

Supplementary Information

Tandem ketone reduction in pepstatin biosynthesis reveals an F₄₂₀H₂–dependent statine pathway

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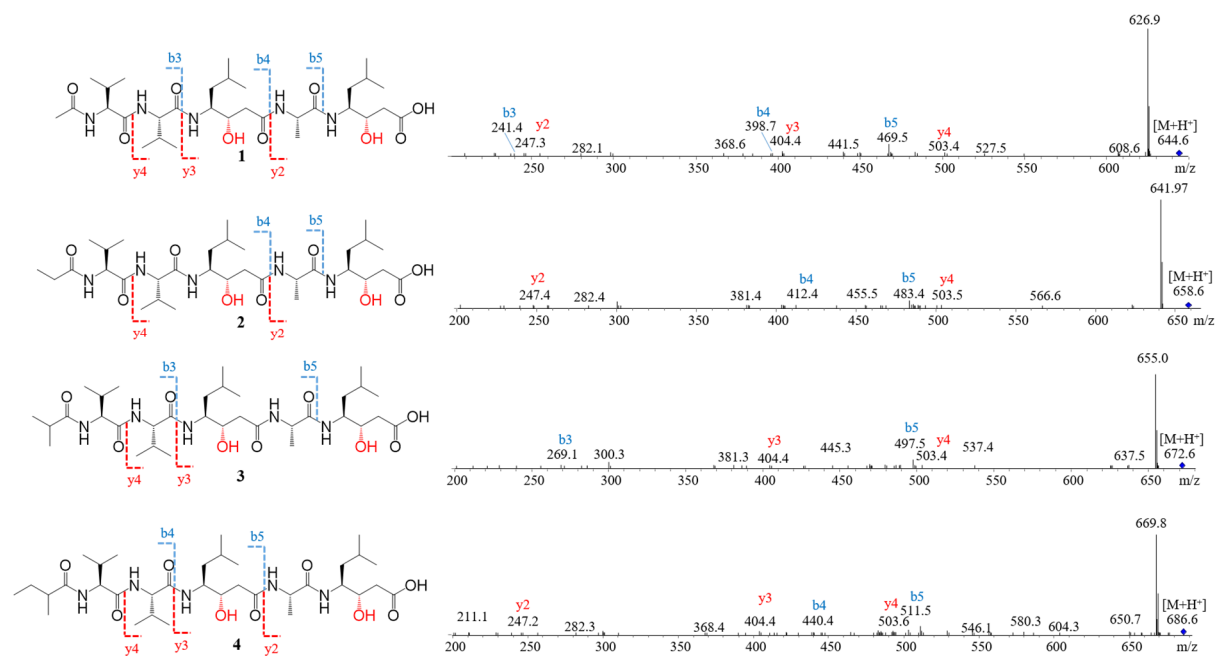
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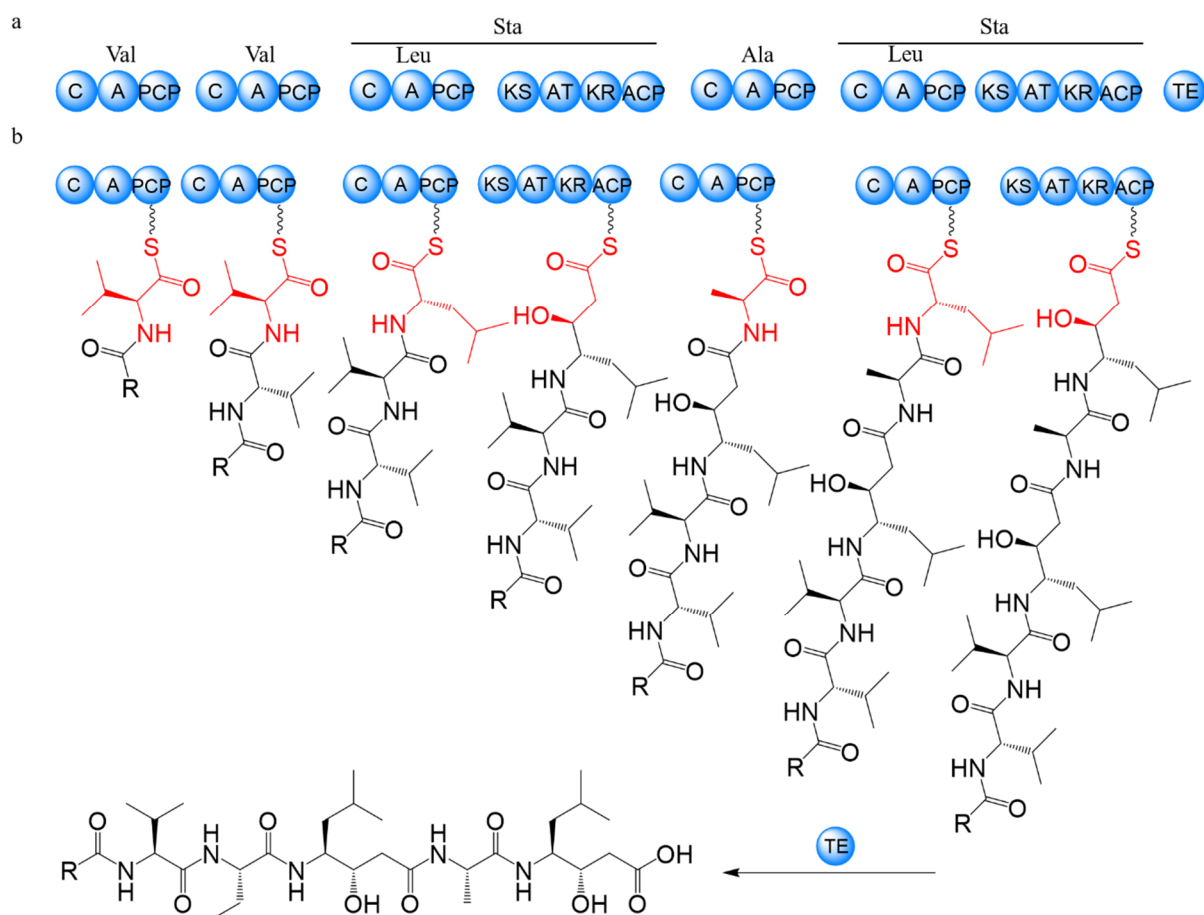
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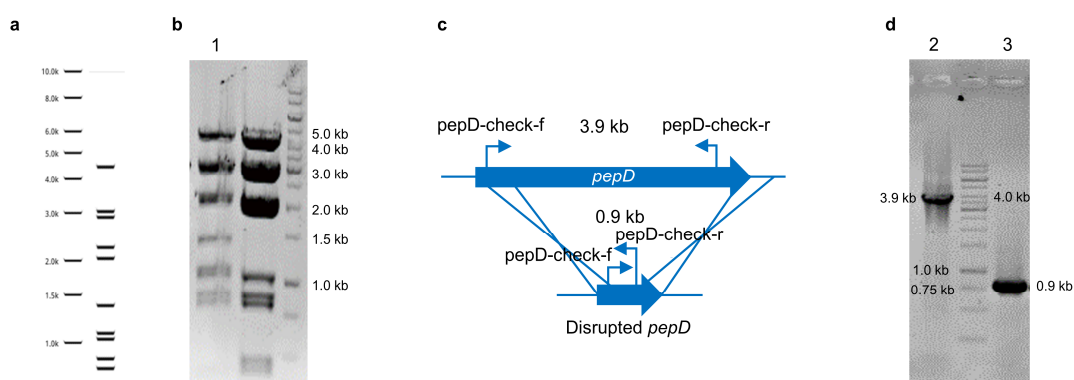


Supplementary Fig. 1. HPLC-MS/MS fragmentation analysis of compounds 1-4.

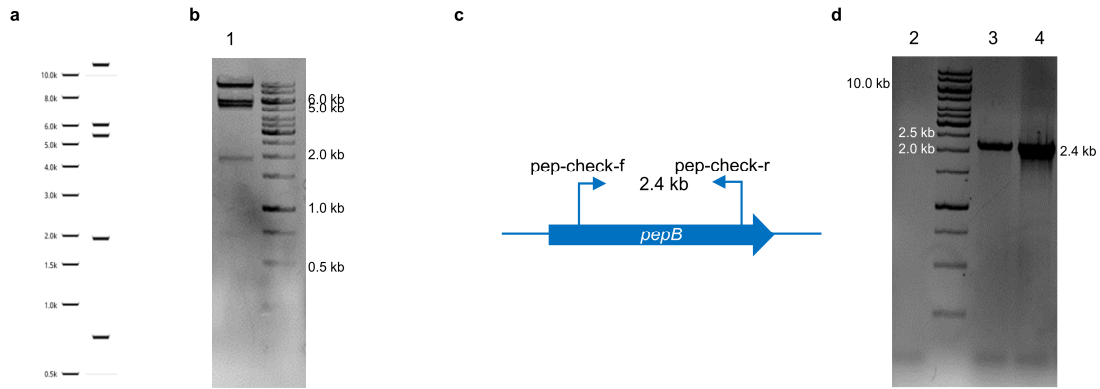


Supplementary Fig. 2. Theoretical NRPS assembly line for pepstatin based on colinearity principles.

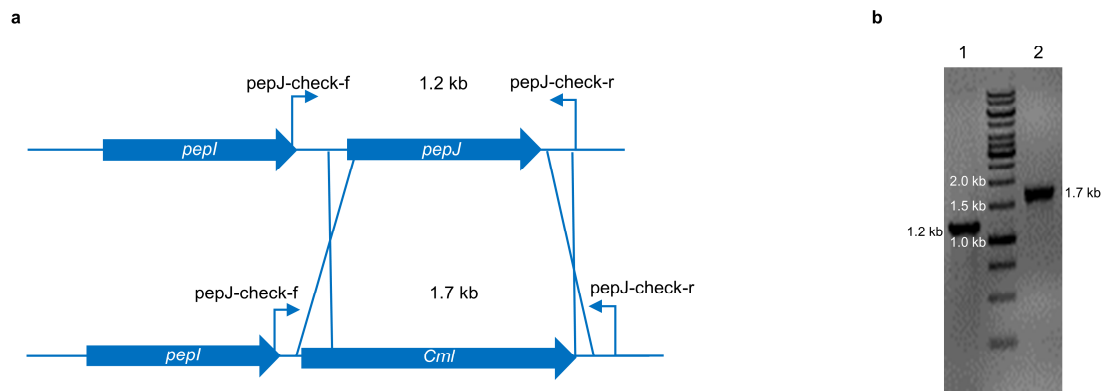
a. Theoretical pepstatin biosynthesis NRPS BGC schematic diagram; **b.** Theoretical pepstatin biosynthesis pathway. C: condensation domain; A: adenylation domain; PCP: peptidyl carrier protein; KS: ketosynthase; AT: acyltransferase; KR: ketoreductase; ACP: acyl carrier protein; TE: thioesterase.



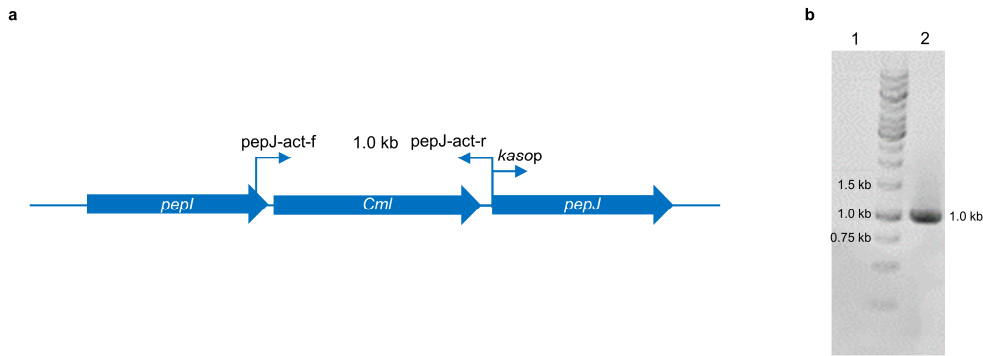
Supplementary Fig. 3. Plasmid pQS9- Δ pepD and mutant *S. catenulae* - Δ pepD verification. **a.** *In silico* digestion of pQS9- Δ pepD with *Sal*I; **b.** 1. pQS9- Δ pepD digested with *Sal*I; **c.** pepD gene disruption schematic diagram; **d.** 2-3. PCR verification with primers pepD-check-f/ pepD-check-r 2. *S. catenulae*; 3. *S. catenulae*- Δ pepD.



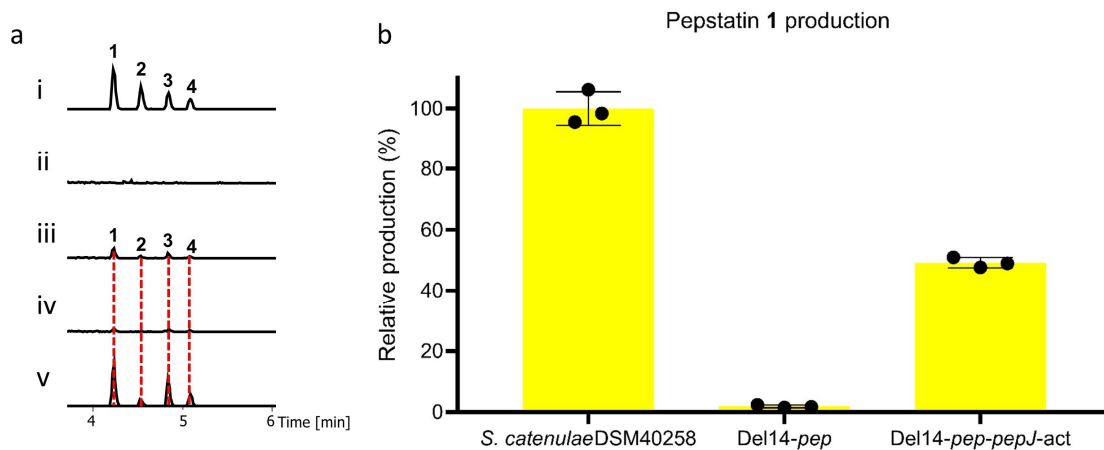
Supplementary Fig. 4. Plasmid p15A-int-pep and mutant *S. albus* Del14-pep verification. **a.** *In silico* digestion of p15A-int-pep with *Nco*I; **b.** 1. p15A-int-pep digested with *Nco*I; **c.** BGC *pep* PCR verification schematic diagram; **d.** 2-4. PCR verification with primers pep-check-f/ pep-check-r 2. *S. albus* Del14; 3. *S. catenulae*; 4. *S. albus* Del14-pep.



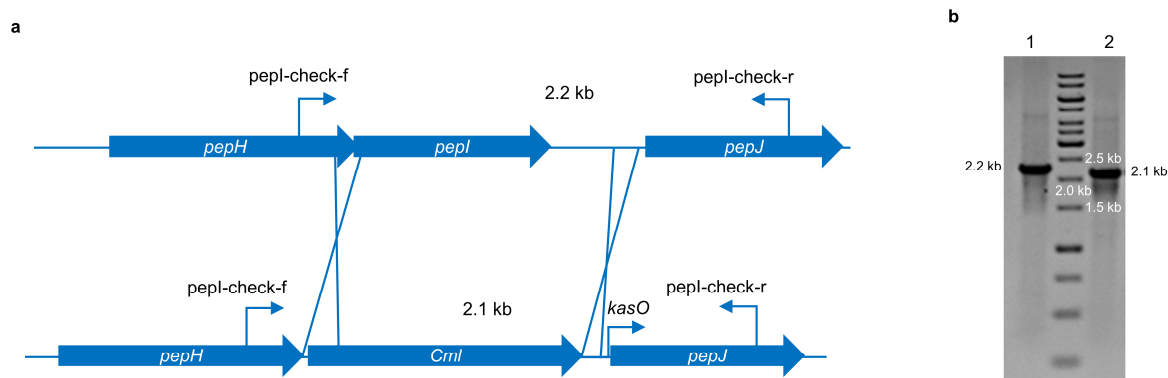
Supplementary Fig. 5. PCR verification of *pepJ* gene deletion mutant. **a.** *pepJ* gene deletion schematic diagram; **b.** *pepJ* gene deletion mutant PCR verification 1. *S. albus* Del14-pep; 2. *S. albus* Del14-pep- Δ *pepJ*.



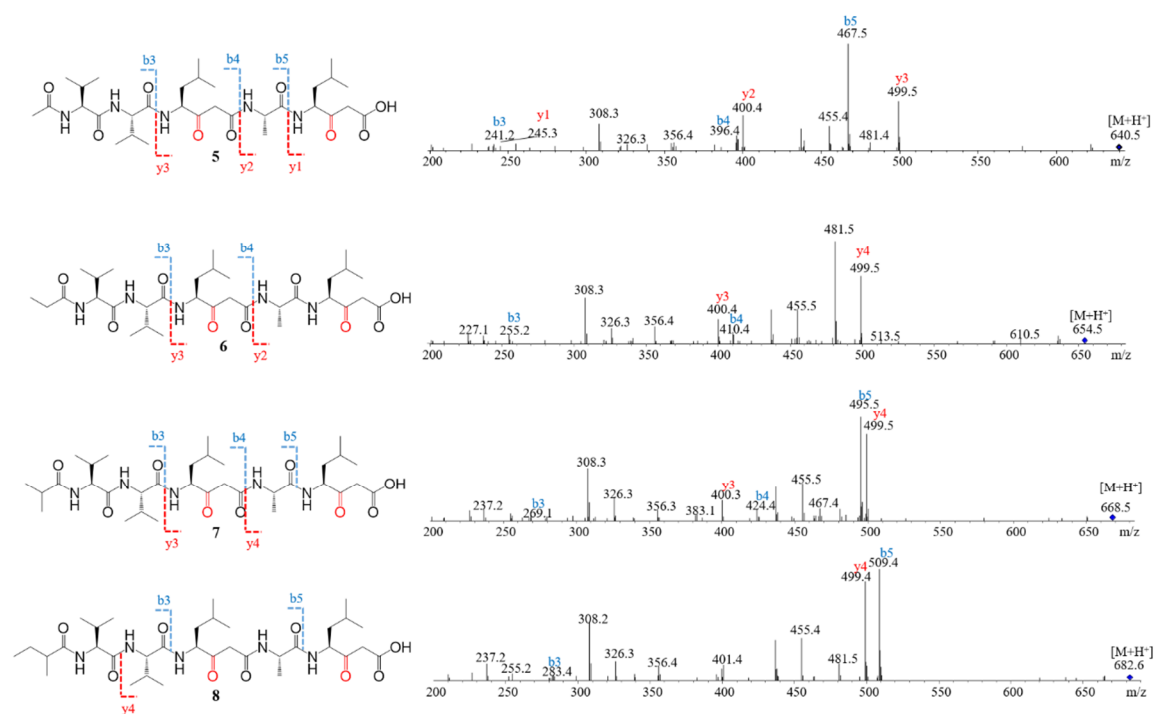
Supplementary Fig. 6. PCR verification of *pepJ* gene activation mutant. **a.** *pepJ* gene activation schematic diagram; **b.** *pepJ* gene activation mutant PCR verification 1. *S. albus* Del14-*pep*; 2. *S. albus* Del14-*pep-pepJ-act*.



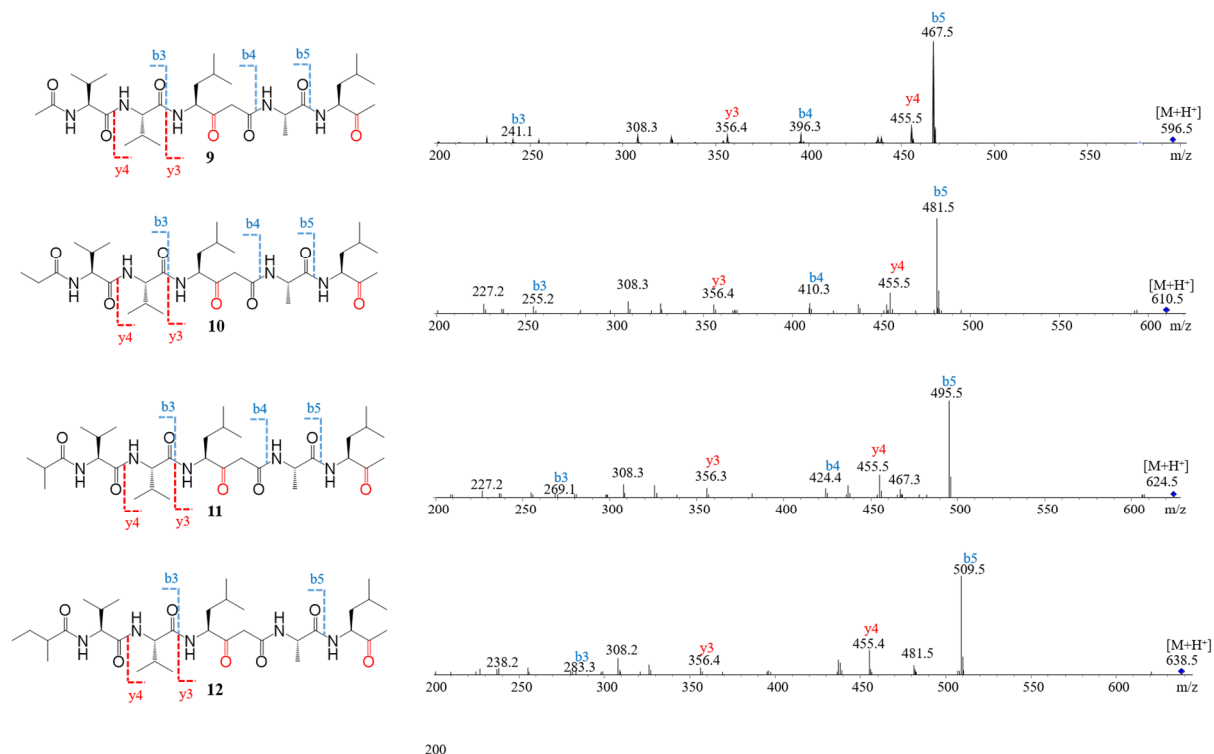
Supplementary Fig. 7. Pepstatin yield in different producers. **a.** UPLC-HRMS analysis (extracted ion chromatogram (EIC)) of Pepstatin congeners (**1-4**: **1**, $[M + H]^+ = 644.42$; **2**, $[M + H]^+ = 658.44$; **3**, $[M + H]^+ = 672.45$ and **4**, $[M + H]^+ = 686.47$) produced by *Streptomyces catenulae* DSM40258 (**i**); Knocking out *pepD* abolished pepstatin **1-4** production (**ii**); Pepstatin congeners (**1-4**) produced by heterologous expression of BGC *pep* in Del14 (Del14-*pep*) (**iii**); The production of **1-4** decreased by *pepJ* deletion in Del14-*pep-ΔpepJ* (**iv**); The production of **1-4** increased in Del14-*pep-pepJ-act* by promoter exchange of *pepJ* (**v**). **b.** The production of pepstatin **1** in the *pepJ* activation mutants *S. albus* Del14-*pep-pepJ-act* (right) was enhanced by 25-fold on average compared to *S. albus* Del14-*pep* (middle) but still lower than in the WT strain *S. catenulae* DSM40258 (left). The production in each culture was determined by calculating the sum of the peak area, while the yield in the WT strain was denoted as $100 \pm 5.5\%$. The yield from heterologously expressed mutant *S. albus* Del14-*pep* is $2 \pm 0.5\%$. By placing regulator gene *pepJ* under the control of a constitutive promoter in the heterologously expressed mutant, the yield of *S. albus* Del14-*pep-pepJ-act* is $49 \pm 1.7\%$. Three biological replicates ($n=3$ independent experiments) were performed. All error bars represent SEM. Source data are provided as a Source Data file.



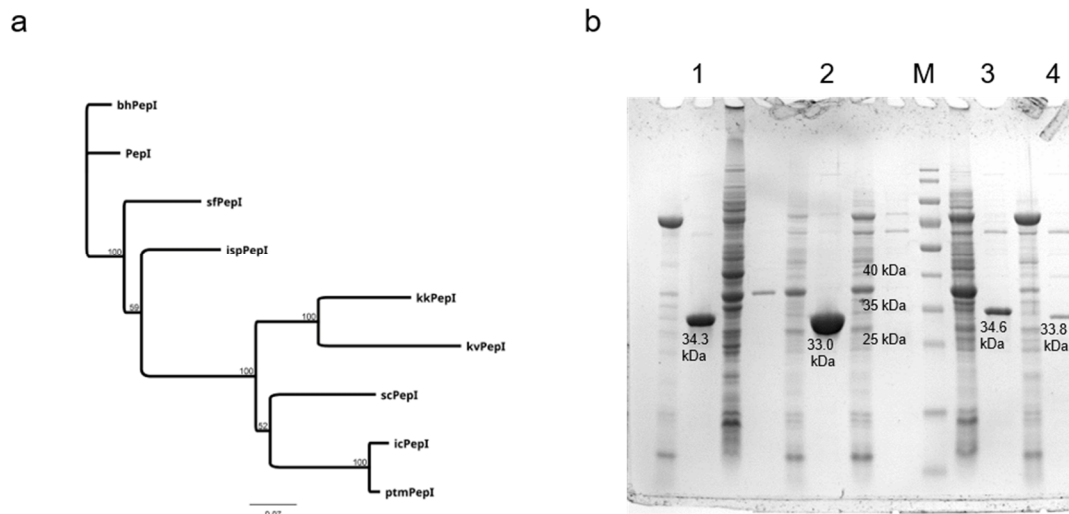
Supplementary Fig. 8. PCR verification of *S. albus* Del14-*pepI*. **a.** *pepI* gene deletion schematic diagram; **b.** *pepI* gene deletion mutant PCR verification 1. *S. albus* Del14-*pep*; 2. *S. albus* Del14-*pep*- Δ *pepI*.



Supplementary Fig. 9. HPLC-MS/MS fragmentation analysis of compounds **5-8**.

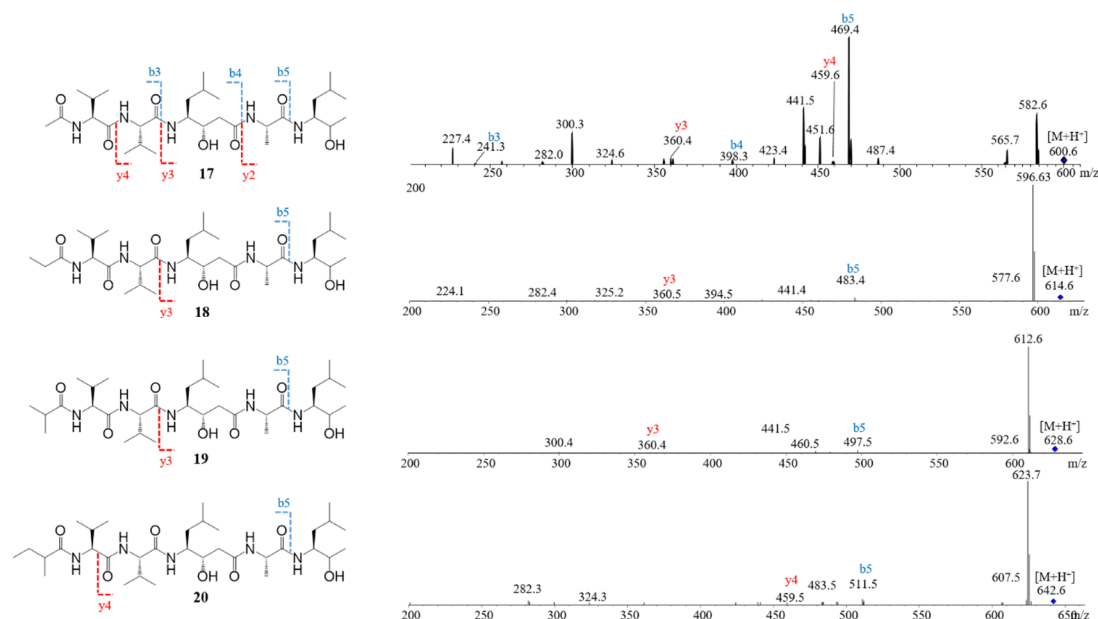


Supplementary Fig. 10. HPLC-MS/MS fragmentation analysis of compounds 9-12.

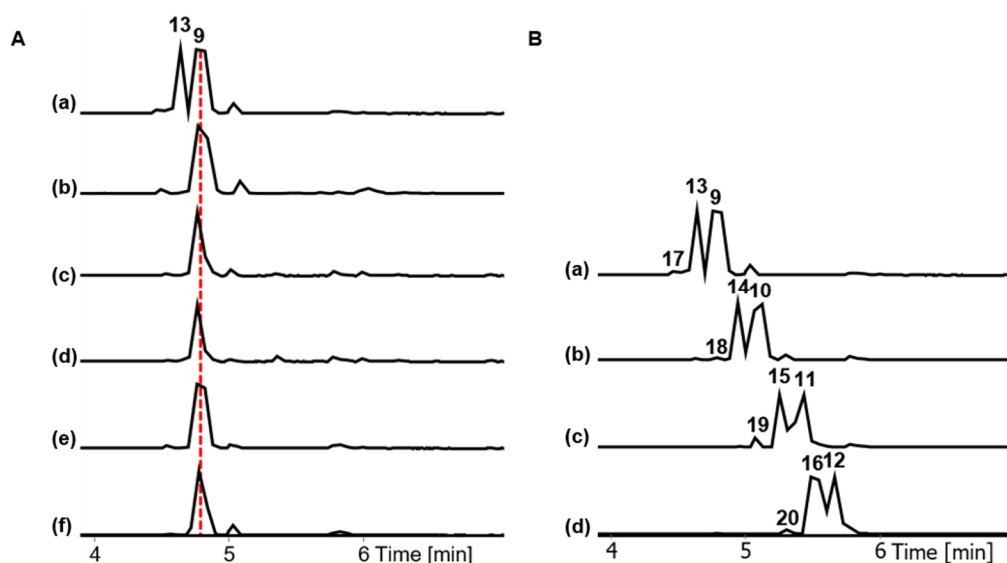


Supplementary Fig. 11. PepI and PepI homologues. **a.** Alignment consensus phylogenetic tree of PepI and analogs (bhPepI from *Streptomyces* sp. BHT-5-2; sfPepI from *Streptomyces* sp. SF28; ispPepI from *Streptomyces varsoviensis* NRRL ISP-5346; kkPepI from *Kitasatospora kifunensis*; kvPepI from *Kitasatospora viridis*; scPepI from *Streptomyces cattleya*; icPepI from *Streptomyces* sp. ICBB 8177; ptmPepI from *Streptomyces* sp. PTM05;) from *pep*-like BGCs. Genetic distance model: Jukes-Cantor, Tree build method: Neighbor-Joining, Resampling method: Bootstrap, Random seed: 443,376, Scale bar: evolutionary distance of amino acid residue per position in the sequence. **b.** SDS-PAGE analysis of

PepI and PepI homologues 1. sfPepI Scientific™ PageRuler™ Prestained Protein Ladder, 10 to 180 kDa. 3. svPepI 34.6 kDa (from *Streptomyces varsoviensis*³); 4. PepI 33.8 kDa.

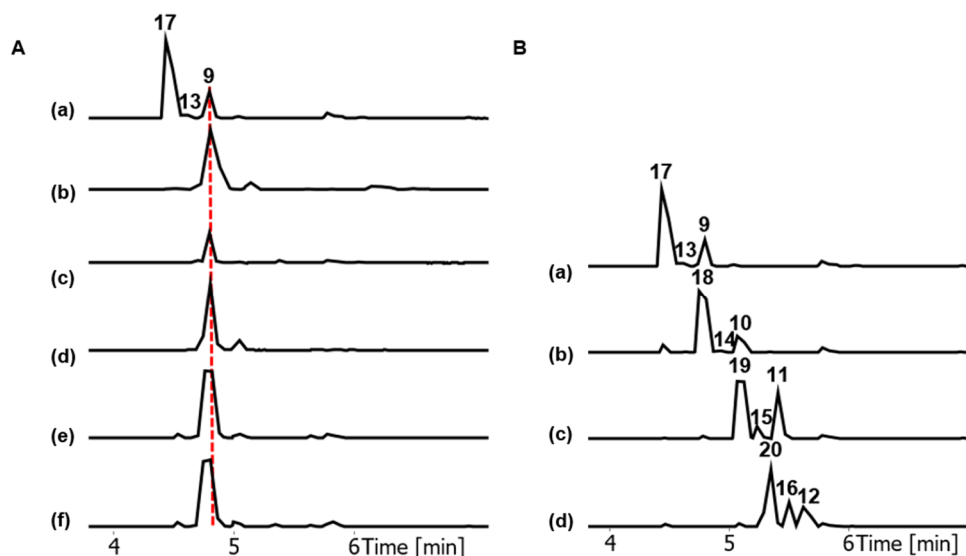


Supplementary Fig. 12. HPLC-MS/MS fragmentation analysis of compounds 17-20.

