Supporting Information

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SI Materials and Methods

Peptide Synthesis. The ^{13}C-labeled CP substrate peptide was synthesized at a 0.2-mmol scale on a custom-modified Applied Biosystems 433A peptide synthesizer using S-DVB (styrene-divinylbenzene) resin carrying an -OCH$_2$-PAM (Applied Biosystems) and a thioester-generating linker following an in situ neutralization protocol for machine-assisted Boc (*tert*-butoxycarbonyl) chemistry (1, 2). Side chain-protecting groups were as follows: Arg(Tos), Asn(Xan), Glu(OcHx), Ser(Bzl), and Tyr (2BrZ). Boc-Phe(Cα-^{13}C) was obtained from euriso-top. The peptide was released from resin by treatment with liquid HF for 1 h at 0 °C in the presence of 5% (vol/vol) *p*-cresol. 10 mL of liquid HF were used for 1 g of peptide-resin. Purification of the crude peptide was achieved using a semipreparative RP-HPLC C4 column (Grace) and a gradient of 40–65% (vol/vol) buffer B (acetonitrile plus 0.08% TFA) in buffer A [water plus 0.1% (vol/vol) TFA] over 60 min. Product containing fractions were identified by electrospray ionization mass spectrometry (ESI-MS) and pooled accordingly. Twenty-four percent of purified peptide was obtained from crude material and dissolved in DMSO for application in digestion assays.

Secretion and Purification of GlbA/CepI. Bacteria were grown in TYEA medium [0.2% (wt/vol) Bacto-tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) NaCl per liter] for 3 d at 28 °C. This culture was used to inoculate TYEB medium (TYEA without NaCl) at a ratio of 1:100. Solid adsorber resin (XAD-16) was added to the culture broth, which was in turn incubated for 3 d. The resin was filtrated and extracted with methanol. After concentrating the sample by evaporation, it was applied to a silica column (Silica Gel 60 by Merck). The column was washed with cyclohexane and eluted with ethylacetate. All fractions were tested by the NMR proteasome assay, and positive fractions were applied to preparative HPLC chromatography (RP-C18, 150 mm, 1-cm i.d., 3.5 μm) with a gradient of 10–70% (vol/vol) acetonitrile within 60 min. Fractions were again tested by the NMR assay; GlbA and CepI eluted at ∼55% (vol/vol) acetonitrile. Mass-spectrometrical analysis was perfomed by ESI-MS (Thermo Fisher Scientific Orbitrap). NMR measurements for structure elucidation were carried out in D_6-DMSO at 500 MHz with respective compound concentrations of 25 mM.

IC_{50} Measurements by Fluorogenic Substrates. In vitro proteasome inhibition assays were performed in 96-well microtiter plates with 10 μg/mL yeast CP in 100 mM Tris (pH 8.0) supplemented with 0.01% SDS. Inhibitors were added in DMSO at various concentrations with three repetitions each. After a reaction time of 30 min, the fluorogenic substrates Suc-Leu-Leu-Val-Tyr-AMC, Z-Ala-Arg-Arg-AMC, or Z-Leu-Leu-Glu-AMC were added for analysis of ChTL, TL, and CL activities, respectively. The assay mixture was incubated for a further 60 min; fluorescence and excitation were monitored with a Varian Cary Eclipse Photofluorometer at 460 and 360 nm, respectively.

Gel Shift Assay. A tagged β5-$(His)_6$ yeast proteasome mutant was incubated at a concentration of 1 mg/mL with inhibitor or DMSO in 100 mM Tris (pH 8.0) for 1 h and applied to a 12% (wt/vol) SDS/PAGE. The β5-$(His)_6$ subunit was stained by Western blot analysis. Covalent and irreversible modification of the subunit is accompanied by a gel shift, whereas noncovalent or reversible inhibitors detach during electrophoresis.

Cocrystallization and Structure Elucidation. Crystals of the yeast CP were grown in hanging drops at 24 °C as described (3). The protein concentration used for crystallization was 40 mg/mL in Tris·HCl (10 mM, pH 7.5) and EDTA (1 mM). The drops contained 3 μL of protein and 2 μL of the reservoir solution [30 mM magnesium acetate, 100 mM morpholino-ethane-sulfonic acid (pH 7.2) and 10% (wt/vol) 2-methyl-2,4-pentanediol]. Crystals were incubated at 2 mM inhibitor concentration for 24 h, followed by soaking in a cryoprotecting buffer [30% (wt/vol) 2-methyl-2,4-pentanediol, 20 mM magnesium acetate, 100 mM morpholino-ethane-sulfonic acid, pH 6.9] and supercooled in a stream of liquid nitrogen gas at 100 K (Oxford Cryo Systems).

