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Synthesis of heterobactins A and B and Nocardia heterobactin

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ABSTRACT

The synthesis of the *Rhodococcus erythropolis* siderophores heterobactins A and B, and the structurally related Nocardia heterobactin, is described. Two approaches for the assembly of these asymmetric ligand donor chelators are explored. In the first approach, a scheme predicated on the biosynthesis of the *Paracoccus denitrificans* siderophore, parabactin, is employed. In this approach, the central donor synthon is added last. In the second scheme, the central donor and the terminal 2,3-dihydroxybenzoyl fragment are first fixed to the ligand's D-ornithine backbone. This is followed by condensation with the cyclic ornithine hydroxamate glycine segment. The schemes offer a flexible approach to other heterobactins. Job's plots suggest that heterobactin A and Nocardia heterobactin form 1:1 ligand/metal complexes, while heterobactin B forms a 3:2 ligand/metal complex.

1. Introduction

Iron serves as a prosthetic for many different redox enzymes,[1] which are essential for life itself. Although it comprises about 5% of the earth's crust, the metal is not easily accessible to biological systems.

The concentration of ferric iron required to support the growth of most microorganisms lies in the range of 5×10^{-8} to 1×10^{-6} mol/L.[2] However, because of the extreme insolubility of ferric hydroxide at physiological pH, about 10^{-18} mol/L, microorganisms had to develop a means of sequestering and transporting this metal.[3] They secrete large quantities of siderophores, low molecular weight chelators, which form complexes with iron(III), providing a transport vector.[4] Most siderophores present with either hydroxamate donors, e.g., desferrioxamine[5] and nocardamine,[6] or catecholamide donors, e.g., petrobactin[7] and vibriobactin[8] (Fig. 1). These particular ligands form 1:1 complexes with Fe(III). Catecholamide chelators typically form tighter Fe(III) complexes, e.g., parabactin[9] (K_f=10^{48} M^{-1}) (Fig. 2), than their hydroxamate counterparts, e.g., desferrioxamine (10^{28} M^{-1}) (Fig. 1).[10]

The current study focuses on the assembly of the heterobactins A and B (Fig. 2), isolated from *Rhodococcus erythropolis*[11] and a heterobactin analogue (Nocardia heterobactin or JBIR-16) derived from a human pathogen *Nocardia tenerifensis*.[12] *R. erythropolis* has drawn considerable attention recently because of the ability of particular strains to metabolize oil, making these organisms attractive candidates as tools for bioremediation.[13] This potential application adds value to the current work. The *Rhodococcus* siderophores are now synthetically accessible for analytical standards or biosynthetic studies. This will help investigators to monitor and understand the organism's iron-regulated growth properties. This is key to optimizing the use of *Rhodococcus* in oil remediation.

Interestingly, the siderophores isolated from both *Rhodococcus* and *Nocardia* are in many ways similar to the ligands predicated on polyamine backbones, e.g., parabactin[9] and agrobactin[14] (Fig. 2). For example, with parabactin, the bidentate fragments, 2,3-dihydroxybenzoyl

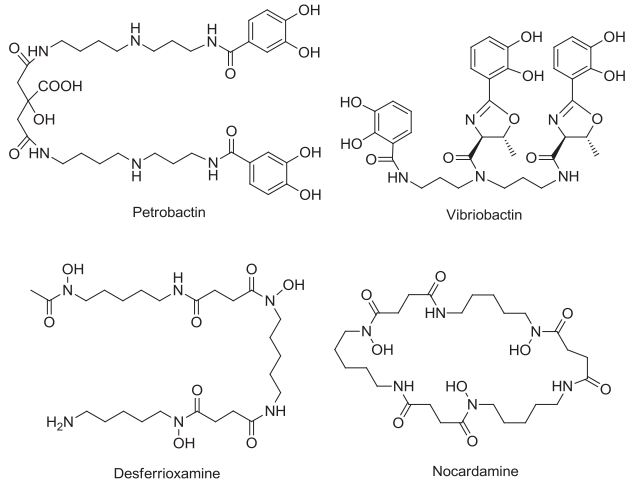

Fig. 1. Catecholamide and hydroxamate siderophores.

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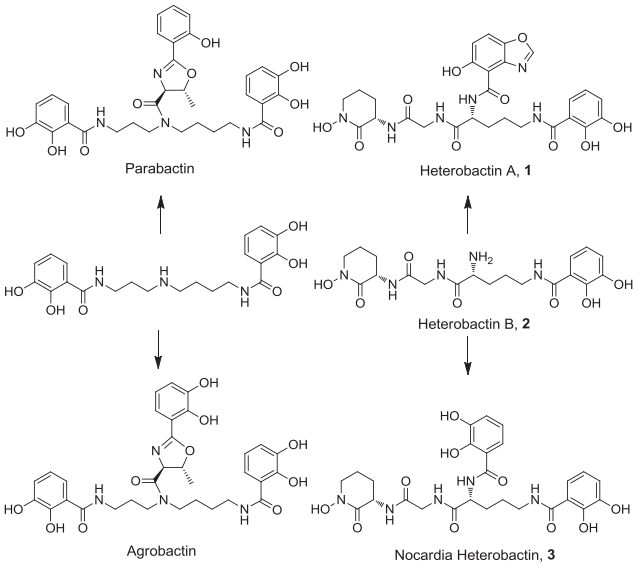


Fig. 2. Derivation of heterobactin A and Nocardia heterobactin from heterobactin B by analogy to parabactin and agrobactin.

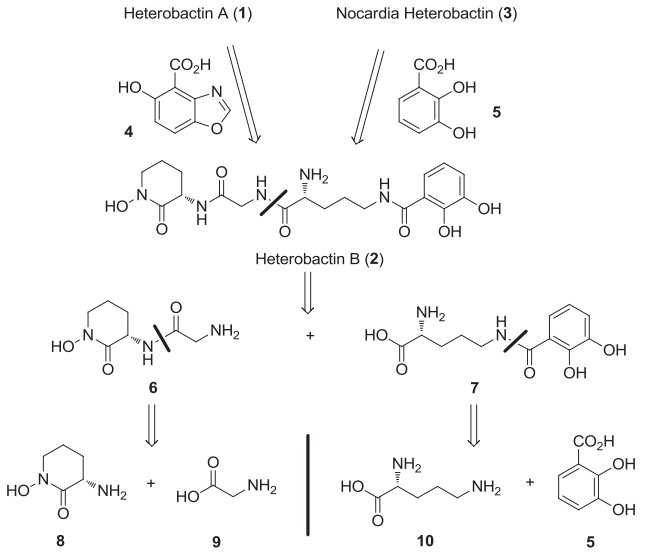
groups, are fixed to a linear polyamine backbone at the terminal nitrogens via amide linkages. The remainder of the hexacoordinate donor array is fixed to the central nitrogen as the amide of 2-(2-hydroxyphenyl) -(4*S*,5*R*)-*trans*-5-methyl-4-oxazolinecarboxylic acid. The donor ligands consist of the terminal 2,3-dihydroxybenzoyl hydroxyls, the central oxazoline nitrogen, and the internal aromatic hydroxyl. Heterobactin A (**1**) and B (**2**), from *Rhodococcus*,[11] and the heterobactin analogue (**3**) from *Nocardia*[12] also present with terminal bidentate fragments, although unsymmetrical: on one end, a 2,3-dihydroxybenzoyl, on the other, a hydroxamate derived from cyclized N^5—OH—L-ornithine. The central donor in heterobactin A (**1**), a 5-hydroxy-4-carboxyl-benzoxazole may employ the aromatic hydroxyl and the amide function, as has been previously observed.[11] A similar central donor scenario, a substituted salicylamide array, can also be invoked with Nocardia heterobactin (**3**) with its central 2,3-dihydroxybenzamide unit.

