Supporting Information

Chemoproteomic profiling of itaconations in *Salmonella*

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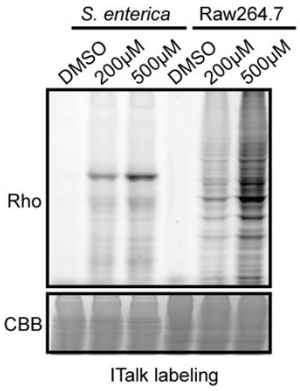


Fig. S1 The labeling of ITalk (C8E) in *S. enterica* lysates and Raw264.7 lysates. The lysates were treated by 200 μM or 500 μM of the ITalk probe at 37 °C for 2 hours. The labeled lysates were reacted with azide-rhodamine (Rho) via CuAAC and the labeling intensity was determined by in-gel fluorescence scanning. Coomassie Brilliant Blue (CBB) staining demonstrates the equal loading.

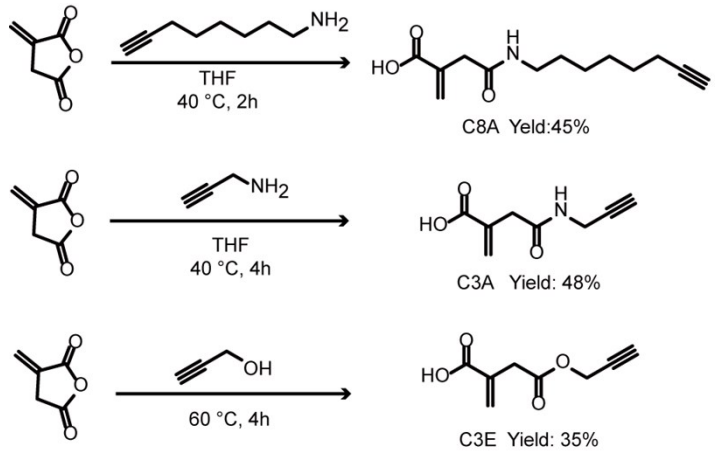


Fig. S2 Synthetic routes and yields of the C3A, C3E and C8A probes.

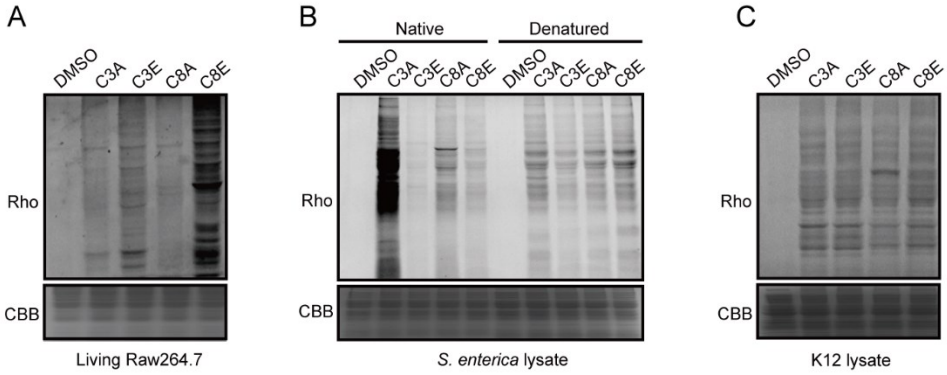


Fig. S3 Evaluation of the labeling of itaconate-derivative probes. (A) *In situ* labeling in Raw264.7 cells. The living cells were treated by different probes for 12 hours. (B) The labeling of native and denatured *S. enterica* lysates by different probes for 4 hours. (C) The labeling in *E. coli* K12 lysates. The lysates were treated with 100 μM of different probes at 37 °C for 2 hours.

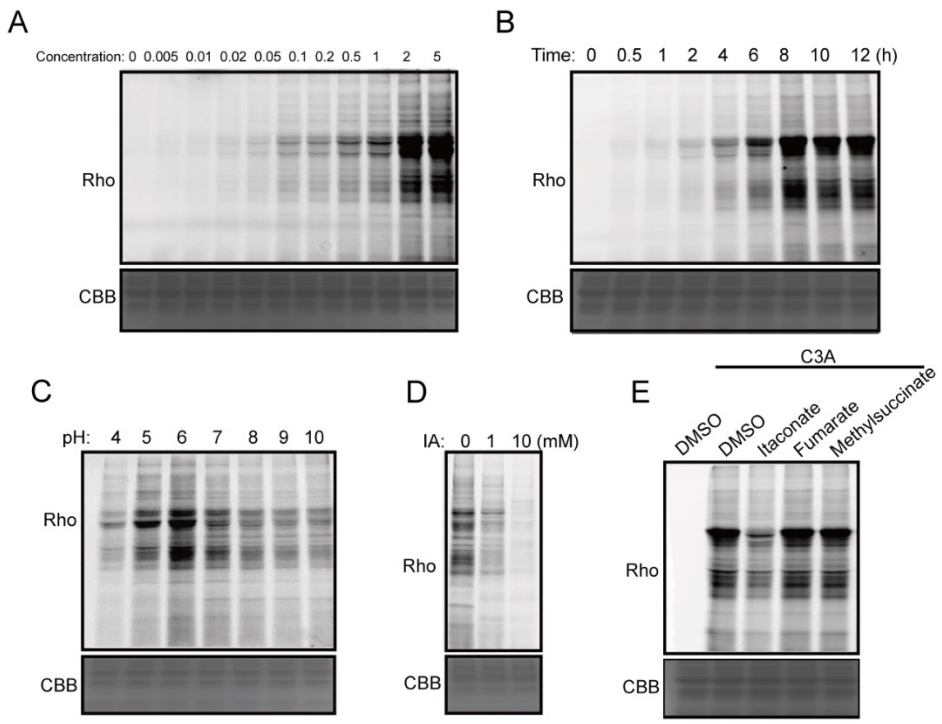


Fig S4. Evaluation of C3A labeling in *S. enterica* lysates. (A) Concentration-dependent labeling of C3A in *S. enterica* lysates. The lysates were treated with different concentrations of C3A at 37 °C for 2 hours. (B) Time-dependent labeling of C3A in *S. enterica* lysates. The lysates were treated with 500 µM of C3A at 37 °C for different times. (C) The pH-dependent labeling of C3A in *S. enterica* lysates. (D) Blocking free cysteines by iodoacetamide (IA) diminished C3A labeling in *S. enterica* lysates. The lysates were pre-treated with various concentrations of IA and then labeled with 500 µM of C3A at 37 °C for 4 hours. (E) Competitive labeling of C3A by itaconate, fumarate and methylsuccinate in *S. enterica* lysate. The lysates were pre-treated with 5 mM of each of the compounds, respectively, and then labeled with 500 µM of C3A for 12 hours.

