Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Precursor-directed biosynthesis of catechol compounds in *Acinetobacter bouvetii* DSM 14964

Zachary L. Reitz, Alison Butler*

Department of Chemistry & Biochemistry

University of California

Santa Barbara, CA 93106-9510

Methods

Genome mining

The protein sequence for VibH was retrieved from UniProtKB (A0A0H3AII5). Homologs were found with phmmer (EMBL-EBI web server),[1] targeting the UniProtKB database and restricting taxonomy to *Acinetobacter*. Results were filtered by domain to find standalone condensation domains.

Culture conditions

Acinetobacter bouvetii DSM 14964 was obtained from DSMZ GmbH, Braunschweig, Germany. For siderophore isolation, *A. bouvetii* DSM 14964 was cultured in 1 L casamino acid minimal medium (5 g/L Bacto low-iron casamino acids, 1.54 g/L K_2HPO_4, and 0.25 g/L $MgSO_4$) in a 2L acid-washed Erlenmeyer flask. For the precursor-directed biosynthesis investigations, *A. bouvetii* DSM 14964 was cultured in 100 mL casamino acid medium in a 250 mL acid-washed Erlenmeyer flask; potential substrates were added directly after autoclave sterilization. Cultures were grown on an orbital shaker (180 rpm) at ambient temperature. Growth was monitored by measuring the optical density at 600 nm.

Siderophore isolation

After 72 h, the bacterial cell culture was centrifuged at 10000 rpm for 25 min to pellet the cells. Decanted supernatant was shaken with Amberlite XAD-4 resin (10% v/v) for 3 hours. The resin was isolated by filtration and washed with 5 column volumes nanopure water followed by 5 column volumes 10% v/v MeOH/H_2O. Organic compounds were eluted with 5 column volumes 90% v/v MeOH/H_2O and concentrated *in vacuo*. Siderophores were purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a semi-preparative C_4 column using a constant 7 mL/min flow rate and a linear methanol gradient (5 – 60%, 2%/min). Pooled fractions were concentrated *in vacuo*.

Siderophore characterization

Extracts were analyzed through positive ion mode ESI-MS or ESI-MS^E on a Waters Xevo G2-XS QTof coupled to a Waters Acquity H-Class UPLC system. A Waters BEH C18 column was used with a gradient of 0% to 60% acetonitrile/water (both with 0.1% w/v formic acid) over 12 minutes. GC-CI accurate mass data was obtained on a Waters GCT Premier Time of Flight mass spectrometer. Gas chromatography was performed using an Agilent 7890A GC equipped with a J&W Scientific DB-5MS narrow bore column (30m, 0.250mm ID, 0.25µm film thickness).

Chemical ionization was achieved using methane reagent gas. Positive mode accurate mass data was calibrated using known fragments of perfluorotributylamine as an internal standard. ESI accurate mass data was obtained on a Waters LCT Premier Time of Flight mass spectrometer. LC-MS grade methanol (Fisher Optima) was used as background eluent and solvent with no additives. Samples were directly infused using an Alliance 2695 Separations Module as solvent pump and autosampler. Positive mode accurate mass data were calibrated using polyethylene glycol or polyethylene glycol monomethyl ether internal standards as appropriate. NMR spectroscopy was carried out on Varian Unity Inova 500 MHz and 600 MHz spectrometers or a Bruker Avance NEO 500 MHz spectrometer equipped with a Prodigy coldprobe. Chemical shifts were referenced through residual DMSO-d_6 solvent peaks at 2.50 (^{1}H) or 39.51 (^{13}C) ppm.

1. S. C. Potter, A. Luciani, S. R. Eddy, Y. Park, R. Lopez and R. D. Finn, HMMER web server: 2018 update, *Nucleic Acids Res.*, 2018, **46**, W200-W204.

Characterization of novel compounds

N,N'-(azanediylbis(ethane-2,1-diyl))bis(2,3-dihydroxybenzamide) (4). ^{1}H NMR (600 MHz, DMSO) δ 12.31 (s, 2H), 9.23 (br s, 2H), 8.93 (br t, J = 5.6 Hz, 2H), 8.72 (br s, 1H), 7.28 (dd, J = 8.2, 1.5 Hz, 2H), 6.93 (dd, J = 7.8, 1.4 Hz, 2H), 6.69 (t, J = 7.9 Hz, 2H), 3.59 (q, J = 5.9 Hz, 4H), 3.17 (m, 4H). **^{13}C NMR** (126 MHz, DMSO) δ 170.76, 149.84, 146.68, 119.48, 118.52, 118.10, 115.58, 46.91, 36.07. **HRMS** for $C_{18}H_{22}N_3O_6^+$: calcd. $[M+H]^+$ 376.1508, found: 376.1507.

2,3-dihydroxy-N-(prop-2-yn-1-yl)benzamide (5). ^{1}H NMR (500 MHz, DMSO) δ 12.31 (s, 1H), 9.21 (s, 1H), 9.14 (br t, J = 5.5 Hz, 1H), 7.26 (dd, J = 8.1, 1.5 Hz, 1H), 6.91 (dd, J = 7.9, 1.5 Hz, 1H), 6.68 (t, J = 8.0 Hz, 1H), 4.06 (dd, J = 5.6, 2.5 Hz, 2H), 3.15 (t, J = 2.5 Hz, 1H). **^{13}C NMR** (126 MHz, DMSO) δ 169.79, 149.85, 146.68, 119.53, 118.66, 117.86, 115.33, 81.18, 73.68, 28.76. **HRMS** for $C_{10}H_{10}NO_3^+$: calcd. $[M+H]^+$ 192.0661, found: 192.0654.

N-allyl-2,3-dihydroxybenzamide (6). ^{1}H NMR (500 MHz, DMSO) δ 12.66 (br s, 1H), 9.16 (br s, 1H), 8.96 (br t, J = 5.9 Hz, 1H), 7.32 (d, J = 8.0 Hz, 1H), 6.92 (d, J = 7.7 Hz, 1H), 6.69 (t, J = 8.0 Hz, 1H), 5.90 (ddt, J = 16.0, 10.5, 5.3 Hz, 1H), 5.18 (d, J = 17.2 Hz, 1H), 5.12 (d, J = 10.3 Hz, 1H), 3.92 (t, J = 5.6 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 170.01, 150.07, 146.68, 135.22, 119.30, 118.46, 117.68, 116.00, 115.41, 41.67. **HRMS** for $C_{10}H_{12}NO_3^+$: calcd. $[M+H]^+$ 194.0817, found: 194.0818.

Table S1. *Acinetobacter* strains putatively containing a siderophore biosynthetic gene cluster with a VibH homolog.

Protein Accession	Organism
A0A4Q7ARR8_9GAMM	*Acinetobacter bouvetii*
N9DJ67_9GAMM	*Acinetobacter bouvetii* DSM 14964 = CIP 107468
A0A1A7R8J8_9GAMM	*Acinetobacter gandensis*
A0A1B2M479_9GAMM	*Acinetobacter larvae*
A0A4Q4H0F1_9GAMM	*Acinetobacter piscicola*
A0A151Y3Z5_9GAMM	*Acinetobacter pragensis*
A0A240ECV6_9GAMM	*Acinetobacter puyangensis*
A0A241WES3_9GAMM	*Acinetobacter* sp. ANC 3813
A0A4R0ECX9_9GAMM	*Acinetobacter* sp. ANC 4249
A0A1H7NGA5_9GAMM	*Acinetobacter* sp. DSM 11652
N9M6P9_9GAMM	*Acinetobacter* sp. NIPH 713
A0A031LTQ2_9GAMM	*Acinetobacter* sp. Ver3
A0A3B7LX09_9GAMM	*Acinetobacter* sp. WCHAc010005
A0A3B7PT29_9GAMM	*Acinetobacter* sp. WCHAc010034
A0A3B7M868_9GAMM	*Acinetobacter* sp. WCHAc010052
A0A3A8ECI6_9GAMM	*Acinetobacter* sp. WCHAc060012

Table S2. The putative siderophore biosynthetic gene cluster of *A. bouvetii* DSM 14964			
Name	**Locus tag**	**Accession**	**Putative function**
bsuB	B090_RS0102660	WP_005009566.1	Ferric siderophore reductase
absH	B090_RS0102665	WP_005009568.1	VibH-like condensation domain
absA	B090_RS0102670	WP_005009570.1	2,3-dihydro-2,3-DHB dehydratase
absC	B090_RS0102675	WP_005009576.1	Isochorismate synthase
absE	B090_RS0102680	WP_005009579.1	2,3-DHB–AMP ligase
absB	B090_RS0102685	WP_005009581.1	Isochorismatase
absG	B090_RS0102690	WP_005009583.1	Aryl carrier protein
absI	B090_RS0102695	WP_005009584.1	Ornithine decarboxylase
bsuA	B090_RS0102700	WP_026253326.1	TonB-dependent receptor

Table S3. Natural and non-natural amines used in precursor directed biosynthesis studies.*		
Substrate	**Single Addition Product**	**Double Addition Product**
Diamines		
Ethylenediamine	**Major**	N.D.†
1,3-Diaminopropane	N.D.	**Major**
Putrescine (1,4-diaminobutane)	N.D.	**Major**
Cadaverine (1,5-diaminopentane)	N.D.	**Major**
1,6-Diaminohexane	**Major**	**Major**
Amino acids		
L-Diaminobutyric acid	N.D.	N.D.
L-Ornithine	N.D.	N.D.
L-Lysine	N.D.	N.D.
Polyamines		
Diethylenetriamine	N.D.	**Major**
Norspermidine	*No growth detected*	
Tris(2-aminoethyl)amine (Tren)	*No growth detected*	
Other amines		
Histamine	N.D.	-
Allylamine	**Major**	-
Propargylamine	**Major**	-
* Substrates were added to casamino acid minimal medium prior to inoculation at a final concentration of 1 mM. † N.D., not detected.		