2. Results and discussion

2.1. Synthetic strategy

From a biosynthetic perspective, it seems reasonable that both heterobactin A (**1**) and Nocardia heterobactin (**3**) derived from heterobactin B (**2**) (Fig. 2). However, although heterobactin B (**2**) was isolated from *R. erythropolis*,[11] it remains to be identified in *Nocardia* sp. It is interesting that the same biosynthetic sequence was shown with both parabactin[9] and agrobactin.[14] Both were derived from the tetracoordinate precursor, N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine.[9] These observations suggested a biomimetic, retrograde synthetic scheme for the heterobactins described in this work. Initially, the key intermediate would indeed be a protected form of ligand **2** (Fig. 2).

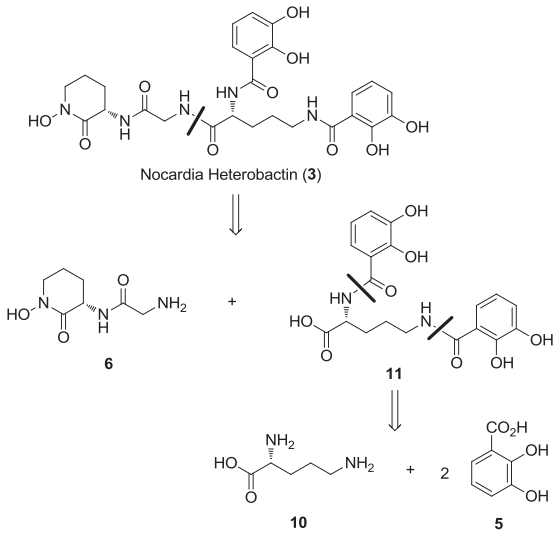
An approach to heterobactins **1**–**3** derives from the following retrosynthetic analysis, which requires the regiospecific formation of three amide bonds (Scheme 1). In the final step the amino group of heterobactin B (**2**) could be acylated with activated 5-hydroxy-4-benzoxazolecarboxylic acid (**4**) to provide heterobactin A (**1**) or 2,3-dihydroxybenzoic acid (**5**) to give Nocardia heterobactin (**3**). The inner amide bond of **2** could be formed by coupling dipeptide **6** with monoacylated D-ornithine **7**. *N*-Acylation of (*S*)-3-amino-1-hydroxy-2-piperidinone (**8**), a cyclic L-ornithine hydroxamate, with glycine (**9**) would furnish **6**; condensation of D-ornithine (**10**) at the 5-amino group with 2,3-dihydroxybenzoic acid (**5**) would give **7**. A



Scheme 1. Retrosynthetic analysis of heterobactins A, B and Nocardia heterobactin.

key to the success of Scheme 1 is the effective use of *N*- and *O*-protecting groups.

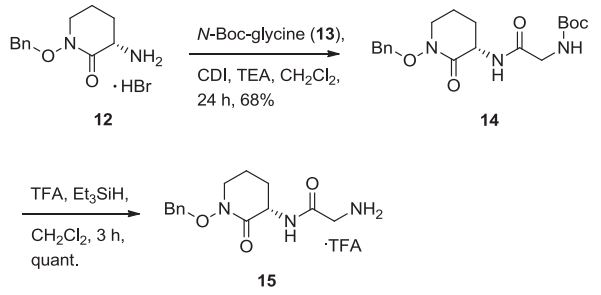
An alternative retrosynthetic view of Nocardia heterobactin (**3**) begins by bis-acylation of D-ornithine (**10**) with activated 2,3-dihydroxybenzoic acid (**5**), generating bis-catecholamide **11** (Scheme 2). Peptide coupling of glycine derivative **6** with carboxylic acid **11** will produce the natural product **3** by a more convergent synthesis than in Scheme 1. As before, the route in Scheme 2 is dependent on optimal fragment protecting groups.



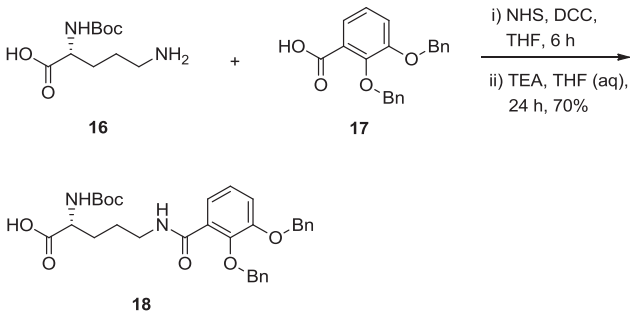
Scheme 2. Alternative retrosynthesis of Nocardia heterobactin.

2.2. Total synthesis

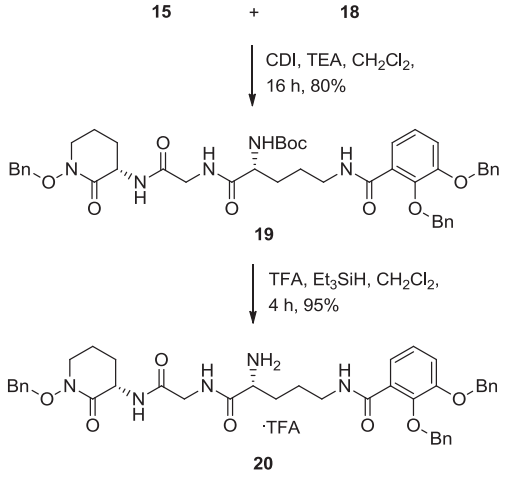
The biomimetic approach is defined by four segments, (1) assembly of the hydroxamate, cyclic N^5-hydroxy-L-ornithine-N^2-glycyl fragment (**15**, Scheme 3), (2) construction of the N^5-(2,3-dibenzyloxy)-D-ornithine segment (**18**, Scheme 4), (3) coupling of the above fragments and deprotection of the central nitrogen to liberate the key intermediate **20** (Scheme 5), and (4) *N*-acylation of tri-*O*-protected heterobactin B (**20**) to fully protected heterobactin A (**21**) or Nocardia heterobactin (**22**, Scheme 6).



Scheme 3. Synthesis of protected cyclic ornithine hydroxamate peptide **15**.



Scheme 4. Synthesis of protected catecholamide fragment **18**.