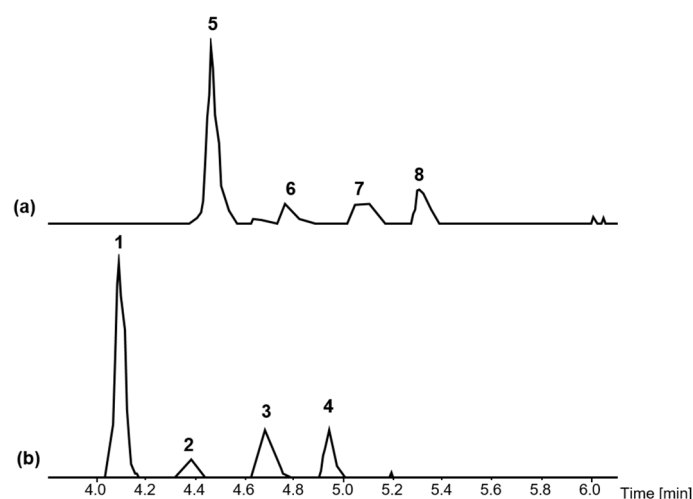


Supplementary Fig. 13. HPLC-MS analysis of PepI *in vitro* reactions. **A** Flavin cofactor preference and F₄₂₀ recycle. (a) PepI+9+F₄₂₀+FGD+G6P; (b) PepI+9+FGD+G6P; (c) PepI+9+FMN+NADH; (d) PepI+9+FAD+NADH; (e) PepI+9+F₄₂₀+FGD; (f) PepI+9+F₄₂₀+G6P. **B.** F₄₂₀H₂-dependent PepI *in vitro* ketoreduction with different substrates (a) 9 transformed to 13, 17; (b) 10 transformed to 14, 18; (c) 11 transformed to 15, 19; (d) 12 transformed to 16, 20. Base peak chromatograms (BPC) were shown. 9, [M + H]⁺ = 596.40; 10, [M + H]⁺ = 610.42; 11, [M + H]⁺ = 624.44; 12, [M + H]⁺ = 638.45; 13, [M + H]⁺ = 596.40; 14, [M + H]⁺ = 610.42; 15, [M + H]⁺ = 624.44; 16, [M + H]⁺ = 638.45; 17, [M + H]⁺ = 596.40; 18, [M + H]⁺ = 610.42; 19, [M + H]⁺ = 624.44; 20, [M + H]⁺ = 638.45.

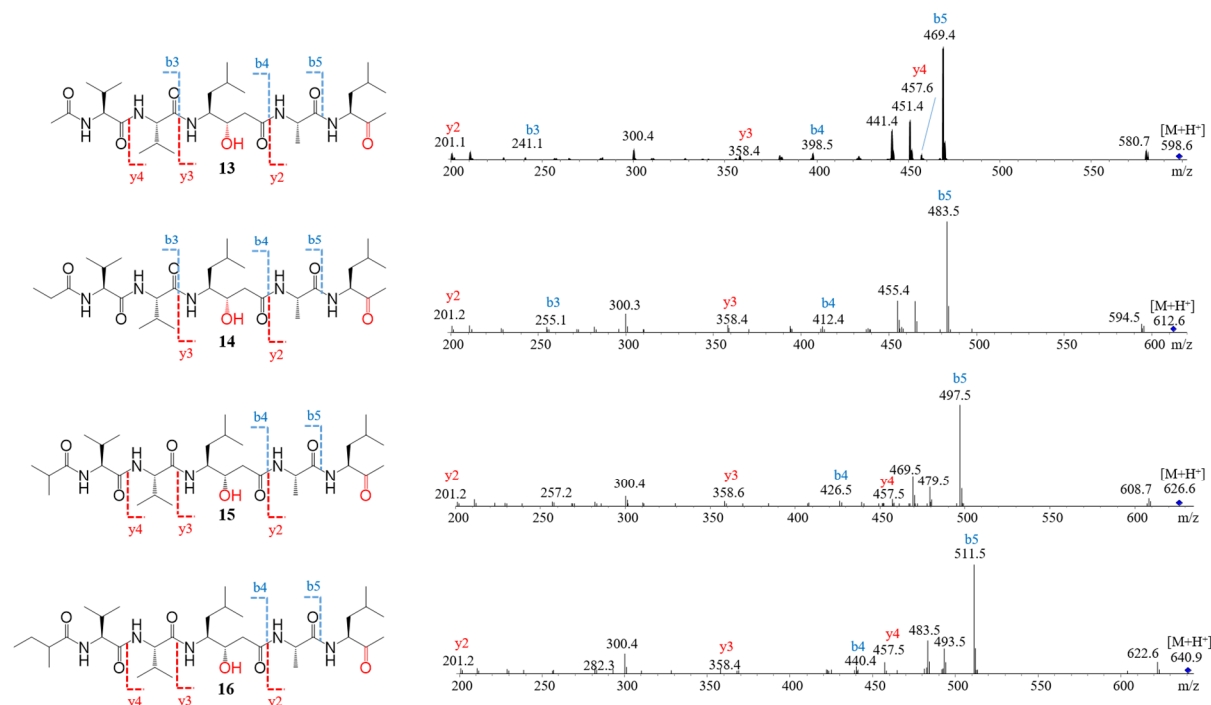
$H]^+ = 598.42$; **14**, $[M + H]^+ = 612.44$; **15**, $[M + H]^+ = 626.45$; **16**, $[M + H]^+ = 640.47$; **17**, $[M + H]^+ = 600.43$; **18**, $[M + H]^+ = 614.45$; **19**, $[M + H]^+ = 628.46$ and **20**, $[M + H]^+ = 642.48$ were analyzed.



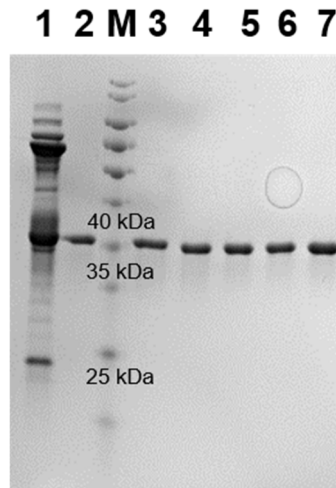
Supplementary Fig. 14. HPLC-MS analysis of kvPepI *in vitro* reactions. **A** Flavin cofactor preference and F_{420} recycle. (a) kvPepI+**9**+ F_{420} +FGD+G6P; (b) kvPepI+**9**+FGD+G6P; (c) kvPepI+**9**+FMN+NADH; (d) kvPepI+**9**+FAD+NADH; (e) kvPepI+**9**+ F_{420} +FGD; (f) kvPepI+**9**+ F_{420} +G6P; **B** $F_{420}H_2$ -dependent kvPepI *in vitro* ketoreduction with different substrates (a) **9** transformed to **13**, **17**; (b) **10** transformed to **14**, **18**; (c) **11** transformed to **15**, **19**; (d) **12** transformed to **16**, **20**. Base peak chromatograms (BPC) were shown. **9**, $[M + H]^+ = 596.40$; **10**, $[M + H]^+ = 610.42$; **11**, $[M + H]^+ = 624.44$; **12**, $[M + H]^+ = 638.45$; **13**, $[M + H]^+ = 598.42$; **14**, $[M + H]^+ = 612.44$; **15**, $[M + H]^+ = 626.45$; **16**, $[M + H]^+ = 640.47$; **17**, $[M + H]^+ = 600.43$; **18**, $[M + H]^+ = 614.45$; **19**, $[M + H]^+ = 628.46$ and **20**, $[M + H]^+ = 642.48$ were analyzed.



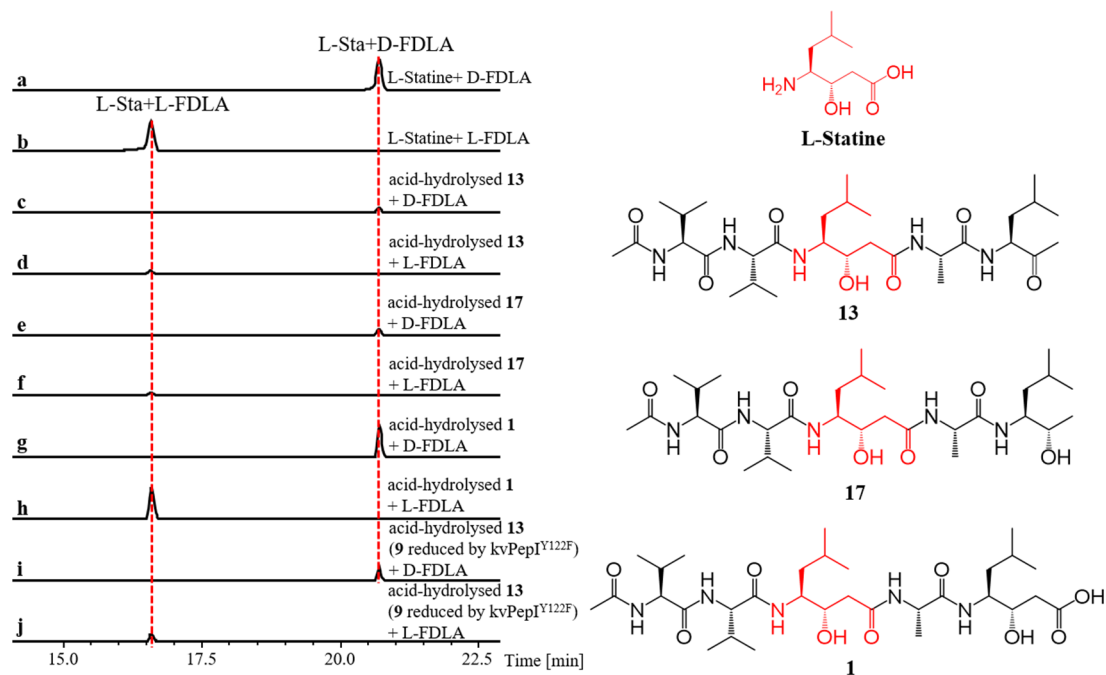
Supplementary Fig. 15. HPLC–MS analysis of PepI reaction using Del14- Δ pepI fermentation broth crude extracts as substrates. **(a).** Del14- Δ pepI crude extract+boiled PepI and cofactor system **(b).** Del14- Δ pepI crude extract+PepI and cofactor system. Extracted ion chromatograms (EIC): **5**, $[M + H]^+ = 640.39$; **6**, $[M + H]^+ = 654.40$; **7**, $[M + H]^+ = 668.42$; **8**, $[M + H]^+ = 682.44$; **1**, $[M + H]^+ = 644.42$; **2**, $[M + H]^+ = 658.44$; **3**, $[M + H]^+ = 672.45$ and **4**, $[M + H]^+ = 686.47$; were analyzed.



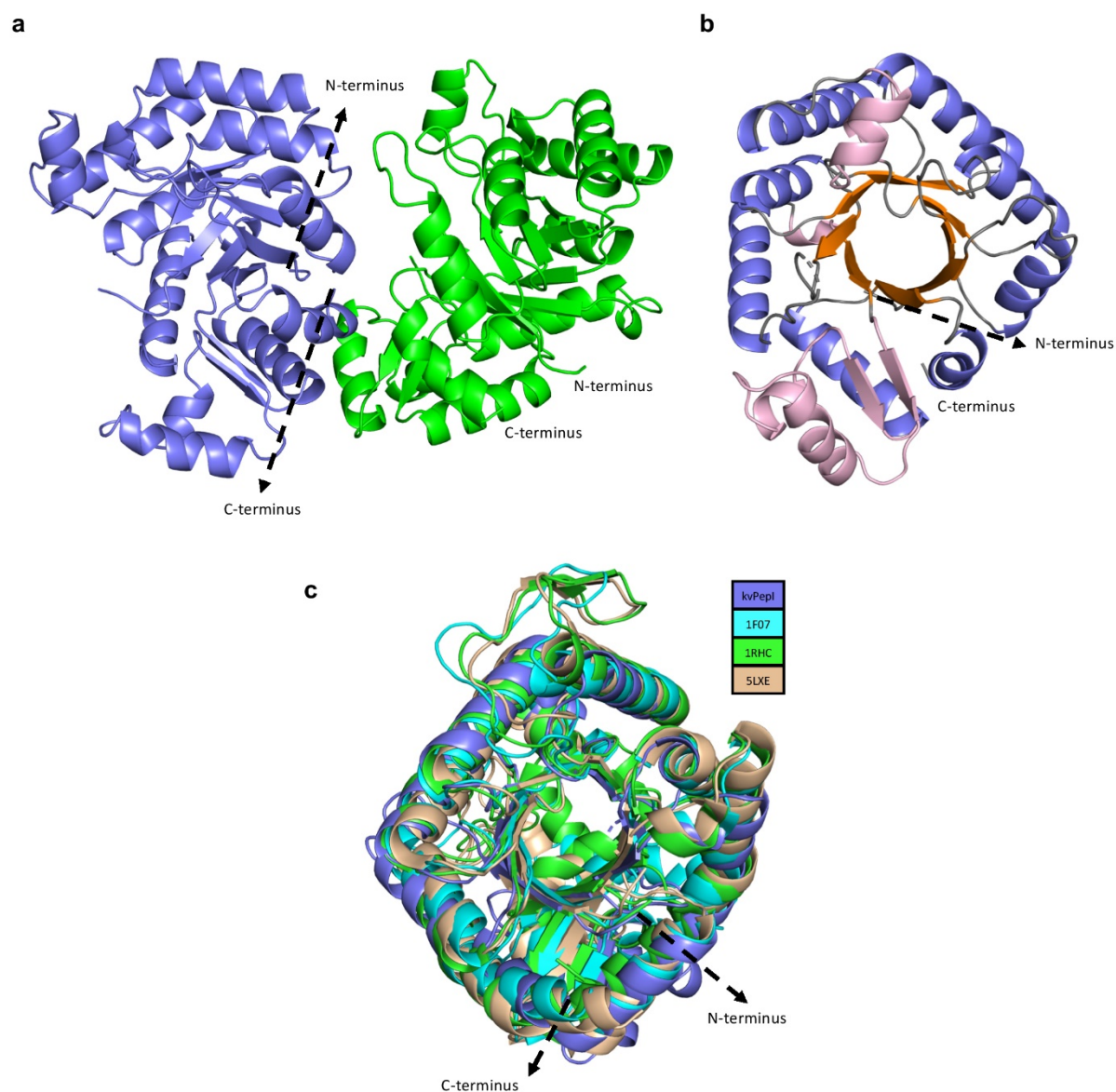
Supplementary Fig. 16. HPLC-MS/MS fragmentation analysis of compounds 13-16.



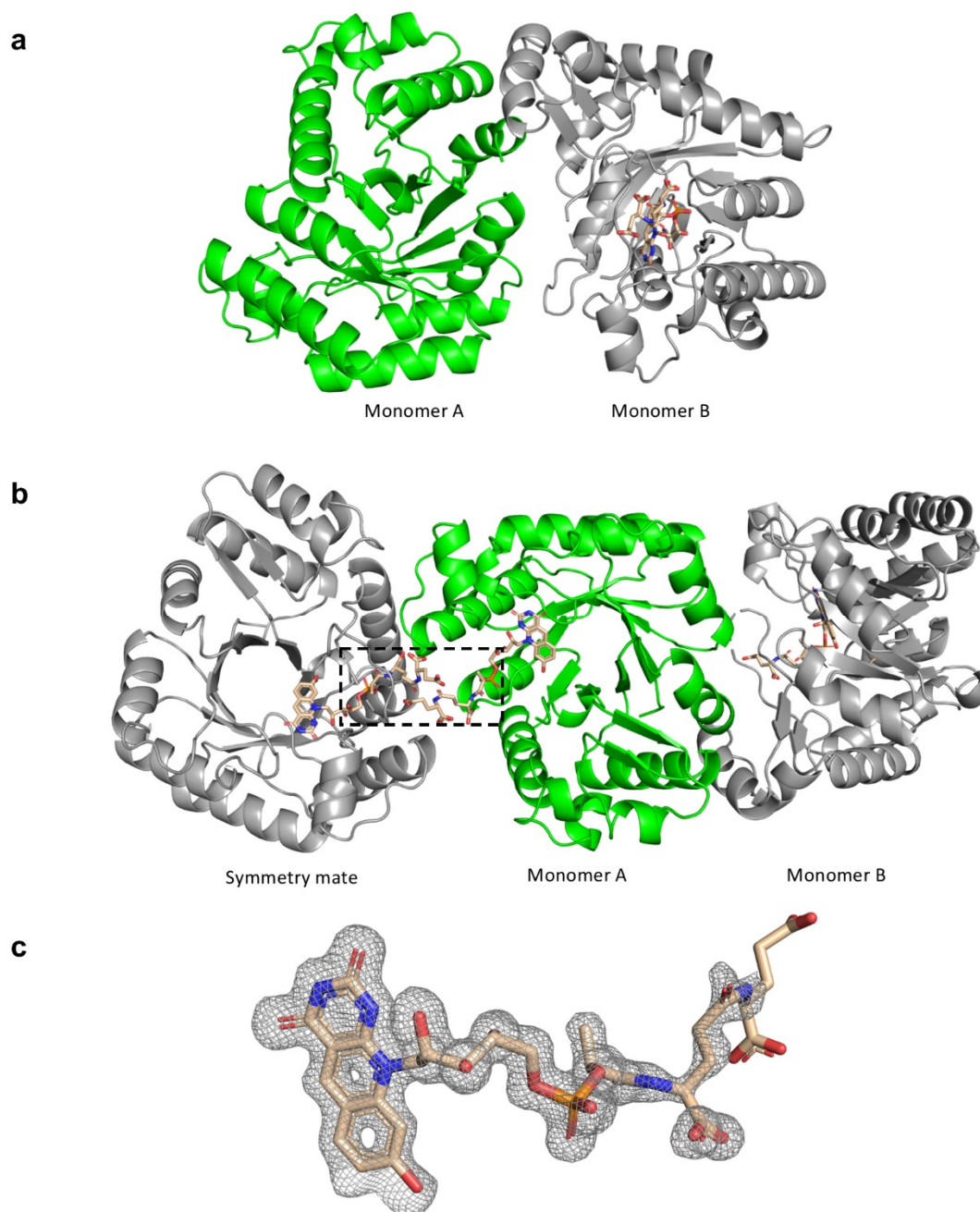
Supplementary Fig. 17. SDS-PAGE analysis of PepI, kvPepI and kvPepI mutants. 1. PepI 33.8 kDa; 2. kvPepI 33.0 kDa; 3. kvPepI^{H62A} 32.7 kDa; 4. kvPepI^{Y122A} 32.6 kDa; 5. kvPepI^{Y122F} 32.7 kDa; 6. kvPepI^{Q229A} 32.6 kDa; 7. kvPepI^{Q289A} 32.9 kDa. M. Thermo Scientific™ PageRuler™ Prestained Protein Ladder, 10 to 180 kDa).



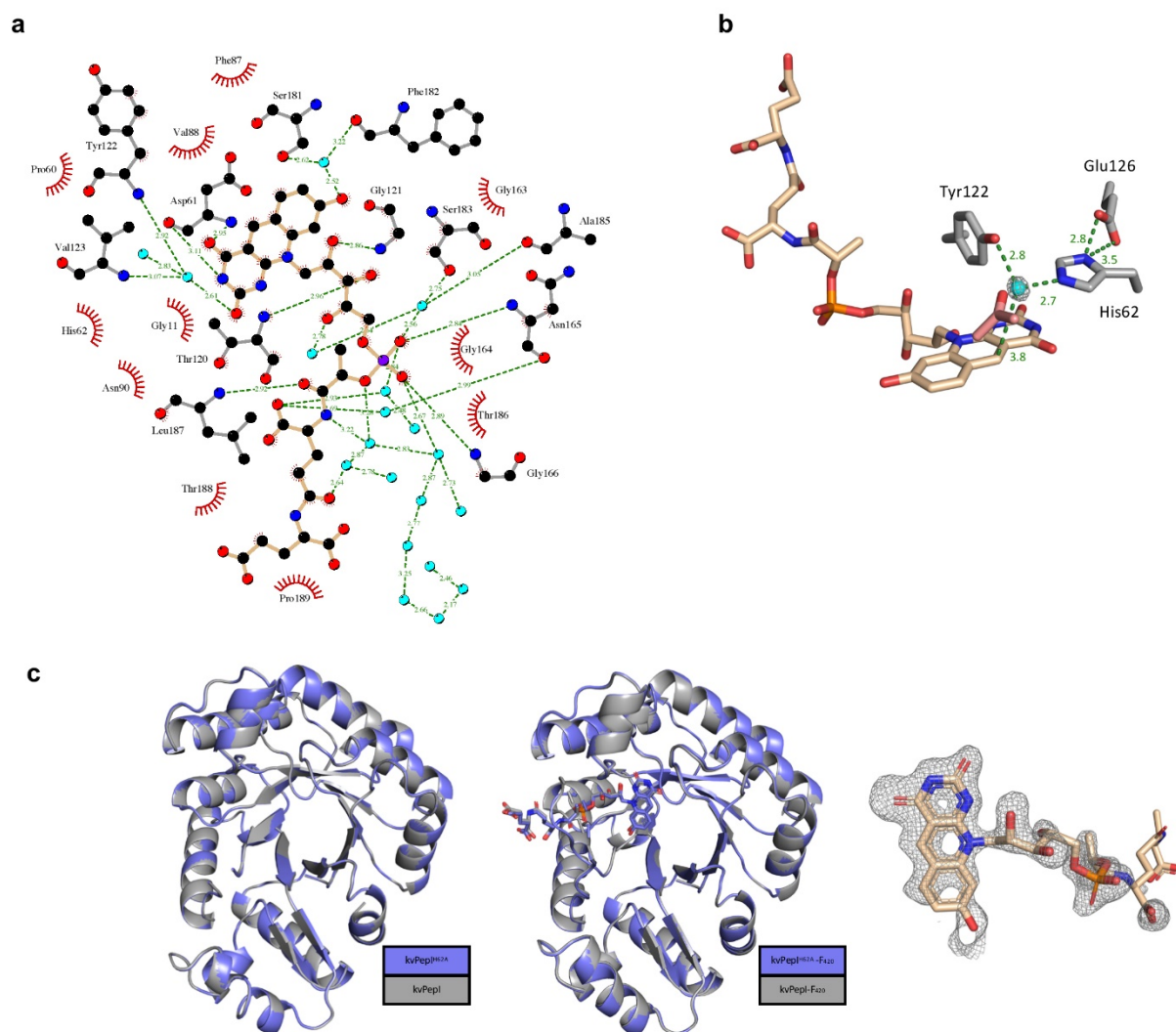
Supplementary Fig. 18. HPLC-MS analysis of statine using the advanced Marfey's method⁴. EIC $[M+H]^+ = 470.22$ **a.** L-statine+D-FDLA; **b.** L-statine+L-FDLA; **c.** acid-hydrolysed **13** (9 reduced by kvPepI)+ D-FDLA; **d.** acid-hydrolysed **13** (9 reduced by kvPepI)+L-FDLA; **e.** acid-hydrolysed **17**+D-FDLA; **f.** acid-hydrolysed **17**+L-FDLA; **g.** acid-hydrolysed **1**+D-FDLA; **h.** acid-hydrolysed **1**+L-FDLA; **i.** **13** (9 reduced by kvPepI^{Y122F})+D-FDLA; **j.** **13** (9 reduced by kvPepI^{Y122F})+L-FDLA.



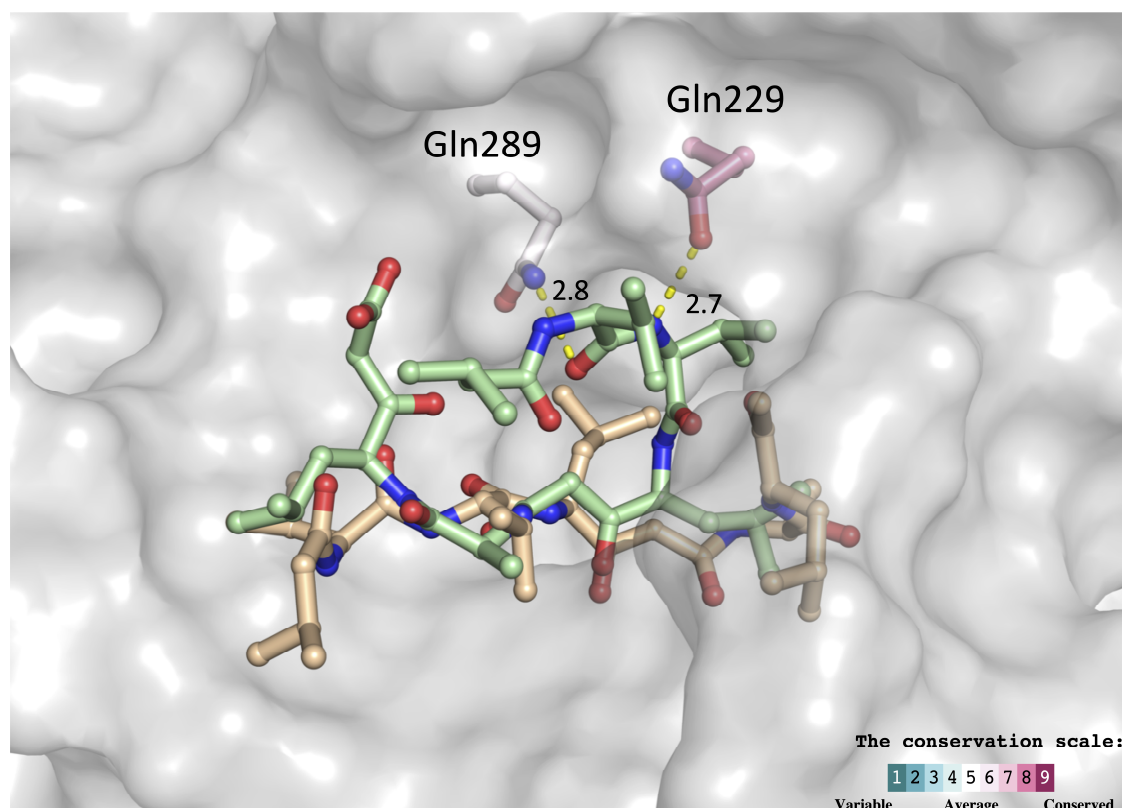
Supplementary Fig. 19. Structural analysis of kvPepI crystal structure. **a**, A dimer comprising the asymmetric unit is shown as ribbon model with the monomers coloured green and slate. **b**, Cartoon representation of kvPepI monomer showing the TIM-barrel fold (colour scheme: α -helices, orange; β -strands, slate; and loops, grey) and the additional structural inserts (pink). **c**, Superposition of kvPepI structure (cyan) with other members of Class I F₄₂₀ – dependent enzymes (PDB IDs: 1F07 (cyan), 1RHC (green) and 5LXE (wheat)) . C α RMSDs were approx. 2.9 Å (1F04) 2.3 Å (1RHC) and 3.2 Å (5LXE) over the entire length of the protein. The N- and C-terminus are indicated.



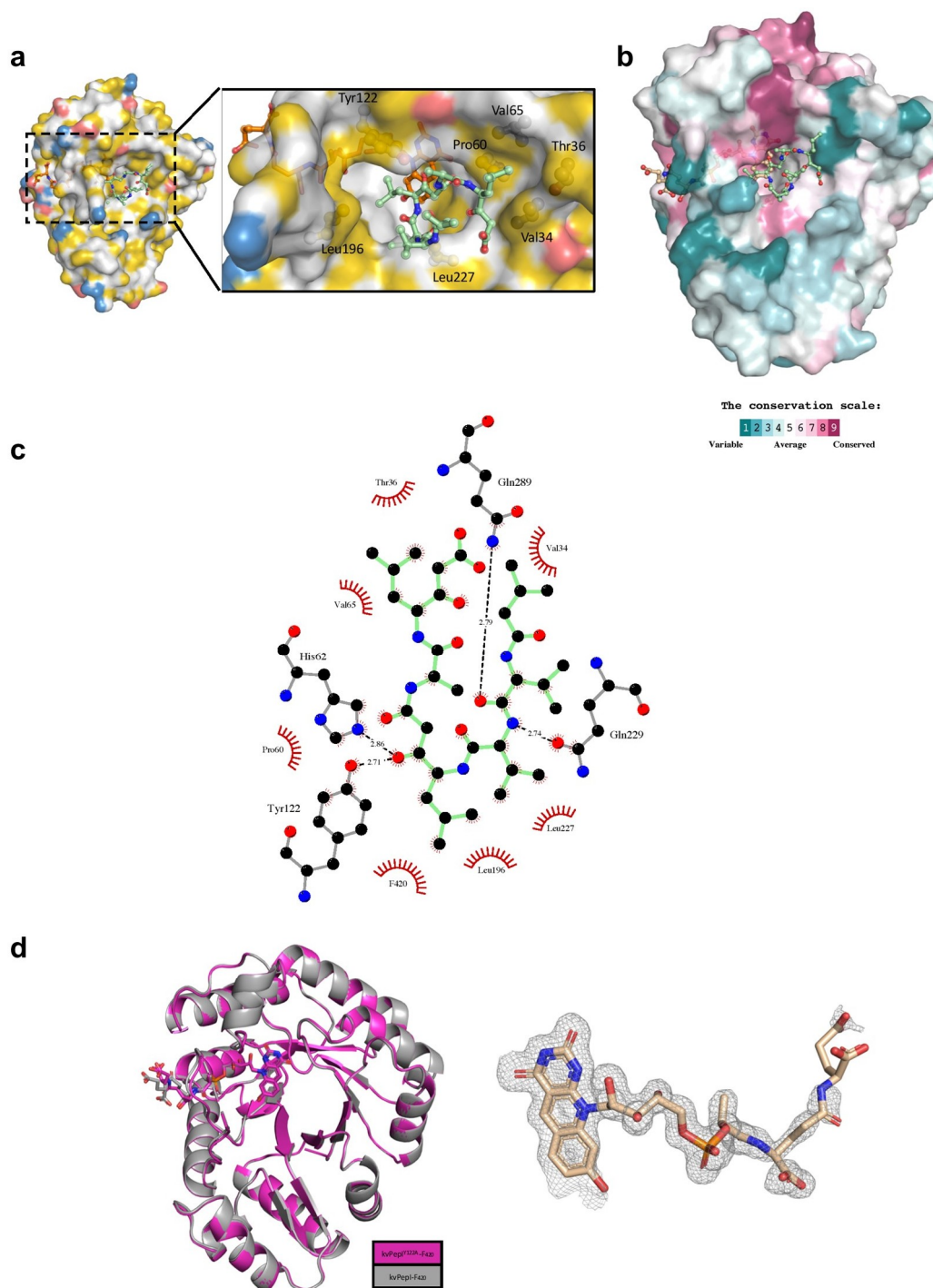
Supplementary Fig. 20. Structural analysis of kvPepI – F₄₂₀ co-crystal structure. **a**, A dimer comprising the asymmetric unit is shown as ribbon model with the monomers (A and B) coloured green and grey. F₄₂₀ (shown as sticks) is only observed in one of the monomers in the asymmetric unit due to crystallization artifact. **b**, Crystallization artifact preventing F₄₂₀ binding. The bound F₄₂₀ in the flanking symmetry related monomer likely leads to a clash with F₄₂₀ (dotted box) modelled in monomer A by superposition of monomer B onto monomer A. **c**, Polder map (grey isomesh) of F₄₂₀ contoured at 3.5 σ .



Supplementary Fig. 21. Structural analysis of kvPepI – F₄₂₀ cocrystal structure and kvPepI^{H62A}. **a**, LigPlot diagram of the interactions between kvPepI (grey) and F₄₂₀ (wheat). Water molecules and atoms are represented as circles (carbon black, oxygen red, nitrogen blue, and water cyan). Hydrogen bonds are shown as dashed green lines with distances given in Å. Hydrophobic interactions are depicted as red spoked arcs. **b**, The ordered water molecule (HOH570, cyan; $2F_O - F_C$ electron density map shown as grey isomesh contoured at 2σ) found in close proximity to F₄₂₀, His62 and Tyr122. Glu126 likely help stabilise the positive charge on His62. Adf bound F₄₂₀-acetone adduct (PDB ID: 1RHC) was superposed to the kvPepI – F₄₂₀ structure. The carbonyl of acetone adduct lies next to the ordered water molecule observed in kvPepI – F₄₂₀ complex structure. For clarity only acetone is shown as stick (pink). **c**, Structural analysis of kvPepI^{H62A} and kvPepI^{H62A} – F₄₂₀ cocrystal structure. Superposition of kvPepI and KvPepI – F₄₂₀ with kvPepI^{H62A} and KvPepI^{H62A} – F₄₂₀ complex structure. C α RMSD were approx. 0.14 Å (kvPepI^{H62A} vs. kvPepI; left) and 0.1 Å (kvPepI^{H62A} – F₄₂₀ vs. kvPepI – F₄₂₀; middle) over the entire length of the protein. Polder map (grey isomesh; contoured at 3σ) of F₄₂₀ bound to kvPepI^{H62A} is shown on the right.

