Supporting Information for:

In vitro biosynthesis of the core scaffold of the thiopeptide thiomuracin

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Experimental Methods.

General materials and methods. Reagents used for molecular biology experiments were purchased from New England BioLabs (Ipswich, MA), Thermo Fisher Scientific (Waltham, MA), or Gold Biotechnology Inc. (St. Louis, MO). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). *Escherichia coli* DH5α and BL21 (DE3) strains were used for plasmid maintenance and protein overexpression, respectively. *Thermobispora bispora* DSM 43833 was obtained from DSMZ (Braunschweig, Germany). *Planobispora rosea* ATCC53733 was obtained from the USDA Agricultural Research Service (ARS) Culture Collection. Plasmid inserts were sequenced at ACGT Inc. (Wheeling, IL). MALDI-TOF-MS analysis was performed using a Bruker UltrafleXtreme MALDI TOF-TOF mass spectrometer (Bruker Daltonics) in reflector positive mode at the University of Illinois School of Chemical Sciences Mass Spectrometry Laboratory. ESI-MS/MS analyses were performed using a SYNAPT ESI quadrupole TOF Mass Spectrometry System (Waters) equipped with an ACQUITY Ultra Performance Liquid Chromatography (UPLC) system (Waters). Kinetic assays were performed using a Varian Cary 4000 UV-Vis spectrophotometer. HiTrap columns for Ni-NTA affinity chromatography were purchased from GE Healthcare.

Molecular biology techniques. Oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA). Primers for *E. coli* $tRNA^{Glu}$ were designed and the dsDNA template for in vitro transcription was prepared as previously described.[1] *Thermobispora bispora* was identified as a potential producer of a thiomuracin-like thiopeptide using BLAST to identify homologs of genes from the relevant biosynthetic gene cluster of thiomuracin producer *Nonomurea* sp. Bp3714-39.[2] Genes optimized for recombinant expression in *Escherichia coli* were synthesized by GenScript (Piscataway, NJ) in pUC57 (kanamycin, Kan) vectors with BamHI and XhoI sites flanking each gene at the 5′ and 3′ ends, respectively. The GenBank locus tag and *E. coli* optimized sequence for each gene is provided in **Table S1**. *E. coli* DH5α were transformed with pUC57-Kan vectors containing each gene for replication and subsequent isolation using a QIAprep Spin Miniprep Kit (Qiagen). The isolated DNA was then treated with BamHI-HF and XhoI-HF (New England Biolabs). The digested genes were separated on a 1% (w/v) agarose gel, purified using a QIAQuick gel extraction kit (Qiagen), and ligated into an appropriately endonuclease-digested and gel-purified pET28 vectors using T4 DNA ligase (NEB). Ligation reactions were used to transform chemically competent DH5α cells, which were plated on Luria-Bertani (LB) agar plates containing 50 μg/mL kanamycin and grown at 37 °C. Colonies were picked at random and grown in LB broth for 16–20 h prior to plasmid isolation using a QIAprep Spin Miniprep Kit. For TbtA and TbtD, the vector encoded a tobacco etch virus (TEV) cleavable, N-terminal maltose binding protein (MBP) affinity tag, while TbtE, TbtF, and TbtG constructs did not encode a TEV proteolysis site. All recombinant constructs featuring an MBP-tag were sequenced using a custom MBP forward primer, whereas constructs encoding His_6-tagged proteins were sequenced using the T7 forward primer. All constructs were reverse- sequenced using a T7 reverse primer as well as an internal primer if necessary (**Table S2**).

The gene encoding PbtD (TbtD ortholog) from *Planobispora rosea* ATCC 23866 was amplified from genomic DNA using the primers listed in **Table S2**. The resulting DNA was purified using a QIAprep Spin Miniprep Kit. The purified DNA was then digested using BamHI-HF and XhoI-HF before being ligated into an appropriately digested pET28 vector encoding an N-terminal MBP tag featuring a TEV proteolysis site. The resulting construct was then used to transform *E. coli*, propagated, and sequenced as described above.

Site-directed mutagenesis on TbtD was performed using the QuikChange method (Agilent) as per the manufacturer's instructions using PfuTurbo DNA polymerase. The primers for each mutant are listed in **Table S2**. The mutations were verified by sequencing using the MBP forward primer or the T7 reverse primer.

The *tbtA* gene was cloned into *pRSFDuet-1* with an *N*-terminal His_6-tag following standard cloning procedures. In addition, the GlySerSer residues preceding the His_6 tag were mutated to a single Pro to suppress gluconoylation.[3] The PCR product was purified by gel extraction on a 1% (w/v) agarose gel using the QIAquick Gel Extraction Kit (Qiagen). The vector *pRSFDuet-1* was digested using BamHI-HF and HindIII-HF (NEB) restriction endonucleases and purified by gel extraction as described above. Insertion of the DNA fragment was achieved by Gibson one-step isothermal DNA assembly as previously described.[4] An aliquot (20 μL) of the Gibson assembly reaction was used to transform *E. coli* DH5α cells using the heat shock method. The cells were plated on LB plates supplemented with kanamycin (50 μg/mL) and single colonies were grown at 37 °C for 12–15 h. The plasmid *His_6-TbtA-pRSFDuet-1*

was isolated using a QIA Spin Miniprep Kit. Insert integrity was verified by sequencing the plasmids with the appropriate primers (**Table S2**).

The gene coding for the dehydratase TbtB was cloned into *pET28a* encoding an N-terminal His_6-tag. The *tbtB* gene was amplified by PCR, and purified by gel extraction from a 1% (w/v) agarose gel using the QIAquick Gel Extraction Kit. The vector *pET28a* and the *tbtB* PCR fragment were digested using BamHI-HF and XhoI-HF (NEB) restriction endonucleases, and purified by gel extraction as described above. The DNA fragment was inserted by Gibson one-step isothermal DNA assembly, and an aliquot of 20 µL of the Gibson assembly reaction was used to transform *E. coli* DH5α cells using the heat shock method. The cells were plated on LB plates supplemented with kanamycin (50 µg/mL) and the plates were incubated at 37 °C for 12–15 h. Single colonies were picked and grown in LB supplemented with kanamycin (50 µg/mL) at 37 °C for 12–15 h and the plasmid *His_6-TbtB-pET28a* was isolated using a QIA prep Spin Miniprep Kit. Insert integrity was verified by sequencing the plasmids with the appropriate primers (**Table S2**). All the other genes and their corresponding His_6-tagged proteins were cloned using the same procedures.

MBP-tagged peptide and enzyme overexpression and purification. *E. coli* BL21(DE3) cells were transformed with a pET28 plasmid encoding the MBP-tagged enzyme or peptide of interest. Cells were grown for 24 h on LB agar plates containing 50 µg/mL kanamycin at 37 °C. Single colonies were used to inoculate 10 mL of LB containing 50 µg/mL kanamycin and grown at 37 °C for 16–18 h. This culture was used to inoculate 1 L of Terrific Broth (24 g/L yeast extract, 12 g/L tryptone, 0.4% glycerol (v/v), 17 mM KH_2PO_4, and 72 mM K_2HPO_4) and grown to an optical density at 600 nm (OD_{600}) of 1.0 before being placed on ice for 15 min. Protein expression was then induced with the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for biosynthetic enzymes or 1 mM IPTG for the TbtA precursor peptide. Expression was allowed to proceed for 16-18 h at 22 °C. For expression of MBP-TbtE and MBP-TbtG, 70 µM riboflavin or 100 µM $ZnCl_2$, respectively, were added at the time of induction. Cells were harvested by centrifugation at 3,000 × *g* for 20 min, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), and centrifuged again. The cell pellet was flash-frozen and stored at -80 °C for a maximum of one week before use.

Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5% glycerol (v/v), and 0.1% Triton X-100) containing 4 mg/mL lysozyme, 2 µM leupeptin, 2 µM benzamidine, and 2 µM E64. After 30 min at 4 °C, cells were further homogenized by sonication (3 × 45 s with 10 min nutation periods at 4 °C). Insoluble debris was removed by centrifugation at 20,000 × *g* for 90 min. The supernatant was then applied to a pre-equilibrated amylose resin (NEB; 20 mL of resin per L of cells). The column was washed with 10 column volumes (CV) of lysis buffer followed by 10 CV of wash buffer (lysis buffer with 400 mM NaCl and lacking Triton X-100). The MBP-tagged proteins were eluted using elution buffer (lysis buffer with 300 mM NaCl, 10 mM maltose, and also lacking Triton X-100) until the eluent no longer contained protein detectable with the Bradford reagent (assayed using 2 µL of eluent and 198 µL of Bradford solution at rt). Eluent was concentrated using an appropriate MWCO Amicon Ultra centrifugal filter (EMD Millipore). A buffer exchange with 10× volume of protein storage buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 2.5% glycerol (v/v)) was performed prior to final concentration and storage. Protein concentrations were assayed using both 280 nm absorbance (theoretical extinction coefficients were calculated using the ExPASy ProtParam tool; http://web.expasy.org/protparam/protpar-ref.html) and a Bradford colorimetric assay. Purity and possible truncation were assessed by Coomassie-stained SDS-PAGE gel (**Figure S1**). All wash, elution, and storage buffers were supplemented with 0.5 mM tris-(2-carboxyethyl)-phosphine (TCEP) except for those used for the preparation of MBP-TbtD (to avoid nucleophilic addition of TCEP to the dehydroalanine residues).

Expression, and purification of His_6-TbtA (1). *E. coli* BL21 (DE3) cells (50 µL) were electroporated with *His_6-TbtA-pRSFDuet-1* (50 ng), plated on LB agar plates supplemented with kanamycin (as above) and grown at 37 °C for 12–15 h. A single colony was used to inoculate 60 mL of LB broth supplemented with kanamycin, grown for 12–15 h at 37 °C, and the culture was used to inoculate 6 L of LB media, supplemented with kanamycin, to an OD_{600} of 0.025. Cultures were grown at 37 °C to an OD_{600} of 1.0. Peptide expression was induced by the addition of IPTG to a final concentration of 1 mM and cultures were grown at 37 °C for 3 h. Peptide purification was performed following a previously described method.[5]

Expression, and purification of His_6-TbtB. *E. coli* BL21 (DE3) cells (50 μL) were electroporated with *His_6-TbtB-pET28a* (50 ng), and cells were plated on LB agar plates supplemented with kanamycin and grown at 37 °C for 12–15 h. A single colony was used to inoculate 20 mL of LB broth supplemented with kanamycin, grown for 12–15 h at 37 °C, and the culture was used to inoculate 2 L of TB media, supplemented with kanamycin, to an OD_{600} of 0.025. Cultures were grown at 37 °C to a final OD_{600} of 0.6–0.8. Protein overexpression was induced by the addition of IPTG to a final concentration of 0.2 mM, and cultures were grown at 18 °C for 18 h. Protein purification was performed following a similar protocol as described before.[6] All other His_6-tagged proteins were purified using the same procedure.

Cyclodehydration of TbtA precursor peptide. Thiazole installation was carried out with 50 μM MBP-tagged TbtA and 1.5 μM of MBP-tagged biosynthetic enzymes (TbtE, TbtF, and TbtG) in synthetase buffer [50 mM Tris pH 8.0, 125 mM NaCl, 2 mM TCEP, 20 mM $MgCl_2$, and 6 mM ATP]. The MBP-tag on the precursor peptide was removed using 250 μM TEV protease, and reactions were allowed to proceed for 3 h at 25 °C; the MBP-tagged biosynthetic enzymes did not contain a TEV cleavage site. Aliquots of 50 μL were desalted via C18 ZipTip (EMD Millipore) according to the manufacturer's instructions, and the peptide was eluted using a saturated solution of sinapinic acid in 50% aq. MeCN. The reaction was checked for completion by MALDI-TOF MS. The depletion of unmodified peptide and the appearance of the hexazole species were indicative of reaction completion.

Purification of 2. The cyclodehydration/dehydrogenation reaction mixtures were applied to Ni-NTA resin (Qiagen) equilibrated with 50 mM Tris-HCl pH 8.0, 150 mM NaCl using 1 mL resin per 1 mL of reaction. The flow-through was collected, and the column was subsequently washed with 5 mL of wash buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl, 75 mM imidazole) per 1 mL of reaction mixture. The total volume was then applied to a Thermo C18 HyperSep cartridge equilibrated first with 10 CV of MeCN followed by 10 CV of 5% aq. MeCN containing 0.3% formic acid. The column was washed with 10 CV of 5% aq. MeCN containing 0.3% formic acid to remove polar contaminants. **2** was eluted from the column using up to 5 CV of 80% aq. MeCN. From a reaction using 26.5 mg TEV-cleaved TbtA, 20.0 mg of crude **2** was isolated (77% yield).

In vitro transcription of *T. bispora* $tRNA^{Glu}$ (CUC). Primers for *T. bispora* $tRNA^{Glu}$ were designed according to a previously described method.[1] The $tRNA^{Glu}$ dsDNA template was generated from two overlapping synthetic deoxyoligonucleotides. To prepare the dsDNA template for in vitro transcription, 5′ overhangs were filled in using the following conditions: NEB Buffer 2 (1×), primers (4 μM each), dNTP (100 μM each), DNA polymerase I large (Klenow) fragment (1 U μg^{-1} DNA) in a final volume of 50 μL. The reaction was incubated at 25 °C for 15 min, quenched with EDTA (10 mM) at 75 °C for 25 min, and dsDNA $tRNA^{Glu}$ template was precipitated with cold EtOH overnight. In vitro transcription was performed using a previously described method.[7] The transcribed $tRNA^{Glu}$ was then purified by acidic phenol extraction using a previously described method.[8]

In vitro dehydration of 2. The following reaction conditions were used for dehydration assays: HEPES pH 7.5 (100 mM), $MgCl_2$ (5 mM), KCl (150 mM), glutamate (10 mM), ATP (5 mM), TbtA hexazole (50 μM), *T. bispora* $tRNA^{Glu}$ (10 μM), *E. coli* GluRS (10 μM), TbtB (5 μM), TbtC (5 μM), and thermostable inorganic pyrophosphatase (TIPP; 0.02 U μL^{-1}) in a final volume of 25 μL. The reaction mixture was incubated at 30 °C for 3 h, centrifuged to remove insoluble material (14,000 × *g*, 5 min, 25 °C), and desalted using C18 ZipTips (EMD Millipore). The sample was mixed in a 1:1 ratio with 2,5-dihydroxybenzoic acid matrix, spotted on a Bruker MALDI plate, and analyzed by MALDI-TOF-MS.

Purification of 3. The reaction mixtures to produce **3** were lyophilized, and the residue was extracted with a volume of 40% aq. MeCN equal to the volume used for the dehydration reaction. The insoluble material was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was purified on a Shimadzu Prominence Preparative Liquid Chromatography system equipped with a Phenomenex Luna C18 column (250 × 10 mm, 10 μm particle size, 100 Å pore size). Acetonitrile and 10 mM aq. NH_4HCO_3 were used as the mobile phases, and a gradient of 2-80% aq. MeCN over 45 min at 1 mL/min was used for separation.

Purification of TbtA-hexazole-tetradehydrate core. HPLC-purified TbtA-hexazole-tetradehydrate was dissolved in 50 mM Tris-HCl (pH 7.5) to a final concentration of 100 μM. GluC endoproteinase was added to a concentration of 0.02 mg/mL, and the reaction mixture was incubated at 37 °C for 1 h, followed by addition of MeCN to 50%

(v/v) to precipitate proteins. The insoluble material was removed by centrifugation at 16,100 × *g* for 10 min, and the supernatant was purified on an Agilent 1260 Infinity HPLC system with a Phenomenex Luna C18 column (250 × 4.6 mm, 10 μm particle size, 100 Å pore size). Acetonitrile and 10 mM NH_4HCO_3 were used as the mobile phases, and a gradient of 2–80% aq. MeCN over 45 min at 1 mL/min was used for separation.

