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Synthesis and functional analysis of deferriferrichrysin derivatives: application to colorimetric pH indicators

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ABSTRACT

Deferriferrichrysin belongs to the siderophore peptide family which are Fe(III)-coordinating cyclic peptides. The common structure of this family is three consecutive hydroxamate moieties, such as N^δ-acetyl-N^δ-hydroxy-L-ornithine (Aho). We have designed two deferriferrichrysin derivatives where three Aho residues were arranged as: *cyclo*(-Aho-Gly-Aho-Gly-Aho-Gly-) and *cyclo*(-Aho-Ser-Aho-Ser-Aho-Ser-). Comparative evaluation of the physicochemical properties of their Fe(III) complexes revealed that naturally occurring deferriferrichrysin formed a more stable Fe(III) complex when compared with the two derivatives. This result shows that three consecutive Aho residues are indispensable for high affinity Fe(III) binding by deferriferrichrysin. Of note, the observed pH-dependent chromogenic response of the Fe(III) complexes of the derivatives suggests that these two derivatives should function as sensitive pH indicators in acidic environments.

Keywords: deferriferrichrysin, ferrichrome peptide, hydroxamate, iron complex, siderophore

1. Introduction

Microbes produce Fe(III) selective chelators, called siderophores,[1] to sequester Fe(III), which is necessary for cellular redox chemisty,[2] but insoluble under physiological conditions. Over 500 siderophores have been isolated and classified according to coordinate groups and scaffolds. Deferriferrichrysin (**1**) is a cyclic hexapeptide siderophore with three N^δ-acetyl-N^δ-hydroxy-L-ornithine (Aho) residues that is produced by *Aspergillus oryzae* (Figure 1).[3] Its Fe(III) complex, ferrichrysin (**2**), is a colorant in rice wine (sake). Deferriferrichrome (**3**)[4] and deferriferrichrome A (**4**)[5] are also hydroxamate-containing cyclic hexapeptides. The common feature of this siderophore family is the presence of three consecutive residues with Fe(III)-coordinating hydroxamate side-chains.

These Fe(III)-siderophore complexes are incorporated into the cytoplasm via ABC type transporters.[1] Using this iron uptake system found in microbes, several siderophore-conjugated pharmaceuticals have been designed for a microbe selective drug delivery system.[6-8] For example, an antibiotic with a triscatecholate siderophore (**5**) was taken up by Gram-negative bacteria species, including *Pseudomonas aeruginosa*. The mycobactin-artemisinin conjugate (**6**) was effective against drug resistant strains of *Mycobacterium tuberculosis*, in which Fe(III)-mediated Fenton-type radical formation from a mycobactin moiety was also the key active ingredient of this anti-tuberculosis conjugate. Thus, Fe(III)-siderophore complexes represent interesting pharmaceutical materials for antimicrobial agents.

In order to investigate the coordinating ability of three consecutive Aho residues in deferriferrichrysin (**1**), we have designed two deferriferrichrysin derivatives, *cyclo*(-Aho-Gly-Aho-Gly-Aho-Gly-) (**7a**) and *cyclo*(-Aho-Ser-Aho-Ser-Aho-Ser-) (**7b**). These two compounds contain three Aho residues interspersed by either Gly or Ser residues derived from deferriferrichrysin. In this article, we report the synthesis of these two deferriferrichrysin derivatives and the spectroscopic analysis of the physicochemical properties of the Fe(III) complexes.

