Supplementary Information

Core-Dependent Post-translational Modifications Guide the Biosynthesis of a New Class of Hypermodified Peptides

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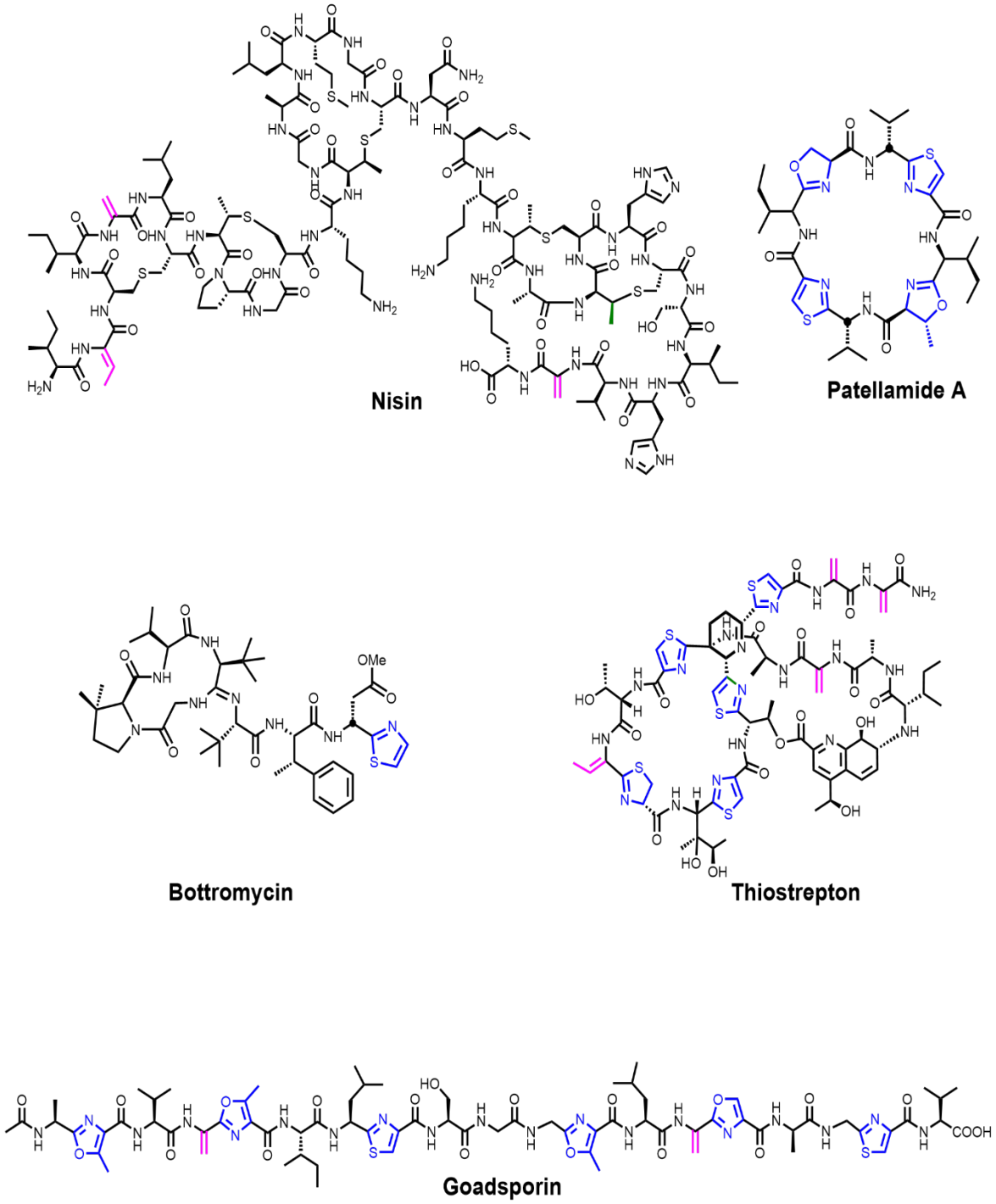
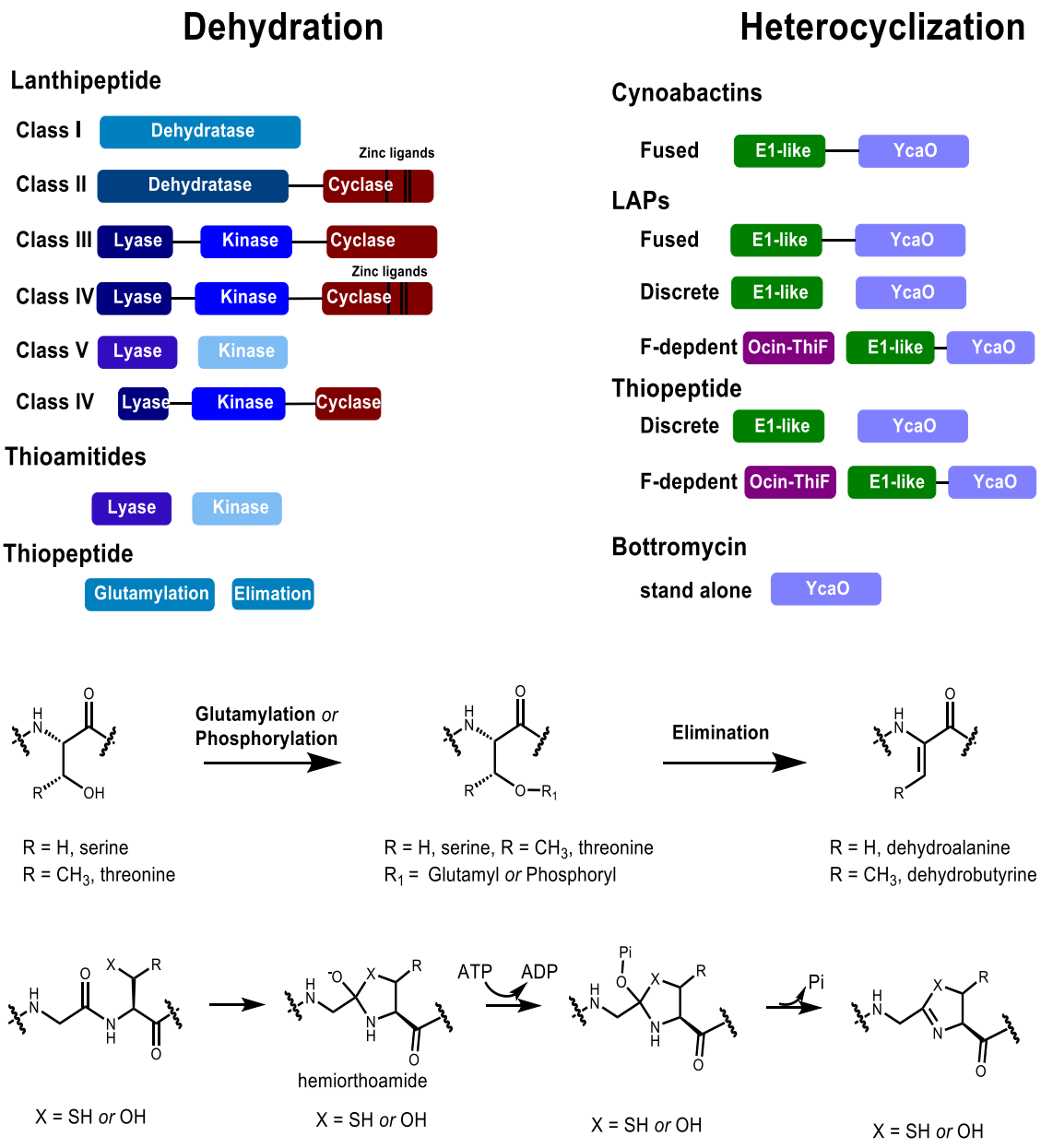
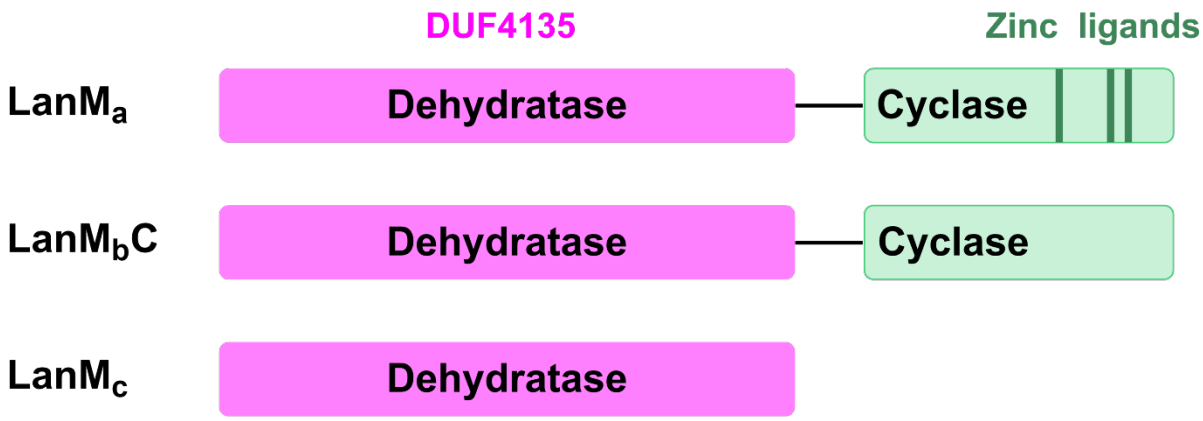


Supplementary Figure 1. Representative dehydroamino acid and azol(in)e containing RiPPs.



Supplementary Figure 2. Biosynthetic origin and mechanism of dehydro amino acid and azol(in)e in RiPPs.

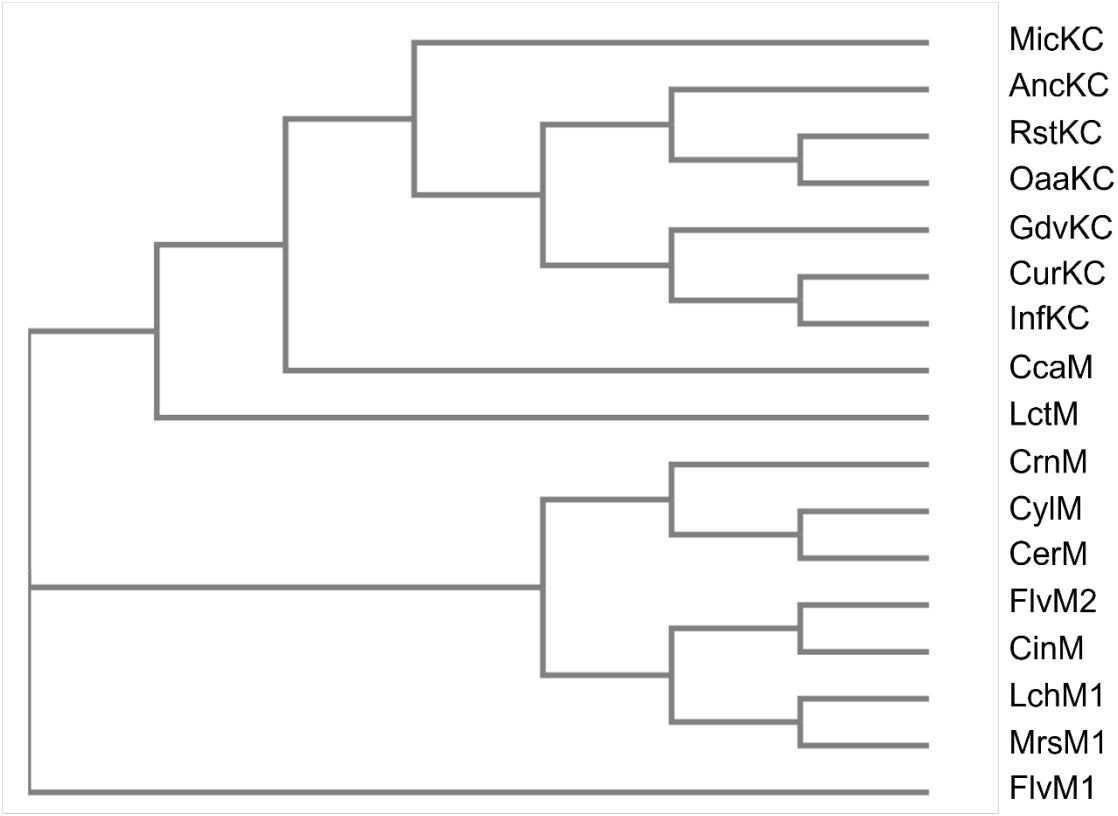


Supplementary Figure 3. Classification of LanM based on the biosynthetic gene organization.

