 °C and 275 °C, respectively. Survey MS
417 scans of precursors were performed from 150 to 2,000 *m/z*, at 60 K resolution (full width of the peak at its
418 half maximum, at 200 *m/z*) with MS parameters as follows: RF-lens, 50%; maximum injection time, 50
419 ms; data type, profile; internal mass calibration EASY-IC™ activated; AGC target: custom; normalized
420 AGC target: 50%. A top speed (0.6 sec) data dependent MS² was performed by isolation at 1.6 Th with
421 the quadrupole, HCD fragmentation at a fixed 30% collision energy and analysis in the orbitrap at 30 K
422 resolution (high resolution MS/MS analysis). The dynamic exclusion duration was set to 2.5 s with a
423 10 ppm tolerance around the selected precursor (isotopes excluded). Other MS/MS parameters were as
424 follows: data type, profile; standard AGC target, internal mass calibration EASY-IC™ activated. Mass
425 spectrometer calibration was performed using the Pierce FlexMix calibration solution (Thermo Scientific).
426 MS data acquisition was carried out utilizing the Xcalibur v. 3.0 software (Thermo Scientific).

427 **Generation of the molecular network and interpretation of MS/MS data.** The raw data obtained from
428 the LC-MS/MS system were converted to mzXML format using the ProteoWizard tool msconvert. All
429 mzXML data were uploaded to the Global Natural Products Social Molecular Networking-platform (GNPS)
430 and analyzed using the workflow as previously published (28), generating a molecular network based on
431 the fragmentation spectra. To create consensus spectra, data were clustered by applying a parent mass
432 tolerance of 1.0 Da and an MS/MS fragment ion tolerance of 0.5 Da; the remaining parameters were set
433 to default. The output of the molecular networks was visualized using Cytoscape (64) and displayed using
434 the settings “preferred layout” with “directed” style. For compounds in the network with known structures,
435 e.g., DFO-B and acyl-DFOs, identified with GNPS IDs, the fragments were assigned to the corresponding
436 molecules by comparison to the published fragmentation patterns. Structure analysis of compounds not
437 giving rise to GNPS hits but linked to known compounds, was based on detailed comparison of MS/MS
438 fragment spectra in order to identify both common and unique fragments.

439 **Statistical analysis.** Analysis of variance (2-way ANOVA) of the data was performed using GraphPad
440 Prism 9.0.2 software. P-values were calculated by Dunnett’s multiple comparison test and are presented
441 in NEJM style: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001(***).

442

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450 Spectrometer.

451

452 **Figure legends**

453 **FIG 1** Representative biosynthetic gene clusters and pathway to NIS desferrioxamine. (A) The BGC for
454 desferrioxamines (DFO-B and -E) in *S. coelicolor*, and the corresponding DFO BGC in *S. ambofaciens*. The same
455 color indicates a shared enzymatic function, and the size of each protein in amino acids is indicated. (B) Currently
456 proposed biosynthetic pathways to DFO-B and -E.

457 **FIG 2** Annotation of the subnetwork related to the DFOs. The m/z value of the protonated parental ion ([M+H]⁺) is
458 specified for each dot. The names of all known compounds are indicated above each dot, and their structures shown.
459 The remaining members of acyl-DFOs+30 (C9 and C15), acyl-DFOs+200+30 (C9 and C15) and acyl-DFOs+200+
460 200+30 (C9, C10 and C15) have not been presented in the network due to their relatively low parental ion densities
461 (as shown in Fig. S2) and the subsequent lack of available MS/MS fragments in MS/MS full scan model. The
462 complete molecular network is shown in Fig. S3.

463 **FIG 3** Comparison of the observed HPLC-MS/MS fragmentation patterns of C12 acyl-DFO and its congeners. The
464 structure and MS/MS fragment assignment of C12 acyl-DFOs are based on previously-published work. Comparison

465 with the MS/MS data obtained on C12 acyl-DFO+30, C12 acyl-DFO+200+30 and C12 acyl-DFO+200+200+30
466 reveals a series of shared fragments (solid underline), as well as divergent fragments (dashed underlines)
467 characteristic of the extended structures. The structure of the portion of the molecules highlighted in green has not
468 been conclusively determined, but the available data are most consistent with the *N*-hydroxy, *N*-acetyl version.