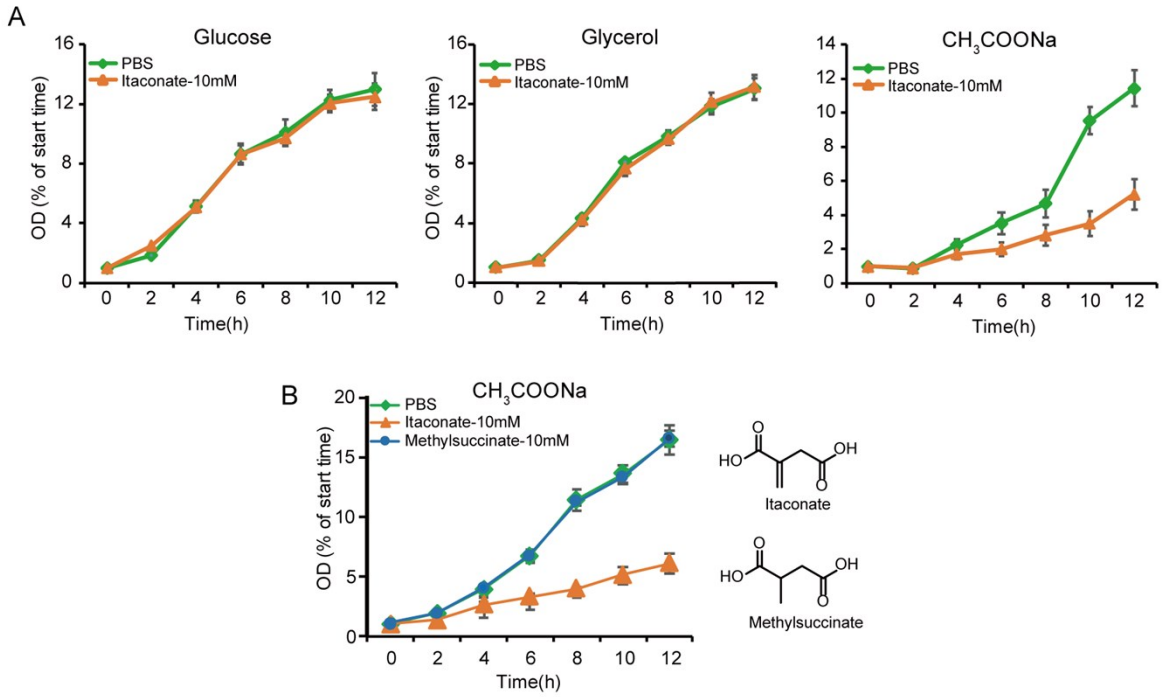


Fig S5. Itaconate inhibited the growth of *S. enterica*. (A) The growth curves of *S. enterica* in media with different carbon sources, including glucose, glycerol or acetate. The media were adjusted to pH 7. Itaconate inhibited the growth of *S. enterica* only when the carbon source of media was replaced by acetate. (B) Compared to itaconate, methylsuccinate has little influence on the growth of *S. enterica*, suggesting that the inhibitory effect comes from the electrophilicity of itaconate.

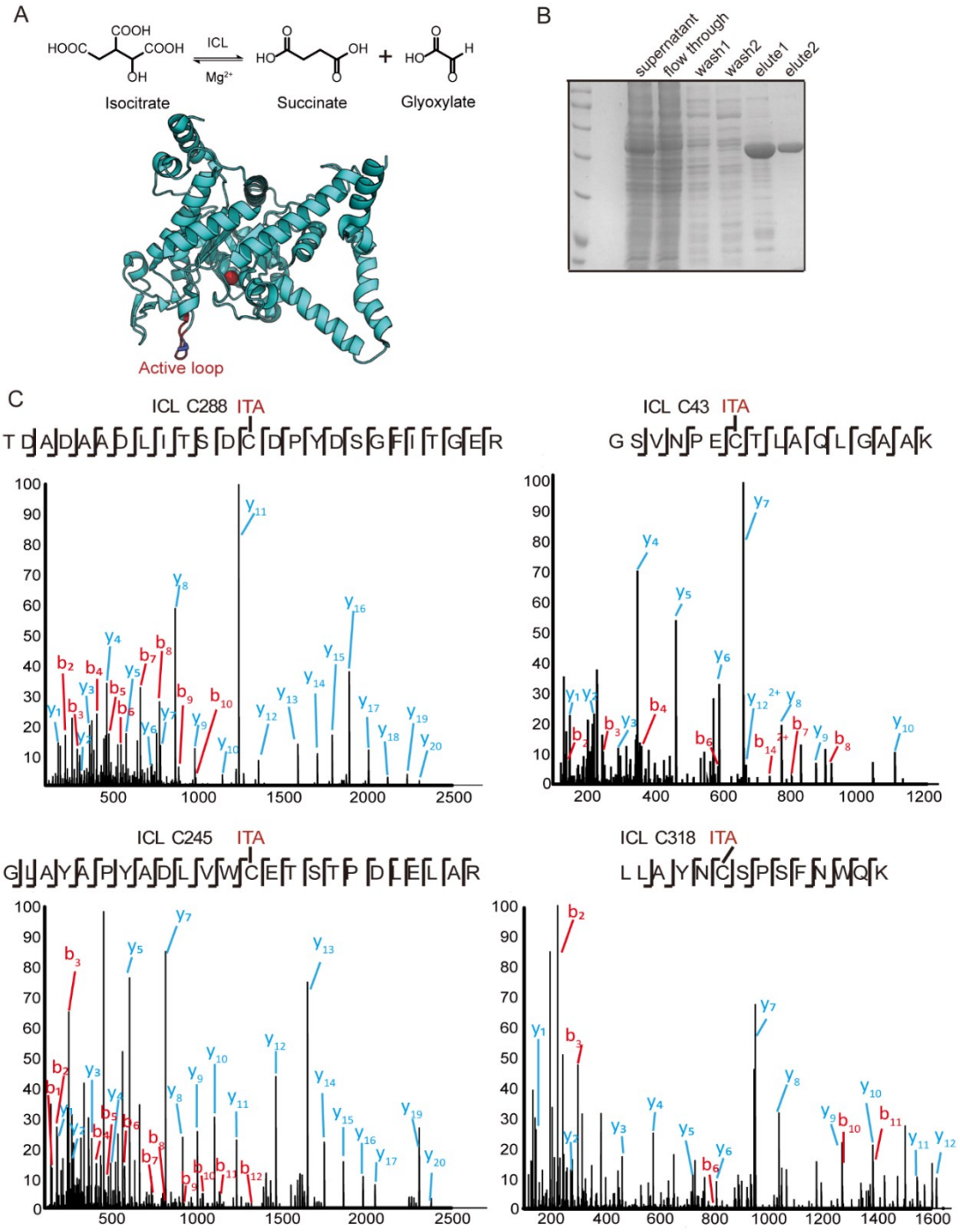
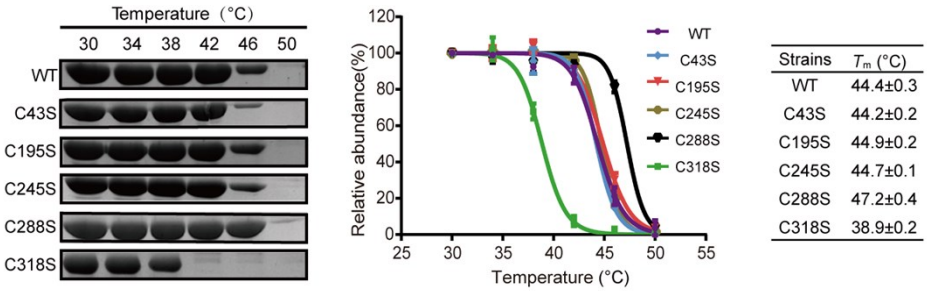


