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Engineering a cleavable disulfide bond into a natural product siderophore using precursor-directed biosynthesis

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REAGENTS

Desferrioxamine B (DFOB), sodium chloride, trizma hydrochloride, Chrome Azurol S (CAS), piperazine, 5-sulfosalicylic acid dihydrate, sodium phosphate (dibasic), threonine, zinc sulfate heptahydrate, cystamine, dithiothreitol (DTT), and gallium(III) acetylacetonate were purchased from Sigma-Aldrich. Difco yeast mold (YM) broth was purchased from BD Biosciences. Potassium dihydrogen phosphate, calcium chloride, magnesium sulfate heptahydrate, ethanol (≥99.5%), and iron(III) chloride hexahydrate were purchased from Ajax Finechem. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acetate disodium (EDTA) were purchased from AMRESCO. Ni(II) Sepharose 6 Fast Flow was purchased from GE Healthcare. MilliQ water was used in all experiments requiring water.

INSTRUMENTATION

Bacterial culturing: All bacterial cultures were grown in plastic Erlenmeyer flasks purchased from VWR and incubated in an Eppendorf New Brunswick Innova 42 Shaking Incubator. All bacterial work that required aseptic technique was completed in a PCII Laminar Flow Cytotoxic Drug Safety Cabinet (Westinghouse Pty Ltd). All autoclaving was performed using a Getinge Group GE 6610 ER-1 steam steriliser (121 °C, 100 kPa, 20 min). Solutions that needed to be sterilised by a method other than autoclaving were sterile filtered using Minisart 0.2 μm syringe filters (Sartorius).

Centrifugation: Samples in 50 mL and 15 mL Falcon tubes were centrifuged using a Beckman GS-15R Centrifuge. Samples in Eppendorf tubes were centrifuged using a Sigma 1-14 Laboratory Table Top Microcentrifuge.

Liquid chromatography-mass spectrometry (LC-MS): All reversed phase LC-MS analysis was performed using an Agilent Technologies system (Santa Clara, CA) consisting of a 1260 series quaternary pump with inbuilt degasser, a 1200 series autosampler and injector (100 μL loop), a temperature-controlled column compartment, a diode array detector, a fraction collector, and a 6120 series single quadrupole mass spectrometer fitted with an electrospray ionisation (ESI) ion source. In all experiments, the diode array detector was set at 220 nm for detection of hydroxamic acids and the capillary voltage of the mass spectrometer was set to 4,000 V. The drying gas flow, temperature, and nebuliser pressure of the mass spectrometer were set to 12 L min^{-1}, 350 °C, and 35 psi, respectively. Agilent OpenLAB chromatography data system ChemStation Edition (B.04.02) software was used for data acquisition and processing. For analysis, samples were run on an analytical Eclipse XDB-C18 reverse-phased prepacked column (particle size: 5 μm; 4.6 i.d. × 150 mm), using a gradient of acetonitrile (ACN) in H_2O, with formic acid (0.1% v/v) added to both solvents in all experiments. The ACN:H_2O gradient, flow rate, and injection volume were modified between different analyses.

LC-MS/MS: Analysis by LC-MS/MS was performed using an Agilent system, which consisted of a 1290 series quaternary pump with inbuilt degasser, a 1200 series autosampler, a temperature-controlled column compartment, a diode array detector, and a 6460 series triple quadrupole mass spectrometer fitted with an ESI ion source. Collision energy voltages were optimised for individual precursor ions and ranged from 14 to 28 V. The fragmentor voltage, drying gas flow, temperature, capillary voltage, and nebuliser pressure of the mass spectrometer were set to 150 V, 10 mL min^{-1}, 300 °C, 4,000 V, and 25 psi, respectively. The column was identical to that used for LC-MS analysis, with formic acid (0.1% v/v) added to both solvents in all experiments. The ACN:H_2O gradient, flow rate, and injection volume were modified between different analyses.

Agilent MassHunter Workstation (B.07.01) software was used for data acquisition and processing.

