NMR Analysis of Metabolic Responses to Extreme Growing Conditions of the Temperature-Dependent Coral Pathogen *Vibrio coralliilyticus*

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To be submitted as a Letter to: ACS Chemical Biology.

ABSTRACT Recent metabolomics research on the temperature-dependent coral pathogen *Vibrio coralliilyticus* has led to the discovery of several compounds produced by the organism as a response to high-density, low nutrient growing conditions. Three compounds identified in the stressed metabolome are maltose, ethanolamine, and the first report of the production of the bioplastic-type compound (BTC) 2-buteonic acid, 2-carboxy-1-methylethyl ester by *V. coralliilyticus*. The presence of maltose and ethanolamine indicate a state of acute nutrient limitation; therefore, we hypothesize that the cell's metabolism turned to its own cell wall, or perhaps neighboring cells, for sources of carbon and nitrogen. The presence of the BTC also supports the acute nutrient limitation idea due to the parallels with polyhydroxyalkanoate (PHA) production in other Gram-negative bacteria including other *Vibrio* species. PHAs are industrially manufactured via fermentation of bacteria in which certain nutrients are limited once cell density has reached a substantial level. The result is a bioplastic polymeric material or PHA formed as a defense mechanism of the bacterial cell to store nutrients in the form of granular polyesters. Although nuclear magnetic resonance (NMR) and mass spectroscopy (MS) data indicate that the BTC produced by *V. coralliilyticus* is a single unit (as opposed to the polymeric PHA), its structure is common with the repeated ester monomer of PHAs. The three metabolites along with the NMR ^{1}H metabolic fingerprints of the nutrient-limited samples are proposed to serve as metabolic markers for extremely stressful growing conditions of *V. coralliilytiucs*.

The temperature dependent coral pathogen *Vibrio coralliilyticus* has been shown to cause coral bleaching (specifically in *Pocillopora damicornis*) at temperatures above 24.5 °C and coral lysis above 27 °C (*1-6*). A hypothesized relationship between virulence of *V. coralliilyticus* and elevated sea water temperatures (*3, 6*) fueled an NMR-based metabolomics investigation of the

organism in its virulent and non-virulent states. Recently reported metabolic profiles of the organism (in the form of 1D ^{1}H NMR spectra) consistently showed metabolic differences between the two states via principal components analysis (PCA); however, an unexpected separation in the PCA data indicated biological variability between parallel bacterial cultures (*7*). Several compounds consistently emerged as the cause of the separations, in both inter- and intra-batch comparisons. These were betaine, glutamate, and succinate; metabolites responsible for cellular osmoregulation and involved in various metabolic pathways such as the citric acid cycle and amino acid production or metabolism (*7, 8*). These compounds, along with many others, are characteristic of normal, healthy *V. coralliilyticus* growth and produce a distinctive 1D ^{1}H NMR metabolic profile for the organism. Here, we report on the discovery of several new compounds produced by the organism as a response to high-density, low nutrient growing conditions. Three compounds identified in the stressed metabolome are maltose, ethanolamine, and the first report of the production of the bioplastic-type compound (BTC) 2-buteonic acid, 2-carboxy-1-methylethyl ester (CAS: 20638-00-0) (*9-14*) by *V. coralliilyticus*. The 1D ^{1}H NMR metabolic profile for the organism as a result of poor growth conditions was dominated by large signals from these newly identified compounds.

The differences between typical *V. coralliilyticus* endometabolome spectra (or metabolic profiles) and those from acutely stressed samples (Figure 1) indicate two different metabolic profiles for the organism. Four samples from a batch growth referred to as “batch 5” were stressed during lyophlization of the quenched and salt-washed cell pellets. The stress involved a complete thawing of the frozen cell pellet prior to or during lyophlization. During this time the cells resumed metabolism that was previously quenched with liquid nitrogen; however, their environment was dramatically different than during cell culture in glycerol artificial sea water

(GASW) media. The cells were in an extremely nutrient-limited environment as the cell pellet thawed, and as a consequence, the cells' metabolism was stressed before the lyophilization was completed. The stressed lyophilized cell pellets were a shiny, gel-like dry material while unstressed lyophilized cell material is flakey and powdery. The ^{1}H NMR spectra collected for the four stressed samples showed the presence of new peaks in very high concentrations compared to the usual peaks in the metabolic fingerprint of unstressed *V. coralliilyticus*. The metabolic profiles of the 12 samples in batch 5 (Figure 1a) in the form of one dimensional proton NMR spectra which were collected on a 700 MHz Bruker instrument exhibit visually obvious spectral variations in samples B05_2009_27_03, _04, _05, and _06, with these spectra exhibiting excessively large peaks not present in the spectra of the un-stressed samples.