In vitro production of 4. Purified **2** was dissolved in DMSO to a concentration of 1 mM. The in vitro production of macrocycle was performed using: HEPES pH 7.5 (100 mM), $MgCl_2$ (5 mM), KCl (150 mM), ATP (5 mM), **2** (100 μM), *T. bispora* $tRNA^{Glu}$ (10 μM), *E. coli* GluRS (10 μM), TbtB (5 μM), TbtC (5 μM), MBP-TbtD (5 μM), and TIPP (0.02 U μL^{-1}). The reaction mixture was allowed to proceed at 30 °C for 4 h.

Purification of 4 (thiomuracin GZ). Reaction mixtures containing crude **4** were lyophilized and extracted with an equal volume of dimethylformamide (DMF) with respect to the original volume of the reaction by heating to 50 °C with occasional agitation for 30 min. The insoluble material was removed by centrifugation at 18,000 × *g* for 15 min. The supernatant was removed and evaporated in a Thermo Scientific Savant SpeedVac system. The resulting material was resuspended in 80% aq. MeCN prior to 3:1 dilution with H_2O. The dissolved material was then purified using a Flexar HPLC system (PerkinElmer) equipped with a Betasil C18 column (4.6 × 250 mm, 5 μm particle size, 100 Å pore size; Thermo Scientific) operating at 1 mL/min. Following a 10 minute hold at 20% aq. MeCN, separation was achieved with a gradient of 20-90% aq. MeCN over 45 min and monitoring at the λ_{max} for thiomuracin GZ of 315 nm. Collected fractions were confirmed to contain thiomuracin GZ through MALDI-TOF MS (*m/z* 1366 Da) analysis as previously described. The appropriate fractions were pooled and dried using the SpeedVac evaporator to yield purified thiomuracin GZ. A reaction using 20.0 mg **2** yielded 1.3 mg of purified thiomuracin GZ, representing a yield of 27% for this step and an overall unoptimized yield of 21% for all biosynthetic steps.

NMR spectroscopy. Samples were prepared by dissolving ca. 1.1 mg of thiomuracin GZ (HPLC-purified and lyophilized) in 180 μL of DMSO-d_6 (99.96 atom % D; Sigma-Aldrich). NMR spectra were obtained on a Bruker 900 MHz AVANCE NMR spectrometer equipped with an inverse 5 mm TCI cryogenic probe with z-axis pulsed field gradient (pfg) capability. Samples were held at 298 K during acquisition. Standard Bruker pulse sequences were used for each of the following experiments: ^{1}H, ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY (60 ms mixing time), ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC (optimized for 6 Hz heteronuclear couplings), and ^{1}H-^{1}H NOESY (750 ms mixing time). Spectra were recorded with Bruker TopSpin 1.3 software, and data was processed using MestReNova 8.1.1. Chemical shifts (δ, ppm) were referenced internally to the solvent peak (DMSO).

Evaluation of thiomuracin GZ antibiotic activity. *Escherichia coli* MC4100, *Pseudomonas aeuroginosa* PA01, *Bacillus anthracis* strain Sterne, *Mycobacterium smegmatis* B-14616, *Enterococcus faecium* U503, and *Staphylococcus aureus* USA300 were grown in 10 mL of brain-heart infusion (BHI) medium at 37 °C. The cultures were adjusted to an OD_{600} of 0.015 in the designated medium before being added to 96-well microplates. Successive two-fold dilutions of thiomuracin GZ in DMSO were added to the cultures (0.03–64 μg/mL). As a positive control, kanamycin (1–32 μg/mL) was added to samples of *E. coli*, *B. anthracis*, and *P. aeruginosa*. Gentamicin was used a positive control for *S. aureus* and *E. faecium* while streptomycin was used for *M. smegmatis*. As a negative control, 1% (v/v) DMSO was added to samples of each strain. Plates were covered and incubated at 37 °C for 20 h with shaking at 180 rpm. The minimum inhibitory concentration (MIC) reported are the values which suppressed all visible growth (n = 3).

Evaluation of thiomuracin GZ antifungal activity. *Saccharomyces cerevisiae*, *Talaromyces stipitatus*, and *Aspergillus niger* were grown for 36 h in 6 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose) at 30 °C. *S. cerevisiae* cultures were diluted to OD_{600} of 0.015 in YPD medium before being added to 96-well microplates. *T. stipitatus* and *A. niger* were not diluted prior to distribution on 96-well microplates. Successive two-fold dilutions of thiomuracin GZ in DMSO were added to cultures (1–128 μg/mL). As a positive control, amphotericin B was added to cultures in dilutions of 0.5–8 μg/mL. As a negative control, 1% DMSO (v/v) was added to samples of each fungal species. Plates were covered and incubated at 30 °C for 36 h with shaking at 180 rpm. The reported MICs are the values which suppressed all visible growth (n = 3).

UPLC-MS of purified thiomuracin GZ. Purified thiomuracin GZ was diluted in 20% aq. MeCN to 10 μM, and LC-MS was performed using a Waters SYNAPT mass spectrometer outfitted with an ACQUITY UPLC, an ACQUITY Bridged Ethyl Hybrid C8 column (2.1 × 50 mm, 1.7 μm particle size, 200 Å; Waters), an ESI ion source, and a quadruple TOF detector. A gradient of 10–90% aq. MeCN with 0.1% formic acid (v/v) over 15 min was used, and the total ion chromatogram (TIC) was recorded (**Figure S8**).

HPLC of purified thiomuracin GZ. 50 μg of purified thiomuracin GZ was diluted in 20% aq. MeCN to a final concentration of 37 μM, and HPLC was performed using a Flexar HPLC system (PerkinElmer) equipped with a BetaBasic C18 column (4.6 × 250 mm; 5 μm particle size; 100 Å pore size; Thermo Scientific) operating at 1 mL/min. Following a 10 minute hold at 20% aq. MeCN, separation was achieved with a gradient of 20–90% MeCN over 45 min; purity was assessed by monitoring 220 and 315 nm (**Figure S8**).

UPLC-MS and MS/MS of ejected His_6-TbtA leader. Purified ejected His_6-TbtA leader after the macrocylization reaction was dissolved in water, and LC-MS/MS was performed using the same procedure as above. A gradient of 2–100% aq. MeCN with 0.1% formic acid (v/v) over 20 min was used. A ramping of cone voltage of 16–19 kV during the scan was performed to generate peptide fragments for MS/MS analysis.

Kinetic assay to probe if TbtB/TbtF accelerate the macrocyclization reaction. HPLC-purified TbtA-hexazole-tetradehydrate was dissolved to a concentration of 200 μM in 50 mM Tris-HCl, 150 mM KCl, pH 7.5. Before starting the reaction, enzymes and the substrate were incubated at 30 °C for 5 min. MBP-TbtF was pre-treated with thrombin in order to release the MBP tag. The reaction was performed with 150 μM of substrate **3** and 5 μM of either protein at 30 °C. Macrocycle formation was monitored by absorbance at 315 nm. Time points were collected every 2 s and initial reaction rates were calculated.

Bioinformatic identification of TbtD residues for mutagenesis. The protein sequence for TbtD (gi: 915296818) was used to identify homologs via BLAST searching. Homologs with E-values < 5E-50 were aligned by sequence and highly conserved residues were targeted for alanine replacement.

Enzymatic activity of alanine-substituted TbtD proteins. The activity of the TbtD mutant proteins were assessed under two different reaction conditions with the first using a relatively high concentration of enzyme and substrate and the second using significantly lower concentrations. For the "high" concentration reaction conditions, tandem dehydration (TbtB/C) and macrocyclization (MBP-TbtD) reactions were set up identically as previously, with the exception that 100 μM of **2** and 5 μM of TbtD mutant proteins were employed. For the "lower" concentration conditions, 15 μM of **2** and 1 μM of TbtD mutant proteins were used. Samples were quenched by the addition of four volumes of MeCN and evaporation to dryness in a Savant SpeedVac system. Samples were then resuspended in a volume of saturated, aqueous guanidine-HCl equal to the original reaction volume, desalted using C18 ZipTips and eluted in a saturated solution of sinapinic acid in 50% aq. MeCN. Each reaction was repeated three times, and the relative peak heights of starting material (**2**) and product (**4**) were compared by MALDI-TOF-MS.

Enzymatic activity of selected TbtD mutant proteins. Seven TbtD mutants were analyzed in more detail by monitoring the peak area of the EIC (extracted-ion chromatogram) of the macrocycle (ESI, *m/z* 1366.2608) produced over time. Experiments with macrocycle standard solutions under the same conditions of the enzymatic activity assay showed that the peak area of the EIC of the macrocycle responded linearly to the macrocycle concentration in a 0.1-25 μM range. The following reaction conditions were used for the assay: HEPES pH 7.5 (50 mM), 15 μM **3**, and 1 μM MBP-TbtD mutant enzyme. The reaction was allowed to proceed at 30 °C, and was quenched by addition of MeCN to 50% (v/v) at different time points. The insoluble material was removed by centrifugation at 16,100 × *g* for 5 min, and the supernatant was subjected to LC-MS using a Waters SYNAPT mass spectrometer outfitted with an ACQUITY UPLC, an ACQUITY Bridged Ethyl Hybrid C18 column (2.1 × 50 mm, 1.7 μm particle size, 200 Å; Waters), an ESI ion source, and a quadruple TOF detector. A gradient of 3–100% aq. MeCN with 0.1% formic acid (v/v) over 20 min was used, and the EIC was recorded.

Table S1: Sequence of *tbt* biosynthetic genes for optimal *E. coli* expression. All sequences are provided 5′ to 3′. Restriction sites for cloning are underlined (5′ BamHI, 3′ XhoI). These gene constructs were synthesized by GenScript (Piscataway, NJ, USA).

tbtA

GGATCCATGGACCTGAATGATCTGCCGATGGATGTTTTTGAACTGGCAGATAGCGGTGTTGCAGTTGAAAGCCTGACCGCAGGT
CATGGTATGACCGAAGTTGGTGCAAGCTGTAATTGCTTTTGTTATATTTGTTGTAGCTGCAGCAGCGCCTAACTCGAG

tbtB

GGATCCATGCGTCTGGTGGAACGTCGCTTTCCGGTTGCCATGACCTCGACGGCTCCGAAAGTCGCAGTGCGTGAATGCGGTCTG
CCTGTGAGCGCGATTGAATCTCTGTGCTGTACCGATAGCTTCGCACTGATCCGTCGCCAGGTTCGCGAAACGGCGTGGCTGAAA
GGCGAAGGTAAACGTCTGGCCGTGGATCTGGGCCTGCTGATTGGTGAACGTGGTGACGGTGATGATGGTCTGCGTCCGGTTCTG
GTCGGTCTGCGTCGCGCACTGCATACCGGTCGTCTGCCGGATGCACGCGAATGGACGCCGCGTGTGGCATCTGCTCTGCCGGCA
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GCTGAAGCACGCGTTAAAGAAAAAGTCCTGGCACAGGTGGCAGCCGATCCGGGTTTCCGTCGCGCTCTGAGTCTGGCATCCCCG
GAACTGGCAGCTGATCTGGACCGTTGGCTGGCAGAACCGGCACGTCGCCCGAAAACCCAAAAACTGCTGCGCCTGGCCAAATAT
GTTGCACGTGCGGCCGTCAAAACGTCACCGTACTCGACCTTTACGAGCATGGGTGTGGCCGTTTGGGAAAACGGCGAAGATTGG
GCAGACGGTGCTATTGTTCGTTTCGCACCGCGCGAACCGCCGTCAGTCATCCTGGAACCGTCGGGTGAATGGCTGCATGGTGCA
CTGCGTGCATGGCTGGCACGTCCGGAAAACCTGGTGCGCAGCCGTCTGCGCCTGAATCCGTCTCTGGTTATTCGCGCTGATAAA
GCGGAATTTCTGGGCTTCCCGCCGCGCGAACCGATTATCCGTATGGGTCTGACCCCGGTGGTTGCAACGGTTCTGCGTCTGGCA
GAACCGGCAGCTGATGCTGACGGCTGGATCGATCCGATGGGTTTTCGTGATCGTCTGGCTCGTGACCTGCCGGCAGAACCGGAA
CAGGTCGACCGTCTGCTGCGCTCCCTGATTGAAGCCGGCGTGCTGGAAGCACACCCGCTGACCCGTGCGGGTCTGCCGGAAACG
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CTGCCGATTCGCATCGTGGTTGCCGAATATCTGCGTGCACGTTACGGTGAACACGCCCGCGTGCCGTTTCTGACCTTCCATCGT
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CCGCCGCTGGCACATAGCCGCCTGCCGCGTCTGCGTGAACTGGCAAAACTGCGTGAAGCAGCCCGCGAACTGGCACTGGGTCGT
CCGGAACACGATGGTATTCAGCGTGTGGACCCGGAAGAACTGATCAAACAAATGGCGACCTGGCCGGAATGGATTGTCGTGCCG
CGTTCTTGCGCGTGTTATGTCCAGCCGGCACCGGAAGGCCGCCTGGTGCTGAACGTTGTCCATGGCGGTCACGGTCGTGGTCTG
CGTCGCCTGAGTCATCTGATCGGCCGTGTGCGCGGTGAAGCGGTTGATCACCCGATGGTCGCCGACGAACCGGAAGGTACCGTG
TACGCGGAACTGAGCGGCTCTCTGGGTTCAACCCTGAATGTCCATGTGCCGGGCACGCGTTATGAAATTGATTACCCGTTTAGT
CCGGGTGATCGTTCCCGCGACCGTCGCCTGCCGCTGTCTGACCTGGAAGTGGTTCTGGCACCGGAAACCGGTCTGGCTGAACTG
CGCAGTCGTCGCCTGGGCTTTCGTGTTATTCCGCTGCATCTGGGTATGGCAGCTGAATTCCAGCTGCCGCCGGCAGCACGTTTT
CTGGAACGTGCCTTCGGCGTGACCTATCTGCTGCACCCGAGTGCACCGCCGCTGCTGCGTATCGGCGAAGTGCCGCCGCCGCAG
GAAGTGACGCGTTACCCGCGCGTTGAAGTCGGTCGTGTCGTGGTTCAACGTCGCCGTTGGCTGGCACCGGCAGGTACCCTGCCG
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tbtC

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tbtD

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tbtE

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tbtF

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tbtG

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Table S2: Oligonucleotide primers used in this study. All sequences are provided 5′ to 3′. F indicates a forward primer while R indicates the reverse. Lowercase m indicates 2′ *O*-methylation of the following residue; methylation suppresses random addition of bases at the end of the RNA by T7 RNA polymerase.[1]