469 **FIG 4** Comparison of the monomer composition of acyl-DFOs+30, acyl-DFOs+200+30 and acyl-DFOs+200+200+30.
470 The structures of the acyl-DFOs were published previously, while the structures of the additional derivatives were
471 predicted on the basis of the MS/MS fragmentation patterns. The building blocks shown in different colors are linked
472 together by amide bonds to form the various DFOs. The “+200” refers to incorporation of an additional HSC unit,
473 while the “+30” is predicted to originate from one of two alternative modifications to the terminal amine group, with the
474 *N*-hydroxy, *N*-acetyl congener being more likely.

475 **FIG 5** Patterns of isotope incorporation into C12 acyl-DFO and its three novel derivatives in the absence (A, C, E, G)
476 and presence (B, D, F, H) of fed $^{13}\text{C}_6$, $^{15}\text{N}_2$ -lysine. The mass spectrum of the parent ion (m/z [M+H] $^+$) is shown in
477 each case. The expected labeling patterns are shown as red dots on the structures. The absence of +7 Da \times 3, +7
478 Da \times 4 and +7 Da \times 5 peaks in D, F and H, respectively, excluded the possibility the compounds incorporate a Lys-
479 derived nitrosylated building block (Fig. S8). C11 acyl-DFO+30 was also detected (m/z values shown in grey in
480 panels A and B) in the spectrum of C12 acyl-DFO+30, as the two metabolites co-eluted (Fig. S2). Similarly, peaks
481 indicated in grey in C and D correspond to C13 acyl-DFO. ND = not detected.

482 **FIG 6** Predicted biosynthetic pathway to all DFO variants described in this study. (A) The genomic organization of the
483 potential gene cluster involved in the biosynthesis of the predicted building block, relative to the enzymes involved in
484 the biosynthesis of schizokenin in *Nostoc* sp. PCC7120 and DFOs in *S. ambofaciens*. Identities on the AA sequence
485 level are shown. Enzymes All0391 (MFS transporter) and DesE and DesF are not shown. (B) Proposed biosynthetic
486 pathway to schizokenin. (C) Proposed biosynthetic pathway to the acyl-DFOs+30, acyl-DFOs+200+30 and acyl-
487 DFOs+200+200+30.

488 **FIG 7** Patterns of isotope incorporation into C12 acyl-DFO and its three novel congeners in the absence (A, C, E, G)
489 and presence (B, D, F, H) of fed sodium acetate-2- ^{13}C . The expected site of labeling of the proposed alternative
490 building block is shown as a red dot, and the observed relative intensity of the +1 peaks highlighted in red. Succinate
491 units, which can also incorporate fed acetate, are indicated in pink. Panel (I) shows the statistical analysis of the +1
492 peaks detected in the MS spectra of C12 acyl-DFO and its three novel congeners under unfed and fed conditions.
493 One set of representative spectra from three replicate experiments is shown, but the relative intensity represents the
494 average of all replicate measurements, as detailed in Fig. S17. The complete data sets for all DFOs (C9–C15) are
495 included in Table S7. Error bars indicate mean \pm s.d., *P*-values were calculated using Dunnett’s multiple comparison
496 test and are presented in NEJM style. According to this analysis, there was a significant difference (*P* = 0.01)
497 between the incorporation levels into C12 acyl-DFO and C12 acyl-DFO+30 based on the +1 peak under fed
498 conditions, whereas there was no significant difference (*P* > 0.99) under unfed conditions.

499 **FIG 8** Representative MS² fragments of C12 acyl-DFO and C12 acyl-DFO+30 after feeding with sodium acetate-2-
500 ^{13}C . The indicated fragments derive from parental ions 716.6359 and 746.5100 in Figs. 7B and 7D, respectively. We
501 chose to focus our analysis on the m/z [M+H] $^+$ = 349/319 and 149/119 pairs, as the origin of the 201/231 fragments is
502 ambiguous (terminal vs. internal fragment in C12 acyl-DFO). One set of representative spectra from three replicate
503 experiments is shown, but the relative intensity represents the average of all replicate measurements. ND = not
504 detected. The complete data sets are provided in Table S8.