Identification and chemical shift assignments for the new peaks in the spectra of the stressed samples verified the presence of three distinct metabolites: 2-butenoic acid, 2-carboxy-1-methylethyl ester (the BTC), maltose, and ethanolamine (Figure 1b). Structural determination of the BTC was achieved via several NMR experiments and validated with empirical calculations of chemical shifts using ChemBioDraw software (version 12.0 CambridgeSoft, Inc.) which resulted in a structure with a chemical formula of $C_8H_{12}O_4$ (Figure 2). The stereochemistry in the predicted name of the BTC, (E)-3-(but-2-enoyloxy)butanoic acid, given by ChemBioDraw was the result of the hydrogens on carbons 6 and 7 being in the *trans* position across the double bond. The predicted chemical shifts for the *trans* (E) stereochemistry are in better agreement with the experimental chemical shifts than those of the *cis* (Z) conformation. Confirmative assignment information for the metabolites was obtained from ^{1}H, ^{13}C, HSQC and HMBC spectra (Figure 3 and Table 1; integrations are given in Supplementary Figure 1). The integrated peaks conform to the stated molecular formula. The ^{1}H-^{13}C HSQC spectrum (Figure

3a) provides ^{1}H and ^{13}C assignments and the ^{1}H-^{13}C HMBC spectrum (Figure 3b) shows connectivities between H's and C's in each spin system. LC-UV-MS analysis of the aqueous extract confirmed the mass (The dominant peak at 195 m/z can be attributed to the adduct of (E)-3-(but-2-enoyloxy)butanoic acid with sodium (23 + 172).) and identification of the BTC (see Supplemental Information).

The three metabolites identified in the microbial stress *V. coralliilyticus* sample all point to the cells coping with a very stressful event: acute nutrient limitation. Maltose, a disaccharide, is formed from two glucose units by an α(1→4) linkage and results from the breakdown of starch for a food source (*15*). The presence of maltose in the stressed *V. coralliilyticus* sample indicates that when the quenched and frozen cells thawed and resumed their metabolism in their now extremely nutrient-limited environment, they began using their own starch stores as a carbon source. Phosphatidylethanolamine (PE) is the major phospholipid in Gram-negative bacteria membranes (*16*). Ethanolamine, which makes up the head group of the PE phospholipid, can be used by bacteria as a carbon, nitrogen, and energy source (*17*). However, the presence of ethanolamine in the stressed *V. coralliilyticus* sample could suggest a breakdown of one or more utilization pathways such as glycerophospholipid metabolism or phosphonate and phosphinate metabolism (*8*). Ethanolamine is produced in these pathways as an end product in phosphonate and phosphinate metabolism and for subsequent use in glycolysis or pyruvate metabolism in glycerophospholipid metabolism. Perhaps the limited resources available to *V. coralliilyticus* cells when they thawed caused the cells to produce a buildup of ethanolamine via phosphonate and phosphinate metabolism; or a breakdown of the activity of the enzymes in glycerophospholipid metabolism responsible for converting ethanolamine into acetaldehyde used in glycolysis or pyruvate metabolism (*8*).. Finally, the largest peaks in the spectrum of the

stressed *V. coralliilyticus* correspond to a bioplastic-type compound (BTC). Very few literature references for this compound were found (*9, 10*), and one identifies the compound as 2-butenoic acid, 2-carboxy-1-methylethyl ester (racemic CAS: 172471-84-0) (*14*). The compound is referred to as an isomer of a crotonic acid dimer by one report (*trans* (E) stereochemistry CAS: 20638-00-0) (*9*). Other similar structures have been reported, for example, as the result of thermal degradation of PHBs (polyhydroxybutyrate) (*18*), in relation to cyclic dimers (*19*), and as bacterial biomarkers (*14, 20*). The BTC is similar in structure to a polyhydroxyalkanoate (PHA). PHAs are produced by bacterial fermentation usually due to very stressful events in which normal nutrient or environmental conditions are altered (*21*). Upon an occurrence of limited nutrients, the bacteria produce light-refracting granular polyesters as storage devices for carbon and energy to be used for survival (*22*). The shiny, gel-like lyophilized cell material for the stressed *V. coralliilyticus* samples and the presence of the BTC in the NMR data indicates a similar condition. The assigned structure (Figure 2) with the chemical formula of $C_8H_{12}O_4$ is composed of two units of the base structure for a PHA, making it a medium-chain length (C5-C14) PHA (*22*).

