

SUPPORTING INFORMATION

Potent human alpha-amylase inhibition by the β -defensin-like protein Helianthamide.

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MATERIALS AND METHODS

Collection and extraction of animal material. *Stichodactyla helianthus* was collected by hand at a depth of 2-3 meters at Cape Capucin, Island of Dominica. Specimens were immediately frozen and transported to Vancouver, British Columbia. Prior to analysis, the *S. helianthus* were exhaustively extracted with MeOH (3 x 300 mL).

Activity guided isolation. The *S. helianthus* methanolic extract from a 154 g *S. helianthus* specimen was further separated on a Diaion HP-20 column, which was eluted with 150 mL portions of 0%, 30%, 50%, 75%, and 100% acetone in water. Greatest activity was traced to the 30% acetone/water fraction (162 mg), which was then chromatographed on a Sephadex LH-20 size exclusion column (28 x 710 mm) eluting with 25% methanol/75% water/0.1% trifluoroacetic acid (TFA). The material (13.1 mg) was then purified using RP-HPLC with a C₈ column eluting with 23% acetonitrile/77% water/0.1% TFA to yield the active peptide (2.0 mg).

Peptide sequencing. Disulfide linkages were reduced with dithiothreitol (100 mM) for 1 hour at 56°C after which the resulting free cysteines were alkylated with iodoacetamide (100 mM) for 1 hour at 25°C in pH 7.5 ammonium bicarbonate buffer (50 mM). MALDI-TOF analysis indicated a change in mass of 350.4 after reduction and alkylation which corresponds to approximately six alkylated cysteines. It could be deduced that the native material possessed three disulfide bonds. Peptide digestions were carried out by incubating reduced and alkylated peptide with trypsin (Promega Gold) in pH 8.5 Tris buffer (50 mM) for 18-24 hours at 37 °C, or with GluC (V8 protease, Roche) in pH 8.5 sodium phosphate buffer (50 mM) for 18-24 hours.

De novo sequencing: Sequencing was performed by LC-MS/MS on an LC Packings capillary LC system (Dionex) coupled to a QSTAR Pulsar quadrupole TOF mass spectrometer (Applied Biosystems). MS data were acquired using Analyst QS software (Applied Biosystems) for information-dependent acquisition. N₂ was used as collision gas and the ionization tip voltage was 2200 V.

Edman sequencing: Digested peptides were separated on a Vydac HPLC microbore C₁₈ column (ODS-300, 7 μm, 1 × 50 mm). The peptide fractions were loaded on a precycled glass fiber filter coated with Biobrene and sequenced by the Edman degradation liquid phase method using a Procise cLC-494 (Applied Biosystems) equipped with an on-line 140D Phenylthio-hydantoin analyser (Applied Biosystem). The phenylthiohydantoin amino acid (PTH-aa) derivatives were determined by comparison with standards analyzed at the start of the sequence analysis.

CD spectroscopy. Analysis was conducted on a Jasco J-815 CD spectrophotometer. Samples of synthetic, natural, and recombinant helianthamide were diluted in distilled water. Analysis was run from 260 to 190 nm for the natural and synthetic material, and 250 to 190 nm for the recombinant material. The resulting data was analyzed on the Dichroweb server(1). All three data sets were analyzed with CONTIN software using reference sets 4, 7, and SP175(2,3). The data of the natural and synthetic material was also analyzed using CDSSTR software with reference sets 4, 7, and SP175. The secondary structural estimates were averaged and presented in Table S1.

Thiol titration assay. A solution of thiol titration buffer was prepared (0.2 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 6 M guanidinium chloride, 20 mM HEPES, 1 mM EDTA, pH 7.4). Helianthamide was added to the solution in final protein concentrations ranging from 2 – 6 μ M(4). The release of 2-nitro-5-thiobenzoate was monitored at 412 nm at 25 °C over the course of 30 minutes. During each measurement, ΔA_{412} was also measured for the thiol titration buffer to take into account the rate of spontaneous hydrolysis of the DTNB reagent. The experiment was also conducted with BSA, which has one free thiol group, as a positive control.