2. Results and discussion

2.1. Synthesis of deferriferrichrysin derivatives

In a previous study, we have established a facile synthetic approach to N^{α}-Fmoc-protected N^{δ}-acetyl-N^{δ}-(*tert*-butoxy)-L-ornithine [Fmoc-Aho(*t*-Bu)-OH].[9] Using this amino acid component as a building block in Fmoc-based solid-phase peptide synthesis (SPPS), deferriferrichrysin (**1**) and deferriferrichrome (**3**) were synthesized.[9] According to this protocol, hexapeptide resins **9a,b** were prepared on 2-chlorotrityl [(2-Cl)Trt] resin (Scheme 1). Peptide elongation was performed by standard Fmoc-based SPPS using *N,N'*-diisopropylcarbodiimide (DIC)/*N*-hydroxybenzotriazole (HOBt). Treatment of the resin with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) gave *t*-Bu-protected linear peptides **10a,b**, which were cyclized using diphenylphosphoryl azide (DPPA)/NaHCO$_3$. Linear peptides **10a,b** were converted to protected cyclic peptides **11a,b**, respectively. In the cyclization process of linear peptide **10b**, two minor side-products were obtained: an epimeric product and a ureal product, which was possibly produced via Curtius rearrangement. These were excluded during HPLC purification. Final deprotection of **11a,b** provided the expected peptides **7a,b**, respectively.

2.2. Evaluation of Fe(III) coordinating activities of deferriferrichrysin and the derivatives

Initially, Fe(III) coordinating activities of the siderophore peptides were evaluated by a competition experiment using ethylenediaminetetraacetic acid (EDTA).[10] After the equimolar peptide and Fe(III) were dissolved in phosphate buffer (pH 7.0), the mixture was treated with increasing concentrations of EDTA. The proportion of the Fe(III)-peptide complex in the presence of EDTA was calculated based on the absorbance intensity at the absorbance maximum of the complex (Figure 2). Ferrichrysin (**2**) formed a stable complex even in the presence of excess EDTA [the formation of 83% of the Fe(III)-peptide complex in the presence of three equivalents of EDTA]. Forty-seven percent of the Fe(III)-deferriferrichrome (**3**) complex remained in the presence of three

equivalents of EDTA. In contrast, derivatives **7a** and **7b** were easily deprived of Fe(III) by EDTA [22% and 14% of the Fe(III)-peptide complexes remained in the presence of equimolar EDTA]. Thus, the order of Fe(III) coordinating activity for the siderophore peptides was deferriferrichrysin (**1**) > deferriferrichrome (**3**) > *cyclo*(-Aho-Gly-Aho-Gly-Aho-Gly-) (**7a**) ≈ *cyclo*(-Aho-Ser-Aho-Ser-Aho-Ser-) (**7b**) at pH 7.0,[11] suggesting that three consecutive Aho residues in deferriferrichrysin and naturally occurring siderophore peptides contributed noticeably to the high Fe(III) coordinating activity.

2.3. Circular dichroism (CD) spectra of ferrichrysin and the derivatives

For a hexacoordinate octahedral metal complex of three hydroxamate ligands, there are two possible geometric isomers, *trans* and *cis*.[14,15] Each geometric isomer consists of Λ and Δ optical isomers.[15] All the reported X-ray crystal structures of hexadentate hydroxamate complexes show the *cis* configuration, and the optical chilarities have been determined by CD analysis.[16-19] For example, it was proven by an X-ray diffraction study that ferrichrome A (**4**) exists in the *cis*-Λ configuration,[20] which was supported by the characteristic 465 nm positive band and the 360 nm negative band in the CD spectrum.[21]

The Fe(III)-**7a** complex showed a similar CD spectrum to that of ferrichrysin (**2**) at pH 7.0 (Figure 3), which shows that this species predominantly adopts the *cis*-Λ configuration. In contrast, the Fe(III)-**7b** complex had a positive band at 370 nm and a negative band at 445 nm, indicating that this complex predominantly exists in the *cis*-Δ configuration. Since the *cyclo*(-Xaa-Gly-Xaa-Gly-Xaa-Gly-) scaffold is assumed to exhibit flexible conformations with a low number of intramolecular hydrogen bonds,[22] the flexibility of peptide **7a** may lead to Fe(III) complex adopting the usual *cis*-Λ configuration. In contrast, three side chains of L-Ser in **7b** would restrict the backbone conformation due to the 1,3-allylic strain-like effect.[23] This more rigid

conformation of **7b** would hinder the usual *cis*-Λ configuration of Fe(III)-**7b** complex, resulting in the characteristic *cis*-Δ type coordinating configuration.