Primer Name	Oligonucleotide Sequence
mbp F (sequencing)	GAGGAAGAGTTGGCGAAAGATCCACGTA
T7 F (sequencing)	TAATACGACTCACTATAGGG
T7 R (sequencing)	GCTAGTTATTGCTCAGCGG
tbtA Gibson F	GCATCACCATCATCACCACAGCCAGATGGACCTGAATGATCTGCC
tbtA Gibson R	GACTTAAGCATTATGCGGCCGCATTAGGCGCTGCTGCAG
tbtB Gibson F	CTGGTGGACAGCAAATGGGTCGCGGAATGCGTCTGGTGGAAC
tbtB Gibson R	GTGGTGGTGCTCGAGTGCGGCCGCATTATTCACCCAGTTCAACCAG
tbtC Gibson F	CTGGTGGACAGCAAATGGGTCGCGGAATGACCCCGCACGAAG
tbtC Gibson R	GTGGTGGTGCTCGAGTGCGGCCGCATTAAACGGTCACTTCCGCTTC
E. coli gluRS Gibson F	CTGGTGGACAGCAAATGGGTCGCGGAATGAAAATCAAAACTCGCTTC
E. coli gluRS Gibson R	GTGGTGGTGCTCGAGTGCGGCCGCTTACTGCTGATTTTCGCG
E. coli tRNA F	AATTCCTGCAGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACC
E. coli tRNA R	mUmGGCGTCCCCTAGGGGATTCGAACCCCTGTTACCGCCGTGAAAGGGCGGTGTCCTGG
T. bispora tRNA(CUC) F	AATTCCTGCAGTAATACGACTCACTATAGGTCCCGTCGTCTAGAGGCCTAGGACGC
T. bispora tRNA(CUC) R	mUmGGTAGTCCCGAGCGGATTCGAACCGCCGTTACCGCCTTGAGAGGGCGGCGTCCTAGG
T. bispora tRNA(UUC) F	AATTCCTGCAGTAATACGACTCACTATAGCCCCCATCGTCTAGCGGTCTAGGACACCGCC
T. bispora tRNA(UUC) R	mUmGGTGCCCCCAACGGGATTCGAACCCGTGCCGCCGCCTTGAAAGGGCGGTGTCC
tbtG Internal	GTGATACCCAAGGCGGTCT
tbtF Internal	TGCACATCACTTTATTCCGCA
tbtB Internal 702	CTTTACGAGCATGGGTGTGG
tbtB Internal 1496	TCCATCGTCACGTTCAGGAA
tbtB Internal 2116	GTTATTCCGCTGCATCTGG
pbtD (BamHI) F	AAGGATCCGTGACCTGGCGACGCTTTGAC
pbtD (XhoI) R	AACTCGAGTCACATGCCTCCTACGGCGATC
tbtD H46A F	ATTTTCTGCGTGCTTGGCGTCGCGGTCCGCACCTGCGCATTTAC
tbtD H46A R	CCGCGACGCCAAGCACGCAGAAAATATGCCATCGGTGCCTG
tbtD W47A F	TTCTGCGTCATGCGCGTCGCGGTCCGCACCTGCGCATTTAC
tbtD W47A R	GGACCGCGACGCGCATGACGCAGAAAATATGCCATCGGTGC
tbtD R49A F	GTCATTGGCGTGCCGGTCCGCACCTGCGCATTTACGTTAG
tbtD R49A R	CAGGTGCGGACCGGCACGCCAATGACGCAGAAAATATGCCATC
tbtD H52A F	GTCGCGGTCCGGCCCTGCGCATTTACGTTAGTACCACGCGTG
tbtD H52A R	GTAAATGCGCAGGGCCGGACCGCGACGCCAATGACGCAG
tbtD R54A F	GTCCGCACCTGGCCATTTACGTTAGTACCACGCGTGAAGCAC
tbtD R54A R	CTAACGTAAATGGCCAGGTGCGGACCGCGACGCCAATGACG
tbtD S85A F	GTGCACGTCCGGCTCCGGGTATGGCTGATCCGTCCGCGTTC
tbtD S85A R	GCCATACCCGGAGCCGGACGTGCACGCAGATAACCGCCCAC
tbtD H98A F	GTTCCTGCCGCTGGCTGAACGCCTGGCCGAACTGGAAGGCGAAG

tbtD H98A R	GCCAGGCGTTCAGCCAGCGGCAGGAACGCGGACGGATCAGC
tbtD E105A F	CTGGCCGAACTGGCAGGCGAAGATGGTCCGCTGATGCCGTGG
tbtD E105A R	CCATCTTCGCCTGCCAGTTCGGCCAGGCGTTCATGCAGCGG
tbtD N118A F	GGTCTCCGGACGCCACGATTCACGCAGAAGGTGAACGTCCGG
tbtD N118A R	GCGTGAATCGTGGCGTCCGGAGACCACGGCATCAGCGGACC
tbtD T144A F	TTTATGCAGACGCCACGCCGAGCGTTTACCACGCGCTGGAAC
tbtD T144A R	ACGCTCGGCGTGGCGTCTGCATAAAAATCGGCCAGCAGAAC
tbtD R189A F	GTACCTCACTGGCTTCGCACGCAGAAGCTTATCTGGCACGTC
tbtD R189A R	TCTGCGTGCGAAGCCAGTGAGGTACGTGCGACCGGCAGACC
tbtD S190A F	CCTCACTGCGTGCGCACGCAGAAGCTTATCTGGCACGTCGC
tbtD S190A R	GCTTCTGCGTGCGCACGCAGTGAGGTACGTGCGACCGGCAG
tbtD H191A F	CACTGCGTTCGGCCGCAGAAGCTTATCTGGCACGTCGCTCC
tbtD H191A R	TAAGCTTCTGCGGCCGAACGCAGTGAGGTACGTGCGACCGG
tbtD E193A F	GTTCGCACGCAGCAGCTTATCTGGCACGTCGCTCCGATGGTG
tbtD E193A R	GCCAGATAAGCTGCTGCGTGCGAACGCAGTGAGGTACGTGC
tbtD S287A F	TGGCTGAAGTTGCTGCGTTTCATCGCGAACTGGAAAGCCGTC
tbtD S287A R	CGATGAAACGCAGCAACTTCAGCCAGGCTCGGCAGATCGCG
tbtD H290A F	GTTTCTGCGTTTGCTCGCGAACTGGAAAGCCGTCCGGAATGG
tbtD H290A R	TCCAGTTCGCGAGCAAACGCAGAAACTTCAGCCAGGCTCGG
tbtD R291A F	CTGCGTTTCATGCCGAACTGGAAAGCCGTCCGGAATGGGCAC
tbtD R291A R	CTTTCCAGTTCGGCATGAAACGCAGAAACTTCAGCCAGGCTC
tbtD N316A F	GTCTGGTTATTGCTTGCACCTACCTGCACCTGACGCGTCTG
tbtD N316A R	CAGGTAGGTGCAAGCAATAACCAGACGATATGCGCCGAATGC
tbtD Y319A F	TTAATTGCACCGCCCTGCACCTGACGCGTCTGGGTCTGACC
tbtD Y319A R	GTCAGGTGCAGGGCGGTGCAATTAATAACCAGACGATATGC
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tbtD R324A R	GTCAGACCCAGAGCCGTCAGGTGCAGGTAGGTGCAATTAATAAC
tbtD R332A F	CCCCGCATCAAGCTTTCCTGGTCTGTCACCTGGCAGCAGATG
tbtD R332A R	CAGACCAGGAAAGCTTGATGCGGGGTCAGACCCAGACGCGTC
tbtD C336A F	GTTTCCTGGTCGCTCACCTGGCAGCAGATGCAGCTGCAGAC
tbtD C336A R	GCTGCCAGGTGAGCGACCAGGAAACGTTGATGCGGGGTCAG
tbtD H337A F	TCCTGGTCTGTGCCCTGGCAGCAGATGCAGCTGCAGACGTG
tbtD H337A R	TCTGCTGCCAGGGCACAGACCAGGAAACGTTGATGCGGGGTC

Figure S1: SDS-PAGE analysis of proteins used in this study. The minor impurity found at ~45 kDa is the result of endogenous protease activity on the MBP fusion proteins. Cleavage occurs in the linker region between MBP and the protein of interest. Enrichment occurs due to the use of amylose resin for affinity purification.

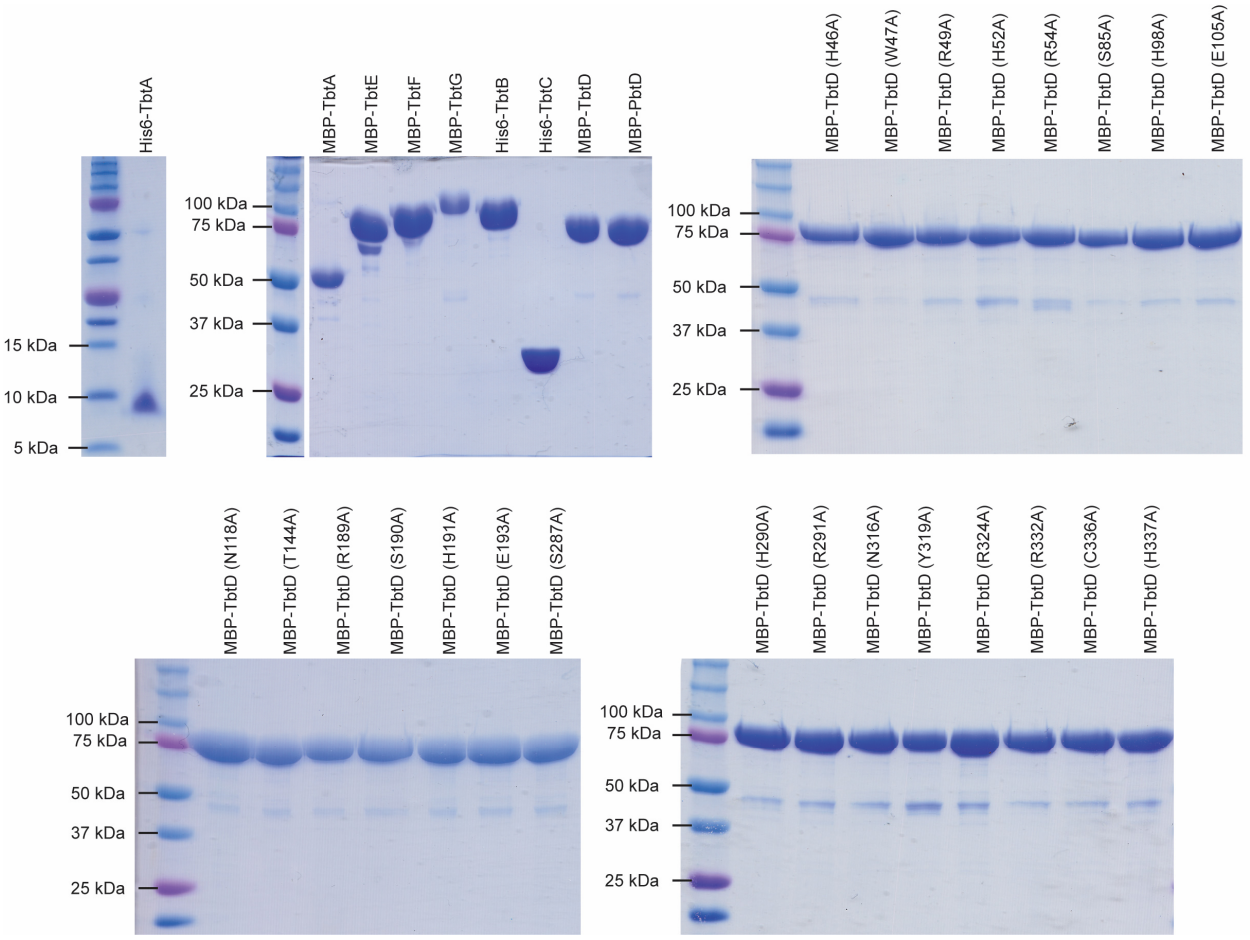


Figure S2: TbtB/C do not process unmodified TbtA. The top MALDI-TOF mass spectrum shows unmodified TbtA precursor peptide (**1**). The middle spectrum spectrum shows **1** treated with TbtB/C in the presence of other necessary reaction components [*E. coli* GluRS and *E. coli* $tRNA^{Glu}$]. The bottom spectrum is an equivalent reaction except with *T. bispora* $tRNA^{Glu}$ (CUC). These data suggest that the split LanB dehydratase (TbtB/C) requires a thiazol(in)e-containing substrate, implicating cyclodehydration (TbtF/G) and dehydrogenation (TbtE) as the initial two steps of thiopeptide biosynthesis.

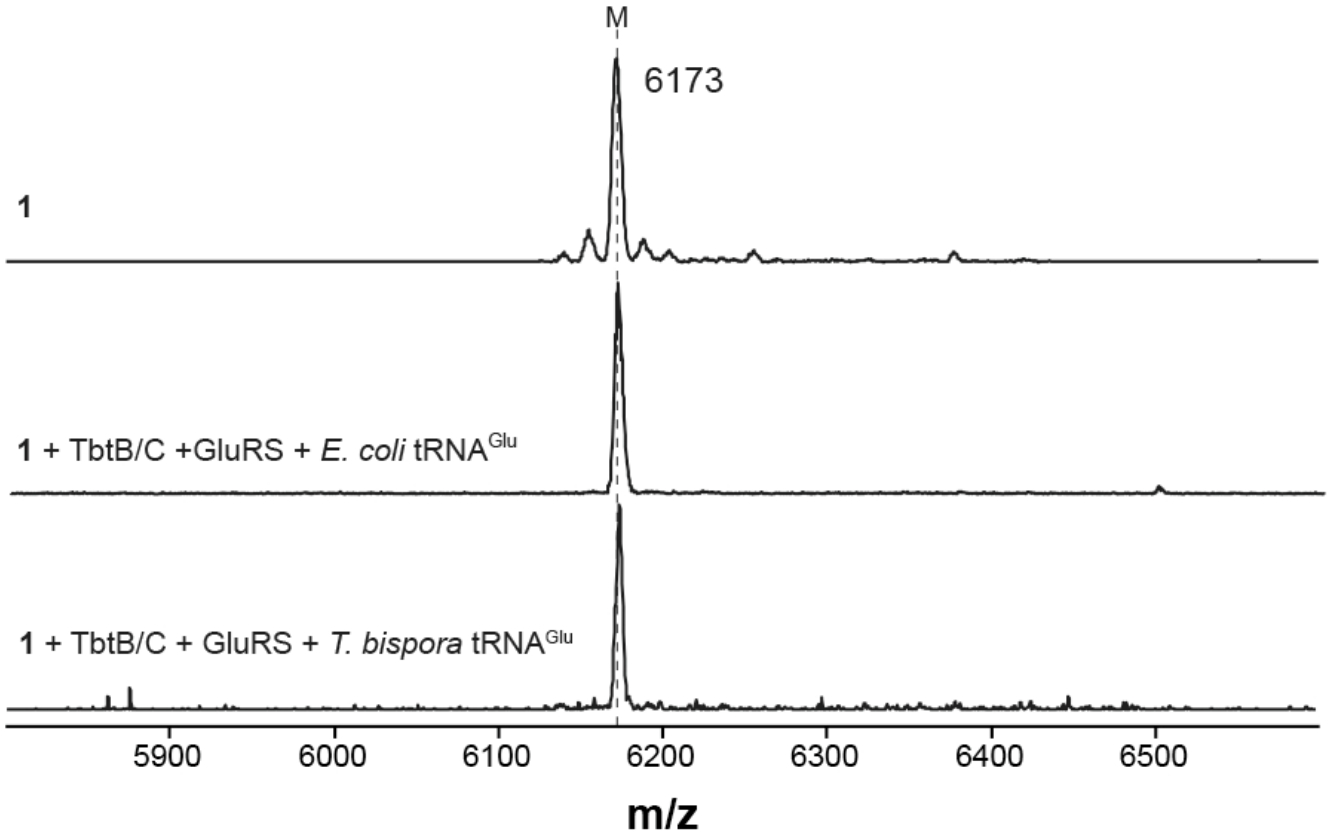


Figure S3: MALDI-TOF-MS analysis of TbtE/F/G reaction requirements. Unmodified TbtA (**1**) is shown in the top spectrum. Reaction of **1** with the F-protein dependent cyclodehydratase (TbtF/G) and ATP results in a six-fold cyclodehydrated product (hexa-thiazoline). Addition of the dehydrogenase (TbtE) to an otherwise identical reaction yields a 6-fold dehydrated and 6-fold dehydrogenated product (hexazole, **2**). Omission of ATP, a requirement of the cyclodehydratase, results in no product formation. Similarly, omission of either TbtF or TbtG results in no product formation. Residues undergoing thiazol(in)e formation in the complete reaction are colored blue in the sequence above the spectra.

