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Streamlined synthesis of (*R*, *R*)-rhizoferrin, (*S*, *S*)-rhizoferrin and (*R*, *S*, *R*)-staphyloferrin A

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ABSTRACT

(*R*, *R*)-Rhizoferrin and (*R*, *S*, *R*)-staphyloferrin A are carboxylate-type siderophores. Their streamlined synthesis has been accomplished starting from (*R*)-citric acid. Key-step of these syntheses is a chemo-enzymatic ester hydrolysis. (*S*, *S*)-rhizoferrin was accessible by a multistep synthesis starting from an (*S*)-citrate derived synthon.

1. Introduction

Iron is an essential element for virtually all living organisms (Lankford, 1973) since iron plays an essential role in photosynthetic and respiratory chains; it is necessary for metalloenzymes, in hydrogenases or redox enzymes as well as in Fe-S-clusters.(Baakza et al., 2005; Beasley and Heinrichs, 2010; Fernandez et al., 2005; Goldoni et al., 1991; Matzanke et al., 1997; Raymond et al., 2003; Seneviratne and Vithanage, 2015) Despite the fact that iron is the fourth most abundant element, its oxyhydroxides are almost insoluble at neutral or alkaline pH, and their equilibrium concentrations of 10^{-17} M are far away from the concentration allowing optimal growth of most organisms at 10^{-6} M to 10^{-8} M. Binding to strong ligands such as hemoglobin in erythrocytes or transferrin in body fluids can even lower the concentration of free available iron to 10^{-24} M. Many microorganisms therefore counteract iron depletion through the synthesis of siderophores, and up to now, more than 500 distinct siderophores are known.(Harris et al., 2007; Seneviratne and Vithanage, 2015; Shenker et al., 1995; Hider and Kong, 2010) These low molecular weight compounds are designed to strongly bind iron to either dissolve iron from minerals or to successfully compete for iron with other organisms, for example with host plants or animals. Furthermore, the presence of siderophores is necessary and even crucial for transport and storage of iron.(Bergeron et al., 1997; Carrano et al., 1996; Dubey and Heinonen, 2013; Lankford, 1973; Neilands and Leong, 1986) The secretion of siderophores and - as a consequence – siderophore-mediated iron deprivation in host organism resulting from the ability of iron acquisition from host tissue is considered a fungal and bacterial virulence trait. (Albarouki et al., 2014; Boughammoura et al., 2007; Greenshields et al., 2007; Haas et al., 2008; Schrettl et al., 2004, 2007) Furthermore, siderophores may stimulate in plants defense responses to pathogens, (Beasley and Heinrichs, 2010; Dubey and Heinonen, 2013) and have been shown to play a role in symbiosis as well as in saprophytic survival and they increase competence of the bacteria in the soil and rhizosphere.

(3*R*, 3′*R*) Rhizoferrin (**11**), its enantiomer (3*S*, 3'*S*) rhizoferrin (**6**) and (*R*, *S*, *R*) staphyloferrin A (**9**) are carboxylate-type siderophores holding a citric acid as the major structural motif. Rhizoferrin outperformes most other phyto-siderophores because of its enormous affinity to iron and transport capabilities. Existing syntheses provide low yields and require tedious purification procedures.

The absolute configuration of **11** had previously been determined from the results of a total synthesis by Bergeron et al. (Bergeron et al., 1997) with an overall yield of 1%. More recently Madsen et al. (Madsen et al., 2015) published a synthesis of (*S*) citric acid synthon. Commercially available rhizoferrin and staphyloferrin A are exclusively produced by fermentation; *e.g. Rhizopus arrhizus* (Shenker et al., 1995), *Cunninghamella elegans* (Tschierske et al., 1996). These procedures are time-consuming, and the separation of analytically pure products from the fermentation broth is tedious albeit these broths contain 50–100 mg/L (Shenker et al., 1995), 800 mg/L (Tschierske et al., 1996) or even 4 g/L. (Tschierske et al., 1996) For staphyloferrin A a solid phase peptide synthesis has been accomplished in good yields, but its scaling up still represents a synthetic challenge.(Pandey et al., 2014)

2. Results and discussion

In our first approach (Scheme 1) following Madsen's strategy

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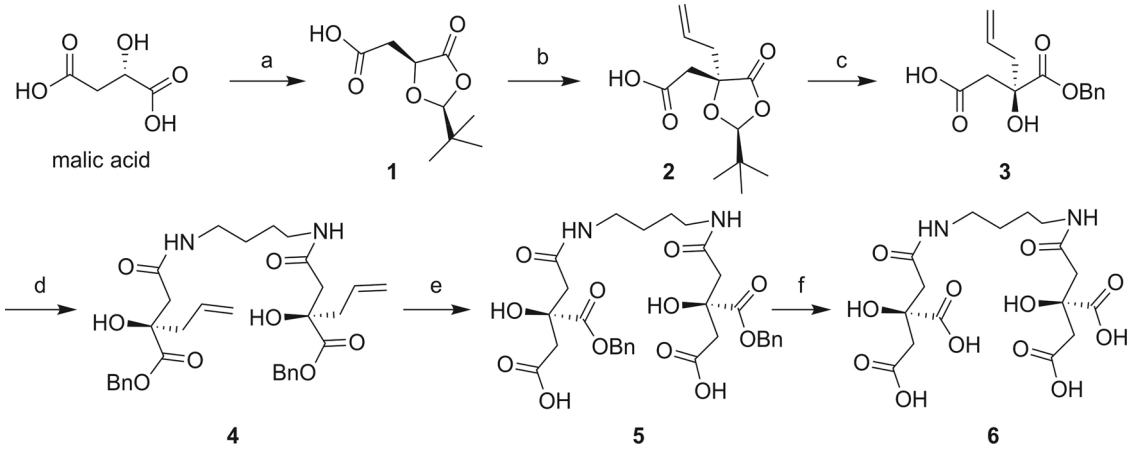


