Supplementary Information

Lasso peptide benenodin-1 is a thermally actuated [1]rotaxane switch

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Methods

Strains and Reagents. *A. benevestitus* was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), and whole genomic DNA was extracted using QIAGEN DNeasy Blood & Tissue Kit (Qiagen). PicoMaxx DNA polymerase (Agilent Technologies) was used for DNA amplification with primers purchased from Integrated DNA Technologies. The restriction enzymes *Hin*dIII, *Eco*RI, *Xba*I, and *Bgl*II (New England Biolabs) were used to clone the gene clusters into the vector. XL-1 blue *E. coli* cells were used for recombinant DNA steps, while BL-21 was used for peptide expression.

Plasmid Construction. Molecular cloning was done according to standard protocols. Gene clusters for benenodin-1 were cloned into pASK75, which harbors a tetracycline-inducible promoter.[1] The gene cluster was designed such that the *benA* gene was flanked on its 5' end by an *Xba*I site and on its 3' end by an *Eco*RI site, allowing for facile swapping of the *benA* gene. The *benB* and *benC* genes were inserted downstream between *Eco*RI and *Kpn*I sites. Benenodin-1 variants were generated using overlap PCR. The *benE* gene, encoding the benenodin-1 isopeptidase, was cloned into pQE60 (Qiagen) which installs a C-terminal His-tag for protein purification. The gene including the His-tag was cut out of this plasmid and ligated into pQE80 (Qiagen) to generate the final expression plasmid. Primers, restriction enzymes, and vectors used for each plasmid are shown in the tables below.

Plasmids constructed in this study

Plasmid Name	**Description**	**Primers**	**Restriction Sites**	**Vector**
pCZ35	*benABC*	1,2,3,4	*Xba*I, *Eco*RI, *Kpn*I	pASK75
pCZ31	*benA*(E14A)*BC*	3,4,5,6	*Xba*I, *Eco*RI	pASK75
pCZ32	*benA*(Q15A)*BC*	3,4,7,8	*Xba*I, *Eco*RI	pASK75
pCZ33	*benA*(K17A)*BC*	3,4,9,10	*Xba*I, *Eco*RI	pASK75
pCZ85	*benA*(T12R)*BC*	3,4,11,12	*Xba*I, *Eco*RI	pASK75
pCZ79	*benA*(atxA22-loop)*BC*	13,14,15,16	*Xba*I, *Eco*RI	pASK75
pMW19	*BenE* with C-terminal His tag	17,18	*Eco*RI, *Bgl*II	pQE60
pCZ67	*BenE* with C-terminal His tag	-	*Eco*RI, *Hin*dIII	pQE80

Sequences of primers used in this study

Primer Name	**Sequence (5’ to 3’)**
Primer 1	TCTCTAGATGGTAGCGTGAGGAGACC
Primer 2	TGGGTACCTTAGGTAGGGCGC
Primer 3	CGCGATTGAGAATTCCATTGGGGCGTACAAACCC
Primer 4	CGCCCCAATGGAATTCTCAATCGCGATCAAGGCC
Primer 5	GCTTCGCTTGCGCCTGCGTC
Primer 6	GACGCAGGCGCAAGCGAAGC
Primer 7	CGGCTTCGCCGCTTCCTGCG
Primer 8	CGCAGGAAGCGGCGAAGCCG
Primer 9	CCCATCGGCGCCGCTTGTTC
Primer 10	GAACAAGCGGCGCCGATGGG
Primer 11	CCCGACAGCATCCTGCGTCAGGAACAAGCGAAG

Primer 12	CTTCGCTTGTTCCTGACGCAGGATGCTGTCGGG
Primer 13	GAGTTATTTTACCACTCCCTATCAG
Primer 14	GCAAGTCGAGGCAAGCGATC
Primer 15	GACAGCGTGAGCGGCCAAGAACAAGCGAAG
Primer 16	TTCTTGGCCGCTCACGCTGTCGGGACGGCC
Primer 17	TGACCCTTTCGAATTCCAGGAGAGGAAATGA
Primer 18	TGAGATCTCTGCCCCGGAGAGGCACTC

Lasso Peptide Expression and Purification. For all peptide expressions, *E. coli* BL21 cells were transformed with the plasmid containing the lasso peptide gene cluster. Overnight cultures were grown in 5 mL of LB with ampicillin (100 µg/mL) at 37 ˚C. Cells were subcultured at an OD_{600} of 0.02 into M9 minimal media, supplemented with 20 amino acids (0.04 g/L each) and ampicillin. Upon reaching an OD600 of 0.2 to 0.3, cultures were induced with 200 µg/L anhydrotetracycline. The cultures were induced for 18 hours at 20 ˚C. The cells were harvested by centrifugation at 8,000 x *g* at 4 ˚C for 10 minutes, then lysed in methanol. The insoluble fraction was removed by centrifugation, and the methanol-soluble fraction, which contains the peptide of interest, was dried under reduced pressure and reconstituted in ultrapure water. The sample was further cleaned up using solid-phase extraction columns (Strata C_8, 6mL). The crude peptide was eluted from the column using methanol, dried under reduced pressure, and then resuspended in 50% acetonitrile (ACN) and 50% water solution. The peptide sample was injected onto a Zorbax 300SB-C18 Semi-Prep HPLC Column (9.4 by 250 mm, Agilent Technologies). Solvent gradient 1 was applied to the column at a flow rate of 4.0 mL/min: 10% ACN for

1 min, increase to 50% ACN over 19 min, increase to 90% ACN over 5 min, remain at 90% ACN for 5 min, decrease to 10% ACN in 2 min. Detection was done at 215 nm. HPLC fractions containing the peptide of interest were collected and lyophilized (Labconco Freezone 4.5/−105 °C). The yield of wild-type benenodin-1ΔC5 was 0.28 mg/L after all purification steps. Peptide identity was confirmed using MALDI-MS. Peptide samples were diluted in a 2.5 mg/mL solution of α-cyano- 4-hydroxycinnamic acid matrix, and spotted onto an Applied Biosystem(ABI) 384 Opti-TOF 123 mm × 81 mm SS plate for MALDI-MS analysis.

Benenodin-1 ΔC5, Conformer 2 Production. Lyophilized benenodin-1 ΔC5 (conformer 1) was reconstituted in ultrapure water, and then incubated at 95 °C in a thermocycler (Bio-Rad DNAEngine) for 3 h allowing the conversion to benenodin-1 ΔC5 conformer 2. The heated sample was injected on HPLC using solvent gradient 1 described above. The benenodin-1 ΔC5 conformers differ in retention time by ~0.5 min. Based on the retention times, the fraction containing benenodin-1ΔC5 conformer 2 was collected. The sample was then freeze-dried and reinjected on HPLC to ensure its purity.

Heating Assay on Lasso Peptides. Lasso peptides were resuspended to a final concentration of 100 μM in ultrapure H_2O. 200 μL of each peptide was placed in a PCR tube and heated in a BioRad DNA Engine Thermal Cycler using the heated lid to minimize evaporation. Temperatures used ranged from 35-95 °C, and time points (each time point was an aliquot of 20 μL) were taken between 0 minutes and 13 days. Samples were cooled to 4 °C and subjected to HPLC. The area under the curve was calculated from the chromatogram and fit to a two state reversible model:

$$\text{conformer 1} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{conformer 2}$$

With the following solutions to the differential equations:

$$\text{Fraction of conformer 1} = \frac{k_1 e^{-(k_1+k_2)t} + k_2}{k_1 + k_2} \quad \text{(eq1)}$$

$$\text{Fraction of conformer 2} = \frac{k_1 - k_1 e^{-(k_1+k_2)t}}{k_1 + k_2} \quad \text{(eq2)}$$

The values of k_1 and k_2 were determined by fitting the experimental data to these equations using the SOLVER function of Microsoft Excel.

NMR Structure Analysis of Benenodin-1 ΔC5, Conformers 1 and 2. The TOCSY (60 ms mixing time, and NOESY (100 ms mixing time) spectra of benenodin-1 ΔC5, conformer 1 (2.5 mg/ml, 100 uL sample, 2.5 mm NMR tube) and benenodin-1ΔC5, conformer 2 (6.5 mg/ml, 360 μL, 5 mm NMR tube) in 95% H_2O and 5% D_2O solution were acquired on a Bruker Avance III 800 MHz spectrometer at 288 K. Structural modeling and energy minimization were performed using CYANA and GROMACS as described previously.[2-3] Briefly, both TOCSY and NOESY spectrum were used for assigning proton chemical shifts. The volumes of cross peaks from NOESY spectrum were integrated using the MestReNova software package (Mestre Lab Research, S.S.L., Santiago de Compostella, Spain). The integrated peaks were used as input for automated NOE assignment using *noeassign* macro in CYANA 2.1, where the ambiguous interactions were resolved and assigned.[4] The refined cross peak volumes were calibrated using

caliba macro in CYANA 2.1, and then converted to upper distance restraints for stimulated annealing and final structure calculation.[5] The top 20 structures were generated from initial 200 random structure seeds. The structures were refined and energy-minimized following the GROMACS structure refining protocol described by Spronk *et al*.[6-9] The atomic coordinates, distance constraints and related NMR experimental conditions have been uploaded in the PDB and BMRB with accession numbers: conformer 1 (PDB: 5TJ1, BMRB: 30188), conformer 2 (PDB: 5TJ0, BMRB: 30187).

BenE Isopeptidase Expression and Purification. *E. coli* BL-21 Δ*slyD* cells bearing the plasmid, pCZ67, encoding BenE isopeptidase were grown overnight in LB media with ampicillin at 37 °C. The cells were subcultured into LB media with ampicillin at OD_{600} 0.02, which was grown at 20 °C with shaking. When the culture reached OD_{600} of 0.6 to 0.8, 1 mM IPTG was added to induce BenE isopeptidase production. The culture was then grown for 5 hours. Cells then were harvested by centrifugation at 8,000 x *g* for 10 min at 4 °C. The cells were lysed via sonication, and the isopeptidase was purified under native conditions on Ni-NTA resin according to the manufacturer's recommendation (Qiagen). Purified protein was analyzed by SDS-PAGE.