2.4. Ultraviolet-visible (UV-vis) absorption spectra of ferrichrysin and the derivatives

UV-vis spectra of Fe(III)-peptide complexes were acquired in pH-controlled buffers between 1.2 and 7.0 to reveal the pH-dependent structural changes of the complexes. The UV-vis spectra of ferrichrysin (**2**) were nearly identical (λ_{max} = 425 nm) over the pH range 1.2–7.0. Both Fe(III)-**7a** and Fe(III)-**7b** in pH 5.0 and 7.0 solutions had similar spectra with absorbance $\lambda_{max} \approx 425$ nm. Interestingly, red shifts of the visible λ_{max} of the spectra of Fe(III)-**7a** and Fe(III)-**7b** were observed at low pH values (Figure 4). This indicates that symmetric Fe(III) complexes with non-sequential Aho residues were easily protonated to change the structure of the complex,[24] which is in contrast to ferrichrysin.

The visual appearance of the Fe(III)-peptide complexes in three pH buffers are shown in Figure 5. A similar orange color was observed between the three ferrichrysin (**2**) solutions at pH 1.2, 3.6 and 7.0. In contrast, both solutions of Fe(III)-**7a** and Fe(III)-**7b** turned red at low pH. These corresponded to the UV-vis spectra of the solutions, suggesting that Fe(III)-**7a** and Fe(III)-**7b** represent potential pH indicators for acidic solutions.

3. Conclusions

In this study, we have carried out synthesis and comparative physicochemical analysis of two deferriferrichrysin analogs: *cyclo*(-Aho-Gly-Aho-Gly-Aho-Gly-) (**7a**) and *cyclo*(-Aho-Ser-Aho-Ser-Aho-Ser-) (**7b**) containing three interspersed Aho residues to investigate the structural basis of ferrichrysin siderophores. The siderophore/EDTA competition experiment revealed that the high Fe(III) coordinating activity of naturally occurring siderophore peptides was attributable to the three consecutive Aho residues. CD analysis demonstrated that the Fe(III)-**7b** complex exhibited a coordinating configuration not observed in other cyclic hexapeptide

siderophores. Notably, characteristic pH-dependent spectral shifts in acidic media were observed for the Fe(III)-complexes of peptides **7a** and **7b**, suggesting that these complexes could be useful as novel pH indicators.

4. Experimental

4.1. Synthesis

4.1.1. General Methods. ^{1}H NMR spectra were recorded using a JEOL ECA-500 spectrometer. Chemical shifts are reported in δ (ppm) relative to residual peak as an internal standard. ^{13}C NMR spectra were referenced to the CD_3CN (in D_2O) or residual peak (in DMSO-d_6) as an internal standard. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Optical rotations were measured with a JASCO P-1020 polarimeter. For flash chromatography, Wakogel C-300E (Wako) was employed. For analytical HPLC, a COSMOSIL 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) was employed with a linear gradient of CH_3CN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min, and eluting products were detected by UV at 220 nm. Preparative HPLC was performed using a COSMOSIL 5C18-ARII column (20 or 10 × 250 mm, Nacalai Tesque Inc.) with a linear gradient of CH_3CN containing 0.1 % (v/v) TFA at a flow rate of 8 or 4 mL/min, respectively.

4.1.2. *cyclo*(-Aho-Gly-Aho-Gly-Aho-Gly-) (7a). The protected linear peptide resin **9a** was constructed by Fmoc-based solid-phase synthesis on H-Gly-(2-Cl)Trt resin (0.64 mmol/g, 158 mg, 0.10 mmol). Fmoc-Gly-OH (0.50 mmol) and Fmoc-Aho(*t*-Bu)-OH (0.30 mmol) were coupled using DIC-HOBt·H_2O (equivalent of Fmoc-amino acid) in DMF. Completion of each coupling reaction was ascertained using the Kaiser ninhydrin test. The Fmoc-protecting group was removed by treating the resin with 20% piperidine in DMF. The resulting protected peptide resin was subjected to HFIP–CH_2Cl_2 (2:8, 20 mL) treatment at room temperature for 2 h. After filtration of the residual resin, the filtrate was concentrated under reduced pressure to give a crude linear peptide **10a**. To a

