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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., desferrioxamine⁵ and nocardamine,⁶ or catecholamide donors, e.g., petrobactin⁷ and vibriobactin⁸ (Figure 1). These particular ligands form 1:1 complexes with Fe(III). Catecholamide chelators typically form tighter Fe(III) complexes, e.g., parabactin⁹ ($K_f = 10^{48} \text{ M}^{-1}$) (Figure 2), than their hydroxamate counterparts, e.g., desferrioxamine (10^{28} M^{-1}) (Figure 1).¹⁰

The current study focuses on the assembly of the heterobactins A and B (Figure 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., parabactin⁹ and agrobactin¹⁴ (Figure 2). For example, with parabactin, the bidentate fragments, 2,3-dihydroxybenzoyl 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**) derive from heterobactin B (**2**) (Figure 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 parabactin⁹ 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** (Figure 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 key to the success of Scheme 1 is the effective use of *N*- and *O*-protecting groups.

An alternate 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.

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).

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 CH₂Cl₂, 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 the *Nocardia* heterobactin (**3**) (Scheme 6) was accessed by first coupling **15** with **18** using CDI/TEA to generate the intermediate **19** in 80% yield. The *tert*-butoxycarbonyl protecting group of **19** was removed by treatment with TFA and triethylsilane in CH₂Cl₂, providing **20** in 95% yield.

When tripeptide **20** was debenzylated under a hydrogen atmosphere over Pd/C in CH₃OH 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 H₂ 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.

Alternatively, acylation of the amine 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 an 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 D-ornithine (**10**) itself with two moles 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 heterobactin 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 heterobactin A and *Nocardia* heterobactin iron complexes and at 508 nm for the heterobactin B iron complex (Figure 3). The Job's plots for the heterobactin A and *Nocardia* heterobactin iron complexes complement the mass spectral data and are consistent with the 1:1 ligand/metal complexes. However, the Job's plot for the heterobactin B iron complex suggests 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 *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 the *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.