Freeze drying: Lyophilisation of samples was performed in 15 mL Falcon tubes using a Labconco FreeZone freeze-dryer (Kansas City, MO, USA).

High Resolution (HR)MS: HRMS analysis was performed using a Bruker Apex qE 7T fourier transform ion cyclotron resonance mass spectrometer with an Apollo(II) ESI/MALDI dual source. This instrument was externally calibrated using PEG 600 and PEG 1500 prior to analysis.

Preparative HPLC: Semi-preparative HPLC purification was performed using an Agilent Technologies system (Santa Clara, CA) consisting of a 1260 series quaternary pump with inbuilt degasser, a 1200 series autosampler and injector (100 µL loop), a temperature-controlled column compartment, a diode array detector and a fraction collector. The diode array detector was set at 220 nm for detection of target hydroxamic acids. Agilent OpenLAB chromatography data system ChemStation Edition (B.04.02) software was used for data acquisition and processing. For purification of DFOB-(SS)1, samples of semi-purified *S. pilosus* supernatant were run on a semi-preparative Eclipse XDB-C18 reverse-phased prepacked column (particle size: 5 µm; 9.4 mm i.d. × 250 mm), using acetonitrile (ACN) in H_2O, with formic acid (0.1% v/v) added to both solvents. A gradient of 0-60% ACN:H_2O was used over 60 minutes, with a flow rate of 1 mL min-1 and injection volume of 100 µL at 35 °C.

METHODOLOGY

Preparing frozen stocks of *S. pilosus*: *S. pilosus* ATCC® 19797 was obtained from the ATCC in the form of a lyophilised culture. To prepare stock cultures, the lyophilised *S. pilosus* culture was rehydrated in sterile YM broth (0.5 mL, 2.1% w/v, pH 6.5). The rehydrated culture was then inoculated into sterile YM broth (100 mL, 2.1%, w/v, pH 6.5) and incubated aerobically on a rotary shaker (160 rpm, 28 °C). After 3 d of incubation, samples of *S. pilosus* culture (1 mL) were transferred into Eppendorf tubes. Dimethylsulfoxide (10% v/v) was added as a cryoprotectant and the bacterial stocks were stored at −80 °C.

Precursor-directed biosynthesis with *S. pilosus*: For preculturing of *S. pilosus*, YM broth (2.1% w/v) was combined with Chelex® resin (1 g per 100 mL) and stirred vigorously (3 h) for removal of adventitious Fe(III) from the medium. The YM broth was decanted from the Chelex® resin and adjusted to pH 6.5 before autoclaving. Frozen stocks of *S. pilosus* (1 mL) were inoculated into plastic Erlenmeyer flasks of the autoclaved YM broth (100 mL) and incubated for 4 d (160 rpm, 28 °C). Cells of the *S. pilosus* precultures were then removed and centrifuged (3,838 × g, 20 min), and the supernatant was decanted. The cells were resuspended in an enriched medium and distributed evenly into plastic Erlenmeyer flasks containing enriched medium (50 mL) for precursor-directed biosynthesis experiments. This enriched medium was made by combining a solution of YM (4.2% w/v), a phosphate buffer solution and a solution of enrichment components in a 2:1:1 volumetric ratio. The phosphate buffer solution contained per litre of water: $K_2HPO_4.3H_2O$ (128 g) and Na_2HPO_4 (6.36 g). The YM and phosphate buffer solutions were each stirred with Chelex® resin (3 h) and adjusted to pH 6.5 before autoclaving. The solution of enrichment components contained per litre of Chelex®-treated water: threonine (0.40 g), $MgSO_4.7H_2O$ (2.40 g), $CaCl_2$ (6.04 g), $ZnSO_4.7H_2O$ (0.016 g), and trizma base (48.5 g). The solution of enrichment components was sterile filtered before combination with the YM and phosphate buffer solutions in a biosafety cabinet. A solution of cystamine (pH 6.5) was sterile

filtered before supplementation to selected *S. pilosus* cultures at a final concentration of 10 mM. *S. pilosus* cultures were incubated for 8-10 d (160 rpm, 28 °C), with siderophore production monitored by Fe(III)-addition assay of subsamples (1 mL) taken throughout the culturing period. Once the concentration of siderophores in the supernatant had plateaued, the *S. pilosus* supernatant of each culture was harvested by centrifugation (3,838 × g, 20 min) for processing by XAD-2 chromatography purification.

