Supplemental Information for Behling, L.A. et al. *J Amer Chem Soc* **2008**

NMR, Mass Spectrometry and Chemical Evidence Reveal a Different Chemical Structure for Methanobactin That Contains Oxazolone Rings

Contents

Background

The published structure for methanbactin

A chemical structure for methanobactin (mb) isolated from *Methylosinus trichorsporium* Ob3b was first published by Kim *et al*, 2004[1]. This structure is shown in Figure S1 and was based on mass spectroscopy data obtained after limited hydrolyses.[2,3] of methanobactin. It also relied on an X-ray determined structure[1] of copper-bound methanobactin for the chemical identification of the two chromophoric residues. These were identified as hydroxyimidazolates and are labeled THI and HTI in Figure S1. This structure also shows the N-terminus capped with an isopropyl ester. We find the isopropyl ester is inconsistent with ^{1}H and ^{13}C NMR resonance assignments (Figure S2 and Table S1), which indicate that this group should be a 3-methylbutanoyl group (Figure 1).

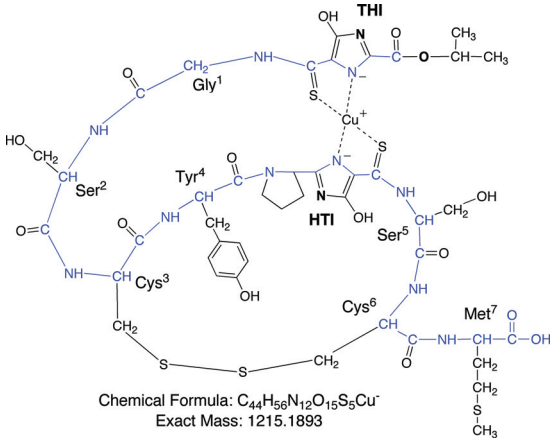


Figure S1. The previously published structure for methanobactin:[1] *N*-2-Isopropylester-(4-thionyl-5-hydroxy-imidzole)-Gly[1]-Ser[2]-Cys[3]-Tyr[4]-pyrrolidine-(4-hydroxy-5-thionyl-imidazole)-Ser[5]-Cys[6]-Met[7]. The model shown has the two hydroxyimidazolate rings ligated to Cu^{1+} to form the $(M\text{-}2H+^{63}Cu)^{1-}$ charged species that is observed by mass spectroscopy. The backbone is traced in blue.

Experimental

Procedures for isolating copper bound methanobactin after exposure to Cu(II).

Methanobactin was isolated from the spent media of *Methylosinus trichorsporium* Ob3b as described by Choi *et al*.[4] For uniformly ^{15}N-labeled methanobactin, $K^{15}NO_3$ (Sigma Chemical Co.) was used in the culture media as the sole nitrogen source. By this procedure, methanobactin is isolated in its copper-free form, which makes it possible to carry out systematic metal ion binding studies.[5,6] These studies revealed that major spectroscopic changes associated with copper binding are complete by around 0.6 Cu per methanobactin. We have used ^{1}H 1D and [^{1}H,^{15}N]-HSQC NMR experiments to monitor Cu ion binding by methanobactin and observe a similar leveling off of changes at around 0.6 Cu:mb (data not shown). For the studies reported in this communication, we used HPLC to isolate the Cu(I)-bound methanobactin formed after exposure to between 0.5 and 1.0 Cu:mb.

To prepare Cu(I)-bound methanobactin, lyophilized, copper-free methanobactin was dissolved in a 10 mM sodium phosphate buffer at pH 6.5. The concentration of the methanobactin was determined by UV/Vis spectrophotometry using a molar extinction coefficient of $\varepsilon_{394} = 16.07$ mM^{-1}cm^{-1} for the unbound methanobactin and $\varepsilon_{394} = 9.75$ mM^{-1}cm^{-1}for the Cu-bound methanobactin.[6] Cu(II) was added as $CuSO_4$ in 0.1 Cu:mb increments and the *pH* readjusted to 6.5 with NaOH after each addition. After reaching between 0.5 and 1.0 Cu:mb, the sample was subjected to reverse phase HPLC on a Hamilton 305 mm x 7 mm PRP-3 column and eluted with a gradient of 1% to 99% MeOH containing 1 mM ammonium acetate buffer, *pH* 5.0. After HPLC, the fraction containing the Cu(I)-bound methanobactin was lyophilized, redissolved in water, and lyophilized a second time.

