Pergamon

Tetrahedron, Vol. 53, No. 2, pp. 427-434, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(96)01061-7

Total Synthesis of Rhizoferrin, An Iron Chelator

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Abstract: The absolute configuration of rhizoferrin, a novel siderophore isolated from the fungus *Rhizopus microsporus* var. *rhizopodiformis*, was determined via its total synthesis. The principal steps in the synthesis of this naturally occurring iron chelator involve a simple coupling of key intermediate (*R*)-1,2-dimethyl citrate with N^1,N^4-dibenzyl-1,4-diaminobutane followed by ester hydrolysis and debenzylation. This is the first example of a total synthesis of a citrate-based siderophore from a chiral citric acid fragment, the structure of which was confirmed by single crystal X-ray diffraction.

Iron is essential for almost all forms of life. However, because of the aqueous insolubility of $Fe(OH)_3$ ($K_{sp} = 2 \times 10^{-39}$),[1] the predominant form of the transition metal in the environment, virtually all life forms have developed rather sophisticated iron chelating and transport systems to utilize the metal. Higher animals tend to utilize proteins to transport and assimilate iron,[2] whereas microorganisms use small nonproteinaceous iron chelators, or siderophores.[3,4]

Although a rather large number of siderophores have been isolated, for the most part they can be separated into two basic structural groups, the hydroxamates and the catecholamides.[5] Molecules of both classes are usually predicated on polyamine backbones, specifically 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), norspermidine, or spermidine, or on their biochemical precursors ornithine or lysine. The presence of such common structural units has led to the efficient total synthesis of many siderophores in this and other laboratories, e.g., parabactin[6] and desferrioxamine (DFO).[7]

N^1,N^4-Bis(1-oxo-3-hydroxy-3,4-dicarboxybutyl)diaminobutane (rhizoferrin) was first isolated from *Rhizopus microsporus* var. *rhizopodiformis*, an organism associated with mucormycosis seen in dialysis patients,[8] and occurs in several Zygomycetes strains of fungi.[9] Like the natural chelators parabactin and DFO, rhizoferrin forms a 1:1 complex with ferric ion;[10] however, the formation constant of the chelate has not been measured. Structure determination of rhizoferrin[8] revealed a putrescine center symmetrically diacylated by citric acid at its 1-carboxylate (Figure 1). Thus, although rhizoferrin contains a polyamine backbone, it is not a member of either class of chelators. Rather it is a hydroxy polycarboxylate, along with rhizobactin[11] and staphyloferrin A,[12] which are predicated on L-lysine and D-ornithine, respectively. Unlike the hydroxamates aerobactin, arthrobactin, schizokinen,[5] and nannochelin,[13] in

which citric acid is symmetrically 1,3-disubstituted, the prochiral carbon of each unsymmetrically functionalized citric acid in rhizoferrin is asymmetric. These two sites of the molecule are in the (*R*)-configuration (Figure 1) according to circular dichroism (CD) spectroscopy in comparison with natural (*R,R*)-tartaric acid.[10]

