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Biomimetic ferrichrome: structural motifs for switching between narrow- and broad-spectrum activities in *P. putida* and *E. coli*

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Experimental part

General

All reagents and solvents used for the synthesis were purchased from Sigma-Aldrich, Merck and Acros. Protected amino acids were purchased from Chem-Impex. Amidoxime iron (III) chelating resin Purolite® S910 was obtained from Purolite® SRL. Magnesium sulfate heptahydrate for the preparation of MKB medium of Bio Ultra grade (min. 99.5%) was purchased from Sigma Aldrich. Commercial reagents were used for synthesis without further purification.

Flash chromatography was performed using Merck Silica gel Kieselgel 60 (0.04-0.06 mm).

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance -300 MHz and a Bruker Evance-400 MHz spectrometer, equipped with QNP probe. Chemical shifts are reported in ppm on the δ scale down field from TMS. All *J* values are given in Hertz.

CuCAs, $FeCl_3$ and $Fe(ClO_4)_3$ stock solutions were standardized by ICP-AES "Arcos" (Spectro Ltd, Kleve City, Germany). Electron-spray mass spectrometry (ESI-MS) was performed for the free ligands on Waters Mass Spectrometer Type Q-TOF or on a SYNAPT High Definition, Q-TOF Mass Spectrometer (Micromass, Manchester, UK). The spectra of Fe(III) – ligand complexes were recorded using a Bruker apex ultra FT-ICR and a micrOTOF-Q mass spectrometers, both with ESI.

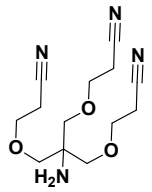
CD spectra were recorded on Chirascan Spectrophotometer (Applied Photophysics).

CV measurements were carried out on a VA 797 Computrace (Metrohm) with hanging drop mercury electrode (HDME) as the working electrode. Auxiliary electrode was platinum electrode, and silver chloride (3M KCl) electrode was used as reference electrode. 0.1M $NaClO_4$ was used as supporting electrolyte.

For potentiometric and UV-Vis titrations all solutions were prepared in doubly distilled water. All stock solutions were prepared using an R200D Sartorius analytical balance (precision 0.01 mg). $Fe(ClO_4)_3$ stock solution was prepared immediately before use from $Fe(ClO_4)_3 \cdot H_2O$ (Aldrich, chlorides < 0.005%) in 1.0×10^{-2} M $HClO_4$ (AppliChem p.a. 70%) and standardized by ICP-AES (Fisons Instruments, 3410 ICP). $HClO_4$ solution was titrated by carbonate-free NaOH (0.1 N Fluka standard solution), standardized by titration with potassium hydrogen phthalate (Merck. p.a.). The absorption measurements (250 nm - 800 nm) were performed on a Varian Cary 300 Bio or a Varian-50 UV-Visble spectrophotometers. In one set of experiments the solutions were measured in quartz cuvettes (Hellma), in another the spectrophotometer was fitted with C-Technologies optical fibers and immersion probe made of quartz (C-Technologies) or metal (Hellma Ultra-Mini Immersion Probe). The free hydrogen ion concentration was measured with a combined glass electrode (Mettler Toledo InLab Semi micro) with polymer filling. Potentiometric titrations were performed using an automatic titrator system Titrando 905 (Metrohm) with a combined glass electrode (Mettler Toledo InLab Semi micro) with polymer filling.

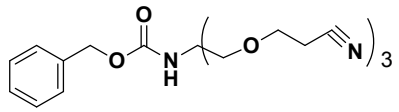
Siderophore growth was recorded on a BioMate3 Spectrophotometer (Thermo Electron Corporation, Wisconsin US). Fluorescence images were taken by a spinning-disc confocal microscope Axio Observer 21 (Carl Zeiss MicroImaging, GmbH Göttingen, Germany) equipped with an PvCam camera and processed by using ZEN 2 software.

Synthetic procedures and characterization data for the prepared synthetic compounds



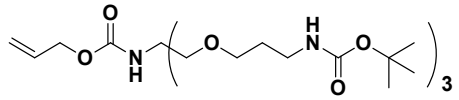
2

Compound 2[1]**: 3,3'-((2-amino-2-((2-cyanoethoxy)methyl)propane-1,3-diyl)bis(oxy))-dipropanenitrile.** 40% KOH (0.15 g) solution was added dropwise to a suspension of tris(hydroxymethyl)aminomethane **1** (3.00 g, 27.5 mmol) in dioxane (3 mL). After the addition was completed, acrylonitrile (4.70 g, 89.3 mmol) was added to the reaction mixture at 0 °C, than the temperature was raised and the mixture was stirred at r.t. overnight. DCM was added to the solution and extracted twice with water. The extractions were dried over Na_2SO_4 and the solvent was evaporated *in vacuo* to yield the crude residue. The residue was purified by silica gel chromatography (gradient, 60:40 $EtOA_C$: DCM → $EtOA_C$ and finally eluted with 5% MeOH in $EtOA_C$) to yield the product (50 %, 3.78 g) as yellowish oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 2.59 (t, J = 6.0 Hz, 6H), 3.41 (s, 6H), 3.66 (t, J = 6.0 Hz, 6H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 18.70, 55.94, 65.59, 72.38, 117.94. **ESI-MS:** m/z 281.20 $[M+H]^+$, 303.20 $[M+Na]^+$, 561.46 $[2M+H]^+$, 583.46 $[2M+Na]^+$.



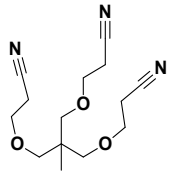
3

Compound 3: benzyl (1,3-bis(2-cyanoethoxy)-2-((2-cyanoethoxy)methyl)propan-2-yl)carbamate. Compound **2** (1.00 g, 3.56 mmol) was suspended in dioxane (10 mL), in parallel $NaHCO_3$ (0.29 g, 7.12 mmol) was dissolved in water (3 mL) and added to the dioxane mixture with stirring. The reaction mixture was cooled to 0 °C, and benzyl chloroformate (0.76 mL, 5.34 mmol) was added dropwise. The resulting mixture was left to stir at 0 °C for additional hour and allowed to warm to r.t. overnight. Water was then added and the aqueous layers extracted twice with EtOAc, and once with saturated $NaHCO_3$ solution. The solvent was dried *in vacuo* to provide the product (100%, 1.50 g) as yellowish oil that was used without further purification. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 2.57 (t, J = 6.0 Hz, 6H), 3.67 (t, J = 6.0 Hz, 6H), 3.79 (s, 6H), 5.06 (s, 2H), 5.14 (s,1H), 7.37 (bs, 5H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 18.81, 58.78, 65.75, 66.56, 69.24, 117.92, 128.21, 128.54, 136.41, 155.07, 203.58.



6

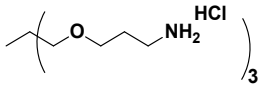
Compound 6[2]**:** Compound **5** (0.93 g, 1.56 mmol) was suspended in dioxane (15 mL), and the mixture was cooled to 0 °C, in parallel allyl chloroformate (0.41 mL, 3.92 mmol) was dissolved in of 4 M NaOH (10 mL), and the resulting solution was added to compound (5) in small portions. Each addition was followed with vigorous stirring. After the additions were finished, the pH was adjusted to 10 with 4 M NaOH. The mixture was stirred at r.t. overnight, than diluted with water, and extracted twice with EtOAc. The residue was purified by silica gel chromatography (gradient, 3:1 DCM: $EtOA_C$→1:1 DCM: $EtOA_C$) to yield the product (40%, 0.42 g) as yellowish oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.38 (s, 27H), 1.63-1.71 (m, 6H), 3.16 (bt, J = 6.1 Hz, 6H), 3.43 (t, J = 5.6 Hz, 6H), 3.60 (s, 6H), 4.45 (d, J = 5.6 Hz, 2H), 4.99 (bs, 1H), 5.09-5.34 (m, 2H), 5.78-5.90 (m, 1H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 28.39, 29.52, 38.22, 58.59, 65.59, 69.47, 69.96, 78.83, 117.52, 132.91, 155.09, 155.96. **ESI-MS:** m/z, 699.53 $[M+Na]^+$.



9

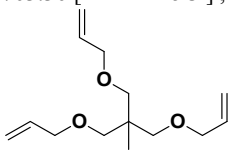
Compound 9[3]**: 3,3'-((2-((2-cyanoethoxy)methyl)-2-methylpropane-1,3-diyl)bis(oxy))-dipropanenitrile.** 7.5 M NaOH (3.5 mL) solution was added by portions to a 1,1,1-tris(hydroxymethyl)ethane **8** (6.00 g, 0.49 mol). After the addition was completed,

acrylonitrile (13.00 g, 0.245 mol) was added to the reaction mixture at 0 °C, than the temperature was raised and the mixture was stirred at r.t. overnight, followed by neutralization with 0.5 M citric acid solution till neutral pH. The aqueous solution was extracted twice by $EtOA_C$. The extractions were dried over Na_2SO_4 and the solvent was removed *in vacuo* to give yellowish oil. The oil was dissolved in Et_2O and washed several times with 1 M NaOH and once with 0.5 M citric acid. The organic layer was dried over Na_2SO_4 and evaporated to yield the product (75%, 10.40 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.98 (s, 3H), 2.60 (t, J = 6.0 Hz, 6H), 3.37 (s, 6H), 3.65 (t, J = 6.0 Hz, 6H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.17, 18.68, 40.84, 65.57, 72.88, 118.09. **ESI-MS:** m/z 302.19 $[M+Na]^+$, 581.43 $[2M+Na]^+$.



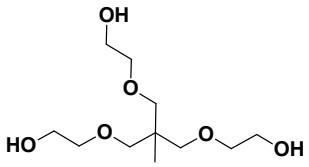
11

Compound 11[4]: 3,3'-((2-((3-aminopropoxy)methyl)-2-methylpropane-1,3-diyl)bis(oxy))bis(propan-1-amine) trihydrochloride. A solution of 4 N HCl in dioxane (4 mL, 16.0 mmol) was added to Boc protected compound **10** (0.95 g, 1.62 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 hours and then was left to stir overnight at r.t. The solvent was removed *in vacuo* to give the product (100%) as yellowish solid. **^{1}H NMR** (300 MHz, DMSO-d6): δ 0.85 (s,3H), 1.74-1.83 (m, 6H), 2.78-2.84 (m, 6H), 3.21 (s,6H), 3.42 (t, J =5.9 Hz, 6H), 8.12 (bs, 9H). **^{13}C NMR** (300 MHz, DMSO-d6): δ 17.31, 26.90, 36.20, 40.14, 67.51, 72.69. **ESI-MS:** m/z, 292.29 $[M+H]^+$, 146.55 $[M+2H]^{2+}$, 362.18 $[M+H+2Cl]^-$, 398.19 $[M+2H+3Cl]^-$, 727.49 $[2M+3H+4Cl]^-$, 763.36 $[2M+4H+5Cl]^-$, 799.50 $[2M+5H+6Cl]^-$, 835.31 $[2M+6H+7Cl]^-$, 859.33 $[2M+5H+7Cl+Na]^-$.



