

Identification of the Biosynthetic Pathway for the Antibiotic Bicyclomycin

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Experimental procedures:

Chemicals were purchased from commercial suppliers and used without further purification. L-leucine-ethyl ester HCl, gluten meal, and potato starch were purchased from Alfa Aesar. Methanol, dichloromethane, acetone, HEPES, KH_2PO_4 , MgSO_4 , hexanes, and ethyl acetate, and $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, Luria Broth, and terrific broth were purchased from Fisher Scientific. Piperidine, DIPEA, α -ketoglutarate, sodium ascorbate, L-aminolevulinic acid, and Amberlite XAD16N were purchased from Sigma-Aldrich. Fmoc-L-isoleucine was purchased from Acros Organics. HATU was purchased from Oakwood Products Inc. NMR solvents were purchased from Cambridge Isotopes Laboratories. Agarose was purchased from Apex Bio. Isopropylthio-D-galactoside (IPTG) was purchased from Goldbio. Tryptic soy broth was purchased from Bacto Laboratories. Pharmamedia was purchased from Archer Daniels Midland. Normal-phase column chromatography was performed on silica gel (230–400 mesh). Reverse phase chromatography was performed on C18-bonded silica, on Phenomenex Kinetex C18, Phenomenex Luna C18 for prep HPLC, Phenomenex Kinetex C18 for semiprep HPLC, and Thermo VYDAC C-18. NMR spectra were recorded either on a Bruker 400 MHz or 600 MHz NMR spectrometer. NMR experiments are reported in δ units, parts per million (ppm), and were referenced to CDCl_3 (δ 7.26 ppm), DMSO (δ 2.50 ppm), MeOD (δ 3.31 ppm) or D_2O (δ 4.79 ppm) as internal standards. High-resolution mass spectra (HRMS) were obtained using an Agilent 6520 Q-TOF mass spectrometer. Specific rotation was measured on a JASCO DIP-1000.

Strain and culture conditions for bicyclomycin production

Streptomyces cinnamoneus (ATCC 21532) was purchased from ATCC (Manassas, VA). The strain was grown on an ISP2 plate for 3 days at 28 °C until sporulation. A single colony was inoculated in 10 mL of tryptic soy broth and the seed culture was grown at 28 °C at 200 rpm. After 3 days of growth, a sample of 1 mL seed culture was used to inoculate 100 mL of fermentation medium (potato starch, 20 g/L; Pharmamedia, 20 g/L; gluten meal, 10 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L; KH_2PO_4 , 10 g/L; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 7.2 g/L). The large culture was grown for 7 days at 28 °C at 200 rpm. A 10 mL aliquot was centrifuged at 4,000 rcf, and the supernatant was absorbed onto XAD16N and washed with water. Organic molecules bound to XAD16N were eluted with 50% acetone/water. The 50% acetone/water elution was dried by rotary evaporation and resuspended in 1 mL of water. A 5 μL sample was analyzed by liquid chromatography (LC) coupled electrospray ionization (ESI) high resolution mass spectrometry (HRMS). The following LC conditions were used: the sample was injected on a Kinetex C18 column (2.6 μm , 150 x 4.60 mm) and run using a isocratic gradient of 2% mobile phase B for 10 min followed by a gradient of 2% to 95% mobile phase B over 15 min (mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile + 0.1% formic acid). Mass spectra were acquired in ESI positive mode using the following parameters: gas temperature 300 °C, drying gas 10 L/min, nebulizer 45 lb/in², fragmentor 175 V, skimmer 65 V. Bicyclomycin was detected in the *S. cinnamoneus* extract by MS ($[\text{M} + \text{H} - \text{H}_2\text{O}]^+$ $\text{C}_{12}\text{H}_{17}\text{N}_2\text{O}_6$, m/z calc'd 285.1081, observed 285.1082).

Genome sequencing

Genomic DNA of *Streptomyces cinnamoneus* (ATCC 21532) was extracted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) and sequenced on a PacBio RS II instrument (UNC High Throughput Sequencing Facility). *De novo* assembly was performed using SMRT Portal and genome annotation was conducted using Prokka 1.10. Frameplot 4.0 was used to predict open reading frames. NCBI BLAST, MultiGeneBlast and HHpred were used to predict possible functions of biosynthetic enzymes encoded by the *bcm* cluster.

Identification of *bcm* cluster in *S. cinnamoneus*

Nucleotide BLAST was performed using 51 cyclodipeptide synthase (CDPS) genes against the draft genome of *S. cinnamoneus*. One ORF (*bcmA*) was identified that encodes for a protein sharing 26% sequence identity to the CDPS, AlbC. Frameplot 4.0 was used to identify ORFs adjacent to *bcmA*. Six oxidases were identified (five Fe(II)/ α -KG oxidases and one P450 oxidase) downstream of BcmG, and an additional gene (BcmH) was identified upstream that is homologous to transport proteins of the major facilitator superfamily.

Cloning of *bcmA*, *C*, *G*, and *H* in pLIC-His vectors

Each *bcm* gene was amplified by PCR using the Q5 high fidelity kit (New England Biolabs) using genomic DNA of *S. cinnamoneus* as template. The DNA fragments of the expected sizes were purified by gel extraction kit (Zymo Research) and annealed with T4 DNA ligase into treated pLIC-His vector as previously described.¹ Cloned genes were verified by DNA sequencing. Sequences of primers are listed in Table S7.

Bicyclomycin resistance of *E. coli* expressing *bcmH*

E. coli BL21 DE3 (Gold) was transformed with pLIC-His plasmid containing *bcmH* or empty vector pLIC-His. Bacterial cultures (5 mL) were inoculated with a sample of 50 μ L of an overnight seed culture and grown in LB medium supplemented with 50 μ g/mL ampicillin and 0.2 mM IPTG. After cultures were grown for 30 minutes at 37°C, varying amounts of bicyclomycin was added to final concentrations ranging from 3 to 800 μ g/mL. Cultures were grown at 37°C for 16 hours, and OD₆₀₀ were measured to indicate growth.

Expression and purification of BcmA, C, and G

E. coli BL21 DE3 (Gold) was transformed with pLIC-His plasmids containing *bcmA*, *C*, or *G*. Bacterial seed cultures were started from a single colony and grown in LB medium supplemented with 50 μ g/mL ampicillin. A sample of 2 mL seed culture was transferred to 1 L of LB medium supplemented with 50 μ g/mL ampicillin. Cultures were grown at 37 °C until the cell density reached an OD₆₀₀ of 0.4–0.6, when protein expression was induced with 0.1 mM IPTG. After growing at 16°C for 16 hours, bacterial cells were harvested at 6,000 rcf and lysed by sonication. The resulting cell debris was removed by centrifugation at 21,000 rcf. Supernatant was loaded on HisPur Ni-NTA resin (ThermoFisher) for affinity purification. The resin was first washed with wash buffer (25 mM HEPES, 30 mM imidazole, 200 mM NaCl, 5 mM β -mercapto ethanol (BME), pH 7.5). Proteins were eluted from the resin using elution buffer (25 mM HEPES, 300 mM imidazole, 200 mM NaCl, 5 mM BME, pH 7.5). Fractions containing desired

protein (visualized on an SDS-PAGE gel) were concentrated. The imidazole-containing buffer in the concentrated protein was removed using a desalting column (GE PD-10) and exchanged with storage buffer (25 mM HEPES, 10% glycerol, 200 mM NaCl, 5 mM BME, pH 7.5). The purity of each protein was determined by SDS-PAGE (Figure S5). Purified proteins were promptly flash frozen in sealed microcentrifuge tubes using liquid N₂.

Isolation of cyclo(L-Ile-L-Leu) (2) and cyclo(L-Val-L-Leu) from *E. coli* overexpressing *bcmA*

A 1 L culture of *E. coli* BL21 DE3 (Gold) harboring the pLIC-His-*bcmA* vector was inoculated with 2 mL of an overnight seed culture started from a single colony. The culture was supplemented with 50 µg/mL ampicillin and grown at 37 °C. When the cell density reached an OD₆₀₀ between 0.4 and 0.6 protein expression was induced with 0.1 mM IPTG. The culture was incubated at 16°C for 16 hours. Cells were centrifuged at 6,000 rcf, and the supernatant was extracted with dichloromethane (DCM, 3 × 250 mL). The organic extract was dried by rotary evaporation and purified by silica column chromatography using a stepwise gradient of 0 to 10 to 20% methanol/DCM. **2** and cyclo(L-Ile-L-Leu) both eluted in 10% methanol/DCM. Fractions containing **2** and cyclo(L-Val-L-Leu) precipitated after three days at room temperature. These fractions were combined, dried by rotary evaporation, and purified by reverse-phase HPLC using a VYDAC C18 column (5 µm, 250 x 4.60 mm) and a gradient of 5–95% acetonitrile/water and 0.1% TFA over 35 min. HPLC fractions containing **2** and cyclo(L-Val-L-Leu) were collected and analyzed by LC-HRMS. The sample was injected on a Kinetex Gemini C18 column (5 µm, 50 x 2.00 mm) and run using a gradient of 5% to 95% mobile phase B over 10 min (mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile + 0.1% formic acid). Purified molecules were further analyzed by NMR spectroscopy (Figures S9–S12).

Cyclo(L-Ile-L-Leu), **2**: ¹H NMR (400 MHz, DMSO-*d*₆) δ: 0.85 (t, *J*=5.8 Hz, 3H), 0.86 (d, *J*= 4.6 Hz, 3H), 0.88 (d, *J*= 6.7 Hz, 3H), 0.92 (d, *J*= 7.0 Hz, 3H), 1.17 (m, 1H), 1.44 (m, 2H), 1.62 (ddd, *J*= 13.8, 8.7, 5.4 Hz, 1H), 1.83 (m, 2H), 3.70 (m, 1H), 3.77 (m, 1H), 8.05 (s, 1H), 8.20 (s, 1H). HRMS (ESI) *m/z* [M + H]⁺ calc'd for C₁₂H₂₃N₂O₂ 227.1754, observed 227.1725. [α]_D²⁴: –45.1° (*c* = 0.1, MeOH). Compound **2** is poorly soluble in most solvents, limiting the accuracy of the specific rotation measurement. The [α]_D²⁴ (solubility 0.1 g/ 100 mL in methanol) is consistent with the literature value in the same solvent (–56.5°).²

Cyclo(L-Val-L-Leu): ¹H NMR (400 MHz, DMSO-*d*₆) δ: 0.85 (d, *J*= 4.8 Hz, 3H), 0.86 (d, *J*= 4.4 Hz, 3H), 0.88 (d, *J*= 6.6 Hz, 3H), 0.95, (t, *J*= 7.0 Hz, 3H), 1.44 (ddd, *J*= 13.8, 8.7, 5.4 Hz, 1H), 1.62 (ddd, *J*= 13.5, 8.8, 4.7 Hz, 1H), 1.84 (m, 1H), 2.11 (m, 1H), 3.62 (m, 1H), 3.77 (m, 1H), 8.08 (s, 1H), 8.21 (s, 1H). HRMS (ESI) *m/z* [M + H]⁺ calc'd for C₁₁H₂₁N₂O₂ 213.1598, observed 213.1586.

Reconstitution of BcmA activity *in vitro*

E. coli BL21 DE3 (Gold) cell lysate, supplemented with 1 mM L-Ile, 1 mM L-Leu, and 1 mM ATP, was incubated at 30 °C for 1 hour. For controls including RNase A, 0.05 ng RNase A was added prior to addition of BcmA. For samples in which fresh tRNA was added, 24 U RNasin was added, and samples were incubated for 5 minutes. A sample of 60 µg yeast tRNA (ThermoFisher) was then added, followed by 10 µM BcmA. Water was added to a final volume

of 100 μL for each reaction. Upon completion of each reaction, 1.5 mL DCM was used to extract organic compounds. DCM extracts were dried by rotary evaporation, resuspended in 100 μL water, diluted 1 to 10 in water, and analyzed by LC-HRMS. The samples were injected on a Kinetex Gemini C18 column (5 μm , 50 x 2.00 mm) and run using a gradient of 5% to 95% mobile phase B over 10 min (mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile + 0.1% formic acid). MS parameters are the same as described above.

Reconstitution of BcmC activity *in vitro*

A sample of 125 μM cyclo(L-Ile-L-Leu) (**2**) was incubated with 2.5 μM BcmC in the presence of 100 μM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, 4 mM α -ketoglutarate (α -KG), 1 mM sodium ascorbate, 5% v/v DMSO, and 50 mM potassium phosphate (pH 7.5) in a 300 μL assay. BcmC was added last to initiate the reaction. An aliquot of 50 μL was taken at each time point and quenched with an equal volume of acetonitrile. The quenched reaction was analyzed by LC-MS and by HPLC for UV analysis. For LC-MS, the samples were injected on a Kinetex Gemini C18 column (5 μm , 50 x 2.00 mm) and run using a gradient of 5% to 95% mobile phase B over 10 min (mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile + 0.1% formic acid). MS parameters are the same as described above. For HPLC, the samples were injected on a Kinetex Gemini C18 column (5 μm , 50 x 2.00 mm) and run using a gradient of 5–10% mobile phase B over 3 minutes, 10–40% mobile phase B over 4 minutes, and 40–95% mobile phase B over 4 minutes (mobile phase A: water; mobile phase B: acetonitrile).

Enzymatic synthesis and purification of BcmC product, **3**

A sample of 125 μM BcmC substrate, **2**, was mixed thoroughly by stirring in a round bottom flask with 4 mM α -KG, 1 mM sodium ascorbate, 100 μM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, and 5% v/v DMSO in 150 mL of 50 mM potassium phosphate buffer at pH 7.5. To this mixture, 2.5 μM BcmC was added to initiate the reaction. The reaction mixture was stirred slowly for eight hours. The reaction mixture was extracted with 150 mL dichloromethane three times. The organic extract was dried by rotary evaporation. The BcmC product, **3**, was purified by reverse-phase preparative HPLC without acid in the mobile phase. Column purification conditions were as follows: samples were injected on Phenomenex Luna C18 (10 μm , 250 x 21.20 mm) and run using a gradient of 5–95% mobile phase B over 30 minutes (mobile phase A: water; mobile phase B: acetonitrile). Acid is not included in the mobile phases to prevent the rearrangement of **3**. Purified **3** was characterized by 1D and 2D NMR and LC-HRMS (Figures S15-S19). Average yield is 72.9% (n = 6).

8-Hydroxy-cyclo(L-Ile-L-Leu), **3**: ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 0.85 (t, J = 7.4 Hz, 3H), 0.93 (d, J = 7.0 Hz, 3H), 1.16 (s, 3H), 1.19 (s, 3H), 1.21 (m, 1H), 1.40 (m, 1H), 1.58 (dd, J = 14.2, 9.6 Hz, 1H), 1.89 (m, 1H), 2.03 (dd, J = 14.2, 3.0 Hz, 1H), 3.80 (s, 1H), 4.11 (br d, J = 9.5 Hz, 1H), 7.52 (s, 1H), 8.11 (s, 1H). HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calc'd for $\text{C}_{12}\text{H}_{23}\text{N}_2\text{O}_3$ 243.1703, observed 243.1727.

Reconstitution of BcmG activity *in vitro*

A sample of 1 mM **3** was incubated with 5 μ M BcmG in the presence of 100 μ M $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, 4 mM α -KG, 1 mM sodium ascorbate, and 50 mM potassium phosphate (pH 7.5) in a 300 μ L assay. BcmG was added last to initiate the reaction. An aliquot of 50 μ L was taken at each time point and quenched by an equal volume of acetonitrile. The quenched reaction was analyzed by LC-HRMS. For data shown in Figure 4, the samples were injected on a Kinetex C18 column (2.6 μ m, 150 x 4.60 mm) and run using an isocratic gradient of 2% mobile phase B for 10 min followed by a gradient of 2% to 95% mobile phase B over 15 min (mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile + 0.1% formic acid). MS parameters are the same as described above. For Figure S8, the samples were injected on a Kinetex Gemini C18 column (5 μ m, 50 x 2.00 mm) and run using a gradient of 5% to 95% mobile phase B over 10 min (mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile + 0.1% formic acid). MS parameters are the same as described above.

Enzymatic synthesis and purification of BcmG product, **4**

A sample of 1 mM BcmG substrate, **3**, was mixed thoroughly by stirring in a round bottom flask with 0.8 mM α -KG, 1 mM sodium ascorbate, 100 μ M $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, in 15 mL of 50 mM potassium phosphate at pH 7.5. A lower amount of α -KG was used to maximize production of **4** and prevent further oxidation. To this mixture 5 μ M BcmG was added to initiate the reaction. The reaction mixture was stirred slowly for four hours. The reaction was quenched with 15 mL acetonitrile to precipitate the proteins, which were removed by centrifugation. The supernatant was dried by rotary evaporation, resuspended in 5 mL of water, and purified by reverse-phase preparative HPLC. Column purification conditions were as follows: samples were injected on Phenomenex Luna C18 (10 μ m, 250 x 21.20 mm) and run using a gradient of 5–40% mobile phase B over 25 minutes; mobile phase A: water; mobile phase B: acetonitrile. Acid is not included in the mobile phases to prevent the rearrangement of **4**. Purified **4** was characterized by 1D and 2D NMR (Figures S20-S24). Average yield is 41.6% (n = 6).

8,9-Dihydroxy-cyclo(L-Ile-L-Leu), **4**: ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ : 0.85 (t, J = 7.4 Hz, 3H), 0.93 (d, J = 7.1 Hz, 3H), 1.08 (s, 3H), 1.21 (m, 1H), 1.40 (m, 1H), 1.58 (dd, J = 14.3, 9.6 Hz, 1H), 1.90 (m, 1H), 2.06 (dd, J = 14.3, 2.7 Hz, 1H), 3.19 (dd, J = 10.7, 5.9 Hz, 1H), 3.23 (dd, J = 10.6, 5.7 Hz, 1H), 3.81 (s, 1H), 4.12 (d, 9.5 Hz, 1H), 4.85 (s, 1H), 4.90 (t, J = 5.7 Hz, 1H), 7.49 (s, 1H), 8.10 (s, 1H).

^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ : 169.54, 167.14, 72.47, 70.16, 58.75, 51.56, 41.24, 37.79, 24.53, 24.04, 15.41, 12.48.

HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calc'd for $\text{C}_{12}\text{H}_{23}\text{N}_2\text{O}_4$ 259.1652, observed 259.1659.

Enzymatic synthesis and purification of BcmG overoxidation products, **5a** and **5b**

A sample of 1 mM BcmG substrate, **3**, was mixed thoroughly by stirring in a round bottom flask with 4 mM α -KG, 1 mM sodium ascorbate, 100 μ M $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, in 15 mL of 50 mM potassium phosphate, pH 7.5. An excess of α -KG was used to maximize production of **5a** and **5b**. To this mixture 5 μ M BcmG was added to initiate the reaction. The reaction mixture was stirred slowly for four hours. The reaction was quenched with 15 mL acetonitrile, dried by rotary

evaporation, resuspended in 5 mL of water, centrifuged to remove precipitated protein, and purified by reverse-phase preparative HPLC. Column purification conditions were as follows: samples were injected on Phenomenex Luna C18 (10 μ m, 250 x 21.20 mm) and run using a gradient of 5–40% mobile phase B over 25 minutes (mobile phase A: water; mobile phase B: acetonitrile). Acid is not included in the mobile phases to prevent the rearrangement of **5a** and **5b**. Purified **5a** and **5b** were characterized by 1D and 2D NMR (Figures S25–S37). Average combined yield of both diastereomers is 54% (n = 2).

8,9-Dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer 1, **5a**: ^1H NMR (600 MHz, DMSO- d_6) δ : 0.85 (t, J = 7.4 Hz, 3H), 1.01 (d, J = 7.1 Hz, 3H), 1.20 (s, 3H), 1.29 (m, 1H), 1.39 (m, 1H), 1.89 (m, 2H), 2.04 (m, 1H), 3.96 (s, 1H), 4.25 (dd, J = 10.3, 7.0, 1H), 4.87 (d, J = 6.4 Hz, 1H), 4.95 (s, 1H), 6.23 (d, J = 6.6 Hz, 1H), 7.95 (s, 1H).