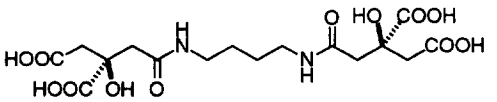


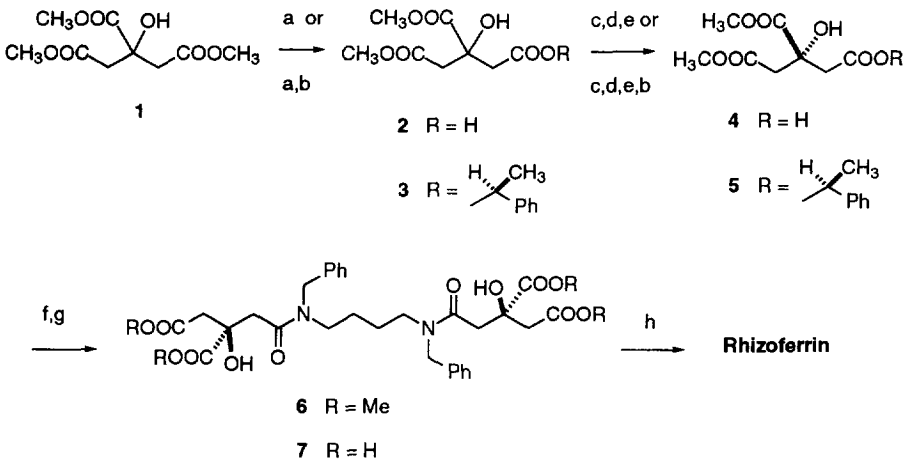
Figure 1. Structure of Rhizoferrin.

The principal challenge to the synthesis of rhizoferrin was to access a citrate synthon of correct configuration for coupling to both termini of putrescine in order to unequivocally define the absolute configuration of the siderophore. The first total synthesis of this natural product is also the first example of the conversion of a chiral citric acid fragment, as confirmed by X-ray diffraction and high field NMR, to a chelator.

RESULTS AND DISCUSSION

The synthesis of rhizoferrin began with trimethyl citrate (**1**), which was converted to 1,2-dimethyl citrate (**2**) by a sterically controlled saponification[14] (Scheme 1). We were able to separate the enantiomers of carboxylic acid **2** by forming their (-)-brucine salts. After five fractional crystallizations from water, the crystalline salt was shown by single crystal X-ray diffraction to contain 1,2-dimethyl citrate in the *R*-configuration (Figure 2). Treatment of the salt with 1 N HCl and extraction with ethyl acetate furnished (*R*)-1,2-dimethyl citrate (**4**).

Both the racemic (**2**) and chiral (**4**) diesters were acylated with (*S*)-(-)-*sec*-phenethyl alcohol (1,3-dicyclohexylcarbodiimide/catalytic DMAP/CH_2Cl_2) to unsymmetrical triesters **3** and **5**, respectively. An examination of the methyl ester region in the 600 MHz NMR spectrum of **3** and **5** showed that the latter contained an enantiomeric excess (ee) of 99%. With the correct enantiomeric acid in hand, N^1,N^4-dibenzyl-1,4-diaminobutane[15] was acylated with **4** (2 equivalents) utilizing diphenylphosphoryl azide (Et_3N/DMF).[16] The diamide **6** was obtained in 26% yield after flash column chromatography, which removed by-products including olefins due to elimination of the tertiary alcohol as indicated by NMR. The methyl esters of **6** were hydrolyzed with sodium hydroxide in aqueous methanol; acidification gave *N, N'*-dibenzyl rhizoferrin (**7**).



[a](a) NaOH/MeOH (39%); (b) (*S*)-(-)-*sec*-phenethyl alcohol/DCC/catalytic DMAP/CH_2Cl_2; (c) (-)-brucine; (d) fractional crystallization; (e) HCl; (f) N^1,N^4-dibenzyl-1,4-diaminobutane/ DPPA/Et_3N/DMF (26%); (g) NaOH/MeOH (77%); (h) Li/NH_3/THF (64%)

Scheme 1[a]

Finally, since *N*-benzyl amides are resistant to hydrogenolysis,[17] deprotection of tetraacid **7** under dissolving metal reduction conditions (Li/NH_3/THF),[18] protonation of the salts on a cation exchange resin column, and purification on a C-18 reversed-phase column furnished the final product, rhizoferrin. The high field NMR and high resolution mass spectrum of the synthetic compound were essentially identical to the published spectra of the natural product.[8] The absolute configurations (*R*,*R*) of the synthetic sample and the natural material are identical, since both exhibited a negative Cotton effect at the same wavelength.[10] It is fortuitous that the correct crystalline diastereomeric (-)-brucine salt of 1,2-dimethyl citrate crystallized out and that no subsequent synthetic step could compromise the stereochemical integrity of the chiral centers.