Fig S6. Validation of itaconation sites on ICL. (A) ICL catalyzes the breakdown of isocitrate into succinate and gloxylate. Shown is the structure of ICL in *E. coli* (PDB:1IGW). The loop that contains the active-site Cys195 is colored in red. (B) Recombinant overexpression and purification of the wild-type ICL. (C) MS/MS spectra of the peptides containing the itaconated Cys43, Cys245, Cys288, Cys318 of ICL, respectively. The corresponding b and y ions are shown. The spectra were generated by the pFind software[1].



Strains	T_m (°C)
WT	44.4±0.3
C43S	44.2±0.2
C195S	44.9±0.2
C245S	44.7±0.1
C288S	47.2±0.4
C318S	38.9±0.2

Fig S7. Protein thermal stability of the wild-type ICL (WT) and its mutants. Compared to WT, the C318S mutant showed significantly reduced thermal stability while the C288S mutant showed slightly increased thermal stability.

Experimental Procedures

Cell culture. Raw264.7 were obtained from ATCC and maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% (vol/vol) dialyzed FBS (Thermo Fisher Scientific), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere at 37 °C with 5% CO_2.

***Salmonella* Growth Analysis**. The *Salmonella enterica* serovar Typhimurium strains (SL1344) were used in this study. Bacterial strains were stored under -80 °C conditions in 25% (vol/vol) glycerol. The frozen bacteria were first streaked and cultivated on LB agar plates overnight at 37°C. A single colony was picked and incubated in 3 ml of LB medium. The next morning, the culture was further diluted 1:20 into fresh LB medium and was grown at 37 °C until OD600 reached 0.9. Then the bacteria were collected by centrifugation of 1000 g for 3 min and resuspended in M9 liquid media with 4mg/ml sodium acetate, glucose or glycerol in the presence of different concentrations of itaconate, methylsuccinate or C3A, respectively. The media pH was adjusted to 7 and the growth rates were measured as OD600 at indicated time points.

Plasmids. Full-length complementary DNAs of ICL, ENO, Orn, ATPG, YEBC, YIEF and YHBO were subcloned from the *S.enterica* DNA into the pBAD/HisA vector. The mutant plasmids were generated by using the PCR-based site-directed mutagenesis with TransStart FastPFU DNA polymerase (Beijing TransGen Biotech Co., Ltd).

Antibodies and Reagents. The antibody used for immunoblotting was mouse monoclonal anti-His antibody (cat. no. HT501, Beijing TransGen Biotech Co., Ltd.). Acid-cleavable azide-biotin tags (DADPS Biotin Azide, cat. no. 1330-5) and TAMRA Azide (AZ109) were purchased from Click Chemistry Tools. Itaconate (cat. no. I29204-100G), methylsuccinic acid (cat. no. M81209-25g) were purchased from Sigma. Fumaric acid (cat. no. F0067) was purchased from TCI (Tokyo Chemical Industry). Propargylamine (cat. no. A42615-25g) was purchased from Innochem. 2-Propyn-1-ol (cat. no. 32421-00) was purchased from Kanto. Itaconic anhydride (cat. no. B25216) was purchased from Alfa Aesar. The ICL enzyme activity assay kit (cat. no. BC2030) was purchased from Solarbio.

C3A labeling of wild-type and mutant proteins. Pallets of bacterial strains with overexpression of wild-type proteins and the corresponding mutants were resuspended in ice-cold PBS buffer containing EDTA-free Pierce Halt™ protease inhibitor cocktail. The cells were lysed by sonication in ice and the cell lysates were collected by centrifugation (20,000 *g*, 30 min) at 4°C to remove the debris. The protein concentration was determined by using the BCA protein assay kit (Pierce). 1 mL lysates (2 mg/mL) were incubated with 100 μM probes at 37°C for 2 hours. The lysates were reacted with 1 mM CuSO4, 100 μM TBTA ligand, 100 μM azide-biotin, and 1 mM TCEP for 1 h at room temperature. The resulting click-labeled lysates were precipitated by 4 mL methanol, 1 mL chloroform and 3 mL Milli-Q water. The precipitated proteins were centrifuged at 4000 *g* for 10 min at 4°C, washed twice with 500 μL cold methanol, and then resuspended in 1 mL PBS containing 1.2% SDS. 30 μL of the samples were saved as “input”. 100 μL streptavidin beads (Thermo Fisher Scientific) were washed for

three times with 1 mL PBS, resuspended in 5 mL PBS, and added to the protein solution. The beads were incubated with the protein solution for 4 h at 29 °C, washed with 5 mL PBS for three times, and 5 mL distilled water for three times. The beads were added with 30 μL loading buffer, heated in 90 °C for 10 min and centrifuged at 2000 *g* for 3 min. The supernatant was saved as the "elution". The "input" and "elution" samples were resolved on 10% SDS-PAGE gels and analyzed by western blotting of 6xHis tag.

Enzyme activity. The ICL was purified according to the reported route[4]. It was purified by Ni-NTA column and Superdex 200 Increase 10/300 GL using a buffer containing PBS (pH 7.4), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mM EDTA. The ICL enzyme activity was measured using an optimized ICL activity assay kit (cat. no. BC2030, Solarbio) based on the work as previously reported[5]. It was monitored by coupling the production of glyoxylate to the oxidation of NADH by using lactate dehydrogenase. For the inhibition experiment, ICL (1 mg/ml) was incubated with different concentrations of compounds in 37 °C, and then the activity assays were performed.