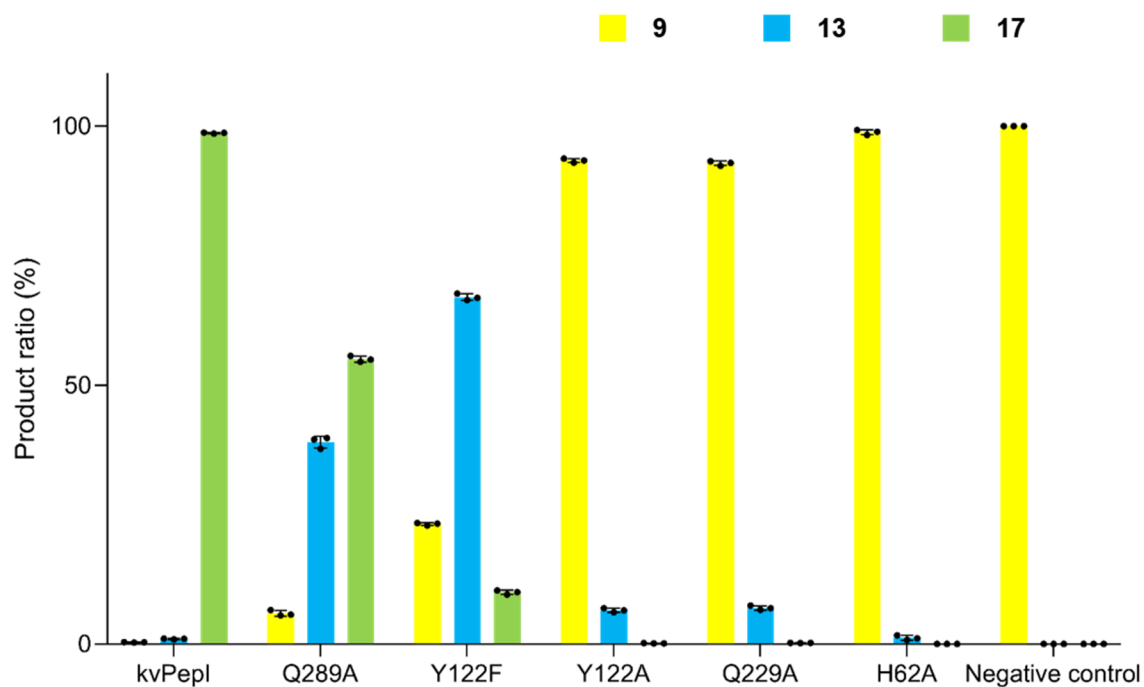


Supplementary Fig. 22. Binding poses of compound **9** in kvPepI. **9** is shown as sticks (conformation: U-shaped, pale green; linear, wheat) while kvPepI substrate binding pocket is shown as surface. The residues (Gln229 and Gln289) involved in H-bond interactions with the amide bond of Val1 of U-shaped **9** are shown as sticks. Hydrogen bond interactions are depicted with dotted yellow lines with distances given in Å. The residues are colored according to the conservation score calculated using the ConSurf server.

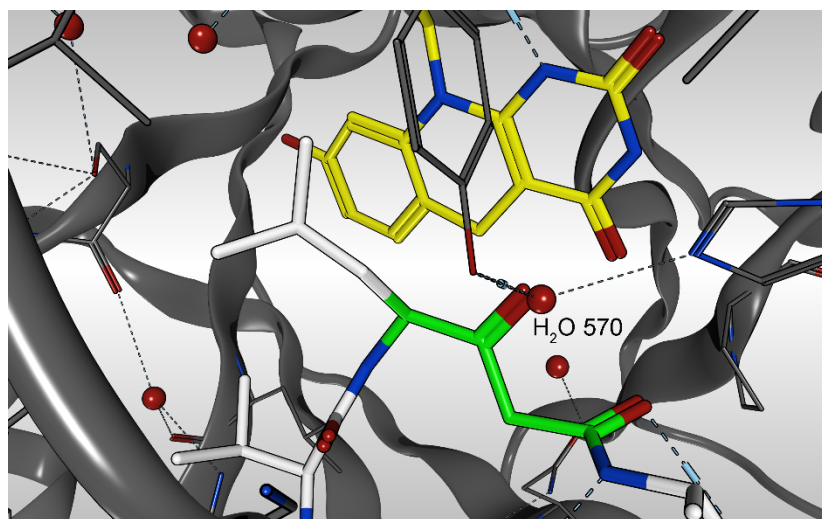


Supplementary Fig. 23. Structural analysis of kvPepI – F₄₂₀ cocystal structure with **9** and kvPepI^{Y122A}–F₄₂₀ complex. **a**, Surface representation of kvPepI–F₄₂₀–**9** model coloured to highlight hydrophobicity and charge using the YRB scheme (hydrocarbons groups without polar substitutions, yellow; negatively charged oxygens of glutamate and aspartate, red; nitrogens of positively charged functional groups of lysine and arginine, blue; all remaining atoms including the polar backbone, white). The close-up view of the substrate binding pocket illustrating the hydrophobicity along with the hydrophobic residues (Val34, Thr36, Pro60, Val65, Tyr122, Leu196 and Leu227) in direct contact with **9** (stick, pale green). **b**, ConSurf^{45,46} map showing the conservation of residues around the kvPepI active site. The calculation was performed on a sample of 150 homologous sequences selected using default settings. F₄₂₀ (orange) along with the modelled **9** (pale green) are shown as sticks. **c**, LigPlot⁺ diagram

of the interactions between the modelled **9** and kvPepI – F₄₂₀. The dashed lines show distances in Å. **d**, Structural analysis of kvPepI^{Y122A} – F₄₂₀ cocrystal structure. Superposition of kvPepI – F₄₂₀ with kvPepI^{Y122A} – F₄₂₀ complex structure. C α RMSD was approx. 0.12 Å (kvPepI^{Y122A} – F₄₂₀ vs. kvPepI - F₄₂₀; left) over the entire length of the protein. Polder map (grey isomesh; contoured at 3 σ) of F₄₂₀ bound to kvPepI^{Y122A} is shown on the right.

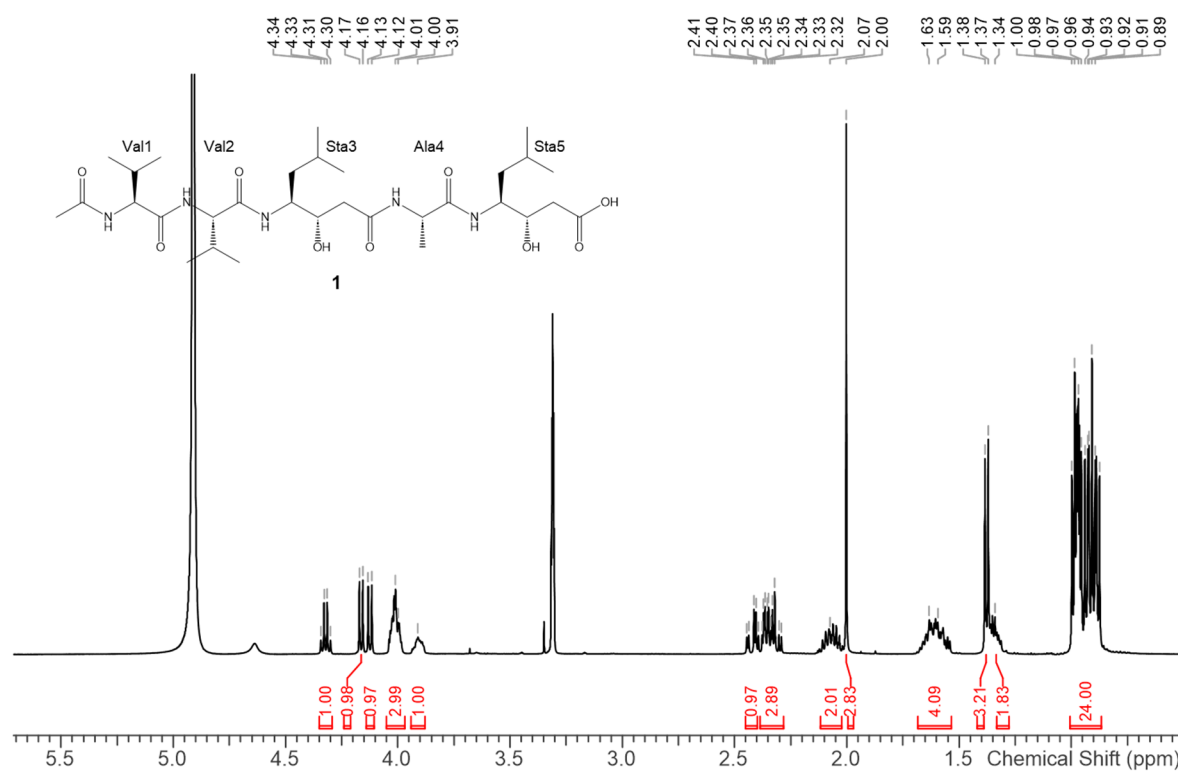


Supplementary Fig. 24. Evaluation of the activity of kvPepI and its mutants on two reduction steps was conducted using HPLC-MS analysis to determine the compound ratio between substrate **9** and products **13** and **17**. Reactions with heat-inactivated enzymes served as controls. Bar heights represent the average compound ratios from three independent reactions, with all data points presented. Three biological replicates (n=3 independent experiments) were performed. All error bars represent the standard error of the mean (SEM). Source data are provided as a Source Data file.

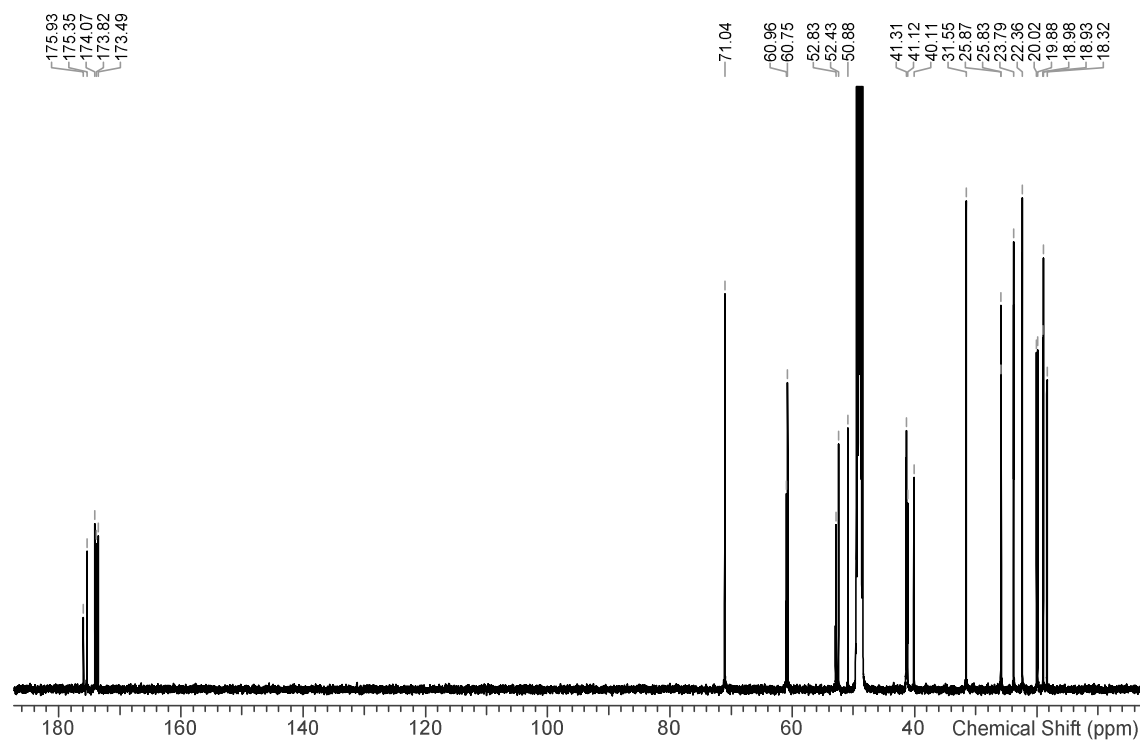


Supplementary Fig. 25. Initial orientation of β -keto amide motif of PreSta3 (green carbons) of compound **9** (white carbons) in the active site in the modelling process. Carbon atoms of cofactor F₄₂₀ are shown in yellow. Ribbon and sidechain atoms of kvPepI protein are shown in dark grey. Oxygen: red. Nitrogen: blue. Hydrogen is left out for clarity. Dotted lines indicate hydrogen bonds.

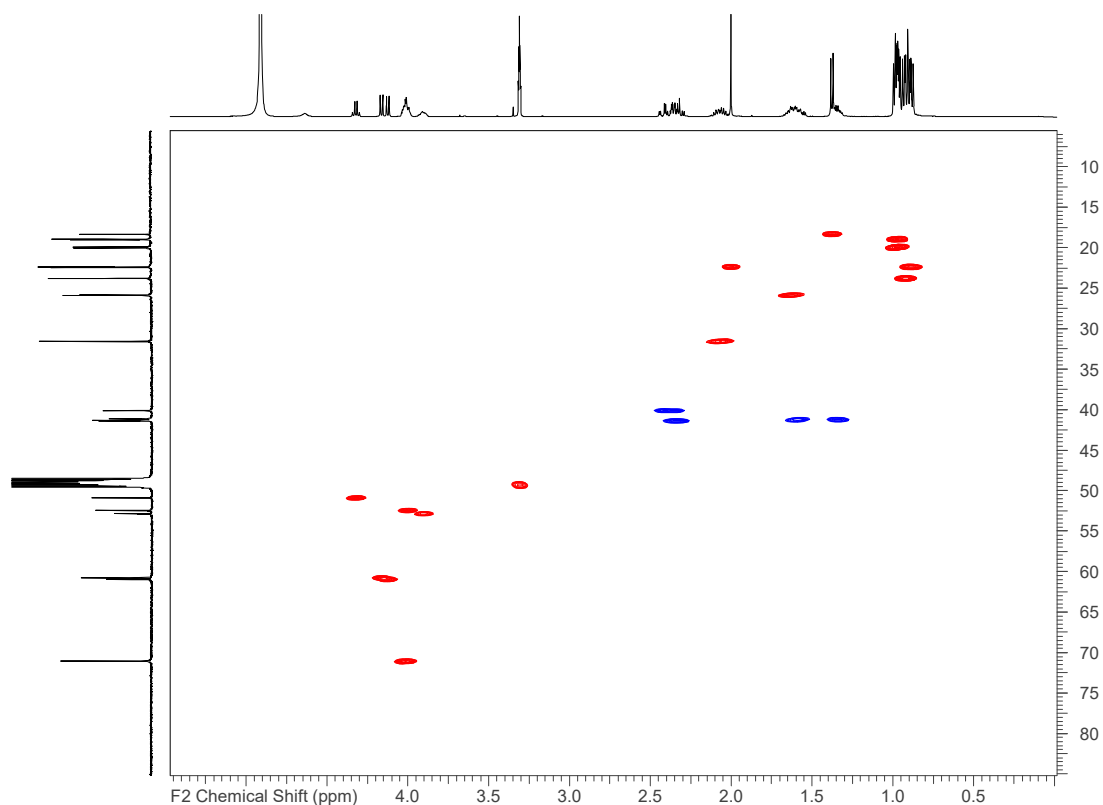
NMR spectra



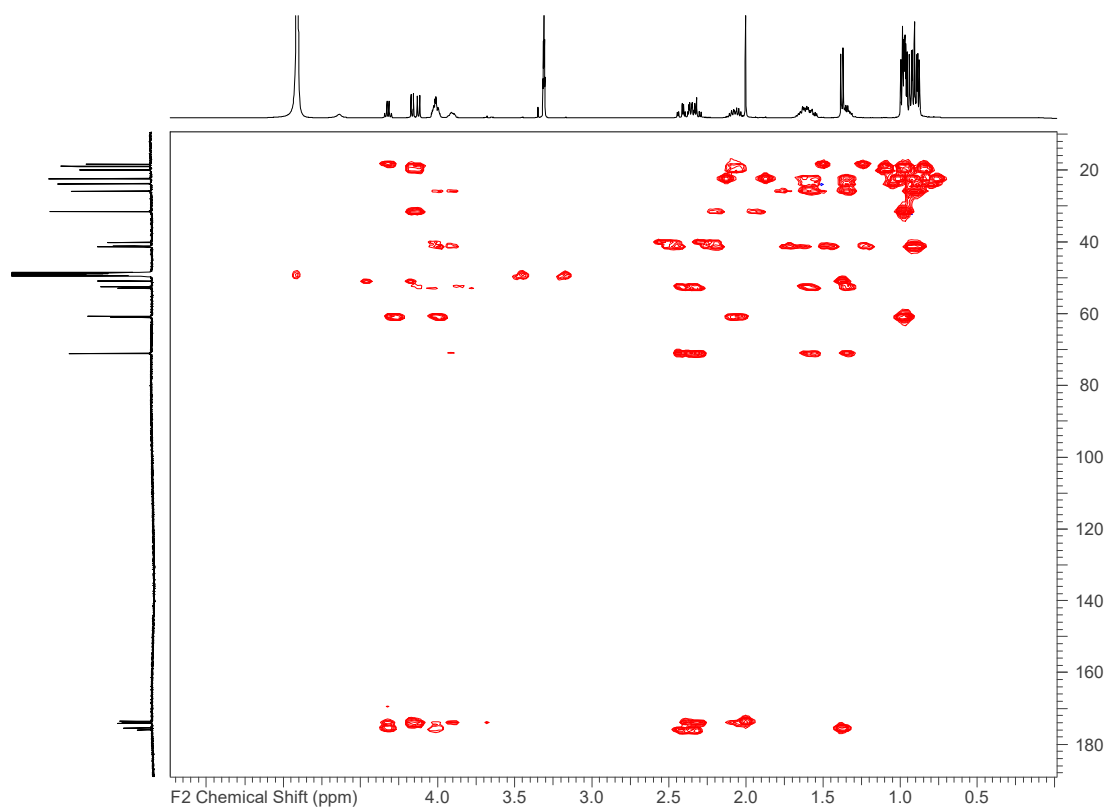
Supplementary Fig. 26. ^1H -NMR spectrum of compound **1** (500 MHz, methanol- d_4).



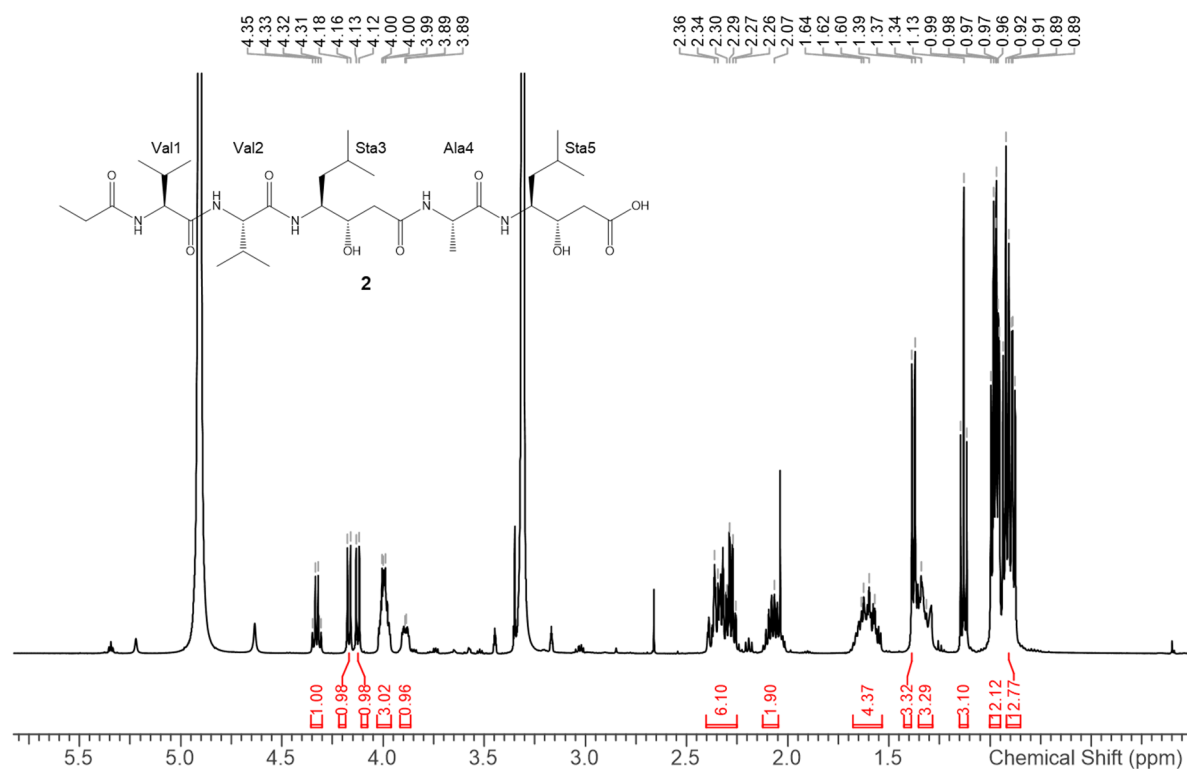
Supplementary Fig. 27. ^{13}C -NMR spectrum of compound **1** (125 MHz, methanol- d_4).



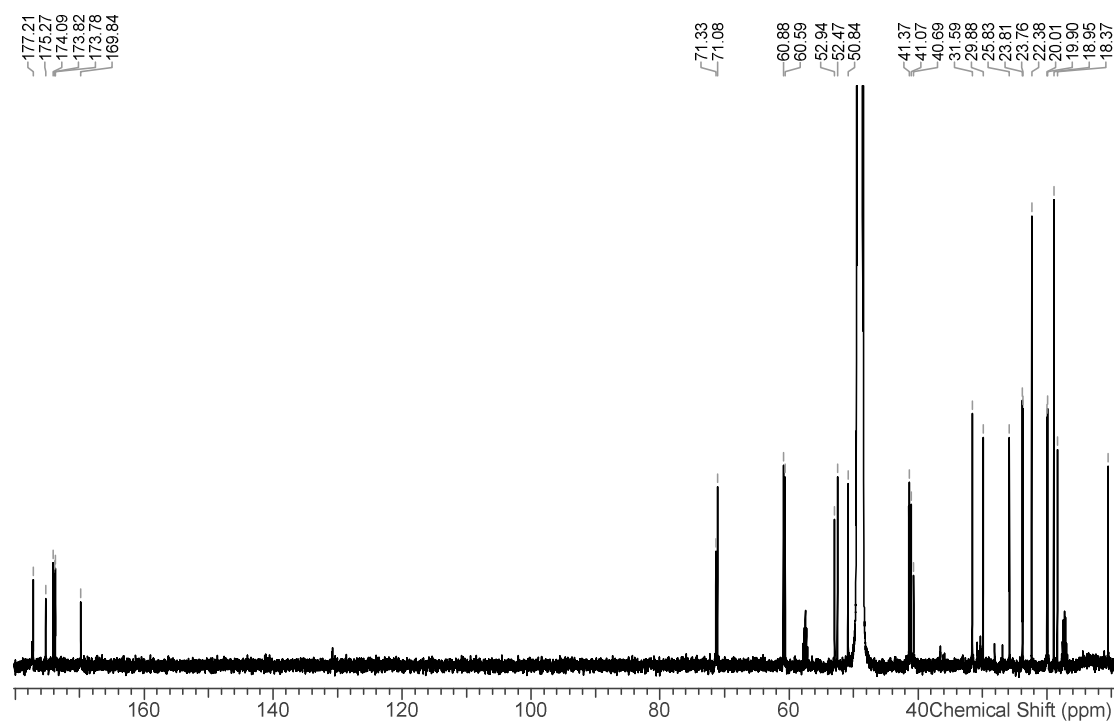
Supplementary Fig. 28. ^1H , ^{13}C -HSQC spectrum of compound **1** (^1H :500 MHz, ^{13}C :125 MHz, methanol- d_4).



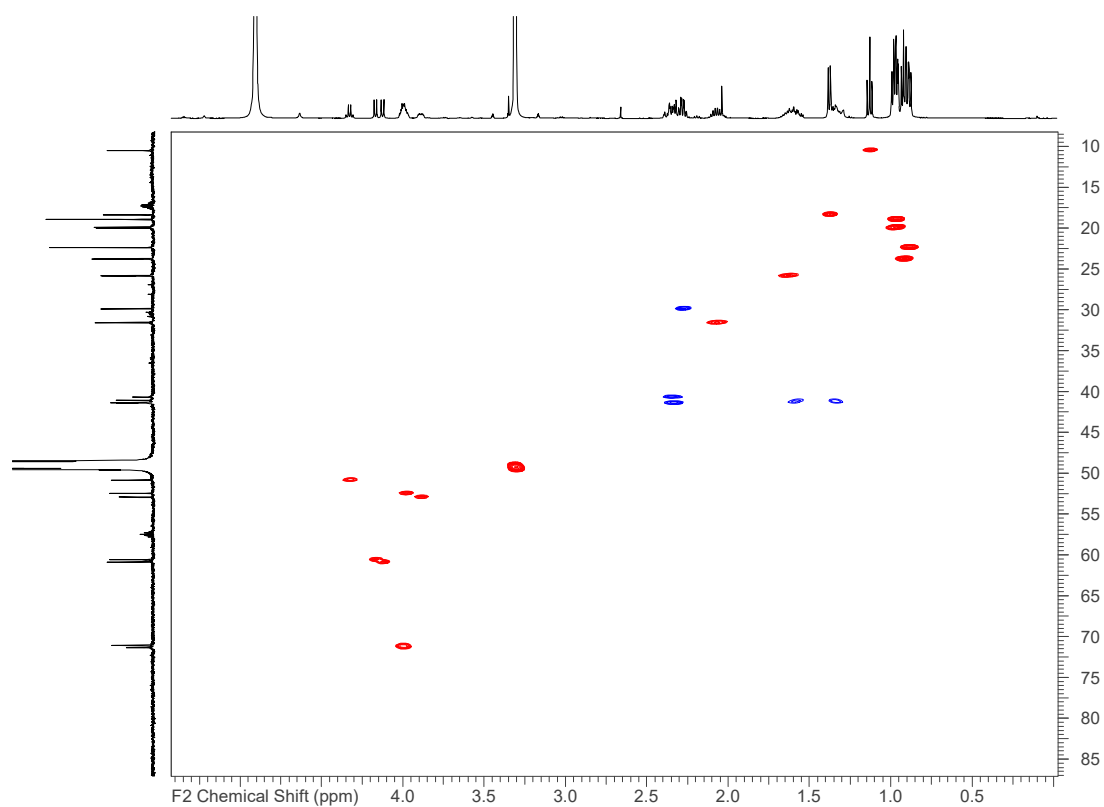
Supplementary Fig. 29. ^1H , ^{13}C -HMBC spectrum of compound **1** (^1H :500 MHz, ^{13}C :125 MHz, methanol- d_4).



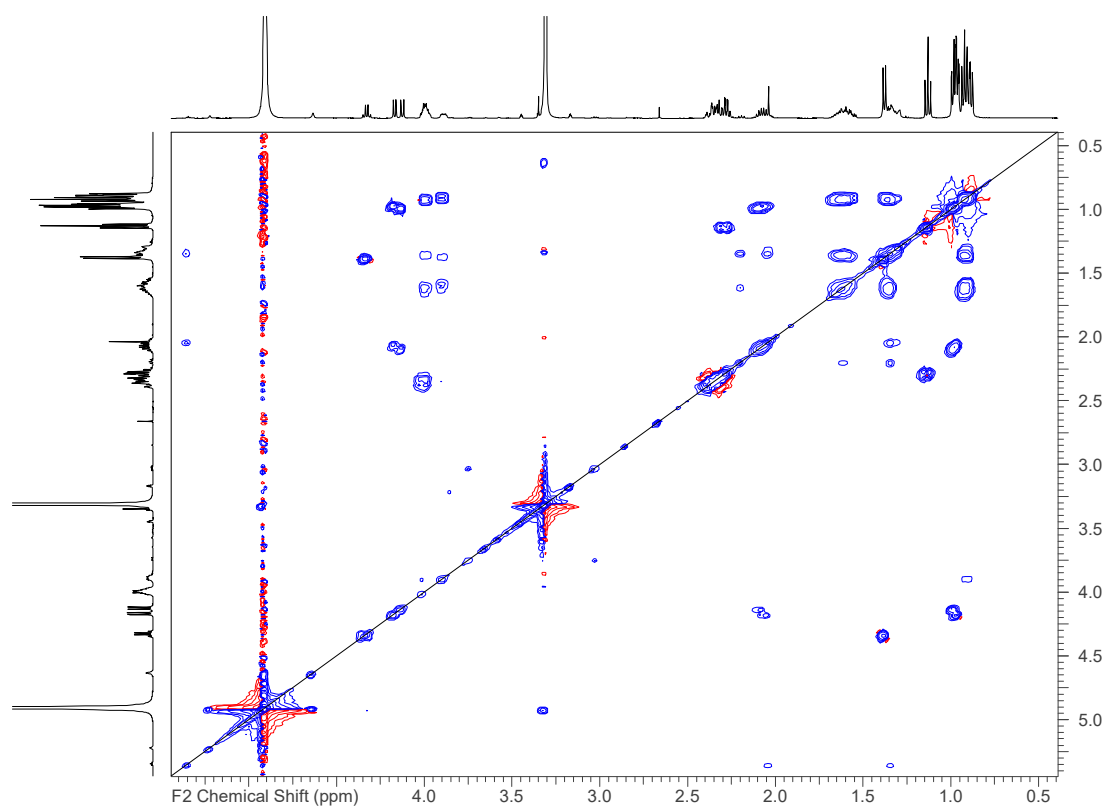
Supplementary Fig. 30. ^1H -NMR spectrum of compound **2** (500 MHz, methanol- d_4).



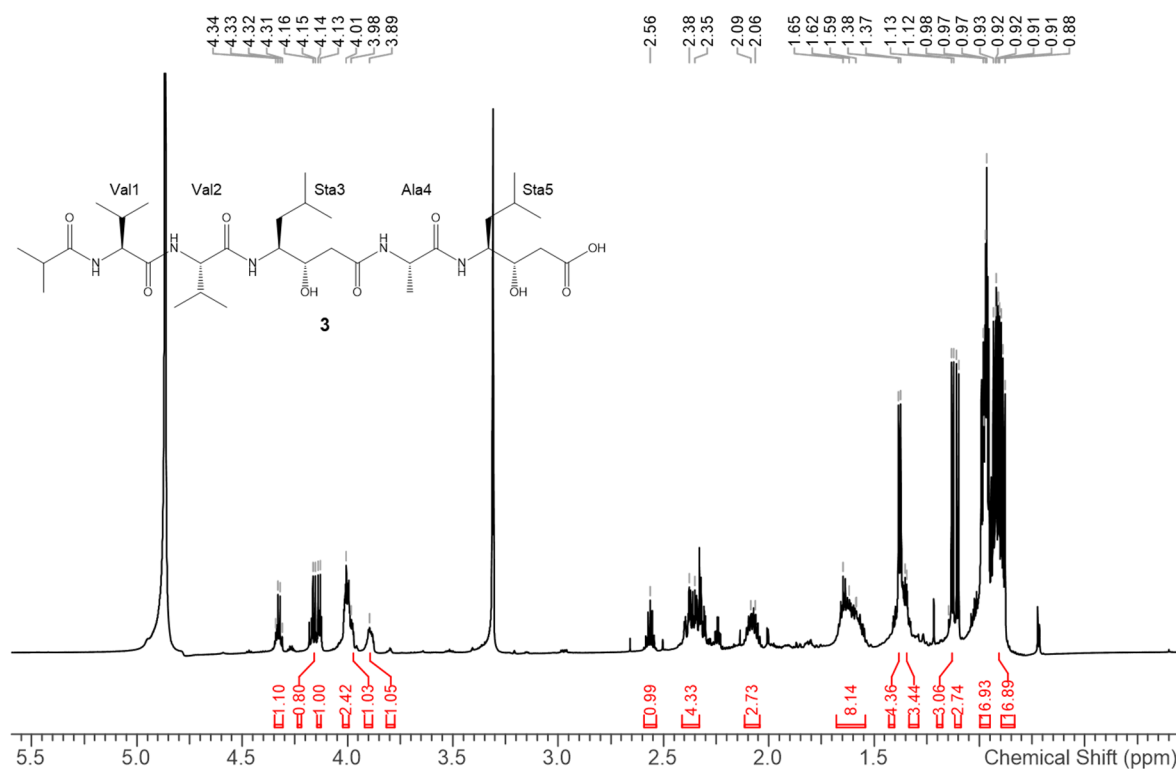
Supplementary Fig. 31. ^{13}C -NMR spectrum of compound **2** (125 MHz, methanol- d_4).