Scheme 1. a) Pivalaldehyde, H_2SO_4, pentane, reflux, 56 h, 49%; b) LiHMDS, THF, allylbromide, −78 °C, 2 h, 56%; c) NaH, BnOH, 0 °C, 1 h, 97%; d) TBTU, HOBt, DMF, 1,4-diaminobutane, 0 °C → r.t., 10 h, 83%; e) $RuCl_3$, $NaIO_4$, MeCN/H_2O, 0 °C, 2 h, 63% f) Pd, H_2, THF, 91%.

(Madsen et al., 2015), malic acid was chosen as a starting material, and an overall yield of 16% of *enantio*-rhizoferrin (**6**) (Drechsel and Winkelmann, 2005; Munzinger et al., 1999) was obtained.

A serious drawback, however, of this sequence is the time-consuming removal of the protection groups. In addition, several attempts to improve the overall yields failed, and the purification required extensive chromatography.

However, the synthesis of (*R*, *R*) rhizoferrin (**11**) and (*R*, *S*, *R*) staphyloferrin A (**9**) (Scheme 2) used *rac*-triethylcitrate; this starting material that can easily be hydrolyzed to (*R*)-diethylcitrate (**7**) (Chenevert et al., 1998) using the hydrolytic enzyme Novozym 435. Coupling of **7** with ornithine ethyl ester or with putrescine gave compounds **9** and **11** in good yields, respectively. Due to its similarity to (*R*) diethylcitrate, ornithine ethyl ester was used to synthesize (*R*, *S*, *R*) staphyloferrin A, which presents a generalization of the synthesis of (*R*) citrate type siderophores. This also gives the advantage to remove the protection group of the ornithine moiety in a single step along with the citrate ethylesters.

At first sight, the cleavage of the ethyl esters in **8** or **10** should not represent a problem, but “common” acidic or basic conditions failed to yield good results, and decomposition of the starting materials was detected by TLC and mass spectrometry. These ethyl esters have been cleaved, however, with an aqueous solution of $Ba(OH)_2$, followed by a careful acidification in the cold with sulfuric acid to precipitate $BaSO_4$, and deferri-rhizoferrin (**11**) and deferri-staphyoferrin A (**9**) were obtained in good yields and at high purity. This acidification has to be performed with caution and with cooling of the reaction mixture. As previously described, (Bergeron et al., 1997; Milner et al., 2013) rhizoferrin and staphyloferrin A form imidorhizoferrin and imidostaphyloferrin under acidic conditions very easily.

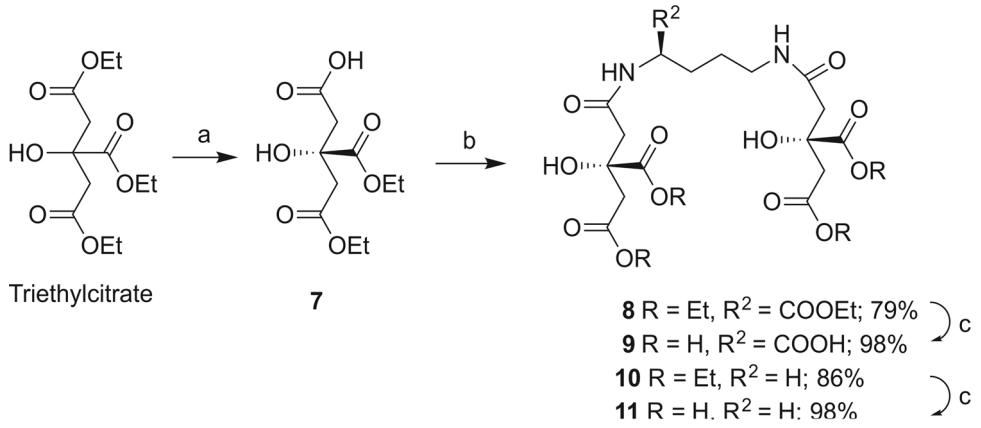
Rhizoferrin (**11**) was identical with an authentic sample (commercially obtained; prepared by extraction). For comparison, CD spectra of our materials and an authentic sample were taken, and these the CD spectra (Munzinger et al., 1999) are depicted in Fig. 1.

