Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Siderophore purification with TiO_2 nanoparticle solid phase extraction

Philipp H. Egbers[⊥], Tilmann Harder[⊥,#], Boris P. Koch[#,+], Jan Tebben[#*]

[⊥] University of Bremen, Faculty of Biology and Chemistry, Leobener Straße 6, 28359 Bremen, Germany

[#] Department of Ecological Chemistry, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

[+] University of Applied Sciences, An der Karlstadt 8, D-27568 Bremerhaven, Germany

The Supporting Information includes following items:

PROTOCOL FOR TIO_2 NP-BASED SPE FROM BACTERIAL CULTURE SUPERNATANTS

Materials
Reagents:
TiO_2 nanopowder 21 nm
NaH_2PO_4
Methanol and formic acid
NaOH
Ultrapure water (Sartorius)
HCl 35%
$HClO_4$
$Fe(ClO_4)_3$
Equipment:
2 mL screw cap micro tubes
Cryomill (or shaker)
Vortex mixer
Centrifuge
1.5 mL micro tubes
Setup:
Acid washing: Soak 2 mL screw cap micro tubes with 5 M HCl for 24 h and then rinse with ultrapure water 5 times. Store acid washed screw cap microtubes closed in clean bags until needed.
Hydroxamate assay solution (Atkin's assay): 5 mmol L^{-1} $Fe(ClO_4)_3$ in 0.1 M $HClO_4$. Mix 1 part of crude sample with 1 part of assay solution. If sample contains hydroxamates brownish/reddish colour appears immediately after mixing the two solutions. If possible, compare this result to a similarly prepared blank.
TiO_2: Prepare a suspension by mixing 10 g TiO_2 nanopowder with 100 mL ultrapure water. Vortex the suspension shortly before use.
Washing solution: Formic acid in methanol (0.02 vol%)
Eluent: NaH_2PO_4 100 mmol L^{-1} in ultrapure water and pH adjusted to 12.6 with NaOH.
Neutralization solution: 100 mmol L^{-1} $FeCl_3$ in aqueous formic acid (0.2 vol%).

Procedure

1. Transfer 0.1 mL TiO_2 suspension into acid washed screw cap micro tube
2. Apply 1 mL of Atkin's assay positive sample to the TiO_2 suspension and vortex occasionally for at least 20 min to achieve complete adsorption.
3. Centrifugation of the screw cap micro tube at 14000 rpm for 2 min.
4. Separate supernatant from the TiO_2 pellet by decanting. Collect the supernatant and check with Atkin's assay. If the supernatant is showing still a positive reddish colouration repeat step 1.-3. to extract the reaming hydroxamates.
5. Rinse TiO_2 with 1 mL ultrapure water and re-suspend the pellet by shaking the sample for 2 min at 30 Hz with a Cryomill. If a Cryomill is not available, it is also possible to achieve dissolution of the pellet manually. After re-suspending, the sample is occasionally vortexed for 5 min.
6. Centrifugation of the screw cap micro tube at 14000 rpm for 2 min and the ultrapure water supernatant is discarded. Perform ultrapure water rinsing steps 5. and 6. again
7. Repeat steps 5.-6. but instead of ultrapure water use washing solution. Supernatant are also discarded.
8. To achieve elution of hydroxamates add 1 mL of eluent and process the TiO_2 pellet as for the previous steps. Collect supernatant in 1.5 mL microtubes. This step is repeated three times to achieve complete elution of bound hydroxamates.
9. Add 0.2 mL of neutralization solution to each 1.5 mL microtube and centrifuge for 20 min at 14000 rpm.
10. Transfer an aliquot of each sample into untreated LC-Vials and let it stand for 48 h to achieve complete complexation of the eluted free hydroxamate ligands.
11. Measure samples via LC-MS to detected isotopic pattern of Fe and therefor confirm the presents of Fe-ligands in the eluate.

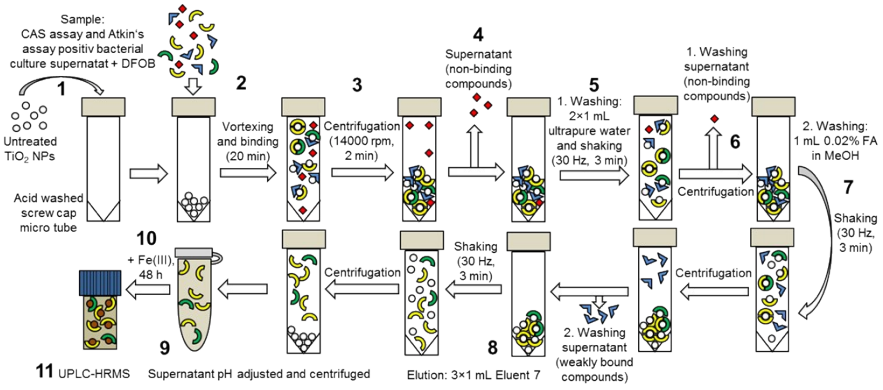

Figure S1. TiO_2 nanoparticle based SPE of hydroxamates from bacterial culture supernatants.

BACTERIAL GROWTH MEDIUM RECIPE

990 ml ultrapure water
Add:

Ingredients	Amount (g)
Casein hydrolysate	2
NH_4Cl	1
Glycerol	6
NaCl	23.926
Na_2SO_4	4.008
KCl	0.677
$NaHCO_3$	0.196
KBr	0.098
$StCl_2 \times 6H_2O$	0.024
H_3BO_3	0.026
NaF	0.003

Next: Chelex treatment
Add 25 g of Chelex to 1 L medium and stir for 1 h.
Separate Chelex from medium by using acid washed lass column.
Collect eluate in acid washed Ultra Yield bottles.