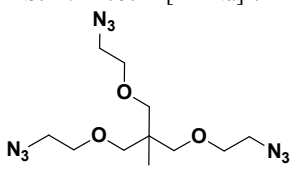
12

Compound 12[5]: 3-(3-(allyloxy)-2-((allyloxy)methyl)-2-methylpropoxy)prop-1-ene. Allyl bromide (10 mL, 0.116 mol) was added to a solution of 1,1,1-tris(hydroxymethyl)ethane **8** (3.00 g, 24.9 mmol) in anhydrous DMF (100 mL). The solution was cooled to 0 °C and sodium hydride (60%, 4.63 g, 0.193 mol) was added to the solution in small portions. The reaction mixture was left to stir overnight at r.t., then poured into water and the product was extracted by $EtOA_C$. The extract was dried over Na_2SO_4 and the solvent was removed *in vacuo* to yield the crude residue. The residue was purified by silica gel chromatography (gradient, DCM →$EtOA_C$) to yield the product (60%, 3.50 g) as yellowish oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.96 (s, 3H), 3.31 (s, 6H), 3.95 (dt, J_1 = 5.3 Hz, J_2 = 1.5 Hz, 6H), 5.10-5.28 (m, 6H), 5.80-5.93 (m, 3H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.57, 40.84, 72.24, 73.06, 116.13, 135.22. **ESI-MS:** m/z 241.18 $[M+H]^+$, 263.19 $[M+Na]^+$.



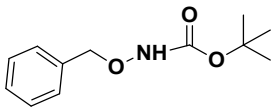
13

Compound 13[5]: 2,2'-((2-((2-hydroxyethoxy)methyl)-2-methylpropane-1,3-diyl)bis(oxy))diethanol. Ozone was bubbled through a solution of **12** (1.30 g, 5.41 mmol) in DCM: MeOH (1:1, 80 mL) at -78 °C until the solution was saturated. Argon was bubbled through the solution till the disappearance of the bluish color, and thiourea was added to the reaction mixture in excess. After the reaction mixture reached r.t., the solvent was removed *in vacuo* and the residue was suspended in EtOH (80 mL). The solution was cooled to 0 °C and sodium borohydride (1.00 g, 26.43 mmol) was added in small portions. The mixture was stirred at r.t. overnight, removal of EtOH provide the crude product, which was purified by silica gel chromatography (gradient, $EtOA_C$ → 5% MeOH in $EtOA_C$) to yield the product (70%, 0.95 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 0.95 (s, 3H), 3.37 (s, 6H), 3.49 (t, J = 4.7 Hz, 6H), 3.65 (t, J = 4.7 Hz, 6H), **^{13}C NMR** (300 MHz, MeOD): δ 16.67, 40.77, 60.84, 72.67, 73.48. **ESI-MS:** m/z 275.11 $[M+Na]^+$.



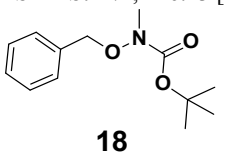
14

Compound 14[6]**: 1,3-bis(2-azidoethoxy)-2-((2-azidoethoxy)methyl)-2-methylpropane**. Diphenyl phosphorazide was added under inert atmosphere to a solution of **13** (0.25 g, 0.99 mmol) in anhydrous DMF (2 mL). The solution was cooled to 0 °C and DBU (0.6 mL, 3.96 mmol) was added to the reaction mixture. The reaction mixture was left to stir for 5 hours at 50 °C, and then overnight at r.t. The mixture was poured into water and the product was extracted by hexane. The extract was dried over Na_2SO_4 and the solvent was removed in vacuo to yield the crude residue. The residue was purified by silica gel chromatography using DCM to yield the product (90%, 0.29 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.01 (s, 3H), 3.31 (t, J = 4.6 Hz, 6H), 3.39 (s, 6H), 3.62 (t, J= 4.6 Hz, 6H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.19, 40.54, 50.79, 70.50, 73.50. **ESI-MS:** m/z, 350.13 $[M+Na]^+$, 675.00$[2M+Na]^+$, 1000.92$[3M+Na]^+$.



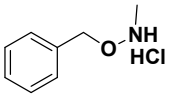
17

Compound 17[7]**: tert-butyl benzyloxycarbamate.** O-Benzylhydroxylamine hydrochloride **16** (6.38 g, 0.040 mol) was suspended in THF/H_2O (1:1, 80 mL) and treated with triethylamine (5.49 mL, 0.040 mol). Di-*tert*-butyl dicarbonate (8.72 g, 0.040 mol) dissolved in THF (20 mL) was added dropwise while stirring for 30 min. After the addition the reaction mixture was stirred at r.t. for additional 3 hours. The mixture was concentrated *in vacuo*. The residue was suspended in EtOAc and washed twice with 0.5 M citric acid and once with water. The organic layer was dried over $MgSO_4$. After evaporation of EtOAc the residue was crystallized from hexane to afford the product (83%, 7.40 g) as colorless crystals. **^{1}H NMR** (250 MHz, $CDCl_3$): δ 1.47 (s, 9H), 4.85 (s, 2H), 7.09 (s, 1H),7.33-7.39 (m, 5H). **^{13}C NMR** (250 MHz, $CDCl_3$): δ 28.13, 78.38, 81.67, 128.42, 129.07, 135.65, 156.66. **ESI-MS:** m/z, 246.13 $[M+Na]^+$, 469.33 $[2M+Na]^+$.



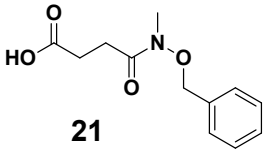
18

Compound 18[8]: **tert-butyl benzyloxy(methyl)carbamate.** Compound **17** (7.40 g, 0.035 mol) was dissolved in anhydrous DMF (50 mL). Sodium hydride (1.57 g, 0.039 mol) was added to the solution in small portions, and the reaction was stirred at r.t for 30 min. After 30 min, methyl iodide (2.45 mL, 0.039 mol) was added by syringe, under inert atmosphere. The mixture was left to stir at r.t. overnight, then poured into water and the product was extracted by hexane. The hexane extract was dried over $MgSO_4$ and concentrated *in vacuo* to give the product (87%, 6.85 g) as yellowish oil. **^{1}H-NMR** (250 MHz, $CDCl_3$): δ 1.49 (s, 9H), 3.04 (s, 3H), 4.82 (s, 2H), 7.30-7.44 (m, 5H). **^{13}C-NMR** (250 MHz, $CDCl_3$): δ 28.18, 36.69, 76.37, 81.10, 128.30, 128.37, 129.33, 135.53, 156.88. **ESI-MS:** m/z, 238.13 $[M+H]^+$, 260.14 $[M+Na]^+$, 497.36 $[2M+Na]^+$.



19

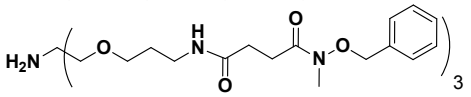
Compound 19[4]**: O-benzyl-N-methylhydroxylamine.** A solution of 4 N HCl in dioxane (21 mL, 0.084 mol) was added to compound **18** (6.80 g, 0.028 mol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 hours and then was left to stir overnight at r.t. The solvent was evaporated and the residue was suspended in a minimal amount of Et_2O. The suspension was heated to boiling and THF was slowly added until the solids dissolved. The reaction mixture was allowed to cool at r.t. and first crystals appeared. Et_2O was slowly poured into the mixture, causing a massive separation of white crystals. The crystals were filtered off and washed with cold Et_2O. The desired product was obtained (75%, 3.68 g), as white crystals. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 2.97 (s, 3H), 5.29 (s, 2H), 7.26-7.44 (m, 5H). **^{13}C NMR** (400 MHz, $CDCl_3$): δ 35.44, 76.59, 128.80, 129.54, 129.62, 132.37. **ESI-MS:** m/z, 138.05 $[M+H]^+$, 160.00 $[M+Na]^+$.



21

Compound 21: 4-((benzyloxy)(methyl)amino)-4-oxobutanoic acid. To a stirred solution of compound **19** (1.60 g, 9.24 mmol) in dioxane (20 mL) was added succinic anhydride (20) (2.77 g, 27.72 mmol), the pH of the solution was adjusted with K_2CO_3 (2.55 g, 18.48 mmol) and the solution was left stirring overnight at r.t. After the reaction finished the solution was poured into water and extracted twice by $EtOA_C$. The combined organic extracts were washed 0.5 M citric acid, and dried over Na_2SO_4. The solvent was removed *in vacuo* to give the crude residue, that was purified by silica gel chromatography (gradient, DCM →2:3

DCM: $EtOA_C$) to yield the product (75%, 1.63 g) as white solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 2.66 (d, J = 6.0 Hz, 2H) 2.72 (d, J = 5.9 Hz, 2H), 3.23 (s, 3H), 4.88 (s, 2H), 7.40 (s, 5H).**^{13}C NMR** (300 MHz, $CDCl_3$): δ 27.10, 28.69, 33.65, 76.29, 128.78, 129.10, 129.34, 134.28, 173.57, 177.66. **ESI-MS:** m/z, 238.13 $[M+H]^+$, 260.14 $[M+Na]^+$.

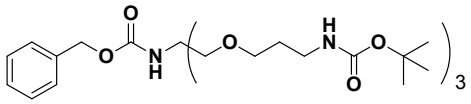


23

Compound 23: Compound **22** (0.10 g, 0.1 mmol) was dissolved in anhydrous DCM (6 mL), phenylsilane (0.024 mL, 0.2 mmol) was added to the mixture. The solution was degassed by bubbling argon through the solvent. After 5 min $Pd(PPh_3)_4$ catalyst (0.003 g, 0.003 mmol) was added, under a strong flow of argon. After the addition, the argon flow was stopped, and the flask was covered with aluminum foil. The reaction mixture was stirred for 3 hours at r.t., under inert atmosphere. After the reaction was finished, the solvent was concentrated *in vacuo* to provide the crude residue, which were purified by silica gel chromatography (gradient, DCM → 9:1 DCM: MeOH). The product (90%, 0.086 g) was obtained as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.70-1.78 (m, 6H), 2.45 (t, J = 6.7 Hz, 6H), 2.77 (bt, J = 6.3 Hz, 6H), 3.17 (s, 9H), 3.27-3.33 (m, 12H), 3.49 (t, J = 5.8 Hz, 6H), 4.86 (s, 6H), 6.71 (bs, 3H), 7.37 (s, 15H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 27.72, 29.01, 30.48, 33.72, 37.13, 56.88, 69.63, 72.16, 76.25, 128.63, 128.87, 129.19, 134.36, 172.17, 174.10. **ESI-MS:** m/z, 950.66 $[M+H]^+$, 972.66 $[M+Na]^+$, 486.90 $[M+H+Na]^{2+}$.

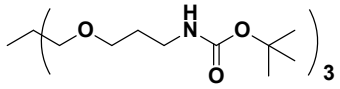
General method for nitrile reduction[9]:

Cobalt (II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) was added to solution of trinitrile **3** or **9** at MeOH. Boc anhydride was added to the solution and the reaction mixture was cooled to 0 °C. Sodium borohydride ($NaBH_4$) was added in small portions, while evolution of hydrogen gas was observed, with appearance of black precipitation. The mixture was stirred at r.t. overnight, MeOH was removed *in vacuo* and the black precipitation was suspended in $EtOA_C$ and filtered off. The organic solution was extracted twice with 1 N $NaHCO_3$ and once with brine. The solution was dried over Na_2SO_4 and evaporated *in vacuo* to give the crude residue.