Rhizoferrin cyclizes upon standing through dehydration to imidorhizoferrin and bis-imidorhizoferrin, which possess one and two five-membered rings, respectively.[10] We observed by NMR that the zero order rate constant for this ring formation at pH 5.0 is 6.9 x 10^{-2} h^{-1}. At pH 3 our findings on the extent of cyclization were similar to the literature,[10] thus the analytical data were obtained before this decomposition occurred.

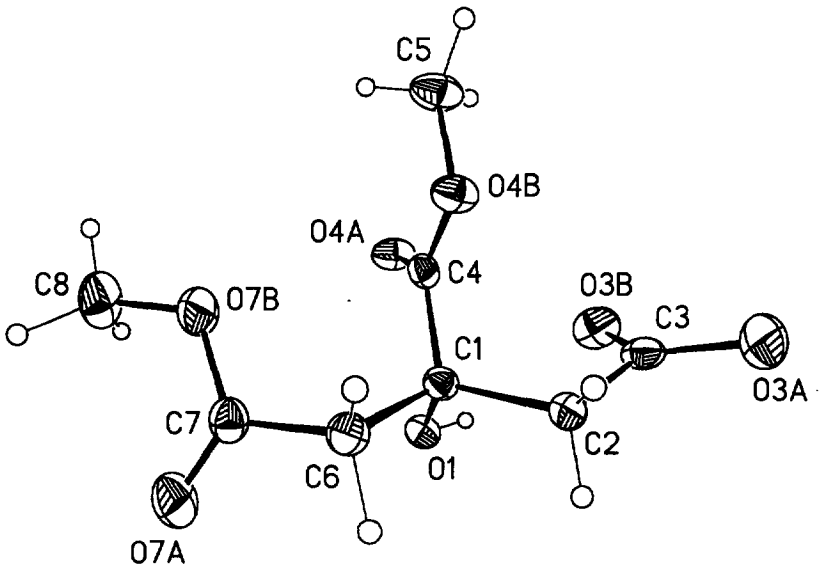


Figure 2. X-Ray of the (*R*)-Enantiomer of 1,2-Dimethyl Citrate, (-)-Brucine Salt.

The synthetic methodology for rhizoferrin is currently being used to prepare the hydroxy polycarboxylated siderophore staphyloferrin A,[12,19] in which D-ornithine is N^{α},N^{δ}-diacylated with citric acid at its 1-carboxylate. Thus the configuration of the citrates of this amino acid chelator will be determined by total synthesis. In addition, analogues of rhizoferrin in which the chain length of the central methylene bridge is varied can now be synthesized for structure-activity studies.

EXPERIMENTAL SECTION

Trimethyl citrate (**1**) was obtained from CTC Organics, Atlanta, GA. Other reagents were purchased from Aldrich Chemical Co. and were used as received. Fisher Optima grade solvents were routinely employed. Silica gel 32-63 (40 μM "flash") from Selecto, Inc. (Kennesaw, GA) or silica gel 60 (70-230 mesh) from EM Science (Darmstadt, Germany) was used for column chromatography. Optical rotations were run in CH_3OH at 589 nm (Na lamp) at room temperature with *c* as g of compound per 100 mL. ^1H NMR spectra were recorded at 300 or 600 MHz and run in the deuterated organic solvent indicated or in D_2O with chemical shifts given in parts per million downfield from tetramethylsilane or 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt, respectively.

X-Ray Diffraction. Data were collected at 173K on a Siemens SMART PLATFORM equipped with a CCD area detector and a graphite monochromator utilizing MoK_{α} radiation ($\lambda = 0.71073$ Å). Cell parameters were refined using up to

6233 reflections. A hemisphere of data (1381 frames) was collected using the ω-scan method (0.3° frame width). The first 50 frames were remeasured at the end of data collection to monitor instrument and crystal stability (maximum correction on I was < 1%). Psi scan absorption corrections were applied based on the entire data set.

