

## Supporting Information

# DMSO-Perturbing Assay for Identifying Promiscuous Enzyme Inhibitors

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### **Protocol for Enzyme Inhibitory Assays.**

**General.** Dimethyl sulfoxide (DMSO) for biochemical research was purchased from Nacalai, and used as received. Milli-Q water was used throughout. Absorbance was measured with a JASCO V-660 spectrometer at 25 °C. Concentration-response curves were plotted, and IC<sub>50</sub> values were calculated by GraphPad Prism 7 software using a sigmoidal concentration-response curve analysis with variable slope.

**β-Lactamase (no detergent/with detergent/with DTT).** β-Lactamase (BL) from *Enterobacter cloacae* was purchased from Sigma (P4399), and BL from *Bacillus cereus* 569/H9 was purchased from Merck (426205). These enzymes were dissolved in and diluted with potassium phosphate buffer containing with 1.0 mg bovine serum albumin mL<sup>-1</sup> just before use. Enzyme solutions were kept on ice during the experiments. The molecular mass of BL from *B. cereus* 569/H9 was taken as 31,500 Da. Nitrocefin was purchased from Cayman Chemical, prepared as a 14 mM stock solution in DMSO, and stored at -20 °C. Triton X-100 (TX-100) was purchased from Wako, and prepared as a 1% (v/v) buffer solution daily. Dithiothreitol (DTT) was purchased from Wako, and prepared as a 10 mM buffer solution daily. The solution was kept on ice during the experiments. The inhibitory assay for BL from *E. cloacae* was performed in potassium phosphate buffer (50 mM, pH 7.0) containing BL (65 nM, final concentration), NaCl (1 M, final concentration), and in the absence and presence of TX-100 (0.05%, v/v, final concentration) or DTT (1 mM, final concentration). With 1 mM DTT, BL from *E. cloacae* retained full enzymatic activity. After 10 min incubation with test compound at 25 °C, the reaction was initiated by addition of nitrocefin (100 μM, final concentration) to give a final volume of 700 μL. Reaction progress was monitored by the absorbance at 492 nm for 5 min. The final concentration of DMSO was less than 4% (v/v). The

inhibitory assay for BL from *B. cereus* 569/H9 was performed in potassium phosphate buffer (50 mM, pH 7.0) containing BL (~0.5  $\mu$ M, final concentration), NaCl (100  $\mu$ M, final concentration), ZnCl<sub>2</sub> (100  $\mu$ M, final concentration), and in the absence and presence of TX-100 (0.05%, v/v, final concentration). After 10 min incubation with test compound at 25 °C, the reaction was initiated by addition of nitrocefin (100  $\mu$ M, final concentration) to give a final volume of 700  $\mu$ L. Reaction progress was monitored by the absorbance at 492 nm for 5 min. The final concentration of DMSO was less than 4% (v/v).

**$\beta$ -Lactamase (with DMSO).** The assay was performed as described above except that enzyme was incubated with DMSO at 25 °C for 5 min before addition of test compound.

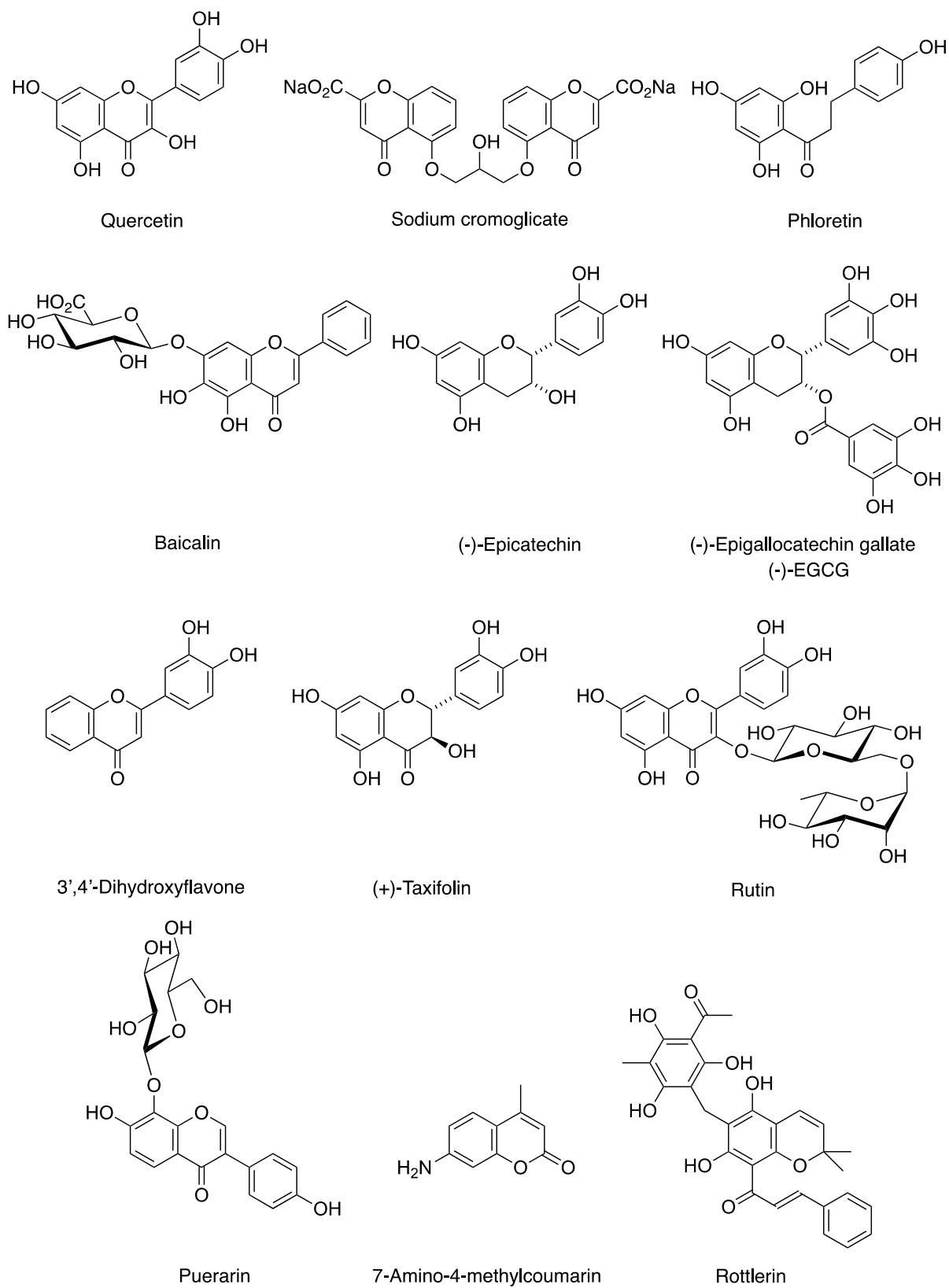
**$\alpha$ -Chymotrypsin (no detergent/with detergent/with DTT).** Bovine pancreatic  $\alpha$ -chymotrypsin (CT) was purchased from Sigma (C4129) and used as received. The molecular mass of CT was taken as 25 kDa. *N*-Succinyl-L-phenylalanine-*p*-nitroanilide (SPpNA) was purchased from Sigma and prepared daily as a 40 mM solution in DMSO. *N*-Succinyl-L-alanine-L-alanine-L-proline-L-phenylalanine-*p*-nitroanilide (SAAPpNA) was purchased from Sigma (S7388), prepared as a 10 mM stock solution in DMSO, and stored at 4 °C. A microbial chymostatin, a mixture of chymostatin A–C, was purchased from Peptide Institute (4063) and used as a positive control. The assay was performed in Tris-HCl buffer (80 mM, pH 7.8) containing CT (6  $\mu$ M, final concentration), CaCl<sub>2</sub> (50 mM, final concentration), and in the absence and presence of TX-100 (0.05%, v/v, final concentration) or DTT (1 mM, final concentration). With 1 mM DTT, the activity of CT was somewhat reduced. After 30 min incubation with test compound at 25 °C, the enzymatic reaction was initiated by addition of SPpNA (400  $\mu$ M, final concentration) to give a final volume of 1 mL. Reaction progress was monitored by the absorbance at 410 nm for 12 min. The final concentration of DMSO was 3%

(v/v). The assay with low concentration of CT was performed in Tris-HCl buffer (80 mM, pH 7.8) containing CT (6 nM, final concentration) and  $\text{CaCl}_2$  (20 mM, final concentration). After 30 min incubation with test compound at 25 °C, the enzymatic reaction was initiated by addition of SAAPPpNA (100  $\mu\text{M}$ , final concentration) to give a final volume of 700  $\mu\text{L}$ . Reaction progress was monitored by the absorbance at 410 nm for 5 min. Enzymes were dissolved in and diluted with assay buffer containing with 1.0 mg bovine serum albumin  $\text{mL}^{-1}$  just before use to prevent enzyme denaturation. Enzyme solutions were kept on ice during the experiments. The final concentration of DMSO was 3% (v/v).