NMR experiments

For NMR analyses, the lyophilized Cu(I)-bound methanobactin was dissolved to a concentration of between 2 and 5 mM in 9 mM sodium phosphate, pH 6.5, containing 10% D_2O, or in 100% D_2O adjusted to pH 6.5. Spectra were collected at either 5 °C or 25 °C on a 400 MHz Bruker Avance II spectrometer equipped with either a z-gradient, inverse detection TXI probe, or z-gradient, broadband BBO probe. Some experiments were also carried out on a 600 MHz Varian VNMRS spectrometer equipped with a z-gradient, inverse detection HCN probe. 1D-^{1}H, [^{1}H-^{1}H]-COSY, [^{1}H-^{1}H]-TOCSY, [^{1}H-^{1}H]-ROESY, [^{1}H-^{13}C]-HSQC, [^{1}H-^{15}N]-HSQC and [^{1}H-^{13}C]- HMBC experiments were collected with the inverse detection probes. ^{15}N-1D direct-detection experiments were collected with the BBO probe. WATERGATE 3919 pulse sequences were used for solvent suppression in the ^{1}H-1D, [^{1}H-^{1}H]-COSY, [^{1}H-^{1}H]-TOCSY, and [^{1}H-^{1}H]-ROESY experiments. The [^{1}H-^{1}H]-ROESY experiments were collected using a mixing time of 200 ms. [^{1}H-^{15}N]- HSQC indirect detection and ^{15}N-1D direct detection experiments were carried out on uniformly ^{15}N-labeled Cu(I)-bound methanobactin samples that were prepared as described above for the unlabeled samples. The spectra obtained from these experiments were processed using either *iNMR*[7] or *NMRPipe*,[8] and analyzed using *Sparky*.[9] The ^{1}H, ^{13}C and ^{15}N assignments obtained from these analyses are shown in Table S1. Figure S2 shows the [^{1}H-^{15}N]-HSQC spectrum, along with ^{1}H and ^{15}N external projections, which were used to assign the seven amide nitrogens and the pyrrolidinyl nitrogen.

Table S1: ^{1}H, ^{13}C, and ^{15}N Resonance Assignments for Methanobactin from *Methylosinus trichosporus* OB3b

		Resonances (ppm)*		
	Atom	^{1}H	^{13}C	^{15}N
3-Methyl-	C^{1}		188.1	
butanoyl	C^{2}		46.3	
	C^{3}		26.0	
	C^{4}		21.6	
	C^{5}		22.2	
	H^{2}	3.07		
	H^{2}	3.11		
	H^{3}	2.11		
	H^{4}	0.82		
	H^{5}	0.73		
				225.9
Oxazolone A	N^{3}			or
				248.5
Gly^{1}	N			121.7
	C		173.5	
	C^{α}		48.6	
	H	9.28		
	H^{α}	4.46		
	H^{α}	4.59		
Ser^{2}	N			117.6
	C		171.6	
	C^{α}		57.5	
	C^{β}		60.6	
	H	9.04		
	H^{α}	4.41		
	H^{β}	3.97		
	H_{β}	4.10		
Cys^{3}	N			117.2
	C		171.0	
	C^{α}		52.6	
	C^{β}		44.8	
	H	7.73		
	H^{α}	5.12		
	H^{β}	2.80		
	H^{β}	3.78		
Tyr^{4}	N			123.5
	C		171.9	
	C^{α}		55.1	
	C^{β}		36.2	
	C^{γ}		126.3	
	C^{δ}		130.1	
	C^{ε}		115.3	
	C^{ζ}		155.2	
	H	7.11		
	H^{α}	4.30		
	H^{β}	2.75		
	H^{β}	2.86		
	H^{δ}	6.57		
	H^{ε}	6.61		

		Resonances (ppm)		
Residue	Atom	^{1}H	^{13}C	^{15}N
Pyrrolidinyl	N^{1}			142.1
	C^{2}		55.5	
	C^{3}		30.8	
	C^{4}		22.1	
	C^{5}		46.2	
	H^{2}	3.71		
	H^{3}	1.46		
	H^{3}	1.87		
	H^{4}	1.75		
	H^{4}	2.15		
	H^{5}	3.28		
	H^{5}	3.58		
				225.9
Oxazolone B	N^{3}			or
				248.5
Ser^{5}	N			117.0
	C		171.9	
	C^{α}		60.5	
	C^{β}		60.7	
	H	8.74		
	H^{α}	4.85		
	H^{β}	3.99		
	H^{β}	4.26		
Cys^{6}	N			118.4
	C		171.4	
	C^{α}		57.0	
	C^{β}		40.7	
	H	8.12		
	H^{α}	4.81		
	H^{β}	3.27		
	H^{β}	3.46		
Met^{7}	N			129.1
	C		177.9	
	C^{α}		56.0	
	C^{β}		31.4	
	C^{γ}		30.1	
	C^{ε}		14.1	
	H	7.80		
	H^{α}	4.12		
	H^{β}	1.98		
	H^{β}	1.98		
	H^{γ}	2.41		
	H^{γ}	2.50		
	H^{ε}	1.92		

*The ^{1}H assignments were made at 25°C and referenced against TSP. The ^{13}C and ^{15}N assignments were made at 25°C.