Disulfide assignment of recombinant material. Intact helianthamide was incubated in 11 M hydrochloric acid at 37 °C for 4 days. The sample was diluted and neutralized before desalting by C18 reverse phase chromatography. The peptide mixture was injected into an Agilent 6460 QQQ LC/MS mass spectrometer. Elution and MS/MS analysis was carried out over a 40 minute period. A script was written to predict all possible fragments for the helianthamide sequence in the event of random hydrolysis and was used to analyze the resulting MS and MS/MS peaks.

Expression of human pancreatic α -amylase. A detailed protocol for the expression and purification of HPA can be found in the work done by Rydberg *et al.* (5) Expression was carried out in *Pichia pastoris*. Colonies were grown in 60 mL BMGY media at 30°C 200 RPM. 20 mL of the overnight culture was added to 600 mL BMGY. After 16 hours the cells were transferred to 300 mL BMMY. After one day 2 mL of 50% methanol was added. The culture was left overnight. The culture supernatant was collected and purified via Phenyl Sepharose and Hitrap Q columns. Deglycosylation was performed with *N*-glycosidase F.

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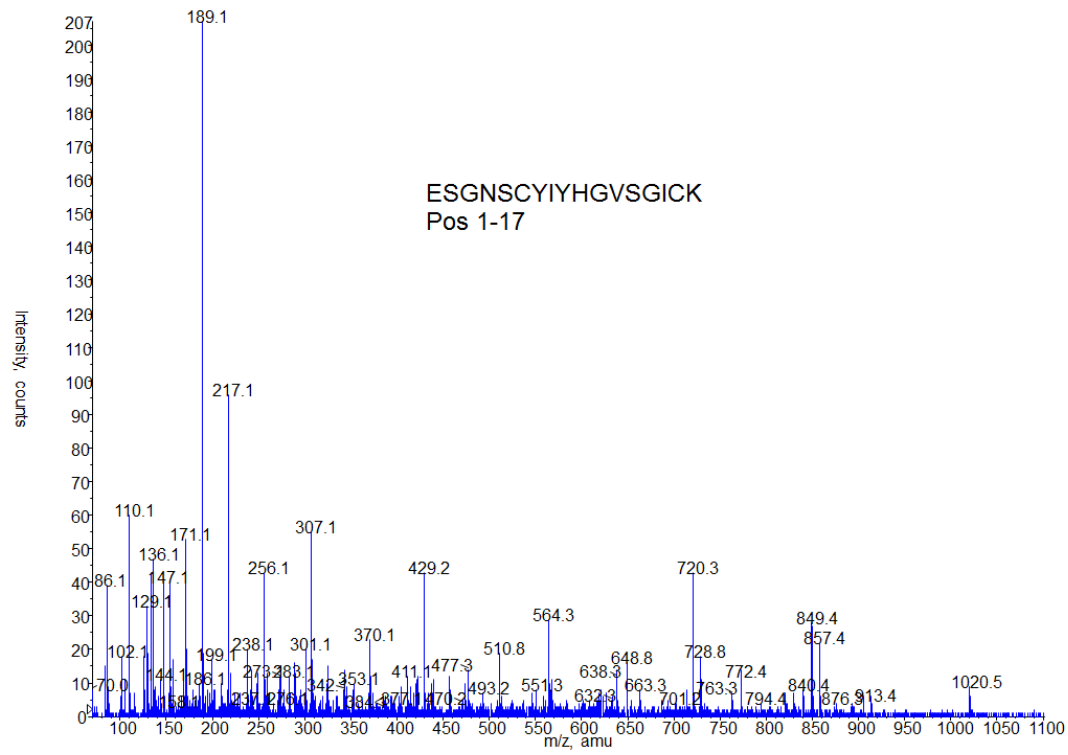
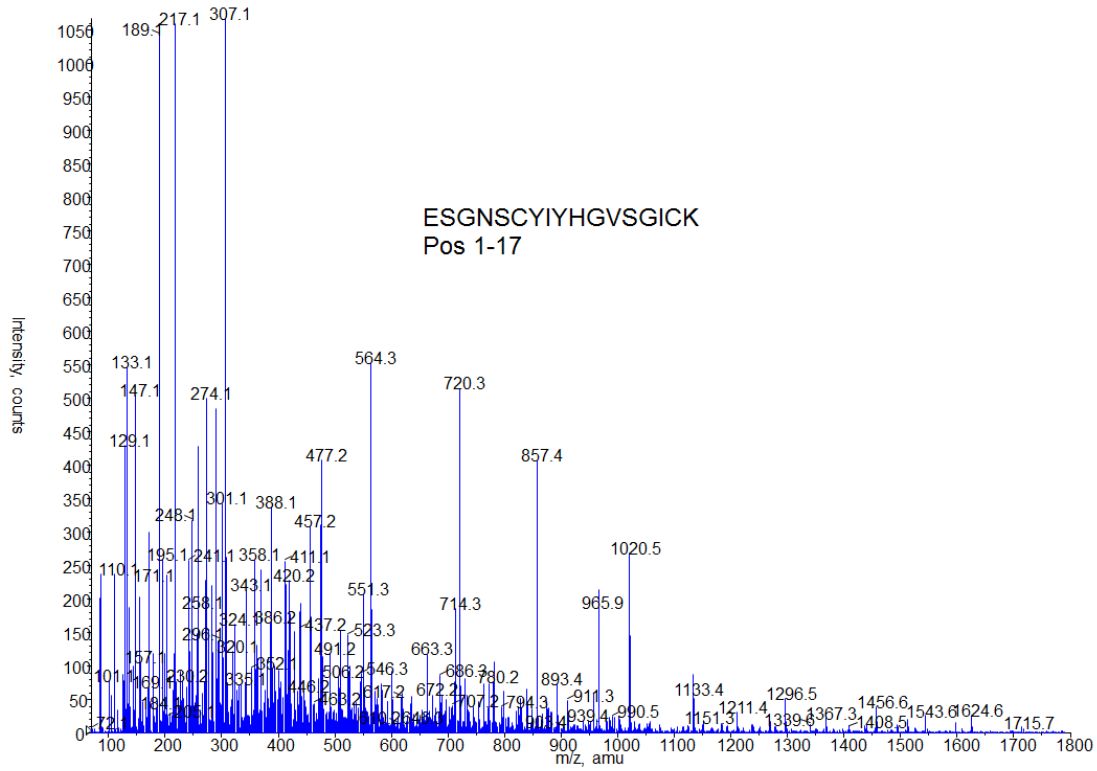


Figure S1. MS/MS data of tryptic digests of natural helianthamide.

