Electronic Supplementary Information

Structural Determinants of Macrocyclization in Substrate-Controlled Lanthipeptide Biosynthetic Pathways

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KEYWORDS. RiPP, lanthipeptide, toxin, hemolytic peptide, cyclic peptide

NOTE: The original ESI associated with this study has been updated based on a published Correction. After publication we became aware that the custom written definition of the non-proteinogenic bis-amino acid methyllanthionine (MeLan) for use by the software package Xplor does not sufficiently define the stereochemistry at C3. As a result, a subset of the seven structures contained a single MeLan with incorrect stereochemistry at C3. We rewrote the definition as shown here in this current revised ESI file, redid all calculations using the original NMR data using the new definition, and manually inspected all 20 lowest energy structures to make sure they all had the correct stereochemistry of MeLan. All of the interactions observed in the original publication were still observed and none of the original conclusions were affected. We replaced the Protein Data Bank (PDB) files for prochlorosins 1.1 and 2.1 and cytolysin L using the same PDB identification numbers as in the original publication (6VHJ, 6VJQ, and 6VGT, respectively). For prochlorosins 2.10 and 2.11, we deposited the new structures with a new PDB ID (7JVF and 7JU9, respectively) and rendered the old pdb files obsolete. The following figures in the ESI changed subtly in the new structures and were replaced in this version of the ESI: S5, S6, S12, S13, S17, S18, S22, S23, S37, S38, S39. The following Tables were updated to reflect the new structures: Tables S2, S4, S6, S8, and S14.

Definition of methyllanthionine for Xplor-NIH

In our previous MeLan definition, a new bond was created in Xplor by removing a beta proton (HB1) from L-Abu or D-Abu, and then forming a new bond with the Cys sulfur. The patch command then created a new bond. Our previous patch command (which we termed ABUS) allowed formation of either stereochemistry at the beta carbon (CB) of methyllanthionine, because the definition did not specify an improper dihedral angle around CB. Therefore, a subset of MeLan in some structures ended up with incorrect stereochemistry at C3.

This problem was corrected by declaring an additional improper dihedral angle at CB for the two known stereochemical configurations of MeLan in prochlorosins and cytolysins. Thus, instead of one patch command we used two patch commands, one for each known stereochemistry (the Xplor declaration of this angle uses "improper") as shown below.

The patch command ABUS ensures that the stereochemistry at CB of L-Abu is (*R*) by adding one line:

```
improper 1HB2 1CA 2SG 1CG      !stereo CB
```

The patch command ABS ensures that the stereochemistry at CB of D-Abu is (S) by adding one line:

```
improper 1HB2 2SG 1CG 1CA      !stereo CB
```

In addition, these two angles were also defined in the parameter file as follows:

```
improper  HA   CT   S   CT      $kchi    0   65.977              ! CB chirality; for L-Abu in CyllL/S of
ABUS linker
improper  HA   S   CT   CT      $kchi    0   -65.977             ! CB chirality; for D-Abu of ABS linker
```

We provide two new files for the edited .top and .par files in the Xplor-NIH 2.51 base package (Updated_protein-3.2.top and Updated_protein-3.2.par).

Figure S1. MALDI ToF mass spectra of the five prochlorosins modified by ProcM in *E. coli* followed by *in vitro* proteolytic leader peptide removal. Pcn1.1 calculated 1783.8 Da, observed 1783.8 Da; Pcn 2.1 calculated 2750.1 Da, observed 2750.6; Pcn 2.8 calculated 2050.8 Da, observed 2050.9 Da; Pcn 2.10 calculated 2021.0 Da, observed 2021.0 Da; Pcn 2.11 calculated 1823.7 Da, observed 1823.9 Da.

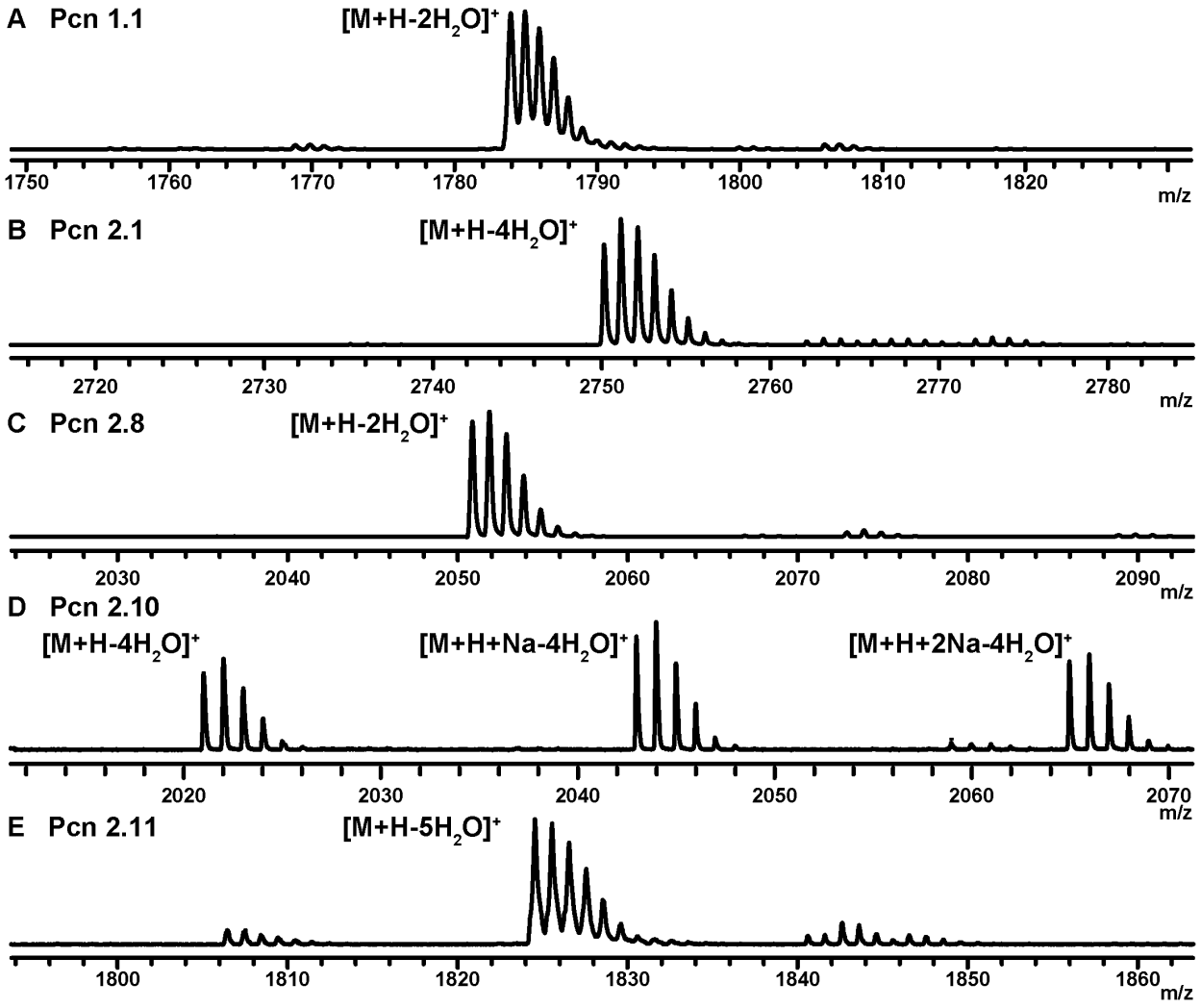

Table S1. Chemical shift assignments for Pcn 2.10 in 90% H_2O/10% D_2O. Cysteine residues involved in thioether linkages are colored in blue, formerly dehydrated residues involved in thioether linkages are colored in red.

	NH	αH	βH	γH	Hδ+other protons / notes
1 Ala	-	4.193	1.580	-	-
2 Gly	8.589	4.088, 4.05	-	-	-
3 Gly	8.335	4.100	-	-	-
4 Dhb	9.247	-	6.585	1.759	-
5 Ile	7.662	4.535	1.925	0.9601 1.185, 1.544	Hδ: 0.874
6 Pro	-	4.432	2.37, 1.998	2.12, 2.029	Hδ (D_2O):3.982, 3.761
7 Ala	8.506	4.342	3.125, 3.07		
8 Leu	7.725	4.319	1.688	1.634	Hδ: 0.924, 0.887
9 Met	8.167	4.547	2.217, 2.048	2.560	
10 Abu	7.764	4.722 overlap with Trp14 Hα	3.550	1.272	-
11 Gly	8.369	4.139, 3.770	-	-	-
12 Cys	7.841	4.480	2.791, 2.700	-	NOE from Hβ to 3.125 and 3.07 ppm (Ala7 Hβ)
13 Gly	8.44	3.896, 3.811	-	-	
14 Trp	7.75	4.736 overlap with Abu10 Hα	3.393, 3.315	-	Hε: 10.14, 7.60 Hδ: 7.237 Hζ: 7.46, 7.14 Hη:7.207
15 Leu	7.864	4.346	1.62, 1.516	1.230	Hδ: 0.84, 0.76
16 Dhb	8.880	--	6.645	1.750	-
17 Gly	7.945	3.973,3.818	-	-	-
18 Leu	7.795	4.344	1.715, 1.605	1.660	Hδ: 0.924, 0.860
19 Cys	8.031	4.580	3.062, 2.729	-	NOE from Hβ to 3.55 and 1.27 ppm (Abu10 Hβ and Hγ)

Table S1 (continued). Chemical shift assignments for Pcn 2.10 in 90% H_2O/10% D_2O.

20 Val	7.847	4.149	2.076	0.9307, 0.896	
21 Arg	8.205	4.371	1.921,1.778	1.634	Hδ:3.20 Hη: 7.150, 6.635

Figure S2. Water-suppressed 70 ms TOCSY spectrum identifying all spin systems of Pcn 2.10.

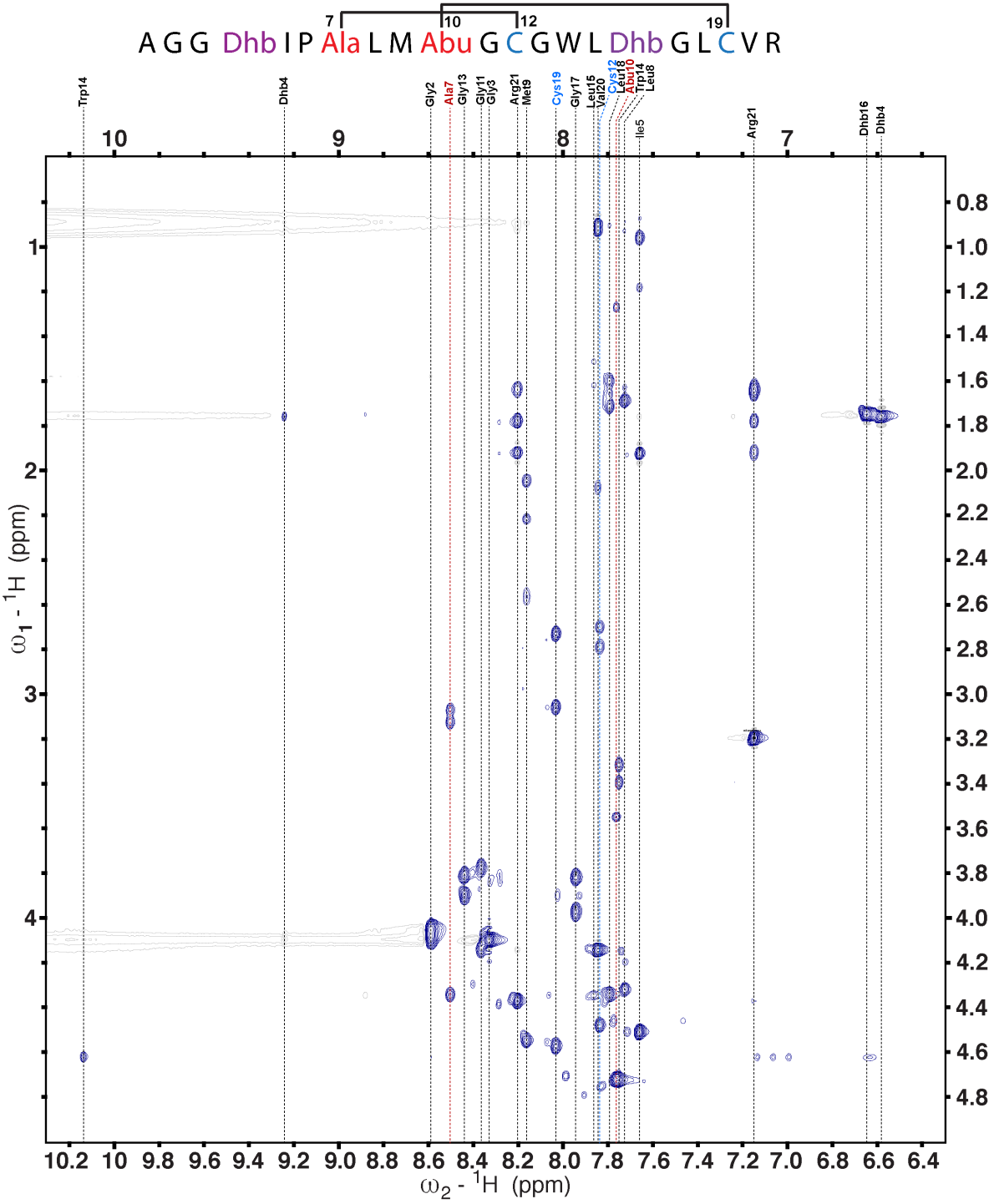

Figure S3. Water-suppressed NOESY spectrum (mixing time = 0.30 s) for amide resonance assignments to amino acids from Pcn 2.10. E.g. "5-4" designates the cross peak between the amide proton of residue 5 at 7.66 ppm and the amide proton of residue 4 at 9.24 ppm.

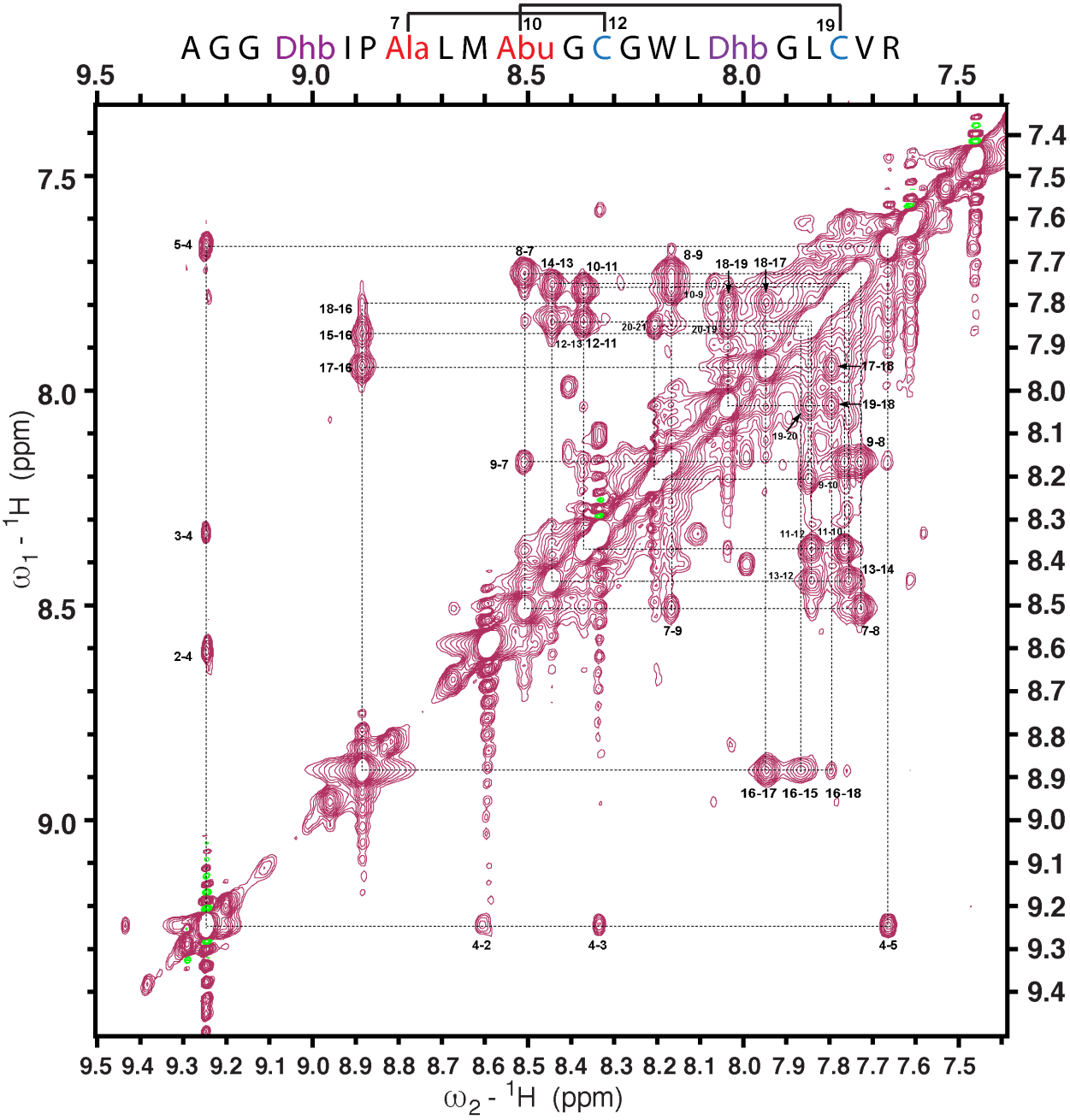

Figure S4. NOESY spectrum of Pcn 2.10 in D_2O (mixing time = 0.30 s) for ring pattern assignment. The nOe cross peaks for ring assignments are labeled on the axes. (A) Structure of Pcn 2.10. (B) Full NOESY spectrum of the aliphatic region relevant to ring assignment.

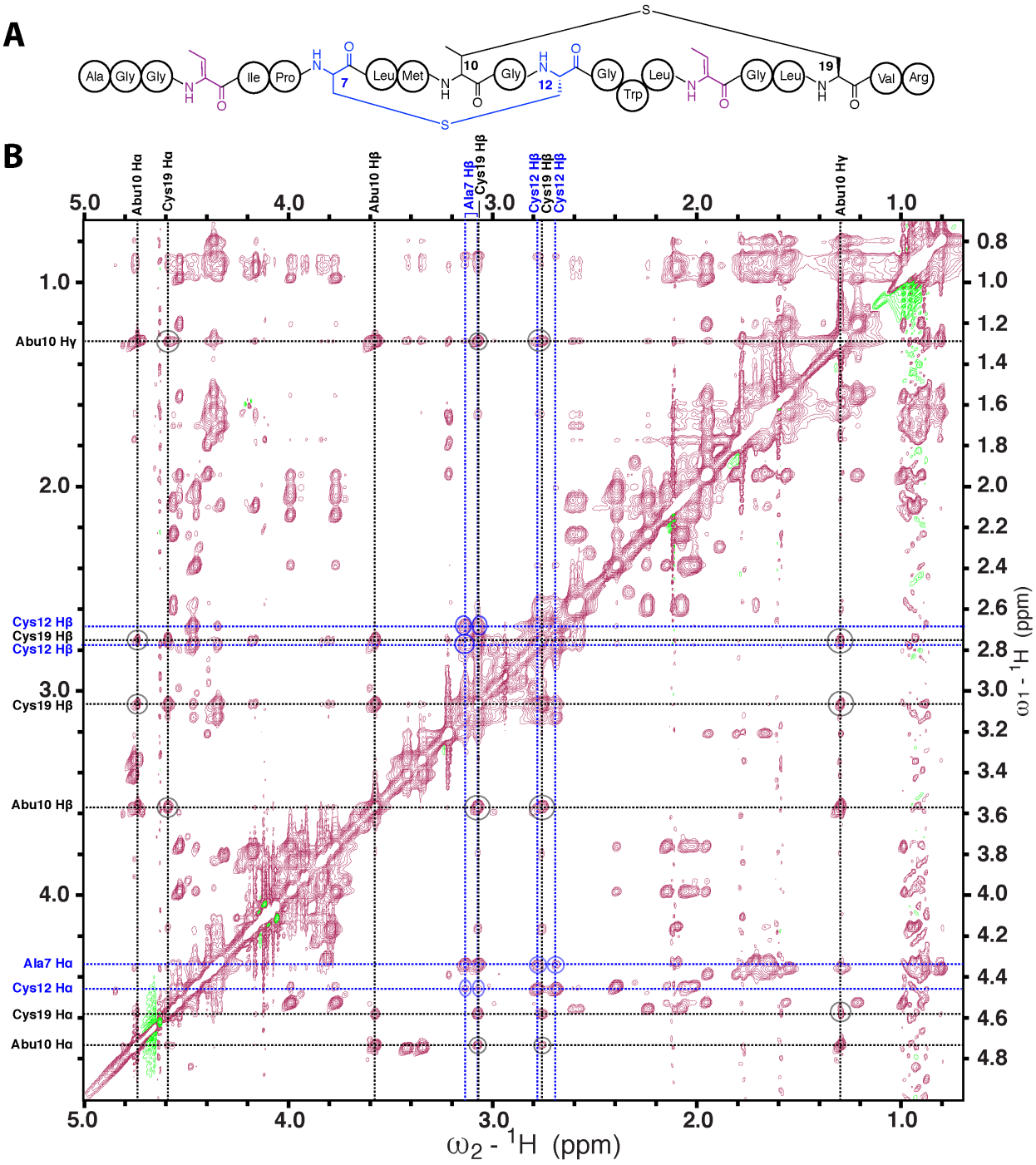

Figure S4 (continued). (C) Diagnostic nOe cross peaks for the lanthionine ring formed between former Ser7 and Cys12 are marked in blue. (D) Diagnostic nOe cross peaks for the methyllanthionine formed between former Thr10 and Cys19 are marked in black.

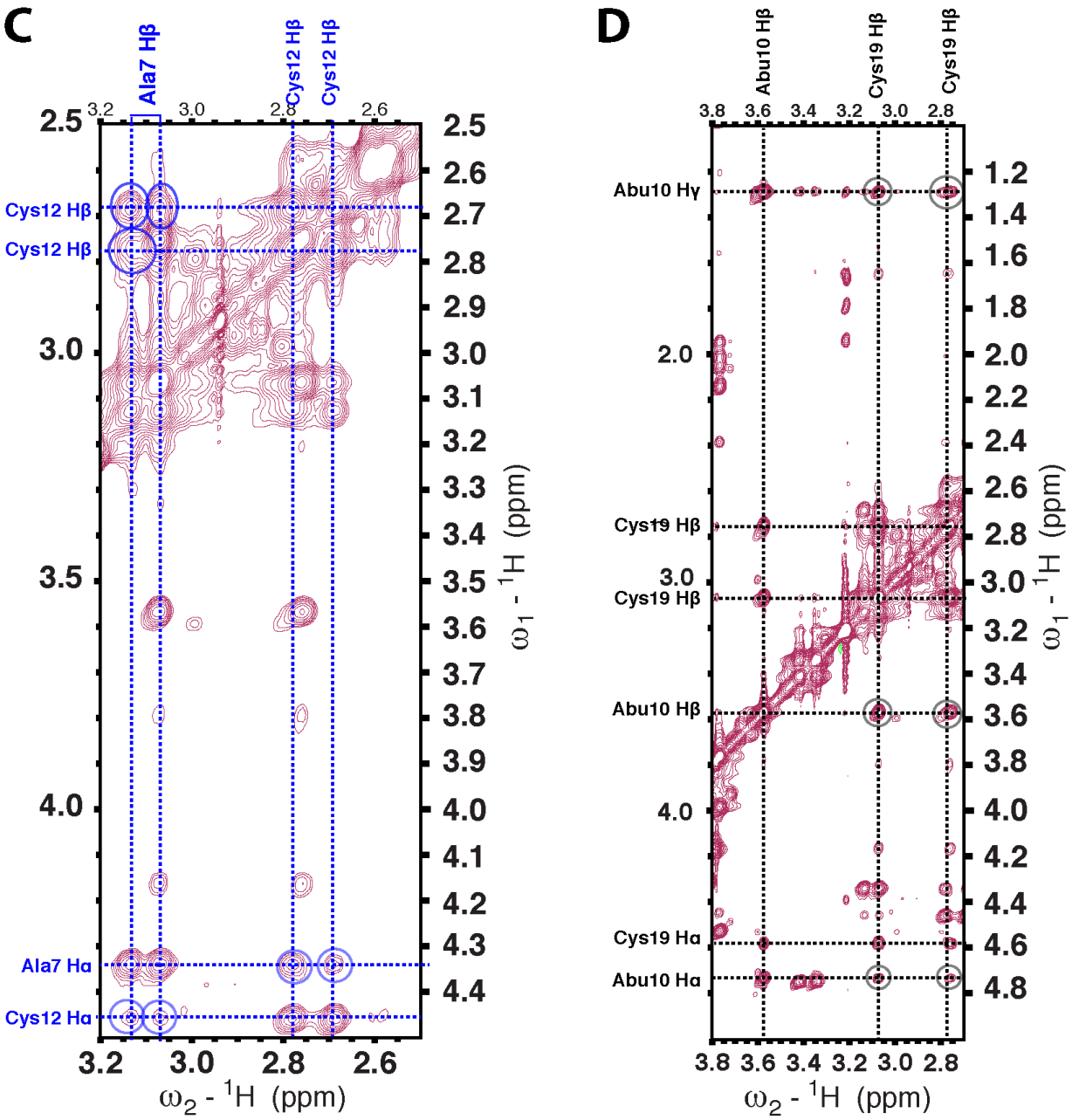

Figure S5. Ramachandran plots for Pcn 2.10. Residues in disallowed areas are predominantly the D-amino acids parts of the thioether rings, cysteines involved in ring formation, and dehydroamino acids. For Pcn 2.10, the D-stereochemistry is assumed. For Pcn 1.1, 2.8, and 2.11 (as well as three other prochlorosins) the D-stereochemistry has been experimentally verified.[1]

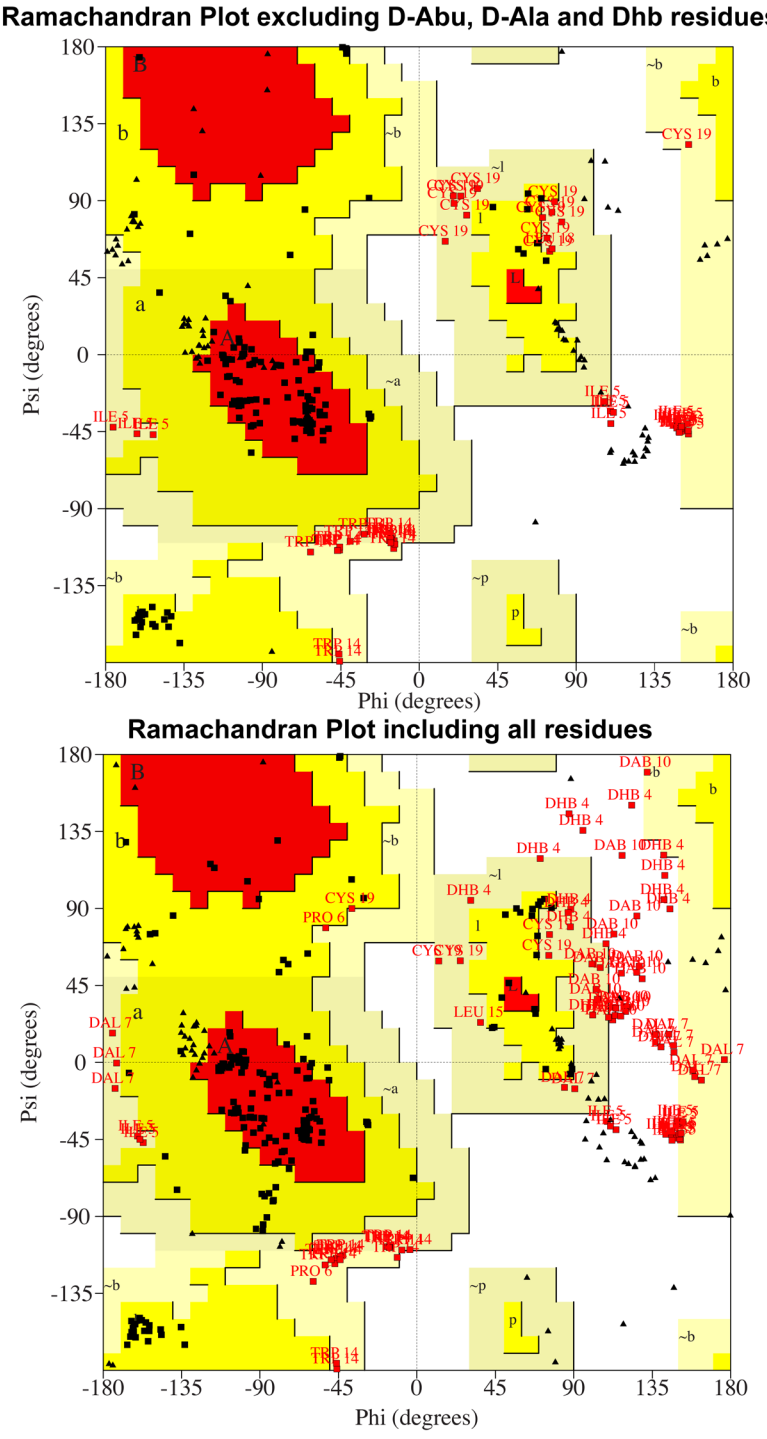

Table S2. Structural statistics for Pcn 2.10.

Procheck Analysis	**All L residues (%)**	**All residues (%)**
Residues in most favored regions [A,B,L]	48.3	35.8
Residues in additional allowed regions [a,b,l,p]	23.3	25.8
Residues in generously allowed regions [~a,~b,~l,~p]	23.3	24.6
Residues in disallowed regions	5.0	13.8
RMSD		
Residues	**Backbone only (Å)**	**All atoms (Å)**
1 to 21	1.23	1.48
Ring A (7 to 12)	0.41	0.92
Ring B (10 to 19)	0.58	0.93
1 to 6	1.94	1.83
Restraint Types Used		
Intra residue nOe	18	
Sequential nOe (|i-j|=1)	78	
Medium range nOe (1<|i-j|≤4)	74	
Long range nOe (|i-j|>4)	58	
Total	228	

Figure S6. Superimposition of the 3D structures of the 20 lowest energy conformers of Pcn 2.10. (A) Loop representation of main chain and thioether linkages between residues 7-12 and 10-19. (B) Representation of the interaction between residue Leu15 and residue Trp14 resulting in upfield shift of the γ, δ protons of Leu15. Trp14 is partially buried by the rest of the peptide. (C) Representation of Leu8 as spheres model (colored in deep teal) shows it is almost fully buried, flanked by the flexible N-terminal region and the thioether-cyclized region spanning residues 7-19.