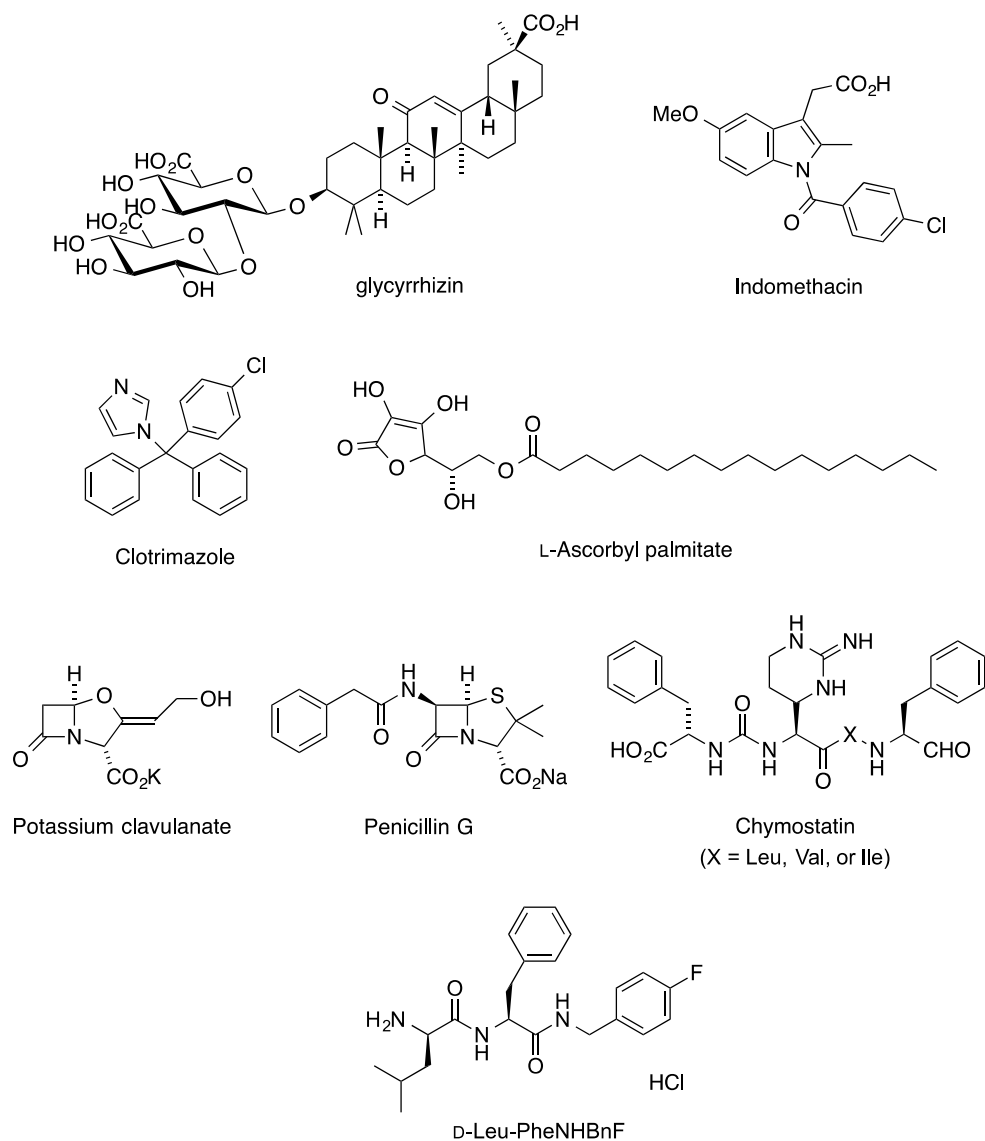
**$\alpha$ -Chymotrypsin (with DMSO).** The assay was performed as described above except that enzyme was incubated with DMSO at 25 °C for 5 min before addition of test compound.

**Dilution Test.** The enzymes at a concentration of 10 times usual concentration were incubated at different buffer/DMSO concentrations at 25 °C for 5 min. Then, the resulting solutions were diluted 10-fold with assay solutions to decrease all assay components to their usual assay concentrations as described above. After 10 min incubation for the BLs or 30 min incubation for CT at 25 °C, the enzymatic reactions were initiated by addition of corresponding substrates. The assays were otherwise performed as stated above.

**Figure S1.** Structures of the test compounds.



**Figure S1. (Continued).**



**Table S1. Enzyme Inhibitory Activity (% inhibition)<sup>a</sup> of The Test Compounds**

Entry	Compound	$\beta$ -Lactamase from <i>E. cloacae</i> <sup>b</sup>	$\alpha$ -Chymotrypsin <sup>c</sup>
1	(-)-Epicatechin	$-3.7 \pm 4.6$	$-0.62 \pm 2.2$
2	(-)-EGCG	$51 \pm 8.7$	$-5.1 \pm 6.2$
3	Baicalin	$39 \pm 4.9$	$22 \pm 4.5$
4	Glycyrrhizin	$40 \pm 9.1$	$3.0 \pm 6.8$
5	Sodium cromoglicate	$1.5 \pm 5.3$	$1.8 \pm 1.6$
6	Indomethacin	$2.7 \pm 5.5$	$6.8 \pm 3.2$
7	L-Ascorbyl palmitate	$7.9 \pm 4.9$	nd <sup>d</sup>
8	Clotrimazole	nd <sup>d</sup>	nd <sup>d</sup>
9	(+)-Taxifolin	$8.5 \pm 6.3$	$-3.1 \pm 0.78$
10	Puerarin	$6.4 \pm 8.4$	$-1.4 \pm 4.4$
11	7-Amino-4-methylcoumarin	$5.8 \pm 2.5$	$3.6 \pm 3.1$

<sup>a</sup>Data are presented as the mean  $\pm$  SE from at least three independent experiments. <sup>b</sup>At 500  $\mu$ M. <sup>c</sup>At 300  $\mu$ M. <sup>d</sup>Not determined due to the insolubility.



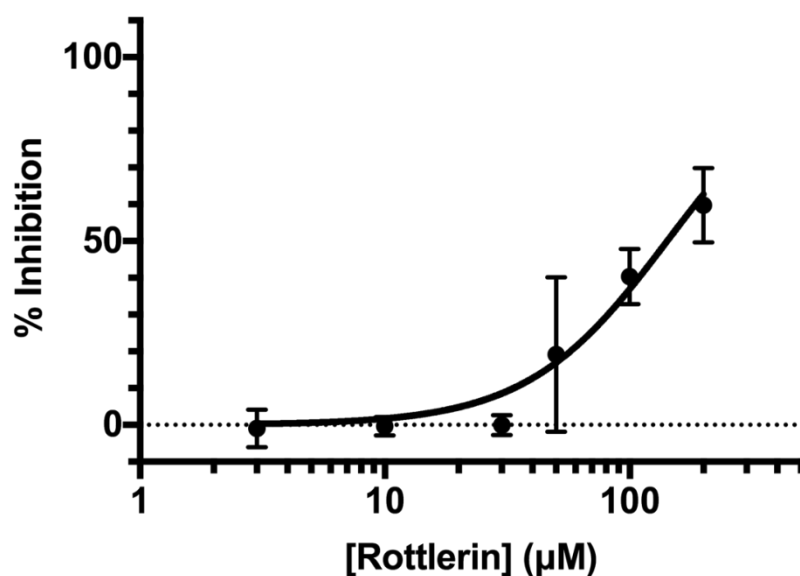
**Table S2. BL from *B. cereus* 569/H9 Inhibitory Activity (IC<sub>50</sub> values,  $\mu$ M)<sup>a</sup> of The Test****Compounds**

Entry	Compound	No detergent	With detergent <sup>b</sup>
1	Quercetin	>500 (13 $\pm$ 4.3) <sup>c</sup>	nt <sup>d</sup>
2	Rutin	>500 (20 $\pm$ 3.7) <sup>c</sup>	nt <sup>d</sup>
3	Phloretin	>500 (10 $\pm$ 2.3) <sup>c</sup>	nt <sup>d</sup>
4	Rottlerin	>500 (33 $\pm$ 4.1) <sup>c</sup>	nt <sup>d</sup>
5	3',4'-Dihydroxyflavone	nd <sup>e</sup>	nt <sup>d</sup>
6	Potassium clavulanate <sup>f</sup>	7.6 $\pm$ 2.0	6.1 $\pm$ 3.5
7	Penicillin G <sup>f</sup>	>500 (6.6 $\pm$ 0.53) <sup>c</sup>	nt <sup>d</sup>
8	(-)-Epicatechin	>500 (0.0 $\pm$ 5.5) <sup>c</sup>	nt <sup>d</sup>
9	(-)-EGCG	>500 (21 $\pm$ 2.0) <sup>c</sup>	nt <sup>d</sup>
10	Baicalin	>500 (2.3 $\pm$ 1.9) <sup>c</sup>	nt <sup>d</sup>
11	Glycyrrhizin	>500 (5.6 $\pm$ 6.9) <sup>c</sup>	nt <sup>d</sup>
12	Sodium cromoglicate	>500 (-17 $\pm$ 2.3) <sup>c</sup>	nt <sup>d</sup>
13	Indomethacin	>500 (10 $\pm$ 1.4) <sup>c</sup>	nt <sup>d</sup>
14	L-Ascorbyl palmitate	>500 (24 $\pm$ 9.2) <sup>c</sup>	nt <sup>d</sup>
15	Clotrimazole	nd <sup>e</sup>	nt <sup>d</sup>
16	(+)-Taxifolin	>500 (10 $\pm$ 2.8) <sup>c</sup>	nt <sup>d</sup>
17	Puerarin	>500 (-1.4 $\pm$ 4.4) <sup>c</sup>	nt <sup>d</sup>
18	7-Amino-4-methylcoumarin	>500 (-3.8 $\pm$ 5.6) <sup>c</sup>	nt <sup>d</sup>