Fe(III)-addition assay: The Fe(III)-addition assay involved photometric analysis in microplates, as follows: Fe(III) assay solution (110 μL, 10 mM ferric perchlorate in 0.2 M perchloric acid) was added to supernatant (220 μL) and a white precipitate of ferric phosphate formed which required centrifugation (21,130 × g, 10 min) for removal. The mixture of the Fe(III) assay solution and *S. pilosus* supernatant (3 replicates of 100 μL) was then incubated for 10 min at room temperature before photometric measurement at 500 nm.

XAD-2 purification of *S. pilosus* supernatant: A protocol for purification of *S. pilosus* supernatant by XAD-2 chromatography was modified from previous methods.[1-3] Amberlite® XAD-2 resin (100 mL) was activated by stirring in methanol (200 mL, 15 min) and removed by filtration. The XAD-2 resin was then washed with water (2 × 200 mL, 15 min) before being suspended in a glass Econo-Column® (2.5 cm i.d. × 30 cm, BioRad) in water and backwashed to give a homogenous resin distribution. The XAD-2 resin was washed with water (200 mL), with a maximum flow rate of 5 mL min^{-1}. The *S. pilosus* supernatant was introduced (50 mL supernatant per column containing 100 mL XAD-2 resin), the column was washed with water (200 mL) and the sample was eluted with aqueous methanol (50% v/v, 200 mL) with fractions of eluent (40 mL) collected. Siderophore content of the eluted fractions was tracked by the CAS assay, with CAS positive fractions pooled and dried *in vacuo* for further purification by Ni(II)-based immobilised metal ion affinity chromatography (IMAC) purification.

CAS assay for detection of siderophores: A CAS assay solution was prepared according to previous literature[4] for analysis of eluting XAD-2 and Ni(II)-based immobilised metal ion affinity chromatography (IMAC) fractions. An aqueous solution of CAS (2.0 mM, 7.5 mL) was added slowly to an aqueous Fe(III) solution (1.5 mL) containing $FeCl_3.6H_2O$ (10 mM) and HCl (0.1 M). An aqueous piperazine solution (1 M, 50 mL, pH 5.6) was prepared and added to the first solution, with water added to a total volume of 100 mL to give the CAS assay solution. A solution of 5-sulfosalicylic acid dihydrate (0.2 M) was also prepared. Subsamples of the eluted XAD-2 fractions (100 μL) were combined with the CAS assay solution (100 μL) and 5-sulfosalicyclic acid dihydrate solution (4 μL) in a 96 well microplate, and the mixture allowed to equilibrate for 4 h to monitor for colour change. A change from blue to pink indicated presence of siderophores, where a greater decrease in absorbance measured at 630 nm correlated to a higher concentration of siderophores.

IMAC purification of *S. pilosus* supernatant: Ni(II)-based IMAC was performed according to previous methodology.[3,5] Ni(II) Sepharose resin (40 mL) was washed with water (200 mL) and then equilibrated with binding buffer (200 mL; 10 mM HEPES, 0.2 M NaCl, pH 9.0). The XAD-2-purified *S. pilosus* supernatant was dissolved in binding buffer (2 mL) and loaded onto the IMAC resin. The resin was washed with binding buffer (200 mL) and the sample eluted with elution buffer (200 mL, 10 mM HEPES, 0.2 M NaCl, pH 5.5) as fractions of eluent (40 mL) were collected and monitored for siderophore production by CAS assay. The CAS positive fractions were pooled and dried *in vacuo* for desalting before LC-MS analysis.

