SUPPORTING INFORMATION

Peptide Macrocyclization Inspired by Non-Ribosomal Imine Natural Products

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Methods for peptide synthesis

Analytical reverse-phase HPLC was performed on a Hitachi D-7000 separations module equipped with a L-4500A photodiode array detector. Peptides were analyzed using a Vydac 218TP54 Protein & Peptide C18 column (5 μm, 4.6 mm x 250 mm) at a flow rate of 1.5 mL min^{-1} using a mobile phase of 99% water/1% acetonitrile containing 0.1% TFA (Solvent A) and 10% water/90% acetonitrile containing 0.07% TFA (Solvent B). Results were analyzed using Hitachi Model D-7000 Chromatography Data Station Software. Low-resolution mass spectra (LRMS) and reaction monitoring were recorded on an Agilent LCMS TOF mass spectrometer using electrospray ionization time-of-flight (ESI-TOF) reflectron experiments.

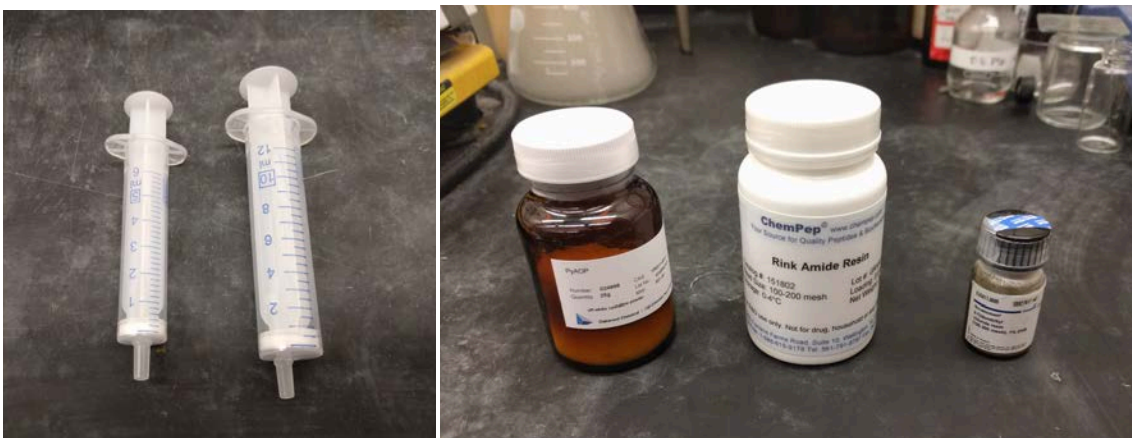
Preparative reverse-phase HPLC was performed using a Hitachi system comprised of an L-7150 pump and L-4000 programmable UV detector operating at a wavelength of 230 nm coupled to a Hitachi D-2500 Chromato-Integrator. Peptides were purified on a Thermo Scientific Bio-basic C18 10 μm preparative column operating at a flow rate of 12 mL min^{-1} using a mobile phase of 99% water/1% acetonitrile containing 0.1% TFA (Solvent A) and 10% water/90% acetonitrile containing 0.07% TFA (Solvent B) and a linear gradient as specified. Peptides were isolated as white solids (unless otherwise noted) following lyophilization.

Several peptide aldehydes were prepared using an Advanced ChemTech Apex 396 DCFWM automated peptide synthesizer. Reaction set-up, progress, and analysis were monitored by Aaptec Multiple Organic Synthesizer (Version 1.60.17T) software. An Innova 2000 portable platform shaker (operating at 145 rpm) was used for the general mixing and agitation of solid-phase reactions.

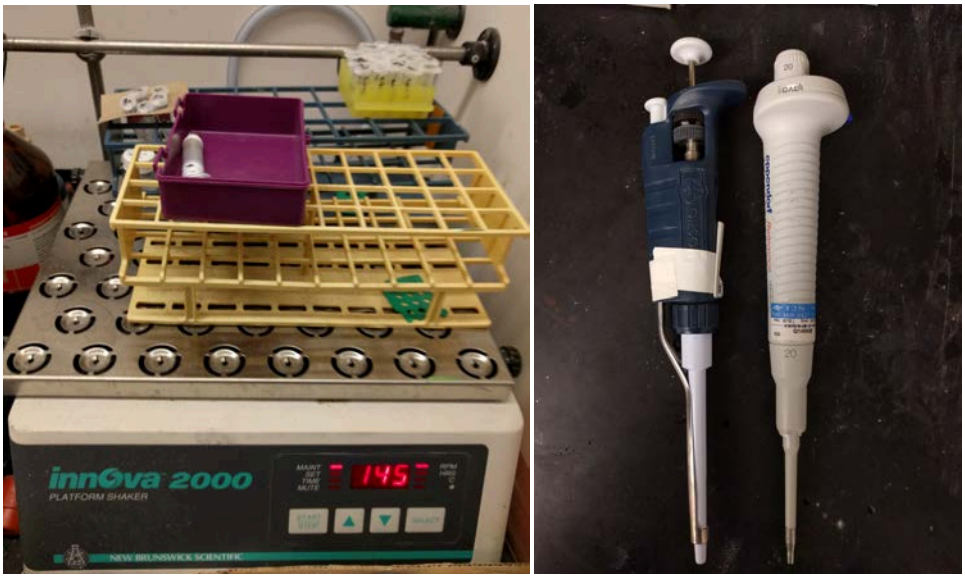
Materials

Commercial materials were used as received unless otherwise noted. Amino acids and coupling reagents were obtained from Novabiochem or Combi-blocks. Rink amide resin (0.8 mmol/g) was purchased from Chempep. Solid-phase reaction vessels and pressure caps were purchased from Torviq. Reagents that were not commercially available were synthesized following literature procedures.

General Pictorial Supporting Information



(Left) Solid-phase reaction vessels purchased from Torviq. **(Right)** PyAOP coupling reagent and commercially available resins (Chempep Rink amide resin and Novabiochem 2-chlorotrityl chloride resin).

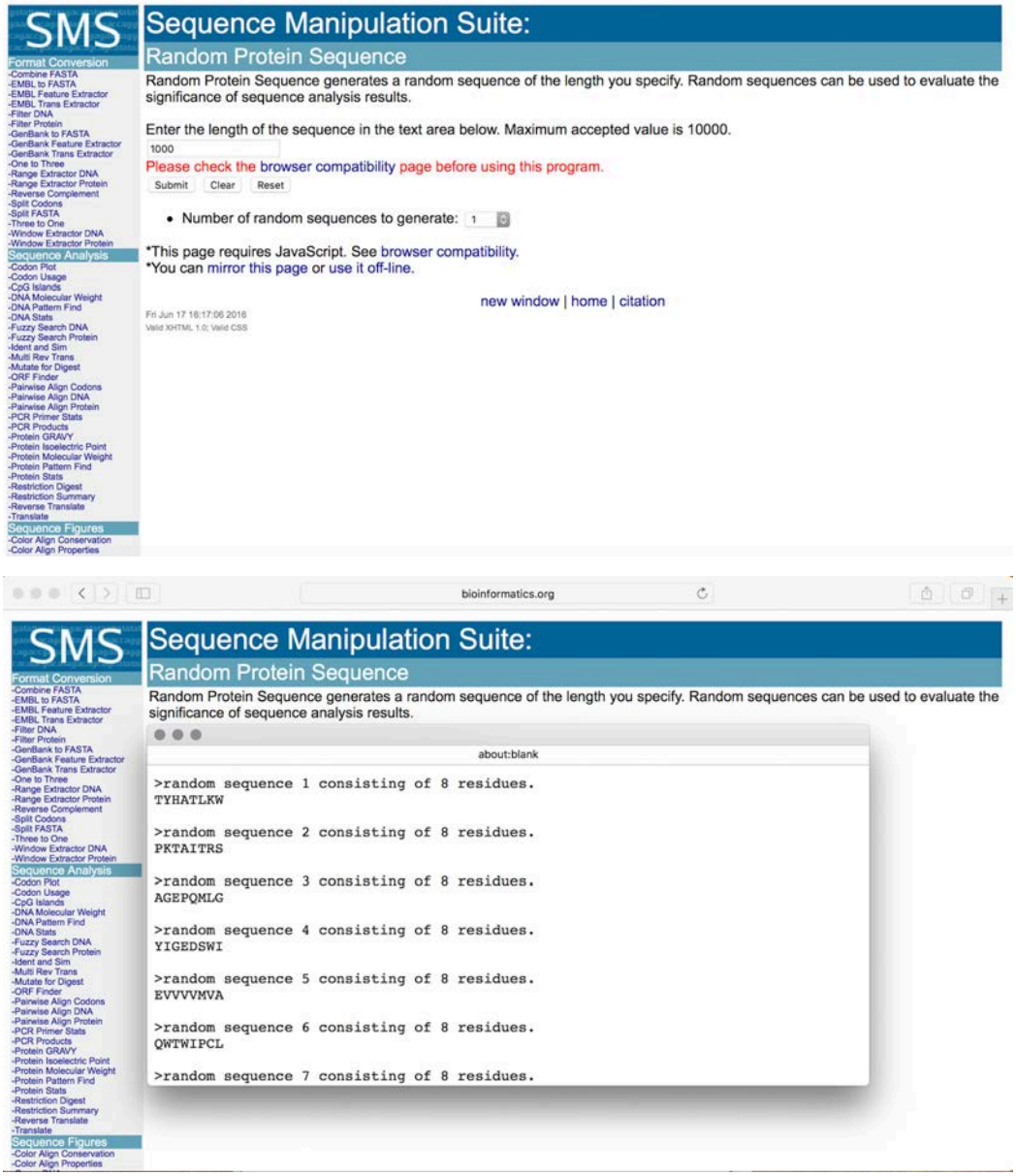


(Left) Orbital shaker for solid-phase peptide synthesis (SPPS). **(Right)** Adjustable pipettes (200 µL and 20 µL) for reaction set-up.

Random Peptide Sequence Generator

In an effort to preclude any bias toward preorganization in our selection of substrates, the *Random Protein Sequence* tool* was used to design indiscriminate residue sequences.

URL: http://www.bioinformatics.org/sms2/random_protein.html

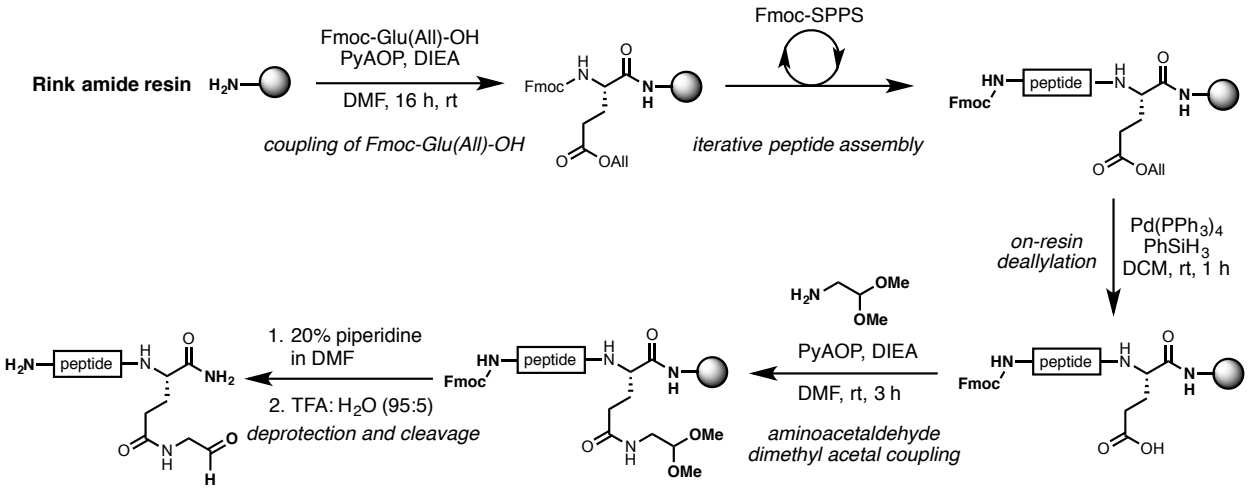


(Top) Screen shot of the *Random Protein Sequence* website homepage. **(Bottom)** Example of the sequence generator at work (number of residues: 8, number of sequences: 50).

*The Sequence Manipulation Suite © 2000, 2004 Paul Stothard

Solid-phase peptide synthesis

General procedure 1. Rink amide resin (glycinal series)



Preloading Rink amide resin – Coupling of Fmoc-Glu(All)-OH

Rink amide resin (1.0 equiv., substitution = 0.8 mmol/g) was swollen in dry DCM for 30 min then washed with DCM (5 x 3 mL) and DMF (5 x 3 mL). A solution of the Fmoc-Glu(All)-OH (4.0 equiv.), PyAOP (4.0 equiv.) and *N*,*N*-diisopropylethylamine (DIEA, 8.0 equiv.) in DMF (final concentration 0.1 M) was added to the resin (1.0 equiv.) and agitated at room temperature. After 16 h, the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL). A capping step was performed as described below and the resin-bound residue was submitted to iterative peptide assembly (Fmoc-SPPS).

The loading efficiency was evaluated through treatment of the resin with 20% piperidine/DMF (3 mL, 2 × 3 min) to deprotect the Fmoc group. The combined deprotection solutions were diluted to 10 mL with 20% piperidine/DMF. An aliquot of this mixture (50 μL) was diluted 200-fold with 20% piperidine/DMF and the UV absorbance of the piperidine-fulvene adduct was measured (λ = 301 nm, ε = 7800 M^{-1} cm^{-1}) to quantify the amount of amino acid loaded onto the resin. The theoretical maximum for the reported yields of all isolated peptides are based on the numerical value obtained from the resin loading.

General iterative peptide assembly (Fmoc-SPPS)

Peptides were elongated using iterative Fmoc-solid-phase peptide synthesis (Fmoc-SPPS), according to the following general protocols:

Deprotection: The resin was treated with 20% piperidine/DMF (3 mL, 2 x 3 min) and washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

General amino acid coupling: A preactivated solution of protected amino acid (4 equiv.), PyBOP (4 equiv.), and *N*-methylmorpholine (NMM) (8 equiv.) in DMF (final concentration 0.1 M) was added to the resin. After 1 h, the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Capping: Acetic anhydride/pyridine (1:9 v/v) was added to the resin (3 mL). After 3 min the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

On-resin deallylation

A solution of $Pd(PPh_3)_4$ (0.88 equiv.) and $PhSiH_3$ (40 equiv.) in dry DCM (final concentration of 0.1 M with respect to resin) was added to the resin (1 equiv.). The resin was shaken for 1 h and the progress of the reaction checked by cleavage of a small portion of resin beads and LC-MS analysis. The procedure was repeated if necessary, and upon completion, the resin was washed with DCM (10x 3 mL) and DMF (10 x 3 mL). To remove residual Pd from the solid support, the resin-bound peptide was washed (2 x 15 min) with a solution of sodium dimethyldithiocarbamate hydrate (0.02 M in DMF). Following Pd removal, the resin was washed with DMF (5 x 3 mL) and DCM (5 x 3 mL).

Aminoacetaldehyde dimethyl acetal coupling

A solution of aminoacetaldehyde dimethyl acetal (10 equiv.), PyAOP (10 equiv.), and DIEA (20 equiv.) in dry DMF (final concentration of 0.1 M with respect to resin) was added to the resin (1 equiv.). The resin was shaken for 3 h and the progress of the reaction checked by cleavage of a small portion of resin beads and LC-MS analysis. The procedure was repeated if necessary and,

upon completion, the resin was washed with DMF (10 x 3 mL), DCM (10 x 3 mL), and DMF (10 x 3 mL).

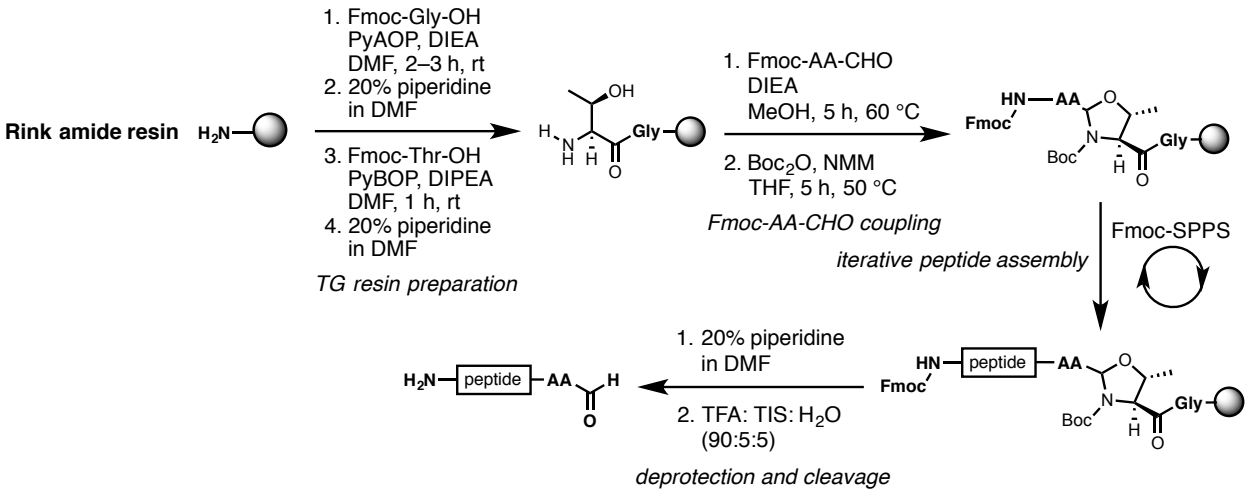
Coupling conditions for Boc-Sec-OH (for the synthesis of selenopeptide dimers)

A solution of Boc-Sec-OH dimer (2.0 eq.), HOAt (4.0 eq.) and DIC (4.0 eq.) in DMF (final concentration 0.1 M) was added to the resin (1.0 eq.) and shaken at rt. After 3 h, the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL).

Cleavage: A mixture of TFA and water (95:5 v/v) was added to the resin. After 2 h, the resin was washed with TFA (3 x 2 mL) and DCM (3 x 2 mL). Note: The scavenger triisopropylsilane (TIS) was excluded from the cleavage mixture to prevent unwanted reduction of the aldehyde.

Work-up: The combined cleavage solution and TFA and DCM washes were concentrated under a stream of nitrogen. The residue was treated with cold Et_2O to precipitate the crude peptide, which was subsequently dissolved in water/acetonitrile containing 0.1% TFA, filtered and purified by reverse-phase HPLC.

General procedure 2. Rink TG resin (α-amino acid series)



Preloading Rink TG resin – Coupling of Fmoc-AA-CHO

Rink amide resin (1.0 equiv., substitution = 0.8 mmol/g) was swollen in dry DCM for 30 min then washed with DCM (5 x 3 mL) and DMF (5 x 3 mL). A solution of Fmoc-Gly-OH (4.0

equiv.), PyAOP (4.0 equiv.), and *N*-methylmorpholine (NMM) (8.0 equiv.) in DMF (final concentration of 0.1 M) was added and the resin agitated on an orbital shaker at rt for 2–3 h. The resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL) and capped with a solution of acetic anhydride/pyridine (1:9 v/v, 3 mL) for 10 min. The resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL), then treated with 20% piperidine/DMF (3 mL, 2 x 3 min) and washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL) to effect Fmoc-deprotection. A preactivated solution of Fmoc-Thr-OH (4.0 equiv.), PyBOP (4.0 equiv.) and *N*-methylmorpholine (NMM) (8 equiv.) in DMF (final concentration 0.1 M) was added to the resin. After 1 h, the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL). The Fmoc group was removed by treatment with 20% piperidine/DMF (3 mL, 2 x 3 min) and washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Note: The capping step was not performed following Fmoc-Thr-OH coupling to avoid potential acetylation of the free threonine hydroxyl side-chain.

Fmoc-AA-CHO was prepared according to literature procedure.[1] A solution of Fmoc-AA-CHO (4.0 equiv.) and DIEA (1% v/v with respect to MeOH) in MeOH (final concentration of 0.1 M with respect to the resin) was added to the resin and the resulting mixture was agitated at 60 °C for 5 h. The resin was then washed with MeOH (5 x 3 mL), DMF (5 x 3 mL), DCM (5 x 3 mL), and THF (5 x 3 mL). A solution of Boc_2O (5.0 equiv.) and NMM (5.0 equiv.) in THF (final concentration of 0.1 M with respect to the resin) was added to the resin and agitated at 50 °C for 5 h. The resin was washed with THF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL). The loading efficiency was evaluated as described in general procedure 1. The resin-bound residue was submitted to iterative peptide assembly (Fmoc-SPPS), either manually (as described above) or through preparation on an automated peptide synthesizer.

Automated Peptide Synthesis: Each of the required Fmoc-AA-OHs were added to labeled 50 mL centrifuge tubes. The amino acids were dissolved in a stock solution of 0.4 M OxymaPure® (Ethyl (hydroxyimino)cyanoacetate) in *N*-methylpyrrolidinone (NMP). A stock solution 1.2 M of *N*,*N'*-diisopropylcarbodiimide (DIC) in NMP was prepared for coupling reactions, which were

run at ca. 6.4 fold excess of amino acid relative to the resin-bound peptide. A separate stock solution of 30% pyrrolidine in NMP was prepared to effect Fmoc-deprotection. The dried resin was added to the instrument well plate (70 μmol per well), which was kept under a slow stream of N_2 gas for the duration of the synthesis. The resin-bound peptide was then submitted to a reswelling period, followed by sequential deprotection–coupling reactions (ca. 2 hours per residue); progress was monitored by Aaptec Multiple Organic Synthesizer software. No capping steps were performed. On termination, the resin was transferred to a disposable PP reaction vessel, washed, and subjected to resin cleavage, as described below.

Note: Throughout the text, general procedure 2a refers to manual elongation of the peptide amino aldehydes; general procedure 2b refers to automated elongation.

Cleavage: A mixture of TFA/TIS/water (90:5:5 v/v/v) was added to the resin. After 2 h, the resin was washed with TFA (3 x 2 mL) and DCM (3 x 2 mL). *Note: With Rink TG resin, reduction of the C-terminal aldehyde was not observed in the presence of the scavenger triisopropylsilane (TIS).*

Work-up: The combined cleavage solution and TFA and DCM washes were concentrated under a stream of nitrogen. The residue was treated with cold Et_2O to precipitate the crude peptide, which was subsequently dissolved in water/acetonitrile containing 0.1% TFA, filtered and purified by reverse-phase HPLC.

The theoretical maximum for the reported yields of all isolated peptides are based on the numerical value obtained from the resin loading.

Notes on aldehyde synthesis:

- Although a commercial NovaSyn TG resin is available, all described C-terminal aldehydes were constructed on a manually prepared Rink amide-based TG resin. Qualitative assessments indicate that the loading efficiency—and accordingly, isolated yields—are significantly higher with this resin linker, and no detrimental effect on peptide purity was observed, except where otherwise noted. Similarly, cost comparisons against the commercial variant reveal economic superiority.

- Oligomerization of the purified peptide aldehydes was observed on standing at reduced temperatures. As such, for ease of analysis, it is recommended that the aldehydes be used within one week of purification. Nonetheless, due to the reversible nature of the imine formation, oligomerized starting materials can, in principle, be employed (for a more detailed examination of this phenomenon, refer to the one-pot preparation of β-carboline **30**).
- Epimerization of C-terminal aldehydes has been documented in the literature upon loading onto TG resin and during routine peptide purifications.[2] In the majority of our synthetic amino aldehydes prepared on Rink TG resin, however, we did not observe epimerization upon analysis of the purified peptide aldehydes by analytical HPLC and NMR. One notable exception was in the preparation of peptide **S12** (a model pentapeptide bearing a D-Leu aldehyde), in which we isolated small amounts of the C-terminal epimer following purification. Interestingly, in the case of the natural product imines, which are thought to spontaneously self-assemble following reductive cleavage from the NRPS, there is likely a self-correcting mechanism (thermodynamic equilibration) that leads to the cyclization of a single C-terminal configuration. Such configurational equilibration in imine macrocyclization processes has previously been observed by Marahiel and coworkers.[3, 4]

Macrocyclization Protocols

General procedure 3. Strecker macrocyclization

To a solution of purified peptide aldehyde in deionized water (final concentration of 1 mM) was added KCN (1.2 equiv.). The resulting solution was stirred at room temperature for 24–48 h (unless otherwise noted) until consumption of starting material, as monitored by LC-MS analysis. The reaction was purified by preparative reverse-phase HPLC (direct inject of crude reaction mixture; eluent as noted) to afford the peptide macrocycle following lyophilization.

Notes on Strecker macrocyclizations:

- Due to the standard scale of the described reactions, KCN was typically added as a stock solution.

- As with most macrocyclization protocols, high dilution conditions are used to prevent unwanted oligomerization. See p. S22 for a thorough concentration study of the related reductive amination reaction.
- Competitive nucleophilic addition to the aldehyde, resulting in formation of the corresponding linear cyanohydrin, accounts for the majority of the mass balance. In some cases, the reversible addition of cyanide to the aldehyde allows for the gradual funneling of material to the thermodynamically more stable α-aminonitrile. This feature of the equilibrium cyclization process is advantageous in slow macrocyclization reactions. In methods that require preactivation (e.g. activated ester formation in traditional amide couplings), competitive hydrolysis generally limits the yield and efficiency of such macrocyclizations.
- Care should be taken when handling the Strecker macrocyclization products in aqueous acidic media (e.g. HPLC buffers containing TFA), as gradual hydrolysis of the α-aminonitrile to regenerate the linear amino aldehyde was observed in some cases. Strecker products should ideally be stored dry at –20 ^{o}C to ensure long-term stability.

General Procedure 4. Reductive amination

To a solution of purified peptide aldehyde (final concentration of 1 mM) in NaOAc/AcOH buffer (0.4 M, pH = 5.5, unless otherwise noted) was added $NaBH_3CN$ (10 equiv.). The resulting solution was stirred at room temperature for 8 – 24 h, until consumption of starting material, as monitored by LC-MS analysis. The reaction was purified by preparative reverse-phase HPLC (direct inject of crude reaction mixture; eluent as noted) to afford the peptide macrocycle following lyophilization.

Notes on reductive amination:

- Due to the standard scale of the described reactions, $NaBH_3CN$ was typically added as a stock solution.
- Competitive reduction of the linear aldehyde is a common byproduct observed in the reductive amination reactions, particularly in peptides that are less predisposed to cyclize. The amount of aldehyde reduction was also found to be highly pH dependent. A brief

study on the effect of pH in the synthesis of Lys-containing macrocycle **22** indicated that lower pH resulted in greater aldehyde reduction (see p. S184 for details). However, in some substrates, reduction was more prevalent at elevated pH. As a general rule, moderately acidic conditions (pH = 5.0–5.5) allowed for efficient formation of the desired macrocycles while minimizing the amount of reduced aldehyde observed.

General Procedure 5. Thiazolidine formation

Tris(2-carboxyethyl)phosphine (TCEP, 10 equiv.) was dissolved in Na_2HPO_4 buffer (0.2 M, final pH = 7.0 – 7.5), and the solution was degassed under argon sparge for 10 minutes. The solution was added to purified peptide aldehyde (final concentration of 1 mM) and the resulting solution stirred at room temperature for 6–18 h, until consumption of starting material, as monitored by LC-MS analysis. The reaction was purified by preparative reverse-phase HPLC (direct inject of crude reaction mixture; eluent as noted) to afford the peptide macrocycle following lyophilization.

Notes on thiazolidine formation:

- In order to facilitate thiazolidine formation, the N-terminal Cys residue must be in reduced form. To unleash the reactive thiol from StBu disulfide-protected Cys residues (e.g. **S23**), the phosphine reductant TCEP is added to the reaction mixture. Upon addition, pungent tBuSH is immediately observed.

General Procedure 6. Selenazolidine formation

Tris(2-carboxyethyl)phosphine (TCEP, 10 equiv.) and sodium ascorbate (50 equiv.) were dissolved in Na_2HPO_4 buffer (0.2 M, final pH = 7.0 – 7.5), and the solution was degassed under argon sparge for 10 minutes. The solution was added to the purified peptide aldehyde (final concentration of 1 mM) and the resulting solution stirred at room temperature for 1–2 h, until consumption of starting material, as monitored by LC-MS analysis. The reaction was purified by preparative reverse-phase HPLC (direct inject of crude reaction mixture; eluent as noted) to afford the peptide macrocycle following lyophilization.

Notes on selenazolidine formation:

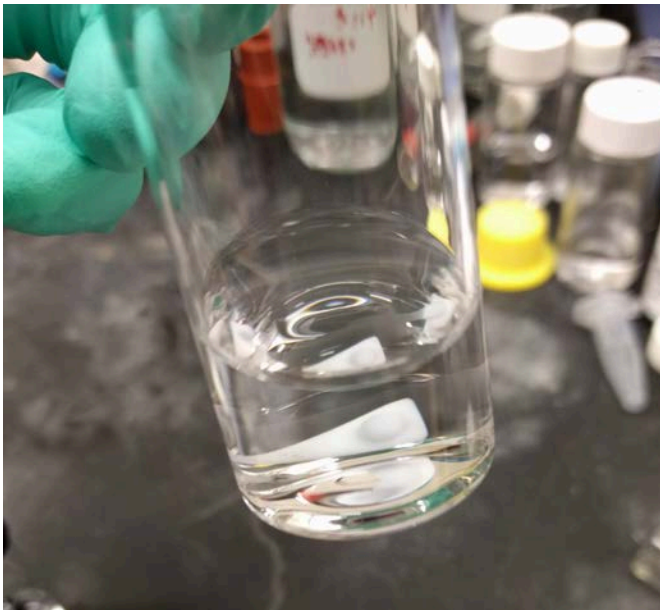
- Selenopeptides exist in solution primarily as the corresponding diselenide dimers. In order to facilitate macrocyclization, the dimer must first be reduced *in situ* by treatment with TCEP. To prevent phosphine-mediated deselenization,[5] sodium ascorbate is added to the reaction mixture.[6, 7]
- The selenazolidine cyclization proceeds much more rapidly than the corresponding thiazolidine cyclization, and generally reaches completion after 1–2 h at rt.
- The selenazolidine product is stable to HPLC purification. However, prolonged exposure to aqueous media may lead to hydrolysis of the selenazolidine to re-form the linear diselenide dimer. The peptide should be stored dry and in the freezer to ensure long-term stability.

Pictorial Supporting Information, Representative Procedures.

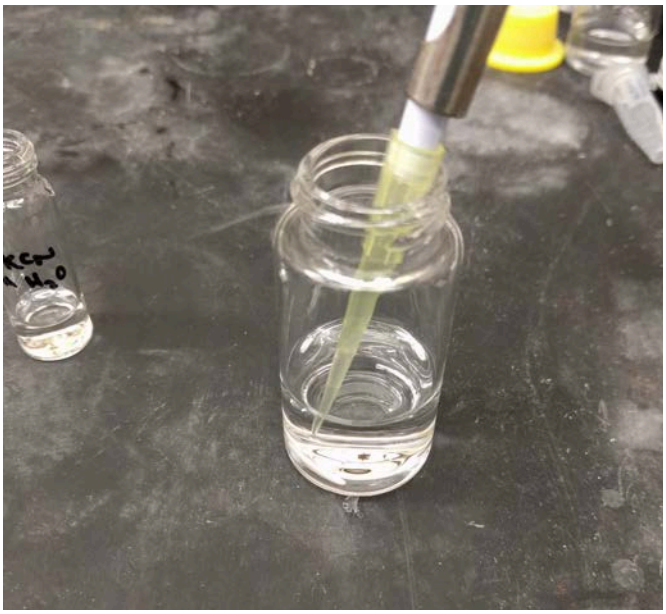
Representative Procedure – Strecker Macrocyclization:

The following pictoral guide is representative of the general techniques employed in each of the macrocyclization reactions described. Reactions are generally carried out in an open flask and in aqueous reaction media, as described in the general procedures.

(Left) Purified peptide amino aldehyde following lyophilization into a 20 mL vial **(Right)** A solution of KCN in water (~1 mg/mL) and the peptide amino aldehyde starting material.

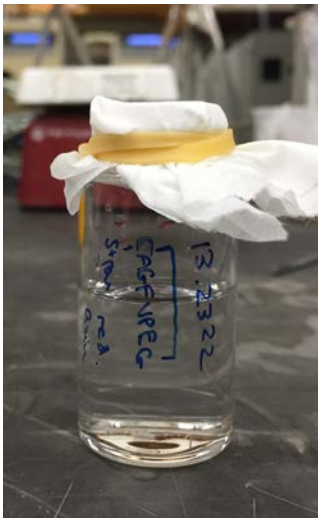


(Left) The peptide is dissolved in water (1 mM final concentration) and the vial charged with a magnetic stir bar **(Right)** An appropriate amount of the solution of KCN in water (1.2 eq. with respect to the peptide) is drawn up into a pipette.



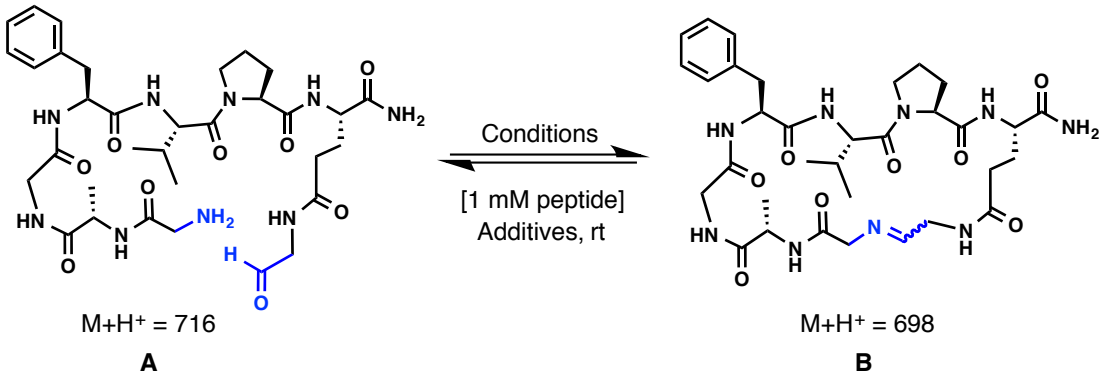

(Left) The KCN solution is added to the peptide. **(Right)** The reaction is stirred at room temperature and monitored by LC-MS. Upon completion, the reaction is purified immediately by preparative reverse-phase HPLC.

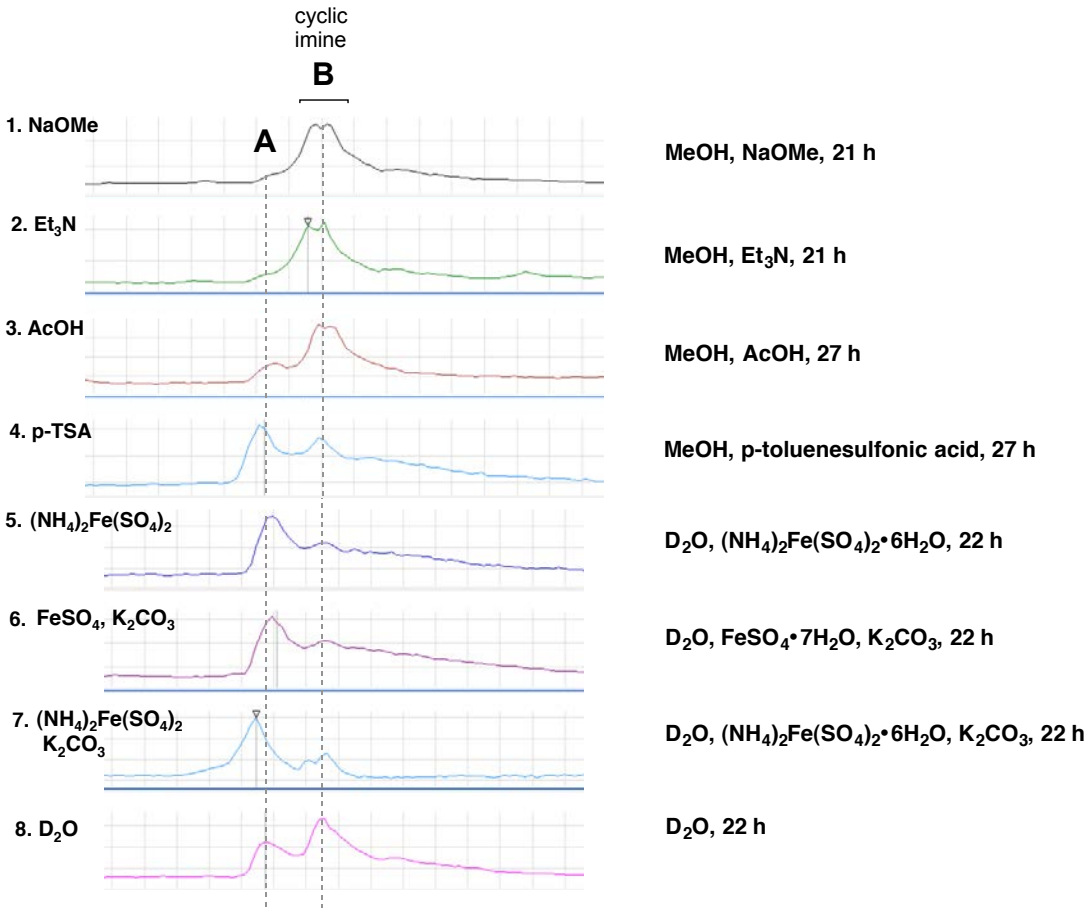
Representative Procedure – Peptide Lyophilization:


(Left) Following HPLC purification, clean fractions are combined, concentrated on a rotovap, and transferred to a 20 mL vial for lyophilization **(Center)** Pre-weighed vial containing combined HPLC fractions. **(Right)** The vial is flash-frozen in liquid nitrogen and placed in a freeze-drier vessel for lypohilization (24 – 48 h).

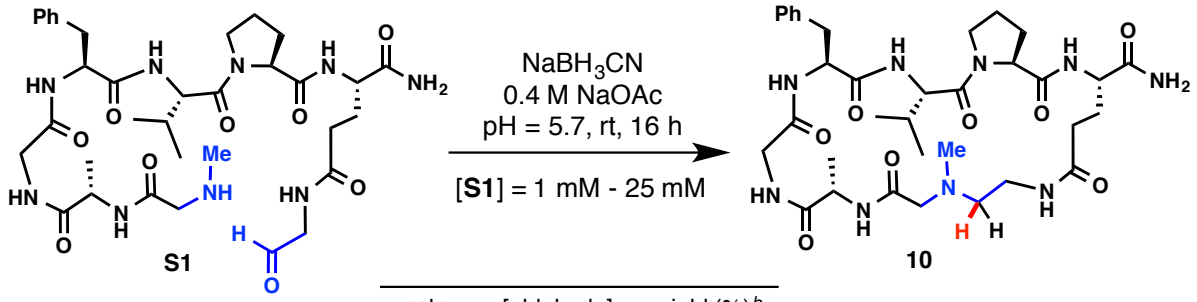
Probing Imine Macrocyclization





TIC traces at various timepoints showing the treatment of peptide **S2** with various additives. Formation of cyclic imine (**B**) is observed most prominently under basic conditions with MeOH as the solvent (entries 1–2). Surprisingly, condensation is also observed in aqueous media (D_2O, entry 8). However, imine formation was not visible upon analysis of the same sample by ^1H NMR. The acidic LC-MS eluent (0.1% formic acid) may be sufficient to catalyze the condensation following injection of the sample onto the column.

Concentration Screen

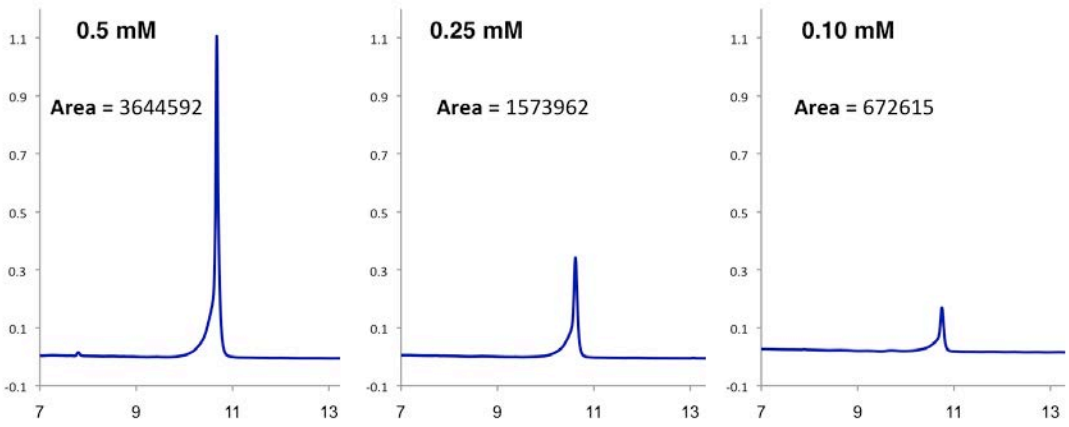


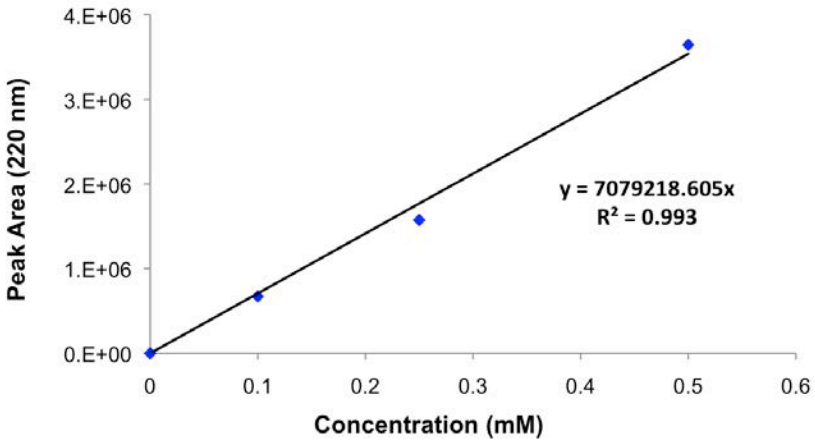
entry	[aldehyde]	yield (%)[b]
1	1 mM	55 (50)[c]
2	2 mM	58
3	5 mM	45
4	10 mM	40
5	25 mM	23

[a] 0.5 μmol scale. [b] Yields determined using analytical HPLC. [c] Isolated yield.

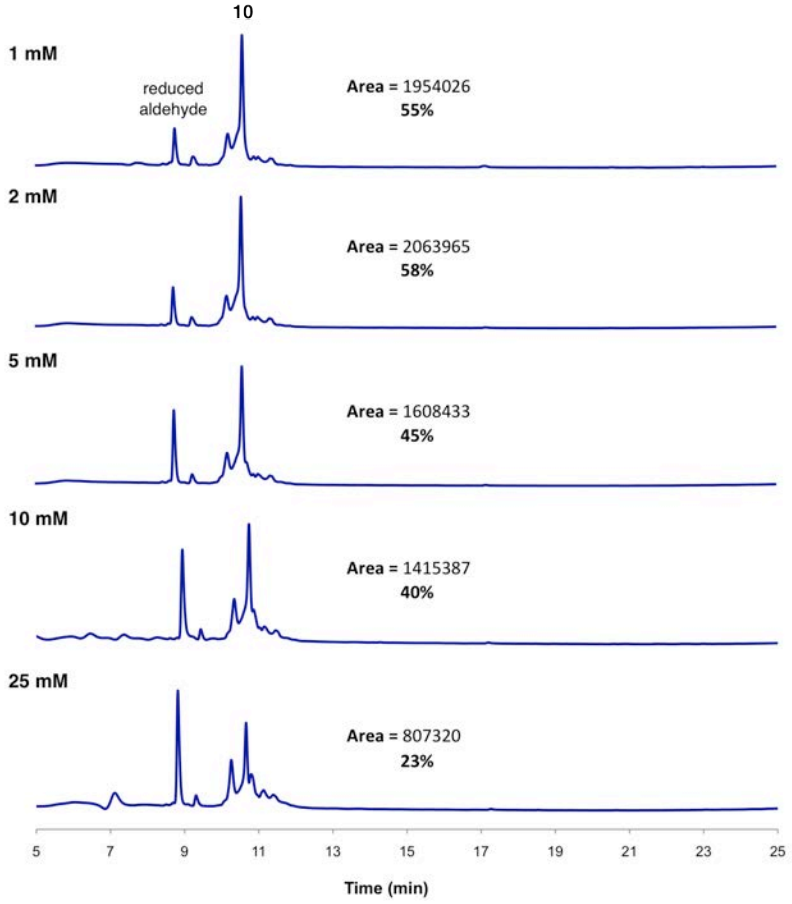
To evaluate the effect of peptide concentration on macrocyclization yield, the reductive amination of peptide **S1** was carried out in 0.4 M NaOAc buffer (pH = 5.7) at 1 mM, 2 mM, 5 mM, 10 mM, and 25 mM concentration with respect to **S1**. The peptide (0.5 μmol scale) was dissolved in buffer and treated with $NaBH_3CN$ (10 equiv.). The reaction was stirred at rt for 16 h, diluted to a concentration of 0.5 mM with respect to the initial concentration of **S1** and evaluated by analytical reverse-phase HPLC (5 to 100% B over 25 min, λ = 220 nm). To estimate reaction yield, the area of the product was integrated and compared to a standard curve derived from the integration (λ = 220 nm) of stock samples of macrocycle **10** prepared at concentrations of 0.5 mM, 0.25 mM, and 0.10 mM.

Standard curve preparation:





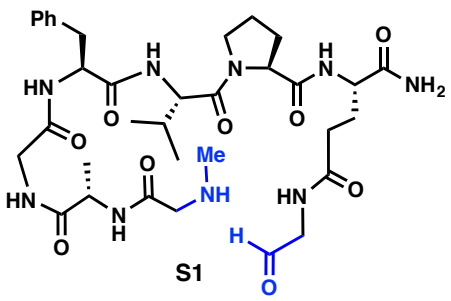
Crude reaction traces:



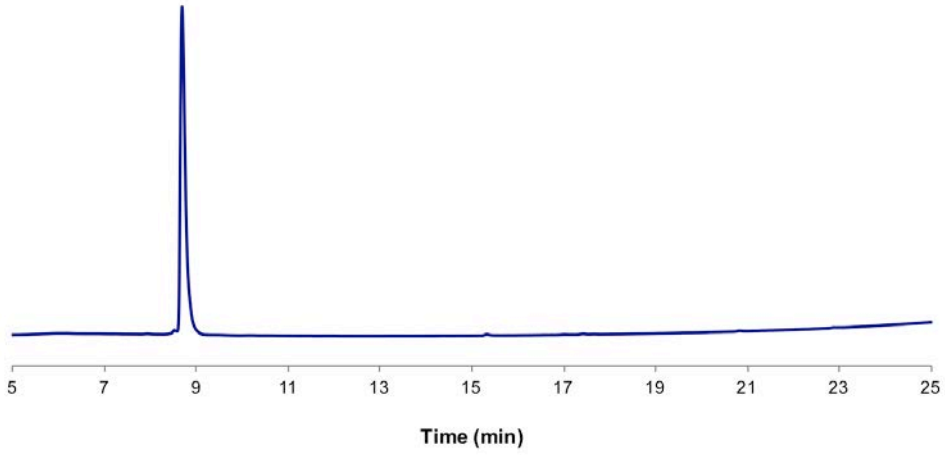
Crude analytical HPLC traces of the reductive amination of amino aldehyde **S1** to form macrocycle **10** at various reaction concentrations (5 to 100% B over 25 min, λ = 220 nm).

Characterization Data for Peptide Aldehydes

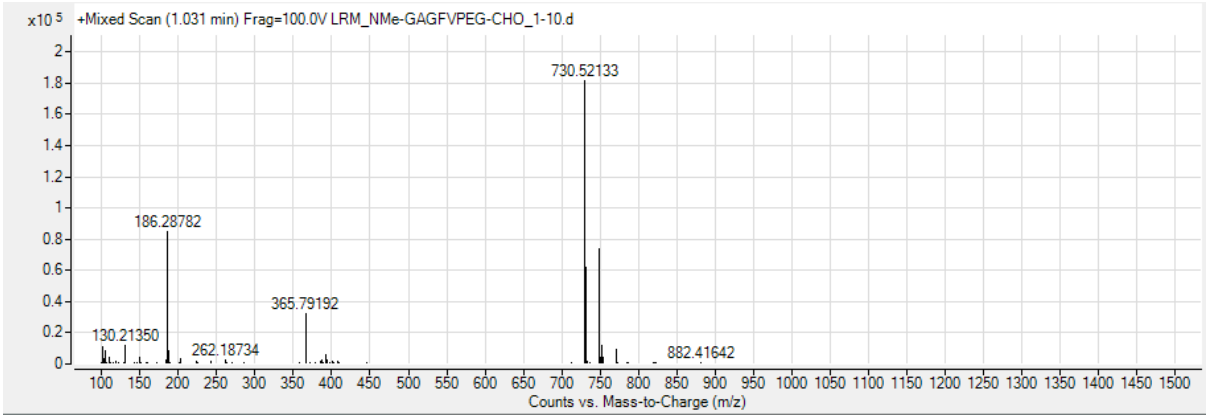
Peptide S1



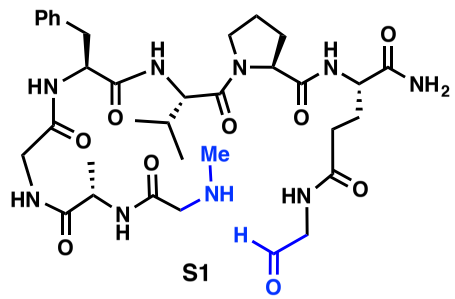
Amino aldehyde **S1** was prepared on a 30 µmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% B to 50% B over 25 min) to afford peptide **S1** (10.0 mg, 46% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S1** (Rt = 8.7 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{34}H_{52}N_9O_9$ $[M+H]^+$ 730.39; found 730.52.



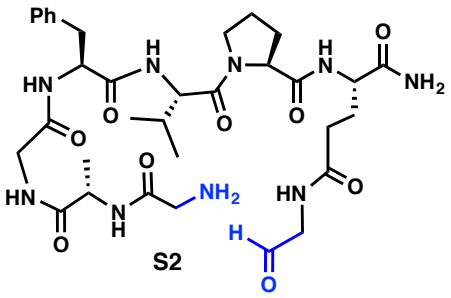
S1

^{1}H, DMSO-d$_6$, 600 MHz

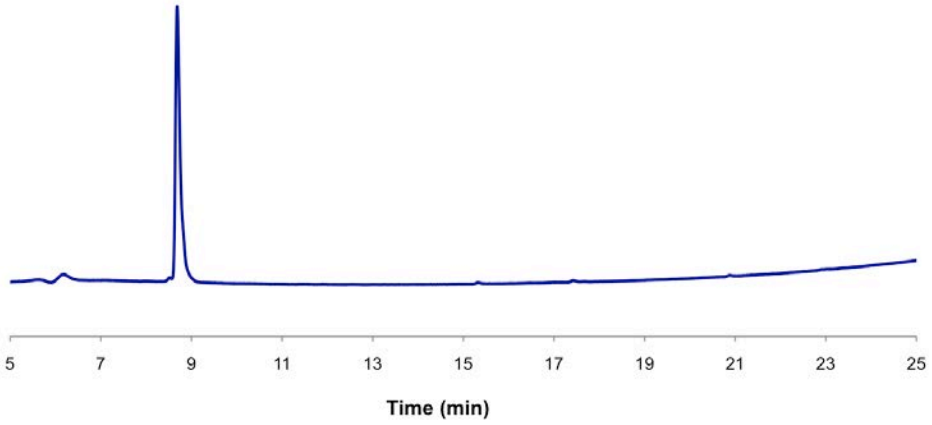
12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5

f1 (ppm)

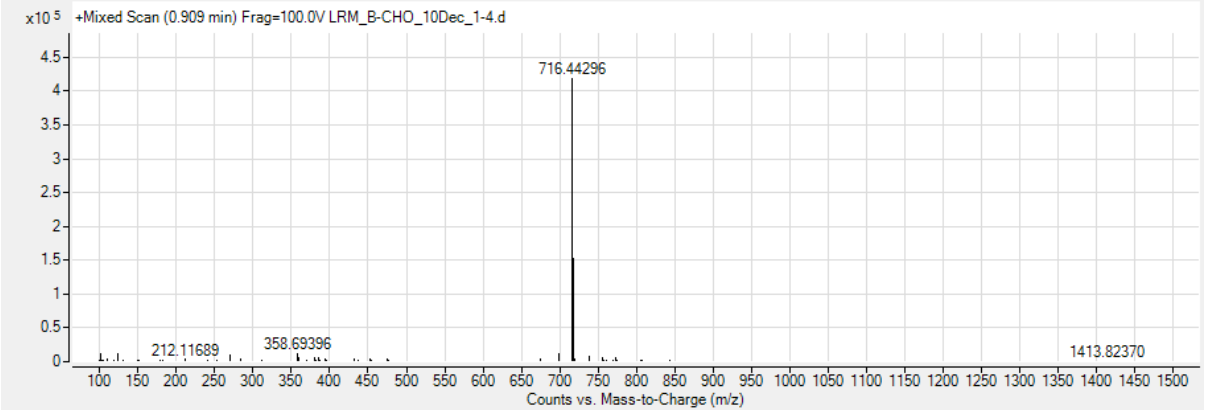
Peptide S2



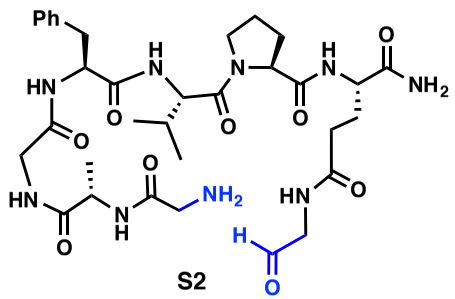
Amino aldehyde **S2** was prepared on a 50 µmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 35% B over 30 min) to afford peptide **S2** (8.0 mg, 22% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S2** (Rt = 8.7 min, 5 to 100% B over 25 min, λ = 230 nm).



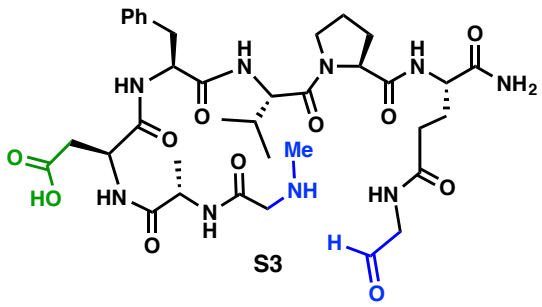
LRMS (ESI-TOF): calc'd for $C_{33}H_{50}N_9O_9$ $[M+H]^+$ 716.37; found 716.44.



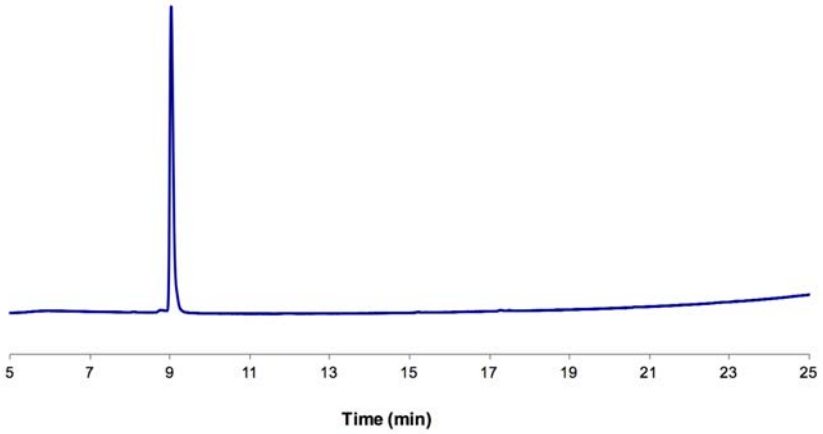
S2

^{1}H, D_2O, 600 MHz

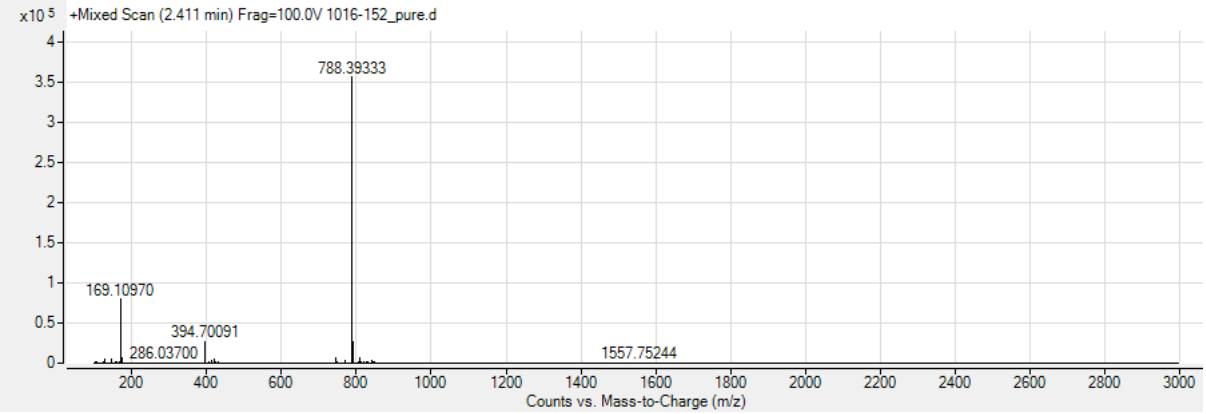
Peptide S3



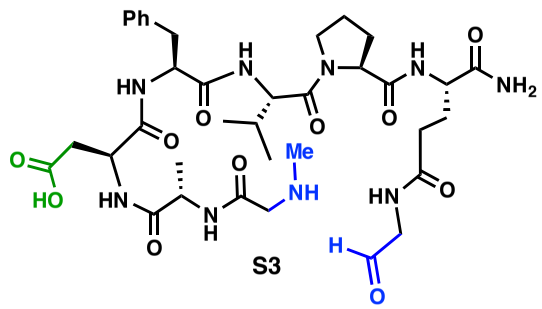
Amino aldehyde **S3** was prepared on a 62 µmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 40% B over 30 min) to afford peptide **S3** (16.9 mg, 35% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S3** (Rt = 8.8 min, 5 to 100% B over 25 min, λ = 230 nm).



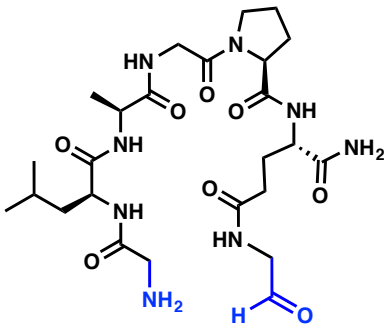
LRMS (ESI-TOF): calc'd for $C_{26}H_{41}N_9O_7$ $[M+H]^+$ 788.39; found 788.39.



S3

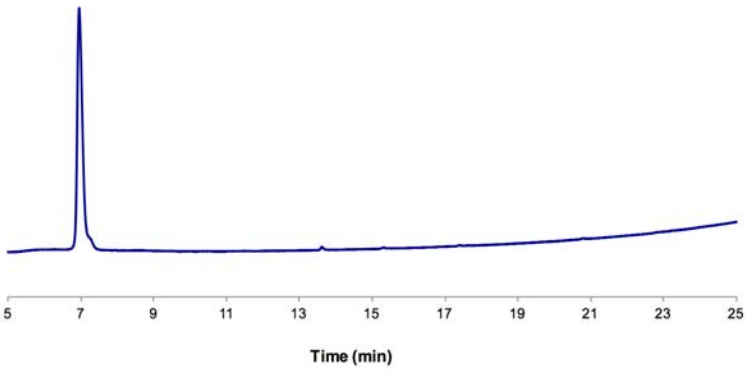
^{1}H, DMSO-d$_{6}$, 600 MHz

Peptide S4

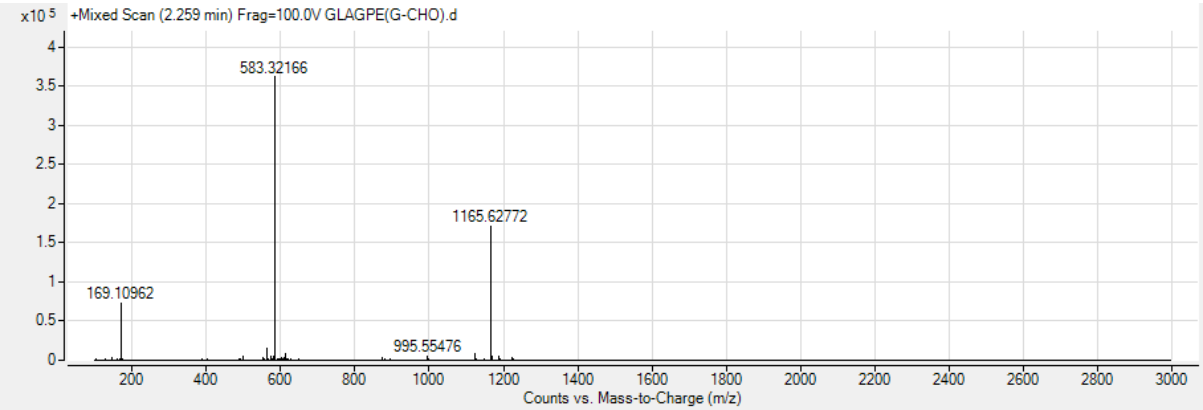


S4

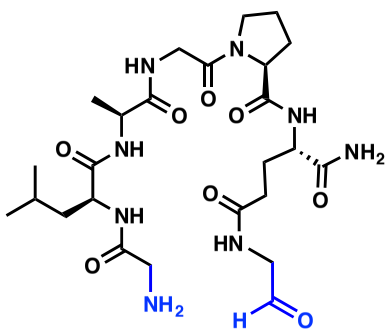
Amino aldehyde **S4** was prepared on a 170 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 40% B over 30 min) to afford peptide **S4** (30.7 mg, 31% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S4** (Rt = 7.0 min, 5 to 100% B over 25 min, λ = 230 nm).



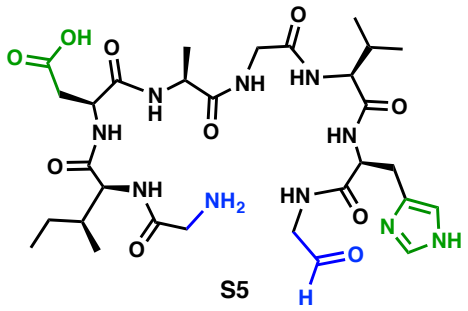
LRMS (ESI-TOF): calc'd for $C_{26}H_{41}N_9O_7$ $[M+H]^+$ 583.32; found 583.32.



S4

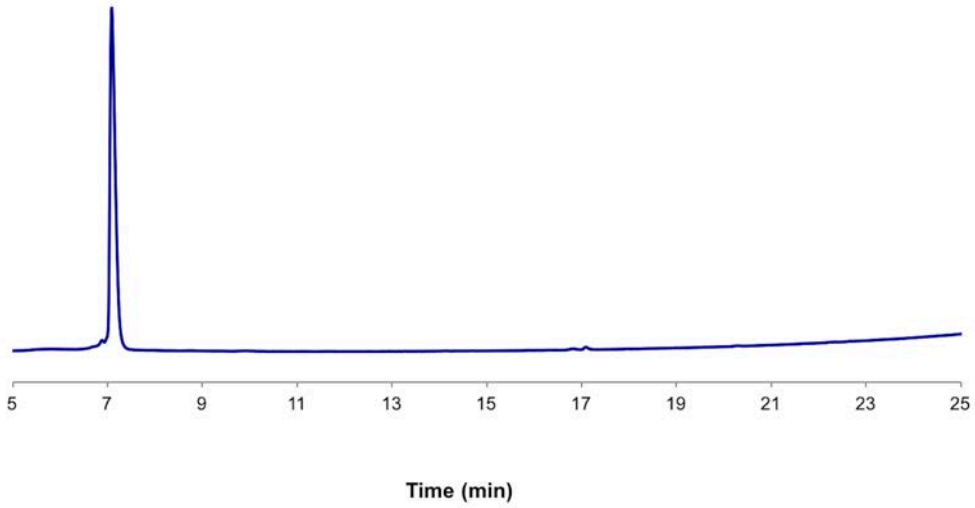
^{1}H, DMSO-d_6, 600 MHz

Peptide S5

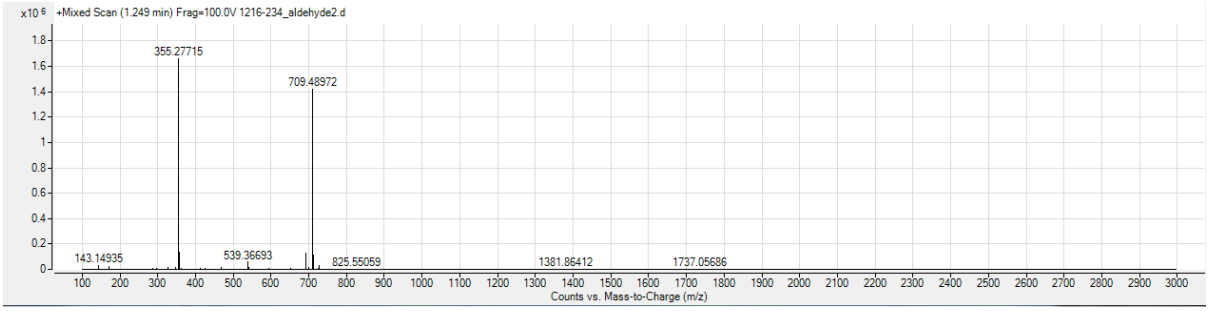


S5

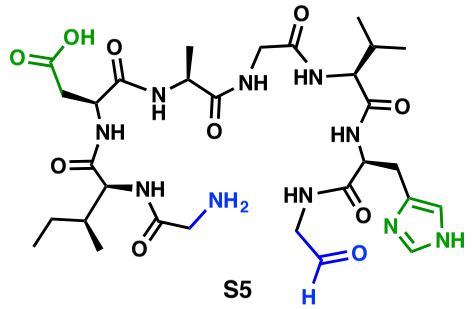
Amino aldehyde **S5** was prepared on a 70 µmol scale according to general procedure 2b. Fmoc-Gly-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 40% B over 30 min) to afford peptide **S5** (31.0 mg, 63% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S5** (Rt = 7.1 min, 5 to 100% B over 25 min, λ = 210 nm).



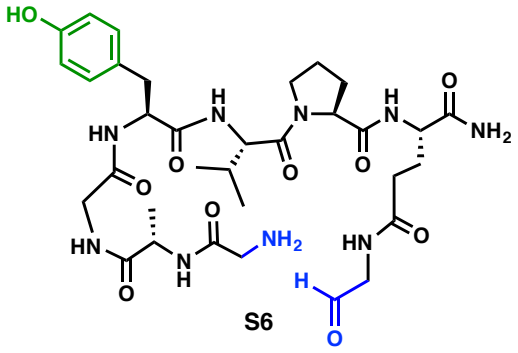
LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 709.36; found 709.49, $[M+2H]^{2+}$ 355.19; found 355.28.