Circular Dichroism. CD spectra were obtained with a Jasco Model J500C spectropolarimeter equipped with a Jasco IF-500II interface and CompuAdd 286 computer; data collection and processing were performed with Jasco DP-500/PC System version 1.28 software. The cell path length was 2.00 cm.

Ultraviolet Spectroscopy. UV spectra were obtained with a Shimadzu UV-2501PC equipped with an AST 486/33 computer datastation. The cell path length was 1.00 cm.

1,2-Dimethyl Citrate (2) was prepared by modification of a published method.[14] Sodium hydroxide (0.1 N, 215 mL) was added to a solution of **1** (10.0 g, 42.7 mmol) in 50% aqueous CH_3OH (200 mL) over 2 h with vigorous stirring at room temperature. The solution was concentrated to about 150 mL and extracted with EtOAc (3 x 150 mL). The aqueous layer was acidified with 1 N HCl (45 mL) and extracted with EtOAc (3 x 150 mL). The organic layer was dried ($MgSO_4$) and concentrated, providing 3.70 g (39%) of **2** as a colorless oil: ^{1}H NMR (d_6-DMSO) δ 5.60 (br s, 1 H, O<u>H</u>), 3.64 (s, 3 H, $CO_2C\underline{H}_3$), 3.57 (s, 3 H, $CO_2C\underline{H}_3$), 2.87 (d, 1 H, J = 15 Hz, 1/2 C<u>H</u>$_2$), 2.81 (d, 1 H, J = 15 Hz, 1/2 C<u>H</u>$_2$), 2.73 (d, 1 H, J = 15 Hz, 1/2 C<u>H</u>$_2$), 2.65 (d, 1 H, J = 15 Hz, 1/2 C<u>H</u>$_2$).

1,2-Dimethyl-3-[(*S*)-*sec*-Phenethyl] Citrate (3). 1,3-Dicyclohexylcarbodiimide (103 mg, 0.5 mmol) was added to a solution of **2** (110 mg, 0.5 mmol), (*S*)-(-)-*sec*-phenethyl alcohol (61 mg, 0.5 mmol), and 4-dimethylaminopyridine (3 mg) in dry CH_2Cl_2 (10 mL) at 0 °C, and the mixture was stirred overnight. The mixture was filtered, and the filtrate was concentrated and purified by flash chromatography (1:2 EtOAc/hexane) resulting in 60 mg (37%) of **3** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.35-7.28 (m, Ph), 5.97 (q, J = 7 Hz, C<u>H</u>Ph), 5.88 (q, J = 7 Hz, C<u>H</u>Ph), 3.77 (s, C<u>H</u>$_3$O), 3.73 (s, C<u>H</u>$_3$O), 3.69 (s, C<u>H</u>$_3$O), 3.68 (s, C<u>H</u>$_3$O), 2.98-2.74 (m, C<u>H</u>$_2$), 1.54 (d, J = 7 Hz, C-C<u>H</u>$_3$), 1.52 (d, J = 7 Hz, C-C<u>H</u>$_3$).

(-)-Brucine Salt of (*R*)-1,2-Dimethyl Citrate. To a solution of (-)-brucine (12.5 g, 31.8 mmol) (CAUTION: toxic) in EtOAc (460 mL) was added **2** (7 g, 31.8 mmol) with vigorous stirring overnight. After filtration the precipitate (10.5 g) was recrystallized from water (5 x) and dried to afford 2.04 g of white crystals: mp 165-168 °C.