^{13}C NMR (150 MHz, DMSO- d_6) δ : 172.05, 167.64, 85.76, 77.39, 59.63, 57.19, 37.87, 34.68, 24.17, 22.79, 15.45, 12.88.

^1H NMR (600 MHz, D $_2$ O) δ : 0.76 (t, J = 7.5 Hz, 3H), 0.93 (d, J = 7.2 Hz, 3H), 1.14 (m, 1H), 1.25 (m, 1H), 1.25 (s, 3H), 1.96 (dd, J = 13.3, 11.2 Hz, 1H), 2.07 (m, 1H), 2.09 (dd, J = 13.2, 6.4 Hz, 1H), 4.06 (t, J = 2.2 Hz, 1H), 4.37 (ddd, J = 11.2, 6.5, 1.9 Hz, 1H), 5.01 (s, 1H).

^{13}C NMR (150 MHz, D $_2$ O - d_2) δ : 173.30, 169.78, 85.60, 77.96, 59.87, 56.76, 36.77, 34.34, 23.61, 20.61, 14.71, 11.40.

HRMS (ESI) m/z [$M + H$] $^+$ calc'd for C $_{12}$ H $_{21}$ N $_2$ O $_4$ 257.1496, observed 257.1498.

8,9-Dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer 2, **5b**: ^1H NMR (600 MHz, D $_2$ O) δ : 0.75 (t, J = 7.4 Hz, 3H), 0.91 (d, J = 7.2, 3H), 1.11 (m, 1H), 1.17 (m, 1H), 1.18 (s, 3H), 2.03 (m, 1H), 2.03 (dd, J = 13.1, 8.6 Hz, 1H), 2.21 (dd, J = 13.2 7.5 Hz, 1H), 4.07 (t, J = 2.3 Hz, 1H), 4.50 (dt, J = 8.1, 1.2 Hz, 1H), 5.12 (s, 1H).

^{13}C NMR (150 MHz, D $_2$ O - d_2) δ : 171.90, 168.40, 84.74, 74.76, 59.64, 55.90, 38.66, 34.90, 23.65, 23.60, 14.58, 11.33.

HRMS (EI) m/z [$M + H$] $^+$ calc'd for C $_{12}$ H $_{21}$ N $_2$ O $_4$ 257.1496, observed 243.1507.

Synthesis of Fmoc-NH- L-Ile-L-Leu -OEt

Synthesis was adapted from previously reported procedure.³ To a round bottom flask on ice was added 20 mL DCM, 500 mg (1.42 mmol) Fmoc-NH- L-Ile-OH, 820.5 mg (2.12 mmol) HATU, and 492 μ L (2.83 mmol) di-isopropylethylamine. After 20 minutes, 277 mg (1.42 mmol) L-Leu-OEt was added. The reaction was stirred at room temperature for 16 hours. The solvent was rotary evaporated, and the crude reaction mixture was purified by silica flash column chromatography (0-40% hexanes/ethyl acetate). The product (450 mg, 64% yield) was obtained as a white powder.

^1H NMR (400 MHz, CDCl $_3$ - d_6) δ : 0.94 (m, 12H), 1.22 (m, 1H), 1.28 (t, 3H), 1.57 (m, 2H), 1.67 (m, 2H), 1.89 (m, 1H), 4.06 (t, J = 7.9 Hz, 1H), 4.21 (m, 2H), 4.25 (m, 1H), 4.42 (m, 2H), 4.62 (m, 1H), 5.42 (d, J = 8.9 Hz, 1H), 6.13 (d, J = 8.2 Hz, 1H), 7.33 (t, J = 7.4 Hz, 2H), 7.42 (t, J = 7.6 Hz, 2H), 7.61 (d, J = 7.5 Hz, 2H), 7.79 (d, J = 7.5 Hz, 2H). HRMS (ESI) m/z [$M + H$] $^+$ calc'd for C $_{29}$ H $_{39}$ N $_2$ O $_5$ 495.2853, observed 495.2840.

Synthesis of cyclo(L-Ile-L-Leu), **2**

To a round bottom flask was added 15 mL of DMF and 880 mg (1.77 mmol) Fmoc-NH-L-Ile-L-Leu-OEt and 1.05 mL (10.62 mmol) piperidine. The reaction was stirred at room temperature for 16 hours. Minimal water was added until all yellow color had disappeared and converted to a thick white powder. This precipitant was filtered and washed with minimal water, followed by washing with minimal 10% MeOH in DCM. The product **2** (43 mg, 11% yield) was obtained as a white powder.

^1H NMR (400 MHz, DMSO- d_6) δ : 0.85 (t, J = 5.6 Hz, 3H), 0.86 (d, J = 4.4 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 0.92 (d, J = 7.0, 3H), 1.17 (m, 1H), 1.43 (m, 2H), 1.63 (ddd, J = 13.4, 8.7, 4.6 Hz, 1H), 1.84 (m, 2H), 3.69 (m, 1H), 3.76 (m, 1H), 8.05 (s, 1H), 8.18 (s, 1H). HRMS (ESI) m/z [$M + H$] $^+$ calc'd for $C_{12}H_{23}N_2O_2$ 227.1754, observed 227.1766. $[\alpha]_D^{24}$: -26.8° (c = 0.1, MeOH)

Phylogenetic analysis of CDPSs

BcmA, the BcmA homologue from *Pseudomonas aeruginosa* ATCC 14886, and the sequences curated by Jacques et al⁴ were aligned with MUSCLE version 3.8.31.⁵ Following removal of identical sequences, a maximum likelihood tree was constructed using 100 iterations of rapid bootstrapping in RAxML version 8.2.4.⁶ The tree was visualized using Interactive Tree Of Life (iTOL) version 3.5.4.⁷

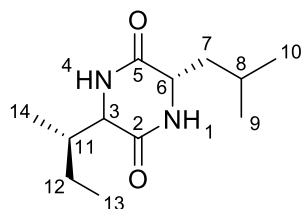
Cloning and overexpression of *bcm*-like cluster in *P. aeruginosa*

Pseudomonas aeruginosa ATCC 14886 genomic DNA was extracted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). This DNA was used as template for PCR to amplify the *bcm* cluster using primers Pbcm fw and Pbcm rv (Table S7). The expression vector, pPSV35, was digested using restriction enzymes BamHI and HindIII. A sample of 100 ng of pPSV35 and 200 ng of PCR product were incubated for 1 hour at 50 °C and assembled using the NEBuilder HiFi DNA assembler mix. The ligated vector was transformed into electrocompetent *E. coli* TOP10 cells. Cloning of the *bcm* cluster into pPSV35 was confirmed by DNA sequencing. Electrocompetent *P. aeruginosa* ATCC 14886 was prepared using the zero recovery time method as previously described,⁸ and transformed separately with two purified plasmids: one containing the *bcm* cluster as well as the empty pPSV35 vector. *P. aeruginosa* ATCC 14886 containing the pPSV35 plasmid were grown in parallel with the pPSV35-*bcm* plasmid in King's Broth (20 g/L Bacto Proteose Peptone #3 (BD Biosciences), 1.5 g/L dipotassium phosphate, 1.5 g/L magnesium sulfate, 1% (v/v) glycerol, pH 7.0) at 37 °C until OD₆₀₀ reached 0.4–0.5. At this point, IPTG was added to a final concentration of 0.5 mM and cultures were grown at 16 °C for 3 days. Cultures were centrifuged, and 20 mL of supernatant was mixed with XAD16N resin, which had been pre-equilibrated in three steps (30 minutes of soaking in (1) water, (2) methanol, and (3) water). After the supernatant and resin mixture was stirred for 1 hour at room temperature to remove hydrophobic molecules, the resin was filtered from the supernatant. The treated supernatant, left with polar molecules, was dried by rotary evaporation, and this dried sample was resuspended in 1 mL methanol. The methanol resuspended sample was centrifuged at 21,000 rcf and diluted 1:10 in water prior to LC-HRMS analysis. A 10 μ L sample was analyzed

by LC-HRMS. Separation was achieved with a Kinetex C18 column (2.6 μ m, 150 x 4.60 mm) in an isocratic gradient of 2% mobile phase B for 10 min followed by a gradient of 2% to 95% mobile phase B over 15 min (mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile + 0.1% formic acid). Mass spectra were acquired using parameters described above. Bicyclomycin was detected in the extract of *P. aeruginosa* containing pPSV35-*bcm*, $[M + H - H_2O]^+$ C₁₂H₁₇N₂O₆, m/z calc'd 285.1081, observed 285.1090 (Figure 4C). For fragmentation analysis, a species with m/z of 285.1081 was selected and fragmented using a collision energy of 25 V.

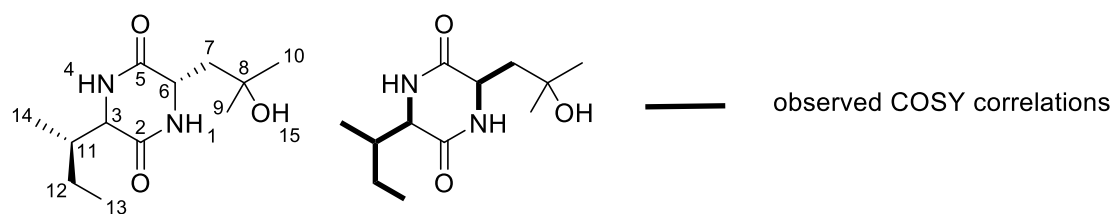
<i>bcm</i> gene	Size, aa	Proposed function	E-value	Superfamily accession
<i>bcmA</i>	242	tRNA-dependent cyclodipeptide synthase	7.25E-9	cl22834
<i>bcmB</i>	326	Fe(II)- and α -KG-dependent oxidase	1.97E-4	cl21496
<i>bcmC</i>	306	Fe(II)- and α -KG-dependent oxidase	8.25E-9	cl21496
<i>bcmD</i>	489	Cytochrome P450	1.71E-8	cl12078
<i>bcmE</i>	315	Fe(II)- and α -KG-dependent oxidase	1.26E-4	cl21496
<i>bcmF</i>	297	Fe(II)- and α -KG-dependent oxidase	2.04E-8	cl21496
<i>bcmG</i>	301	Fe(II)- and α -KG-dependent oxidase	1.12E-11	cl21496
<i>bcmH</i>	474	Major facilitator superfamily transporter	1.93E-26	cl21472

Table S1. Predicted functions of *bcm* gene functions by translated nucleotide-protein (blastx) local sequence alignment analysis.



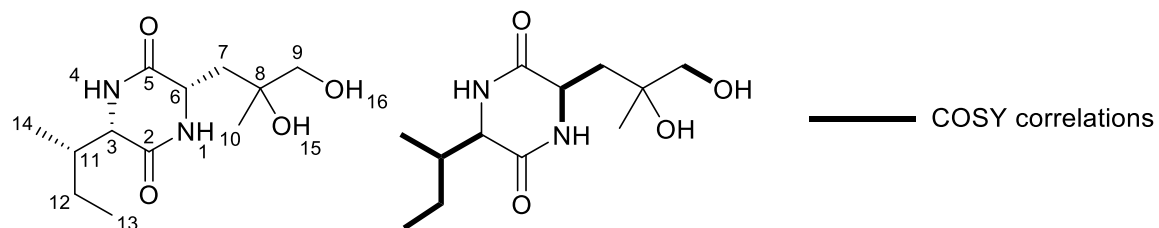
Position	<i>in vivo</i> 2		Synthetic 2	
	δ_H (ppm)	Mult. (<i>J</i> in Hz)	δ_H (ppm)	Mult. (<i>J</i> in Hz)
1	8.20	s	8.18	s
2				
3	3.70	m	3.69	m
4	8.05	s	8.05	s
5				
6	3.77	m	3.76	m
7a	1.62	ddd, (13.8, 8.7, 5.4)	1.63	ddd (13.4, 8.7, 4.6)
7b	1.44	m	1.43	m
8	1.83	m	1.84	m
9	0.88	d (6.7)	0.88	d (6.6)
10	0.86	d (4.6)	0.86	d (4.4)
11	1.83	m	1.84	m
12a	1.17	m	1.17	m
12b	1.44	m	1.43	m
13	0.85	t (5.8)	0.85	t (5.6)
14	0.92	d (7.0)	0.92	d (7.0)

Table S2. NMR shifts of **2** in DMSO-*d*₆ (400 MHz) isolated from *in vivo* expression of *bcmA* and synthesized **2**.



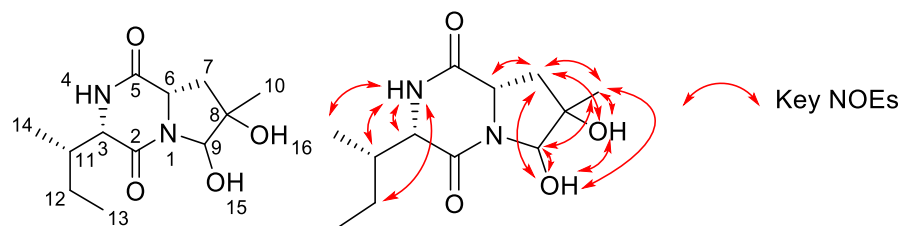
Position	δ_{H} (ppm)	Mult. (J in Hz)	δ_{C} (ppm)
1	7.52	s	
2			167.21
3	3.80	s	58.76
4	8.11	s	
5			169.46
6	4.11	br d (9.5)	52.09
7a	1.58	dd (14.2, 9.6)	45.46
7b	2.03	dd (14.2, 3.0)	
8			69.91
9	1.19	s	31.60
10	1.16	s	28.34
11	1.89	m	37.79
12a	1.21	m	24.57
12b	1.40	m	
13	0.85	t (7.4)	12.44
14	0.93	d (7.0)	15.42
15	Not observed		

Table S3. NMR shifts and 2D correlations of **3** in DMSO- d_6 (600 MHz for ^1H , 150 MHz for ^{13}C) generated from *in vitro* reconstitution of BcmC.



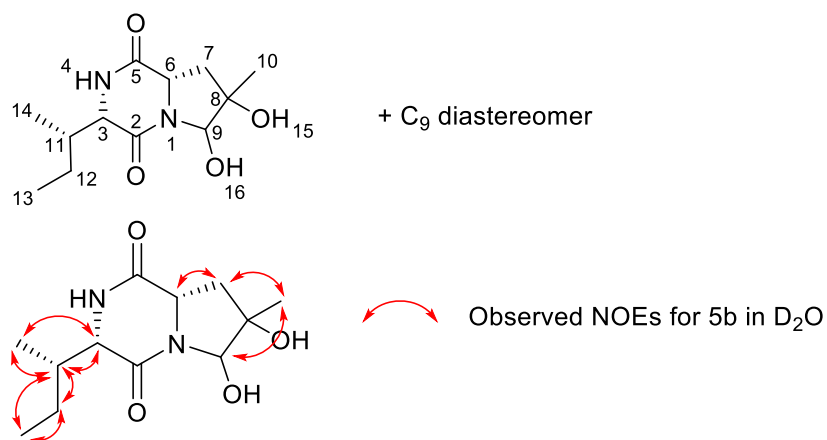
Position	δ_{H} (ppm)	Mult. (J in Hz)	δ_{C} (ppm)	HMBC
1	8.10	s		2,6
2			167.16	
3	3.82	s	58.75	2,12
4	7.49	s		3,5
5			169.54	
6	4.12	d (9.5)	51.56	5
7a	2.06	dd (14.3, 2.7)	42.24	6,8,10
7b	1.58	dd (14.3, 9.6)		
8			72.47	
9a	3.19	dd (10.7, 5.9)	70.16	7,8,10
9b	3.23	dd (10.6, 5.7)		
10	1.08	s	24.04	7,8,9
11	1.90	m	37.79	
12a	1.21	m	24.53	
12b	1.40	m		
13	0.85	t (7.4)	12.48	11,12
14	0.93	d (7.1)	15.41	3,11,12
15	4.85	s		
16	4.90	t (5.7)		

Table S4. NMR shifts and 2D correlations of 8,9-dihydroxy-cyclo(L-Ile-L-Leu) (**4**) in DMSO- d_6 (600 MHz for ^1H , 150 MHz for ^{13}C) generated from *in vitro* reconstitution of BcmG.



5a				
Position	δ_{H} (ppm)	Mult. (<i>J</i> in Hz)	δ_{C} (ppm)	HMBC
1				
2			167.64	
3	3.96	s	59.63	2,11,12,14
4	7.95	s		2,3,6
5			172.05	
6	4.25	dd (10.3, 7.0)	57.19	7
7	1.89	m	37.87	5,6,8,9
8			77.39	
9	4.87	d (6.4)	85.76	6,7,8
10	1.20	s	24.17	7,8,9
11	2.04	m	34.90	
12a	1.29	m	22.79	
12b	1.39	m		
13	0.85	t, (7.4)	12.88	11,12
14	1.01	d (7.1)	15.45	11,12
15	4.95	s		7,9
16	6.23	d (6.6)		8,9

Table S5. NMR shifts and 2D correlations of 8,9-dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer 1 (**5a**) in DMSO-*d*₆ (600 MHz for ¹H, 150 MHz for ¹³C) generated from *in vitro* reconstitution of BcmG.



Position	5a			5b		
	δ_{H} (ppm)	Mult. (J in Hz)	δ_{C} (ppm)	δ_{H} (ppm)	Mult. (J in Hz)	δ_{C} (ppm)
1						
2			173.30			171.90
3	4.06	t (2.2)	59.87	4.07	t (2.3)	59.64
4						
5			169.78			168.40
6	4.37	ddd (11.2, 6.5, 1.9)	56.76	4.50	dt (8.1, 1.2)	55.90
7a	1.96	dd (13.3, 11.2)	36.77	2.03	dd (13.1, 8.6)	38.66
7b	2.09	dd (13.2, 6.4)		2.21	dd (13.2, 7.5)	
8			77.96			74.76
9	5.01	s	85.60	5.12	s	84.74
10	1.25	s	20.61	1.18	s	23.65
11	2.07	m	34.34	2.03	m	34.9
12a	1.14	m	23.61	1.11	m	23.60
12b	1.25	m		1.17	m	
13	0.76	t (7.5)	11.40	0.75	t (7.4)	11.33
14	0.93	d (7.2)	14.71	0.91	d (7.2)	14.58

Table S6. NMR shifts of both diastereomers of 8,9-dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) (**5a** and **5b**) in D₂O (600 MHz for ¹H, 150 MHz for ¹³C) generated from *in vitro* reconstitution of BcmG.

Primer name	Sequence
bcmG fw	TACTTCCAATCCAATGCGATGAGCACGGCACAGGGATACG
bcmG rv	TTATCCACTTCCAATGCGCTATCAGTACAGGCCGGCGGTGTCC
bcmC fw	TACTTCCAATCCAATGCGGTGAGCACTGAGACGCTGCGCC
bcmC rv	TTATCCACTTCCAATGCGCTATCATGCCCTACCCCGTCTTCC
bcmA fw	TACTTCCAATCCAATGCGATGGAGCCTCGGGAAGAACC
bcmA rv	TTATCCACTTCCAATGCGCTATCATCGCCCTTTCAGGTGGA
bcmH fw	ACTTCCAATCCAATGCGGTGCTGAAGGGACCTCCTG
bcmH rv	TTATCCACTTCCAATGCGCTACTACCTCCGGCCCGTCCG
Pbcm fw	AATTCGAGCTCGGTACCCGGGGATCCATGAACCTGATAGCGGAAGAT TTCAGC
Pbcm rv	GTAAAACGACGGGCCAGTGCCAAGCTTCAATCGCGCAACGGCAG

Table S7. List of primers used in this study.