His_6-TbtA: PHHHHHHSQVDLNDLPMDVFELADSGVAVESLTAGHGMTEVGA*SCNCFCYICCSCSSA

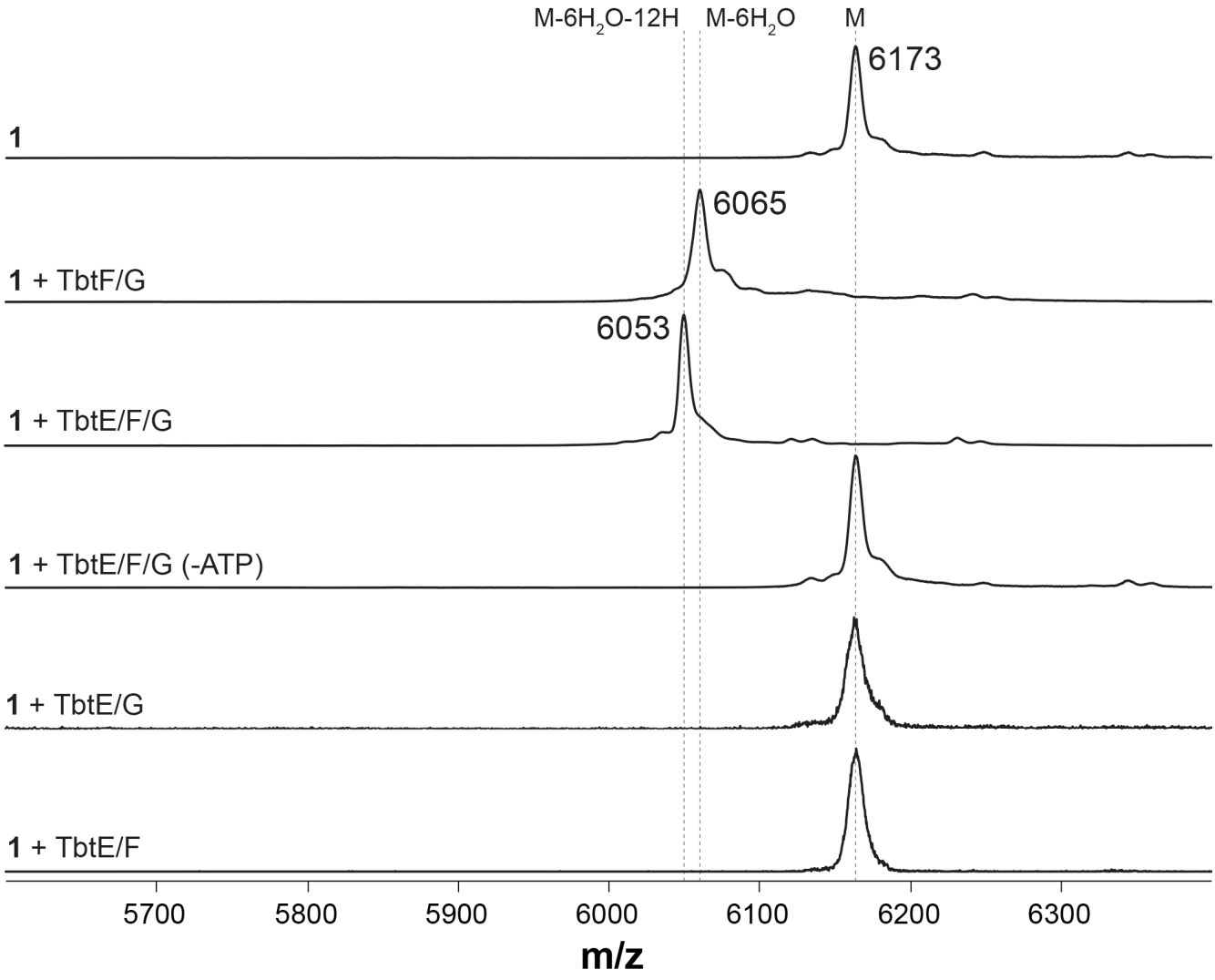
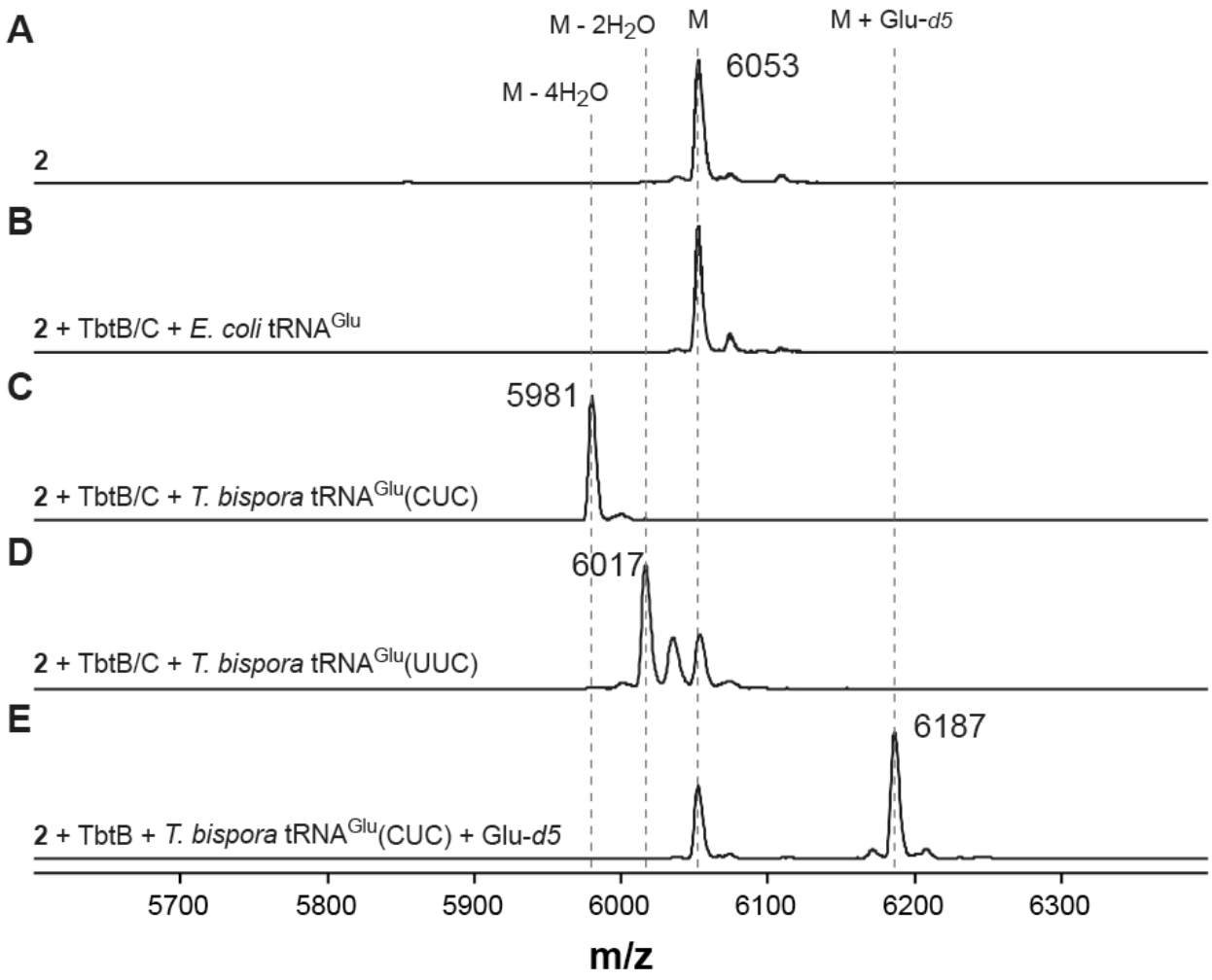


Figure S4: TbtB/C is selective for a particular tRNA^{Glu}. (A) MALDI-TOF mass spectrum of **2** (starting material). (B) *E. coli* tRNA^{Glu} (UUC) is not accepted by TbtB/C. (C) In contrast, four dehydrations are observed upon treating **2** with TbtB/C and tRNA^{Glu}(CUC) from *T. bispora*. (D) An identical reaction with the alternative tRNA^{Glu}(UUC) from *T. bispora* yielded a mixture of dehydrated species, primarily the didehydrated peptide. (E) A reaction that omitted TbtC (elimination domain), while employing the optimal tRNA and d_5-Glu, yielded a mono-glutamylated species. (F) Sequence alignment of the three pertinent tRNA^{Glu}. Top, *T. bispora* tRNA^{Glu}(CUC); middle, *T. bispora* tRNA^{Glu}(UUC); bottom, *E. coli* tRNA^{Glu}.

His$_6$-TbtA: PHHHHHHSQVDLNDLPMDVFELADSGVAVESLTAGHGMTEVGA*SCNCFCYICCSCSSA



F

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GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTTTCACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGCCA
*  *** ******** ** * ***** ******* *** *****   *   ***********  * ***    ***
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Figure S5: MALDI-TOF-MS analysis of TbtB/C reaction requirements. Shown in the top spectrum is hexazole **2**, which is the six-fold cyclodehydration and dehydrogenation product of TbtA. Reaction of **2** with the split LanB dehydratase (TbtB/C) in the presence of *E. coli* GluRS and *T. bispora* $tRNA^{Glu}$(CUC) results in a four-fold dehydrated product (**3**). The formation of **3** was dependent on the presence of all reaction components, as individual omission of TbtB/C, *E. coli* GluRS, or *T. bispora* $tRNA^{Glu}$(CUC) did not result in any change in mass. Residues modified to a thiazole are shown in blue, and residues that are transformed to dehydroalanine are colored green. All reactions also contained Glu, ATP, and $MgCl_2$ (see methods).

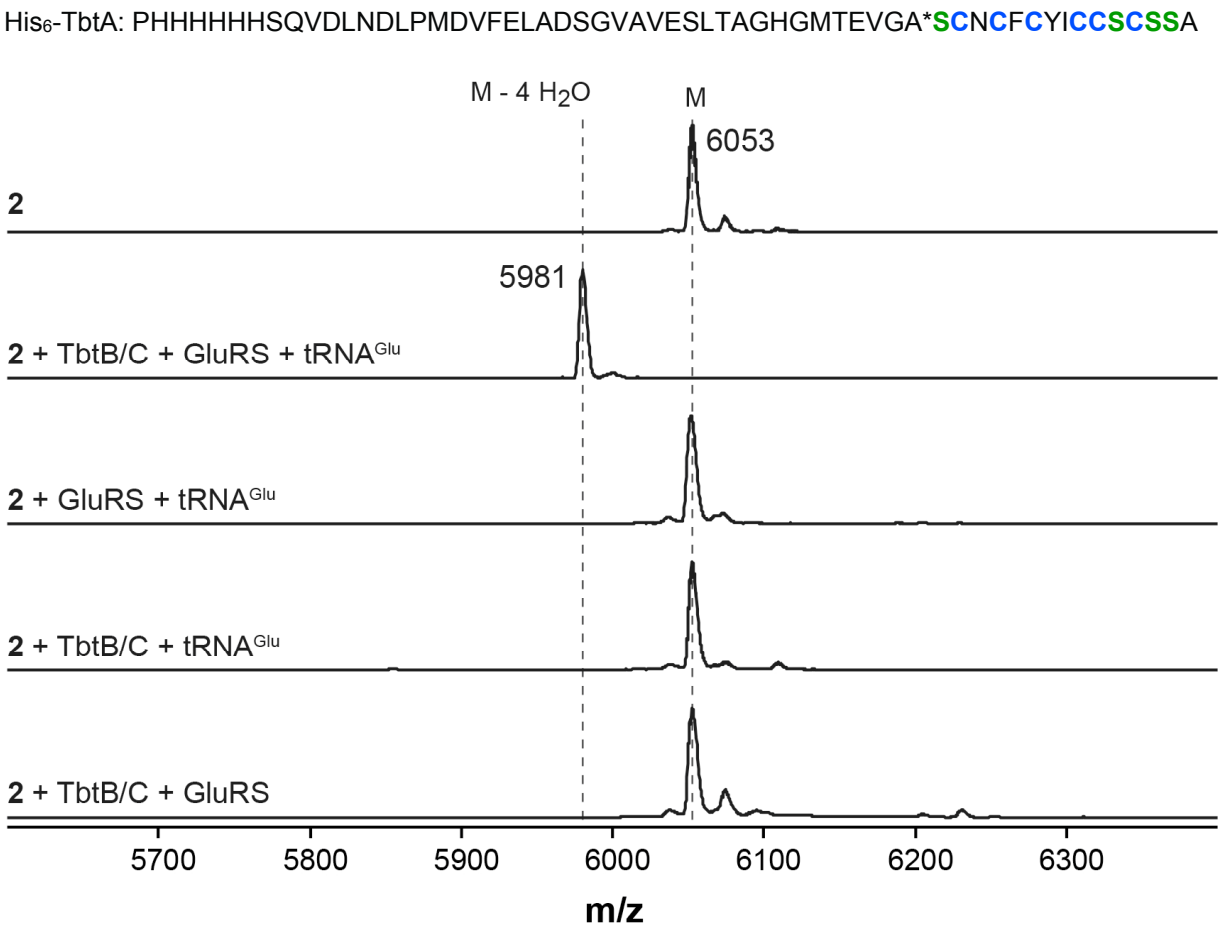


Figure S6: TbtD requires the leader peptide for activity. (A) HR-ESI-MS of the TbtA leader peptide generated by macrocyclization (theoretical $[M+H]^+$, 4593.143 Da, 0.4 ppm). The sequence of the His_6-TbtA-derived substrate **3** is given above panel A with modifications present color-coded (blue, thiazoles; green, dehydroalanines). The asterisk designates the end of the leader peptide. Shown italicized are additional N-terminal residues present on the substrate (His-tag and cloning remnants). (B) MALDI-TOF-MS of **3** after treatment with endoproteinase GluC. This treatment removes all but three residues (VGA, boldfaced and underlined) of the leader peptide. (C) Reaction of GluC-treated **3** with TbtD. No reaction was observed.