4

Compound 4: Following general procedure**,** $CoCl_2 \cdot 6H_2O$ (3.43 g, 14.4 mmol), trinitrile **3** (1.50 g, 3.6 mmol), Boc anhydride (3.14 g, 14.4 mmol), $NaBH_4$ (2.80 g, 74 mmol) were stirred in MeOH (40 mL) overnight. The crude residue was purified by silica gel flash chromatography (DCM→ acetone) to give the product (70%, 1.87 g), as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.43 (s, 27H), 1.67-1.75 (m, 6H), 3.15-3.22 (m, 6H), 3.49 (t, J = 5.7 Hz, 6H), 3.66 (s, 6H), 4.94(bs, 3H), 5.05 (s, 2H), 5.36 (bs, 1H), 7.43-7.28 (m, 5H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 28.64, 29.55, 38.27, 58.67, 66.28, 69.54, 69.99, 78.87, 128.03, 128.10, 128.47, 136.98, 155.25, 155.99.

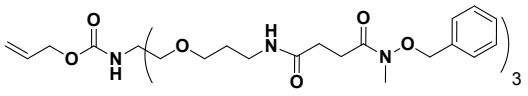


10

Compound 10: Following general procedure, $CoCl_2 \cdot 6H_2O$ (14.27 g, 0.060 mol), trinitrile **9** (5.65 g, 0.020 mol), Boc anhydride (17.82 g, 0.080 mol), $NaBH_4$ (19.68 g, 0.520 mol) were stirred in MeOH (120 mL) overnight. The crude residue was purified by silica gel flash chromatography ($EtOA_C$: hexane 55:45) to yield the product (55%, 6.50 g), as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.82 (s, 3H), 1.31 (s, 27H), 1.61-1.67 (m, 6H), 3.08-3.15 (m, 12H), 3.36 (t, J = 5.6 Hz, 6H), 5.08 (bs, 3H). **^{13}C NMR** (300 MHz, DMSO-d6): δ 17.25, 28.12, 29.46, 37.28, 40.39, 68.50, 72.81, 77.27, 155.43. **ESI-MS:** m/z, 614.33 $[M+Na]^+$, 630.31$[M+K]^+$.

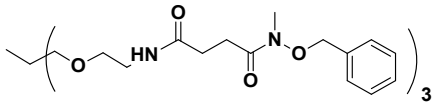
General method for coupling of amino acid carboxylate to triamine via DIC:

The required carboxylic acid was suspended in a solution of anhydrous DCM: DMF (1:1 ratio). The solution was cooled to 0 °C, HOAt and DIC were added. After stirring the suspension for 5 minutes, the appropriate amine/ hydroxylamine was added and the pH of the reaction mixture was adjusted to 9 using iPr_2NEt. The reaction was left to stir at r.t. overnight. After completing the reaction, the solvent was removed *in vacuo* and the residue was suspended in EtOAc, and washed twice with saturated $NaHCO_3$ solution. The organic extract was dried over Na_2SO_4 and evaporated *in vacuo* to yield the crude products, which were purified by silica gel chromatography (gradient, DCM → acetone).



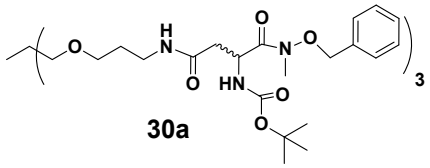
22

Compound 22: Following general procedure, triamine **7** (3.00 g, 4.28 mmol), compound **21** (3.65 g, 15 mmol), DIC (2.15 g, 17 mmol), HOAt (0.11 g, 0.8 mmol), iPr_2NEt (till basic pH) were stirred in DCM: DMF (1:1, 100 mL) overnight. The crude residue was purified by silica gel chromatography (gradient, DCM → acetone) to give the product (30%, 1.20 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.69-1.77 (m, 6H), 2.45 (t, J = 6.7 Hz, 6H), 2.76 (bt, J = 6.3 Hz, 6H), 3.17 (s, 9H), 3.26-3.32 (m, 6H), 3.49 (t, J= 5.8 Hz, 6H), 3.65 (s, 6H), 4.48 (d, J = 5.6 Hz, 2H), 4.87 (s, 6H), 5.14-5.30 (m, 2H), 5.57 (s, 1H), 5.80-5.93 (m, 1H), 6.64 (bt, J = 5.2 Hz, 3H), 7.39 (s, 15H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 27.84, 29.11, 30.59, 33.64, 37.30, 58.72, 65.22, 69.74, 70.09, 76.36, 117.70, 128.70, 128.94, 129.27, 133.01, 134.44, 155.23, 172.19, 174.12. **ESI-MS:** m/z, 1056.69 $[M+Na]^+$, 539.84 $[M+2Na]^{2+}$.



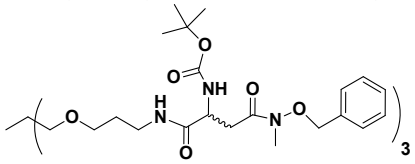
25

Compound 25: Following general procedure, triamine **15** (0.04 g, 0.168 mmol), compound **21** (0.16 g, 0.674 mmol), DIC (0.089 g, 0.708 mmol), HOAt (4 mg, 0.03 mmol), iPr_2NEt (till basic pH) were stirred in DCM: DMF (1:1, 6 mL) overnight. The crude residue was purified by silica gel chromatography (gradient, DCM → acetone) to give the product (45%, 0.065 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.93 (s, 3H), 2.49 (t, J = 6.6 Hz, 6H), 2.78 (bt, J = 6.2 Hz, 6H), 3.19 (s, 9H), 3.31 (s, 6H), 3.41-3.54 (m, 12H), 4.87 (s, 6H), 6.56 (bs, 3H), 7.37 (s, 15H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.68, 27.85, 29.35, 30.64, 39.31, 41.07, 70.07, 73.52, 76.37, 128.78, 129.03, 129.35, 134.48, 172.29, 174.19. **ESI-MS:** m/z, 929.43 $[M+Na]^+$, 476.37 $[M+2Na]^{2+}$.



30a

Compound 30a: Following general procedure, compound **29a** (0.43 g, 0.46 mmol), compound **19** (0.27 g, 1.56 mmol), DIC (0.20 g, 1.56 mmol), HOAt (12 mg, 0.09 mmol), iPr_2NEt (till basic pH) were stirred in DCM: DMF (1:1, 4 mL) overnight. The crude residue was purified by silica gel chromatography (gradient, DCM → acetone) to give the product (25%, 0.14 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.89 (s, 3H), 1.42 (s, 27H), 1.78-1.86 (m, 6H), 2.70-3.08 (m, 6H), 3.11 (s, 9H), 3.22 (s, 6H), 3.39 (t, J = 6.2 Hz, 6H), 3.60 (t, J = 7.0 Hz, 6H), 4.29 (bs, 3H), 4.74 (s, 6H), 5.63 (bs, 3H), 7.26-7.35 (m, 15H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.58, 28.15, 28.84, 33.75, 37.11, 38.53, 40.46, 48.20, 69.52, 73.42, 77.19, 79.53, 128.48, 128.76, 129.32, 133.96, 155.28, 169.13, 172.37. **ESI-MS:** m/z, 1317.15 $[M+Na]^+$, 669.93 $[M+2Na]^{2+}$.

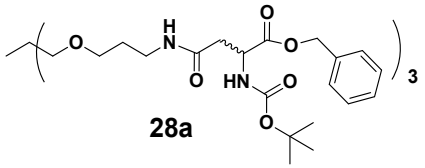


30b

Compound 30b: Following general procedure, compound **29b** (0.43 g, 0.46 mmol), compound **19** (0.27 g, 1.56 mmol), DIC (0.2 g, 1.56 mmol), HOAt (12 mg, 0.09 mmol), iPr_2NEt (till basic pH) were stirred in DCM: DMF (1:1, 4 mL) overnight. The crude residue was purified by silica gel chromatography (gradient, DCM → acetone) to give the product (35%, 0.20 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.93 (s, 3H), 1.45 (s, 27H), 1.70-1.78 (m, 6H), 2.51-2.84 (m, 6H), 3.19 (s, 9H), 3.26 (s, 6H), 3.29-3.35 (m, 6H), 3.43 (t, J = 6.0 Hz, 6H), 4.51 (bs, 3H), 4.89 (s, 6H), 6.01 (bs, 3H), 6.92 (bs, 3H), 7.39 (s, 15H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.66, 28.56, 29.47, 33.35, 34.52, 37.19, 40.86, 50.73, 69.02, 73.68, 76.36, 80.09, 128.74, 129.04, 129.27, 134.20, 155.63, 171.17, 172.91. **ESI-MS:** m/z, 1316.64 $[M+Na]^+$, 670.05 $[M+2Na]^{2+}$.

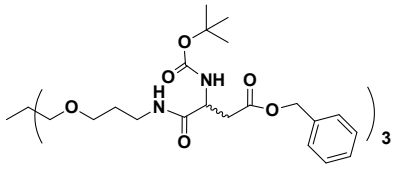
General method of coupling of amino acid carboxylate to triamine via CDI[10]:

CDI was added to a 0 °C solution of acid in THF. After 15 min at 0 °C and 30 min at r.t., the reaction mixture was cooled to 0 °C and amine was added in one portion. The pH of the reaction mixture was adjusted to 9 using iPr_2NEt, and left for stirring at r.t. overnight. After the reaction was completed, the solvent was evaporated and the residue was suspended in EtOAc and washed twice with 3 N HCl, once with solution of saturated $NaHCO_3$ and once with brine. The organic extract was dried over Na_2SO_4 and evaporated *in vacuo* to yield the crude product, which were purified by silica gel chromatography.



28a

Compound 28a (L/D isomers): Following general procedure, protected amino acid: L/D-t-Boc -aspartic acid α-benzyl ester **27a** (0.95 g, 2.97 mmol), triamine **11** (0.29 g, 0.74 mmol), CDI (0.49 g, 3.05 mmol), iPr_2NEt (till basic pH) were stirred in THF (3 mL) overnight. The crude product was purified by silica gel chromatography (gradient, DCM → 2:8 DCM: EtOAc) to yield the product (0.40 g, 45%) as white solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.88 (s, 3H), 1.38 (s, 27H), 1.64-1.72 (m, 6H), 2.51-2.88 (m, 6H), 3.21-3.28 (m, 12H), 3.41 (t, J = 5.5Hz, 6H), 4.49-4.52 (m, 3H), 5.12 5.17 (ABq, J_1 = 12.4 Hz, J_2=3.5 Hz 6H,), 5.85 (bs, 3H), 6.40 (bs, 3H), 7.30 (s, 15H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.87, 28.26, 28.94, 37.76, 40.59, 50.57, 67.19, 70.02, 73.85, 79.86, 128.02, 128.18, 128.45, 135.47, 155.69, 169.67, 171.47. **ESI-MS:** m/z, 1229.57 $[M+Na]^+$, 1245.54 $[M+K]^+$, 626.44 $[M+2Na]^{2+}$.