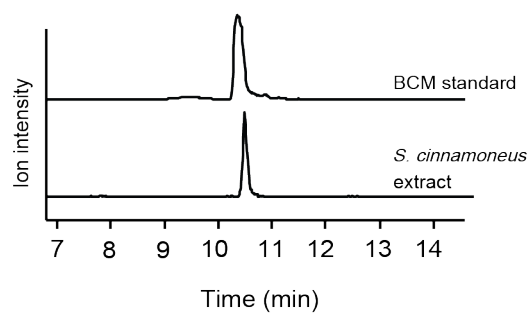


Figure S1. Bicyclomycin production from *S. cinnamoneus* ATCC 21532. EICs of bicyclomycin $[M + H - H_2O]^+$ detected in synthetic standard and in the extract of *S. cinnamoneus*.

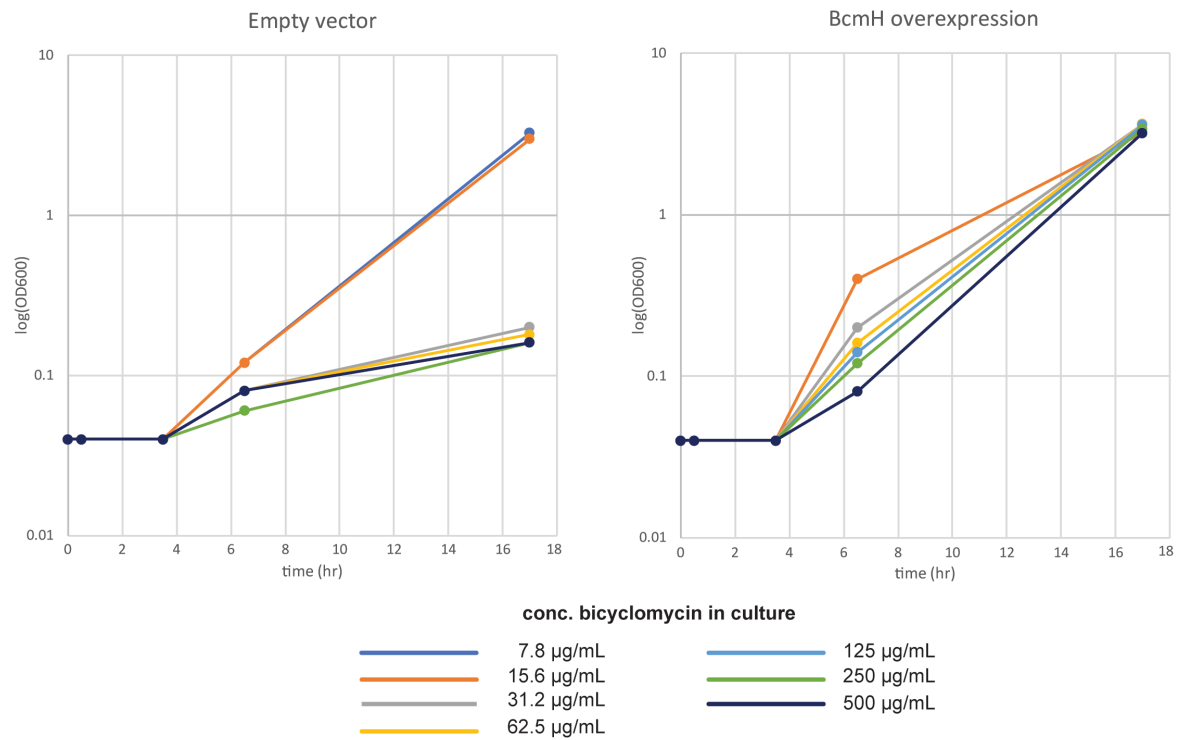


Figure S2. BcmH overexpression confers bicyclomycin resistance. Growth curves of *E. coli* in the presence of varying concentration of bicyclomycin were monitored at OD₆₀₀ and plotted against time. Growth curve of *E. coli* BL21 DE3 (Gold) containing empty vector is shown on the left and that of *E. coli* overexpressing BcmH is shown on the right. The experiment was repeated twice with similar results; representative data is shown.

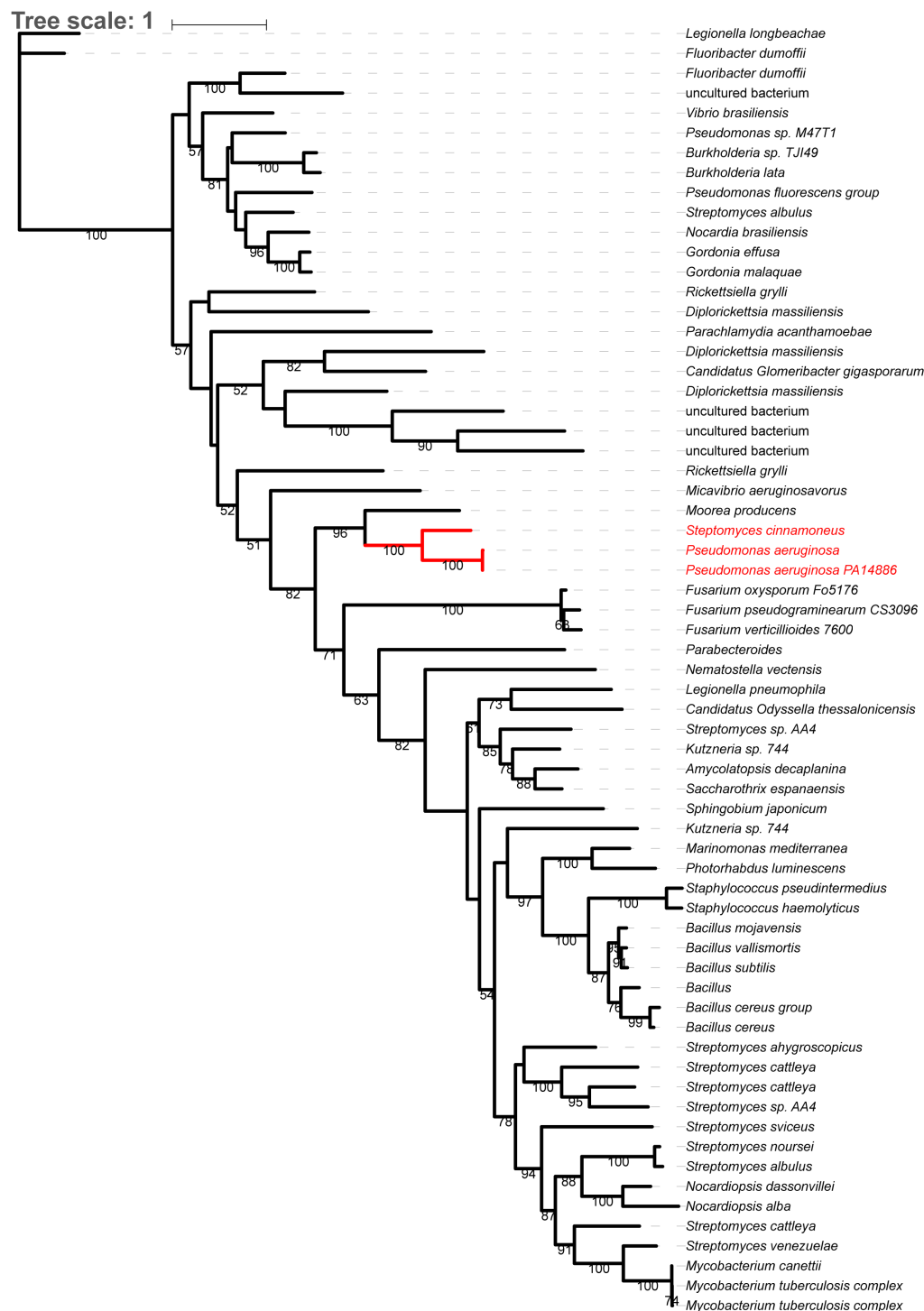


Figure S3. Phylogenetic tree of CDPSs. Maximum Likelihood Tree of amino acid sequences of BcmA and the curated CDPS proteins described by Jacques *et al.*⁴ In red is the clade containing BcmA from *S. cinnamoneus* and the homologues from *P. aeruginosa*.

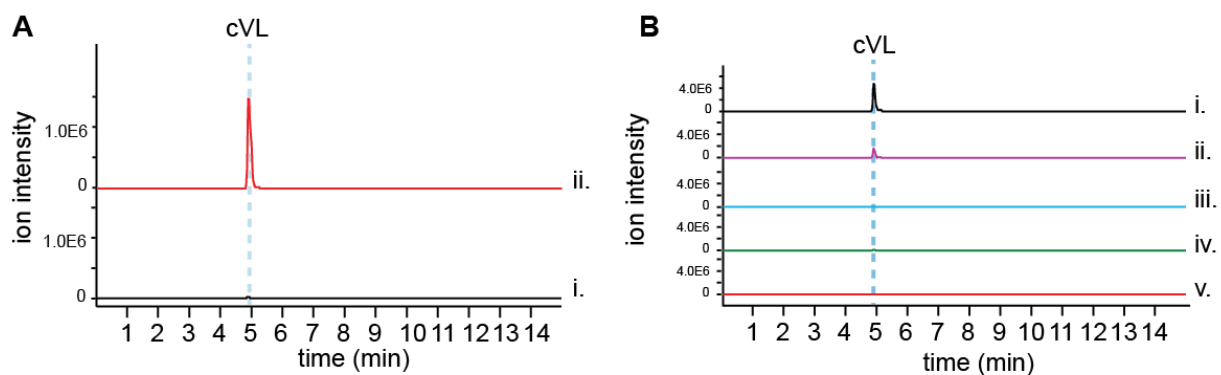


Figure S4. Cyclo(L-Val-L-Leu), cVL, is produced by *E. coli* expressing *bcmA*. (A) EICs of cVL (m/z 213.1598 [M+H]⁺) from the supernatant of *E. coli* harboring (i.) empty vector pLIC-His or (ii.) pLIC-His-*bcmA*. (B) EICs of cVL from *in vitro* BcmA reactions. *E. coli* cell lysate was (i) incubated with BcmA; (ii) incubated with RNase A first, then with RNasin, followed by the addition of yeast tRNA mix and BcmA. Negative controls include (iii) omission of BcmA, (iv) omission of RNasin, and (v) cell lysate alone.

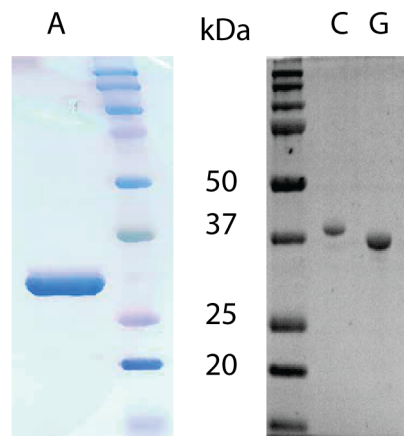


Figure S5. SDS-PAGE gels of purified BcmA, C, and G. All proteins contained *N*-terminal hexahistidine tags.

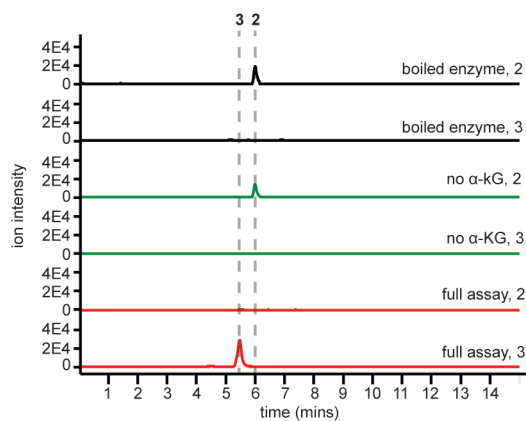


Figure S6. BcmC-catalyzed conversion of **2** to **3**. EICs of **2** (m/z , 227.2754 $[M + H]^+$) and **3** (m/z , 243.1713 $[M + H]^+$) were detected by LC-HRMS. Inclusion of boiled BcmC enzyme or exclusion of α -KG were used as controls. **3** was not produced in the absence of α -KG or in the presence of boiled BcmC.

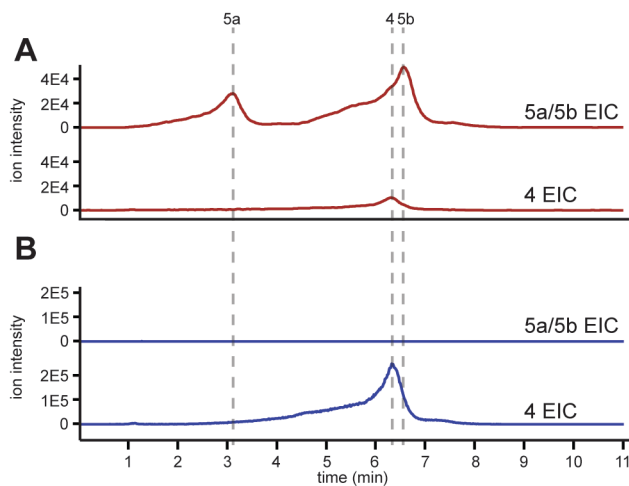


Figure S7. BcmG-catalyzed conversion of **4** to **5a** and **5b**. (A) Extracted ion chromatograms of **4** (m/z , 259.1652 $[M + H]^+$) and **5a** and **5b** (m/z , 257.1496 $[M + H]^+$) from reactions of **4** with BcmG. Incubation of **4** with BcmG converts **4** to **5a** and **5b**. (B) No conversion of **4** to **5a** or **5b** is observed when BcmG is not added.

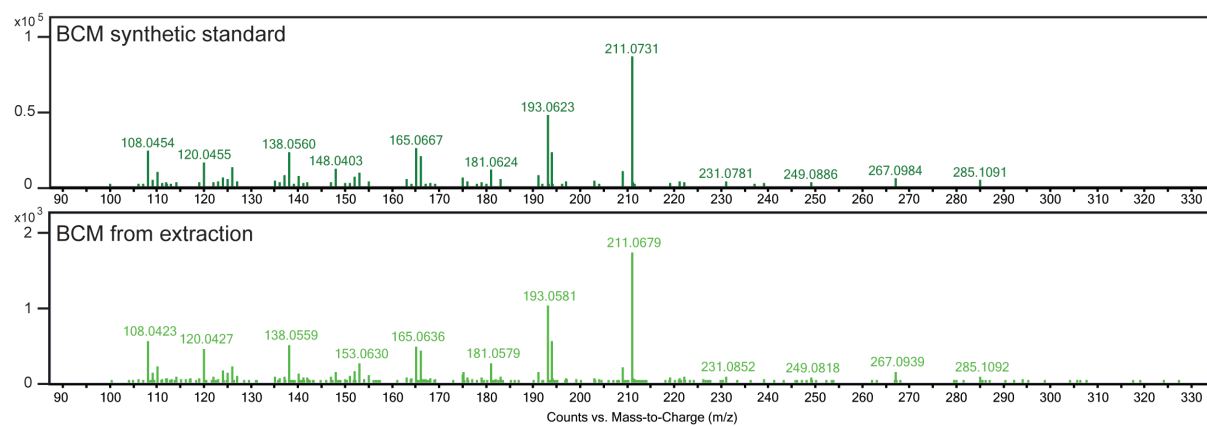


Figure S8. Tandem MS spectra of bicyclomycin overexpressed in *P. aeruginosa* in comparison with the bicyclomycin standard.

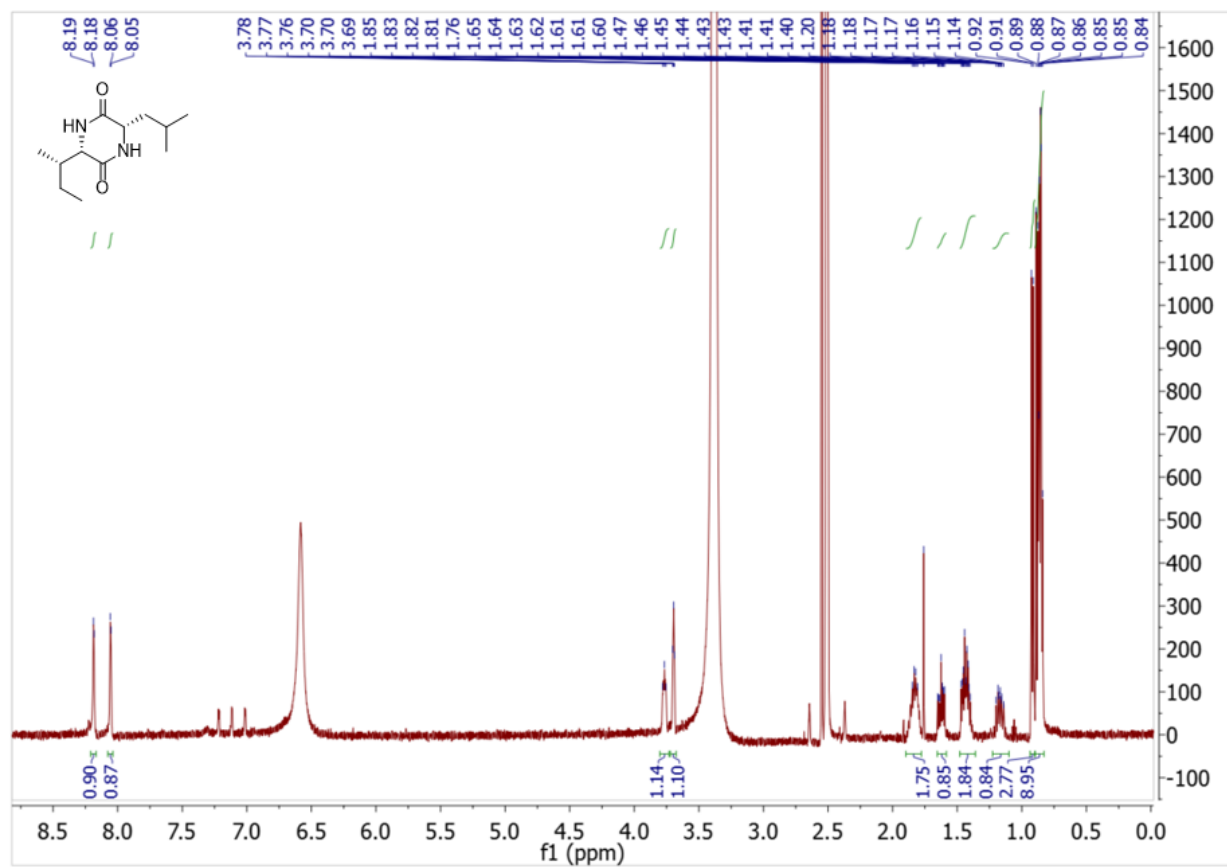


Figure S9. ^1H NMR spectrum of **2** in $\text{DMSO}-d_6$ (400 MHz) isolated from *E. coli* expressing *bcmA*.