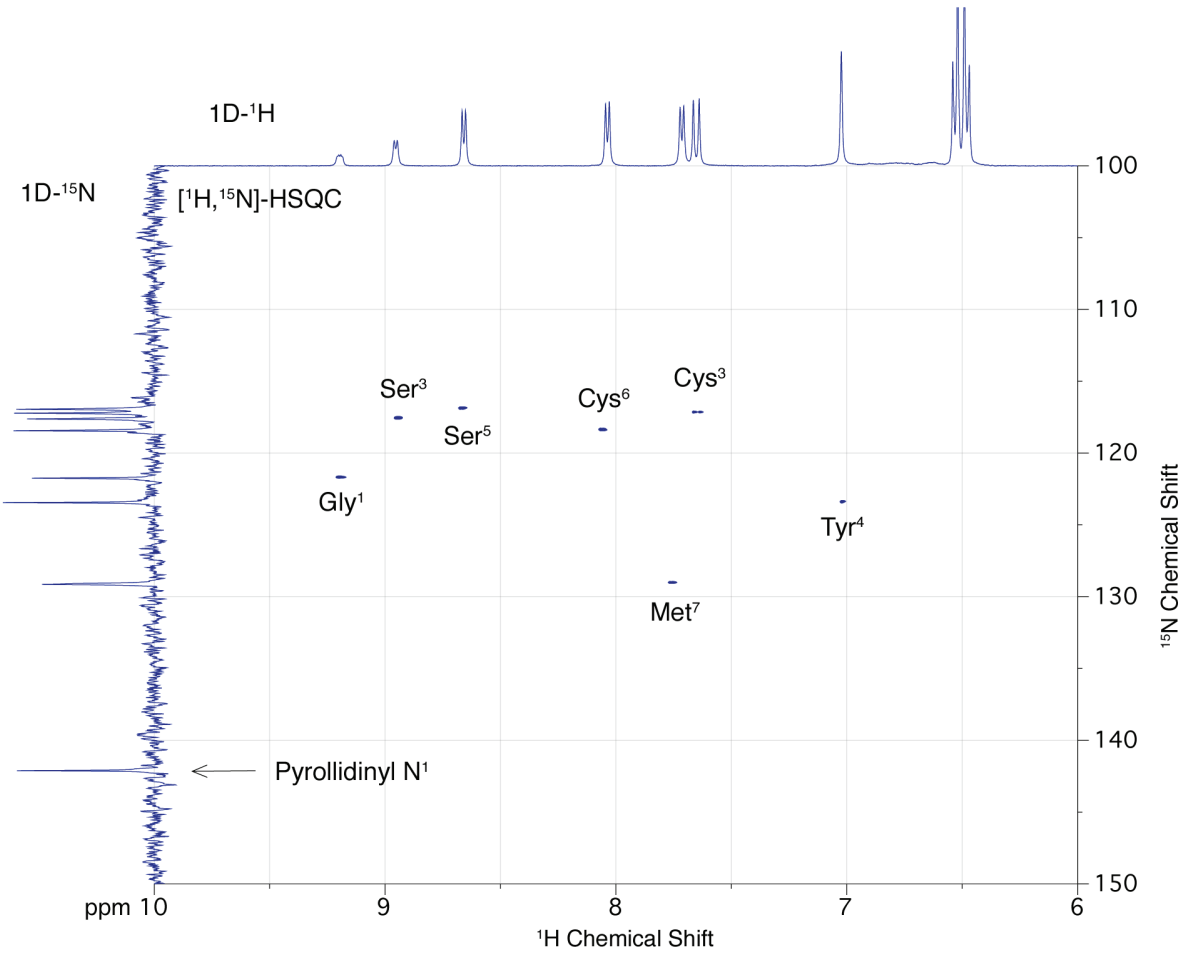
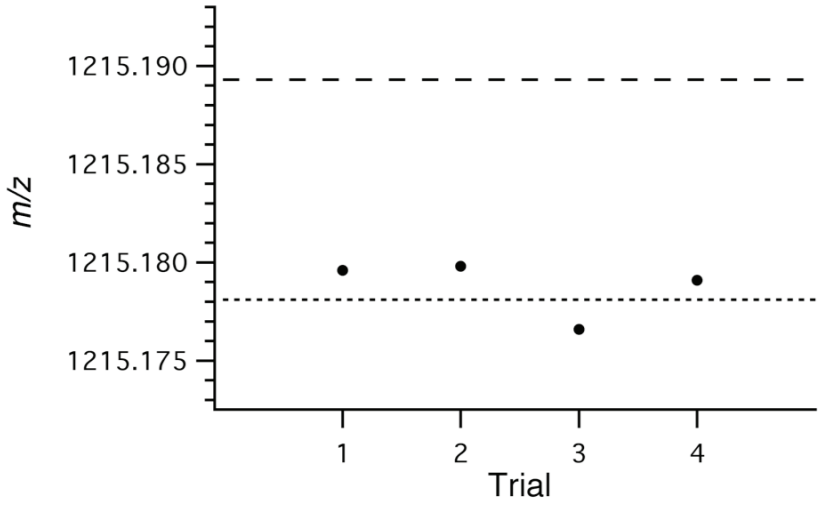


