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Letter

Activity and Biocatalytic Potential of an Indolylamide Generating Thioesterase

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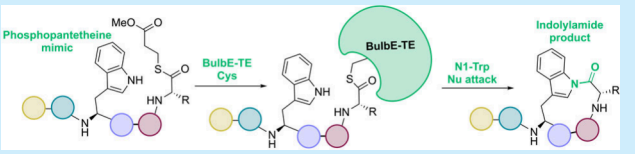
ABSTRACT: The chemical synthesis of *N*-acyl indoles is hindered by the poor nucleophilicity of indolic nitrogen, necessitating the use of strongly basic reaction conditions that encumber elaboration of highly functionalized scaffolds. Herein, we describe the total chemoenzymatic synthesis of the bulbiferamide natural products by the biochemical activity reconstitution of a nonribosomal peptide synthetase assembly line-derived (NRPS-derived) thioesterase that neatly installs the macrocyclizing indolylamide. The enzyme represents a starting point for biocatalytic access to macrocyclic indolylamide peptides and natural products.



Peptidic natural products furnished by nonribosomal peptide synthetases (NRPSs) are frequently endowed with desirable pharmacological activities. Among these molecules, an often-observed structural feature is macrocyclization. Macrolactams such as cyclosporine, macrolactones such as daptomycin, and peptides macrocyclized by amino acid side chain couplings, such as vancomycin, are examples wherein macrocyclization lends rigidity, proteolytic stability, membrane permeability, and target-engaging conformations to these peptidic natural products.[1]

For NRPS-derived peptides, the macrocyclization catalyst is usually the terminal thioesterase (TE) domain which also offloads the peptide from the NRPS assembly line. The peptide is transesterified from the phosphopantheine thiol of the carrier protein (CP) to generate an acyl intermediate. The TE domain can then use exogenous nucleophiles to release the peptide chain (in red, Figure 1A). Alternatively, the TE can employ intramolecular nucleophiles, such as the N-terminal amine or nucleophilic amino acid side chains to generate macrocyclic products (in blue, Figure 1A).[2,3]

The discovery of the bulbiferamides, ureidopeptides produced by marine *Microbulbifer* bacteria, led to the observation of a 15-atom macrocycle afforded by amide bond formation with the N-1 position of the tryptophan side chain indole (Figure 1B).[4,5] Indolylamides are well represented among fungal NRPS-derived alkaloids.[6−9] In fungi, terminal condensation (C_T), rather than TE domains, have been implicated in the formation of the acyl indole bond.[10,11]

The production of the bulbiferamides has been attributed to the *bulb* BGCs detected within the *Microbulbifer* spp. genomes. Consistent with bacterial NRPS assembly line architecture, a TE domain at the C-terminus of the BulbE NRPS, henceforth referred to as the BulbE-TE, is positioned at the terminus of the Bulb NRPS assembly line (Figure S1). Thus, the BulbE-TE could conceivably release the peptide from the NRPS assembly line via indolylamide cyclization.[4]

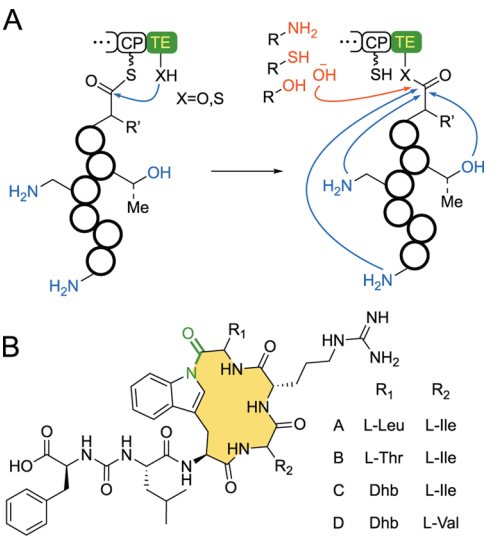


Figure 1. (A) Typical activity of NRPS TE domains wherein they employ inter- or intramolecular nucleophiles to offload the peptide chain. (B) Bulbiferamides A−D; Dhb: dehydrobutyrine. The site of cyclization is highlighted in green.

The use of a tryptophan indole side chain nitrogen as a nucleophile for peptide macrocyclization by TEs is unprecedented. Therefore, it was unclear if the BulbE-TE domain was indeed responsible for the formation of the indolylamide macrolactam in bulbiferamides, necessitating experimental

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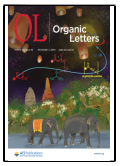
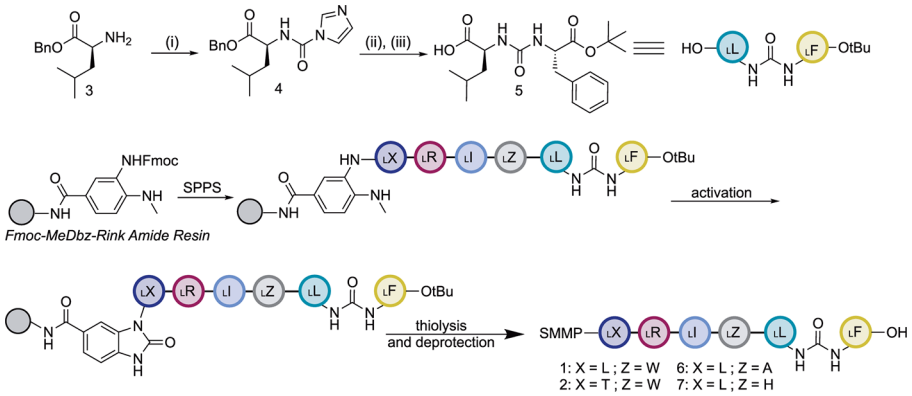


Scheme 1. Synthesis of BulbE-TE Substrate Mimics[a]



[a](i) 1.1 equiv. carbonyldiimidazole (CDI), 0.04 equiv. 4-dimethylaminopyridine (DMAP), 3 equiv. triethylamine (TEA), at RT in CH_2Cl_2. (ii) 1.2 equiv. L-phenylalanine *tert*-butyl ester hydrochloride, 2.5 equiv. TEA, at RT in CH_2Cl_2. 83% yield over two steps. (iii) H_2, Pd−C (10 mol %), at RT in MeOH. 63% yield. SPPS: solid phase peptide synthesis; SMMP: methyl 3-mercaptoproprionate. Exact conditions can be found in the Supporting Information.

validation. This validation could additionally provide a new biocatalyst for a synthetically challenging class of macrocyclizations.

To verify the proposed route for bulbiferamide macrocycle formation, the nucleotide sequence encoding the BulbE-TE domain from *Microbulbifer* sp. MLAF003 was expressed in *Escherichia coli* and the recombinant enzyme purified (Figure S2). Next, we synthesized the peptidic substrates **1** and **2** (Scheme 1), as dictated by the bulbiferamide biosynthetic logic (Figure S1). Here, a linear hexapeptide substrate with a ureido linkage between the L-Phe[1] and L-Leu[2] residues must be thioesterified to an upstream CP phosphopantetheine appendage.

Previous syntheses of peptides featuring an N-terminal urea dipeptide have primarily focused on a class of closely related aldehyde protease inhibitors: GE 20372 and (S)-α- and (R)-β-MAPI (MAPI: Microbial Alkaline Protease Inhibitors).[12−14] Using a similar approach, the ureidodipeptide **5** was generated off-resin via activation of L-leucine benzyl ester (**3**) with carbonyldiimidazole (CDI) to afford **4**. The crude **4** was then coupled with L-phenylalanine methyl ester in the presence of triethylamine (Figure S3). Removal of the benzyl ester via hydrogenation afforded **5** in suitable purity for solid-phase peptide synthesis (SPPS) (Figure S4). The SPPS of the ureidohexapeptides was accomplished utilizing a safety catch strategy.[15] The MeDbz linker, which was created by Dawson and co-workers, enabled on-resin activation of the C-terminal amino acid followed by cleavage with a nucleophilic thiol.[16] This strategy avoids epimerization due to oxazolone formation. Cleavage of the peptides using methyl 3-mercaptoproprionate (SMMP) furnished **1** and **2**; the SMMP moiety serves as a surrogate for the CP phosphopantetheine (Figures S5−S6).[15]

Incubation of **1** and **2** with purified BulbE-TE resulted in production of the natural products bulbiferamides A and B, respectively. The respective thioester hydrolysis side products were also observed (Figures 2 and S7−S8). The retention times and mass spectrometric fragmentation patterns of the macrocyclized products were identical to the bulbiferamide natural product standards (Figure S9). Macrocyclization of **1** proceeded with kinetic parameters k_{cat} 0.16 ± 0.02 min^{-1} and K_M 100 ± 29 μM (Figure S10). No macrocyclized product formation was observed in the absence of the enzyme, or when the active site catalytic Cys was replaced with Ser or Ala (*vide infra*, Figure S11). Taken together, these data establish a chemoenzymatic route to access bulbiferamide natural products while unveiling a novel macrocyclic indolylamide forming activity for TEs.

While the activity of BulbE-TE was thusly validated, the product yields were modest (Table S1). Other marine peptide macrocyclases have likewise been demonstrated to possess reduced catalytic activities.[17,18] Of note, the yield of bulbiferamide B starting from **2** was lower than that of bulbiferamide A production from **1** (Figure 2). The bacterium *Microbulbifer* sp. MLAF003 does not produce bulbiferamide B—this natural product was isolated from a different strain—*Microbulbifer* sp. VAAF005 which contains a similar *bulb* BGC.[4] The thioesterase domain from *Microbulbifer* sp. VAAF005 was cloned and expressed. Incubation of **2** with *Microbulbifer* sp. VAAF005-derived BulbE-TE resulted in a near 4-fold increase in bulbiferamide B yield, pointing to the fine-tuning of the TE active site for the different substrates (Figures S12−S14 and Table S1). Replacement of the substrate Trp residue with Ala, corresponding to the thiotemplated ureidopeptide substrate **6**, expectedly did not yield any macrocyclic products (Figures S15−S16). Replacement of Trp with the more nucleophilic His in ureidopeptide substrate **7** also did not yield any macrocyclic products (Figures S17−S18). This is likely due to poor enzymatic recognition of the His containing substrate in the TE active site.

The active site of the BulbE-TE domain was rationalized to possess the Cys961/Asp988/His1097 catalytic triad (amino acid numbering per the *Microbulbifer* sp. MLAF003 BGC). The BulbE-TE-catalyzed transformation is thus expected to proceed via transthioesterification of the substrate peptide to the Cys961-Sγ, followed by resolution of the acyl thioester by the substrate Trp side chain indole via the formation of a tetrahedral thioketal intermediate.[19] Catalytic Cys residues in TE active sites are suggestive of challenging transformations.[2] As mentioned above, Ser could not replace Cys in the BulbE-TE active site in line with similar observations for the obafluorin and sulfazecin biosynthetic TE domains—ObiF-TE and SulM-TE—which generate strained 4-atom lactone and lactam products, respectively.[20,21] Of note, SulM-TE employs an unusual sulfamated amine as the lactam-forming nucleophile. For bulbiferamide biosynthesis, the nucleophilicity of the

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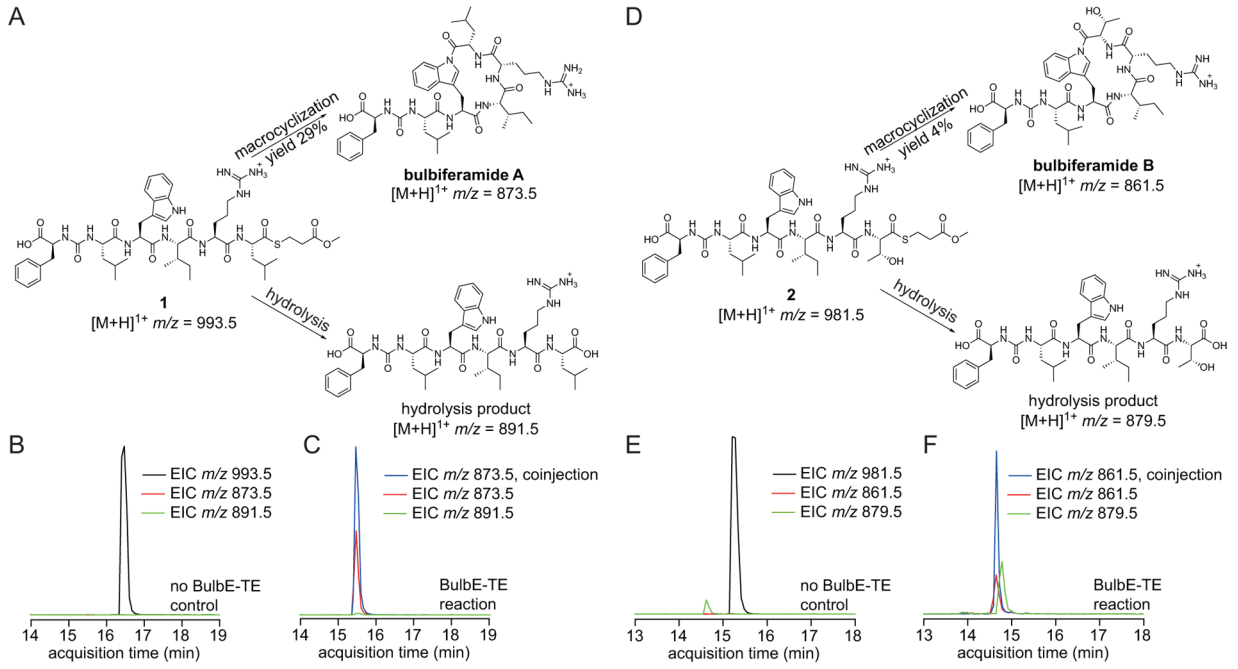

Figure 2. *In vitro* enzymatic activity of BulbE-TE. (A) Macrocyclized and thioester hydrolysis products for substrate **1**. (B) Extracted ion chromatograms (EICs) of **1**, hydrolyzed, and macrocyclized products in the reaction where the BulbE-TE was omitted. (C) EICs of **1**, hydrolyzed, and macrocyclized products in the reaction in the presence of BulbE-TE. The "coinjection" EIC refers to a spiking experiment in which bulbiferamide A was added to the quenched enzymatic reaction to confirm coelution with the macrocyclized enzymatic product. (D) Macrocyclized and thioester hydrolysis products for substrate **2**. (E) EICs demonstrating the presence of **2**, hydrolyzed, and macrocyclized products in the reaction where the BulbE-TE was omitted. (F) EICs of **2**, hydrolyzed, and macrocyclized products in the reaction in the presence of BulbE-TE. As above, the "coinjection" EIC refers to a spiking experiment in which bulbiferamide B was added to the quenched enzymatic reaction to confirm coelution with the macrocyclized enzymatic product.

indole-N is similarly compromised. In light of these observations, the choice of Cys as the preferred active site nucleophile can be rationalized as the acyl-thioester intermediate is much more activated than an acyl-oxoester intermediate for aminolysis.[22,23] Contrary to aminolysis, oxoesters and thioesters have similar reactivity toward the hydrolysis.[22] Indeed, while the BulbE-TE Cys961 → Ser mutant does not generate detectable macrocyclized products, it does generate the thioester hydrolysis product in much greater abundance than the wild type enzyme or the Cys961 → Ala mutant (Figure S19).

The AlphaFold3-generated model of the BulbE-TE demonstrates a canonical α/β hydrolase fold with the catalytic Cys and His residues positioned on loops at the end of the β5 and β8 strands of the central β-sheet (Figure S20). Unlike the other Cys-containing NRPS TEs ObiF-TE and the SulM-TE, the catalytic Asp residue in the BulbE-TE is positioned on the loop at the end of the β6 strand, and not the β7 strand.[24,25]

Next, we explored the biocatalytic potential of the BulbE-TE. The physiological product furnished by the BulbE-TE is a 15-atom macrolactam ring (Figure 1B). Expanding or contracting the indolylamide macrocycle, queried using ureidopeptide thioesters **8** and **9** as substrates, respectively, was not successful as only hydrolyzed products were observed in each case (Figure 3, Figures S21−S24).

The bulbiferamides demonstrate the invariant presence of the Arg residue as a constituent of the macrolactam ring. Ureidopeptide thioester **10**, wherein the Arg residue was replaced with 1,3-diaminopropionic acid (Dap), was accepted as substrate by the BulbE-TE furnishing the appropriately cyclized macrocyclic product in 3% yield (Figures 3 and S25−S27). However, the thioester hydrolysis product dominated the macrocyclized product (Table S1). The other invariant structural feature in bulbiferamides, the ureido coupling between Phe1 and Leu2 residues, was dispensable with molecule **11** serving as a viable substrate for BulbE-TE, leading to macrocyclic product formation in yields comparable to substrate **1** (Figures 3 and S28−S30). This implies that the ureido group was not required for substrate recognition. Taken together with the fact that other indolylamide-forming enzymes require a CP-loaded substrate, this highlights the ability of the BulbE-TE to serve as a more general biocatalyst. Replacement of the poor indole-N nucleophile in **11** with an ornithine-derived primary amine in molecule **12** yielded an enhanced product yield (Figures 3 and S31−S34, Table S1).

The macrocyclization of **12** mimics the biosynthesis of cyanobacterial ureidopeptidic natural products that are macrocyclized via amide bond formation with Lys side chain primary amines.[26] Unlike cyclization of **1** and **2**, **12** yielded a macrocyclized product even in the absence of the enzyme which likely alludes to the preorganization of the substrate for intramolecular thioester displacement by a much stronger primary amine nucleophile (Figure S33, Table S1). Decreasing the reaction pH—from 7.5 to 6.0—abolished the noncatalytic product formation and the overall product yield also decreased pointing to the reactivity of the macrocyclizing nucleophile being a primary determinant (Figure S35, Table S1). Increasing the reaction pH—from 7.5 to 9.0—led to thioester hydrolysis being the dominating reaction outcome (Figure S36, Table S1).

The ability of BulbE-TE to acylate the relatively non-nucleophilic tryptophan nitrogen is exciting. Most synthetic

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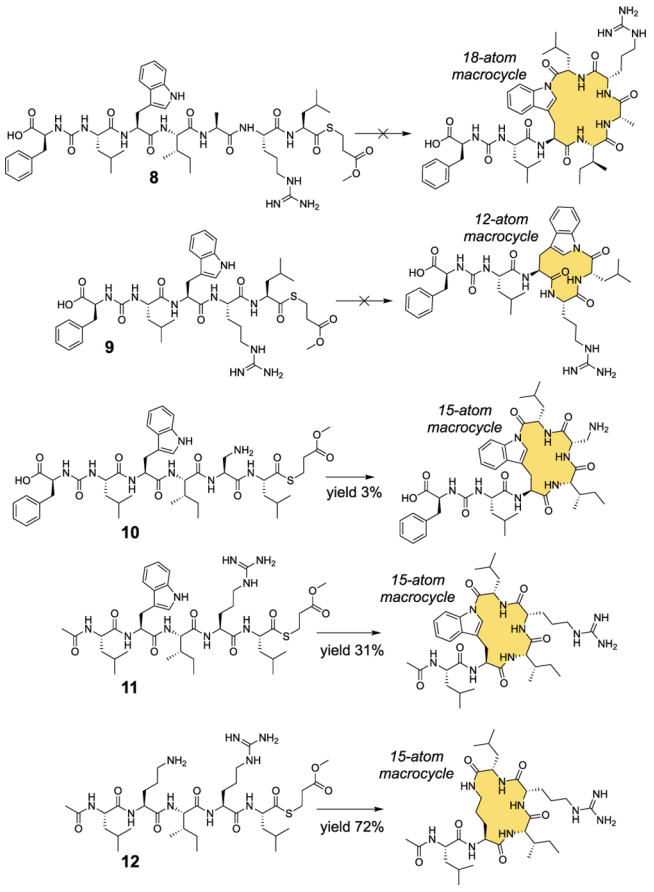

Figure 3. *In vitro* enzymatic biocatalytic potential of BulbE-TE. Substrates **8** and **9** did not yield macrocyclized products. Substrates **10**–**12** did yield macrocyclized peptides demonstrating that the Arg side chain and the ureidopeptide linkage were not required for substrate recognition by the BulbE-TE.

strategies for acylation of tryptophan require protection of other nucleophilic residues and cannot happen in nucleophilic solvents such as water.[27,28] Additionally, they typically require the use of strong, often stoichiometric bases, limiting the functional group tolerance of the reactions. The total synthesis of the fungal macrocyclic indolylamide natural product psychrophilin E has been achieved; the timing for the installation of the indolylamide in the chemical synthesis and in the biosynthetic route is entirely opposite.[29] While indolylamide installation is the first step in chemical synthesis of psychrophilin E, it is the very last transformation in bulbiferamide biosynthesis. Taken together, BulbE-TE facilitates a synthetically challenging peptide macrocyclization to a 15-membered ring that has not been previously achievable. Future efforts for enzyme evolution are likely to further expand the substrate scope, thus providing a highly useful biocatalyst.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.4c03648.

Comprehensive description of materials and methods used in this study, synthetic schemes, compound characterization data, ^{1}H & ^{13}C NMR spectra, and descriptions of enzyme reaction outcomes (PDF)

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Author Contributions

#W.Z. and Z.L.B. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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SUPPLEMENTARY INFORMATION FOR:

Activity and biocatalytic potential of an indolylamide generating thioesterase

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SUPPLEMENTARY MATERIALS AND METHODS

General materials and instrumentation

All chemicals, solvents, and media components were obtained commercially from Sigma-Aldrich, Fisher Scientific, and VWR, and used without further purification. Phusion high-fidelity DNA polymerase and Gibson assembly Master Mix were purchased from New England Biolabs. PrimeSTAR DNA polymerase Master Mix was purchased from Takaro Bio. Mass spectra were recorded on an Agilent 6530C high resolution time of flight (ToF) mass spectrometer with an electrospray ionization (ESI) source coupled to an Agilent 1260 high-performance liquid chromatography system equipped with a diode array detector. NMR data were recorded on a Bruker Avance-III-800 with a QCI cryoprobe or a Bruker NEO500 with a BBFO cryoprobe.

Cloning, expression and purification of BulbE-TE domains from *Microbulbifer* sp. MLAF003 and *Microbulbifer* sp. VAAF005

On the basis of primary protein sequence similarity, the domain boundaries for BulbE-TE domain were mapped to *Microbulbifer* sp. MLAF003 BulbE residues 866–1135. The DNA fragment encoding TE domain was amplified from the genomic DNA of *Microbulbifer* sp. MLAF003 with Phusion high fidelity DNA polymerase. The amplified DNA fragment was inserted into the pET28(+) vector using Gibson assembly to furnish an N-His_6 tag fused construct. The sequence of the plasmid was confirmed by nanopore sequencing. The plasmid was then transformed into *Escherichia coli* BL21Gold(DE3) for protein expression.

Overnight culture was inoculated into 1 L terrific broth supplemented with 50 μg/mL kanamycin. The cells were grown at 30 ˚C until OD_{600} reached 0.4–0.5. The incubation temperature was then reduced to 18 ˚C. At an OD_{600} of 0.7–0.8, protein expression was induced by the addition of 0.1 mM IPTG. Induced cultures were allowed to grow at 18 ˚C for an addition of 18 h before being harvested by centrifugation. Cell pellets were resuspended in 50 mL binding buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and lysed by homogenization. The lysate was clarified by centrifugation (40,000 × g, 45 min), and then applied to a 5 mL HisTrap HP column. The column was washed with 10 column volumes of wash buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 30 mM imidazole), and eluted using ÄKTAprime plus FPLC system with a linear gradient to 100% of elution buffer (20 mM Tris-HCl (pH 8.0), 250 mM imidazole, 500 mM NaCl) over 6 column volumes. Purity of eluent protein fractions was checked by SDS-PAGE. The fractions containing desired proteins were pooled and dialyzed in 2 L binding buffer overnight. Freshly prepared proteins were used for assays. The BulbE-TE from *Microbulbifer* sp. VAAF005 strain

(referred to as BulbE-TE2 in some of the figures below) was prepared in an identical manner. The sequences for the two BulbE-TE domains are listed below.

Amino acid sequences of the BulbE-TE and BulbE-TE2

BulbE-TE from *Microbulbifer* sp. MLAF003

MENEIVDFLKDYNEFRKFMTFNGDSNKTGLFLIPAAAGPETFIPLVEKLDIDRPVQLLENIQVYSG
RQIRLNHLIDYYLAVIRKKQPGGPYFLGGYCEGAMVSLGIAQKLEALGEQVEMLFLIDPVVITIEQ
TMIDTIKQDSRLLECGRFEAEMVDTFLFYAEYVKSLHPYGGPAIFFEGSSVSDEATPTQTLALIND
YIDIQGVFKKGFSTPKNGFEDLLLNCDYISIKAKHERVMIEDETLNTIAMAINRKLSTGQQTYLAP
EAQTTEM

BulbE-TE2 from *Microbulbifer* sp. VAAF005

MDEIVNFLTGYNEFNKLMTFNEASNKTGLFLIPAAAGPETFTPLVEKLDINRPVHLLENIQVYSGR
QIRLNYLIDYYFAVIRKKQASGPYLLGGYCEGAMVSLGIAQKLIALGERVELLFLIDPVVITIEQNL
IDTIKQDPRLPKCGRFEAEMVDTFLFYAEYVKSLQPYSGPVVFFEGSSVSDEATPTQILAVINDYV
DIQELFNKGFSTPKNGFESLLLNCDYIAIDAKHERIMIEDETLNTMANVINLKFSSSYDNYLETETH
S

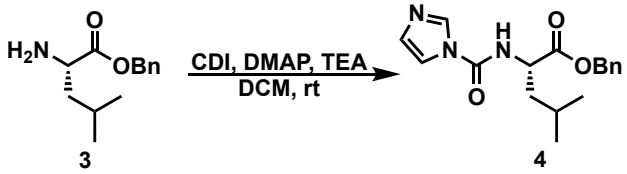
Enzymatic assays

Enzymatic assays were performed at 30 ˚C for 12 h, in a total volumn of 100 μL containing 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1 mM DTT, 100 μM substrates, 1 μM wild type or mutated BulbE-TE enzymes. Additionally, substrate **12** was also tested in buffers at pH 6.0 and 9.0. Reactions were quenched by addition of equal volume of MeOH supplemented with 2% (v/v) formic acid (FA). Negative control reactions omitted the enzyme. Quenched aliquots were centrifuged at 18,000×g for 30 min at room temperature before analysis by LCMS. Chromatographic separations were performed using an Agilent Poroshell 120 EC-C_{18} reverse phase HPLC column (100 × 4.6 mm, 5.0 μm) at a flow rate of 0.3 mL/min. The mobile phase was composed of H_2O (A) and MeCN (B) both supplemented with 0.1% (v/v) FA. A flow rate of 0.3 mL/min was used with the following gradient: 0–3 min: 5% B, 3–15 min: linear gradient to 100% B, 15–18 min: 100% B, 18–20 min: linear gradient to 5% B, 20–22 min: 5% B. Data were acquired in the positive ionization mode with *m/z* 100–3000 Da.

Kinetic characterization of BulbE-TE activity

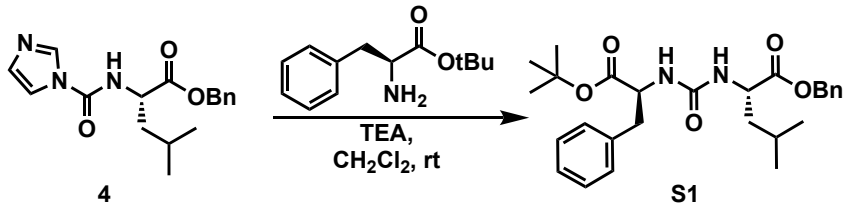
The time-course experiments for BulbE-TE (from *Microbulbifer* sp. MLAF003) kinetics were performed at 30 ˚C for 12 h in a total volume of 200 μL containing 100 μM substrate **1**, 1 μM BulbE-TE enzyme, 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 1 mM DTT. At 1, 5, 10, 30, 60, 120, 240, 480, 960 min, a 20 μL aliquot of enzyme assay was withdrawn, quenched by the addition of 20 μL MeOH with 2% formic acid and analyzed by HPLC to determine the linear response time for the discontinuous assay. Assays with different concentrations (10, 25, 50, 100, 200, 500 μM) of substrate **1** were then conducted at 30 ˚C for 10 min. The initial velocity of product formation was calculated. The resulting curve was fit using OriginPro 2018 (9.5) to extract K_M and k_{cat}.