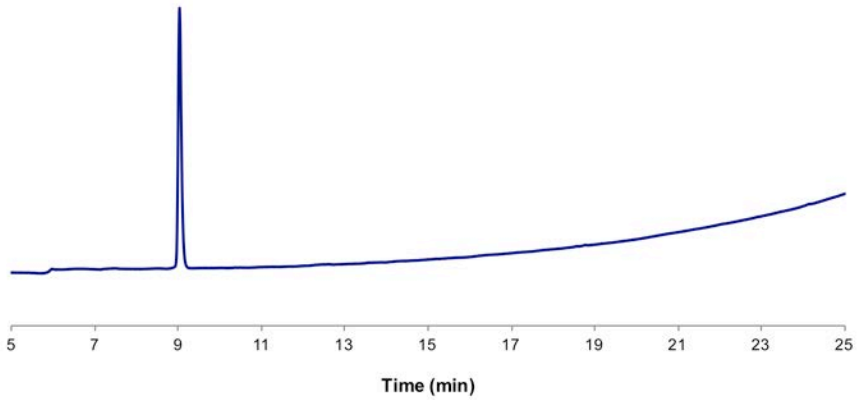
S5

^1H, D_2O, 600 MHz

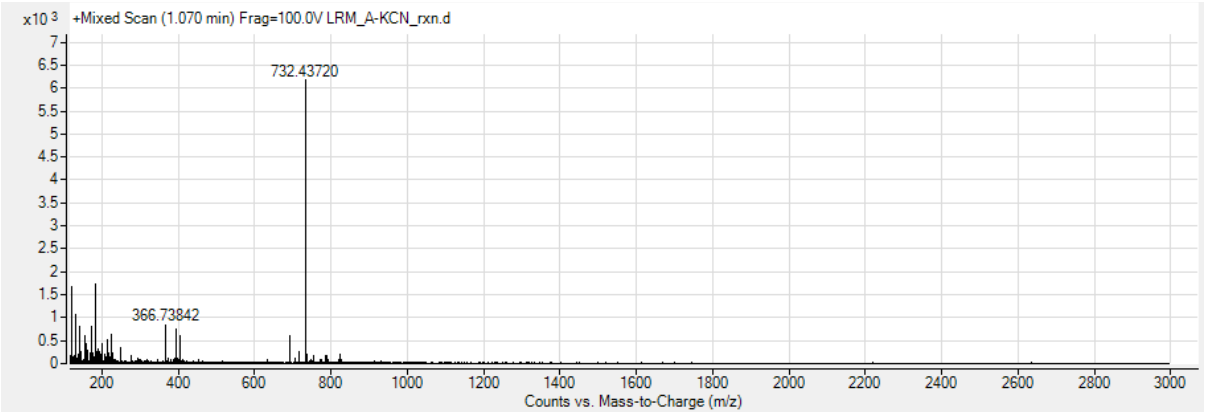
Peptide S6



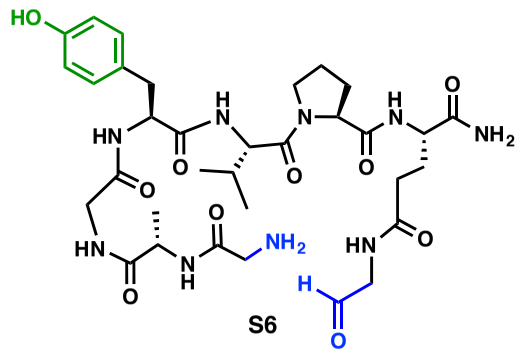
Amino aldehyde **S6** was prepared on a 25 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 40% B over 30 min) to afford peptide **S6** (3.2 mg, 17% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S6** (Rt = 9.0 min, 0 to 100% B over 25 min, λ = 230 nm).



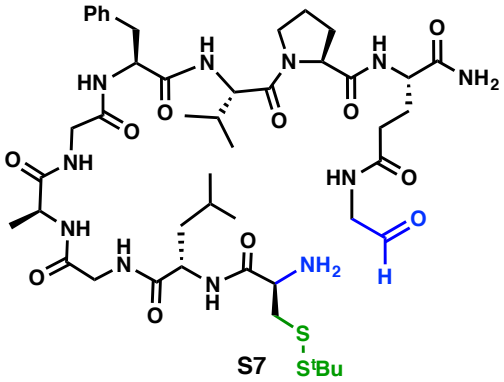
LRMS (ESI-TOF): calc'd for $C_{33}H_{50}N_9O_{10}$ $[M+H]^+$ 732.37; found 732.44.



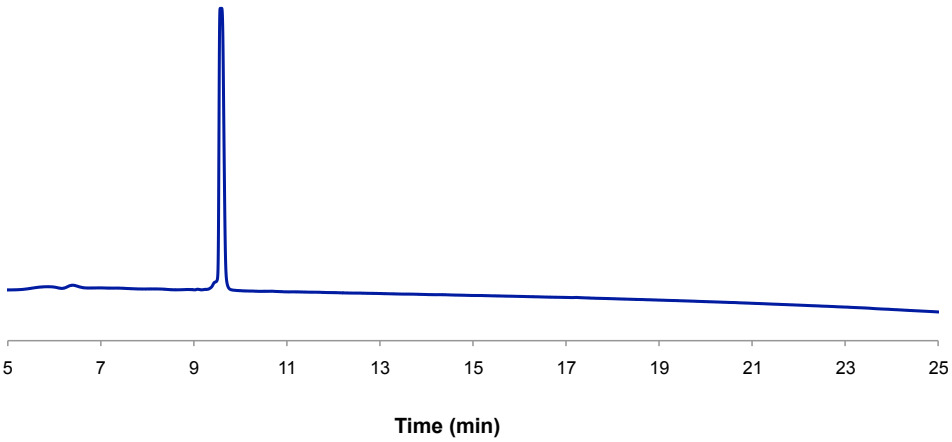
S6

^{1}H, D_2O, 600 MHz

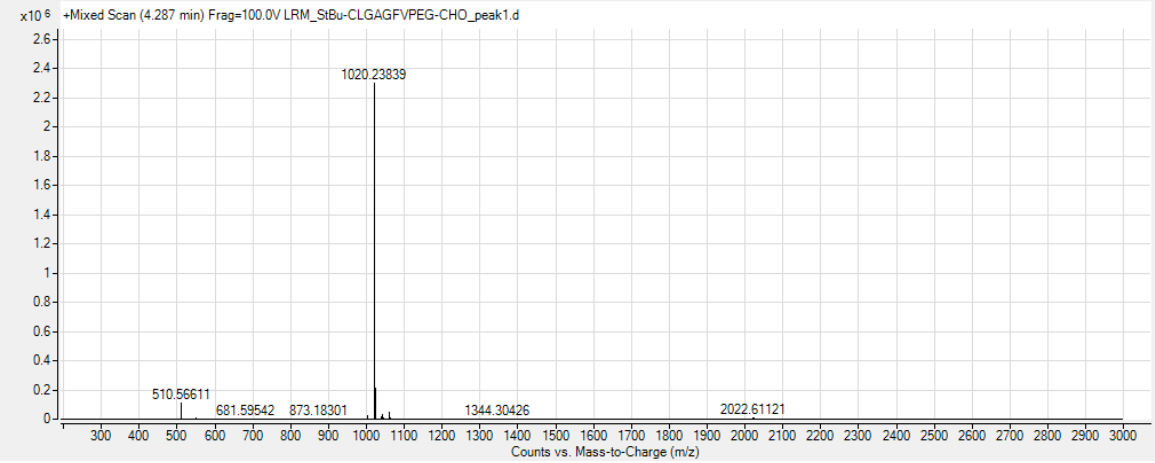
Peptide S7



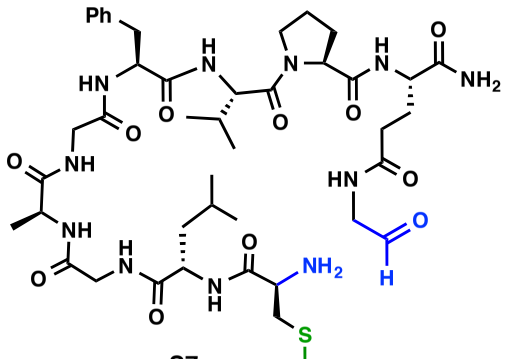
Amino aldehyde **S7** was prepared on a 50 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% B to 55% B over 30 min) to afford peptide **S7** (8.1 mg) and a peptide epimer at the Cys(S^{t}Bu) residue (4.6 mg) in 25% combined yield based on the original resin loading and as white solids following lyophilization.



Purified peptide **S7** (Rt = 9.6 min, 5 to 100% B over 25 min, λ = 210 nm).



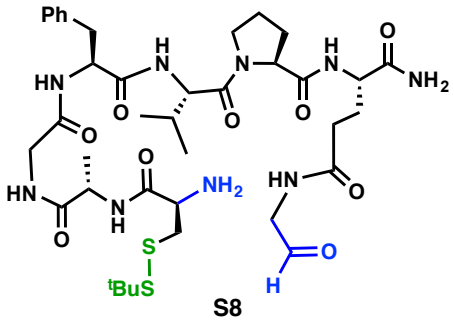
LRMS (ESI-TOF): calc'd for $C_{46}H_{74}N_{11}O_{11}S_2$ $[M+H]^+$ 1020.50; found 1020.24.



S7

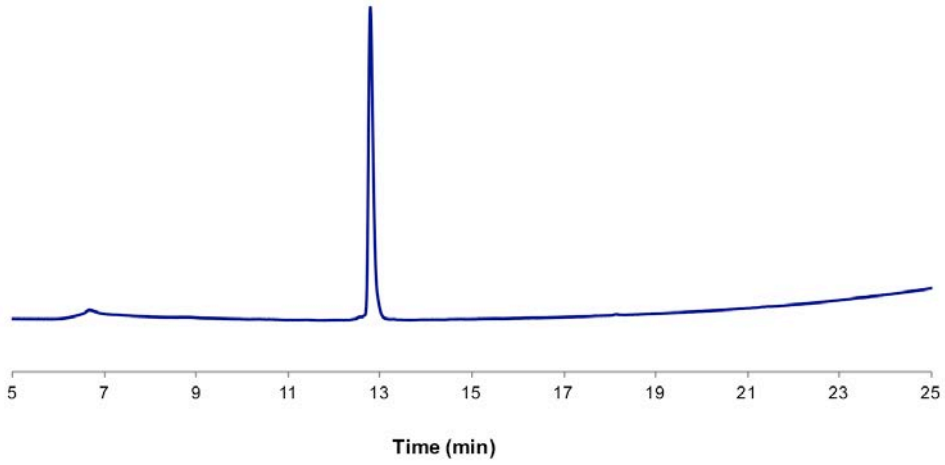
^{1}H, D_2O, 600 MHz

Peptide S8

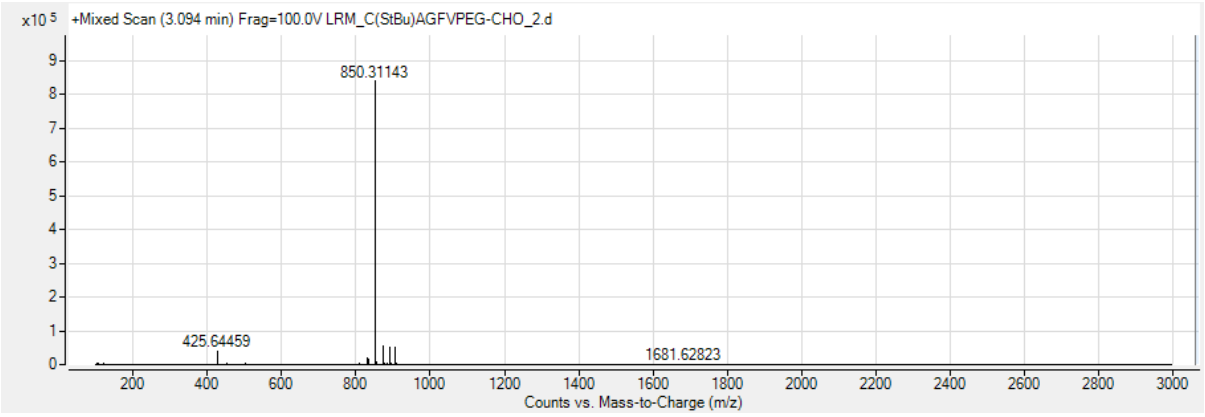


S8

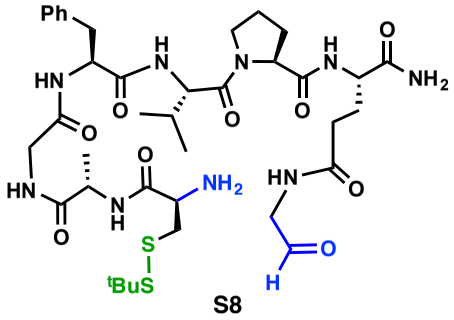
Amino aldehyde **S8** was prepared on a 23 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% B to 60% B over 30 min) to afford peptide **S8** (6.2 mg, 32% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S8** (Rt = 12.8 min, 5 to 100% B over 25 min, λ = 230 nm).



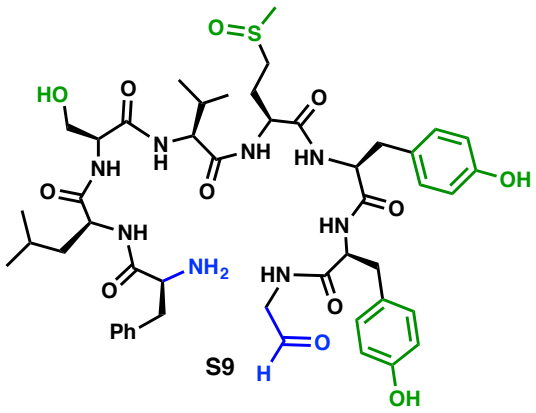
LRMS (ESI-TOF): calc'd for $C_{38}H_{60}N_9O_9S_2$ $[M+H]^+$ 850.40; found 850.31.



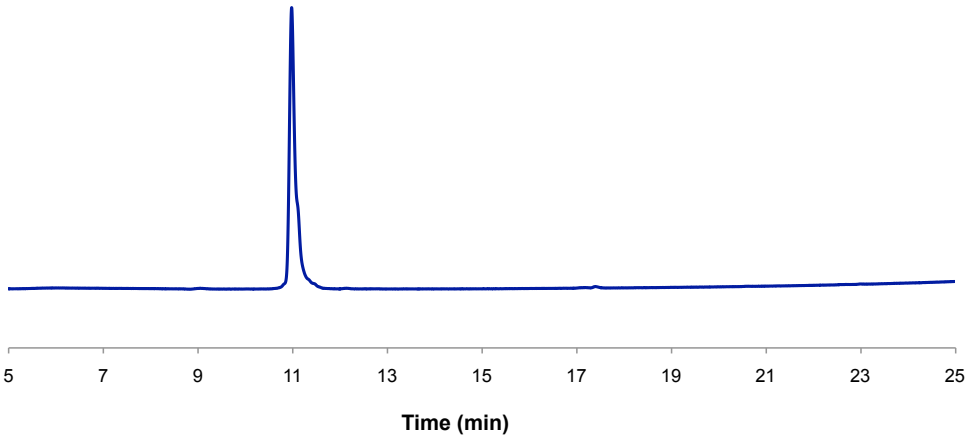
S8

^{1}H, DMSO-d$_6$, 600 MHz

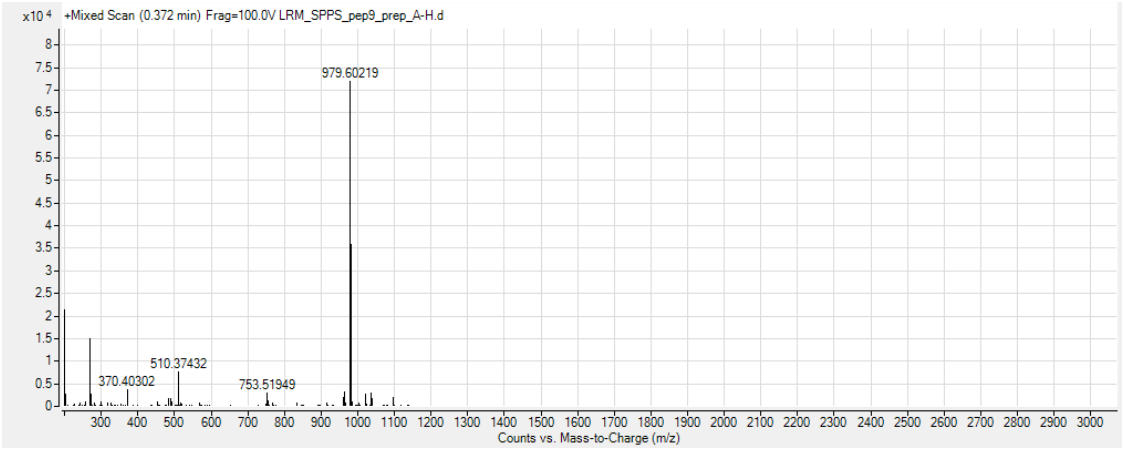
Peptide S9



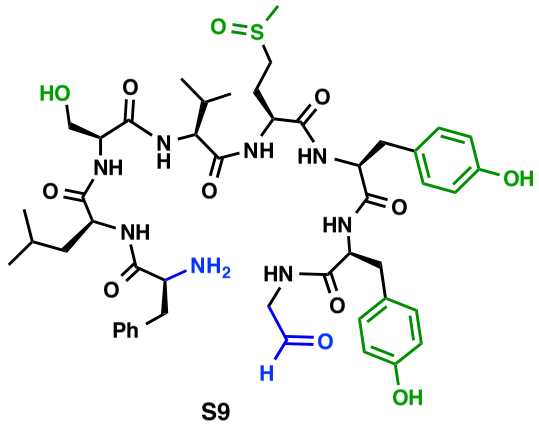
Amino aldehyde **S9** was prepared on a 35 μmol scale according to general procedure 2b. Fmoc-Gly-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% B to 60% B over 30 min) to afford peptide **S9** (6.8 mg) and a reduced Met variant (2.0 mg) in 26% combined yield based on the original resin loading and as white solids following lyophilization.



Purified peptide **S9** (Rt = 11.0 min, 5 to 100% B over 25 min, λ = 230 nm).



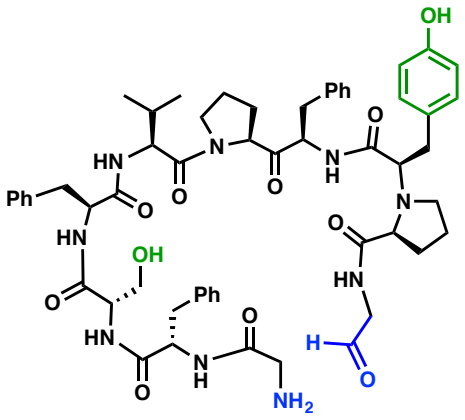
LRMS (ESI-TOF): calc'd for $C_{48}H_{67}N_8O_{12}S$ $[M+H]^+$ 979.46; found 979.60.



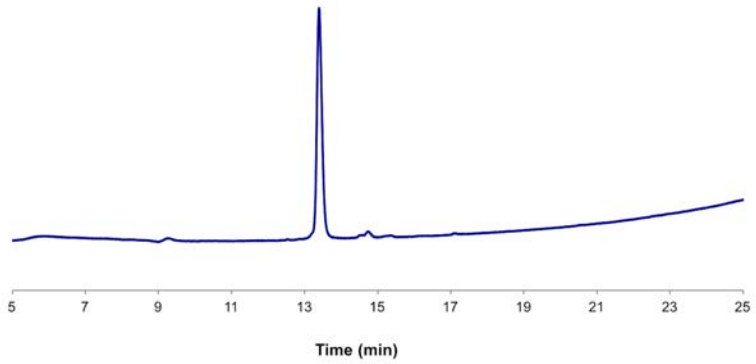
S9

^{1}H, D_2O/MeOD (2:1 v/v), 600 MHz

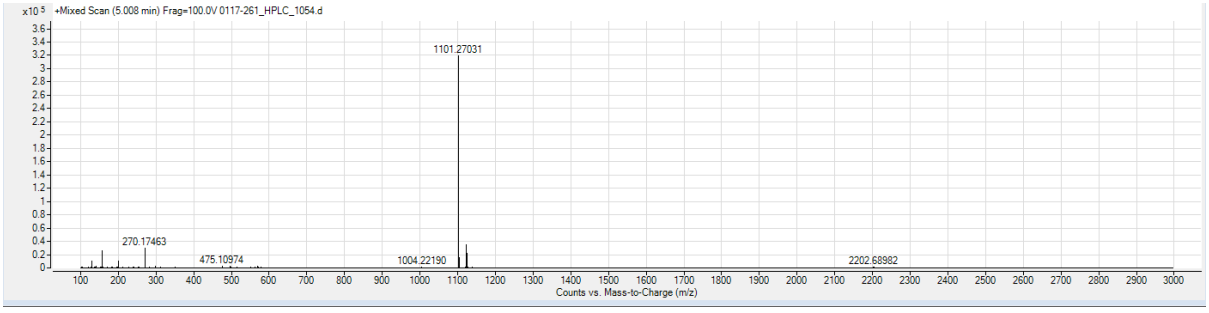
Peptide S10



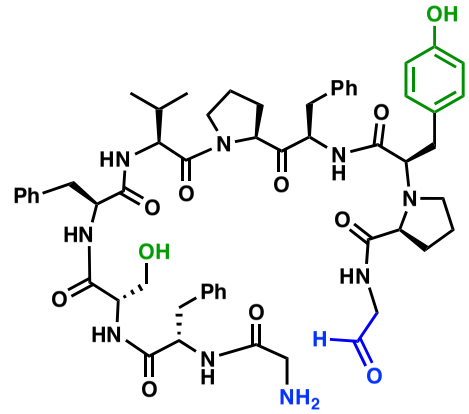
Amino aldehyde **S10** was prepared on a 35 μmol scale according to general procedure 2b. Fmoc-Gly-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (35% B for 5 min, then 35% B to 75% B over 30 min) to afford peptide **S10** (17.1 mg, 45% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S10** (Rt = 13.4 min, 5 to 100% B over 25 min, λ = 210 nm).



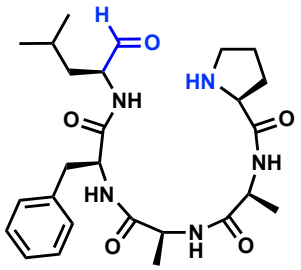
LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 1101.54; found 1101.27.



S10

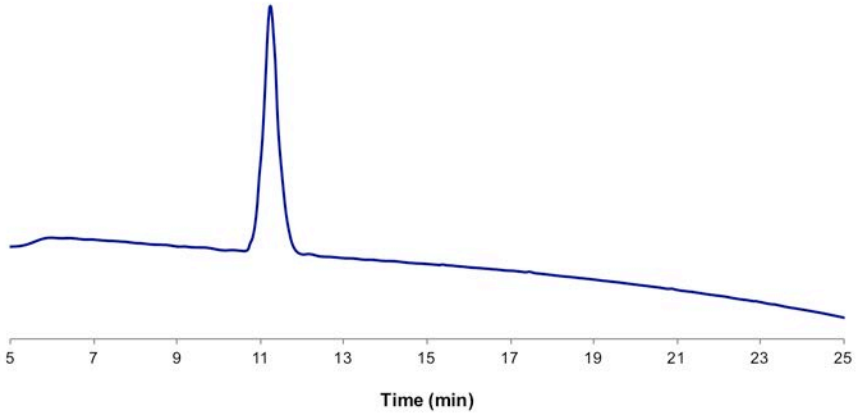
^{1}H, D_2O, 600 MHz

Peptide S11

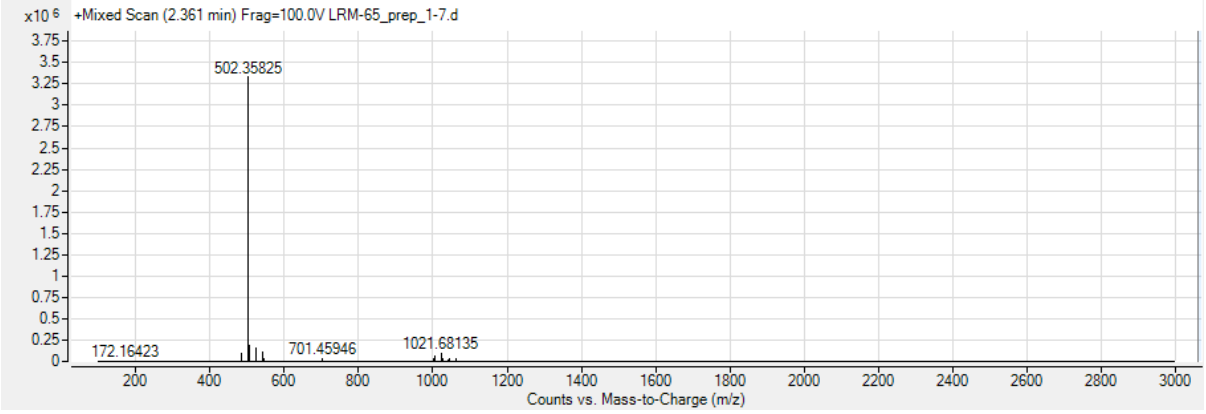


S11

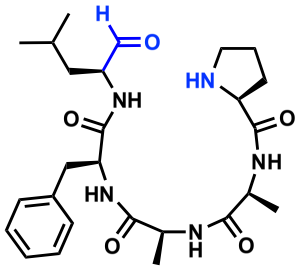
Amino aldehyde **S11** was prepared on a 37 μmol scale according to general procedure 2a. Fmoc-Leu-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% B to 55% B over 25 min) to afford peptide **S11** (13.0 mg, 70% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S11** (Rt = 11.2 min, 5 to 100% B over 25 min, λ = 210 nm).



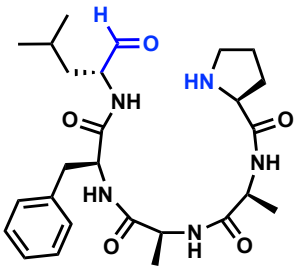
LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 502.30; found 502.36.



S11

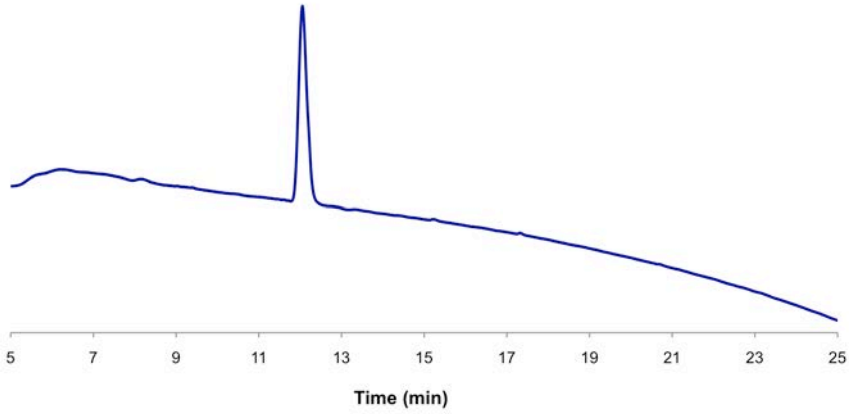
^{1}H, DMSO-d$_{6}$, 500 MHz

Peptide S12

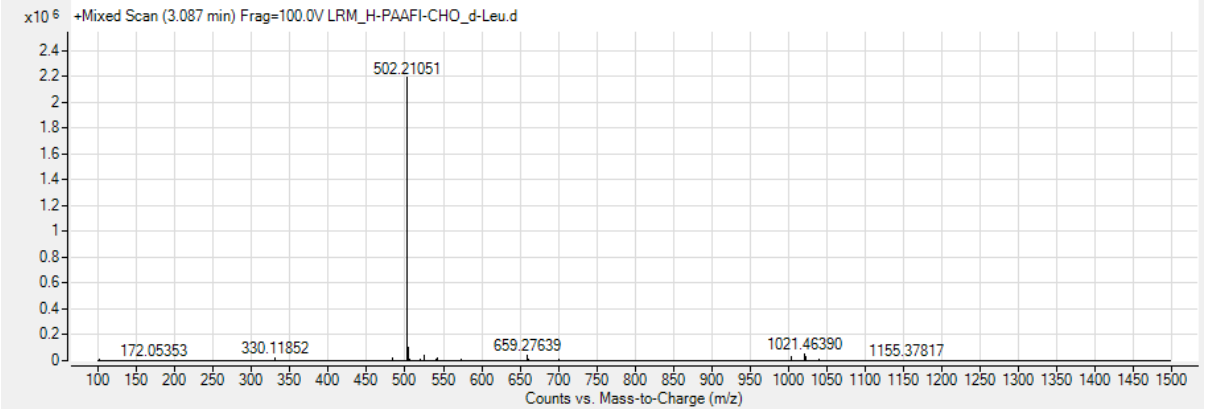


S12

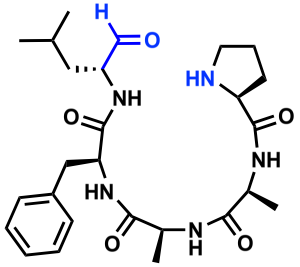
Amino aldehyde **S12** was prepared on a 47.5 µmol scale according to general procedure 2a. Fmoc-D-Leu-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% B to 55% B over 30 min) to afford peptide **S12** (11.5 mg, 48% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S12** (Rt = 12.1 min, 5 to 100% B over 25 min, λ = 210 nm).



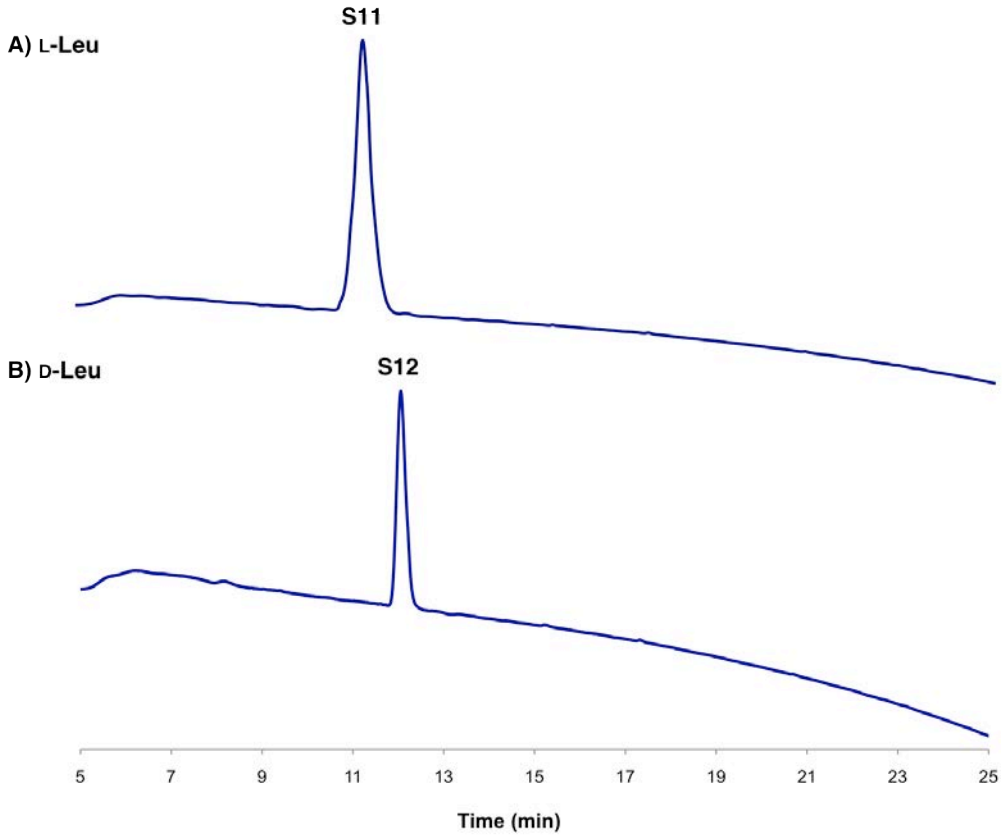
LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 502.30; found 502.21.



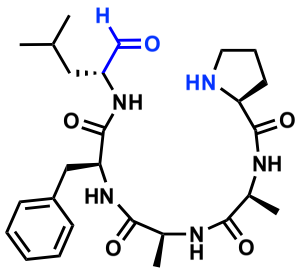
S12

^{1}H, DMSO-d$_{6}$, 600 MHz

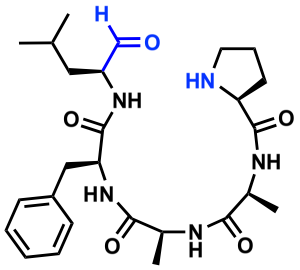
HPLC overlay of peptides S11 and S12



A) Purified amino aldehyde **S11**, H-PAAFL-CHO (Rt = 11.2 min, 5 to 100% B over 25 min, λ = 210 nm). **B)** Purified amino aldehyde **S12**, H-PAAFL-CHO (Rt = 12.1 min, 5 to 100% B over 25 min, λ = 210 nm).


H
O
NH
O
HN
HN
O
NH
O
N
H
O

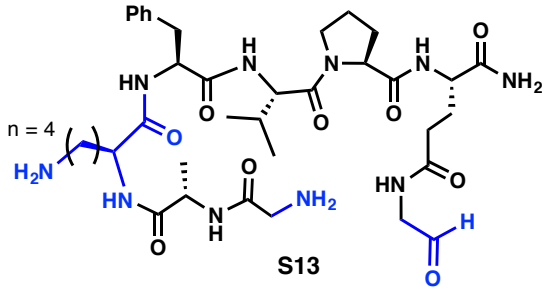
S12


H
O
NH
O
HN
HN
O
NH
O
N
H
O

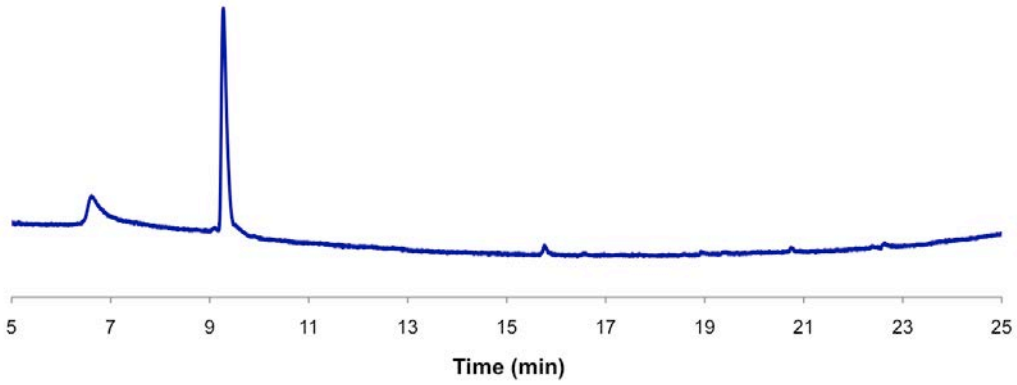
S11

10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

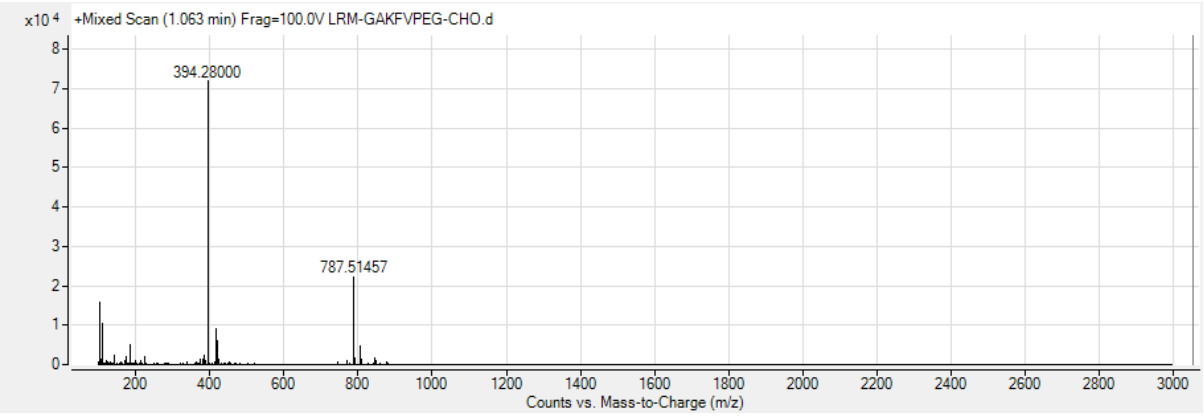
Peptide S13



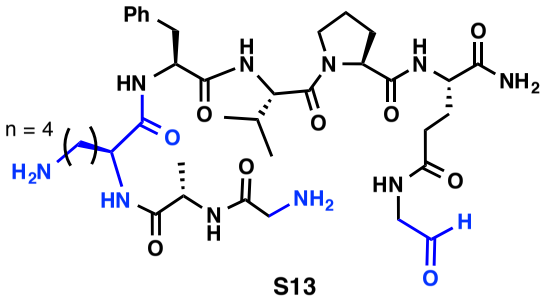
Amino aldehyde **S13** was prepared on a 50 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (0% B for 5 min, then 0% B to 70% B over 30 min) to afford peptide **S13** (16.5 mg, 42% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S13** (Rt = 9.3 min, 0 to 100% B over 25 min, λ = 254 nm).



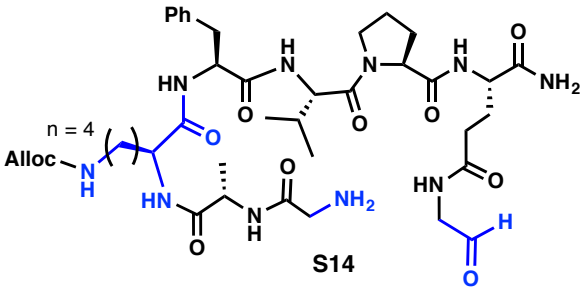
LRMS (ESI-TOF): calc'd for $C_{37}H_{59}N_{10}O_9$ $[M+H]^+$ 787.45; found 787.51, $[M+2H]^{2+}$ 394.23; found 394.28.



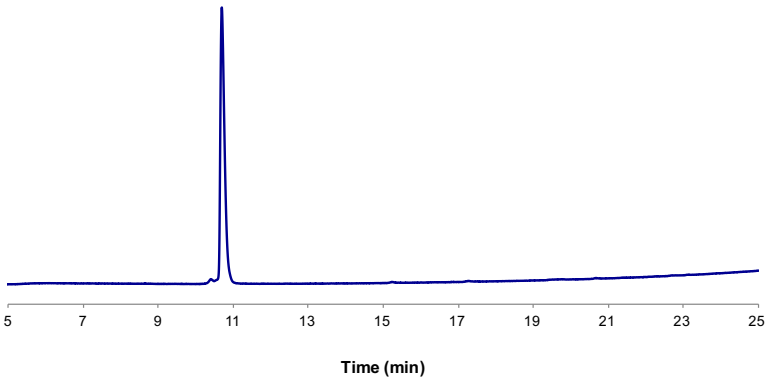
S13

^{1}H, D_2O, 600 MHz

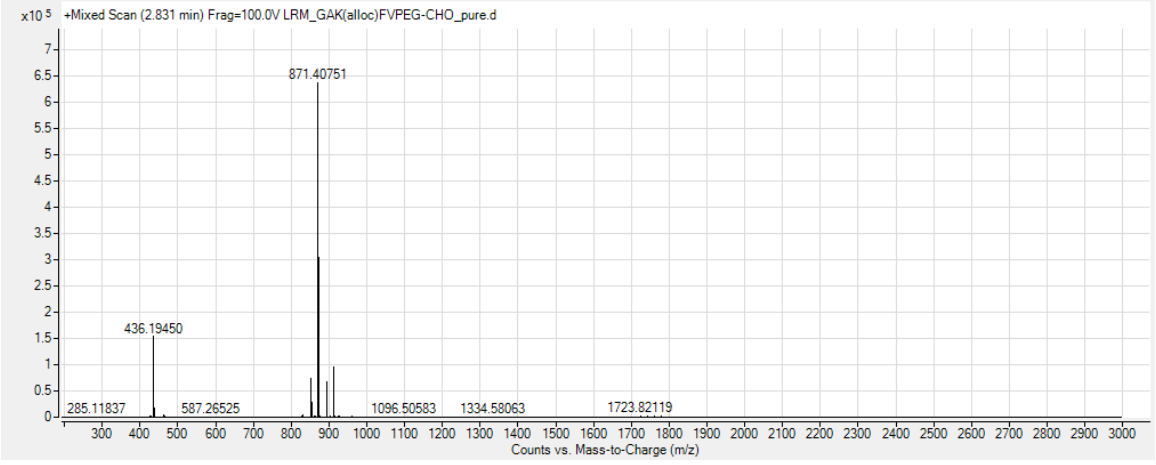
Peptide S14



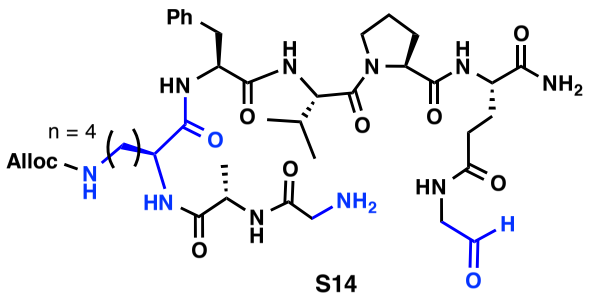
Amino aldehyde **S14** was prepared on a 50 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (0% B for 5 min, then 0% B to 70% B over 30 min) to afford peptide **S14** (16.5 mg, 38% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S14** (Rt = 10.7 min, 5 to 100% B over 25 min, λ = 230 nm).



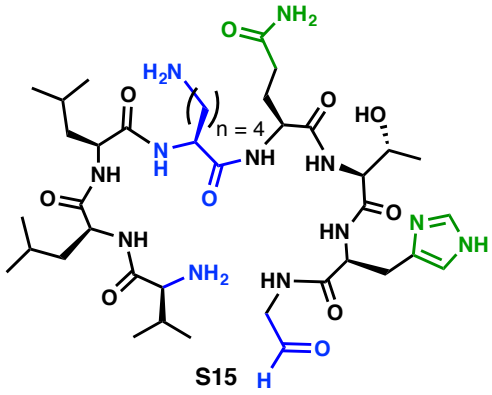
LRMS (ESI-TOF): calc'd for $C_{41}H_{63}N_{10}O_{11}$ $[M+H]^+$ 871.47; found 871.41.



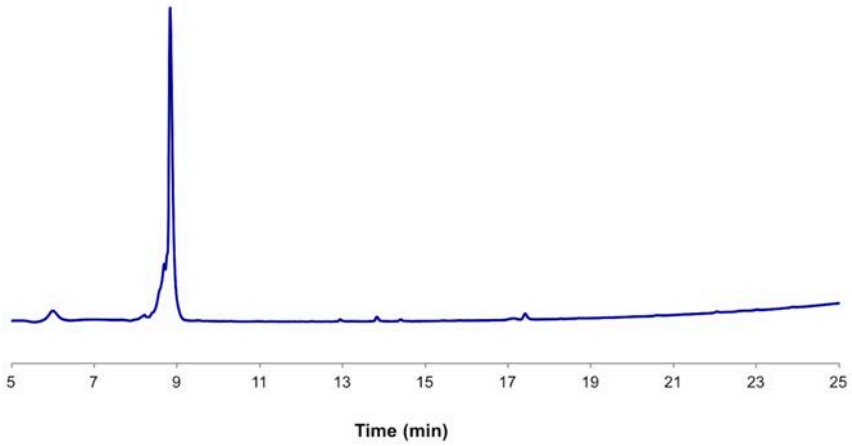
S14

^{1}H, DMSO-d$_6$, 600 MHz

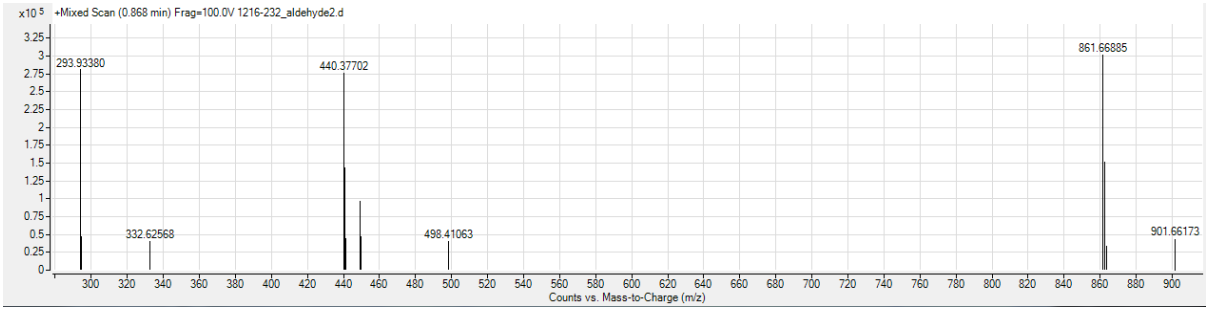
Peptide S15



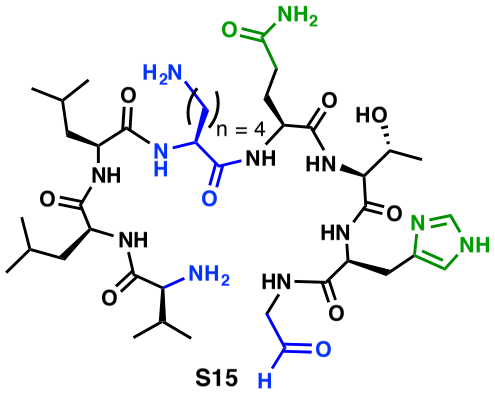
Amino aldehyde **S15** was prepared on a 70 μmol scale according to general procedure 2b. Fmoc-Gly-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (10% B for 5 min, then 10% B to 60% B over 30 min) to afford peptide **S15** (31.2 mg, 51% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S15** (Rt = 8.8 min, 5 to 100% B over 25 min, λ = 210 nm).



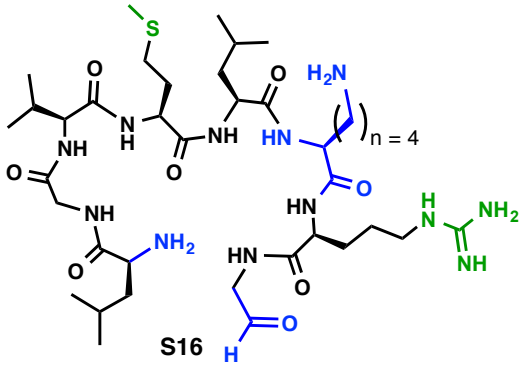
LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 861.53; found 861.67.



S15

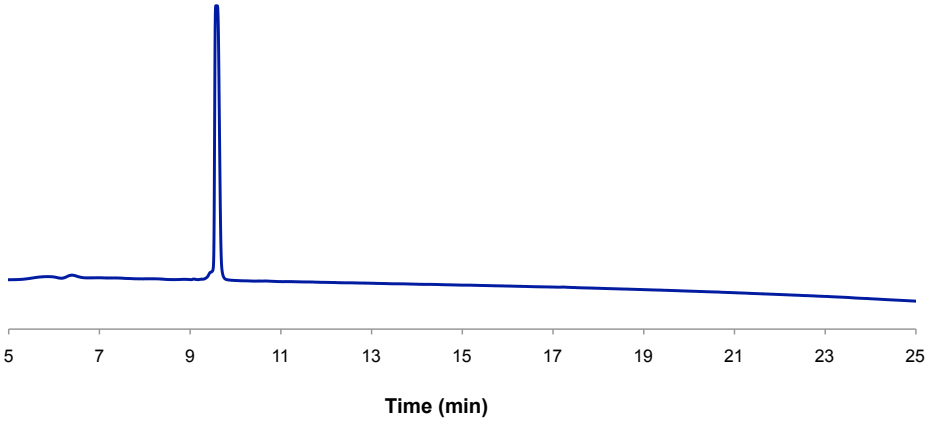
^{1}H, D_2O, 600 MHz

Peptide S16

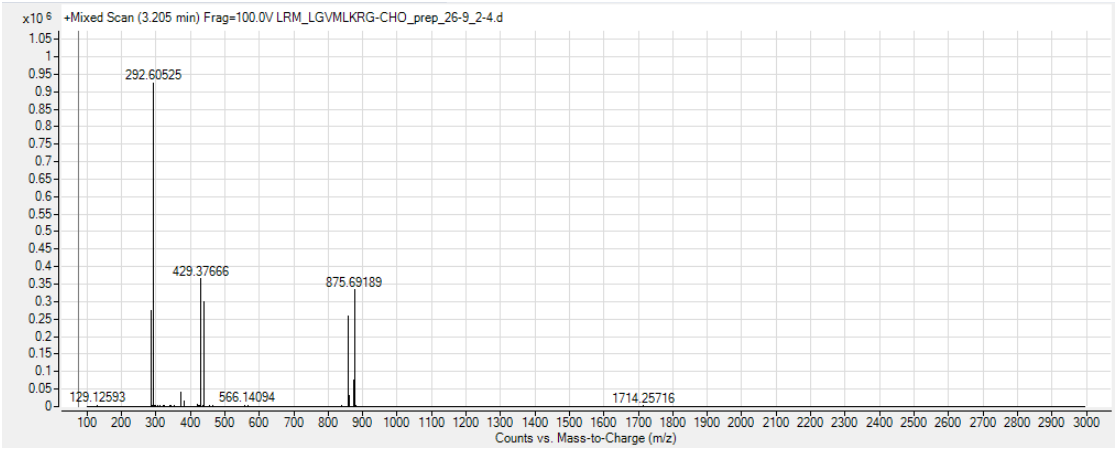


S16

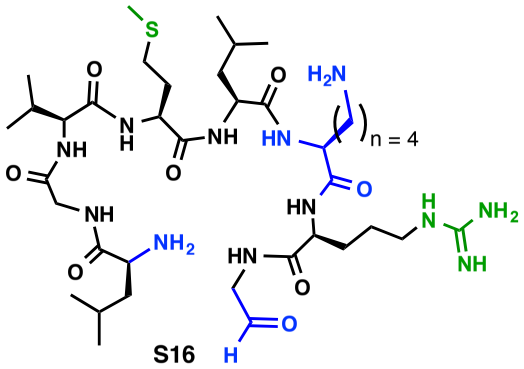
Amino aldehyde **S16** was prepared on a 35 μmol scale according to general procedure 2b. Fmoc-Gly-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 45% B over 35 min) to afford peptide **S16** (3.1 mg) and an oxidized Met variant (3.5 mg) in 22% combined yield based on the original resin loading and as white solids following lyophilization.



Purified peptide **S16** (Rt = 9.6 min, 5 to 100% B over 25 min, λ = 210 nm).



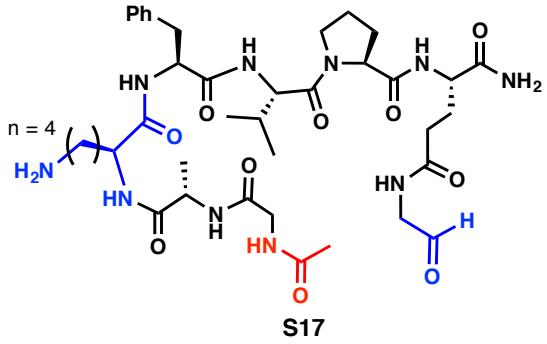
LRMS (ESI-TOF): calc'd for $C_{38}H_{73}N_{12}O_8S$ $[M+H]^+$ 857.54; found 875.69 (aldehyde hydrate), $[M+2H]^{2+}$ 429.27; found 429.38, $[M+3H]^{3+}$ 286.52; found 292.60 (aldehyde hydrate).



S16

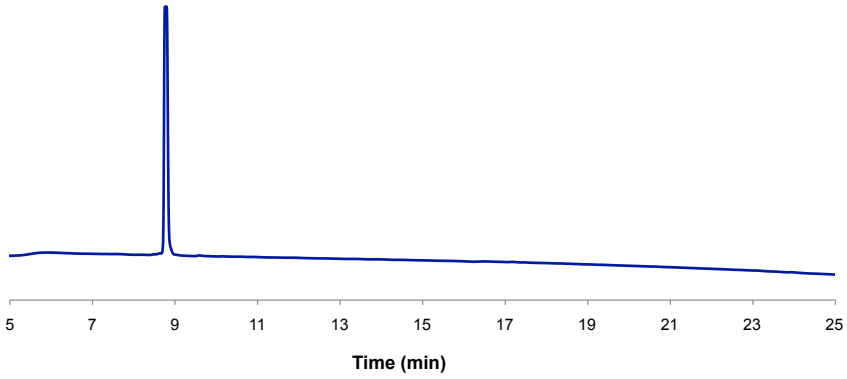
^1H, D_2O, 600 MHz

Peptide S17

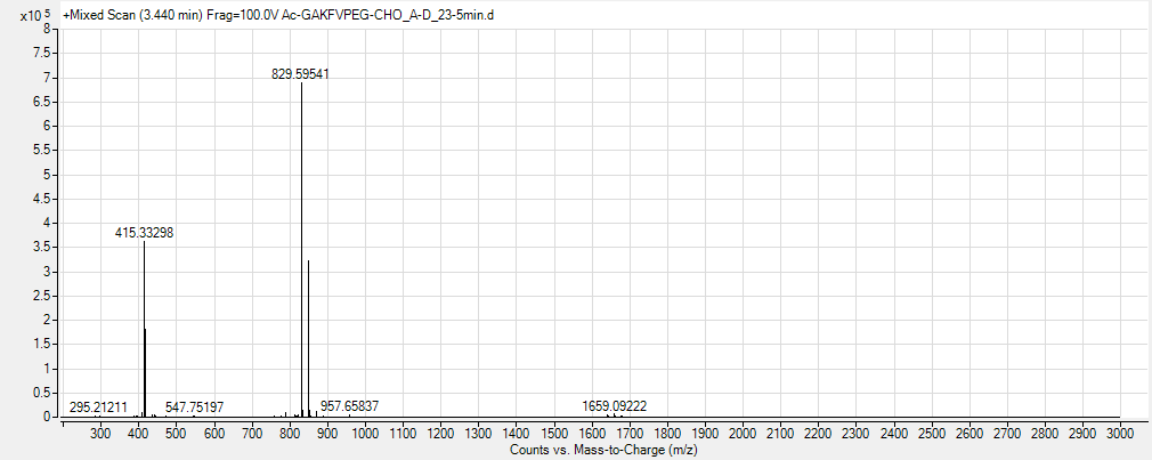


S17

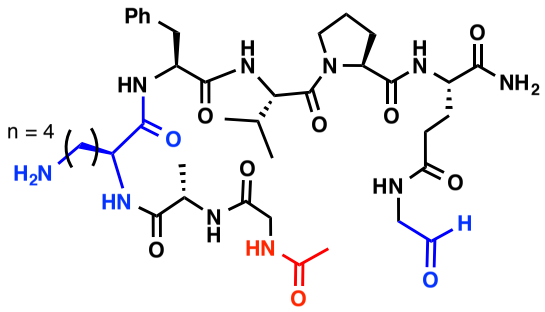
Amino aldehyde **S17** was prepared on a 40 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 40% B over 25 min) to afford peptide **S17** (1.3 mg, 4% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S17** (Rt = 8.8 min, 5 to 100% B over 25 min, λ = 210 nm).



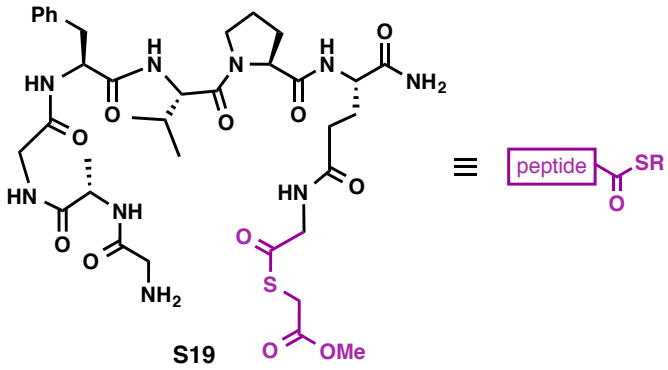
LRMS (ESI-TOF): calc'd for $C_{39}H_{61}N_{10}O_{10}$ $[M+H]^+$ 829.46; found 829.60.



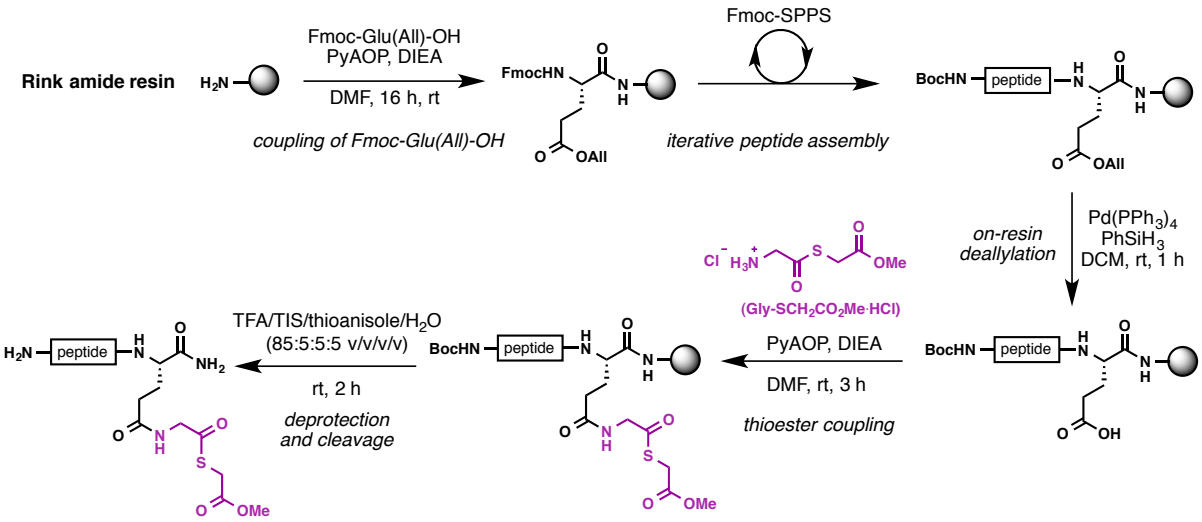
S17

^{1}H, D_2O, 600 MHz

Peptide S19



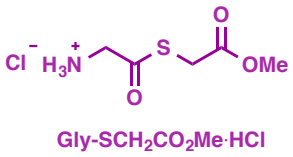
Peptide **S19** was prepared on Rink amide resin using Fmoc-SPPS according to the following reaction scheme:



Preparation of Gly-SCH_2CO_2Me•HCl:

Gly-SCH_2CO_2Me•HCl was prepared from Boc-Gly-OH according to a published protocol,[8] by employing methyl thioglycolate in the thioesterification step. Briefly, Boc-Gly-OH (1.00 g, 5.71 mmol) was dissolved in THF (20 mL) and treated with methyl thioglycolate (1.0 equiv.). The reaction was cooled to 0 ^{o}C and then treated with HOBt (1.2 equiv.) and DIC (1.2 equiv.). The reaction was warmed to rt and stirred for 20 h. The crude reaction mixture was filtered through a plug of Celite, eluted with DCM, and concentrated *in vacuo*. The residue was resuspended in EtOAc (40 mL) and washed with 5% $NaHCO_3$ (30 mL), 0.1 M HCl (30 mL), and brine (30 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated, and subsequently purified by flash column chromatography (1:2 EtOAc/Hexanes) to afford the Boc-protected thioester

(1.301 g, 86% yield). A portion of the thioester (0.951 g, 3.61 mmol) was dissolved in DCM (2 mL) and treated with 4 M HCl in dioxane (1.5 mL) to remove the Boc group. After stirring at rt for 3 h, a second aliquot of 4 M HCl in dioxane (1 mL) was added and the reaction stirred for an additional 3 h. The reaction mixture was concentrated *in vacuo* and coevaporated twice with EtOAc to afford the target thioester as the HCl salt in quantitative yield.



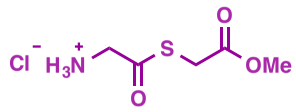
Gly-SCH$_2$CO$_2$Me·HCl

Physical State: white solid

^{1}H NMR (600 MHz, D_2O) δ 4.28 (s, 2H), 3.97 (s, 2H), 3.80 (s, 3H).

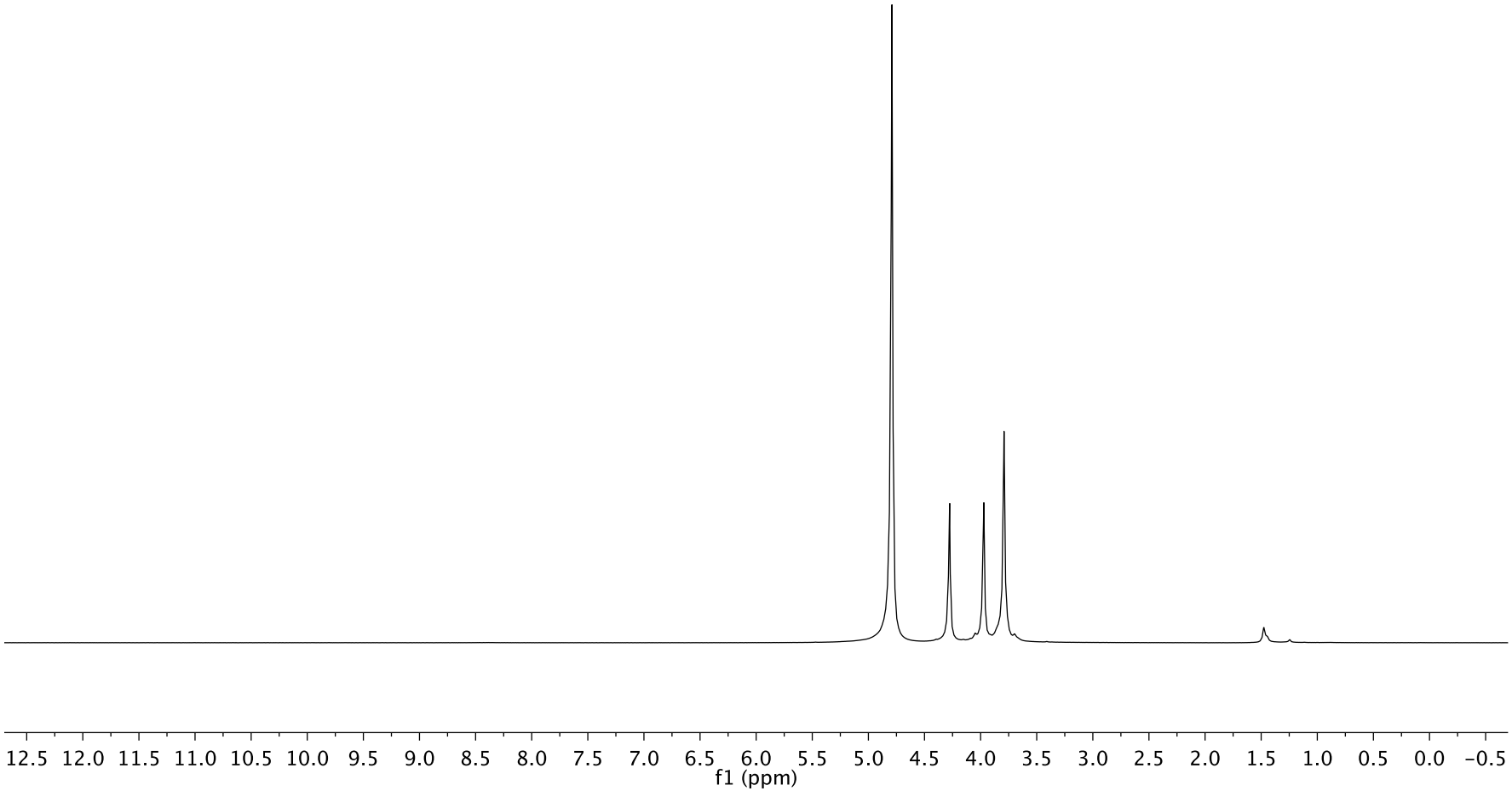
^{13}C NMR (151 MHz, D_2O) δ 193.8, 171.1, 53.5, 46.8, 30.9.

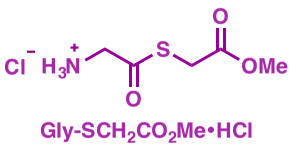
HRMS (ESI-TOF): calc'd for $C_5H_{10}NO_3S$ $[M+H]^+$ 164.0376; found 164.0376.



Gly-SCH$_2$CO$_2$Me·HCl

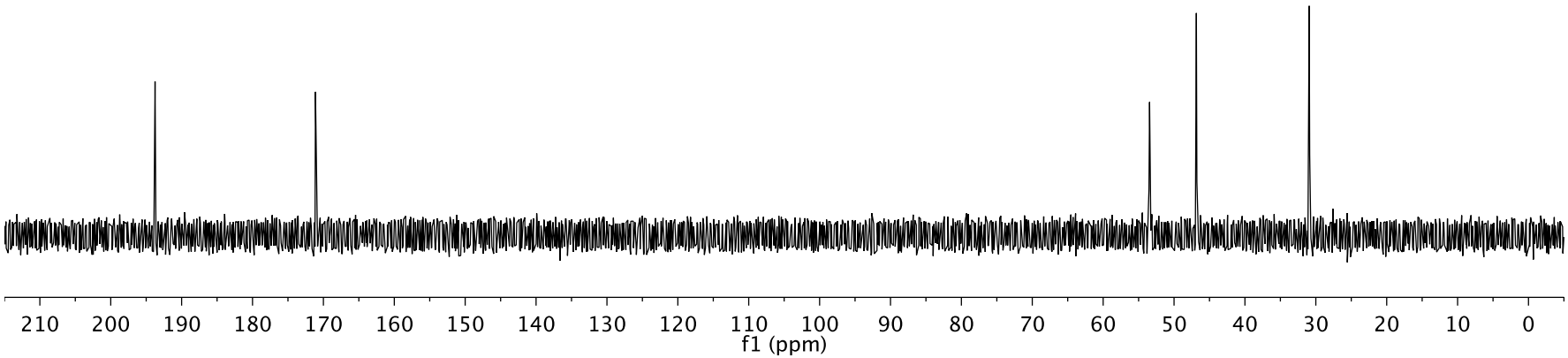
^{1}H, D$_2$O, 600 MHz





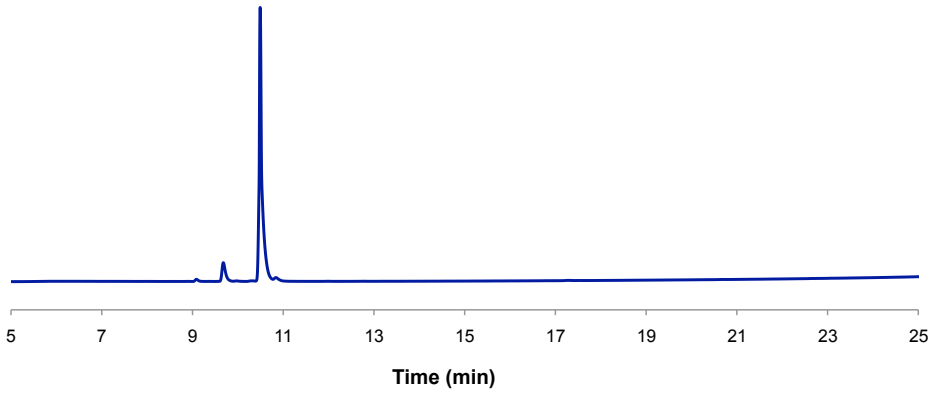
Gly-SCH$_2$CO$_2$Me•HCl

^{13}C, D$_2$O, 151 MHz

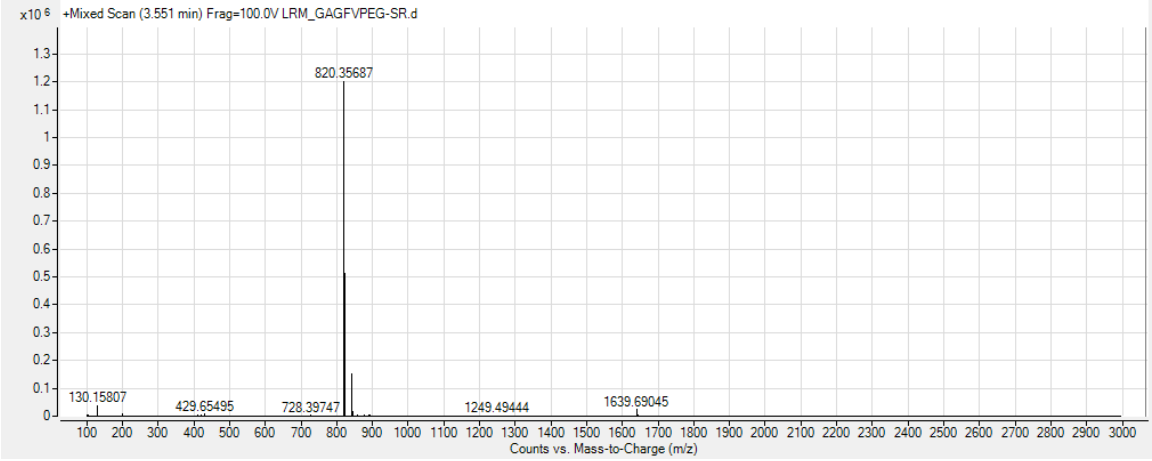


*Preparation of peptide thioester **S19***:

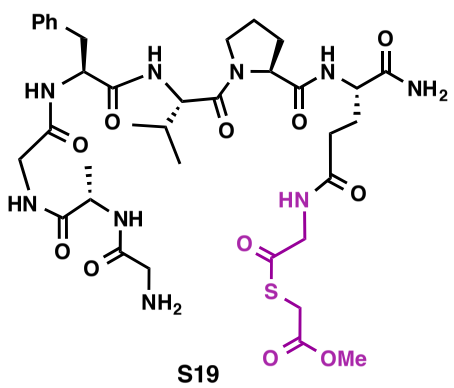
General iterative Fmoc-SPPS was used to prepare the precursor peptide, Boc-GAGFVPE(All)-Rink. The resin-bound peptide was deallylated using $Pd(PPh_3)_4$ and $PhSiH_3$ as described in the general solid-phase protocols. The preformed amino acid thioester, Gly-SCH_2CO_2Me•HCl, was next coupled to the resin (20 μmol scale). The thioester (10 equiv.) was dissolved in DMF (0.55 mL) and treated with DIEA (20 equiv.). PyAOP (10 equiv.) was added as a solid to the reaction vessel, followed by the addition of the solution of thioester. The resin was shaken at rt for 2 h then washed with DMF, and DCM. The peptide was cleaved from the resin upon treatment with a mixture of TFA/TIS/thioanisole/water (85:5:5:5 v/v/v/v) at rt for 2 h. The cleavage solution was concentrated under a stream of nitrogen and the crude peptide precipitated with cold ether. Purification by preparative reverse-phase HPLC (20% for 5 min, 20% to 70% over 30 min) afforded thioester **S19** (6.7 mg, 41% yield) as a white solid following lyophilization.