Desalting of IMAC-purified *S. pilosus* supernatant: To desalt the IMAC-purified *S. pilosus* supernatant prior to MS analysis, sequential washes of the residue with methanol were used to separate the target siderophores from undissolved salt. This step was optimised so that each sample of IMAC-purified supernatant was mixed thoroughly with HPLC-grade methanol (6 × 10 mL), with each fraction of methanol taken into a separate container between each wash. The fractions containing siderophores were pooled and dried *in vacuo* for semi-preparative HPLC purification.

LC-MS and LC-MS/MS analysis: The semipurified *S. pilosus* supernatant was analysed by LC-MS to characterise DFOB and disulfide-containing analogues of DFOB using a 0-30% ACN:H_2O gradient over 60 min with a 0.2 mL min^{-1} flow rate and 2 µL injection volume. Individual peaks were then analysed by LC-MS/MS fragmentation with the same column and identical LC conditions. To confirm Fe(III)-binding capacity of siderophores, supernatant samples were incubated with Fe(III) assay solution in a 1:1 ratio prior to LC-MS analysis.

***In situ* reduction of** DFOB-$(SS)_1$[001] (**2**): The semipurified *S. pilosus* supernatant from a cystamine (10 mM)-supplemented culture or a control culture was incubated with the reducing agent dithiothreitol (DTT) to monitor for the reduction of DFOB-$(SS)_1$[001] (**2**) or DFOB (**1**), respectively. The same supernatant samples were incubated prior to the addition of DTT, with Fe(III)Cl_3 (10 mM) or Ga(III)$(acac)_3$ (10 mM) for formation of the Fe(III)-DFOB-$(SS)_1$[001] (Fe(III)-**2**), Ga(III)-DFOB-$(SS)_1$[001] (Ga(III)-**2**), Fe(III)-DFOB (Fe(III)-**1**), or Ga(III)-DFOB (Ga(III)-**1**) complexes. These Fe(III)- or Ga(III)-containing mixtures were also combined with DTT to monitor for reduction of DFOB-$(SS)_1$[001] (**2**), with DFOB (**1**) included as a control. Samples were prepared in a 1:1:1 ratio of supernatant (20 µL), DTT (20 µL) and either Fe(III) or Ga(III) (20 µL). This gave a final concentration of DTT of about 10 mM. In an experiment conducted subsequently, the final concentration of DTT was reduced to 2 mM. As negative controls, the same samples were analysed with water (20 µL) in place of DTT. The supernatant from the cystamine-supplemented and control cultures were also solely incubated with water (40 µL) to acquire mass chromatograms for the supernatant alone. All samples were maintained at pH 7.0. Incubations were left at 25 °C for 1 h prior to LC-MS analysis. LC-MS analysis was used in selected ion monitoring (SIM) mode to track reduction of DFOB-$(SS)_1$[001] (m/z = 611.2) (**2**), Fe(III)-DFOB-$(SS)_1$[001] (m/z = 664.2) (Fe(III)-**2**), Ga(III)-DFOB-$(SS)_1$[001] (m/z = 677.2) (Ga(III)-**2**), DFOB (m/z = 561.3) (**1**), Fe(III)-DFOB (m/z = 614.3) (Fe(III)-**1**), and Ga(III)-DFOB (m/z = 627.3) (Ga(III)-**1**), and for production of the major fragment of DFOB-$(SS)_1$[001] after reduction (m/z = 536.3) (**9**) and the complexes that it would form with Fe(III) (m/z = 589.2) (Fe(III)-**9**) or Ga(III) (m/z = 602.2) (Ga(III)-**9**). LC-MS analysis was performed with a 0-30% ACN:H_2O gradient over 60 min, a flow rate of 0.2 mL min^{-1}, and a 10 µL injection volume.