Synthesis of Urea Dipeptide



In a flame-dried round-bottom flask, L-leucine benzyl ester p-toluenesulfonate **3** (5 g, 12.7 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (50 mL) and cooled to 0 °C. Triethylamine (TEA, 5.2 mL, 38 mmol, 3 equiv.) and 4-dimethylaminopyridine (DMAP, 61 mg, 0.51 mmol, 0.04 equiv.) were added. After stirring for 5 min, carbonyldiimidazole (CDI, 2.3 g, 14 mmol, 1.1 equiv.) was added, and the mixture was stirred overnight while warming to room temperature. The reaction was then diluted with an additional 50 mL of CH_2Cl_2. The organic layer was washed with sodium bicarbonate (2 × 50 mL), dH_2O (1 × 50 mL), and brine (3 × 50 mL). It was then dried over sodium sulfate and concentrated in vacuo to afford **4** as a light yellow oil. The reaction product (3.6 g, 11.4 mmol) was verified by proton NMR and used crude in the next reaction.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.80 (d, J = 7.6, 1H), 8.27 (d, J = 1.2, 1H), 7.71 (q, J = 1.4, 1H), 7.45–7.29 (m, 5H), 7.03 (s, 1H), 5.16 (s, 2H), 4.42 (ddd, J = 10.9, 7.6, 4.5, 1H), 1.81–1.55 (m, 3H), 0.89 (m, 6H).



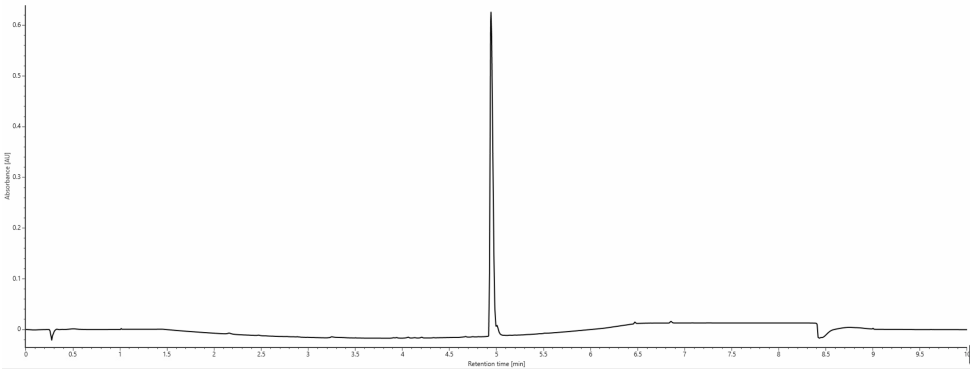
In a flame-dried round-bottom flask, L-phenylalanine tert-butyl ester hydrochloride (3.5 g, 13.7 mmol, 1.2 equiv.) was suspended in dry CH_2Cl_2 (40 mL). Compound **4** (3.6 g, 11.4 mmol) was dissolved in 10 mL of dry CH_2Cl_2 and added to the flask. The mixture was cooled to 0 °C, and TEA (3.9 mL, 28.5 mmol, 2.5 equiv.) was added. The reaction mixture was stirred overnight while warming to room temperature. Upon completion, the reaction was diluted with an additional 50 mL of CH_2Cl_2. The organic layer was washed with sodium bicarbonate (2 × 50 mL), dH_2O (1 × 50 mL), and brine (3 × 50 mL). It was then dried over sodium sulfate, concentrated in vacuo, and purified by flash chromatography (30% EtOAc/Hexanes) to give the diester **S1** as a clear, viscous oil (4.4 g, 9.5 mmol, 83%). Rf = 0.50 (30% EtOAc/Hexanes).

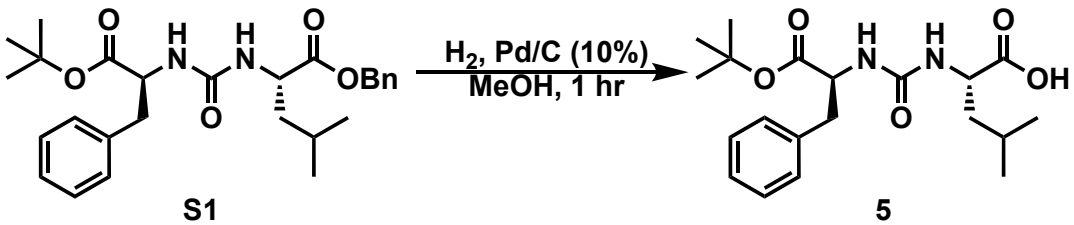
^{1}H NMR (800 MHz, DMSO-d_6): δ = 7.39–7.30 (m, 5H), 7.27 (t, J = 7.5, 2H), 7.24–7.16 (m, 3H), 6.58 (d, J = 8.2, 1H), 6.19 (d, J = 8.1, 1H), 5.11 (d, J = 3.1, 2H), 4.28 (q, J = 7.0, 1H), 4.21 (td, J = 8.5, 6.0, 1H), 2.90 (d, J = 6.7, 2H), 1.62 (m, 1H), 1.50–1.42 (m, 2H), 1.32 (s, 9H), 0.86 (m, 6H).

^{13}C NMR (201 MHz, DMSO-d_6): δ = 173.7, 171.7, 157.29, 137.4, 136.4, 129.7, 128.8, 128.5, 128.4, 128.19, 126.9, 80.9, 79.6, 66.1, 54.9, 51.4, 41.1, 38.2, 27.9, 24.6, 23.1, 21.9.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{27}H_{37}N_2O_5$ 469.2697; Found 469.2640.

UPLC Trace: Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 5% to 95% over 5 min at a flow rate of 0.5 mL/min. The column was equilibrated with 5% mobile phase B for 1 min before and 2 min after the gradient. Monitoring at wavelength of 254 nm.





S1 **5**

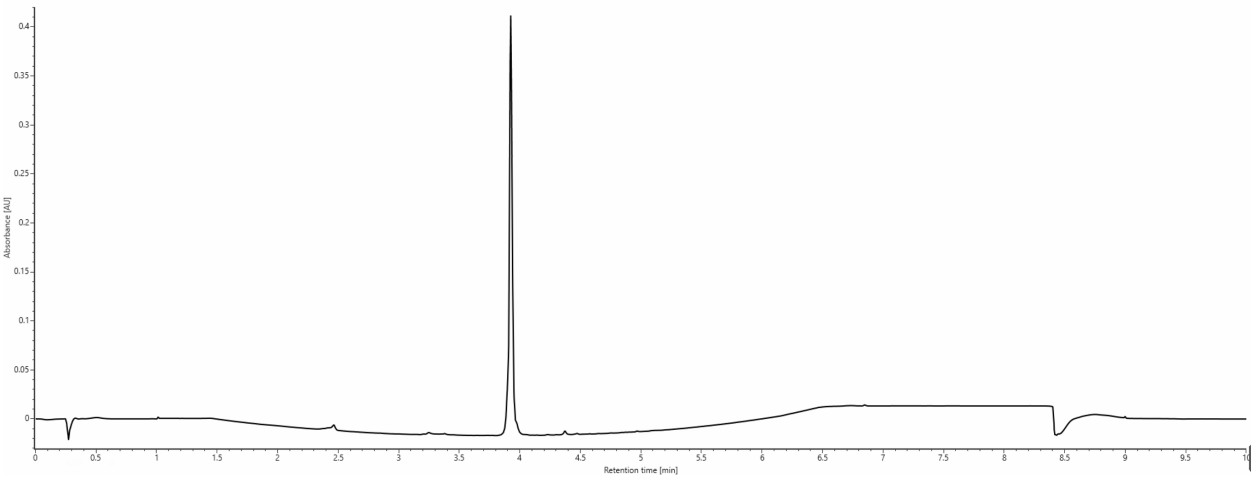
The protected urea diester **S1** (5.3 g, 11.3 mmol) was dissolved in methanol and sparged with N_2 (2 × 10 min) and 110 mg Pd-C (10 mol%) was added. The solution was then sparged with H_2 (10 min) and left to hydrogenate for 1 h. The reaction mixture was then filtered over celite and concentrated in vacuo to give **5** as a crystalline white solid (2.7 g, 7.1 mmol, 63%) which was deemed sufficiently pure to use for SPPS without additional purificaiton.

^{1}H NMR (800 MHz, DMSO-d_6): δ = 7.24 (m, 5H), 6.41 (s, 1H), 6.19 (s, 1H), 4.26 (s, 1H), 4.08 (s, 1H), 2.89 (s, 2H), 1.64 (s, 1H), 1.32 (s, 11H), 0.89 (s, 6H).

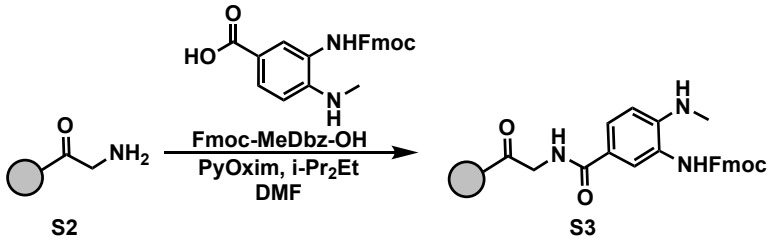
^{13}C NMR (201 MHz, DMSO-d_6): δ = 175.5, 171.8, 157.4, 137.4, 129.7, 128.5, 126.9, 80.8, 55.0, 51.2, 41.6, 38.1, 27.9, 24.6, 23.3, 21.9.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{20}H_{31}N_2O_5$ 379.2227; Found 379.2170.

UPLC Trace: Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 5% to 95% over 5 min at a flow rate of 0.5 mL/min. The column was equilibrated with 5% mobile phase B for 1 min before and 2 min after the gradient. Monitoring at wavelength of 254 nm.

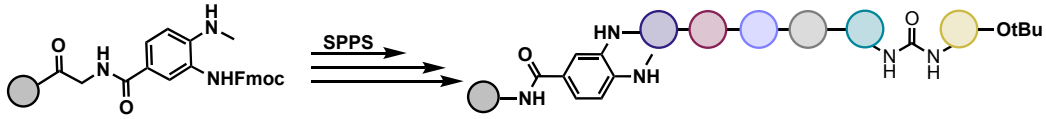


Conventional peptide thioester synthesis at the NDbz "safety-catch" resin



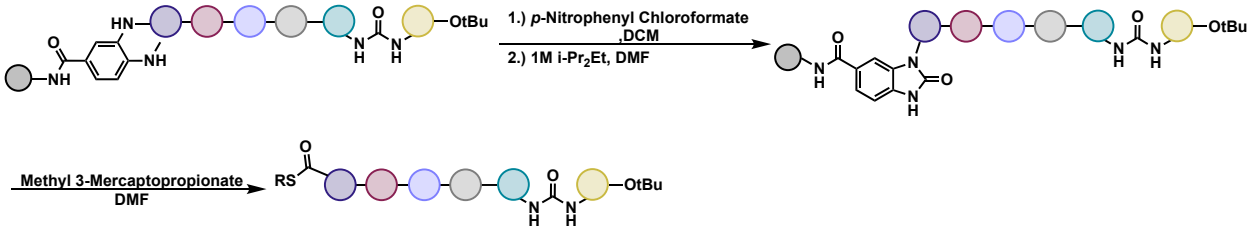
S2 S3

Attachment of Fmoc-MeDbz-OH: A 5 mL fritted polypropylene syringe (Torviq) containing 0.1 mmol of Fmoc-Gly Rink amide resin (0.592 mmol/g loading) **S2** (Chem-Impex, MFCD00801253) was washed with DMF (3 × 5 mL) and allowed to swell for 15 min. A solution of Fmoc-MeDbz-OH (synthesized according to a previously published route)[1] (0.3 mmol), Pyoxim (0.5 mmol), and diisopropylethylamine (DIPEA, 0.7 mmol) in 3 mL of DMF was then added to the resin and allowed to shake for 1 h. The resin was filtered and washed with CH_2Cl_2 (3 × 5 mL) and DMF (3 × 5 mL). Loading efficiency was assumed to be 100% based on a negative ninhydrin test, and the resin was subsequently used in solid-phase peptide synthesis.

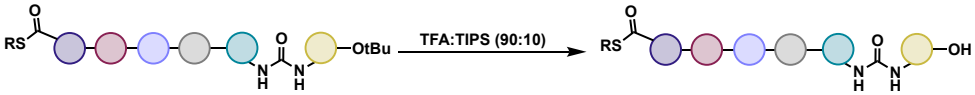


Solid-phase peptide synthesis (SPPS): All manual SPPS and cleavage steps were carried out using 5 mL fritted polypropylene syringes (Torviq) as reaction vessels. Pre-loaded Fmoc-MeDbz resin (0.1 mmol) was swelled in DMF for 15 min, drained, and treated with piperidine-DMF (1:4, 4 mL, 1 × 15 min). The resin was then filtered and washed with DMF (2 × 3 mL) and CH_2Cl_2 (2 × 3 mL). In a separate flask, Pyoxim (0.5 mmol) was added to a solution of Fmoc-AA-OH (0.5 mmol) and diisopropylethylamine (0.7

mmol) in DMF (3 mL). The resulting solution was added to the resin, and the mixture was agitated for 1 h. The resin was then filtered and washed with DMF (3 × 2 mL) and CH_2Cl_2 (3 × 2 mL). The Kaiser ninhydrin test was performed to determine reaction completion. Deprotection and coupling cycles were repeated until the desired peptide sequence was complete. The final protected urea dipeptide **5** (0.3 mmol) was attached following the same procedure as above with a lower equivalents of peptide used.



Activation and thiolysis: The resin was washed with CH_2Cl_2 (3 × 5 mL) and allowed to swell for 15 min. The resin was then treated with 1 mL of 0.5 M 4-nitrophenyl chloroformate (TCI America) in CH_2Cl_2 and shaken for 2 h. The resin was subsequently washed with CH_2Cl_2 (3 × 5 mL) and DMF (3 × 5 mL). A solution of 3 mL of 0.5 M DIPEA in DMF was added to the resin and allowed to react for 15 min. The resin was then filtered, washed with DMF (1 × 5 mL), and exposed to 3 mL fresh 0.5 M DIPEA in DMF solution, repeating this process until the solution no longer turned yellow (typically 4 times). The resin was then swollen in 3 mL DMF for 15 min, filtered, and treated with a 50:50 solution of thiol (2.5 mmol) in DMF, and shaken for 24 h. The resin was then filtered and washed with DMF (3 × 1 mL). The combined filtrate and washes were collected in a 20 mL scintillation vial and subjected to rotary evaporation and lyophilization.

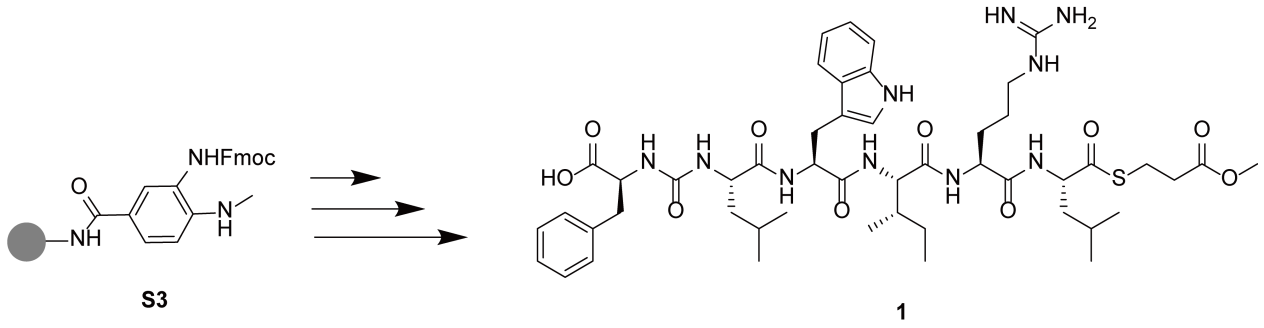


Global deprotection of peptide thioesters: The crude peptide was subjected to global deprotection by treating it with a mixture of 5 mL trifluoroacetic acid (TFA) and triisopropylsilane (TIPS) in a ratio of 90:10 for 1.5 h. The TFA solution was then removed by a stream of air, and the peptide was precipitated by addition of diethyl ether. The resulting precipitate was collected by centrifugation, and the supernatant was discarded. The peptide was then lyophilized to obtain a dry powder. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of H_2O with 0.05% TFA (A) and acetonitrile with 0.05% TFA (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The purified product was then characterized by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) to ensure a purity >95% (UV 214 nm).

Peptide Thioesters Used in This Study

In this study, UPLC and/or analytical HPLC were utilized to assess the purity of the peptide prior to assay or purification by monitoring absorbance at 214 nm. The analytical HPLC analysis was performed on a Luna Omega 5 µm Polar C_{18} 100 Å 150 × 4.6 mm (Phenomenex) column, while the UPLC analysis was carried out on a CORTECS T3 Column, 120 Å, 1.6 µm, 2.1 mm × 50 mm (Waters) column. For purification, semi-preparative HPLC was employed using a Luna Omega 5 µm Polar C_{18} 100 Å 150 × 21.2 mm (Phenomenex) column. The specific gradient and flow rate for each peptide can be found in their respective sections.

Synthesis of 1



Peptide **1** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **S3** loaded resin (0.10 mmol), which was then coupled with the following amino acids: Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ile-OH, Fmoc-L-Trp(Boc)-OH, and **5**. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **1** as an off-white solid (45 mg, 43% yield).

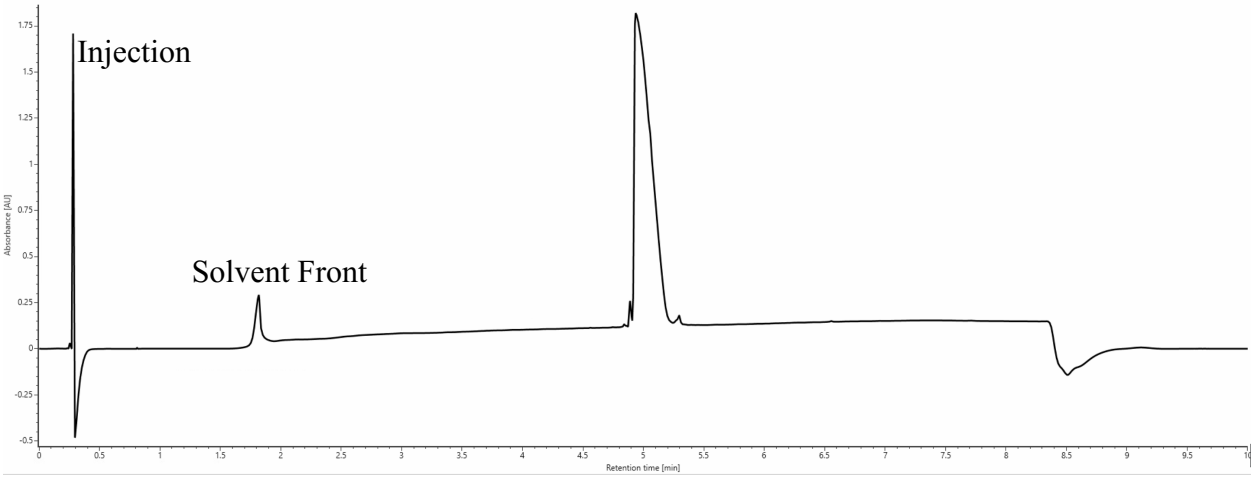
^{1}H NMR (800 MHz, DMSO-d_6): δ = 10.76 (d, J = 2.5, 1H), 8.52 (d, J = 7.8, 1H), 8.13–8.00 (m, 2H), 7.73 (d, J = 8.5, 1H), 7.64 (d, J = 6.0, 1H), 7.54 (d, J = 7.9, 1H), 7.31 (d, J = 8.1, 1H), 7.26 (t, J = 7.5, 2H), 7.22–7.14 (m, 3H), 7.11 (d, J = 2.4, 1H), 7.08–7.02 (m, 1H), 6.95 (t, J = 7.2, 1H), 6.35 (d, J = 8.0, 1H), 6.20 (d, J = 8.1, 1H), 4.59 (td, J = 8.0, 5.1, 1H), 4.43–4.29 (m, 3H), 4.26–4.20 (m, 1H), 4.14–4.07 (m, 1H), 3.61 (s, 3H), 3.13 (tt, J = 12.5, 5.9, 3H), 2.98 (dtd, J = 13.5, 7.6, 4.1, 4H), 2.56 (t, J = 7.1, 2H), 1.81–1.73 (m, 1H),

1.73–1.61 (m, 2H), 1.63–1.45 (m, 6H), 1.43–1.29 (m, 2H), 1.25 (ddd, J = 14.3, 9.9, 5.5, 1H), 1.05 (ddt, J = 16.0, 8.9, 5.1, 1H), 0.92–0.66 (m, 19H).

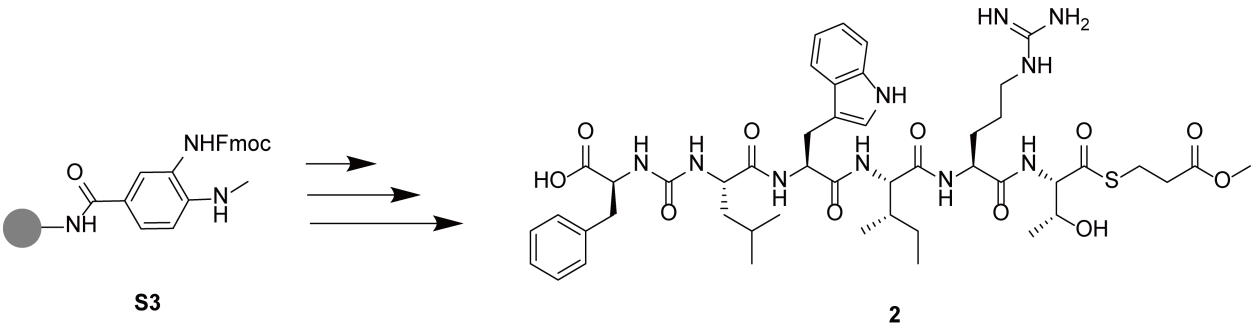
^{13}C NMR (201 MHz, DMSO-d_6): δ = 206.9, 201.7, 174.1, 173.4, 172.1, 172.0, 171.5, 171.0, 157.5, 157.2, 137.7, 136.4, 129.7, 128.6, 127.8, 126.8, 123.7, 121.2, 118.7, 118.6, 111.6, 110.4, 57.8, 57.1, 54.3, 53.5, 52.4, 52.0, 52.0, 42.5, 40.8, 37.9, 37.4, 33.8, 31.1, 29.2, 27.4, 25.5, 24.5, 24.4, 24.4, 23.7, 23.5, 23.3, 22.2, 21.2, 15.5, 11.5.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{49}H_{73}N_{10}O_{10}S$ 993.5226; Found 993.5132.

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 6 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be 98% monitoring at wavelength of 214 nm.



Synthesis of 2



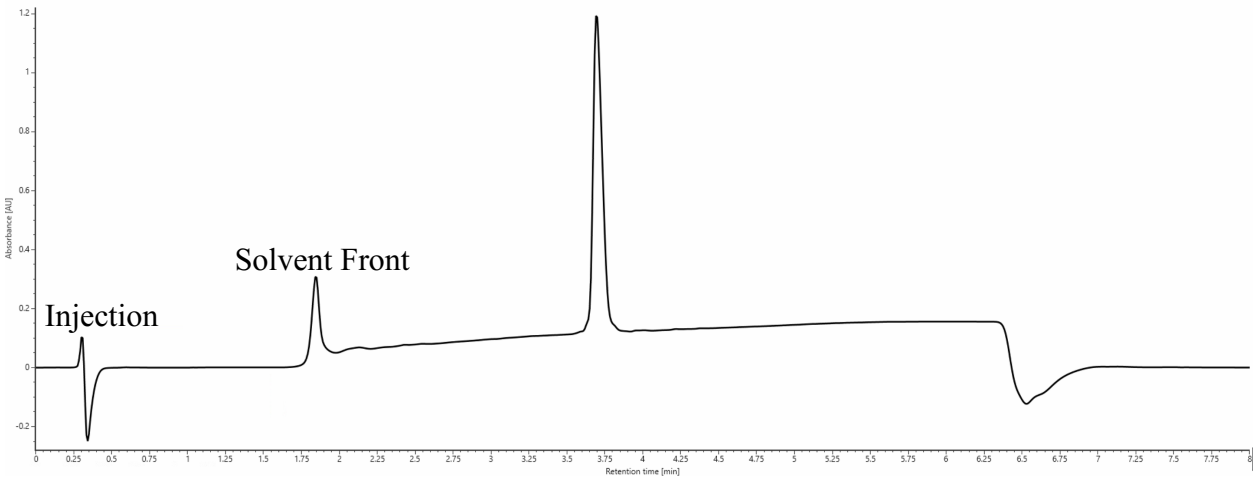
Peptide **2** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **S3** loaded resin (0.10 mmol), which was coupled to the following amino acids: Fmoc-L-Thr(tBu)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ile-OH, Fmoc-L-Trp(Boc)-OH, and **5**. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **2** an off-white solid (16 mg, 15% yield).

^{1}H NMR (800 MHz, DMSO-d_6): δ = 10.76 (d, J = 2.4, 1H), 8.23 (d, J = 8.6, 1H), 8.11 (d, J = 7.8, 1H), 8.05 (d, J = 7.9, 1H), 7.77 (d, J = 8.6, 1H), 7.53 (d, J = 8.0, 1H), 7.30 (t, J = 6.1, 1H), 7.21 (s, 2H), 7.18 (dd, J = 14.5, 7.3, 4H), 7.10 (d, J = 2.3, 2H), 7.04 (t, J = 7.5, 2H), 6.94 (q, J = 7.2, 2H), 6.34 (d, J = 8.1, 1H), 6.18 (d, J = 8.1, 1H), 5.08 (d, J = 4.9, 1H), 4.56 (m, 2H), 4.38–3.98 (m, 5H), 3.61 (d, J = 11.3, 4H), 3.36 (s, 8H), 3.19–3.03 (m, 4H), 2.75–3.03 (m, 7H), 1.85–1.73 (m, 1H), 1.70 (dtd, J = 10.1, 6.9, 3.4, 1H), 1.63–1.45 (m, 5H), 1.24 (ddd, J = 14.3, 9.8, 5.4, 1H), 1.12–0.95 (m, 4H), 0.79 (m, 14H).