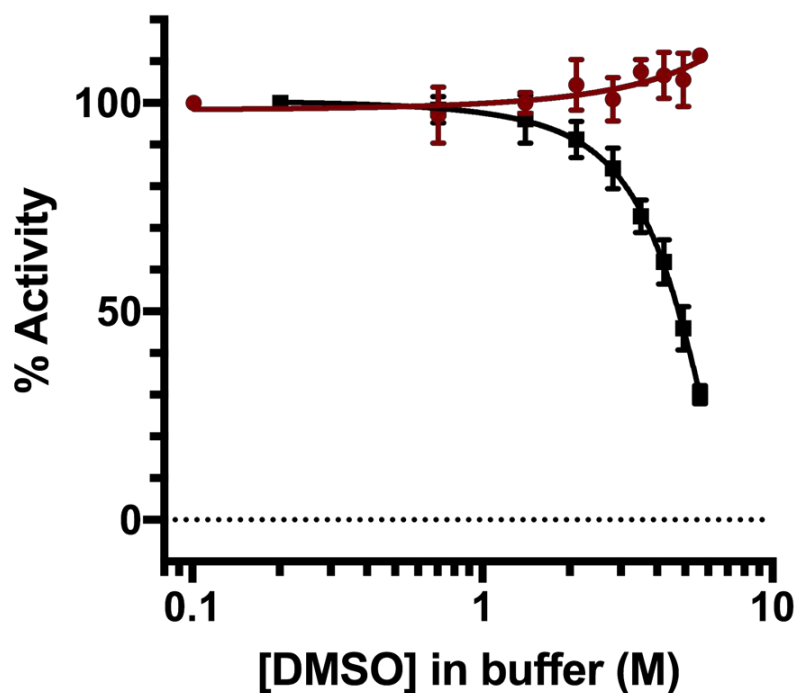
<sup>a</sup>Data are presented as the mean  $\pm$  SE from at least three independent experiments. <sup>b</sup>In the presence of 0.05% (v/v) Triton X-100. <sup>c</sup>Figures in parentheses are % inhibition at 500  $\mu$ M.

<sup>d</sup>Not tested. <sup>e</sup>Not determined due to the insolubility. <sup>f</sup>Positive control.

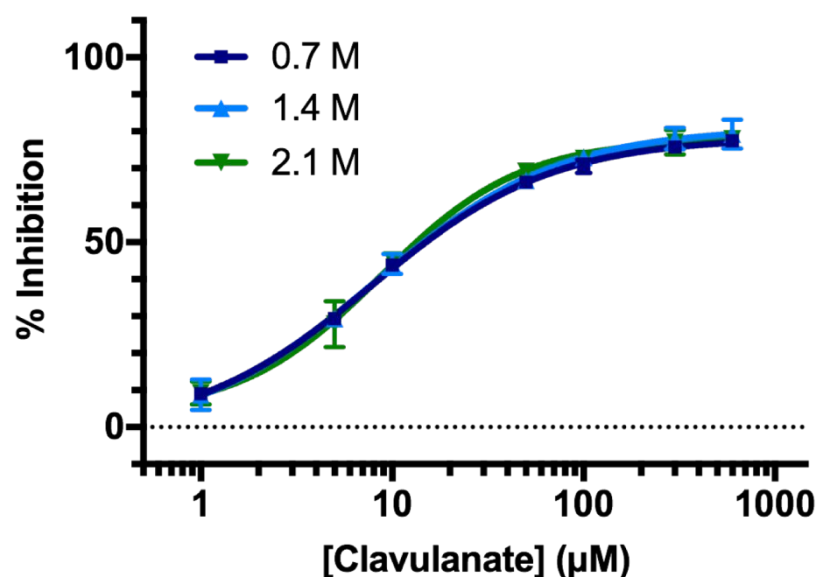
**Figure S2.** Concentration-response curves of rottlerin in the presence of Triton X-100 (0.05%, v/v). Data are presented as the mean  $\pm$  SD from at least three independent experiments.



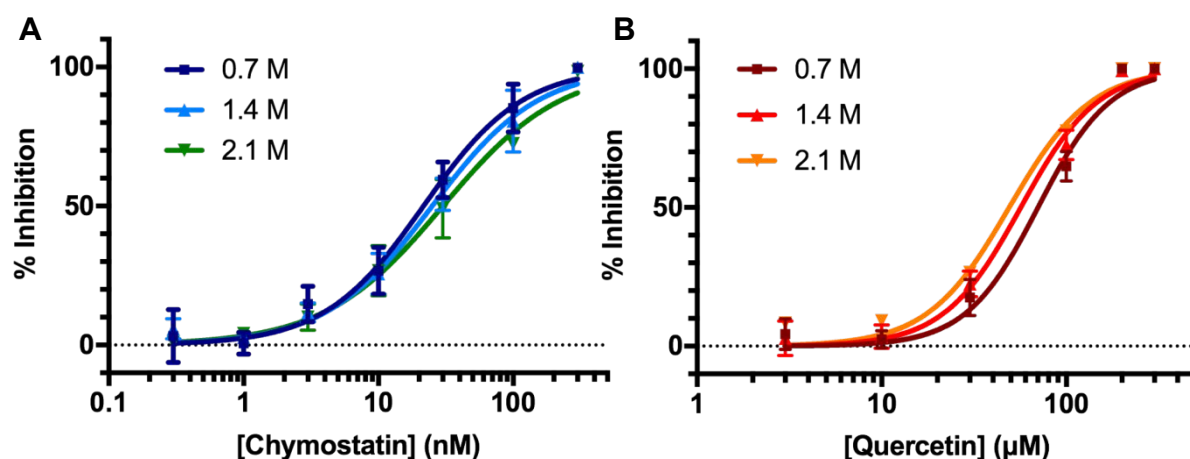
**Figure S3.** Enzymatic activity of BL from *B. cereus* 569/H9 at different concentration of DMSO. Black lines indicate the intrinsic activities, and red lines indicate the recovered activities upon 10-fold dilutions. Data are presented as the mean  $\pm$  SD from at least three independent experiments.



**Figure S4.** Concentration-response curves of potassium clavulanate in the DMSO-perturbing assay of BL from *B. cereus* 569/H9. Data are presented as the mean  $\pm$  SD from at least three independent experiments.