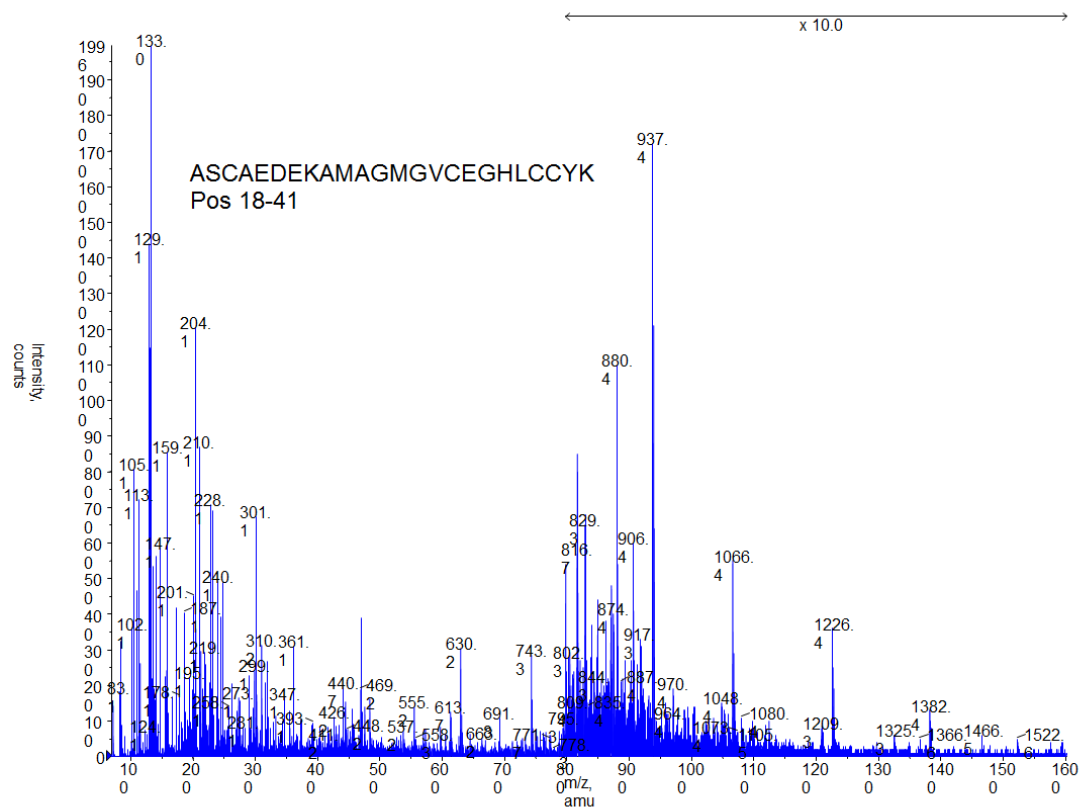
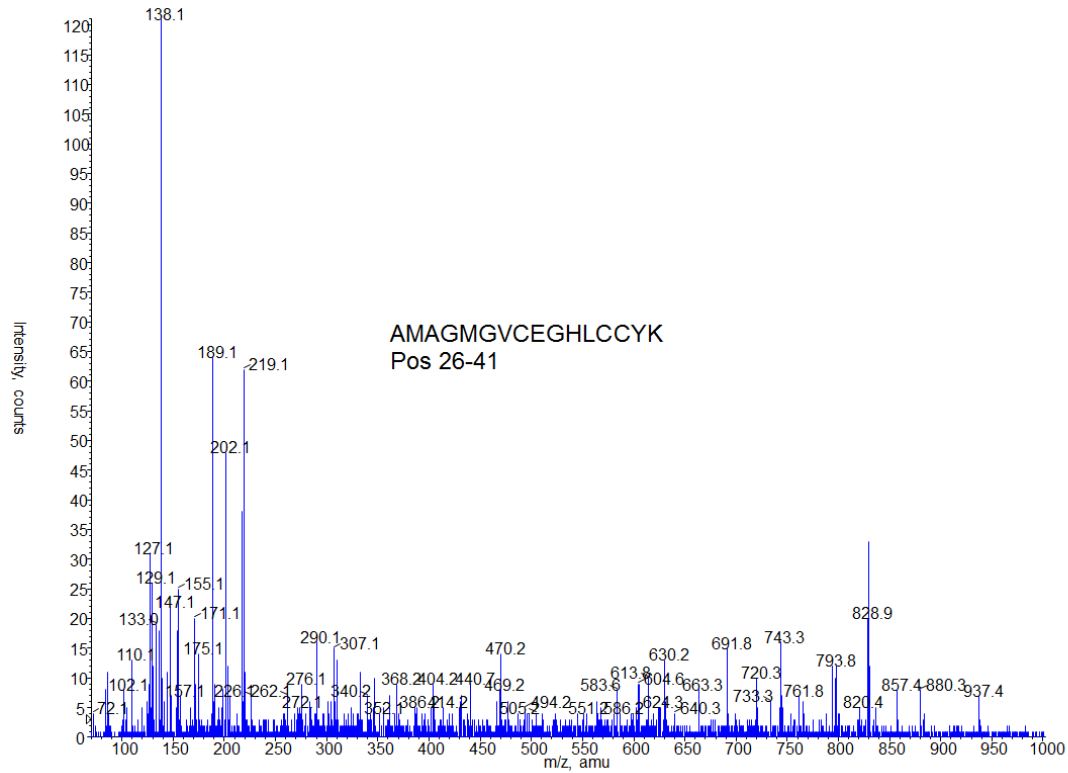