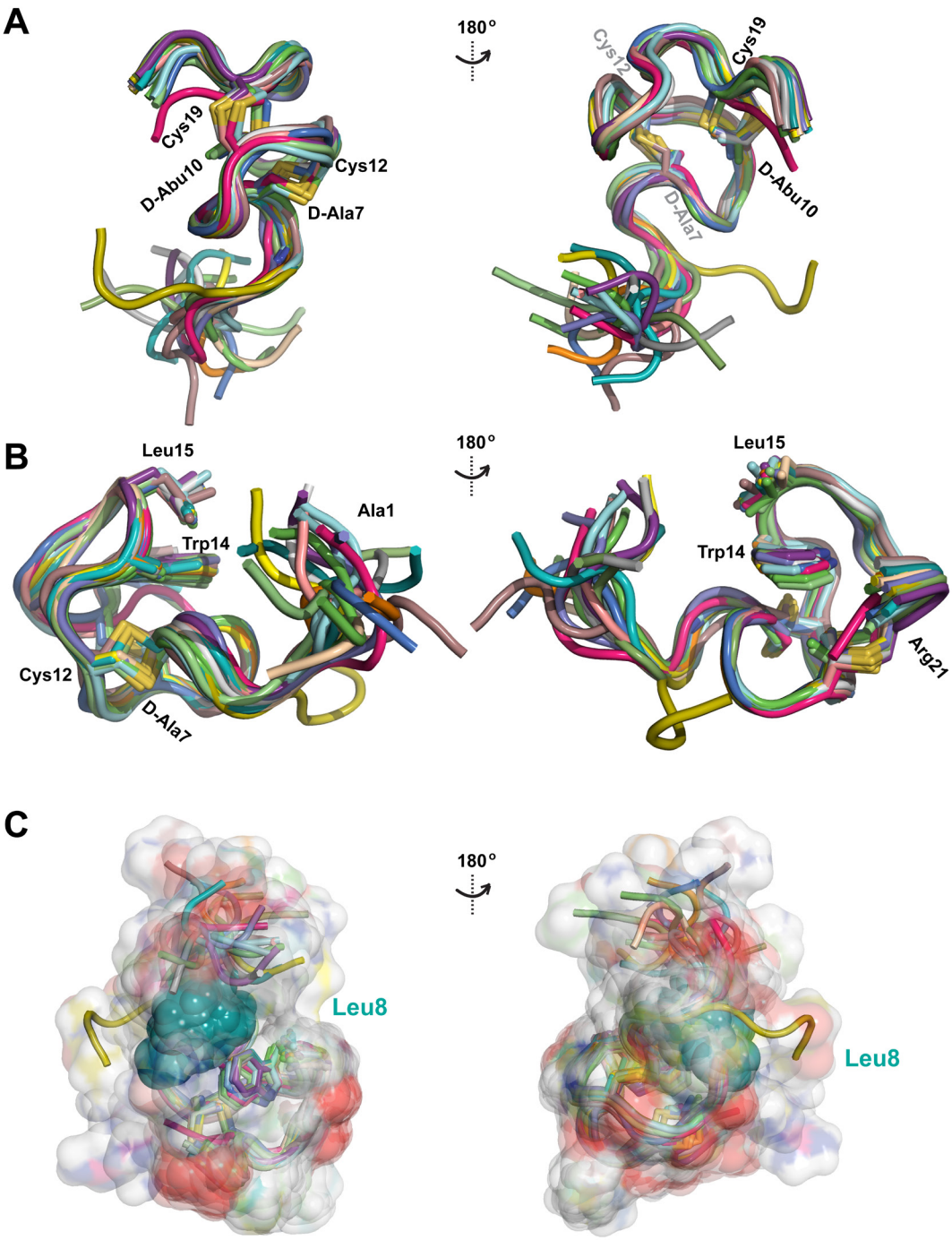

Table S3. Chemical shift assignments for Pcn 2.1 in 90% H_2O/10% D_2O. Cysteine residues involved in thioether linkages are colored in blue, formerly dehydrated residues involved in thioether linkages are colored in red.

	HN	αH	βH	γH	Hδ+other protons	Notes
2 Cys	8.96	4.799	3.022,2.918	-	-	-
3 Ile	9.227	3.912	2.240	1.540, 1.159	0.880, 0.993	-
4 Abu	7.846	4.332	3.720	1.350		NOE from Hβ to 3.150, 3.250 (Cys1Hβ)
5 Gly	8.460	4.291, 3.944	-	-	-	-
6 Glu	8.671	4.556	1.907	2.290, 2.460	-	-
7 Ser	8.044	4.770	3.910, 3.940	-	-	-
8 Pro	-	4.463	2.319	1.980, 2.060	3.790, 3.890	-
9 Gly	8.438	3.960	-	-	-	-
10 Ala	7.908	4.541	2.980, 3.080	-	-	NOE from Hα to 3.022, 2.918 (Cys2Hβ)
11 Ala	8.525	4.545	1.347	-	-	-
12 Pro	-	4.507 overlap with Abu19HA	2.308, 1.890	2.014, 2.060	3.796, 3.608	-
13 Abu	8.575	4.744	3.835	1.260	-	NOE from Hβ to 3.125(Cys18Hβ)
14 Asn	8.413	4.947	2.905, 2.590		Hδ:7.406, 6.875	-
15 Asp	7.578	4.848	3.296, 2.797	-	-	-
16 Tyr	8.555	4.803	3.035, 2.858		Hδ: 7.11; Hε: 6.830	-
17 Lys	7.322	3.871	1.770, 1.673	0.993, 0.909	Hδ: 1.570 Hε: 2.950 Hζ: 7.536	-
18 Cys	7.958	4.683	3.125	-	-	NOE from Hβ to 3.83 (Abu 13)
19 Abu	7.699	4.504	3.487	1.321		NOE from Hγ to 2.75,2.50 (Cys27Hβ)
20 Lys	7.730	4.562	1.915, 1.511	1.474, 1.307	Hδ: 1.725 Hε:3.020 Hζ: 7.520	-
21 Gly	8.248	4.158, 3.857	-	-	-	
22 Arg	8.284	4.645	1.868, 1.683	1.606, 1.544	Hδ: 3.168; Hη: 7.168	-
23 Gly	8.264	3.920, 4.179	-	-	-	-
24 Pro	-	4.369	2.289, 1.947	2.000, 2.060	Hδ: 3.604	-
25 Gly	8.678	4.102, 3.902	-	-	-	-
26 Gly	8.298	4.300, 3.626	-	-	-	-
27 Cys	7.874	4.387	2.750, 2.500	-	-	-
28 Tyr	8.147	4.587	3.176, 2.857	-	Hδ: 7.087; Hε: 6.844	-

Figure S7. Water-suppressed 70 ms TOCSY spectrum identifying the spin systems of Pcn 2.1. (A) Amide region of the spectrum of the sample in 90% H_2O/10% D_2O.

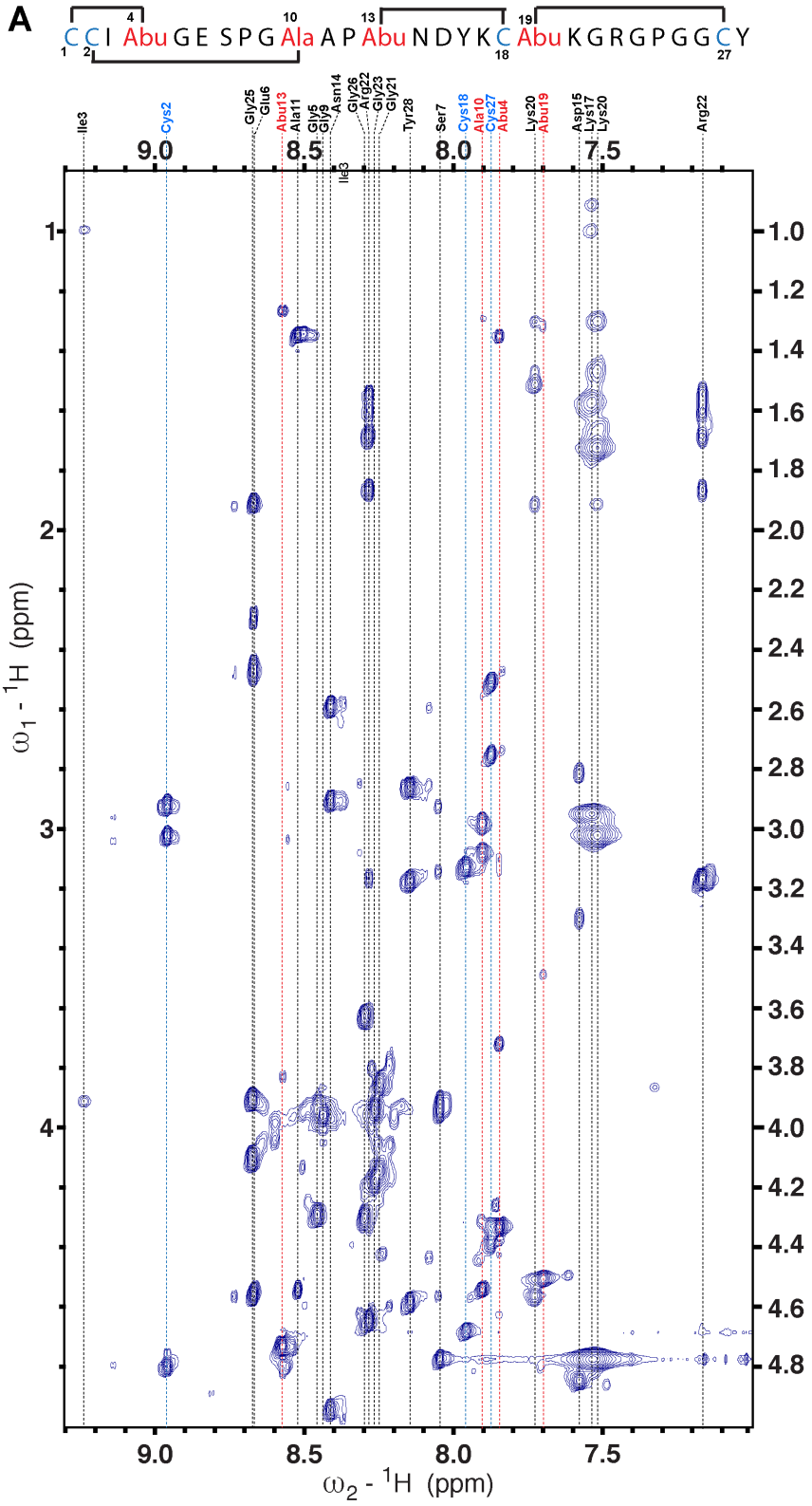

Figure S7 (continued) (B) Aliphatic region of the water-suppressed 70 ms TOCSY spectrum recorded in D_2O for Pcn 2.1. Chemical shifts for the Lys17 sidechain are more upfield than shifts coming from a random coil lysine sidechain suggesting this residue is experiencing a shielding effect.

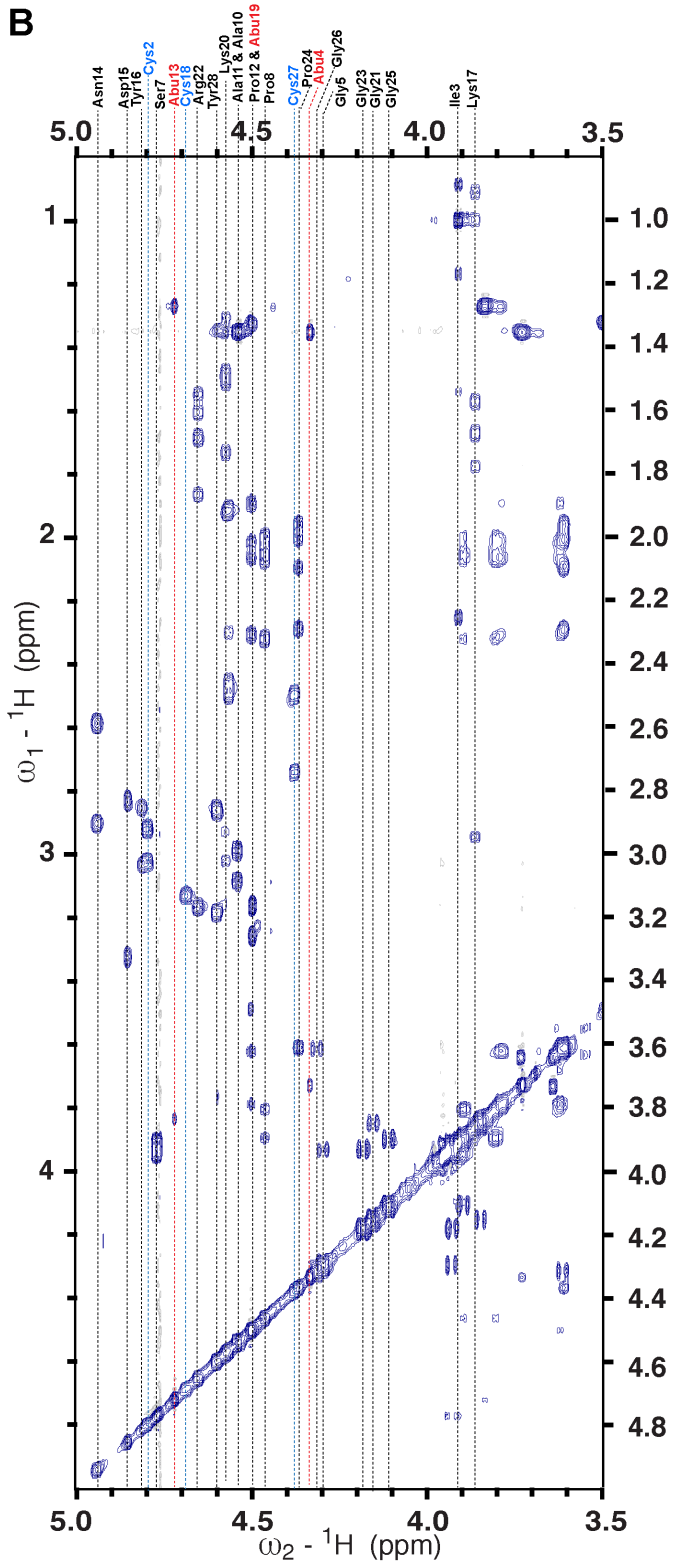

Figure S8. Water suppressed NOESY spectrum (mixing time = 0.30 s) for amide resonance assignments to amino acids from Pcn 2.1.

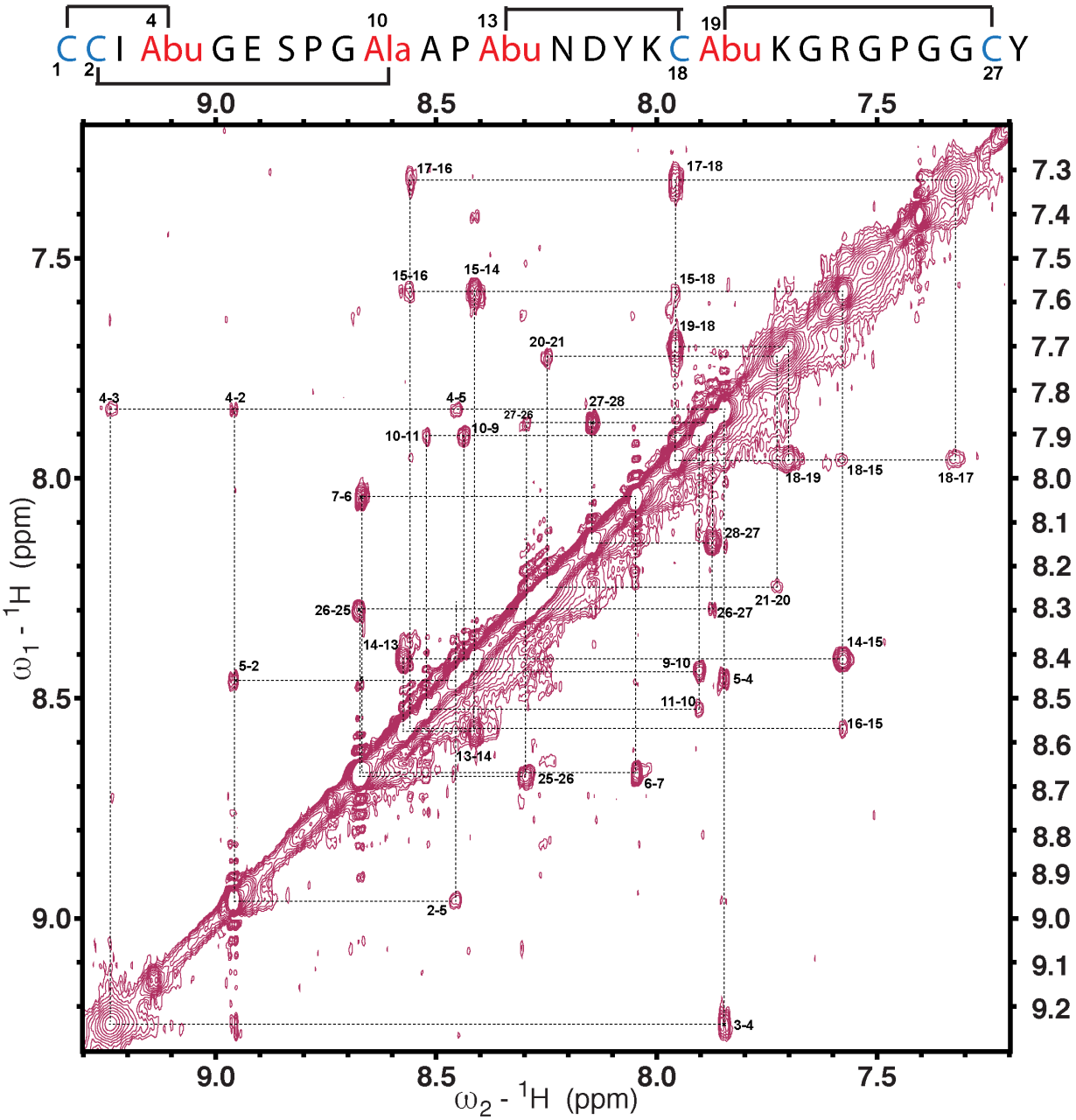

Figure S9. Water-suppressed NOESY spectrum (D_2O, mixing time = 0.30 s) for establishing connectivity of Pro residues of Pcn 2.1. TOCSY contours are shown in navy blue, NOESY contours are shown in maroon. The assignments related to the proline residues are the only ones marked. (A) Structure of Pcn 2.1. (B) nOe between Pro8 and Ser7. (C) nOe cross peaks establishing connectivity between Pro12-Ala11 and Pro12-Abu13.

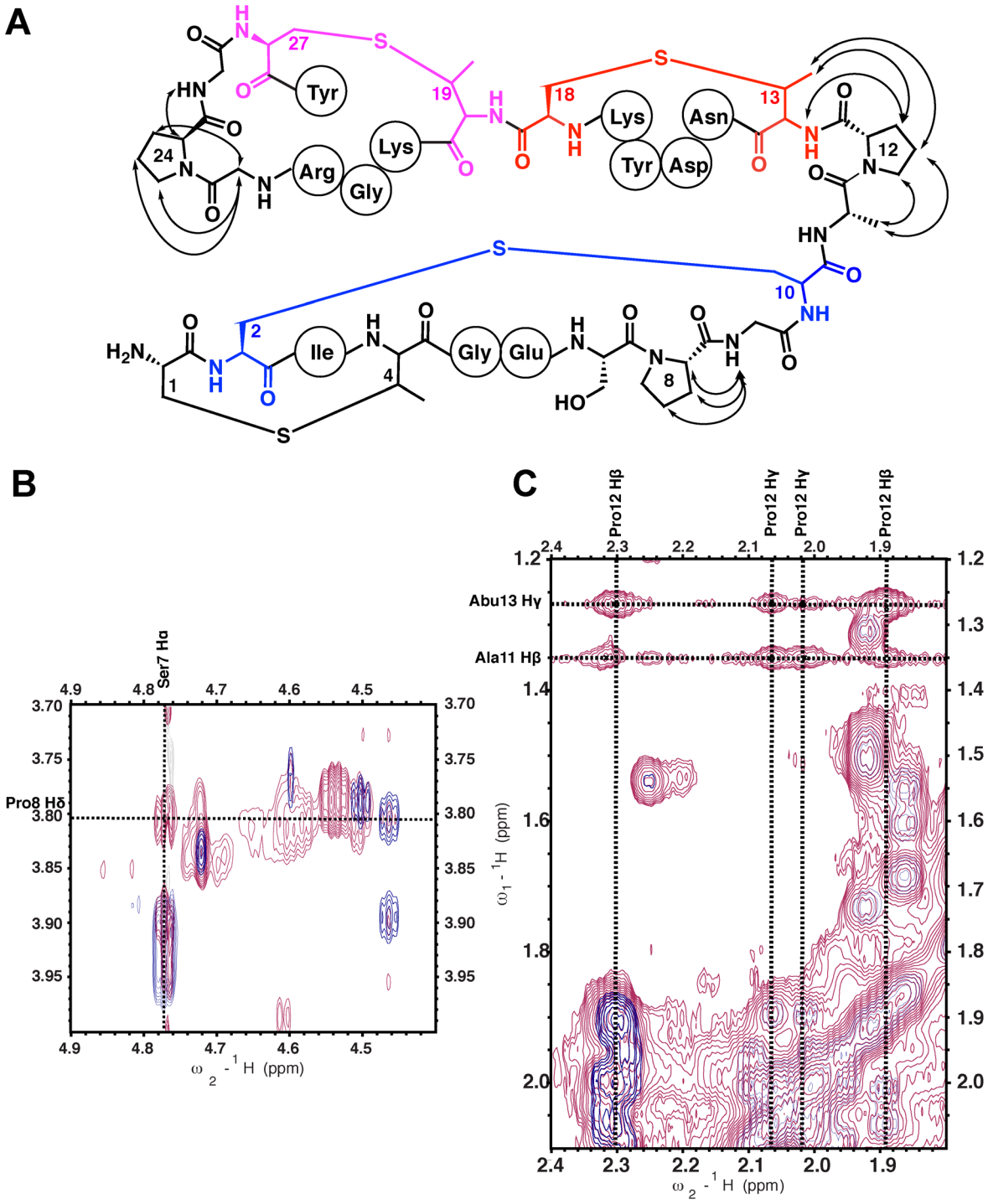

Figure S9 (continued) (D) nOe crosspeaks establishing connectivity between Pro12-Abu13 and Pro8-Gly9 in Pcn 2.1.

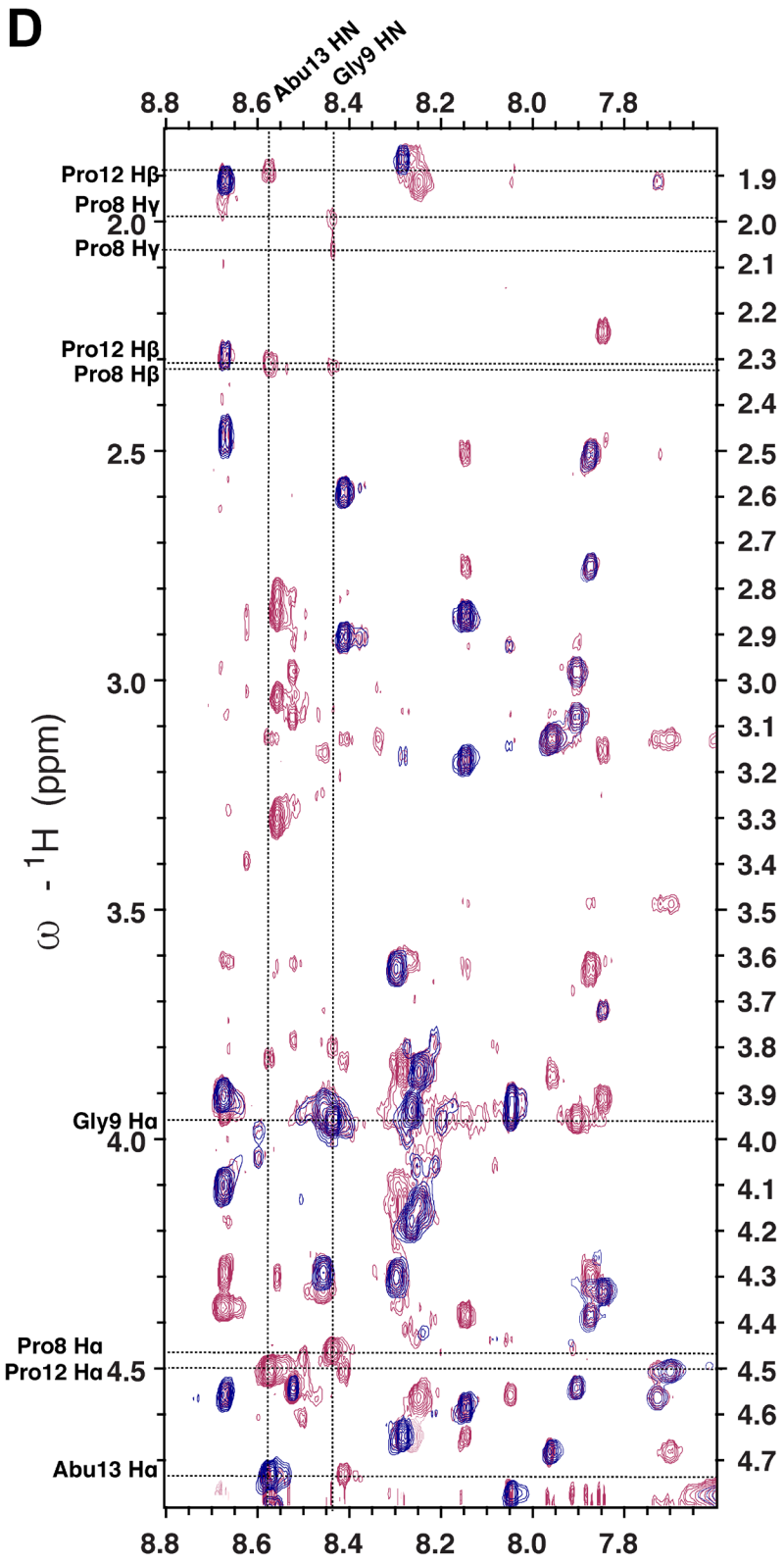

Figure S9 (continued). (E) nOe cross peaks establishing connectivity between Pro24-Gly23 in Pcn 2.1.

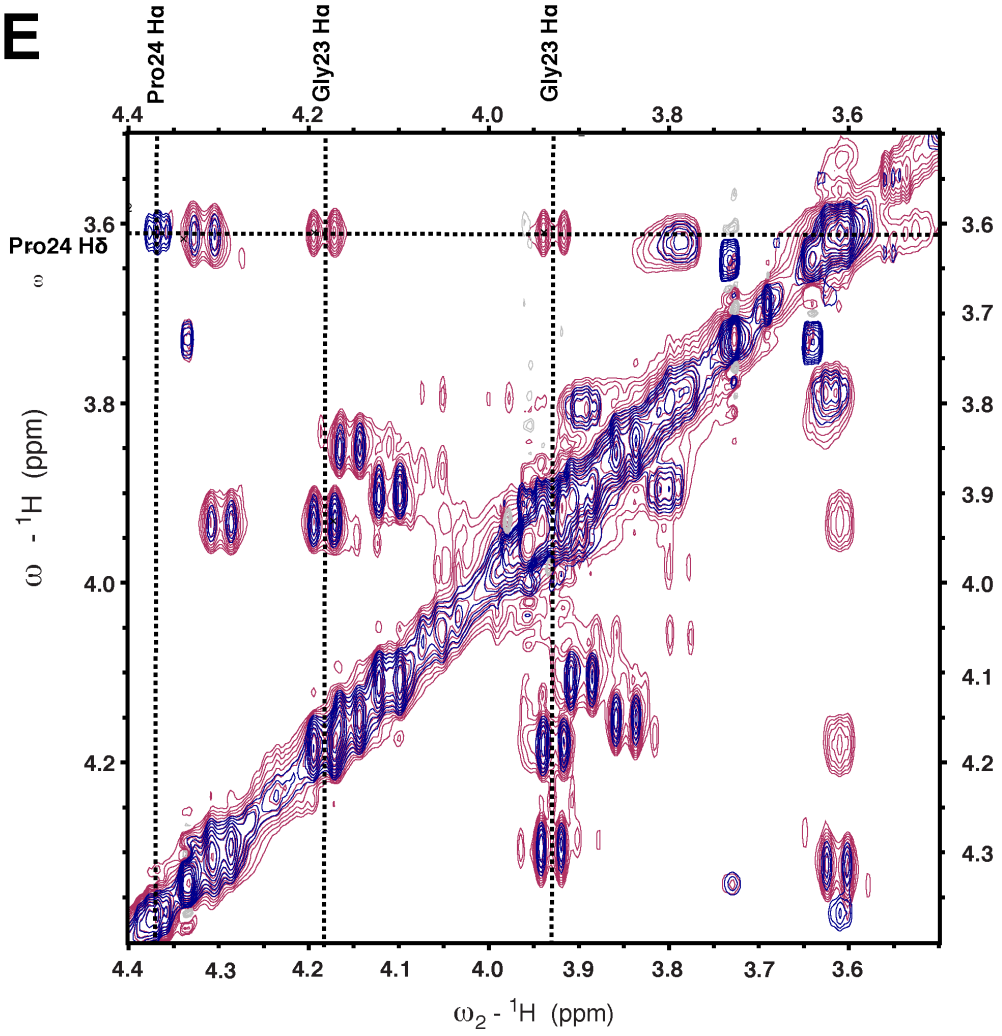

Figure S10. Water-suppressed NOESY spectrum of Pcn 2.1 (D_2O, mixing time = 0.30 s) for ring pattern assignments. TOCSY contours are shown in navy blue. NOESY contours are shown in maroon. Methyllanthionine linkage between former residues Cys1 and Thr4 is colored in black. Lanthionine linkage between former residues Cys2 and Ser10 is colored in blue. Methyllanthionine linkage between former residues Thr13 and Cys18 is colored in red. Methyllanthionine linkage between residues 19-27 is colored in pink. The assignments are also found in separate panels in **Figure S11** for clarity.

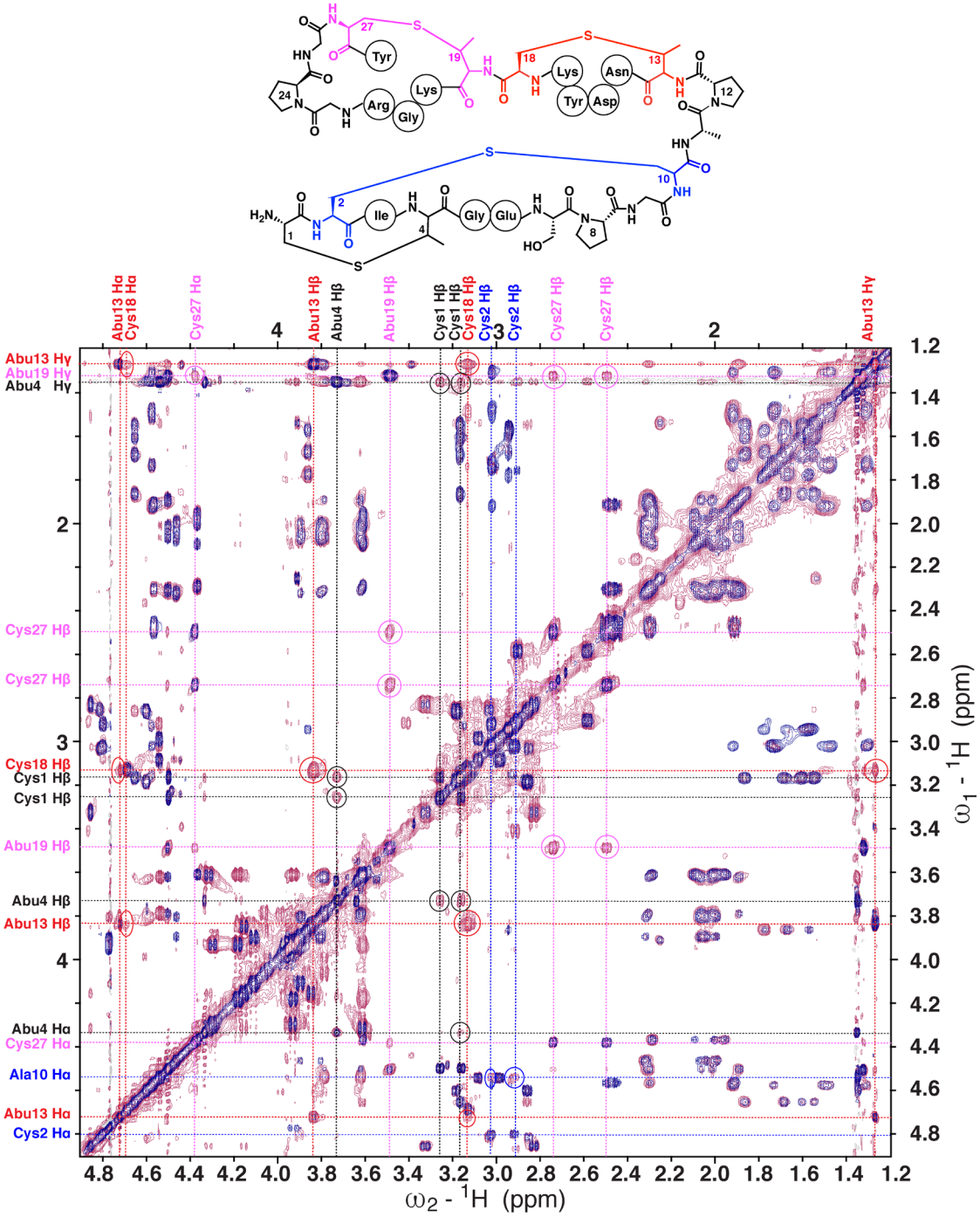

Figure S11. Water-suppressed NOESY spectrum of Pcn 2.1 (D_2O, mixing time = 0.30 s) for ring pattern assignments with each thioether assignment depicted in separate panels. TOCSY contours are shown in navy blue. NOESY contours are shown in maroon. (A) Structure of Pcn 2.1. (B) Methyllanthionine linkage between residues 1-4 is colored in black. Diagnostic nOe cross peaks are circled black.