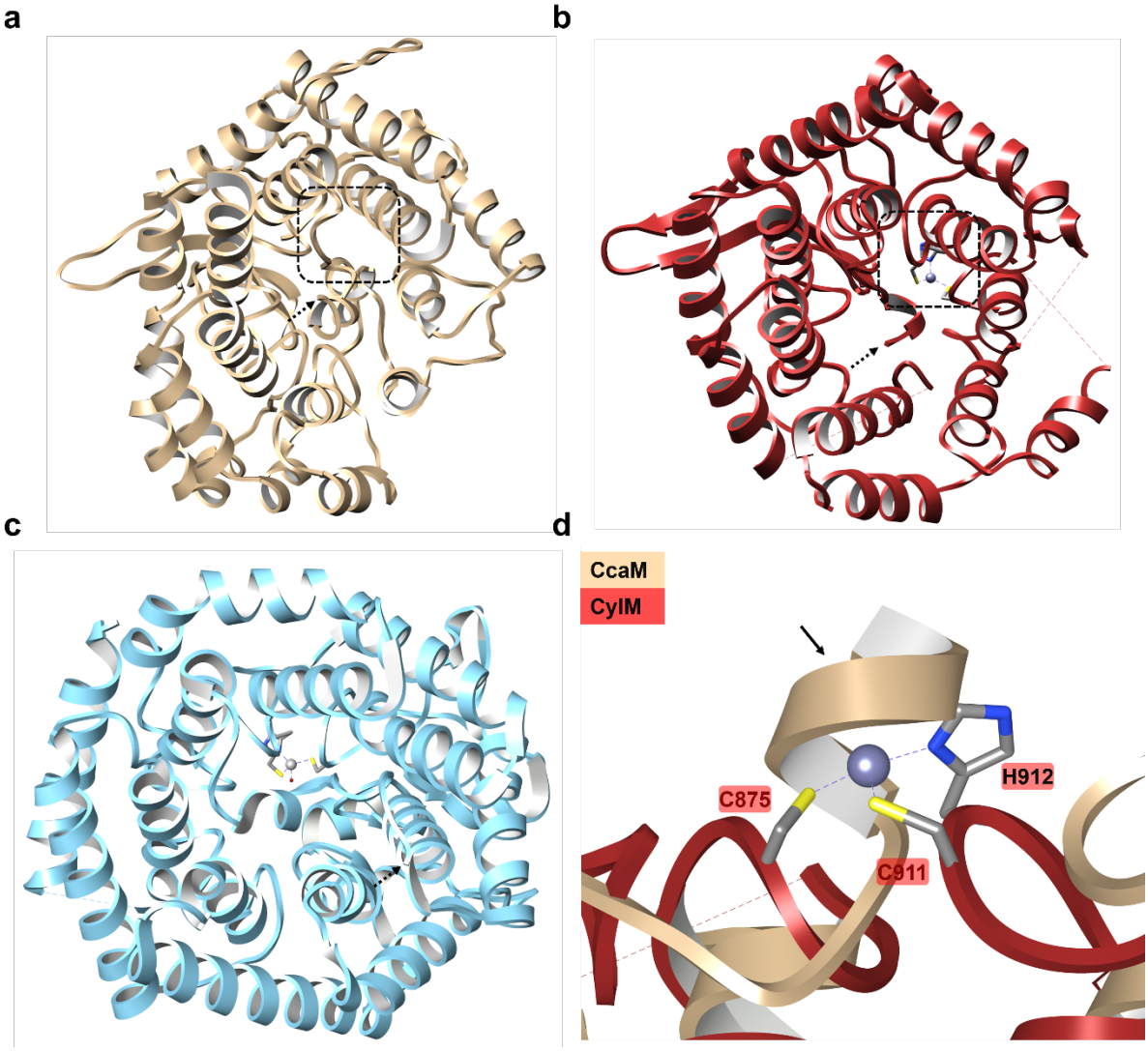
a


**
CylM YDDNIS----NIDINKTI-EYK-------NKDCLCHGNAGTLEGLIQLAK--------KD
A0A2N3KQU4 AGLL-----ESRQLRPLVIQSAQAKSKFFDDSISCLDLLVCCRLALALFKQ---------
A0A5R9M7I1 AVSLGTGAVHPGDLAWLTPPVT----AAEIAALGTRELVAAAGEALTSAEAGLLHTLDDD
A0A6B2VR55 AVSLGTGAVHPGDLAGLTPPVT----AAEIAALGTRELVAAAGEALTSAEAGLLHTLDDD
A0A6B2WA28 AVSLGTGAVHPGDLAGLTPPVT----AAEIAALGTRELVAAAGEALTSAEAGLLHTLDDD
A0A1I2LQC0 AVSLGAAALDSRQLGLLAAPVG----LAQVAGLGTAELVATAGEALTAAELGLVHSLPEG
A0A066YQS6 ----------
A0A1Q5EUS0 AVSLGADAVDGADLGALAPPVT----GPELAALTGRALLSAATEALTAAEAGLVHTLPEA
A0A7H8KY55 AVSLGADAVDQADLGALAPPVT----GPELAALTGRALLSAATEALTAAEAGLVHTLPED
E4NFR7 AVSLGADAVDQDDLGVLAPPVT----GPELAALTGRALLSAATEALTAAEAGLVHTLPEE
A0A419YLJ0 AVSLGADAVDQADLGALAPPVT----GPELAALTGRALLSAATEALTAAEAGLVHTLPEG
A0A327T358 AVSLGADAVDQADLGALAPPVT----GPELAALTGRALLSAATEALTAAEAGLVHTLPED
A0A3N4S9A4 AVSLGADAVDGADLGALAPPVT----GPELAALTGRALLAAATEALTAAEAGLVHTLPED
A0A8G1X9V3 AVSLGADAVDGADLGALAPPVT----GPELAALTGRALLAAATEALTAAEAGLVHTLPED
A0A561EXY4 AVSLGAGAVNPADLGELAPPVG----ESQILGLSTRALVATAGEALTAAEAGLLHTLPEG
A0A2A3I1T1 AVSLGAGSVDPAELGALTPPVG----AAGIAGRTTRDLVATAGEALTAAEAGLLHTVPEG
A0A0Q8PMZ3 AVALGAGIVDPAELAALTPPVS----ERQITGRSTRDLVATAGEALTAAEAGLLHTVPDP
A0A7W7SDA2 AVALGAGIVDPAELAALTPPVS----ARQISGRSTRDLVATAGEALTAAEAGLLHTVPEA
A0A097CRM5 ----------
F4FHE1 AAALGRAR--P-----AQAQTP----T-PAGSPSSVDLLDRIELGLAAWR----------
A0A1G9HJZ5 DHLLTGDV--SAELRERVARQC----A-PRARRSCERLVVDAELALHTAR----------
A0A4U3MMZ2 AHALTGEV--PADLRDHVARHC----A-PTPARSCAALVVDAEMAIHTAR----------
A0A8J3W1S2 AHLLTGEV--PGGLRERVARHC----A-PDPGRSCVRLVVDAEVALHTAR----------
A0A1H6EW28 AHLLTGEV--PGDLRERVARYC----A-ADAARSCARLVVDTEVALHTAR----------
A0A7W9LCK1 AHLLTGEV--PGDLRERVARYC----A-PDAARSCARLVVDAEVALHTAR----------
A0A2D8UIJ3 LELN-K---NNKSIRSYYDTFL----TKPPKKLRTTQLIEYADLSLNLYEI---------
A0A2E6EXT3 LELN-K---NNKSIRSYYDTFL----TKPPKKLRTTQLIEYADLSLNLYEI---------
A0A0Q4EB58 RY--------DQNSADVYAECL----SRDIRQLTVQQLIDYAELALNLSDI---------
A0A1I4VP56 RY--------DQKSADAYKTCL----SRDVGQLTTQQLIDYAELALNLSEI---------
A0A1M5H7Z5 RY--------DQNSADVYAARL----SRDIRQLTVQQLIDHAELALNLSDI---------
A0A1N7HZ13 RY--------DQNSADVYQAFL----SRDTRQLTTQQLIDYAELALNLSDI---------
CcaM RY--------DQNSANVYQAFL----SRDIRQLTVQQLIDYAELALNLSDI---------
A0A376DRU0 RY--------DQNSANVYQAFL----SRDIRQLTVQQLIDYAELALNLSDI---------
A0A1E2WG51 IQSTRI---NAEQVLRKIDEYL----AVERLATDTVGLLDDLEVAITAFQV---------
A0A0K1EEA4 DELGVR---SLPELFARVDAYL----DVDPGSLGSRRLLGALELALVAYRR---------
A0A848LRJ3 ADGTAH---PALP--TSIRRVL-------RRAATASTSLEAVELALTAGRE---------
A0A2T4V8J4 G--------QGDGLRARVLHAL----EGAAS-GSTSARLDGLELALVADSS---------
A0A1L9AYM3 AREQAR---PGDSLRPRVLRAL----EEARAASSVAVQLDAIELALDAART---------
A0A250IW45 AREQER---PDGSLRPRVLRAL----EEARAASSVAVQLDAIELALDAAQT---------
S9PA60 AREQGQ---PGGSLRPRVLRAL----EEAHAASSVAVQLDAIELALDAAQT---------

b

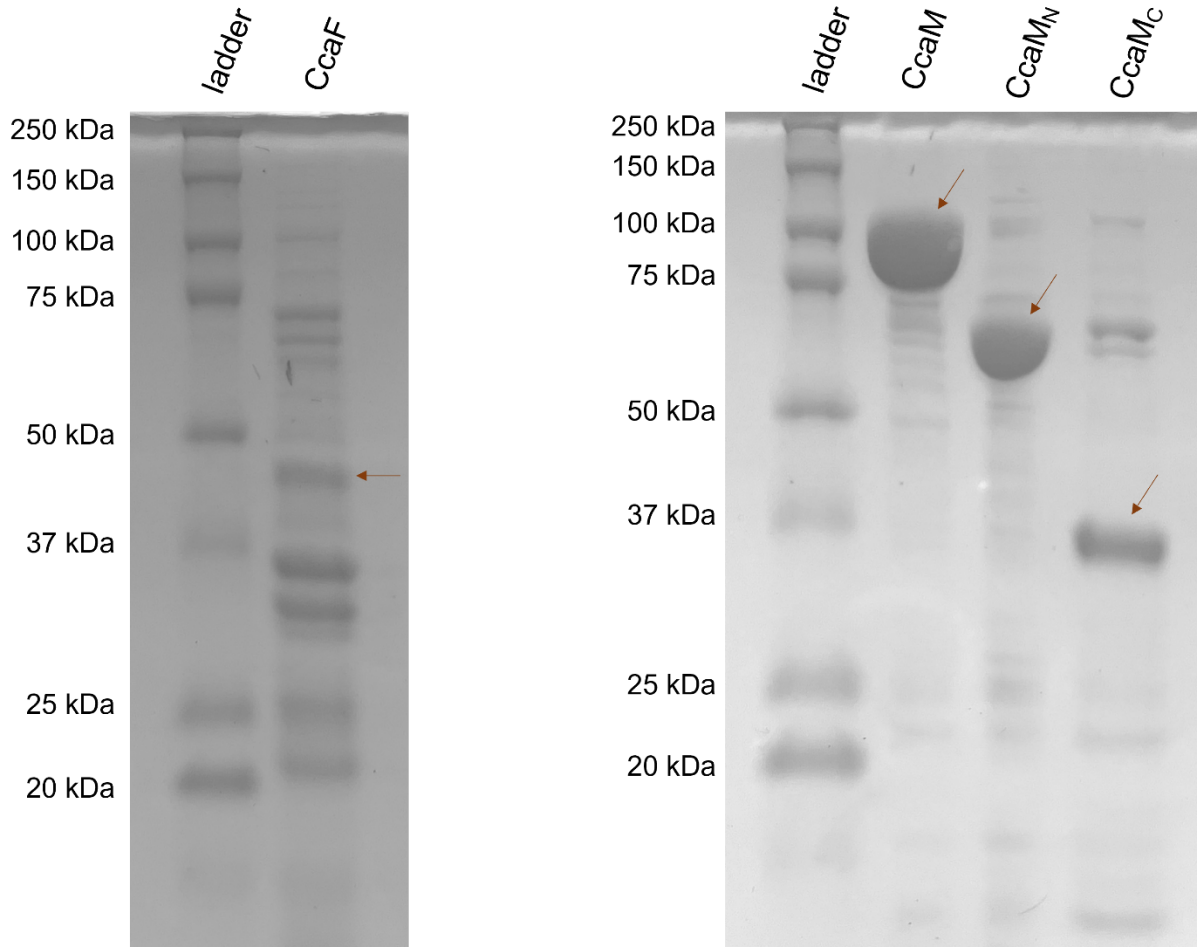


Supplementary Figure 4. Multiple sequence alignment and phylogenetic tree analysis. (**a**) Multiple sequence alignment of canonical zinc-binding CylM[1] with sequences of representative metal-independent $LanM_bCs$. The asterisks designate the two conserved zinc binding sites in the cyclase domain of CylM (C911/H912). (**b**) Simple phylogenetic tree analysis of CcaM cyclase domain and cyclase domain of characterized conical Class II lanthipeptide synthetase LanM and class III lanthipeptide synthetase LanKC, it shows that CcaM cyclase domain is closer to that of LanKC.

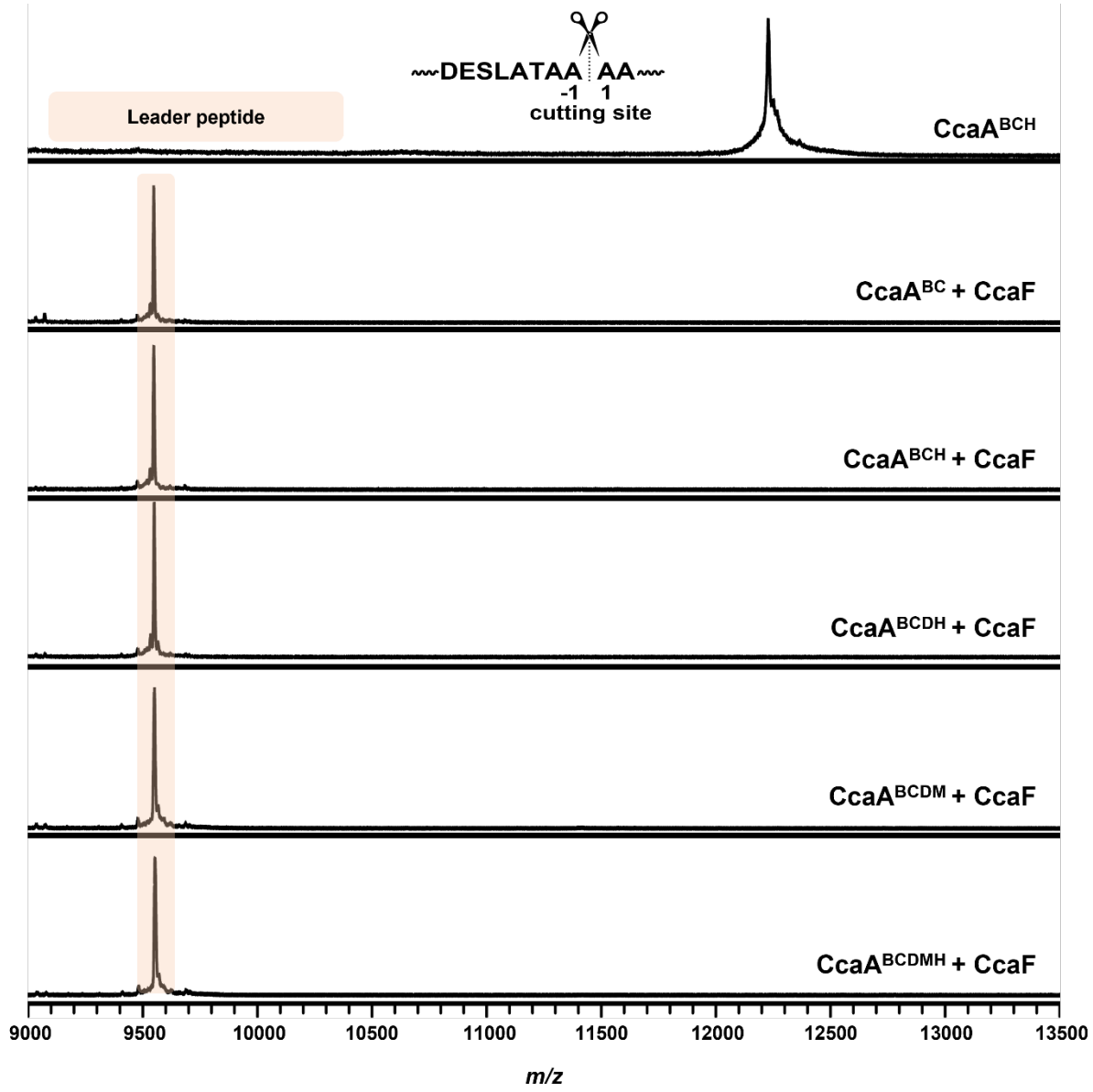


Supplementary Figure 5. Structural comparison of the cyclase domain of CcaM (predicted by AlphaFold[3]), CylM[1] (5DZT) and NisC[2] (2G02). Overall view of the cyclase domains CcaM (predicted) (**a**), CylM (**b**) and NisC (**c**). The dash arrow points to the C termini of the protein. Box indicates that the zoom-in region of CcaM and CylM. (**d**) Superposition of the model structure of CcaM with that of CylM shows that the zinc binding residues are not conserved in CcaM, and the conserved zinc binding pocket is occupied by a helix (pointed by an arrow) in the predicted CcaM structure.