The diastereomeric salt crystallizes in the monoclinic space group C2 and has cell dimensions: a = 13.8947 (3), b = 12.4224 (3), and c = 17.5408 (3) Å; α = 90°, β = 104.556 (1)°, and γ = 90°. The structure was solved by the Direct Methods in SHELXTL[20] and was refined using full matrix least squares. The non-H atoms were treated anisotropically. The methyl hydrogen atoms were calculated in ideal positions and were riding on their respective carbon atoms; the rest of the H atoms were refined

without constraints. Two water molecules were located in the asymmetric unit. One was refined with full occupancy, and its H atoms were located. The other, located on a 2-fold axis of rotation, was refined to a 30% occupancy. An absolute configuration of (*R*) was assigned to the citrate portion of the salt (Figure 2) based on knowledge of the stereochemistry of brucine. Parameters (521) were refined in the final cycle of refinement using 3855 reflections with $I > 2\sigma(I)$ to yield R_1 and wR_2 of 0.0434 and 0.1040, respectively. Refinement was done using F^2.

(*R*)-1,2-Dimethyl Citrate (4). HCl (1 N, 4 mL) was added to a solution of the (-)-brucine salt of (*R*)-1,2-dimethyl citrate (2.04 g, 3.32 mmol) in water (50 mL), and stirring was continued for 5 min. Extraction with EtOAc (3 x 50 mL), drying over Na_2SO_4, and concentration gave 630 mg (86%) of **4** as a colorless oil: [α] +4.0 (*c* 1.00); the NMR was identical to **2**.

(*S*)-1,2-Dimethyl-3-[(*S*)-*sec*-Phenethyl] Citrate (5). Esterification of **4** with (*S*)-(-)-*sec*-phenethyl alcohol by the method of **3** gave **5**. The ratio of $C\underline{H}_3O$ peaks δ 3.77 and 3.69 to δ 3.73 and 3.68 in the 600 MHz ^1H NMR ($CDCl_3$) showed an ee of 99%.

***N*, *N'*-Dibenzyl Rhizoferrin, Tetramethyl Ester (6).** Diphenylphosphoryl azide (760 mg, 2.76 mmol) and NEt_3 (1.5 mL, 11 mmol) were added to a solution of **4** (610 mg, 2.77 mmol) and N^1,N^4-dibenzyl-1,4-diaminobutane[15] (370 mg, 1.38 mmol) in DMF (20 mL) at 0 °C under nitrogen. The solution was stirred at 0 °C for 1 h and then at room temperature for 23 h. After solvents were removed under high vaccum, the residue was taken up in EtOAc (25 mL) and was washed with saturated $NaHCO_3$ (25 mL), water (25 mL), 0.5 N HCl (25 mL), and water (25 mL). The organic layer was dried ($MgSO_4$) and concentrated. Flash chromatography, eluting with 4:1 EtOAc/hexane, generated 240 mg (26%) of **6** as a pale yellow oil: [α] +8.25 (*c* 1.00); ^1H NMR ($CDCl_3$) δ 7.42-7.24 (m, 10 H), 4.65-4.48 (m, 4 H), 3.81 (s, 3 H, $OC\underline{H}_3$), 3.79 (s, 3 H, $OC\underline{H}_3$), 3.69 (s, 3 H, $OC\underline{H}_3$), 3.65 (s, 3 H, $OC\underline{H}_3$), 3.40-3.12 (m, 4 H), 3.10-2.67 (m, 8 H), 1.57-1.41 (m, 4 H). Anal. Calcd. for $C_{34}H_{44}N_2O_{12}$: C, 60.70; H, 6.59; N, 4.16. Found: C, 60.64; H, 6.61; N, 4.15.