Data of the CP:CepI and the CP:GlbA complexes were collected to 2.8 and 3.0 Å, respectively, using synchrotron radiation (λ = 1.0 Å) at the X06SA-beamline (Swiss Light Source, Villingen, Switzerland). The space group belongs to $P2_1$ with cell dimensions of a = 134 Å, b = 301 Å, c = 144 Å, and β =112° (Table S3). X-ray intensities were evaluated by using XDS (4) and data reduction was performed with XSCALE (4). The anisotropy of diffraction was corrected by an overall anisotropic temperature factor by comparing observed and calculated structure amplitudes using the program CNS (5). Electron density was improved by averaging and back-transforming the reflections 10 times over the twofold noncrystallographic symmetry axis using the program package MAIN (6). Conventional crystallographic rigid body, positional, and temperature factor refinements were carried out with CNS using the yeast CP structure as starting model (PDB accession code 1RYP) (3). Model building was performed with the program MAIN (6). Apart from the bound inhibitor molecules, structural changes were only noted in the specificity pockets. Temperature factor refinement indicates full occupancies of all inhibitor binding sites.

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Analysis of Peptide Cleavage Pattern and Specificity. The peptide cleavage site was determined by liquid chromatography–mass spectrometry (LC-MS) analysis of the digestion products in an in vitro assay containing 10 μg/mL yeast CP, 100 mM Tris (pH 8.0), 0.01% SDS, 10% (wt/vol) DMSO, and 2 mM peptide, which was incubated for 6 h at 37 °C. Confirming the specificity of the probe molecule toward the β5 subunit, hydrolysis was prevented in the same assay after addition of the synthetic boronic acid inhibitor MG262 (Enzo Life Sciences), which exhibits inhibition merely of the ChTL activity.

Induction of Biosynthesis of GlbA and CepI in *Photorhabdus luminescens*. Initially, an NMR screen of *Photorhabdus* cultures was carried out to detect suitable growth conditions for inhibitor secretion. However, application of standard media and variation of their respective composition, as well as complementation with various additives, failed to initiate the pathogenic phase in *Photorhabdus* (Table S1). Furthermore, mimicking previously described conditions for producing SylA and GlbA from *P. syringae* and *Burkholderiales* strain K481-B101 did not yield any positive result. We therefore performed inoculation experiments by transmission of precultured *Photorhabdus* bacteria to differently conditioned media to analyze the effects of environmental shifts. An abrupt change of osmolarity, going from 170 to 0 mM NaCl, resulted in complete inhibition of the proteasome in our NMR assay, thus identifying the conditions to trigger the virulent phase. In agreement with previous findings (7, 8), we could demonstrate that there is no consensus between the different molecular stimuli among the organisms producing SylA-related compounds. To further confirm the quality

of our NMR-based assay, we performed LC-MS runs of *Photorhabdus* cultures with or without submission to an osmotic shock and could show that under common growth conditions none of the inhibitors is detectable (Fig. S2). In conclusion, our data indicate that secretion of GlbA and CepI is limited to a well-defined environmental shift, reflecting the distinct life stages of *Photorhabdus*.

IC_{50} Measurements of the CL and TL Activities. Just as in the case of the ChTL activity, inhibition of the β2 subunit is enhanced for CepI (Fig. S4), thus reflecting increased hydrophobic interactions between its aliphatic fatty acid chain and the adjacent β3 subunit. In contrast, neither of the compounds binds to β1, which is again in agreement with the in vitro assay results.