Figure S2: 400 MHz [^{1}H,^{15}N]-HSQC spectrum of uniformly ^{15}N-labeled methanobactin in 9 mM phosphate/10% D_2O, *pH*6.5, 25°C. The sample was exposed to Cu(II) at 0.50 Cu:mb prior to isolation by HPLC. The ^{15}N projection was collected separately in a 1D-^{15}N direct detection experiment on the same sample. The 1D-^{1}H projection was collected separately on an unlabeled sample of methanobactin.

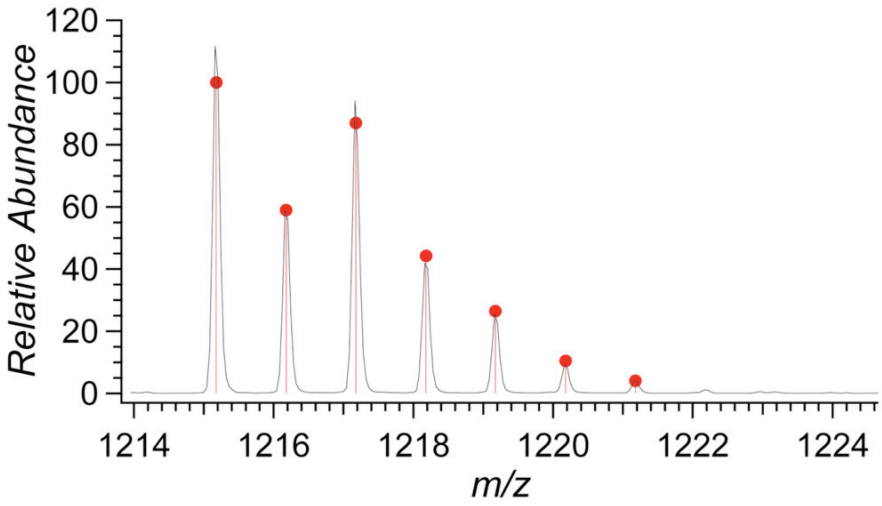
ESI-TOF mass spectroscopy

Accurate mass experiments were carried out using an Agilent 6210 ESI-TOF mass spectrometer operating in negative ion mode. Samples dissolved in 70% acetonitrile were injected directly into the electrospray source of the mass spectrometer. The sample used to collect the data shown in Figure S4 also contained a mixture of 10 reference masses with *m/z's* of between 300 and 2800. The solution containing these references masses was supplied as a calibrating solution for the spectrometer (Agilent ESI-TOF Tuning Mix). The specification of accuracy for this instrument, when using internal reference masses, is 2 ppm. Before running a methanobactin sample, the mass spectrometer was tuned and calibrated using the ES TOF Tuning Mix. After collecting a mass spectrum, the spectra were re-calibrated using 7 of the internal reference masses. These had *m/z's* ranging from 302 to 2234. Figure S3a shows the *m/z* values

obtained from four trials and compares them to the *m/z* values expected for the previously published and the proposed structures for methanobactin. Figure S3b shows a representative example of a mass spectrum for Cu(I)-bound methanobactin and compares it to the isotropic distribution expected for molecule with the proposed chemical formula shown in Figure 1.



a)



b)

Figure S3: **a)** The accurate mass determination of $(M\text{-}2H+^{63}Cu)^{1-}$ species of Cu(I)-bound methanobactin. The results of four trials compared to the expected mass of the published structure (dashed line) and the proposed structure (dotted line). **b)** The mass spectrum for trial 1 shown in a). The red dots and lines indicate the expected isotope pattern for a molecule with the same molecular formula