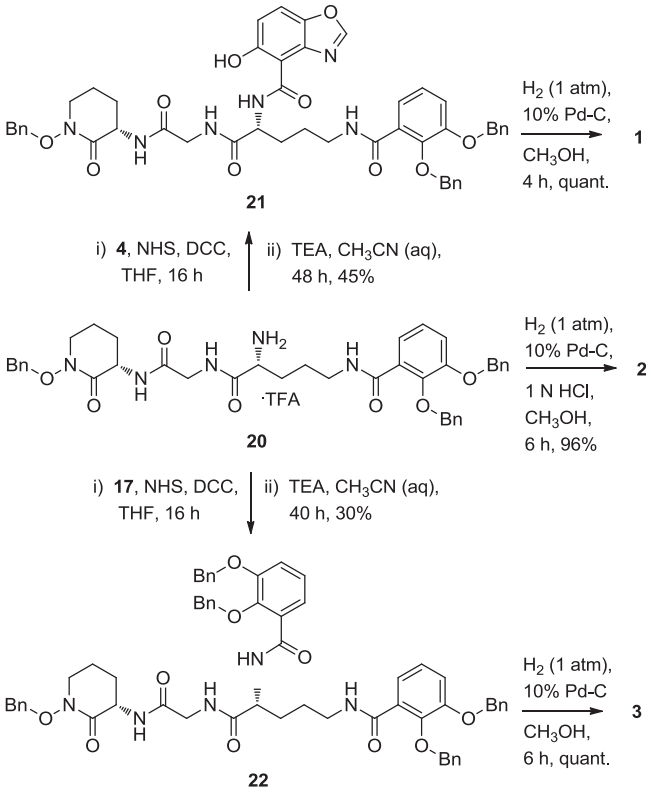


Scheme 5. Synthesis of the key intermediate, **20**.

Synthesis of the cyclic hydroxamate of L-ornithine/glycine (**15**) (Scheme 3) fragment was accomplished by a 1,1′-carbonyldiimidazole (CDI) mediated coupling of (*S*)-3-amino-1-benzyloxy-2-piperidinone hydrobromide (**12**)[15] with *N*-(*tert*-butoxycarbonyl)glycine (**13**) in triethylamine (TEA) and CH_2Cl_2, providing peptide **14** in 68% yield. The peptide was subjected to a trifluoroacetic acid (TFA) removal of the Boc-protecting group in the presence of triethylsilane to provide *O*-benzylated hydroxamate **15** as its TFA salt in quantitative yield.

In order to construct the terminal catecholamide of heterobactins **1**–**3**, 2,3-bis(benzyloxy)benzoic acid (**17**)[16] was activated as its *N*-hydroxysuccinimide (NHS) ester using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and coupled with D-N^2-Boc ornithine (**16**), producing N^2-Boc-N^5-(2,3-dibenzyloxybenzoyl)-D-ornithine (**18**) in 70% yield (Scheme 4).

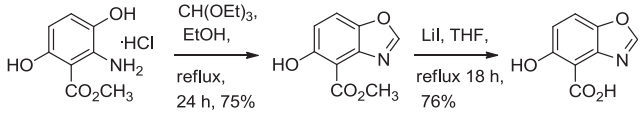
The key intermediate (**20**, Scheme 5) required for the assembly of heterobactin A (**1**), heterobactin B (**2**), and Nocardia heterobactin (**3**) (Scheme 6) was accessed by first coupling **15** with **18** using CDI/TEA in CH_2Cl_2 to generate the intermediate **19** in 80% yield. The *tert*-butoxycarbonyl protecting group of **19** was removed by treatment with TFA and triethylsilane in CH_2Cl_2, providing **20** in 95% yield.



Scheme 6. Synthesis of heterobactins A (**1**), B (**2**), and Nocardia heterobactin (**3**).

When tripeptide **20** was debenzylated under a hydrogen atmosphere over Pd/C in CH_3OH and a catalytic amount of 1 N HCl, heterobactin B (**2**) was obtained in 96% yield (Scheme 6). *N*-Acylation of intermediate **20** with 5-hydroxy-4-benzoxazolecarboxylic acid (**4**) as its NHS ester provided peptide **21** (45% yield). Treatment of **21** with H_2 over Pd/C, led to heterobactin A (**1**) quantitatively.

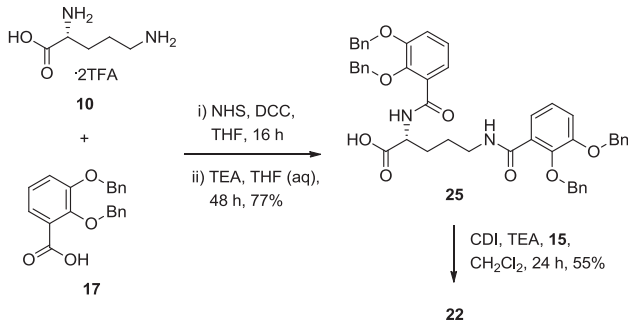
5-Hydroxy-4-benzoxazolecarboxylic acid (**4**) was generated from methyl 2-amino-3,6-dihydroxybenzoate (**23**)[17] as in Scheme 7. Methyl ester **23** was converted into oxazole **24** in 75% yield with triethyl orthoformate in refluxing EtOH. While saponification of the methyl ester of **24** with LiOH failed, treatment of **24** with LiI in refluxing THF for 18 h provided carboxylic acid **4** in 76% yield.



Scheme 7. Synthesis of 5-hydroxy-4-benzoxazolecarboxylic acid (**4**).

Alternatively, acylation of the amino group of **20** with 2,3-dibenzyloxybenzoic acid (**17**) as its NHS ester provided penta-*O*-protected peptide **22** (30% yield). Hydrogenolysis of **22** over Pd/C led to the Nocardia heterobactin (**3**) quantitatively.

The moderate yields (30–45%) associated with the formation of intermediates **21** or **22** by the methodology of Scheme 6 prompted us to consider another route to both heterobactin A (**1**) and Nocardia heterobactin (**3**), shown in Scheme 8. The alternative synthesis of the latter siderophore began with the condensation of



Scheme 8. Alternate synthesis of **22**.

D-ornithine (**10**) itself with 2 mol of 2,3-dibenzyloxybenzoic acid (**17**) activated as NHS ester to produce diamide acid **25** in 77% yield. The carboxylate group of **25** was then coupled with the amine of **15** to produce the benzyl-protected precursor (**22**) to the Nocardia siderophore in 55% yield. As the debenzylation of **22** was quantitative, the overall yield of Nocardia heterobactin (**3**) from D-ornithine (**10**) via Scheme 8 was 37%, while the previous route from D-N^2-Boc ornithine (**16**) provided **3** in only a 16% overall yield.

A similar approach was also invoked for the assembly of heterobactin A (**1**) but failed. The *N*-Boc protecting group of amide **18** (Scheme 4) was quantitatively removed with TFA. Unfortunately, we were unable to condense oxazolecarboxylic acid **4** with the resulting primary amine using either DCC or CDI.

2.3. Stoichiometry

In the original papers describing the structures of heterobactins A (**1**) and B (**2**)[11] and Nocardia heterobactin (**3**),[12] the authors offer some preliminary evidence regarding the stoichiometry of the siderophore/iron complexes. The evidence, largely mass spectral data, is in keeping with the idea that heterobactins A and B and Nocardia heterobactin all form 1:1 ligand/metal complexes. Winkelmann,[11] further speculates that the tetracoordinate heterobactin B may also form a 3:2 ligand/metal complex. While the mass spectral data is certainly consistent with the 1:1 ligand/metal complexes, more complete support for the solution chemistry seemed necessary.