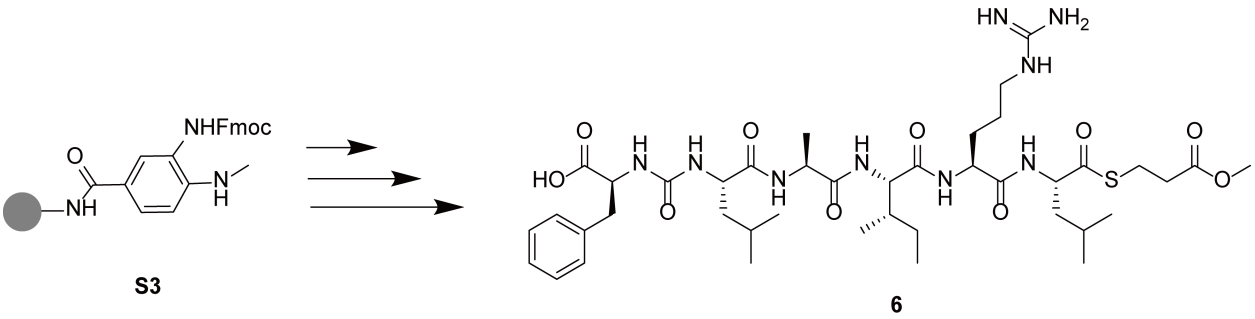
^{13}C NMR (201 MHz, DMSO-d_6): δ = 200.5, 174.2, 173.4, 172.7, 172.0, 171.5, 171.2, 157.5, 157.2, 137.7, 136.4, 129.7, 128.6, 127.8, 126.8, 123.7, 121.2, 118.8, 118.6, 111.6, 110.4, 66.5, 64.8, 57.2, 54.4, 53.4, 52.3, 51.9, 42.5, 40.8, 37.9, 37.3, 33.8, 33.6, 33.0, 29.2, 27.4, 25.4, 24.5, 24.4, 23.8, 23.5, 22.2, 20.5, 15.6, 11.5.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{47}H_{69}N_{10}O_{11}S$ 981.4863; Found 981.4830.

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 4 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be >99% monitoring at wavelength of 214 nm.


Injection
Solvent Front
Absorbance [AU]
Retention time [min]

Synthesis of 6



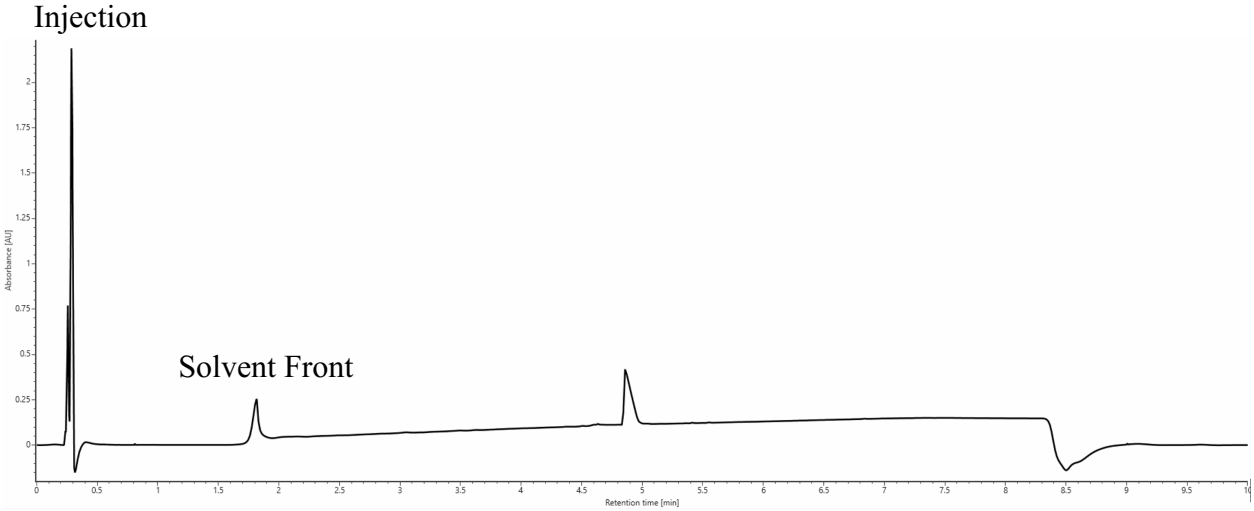
Peptide **6** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **S3** loaded resin (0.10 mmol), which was then coupled with the following amino acids: Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ile-OH, Fmoc-L-Ala-OH, and **5**. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **6** an off-white solid (36 mg, 36% yield).

^{1}H NMR (800 MHz, DMSO-d_6): δ = 8.51 (s, 1H), 8.08 (d, J = 6.6, 2H), 7.74 (s, 1H), 7.63 (s, 1H), 7.27 (s, 3H), 7.20 (s, 4H), 6.36 (s, 1H), 6.21 (s, 1H), 4.46–4.25 (m, 5H), 4.19 (s, 1H), 4.12 (s, 1H), 3.61 (s, 4H), 3.10 (s, 2H), 2.98 (s, 4H), 2.87 (s, 1H), 2.56 (s, 3H), 1.81–1.43 (m, 11H), 1.34 (m, 4H), 1.17 (s, 4H), 1.05 (s, 1H), 0.96–0.73 (m, 23H).

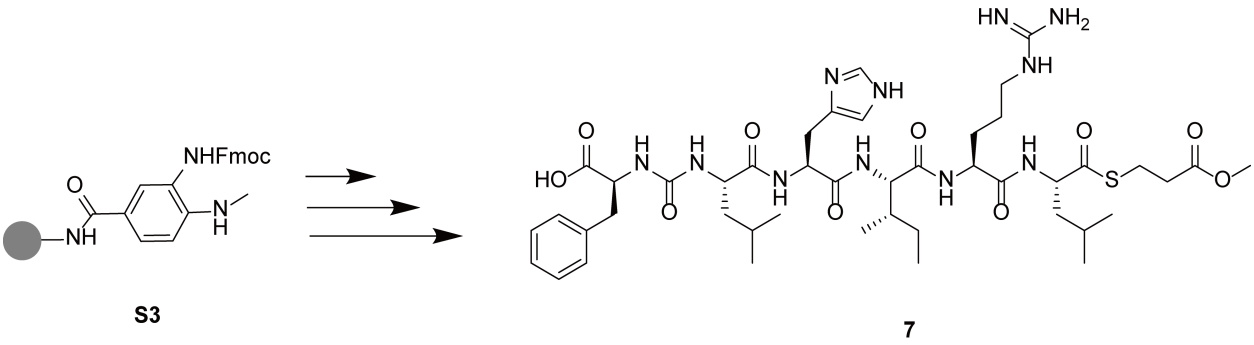
^{13}C NMR (201 MHz, DMSO-d_6): δ = 201.8, 174.4, 173.4, 172.4, 172.1, 172.0, 171.2, 157.7, 157.3, 138.0, 129.7, 128.6, 126.8, 57.9, 57.1, 54.6, 52.4, 52.0, 51.9, 48.6, 42.5, 40.9, 38.0, 37.3, 33.9, 29.3, 25.6, 24.6, 24.5, 24.4, 23.7, 23.6, 23.4, 22.3, 21.3, 18.0, 15.7, 11.6.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{41}H_{68}N_9O_{10}S$ 878.4804; Found 878.4862

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 6 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be 95%.



Synthesis of 7



S3 **7**

Peptide **7** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **S3** loaded resin (0.10 mmol), which was then coupled with the following amino acids: Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ile-OH, Fmoc-L-His(Trt)-OH, and **5**. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **7** as an off-white solid (28 mg, 26% yield).

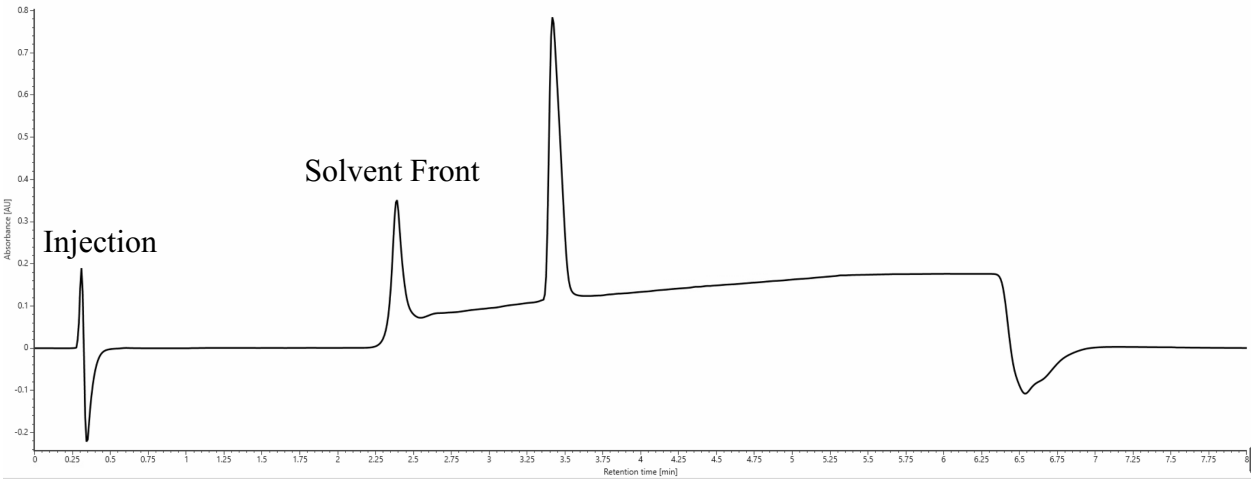
^{1}H NMR (800 MHz, DMSO-d_6): δ = 8.51 (s, 1H), 8.34 (s, 1H), 8.21–8.15 (m, 1H), 7.76 (t, J = 5.8, 1H), 7.66 (d, J = 8.3, 1H), 7.27 (t, J = 7.5, 3H), 7.19 (dt, J = 13.7, 6.6, 5H), 6.45 (d, J = 7.4, 1H), 6.26 (d, J = 8.1, 1H), 4.57 (q, J = 7.3, 1H), 4.37 (ddd, J = 11.4, 7.7, 4.3, 1H), 4.36–4.30 (m, 2H), 4.20 (t, J = 7.4, 1H), 4.05 (h, J = 5.8, 1H), 3.60 (s, 4H), 3.11 (q, J = 7.3, 2H), 2.97 (s, 7H), 2.55 (t, J = 7.1, 2H), 2.52–2.48 (m,

3H), 1.75 (dt, J = 16.9, 6.6, 1H), 1.69 (dtd, J = 10.2, 6.7, 3.4, 1H), 1.66–1.46 (m, 8H), 1.33 (dtd, J = 17.7, 10.8, 4.6, 4H), 1.06–0.98 (m, 1H), 0.92–0.84 (m, 7H), 0.83 (d, J = 6.5, 4H), 0.81–0.73 (m, 11H).

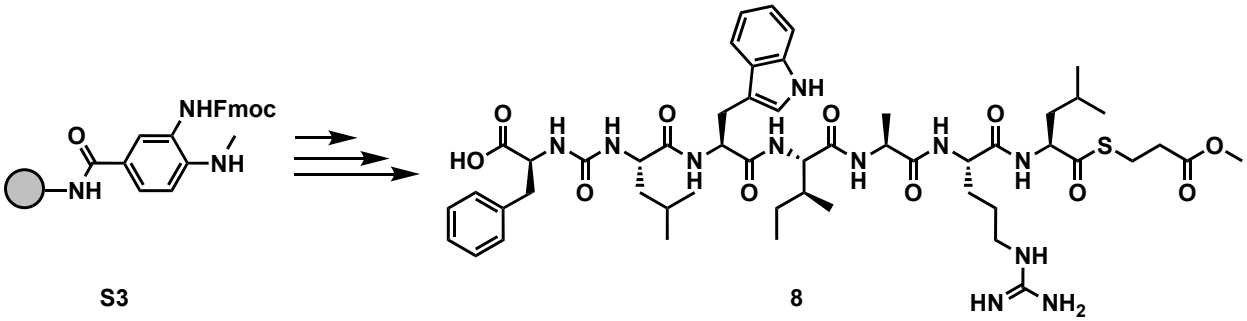
^{13}C NMR (201 MHz, DMSO-d_6): δ = 201.8, 174.2, 173.8, 172.1, 172.0, 171.2, 170.4, 157.9, 157.3, 137.8, 134.4, 129.8, 128.7, 126.9, 118.4, 116.9, 57.9, 57.2, 54.4, 52.5, 52.3, 52.2, 52.0, 42.0, 40.9, 38.0, 37.4, 33.9, 29.2, 27.5, 25.6, 24.5, 24.5, 24.4, 23.7, 23.5, 23.4, 22.2, 21.3, 15.6, 11.6.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{44}H_{70}N_{11}O_{10}S$ 944.5022; Found 944.5183.

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 4 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be 99%.



Synthesis of 8



Peptide **8** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **S3** loaded resin (0.10 mmol), which was then coupled with the following amino acids:

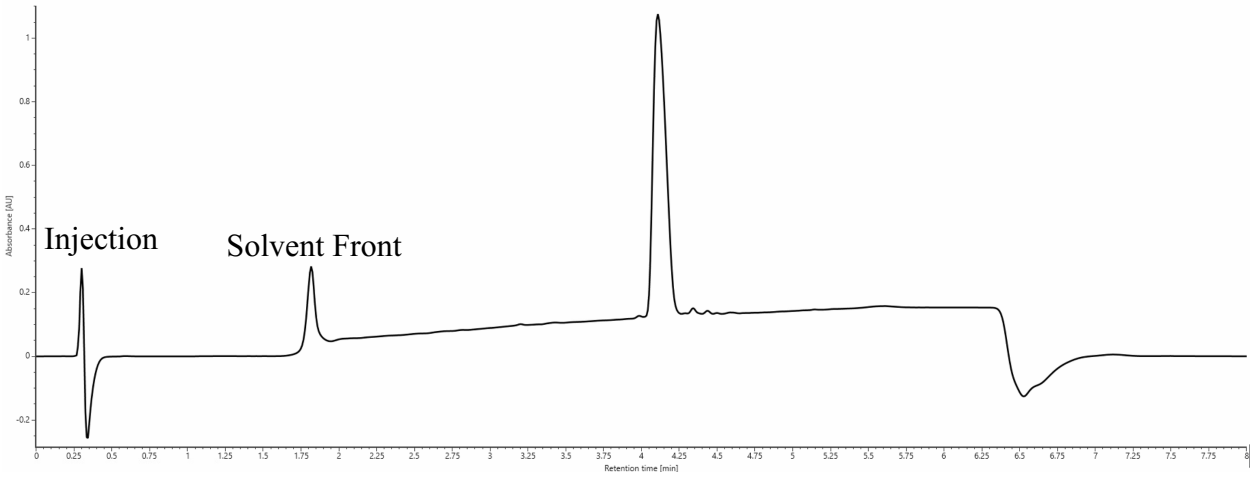
Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ala-OH, Fmoc-L-Ile-OH, Fmoc-L-Trp(Boc)-OH, and **5**. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **8** as an off-white solid (22 mg, 18% yield).

^{1}H NMR (800 MHz, DMSO-d_6): δ = 10.77 (s, 1H), 8.48 (d, J = 7.8, 1H), 8.09–7.84 (m, 3H), 7.81–7.58 (m, 2H), 7.52 (d, J = 7.9, 1H), 7.30 (d, J = 8.1, 1H), 7.25 (q, J = 8.4, 2H), 7.18 (p, J = 8.6, 4H), 7.09 (s, 1H), 7.04 (t, J = 7.5, 2H), 6.94 (t, J = 7.4, 2H), 6.37 (d, J = 8.0, 1H), 6.21 (d, J = 8.2, 1H), 4.58 (q, J = 7.2, 1H), 4.32 (m, 4H), 4.19–4.01 (m, 2H), 3.59 (d, J = 8.8, 3H), 3.37 (s, 4H), 3.17–3.03 (m, 3H), 3.01–2.76 (m, 6H), 1.83–0.93 (m, 17H), 0.93–0.61 (m, 20H).

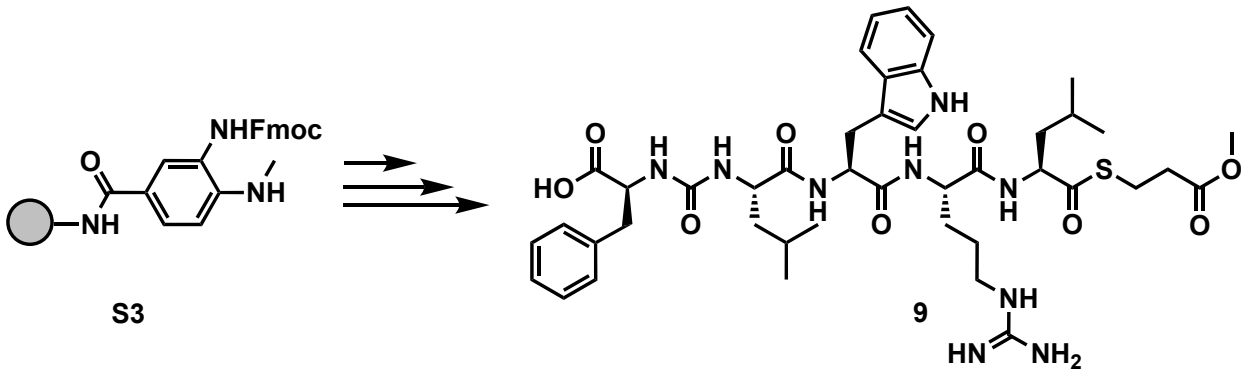
^{13}C NMR (201 MHz, DMSO-d_6): δ = 201.8, 174.1, 173.4, 172.4, 172.1, 172.0, 171.5, 171.0, 157.5, 157.2, 137.6, 136.4, 129.7, 128.6, 127.8, 126.8, 123.8, 121.2, 118.7, 118.6, 111.6, 110.3, 57.9, 57.2, 54.3, 53.6, 52.3, 52.1, 52.0, 48.7, 42.4, 40.8, 37.9, 37.1, 33.8, 29.2, 27.4, 25.4, 24.6, 24.4, 23.6, 23.5, 23.2, 22.2, 21.4, 18.3, 15.6, 11.4.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{52}H_{78}N_{11}O_{11}S$ 1064.5597; Found 1064.5587.

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 4 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be 97%.



Synthesis of 9



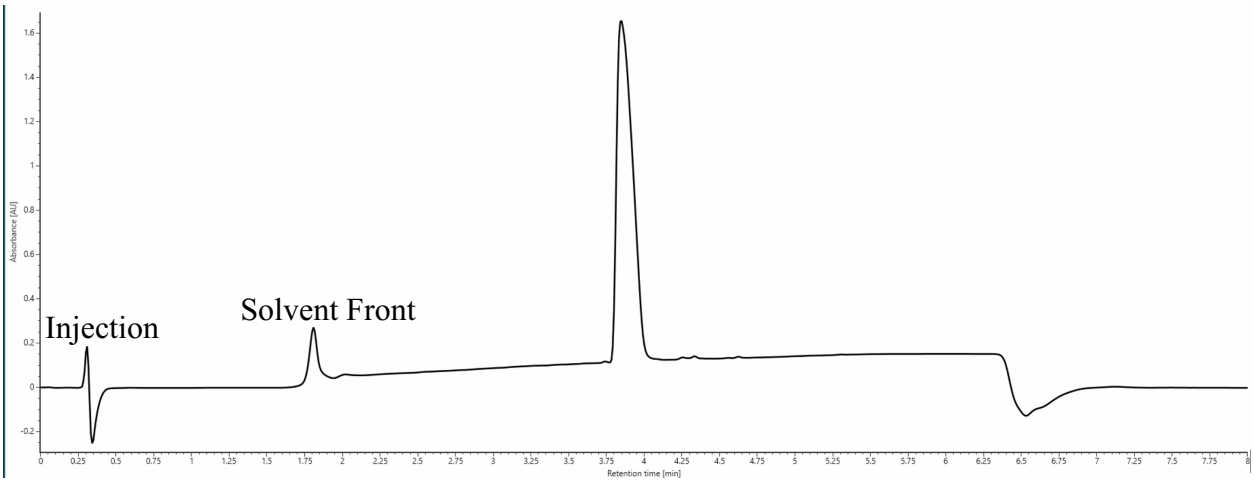
Peptide **9** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **S3** loaded resin (0.10 mmol), which was coupled with the following amino acids: Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Trp(Boc)-OH, and **5**. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **9** as an off-white solid (16 mg, 16% yield).

^{1}H NMR (800 MHz, DMSO-d_6): δ = 10.80 (s, 1H), 8.46 (s, 1H), 8.11 (s, 1H), 7.91 (s, 1H), 7.64 (s, 1H), 7.53 (s, 1H), 7.31 (s, 1H), 7.26 (s, 2H), 7.18 (s, 3H), 7.11 (s, 1H), 7.04 (s, 1H), 6.94 (s, 2H), 6.35 (s, 1H), 6.24 (s, 1H), 4.55 (s, 1H), 4.35 (m, 3H), 4.04 (s, 1H), 3.59 (s, 3H), 3.17–3.05 (m, 3H), 2.98 (s, 4H), 2.89 (dd, J = 13.8, 7.2, 1H), 2.56 (s, 2H), 1.77 (s, 1H), 1.66 (s, 1H), 1.61–1.45 (m, 6H), 1.33 (s, 1H), 1.22 (s, 1H), 0.89 (s, 3H), 0.81 (m, 9H).

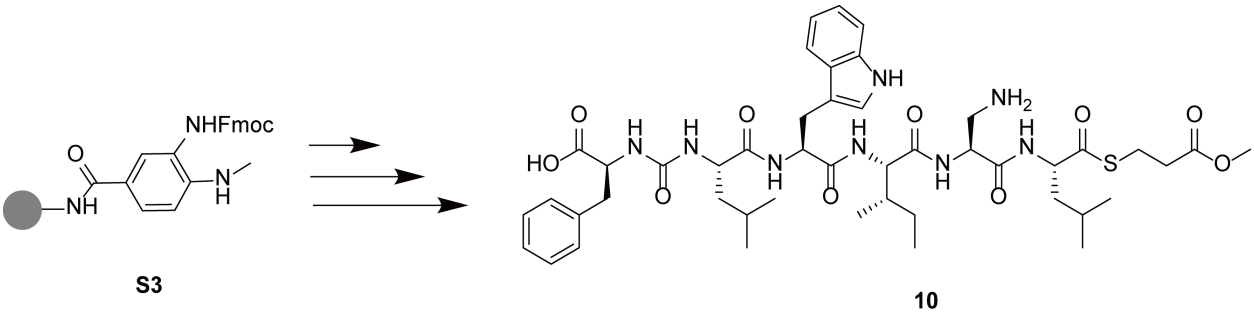
^{13}C NMR (201 MHz, DMSO-d_6): δ = 201.8, 174.0, 173.4, 172.1, 172.0, 171.7, 157.6, 157.2, 137.6, 136.4, 129.7, 128.6, 127.8, 126.8, 123.9, 121.2, 118.7, 118.5, 111.7, 110.2, 57.9, 54.3, 53.6, 52.3, 52.2, 51.9, 42.1, 40.2, 39.6, 37.9, 33.8, 29.2, 27.8, 25.3, 24.4, 24.4, 23.6, 23.5, 23.3, 22.1, 21.4.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{43}H_{62}N_9O_9S$ 880.4386; Found 880.4364.

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 4 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be 99%.



Synthesis of 10



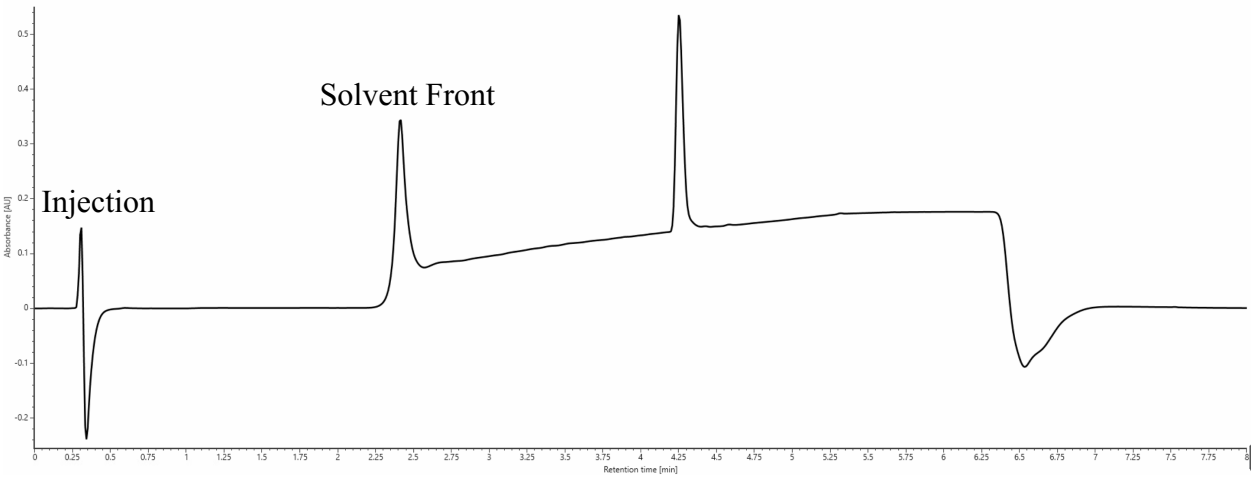
Peptide **10** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **S3** loaded resin (0.10 mmol), which was then coupled with the following amino acids: Fmoc-L-Leu-OH, Fmoc-L-Dap(Boc)-OH, Fmoc-L-Ile-OH, Fmoc-L-Trp(Boc)-OH, and **5**. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **10** as an off-white solid (5 mg, 5% yield).

^{1}H NMR (800 MHz, DMSO-d_6): δ = 10.91–10.72 (m, 1H), 8.69 (d, J = 7.8, 1H), 8.35 (s, 1H), 8.16 (d, J = 7.5, 1H), 7.69 (d, J = 7.8, 1H), 7.54 (d, J = 7.9, 1H), 7.37–6.88 (m, 9H), 6.43 (d, J = 7.4, 1H), 6.28 (d, J = 8.1, 1H), 4.70–4.51 (m, 2H), 4.44–4.19 (m, 3H), 4.08 (q, J = 7.5, 1H), 3.61 (s, 4H), 3.21 (m, 3H), 3.06–2.83 (m, 6H), 2.58 (td, J = 7.0, 2.2, 3H), 1.79 (dtt, J = 13.2, 10.0, 5.2, 1H), 1.69–1.46 (m, 4H), 1.44–1.21 (m, 3H), 1.10–1.01 (m, 1H), 0.95–0.70 (m, 19H).

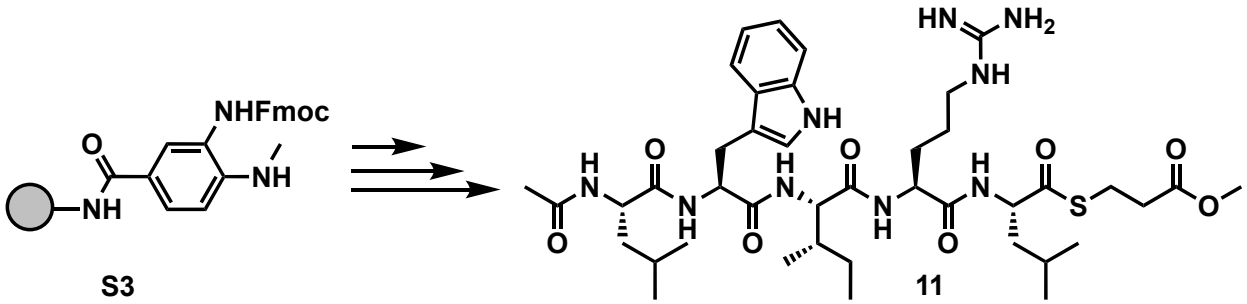
^{13}C NMR (201 MHz, DMSO-d_6): δ = 201.3, 174.3, 173.9, 172.1, 172.0, 171.7 169.5, 157.8, 137.8, 136.5, 129.8, 128.6, 127.8, 126.9, 123.9, 121.3, 118.7, 118.6, 111.8, 110.6, 58.2, 57.6, 54.5, 53.8, 52.4, 52.0, 51.0, 42.4, 38.0, 37.2, 33.8, 27.2, 24.5, 24.4, 23.8, 23.5, 23.3, 22.3, 21.4, 15.6, 11.7.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{46}H_{67}N_8O_{10}S$ 923.4695; Found 923.3919.