Purified peptide **S19** (Rt = 10.5 min, 5 to 100% B over 25 min, λ = 230 nm).



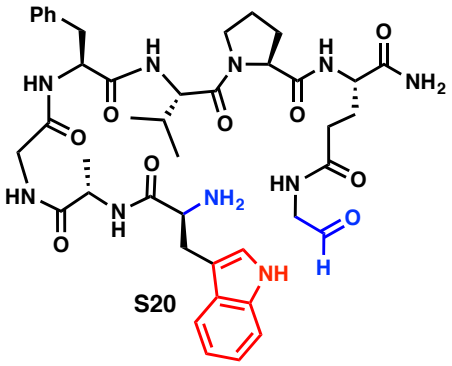
LRMS (ESI-TOF): calc'd for $C_{36}H_{54}N_9O_{11}S$ $[M+H]^+$ 820.37; found 820.36.



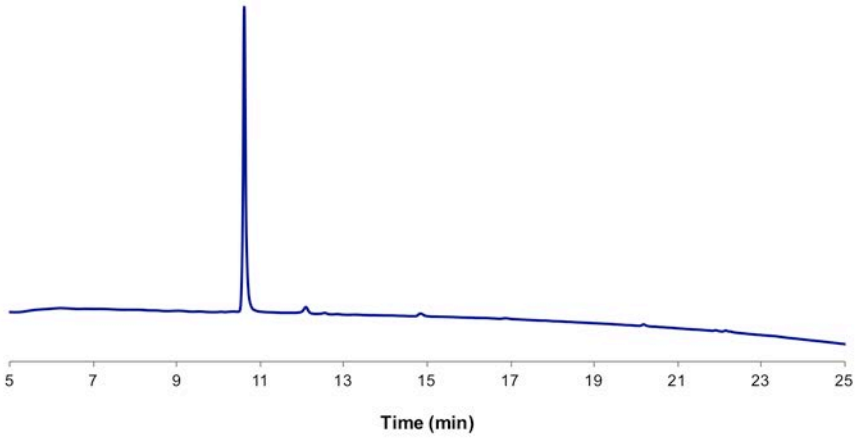
S19

^{1}H, D_2O, 600 MHz

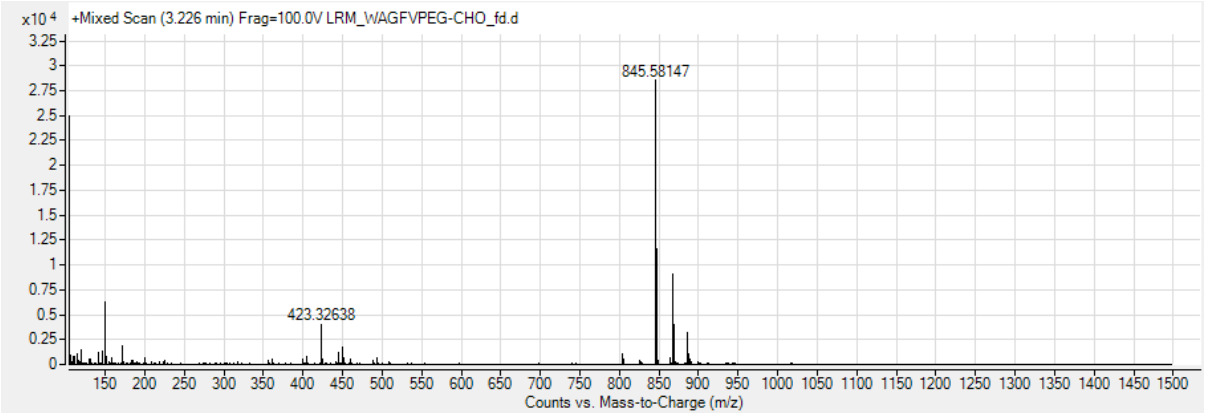
Peptide S20



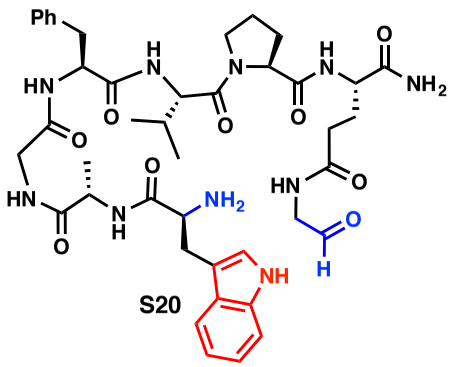
Amino aldehyde **S20** was prepared on a 34 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% B to 55% B over 25 min) to afford peptide **S20** (6.2 mg, 22% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S20** (Rt = 10.6 min, 5 to 100% B over 25 min, λ = 210 nm).



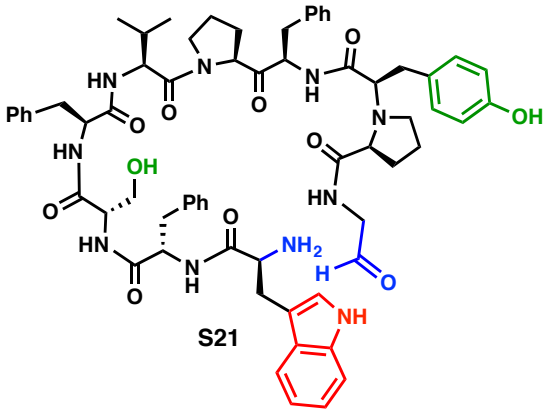
LRMS (ESI-TOF): calc'd for $C_{42}H_{57}N_{10}O_9$ $[M+H]^+$ 845.43; found 845.58.



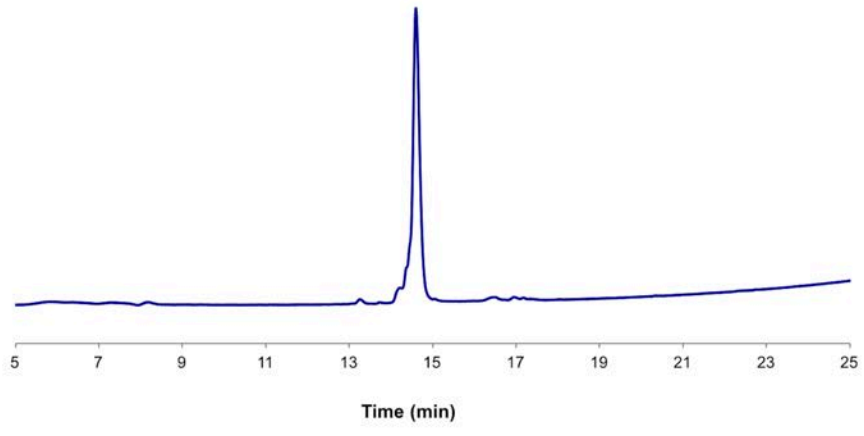
S20

^{1}H, DMSO-d$_6$, 600 MHz

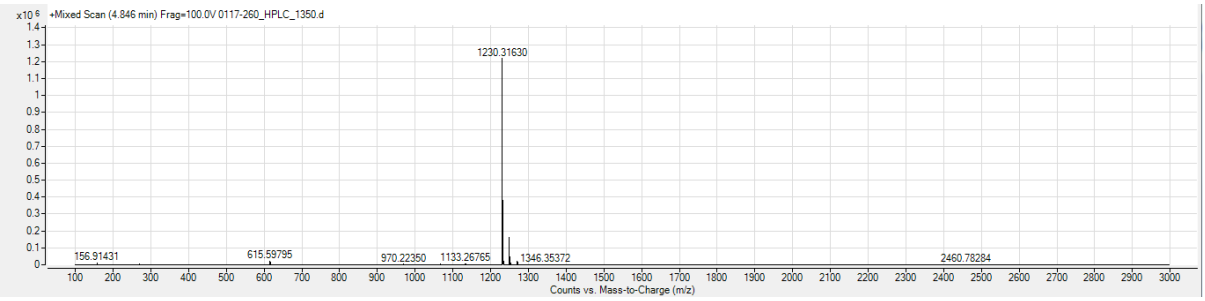
Peptide S21



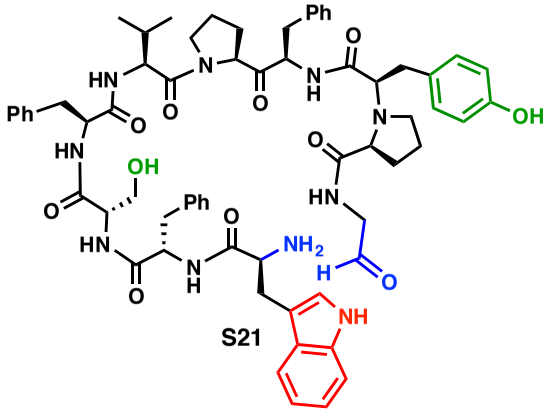
Amino aldehyde **S21** was prepared on a 35 μmol scale according to general procedure 2b. Fmoc-Gly-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (35% B for 5 min, then 35% B to 75% B over 30 min) to afford peptide **S21**(16.3 mg, 38% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S21** (Rt = 14.6 min, 5 to 100% B over 25 min, λ = 210 nm).



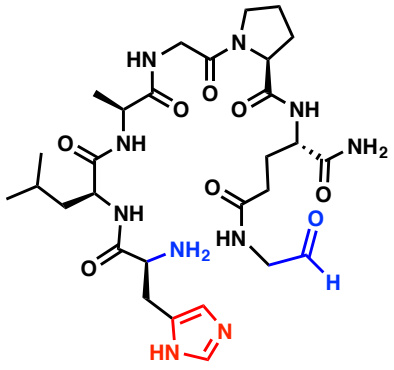
LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 1230.60; found 1230.32.



S21

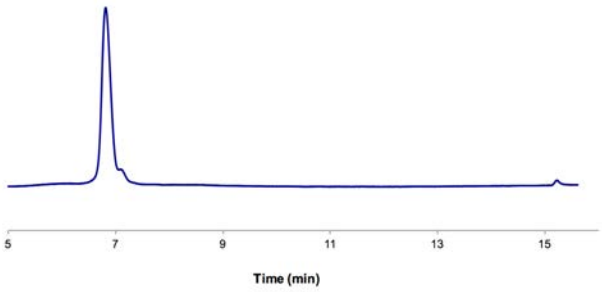
^{1}H, DMSO-d_6/D_2O (20:1 v/v), 600 MHz

Peptide S22

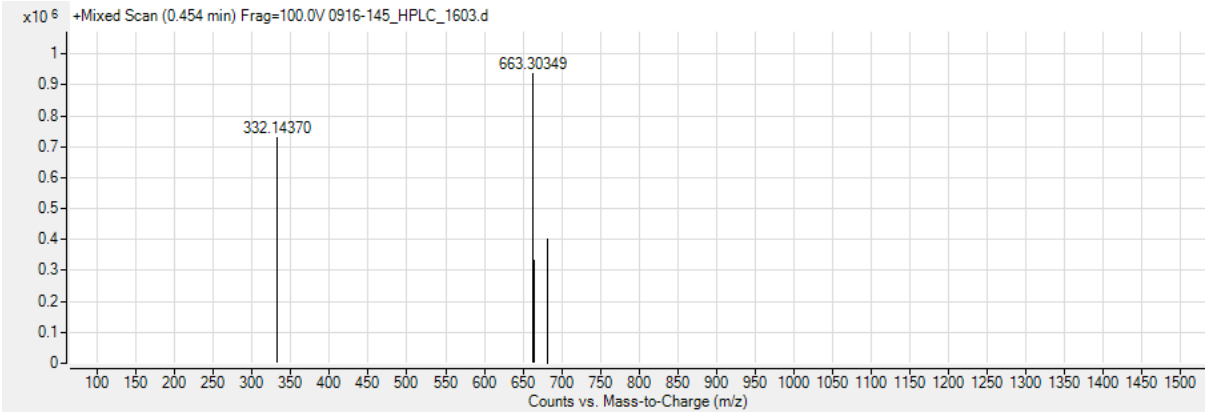


S22

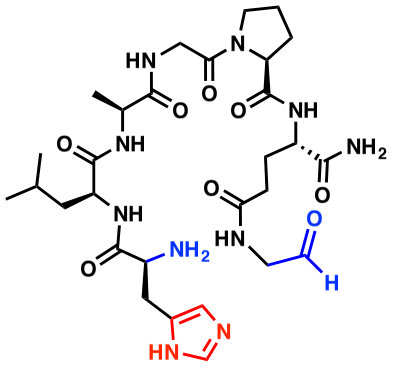
Amino aldehyde **S22** was prepared on a 170 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 40% B over 30 min) to afford peptide **S22** (31.6 mg, 28% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S22** (Rt = 6.80 min, 5 to 100% B over 25 min, λ = 210 nm).



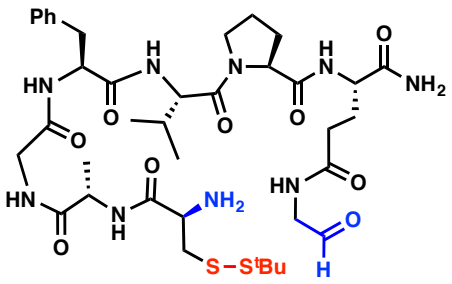
LRMS (ESI-TOF): calc'd for $C_{26}H_{41}N_9O_7$ $[M+H]^+$ 663.36; found 663.30, $[M+2H]^{2+}$ 332.18; found 332.14.



S22

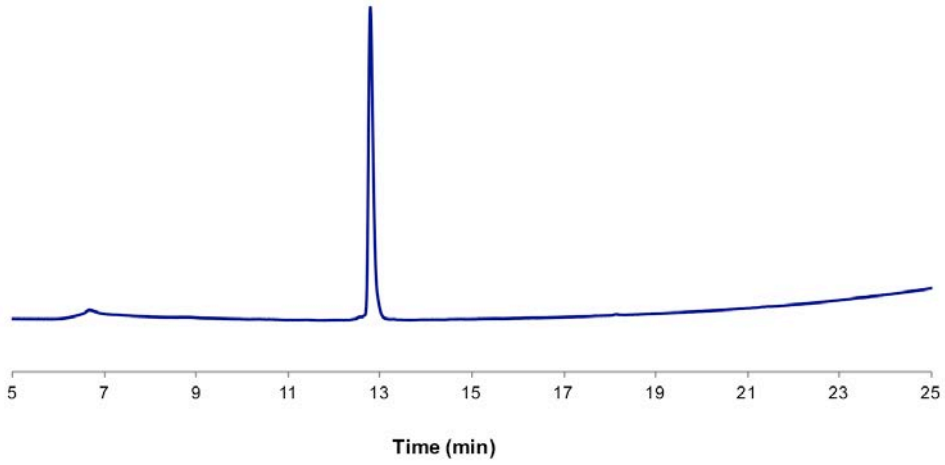
^{1}H, DMSO-d$_6$, 600 MHz

Peptide S23

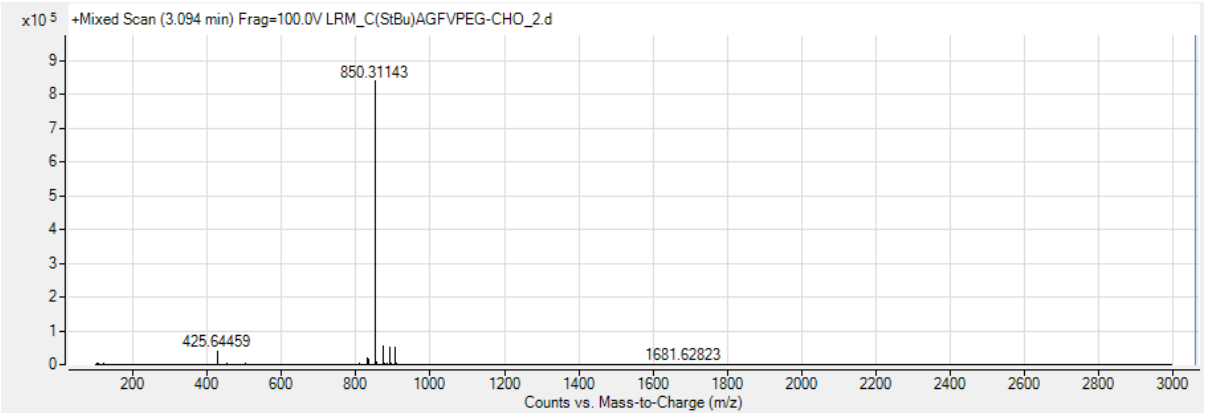


S23

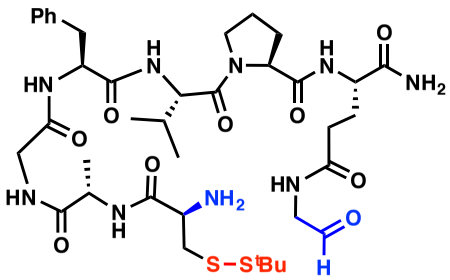
Amino aldehyde **S23** was prepared on a 23 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% B to 60% B over 30 min) to afford peptide **S23** (6.2 mg, 32% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S23** (Rt = 12.8 min, 5 to 100% B over 25 min, λ = 230 nm).



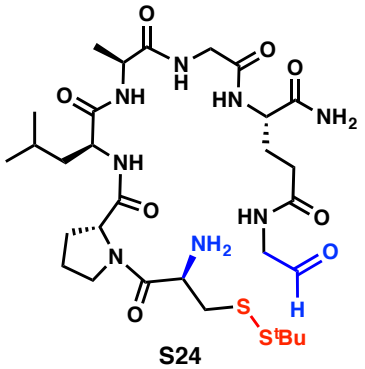
LRMS (ESI-TOF): calc'd for $C_{38}H_{60}N_9O_9S_2$ $[M+H]^+$ 850.40; found 850.31.



S23

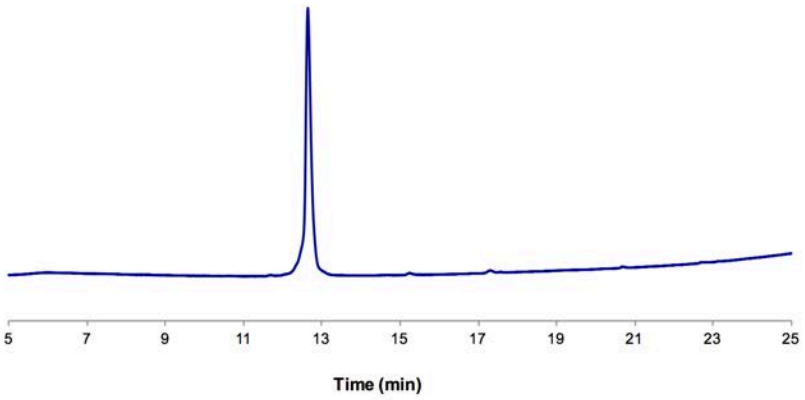
^{1}H, DMSO-d$_6$, 600 MHz

Peptide S24

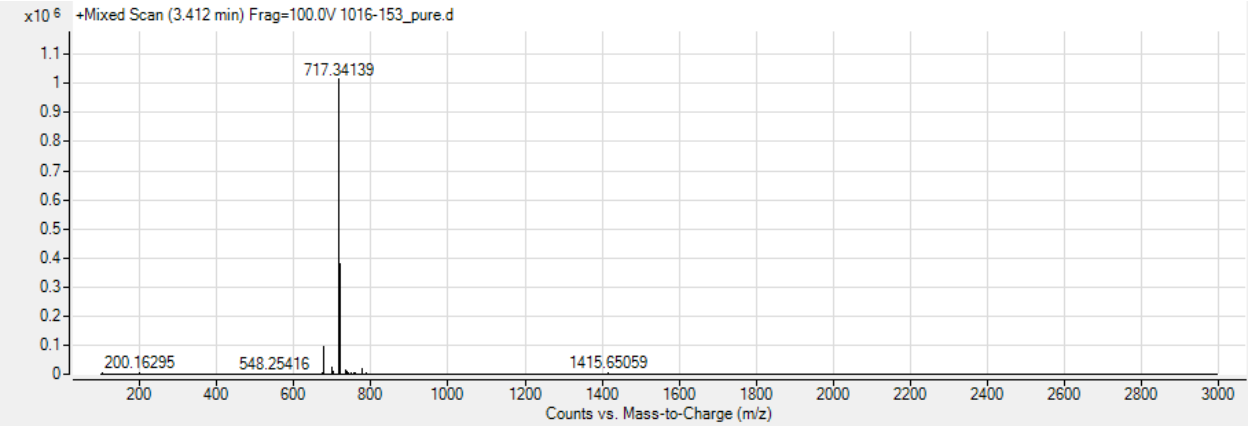


S24

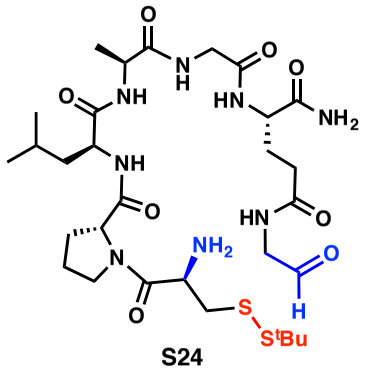
Amino aldehyde **S24** was prepared on a 113 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% B to 40% B over 30 min) to afford peptide **S24** (27.1mg, 34% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S24** (Rt = 12.64 min, 5 to 100% B over 25 min, λ = 230 nm).



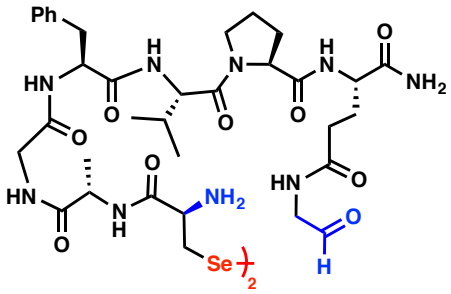
LRMS (ESI-TOF): calc'd for $C_{26}H_{41}N_9O_7$ $[M+H]^+$ 717.34; found 717.34.



S24

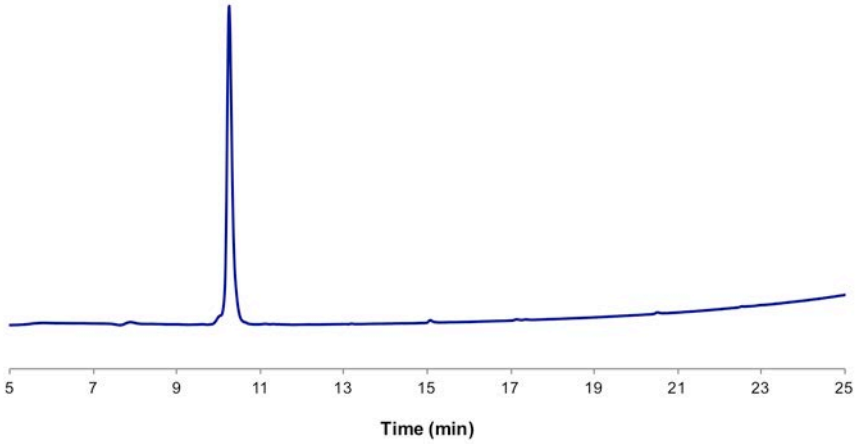
^{1}H, DMSO-d_6, 600 MHz

Peptide S25

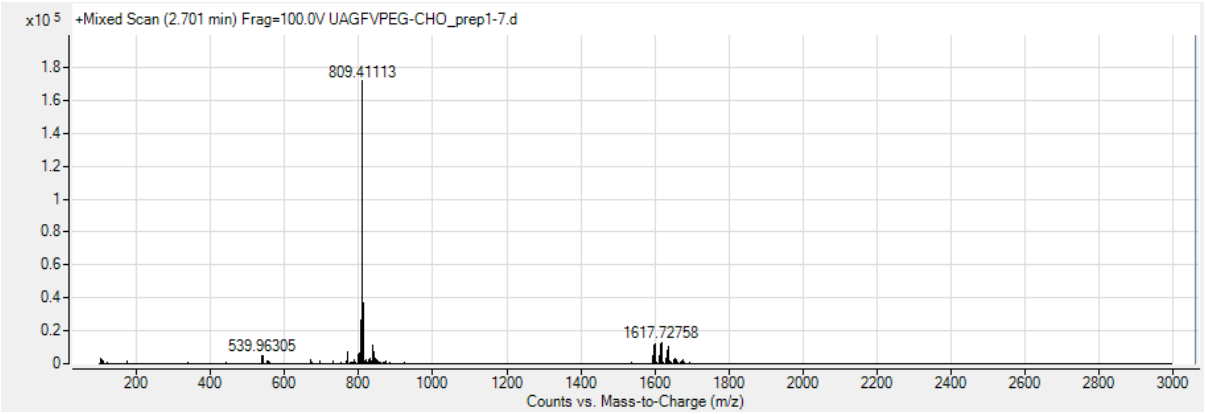


S25

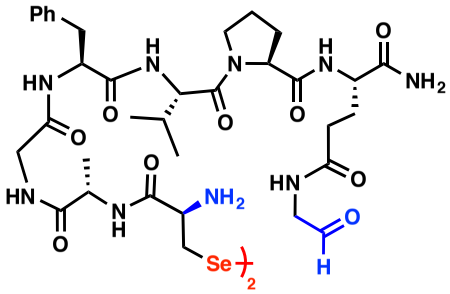
Amino aldehyde **S25** was prepared on a 40 μmol scale on Rink amide resin using standard Fmoc-SPPS. The aldehyde was incorporated according to general procedure 1. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% B to 50% B over 25 min) to afford peptide **S25** (5.8 mg, 18% yield based on the original resin loading) as a white solid following lyophilization.



Purified peptide **S25** (Rt = 10.2 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{68}H_{101}N_{18}O_{18}Se_2$ $[M+H]^+$ 1617.59; found 1617.73, $[M+2H]^{2+}$ 809.30; found 809.41.

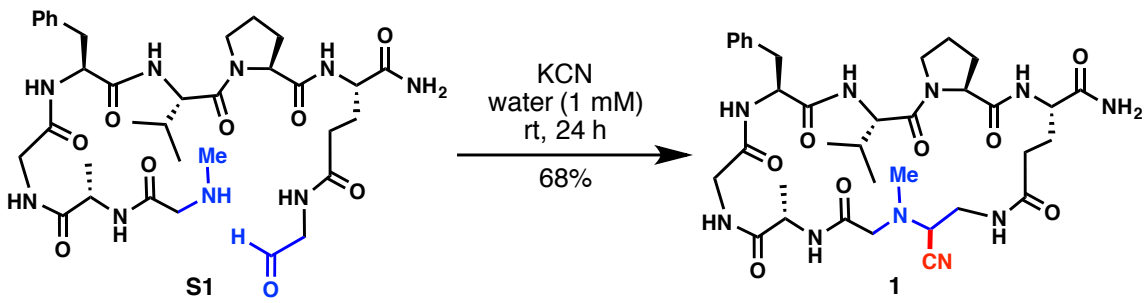


S25

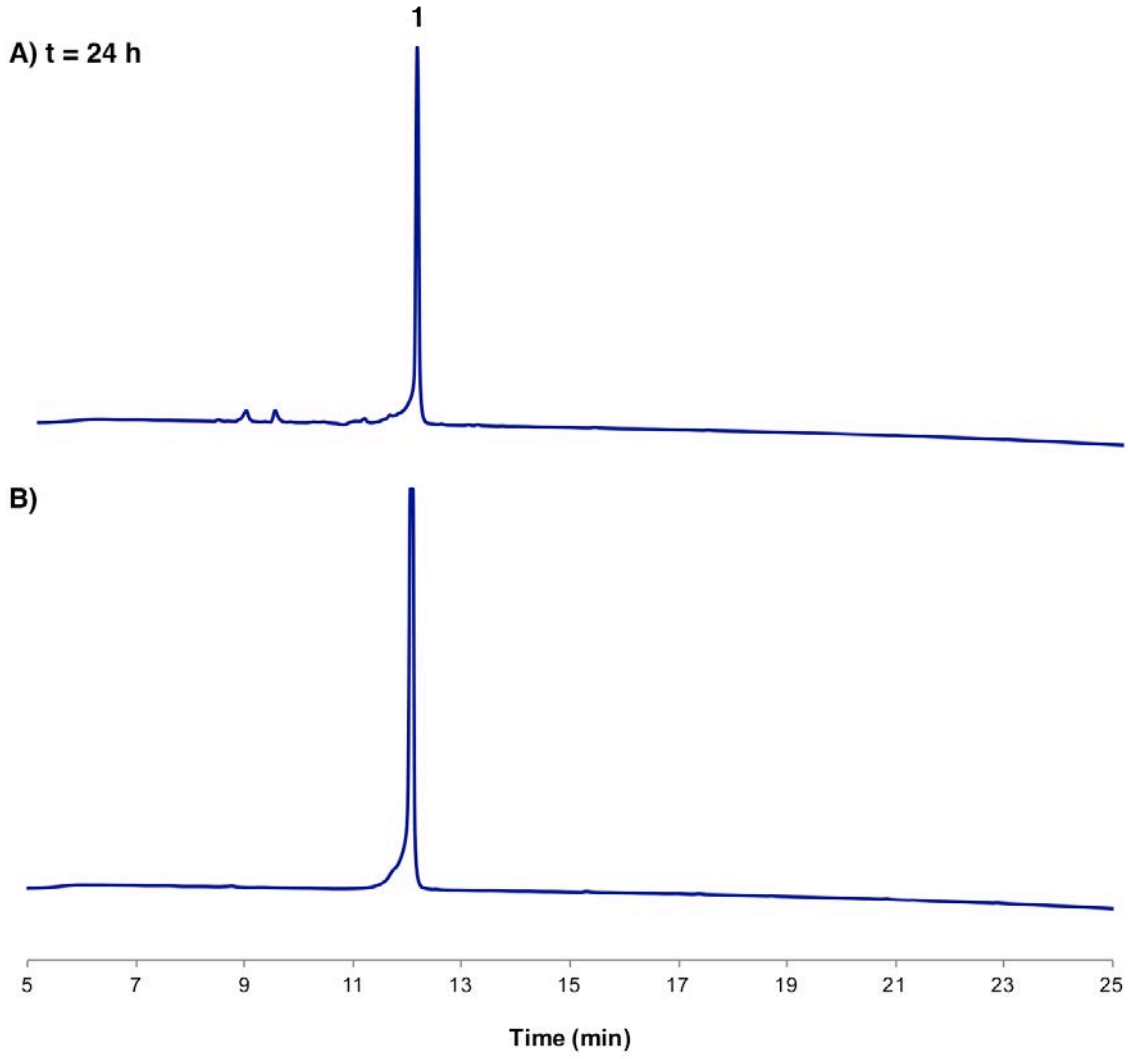
^{1}H, DMSO-d$_{6}$, 600 MHz

Characterization Data for Strecker Macrocyclization

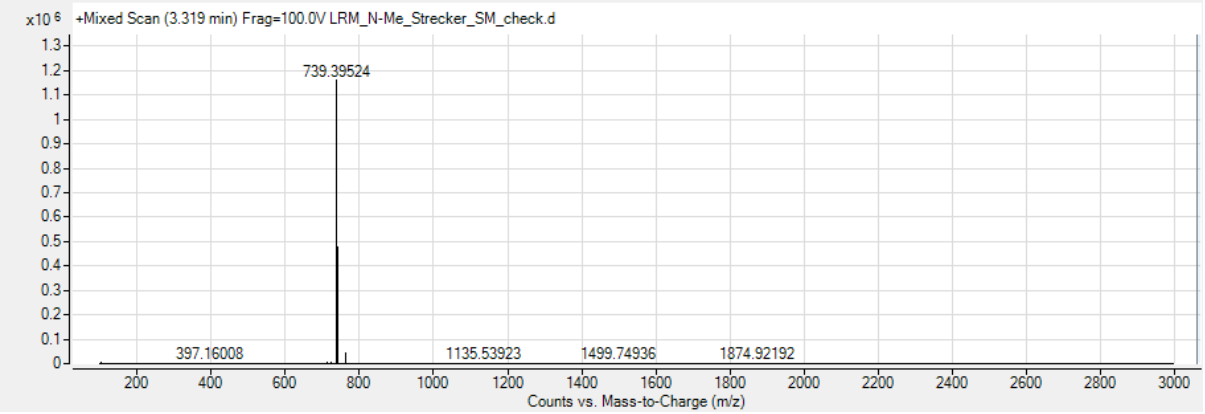
Peptide 1



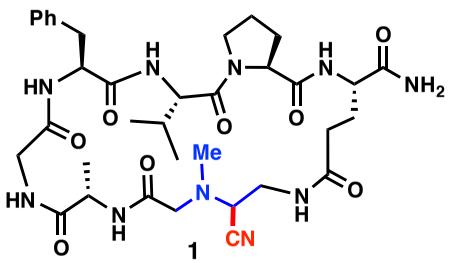
Peptide **1** was prepared according to general procedure 3 from linear amino aldehyde **S1** (5.8 mg, 7.9 μmol). At t = 24 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% to 50% B over 30 min) to afford peptide macrocycle **1** (4.0 mg, 68% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **1** from amino aldehyde **S1** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **1** (Rt = 12.1 min, 5 to 100% B over 25 min, λ = 210 nm).

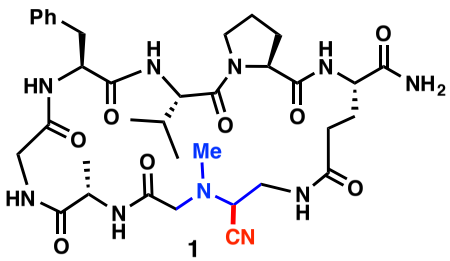


LRMS (ESI-TOF): calc'd for $C_{35}H_{51}N_{10}O_8$ $[M+H]^+$ 739.39; found 739.40.



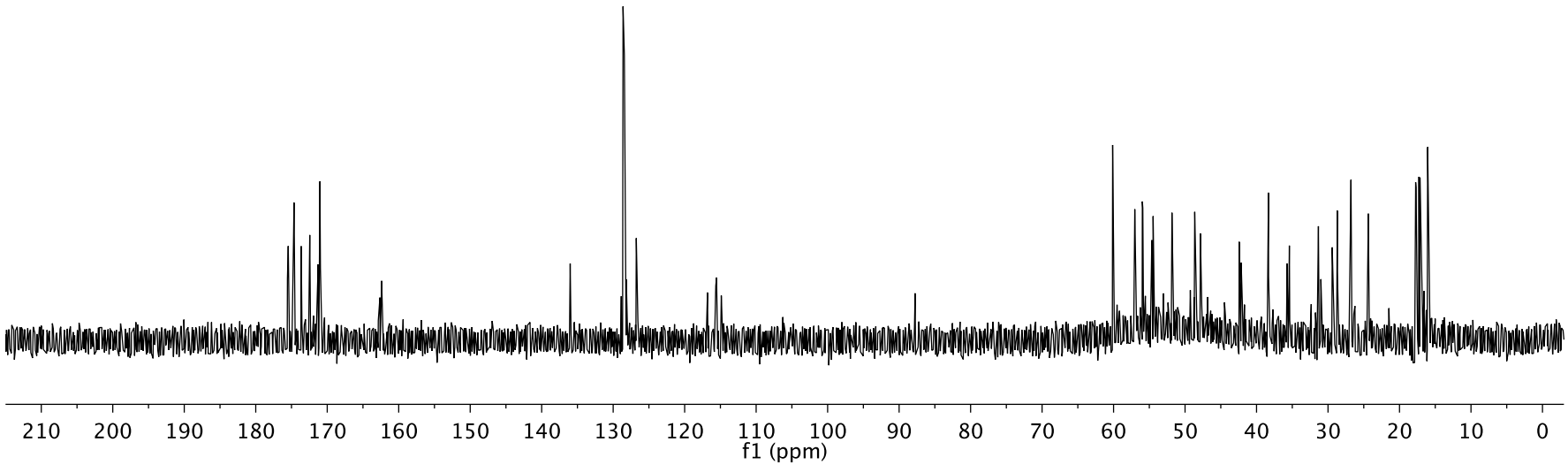
1

^{1}H, D_2O, 600 MHz

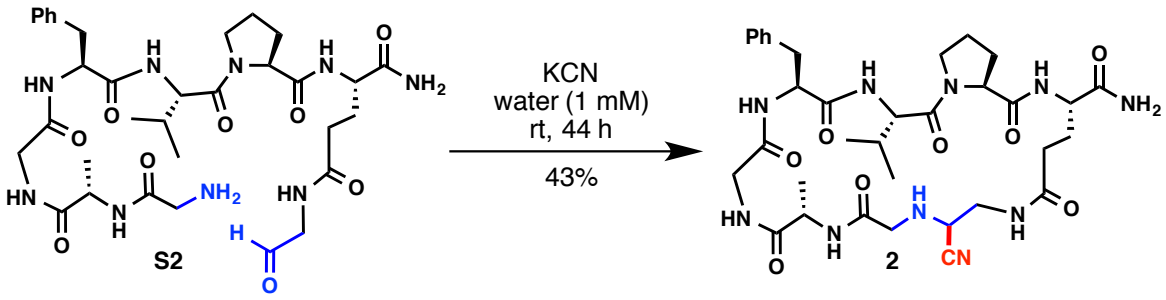


1

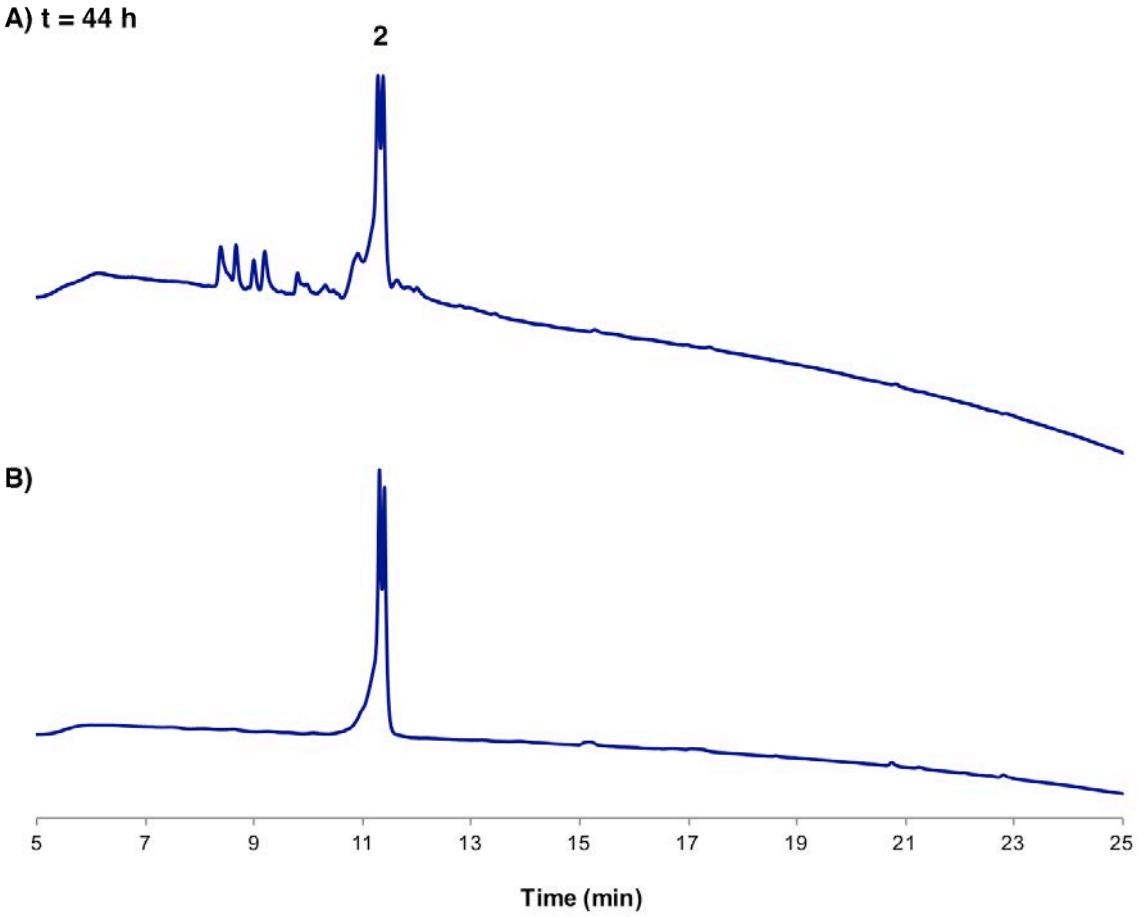
^{13}C, D_2O, 151 MHz



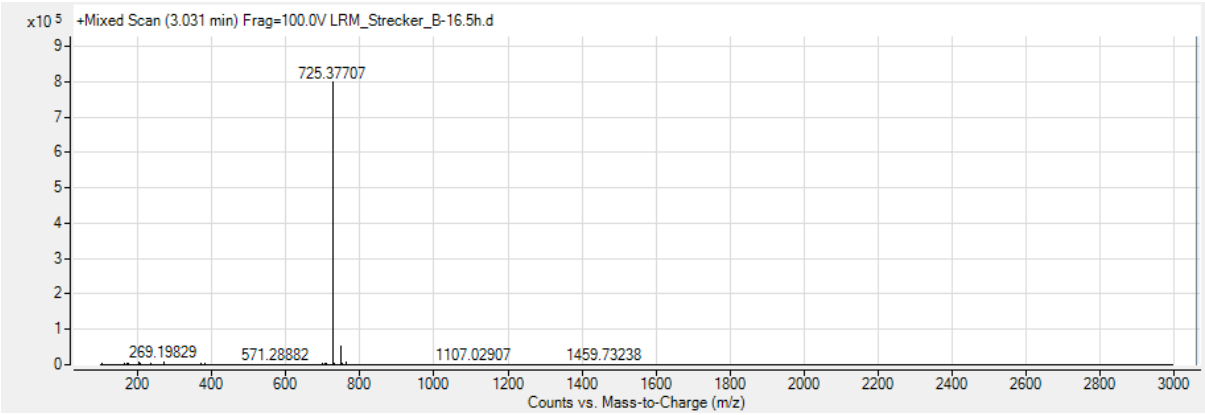
Peptide 2



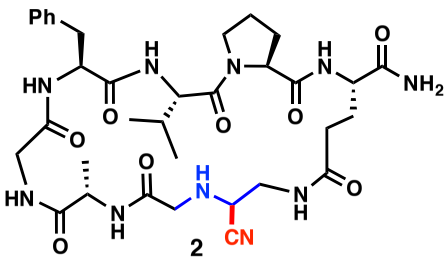
Peptide **2** was prepared according to general procedure 3 from linear amino aldehyde **S2** (3.7 mg, 5.2 µmol). At t = 44 h, the reaction was purified by preparative reverse-phase HPLC (5% B for 5 min, 5% B to 20% B over 5 min, then 20% to 55% B over 30 min) to afford peptide macrocycle **2** (1.6 mg, 43% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **2** from amino aldehyde **S2** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **2** (Rt = 11.3, 11.4 min, 5 to 100% B over 25 min, λ = 210 nm).



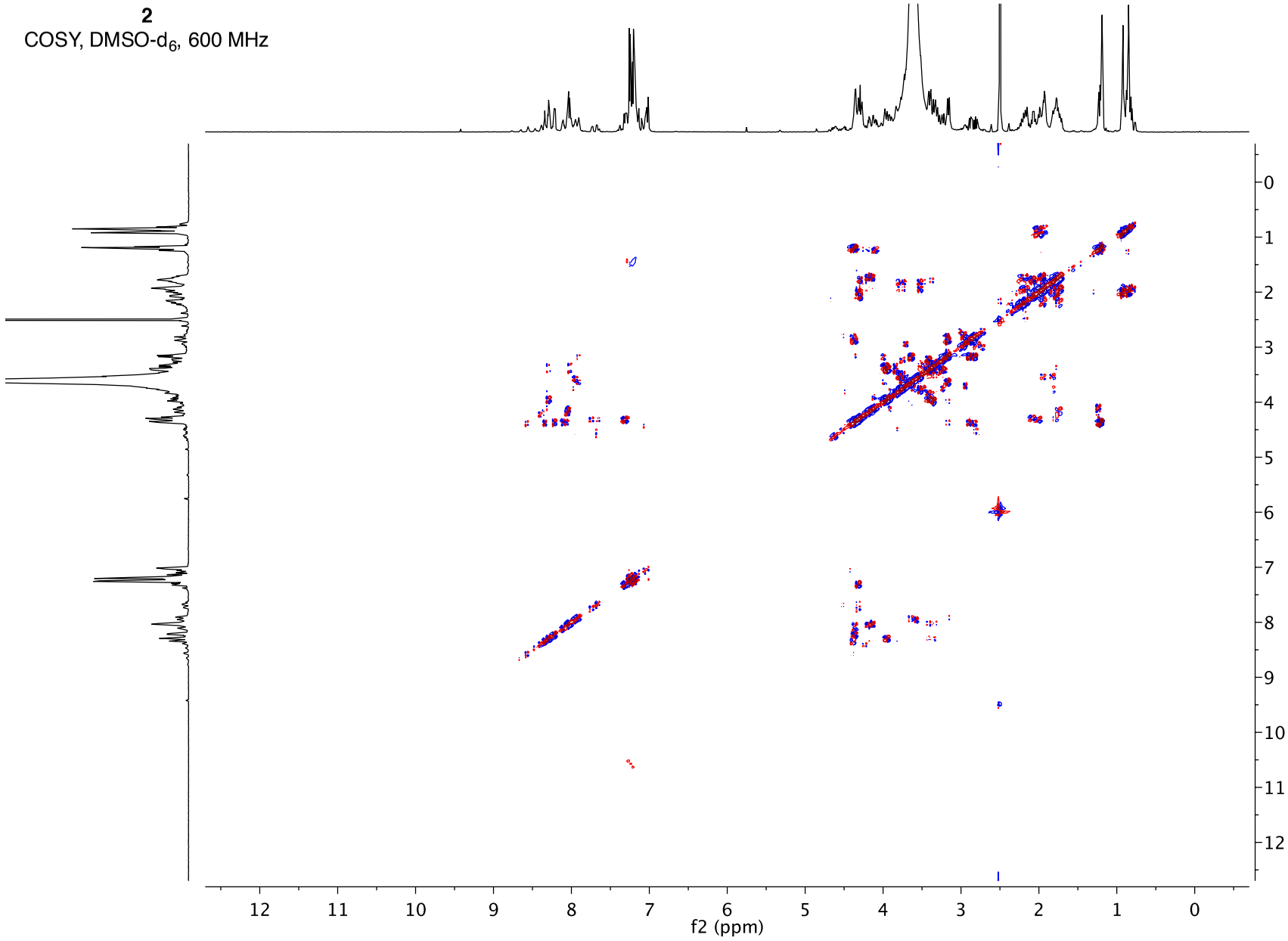
LRMS (ESI-TOF): calc'd for $C_{34}H_{49}N_{10}O_8$ $[M+H]^+$ 725.37; found 725.38.



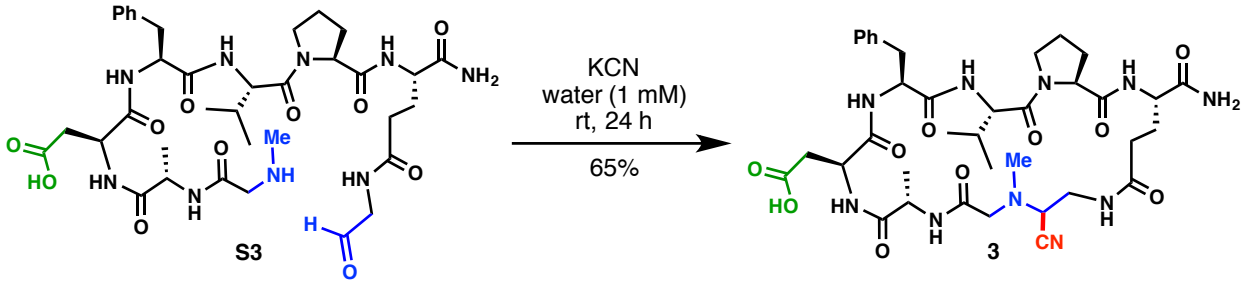
^{1}H, DMSO-d$_6$, 600 MHz

2

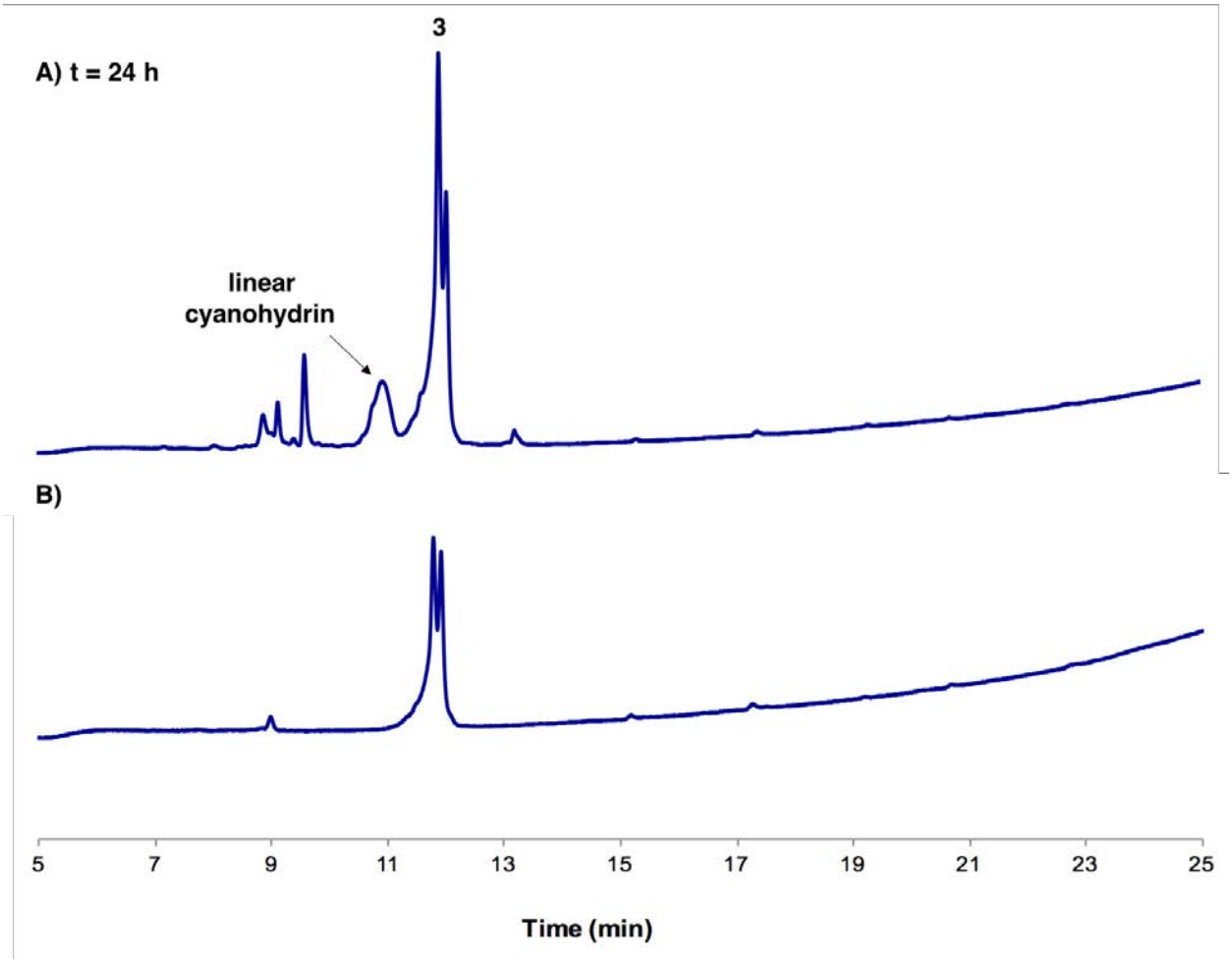
COSY, DMSO-d_6, 600 MHz



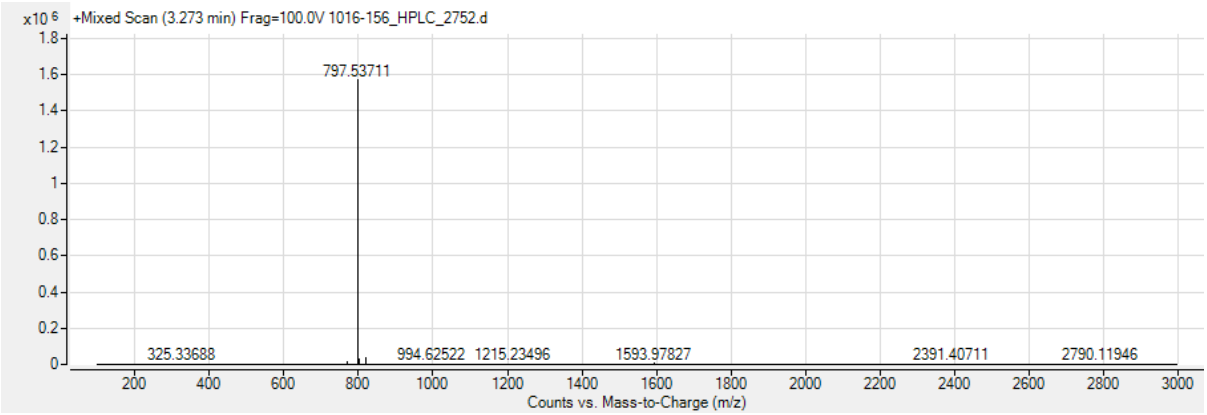
Peptide 3



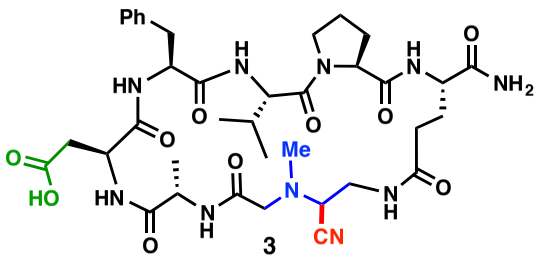
Peptide **3** was prepared according to general procedure 3 from linear amino aldehyde **S3** (10.0 mg, 13 μmol). At t = 24 h, the reaction was purified by preparative reverse-phase HPLC (10% B for 5 min, then 10% B to 55% B over 30 min) to afford peptide macrocycle **3** (6.6 mg, 65% yield) as an inseparable mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **3** from amino aldehyde **S3** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **3** (Rt = 11.8, 11.9 min, 5 to 100% B over 25 min, λ = 230 nm).
Note: Diastereomers were inseparable by preparative reverse-phase HPLC.



LRMS (ESI-TOF): calc'd for $C_{37}H_{52}N_{10}O_{10}$ $[M+H]^+$ 797.39; found 797.54.

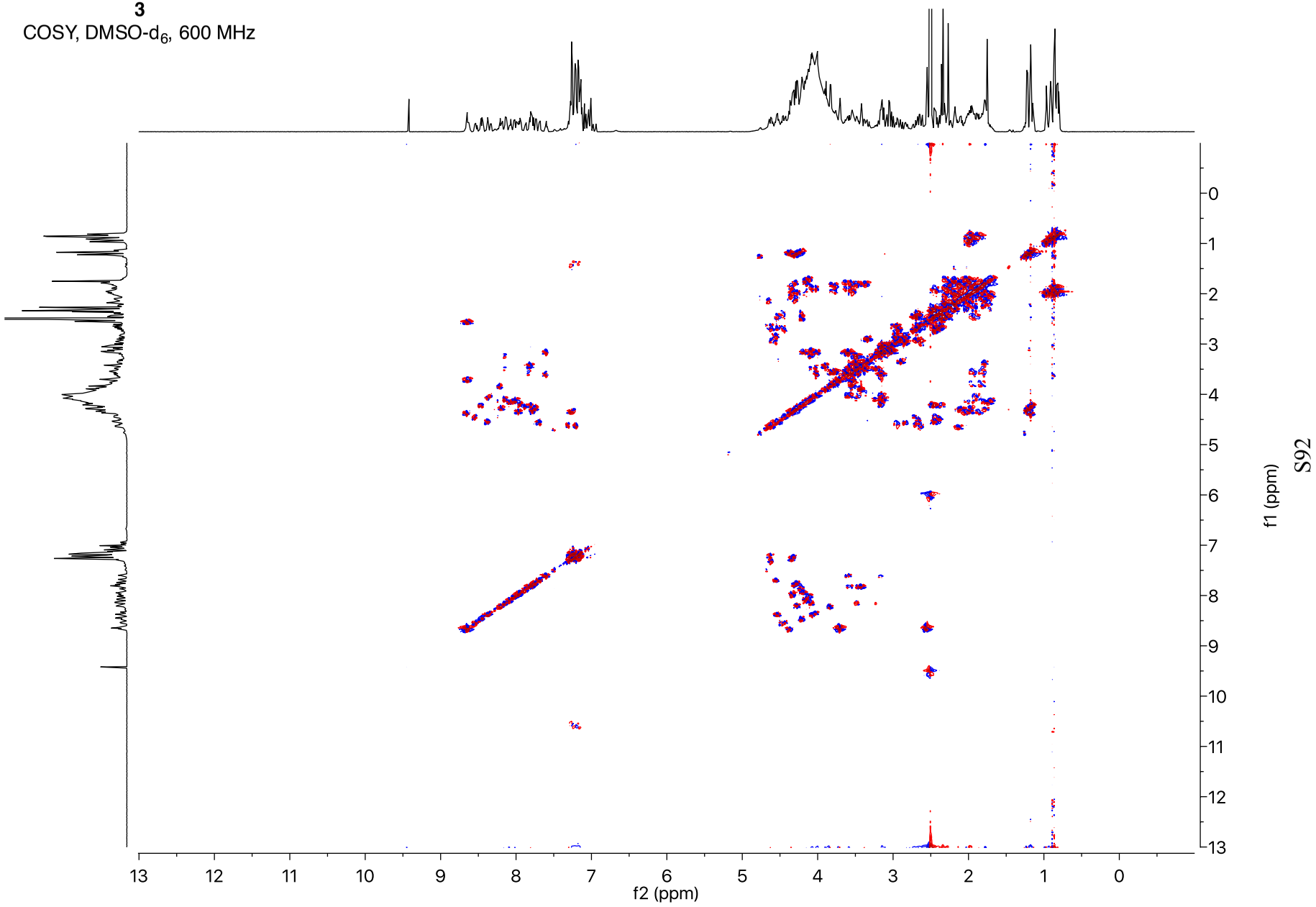


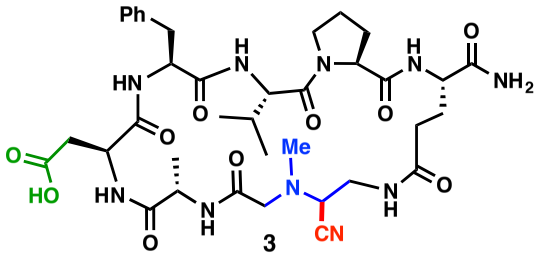
3

^{1}H, DMSO-d$_{6}$, 600 MHz

3

COSY, DMSO-d_6, 600 MHz

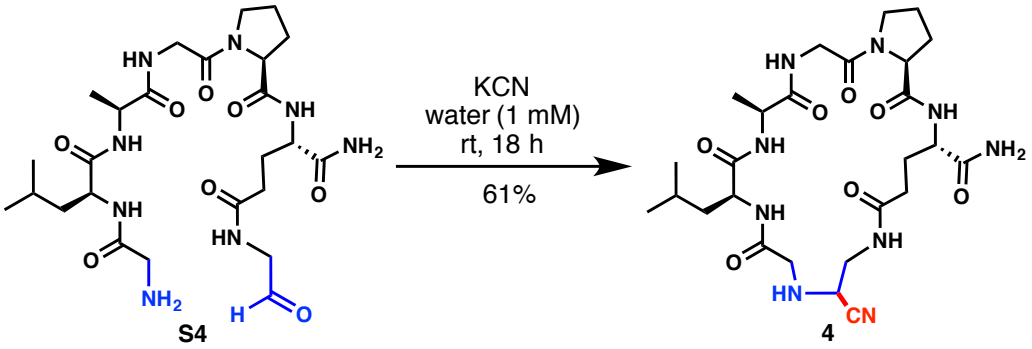




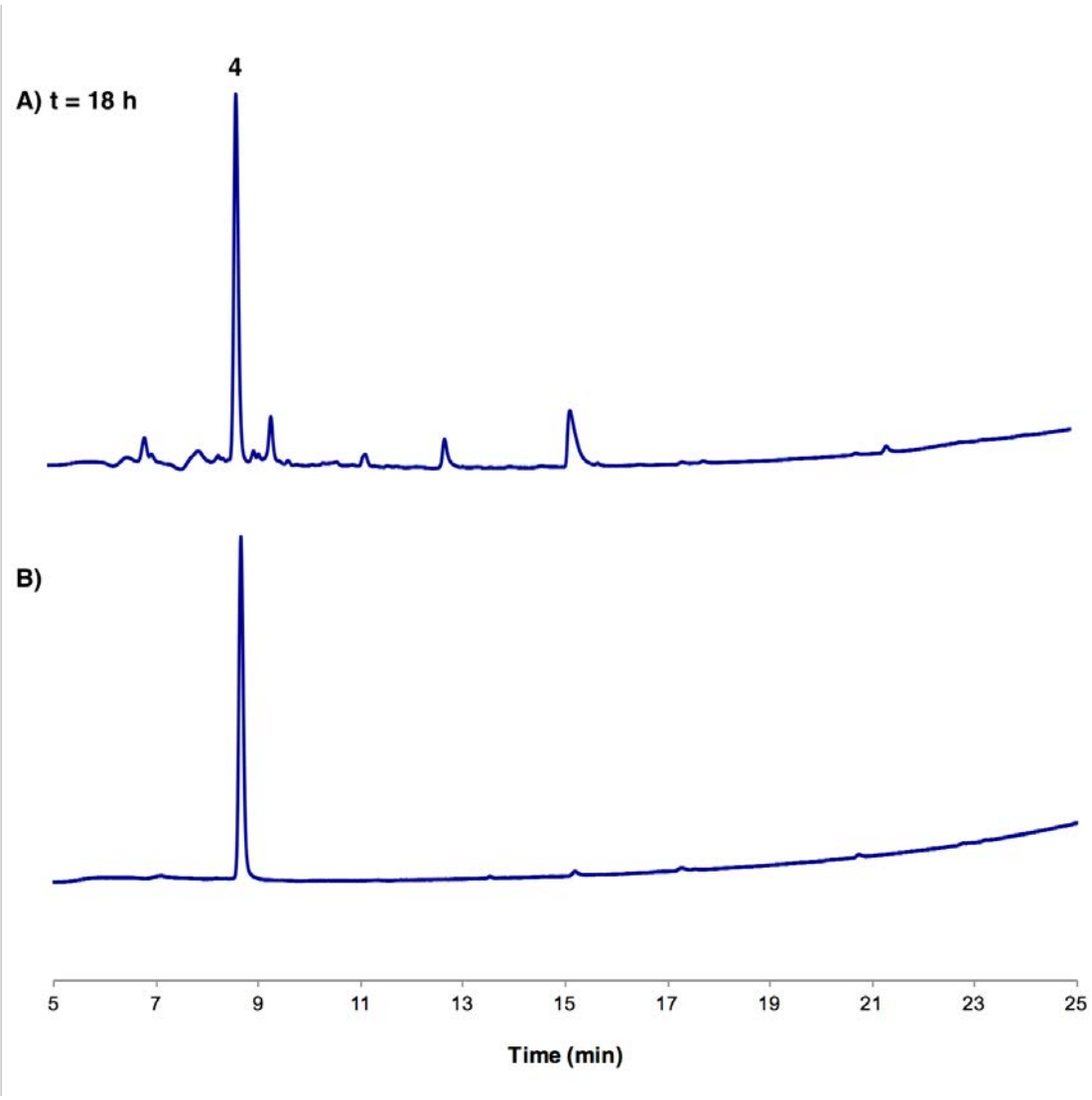
3

^{13}C, DMSO-d$_6$, 151 MHz

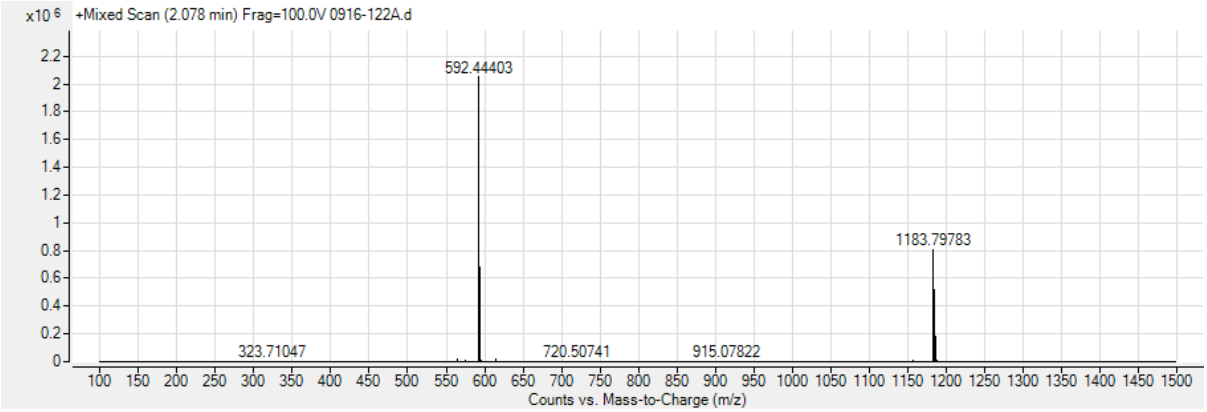
Peptide 4



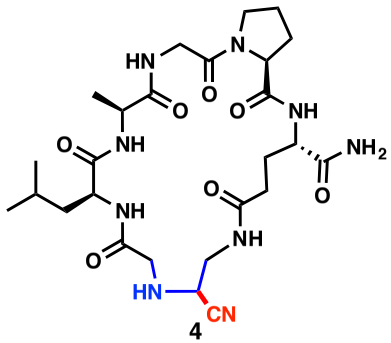
Peptide **4** was prepared according to general procedure 3 from linear amino aldehyde **S4** (10.0 mg, 17 μmol). At t = 18 h, the reaction was purified by preparative reverse-phase HPLC (10% B for 5 min, 10% B to 50% B over 30 min) to afford peptide macrocycle **4** (6.1 mg, 61% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **4** from amino aldehyde **S4** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **4** (Rt = 8.7 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{26}H_{41}N_9O_7$ $[M+H]^+$ 592.32; found 592.44.