Subsequent efforts to grow *V. coralliilyticus* for investigations of biological metabolic variability are ongoing and have thus far resulted in evidence of the presence of the newly identified stress metabolites in batches that did not grow properly. These batches did not grow properly for as yet undetermined reasons; however, the possibility of nutrient limitation is a present and common theme, which leads to the hypothesis that these compounds could be used as biomarkers for stress in *V. coralliilyticus* cultures.

In conclusion, the observation of a bioplastic-type compound produced by *Vibrio coralliilyticus* has shown that this organism produces three distinct metabolites in response to

extreme nutrient limitation for a prolonged period of time. All three metabolites are present consistently in stressed *V. coralliilyticus* bacterial cultures and, therefore, provide an indication of stressful growing conditions. The stressful conditions produced in this study are similar to the fermentation of bacteria and production of PHAs, which has a large industrial base and is the foundation for several companies (*21*). The type of PHA produced is strain (and species) specific (*22*) and, to the best of our knowledge, this is the first report of the BTC 2-butenoic acid, 2-carboxy-1-methylethyl ester (or maltose or ethanolamine, for that matter) produced by *V. coralliilyticus*.

METHODS

The procedure described in the *Sample Preparation* section was performed multiple times and each iteration is referred to as a batch. The details for two batches denoted as B05_2009 and B09_2009 (grown as a control material) are discussed below. The four B05_2009 samples discussed were grown at 27 °C, as was the control material batch B09_2009. The numbering scheme for a batch is Bbb_yyyy_TT_ss with bb being a particular growth batch, yyyy denoting the year of growth, TT denoting the growing temperature and ss representing a specific sample in that batch.

Sample Preparation

Cell Growth. *V. coralliilyticus* growths were started from a (thawed) -80 °C freezer stock and inoculated onto two Glycerol Artificial Seawater (GASW) agar plates. One plate was grown at 24 °C and the other at 27 °C for 24 h in the dark. Further cell growth was accomplished using GASW growth media. For each growth temperature, six individual colonies from each plate

were transferred into six individual 250 ml flasks with 50 ml GASW and grown in shakers (at 200 rpm) at their respective temperatures for 24 h in the dark. At this point cell pellets were harvested and collected as six individual samples for each temperature. B05_2009 and B09_2009 were grown as described here; however, B09_2009 was grown at 27 °C only with all samples combined and homogenized into one large pellet and used for quality control and microbial stress testing purposes.

Quality Control of Growth. To ensure contamination-free growth of the *V. coralliilyticus* cultures, GASW plates (referred to as purity plates) were streaked with 20 µl of the final growth (prior to quenching) and left in the incubator (27 °C) for at least 24 h. Healthy colonies are small, round, and somewhat transparent. Some cell cultures for subsequent cell growth trials exhibited very little or no growth indicating that the particular sample's growth was hindered.

Cell Quenching and Collection. The cell cultures' metabolism was quenched and cell pellets collected as described previously (*7*). Ten milliliters of spent media for each cell culture was reserved after quenching and stored at -40 °C. The final cell pellets were flash frozen in liquid nitrogen and stored at -80 °C for 3 h. Finally, the cells were lyophilized overnight and stored at -80 °C until extraction. Four B05_2009 samples (B05_2009_27_03, B05_2009_27_04, B05_2009_27_05, and B05_2009_27_06) were thawed to room temperature prior to complete lyophilization. The twelve B09_2009 cell cultures (all at 27 °C) were combined after quenching and prior to centrifugation in order to achieve a homogeneous batch growth which we use as a control material (CM).