Methanolysis of uncomplexed methanobactin

Methanolysis of uncomplexed methanobactin was carried out by dissolving lyophilized methanobactin in methanolic HCl. Saturated Methanolic HCl was produced by bubbling HCl gas through methanol for 5 minutes. The saturated methanolic HCl was then diluted to 1/10, 1/100 and 1/1000 with MeOH. 1 mg samples of methanobactin, were dissolved in 1 mL MeOH, or 1 mL aliquots of the diluted and undiluted saturated methanolic HCl. The mixtures were allowed to react at room temperature for 15 minutes and then rotary-evaporated to a dry film at 30°C. The evaporation took approximately 3-4 minutes. The dried films were taken up in 1 mL H_2O and the *pH* adjusted to between 6.7 and 6.9 with 100 mM NaOH. The samples were then diluted to 1/10 with water and analyzed by UV/Vis spectrophotometry and ESI-TOF mass spectroscopy. Figure S4 shows an overlay of the UV/Vis spectra from these samples. The spectrum for the sample that contained no HCl is characteristic of uncomplexed methanobactin. As the HCl concentration is increased it leads first to the loss of the oxazolone B ring, which is associated with the absorbance at 340 nm,[5] followed with the loss at higher methanolic HCl concentrations of the oxazolone A ring, which is associated with the absorbance at 394 nm.[5]

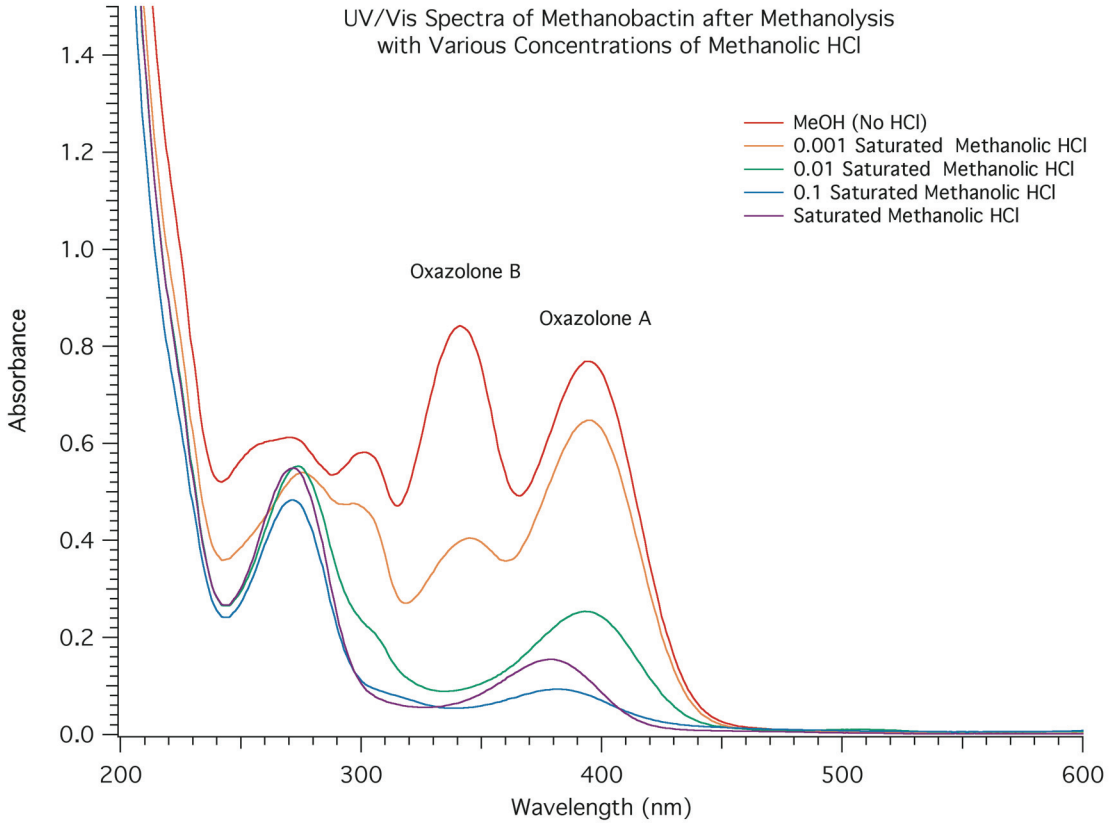


Figure S4: The UV/Vis spectra of methanobactin after methanolysis with various concentrations of methanolic HCl. The assignment of the the A_{394} to the N-terminal chromophoric residue and A_{340} to the other chromophoric residue was made based on the more extended conjugated system for the N-terminal residue.[5]