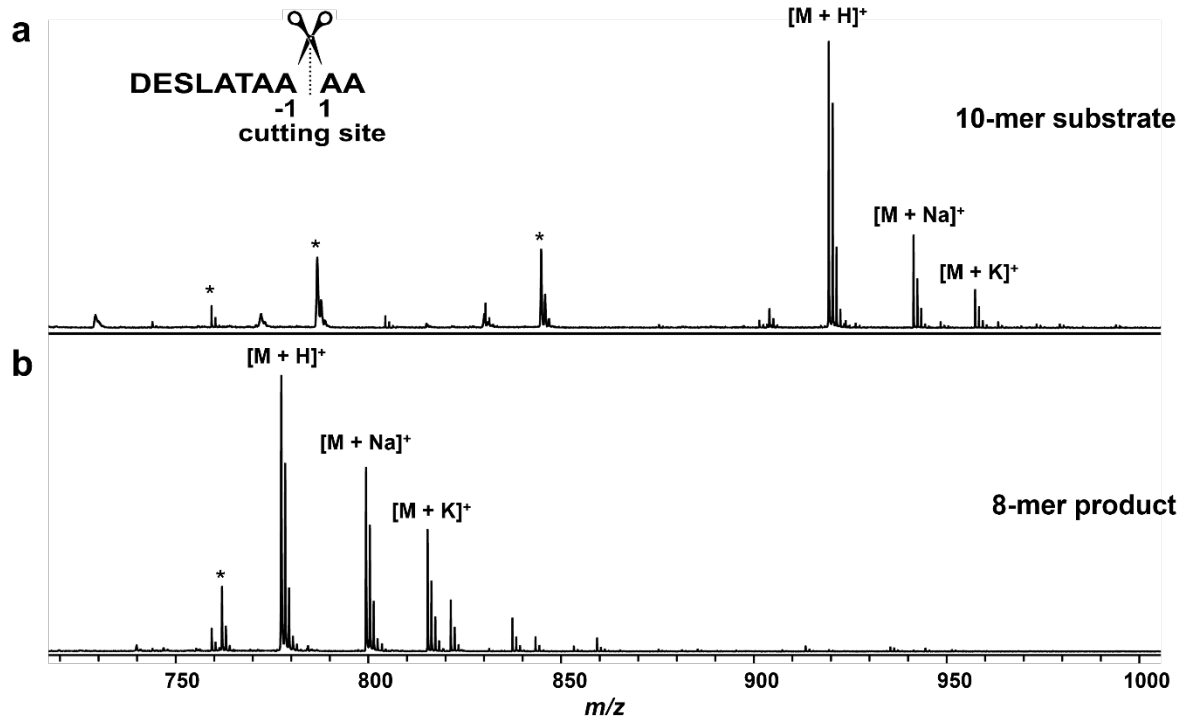
Supplementary Figure 6. Sequence alignment of CcaD and Radical SAM epimerase PoyD[4].



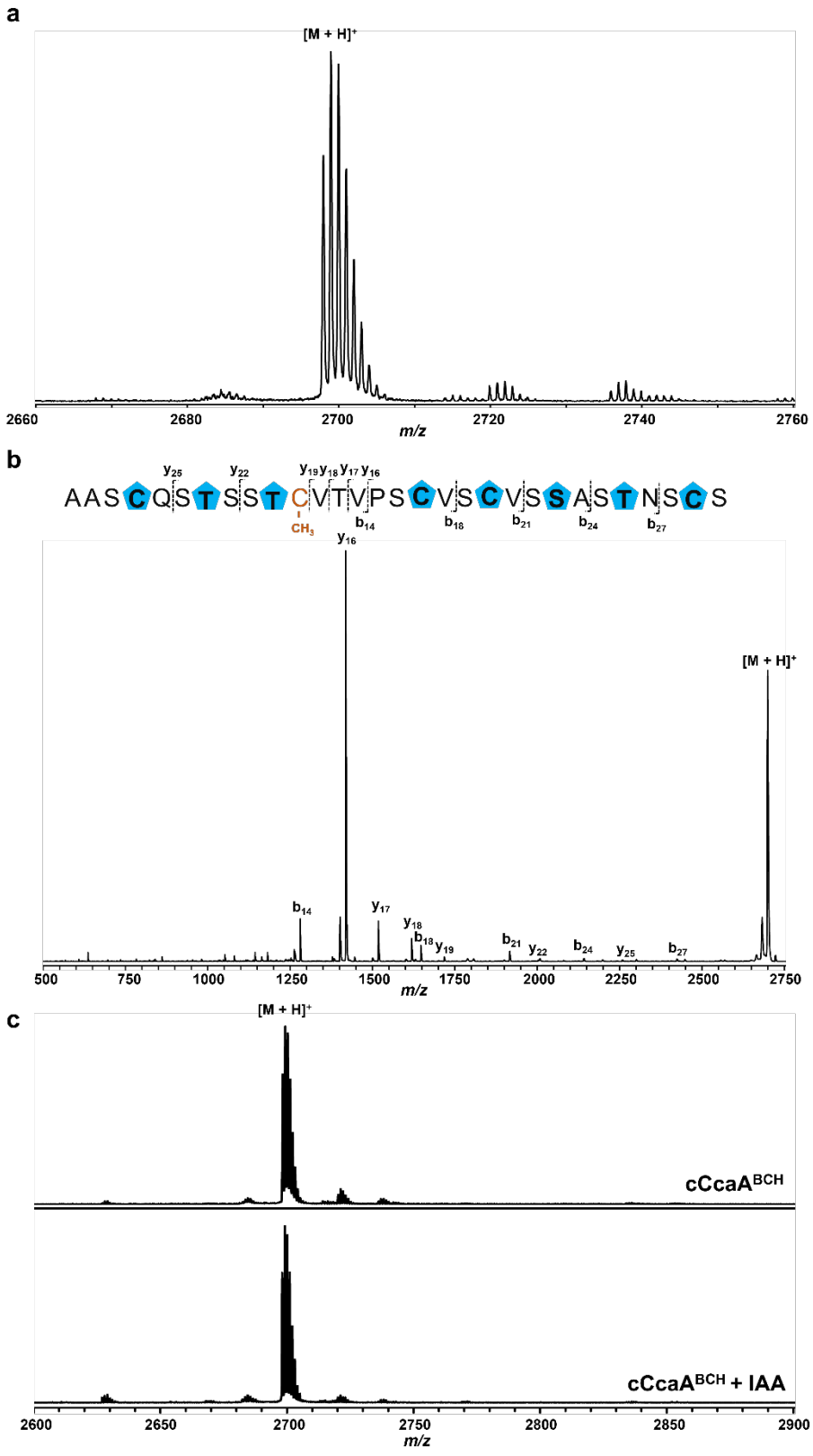
Supplementary Figure 7. SDS-PAGE analysis of purified proteins used in this study.



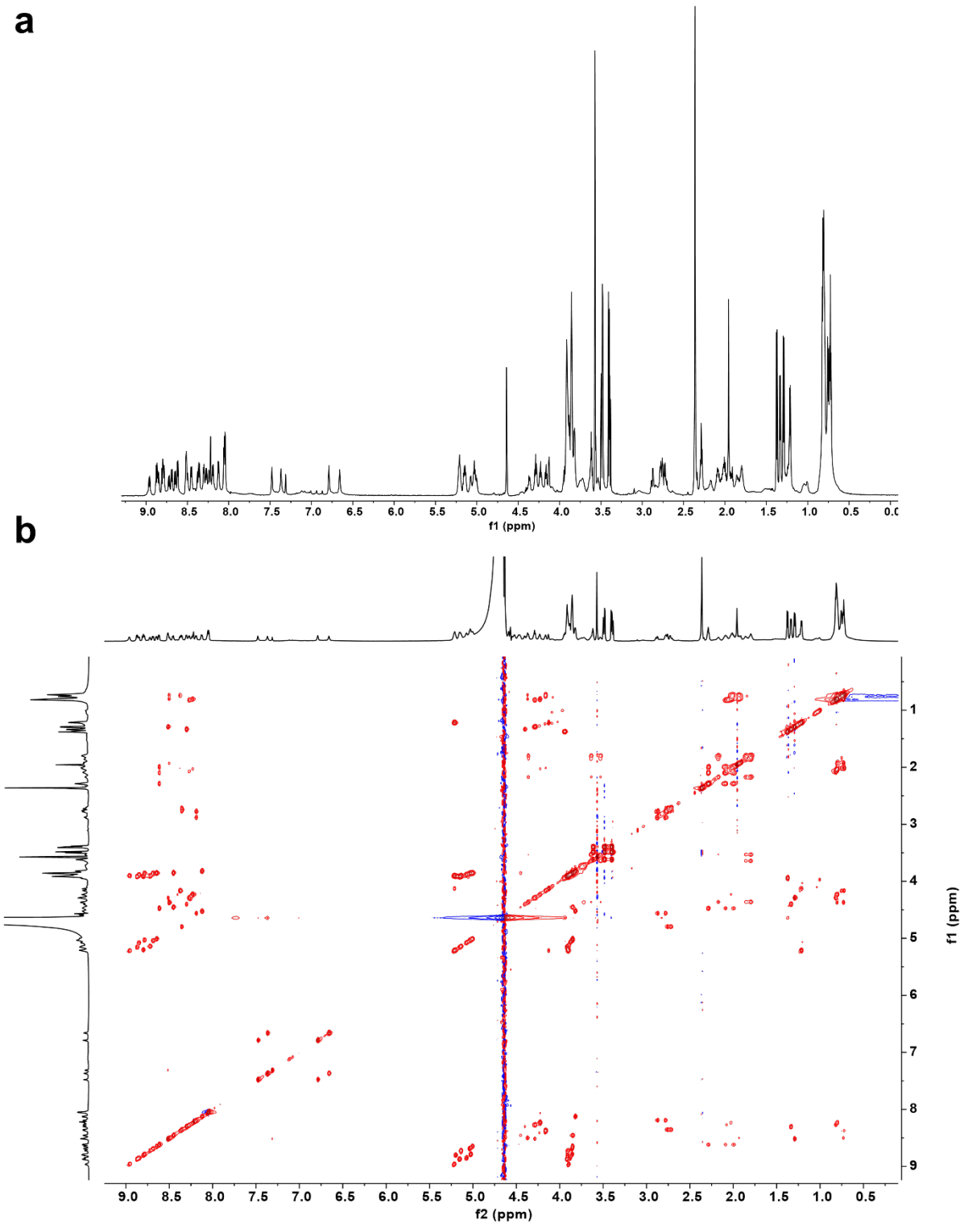
Supplementary Figure 8. In vitro removal of leader peptide of different modified precursor peptides by CcaF. The cutting site is shown in cartoon.



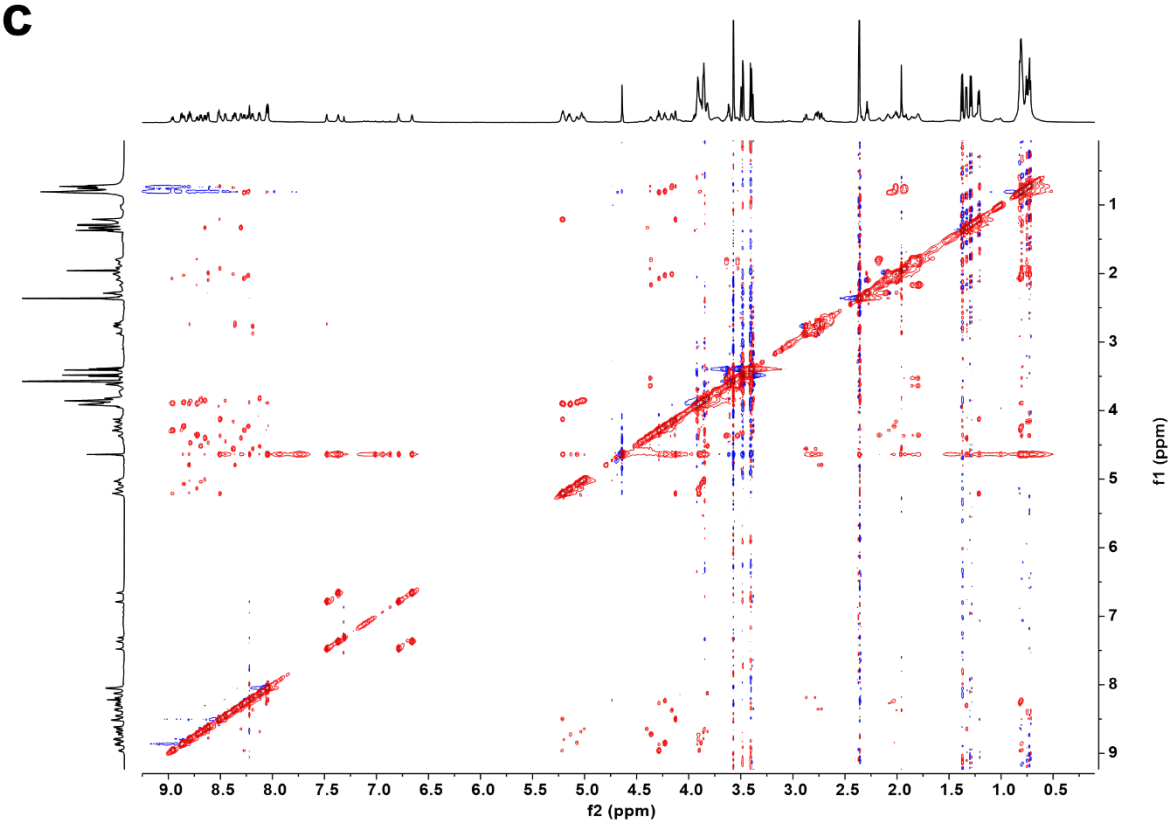
Supplementary Figure 9. **In vitro CcaF assay of a syntheic10-mer peptide (DELSATAAAA)**. (**a**) 10-mer peptide substrate. (**b**) In vitro CcaF assay of 10-mer peptide substrate. (∗) Denotes impurities which cannot be removed by HPLC.



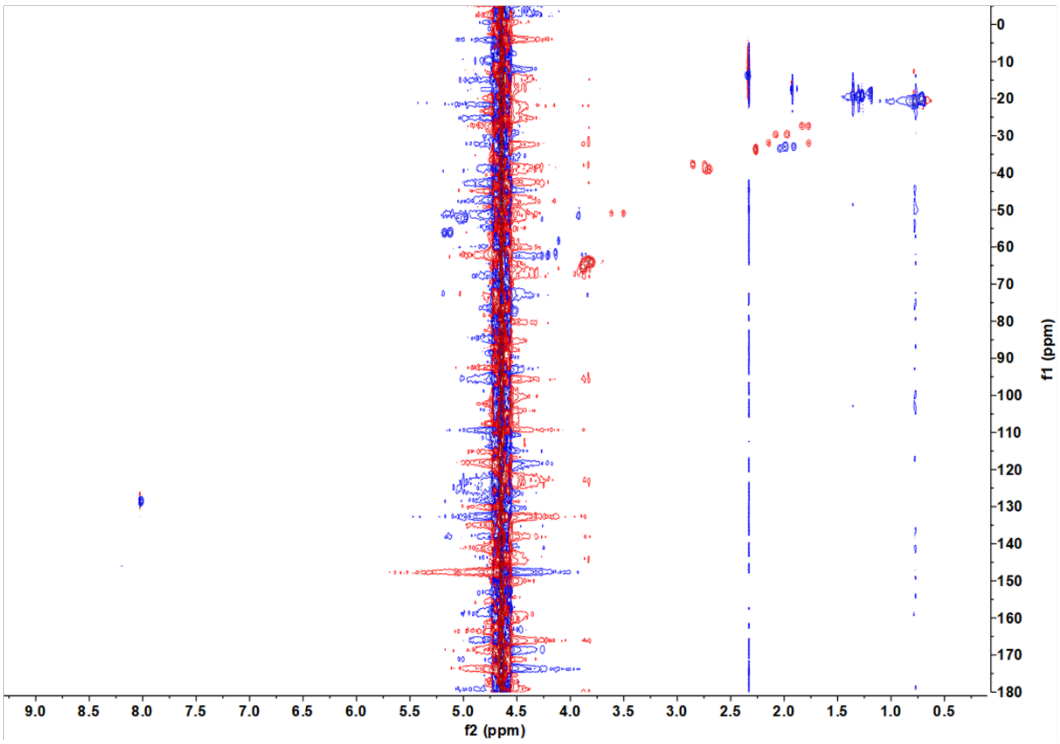
Supplementary Figure 10. **MALDI-TOF mass spectra of cCcaA^{BCH}**. (**a**) MALDI-TOF MS of cCcaA^{BCH}. (**b**) MALDI-TOF MS/MS of cCcaA^{BCH}. (**c**) IAA alkylation assay shows that no free cysteine in cCcaA^{BCH}.