505

506

507 **Reference**

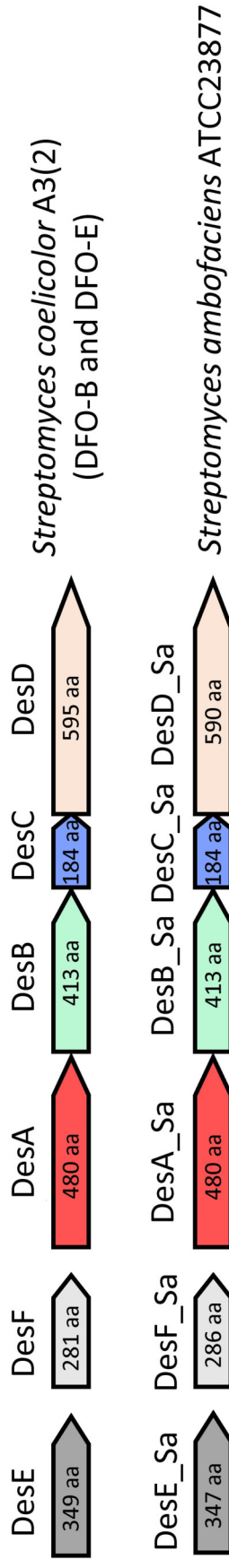
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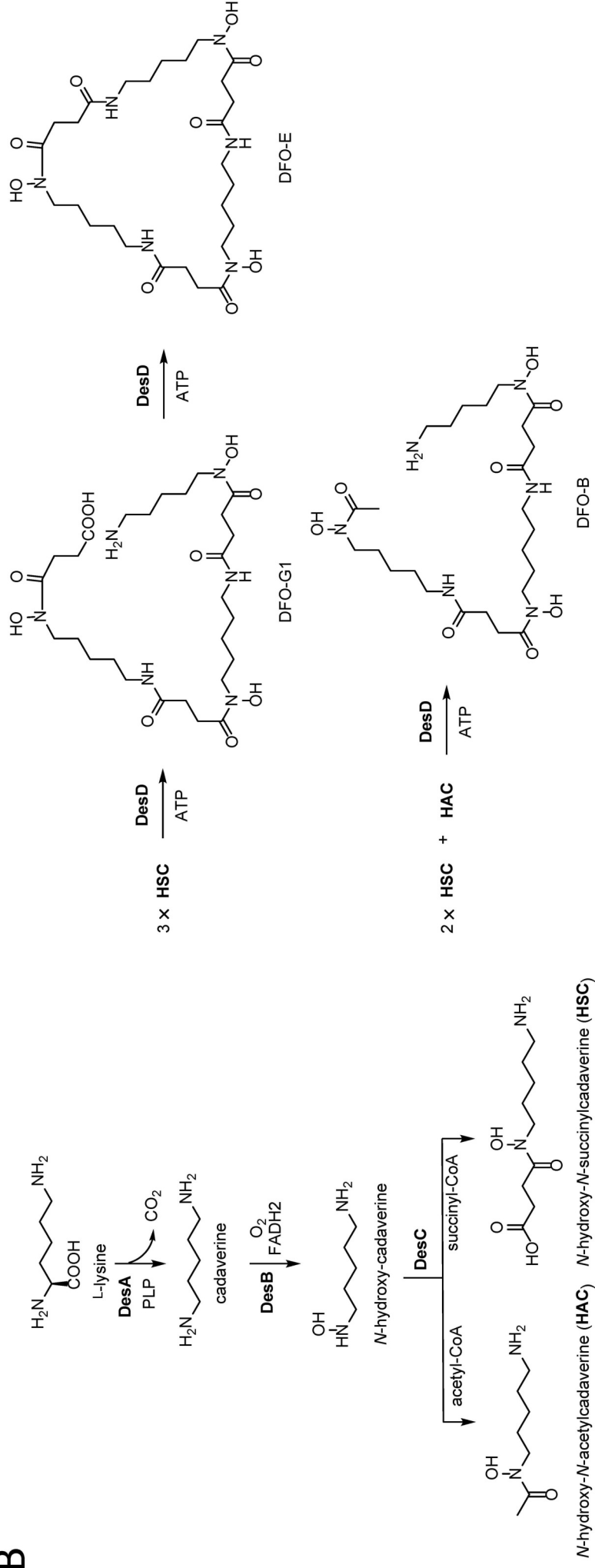
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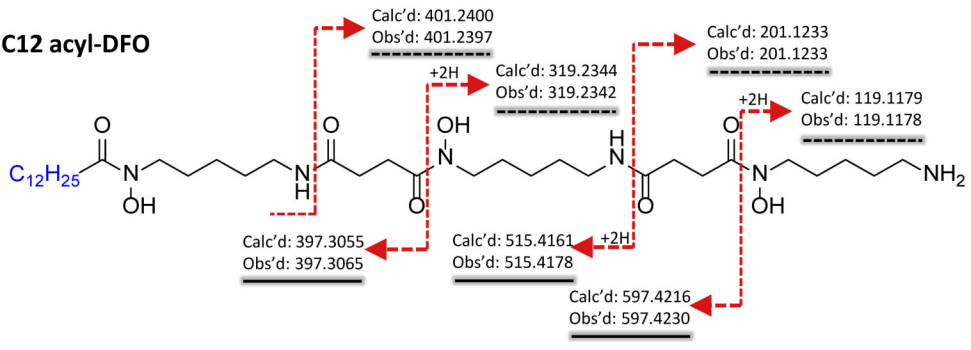
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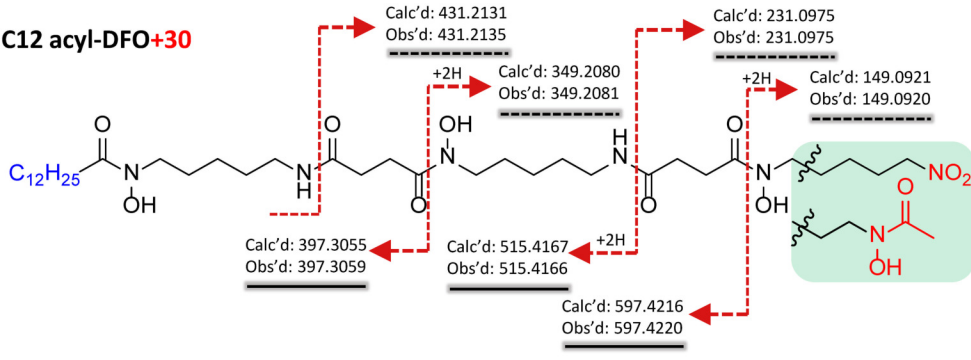
B