3. Conclusion

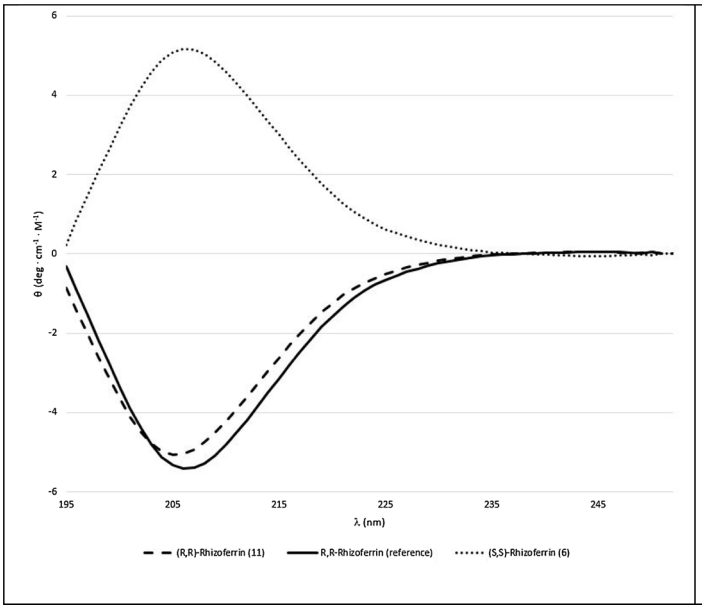
In this study, convenient syntheses of (*S*, *S*)-rhizoferrin (**6**), (*R*, *R*)-rhizoferrin (**11**) and (*R*, *S*, *R*)-staphyloferrin A (**9**) were performed. Thus, *enantio*-rhizoferrin was accessed starting from the corresponding (*S*)-citrate synthon **3**. In a very straightforward manner, an enzymatic enantioselective ester hydrolysis of triethyl citrate was used to obtain the key intermediate, (*R*)-diethyl citrate (**7**); this compound was then effectively coupled in TBTU catalyzed reactions yielding the title compounds in just three reaction steps in excellent overall yields and at high purity.

4. Experimental section

General methods are described in the supplementary materials file; this file also contains the depicted ^1H and ^{13}C NMR spectra. All lab equipment was purged with an aqueous solution of EDTA to remove



Scheme 2. a) Novozym 435, H_2O, pH = 7.4, 40 °C, 10 h, 45%; b) TBTU, HOBt, DMF, 1,4-diaminobutane or ornithine ethylester, 0 °C → r.t., 10 h; c) $Ba(OH)_2$, H_2SO_4, 1 h, 0 °C, 98%.



Compound	θ (deg · cm^{-1} · M^{-1}) (206 nm)
(*R,R*)-Rhizoferrin (reference)	5.42
(*R,R*)-Rhizoferrin (**11**)	5.04
(*S,S*)-Rhizoferrin (**6**)	5.17

Fig. 1. CD spectra of synthetic **11** and authentic **11** as well as of enantiomeric **6**: The measurements were performed on a J-810 instrument (JASCO Corp., Rev. 1.00) at 20 °C using a cuvette with a space length of 1 mm and a sample concentration of 0.4 mg/mL in D_2O at pH = 4.8. The spectra were measured using wavelengths ranging from 250 to 195 nm and a scan rate of 1 nm per second performing 50 accumulations. The absorption is reported after subtraction of the spectrum of the blank solvent from the sample spectra.

any traces of iron. HPLC analysis (C_{18} reversed-phase, Nucleosil, 5 µm, 240 x 4.6 mm; MeCN/H_2O (3 → 8%; + 0.1% TFA) showed products of > 98% purity.

4.1. 2-((2S, 4S) 2-tert-Butyl-5-oxo-1, 3-dioxolan-4-yl) acetic acid (1)

To a suspension of (*S*) malic acid (10.0 g, 74.6 mmol) in pentane (150 mL) pivalaldehyde (13 mL, 120 mmol), *p*-toluenesulfonic acid (1.2 g, 5.8 mmol) and sulfuric acid (3 drops) were added. [40] The mixture was heated in a Dean-Stark trap under reflux for 56 h. The resulting suspension was filtered. The solid cake was dissolved in DCM and washed with 8% aqueous phosphoric acid (2 × 20 mL). The combined organic phases were dried ($MgSO_4$), and the solvent was removed under diminished pressure; re-crystallisation from DCM gave **1** (9.87 g, 49%)(Hoye et al., 1987; Seebach et al., 1984; Vahl Gabrielsen, 1975; Xu et al., 2005) as a white solid; m.p. 103–106 °C (lit.: 104–106 °C (Huang et al., 2006); R_f = 0.26 (silica gel, *n*-hexane/ethyl acetate, 9:1); IR (ATR): *ν* = 3385*w*, 2964*m*, 2880*m*, 2663*w*, 2595*w*, 1702*vs*, 1482*vw*, 1411*m*, 1287*s*, 1262*s*, 1225*s*, 1178*s*, 1114*s*, 1096*s*, 1039*w*, 932*m*, 883*m*, 759*m*, 660*m*, 638*m* cm^{-1}; $[\alpha]_D$ = -0.57° (c 0.385, $CHCl_3$); ^{1}H NMR (500 MHz, $CHCl_3$): δ = 10.33 (*s*, 1H, COOH), 5.20 (*d*, *J* = 1.2 Hz, 1H, H-2), 4.66 (*ddd*, *J* = 7.3, 3.7, 1.2 Hz, 1H, H-4), 3.02 (*dd*, *J* = 17.2, 3.7 Hz, 1H, H-6a), 2.83 (*dd*, *J* = 17.2, 7.3 Hz, 1H, H-6b), 0.98 (s, 9H, *tert*-butyl) ppm; ^{13}C NMR (100 MHz, $CHCl_3$): δ = 170.26 (C-5), 167.33 (C-7), 105.15 (C-2), 66.68 (C-4), 30.64 (C-6), 29.49 (Cq-*t*-Bu), 18.66 (CH_3-*t*-Bu) ppm; MS (ESI, MeOH): *m/z* = 203.1 (28%, $[M+H]^+$), 225.1 (100%, $[M + Na]^+$);analysis calcd for $C_9H_{14}O_5$ (202.20): C 53.46, H 6.98; found: C 53.27, H 7.11.