Figure S2. MS/MS data of tryptic digests of natural helianthamide (continued).

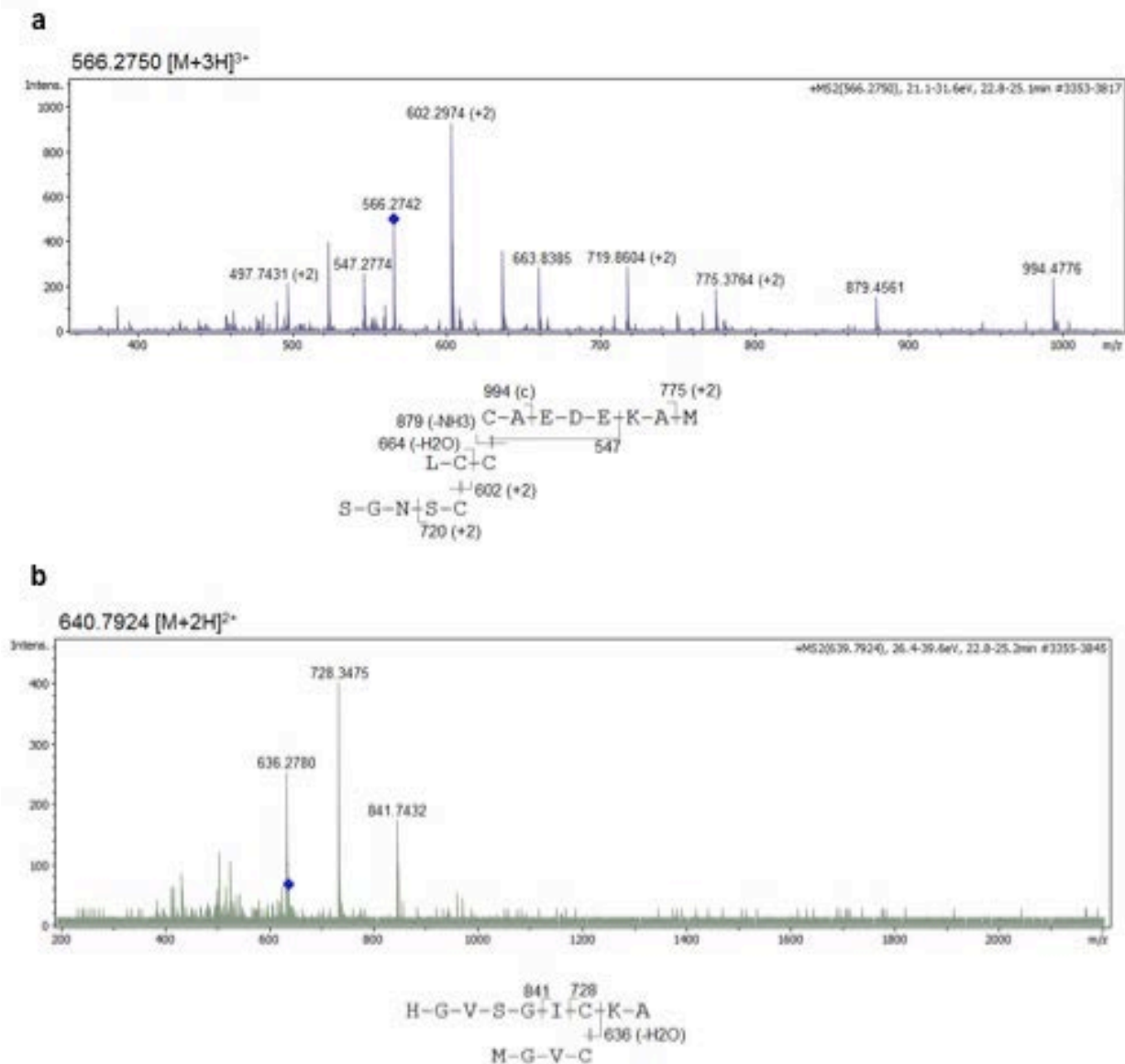


Figure S3. MS/MS data of selected peaks and their corresponding sequences for