Biosynthetic Functionalization of Nonribosomal Peptides

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Table of Contents

Materials & Methods

General. All reagents were used as received and all solvents were HPLC grade. High resolution mass spectrometry (HRMS) was performed on a Bruker maXis UHR-TOF by electrospray ionization (ESI) at the Mass Spectrometry Service of the Laboratory of Organic Chemistry at ETH Zurich. NMR spectra were recorded on a Bruker Avance-III 600 MHz spectrometer at the NMR Service at the LOC, ETH Zurich. ^{1}H-NMR spectra were recorded relative to residual solvent peak (DMSO-d_6 δ_H 2.50 ppm) and reported as follows: chemical shift (ppm), multiplicity, coupling constant (Hz), and integration. Multiplicity abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. ^{13}C-NMR spectra were recorded relative to residual solvent peaks (DMSO-d_6 δ_C 39.52 ppm).

Cloning. Expression media and buffers were prepared using purified H_2O (Nanopure system, Barnstead). Media and buffer components, kits, and enzymes were used as received from specified commercial suppliers. Commercial enzymes were purchased from NEB if not stated otherwise.

Oligonucleotide primers were purchased from Microsynth AG (Switzerland) and all PCRs utilized Phusion HF polymerase (NEB) according to the manufacturer's instructions with the supplied HF buffer (50μL total volume, 50 ng plasmid DNA, 0.5 μM primer, 0.2 mM dNTPs (Sigma), 1μL Phusion HF). DNA was either purified via agarose gels (1%) and extracted using a Gel DNA Recovery Kit (Zymo Research) or via spin columns using a DNA clean and concentrator kit (Zymo Research). Transformations were conducted with electrocompetent *Escherichia coli* HM0079[1] cells (100 μL, ~100 ng DNA), followed by immediate SOC (1 mL) rescue and incubation at 37 °C, 400 rpm for 1 h before plating onto LB agar containing the respective antibiotic. Single colonies were selected and grown in overnight cultures using LB Miller broth (Merck) containing an appropriate antibiotic. After harvesting cells by centrifugation, plasmid DNA was isolated with a ZR Plasmid Miniprep Classic kit (Zymo Research) according to the manufacturer's specifications. All cloned variants were verified by Sanger sequencing at Microsynth AG (Switzerland).

The cloning of plasmids **pSU18_tycA** and **pSU18_tycA_W227S** was described previously.[1,2] Plasmid **pTrc99a_tycB** for the production of C-terminally His_6-tagged TycB was constructed by Gibson assembly[3] of four fragments (pTrc99a, tycB_1, tycB_2, and tycB_3) with 20 bp overlaps, each using a Gibson Assembly cloning kit (NEB) according to manufacturer specifications. The individual fragments were designed to introduce XhoI, SacI, and NotI restriction sites by silent mutation to facilitate subsequent cloning; the ligation sites were chosen accordingly. Fragment A (4,100 bp) was PCR amplified from plasmid pTrc99a[1] with primer pair pTrc99a_f / pTrc99a_r. Fragments tycB_1 (4,700 bp), tycB_2 (3,000 bp), and tycB_3 (3,100 bp) were PCR amplified from *Brevibacillus parabrevis* genomic DNA (DSM 362, tyrocidine producer) with primer pairs tycB_1_f / tycB_1_r, tycB_2_f / tycB_2_r and tycB_3_f / tycB_3_r, respectively. *B. parabrevis* genomic DNA was isolated and purified by isopropanol precipitation as described previously.[1] Sequencing of the resulting plasmid showed that the TycB sequence corresponds to GenBank entry HBZ81682.

To reprogram TycB-A3 for *O*-propargyl-L-Tyr, two fragments were PCR amplified from pTrc99a_tycB with primer pairs tycB_3_f / tycB_W2742S_r and tycB_W2742S_f / tycB_3_r, respectively. The two fragments were assembled by PCR with primer pair tycB_3_f / tycB_3_r and introduced into pTrc99a_tycB via SacI and NotI restriction sites to yield plasmid **pTrc99a_tycB_W2742S**.

For the recombinant production of His_6-tagged TycC, plasmid **pTrc99a_tycC** was constructed as described for pTrc99a_tycB by Gibson assembly of four fragments. Fragments tycC_1, tycC_2, and tycC_3 were PCR-amplified from *B. parabrevis* gDNA using primer pairs tycC_1_f / tycC_1_r, tycC_2_f / tycC_2_r, and tycC_3_f / tycC_3_r, respectively. Sequencing of the resulting plasmid showed that the TycC sequence corresponds to GenBank entry HBZ81681.

Primer	**Sequence 5'-3'**
pTrc99a_f	GGA TCC AGA TCT CAT CAC CAT CAC
pTrc99a_r	CAT GGT ACC TTT CCT GTG TGA AAT TG
tycB_1_f	CAA TTT CAC ACA GGA AAG GTA CCA TGA GTG TAT TTA GCA AAG
tycB_1_r	CGC CAT CTC GAG CGA TCT TTG CGC CAT GAT C
tycB_2_f	GCA AAG ATC GCT CGA GAT G
tycB_2_r	CTC TTT CAT TGA GCT CCC
tycB_3_f	GGG AGC TCA ATG AAA GAG
tycB_3_r	GTG ATG GTG ATG AGA TCT GG
tycB_W2742S_f	ATC GGT GTC GGA TAT GTT TGG C
tycB_W2742S_r	AAC ATA TCC GAC ACC GAT GCG TC
tycC_1_f	CAA TTT CAC ACA GGA AAG GTA CCA TGA AAA AGC AGG AAA ACA TC
tycC_1_r	GAG TGG AAA CTA GTG CGC AAC GAT TCG
tycC_2_f	TTG CGC ACT AGT TTC CAC TCC GTG CAA G
tycC_2_r	CAT ACA AGC TAG CAA TTT CCT CGA CGA TG
tycC_3_f	GGA AAT TGC TAG CTT GTA TGC AGG AAA AC
tycC_3_r	GTG ATG GTG ATG AGA TCT GGA TCC TTT CAG GAT GAA CAG TTC TTG

Protein production. Protein production was performed in modified Studier medium[4] "ZYM-G".

Modified Studier medium

ZY	tryptone (Merck, 1% w/w); yeast extract (Merck, 0.5% w/w)
50 x M solution (20 mL/L)	Na_2HPO_4 (Merck, 25 mM); KH_2PO_4 (Merck, 25 mM); NH_4Cl (Merck, 50 mM) Na_2SO_4 (Merck, 5 mM)
100 x G solution (10 mL/L)	glycerol (Sigma, 50% v/v)
500 x Mg(II) solution (2 mL/L)	$MgSO_4$ (Merck, 1 M)

The ZY component was autoclaved, whereas the M, G, and Mg(II) components were sterile filtered (TPP, 0.22 μm) before use (note: LB Miller broth (Merck) could be substituted for ZY with no discernible difference). Isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin (amp), and chloramphenicol (cam) were obtained from Apollo scientific.

HM0079 cells[1] harboring the expression plasmids were taken from glycerol cell stocks stored at -80 °C and grown overnight at 37 °C in LB Miller broth (5 mL) containing the respective antibiotic. The following morning, ZYM-G (800 mL in a 2 L baffled flask) containing the respective antibiotic was inoculated with the overnight culture (1/500 v/v) and shaken at 180 rpm and 37 °C until an OD600 of 2 was reached, at which point the cultures were cooled to 20 °C. After reaching 20 °C, cell cultures were induced with IPTG (100 µM) and shaken at 180 rpm and 20 °C for ~20 h. Cells were pelleted at 5,000 x g and 4 °C for 25 minutes, transferred to 50 mL falcon tube(s), and frozen at -20 °C.

Protein purification. Imidazole, NaCl, HEPES, and Tris base were obtained from Merck. The pH of all buffers was adjusted using a WTW bench pH/mV meter (routine meter pH526) calibrated according to manufacturer specifications. Cell pellets were thawed and suspended in lysis buffer [3 mL / g cells, Tris (50 mM), NaCl (500 mM), glycerol (10% v/v), pH 8.0] via vortex. For lysis, the cell suspension was treated by addition of lysozyme (Sigma, 2 mg/mL), polymyxin B (Apollo, 2 mg/mL), RNase A (Sigma, 0.04 mg/mL) and DNase I (Sigma, 0.04 mg/mL) at 4 °C for 30 min before sonication (Dr. Heilscher, UP 200s sonic dismembrator, total sonication time of 6 min at 100% power with care taken to keep internal temperature <15 °C). Cellular debris was pelleted in a pre-cooled (4 °C) centrifuge at 15,000 x g for 25 min and the supernatant was applied to Ni-NTA resin (Qiagen, 1 mL / 4 g cells, pre-equilibrated with five column volumes of lysis buffer). Wash buffer [10 column volumes, Tris (50 mM), NaCl (500 mM), imidazole (20 mM), glycerol (10% v/v), pH 8.0] was added and the column was gently pressurized with a syringe. The enzyme of interest was eluted with elution buffer [3 column volumes, Tris (50 mM), NaCl (500 mM), imidazole (300 mM), glycerol (10% v/v), pH 8.0].

TycA variants were further purified to reduce the endogenous amino acid background in the ^{32}P-PP$_i$/ATP exchange assay. To that end, buffer was exchanged to FPLC buffer [Tris (20 mM), NaCl (20 mM), glycerol (5% v/v), pH 8.0] using centrifugal filter units (Merck, Amicon Ultra-15) and the proteins were purified by anion-exchange chromatography (GE Health- care, MonoQ 10/100, linear gradient from 0.05 to 0.5 M NaCl). All other proteins were buffer exchanged to storage buffer [HEPES (50 mM), NaCl (100 mM), $MgCl_2$ (10 mM), glycerol (10% v/v), pH 8.0] and aliquoted. Purified proteins were immediately flash frozen in liquid N_2 and stored at -80 °C until use. Protein concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher) and corrected by the calculated extinction coefficient (ProtParam, http://web.expasy.org/protparam/). Protein purity was assessed by SDS-PAGE using a Phast system and 7.5% gels (GE), according to the manufacturer's specifications.

Adenylation kinetics. Adenylation kinetics were determined with a pyrophosphate exchange assay as described in Kries et al.[2]. The L-Phe analogs *p*-bromo-L-Phe, *p*-iodo-L-Phe and *p*-benzoyl-L-Phe were purchased from Chem-Impex. *O*-Propargyl-L-Tyr was synthesized as previously described.[2]

In vitro tyrocidine biosynthesis. Enzymatic reactions for in vitro tyrocidine production were performed in Bis-Tris propane buffer (100 mM, 100 mM NaCl, 10 mM $MgCl_2$, 4 mM tris(2-carboxyethyl)phosphine (TCEP), 50 mM ATP, 1 mg/mL BSA (Sigma), 0.1 units/mL inorganic pyrophosphatase from baker's yeast (Merck), pH 8) with TycA or W227S TycA (1 µM), TycB or W2742S TycB (1 µM), TycC (1 µM) and all substrate amino acids (4 mM L-Phe, 2 mM L-Pro, 2 mM L-Asn, 2 mM L-Gln, 2 mM L-Tyr, 2 mM L-Val, 2 mM L-Orn, 2 mM L-Leu, and 2 mM L-Phe or analog). Reactions were started by TycC addition and incubated at 37 °C in a water bath. After 6-7 h, the samples (20 µL) were quenched with 180 µL MeOH, vortexed, clarified by centrifugation (21,000 x g, 10 min), and analyzed by HPLC (Ultimate 3000, Dionex) or LC-MS.

For HPLC analyses, a 5 µL aliquot was injected onto a Dr. Maisch Reprosil-Gold C18 column (100 x 2 mm, 3 µm), monitored at 220 nm, 254 nm and 280 nm. Products were separated by gradient elution: solvent A = H_2O + 0.1% TFA, solvent B = MeCN + 0.1% TFA, flow rate = 1 mL/min, 0-0.5 min = 5% B, 0.5-1.5 min ramp to 55% B, 1.5-4.0 min ramp to 82.5% B, 4.0-5.2 min = 99% B, 5.2-5.6 min ramp to 5 % B, 5.6-6.5 min re-equilibration = 5% B. LC-MS (Waters H-class UPLC/SQD-2) analyses were performed on an Acquity UPLC BEH C-18 column (50 x 2.1 mm, 1.7 µm), 1 µL injection, monitoring ESI^+ for $[M+2H]^{2+}$, solvent A = H_2O + 0.1% FA, solvent B = MeCN + 0.1% FA, flow rate = 1 mL/min, initial conditions = 5% B, 0-1.5 min ramp to 80% B, 1.5-2 min ramp to 100% B, 2-2.2 min = 100% B, 2.2-2.3 min ramp to 5% B, 2.3-3 min re-equilibration = 5% B. Reaction products were identified by liquid chromatography high-resolution mass spectrometry (LC-HRMS) on a Bruker maXis UHR-TOF by electrospray ionization using an Eclipse Plus C18 column (Agilent), monitoring m/z 100-5000 with m/z 622.029 as a reference: solvent A = H_2O + 0.1% formic acid, solvent B = MeCN + 0.1% formic acid, flow rate = 0.6 mL/min, 0-1 min = 5% B, 1-2 min ramp to 55% B, 2-7.5 min ramp to 85% B, 7.51-9 min = 99% B, 9.01-14 min = 5% B.

Rate constants (k_{obs}) and total turnover numbers for formation of tyrocidine A (**1**) and analogues **2** and **7** were determined in time course experiments. The reactions were performed in a total volume of 200 µL. After 15, 30, 45, 60, 75, 90 min and 6-7 h, 20 µL aliquots were worked up and analyzed as described above. Authentic standards were used for product quantification at 220 nm. Three biological replicates were used to determine total turnovers and k_{obs} values. Because compound **2** could not be separated from wild-type tyrocidine A (**1**) under the standard UPLC conditions, the product ratio was determined independently by separating the mixture on a Reprospher 100 Phenyl-hexyl column (125 x 4.6 mm, 5 µm; Dr. Maisch GmbH HPLC), 5 µL injection, monitored at 220 nm, 254 nm and 280 nm, solvent A = H2O + 0.1% TFA, solvent B = MeCN + 0.1% TFA, flow rate = 1 mL/min, 0-1 min = 5% B, 1-3 min ramp to 55% B, 3-16 min ramp to 95% B, 16-19 min 95% B, 19-19.5 ramp to 5% B, 19.5-22 min re-equilibration = 5% B (see Figure S4).

Preparative biosynthesis of tyrocidine A (1) and propargylated analogues 2 and 7. Larger scale NRP biosynthesis was performed as described above but in a total volume of 300 mL for **1**, 25 mL for **2** and 130 mL for **7**. For compound **2**, the reaction was supplemented with 20 mM *O*-propargyl-L-Tyr to suppress side product formation. All large-scale reactions were incubated at 37 °C for 8-10 hours and aliquots of the reactions were quenched with MeOH, and analyzed by HPLC and LC-MS as described above.

After 8-10 hours, precipitated tyrocidines were isolated by centrifugation and the supernatants were discarded. The solid products were dissolved in 1:1 DMSO:MeOH and remaining enzymes were removed by centrifugation (7,000 x g, 20 min). The resulting supernatants were filtered over a syringe filter (Macherey-Nagel, 0.45 µm, PTFE) and purified by preparative HPLC (Waters) on a Gemini 5µm NX-C18 110 Å LC column (250 x 21.2 mm, Phenomenex Inc. Basel, Switzerland), 10 mL injection, monitoring with a diode array detector at 220 nm and 254 nm, solvent A = H_2O + 0.1% TFA, solvent B = MeCN + 0.1% TFA, flow rate = 25 mL/min, 0-2.5 min = 5% B, 2.5-7.0 min ramp to 55% B, 7.0-18.5 min = 82.5% B, 18.5-19 min ramp to 99% B, 19-24 min = 99% B, 24-26 min ramp to 5% B, 26-30 min re-equilibration = 5% B. Individual fractions were analyzed by HPLC and LC-MS, pure fractions were combined and the solvent was removed under high vacuum to yield the (modified) tyrocidine analogue.

Tyrocidine A (1): 26 mg; HRMS: calculated $[M+H]^+$ 1270.6619, found 1270.6600.