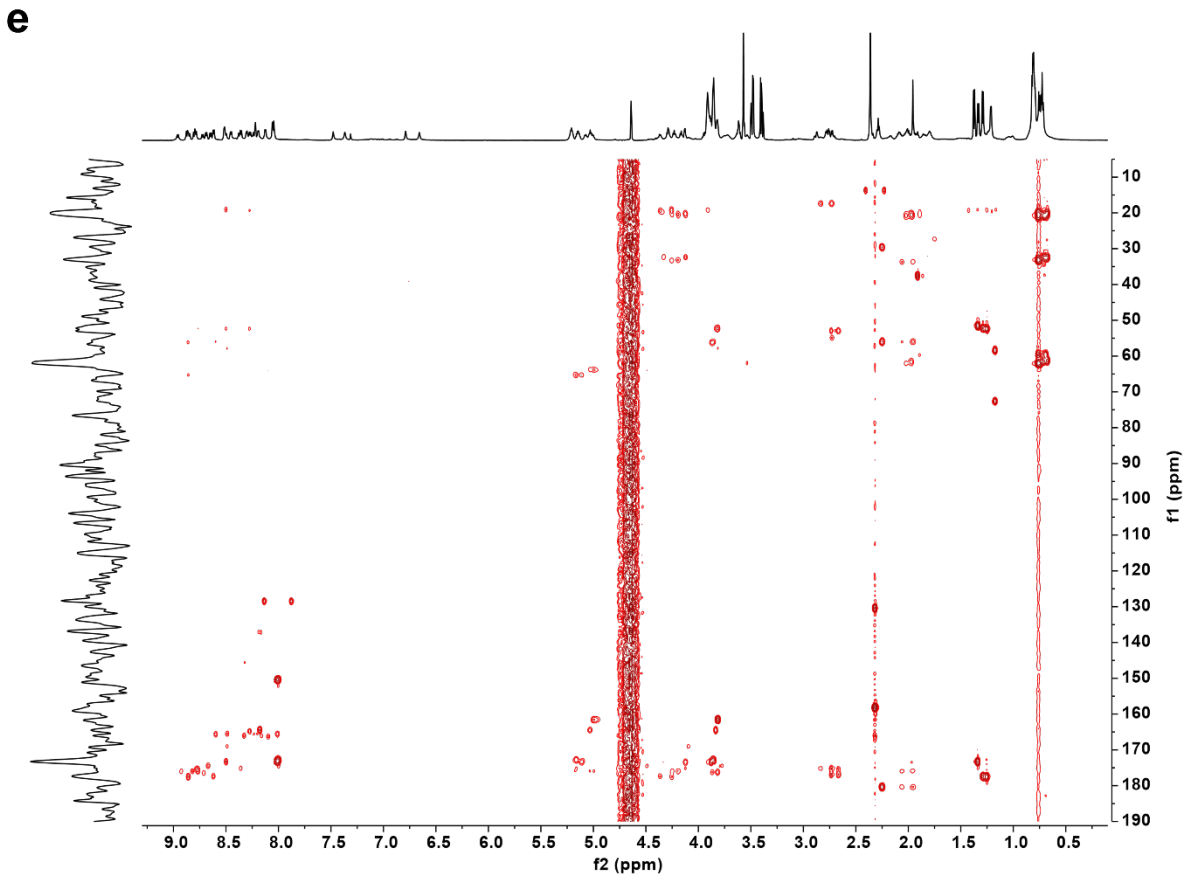

a
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)
b
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f2 (ppm)
1 2 3 4 5 6 7 8 9
f1 (ppm)

c

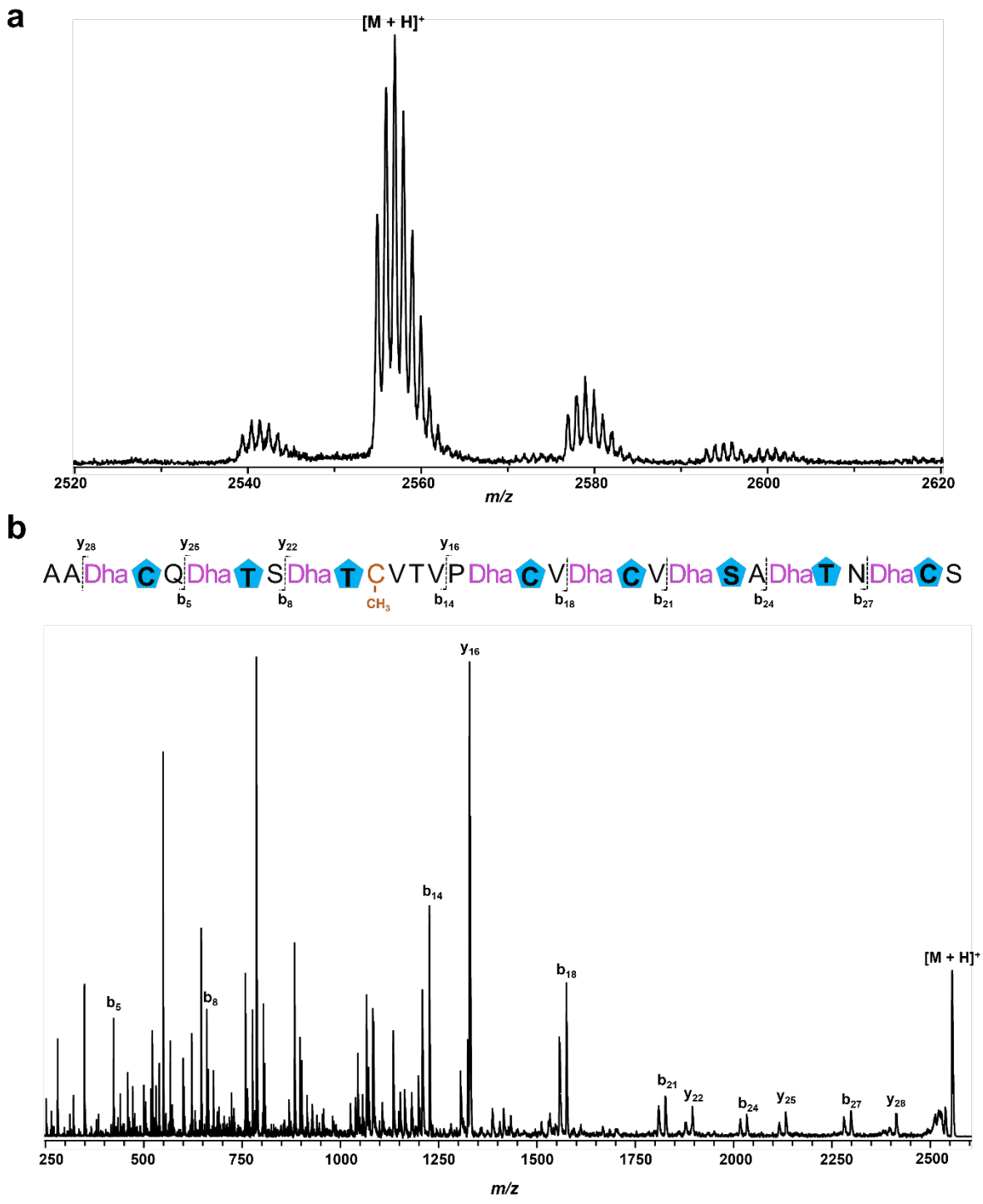


d

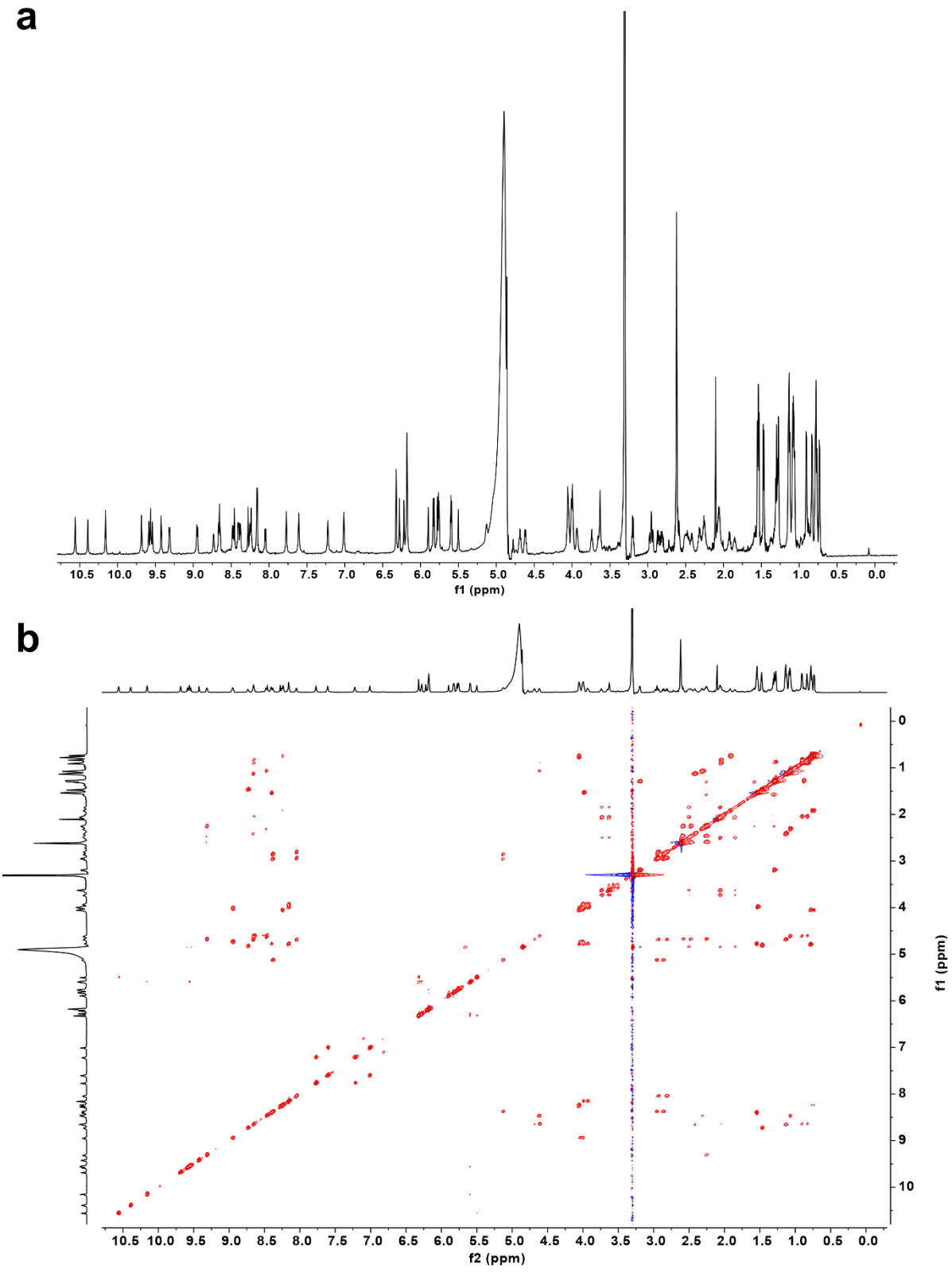


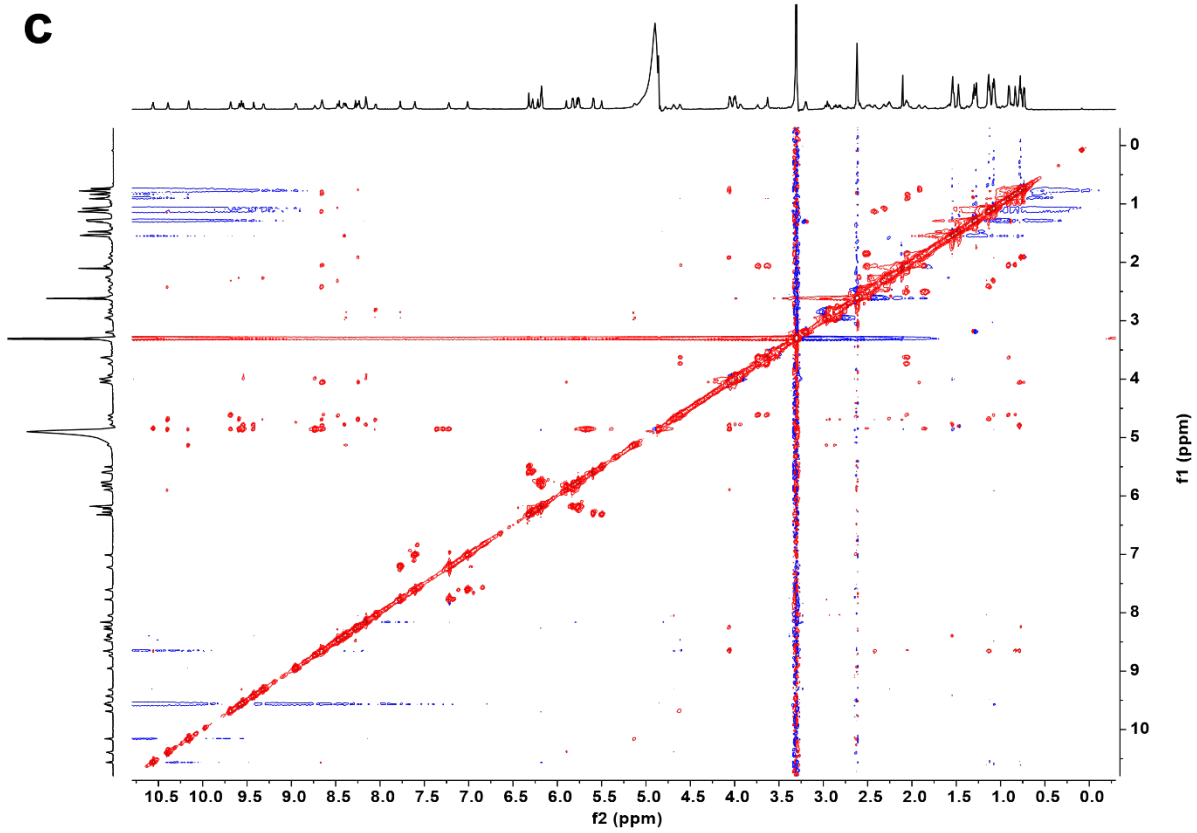


Supplementary Figure 11. **NMR spectra of cCcaA^{BCH} in 9:1 (v/v) H_2O/D_2O at 25 °C**. (**a**) ^{1}H NMR spectrum. (**b**) ^{1}H-^{1}H TOCSY spectrum. (**c**) ^{1}H-^{1}H NOESY spectrum. (**d**) ^{1}H-^{13}C HSQC spectrum. (**e**) ^{1}H-^{13}C HMBC spectrum.