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 4 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be 98%.



Synthesis of 11



Peptide **11** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **11** loaded resin (0.10 mmol), which was coupled with the following amino acids: Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ile-OH, Fmoc-L-Trp(Boc)-OH, and Fmoc-L-Leu-OH. The N-terminal Fmoc protecting group was removed with 20% piperidine-DMF, and acetylated with 50 equivalents (5 mmol) acetic anhydride and 50 equivalents of pyridine (5 mmol) in DMF for 1 h. Methyl 3-

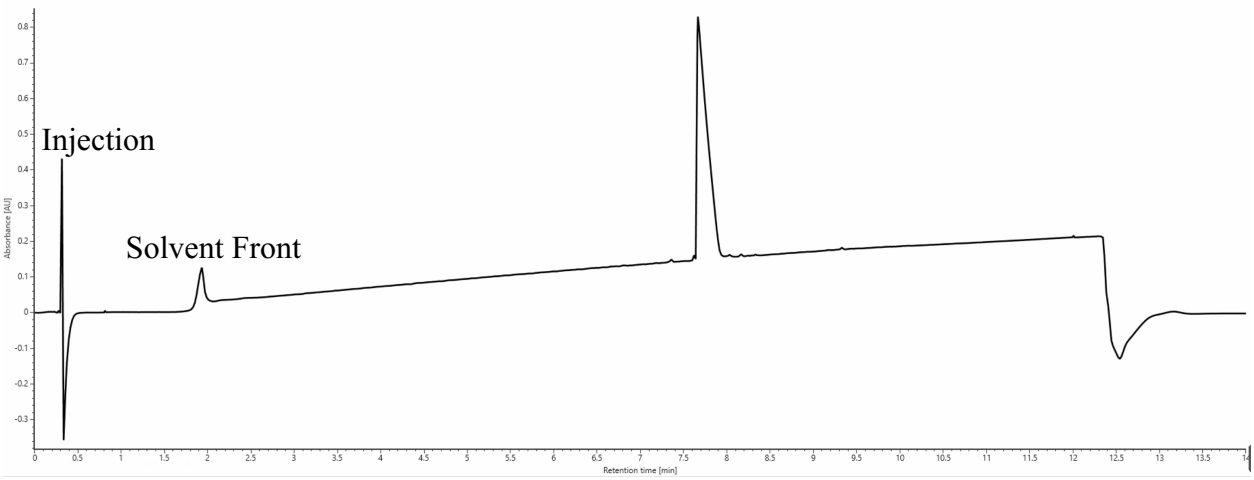
mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **11** as an off-white solid (45 mg, 47% yield).

^{1}H NMR (800 MHz, DMSO-d_6): δ = 10.85–10.78 (m, 1H), 8.52 (d, J = 7.8, 1H), 8.17–7.95 (m, 3H), 7.66 (d, J = 8.6, 2H), 7.53 (d, J = 7.9, 1H), 7.31 (d, J = 8.1, 2H), 7.13–6.89 (m, 4H), 4.54 (td, J = 8.5, 4.5, 1H), 4.47–4.35 (m, 2H), 4.34–4.19 (m, 2H), 3.60 (d, J = 2.1, 3H), 3.35 (t, J = 3.6, 5H), 3.12 (tt, J = 13.3, 6.0, 3H), 3.06–2.91 (m, 3H), 1.80 (s, 4H), 1.71–1.60 (m, 2H), 1.53 (m, 6H), 1.44–1.30 (m, 3H), 1.04 (ddd, J = 13.2, 9.2, 6.9, 1H), 1.00–0.68 (m, 19H).

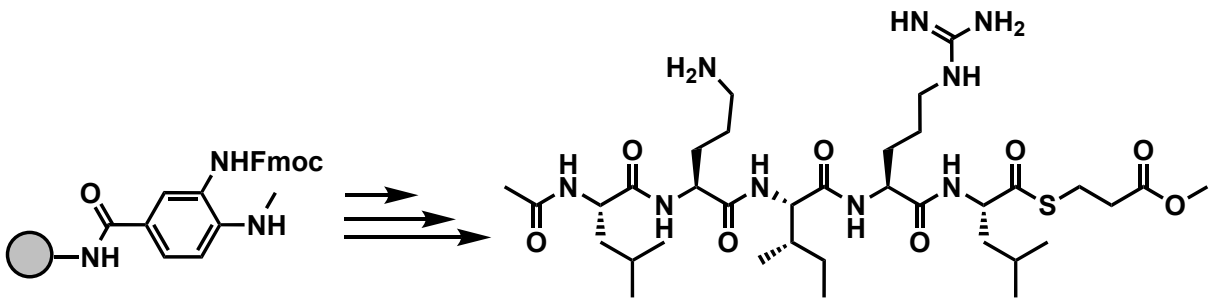
^{13}C NMR (201 MHz, DMSO-d_6): δ = 201.8, 172.7, 172.1, 172.0, 171.5, 171.0, 169.7, 157.2, 136.4, 127.7, 123.8, 121.2, 118.7, 118.6, 111.6, 110.4, 57.8, 57.1, 53.5, 52.4, 52.0, 51.4, 41.0, 40.8, 37.4, 33.8, 29.2, 27.3, 25.5, 24.5, 24.5, 24.4, 23.7, 23.3, 22.8, 22.0, 21.2, 15.5, 11.5.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{41}H_{66}N_9O_8S$ 844.4750; Found 844.4798.

UPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 11 min at a flow rate of 0.5 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 2 min after the gradient. The peptide purity was determined to be 98%.



Synthesis of 12



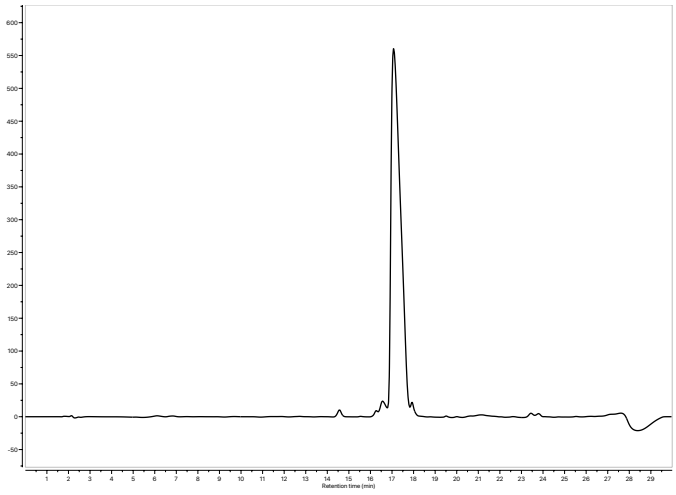
Peptide **12** was synthesized using the NDbz "safety-catch" resin. The synthesis was initiated from Fmoc-MeDbz-OH **12** loaded resin (0.20 mmol), which was coupled with the following amino acids: Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ile-OH, Fmoc-L-Orn(Boc)-OH, and Fmoc-L-Leu-OH. The N-terminal Fmoc protecting group was removed with 20% piperidine-DMF, and acetylated with 50 equivalents (5 mmol) acetic anhydride and 50 equivalents of pyridine (5 mmol) in DMF for 1 h. Methyl 3-mercaptopropionate was used as the cleaving thiol. The crude peptide was purified via reverse-phase semi-preparative HPLC using mobile phases of acetonitrile + 0.05% TFA (B) and H_2O + 0.05% TFA (A). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 70% over 22 min at a flow rate of 20 mL/min. The column was equilibrated with 0% mobile phase B for 5 min after and 1 min before the gradient to yield the TFA salt of **12** as an off-white solid (32 mg, 16% yield).

^{1}H NMR (800 MHz, DMSO-d_6): δ = 8.56 (d, J = 7.8, 1H), 8.18 (d, J = 8.2, 1H), 8.06 (m, 2H), 7.77 (q, J = 5.8, 4H), 7.58 (d, J = 8.7, 1H), 4.44–4.20 (m, 5H), 3.60 (s, 3H), 3.17–3.07 (m, 2H), 2.98 (td, J = 7.0, 2.8, 2H), 2.78 (h, J = 5.9, 2H), 2.56 (t, J = 7.1, 5H), 1.81–1.36 (m, 18H), 0.93–0.74 (m, 20H).

^{13}C NMR (201 MHz, DMSO-d_6): δ = 201.8, 173.1, 172.2, 172.1, 171.4, 171.1, 169.8, 157.3, 57.9, 57.0, 52.4, 52.3, 52.0, 51.6, 41.3, 40.9, 38.9, 37.6, 33.9, 29.3, 28.7, 25.6, 24.7, 24.5, 24.2, 23.7, 23.5, 23.4, 22.9, 22.0, 21.3, 15.6, 11.6.

Mass spec: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{35}H_{66}N_9O_8S$ 772.4750; Found 772.4739.

HPLC Trace Obtained using mobile phases of H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Samples were eluted using a gradient mode with mobile phase B ranging from 0% to 60% over 21 min at a flow rate of 1 mL/min. The column was equilibrated with 0% mobile phase B for 1 min before and 5 min after the gradient. The peptide purity was determined to be 95%.


600
550
500
450
400
350
300
250
200
150
100
50
0
-50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Retention time (min)

SUPPLEMENTARY TABLES

Table S1: Enzymatic conversion yields for cyclic and hydrolysis products by the two BulbE-TE enzymes, C961S BulbE-TE, and no enzyme control

BulbE-TE from *Microbulbifer* sp. MLAF003		
Substrates	Cyclic product yields	Hydrolysis product yields
1	29%	0.5%
2	4%	7%
6	product not detected	18%
7	product not detected	45%
8	product not detected	1%
9	product not detected	5%
10	3%	47%
11	31%	7%
12 (pH 6.0)	13%	4%
12 (pH 7.5)	72%	26%
12 (pH 9.0)	35%	63%
C961S mutant enzyme		
12 (pH 6.0)	0.5%	10%
12 (pH 7.5)	55%	43%
12 (pH 9.0)	10%	89%
Without enzyme		
12 (pH 6.0)	product not detected	0.1%
12 (pH 7.5)	69%	29%
12 (pH 9.0)	9%	89%
BulbE-TE from *Microbulbifer* sp. VAAF005		
2	15%	77%

SUPPLEMENTARY FIGURES

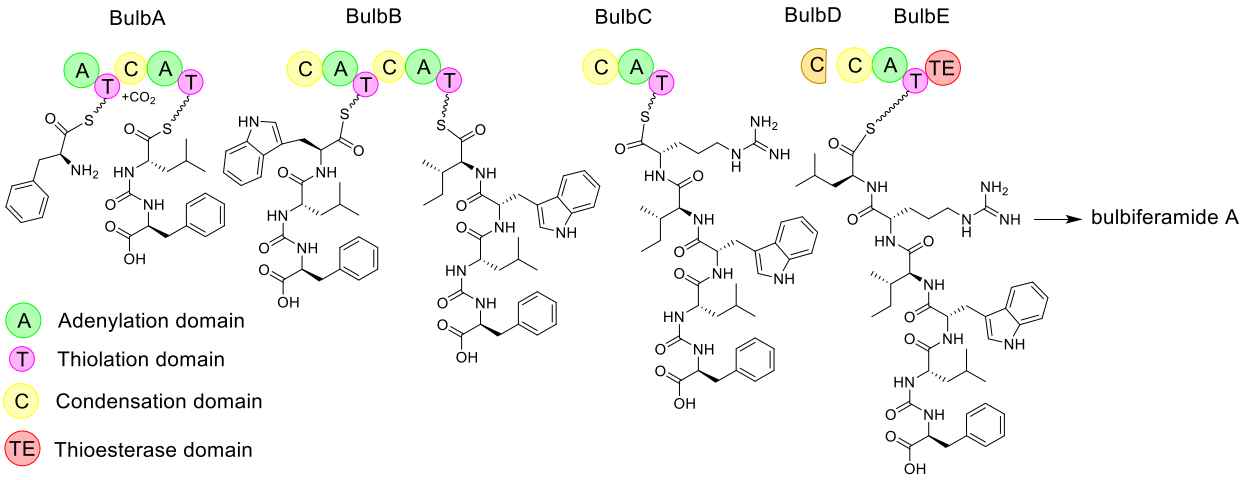


Figure S1: Proposed biosynthetic assembly line of bulbiferamide A. Note the presence of the TE domain at the end of the BulbE NRPS, which is referred to as the BulbE-TE in this report.

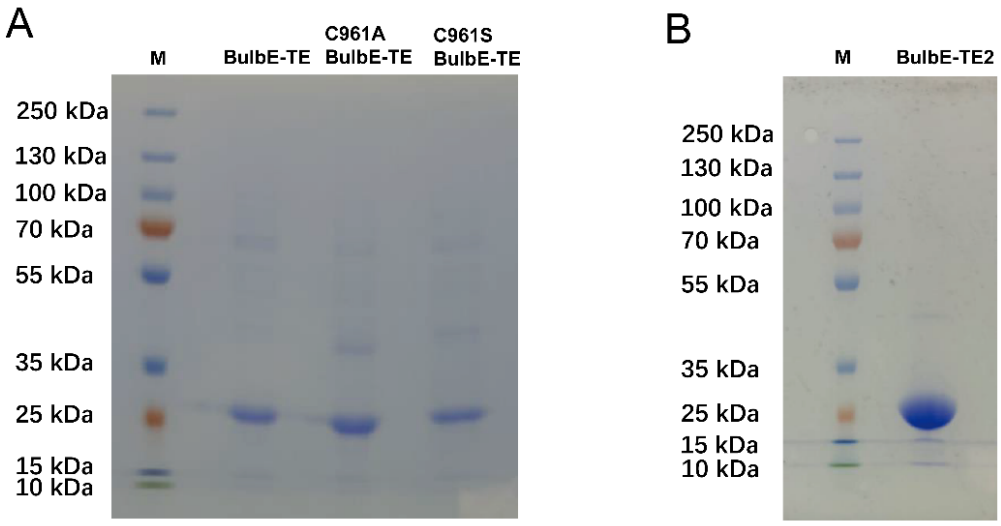


Figure S2: (**A**) SDS-PAGE of purified recombinant wild type and mutant forms of BulbE-TE from *Microbulbifer* sp. MLAF003. Expected molecular weight of protein is approximately 30.5 kDa. (**B**) SDS-PAGE of purified recombinant wild type BulbE-TE from *Microbulbifer* sp. VAAF005 (referred to as BulbE-TE2 in the figure). Expected molecular weight of protein is approximately 30.0 kDa.

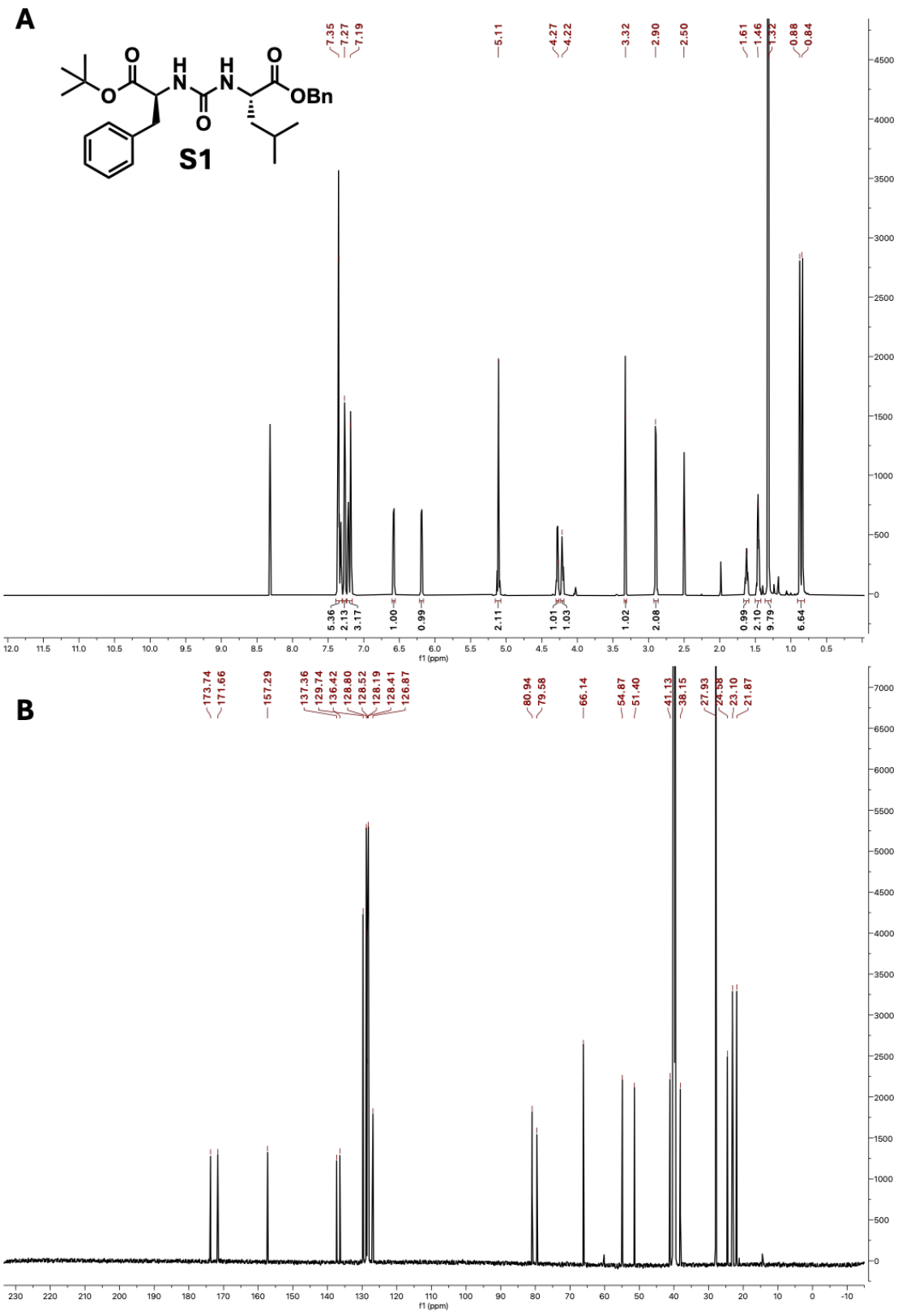


Figure S3: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **S1** in DMSO-d_6.

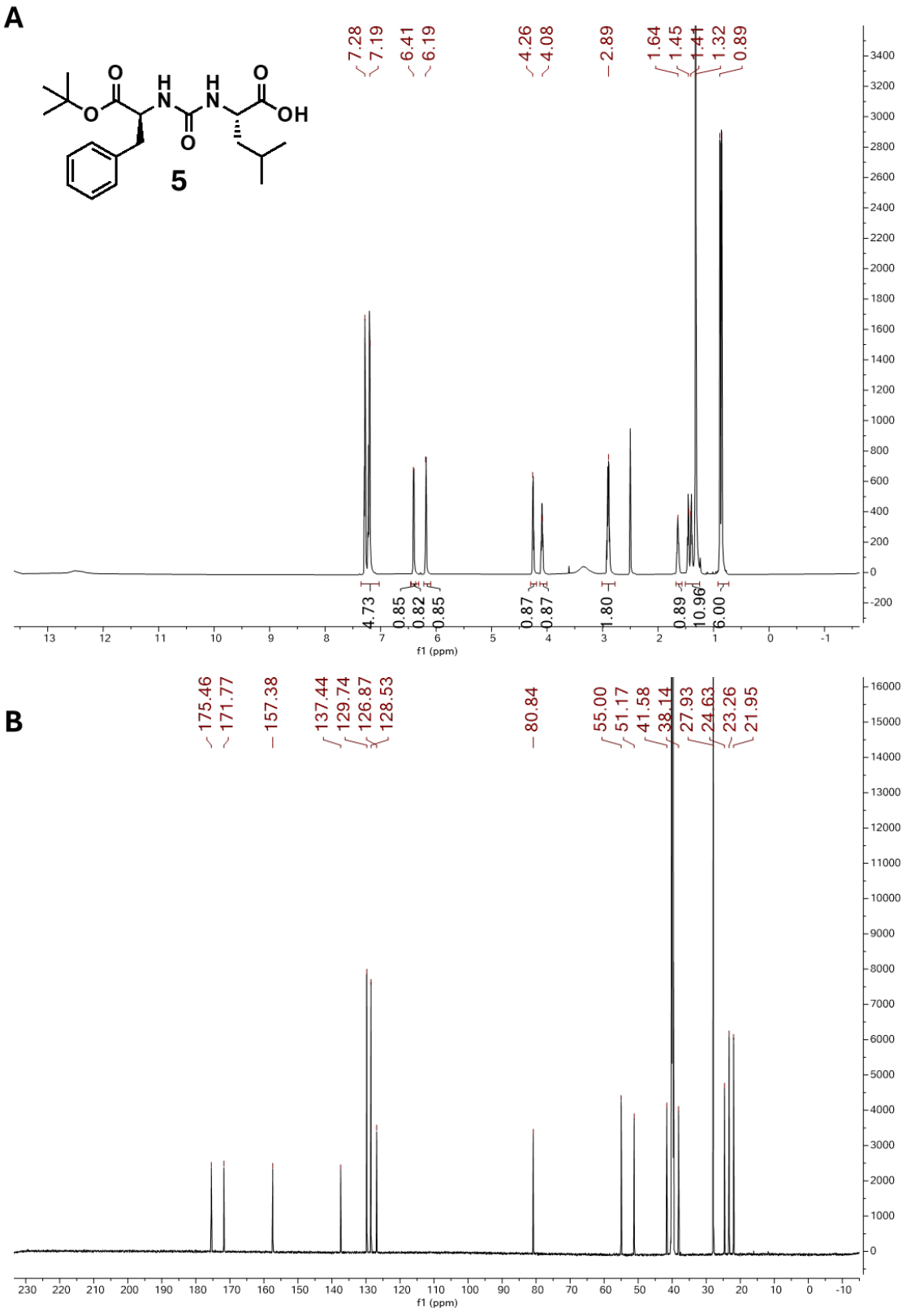


Figure S4: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **5** in DMSO-d_6.

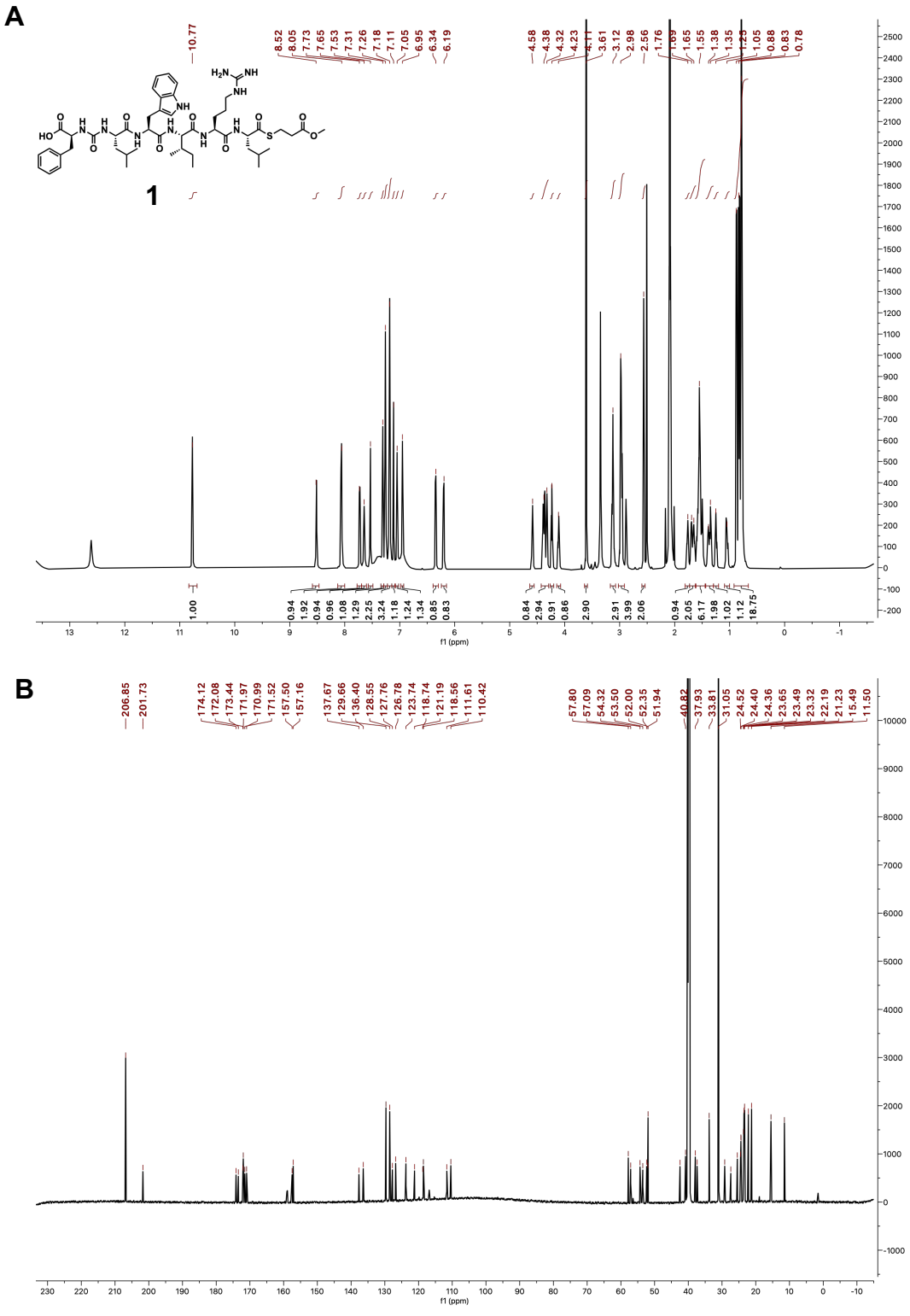


Figure S5: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **1** in DMSO-*d*$_6$.

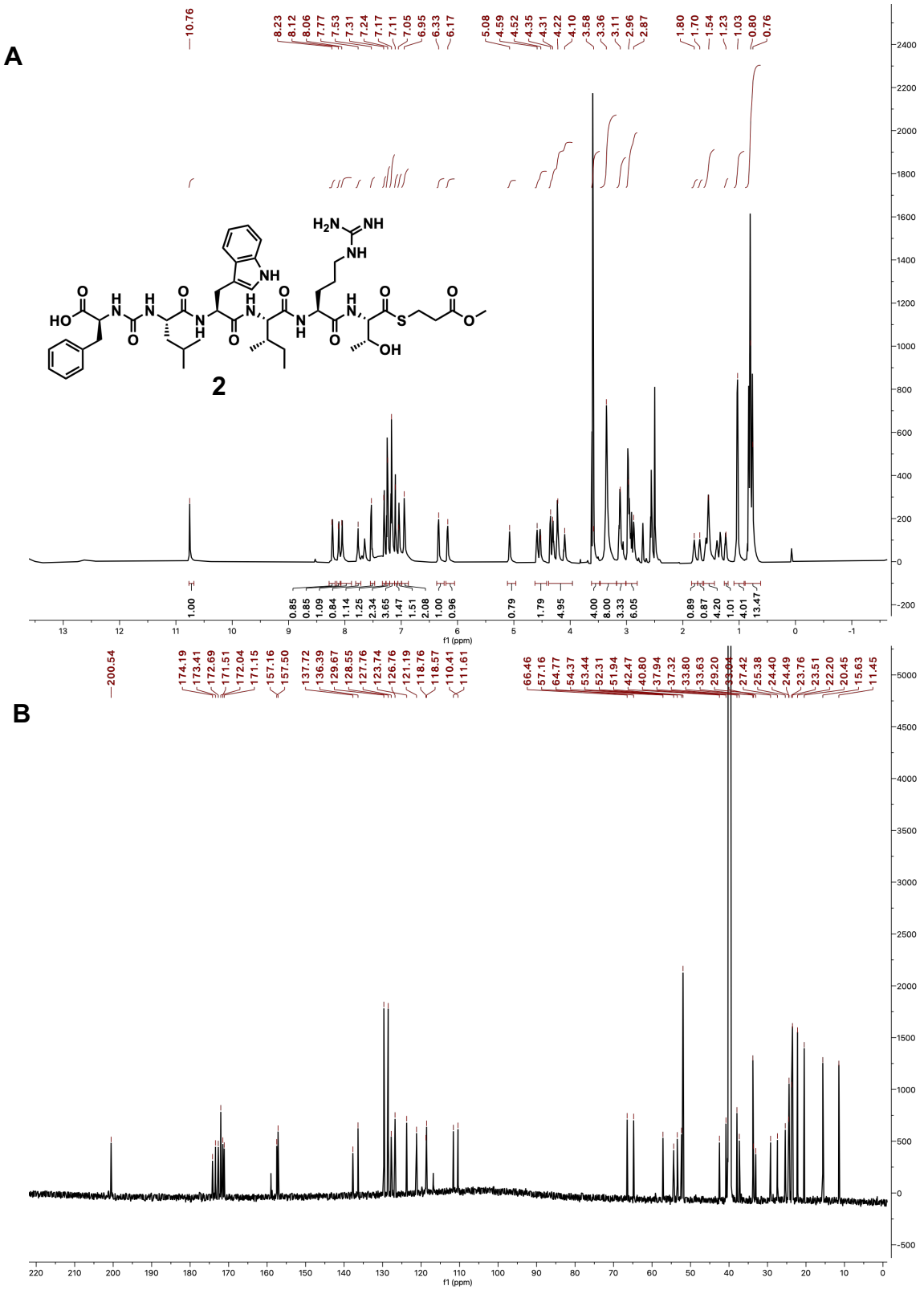


Figure S6: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **2** in DMSO-d_6.

