Total Synthesis of Vulnibactin: A Natural Product Iron Chelator

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Received 15 November 2006; revised 20 December 2006

Abstract: A short, high-yield, flexible synthesis is described for accessing vulnibactin and related siderophores, e.g., vibriobactin analogues and homologues. The spectral properties of the synthetic vulnibactin are identical with those reported for the natural product, thus confirming the proposed structure of vulnibactin.

Key words: chelates, iron, medicinal chemistry, natural products, receptors

The concentration of ferric ion required to support the growth of most microorganisms lies in the range of 5×10^{-8} to 1×10^{-6} mol/L. However, because of the extreme insolubility of ferric hydroxide at physiological pH, about 10^{-18} mol/L,[1] microorganisms have developed a means of solubilizing and transporting this metal. Many microbes excrete large quantities of low molecular weight chelators, siderophores, which preferentially bind ferric iron, rendering the metal accessible to the organism.[2]

In 1975, Tait[3] isolated the first hexacoordinate catecholamide iron chelator predicated on a polyamine backbone, N^4-[2-(2-hydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamido]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (parabactin) (Figure 1). We described the synthesis of this ligand and a number of other polyamine-based catecholamide ligands that followed, e.g., N^4-[2-(2,3-dihydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamido]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (agrobactin),[4] N^4-[2-(2,3-dihydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamido]-N^1,N^7-bis(2,3-dihydroxybenzoyl)norspermidine (fluviabactin),[4] and *N*-[3-(2,3-dihydroxybenzamido)propyl]-1,3-bis[2-(2,3-dihydroxyphenyl)-*trans*-5-methyl-2-oxazoline-4-carboxamido]propane (vibriobactin).[5] This current study focuses on the assembly of a new siderophore, *N*-[3-(2,3-dihydroxybenzamido)propyl]-1,3-bis[2-(2-hydroxyphenyl)-*trans*-5-methyl-2-oxazoline-4-carboxamido]propane (vulnibactin), isolated from *vibrio vulnificus*.[6]

Since the time of parabactin's isolation, considerable interest has been focused on the biological and physical properties of these catecholamide-based siderophores and their ferric chelates.[3,7] The coordination chemistry of a number of catecholamide iron complexes has been thoroughly investigated. It has been determined that the for-