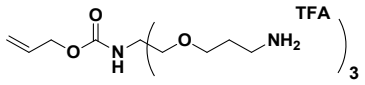


28b

Compound 28b (L/D isomers): Following general procedure, protected amino acid: L/D- t-Boc -aspartic acid β-benzyl ester **27b** (0.95 g, 2.97 mmol), compound triamine **11** (0.29 g, 0.74 mmol), CDI (0.49 g, 3.05 mmol), iPr_2NEt (till basic pH) were stirred in THF (3 mL) overnight. The crude product was purified by silica gel chromatography (gradient, 3:7 ACN: DCM → 1:1 ACN: DCM) to yield the product (0.26 g, 30%) as white solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.92 (s, 3H), 1.43 (s, 27H), 1.67-1.72 (m, 6H), 2.71-3.00 (m, 6H), 3.35-3.19 (m, 12H), 3.41 (t, J = 5.9 Hz, 6H), 4.49 (bs, 3H), 5.08, 5.13 (ABq, J_1= 9 Hz, J_2=3 Hz 6H), 5.74 (bs, 3H), 6.74 (bs, 3H), 7.33 (s, 15H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.48, 28.07, 29.02, 36.24, 37.03, 40.52, 50.59, 66.40, 68.78, 73.32, 79.96, 127.95, 128.06, 128.31, 135.26, 155.31, 170.47, 171.17. **ESI-MS:** m/z, 1229.57 $[M+Na]^+$, 1245.48 $[M+K]^+$, 626.57 $[M+2Na]^{2+}$.

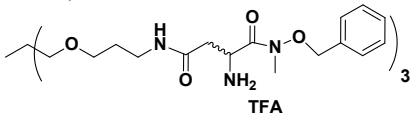
<u>General method for removal of *t*-butyl by TFA</u>:

Boc protected compound was cooled to 0 °C, and dissolved in 1:1 DCM: TFA solution, followed by addition of a drop of Triisopropylsilane. The reaction mixture was stirred at r.t. overnight; the solvent was removed *in vacuo* to yield the product.



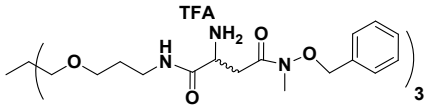
7

Compound 7: Following general procedure, compound **6** (0.34 g, 0.51 mmol) was dissolved in DCM: TFA (1:1, 4 mL). The product was obtained (100%, 0.36 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 1.87-1.95 (m, 6H), 3.03 (t, J = 7.1 Hz, 6H), 3.56 (t, J = 5.7 Hz, 6H), 3.66 (s, 6H), 4.49 (d, J = 5.4 Hz, 2H), 5.17-5.32 (m, 2H), 5.86-5.99 (m, 1H) **^{13}C NMR** (300 MHz, MeOD): δ 28.47, 39.09, 60.10, 66.25, 70.11, 71.61, 117.74, 134.44, 157.53. **ESI-MS:** m/z, 377.33 $[M+H]^+$, 399.33 $[M+Na]^+$.



31a

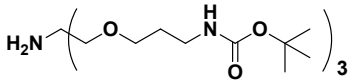
Compound 31a (L/D isomers): Following general procedure, compound **30a** (0.25 g, 0.19 mmol) was dissolved in DCM: TFA (2 mL). The product was obtained (100%, 0.25 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 0.93 (s, 3H), 1.70-1.74 (m, 6H), 2.60-2.83 (m, 6H), 3.21-3.27 (m, 12H), 3.32 (s, 9H), 3.41 (t, J = 6.1 Hz, 6H), 4.52 (dd, J_1 = 8.83, J_2=3.40 Hz, 3H), 4.95, 5.08 (ABq, J_1=25.6 Hz, J_2= 10.3 Hz, 6H), 7.41-7.46 (m, 15H). **^{13}C NMR** (300 MHz, MeOD): δ 18.06, 30.39, 34.04, 35.19, 38.01, 41.93, 50.14, 69.92, 74.65, 77.71, 129.95, 130.41, 130.86, 135.32, 169.75, 170.34. **ESI-MS:** m/z, 994.27 $[M+H]^+$, 497.87 $[M+2H]^{2+}$,332.24 $[M+3H]^{3+}$.



31b

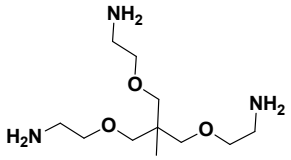
Compound 31b (L/D isomers): Following general procedure, compound **30b** (0.25 g, 0.19 mmol) was dissolved in DCM: TFA (2 mL). The product was obtained (100%, 0.25 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 0.93 (s, 3H), 1.73-1.77 (m, 6H), 2.92-3.01 (m, 6H), 3.26 (s, 9H), 3.29 (s, 6H), 3.31-3.33 (m, 6H), 3.43 (t, J = 5.9 Hz, 6H), 4.16 (t, J = 5.9, 3H), 4.94 (s, 6H), 7.40-7.45(m, 15H). **^{13}C NMR** (300 MHz, MeOD): δ 18.08, 30.34, 33.71, 34.75, 38.14, 41.95, 50.94, 69.71, 74.66, 77.39, 129.83, 130.23, 130.74, 135.76, 169.41, 171.80. **ESI-MS:** m/z, 994.33 $[M+H]^+$, 1016.26$[M+Na]^+$, 1032.17 $[M+K]^+$, 497.87 $[M+2H]^{2+}$,508.90 $[M+H+Na]^{2+}$, 519.87 $[M+2Na]^{2+}$.

<u>General method for hydrogenolysis:</u>
10% Pd on carbon catalyst was added to a solution of required compound in either MeOH or EtOH. The reaction was stirred overnight under 1 atmosphere (atm) of H_2, followed by filtration of the catalyst and evaporation of the solvent.



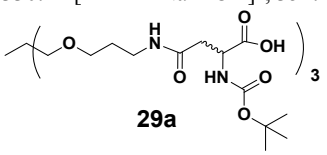
5

Compound 5: Following general procedure, 10% Pd on carbon catalyst (30 mg) and compound **4** (1.00 g, 1.37 mmol) were dissolved in EtOH (50 mL). The reaction was stirred for 2 days under 1 atm of H_2. The product was obtained (100%, 0.81 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 1.45 (s, 18H), 1.70-1.78 (m, 6H), 3.14 (t, J= 6.3, 6H), 3.43 (s, 6H), 3.50 (t, J=5.9, 6H). **^{13}C NMR** (300 MHz, MeOD): δ 27.50, 29.53, 37.14, 57.17, 68.65, 70.88, 78.50, 157.11. **ESI-MS:** m/z, 593.54 $[M+H]^+$, 615.53 $[M+Na]^+$.



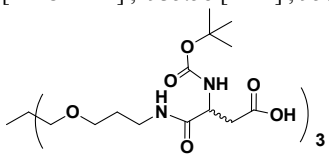
15

Compound 15: 2,2'-((2-((2-aminoethoxy)methyl)-2-methylpropane-1,3-diyl)bis(oxy)diethanamine. Following general procedure, 10% Pd on carbon catalyst (30 mg) and compound **14** (0.30 g, 0.91 mmol) were dissolved in EtOH (15 mL). The reaction was stirred for 2 days under 1 atm of H_2. The product was obtained (100%, 0.23 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 0.98 (s, 3H), 2.79 (t, J=5.3 Hz, 6H), 3.36 (s, 6H), 3.46 (t, J = 5.3 Hz, 6H). **^{13}C NMR** (300 MHz, MeOD): δ 18.10, 42.05, 42.08, 73.44, 74.66. **ESI-MS:** m/z, 250.18 $[M+H]^+$, 272.19 $[M+Na]^+$, 290.20 $[M+H+Na+OH]^+$, 312.21 $[M+2Na+OH]^+$, 330.22 $[M+H+2Na+2OH]^+$, 352.22 $[M+3Na+2OH]^+$, 370.23 $[M+H+3Na+3OH]^+$, 392.23 $[M+4Na+3OH]^+$.



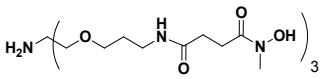
29a

Compound 29a (L/D isomers): Following general procedure, 10% Pd on carbon catalyst (10 mg) and compound **28a** (0.30 g, 0.24 mmol) were dissolved in MeOH (20 mL). The reaction was stirred for 24 hours under 1 atm of H_2. The product was obtained (100%, 0.23 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.87 (s, 3H), 1.39 (s, 27H), 1.77-1.81 (m, 6H), 2.69-3.05 (m, 6H), 3.19 (s, 6H), 3.36 (t, J = 5.8 Hz, 6H), 3.56 (t, J = 6.9 Hz, 6H), 4.26 (bs, 3H), 5.77 (bs, 3H), 7.36 (bs, 3H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.65, 28.15, 28.71, 37.49, 37.69, 40.53, 50.29, 69.29, 73.67, 80.05, 155.81, 170.97, 173.31. **ESI-MS:** m/z, 917.58 $[M+OH-2H]^-$, 935.58 $[M-H]^-$, 957.51 $[M+Na-2H]^-$, 973.48 $[M+Na+OH-3H]^-$, 979.50 $[M+2Na-3H]^-$, 995.60 $[M+2Na+OH-4H]^-$.



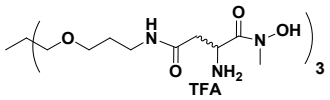
29b

Compound 29b (L/D isomers): Following general procedure, 10% Pd on carbon catalyst (10 mg) and compound **28a** (0.30 g, 0.24 mmol) were dissolved in MeOH (20 mL). The reaction was stirred for 24 hours under 1 atm of H_2. The product was obtained (100%, 0.23 g) as colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.90 (s, 3H), 1.43 (s, 27H), 1.72-1.76 (m, 6H), 2.66-2.97 (m, 6H), 3.23-3.33(m, 12H), 3.44-3.46 (m, 6H), 4.48 (bs, 3H), 5.90 (bs, 3H), 7.06 (bs, 3H). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 17.64, 28.18, 28.85, 36.45, 37.19, 40.63, 50.68, 68.90, 73.54, 80.38, 155.64, 171.39, 173.83. **ESI-MS:** m/z, 959.47 $[M+Na]^+$, 491.27 $[M+2Na]^{2+}$, 917.58 $[M+OH-2H]^-$, 935.45 $[M-H]^-$, 957.45 $[M+Na-2H]^-$.



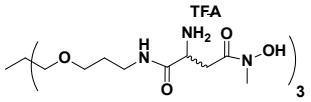
T1

Compound T1: Following general procedure, 10% Pd on carbon catalyst (20 mg) and compound **23** (0.40 g, 0.42 mmol) were dissolved in EtOH (40 mL). The reaction was stirred for 4 hours under 1 atm of H_2. The product was obtained (100%, 0.28 g) as colorless oil. **^{1}H NMR** (400 MHz, MeOD): δ 1.3-1.78 (m, 6H), 2.44 (t, J = 7.0 Hz, 6H), 2.76 (t, J = 7.0 Hz, 6H), 3.18 (s, 9H), 3.26 (t, J = 6.8 Hz, 6H), 3.44 (s, 6H), 3.49 (t, J = 5.9 Hz, 6H). **DEPTQ NMR** (400 MHz, MeOD): 28.74, 30.39, 31.48, 36.38, 37.60, 58.61, 70.01, 72.24, 174.58, 174.98. **HRMS**: m/z, $C_{28}H_{53}N_7O_{12}$, required 679.3752, found 680.3826 $[M+H]^+$, 702.3635 $[M+Na]^+$.