N-hydroxy-N-acetyl/cadaverine (**HAC**) N-hydroxy-N-succinylcadaverine (**HSC**)

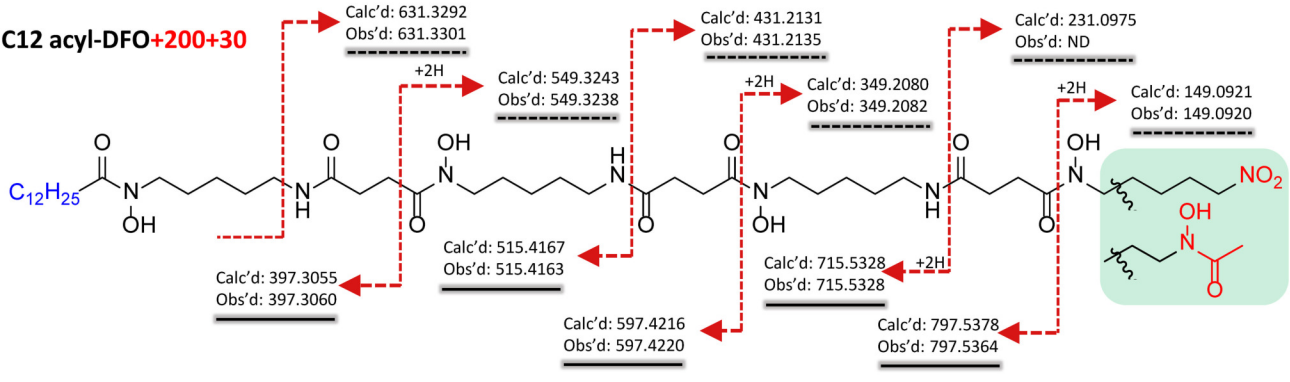
C12 acyl-DFO



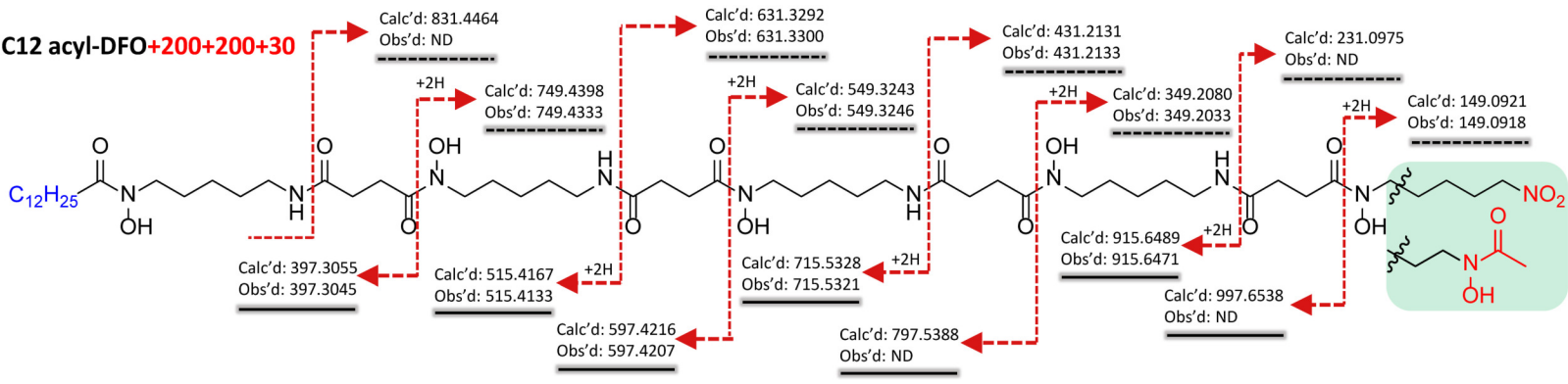
C12 acyl-DFO+30