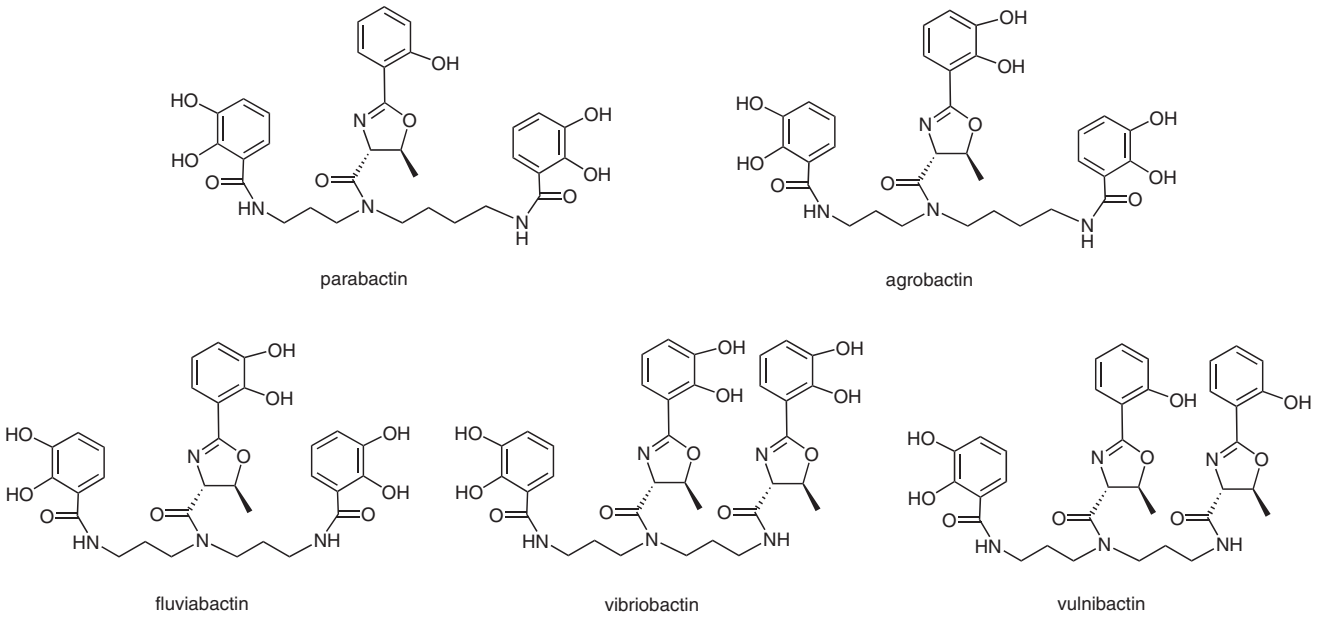


Figure 1 Hexacoordinate catecholamide iron chelators predicated on a polyamine backbone

SYNTHESIS 2007, No. 7, pp 1033–1037
Advanced online publication: 28.02.2007
DOI: 10.1055/s-2007-965960; Art ID: M06806SS

mation constants for the 1:1 iron(III) complexes of many of these ligands at alkaline pH are in the range of 10^{48}. The biological studies of these ligands revolved around two different issues: understanding the details of how microorganisms assembled and utilized these chelators for iron assimilation and their application as deferration agents in various iron overload diseases.

In fact, parabactin was shown to be nearly 300% more effective at removing iron from a rodent model than desferrioxamine, a clinically accepted agent for iron decorporation. Unfortunately, as with desferrioxamine, catecholamides do not work well orally, and require subcutaneous administration.

There are three notable structural features of the polyamine catecholamide ligands: the nature of the polyamine backbones, the ligand donor groups, and the symmetry of the donor groups (Figure 1). The first two catecholamides, parabactin and agrobactin, utilize spermidine, an unsymmetrical polyamine, as a backbone; each presents with a 2,3-dihydroxybenzoyl donor at the terminal nitrogens. Parabactin has a 2-(2-hydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamide fragment fixed to the central nitrogen, while agrobactin has a 2-(2,3-dihydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamide fixed to the central nitrogen. The latter three ligands, vibriobactin, fluviabactin, and vulnibactin employ the symmetrical polyamine norspermidine with a 3,3-methylene backbone as a platform. Fluviabactin, like parabactin and agrobactin, is symmetrical with regards to the N-terminal 2,3-dihydroxybenzoyl donors. It presents with the same central nitrogen donor as agrobactin.