In order to further substantiate the nature of the metal complexes, Job's plots were run for all three ligands at pH 7.4. The total siderophore plus iron concentrations were kept constant while the mole fraction was varied. The absorbance max was read at 520 nm for the heterobactin A and Nocardia heterobactin iron complexes and at 508 nm for the heterobactin B iron complex (Fig. 3). The Job's plots for the heterobactin A and Nocardia heterobactin iron complexes complement the mass spectral data and are consistent with 1:1 ligand/metal complexes. However, the Job's plot for the heterobactin B iron complex suggests that a 3:2 ligand/metal chelator exists at pH 7.4.

3. Conclusion

Siderophores from *R. erythropolis*, heterobactins A (**1**) and B (**2**), and from *N. tenerifensis*, Nocardia heterobactin (**3**), are now synthetically accessible. Two approaches were investigated. One scheme is based on the likely biosynthesis of heterobactin A and Nocardia heterobactin, a biomimetic design (Scheme 1). With this methodology, the central donor fragments for both heterobactin A and the Nocardia heterobactin are added to the heterobactin B backbone last.

In the second design, the cyclic L-ornithine hydroxamate 'end' of the ligand is coupled to the N^2,N^5-bis(2,3-dihydroxybenzoyl)-D-ornithine 'end' of the siderophore (Scheme 2). In this instance, all of the donor fragments are already in place. The latter scheme worked well for Nocardia heterobactin (**3**) but was not viable for heterobactin A (**1**). The former approach (Scheme 1) provided all of the heterobactins and would also permit the generation of synthetic heterobactin analogues for biological testing, especially in vivo metal decorporation. Specifically, amine **20** (Scheme 6) could be reacted with a wide range of activated carboxylic acids followed by removal of the *O*-benzyl protecting groups.

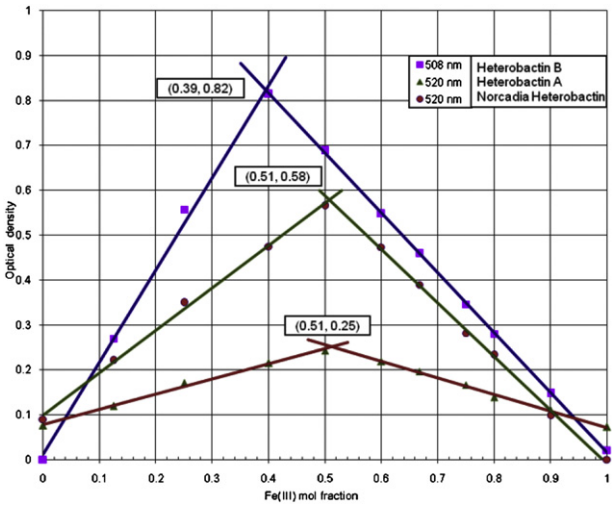


Fig. 3. Job's plots of the Fe(III) complex of heterobactins A (**1**), B (**2**), and Nocardia heterobactin (**3**). Solutions containing different ligand/Fe(III) ratios were prepared such that [ligand]+[Fe(III)]=0.9 mM at pH 7.4.

With the ligands available in sufficient quantity, we were able to evaluate the iron complex stoichiometries utilizing Job's plots. Both heterobactin A (**1**) and Nocardia heterobactin (**3**) formed 1:1 ligand/metal complexes. Heterobactin B (**2**) was shown to form a 3:2 ligand/metal complex. These findings support previous structural studies predicated on mass spectral data.[11] The included work will be valuable for investigators interested in the potential oil spill bioremediation properties of *Rhodococcus*. They now have facile access to heterobactins A (**1**) and B (**2**), analytical standards, and biosynthetic intermediates that will help define the role of iron in microbial growth.

4. Experimental

4.1. General procedures

Reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Fisher Optima-grade solvents were routinely used, and THF was distilled from sodium/benzophenone. Reactions were run under a nitrogen atmosphere, and the organic extracts were dried with sodium sulfate and then filtered. Silica gel 70–230 from Fisher Scientific (Pittsburgh, PA) was utilized for column chromatography, and silica gel 40–63 from SiliCycle, Inc. (Quebec City, Quebec, Canada) was used for flash column chromatography. Compounds **1**–**3** were chromatographed using Sephadex LH-20, which was obtained from Amersham Bioscience (Piscataway, NJ). Fractions were spotted on a silica gel TLC plate and sprayed with 1% ferric chloride in ethanol. Distilled solvents and glassware that had been presoaked in 3 N HCl for 15 min, washed with distilled water and distilled ethanol, and oven dried were used in the isolation of **1**–**3**. Optical rotations were run at 589 nm (sodium D line) at 20 °C utilizing a Perkin–Elmer 341 polarimeter, with *c* being concentration in grams of compound per 100 mL of solution ($CHCl_3$ not indicated). The iron content of the Fe(III)-NTA solution was verified

using a Perkin–Elmer 5100 PC Atomic Absorption Spectrophotometer (AAS). Data for the Job's plots were recorded on a UV-2550 UV–vis spectrophotometer. High resolution mass spectra were obtained on an Agilent 6210 TOF mass spectrometer using electrospray ionization (ESI). The base peaks are reported for the high resolution mass spectra. NMR spectra were obtained at 400 MHz (^{1}H) or 100 MHz (^{13}C) on a Varian Mercury 400 BB instrument. Chemical shifts (δ) for ^{1}H spectra are given in parts per million downfield from tetramethylsilane for organic solvents ($CDCl_3$ not indicated) or sodium 3-(trimethylsilyl)proponate-2,2,3,3-d_4 for D_2O. Chemical shifts (δ) for ^{13}C NMR spectra are given in parts per million referenced to 1,4-dioxane (δ 67.19) in D_2O or to the residual solvent resonance in $CDCl_3$ (δ 77.16) (not indicated) or CD_3OD (δ 49.00). Coupling constants (J) are in hertz. R_f values were calculated from silica gel TLC plates run on the column chromatography solvent unless otherwise specified. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected.

4.2. Procedures

*4.2.1. tert-Butyl (S)-[2-[[1-(benzyloxy)-2-oxopiperidin-3-yl]-amino]-2-oxoethyl]carbamate (**14**).* CDI (0.162 g, 1.0 mmol) was added to a solution of **13** (0.175 g, 1.0 mmol) in CH_2Cl_2 (5 mL). After stirring for 1 h, a solution of **12** (0.301 g, 1.0 mmol) in CH_2Cl_2 (2 mL) and TEA (0.202 g, 2.0 mmol) was added. The solution was stirred for 24 h at room temperature and diluted with CH_2Cl_2 (25 mL). The organic layer was washed with 8% $NaHCO_3$ (25 mL), 0.5 M citric acid (25 mL), and saturated NaCl (25 mL) and was concentrated by rotary evaporation. Flash chromatography using 5% CH_3OH/CH_2Cl_2 (R_f 0.5) generated 0.256 g (68%) of **14** as a viscous oil: [α] +98.0 (*c* 0.10). ^{1}H NMR δ 1.46 (s, 9H), 1.82–1.89 (m, 2H), 2.34–2.42 (m, 1H), 3.31–3.38 (m, 1H), 3.39–3.48 (m, 1H), 3.81 (dd, 1H, J=16.8, 4.8), 3.94 (dd, 1H, J=17.2, 4.8), 4.40 (quintet, 1H, J=6.0), 4.92 (2d, 2H, J=10.8, 10.8), 5.26 (br s, 1H), 6.98 (d, 1H, J=6.0), 7.36–7.43 (m, 5H). ^{13}C NMR δ 21.0, 27.8, 28.5, 44.3, 51.4, 51.4, 76.2, 80.4, 128.7, 129.0, 129.7, 135.2, 156.1, 167.9, 169.9. HRMS *m*/*z* calcd for $C_{19}H_{27}N_3O_5$ 378.2023 $[M+H]^+$, 400.1843 $[M+Na]^+$, 777.3794 $[2M+Na]^+$; found 378.2021 $[M+H]^+$, 400.1838 $[M+Na]^+$, 777.3794 $[2M+Na]^+$.