***In vitro* proteins labeling by different probes.** For the labeling of proteins in *S.enterica* lysates, frozen *S.enterica* were resuspended in ice-cold PBS buffer containing EDTA-free Pierce Halt™ protease inhibitor cocktail. The cells were lysed by sonication in ice and cell lysates were collected by centrifugation (20,000 *g*, 30 min) at 4 °C to remove the debris. The protein concentration was determined by using the BCA protein assay kit (Pierce). 100 μL of cell lysates (2 mg/ml) were incubated with 500 μM probes at 37°C for 1 h. The resulting lysates were precipitated by 400 μL methanol, 100 μL chloroform and 300 μL Milli-Q water. The precipitated proteins were centrifuged at 8000 *g* for 5 min at 4 °C and washed twice with 500 μL cold methanol. For visualizing the probe labeling efficiency by in-gel fluorescence, the precipitated proteins were resuspended in 50 μL PBS containing 0.4% SDS, 1 mM CuSO4, 100 μM TBTA ligand, 100 μM Rhodamine-N3, and 1 mM TCEP for 1 h at room temperature. The reacted samples were resolved on 10% SDS-PAGE gels and imaged by ChemiDoc XRS+ (Bio-Rad). The gels were then stained by Coomassie brilliant blue to demonstrate equal loading.

Quantitative profiling of itaconated protein targets by C3A. For the identification of itaconation targets by C3A, frozen *S.enterica* were resuspended in ice-cold PBS buffer containing EDTA-free Pierce Halt™ protease inhibitor cocktail. The cells were lysed by sonication in ice and cell lysates were collected by centrifugation (20,000 *g*, 30 min) at 4°C to remove the debris. The protein concentration was determined by using the BCA protein assay kit (Pierce). 3mL lysates (2 mg/mL) were divided into 3 alliquots, each of which was incubated with 5mM itaconate or PBS for 3 hours and then incubated with 500 μM probes or PBS at 37°C for 12 h (see Figure 2A). The resulting lysates were precipitated by 4ml methanol, 1ml chloroform and 3ml Milli-Q water. The precipitated proteins were centrifuged at 4000 *g* for 10 min at 4°C and washed twice with 500 μL cold methanol, and resuspended in 1 mL PBS containing 0.4% SDS. 1 mL cell lysates (2 mg/mL) were reacted with 1 mM CuSO4, 100 μM TBTA ligand, 100 μM azide-biotin, and 1 mM TCEP for 1 h at room temperature. The resulting click-labeled lysates were precipitated by 4mL methanol, 1mL chloroform and 3mL Milli-Q

water. The precipitated proteins were centrifuged at 4000 *g* for 10 min at 4°C, washed twice with 500 μL cold methanol, and then resuspended in 1 mL PBS containing 1.2% SDS. 100 μL streptavidin beads (Thermo Fisher Scientific) were washed for three times with 1 mL PBS, and resuspended in 5 mL PBS, which was added to the protein solution. The beads were incubated with the protein solution for 4 h at 29 °C, and then washed with 5 mL PBS for three times, and 5 mL distilled water for three times. The resulting beads were resuspended in 500 μL TEAB containing 6 M urea, incubated in 10 mM DTT at 37 °C for 30 min, and added with 20 mM iodoacetamide for 30 min at 35 °C in the dark. The beads were then collected by centrifugation and resuspended in 200 μL PBS containing 2 M urea, 1 mM $CaCl_2$ and 10 ng/uL trypsin (Promega). Trypsin digestion was performed at 37 °C with rotation overnight and the beads were washed with 200 μL distilled water for three times.

For dimethyl labeling, per 100 μL peptides were reacted with 4 μL of 4% "light" formaldehyde (Sigma), "medium" formaldehyde (CD2O) and "heavy" formaldehyde (C13D2O) (Sigma), respectively. The resulting solution were treated with 4 μL of 0.6M sodium cyanoborohydride (Sigma) and incubated at RT for 1 h. The reaction was quenched by adding 16 μL of 1% ammonia and 8 μL of 5% formic acid. The "light", "medium" and "heavy" samples were combined and subjected for fraction. The peptides were separated into 20 fractions by a high-pH reverse phase C18 column (Agela Technologies) using Agilent HPLC system. Mobile phase A: 2% ACN–98% H2O (adjusted to pH 10 with NH3·H2O); B: 98% ACN–2% H2O (adjusted to pH 10 with NH3·H2O). Samples were separated using a 20-min gradient of buffer B at a flow rate of 1.5 mL/min, as follows: 0 min 0% B; 0.1 min 5% B; 1 min 8% B; 7 min 14% B; 12 min 24% B; 16 min 40% B; 18 min 95% B; 20 min 15% B. The columns were operated at 25 °C and the temperature was controlled by a built-in column heater. The 20 fractions were combined into 12 fractions, dried in a SpeedVac and subjected to LC-MS/MS analysis.

Identification of itaconation sites by C3A. For the identification of itaconation sites by C3A, frozen *S.enterica* were resuspended in ice-cold PBS buffer containing EDTA-free Pierce HaltTM protease inhibitor cocktail. The cells were lysed by sonication in ice and cell lysates were collected by centrifugation (20,000 g, 30 min) at 4°C to remove the debris. The protein concentration was determined by using the BCA protein assay kit (Pierce). 1mL lysates (2 mg/mL) were incubate with 500 μM probes at 37°C for 12 h. The resulting lysates were precipitated by 4mL methanol, 1mL chloroform and 3mL Milli-Q water. The precipitated proteins were centrifuged at 4000 g for 10 min at 4°C, washed twice with 500 μL cold methanol, and then resuspended in 1 mL PBS containing 0.4% SDS. 1 mL cell lysates (2 mg/mL) were reacted with 1 mM CuSO4, 100 μM TBTA ligand, 100 μM acid-cleavable azide-biotin, and 1 mM TCEP for 1 h at room temperature. The resulting click-labeled lysates were precipitated by 4mL methanol, 1mL chloroform and 3mL Milli-Q water. The precipitated proteins were centrifuged at 4000 g for 10 min at 4°C, washed twice with 500 μL cold methanol, and then resuspended in 1 mL PBS containing 1.2% SDS. The streptavidin enrichment and on-beads Trypsin digestion were performed as described above. Release of the modified peptides from the beads was carried out by incubating the beads with 200 μL of 2% formic acid/water for 1 h with gentle rotation. After centrifugation, the supernatant was collected. Then the cleavage process was repeated and the supernatants were combined. In addition, the beads were

washed with 50% acetonitrile/water containing 1% formic acid (400 µL), and the washes were combined with the supernatant to form the cleavage fraction. Sample was dried in a vacuum centrifuge and stored at −30 °C until analysis by LC-MS/MS.