Add: 10.83 g $MCl_2 \times 6H_2O$ (iron free)
1.519 g $CaCl_2 \times 2H_2O$ (iron free)

Adjust pH to 7.6
Add 1 ml of supplement solution*
Fill up to 1 L
Autoclave sterilization

*Supplement solution

Ingredients	Amount/Volume
25% HCl	13.0 mL
Titriplex-(III) (Na_2EDTA)	5.2 g
$CoCl_2 \times 6H_2O$	190 mg
$ZnSO_4 \times 7H_2O$	144 mg
$MnCl_2 \times 4H_2O$	100 mg
$Na_2MoO_4 \times 2H_2O$	36 mg
H_3BO_3	30 mg
$NiCl_2 \times 6H_2O$	24 mg
$CuCl_2 \times 2H_2O$	2 mg
Ultrapure water	1000 mL

SALINE AQUEOUS MATRIX RECIPE

990 mL of ultrapure water
Add:

Ingredients	Amount/Volume
NaCl	20.8 g
$MCl_2 \times 6H_2O$	9.6 g
Na_2SO_4	3.5 g
$CaCl_2$ (1 M)	9 mL
KCl	0.6 g

Adjust pH to 8.0 – 8.2
Add 1 ml of supplement solution*
Fill up to 1 L

DATA & RESULTS

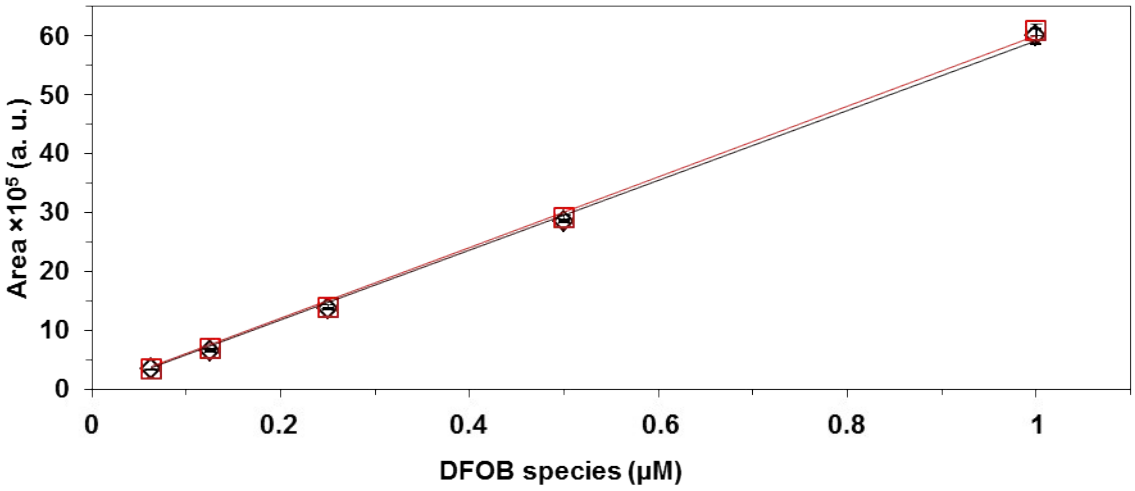

Fig. S2 Calibration of (black diamonds) FOB ($[M+H]^+$ = $[^{56}Fe(III)\ C_{25}H_{46}N_6O_8]^+$, m/z 614) and (red squares) AlOB ($[M+H]^+$ = $[^{27}Al(III)\ C_{25}H_{46}N_6O_8]^+$, m/z 585) in ultrapure water. FOB: LOD = 56.451 nmol L^{-1}; LOQ = 112.902 nmol L^{-1}; R^2 = 0.9997; N = 5. AlOB: LOD = 52.118 nmol L^{-1}; LOQ = 104.235 nmol L^{-1}; R^2 = 0.9997; N = 5. Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to DIN standard 32645. Three different calibrations were prepared and average area value was used for calculations.

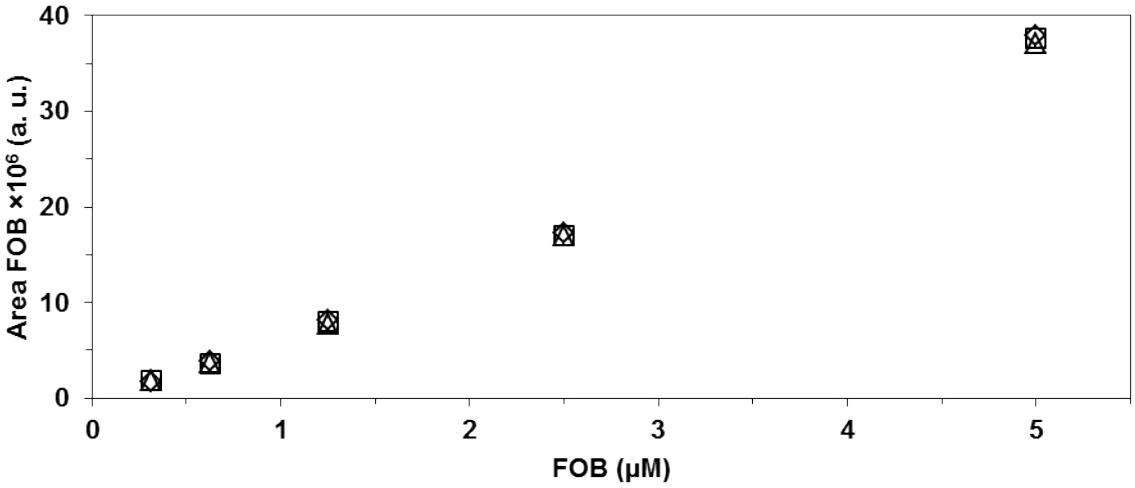

Fig. S3 Calibration of FOB ($[M+H]^+$ = $[^{56}Fe(III)\ C_{25}H_{46}N_6O_8]^+$, m/z 614) in saline aqueous matrix (diamonds), (squares) neutralized Eluent 7 (squares) and in ultrapure water (triangles).