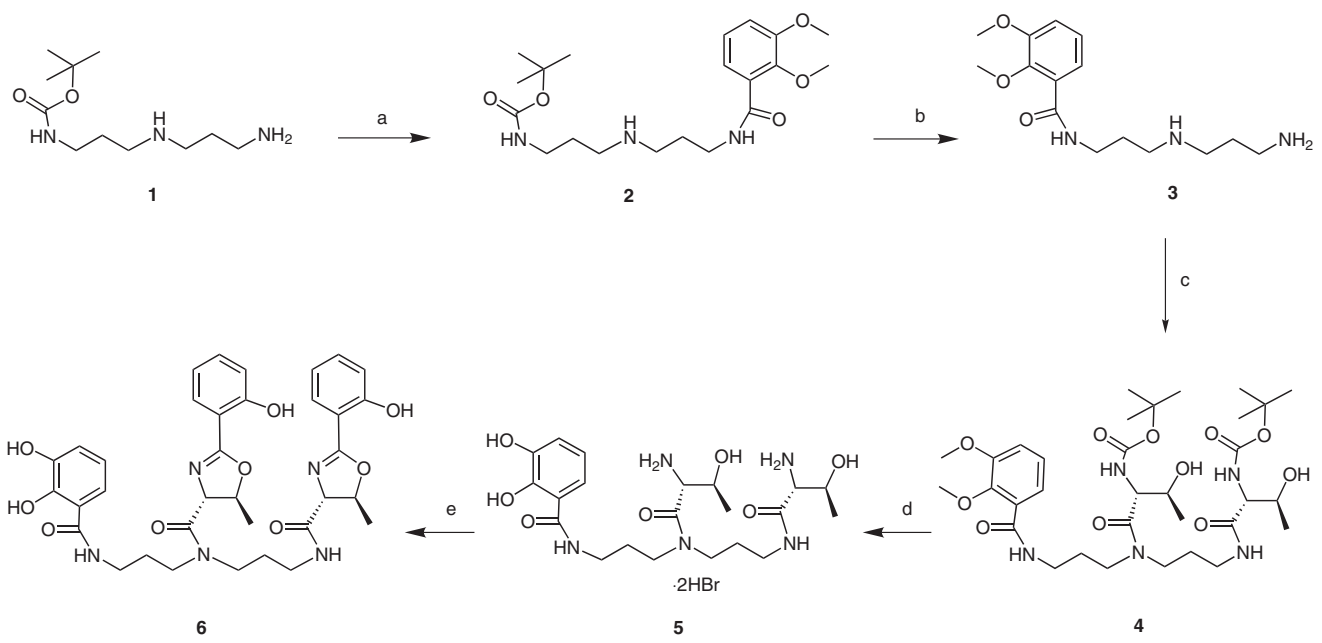
Both vibriobactin and vulnibactin are unsymmetrical with respect to the ligating groups. Both have a single 2,3-dihydroxybenzoyl donor fixed to a terminal nitrogen on norspermidine. The remaining two nitrogens in vibriobactin are coupled to 2-(2,3-dihydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamide groups, while vulnibactin utilizes a 2-(2-hydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamide fragment on the remaining two nitrogens.

The first two ligands are derived from soil microorganisms, parabactin from *Paracoccus dentrificans* and agrobactin from *Agrobacterium tumefaciens.* The last three ligands are derived from human pathogens, vibriobactin from *Vibrio cholera*, fluviabactin from *Vibrio fluvialis* and vulnibactin from *Vibrio vulnificus*. Both *Vibrio fluvialis* and *Vibrio vulnificus* are halophilic opportunistic pathogens.

Recent studies with a CRP mutant of *Vibrio vulnificus* showed that transcriptional suppression of genes responsible for vulnibactin synthesis and vulnibactin receptor proteins, *vis* and *vuuA*, resulted in less pathogenic organisms. The CRP mutant was unable to utilize transferrin-bound iron and its growth was severely retarded on both transferrin-bound iron and cirrhotic ascites.[8] This finding makes the vulnibactin transporter an attractive target in therapeutic design strategies, e.g., assembly of competitive inhibitors and/or of irreversible covalent inhibitors of transport.[9] However, a high-yield, flexible synthetic method providing facile access to the vulnibactin platform is first required.

Vulnibactin was first isolated from cultures of *Vibrio vulnificus* in a very small quantity and its structure assessed on the basis of NMR and mass spectral data.[8] The current synthesis provides facile access to large quantities of the siderophore in addition to confirming the structural assignment. As with vibriobactin (Figure 1), the positional asymmetry of donor groups of vulnibactin sets the strategy for the synthesis. The primary difference between vibriobactin and vulnibactin is that vulnibactin has 2-hydroxybenzoyl groups fixed to the oxazoline, while vibriobactin has 2,3-dihydroxybenzoyl in this position. Briefly, the synthesis of vibriobactin began with N^5-benzyl-N^1-(*tert*-butoxycarbonyl)spermidine. This reagent was acylated with 2,3-dimethoxybenzoyl chloride. The resulting amide was debenzylated, the Boc group removed, and the free nitrogens acylated with L-*N*-*tert*-butoxycarbonylthreonine. The threonyl Boc groups were removed and the threonyl fragment condensed with the ethyl imidate of 2,3-dihydroxybenzoic acid to yield vibriobactin in an overall yield of 31%. However, the initial reagent, N^4-benzoyl-N^1-(*tert*-butoxycarbonyl)norspermidine, although accessible in 63% overall yield, requires a six-step synthesis. This of course reduces the overall yield to 20% making the assembly procedure cumbersome when large quantities of vibriobactin are needed.