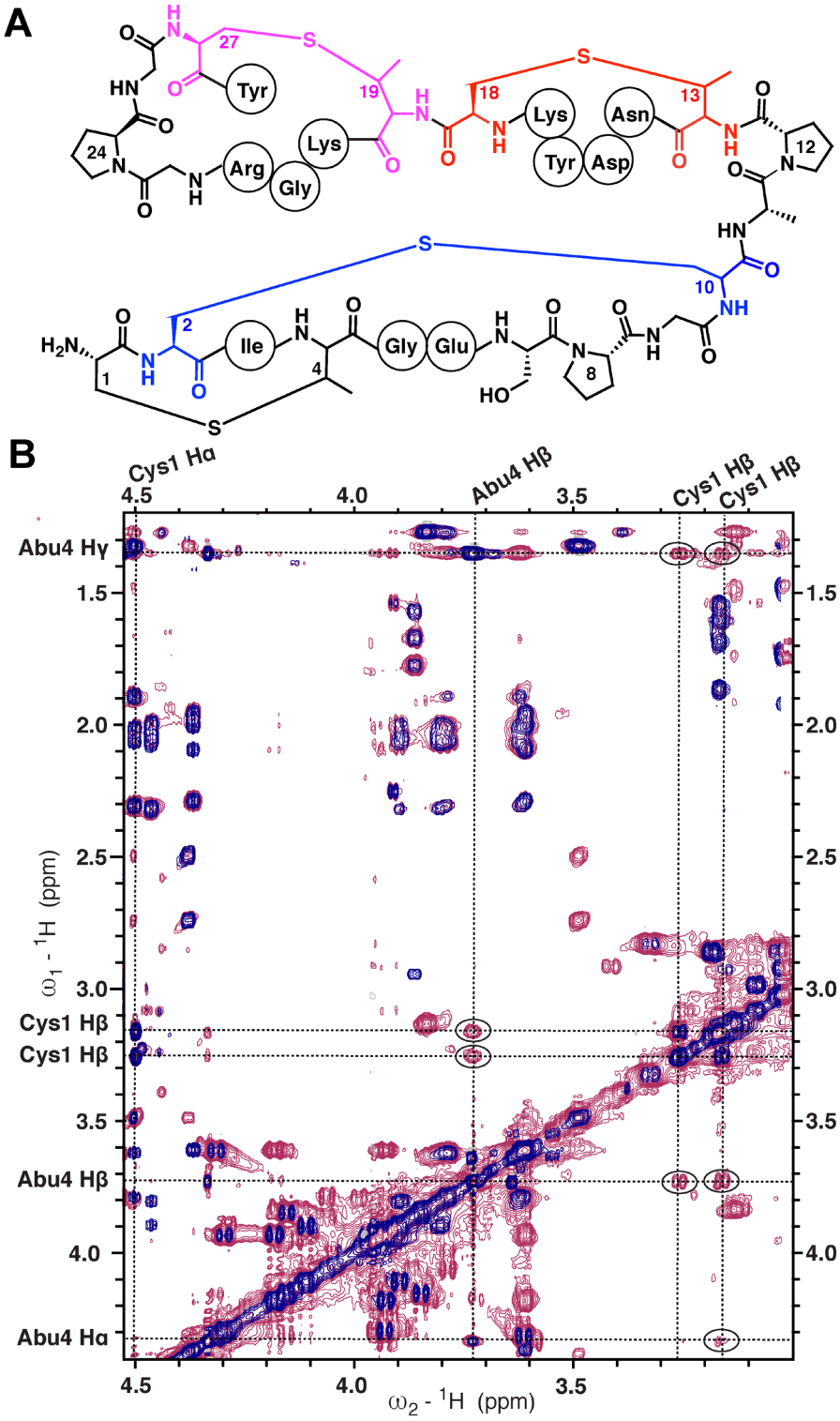

Figure S11 (continued). (C) Lanthionine linkage between residues 2-10 of Pcn 2.1 is colored in blue. Diagnostic nOe crosspeaks are circled blue.

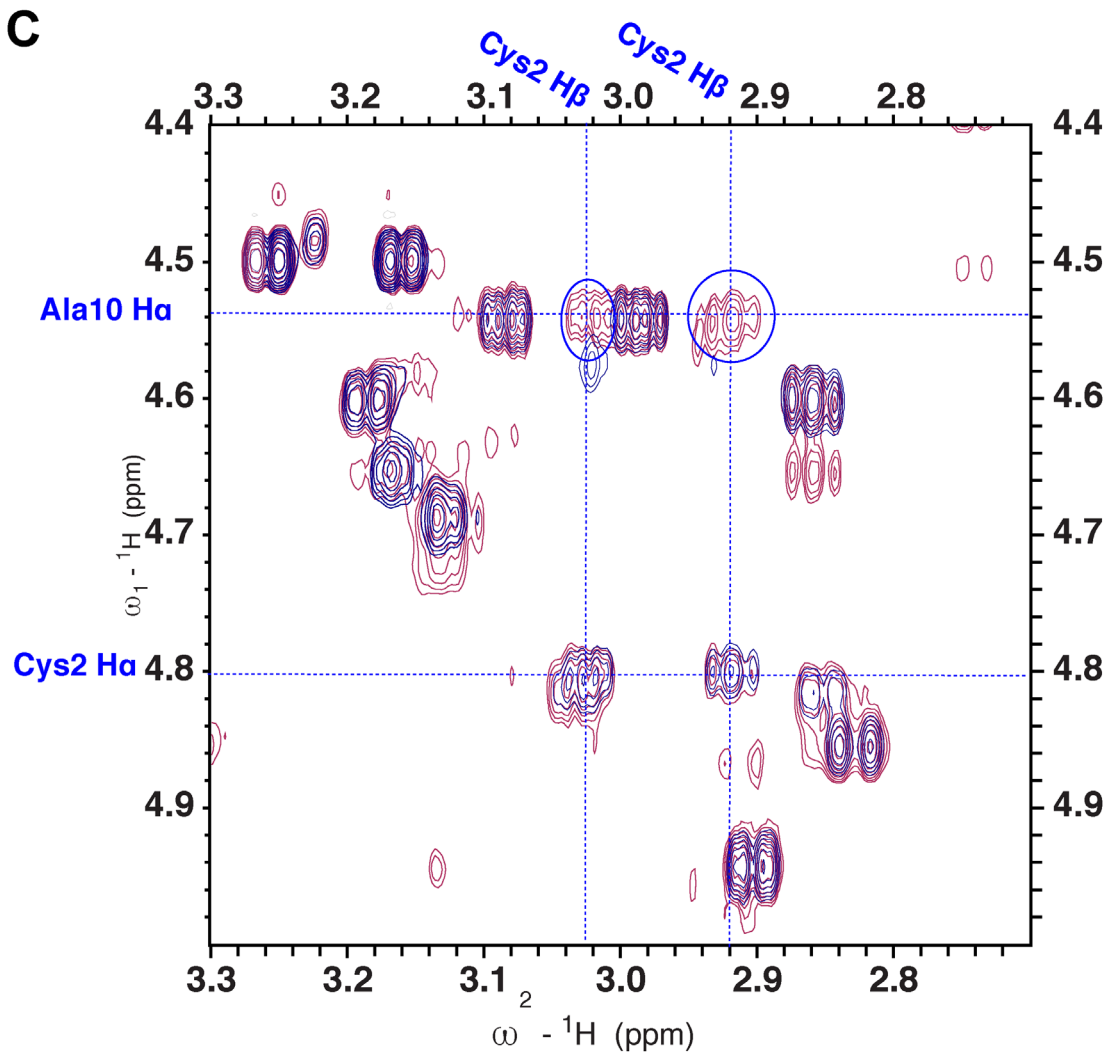

Figure S11 (continued). (D) Methyllanthionine linkage between residues 13-18 of Pcn 2.1 is colored in red. Diagnostic nOe crosspeaks are circled red.

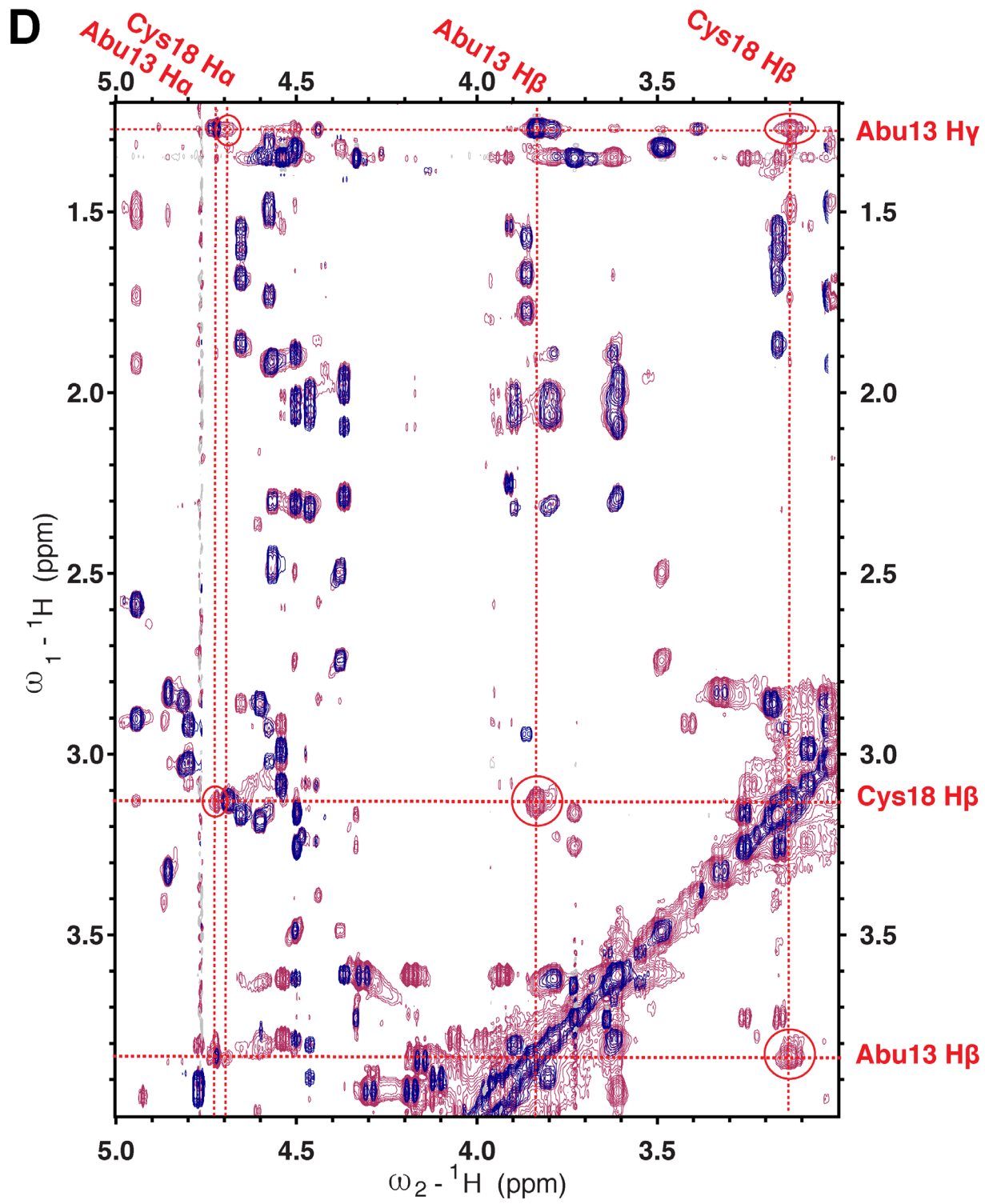

Figure S11 (continued). (E) Methyllanthionine linkage between residues 19-27 of Pcn 2.1 is colored in pink. Diagnostic nOe cross peaks are circled pink.

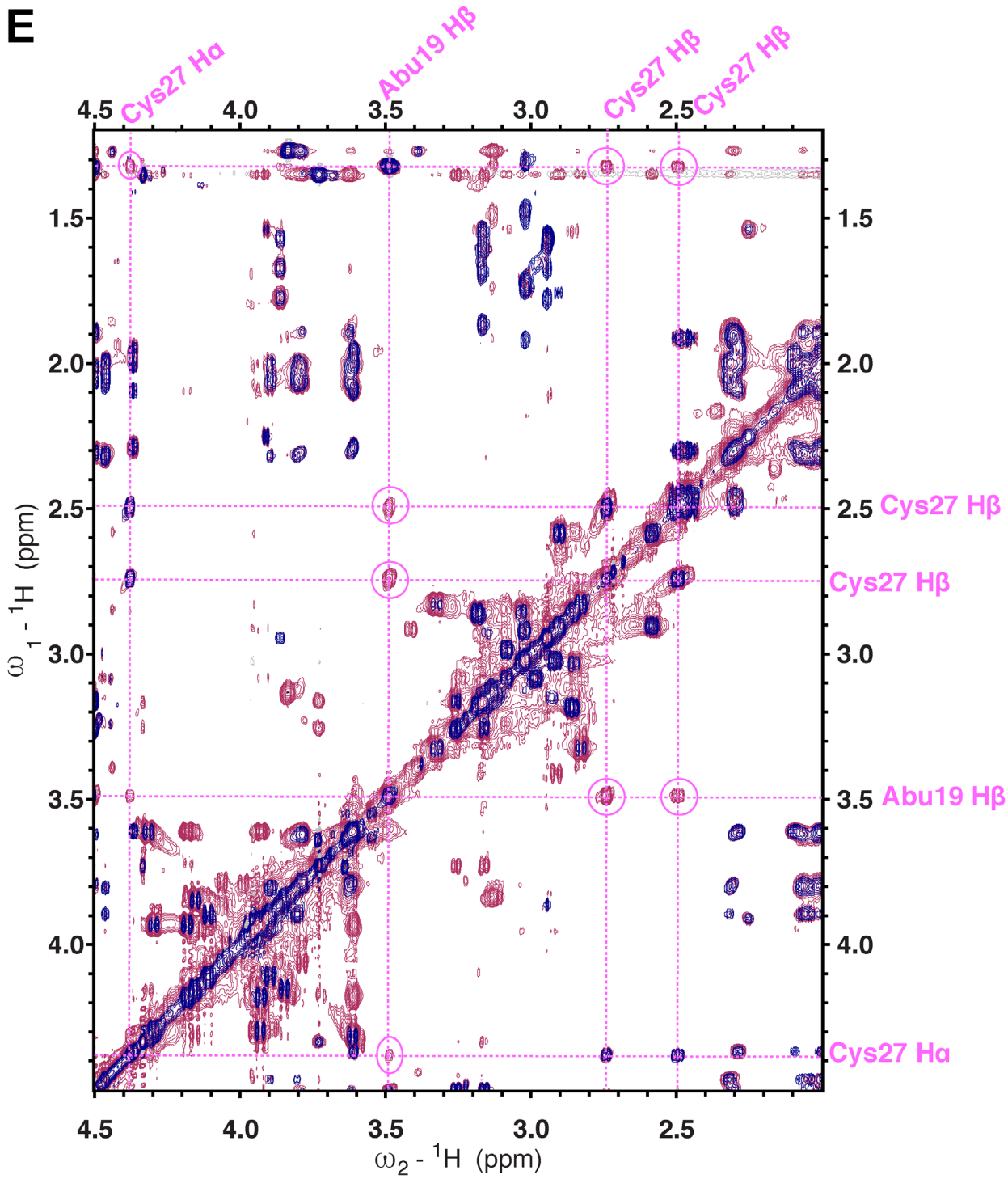

Figure S12. Ramachandran plots for Pcn 2.1. D-amino acid residues that are part of the thioether structures are in disallowed and generously allowed regions. For Pcn 2.1, the D-stereochemistry is assumed. For Pcn 1.1, 2.8, and 2.11 (as well as three other prochlorosins) the D-stereochemistry has been experimentally verified.[1]

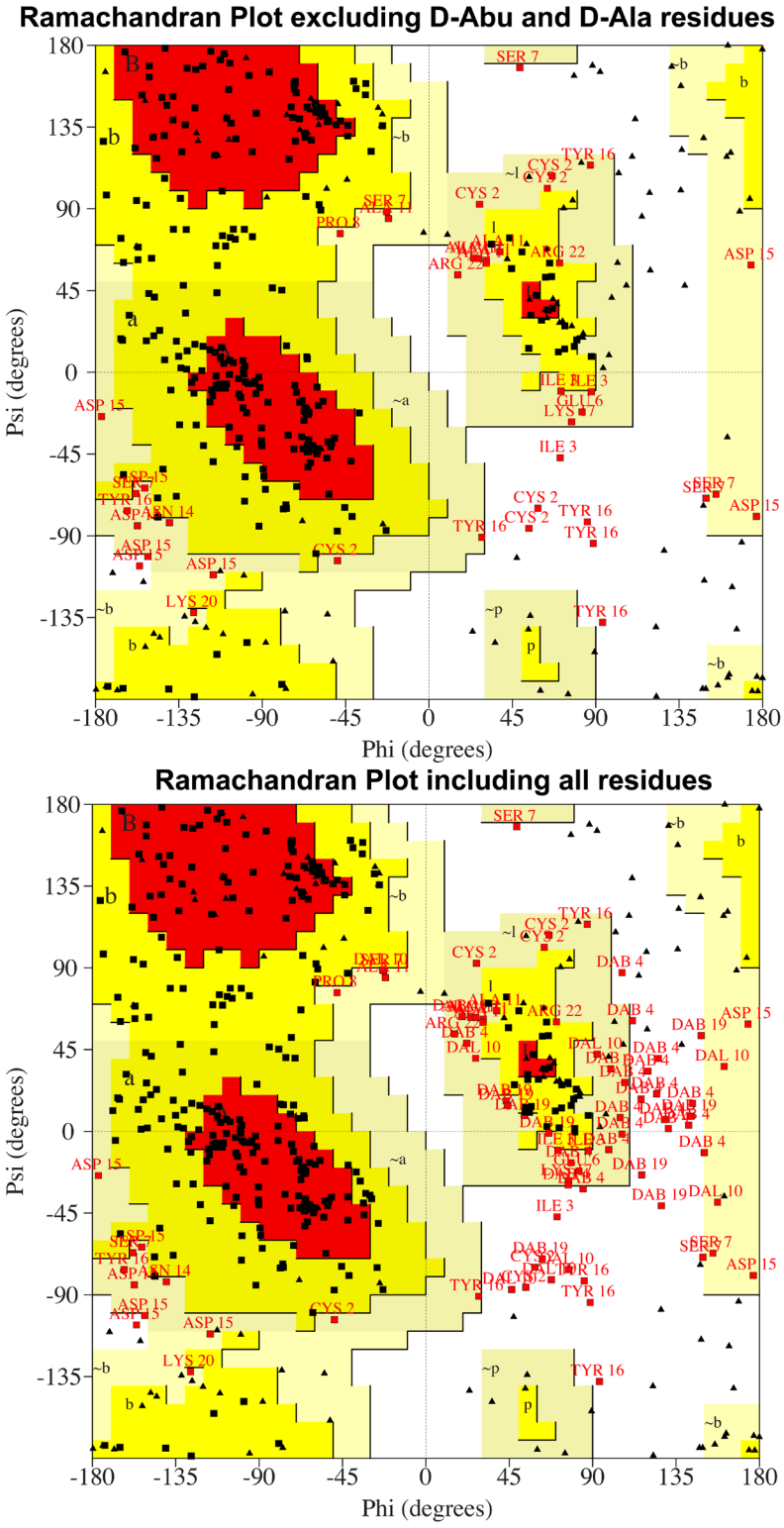

Table S4. Structural statistics for Pcn 2.1.

Procheck Analysis	**All L residues (%)**	**All residues (%)**
Residues in most favored regions [A,B,L]	46.2	37.1
Residues in additional allowed regions [a,b,l,p]	38.5	40.0
Residues in generously allowed regions [~a,~b,~l,~p]	11.5	14.4
Residues in disallowed regions	3.8	8.5
RMSD		
Residues	**Backbone only (Å)**	**All atoms (Å)**
1 to 28	1.65	2.48
Ring A (1 to 4)	1.49	1.82
Ring B (2 to 10)	1.41	1.99
Ring C (13 to 18)	1.16	1.89
Ring D (19 to 27)	2.15	3.35
Restraint Types Used		
Intra residue nOe	129	
Sequential nOe (|i-j|=1)	118	
Medium range nOe (1<|i-j|≤4)	41	
Long range nOe (|i-j|>4)	48	
Total	336	

Figure S13. Superimposition of the 20 lowest energy conformers of Pcn 2.1.

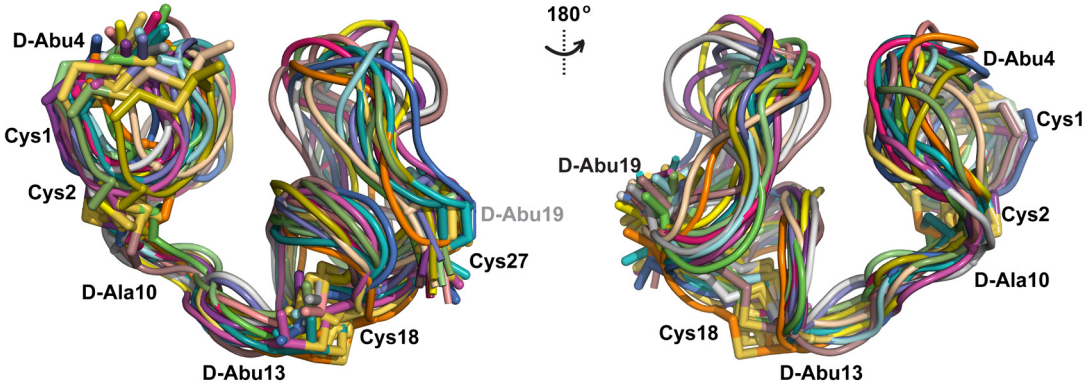

Table S5. Chemical shift assignments for Pcn 2.11 in 90% H_2O/10% D_2O.

	NH	αH	βH	γH	Hδ / Notes
1 Gly	-	-			-
2 Arg	8.45	4.24	1.66, 1.60	3.04	-
3 Ile	8.25	4.04	1.72	1.04, 1.32	Hδ: 0.75, 0.72
4 Asp	8.43	4.55	2.70, 2.76	-	-
5 Dhb	9.16	-	6.67	1.59	-
6 Cys	7.92	4.70	2.90, 2.67	-	nOe from 2.89 ppm (Hβ) to 1.24 (D-Abu12Hγ)
7 Pro	-	4.24	1.84, 2.16		Hδ: 3.04
8 Ala	8.23	4.12	1.25	-	-
9 Gly	8.12	4.05, 3.73	-	-	-
10 Gly	8.17	3.80, 3.93	-	-	-
11 Gly	8.46	3.72, 3.83	-	-	-
12 D-Abu	7.74	4.36	3.21	1.24	-
13 D-Ala	8.39	4.41	2.81, 2.93	-	-
14 Glu	8.05	4.15	1.90, 1.96	-	-
15 Gln	8.39	4.14	1.83, 2.03	2.26	-
16 D-Abu	6.97	4.74	3.20	1.25	-
17 Gly	8.37	4.40 3.79	-	-	-
18 Dhb	9.76	-no H	6.51	1.66	-
19 Cys	7.60	4.54	3.31, 2.56	-	nOe from 3.31 ppm (Hβ) to 2.81, 2.93 (D-Ala13Hβ)
20 Cys	7.73	4.07	3.37, 2.81	-	nOe from 3.37 ppm (Hβ) to 1.25 (D-Abu16Hγ)

Figure S14. (A) Water-suppressed TOCSY spectrum (mixing time = 80 ms) identifying all the spin systems in Pcn 2.11.

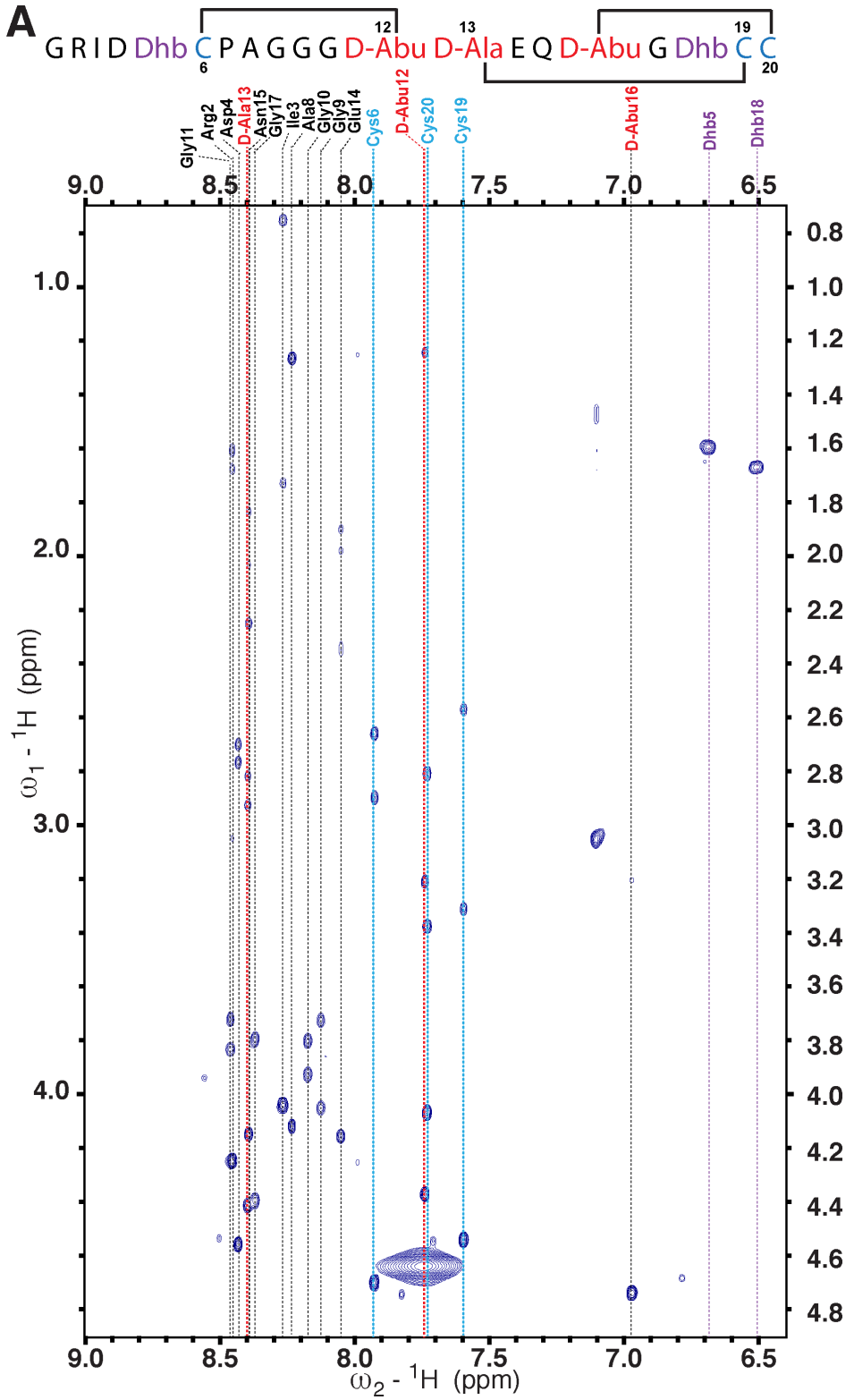

Figure S14 (continued) (B) Water-suppressed NOESY (mixing time = 300 ms) identifying the γ protons of the dehydrobutyrine residues.

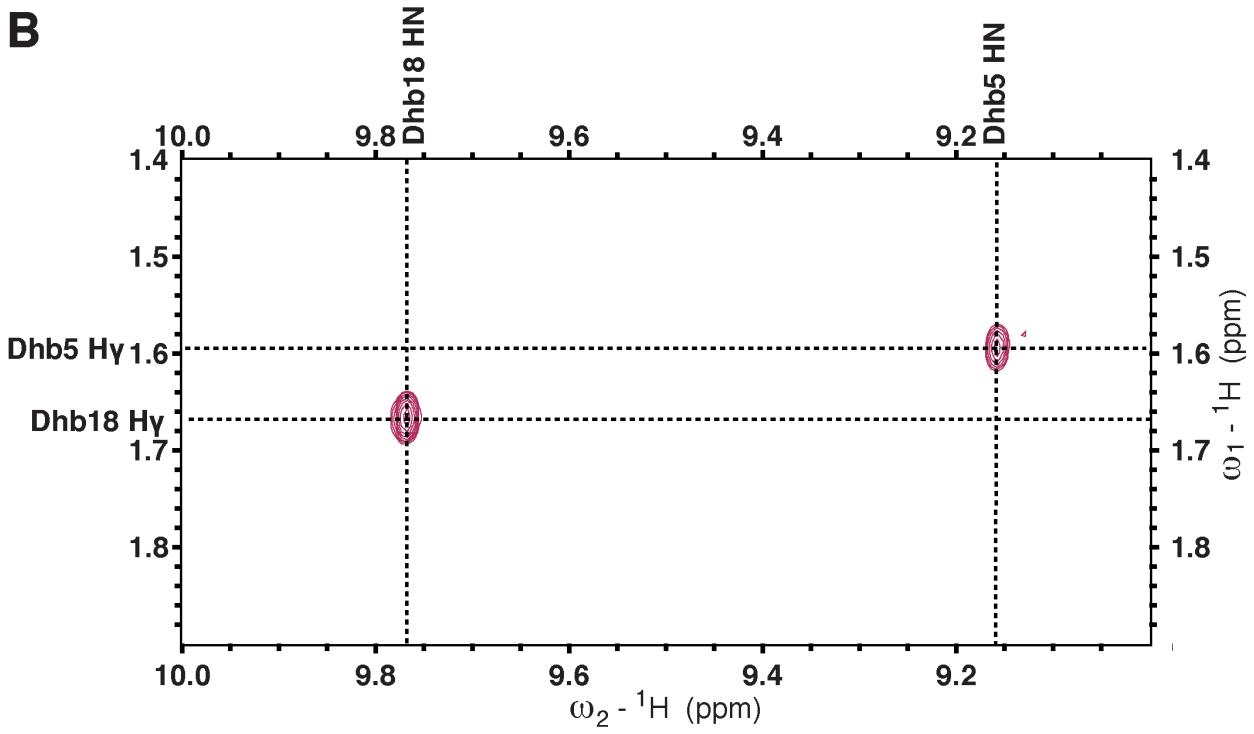

Figure S15. Water-suppressed NOESY spectrum (mixing time = 0.30 s) for amide resonance assignments to amino acids from Pcn 2.11.