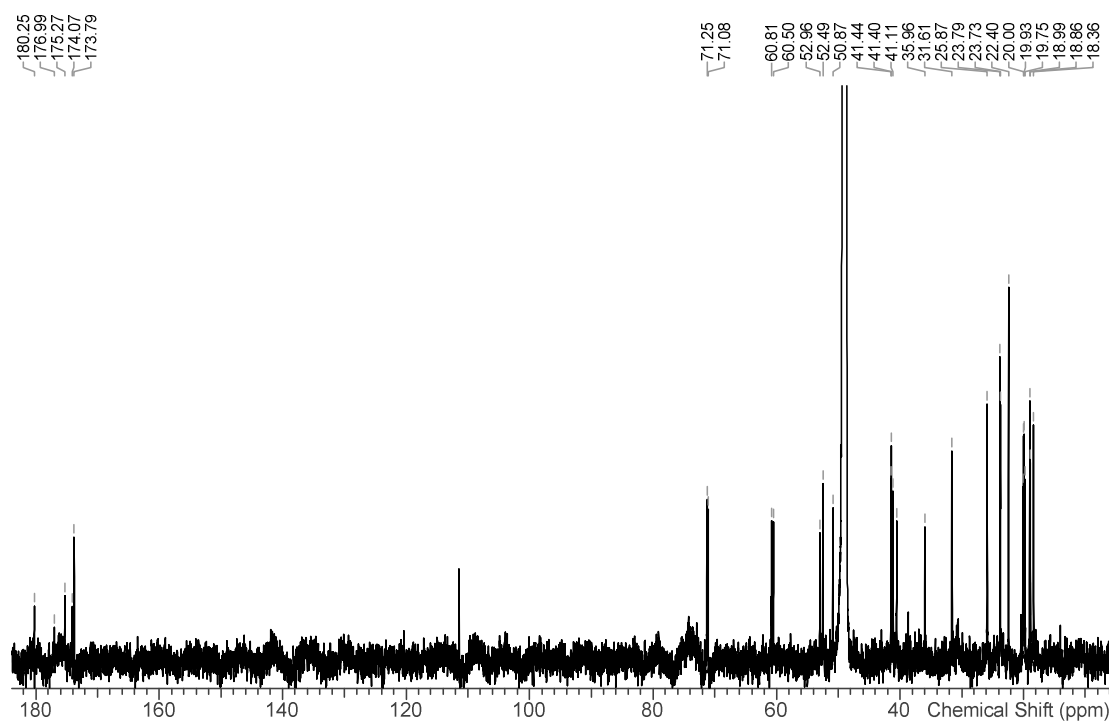
Supplementary Fig. 32. ^1H , ^{13}C -HSQC spectrum of compound **2** (^1H :500 MHz, ^{13}C :125 MHz, methanol- d_4).



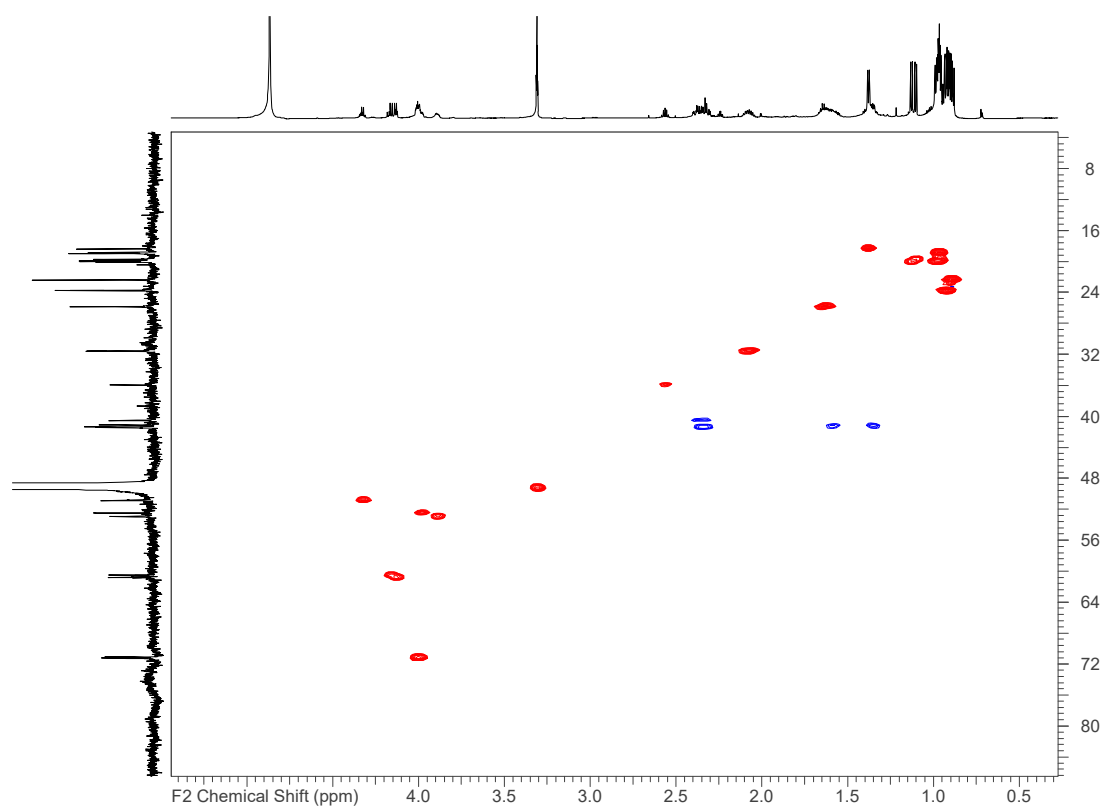
Supplementary Fig. 33. ^1H , ^1H -TCOSY spectrum of compound **2** (500 MHz, methanol- d_4).



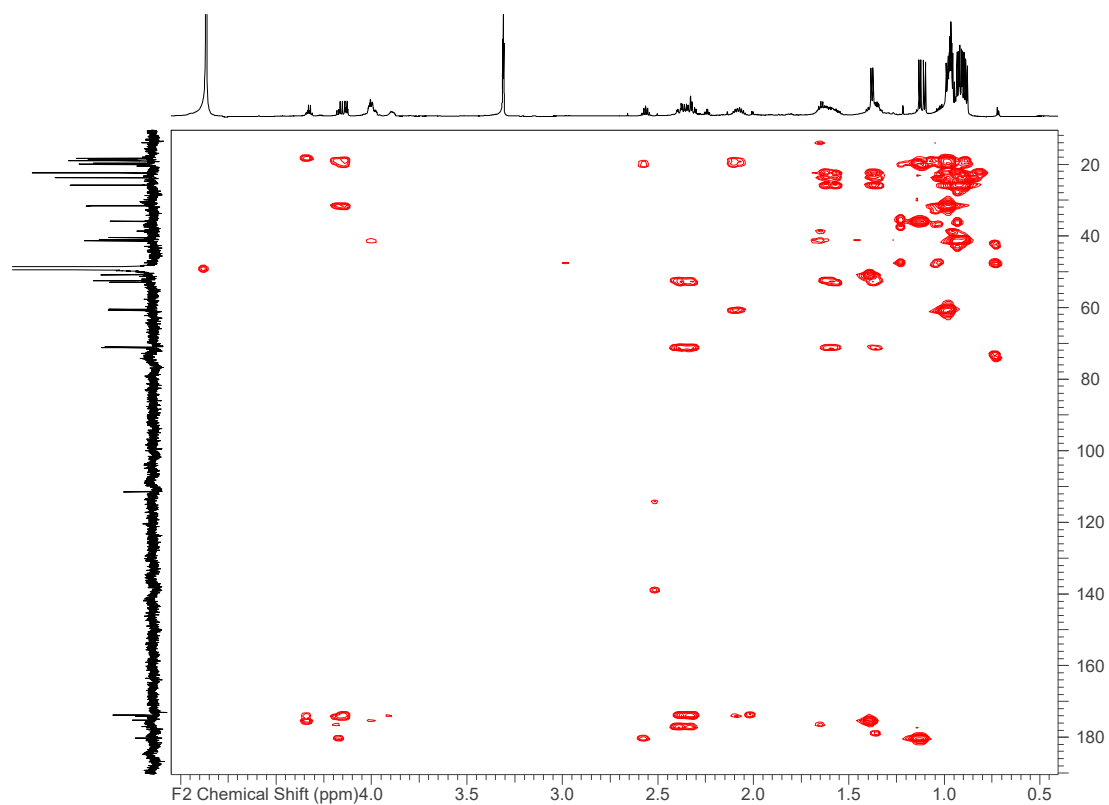
Supplementary Fig. 34. ^1H -NMR spectrum of compound **3** (700 MHz, methanol- d_4).



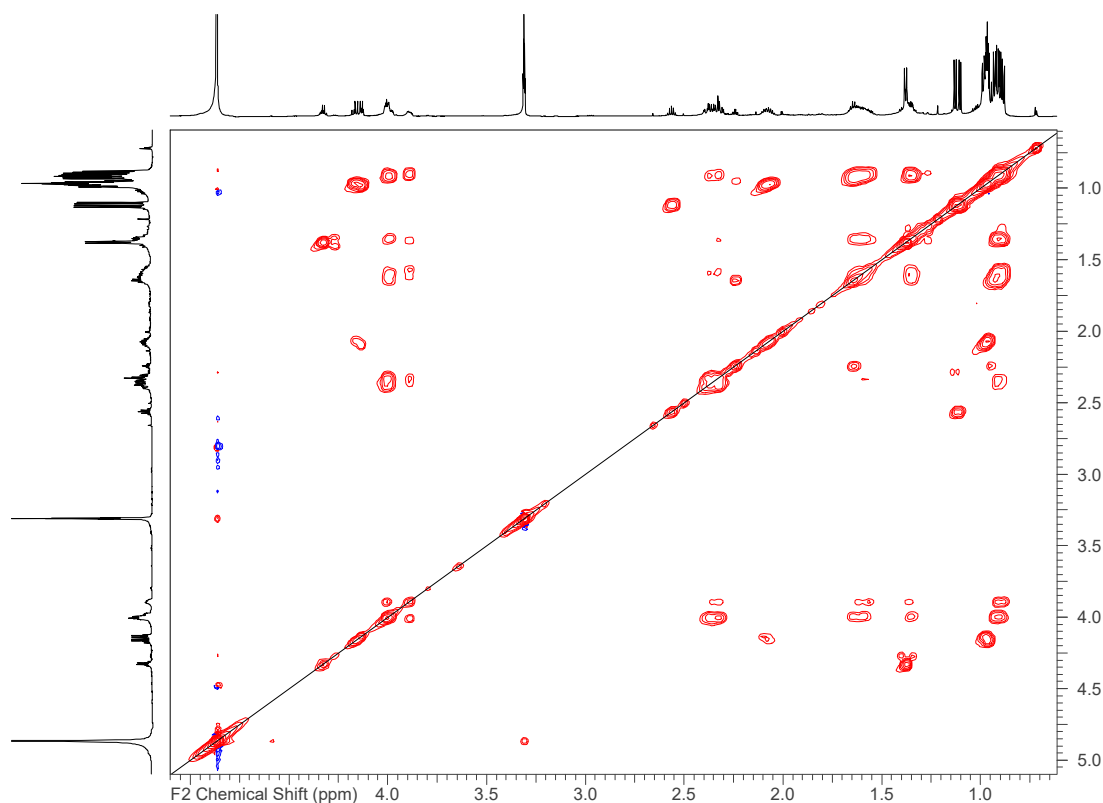
Supplementary Fig. 35. ^{13}C -NMR spectrum of compound **3** (175 MHz, methanol- d_4).



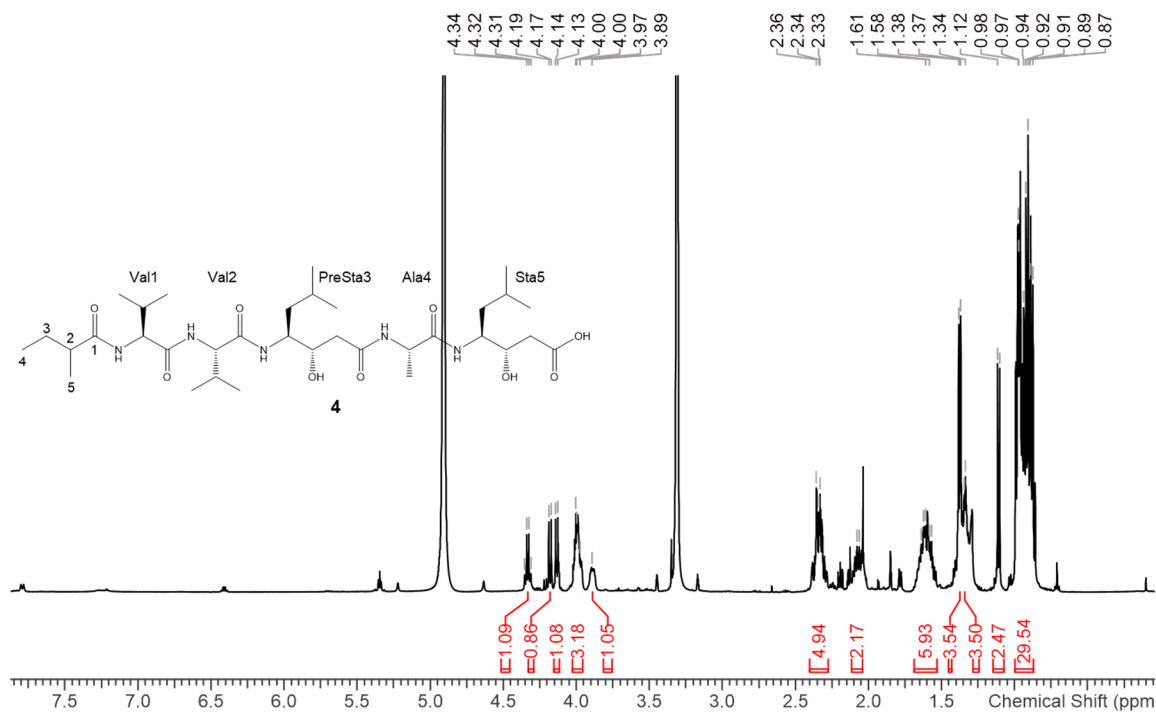
Supplementary Fig. 36. ^1H , ^{13}C -HSQC spectrum of compound **3** (^1H :700 MHz, ^{13}C :175 MHz, methanol- d_4).



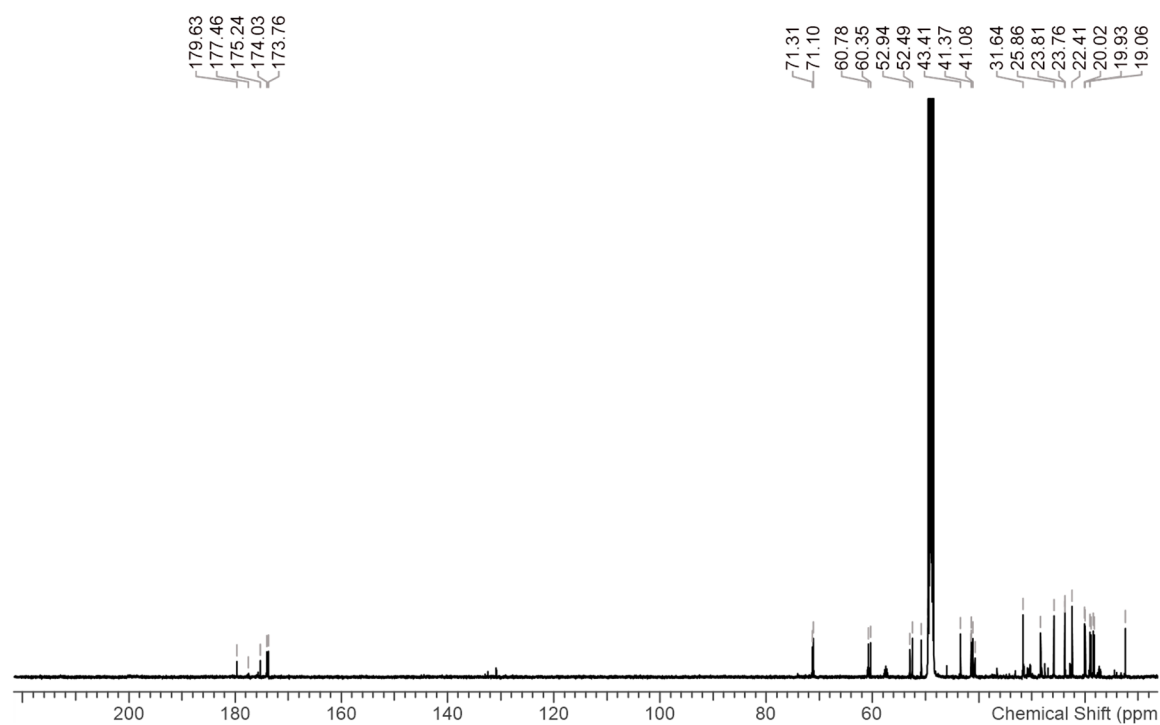
Supplementary Fig. 37. ^1H , ^1H -HMBC spectrum of compound **3** (^1H :700 MHz, ^{13}C :175 MHz, methanol- d_4).



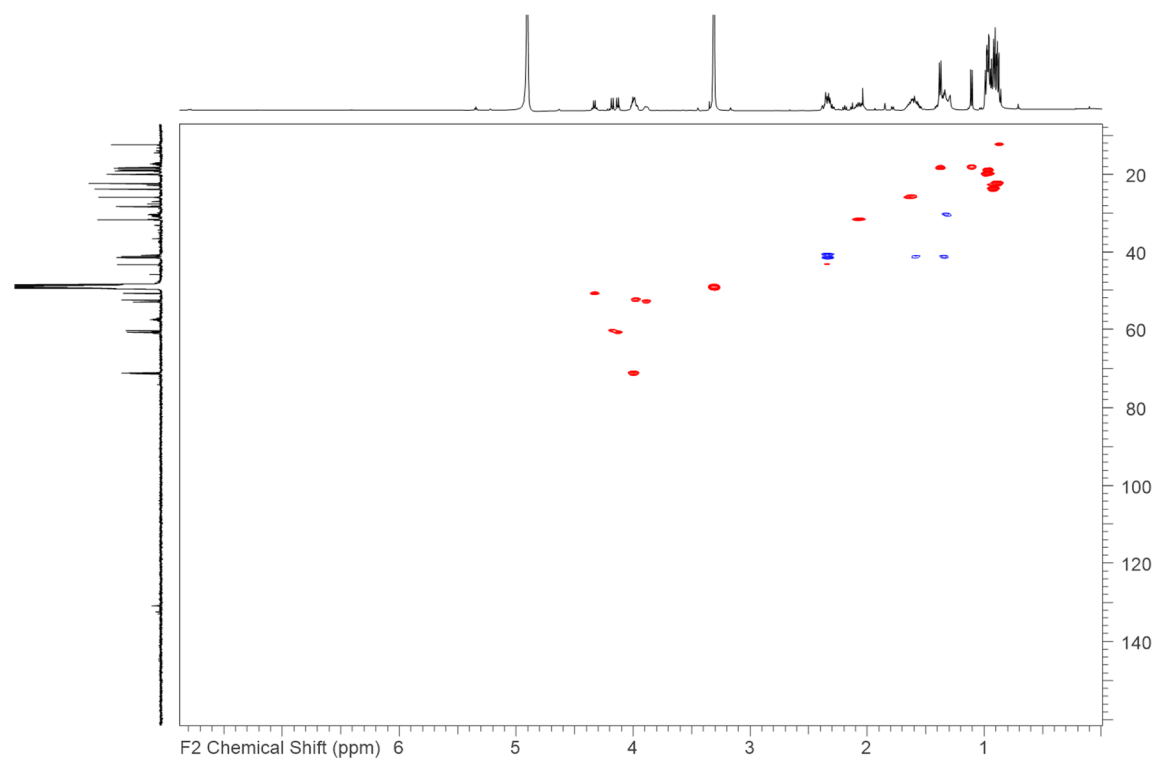
Supplementary Fig. 38. $^1\text{H},^1\text{H}$ -TCOSY spectrum of compound **3** (700 MHz, methanol- d_4).



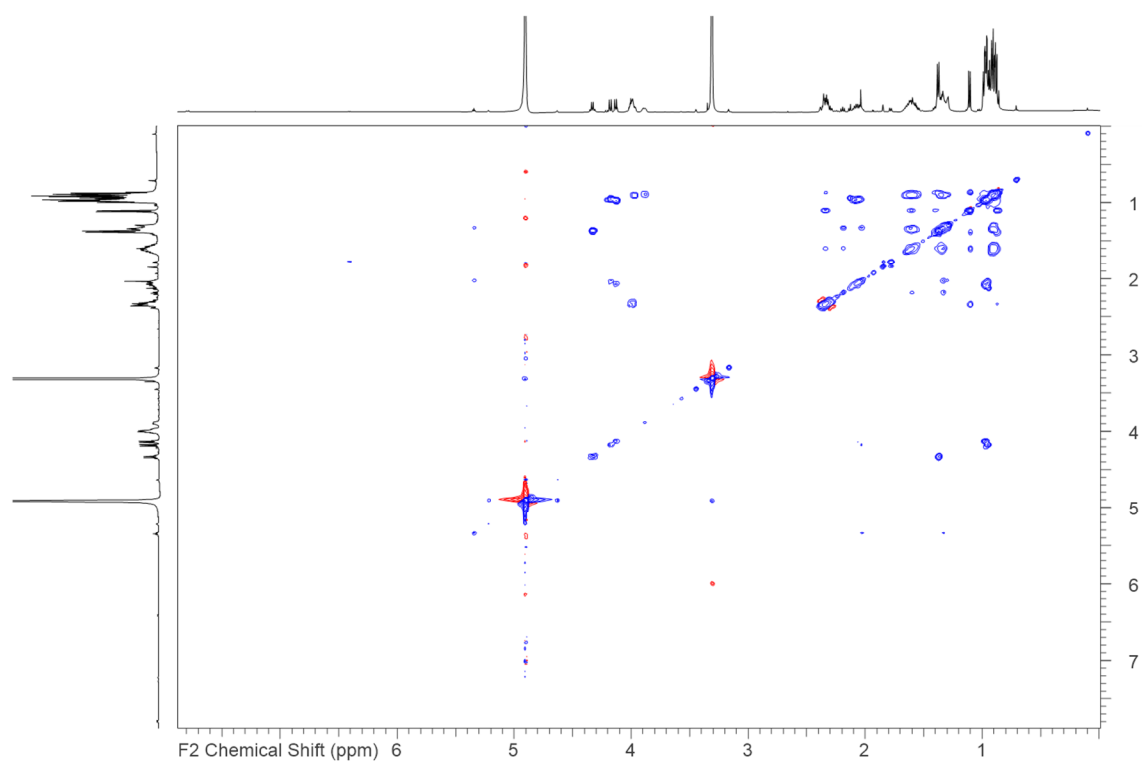
Supplementary Fig. 39. ^1H -NMR spectrum of compound **4** (500 MHz, methanol- d_4).



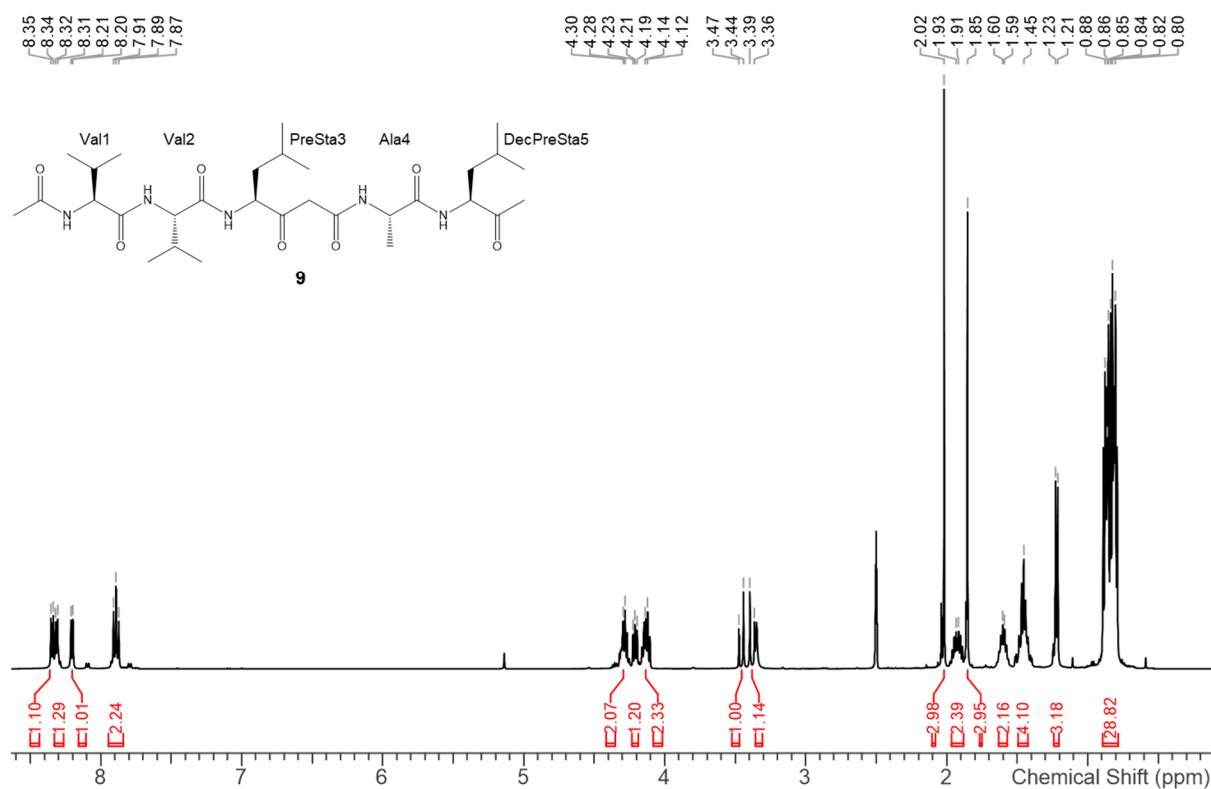
Supplementary Fig. 40. ^{13}C -NMR spectrum of compound **4** (125 MHz, methanol- d_4).



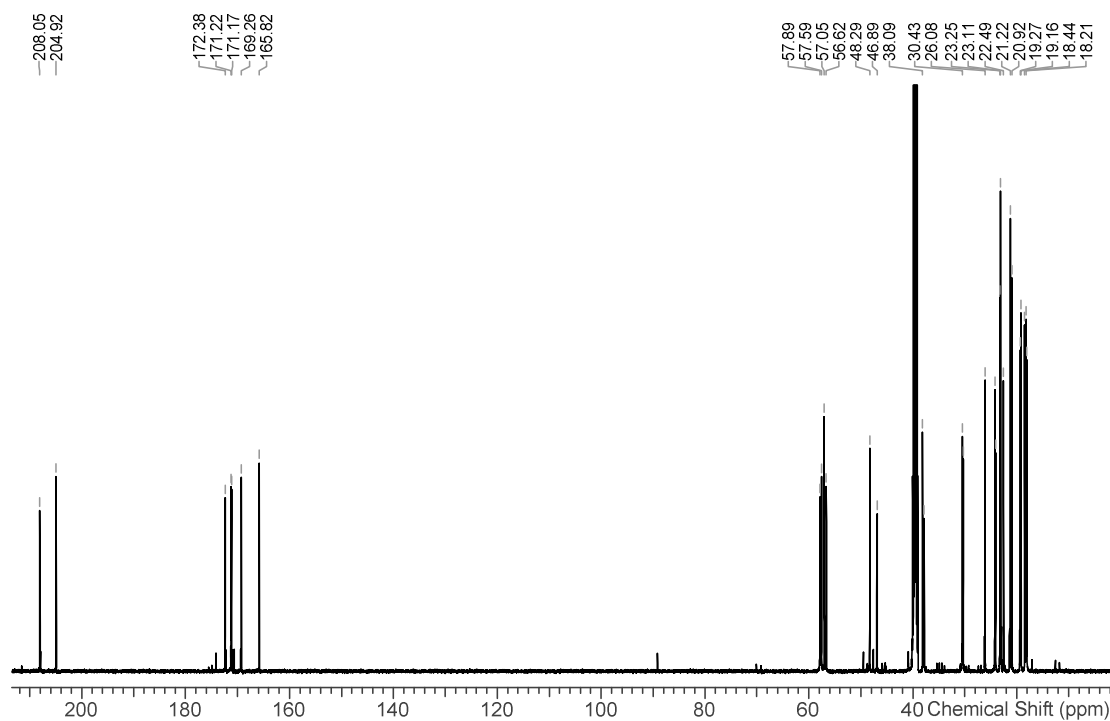
Supplementary Fig. 41. ^1H , ^{13}C -HSQC spectrum of compound **4** (^1H :500 MHz, ^{13}C :125 MHz, methanol- d_4).



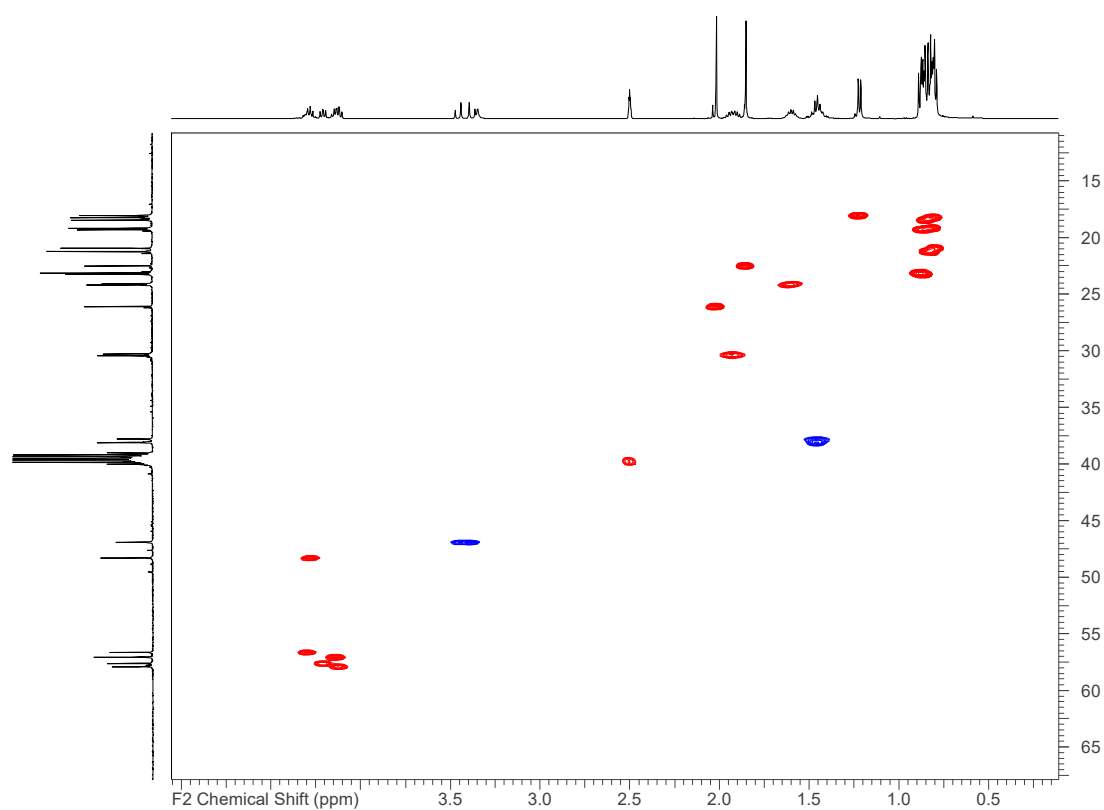
Supplementary Fig. 42. ^1H , ^1H -TCOSY spectrum of compound **4** (500 MHz, methanol- d_4).