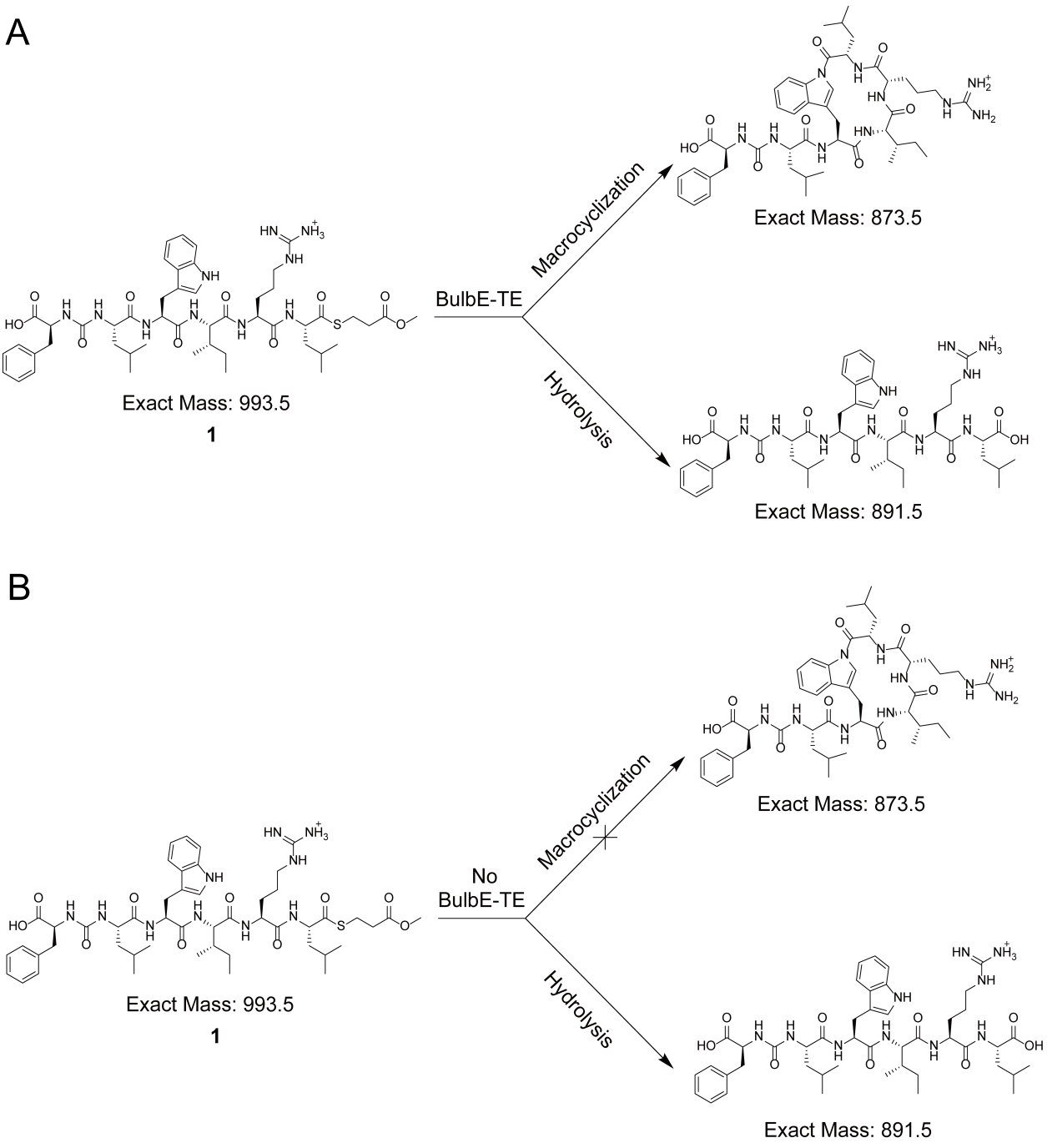


Figure S7: (**A**) Scheme for enzymatic reaction of substrate **1** with BulbE-TE to yield cyclic product bulbiferamide A and a thioester hydrolysis product. (**B**) Negative control of enzymatic reaction of substrate **1** without BulbE-TE did not yield cyclic product bulbiferamide A, only hydrolysis product.

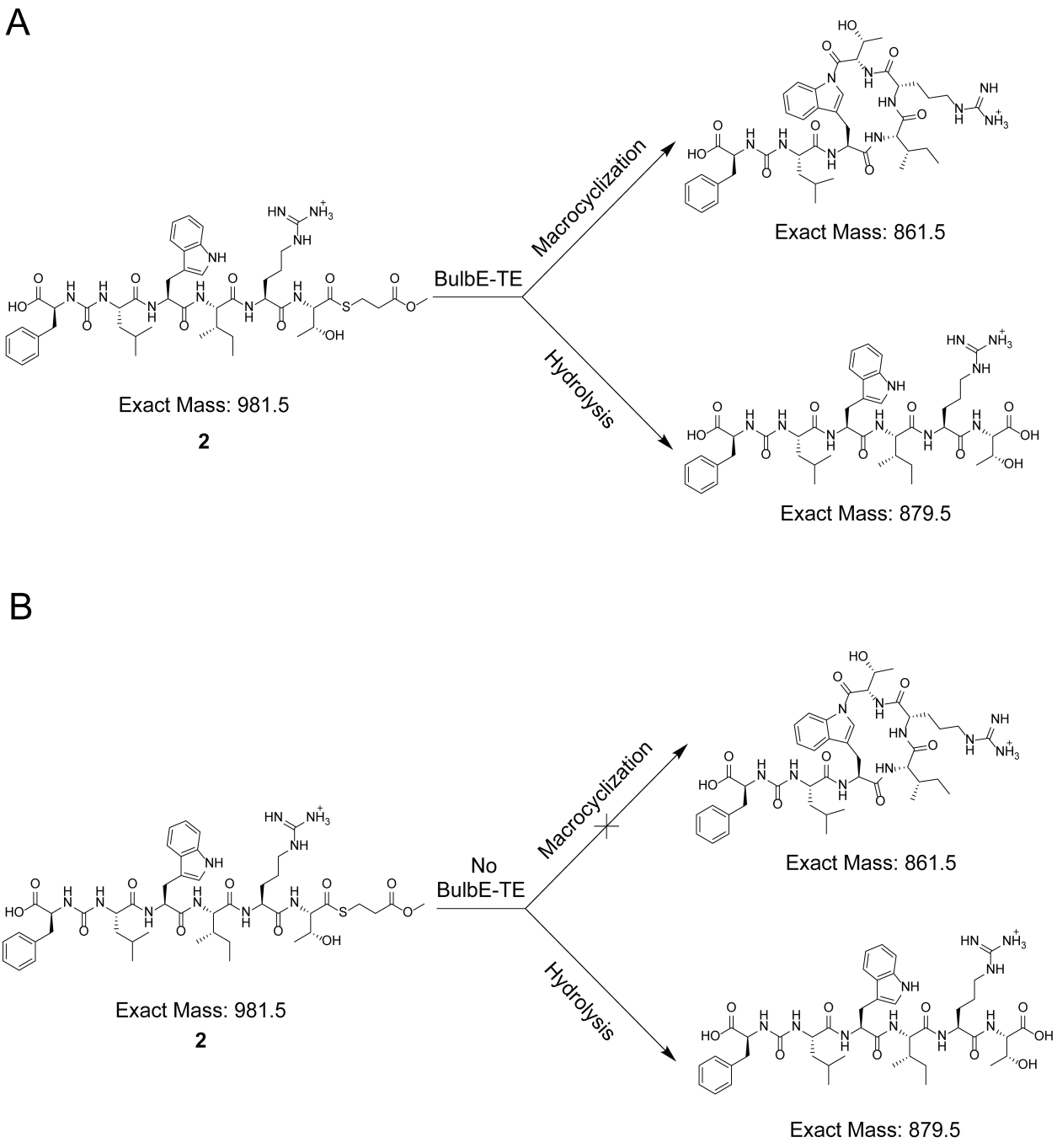


Figure S8: (**A**) Scheme for enzymatic reaction of substrate **2** with BulbE-TE to yield cyclic product bulbiferamide B and a thioester hydrolysis product. (**B**) Negative control of enzymatic reaction of substrate **2** without BulbE-TE did not yield cyclic product bulbiferamide B, only hydrolysis product.

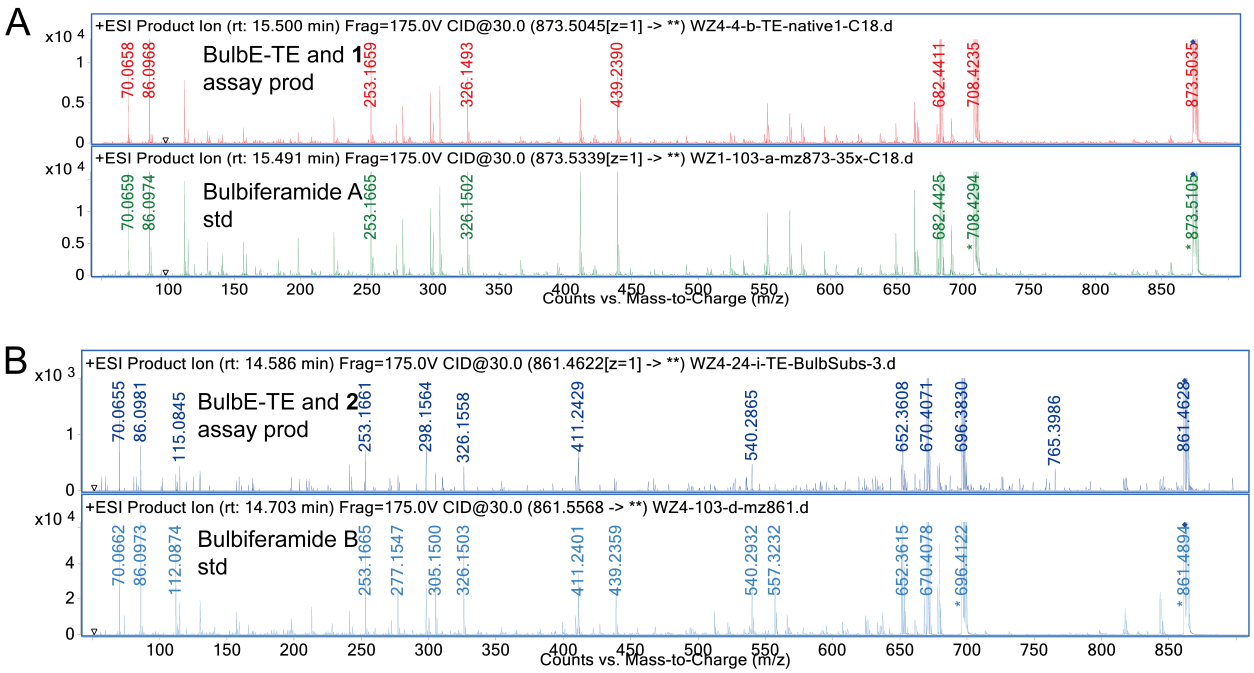


Figure S9: (**A**) MS^2 spectra for enzymatic product generated by BulbE-TE from **1** (top), and bulbiferamide A standard (bottom). (**B**) MS^2 spectra for enzymatic product generated by BulbE-TE starting from **2** (top), and bulbiferamide B standard (bottom). $[M+H]^{1+}$ precursor ions were chosen for fragmentation in each case with identical fragmentation energies.

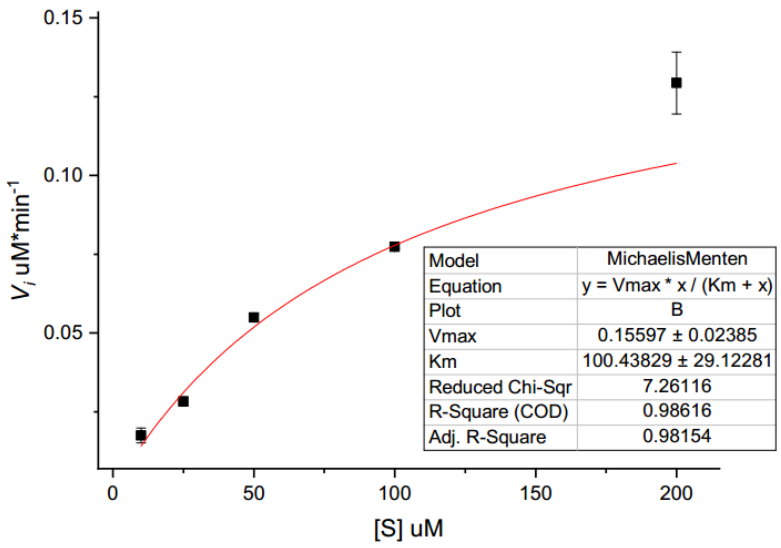


Figure S10. Michaels-Menten kinetics curve for macrocylization of **1** by BulbE-TE.

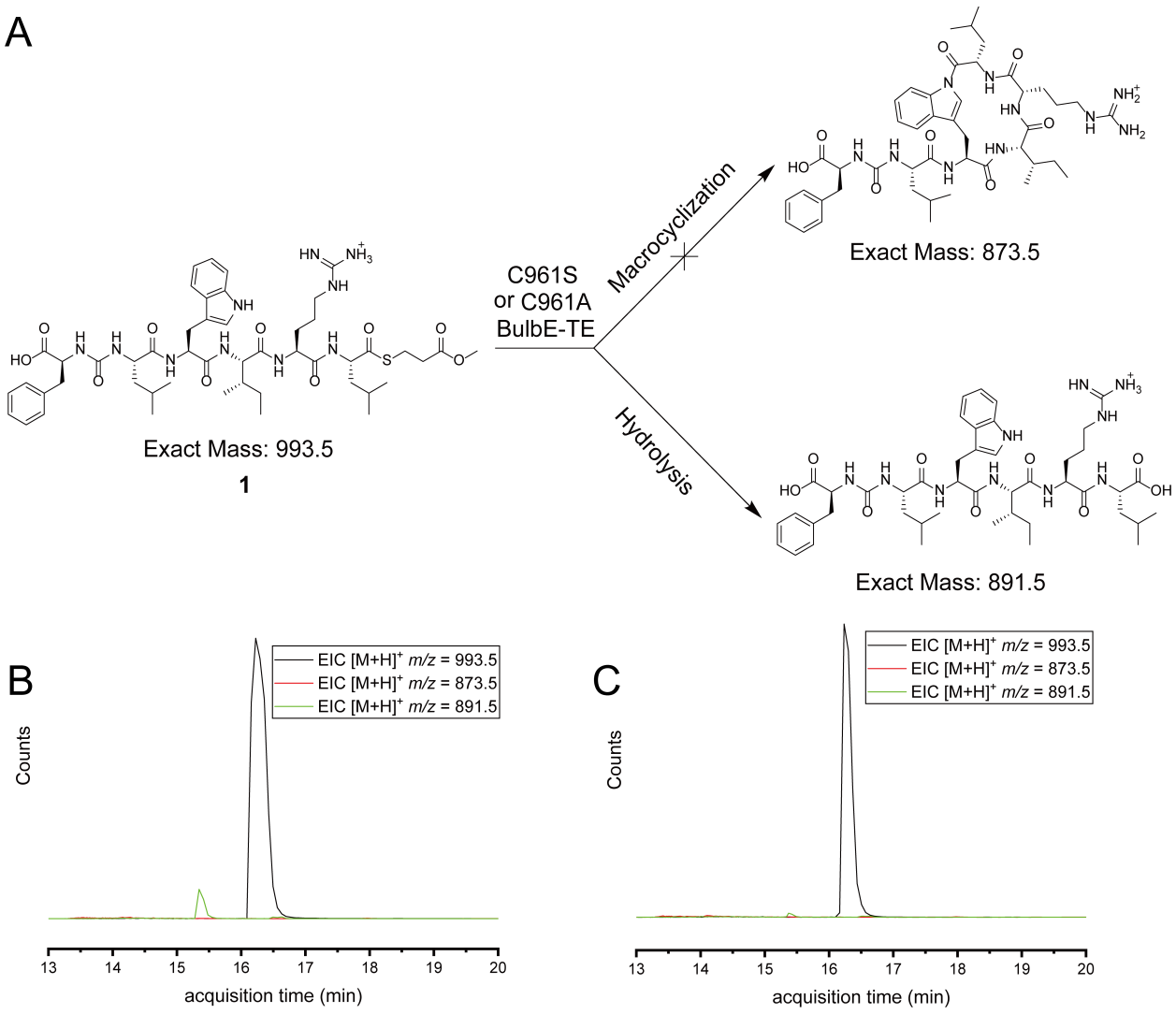


Figure S11: (**A**) Enzymatic reaction of substrate **1** with C961S or C961A mutated BulbE-TE did not yield cyclic product bulbiferamide A, only hydrolysis product. (**B**) LCMS analysis of C961S mutated BulbE-TE reaction with **1**. EICs showing the presence of the substrate **1** (black) and the thioester hydrolysis product (green). No cyclic product was observed (red). (**C**) LCMS analysis of the C961A mutated BulbE-TE reaction with **1**. EICs showing the presence of the substrate **1** (black) and the thioester hydrolysis product (green). No cyclic product was observed (red).

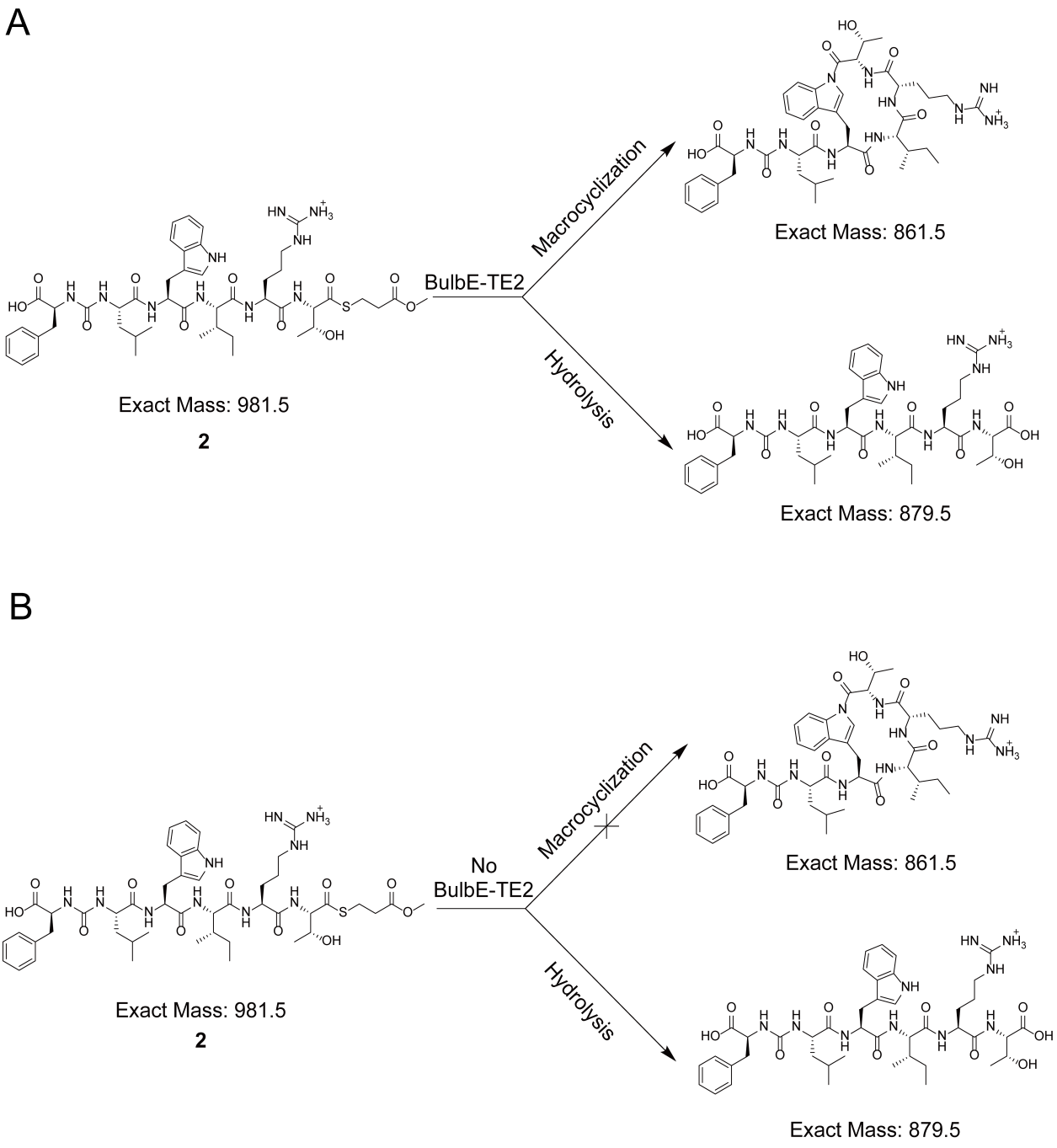


Figure S12: (**A**) Scheme for enzymatic reaction of substrate **2** with *Microbulbifer* sp. VAAF005-derived BulbE-TE (referred to as BulbE-TE2 in the figure) to yield cyclic product bulbiferamide B and a thioester hydrolysis product. (**B**) Negative control of enzymatic reaction of substrate **2** while omitting the enzyme did not yield cyclic product bulbiferamide B, only hydrolysis product was observed in this reaction.

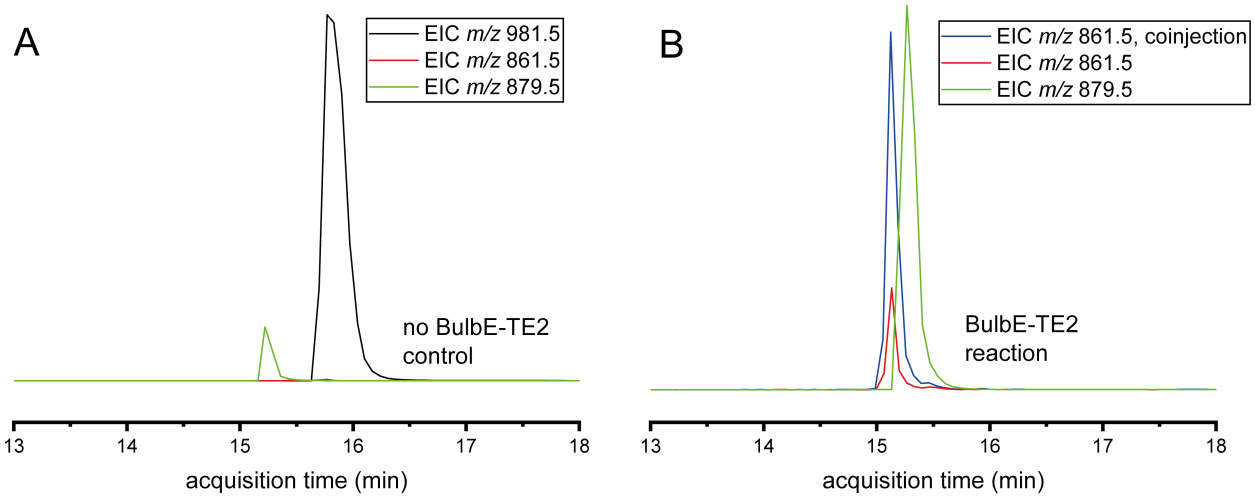


Figure S13: (**A**) EICs demonstrating the presence of **2**, hydrolyzed, and macrocyclized products in the reaction where the BulbE-TE from *Microbulbifer* sp. VAAF005 (also referred to as BulbE-TE2) was omitted. (**B**) EICs for hydrolyzed and macrocyclized products in the reaction in the presence of BulbE-TE2. The "coinjection" EIC refers to a spiking experiment in which bulbiferamide B was added to the quenched enzymatic reaction to confirm co-elution with the macrocyclized enzymatic product.

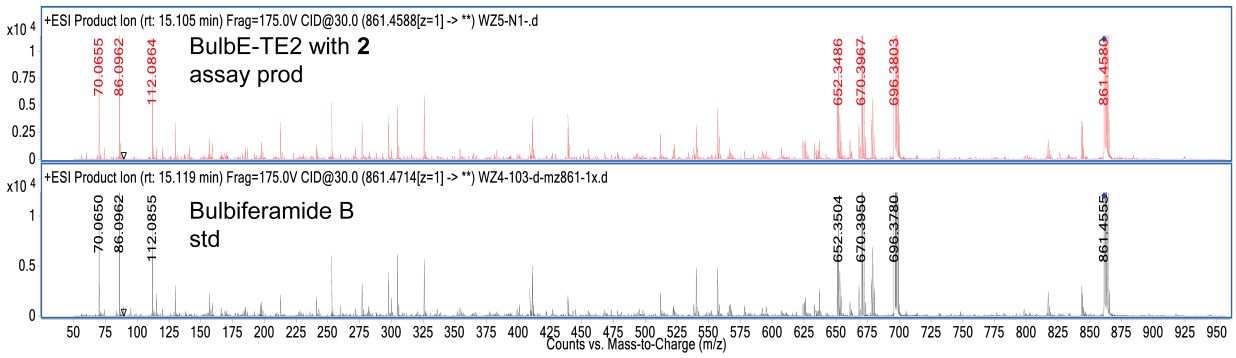


Figure S14: MS^2 spectra for enzymatic product generated by BulbE-TE2 starting from **2** (top), and bulbiferamide B standard (bottom). $[M+H]^{1+}$ precursor ions were chosen for fragmentation in each case with identical fragmentation energies.