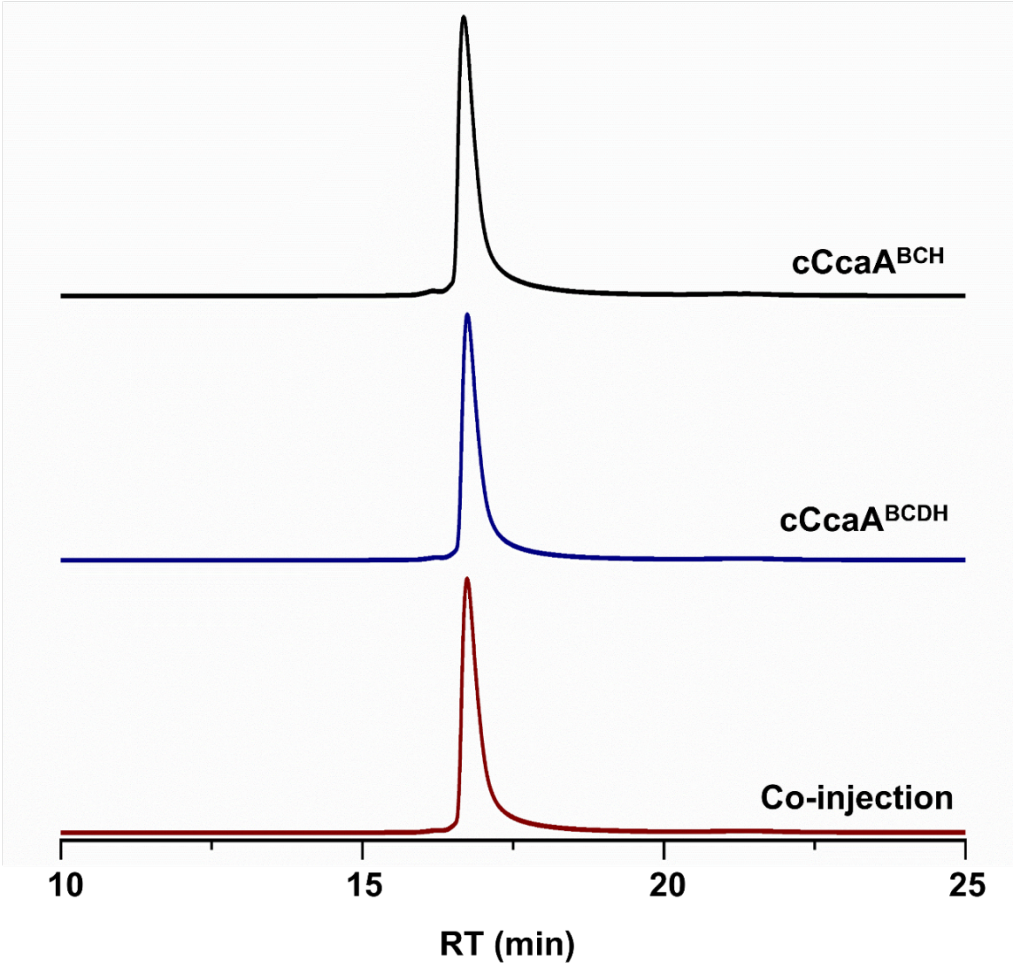
Supplementary Figure 12. **MALDI-TOF mass spectra of carnazolamide**. (**a**) MALDI-TOF MS of carnazolamide. (**b**) MALDI-TOF MS/MS of carnazolamide.

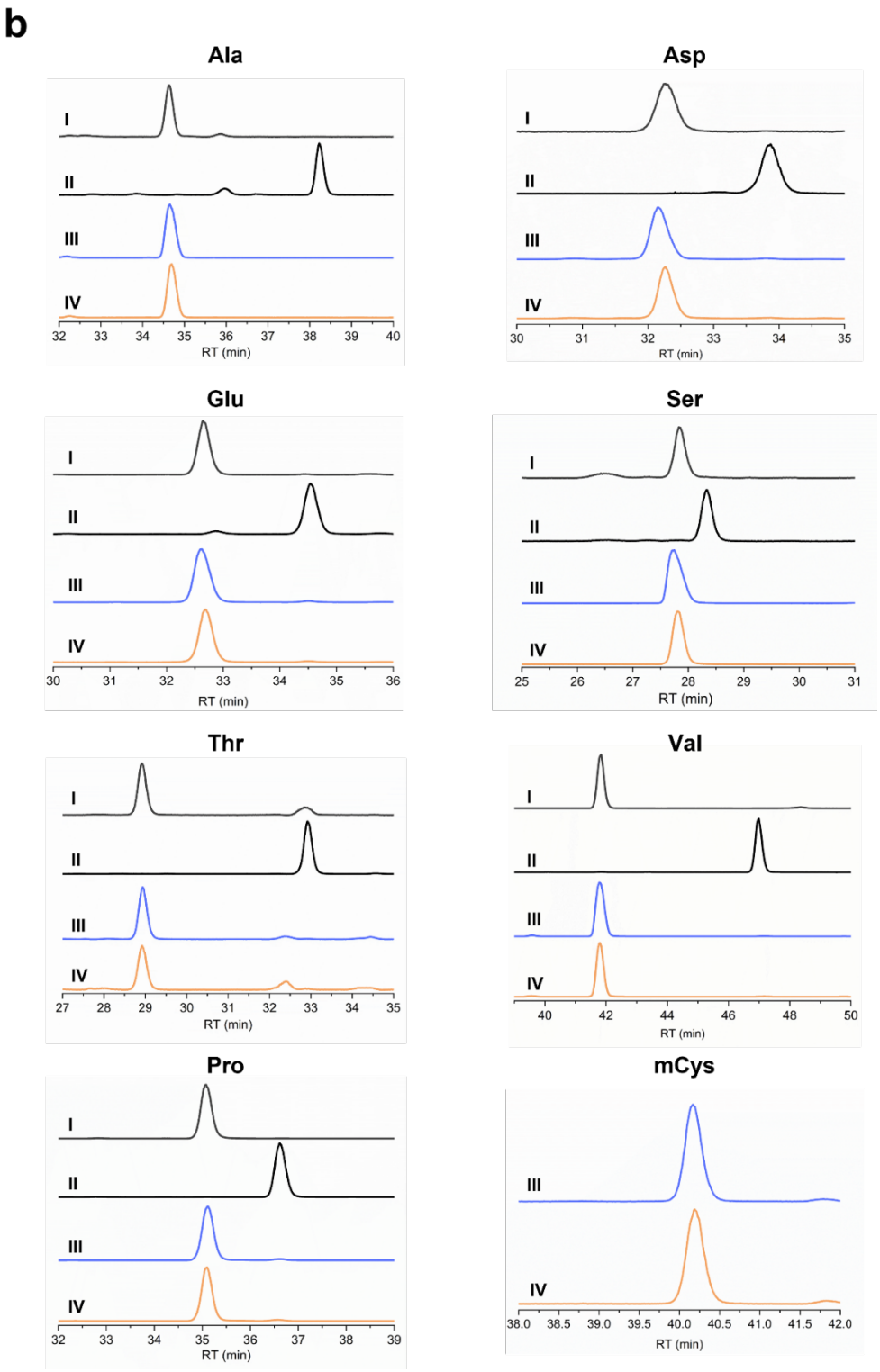

a
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)
b
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f2 (ppm)
0 1 2 3 4 5 6 7 8 9 10
f1 (ppm)



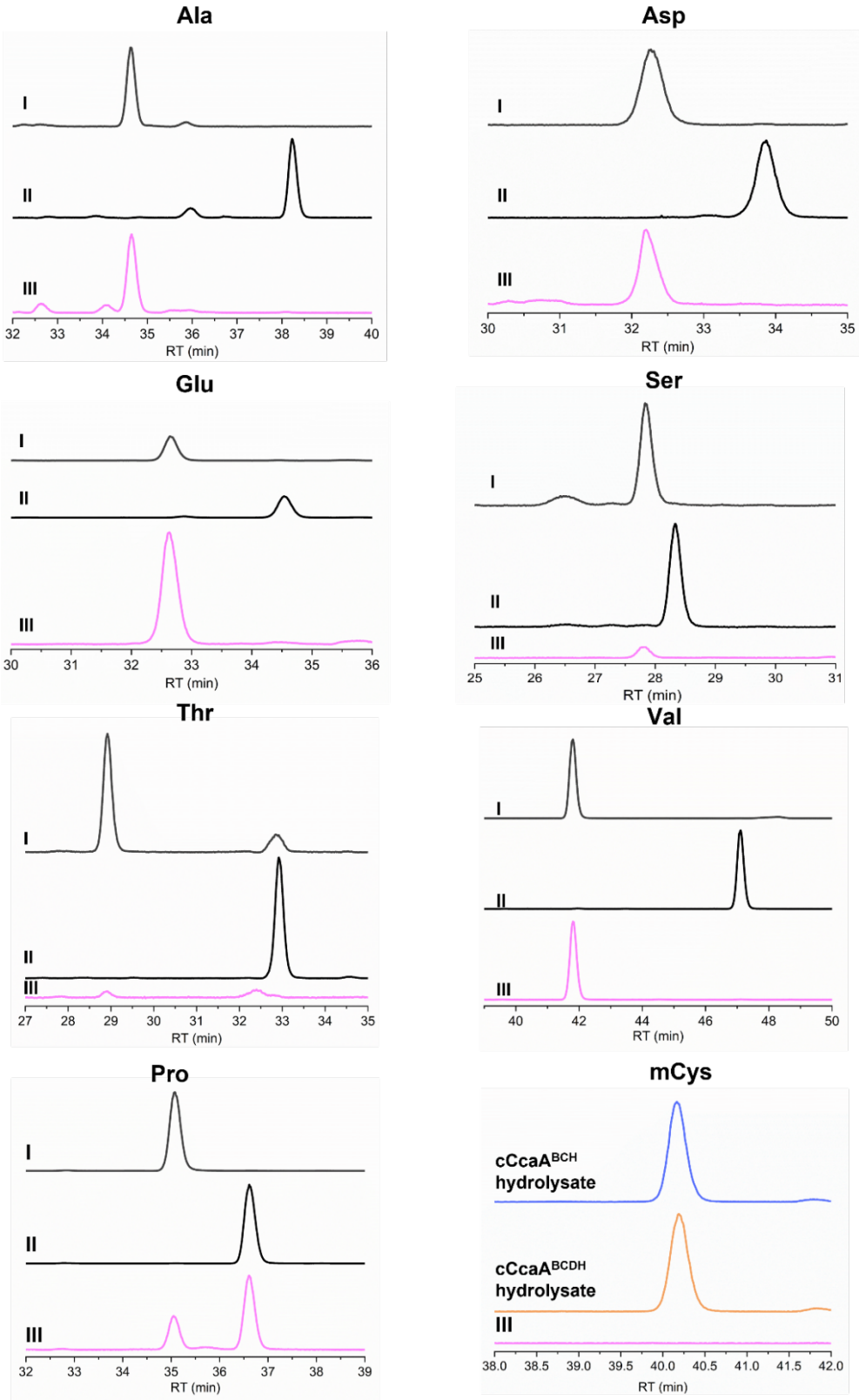
Supplementary Figure 13. **NMR spectra of carnazolamide in CD_3OH at 25 °C**. (**a**) ^1H NMR spectrum. (**b**) ^1H-^1H TOCSY spectrum. (**c**) ^1H-^1H NOESY spectrum.

a

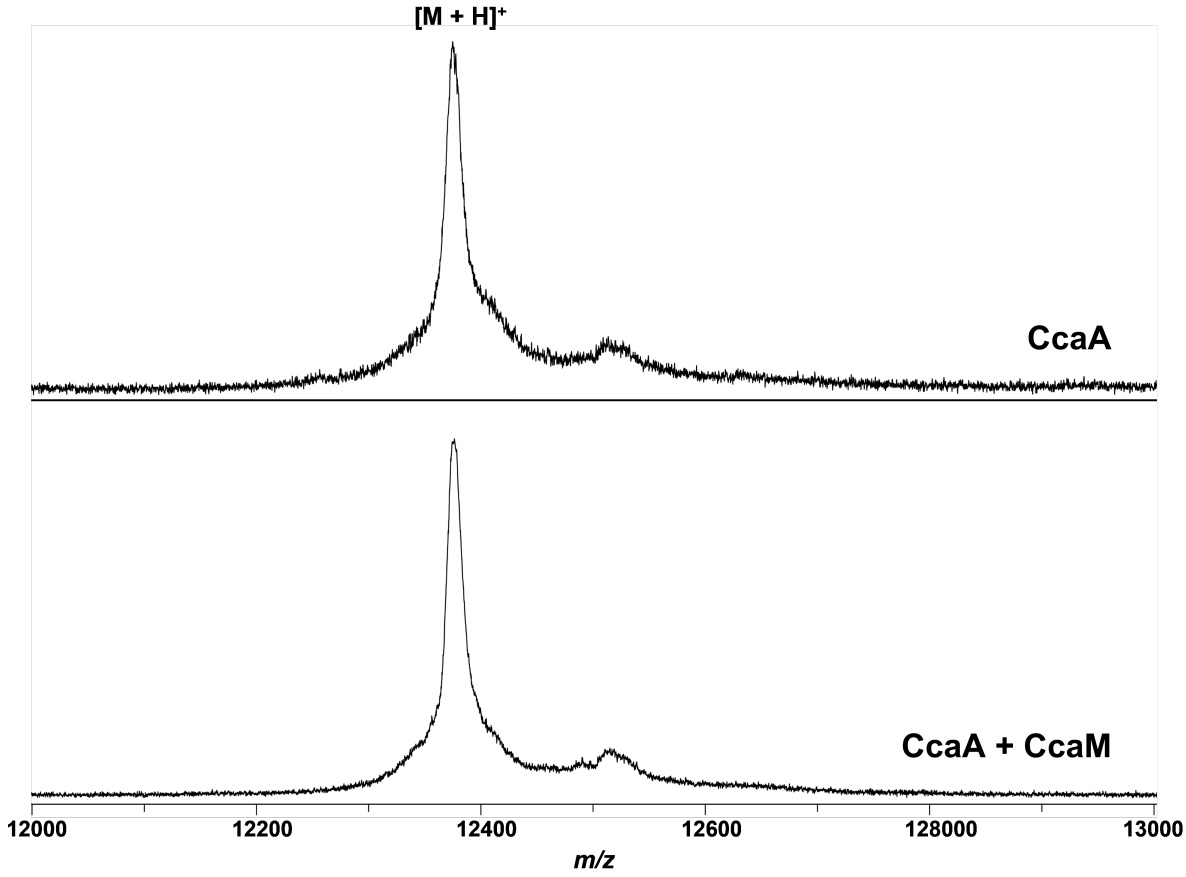




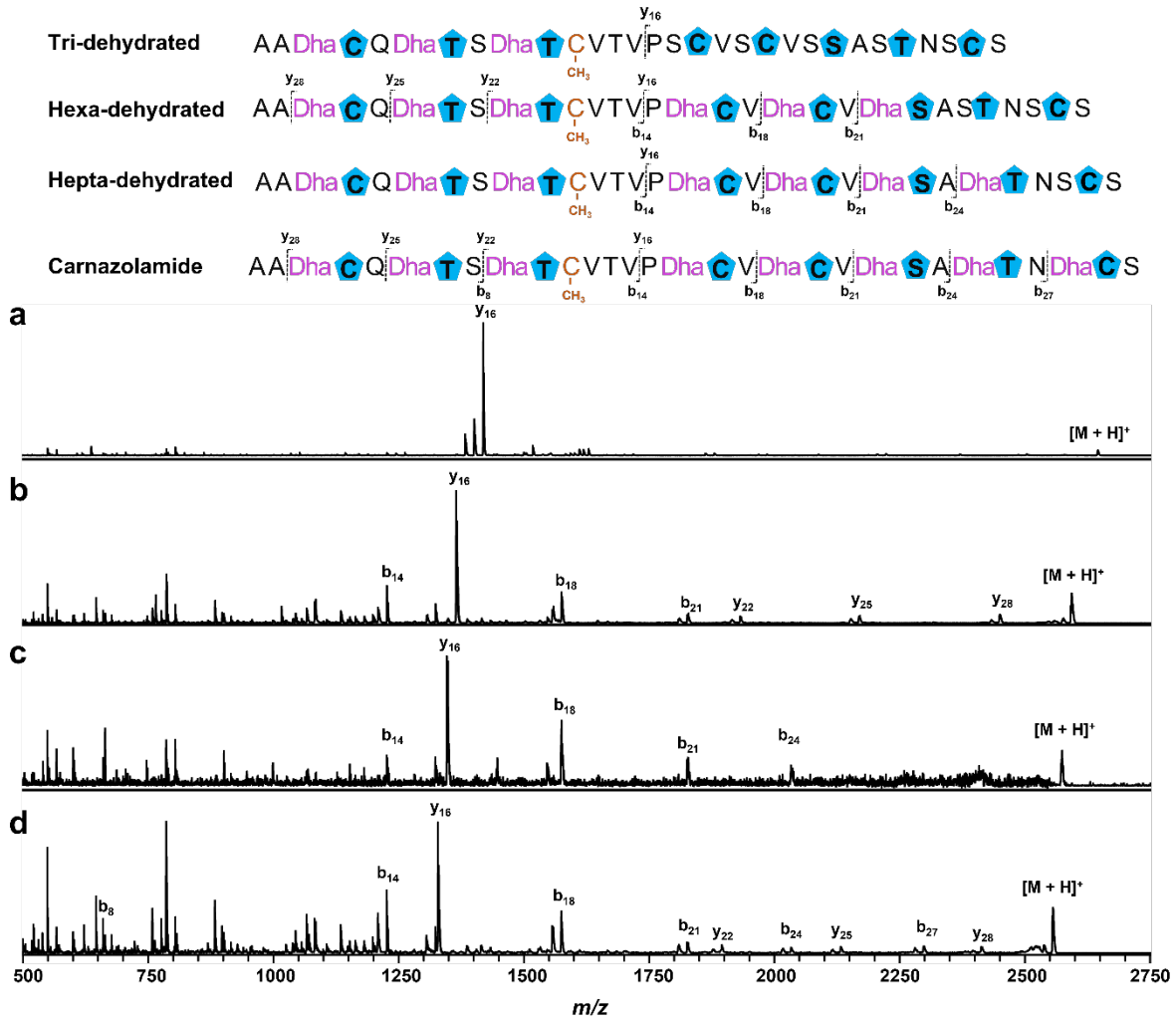
Supplementary Figure 14. HPLC analysis cCcaA^{BCH} and cCcaA^{BCDH} and Marfey's assay of cCcaA^{BCH} hydrolysate and cCcaA^{BCDH} hydrolysate. (**a**) HPLC analysis of cCcaA^{BCH} and cCcaA^{BCDH}. (**b**) Marfey's assay of cCcaA^{BCH} hydrolysate and cCcaA^{BCDH} hydrolysate. L-amino acid standard (I), D-amino acid standard (II), cCcaA^{BCH} hydrolysate (III) and cCcaA^{BCDH} hydrolysate (IV). mCys means *S*-methylated cysteine.