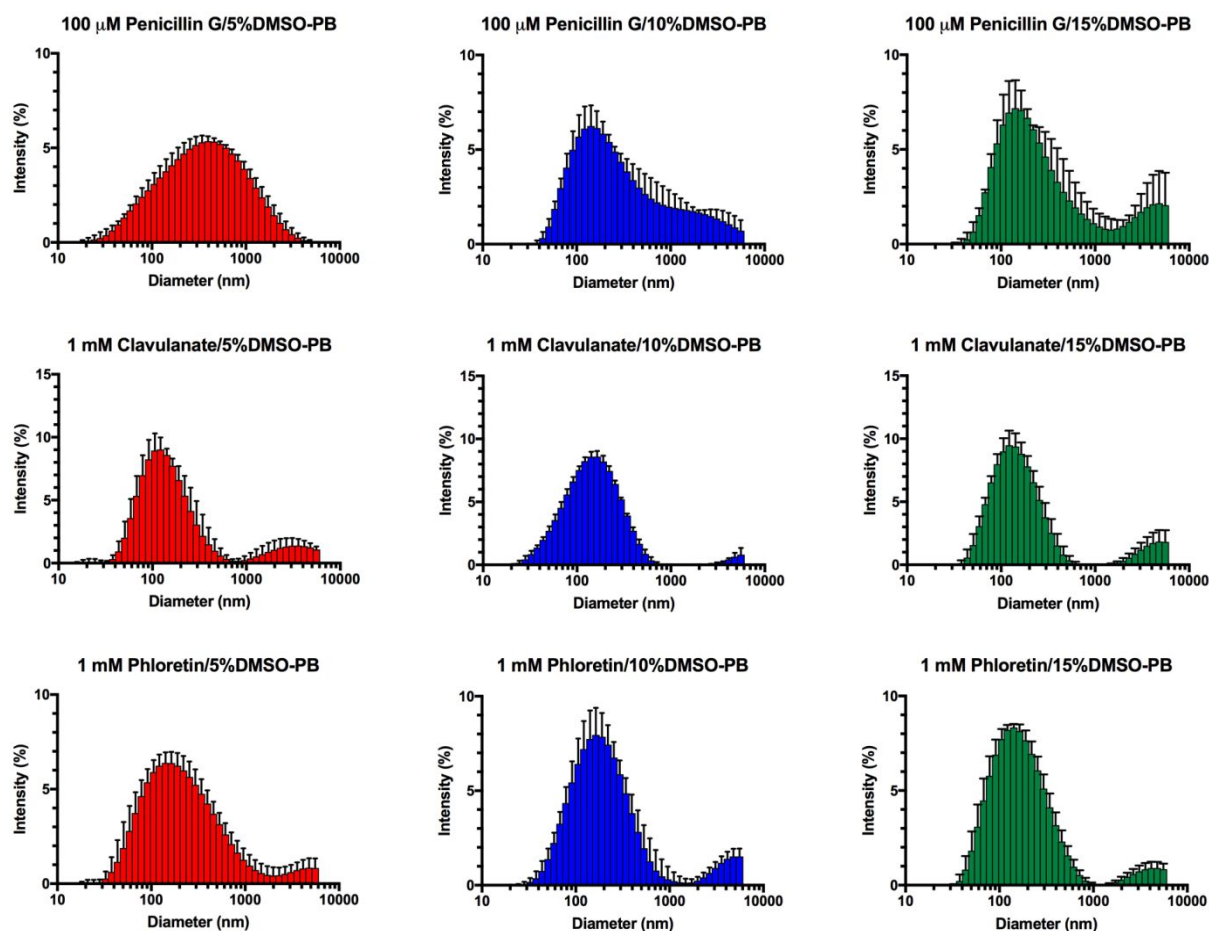


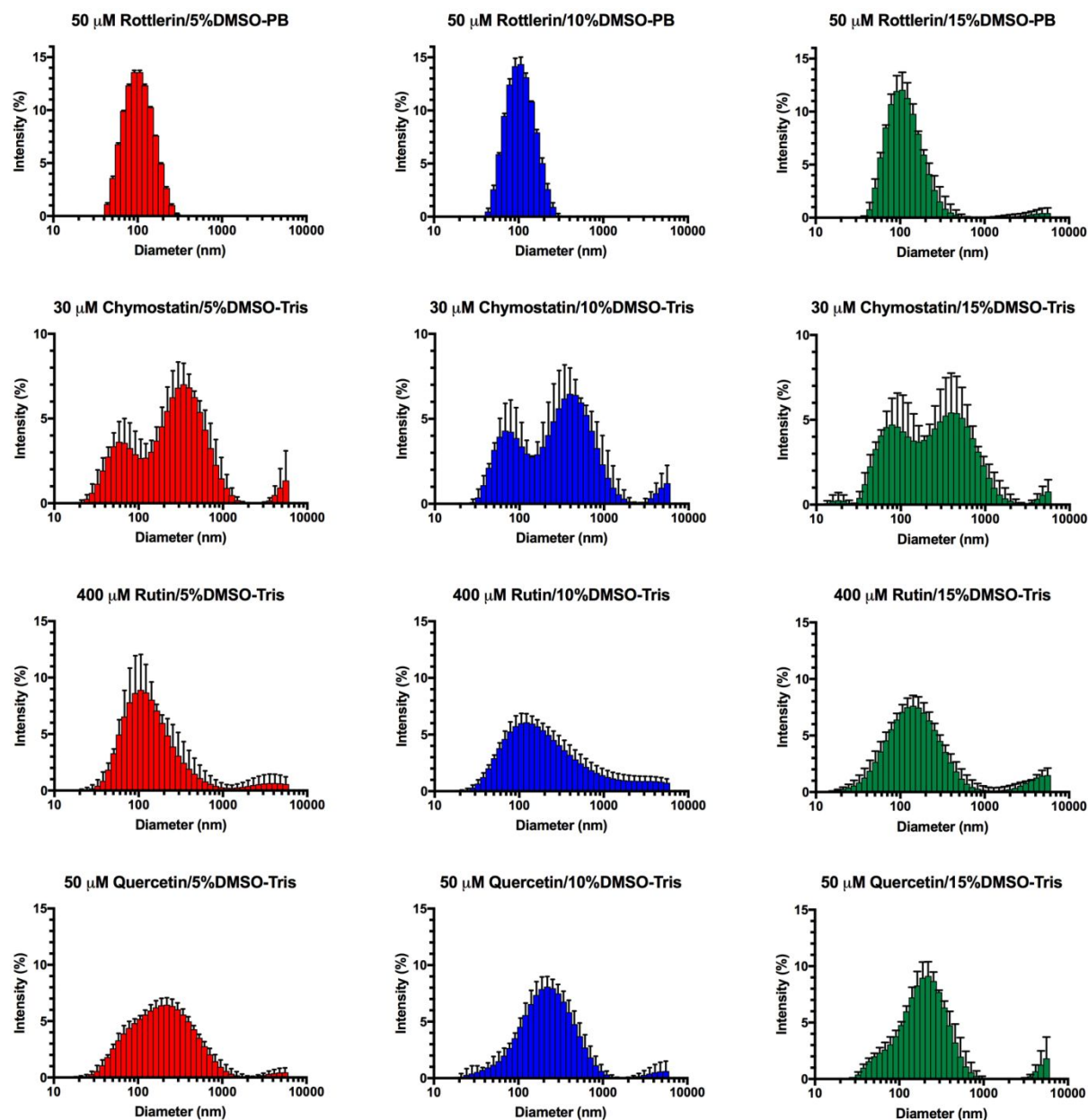
**Figure S5.** Concentration-response curves of chymostatin (A) and quercetin (B) in the DMSO-perturbing assay at the reduced CT concentration (6 nM). The legends in each panel indicate a final concentration of DMSO. Data are presented as the mean  $\pm$  SD from at least three independent experiments.



**Dynamic Light Scattering.** Dynamic light scattering (DLS) was measured on a Malvern Zetasizer Nano ZS equipped with a quartz cuvette of 3 mm path length at 25 °C. Assay buffers, DMSO, and DMSO stock solutions of test compounds were filtered through a 0.2 µm filter (Sartorius Minisart RC) before preparing test solutions. The values reported are the mean ± SD obtained from at least three measurements.

**Figure S6.** Particle size distributions of enzyme inhibitors at different aqueous DMSO concentrations.

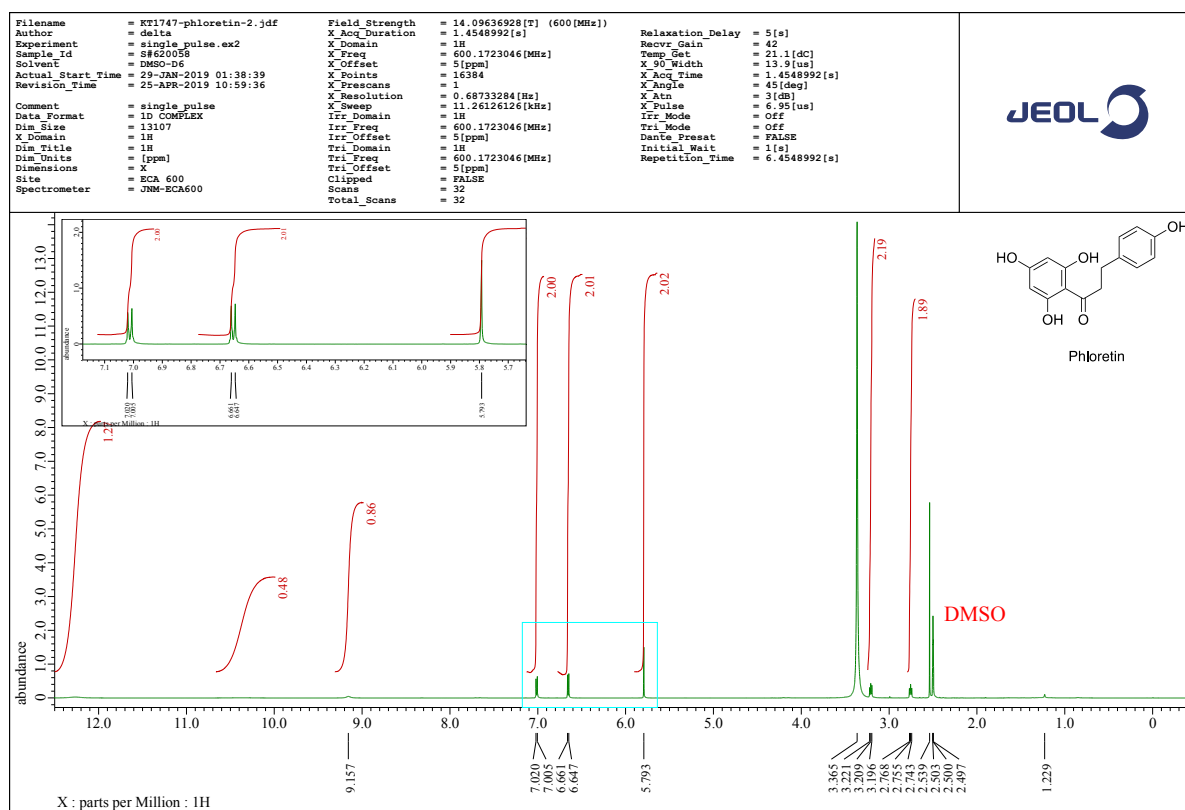




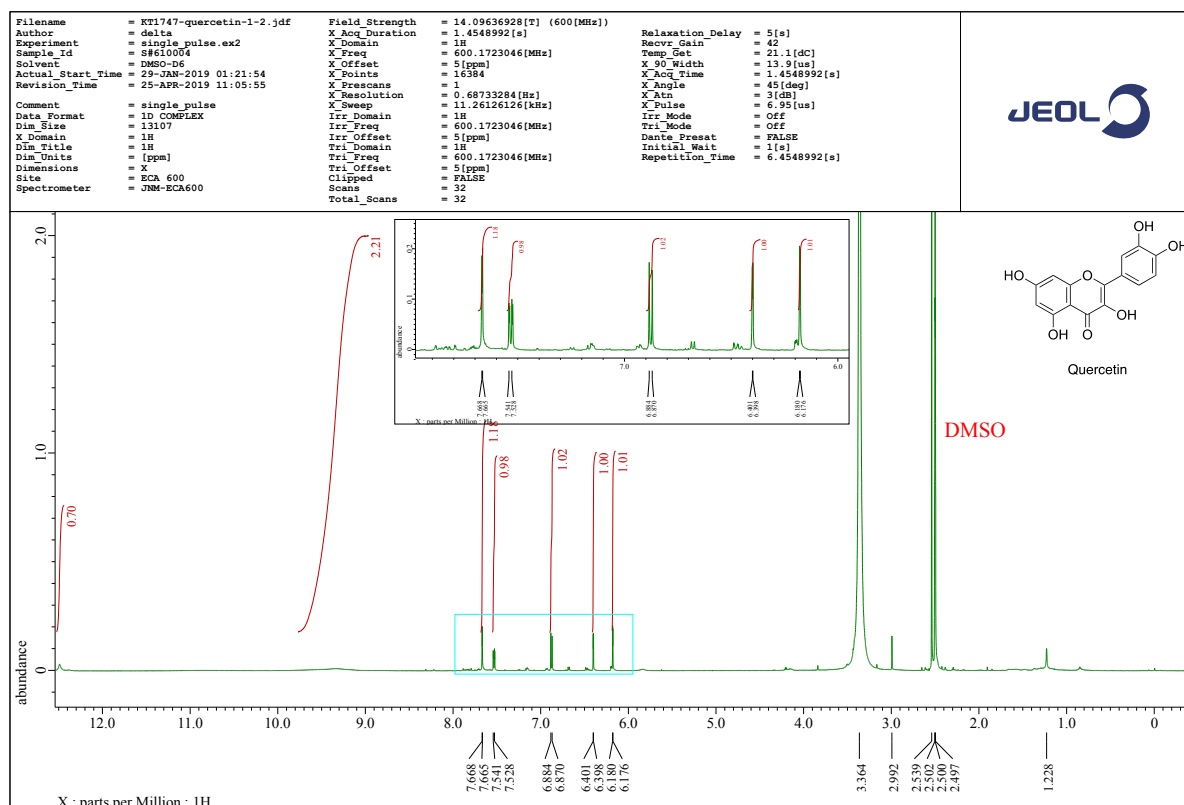
**Quality Control Analysis.** NMR spectra were recorded on JEOL JNM-ECA600 spectrometer with tetramethylsilane as an internal standard. The inhibitors were incubated under the assay conditions. Then, the resulting incubation mixtures were extracted with ethyl acetate three times, and the combined extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. The inhibitors were quantitatively recovered. Purity was analyzed by <sup>1</sup>H NMR in DMSO-*d*<sub>6</sub>.