BenE and AtxE2 Isopeptidase Assay. For a typical isopeptidase assay, 100 μM of peptide substrate and 100 nM of enzyme were mixed in the assay buffer (1 x PBS, pH 7.5) with a total volume of 100 μL. The reaction was incubated at 20 °C for 16 h. 95 μL of the reaction mixture was injected on a Zorbax 300SB-C18 Semi-Prep HPLC Column using solvent gradient 1 described above in the Lasso Peptide Expression and Purification section. Based on the difference in retention time of lasso peptide and linearized lasso peptide, isopeptidase digestion can be monitored by integrating the

peaks corresponding to lasso peptide and linearized lasso peptide. To further confirm linearization, 1 µL of reaction mixture was diluted 10 fold with 2.5 mg/mL solution of α-cyano- 4-hydroxycinnamic acid matrix, and spotted onto an Applied Biosystem(ABI) 384 Opti-TOF 123 mm × 81 mm SS plate for MALDI-MS analysis.

Carboxypeptidase Assay. For a typical carboxypeptidase assay, 50 µM of the peptide substrate was digested with 1 U carboxypeptidase B (Sigma-Aldrich) and 1 U carboxypeptidase Y (Affymetrix) in carboxypeptidase digestion buffer (50 mM sodium acetate, pH = 6.0) with a total volume of 20 µL for 16 hours at 20 °C. 18 µL of the digested mixture was injected on HPLC as described above. 1 µL of the digested sample was further analyzed by MALDI-MS as described above.

Trypsin Assay. Trypsin digestion was conducted in trypsin digestion buffer (50 mM NH_4HCO_3). T12R benenodin-1 ΔC5 was treated with 0.1 µg of sequencing grade trypsin (Promega) in a total volume of 80 µL. The reaction was carried out for 16 hours at 37 °C. 60 µL was analyzed on HPLC and peaks of interest were collected. These peaks were subsequently analyzed using an LC-MS (Agilent 6530 Accurate-Mass Q-TOF LC/MS).

Supplementary References

(1) Skerra, A. *Gene* **1994,** *151*, 131-135.
(2) Maksimov, M. O.; Koos, J. D.; Zong, C.; Lisko, B.; Link, A. J. *J. Biol. Chem.* **2015,** *290*, 30806-30812.
(3) Maksimov, M. O.; Link, A. J. *J. Am. Chem. Soc.* **2013,** *135*, 12038-12047.
(4) Mumenthaler, C.; Braun, W. *J. Mol. Biol.* **1995,** *254*, 465-480.
(5) Mumenthaler, C.; Guntert, P.; Braun, W.; Wuthrich, K. *J. Biomol. NMR* **1997,** *10*, 351-362.
(6) Spronk, C.; Nabuurs, S. B.; Krieger, E.; Vriend, G.; Vuister, G. W. *Prog. Nucl. Magn. Reson. Spectrosc.* **2004,** *45*, 315-337.

(7) Spronk, C.; Linge, J. P.; Hilbers, C. W.; Vuister, G. W. *J. Biomol. NMR* **2002,** *22*, 281-289.
(8) Linge, J. P.; Williams, M. A.; Spronk, C.; Bonvin, A.; Nilges, M. *Proteins-Structure Function and Bioinformatics* **2003,** *50*, 496-506.
(9) Spronk, C.; Nabuurs, S. B.; Bonvin, A.; Krieger, E.; Vuister, G. W.; Vriend, G. *J. Biomol. NMR* **2003,** *25*, 225-234.

Supplementary Figures

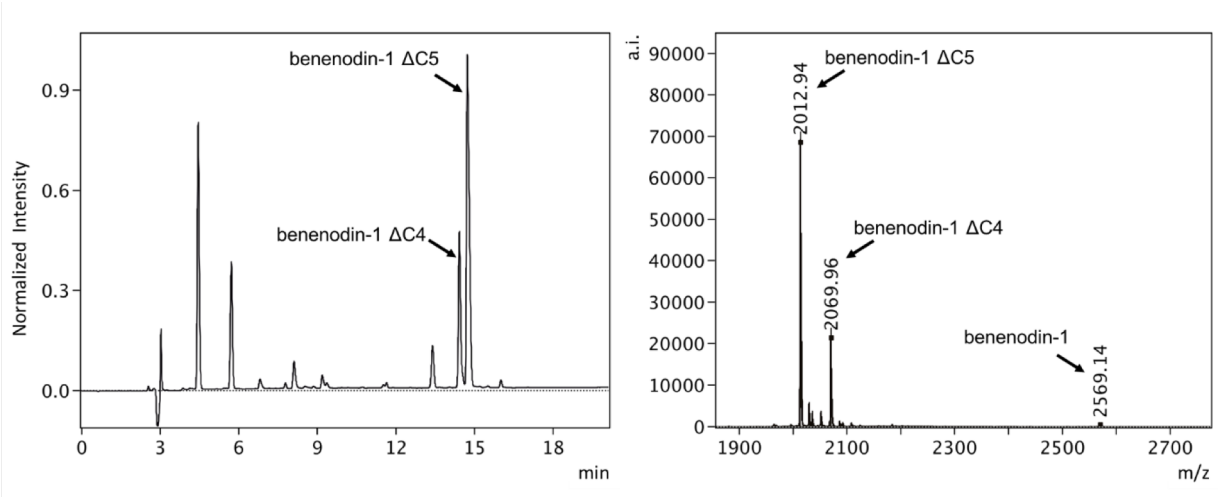


Figure S1: HPLC (left) and MALDI-MS (right) of cell extracts producing benenodin-1. The predominant form of benenodin-1 is the ΔC5 variant which lacks the final 5 C-terminal amino acids. Theoretical masses (singly protonated): benenodin-1: 2569.28 Da, benenodin-1 ΔC4: 2070.04 Da, benenodin-1 ΔC5: 2013.02.

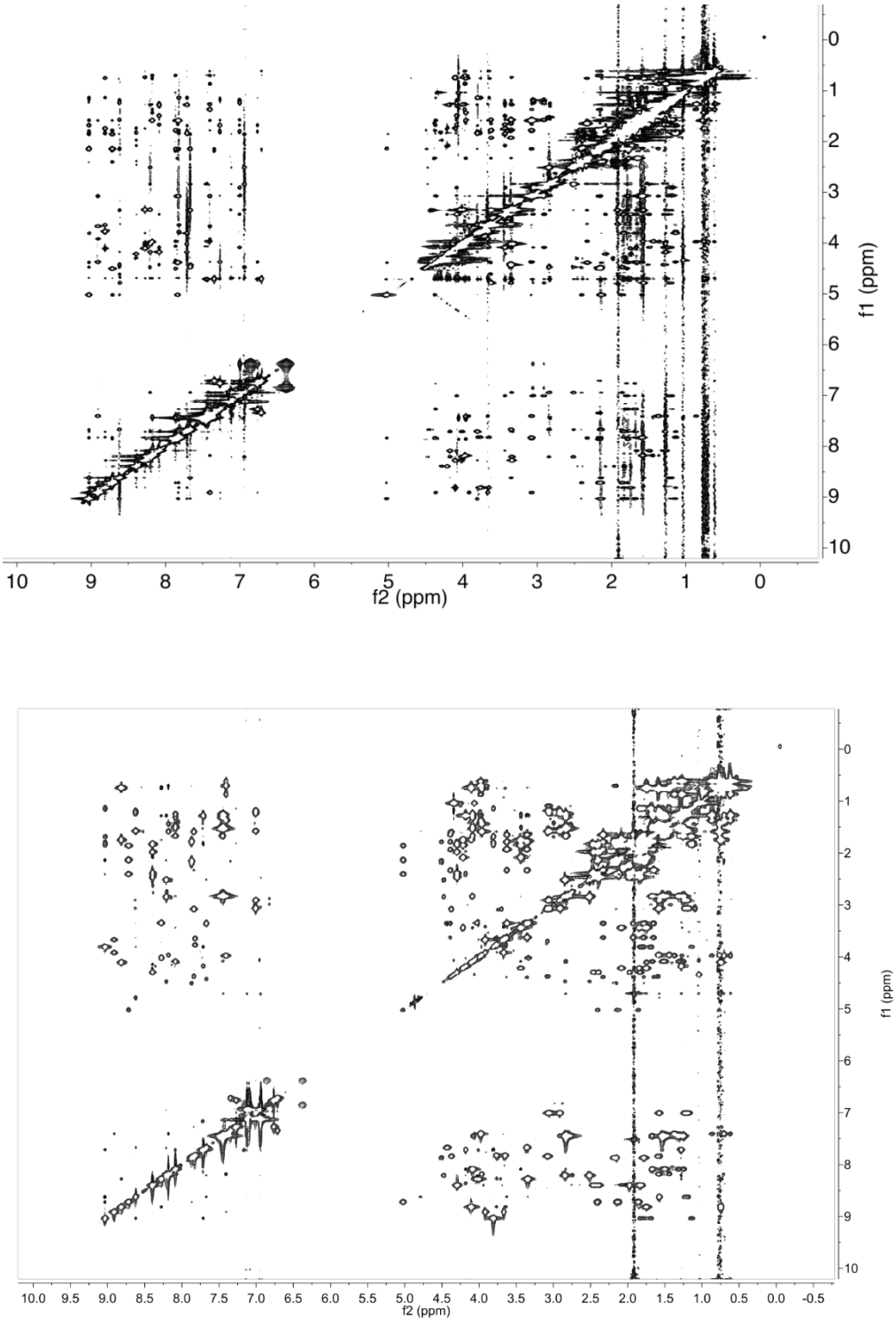


Figure S2: NOESY (top) and TOCSY (bottom) spectra of benenodin-1 ΔC5, conformer 1 (native state).