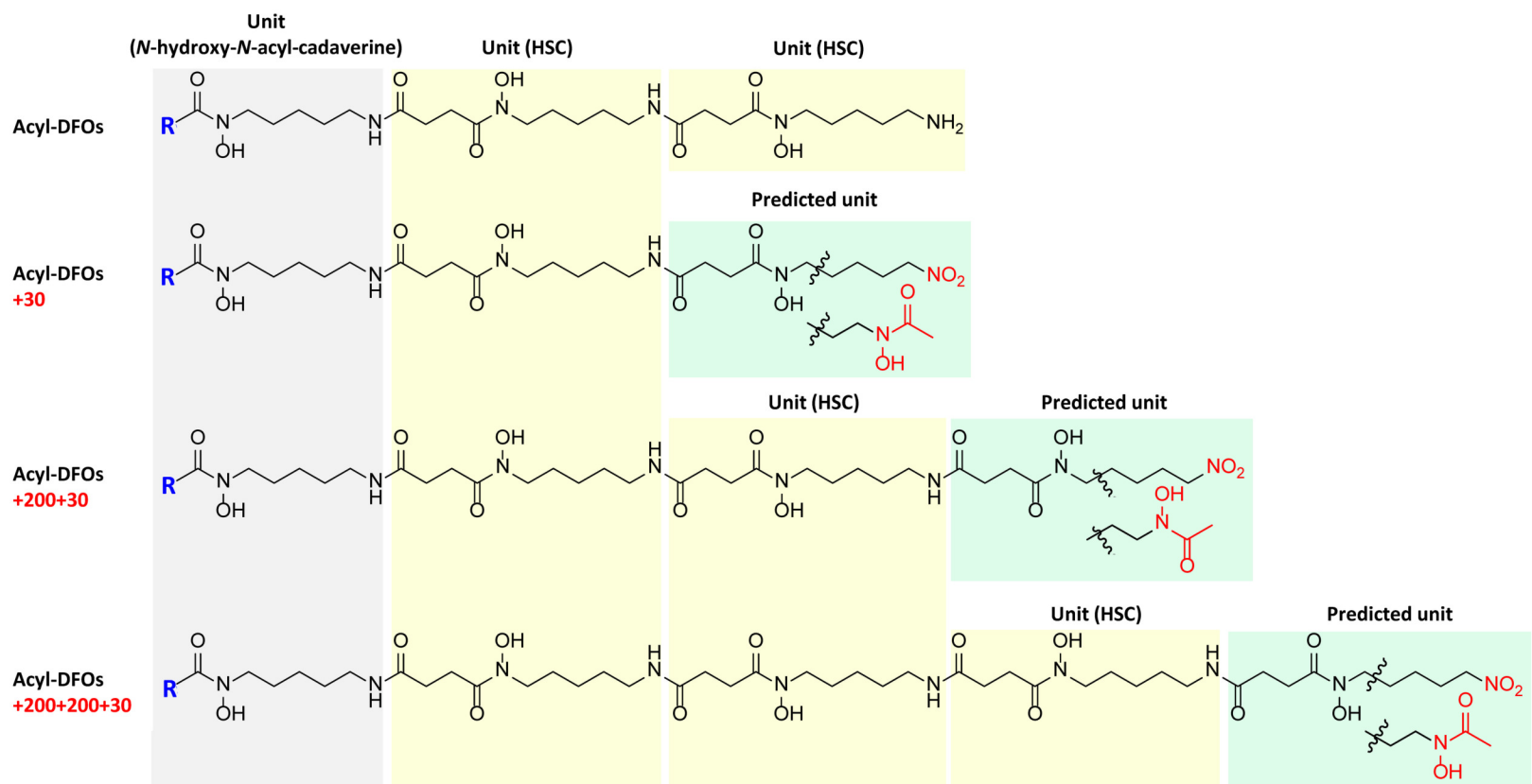


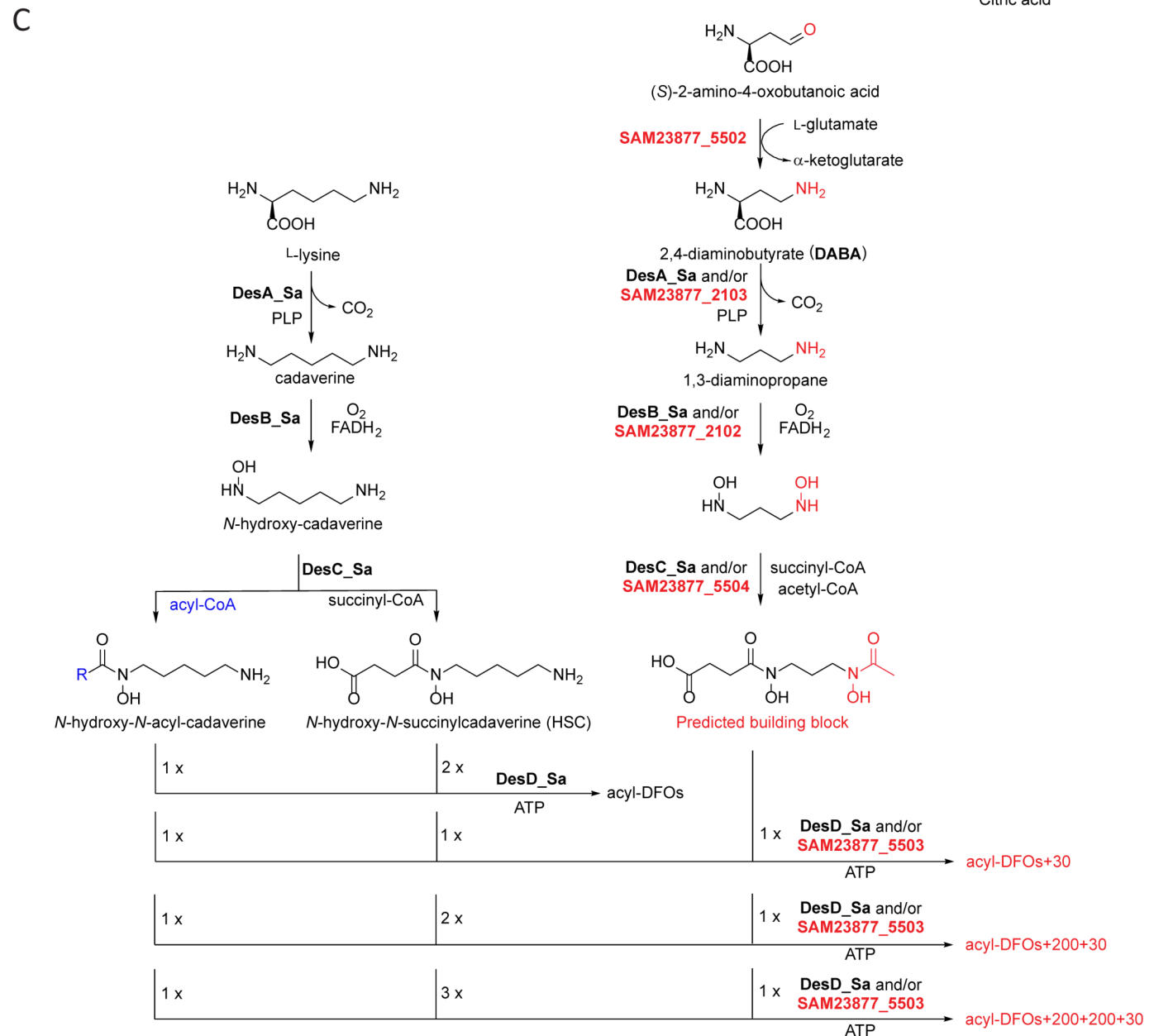