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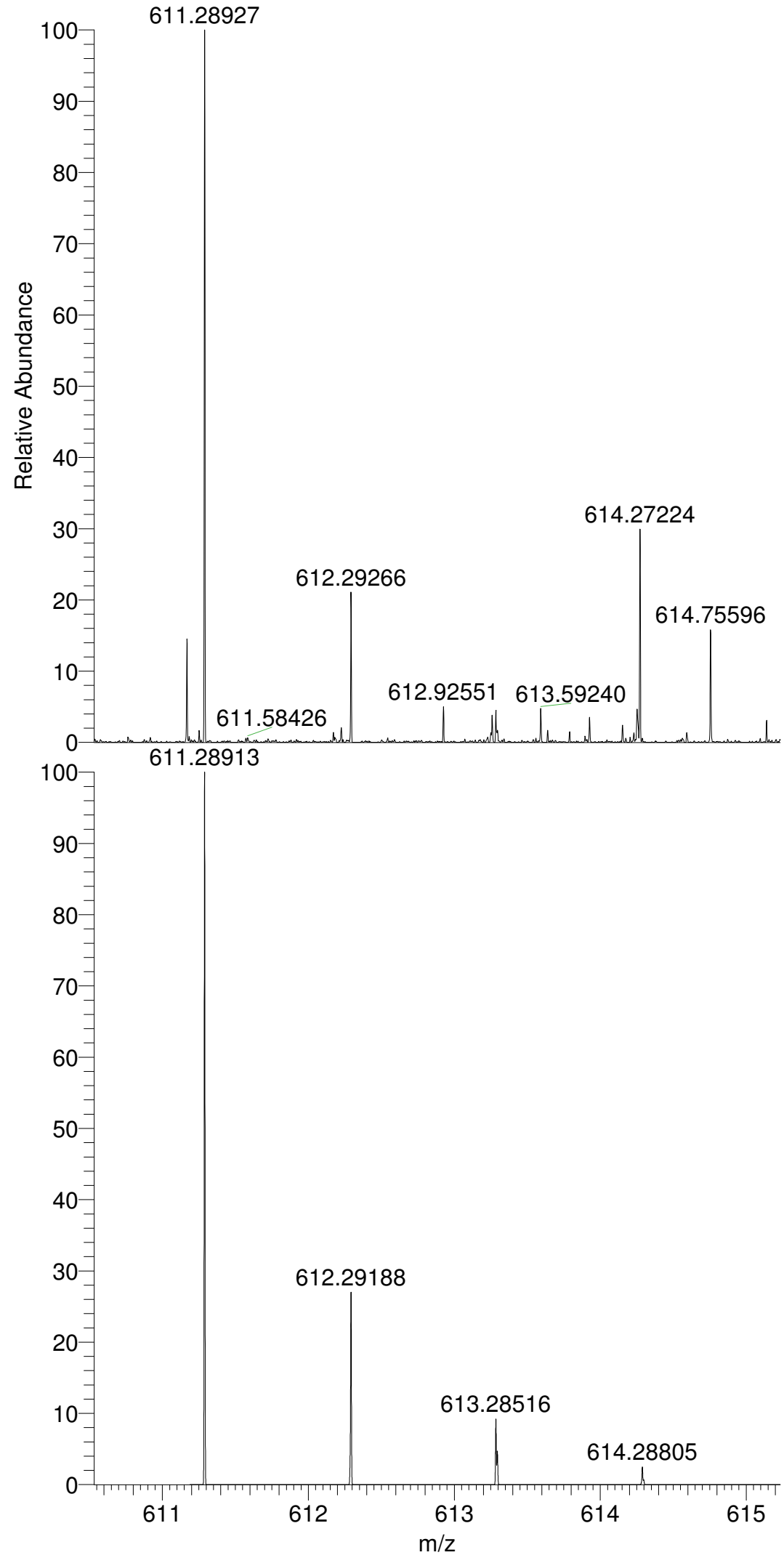

Fig. S1. Experimental (upper) and calculated (lower) HRMS of **2**.