*4.2.2. (S)-2-Amino-N-[1-(benzyloxy)-2-oxopiperidin-3-yl]-acetamide trifluoroacetate (**15**).* TFA (1.96 mL, 26.4 mmol) was added to **14** (0.66 g, 1.75 mmol) in CH_2Cl_2 (2 mL) with ice bath cooling followed by triethylsilane (0.70 mL, 4.4 mmol), and the solution was stirred for 1 h at 0 °C and 2 h at room temperature. Volatiles were removed under reduced pressure. The procedure was repeated with toluene, and the residue was dried under high vacuum to give 0.65 g (quantitative) of **15** as a white solid, mp 91–92 °C: [α] +5.2 (*c* 0.115, H_2O). ^{1}H NMR (D_2O) δ 1.72–2.10 (m, 4H), 3.54–3.62 (m, 2H), 3.84 (s, 2H), 4.46–4.52 (m, 1H), 4.97 (s, 2H), 7.40–7.54 (m, 5H). ^{13}C NMR (D_2O) δ 23.2, 29.4, 43.4, 52.9, 53.6, 78.6, 119.3 (q, J=290.2), 131.7, 132.3, 133.0, 137.2, 166.0 (q, J=35.2), 169.8, 170.7. HRMS *m*/*z* calcd for $C_{14}H_{19}N_3O_3$ (free amine) 278.1499 $[M+H]^+$, 300.1319 $[M+Na]^+$, 555.2926 $[2M+H]^+$, 577.2725 $[2M+Na]^+$; found 278.1486 $[M+H]^+$, 300.1305 $[M+Na]^+$, 555.2905 $[2M+H]^+$, 577.2724 $[2M+Na]^+$.

*4.2.3. (R)-5-[2,3-Bis(benzyloxy)benzamido]-2-[(tert-butoxy-carbonyl)amino]pentanoic acid (**18**).* A solution of DCC (0.206 g, 1.0 mmol) in THF (1 mL) was added dropwise to a mixture of **17** (0.334 g, 1.0 mmol) and NHS (0.115 g, 1.0 mmol) in THF (2 mL) at 0 °C. The mixture was stirred for 6 h at room temperature and was filtered. The filtrate was added to a solution of **16** (0.232 g, 1.0 mmol) and $KHCO_3$ (0.20 g, 2.0 mmol) in 50% aqueous THF (20 mL) at pH ~8. After the reaction mixture was stirred at room temperature for 24 h, THF was removed by rotary evaporation. The residue was treated with 0.5 M citric acid (20 mL) and extracted with EtOAc (2×25 mL). The combined organic portion was washed with H_2O (25 mL) and saturated NaCl (25 mL) and concentrated under reduced pressure. Column chromatography with 10% $CH_3OH/CHCl_3$ (R_f 0.55) provided 0.39 g (70%) of **18** as a white solid, mp 65–66 °C: [α] −9.0 (*c* 0.2). ^{1}H NMR δ 1.36–1.95 (m+s, 13H), 3.12–3.20 (m, 1H), 3.32–3.44 (m, 1H), 4.34 (q, 1H, J=4.4), 5.09 (s, 2H), 5.15 (s, 2H), 7.15–7.18 (m, 2H), 7.30–7.50 (m, 10H), 7.68–7.73 (m, 1H), 8.17 (br s, 1H). ^{13}C NMR δ 25.0, 25.6, 28.5, 30.1, 33.8, 39.1, 52,8, 71.5, 76.7, 80.1, 117.4, 123.5, 124.6, 126.7, 127.8, 128.4, 128.8, 128.9, 129.0, 129.0, 136.4, 136.5, 147.1, 151.8, 155.9, 166.1, 174.9. HRMS *m*/*z* calcd for $C_{31}H_{36}N_2O_7$ 549.2595 $[M+H]^+$, 571.2415 $[M+Na]^+$, 1119.4937 $[2M+Na]^+$; found 549.2584 $[M+H]^+$, 571.2410 $[M+Na]^+$, 1119.4888 $[2M+Na]^+$.

*4.2.4. tert-Butyl [(R)-1-[[2-[[(S)-1-(benzyloxy)-2-oxopiperidin-3-yl]amino]-2-oxoethyl]amino]-5-[2,3-bis(benzyloxy)benzamido]-1-oxopentan-2-yl]carbamate (**19**).* CDI (0.109 g, 0.67 mmol) was added to a solution of **18** (0.370 g, 0.67 mmol) in CH_2Cl_2 (2 mL) and stirred for 1 h at room temperature. The resulting solution was cooled to 0 °C and was added to a suspension of **15** (0.262 g, 0.67 mmol) and TEA (0.14 mL, 1.34 mmol) at 0 °C. The solution was stirred for 15 h at room temperature, followed by the addition of CH_2Cl_2 (20 mL). The reaction mixture was washed with 8% $NaHCO_3$ (25 mL), 0.5 M citric acid (25 mL), saturated NaCl (25 mL). The organic phase was concentrated by rotary evaporation. Flash chromatography eluting with 5% CH_3OH/CH_2Cl_2 (R_f 0.4) afforded 0.43 g (80%) of **19** as a colorless solid, mp 72–73 °C: [α] +44.35 (*c* 0.115). ^{1}H NMR δ 1.43 (s, 9H), 1.48–1.58 (m, 2H), 1.72–1.84 (m, 6H), 2.21–2.25 (m, 1H), 3.03–3.16 (m, 1H), 3.24–3.30 (m, 1H), 3.33–3.42 (m, 1H), 3.49–3.58 (m, 1H), 3.90 (dd, 1H, J=5.6, 16.8), 4.04 (dd, 1H, J=5.6, 16.8), 4.18–4.27 (m, 1H), 4.28–4.35 (m, 1H), 4.82 (2d, 2H, J=10.4, 10.4), 5.08 (s, 2H), 5.14 (s, 2H), 5.30 (br s, 1H), 7.01 (br s, 1H), 7.14 (d, 2H, J=5.2), 7.27–7.48 (m, 15H), 7.67 (t, 1H, J=4.8), 8.10 (br s, 1H). ^{13}C NMR δ 21.1, 26.1, 27.6, 28.5, 30.1, 38.6, 43.2, 51.3, 53.9, 71.4, 76.0, 76.6, 77.6, 80.3, 117.1, 123.3, 124.6, 125.4, 127.4, 127.8, 128.4, 128.4, 128.6, 128.8, 128.9, 129.0, 129.0, 129.2, 129.7, 135.3, 136.5, 146.9, 151.8, 165.9, 167.5, 169.3, 173.0. HRMS *m*/*z* calcd for $C_{45}H_{53}N_5O_9$ 808.3916 $[M+H]^+$, 830.3736 $[M+Na]^+$; found 808.3886 $[M+H]^+$, 830.3706 $[M+Na]^+$.