169 *Endometabolite Extraction*. Polar endo-metabolites were extracted from the lyophilized *V.*
170 *coralliilyticus* cells as described previously (*7*) for all batches with one exception. The
171 temperature of the solvent extraction for the 2010 samples and B09_2009 (control material) was
172 changed to 50 °C after additional methods development showed this temperature gave more
173 consistent results.
174 *NMR Data Acquisition*
175 One- and two-dimensional high-resolution ^{1}H NMR spectra were acquired on a Bruker
176 700 MHz spectrometer equipped with a TCI cryoprobe as described previously (*7*). Experiments
177 used for metabolite identification purposes include the one-dimensional (1D) ^{1}H (noesypr1dpr
178 Bruker pulse sequence) and ^{13}C (zgpg30 Bruker pulse sequence); and the two-dimensional (2D)
179 ^{13}C-HSQC (hsqcedetgpsisp2.2 Bruker pulse sequence), ^{13}C-HMBC (hmbcgpl2ndqf Bruker pulse
180 sequence), and ^{13}C-HSQC-TOCSY (hsqcdiedetgpsisp.1 Bruker pulse sequence).
181
182 *LC-UV-MS Data Acquisition*
183 Samples of the extract (20 μL injections) were eluted through an Agilent Zorbax Eclipse
184 XDB-C18 4.6 x 150 mm, 5 μm column at 35°C. Chromatographic mobile phase flow was 0.5
185 ml/min with a linear gradient profile from 30% acetonitrile/ 70% water to 70% acetonitrile/30%
186 water over 10 minutes. Data were collected from time 1 to 10 minutes. Mass spectral detection
187 (MSD) was performed with a Bruker Esquire 6000 ion trap mass spectrometer with an
188 electrospray ionization source. Masses were calibrated by the manufacturer's method as defined
189 in the Hystar software using a test mixture of substituted phosphazines in acetonitrile infused
190 directly into the ESI source.
191

Metabolite Identification

Ethanolamine and maltose were identified in the microbial stress samples using comparisons between processed one-dimensional (1D) ^{1}H NMR spectral libraries of standard spectra: one developed in-house, SBASE-1-1-2 from AMIX (version 3.8.3, Bruker Biospin GmbH), Chenomx 700 MHz standard library (Chenomx NMR Suite v 6.1 (Chenomx, Inc.)), and online databases (such as the MMC Database (*23*) and HMDB (*24*)). The third metabolite produced in the microbial stress incident samples was the BTC identified to be 2-butenoic acid, 2-carboxy-1-methylethyl ester (*trans* (E) stereochemistry CAS: 20638-00-0) after its structure was determined using ^{13}C, ^{13}C-HSQC, ^{13}C-HMBC, and ^{13}C-HSQC-TOCSY spectra. These spectra provided carbon chemical shifts and H-C correlations that allowed for structure determination. ChemBioDraw software (version 12.0 CambridgeSoft, Inc.) provided confirmation of the extrapolated structure.

Acknowledgments: We acknowledge the support of the Hollings Marine Laboratory NMR Facility. *V. coralliilyticus* samples were grown by our microbiologist partners Maria I. Vizcaino and Pamela J. Morris at the Hollings Marine Laboratory. Commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Supporting Information Available: This material is available free of charge via the Internet.

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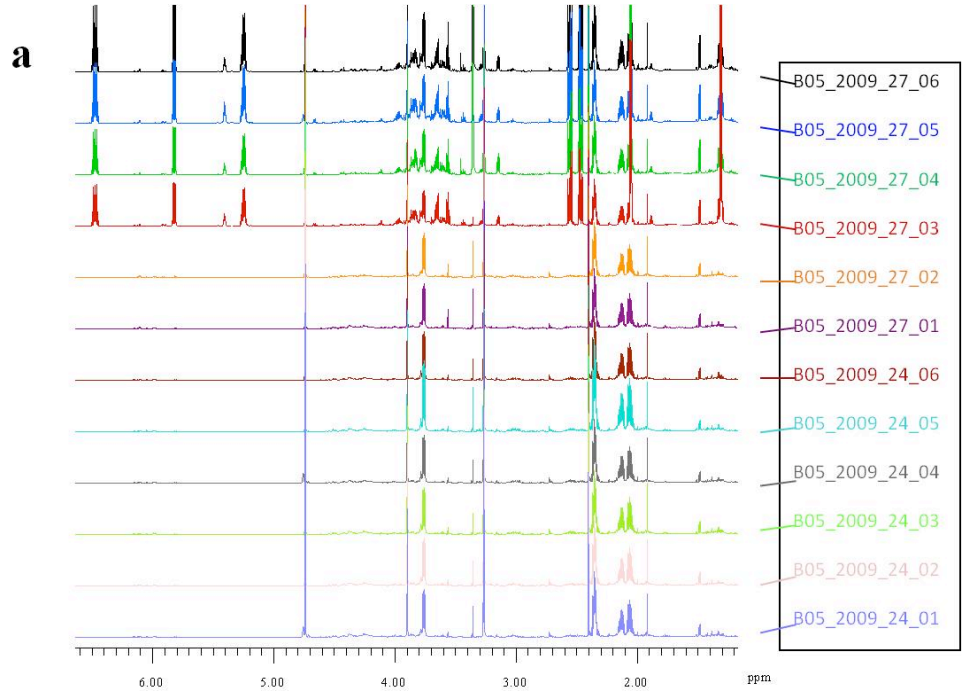
Figure 1. a) ^{1}H metabolic profiles of B05_2009. b) ^{1}H spectral comparison of a normal and a stressed sample (B05_2009_24_01, red and B05_2009_27_06, blue). Stressed sample includes maltose, ethanolamine, and the BTC.