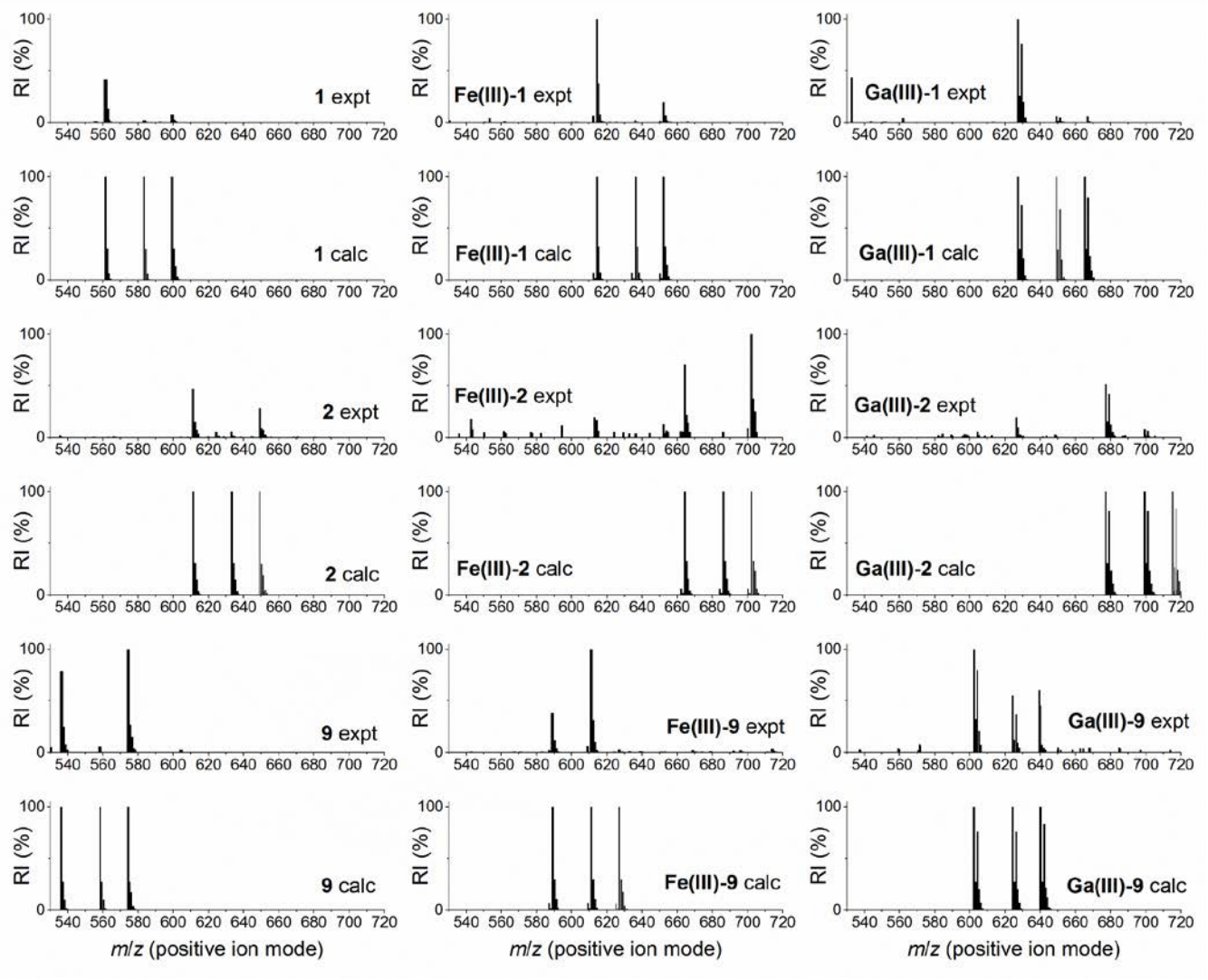

Fig. S2. MS isotope patterns for **1**, **2**, **9**, Fe(III)-**1**, Fe(III)-**2**, Fe(III)-**9**, Ga(III)-**1**, Ga(III)-**2**, Ga(III)-**9**, shown as experimental (expt) or calculated (calc) data for $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ adducts.