4.2. 2-((2S,4S) 4-Allyl-2-(tert-butyl)-5-oxo-1,3-dioxolan-4-yl)-acetic acid (2)

To a solution of **1** (2.8 g, 13.9 mmol) in THF (150 mL) LiHMDS (1 M in THF, 27.8 mL, 27.8 mmol) was added slowly at −90 °C. The reaction was stirred for 1 h, then allylbromide (2.41 mL, 27.8 mmol) was slowly added over a period of 15 min, and the temperature was allowed to raise to −10 °C. The resulting solution was diluted with DCM followed by usual aqueous workup. The residue was purified by column chromatography (*n*-hexane/ethyl acetate, 9:1) to afford **2** (1.89 g, 56%) (Huang et al., 2006; Madsen et al., 2015; Seebach et al., 1984) as colorless oil; R_f = 0.47 (silica gel, n-hexane/ethyl acetate, 9:1); IR (ATR): *ν* = 3437*w*, 2965*m*, 2910*w*, 2877*w*, 2667*vw*, 2582*vw*, 1795*s*, 1718*vs*, 1643*w*, 1485*m*, 1467*w*, 1460*w*, 1436*m*, 1410*m*, 1380*w*, 1367*m*, 1353*m*, 1285*m*, 1252*m*, 1184*s*, 1161*vs*, 1116*m*, 1084*s*, 1042*w*, 996*m*, 967*s*, 927*s*, 624*m* cm^{-1}; $[\alpha]_D$ = +39.3° (c 0.305, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.76 (*s*, 1H, COOH), 5.87 – 5.68 (m, 1H, H-9), 5.26 – 5.18 (m, 3H, H-2 + H-10a + H-10b), 2.90 – 2.78 (AB, *J* = 17 Hz, 2H, H-6a + H-6b), 2.56 (m, 2H, H-8a + H-8b), 0.94 (s, 9H, CH_3-*t*-Bu) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.81 (C-5), 173.50 (C-7), 130.16 (C-9), 121.37 (C-10), 108.54 (C-2), 79.94 (C-4), 39.67 (C-6), 38.27 (C-8), 34.51 (Cq-*t*-Bu), 23.71 (CH_3-*t*-Bu) ppm; MS (ESI, MeOH): *m/z* = 242.9 (20%, $[M+H]^+$), 260.0 (38%, $[M + NH_4]^+$), 265.0 (100%, $[M + Na]^+$); analysis calcd for $C_{12}H_{18}O_2$ (242.27): C 59.49, H 7.49; found: C 59.27, H 7.62.

4.3. (3S) 3-((Benzyloxy)carbonyl)-3-hydroxyhex-5-enoic acid (3)

To a solution of **2** (1.4 g, 5.8 mmol) in THF (50 mL) at 0 °C, benzyl alcohol (0.9 mL, 8.7 mmol) and sodium hydride (60%, 0.6 g, 15.2 mmol) were added. The solution was stirred for 1 h before quenching with sodium bicarbonate (15 mL). Usual aqueous workup gave **3** (1.50 g, 97%) (Eckelbarger et al., 2008) being pure enough for the transformations to follow. An analytical sample showed: colorless oil; R_f = 0.43 (silica gel, $CHCl_3$/MeOH, 9:1); IR (ATR): *ν* = 3456*w*, 2981*w*, 1729*vs*, 1641*w*, 1587*w*, 1499*w*, 1456*m*, 1434*m*, 1416*m*, 1398*m*, 1215*vs*, 1190*vs*, 1155*s*, 1095*m*, 1076*m*, 1039*m*, 1029*m*, 997*m*, 920*m*, 751*s*, 697*vs*, 627*m* cm^{-1}; $[\alpha]_D$ = −5.8° (c 0.32, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41 – 7.31 (*m*, 5H, H-10 + H-10' + H-11 + H-11' + H-12), 5.75 (*ddt*, *J* = 17.4, 10.2, 7.3 Hz, 1H, H-5), 5.22 (*d*, *J* = 12 Hz, 1H, H-6a), 5.19 (*d*, *J* = 12 Hz, 1H, H-6b), 5.15 – 5.04 (*m*, 2H, H-8a + H-8b), 3.00 (*d*, *J* = 16.6 Hz, 1H, H-2a), 2.77 (*d*, *J* = 16.6 Hz, 1H, H-2b), 2.50 – 2.41 (*m*, 2H, H-4) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 175.9 (C-1), 174.4 (C-7), 135.2 (C-9), 131.2 (C-5), 128.7 (C-10 + C-10' + C-11 + C-11' + C-12), 120.0 (C-6), 75.0 (C-3), 68.0 (C-8), 43.8 (C-2), 42.7 (C-4) ppm; MS (ESI, MeOH): *m/z* = 264.9 (20%, $[M+H]^+$), 282.0 (52%, $[M + NH_4]^+$), 287.1 (100%, $[M + Na]^+$);

analysis calcd for $C_{14}H_{16}O_5$ (264.27): C 63.63, H 6.10; found: C 63.35, H 6.27.