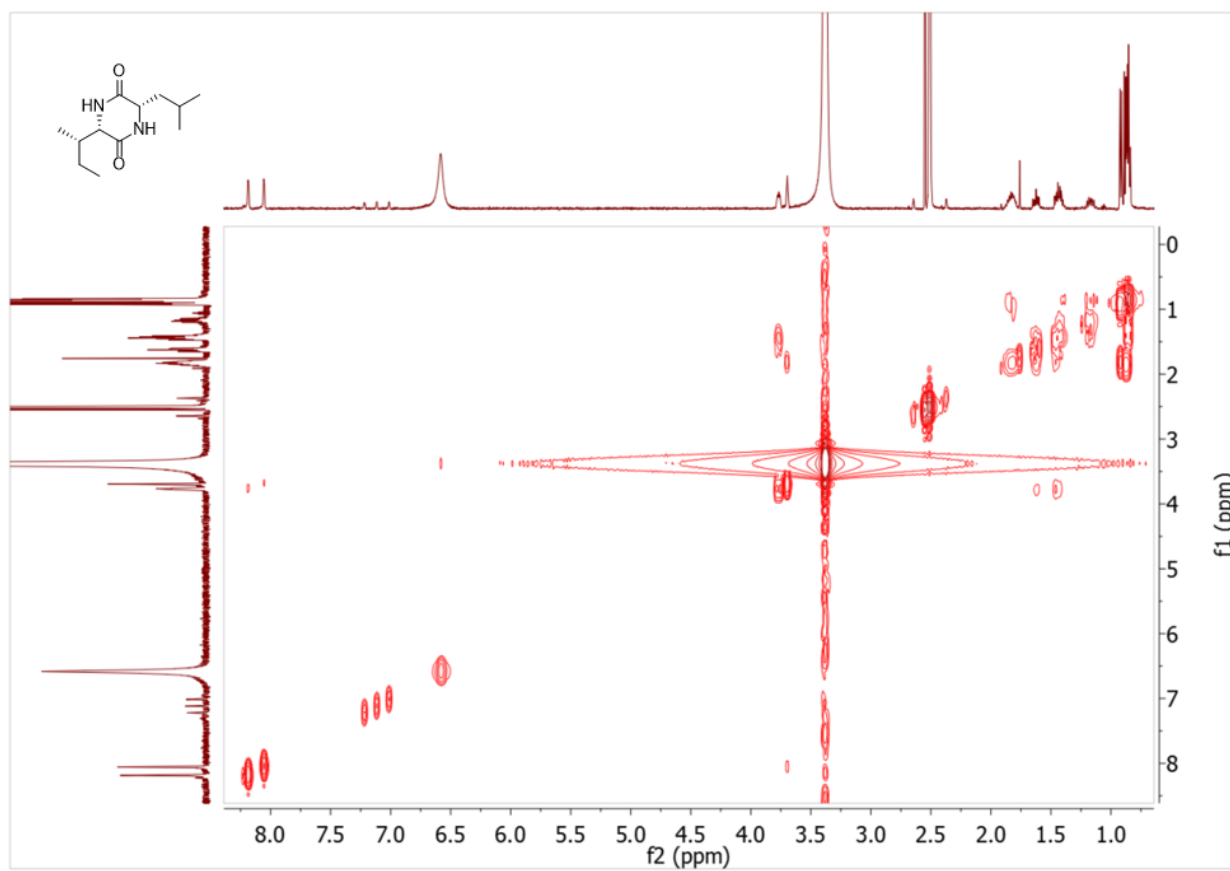


Figure S10. ^1H , ^1H -COSY NMR spectrum of **2** in $\text{DMSO-}d_6$ (400 MHz) isolated from *E. coli* expressing *bcmA*.

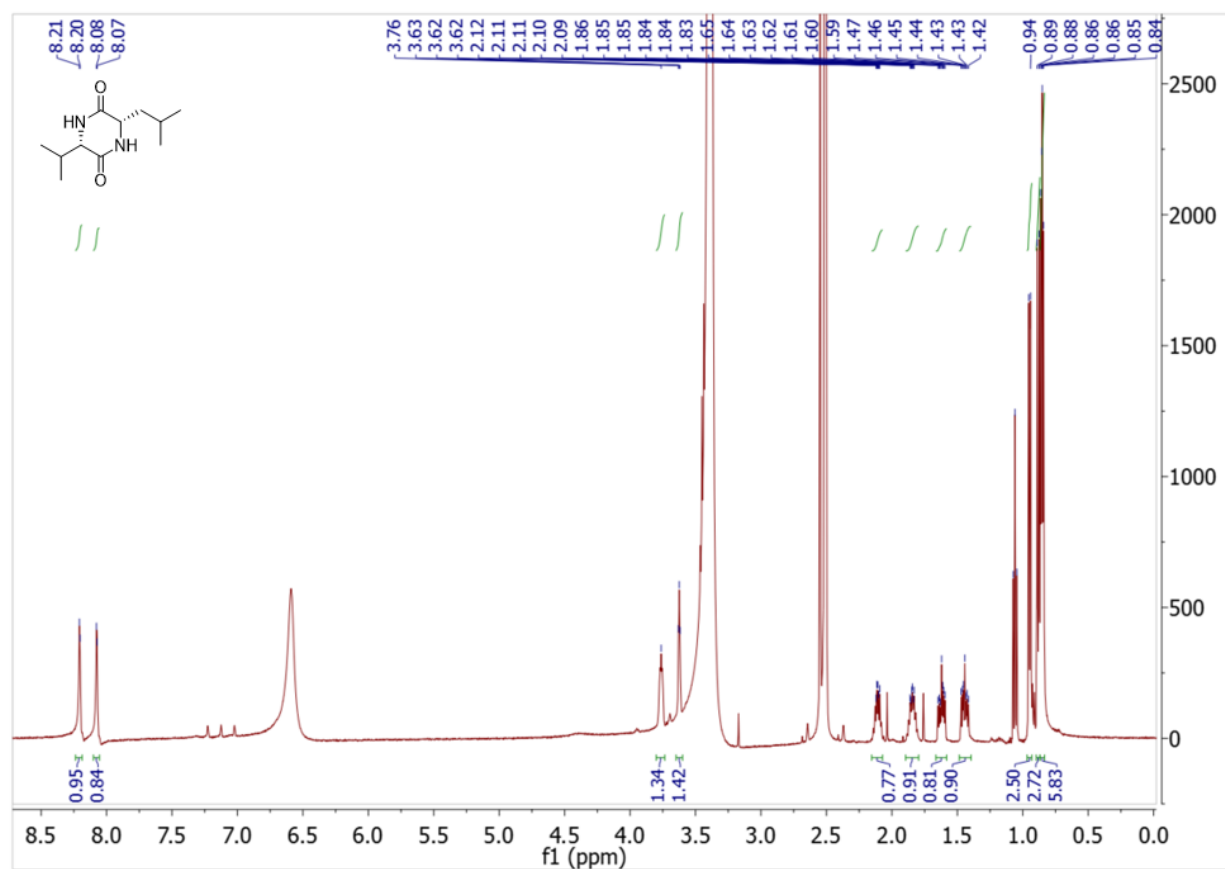


Figure S11. ^1H NMR spectrum of cyclo(L-Val-L-Leu) in $\text{DMSO-}d_6$ (400 MHz) isolated from *E. coli* expressing *bcmA*.

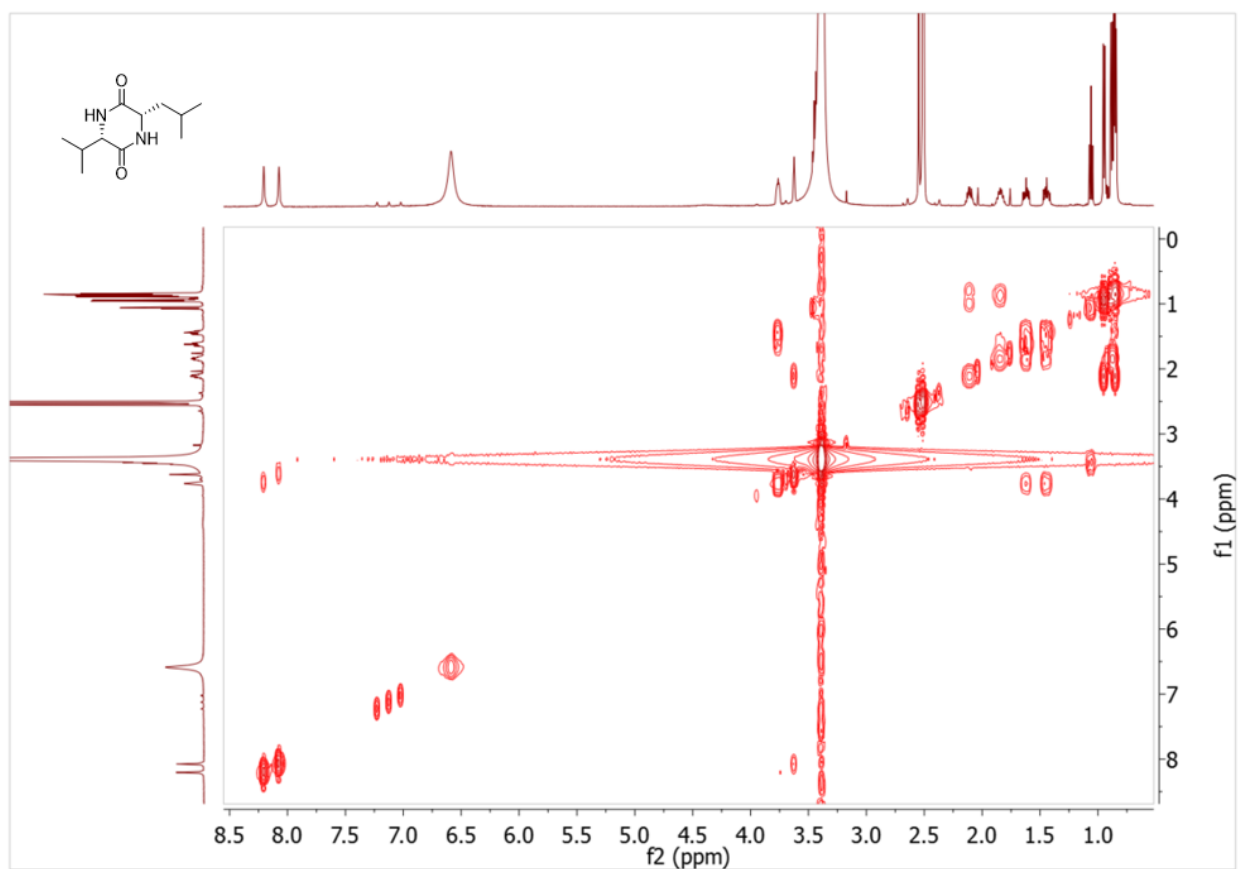


Figure S12. ^1H , ^1H -COSY NMR spectrum of cyclo(L-Val-L-Leu) in $\text{DMSO-}d_6$ (400 MHz) isolated from *E. coli* expressing *bcmA*.

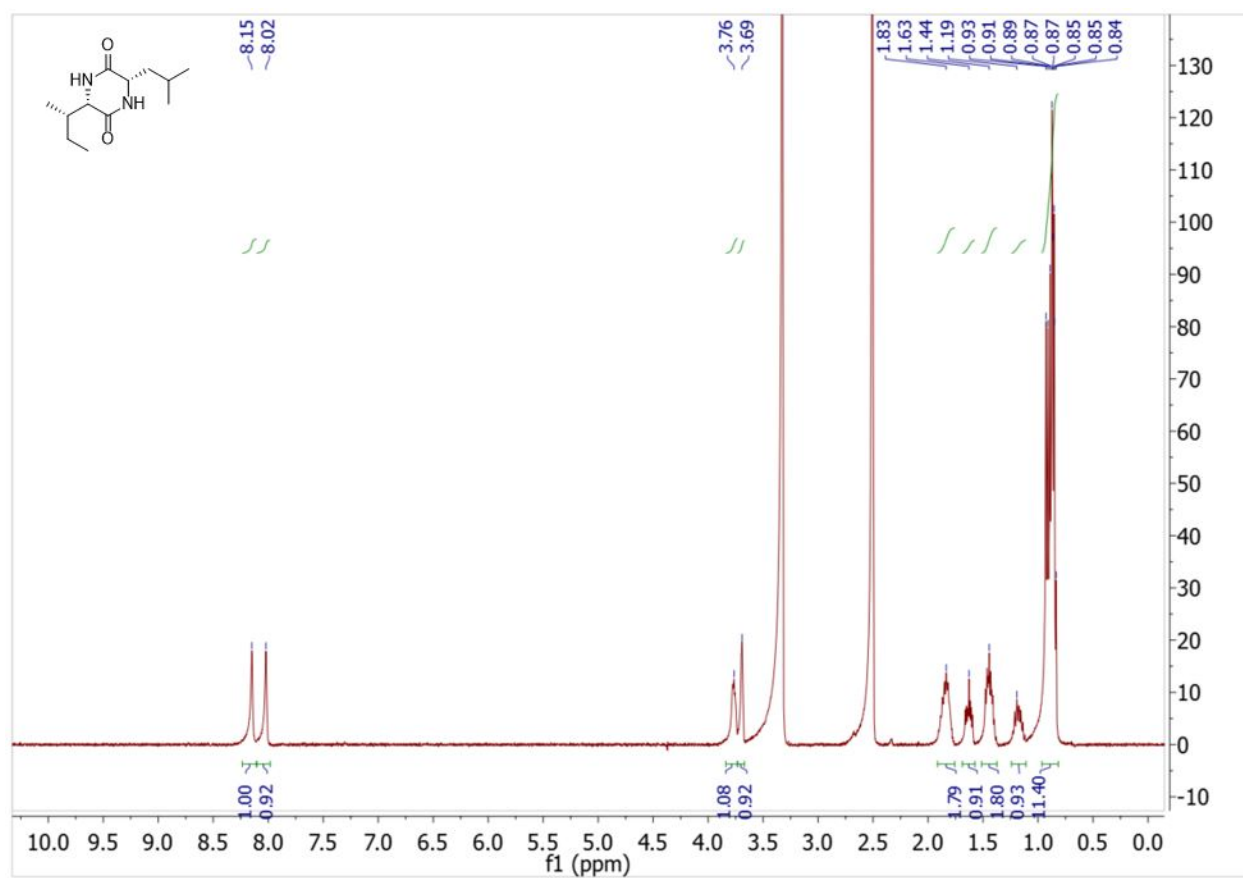


Figure S13. ^1H NMR spectrum of cyclo(L-Ile-L-Leu) (**2**) synthetic standard in $\text{DMSO-}d_6$ (400 MHz).

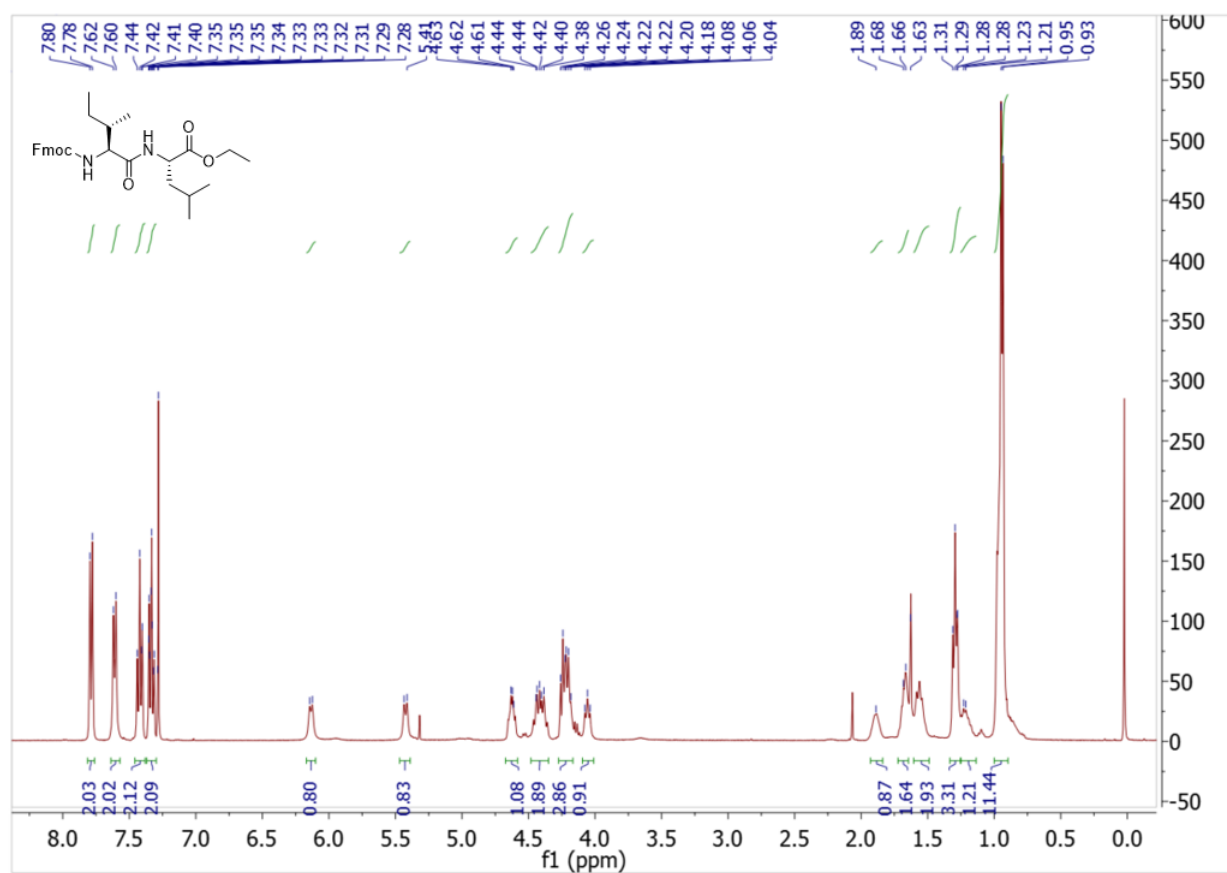


Figure S14. ^1H NMR spectrum of Fmoc-NH-L-Ile-L-Leu-OEt in CDCl_3 (400 MHz).

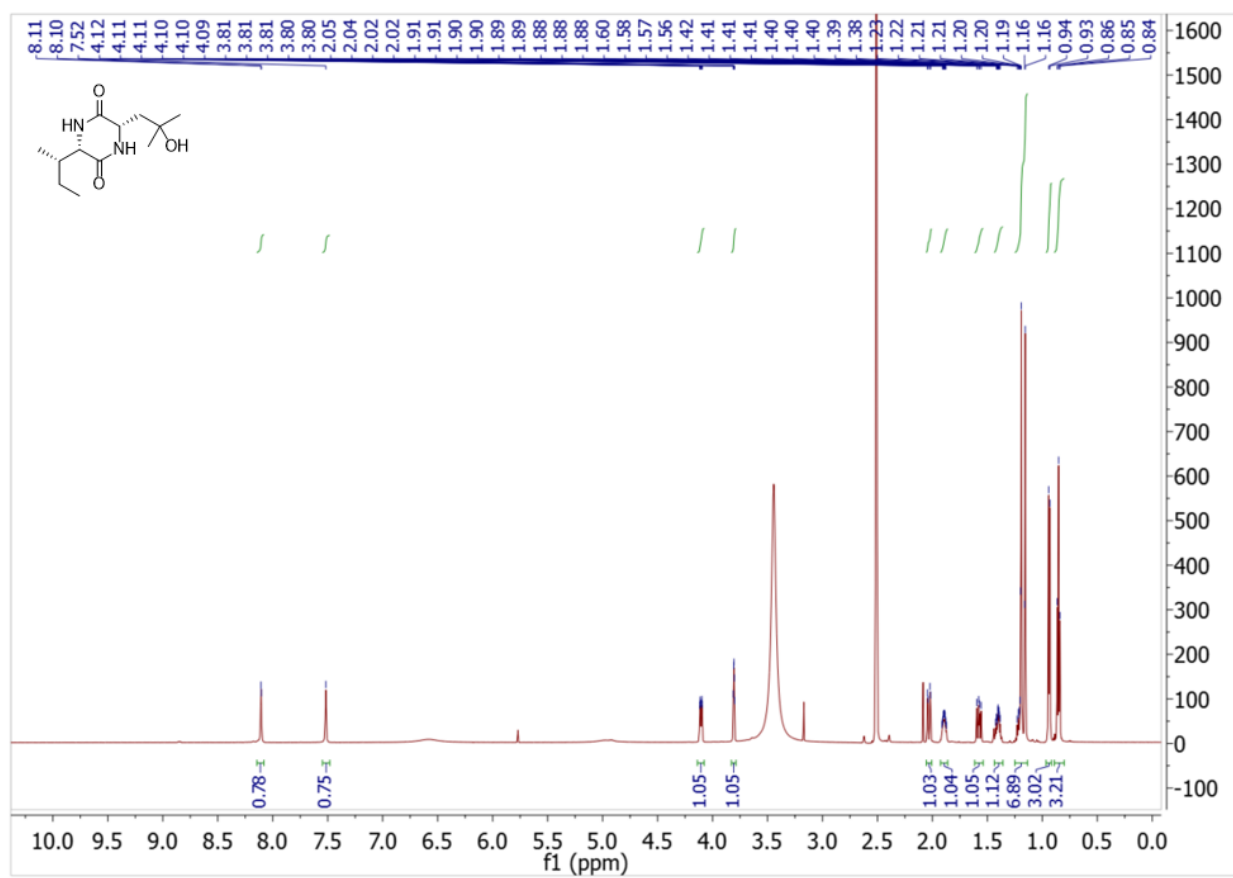


Figure S15. ¹H NMR spectrum of 8-hydroxy-cyclo(L-Ile-L-Leu) (**3**) in DMSO-*d*₆ (600 MHz) generated by BcmC *in vitro*.