*4.2.5. N-[(R)-4-Amino-5-[[2-[[(S)-1-(benzyloxy)-2-oxopiperidin-3-yl]amino]-2-oxoethyl]amino]-5-oxopentyl]-2,3-bis(benzyloxy) benzamide trifluoroacetate (**20**).* TFA (0.45 mL, 6.0 mmol) and triethylsilane (0.16 mL, 1.0 mmol) were successively added to **19** (0.320 g, 0.4 mmol) in CH_2Cl_2 (2 mL) with ice bath cooling, and the solution was stirred at 0 °C for 1 h and at room temperature for 3 h. After the removal of volatiles by rotary evaporation, the residue was dried under high vacuum to give 0.306 g (95%) of **20** as a white solid, mp 94–95 °C: [α] −2.5 (*c* 0.12, CH_3OH). ^{1}H NMR δ 1.54–1.98 (m, 8H), 2.94–3.05 (m, 1H), 3.16–3.24 (m, 1H), 3.32–3.41 (m, 2H), 3.72–3.80 (m, 1H), 4.11–4.17 (m, 1H), 4.24–4.30 (m, 1H), 4.43–4.52 (m, 1H), 4.79 (2d, 2H, J=10.4, 10.4), 5.04 (s, 2H), 5.12 (s, 2H), 7.06–7.12 (m, 2H), 7.27–7.33 (m, 13H), 7.36 (m, 5H), 7.56 (dd, 1H, J=2.0, 7.6), 8.10 (d, 1H, J=7.6), 8.19 (t, 1H, J=6.4), 8.67 (br s, 1H). ^{13}C NMR δ 21.0, 25.2, 27.2, 28.4, 29.9, 37.8, 43.3, 50.7, 52.7, 71.4, 75.7, 76.7, 116.4 (q, J=290.6), 117.4, 122.9, 124.6, 126.8, 127.5, 127.8, 128.0, 128.5, 128.6, 128.7, 128.8, 128.9, 129.0, 129.0, 129.6, 129.7, 134.9, 136.3, 136.4, 147.0, 151.9, 161.6 (q, J=35.9), 166.5, 167.7, 169.7, 171.0. HRMS *m*/*z* calcd for $C_{40}H_{46}N_5O_7$ (free amine) 708.3353 $[M+H]^+$, 730.3217 $[M+Na]^+$; found 708.3390 $[M+H]^+$, 730.3220 $[M+Na]^+$.

*4.2.6. N-[(R)-1-[[2-[[(S)-1-(Benzyloxy)-2-oxopiperidin-3-yl]-amino]-2-oxoethyl]amino]-5-[2,3-bis(benzyloxy)benzamido]-1-oxopentan-2-yl]-5-hydroxy-4-benzoxazolecarboxamide (**21**).* A solution of DCC (0.166 g, 0.81 mmol) in THF (1 mL) was added dropwise to a mixture of **4** (0.143 g, 0.81 mmol) and NHS (0.092 g, 0.81 mmol) in THF (2 mL) at 0 °C. The reaction mixture was stirred overnight at room temperature and was filtered. The filtrate was concentrated to

dryness under reduced pressure. The residue was added to a solution of **20** (0.64 g, 0.80 mmol) and TEA (0.160 g, 1.6 mmol) in 5% aqueous CH_3CN (20 mL) followed by stirring at room temperature for 48 h. Volatiles were removed under reduced pressure, and the residue was treated with 0.25 M citric acid (20 mL) and extracted with EtOAc (2×25 mL). The combined organic extracts were washed with H_2O (25 mL) and saturated NaCl (25 mL), and concentrated. Column chromatography with 8% CH_3OH/CH_2Cl_2 (R_f 0.6) generated 0.316 g (45%) of **21** as a viscous oil: [α] −5.0 (*c* 0.11). ^{1}H NMR δ 1.50–1.70 (m, 3H), 1.71–2.10 (m, 4H), 2.24–2.31 (m, 1H), 3.12–3.21 (m, 1H), 3.24–3.32 (m, 1H), 3.36–3.43 (m, 1H), 3.61–3.71 (m, 1H), 3.91 (dd, 1H, *J*=16.8, 5.6), 4.11 (dd, 1H, *J*=16.8, 6.0), 4.31 (quintet, 1H, *J*=5.6), 4.83 (2d, 2H, *J*=10.0, 10.0), 5.07 (s, 2H), 5.14 (s, 2H), 6.98 (d, 1H, *J*=9.2), 7.05 (d, 1H, *J*=6.8), 7.12–7.15 (m, 2H), 7.27–7.47 (m, 15H), 7.50 (t, 1H, *J*=6.0), 7.55 (d, 1H, *J*=9.2), 7.66 (t, 1H, *J*=5.6), 8.09 (s, 1H), 8.12 (t, 1H, *J*=5.6), 9.62 (d, 1H, *J*=7.2), 12.61 (s, 1H). ^{13}C NMR δ 21.0, 26.1, 27.6, 30.2, 38.5, 43.3, 51.3, 51.4, 52.7, 71.4, 76.0, 76.6, 104.3, 116.3, 116.8, 117.0, 123.2, 124.6, 127.5, 127.8, 128.4, 128.6, 128.8, 128.8, 128.9, 129.0, 129.7, 135.3, 136.4, 136.5, 137.8, 143.0, 146.9, 151.8, 153.7, 160.5, 165.9, 167.5, 169.2, 169.2, 172.0. HRMS *m*/*z* calcd for $C_{48}H_{48}N_6O_{10}$ 891.3324 $[M+H]^+$, 913.3144 $[M+Na]^+$; found 891.3317 $[M+H]^+$, 913.3129 $[M+Na]^+$.

*4.2.7. N,N′-[(R)-5-[[2-[[(S)-1-(Benzyloxy)-2-oxopiperidin-3-yl]-amino]-2-oxoethyl]amino]-5-oxopentane-1,4-diyl]bis[2,3-bis-(benzyloxy)benzamide] (**22**).* A solution of DCC (0.103 g, 0.5 mmol) in THF (1 mL) was added dropwise to a mixture of **17** (0.167 g, 0.5 mmol) and NHS (0.058 g, 0.5 mmol) in THF (2 mL) at 0 °C, and the reaction mixture was stirred at room temperature for 16 h, filtered, and concentrated to dryness under reduced pressure. The residue was added to a solution of **20** (0.402 g, 0.5 mmol) and TEA (0.101 g, 1.0 mmol) in 10% aqueous CH_3CN (20 mL). After the mixture was stirred at room temperature for 40 h, volatiles were removed by rotary evaporation. The residue was treated with 0.5 M citric acid (20 mL) and extracted with EtOAc (2×25 mL). Organic extracts were washed with H_2O (25 mL) and saturated NaCl (25 mL), and concentrated in vacuo. Column chromatography with 10% $CH_3OH/CHCl_3$ generated 0.153 g (30%) of **22** as a glassy solid.