mixture of the resulting linear peptide in DMF (40 mL), DPPA (53.9 μL, 0.25 mmol) and NaHCO$_3$ (42.0 mg, 0.50 mmol) were added at –40 °C. The mixture was stirred for 20.5 h with warming to room temperature. After filtration, the filtrate was concentrated under reduced pressure, followed by flash chromatography over silica gel with CHCl$_3$–MeOH (9:1) to give the crude protected cyclic peptide **11a**. The crude peptide was treated with CH$_2$Cl$_2$–TFA–thioanisole (10:10:1, 3.65 mL) at room temperature for 13 h. The mixture was poured into ice-cold dry Et$_2$O (30 mL). The resulting powder was collected by centrifugation and washed three times with ice-cold dry Et$_2$O. The crude product was purified by preparative HPLC to afford the title compound as colorless freeze-dried powder (13.1 mg, 19% from H-Gly-(2-Cl)Trt resin): $[\alpha]^{27}_D$ –11.5 (c 0.82, H$_2$O); ^{1}H-NMR (500 MHz, D$_2$O) δ: 1.57-1.96 (m, 12H), 2.07-2.18 (m, 9H), 3.56-3.71 (m, 6H), 3.90 (d, J = 16.6 Hz, 3H), 3.98 (d, J = 16.6 Hz, 3H), 4.35 (dd, J = 6.3, 6.3 Hz, 3H); ^{13}C NMR (125 MHz, D$_2$O) δ: 19.9 (3C), 22.8 (3C), 28.0 (3C), 43.3 (3C), 47.8 (3C), 54.0 (3C), 171.8 (3C), 174.4 (6C); HRMS (FAB): m/z calcd for C$_{27}$H$_{46}$N$_9$O$_{12}$ [M + H]$^+$ 688.3260; found: 688.3258.

4.1.3. *cyclo*(-Aho(*t*-Bu)-Ser(*t*-Bu)-Aho(*t*-Bu)-Ser(*t*-Bu)-Aho(*t*-Bu)-Ser(*t*-Bu)-) (11b). By the identical procedure for the synthesis of peptide **11a** and preparative HPLC purification, the expected peptide **11b** was obtained as white freeze-dried powder (21.6 mg, 19% from H-Ser(*t*-Bu)-(2-Cl)Trt resin): $[\alpha]^{26}_D$ –14.0 (c 0.94, MeOH); ^{1}H-NMR (500 MHz, DMSO-d_6, 100 °C) δ: 1.17 (s, 27H), 1.28 (s, 27H), 1.56-1.81 (m, 12H), 2.02 (s, 9H), 3.50-3.71 (m, 12H), 4.04-4.16 (m, 6H), 7.62 (d, J = 6.9 Hz, 3H), 7.70 (d, J = 5.7 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6, 100 °C) δ: 20.6 (3C), 22.2 (3C), 26.8 (9C), 26.9 (9C), 28.0 (3C), 48.9 (3C), 53.3 (3C), 54.3 (3C), 60.3 (3C), 72.7 (3C), 81.6 (3C), 169.0 (3C), 170.5 (3C), 173.5 (3C); HRMS (FAB): m/z calcd for C$_{54}$H$_{100}$N$_9$O$_{15}$ [M + H]$^+$ 1114.7333; found: 1114.7334.

4.1.4. *cyclo*(-Aho-Ser-Aho-Ser-Aho-Ser-) (7b). By the identical procedure for the synthesis of peptide **7a**, the expected peptide **7b** was obtained as light yellow freeze-dried powder (12.3 mg,

16% from H-Ser(*t*-Bu)-(2-Cl)Trt resin): $[\alpha]^{26}_D$ −14.9 (*c* 0.90, H_2O); ^{1}H-NMR (500 MHz, D_2O) δ: 1.58-2.04 (m, 12H), 2.06-2.18 (m, 9H), 3.57-3.73 (m, 6H), 3.88 (dd, *J* = 11.5, 4.6 Hz, 3H), 3.96 (dd, *J* = 11.5, 4.6 Hz, 3H), 4.36 (dd, *J* = 8.6, 5.2 Hz, 3H), 4.43 (dd, *J* = 4.6, 4.6 Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ: 19.8 (3C), 22.9 (3C), 28.1 (3C), 47.8 (3C), 54.5 (3C), 56.8 (3C), 61.3 (3C), 172.0 (3C), 174.0 (3C), 174.4 (3C); HRMS (FAB): *m/z* calcd for $C_{30}H_{52}N_9O_{15}$ $[M + H]^+$ 778.3577; found: 778.3571.