**Figure S7.** NMR spectra of the recovered phloretin, quercetin, and rottlerin in the QC analysis.

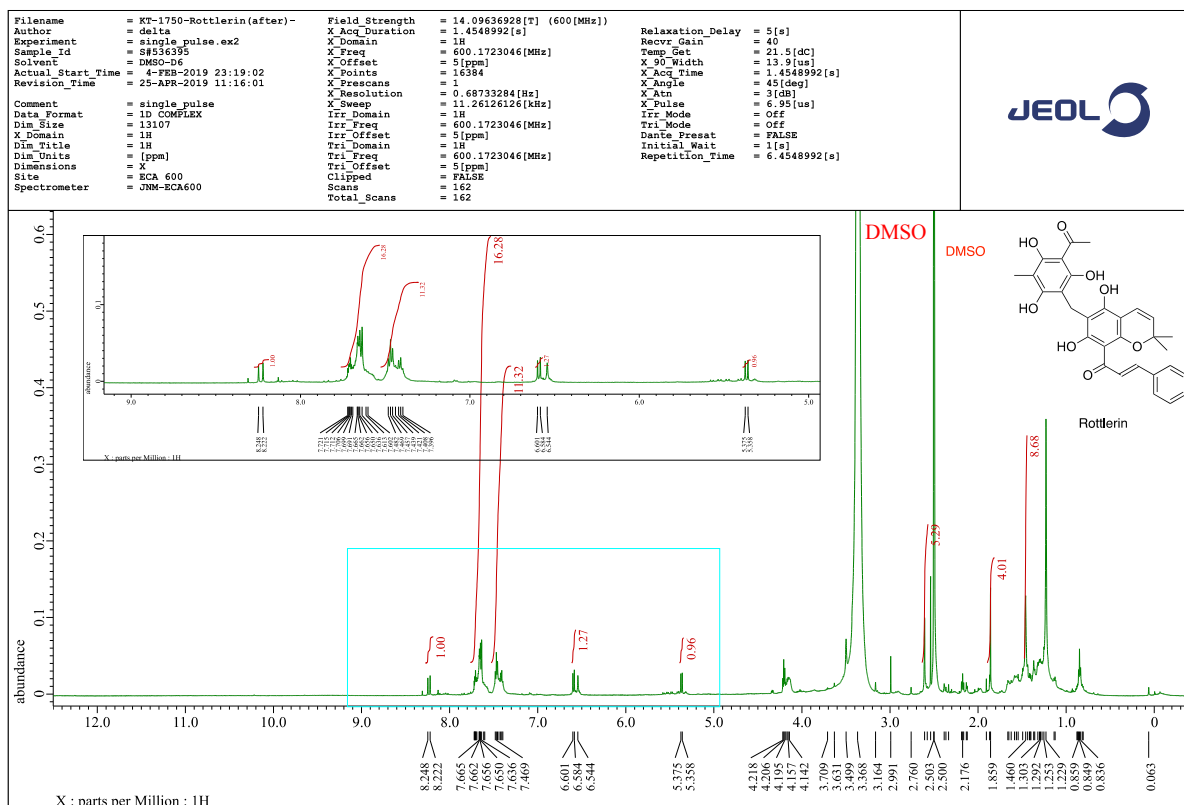
### (A) Phloretin



## (B) Quercetin



## (C) Rottlerin



## **Protocols for spectroscopic analyses.**

**General.** DMSO for spectrophotometry was purchased from Tokyo Chemical Industry, and used as received. Milli-Q water was used throughout.

**Fluorescence.** Fluorescence spectra were measured on a JASCO FP-8500 spectrofluorometer equipped with a quartz cuvette of 10 mm path length at 25 °C for CL and the BLs. After 5 min incubation at 25 °C for CT (0.15 mg mL<sup>-1</sup>, final concentration) and the BLs (0.14 mg mL<sup>-1</sup>, final concentration) in assay buffer with different concentrations of DMSO, guanidine hydrochloride (GdnHCl), and urea, the emission spectra were recorded from 300 to 500 nm, with 295 nm excitation. Three spectra were accumulated and averaged, and the corresponding blank spectra (without enzyme) were subtracted. The emission spectra of the BLs were smoothed with a 25-point Savitzky-Golay filter.

**Circular Dichroism.** Circular dichroism (CD) spectra of CT were measured on a JASCO J-725 spectropolarimeter with a quartz cuvette of 10 mm path length at 25 °C. After 5 min incubation of CT (6 μM, final concentration) in potassium phosphate buffer (10 mM, pH 7.8) with different concentrations of DMSO, GdnHCl, and urea at 25 °C, CD spectra were recorded from 260 to 310 nm. Four spectra were accumulated and averaged, and the corresponding blank spectra (without CT) were subtracted. The spectra data are expressed as the mean residue ellipticity [θ] in deg cm<sup>2</sup> dmole<sup>-1</sup>.

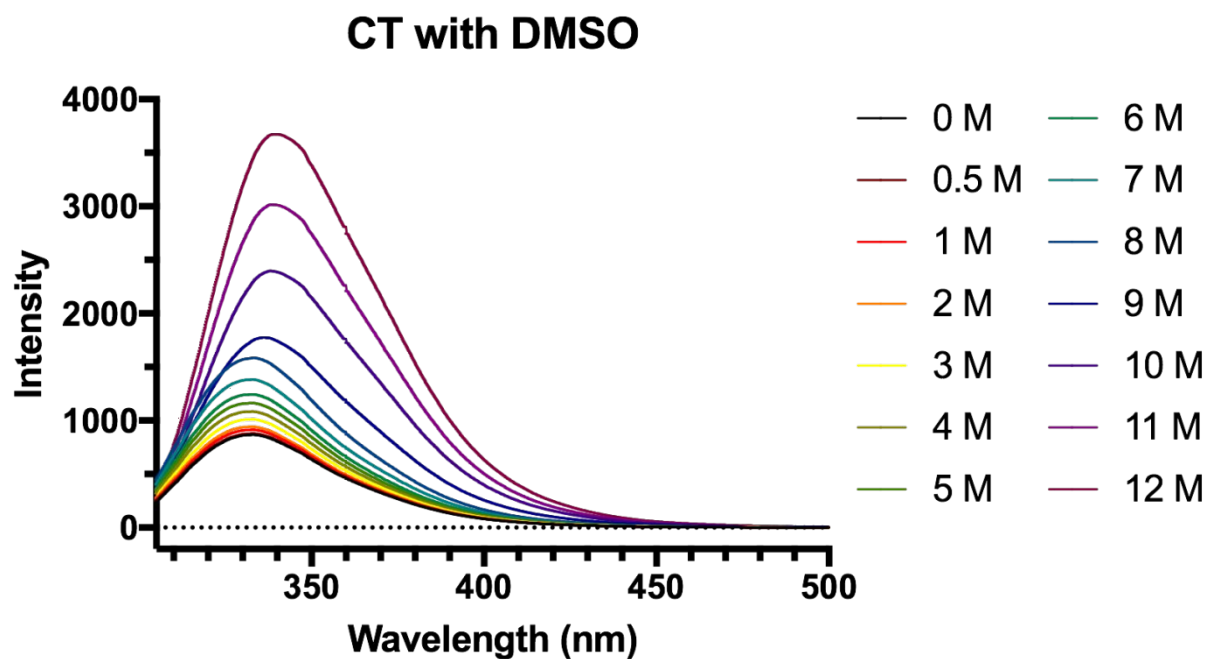
**Infrared.** Deuterated CT, KD<sub>2</sub>PO<sub>4</sub>, and K<sub>2</sub>DPO<sub>4</sub> were prepared by dissolving CT, KH<sub>2</sub>PO<sub>4</sub>, and K<sub>2</sub>HPO<sub>4</sub> in D<sub>2</sub>O and freeze-drying them three times. An empirical D<sub>2</sub>O correction of 0.45 was added to the observed meter reading to convert the operational pH to an equivalent value on the pD scale. Infrared (IR) spectra of CT were measured on SHIMADZU IR Affinity-1 with CaF<sub>2</sub> windows and a 0.05-mm spacer at room temperature. After 5 min incubation of deuterated