disulfide analysis of helianthamide. The data confirm the 1-5,2-4,3-6 connectivity seen in the crystal structure.

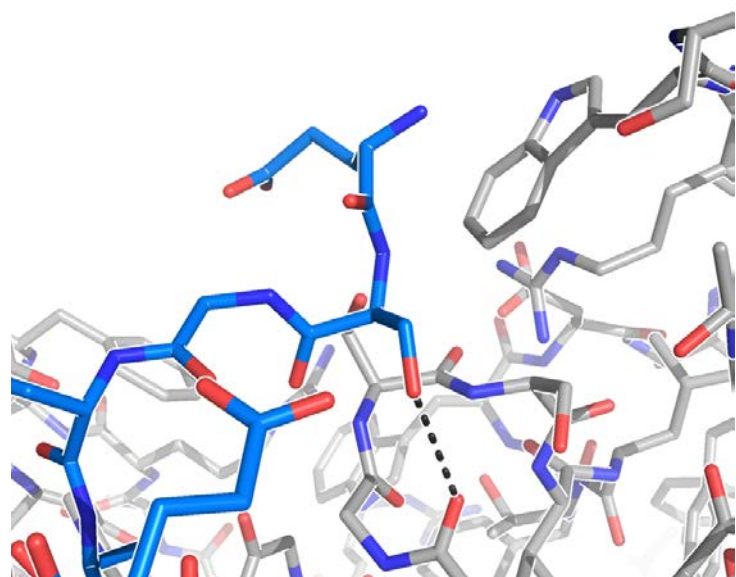


Figure S4. N-terminal region of helianthamide.