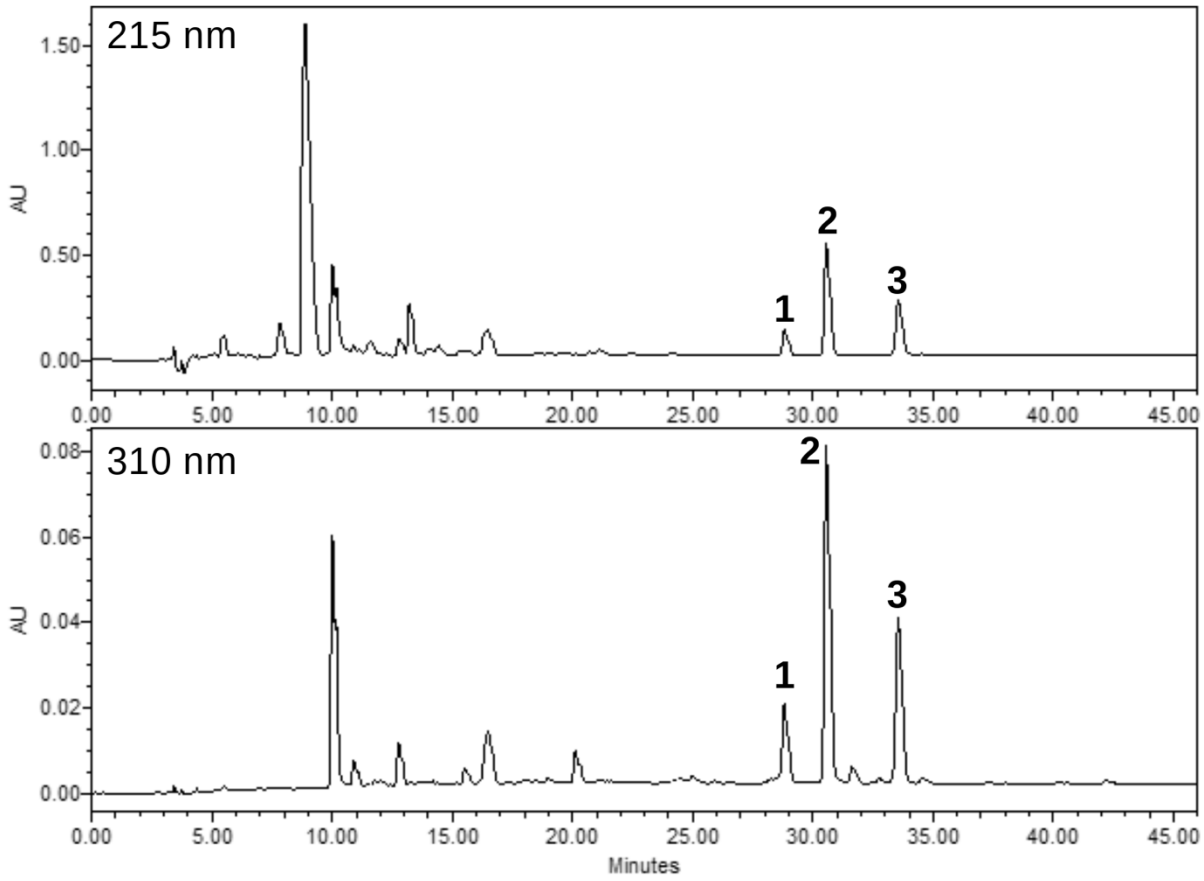

Figure S1. HPLC–UV/Vis chromatogram of *A. bouvetii* DSM 14964 supernatant extract from growth in low iron medium. Top: chromatogram monitored at 215 nm. Bottom: chromatogram monitored at 310 nm. Peaks corresponding to natural siderophores **1**, **2**, and **3** are labeled.

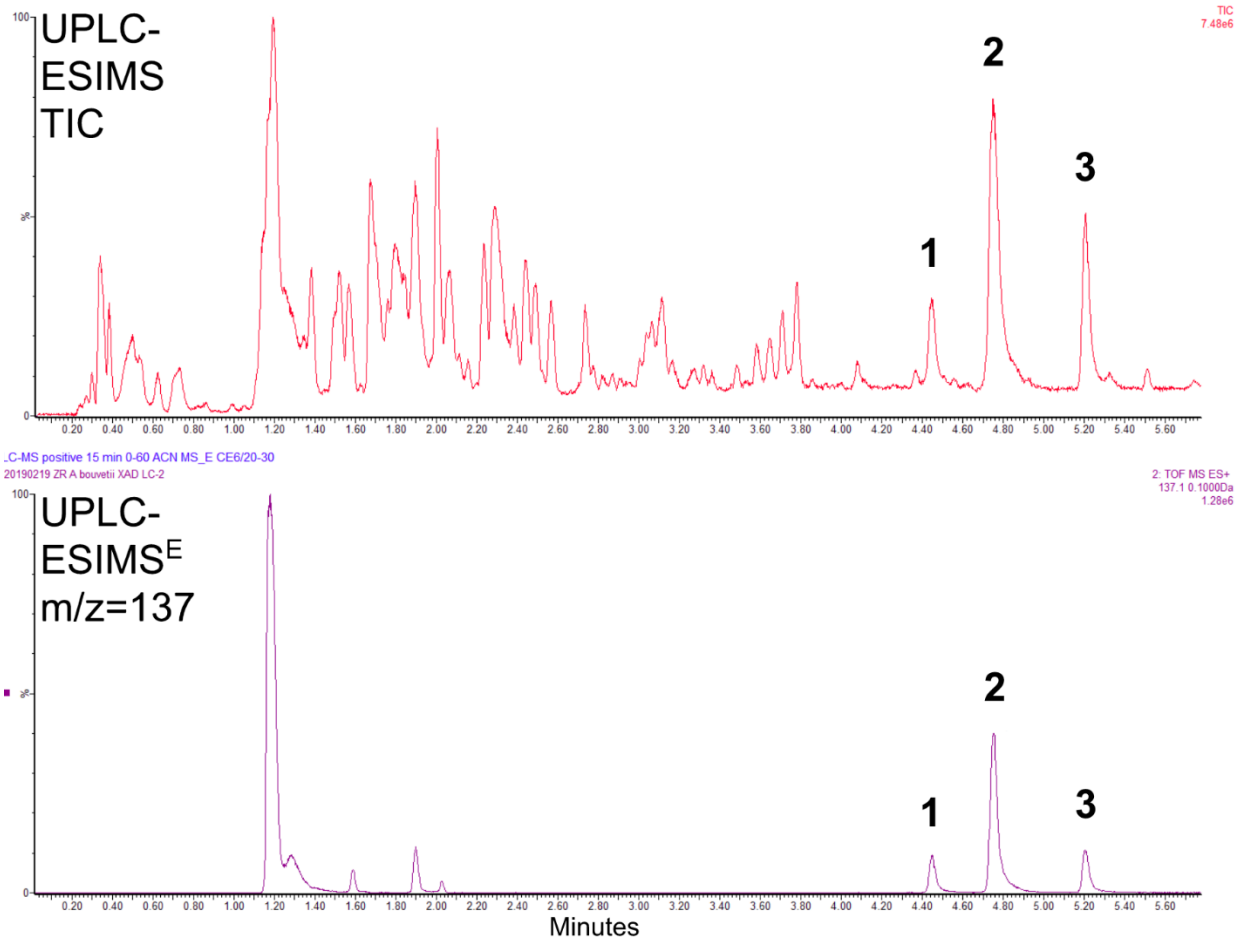

Figure S2. UPLC–ESIMS spectra of *A. bouvetii* DSM 14964 supernatant extract from a low iron medium. Top: Total ion chromatogram. Bottom: MS^E high-collision-energy extracted ion chromatogram for 137 m/z. Peaks corresponding to natural siderophores **1**, **2**, and **3** are labeled.

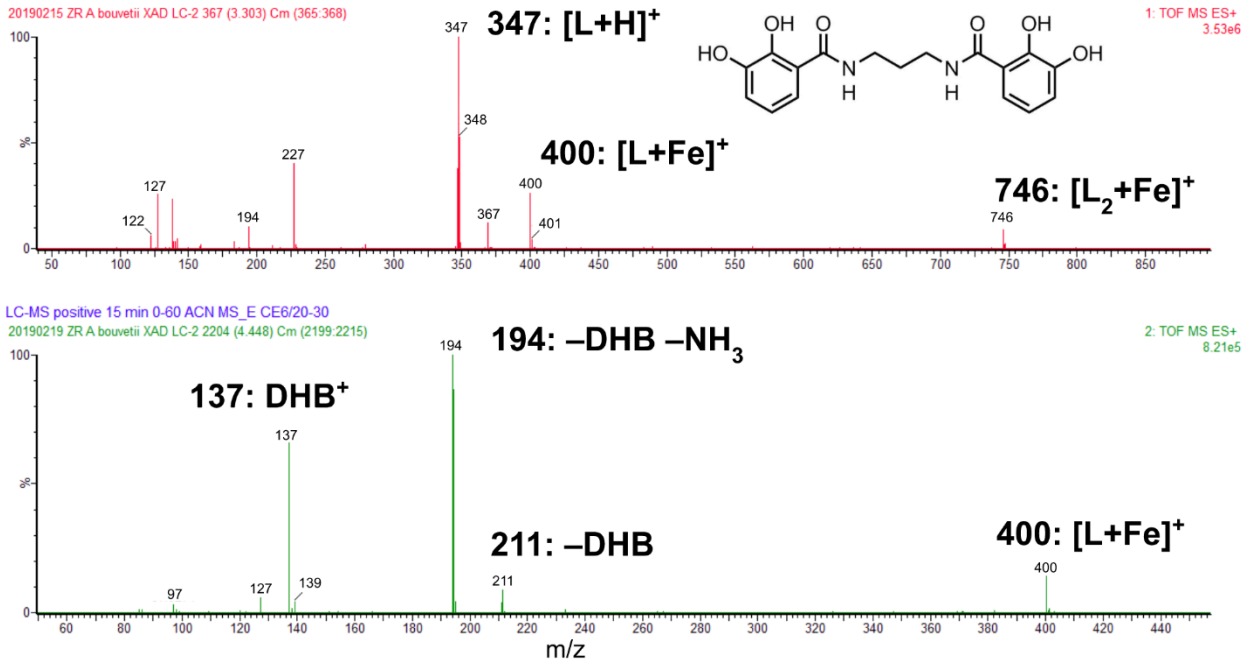

Figure S3. Mass spectrometry and fragmentation of propanochelin (**1**). Top: ESIMS spectrum. Bottom: high-collision-energy MS^E fragmentation.