^{1}H NMR (600 MHz, DMSO-d_6) δ 9.27 (d, J = 3.7 Hz, 1H), 9.17 (s, 1H), 9.04 (m, 2H), 8.89 (d, J = 9.3 Hz, 1H), 8.71 (d, J = 4.7 Hz, 1H), 8.42 (d, J = 9.7 Hz, 1H), 8.02 (s, 1H), 7.91 (d, J = 8.1 Hz, 1H), 7.48 - 7.37 (m, 5H), 7.27 – 7.14 (m, 13H), 7.13 – 7.02 (m, 4H), 6.94 – 6.88 (m, 3H), 6.62 (d, J = 8.2 Hz, 2H), 5.58 (m, 1H), 5.28 (m, 1H), 4.58 – 4.44 (m, 4H), 4.33 – 4.27 (m, 2H), 4.06 (d, J = 8.0 Hz, 1H), 3.83 – 3.79 (m, 1H), 3.37 – 3.27 (m, 2H), 3.08 – 2.67 (m, 9H), 2.25 – 2.10 (m, 3H), 2.04 – 1.95 (m, 2H), 1.90 – 1.60 (m, 7H), 1.52 – 1.32 (m, 3H), 1.28 – 1.11 (m, 2H), 1.06 – 0.98 (m, 1H), 0.95 – 0.85 (m, 12H), 0.42 - 0.31 (m, 1H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 174.5, 173.0, 172.2, 171.9, 171.0, 170.8, 170.8, 170.6, 170.3, 170.2, 169.8, 169.2, 155.7, 138.5, 137.3, 136.1, 129.7, 129.3, 129.2, 128.9, 128.2, 128.1, 127.9, 127.8, 126.9, 126.2, 126.1, 114.9, 59.6, 56.8, 56.2, 55.0, 54.1, 53.5, 52.7, 50.3, 50.1, 49.1, 45.7, 41.5, 40.1, 38.6, 37.1, 36.9, 35.5, 35.0, 31.5, 31.1, 31.0, 28.5, 25.7, 24.6, 23.0, 22.8, 22.2, 21.9, 18.7, 17.9.

Propargylated tyrocidine analogue 2: 0.6 mg; HRMS: calculated $[M+Na]^+$ 1346.6544, found 1346.6525. An additional 3.7 mg **2** containing ~8% **1** was also obtained.

^{1}H NMR (600 MHz, DMSO-d_6) δ 9.30 (br s, 3H), 9.06 (d, J = 6.8 Hz, 1H), 8.91 (d, J = 9.8 Hz, 1H), 8.76 (d, J = 9.2 Hz, 1H), 8.68 (d, J = 4.2 Hz, 1H), 8.52 (s, 1H), 8.45 (d, J = 9.5 Hz, 1H), 8.13 – 7.96 (m, 2H), 7.43 - 7.01 (m, 17H), 6.95 – 6.79 (m, 5H), 6.62 (d, J = 8.5 Hz, 2H), 5.68 – 5.59 (m, 1H), 5.33 - 5.24 (m, 1H), 4.72 (d, J = 2.3 Hz, 2H), 4.61 – 4.43 (m, 4H), 4.33 – 4.26 (m, 1H), 4.25 – 4.18 (m, 1H), 4.05 (d, J = 8.0 Hz, 1H), 3.80 – 3.76 (m, 1H), 3.50 – 3.47 (m, 1H), 3.35 –

3.32 (m, 1H), 3.25 – 3.22 (m, 1H), 3.09 – 2.55 (m, 10H), 2.26 – 2.13 (m, 2H), 2.07 – 1.95 (m, 3H), 1.93 – 1.83 (m, 1H), 1.66 – 1.59 (m, 5H), 1.51 – 1.36 (m, 4H), 1.31 – 1.27 (m, 1H), 1.06 – 1.00 (m, 1H), 0.96 – 0.86 (m, 12H), 0.38 – 0.28 (m, 1H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 174.5, 173.0, 171.9, 171.8, 171.0, 170.8, 170.7, 170.6, 170.4, 170.1, 169.4, 169.4, 156.0, 155.8, 138.6, 137.4, 130.3, 129.7, 129.3, 129.0, 128.9, 128.1, 127.8, 127.7, 126.0, 126.0, 114.9, 114.6, 79.2, 78.0, 59.3, 56.9, 56.2, 55.3, 55.1, 54.3, 53.3, 52.4, 51.1, 50.3, 49.1, 45.5, 41.7, 40.4, 40.3, 37.1, 37.0, 35.0, 34.7, 31.5, 31.3, 31.2, 28.4, 25.7, 24.6, 24.0, 23.0, 22.2, 21.9, 18.8, 17.7.

Propargylated tyrocidine analogue 7: 25 mg; HRMS: calculated $[M+H]^+$ 1324.6725, found 1324.6696. An additional 20 mg **7** containing ~3% **1** was also obtained.

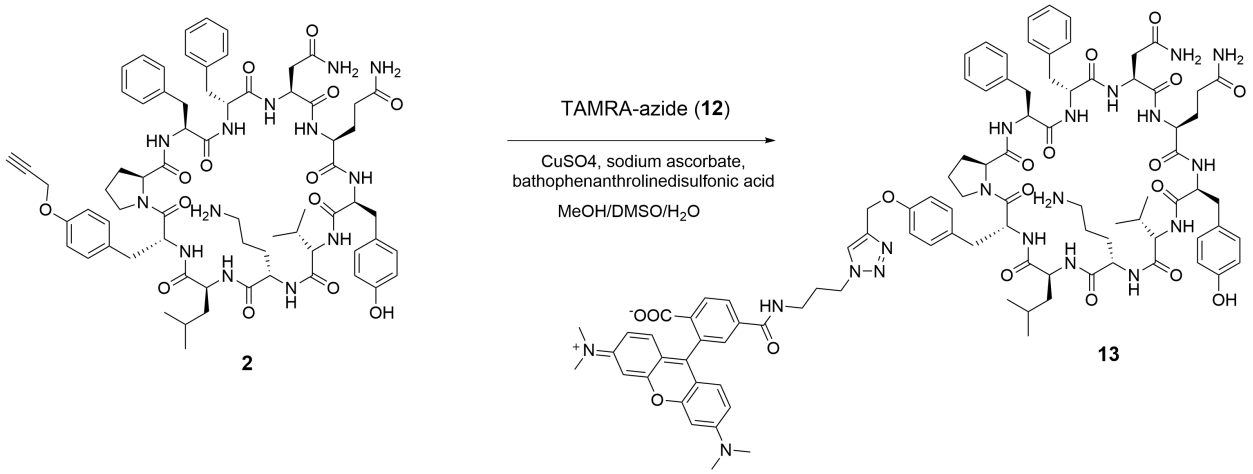
^{1}H NMR (600 MHz, DMSO-d_6) δ 9.27 (d, J = 3.7 Hz, 1H), 9.18 (s, 1H), 9.04 – 8.98 (m, 2H), 8.88 (d, J = 9.3 Hz, 1H), 8.70 (d, J = 4.7 Hz, 1H), 8.42 (d, J = 9.7 Hz, 1H), 8.05 – 7.94 (m, 2H), 7.49 – 7.38 (m, 5H), 7.27 – 7.15 (m, 11H), 7.13 – 7.06 (m, 3H), 6.95 – 6.88 (m, 3H), 6.71 – 6.66 (m, 2H), 6.62 (d, J = 8.0 Hz, 2H), 5.58 – 5.50 (m, 1H), 5.32 – 5.23 (m, 1H), 4.60 – 4.47 (m, 6H), 4.34 – 4.27 (m, 2H), 4.07 (d, J = 8.2 Hz, 1H), 3.83 – 3.79 (m, 1H), 3.37 – 3.27 (m, 2H), 3.19 (t, J = 2.4 Hz, 1H), 3.05 – 2.72 (m, 8H), 2.71 – 2.63 (m, 1H), 2.29 (d, J = 8.1 Hz, 2H), 2.21 – 2.13 (m, 1H), 2.05 - 1.94 (m, 2H), 1.90 – 1.60 (m, 7H), 1.49 – 1.39 (m, 3H), 1.29 – 1.14 (m, 2H), 1.08 - 0.98 (m, 1H), 0.96 – 0.87 (m, 12H), 0.43 - 0.33 (m, 1H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 174.5, 173.0, 172.3, 171.9, 171.0, 170.8, 170.7, 170.6, 170.3, 170.2, 169.9, 169.2, 155.8, 155.7, 138.5, 136.1, 130.0, 129.9, 129.7, 129.3, 128.9, 128.2, 128.0, 127.9, 126.9, 126.1, 114.9, 114.2, 78.9, 77.6, 59.6, 56.8, 56.2, 55.5, 55.0, 54.1, 53.4, 52.6, 50.3, 49.1, 45.8, 41.3, 40.1, 38.6, 37.1, 37.0, 35.5, 35.0, 31.5, 31.1, 31.0, 29.2, 28.5, 25.7, 24.7, 23.2, 22.8, 22.1, 21.9, 18.7, 18.0.

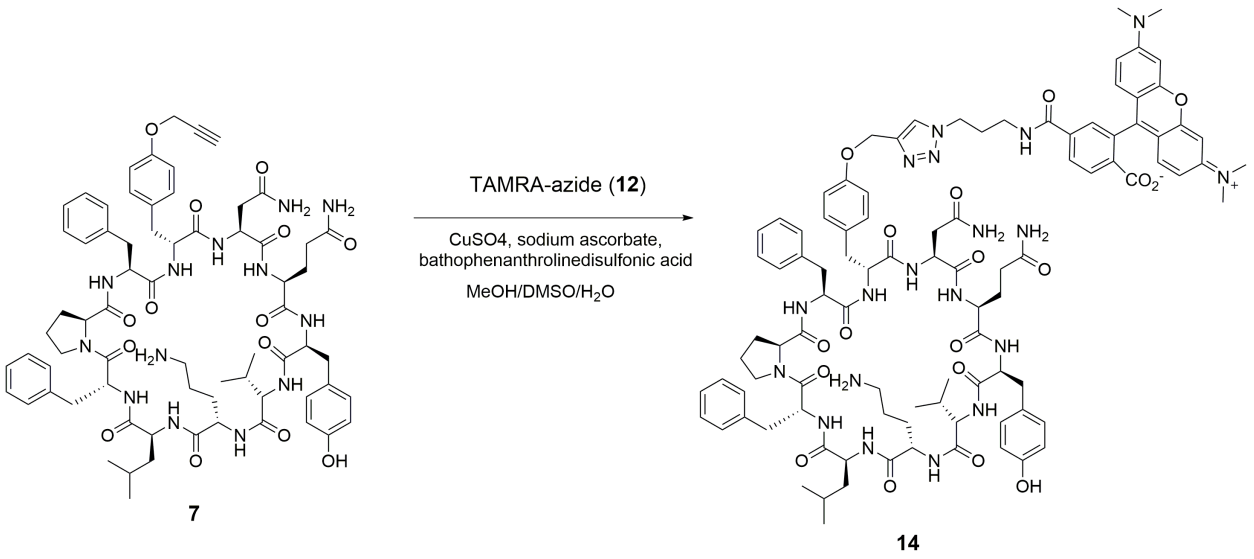
Bioactivity assays. Bioactivity was tested against *E. coli HM0079,*[1] *Acinetobacter baumannii* (ATCC 19606), *Bacillus subtilis* JH642,[5] and *Staphylococcus aureus* (ATCC 43300). A single colony of each strain was regrown in 5 mL Mueller Hinton Broth (MHB) at 37 °C and 180 rpm overnight. The next morning, each culture was diluted to ~1 x 10^6 CFU/mL in MHB. Serial dilutions of the tyrocidines from 200 μM to 0.4 μM were prepared in MHB supplemented with 12% DMSO. In 96-well plates, 50 μL of the respective tyrocidine dilution were added to 50 μL of respective strain in MHB (6% final DMSO concentration) and incubated for 14 hours at 37 °C and 180 rpm. Inhibition of growth was evaluated by eye and by OD_{600} measurement on a SpectraMax 250 plate reader (Molecular Devices). Each well was prepared in duplicate and controls with MHB only or MHB supplemented with 6% DMSO were included in each row. As a sterile control, each serial dilution of the tyrocidines was prepared in duplicate in MHB containing 6% DMSO without addition of a bacterial strain. The experiment for each tyrocidine analog was conducted in duplicate or triplicate.

Human blood was purchased from Interregionale Blutspende SRK AG Bern. Erythrocytes were isolated by centrifugation at 500 x g and 4 °C for 5 min, washed three times with 150 mM NaCl and two times with PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and diluted 50-fold in PBS. Serial dilutions of the tyrocidines from 2 mM to 4 µM were prepared in 6:4 PBS:MeOH. In 96-well plates, 5 µL of respective tyrocidine dilution were added to 95 µL of erythrocytes in PBS (2% final MeOH concentration) and incubated for 1 hour at 37 °C and 700 rpm. Intact erythrocytes were separated from hemoglobin in solution by centrifugation at 500 x g and RT for 10 min. The supernatant was transferred to a fresh 96-well plate and hemolysis was monitored by absorbance of the solution at 405 nm on a SpectraMax 250 platereader (Molecular Devices). Each well was prepared in duplicate and controls with erythrocytes in PBS only, erythrocytes in PBS containing 2% MeOH and erythrocytes in PBS containing 1% Triton X-100 were included for each dilution series. The experiment for each tyrocidine analog was conducted in duplicate.

Bioorthogonal labeling of propargylated tyrocidine analogues



TAMRA-modified tyrocidine 13: Tyrocidine analogue **2** (2.0 mg, 1.5 µmol, 1 eq., dissolved in 151 µL DMSO) and 6-TAMRA-azide (**12**, Lumiprobe, 1.2 mg, 2.3 µmol, 1.5 eq. dissolved in 114 µL MeOH) were mixed in a 1.5 mL glass vial. 151 μL of click mix [$CuSO_4$ (Sigma, 0.32 mg, 2.0 μmol, 1.3 equiv), sodium ascorbate (ABCR, 3.2 mg 16 μmol, 11 equiv) and bathophenanthrolinedisulfonic acid disodium salt hydrate (ABCR, 2.0 mg 3.2 µmol, 2.1 eq.) in water] were added to the solution and the reaction was run for 30 min at RT, quenched by dilution in MeOH, and stored at -20 °C until purification. Preparative HPLC was performed as described above. Although the reaction ran to completion, separation of **13** from the corresponding tyrosine analogue **6**, which was formed as a minor side product during the click reaction, proved difficult. Fractions containing pure product were combined and the solvent was removed under high vacuum to afford pure **13** (0.81 mg, 0.44 μmol, 29%) as a pink solid. **HRMS**: calculated $[M+2H]^{2+}$ 918.9485, found 918.9459.



TAMRA-modified tyrocidine 14: Tyrocidine analogue **7** (7.9 mg, 6 μmol, 1 eq. dissolved in 600 µL DMSO) and 6-TAMRA-azide (**12**, Lumiprobe, 4.6 mg, 9 μmol, 1.1 eq. dissolved in 450 µL MeOH) were mixed in a 5 mL glass vial. 600 μL of click mix [$CuSO_4$ (Sigma, 0.8 mg, 4.8 μmol, 0.6 equiv), sodium ascorbate (Sigma, 8.0 mg 40 μmol, 6.8 equiv) and bathophenanthrolinedisulfonic acid disodium salt hydrate (Sigma, 4.6 mg 7.8 µmol, 1 eq.) in water] were added to the solution and the reaction was run for 15 min at RT. The reaction was quenched by dilution in MeOH and the solution stored at -20 °C until purification. Preparative HPLC was performed as described above. Although the reaction ran to completion, separation of **14** from the corresponding tyrosine analogue **11**, which was formed as a minor side product during the click reaction, proved difficult. Fractions containing pure product were combined and solvent was removed under high vacuum to afford **14** (3.0 mg, 1.6 μmol, 26%) as a pink solid. **HRMS**: calculated $[M+2H]^{2+}$ 918.9485, found 918.9479.

^{1}H NMR (600 MHz, DMSO-d_6) δ 9.27 (d, J = 3.5 Hz, 1H), 9.18 (s, 1H), 9.05 – 8.98 (m, 2H), 8.86 (d, J = 9.4 Hz, 1H), 8.79 (br s, 1H, TAMRA), 8.71 (d, J = 4.1 Hz, 1H), 8.40 (d, J = 9.6 Hz, 1H), 8.35 – 8.10 (br m, 2H, TAMRA), 8.08 – 7.95 (m, 3H), 8.09 – 7.74 (br m, 1H, TAMRA), 7.48 – 7.36 (m, 5H), 7.28 – 7.17 (m, 10H), 7.11 – 7.05 (m, 4H), 6.95 – 6.89 (m, 3H), 6.74 (d, J = 8.6 Hz, 2H), 6.64 – 6.60 (m, 2H), 7.22 – 6.67 (br m, 6H, TAMRA), 5.57 – 5.50 (m, 1H), 5.30 – 5.23 (m, 1H), 4.92 – 4.85 (m, 2H), 4.57 – 4.46 (m, 4H), 4.37 – 4.26 (m, 4H, two of TAMRA), 4.07 (d, J = 8.3 Hz, 1H), 3.81 (q, J = 6.2 Hz, 1H), 3.32 – 3.30 (m, 2H), 3.27 – 2.70 (br m, 14H, TAMRA), 3.08 – 2.63 (m, 9H), 2.35 – 2.27 (m, 2H), 2.21 – 2.14 (m, 1H), 2.06 – 1.93 (m, 4H, two of TAMRA), 1.89 – 1.75 (m, 2H), 1.74 – 1.59 (m, 5H), 1.48 – 1.36 (m, 3H), 1.28 – 1.14 (m, 2H), 1.08 – 1.00 (m, 1H), 0.92 – 0.75 (m, 12H), 0.43 – 0.33 (m, 1H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 174.5, 173.0, 172.3, 171.9, 171.0, 170.8, 170.7, 170.7, 170.3, 170.2, 169.8, 169.2, 164.7, 156.7, 155.7, 142.6, 138.5, 136.1, 130.1, 129.7, 129.6, 129.3, 128.8, 128.2, 128.0, 127.9, 126.9, 126.1, 124.1, 114.9, 114.0, 61.4, 59.6, 56.8, 56.2, 55.0, 54.1, 53.4, 52.6, 50.4, 50.3, 49.1, 47.3, 45.7, 41.3, 39.1, 38.54, 37.1, 37.0, 36.7, 35.5, 35.0, 31.4, 31.1, 31.0, 29.7, 28.5, 25.7, 24.6, 23.1, 22.8, 22.0, 21.9, 18.7, 17.9.