His_6-TbtA: *PHHHHHHSQ*VDLNDLPMDVFELADSGVAVESLTAGHGMTE**VGA*****SC**N**C**F**C**Y**C**I**CCSCSS**A

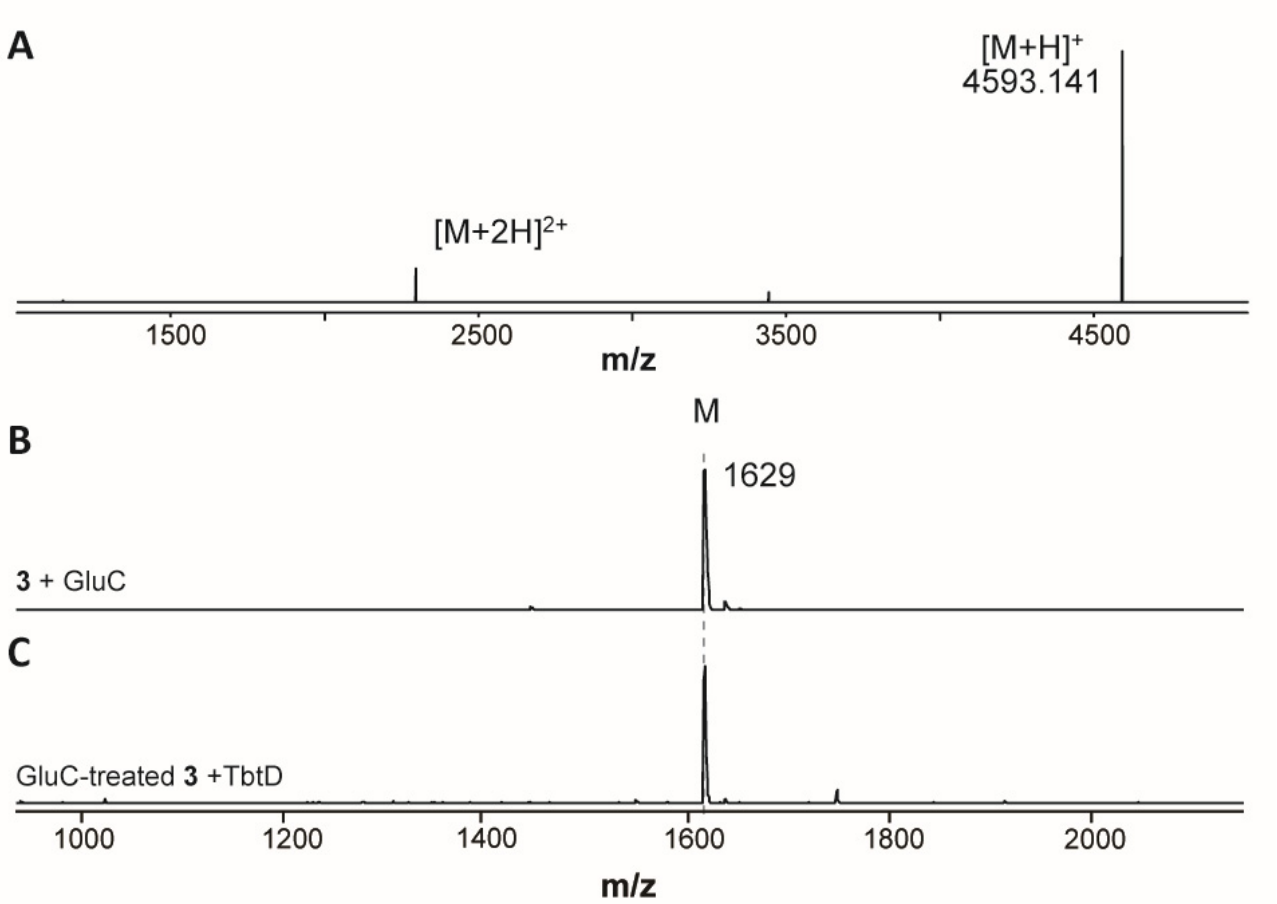


Figure S7: TbtD activity is not enhanced by known leader peptide-binding Tbt proteins. Addition of TbtB or TbtF did not increase the rate of macrocycle formation by TbtD as monitored by UV-Vis spectroscopy at 315 nm (owing to the generation of the tri-thiazole-substituted pyridine). Both TbtB and TbtF contain RiPP leader peptide Recognition Elements (RREs)[9] while TbtD does not appear to, despite its activity being leader peptide-dependent (Figure S6).

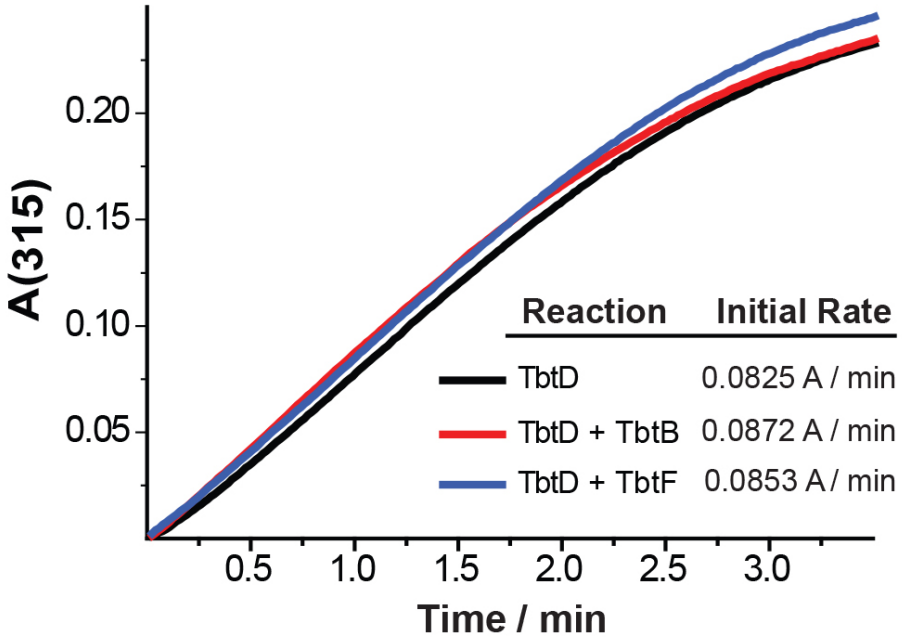


Figure S8: Purity analysis of **4** (macrocyclized thiomuracin GZ). (A) Total ion chromatogram (TIC) monitoring m/z 50–2000 of HPLC-purified **4**. (B) Extracted ion chromatogram of the *m/2* (EIC, *m/z* 683.61 Da) for **4**. (C) Analytical HPLC analysis of purified **4** showing UV traces for absorbance at 220 and 315 nm. Hydrolysis of the *C*-terminal dehydroalanine tail of **4** occurs on-column, as indicated by asterisks and verified by re-injection of the 42.4 min eluting product. Including the *in situ* hydrolysis peaks, the purity was judged to be ~95%, as measured by area-under-the-curve analysis. Retention times (min) are given for **4** and the *in situ* hydrolysis products.

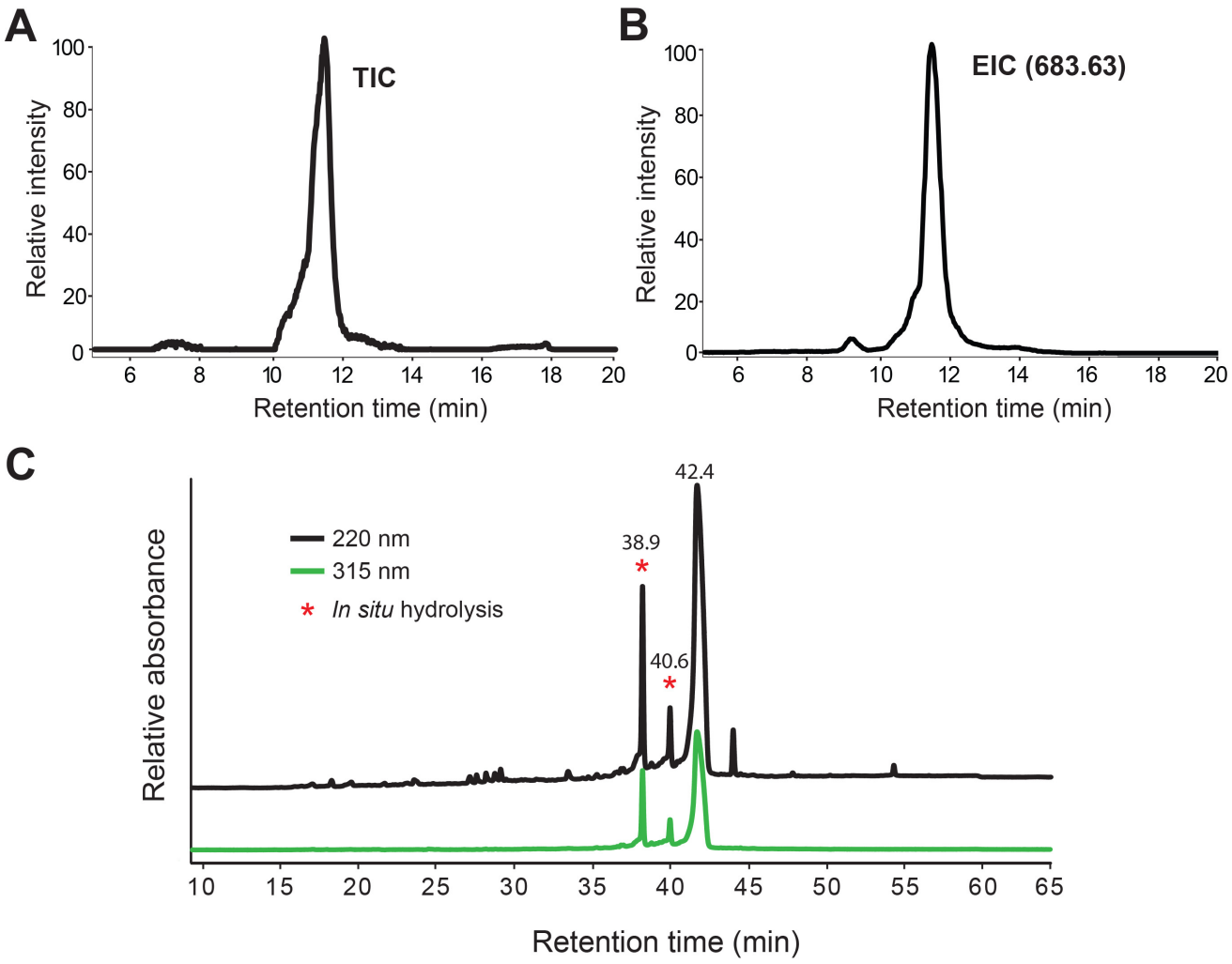


Figure S9: HR-ESI-MS and MS/MS analysis of **4** (macrocyclized product). (A) The molecular formula of the protonated form of **4** was deduced from HR-ESI-MS to be $C_{61}H_{56}N_{15}O_{11}S_6$. (B) Tandem MS of *m/z* 1366 Da gives a fragmentation pattern consistent with the expected structure.

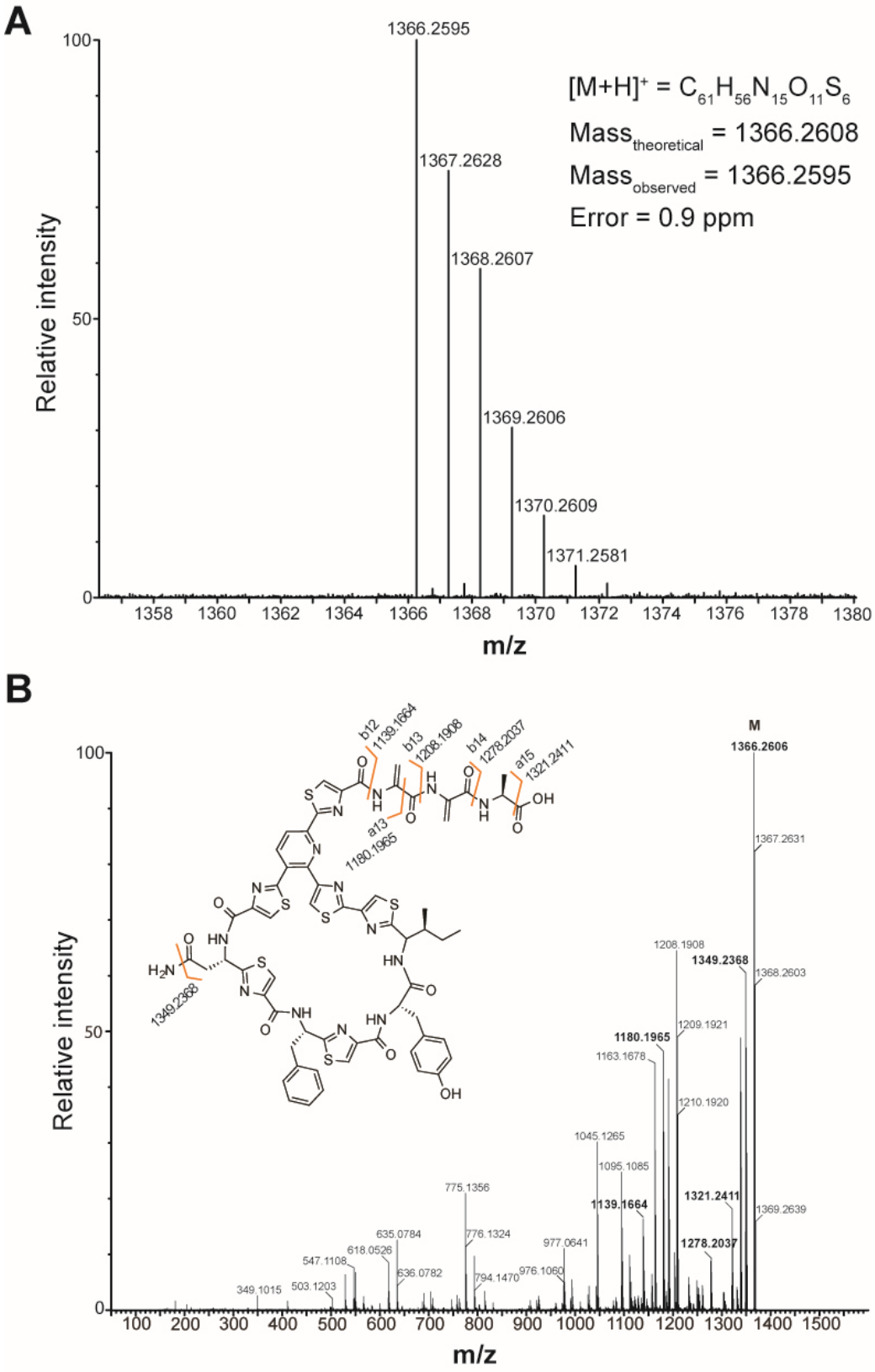


Figure S10: Structurally overlaid assignments for atom numbering and $^{1}H/^{13}C$ NMR chemical shifts of **4**.