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 heterobactin 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), and Fisher Optima-grade solvents were routinely used and THF was distilled from sodium/benzophenone. Reactions were run under a nitrogen atmosphere, and 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 was 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 (CHCl₃ 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 (¹H) or 100 MHz (¹³C) on a Varian Mercury 400 BB instrument. Chemical shifts (δ) for ¹H spectra are given in parts per million downfield from tetramethylsilane for organic solvents (CDCl₃ not indicated) or sodium 3-(trimethylsilyl)propanate-2,2,3,3-d₄ for D₂O. Chemical shifts (δ) for ¹³C NMR spectra are given in parts per million referenced to 1,4-dioxane (δ 67.19) in D₂O or to the residual solvent resonance in CDCl₃ (δ 77.16) (not indicated) or CD₃OD (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 CH₂Cl₂ (5 mL). After stirring for 1 h, a solution of **12** (0.301 g, 1.0 mmol) in CH₂Cl₂ (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 CH₂Cl₂ (25 mL). The organic layer was washed with 8% NaHCO₃ (25 mL), 0.5 M citric acid (25 mL), and saturated NaCl (25 mL) and was concentrated by rotary evaporation. Flash chromatography using 5 % CH₃OH/CH₂Cl₂ (*R_f* 0.5) generated 0.256 mg (68%) of **14** as viscous oil: [α]_D +98.0 (*c* 0.10). ¹H NMR δ 1.46 (s, 9 H), 1.82–1.89 (m, 2 H), 2.34–2.42 (m, 1 H), 3.31–3.38 (m, 1 H), 3.39–3.48 (m, 1 H), 3.81 (dd, 1 H, *J* = 16.8, 4.8), 3.94 (dd, 1 H, *J* = 17.2, 4.8), 4.40 (quintet, 1 H, *J* = 6.0), 4.92 (2d, 2 H, *J* = 10.8, 10.8), 5.26 (br s, 1H), 6.98 (d, 1H, *J* = 6.0), 7.36–7.43 (m, 5 H). ¹³C 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 C₁₉H₂₇N₃O₅ 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 CH₂Cl₂ (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 (quant.) of **15** as white solid, m.p.: 91–92 °C: [α]_D +5.2 (*c* 0.115, H₂O). ¹H NMR (D₂O) δ 1.72–2.10 (m, 4 H), 3.54–3.62 (m, 2 H), 3.84 (s, 2 H), 4.46–4.52 (m, 1 H), 4.97 (s, 2H), 7.40–7.54 (m, 5 H). ¹³C NMR (D₂O) 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 C₁₄H₁₉N₃O₃ (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 KHCO₃ (0.20 g, 2.0 mmol) in 50% aq. 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 was extracted with EtOAc (2 X 25 mL). The combined organic portion was washed with H₂O (25 mL) and saturated NaCl (25 mL) and concentrated under reduced pressure. Column chromatography with 10 % CH₃OH/CHCl₃ (*R_f* 0.55) provided 0.39 g (70%) of **18** as a white solid. m.p.: 65–66 °C: [α]_D –9.0 (c 0.2). ¹H NMR δ 1.36–1.95 (m + s, 13 H), 3.12–3.20 (m, 1 H), 3.32–3.44 (m, 1 H), 4.34 (q, 1 H, *J* = 4.4), 5.09 (s, 2 H), 5.15 (s, 2 H), 7.15–7.18 (m, 2 H), 7.30–7.50 (m, 10 H), 7.68–7.73 (m, 1 H), 8.17 (br s, 1 H). ¹³C 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 C₃₁H₃₆N₂O₇: 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 CH₂Cl₂ (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 CH₂Cl₂ (20 mL). The reaction mixture was washed with 8% NaHCO₃ (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% CH₃OH/CH₂Cl₂ (*R_f* 0.4) afforded 0.43 g (80%) of **19** as colorless solid. m.p.: 72–73 °C: [α]_D +44.35 (c 0.115). ¹H NMR δ 1.43 (s, 9 H), 1.48–1.58 (m, 2 H), 1.72–1.84 (m, 6 H), 2.21–2.25 (m, 1 H), 3.03–3.16 (m, 1 H), 3.24–3.30 (m, 1 H), 3.33–3.42 (m, 1 H), 3.49–3.58 (m, 1 H), 3.90 (dd, 1 H, *J* = 5.6, 16.8), 4.04 (dd, 1 H, *J* = 5.6, 16.8), 4.18–4.27 (m, 1 H), 4.28–4.35 (m, 1 H), 4.82 (2d, 2 H, *J* = 10.4, 10.4), 5.08 (s, 2 H), 5.14 (s, 2 H), 5.30 (br s, 1 H), 7.01 (brs, 1 H), 7.14 (d, 2 H, *J* = 5.2), 7.27–7.48 (m, 15 H), 7.67 (t, 1 H, *J* = 4.8), 8.10 (br s, 1H). ¹³C 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 C₄₅H₅₃N₅O₉: 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-oxo piperidin-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 CH₂Cl₂ (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, m.p.: 94–95 °C: [α]_D –2.5 (c 0.12, CH₃OH). ¹H NMR δ 1.54–1.98 (m, 8 H), 2.94–3.05 (m, 1 H), 3.16–3.24 (m, 1 H), 3.32–3.41 (m, 2 H), 3.72–3.80 (m, 1 H), 4.11–4.17 (m, 1 H), 4.24–4.30 (m, 1 H), 4.43–4.52 (m, 1 H), 4.79 (2d, 2 H, *J* = 10.4, 10.4), 5.04 (s, 2 H), 5.12 (s, 2 H), 7.06–7.12 (m, 2 H), 7.27–7.33 (m, 13 H), 7.36 (m, 5 H), 7.56 (dd, 1 H, *J* = 2.0, 7.6), 8.10 (d, 1 H, *J* = 7.6), 8.19 (t, 1 H, *J* = 6.4), 8.67 (br s, 1 H). ¹³C 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% aq. 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: $[\alpha]_D -5.0$ (c 0.11). 1H NMR δ 1.50–1.70 (m, 3 H), 1.71–2.10 (m, 4 H), 2.24–2.31 (m, 1 H), 3.12–3.21 (m, 1 H), 3.24–3.32 (m, 1 H), 3.36–3.43 (m, 1 H), 3.61–3.71 (m, 1 H), 3.91 (dd, 1 H, $J = 16.8, 5.6$), 4.11 (dd, 1 H, $J = 16.8, 6.0$), 4.31 (quintet, 1 H, $J = 5.6$), 4.83 (2d, 2 H, $J = 10.0, 10.0$), 5.07 (s, 2 H), 5.14 (s, 2 H), 6.98 (d, 1 H, $J = 9.2$), 7.05 (d, 1 H, $J = 6.8$), 7.12–7.15 (m, 2 H), 7.27–7.47 (m, 15 H), 7.50 (t, 1 H, $J = 6.0$), 7.55 (d, 1 H, $J = 9.2$), 7.66 (t, 1 H, $J = 5.6$), 8.09 (s, 1 H), 8.12 (t, 1 H, $J = 5.6$), 9.62 (d, 1 H, $J = 7.2$), 12.61 (s, 1 H). ^{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% aq. 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, m.p.: 67–68 °C: $[\alpha]_D +30.67$ (c 0.15). 1H NMR δ 1.50–1.70 (m, 5 H), 1.71–1.94 (m, 2 H), 2.19–2.24 (m, 1 H), 2.96–3.08 (m, 1 H), 3.24–3.44 (m, 3 H), 3.83 (dd, 1 H, $J = 16.8, 5.6$), 4.04 (dd, 1 H, $J = 16.8, 6.0$), 4.31–4.42 (m, 1 H), 4.83 (2d, 2 H, $J = 10.0, 10.0$),