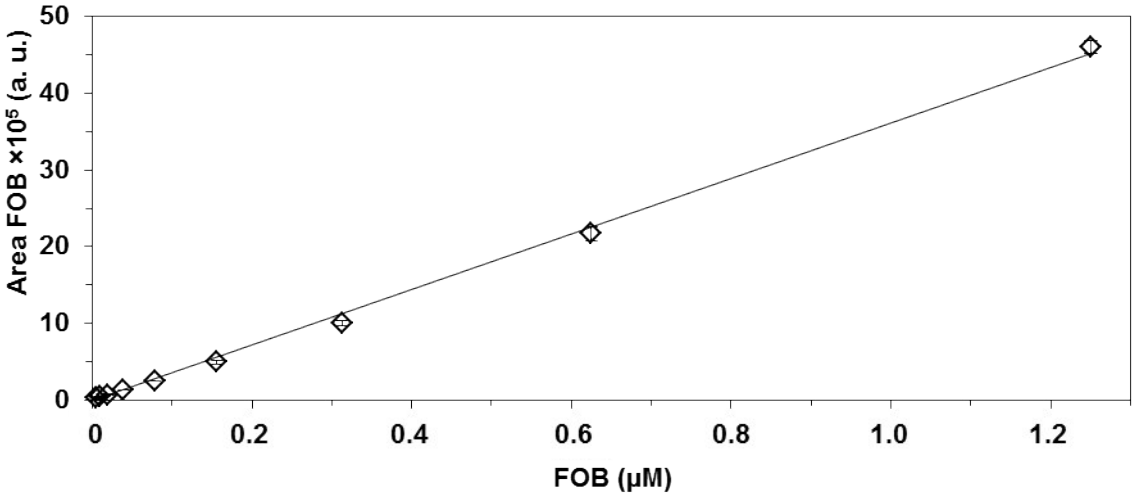

Fig. S4 Calibration of FOB ($[M+H]^+$ = $[^{56}Fe(III)\ C_{25}H_{46}N_6O_8]^+$, m/z 614) in ultrapure water used for quantification of method development and bacterial processing experiments. LOD = 56.572 nmol L^{-1}; LOQ = 113.144 nmol L^{-1}; R^2 = 0.9992; N = 9. Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to DIN standard 32645. Three different calibrations were prepared and average area value was used for calculations.

Table S1 Adsorption efficiency of DFOB before eluent optimization experiments

Adsorption efficiency[a] (%)			Experiment
Triplicate 1	Triplicate 2	Triplicate 3	
99.7	99.6	99.8	Used for elution experiment with E1
99.4	99.8	99.6	Used for elution experiment with E2
99.4	96.4	99.3	Used for elution experiment with E3
99.3	99.4	99.5	Used for elution experiment with E4
99.8	99.1	99.6	Used for elution experiment with E5
99.8	99.6	99.6	Used for elution experiment with E6
99.8	99.4	99.9	Used for elution experiment with E7
-0.7 (FOB)	0.4 (FOB)	0.3 (FOB)	Used for elution experiment with E7

(a) Adsorption efficiency is defined as the ratio of AlOB and FOB concentration detected in the supernatant decreased to the AlOB and FOB concentration in the initial solution before extraction expressed in %. Supernatants and aliquots of initial solution were spiked with $FeCl_3$ before LC-HRMS measurement (Section: Standardized siderophore adsorption).