4.4. Dibenzyl (2S, 2'S) 2,2'-((butane-1,4-diylbis(azanediyl))bis(2-oxoethane-2,1-diyl))bis(2-hydroxypent-4-enoate) (4)

To an ice-cold solution of 3 (1.0 g, 3.8 mmol) in DCM/DMF (1:1; 25 mL), TBTU (1.34 g, 4.18 mmol), HOBt (565 mg, 4.18 mmol) and DIPEA (2.15 mL, 12.5 mmol) were, and the mixture was stirred for 5 min. 1,4-Diaminobutane (0.15 mL, 1.5 mmol) was added. After stirring for 10 h, the solvent was removed. Usual aqueous work-up followed by column chromatography (silica gel, *n*-hexane/ethyl acetate, 8:1) gave **4** (722 mg, 83%) as a highly viscous, colorless oil; R_f = 0.63 (silica gel, *n*-hexane/ethyl acetate, 9:1); IR (ATR): 3332*w*, 2939*w*, 2870*w*, 1733*s*, 1640*vs*, 1544*s*, 1499*w*, 1455*m*, 1437*m*, 1411*m*, 1374*m*, 1321*m*, 1213*s*, 1188*vs*, 1148*s*, 1081*m*, 1029*m*, 998*m*, 917*m*, 738*m*, 697*vs*, 660*m*, 586*m* cm^{-1}; $[\alpha]_D$ = -1.5° (c 0.3, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.38 – 7.30 (m, 10H, H_{Ar}), 6.24 (*s*, NH, 2 H), 5.73 (*ddt*, *J* = 17.4, 10.2, 7.3 Hz, 2H, H-5 + H-5'), 5.25 – 5.16 (*m*, 4H, H-8 + H-8'), 5.12 – 5.01 (m, 4 H, H-6a + H-6b + H6'-a + H-6'b), 3.28 – 3.06 (*m*, 4H, H-13 + H-16), 2.77 (*d*, *J* =15.0 Hz, 2H, H-2a + H-2a'), 2.55 (*d*, *J* =15.0 Hz, 2H, H-2b + H-2'b), 2.46 (*qd*, *J* = 13.8, 7.4 Hz, 4H, H-4 + H-4'), 1.47 – 1.40 (*m*, 4H, H-14 + H-15) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.7 (C-7 + C7'), 170.4 (C-1 + C-1'), 135.4 (C-9 + C9'), 131.4 (C5 + C-5'), 128.6 (C10 + C10 '+ C10' '+ C10"'), 128.4 (C11 + C11 '+ C11' '+ C11" '+ C12), 119.6 (C-6), 75.8 (C-3), 67.6 (C-8), 43.8 (C-2 + C-2'), 43.7 (C-4 + C-4'), 38.7 (C-13 + C16), 26.5 (C-14 + C-15) MS (ESI, MeOH): *m/z* = 581.2 (32%, $[M+H]^+$), 603.3 (100%, $[M + Na]^+$); analysis calcd for $C_{32}H_{40}N_2O_8$ (580.67): C 66.19, H 6.94, N 4.82; found: C 65.87, H 7.13, N 4.69.

4.5. (S)-5-[(4-[(S)-4-(Benzyloxy)-3-(carboxymethyl)-3-hydroxy-4-oxobutanamido)butyl]amino]-3-[(benzyloxy)carbonyl]-3-hydroxy-5-oxopentanoic acid (5)

To a solution of 4 (300 mg, 0.52 mmol) in acetonitrile/water (1:1, 25 mL) at 0 °C ruthenium chloride trihydrate (20 mg, 0.08 mmol) and sodium metaperiodate (450 mg, 2.1 mmol) were added. The reaction was allowed to stir for 2 h at 0 °C followed by aqueous workup and column chromatography (silica gel, 1) $CHCl_3$, 2) MeOH) to yield 5 (210 mg, 63%) as a colorless highly viscous oil; R_f = 0.11 (silica gel, n-hexane/ethyl acetate, 9:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.38 – 7.30 (m, 10H, H_{Ar}), 5.73 (*d*, *J* = 2.1, 4H, H-7 + H-7'), 3.13 – 3.08 (*m*, 4H, H-8 + H-8'), 2.92 (*d*, *J* =15.9 Hz, 2H, H-2a + H-2a'), 2.87 (*d*, *J* =17.0 Hz, 2H, H-2b + H-2'b), 2.46 (*m*, 4H, H-4 + H-4'), 1.46 – 1.42 (*m*, 4H, H-9 + H-9') ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.5 (C-1 + C1'), 171.9 (C-6 + C-6'), 170.3 (C-5 + C-5'), 135.7 (C-10 + C10'), 128.1 (C10 + C10 '+ C10' '+ C10"'), 128.0 (C11 + C11 '+ C11' '+ C11" '+ C12), 73.5 (C-3), 67.1 (C-7), 43.8 (C-2 + C-2'), 42.8 (C-4 + C-4'), 38.5 (C-8 + C8'), 26.50 (C-19 + C-9') ppm; MS (ESI, MeOH): *m/z* = 617.4 (26%, $[M+H]^+$), 639.5 (100%, $[M + Na]^+$); analysis calcd for $C_{30}H_{36}N_2O_{12}$ (616.61): C 58.44, H 5.88, N 4.54; found: C 58.17, H 6.04, N 4.37.