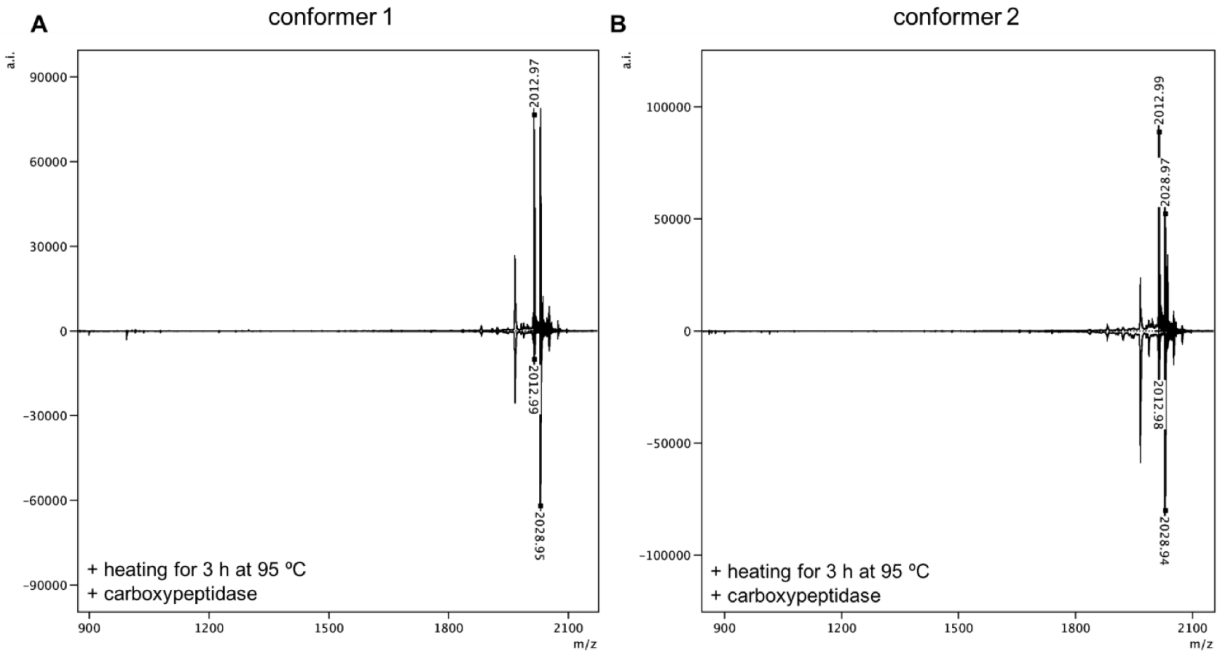


Figure S3: Carboxypeptidase assays on benenodin-1 ΔC5, conformers 1 and 2. Top panel: mass spectrum of purified peptide before digestion with carboxypeptidase. Bottom panel: mass spectrum of peptide that has been heated at 95 °C for 3 h and subsequently treated with carboxypeptidase. Neither conformer 1 or 2 exhibit any cleavage, suggesting that both conformers are threaded. The +16 Da adduct on peptide is due to the oxidation of Met 19 of benenodin-1.

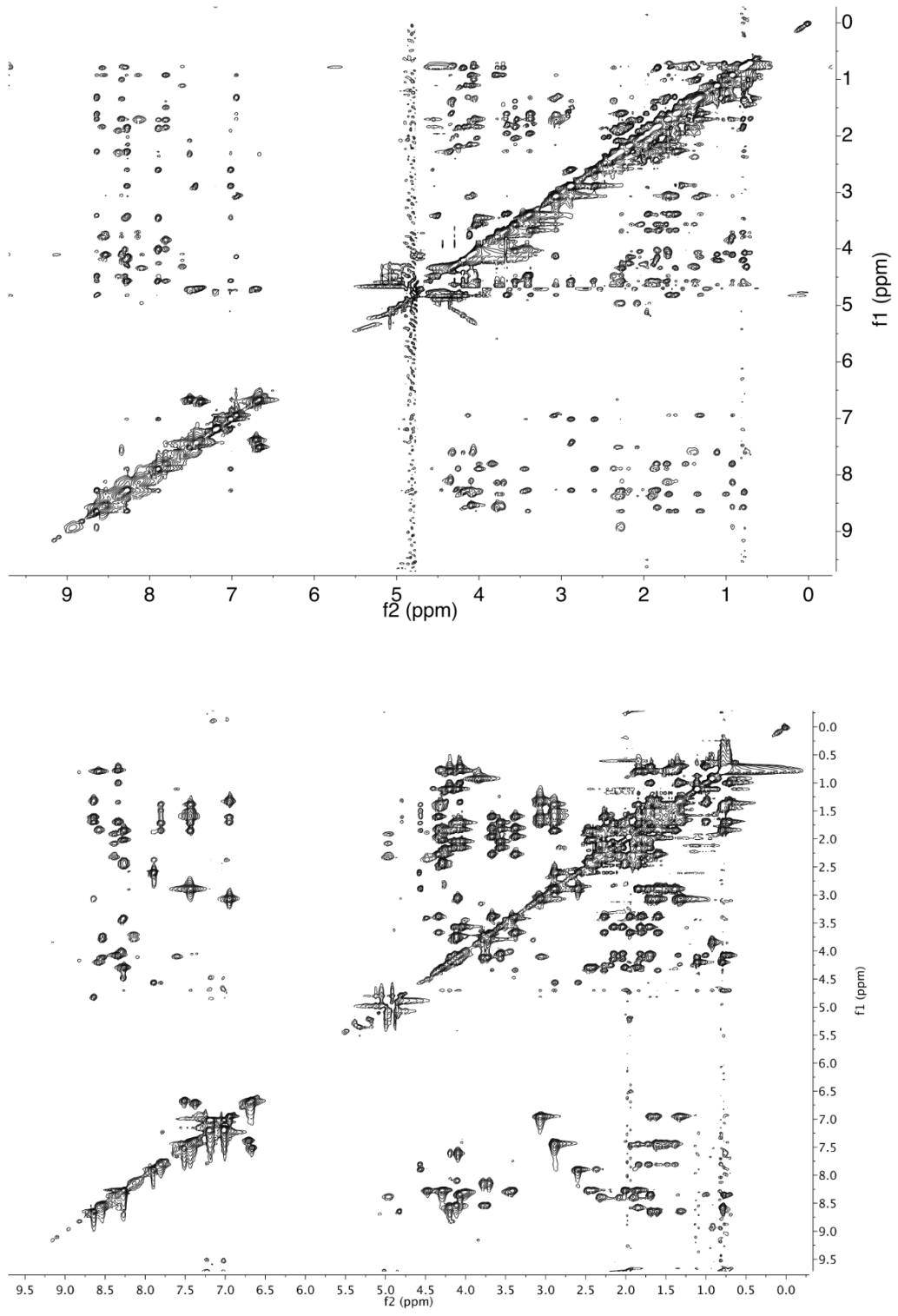


Figure S4: NOESY (top) and TOCSY (bottom) spectra of benenodin-1 ΔC5, conformer 2.

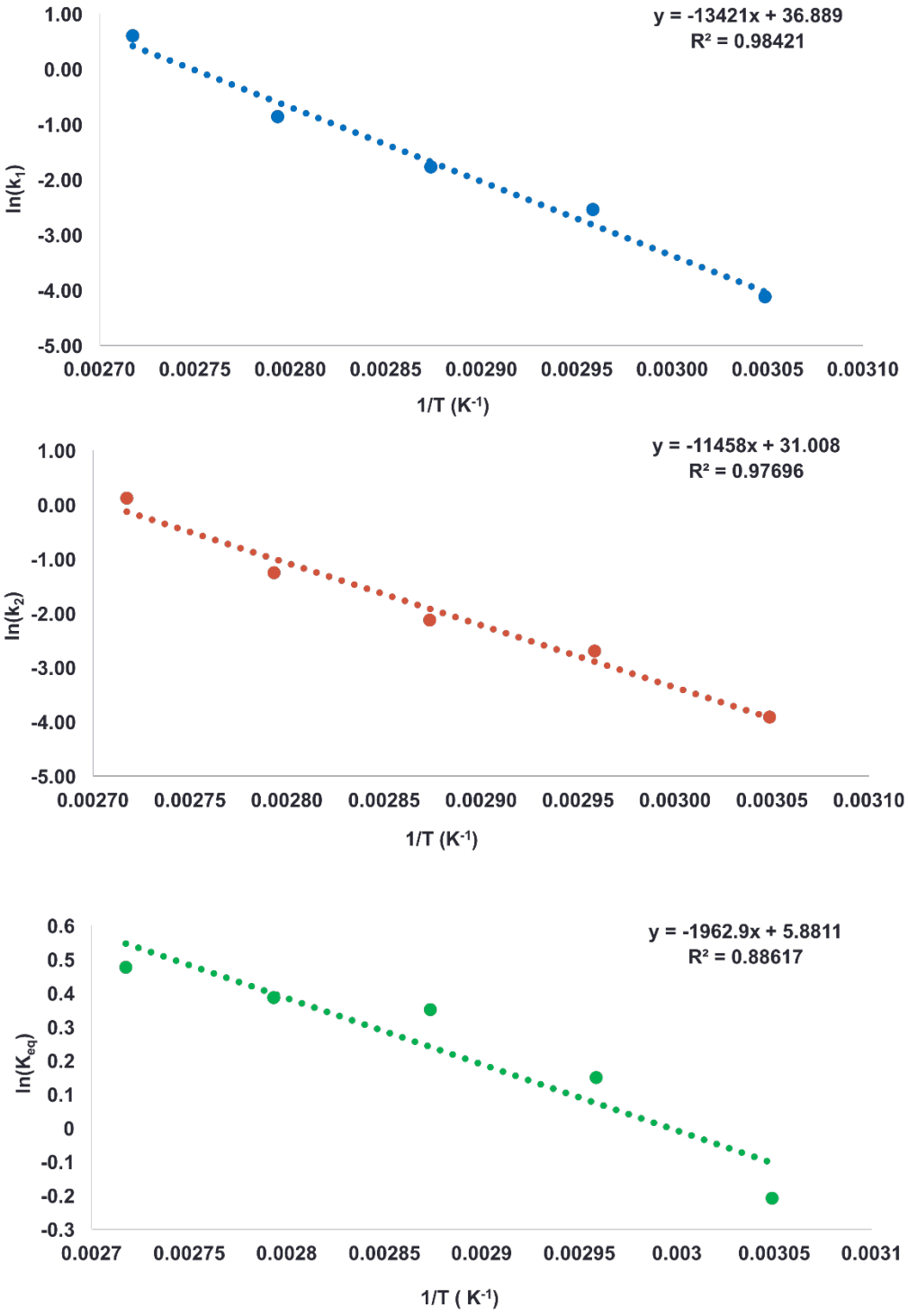


Figure S5: Arrhenius (top and middle) and van't Hoff (bottom) plots of conformational switching data. The source data for these plots is in Table S2.