Supplementary Figure 15. Marfey's assay of carnazolamide hydrolysate. L-amino acid standard (I), D-amino acid standard (II) and carnazolamide hydrolysate (III). The *S*-methylated cysteine was not shown for the intensity is too low to be detected.

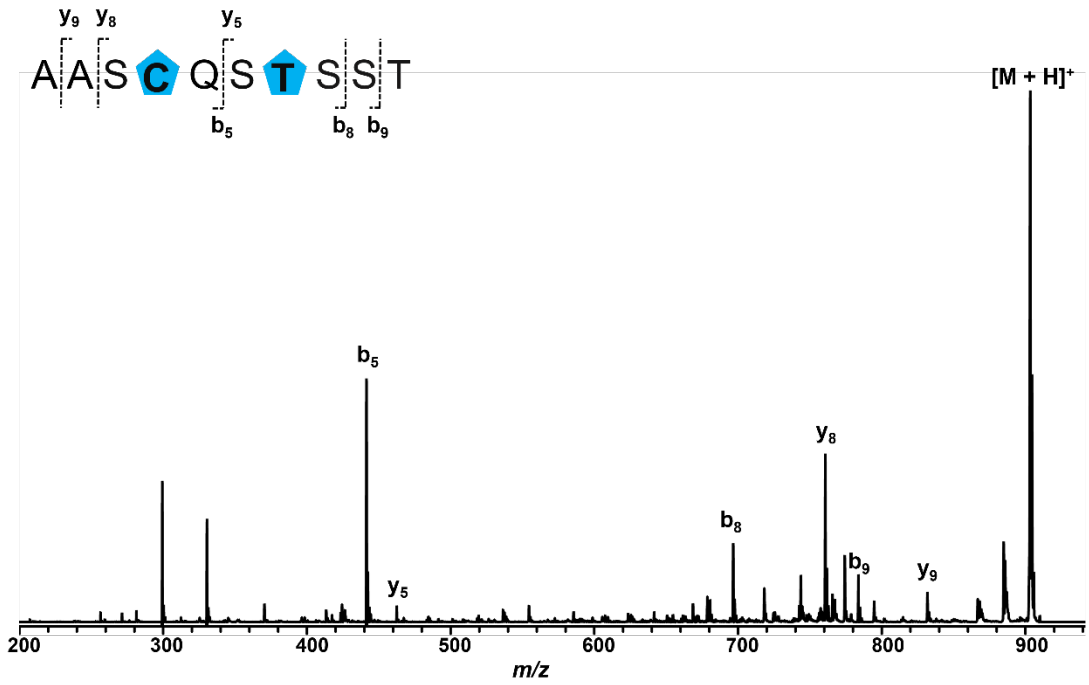


Supplementary Figure 16. In vitro assay of full length CcaA with CcaM. CcaM does not catalyze dehydration of the unmodified precursor peptide.



Supplementary Figure 17. MALDI-TOF MS/MS of the tri (**a**), hexa (**b**), hepta (**c**) dehydrated ocatazole containing core peptide and carnazolamide (**d**).

a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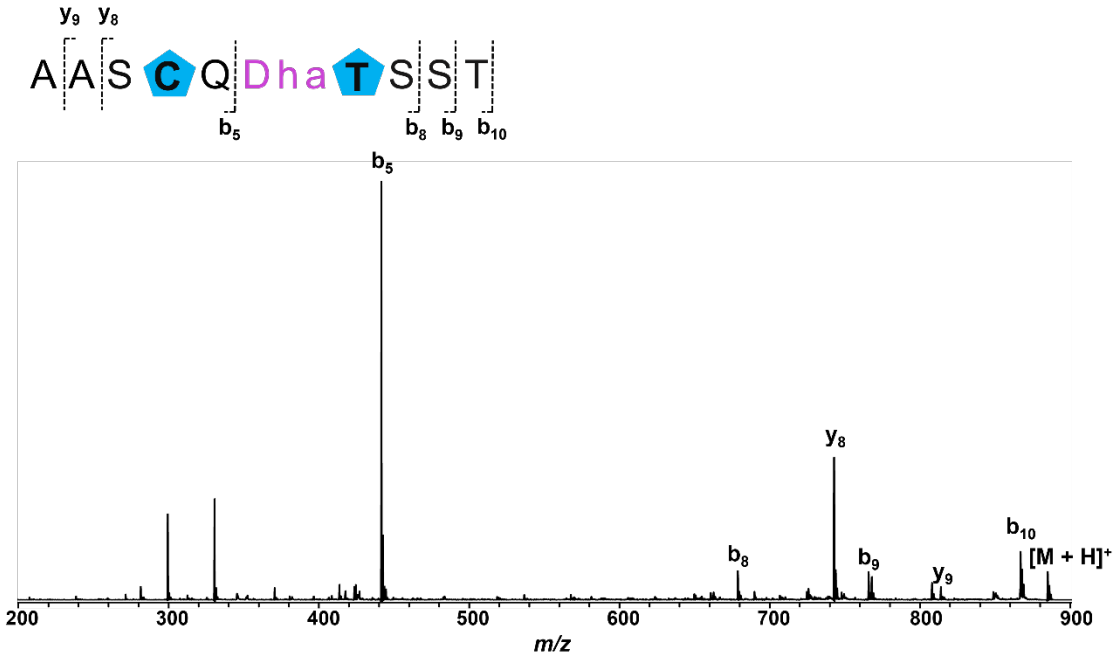
y5
A A S C Q S T S S T
b5
b8 b9
[M + H]+
b5
y8
b8
b9
y9
y5
200 300 400 500 600 700 800 900
m/z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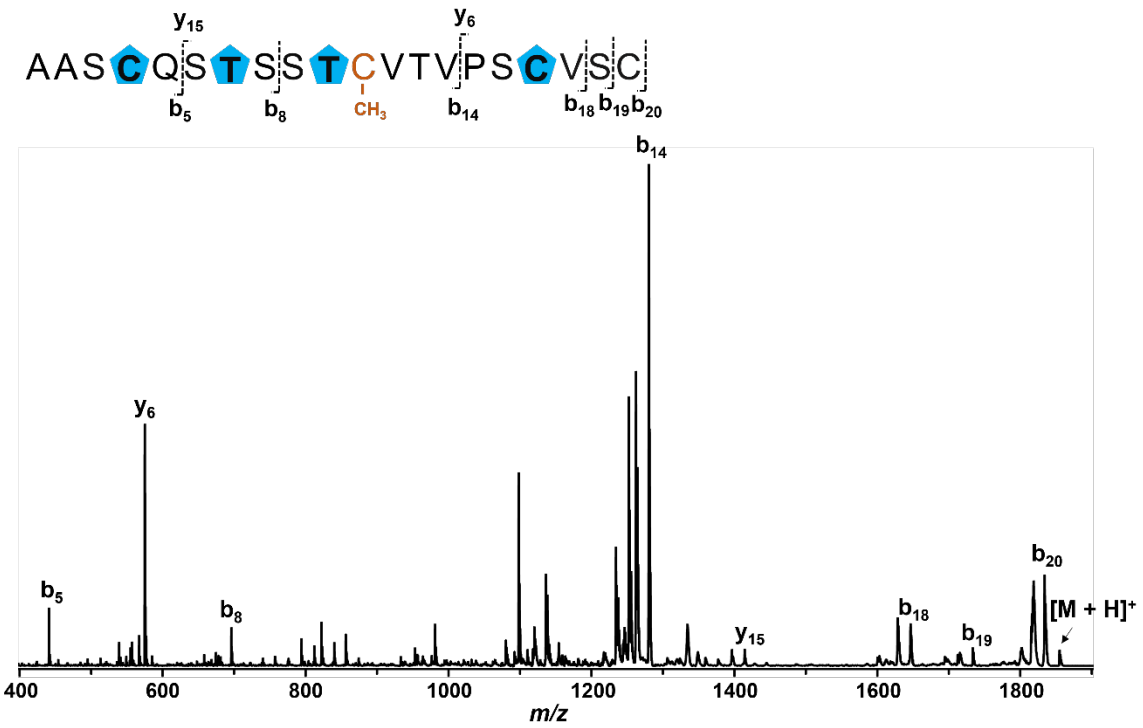
b5
b8 b9 b10
b5
y8
b8
b9
y9
b10
[M + H]+
200 300 400 500 600 700 800 900
m/z

b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y15
y6
b5
b8
CH3
b14
b18 b19 b20
b14
y6
b5
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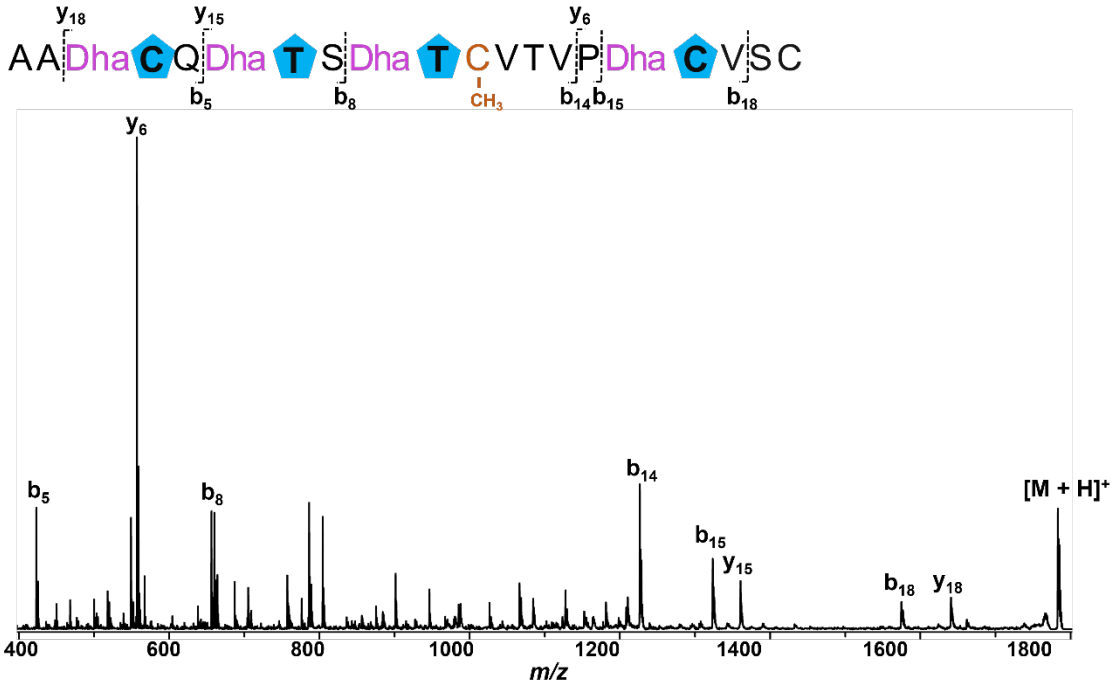
b19
b20
[M + H]+
400 600 800 1000 1200 1400 1600 1800
m/z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y15
y6
b5
b8
CH3
b14 b15
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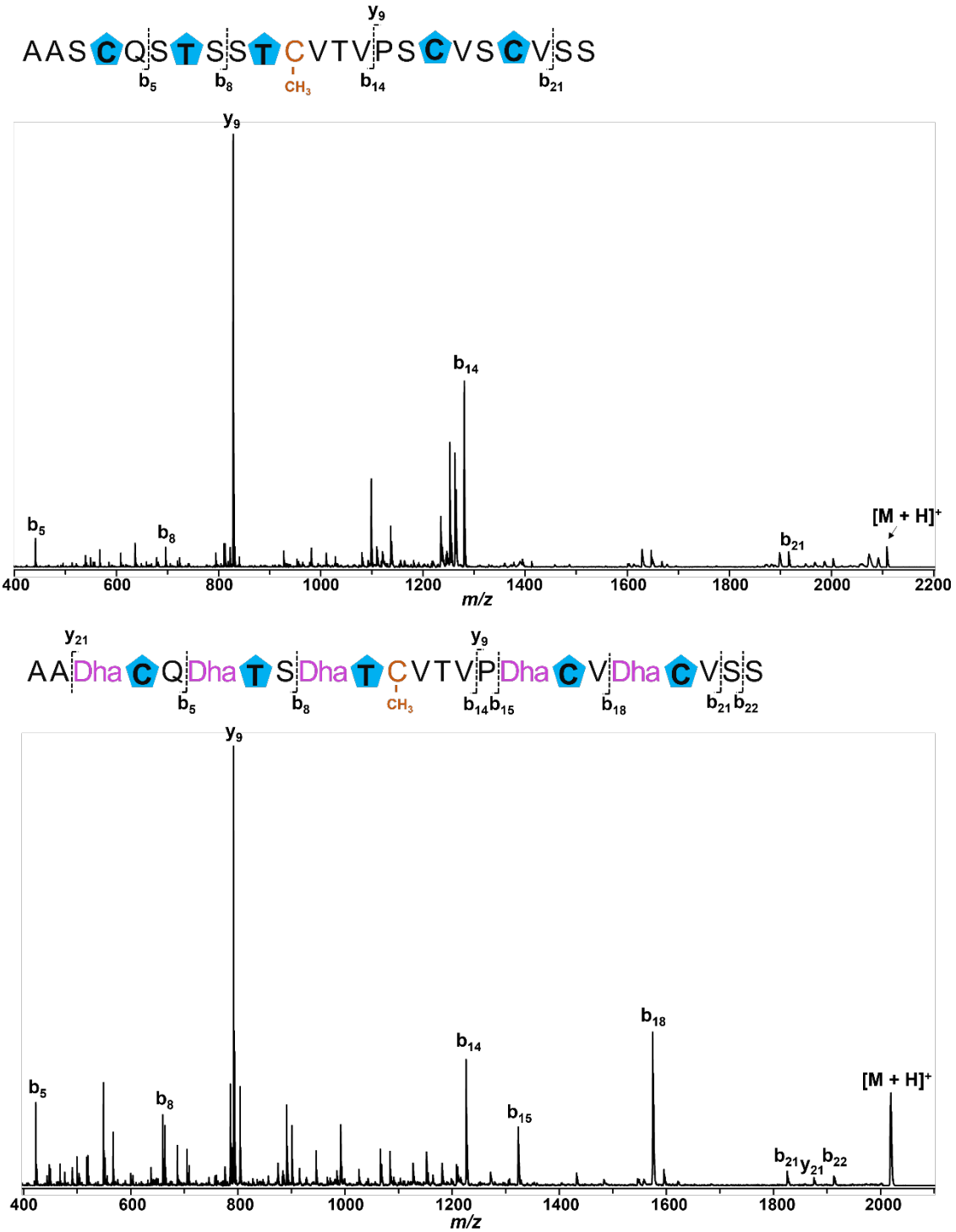
y18
[M + H]+
400 600 800 1000 1200 1400 1600 1800
m/z