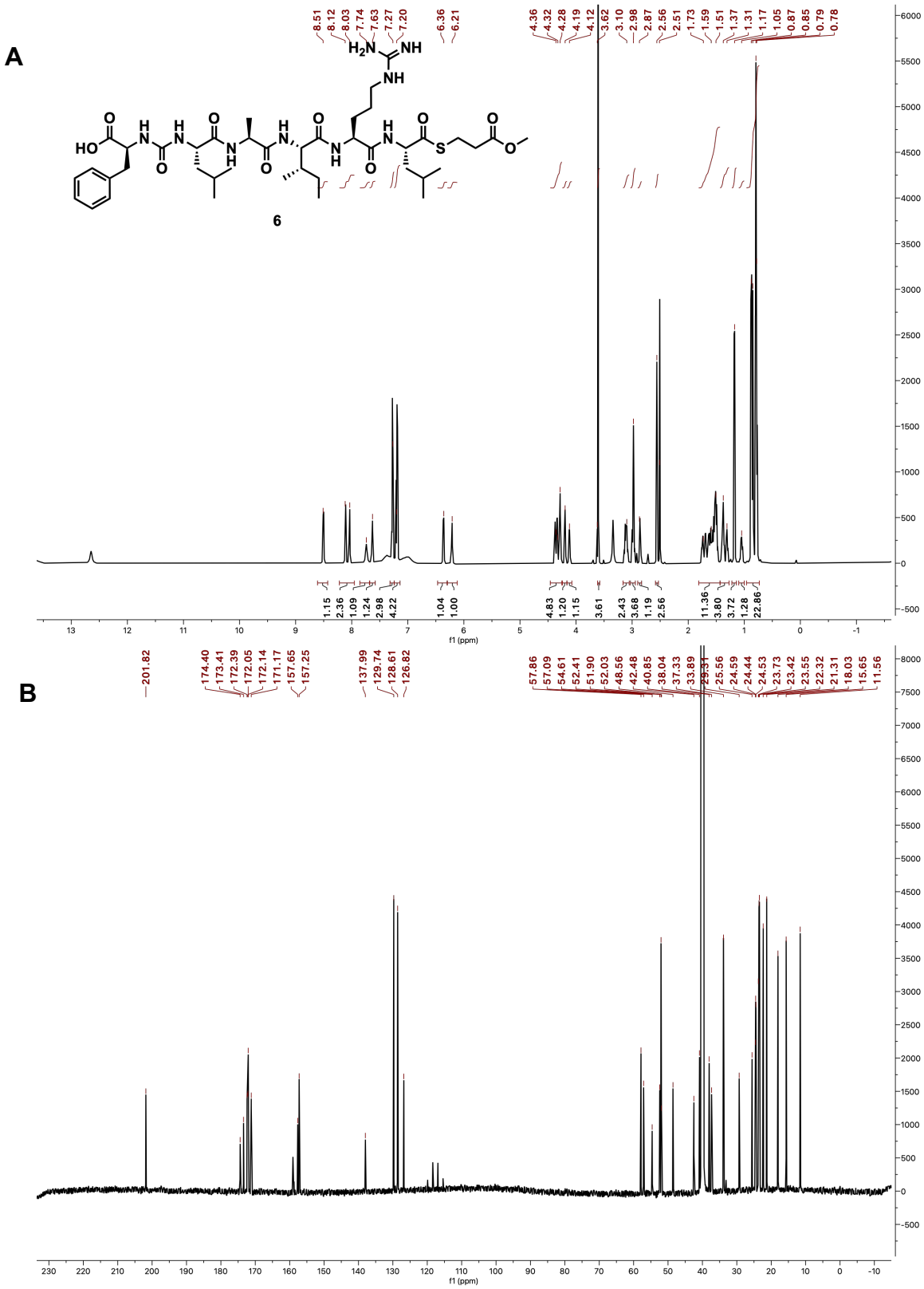


Figure S15: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **6** in DMSO-d_6.

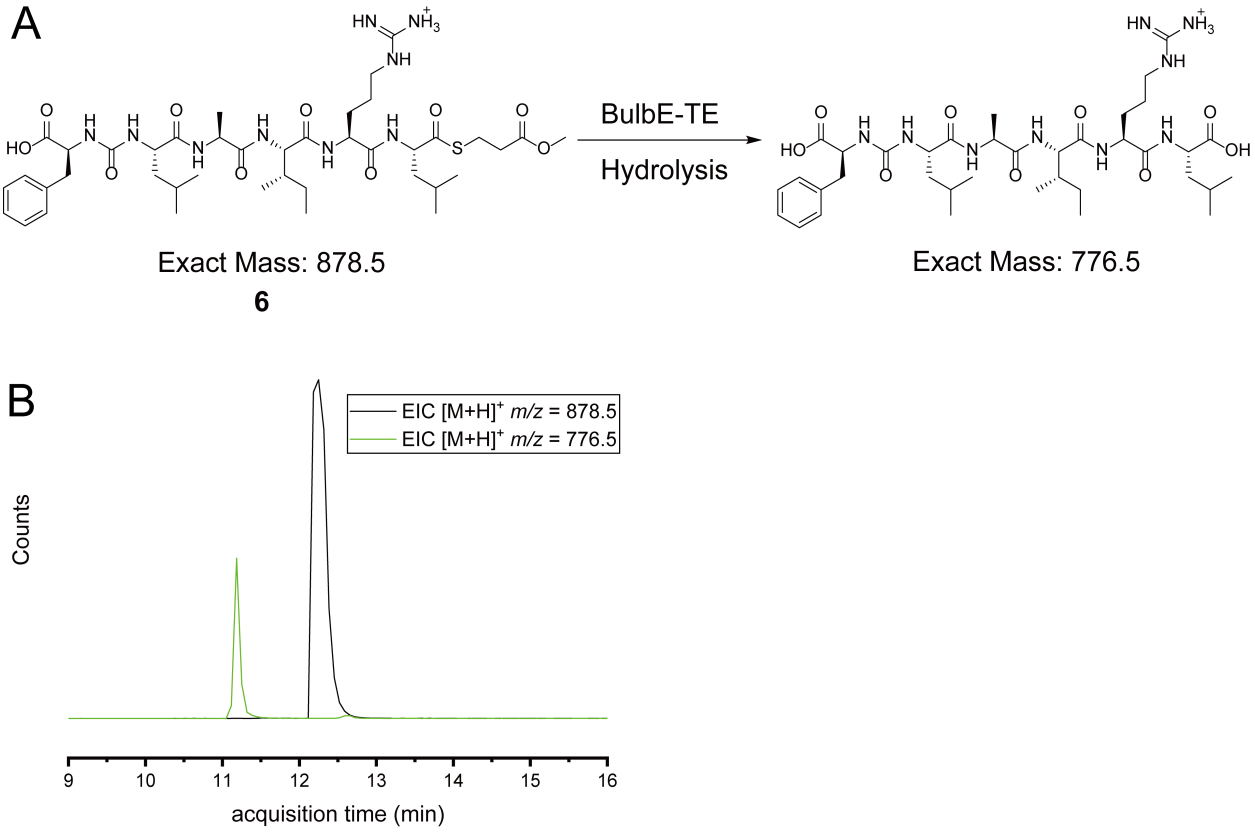


Figure S16: (**A**) Enzymatic reaction of Trp→Ala substrate **6** with BulbE-TE did not yield cyclic product, only hydrolysis product. (**B**) EICs showing the presence of the substrate **6** (black) and the thioester hydrolysis product (green).

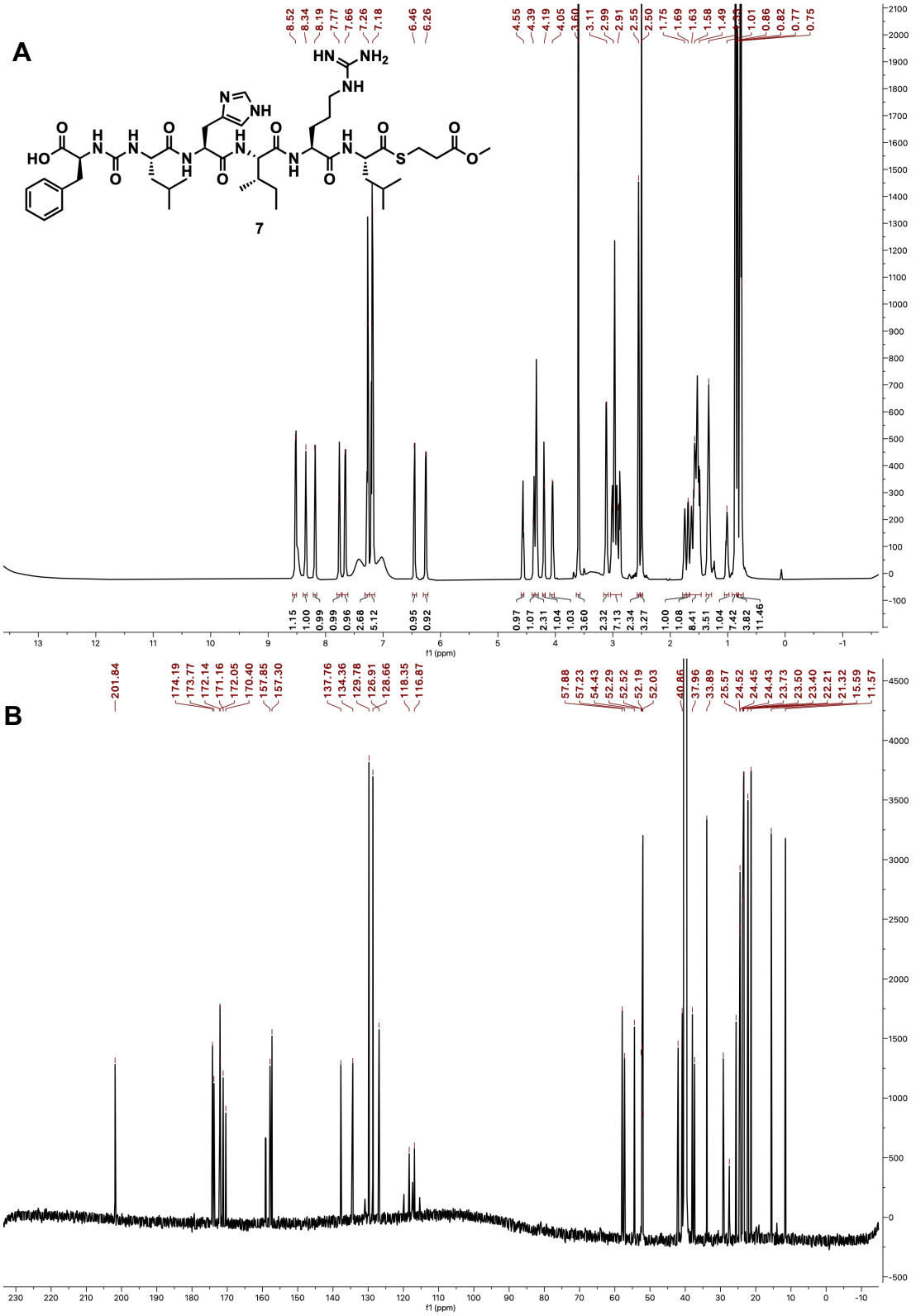


Figure S17: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **7** in DMSO-d_6.

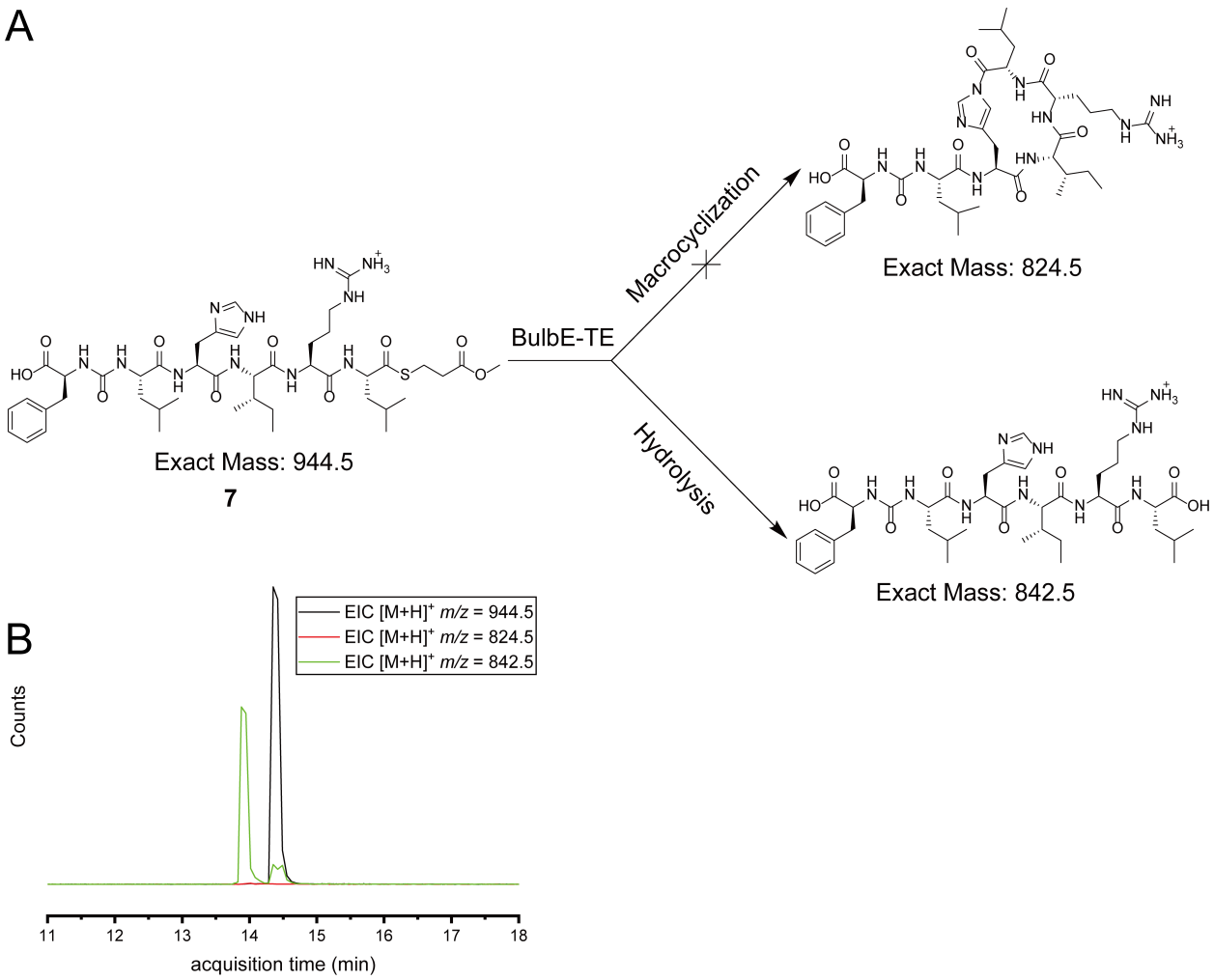


Figure S18: (**A**) Enzymatic reaction of Trp→His substrate **7** with BulbE-TE did not yield cyclic product, only hydrolysis product. (**B**) EICs showing the presence of the substrate **7** (black) and the thioester hydrolysis product (green). No cyclic product (red) was observed.

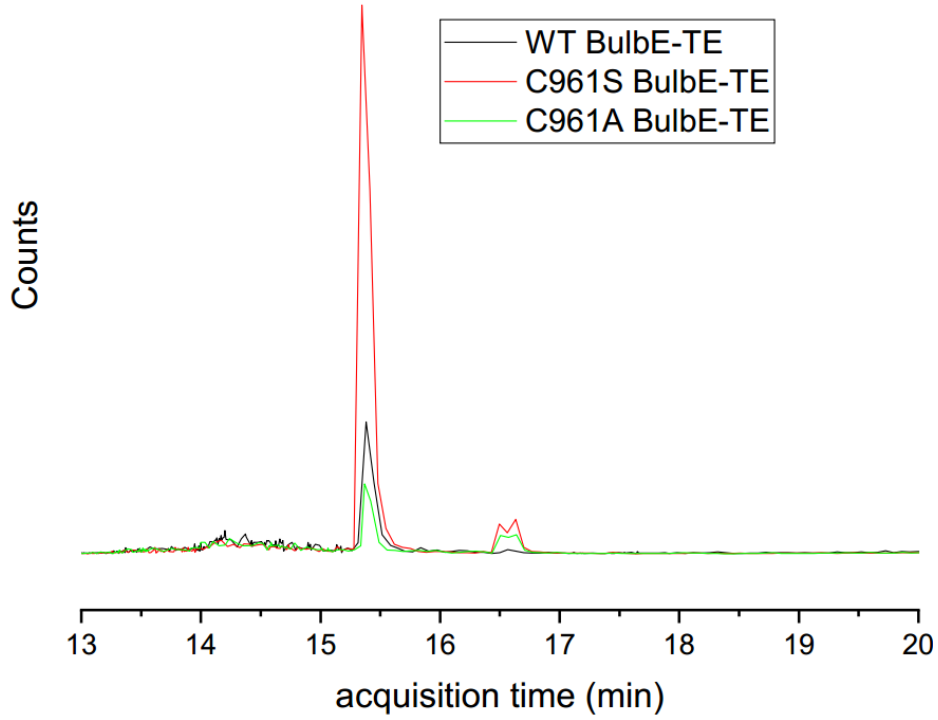


Figure S19: EICs demonstrating abundance of thioester hydrolysis product starting from substrate compound **1** using wild type and mutant forms of BulbE-TE.

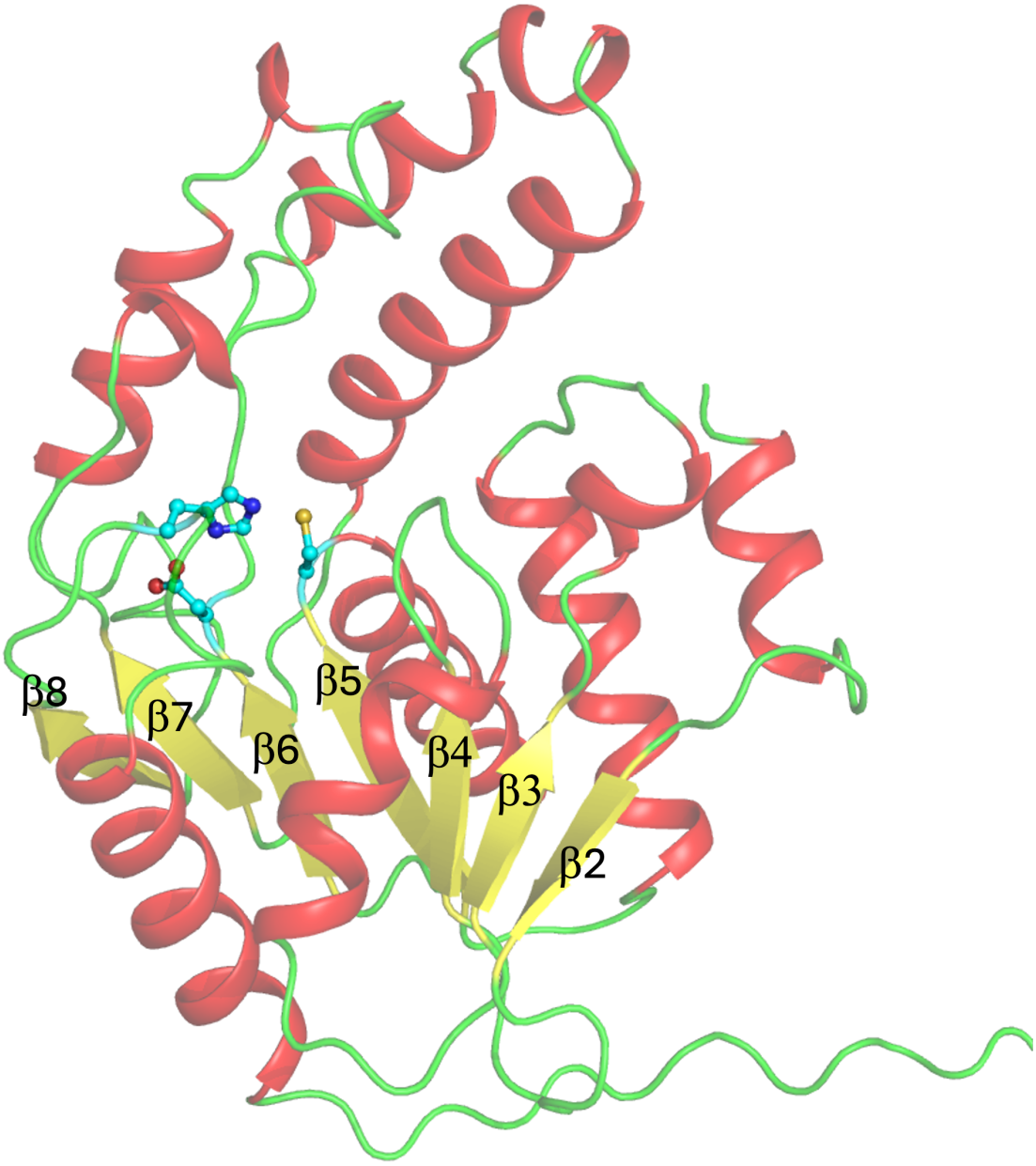


Figure S20: Cartoon representation of the AlphaFold3-generated model of the BulbE-TE with the alpha helices colored red, beta strands colored yellow, and the loop region shown in green. The catalytic triad residues are shown in stick-ball representation with carbon atoms colored cyan. Strands of the central beta-sheet are annotated.

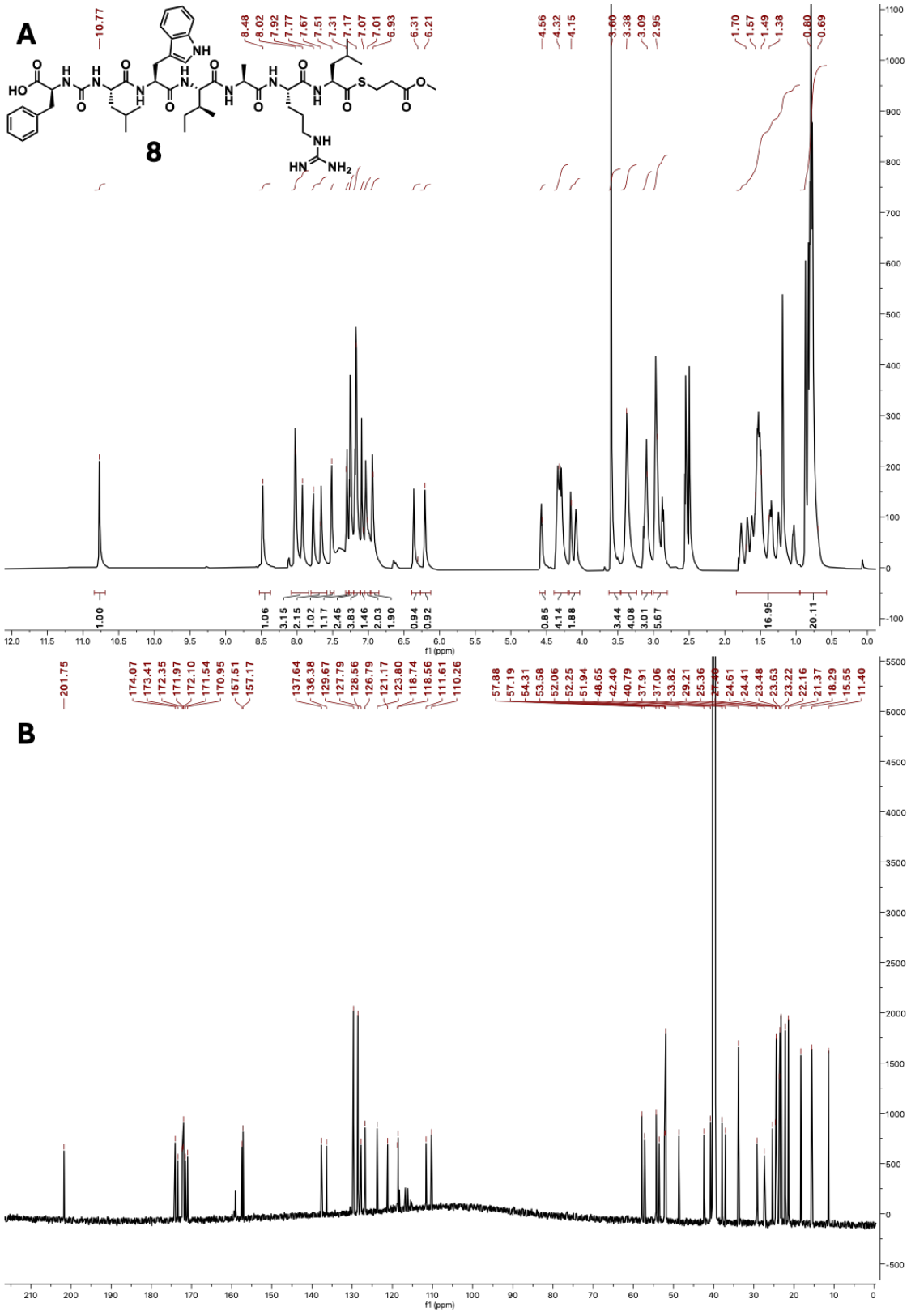


Figure S21: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **8** in DMSO-d_6.

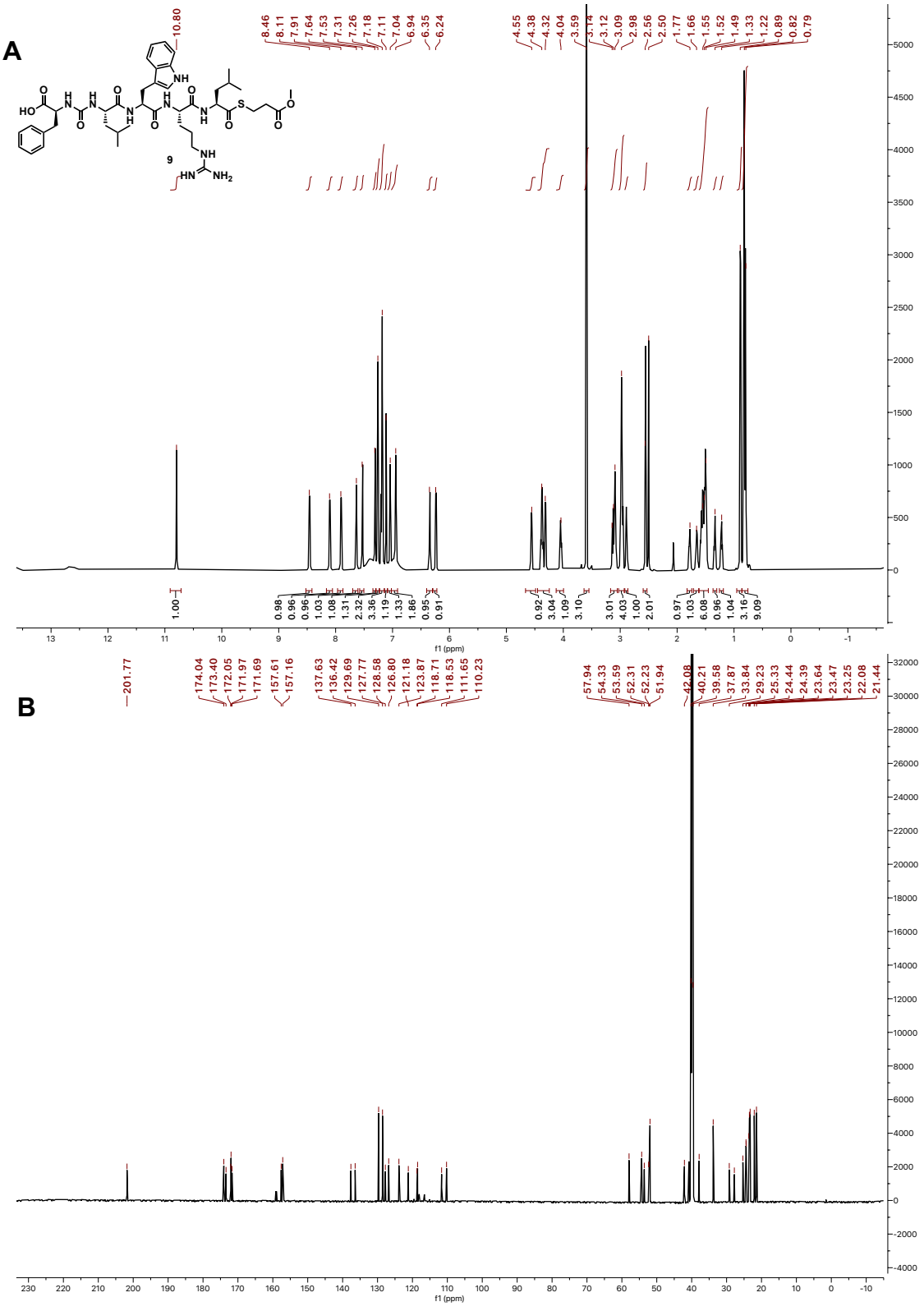


Figure S22: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **9** in DMSO-d_6.