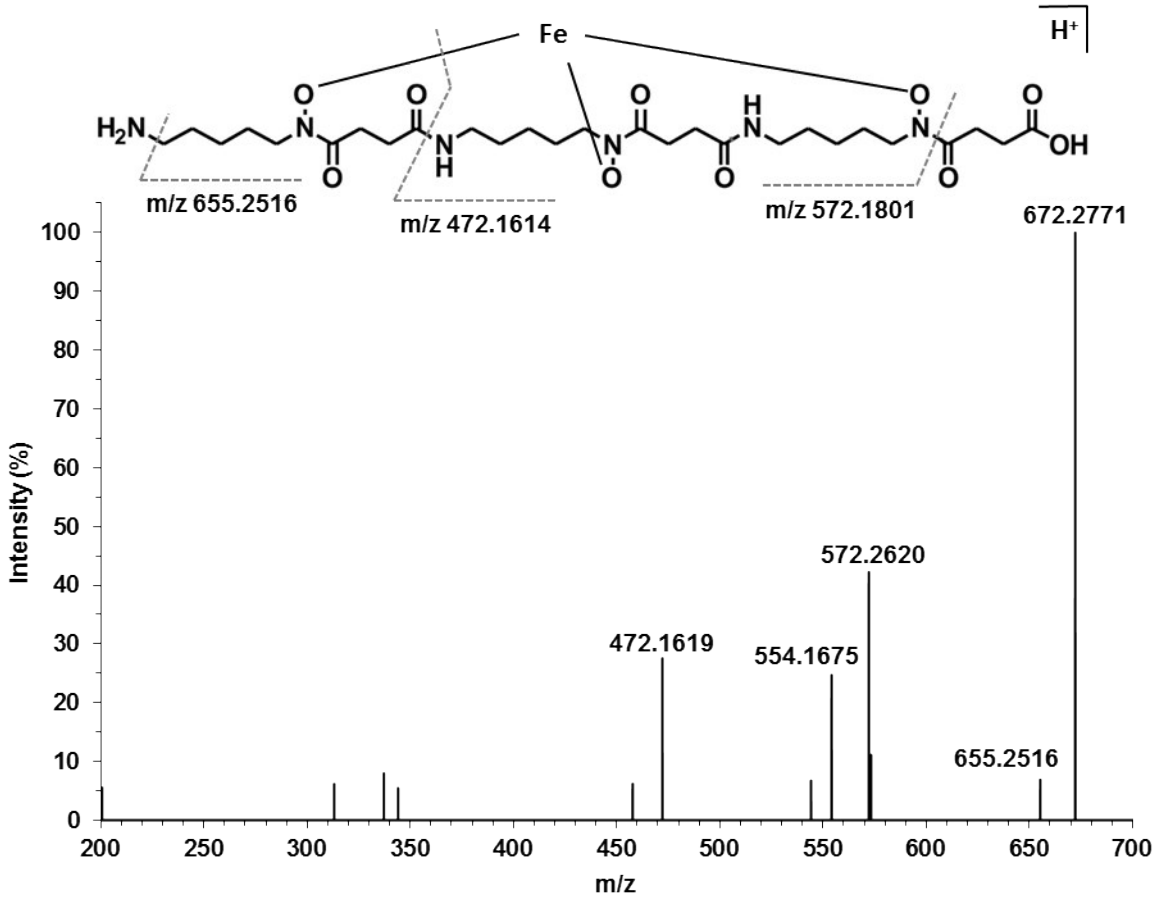

Fig. S5 MS^2 spectra and structure of FOG1 ($[M+H]^+ = [^{56}Fe(III)\ C_{27}H_{48}N_6O_{10}]^+$, m/z 672) showing the main cleavages accounting for product ions observed (grey dotted lines) (similar to results of Mawji et al.[1]). Desaturation/proton removal sites are general and will need further evaluation for exact determination of location (according to Sidebottom et al.[2]).

Table S2 Theoretical and measured m/z and corresponding sum formula of FOG1 (m/z 672, MS^2).

Measured m/z $[M+H]^+$	Theoretical m/z $[M+H]^+$	Δppm	Sum formula $[M+H]^+$
472.16193	472.16149	0.93	$C_{18}H_{32}O_7N_4Fe$
554.16748	554.16697	0.81	$C_{22}H_{34}O_9N_4Fe$
572.26202	572.26154	0.84	$C_{23}H_{44}O_7N_6Fe$
655.25159	655.25104	0.85	$C_{27}H_{45}O_{10}N_5Fe$
672.27710	672.27759	0.37	$C_{27}H_{48}O_{10}N_6Fe$

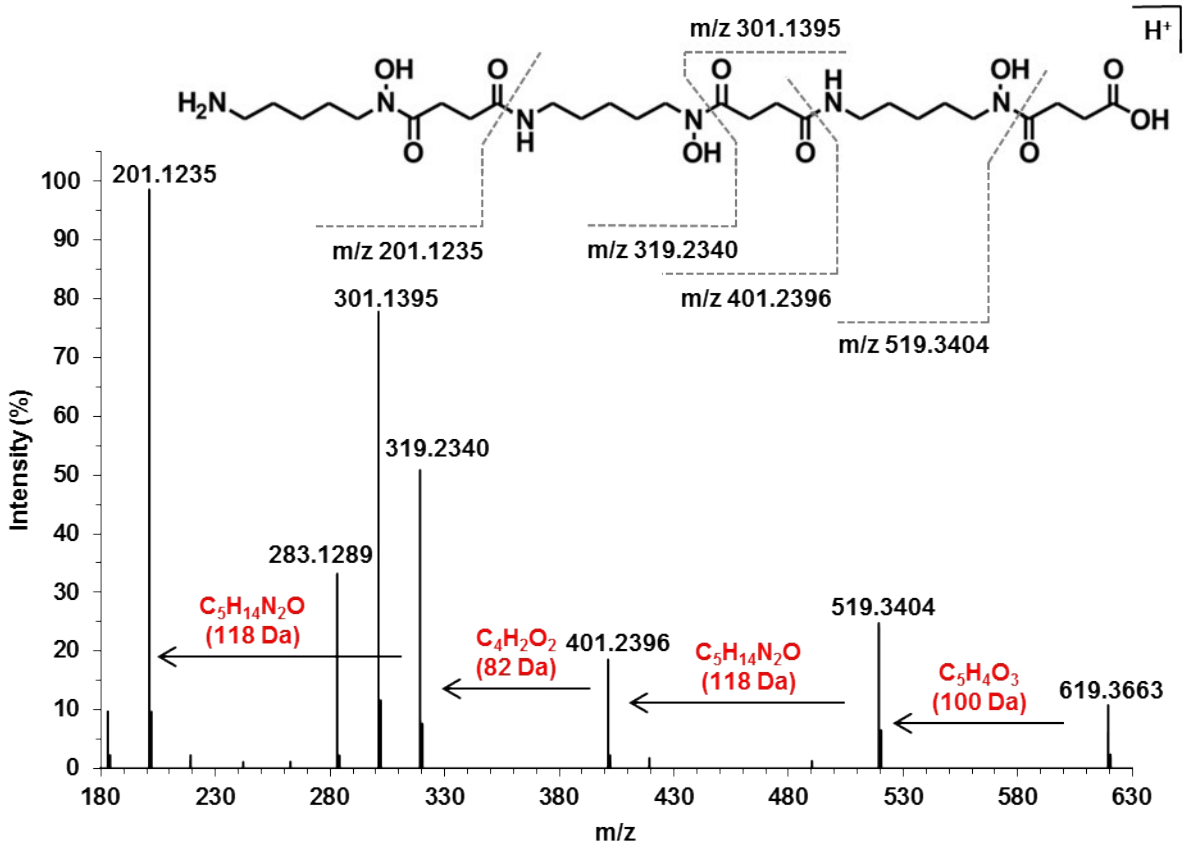

Fig. S6 MS^2 spectra and structure of DFOG1 ($[M+H]^+ = [C_{27}H_{51}O_{10}N_6]^+$, m/z 619), showing the main cleavages accounting for product ions observed (grey dotted lines) (compare to Feistner et al.[3,4]). Desaturation/proton removal sites are general and will need further evaluation for exact determination of location (according to Sidebottom et al.[2]).