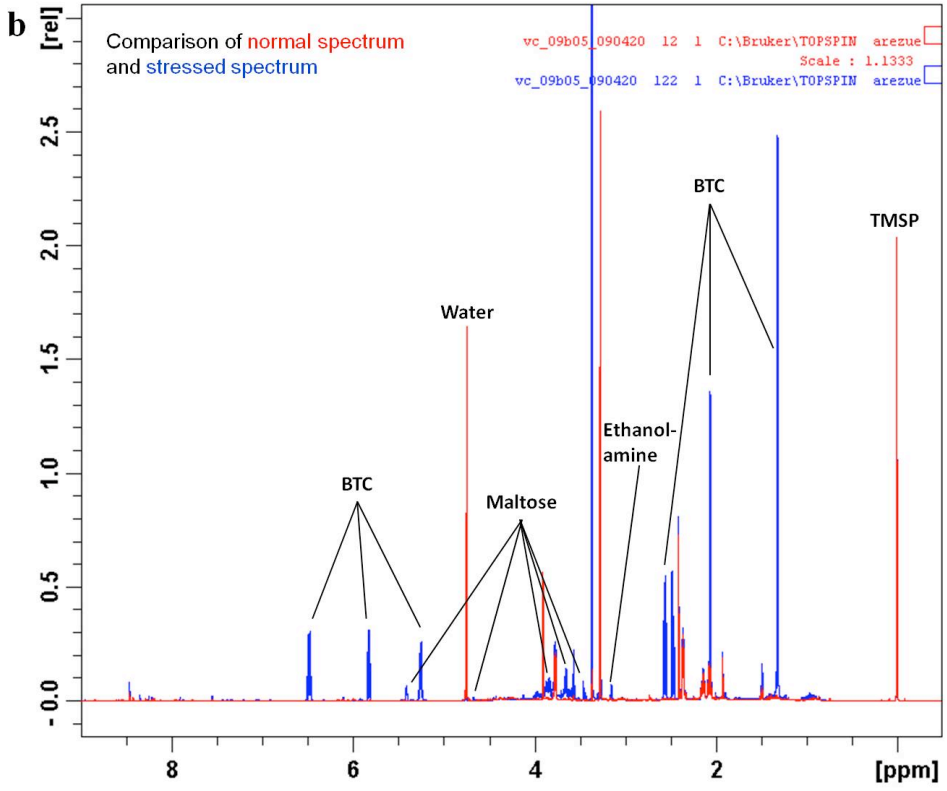


Figure 2. BTC structure and predicted ^{1}H and ^{13}C NMR spectra. See also Table 1.