1. Camarero JA, Muir TW (2001) Native chemical ligation of polypeptides. *Curr Protoc Protein Sci* 18:18.4.
2. Schnölzer M, Alewood P, Jones A, Alewood D, Kent SB (1992) In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int J Pept Protein Res* 40(3–4):180–193.
3. Groll M, et al. (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 386(6624):463–471.
4. Kabsch W (2010) XDS. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):125–132.
5. Brünger AT, et al. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 54(Pt 5): 905–921.
6. Turk D (1992) *Improvement of a Program for Molecular Graphics and Manipulation of Electron Densities and Its Application for Protein Structure Determination* (Technische Universität München, Munich).
7. Waspi U, Blanc D, Winkler T, Ruedi P, Dudler R (1998) Syringolin, a novel peptide elicitor from P*seudomonas syringae pv. syringae* that induces resistance to *Pyricularia oryzae* in rice. *Mol Plant Microbe Interact* 11(8):727–733.
8. Oka M, et al. (1988) Glidobactins A, B and C, new antitumor antibiotics. I. Production, isolation, chemical properties and biological activity. *J Antibiot (Tokyo)* 41(10): 1331–1337.

Fig. S1. Colored culture broth of *P. luminescens*. Secretions and extracts of microorganisms are often colored, making UV-Vis–based assay techniques prone to color quenching artifacts. The culture broth of *Photorhabdus* has a dark shade of red (*Left*), rendering UV-Vis–based techniques impossible. Chromatographic separation on a silica column (*Right*) reveals that a whole array of colored substances is produced by *Photorhabdus*.