Table S3 Theoretical and measured m/z and corresponding sum formula of DFOG1 (m/z 619, MS^2).

Measured m/z $[M+H]^+$	Theoretical m/z $[M+H]^+$	Δppm	Sum formula $[M+H]^+$
201.12346	201.12337	0.44	$C_9H_{17}O_3N_2$
283.12888	283.12885	0.09	$C_{13}H_{19}O_5N_2$
301.13947	301.13941	0.17	$C_{13}H_{21}O_6N_2$
319.23401	319.23398	0.08	$C_{14}H_{31}O_4N_4$
401.23962	401.23946	0.41	$C_{18}H_{33}O_6N_4$
519.34040	519.35007	0.63	$C_{23}H_{47}O_7N_6$
619.36633	619.36612	0.34	$C_{27}H_{51}O_{10}N_6$

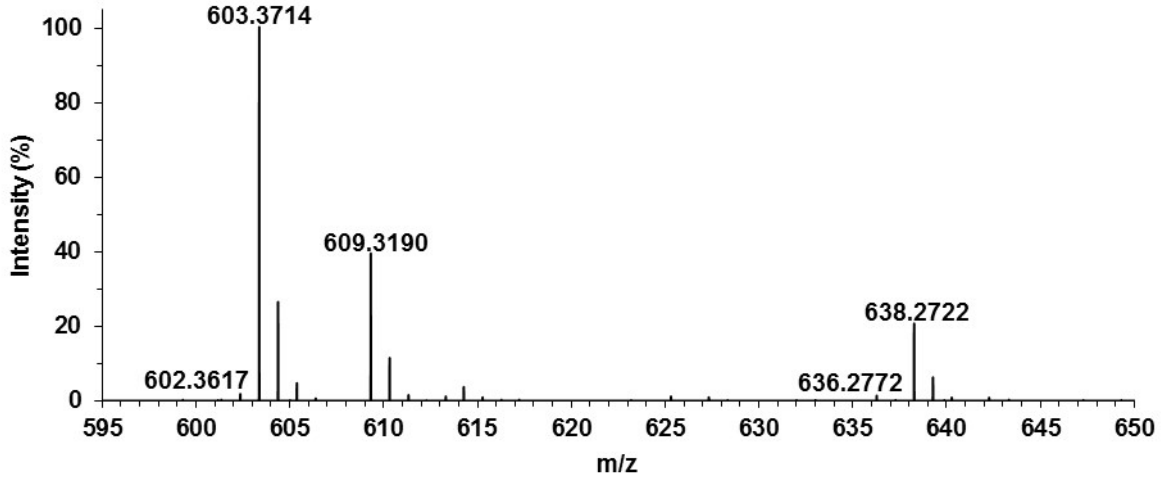

Fig. S7 MS spectra of TiO_2 E7 eluate at 2.82 min.