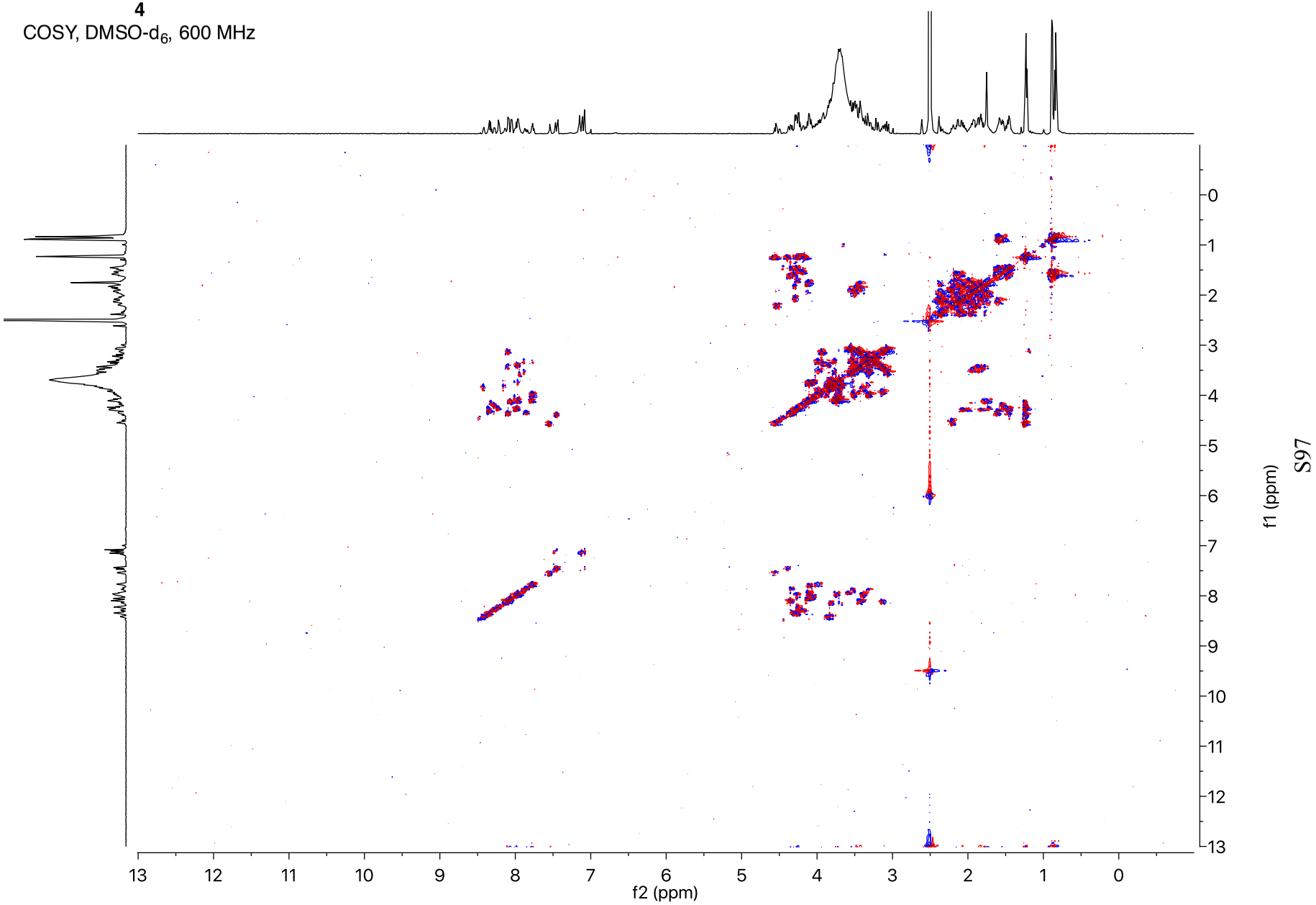


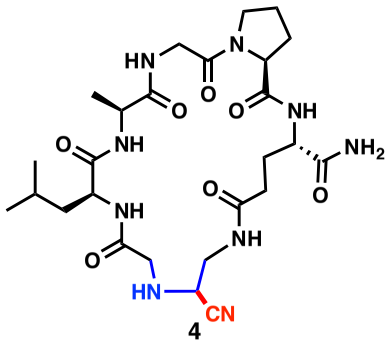
4

^{1}H, DMSO-d$_6$, 600 MHz

4

COSY, DMSO-d_6, 600 MHz

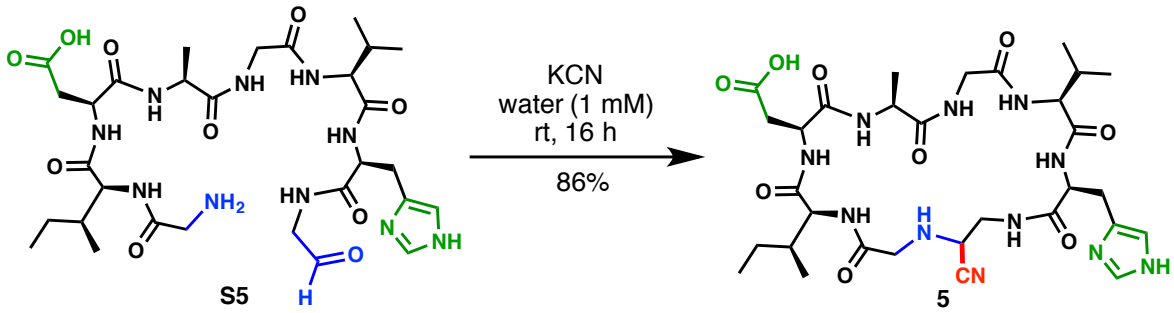




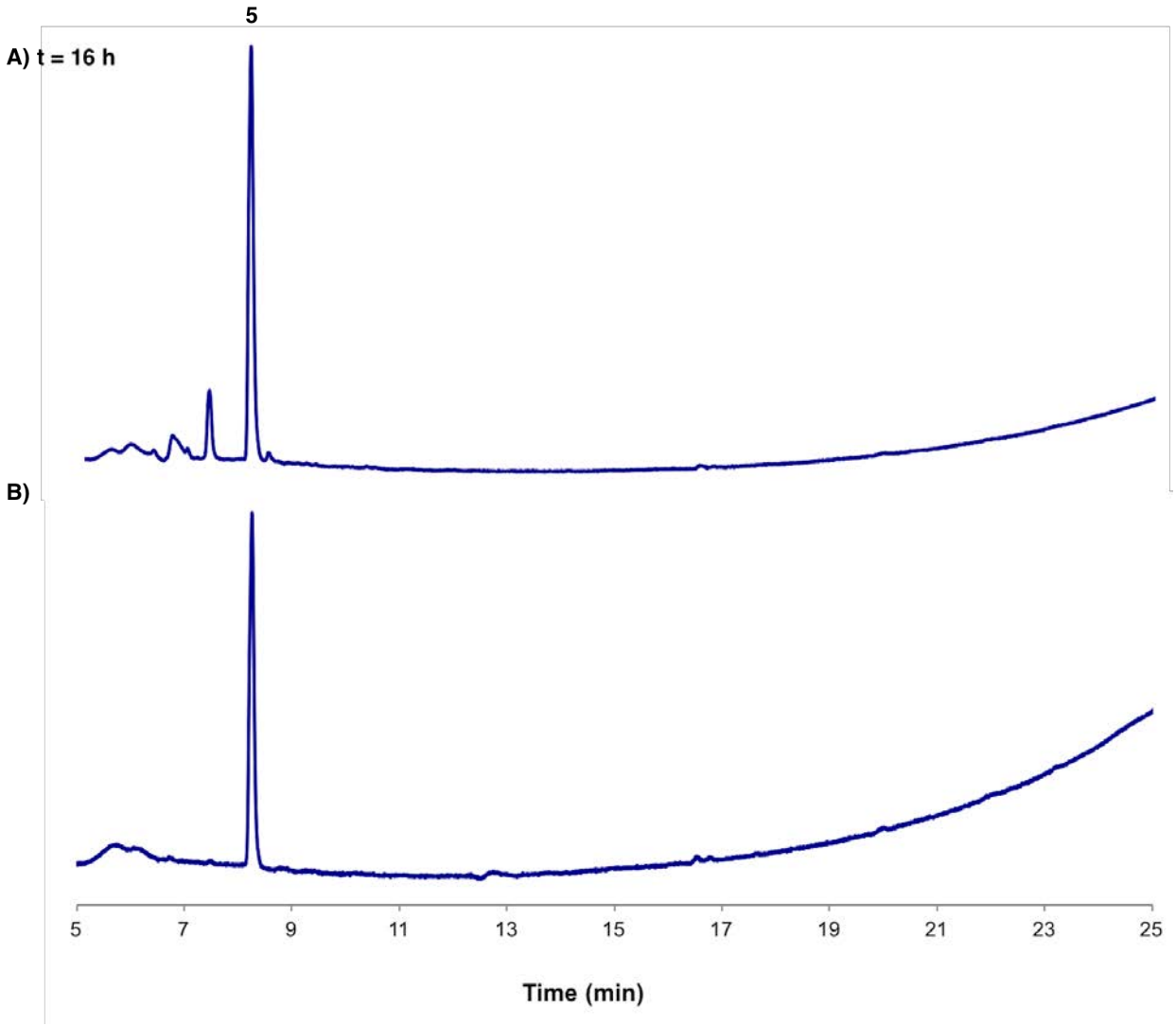
4

^{13}C, DMSO-d$_6$, 151 MHz

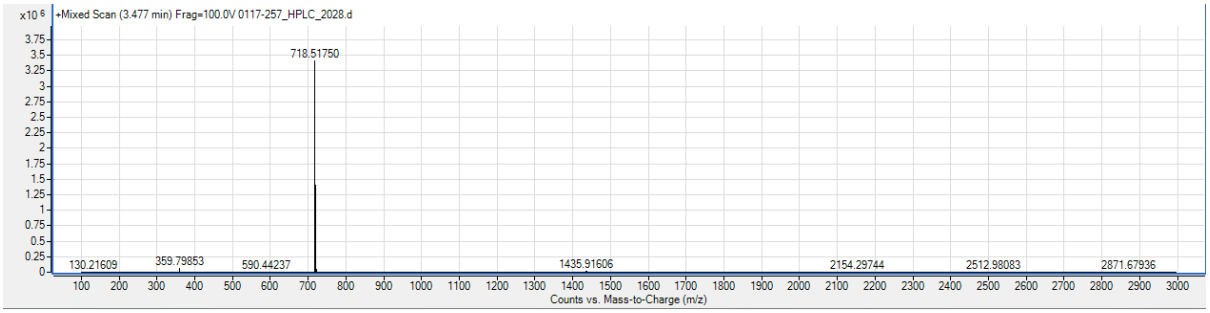
Peptide 5



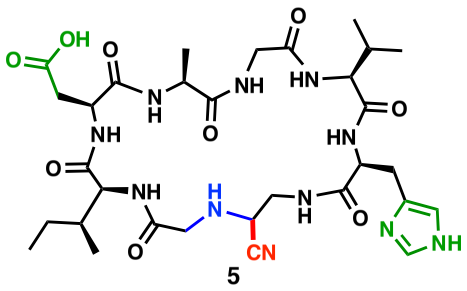
Peptide **5** was prepared according to general procedure 3 from linear amino aldehyde **S5** (5.6 mg, 7.90 μmol). At t = 16 h, the reaction was purified by preparative reverse-phase HPLC (10% B for 5 min, then 10% to 45% B over 30 min) to afford peptide macrocycle **5** (4.9 mg, 86% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **5** from amino aldehyde **S5** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **5** (Rt = 8.3 min, 5 to 100% B over 25 min, λ = 210 nm).

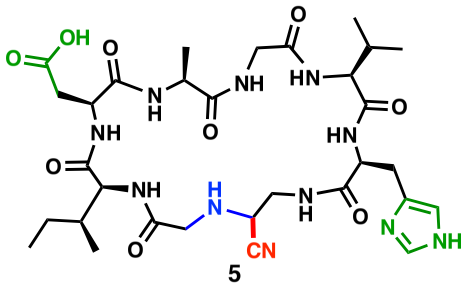


LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 718.36; found 718.52.



5

^{1}H, DMSO-d$_6$/D$_2$O (20:1 v/v), 600 MHz

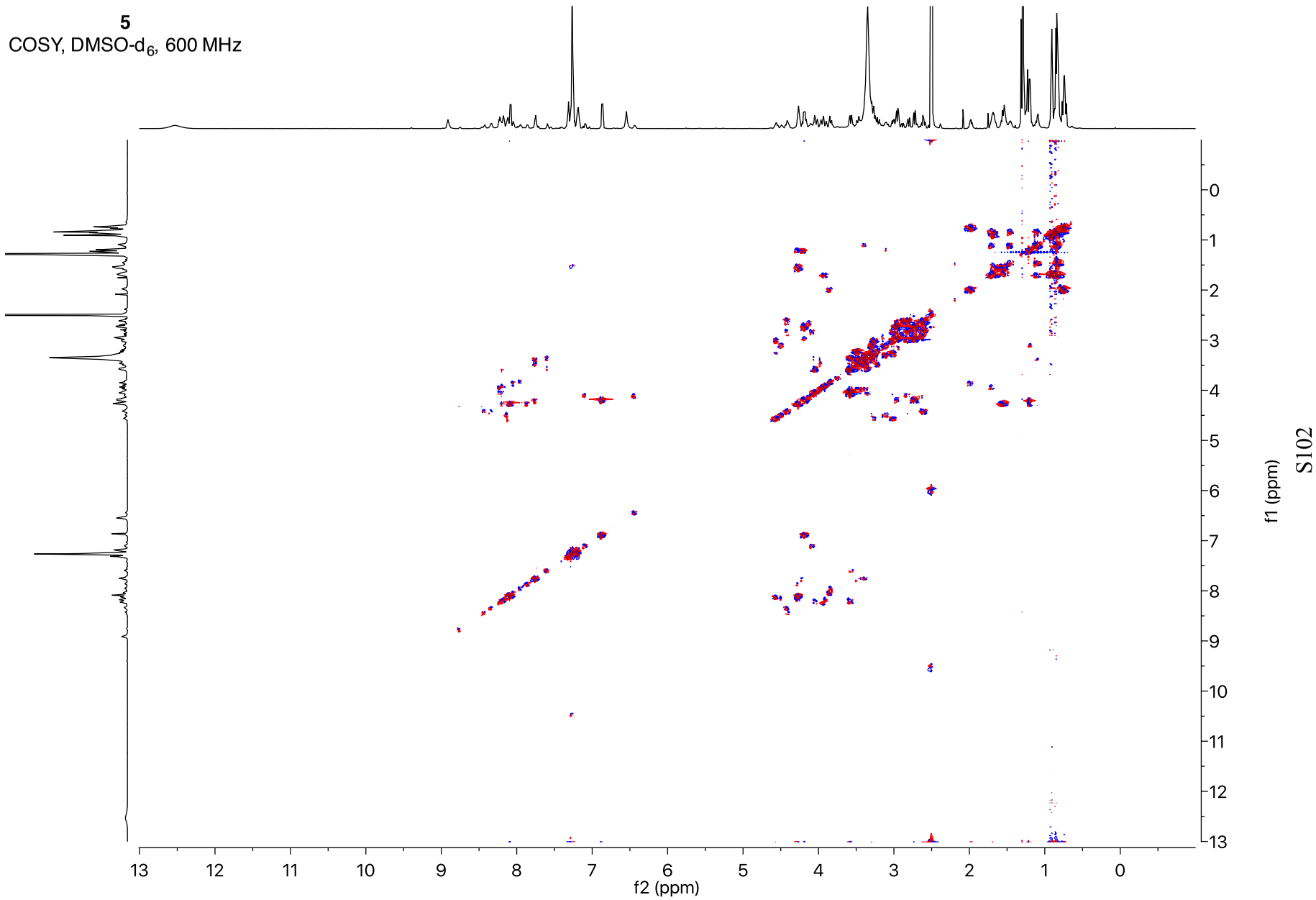


5

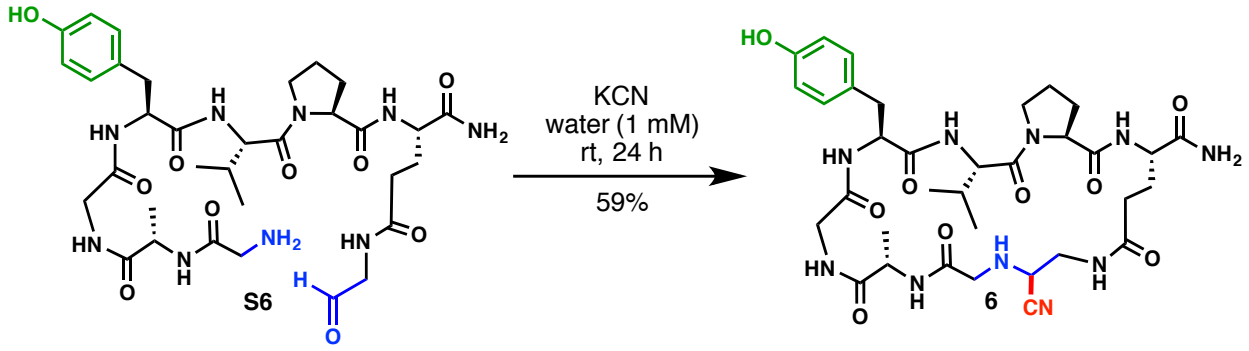
^{1}H, DMSO-d$_6$, 600 MHz

5

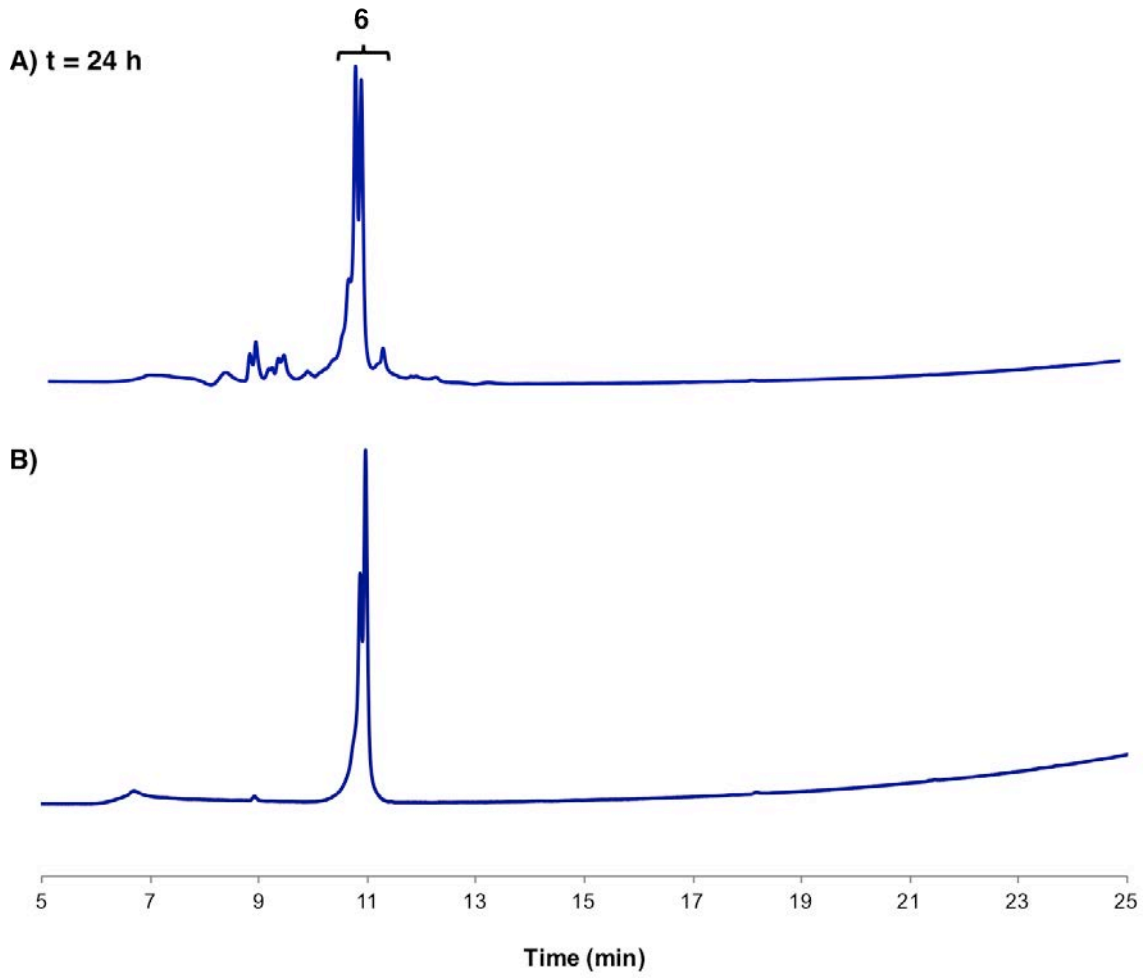
COSY, DMSO-d_6, 600 MHz



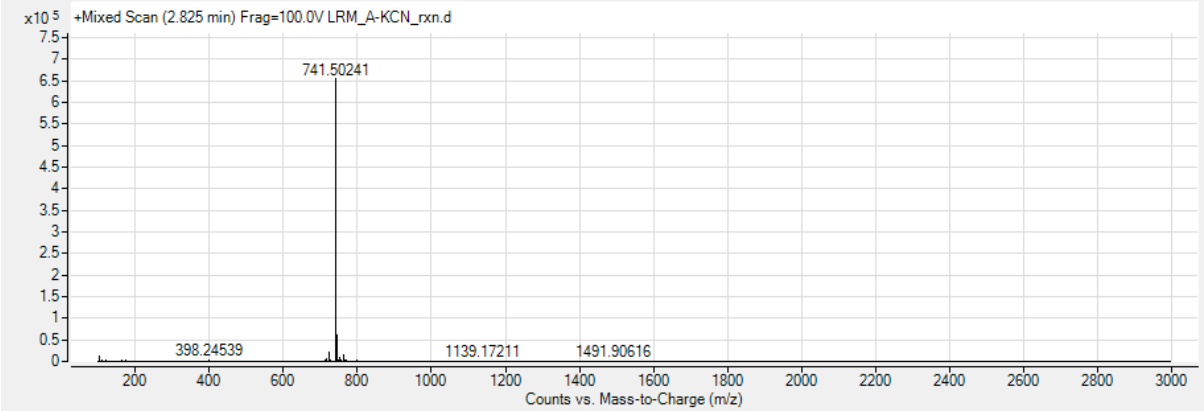
Peptide 6



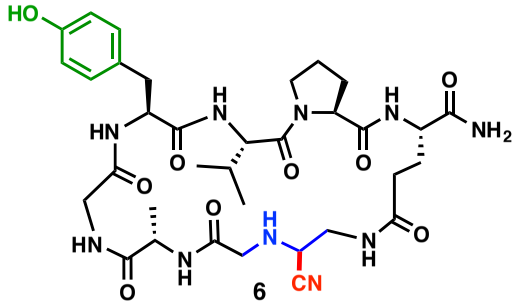
Peptide **6** was prepared according to general procedure 3 from linear amino aldehyde **S6** (5.0 mg, 6.8 μmol). At t = 24 h, the reaction was purified by preparative reverse-phase HPLC (5% B for 5 min, 5% B to 15% B over 5 min, then 15% to 45% B over 30 min) to afford peptide macrocycle **6** (3.0 mg, 59% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **6** from amino aldehyde **S6** following Strecker macrocyclization (0 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **6** (Rt = 10.7, 10.8 min, 0 to 100% B over 25 min, λ = 230 nm).



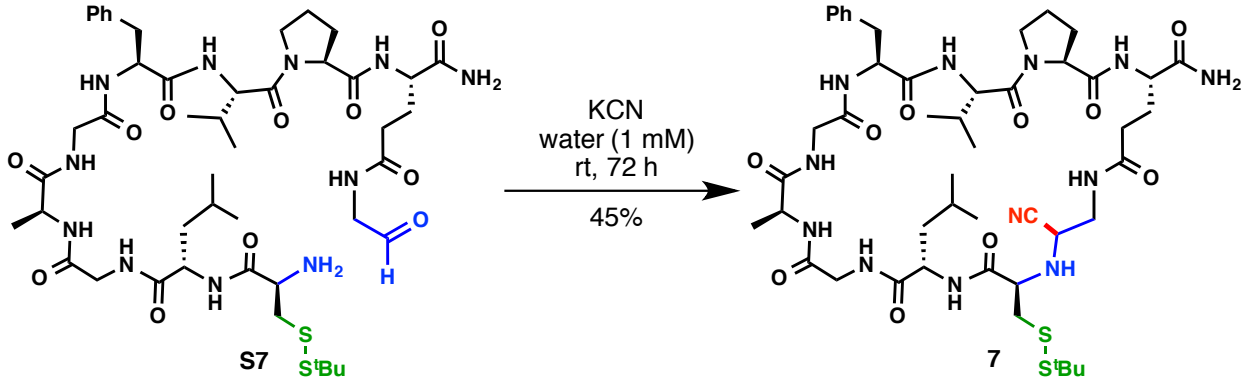
LRMS (ESI-TOF): calc'd for $C_{34}H_{49}N_{10}O_9$ $[M+H]^+$ 741.37; found 741.50.



6

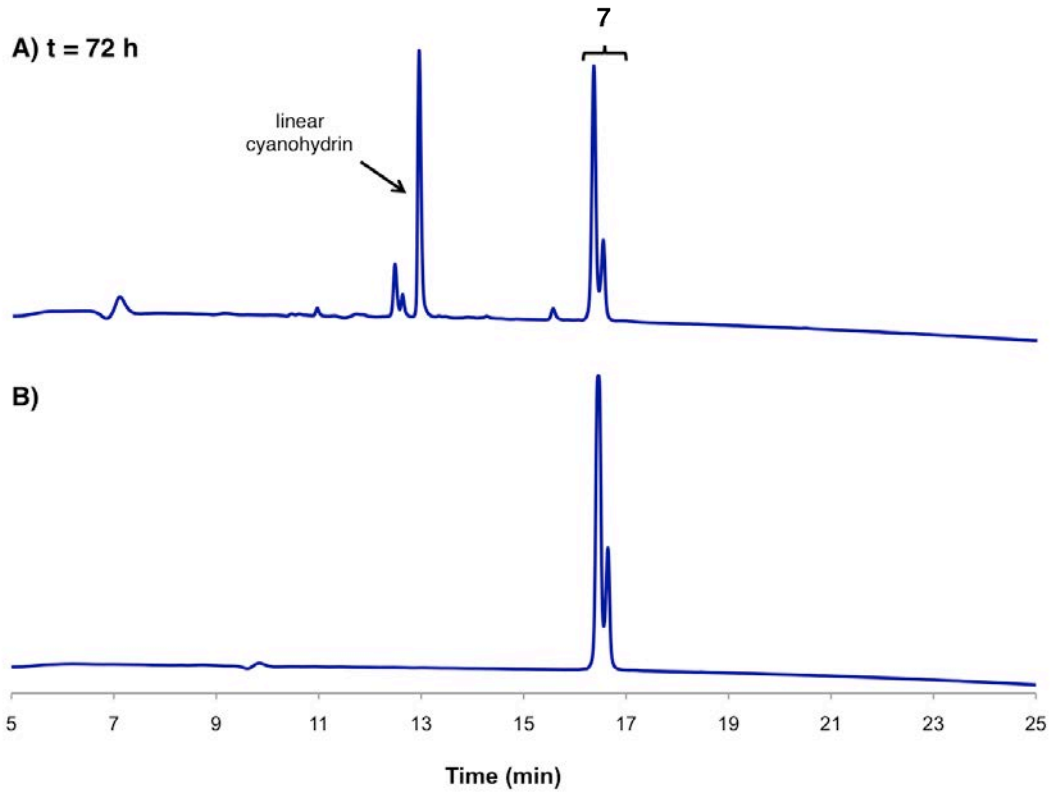
^{1}H, D_2O, 600 MHz

Peptide 7

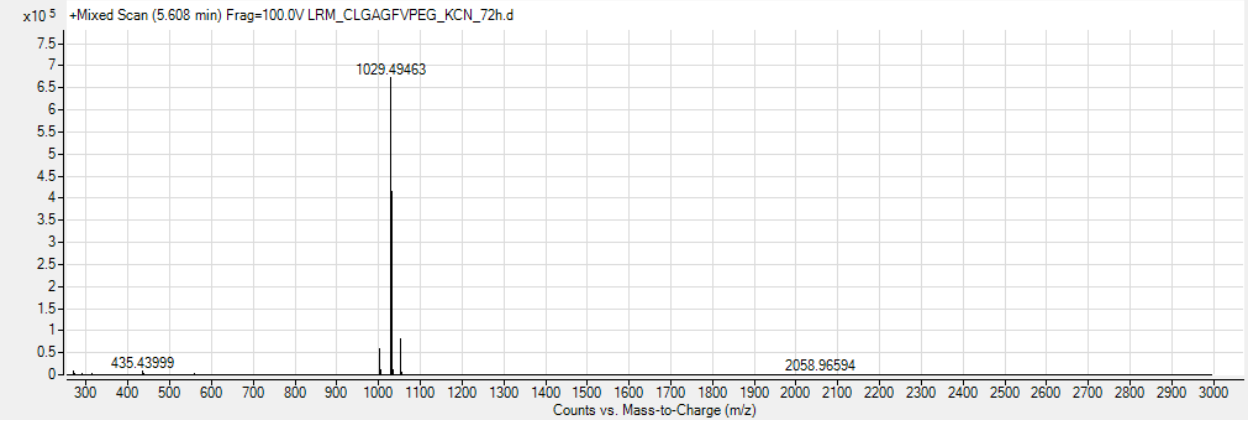


Peptide **7** was prepared according to general procedure 3 from linear amino aldehyde **S7** (3.6 mg, 3.5 μmol). At t = 48 h, an additional portion of KCN (1.2 equiv.) was added to the reaction mixture. At t = 72 h, the reaction was purified by preparative reverse-phase HPLC (25% B for 5 min, then 25% to 70% B over 35 min) to afford peptide macrocycle **7** (1.6 mg, 45% yield) as a mixture of diastereomers (3:1 *d.r.*) and as a white solid following lyophilization.

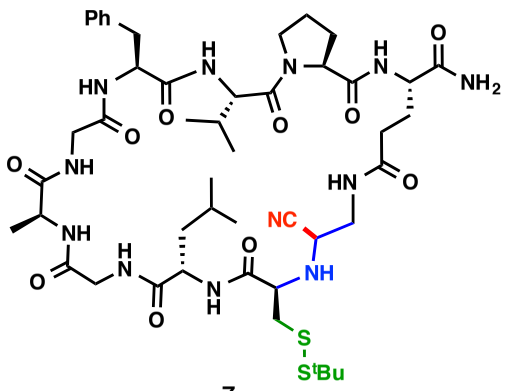
Note: Linear cyanohydrin (1.2 mg) was also isolated from the crude reaction mixture. As the Strecker macrocyclization is an equilibrium process with cyanohydrin formation more readily reversible than α-aminonitrile formation, higher yields of the Strecker adduct may be obtained with prolonged reaction times.



A) Crude analytical HPLC trace of the formation of **7** from amino aldehyde **S7** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **7** (Rt = 16.5, 16.6 min, 5 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{47}H_{73}N_{12}O_{10}S_2$ $[M+H]^+$ 1029.50; found 1029.49.

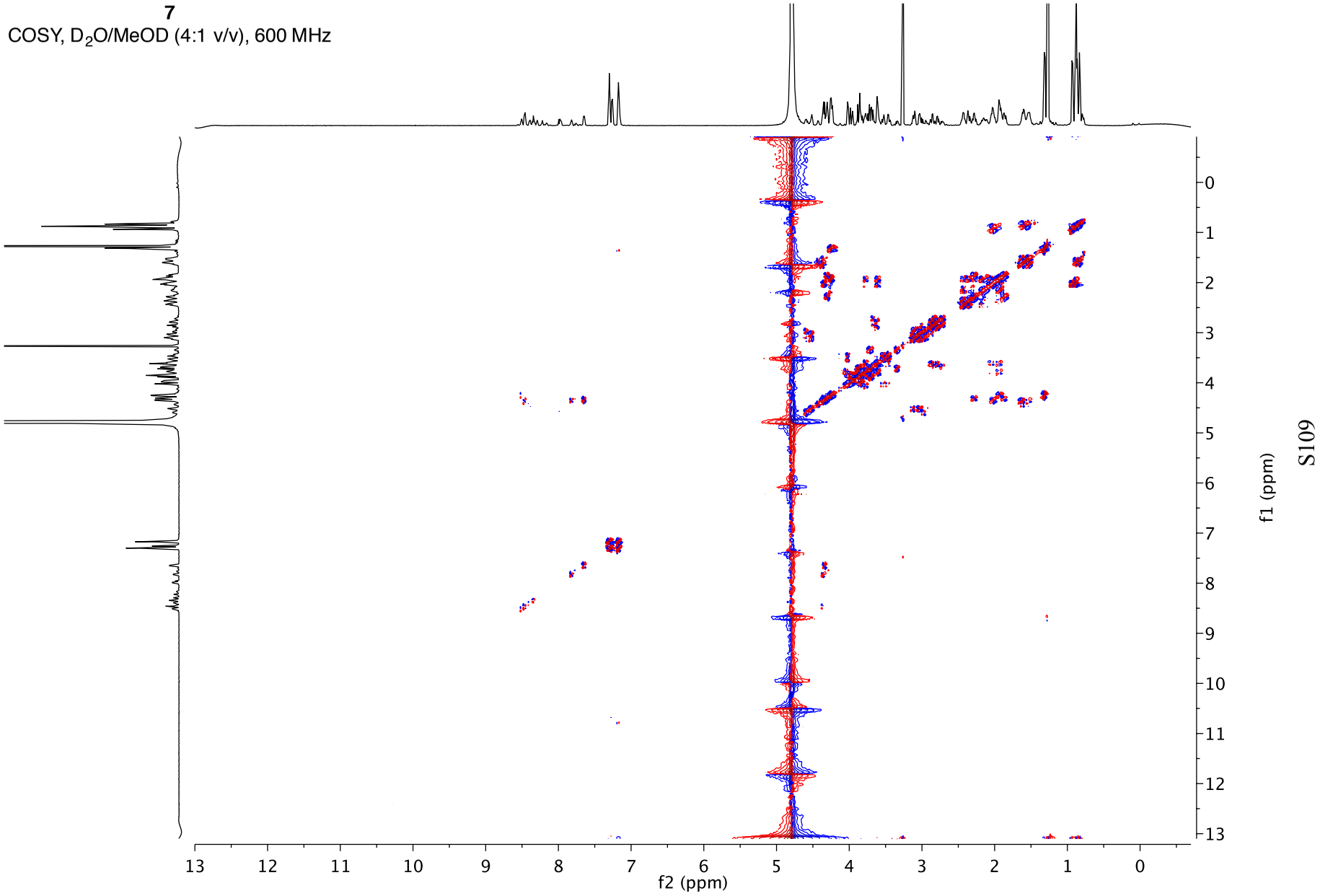


7

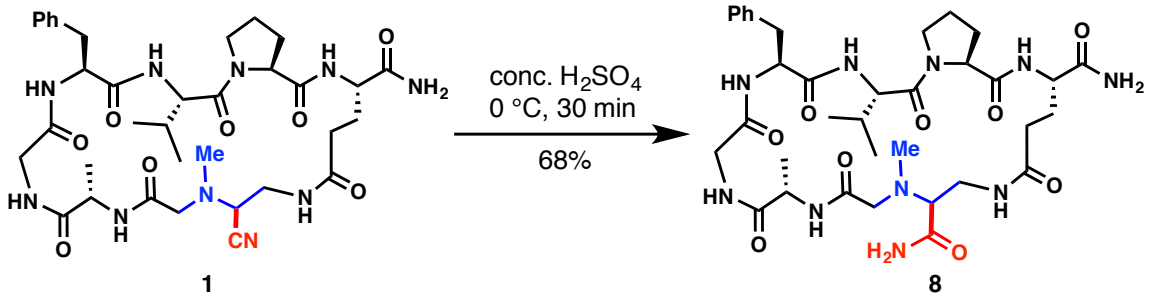
^{1}H, D_2O/MeOD (4:1 v/v), 600 MHz

7

COSY, D_2O/MeOD (4:1 v/v), 600 MHz

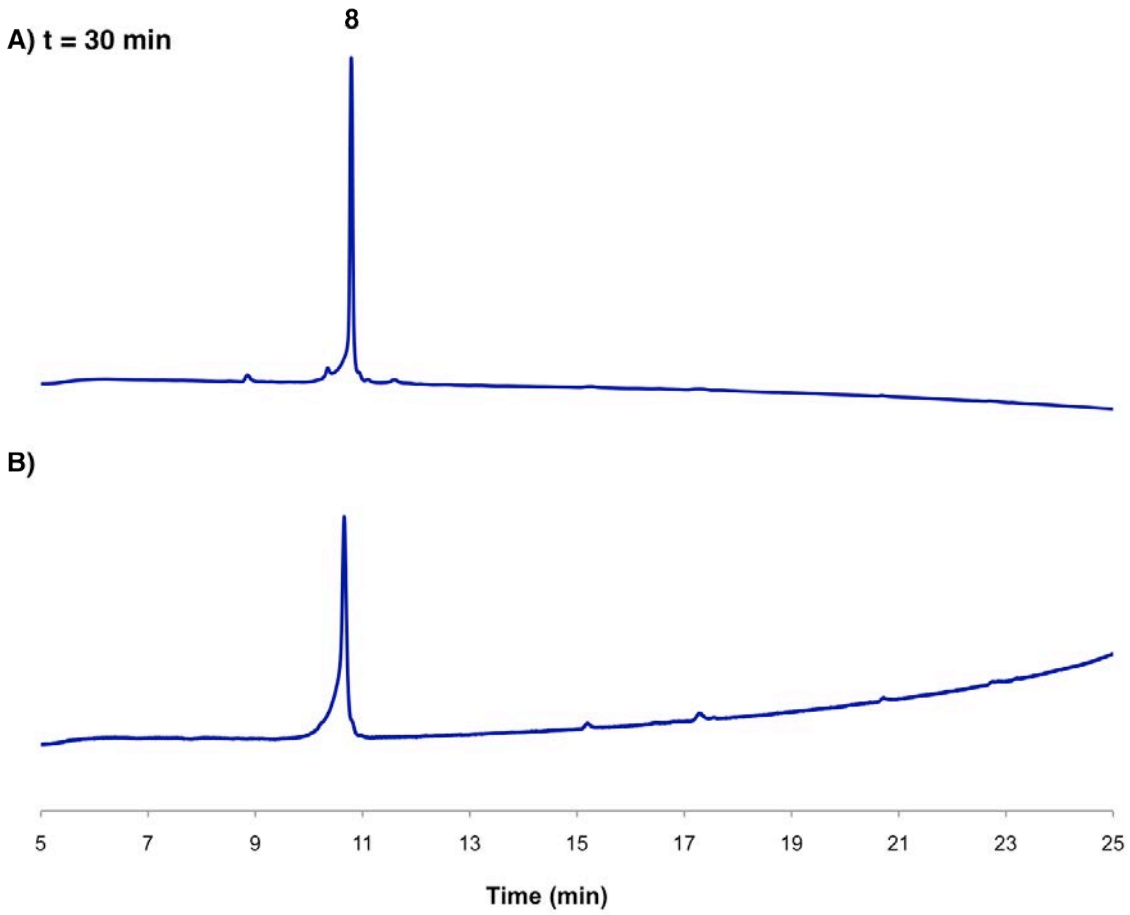


Peptide 8

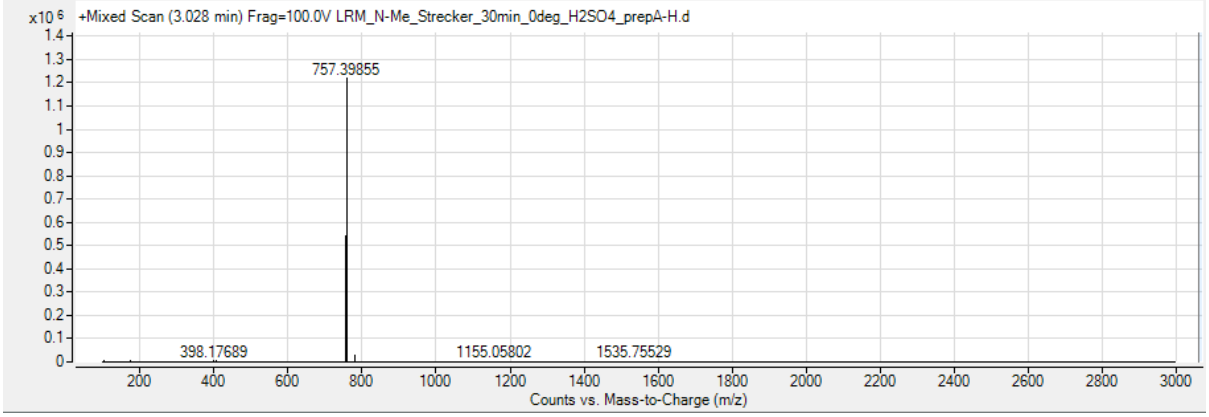


1 8

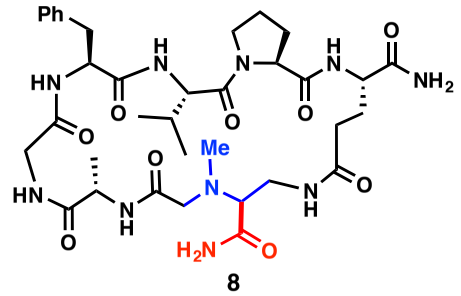
Peptide **8** was prepared from macrocycle **1** (4.0 mg, 5.4 μmol) by treatment with ice-cold conc. H_2SO_4 (1.25 mL). The reaction was stirred at 0 °C for 30 min at which point LC-MS analysis indicated complete consumption of the starting peptide. The reaction mixture was kept at 0 °C and slowly diluted with 10 mL of water before purification by preparative reverse-phase HPLC (15% B for 5 min, then 15% to 50% B over 25 min) to afford peptide macrocycle **8** (2.8 mg, 68% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **8** from macrocycle **1** following acidic hydrolysis (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **8** (Rt = 10.7 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{35}H_{53}N_{10}O_9$ $[M+H]^+$ 757.40; found 757.40.

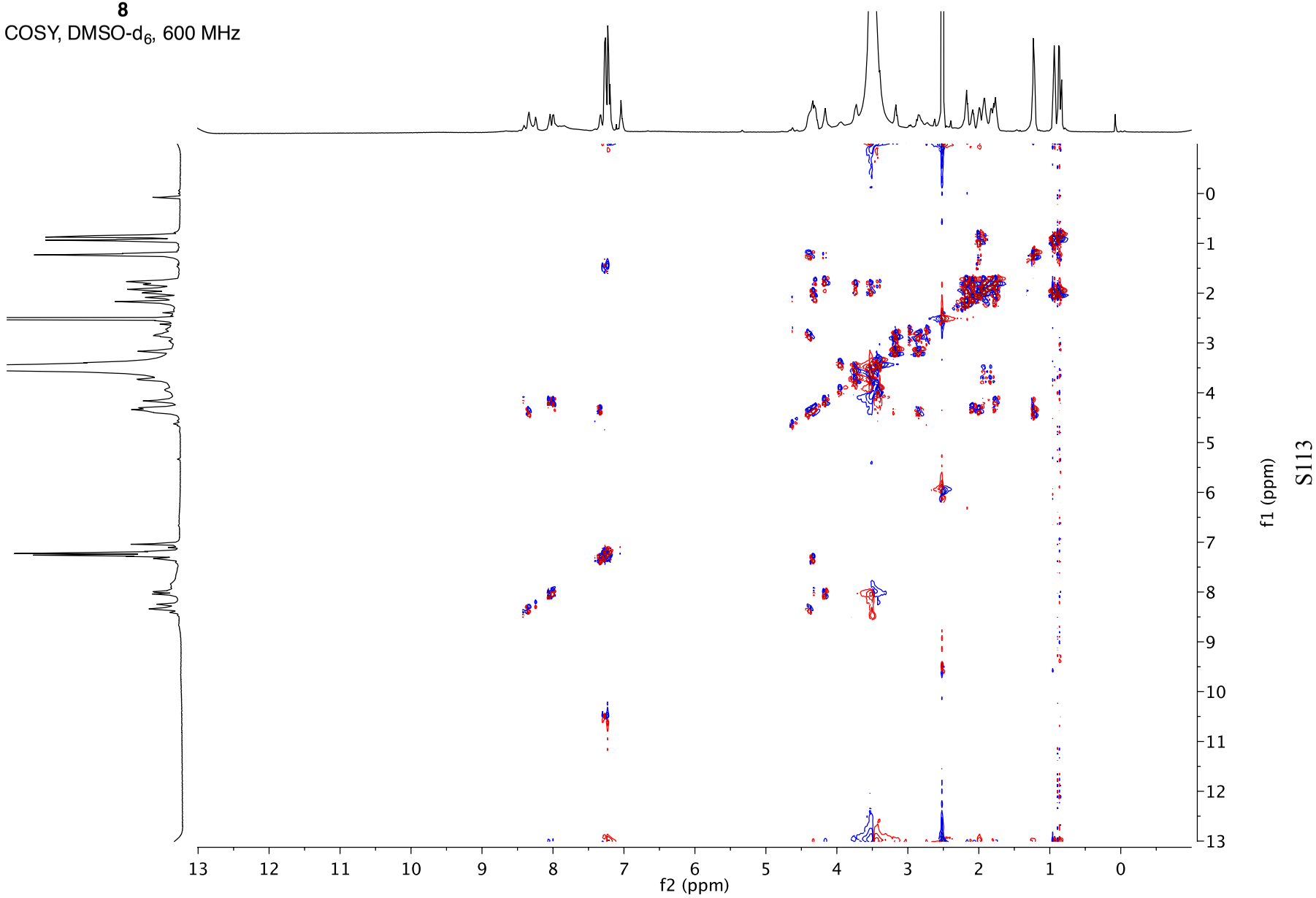


8

^{1}H, DMSO-d_6/D_2O (20:1 v/v), 600 MHz

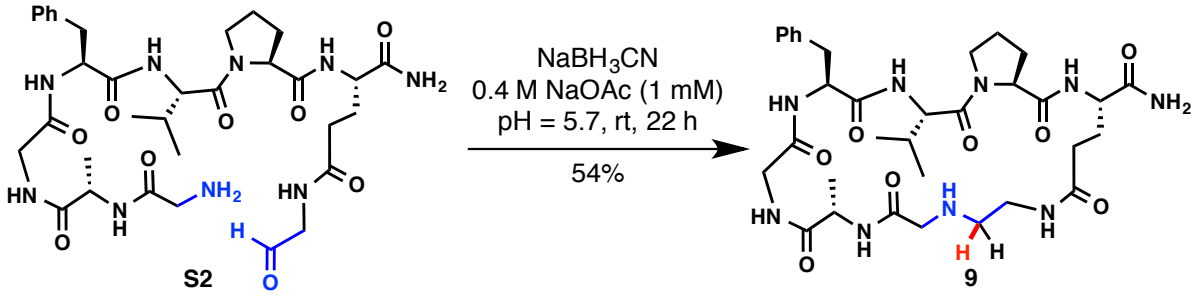
8

COSY, DMSO-d_6, 600 MHz

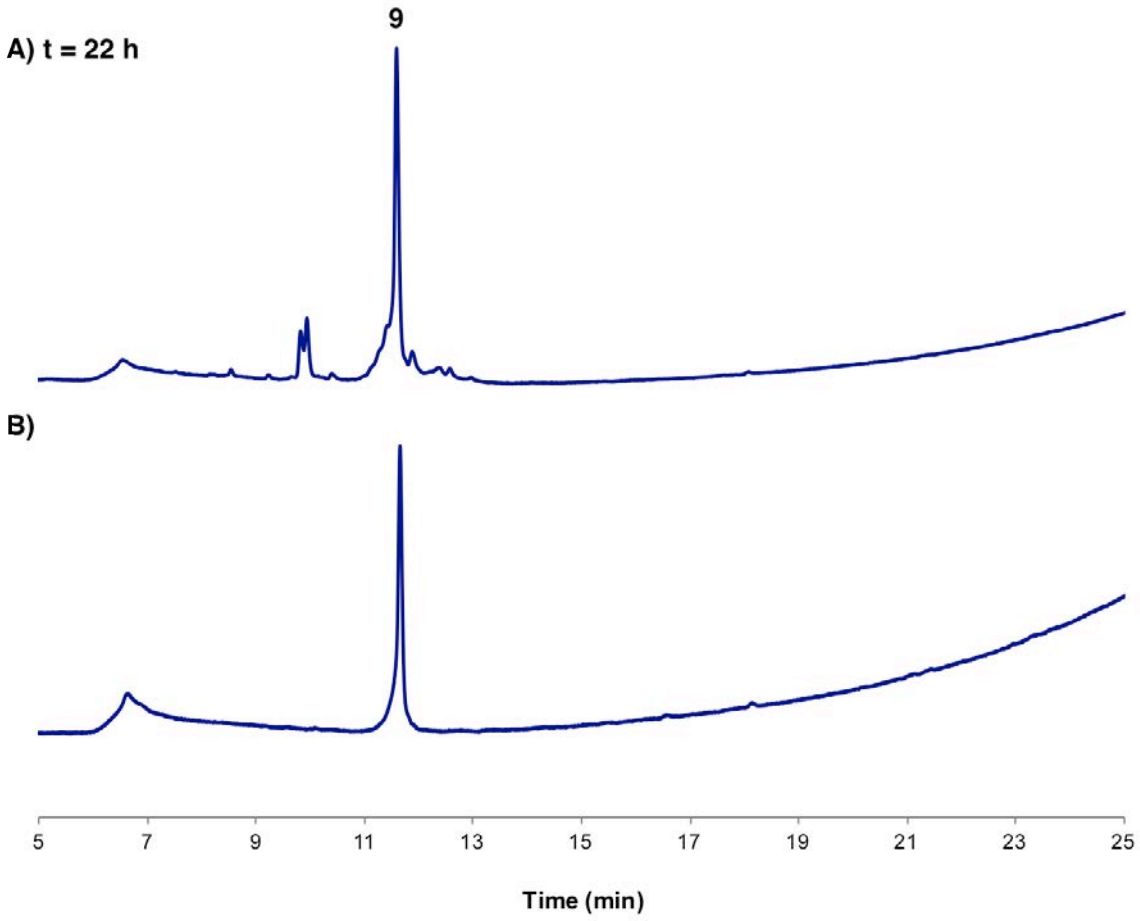


Characterization Data for Reductive Amination

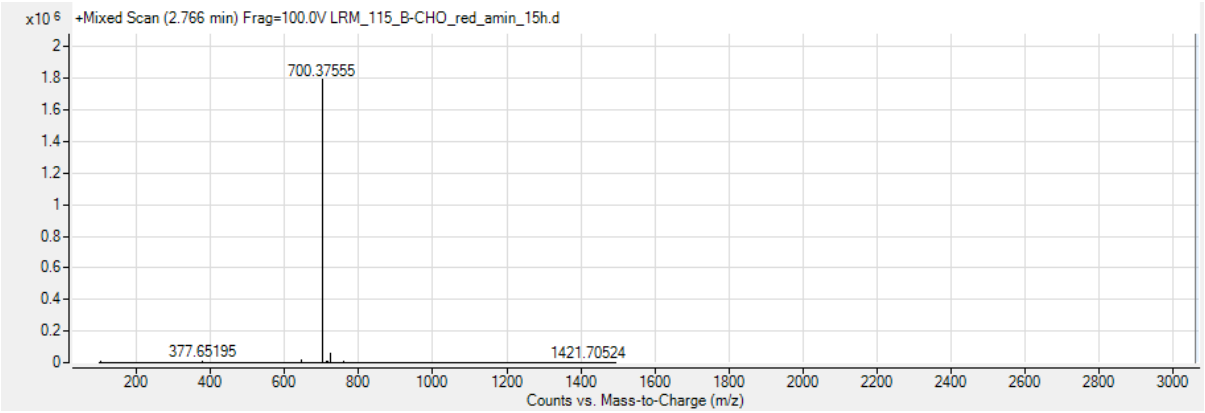
Peptide 9



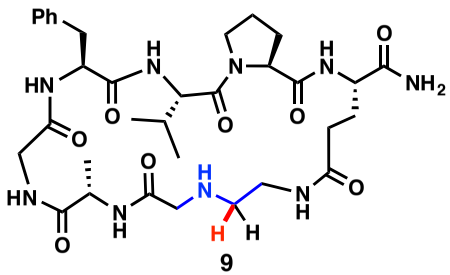
Peptide **9** was prepared according to general procedure 4 from linear amino aldehyde **S2** (7.0 mg, 9.8 μmol). At t = 22 h, the reaction was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% to 50% B over 30 min) to afford peptide macrocycle **9** (3.7 mg, 54% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **9** from amino aldehyde **S2** following reductive amination (0 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **9** (Rt = 11.7 min, 0 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{33}H_{50}N_9O_8$ $[M+H]^+$ 700.38; found 700.38.

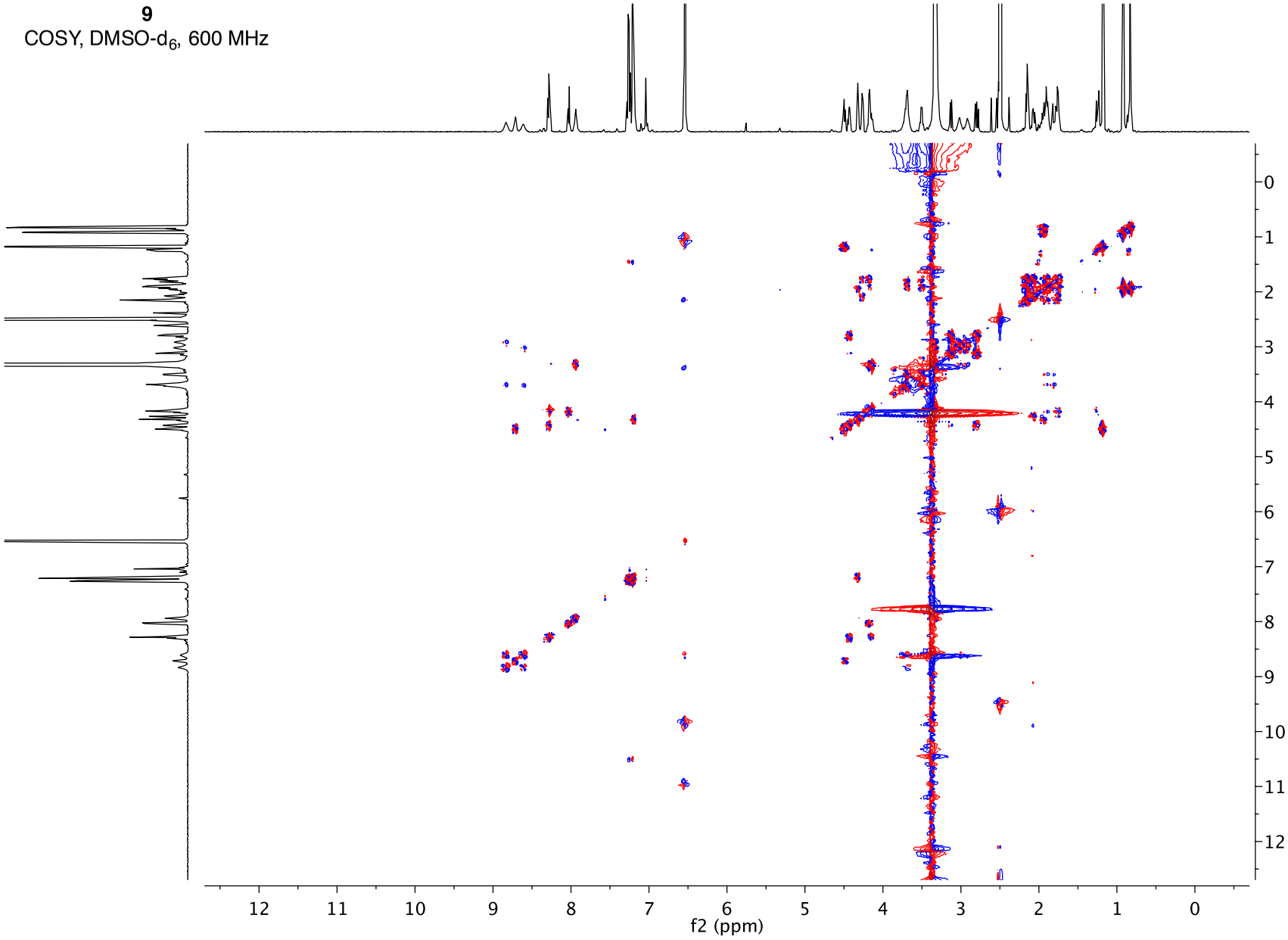


9

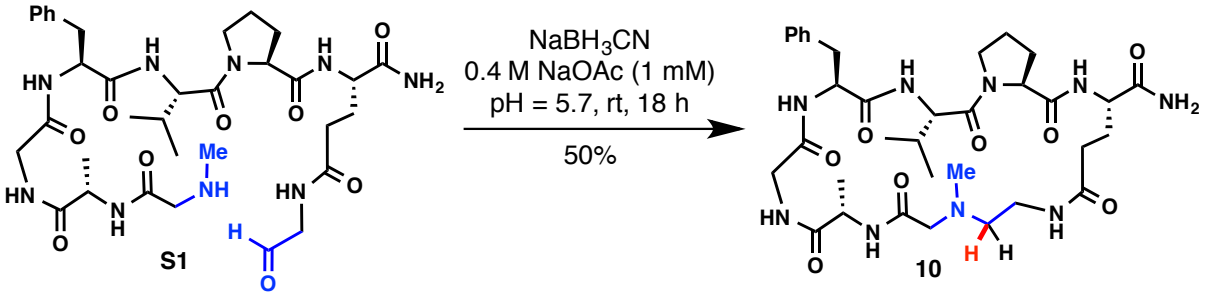
^{1}H, DMSO-d$_6$, 600 MHz

9

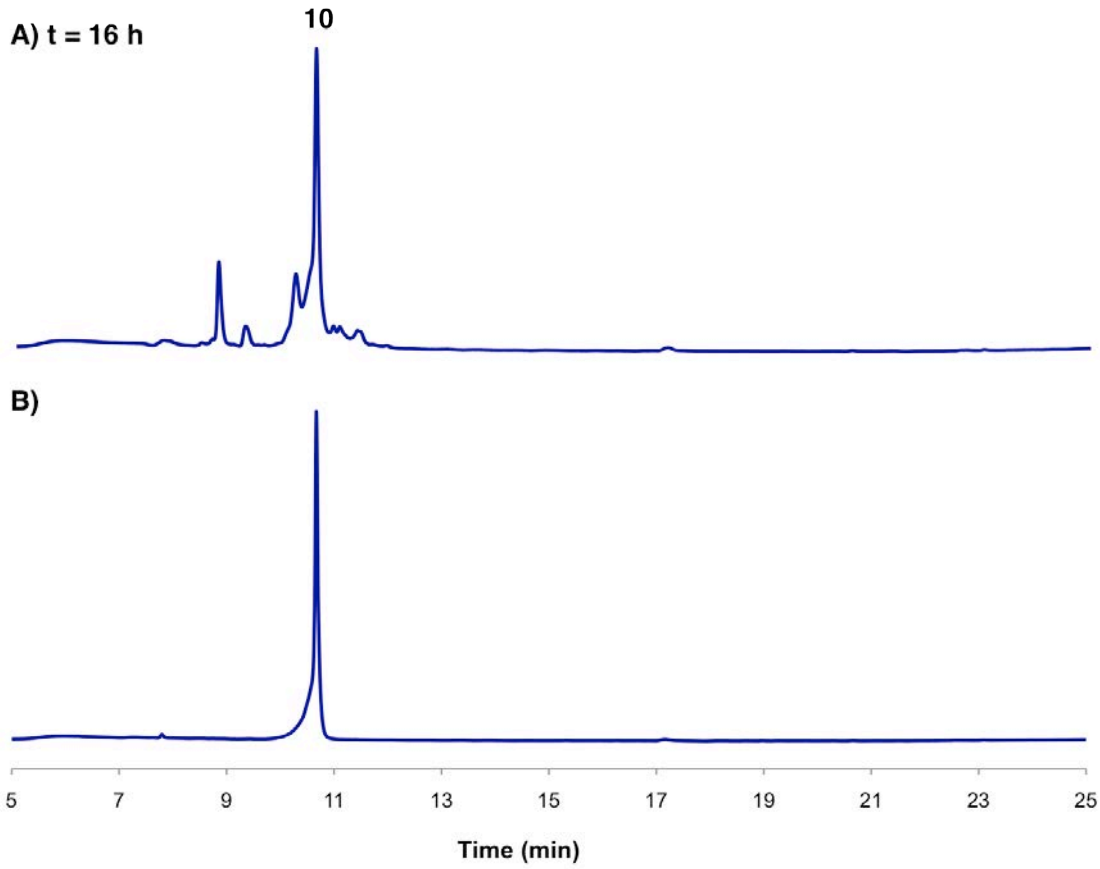
COSY, DMSO-d_6, 600 MHz



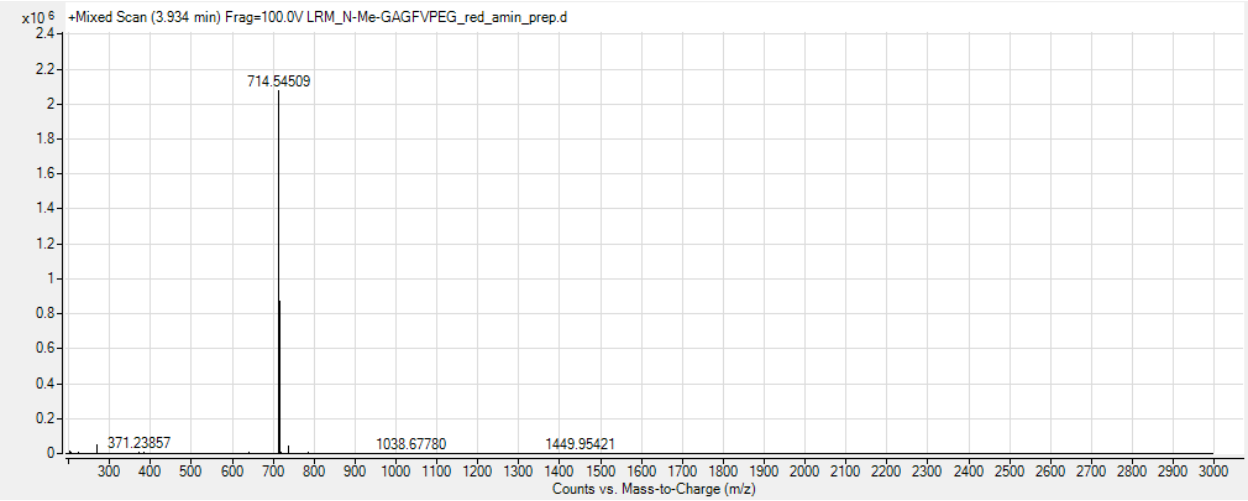
Peptide 10



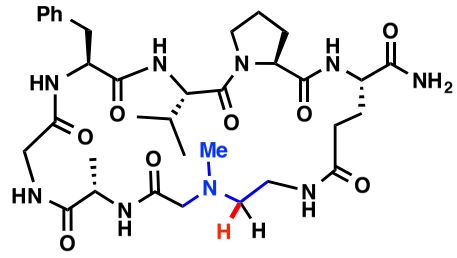
Peptide **10** was prepared according to general procedure 4 from linear amino aldehyde **S1** (4.3 mg, 5.9 μmol). At t = 18 h, the reaction was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% to 50% B over 35 min) to afford peptide macrocycle **10** (2.1 mg, 50% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **10** from amino aldehyde **S1** following reductive amination (5 to 100% B over 25 min, λ = 220 nm). **B)** Purified peptide **10** (Rt = 10.7 min, 5 to 100% B over 25 min, λ = 220 nm).



LRMS (ESI-TOF): calc'd for $C_{34}H_{52}N_9O_8$ $[M+H]^+$ 714.39; found 714.55.

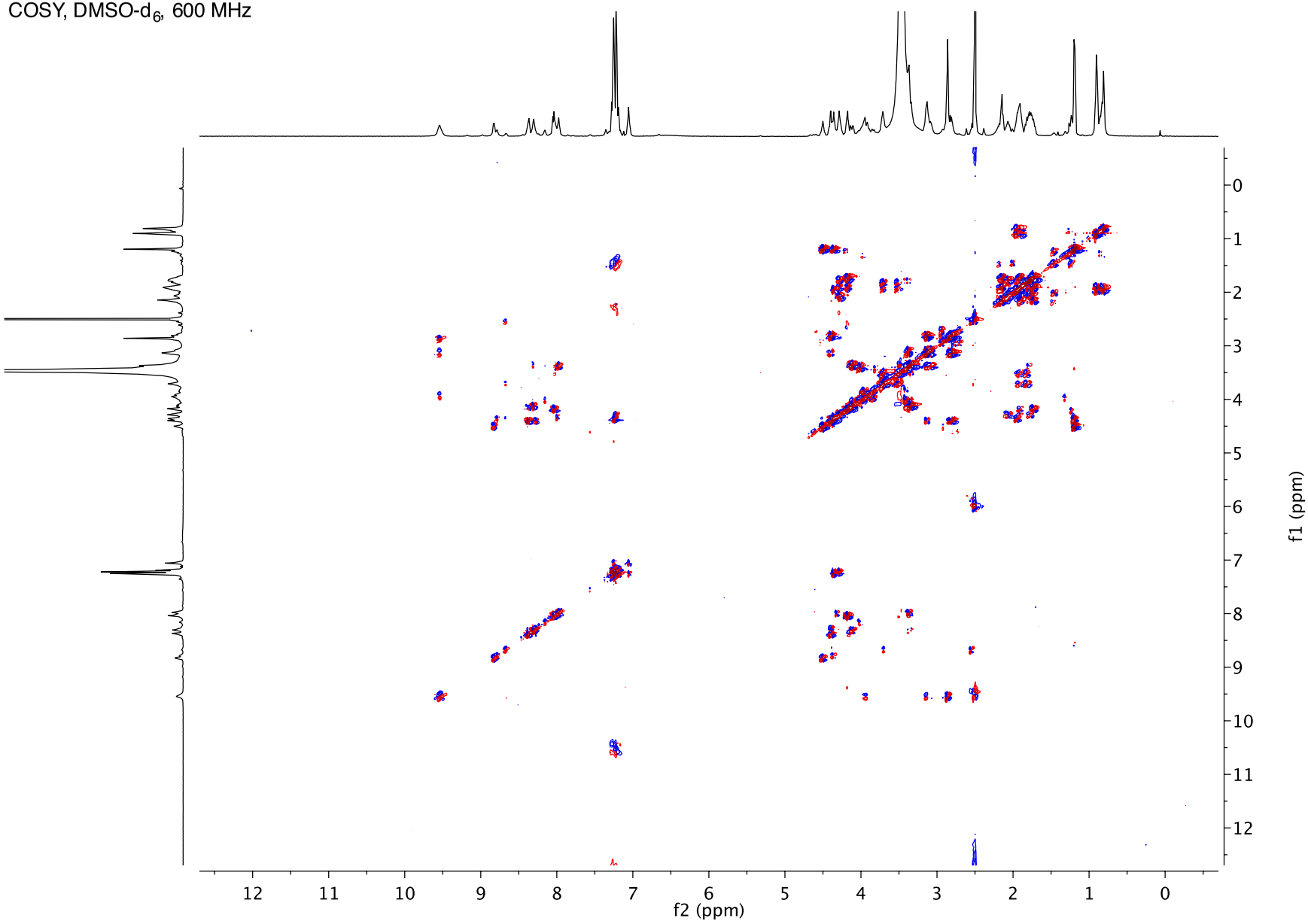


10

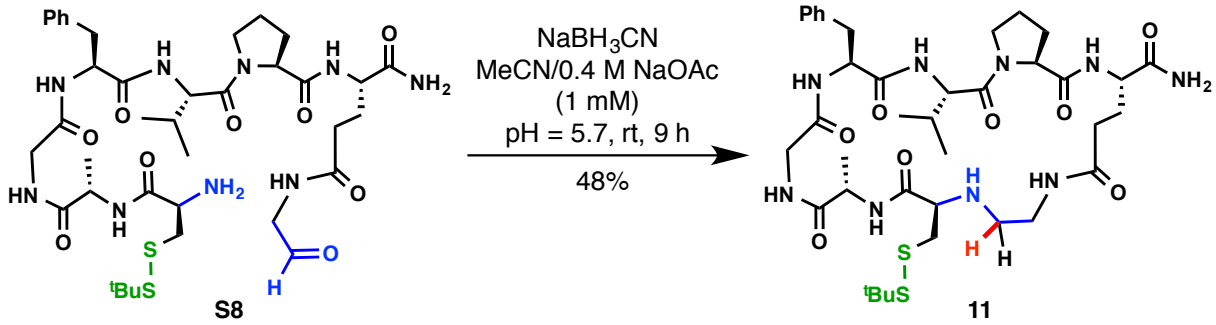
^{1}H, DMSO-d_6, 600 MHz

10

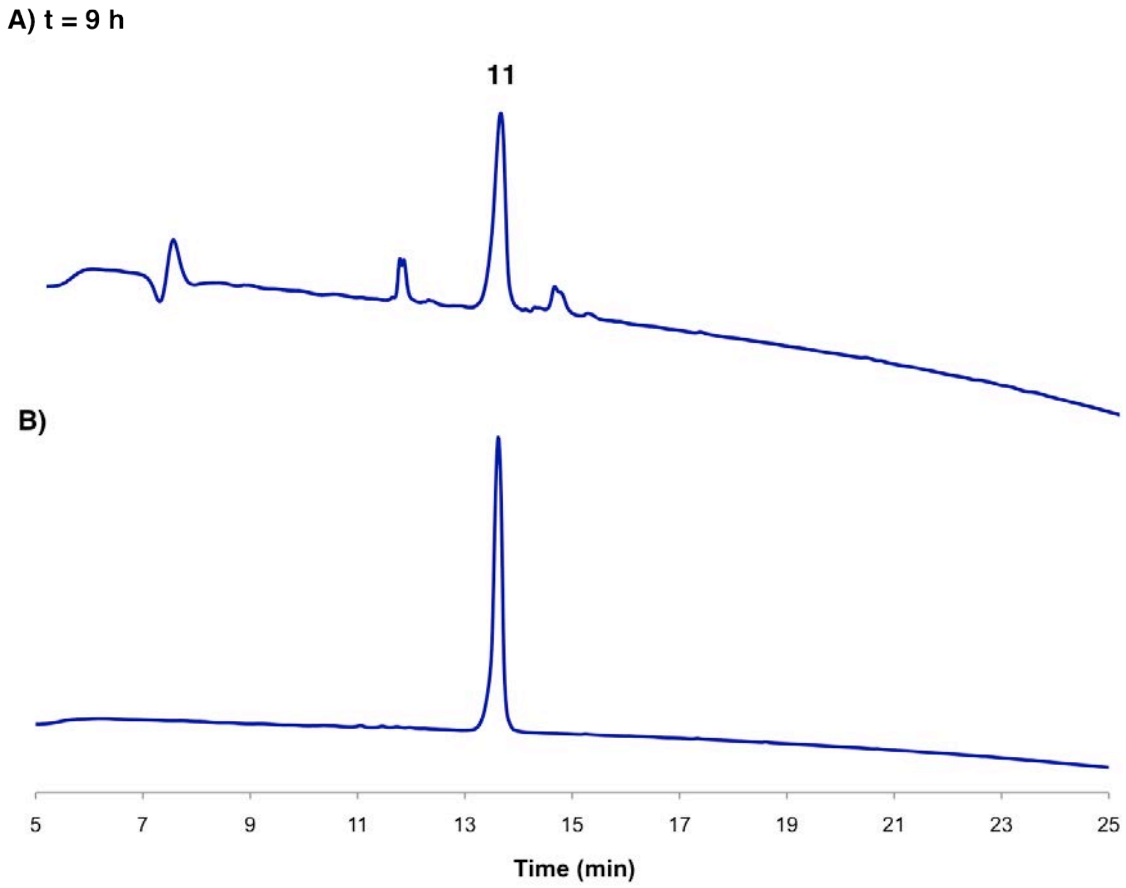
COSY, DMSO-d_6, 600 MHz



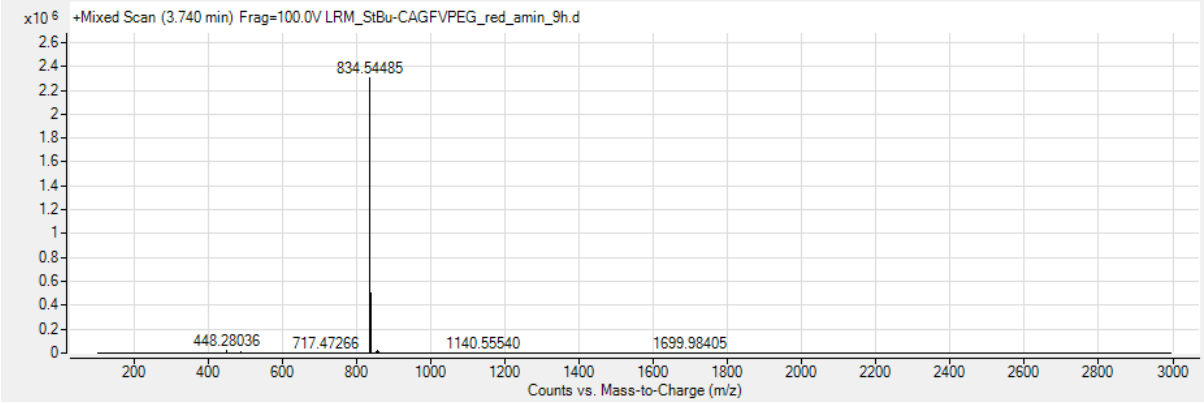
Peptide 11



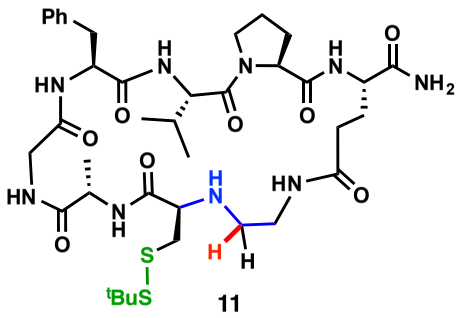
Peptide **11** was prepared according to general procedure 4 from linear amino aldehyde **S8** (3.6 mg, 4.2 μmol). The peptide was solubilized in a 1:3 (v/v) mixture of MeCN/0.4 M NaOAc buffer (pH = 5.7). At t = 9 h, the reaction was purified by preparative reverse-phase HPLC (30% B for 5 min, then 30% to 60% B over 25 min) to afford peptide macrocycle **11** (1.7 mg, 48% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **11** from amino aldehyde **S8** following reductive amination (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **11** (Rt = 13.6 min, 5 to 100% B over 25 min, λ = 210 nm).