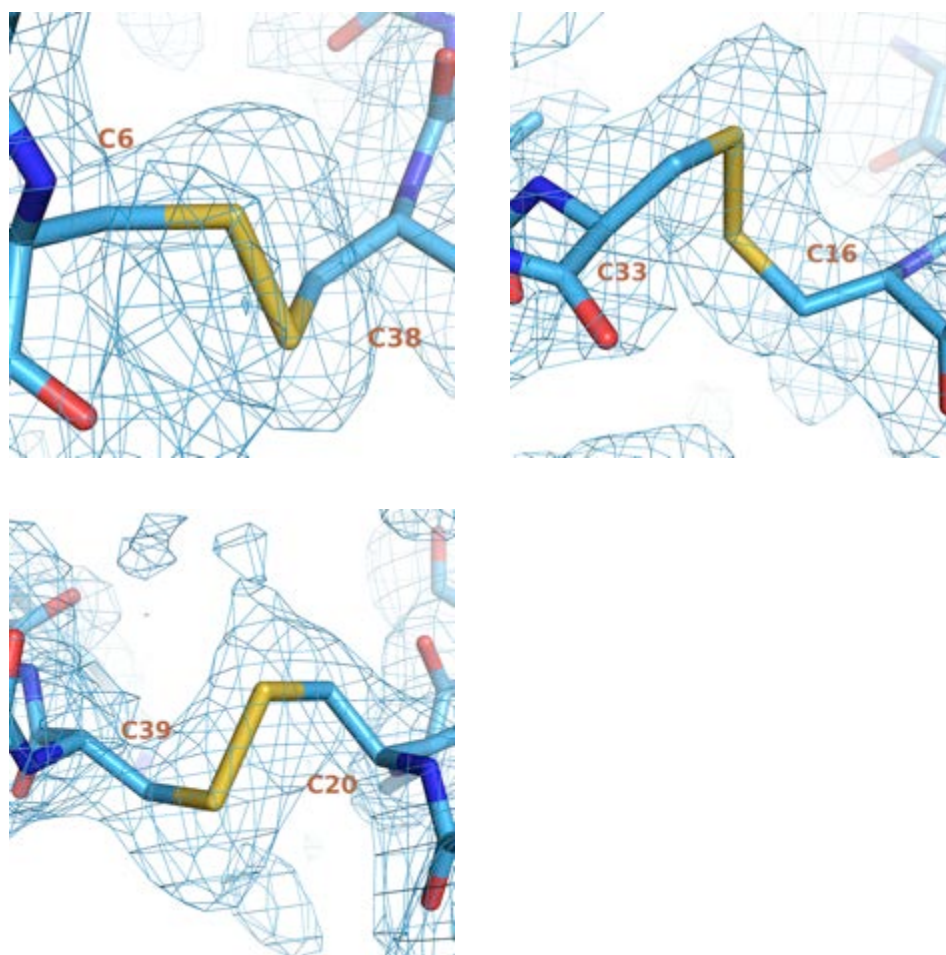


Figure S5. Omit maps of disulfide bridging in the helianthamide/PPA crystal structure.

ATG AAA CAA TCC ACC ATC GCA CTG GCC CTG CTG CCG CTG CTG 42
 TTC ACG CCG GTT ACA AAG CCG CTG GTG CAT CAT CAT CAT CAT 84
 CAC TCG AGT GGC GCA CAG GTT ATT AAC ACC TTT GAT GGT GTT 126
 GCT GAC TAT CTG CAA ACG TAC CAT AAA CTG CCG GAT AAT TAT 168
 ATC ACC AAA TCA GAA GCA CAG GCC CTG GGT TGG GTC GCA TCG 210
 AAA GGT AAC CTG GCA GAT GTG GCT CCG GGC AAA AGT ATT GGC 252
 GGT GAC ATC TTC TCC AAT CGT GAA GGT AAA CTG CCG GGC AAA 294
 TCT GGT CGT ACC TGG CGC GAA GCG GAT ATT AAC TAT ACG TCA 336
 GGC TTT CGT AAT TCG GAT CGC ATT CTG TAC AGC TCT GAC TGG 378
 CTG ATC TAT AAA ACC ACG GAC GCC TAC CAA ACC TTC ACG AAA 420
 ATT CGT ATC GAA GGC CGC GAA AGT GGT AAC TCC TGC TAT ATT 462
 TAC CAC GGC GTT AGC GGT ATC TGC AAA GCG TCT TGT GCC GAA 504
 GAT GAA AAA GCA ATG GCA GGC ATG GGC GTG TGT GAA GGT CAT 546
 CTG TGT TGT TAC AAA ACC CCG TGG TGA TAG

Figure S6. Sequence of the barnase'-helianthamide gene optimized for expression in *E. coli*.

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      10           20           30           40           50
MKQSTIALAL LPLLFTPVTK AAGHHHHHS SGAQVINTFD GVADYLQTYH

      60           70           80           90           100
KLPDNYITKS EAQALGWVAS KGNLADVAPG KSIIGDIFSN REGKLPGKSG

      110          120          130          140          150
RTWREADINY TSGFRNSDRI LYSSDWLIYK TTDAYQTFTK IRENLYFQSE

      160          170          180          190
SGNSCYIYHG VSGICKASCA EDEKAMAGMG VCEGHLCCYK TPW

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Figure S7. Peptide sequence of the complete barnase'-helianthamide fusion protein. His-tag is highlighted in green. The active site mutation of barnase is highlighted in pink. The TEV protease recognition site is highlighted in yellow.