(E)-3-(but-2-enoyloxy)butanoic acid

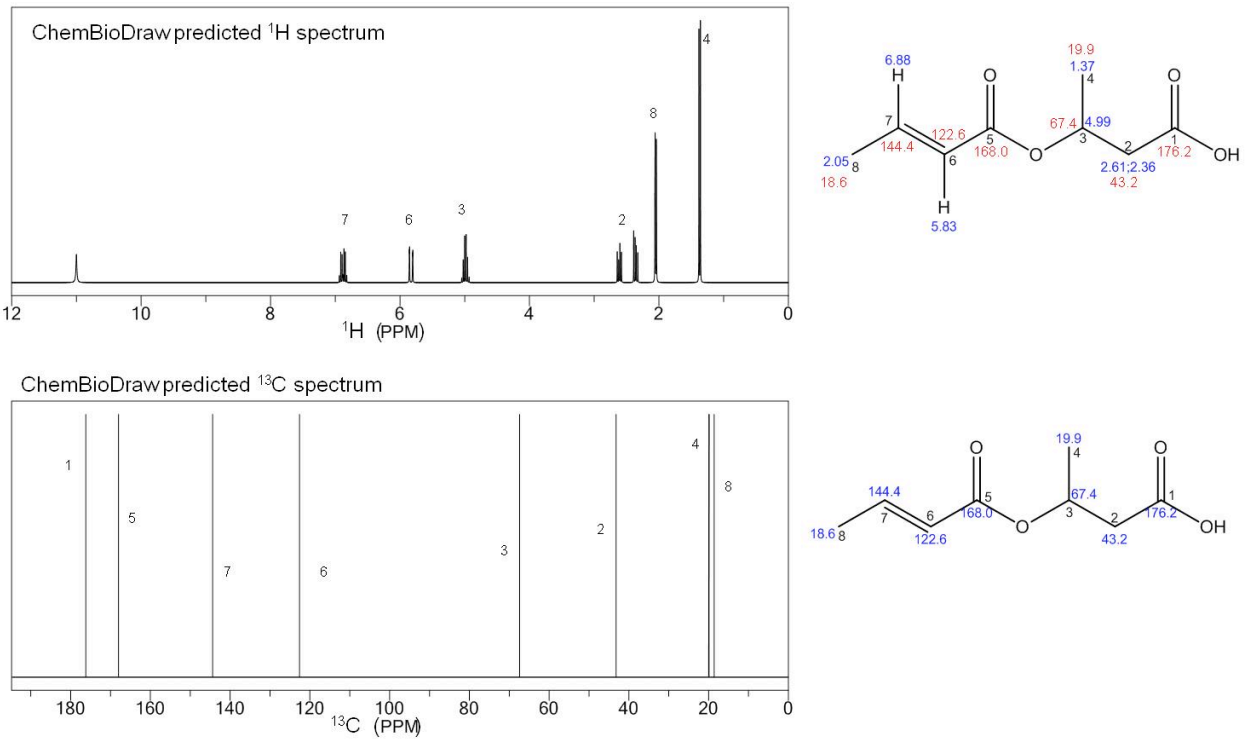


Figure 3. a) ^{1}H-^{13}C HSQC of a stressed *V. coralliilyticus* sample (blue: CH_3 and CH; green: CH_2) showing chemical shift assignments for (E)-3-(but-2-enoyloxy)butanoic acid, maltose, and ethanolamine. b) ^{1}H-^{13}C HMBC spectrum a stressed *V. coralliilyticus* sample showing connectivities (via blue lines) and two spin systems for (E)-3-(but-2-enoyloxy)butanoic acid. Numbering system is from the predicted structure shown in Figure 2 (and further described in Table 1), which includes predicted chemical shifts that correspond to the actual chemical shifts in the spectrum. External projections for a) and b): (top) ^{1}H spectrum of a stressed *V. coralliilyticus* sample and (left) ^{13}C spectrum of an stressed *V. coralliilyticus* sample.