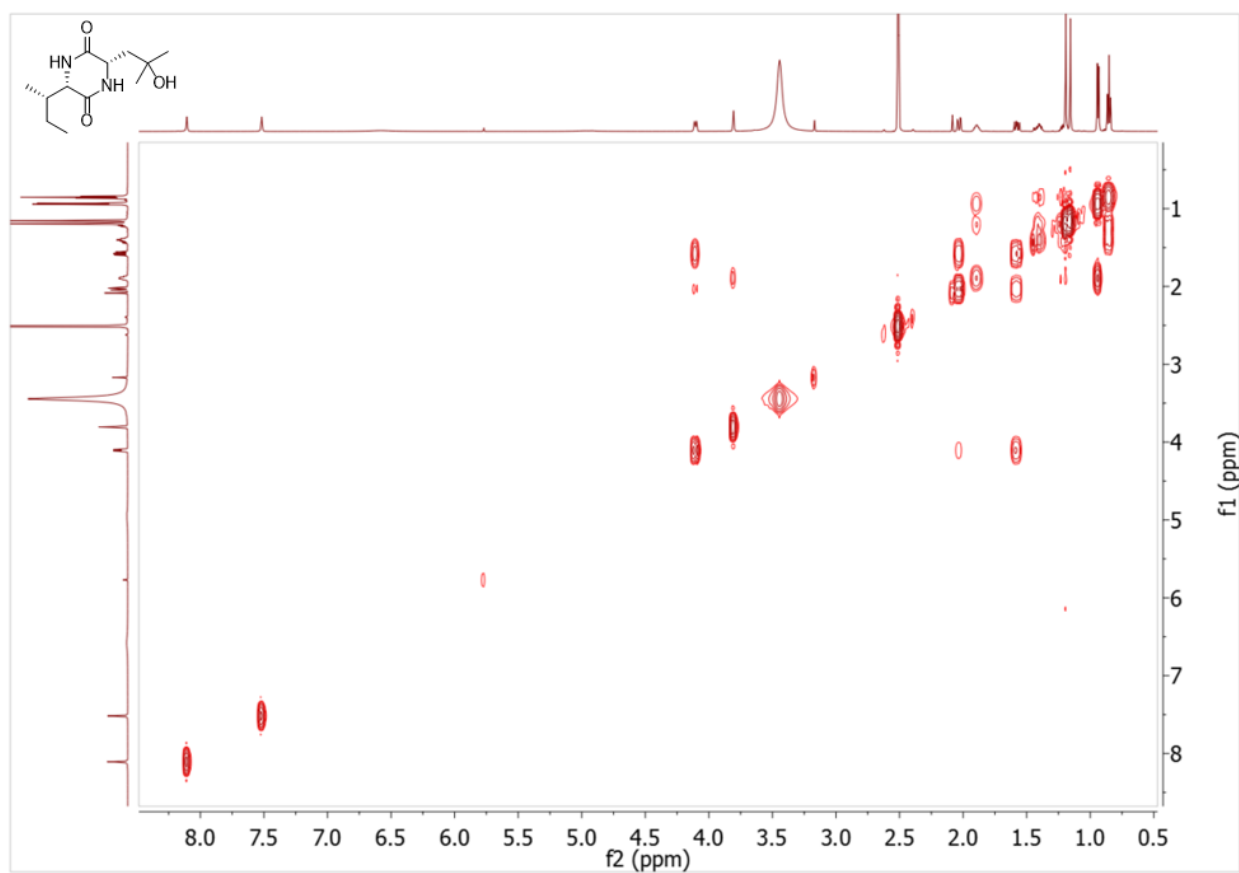


Figure S16. ^1H , ^1H -COSY NMR spectrum of 8-hydroxy-cyclo(L-Ile-L-Leu) (**3**) in $\text{DMSO}-d_6$ (600 MHz) generated by BcmC *in vitro*.

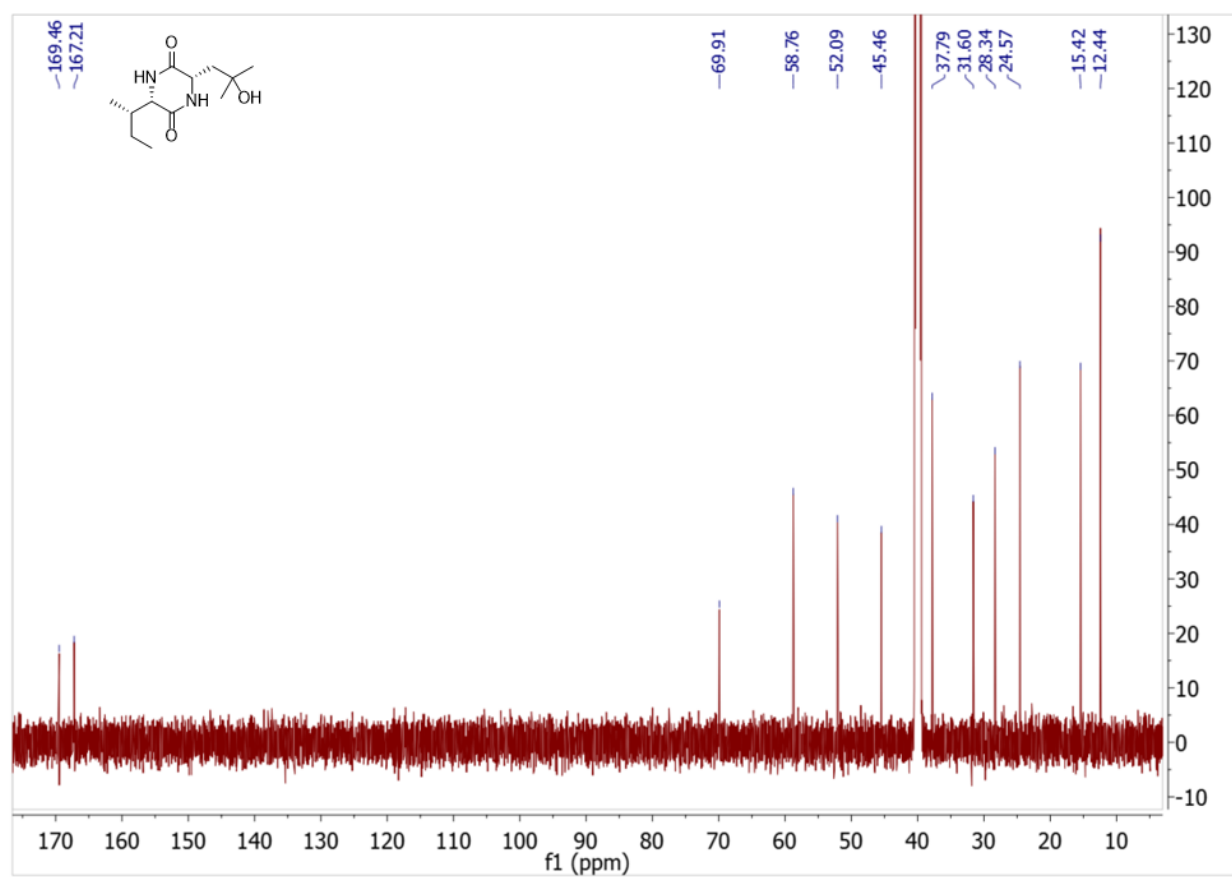


Figure S17. ^{13}C NMR spectrum of 8-hydroxy-cyclo(L-Ile-L-Leu) (**3**) in $\text{DMSO}-d_6$ (150 MHz) generated by BcmC *in vitro*.

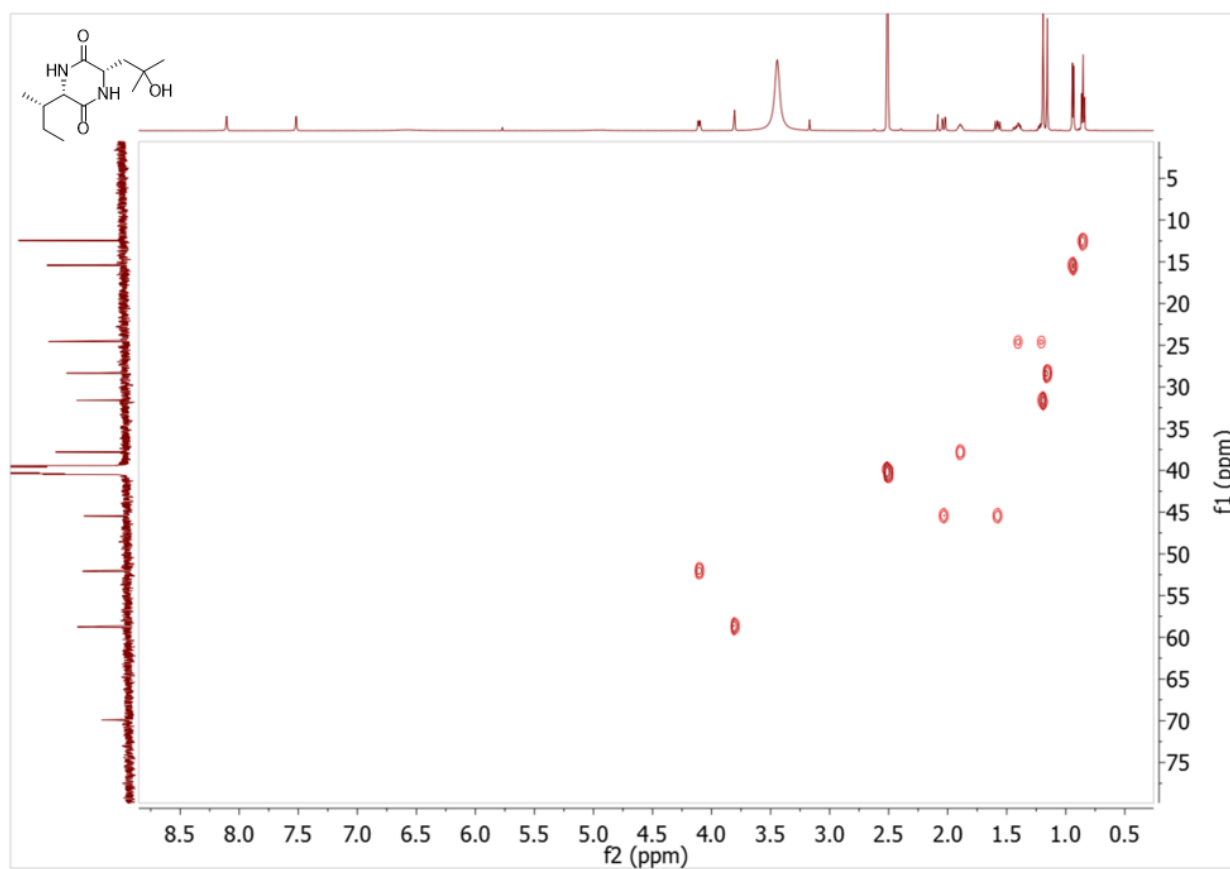


Figure S18. ^1H , ^{13}C -HSQC NMR spectrum of 8-hydroxy-cyclo(L-Ile-L-Leu) (**3**) in $\text{DMSO-}d_6$ (600 MHz) generated by BcmC *in vitro*.

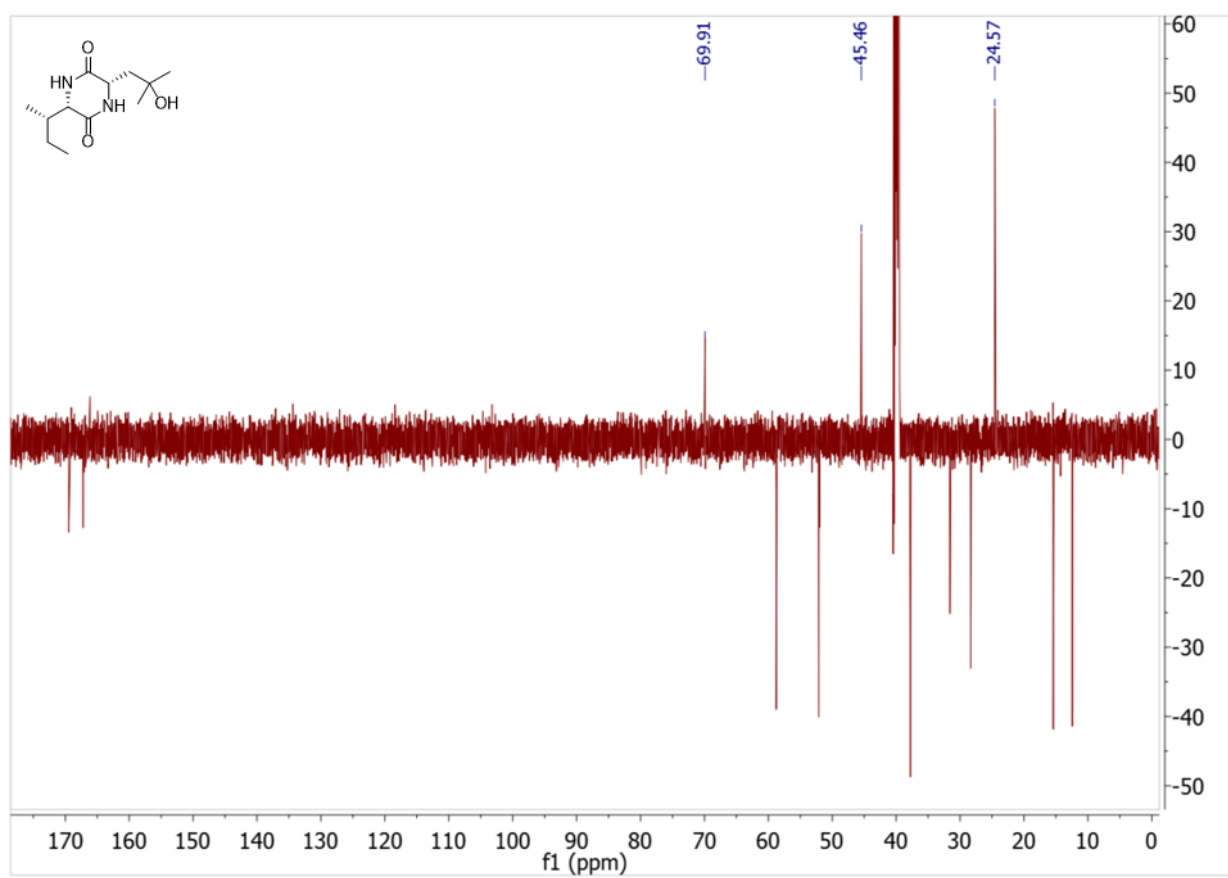


Figure S19. ^{13}C APT NMR spectrum of 8-hydroxy-cyclo(L-Ile-L-Leu) (**3**) in $\text{DMSO-}d_6$ (150 MHz) generated by BcmC *in vitro*.

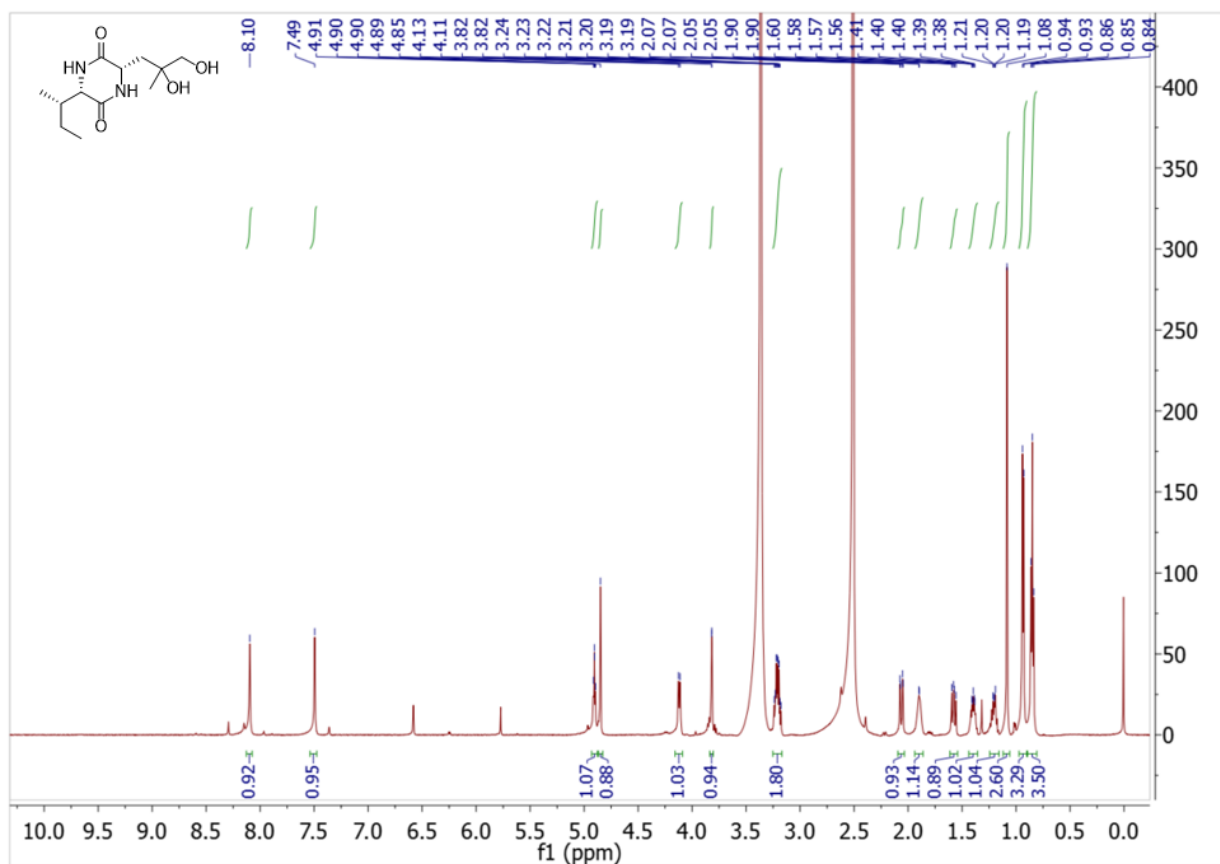


Figure S20. ¹H NMR spectrum of 8,9-dihydroxy-cyclo(L-Ile-L-Leu) (**4**) in DMSO-*d*₆ (600 MHz) generated by BcmG *in vitro*.