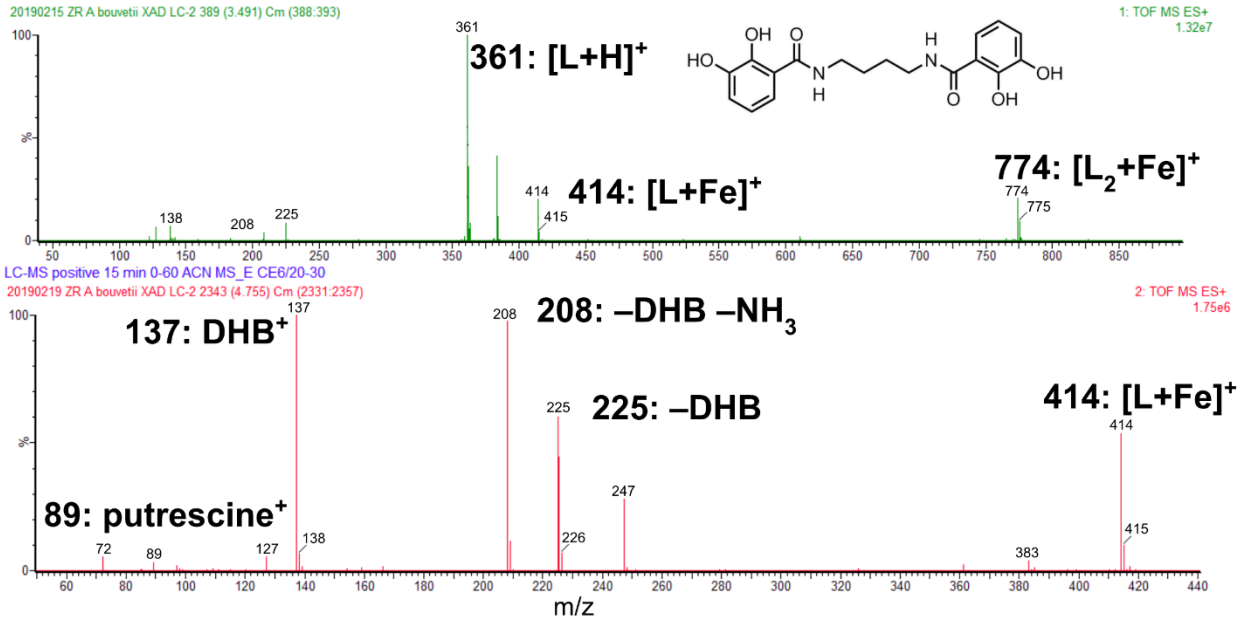

Figure S4. Mass spectrometry and fragmentation of butanochelin (**2**). Top: ESIMS spectrum. Bottom: high-collision-energy MS^E fragmentation.

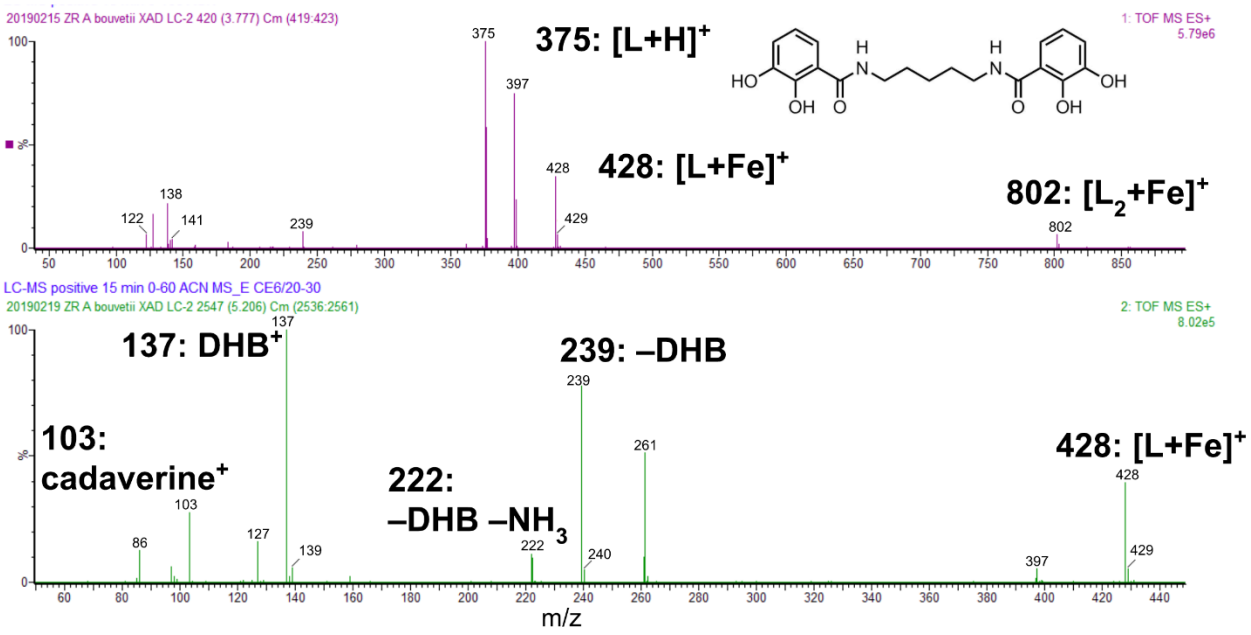

Figure S5. Mass spectrometry and fragmentation of pentanochelin (**3**). Top: ESIMS spectrum. Bottom: high-collision-energy MS^E fragmentation.

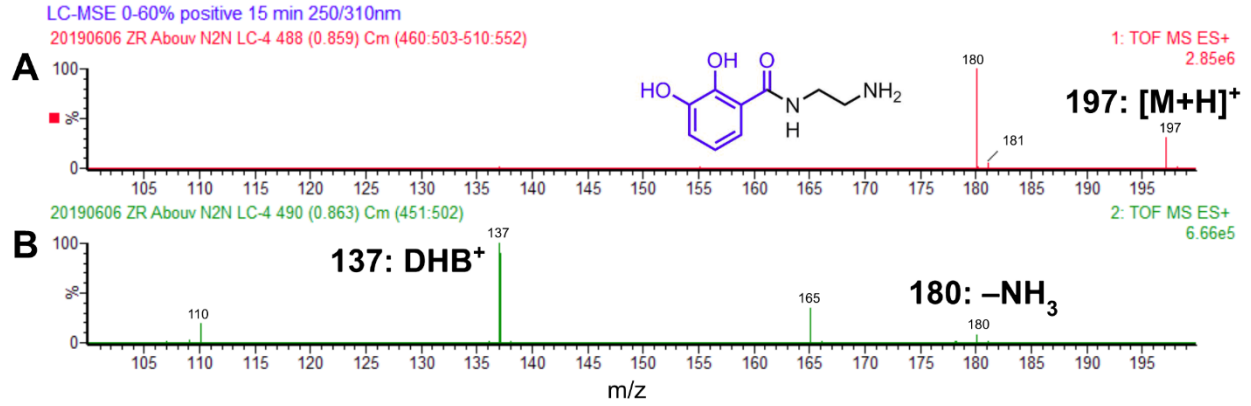

Figure S6. Mass spectrometry and fragmentation of the catecholic product unique to ethylenediamine supplementation in the growth medium. A: ESIMS spectrum and proposed structure. B: high-collision-energy MS^E fragmentation.

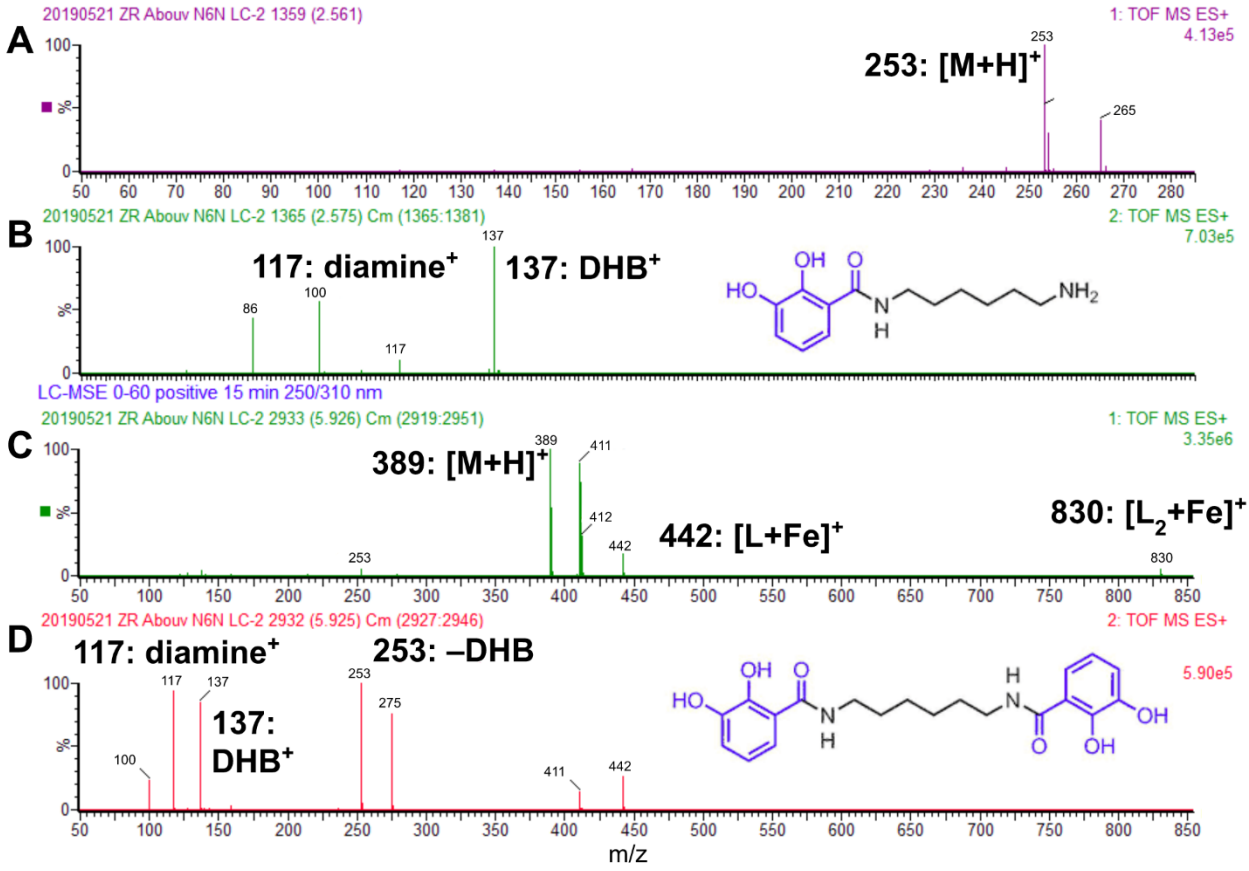

Figure S7. Mass spectrometry and fragmentation of the catecholic products unique to 1,6-diaminohexane supplementation in the growth medium. A: ESIMS spectrum of the single 2,3-DHB addition product. B: high-collision-energy MS^E fragmentation and proposed structure. C: ESIMS spectrum of the double 2,3-DHB addition product. D: high-collision-energy MS^E fragmentation and proposed structure.