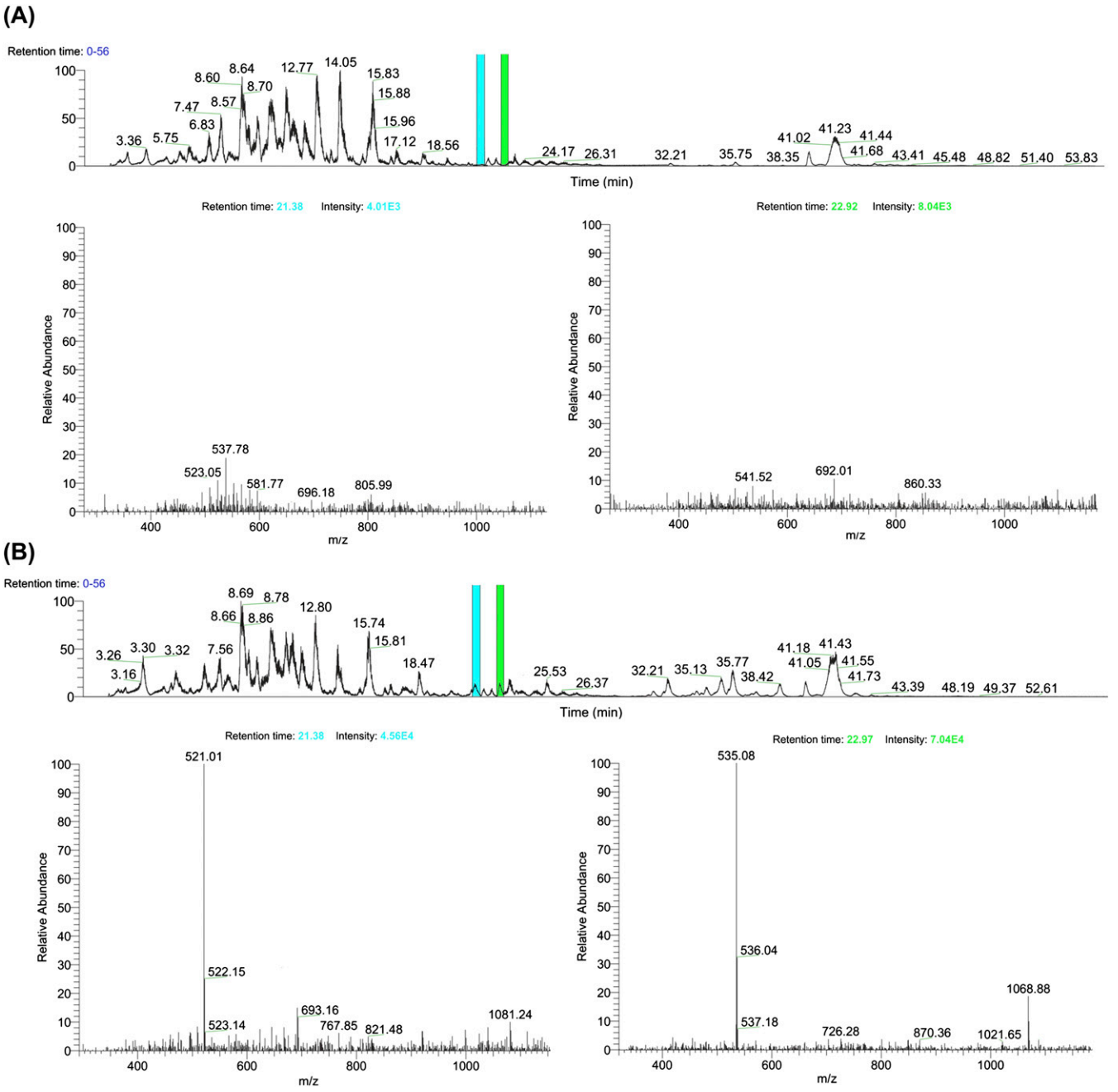

Fig. S2. Comparison between GlbA and CepI secretion in standard medium and after osmotic shock. LC-MS runs of saturated *Photorhabdus* cultures grown in 2xTY (*A*) or TYEA/TYEB induction media (*B*). In agreement with our NMR results, both samples display significant differences in the elution interval between 20 and 40 min. The proteasome inhibitors GlbA (21.38 min, blue bar) and CepI (22.92 min, green bar) were only detected in the culture treated with TYEB (*B*).

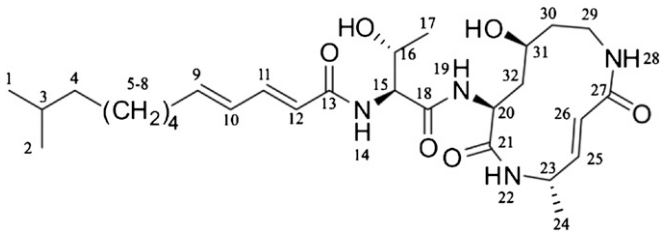

Fig. S3. Molecular structure of CepI with atom labeling.

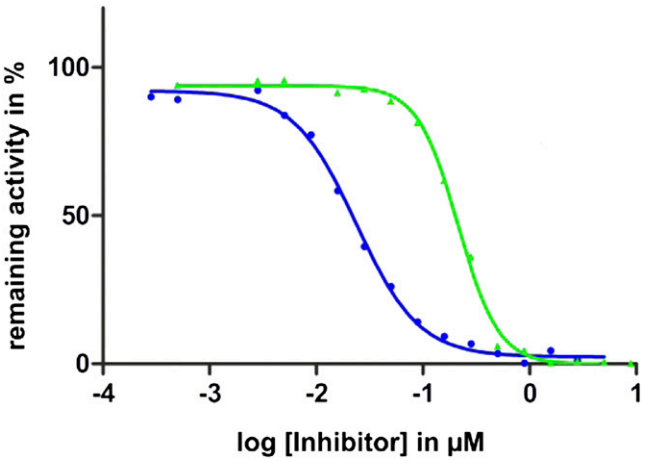

Fig. S4. Inhibition of the TL activity. The IC_{50} value of CepI (blue curve) is six times decreased compared with GlbA (green).

Table S1. Screening of different media and additives

Media	Parameters and additives
Standard media	LB, 2×TY, CY, SRM, TYEA, PDB, DSMZ media 1, 54, 220
Changed parameters in these media	Sugar, casitone, peptone, yeast extract, and salt ingredients
Additives	Fructose, maltose, arbutin, sucrose, lactose, L-proline, L-arginine Iron(III) chloride, iron(II) chloride, soluble and insoluble magnesium, and calcium salts
Other parameters	Temperature (18–34 °C in steps of 2 °C), rotation speed, incubation time (1–10 d), inoculation density

Table S2. NMR spectroscopic data (D_6-DMSO) for CepI (a) and GlbA (b) recorded at 500 MHz

