SUPPLEMENTARY MATERIALS

The Biological Action and Structural Characterization of Eryngitin 3 and 4, Ribotoxin-like Proteins from *Pleurotus eryngii* Fruiting Bodies

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Table S1. Cytotoxicity of eryngitin 3 and 4. HeLa or COLO 320 cells were grown in RPMI 1640 medium and incubated with different toxin concentrations for 48 or 72 h, and cell viability was evaluated by a colorimetric assay, as indicated in Materials and Methods section. Data represent the mean of IC_{50} (the concentration of toxins causing a 50% reduction in viability) obtained from two experiments performed in duplicate.

		IC_{50} (nM)	
Cell line	Time (hour)	Eryngitin 3	Eryngitin 4
HeLa	48	15	640
HeLa	72	3.0	230
COLO 320	48	1200	>3100
COLO 320	72	420	1700

Table S2. Experimental relative molecular masses of tryptic (T), endoproteinase Glu-C (GC), pepsin (P) and chymotryptic (C) peptides from eryngitin 4 determined by MALDI-ToF mass spectrometry.

Peptide	Sequence position	Experimental molecular mass [a]	Theoretical molecular mass	Δ (Da)	Missed cleavage at	Notes
trypsin peptides						
T-1	1-10	1122.54	1122.54	-		*N-terminal*
T-2	16-22	855.43	855.42	0.01		
T-3	23-35	1533.94	1533.83	0.11	K28	
T-3' [b]	29-35	851.50	851.50	-		Cys-PE
T-4	36-40	563.27	563.32	0.05		
T-5	41-50	1043.61	1043.60	0.01		
T-6	61-72	1237.61	1237.60	0.01	K66	
T-7	73-79	786.42	786.42	-		
T-8	97-103	699.39	699.41	0.02		
T-9	104-108	681.34	681.36	0.02	K105	
T-10	110-120	1269.73	1279.72	0.01	K115	
T-10' [b]	116-120	586.24	586.29	0.05		
T-11	121-132	1465.82	1465.74	0.08		*C-terminal*
endoproteinase Glu-C peptides						
GC-1	1-11	1252.60	1251.58	1.02	E2	*N-terminal*
GC-1' [b]	3-11	1065.56	1065.52	0.04		
GC-2	12-24	1374.82	1377.72	2.90		
GC-3	105-112	1049.61	1049.63	0.02		
GC-4	113-132	2361.86	2361.22	0.64		*C-terminal*
pepsin						
P-1	26-48	2502.45	2502.43	0.02	*alternative cleavage*	
P-1' [b]	32-41	1127.61	1127.59	0.02	*alternative cleavage*	
P-1'' [b]	32-45	1568.85	1568.82	0.03	*alternative cleavage*	
P-1''' [b]	32-47	1724.93	1724.91	0.02	*alternative cleavage*	
P-2	39-59	2235.29	2236.20	0.91	*alternative cleavage*	
P-3	52-61	1088.59	1088.58	0.01	*alternative cleavage*	
P-3' [b]	59-77	2061.09	2061.04	0.05	*alternative cleavage*	
P-3'' [b]	68-79	1244.69	1244.68	0.01	*alternative cleavage*	
P-4	79-88	1107.48	1107.57	0.09	*alternative cleavage*	
P-5	83-96	1459.83	1459.77	0.06	*alternative cleavage*	

P-6	112-124	1588.86	1588.85	0.01	*canonical cleavage*	
P-6'	114-124	1372.82	1372.78	0.04	*alternative cleavage*	
P-7	125-132	920.43	920.44	0.01	*canonical cleavage*	*C-terminal*
P-7' [b]	124-132	1067.53	1067.50	0.03	*alternative cleavage*	*C-terminal*
P-7'' [b]	112-132	2491.47	2490.27	1.2	F124; *canonical cleavage*	*C-terminal*
chymotrypsin						
C-1	1-12	1364.59	1364.67	0.08		
C-2	21-29	113.49	113.56	0.07		
C-3	32-42	1314.55	1313.66	0.89	Y32	
C-4	43-76	3535.75	3532.93	2.82	average	
C-5	99-107	1049.45	1049.54	0.09		
C-6	108-123	1924.99	1924.14	0.85	L111	
C-6' [b]	112-123	1441.70	1441.78	0.08		
C-7	127-132	548.11	548.28	0.17		*C-terminal*

[a] $[M+H^+]^+$ experimental molecular mass values obtained by MALDI-ToF MS. The monoisotopic molecular masses have been considered, except for the C-4 peptide for which the average molecular mass is reported.
[b] Not reported in Fig. 4A.