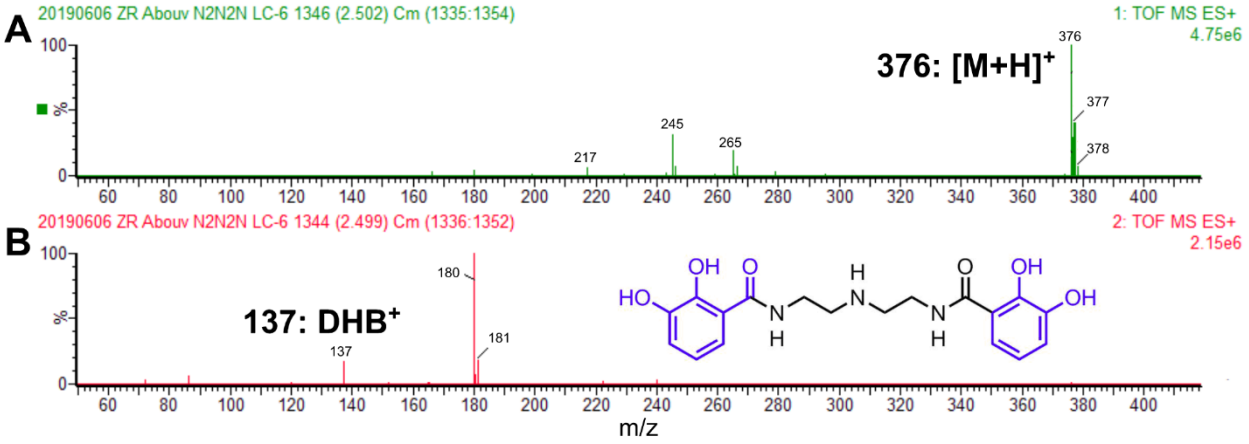

Figure S8. Mass spectrometry and fragmentation of the catecholic product **4** unique to diethylene triamine supplementation. A: ESIMS spectrum. B: high-collision-energy MS^E fragmentation and proposed structure.

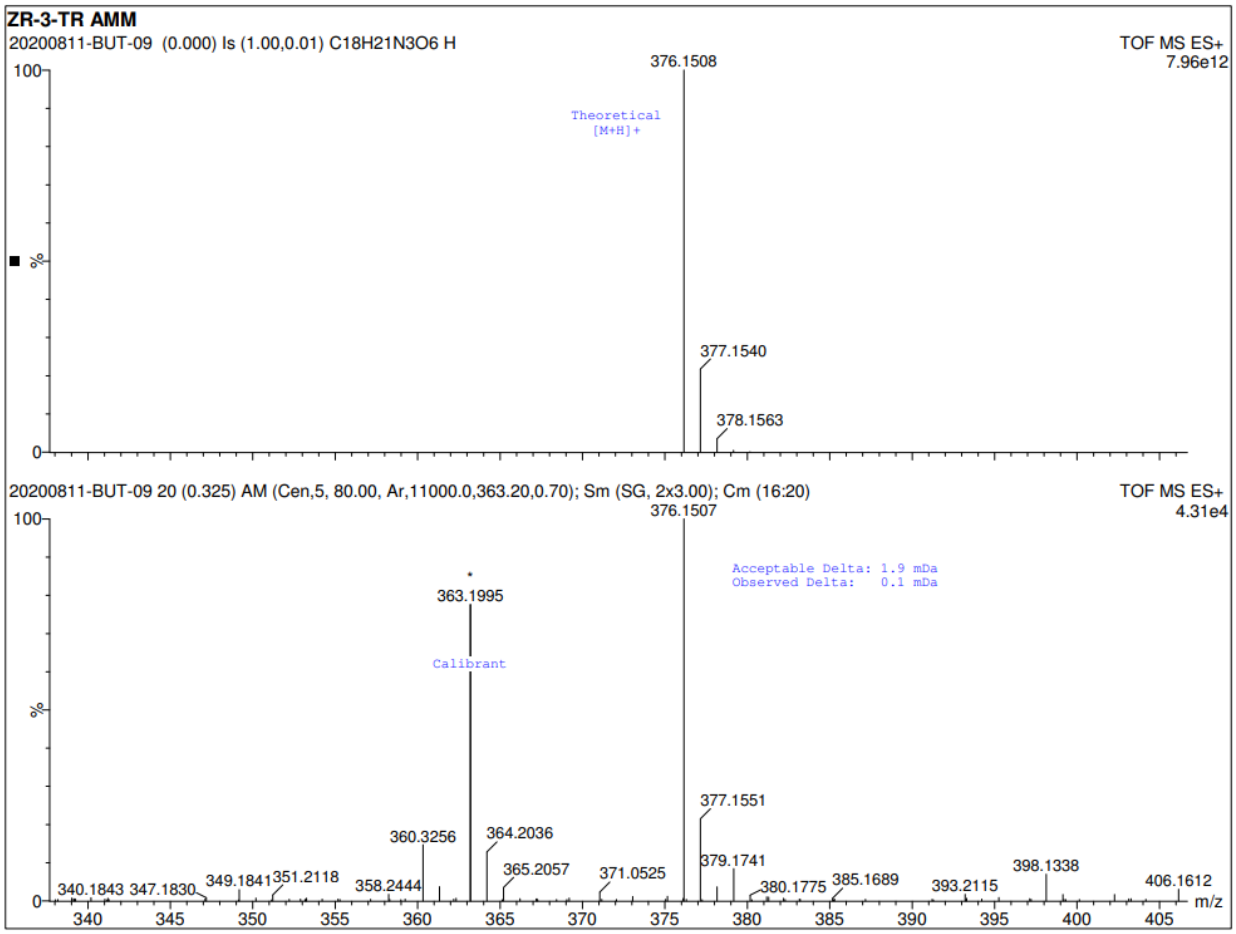

Figure S9. High-resolution mass spectrometry (positive-mode ESI) of the catecholic **4** product unique to diethylene triamine supplementation.

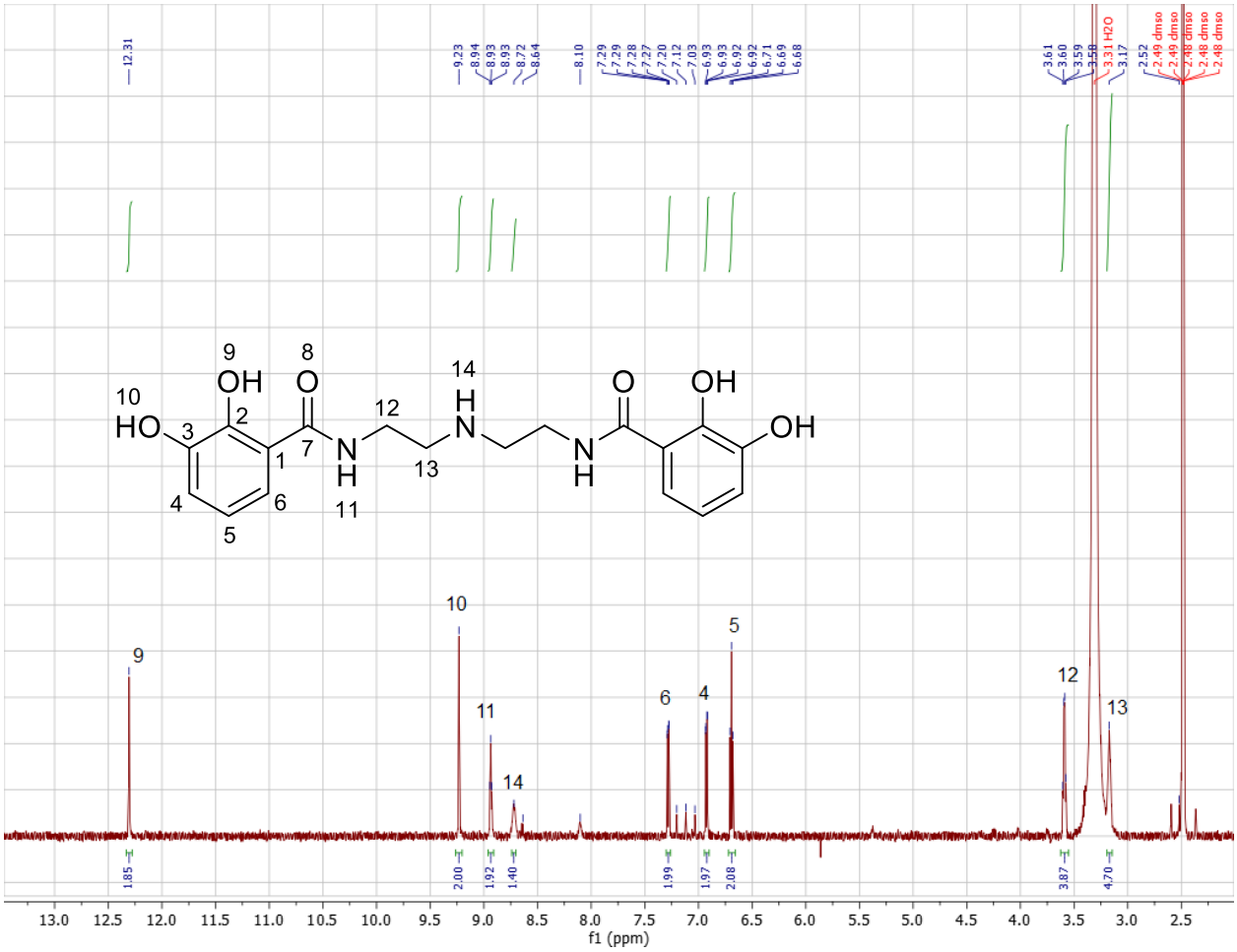

Figure S10. ^{1}H NMR (DMSO-d_6; 500 MHz) of the catecholic product **4** unique to diethylene triamine supplementation.