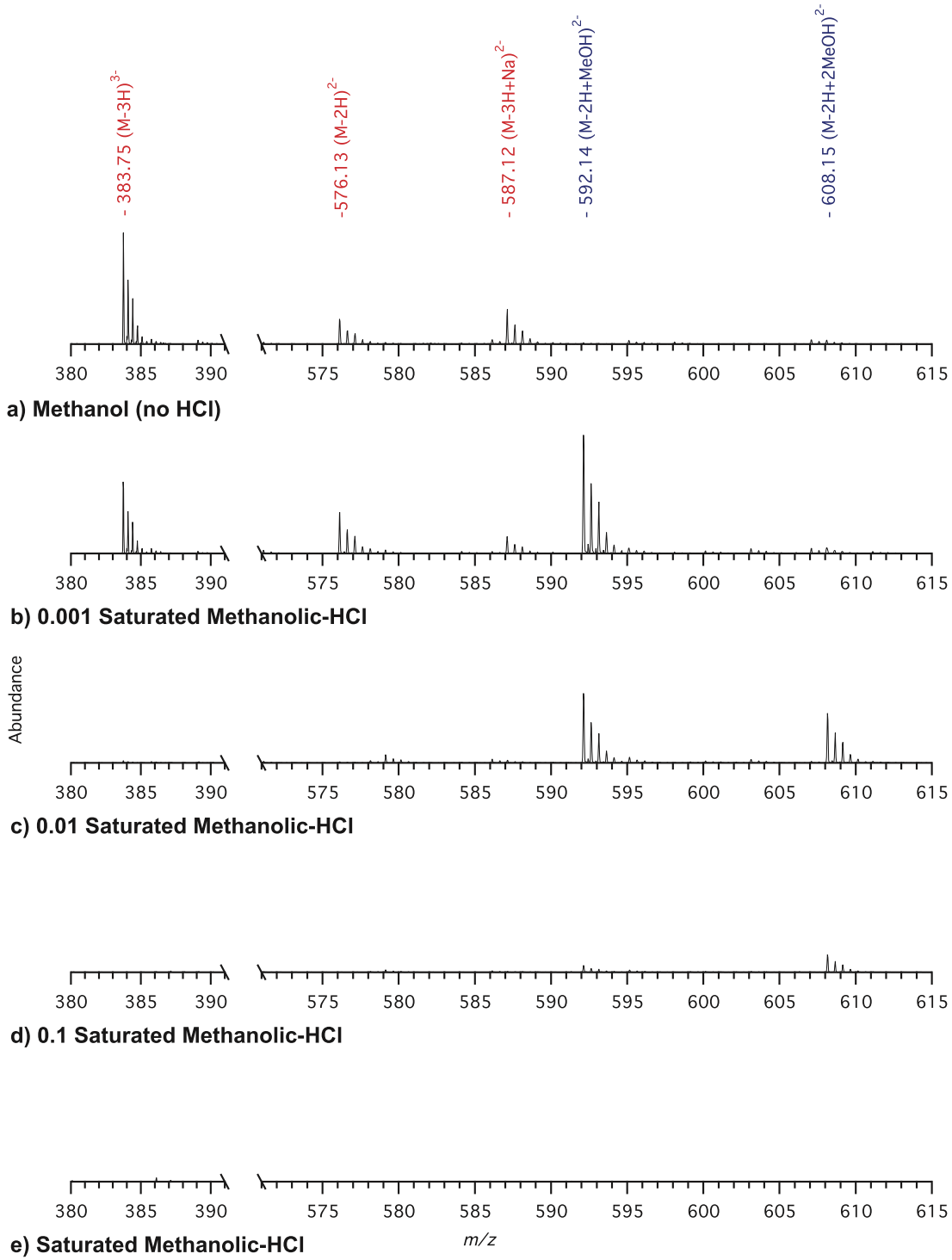


Figure S5: The ESI-TOF mass spectra for the same samples shown in Figure S4. The observed *m/z* values for the methanobactin species, [$(M\text{-}3H)^{3-}$, $(M\text{-}2H)^{2=}$, and $(M\text{-}3H+Na)^{2-}$] are labeled in red, while those for the products of the methanolysis reaction, [$(M\text{-}2H+MeOH)^{2-}$ and $(M\text{-}2H+2MeOH)^{2-}$] are labeled in blue. In the 0.1 saturated methanolic-HCl and the saturated methanolic-HCl reactions the methanobactin species were replaced by species with lower masses (not shown).