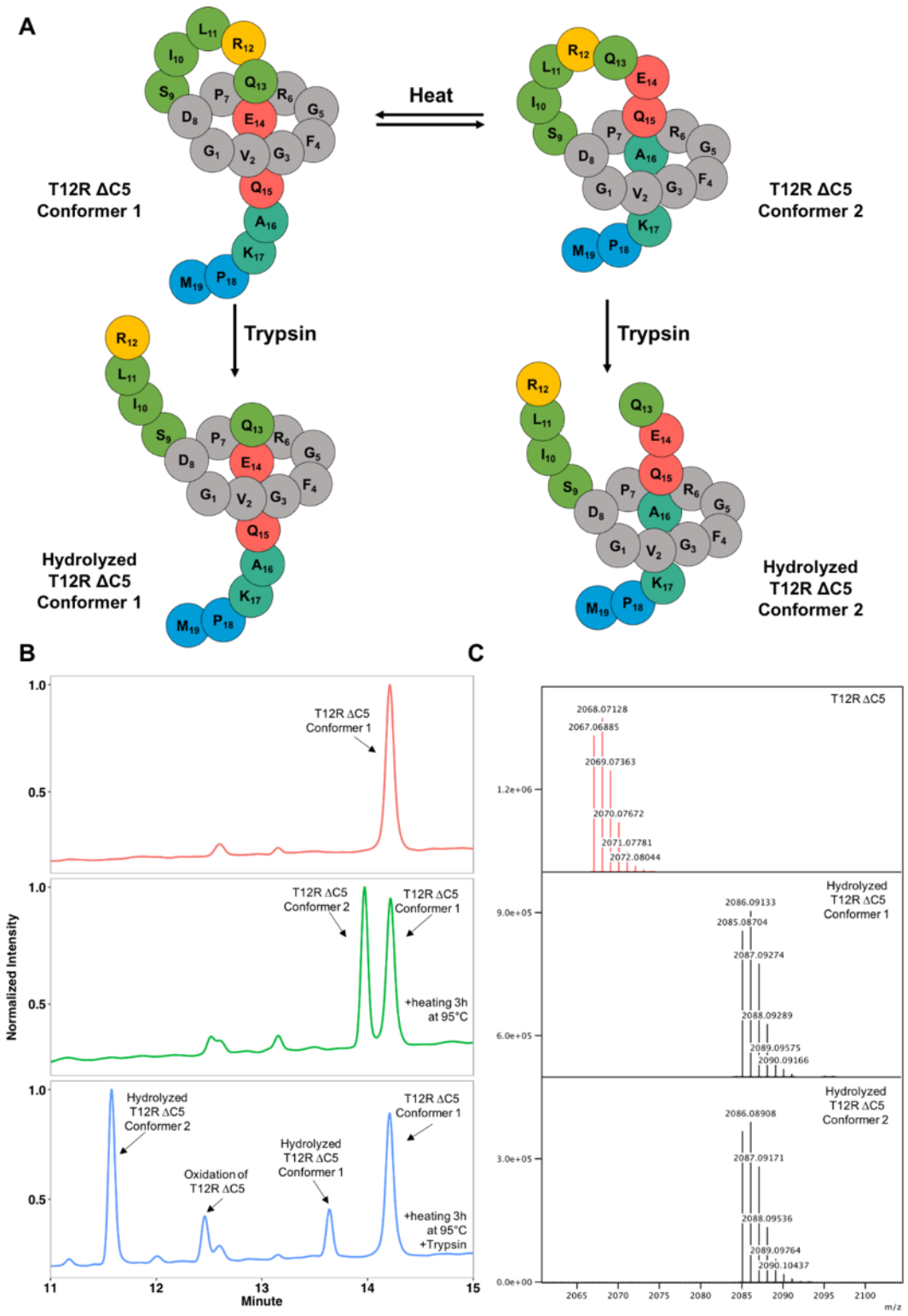


Figure S6: Caption on following page

Figure S6: Generating a protease-cleavable variant of benenodin-1. A: The T12R benenodin-1 ΔC5 variant establishes an equilibrium between two conformers upon heating with a similar ratio to the wild-type peptide. These conformers can be cleaved by trypsin to generate [2]rotaxanes. B: HPLC traces of T12R benenodin-1 DC5 as produced by *E. coli* (red trace), after heating for 3 h at 95 °C (green trace), and after heating and trypsin cleavage at 37 °C for 16 h (blue trace). While conformer 2 is cleaved completely by trypsin under these conditions, only ~25% of conformer 1 is cleaved. C: LC-MS spectra of peaks collected from the blue trace in part B.

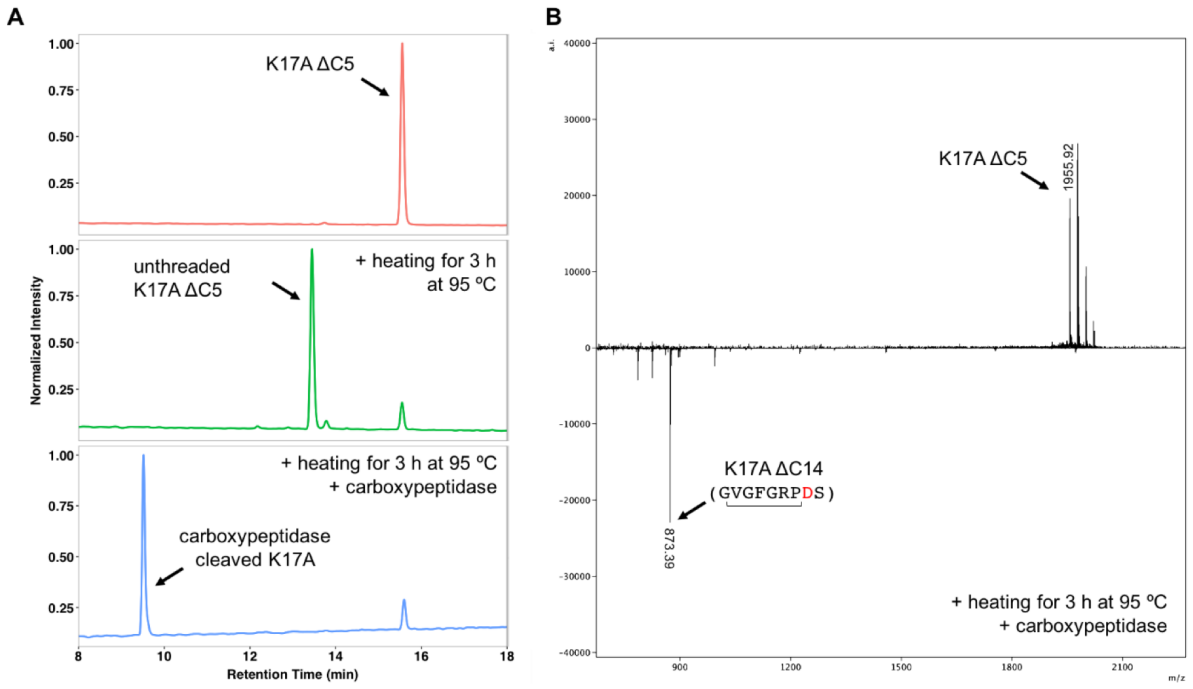


Figure S7: The K17A variant of benenodin-1 ΔC5 unthreads upon heating at 95 °C. A: HPLC traces of untreated benenodin-1 ΔC5 K17A (red), benenodin-1 ΔC5 K17A heated at 95 °C for 3 h (green), and heated benenodin-1 ΔC5 K17A that has been treated with carboxypeptidase (blue). B: mass spectra corresponding to intact benenodin-1 ΔC5 K17A (top) and heated, carboxypeptidase-cleaved peptide (bottom). Theoretical mass (MH^+) of benenodin-1 ΔC5 K17A: 1955.96 Da, benenodin-1 ΔC14 K17A 873.41 Da. The fact that carboxypeptidase can cleave the C-terminal residues of the peptide is an indication that the peptide has unthreaded.

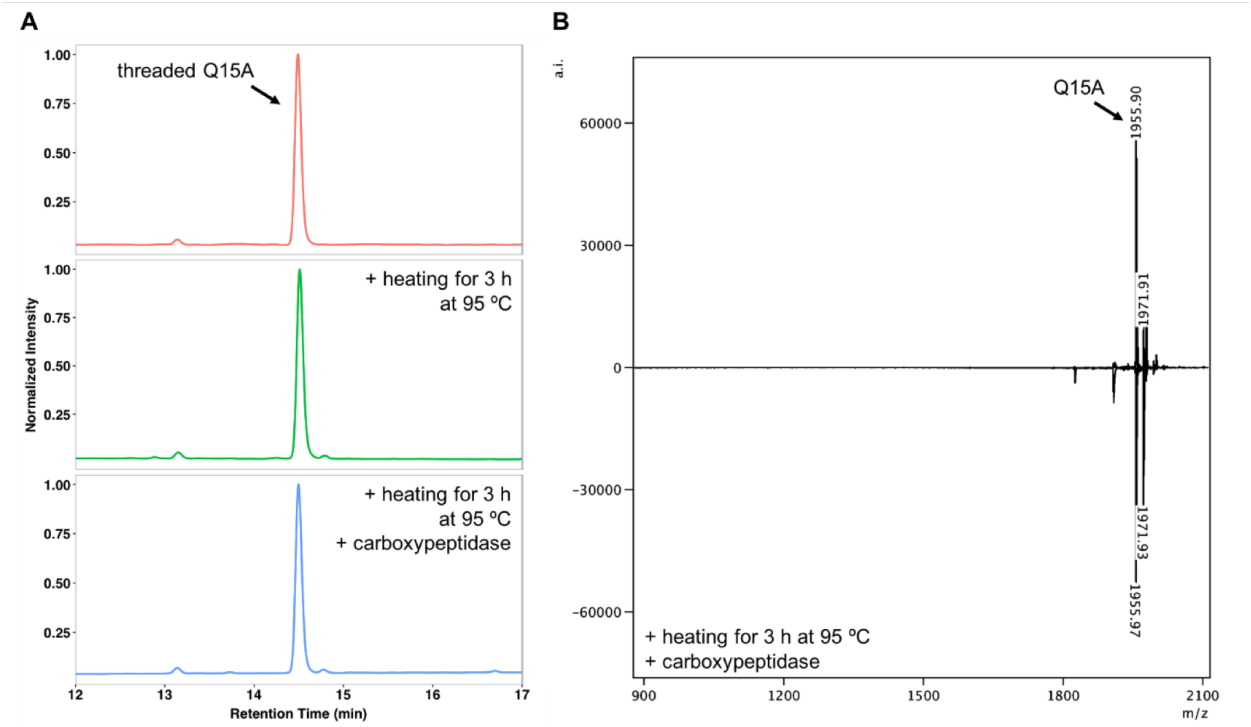


Figure S8: The Q15A variant of benenodin-1 ΔC5 does not undergo a conformation change upon heating at 95 °C. Traces and spectra are as described in the Figure S7 caption. Neither heating nor carboxypeptidase treatment has an effect on Q15A benenodin-1 ΔC5, showing that the peptide remains in a native-like threaded state. The +16 Da adduct on peptide is due to the oxidation of Met 19 of benenodin-1. Theoretical mass of benenodin-1 ΔC5 Q15A (MH^+): 1955.99 Da.