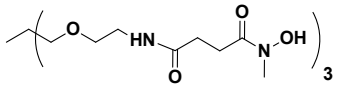
T2

Compound T2 (L/D isomers):: Following general procedure, 10% Pd on carbon catalyst (5 mg) and compound **31a** (0.10 g, 0.07 mmol) were dissolved in MeOH (10 mL). The reaction was stirred for 24 hours under 1 atm of H_2. The product was obtained (100%, 0.08 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 0.92 (s, 3H), 1.72-1.80 (m, 6H), 2.67-2.98 (m, 6H), 3.26-3.27 (m, 15H), 3.43 (t, J = 6.0 Hz, 6H), 4.67 (dd, J_1 = 8.95, J_2= 3.18 Hz, 3H). **^{13}C NMR** (300 MHz, MeOD): δ 18.01, 30.40, 35.17, 36.68, 38.04, 41.94, 49.78, 69.95, 74.67, 168.81, 170.79. **HRMS**: m/z, $C_{29}H_{57}N_9O_{12}$, required 723.4127, found 746.4030 $[M+Na]^+$.



T3

Compound T3 (L/D isomers):: Following general procedure, 10% Pd on carbon catalyst (5 mg) and compound **31b** (0.10 g, 0.07 mmol) were dissolved in MeOH (10 mL). The reaction was stirred for 24 hours under 1 atm of H_2. The product was obtained (100%, 0.08 g) as colorless oil. **^{1}H NMR** (300 MHz, MeOD): δ 0.92 (s, 3H), 1.74-1.82 (m, 6H), 2.92-3.09 (m, 6H), 3.22 (s, 9H), 3.27-3.34 (m, 12H), 3.45 (t, J = 6.0 Hz, 6H), 4.16-4.20 (m, 3H). **DEPTQ-NMR** (400 MHz, MeOD): δ 18.01, 30.31, 34.53, 36.26, 38.13, 41.96, 51.10, 69.70, 74.66, 169.55, 170.67. **HRMS**: m/z, $C_{29}H_{57}N_9O_{12}$, required 723.4127, found 746.4027 $[M+Na]^+$.



T4

Compound T4: Following general procedure, 10% Pd on carbon catalyst (10 mg) and compound **25** (0.10 g, 0.11 mmol) were dissolved in EtOH (15 mL). The reaction was stirred for 3 hours under 1 atm of H_2. The product was obtained (100%, 0.070 g) as colorless oil. **^{1}H NMR** (400 MHz, MeOD): δ 0.94 (s, 3H), 2.48 (t, J = 7.1 Hz, 6H), 2.77 (t, J = 7.1 Hz, 6H), 3.20 (s, 9H), 3.32-3.36 (m, 12H), 3.48 (t, J = 5.4 Hz, 6H). **DEPTQ NMR** (400 MHz, MeOD): δ 17.83, 28.72, 31.43, 36.32, 40.50, 42.10, 70.90, 74.56, 174.54, 175.02. **HRMS:** m/z, $C_{26}H_{48}N_6O_{12}$, required 636.3330 found 659.3226 $[M+Na]^+$.

Physicochemical properties

Potentiometric titrations

The ionic strength was fixed at I = 0.1 M with $NaClO_4$. The combined glass electrode (Mettler Toledo InLab Semi micro) with polymer filling was calibrated as hydrogen concentration probe by titrating known amounts of $HClO_4$ ($1.0\cdot10^{-2}$ M) with CO_2-free NaOH solution ($1.0\cdot10^{-2}$ M).[11] A stream of Argon, pre-saturated with water vapor, was passed over the surface of the solution cell, thermostated at 25.0 ± 0.2 °C. Ligands were dissolved in water solution of 0.1M $NaClO_4$ to obtain concentration of 3.0 mM. The complexes were measured in metal to ligand molar ratio 1:1.

Competition experiments with EDTA

Spectrophotometric experiments involving Fe(III) competition equilibria with tripodal ligands and EDTA were carried out at pH of 8, in 0.1 M $NaClO_4$ (Fluka, purum p.a.). The stock solution of Fe(III)-tripodal analog, prepared in pH around 3, was divided into several aliquots to which up to a 10-fold excess of EDTA was added and pH of every sample was adjusted to 8. The samples were allowed to equilibrate for several days at 25 °C, until the equilibrium was attained. Spectra of each sample were recorded every day.

pH-dependent UV-Visible titrations

To probe the coordination properties of new ferrichrome analogs with Fe(III) UV-visible spectrophotometric experiments as a function of pH were carried out. Two series of experiments were performed: (i) in the pH range of 0.1-2, and (ii) between pH 2 and 11, but data was analyzed only up to pH 8.2 as above this pH the hydrolysis of complex occurred, causing a decrease of the intensity of spectra.

In the (i) series, the stock solution of iron complexes was divided into various batches, with constant total volume of 2 mL. Ionic strength was adjusted to 1 M with addition of $NaClO_4$, and pH was controlled by the concentration of the perchloric acid (range 0.1-2). After preparation, each solution was allowed to equilibrate for 50-60 min, and then its visible spectrum was recorded using a Varian CARY 300 UV/Vis spectrophotometer.

In the (ii) set of experiments, 15 mL of solution containing a 1:1 molar ratio of Fe(III):tripod, where ferric concentration was around 0.20 mM, were introduced into a jacketed cell (Metrohm) maintained at 25.0 ± 0.2 °C. The titration of the complex (2 < pH < 11) was carried out by addition of known volumes of $1.0 \cdot 10^{-2}$ M sodium hydroxide by the Titrando 905 titrator equipped with a combined glass electrode (Mettler Toledo InLab Semi micro) with polymer filling. Simultaneous pH and UV-visible measurements were recorded using a Varian CARY 50 UV/Vis spectrophotometer fitted with C-Technologies optical fibers and immersion probe made of quartz (C-Technologies) or metal (Hellma Ultra-Mini Immersion Probe).

Analysis and processing of the potentiometric and spectroscopic data

The potentiometric data (about 140 points collected over the pH range 2-11) were refined with the Superquad[12] or Hyperquad 2006[13] programs[14].

The successive protonation constants, defined by equations (S1) and (S2), were calculated from the cumulative constants determined with the Superquad program. For the sake of simplicity charges are omitted in all chemical equilibria given here.

$$H_{n-1}L + H \leftrightarrow H_nL \qquad \text{(S1)}$$

$$K_n^H = [H_nL]/[H_{n-1}L][H] \qquad \text{(S2)}$$

Complex formation constants, presented in Table S3 as $\log\beta[FeH_mL_n]$, and defined by equations (S3) and (S4) were determined from combination of potentiometric and UV-Vis spectrophotometric titration data.

$$Fe^{3+}_{(aq)} + L^{3-} \xleftrightarrow{K_{ML}} FeL \qquad \text{(S3)}$$

$$K_{ML} = \frac{[FeL]}{[Fe^{3+}_{(aq)}][L^{3-}]} \qquad \text{(S4)}$$

The uncertainties in the log*K* values correspond to the added standard deviations in the cumulative constants. The distribution curves of the protonated species of ligand/complexes as a function of pH were calculated using the Hyss program.[15]

All of the spectrophotometric data were analyzed using the SPECFIT/32[16-19] software program which adjusts the molar absorptivities and the stability constants of the species formed at equilibrium. SPECFIT uses factor analysis to reduce the absorbance matrix and to extract the eigenvalues prior to the multi-wavelength fit of the reduced data set according to the Marquardt algorithm[20, 21] Uncertainties in logβ and were calculated from the standard deviation.

The stability of ferric ion complexes with EDTA is of sufficient strength ($\beta_{110}^{FeEDTA} = 10^{25.0}$)[22] to compete effectively with the tris-hydroxamates for ferric ion at intermediate pH values (5-8) if an excess of EDTA is used. The competition equilibrium is described by equations (S5) and (S6).

$$Fe^{III}L + EDTA \xleftrightarrow{K} Fe^{III}EDTA + L \qquad \text{(S5)}$$

$$K = \frac{[Fe^{III}EDTA][L]}{[Fe^{III}L][EDTA]} = \frac{\beta_{110}^{FeEDTA}}{\beta_{110}^{Fe(III)L}} \qquad \text{(S6)}$$

The competition data were refined to obtain the overall Fe(III)-binding constant (log*K*) for analogs using a model involving two ligands and one metal. The protonation constants and Fe(III) formation constants for EDTA[22] and the protonation constants of the analogs (Table S1), were used as fixed parameters during data analysis.
The concentration of iron-analog complex for each derivative, FeL, was calculated from the absorbance maximum at around 420 nm (where FeL is the only light-absorbing species) and previously determined extinction coefficients. The concentrations of other species in equation (S6) were calculated from mass balance equations using the experimental pH values:

$$[Fe]_{tot} = \alpha_{FeL}[FeL] + \alpha_{FeEDTA}[FeEDTA] \qquad (S7)^a$$

$$[L]_{tot} = \alpha_{FeL}[FeL] + \alpha_{L}[L] \qquad (S8)^a$$

$$[EDTA]_{tot} = \alpha_{FeEDTA}[FeEDTA] + \alpha_{EDTA}[EDTA] \qquad (S9)^a$$

[a]α is the usual Ringbom's coefficient.[23]

Supplementary data and discussion

Ligand's protonation constants

Protonation constants of studied ligands, defined by equations (S1) and (S2), were determined by potentiometric titrations and obtained values are collected in Table S1.

Table S1. Protonation (logβ^{H}) constants for studied ligands[a]

Assignments	Compound				
	T1	T2	T3	T4	Ferrichrome[b]
$log\beta_1^H$	9.67(2)	10.11(1)	10.08(2)	9.50(1)	9.83
$log\beta_2^H$	18.55(1)	19.30(1)	19.06(2)	18.47(1)	18.83
$log\beta_3^H$	27.07(2)	28.05(1)	27.65(2)	26.73(1)	26.94
$log\beta_4^H$	34.75(2)	35.79(1)	34.93(2)		
$log\beta_5^H$		42.78(1)	41.68(3)		
$log\beta_6^H$		49.17(1)	47.71(3)		
pK_{NHOH}	9.67[c]	10.11[c]	10.08[c]	9.50	9.83
pK_{NHOH}	8.88[c]	9.19[c]	8.98[c]	8.97	9.00
pK_{NHOH}	8.52[c]	8.75[c]	8.59[c]	8.26	8.11
$pK_{NH_4^+}$	7.68[c]	7.74[c]	7.28[c]		
$pK_{NH_4^+}$		6.99[c]	6.75[c]		
$pK_{NH_4^+}$		6.39[c]	6.03[c]		

[a]Conditions: T=25˚C, I = 0.10M $NaClO_4$; [b]Anderegg et al.[24]; [c]Presumed attribution.

Tripodal analogs T1-4 possess three arms terminated by hyroxamate moieties. T1 possess additional amino group bound to apical carbon atom, and both, T2 and T3, have an amino group on each arm, nearby the hydroxamate. For T4 the protonation constants are within the scope for hydroxamates known from literature, such as natural trihydroxamate desferrichrome[24, 25], desferricrocin[26] or desferrichrysin[24], or synthetic retrohydroxamate TRENDROX[27]. The constants are a bit unevenly separated from each other with $logK_1^H - logK_2^H$ = 0.53 and $logK_2^H - logK_3^H$ = 0.71, but these values are only slightly higher than for three identical independent units (Δlog$K$ = 0.48)[28]. This suggests that any intramolecular interaction do not occur in this molecule.