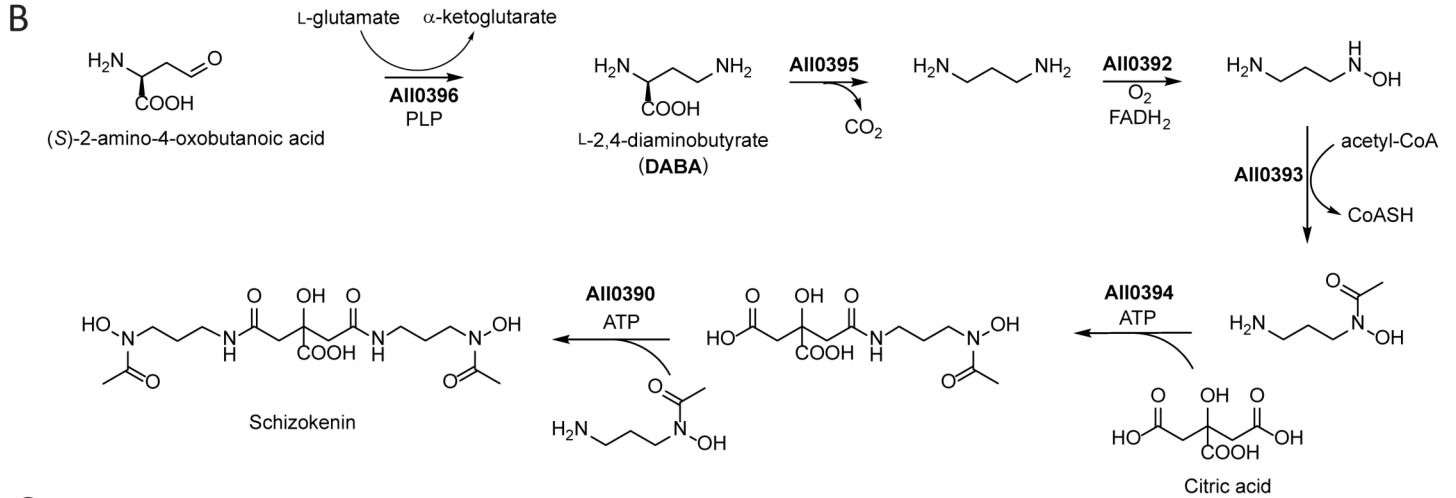
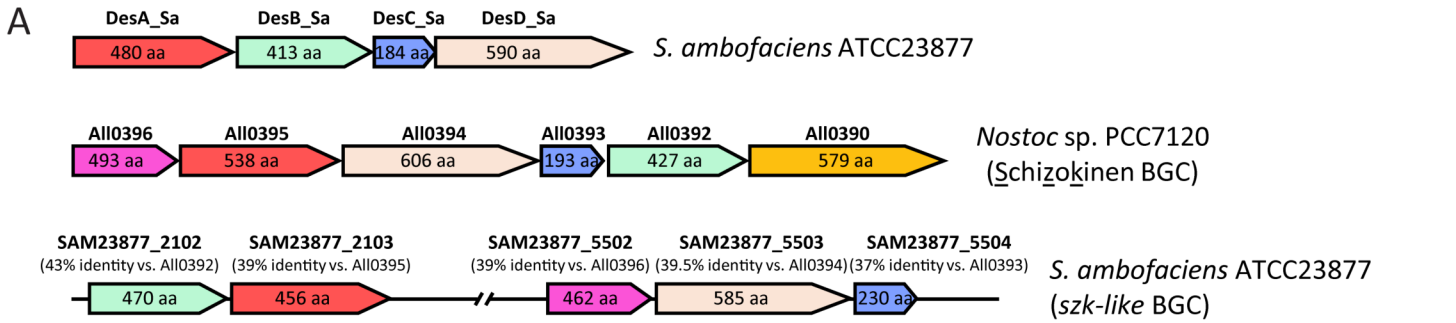
C12 acyl-DFO+200+30