LC-MS/MS analysis. Samples were analyzed by LC-MS/MS on a Q Exactive series Orbitrap mass spectrometers (Thermo Fisher Scientific) coupled with EasyNano-LC. Under the positive-ion mode, full-scan mass spectra were acquired over the m/z range from 350 to 1800 using the Orbitrap mass analyzer with mass resolution of 70000. MS/MS fragmentation is performed in a data-dependent mode, of which the TOP 20 most intense ions are selected for MS2 analysis a resolution of 17500 using collision mode of HCD. Other important parameters: isolation window, 2.0 m/z units; default charge, 2+; normalized collision energy, 28%; maximum IT, 50 ms; and dynamic exclusion, 20.0 s.

Data analysis. For the dimethyl labeling-based experiments, LC-MS/MS data was first analyzed by ProLuCID[6] with static modification of cysteine (+57.0215 Da). The isotopic modifications (28.0313, 32.0564 and 36.0757 Da for light, medium and heavy labeling respectively) are set as variable modifications on the N-terminal of a peptide and lysines. The ratios of reductive dimethylation were quantified by the CIMAGE software as described previously[7]. For the identification of itaconation sites, LC-MS/MS data was analyzed by ProLuCID with static modification of cysteine (+57.0215 Da). +253.14265 Da on cysteine and +310.16415 Da on lysine, histidine and serine are set as variable modifications.

Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request.

Compound Synthesis

Synthesis of ITalk and C3A

The synthesis of ITalk was performed as previously reported[2]. For the synthesis of C3A, itaconate anhydride (100 mg, 0.892 mmol, 1.0 eq) and propargylamine (51.4mg, 0.936 mmol, 1.05 eq) were added in 3ml tetrahydrofuran and heated to 40°C for 4 hours. After cooling to RT, the solution was concentrated under vacuum and recrystallized in dichloromethane. The resulting solid was purified by silica chromatography column with mobile phase (5~6% methanol in dichloromethane) to afford C3A as a white solid (42.8 mg, 48%).

^{1}H NMR (400 MHz, DMSO-d6) δ = 12.48 (s, 1H), 8.31 (s, 1H), 6.11 (s, 1H), 5.66 (s, 1H), 3.83 (d, J=4.0, 2H), 3.34 (s, 1H), 3.10 (s, 2H).

^{13}C NMR (101 MHz, DMSO-d6) δ = 169.63, 168.01, 136.29, 127.58, 81.66, 73.39, 38.60, 28.39.

ESI MS: calcd. for $C_8H_8NO_3$- $[M-H]^-$ m/z, 166.05097; found, 166.05140.

Synthesis of C8A

For the synthesis of C8A, 8-aminooct-1-yne was prepared as previously reported[3]. To a solution of itaconate anhydride (81.4 mg, 0.73mmol, 1.0 eq) in 2 mL dry tetrahydrofuran was added 8-aminooct-1-yne (100mg, 0.80 mmol, 1.10 eq). After stirring for 2 hours at 40°C, the solution was concentrated under vacuum and recrystallized in dichloromethane. The resulting solid was purified by silica chromatography column with mobile phase (2.5% methanol in dichloromethane) to afford C8A as a white solid (76.5 mg, 45%).

^{1}H NMR (400 MHz, DMSO-d6) δ 12.44 (s, 1H), 7.80 (t, J = 5.7 Hz, 1H), 6.09 (d, J = 1.8 Hz, 1H), 5.64 (d, J = 1.7 Hz, 1H), 3.06 (s, 2H), 3.01 (dt, J = 5.7, 6.7 Hz, 2H), 2.73 (t, J = 2.7 Hz, 1H), 2.14 (td, J = 6.9, 2.7 Hz, 2H), 1.48 – 1.20 (m, 8H).

^{13}C NMR (101 MHz, DMSO-d6) δ 169.62, 168.12, 136.69, 127.21, 84.97, 71.48, 38.99, 39.00, 29.44, 28.38, 28.33, 26.28, 18.11.

ESI MS: calcd. for $C_{13}H_{20}NO_3$+ $[M+H]^+$ m/z, 238.14432; found, 238.14352.

Synthesis of C3E

For the synthesis of C3E, itaconate anhydride (112 mg, 1mmol, 1.0 eq) and 2-propyn-1-ol (67.2mg, 1.2mmol, 1.2 eq) were heated to 60°C for 4 hours. After cooling to RT, the solution was concentrated under vacuum to remove excess propargyl alcohol, then recrystallized in dichloromethane. The resulting solid was purified by silica chromatography column with mobile phase (2.5% methanol in dichloromethane) and recrystallized in cold ether to afford C3E as white crystals (58.8 mg, 35%).

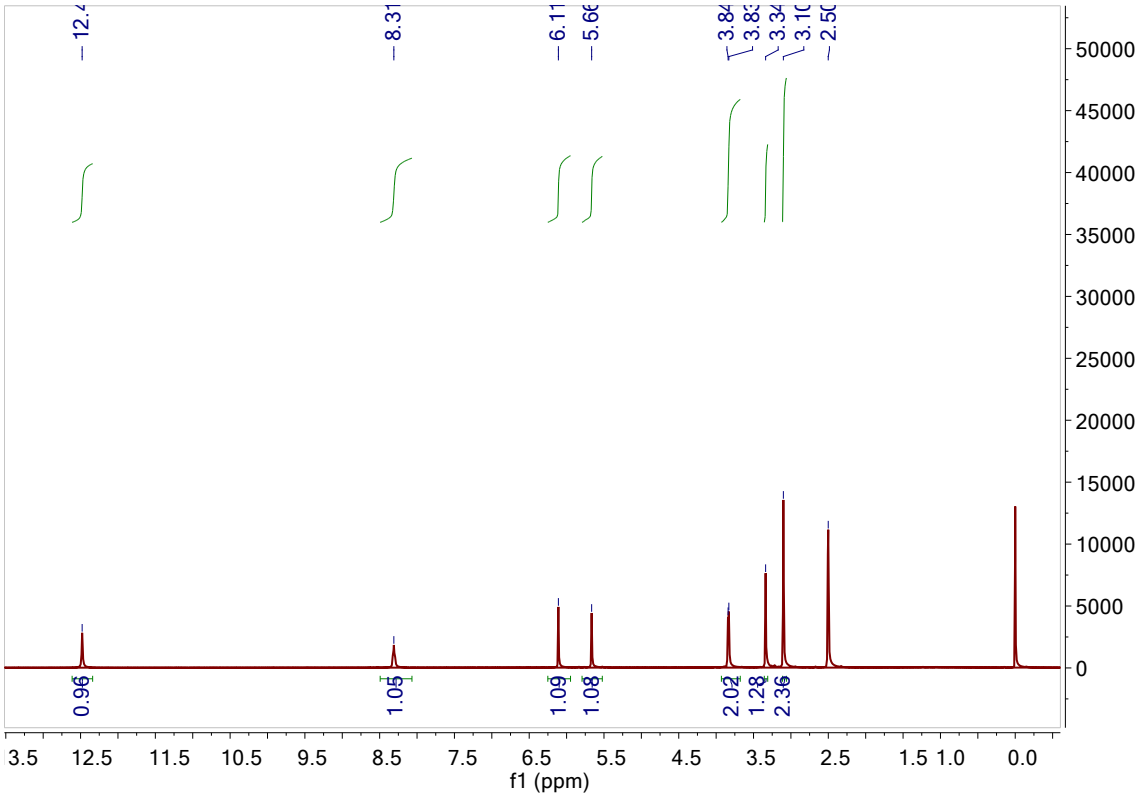
^{1}H NMR (400 MHz, DMSO-d6) δ 12.68 (s, 1H), 6.18 (s, 1H), 5.80 (s, 1H), 4.69 (s, 2H), 3.55 (s, 1H), 3.36 (s, 2H).