Table S1. Catalytic parameters for pyrophosphate exchange[a]

TycA	Substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}\ min^{-1}$)
wt	L-Phe [b]	300 ± 20	0.011 ± 0.002	27,000 ± 8,000
	O-propargyl-L-Tyr	n.d.[c]	n.d.[c]	2.3 ± 0.9
	p-bromo-L-Phe	n.d.[c]	n.d.[c]	250 ± 90
	p-iodo-L-Phe	n.d.[c]	n.d.[c]	72 ± 32[d]
	p-benzoyl-L-Phe	n.d.[c]	n.d.[c]	0.35 ± 0.10
W227S	L-Phe	260 ± 10	2.6 ± 0.3	100 ± 20
	O-propargyl-L-Tyr	325 ± 4	0.5 ± 0.1	690 ± 160
	p-bromo-L-Phe	350 ± 20	0.058 ± 0.002	6,000 ± 400
	p-iodo-L-Phe	380 ± 50	0.026 ± 0.003	15,000 ± 2000
	p-benzoyl-L-Phe	190 ± 20	1.6 ± 0.5	120 ± 40

[a] Values represent the average ± s.d. of two biological replicas if not stated otherwise.
[b] Values are from reference 2.
[c] n.d. = not determined due to the absence of substrate saturation.
[d] Errors are error of fit for a single experiment.

Table S2. LC-HRMS analysis. Summary of tyrocidine analogs produced by *in vitro* reactions with the reprogrammed synthetase W227S TycA/TycB/TycC when supplemented with the indicated amino acids.

supplemented amino acid	retention time (min)	relative intensity	compound	calculated $[M+H]^+$	found $[M+H]^+$
L-Phe	4.23	0.87	**6**	1286.6568	1286.6556
	4.48	1.00	**1**	1270.6619	1270.6597
O-propargyl-L-Tyr [a]	4.23	0.66	**6**	1286.6568	1286.6561
	4.48	0.50	**1**	1270.6619	1270.6629
	4.52	1.00	**2**	1324.6725	1324.6708
p-bromo-L-Phe [a]	4.23	0.04	**6**	1286.6568	1286.6581
	4.48	0.02	**1**	1270.6619	1270.6631
	4.65	1.00	**3**	1350.5723	1350.5718
	4.93	0.12	‡	1428.4809	1428.4838
p-iodo-L-Phe [a]	4.23	0.02	**6**	1286.6568	1286.6584
	4.48	0.02	**1**	1270.6619	1270.6635
	4.73	1.00	**4**	1396.5585	1396.5553
	5.14	0.10	‡	1522.4552	1522.4565
p-benzoyl-L-Phe [a]	4.23	1.00	**6**	1286.6568	1286.6564
	4.48	0.65	**1**	1270.6619	1270.6621
	4.65	0.05	**5**	1374.6881	1374.6891

‡ indicates a tyrocidine analog with two Phe replaced by the respective functionalized amino acid.

[a] The DKP formed between the supplemented amino acid and Pro seen in the chromatograms (Figure 3B) were identified by LC-MS.

Table S3. LC-HRMS analysis. Summary of tyrocidine analogs produced by *in vitro* reactions with the reprogrammed synthetase TycA/W2742S TycB/TycC when supplemented with the indicated amino acids.

supplemented amino acid	retention time (min)	relative intensity	compound	calculated $[M+H]^+$	found $[M+H]^+$
L-Phe	4.12	0.13	**11**	1286.6556	1286.6574
	4.49	1.00	**1**	1270.6597	1270.6572
O-propargyl-L-Tyr	4.12	0.01	**11**	1286.6561	1286.6579
	4.39	1.00	**7**	1324.6708	1324.6687
	4.48	0.06	**1**	1270.6629	1270.6633
p-bromo-Phe	4.48	0.01	**1**	1270.6631	1270.6632
	4.64	1.00	**8**	1350.5718	1350.571
	4.92	0.13	‡	1428.4838	1428.4831
p-iodo-Phe	4.68	1.00	**9**	1396.5553	1396.5554
	5.07	0.11	‡	1522.4565	1522.4559
p-benzoyl-Phe	4.12	0.07	**11**	1286.6564	1286.6585
	4.48	1.00	**10**	1374.6891	1374.6877
	4.48	0.59	**1**	1270.6621	1270.6628

‡ indicates a tyrocidine analog with two Phe replaced by the respective functionalized amino acid.

Table S4. Specificity codes for select homologs of the TycA A domain possessing a delimiting Trp residue.

NRP	A domain	Specificity code[a]	NCBI accession #
tyrocidine	TycA-A1	A W T I A A I C	P09095
	TycB-A3	A W T I A G V C	HBZ81682
gramicidin S	GrsA-A1	A W T I A A I C	WP_043064678
glycopeptidolipid	Mps1-A1	A W T A V A I C	BAR79916
	Mps2-A1	V W C I A A I Y	WP_011726917
	Mps2-A2	V W Y V A A I Y	WP_011726917
octapeptin	OctB-A1	A W I I G A I V	CUX79061
pelgipeptin	PlpE-A3	A W T I A A I C	AFJ14794
polymyxin	PmxA-A1	A W T I A A I A	AEZ51516
linear gramicidin	LgrD-A1	A W T I A A I C	WP_160243239
	LgrD-A3	A W T I A A I C	WP_160243239
thaxtomin	TxtA-A1	A W T V A A V C	WP_010350605
barbamide	BarG-A1	A W T V A A V C	WP_008178615
virginiamycin	VisF-A2	A W T V A A V C	BAK86398
mannopeptimycin	MppA-A3	G W T G A I L S	AAU34202
aureobasidin	Aba1-A3	A W V L A G I Q	ACJ04424
	Aba1-A4	A W V L A G I Q	ACJ04424
lysocin	LesA-A5	A W T I A A V C	BAV56270
tridecaptin	TriD-A9	A W T F A G V D	WP_051114643
salinamide	Sln7-A2	A W T V A A V C	ABW84365
cyanopeptolin	OciB-A4	A W T I A G V C	BAK86398

[a] Amino acids corresponding to the specificity conferring positions 224, 227, 266, 287, 289, 310, 318 and 319 in TycA-A.[6,7] Position 227 in TycA-A corresponds to position 239 in GrsA-A.

W227S TycA (residues 1-1088)

XVANQANLID NKRELEQHAL VPYAQGKSIH QLFEEQAEAF PDRVAIVFEN RRLSYQELNR KANQLARALL
EKGVQTDSIV GVMMEKSIEN VIAILAVLKA GGAYVPIDIE YPRDRIQYIL QDSQTKIVLT QKSVSQLVHD
VGYSGEVVVL DEEQLDARET ANLHQPSKPT DLAYVIYTSG TTGKPKGTML EHKGIANLQS FFQNSFGVTE
QDRIGLFASM SFDASVSEMF MALLSGASLY ILSKQTIHDF AAFEHYLSEN ELTIITLPPT YLTHLTPERI
TSLRIMITAG SASSAPLVNK WKDKLRYINA YGPTETSICA TIWEAPSNQL SVQSVPIGKP IQNTHIYIVN
EDLQLLPTGS EGELCIGGVG LARGYWNRPD LTAEKFVDNP FVPGEKMYRT GDLAKWLTDG TIEFLGRIDH
QVKIRGHRIE LGEIESVLLA HEHITEAVVI AREDQHAGQY LCAYYISQQE ATPAQLRDYA AQKLPAYMLP
SYFVKLDKMP LTPNDKIDRK ALPEPDLTAN QSQAAYHPPR TETESILVSI WQNVLGIEKI GIRDNFYSLG
GDSIQAIQVV ARLHSYQLKL ETKDLLNYPT IEQVALFVKS TTRKSDQGII AGNVPLTPIQ KWFFGKNFTN
TGHWNQSSVL YRPEGFDPKV IQSVMDKIIE HHDALRMVYQ HENGNVVQHN RGLGGQLYDF FSYNLTAQPD
VQQAIEAETQ RLHSSMNLQE GPLVKVALFQ TLHGDHLFLA IHHLVVDGIS WRILFEDLAT GYAQALAGQA
ISLPEKTDSF QSWSQWLQEY ANEADLLSEI PYWESLESQA KNVSLPKDYE VTDCKQKSVR NMRIRLHPEE
TEQLLKHANQ AYQTEINDLL LAALGLAFAE WSKLAQIVIH LEGHGREDII EQANVARTVG WFTSQYPVLL
DLKQTAPLSD YIKLTKENMR KIPRKGIGYD ILKHVTLPEN RGSLSFRVQP EVTFNYLGQF DADMRTELFT
RSPYSGGNTL GADGKNNLSP ESEVYTALNI TGLIEGGELV LTFSYSSEQY REESIQQLSQ SYQKHLLAII
AHCTEKKEVE RTPSDFSVKG LQMEEMDDIF ELLANTLR

A His_6-tag is appended to the N-terminus of the protein via an SGRS linker: X = MHHHHHHSGRS; residue 227 is highlighted in red

Module 3 A, T and E domains of W2742S TycB (residues 2521-3588)

MLTAAEKQML LVAFNDTHRE YRADQTIQQL FEELAEKMPE HTALVFEEKR MSFRELNERA NQLAAVLREK
GVGPAQIVAL LVERSAEMVI ATLATLKAGG AFLPVDPDYP EERIRYMLED SQAKLVVTHA HLLHKVSSQS
EVVDVDDPGS YATQTDNLPC ANTPSDLAYI IYTSGTTGKP KGVMLEHKGV ANLQAVFAHH LGVTPQDRAG
HFASISFDAS VSDMFGPLLS GATLYVLSRD VINDFQRFAE YVRDNAITFL TLPPTYAIYL EPEQVPSLRT
LITAGSASSV ALVDKWKEKV TYVNGYGPTE STVCATLWKA KPDEPVETIT IGKPIQNTKL YIVDDQLQLK
APGQMGELCI SGLSLARGYW NRPELTAEKF VDNPFVPGTK MYRTGDLARW LPDGTIEYLG RIDHQVKIRG
HRVELGEVES VLLRYDTVKE AAAITHEDDR GQAYLCAYYV AEGEATPAQL RAYMENELPN YMVPAFFIQL
EKMPLTPNDK IDRKALPKPN QEENRTEQYA APQTELEQLL AGIWADVLGI KQVGTQDNFF ELGGDSIKAI
QVSTRLNASG WTLAMKELFQ YPTIEEAALR VIPNSRESEQ GVVEGEIALT PIQKWFFANN FTDRHHWNQA
VMLFREDGFD EGLVRQAFQQ IVEHHDALRM VYKQEDGAIK QINRGLTDER FRFYSYDLKN HANSEARILE
LSDQIQSSID LEHGPLVHVA LFATKDGDHL LVAIHHLVVD GVSWRILFED FSSAYSQALH QQEIVLPKKT
DSFKDWAAQL QKYADSDELL REVAYWHNLE TTTTTAALPT DFVTADRKQK HTRTLSFALT VPQTENLLRH
VHHAYHTEMN DLLLTALGLA VKDWAHTNGV VINLEGHGRE DIQNEMNVTR TIGWFTSQYP VVLDMEKAED
LPYQIKQTKE NLRRIPKKGI GYEILRTLTT SQLQPPLAFT LRPEISFNYL GQFESDGKTG GFTFSPLGTG
QLFSPESERV FLLDISAMIE DGELRISVGY SRLQYEEKTI ASLADSYRKH LLGIIEHCMA KEEGEYTPSD
LGDEELSMEE LENILEWIGS RSHHHHHH

A His_6-tag is appended to the C-terminus of the protein via a GSRS linker; residue 2742 is highlighted in red.

Figure S1. Amino acid sequences of the modified tyrocidine synthetases

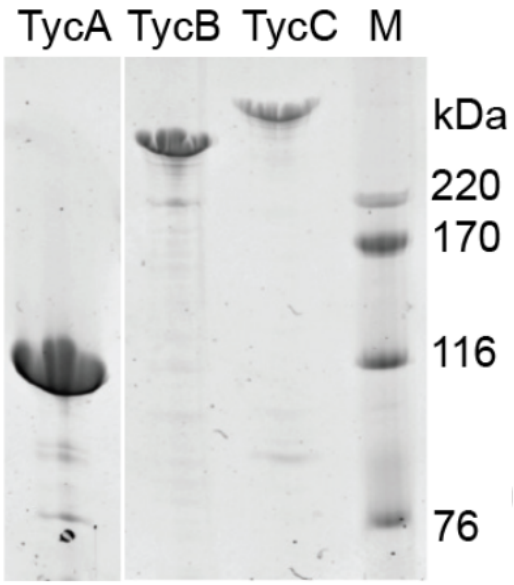


Figure S2. Protein production. 7.5% SDS-PAGE gels showing purified His_6-tagged TycA, TycB and TycC produced in *E. coli* HM0079; M = marker

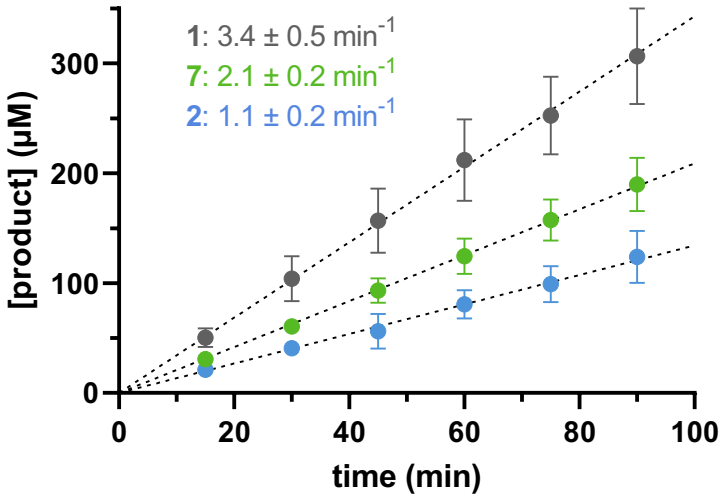


Figure S3. Kinetics for tyrocidine formation. In vitro biosynthesis of **1** (gray), **2** (blue), and **7** (green) by the wildtype synthetase, W227S TycA/TycB/TycC and TycA/W2742S TycB/TycC, respectively, was linear over the first 90 min and used to calculate k_{obs} values. As shown in Figure S4, W227S TycA/TycB/TycC produced a ~3:1 mixture of **2** and **1** and the data were corrected accordingly.

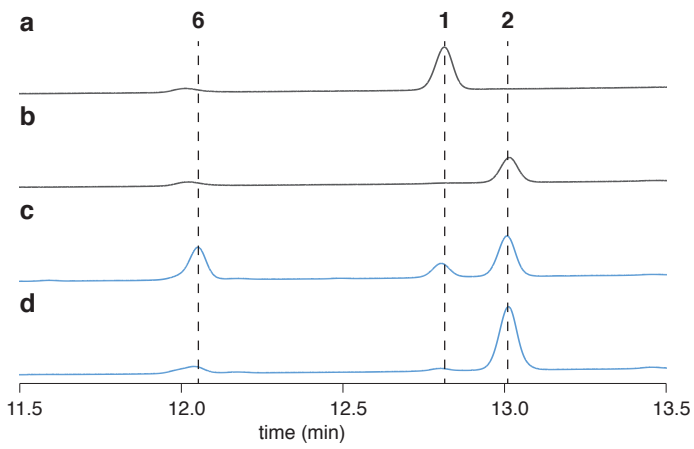


Figure S4. Selectivity of W227S TycA. HPLC chromatograms of authentic standards of (**a**) tyrocidine A (**1**) and (**b**) **2** and the products of in vitro reactions with the W227S TycA/TycB/TycC synthetase supplemented with (**c**) 2 mM or (**d**) 20 mM *O*-propargyl-L-Tyr, respectively. Tyrocidines **1** or **6** are formed as side product by incorporation of L-Phe or L-Tyr at position 1. The reactions and authentic standards were injected onto a Reprospher 100 Phenyl-hexyl column to achieve baseline separation of **1** and **2**; note that the column and conditions differ from those described in Figure 3 of the main text.