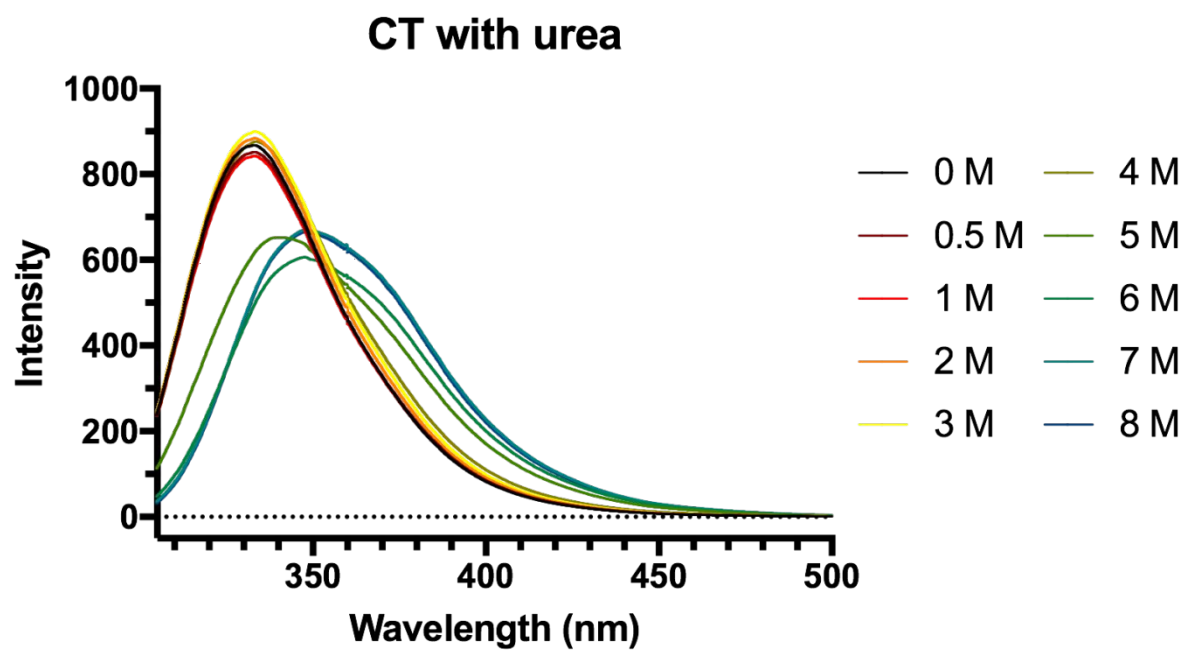
CT (6  $\mu$ M, final concentration) in deuterated potassium phosphate buffer with different DMSO concentrations at 25 °C, IR spectra were recorded from 2000 to 1300  $\text{cm}^{-1}$ . 256 spectra were accumulated and averaged, and the corresponding blank spectra (without CT) were subtracted. All spectra were smoothed with a 25-point Savitsky-Golay filter.

**Figure S8.** Emission spectra of CT and BLs at different concentration of DMSO, urea and GdnHCl. The samples were excited with 295 nm and the emission spectra were recorded from 300 to 500 nm. Three spectra were accumulated and averaged, and the blank spectra (without enzyme) were subtracted. The spectra of the BLs were smoothed with a 25-point Savitzky-Golay filter.

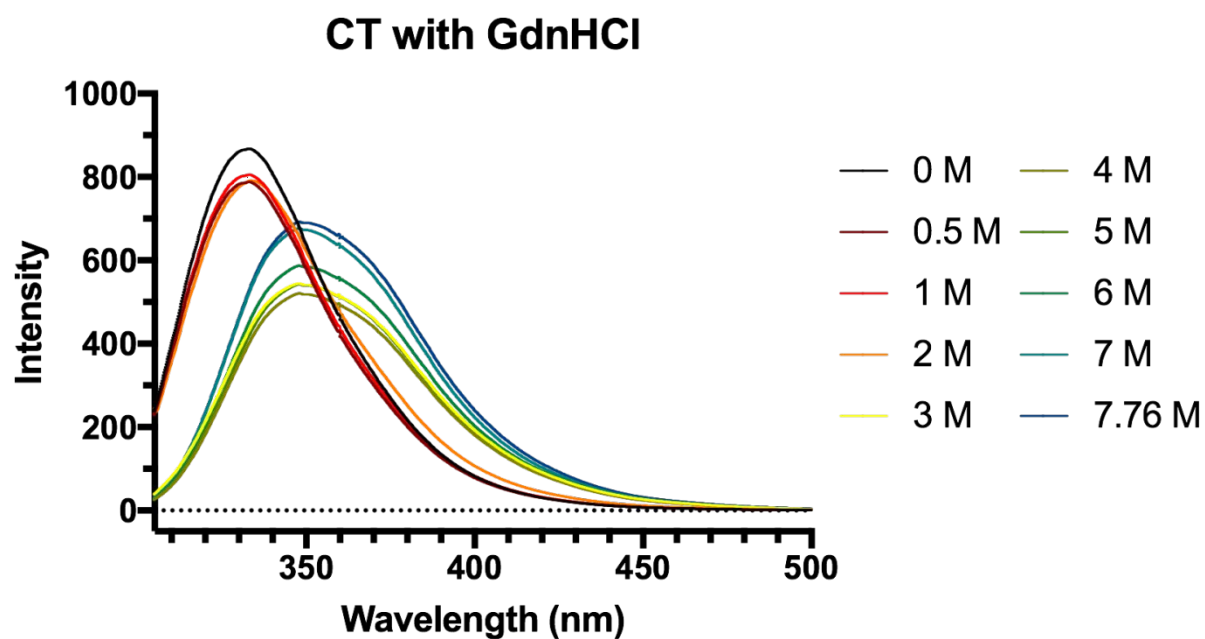
(A) CT at different concentration of DMSO.



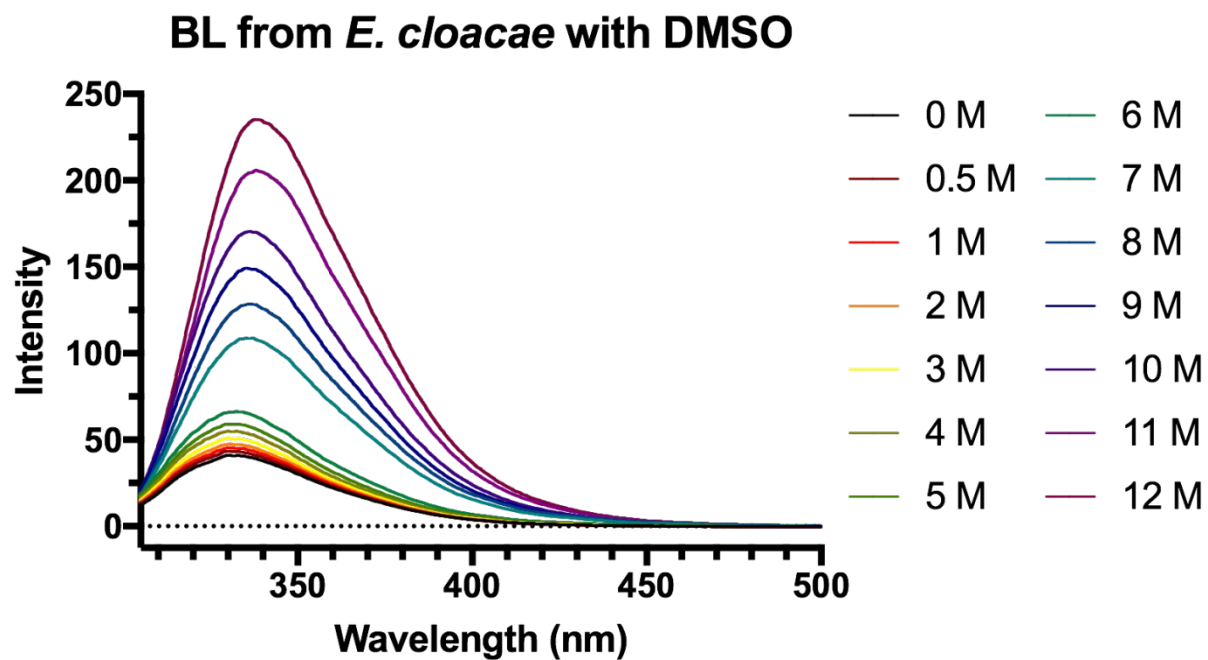
(B) CT at different concentration of urea.