4.6. (3S, 3'S) Rhizoferrin (6)

Hydrogenation (45 psi) of a solution of **5** (150 mg, 0.26 mmol) in THF (30 mL) in the presence of 10% Pd/C (20 mg) followed by purification by column chromatography (C-18, MeOH) gave **6** (105 mg, 91%) as colorless glassy sticky solid; IR (KBr): ν = 3384*s*, 2922*vs*, 2852*s*, 1724*vs*, 1640*vs*, 1432*vs*, 1384*vs*, 1332*s*, 1230*s*, 1120*s* cm^{-1}; $[\alpha]_D$ = +5.48° (c 0.21, H_2O); ^{1}H NMR (500 MHz, D_2O): δ = 3.15 - 3.05 (*m*, 4H, H-7 + H-7'), 2.94 (*d*, *J* =16.0 Hz, 2H, H-2a + H-2'a), 2.72 (*d*, *J* =16.0 Hz, 2H, H-2b + H-2'b), 2.63 (*d*, *J* =14.1 Hz, H-4a + H-4'a), 2.58 (*d*, *J* =14.5 Hz, 2H, H-4b + H-4'b), 1.54 - 1.49 (*m*, 4H, H-8 + H-8') ppm; ^{13}C NMR (125 MHz, D_2O): δ = 177.5 (C6 + C6'), 171.5 (C1 + C1'), 169.8 (C5 + C5'), 74.1 (C3 + C3'), 44.9 (C4 + C4'), 43.4 (C2 + C2'), 39.2 (C7 + C7'), 25.9 (C8 + C8') ppm; MS (ESI, MeOH): m/z = 435.1 (100%, [M−H]-), 909.1 (18%, $[2M-2H + K]^-$); analysis calcd for $C_{16}H_{24}N_2O_{12}$ (436.37): C 44.04, H 5.54, N 6.42; found: C 43.84, H 5.75, N 6.25.

4.7. (3R) 5-Ethoxy-3-(ethoxycarbonyl)-3-hydroxy-5-oxopentanoic acid (7)

To a suspension of triethylcitrate (10 g, 36.2 mmol) in phosphate buffer pH = 7.4 (250 mL) Novozym was added (2.5 g), and the mixture was stirred at 45 °C; the pH was adjusted with 1 M NaOH at 7.4. After 1 equivalent of NaOH has been added, the reaction was allowed to cool to room temperature. The mixture was extracted with diethyl ether (2 × 200 mL), acidified with 2 M HCl until pH < 4 and extracted with ethyl acetate (4 × 200 mL). The combined organic phases were dried ($MgSO_4$), and the filtrate was concentrated under reduced pressure to yield **7** (4.03 g, 45%) as a colorless oil; R_f = 0.38 (silica gel, $CHCl_3$/MeOH, 9:1); $[\alpha]_D$ = +5.31° (c 0.35, MeOH); IR (film): ν = 3482*s*, 2988*s*, 1740*vs*, 1374*s*, 1326*s*, 1214*vs*, 1184*s*, 1128*s*, 1096*s*, 1024*s* cm^{-1}; ^{1}H NMR (400 MHz, DMSO): δ = 4.10 (*q*, *J* =7.1 Hz, 2H, H-7), 4.02 (*q*, *J* =7.1 Hz, 2H, H-9), 2.84 (*d*, *J* =15.0 Hz, 1H, H-2a), 2.79 (*d*, *J* =15.6 Hz, 1H, H-4a), 2.70 (*d*, *J* =15.0 Hz, 1H, H-2b), 2.65 (*d*, *J* =15.5 Hz, 1H, H-4b), 1.19 (*t*, *J* =7.1 Hz, 3H, H-8), 1.15 (*t*, *J* =7.1 Hz, 3H, H-10) ppm; ^{13}C NMR (100 MHz, DMSO): δ = 172.6 (C-1), 171.0 (C-5), 169.3 (C-6), 72.9 (C-3), 60.7 (C-7), 60.0 (C-9), 43.0 (C2 + C4), 14.0 (C-8), 13.9 (C-10) ppm; MS (ESI, MeOH): *m/z* = 248.9 (68%, $[M+H]^+$), 266.0 (58%, $[M + NH_4]^+$), 271.1 (100%, $[M + Na]^+$); analysis calcd for $C_{10}H_{16}O_7$ (248.23): C 48.39, H 6.50; found: C 48.09, H 6.66.