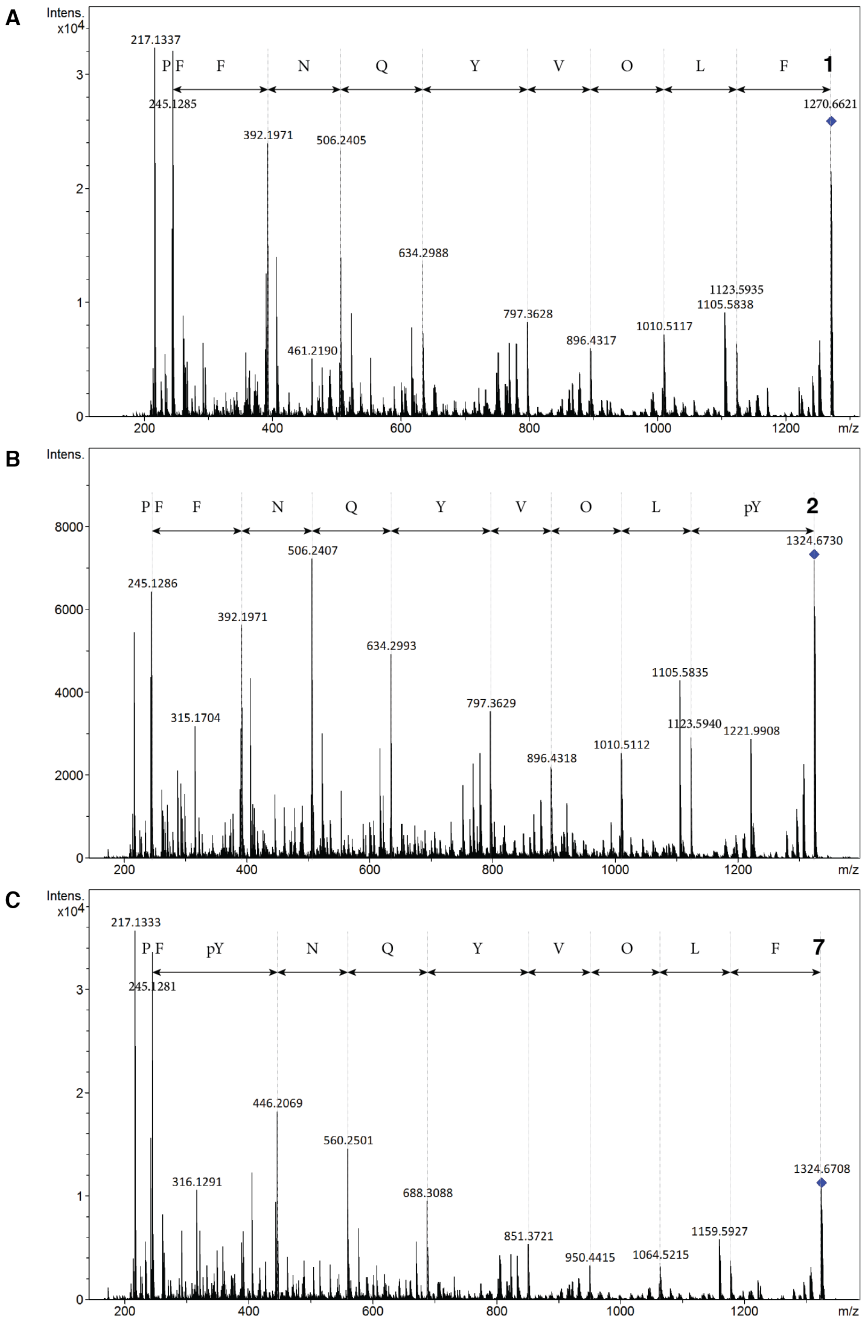
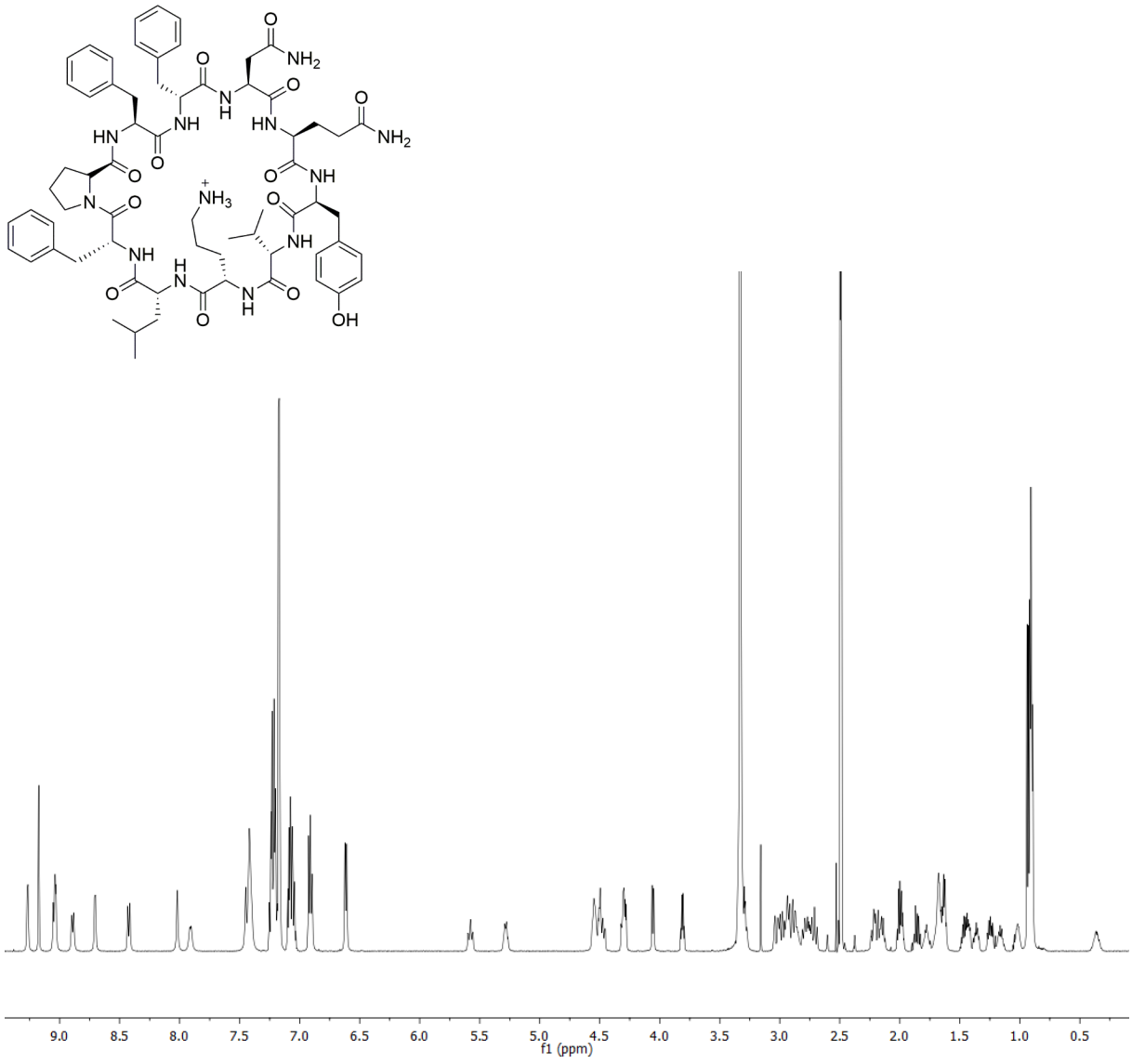


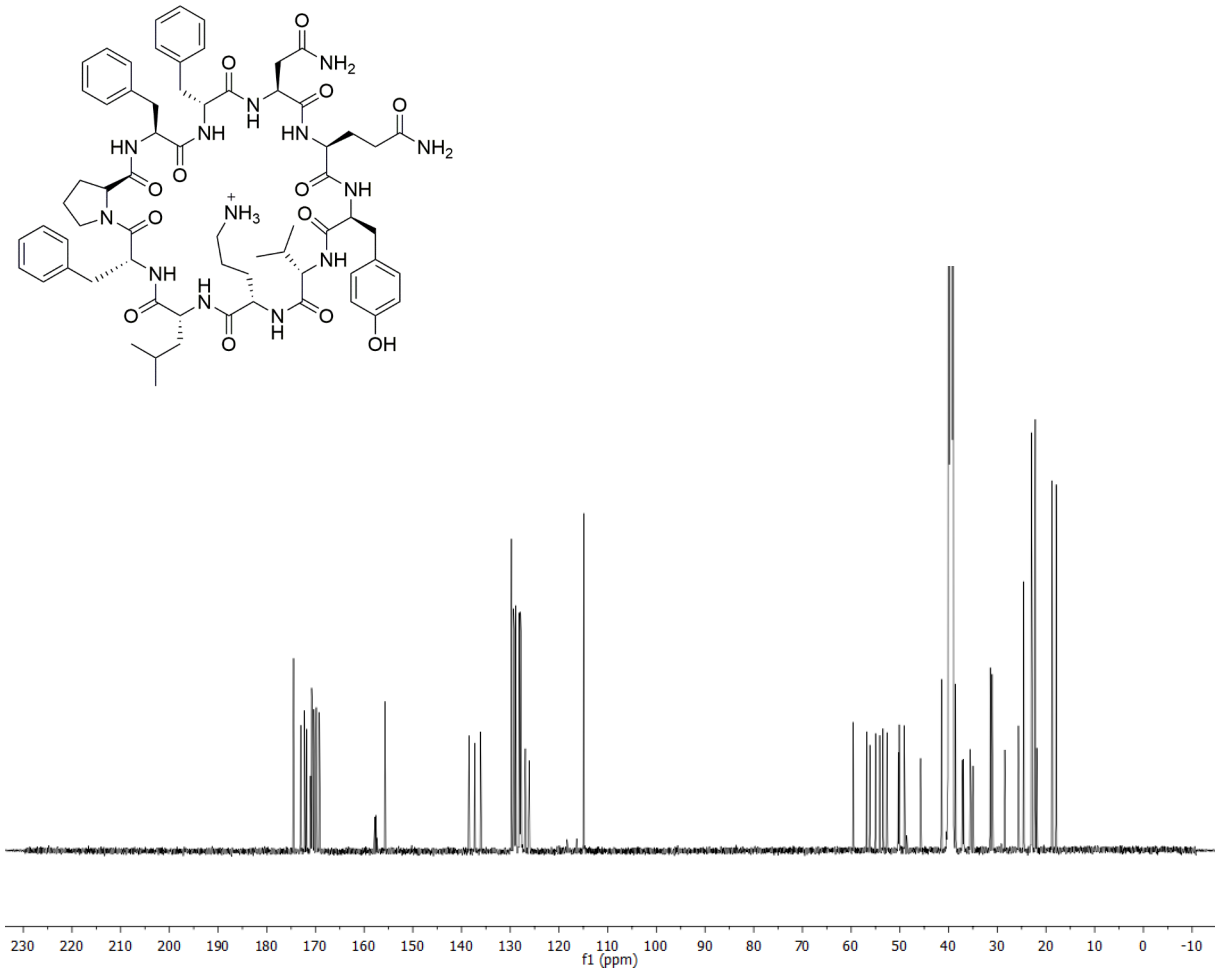
Figure S5. MS/MS analysis of (A) tyrocidine A (1), (B) 2, and (C) 7. The found b-ion series and respective amino acid sequence is shown for each compound. O = ornithine, pY = *O*-propargyl-tyrosine.

NMR spectra

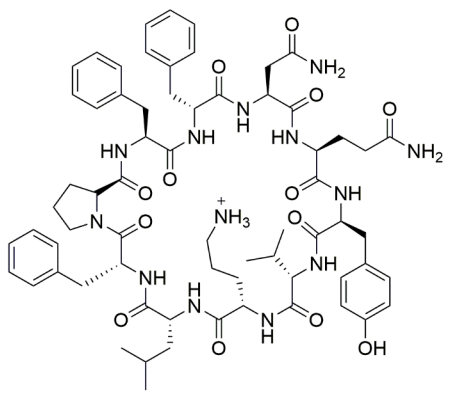
Tyrocidine A (1): ^{1}H-NMR (DMSO-*d*6)

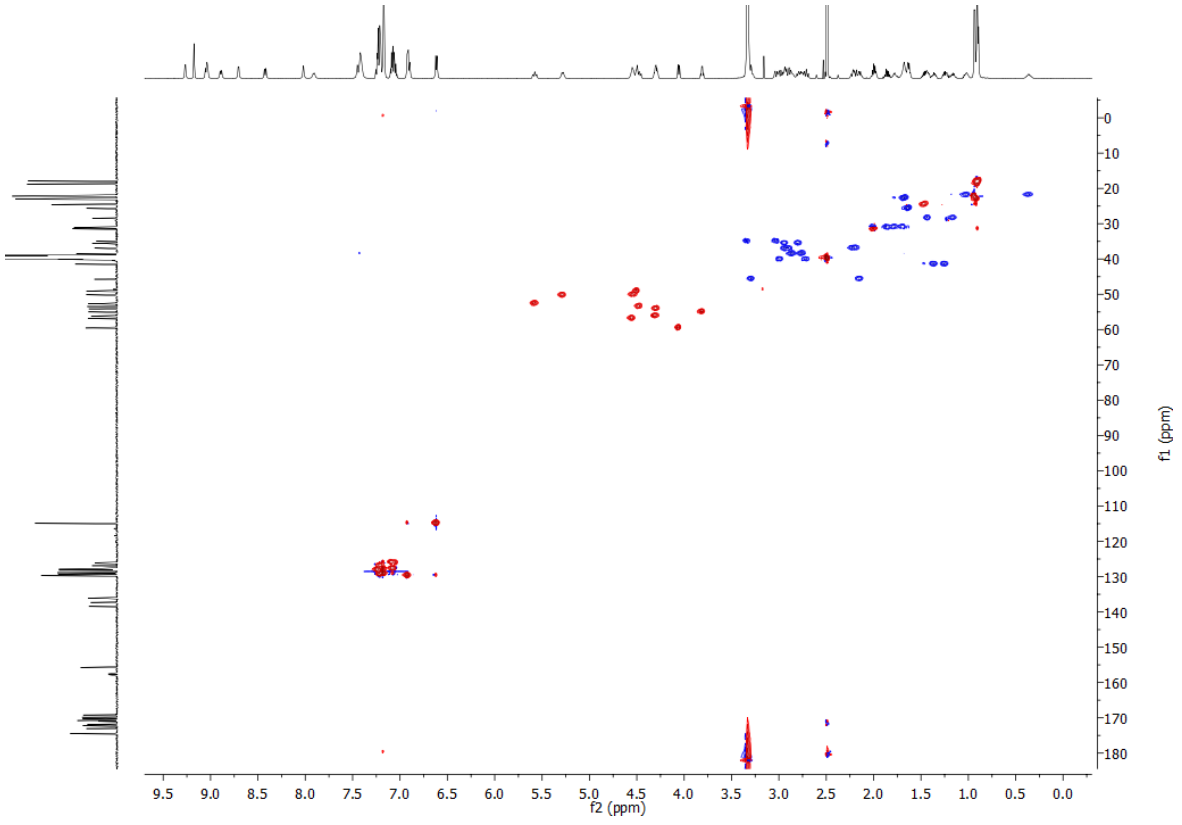


Tyrocidine A (1): ^{13}C-NMR (DMSO-d_6)