^{13}C NMR (101 MHz, DMSO-d6) δ 170.30, 167.63, 134.88, 128.86, 78.78, 78.21, 52.39, 37.25.

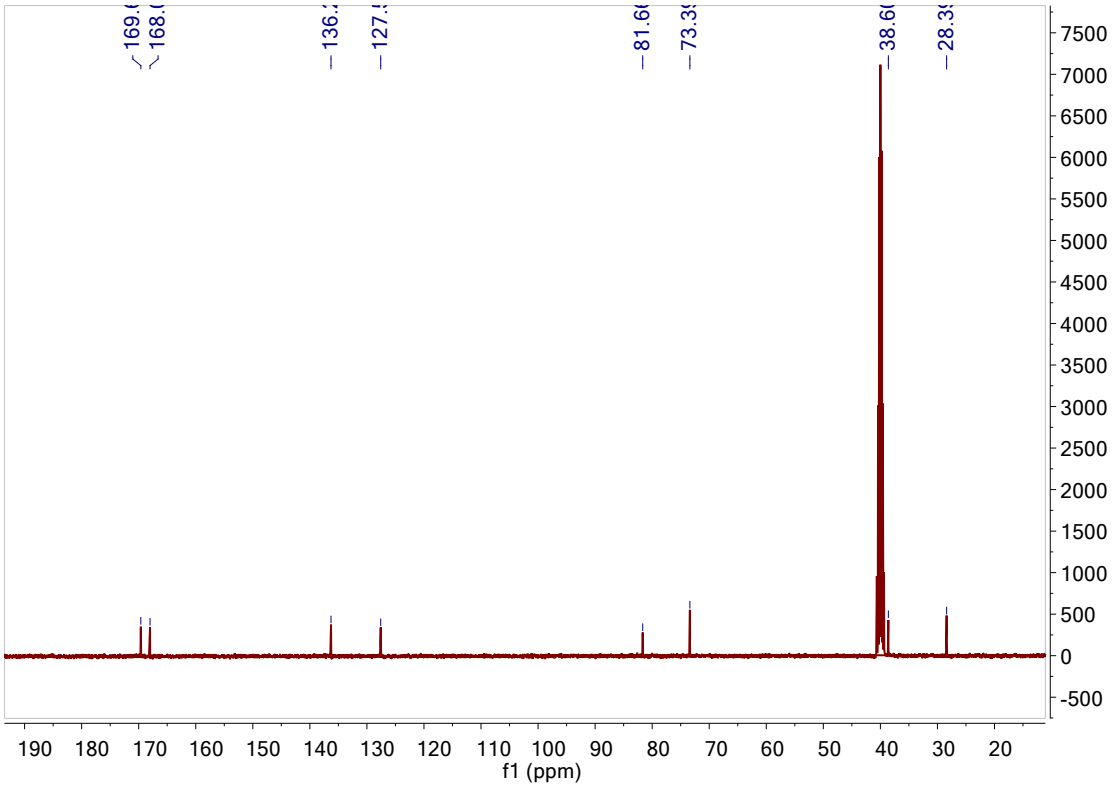
ESI MS: calcd. for $C_8H_9NO_4$ $[M+H]^+$ m/z, 169.05008; found, 169.04926.

Compound characterizations

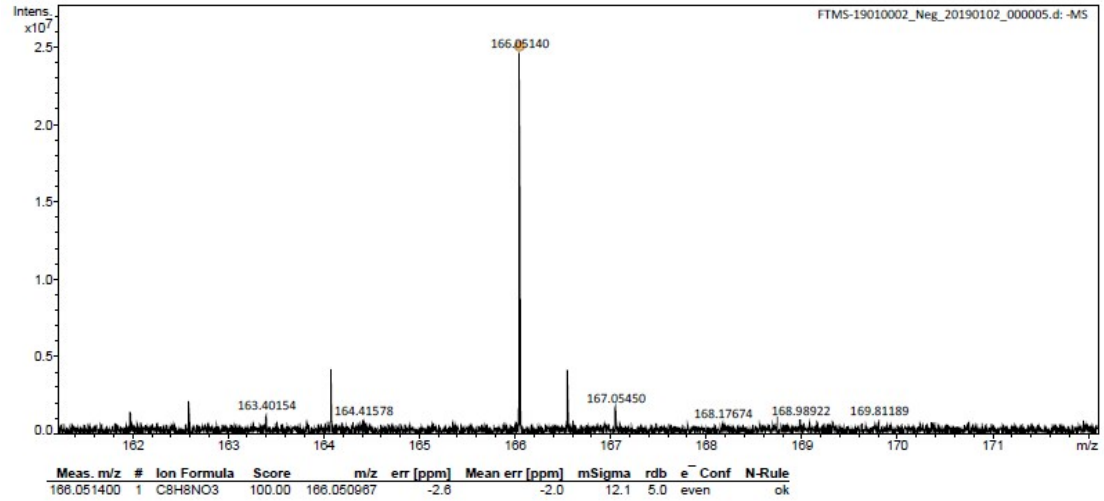
^{1}H NMR spectrum of C3A (400 MHz, DMSO-d6).