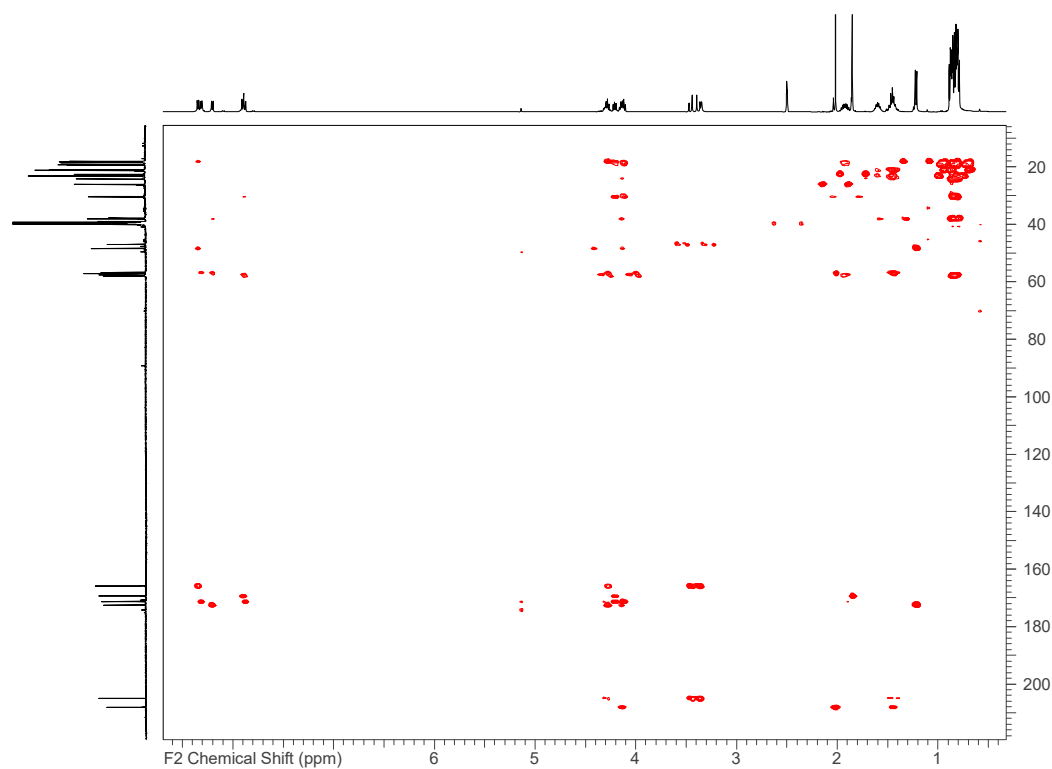
Supplementary Fig. 43. ¹H-NMR spectrum of compound 9 (500 MHz, DMSO-*d*₆).



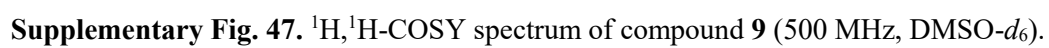
Supplementary Fig. 44. ¹³C-NMR spectrum of compound 9 (125 MHz, DMSO-*d*₆).

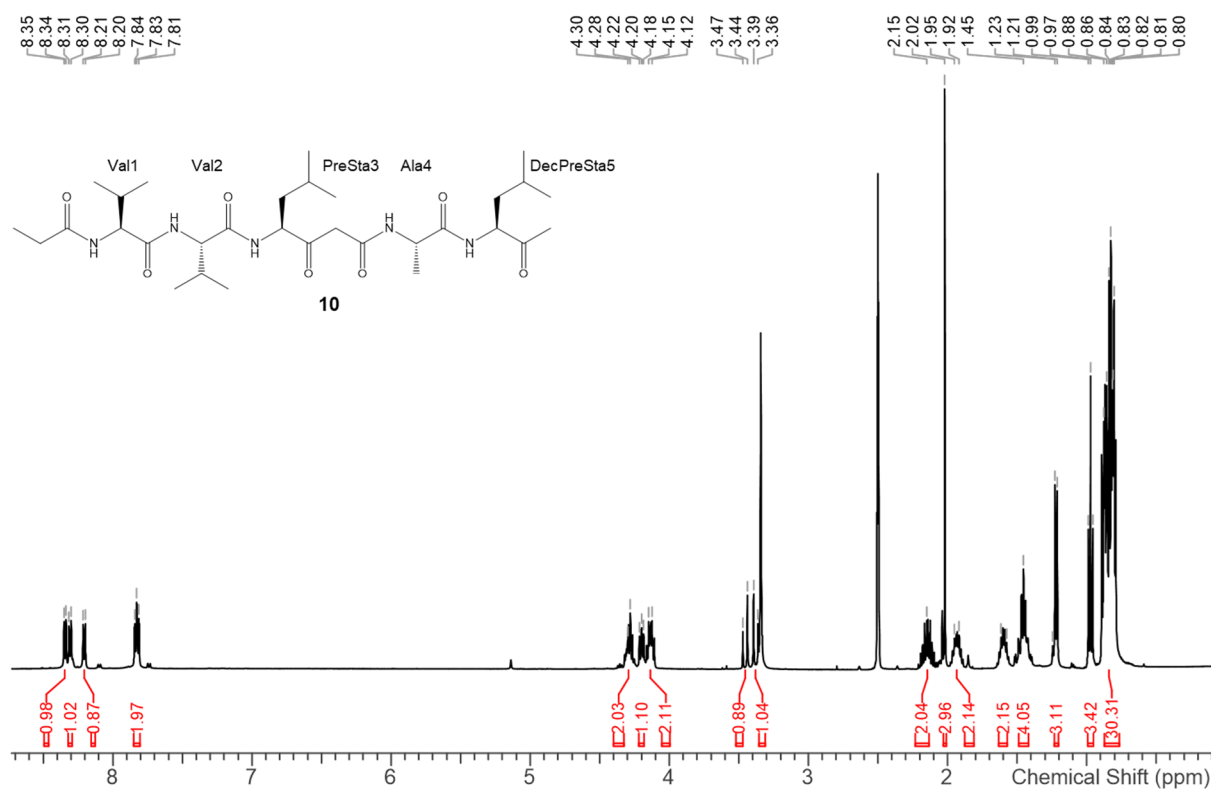


Supplementary Fig. 45. ^1H , ^{13}C -HSQC spectrum of compound **9** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

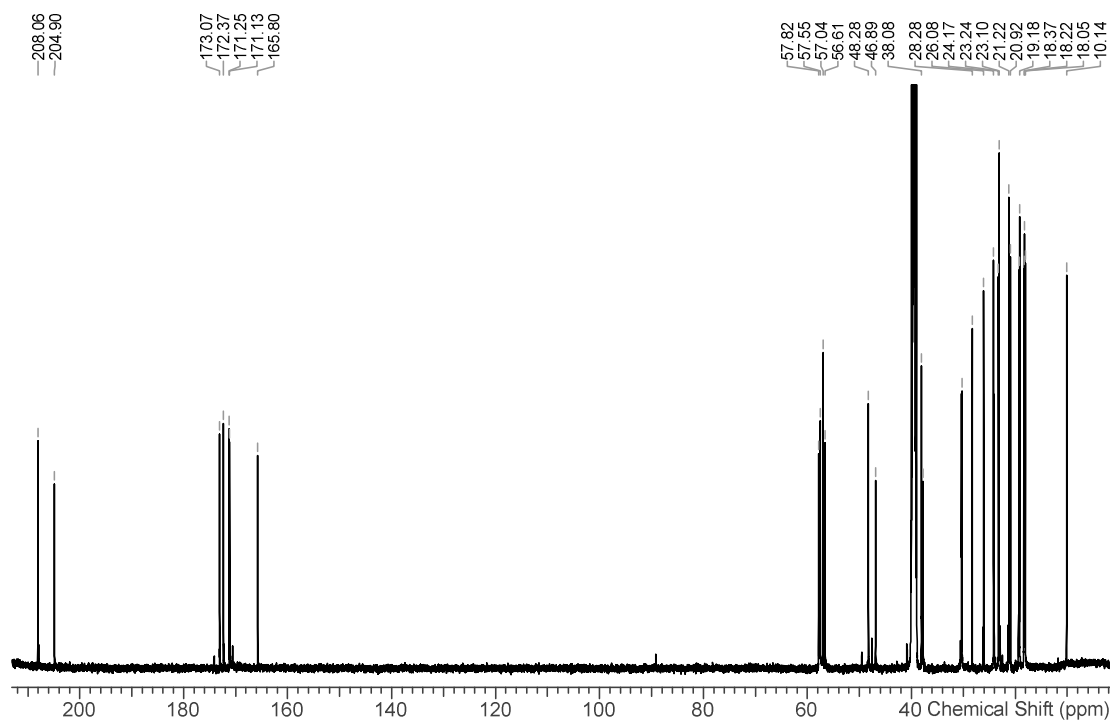


Supplementary Fig. 46. ^1H , ^{13}C -HMBC spectrum of compound **9** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

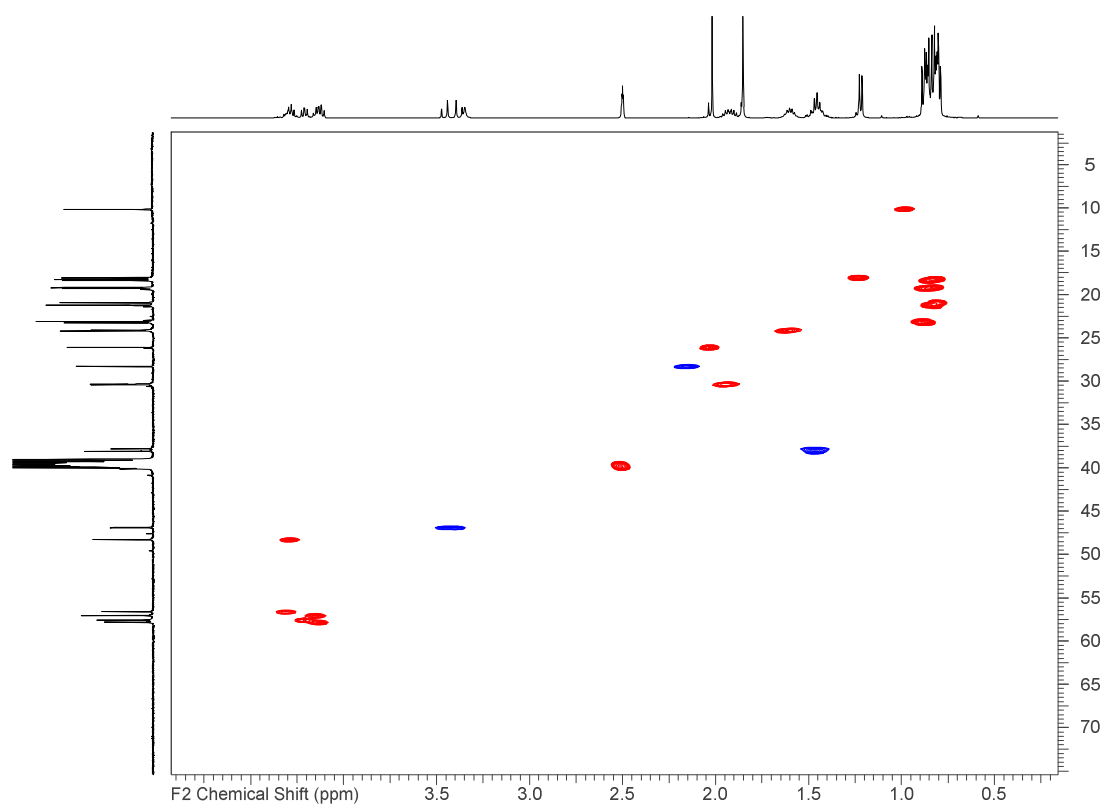




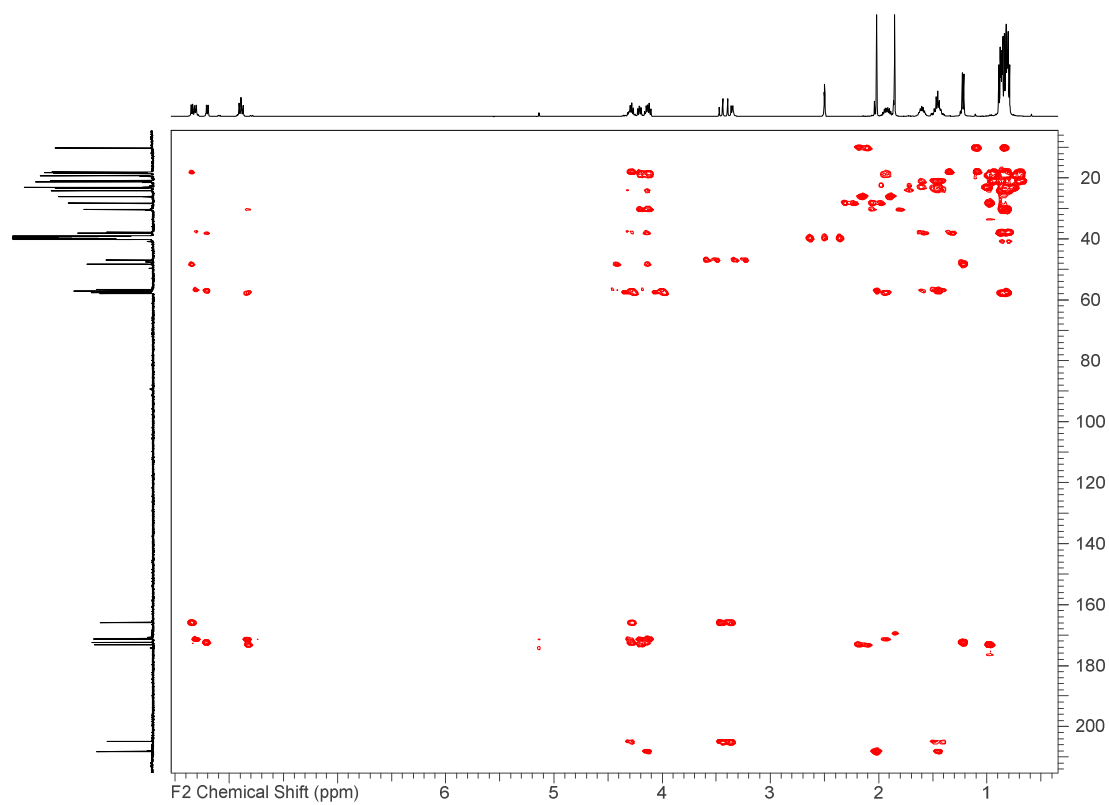
Supplementary Fig. 48. ¹H-NMR spectrum of compound **10** (500 MHz, DMSO-*d*₆).



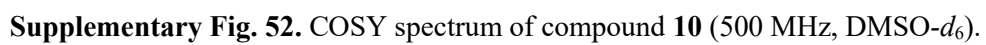
Supplementary Fig. 49. ¹³C-NMR spectrum of compound **10** (125 MHz, DMSO-*d*₆).

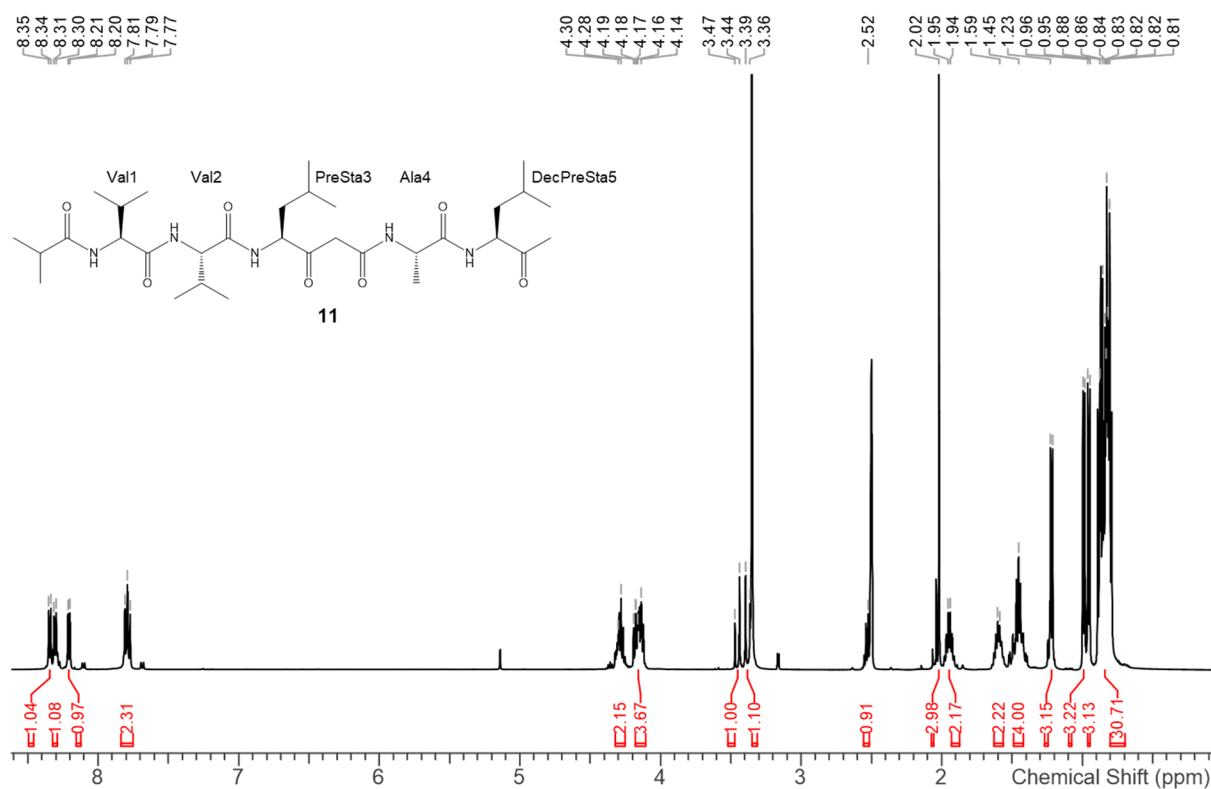


Supplementary Fig. 50. ^1H , ^{13}C -HSQC spectrum of compound **10** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

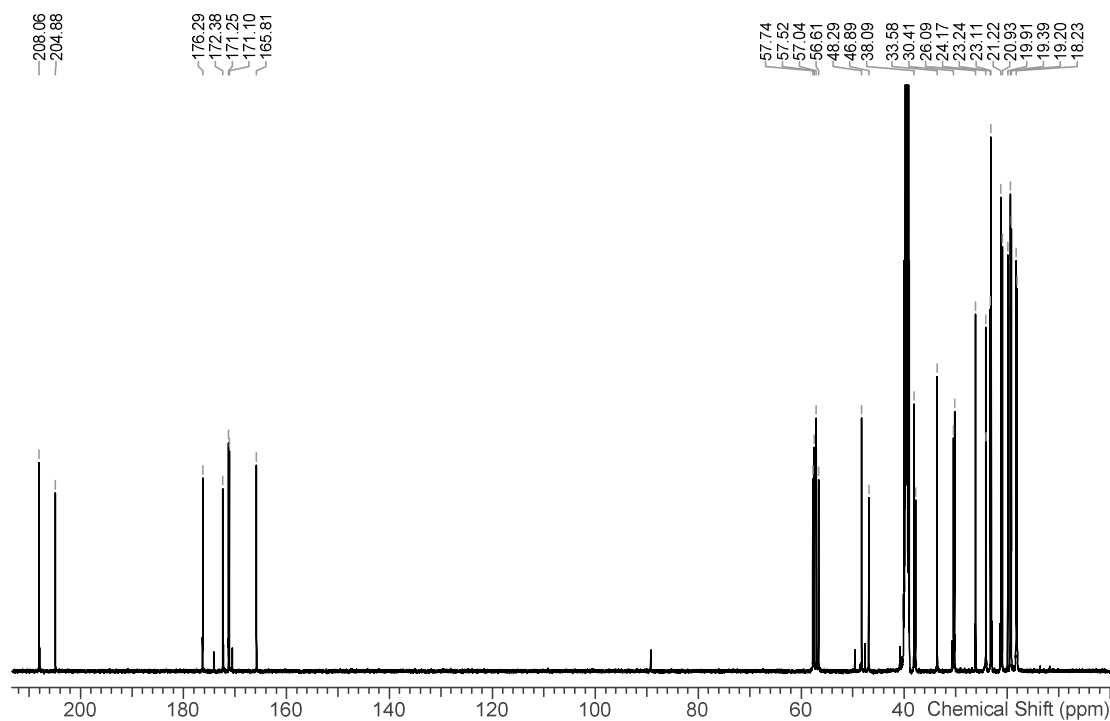


Supplementary Fig. 51. ^1H , ^{13}C -HMBC spectrum of compound **10** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

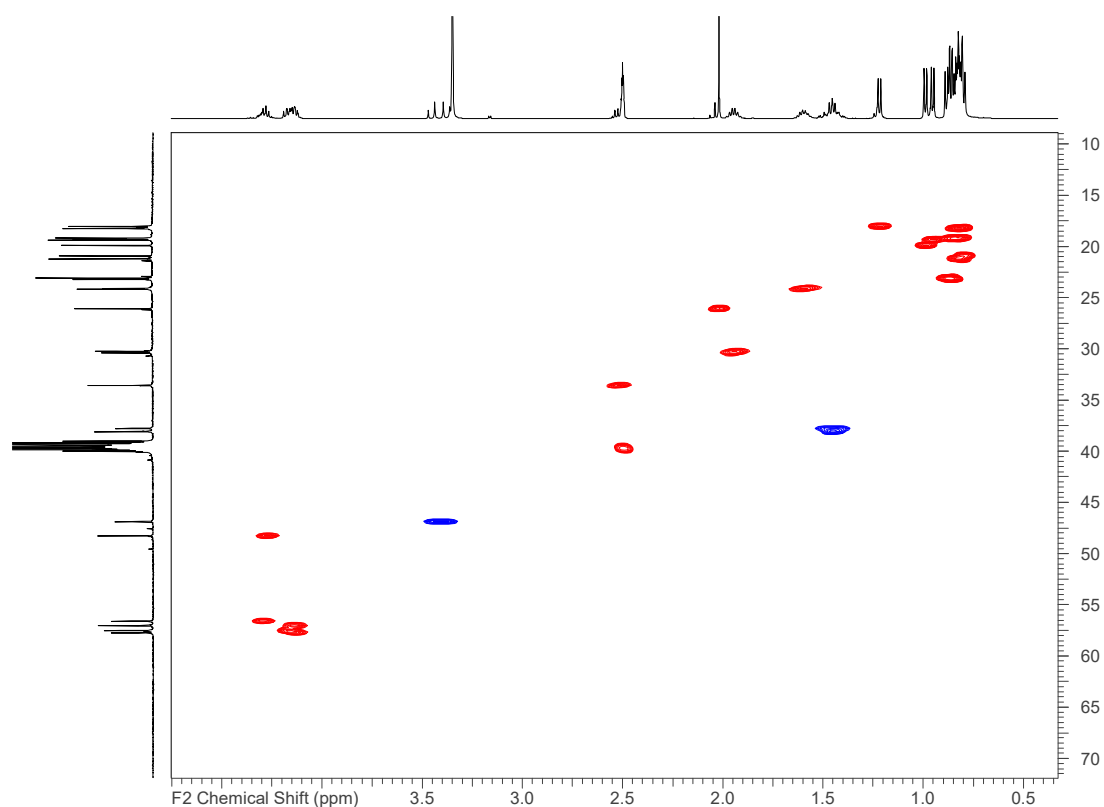




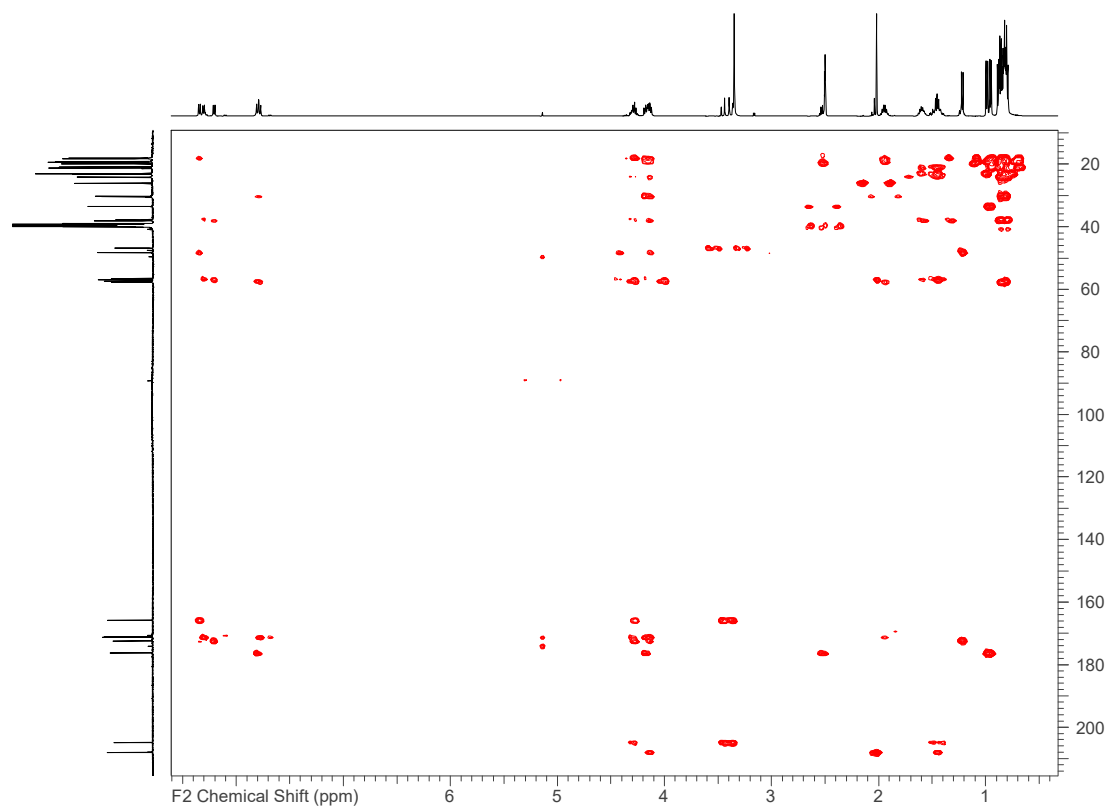
Supplementary Fig. 53. ¹H-NMR spectrum of compound **11** (500 MHz, DMSO-*d*₆).



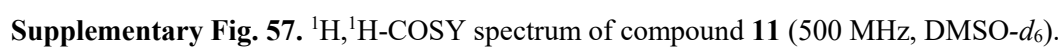
Supplementary Fig. 54. ¹³C-NMR spectrum of compound **11** (125 MHz, DMSO-*d*₆).

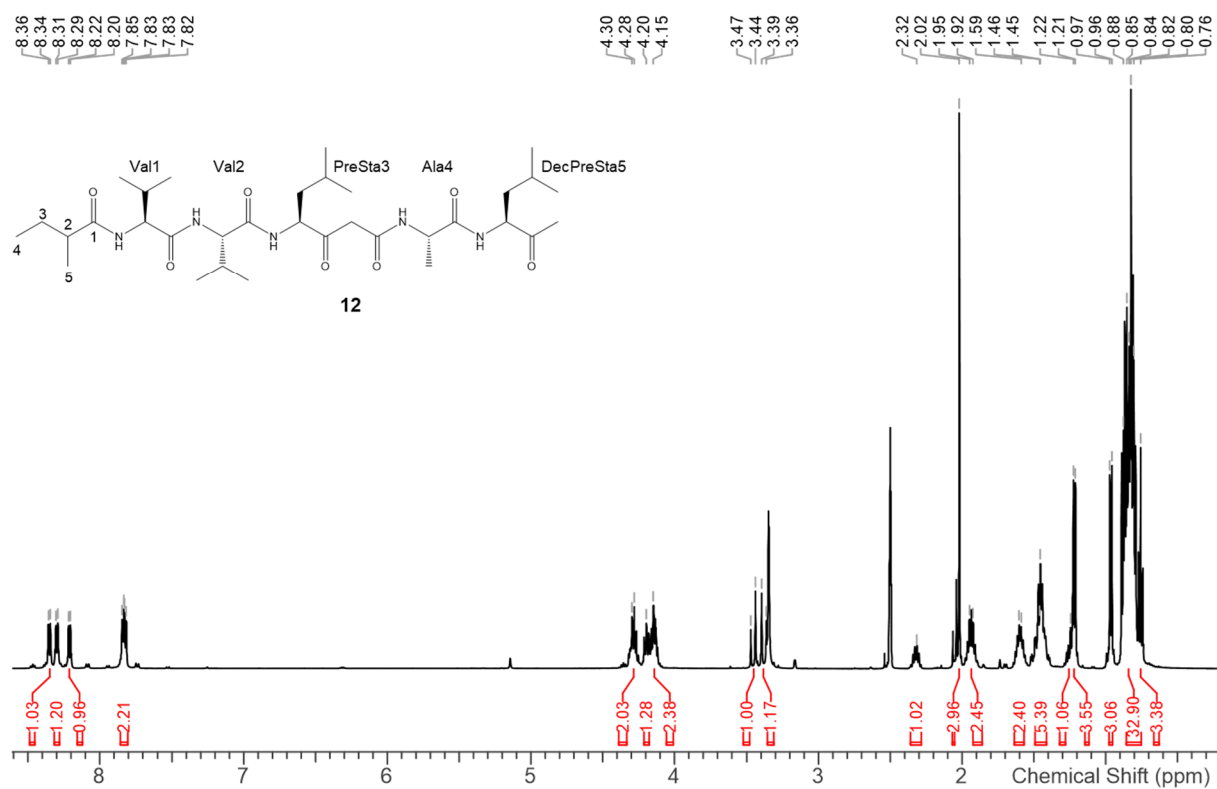


Supplementary Fig. 55. ^1H , ^{13}C -HSQC spectrum of compound **11** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

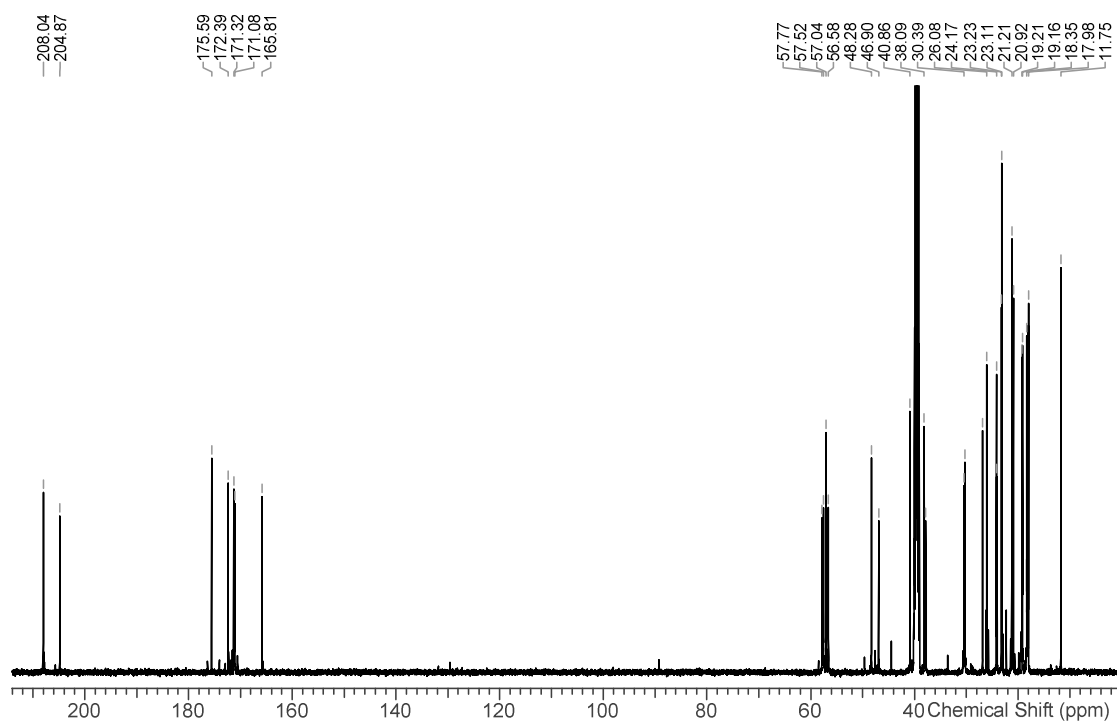


Supplementary Fig. 56. ^1H , ^{13}C -HMBC spectrum of compound **11** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