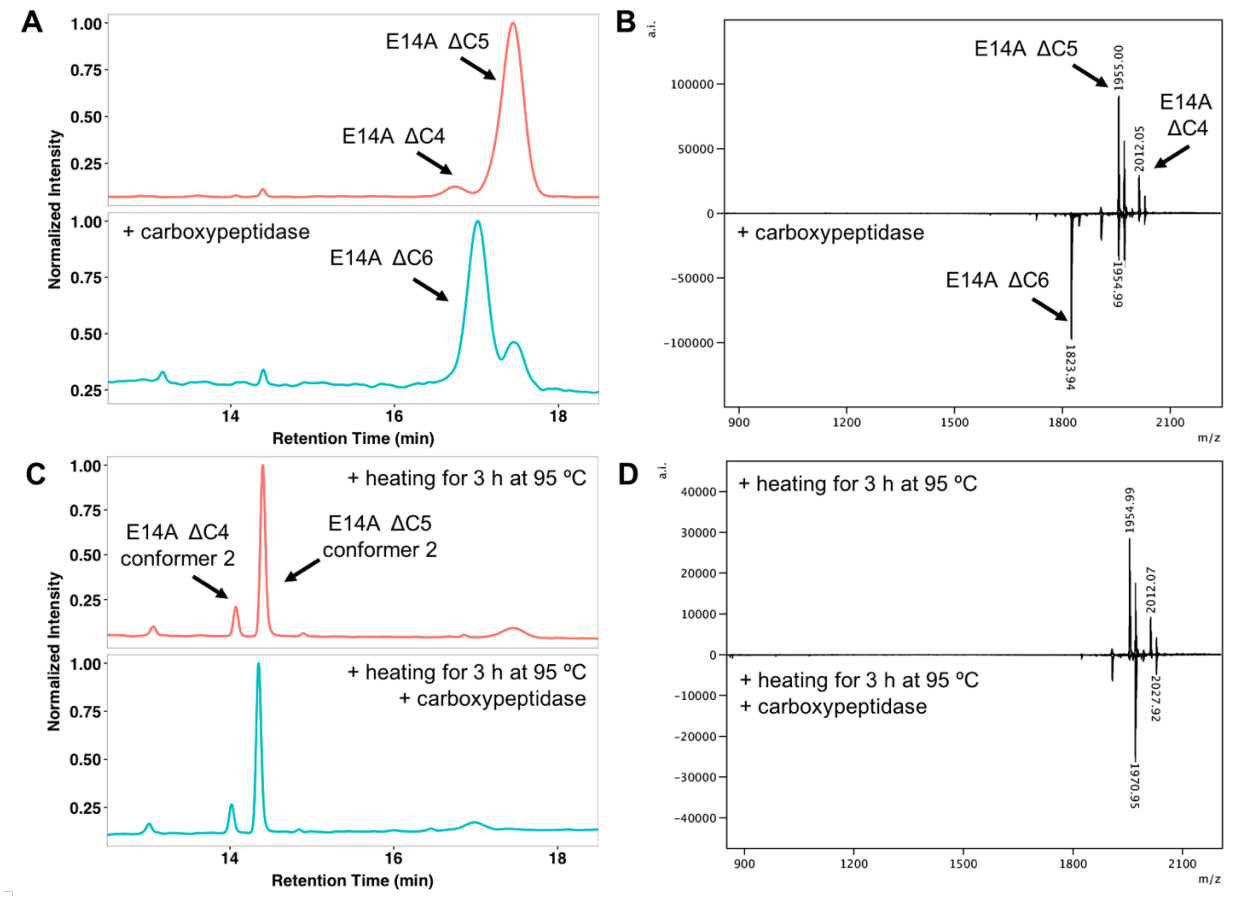


Figure S9: The E14A benenodin-1 ΔC5 variant switches to conformer 2 upon heating. A: E14A variants of benenodin-1 run as broad peaks on the HPLC (red trace). Carboxypeptidase treatment of the mixture of E14A benenodin-1 ΔC4 and E14A benenodin-1 ΔC5 results in a new peak (turquoise trace). B: Mass spectra of the mixture of E14A benenodin-1 ΔC4 and E14A benenodin-1 ΔC5 before (top spectrum) and after (bottom spectrum) treatment with carboxypeptidase. The major product after carboxypeptidase treatment is ΔC6 variant, suggesting that the peptide is still threaded. Theoretical masses (MH^+) of benenodin-1 ΔC4 E14A: 2012.03 Da, benenodin-1 ΔC5 E14A: 1955.01 Da, benenodin-1 ΔC6 E14A: 1823.97 Da. C: As in part A, but the mixture of E14A benenodin-1 ΔC4 and E14A benenodin-1 ΔC5 has been heated to 95 °C for 3 h. The peaks for E14A benenodin-1 ΔC4 and E14A benenodin-1 ΔC5 are sharpened (red trace) and carboxypeptidase does not cleave the peptide (turquoise trace), suggesting that these benenodin-1 variants are in a state resembling conformer 2. D: Mass spectra showing that carboxypeptidase does not cleave heated E14A benenodin-1 ΔC4 and E14A benenodin-1 ΔC5. The +16 Da adduct on the peptide is due to the oxidation of Met 19 of benenodin-1.

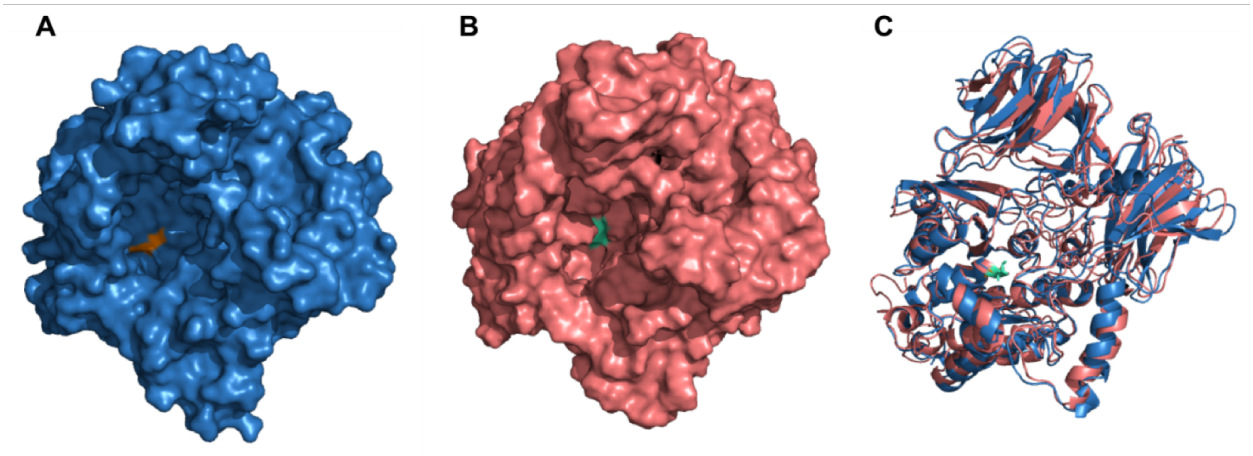


Figure S10: Homology modeling of the benenodin-1 isopeptidase BenE. A: Isopeptidase AtxE2, which hydrolyzes astexin-2 and astexin-3 as substrates. Drawn from PDB coordinates 5TXC. The active site serine is highlighted in orange at the bottom of the substrate cleft. B: Homology model of BenE derived from the coordinates of AtxE2. The active site serine is highlighted in green. The substrate cleft in BenE is smaller than that of AtxE2. C: Alignment of the crystal structure of AtxE2 with the homology model for BenE. The active site serine is shown as sticks. Figures generated using Pymol.

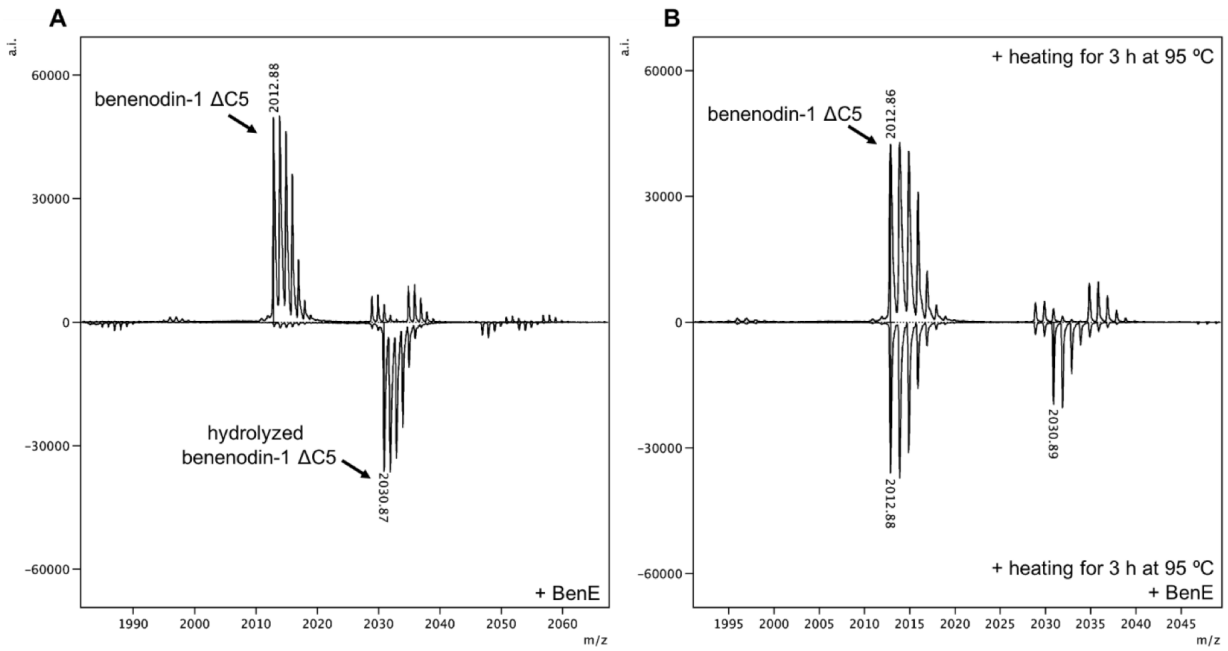


Figure S11: Mass spectra of isopeptidase cleavage reactions on benenodin-1 ΔC5. These spectra correspond to the HPLC traces in Figure 6A. A: Benenodin-1 ΔC5 conformer 1 is hydrolyzed completely by BenE. B: A mixture of the two conformers of benenodin-1 ΔC5 is only partially hydrolyzed by BenE.