4.8. Tetraethyl 2,2'-((((R)-5-ethoxy-5-oxopentane-1,4-diyl)bis(azanediyl))bis(2-oxoethane-2,1-diyl))(2R,2'R)-bis(2-hydroxysuccinate) (8)

To a solution of 7 (1.0 g, 3.8 mmol) in DMF (25 mL), TBTU (1.42 g, 4.43 mmol), HOBt (600 mg, 4.43 mmol) and DIPEA (3.05 mL, 17.7 mmol) were added at 0 °C. After 5 min of stirring, L-ornithine ethylester (256 mg, 1.6 mmol) was added, and stirring was continued overnight. Usual aqueous work-up followed by chromatography (silica gel, $CHCl_3$) gave give **8** (772 mg, 88%) as a highly viscous colorless liquid; R_f = 0.42 (silica gel, $CHCl_3$/ MeOH, 9:1); $[\alpha]_D$ = +7.3° (c 0.3, $CHCl_3$); IR (KBr): ν = 3355*br*, 2982*s*, 1732*s*, 1647*s*, 1543*s*, 1370*s*, 1183*m*, 1095*m*, 1024*s*, 861*m* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 6.96 (*s*, 1H, NH), 6.63 (*s*, 1H, NH), 4.51 (*m*, 1H, H-7), 4.23 (*q*, *J* =7.0 Hz, 4H, H-9 + H-9'), 4.16 (q, *J* =7.2 Hz, 2H, H-14), 4.11 (q, *J* = 7.1, 4H, H-11 + H-11'), 3.25 (m, 2H, H-7'), 2.93 – 2.57 (m, 8H, H-2 + H-2' + H4 + H4'), 1.92 – 1.82 (m, 1H, H-8a), 1.70 – 1.60 (m, 1H, H-8b), 1.54 (m, 2H, H-8'), 1.27 (t, *J* =7.0 Hz, 6H, H-10 + H-10'), 1.24 (t, *J* =7.1 Hz, 3H, H-15), 1.22 (t, *J* =7.2 Hz, 6H, H-12 + H-12') ppm; ^{13}C NMR (125 MHz, DMSO): δ = 173.6 (C-6), 173.5 (C-6'), 171.8 (C-13), 170.1 (C-1), 170.0 (C-1'), 169.6 (C-5), 169.3 (C-5'), 73.7 (C-3 + C3'), 62.3 (C-9 + C-9'), 61.6 (C-14), 61.0 (C-11 + C-11'), 51.9 (C-7), 44.2 (C-4), 44.1 (C-4'), 43.0 (C-2), 42.9 (C-2'), 38.7 (C-7'), 29.4 (C-8), 25.3 (C-8'), 14.0 (C-10 + C-10' + C-12 + C-12' + C-15) ppm; MS (ESI, MeOH): *m/z* = 621.1 (%, $[M+H]^+$), 643.3 (100%, $[M + Na]^+$); analysis calcd for $C_27H_{44}N_2O_{14}$ (620.65): C 52.25, H 7.15, N 4.51; found: C 51.96, H 7.32, N 4.30.

4.9. 2-(2-(((S)-1-Carboxy-4-((R)-3,4-dicarboxy-3-hydroxybutanamido)butyl)amino)-2-oxoethyl)-2-hydroxysuccinic acid (9)

To a suspension of **8** (500 mg, 0.8 mmol) in deionized water (10 mL), an aqueous solution of $Ba(OH)_2$ (0.2 M, 5 mL) was added. After stirring for 1 h, **0.5** M sulfuric acid was added until pH = 7. The precipitated $BaSO_4$ was removed by centrifugation and washed with

deionized water (3 × 20 mL). Lyophilization of the aqueous phase gave 9 (375 mg, 98%) as a hygroscopic colorless powder; $[\alpha]_D = +66.0°$ (c 0.295, H_2O); IR (KBr): ν = 3381*br*, 2939*s*, 1704*s*, 1625*s*, 1551*s*, 1180*s*, 1046*m*, 889*m*, 781*m*, 578*m* cm^{-1}; ^1H NMR (500 MHz, D_2O) δ = 4.28 – 4.21 (m, 1H, H-7), 3.12–3.03 (m, 2H, H-z'), 2.90–2.54 (m, 8H, H-2 + H-2' + H-4 + H-4'), 1.82–1.74 (m, 1H, H-8a), 1.66 – 1.57 (m, 1H, H-8b), 1.53 – 1.41 (m, 2H, H-8') ppm; ^{13}C NMR (125 MHz, D_2O) δ = 176.6 (C-6), 176.5 (C-6'), 175.32 (C-9), 173.45 (C-1), 173.41 (C-1'), 171.17 (C-5), 170.97 (C-5'), 73.60 (C-3), 73.60 (C-3'), 52.37 (C-7), 44.61 (C-4), 44.03 (C-4'), 43.11 (C-2), 43.02 (C-2'), 38.55 (C-7'), 27.81 (C-8), 24.65 (C-8') ppm; *m/z* = 479.1 (100%, [M − H]-), 997.1 (12%, $[2M-2H + K]^-$); analysis calcd for $C_{17}H_{24}N_2O_{14}$ (480.38): C 42.50, H 5.04, N 5.83; found: C 42.31, H 5.29, N 5.61.