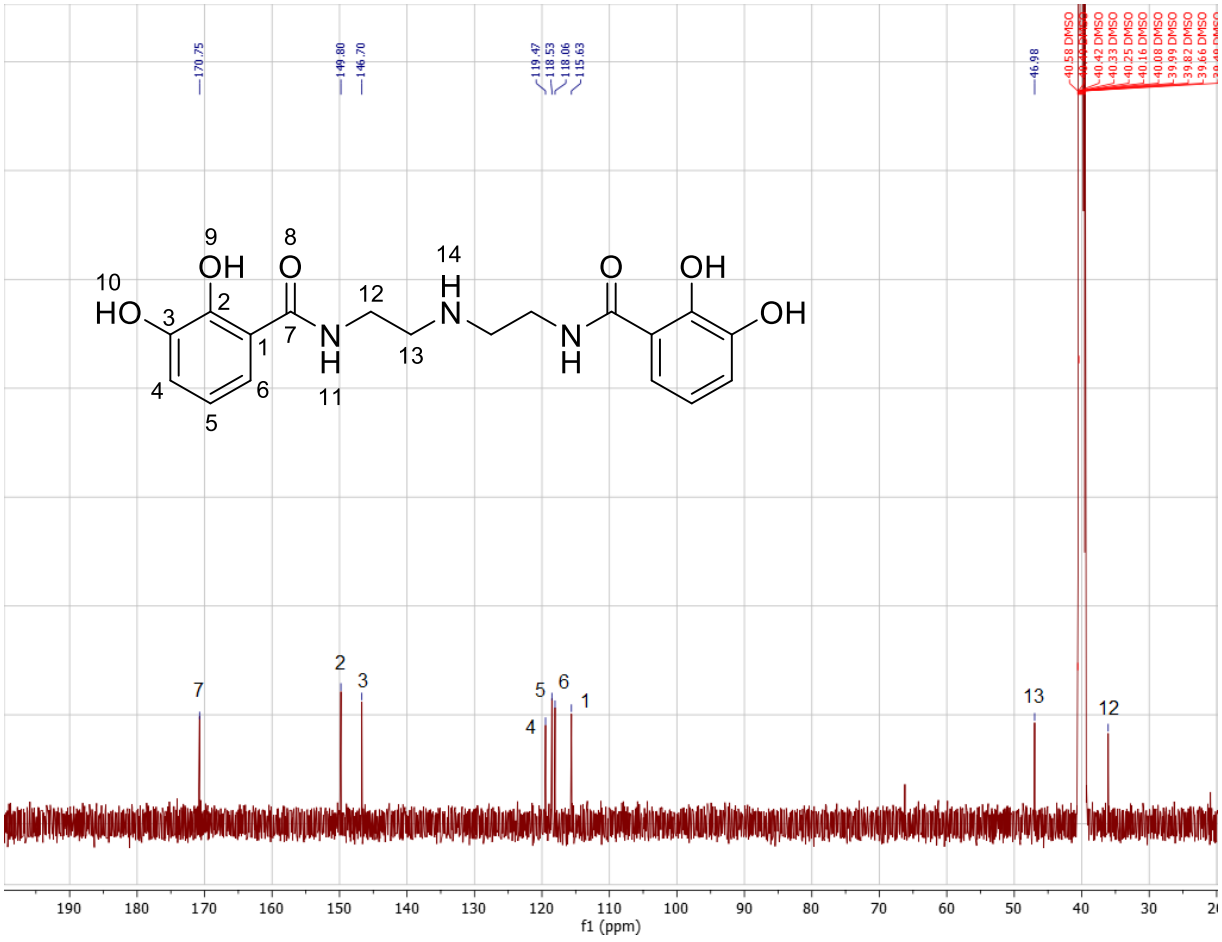

Figure S11. ^{13}C NMR (DMSO-d_6; 126 MHz) of the catecholic product **4** unique to diethylene triamine supplementation.

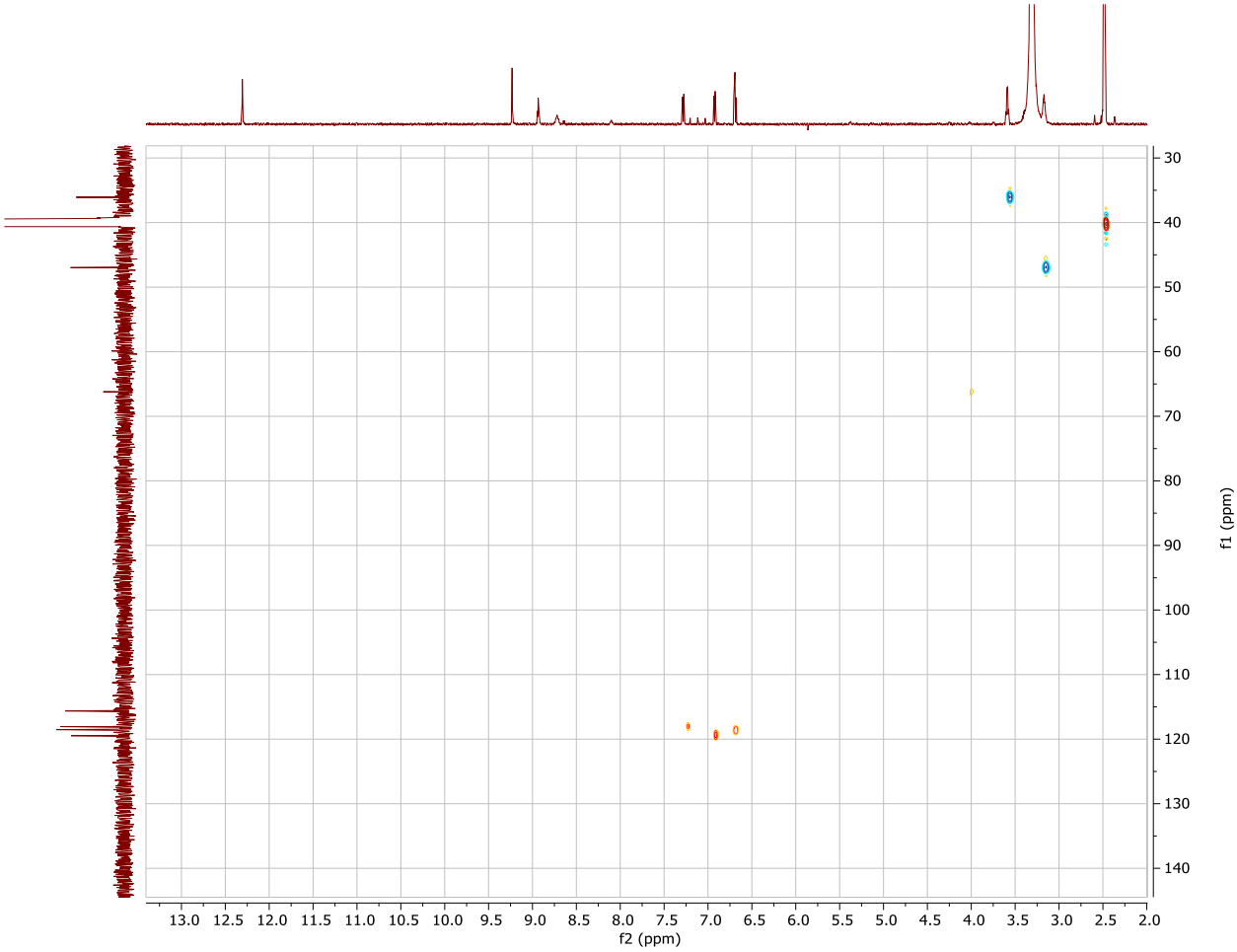

Figure S12. ^{1}H-^{13}C HSQC NMR (DMSO-d_6; 600 MHz) of the catecholic product **4** unique to diethylene triamine supplementation.

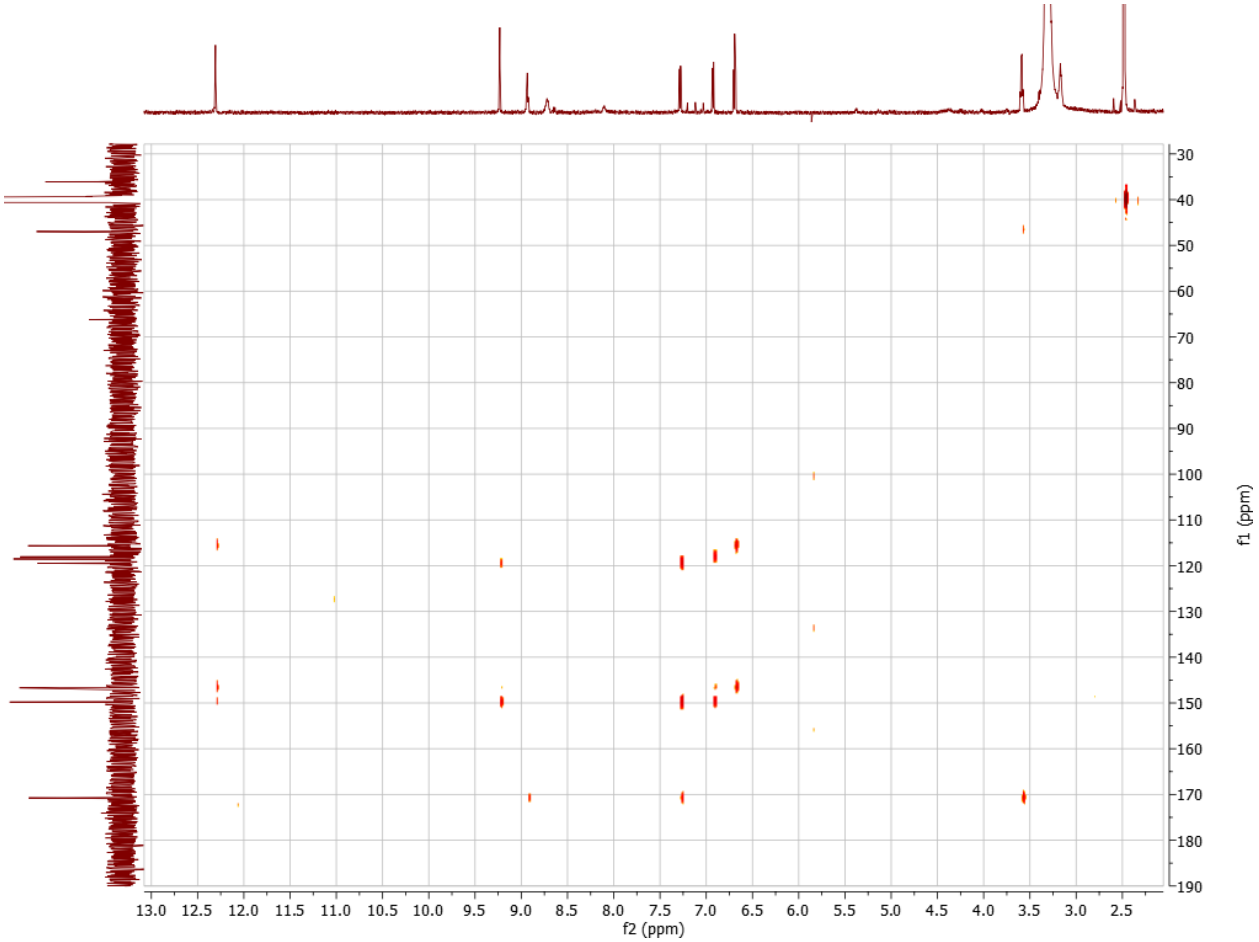

Figure S13. ^{1}H-^{13}C HMBC NMR (DMSO-d_6; 600 MHz) of the catecholic product **4** unique to diethylene triamine supplementation.