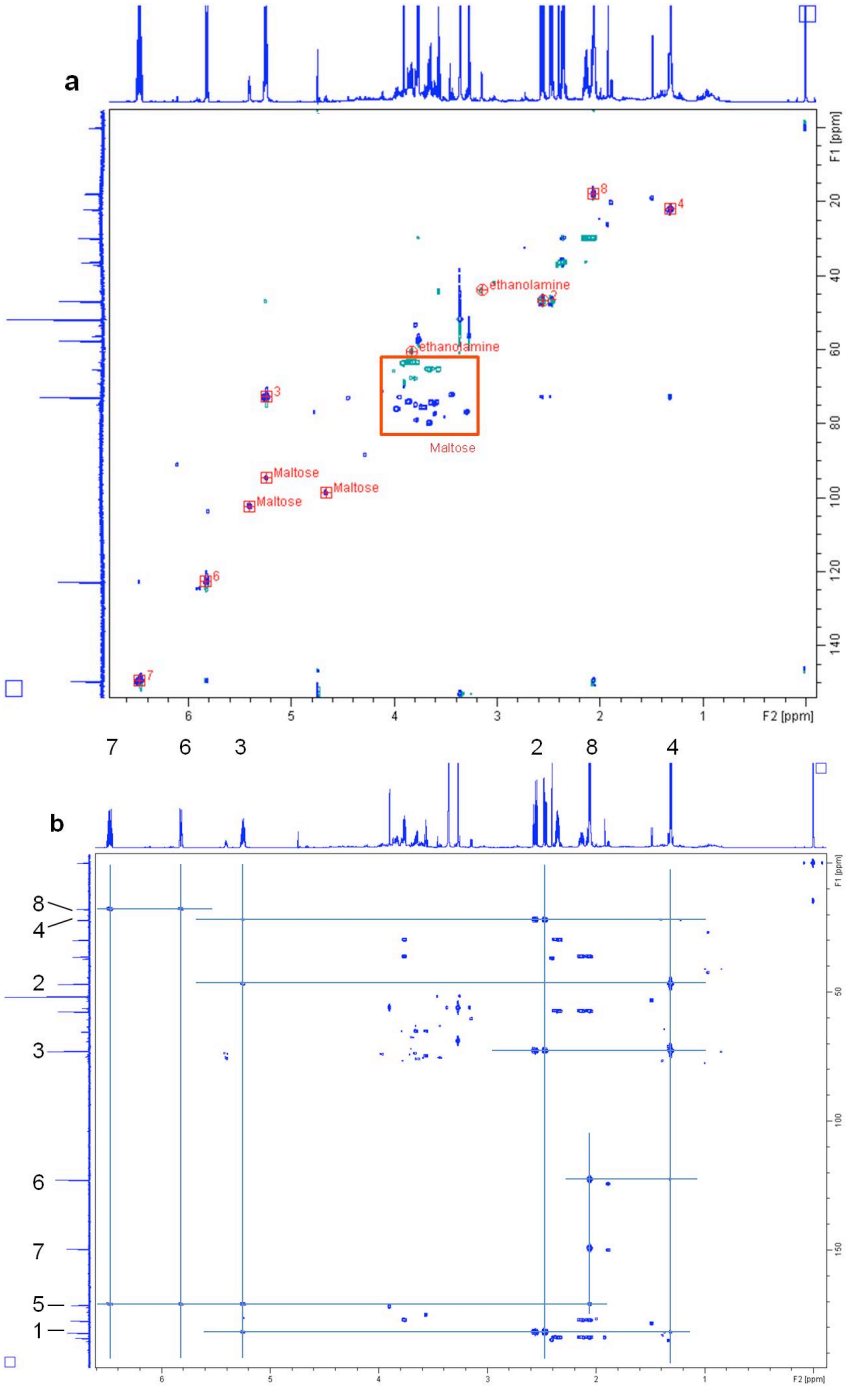


Table 1. Chemical shifts (predicted and experimental), multiplicity, functional group, and connectivities for (E)-3-(but-2-enoyloxy)butanoic acid.

Carbon #	Predicted[a] Chemical Shift (ppm)		Experimental[b] Chemical Shift (ppm)		^{1}H multiplicity; functional group[c]	one-bond ^{1}H connectivity[d]	two-bond ^{1}H connectivity	3+-bond ^{1}H connectivity
	^{1}H	^{13}C	^{1}H	^{13}C				
1	n/a	176.2	n/a	182.1	n/a; C=O	2	3	4
2	2.36; 2.61	43.2	2.47; 2.56	46.9	qq; CH_2	3	4	
3	4.99	67.4	5.25	72.9	m; CH	2, 4		
4	1.37	19.9	1.31	22.1	d; CH_3	3	2	
5	n/a	168.0	n/a	171.3	n/a; C=O	6	3, 7	8
6	5.83	122.6	5.82	122.9	m; CH	4	8	
7	6.88	144.4	6.48	149.5	m; CH	8		
8	2.05	18.6	2.06	17.9	dd; CH_3	7	6	

[a] Predicted chemical shifts obtained from ChemBioDraw

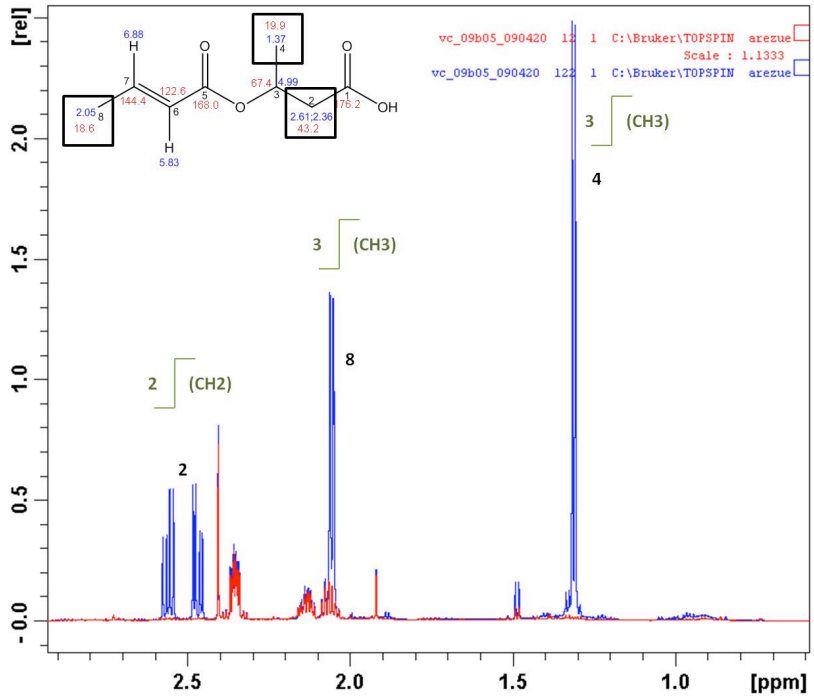
[b] Experimental chemical shifts obtained from ^{1}H, ^{13}C, and ^{1}H-^{13}C-HSQC experiments