Due to the presence of amino group(s) in T1, T2 and T3 analogs the protonation scheme of the ligands become more complex. The electron-withdrawing character of both, the -NH_3^+ and -NHOH groups affects the acidity of the other group in comparison with that in the related non-substituted compound T4. Because of the possible overlap of the dissociation processes, the pK values determined, cannot be ascribed unambiguously to either of the groups and only presumed assignment is presented in Table S1. As it was previously reported on the example of α- and β-alaninehydroxamic acids,[29] the amino group may be more acidic than hydroxamic one, or *vice versa*. The determination of both macro and micro constants, by ^{13}C- NMR and pH-metric studies, showed that the NH_3^+ group was more acidic than hydroxamic one for α-alaninehydroxamic acid, while the NHOH group was more acidic than the amino one for β-derivative. Thus, to thoroughly determine the protonation processes of studied compounds, the pH-dependent NMR titrations would be needed. However, such a detailed analysis is not necessary for the determination of stability of iron complexes and therefore was not performed.

Physicochemical analysis of complexes

ESI-MS measurements

The ESI-MS spectra of the reaction mixture of $Fe(ClO_4)_3$/ligand with a 1:1 ratio (Table S2) are characterized by the presence of a few major peaks, successfully attributed to the mononuclear species. Experiments were performed in methanol-water system (50:50 v/v) for better solvent evaporation.

Table S2. Intensity maxima of the major Fe(III)-L complexes and adduct ions observed by ESI-MS.

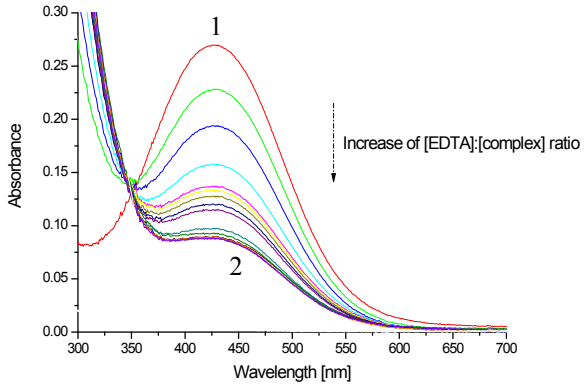
Complexes	Pseudomolcular ion	m/z experimental	m/z simulated
T1 – Fe(III)	$\{[FeL] + [H]^+\}^+$	733.30	733.29
	$\{[FeL] + [Na]^+\}^+$	755.28	755.28
T2 – Fe(III)	$\{[FeL] + [H]^+\}^+$	777.34	777.33
	$\{[FeL] + [Na]^+\}^+$	799.32	799.31
	$\{[FeL] + [H]^+ + [K]^+ + [ClO_4]^-\}^+$	915.25	915.24
T3 – Fe(III)	$\{[FeL] + [H]^+\}^+$	777.34	777.33
	$\{[FeL] + [Na]^+\}^+$	799.32	799.31
	$\{[FeL] + [H]^+ + [K]^+ + [ClO_4]^-\}^+$	915.25	915.24
	$\{[FeL] + 2[Na]^+ + [ClO_4]^-\}^+$	921.26	921.25
T4 – Fe(III)	$\{[FeL] + [H]^+\}^+$	690.26	690.25
	$\{[FeL] + [Na]^+\}^+$	712.24	712.23

All peak assignments were based on the comparison between the calculated and experimental isotope patterns and correlate very well, with the species identified by the potentiometric and UV-Vis experiments (*vide infra*).

Determination of stability constants

Stability constants of studied complexes (Table S3) were determined by EDTA titration (Figure S1), or by pH-dependent spectrophotometric and potentiometric titrations (*vide supra*). The stepwise deprotonation constants of amino groups, $pK_{NH_4^+}$, were determined by potentiometry (Table S3).

a



b

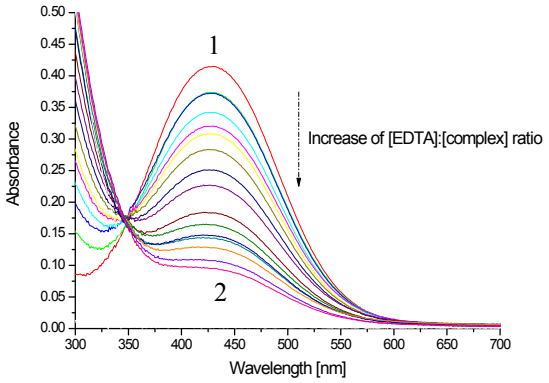


Figure S1. The UV-Vis spectra of EDTA titration of: a. Fe(III) – T1 and b. Fe(III) – T4; $c_{Fe(III)} = 1.5 \cdot 10^{-4}$ M; metal to ligand molar ratio 1:1, EDTA to metal ligand ratio from 0:1 (1) to 10:1 (2); I = 0.1 M $NaClO_4$.

The calculated values of stability constants, based on EDTA titration and pH-dependent potentiometric and spectrophotometric titration are given in Table S3.

Table S3. Stability (logβ) constants for studied complexes with iron (III) ions[a]

Assignments	Compound				
	T1	T2	T3	T4	Ferrichrome[b]
$log\beta_{FeH_4L}$		48.92(8)	48.98(3)		
$log\beta_{FeH_3L}$		45.52(3)	46.19(2)		
$log\beta_{FeH_2L}$	37.46(3)	40.58(4)	40.45(5)		
$log\beta_{FeHL}$	35.14(2)	34.71(4)	34.21(4)	29.82(3)	
$log\beta_{FeL}$	27.45(9)	27.92(6)	26.91(6)	27.34(1)	29.07
	27.96(8)[c]			27.8(1)[c]	
pK_{NHOH}	2.32(3)	3.40(8)	2.79(3)	2.48(3)	1.49
$pK_{NH_4^+}$	7.69(2)	4.94(3)	5.74(2)		
$pK_{NH_4^+}$		5.87(4)	6.24(5)		
$pK_{NH_4^+}$		6.79(4)	7.30(4)		
pFe	23.5	22.4	22.5	23.6	25.2

[a]Conditions: T=25°C, I = 0.10M $NaClO_4$; [b]Anderegg et al.[24]; [c]Constants calculated from EDTA titration. Conditions: $c_{lig} = c_{Fe(III)} = 1.5\cdot10^{-4}$M, $c_{EDTA} = 0\text{-}1.5\cdot10^{-3}$M, I = 0.10M $NaClO_4$.

The UV-visible spectra of the ferric –T4 complex as a function of pH, chosen as a representative example, are shown in Figure S2. The pH-dependent spectral profile indicates the presence of only two absorbing species within the measured pH range (0.1-8, Figure S2c). At pH = 0.1 the solution was light red, due to the LMCT band with λ_{max} at around 479 nm. With the increase of pH the band underwent slight hypsochromic shift, resulting in λ_{max}=472 nm and ε=2130 $M^{-1}cm^{-1}$ (Figure S2a, Figure S2b, Table S4). From pH around 1.5 the band shifted hypsochromically, giving an isosbestic point throughout the range of pH 1.5-8 (Figure S2b). The solution turned yellow-orange, and was characterized by λ_{max} = 430 nm and ε = 2750 $M^{-1}cm^{-1}$ (Table S4). Such values correspond to the trihydroxamate complex.

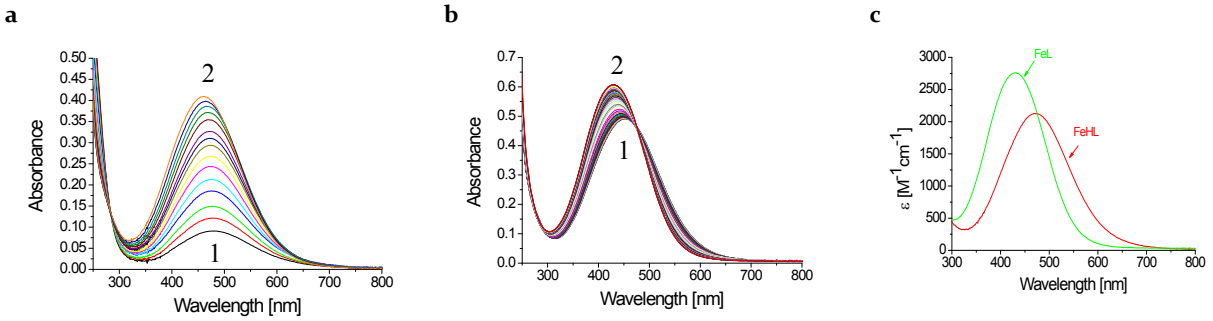


Figure S2. The UV-Vis spectra of Fe(III)-T4 titrations: a. c_{Fe} = $1.8\cdot10^{-4}$ M, pH range (1) 0.1- (2) 2.0; b. $c_{Fe(III)}$ = $2.3\cdot10^{-4}$ M (1) 2.22- (2) 8.77. c. Electronic spectra of Fe(III)-T4 complex forms; ligand to metal molar ratio 1:1; I = 0.1 M $NaClO_4$.

Table S4. Spectroscopic parameters for studied complexes[a]

Complex form	Compound							
	T1		T2		T3		T4	
	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]
FeH_4L			470	940	471	1720		
FeH_3L			430	1440	437	2180		
FeH_2L	472	2280	-	-	-	-		
FeHL	-	-	-	-	-	-	472	2130
FeL	435	2845	427	2162	431	2310	430	2750

[a]Conditions: T = 25°C, I = 1.0M $NaClO_4$ or I = 0.10M $NaClO_4$. Experimental errors: λ_{max} = ±2nm, ε = ±5%

In the UV-Vis spectra of T2 analog ferric complex (Figure S3), the starting band (λ_{max} = 475) was shifted to lower wavelength giving finally the band at λ_{max} = 470 nm and ε = 940 $M^{-1}cm^{-1}$. In the case of T3 analog (Figure S4), similar band shift was observed (from 479 to 471 nm), but the ε was higher (1720 $M^{-1}cm^{-1}$). The obtained spectroscopic data show that for T2 analog the ε values are far from the one characterizing T1 and T4 trihydroxamate species (Table S4). With further increase of pH, the absorbance of Fe(III)-T2 system increased significantly, with only slight hypsochromic shifts (ε_{427} = 2162 $M^{-1}cm^{-1}$, Table S4, Figure S3). Much lower increase of absorbance was observed for T3 ferric complex (Table S4, Figure S4). The increase of absorbance was observed as an effect of the deprotonation of $-NH_3^+$ groups, and the final parameters were assigned to FeL species (ε_{430} = 1440 $M^{-1}cm^{-1}$ for T2 and ε_{437}= 2180 $M^{-1}cm^{-1}$ for T3).

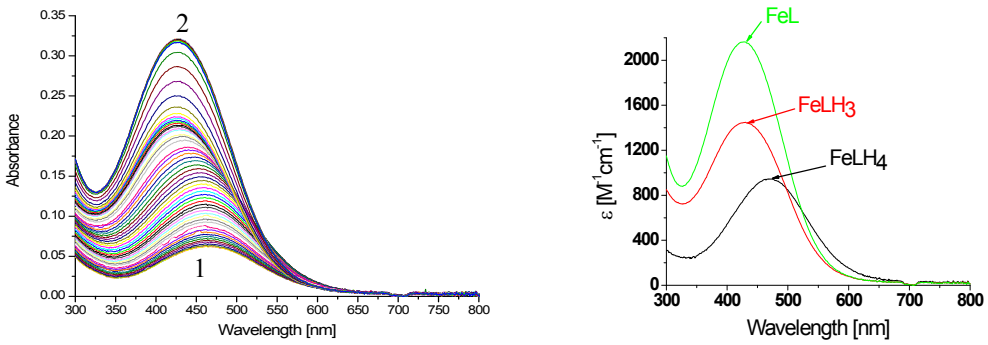


Figure S3. UV-Vis experimental spectra (left) and electronic spectra (right) for T2 analog. pH range (1) 2.11 – (2) 8.30; metal to ligand ratio 1:1; $c_{Fe(III)} = 1.7 \cdot 10^{-4}$ M; I = 0.1 M $NaClO_4$.