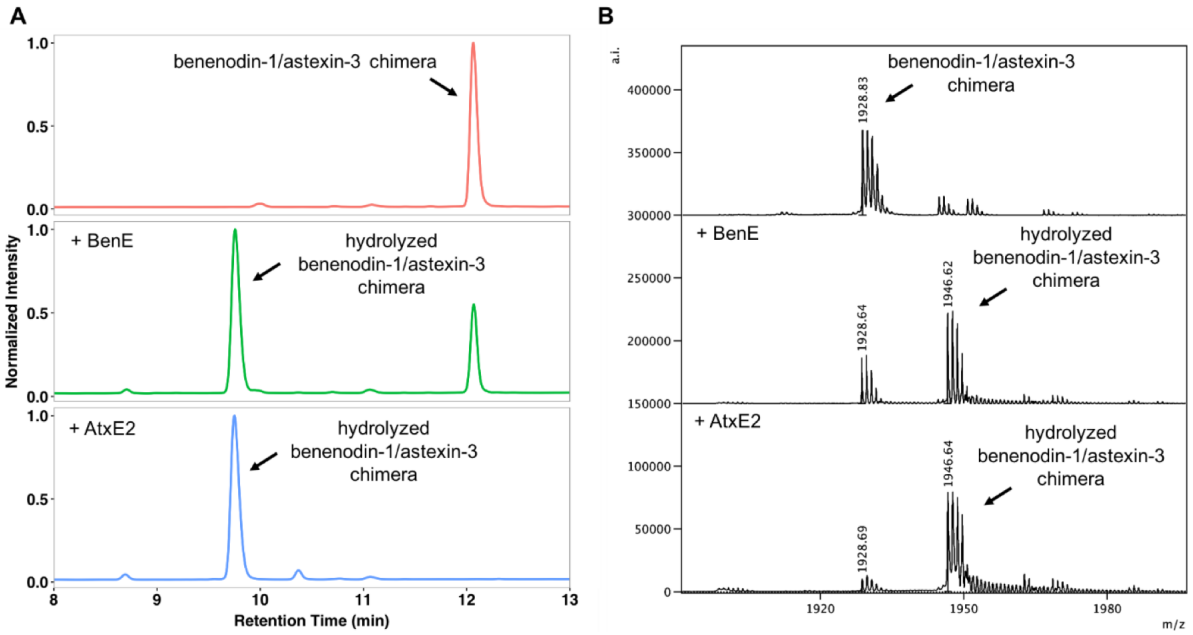


Figure S12: Hydrolysis of a benenodin-1/astexin-3 chimera by BenE and AtxE2. The chimera consists of the loop of astexin-3 grafted on to benenodin-1 ΔC5 (see Figure 6B). A: The intact lasso peptide (red trace) is cleaved partially by BenE over a 16 h reaction time (green trace) and is cleaved completely by AtxE2 (blue trace). B: Mass spectra corresponding to the HPLC traces in panel A. Theoretical mass of benenodin-1/astexin-3 chimera (MH^+): 1928.92.

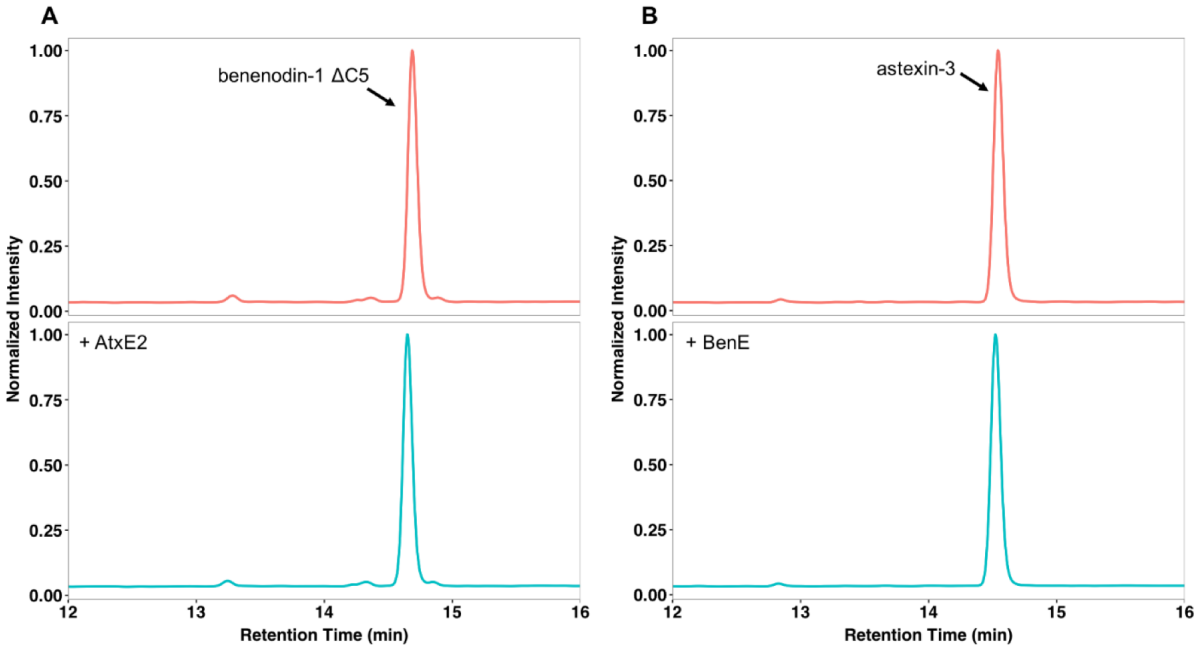


Figure S13: Control isopeptidase hydrolysis experiments. A: Benenodin-1 ΔC5 is not cleaved by AtxE2 within 16 h. B: Astexin-3 is not cleaved by BenE within 16 h. The enzyme concentration in these experiments is 100 nM and substrate concentration is 100 μM, conditions that lead to full hydrolysis of the correct substrate within 16 h.

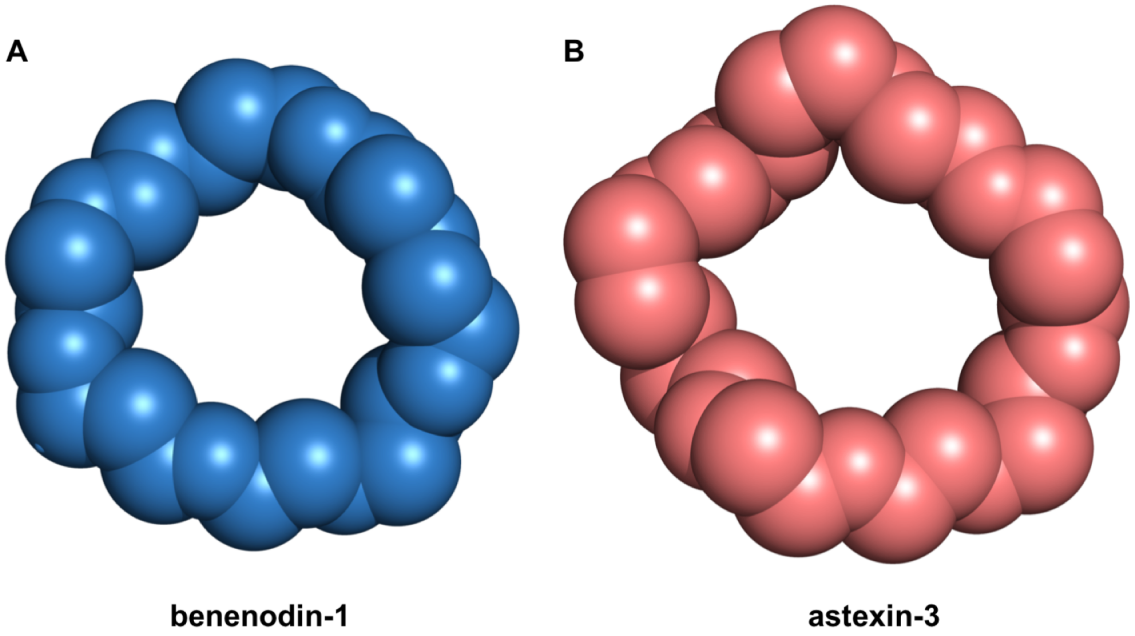


Figure S14: Comparison of the backbone of the isopeptide-bonded rings of benenodin-1 and astexin-3 (PDB: 2M8F). The ring sequences are GVGFGRPD for benenodin-1 and GPTPMVGLD for astexin-3 (see Figure 1B for full peptide sequences). Figures generated using Pymol.