Table S3. Experimental molecular mass values of tryptic (T), endoproteinase Glu-C (GC), pepsin (P) and chymotryptic (C) peptides from eryngitin 3 determined by MALDI-ToF mass spectrometry.

Peptide	Sequence position	Experimental molecular mass [a]	Theoretical molecular mass	Δ (Da)	Missed cleavage at	Notes
trypsin peptides						
T-1$_{(a)}$	1-16	1798.25	1797.90	0.35	K11	*N-terminal of component a in eryngitin 3*
T-1' [b]	1-11	1269.75	1269.61	0.14		*N-terminal of component a in eryngitin 3*
T-1$_{(b)}$	2-11	1122.59	1122.54	0.05		*N-terminal of component b in eryngitin 3*
T-2	17-23	855.45	855.42	0.03		Cys-PE
T-3	24-36	1533.96	1533.83	0.13	K29	
T-4	37-41	563.29	563.32	0.03		
T-5	62-73	1237.63	1237.59	0.04	K67	
T-6	74-80	786.44	786.43	0.01		
T-7	98-104	699.41	699.41	-		
T-8	105-110	808.42	809.45	1.03	K106 and K109	
T-9	110-121	1397.88	1397.81	0.07	K110 and K116	
T-9' [b]	110-116	830.56	830.53	0.03	K110	
T-10 [b]	117-121	586.26	586.29	0.03		
endoproteinase Glu-C peptides						
GC-1	1-12	1398.74	1398.65	0.09	E3	*N-terminal of component a in eryngitin 3*
GC-1' [b]	4-12	1065.54	1065.52	0.02		
GC-2	13-25	1375.82	1377.72	1.90		
GC-3	106-113	1049.67	1049.63	0.04		
GC-4	114-132	2206.68	2205.12	1.56		*C-terminal*
pepsin						
P-1	14-32	2044.09	2044.13	0.04	L30; *canonical cleavage*	Cys-PE
P-2	33-46	1568.85	1568.82	0.03	*alternative cleavage*	
P-2' [b]	33-48	1724.96	1724.91	0.05	*alternative cleavage*	
P-3	45-63	1973.15	1973.07	0.08	*alternative cleavage*	
P-4	83-96	1459.85	1459.77	0.08	*alternative cleavage*	

P-5	112-124	1588.89	1588.85	0.04	*canonical cleavage*	
P-5' [b]	115-125	1372.82	1372.78	0.04	*alternative cleavage*	
P-5'' [b]	120-125	817.44	817.47	0.03	*alternative cleavage*	
chymotrypsin						
C-1	31-43	1525.95	1525.82	0.13	L32 and Y33	
C-2	43-76	3537.49	3532.93	4.56	average	
C-3	100-108	1049.47	1049.54	0.07		
C-4	109-124	1925.42	1924.14	1.28	L112	
C-4' [b]	113-124	1441.78	1441.78	--		

[a] $[M+H^+]^+$ experimental molecular mass values obtained by MALDI-ToF MS. The monoisotopic molecular masses have been considered, except for the C-2 peptide for which the average molecular mass is reported.

[b] Not reported in Fig. 4B.

Subscripts letters in brackets for peptide T-1 indicate the two components (a and b) retrieved in different amount in eryngitin 3 mixture after RP-HPLC, see main text.