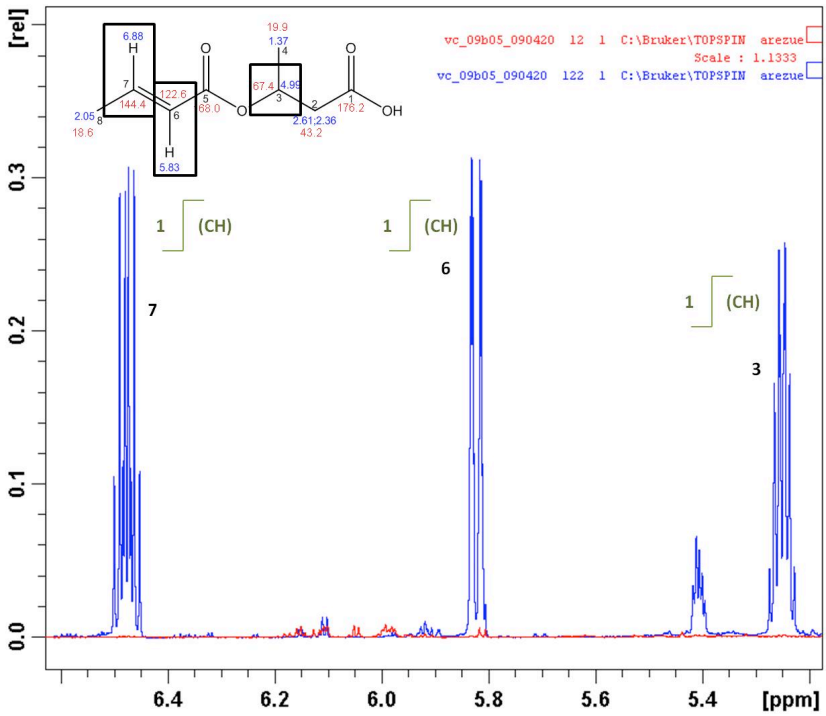
[c] ^{1}H multiplicity and functional group information obtained from shape and integration of peaks in ^{1}H experiment

[d] Single and multiple bond connectivities obtained from ^{1}H-^{13}C-HMBC experiment

Supplemental Information

Supplementary Figure 1. ^{1}H spectral comparison of an unstressed *V. coralliilyticus* sample (red) and a stressed *V. coralliilyticus* sample (blue) showing peak integrals.





LC-UV-MS analysis of the aqueous extract.

In repeated liquid chromatography runs of the aqueous extract solution, elution yielded one peak at 3.5 minutes as seen in the UV chromatogram (λ_{max} = 200 nm). Observing in the MS positive ionization mode, two chromatographic peaks were observed. A small peak at 3.1 minutes shows a base peak at 262.9 m/z and a peak at half the intensity at 142.9 m/z. The vastly larger chromatographic peak as seen in the MSD has a base peak of 195.0 m/z. Replicate runs scanning to 600 m/z and to 3000 m/z yielded identical results. The mass of 195 m/z can be attributed to the adduct of (E)-3-(but-2-enoyloxy)butanoic acid with sodium (23 + 172). Observing in the MS positive mode with automatic MS2, the base mass of 195.3 (±0.1) m/z was isolated from the primary chromatographic peak. Fragmenting at 1 volt, all runs yielded a mass of 109.3 (±0.1) m/z. Three of four runs also yielded a mass of 236.0 (±0.1) m/z. The fragment mass of 109 m/z can be attributed to a mass fragment corresponding to half of the dimeric compound coordinating with sodium (23 + 172/2). In negative ionization mode MS runs, no reproducible peaks were found.