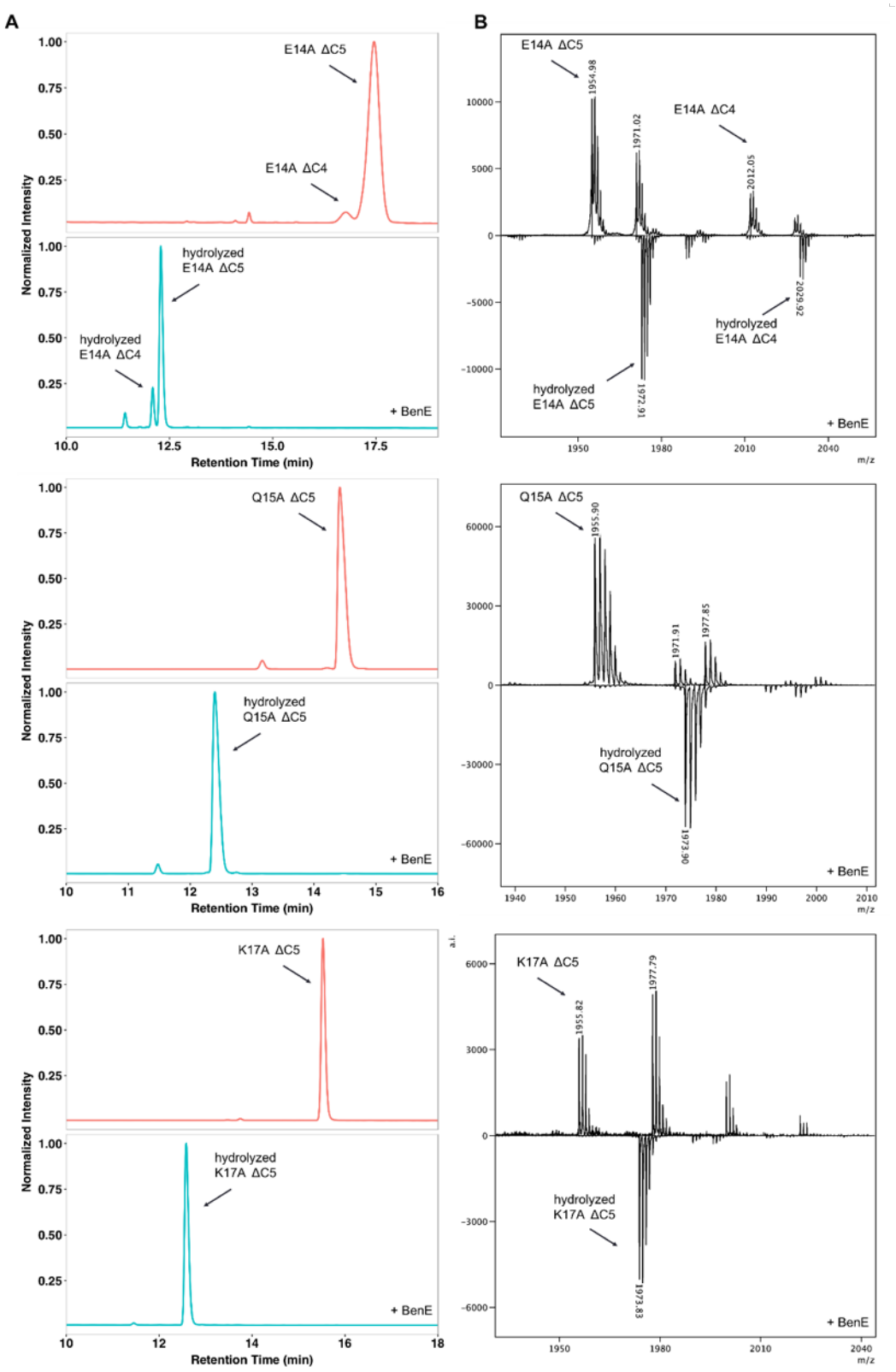


Figure S15: caption on following page

Figure S15: Isopeptidase reactions on benenodin-1 ΔC5 alanine variants. A: As produced by the bacteria in the native conformer 1 state, each of the three alanine variants E14A, Q15A, and K17A are hydrolyzed completely by BenE. B: Mass spectra of the HPLC traces in panel showing complete hydrolysis of the three alanine variants.

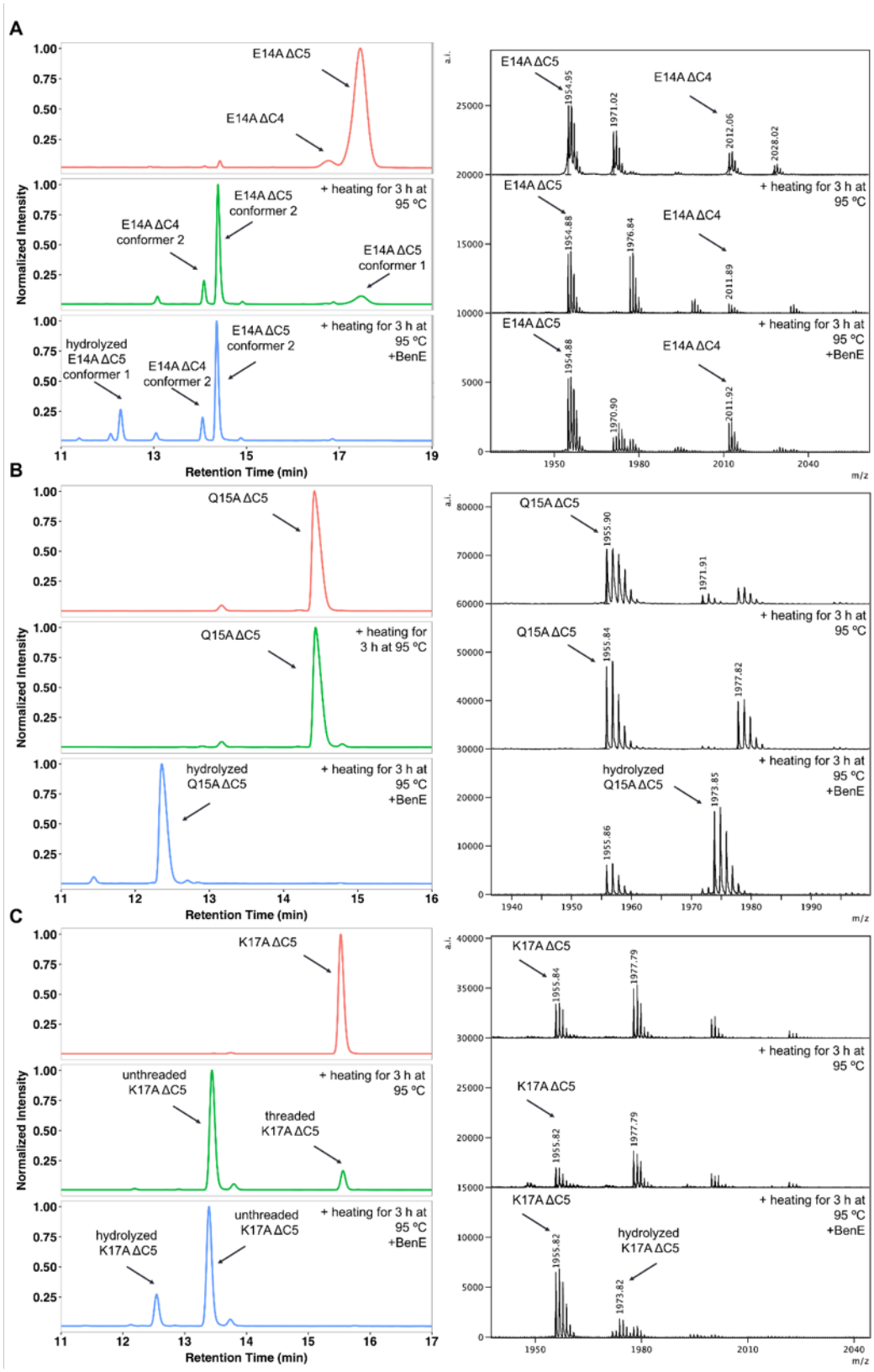


Figure S16: Caption on following page

Figure S16: Isopeptidase reaction on alanine variants of benenodin-1 after heating. A: After heating, E14A benenodin-1 switches to conformer 2 and is not cleaved by the isopeptidase. B: After heating, Q15A benenodin-1 remains in a conformer 1 state and is completely cleaved by the isopeptidase. C: After heating, most of K17A benenodin-1 has unthreaded and cannot be cleaved by the isopeptidase. A small fraction of the K17A variant remains threaded and is successfully hydrolyzed by the isopeptidase.

Table S1: Proton assignments for Conformers 1 and 2

Benenodin-1 ΔC5 Conformer 1		
Residue	**Hydrogen**	**Chemical Shift**
GLY-1	H	7.82
GLY-1	HA2	3.77
GLY-1	HA3	3.64
VAL-2	H	8.81
VAL-2	HA	4.11
VAL-2	HB	1.74
VAL-2	QQG	0.74
GLY-3	H	8.28
GLY-3	HA2	4.02
GLY-3	HA3	3.34
PHE-4	H	8.21
PHE-4	HA	4.47
PHE-4	HB2	2.84
PHE-4	HB3	2.51
PHE-4	QD	6.94
PHE-4	QE	7.12
PHE-4	HZ	7.09
GLY-5	H	7.67
GLY-5	HA2	4.42
GLY-5	HA3	3.35
ARG-6	H	8.62
ARG-6	HA	4.79
ARG-6	QB	1.57
ARG-6	HG2	1.21
ARG-6	HG3	1.19
ARG-6	HD2	3.06
ARG-6	HD3	2.91
ARG-6	HE	7.00
ARG-6	QH1	6.76
ARG-6	QH2	6.71
PRO-7	HA	4.39
PRO-7	HB2	2.33
PRO-7	HB3	1.65
PRO-7	HG2	1.93
PRO-7	HG3	1.78

Benenodin-1 ΔC5 Conformer 2		
Residue	**Hydrogen**	**Chemical Shift**
GLY-1	H	8.14
GLY-1	HA2	3.79
GLY-1	HA3	3.71
VAL-2	H	8.58
VAL-2	HA	4.20
VAL-2	HB	1.84
VAL-2	QQG	0.79
GLY-3	H	8.30
GLY-3	HA2	4.01
PHE-4	H	7.89
PHE-4	HA	4.56
PHE-4	QB	2.60
PHE-4	QD	7.19
PHE-4	QE	7.02
GLY-5	H	8.27
GLY-5	HA2	4.48
GLY-5	HA3	3.42
ARG-6	H	8.64
ARG-6	HA	4.82
ARG-6	HB2	1.70
ARG-6	HB3	1.61
ARG-6	QG	1.32
ARG-6	QD	3.07
ARG-6	QH1	6.95
ARG-6	QH2	6.95
PRO-7	HA	4.34
PRO-7	HB2	2.27
PRO-7	HB3	1.96
PRO-7	HG2	1.81
PRO-7	HG3	1.60
PRO-7	HD2	3.67
PRO-7	HD3	3.38
ASP-8	H	8.09
ASP-8	HA	4.11
ASP-8	QB	1.71