4.2. EDTA/peptide competition experiment

Peptide and $Fe(NO_3)_3$ [18.0 mM in 0.1 mM HNO_3] (0.2 mM each) were dissolved in 100 mM phosphate buffer (pH 7.0). To this Fe(III)-peptide complex solution, 0.10 to 3.0 equiv of EDTA was added. After incubation at 25 °C for 2–3 days, UV-vis spectra were measured. The proportion of the Fe(III)-peptide complex was calculated as: [425 nm absorbance of the sample solution]/[425 nm absorbance of the control sample (no EDTA)].

4.3. CD spectra

CD spectra were recorded on a JASCO J-720 circular dichroism spectrometer at 20 °C. Peptide and $Fe(NO_3)_3$ [18.0 mM in 0.1 mM HNO_3] (0.5 mM each) were dissolved in 100 mM phosphate buffer (pH 7.0).

4.4. UV-vis spectra

UV-vis spectra were recorded on a Shimadzu UV-2450 UV-vis spectrophotometer. Peptide and $Fe(NO_3)_3$ [18.0 mM in 0.1 mM HNO_3] (0.2 mM each) were dissolved in pH-controlled buffers: 50 mM HCl-KCl buffer (for pH 1.2, 1.6 and 2.0); 50 mM glycine-HCl buffer (for pH 2.4, 2.8, 3.2 and 3.6); 100 mM acetate buffer (for pH 5.0); and 100 mM phosphate buffer (for pH 7.0).

Acknowledgements

Supplementary data

Supplementary data associated with this article can be found, in the online version, at ########.

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11. Of note, their Fe(III) coordinating activities were previously reported; deferriferrichrysin (**1**, pK = 30.0),[23] deferriferrichrome (**3**, pK = 29.1),[23] *cyclo*(-Aho-Gly-Aho-Gly-Aho-Gly-) (**7a**, pK = 31.8).[24]

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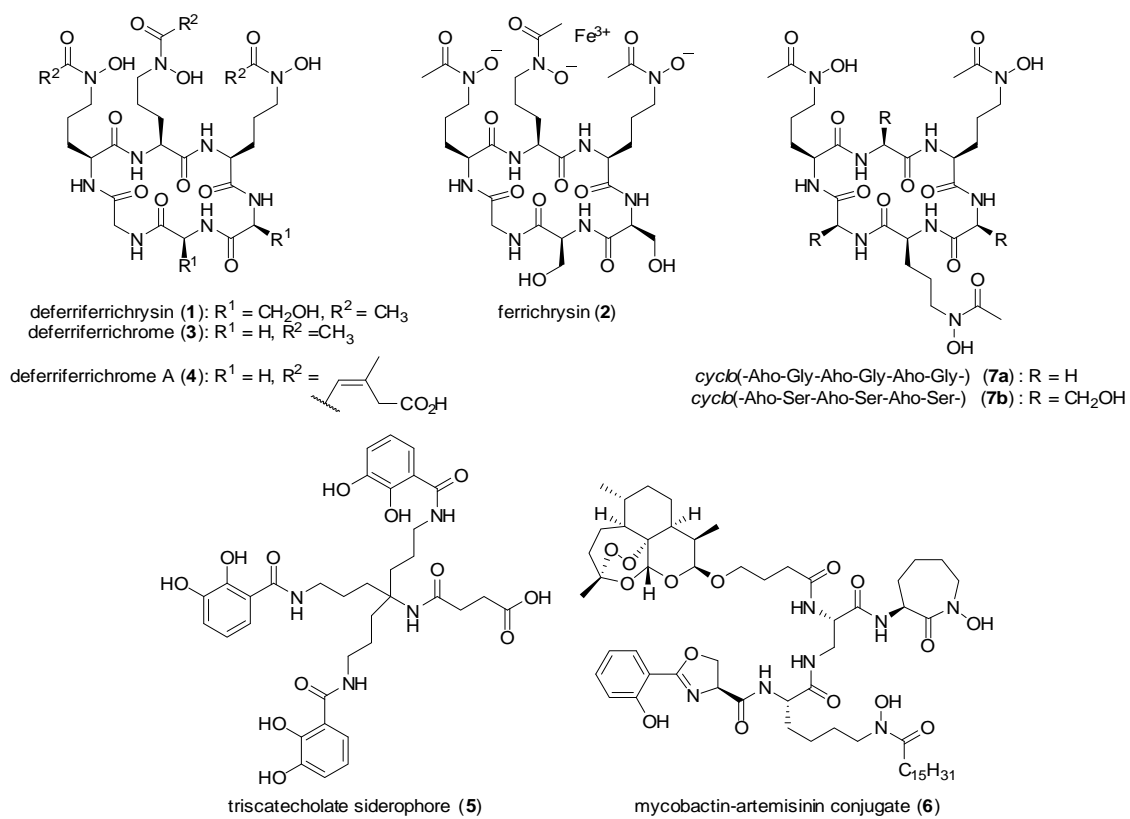24. Akiyama, M.; Katoh, A.; Mutoh, T. *J. Org. Chem.* **1988**, *53*, 6089.