5.05 (s, 2 H), 5.11 (s, 2 H), 5.15 (s, 2 H), 5.13–5.20 (m, 2 H), 6.89 (t, 1 H, $J = 6.0$), 7.10–7.16 (m, 5 H), 7.27–7.46 (m, 23 H), 7.64–7.72 (m, 2 H), 7.93 (t, 1 H, $J = 5.2$), 8.39 (d, 1 H, $J = 5.6$). ^{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 $\text{C}_{61}\text{H}_{61}\text{N}_5\text{O}_{10}$ 1046.4311 $[\text{M}+\text{Na}]^+$, 2070.8761 $[\text{M}+\text{Na}]^+$; found 1046.4356 $[\text{M}+\text{Na}]^+$, 2070.8798 $[\text{M}+\text{Na}]^+$.

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% $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$ (R_f 0.65 in 10% $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$) provided 0.265 g (75%) of **24** as a yellow solid, m.p.: 134–135 °C: ^1H NMR δ 4.13 (s, 3 H), 7.07 (d, 1 H, $J = 8.8$), 7.7 (d, 1 H, $J = 8.8$), 8.19 (s, 1 H), 11.30 (s, 1 H). ^{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 $\text{C}_9\text{H}_7\text{NO}_4$ 216.0267 $[\text{M}+\text{Na}]^+$, 238.0087 $[\text{M}-\text{H}+2\text{Na}]^+$, 409.0642 $[\text{M}+\text{Na}]^+$; found 216.0267 $[\text{M}+\text{Na}]^+$, 238.0080 $[\text{M}-\text{H}+2\text{Na}]^+$, 409.0629 $[\text{M}+\text{Na}]^+$.

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 by 1N HCl. The mixture was extracted with EtOAc (2×20 mL), and the organic phase was concentrated under reduced pressure. Column chromatography eluting with 10% $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$ (R_f 0.28) afforded 0.162 g (76%) of **4** as light yellow solid, m.p.: 171–172 °C: ^1H NMR (CD_3OD): δ 7.05 (d, 1 H, $J = 9.2$), 7.81 (d, 1 H, $J = 8.8$), 8.56 (s, 1 H). ^{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 $\text{C}_8\text{H}_5\text{NO}_4$ 223.9930 $[\text{M}-\text{H}+2\text{Na}]^+$, 245.9750 $[\text{M}-2\text{H}+3\text{Na}]^+$; found 223.9932 $[\text{M}-\text{H}+2\text{Na}]^+$, 245.9744 $[\text{M}-2\text{H}+3\text{Na}]^+$.

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% aq. 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 % $\text{CH}_3\text{OH}/\text{CHCl}_3$ (R_f 0.33) provided 0.294 g (77%) of **25** as a colorless solid, m.p. 45–46 °C: $[\alpha]_D^{25} +15.65$ (c 0.65, CH_3OH). ^1H NMR δ 1.20–1.46 (m, 2 H), 1.50–1.94 (m, 2 H), 3.08 (quintet, 1 H, $J = 6.4$), 3.19 (quintet, 1 H, $J = 6.4$), 4.66 (d, 1 H, $J = 4.8$), 5.01 (s, 2 H), 5.06–5.17 (m+s, 6 H), 7.08–7.46 (m, 26 H), 7.68–7.77 (m, 2 H), 7.96 (br s, 1 H), 8.55 (d, 1 H, $J = 7.6$). ^{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 $\text{C}_{47}\text{H}_{44}\text{N}_2\text{O}_8$ 765.3170 $[\text{M}+\text{H}]^+$, 787.2990 $[\text{M}+\text{Na}]^+$, 809.2809 $[\text{M}-\text{H}+2\text{Na}]^+$; found 765.3154 $[\text{M}+\text{H}]^+$, 787.2987 $[\text{M}+\text{Na}]^+$, 809.2797 $[\text{M}-\text{H}+2\text{Na}]^+$.