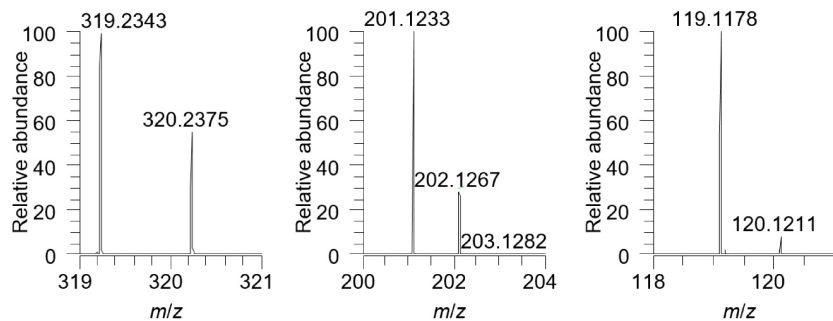
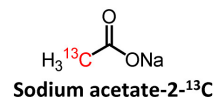
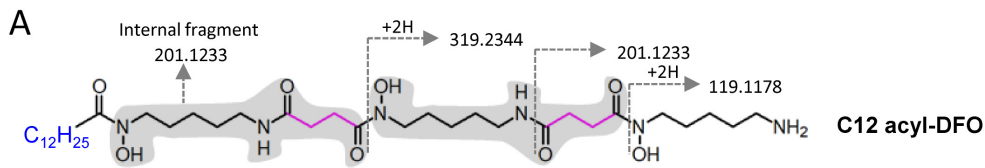


C12 acyl-DFO+200+200+30

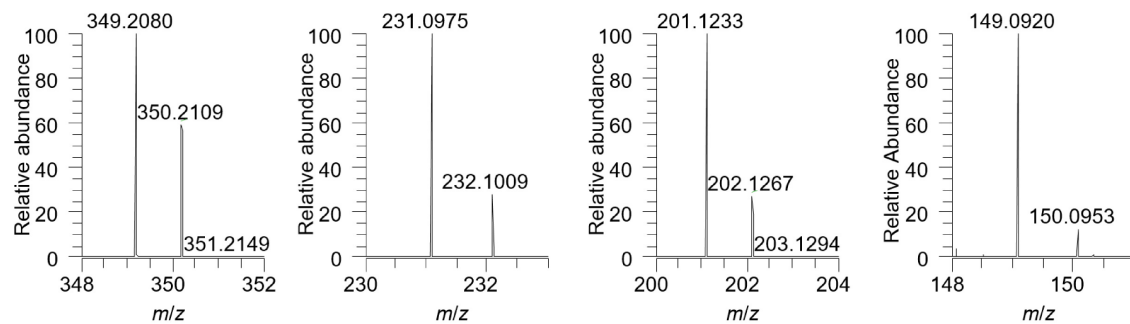
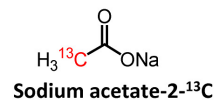
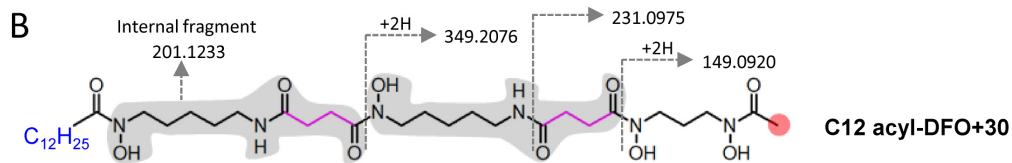








Frag. (Calc'd m/z)	Relative intensity (%)
319.2344	100.0 ± 0.0
320.2375	55.0 ± 0.5
321.2395	ND
201.1233	100.0 ± 0.0
202.1267	27.1 ± 0.5
203.1296	0.1 ± 0.0
119.1178	100.0 ± 0.0
120.1212	6.0 ± 0.0



Frag. (Calc'd m/z)	Relative intensity (%)
349.2080	100.0 ± 0.0
350.2110	60.0 ± 0.6
351.2149	0.5 ± 0.2
231.0975	100.0 ± 0.0
232.1008	29.1 ± 0.6
201.1233	100.0 ± 0.0
202.1267	28.0 ± 0.1
203.1296	0.5 ± 0.0
149.0920	100.0 ± 0.0
150.0955	10.2 ± 0.5