Figure S1

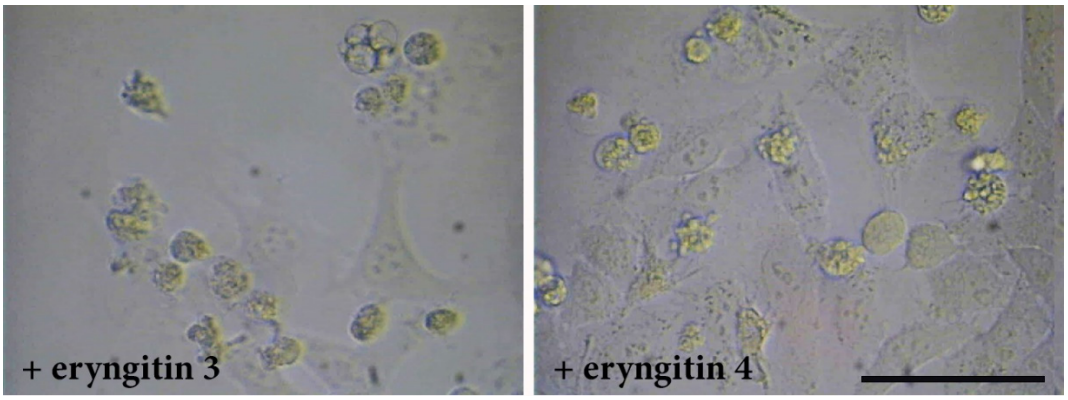


Figure S1. Morphological changes visualized microscopically when HeLa cells were incubated with eryngitin 3 (0.3 μM) or 4 (3.0 μM) after 48 h of incubation. Bar, 50 μm.

Figure S2

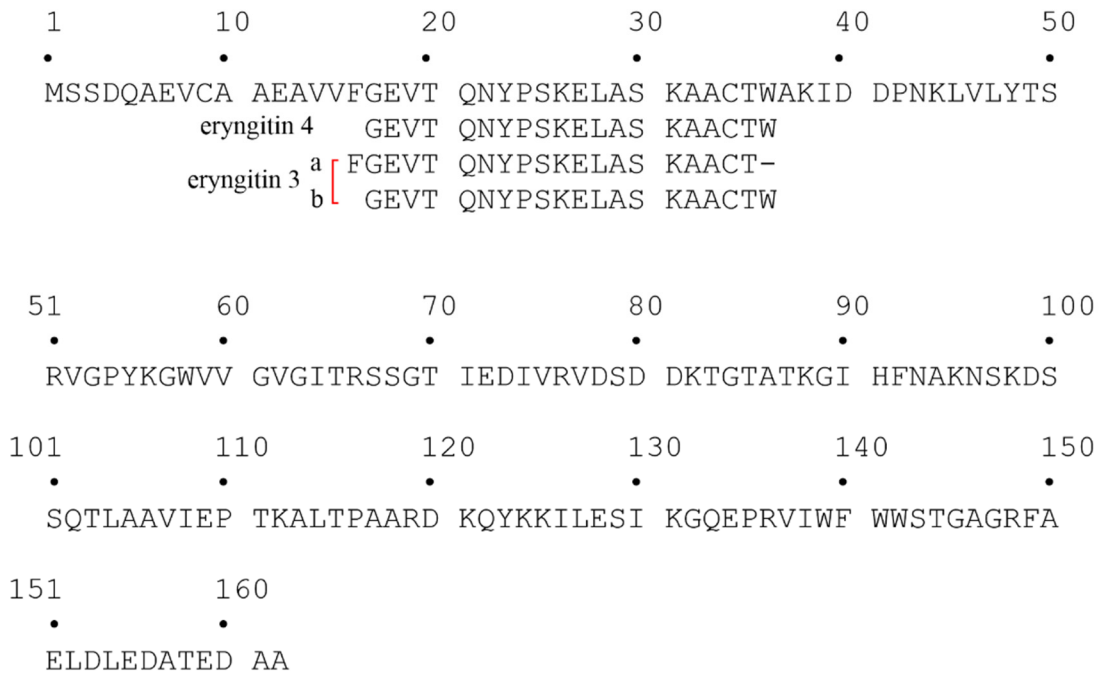


Figure S2. Amino acid sequence of hypothetical protein BDN71DRAFT_1455417 (AC: KAF9489889.1) retrieved in *P. eryngii* genome used as a reference protein for structural characterization of eryngitin 4 and 3 by MALDI-ToF MS analysis and peptides mapping. In addition, the experimental N-terminal amino acid sequences of both eryngitins obtained by automatic Edman degradation are reported. For eryngitin 3, two N-terminal amino acid sequences, named component a and b, were detected.