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 CH₃OH (5 mL), and the mixture was stirred under H₂ at atmospheric pressure for 6 h. The mixture was filtered through Celite, and the solids were washed with CH₃OH (3 × 5 mL). The combined filtrate was concentrated by rotary evaporation. The residue was dissolved in CH₃OH (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 H₂O/EtOH/toluene. The iron active fractions were combined and concentrated to afford 0.091 g (96%) of **2** as a white solid, m.p.: 147–148 °C: [α] –33.64 (c 0.11, CH₃OH). ¹H NMR (CD₃OD) δ 1.71–1.82 (m, 3 H), 1.89 (m, 5 H), 3.45 (t, 2 H, *J* = 6.4), 3.56–3.64 (m, 2 H), 3.94 (t, 1 H, *J* = 6.4), 3.97 (s, 2 H), 4.45–4.49 (m, 1 H), 6.72 (t, 1 H, *J* = 7.6), 6.92 (d, 1 H, *J* = 8.0), 7.15 (d, 1 H, *J* = 7.2). ¹³C NMR (CD₃OD) 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 C₁₉H₂₇N₅O₇ (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 CH₃OH (5 mL), and the mixture was stirred under H₂ at atmospheric pressure for 4 h. The mixture was filtered through Celite, and the solids were washed with CH₃OH (3 × 5 mL). The combined filtrate was concentrated by rotary evaporation. The residue was dissolved in CH₃OH (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 (quant) of **1** as a white solid, m.p.: 140–141 °C: [α] –7.5 (c 0.24, CH₃OH). ¹H NMR (CD₃OD) δ 1.69–2.13 (m, 8 H), 3.46–3.63 (m, 4 H), 3.90 (m, 2 H), 4.38–4.46 (m, 1 H), 4.63–4.67 (m, 1 H), 6.34 (t, 1 H, *J* = 7.6), 6.74 (d, 1 H, *J* = 7.6), 6.93 (d, 1H, *J* = 8.8), 7.29 (d, 1 H, *J* = 8.0), 7.62 (d, 1H, *J* = 9.2), 8.35 (s, 1 H). ¹³C NMR (CD₃OD) 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 C₂₆H₃₁N₅O₁₀ 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 CH₃OH (10 mL), and the mixture was stirred under H₂ at atmospheric pressure for 6 h. The mixture was filtered through Celite, and the residue was washed with CH₃OH (3 × 5 mL). The combined filtrate was concentrated by rotary evaporation. The residue was dissolved in CH₃OH (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 H₂O/EtOH/toluene. The iron active fractions were combined and concentrated to afford 0.229 g (quant.) of **3** as fluffy pale solid, m.p. 135–136 °C: [α] –12.0 (c 0.15, CH₃OH). ¹H NMR (CD₃OD) δ 1.70–1.84 (m, 2 H), 1.85–2.10 (m, 6 H), 3.38–3.52 (m, 2 H), 3.52–3.68 (m, 2 H), 3.82–4.02 (m, 2 H), 4.42–4.54 (m, 1 H), 4.54–4.66 (m, 1 H), 6.68–6.77 (m, 2 H), 6.87–6.96 (m, 2 H), 7.20 (d, 1 H, *J* = 7.2), 7.35 (d, 1 H, *J* = 7.2). ¹³C NMR (CD₃OD) 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 C₂₆H₃₁N₅O₁₀ 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% CH₃OH (v/v) was used to maintain 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-CH₃OH mixture. The Fe(III)-NTA stock solution was prepared by mixing equal volumes of 90 mM of FeCl₃ and 180 mM trisodium NTA. The iron content of the Fe(III)-NTA solution was verified by AAS.

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Figure 1.
Catecholamide and hydroxamate siderophores.

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

Figure 3.

Job's plot 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 $[\text{ligand}] + [\text{Fe(III)}] = 0.9 \text{ mM}$ at pH 7.4.

Scheme 1.
Retrosynthetic analysis of Heterobactins A, B and Nocardia Heterobactin

Scheme 2.
Alternate Retrosynthesis of Nocardia Heterobactin

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**.

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

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

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