Figure S5 shows the corresponding ESI-TOF mass spectra for the samples used in obtaining the UV/Vis spectra shown in Figure S5. The sample that contained no HCl showed species that are all associated with uncomplexed methanobactin, $(M-3H)^{3-}$, $(M-2H)^{2-}$ and $(M-3H+Na)^{2-}$. As the HCl concentration was increased, these species were replaced by two new species with higher masses, first one with a charge of 2- at *m/z* 592.14, followed by one with a charge of 2- at *m/z* 608.15. Figure S6 shows that these two species correspond to what is expected for methanobactin which contains oxazolone rings that have undergone methanolysis,[10] first at one of its oxazolone rings, $(M-2H+MeOH)^{2-}$, followed by both rings $(M-2H+2MeOH)^{2-}$. At even higher concentrations of methanolic HCl, species with lower masses replaced these species, indicating further acid degradation of the methanobactin.

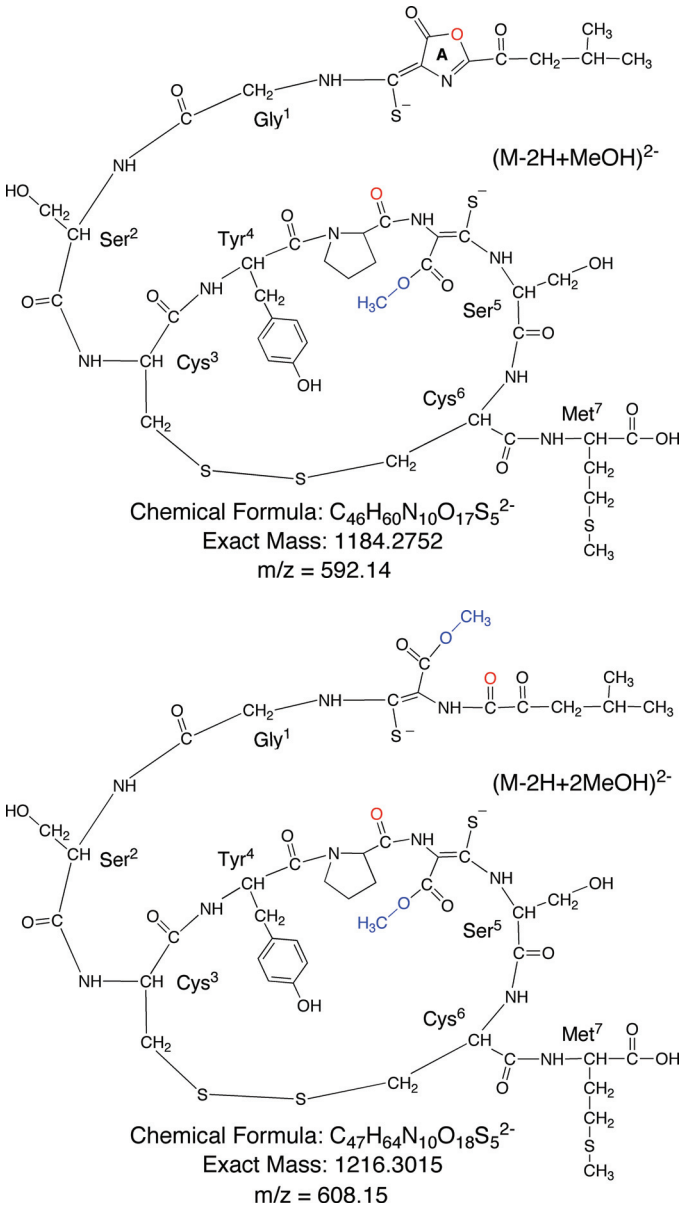
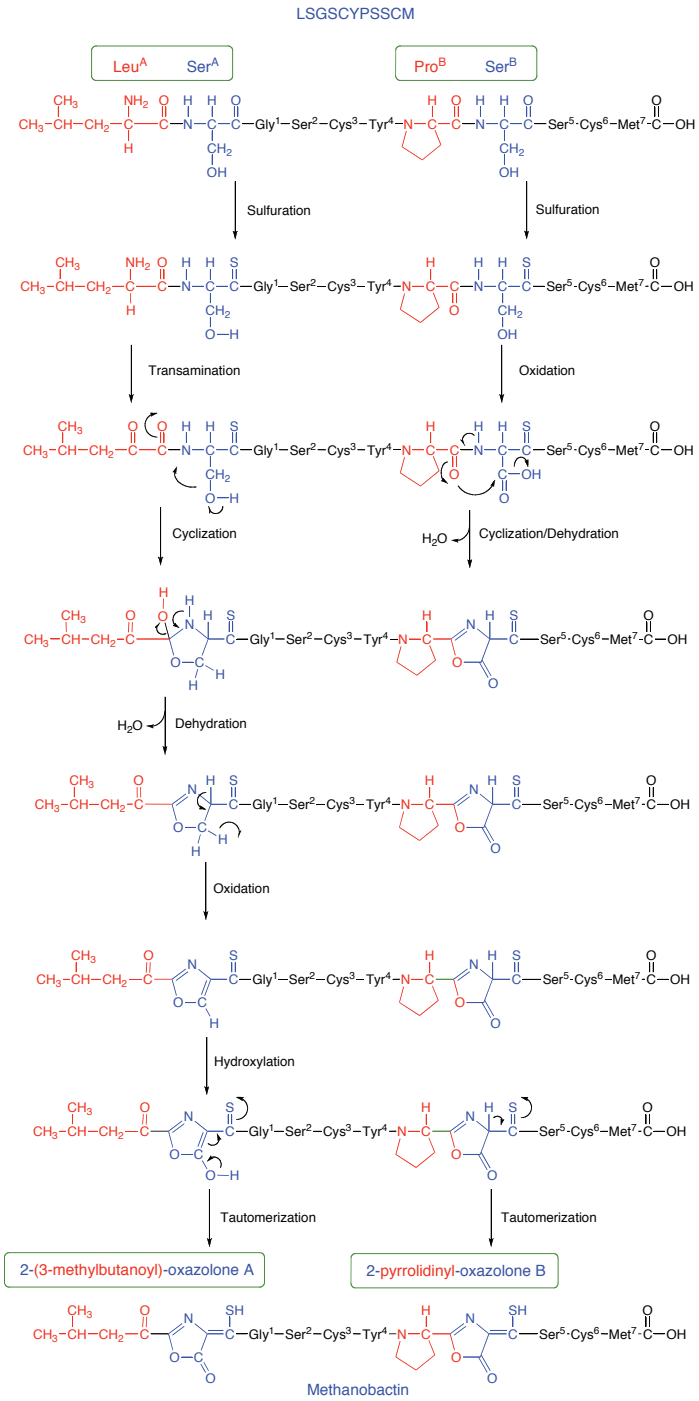