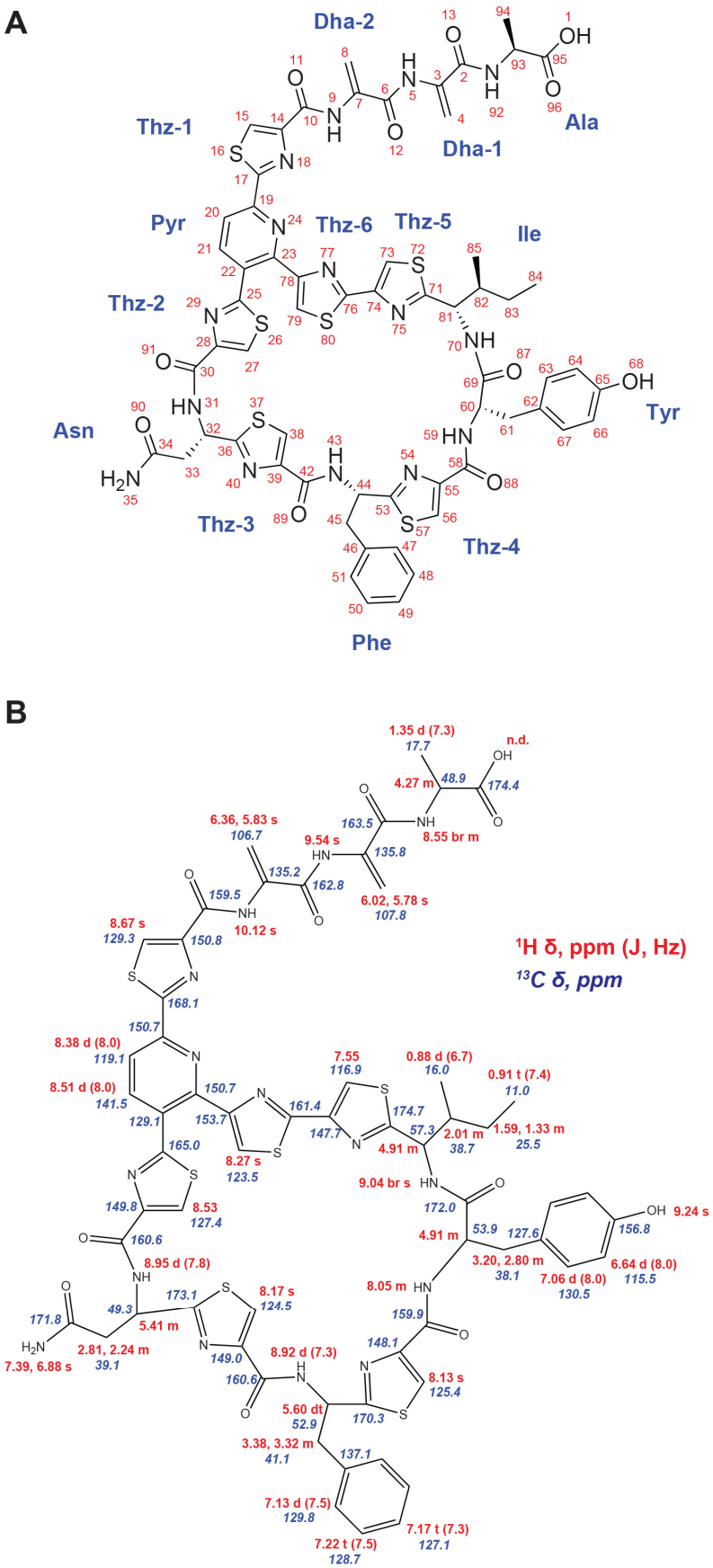


Table S3: ^{1}H and ^{13}C NMR chemical shift assignments of **4**.

Position	Group	^{13}C δ (ppm)	^{1}H δ (ppm)	m (J, Hz)	Residue
92	NH	—	8.55	br m	Ala
93	CH	48.9	4.27	m	
94	CH3	17.7	1.35	d (7.3)	
95	C=O	174.4	—	—	
2	C=O	163.5	—	—	Dha-1
3	C	135.8	—	—	
4	CH2	107.8	6.02	s	
			5.78	s	
5	NH	—	9.54	s	
6	C=O	162.8	—	—	Dha-2
7	C	135.2	—	—	
8	CH2	106.7	6.36	s	
			5.83	s	
9	NH	—	10.12	s	
10	C=O	159.5	—	—	Thz-1
14	C	150.8	—	—	
15	CH	129.3	8.67	s	
17	C	168.1	—	—	
19	C	150.7	—	—	Pyr
20	CH	119.1	8.38	d (8.0)	
21	CH	141.5	8.51	d (8.0)	
22	C	128.9	—	—	
23	C	150.7	—	—	
25	C	164.8	—	—	Thz-2
27	CH	127.4	8.53	s	
28	C	149.8	—	—	
30	C=O	160.6	—	—	
31	NH	—	8.95	d (7.8)	Asn
32	CH	49.3	5.41	m	
33	CH2	39.1	2.24	m	
			2.81	m	
34	C=O	171.8	—	—	
35	NH2	—	7.39	s	
			6.88	s	
36	C	173.1	—	—	Thz-3
38	CH	124.5	8.17	s	
39	C	149	—	—	
42	C=O	160.6	—	—	

Position	Group	^{13}C δ (ppm)	^{1}H δ (ppm)	m (J, Hz)	Residue
43	NH	—	8.92	d (7.3)	Phe
44	CH	52.9	5.60	q (7.0)	
45	CH2	41.1	3.38	m	
			3.32	m	
46	C	137.1	—	—	
47	CH	129.8	7.13	d (7.5)	
48	CH	128.7	7.22	t (7.5)	
49	CH	127.1	7.17	t (7.3)	
50	CH	128.7	7.22	t (7.5)	
51	CH	129.8	7.13	d (7.5)	
53	C	170.3	—	—	Thz-4
55	C	148.1	—	—	
56	CH	153.7	8.13	s	
58	C=O	159.9	—	—	
59	NH	—	8.05	m	Tyr
60	CH	53.9	4.91	m	
61	CH2	38.1	3.20	m	
			2.80	m	
62	C	127.6	—	—	
63	CH	130.5	7.06	d (obs)	
64	CH	115.5	6.64	d (8.0)	
65	C	156.8	—	—	
66	CH	115.5	6.64	d (8.0)	
67	CH	130.5	7.06	d (obs)	
68	OH	—	9.24	s	
69	C=O	160	—	—	
71	C	174.7	—	—	Thz-5
73	CH	116.9	7.55	s	
74	C	147.7	—	—	
76	C	161.4	—	—	Thz-6
78	C	153.7	—	—	
79	CH	123.5	8.27	s	
70	NH	—	9.04	br s	Ile
81	CH	57.3	4.91	m	
82	CH	38.7	2.01	m	
83	CH2	25.5	1.59	m	
			1.33	m	
84	CH3	11.0	0.91	t (7.4)	
85	CH3	16.0	0.88	d (6.7)	

Figure S11: ^{1}H NMR (900 MHz, DMSO-d_6) spectrum of **4**.

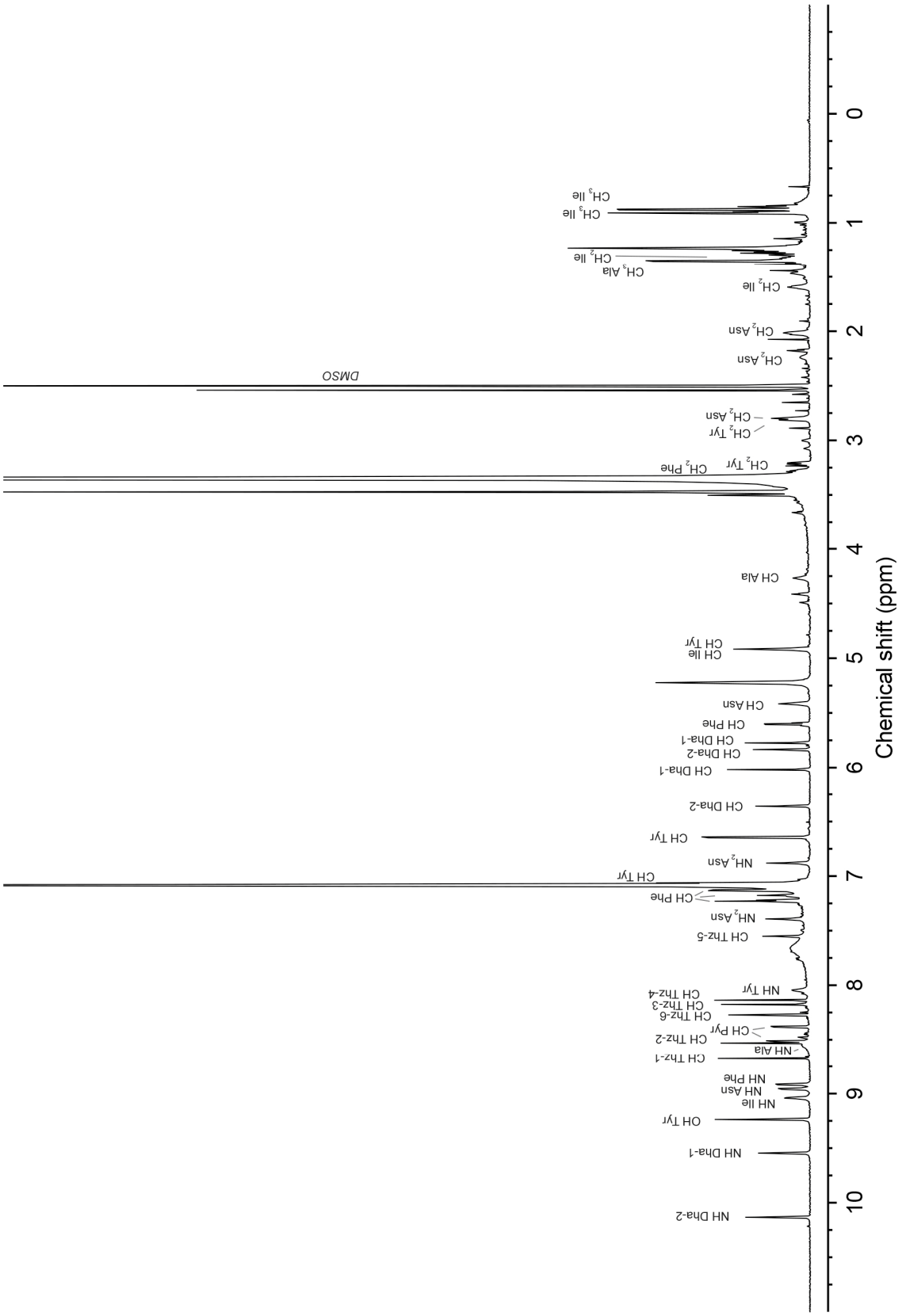


Figure S12: ^{1}H-^{1}H COSY (900 MHz, DMSO-d_6) spectrum of **4**.

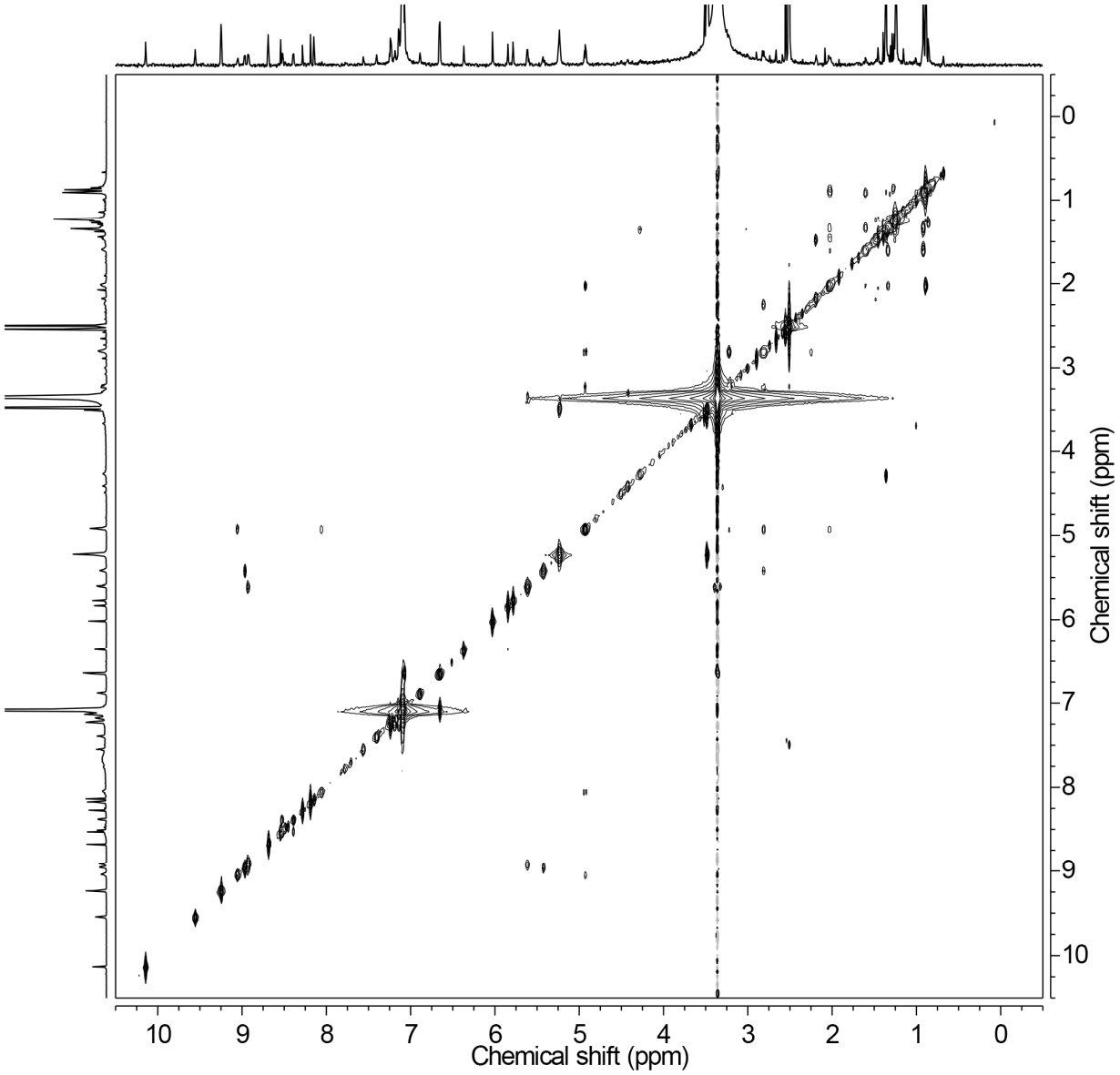


Figure S13: ^{1}H-^{1}H TOCSY (900 MHz, DMSO-d_6) spectrum of **4**.

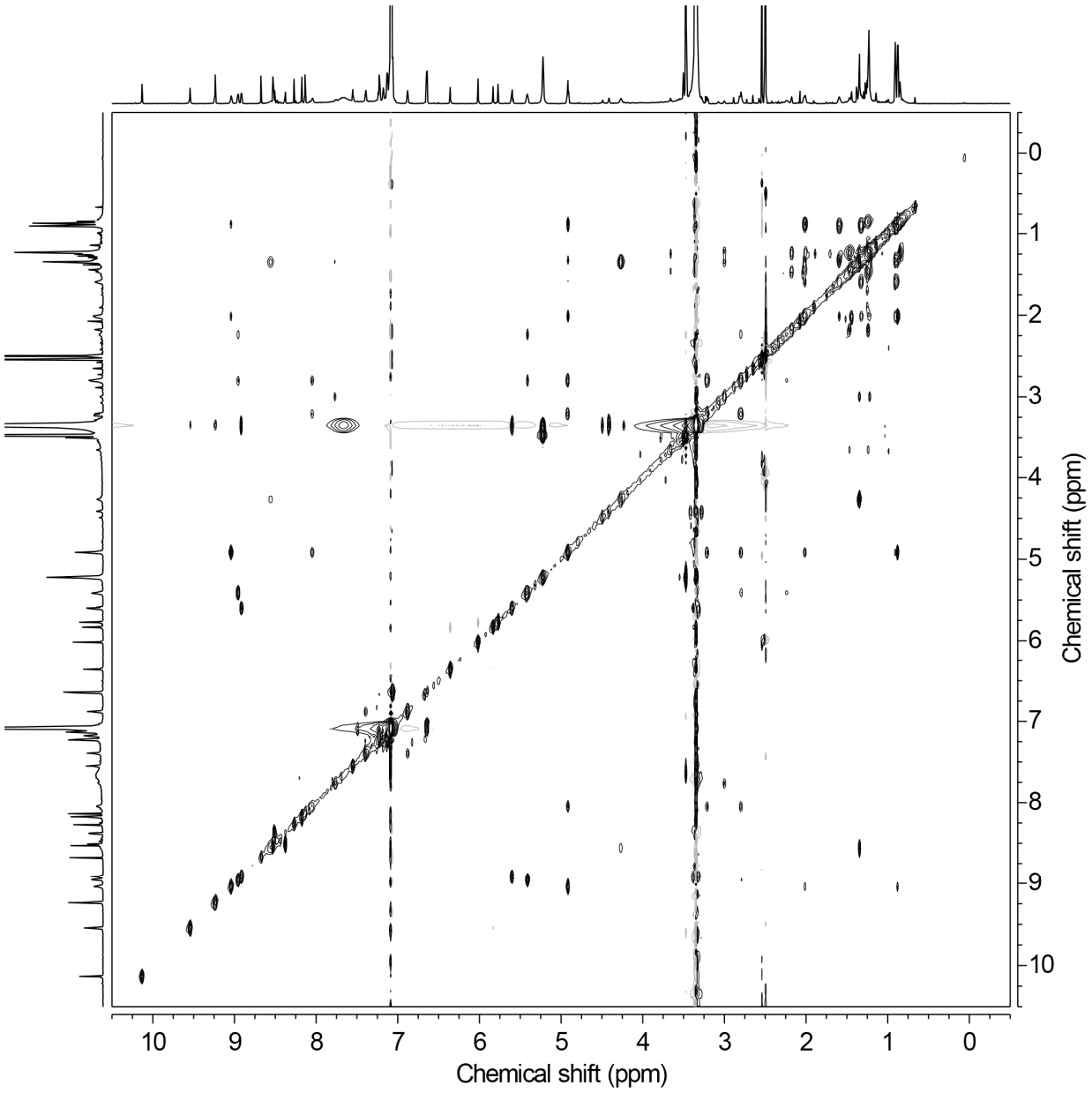


Figure S14: ^{1}H-^{13}C HMBC (900 MHz, DMSO-d_6) spectrum of **4**.