The current synthesis of vulnibactin (Scheme 1) begins with a starting material accessible in one step in 80% yield, *N*-(*tert*-butoxycarbonyl)norspermidine (**1**).[10] This protected triamine is terminally N-acylated with 2,3-dimethoxybenzoic acid in the presence of carbonyl diimidazole to produce the diamide **2** in 99% yield. The *t*-Boc protecting group is next removed with trifluoroacetic acid, providing monamide **3** in 97% yield. This monamide diamine is acylated with *N*-Boc-threonine providing the triamide **4** in 99% yield. The methyl and *t*-Boc protecting group of **4** are removed simultaneously with BBr_3 to generate catechol **5** in 90% yield. Finally, the ethyl imidate[11] of salicylic acid is condensed with the triamide **5** to provide the final product in 90% yield. It is this step which allows for access to a variety of different and potentially useful tools. The aromatic imidates are easily accessible and can be functionalized with any number of useful electrophiles, e.g., halides, aldehydes, or photochemically activatable groups, e.g., azides. The ^{1}H NMR spectra and the optical rotations were essentially identical with the reported data, thus confirming the structure of vulnibactin. The overall yield of the five-step synthesis beginning with amide **1** is 76%, over twice that of our previous seven-step vibriobactin synthesis. As described above, factoring the starting material problem in the initial vibriobactin synthesis, vibriobactin was generated in 20% yield. The same consideration for the vulnibactin leads to a 60% yield. The vulnibactin approach could also be used for assembly of



Scheme 1 Synthesis of vulnibactin (**6**). *Reagents and conditions*: (a) 2,3-dimethoxybenzoic acid, CDI, CH_2Cl_2 (99%); (b) TFA, CH_2Cl_2, 0 °C (97%); (c) *N*-Boc-threonate, DMF, 72 h (99%); (d) BBr_3, CH_2Cl_2, –78 °C (90%); (e) ethyl 2-hydroxybenzimidate, EtOH, 70 °C, 36 h (90%).

vibriobactin and lends itself nicely to scale-up. Assembly of the D-threonyl analogues is currently underway.

The stoichiometry of the ferric complex of vulnibactin was determined at λ_{max} 558nm. The Job's plot of the complex is in keeping with a 1:1 ligand-to-metal stoichiometry (Figure 2).

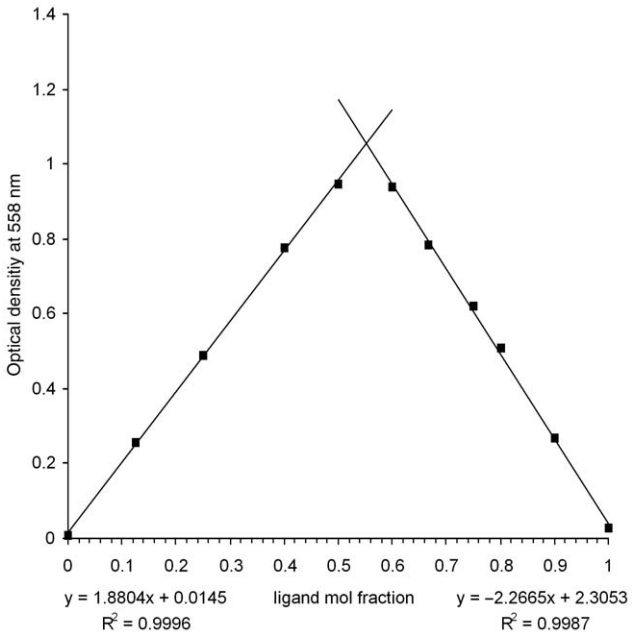


Figure 2 Job's plot of the iron(III) complex of vulnibactin. Briefly, solutions containing different ligand/Fe (III) ratios were prepared such that [ligand] + [Fe(III)] = 1.0 mM. The data points were fitted to the mole fraction (1) from 0 to 0.5 and (2) from 0.5 to 1.00, r^2 = 0.9996 and 0.9987, respectively. The theoretical mole fraction for a 1:1 ligand-to-iron complex is 0.5. A linear intercept maximum of 0.55 was found at 558 nm.