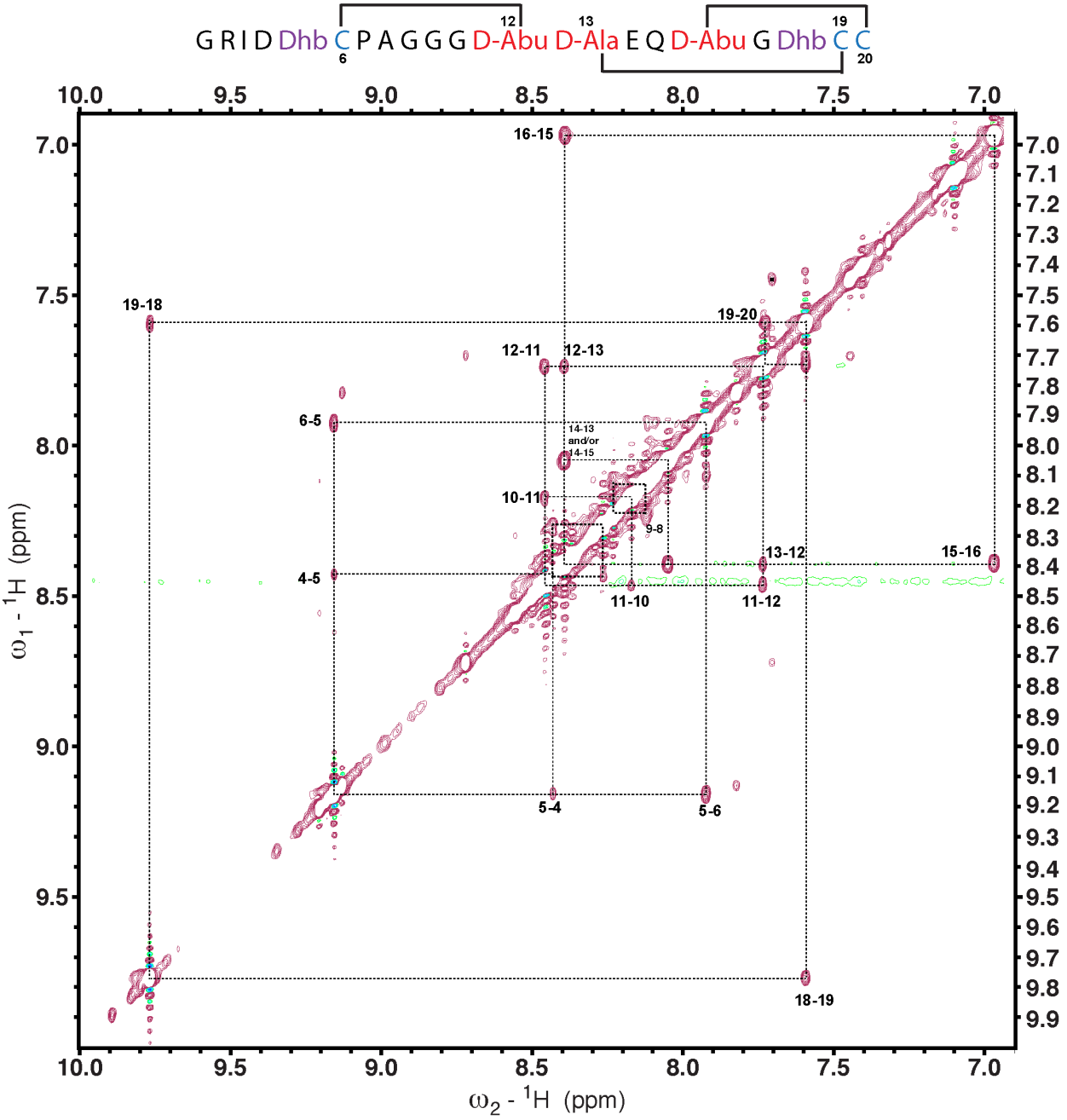

Figure S16. (A) Structure of Pcn 2.11. (B) NOESY spectrum of Pcn 2.11 in D_2O (mixing time = 0.30 s) for ring pattern assignment. The nOe cross peaks for ring assignments are labeled. NOESY contours are shown in maroon, TOCSY contours are shown in navy blue. Lanthionine assignments between former residues Cys6 and Thr12 are colored in black. Lanthionine assignments between former residues Ser13 and Cys19 are colored in blue. Methyllanthionine assignments between former residues Thr16 and Cys20 are colored in red.

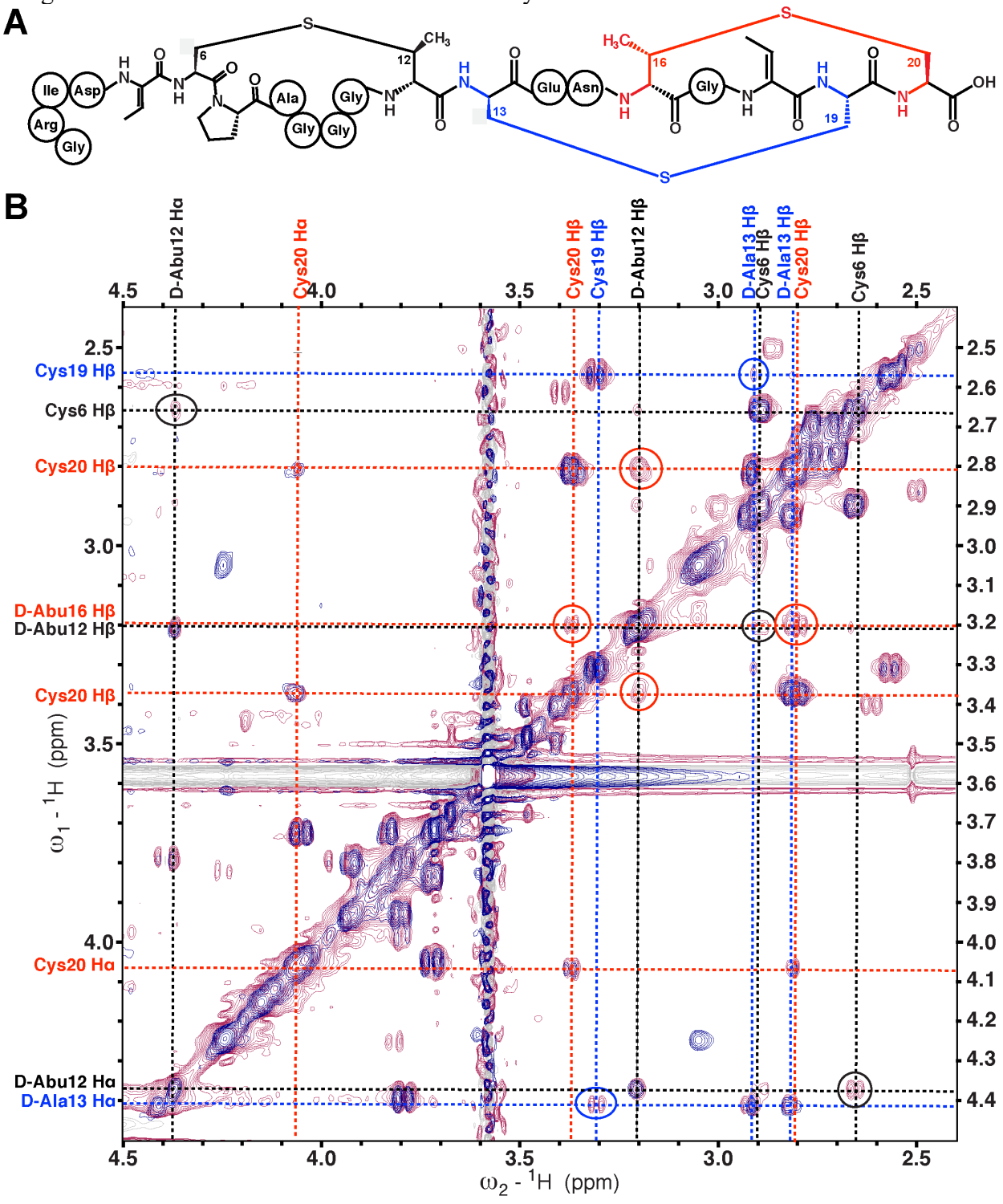

Figure S16 (continued). (C) NOESY spectrum of Pcn 2.11 in D_2O (mixing time = 0.30 s) for ring pattern assignment with the γ protons of D-amino butyric acid shown. The nOe cross peaks for ring assignments are labeled. NOESY contours are shown in maroon, TOCSY contours are shown in navy blue. Lanthionine assignments between former residues Cys6 and Thr12 are colored in black. Methyllanthionine assignments between former residues Thr16 and Cys20 are colored in red.

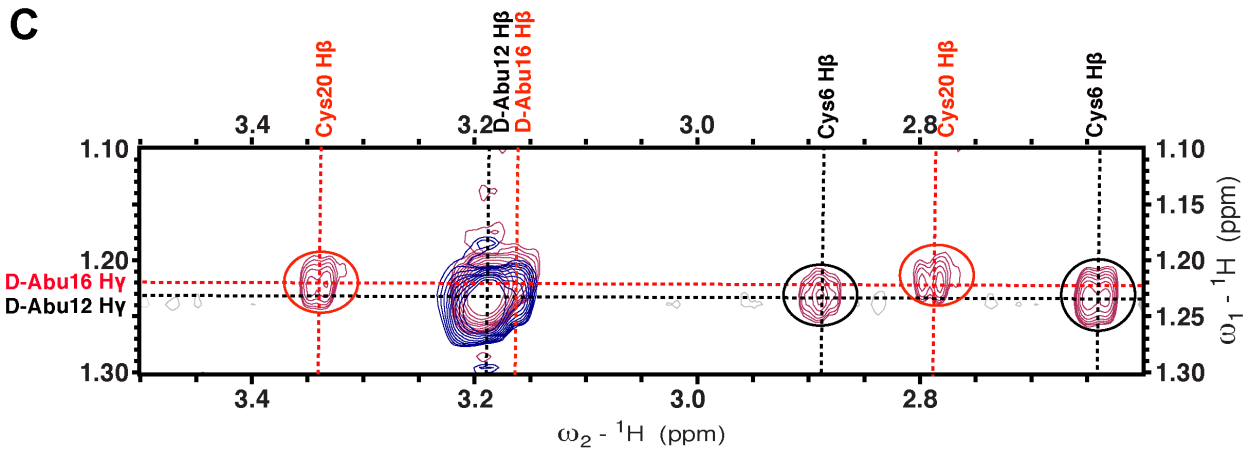

Figure S17. Ramachandran plots for Pcn 2.11. D-Amino acid residues present as part of the thioether crosslinks and dehydroamino acid residues are found in disallowed regions.

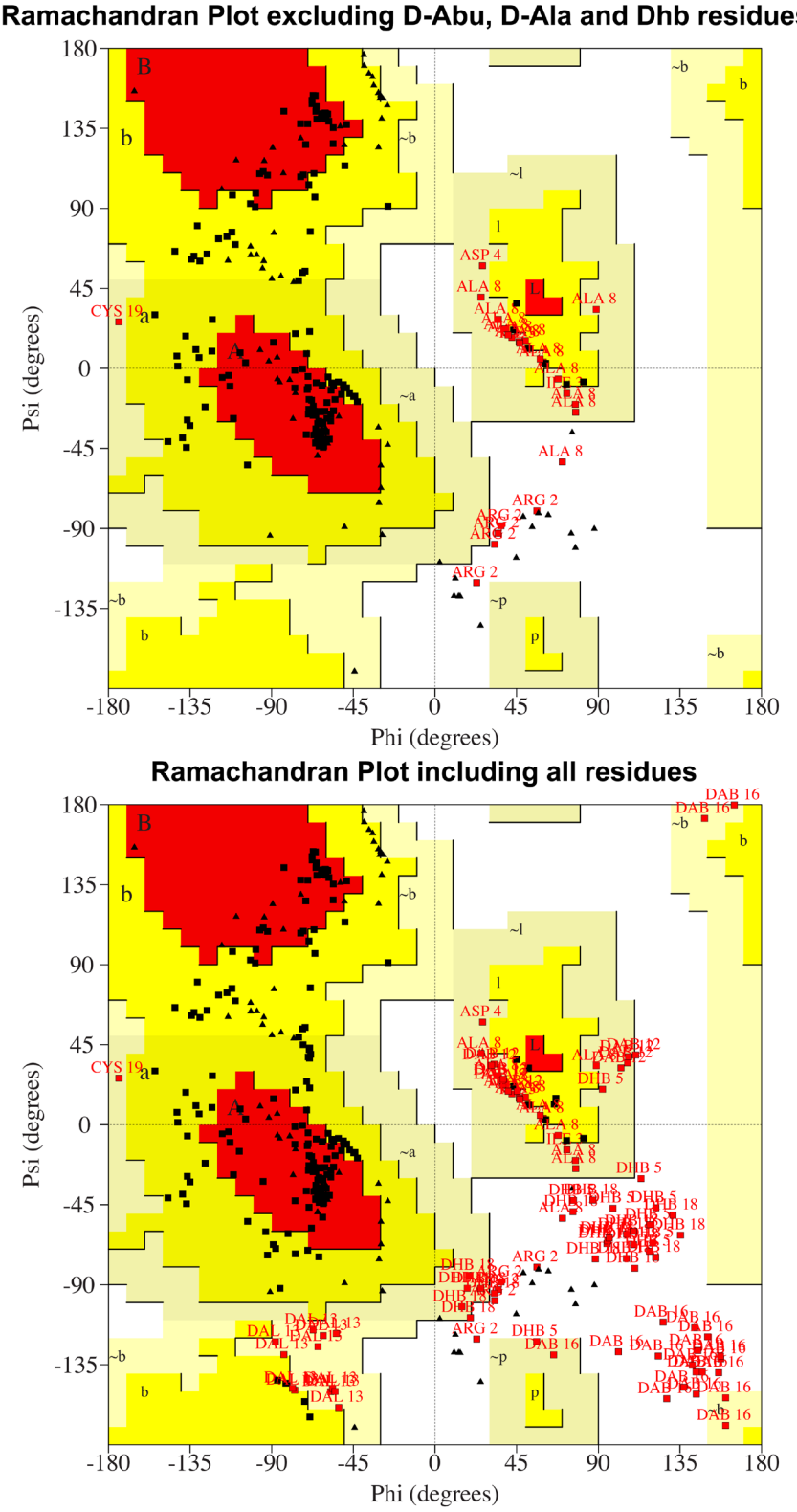

Table S6. Structural statistics for Pcn 2.11.

Procheck Analysis	**All L residues (%)**	**All residues (%)**
Residues in most favored regions [A,B,L]	53.8	35.8
Residues in additional allowed regions [a,b,l,p]	31.2	27.7
Residues in generously allowed regions [~a,~b,~l,~p]	11.2	19.6
Residues in disallowed regions	3.8	16.9
RMSD		
Residues	**Backbone only (Å)**	**All atoms (Å)**
1 to 20	1.31	2.09
1 to 5	2.14	3.09
6 to 20	0.83	1.30
Ring A (6 to 12)	0.83	1.19
Ring B (13 to 19)	0.85	1.37
Ring C (16 to 20)	0.92	0.95
Restraint Types Used		
Intra residue nOe	64	
Sequential nOe (|i-j|=1)	44	
Medium nOe (1<|i-j|≤4)	14	
Long range nOe (|i-j|>4)	36	
Hydrogen bond restraints	4	
Total	152	

Figure S18. Superimposition of the 20 lowest energy structures of Pcn 2.11. (A) Residues involved in thioethers are marked. The methyllanthionine linkage between Cys6 and former Thr12 and the lanthionine linkage between Cys19 and former Ser13 are visible in the first depiction. (B) Methyllanthionine between Cys20 and former Thr16 and between Cys6 and former Thr12 are visible in the second depiction. The structure shows high flexibility in the N-terminal region and between residues 6-12 and more rigidity in the residues spanning the intertwined thioether rings between residues 13-20.

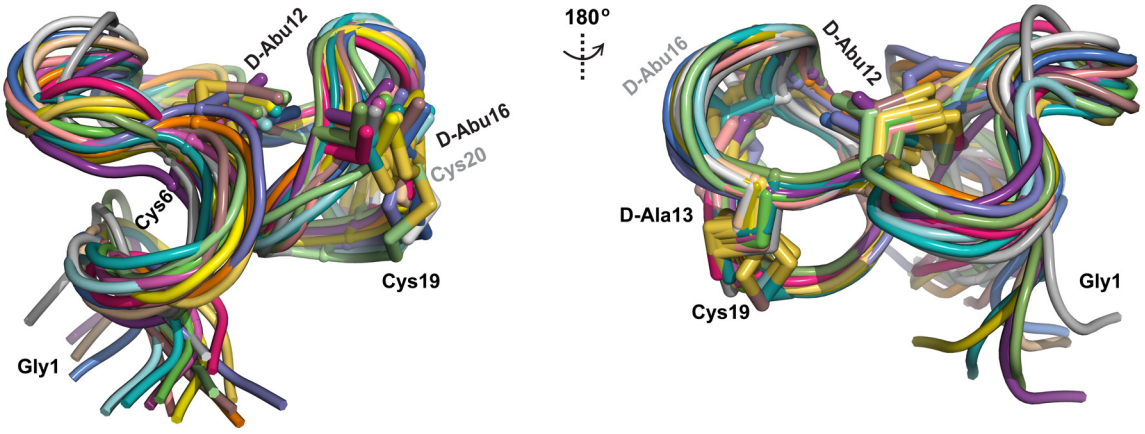

Table S7. Chemical shift assignments for Pcn 1.1 in 90% H_2O / 10% D_2O.

	NH	αH	βH	γH	Hδ+other protons
1 Phe	-	-	-	-	-
2 Phe	8.414	4.88	2.962, 3.029		Hδ: 7.17 Hε: 7.308 Hζ: 7.274
3 Cys	8.398	4.63	2.700, 3.038	-	-
4 Val	8.512	4.507	2.184	0.921, 0.853	-
5 Gln	9.211	3.949	2.011	2.29	
6 Gly	8.656	4.056, 3.678	-	-	-
7 D-Abu	7.61	4.178	3.71	1.353	-
8 Ala	8.063	4.28	1.415	-	-
9 Asn	8.600	4.674	2.734, 2.666		
10 Arg	8.339	4.385	1.769, 1.702	1.551	Hδ: 3.126
11 Phe	8.586		3.051, 2.957		Hδ: 7.200 Hε:7.32 Hζ:7.270
12 D-Abu	8562	4.32	3.657	0.72	
13 Ile	7.767	4.156	1.764	0.82 1.27, 1.02	Hδ: 0.726
14 Asn	8.426	4.628	2.885, 2.805	-	
15 Val (V)	7.826	4.077	2.141	0.921, 0.883	
16 Cys	8.18	4.188	3.03, 2.575		

Figure S19. Water-suppressed TOCSY spectrum (mixing time = 70 ms) identifying all the spin systems in Pcn 1.1.

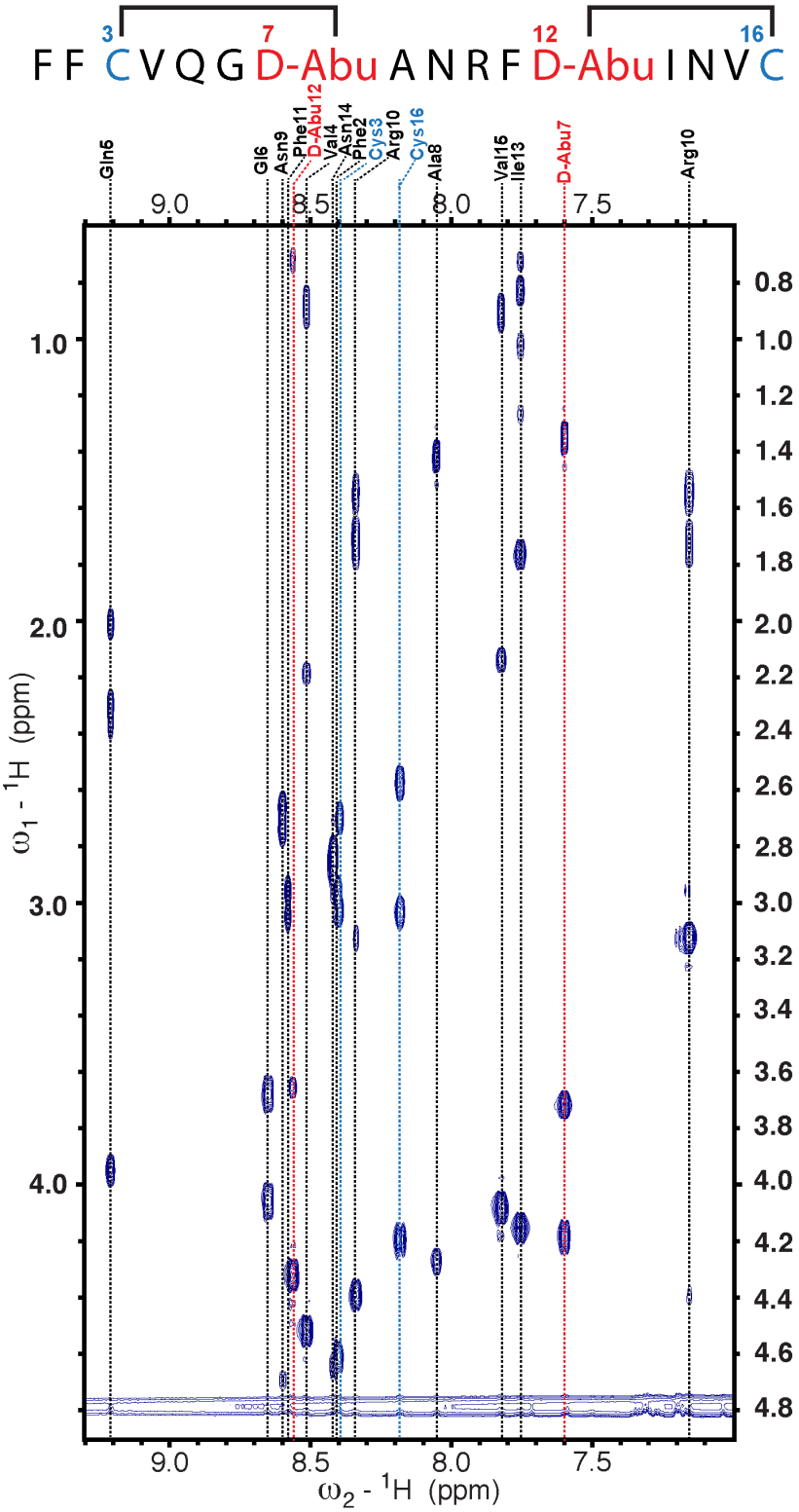

Figure S20. Water suppressed NOESY spectrum (mixing time = 0.30 s) for amide resonance assignments to amino acids from Pcn 1.1.

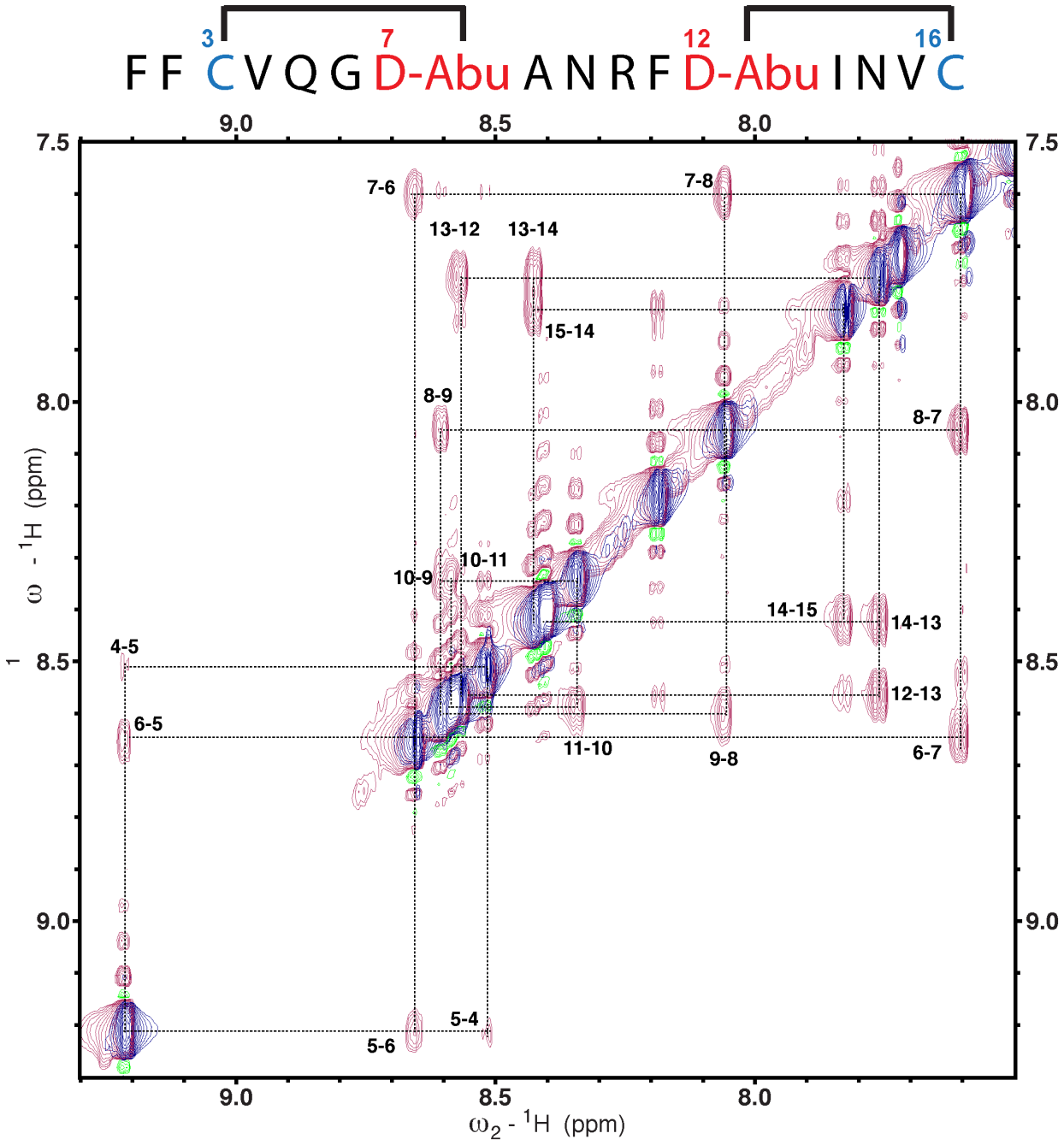

Figure S21. NOESY spectrum of Pcn 1.1 in D_2O (mixing time = 0.30 s) for ring pattern assignment. The nOe cross peaks for ring assignments are labeled. NOESY contours are shown in maroon, TOCSY contours are shown in navy blue. Methyllanthionine assignments between former residues Thr7 and Cys3 are colored in black. Methyllanthionine assignments between former residues Thr12 and Cys16 are colored in blue.

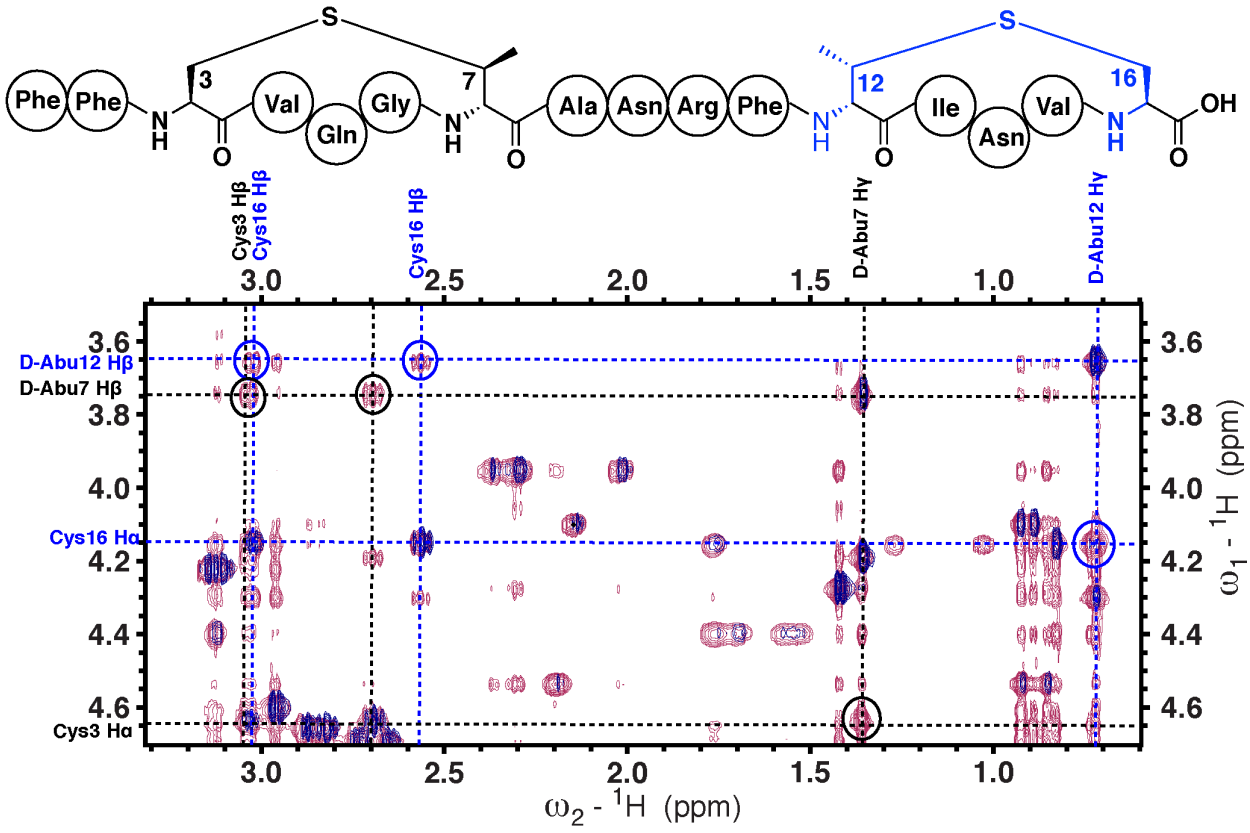

Figure S22. Ramachandran plot for Pcn 1.1. D-Amino acid residues and cysteine residues in thioether linkages are found in disallowed areas.