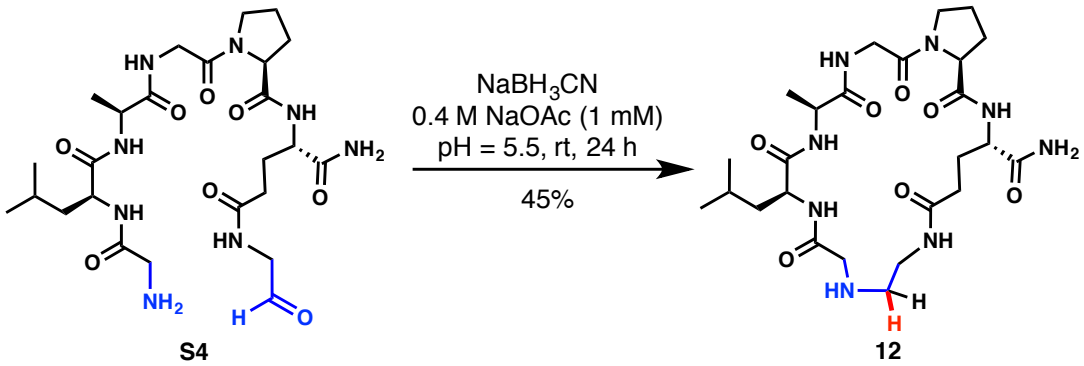
LRMS (ESI-TOF): calc'd for $C_{38}H_{60}N_9O_8S_2$ $[M+H]^+$ 834.40; found 834.54.



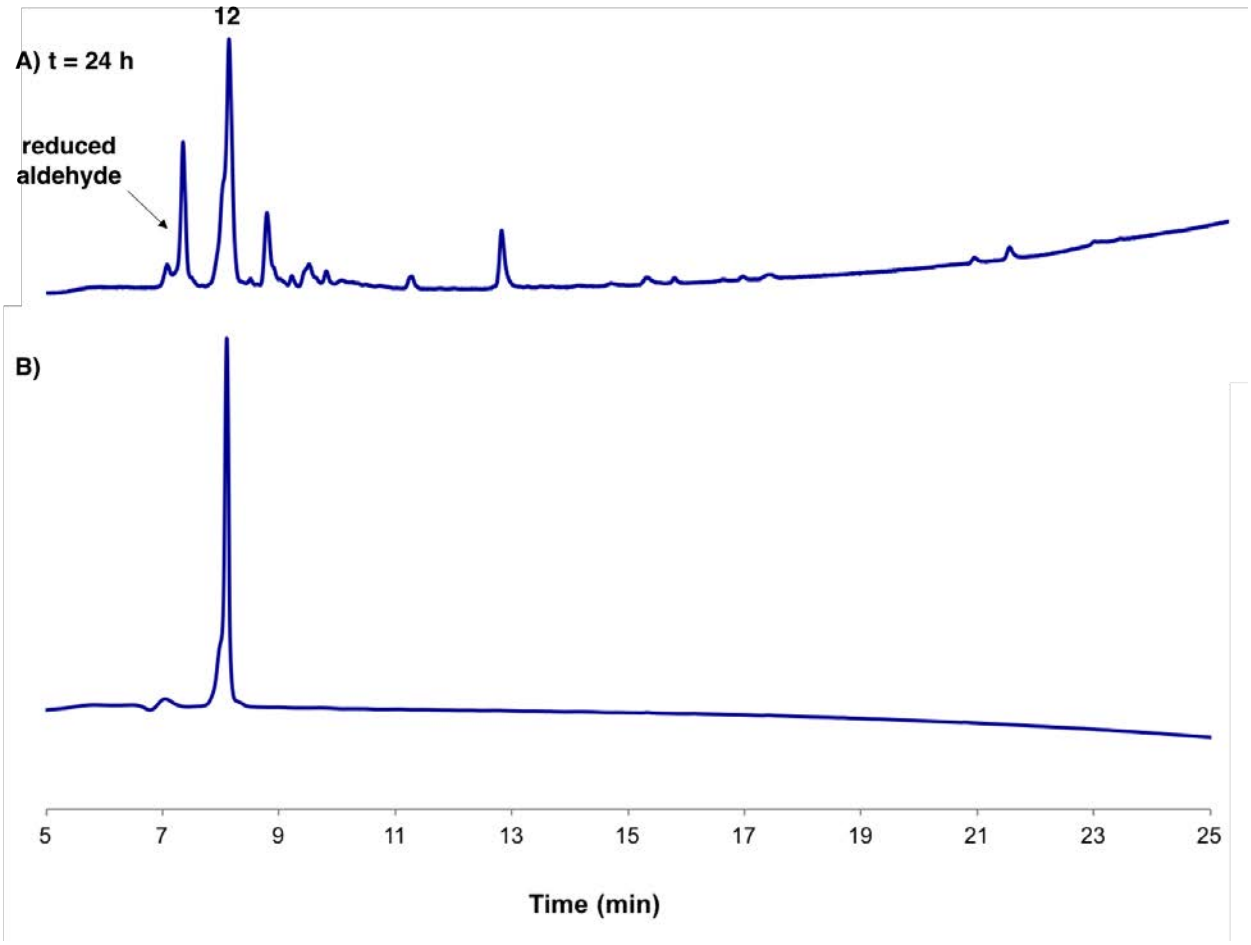
11

^{1}H, DMSO-d_6, 600 MHz

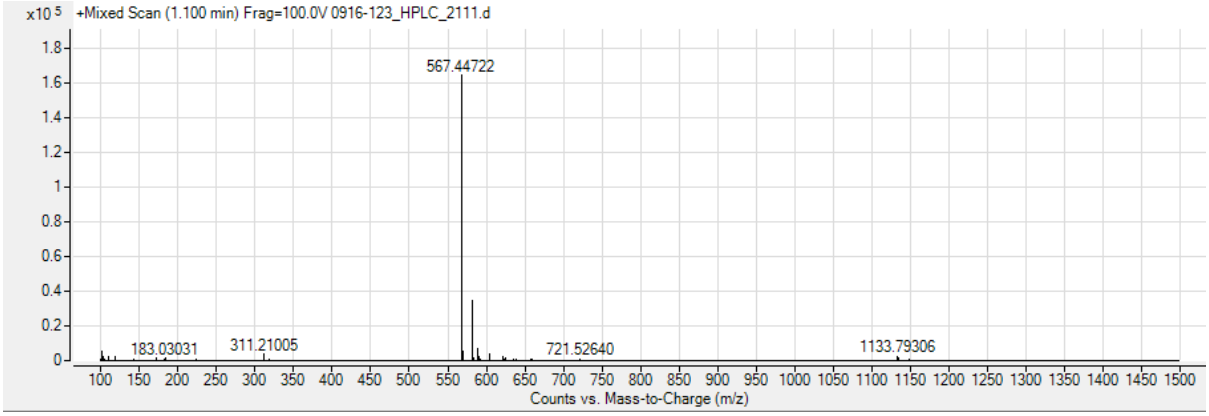
Peptide 12



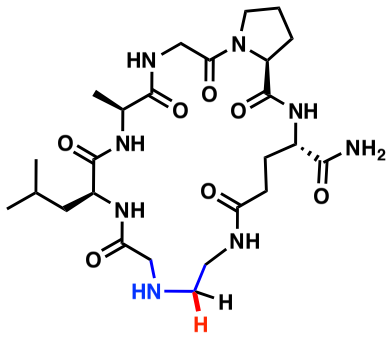
Peptide **12** was prepared according to general procedure 4 from linear amino aldehyde **S4** (10.0 mg, 17 μmol). At t = 24 h, the reaction was purified by preparative reverse-phase HPLC (5% B for 5 min, 5% B to 40% B over 30 min) to afford peptide macrocycle **12** (4.4 mg, 45% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **12** from amino aldehyde **S4** following reductive amination (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **12** (Rt = 8.1 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{25}H_{42}N_8O_7$ $[M+H]^+$ 567.32; found 567.45.

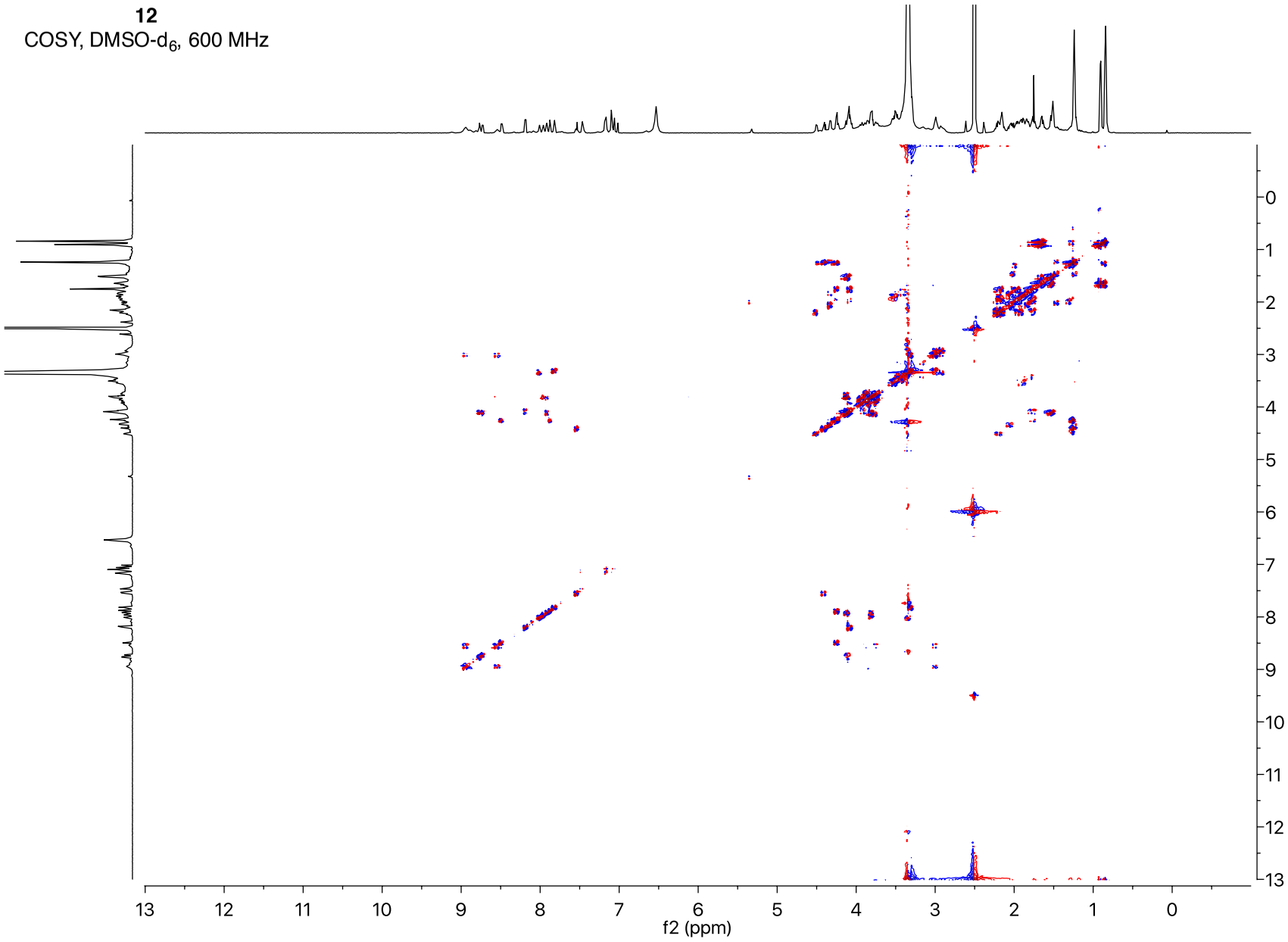


12

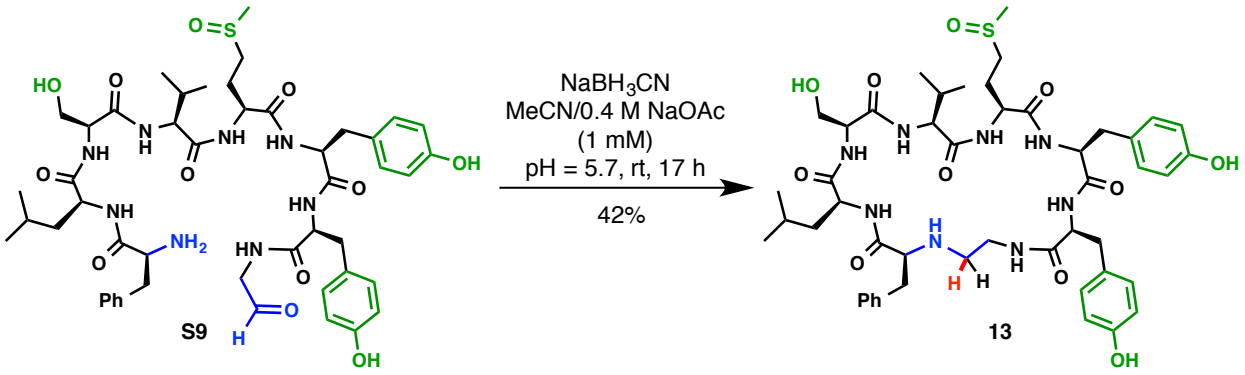
^{1}H, DMSO-d_6, 600 MHz

12

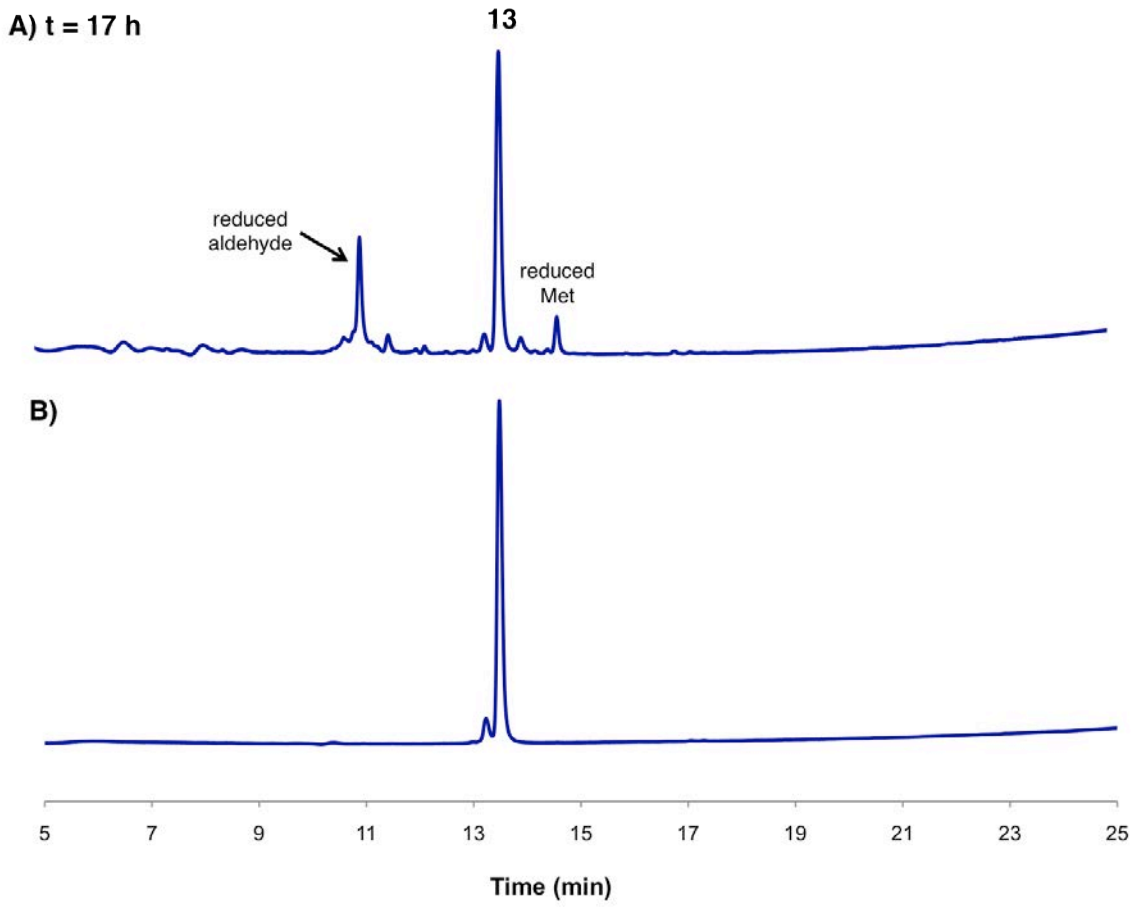
COSY, DMSO-d_6, 600 MHz



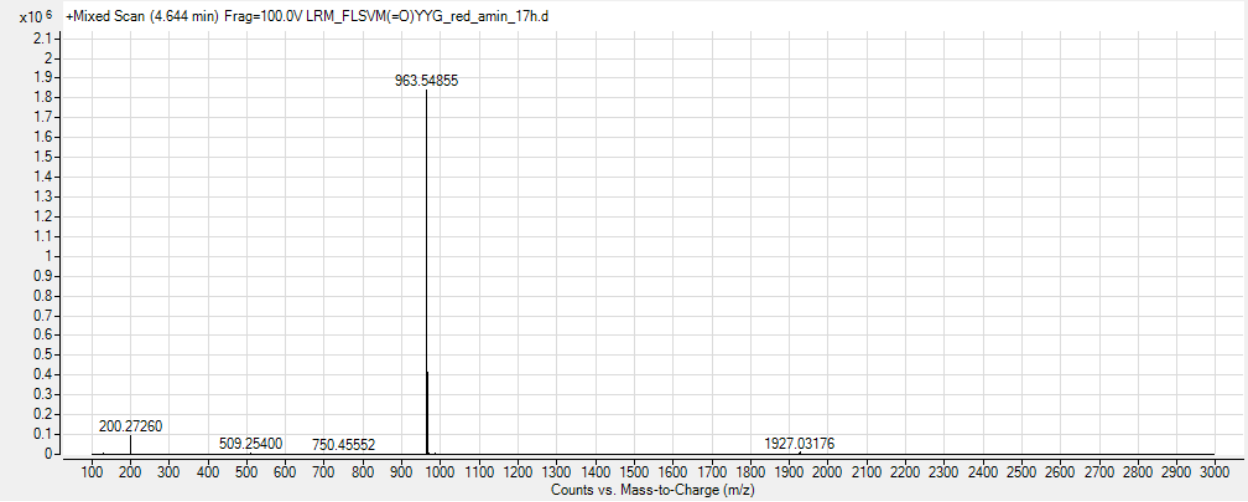
Peptide 13



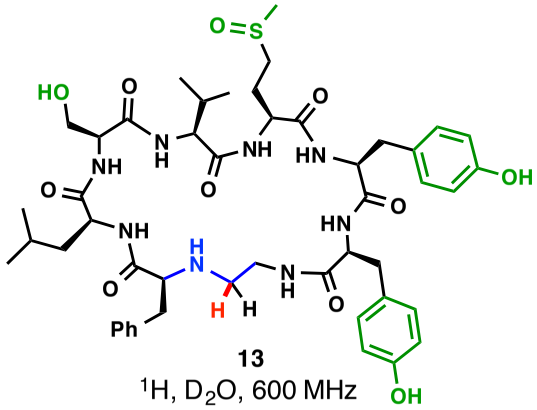
Peptide **13** was prepared according to general procedure 4 from linear amino aldehyde **S9** (4.4 mg, 4.5 μmol). The peptide was solubilized in a 2:3 (v/v) mixture of MeCN/0.4 M NaOAc buffer (pH = 5.7). At t = 17 h, the reaction was purified by preparative reverse-phase HPLC (25% B for 5 min, then 25% to 60% B over 30 min) to afford peptide macrocycle **13** (1.8 mg, 42% yield) as a white solid following lyophilization. A small amount of macrocyclic product bearing a reduced Met residue was also isolated (0.5 mg, 12% yield).



A) Crude analytical HPLC trace of the formation of **13** from amino aldehyde **S9** following reductive amination (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **13** (Rt = 13.5 min, 5 to 100% B over 25 min, λ = 230 nm).



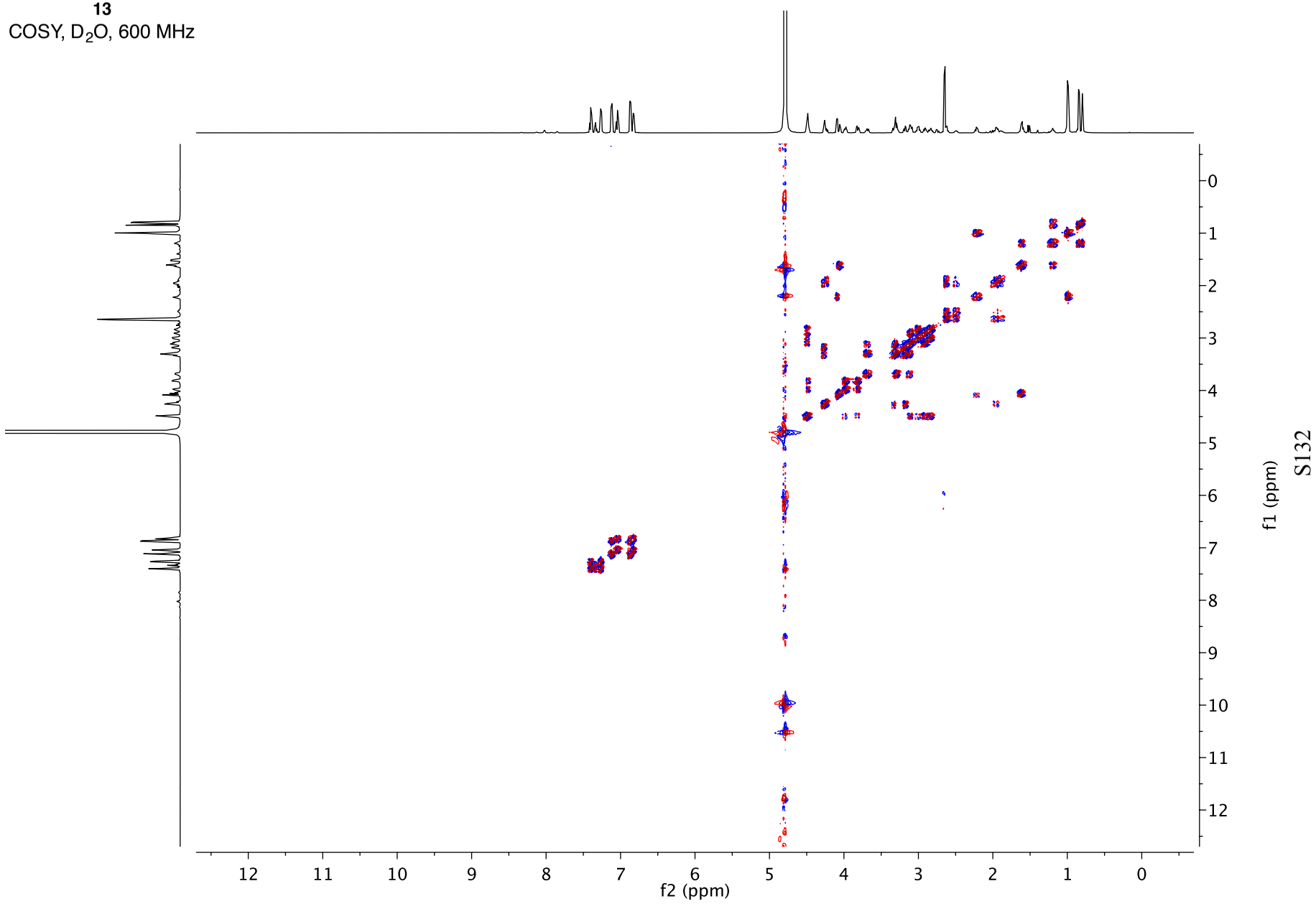
LRMS (ESI-TOF): calc'd for $C_{48}H_{67}N_8O_{11}S$ $[M+H]^+$ 963.46; found 963.55.


13
^{1}H, D_2O, 600 MHz

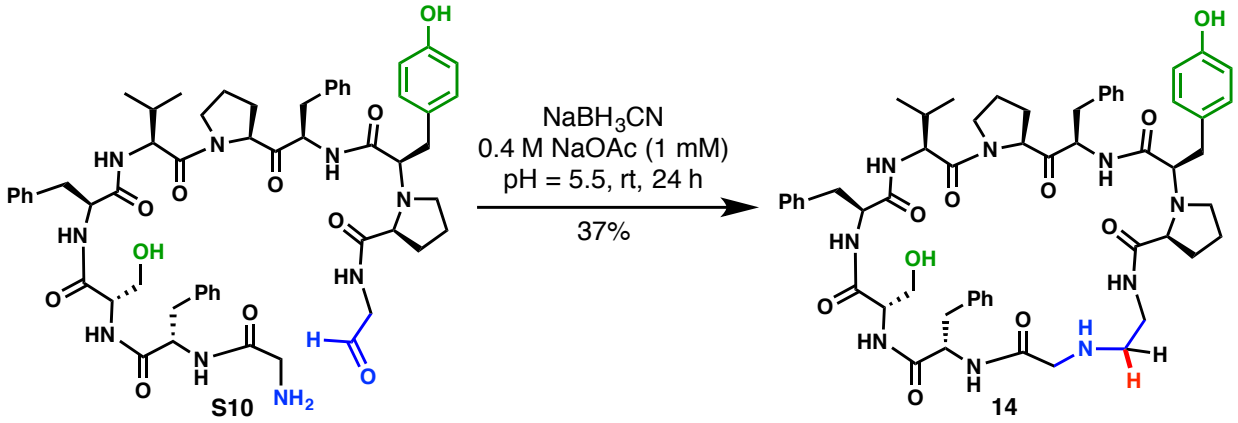
12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

13

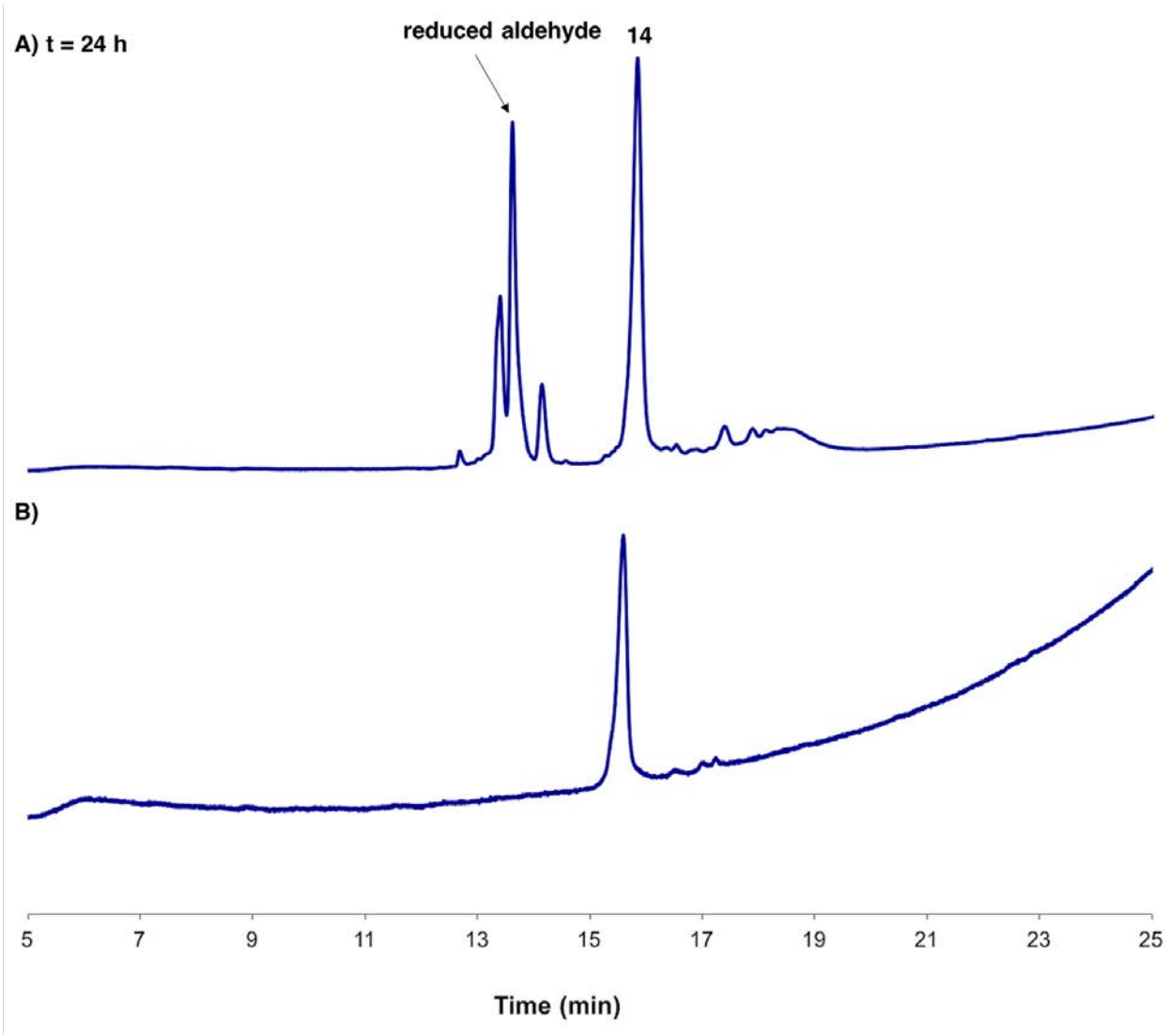
COSY, D_2O, 600 MHz



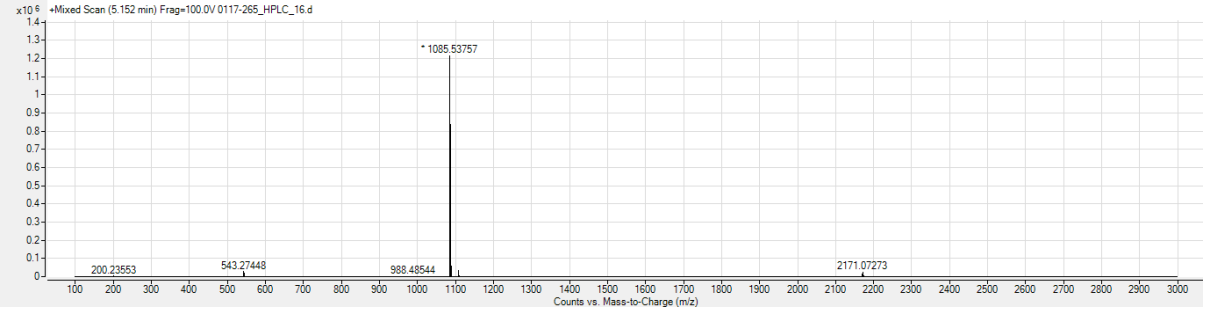
Peptide 14



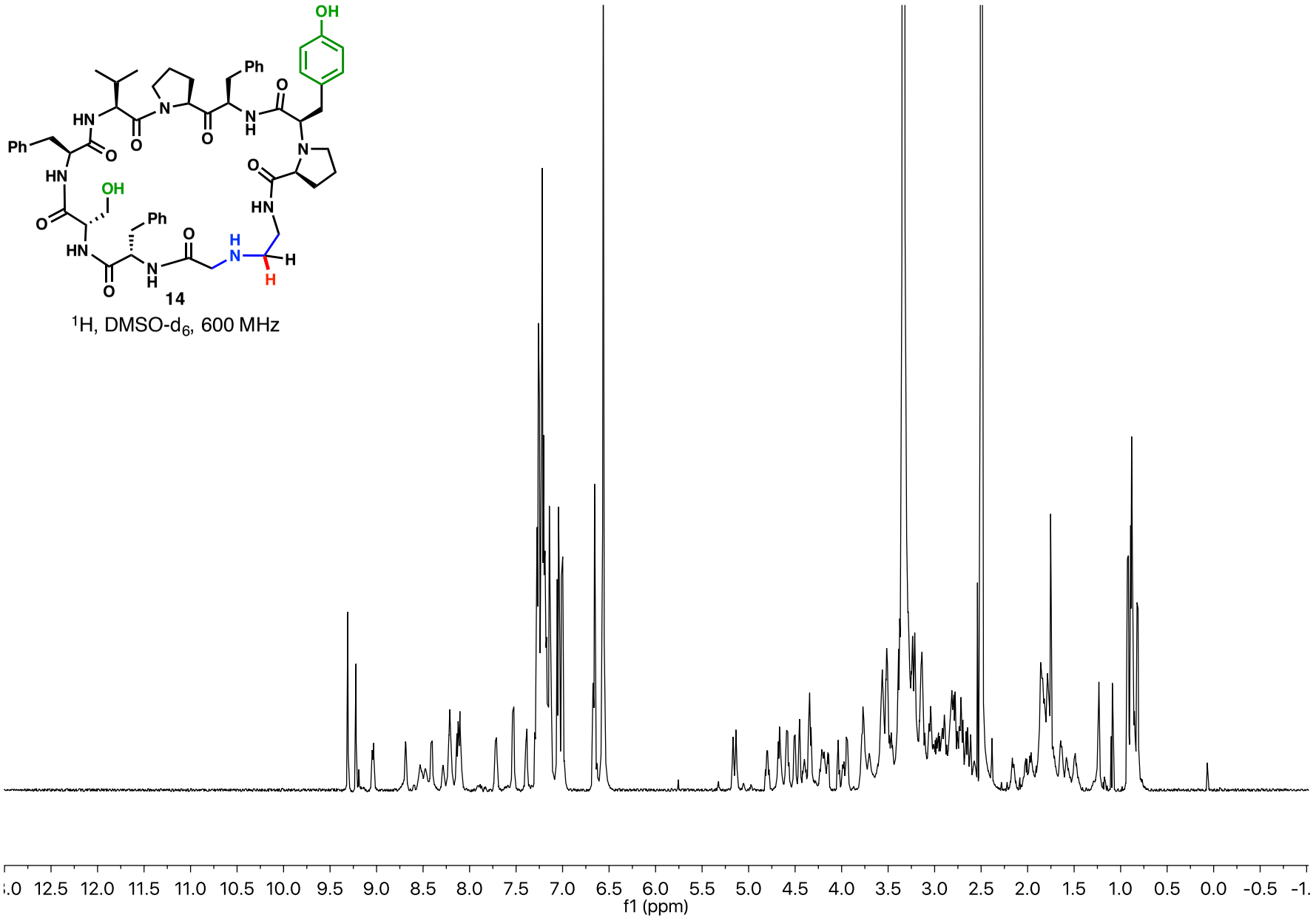
Peptide **14** was prepared according to general procedure 4 from linear amino aldehyde **S10** (5.1 mg, 4.63 μmol). At t = 24 h, the reaction was purified by preparative reverse-phase HPLC (40% B for 5 min, then 40% to 80% B over 30 min) to afford peptide macrocycle **14** (1.9 mg, 37% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **14** from amino aldehyde **S10** following reductive amination (0 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **14** (Rt = 15.6 min, 0 to 100% B over 25 min, λ = 210 nm).

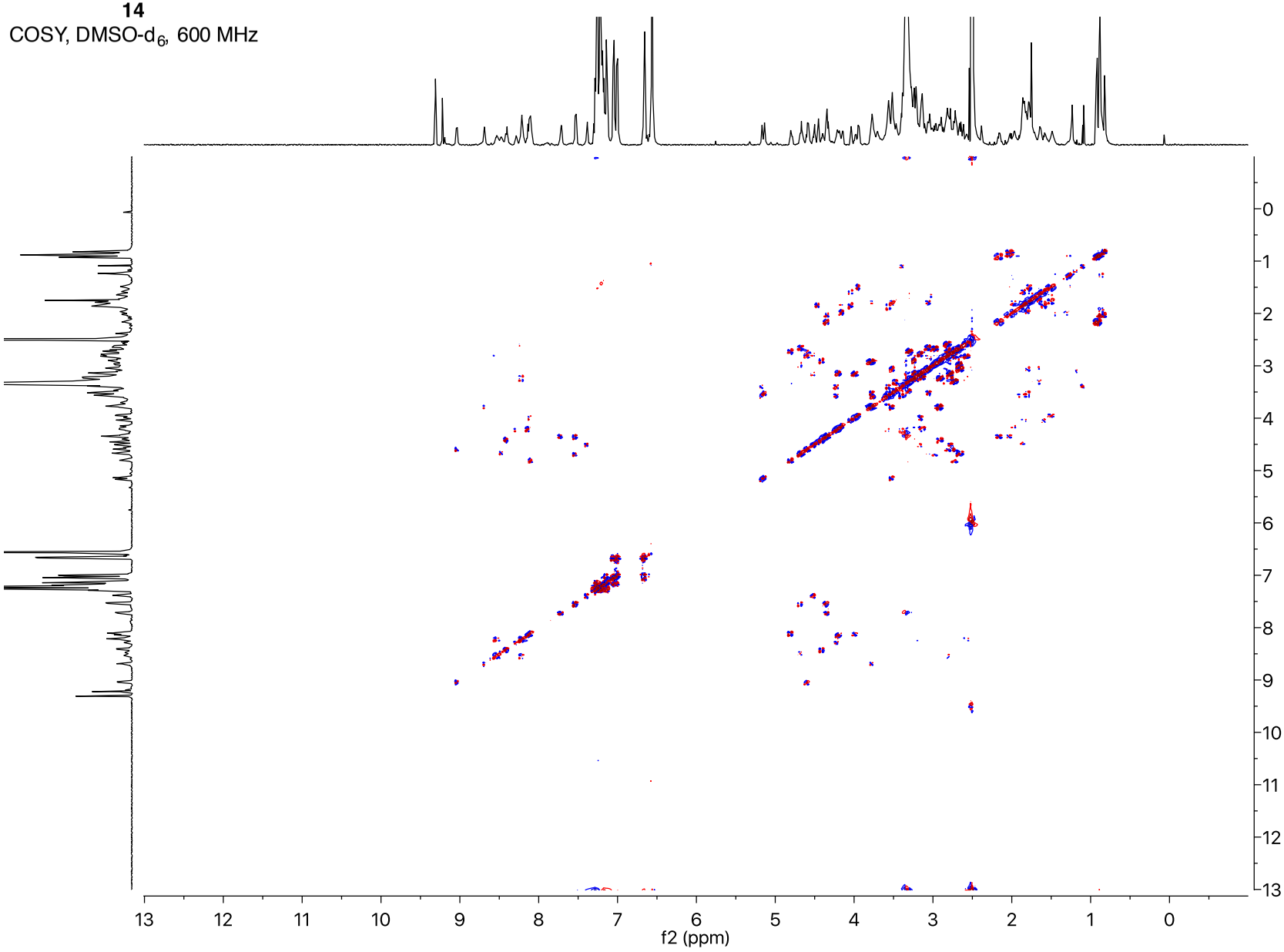


LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 1085.55; found 1085.54.

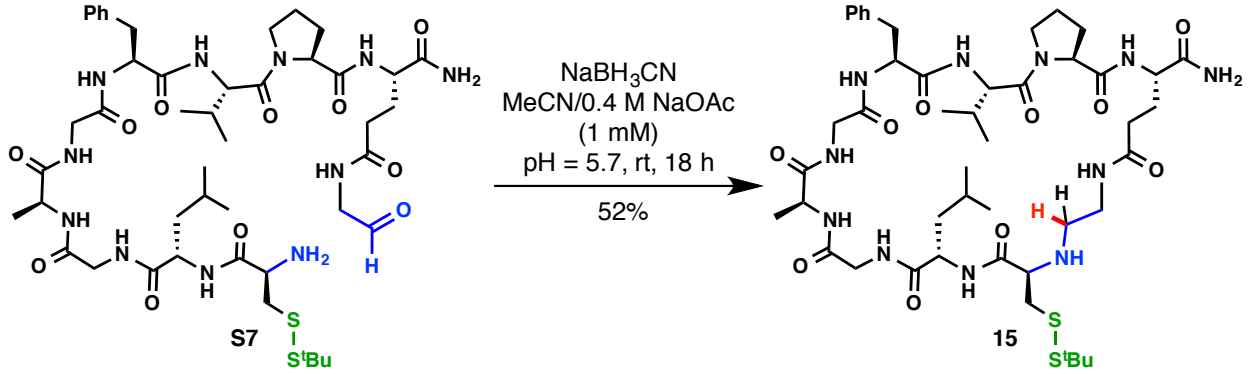

OH
Ph
HN
N
O
Ph
HN
OH
Ph
HN
N
H
O
H
N
H
H
O
HN
14
1H, DMSO-d6, 600 MHz
f1 (ppm)

14

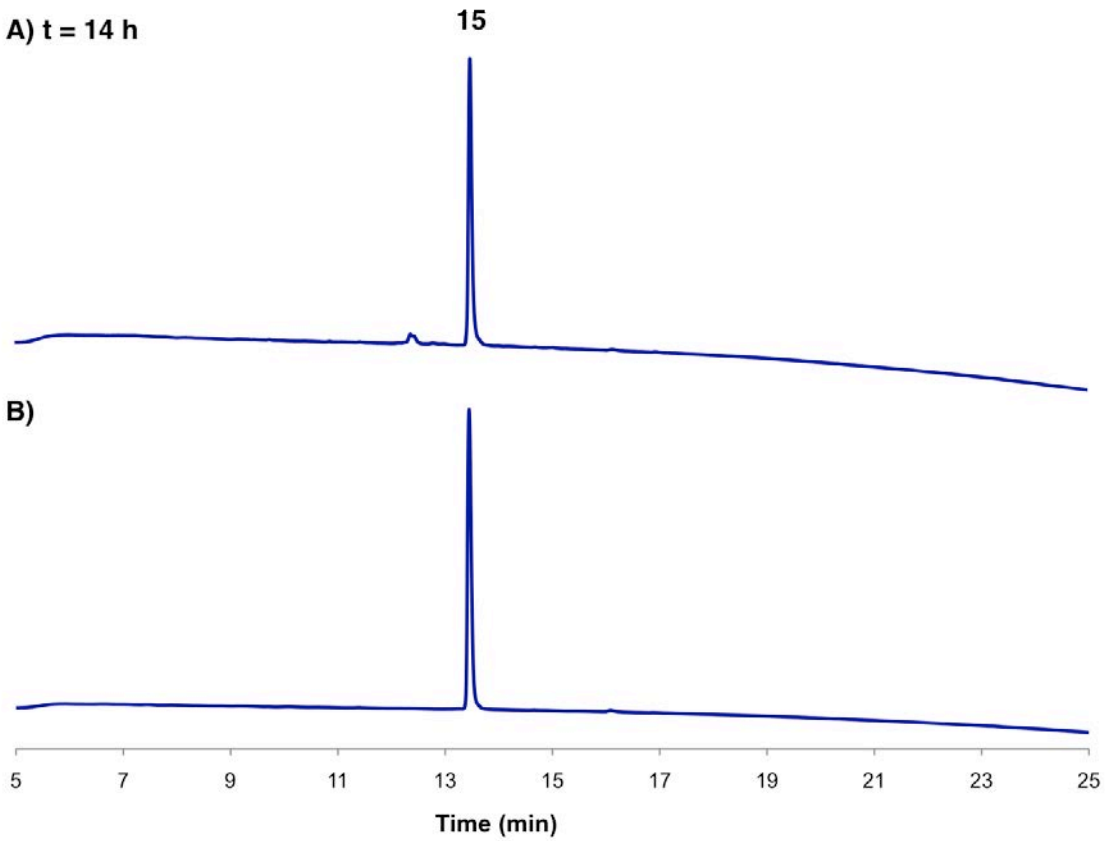
COSY, DMSO-d_6, 600 MHz



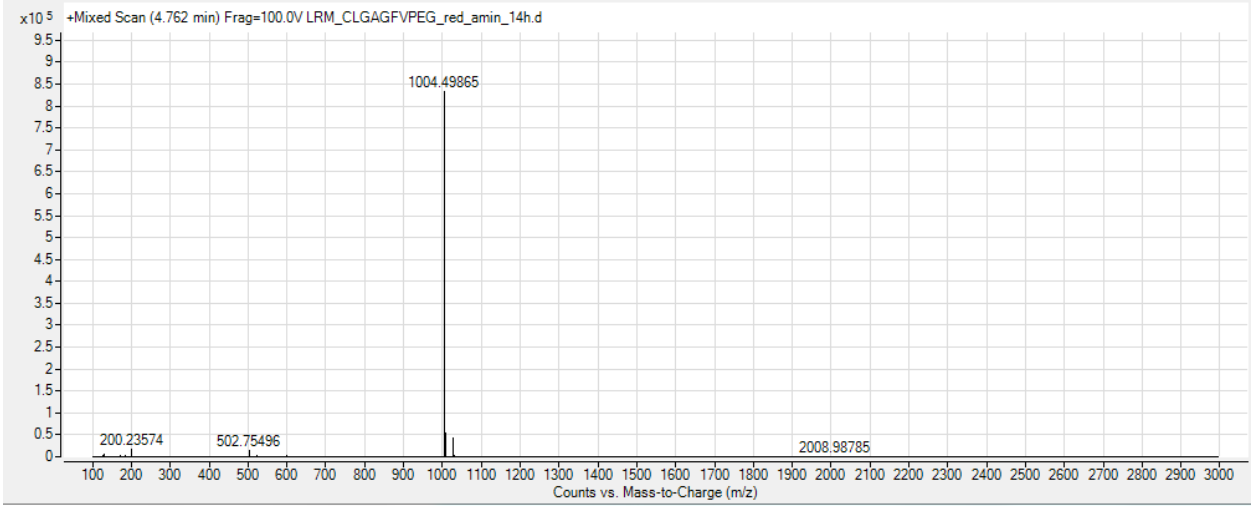
Peptide 15



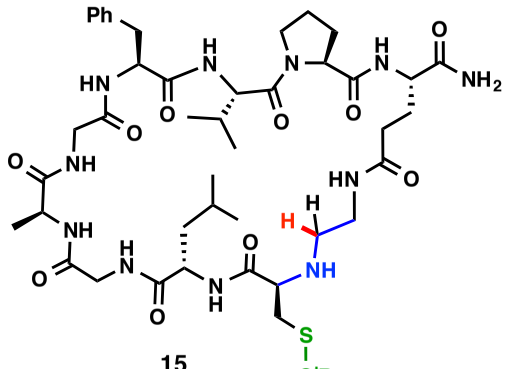
Peptide **15** was prepared according to general procedure 4 from linear amino aldehyde **S7** (4.5 mg, 4.4 μmol). The peptide was solubilized in a 1:3.6 (v/v) mixture of MeCN/0.4 M NaOAc buffer (pH = 5.7). At t = 18 h, the reaction was purified by preparative reverse-phase HPLC (25% B for 5 min, then 25% to 60% B over 30 min) to afford peptide macrocycle **15** (2.3 mg, 52% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **15** from amino aldehyde **S7** following reductive amination (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **15** (Rt = 13.4 min, 5 to 100% B over 25 min, λ = 210 nm).



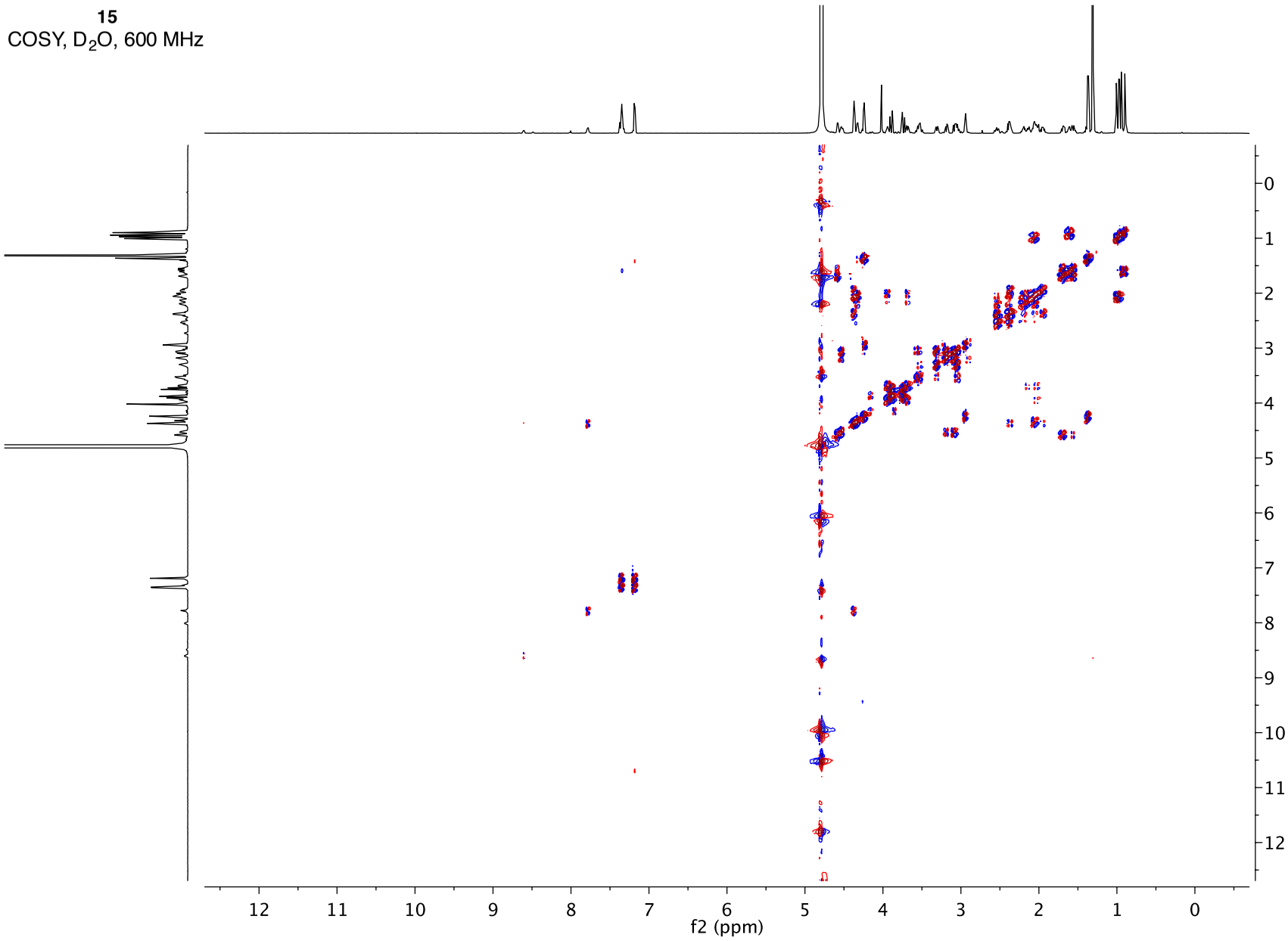
LRMS (ESI-TOF): calc'd for $C_{46}H_{74}N_{11}O_{10}S_2$ $[M+H]^+$ 1004.51; found 1004.50.



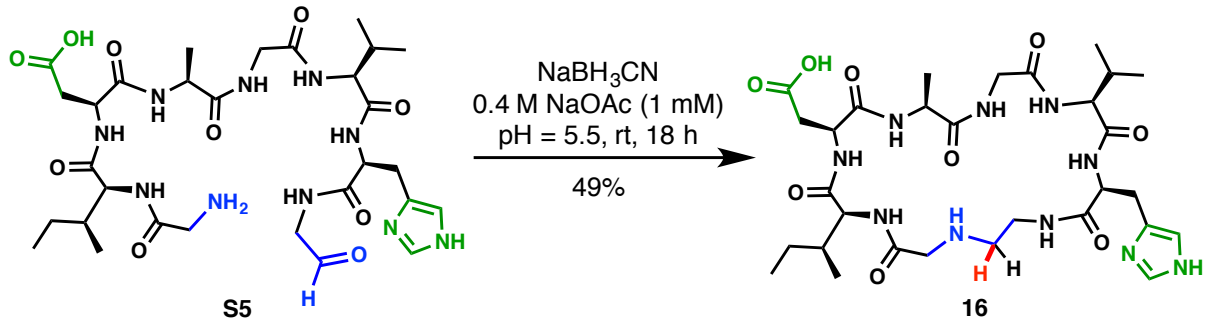
15

^{1}H, D_2O, 600 MHz

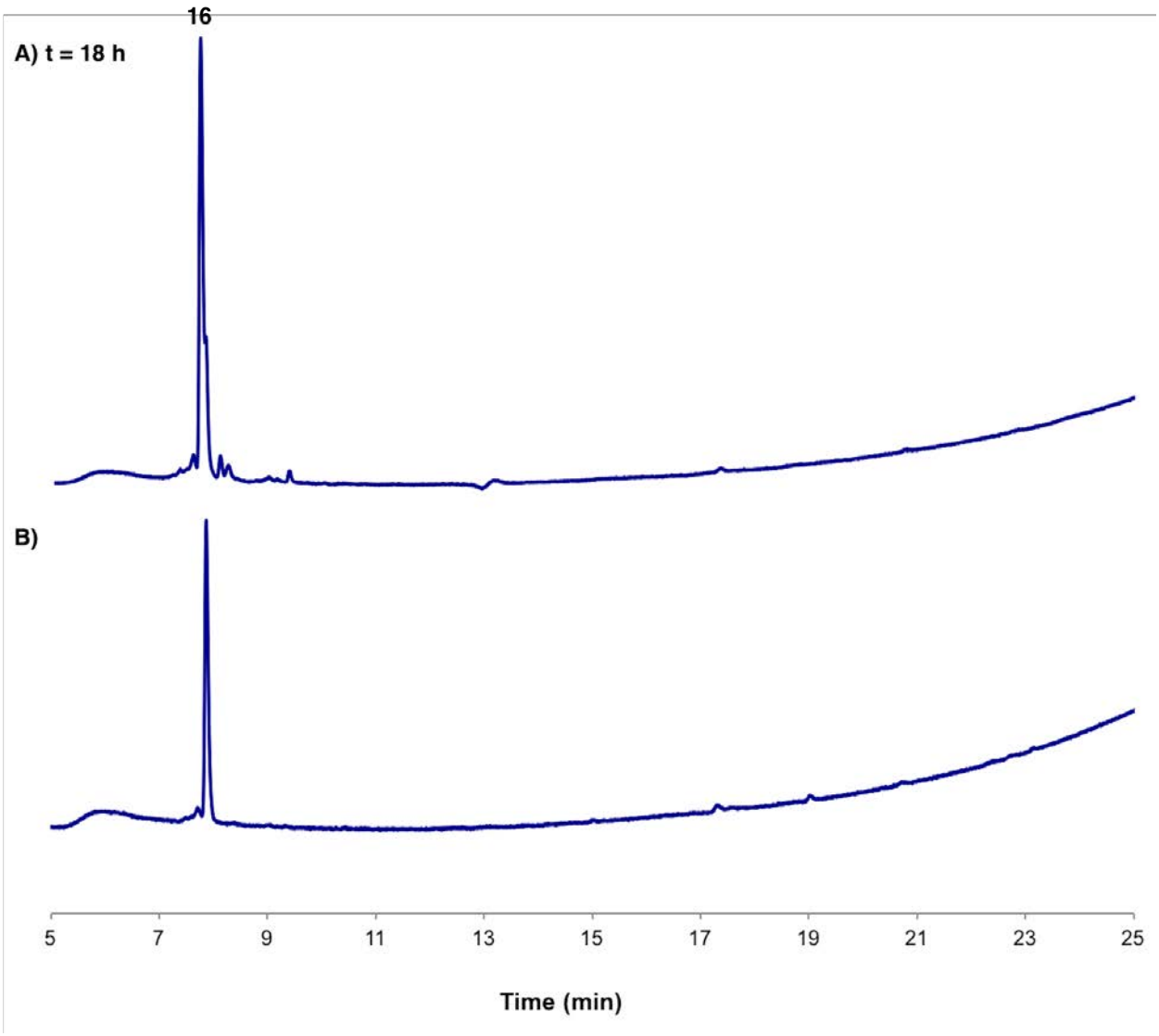
15
COSY, D_2O, 600 MHz



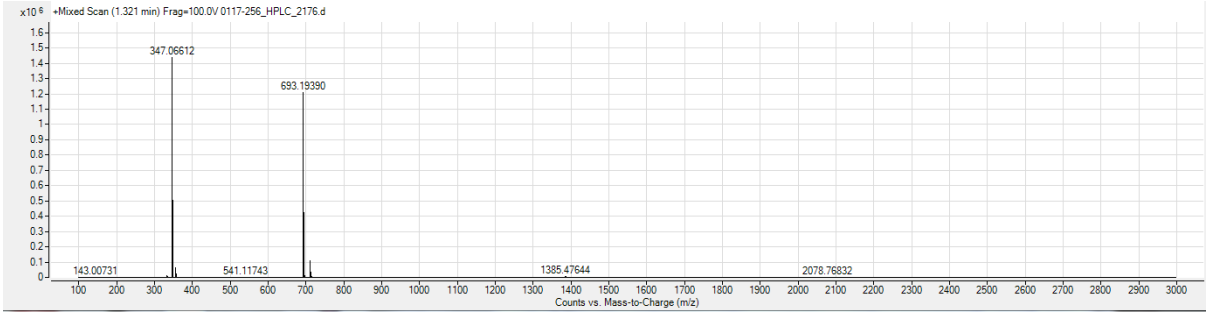
Peptide 16



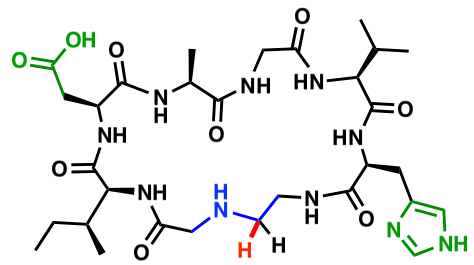
Peptide **16** was prepared according to general procedure 4 from linear amino aldehyde **S5** (4.2 mg, 5.93 μmol). At t = 18 h, the reaction was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% to 40% B over 30 min) to afford peptide macrocycle **16** (2.0 mg, 49% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **16** from amino aldehyde **S5** following reductive amination (0 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **16** (Rt = 7.86 min, 0 to 100% B over 25 min, λ = 230 nm).

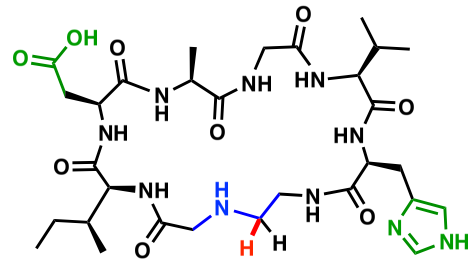


LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 693.37; found 693.19, $[M+2H]^{2+}$ 347.19; found 347.07.



16

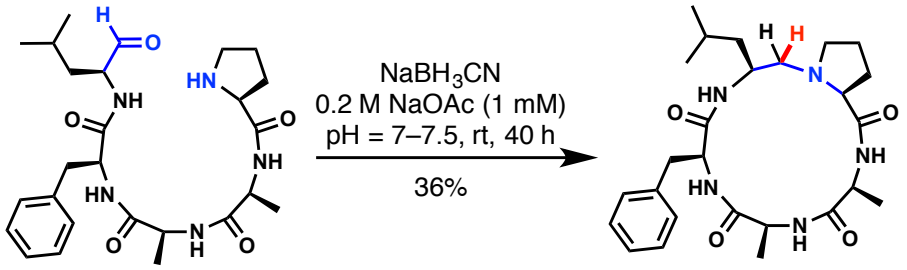
^{1}H, DMSO-d$_6$/D$_2$O (20:1 v/v), 600 MHz



16

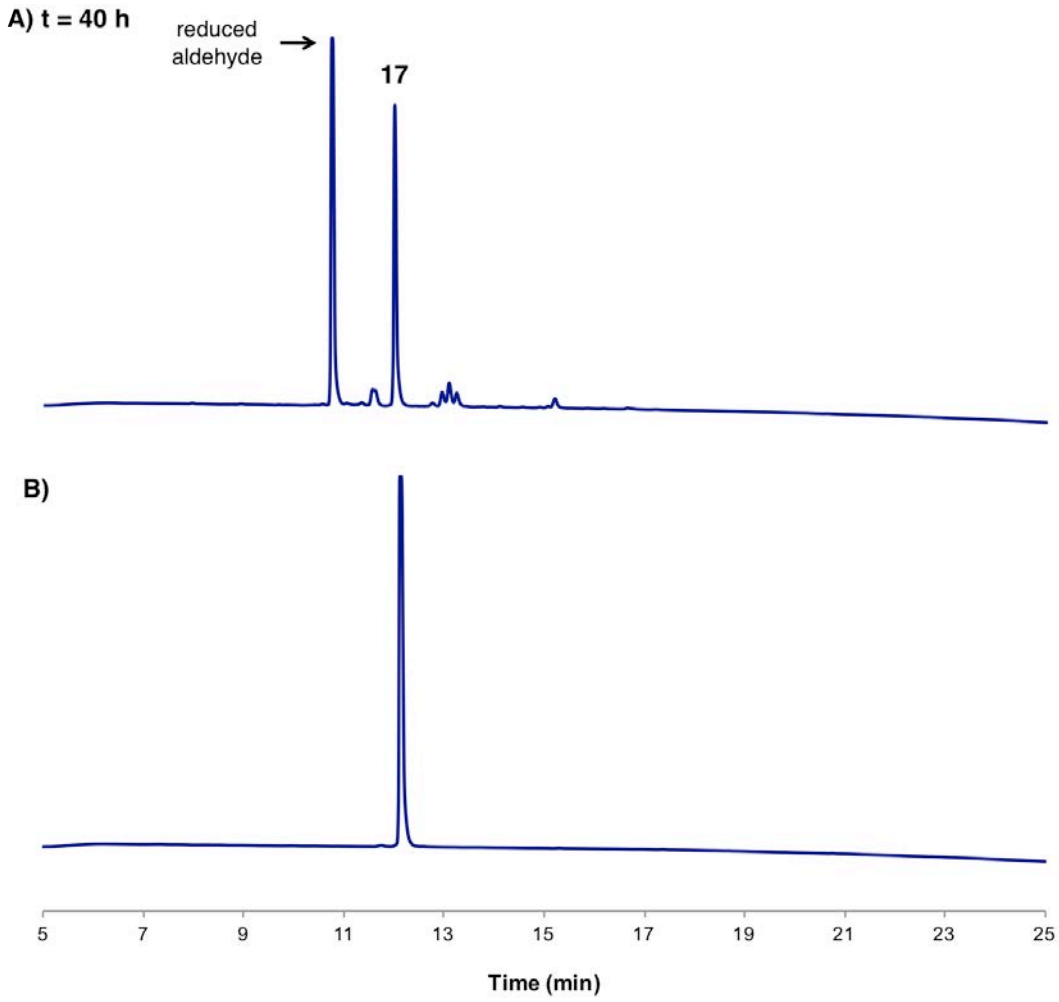
^{1}H, DMSO-d$_6$, 600 MHz

Peptide 17

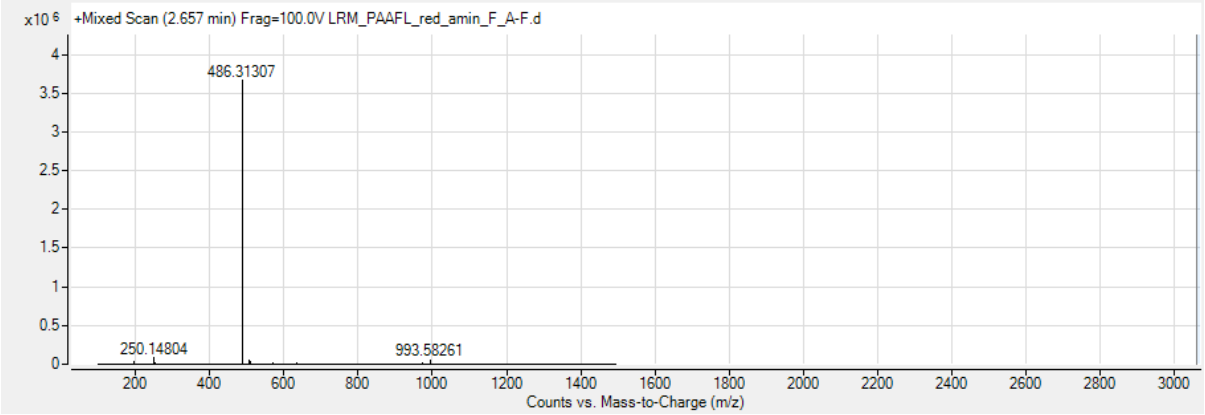


S11 17

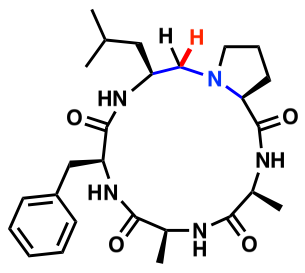
Peptide **17** was prepared according to general procedure 4 from linear amino aldehyde **S11** (9.5 mg, 19.0 μmol). The peptide was solubilized in 0.2 M NaOAc buffer (pH = 7–7.5). At t = 40 h, the reaction was purified by preparative reverse-phase HPLC (15% B for 5 min, 15% to 55% B over 25 min, then 55% to 70% B over 10 min) to afford peptide macrocycle **17** (3.3 mg, 36% yield) as a white solid following lyophilization. Reduction of the starting aldehyde was also observed (3.1 mg, 36% yield).