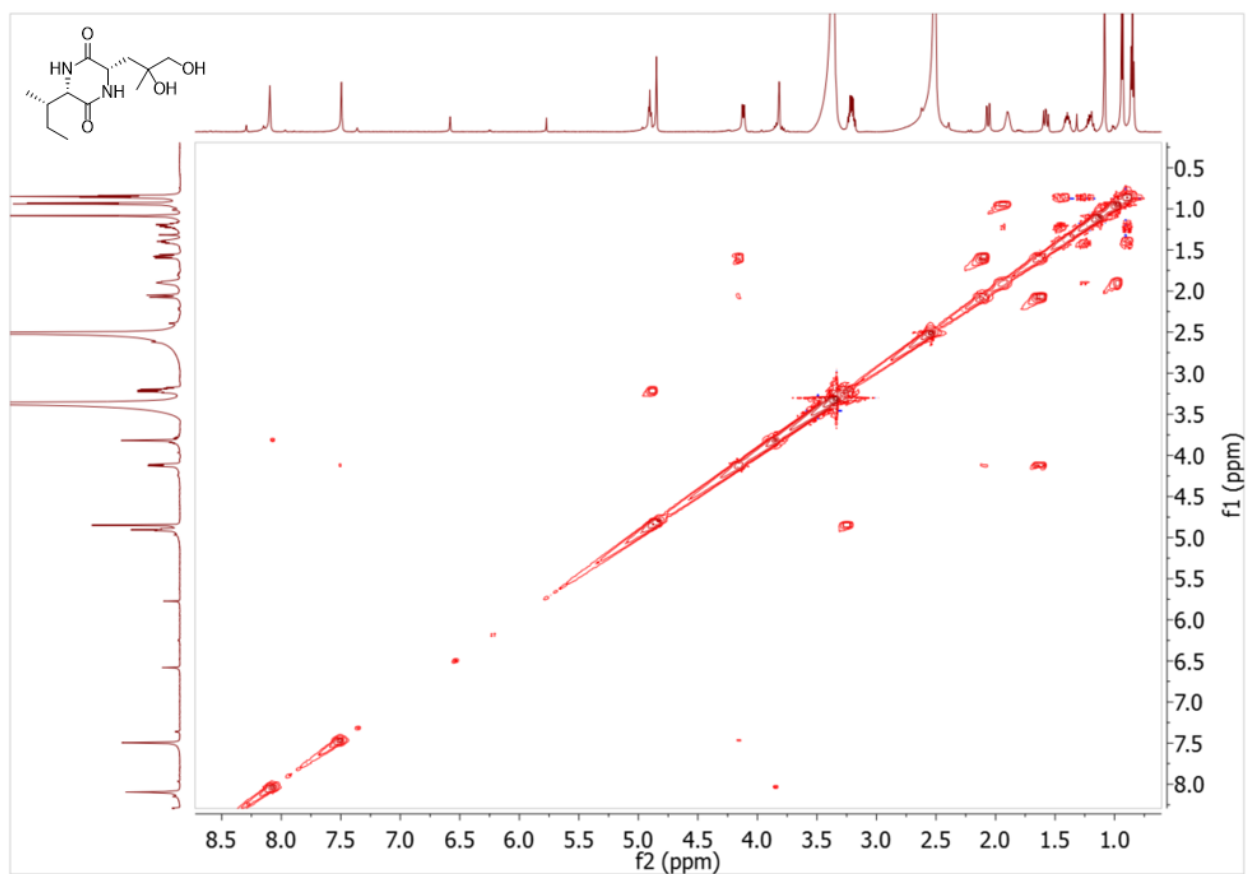


Figure S21. ^1H , ^1H -COSY NMR spectrum of 8,9-dihydroxy-cyclo(L-Ile-L-Leu) (**4**) in $\text{DMSO}-d_6$ (600 MHz) generated by BcmG *in vitro*. Spectrum was processed using standard MestReNova COSY-like symmetrization.

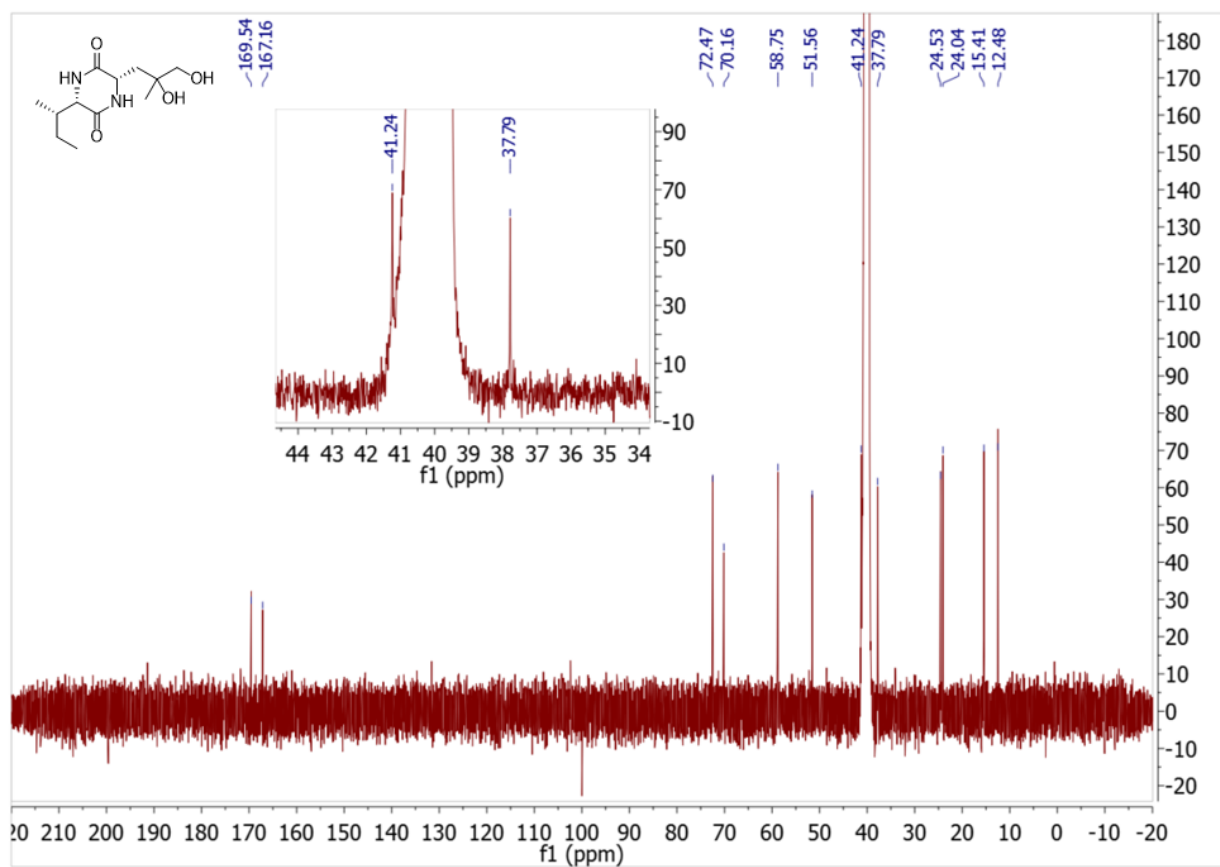


Figure S22. ¹³C NMR spectrum of 8,9-dihydroxy-cyclo(L-Ile-L-Leu) (**4**) in DMSO-*d*₆ (150 MHz) generated by BcmG *in vitro*.

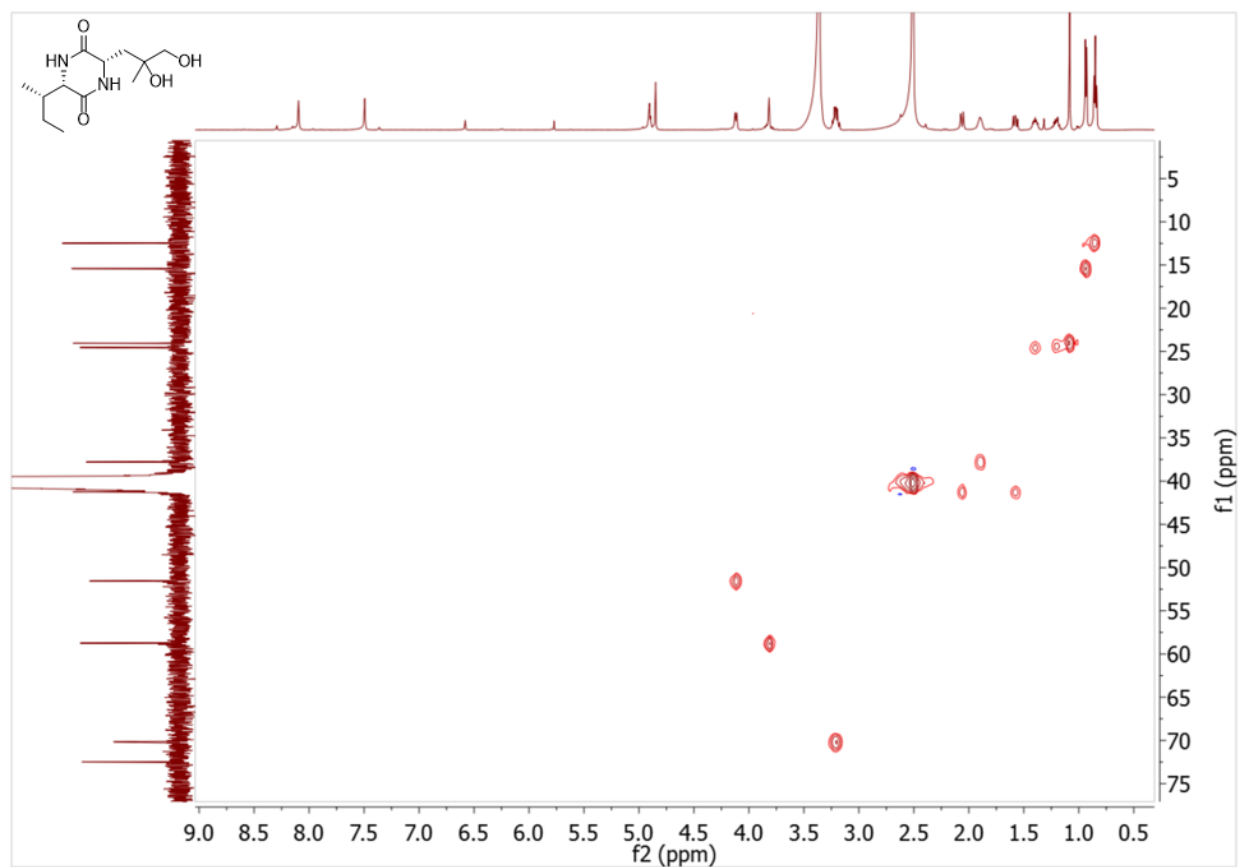


Figure S23. ^1H , ^{13}C -HSQC NMR spectrum of 8,9-dihydroxy-cyclo(L-Ile-L-Leu) (4) in $\text{DMSO-}d_6$ (600 MHz) generated by BcmG *in vitro*.

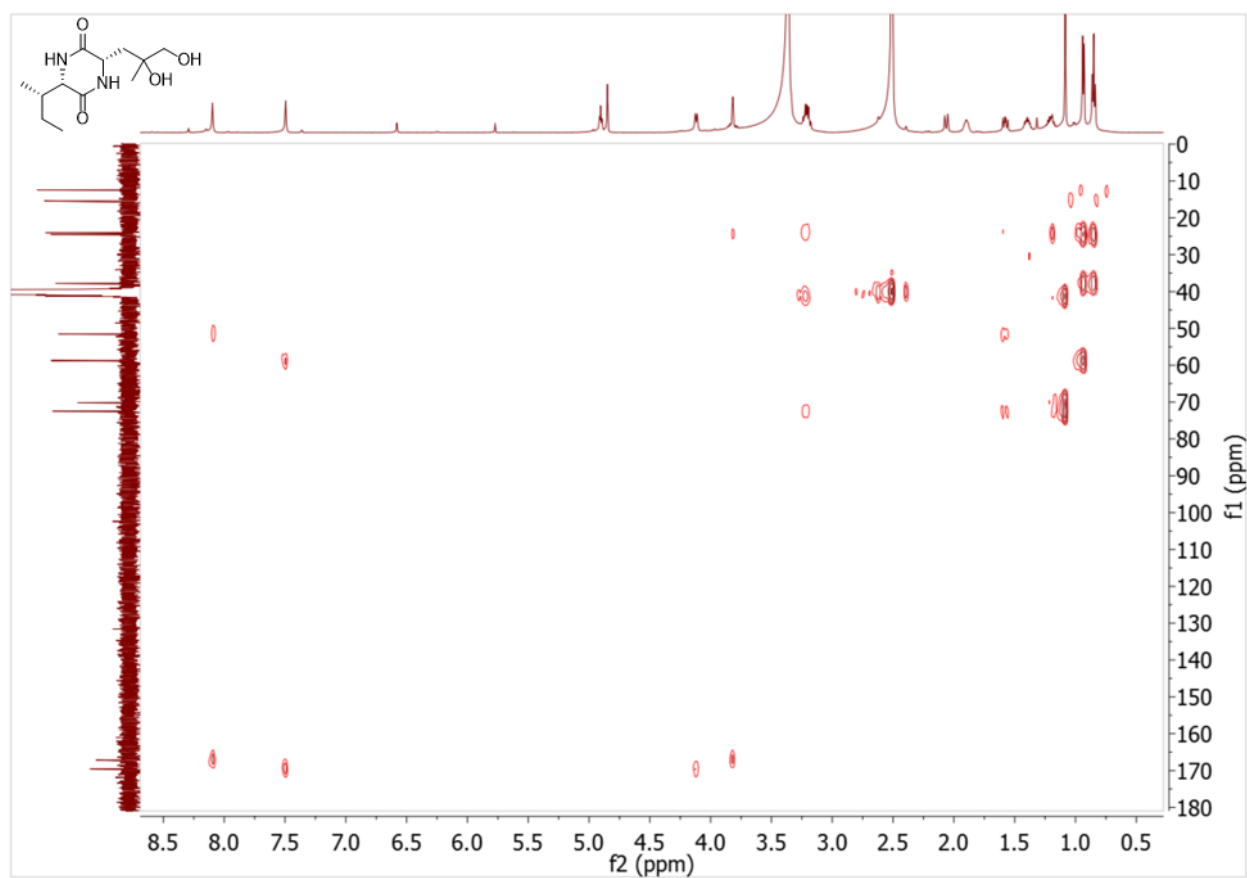


Figure S24. ^1H , ^{13}C -HMBC NMR spectrum of 8,9-dihydroxy-cyclo(L-Ile-L-Leu) (**4**) in $\text{DMSO-}d_6$ (600 MHz) generated by BcmG *in vitro*.

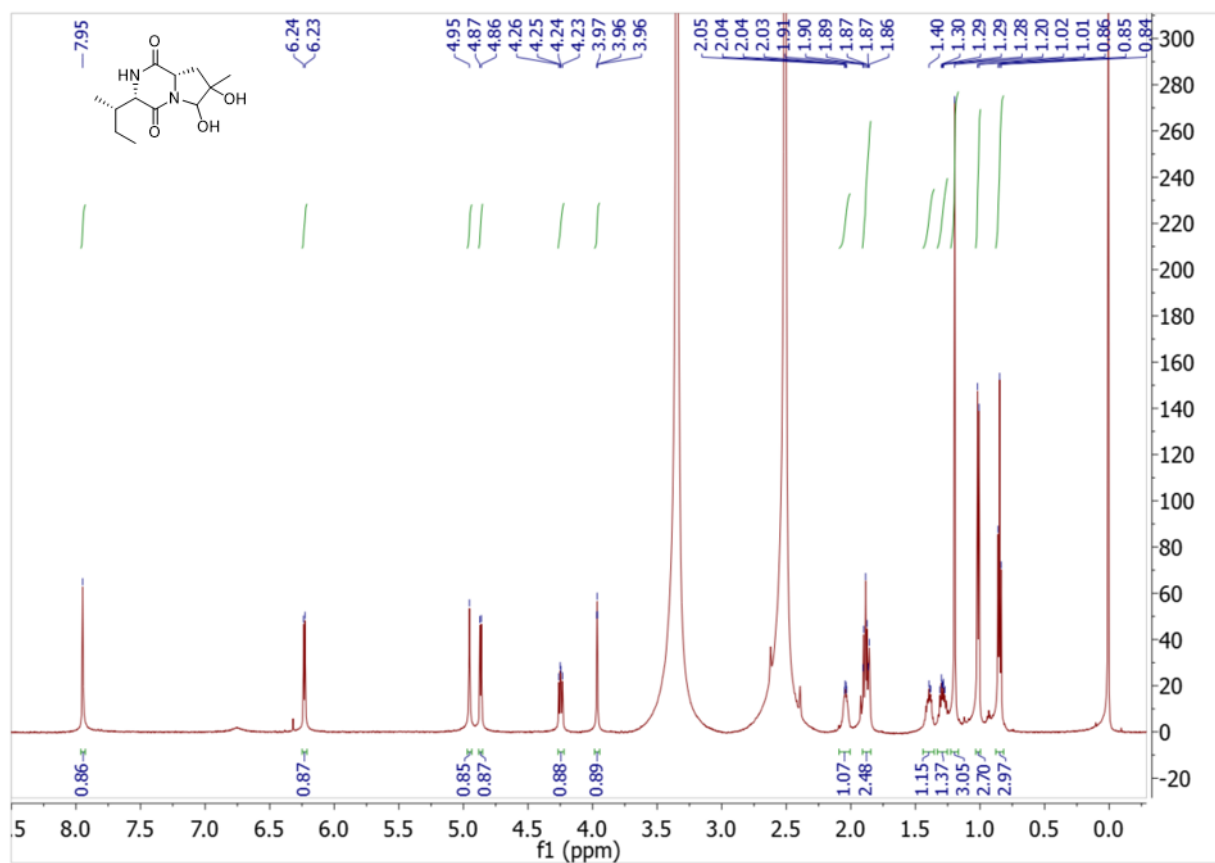


Figure S25. ¹H NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in DMSO-*d*₆ (600 MHz) generated from *in vitro* reconstitution of BcmG.

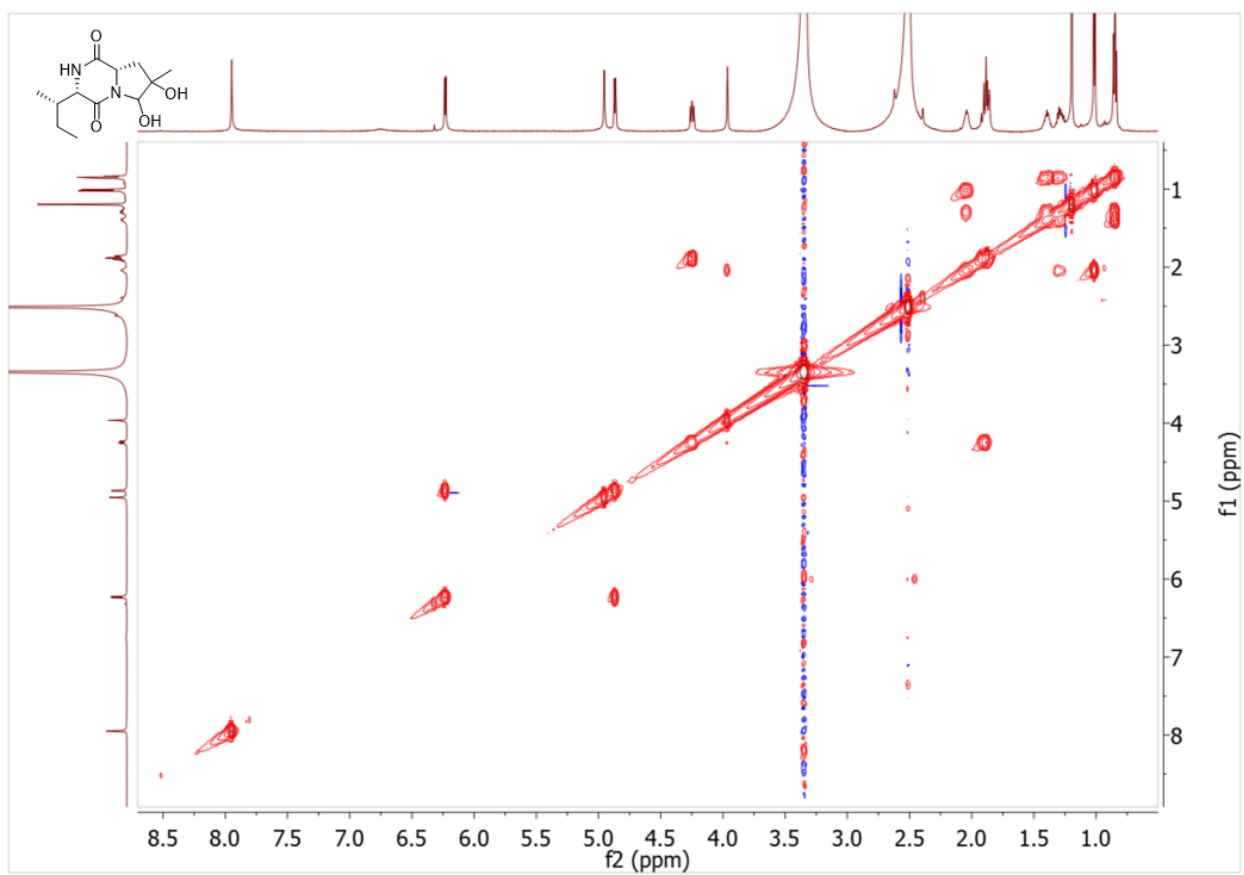


Figure S26. ^1H , ^1H -COSY NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in $\text{DMSO-}d_6$ (600 MHz) generated from *in vitro* reconstitution of BcmG.