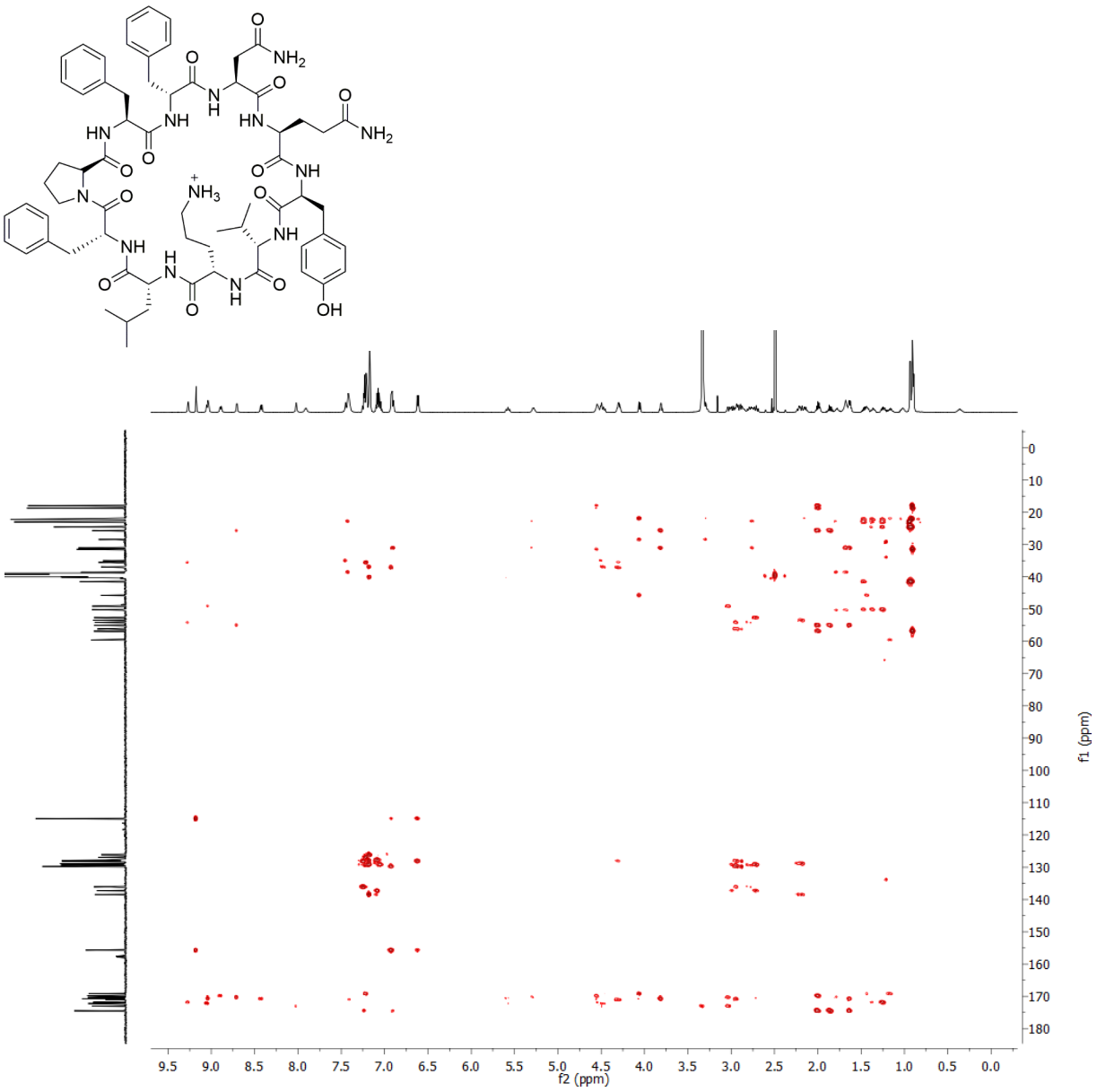


Tyrocidine A (1): HSQC (DMSO-d_6)

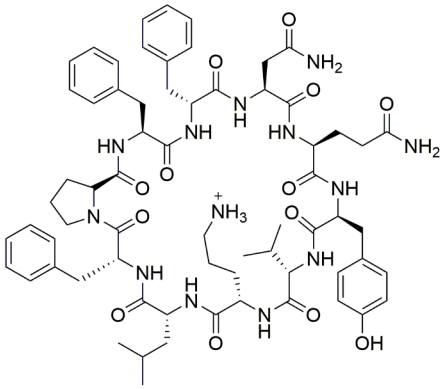


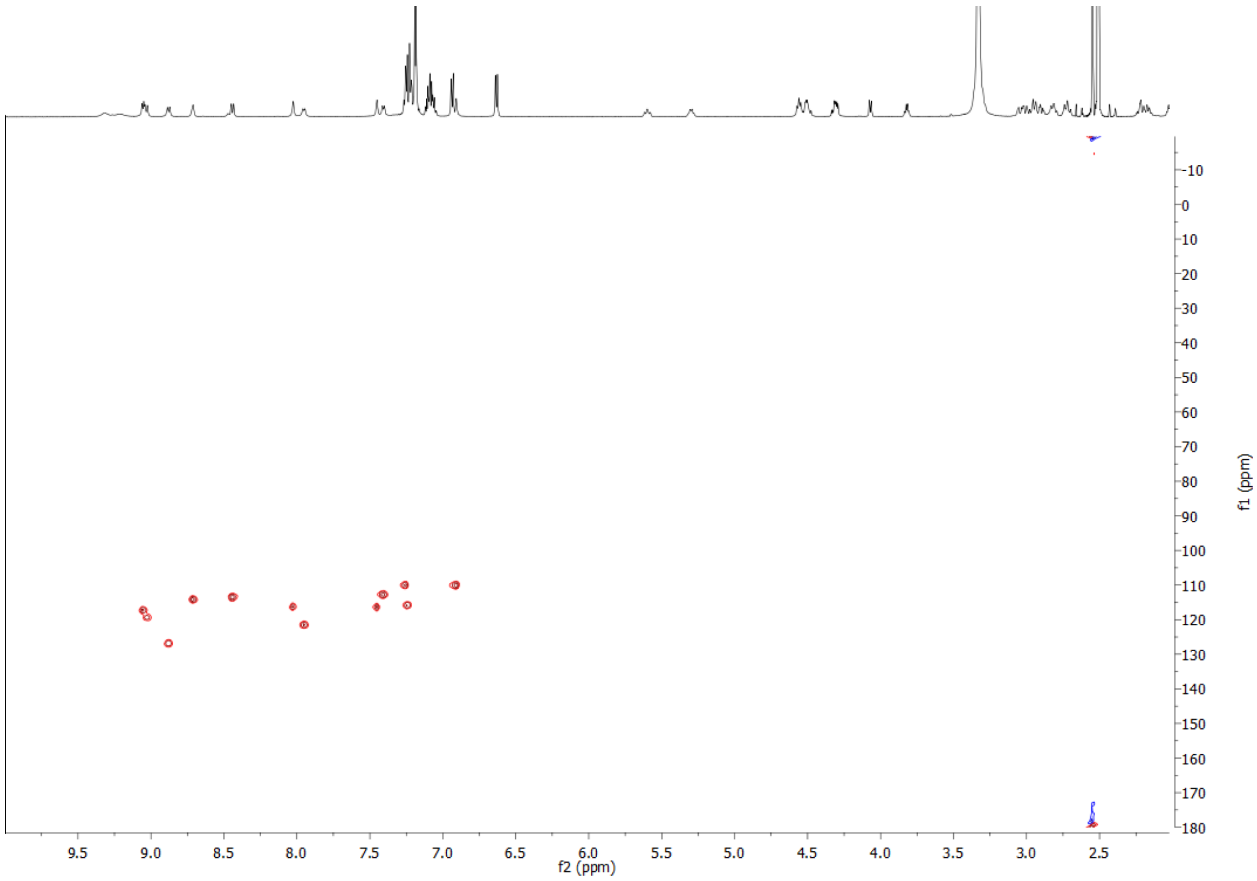


Tyrocidine A (1): HMBC (DMSO-d_6)

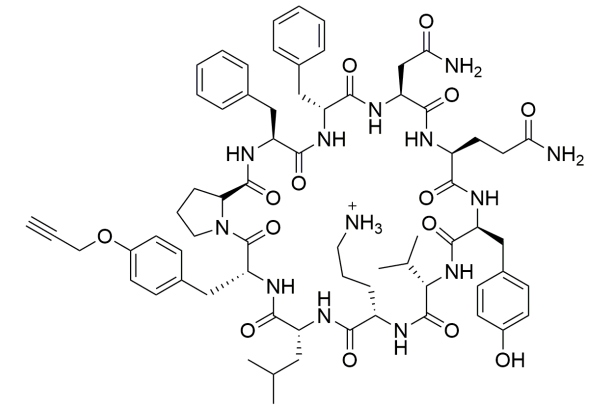


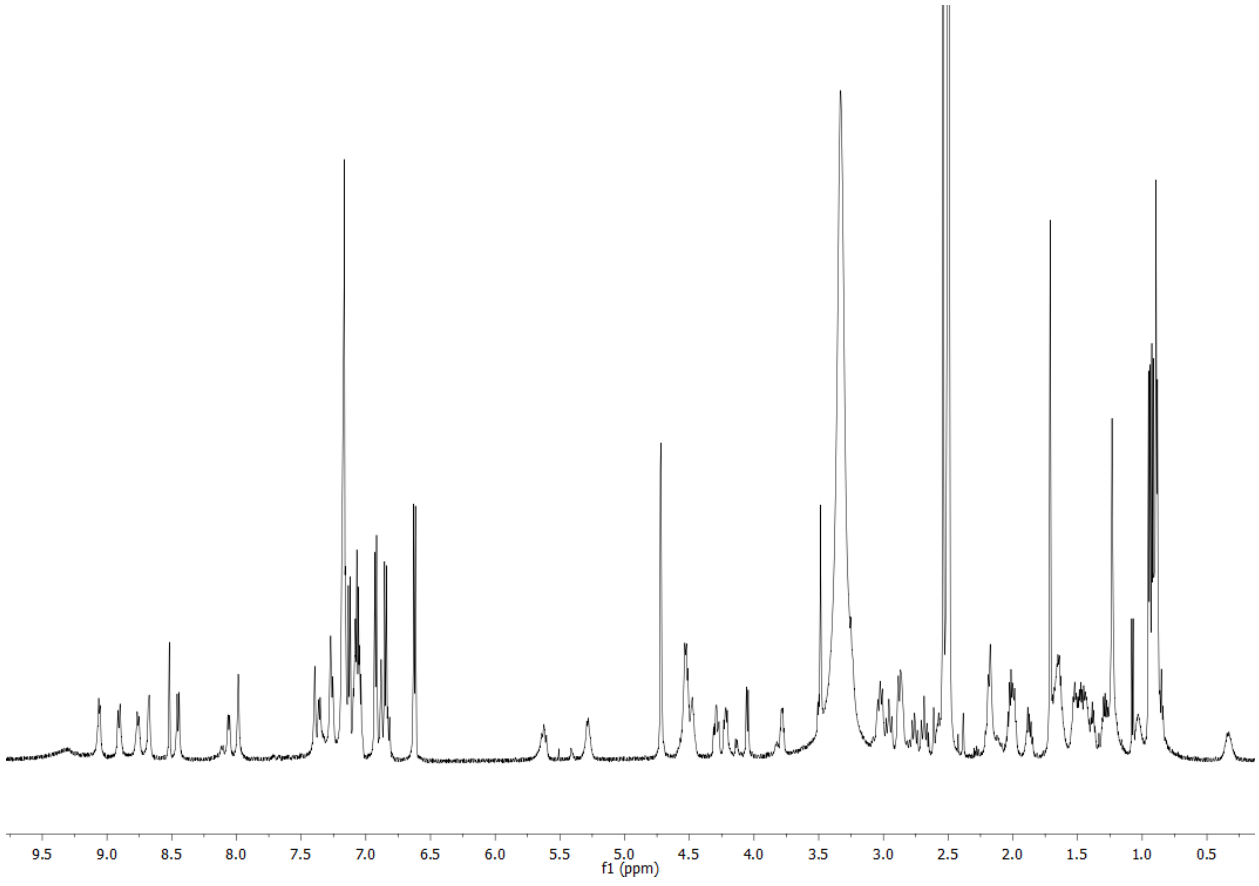
Tyrocidine A (1): ^{15}N-HSQC (DMSO-d_6)



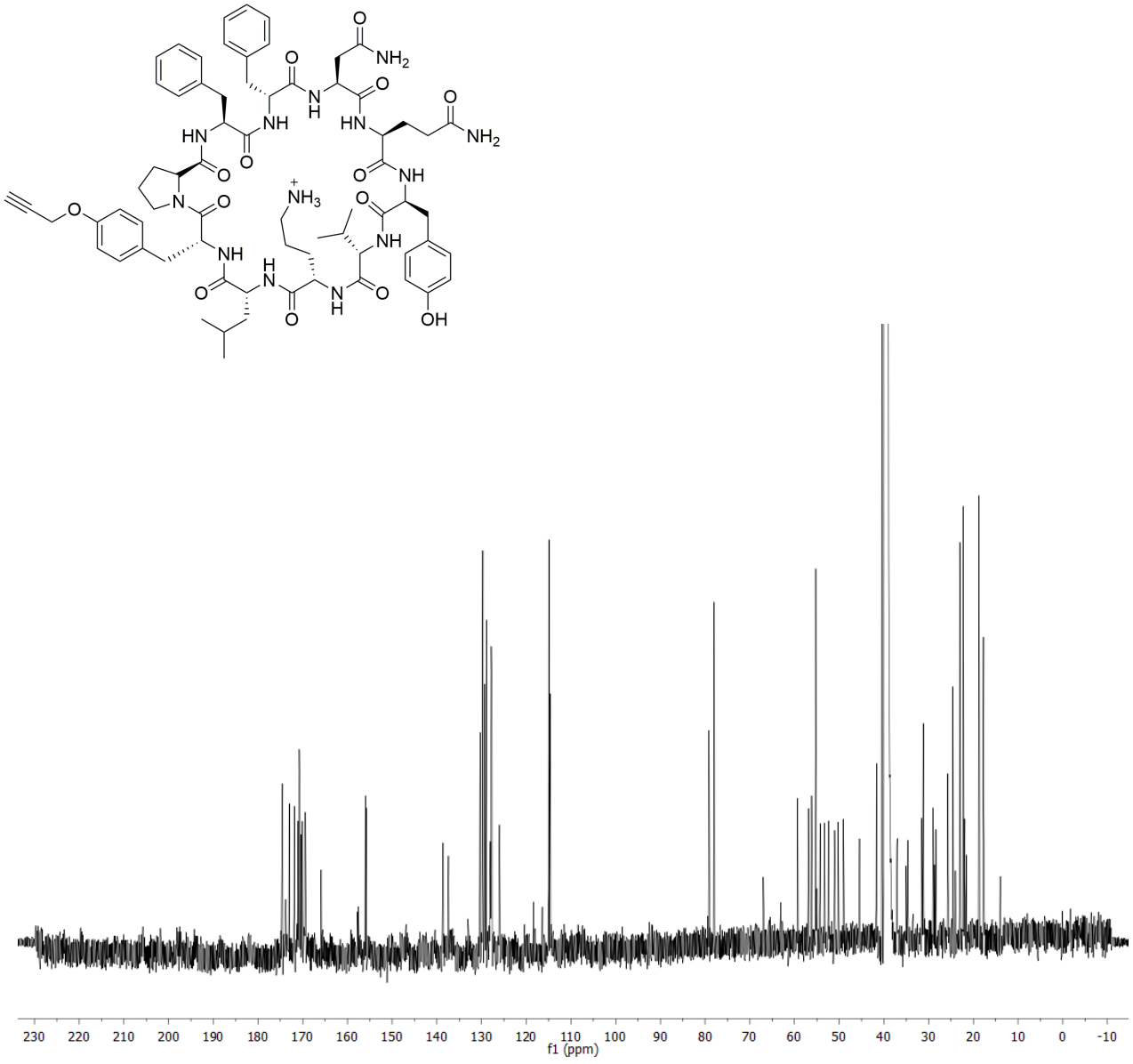


Propargylated tyrocidine A (2): ^{1}H-NMR (DMSO-*d*6)