Table S1. Secondary structural analysis of the CD spectra (Fig 1b) of the natural, synthetic, and recombinant helianthamide

	Proportion of each secondary structural estimate			
	α -helix	β -sheet	turns	other
Synthetic	0.06	0.32	0.18	0.44
Natural	0.19	0.32	0.18	0.31
Recombinant	0.11	0.33	0.23	0.33

Table S2. Select LC-MS/MS peaks and their corresponding sequences for disulfide assignment

M	Parent peak	Sequence
1198.42	600.21 [M+2H] ²⁺	IYHGVSGIC CE
1279.58	640.79 [M+2H] ²⁺	HGVSGICKA MGVC
1286.70	429.90 [M+3H] ³⁺	GICK KAMAGMGVC
1311.70	656.85 [M+2H] ²⁺	CYIYHGV LCC C
1327.68	443.56 [M+3H] ³⁺	VC ICKASCAE CY
1347.66	449.22 [M+3H] ³⁺	NSCYI LCCYK C
1416.68	709.34 [M+2H] ²⁺	GIC EKAMAGMGVCE
1439.60	480.88 [M+3H] ³⁺	SC EGHLCCY SCAE
1534.56	768.28 [M+2H] ²⁺	ESGNSC GHLCC SCAE
1551.70	776.85 [M+2H] ²⁺	GNSC GHLCCY KASCA
1606.82	804.41 [M+2H] ²⁺	CY GVCEGHL SGICK
1631.78	816.89 [M+2H] ²⁺	SESGNSC LCCYK CAE
1698.81	566.27 [M+3H] ³⁺	SGNSC LCC CAEDEKAM
1760.80	881.40 [M+2H] ²⁺	GNSC LCCYKT SCAEDE
1818.97	607.32 [M+3H] ³⁺	SESGNSCYIYHG CC SCA

Table S3. Inhibitory specificity of helianthamide

Enzyme	K_i
Porcine pancreatic α -amylase	0.1 nM
Human maltase-glucoamylase	N.I.
<i>Roseburia inulinivorans</i> α -amylase A	N.I.
<i>Butyrivibrio fibrisolvens</i> α -amylase B	N.I.
<i>Bacillus licheniformis</i> α -amylase	N.I.
Bovine liver β -galactosidase	N.I.
Green coffee bean α -galactosidase	N.I.
Jack bean α -mannosidase	N.I.
Brewer's yeast α -glucosidase	N.I.
Almond β -glucosidase	N.I.

N.I. indicates no inhibition observed at a concentration of 1 μ M.

Table S4. Summary of Structure Determination Statistics

Data Collection Parameters	helianthamide/ PPA Inhibitor Complex
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (Å)	
a	43.75
b	103.28
c	111.90
Total no. reflections collected	116266 (8116)
No. of unique reflections ^a	16310 (1177)
Mean $I/\sigma I^a$	17.3 (7.5)
Multiplicity ^a	7.1 (6.8)
Merging R-factor (%) ^a	10.4 (29.1)
Maximum resolution (Å)	2.60
Structure Refinement Values	
Number of reflections	15472
Resolution range (Å)	49.19 - 2.6
Completeness (%) ^a	95.2 (94.7)
No. protein atoms	3904
No. inhibitor atoms	322
No. solvent atoms	219
Average thermal factors (Å ²)	
Protein atoms	16.9
Inhibitor atoms	21.3
Solvent atoms	35.5
Overall	17.2
Final R-free value (%) ^b	22.6
Final R-factor (%)	18.1
Structure Stereochemistry	
r.m.s. deviations	
bonds (Å)	0.004
angles (°)	0.882

^a Values in parentheses refer to the highest resolution shell: 2.73-2.6 Å.

^b 5% of the data was set aside to calculate R-free.