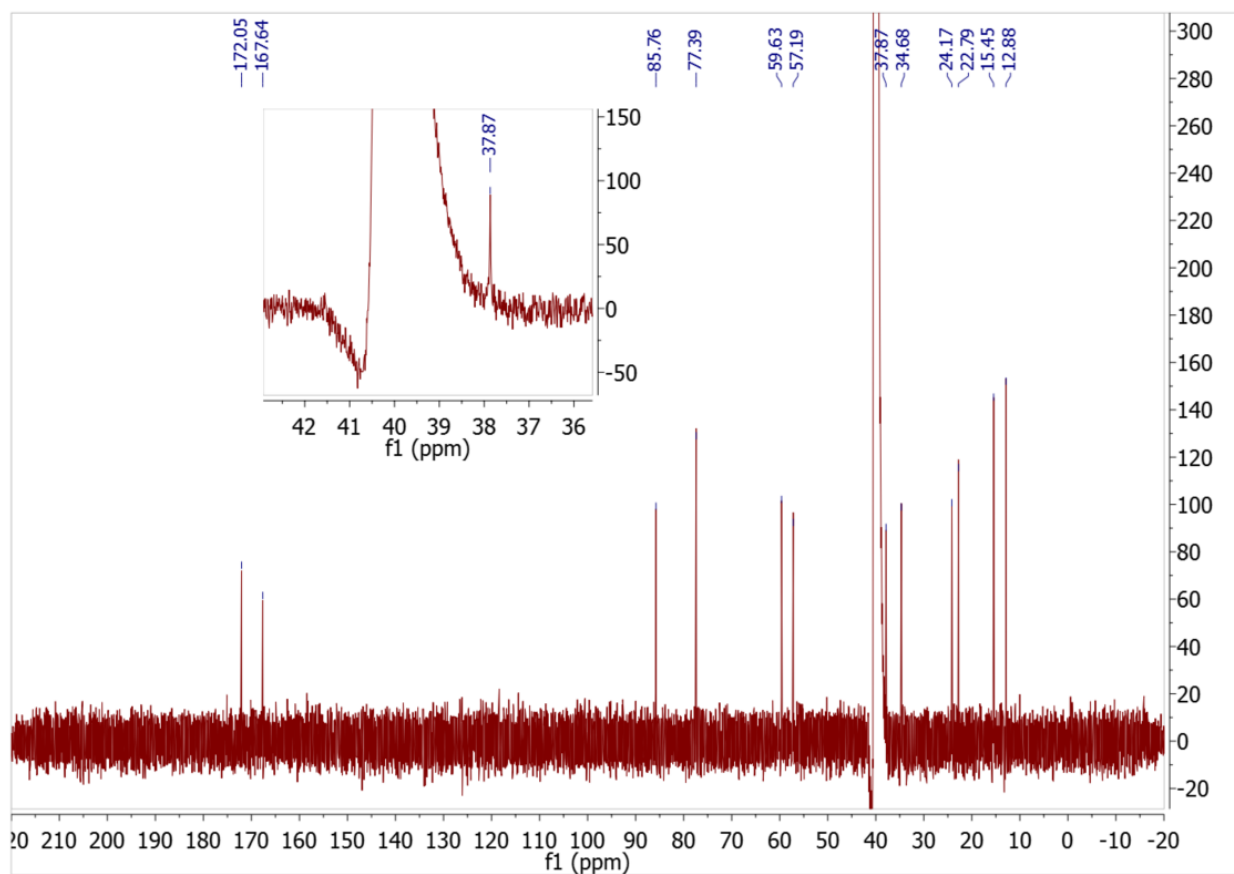


Figure S27. ^{13}C NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in DMSO-*d*₆ (150 MHz) generated from *in vitro* reconstitution of BcmG.

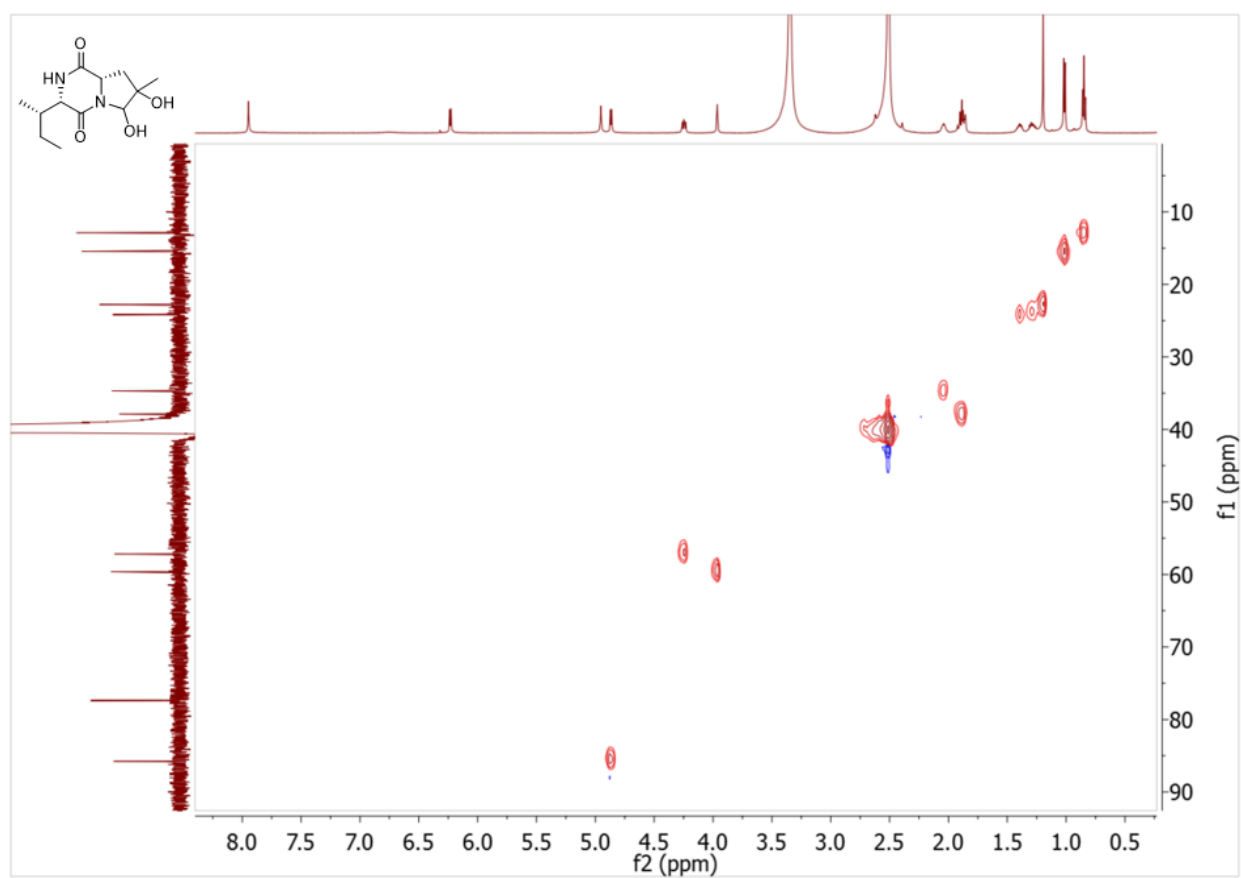


Figure S28. ^1H , ^{13}C -HSQC NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in $\text{DMSO}-d_6$ (600 MHz) generated from *in vitro* reconstitution of BcmG.

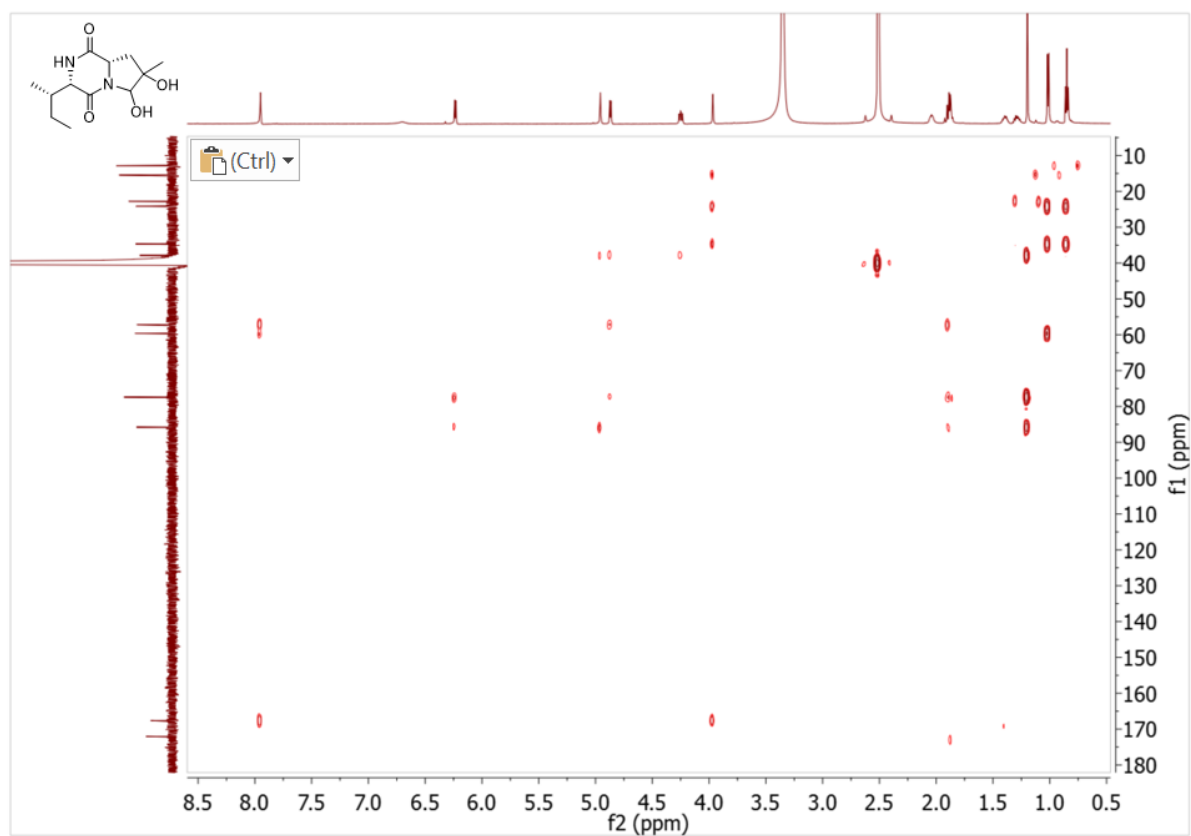


Figure S29. ^1H , ^{13}C -HMBC NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in $\text{DMSO}-d_6$ (600 MHz) generated from *in vitro* reconstitution of BcmG.

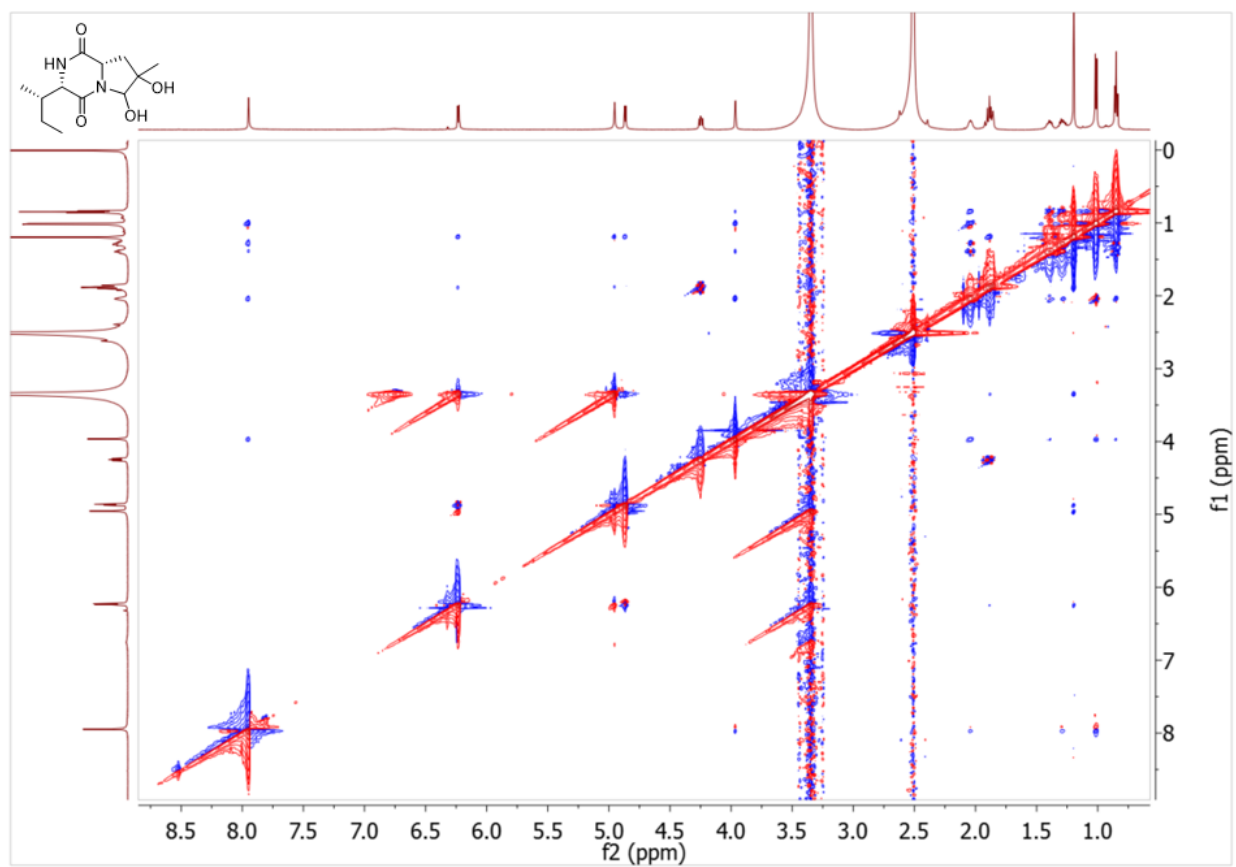


Figure S30. ^1H , ^1H -NOESY NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in $\text{DMSO}-d_6$ (600 MHz) generated from *in vitro* reconstitution of BcmG.

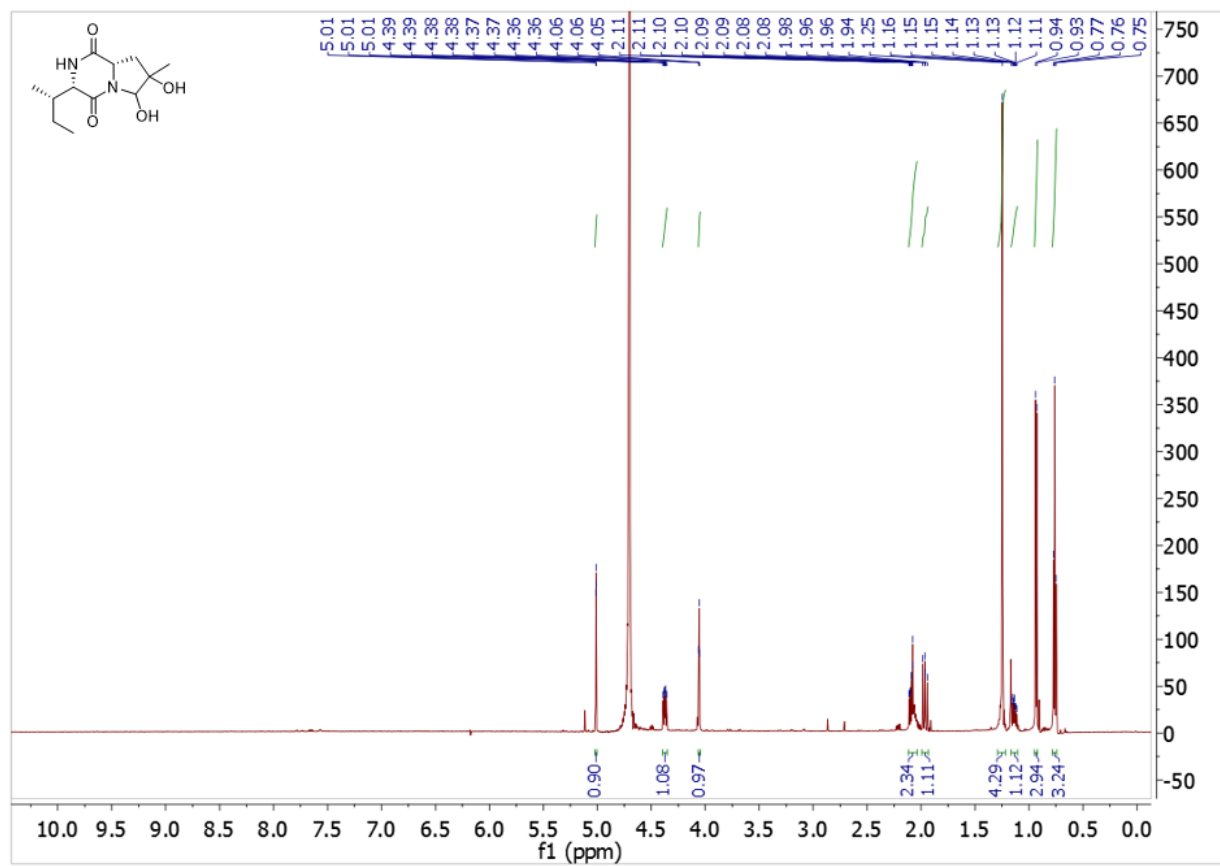


Figure S31. ¹H NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in D₂O (600 MHz) generated from *in vitro* reconstitution of BcmG.