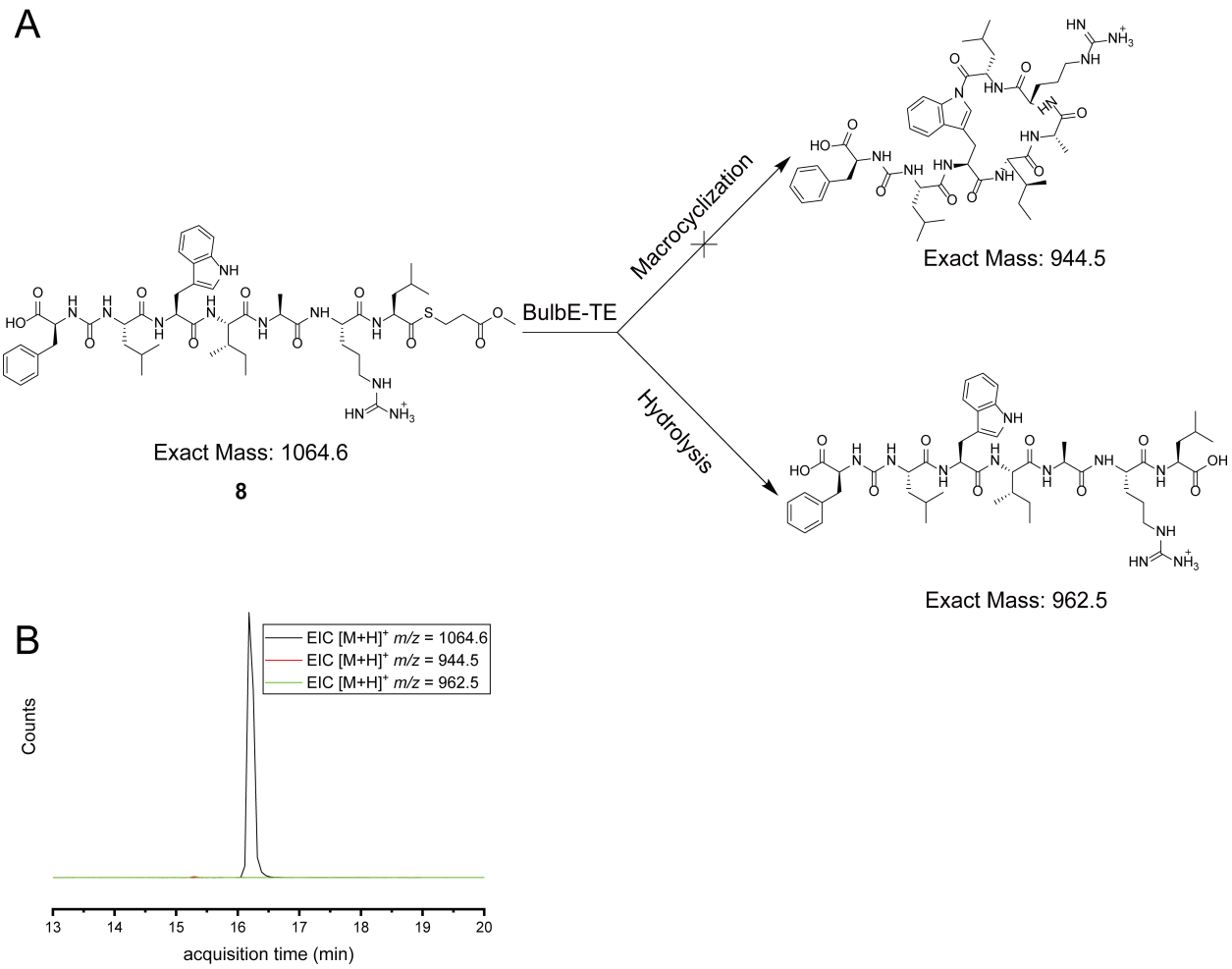


Figure S23: (**A**) Enzymatic reaction of expanded substrate **8** with BulbE-TE did not yield cyclic product, only hydrolysis product. (**B**) EICs showing the presence of the substrate **8** (black) and the thioester hydrolysis product (green). No cyclic product was observed (red). The scale of the EIC y-axis precludes observation of the hydrolysis product.

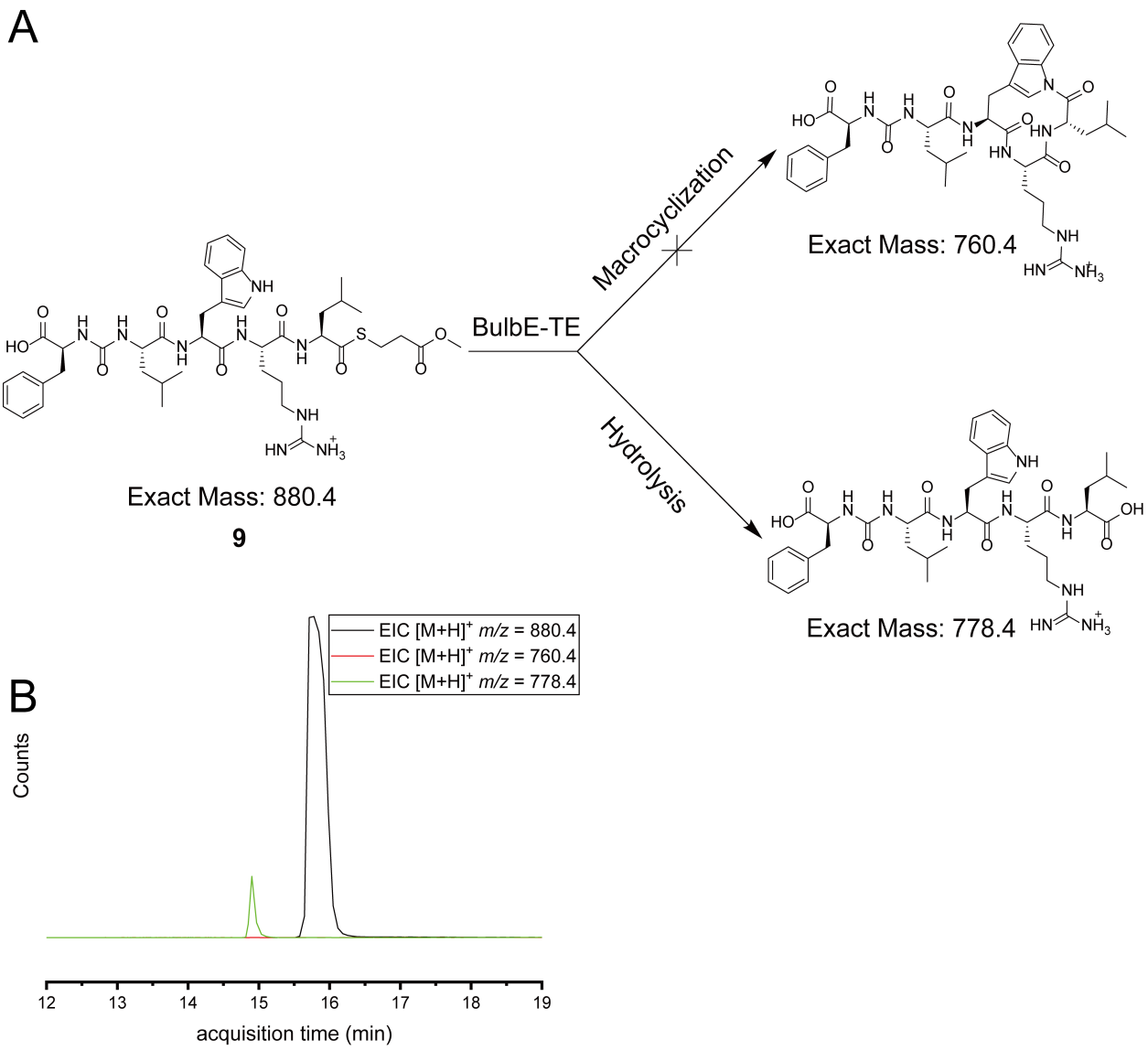


Figure S24: (**A**) Enzymatic reaction of contracted substrate **9** with BulbE-TE did not yield cyclic product, only hydrolysis product. (**B**) EICs showing the presence of the substrate **9** (black) and the thioester hydrolysis product (green). No cyclic product was observed (red).

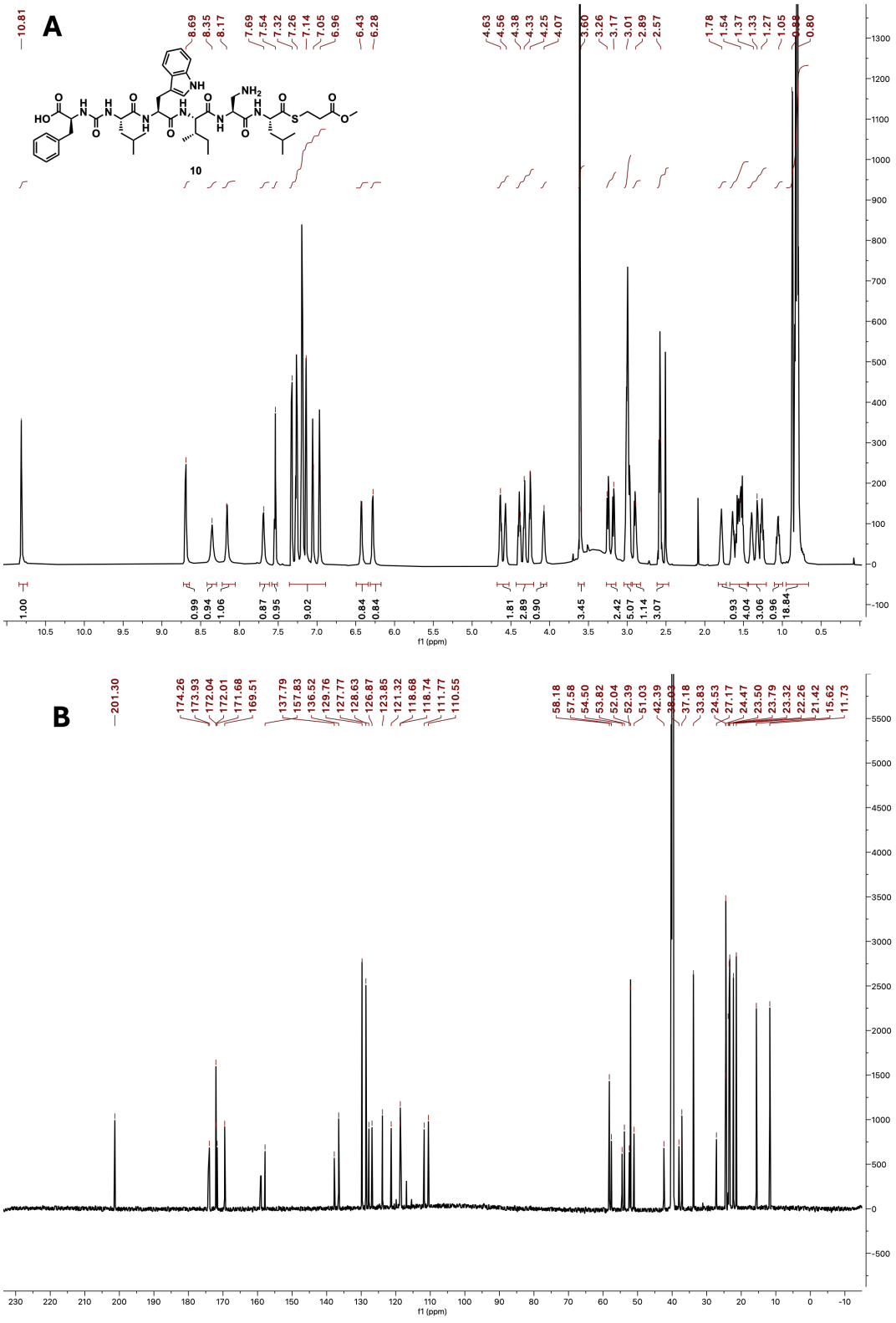


Figure S25: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **10** in DMSO-d_6.

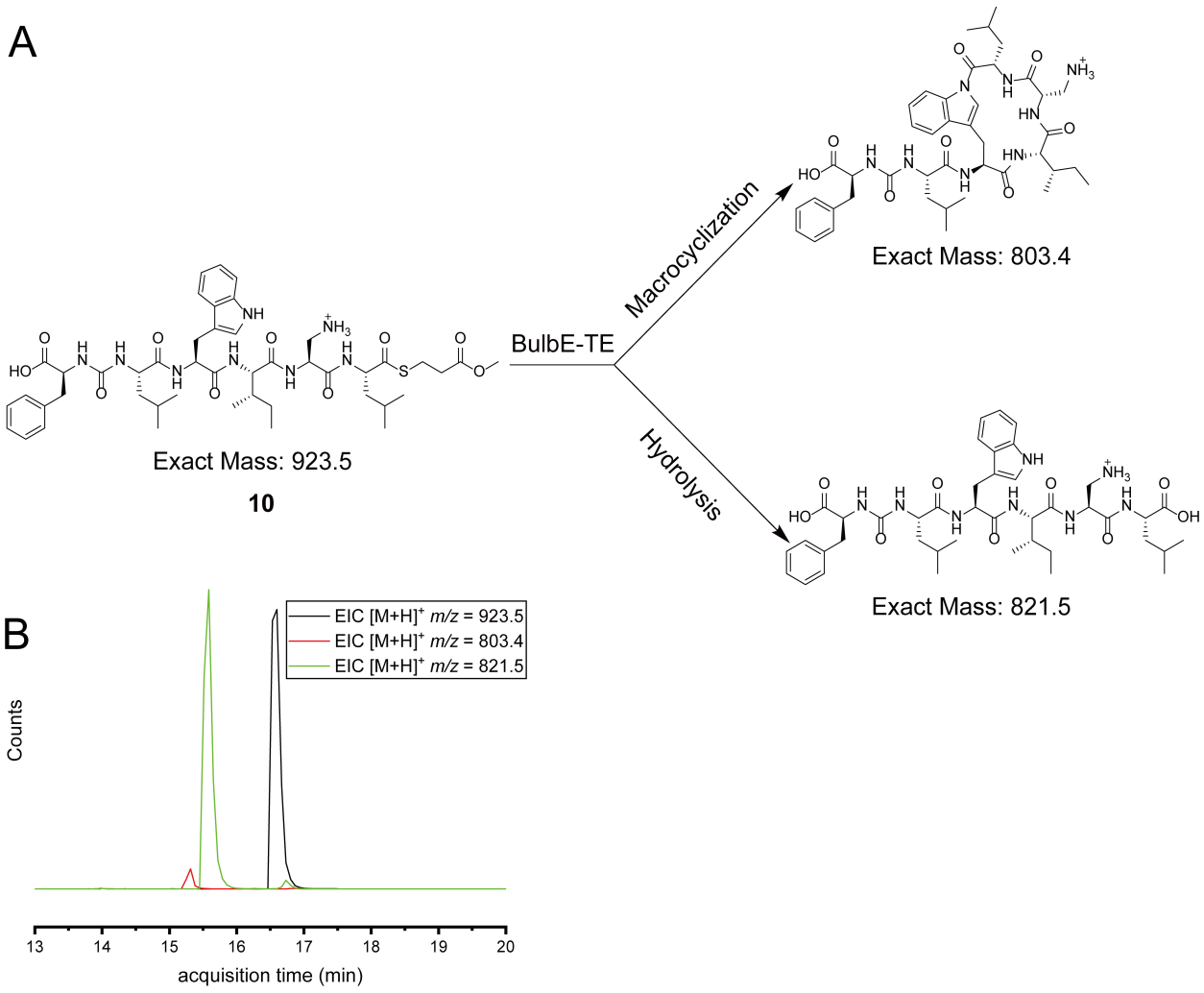


Figure S26: (**A**) Enzymatic reaction of Arg→Dap substrate **10** with BulbE-TE yielded cyclic and hydrolysis products. (**B**) EICs showing the presence of the substrate **10** (black), cyclic product (red), and the thioester hydrolysis product (green).

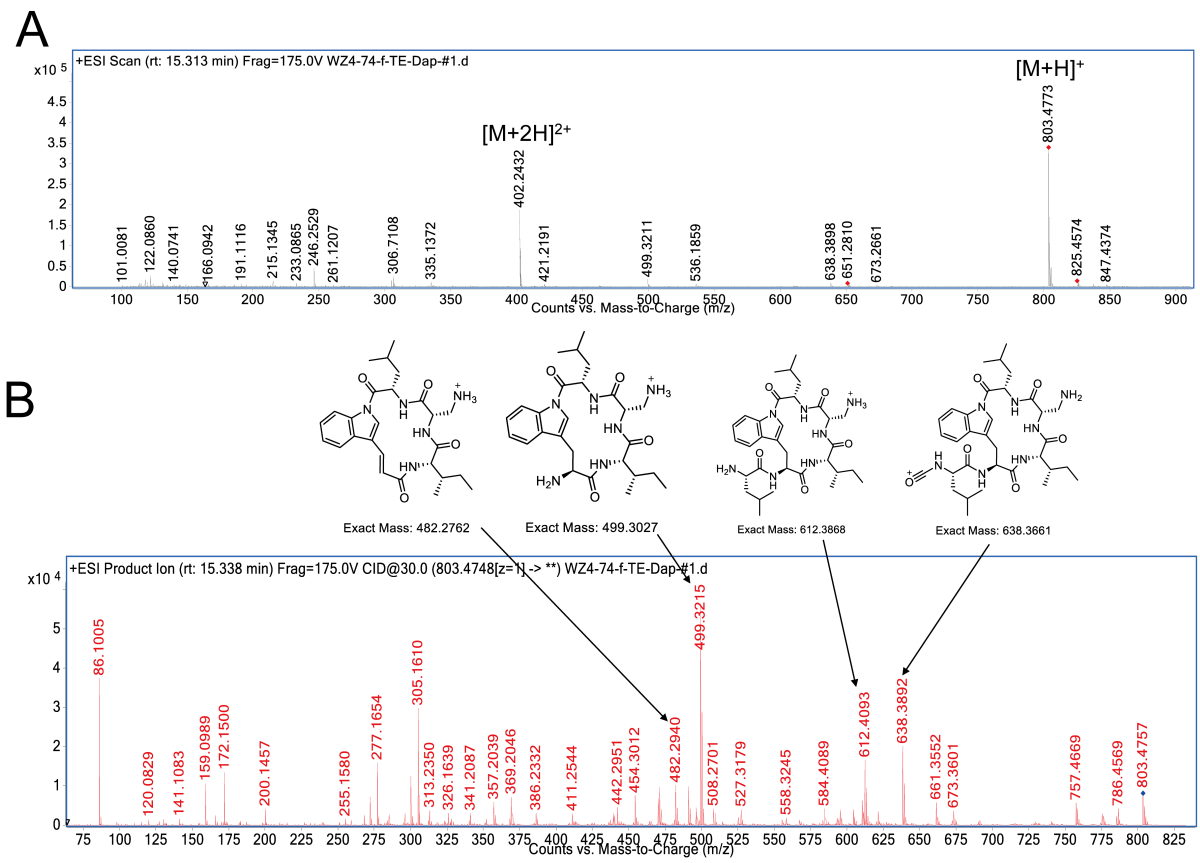


Figure S27: (**A**) MS^1 spectra for the expected macrocyclic product generated by BulbE-TE using **10** as the substrate. Both $[M+H]^{1+}$ and $[M+2H]^{2+}$ ions are observed, as is typical for bulbiferamides. (**B**) MS^2 spectra for the $[M+H]^{1+}$ parent ion with key daughter ions structurally annotated.

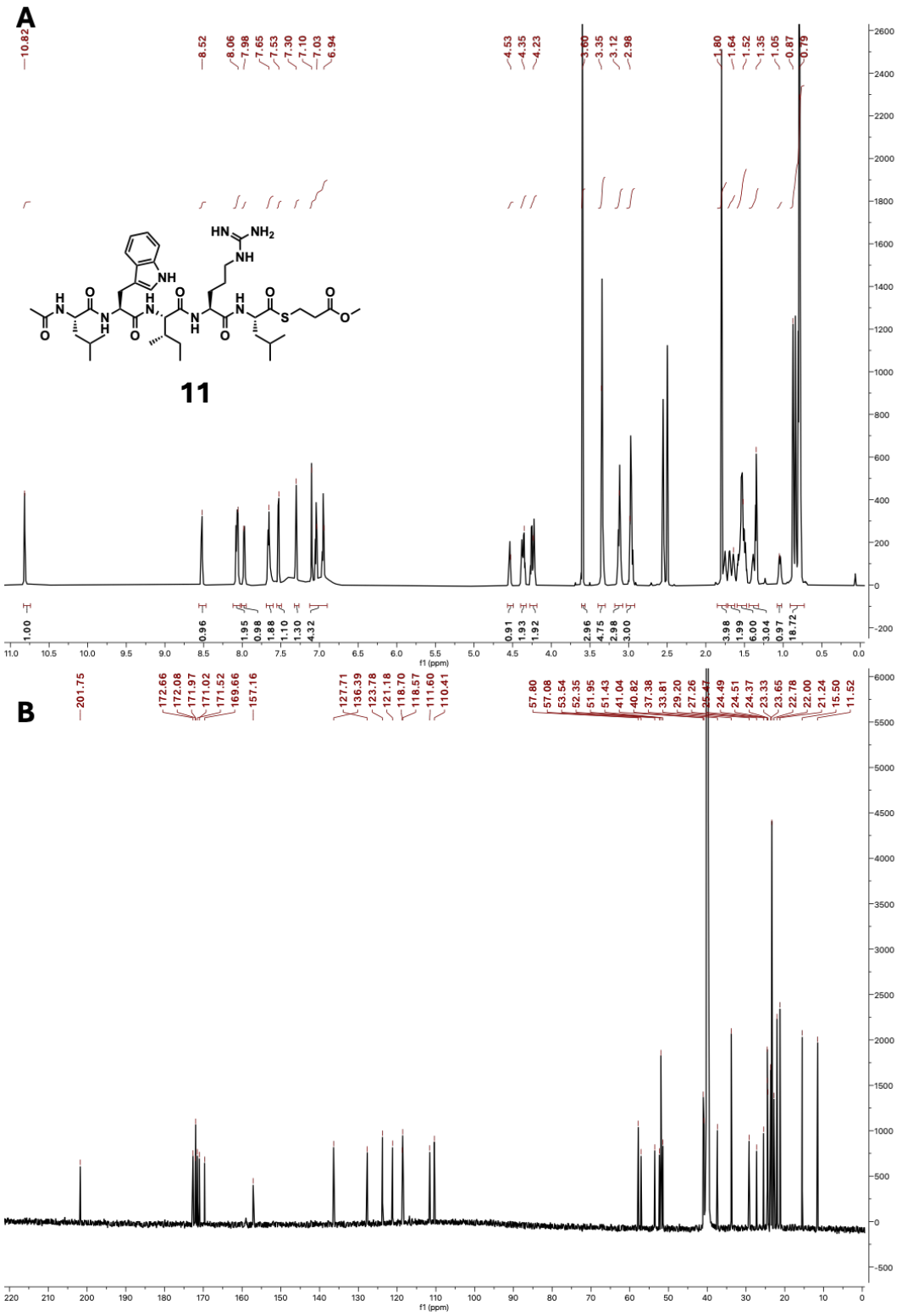


Figure S28: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **11** in DMSO-d_6.

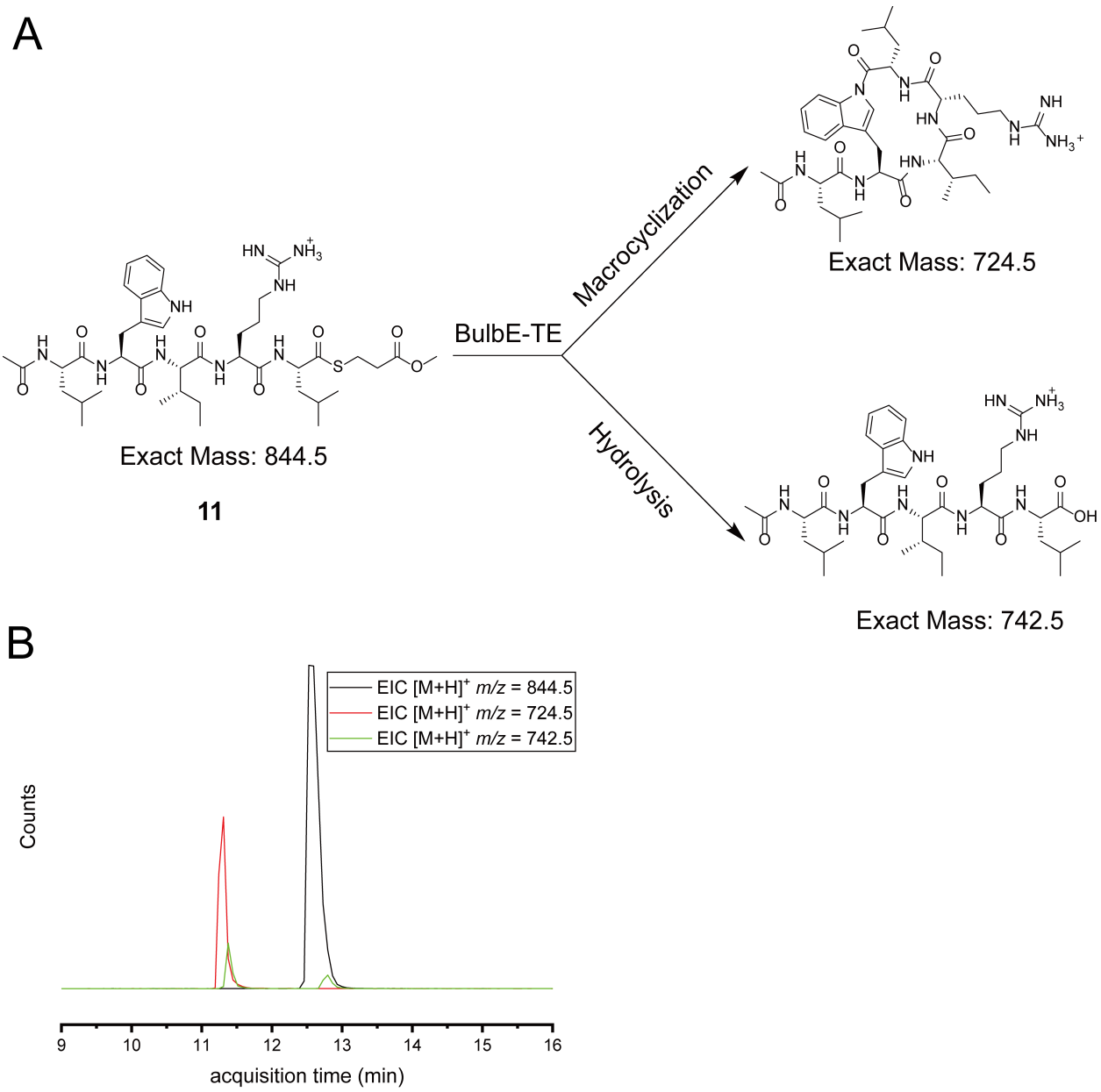


Figure S29: (**A**) Enzymatic reaction of substrate **11** with BulbE-TE yielded cyclic and hydrolysis products. (**B**) EICs showing the presence of the substrate **11** (black), cyclic product (red), and the thioester hydrolysis product (green).