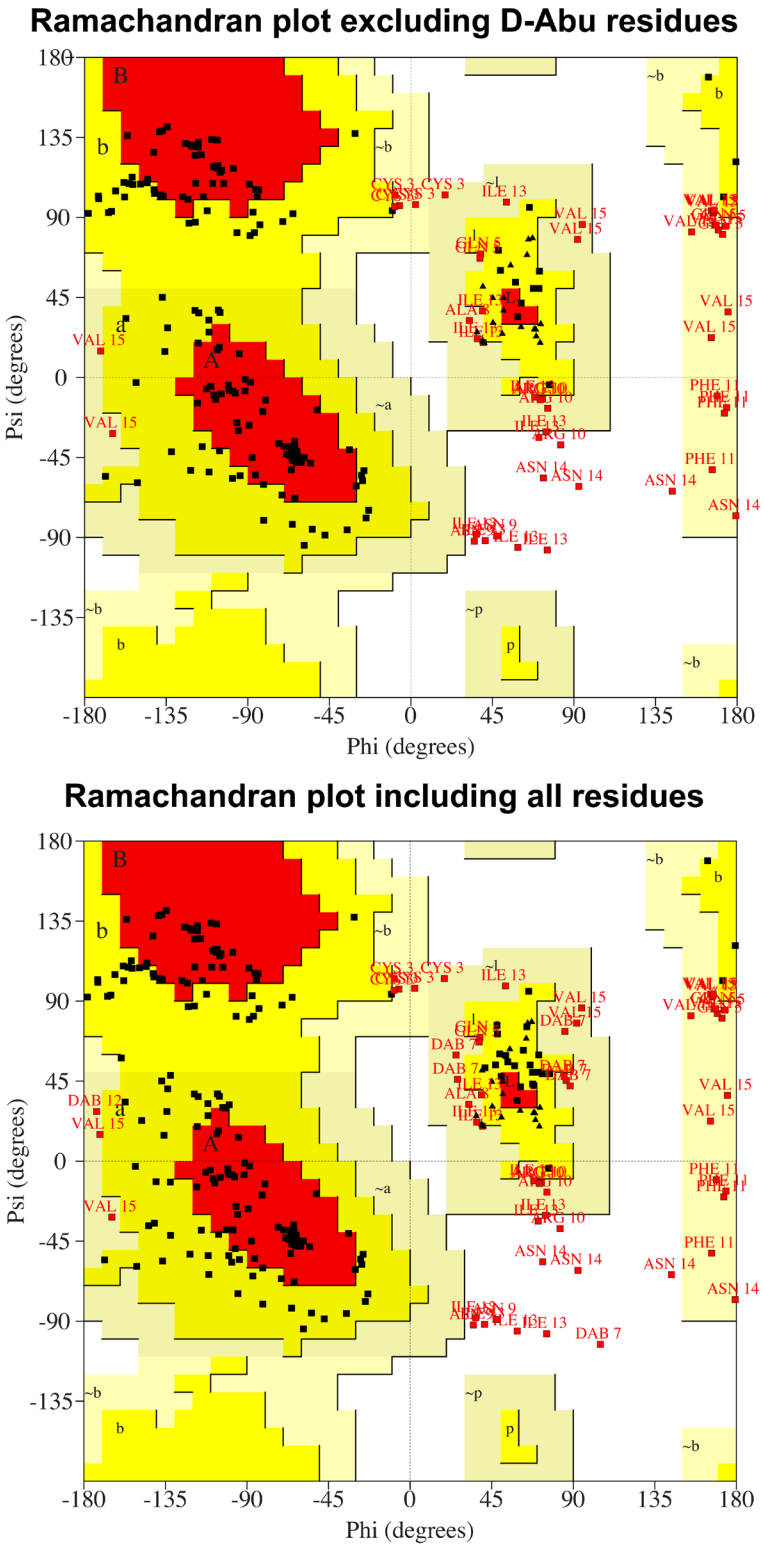

Table S8. Structural statistics for Pcn 1.1.

Procheck Analysis	**All L residues (%)**	**All residues (%)**
Residues in most favored regions [A,B,L]	45.5	40.4
Residues in additional allowed regions [a,b,l,p]	33.2	38.5
Residues in generously allowed regions [~a,~b,~l,~p]	15.9	16.2
Residues in disallowed regions	5.5	5.0
RMSD		
Residues	**Backbone only (Å)**	**All atoms (Å)**
1 to 16	0.89	1.23
Ring A (3 to 7)	0.74	0.99
Ring B (12 to 16)	1.14	1.48
8 to 11	0.81	1.27
Restraint Types Used		
Intra residue nOe	100	
Sequential nOe (|i-j|=1)	72	
Medium nOe (1<|i-j|≤4)	65	
Long range nOe (|i-j|>4)	42	
Total	279	

Figure S23. Superimposition of the 20 lowest energy structures of Pcn 1.1. Residues involved in methyllanthionine linkages are marked in all panels. (A) Backbone represented as a loop with the two methyllanthionine linkages found at the termini of the peptide. (B) Depiction of the interaction between the sidechain of Asn9 which is flanked by Phe2 and Phe11.

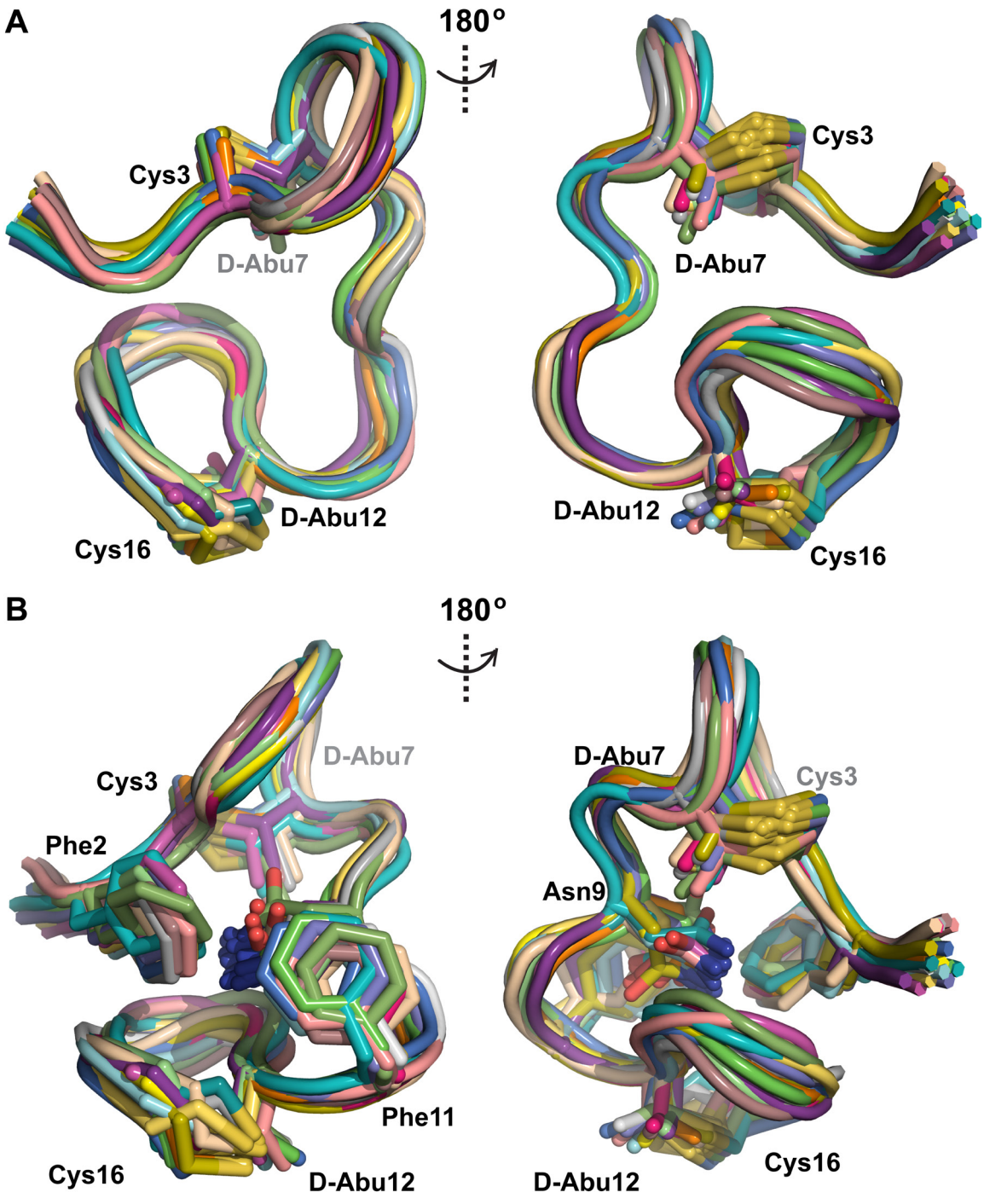

Table S9. Chemical shift assignments for Pcn 2.8 in 90% H_2O/10% D_2O.

	NH	αH	βH	γH	Hδ /Notes
1 Ala	-	4.210 Cα: 51.870	1.650 Cβ: 19.570	-	-
2 Ala	8.685 N: 123.3	4.412 Cα: 52.091	1.425 Cβ: 19.579	-	-
3 Cys	8.620 N:119.8	4.591 Cα: 55.799	3.178, 2.929 Cβ: 35.954	-	Cys3 HN has NOE with D-Ala9 Hβ
4 His	9.23 N:124.1	4.910 Cα: 55.500	3.470, 3.230 Cβ: 28.919	-	Hδ: 7.373
5 Asn	8.173 N: 118.5	4.837 Cα: 53.300	3.040, 2.788 Cβ: 39.205	-	Hδ: 7.708, 7.089 Nδ: 112.9
6 His	8.408 N:117.4	4.626 Cα: 55.760	3.335 Cβ:29.550	-	Hδ: 7.421 Hε: 7.340
7 Ala	8.657 N:126.7	4.435 Cα: 53.534	1.446 Cβ: 17.946	-	Ala7 Hα has NOE with Pro8 Hα
8 Pro	-	4.723 Cα: 64.060	2.406 Cβ: 34.470	2.068, 1.838 Cγ: 24.794	Hδ: 3.730, 3.640 Cδ: 50.544
9 D-Ala	8.377 N:122.7	4.844 Cα: 55.540	3.130, 2.908 Cβ: 35.970	-	-
10 Met	8.622 N:123.9	4.877	1.917 Cβ: 32.605	2.530, 2.460 Cγ: 32.541	Methyl group on S overlaps with M10 Hβ
11 Pro	-	4.794 Cα: 61.904	2.434, 1.990 Cβ: 31.069	2.065 Cγ: 28.230	Hδ: 3.507, 3.846 Cδ: 50.826
12 Pro	-	4.540 Cα: 63.900	2.439, 2.044 Cβ: 32.244	2.212, 2.160 Cγ: 27.931	Hδ: 3.915, 3.745 Cδ: 50.782
13 D-Ala	8.518 N:118.7	4.590 Cα: 56.680	3.079, 2.985 Cβ: 37.330	-	-
14 Tyr	8.125 N:120.9	4.320	3.066, 2.870	-	Hδ: 6.737; Cδ: 133.421 Hε: 6.680; Cε:118.405
15 Trp	7.936 N:119.0	4.715 Cα: 57.430	3.330 Cβ: 29.141	-	Hε: 10.250; Cε: 121.417 7.727 Hδ: 7.358; Cδ: 127.456 Hζ: 7.580, 7.269; Cζ: 122.517, 115.030 Hη: 7.340; Cη: 125.129 Nε:129.363
16 Glu	8.150 N:120.7	4.196 Cα: 57.126	2.064, 1.966 Cβ: 32.683	2.55, 2.204 Cγ: 32.690	-
17 Gly	8.332 N:109.6	4.140, 3.929 Cα: 45.740	-	-	-

Table S9 (continued). Chemical shift assignments for Pcn 2.8 in 90% H_2O/10% D_2O.

18 Glu	8.157 N:118.9	4.607	2.340, 2.140 Cβ: 29.226	2.583 Cγ: 33.087	-
19 Cys	8.413 N:121.5	4.797	3.188, 3.005 Cβ: 37.357	-	-

Figure S24. TOCSY spectrum (mixing time=70 ms) identifying all spin systems of Pcn 2.8.

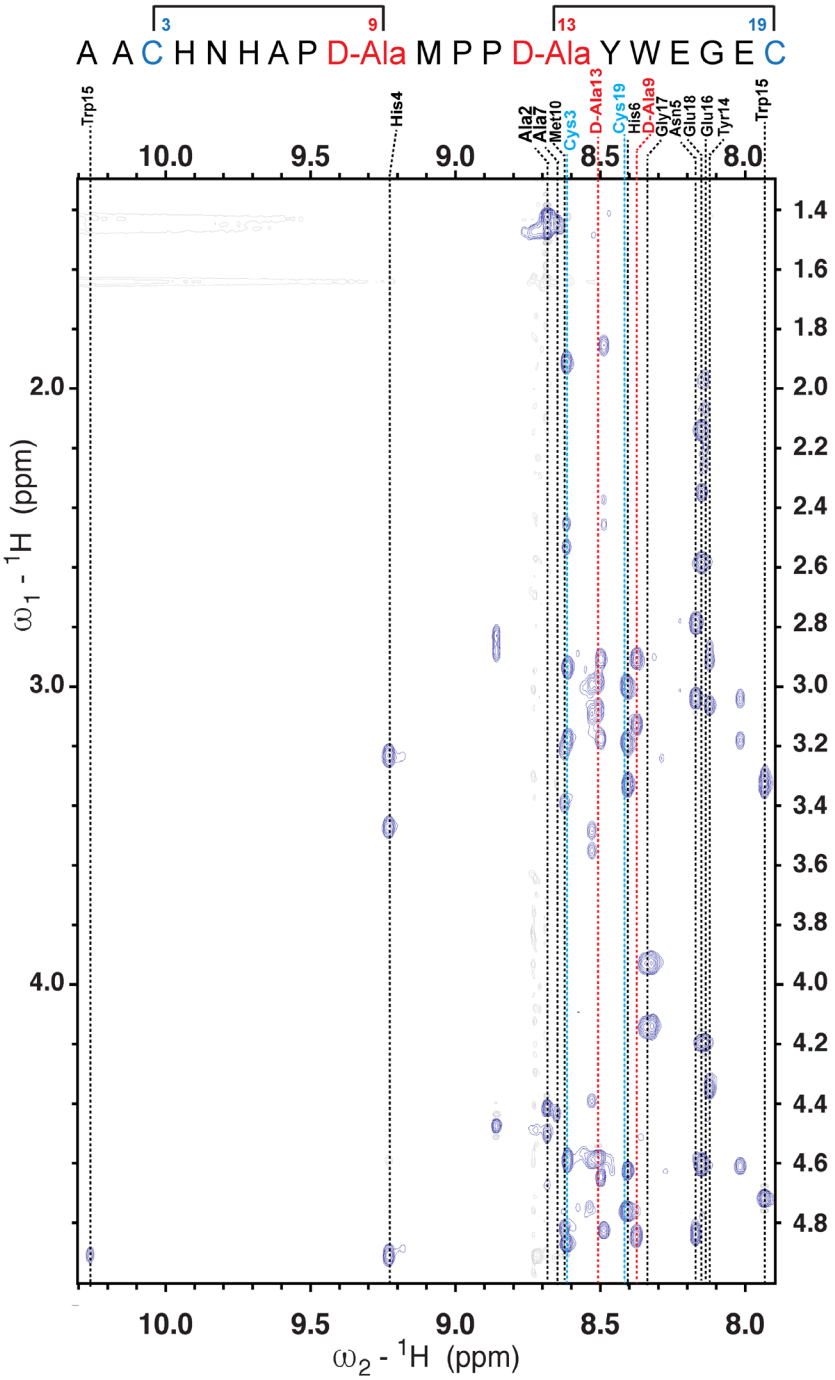

Figure S25. Water-suppressed NOESY spectrum (mixing time = 0.30 s) of Pcn 2.8. NOESY contours are shown in maroon, TOCSY contours are shown in navy blue. (A) Structure of Pcn 2.8. (B) Water suppressed NOESY spectrum for amide resonance assignments to amino acids from Pcn 2.8.

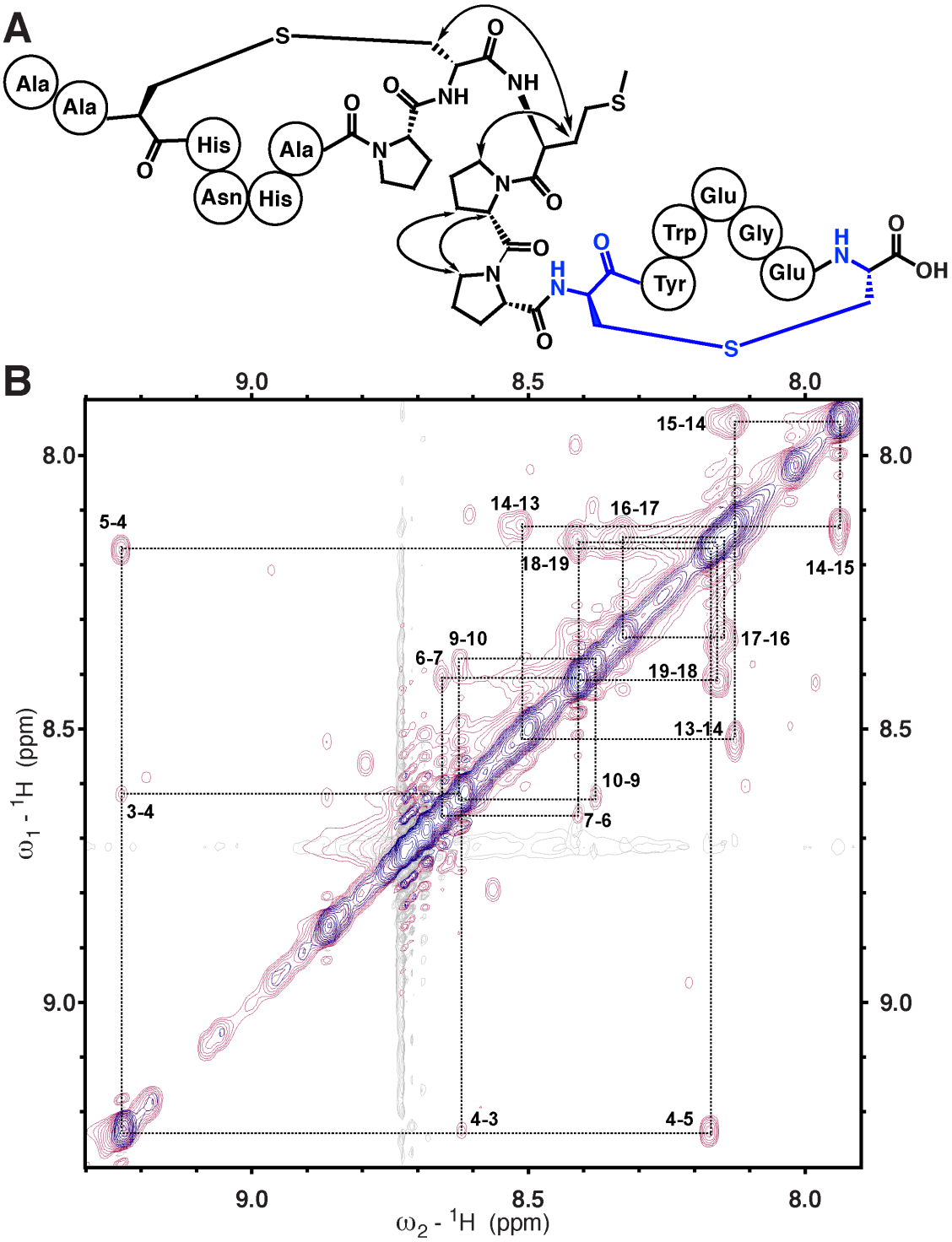

Figure S25 (continued). (C) nOe cross peaks establishing connectivity of Met10 to D-Ala9 in Pcn 2.8. (D) nOe cross peaks establishing connectivity of Met10 to Pro11 and Pro11 to Pro12 in Pcn 2.8.

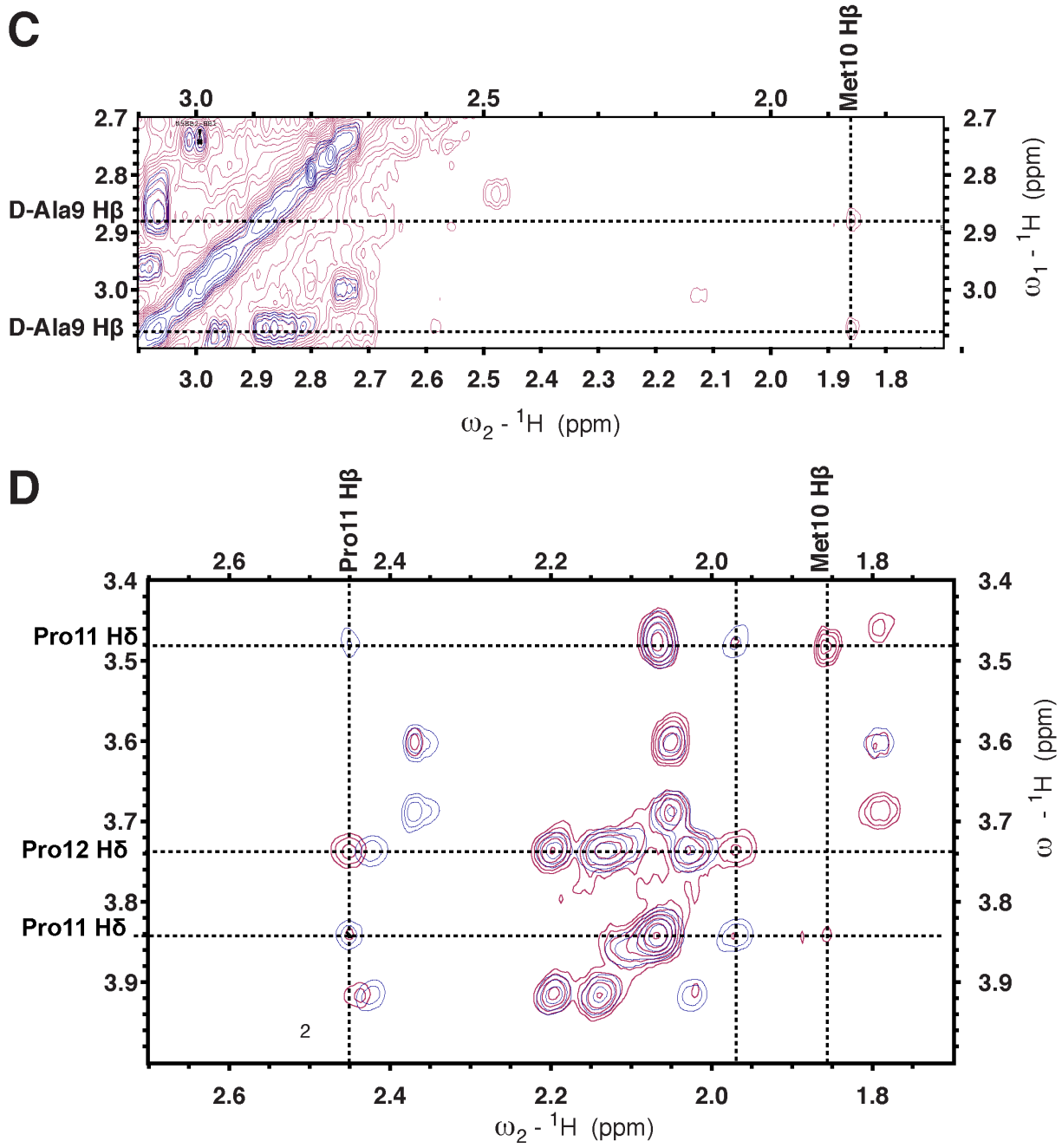

Figure S25 (continued). (E) nOe cross peaks establishing connectivity of Pro11 to Pro12 in Pcn 2.8.

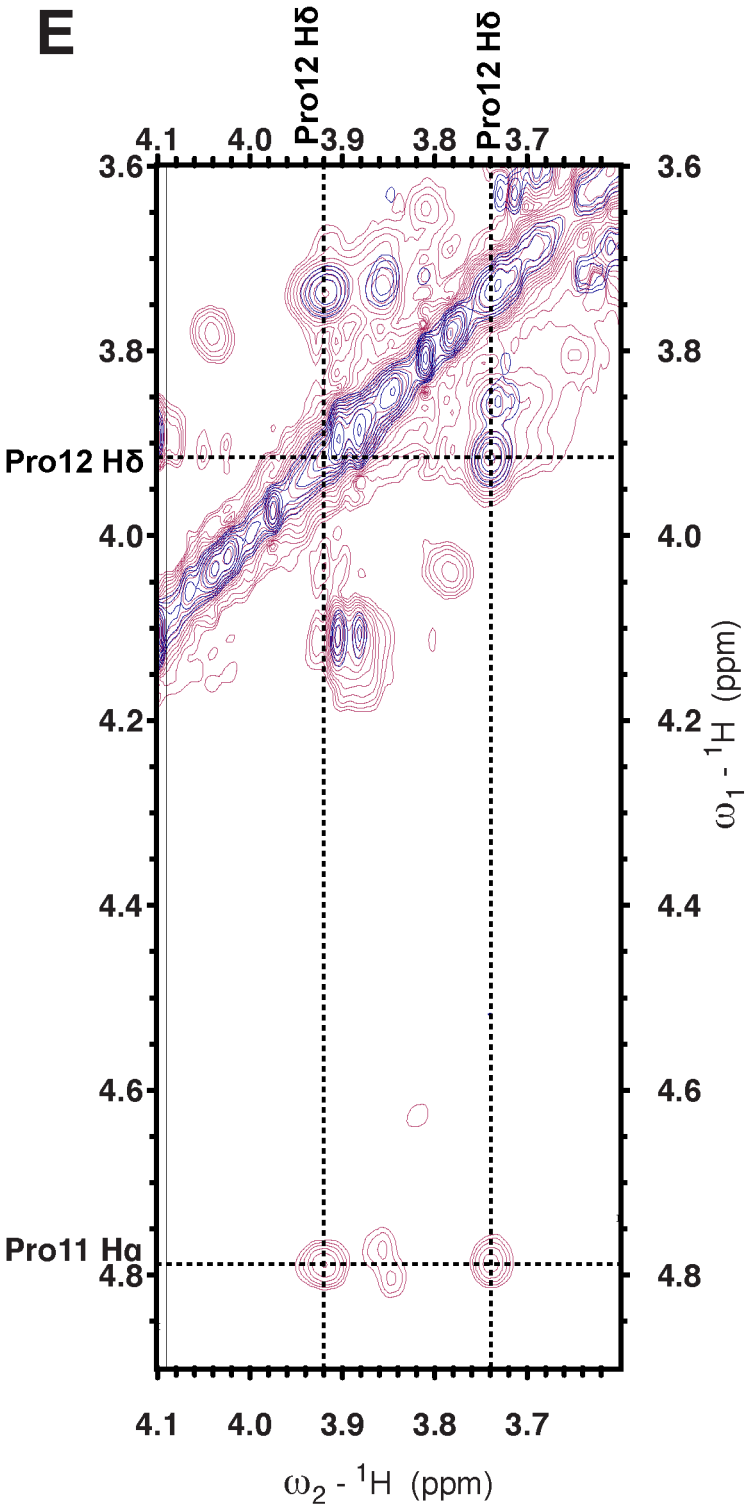

Figure S25 (continued) (F) Water-suppressed NOESY spectrum of Pcn 2.8 (D_2O, mixing time = 0.30 s) for ring pattern assignments. Lanthionine assignments between former residues Cys3 and Ser9 are colored in black. Lanthionine assignments between former residues Ser13 and Cys19 are colored in blue.

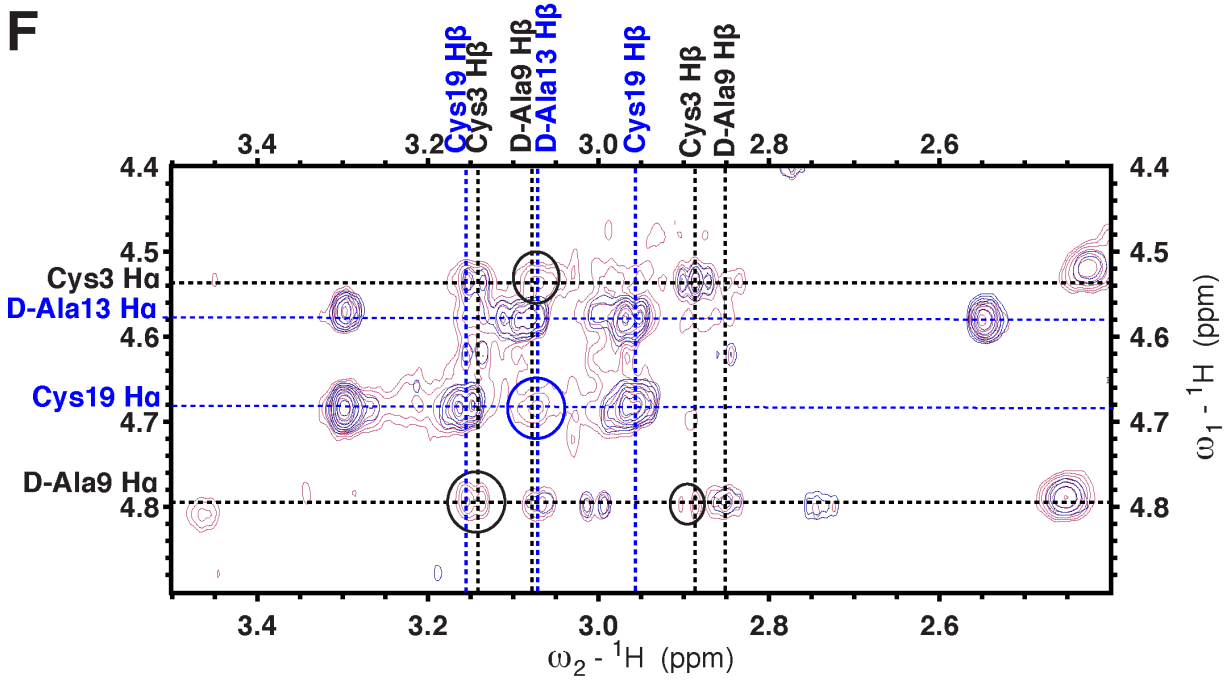

Figure S26. (A) ^{15}N HMQC spectrum for Pcn 2.8 acquired in 90% H_2O/10% D_2O. (B) ^{13}C HSQC spectrum of Pcn 2.8 acquired in 100% D_2O focused on the Cα-Hα region. Minor trans conformations as indicated by interactions between Ala7 and Pro8 are marked.

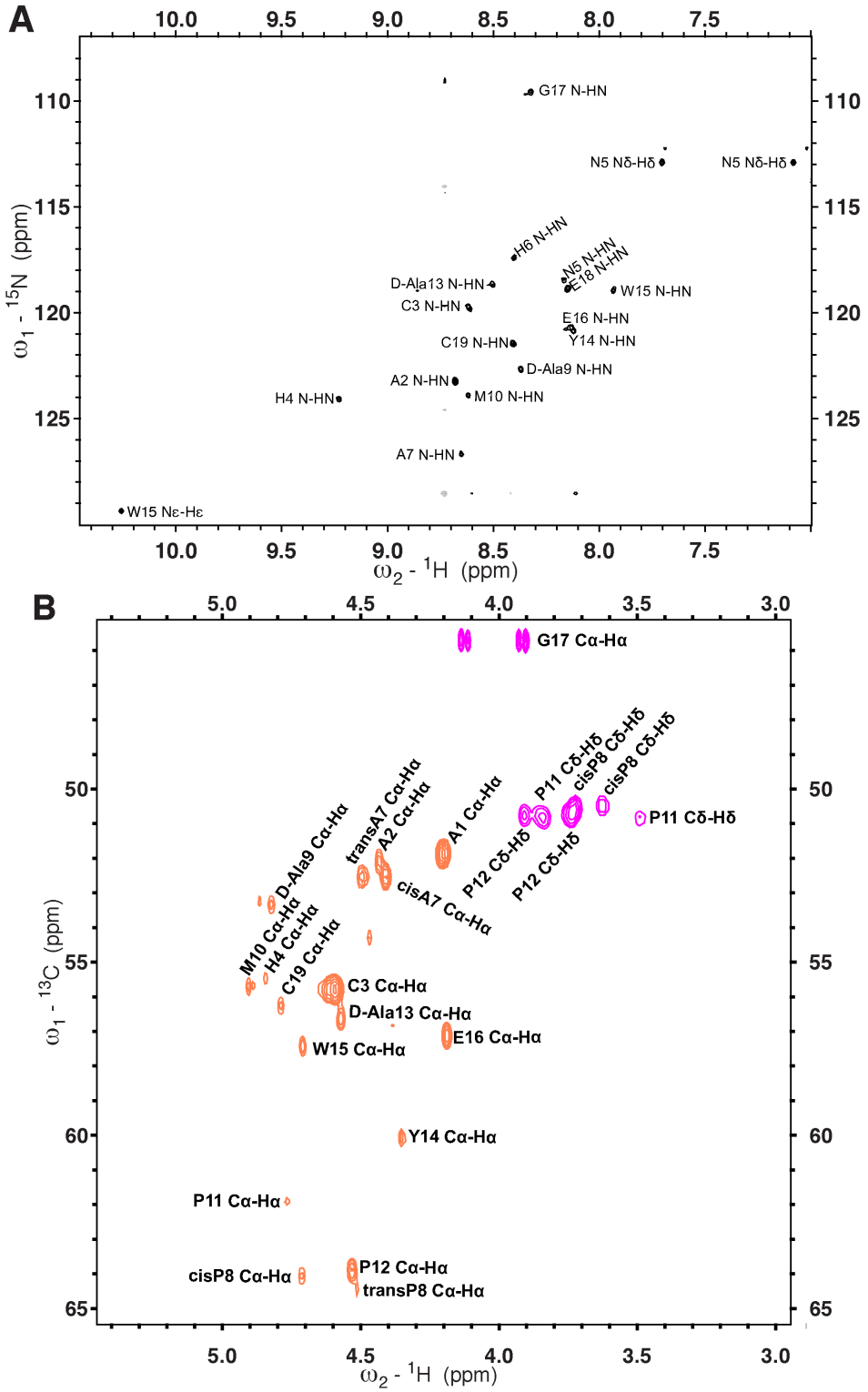

Figure S26 (continued). (C) ^{13}C HSQC spectrum of Pcn 2.8 acquired in 100% D_2O focused on the Cβ-Cε region.