***N*, *N'*-Dibenzyl Rhizoferrin (7).** A solution of **6** (170 mg, 0.253 mmol) in CH_3OH (7 mL) and 1 N NaOH (7 mL) was stirred at room temperature for 5 h. HCl (1 N, 8 mL) was added, and the solution was concentrated to about 15 mL. After extraction with EtOAc (3 x 15 mL), the organic layer was dried (Na_2SO_4) and concentrated to give 120 mg (77%) of **7** as a colorless glass: [α] +12.27 (*c* 1.00); ^1H NMR (CD_3OD) δ 7.42-7.20 (m, 10 H, 2 Ph), 4.67-4.47 (m, 4 H, $C\underline{H}_2Ph$), 3.35-3.23 (m, 4 H, 2 $NC\underline{H}_2$), 3.19-2.69 (m, 8 H, 4 $C\underline{H}_2CO$), 1.58-1.41 (m, 4 H, 2 $C\underline{H}_2$). Anal. Calcd. for $C_{30}H_{36}N_2O_{12}\cdot H_2O$: C, 56.78; H, 6.04; N, 4.41. Found: C, 56.88; H, 6.08; N, 4.34.

Rhizoferrin. A solution of **7** (110 mg, 0.178 mmol) in distilled THF (1.5 mL) was added to Li (33 mg, 4.8 mmol) in NH_3 (100 mL), and the mixture was maintained at -78 °C for 3 h. Aqueous CH_3OH (50%, 10 mL) was added until the blue

color disappeared. Ammonia was evaporated, and the residue was taken up in water (50 mL) and concentrated to dryness (3 x). The colorless residue was dissolved in water and filtered through a cation exchange resin column (Bio Rad, AG 50W-X8). The eluant containing product (pH = 3) was extracted with EtOAc (50 mL), which was concentrated to dryness. The residue was dissolved in distilled EtOH (2 mL), filtered, and concentrated to yield 50 mg (64%) of rhizoferrin as a colorless glass: HRMS (FAB, *m*-nitrobenzyl alcohol matrix) calcd. for $C_{16}H_{25}N_2O_{12}$ 437.1407 (M + H), found 437.1407 (base). Anal. Calcd. for $C_{16}H_{24}N_2O_{12}{\cdot}H_2O$: C, 42.29; H, 5.77; N, 6.17. Found: C, 42.49; H, 5.80; N, 5.84.

A solution of crude product (10 mg) was purified by reversed-phase HPLC[10] (C-18 preparative column, 21.4 mm x 25 cm, obtained from Rainin). The initial mobile phase concentration of 3% CH_3CN in 0.1% TFA was held for 15 min, followed by gradient elution of 3-11% CH_3CN in 0.1% TFA over 35 min, then held at 11% CH_3CN in 0.1% TFA for 20 min. Flow rate was maintained at 4 mL/min. Retention time was 56 min. Lyophilization gave 4.32 mg (9.90 μmol) of purified rhizoferrin as a colorless glass: [α] -16.7 (26 °C) (*c* 0.1613); ^{1}H NMR (D_2O) δ 3.21-3.15 (m, 4 H), 3.02 (d, 2 H, *J* = 16.0 Hz), 2.79 (d, 2 H, *J* = 16.0 Hz), 2.76 (d, 2 H, *J* = 14.6 Hz), 2.65 (d, 2 H, *J* = 14.6 Hz), 1.53-1.47 (m, 4 H).

A stock solution was prepared by dissolving the purified product in 50.00 mL distilled water; a 10.00 mL aliquot was diluted to 20.00 mL and adjusted to pH = 3.02 with 1.90 mL of 0.010 N HCl (final rhizoferrin concentration = 9.04×10^{-5} M). CD and UV spectra were taken immediately after pH adjustment. All spectra were baseline corrected with a distilled water blank, which was acidified as above.

CD Results. The CD spectrum of rhizoferrin exhibited a negative Cotton effect from 200 to 220 nm, with a single minimum at 205 nm, Δε = -2.7 compared to a recorded single minimum[10] at 204 nm, Δε = -4.3.

UV Results.	nm	ε	ε[10]
	196	12200	(13900)
	200	10800	(13150)
	210	5230	(5600)
	215	2770	(3000)
	220	1200	(1400)

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(*Received in USA* 10 *September* 1996; *accepted* 15 *November* 1996)