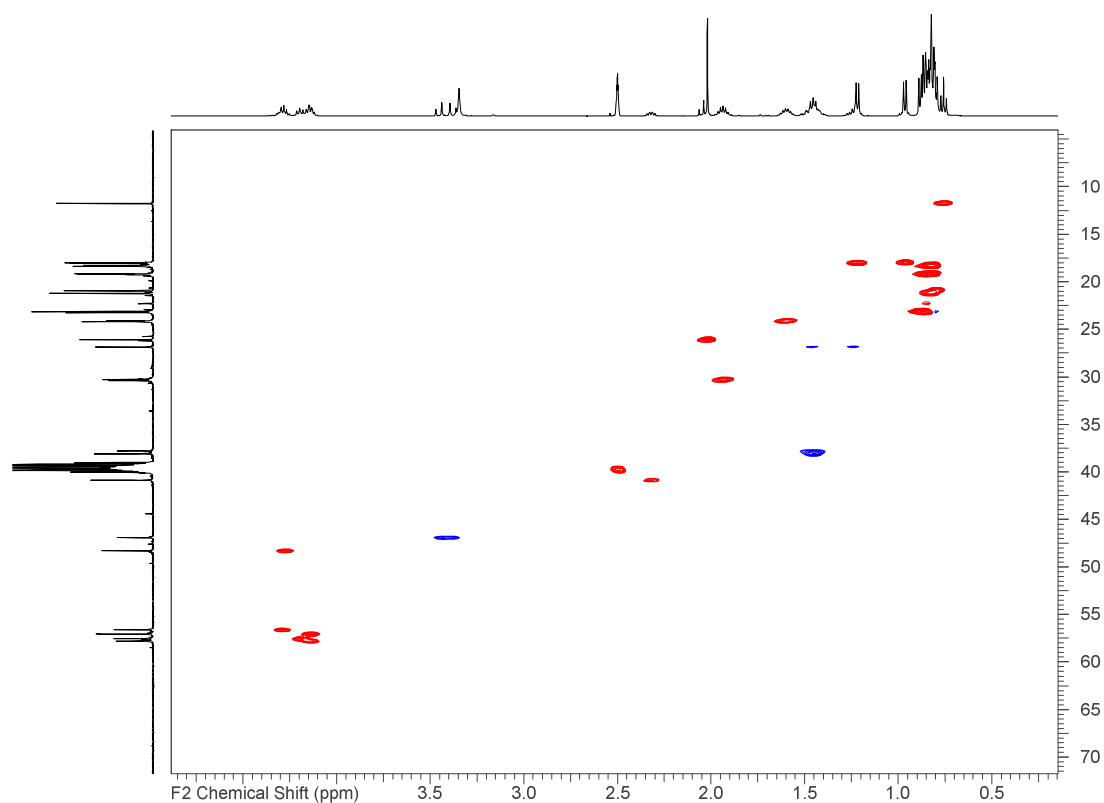




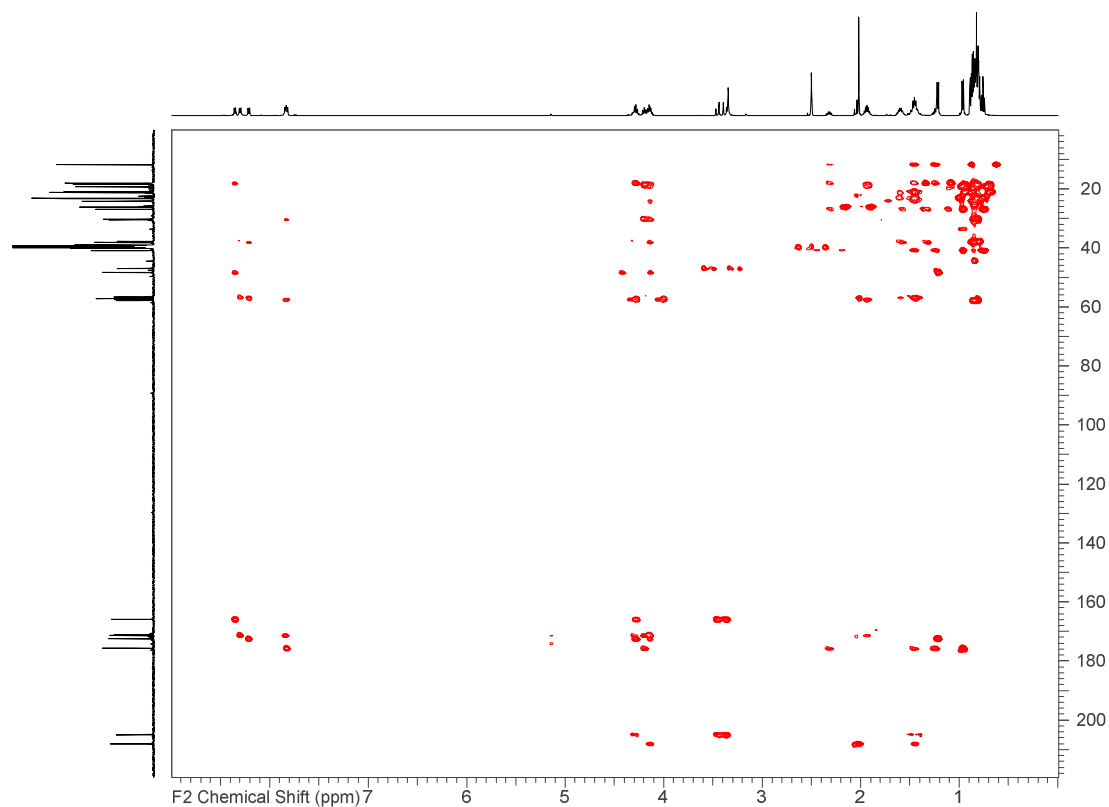
Supplementary Fig. 58. ^1H -NMR spectrum of compound **12** (500 MHz, $\text{DMSO-}d_6$).



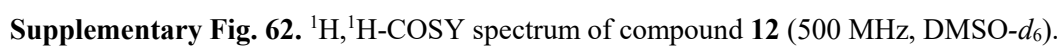
Supplementary Fig. 59. ^{13}C -NMR spectrum of compound **12** (125 MHz, $\text{DMSO-}d_6$).

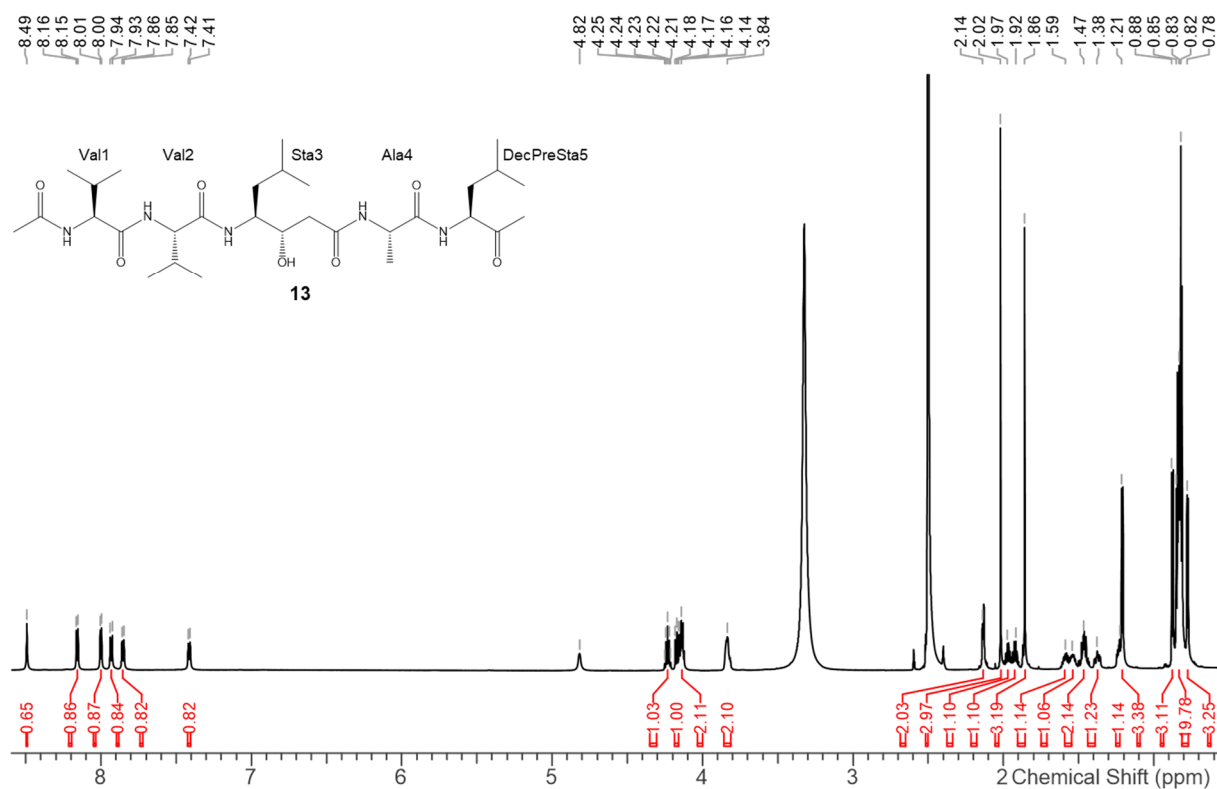


Supplementary Fig. 60. ^1H , ^{13}C -HSQC spectrum of compound **12** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

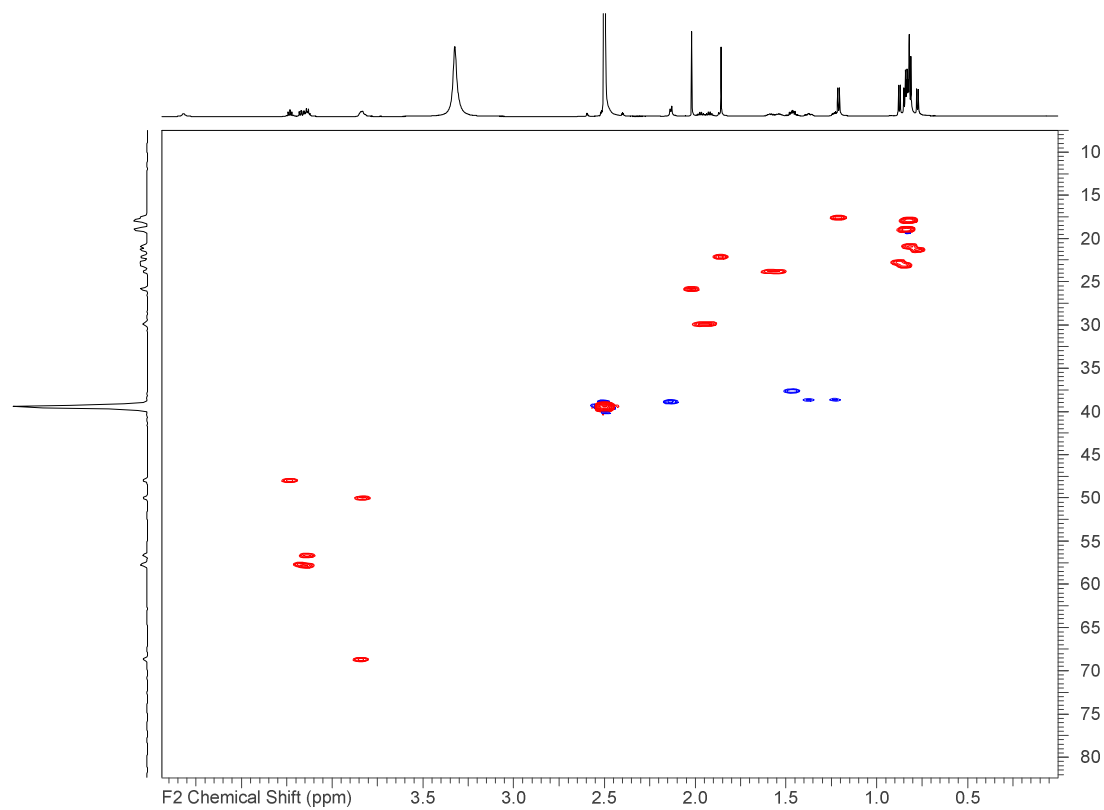


Supplementary Fig. 61. ^1H , ^{13}C -HMBC spectrum of compound **12** (^1H :500 MHz, ^{13}C :125 MHz, DMSO- d_6).

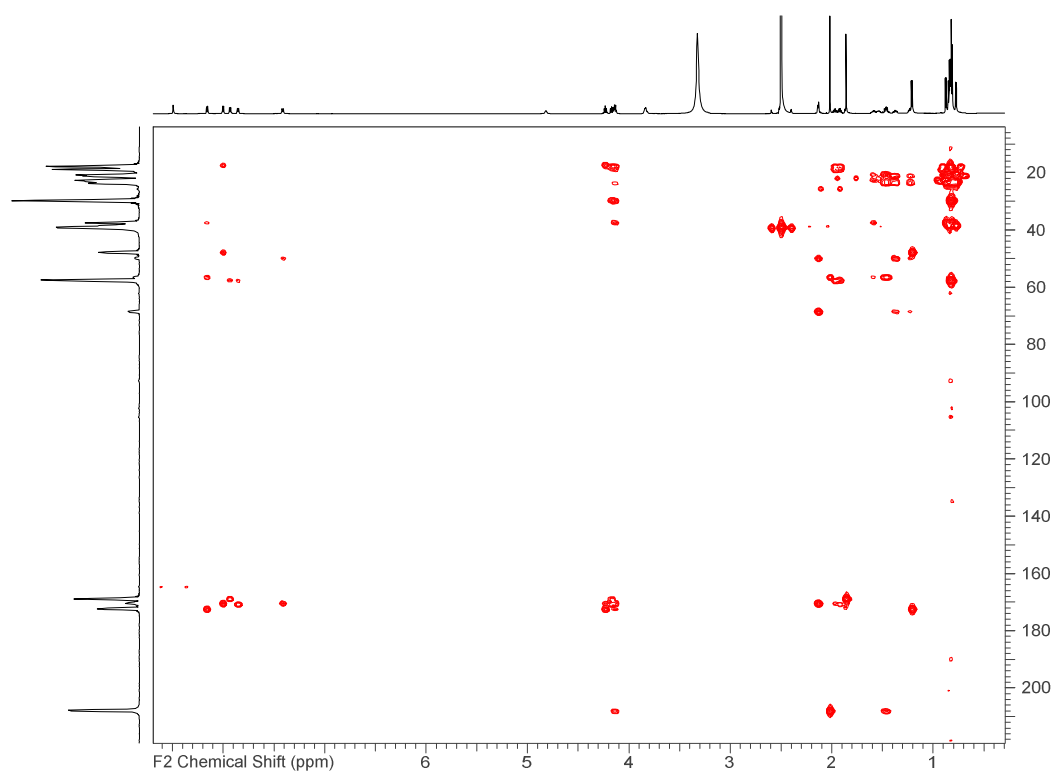




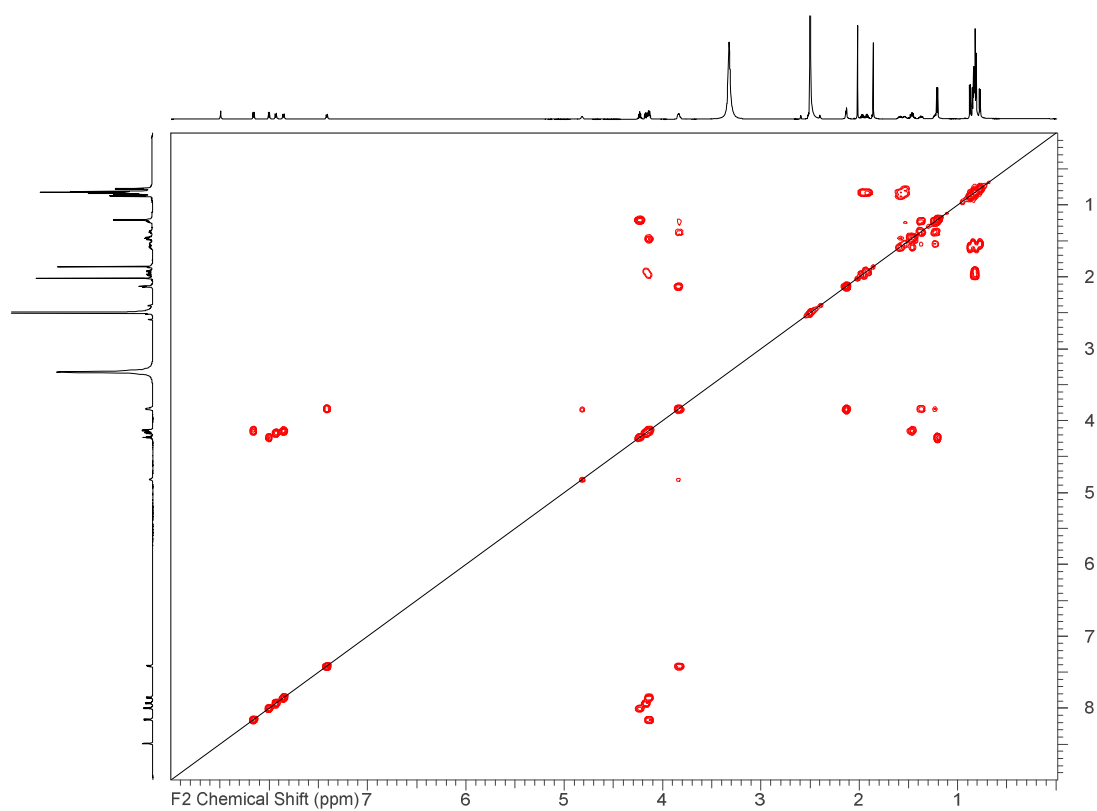
Supplementary Fig. 63. ^1H -NMR spectrum of compound **13** (700 MHz, $\text{DMSO-}d_6$).



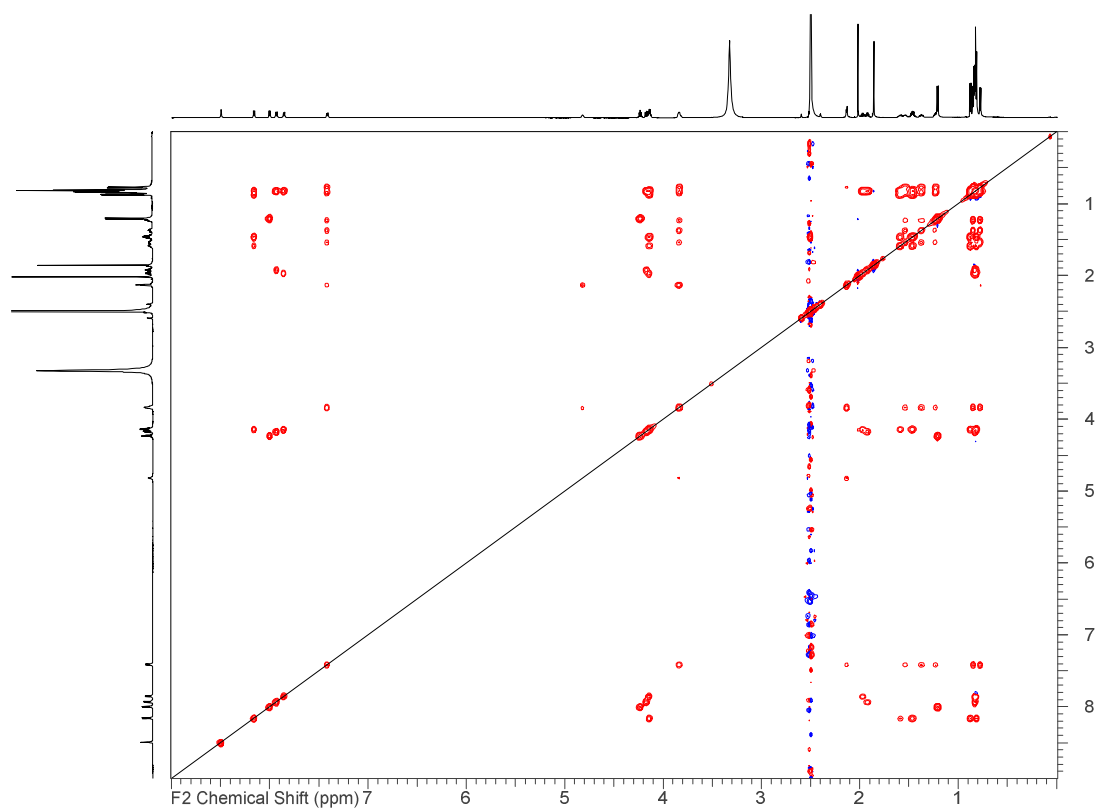
Supplementary Fig. 64. ^1H , ^{13}C -HSQC spectrum of compound **13** (^1H :700 MHz, ^{13}C :175 MHz, $\text{DMSO-}d_6$).



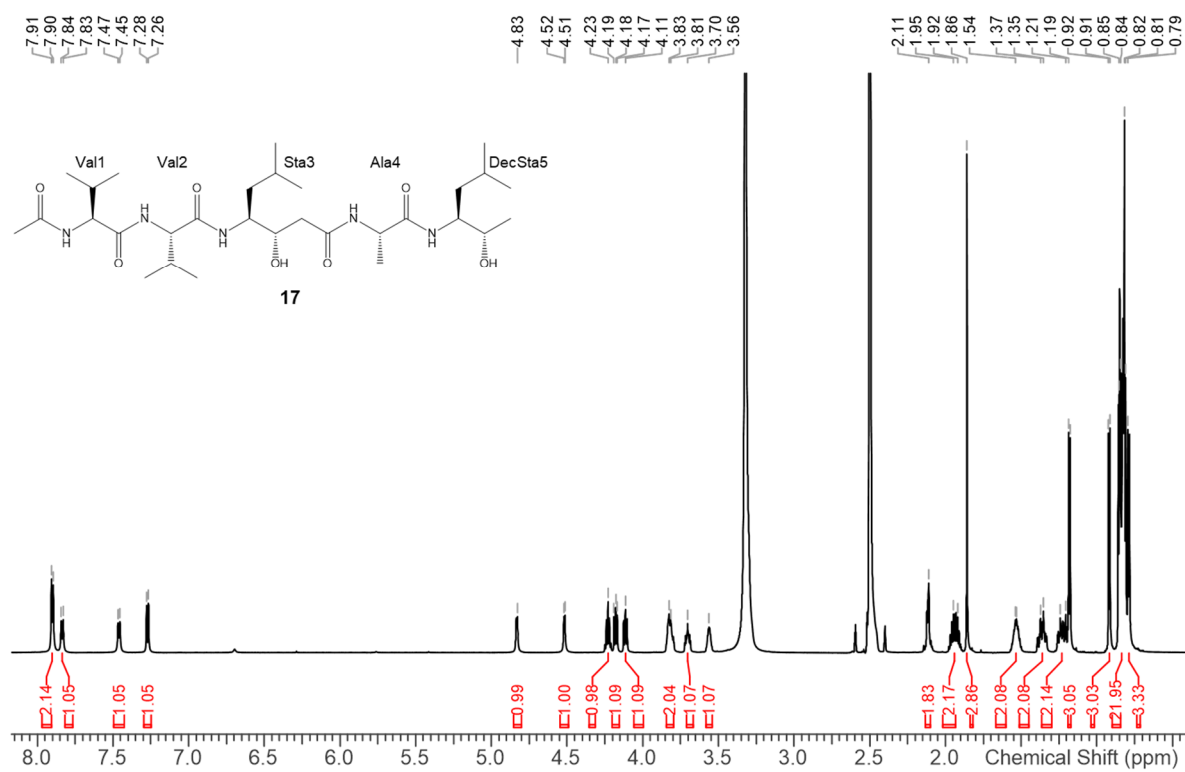
Supplementary Fig. 65. ^1H , ^{13}C -HMBC spectrum of compound **13** (^1H :700 MHz, ^{13}C :175 MHz, $\text{DMSO}-d_6$).



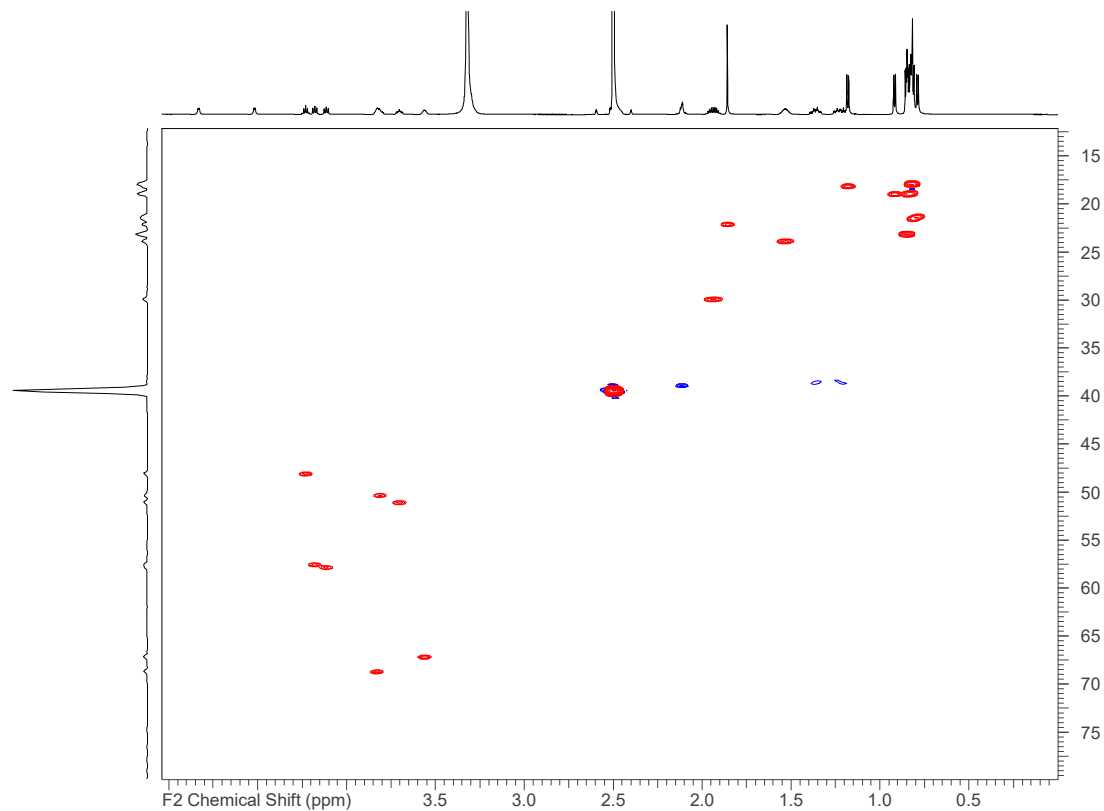
Supplementary Fig. 66. ^1H , ^1H -COSY spectrum of compound **13** (700 MHz, $\text{DMSO}-d_6$).



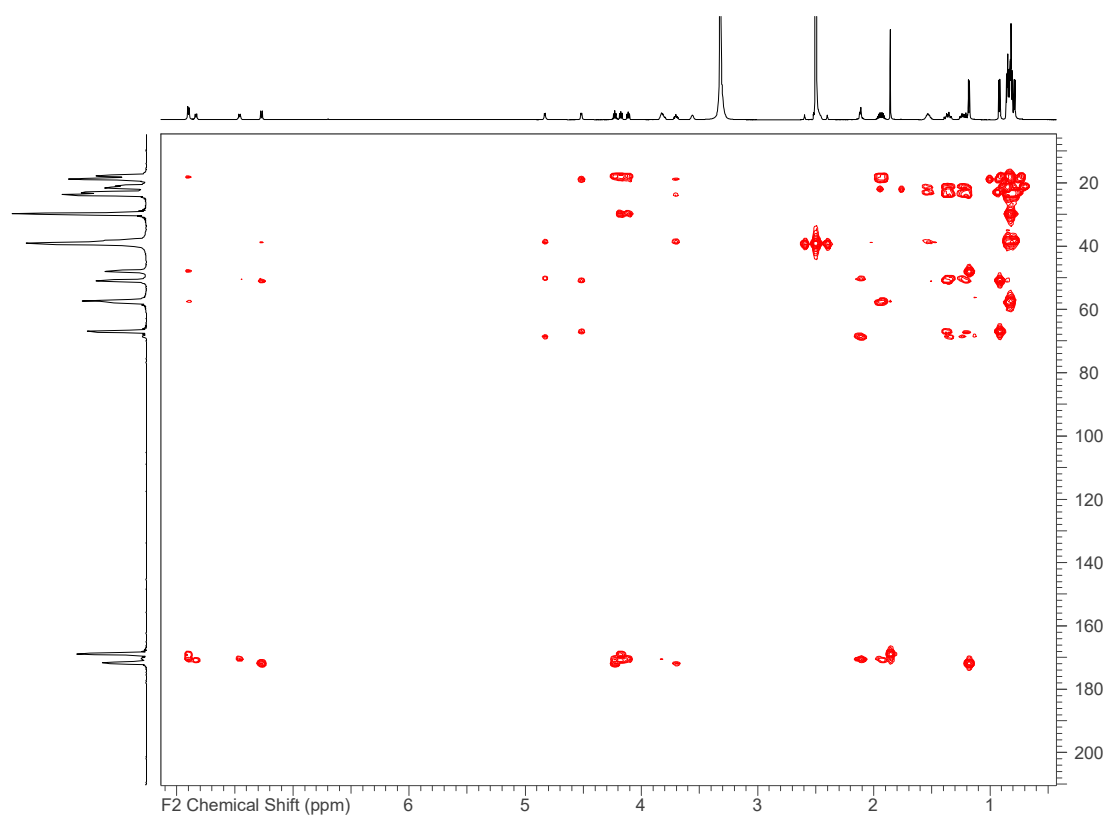
Supplementary Fig. 67. ^1H , ^1H -TOCSY spectrum of compound **13** (700 MHz, $\text{DMSO}-d_6$).



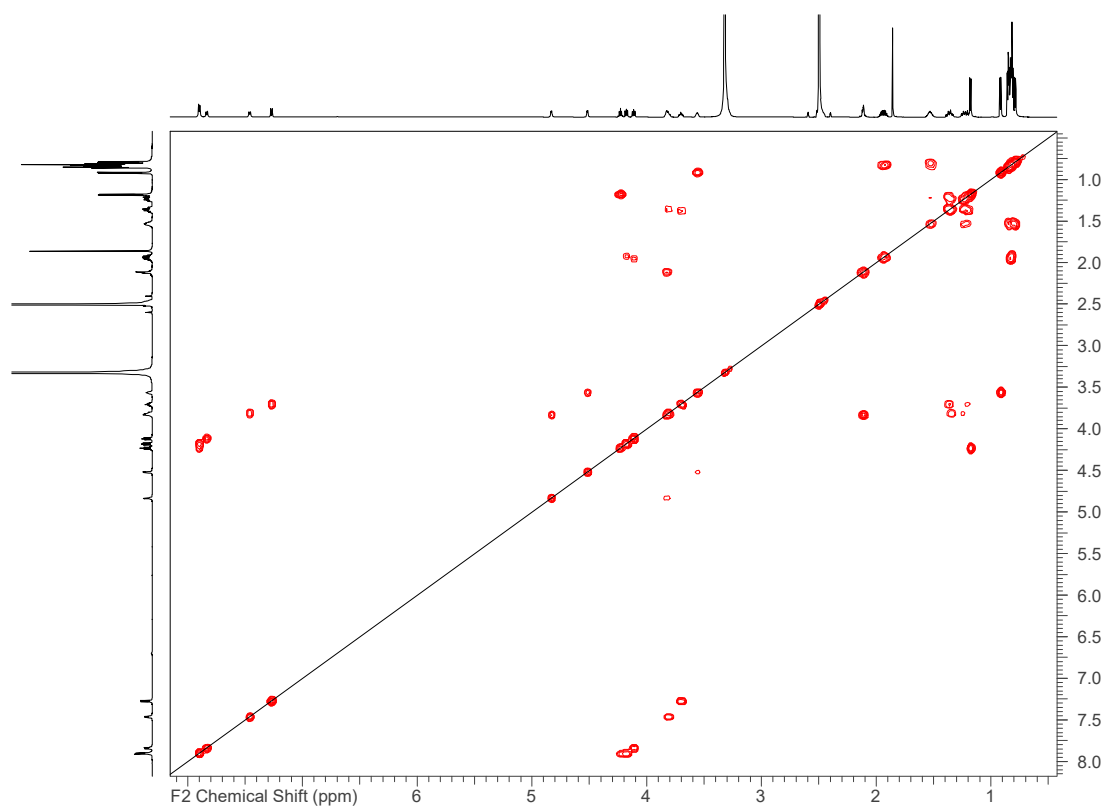
Supplementary Fig. 68. ^1H NMR spectrum of compound 17 (700 MHz, $\text{DMSO-}d_6$).