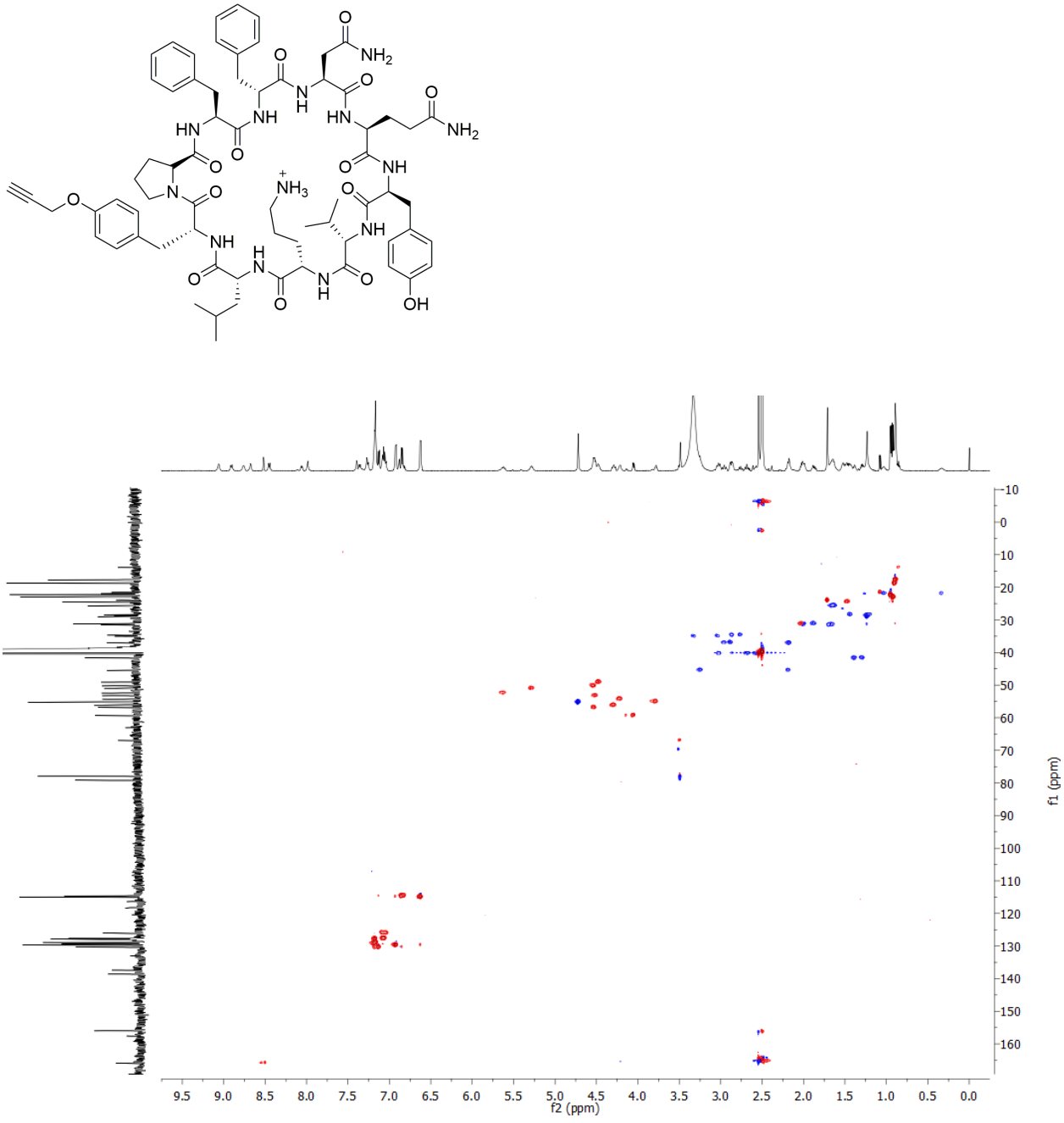




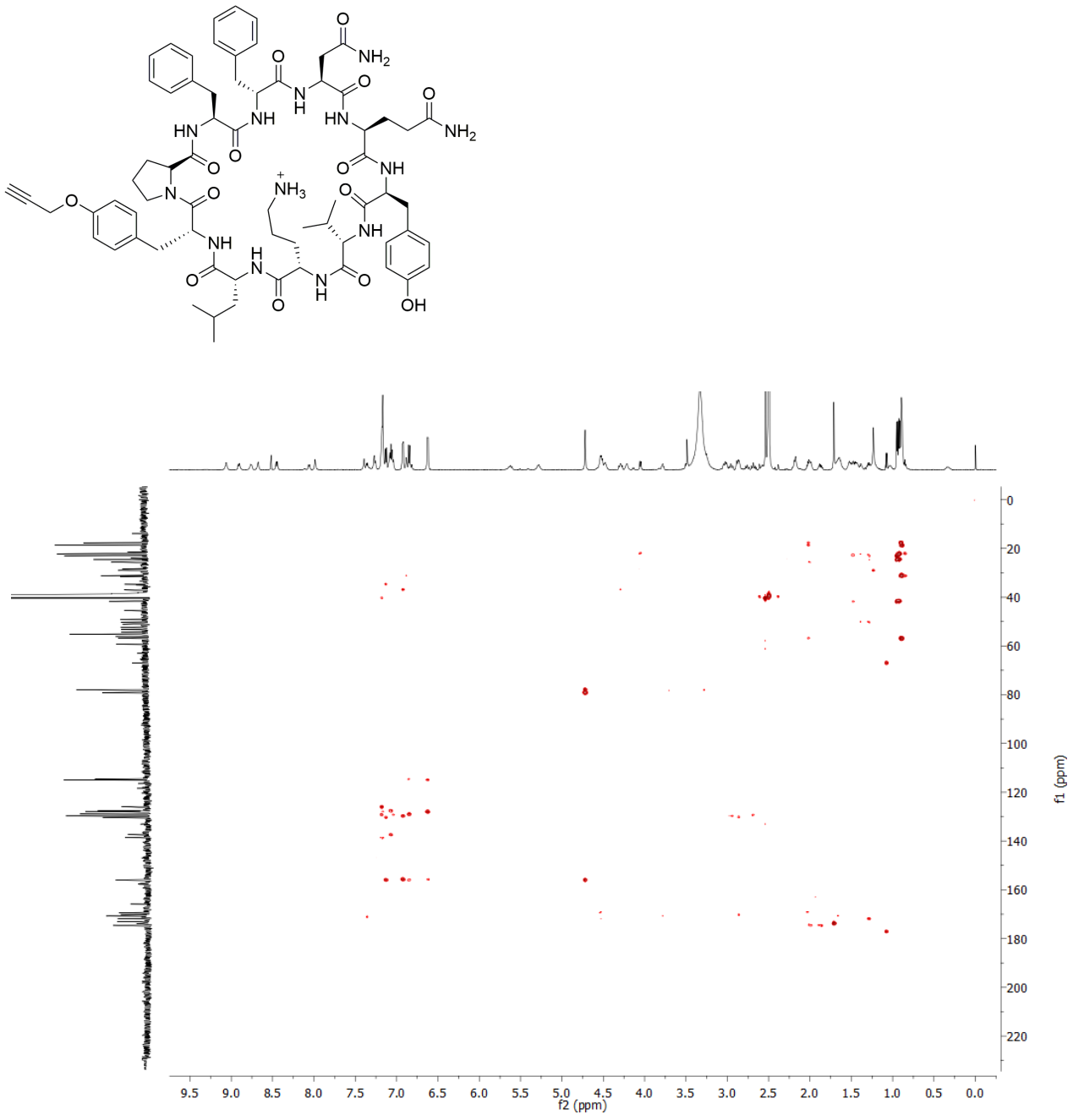
Propargylated tyrocidine A (2): ^{13}C-NMR (DMSO-d_6)



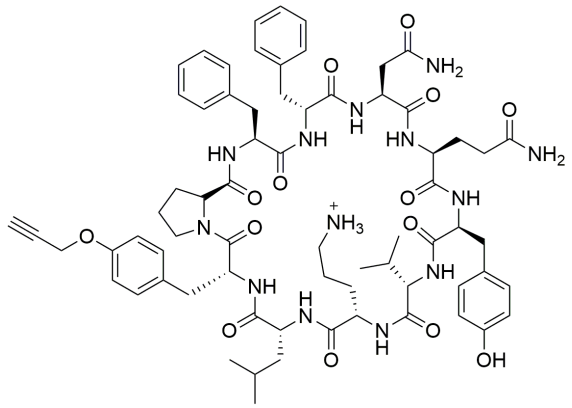
Propargylated tyrocidine A (2): HSQC (DMSO-d_6)

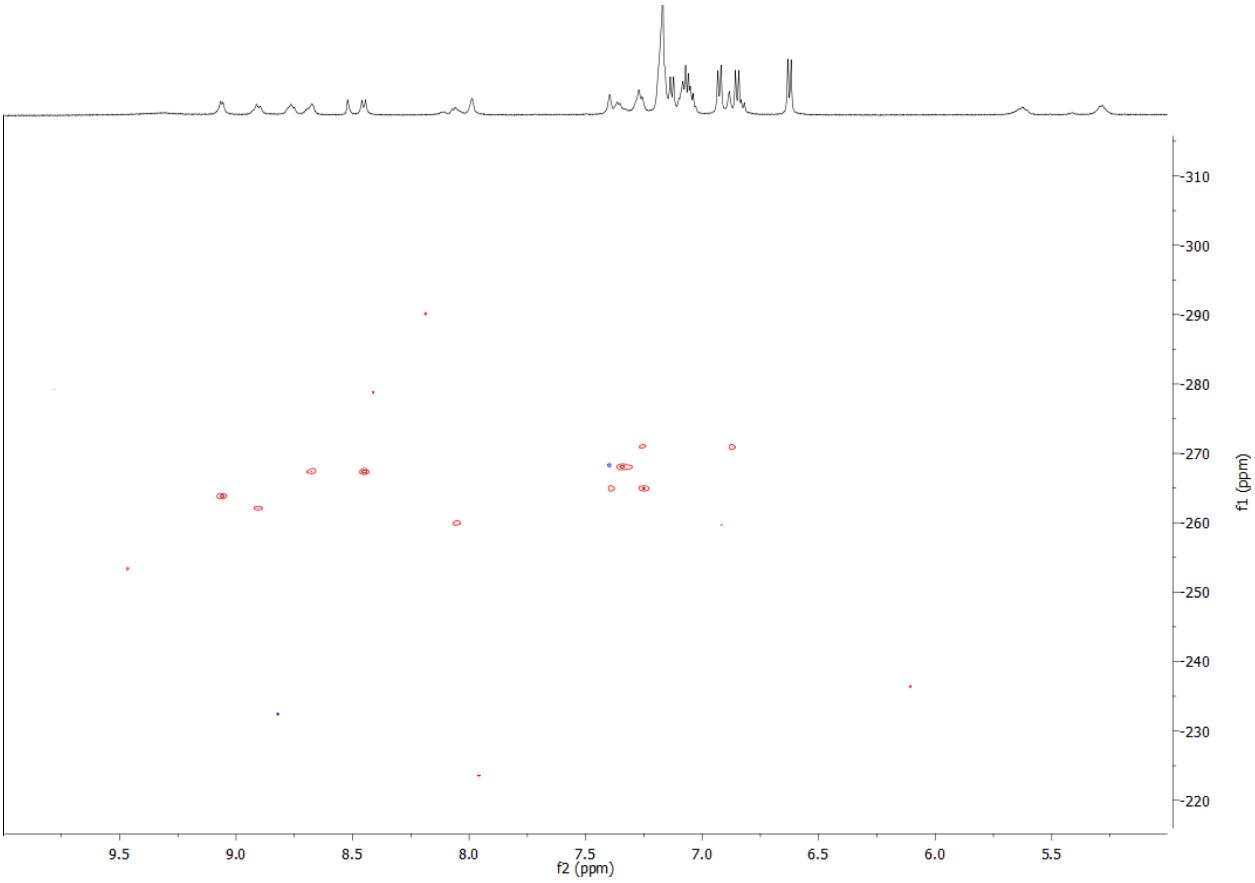


Propargylated tyrocidine A (2): HMBC (DMSO-d_6)

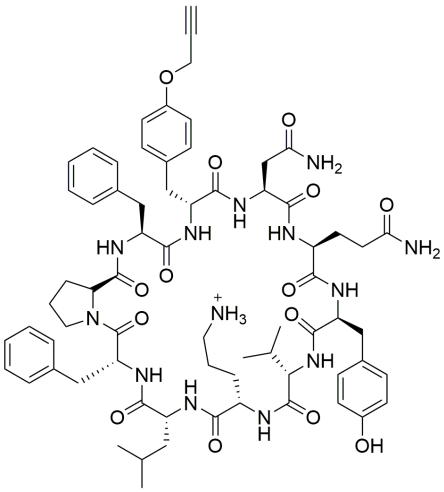


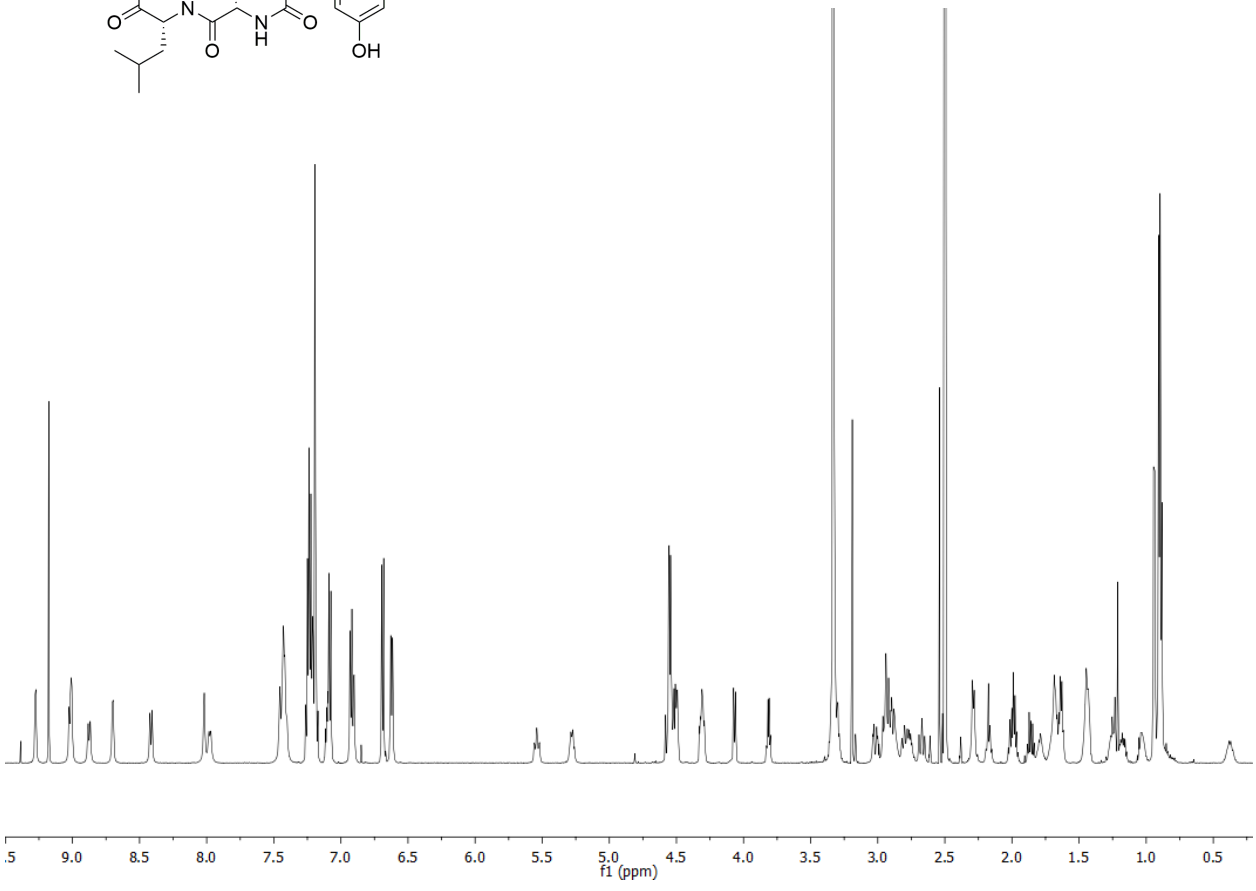
Propargylated tyrocidine A (2): ^{15}N-HSQC (DMSO-d_6)



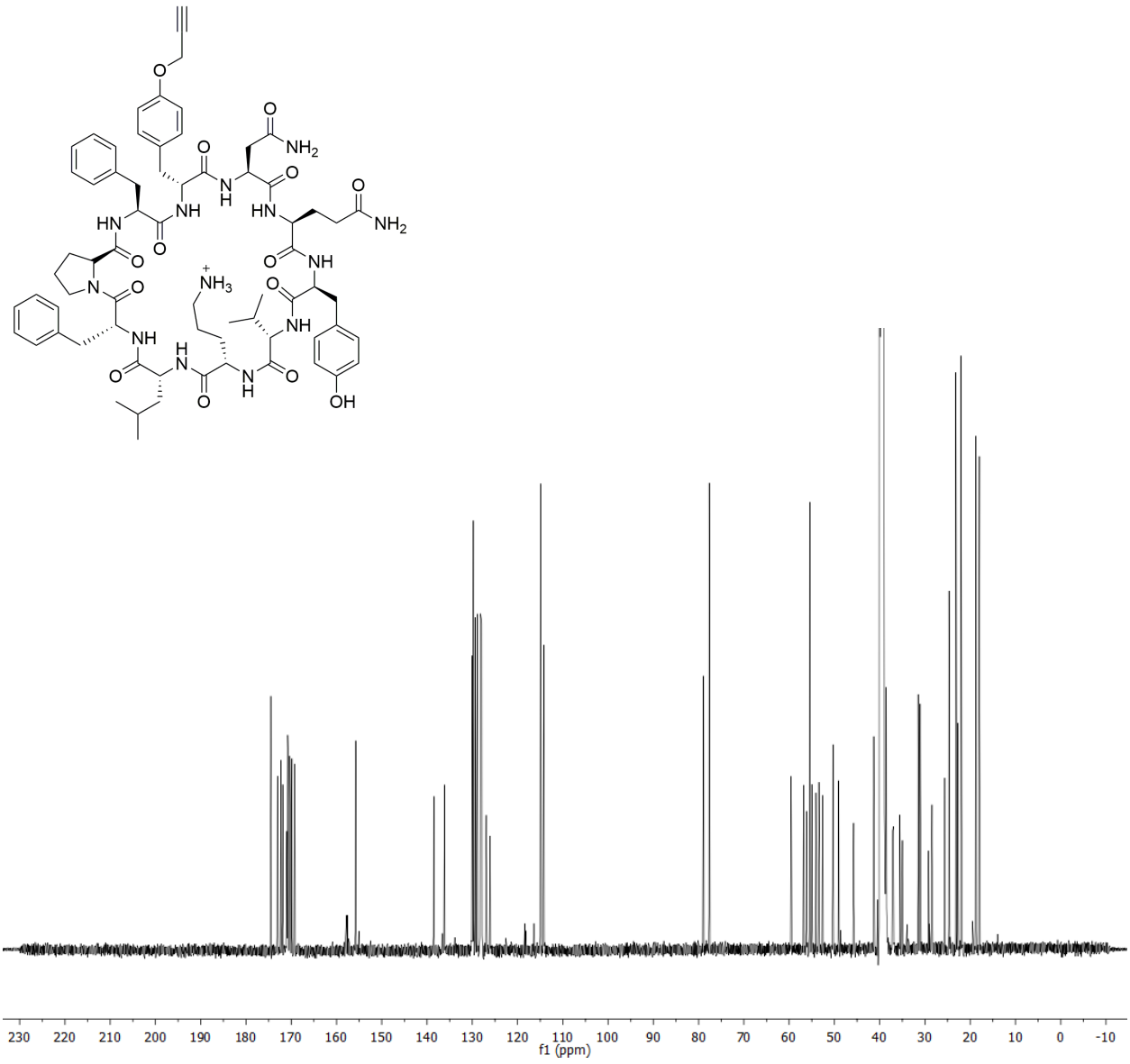


Propargylated tyrocidine A (7): ^{1}H-NMR (DMSO-*d*6)