In conclusion, a short, high-yield, flexible synthesis is available for accessing vulnibactin and related siderophores, e.g., vibriobactin analogues and homologues. The spectral properties of the synthetic vulnibactin are identical with those reported for the natural product, thus confirming the proposed structure of vulnibactin.

Reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), and Fisher Optima-grade solvents were routinely used. Organic extracts were dried with Na_2SO_4 and filtered. DMF was distilled under an inert atmosphere. Distilled solvents and glassware that had been presoaked in 3 N HCl for 15 min were employed in reactions involving chelators. Silica gel 70–230 from Fisher Scientific was utilized for column chromatography, and silica gel 32–63 from Selecto Scientific, Inc. (Suwanee, GA) was used for flash column chromatography. Sephadex LH-20 was obtained from Amersham Biosciences (Piscataway, NJ). Melting points are uncorrected. Optical rotation was run at 589 nm (sodium D line) utilizing a PerkinElmer 341 polarimeter with *c* as g of compound/100 mL of solution. NMR spectra were obtained at 400 MHz (^{1}H) or 100 MHz (^{13}C) on a Varian Mercury-400BB spectrometer. Chemical shifts (δ) for ^{1}H spectra are given in parts per million downfield from tetramethylsilane for organic solvents ($CDCl_3$ not indicated). Chemical shifts (δ) for ^{13}C spectra are given in parts per million referenced to the residual solvent resonance in $CDCl_3$ (δ = 77.16) or CD_3OD (δ = 49.00). Coupling constants (*J*) are in Hertz. The base peaks are reported for the ESI-FTICR mass spectra.

N^1-(*tert*-Butoxycarbonyl)norspermidine (**1**) was prepared as previously described.[10]

N^1-(*tert*-Butoxycarbonyl)-N^8-(2,3-dimethoxybenzoyl)norspermidine (2)

1,1′-Carbonyldiimidazole (2.80 g, 17.29 mmol) was added to a solution of 2,3-dimethoxybenzoic acid (3.15 g, 17.29 mmol) in CH_2Cl_2 (25 mL). After stirring for 1 h, the mixture was added to a solution of N^1-(*tert*-butoxycarbonyl)norspermidine (**1**; 4.0 g, 17.29 mmol) in CH_2Cl_2 (20 mL) at 0 °C. The solution was stirred for 16 h (0 °C to r.t.), and was washed with aq 2 N NaOH (25 mL) and evap-

orated in vacuo. Chromatography on silica gel eluting with 0.5% NH_4OH–MeOH gave 6.70 g (99%) of **2** as a viscous oil.

^{1}H NMR: δ = 1.43 (s, 9 H), 1.65 (quint, *J* = 6.6 Hz, 2 H), 1.79 (quint, *J* = 6.6 Hz, 2 H), 2.63–2.72 (m, 4 H), 3.20 (q, *J* = 6.0 Hz, 2 H), 3.55 (q, *J* = 6.4, 2 H), 3.88 (s, 3 H), 3.89 (s, 3 H), 5.37 (br s, 1 H), 7.03 (dd, *J* = 8.0, 1.6 Hz, 1 H), 7.14 (t, *J* = 8.0 Hz, 1 H), 7.66 (dd, *J* = 7.6, 1.6 Hz, 1 H), 8.15 (br s, 1 H).

^{13}C NMR: δ = 28.5, 29.8, 29.9, 37.7, 39.0, 47.3, 47.6, 56.1, 61.3, 78.8, 115.2, 122.7, 124.5, 127.1, 147.4, 152.6, 156.2, 165.3.

HRMS: *m*/*z* calcd for $C_{20}H_{33}N_3O_5$: 396.2493 (M + H); found: 396.2482.