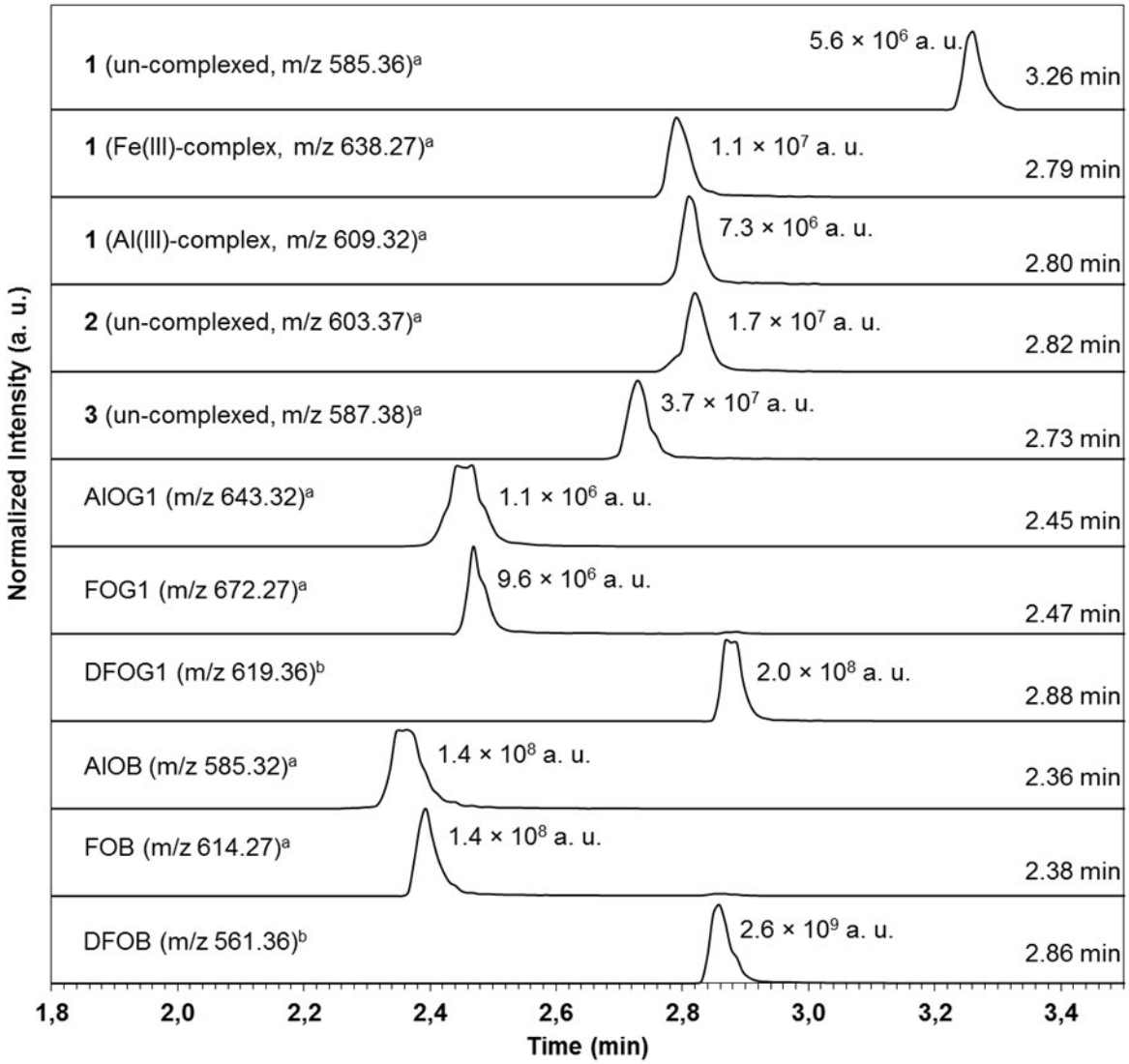

Fig. S8 Stacked normalized extracted ion chromatograms (nominal mass ± 0.01 Da) of peaks detected in (a) the $FeCl_3$ saturated TiO_2 E7 eluate (see also Figure 5D) and (b) the raw bacterial culture supernatant.

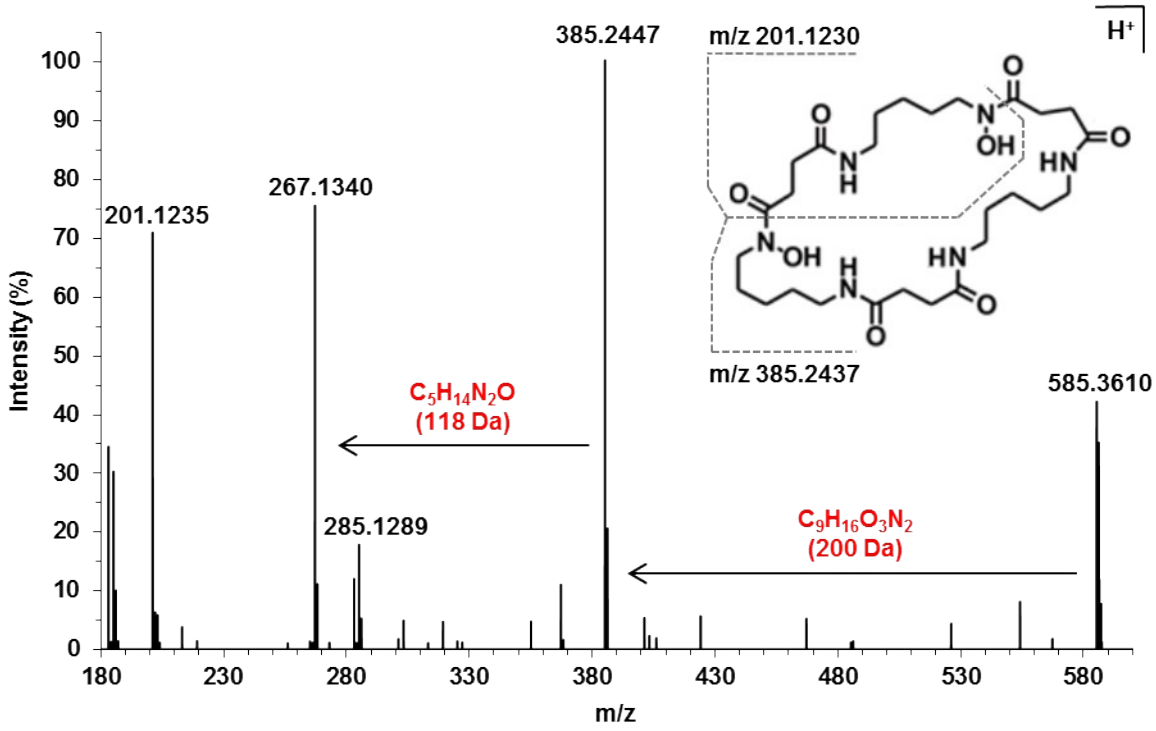

Fig. S9 MS^2 spectra and the proposed structure of **1** ($[M+H]^+$ = $[C_{27}H_{49}O_8N_6]^+$, m/z 585), showing the main cleavages accounting for product ions observed (grey dotted lines) Desaturation/proton removal sites are general and will need further evaluation for exact determination of location (according to Sidebottom et al.[2]).

Table S4 Theoretical and measured m/z and corresponding sum formula of 1 (m/z 585, MS^2).