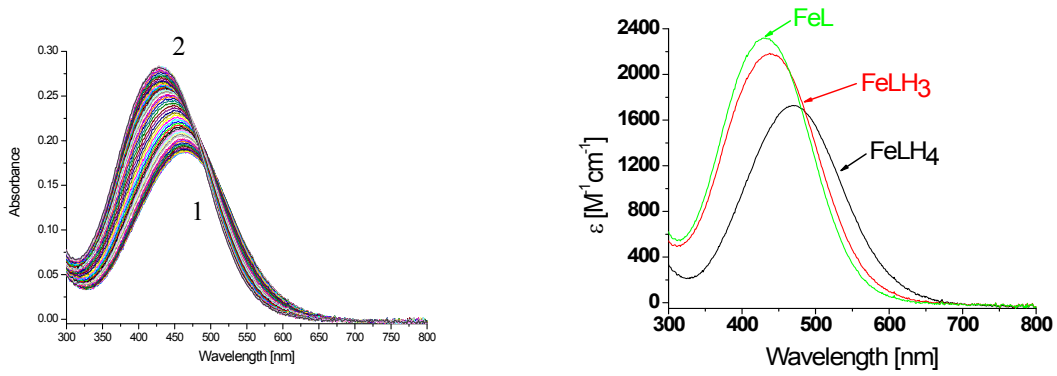


Figure S4. UV-Vis experimental spectra (left) and electronic spectra (right) for T3 analog. pH range (1) 2.03 – (2) 8.40; metal to ligand ratio 1:1; $c_{Fe(III)} = 1.3 \cdot 10^{-4}$ M; I = 0.1 M $NaClO_4$.

The pH-dependent distribution plots (Figure S5) were prepared basing on calculated stability constants for all analyzed compounds.

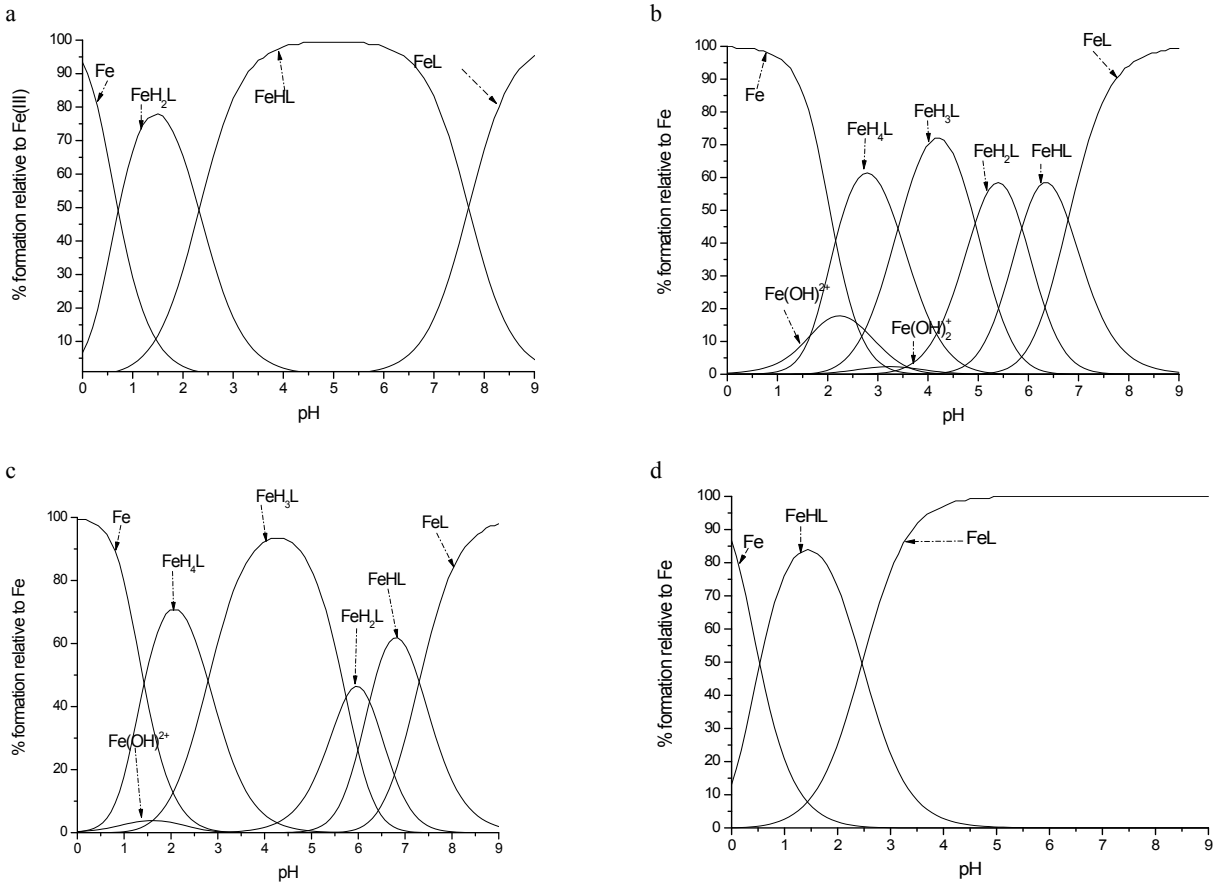
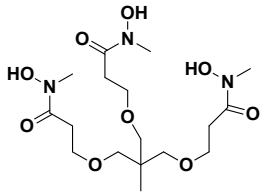
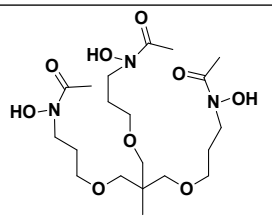
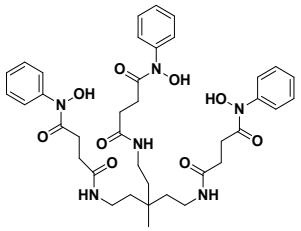
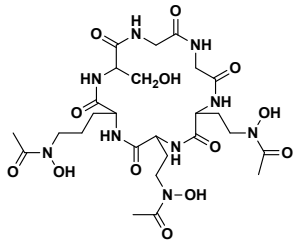
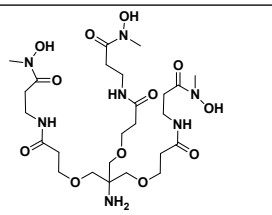
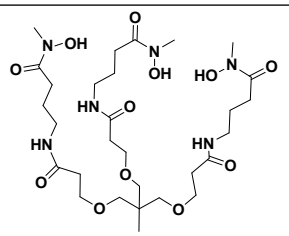


Figure S5. Plots of pH-dependent distribution of complexes: a. for Fe(III)-T1 system; b. for Fe(III)-T2 system; c. for Fe(III)-T3 system; d. for Fe(III)-T4 system; $c_{Fe(III)} = 2 \cdot 10^{-4}$ M, metal to ligand ratio 1:1.

To eliminate the differences in ligand protonation constants, which may influence logK_{FeL}, and to compare the iron chelation efficacy between the studied ligands, and with parent ferrichrome and other strong ferrichrome-based chelators, the free hexaaquairon(III) concentrations were calculated (pFe, Table S1). Some of the ferrichrome-based chelators along with their pFe values are presented in Table S5 in order to show, that the chelating properties of all studied analogs remain in the range for effective iron chelators.

Table S5. Chemical structures and pFe values of trihydroxamate siderophores analogs.

Compound	Structure	pFe
M1		24.2[30]
M2		22.4[30]
Trendrox		27.8[27]
Desferricrocin		26.5[26]
β-Ala		23.3[31]
GABA		23.7[31]

CV measurements

Concentration of Fe(III) ions was 0.5 mM and metal to ligand molar ratio was 1:1. 1.5 mL of solution were measured at pH 8 and 25˚C, in the potential range from around -0.1V to -0.8V, starting from the reduction direction.
Quasireversible voltammogram of T4 – Fe(III) complex, chosen as representative example is presented in Figure S6.

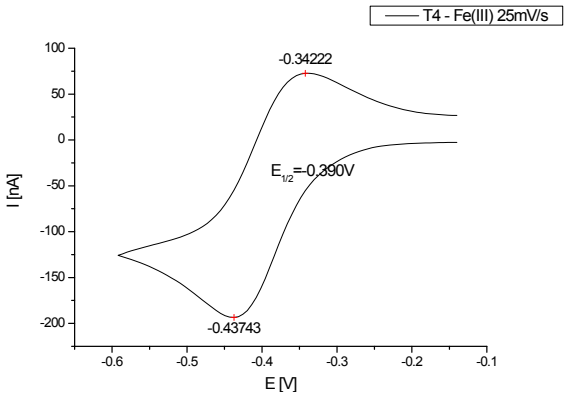


Figure S6. Voltammogram of Fe(III) – T4; ΔE = 95 mV; I = 0.1 M $NaClO_4$, T = 25˚C; metal to ligand ratio 1:1, $c_{Fe} = 0.5 \cdot 10^{-3}$ M.

Additionally, for each complex the stability constant for Fe(II) forms was calculated using the eq. S10.

$$E^{0}_{complex} - E^{0}_{aq} = -59.15 log(\beta^{III}/\beta^{II})^{a,b} \quad \text{(S10)}$$

[a]E^0_{aq}=771mV[32]
[b]Spasojevic et al.[33]

Siderophore analog calibration and CD measurements

Solutions of the analogs were accurately calibrated using copper (II) complex of chrome azurol S[34] (CAS; the CuCAS complex was calibrated using inductively coupled plasma- atomic emission spectrometer (ICP-AES). When CAS dye is added to the siderophore solution, the siderophore scavenges the copper from the Cu-CAS complex, and the color of the solution changes from purple to pale yellow. Using the same method as M. Shenkerand co-workers,[35] the concentration of the remaining Cu-complex was measured at 582 nm. Based on the concentration of the copper bound to the siderophore, the concentration of the siderophore stock solution was calculated.

The calibrated stock solution of the chiral analogs was dissolved in Tris-$HClO_4$ buffer (pH = 7.8, *I* = 0.1M $NaClO_4$), to a final concentration of 0.25 mM. A fresh stock of Fe(III) solution was prepared by dissolving Fe(III) perchlorate in $HClO_4$ (0.1 N) to a concentration of 25 mM (calibrated using ICP-AES). To each siderophore sample, Fe(III) perchlorate solution was added to a final concentration of 0.25 mM. To obtain equilibrium, the samples were left overnight, and CD spectra were recorded.

Biological activity experiments

Siderophore growth promotion experiments

The solutions of the analogs were calibrated using Cu-CAS. After calibration, the siderophore solution was adjusted to a final concentration of 10^{-3} M using double-distilled water (DDW) and $FeCl_3$ stock solution ($1.73 \cdot 10^{-2}$ M; calibrated using ICP-AES) in HCl (1 N). The $FeCl_3$ solution was added to the siderophore to achieve a concentration up to 90% of the siderophore concentration. To ensure sterility, the iron complex solution was filtered through a 0.2 µm filter into a sterile Eppendorf tube.