N^1-(2,3-Dimethoxybenzoyl)norspermidine (3)

Freshly distilled trifluoroacetic acid (48 mL) in CH_2Cl_2 (10 mL) was added dropwise to a flask containing **2** (6.30 g, 16.10 mmol) in CH_2Cl_2 (30 mL) under N_2 with ice-bath cooling and the mixture was stirred at 0 °C for 1 h and at r.t. for 1 h. Solvents were removed under vacuum to give a light brown oil. The oil was taken up in CH_2Cl_2 (100 mL) and extracted with aq sat. K_2CO_3 (2 × 25 mL). The combined organic layers were evaporated in vacuo to give 4.61 g (97%) of **3**.

^{1}H NMR: δ = 1.66 (quint, *J* = 6.8 Hz, 2 H), 1.81 (quint, *J* = 6.8 Hz, 2 H), 2.70–2.81 (m, 6 H), 3.55 (q, *J* = 6.4 Hz, 2 H), 3.89 (s, 3 H), 3.90 (s, 3 H), 7.03 (dd, *J* = 8.0, 1.6 Hz, 1 H), 7.15 (t, *J* = 8.0 Hz, 1 H), 7.66 (dd, *J* = 8.0, 1.6 Hz, 1 H), 8.12 (br s, 1 H).

^{13}C NMR: δ = 29.2, 30.5, 37.2, 40.8, 46.6, 48.4, 56.1, 61.4, 115.4, 122.6, 124.5, 152.6, 161.9, 162.6, 165.9.

HRMS: *m*/*z* calcd for $C_{15}H_{25}N_3O_3$: 296.1969 (M + H); found: 296.1965.

N^1,*N*^{4}-Bis[(L)-*N*-*tert*-butoxycarbonylthreonyl]-*N*^{7}-(2,3-dimethoxybenzoyl)norspermidine (4)

A solution of freshly prepared (L)-*N*-hydroxysuccinimido-*N*-(*tert*-butoxycarbonyl)threonate[12] (3.48 g, 11.0 mmol) in DMF (25 mL) was added to a solution of **3** (1.48g, 5.0 mmol) in anhyd DMF (25 mL). After stirring for 72 h, the solvent was removed under vacuum and the residue was taken up in CH_2Cl_2 (100 mL). The CH_2Cl_2 layer was washed with aq 5% K_2CO_3 (3 × 50 mL), distilled H_2O (50 mL), brine, and concentrated in vacuo. Flash chromatography eluting with 10% EtOH–EtOAc afforded 3.45 g (99%) of **4** as a white foam.

^{1}H NMR: δ = 1.15–1.24 (m, 6 H), 1.45 (s, 18 H), 1.62–2.01 (m, 4 H), 3.20–3.74 (m, 8 H), 3.89 (s, 3 H), 3.91 (s, 3 H), 3.96–4.59 (m, 6 H), 5.60 (d, *J* = 9.2 Hz, 2 H), 7.03–7.06 (m, 1 H), 7.11–7.17 (m, 1 H), 7.62–7.66 (m, 1 H), 8.35 (br, 1 H).

^{13}C NMR: δ = 18.9, 19.3, 27.8, 28.3, 28.4, 28.8, 36.3, 37.3, 42.9, 45.2, 53.5, 56.1, 59.0, 61.4, 67.3, 68.6, 80.1, 80.5, 115.4, 122.6, 124.4, 126.7, 147.6, 152.6, 156.4, 165.6, 171.3, 172.1, 173.0.

HRMS: *m*/*z* calcd for $C_{35}H_{55}N_5O_{11}$ 698.3971 (M + H); found: 698.3960.

N^1,*N*^{4}-Bis[(L)-threonyl]-*N*^{7}-(2,3-dimethoxybenzoyl)norspermidine Dihydrobromide (5)

A 1 M solution of BBr_3 (62.3 mL, 62.3 mmol) was added dropwise at –78 °C to a solution of **4** (2.90 g, 4.16 mmol) in CH_2Cl_2 (50 mL). After complete addition of BBr_3, the mixture was allowed to warm to r.t. and stirred for an additional 20 h. The mixture was cooled to 0 °C and cautiously treated dropwise with H_2O (75 mL). After 3 h of vigorous stirring, the aqueous layer was removed and evaporated in vacuo at 25 °C to give a light brown solid. This residue was taken up in EtOH (25 mL) and concentrated several times. The resulting brown solid was easily purified on Sephadex LH-20 eluting with 20% EtOH–toluene to give 2.35 g (90%) of **5** as a brown solid.