Figure S3

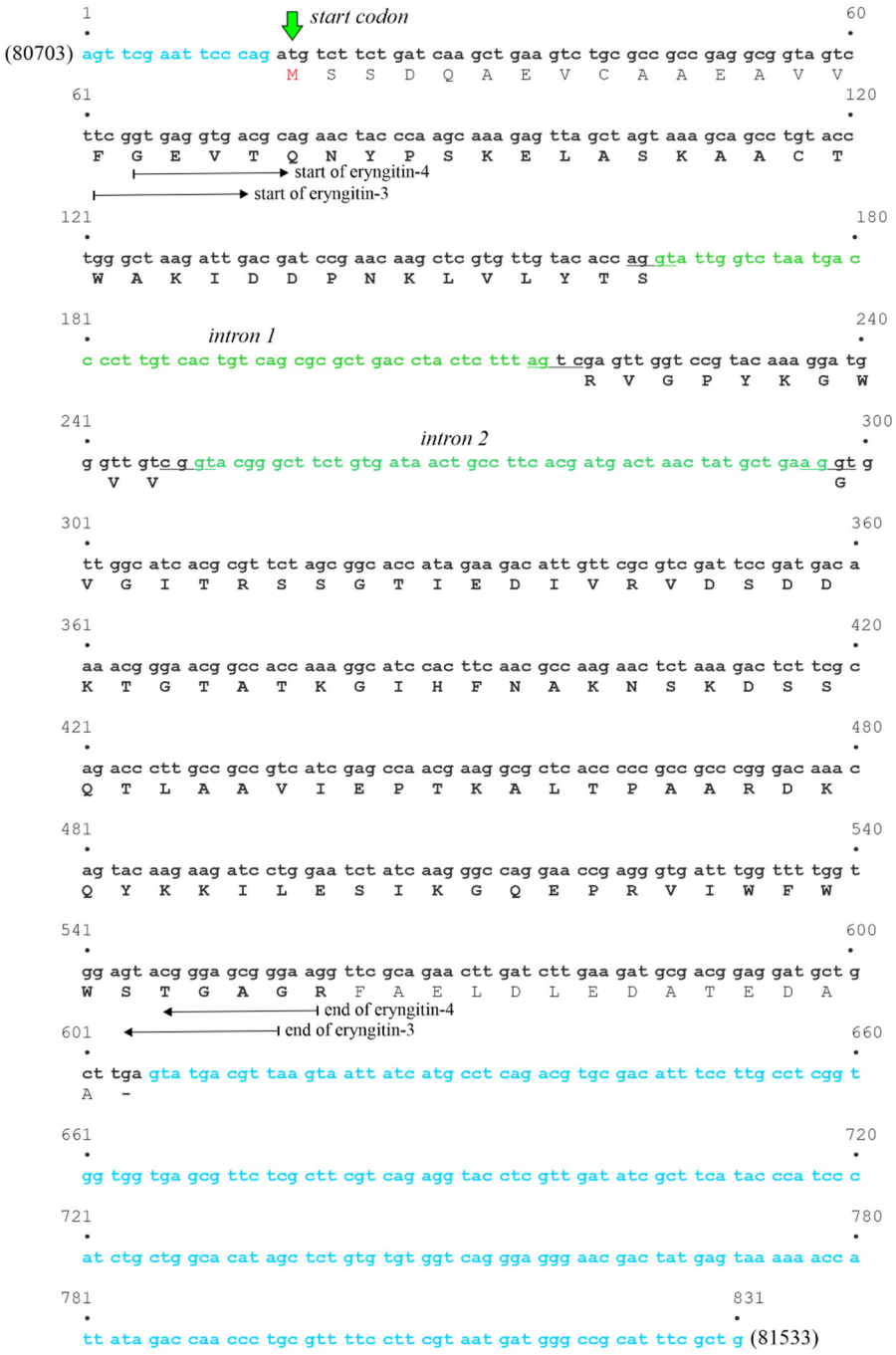


Figure S3. Nucleotide sequence (831 bp) of 'jgi|Pleery1|1455417', present in *P. eryngii* ATCC 90797 genome, containing transcript region (730 bp) of both eryngitins. The 'atg' codon is highlighted by the green arrow; exons, introns and splice junctions are reported in black, green or underlined, respectively. The first (start) and last (end) residues of both purified eryngitin 4 and 3 are shown. Numbers in round brackets are referred to the specific region of scaffold_136:80703-81533 in *P. eryngii* genome.