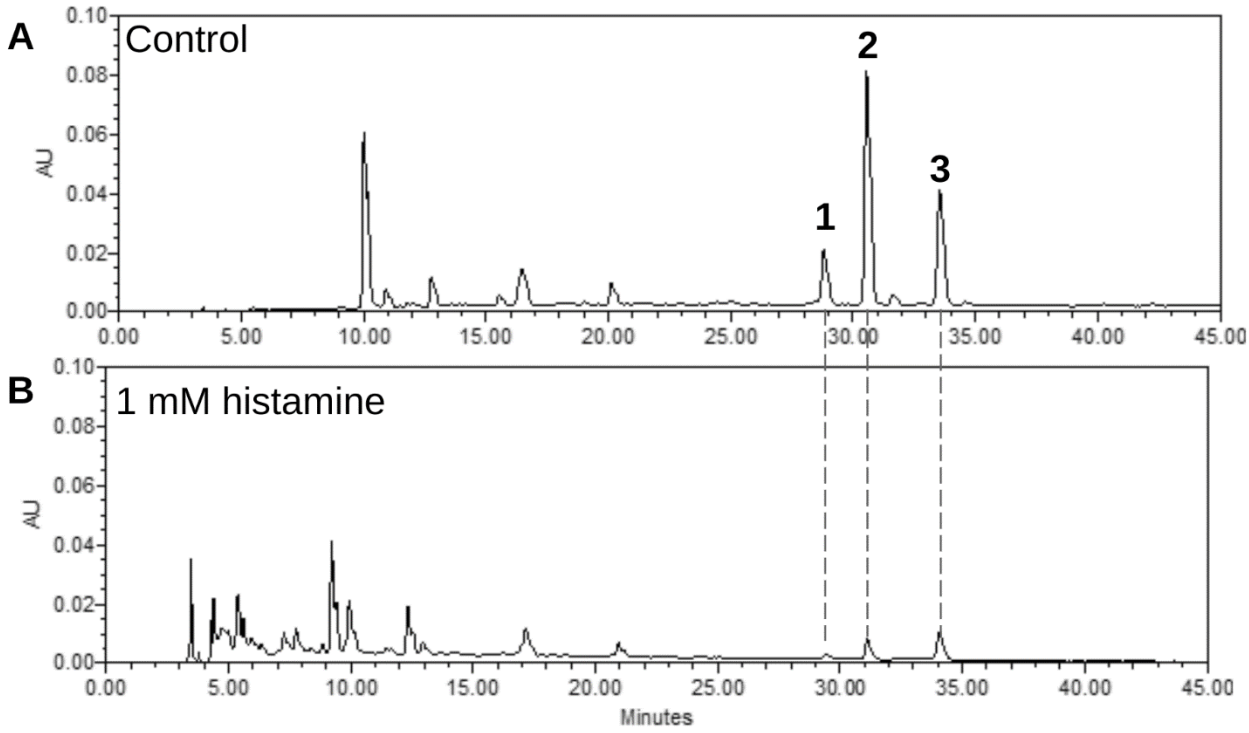

Figure S14. Supplementation of *A. bouvetii* DSM 14964 growth with functionalized amines. HPLC of the bacterial supernatant monitored at 310 nm of *A. bouvetii* DSM 14964 grown with (A) no added substrate or (B) 1 mM histamine. Peaks corresponding to natural siderophores **1**, **2**, and **3** are labeled.

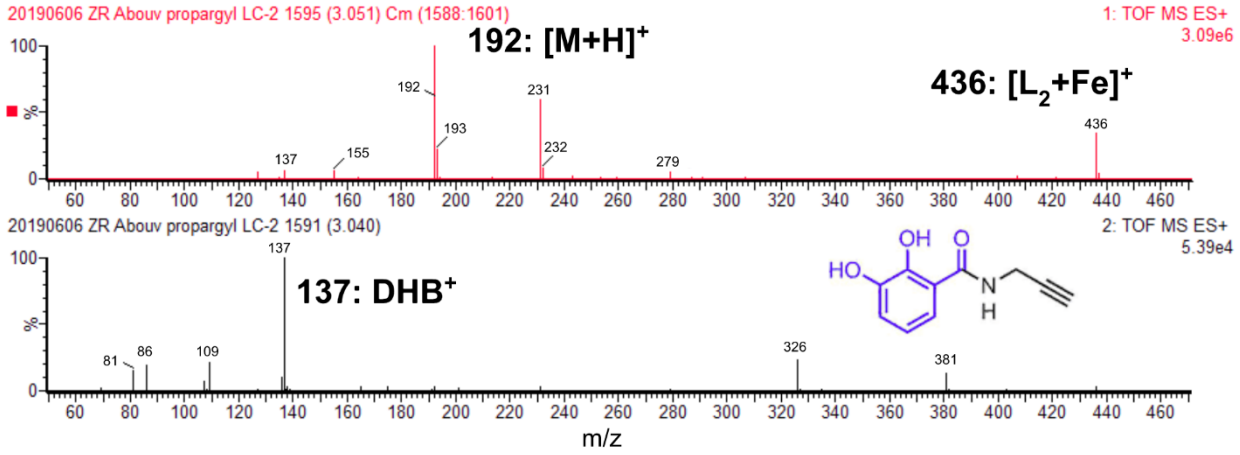

Figure S15. Mass spectrometry and fragmentation of the catecholic product **5** unique to propargylamine supplementation. A: ESIMS spectrum. B: high-collision-energy MS^E fragmentation and proposed structure.

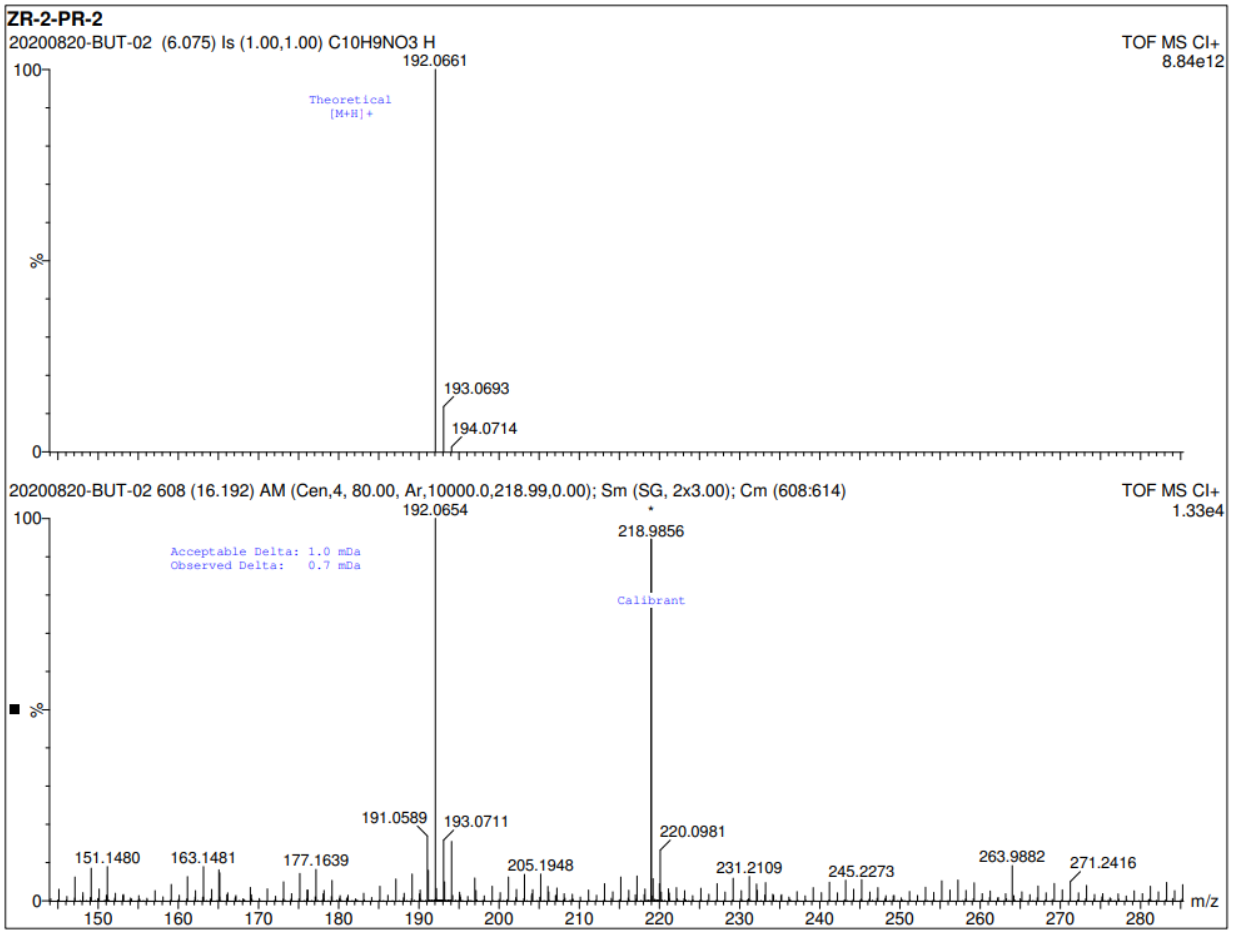

Figure S16. High-resolution mass spectrometry (positive-mode CI) of the catecholic product **5** unique to propargylamine supplementation.