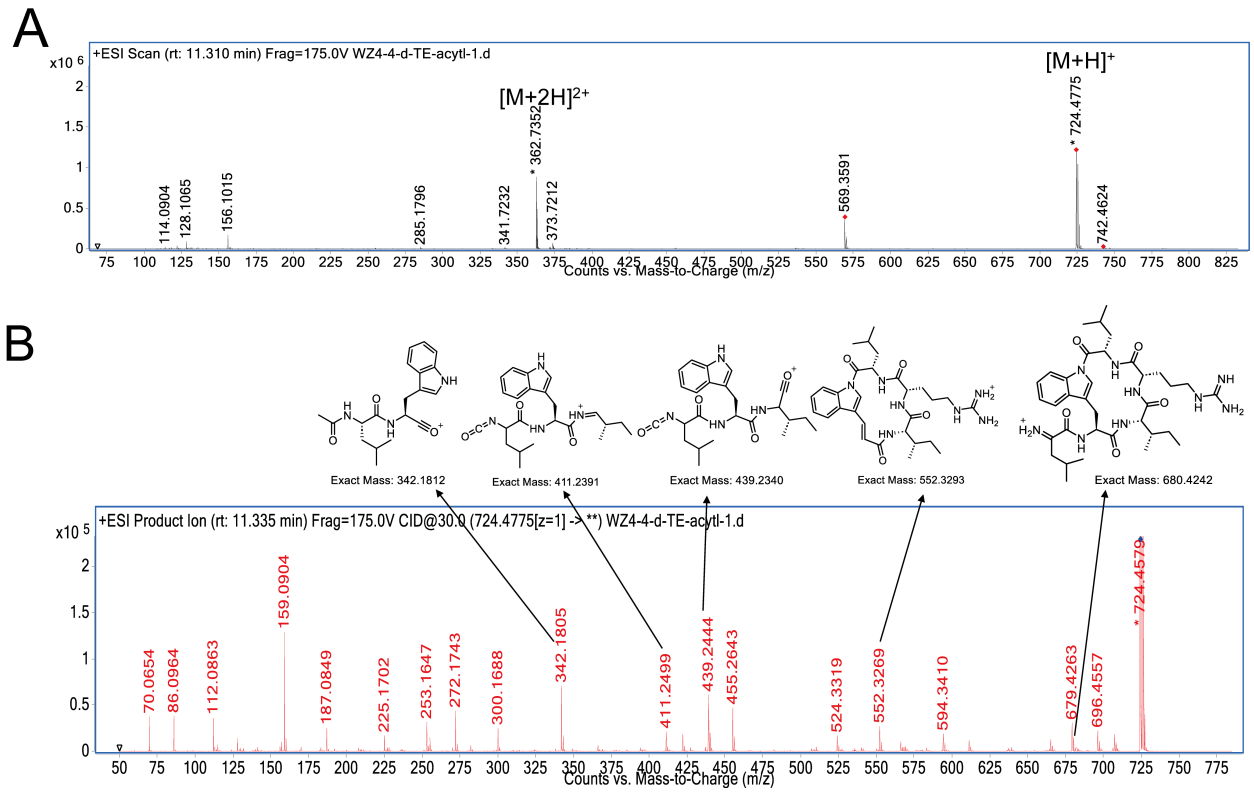


Figure S30: (**A**) MS^1 spectra for the expected macrocyclic product generated by BulbE-TE using **11** as the substrate. Both $[M+H]^{1+}$ and $[M+2H]^{2+}$ ions are observed, as is typical for bulbiferamides. (**B**) MS^2 spectra for the $[M+H]^{1+}$ parent ion with key daughter ions structurally annotated.

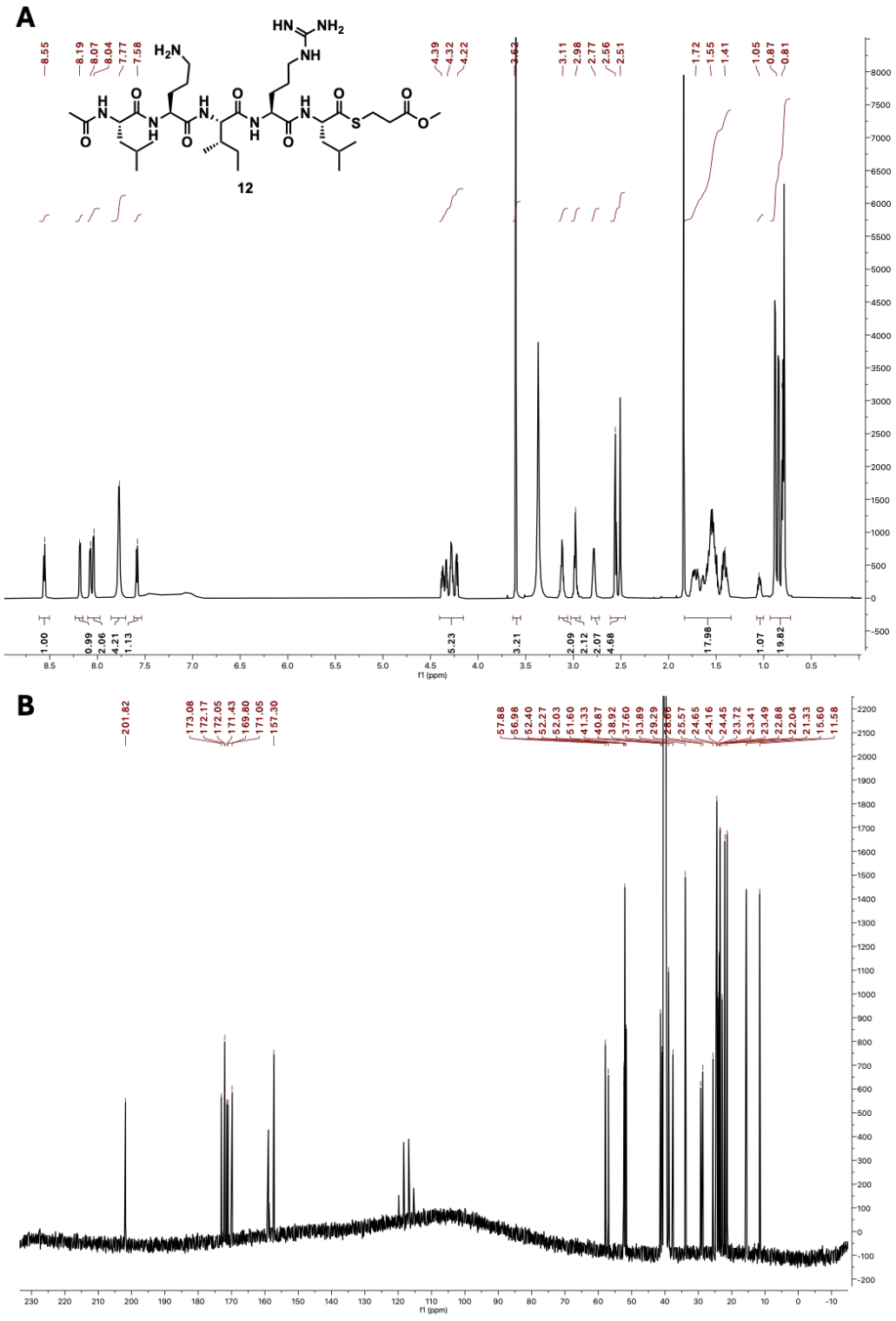


Figure S31: (**A**) ^{1}H (800 MHz) and (**B**) ^{13}C (201 MHz) NMR spectra of **12** in DMSO-d_6.

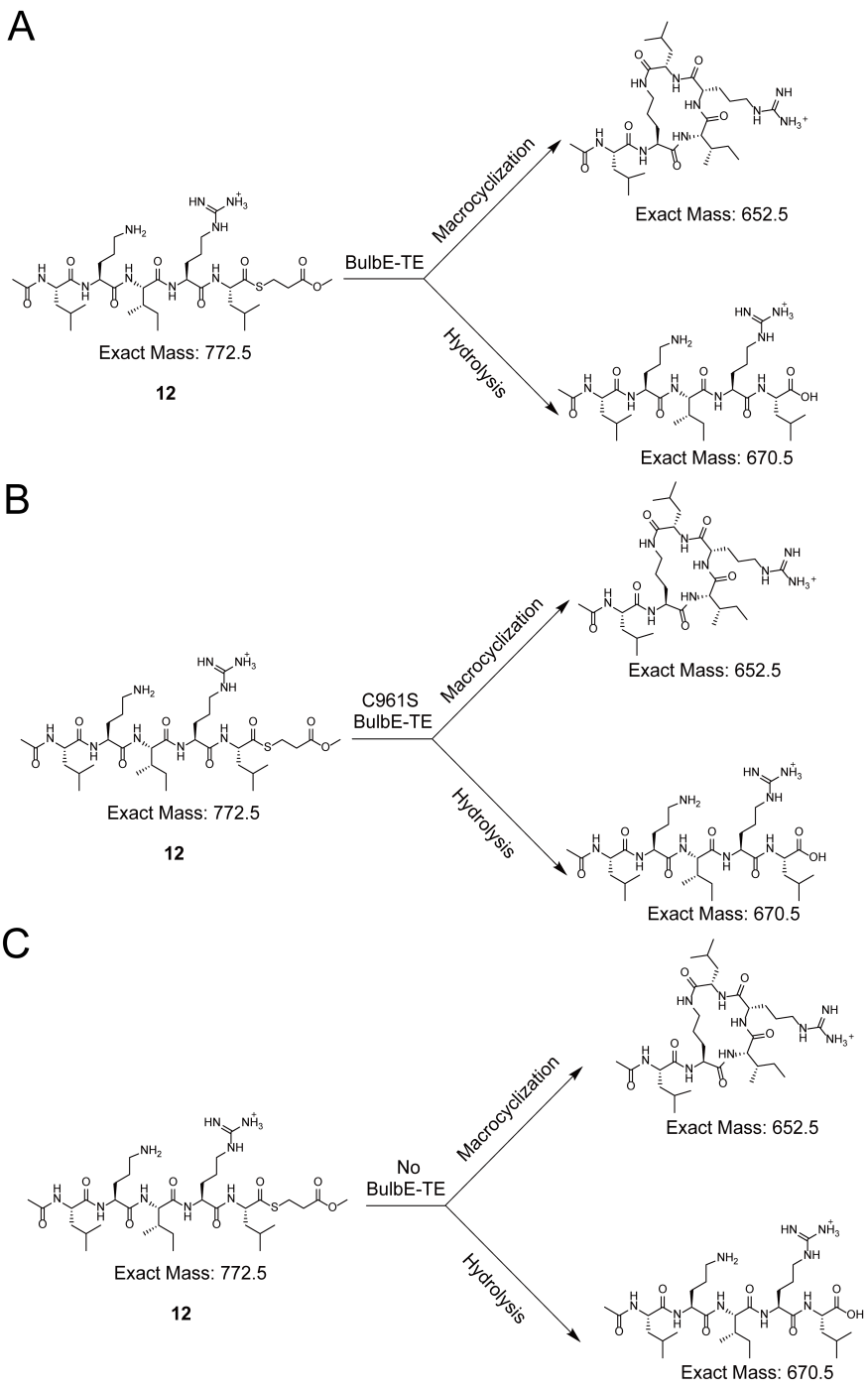


Figure S32: (**A**) Scheme for enzymatic reaction of substrate **12** with BulbE-TE to yield cyclic product and a thioester hydrolysis product. (**B**) Scheme for enzymatic reaction of substrate **12** with C961S BulbE-TE to yield cyclic product and a thioester hydrolysis product. (**C**) Scheme for a negative control reaction in which the enzyme is omitted.

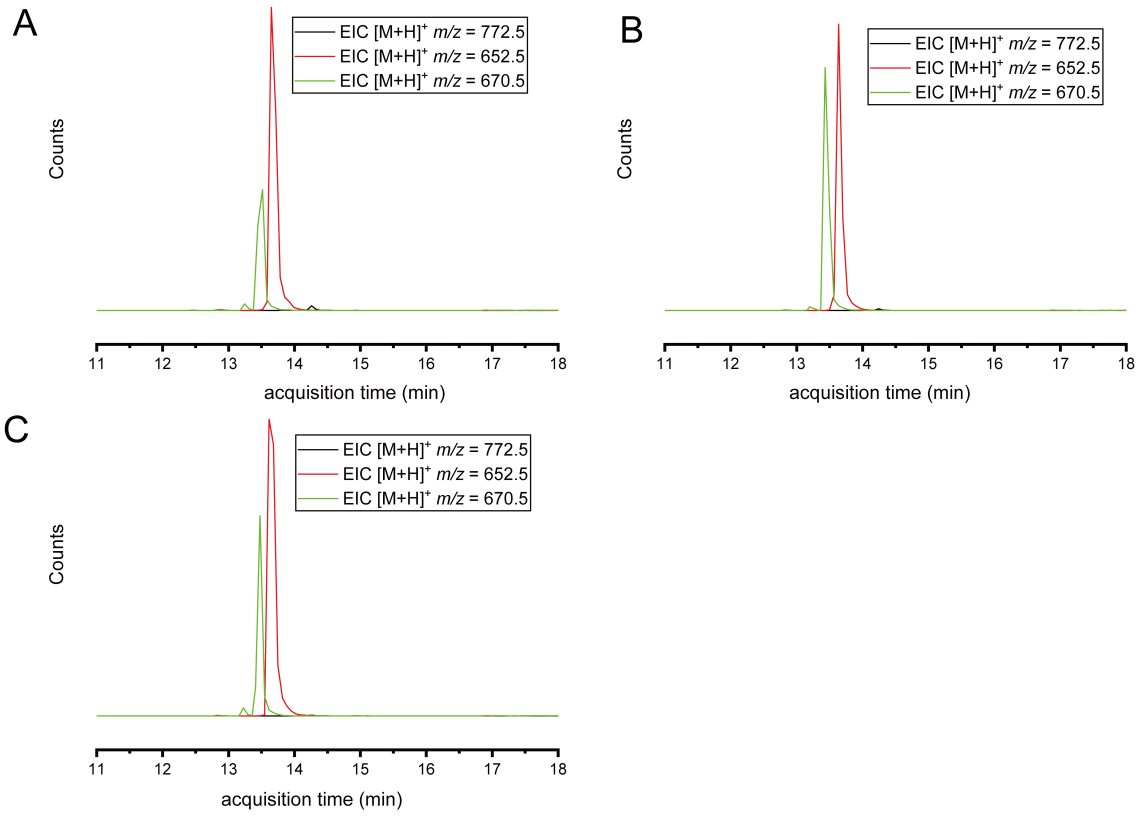


Figure S33: (**A**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** with BulbE-TE at pH 7.5. (**B**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** with C961S BulbE-TE at pH 7.5. (**C**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** without enzyme at pH 7.5.

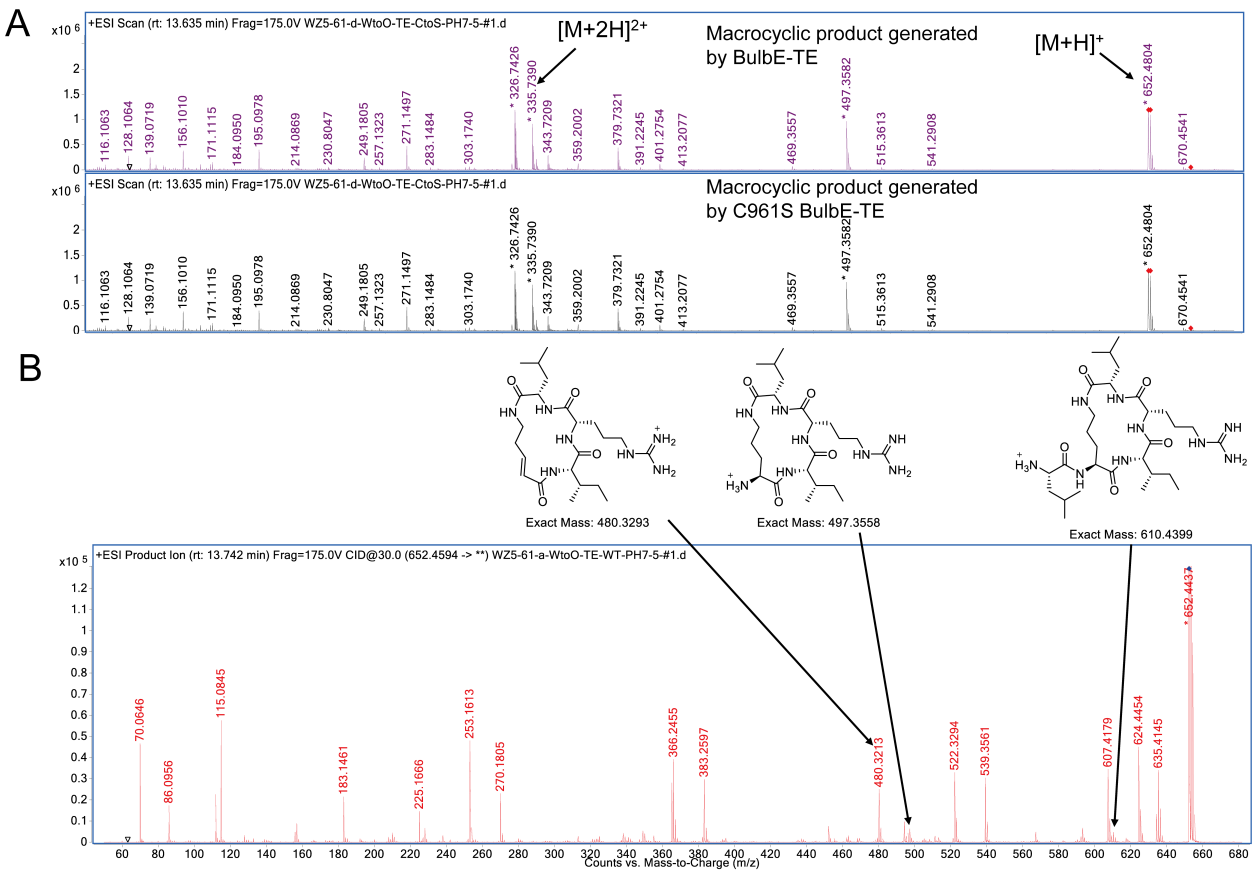


Figure S34: (**A**) MS^1 spectra for the expected macrocyclic product generated by BulbE-TE (top) and C961S BulbE-TE (bottom) using **12** as the substrate. Both $[M+H]^{1+}$ and $[M+2H]^{2+}$ ions are observed, as is typical for bulbiferamides. (**B**) MS^2 spectra for the $[M+H]^{1+}$ parent ion with key daughter ions structurally annotated for the macrocyclic product.

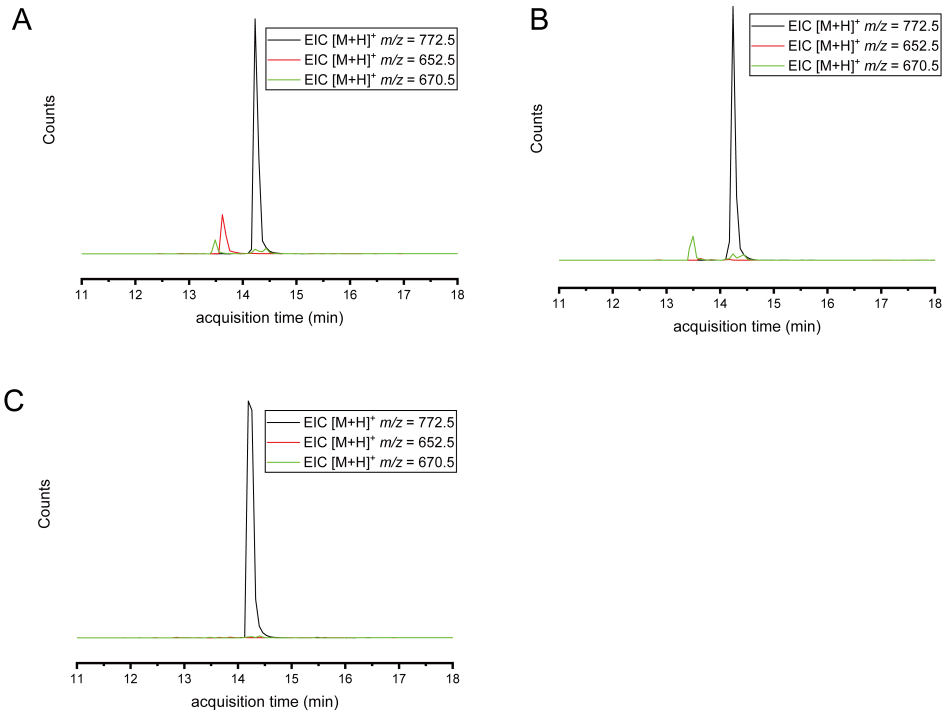


Figure S35: (**A**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** with BulbE-TE at pH 6.0. (**B**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** with C961S BulbE-TE at pH 6.0. (**C**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** with C961S without enzyme at pH 6.0.

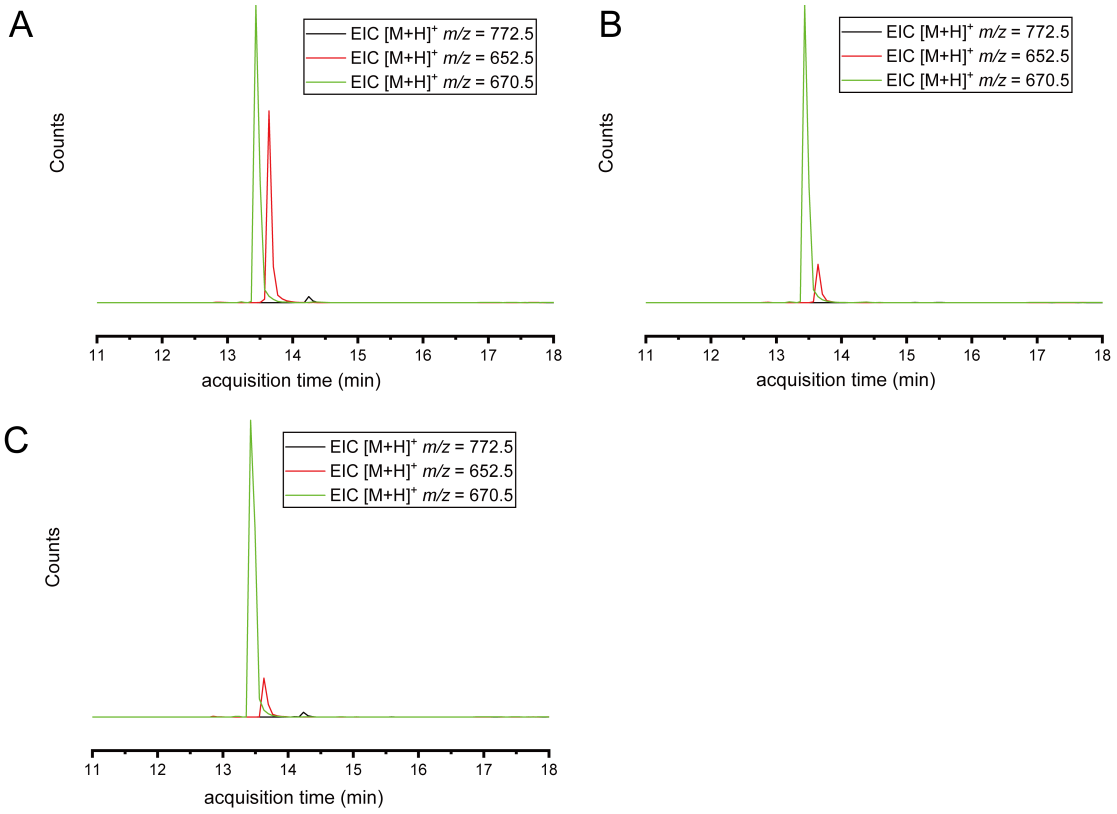


Figure S36: (**A**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** with BulbE-TE at pH 9.0. (**B**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** with C961S BulbE-TE at pH 9.0. (**C**) EICs showing the presence of the substrate **12** (black), cyclic product (red), and the thioester hydrolysis product (green) in enzymatic reaction substrate **12** without enzyme at pH 9.0.

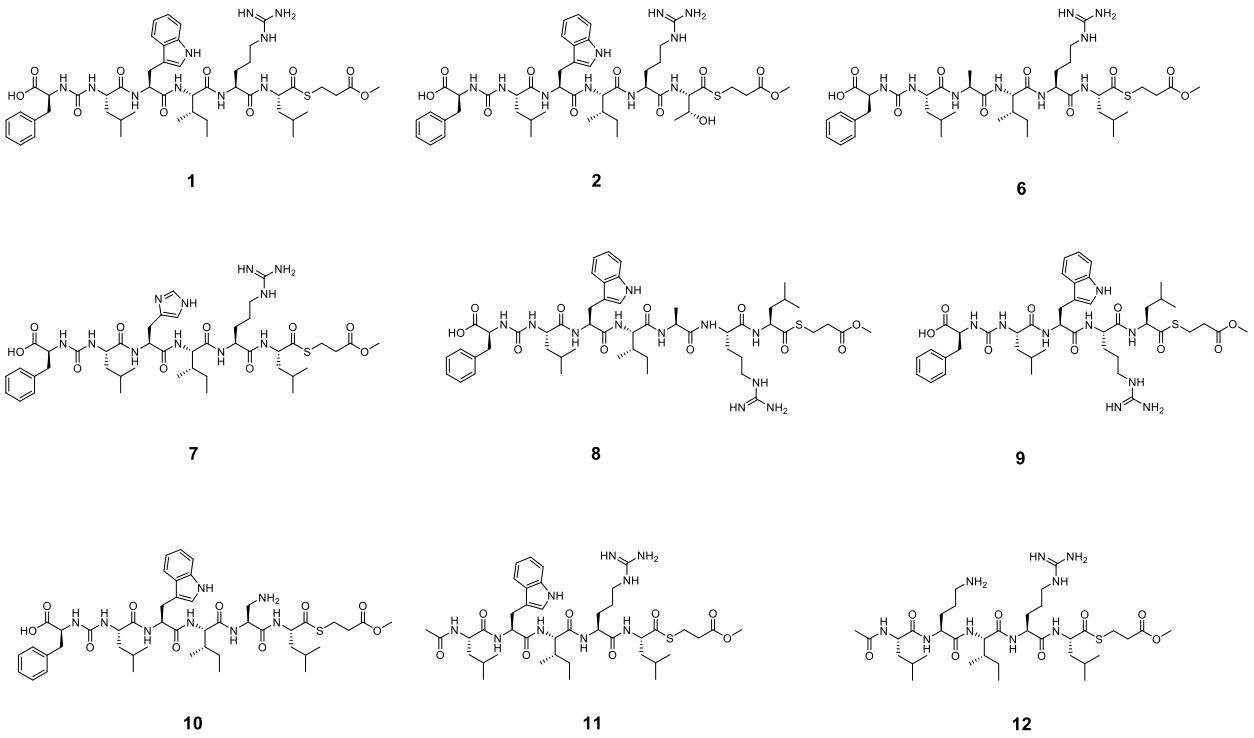


Figure S37: Chemical structures of substrates tested in this study.

SUPPLEMENTARY REFERENCES

(1) Blanco-Canosa, J. B.; Nardone, B.; Albericio, F.; Dawson, P. E. Chemical Protein Synthesis Using a Second-generation N-acylurea Linker for the Preparation of Peptide-thioester Precursors. *J. Am. Chem. Soc.* **2015**, *137*, 7197–7209.