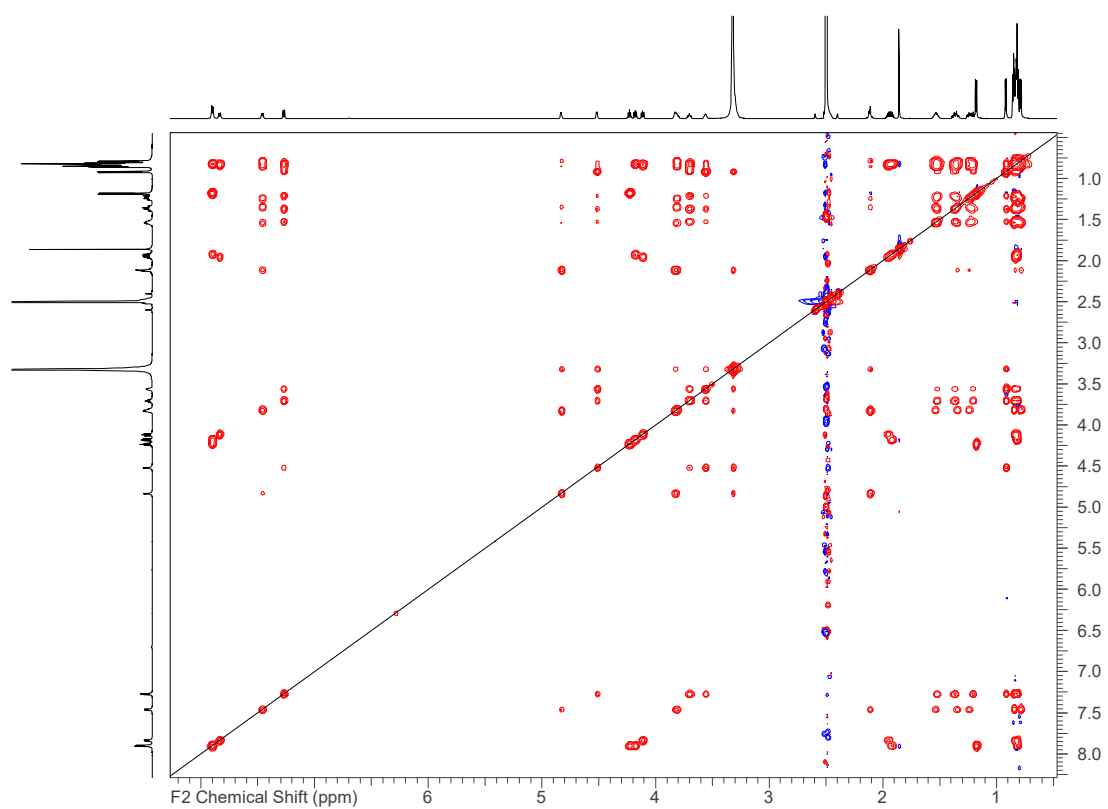
Supplementary Fig. 69. ^1H , ^{13}C -HSQC spectrum of compound 17 (^1H : 700 MHz, ^{13}C : 175 MHz, $\text{DMSO-}d_6$).



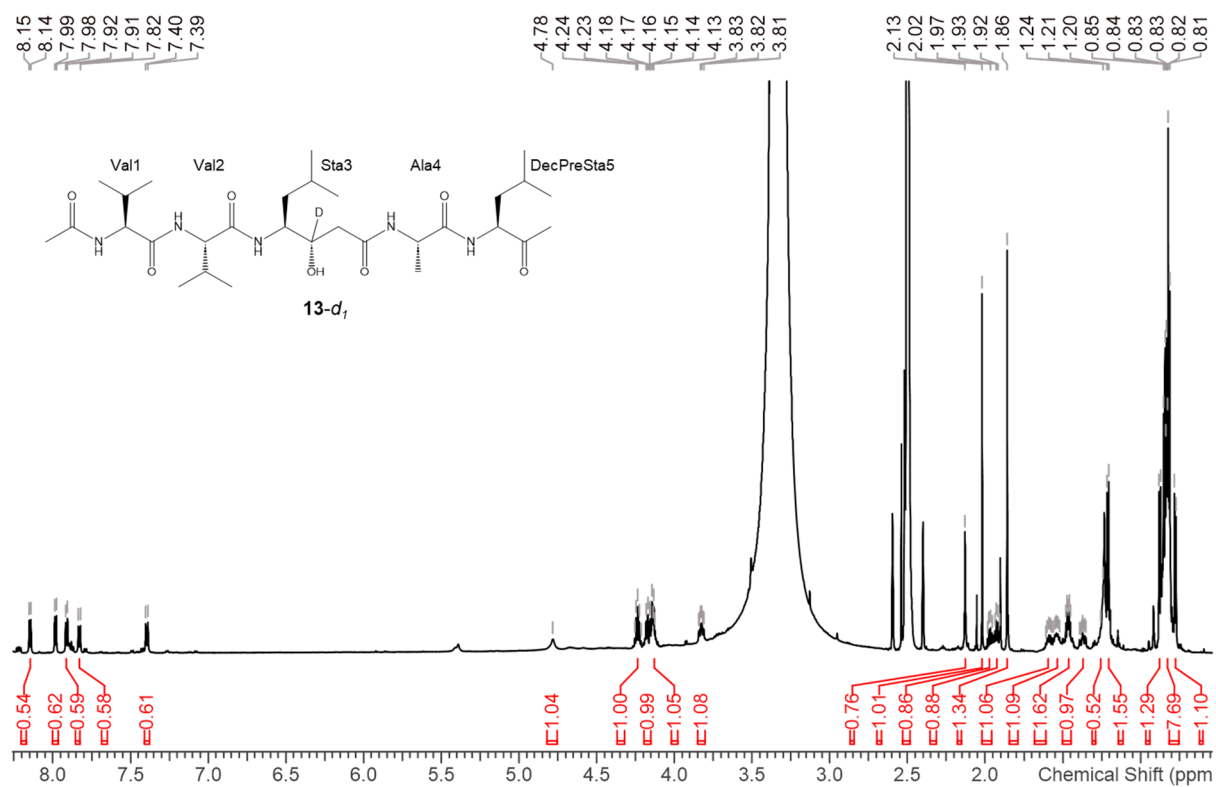
Supplementary Fig. 70. $^1\text{H},^{13}\text{C}$ -HMBC spectrum of compound **17** (^1H :700 MHz, ^{13}C :175 MHz, $\text{DMSO}-d_6$).



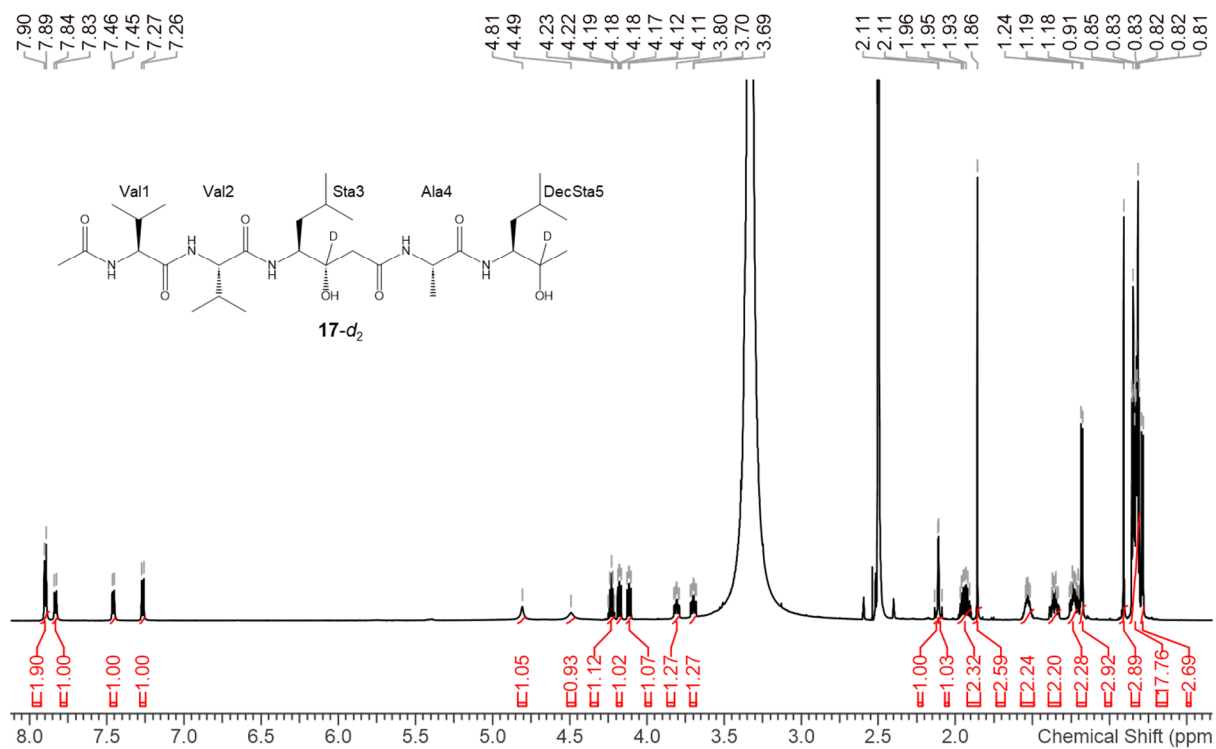
Supplementary Fig. 71. $^1\text{H},^1\text{H}$ -COSY spectrum of compound **17** (700 MHz, $\text{DMSO}-d_6$).



Supplementary Fig. 72. $^1\text{H}, ^1\text{H}$ -TOCSY spectrum of compound **17** (700 MHz, $\text{DMSO}-d_6$).



Supplementary Fig. 73. ^1H NMR spectrum of compound **13-d₁** (700 MHz, DMSO- d_6).



Supplementary Fig. 74. ^1H NMR spectrum of compound **17-d₂** (700 MHz, DMSO- d_6).

Supplementary Tables

Supplementary Table 1. Bacterial strains used in this study.

Strains	Characteristic(s)	Source
<i>E. coli</i>		
DH10B	<i>F</i> – <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ⁵ <i>endA1 recA1</i> ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> <i>rpsL</i> (<i>StrR</i>) <i>nupG</i> λ - Host for general cloning	
GB08-red	Host for Red-ET, <i>E. coli</i> GB2005, ^{6,7} PBAD- $\alpha\beta\gamma$ A, <i>AlacZ</i> ; <i>redα</i> , <i>redβ</i> , <i>redγ</i> , and <i>recA</i> regulated by the arabinose induced Promoter is integrated at the <i>ycbC</i> site on chromosome, and <i>lacZ</i> is knocked out	
BL21(DE3)	<i>fhuA2</i> [<i>lon</i>] <i>ompT gal</i> (λ DE3) [<i>dcm</i>] ⁸ Δ <i>hsdS</i> λ DE3 = λ <i>sBamHI</i> o Δ <i>EcoRI-B</i> <i>int::(lacI::PlacUV5::T7 gene1)</i> <i>i21</i> Δ <i>nin5</i> For protein expression	
HB101/pRK2013	Replicon: <i>ColE1</i> . Vector for <i>E. coli</i> . ⁹ Carries Tn 903. Contains <i>RK2</i> transfer genes. Helper plasmid for mobilisation of non-self-transmissible plasmids. For tri-parental conjugation	
ET12567(pUZ8002)	Donor strain for conjugation ¹⁰ (methylation deficient)	
<i>Streptomyces</i>		
<i>S. albus</i> Delta14	Heterologous expression host	¹¹
<i>S. catenulae</i> DSM40258	Pepstatin producing strain	DSM40258
<i>S. catenulae</i> - Δ <i>pepD</i>	<i>pepD</i> gene knock out	This study
<i>S. albus</i> Del14- <i>pep</i>	<i>pep</i> gene cluster	This study
<i>S. albus</i> Del14- <i>pep</i> - Δ <i>pepJ</i>	<i>pep</i> gene cluster, <i>pepJ</i> gene knock out	This study
<i>S. albus</i> Del14- <i>pep</i> - <i>pepJ</i> -act	<i>pep</i> gene cluster, <i>kasOp</i> activate <i>pepJ</i>	This study
<i>S. albus</i> Del14- <i>pep</i> - Δ <i>pepI</i>	<i>pep</i> gene cluster, <i>kasOp</i> activate <i>pepJ</i> , <i>pepI</i> knock out	This study

Supplementary Table 2. Plasmids used in this study.

Plasmids	Characteristic(s)	Source
pQS9	<i>ori</i> (<i>ColE1</i>), <i>rep</i> (<i>pIJ101</i>), <i>oriT</i> (<i>RK2</i>), <i>aac</i> (3) <i>IV</i> , <i>E. coli</i> - <i>Streptomyces</i> shuttle vector for gene editing <i>NcoI</i> - <i>XbaI</i> sgRNA cloning cassette, <i>ptipA</i> , <i>tsr</i> , <i>StuI</i> , <i>gusA</i> , <i>idgS</i>	¹²
pR6K-oriT-phiC31	<i>oriT</i> , integrase <i>phiC31</i> and apramycin resistance genes with a pR6k origin	
p15A-cm-tetR-tetO-hyg-ccdB	<i>ccdB</i> , chloramphenicol, hygromycin and tetracycline resistance genes with a p15A origin	
pCold I	<i>cspA</i> promoter, <i>amp^r</i> , <i>ColE1</i> origin of replication	Takara
pET-28b	<i>pBR322</i> derivative, <i>T7</i> terminator, N-6×His tag, N-Thrombin site, N-T7 promoter, C-6×His	
psgQS9- Δ <i>pepD</i>	pQS9 derivative	This study
pQS9- Δ <i>pepD</i>	pQS9 derivative, <i>pepD</i> <i>in situ</i> deletion	This study
p15A- <i>pep</i>	<i>p15A ori</i> , harboring <i>pep</i> BGC	This study
p15A-int- <i>pep</i>	<i>p15A ori</i> , integrase <i>phiC31</i> and apramycin resistance genes, <i>pep</i> BGC heterologous expression	This study
p15A-int- <i>pep</i> - Δ <i>pepJ</i>	p15A-int- <i>pep</i> derivative, gene <i>pepJ</i> deletion	This study
p15A-int- <i>pep</i> -kasop- <i>pepJ</i>	p15A-int- <i>pep</i> derivative, promoter <i>kasOp</i> activate gene <i>pepJ</i>	This study
p15A-int- <i>pep</i> - Δ <i>pepI</i>	p15A-int- <i>pep</i> -kasop- <i>pepJ</i> derivative, <i>pepI</i> deletion	This study
pCold-pepI	pCold derivative, <i>PepI</i> expression plasmid	This study
pET28b-kvpepI	pET-28b derivative, kvPepI expression plasmid	This study
pET28b-kvpepI-H62A	pET-28b derivative, kvPepI mutation	This study
pET28b-kvpepI-Y122F	pET-28b derivative, kvPepI mutation	This study
pET28b-kvpepI-Y122A	pET-28b derivative, kvPepI mutation	This study
pET28b-kvpepI-Q229A	pET-28b derivative, kvPepI mutation	This study
pET28b-kvpepI-Q289A	pET-28b derivative, kvPepI mutation	This study

Supplementary Table 3. Primers used in this study. Restriction sites are underlined.

Primer	Sequence (5' to 3') ^a	Description
sgpepD-f	CATGG <u>ATACGCGGTGAGTTGTATTCGTTT</u> TAGAG	sgRNA for cas9
sgpepD-r	CTAGCTCTAAAAC <u>GAATACA</u> ACTCACC <u>GCGTATC</u>	
pepD-L-f	<u>TCTCGTCGAAGGCACTAGAGGGTGACATCTCGTACATCGAG</u>	Homologous recombination repair template
pepD-L-r	<u>GATGCGGTTCAAGGACCAGT</u>	
pepD-R-f	<u>ACTGGTCCCTGAACCGCATC</u> GGCGACATCTTCCACCACCC	Homologous recombination repair template
pepD-R-r	<u>GGTCGATCCCCGCATATAGGGCTC</u> ACCGCCGAAGATGACG	
pepD-check-f	CACACCGGACCTGACCGAAG	<i>pepD</i> gene knock out mutant verification
pepD-check-r	CTGTGGCCGAAGAGGACCAC	
p15A-f	CTCGAGAGATCCGAAAACCCCAAGTT	Heterologous expression plasmid backbone amplification
p15A-r	CTCGAGAGATCCTTTCTCCTCTTTAG	
pepJ-ko-f	<u>GAGGGGGCCATTGTGCGGTTGGCGTCGTACGAGGAGATAA</u> ATTTCTGC CATTTCATCCGCTT	Chloramphenicol resistance gene selection marker
pepJ-ko-r	<u>CAGGTTGTGGCGGGCCACCAGATCGTGGATGACGCTCTT</u> GTTGATAC CGGGAAGCCCTG	
pepJ-act-f	<u>GTGACTGAAAACGATCATCCCGAAGACGCCATATCTCAGA</u> AATTTCTGC CATTTCATCCGCTT	Promoter <i>kasOp</i> and Chloramphenicol resistance gene cassette and verification
pepJ-act-r	<u>GTCATCGGCGAGCACGGCTACGGCAATCGAGCTTCCCAAA</u> ACTCCCC AGTCCTGCACG	
pepJ-check-f	TACATCAGTGTGCATGAATC	<i>pepJ</i> gene knock out mutant verification
pepJ-check-r	GTGTAAGTAGACCAGAACAG	
pepI-ko-f	<u>GAATGGACGGACAAGTGCCGTGCCGCGGAAGCGCTCGGCA</u> ATTTCTG CCATTTCATCCGC	Chloramphenicol resistance gene selection marker
pepI-ko-r	<u>GTCATCGGCGAGCACGGCTACGGCAATCGAGCTTCCCAAA</u> ACTCCCC AGTCCTGCACG	

pepI-check-f	CATCACCTCCCTGACCTCGCC	<i>pepI</i> gene knock out mutant verification
pepI-check-r	GTGCGGTTTCTCAGGTTGTG	
pep-check-f	CACGTTGTGCCAGAACTCCTC	BGC <i>pep</i> heterologous expression mutant verification
pep-check-r	TCGTGTGGTCCAGGAGCGCG	
pepI-f	<u>ATCATCATATCGAAGGTAGGAACGCTTTCGGTTTCGGTGT</u>	Codon-optimized <i>pepI</i> gene amplification
pepI-r	<u>GACTGCAGGTCGACAAGCTTGAATTCTTAACGACCAGCCAGAGCTT</u>	
kvpepI-f	<u>GCCTGGTGCCGCGCGGCAGCACACCCTGCGTCCGTTCCG</u>	<i>kvpepI</i> gene amplification
kvpepI-r	<u>TCGAGTGCGGCCGCAAGCTTTTAACCACGAGCAGAAACCCA</u>	
H62A-r	ATGAACGGAGAGTGAACACCCAG CG CGTCCGGAACAGCGATAACGTC	H62A mutation
H62A-f	ACGACGTTATCGCTGTTCCGGAC CG CTGGGTGTTCACTCTCCGTTCA	
Y122F-r	GCGGTTTCGAATTCAGCACGAACA AAA ACCGGTACCCAGACCAGCTTC	Y122F mutation
Y122F-f	TTGAAGCTGGTCTGGGTACCGGTT TTT GTTCGTGCTGAATTCGAAACCG	
Y122A-r	GCGGTTTCGAATTCAGCACGAAC CG CACCGGTACCCAGACCAGCTTC	Y122A mutation
Y122A-f	TTGAAGCTGGTCTGGGTACCGG TG CGGTTCTGCTGAATTCGAAACCG	
Q229A-r	TCGTGCGTAGCGATAACAGAC CG CAACCAGGGTGTTACGTTCAA	Q229A mutation
Q229A-f	TTGAACGTAACACCCTGGTT G CGTCTGTTATCGCTACCGACGA	
pET-28b-f	TGATGTCTCTGACCAGACAC	site mutation gene expression backbone
pET-28b-r	GAAATCACCATGAGTGACGA	
Q289A-r	AACGCCGCGAGGTAACGTTCC G CAACGCAAACGTAAGAGAAGC	Q289A mutation
Q289A-f	GCTTCTCTTACGTTTGCGTT G CGGAACGTTACCTCGCGGCGTT	

*Gene-specific guiding sequences for CRISPR-Cas9 gene editing are double-underlined. The homologous arms for Gibson assembly or Red-ET are underlined, the mutation sites in kvPepl mutants are marked in **bold**.

Supplementary Table 4. Gene annotation of *pep* BGC.

Gene	Size (aa)	Protein homolog	Proposed function		Putative catalytic domains
<i>pepA</i>	95	WP_051739820.1	phosphopantetheine binding protein		PCP
<i>pepB</i>	1100	WP_157848057.1	NRPS		C, A, PCP
<i>pepC</i>	898	WP_051739811.1	Type I PKS		KS, AT, PCP
<i>pepD</i>	1093	WP_051739812.1	NRPS		C, A, PCP
<i>pepE</i>	278	WP_078654176.1	thioesterase		TE
<i>pepF</i>	63	WP_030286418.1	mbtH		
<i>pepG</i>	530	WP_051739813.1	NRPS		A
<i>pepH</i>	1036	WP_078654178.1	NRPS		C, PCP
<i>pepI</i>	295	WP_030286424.1	methylenetetrahydromethanopterin reductase		
<i>pepJ</i>	212	WP_051739822.1	helix-turn-helix regulator	transcriptional	

Supplementary Table 5. Substrate specificity analysis of the three A domains from *pep* NRPSs.

A domain	ORF	stachelhaus_code ^a	Predicted amino acid ^b	Biosynthesis assumed amino acid ^c
A1	<i>pepB</i>	DAWFLGNVVK	Leu	Leu
A2	<i>pepD</i>	DVWHLSALEK	Ala	Ala
A3	<i>pepG</i>	DFWNIGMVFK	Thr	Val

^a Residues defined according to Stachelhaus et al.¹³

^b Predicted by antiSMASH bacteria version gene cluster analysis according to Stachelhaus prediction.¹⁴

^c Amino acids incorporated in the pepstatin structures.

Supplementary Table 6. Classification of *pep*-like BGCs.

Strain	BGC Type (according to the feature of PKS)
<i>Streptomyces catenulae</i> DSM 40258	<i>cis-AT</i> PKS
<i>Streptomyces</i> sp. SF28	<i>cis-AT</i> PKS
<i>Streptomyces</i> sp. BHT-5-2	<i>cis-AT</i> PKS
<i>Streptomyces</i> sp. PTM05	<i>cis-AT</i> PKS
<i>Streptomyces varsoviensis</i> NRRL ISP-5346	<i>cis-AT</i> PKS
<i>Kitasatospora kifunensis</i> DSM 41654	<i>trans-AT</i> PKS
<i>Kitasatospora viridis</i> DSM 44826	<i>trans-AT</i> PKS
<i>Streptomyces cattleya</i> DSM 46488	without PKS
<i>Streptomyces</i> sp. ICBB 8177	without PKS

Supplementary Table 7. X-ray data collection and refinement statistics.

	kvPepI_apo	kvPepI_F420	kvPepI ^{H62A}	kvPepI ^{H62A} _F420	kvPepI ^{Y122A} _F420
PDB ID	9G64	9GM0	9GKH	9GND	9GNC
Data collection					
Space group	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Cell dimension					
a, b, c (Å)	56.14, 94.79, 111.05	56.35, 94.27, 111.02	56.21, 94.28, 110.82	56.52, 94.27, 110.64	56.31, 94.59, 110.76
α , β , γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.00, 90.00, 90.00
Wavelength (Å)	1.033	1.033	1.033	1.033	1.033
Resolution	48.31 – 1.65 (1.68 – 1.65)*	48.37 – 1.65 (1.68 – 1.65)*	48.28 – 1.70 (1.73 – 1.70)	48.48 – 1.80 (1.84 – 1.80)	48.39 – 1.65 (1.68 – 1.65)
R _{sym} Or R _{merge}	0.123 (1.756)	0.086 (1.431)	0.084 (1.450)	0.243 (2.155)	0.154 (1.797)
R _{pim}	0.024 (0.346)	0.035 (0.587)	0.020 (0.383)	0.067 (0.585)	0.037 (0.426)
CC (1/2)	0.999 (0.830)	0.999 (0.703)	1.000 (0.890)	0.998 (0.820)	0.999 (0.847)
I / σ I	19.4 (2.5)	17.1 (1.9)	28.5 (2.7)	12.3 (2.3)	21.2 (3.5)
Completeness (%)	99.5 (99.7)	100.0 (100.0)	100.0 (100.0)	99.9 (100.0)	99.5 (99.3)
Redundancy	26.8 (27.0)	13.4 (13.5)	34.3 (28.9)	26.3 (27.6)	34.3 (34.9)
Refinement					
Resolution (Å)	48.31 – 1.65	48.37 – 1.65	48.28 – 1.70	48.48 – 1.80	48.39 – 1.65
No. reflection	71666 (3528)	71956 (3513)	65590 (3416)	55564 (3272)	71529 (3484)
R _{work} / R _{free}	0.175 / 0.191	0.169 / 0.188	0.181 / 0.199	0.177 / 0.209	0.169 / 0.192
No. atoms	4583	4721	4472	4602	4771
Protein	4095	4118	4079	4104	4104

Ligands	-	53	-	53	53
Solvent	488	550	393	445	611
Protein residues	539	545	538	544	545
B-factors	26.39	26.79	28.88	30.35	25.27
Protein	25.12	25.30	28.06	29.11	23.59
Ligands	-	35.24	-	66.85	36.75
Water	37.08	37.12	37.38	37.46	35.54
R. m. s deviations					
Bond length (Å)	0.008	0.009	0.011	0.010	0.009
Bond angels (°)	0.93	1.08	1.09	1.05	1.07
MolProbity score	0.96	0.96	1.03	0.97	0.84

*Values in parentheses are for highest-resolution shell

NMR Tables

Supplementary Table 8. NMR spectroscopic data of compounds **1-4** (methanol-*d*₄).

No.	1^a		2^a		3^b		4^a	
	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}
Val1								
1		174.1		174.1		174.1		174.0
2	4.16 (1H, d, 7.7)	60.7	4.17 (1H, d, 7.8)	60.6	4.16 (1H, d, 8.0)	60.5	4.18 (1H, d, 8.3)	60.4
3	2.07 (1H, m)	31.6	2.07 (1H, m)	31.6	2.06 (1H, m)	31.6	2.06 (1H, m)	31.6
4	0.98 (3H, d, 6.4)	18.9	0.98 (3H, d, 6.6)	19.0	0.97 (3H, m °)	18.9	0.97 (3H, m °)	18.9
5	0.96 (3H, d, 6.8)	19.9	0.96 (3H, d, 6.7)	19.9	0.98 (3H, m °)	19.9	0.98 (3H, m °)	19.9
Val2								
1		173.8		173.8		173.8		173.8
2	4.12 (1H, d, 7.9)	61.0	4.12 (1H, d, 7.9)	60.9	4.13 (1H, d, 7.7)	60.8	4.13 (1H, d, 7.7)	60.8
3	2.07 (1H, m)	31.6	2.07 (1H, m)	31.6	2.09 (1H, m)	31.6	2.08 (1H, m)	31.6
4	0.96 (3H, d, 6.7)	19.0	0.96 (3H, d, 6.8)	19.0	0.97 (3H, m °)	19.0	0.97 (3H, m °)	19.0
5	0.99 (3H, d, 6.2)	20.0	0.99 (3H, d, 6.3)	20.0	0.98 (3H, m °)	20.0	0.98 (3H, m °)	20.0
Sta3								
1		173.8		173.8		173.8		173.7
2	2.33 (1H, m °) 2.42 (1H, dd, 15.9, 4.3)	41.4	2.34 (2H, m °)	41.4	2.35 (2H, m °)	41.4	2.34 (2H, m °)	41.4
3	4.01 (1H, m)	71.1	4.00 (1H, m)	71.1	4.01 (1H, m)	71.1	4.00 (1H, m)	71.3
4	3.91 (1H, m)	52.8	3.89 (1H, m)	52.9	3.89 (1H, m)	52.9	3.89 (1H, m)	52.9
5	1.34 (1H, m) 1.59 (1H, m)	41.1	1.34 (1H, m) 1.57 (1H, m)	41.1	1.36 (1H, m) 1.58 (1H, m)	41.1	1.34 (1H, m) 1.57 (1H, m)	41.1
6	1.63 (1H, m)	25.9	1.63 (1H, m)	25.9	1.65 (1H, m)	25.9	1.64 (1H, m)	25.8
7	0.91 (3H, d, 6.7)	23.8	0.91 (3H, d, 6.9)	23.8	0.91 (3H, d, 6.6)	23.8	0.91 (3H, m °)	23.8
8	0.88 (3H, d, 6.4)	22.4	0.88 (3H, d, 6.5)	22.4	0.88 (3H, d, 6.5)	22.4	0.88 (3H, m °)	22.4
Ala4								
1		175.4		175.3		175.3		175.2

2	4.33 (1H, q, 7.2)	50.9	4.33 (1H, q, 7.2)	50.8	4.33 (1H, q, 7.2)	50.9	4.33 (1H, q, 7.2)	50.8
3	1.38 (3H, d, 7.1)	18.3	1.38 (3H, d, 7.2)	18.4	1.38 (3H, d, 7.2)	18.4	1.38 (3H, d, 7.2)	18.4
Sta5								
1		175.9		177.2		177.0		177.5
2	2.31 (1H, dd, 5.6, 14.7) 2.35 (1H, m ^c)	40.1	2.36 (2H, m ^c)	40.7	2.38 (2H, m ^c)	40.5	2.33 (2H, m ^c)	40.7
3	4.01 (1H, m)	71.1	4.00 (1H, m)	71.3	4.01 (1H, m)	71.2	4.00 (1H, m)	71.1
4	4.00 (1H, m)	52.4	3.98 (1H, m)	52.5	3.98 (1H, m)	52.5	3.97 (1H, m)	52.5
5	1.34 (1H, m) 1.59 (1H, m)	41.1	1.31 (1H, m) 1.60 (1H, m)	41.4	1.35 (1H, m) 1.58 (1H, m)	41.4	1.35 (1H, m) 1.58 (1H, m)	41.4
6	1.63 (1H, m)	25.8	1.63 (1H, m)	25.8	1.62 (1H, m)	25.8	1.62 (1H, m)	25.9
7	0.93 (3H, d, 6.6)	23.8	0.93 (3H, d, 6.8)	23.8	0.93 (3H, d, 6.7)	23.7	0.93 (3H, m ^c)	23.8
8	0.90 (3H, d, 6.6)	22.4	0.90 (3H, d, 6.7)	22.4	0.90 (3H, d, 6.5)	22.4	0.90 (3H, m ^c)	22.4
FA								
1		173.5		169.8		180.2		179.6
2	2.00 (3H, s)	22.4	2.28 (2H, q, 7.7)	29.9	2.56 (1H, m)	36.0	2.36 (1H, m)	43.4
3			1.13 (3H, t, 7.7)	10.5	1.10 (3H, d, 6.9)	19.8	1.38 (1H, m) 1.61 (1H, m)	28.3
4					1.14 (3H, d, 6.9)	20.1	0.87 (3H, m ^c)	12.4
5							1.11 (3H, d, 6.9)	18.2

^aMeasured at 500 MHz for ¹H NMR

^bMeasured at 700 MHz for ¹H NMR

^c*J*-value not determined due to overlapped signals

Supplementary Table 9. NMR spectroscopic data of compounds **9-12** (^1H : 500 MHz, ^{13}C : 125 MHz, DMSO- d_6).