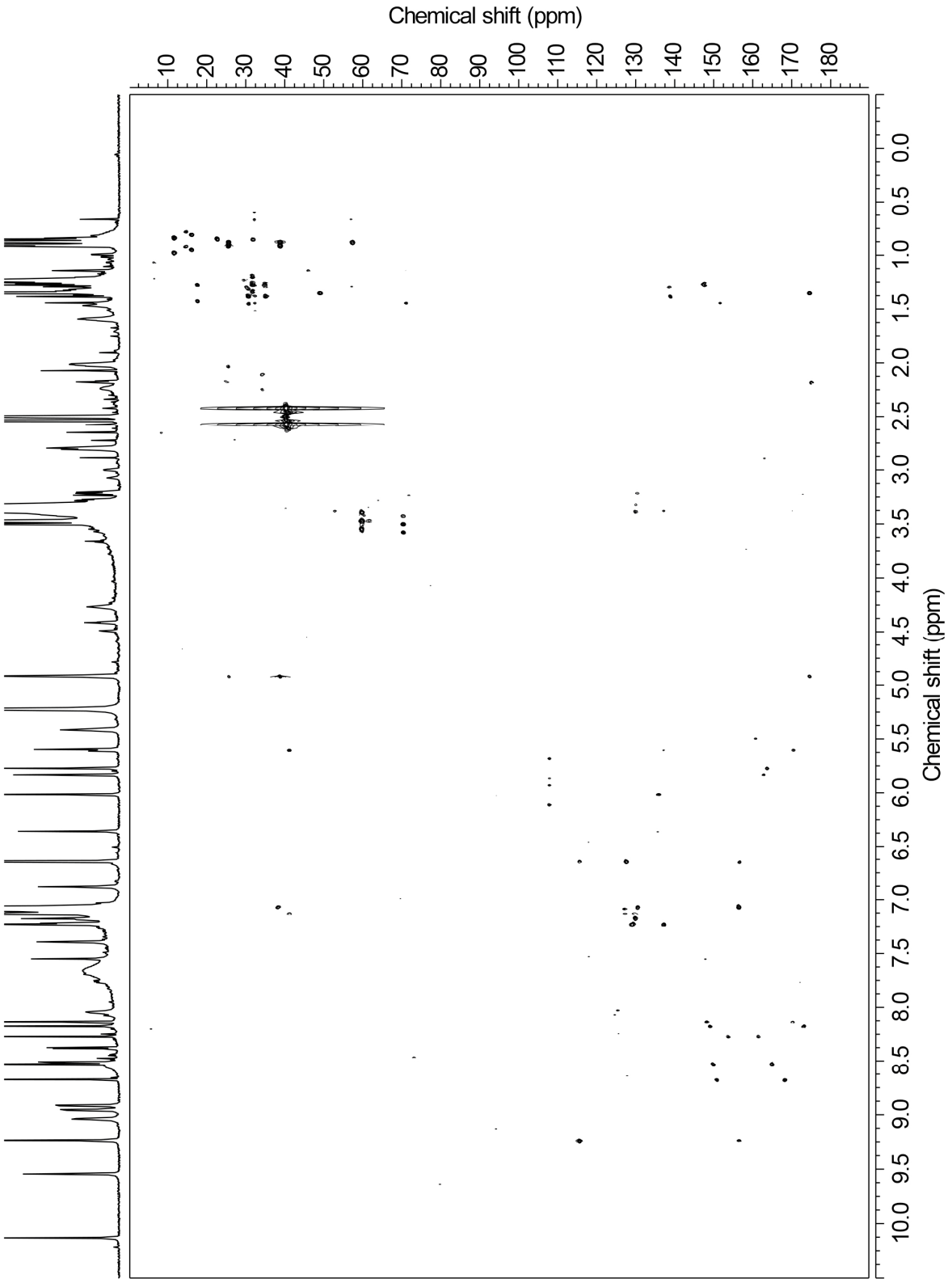


Figure S15: ^{1}H-^{13}C HSQC (900 MHz, DMSO-d_6) spectrum of **4** (downfield).

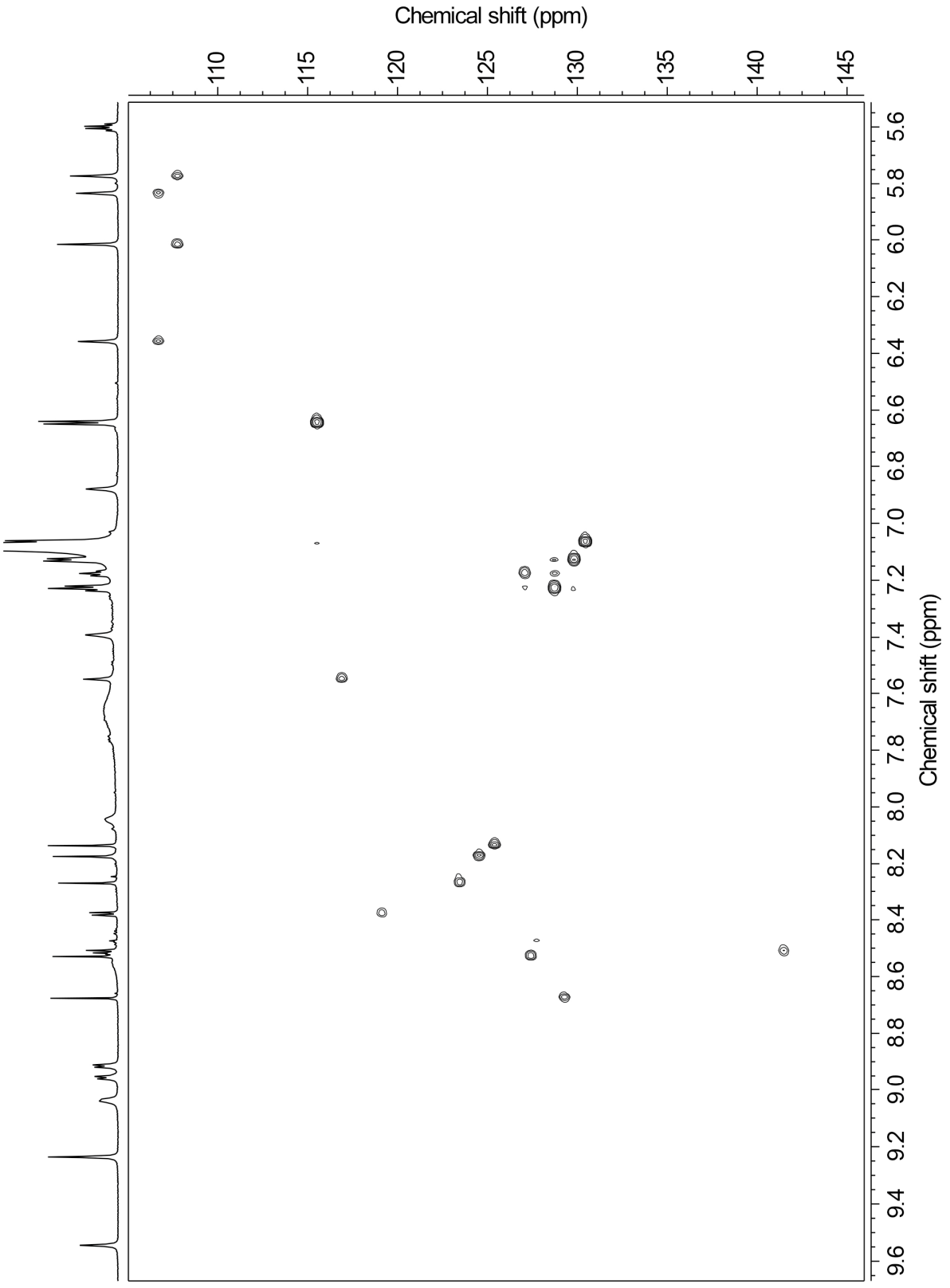


Figure S16: ^{1}H-^{13}C HSQC (900 MHz, DMSO-d_6) spectrum of **4** (upfield).

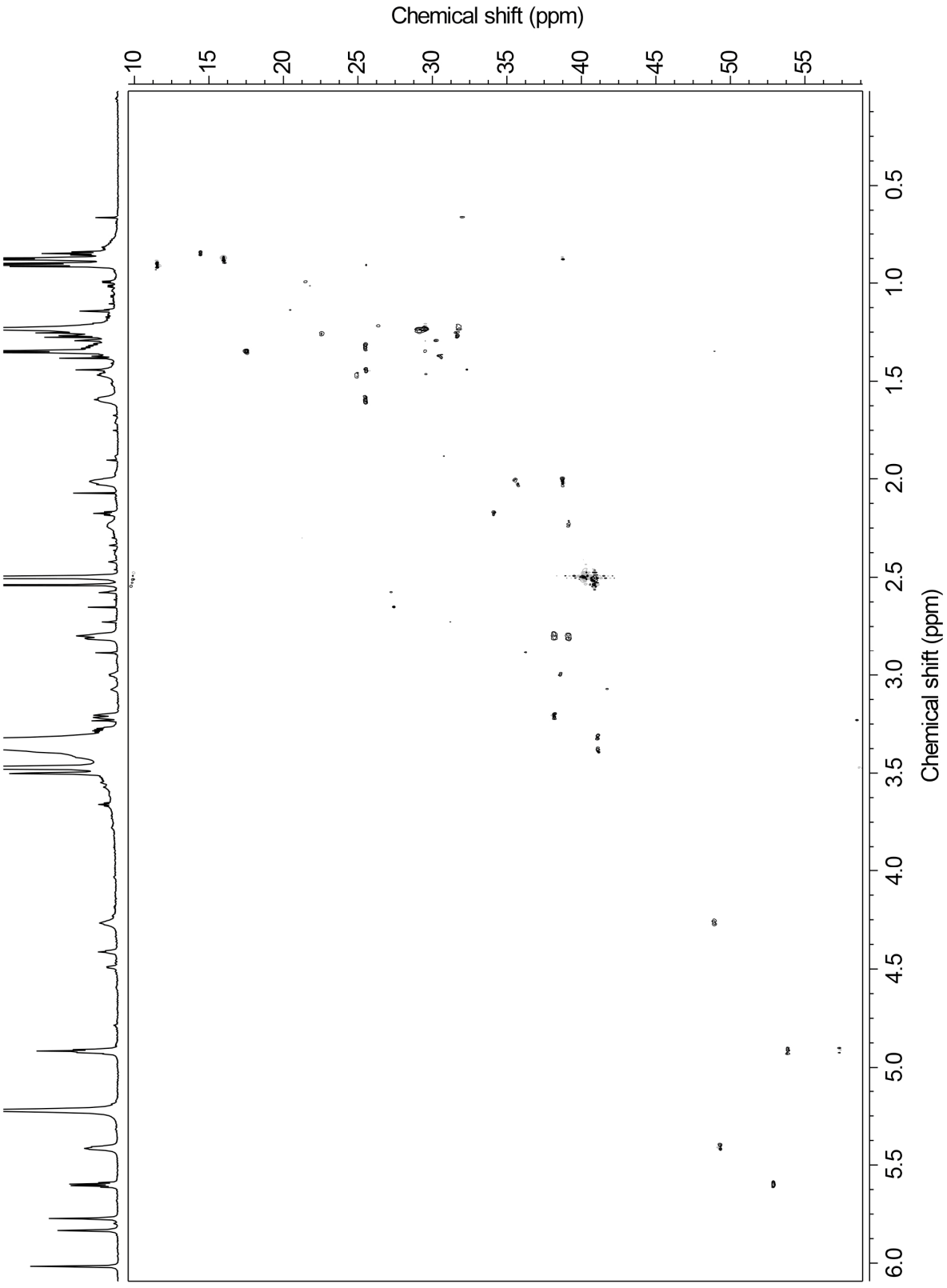


Figure S17: ^{1}H-^{1}H NOESY (900 MHz, DMSO-d_6) spectrum of **4**.