Measured m/z $[M+H]^+$	Theoretical m/z $[M+H]^+$	Δppm	Sum formula $[M+H]^+$
201.12347	201.12337	0.52	$C_9H_{17}O_3N_2$
267.13400	267.13393	0.26	$C_{13}H_{19}O_4N_2$
283.12894	283.12885	0.31	$C_{13}H_{19}O_5N_2$
285.14456	285.14450	0.22	$C_{13}H_{21}O_5N_2$
385.24469	385.24455	0.37	$C_{18}H_{33}O_5N_4$
585.36096	585.36064	0.55	$C_{27}H_{49}O_8N_6$

Table S5 Theoretical m/z and corresponding sum formula of DFOE (m/z 601) and expected fragment ions.[3,4]

Theoretical m/z $[M+H]^+$	Sum formula $[M+H]^+$
201.12337	$C_9H_{17}O_3N_2$
283.12885	$C_{13}H_{19}O_5N_2$
301.13941	$C_{13}H_{19}O_6N_2$
401.23946	$C_{18}H_{33}O_6N_4$
601.35555	$C_{27}H_{49}O_9N_6$

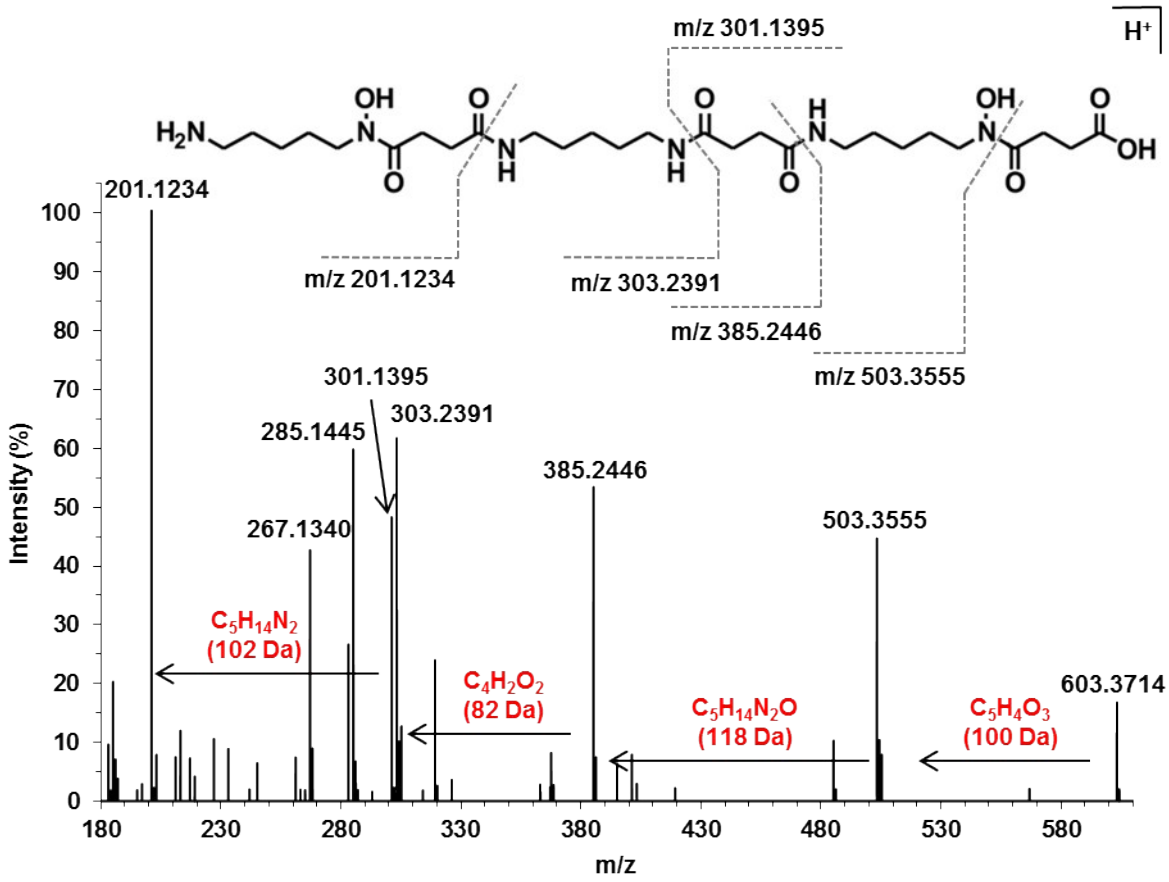

Fig S10 MS^2 spectra and proposed structure of **2** ($[M+H]^+ = [C_{27}H_{51}O_9N_6]^+$, m/z 603), showing the main cleavages accounting for product ions observed (grey dotted lines) Desaturation/proton removal sites are general and will need further evaluation for exact determination of location (according to Sidebottom et al.[2]).

Table S6 Theoretical and measured m/z and corresponding sum formula of 2 (m/z 603, MS^2).

Measured m/z $[M+H]^+$	Theoretical m/z $[M+H]^+$	Δppm	Sum formula $[M+H]^+$
201.12344	201.12337	0.38	$C_9H_{17}O_3N_2$
267.13396	267.13393	0.09	$C_{13}H_{19}O_4N_2$
285.14452	285.1445	0.09	$C_{13}H_{21}O_5N_2$
301.13951	301.13941	0.34	$C_{13}H_{21}O_6N_2$
303.23910	303.23907	0.12	$C_{14}H_{31}O_3N_4$
385.24464	385.24455	0.25	$C_{18}H_{33}O_5N_4$
503.35549	503.35516	0.66	$C_{23}H_{47}O_6N_6$
603.37141	603.37120	0.35	$C_{27}H_{51}O_9N_6$