Figure S4

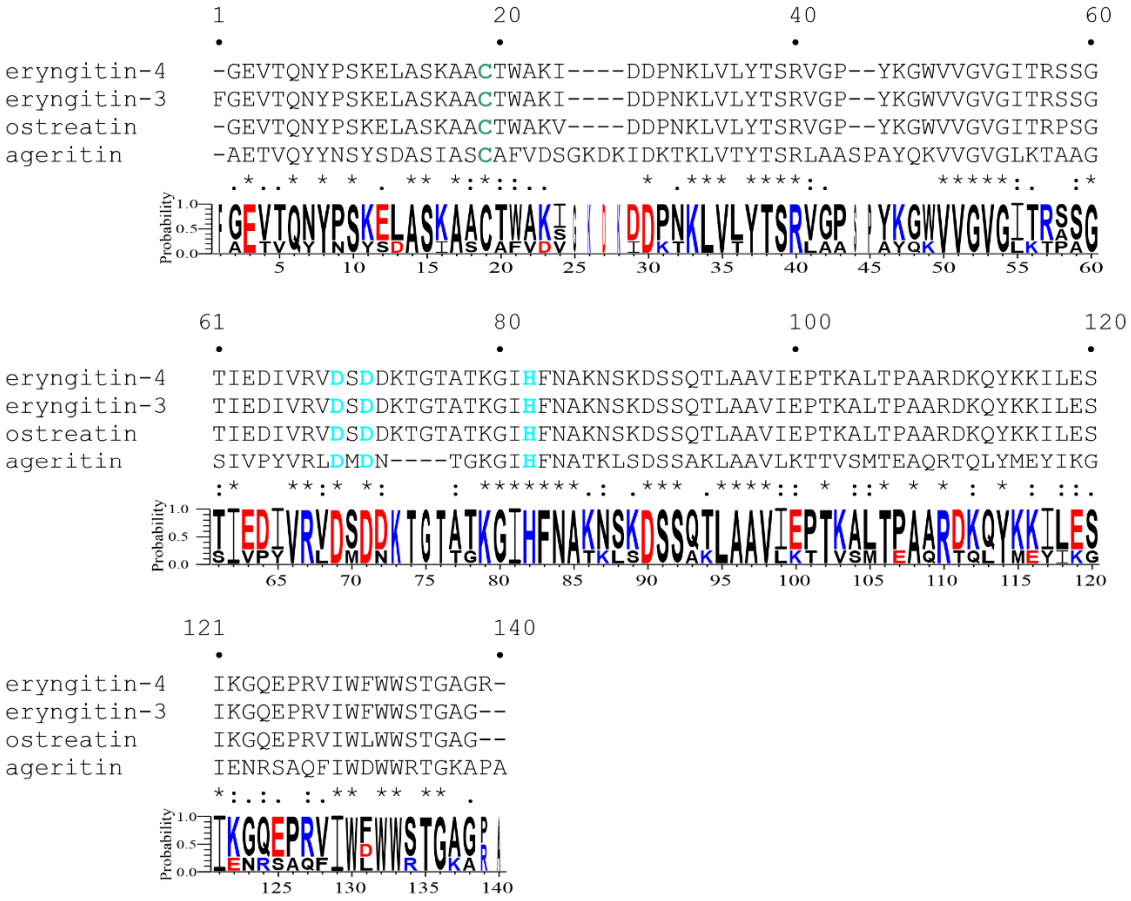


Figure S4. Amino acid sequence alignment of eryngitin 4, eryngitin 3, ostreatin and ageritin. Ostreatin and ageritin are two well-characterized RL-Ps isolated from *P. ostreatus* and *C. aegerita* fruiting bodies, respectively. The standard one-letter code was used for the amino acid residues. Identical residues (*), conserved substitutions (:) and semiconserved substitutions (.) are reported. Amino acid residues of the catalytic site and the single free cysteinyl residue are reported in cyan and green, respectively. Among the amino acid blocks, the Logo representation of alignment is reported. Letter height is proportional to the conservation of that amino acid at that position in the alignment with respect to all the amino acids; letter width is proportional to the conservation of that amino acid but includes gaps. In red and blue, the residues with negative or positive charges, respectively.