c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y9
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600
800
1000
1200
1400
1600
1800
2000
2200
m/z
y21
y9
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b5
b8
CH3
b14
b15
b18
b21
b22
y9
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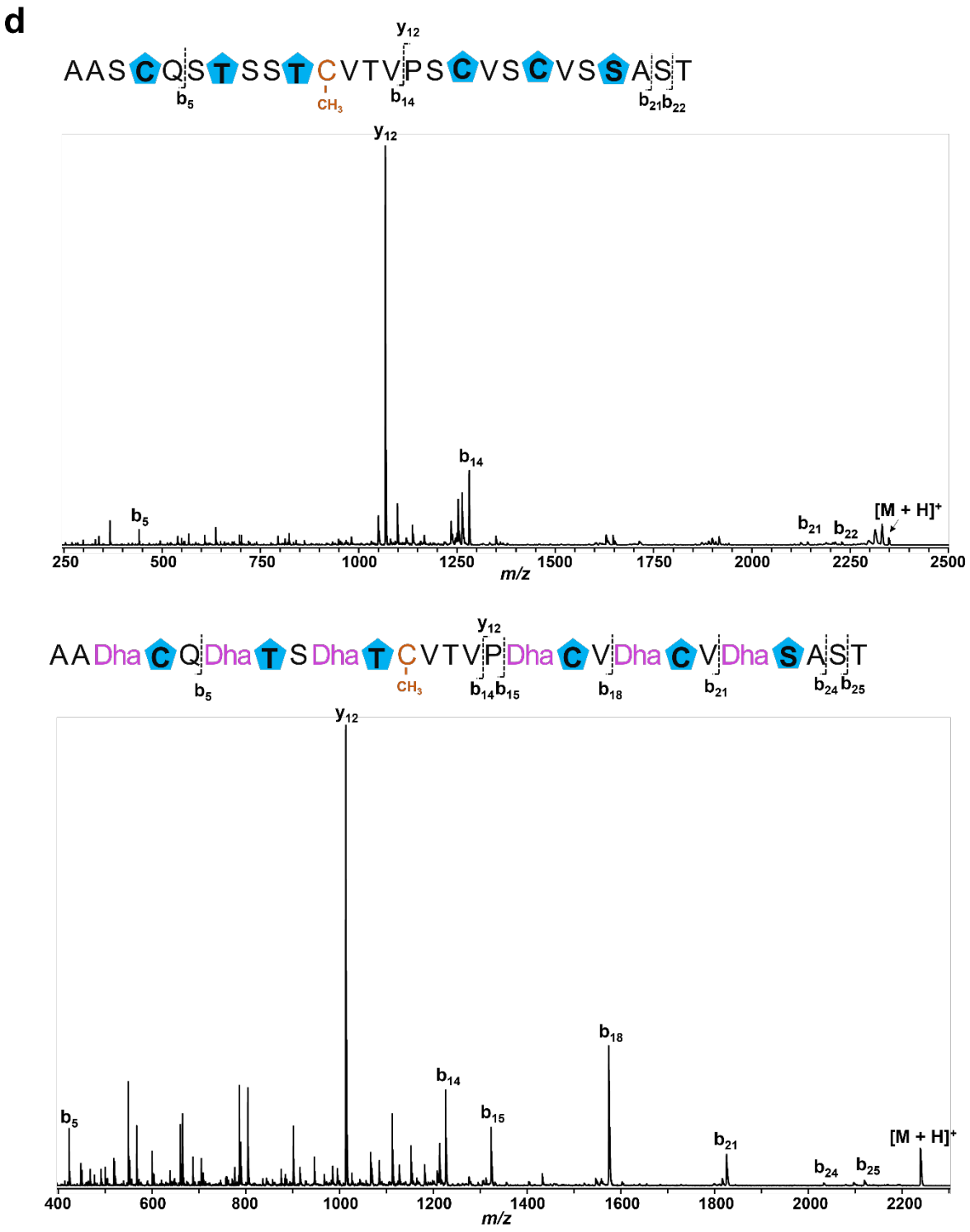
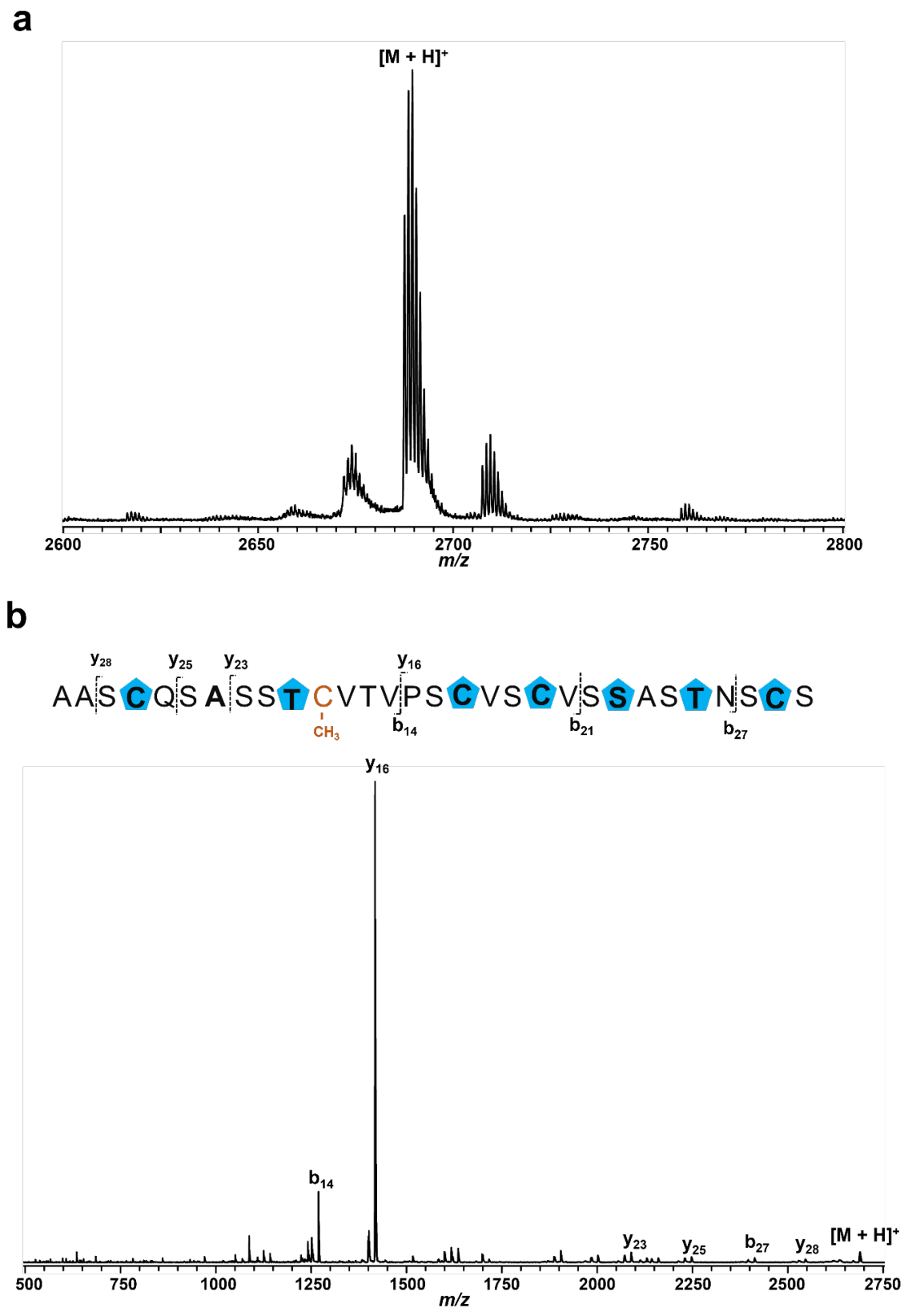
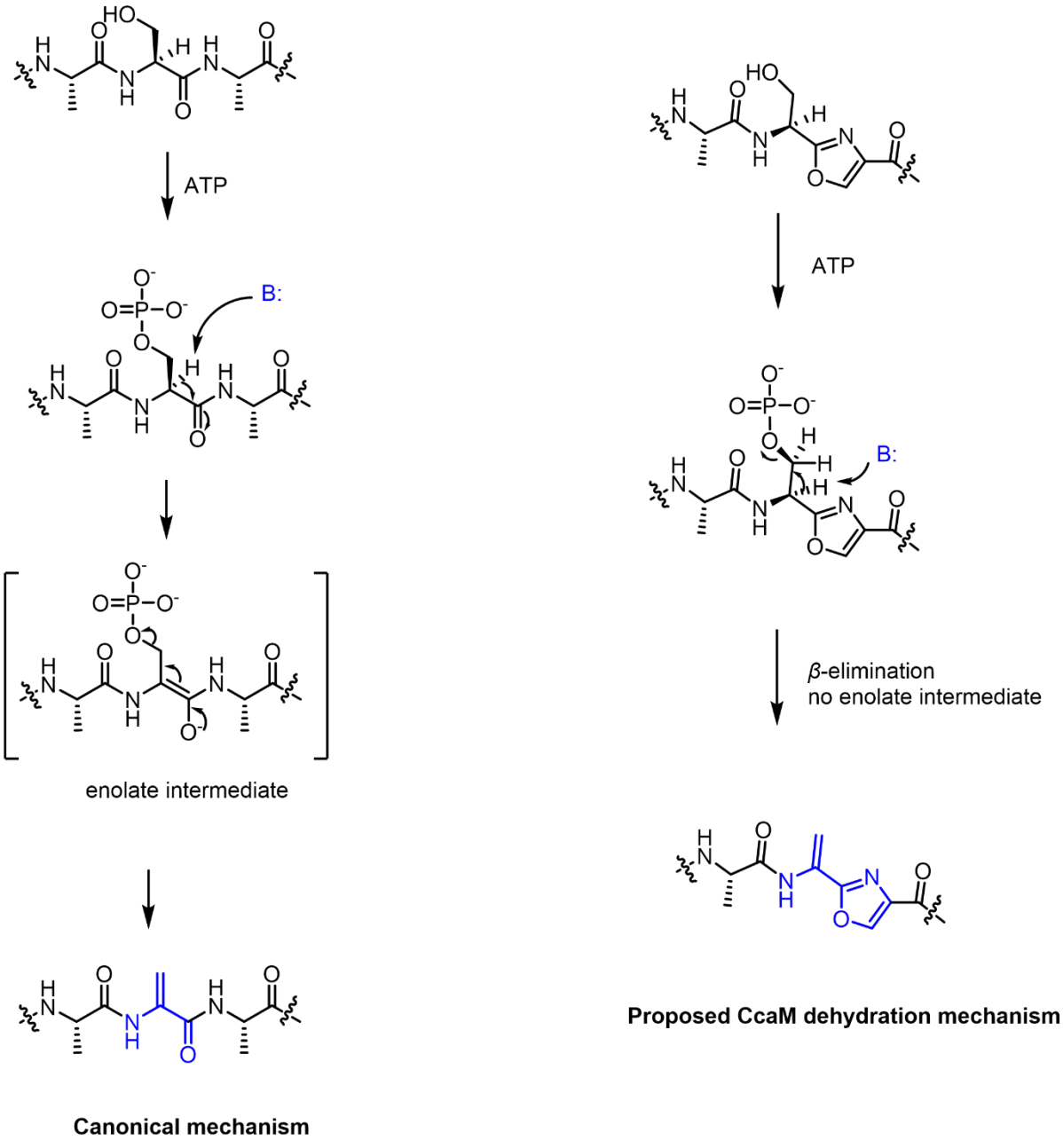


Supplementary Figure 18. MALDI-TOF MS/MS of cCcaA^{BCH} truncants and in vitro CcaM catalyzed dehydrated products. (**a**) MALDI-TOF MS/MS spectrum of cCcaA(1-10)^{BCH} and mono-dehydrated cCcaA(1-10)^{BCH}. (**b**) MALDI-TOF MS/MS spectrum of cCcaA(1-20)^{BCH} and tetra-dehydrated cCcaA(1-20)^{BCH}. (**c**) MALDI-TOF MS/MS spectrum of cCcaA(1-23)^{BCH} and penta-dehydrated cCcaA(1-23)^{BCH}. (**d**) MALDI-TOF MS/MS spectrum of cCcaA(1-26)^{BCH} and hexa-dehydrated cCcaA(1-26)^{BCH}.

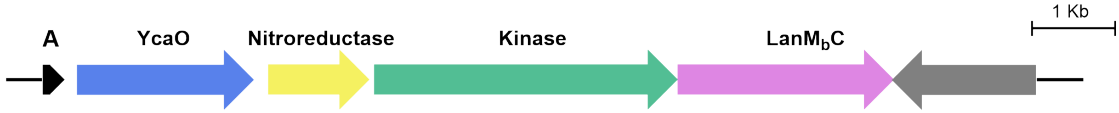


Supplementary Figure 19. MALDI-TOF mass spectra of cCcaA(T7A)^{BCH}. (**a**) MALDI-TOF MS of cCcaA(T7A)^{BCH}. **(b)** MALDI-TOF MS/MS of cCcaA(T7A)^{BCH}.



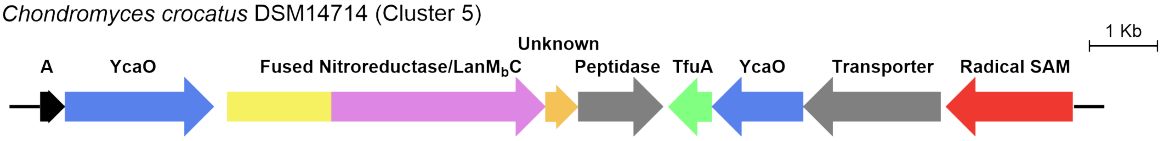
Supplementary Figure 20. Proposed dehydration mechanism of CcaM.