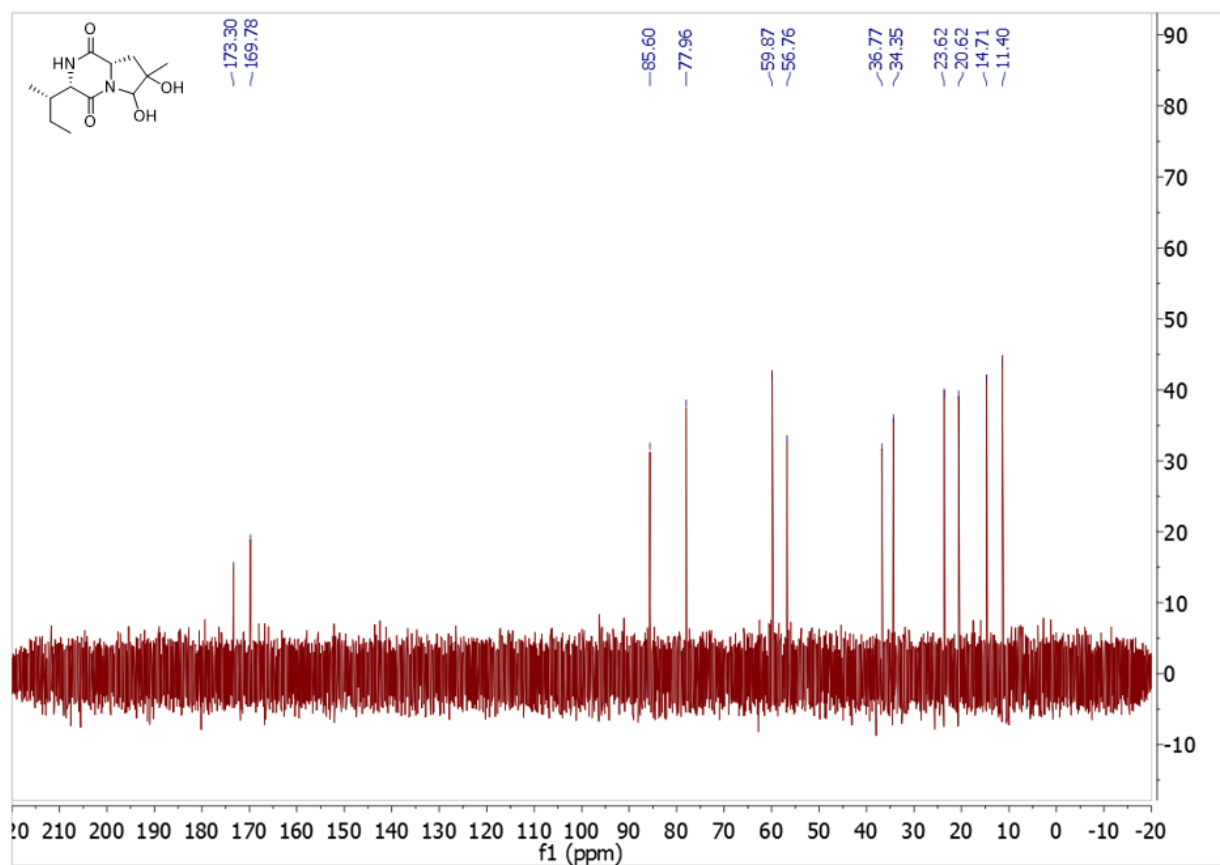


Figure S32. ^{13}C NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-isoleucine-L-proline) diastereomer 1 (**5a**) in D_2O (150 MHz) generated from *in vitro* reconstitution of BcmG.

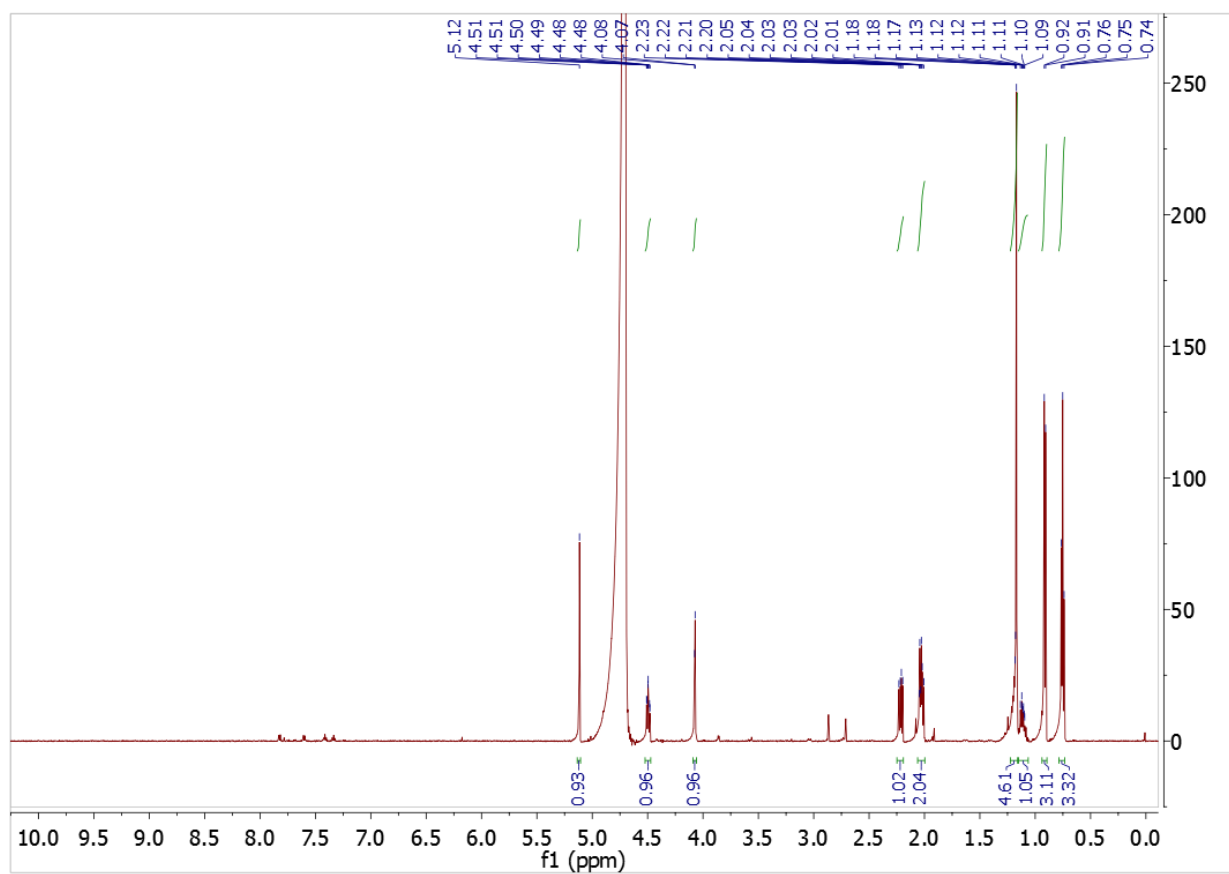


Figure S33. ^1H NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer **2** (**5b**) in D_2O (600 MHz) generated from *in vitro* reconstitution of BcmG.

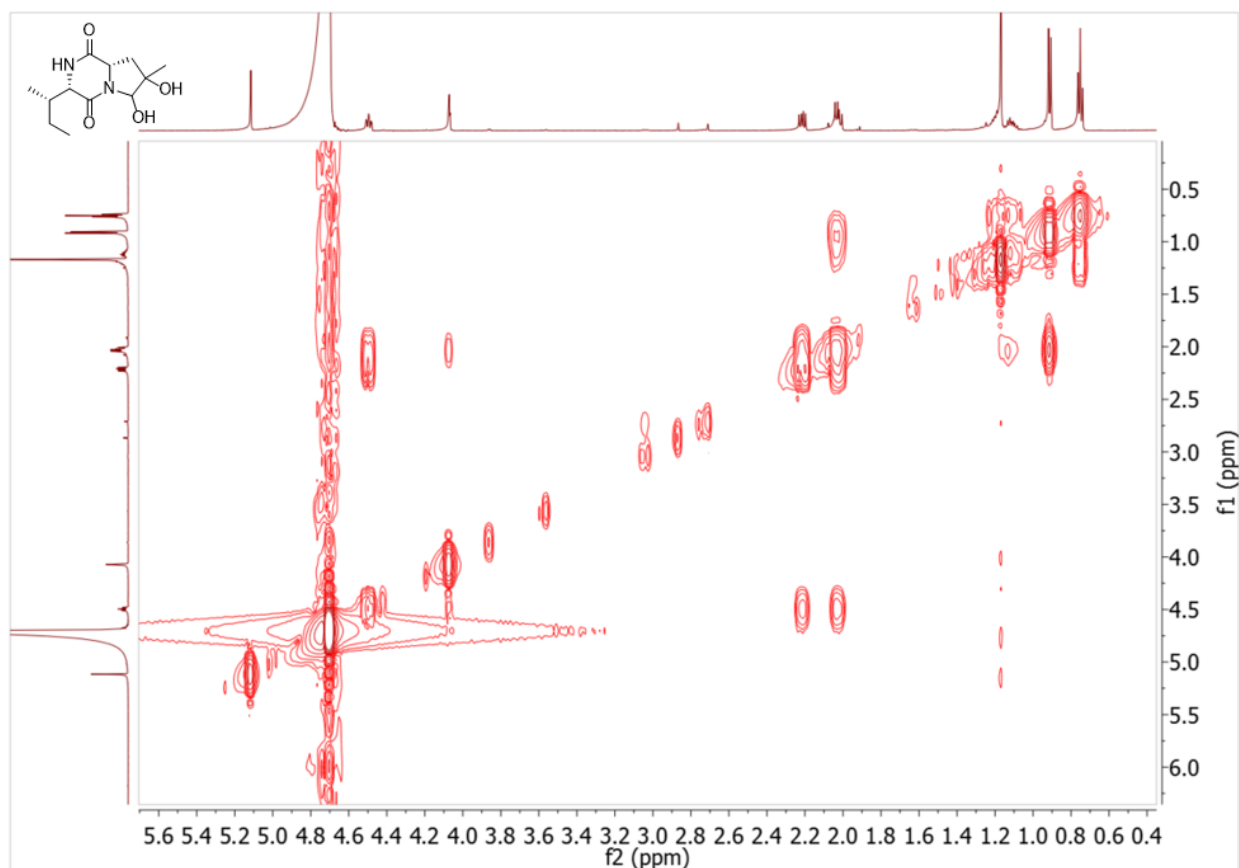


Figure S34. ^1H , ^1H -COSY NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer 2 (**5b**) in D_2O (600 MHz) generated from *in vitro* reconstitution of BcmG.

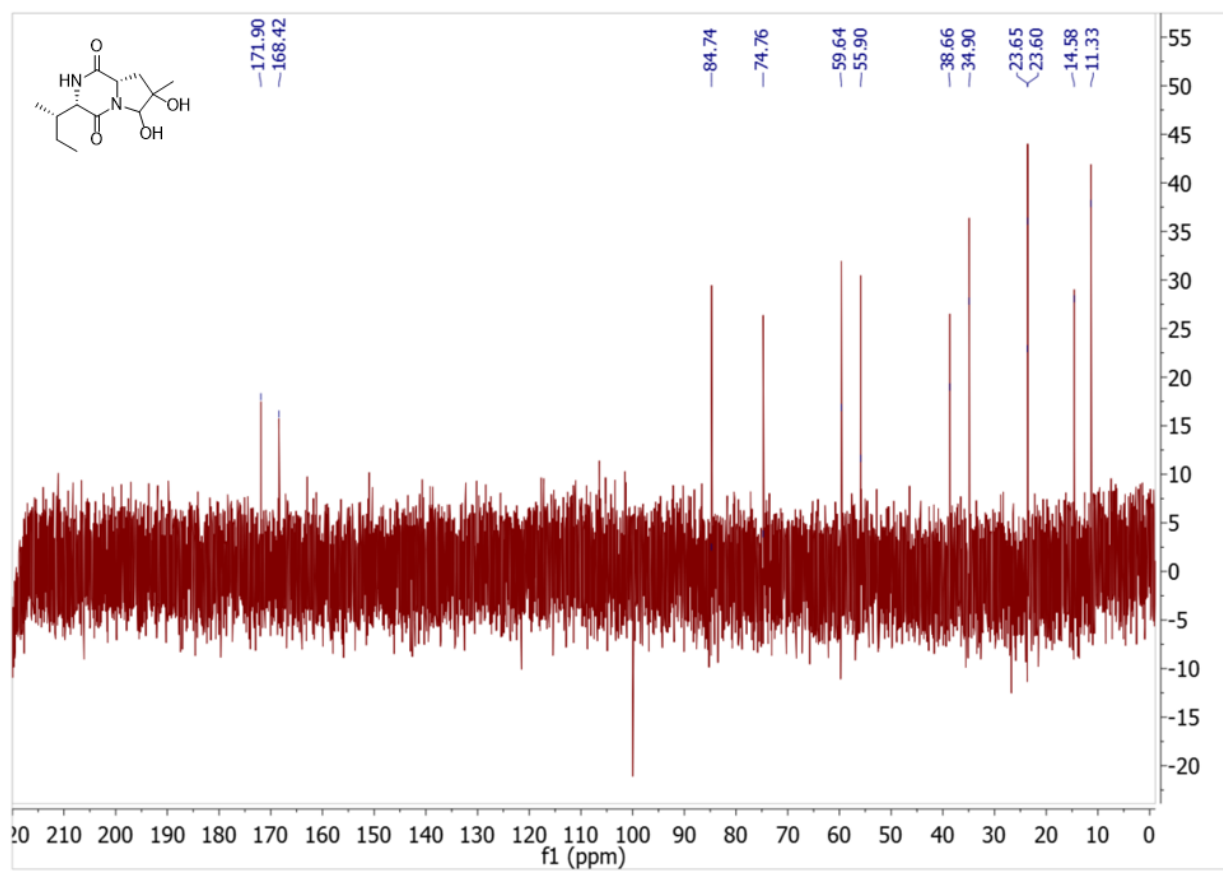


Figure S35. ¹³C NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer 2 (**5b**) in D₂O (150 MHz) generated from *in vitro* reconstitution of BcmG.

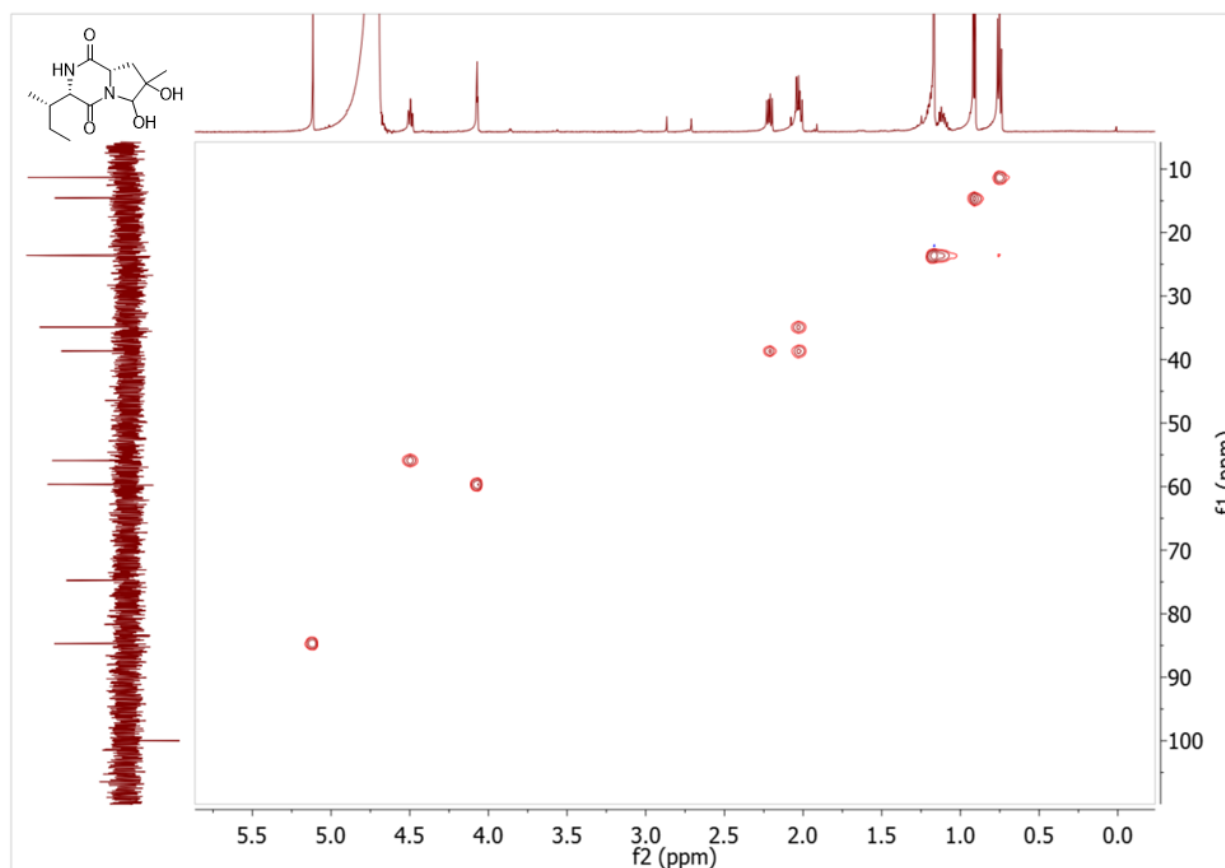


Figure S36. ^1H , ^{13}C -HSQC NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer 2 (**5b**) in D_2O (600 MHz) generated from *in vitro* reconstitution of BcmG.

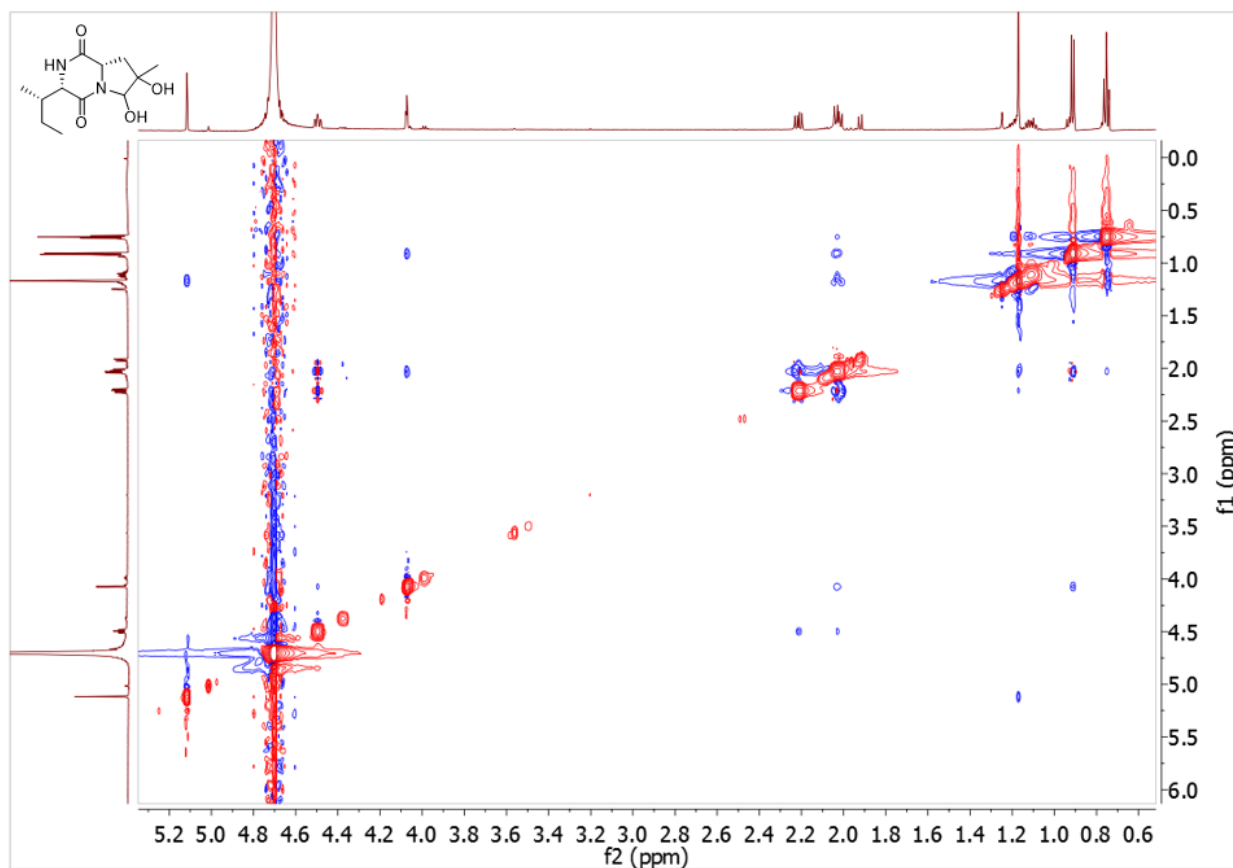


Figure S37. ¹H, ¹H-NOESY NMR spectrum of 8,9-dihydroxy-8-methyl-cyclo(L-Ile-L-Pro) diastereomer **2 (5b)** in D₂O (600 MHz) generated from *in vitro* reconstitution of BcmG.

Supporting references

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