Figure S6: The chemical structures and *m/z* values for the expected products from the methanolysis of methanobactin containing two oxazolone rings.

Suggested pathways for the biosynthesis of methanobactin

The chemical structure that we propose for methanobactin, which contains alkylidene oxazolone rings, suggests a couple of pathways for the biosynthesis of methanobactin from an oligopeptide. Oxazoline rings, which are related to oxazolone rings, are commonly found in other bactins and siderophores.[11] These molecules are synthesized by non-ribosomal peptide synthetases and incorporate oxazoline rings into the backbone of an oligopeptide by reacting the sidechain hydroxyl group of a serine with the carbonyl carbon of the preceding residue in the sequence. The hydroxylation and oxidation of the oxazoline ring in the proposed biosynthesis of methanobactin would require some modifications to this pathway to incorporate the oxo substituent on the ring. An example of how this strategy could lead to the synthesis of the 3-methylbutanoyl-oxazolone A ring is shown on the left-hand side of Scheme S1.

Another possible pathway is suggested by a side reaction that is observed in solid-state peptide synthesis and that leads to the incorporation of an oxazolone ring at the C-terminus of a peptide.[12] The reaction involves reacting an amide carbonyl oxygen with the the carbonyl carbon of the next residue in the sequence. On the right-hand side of Scheme S1 we show how a modification of this reaction, to involve a side chain carbonyl formed from the oxidation of a serine side chain, could lead to the incorporation of oxazolone B ring into methanobactin. The incorporation of the 3-methylbutanoyl group at the N-terminus could arise from a transamination of a leucine residue, while no further modification is required to incorporate the pyrrolidine ring from a proline residue. Sulfuration of the two amide carbonyls attached to the two oxazolone rings would produce thiocarbonyls, which could then tautomerize to the enethiols shown in the proposed structure.



Scheme S1: The proposed structure shown in Figure 1 suggests a couple of pathways for the synthesis methanobactin from the 11 residue oligopeptide LSGSCYPSSCM. Using a modification of the reactions that lead to the biosynthesis oxazoline rings in bactins and siderophores,[11] Leu^A and Ser^A are shown combining to form the 3-methylbutanoyl group and the oxazolone A ring. And using a modification of a side reaction that is observed in solid-state peptide synthesis,[12] Pro^B and Ser^B are shown combining to form the pyrollidinyl group and the oxazolone B ring.

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