Atom number	δ_H, mult. (J [Hz])	δ_C	Type	COSY	HMBC
1a, 2a	0.84, d (3.25)	23.03	CH_3	3a	1a, 2a, 3a, 4a
3a	1.50, m	27.82	CH	1a, 2a, 4a	1a, 2a, 4,a 5a
4a	1.15, m	38.86	CH_2	3a, 5a	1a, 2a, 3a, 5a, 6a
1b	0.86, t (7.0)	14.46	CH_3	3	3b, 4b
3b	1.26, m	31.68	CH_2	1b	1b
4b	1.26, m	26.21	CH_2		
5	1.26, m	27.08	CH_2	4a	
6	1.26, m	29.21	CH_2		
7	1.39, m	28.81	CH_2	8	5, 6, 8
8	2.13, m	32.76	CH_2	7, 9	7, 9, 10
9	6.11, m	142.68	CH	8, 10	7, 8, 10, 11
10	6.19, m	129.49	CH	9, 11	8, 9, 11
11	7.00, dd (15.0, 10.0)	140.24	CH	10, 12	9, 10, 12, 13
12	6.13, m	123.49	CH	11	11, 13
13	—	165.88	C(O)		11, 12
14	7.91, d (9.0)	—	NH	15	13, 15
15	4.29, m	58.49	CH	14, 16	16, 17, 18
16	3.97, m	67.18	CH	15, 17	
17	1.0, d (10)	20.46	CH_3	16	15, 16
18	—	169.86	C(O)		15, 19
19	7.76, d (7.0)	—	NH	20	18, 20, 21
20	4.34, m	51.66	CH	19, 32	
21	—	171.44	C(O)		19
22	8.69	—	NH	23	
23	4.37, m	45.21	CH	22, 24, 25	
24	1.22, m	19.05	CH_3	23	23
25	6.41, d (15.0)	143.56	CH	26	
26	6.19, m	140.23	CH	25	26
27	—	168.09	C(O)		25, 29
28	7.44, t (5.5)	—	NH	29	26, 29, 30
29	3.02, m	40.40	CH_2	28, 30	27, 28, 30
30	1.45, m	40.07	CH_2	29, 31	29
31	3.58, m	67.57	CH	30, 32	30
32	1.85, m 1.58, d (11.5)	42.93	CH_2	31, 32, 20	

The peak assignments refer to the atom numbering in Fig. S3. COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; mult., multiplet.

Table S3. Collection and structural refinement statistics of the crystallographic datasets

Crystallographic data	CP:CepI*	CP:GlbA*
Crystal parameters		
Space group	$P2_1$	$P2_1$
Cell constants (Å)/°	*a* = 135,7	134.8
(dataset was collected from	*b* = 301,0	300.4
1 crystal/1 CP per AU)	*c* = 144,8	144.5
	β = 112,9	112.7
Data collection		
Beamline	X06SA, SLS	
Wavelength, Å	1.0	
Resolution range, Å†	25–2.8	15–3.0
No. observations	837,332	724,190
No. unique reflections‡	256,455	209,581
Completeness, %†	97,8 (99,0)	99.3 (99.7)
R_{merge}†,§	7,7 (61,4)	6.7 (47.2)
I/σ (I)†	13,0 (2,4)	15.2(3.2)
Refinement (CNS)		
Resolution range, Å	15–2,8	15–3.0
No. reflections working set	242,408	197,883
No. reflections test set	12,718	10,363
No. nonhydrogen	49,548	49,548
No. of ligand atoms	140	136
Water	1,320	1,290
R_{work}/R_{free}, %¶	21.3/24.3	19.8/22.7
rmsd bond (Å)/(°)‖	0.007/1.32	0.007/1.32
Ramachandran plot, %**	95.0/4.7/0.3	94.7/4.8/0.5

Despite the existing structural information of the CP:GlbA complex (PDB ID code 3BDM), we performed crystal structure analysis also with the isolated natural compound from *Photorhabdus* for direct comparison because the crystallization conditions were slightly modified.

*Dataset has been collected on a single crystal.

†Values in parentheses of resolution range, completeness, R_{merge}, and I/σ (I) correspond to the last resolution shell.

‡Friedel pairs were treated as identical reflections.

§$R_{merge}(I) = \Sigma_{hkl}\Sigma_j|[I(hkl)_j - I(hkl)]|/\Sigma_{hkl}\ I_{hkl}$, where $I(hkl)_j$ is the measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity.

¶$R = \Sigma_{hkl}||F_{obs}| - |F_{calc}||/\Sigma_{hkl}|F_{obs}|$, where R_{free} is calculated without a sigma cut off for a randomly chosen 5% of reflections, which were not used for structure refinement, and R_{work} is calculated for the remaining reflections.

‖Deviations from ideal bond lengths/angles.

**Number of residues in favored region/allowed region/outlier region.