^{1}H NMR (CD_3OD): δ = 1.25–1.33 (m, 6 H), 1.79–2.02 (m, 4 H), 3.29–4.32 (m, 12 H), 6.70–6.76 (m, 1 H), 6.92–6.95 (m, 1 H), 7.21–7.26 (m, 1 H).

^{13}C NMR (CD_3OD): δ = 20.2, 20.4, 28.2, 29.6, 37.9, 38.1, 44.8, 46.9, 56.8, 60.4, 67.4, 67.8, 116.7, 118.8, 119.6, 119.7, 147.3, 150.1, 168.6, 168.8, 171.6.

HRMS: *m*/*z* calcd for $C_{21}H_{35}N_5O_7$: 470.2609 (M + H, free amine); found: 470.2600.

N-[3-(2,3-Dihydroxybenzamido)propyl]-1,3-bis[2-(2-hydroxyphenyl)-*trans*-5-methyl-2-oxazoline-4-carboxamido]propane (Vulnibactin) (6)

Ethyl 2-hydroxybenzimidate[11] (0.85 g, 5.12 mmol) was added to a solution of **5** (1.01 g, 1.60 mmol) in anhyd EtOH (25 mL). The mixture was heated at reflux under N_2 for 36 h and then concentrated in vacuo. Column chromatography on LH-20 (eluting with 10% EtOH–toluene) afforded 0.97 g (90%) of **6** as a grey solid. The spectral characteristics were identical with those reported in the literature;[6] mp 94–97 °C (Lit.[6] mp 93–97 °C); $[\alpha]^{25}$ +92.5 (*c* = 0.80, CH_3OH).

^{1}H NMR (CD_3OD): δ = 1.40 (2 d, *J* = 6.4, 3 H), 1.51 (2 d, *J* = 6.4 Hz, 3 H), 1.76–1.88 (m, 2 H), 1.91–2.09 (m, 2 H), 3.12–3.85 (m, 8 H), 4.42 (2 d, *J* = 7.2 Hz, 1 H), 4.77 (2 d, *J* = 6.4 Hz, 1 H), 4.91 (2 quint, *J* = 6.4 Hz, 1 H), 5.24 (2 quint, *J* = 6.4 Hz, 1 H), 6.65 (2 t, *J* = 8.0 Hz, 1 H), 6.78–6.94 (m, 5 H), 7.17–7.21 (m, 1 H), 7.28–7.40 (m, 2 H), 7.57–7.65 (m, 2 H).

^{13}C NMR (CD_3OD): δ = 20.2, 21.4, 28.3, 30.1, 37.6, 37.8, 44.6, 46.6, 72.9, 75.7, 79.7, 80.6, 111.5, 111.6, 116.7, 117.6, 117.7, 118.5, 119.6, 119.7, 119.9, 120.0, 129.4, 129.6, 135.0, 135.1, 147.4, 150.3, 160.9, 161.1, 167.4, 167.9, 171.1, 171.6, 173.2.

HRMS: *m*/*z* calcd for $C_{35}H_{39}N_5O_9$: 675.2891 (M + 2 H); found: 675.2871.

Determination of Stoichiometry of Ligand–Fe(III) Complex (Job's Plot)

The stoichiometry of ligand–Fe(III) complex of vulnibactin was determined spectrophotometrically from Job's plot. Solutions were monitored at the visible λ_{max} (558 nm). A 25 mM MOPS buffer with 50% MeOH (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 (pH 7.4) and 0.9 mM Fe(III) nitriloacetate (NTA) in MOPS–MeOH solution (pH 7.4). 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 MOPS–MeOH (50:50) mixture. The Fe(III)-NTA stock solution was prepared by mixing equal volumes of 90 mM of $FeCl_2$ and 180 mM trisodium NTA. The iron content was verified by ICP-MS.

Acknowledgment

Funding was provided by the National Institutes of Health Grant No. R37-DK49108. We acknowledge the spectroscopy services in the Chemistry Department, University of Florida, for the mass spectrometry analyses.

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