Alternate Method: CDI (0.041 g, 0.25 mmol) was added to a solution of **25** (0.191 g, 0.25 mmol) in CH_2Cl_2 (1 mL) and stirred for 1 h at room temperature. The resulting solution was added to a suspension of **15** (0.94 g, 0.25 mmol) and TEA (0.07 mL, 0.67 mmol) at 0 °C. The reaction mixture was stirred for 24 h at room temperature, diluted with CH_2Cl_2 (10 mL), and washed with 8% $NaHCO_3$ (10 mL), 0.5 M citric acid (10 mL), and saturated NaCl (10 mL). Concentration of the organic phase under reduced pressure and flash chromatography, eluting with 10% $CH_3OH/CHCl_3$ (R_f 0.6) afforded 0.141 g (55%) of **22** as a glassy solid, mp 67–68 °C: [α] +30.67 (*c* 0.15). ^{1}H NMR δ 1.50–1.70 (m, 5H), 1.71–1.94 (m, 2H), 2.19–2.24 (m, 1H), 2.96–3.08 (m, 1H), 3.24–3.44 (m, 3H), 3.83 (dd, 1H, *J*=16.8, 5.6), 4.04 (dd, 1H, *J*=16.8, 6.0), 4.31–4.42 (m, 1H), 4.83 (2d, 2H, *J*=10.0, 10.0), 5.05 (s, 2H), 5.11 (s, 2H), 5.15 (s, 2H), 5.13–5.20 (m, 2H), 6.89 (t, 1H, *J*=6.0), 7.10–7.16 (m, 5H), 7.27–7.46 (m, 23H), 7.64–7.72 (m, 2H), 7.93 (t, 1H, *J*=5.2), 8.39 (d, 1H, *J*=5.6). ^{13}C NMR δ 21.2, 26.1, 27.7, 28.9, 30.0, 38.6, 38.8, 43.3, 51.3, 51.3, 54.3, 71.5, 71.6, 76.0, 76.7, 117.2, 117.8, 123.5, 124.6, 124.7, 126.9, 127.5, 127.9, 127.9, 128.5, 128.7, 128.9, 129.0, 129.0, 129.3, 129.8, 135.6, 136.5, 136.6, 136.6, 147.0, 147.4, 151.9, 151.9, 165.6, 166.5, 167.4, 169.5, 172.2. HRMS *m*/*z* calcd for $C_{61}H_{61}N_5O_{10}$ 1046.4311 $[M+Na]^+$, 2070.8761 $[2M+Na]^+$; found 1046.4356 $[M+Na]^+$, 2070.8798 $[2M+Na]^+$.

*4.2.8. Methyl 5-hydroxy-4-benzoxazolecarboxylate (**24**).* Triethyl orthoformate (1.21 mL, 7.29 mmol) was added to a solution of **23** (0.4 g, 1.82 mmol) in EtOH (2 mL). The reaction mixture was refluxed for 24 h, cooled to room temperature and diluted with acetone (20 mL). After treatment with charcoal, the mixture was filtered through a bed of Celite and silica gel, and the filtrate was concentrated under reduced pressure. Column chromatography eluting with 5% CH_3OH/CH_2Cl_2 (R_f 0.65 in 10% CH_3OH/CH_2Cl_2) provided 0.265 g (75%) of **24** as a yellow solid, mp 134–135 °C: ^{1}H NMR δ 4.13 (s, 3H), 7.07 (d, 1H, *J*=8.8), 7.7 (d, 1H, *J*=8.8), 8.19 (s, 1H), 11.30 (s, 1H). ^{13}C NMR δ 53.1, 103.9, 116.3, 118.0, 139.1, 143.9, 154.4, 161.0, 170.5. HRMS *m*/*z* calcd for $C_9H_7NO_4$ 216.0267 $[M+Na]^+$, 238.0087 $[M-H+2Na]^+$, 409.0642 $[2M+Na]^+$; found 216.0267 $[M+Na]^+$, 238.0080 $[M-H+2Na]^+$, 409.0629 $[2M+Na]^+$.

*4.2.9. 5-Hydroxy-4-benzoxazolecarboxylic acid (**4**).* Anhydrous LiI (0.47 g, 11.9 mmol) was added to a solution of **24** (0.23 g, 1.19 mmol) in THF (20 mL), and the mixture was refluxed for 18 h in the dark. The solvent was removed by rotary evaporation. The residue was dissolved in H_2O (10 mL), and the pH was adjusted to ~2 with 1 N HCl. The mixture was extracted with EtOAc (2×20 mL), and the organic phase was concentrated under reduced pressure. Column chromatography eluting with 10% CH_3OH/CH_2Cl_2 (R_f 0.28) afforded 0.162 g (76%) of **4** as light yellow solid, mp 171–172 °C: ^{1}H NMR (CD_3OD): δ 7.05 (d, 1H, *J*=9.2), 7.81 (d, 1H, *J*=8.8), 8.56 (s, 1H). ^{13}C NMR (CD_3OD) δ=104.6, 117.1, 118.8, 139.9, 145.0, 156.8, 162.3, 172.3. HRMS *m*/*z* calcd for $C_8H_5NO_4$ 223.9930 $[M-H+2Na]$, 245.9750 $[M-2H+3Na]^+$; found 223.9932 $[M-H+2Na]$, 245.9744 $[M-2H+3Na]^+$.

*4.2.10. (R)-2,5-Bis[2,3-bis(benzyloxy)benzamido]pentanoic acid (**25**).* A solution of DCC (0.206 g, 1.0 mmol) in THF (3 mL) was added dropwise to a mixture of **17** (0.334 g, 1.0 mmol) and NHS (0.115 g, 1.0 mmol) in THF (3 mL) at 0 °C. The solution was stirred for 16 h at room temperature and was filtered. The filtrate was added to a solution of **10** (0.116 g, 0.5 mmol) and TEA (0.202 g, 2.0 mmol) in 10% aqueous THF (20 mL). After the mixture was stirred at room temperature for 48 h, volatiles were removed under reduced pressure. The residue was dissolved in 0.5 M citric acid (15 mL) and extracted with EtOAc (2×20 mL). The combined organic portion was washed with H_2O (20 mL) and saturated NaCl (25 mL) and then concentrated. Column chromatography with 12% $CH_3OH/CHCl_3$ (R_f 0.33) provided 0.294 g (77%) of **25** as a colorless solid, mp 45–46 °C: [α] +15.65 (*c* 0.65, CH_3OH). ^{1}H NMR δ 1.20–1.46 (m, 2H), 1.50–1.94 (m, 2H), 3.08 (quintet, 1H, *J*=6.4), 3.19 (quintet, 1H, *J*=6.4), 4.66 (d, 1H, *J*=4.8), 5.01 (s, 2H), 5.06–5.17 (m+s, 6H), 7.08–7.46 (m, 26H), 7.68–7.77 (m, 2H), 7.96 (br s, 1H), 8.55 (d, 1H, *J*=7.6). ^{13}C NMR δ 25.7, 29.4, 39.2, 52.5, 71.3, 71.4, 76.3, 76.5, 117.2, 117.5, 123.4, 123.4, 124.5, 126.5, 127.0, 127.7, 127.8, 127.9, 128.3, 128.6, 128.6, 128.7, 128.8, 128.8, 128.9, 129.0, 129.0, 136.2, 136.3, 136.4, 136.5, 147.0, 147.1, 151.7, 151.8, 165.6, 168.6, 174.4. HRMS *m*/*z* calcd for $C_{47}H_{44}N_2O_8$ 765.3170 $[M+H]^+$, 787.2990 $[M+Na]^+$, 809.2809 $[M-H+2Na]^+$; found 765.3154 $[M+H]^+$, 787.2987 $[M+Na]^+$, 809.2797 $[M-H+2Na]^+$.