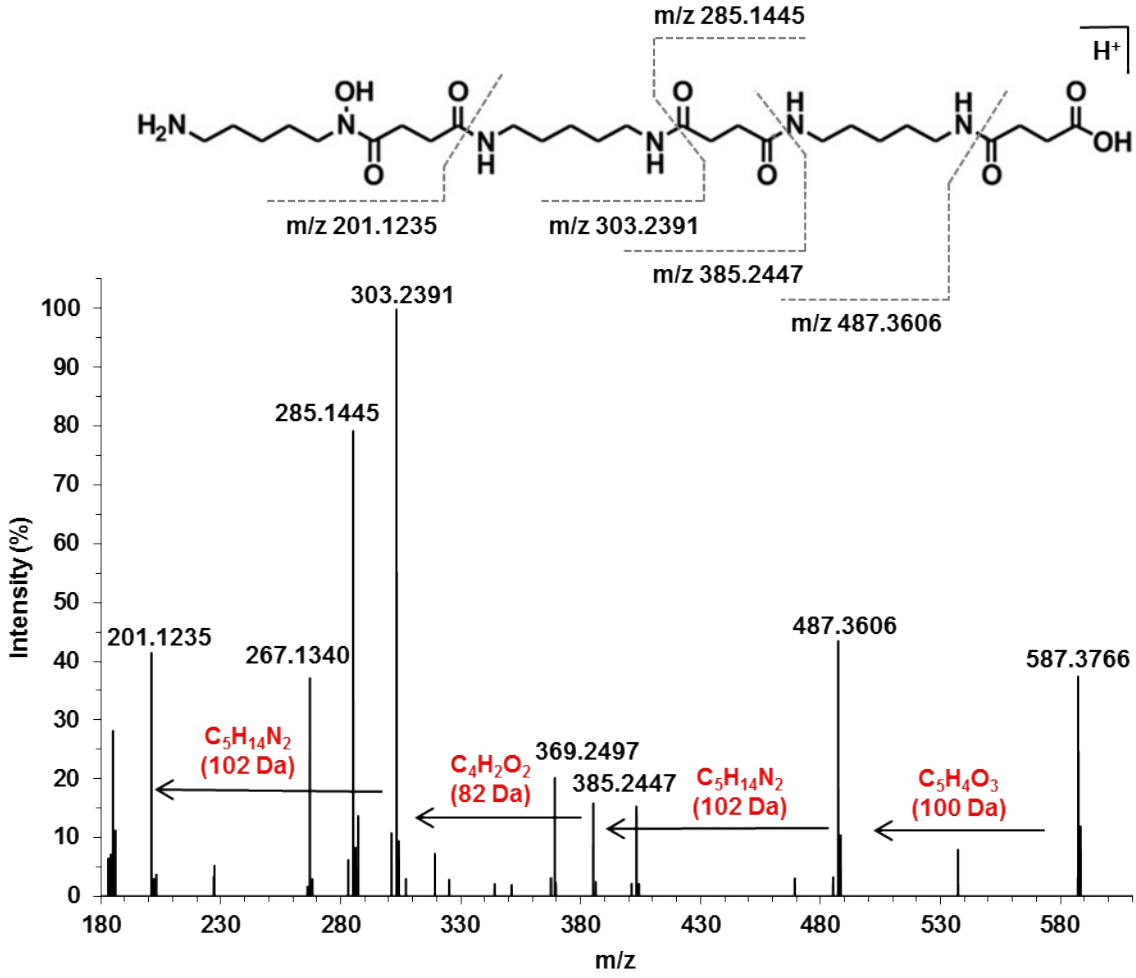

Fig. S11 MS^2 spectra and proposed structure of **3** ($[M+H]^+ = [C_{27}H_{51}O_8N_6]^+$, m/z 587), showing the main cleavages accounting for product ions observed (grey dotted lines) Desaturation/proton removal sites are general and will need further evaluation for exact determination of location (according to Sidebottom et al.[2]).

Table S7 Theoretical and measured m/z and corresponding sum formula of 3 (m/z 587, MS^2).

Measured m/z $[M+H]^+$	Theoretical m/z $[M+H]^+$	Δppm	Sum formula $[M+H]^+$
201.12347	201.12337	0.52	$C_9H_{17}O_3N_2$
267.13397	267.13393	0.14	$C_{13}H_{19}O_4N_2$
285.14453	285.1445	0.11	$C_{13}H_{21}O_5N_2$
303.23911	303.23907	0.12	$C_{14}H_{31}O_3N_4$
369.24973	369.24963	0.25	$C_{18}H_{33}O_4N_4$
385.24469	385.24455	0.37	$C_{18}H_{33}O_5N_4$
487.36063	487.36025	0.64	$C_{23}H_{47}O_5N_6$
587.37659	587.37629	0.51	$C_{27}H_{51}O_8N_6$

Table S8 TiO_2 NPs and IMAC binding capacities for SPE from saline aqueous matrices.

Method	Analyte	Binding Capacity
TiO_2 NP	DFOB	15.7 ± 0.2 µmol/mg TiO_2[a]
TiO_2 NP	TMP	17.4 ± 0.9 nmol/mg TiO_2[5]
TiO_2 NP	TPP	27.5 ± 2.8 nmol/mg TiO_2[5]
TiO_2 NP	DFOB	8.8 ± 0.1 µmol/mg TiO_2[b]
Ni(II)-IDA	DFOB	3.5 µmol/mL resin[6,7]
Yb(III)-BEDTRA	DFOB	8.0 µmol/mL resin[6]

TMP = Thiamine monophosphate; TPP = Thiamine pyrophosphate; (a) calculated from Figure 3 according to Krenkova et al.[8]; (b) calculated from Figure 3 according to Gu et al.[6]

Table S9 Summary of neutral losses with corresponding sum formula, parent ions and parent ion structure feature.

Neutral loss / Da	Sum formula [M]	Parent ion $[M+H]^+$	Parent ion structure
82	$C_4H_2O_2$	DFOG1 (m/z 619); **2** (m/z 603); **3** (m/z 587)	Linear
100	$C_5H_4O_3$	DFOG1 (m/z 619); **2** (m/z 603); **3** (m/z 587)	Linear
102	$C_5H_{14}N_2$	**2** (m/z 603); **3** (m/z 587)	Linear
118	$C_5H_{14}N_2O$	DFOG1 (m/z 619); **2** (m/z 603); **3** (m/z 587)	Linear
200	$C_9H_{16}N_2O_3$	DFOE (m/z 601); **1** (m/z 585)	Cyclic

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