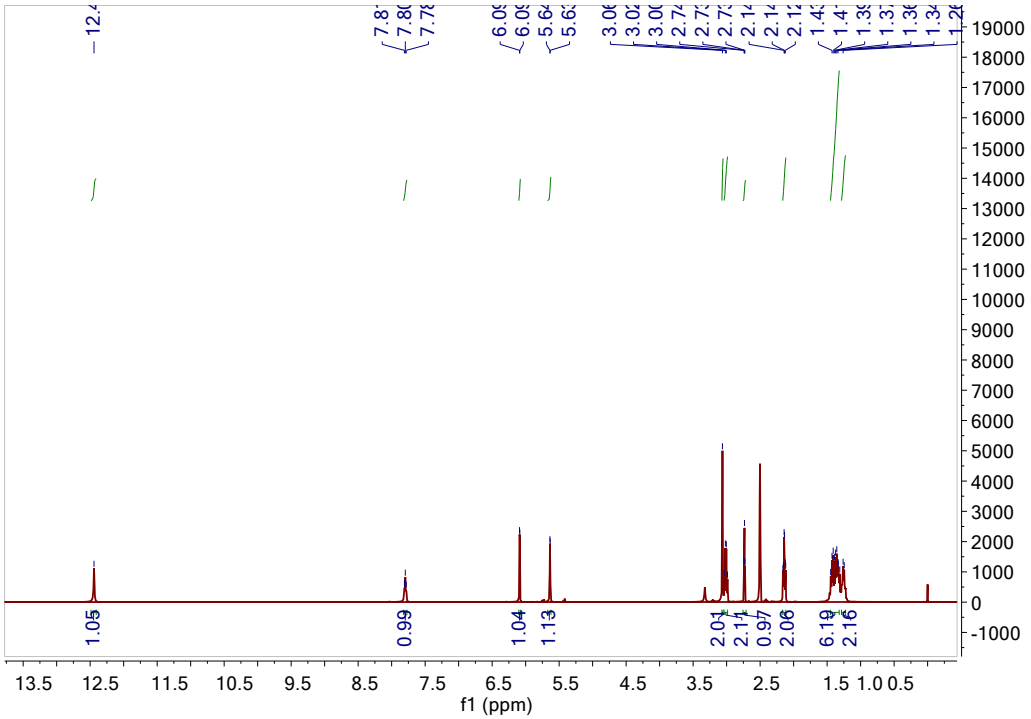
^{13}C NMR spectrum of C3A (101 MHz, DMSO-d6).



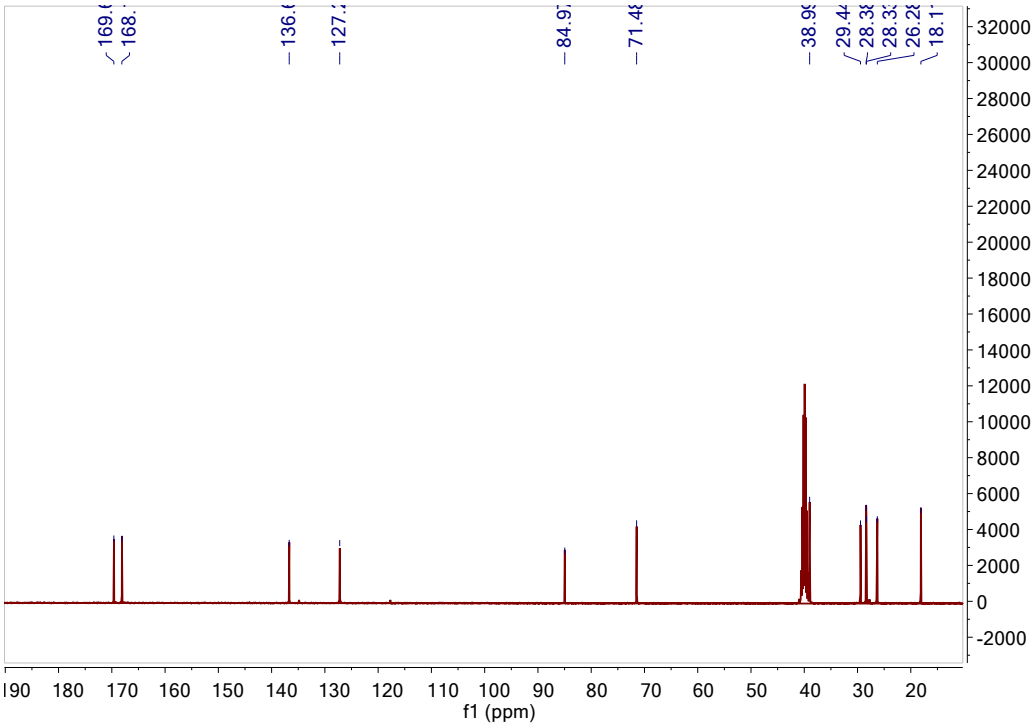
HRMS of C3A



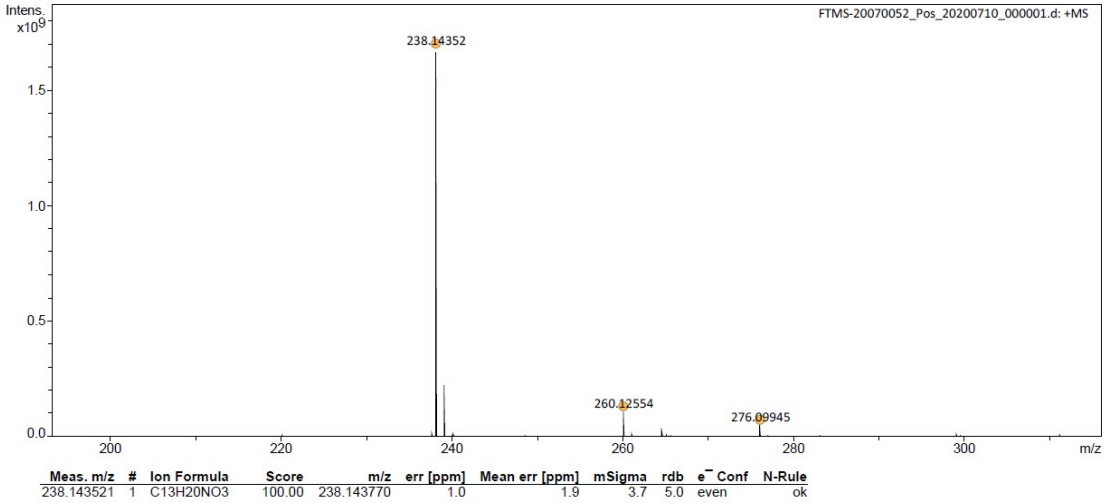
^{1}H NMR spectrum of C8A (400 MHz, DMSO-d6).



^{13}C NMR spectrum of C8A (101 MHz, DMSO-d6).