deferriferrichrysin (**1**): R^1 = CH$_2$OH, R^2 = CH$_3$
deferriferrichrome (**3**): R^1 = H, R^2 =CH$_3$

ferrichrysin (**2**)

deferriferrichrome A (**4**): R^1 = H, R^2 = [structure with CO$_2$H]

cyclo(-Aho-Gly-Aho-Gly-Aho-Gly-) (**7a**) : R = H
cyclo(-Aho-Ser-Aho-Ser-Aho-Ser-) (**7b**) : R = CH$_2$OH

triscatecholate siderophore (**5**)

mycobactin-artemisinin conjugate (**6**)

Figure 1. Structures of siderophore and the derivatives.

13
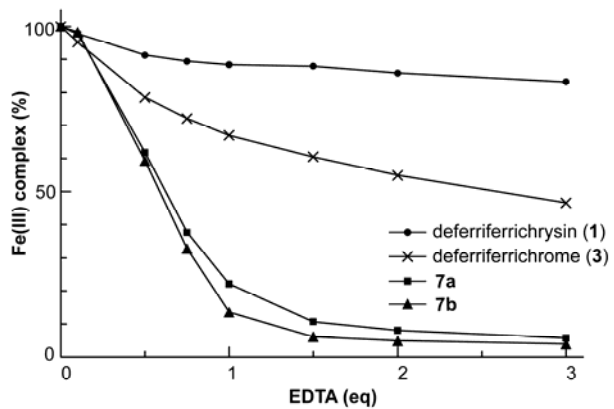
Figure 2. Competition experiments between siderophore peptides and EDTA for Fe(III) coordinating activity at pH 7.0.
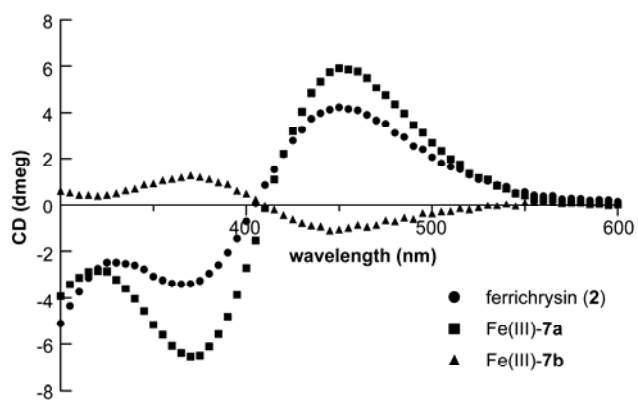
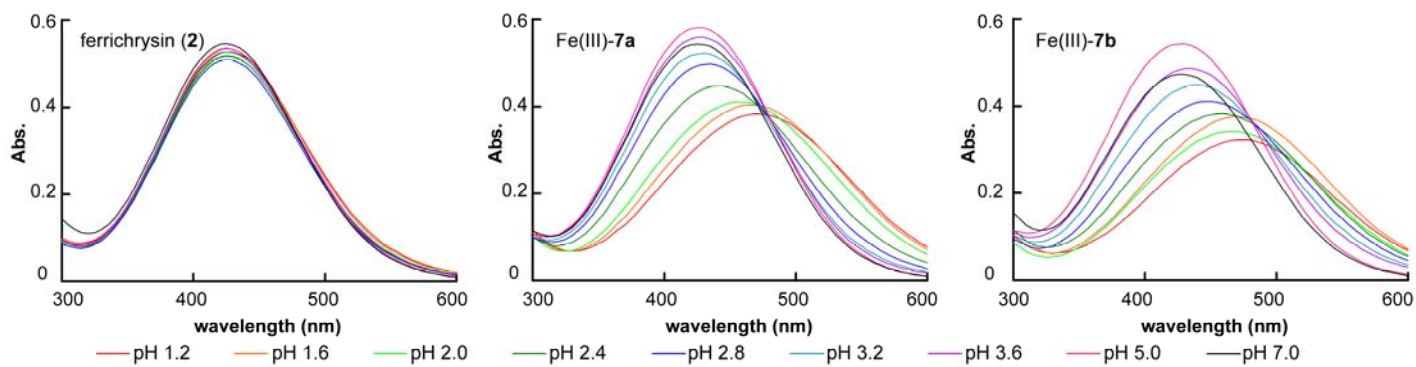**Figure 3.** CD spectra of Fe(III) complexes of siderophore peptides.