No.	9		10		11		12	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
Val1								
1		171.2		171.2		171.3		171.3
2	4.21 (1H, dd, 8.7, 6.9)	57.6	4.20 (1H, dd, 8.7, 7.1)	57.6	4.18 (1H, dd, 7.1, 8.7)	57.5	4.20 (1H, dd, 7.6, 8.7)	57.5
3	1.91 (1H, m)	30.4	1.92 (1H, m)	30.4	1.94 (1H, m)	30.2	1.92 (1H, m)	30.3
4	0.81 (3H, m $^{\circ}$)	18.1	0.81 (3H, m $^{\circ}$)	18.2	0.82 (3H, m $^{\circ}$)	18.2	0.83 (3H, m $^{\circ}$)	18.3
5	0.82 (3H, m $^{\circ}$)	19.2	0.82 (3H, m $^{\circ}$)	19.2	0.83 (3H, m $^{\circ}$)	19.2	0.84 (3H, m $^{\circ}$)	19.2
NH	7.90 (1H, d, 8.9)		7.82 (1H, d, 8.9)		7.80 (1H, d, 8.8)		7.82 (1H, d, 8.8)	
Val2								
1		171.2		171.1		171.1		171.1
2	4.12 (1H, m $^{\circ}$)	57.9	4.12 (1H, m $^{\circ}$)	57.8	4.14 (1H, m)	57.7	4.15 (1H, m)	57.8
3	1.93 (1H, m)	30.3	1.95 (1H, m)	30.3	1.95 (1H, m)	30.4	1.95 (1H, m)	30.4
4	0.84 (3H, m $^{\circ}$)	18.2	0.84 (3H, m $^{\circ}$)	18.4	0.82 (3H, m $^{\circ}$)	18.3	0.83 (3H, m $^{\circ}$)	18.3
5	0.85 (3H, m $^{\circ}$)	19.3	0.85 (3H, m $^{\circ}$)	19.3	0.84 (3H, m $^{\circ}$)	19.2	0.84 (3H, m $^{\circ}$)	19.2
NH	7.88 (1H, d, 9.0)		7.84 (1H, d, 9.0)		7.78 (1H, d, 9.0)		7.78 (1H, d, 8.9)	
PreSta3								
1		165.8		165.8		165.8		165.8
2	3.45 (1H, d, 16.0)	46.9	3.45 (1H, d, 16.0)	46.9	3.45 (1H, d, 15.9)	46.9	3.45 (1H, d, 15.9)	46.9
	3.38 (1H, d, 16.0)		3.38 (1H, d, 16.0)		3.38 (1H, d, 15.9)		3.38 (1H, d, 15.9)	
3		204.9		204.9		204.9		204.9
4	4.30 (1H, m)	56.6	4.30 (1H, m)	56.6	4.30 (1H, m)	56.6	4.30 (1H, m)	56.6
5	1.45 (2H, m)	37.8	1.45 (2H, m)	37.8	1.45 (2H, m)	37.8	1.45 (2H, m)	37.8
6	1.59 (1H, m)	24.1	1.58 (1H, m)	24.1	1.59 (1H, m)	24.1	1.59 (1H, m)	24.1
7	0.86 (3H, m $^{\circ}$)	23.2	0.86 (3H, m $^{\circ}$)	23.2	0.86 (3H, m $^{\circ}$)	23.2	0.86 (3H, m $^{\circ}$)	23.2

8	0.80 (3H, m ^c)	20.9	0.80 (3H, m ^c)	20.9	0.80 (3H, m ^c)	20.9	0.80 (3H, m ^c)	20.9
NH	8.31 (1H, d, 7.7)		8.31 (1H, d, 7.7)		8.30 (1H, d, 7.8)		8.30 (1H, d, 7.8)	
Ala4								
1		172.4		172.4		172.4		172.4
2	4.28 (1H, m)	48.3	4.28 (1H, m)	48.3	4.28 (1H, m)	48.3	4.28 (1H, m)	48.3
3	1.22 (3H, d, 7.1)	18.1	1.22 (3H, d, 7.1)	18.1	1.22 (3H, d, 7.1)	18.1	1.22 (3H, d, 7.1)	18.0
NH	8.34 (1H, d, 7.3)		8.34 (1H, d, 7.3)		8.34 (1H, d, 7.4)		8.35 (1H, d, 7.3)	
DecPreSta5								
1	2.02 (3H, s)	26.1	2.02 (3H, s)	26.1	2.02 (3H, s)	26.1	2.02 (3H, s)	26.1
2		208.1		208.1		208.1		208.0
3	4.14 (1H, m)	57.1	4.15 (1H, m)	57.0	4.14 (1H, m)	57.0	4.15 (1H, m)	57.0
4	1.45 (2H, m)	38.1	1.45 (2H, m)	38.1	1.45 (2H, m)	38.1	1.45 (2H, m)	38.1
5	1.60 (1H, m)	24.2	1.61 (1H, m)	24.2	1.60 (1H, m)	24.2	1.60 (1H, m)	24.2
6	0.88 (3H, m ^c)	23.1	0.88 (3H, m ^c)	23.1	0.88 (3H, m ^c)	23.1	0.88 (3H, m ^c)	23.1
7	0.83 (3H, m ^c)	21.2	0.83 (3H, m ^c)	21.2	0.83 (3H, m ^c)	21.2	0.83 (3H, m ^c)	21.2
NH	8.20 (1H, d, 7.6)		8.20 (1H, d, 7.6)		8.20 (1H, d, 7.6)		8.21 (1H, d, 7.6)	
FA								
1		169.3		173.1		176.3		175.6
2	1.85 (3H, s)	22.5	2.15 (2H, q, 7.5)	28.3	2.52 (1H, m)	33.6	2.32 (1H, m)	40.9
3			0.97 (3H, t, 7.6)	10.1	0.99 (3H, d, 6.8)	19.9	1.46 (1H, m) 1.25 (1H, m)	26.9
4					0.95 (3H, d, 6.8)	19.4	0.76 (3H, t, 7.4)	11.7
5							0.96 (3H, d, 6.8)	18.0

^cJ-value not determined due to overlapped signals

Supplementary Table 10. NMR spectroscopic data of compounds **13**, **13-*d*₁**, **17** and **17-*d*₂** (¹H: 700 MHz, ¹³C: 175 MHz, DMSO-*d*₆).

No.	13		13-<i>d</i>₁	17		17-<i>d</i>₂
	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)
Val1						
1		170.9			170.8	
2	4.17 (1H, dd, 7.2, 8.8)	57.7	4.17 (1H, m)	4.18 (1H, dd, 7.1, 8.6)	57.6	4.18 (1H, dd, 7.0, 8.7)
3	1.92 (1H, m)	29.9	1.92 (1H, m)	1.92 (1H, m)	30.0	1.92 (1H, m)
4	0.83 (3H, m ^c)	18.0	0.83 (3H, m ^c)	0.82 (3H, m ^c)	17.9	0.82 (3H, m ^c)
5	0.83 (3H, m ^c)	18.9	0.83 (3H, m ^c)	0.82 (3H, m ^c)	18.9	0.82 (3H, m ^c)
NH	7.93 (1H, d, 8.7)		7.91 (1H, d, 8.7)	7.90 (1H, br d, 7.8)		7.90 (1H, d, 7.6)
Val2						
1		170.5			170.3	
2	4.14 (1H, m)	57.8	4.14 (1H, m)	4.11 (1H, dd, 7.6, 8.9)	57.8	4.11 (1H, dd, 7.5, 9.0)
3	1.97 (1H, m)	29.9	1.97 (1H, m)	1.95 (1H, m)	30.0	1.95 (1H, m)
4	0.82 (3H, m ^c)	17.9	0.82 (3H, m ^c)	0.84 (3H, m ^c)	18.0	0.84 (3H, m ^c)
5	0.84 (3H, m ^c)	19.1	0.84 (3H, m ^c)	0.84 (3H, m ^c)	19.0	0.84 (3H, m ^c)
NH	7.85 (1H, d, 9.0)		7.83 (1H, d, 9.1)	7.84 (1H, d, 8.9)		7.83 (1H, d, 9.0)
Sta3						
1		170.5			170.4	
2	2.14 (2H, m)	39.0	2.13 (2H, br s)	2.11 (2H, m)	39.0	2.10 (1H, d, 14.5) 2.12 (1H, d, 14.5)
3	3.84 (1H, m)	68.7		3.83 (1H, m)	68.7	
4	3.84 (1H, m)	50.1	3.83 (1H, dt, 4.3, 9.8)	3.81 (1H, m)	50.4	3.81 (1H, m)
5	1.23 (1H, m)	38.8	1.24 (1H, m)	1.24 (1H, m)	38.5	1.24 (1H, m)
	1.38 (1H, m)		1.37 (1H, m)	1.35 (1H, m)		1.35 (1H, m)
6	1.54 (1H, m)	23.9	1.54 (1H, m)	1.54 (1H, m)	24.0	1.54 (1H, m)
7	0.78 (3H, d, 6.5)	21.4	0.78 (3H, d, 6.5)	0.79 (3H, d, 6.5)	21.4	0.79 (3H, d, 6.4)
8	0.85 (3H, m ^c)	23.2	0.85 (3H, m ^c)	0.85 (3H, m ^c)	23.2	0.85 (3H, m ^c)
NH	7.41 (1H, d, 9.0)		7.39 (1H, d, 9.2)	7.46 (1H, d, 8.8)		7.46 (1H, d, 9.0)
3-OH	4.82 (1H, br s)		4.78 (1H, br s)	4.83 (1H, d, 4.8)		4.81 (1H, br s)
Ala4						
1		172.4			171.8	
2	4.23 (1H, dq, 7.1)	48.1	4.23 (1H, dq, 7.1)	4.23 (1H, dq, 7.1)	48.1	4.23 (1H, dq, 7.0)
3	1.21 (3H, d, 7.1)	17.7	1.21 (3H, d, 7.1)	1.18 (3H, d, 7.1)	18.2	1.18 (3H, d, 7.0)
NH	8.00 (1H, d, 7.1)		7.98 (1H, d, 7.2)	7.90 (1H, br d, 7.8)		7.90 (1H, d, 7.6)
Dec(Pre)Sta5						

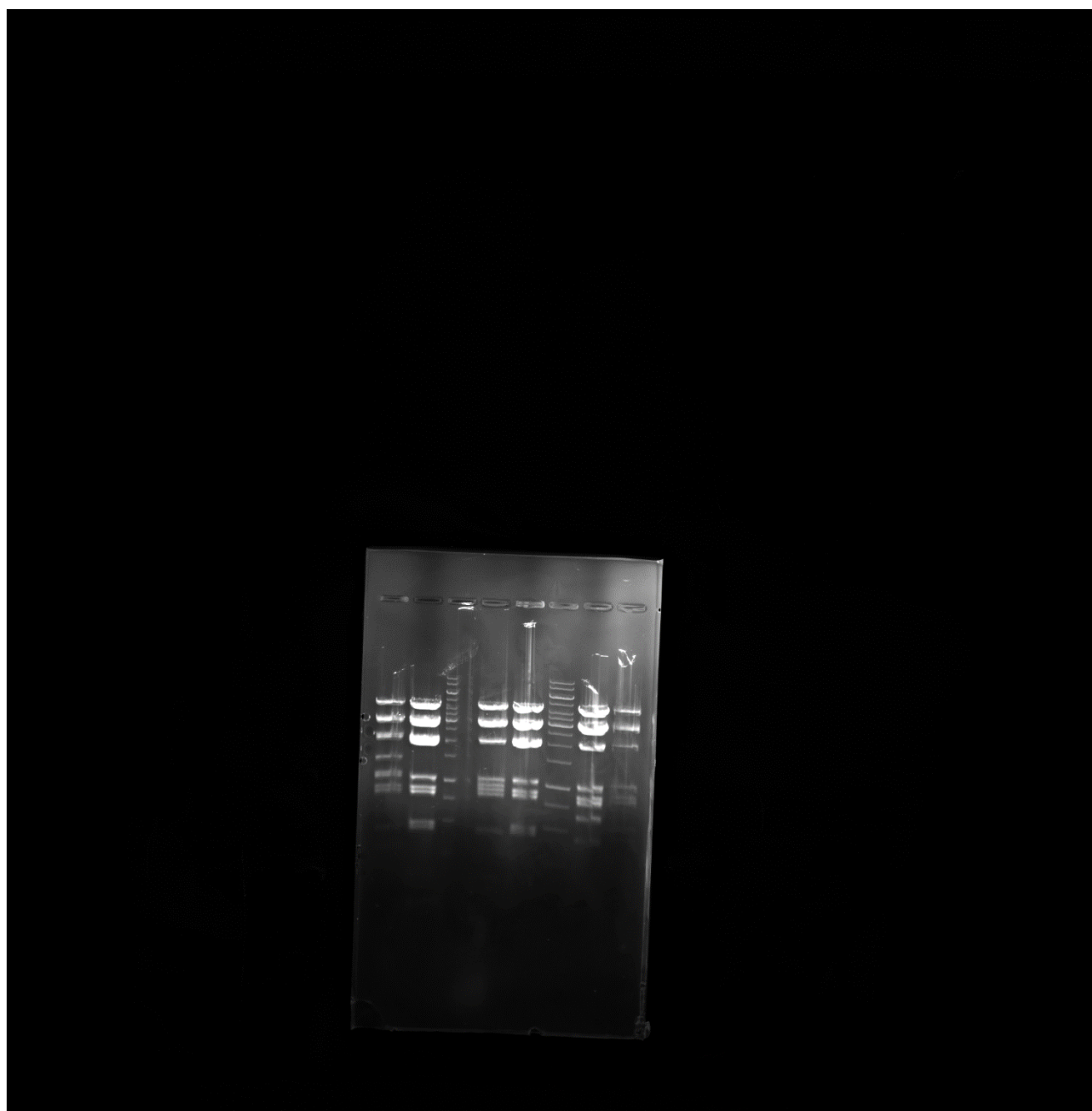
1	2.02 (3H, s)	26.0	2.02 (3H, s)	0.92 (3H, d, 6.3)	19.1	0.91 (3H, s)
2		207.9		3.56 (1H, m)	67.2	
3	4.14 (1H, m)	56.7	4.14 (1H, m)	3.70 (1H, m)	51.1	3.70 (1H, m)
4	1.47 (2H, m)	37.7	1.46 (2H, m)	1.21 (1H, m)	38.7	1.21 (1H, m)
				1.37 (1H, m)		1.37 (1H, m)
5	1.59 (1H, m)	23.9	1.59 (1H, m)	1.53 (1H, m)	24.0	1.53 (1H, m)
6	0.82 (3H, m ^c)	20.9	0.82 (3H, m ^c)	0.81 (3H, m ^c)	21.6	0.81 (3H, m ^c)
7	0.88 (3H, d, 6.7)	22.8	0.88 (3H, d, 6.7)	0.85 (3H, m ^c)	23.2	0.85 (3H, m ^c)
NH	8.16 (1H, d, 7.8)		8.15 (1H, d, 7.8)	7.27 (1H, d, 9.2)		7.27 (1H, d, 9.3)
2-OH				4.52 (1H, d, 4.7)		4.49 (1H, br s)
FA						
1		169.0			169.0	
2	1.86 (3H, s)	22.2	1.86 (3H, s)	1.86 (3H, s)	22.3	1.86 (3H, s)

^cJ-value not determined due to overlapped signals

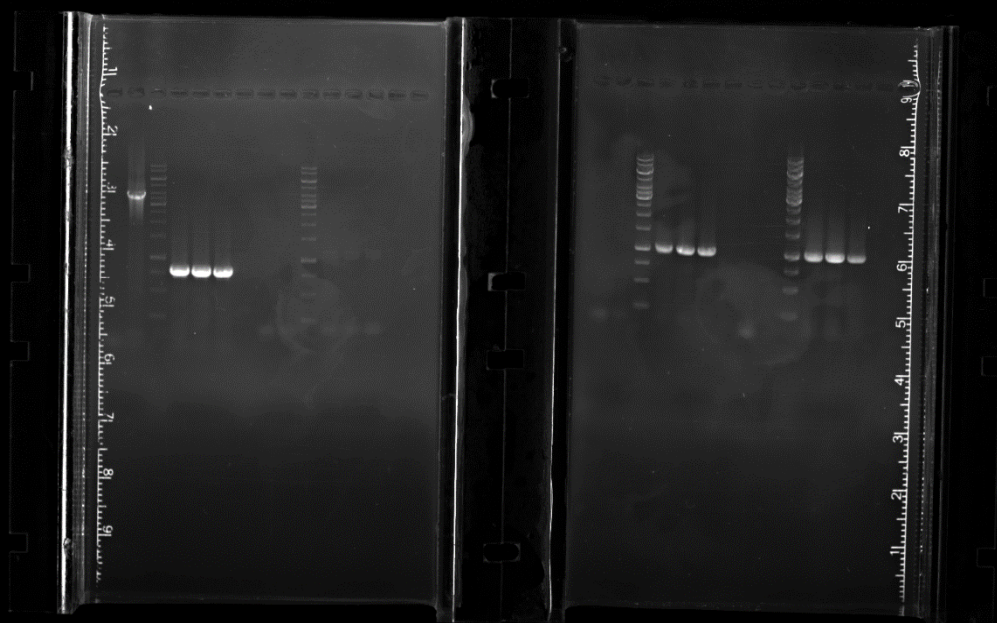
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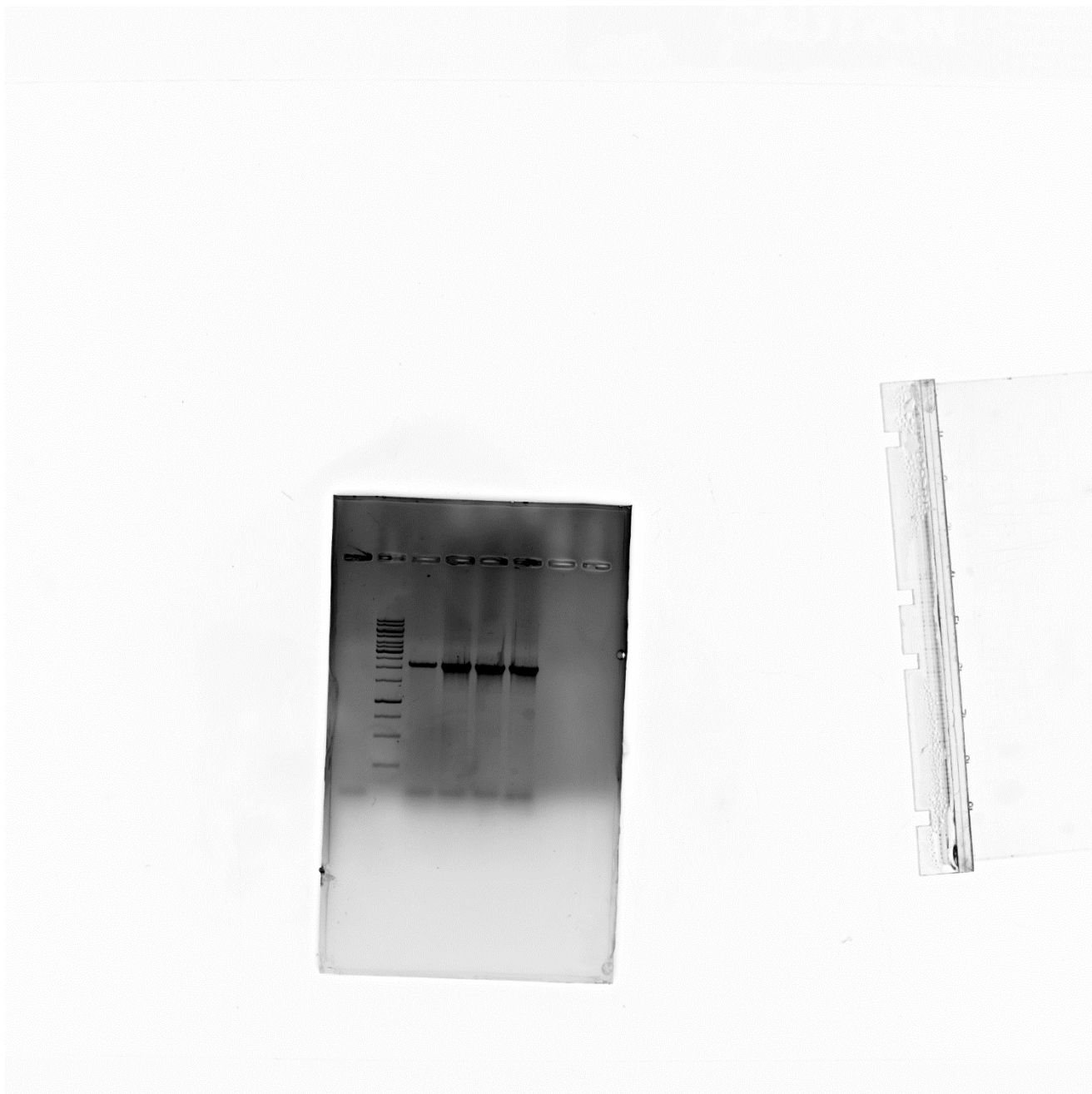
Source data of **Supplementary Fig. 3-b**. Plasmid pQS9- $\Delta pepD$ verification.



Source data of **Supplementary Fig. 3d**. Mutant *S. catenulae* - Δ *pepD* verification and **Supplementary Fig. 6b**. PCR verification of *pepJ* gene activation mutant.



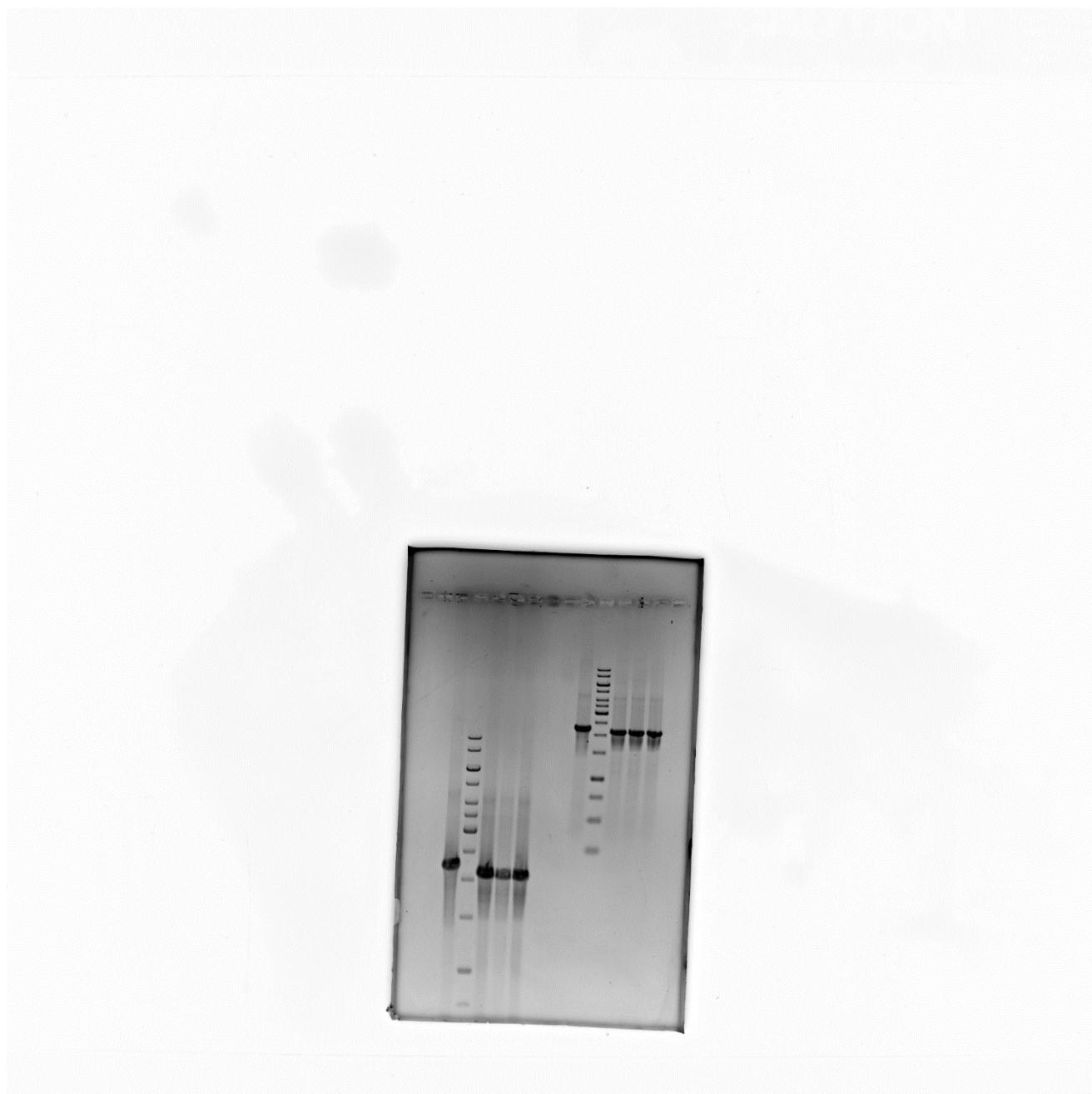
Source data of **Supplementary Fig. 4b**. Plasmid p15A-int-pep verification



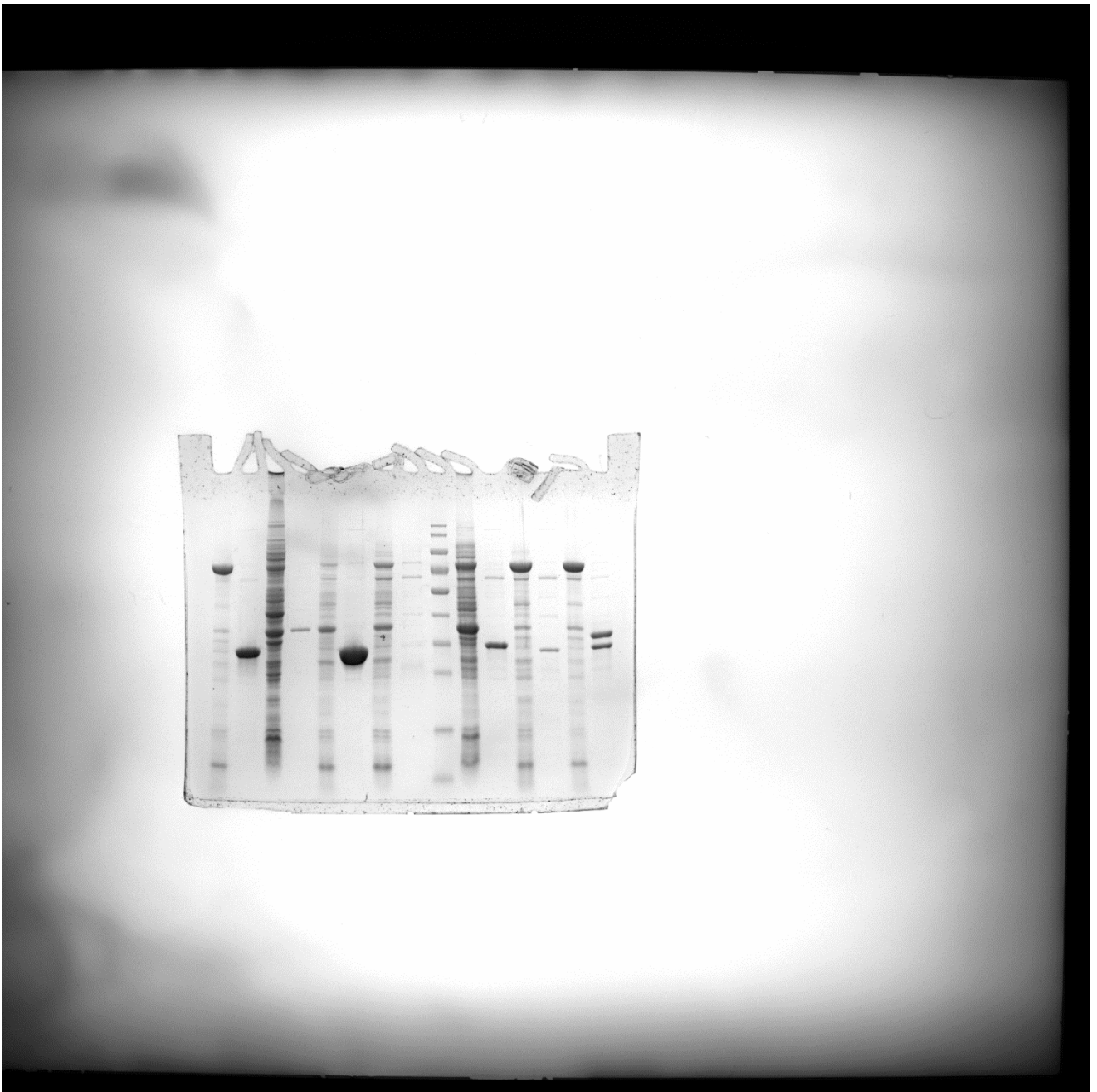
Source data of **Supplementary Fig. 4d**. Mutant *S. albus* Del14-pep PCR verification



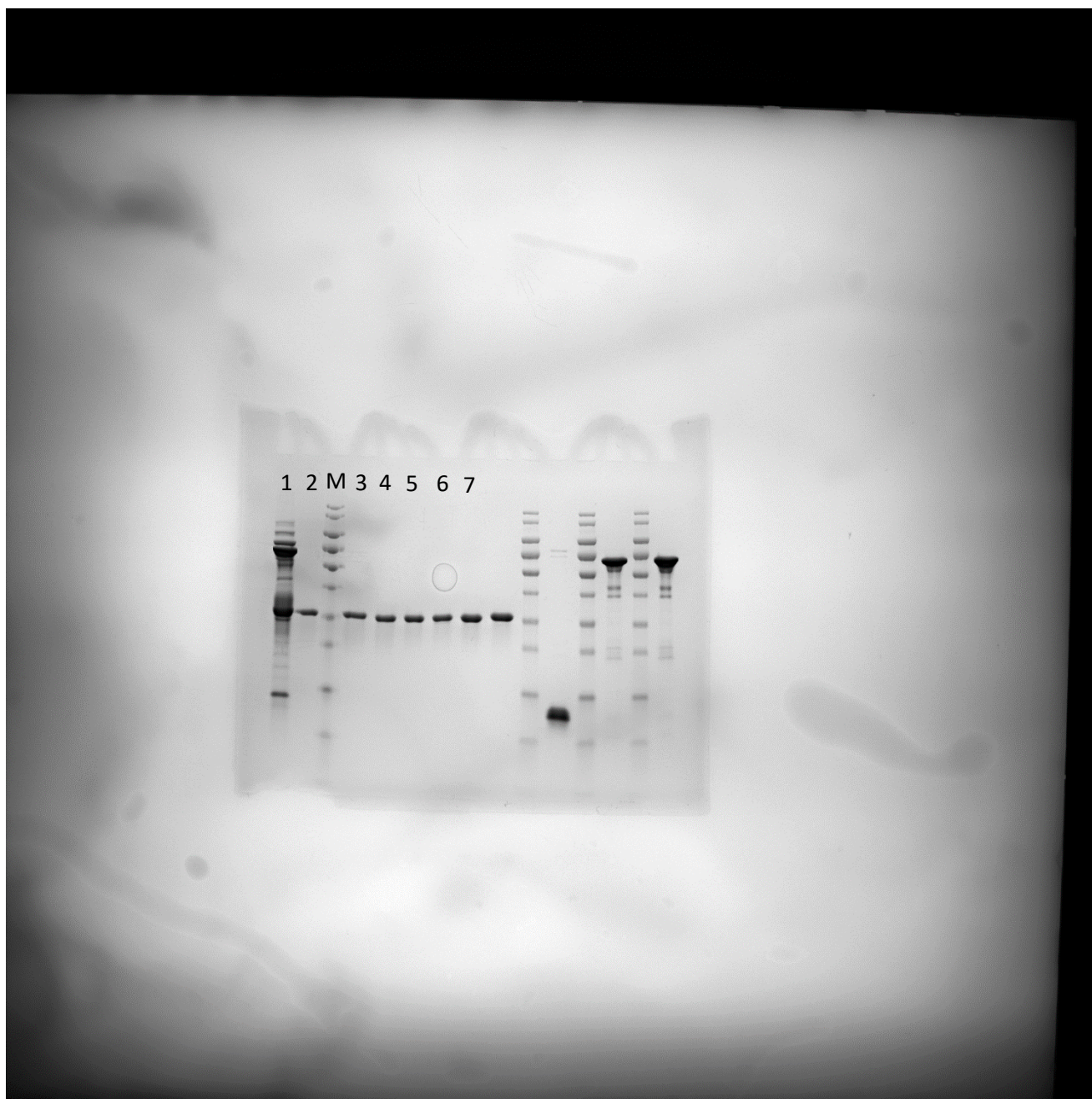
Source data of **Supplementary Fig. 5b**. PCR verification of *pepJ* gene deletion mutant.



Source data of **Supplementary Fig. 8b**. PCR verification of *S. albus* Del14-*pep*- Δ *pepI*.



Source data of **Supplementary Fig. 11b**. SDS-PAGE analysis of PepI and PepI homologues.



Source data of **Supplementary Fig. 17**. SDS-PAGE analysis of PepI, kvPepI and kvPepI mutants.