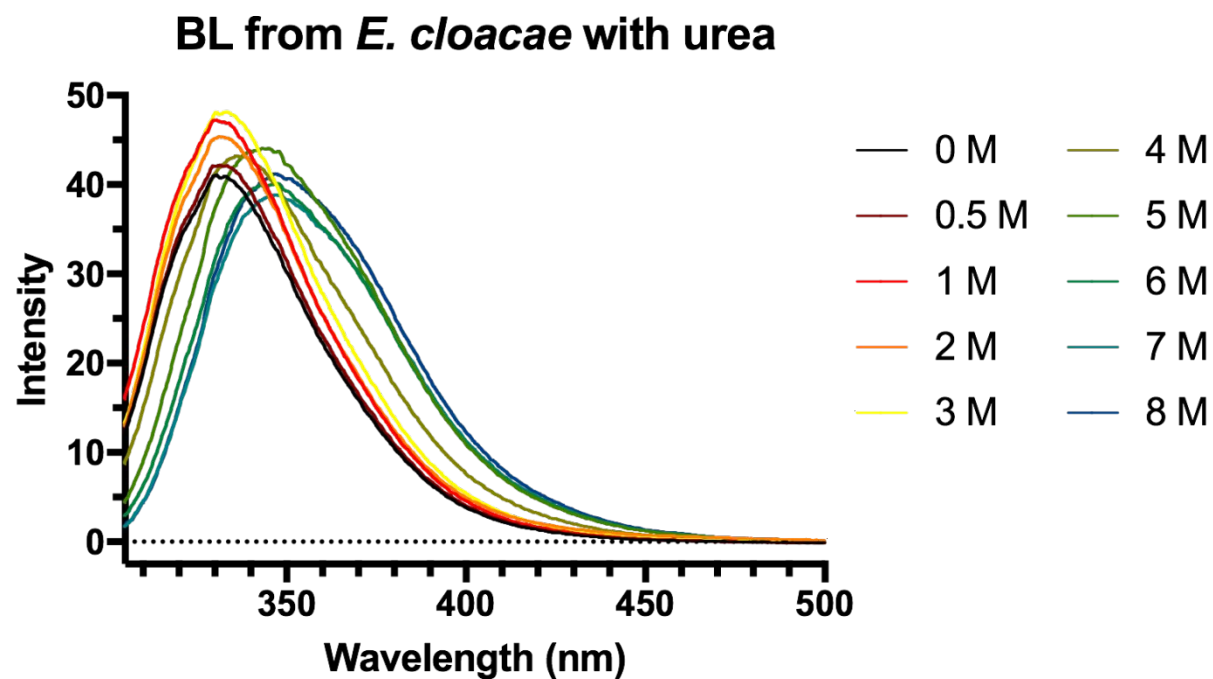
(C) CT at different concentration of GdnHCl.



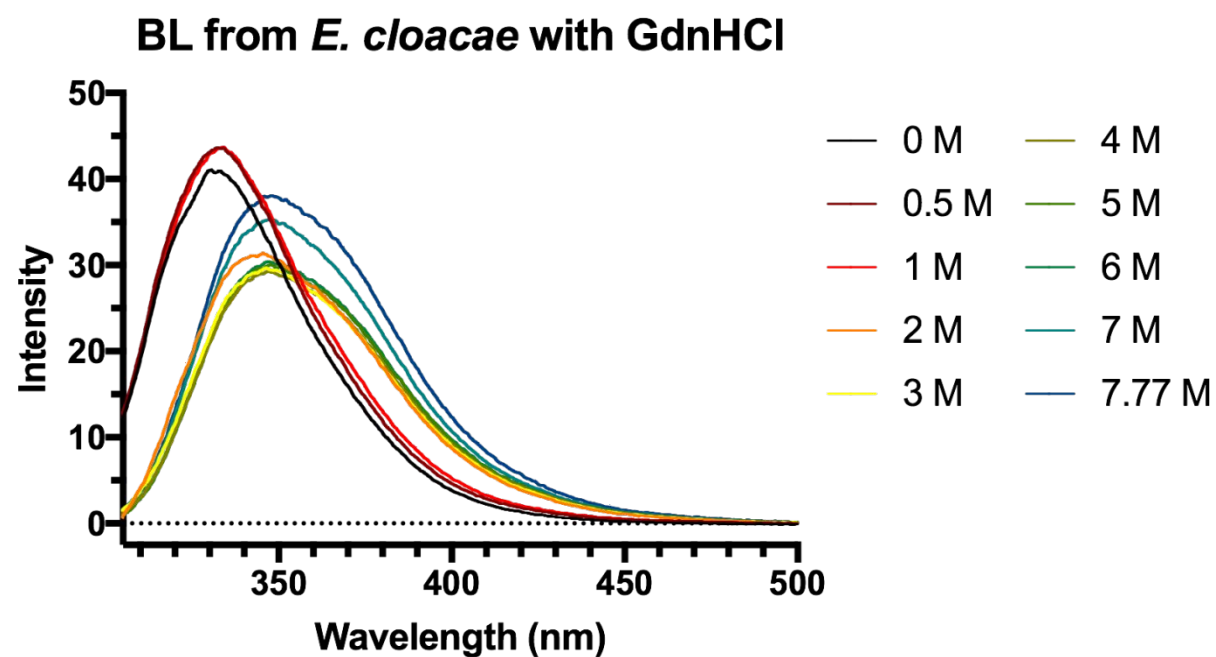
(D) BL from *E. cloacae* at different concentration of DMSO.



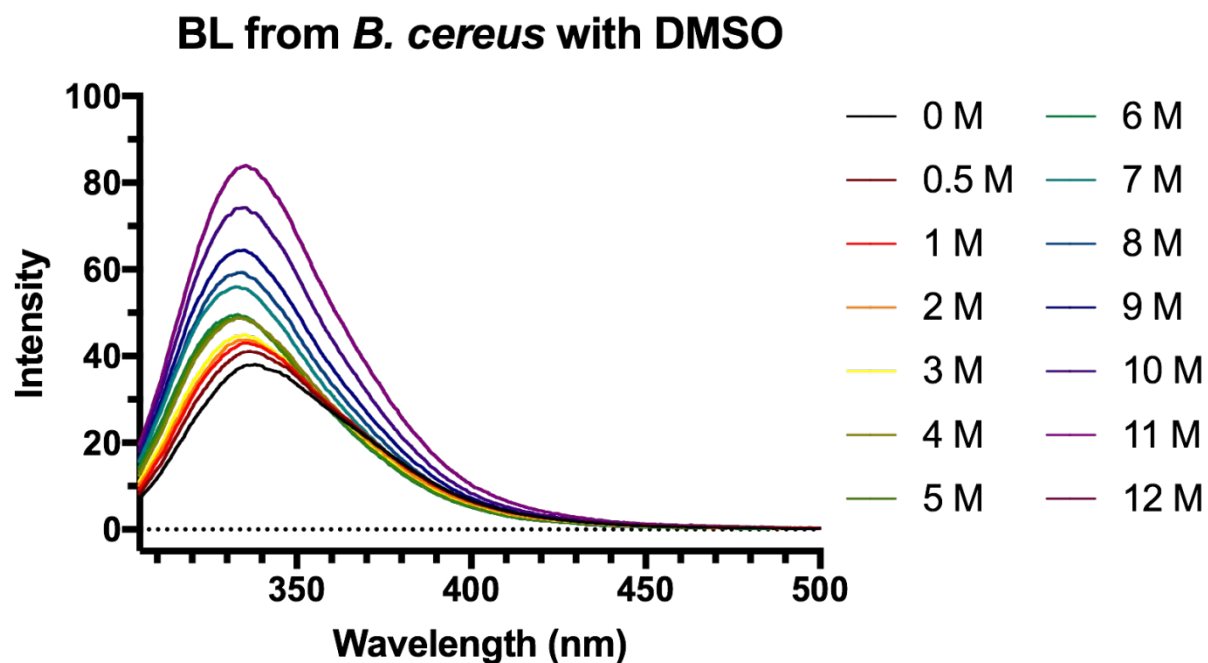
(E) BL from *E. cloacae* at different concentration of urea.



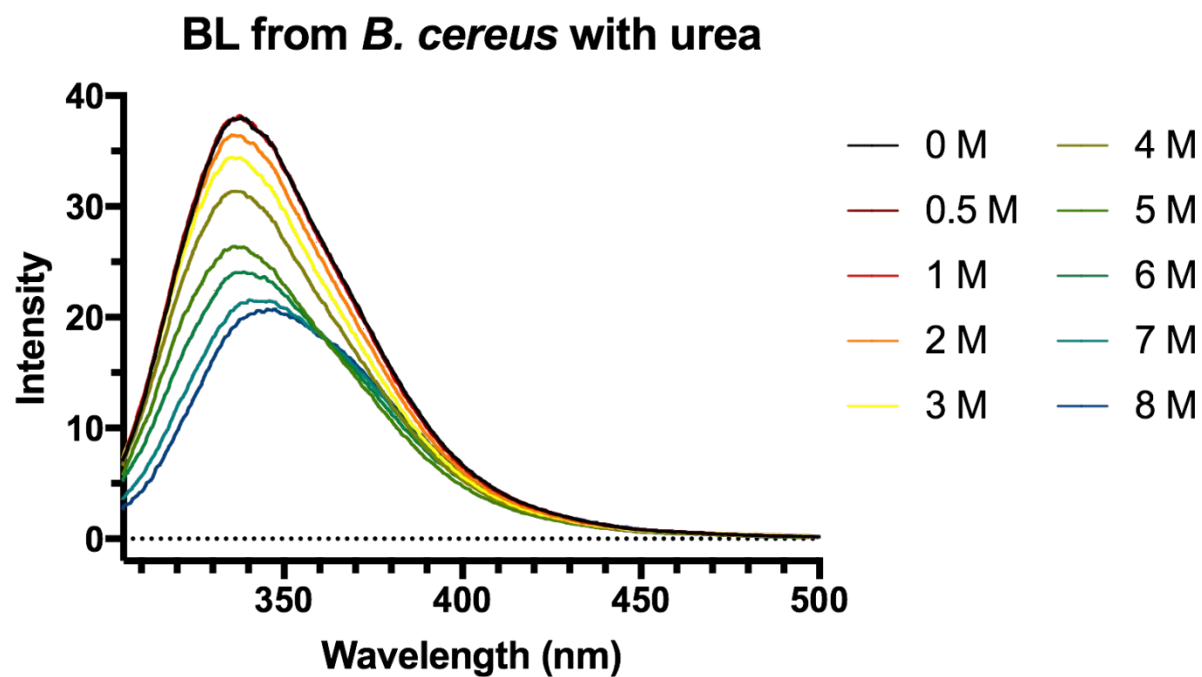
(F) BL from *E. cloacae* at different concentration of GdnHCl.