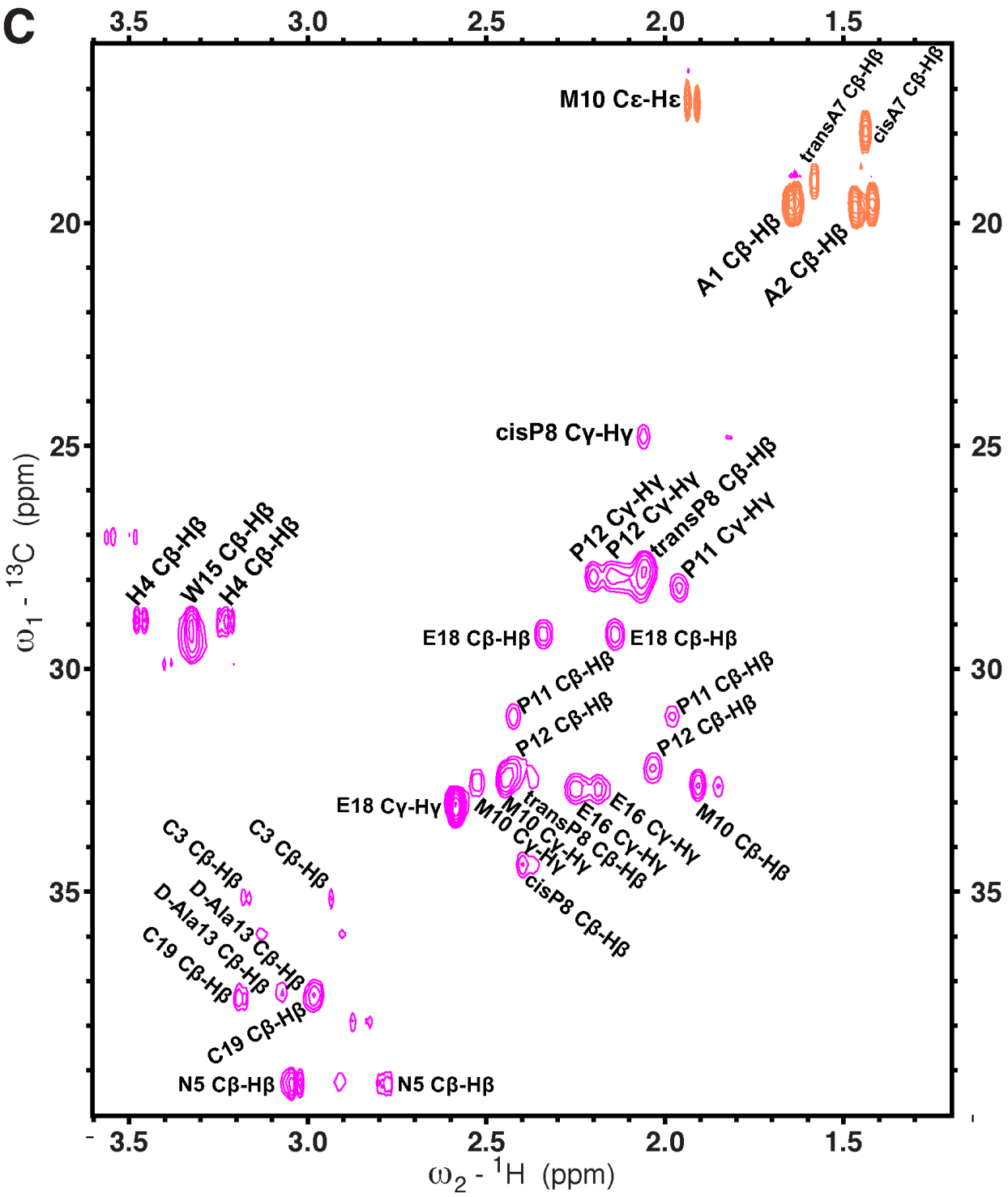

Figure S26 (continued) (D) Water-suppressed NOESY spectrum (mixing time = 0.30 s) of Pcn 2.8 acquired in 100% D_2O. NOESY contours are shown in maroon, TOCSY contours are shown in navy blue. The diagnostic marked nOe between the α protons confirms the peptide bond between Ala7 and Pro8 is *cis*.

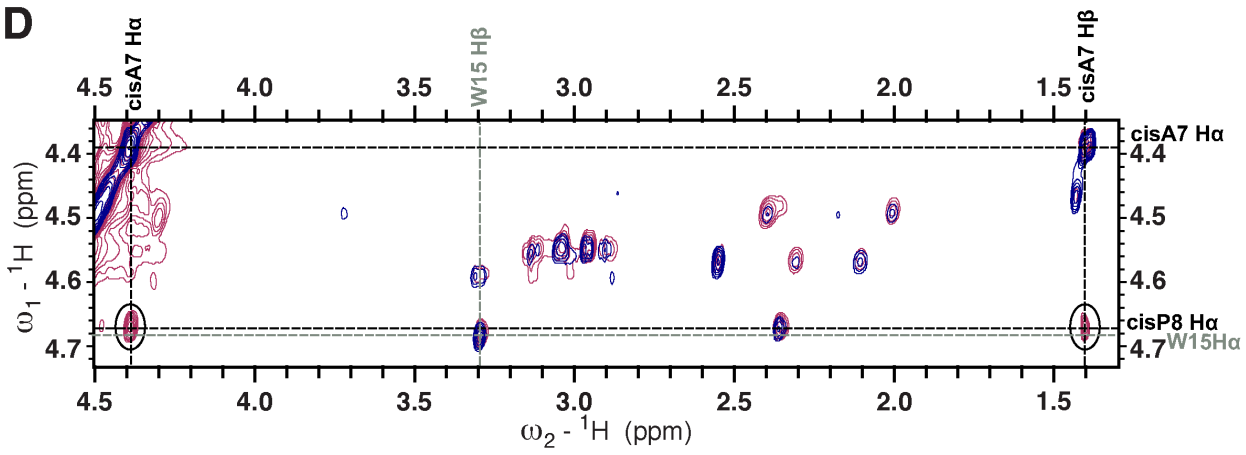

Figure S27. Ramachandran plots for Pcn 2.8. Ala7 is involved in a *cis*-peptide bond with Pro8 and is found in the disallowed areas of the Ramachandran plot.

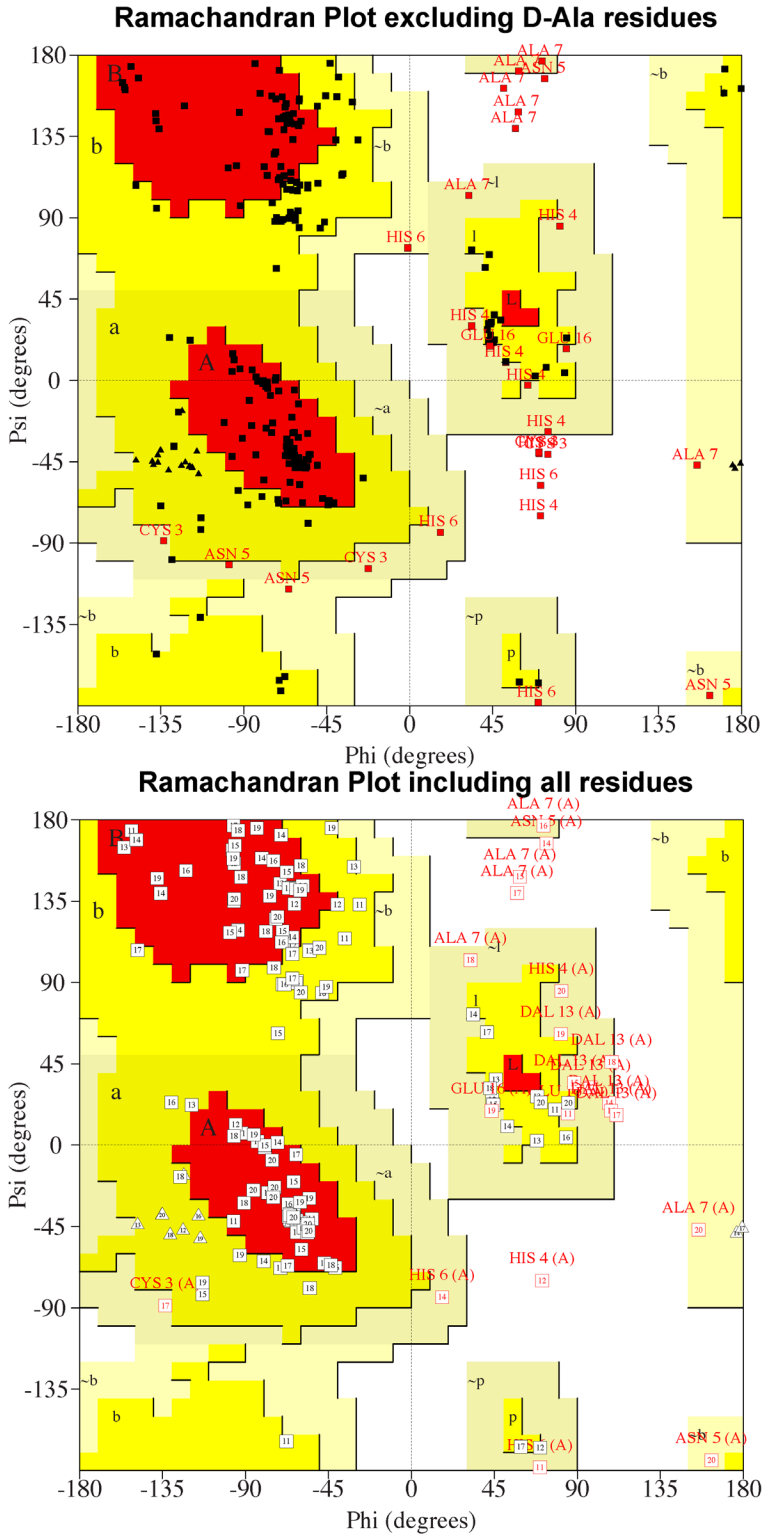

Table S10. Structural statistics for Pcn 2.8.

Procheck Analysis	**All L residues (%)**	**All residues (%)**
Residues in most favored regions [A,B,L]	60.9	60.8
Residues in additional allowed regions [a,b,l,p]	26.4	19.6
Residues in generously allowed regions [~a,~b,~l,~p]	8.6	17.7
Residues in disallowed regions	4.1	1.9
RMSD		
Residues	**Backbone only (Å)**	**All atoms (Å)**
1 to 19	1.32	1.96
Ring A (3 to 9)	1.57	2.63
Ring B (13 to 19)	1.07	1.31
10 to 12	0.67	0.91
Restraint Types Used		
Intra residue nOe	33	
Sequential nOe (|i-j|=1)	59	
Medium range nOe (1<|i-j|≤4)	44	
Long range nOe (|i-j|>4)	9	
Hydrogen bonding restraints	3	
Dihedral restraints	5	
Total	153	

Figure S28. Lowest energy structure ensemble for Pcn 2.8. (A) Superimposition of the 20 lowest energy 3D structures of Pcn 2.8; residues involved in thioether linkages are specified. (B) Superimposition of the 3D structures of Pcn 2.8 illustrating aromatic residues Tyr14 and Trp15 buried by thioether cyclized regions 3-9, 13-19 and the Met-Pro-Pro linker. (C) Superimposition of the 3D structures of Pcn 2.8 illustrating the proline residues. Pro11,12 restrict the conformation of the inter-thioether ring region.

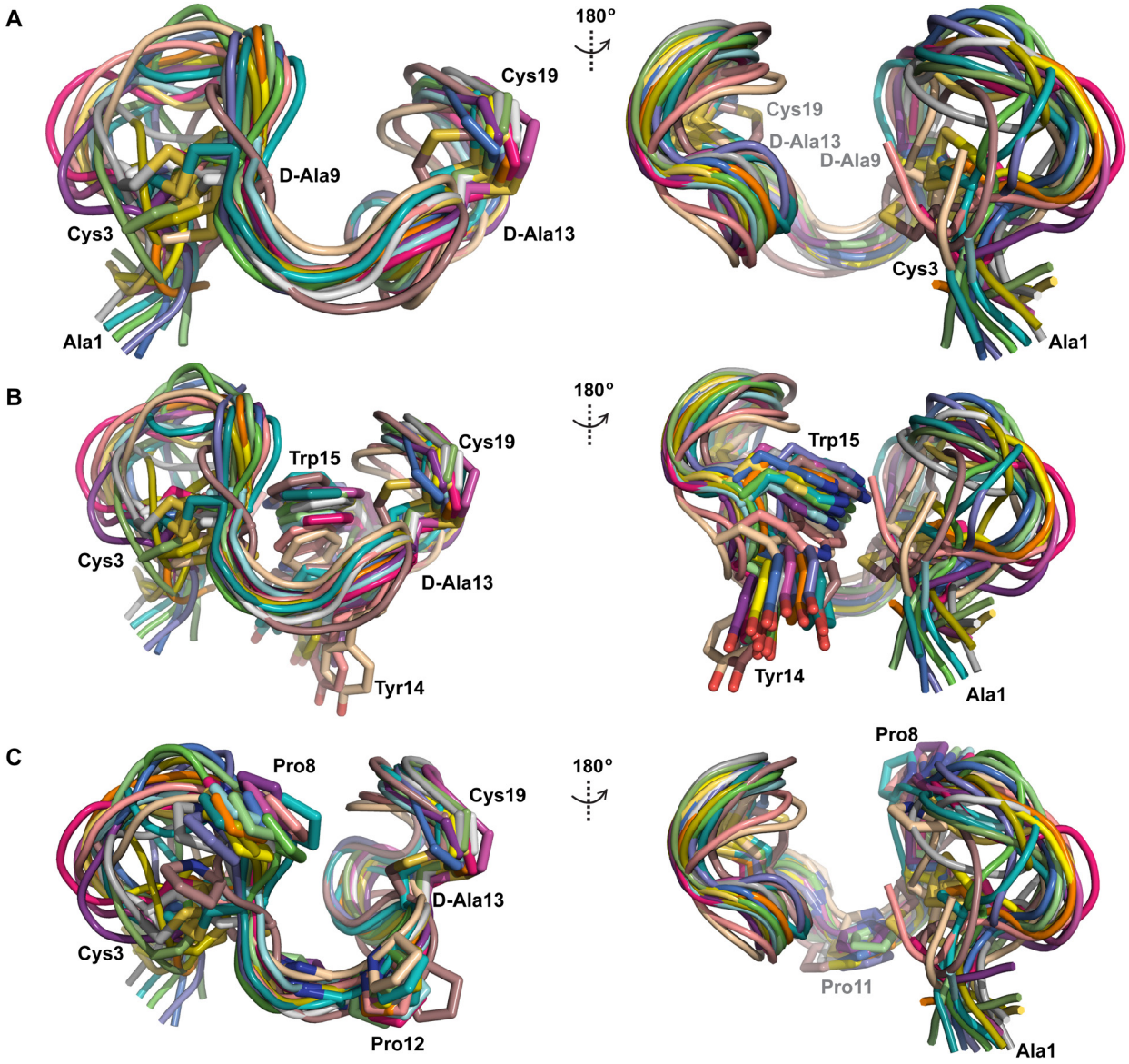

Figure S28 (continued). (D) View of the hydrogen bonding network between the thioether linkage spanning residues 13-19 and the Met-Pro-Pro region in 5 of the 20 lowest energy structures of Pcn 2.8.

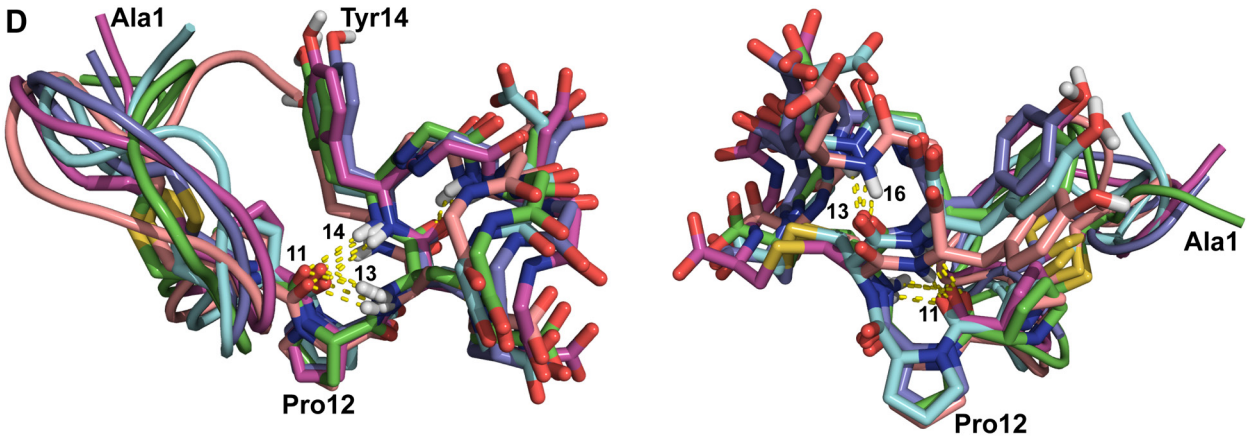

Table S11. Chemical shift assignments for CylL$_S$" in CD_3OH.

	HN	αH	βH	γH	Hδ+other protons	Notes
1 L-Abu		4.255 Cα: 61.800	3.855 Cβ: 47.300	1.408	-	-
2 Dhb		- Cα: 125.600	5.929 Cβ: 13.900	1.829	-	-
3 Pro	-	4.418 Cα: 64.500	2.415, 2.127 Cβ: 31.900	2.073	3.977, 3.768	-
4 Ala	8.209 N:119.1	4.258 Cα: 53.500	1.512 Cβ: 18.400	-	-	-
5 Cys	7.846 N:113.0	4.449 Cα: 58.200	3.329, 2.610 Cβ: 33.400	-	-	NOE from Cys5 HN to L-Abu1 Hα, β, γ
6 Phe	8.170 N:120.2	4.393 Cα: 59.700	3.295, 3.216 Cβ: 38.600	-	Hδ: 7.270 Hε: 7.260 Hζ: 7.209	-
7 Dhb	9.428 N:118.9	- Cα:135.800	6.295 Cβ: 14.600	1.670	-	-
8 Ile	7.891 N:117.3	3.906 Cα: 63.000	2.045 Cβ: 28.100	1.816 1.286, 1.222	Hδ: 0.949	-
9 Gly	8.067 N:110.2	3.873, 3.713 Cα: 45.700	-	-	-	-
10 Leu	8.071 N:119.7	4.076 Cα: 55.700	1.650 Cβ: 42.500	1.435	Hδ: 0.768	-
11 Gly	8.285 N:107.0	3.919, 3.794 Cα: 45.800	-	-	-	-
12 Val	8.483 N:118.1	3.701 Cα: 64.200	2.203 Cβ: 32.000	1.108, 0.982	-	-
13 Gly	8.592 N:108.6	3.950, 3.833	-	-	-	-
14 Ala	8.439 N:122.5	4.235 Cα: 53.700	1.564 Cβ: 18.500	-	-	-
15 Leu	8.081 N:117.4	4.068 Cα: 56.200	1.91, 1.797 Cβ: 42.100	1.665	Hδ: 0.939, 0.906	-

Table S11 (continued). Chemical shift assignments for CylL$_S$" in CD_3OH.

16 Phe	8.580 N:120.1	4.176 Cα: 60.200	3.330, 3.226 Cβ: 39.300	-	Hδ: 7.336 Hε: 7.187 Hζ: 7.088	-
17 D-Ala	9.188 N:122.1	4.025 Cα: 57.200	2.67 Cβ: 34.700	-	-	-
18 Ala	8.590 N: 122.5	3.971 Cα: 54.400	1.486 Cβ: 18.600	-	-	-
19 Lys	7.251 N:113.7	4.045 Cα: 57.000	1.234 Cβ: 33.900	0.931, 0.793	Hδ: 1.400; Hε: 2.707; Hζ: 7.750	-
20 Phe	8.038 N:114.4	4.849 Cα: 57.500	3.172, 2.091 Cβ: 40.200	-	Hδ: 6.918 Hε: 7.135	-
21 Cys	9.333 N:120.4	4.493 Cα: 57.500	3.636, 2.665 Cβ: 36.400	-	-	NOE from Cys21 HN to D-Ala17 Hα, β

Figure S29. (A) Amide region of the methanol-suppressed TOCSY spectrum (mixing time = 80 ms) identifying all spin systems of CylLs".

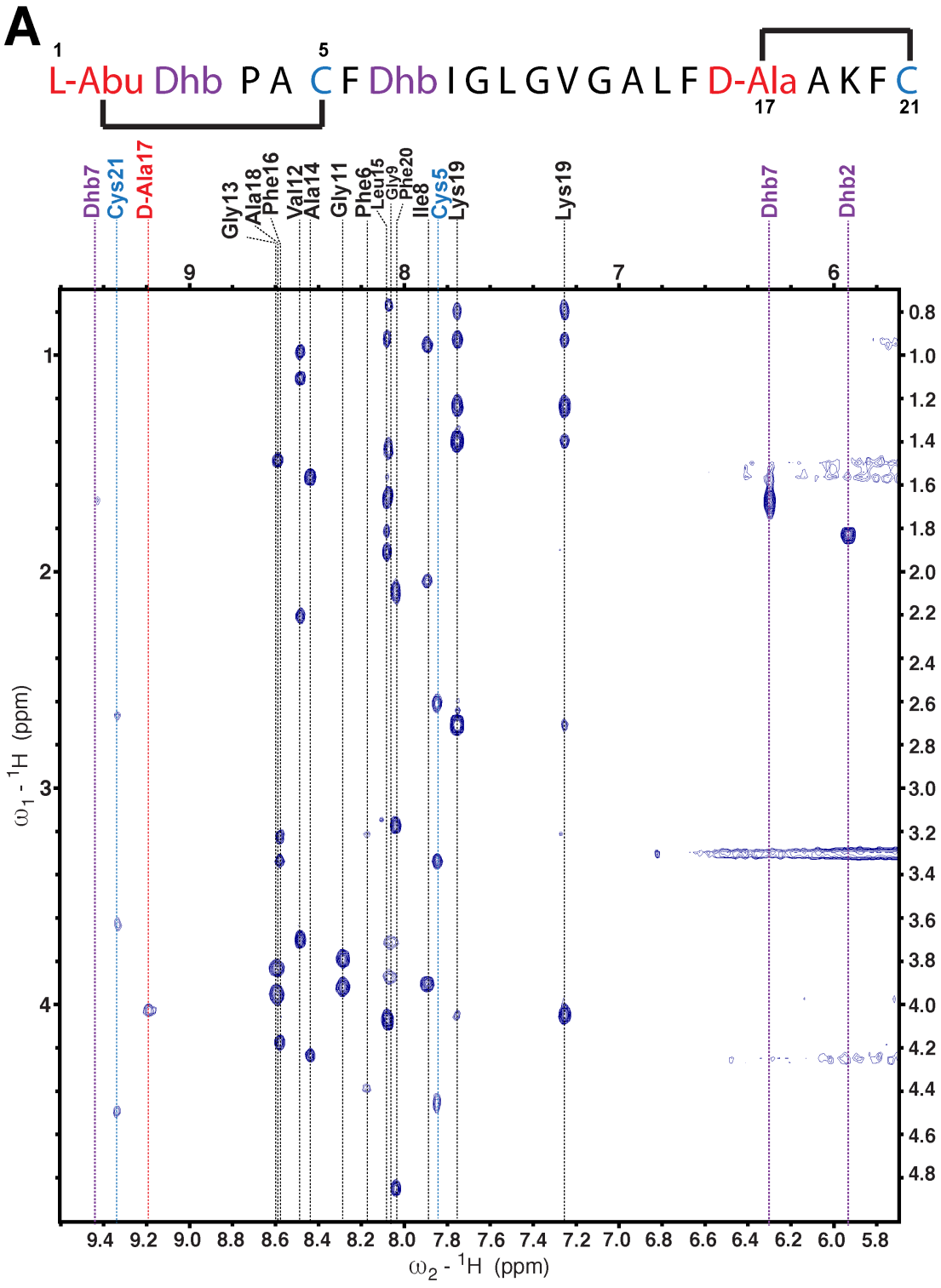

Figure S29 (continued). (B) Aliphatic region of methanol-suppressed TOCSY spectrum (mixing time = 80 ms) identifying residues of CylLs".

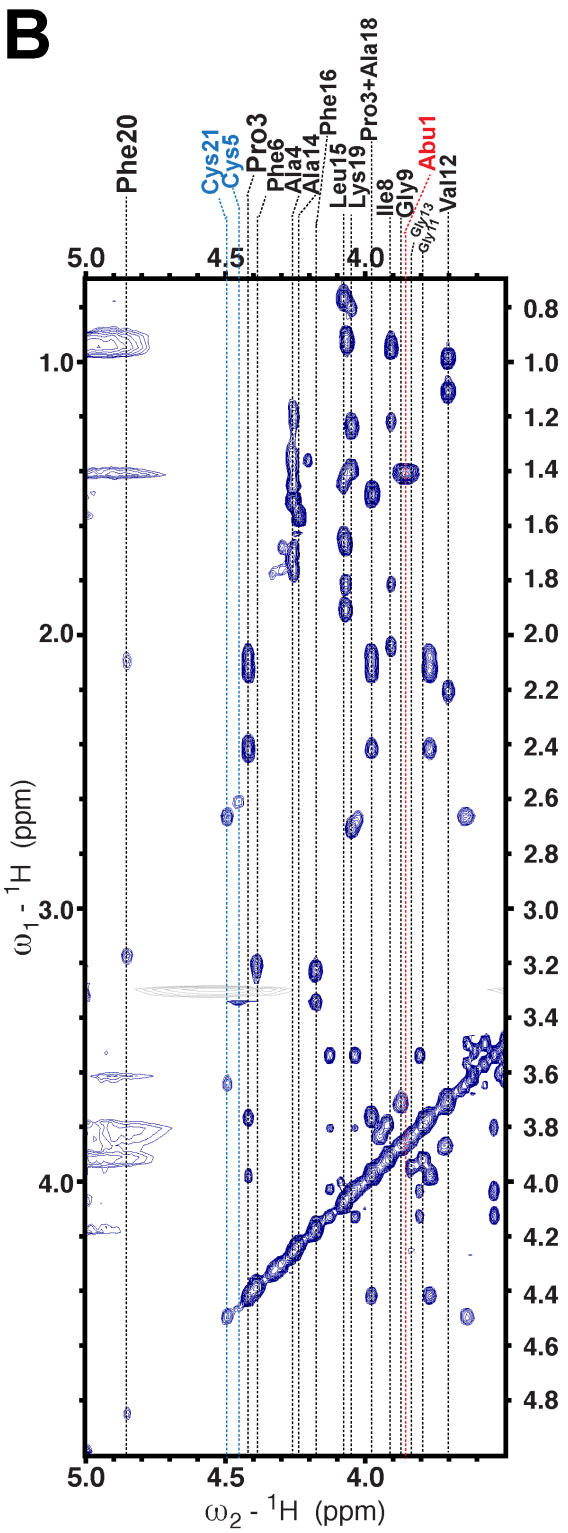

Figure S30. NOESY spectrum (mixing time = 0.30 s) for amide resonance assignments to amino acids from CylLs". NOESY contours are shown in maroon, TOCSY contours are shown in navy blue.

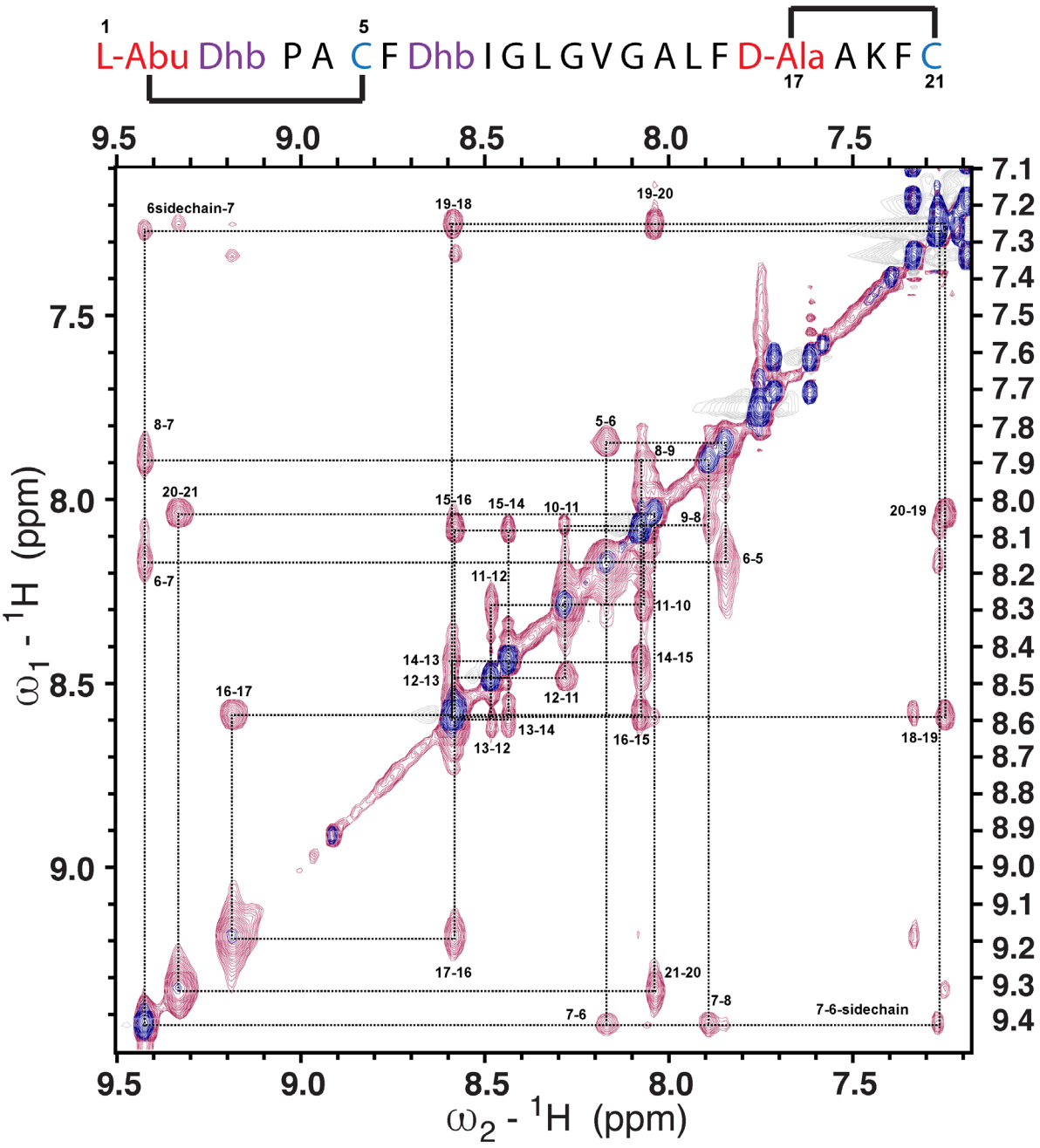

Figure S31. Methanol-suppressed NOESY spectrum of CylLs" in CD_3OH (mixing time = 0.30 s) for ring pattern assignment. NOESY contours are shown in maroon, TOCSY contours are shown in navy blue. (A) Structure of CylLs" (B) nOe signals establishing connection of Dhb2 to Pro3. (C) nOe signals for ring assignments are labeled in the aliphatic region of the spectrum.