*4.2.11. Heterobactin B (**2**).* Pd–C (10%, 0.75 g) and a catalytic amount of 1 N HCl were added to a solution of **20** (0.141 g, 0.2 mmol) in CH_3OH (5 mL), and the mixture was stirred under H_2 at atmospheric pressure for 6 h. The mixture was filtered through Celite, and the solids were washed with CH_3OH (3×5 mL). The combined filtrate was concentrated by rotary evaporation. The residue was dissolved in CH_3OH (10 mL), and Sephadex LH-20 (0.60 g) was introduced. After 4 h, the solvent was removed under reduced pressure. The solid was loaded onto a preswelled LH-20 (3.0 g) column and was eluted with 1:14:85 H_2O/EtOH/toluene. The iron active fractions were combined and concentrated to afford 0.091 g (96%) of **2** as a white solid, mp 147–148 °C: [α] −33.64 (*c* 0.11, CH_3OH). ^{1}H NMR (CD_3OD) δ 1.71–1.82 (m, 3H), 1.89 (m, 5H), 3.45 (t, 2H, *J*=6.4), 3.56–3.64 (m, 2H), 3.94 (t, 1H, *J*=6.4), 3.97 (s, 2H), 4.45–4.49 (m, 1H), 6.72 (t, 1H, *J*=7.6), 6.92 (d, 1H, *J*=8.0), 7.15 (d, 1H, *J*=7.2). ^{13}C NMR (CD_3OD) δ 21.7, 26.0, 28.7, 29.9, 39.6, 43.2, 51.5, 52.6, 54.3, 116.7, 118.7, 119.6, 147.4, 150.2, 167.2, 170.6, 170.9, 171.7. HRMS *m*/*z* calcd for $C_{19}H_{27}N_5O_7$ (free amine) 438.1983

$[M+H]^+$, 460.1803 $[M+Na]^+$, 875.3894 $[2M+H]^+$; found 438.1978 $[M+H]^+$, 460.1793 $[M+Na]^+$, 875.3859 $[2M+H]^+$.

*4.2.12. Heterobactin A (**1**).* Pd–C (10%, 0.60 g) was added to a solution of **21** (0.130 g 0.15 mmol) in CH_3OH (5 mL), and the mixture was stirred under H_2 at atmospheric pressure for 4 h. The mixture was filtered through Celite, and the solids were washed with CH_3OH (3×5 mL). The combined filtrate was concentrated by rotary evaporation. The residue was dissolved in CH_3OH (10 mL), and LH-20 (0.5 g) was added. After 4 h, the solvent was removed under reduced pressure. The solid was loaded onto a preswelled LH-20 (2.5 g) column, which was eluted with 2–50% EtOH in toluene. The iron active fractions were combined and concentrated to afford 0.089 g (quantitative) of **1** as a white solid, mp 140–141 °C: [α] −7.5 (*c* 0.24, CH_3OH). ^1H NMR (CD_3OD) δ 1.69–2.13 (m, 8H), 3.46–3.63 (m, 4H), 3.90 (m, 2H,), 4.38–4.46 (m, 1H), 4.63–4.67 (m, 1H), 6.34 (t, 1H, *J*=7.6), 6.74 (d, 1H, *J*=7.6), 6.93 (d, 1H, *J*=8.8), 7.29 (d, 1H, *J*=8.0), 7.62 (d, 1H, *J*=9.2), 8.35 (s, 1H). ^{13}C NMR (CD_3OD) δ 21.7, 27.3, 28.7, 30.9, 39.4, 43.9, 51.5, 53.1, 54.9, 113.7, 115.0, 116.8, 118.3, 118.9, 118.9, 120.6, 139.6, 139.6, 143.7, 150.4, 155.8, 158.2, 164.8, 170.5, 171.6, 171.8, 175.0. HRMS *m*/*z* calcd for $C_{26}H_{31}N_5O_{10}$ 597.1963 $[M-H]^-$, 599.2096 $[M+H]^+$; found 597.1967 $[M-H]^-$, 599.2028 $[M+H]^+$.

*4.2.13. Nocardia heterobactin (**3**).* Pd–C (10%, 0.20 g) was added to a solution of **22** (0.283 g 0.4 mmol) in CH_3OH (10 mL), and the mixture was stirred under H_2 at atmospheric pressure for 6 h. The mixture was filtered through Celite, and the residue was washed with CH_3OH (3×5 mL). The combined filtrate was concentrated by rotary evaporation. The residue was dissolved in CH_3OH (10 mL), and LH-20 (1.0 g) was added. After 4 h, the solvent was removed under reduced pressure. The solid was loaded onto a preswelled LH-20 (5.0 g) column, which was eluted with 1:10:89 H_2O/EtOH/toluene. The iron active fractions were combined and concentrated to afford 0.229 g (quantitative) of **3** as a fluffy pale solid, mp 135–136 °C: [α] −12.0 (*c* 0.15, CH_3OH). ^1H NMR (CD_3OD) δ 1.70–1.84 (m, 2H), 1.85–2.10 (m, 6H), 3.38–3.52 (m, 2H), 3.52–3.68 (m, 2H), 3.82–4.02 (m, 2H), 4.42–4.54 (m, 1H), 4.54–4.66 (m, 1H), 6.68–6.77 (m, 2H), 6.87–6.96 (m, 2H), 7.20 (d, 1H, *J*=7.2)), 7.35 (d, 1H, *J*=7.2). ^{13}C NMR (CD_3OD) δ 21.9, 27.2, 28.9, 30.3, 40.2, 43.8, 51.6, 52.7, 55.5, 116.9, 117.3, 118.8, 119.6, 119.7, 119.8, 120.0, 120.1, 147.4, 147.5, 149.8, 150.5, 167.3, 171.5, 171.6, 174.5, 174.9. HRMS *m*/*z* calcd for $C_{26}H_{31}N_5O_{10}$ 574.2144 $[M+H]^+$, 596.1963 $[M+Na]^+$; found 574.2143 $[M+H]^+$, 596.1966 $[M+Na]^+$.

4.3. Job's plots for heterobactins A (1), B (2) and Nocardia heterobactin (3)

The stoichiometries of the ligand-Fe(III) complexes of **1**–**3** were determined spectrophotometrically using Job's plots. Solutions were monitored at the visible λ_{max} of the Fe(III) complexes (508 nm for **2** and 520 nm for **1** and **3**). A 25 mM MOPS buffer with 50% CH_3OH (v/v) was used to maintain the pH at 7.4. Solutions containing different ligand/Fe(III) ratios were prepared by mixing appropriate volumes of 0.9 mM ligand solution and 0.9 mM Fe(III)-nitriloacetate (NTA) in MOPS-MeOH solution. The 0.9 mM Fe(III)-NTA solution was prepared immediately prior to use by dilution of a 45 mM Fe(III)-NTA stock solution with the MOPS/CH_3OH mixture. The Fe(III)-NTA stock solution was prepared by mixing equal volumes of 90 mM $FeCl_3$ and 180 mM trisodium NTA. The iron content of the Fe(III)-NTA solution was verified by AAS.

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