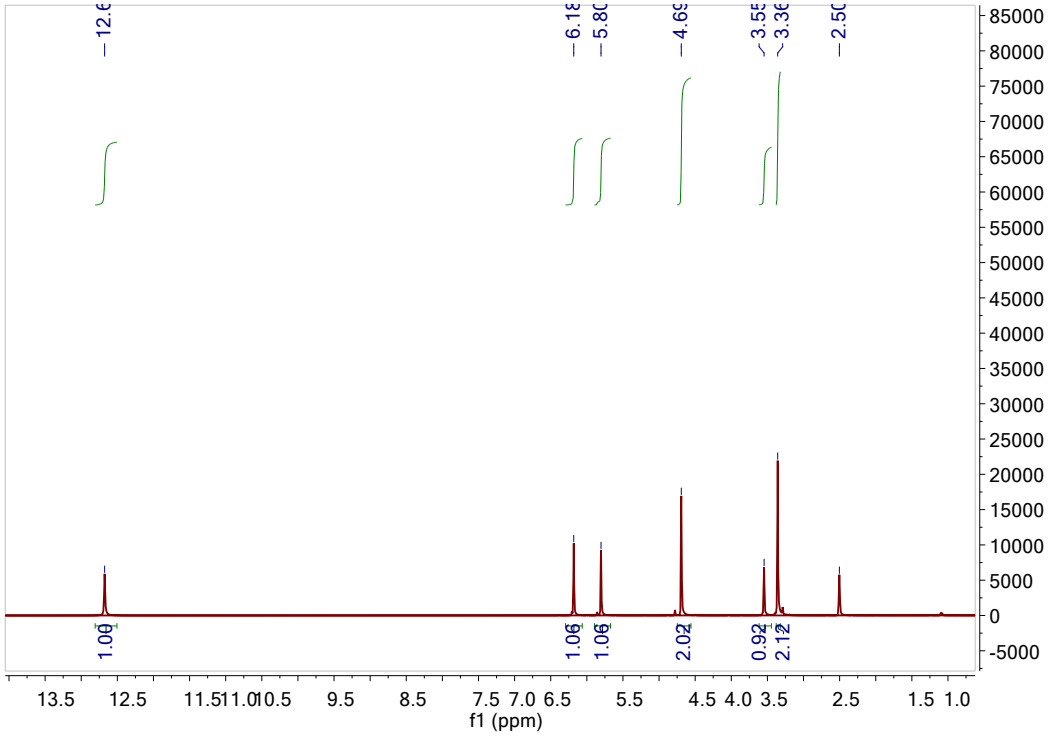


HRMS of C8A

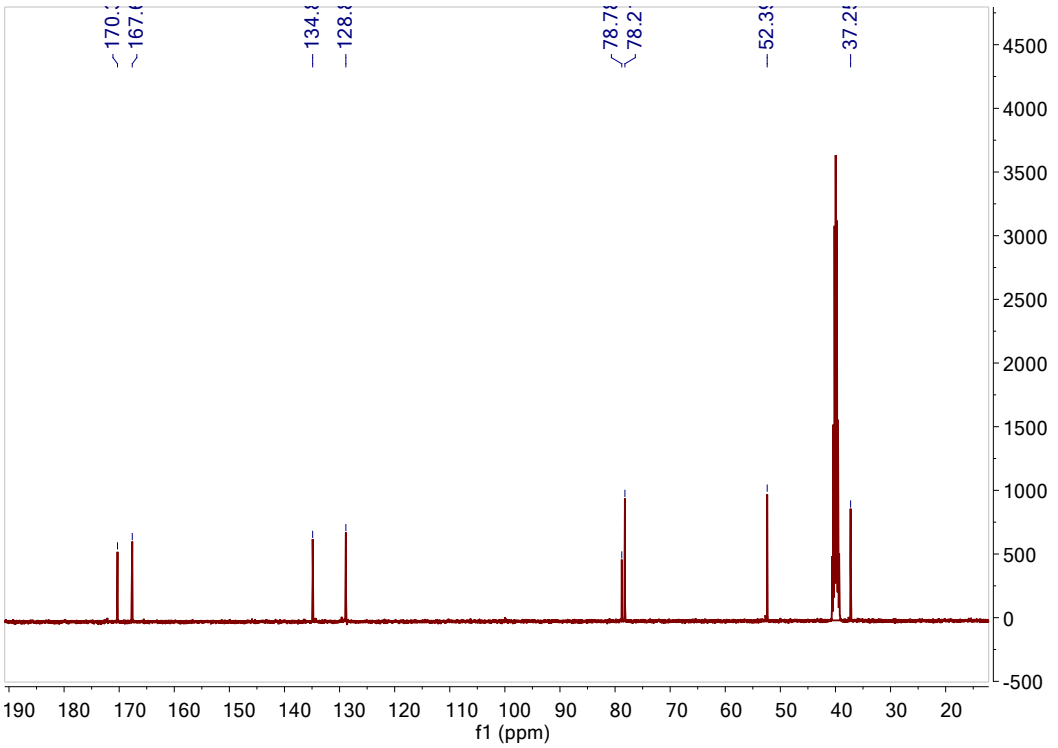


Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
238.143521	1	C13H20NO3	100.00	238.143770	1.0	1.9	3.7	5.0	even	ok

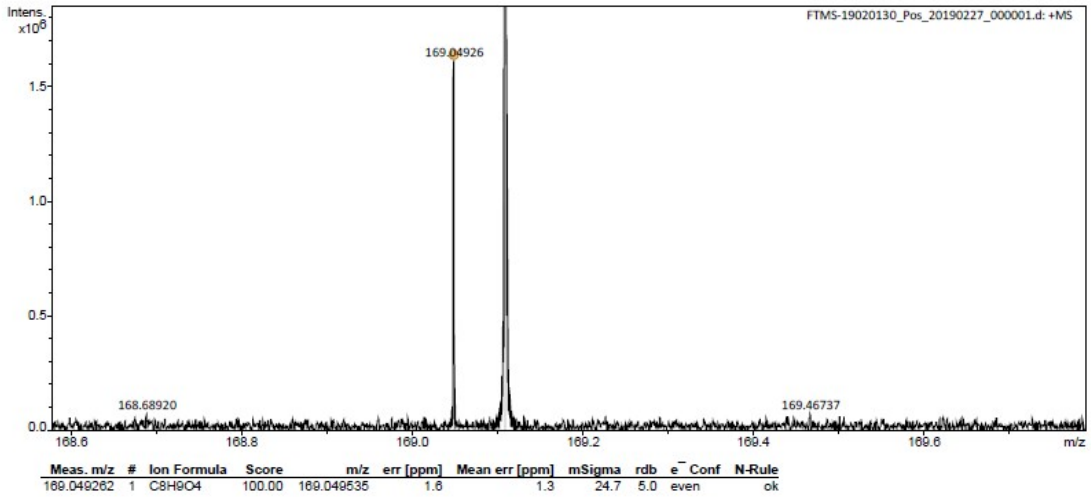
^{1}H NMR spectrum of C3E (400 MHz, DMSO-d6).



^{13}C NMR spectrum of C3E (101 MHz, DMSO-d6).


170.
167.
134.
128.
78.7
78.2
52.3
37.2
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20
f1 (ppm)

HRMS of C3E


Intens.
x10^6
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169.0
169.2
169.4
169.6
m/z
Meas. m/z # Ion Formula Score m/z err [ppm] Mean err [ppm] mSigma rdb e⁻ Conf N-Rule
169.049262 1 C8H9O4 100.00 169.049535 1.6 1.3 24.7 5.0 even ok

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