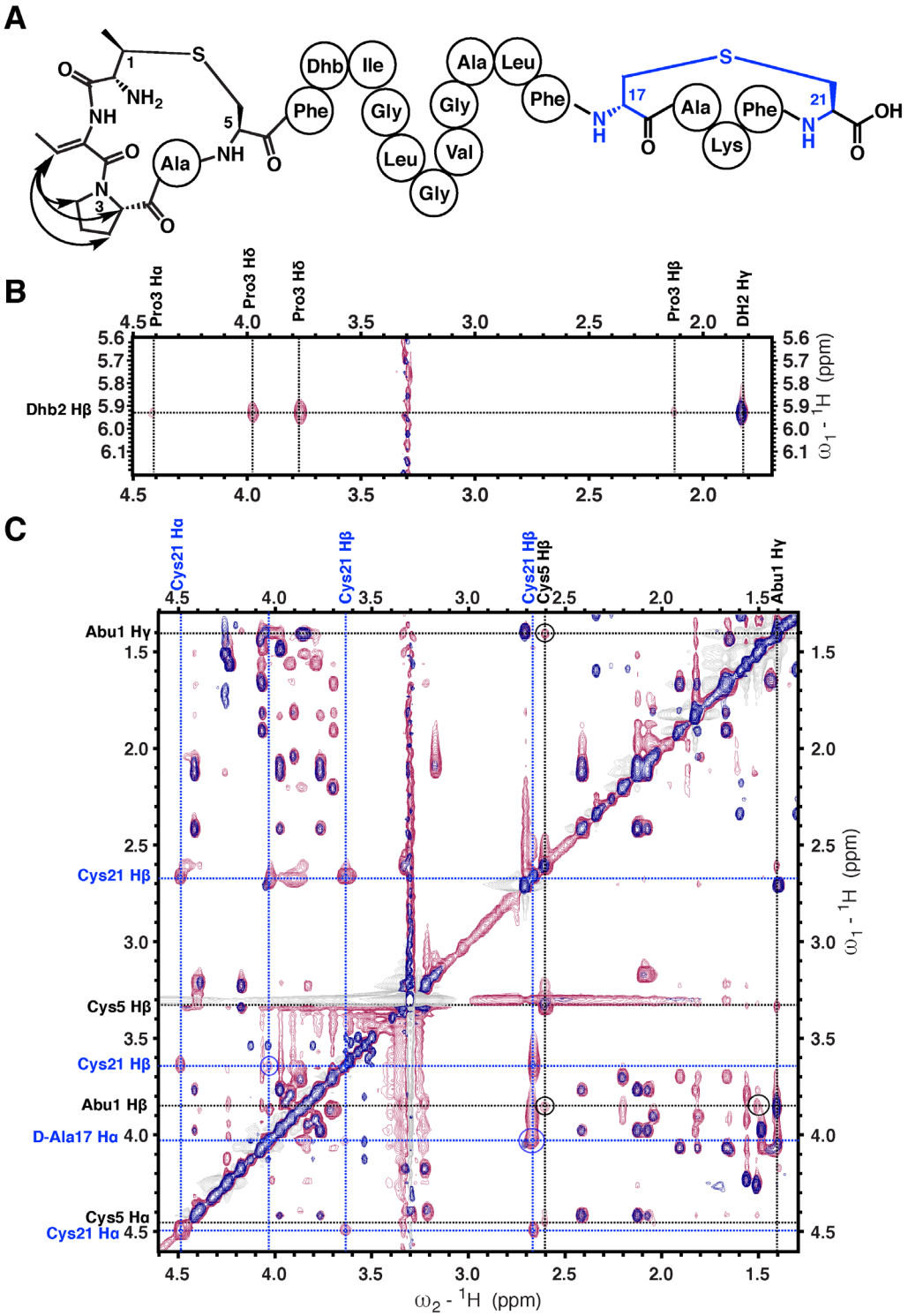

Figure S31 (continued) (D) nOe signals for assignment of the methyllanthionine ring between residues 1 and 5 are colored in black. (E) nOe signals for assignment of the lanthionine ring between residues 17 and 21 are colored in blue.

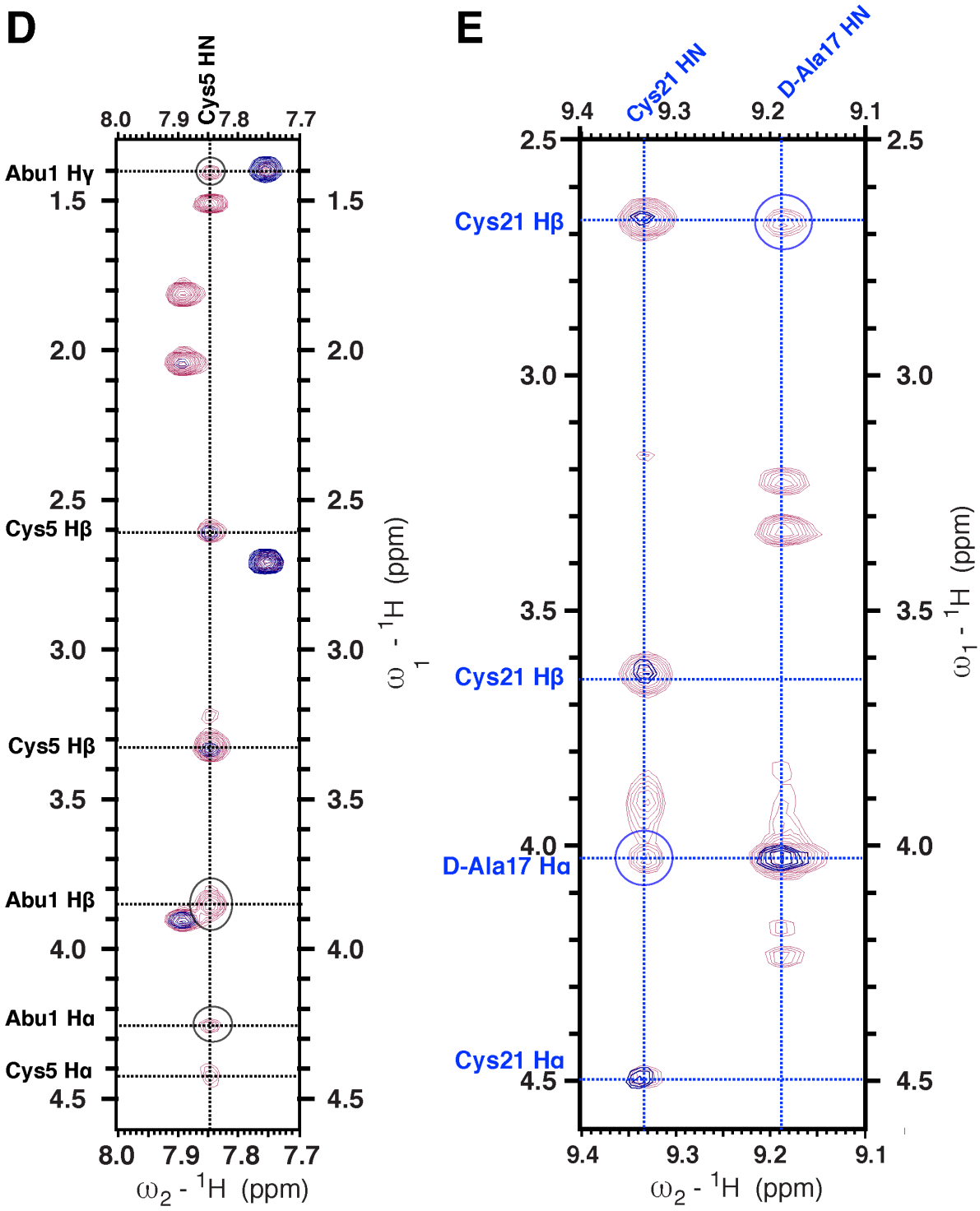

Figure S32. Ramachandran plots for CylLs". Dehydrobutyrine residues are found in disallowed areas of the Ramachandran plot.

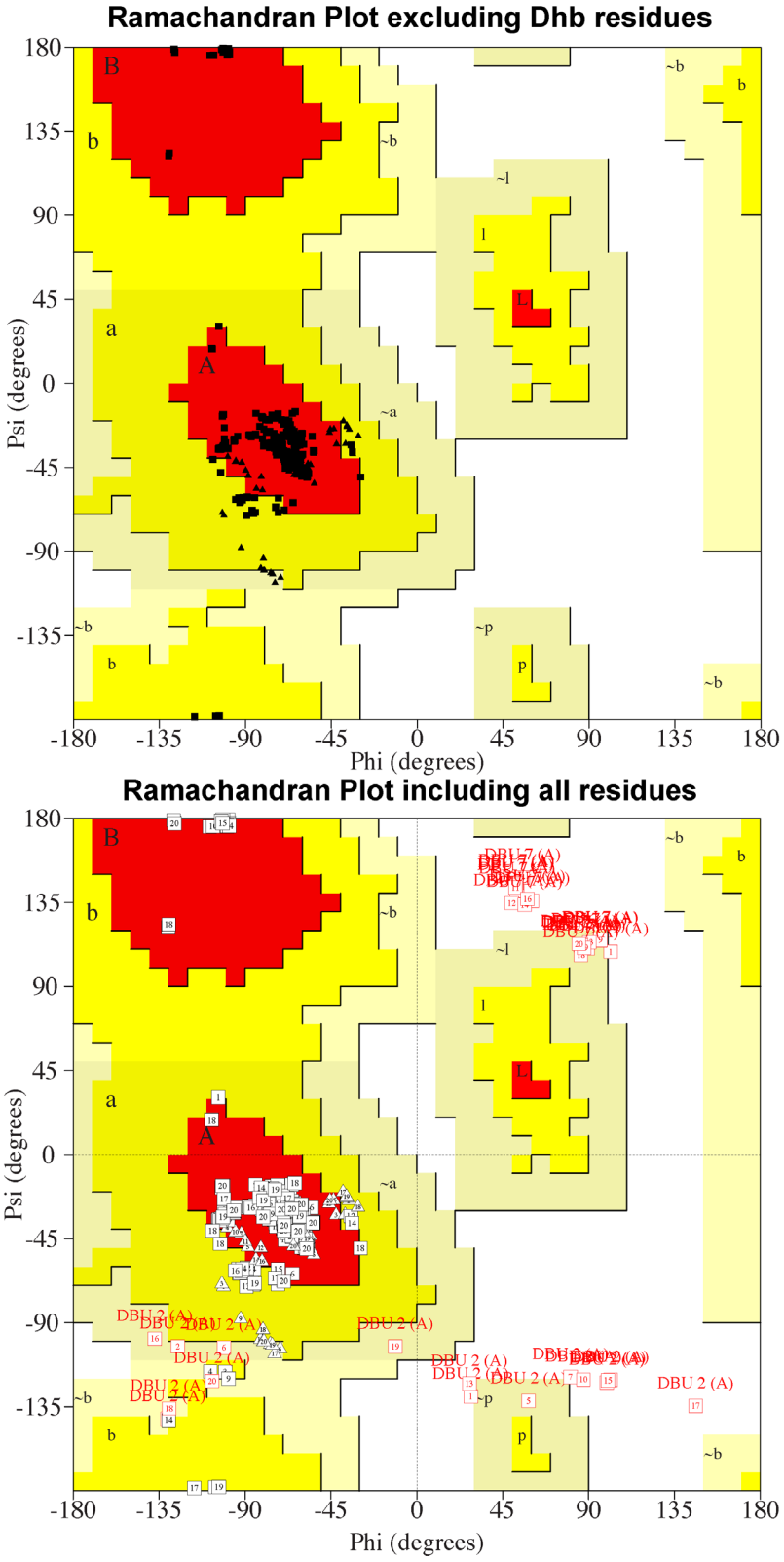

Table S12. Structural Statistics for CylLs".

Procheck Analysis	All residues excluding Dhb (%)	All residues (%)
Residues in most favored regions [A,B,L]	89.6	77.7
Residues in additional allowed regions [a,b,l,p]	10.4	10.3
Residues in generously allowed regions [~a,~b,~l,~p]	0	6.0
Residues in disallowed regions	0	6.0
RMSD		
Residues	**Backbone only (Å)**	**All atoms (Å)**
1 to 21	1.03	1.64
Ring A (1 to 5)	1.18	1.73
Ring B (17 to 21)	1.24	1.56
6 to 16	0.86	1.64
Restraint Types Used		
Intra residue nOe	119	
Sequential nOe (|i-j|=1)	92	
Medium nOe (1<|i-j|≤4)	60	
Long range nOe (|i-j|>4)	8	
Dihedral restraints	8	
Total	287	

Figure S33. Superimposition of the 20 lowest energy structures of CylLs". (A) Residues involved in thioethers and dehydroamino acid residues are marked. (B) Hydrogen bonds throughout the α helical region spanning residues 7 to 20 are shown as yellow dashed lines.

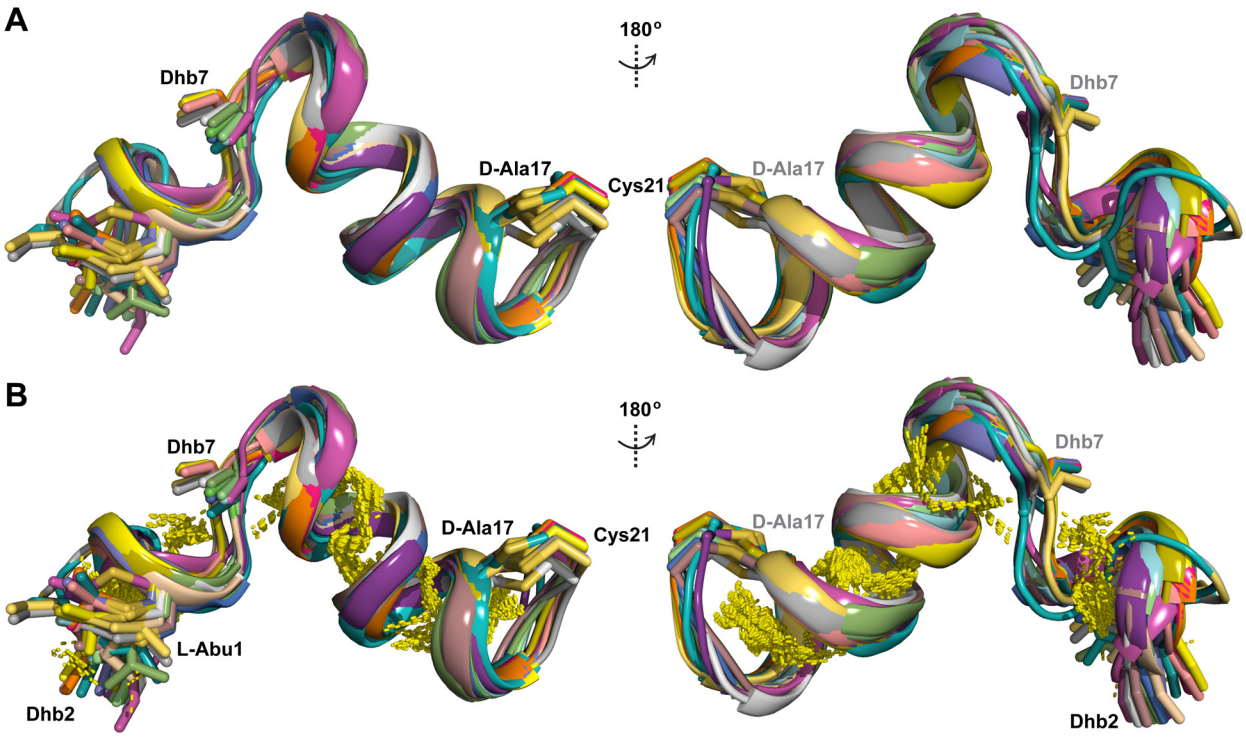

Table S13. Chemical shift assignments for CylL$_L$" in CD_3OH.

	HN	αH	βH	γH	Hδ+other protons	Notes
1 L-Abu		4.105		1.33		-
2 Dhb	n.d.	n.d.	5833			-
3 Pro	-	4.369	2.403, 2.089	2.121	4.014, 3.758	-
4 Val	7.527	4.369	2.285	1.100, 1.019	-	-
5 Cys	7.521	3.680	3.182, 2.560	-	-	NOE from Cys5 Hβ to L-Abu1 Hβ, γ
6 Ala	8.217	4.027	1.461			-
7 Val	7.824	3.570	2.163	1.116, 0.953	-	-
8 Ala	8.049	4.097	1.393			-
9 Ala	8.214	4.149	1.442		-	-
10 Dhb	9.171	-	6.291	1.760		-
11 Ala	8.285	4.113	1.572	-	-	-
12 Ala	8.040	4.059	1.501	-	-	-
13 Ala	8.043	4.024	1.384	-	-	-
14 L-Ala	7.945	4.308	3.326, 3.0	-	-	NOE from L-Ala14 HN to Cys18 Hβ
15 Dha	9.388	-	5.635	-	-	-
16 Ala	8.944	4.126	1.482			-
17 Ala	8.314	4.210	1.637	-	-	-
18 Cys	8.017	3.955	3.233, 2.885	-	-	NOE from Cys18 HN to L-Ala14 Hβ
19 Gly	8.447	3.861, 3.770				-
20 Trp	8.365	4.484	3.405, 3.328	-	Hδ: 7.141; Hε: 10.35, 7.54; Hζ: 7.279, 6.969; Hη: 7.052	-
21 Val	8.172	3.772	2.173	1.119, 0.972	-	-

Table S13 (continued). Chemical shift assignments for CylL$_L$" in CD_3OH.

22 Gly	8.435	3.860, 3.770	-	-	-	-
23 Gly	8.419		-	-	-	
24 Gly	8.283	3.875	-	-	-	-
25 Ile	8.274	3.835	1.955	1.722, 1.128	1.106, 0.851	-
26 Phe	8.303	4.221	3.205	-	Hδ: 7.225; Hε: 7.220 Hζ: 7.000	-
27 Thr	8.180	3.726	4.23	1.188	-	-
28 Gly	8.181	3.844	-	-	-	-
29 Val	8.307	3.614	2.303	1.100, 0.914	-	-
30 Dhb	9.342	-	6.135	1.360	-	-
31 Val	8.137	3.752	2.313	1.099, 0.951	-	-
32 Val	7.663	3.602	2.298	1.102, 0.957	-	-
33 Val	8.433	3.497	2.066	0.932, 0.906		
34 D-Ala	8.717	3.996	2.596	-	-	NOE from D-Ala34 Hα to Cys38 Hα,β
35 Leu	8.641	4.069	2.016, 1.478	1.971	0.906	-
36 Lys	7.565	4.290	1.671	1.392	Hδ: 1.554 Hε: 2.850, 2.803	-
37 His	8.360	4.985	3.405, 2.904		Hδ: 7.288 Hε:8.771,8.525 Hζ: 8.774	-
38 Cys	9.478	4.619	3.637, 2.741			NOE from Cys38 Hβ to D-Ala34 Hα,β

Figure S34. Methanol-suppressed TOCSY spectrum (mixing time = 80 ms) identifying residues in CylLL".

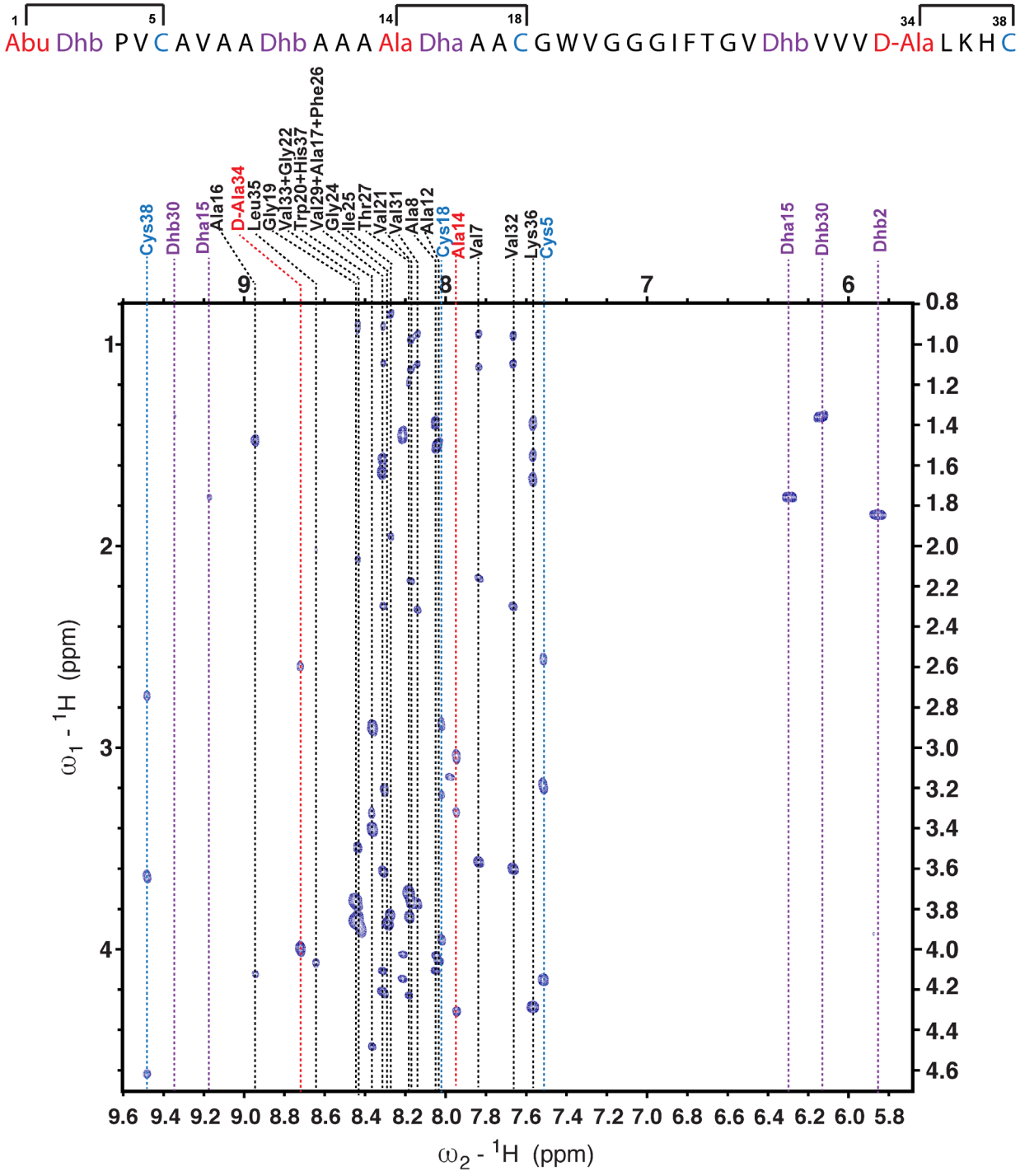

Figure S35. NOESY spectrum (mixing time = 0.35 s) for amide resonance assignments to amino acids from CylL$_L$".

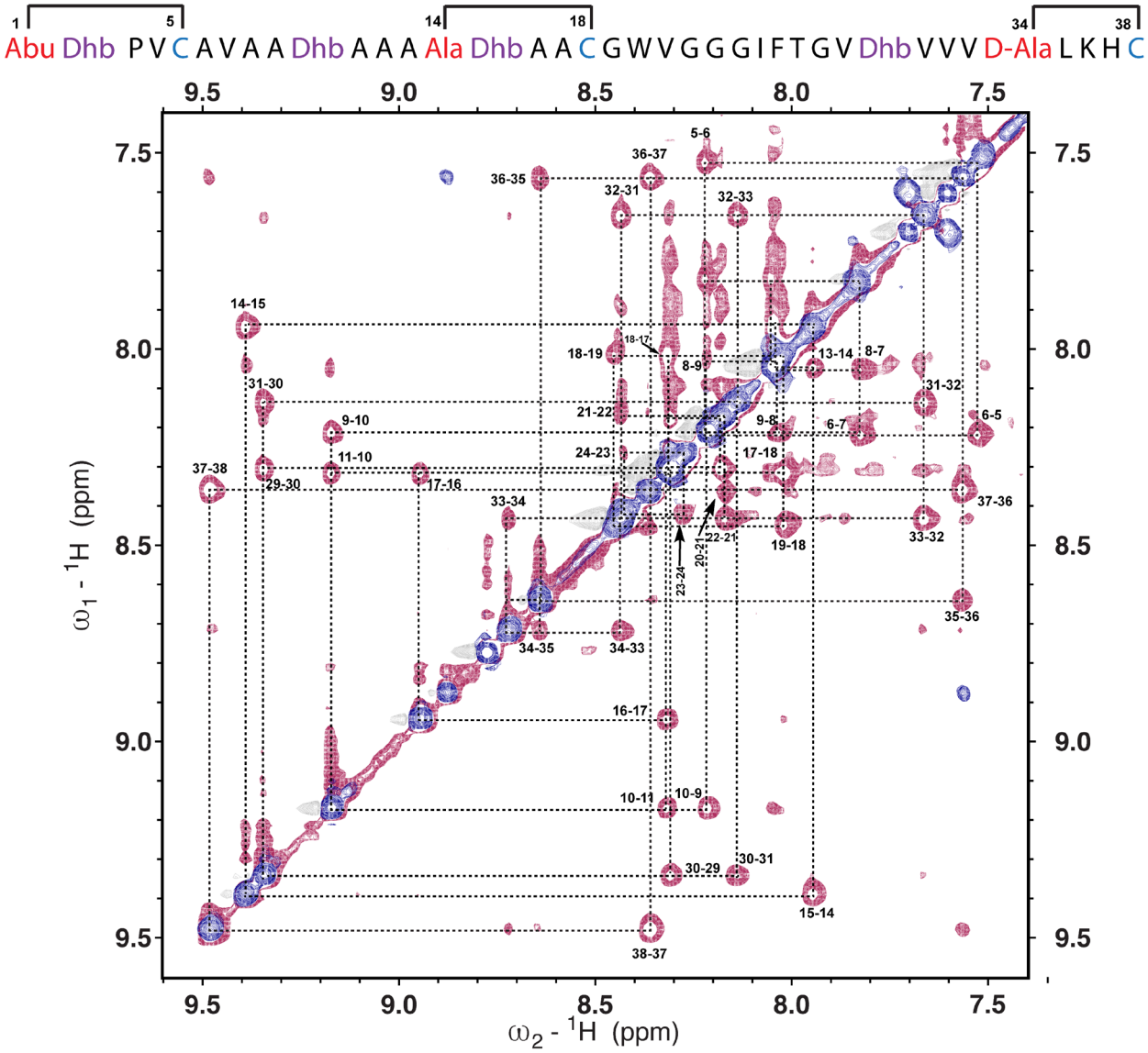

Figure S36. Methanol-suppressed NOESY spectrum of CylL$_L$" in CD_3OH (mixing time = 0.35 s) for ring pattern assignment. NOESY contours are shown in maroon, TOCSY contours are shown in navy blue. (A) Structure of CylL$_L$" (B) nOe signals for assignment of the methyllanthionine ring between residues 1 and 5 are colored in black. (C) nOe signals for assignment of the lanthionine between residues 14 and 18 are colored in blue.

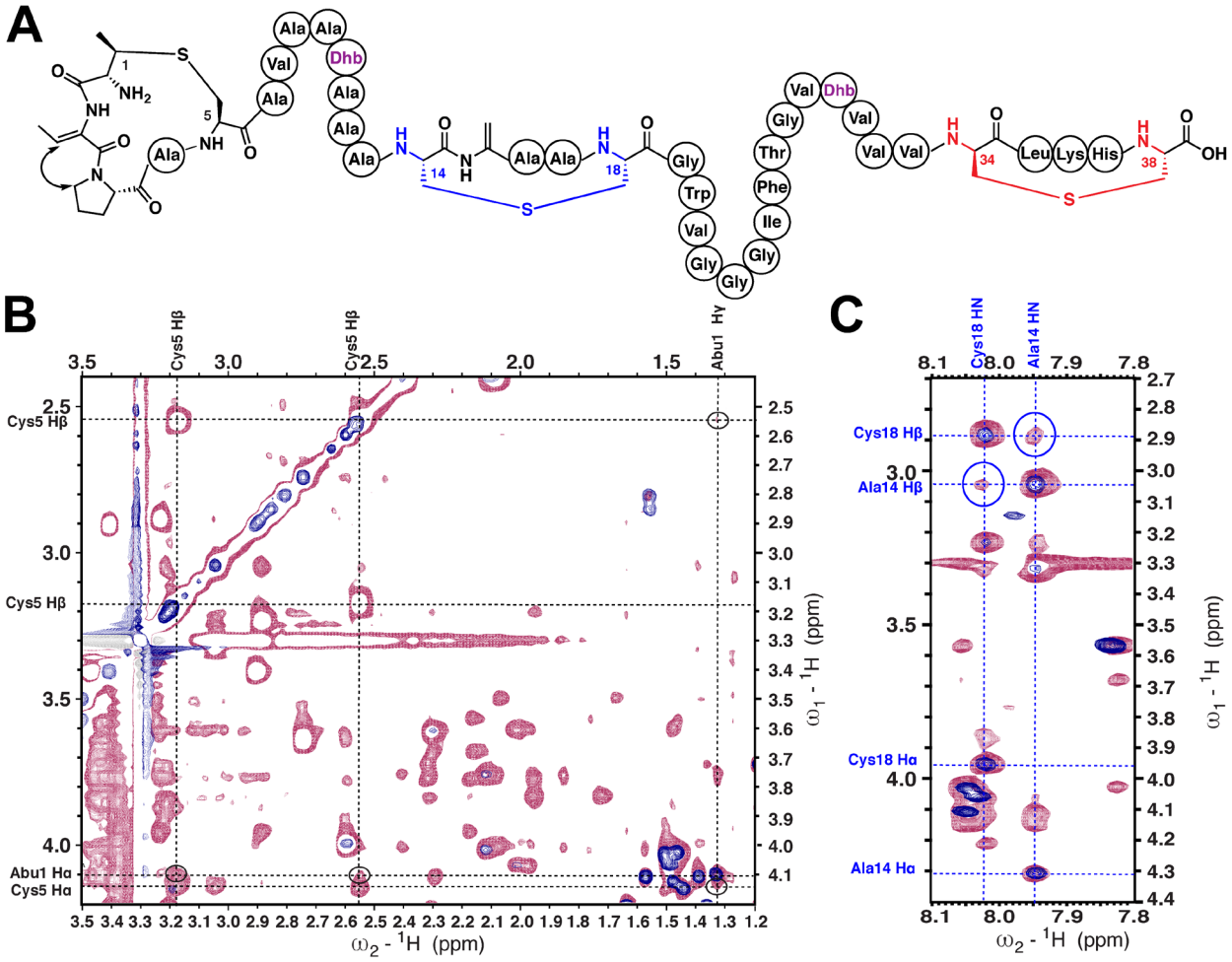

Figure S36 (continued) (D) nOe signals for assignment of the lanthionine involving residues 14 and 18 in CylL$_L$" are colored in blue.