Figure S5

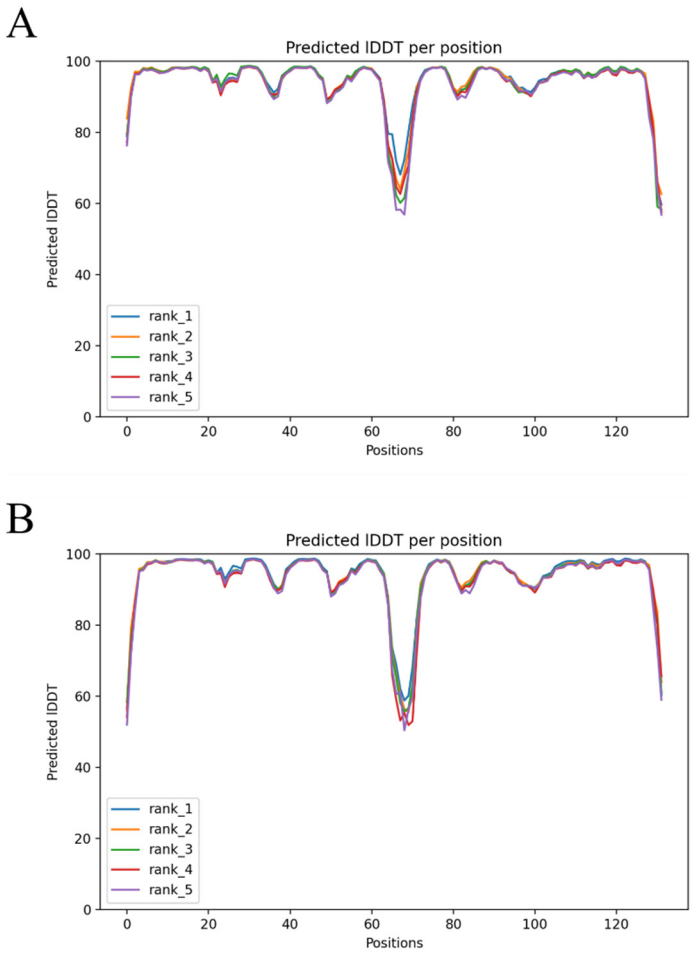


Figure S5. In (**A**) and (**B**), graphical representations of the Local Distance Difference Test (lDDT) of eryngitin 4 and 3, respectively. IDDT is a superposition-free score robust tool used to evaluate the local distance differences of all atoms in the resulting model, including validation of stereochemical plausibility. In particular, the graph should be interpreted as follows: i) regions with pLDDT > 90 are expected to be modelled to high accuracy; ii) regions with pLDDT between 70 and 90 have expected to be modelled well; iii) regions with pLDDT between 50 and 70 are low confidence and should be treated with caution; and iv) regions with pLDDT < 50 have often a ribbon-like appearance and should not be interpreted.