A) Crude analytical HPLC trace of the formation of **17** from amino aldehyde **S11** following reductive amination (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **17** (Rt = 12.1 min, 5 to 100% B over 25 min, λ = 210 nm).

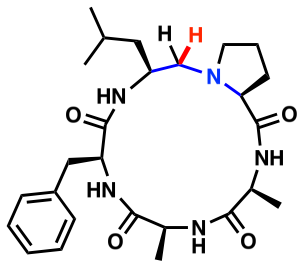


LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_4$ $[M+H]^+$ 486.31; found 486.31.



17

^{1}H, DMSO-d_6/D_2O (20:1 v/v), 600 MHz

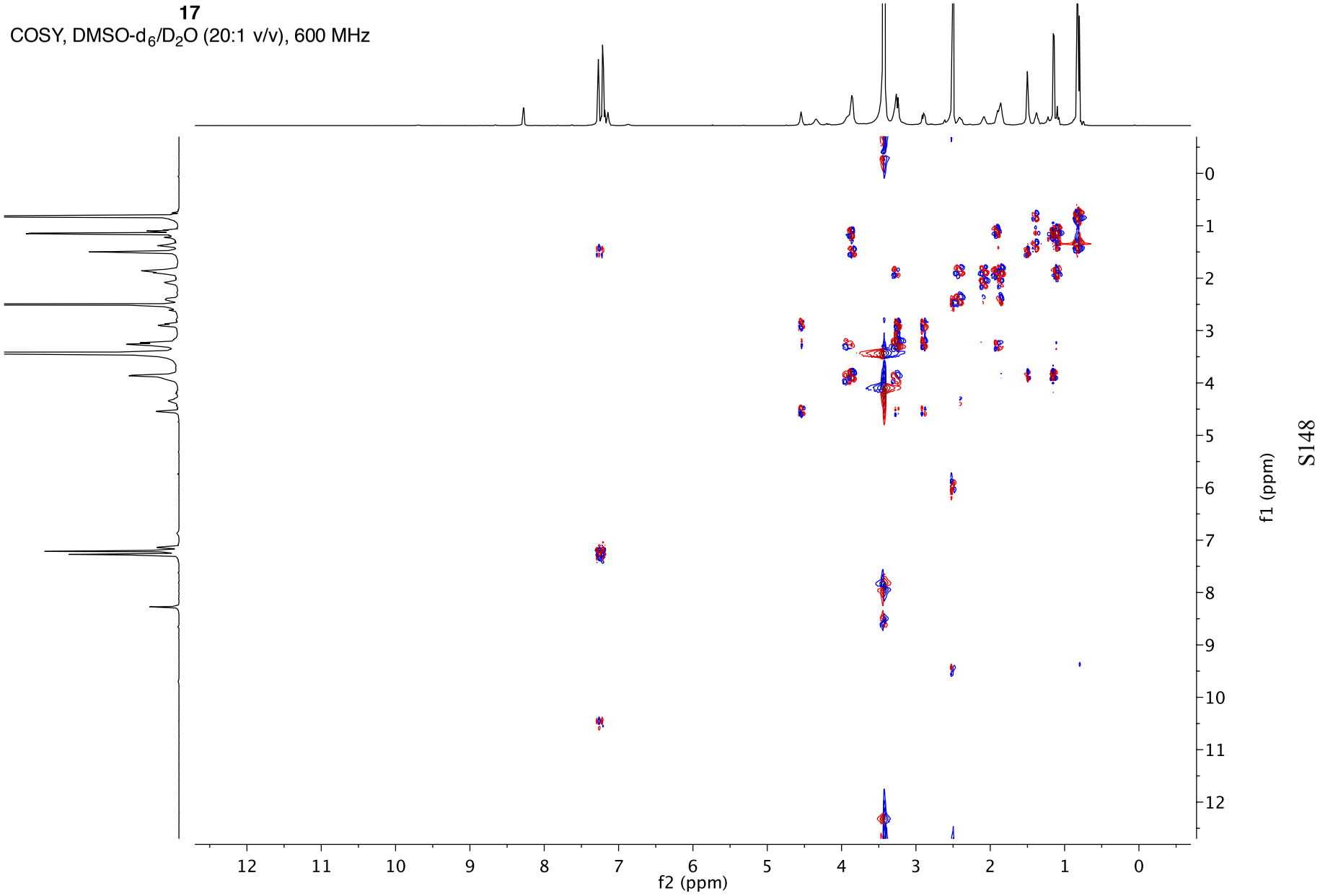


17

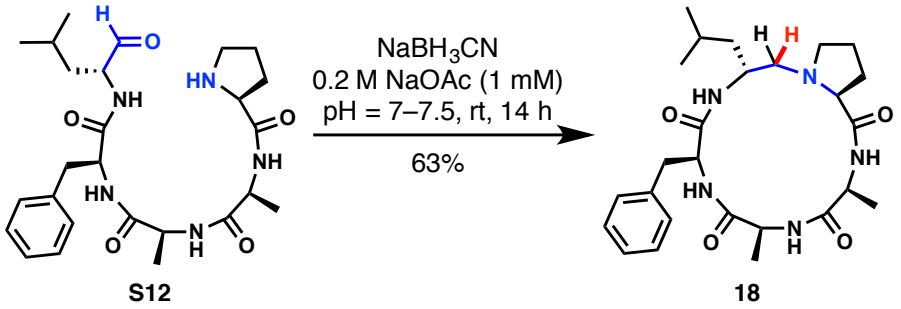
^{13}C, DMSO-d_6/D_2O (20:1 v/v), 151 MHz

17

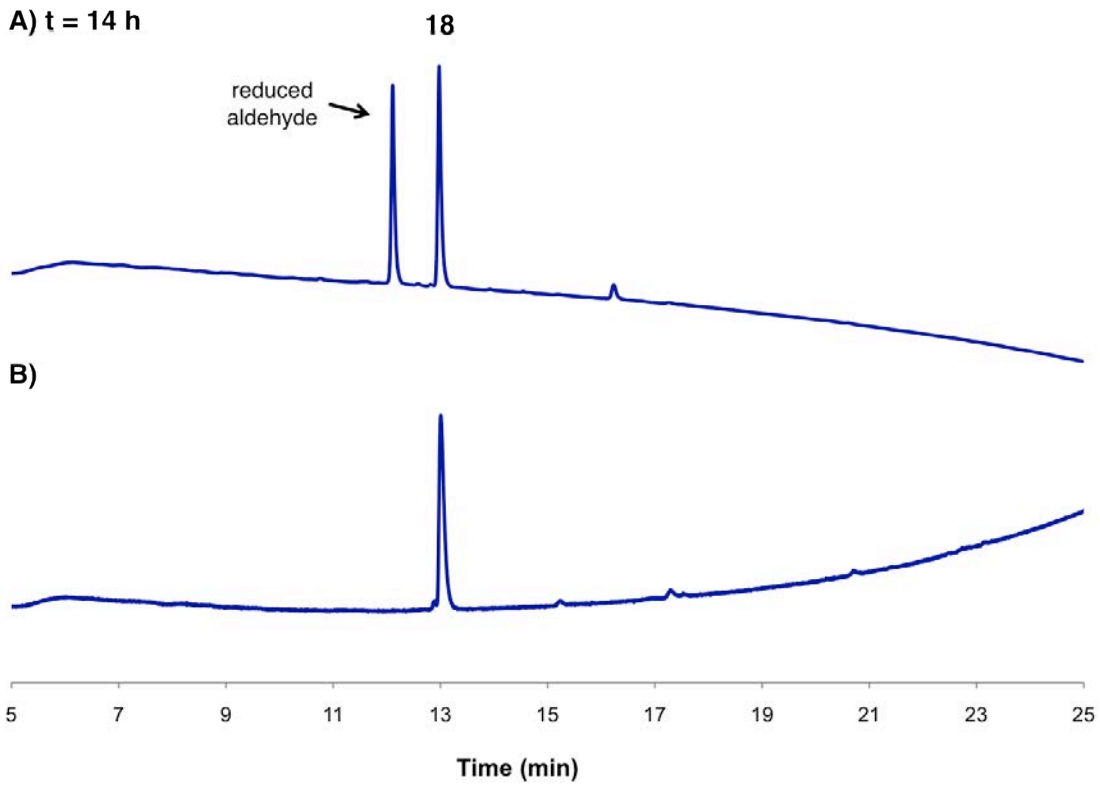
COSY, DMSO-d_6/D_2O (20:1 v/v), 600 MHz



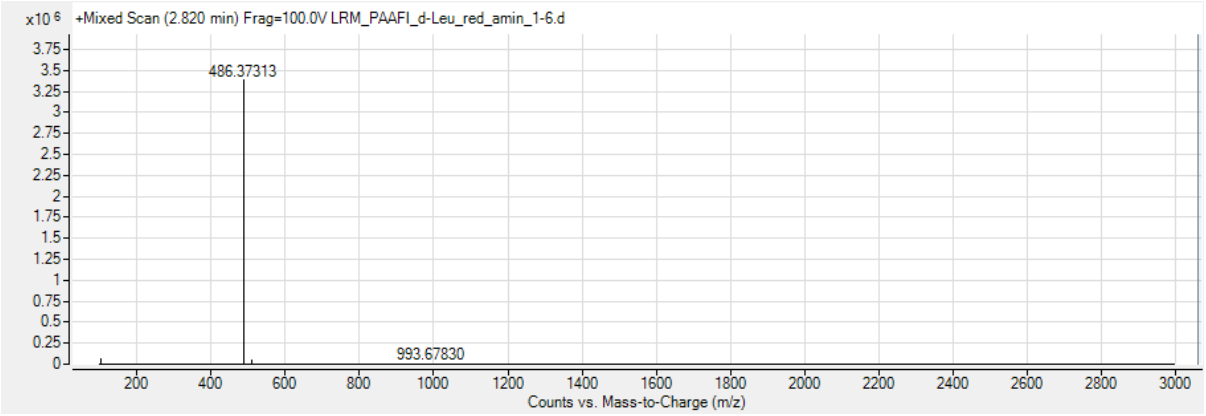
Peptide 18



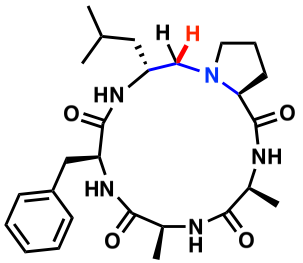
Peptide **18** was prepared according to general procedure 4 from linear amino aldehyde **S12** (6.1 mg, 12.2 μmol). The peptide was solubilized in 0.2 M NaOAc buffer (pH = 7–7.5). At t = 14 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% to 50% B over 25 min) to afford peptide macrocycle **18** (3.7 mg, 63% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **18** from amino aldehyde **S12** following reductive amination (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **18** (Rt = 13.0 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_4$ $[M+H]^+$ 486.31; found 486.37.

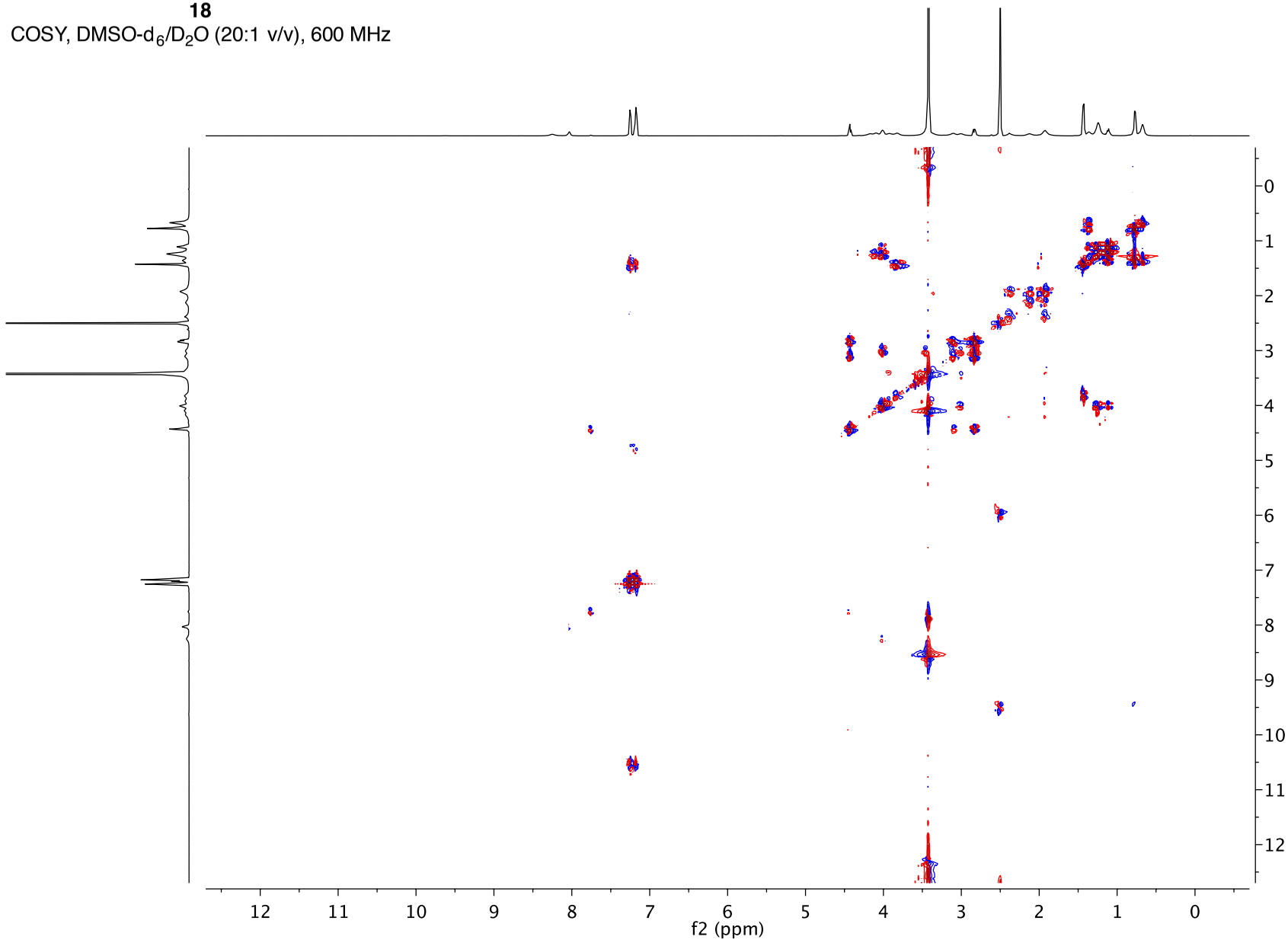


18

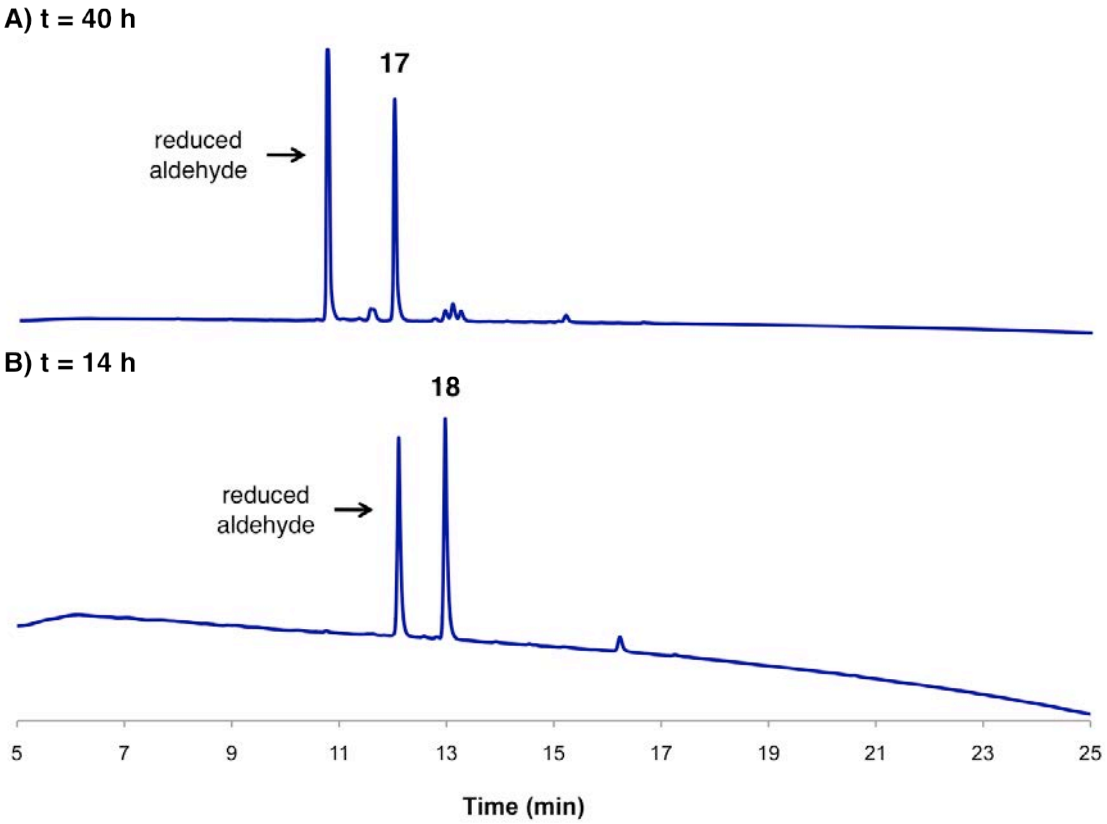
^{1}H, DMSO-d_6/D_2O (20:1 v/v), 600 MHz

18

COSY, DMSO-d_6/D_2O (20:1 v/v), 600 MHz



Comparative macrocyclization: Peptide 17 and 18

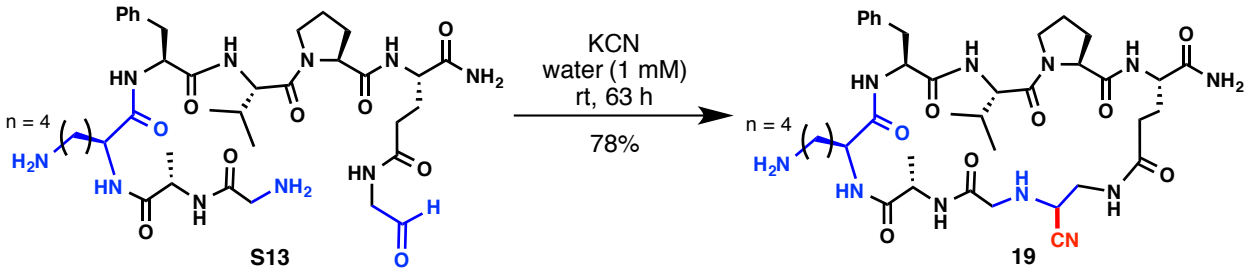


A) Crude analytical HPLC trace of the formation of **17** from amino aldehyde **S11** at t = 40 h (5 to 100% B over 25 min, λ = 210 nm). **B)** Crude analytical HPLC trace of the formation of **18** from amino aldehyde **S12** at t = 14 h (5 to 100% B over 25 min, λ = 210 nm).

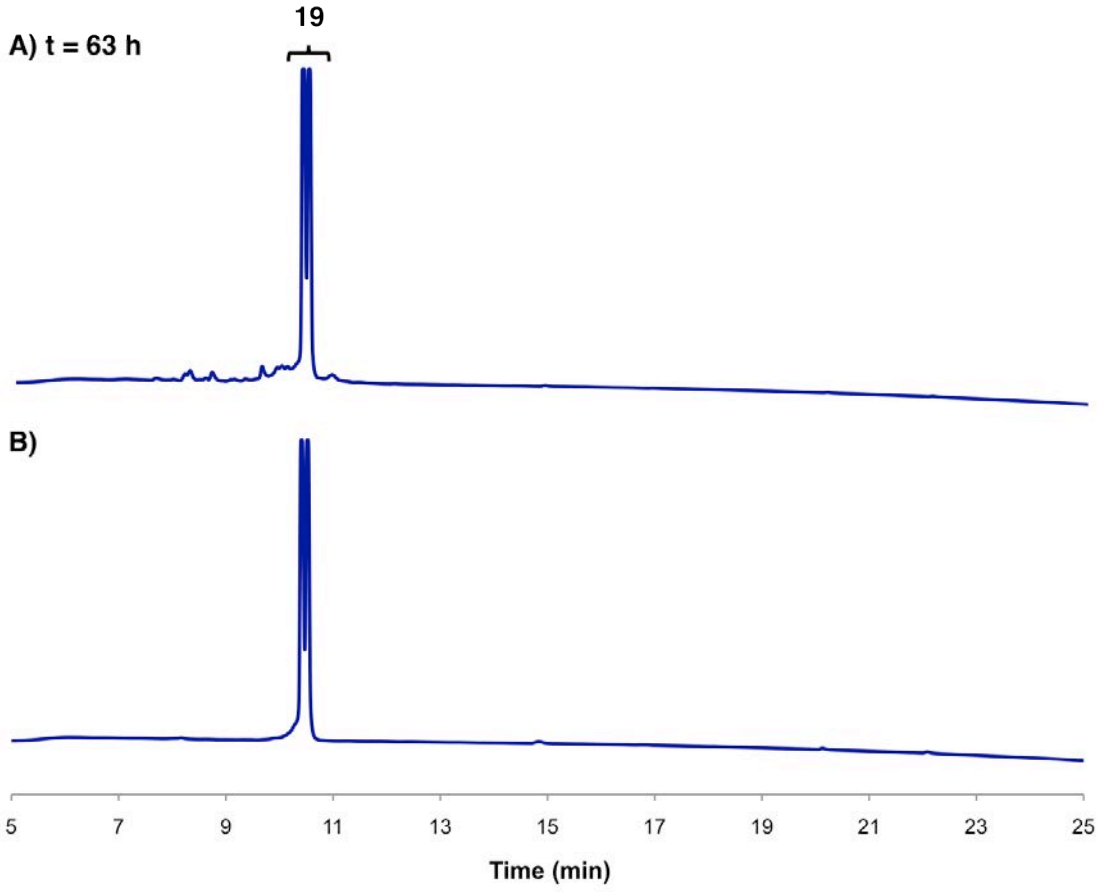
Note the enhanced rate of reaction for peptide **S12**, bearing a D-Leu aldehyde as the C-terminal residue. Based on HPLC analysis, the slower macrocyclization (**S11** to **17**) was also more prone to aldehyde reduction, cyanohydrin formation and dimerization (see minor byproducts in figure A, above). Based on retention time, a small amount of linear **S11** may have epimerized at the C-terminal aldehyde to afford macrocycle **18**. However, this potential byproduct was not substantial enough to be isolated for analysis following HPLC purification.

Characterization Data for Internal Lysine Studies

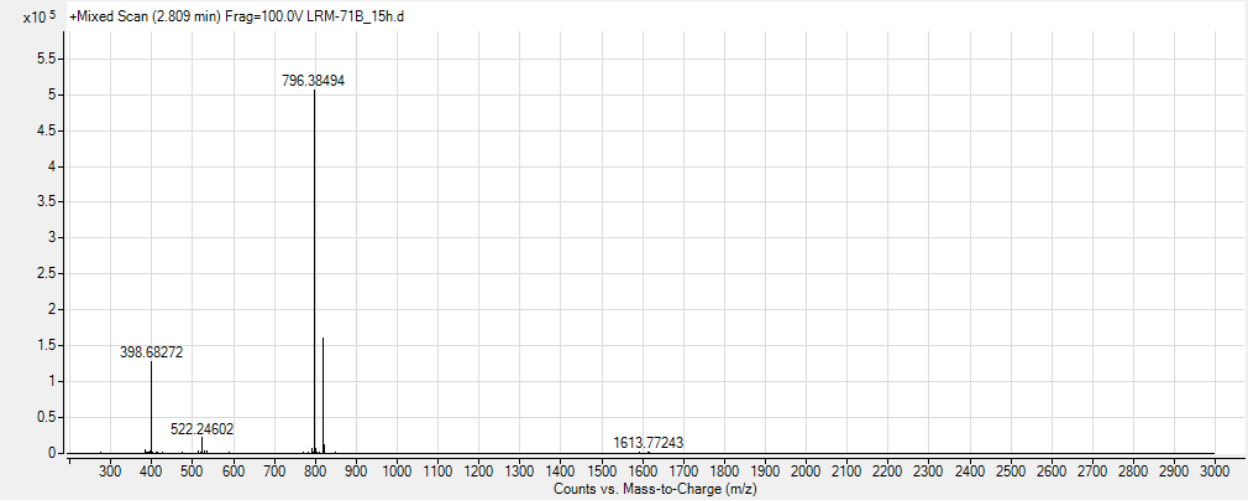
Peptide 19



Peptide **19** was prepared according to general procedure 3 from linear amino aldehyde **S13** (5.1 mg, 6.5 µmol). At t = 63 h, the reaction was purified by preparative reverse-phase HPLC (10% B for 5 min, then 10% to 45% B over 30 min) to afford peptide macrocycle **19** (4.0 mg, 78% yield) as a mixture of diastereomers and as a white solid following lyophilization.

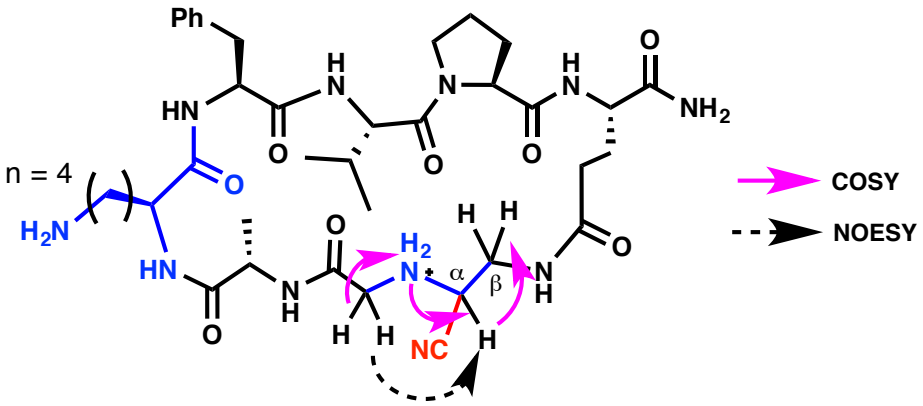


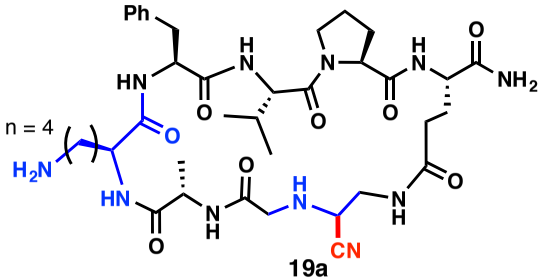
A) Crude analytical HPLC trace of the formation of **19** from amino aldehyde **S13** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **19** (Rt = 10.4, 10.5 min, 5 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{38}H_{58}N_{11}O_8$ $[M+H]^+$ 796.45; found 796.38.

Partial 2D NMR Correlations:



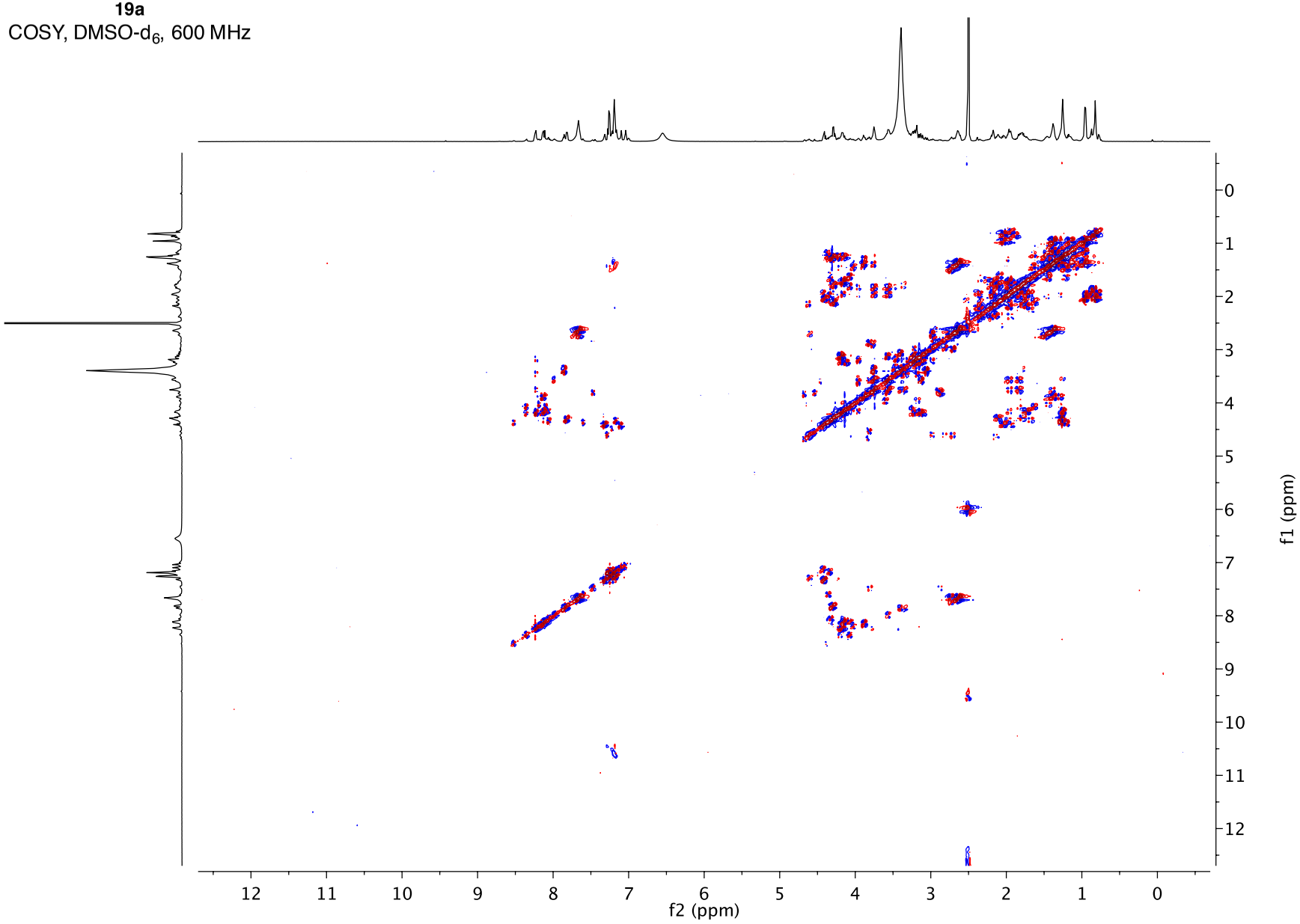


19a

^{1}H, DMSO-d_6, 600 MHz

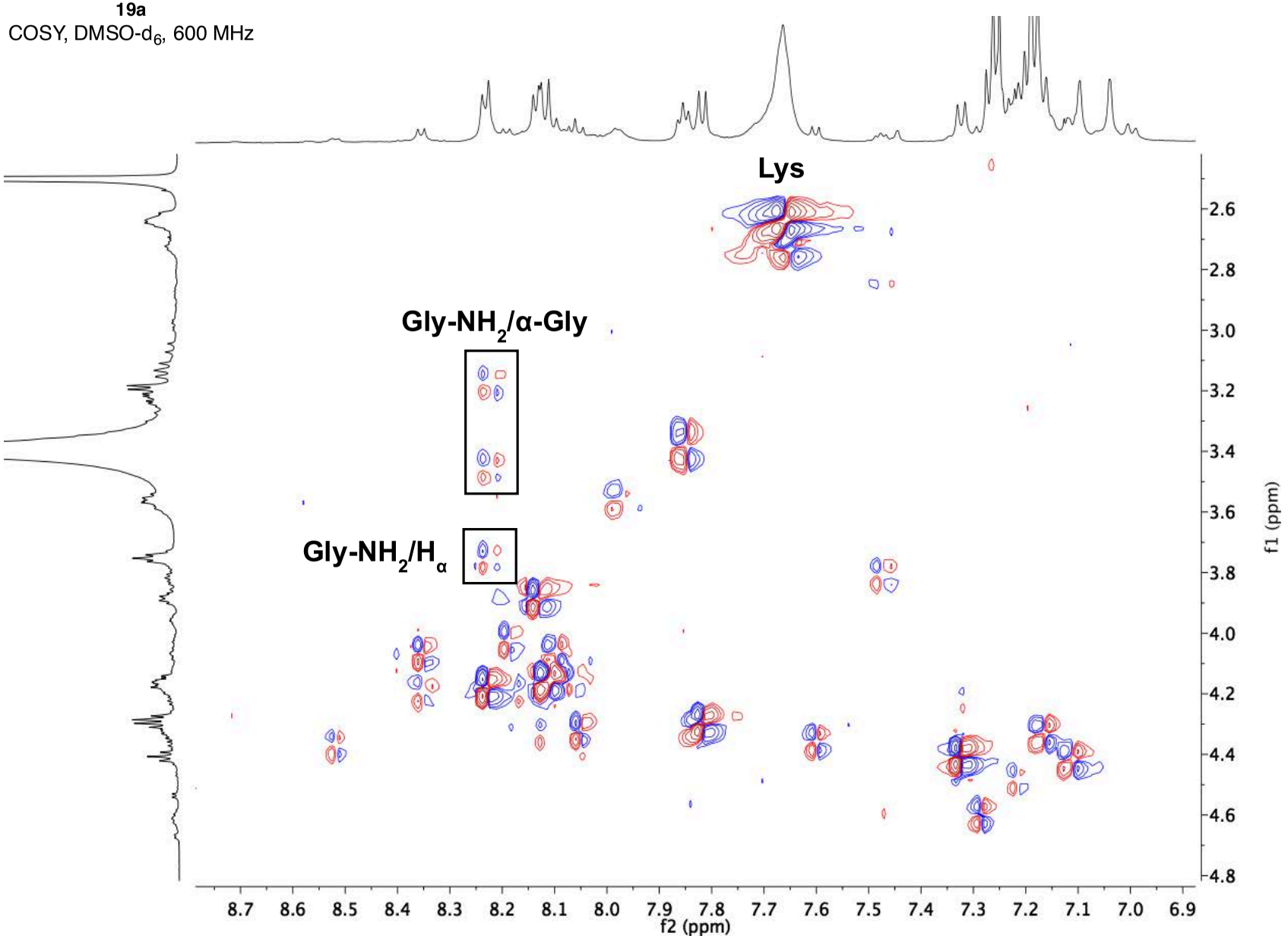
19a

COSY, DMSO-d_6, 600 MHz



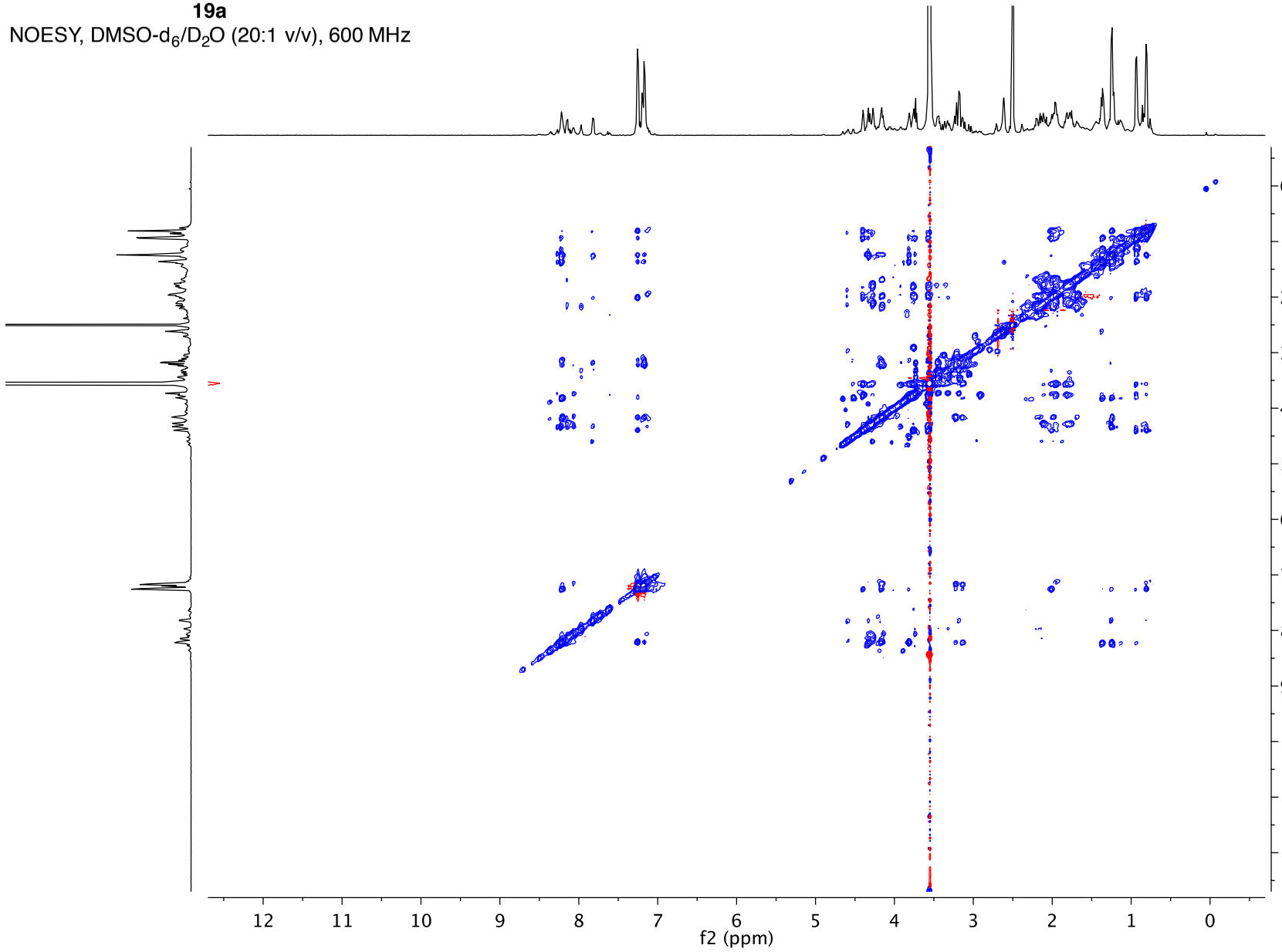
19a

COSY, DMSO-d_6, 600 MHz



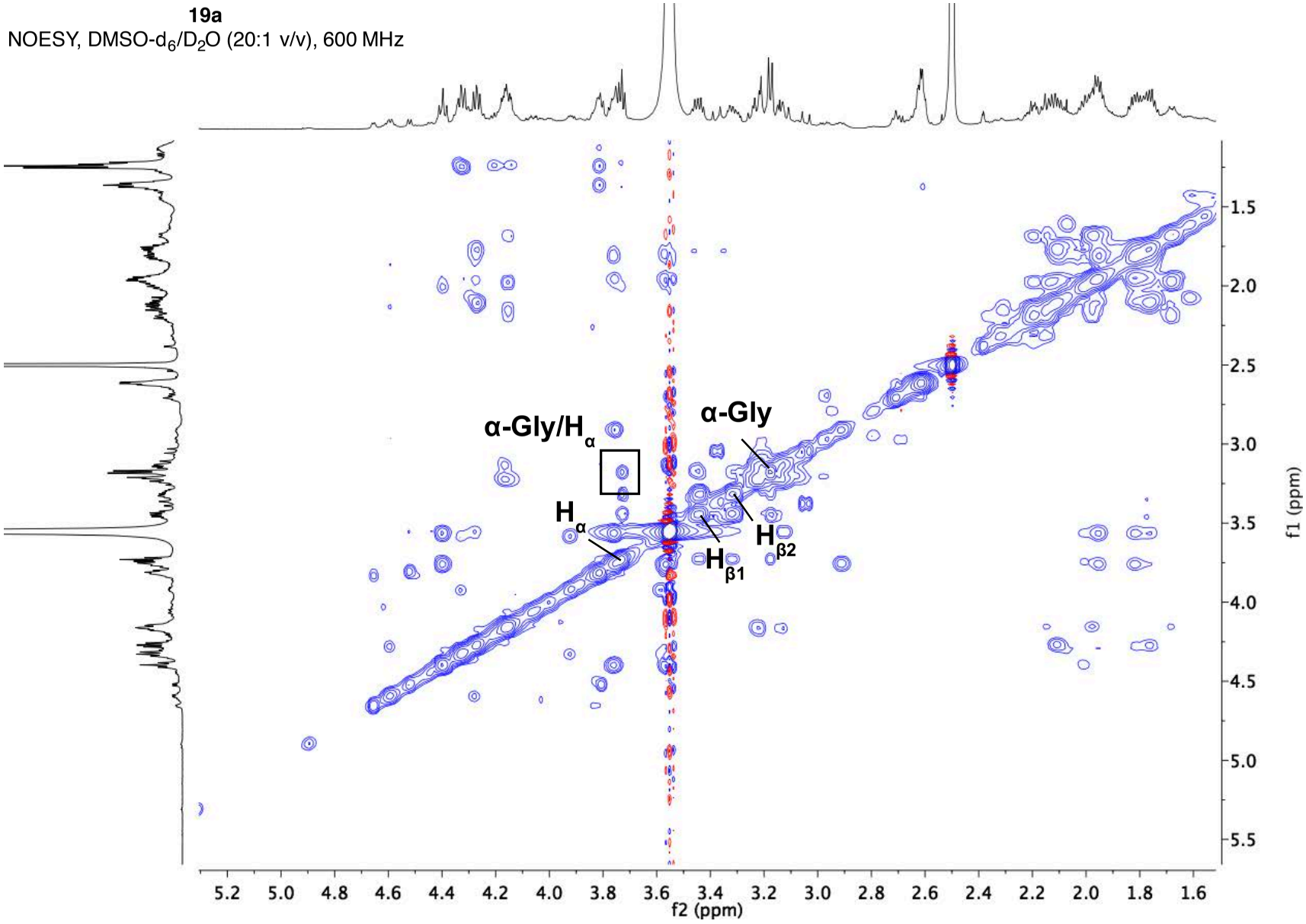
19a

NOESY, DMSO-d_6/D_2O (20:1 v/v), 600 MHz

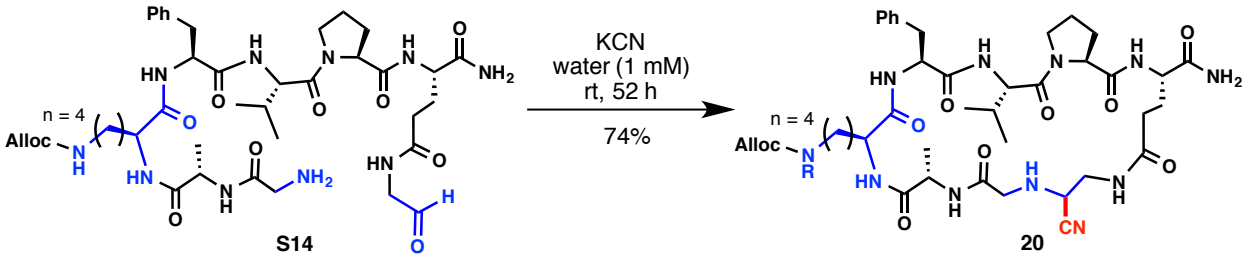


19a

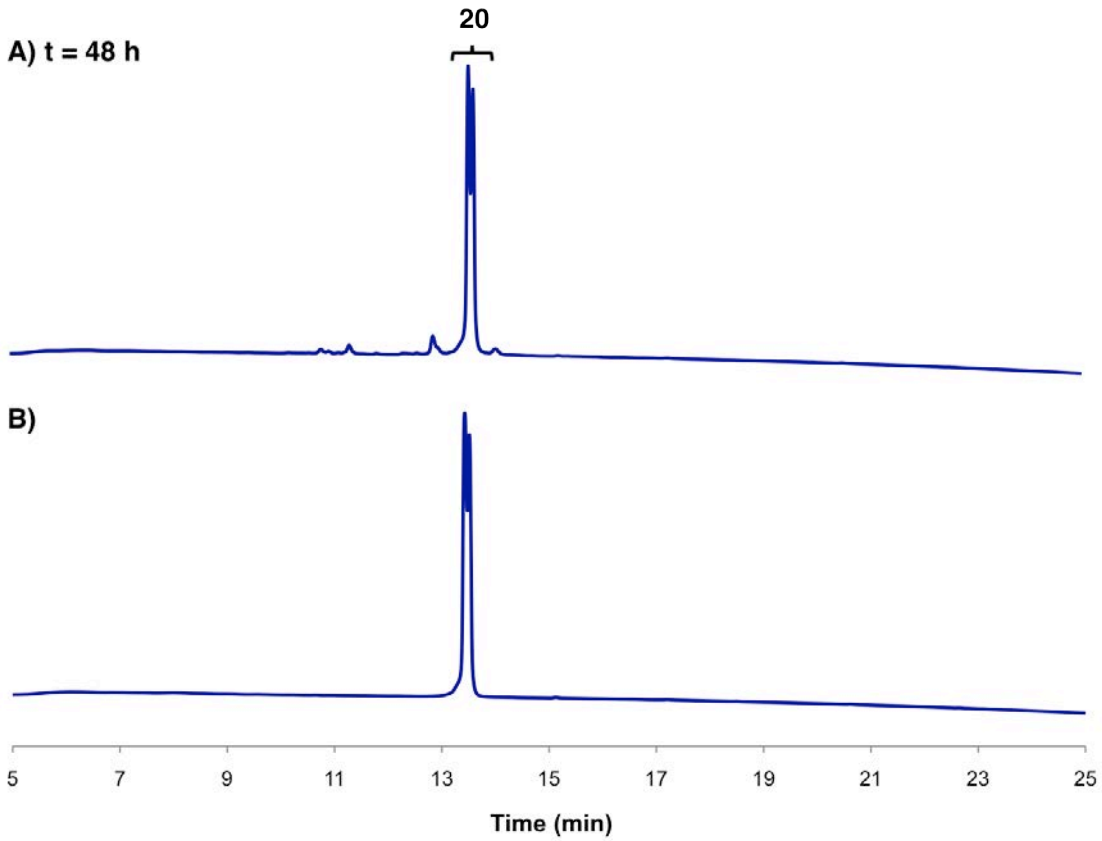
NOESY, DMSO-d_6/D_2O (20:1 v/v), 600 MHz



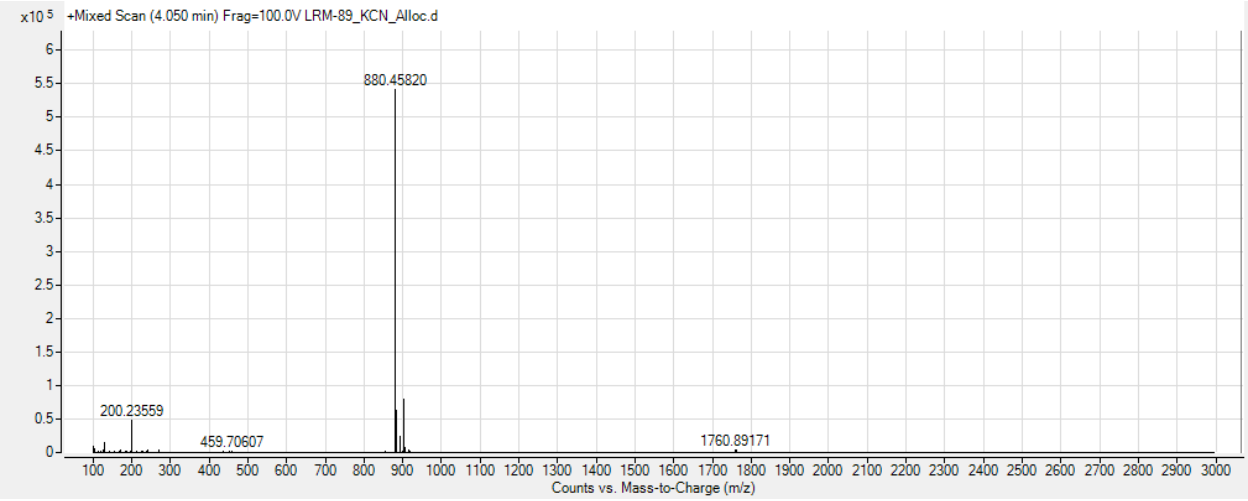
Peptide 20



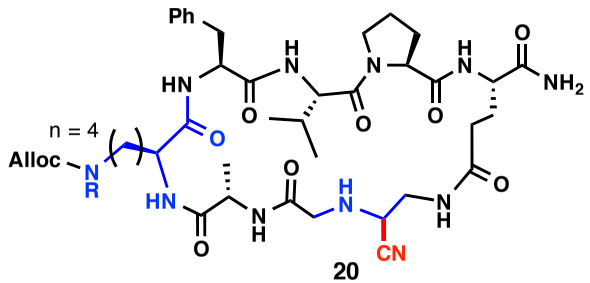
Peptide **20** was prepared according to general procedure 3 from linear amino aldehyde **S14** (3.9 mg, 4.5 μmol). At t = 52 h, the reaction was purified by preparative reverse-phase HPLC (25% B for 5 min, then 25% to 60% B over 30 min) to afford peptide macrocycle **20** (2.9 mg, 74% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **20** from amino aldehyde **S14** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **20** (Rt = 13.4, 13.5 min, 5 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{42}H_{62}N_{11}O_{10}$ $[M+H]^+$ 880.47; found 880.46.

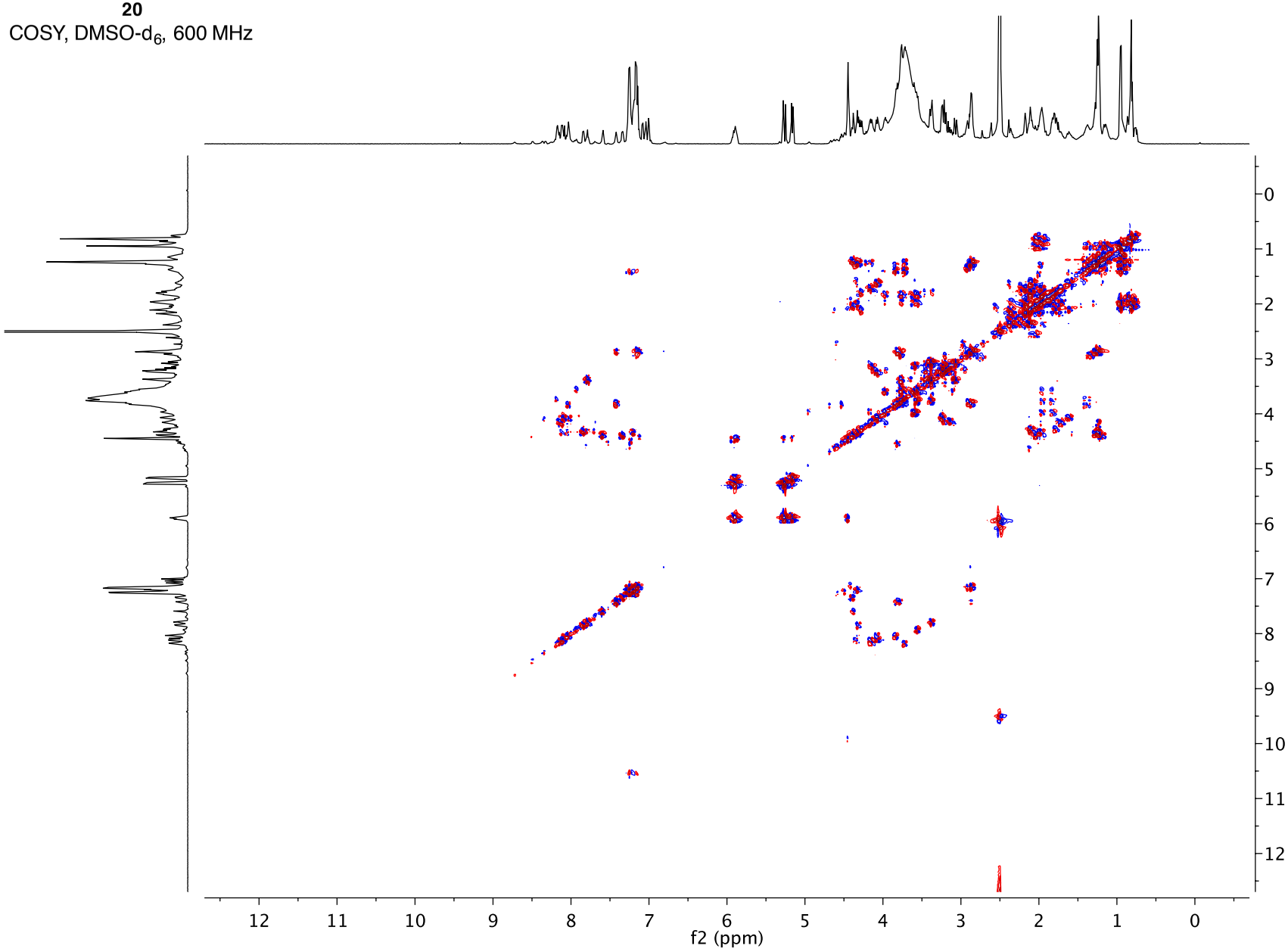


20

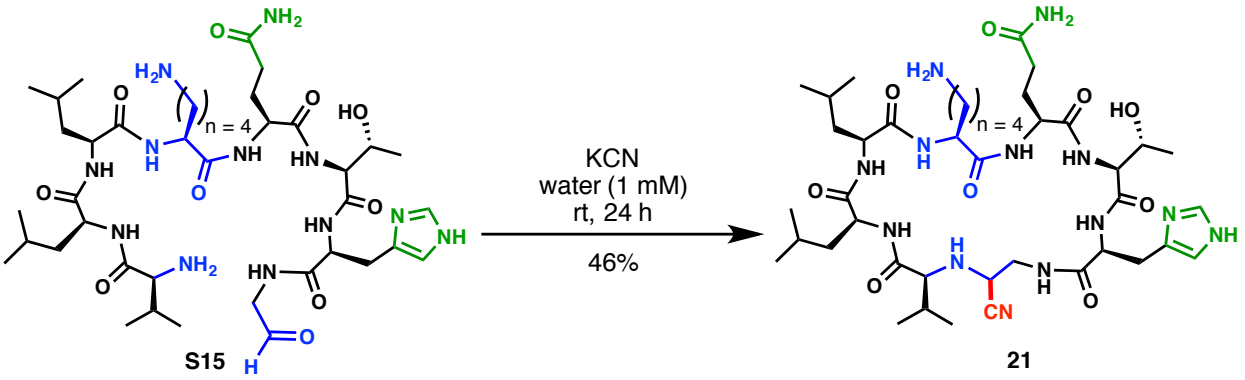
^{1}H, DMSO-d$_6$, 600 MHz

20

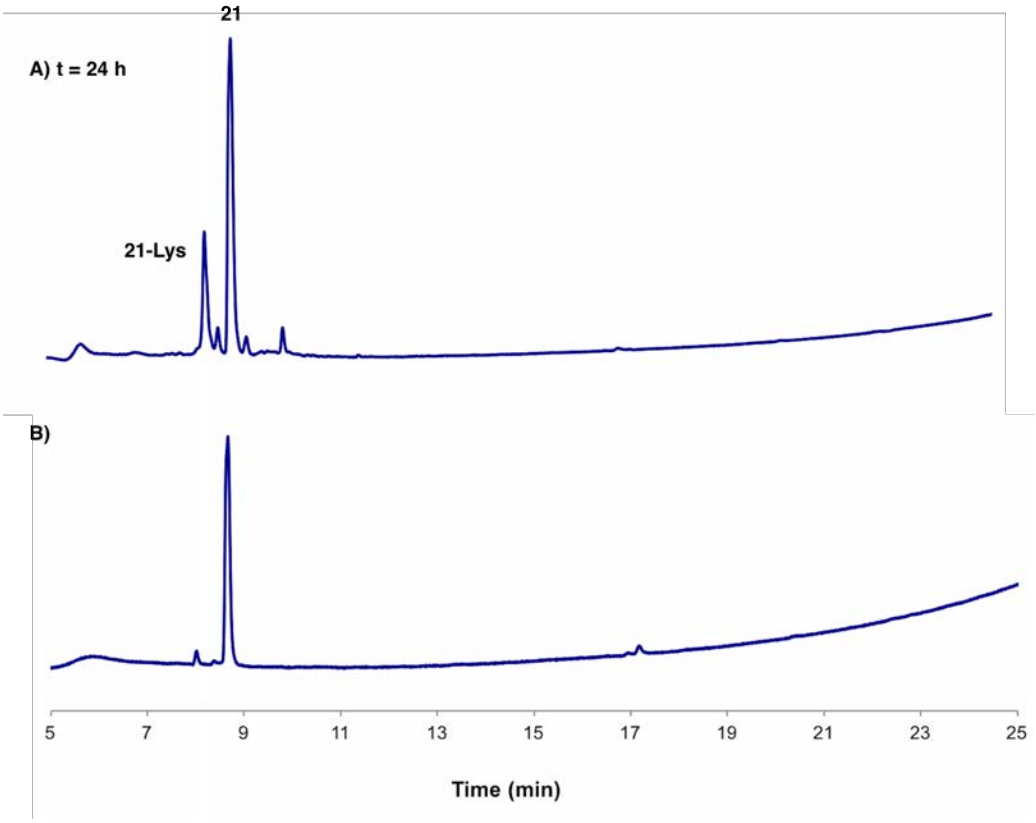
COSY, DMSO-d_6, 600 MHz



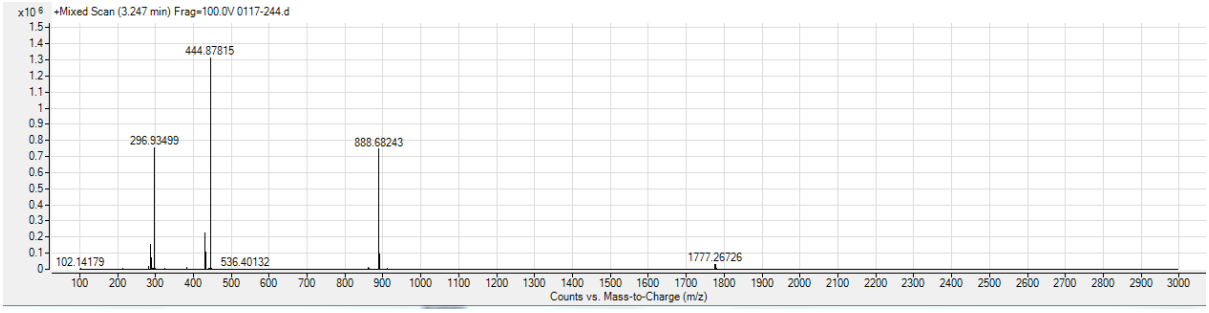
Peptide 21



Peptide **21** was prepared according to general procedure 3 from linear amino aldehyde **S15** (7.0 mg, 7.96 μmol). At t = 24 h, the reaction was purified by preparative reverse-phase HPLC (10% B for 5 min, then 10% to 45% B over 30 min) to afford peptide macrocycle **21** (4.6 mg, 46% yield) as a mixture of diastereomers and as a white solid following lyophilization. *Note: The lysine-εNH$_2$ cyclized compound was also isolated as a minor product (2.3:1* **21**:**21-Lys***).*



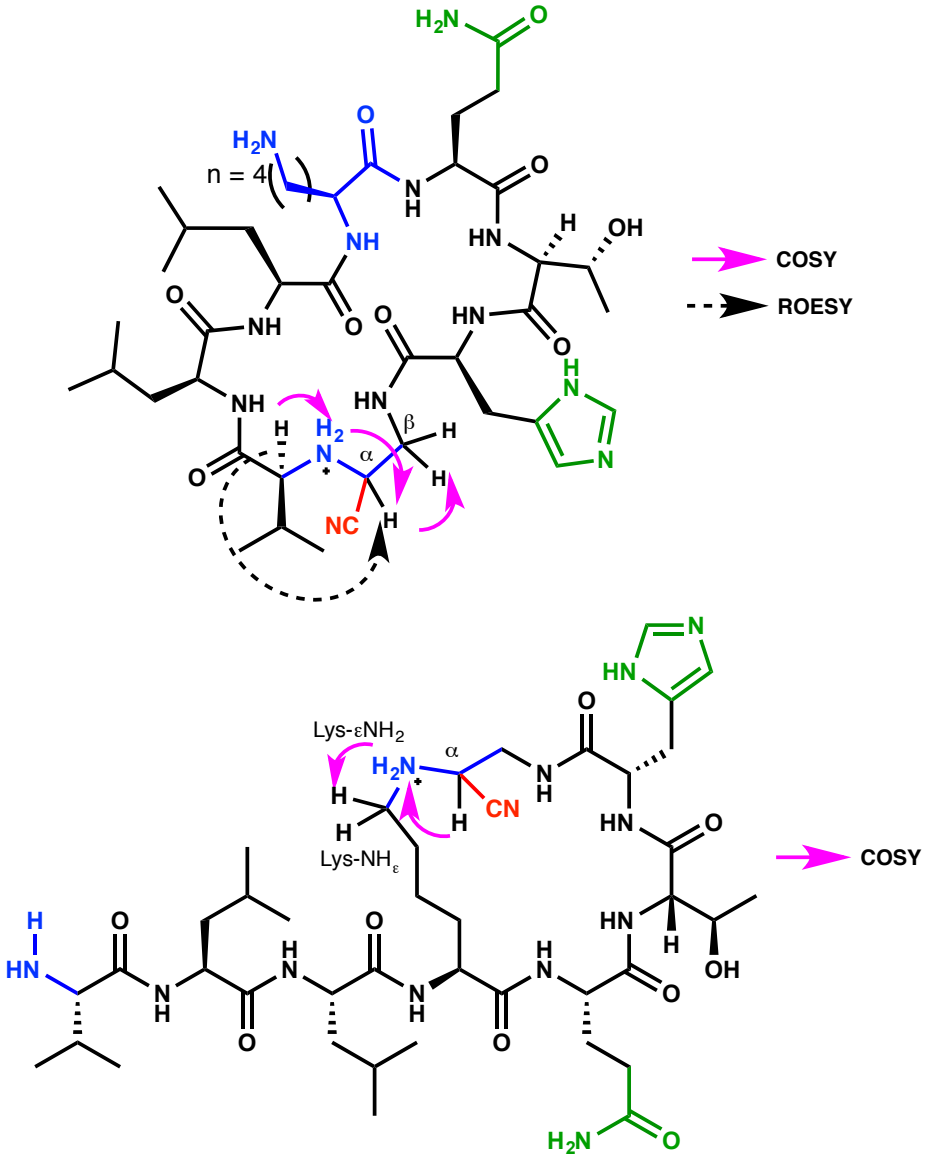
A) Crude analytical HPLC trace of the formation of **21** from amino aldehyde **S15** following Strecker macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **21** (Rt = 8.7 min, 5 to 100% B over 25 min, λ = 210 nm).

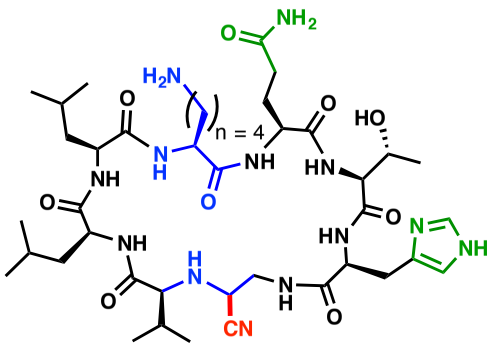


LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 888.54; found 888.68, $[M+2H]^{2+}$ 444.77; found 444.89.