The effectiveness of the synthetic siderophores was evaluated by bacterial growth promotion experiments in a liquid medium: each analog was tested in triplicate on two bacterial strains (*P. putida* JM218[36] or *E. coli* UT5600[37]).
The appropriate bacterial strain was taken from a frozen glycerol stub (2–3 µL) and cultivated in sterile Lysogeny broth (LB; 3 mL) medium overnight at 30 ºC. About 2–3 µL of the bacterial stock grown in LB was inoculated into sterile Modified King B (MKB) medium (3 mL), and incubated under the same conditions for an additional 24 hours. The optical density (OD) of the bacterial MKB solution was measured at 600 nm.
Note: Due to the resistance of *E .coli* UT5600 to streptomycin antibiotic, both the LB and MKB media were supplemented with streptomycin (final concentration 0.1 mM) prior to bacterial incubation.

MKB medium was prepared as follows: Bacto peptone (5.00 g), glycerol (2.50 g) and K_2HPO_4 (1.20 g) were dissolved in DDW (800 mL). In parallel, $MgSO_4$ heptahydrate (1.50 g) was dissolved in DDW (200 mL). The prepared solutions were autoclaved separately and then mixed carefully to avoid magnesium precipitation.
Iron-depleted MKB medium was prepared as follows: two separate solutions, a solution of $MgSO_4$ heptahydrate and a solution of Bacto peptone, glycerol and K_2HPO_4, were prepared as described above. To the solution of Bacto peptone was added "Purolite S910" resin (20 g), and the medium was stirred for 4 hours. The resin was removed by filtration. Both solutions were transferred to separate iron-free bottles (treated previously with 6 N HCl). The prepared solutions were autoclaved and then mixed carefully to avoid magnesium precipitation.

To exclude external iron sources, the appropriate Erlenmeyer flasks used for bacterial growth were filled with HCl (6N) and left for a few hours to remove any iron traces. After rinsing the Erlenmeyer flasks with distilled water, they were plugged with suitable lignin corks and autoclaved. To each flask were added: iron-depleted MKB medium (30 mL), the appropriate analog-Fe(III) complex at a final concentration of $1 \cdot 10^{-6}$ M, and the appropriate bacterial strain to achieve an initial OD corresponding to 0.02 for *E. coli* and 0.01 for *P. putida*. The bacterial strains were incubated at 30˚C. The growth was monitored by measuring OD every hour for *P. putida* and every 2 hours for *E.coli.* The experiment was concluded when the bacteria reached the stationary phase. In addition to the synthetic analogs, natural ferrichrome was cultivated as a positive control, and sterile DDW served as a negative control. Based on the results of the bacterial OD, bacterial growth curves were plotted (Figures S7 and S8).

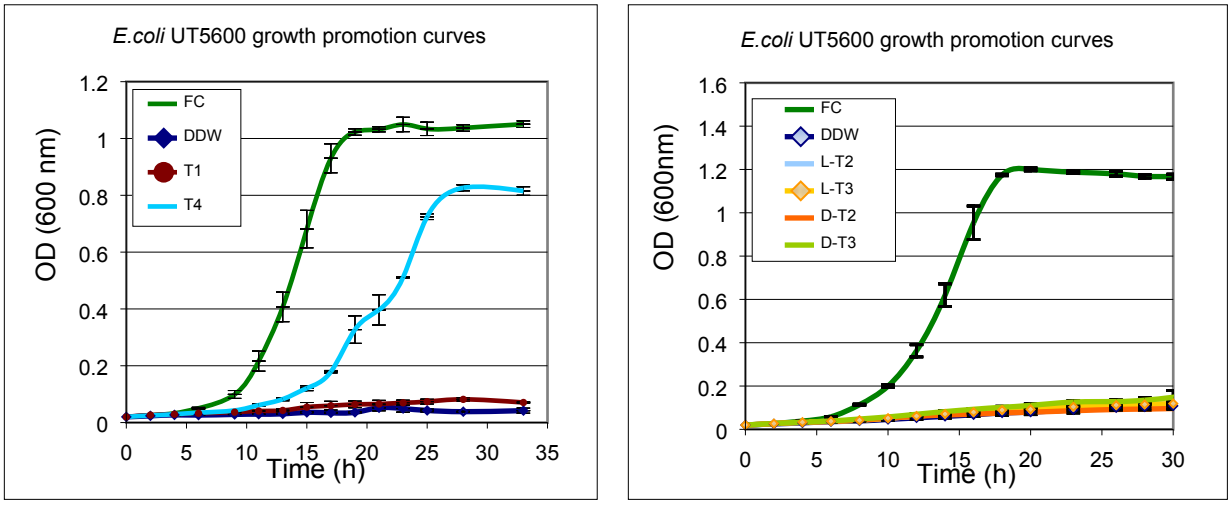


Figure S7. *E.coli* UT5600 growth curves in liquid MKB medium. Vertical bars indicate the statistical error. Each analog-Fe(III) solution (30 µL) was added to iron-depleted MKB medium (30 mL) to achieve a final concentration of 10^{-6} M. DDW= double-distilled water, FC=ferrichrome, T1-T4=artificial analogs.

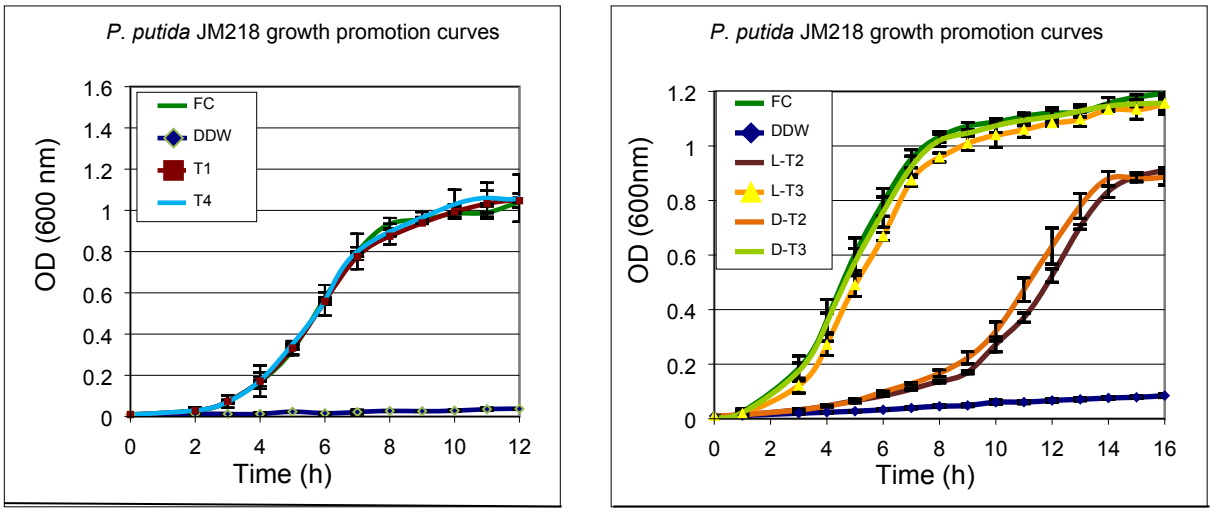


Figure S8. *P.putida* JM218 growth curves in liquid MKB medium. Vertical bars indicate the statistical error. Each analog-Fe(III) solution (30 µL) was added to iron-depleted MKB medium (30 mL) to achieve a final concentration of 10^{-6} M. DDW= double-distilled water, FC=ferrichrome, T1-T4=artificial analogs.

Iron utilization from a fluorescently labeled ferrichrome analog

To visualize the uptake of artificial siderophores by *E. coli*, we used an analog labeled with a fluorescent probe. The β-Ala analog linked to 4-amino naphthalimide (Figure S9a) was kindly provided by Dr. Joseph Englander. To monitor its uptake, two strains were used; UT5600[37] and K-12[38] (ΔfhuA mutation).

The bacterial strains were taken from a frozen glycerol stub (2–3 µL) and cultivated in sterile LB (3 mL) medium for 10 hour at 30 °C. About 2–3 µL of the bacterial stock grown in LB was inoculated into sterile MKB medium (3 mL), and incubated under the same conditions for an additional 24 hours. Both the LB and MKB media were supplemented with antibiotics prior to bacterial incubation; streptomycin in the case of UT5600 and Kanamycin in the case of K-12 (final concentration 0.1 mM).

Strain K-12 is able to synthesize enterobactin. To prevent iron scavenging by enterobactin, a sample from the bacterial culture of K-12 (1 mL) was placed in an Eppendorf tube and concentrated by centrifugation. The cell pellet of K-12 was washed with MKB medium twice to remove traces of enterobactin, and then resuspended in MKB (1 mL) immediately prior to fluorescence monitoring.

Stock solution of the fluorescent analog was dissolved in DDW to achieve a concentration of $4\cdot10^{-4}$ M, iron was added in a 1:1 ratio from $FeCl_3$ stock solution ($1.73\cdot10^{-2}$ M; calibrated using ICP-AES) in HCl (1 N). The fluorescent analog from stock solution (10 µL) was added to the bacterial culture of UT5600 and K-12 (1 mL) to a final concentration of $4\cdot10^{-6}$ M. For imaging, the cell cultures (10 µL) were immobilized on a coverslip with 1% agarose (30 µL). The coverslip was imaged on a confocal microscope using EGFP and TL Brightfield channels. Epi-fluorescent excitation was performed using a DAPI filter at 488 nm and emission was recorded using a GFP filter at 509 nm (Figure S9b and S9c). All images in were acquired under identical conditions. Images were subsequently analyzed using ZEN 2 software.

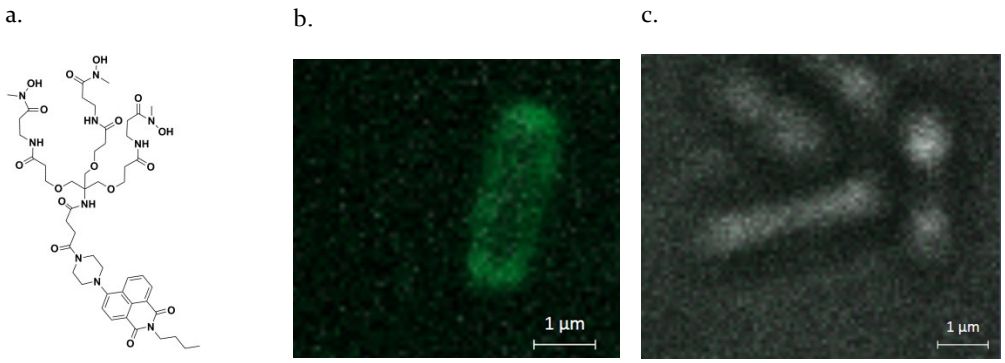


Figure S9. a. Chemical structure of β-Ala linked to fluorescent 4-amino naphthalimide. b-c. Merge of the images obtained from the TL Brightfield and EGFP channels: b. Confocal micrograph of *E. coli* UT5600 treated with the β-Ala naphthalimide analog (10 µL). c. Confocal micrograph of *E. coli* K-12 (ΔfhuA mutant) treated with the β-Ala naphthalimide analog (10 µL). Images were acquired with an Axio Observer 21 microscope and processed using ZEN 2 software.

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