Figure 4. UV-vis spectra of Fe(III) complexes of siderophore peptides.
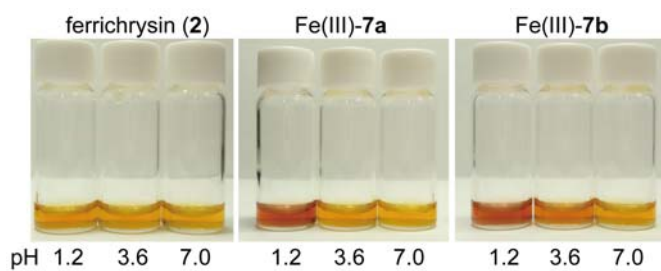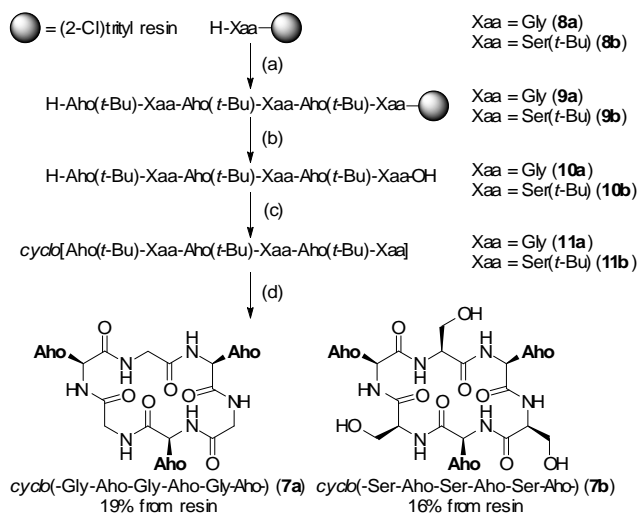
Figure 5. Appearance of Fe(III)-peptide complex solutions.

Peptide and Fe(III) (1.0 mM each) were dissolved in pH-controlled buffers: 50 mM HCl-KCl buffer (for pH 1.2); 50 mM glycine-HCl buffer (for pH 3.6); and 100 mM phosphate buffer (for pH 7.0).

Scheme 1. Synthesis of deferriferrichrysin derivatives.

Reagents and conditions: (a) Fmoc-based SPPS; (b) HFIP, CH_2Cl_2, rt, 2 h; (c) DPPA, NaHCO$_3$, DMF, −40 °C to rt; (d) TFA, CH_2Cl_2, thioanisole, rt (19% from **8a**, 16% from **8b**); Aho = N^δ-acetyl-N^δ-hydroxy-L-ornithine.