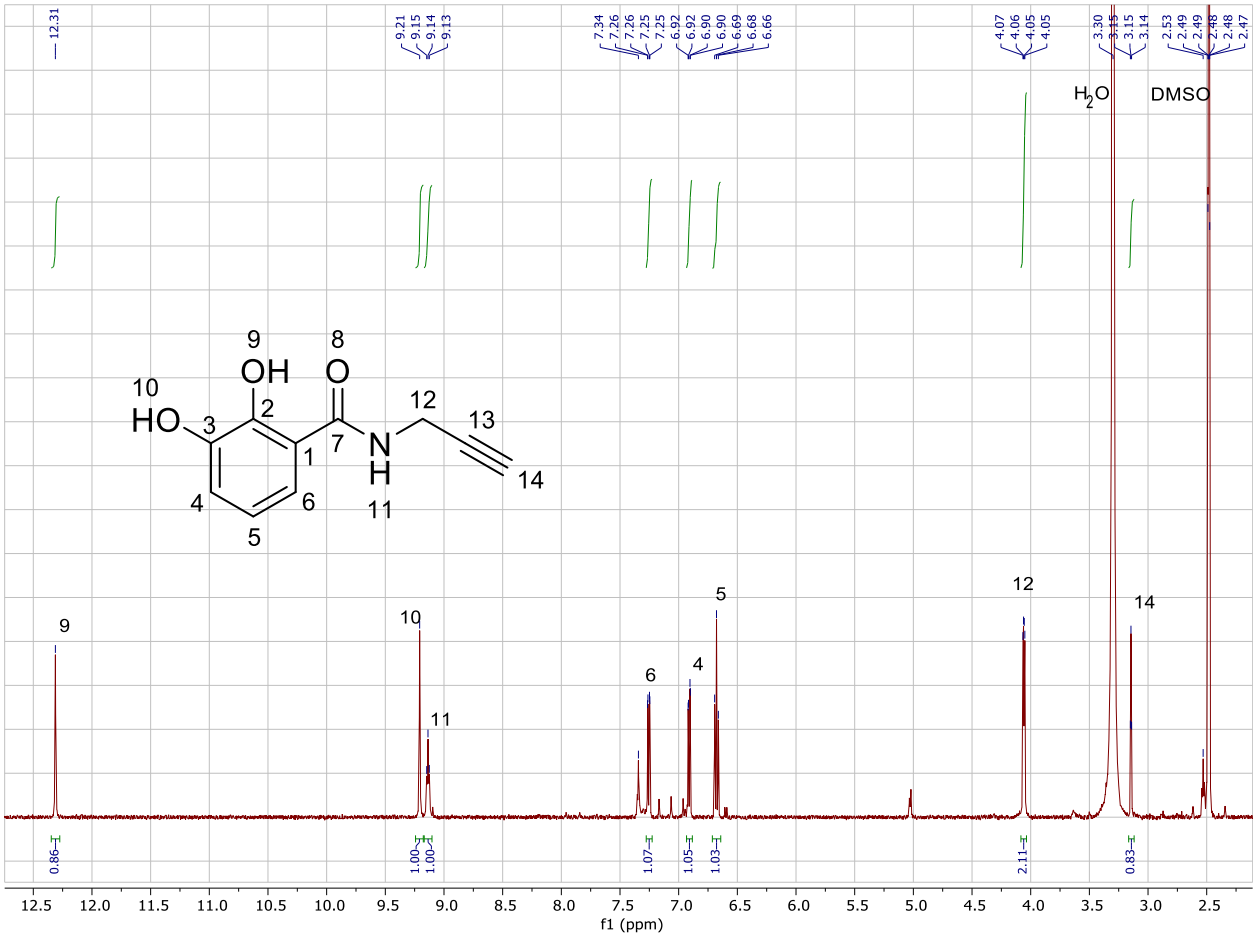

Figure S17. ^{1}H NMR (DMSO-d_6; 500 MHz) of the catecholic product **5** unique to propargylamine supplementation.

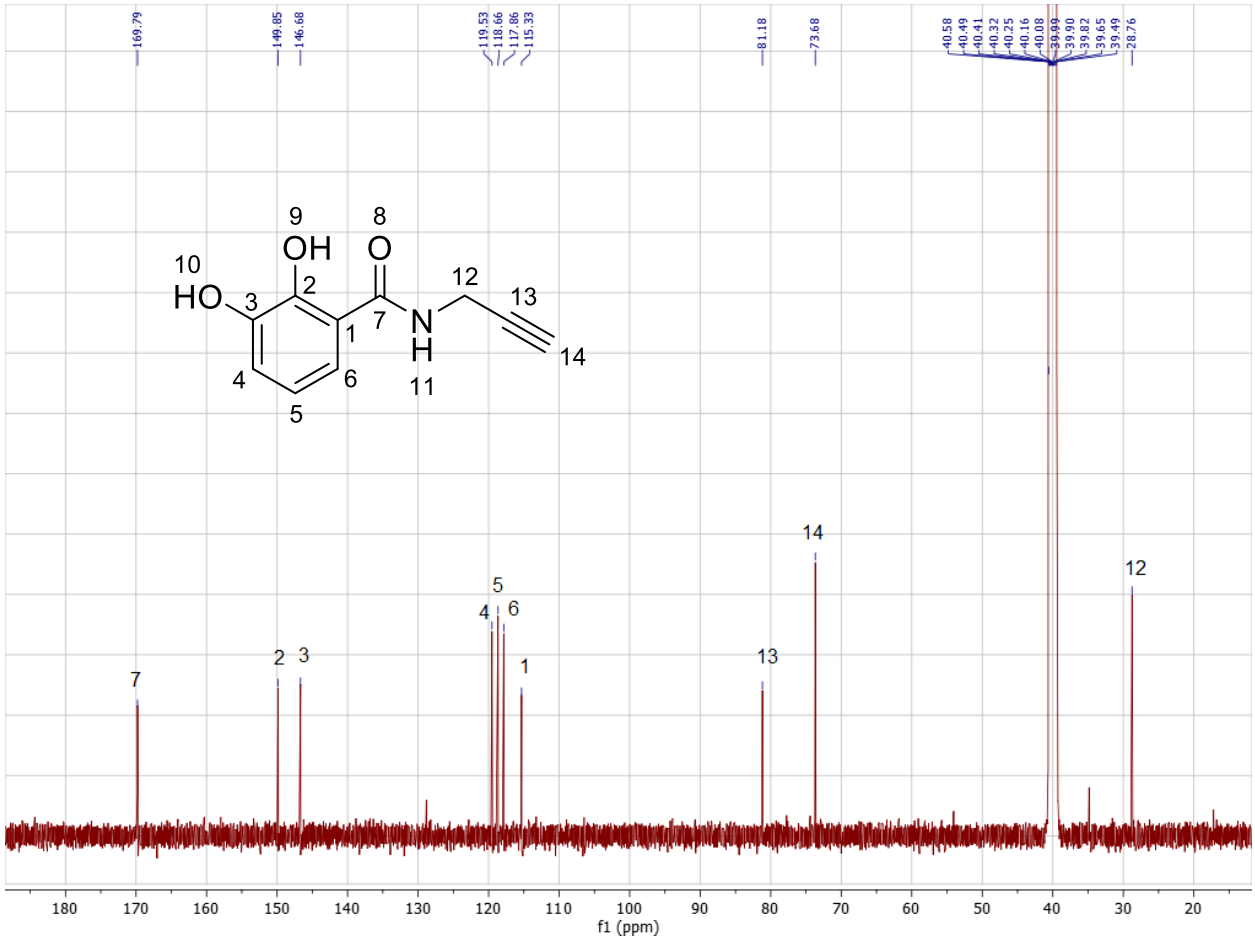

Figure S18. ^{13}C NMR (DMSO-d_6; 126 MHz) of the catecholic product **5** unique to propargylamine supplementation.

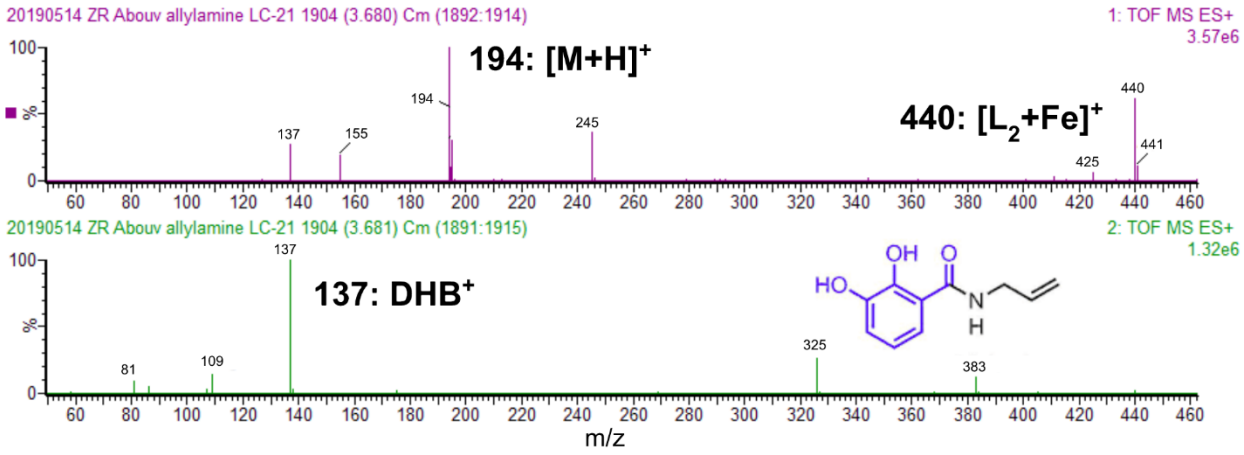

Figure S19. Mass spectrometry and fragmentation of the catecholic product **6** unique to allylamine supplementation. A: ESIMS spectrum. B: high-collision-energy MS^E fragmentation and proposed structure.