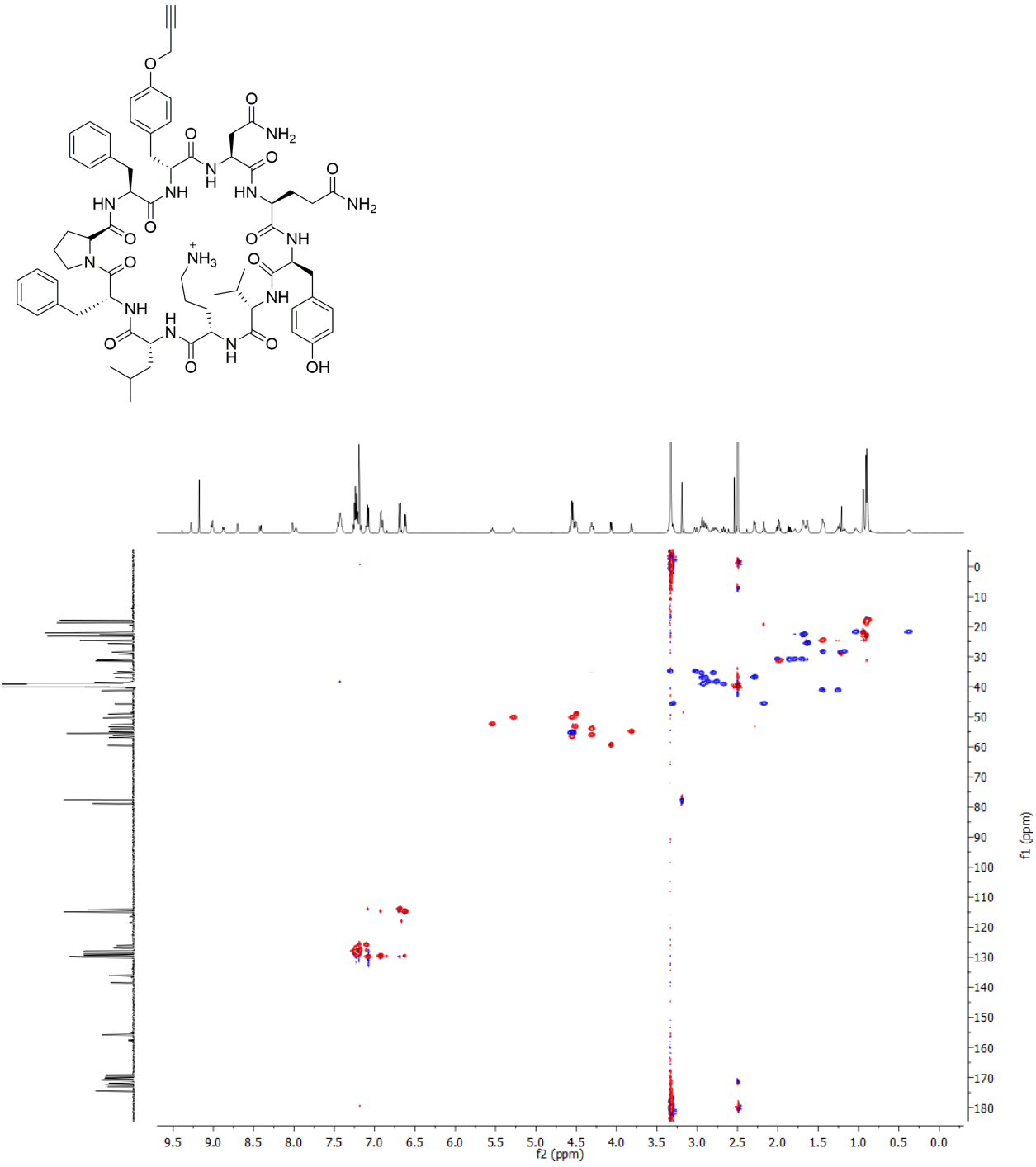




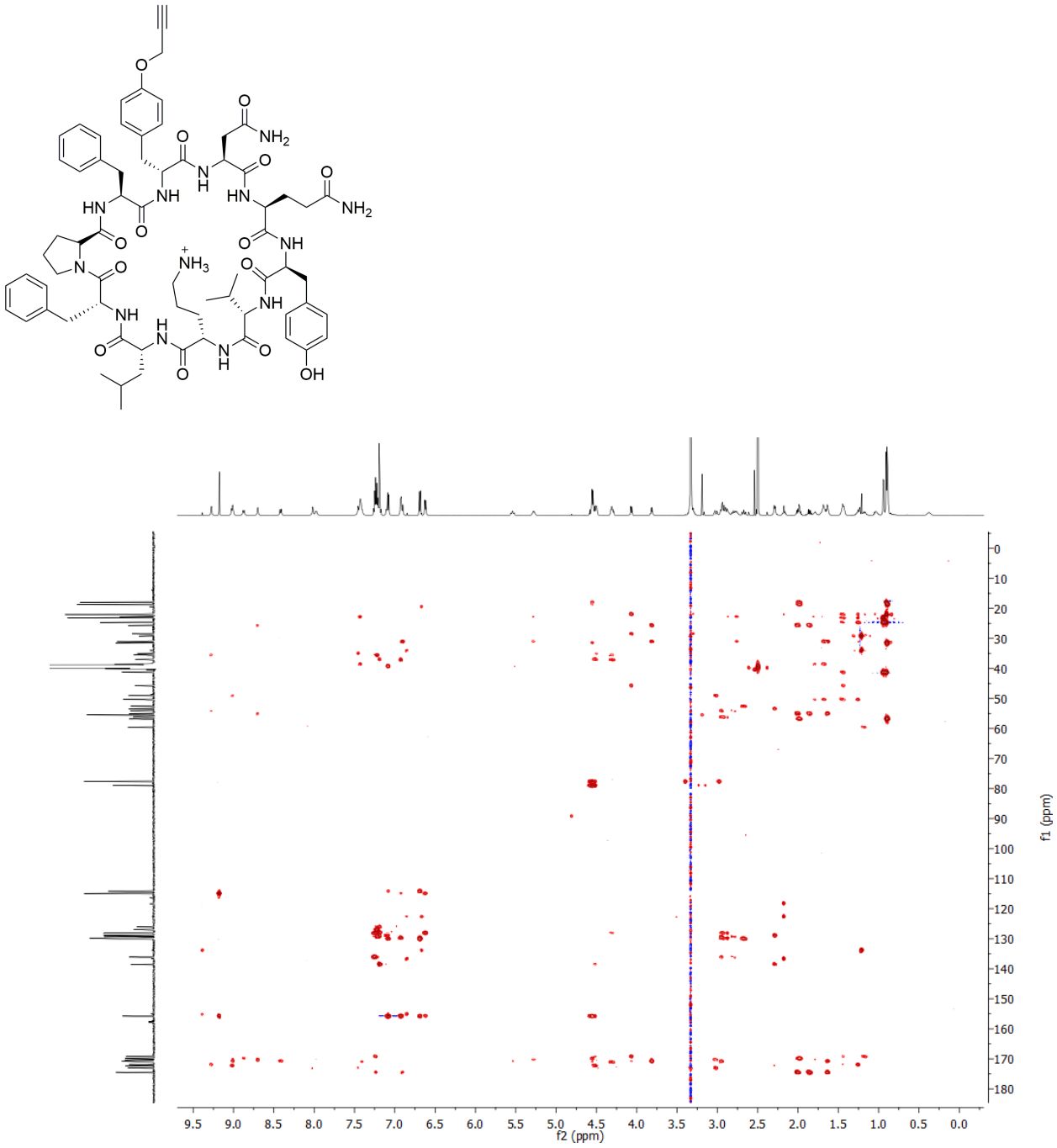
Propargylated tyrocidine A (7): ^{13}C-NMR (DMSO-d_6)



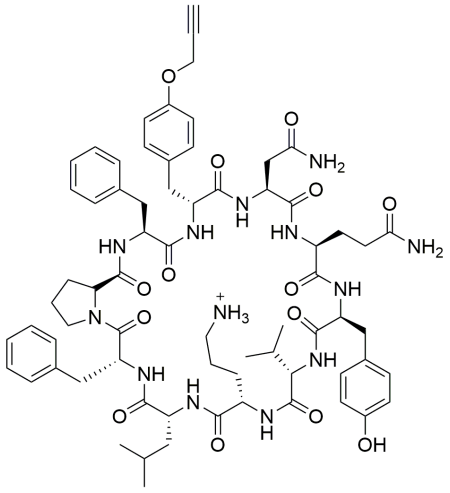
Propargylated tyrocidine A (7): HSQC (DMSO-d_6)

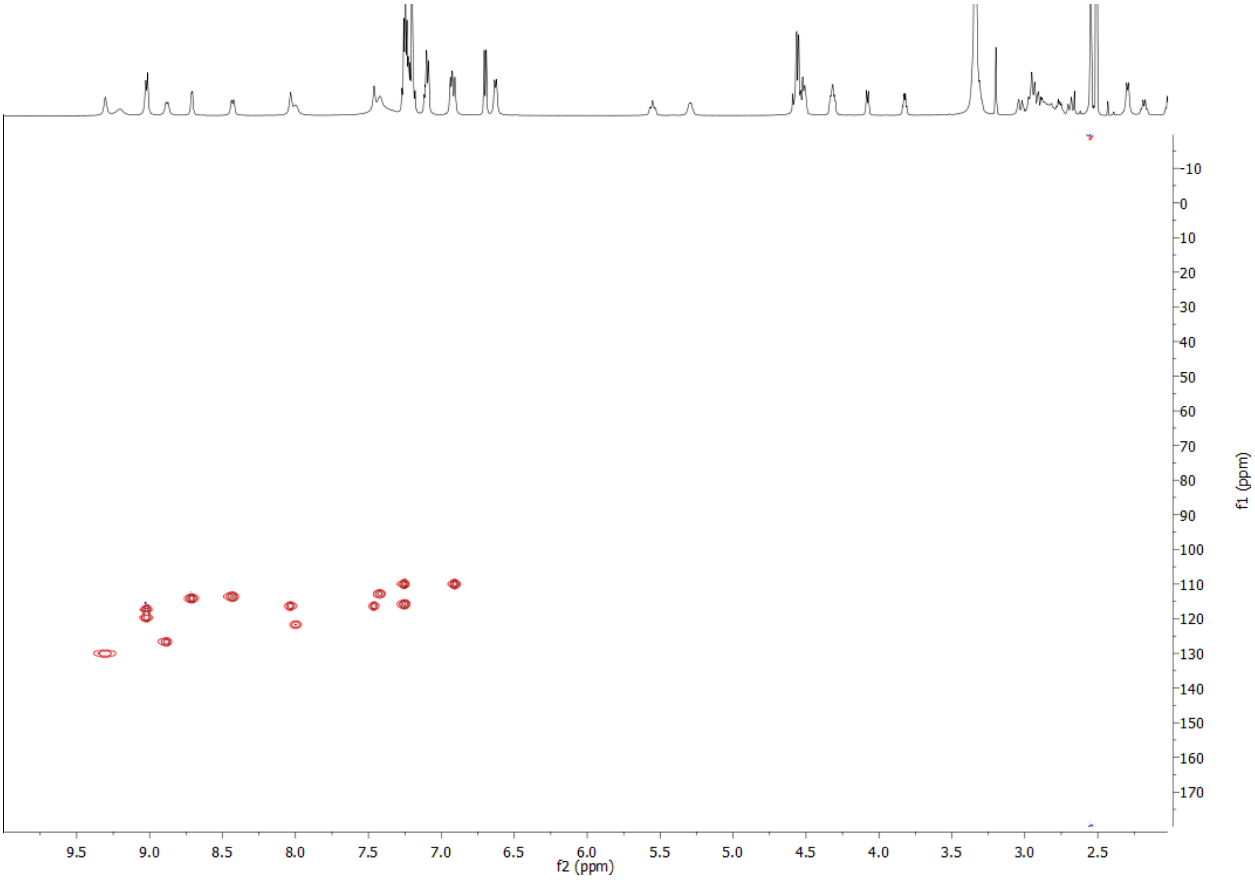


Propargylated tyrocidine A (7): HMBC (DMSO-d_6)

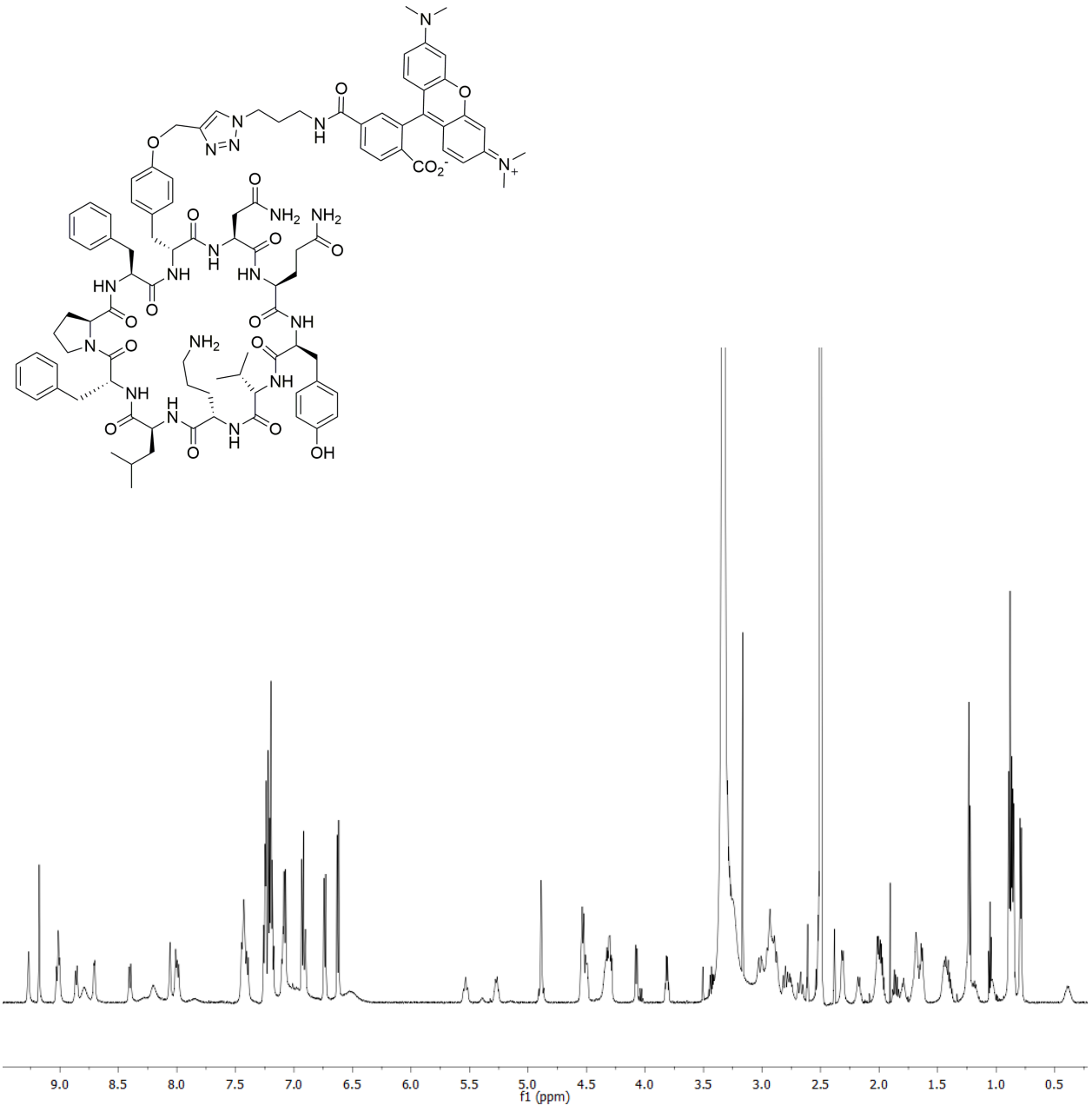


Propargylated tyrocidine A (7): ^{15}N-HSQC (DMSO-d_6)