Partial 2D NMR Correlations:

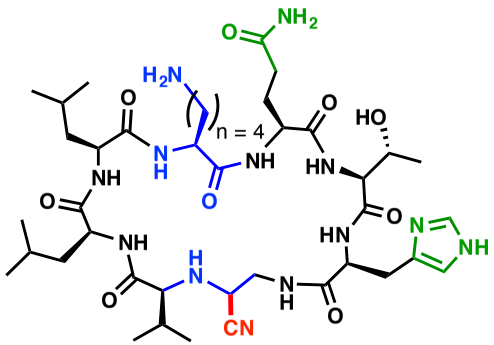
(top: compound **21**; bottom: compound **21-Lys**)





21

^{1}H, DMSO-d$_6$, 600 MHz

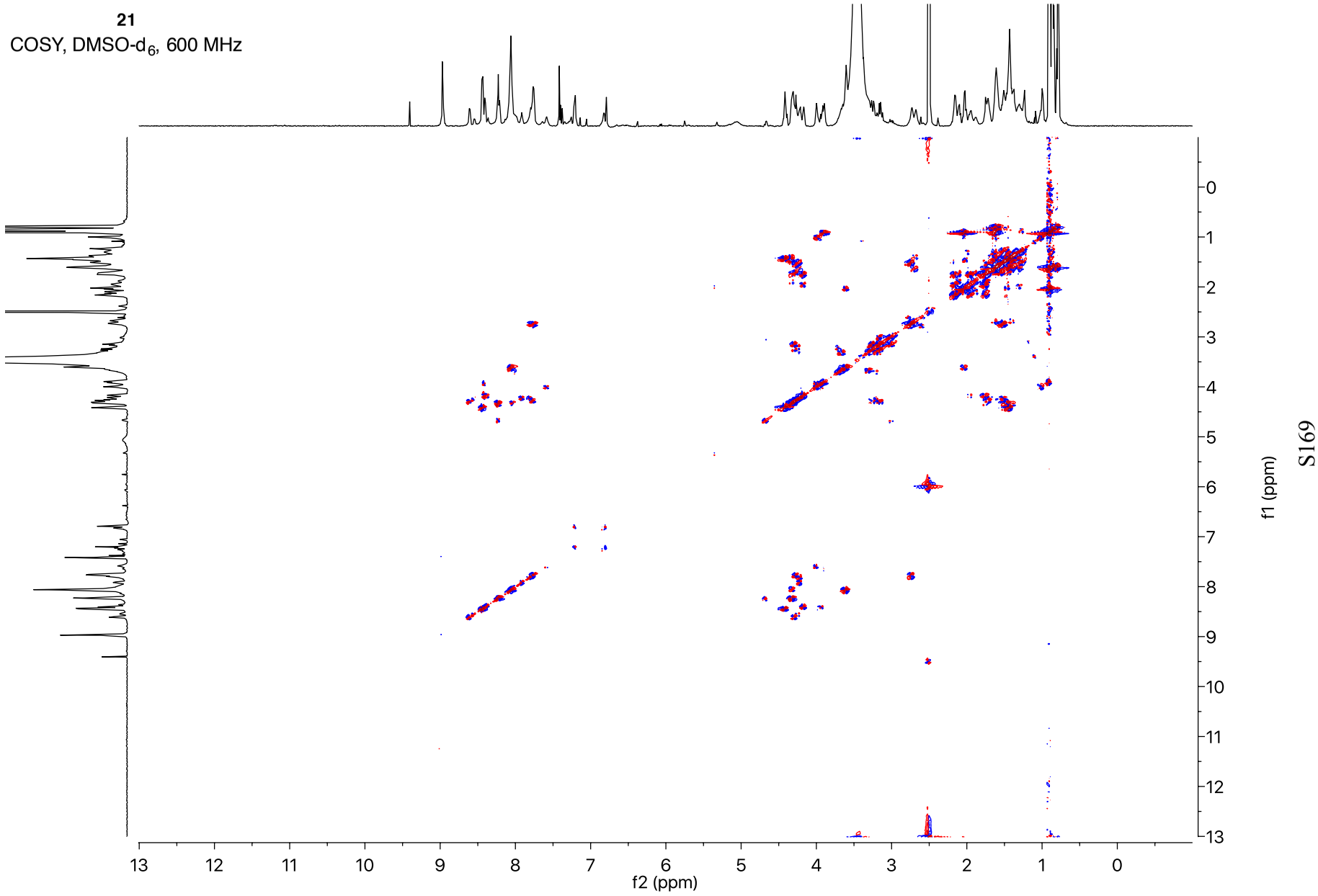


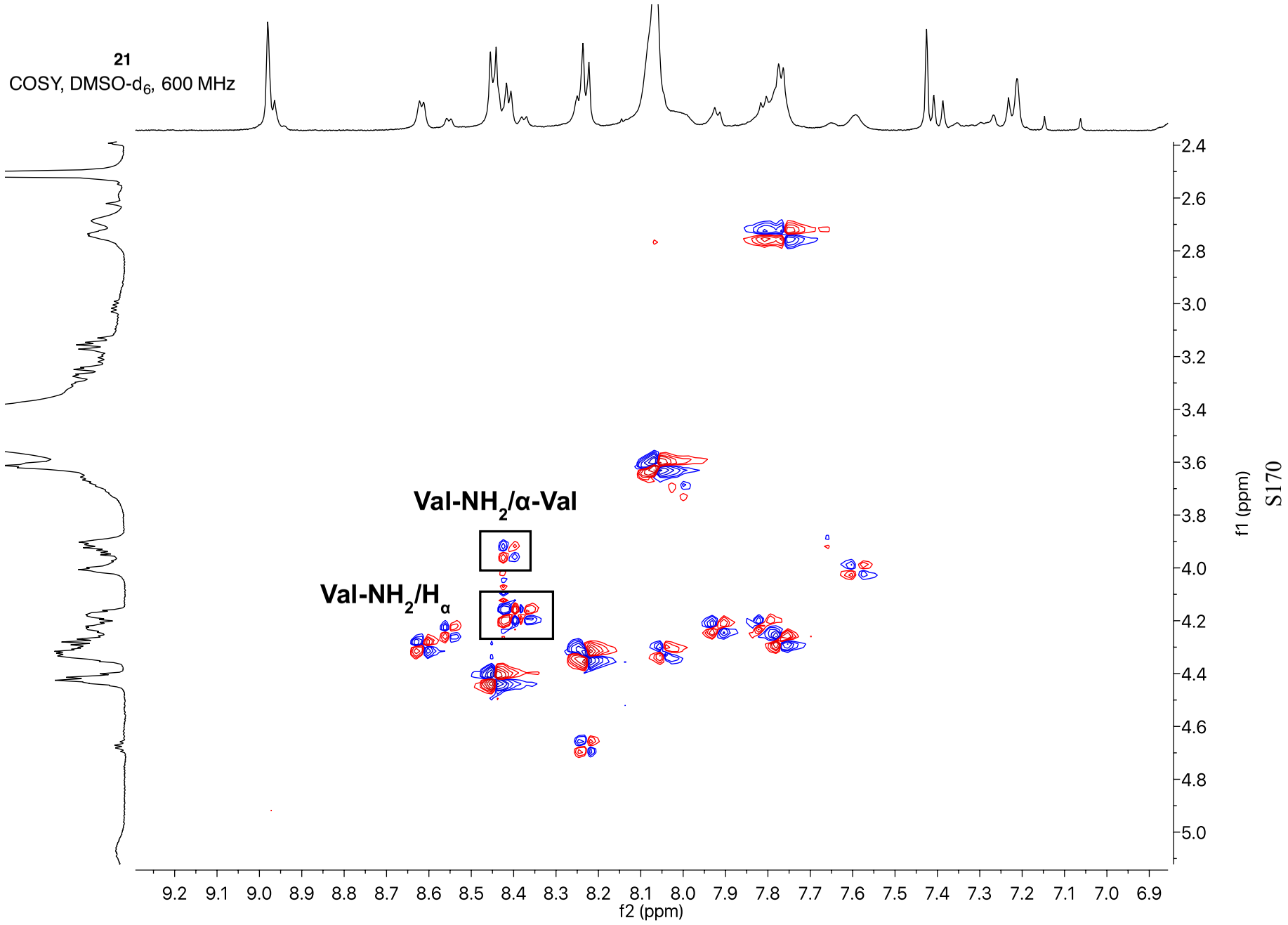
21

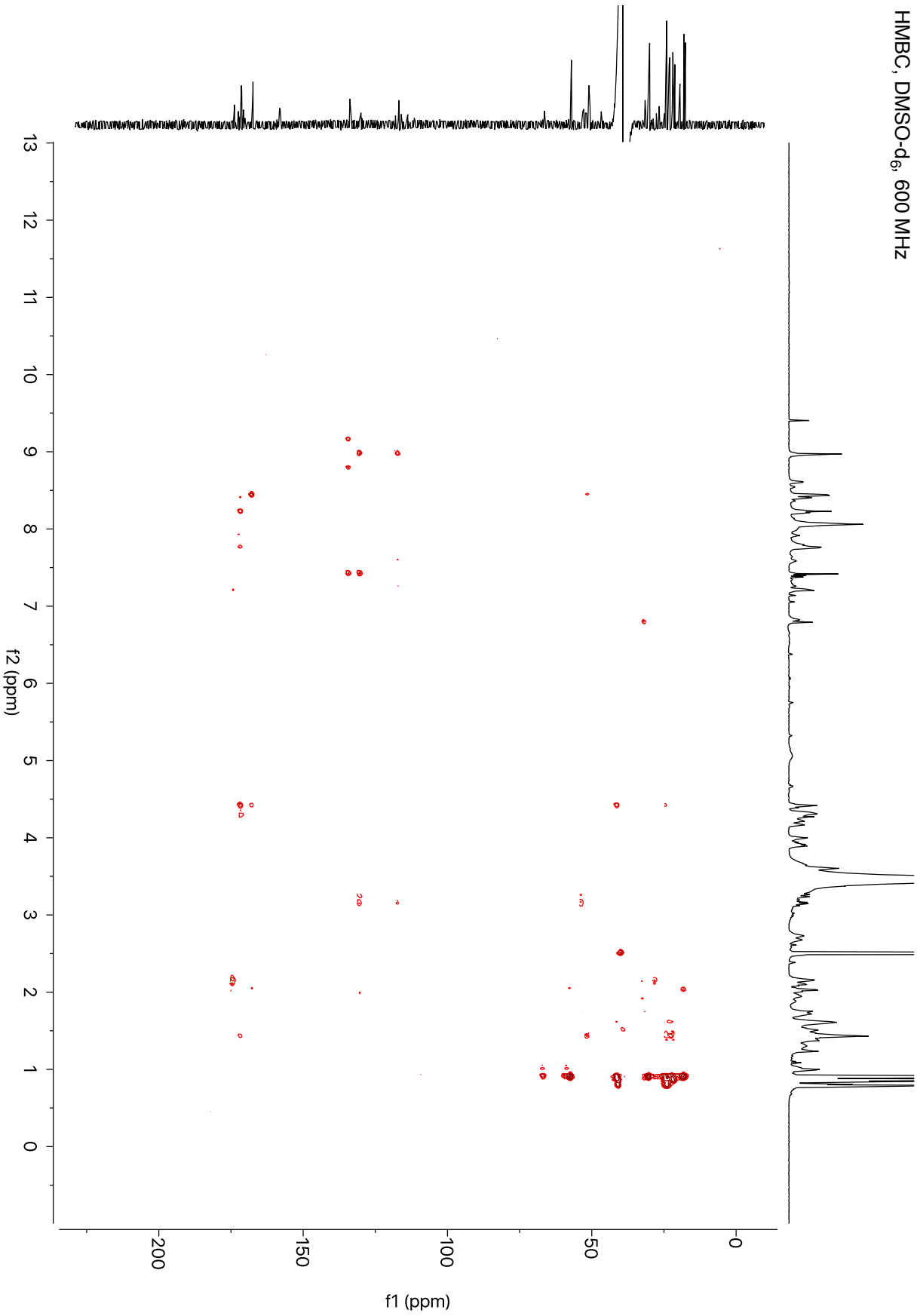
^{13}C, DMSO-d_6, 151 MHz

21

COSY, DMSO-d_6, 600 MHz

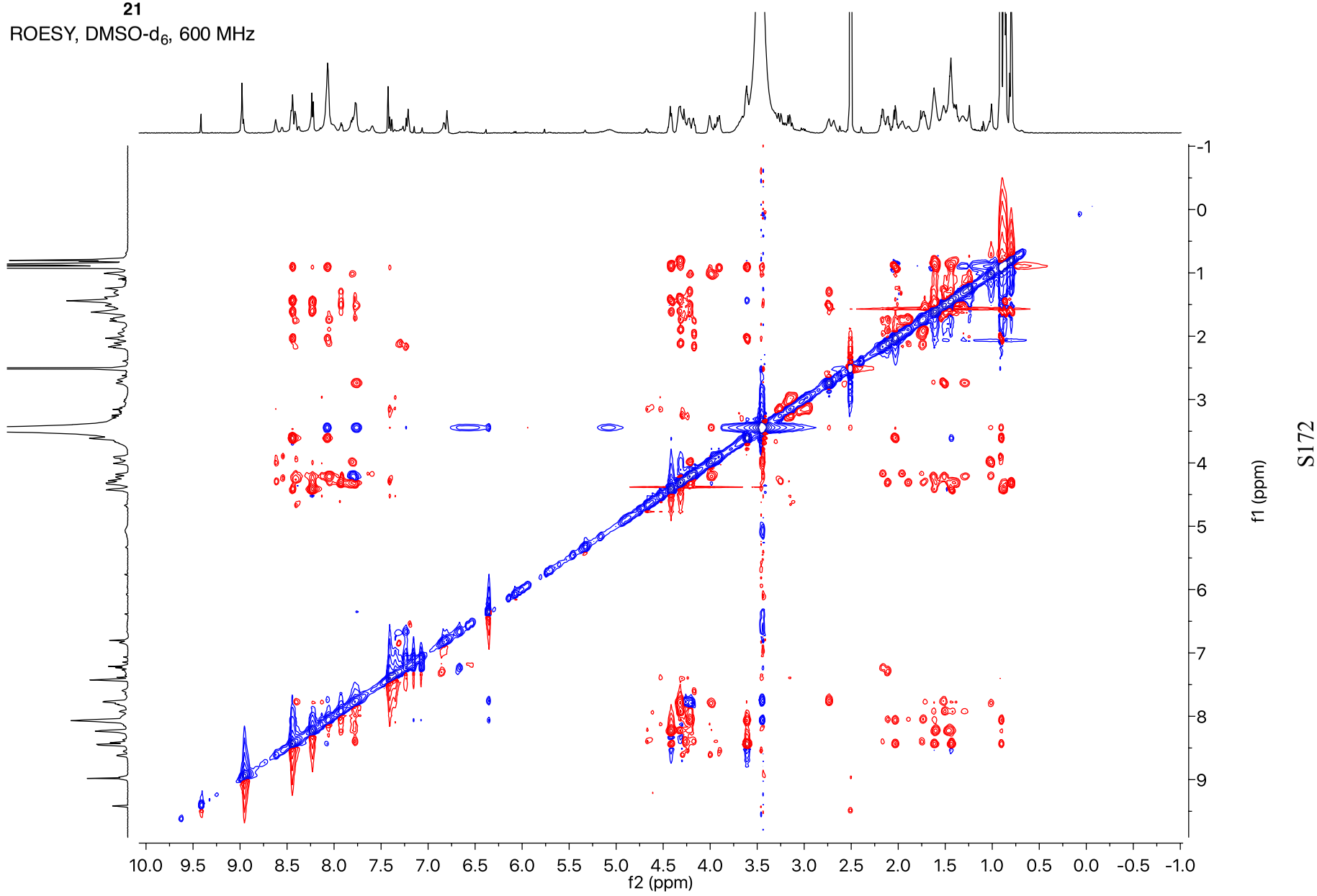



21
COSY, DMSO-d6, 600 MHz
Val-NH2/α-Val
Val-NH2/Hα
f2 (ppm)
f1 (ppm)
9.2 9.1 9.0 8.9 8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9
2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0


21
HMBC, DMSO-d6, 600 MHz
f2 (ppm)
f1 (ppm)

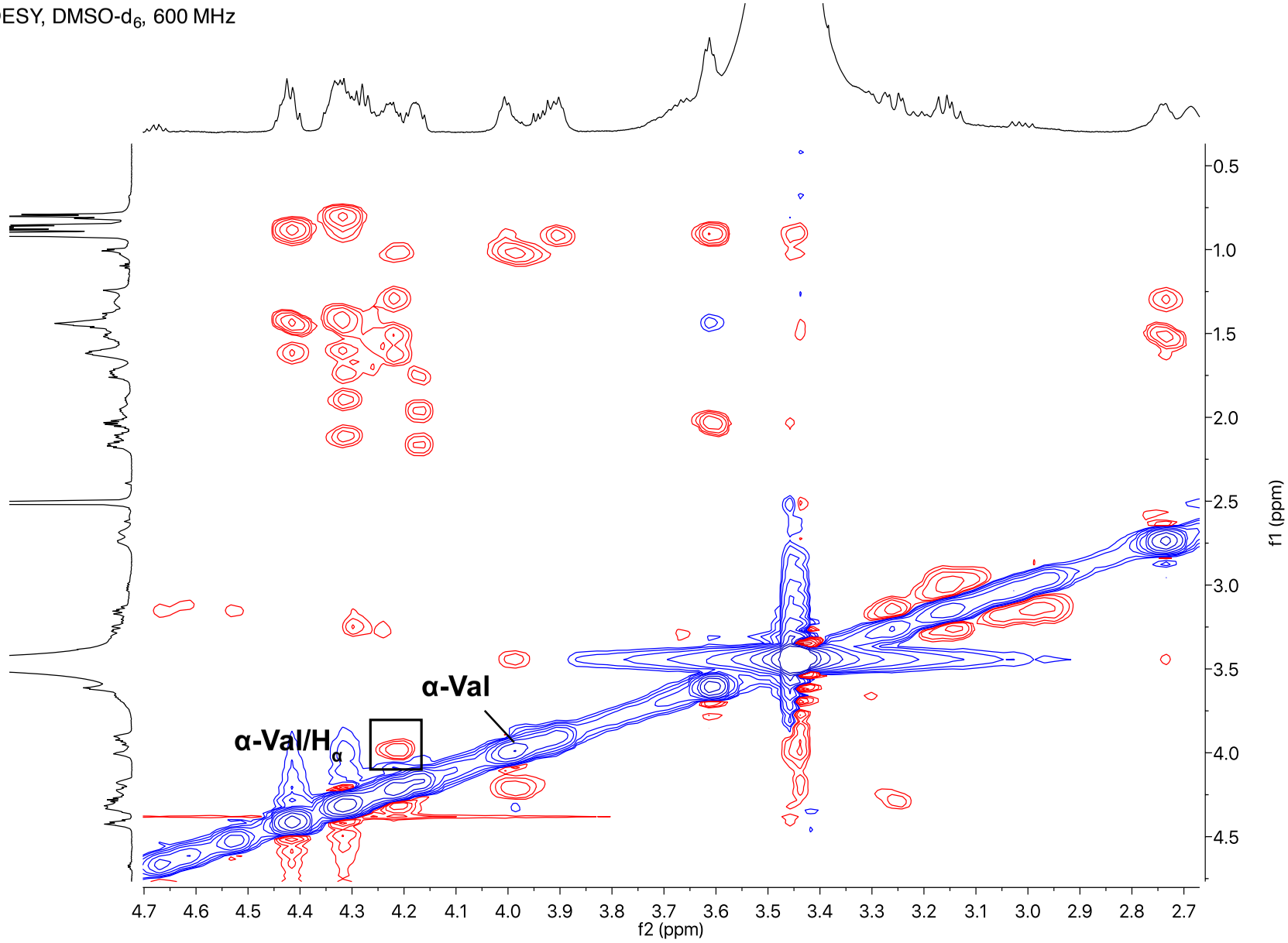
21

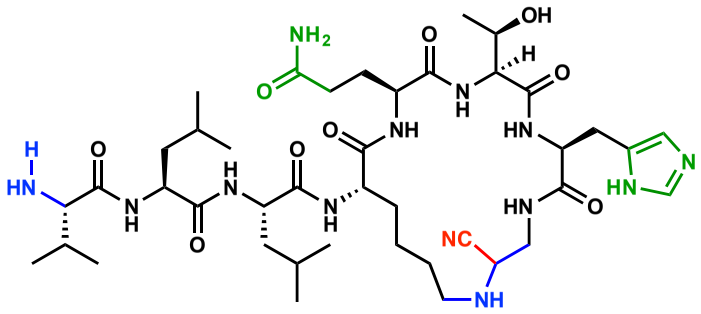
ROESY, DMSO-d_6, 600 MHz



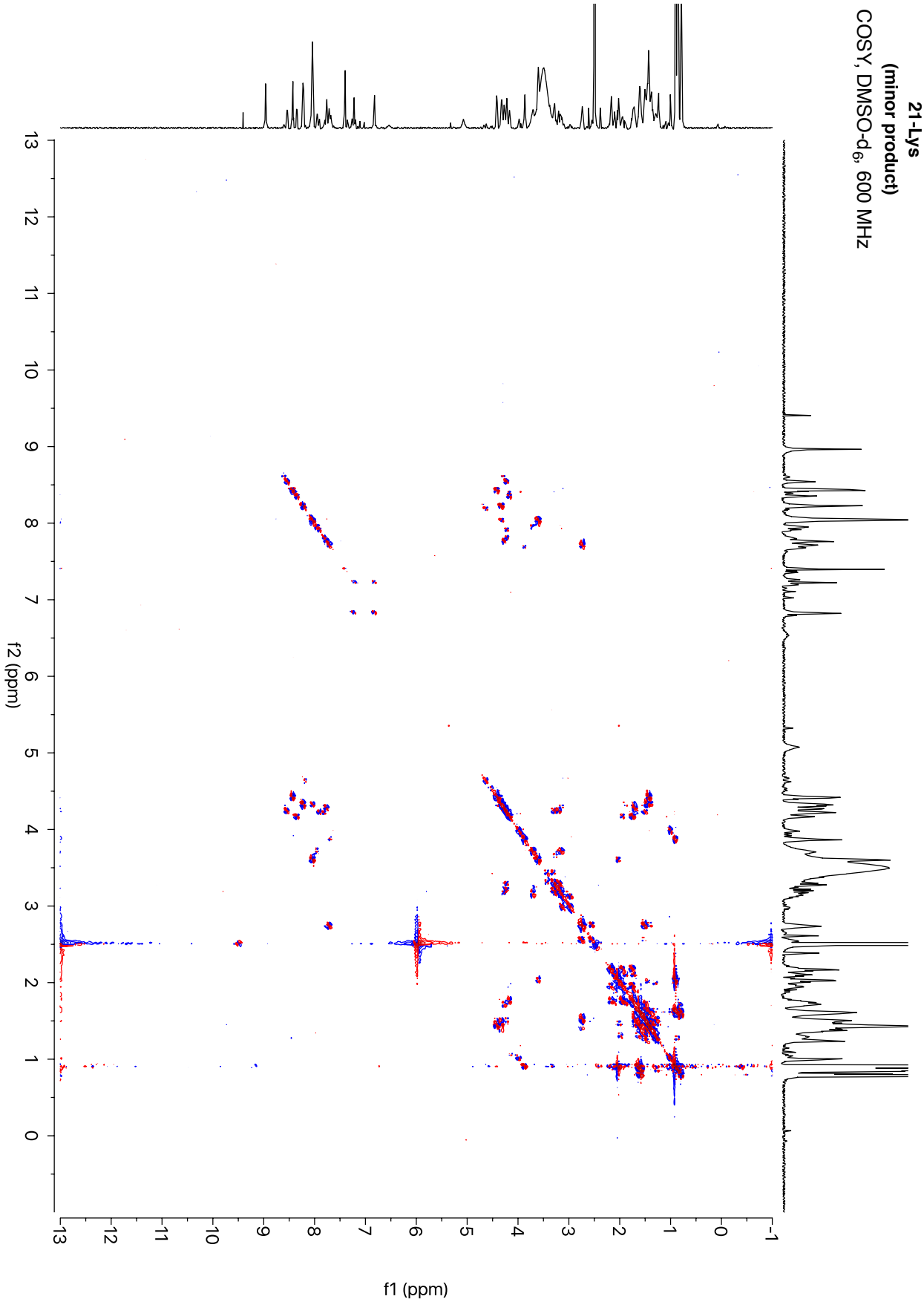
21

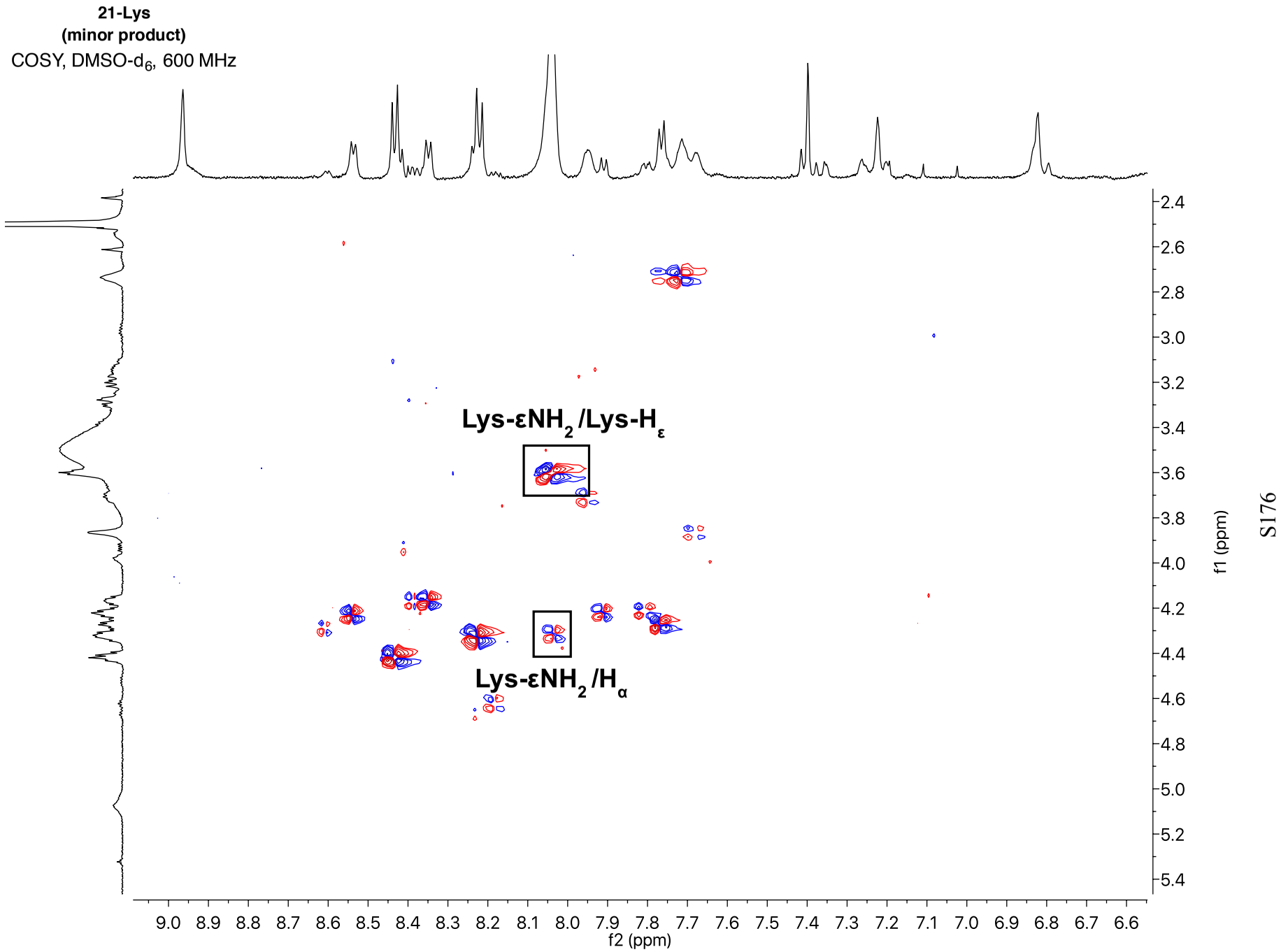
ROESY, DMSO-d_6, 600 MHz



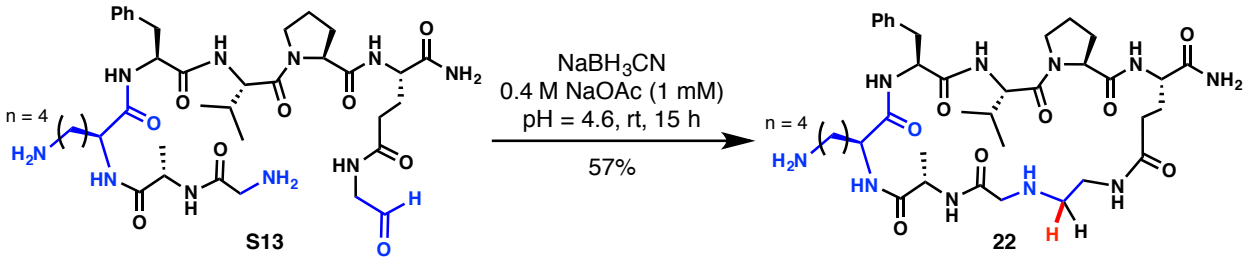


21-Lys
(minor product)
^{1}H, DMSO-d$_{6}$, 600 MHz

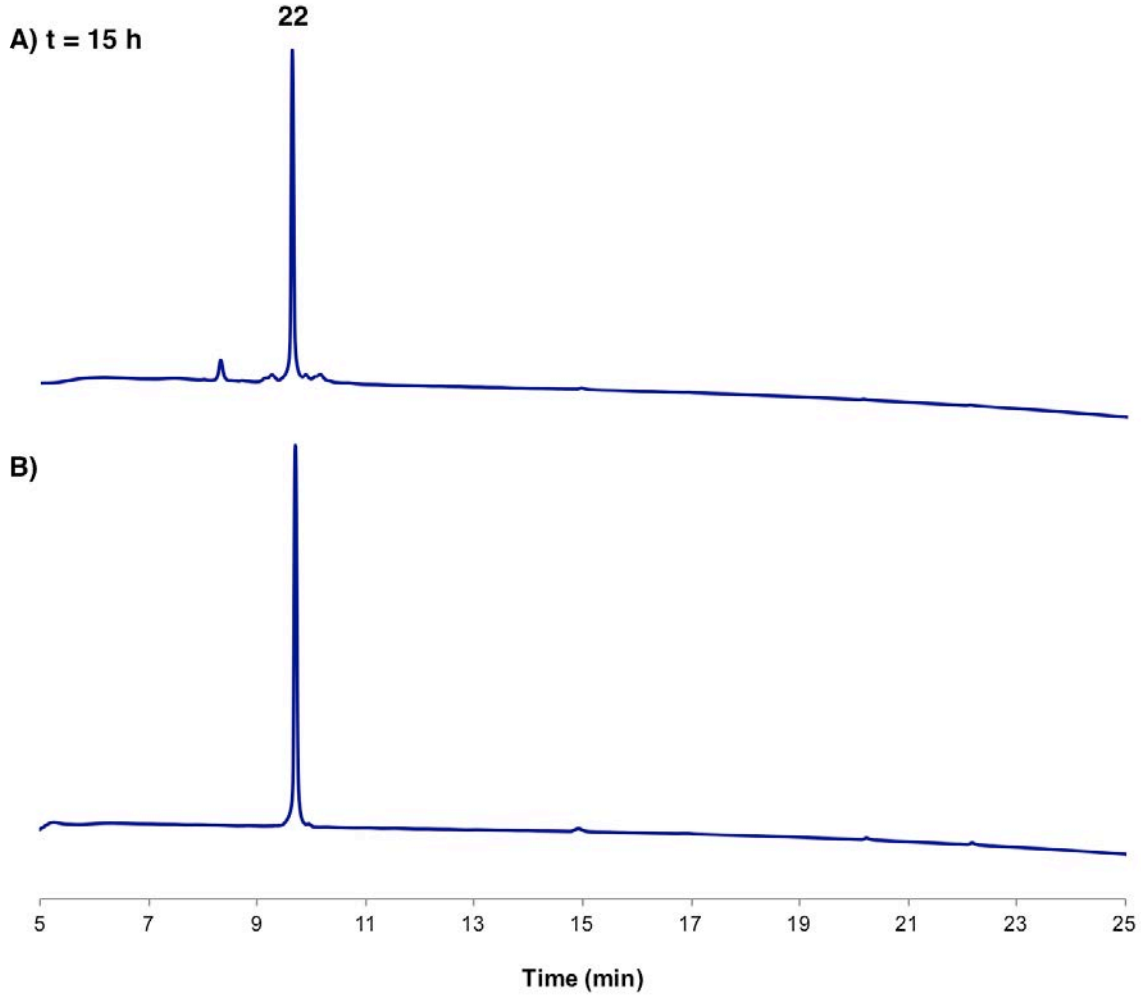

21-Lys
(minor product)
COSY, DMSO-d6, 600 MHz
f2 (ppm)
f1 (ppm)


21-Lys
(minor product)
COSY, DMSO-d6, 600 MHz
Lys-εNH2 /Lys-Hε
Lys-εNH2 /Hα
f2 (ppm)
f1 (ppm)
9.0 8.9 8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9 6.8 6.7 6.6
2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4

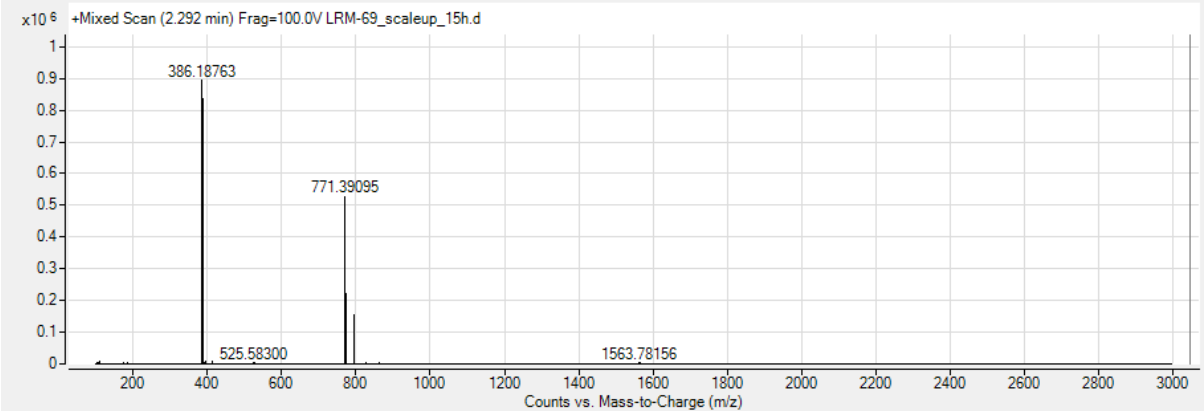
Peptide 22



Peptide **22** was prepared according to general procedure 4 from linear amino aldehyde **S13** (4.3 mg, 5.5 µmol). At t = 15 h, the reaction was purified by preparative reverse-phase HPLC (5% B for 5 min, then 5% to 45% B over 30 min) to afford peptide macrocycle **22** (2.4 mg, 57% yield) as a white solid following lyophilization.

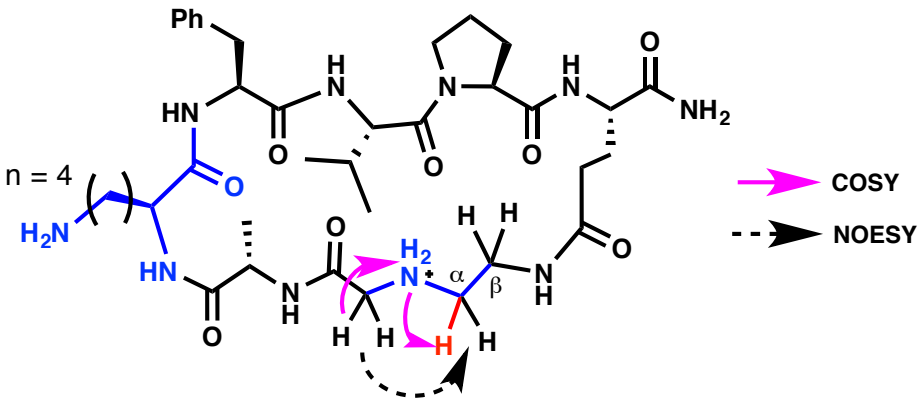


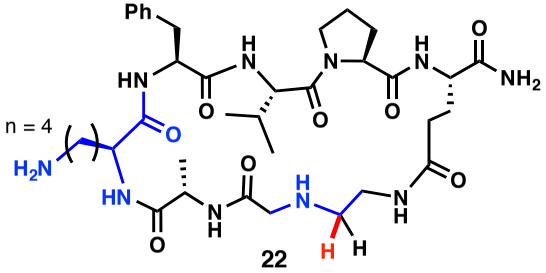
A) Crude analytical HPLC trace of the formation of **22** from amino aldehyde **S13** following reductive amination (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **22** (Rt = 9.7 min, 5 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{37}H_{59}N_{10}O_8$ $[M+H]^+$ 771.45; found 771.39, $[M+2H]^{2+}$ 386.23; found 386.19.

Partial 2D NMR Correlations:



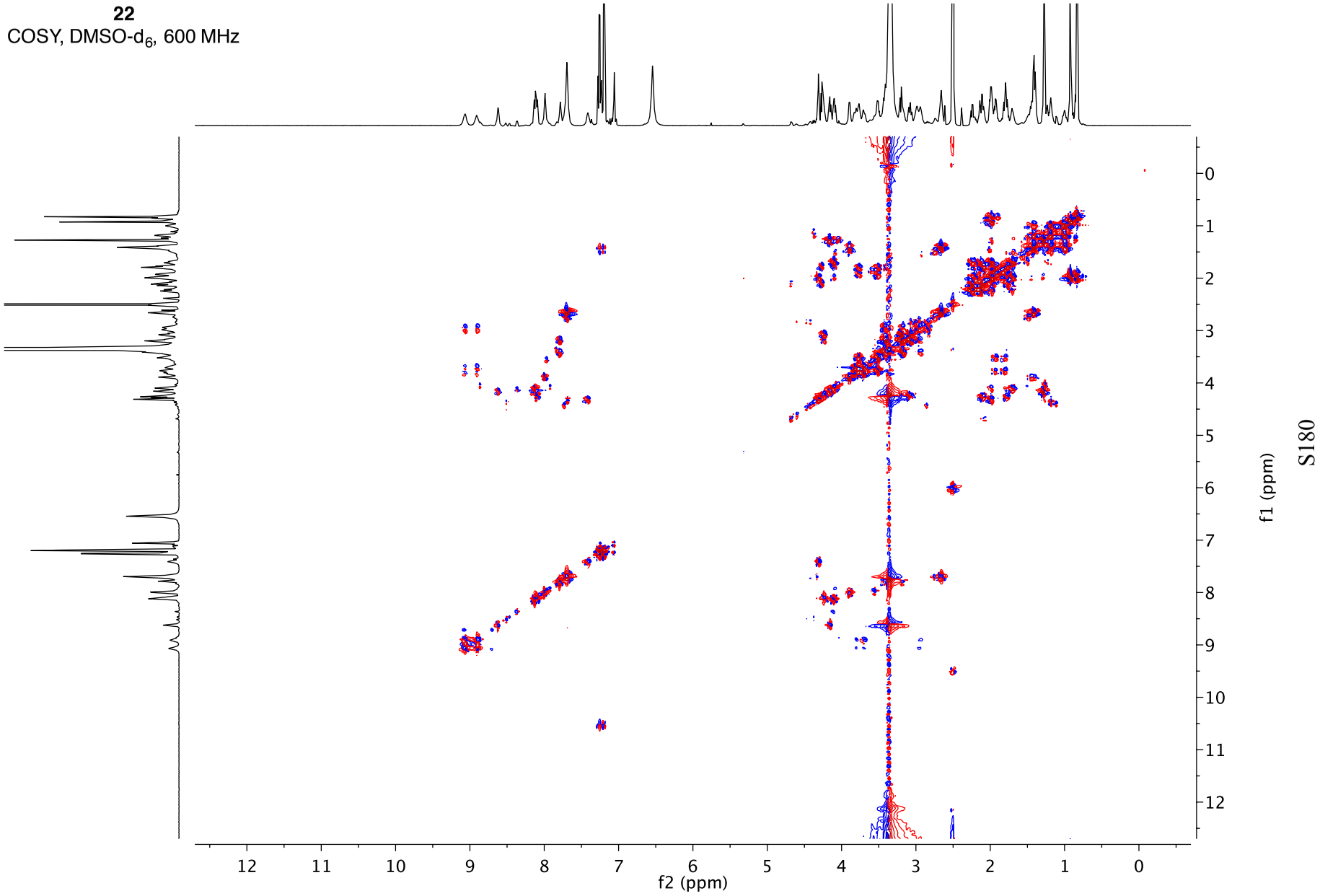


22

^{1}H, DMSO-d$_6$, 600 MHz

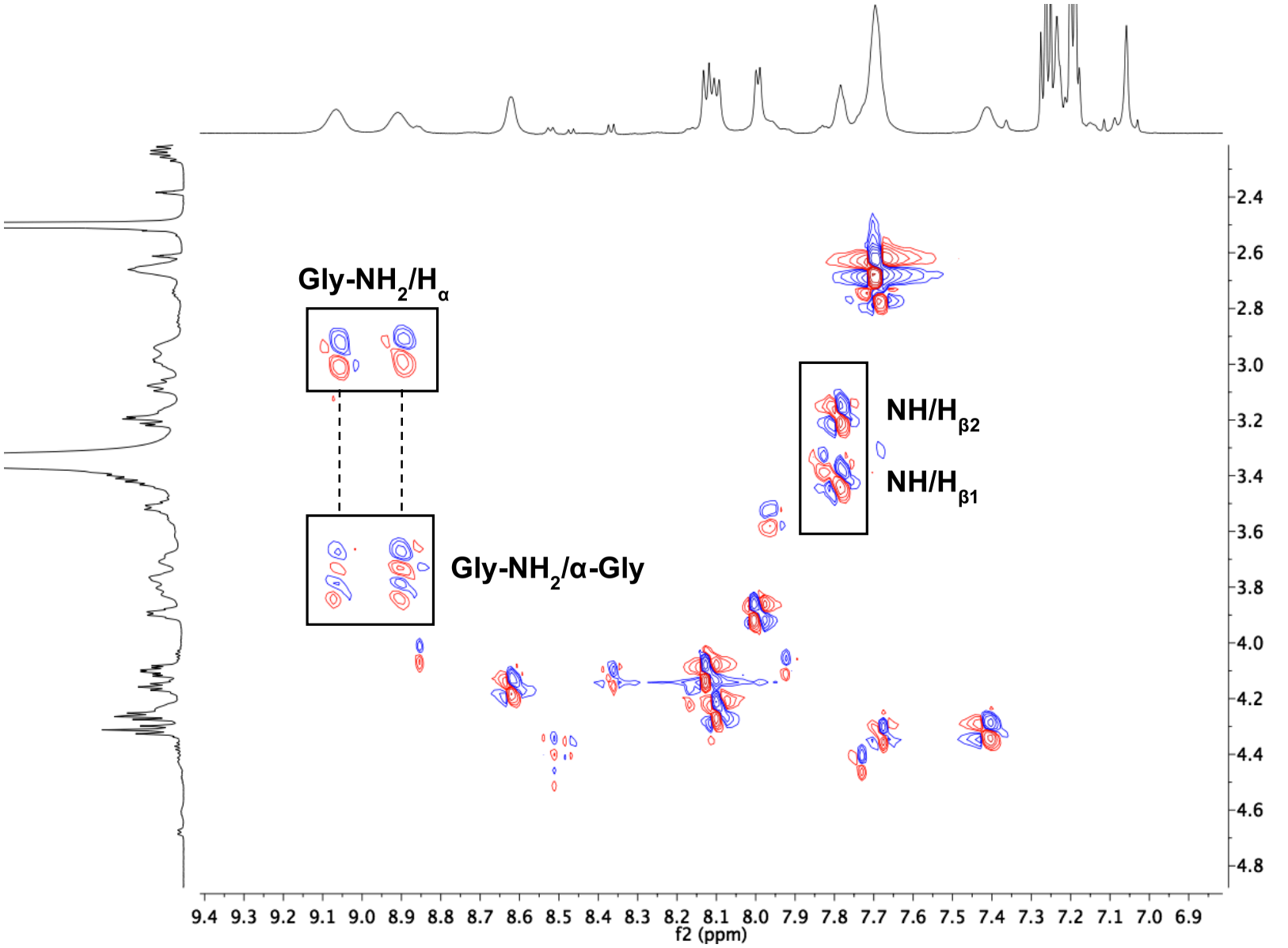
22

COSY, DMSO-d_6, 600 MHz



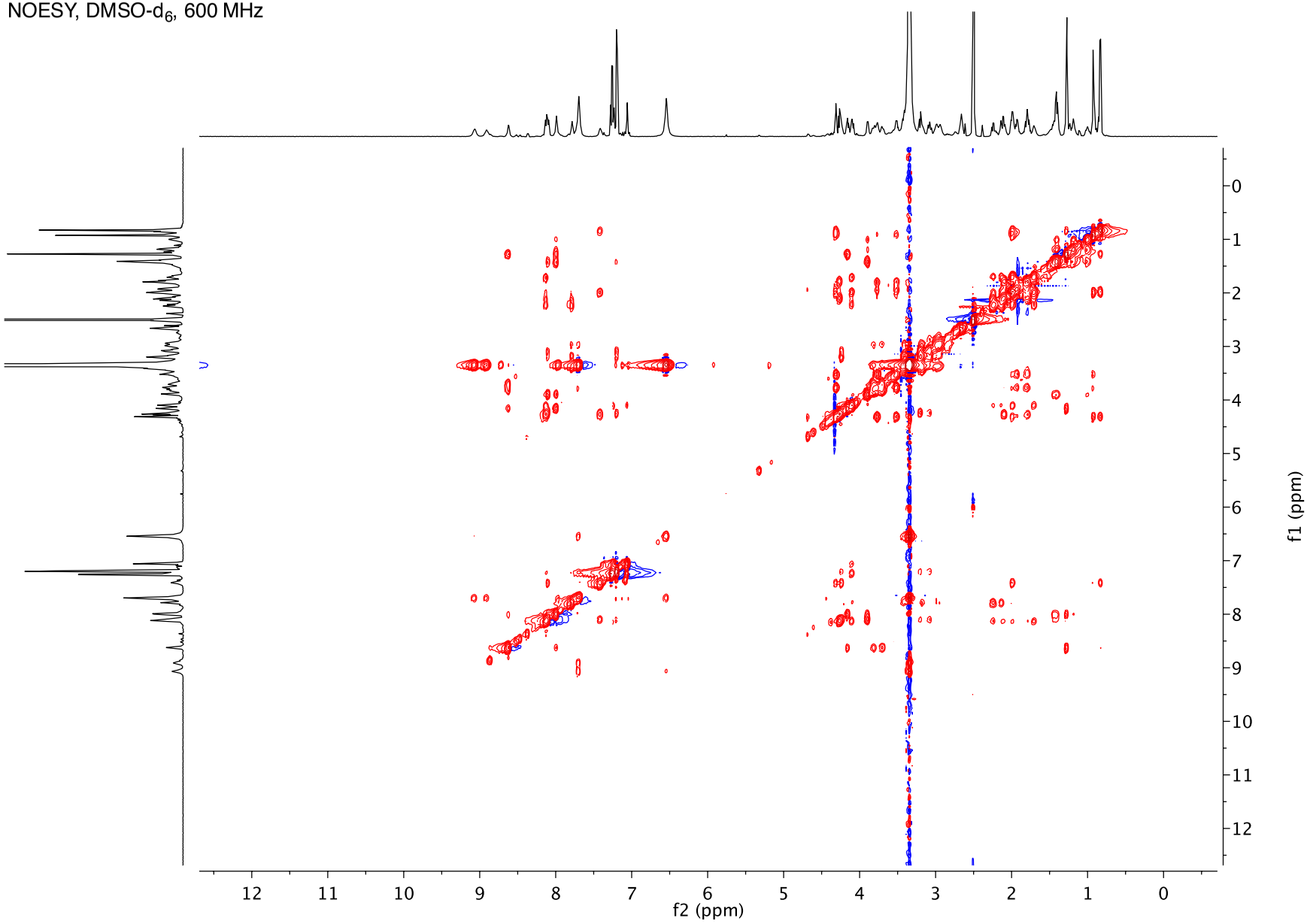
22

COSY, DMSO-d_6, 600 MHz



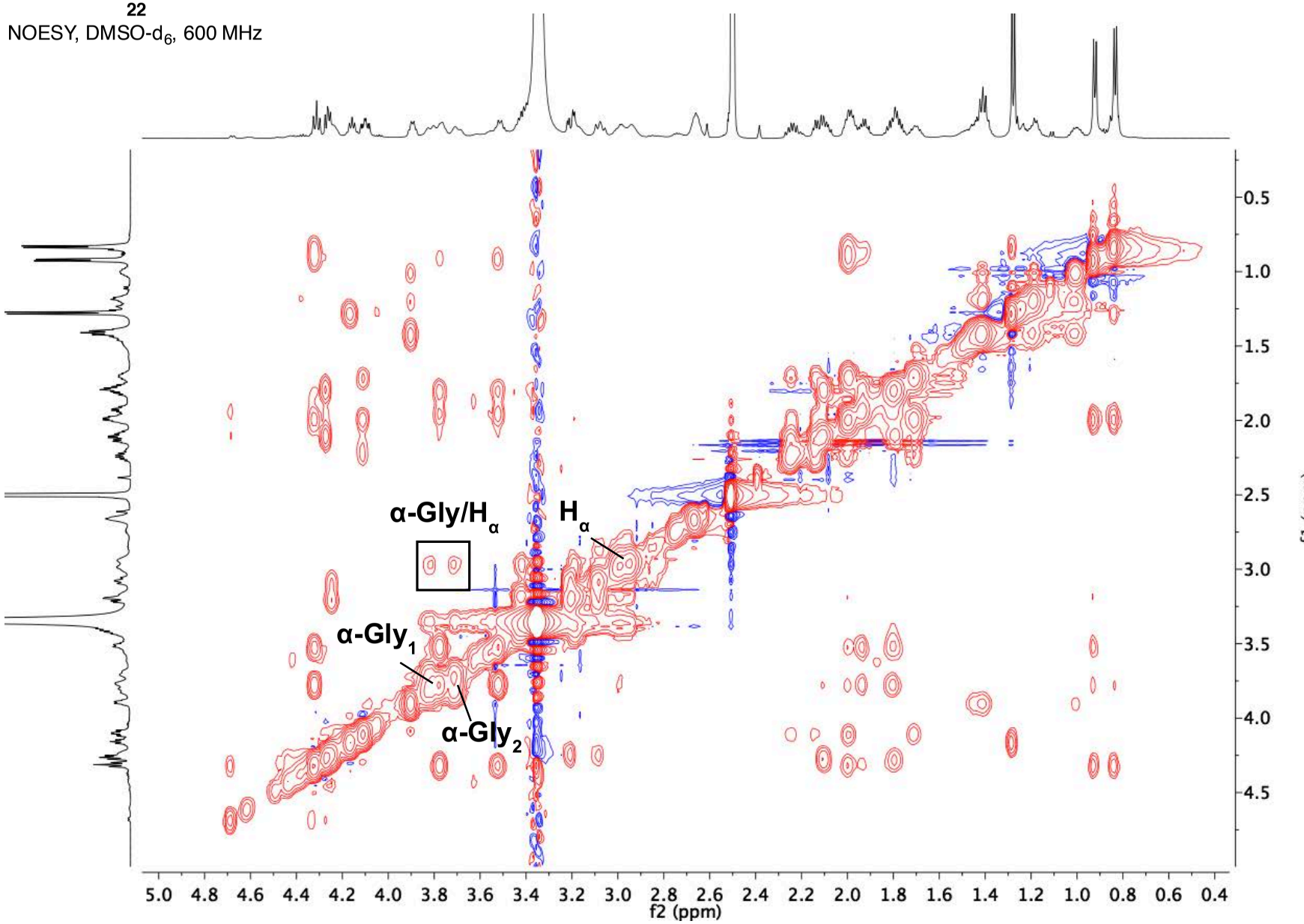
22

NOESY, DMSO-d_6, 600 MHz



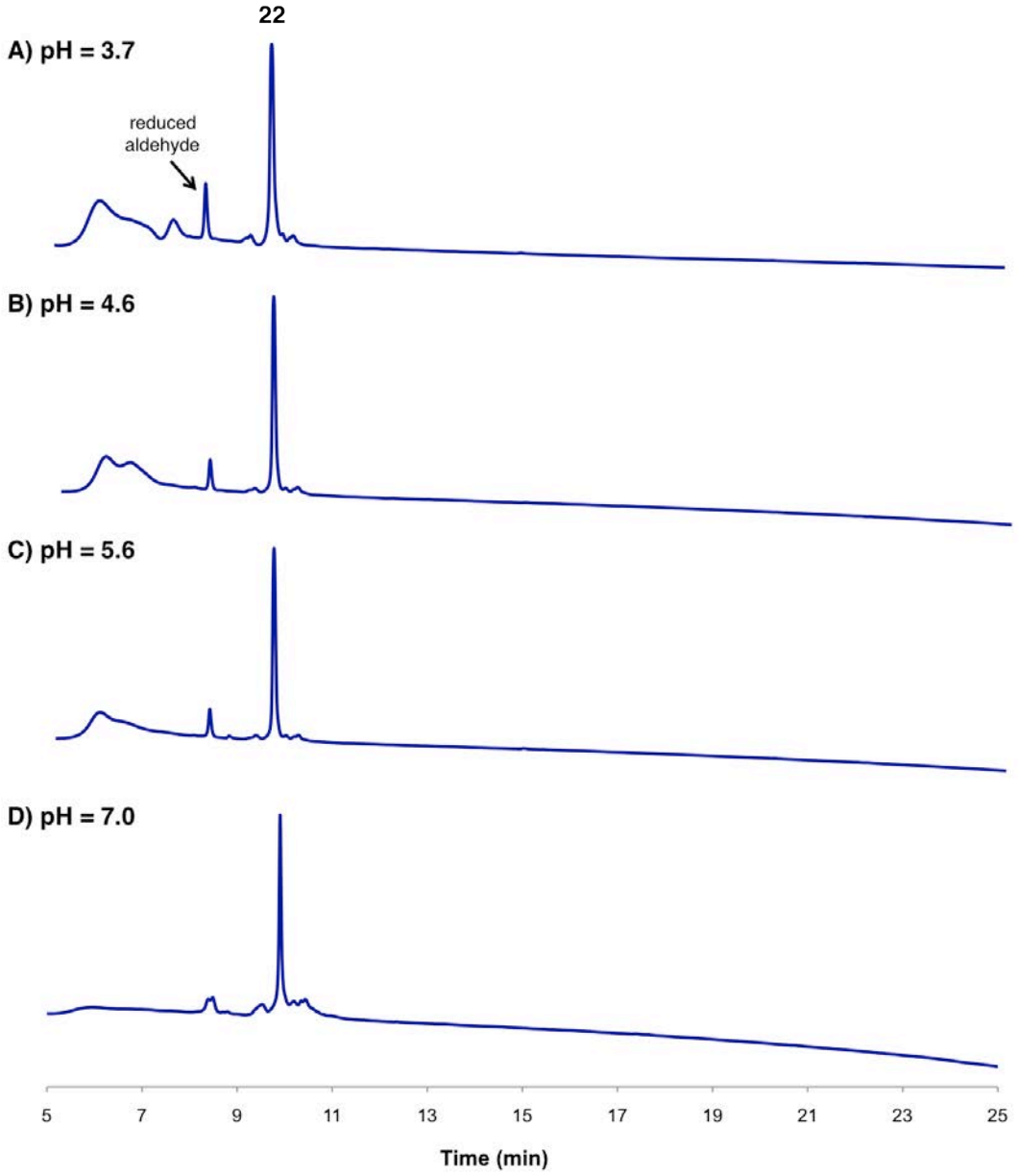
22

NOESY, DMSO-d_6, 600 MHz



pH Screen

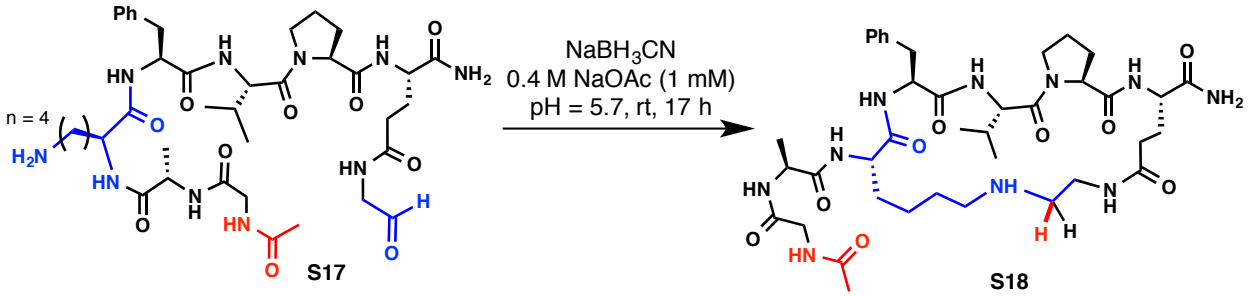
The reductive amination of peptide **S13** to form macrocycle **22** was studied at a variety of different pH conditions (0.4 M NaOAc buffer, adjusted to pH 3.7, 4.6, and 5.6 and 0.2 M Na_2HPO_4 buffer, adjusted to pH 7.0). All reactions yielded the same product macrocycle, but the relative amount of reduced aldehyde was sensitive to changes in pH, with the largest amount of aldehyde reduction observed at pH = 3.7. At pH = 7, minor macrocyclic byproducts were also observed. These minor byproducts were not sufficient in intensity to isolate by HPLC.



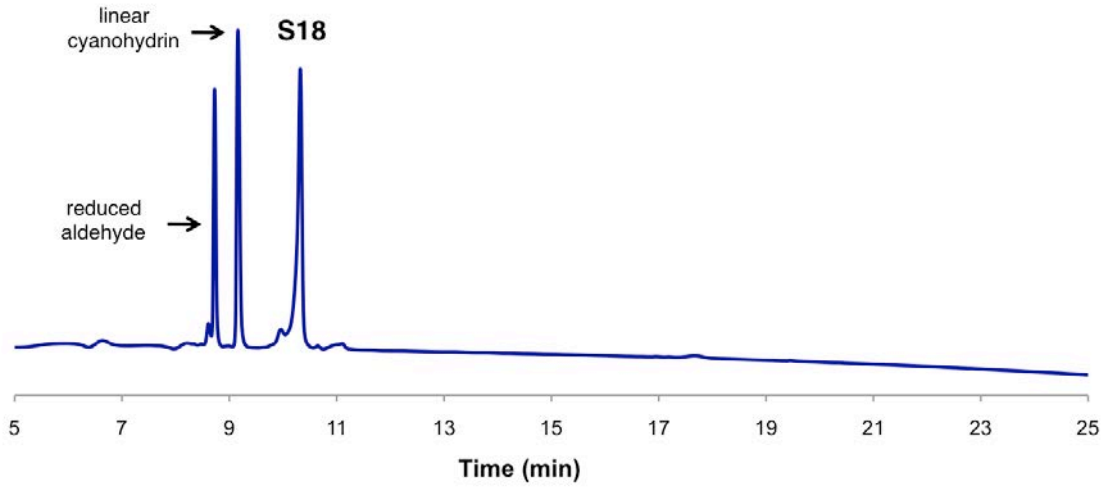
Crude analytical HPLC traces (5 to 100% B over 25 min, λ = 210 nm) of the formation of **22** from amino aldehyde **S13** following reductive amination. The reaction was performed in 0.4 M

NaOAc buffer (pH = 3.7, 4.6, or 5.6, 1 mM concentration) or in 0.2 M Na_2HPO_4 buffer (pH = 7.0, 1 mM concentration). Reactions were run for 16 h at **A)** pH = 3.7, **B)** pH = 4.6, **C)** pH = 5.6, and **D)** pH = 7.0.

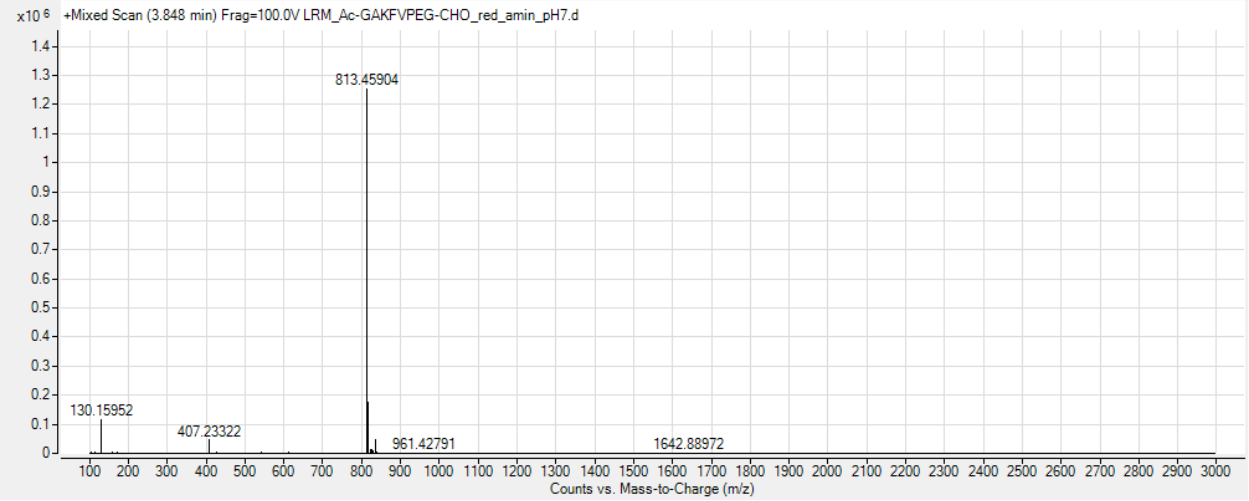
*Note: Cyclization of **S13** at the α-amine outcompetes cyclization at the ε-amine lysine side-chain at a variety of reaction pH conditions. To ensure that cyclization at the α-amine does not result from an absolute conformational preference over the side-chain lysine, an N-acetylated linear peptide aldehyde, **S17**, was prepared and subjected to the reductive amination conditions. Without competitive α-amine cyclization, reaction at the lysine side-chain was observed by analytical HPLC and LC-MS, although the reaction at pH = 5.7 was considerably slower, resulting in the formation of substantial reduced aldehyde and linear cyanohydrin:*



Peptide **S18** was prepared on an analytical scale according to general procedure 4 from linear amino aldehyde **S17**. At t = 17 h, the reaction was monitored by LC-MS and analytical HPLC to determine the extent of macrocyclization.

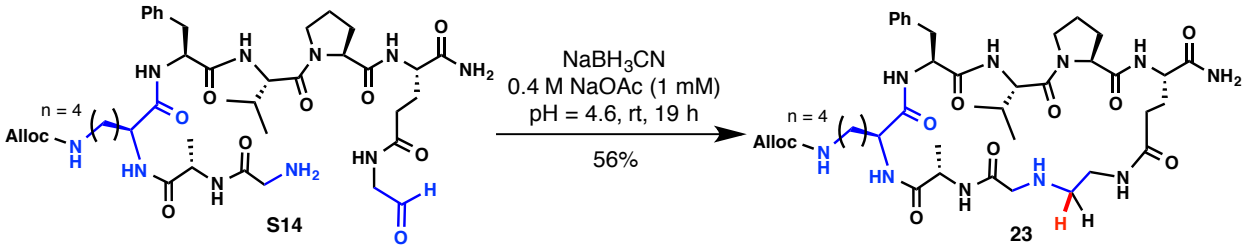


Crude analytical HPLC trace (5 to 100% B over 25 min, λ = 210 nm) of the formation of **S18** from amino aldehyde **S17** following reductive amination.

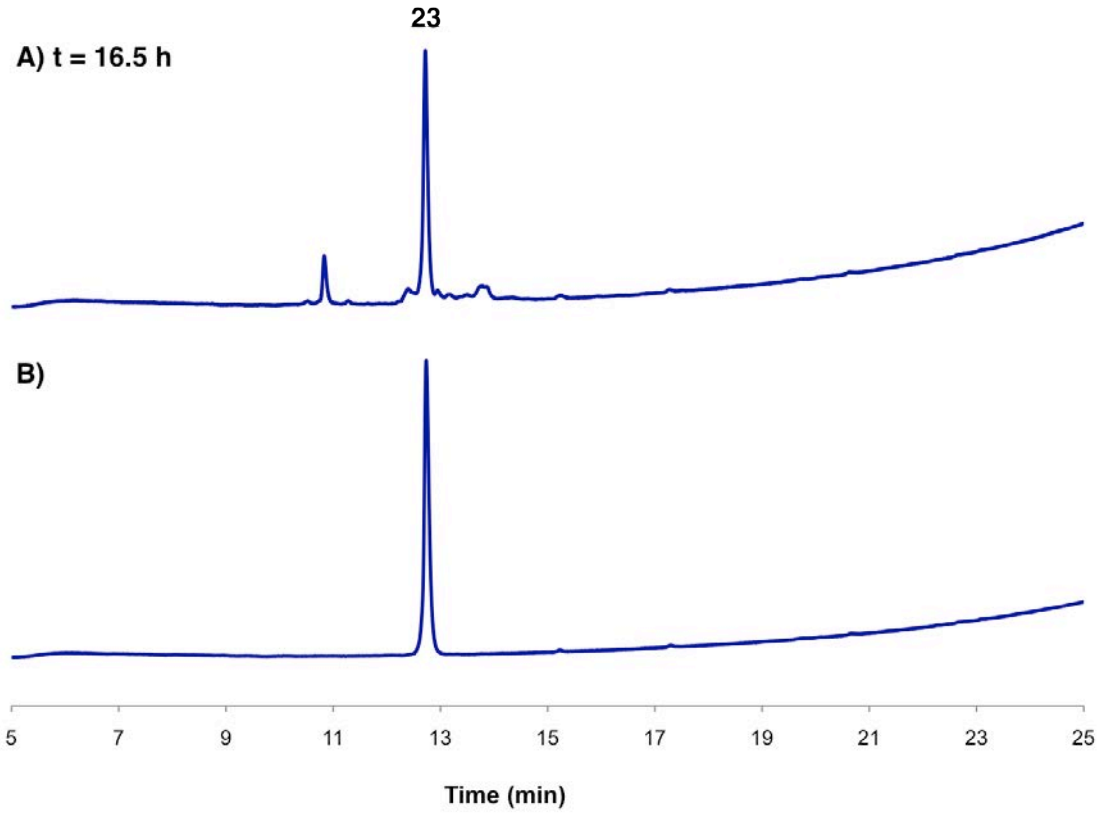


LRMS (ESI-TOF): calc'd for $C_{39}H_{61}N_{10}O_9$ $[M+H]^+$ 813.46; found 813.46.

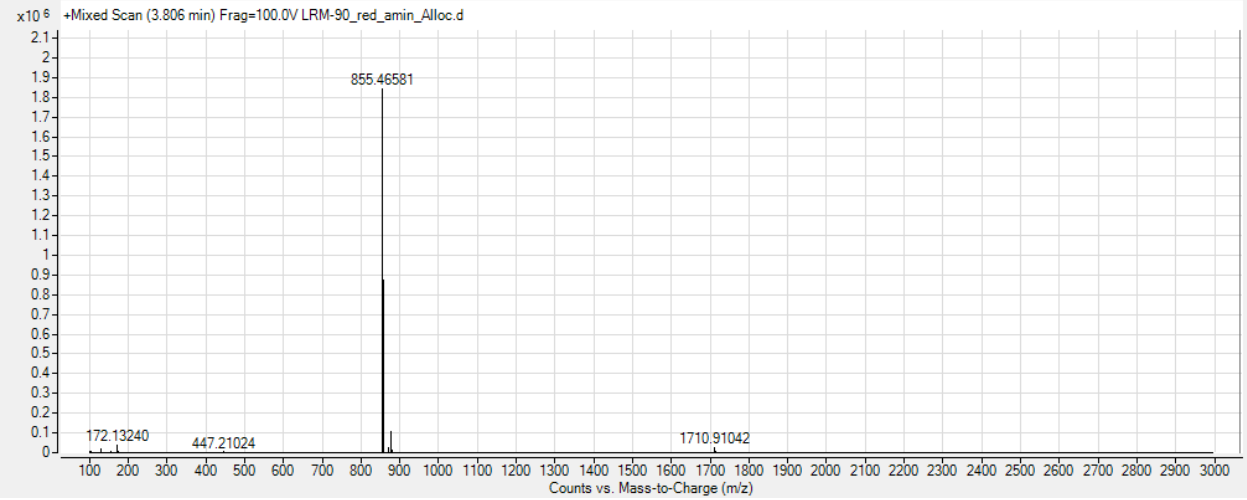
Peptide 23



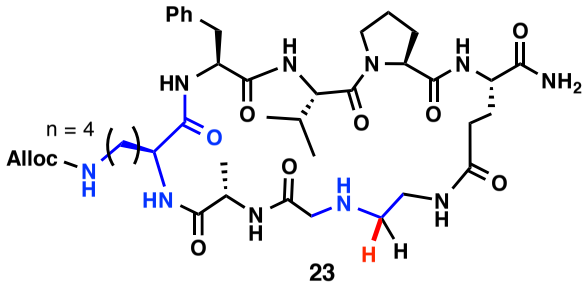
Peptide **23** was prepared according to general procedure 4 from linear amino aldehyde **S14** (4.4 mg, 5.1 μmol). At t = 19 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% to 55% B over 25 min) to afford peptide macrocycle **23** (2.4 mg, 56% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **23** from amino aldehyde **S14** following reductive amination (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **23** (Rt = 12.7 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{41}H_{63}N_{10}O_{10}$ $[M+H]^+$ 855.47; found 855.47.