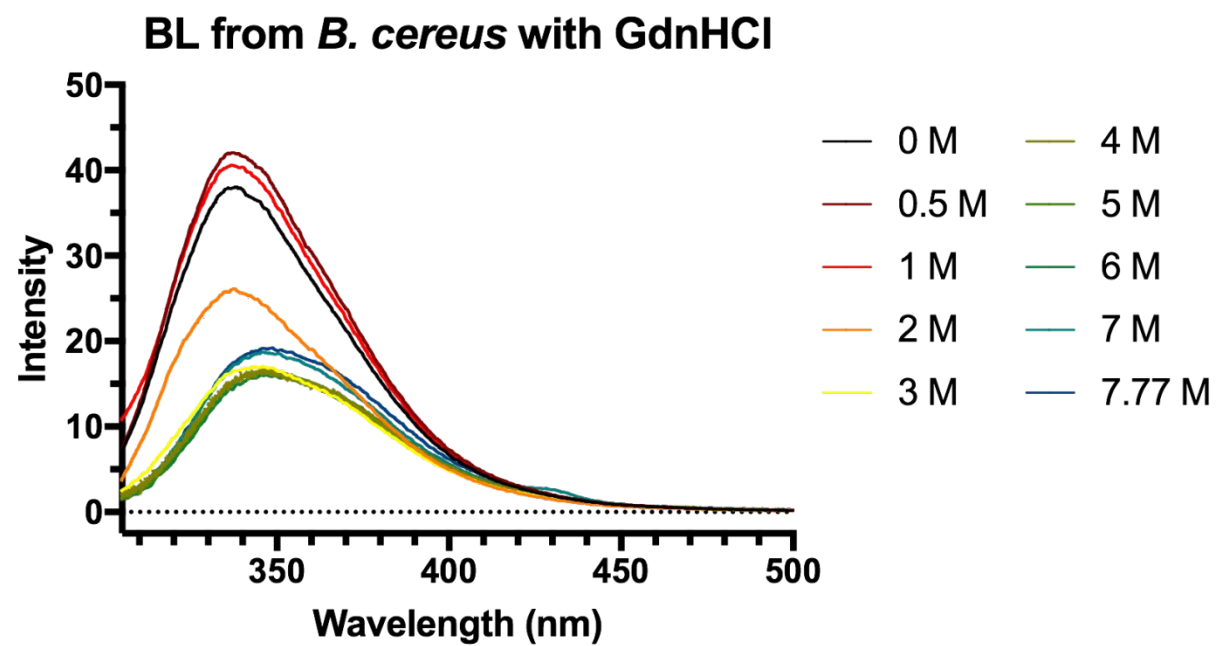
(G) BL from *B. cereus* at different concentration of DMSO.



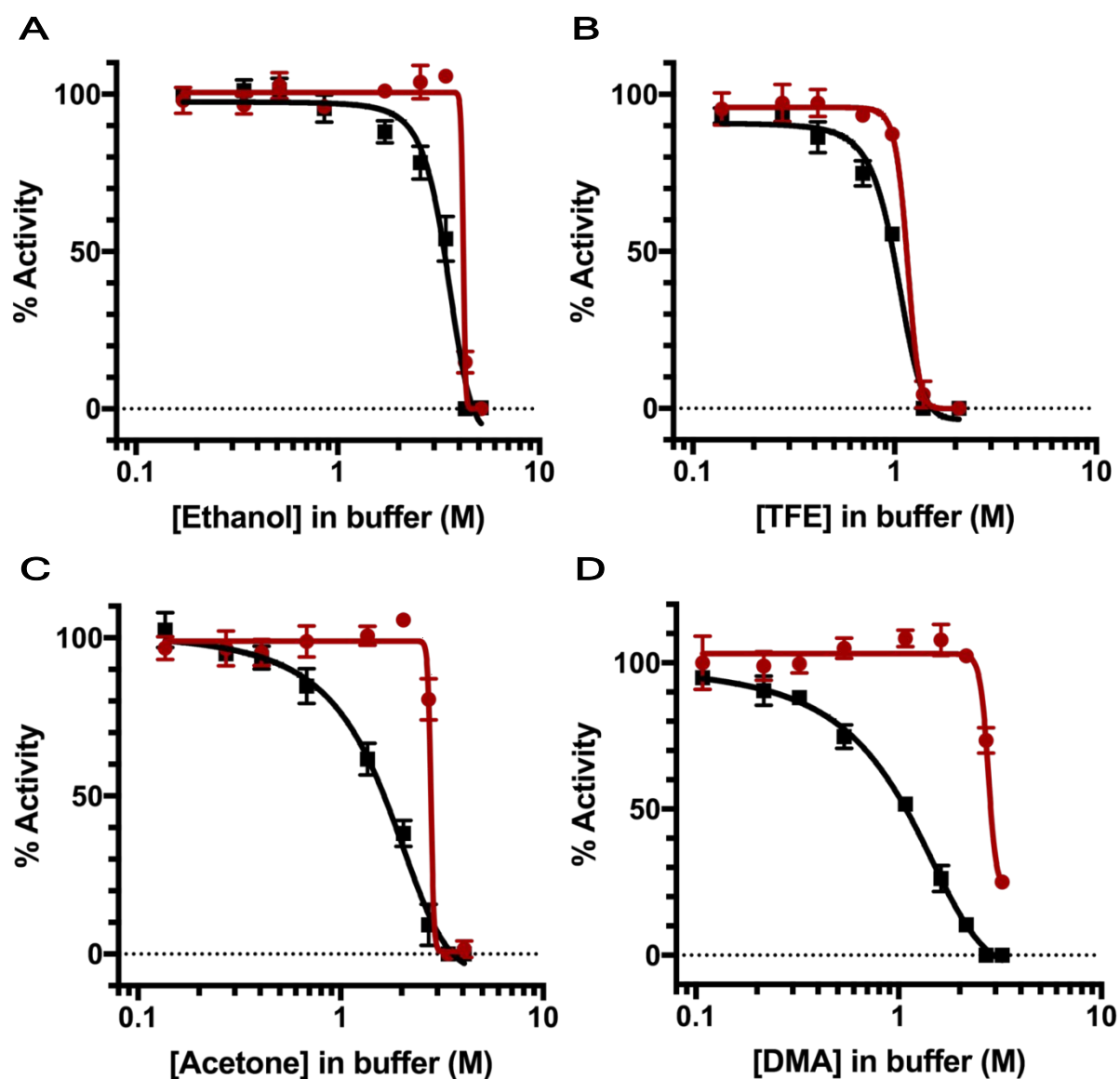
(H) BL from *B. cereus* at different concentration of urea.



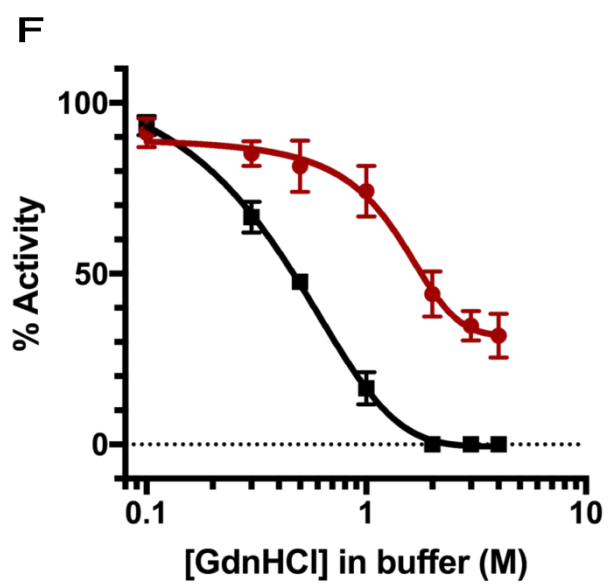
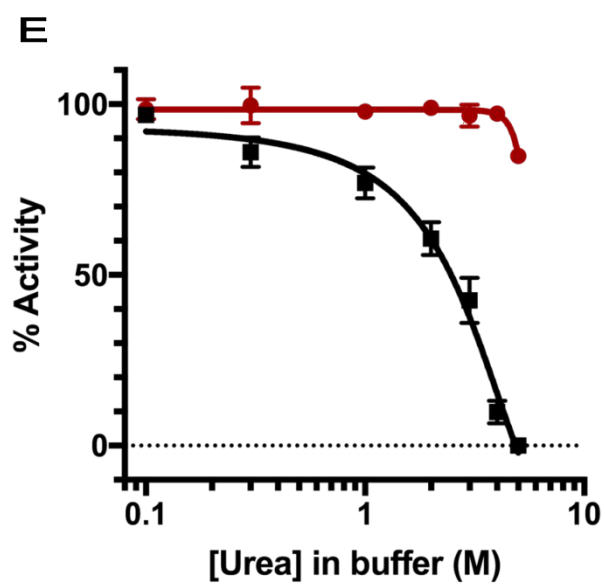
(I) BL from *B. cereus* at different concentration of GdnHCl.



**Figure S9.** Enzymatic activity of BL from *E. cloacae* at different concentration of ethanol (A), trifluoroethanol (TFE) (B), acetone (C), *N,N*-dimethylacetamide (DMA) (D), urea (E), and GdnHCl (F). Black lines indicate the intrinsic activities, and red lines indicate the recovered activities upon 10-fold dilutions. Data are presented as the mean  $\pm$  SD from at least three independent experiments.







**Figure S10.** Concentration-response curves of penicillin G (A and C) and phloretin (B and D) in the presence of DMA and GdnHCl, respectively. The legends in panels C–F indicate concentrations of DMA or GdnHCl in final solution. Data are presented as the mean  $\pm$  SD from at least three independent experiments.