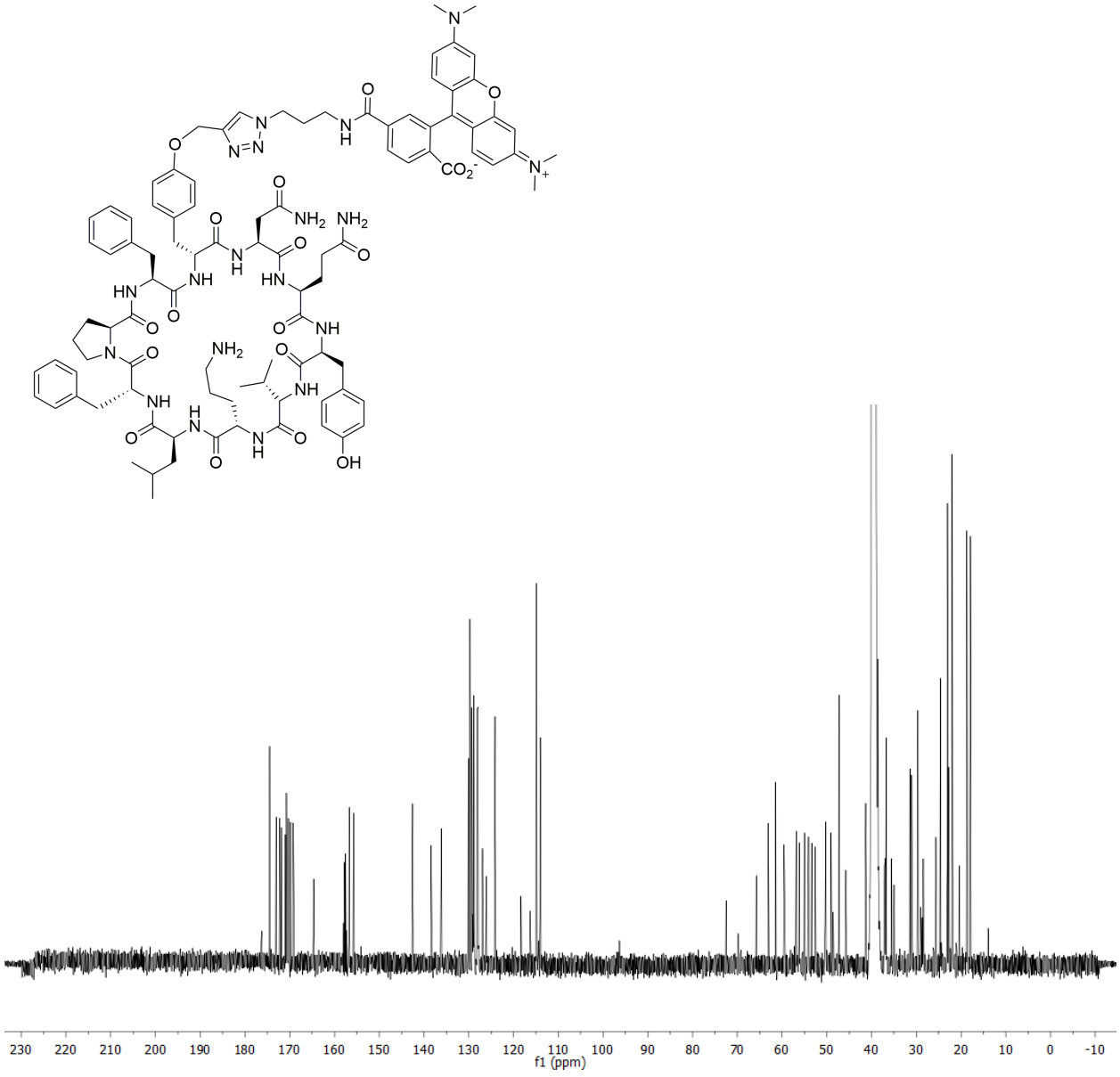




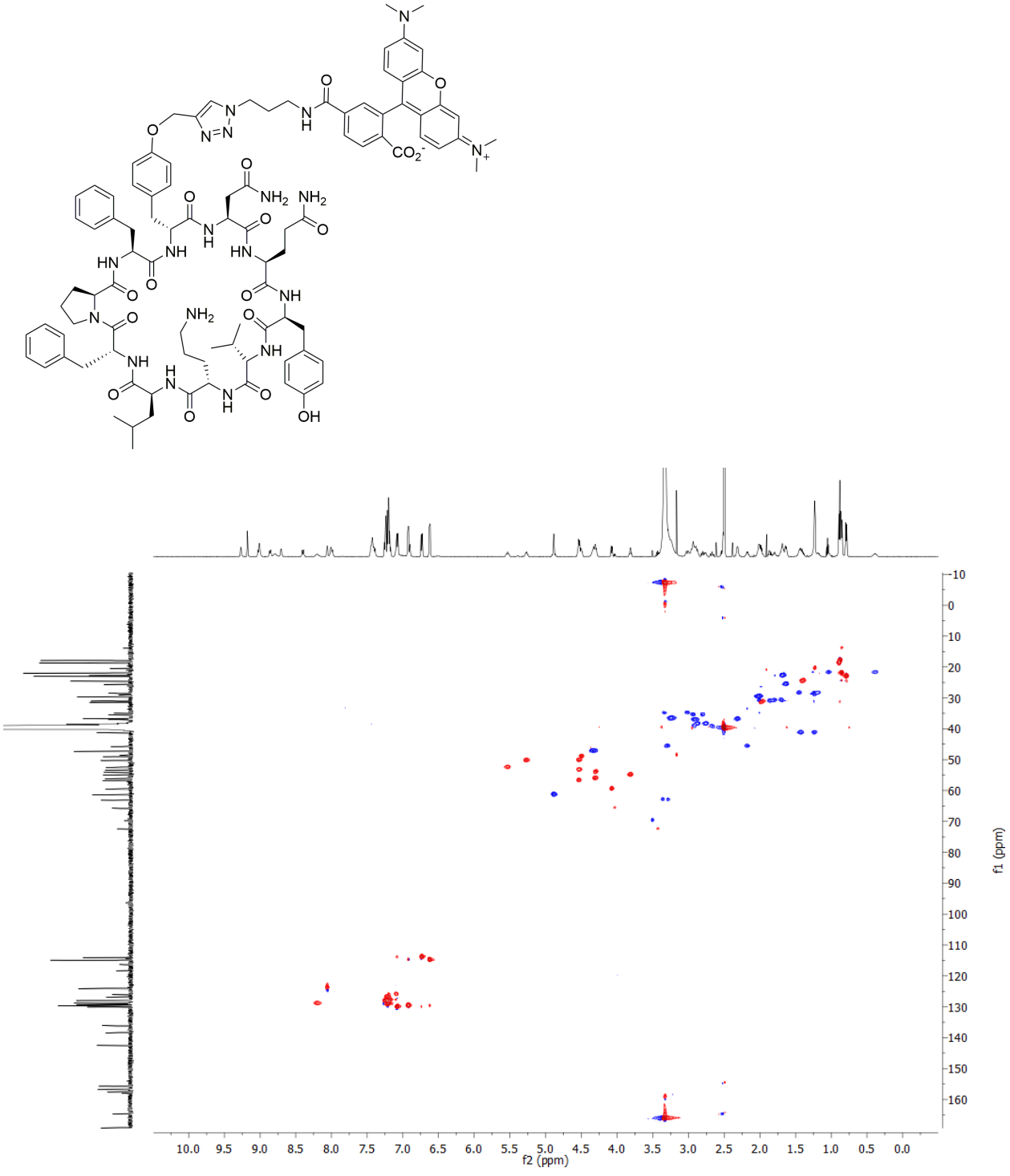
TAMRA-modified tyrocidine A (14): ^{1}H-NMR (DMSO-*d*6)



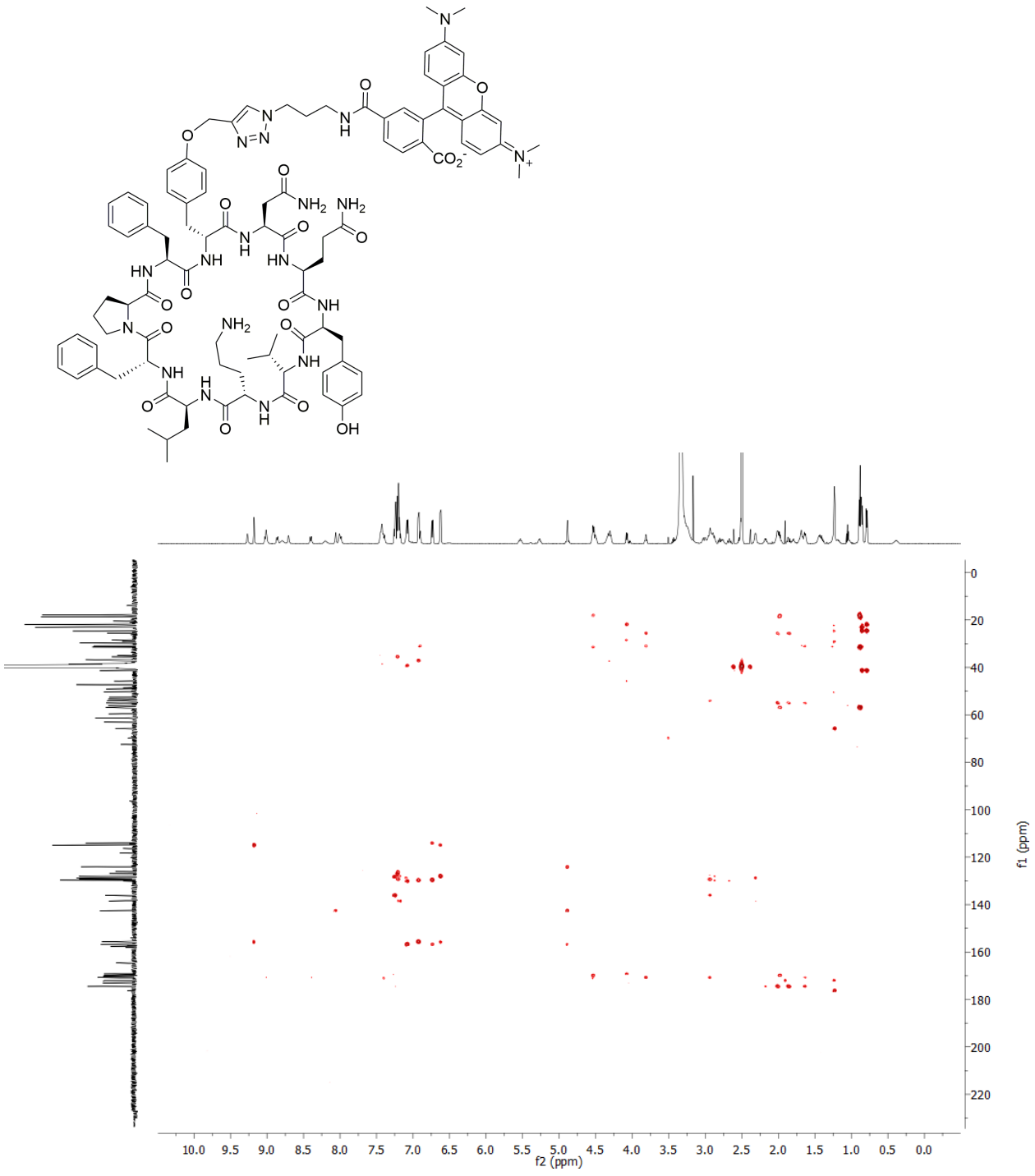
TAMRA-modified tyrocidine A (14): ^{13}C-NMR (DMSO-d_6)



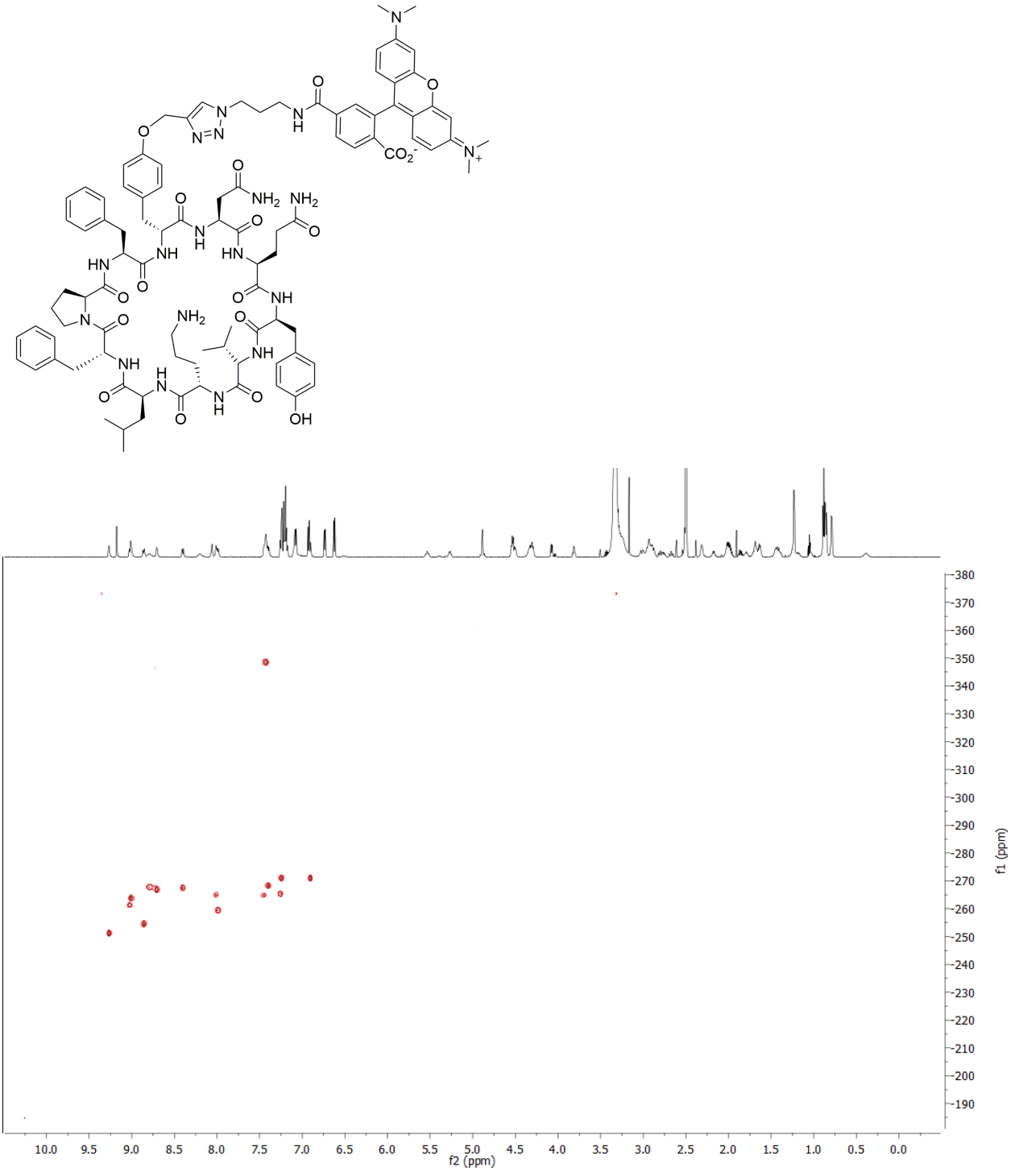
TAMRA-modified tyrocidine A (14): HSQC (DMSO-d_6)



TAMRA-modified tyrocidine A (14): HMBC (DMSO-d_6)



TAMRA-modified tyrocidine A (14): ^{15}N-HSQC (DMSO-d_6)



References

(1) Gruenewald, S.; Mootz, H. D.; Stehmeier, P.; Stachelhaus, T. In Vivo Production of Artificial Nonribosomal Peptide Products in the Heterologous Host *Escherichia coli*. *Appl. Environ. Microbiol.* **2004**, *70*, 3282–3291.

(2) Kries, H.; Wachtel, R.; Pabst, A.; Wanner, B.; Niquille, D.; Hilvert, D. Reprogramming Nonribosomal Peptide Synthetases for "Clickable" Amino Acids. *Angew. Chem., Int. Ed.* **2014**, *53*, 10105–10108.

(3) Gibson, D. G.; Young, L.; Chuang, R.-Y.; Venter, J. C.; Hutchison, C. A., III; Smith, H. O. Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases. *Nat. Methods* **2009**, *6*, 343–345.

(4) Studier, F. W. Protein Production by Auto-Induction in High-Density Shaking Cultures. *Protein Expr. Purif.* **2005**, *41*, 207–234.

(5) Nakano, M. M.; Marahiel, M. A.; Zuber, P. Identification of a Genetic Locus Required for Biosynthesis of the Lipopeptide Antibiotic Surfactin in *Bacillus subtilis*. *J. Bacteriol.* **1988**, *170*, 5662-5668.

(6) Stachelhaus, T.; Mootz, H. D.; Marahiel, M. A. The Specificity-Conferring Code of Adenylation Domains in Nonribosomal Peptide Synthetases. *Chem. Biol.* **1999**, *6*, 493–505.

(7) Challis, G. L.; Ravel, J.; Townsend, C. A. Predictive, Structure-Based Model of Amino Acid Recognition by Nonribosomal Peptide Synthetase Adenylation Domains. *Chem. Biol.* **2000**, *7*, 211–224.