4.10. (2R,2'R) Rhizoferrin-tetraethylester (10)

To a solution of 7 (1.0 g, 3.8 mmol) in DMF (25 mL), TBTU (1.42 g, 4.43 mmol), HOBt (600 mg, 4.43 mmol) and DIPEA (3.05 mL, 17.7 mmol) were added at 0 °C. After 5 min stirring, 1,4-diaminobutane (0.16 mL, 1.6 mmol) was added, and stirring was continued overnight. Usual aqueous work-up followed by column chromatography (silica gel, $CHCl_3$/MeOH, 9:1) gave **10** (772 mg, 88%) as a colorless solid (772 mg, 1.41 mmol, 88%); m.p. 81.3 °C; R_f = 0.55 (silica gel, $CHCl_3$/MeOH, 9:1); $[\alpha]_D = +4.45°$ (c 0.31, DMSO); IR (KBr): ν = 3424*br*, 2986*m*, 2940*m*, 1732*s*, 1646*s*, 1560*m*, 1384*vs*, 1228*s*, 1096*m*, 1080*w*, 1024*m* cm^{-1}; ^1H NMR (500 MHz, DMSO): δ = 7.94 (*t*, *J* = 5.6 Hz, 2H, NH), 4.07 (*q*, *J* = 7.1 Hz, 4H, H-9 + H-9'), 4.01 (*q*, *J* = 7.1 Hz, 4H, H-11 + H-11'), 3.03 - 2.95 (*m*, 4H, H-7 + H-7'), 2.82 (*d*, *J* = 15.1 Hz, 2H, H-2 + H-2'), 2.65 (*d*, *J* = 15.2 Hz, 2H, H-2 + H-2'), 2.61 (*d*, *J* = 14.6 Hz, 2H, H-4 + H-4'), 2.48 (*d*, *J* = 14.7 Hz, 2H, H-4 + H-4'), 1.36 - 1.33 (*m*, 4H, H-8 + H-8'), 1.17 (*t*, *J* = 4.8 Hz, 6H, H-10 + H-10'), 1.17 (*t*, *J* = 4.8 Hz, 6H, H-12 + H-12') ppm; ^{13}C NMR (125 MHz, DMSO): δ = 172.9 (C6 + C6'), 169.4 (C1 + C1'), 169.1 (C5 + C5'), 73.4 (C3 + C3'), 60.6 (C11 + C11'), 60.0 (C9 + C9'), 43.2(C2 + C2' + C4 + C4'), 38.1 (C7 + C7'), 26.4 (C8 + C8'), 14.0 (C12 + C12'), 13.9 (C10 + C10') ppm; MS (ESI, MeOH): *m/z* = 286.2 (20%, $[M + Na + H]^{2+}$), 294.2 (32%, $[M + K + H]^{2+}$), 571.3 (100%, $[M + Na]^+$); analysis calcd for $C_{24}H_{40}N_2O_{12}$ (548.59): C 52.55, H 7.35, N 5.11; found: C 52.27, H 7.56, N 4.96.

4.11. (3R, 3'R) Rhizoferrin (11)

Compound **10** (400 mg, 0.73 mmol) was dissolved in deionized water (10 mL) and an aqueous solution of $Ba(OH)_2$ (0.2 M, 5 mL) was added. After stirring at 0 °C for 1 h, 0.5 M sulfuric acid was added until pH = 7. The precipitated $BaSO_4$ was removed by centrifugation and washed with deionized water (3 × 20 mL). Lyophilization of the aqueous phase gave 11 (310 mg, 98%) as a hygroscopic colorless powder; m.p. 48–52 °C; IR (KBr): ν = 3384*s*, 2922*vs*, 2852*s*, 1724*vs*, 1640*vs*, 1432*vs*, 1384*vs*, 1332*s*, 1230*s*, 1120*s* cm^{-1}; $[\alpha]_D = -5.73°$ (c 0.227, $CHCl_3$); ^1H NMR (500 MHz, D_2O): δ = 3.15–3.05 (*m*, 4H, H-7 + H-7'), 2.92 (*d*, *J* = 16.1 Hz, 2H, H-2a + H-2'a), 2.69 (*d*, *J* = 16.1 Hz, 2H, H-2b + H-2'b), 2.66 (*d*, *J* = 14.1 Hz, H-4a + H-4'a), 2.56 (*d*, *J* = 14.5 Hz, 2H, H-4b + H-4'b), 1.57–1.48 (*m*, 4H, H-8 + H-8') ppm; ^{13}C NMR (125 MHz, D_2O): δ = 177.2 (C6 + C6'), 171.2 (C1 + C1'), 169.5 (C5 + C5'), 73.8 (C3 + C3'), 44.7 (C4 + C4'), 43.3 (C2 + C2'), 38.9 (C7 + C7'), 25.7 (C8 + C8') ppm; MS (ESI, MeOH): *m/z* = 435.1 (100%, [M − H]-), 909.1 (18%, $[2M-2H + K]^-$); analysis calcd for $C_{16}H_{24}N_2O_{12}$ (436.37): C 44.04, H 5.54, N 6.42; found: C 43.82, H 5.71, N 6.25.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2019.07.012.

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