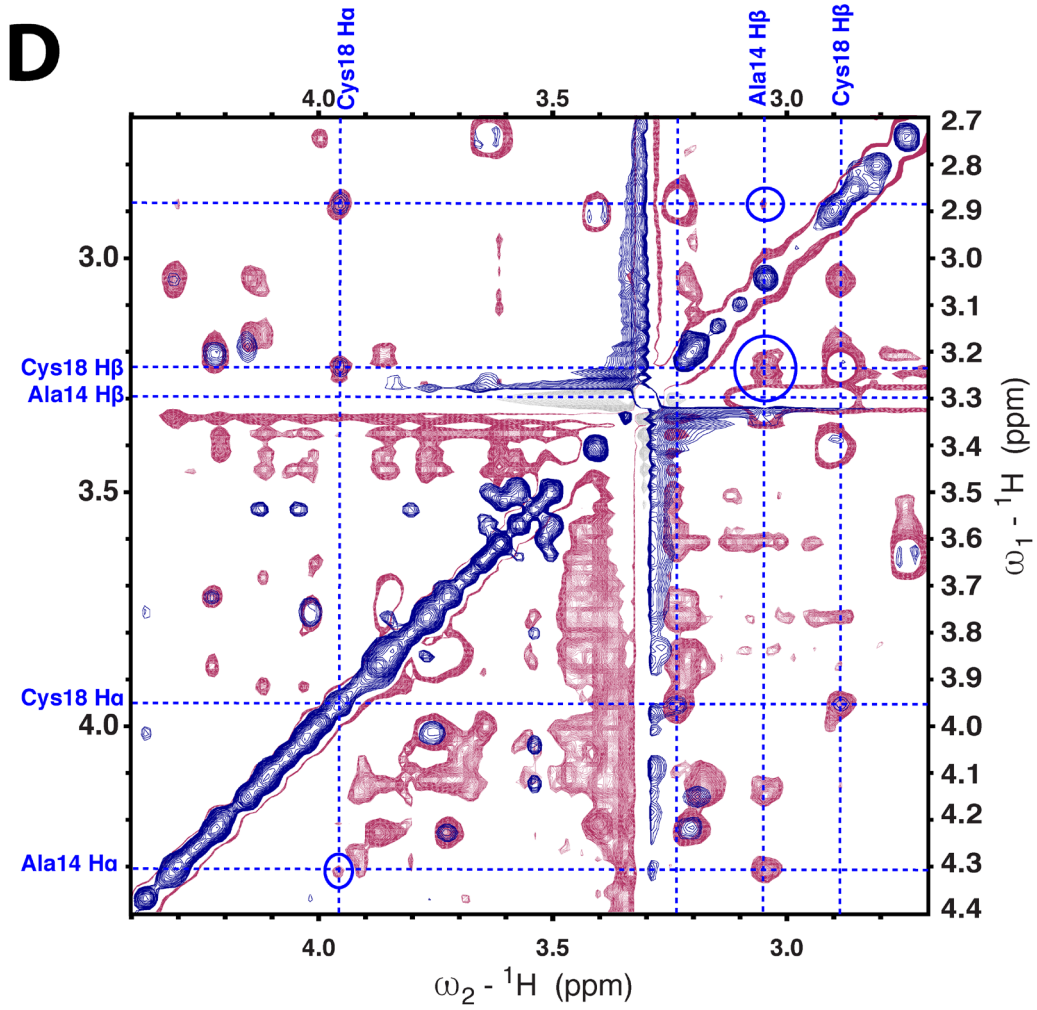

Figure S36 (continued) (E) nOe signals for assignment of the lanthionine ring involving residues 34 and 38 in CylL$_L$" are colored in red.

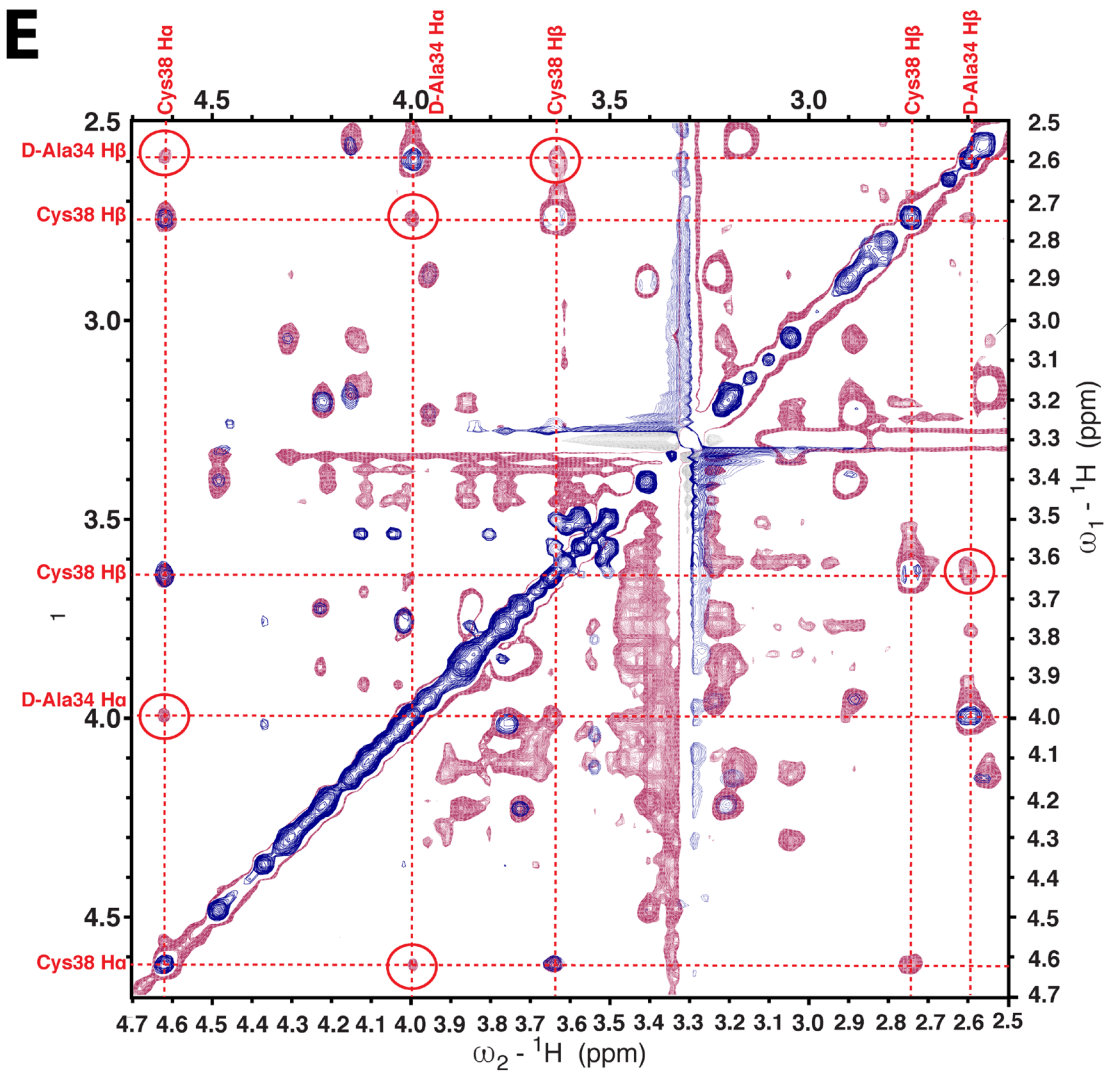

Figure S36 (continued) (F) nOe signals for assignment of the lanthionine ring involving residues 34 and 38 in CylL$_L$" are colored in red **(**G) nOe signals establishing connection of Dhb2 to Pro3 in CylL$_L$".

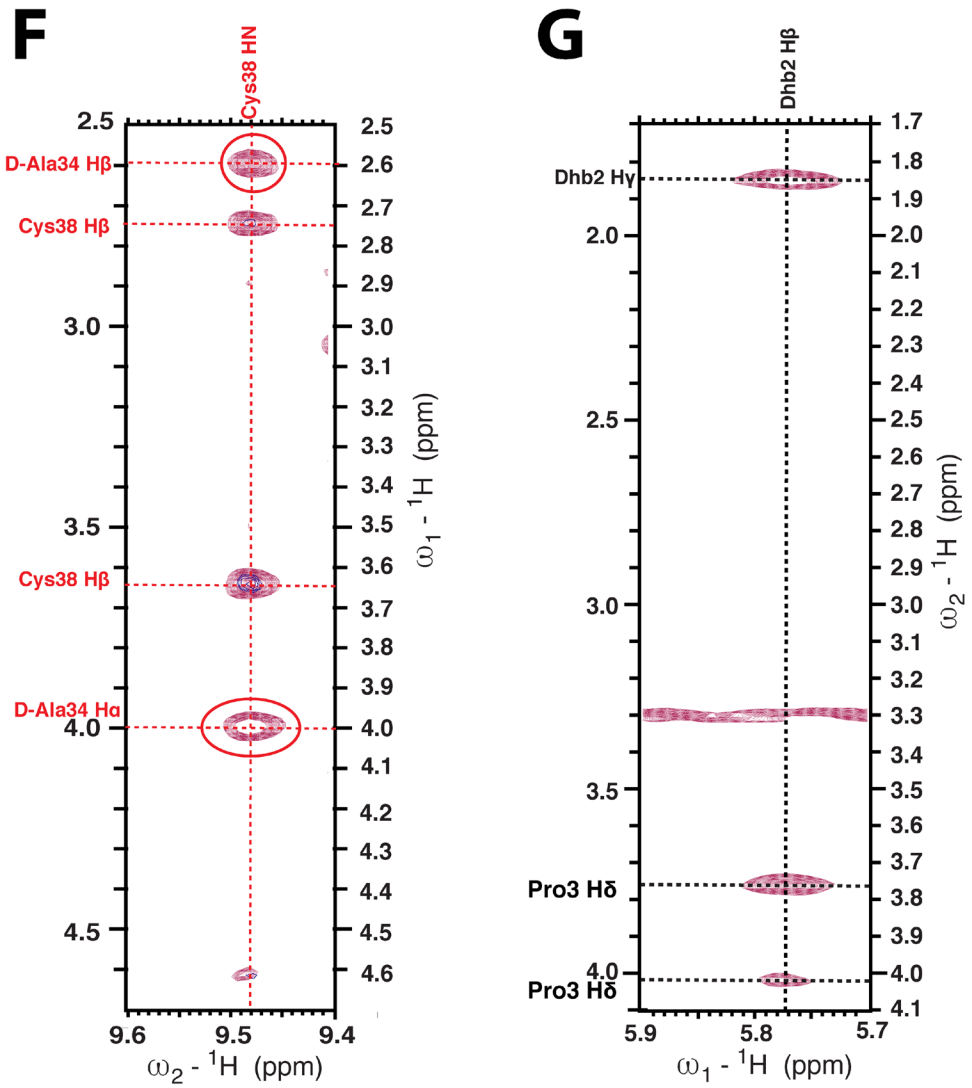

Figure S36 (continued). (H) nOe signals establishing connectivity between Dha15 β protons and Ala16. Weak nOes are indicated with grey arrows, while strong correlations are indicated with black arrows.

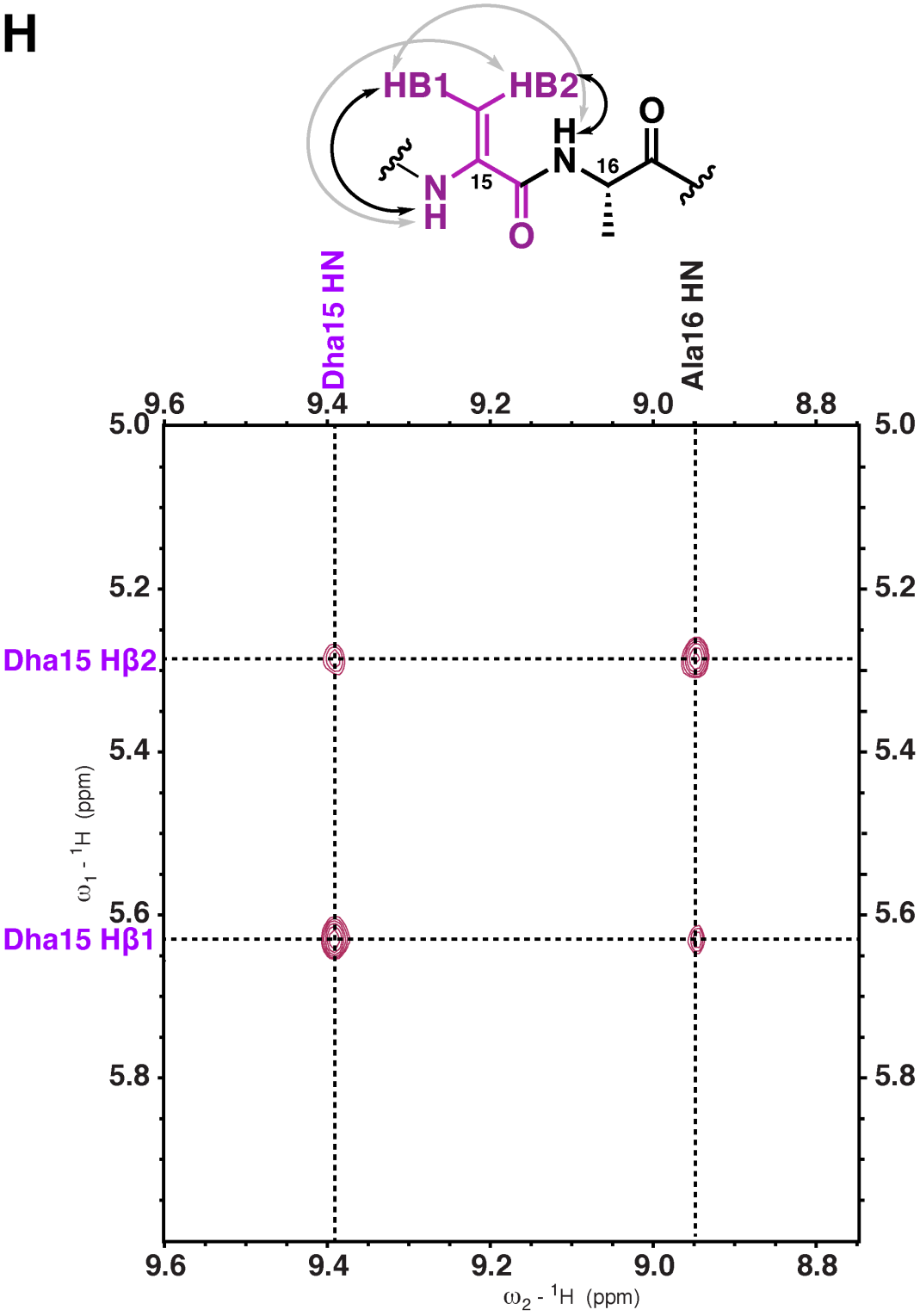

Figure S37. Ramachandran plot for CylL$_L$". Residues Dhb10, 30 and Dha15 are found in disallowed and generously allowed areas.

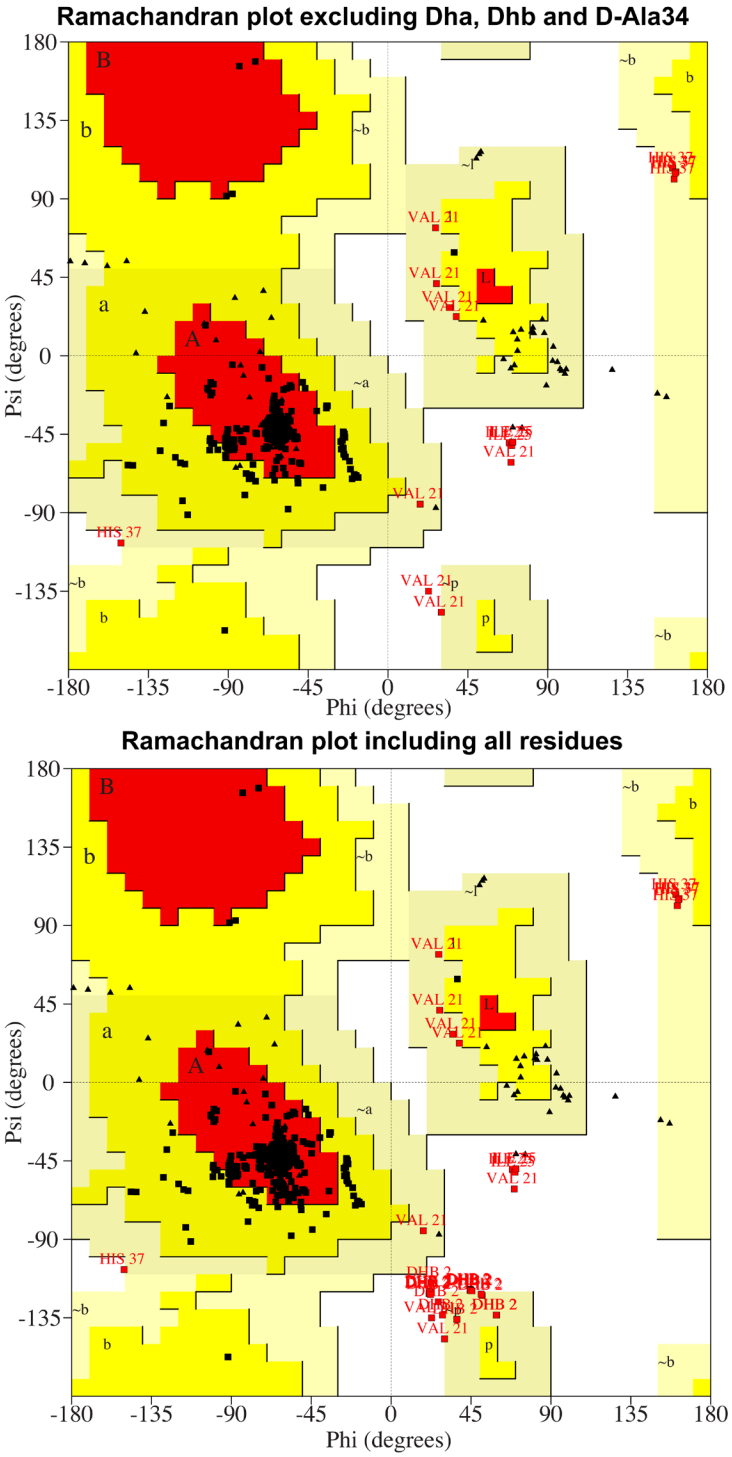

Table S14. Structural statistics for $CylL_L$".

Procheck Analysis	**All L residues (%)**	**All residues (%)**
Residues in most favored regions [A,B,L]	84.6	83.0
Residues in additional allowed regions [a,b,l,p]	12.3	11.0
Residues in generously allowed regions [~a,~b,~l,~p]	1.9	2.5
Residues in disallowed regions	1.2	3.5
RMSD Residues	**Backbone only (Å)**	**All atoms (Å)**
1 to 38 using average structure as reference	4.74	5.69
Ring A (1 to 5) using average structure as reference	5.87	6.78
Ring B (14 to 18) using average structure as reference	3.98	4.25
Ring C (34 to 38) using average structure as reference	7.28	8.17
Helix A (2 to 12) using average structure as reference	4.23	5.10
Helix A (2 to 12) using Helix A as reference only	0.41	0.99
Helix B (16 to 20) using Helix B as reference only	0.62	2.17
Helix C (24 to 38) using average structure as reference	7.62	8.80
Helix C (24 to 38) using Helix C as reference only	1.10	1.57
Restraint Types Used		
Intra residue nOe	169	
Sequential nOe ($\|i-j\|=1$)	95	
Medium range nOe ($1<\|i-j\|\leq 4$)	98	
Long range nOe ($\|i-j\|>4$)	0	
Dihedral restraints	11	
Hydrogen bonding restraints	20	
Total	395	

Figure S38. Superimposition of the 20 lowest energy structures of CylL$_L$". Residues involved in thioethers and dehydroamino acid residues are marked. (A, B) Two depictions of the ensemble with the peptide backbone aligned between residues 2 and 20. The region of the peptide between residues 2 and 20 is a well-defined helix with an interruption at the bond between former Ser14 and former Ser15 where a thioether and a Dha are present in CylL$_L$". (C, D) Two depictions of the ensemble with the peptide backbone aligned between residues 24 and 38. The region of the peptide between residues 24 and 38 is helical.

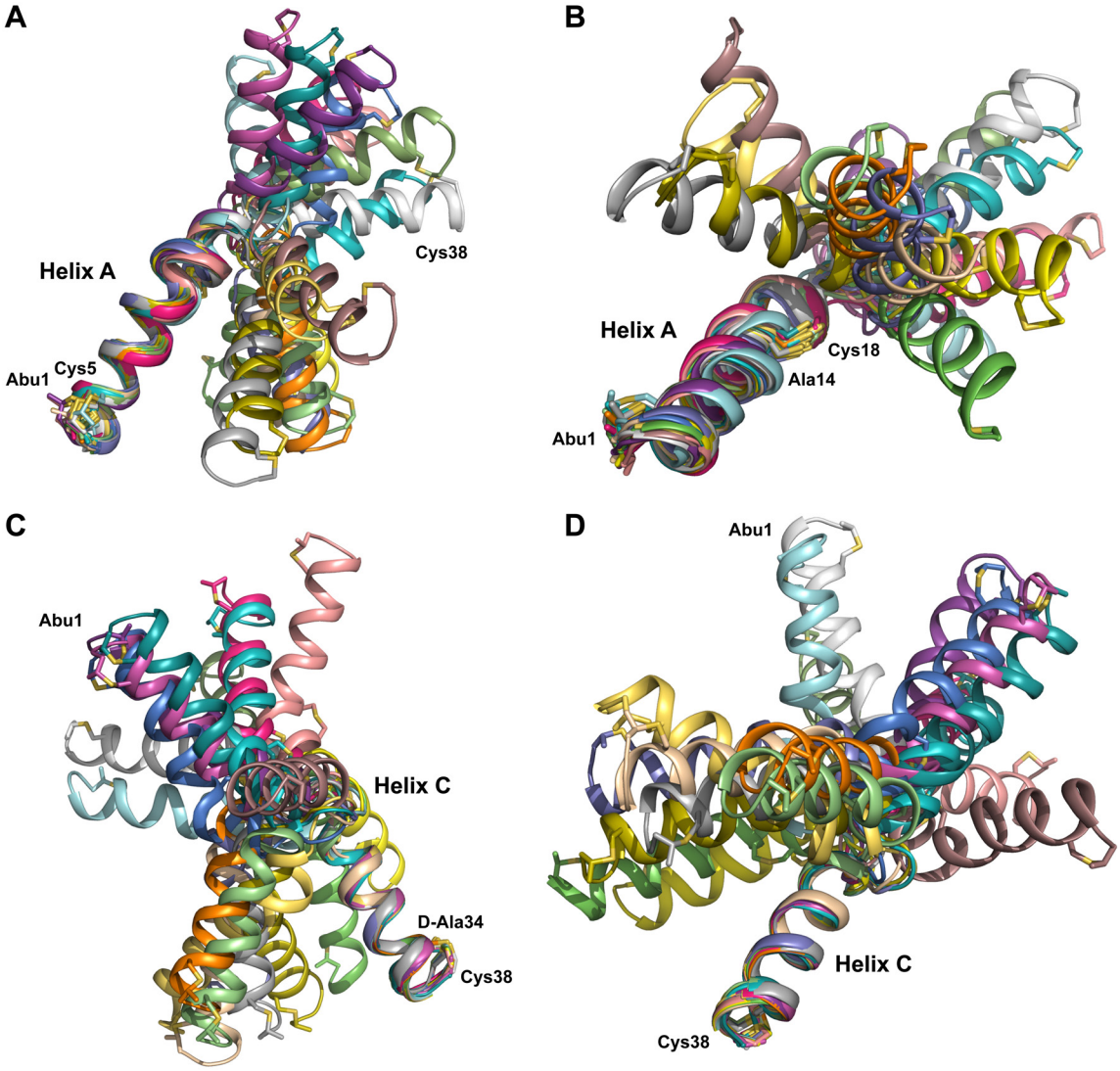

Figure S39. Hydrogen bonding in CylL$_L$". The N- and C-termini are marked. (A) Depiction of the 20 lowest energy structures aligned over residues 2 to 20. Hydrogen bonds spanning residues 2 to 20 are highlighted in yellow dashed lines, Hydrogen bonds in other parts of the structure are not highlighted for clarity. (B) Depiction of the 20 lowest energy structures aligned over residues 24 to 38. Hydrogen bonds spanning residues 24 to 38 are highlighted in yellow dashed lines. Hydrogen bonds in other parts of the structure are not highlighted for clarity.

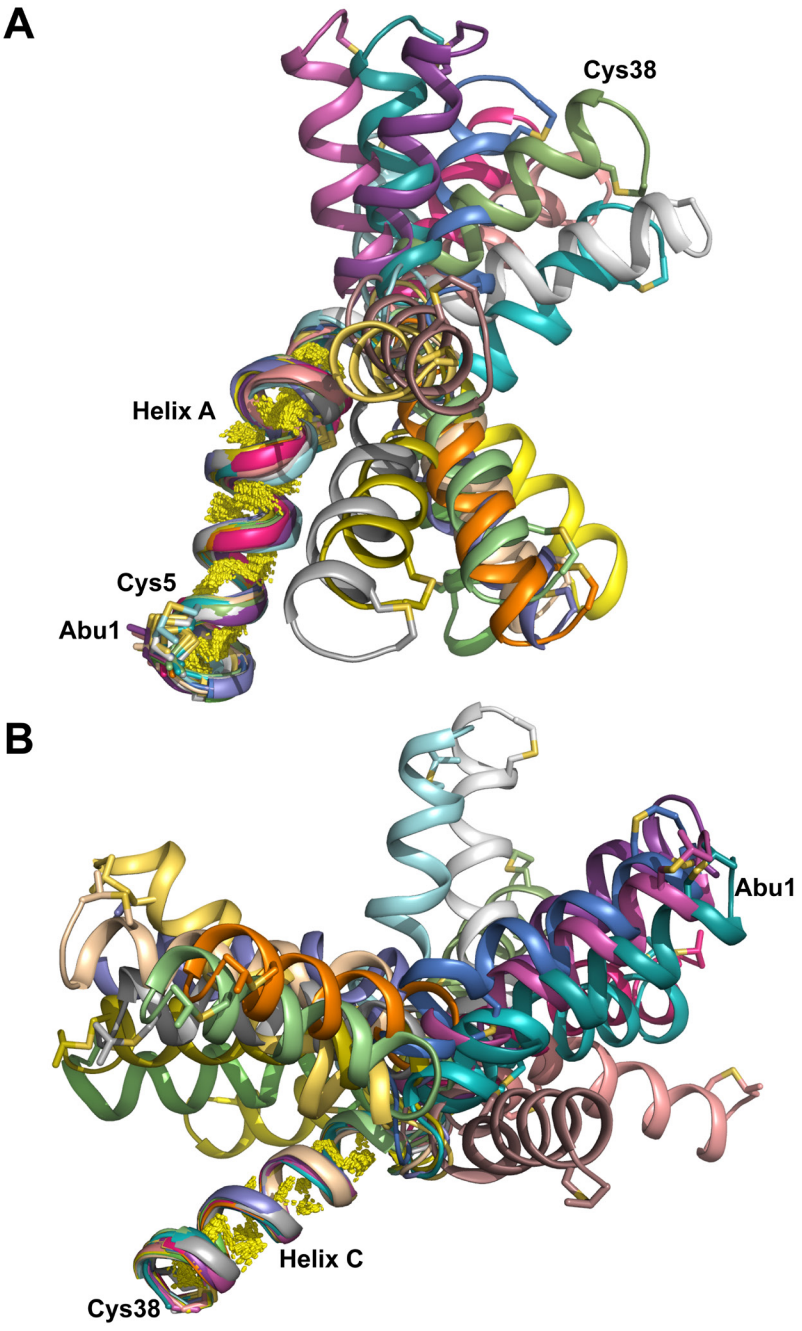

Table S15. Summary of DNA constructs used in this work and previous studies.

	Identifiers	Reference
pRSFDuet His6-ProcA1.1 G-1E (MCSI) – ProcM (MCSII)	pRSFDuet 1.1 / M	[1]
pRSFDuet His6-ProcA2.1 (MCSI) – ProcM (MCSII)	pRSFDuet 2.1/M	[2]
pRSFDuet His6-ProcA2.8 (MCSI) – ProcM (MCSII)	pRSFDuet 2.8/M	[3]
pRSFDuet His6-ProcA2.10 (MCSI) – ProcM (MCSII)	pRSFDuet 2.10/M	[2]
pRSFDuet His6-ProcA2.11 G-1K (MCSI) – ProcM (MCSII)	pRSFDuet 2.11/M	[3]
pRSFDuet His6-CylLL (MCSI) – CylM (MCSII)	pRSFDuet CylLL/M	[4]
pRSFDuet His6-CylLS(MCSI) – CylM (MCSII)	pRSFDuet CylLS/M	[4]
pETDuet His6-LahT150 (MCSI)	pETDuet LahT	[2]
pRSFDuet His6-CylA (MCSI)	pRSFDuet CylA	[4, 5]

Table S16. Primers and synthetic genes used in the cloning of ProcA 2.1 and 2.10 constructs. Homology with pRSFDuet-1 vector backbone is displayed as lowercase letters.

Primer or Synthetic Gene name	Sequence (5'-3')
ProcA2.1EcoRI_fp	ccatcaccatcatcaccacagccaggatccgATGTCAGAAGAACAACTCAAAGC
ProcA2.1NotI_rp	gtacaatacgattactttctgttcgacttaagcattatTCAGTAGCAGCCTCCTGG
ProcA2.10NotI_rp	gtacaatacgattactttctgttcgacttaagcattatTCACCTCACACACAACCCAGTC
ProcA2.10EcoRI_fp	ccatcaccatcatcaccacagccaggatccgATGTCAGAAGAACAACTGAAGGC
ProcA2.10_gene	ATGTCAGAAGAACAACTGAAGGCATTCATCGCCAAAGTTCAGGCA GATAGCTCACTGCAGGAACAGCTCAAAGCAGAAGGTGCTGACCCT GTTTCTATTGCAAAGGCTGCTGGGTTCACGATTACCACAGAAGATC TAAACTCTCATCGCCAAAACCTGTCTGATGAGGAGCTAGAAGGGG CGGCTGGTGGGGCTGGTGGAACAATTCCTTCCCTTATGACTGGATG TGGATGGTTGACTGGGTTGTGTGTGAGGTGA
ProcA2.1_gene	ATGTCAGAAGAACAACTCAAAGCATTCATTGCCAAGGTTCAAGCC GACAGCTCACTGCAGGAACAACTCA AAGCAGAAGGAGCTGATGTTGTTGCTATTGCAAAGGCTGCAGGGT TTTCGATCACCACAGAAGATTGGGA CCAGAGACCCGTAAGAACCTTGTCAGACGAGGAGCTGGAGGGCGC AGCCGGAGGGTGCTGCATCACCGGG GAGTCACCAGGAAGTGCACCAACTAACGACTACAAGTGCACCAAA GGCCGGGGACCAGGAGGCTGCTACTGA

References

1. W. Tang and W. A. van der Donk, *Biochemistry*, 2012, **51**, 4271-4279.
2. S. C. Bobeica, S. H. Dong, L. Huo, N. Mazo, M. I. McLaughlin, G. Jiménéz-Osés, S. K. Nair and W. A. van der Donk, *eLife*, 2019, **8**, e42305.
3. Y. Shi, X. Yang, N. Garg and W. A. van der Donk, *J. Am. Chem. Soc.*, 2011, **133**, 2338-2341.
4. W. Tang and W. A. van der Donk, *Nat. Chem. Biol.*, 2013, **9**, 157-159.
5. W. Tang, S. C. Bobeica, L. Wang and W. A. van der Donk, *J. Ind. Microbiol. Biotechnol.*, 2019, **46**, 537-549.