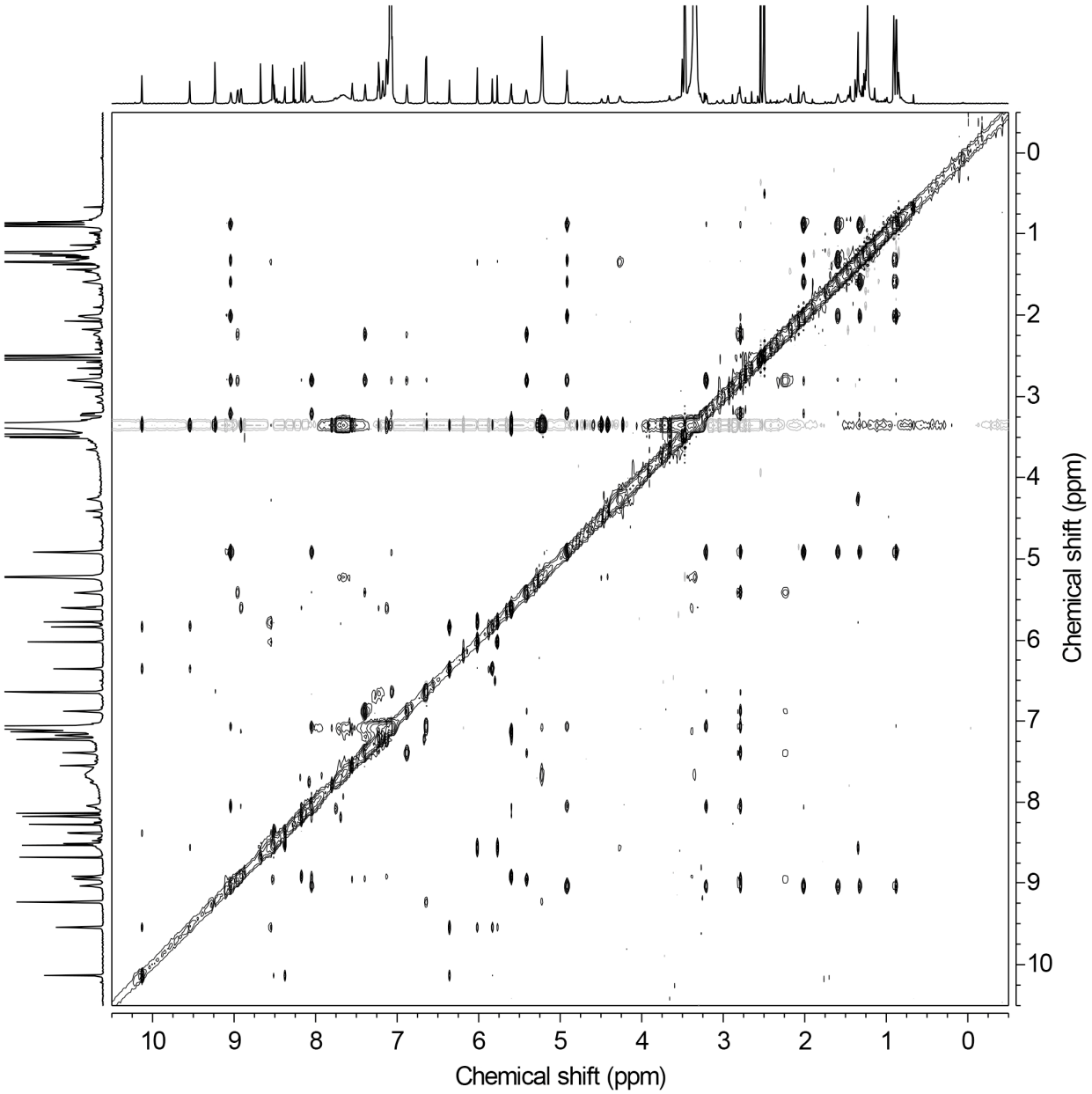
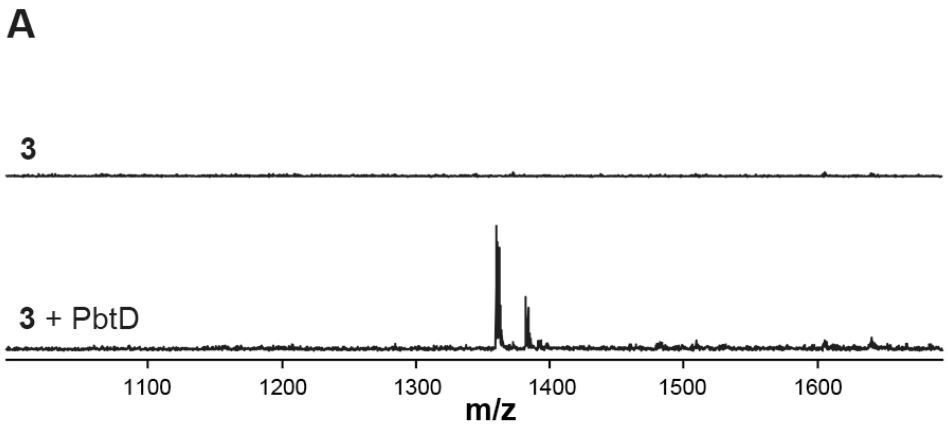


Figure S18: PbtD accepts **3** as a substrate. (A) The amino acid composition of His_6-TbtA is shown. In peptide **3** the residues shown in blue are thiazoles and in green are dehydroalanines. In the top spectrum, **3** is not visible owing to the mass window shown. Reaction of **3** with PbtD (homolog of TbtD from the GE2270A biosynthetic gene cluster) shows the formation of *m/z* 1366 Da (compare to Figure 2E) and the corresponding sodium adduct. (B) The aligned amino acid sequences of the thiomuracin GZ and GE2270A precursor peptides, labeled TbtA and PbtA, respectively. (C) Structural comparison between thiomuracin GZ and GE2270A. While structurally related, there exist key differences in amino acid composition and post-translational modifications.

His_6-TbtA: PHHHHHHSQVDLNDLPMDVFELADSGVAVESLTAGHGMTEVGA*SCNCFCYICCSCSSA



B

```
TbtA  -----MDLNDLPMDVFELADSGVAVESLTAGHGMTEVGASCNCFCYICCSCSSA-
PbtA  MSEMELNLNDLPMDVFEMADSGMEVESLTAGHGMPEVGASCNCVCGFCCSCSPSA
        ::**********:****: ********** ********.* :***** :
```

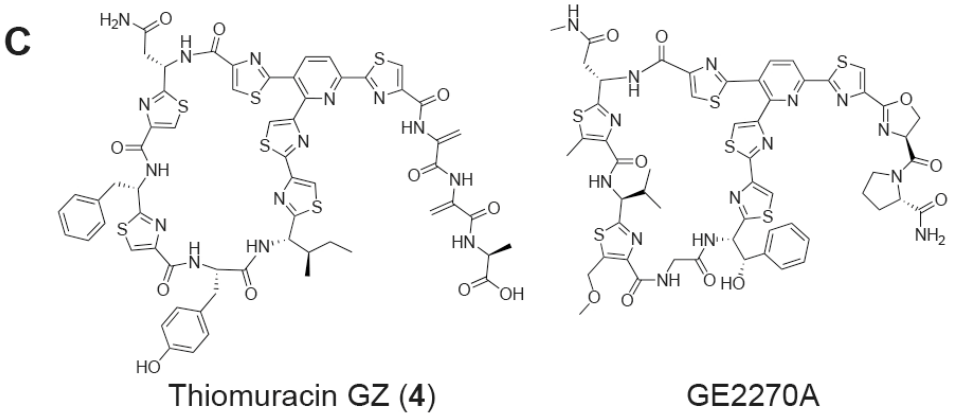


Figure S19: Sequence alignment of TbtD homologs. GI numbers are given to the left of the alignment (green indicates the sequence from the canonical thiomuracin producer, *Nonomuraea* sp. Bp3714-39; blue indicates the sequence from the GE2270A producer, *Planobispora rosea*). Shown in red are residues replaced with alanine for enzymatic activity assessment.

```
TbtD              MMAAGERWWRFRVDYHAGPM-DDLILDGVRPAFAAFAAQAPMAYFLRHWRRGPHLRIYVS
gi:242129450      -----MTWTRLRVDYHDGPM-DDLILDALRPAWHEI-----RGYFLRHWVCGPHLRIFVD
gi:552968830      -----MTWRRFDVAYHDPDL-DRLILAA-RPLLS---ESPGRSWFQRHWVRGPHLELWFD
gi:749676247      -----MEWHSLHVVYYANDK-DRLLLEGVRPLLRRLAPEVAATSFTRHWRLGPHVRIHVK
gi:657940957      MSGAAATWHSIHVHHHDESTEPALVLHAVRPAFEAVRASVDGAWFGRHWLRGPHLRLNFR
gi:242129434      -----MSWRRVDVAYHDPDL-DGLILAT-RPLLA---GTPGRGWFQRHWVRGPHLELWFD
                        *  . * ::       *:*   **             * ***  ***:.: .

TbtD              TTREALEAVVRPAIEHVVGGYLRARPSPGMADPSAFLPLHERLAELEGEDGPLMPWSPDN
gi:242129450      GDATPIV--------RAVERHLAACPSAGTTDPQALLPLHERLAELEGERGPLLPWAPDN
gi:552968830      HPEPS-----WERVREVLGTHLRAHPSRTRIDPDRLLPQHRRLALAEQIDEPLLPFYDDN
gi:749676247      CAPAAVPALVRPAAEEIVGGFLARHPSCTHLVPEDHLDEHRRLAELEEDDRPLLPWRPDN
gi:657940957      TTADAWASKVRPQVVAVLETYVRDHPSTVRLDEAALAPVHERLAELEMETGPRHPWVPDN
gi:242129434      AAQPS-----WERIRDVLEPWLRVNPSRARIDRDRLLAQHRHLAAAERIDEPLLPFYADN
                                  :   :   **           *.:**  *    *  *:  **

TbtD              TIHAEGERPEPL----TVRDVLLADFYADTTPSVYHALERVRSGASLPTIAFDLVVATAH
gi:242129450      TVTAEP--PGLD----TELDRFLADFYADTTEAAFDALGRVRAGTPLPGIAFDLVVATAH
gi:552968830      TLHRAVPRSRVHVLGSAAAEDLFHDFHAAASTAAFDQLDAVVAGESRLGLAFELMIAAAH
gi:749676247      TIHQAPFERRAHVLGSEEVADLLADFHAATADPAFRMTEAVAGGTPRLGLAFDLMVATAH
gi:657940957      TVLEQPYDHRLPVLGSMRGSEVLADFLSDTNDLAFAMYEHLQGGGALPVLALDLMWTTAS
gi:242129434      TLHRAAPRSRAHVLGGPAAEELFHDFHTTASAVAYDELDAVRAGESRLVMALDLMVAAAH
                  *:                   .: ** : :   .:     : .*     :*::*: ::*

TbtD              A------LSTGGLPVARTSLRSHAEAYLARRSDGVRLRELWRDHYARNREAFTERLIAVA
gi:242129450      D------LSEGGLPTARTSLRSHAEAYLSRLPGGVR--AKWQAHYERNQEPLTARIKALT
gi:552968830      A------HAEGGITGGFVSFRSHAEAFLAGAA---GLRERWEAEYRTRAEALRAQVAAVV
gi:749676247      A------LSGVGITTGFMSFRSHAEAFLTTHPEAHRLRPSWDAHYRGHAASLCDRVGRVT
gi:657940957      VAAVPFDEHSAPIARGFLSLRSHADAFLSRTHDPAAYRAAFDERYRRQADALTTRLRTVE
gi:242129434      A------HAEGGVRGGFVSFRSHAEAFLASAP---GLRERWDAEYAARAGALRARITAVV
                            :  .  *:****:*:*:           :  .*  .   :  ::  :

TbtD              SSAE-----SAENGAHLPHVREWVRRLRPIRERARALLESGELTLEYASPAEGARDLPSL
gi:242129450      GAG---------------EPGAWLRTIRATRDRGRTLIDEGRLSLGYAT--DGPSTRPPL
gi:552968830      TG--------------TPRGRAWTGLLDGFAGRGDELIASGALTVEPASPT-AAAEP---
gi:749676247      GALD-----DGS--GGVGFTGEWTELMRSFRRRGRALLAEGRLPSETAFAPGADGRPPPL
gi:657940957      SVLSAEPGSPEAADATVPFVREWAEAVRRCQRAAYPLLASGELTLSGAGRA----PRLPT
gi:242129434      AG--------------IPRGRAWAGLLDRFADRGDELIASGALLVEPAGPD-AVARP---
                                     *   :      .  *: .* *    *

TbtD              AEVSAFHRELESRPEW-ARLRDSPAFGAYRLVINCTYLHLTRLGLTPHQRFLVCHLAADA
gi:242129450      AAVSPFHRNLETDERW-LALKDTPAFAAYRLALNCAYLHLTRLGLTPDQRFLICHLAASA
gi:552968830      --DTEFHRALRANRTWHDEVLRSPSFRRYRLLLNLTYLQMSRLGVTAVQRSLLCHFAASA
gi:749676247      AGASPYHRAAYRNPAV-MASMTAEWFVLYRLMLNYTYLFMTQLGVTPVERYLLCHLTAHA
gi:657940957      RQLSDFHRLLQTDHGHDGFLRDDHWFASFRLVVNYLYVHLNRLGLKPVDRGLLCHLAAGT
gi:242129434      --DTAFHRALRGNRTWHEEVLRSAPFRRYRLLLNLTYLQLSRLGVNAVQRALLCHFAASA
                     : :**                *  :** :*  *: :.:**:.  :* *:**::* :

TbtD              AADVYGIAAHEEVATR-------------------------
gi:242129450      ADDVYRAVAS-------------------------------
gi:552968830      VEEEYGVSAIEIAVGGM------------------------
gi:749676247      VEELYGVSAMEQITRAPPEPVLDAGGEPR------------
gi:657940957      VETVHGVTAEDAFRRHVVSASP-TEDTPEWSRLSKEWAEGR
gi:242129434      VEQEYGVSAIEIAMGGA------------------------
                  .   :   *
```

Table S4: Yield and activity of alanine-substituted TbtD proteins. Twenty-three positions of TbtD were targeted for alanine replacement by site-directed mutagenesis using the alignment of Fig. S18. These proteins were expressed in *E. coli* as MBP fusions, purified to homogeneity (Fig. S1), then assayed for enzymatic activity using a MALDI-TOF-MS endpoint assay. In condition **A**, high concentrations of enzyme and substrate were used to assess the formation of **4**. In condition **B**, both the enzyme and substrate concentration were lowered. Precise conditions follow for **A**: HEPES pH 7.5 (100 mM), $MgCl_2$ (5 mM), KCl (150 mM), ATP (5 mM), **2** (hexazole, 100 μM), *T. bispora* $tRNA^{Glu}$ (10 μM), *E. coli* GluRS (10 μM), TbtB (5 μM), TbtC (5 μM), MBP-TbtD (5 μM), and TIPP (0.02 U/μL). The reaction was allowed to proceed at 30 °C for 4 h. Conditions for **B** were identical to **A** except for the following changes: **2** (hexazole, 15 μM; lowered 6.67-fold), *T. bispora* $tRNA^{Glu}$ (10 μM), *E. coli* GluRS (10 μM), TbtB (5 μM), TbtC (5 μM), MBP-TbtD (1 μM; lowered 5-fold), and TIPP (0.02 U μL^{-1}). The reaction was allowed to proceed at 30 °C for 4 h. +++ indicates enzyme activity roughly equal to wild-type (full conversion to product); ++ indicates modestly reduced enzyme activity (~50% conversion to product); + indicates severely reduced enzyme activity (low but detectable product formation); - indicates no detectable enzyme activity (no observed product formation). For a more quantitative analysis of the proteins that displayed reduced activity, see Table S5. Isolated protein yields are given as a crude measure for protein stability (structurally destabilizing substitutions typically give lower yields and visible degradation by SDS-PAGE).

TbtD Protein	Protein Yield (mg/L)	Relative Product (condition A)	Relative Product (condition B)
Wild-type	14	+++	+++
H46A	8	+++	+++
W47A	**17**	**+++**	**-**
R49A	8	+++	+++
H52A	8	+++	+++
R54A	21	+++	+++
S85A	13	+++	+++
H98A	8	+++	+++
E105A	**11**	**+++**	**-**
N118A	8	+++	+++
T144A	13	+++	+++
R189A	11	+++	+++
S190A	8	+++	+++
H191A	**4**	**+++**	**-**
E193A	11	+++	+++
S287A	**13**	**+++**	**-**
H290A	**10**	**+++**	**++**
R291A	10	+++	+++
N316A	13	+++	+++
Y319A	**11**	**+**	**-**
R324A	10	+++	+++
R332A	**16**	**-**	**-**
C336A	10	+++	+++
H337A	16	+++	+++

Table S5: Enzymatic activity of selected TbtD mutant proteins**.** Seven TbtD mutants were selected based on phenotype in the end-point MALDI-TOF MS assay (Table S4). Conversion efficiency was monitored by comparing EIC intensity to that of wild-type at 90 min (i.e., fully converted). As the wild-type TbtD performed the reaction much faster than the selected mutants, 200 nM enzyme was used for the assay compared to 1 μM for the mutant enzymes. All experiments were performed in duplicate. ND = not detected.

TbtD protein	% conversion 30 min	% conversion 60 min	% conversion 90 min
Wild-type	77 ± 2	87 ± 1	100
W47A	36 ± 3	55 ± 2	64 ± 1
E105A	25 ± 4	47 ± 7	54 ± 11
H191A	36 ± 3	57 ± 1	67 ± 4
S287A	14 ± 4	23 ± 1	39 ± 11
H290A	14 ± 3	33 ± 11	42 ± 4
Y319A	2 ± 1	5.4 ± 3.7	11 ± 1
R332A	ND	ND	ND

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