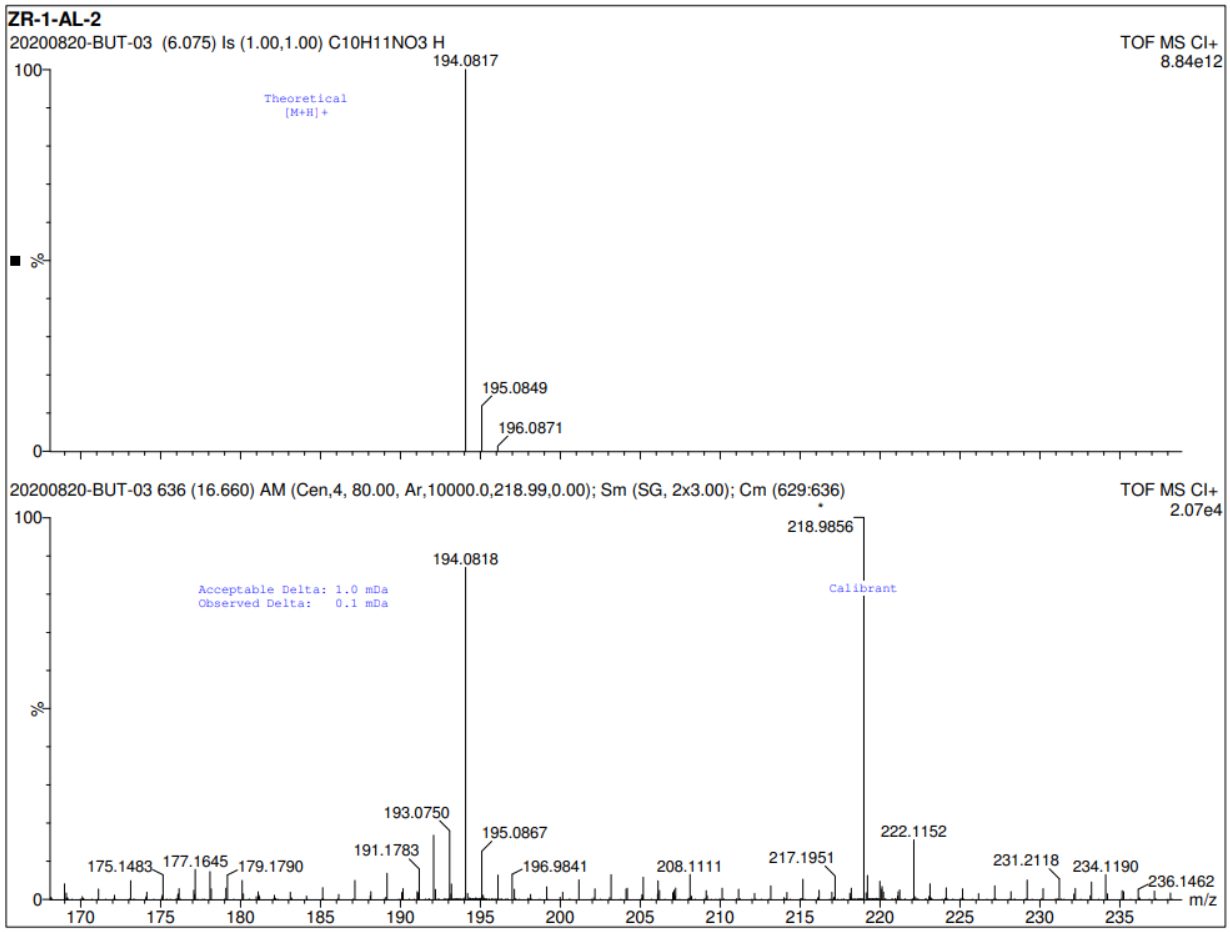

Figure S20. High-resolution mass spectrometry (positive-mode CI) of the catecholic product **6** unique to allylamine supplementation.

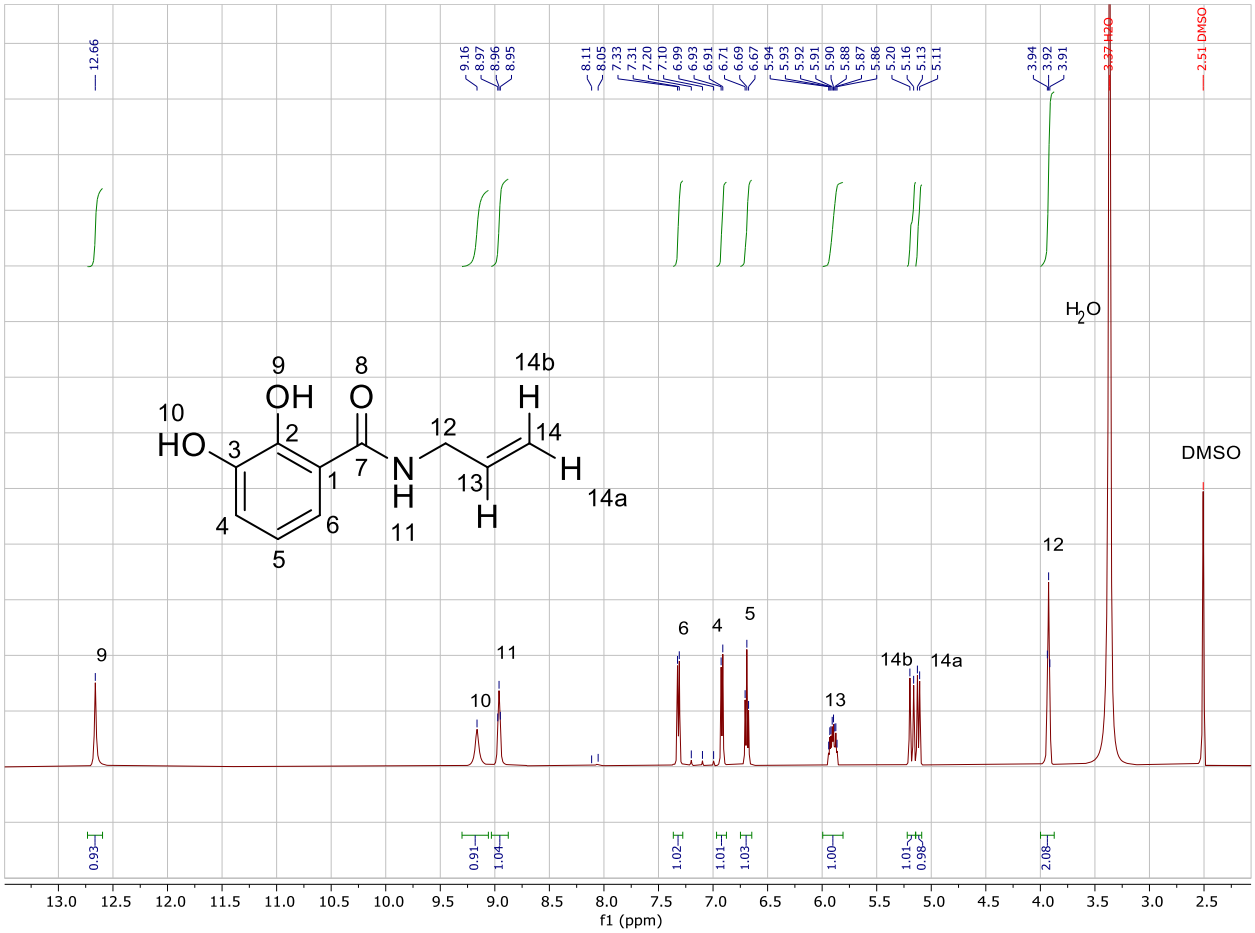

Figure S20. ^{1}H NMR (DMSO-d_6; 500 MHz) of the catecholic product **6** unique to allylamine supplementation.

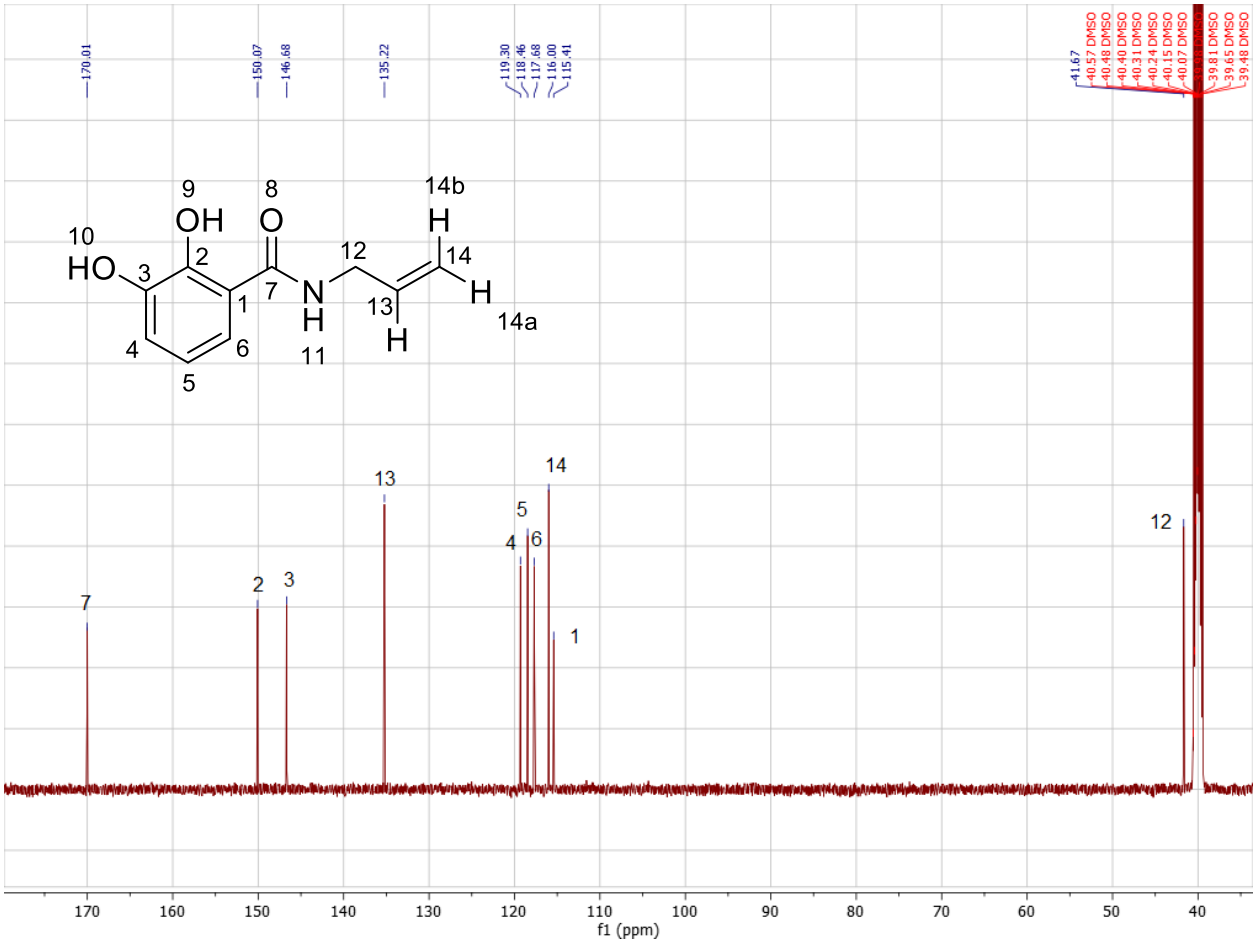

Figure S21. ^{13}C NMR (DMSO-d_6; 126 MHz) of the catecholic product **6** unique to allylamine supplementation.