Cystobacter fuscus DSM2262 (Cluster 5)



A: MERLKRDPLSVAKEYDLPIIEDAAYQVVVYDNPASVISVVLSIPPKPGDLQDESLETLSSYAERTSCANSSTA

Chondromyces crocatus DSM14714 (Cluster 5)

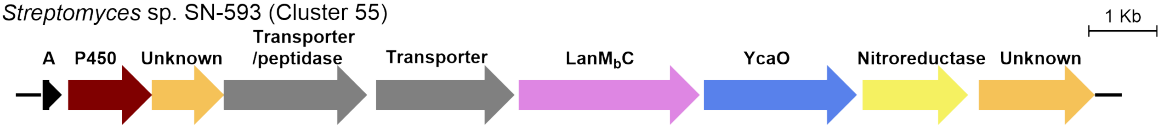


A: MSQQTQDDVLRIMAKVTAQAQADAEFKAEYLANPTEVLRRSGLIIPDNVTFKVQPANSVSPEYQVASGDVVYLVLPEVEELVQDESMATAAAASCETTASTAGTVSTCASSASTASSNSCS

uncultured *Candidatus Thioglobus* sp. (Cluster 39)

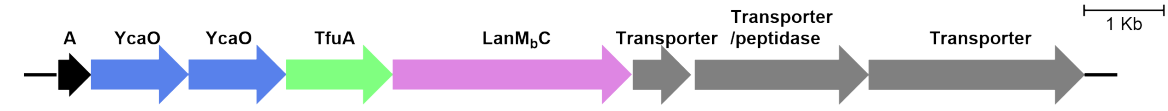
A1: MEQARINELNALMHDKSFRKQLTEDPKSCADKIGFPKEVGYELKVVKHTKDTYYFVIYEDSSNIPDDILAGVNAAGPMATLGTAGSLGTATSTVSTVGSAGSALVINNKVYS
A2: MKNMTDFNKIIEKLLDKEVRGKLLSNNPDRALLAEFGYQIDADTQVKVVASTKKVTYIVMSDDSIDLDLANVSAAGCQGTFSTAGSLCSSLSTAGSSSTI
A3: MEHNINTFADKLINKEFREKLLGDNPGLSLLSDFGYTFDEGQEVKVIASTKEVTYIVMPDDAELELDKVAAGMSIGAGTAGSVGTLCSSLSSASTAGTAINL
A4: MSNNTEKFFEDTKTNPALADAMKNANNENDVIAIGEKFGYTLTLDEVVKQMQTTYVDWSEQLKSISAAGCLTRDPGAYTVSSAEWNNYWNHYGGSYGGE (Nif11-like leader)

Streptomyces sp. SN-593 (Cluster 55)



A: MSDIQLTAEERRSFGALVAQLWSDEELATRYRDEPVAVLAEYGISAQEALPVPPAPVEEISDESLGLLGAVGGLSTCGSASSFSCPGCTASTVGSGTCCGSQPTLPVS

Nakamurella sp. PAMC28650 (Cluster 59)



A: MEKDRAIALVKMLAADPALRERLTSATESNRLAILTELGYADVTPADVLASASLWVPQAVEEIDDEQLASVAGGGITGDNPTIITTTTVTVGAAAAAAT

Supplementary Figure 21. Representative biosynthetic gene cluster containing the novel LanM$_b$C and a YcaO.

Supplementary Table 1. Plasmids constructed in this study.

Plasmid	Insert gene	Vector	Cloning site
pCcaA-RSF	*ccaA*	pRSF-Duet-TEV	*Bam*HI
pCcaAH-RSF	*ccaA*, *ccaH*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaAD-RSF	*ccaA*, *ccaD*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaAM-RSF	*ccaA*, *ccaM*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaA(1-10)H-RSF	*ccaA (*1-10*)*, *ccaH*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaA(1-20)H-RSF	*ccaA (1-20)*, *ccaH*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaA(1-23)H-RSF	*ccaA (1-23)*, *ccaH*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaA(1-26)H-RSF	*ccaA (1-26)*, *ccaH*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaA(T7A)H-RSF	*ccaA (T7A)*, *ccaH*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaA(M1C)H-RSF	*ccaA (M1C)*, *ccaH*	pRSF-Duet-TEV	*Bam*HI, *Nde*I
pCcaBC-CDF	*ccaB*, *ccaC*	pCDF-Duet-TEV	*Nco*I, *Nde*I
pCcaM-ET	*ccaM*	pET-Duet-TEV	*Nco*I
pCcaD-ET	*ccaD*	pET-Duet-TEV	*Nde*I
pCcaH-ET	*ccaH*	pET-Duet-TEV	*Nde*I
pCcaMD-ET	*ccaM*, *ccaD*	pET-Duet-TEV	*Nco*I, *Nde*I
pCcaMH-ET	*ccaM*, *ccaH*	pET-Duet-TEV	*Nco*I, *Nde*I
pCcaF-LIC	*ccaF*	pET-His6-TEV-LIC	*Ssp*I
pCcaM-LIC	*ccaM*	pET-His6-TEV-LIC	*Ssp*I
pCcaM$_N$-LIC	*ccaM$_N$(1-645)*	pET-His6-TEV-LIC	*Ssp*I
pCcaM$_C$-LIC	*ccaM$_C$(645-1015)*	pET-His6-TEV-LIC	*Ssp*I

Supplementary Table 2. Combination of plasmids for heterologous expression in *E. coli* BL21 (DE3).

Combination#	Enzymes	Antibiotics
pCcaA-RSF	His-tagged CcaA	kan
pCcaAM- RSF	His-tagged CcaA, CcaM	kan
pCcaA-RSF, pCcaBC-CDF	His-tagged CcaA, CcaB and CcaC	kan, spec
pCcaAD-RSF, pCcaBC-CDF	His-tagged CcaA, CcaB, CcaC and CcaD	kan, spec
pCcaAH-RSF, pCcaBC-CDF	His-tagged CcaA, CcaB, CcaC and CcaH	kan, spec
pCcaA(1-10)H-RSF, pCcaBC-CDF	His-tagged CcaA(1-10), CcaB, CcaC and CcaH	kan, spec
pCcaA(1-20)H-RSF, pCcaBC-CDF	His-tagged CcaA(1-20), CcaB, CcaC and CcaH	kan, spec
pCcaA(1-23)H-RSF, pCcaBC-CDF	His-tagged CcaA(1-23), CcaB, CcaC and CcaH	kan, spec
pCcaA(1-26)H-RSF, pCcaBC-CDF	His-tagged CcaA(1-26), CcaB, CcaC and CcaH	kan, spec
pCcaA(T7A)H-RSF, pCcaBC-CDF	His-tagged CcaA(T7A), CcaB, CcaC and CcaH	kan, spec
pCcaA(M1C)H-RSF, pCcaBC-CDF	His-tagged CcaA(M1C), CcaB, CcaC and CcaH	kan, spec
pCcaAH-RSF, pCcaBC-CDF, pCcaD-ET	His-tagged CcaA, CcaB, CcaC, CcaH and CcaD	kan, spec, amp
pCcaAD-RSF, pCcaBC-CDF, pCcaH-ET	His-tagged CcaA, CcaB, CcaC, CcaD and CcaH	kan, spec, amp
pCcaAD-RSF, pCcaBC-CDF, pCcaM-ET	His-tagged CcaA, CcaB, CcaC, CcaD and CcaM	kan, spec, amp
pCcaAD-RSF, pCcaBC-CDF, pCcaMH-ET	His-tagged CcaA, CcaB, CcaC, CcaD, CcaH and CcaM	kan, spec, amp
pCcaAH-RSF, pCcaBC-CDF, pCcaMD-ET	His-tagged CcaA, CcaB, CcaC, CcaH, CcaD and CcaM	kan, spec, amp

[#]Note: When the radical SAM protein CcaD was included in the co-expression system, pACYC-sufABCDSE (Chloramphenicol resistance) was also co-expressed to enhance the Fe-S cluster formation.

Supplementary Table 3. Primers used in this study.

Primer Name	Sequence (5' → 3')
CcaA-RSF1-FP	TACTTCCAAAGCCAGATGGAAAACCAAGCCGTA
CcaA-RSF1-RP	GCTCGAATTCGGATCTTAGCTGCAAGAATTTGT
CcaD-RSF2-FP	GAAGGAGATATACATATGCAAAAGAGCCTGAGT
CcaD-RSF2-RP	ATTGAGATCTGCCATTTAACATCCATAAAAGAAGCC
CcaM-RSF2-FP	GAAGGAGATATACATATGGATGTTAACGAAATA
CcaM-RSF2-RP	ATTGAGATCTGCCATTTATAGATTTTTGAAAGAATT
CcaH-RSF2-FP	GAAGGAGATATACATATGGATAACAGTGAAAAATTAACC
CcaH-RSF2-RP	ATTGAGATCTGCCATTTAAAGAGGCAGAGCGTT
CcaB-CDF1-FP	ATAAGGAGATATACCATGCGTATCCCGAAATTT
CcaB-CDF1-RP	ATGGCTGCTGCCCATTTAAATAAAGATGGAAATCGG
CcaC-CDF2-FP	GAAGGAGATATACATATGACCATGGAAAATAT
CcaC-CDF2-RP	ATTGAGATCTGCCATTTATAGATGGTTTCCTAAAAT
CcaM-ET1-FP	AGAAGGAGATATACCATGGATGTTAACGAAATA
CcaM-ET1-RP	ATGGCTGCTGCCCAT TTATAGATTTTTGAAAGAATT
CcaD-ET2-FP	GAAGGAGATATACATATGCAAAAGAGCCTGAGT
CcaD-ET2-FP	ATTGAGATCTGCCATTTAACATCCATAAAAGAAGCC
CcaH-ET2-FP	GAAGGAGATATACATATGGATAACAGTGAAAAATTAACC
CcaH-ET2-RP	ATTGAGATCTGCCATTTAAAGAGGCAGAGCGTT
CcaA-T7A-FP	TGCCAAAGCGCATCAAGTACCTGT
CcaA-T7A-RP	GGTACTTGATGCGCTTTGGCAGCT
CcaA-M1C-FP	CAAAGCCAGTGTGAAAACCAAGCC
CcaA-M1C -RP	TTGGTTTTCACACTGGCTTTGGAAGTA
CcaA-(1-10)-FP	TCAAGTACCTAAGTTACTGTACCA
CcaA-(1-10)-RP	TACAGTAACTTAGGTACTTGATGT
CcaA-(1-20)-FP	GTAAGTTGCTAATCAAGCGCATCT
CcaA-(1-20)-RP	TGCGCTTGATTAGCAACTTACACA
CcaA-(1-23)-FP	GTATCAAGCTAATCTACAAATTCT
CcaA-(1-23)-RP	ATTTGTAGATTAGCTTGATACGCA
CcaA-(1-26)-FP	GCATCTACATAATCTTGCAGCTAA
CcaA-(1-26)-RP	GCTGCAAGA TTA TGTAGATGCGCT
CcaF-His-FP	CTGTACTTCCAATCCAATGCAATGGCAGTTAAAATCGTTATT
CcaF-His-RP	CCGTTATCCACTTCCAATTTAGCTGCTTAAATTGATTCTG
CcaM-His-FP	CTGTACTTCCAATCCAATGCAATGGATGTTAACGAAATA
CcaM-His-RP	CCGTTATCCACTTCCAATTTATAGATTTTTGAAAGAATT
$CcaM_N$-His-FP	CTGTACTTCCAATCCAATGCAATGGATGTTAACGAAATA
$CcaM_N$-His-RP	CCGTTATCCACTTCCAATTTAATCCCCGCAATAGAAGGT
$CcaM_C$-His-FP	CTGTACTTCCAATCCAATGCAATGGATCATGAAGCACCTGTAGGC
$CcaM_C$-His-RP	CCGTTATCCACTTCCAATTTATAGATTTTTGAAAGAATT

Supplementary Table 4. ^{1}H chemical shifts of cCcaA^{BCH} in 9:1 (v/v) H_2O/ D_2O at 25 °C.

number	AA	NH	αH	βH	γH	δH	others
1	Ala		3.95	1.37			
2	A	8.51	4.29	1.29			
3	Ser	8.87	5.16	3.92			
4	thiazole				8.05		
5	Gln	8.62	4.48	2.1,1.99	2.29	NH_2: 7.37, 6.66	
6	Ser	8.78	5.03	3.86			
7	methyloxazole				2.36		
8	Ser	8.12	4.53	3.83			
9	Ser	8.69	5.04	3.86			
10	methyloxazole				2.36		
11	Cys-S-CH_3[#]	8.19	4.58	2.88, 2.78	1.96		
12	Val	8.37	4.17	2.02	0.75, 0.72		
13	Thr	N/A	4.10	5.21	1.21		
14	Val	8.50	4.38	1.94	0.80, 0.73		
15	Pro (*trans*)		4.36	2.17, 1.79	1.84, 1.80	3.64, 3.53	
16	Ser	8.72	5.14	3.91			
17	thiazole				8.06		
18	Val	8.23	4.24	2.03	0.81, 0.79		
19	Ser	8.85	5.08	3.88			
20	thiazole				8.045		
21	Val	8.27	4.29	2.08	0.82		
22	Ser	8.96	5.22	3.90			
23	oxazole				8.22		
24	Ala	8.30	4.41	1.33			
25	Ser	8.64	5.01	3.85			
26	methyloxazole				2.36		
27	Asn	8.35	4.80	2.75	NH_2: 7.48, 6.79		
28	Ser	8.81	5.21	3.91			
29	thiazole				8.041		
30	Ser	8.45	4.47	3.86			

^{1}H was referenced to H_2O at 4.80 ppm.

Observed STD peaks shown in green background (the indistinguishable proton of methyloxazole shown in teal background).

[#]: *S*-methylated Cys

Supplementary Table 5. ^{1}H chemical shifts of carnazolamide in CD_3OH at 25 ℃.

Number	AA	NH	αH	βH	γH	δH
1	Ala		3.98	1.53		
2	Ala	8.728	4.82	1.465		
3	Dha	9.56		6.32, 5.583		
4	thiazole			8.24		
5	Gln	9.309	4.678	2.25	2.585, 2.459	7.60, 7.01 (NH_2)
6	Dha	9.58		6.18, 5.74		
7	methyloxazole			2.61		
8	Ser	8.15	4.78	3.99, 3.93		
9	Dha	9.53		6.20, 5.76		
10	methyloxazole			2.62		
11	Cys-S-CH_3[#]	8.04	4.68	2.93, 2.80		2.10
12	Val	8.24	4.03	1.90	0.73, 0.76	
13	Thr	N/A	4.78	4.05	0.784	
14	Val	8.646	4.608	2.05	0.90, 0.82	
15	Pro (*trans*)		4.84	2.50, 1.84	2.05	3.733, 3.652
16	Dha	10.55		6.30, 5.50		
17	thiazole					
18	Val	8.66	4.673	2.411	1.12	
19	Dha	10.39		5.89, 5.82		
20	thiazole			8.28		
21	Val	8.47	4.61	2.30	1.06	
22	Dha	9.68		6.17,5.83		
23	oxazole			8.46		
24	Ala	8.402	4.772	1.54		
25	Dha	9.42		6.164, 5.75		
26	methyloxazole					
27	Asn	8.378	5.127	2.95, 2.85	7.77, 7.22 (NH_2)	
28	Dha	10.15		6.26, 5.58		
29	thiazole					
30	Ser	8.94	4.74	4.03		

[#]: *S*-methylated Cys.

^{1}H was referenced to CD_3OH at 3.31 ppm.

Supplementary Table 6. Biological assay of carnazolamide.

Antibacterial Assay		
Target strain	**Gram**	**Carnazolamide[#]**
Escherichia coli ATC25592	-	No
Staphylococcus epidermidis 1457	+	No
Bacillus subtilis ATCC 6633	+	No
Micrococcus luteus	+	No
Lactococcus lactis	+	No
Staphylococcus carnosus TM300	+	No

Cytotoxicity Assay	
	Carnazolamide*
VCaP cell line	> 20 μM
Caki-1 cell line	> 20 μM
MDA-MB-231 cell line	> 20 μM

[#]: Carnazolamide was dissolved in methanol as 1 mM stock solution, 5μl stock solution (5 nmol) was used to perform antibacterial activity assay with disc diffusion test, no antibacterial activity was observed.

[*]: Cytotoxicity assay was performed with the cell viability test using the alamarBlue assay method. No activities were observed with 20 μM top dose.

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3. Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583-589 (2021).
4. Freeman, M. F. *et al.* Metagenome mining reveals polytheonamides as posttranslationally modified ribosomal peptides. *Science* **338**, 387–390 (2012).

Uncropped SDS-PAGE protein gel