PRO-7	HD2	3.63
PRO-7	HD3	3.35
ASP-8	H	7.84
ASP-8	HA	4.37
ASP-8	HB2	3.08
ASP-8	HB3	1.59
SER-9	H	8.91
SER-9	HA	3.92
SER-9	QB	3.67
ILE-10	H	7.40
ILE-10	HA	3.98
ILE-10	HB	1.37
ILE-10	HG12	1.27
ILE-10	HG13	0.86
ILE-10	QG	0.70
ILE-10	QD	0.62
LEU-11	H	8.18
LEU-11	HA	3.96
LEU-11	QB	1.58
LEU-11	HG	1.43
LEU-11	QQD	0.76
THR-12	H	7.45
THR-12	HA	4.34
THR-12	HB	4.06
THR-12	QG	1.04
GLN-13	H	7.87
GLN-13	HA	4.50
GLN-13	QB	1.78
GLN-13	QG	2.15
GLN-13	HE21	7.34
GLN-13	HE22	6.71
GLU-14	H	8.72
GLU-14	HA	5.02
GLU-14	QB	1.85
GLU-14	HG2	2.40
GLU-14	HG3	2.13
GLN-15	H	9.03
GLN-15	HA	3.81
GLN-15	QB	1.14

SER-9	H	8.53
SER-9	HA	4.12
SER-9	HB2	3.79
SER-9	HB3	3.73
ILE-10	H	8.34
ILE-10	HA	4.07
ILE-10	HB	1.70
ILE-10	HG12	1.34
ILE-10	HG13	1.00
ILE-10	HG	0.78
ILE-10	HD	0.71
LEU-11	HA	4.31
LEU-11	QB	1.38
LEU-11	QQD	0.77
THR-12	H	7.60
THR-12	HA	4.20
THR-12	HB	4.10
THR-12	HG	1.11
GLN-13	QB	2.18
GLN-13	QG	2.27
GLN-13	HE21	7.74
GLN-13	HE22	6.71
GLU-14	H	7.89
GLU-14	HA	4.48
GLU-14	QB	1.88
GLU-14	QG	2.37
GLN-15	H	8.38
GLN-15	HA	4.96
GLN-15	HB2	2.08
GLN-15	HB3	1.91
GLN-15	QG	2.32
GLN-15	HE21	7.51
GLN-15	HE22	6.67
ALA-16	H	9.15
ALA-16	HA	3.84
ALA-16	HB	0.92
LYS-17	H	7.80
LYS-17	HA	4.57
LYS-17	HB2	1.85

GLN-15	HG2	1.82
GLN-15	HG3	1.68
GLN-15	HE21	7.27
GLN-15	HE22	6.76
ALA-16	H	7.71
ALA-16	HA	4.18
ALA-16	QB	1.28
LYS-17	H	8.08
LYS-17	HA	4.08
LYS-17	HB2	1.53
LYS-17	HB3	1.49
LYS-17	HG2	1.31
LYS-17	HG3	1.25
LYS-17	QD	1.66
LYS-17	QE	2.83
LYS-17	HZ	7.44
PRO-18	HA	4.21
PRO-18	QB	2.08
PRO-18	HG2	1.85
PRO-18	HG3	1.75
PRO-18	QD	3.43
MET-19	H	8.39
MET-19	HA	4.30
MET-19	HB2	1.98
MET-19	HB3	1.83
MET-19	HG2	2.46
MET-19	HG3	2.39
MET-19	QE	1.91

LYS-17	HB3	1.72
LYS-17	HG2	1.49
LYS-17	HG3	1.38
LYS-17	HD2	1.62
LYS-17	HD3	1.57
LYS-17	QE	2.89
LYS-17	HZ	7.43
PRO-18	HA	4.14
PRO-18	HB2	2.15
PRO-18	HB3	2.04
PRO-18	HG2	1.83
PRO-18	HG3	1.71
PRO-18	HD2	4.05
PRO-18	HD3	3.57
MET-19	H	8.27
MET-19	HA	4.29
MET-19	QB	2.02
MET-19	HG2	2.47
MET-19	HG3	2.41
MET-19	QE	1.85

A

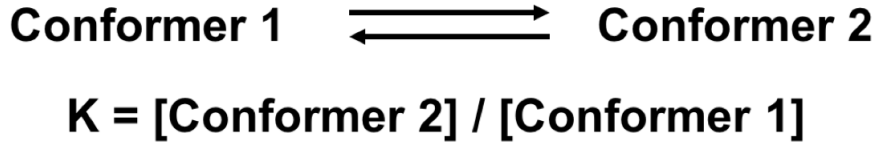
Assignment 1	Assignment 2	Interaction
1-GLY-H	14-GLU-H	Long
4-PHE-QD	14-GLU-HG2	Long
4-PHE-QE	14-GLU-HG2	Long
4-PHE-QE	14-GLU-HG3	Long
5-GLY-H	14-GLU-HA	Long
6-ARG-H	14-GLU-HA	Middle
7-PRO-HA	14-GLU-HA	Middle
8-ASP-H	14-GLU-HA	Middle
1-GLY-H	15-GLN-H	Middle
1-GLY-H	15-GLN-HA	Long
1-GLY-H	15-GLN-QB	Middle
1-GLY-HA2	15-GLN-QB	Middle
1-GLY-HA3	15-GLN-QB	Middle
2-VAL-H	15-GLN-QB	Middle
3-GLY-H	15-GLN-HA	Middle
4-PHE-HA	15-GLN-H	Middle
5-GLY-H	15-GLN-H	Middle
6-ARG-H	15-GLN-H	Middle
6-ARG-H	15-GLN-HG3	Long
6-ARG-HD2	15-GLN-HE21	Long
6-ARG-HE	15-GLN-HG3	Long
6-ARG-HG3	15-GLN-H	Middle
6-ARG-HG3	15-GLN-HE21	Middle
7-PRO-HA	15-GLN-H	Middle
8-ASP-HA	15-GLN-HE21	Middle
8-ASP-HA	15-GLN-QB	Long
2-VAL-H	16-ALA-H	Long
2-VAL-QQG	16-ALA-H	Long
3-GLY-HA2	16-ALA-H	Middle
4-PHE-H	16-ALA-HA	Middle
6-ARG-H	16-ALA-HA	Middle
7-PRO-HD3	16-ALA-HA	Long
5-GLY-HA2	17-LYS-H	Middle
6-ARG-H	17-LYS-HG3	Middle
6-ARG-HD2	17-LYS-HA	Middle
6-ARG-HD2	17-LYS-HG3	Middle
6-ARG-HD3	17-LYS-HA	Middle
6-ARG-HD3	17-LYS-HG3	Middle
6-ARG-HE	17-LYS-HA	Middle
6-ARG-HE	17-LYS-HG3	Middle
6-ARG-HG3	17-LYS-HA	Middle
6-ARG-QB	17-LYS-H	Long
6-ARG-QB	17-LYS-HA	Long

B

Assignment 1	Assignment 2	Interaction
2-VAL-QQG	14-GLU-H	Long
4-PHE-QD	15-GLN-HB3	Long
4-PHE-QD	15-GLN-QG	Long
4-PHE-QE	15-GLN-HB3	Long
5-GLY-H	15-GLN-HE22	Long
6-ARG-H	15-GLN-HA	Long
6-ARG-H	15-GLN-HB2	Long
6-ARG-H	15-GLN-HB3	Long
7-PRO-HA	15-GLN-HB2	Long
7-PRO-HB2	15-GLN-HB2	Middle
8-ASP-H	15-GLN-HA	Middle
1-GLY-H	16-ALA-QB	Middle
1-GLY-HA3	16-ALA-QB	Middle
2-VAL-HA	16-ALA-H	Long
5-GLY-H	16-ALA-QB	Long
6-ARG-H	16-ALA-QB	Long
6-ARG-HB2	16-ALA-QB	Long
6-ARG-QD	16-ALA-QB	Long
6-ARG-QG	16-ALA-QB	Middle
6-ARG-QH1	16-ALA-QB	Long
7-PRO-HA	16-ALA-QB	Long
7-PRO-HB2	16-ALA-QB	Long
2-VAL-QQG	17-LYS-H	Long
2-VAL-QQG	17-LYS-HG2	Middle
2-VAL-QQG	17-LYS-QE	Long
3-GLY-HA2	17-LYS-H	Middle
4-PHE-H	17-LYS-HA	Middle
4-PHE-H	17-LYS-HB2	Middle
4-PHE-H	17-LYS-HB3	Middle
4-PHE-H	17-LYS-HG3	Long
4-PHE-HA	17-LYS-H	Middle
4-PHE-QB	17-LYS-QE	Middle
4-PHE-QE	17-LYS-QE	Middle
5-GLY-H	17-LYS-HB2	Long
5-GLY-H	17-LYS-QE	Middle
6-ARG-H	17-LYS-H	Long
6-ARG-H	17-LYS-HA	Middle
6-ARG-H	17-LYS-QE	Long
6-ARG-HA	17-LYS-H	Long
6-ARG-QG	17-LYS-HD2	Middle
7-PRO-HB2	17-LYS-QE	Long

Middle Range Distance: 2.4 Å < d < 5.5 Å
Long Range Distance: 5.5 Å < d

Table S2: Lists of NOEs observed between ring residues of benenodin-1 ΔC5 and steric lock residues Glu-14 (blue), Gln-15 (yellow), Ala-16 (green), and Lys-17 (gray) for conformer 1 (A) and conformer 2 (B). Distance restraints are classified as in CYANA as medium range (2.4 – 5.5 Å) or long range (greater than 5.5 Å).



Temperature (° C)	k_1 (h^{-1})	k_2 (h^{-1})	K_{model}	$K_{observed}$
95	1.822	1.131	1.61	1.68
85	0.420	0.285	1.47	1.57
75	0.169	0.119	1.42	1.50
65	0.079	0.068	1.16	1.24
55	0.016	0.020	0.81	0.80

Table S3: Kinetic data of benenodin-1 conformational switching. Data were fit to a two-state model (see Methods for details) to determine k_1 and k_2. K_{model} was calculated as k_1/k_2. The experimental values ($K_{observed}$) as presented for comparison to K_{model}.