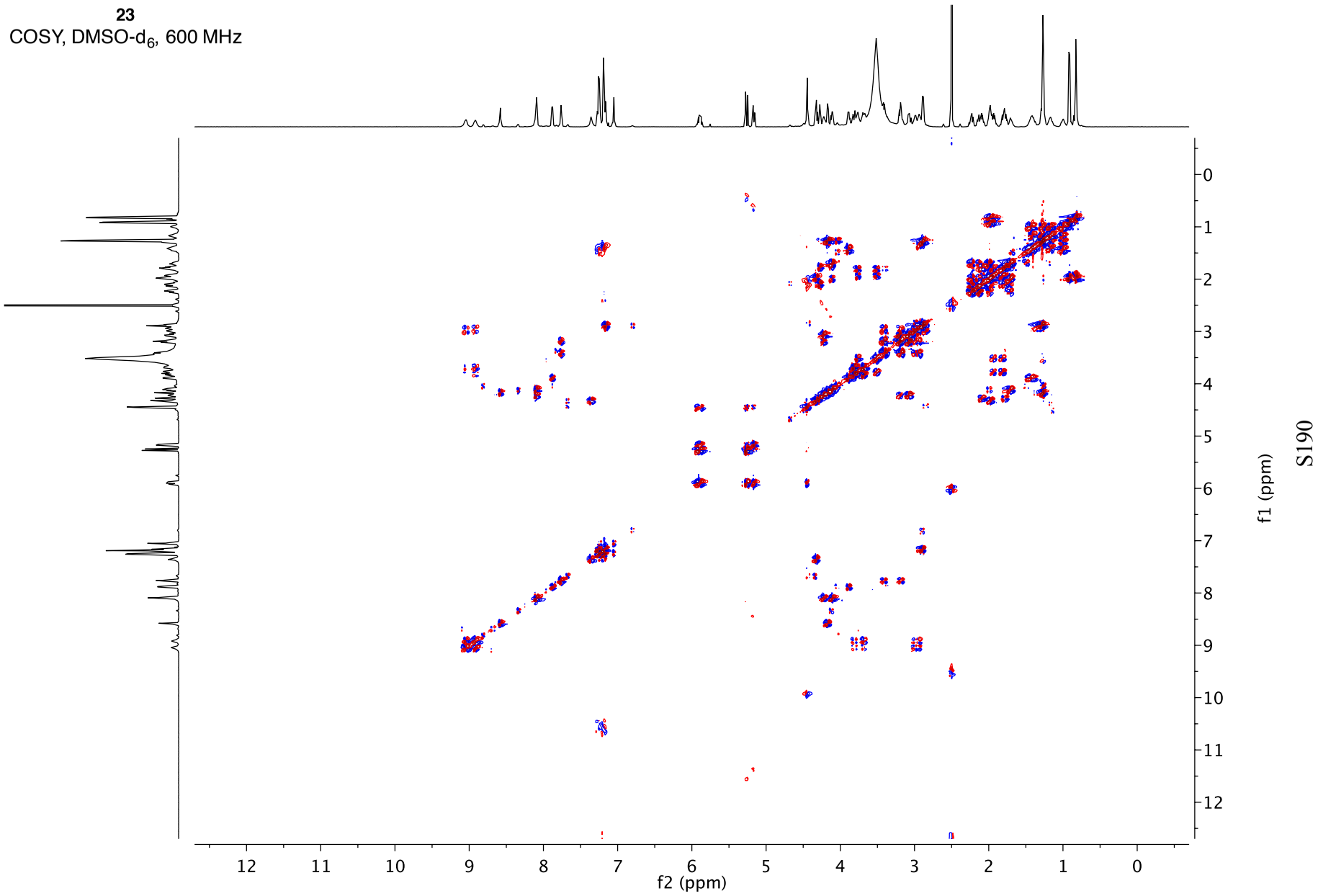


23

^{1}H, DMSO-d$_6$, 600 MHz

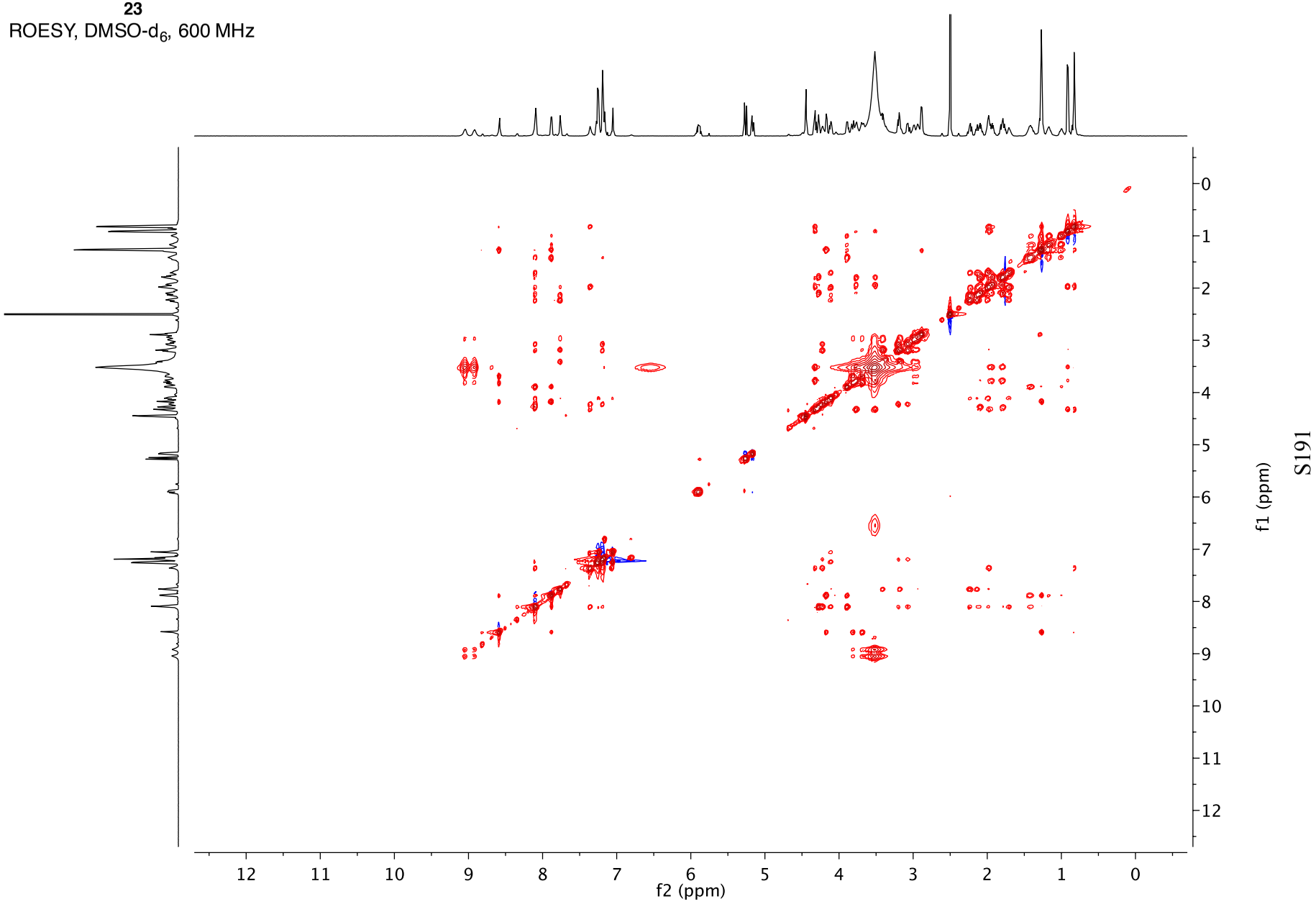
23

COSY, DMSO-d_6, 600 MHz

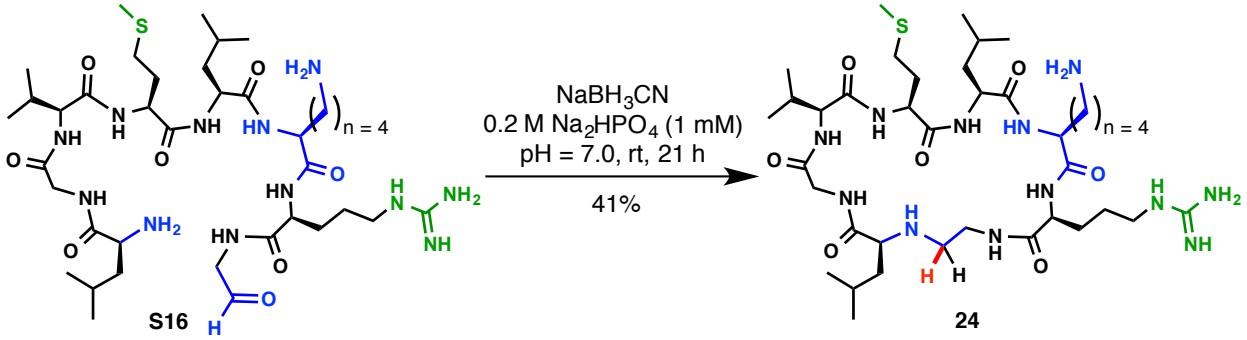


23

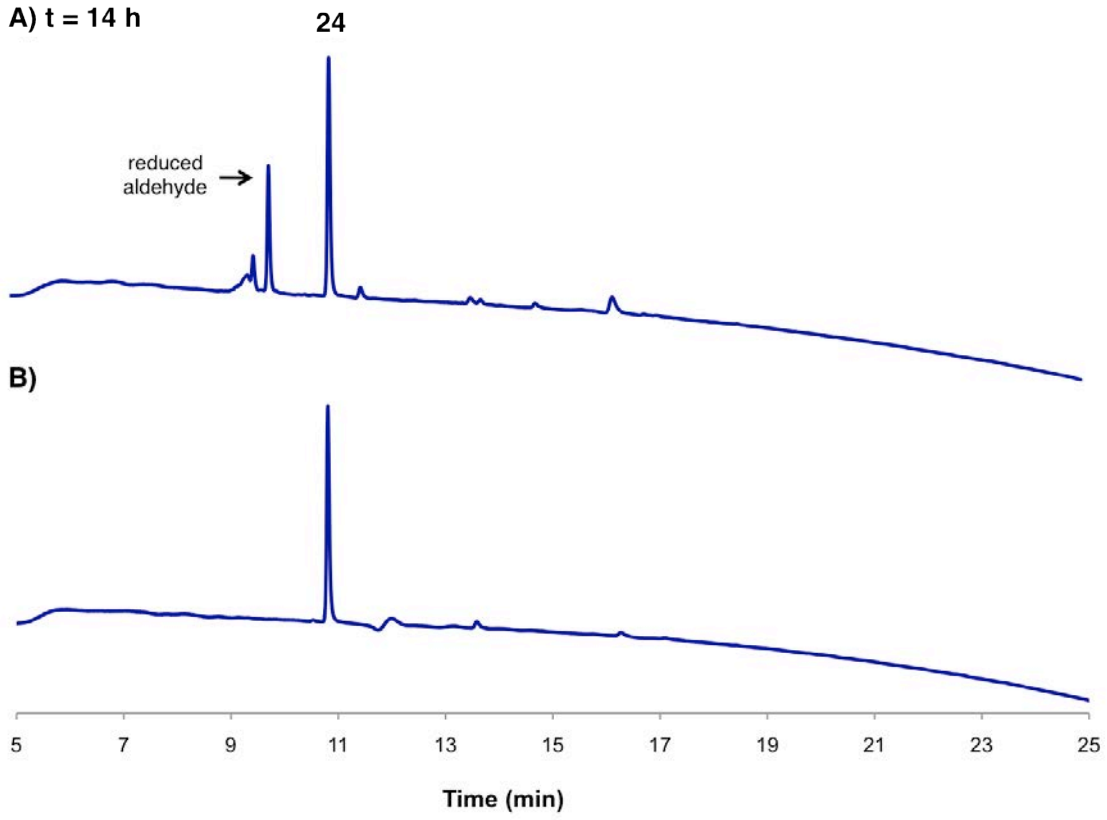
ROESY, DMSO-d_6, 600 MHz



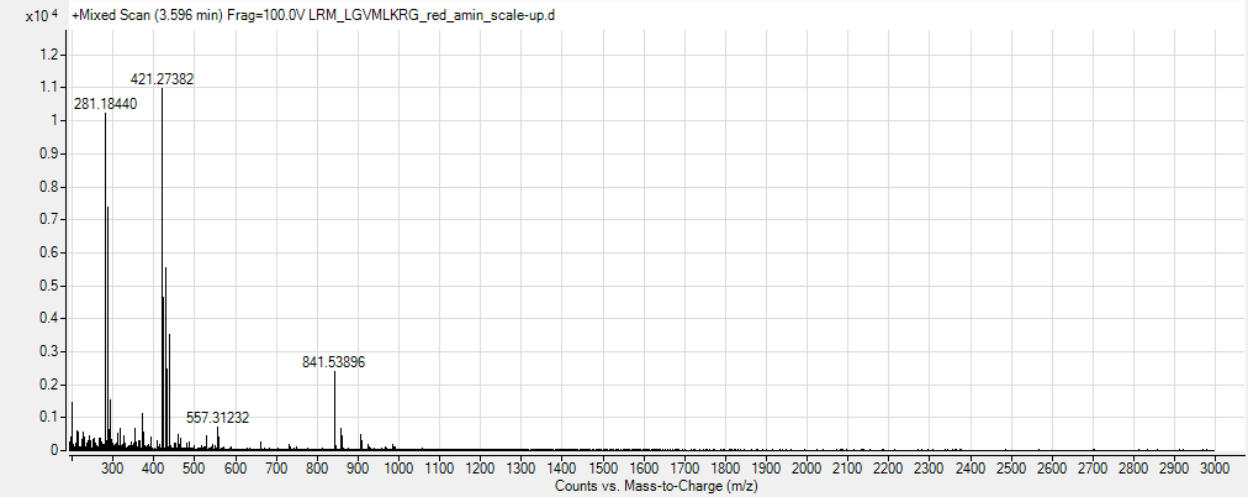
Peptide 24



Peptide **24** was prepared according to general procedure 4 from linear amino aldehyde **S16** (2.5 mg, 2.9 μmol). The peptide aldehyde was solubilized in 0.2 M Na_2HPO_4 (pH = 7.0). At t = 21 h, the reaction was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% to 50% B over 30 min) to afford peptide macrocycle **24** (1.0 mg, 41% yield) as a white solid following lyophilization.

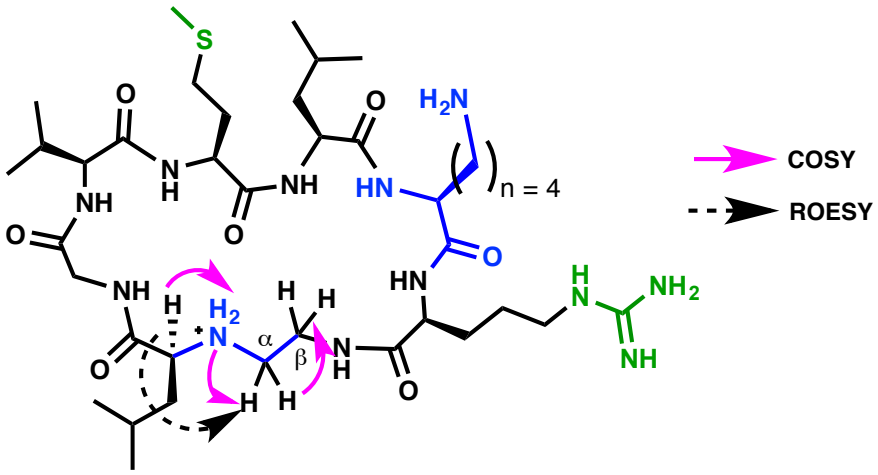


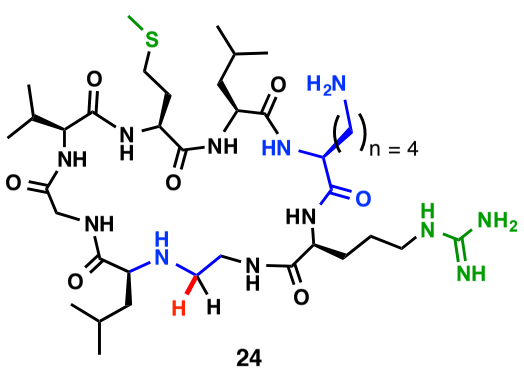
A) Crude analytical HPLC trace of the formation of **24** from amino aldehyde **S16** following reductive amination (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **24** (Rt = 10.8 min, 5 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{38}H_{73}N_{12}O_7S$ $[M+H]^+$ 841.54; found 841.54, $[M+2H]^{2+}$ 421.28; found 421.27, $[M+3H]^{3+}$ 281.19; found 281.18.

Partial 2D NMR Correlations:



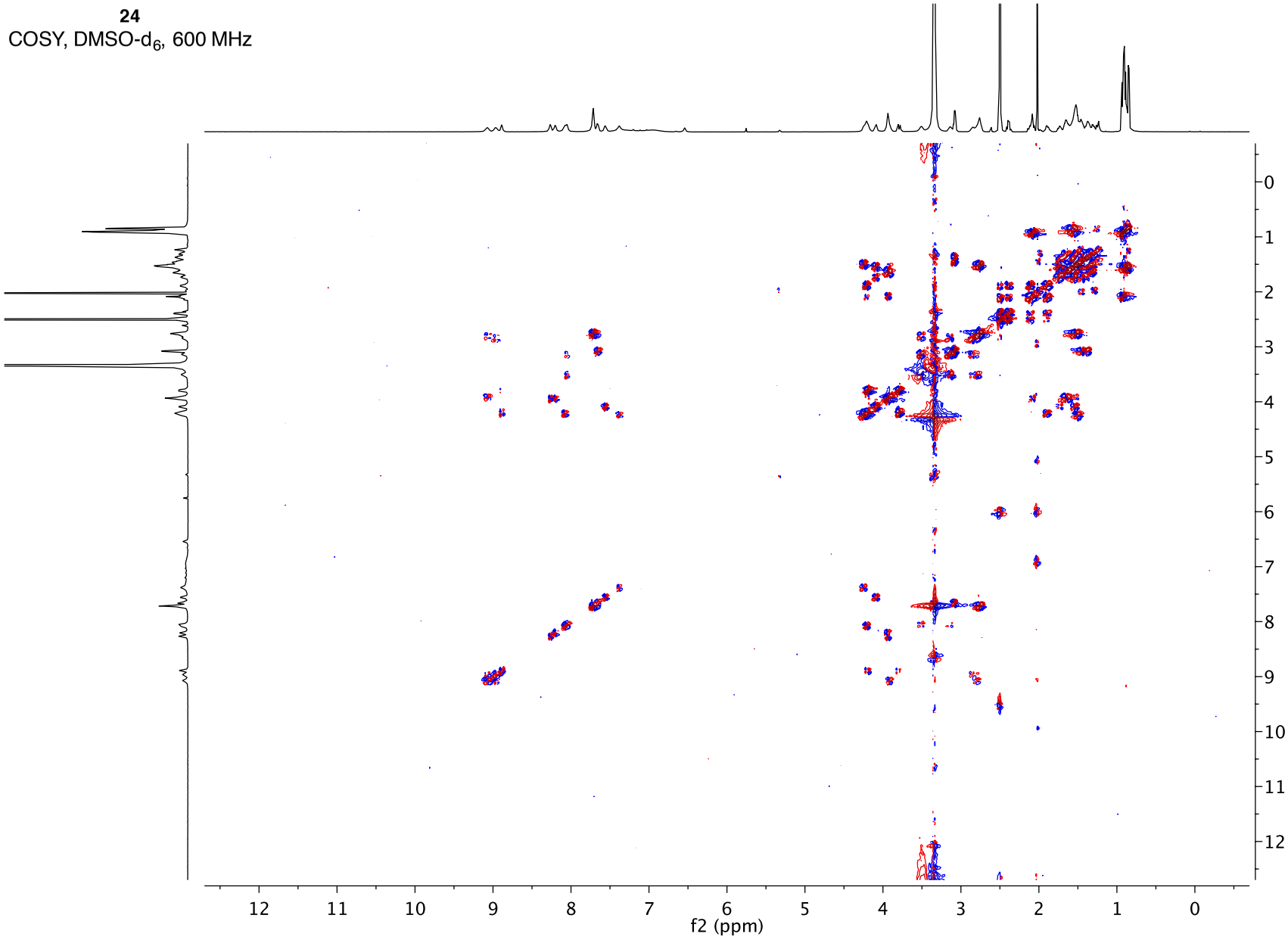


24

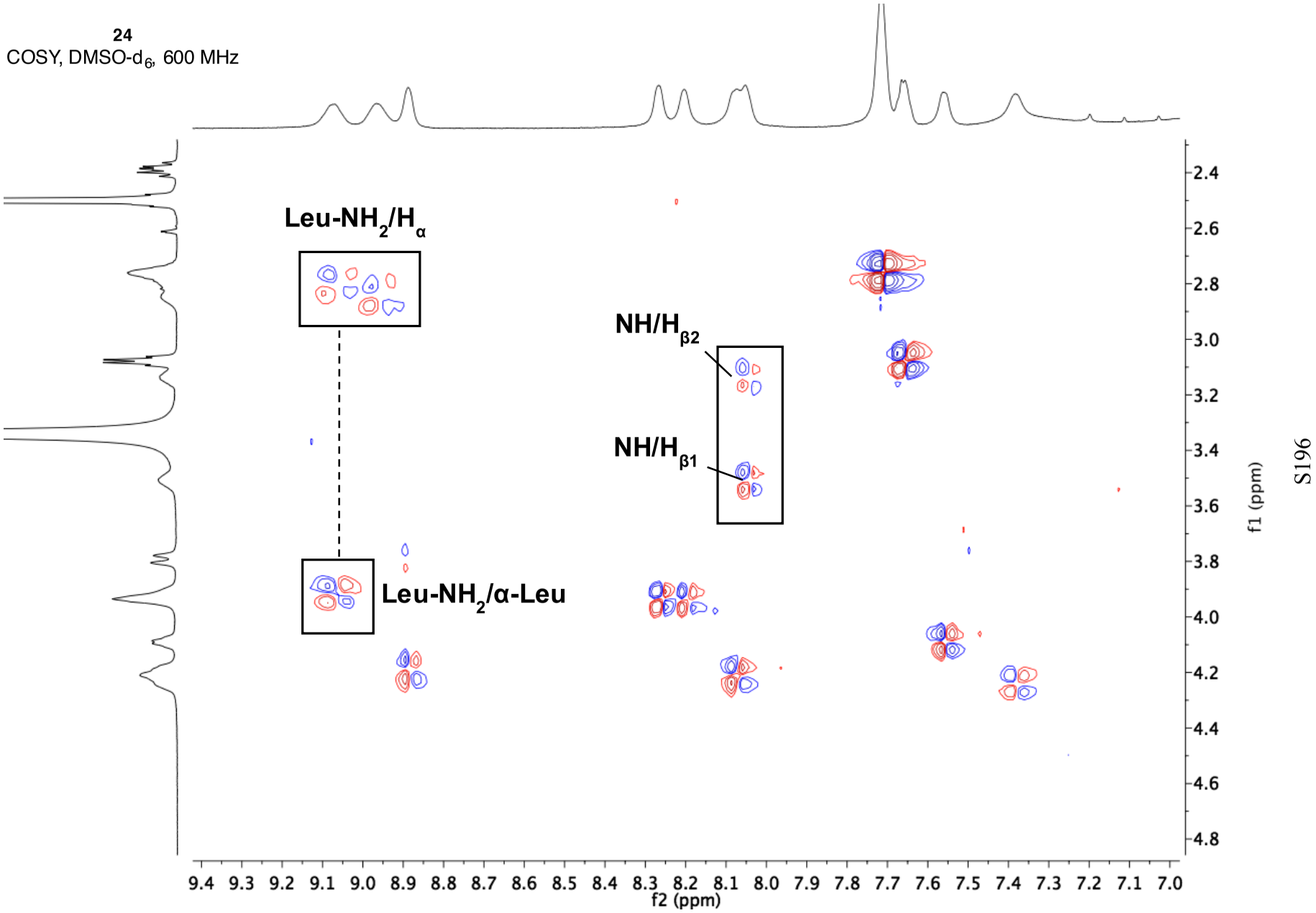
^{1}H, DMSO-d$_6$, 600 MHz

24

COSY, DMSO-d_6, 600 MHz

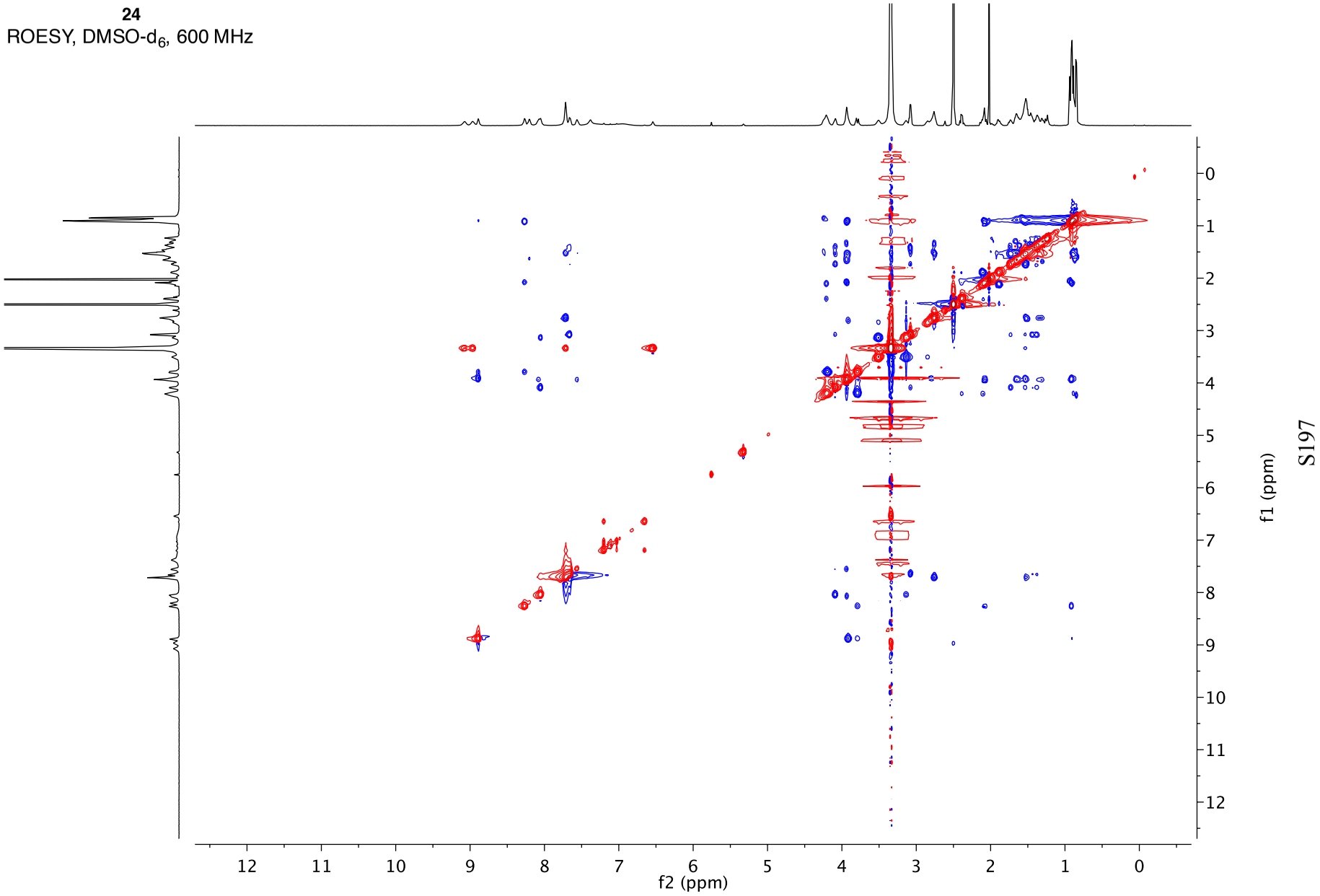


24
COSY, DMSO-d_6, 600 MHz



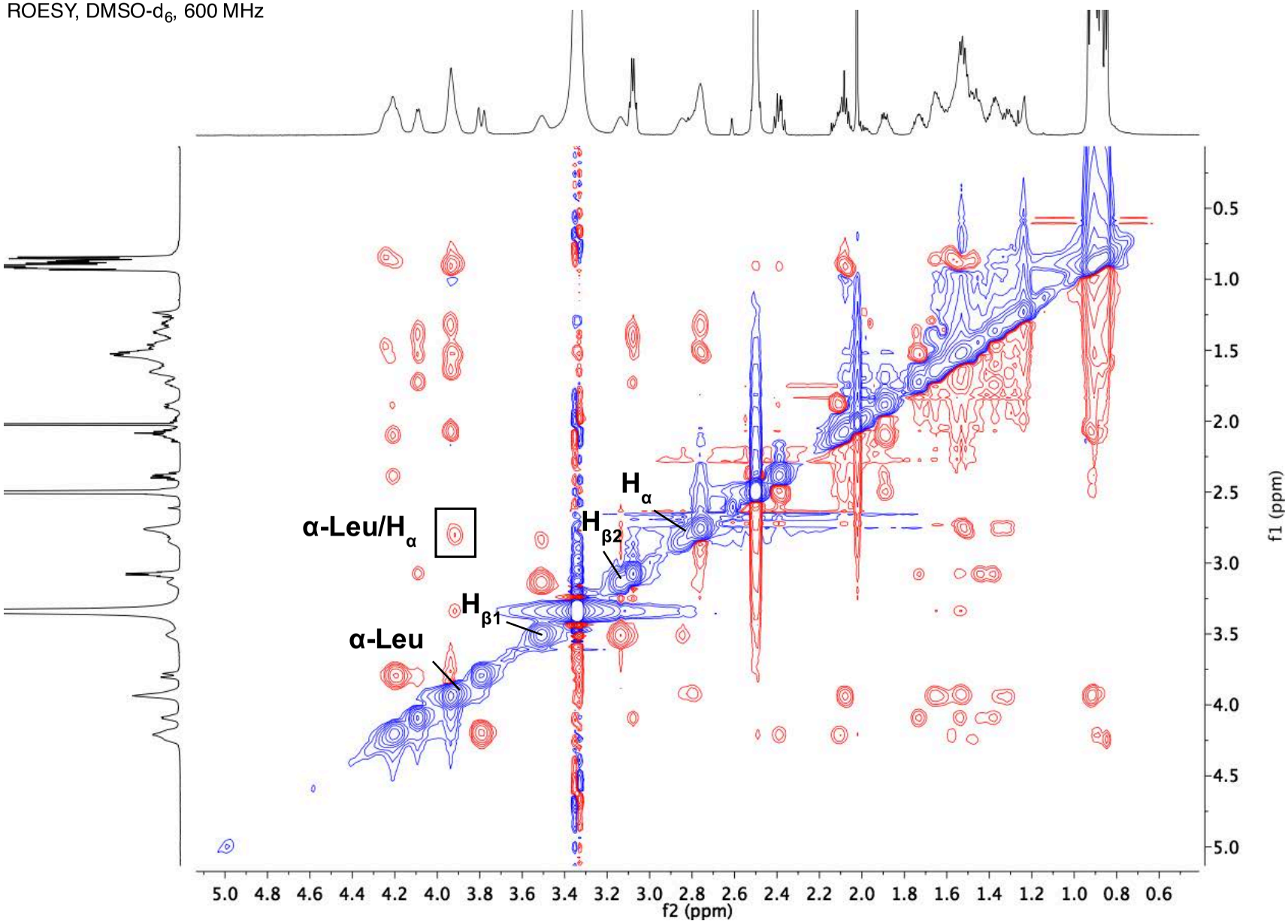
24

ROESY, DMSO-d_6, 600 MHz



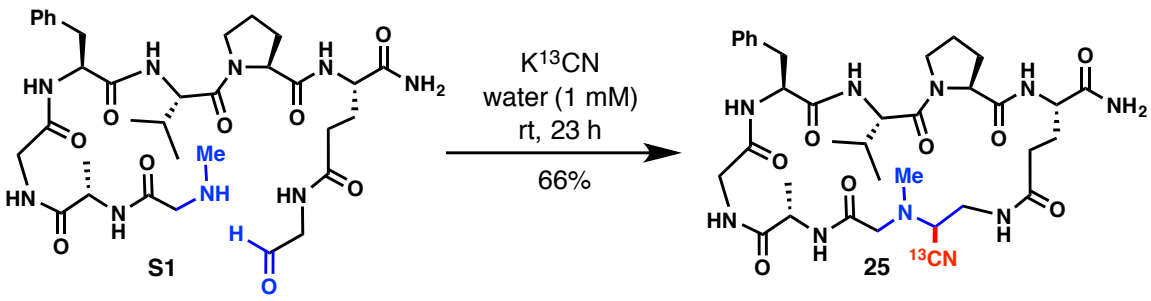
24

ROESY, DMSO-d_6, 600 MHz

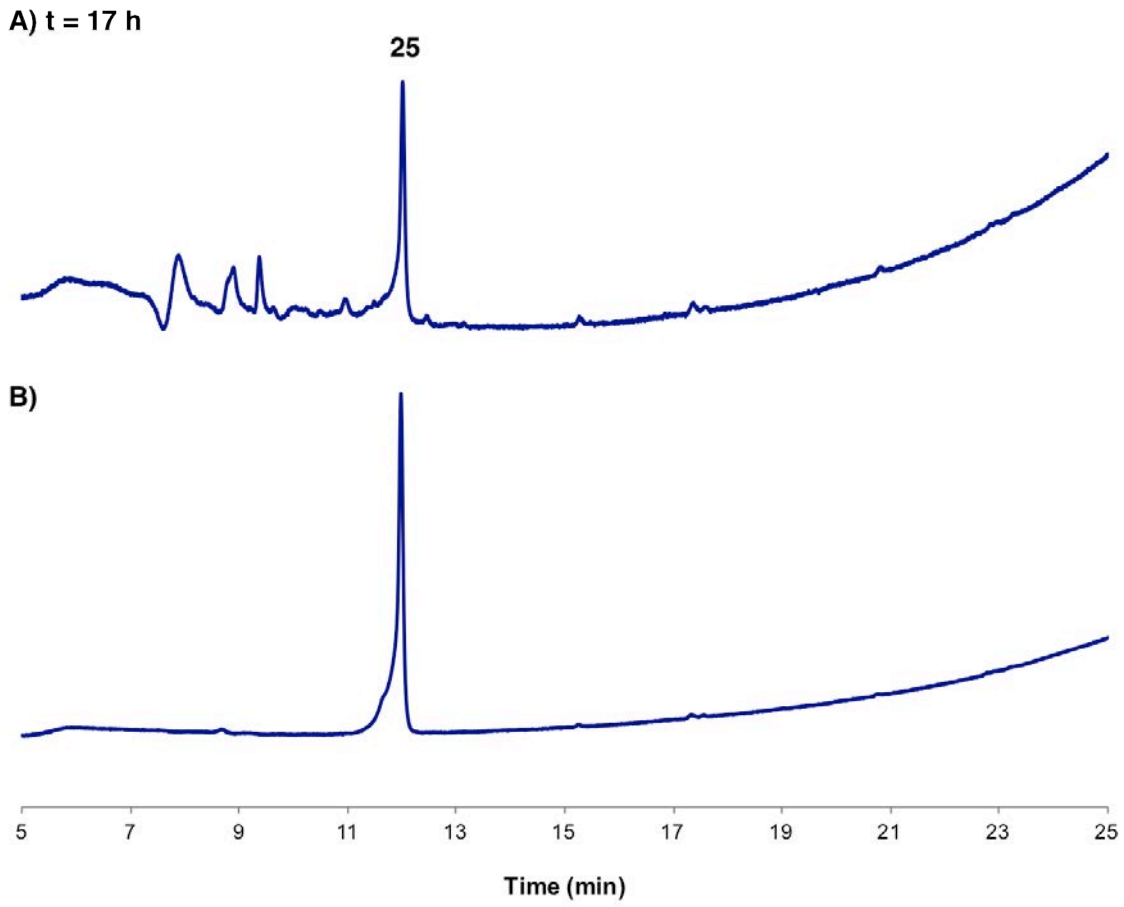


Characterization Data for Introduction of Bioorthogonal Handles

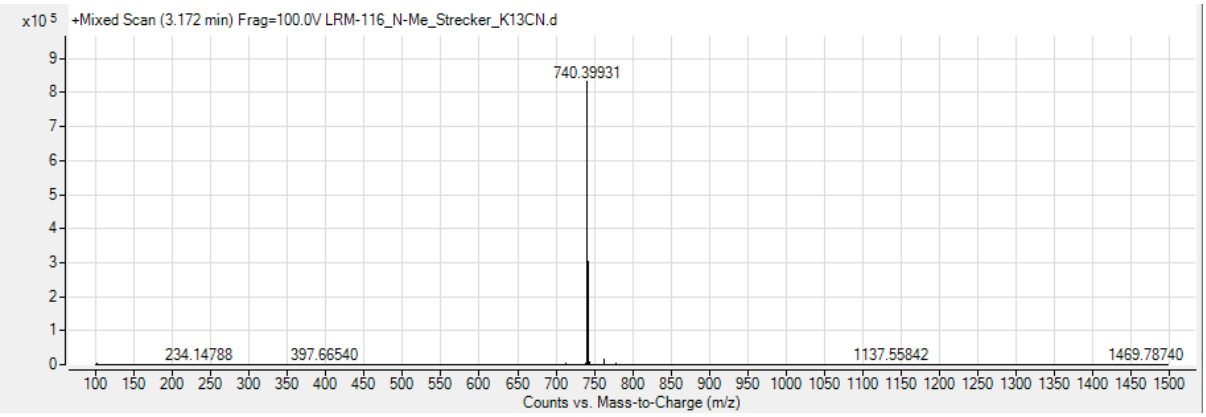
Peptide 25



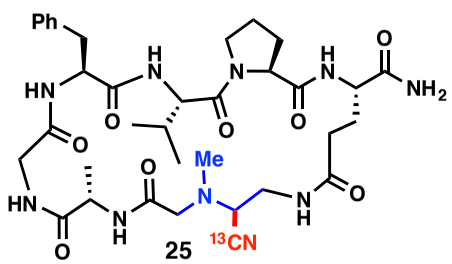
Peptide **25** was prepared with $K^{13}CN$ according to general procedure 3 from linear amino aldehyde **S1** (1.5 mg, 2.1 μmol). At t = 23 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% to 50% B over 30 min) to afford isotope-labeled peptide macrocycle **25** (1.0 mg, 66% yield) as a mixture of diastereomers and as a white solid following lyophilization.



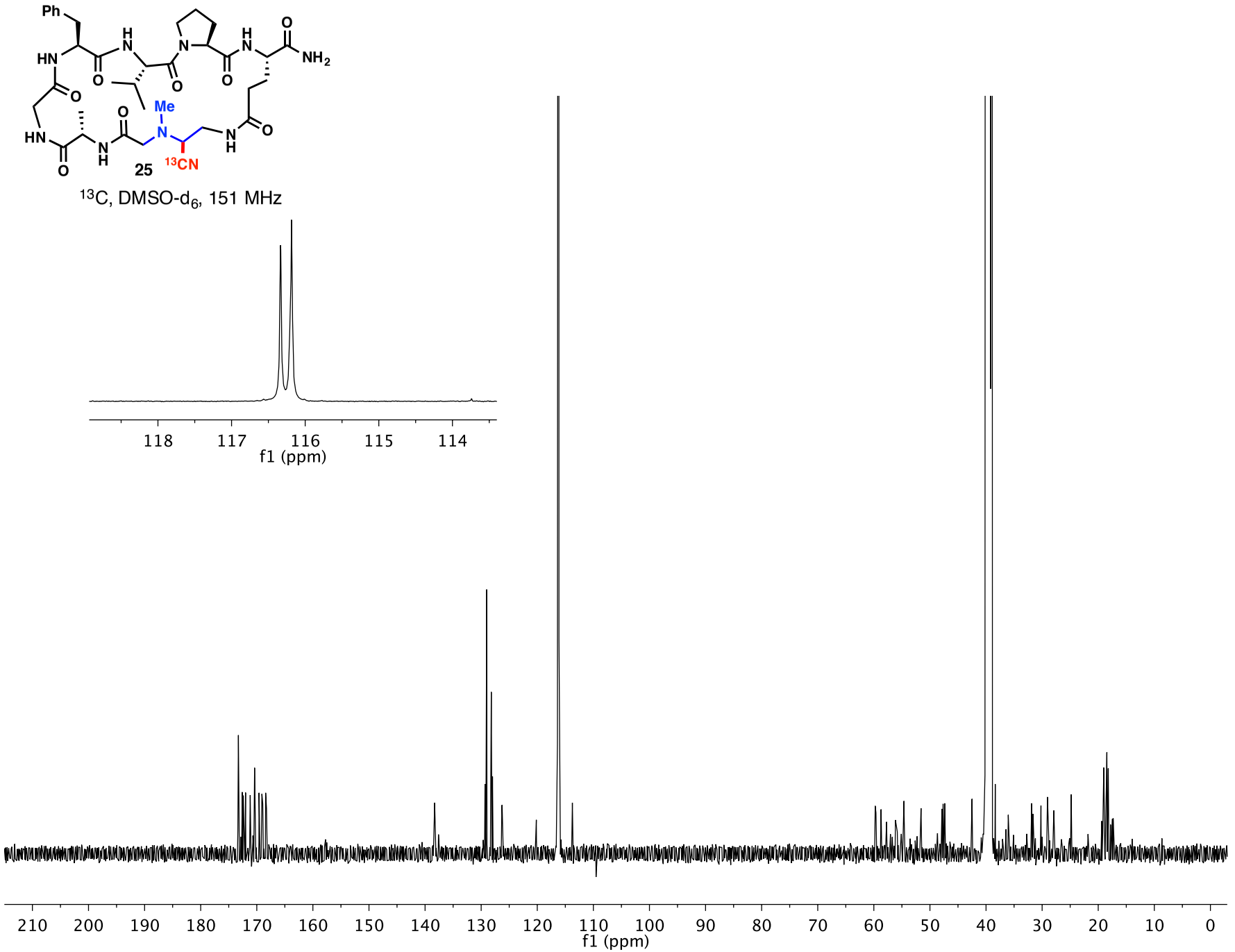
A) Crude analytical HPLC trace of the formation of **25** from amino aldehyde **S1** following Strecker macrocyclization with $K^{13}CN$ (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **25** (Rt = 12.0 min, 5 to 100% B over 25 min, λ = 230 nm).



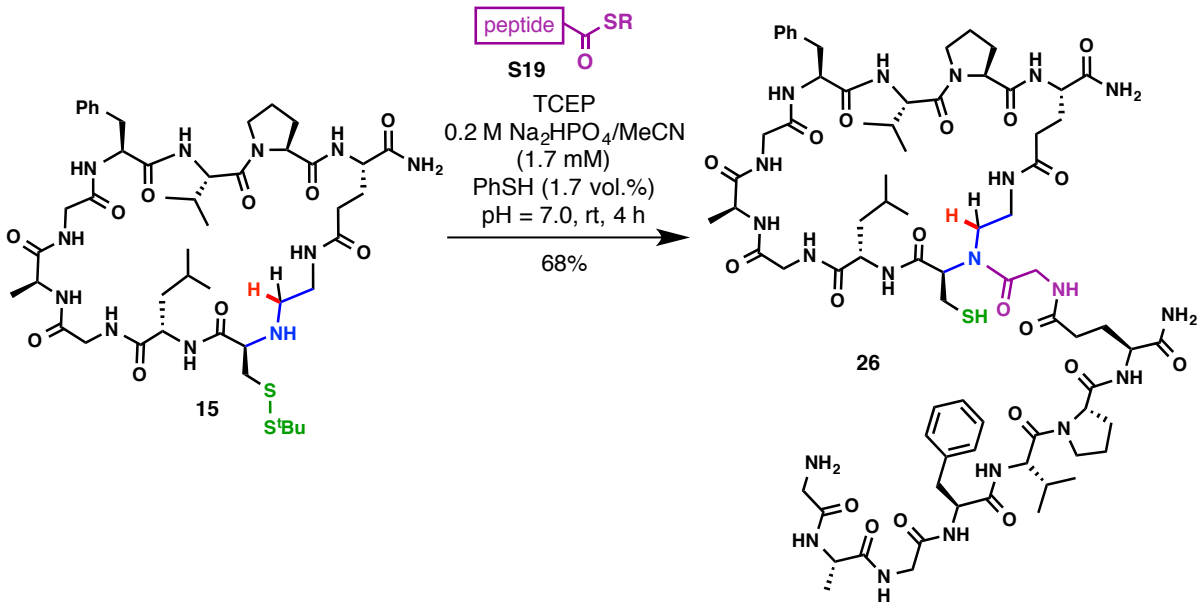
LRMS (ESI-TOF): calc'd for $C_{34}{}^{13}CH_{51}N_{10}O_8$ $[M+H]^+$ 740.39; found 740.40.



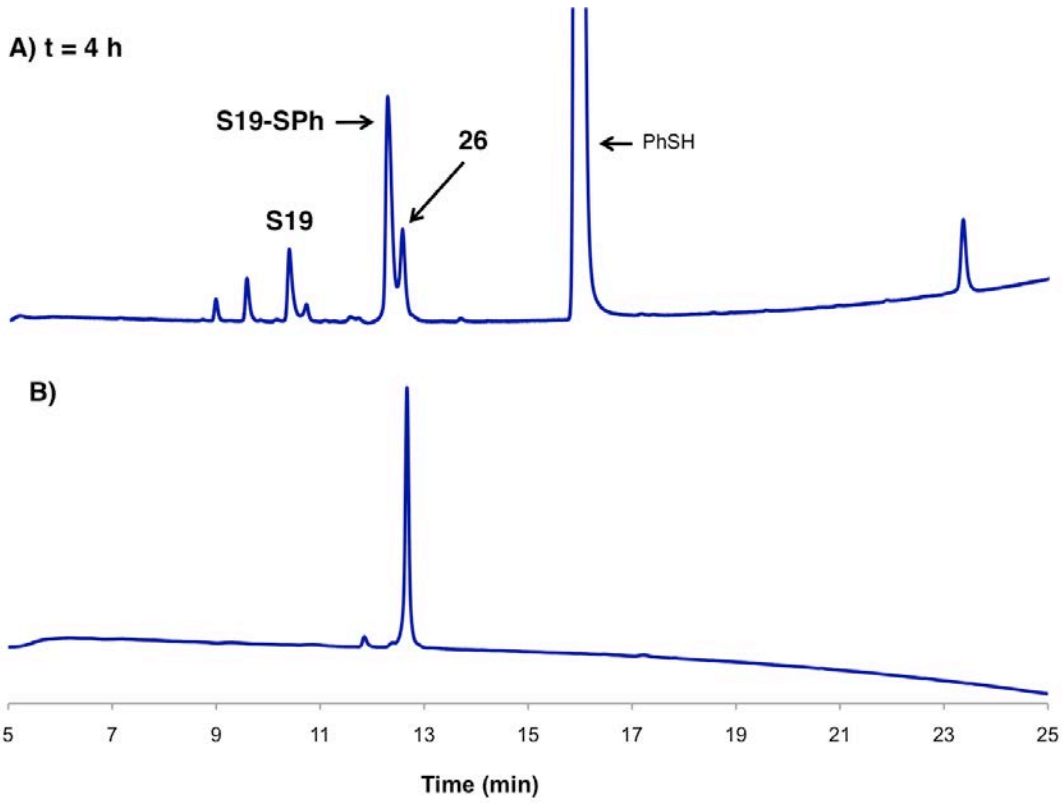
^{1}H, DMSO-d$_{6}$, 600 MHz


Ph
HN
H
N
O
O
N
H
N
O
NH2
O
O
O
Me
N
HN
O
N
H
O
N
H
O
25
13CN
13C, DMSO-d6, 151 MHz
118
117
116
115
114
f1 (ppm)
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

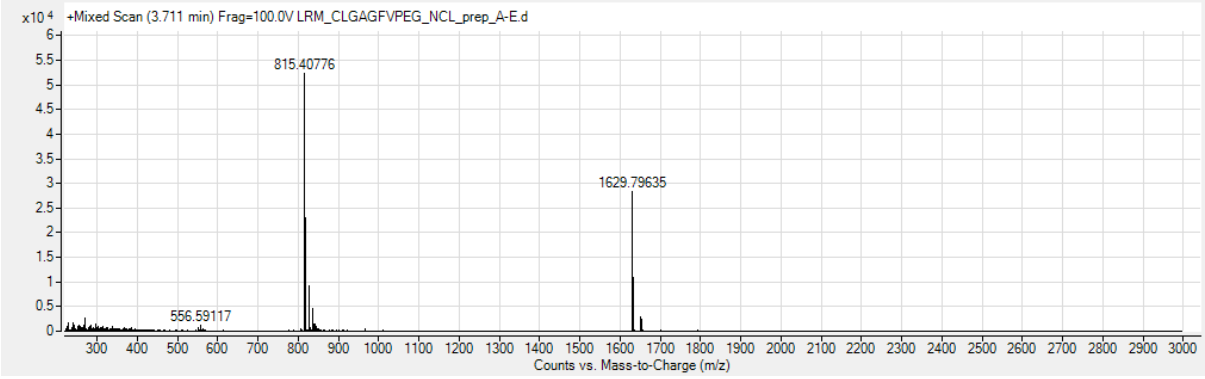
Peptide 26



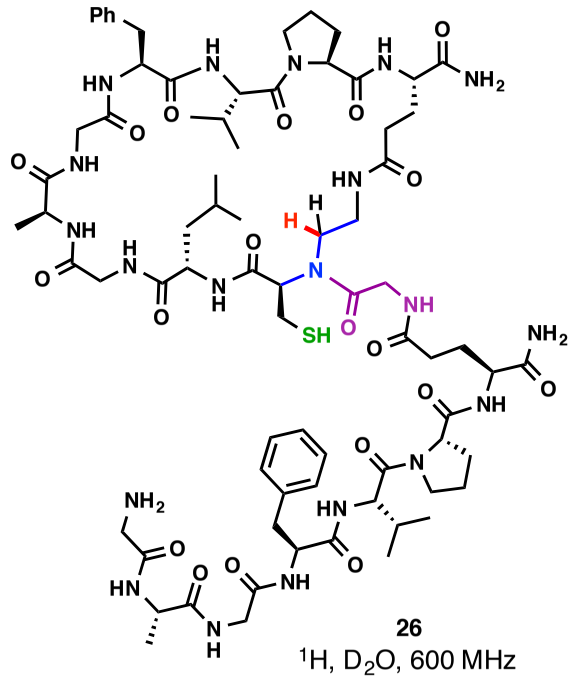
Peptide **26** was prepared by ligation of macrocycle **15** and peptide thioester **S19**. Ligation buffer was first prepared by dissolving the phosphine reductant *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP) in aqueous 0.2 M Na_2HPO_4 to provide a 50 mM solution of TCEP. The final pH of the buffer was adjusted to 7.0 using 1 M NaOH. The buffer was then sparged with argon for 5 min. Peptide thioester **S19** (0.9 mg, 1.1 μmol) was dissolved in ligation buffer (498 μL) and added to peptide macrocycle **15** (1.0 mg, 1.0 μmol) in a 1.5 mL eppendorf vial. MeCN (100 μL) was added to the reaction mixture to aid in solubilizing macrocycle **15**, thus providing a final reaction concentration of 1.7 mM with respect to peptide **15**. Thiophenol (10 μL, 1.7 vol.%) was added and the reaction mixture was stirred at rt and monitored by LC-MS. At t = 4 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, then 20% to 50% B over 30 min) to afford ligation product **26** (1.1 mg, 68% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **26** from macrocycle **15** and peptide thioester **S19** following native chemical ligation (5 to 100% B over 25 min, λ = 230 nm). *Note:* **S19-SPh** represents the thiophenyl thioester variant of **S19** which forms upon transthioesterification of **S19** in the presence of excess PhSH. **B)** Purified peptide **26** (Rt = 12.7 min, 5 to 100% B over 25 min, λ = 210 nm).



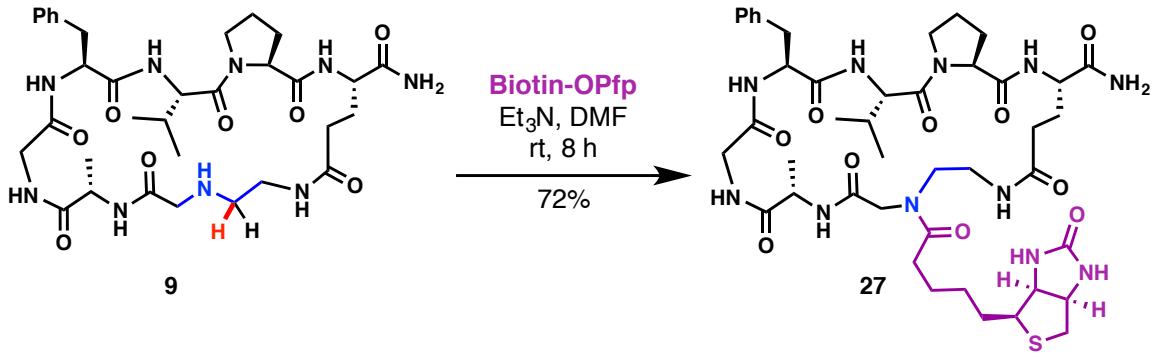
LRMS (ESI-TOF): calc'd for $C_{75}H_{113}N_{20}O_{19}S$ $[M+H]^+$ 1629.82; found 1629.80, $[M+2H]^{2+}$ 815.41; found 815.41



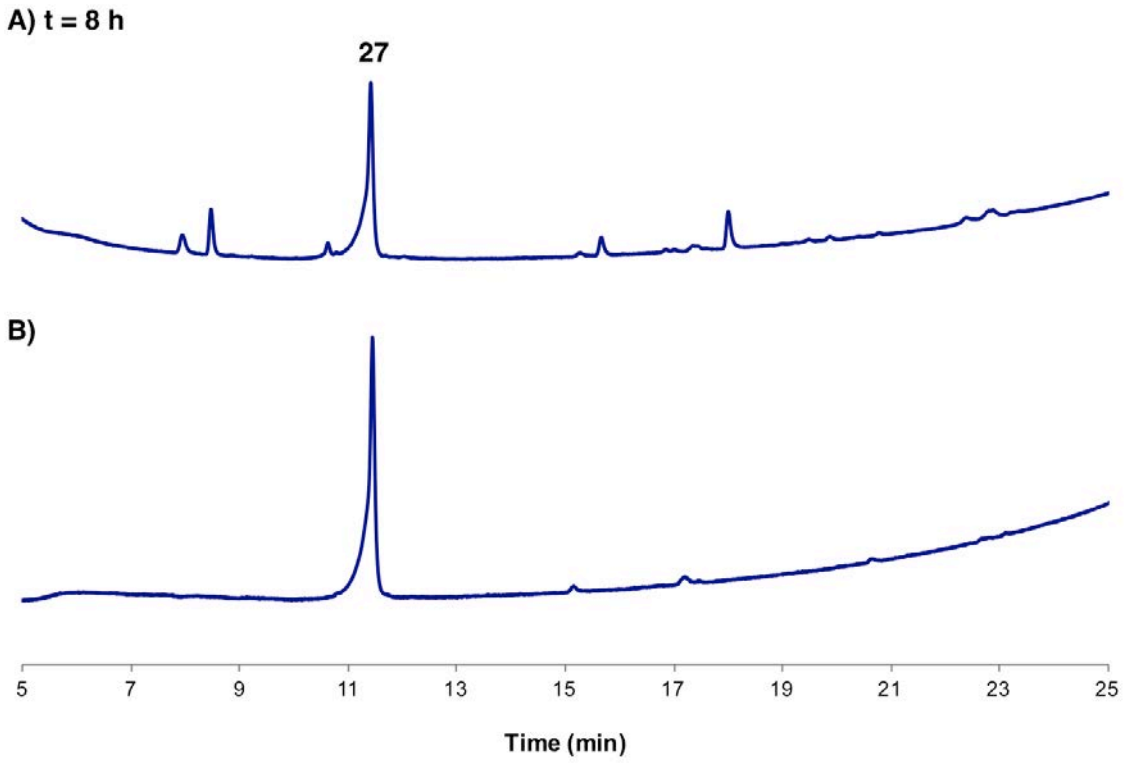
26

^{1}H, D_2O, 600 MHz

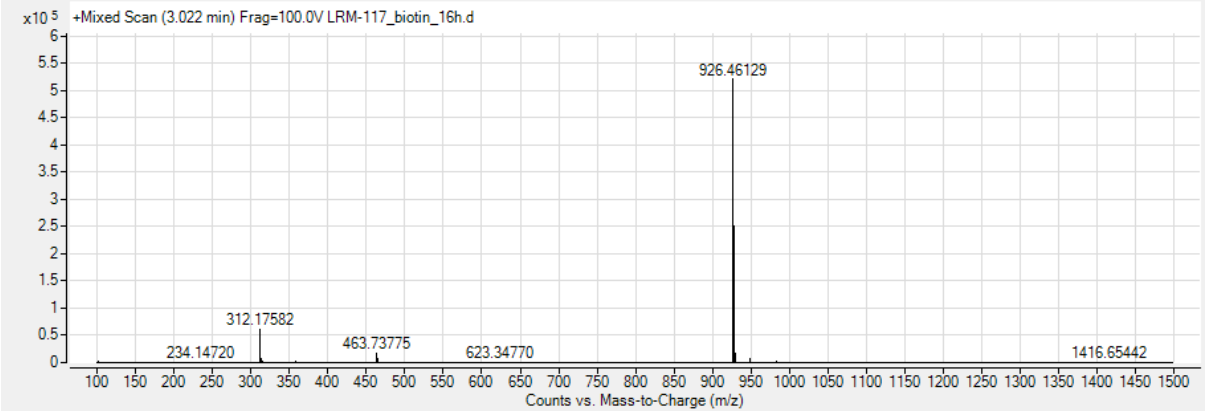
Peptide 27



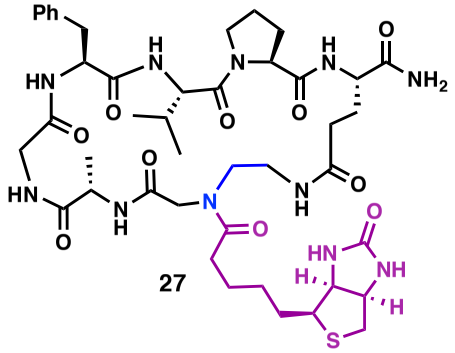
Biotin-labeled peptide **27** was prepared from macrocycle **9** (3.5 mg, 5.0 μmol). To a solution of peptide **9** in dry DMF (1 mL) was added Et_3N (1.4 μL, 2.0 eq.) followed by biotin-OPfp (10.3 mg, 5.0 equiv.).[9] The reaction was stirred at rt and monitored by LC-MS. At t = 8 h, the reaction was diluted with water (5 mL) and unreacted biotin-OPfp crashed out of solution. The crude mixture was partitioned between water and DCM. The aqueous layer was separated and purified by preparative reverse-phase HPLC (5% B for 5 min, 5% to 20% B over 5 min, then 20% to 50% B over 25 min) to afford peptide macrocycle **27** (3.3 mg, 72% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **27** from macrocycle **9** following treatment with biotin-OPfp (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **27** (Rt = 11.4 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{43}H_{64}N_{11}O_{10}S$ $[M+H]^+$ 926.46; found 926.46.

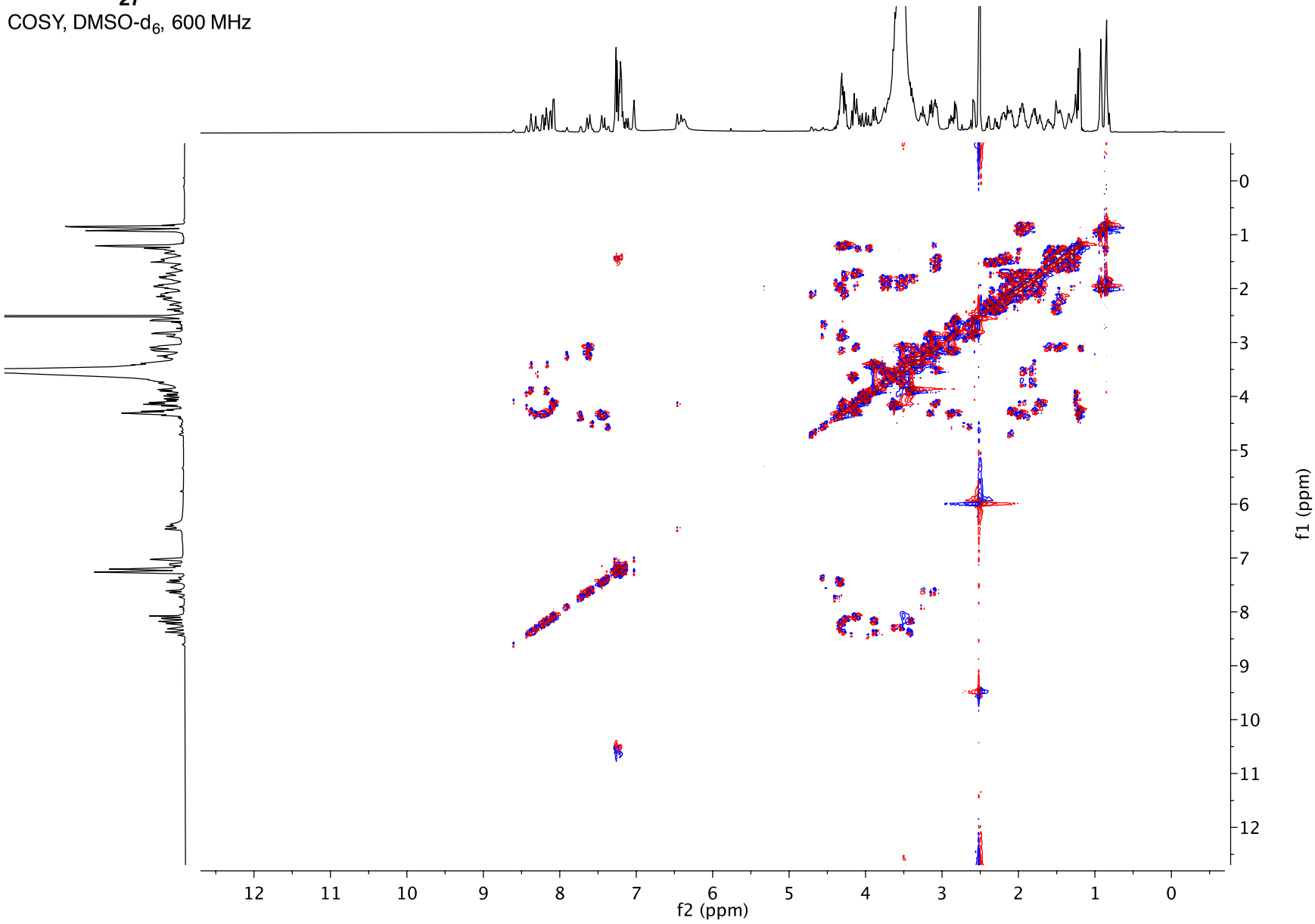


27

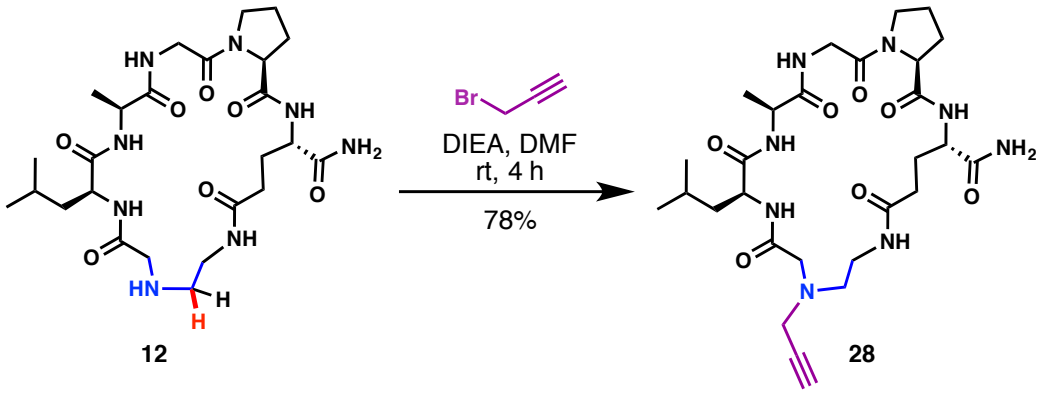
^{1}H, DMSO-d_6/D_2O (20:1 v/v), 600 MHz

27

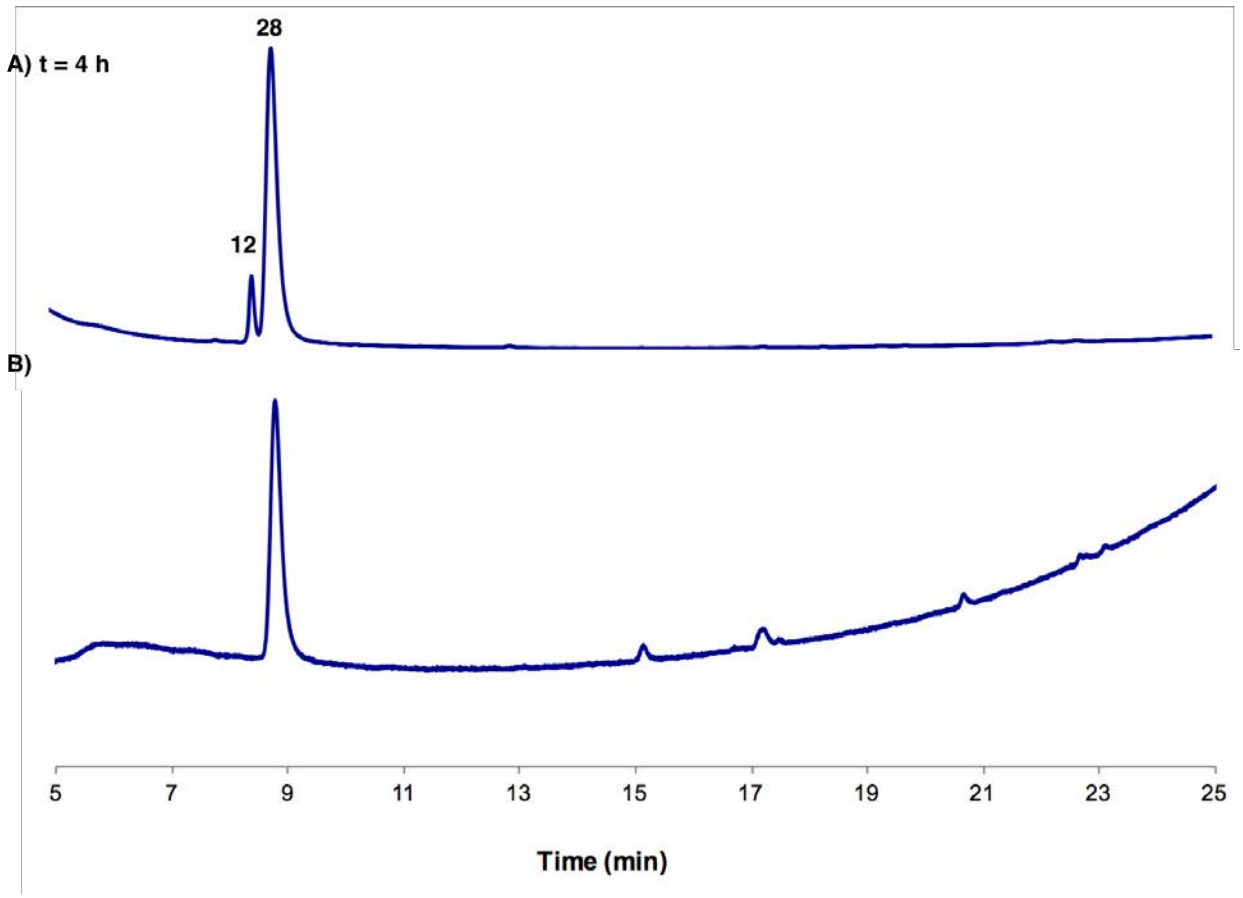
COSY, DMSO-d_6, 600 MHz



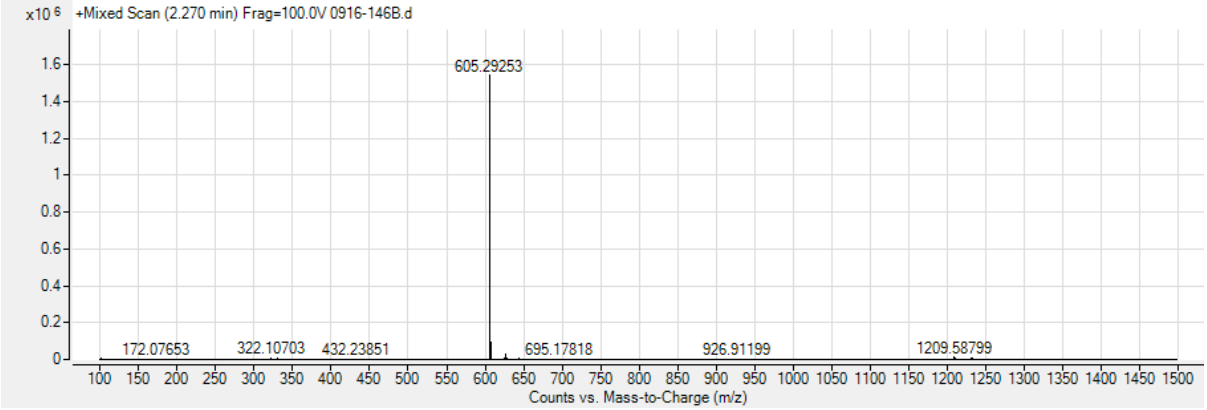
Peptide 28



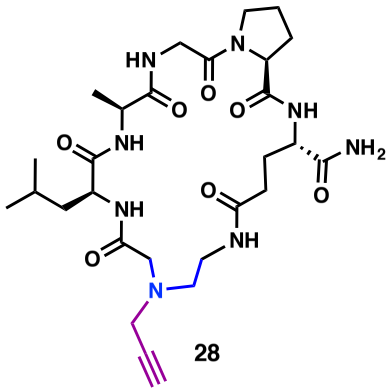
To a solution of purified peptide **12** (2.4 mg, 4.24 μmol) in DMF (1 mM) was added DIEA (2 equiv.), and the resulting solution was allowed to stir at room temperature for 5 min. Propargyl bromide (10 equiv.) was then added, and the reaction mixture was monitored by LC-MS analysis. At t = 4 h, the reaction was purified by preparative reverse-phase HPLC (10% B for 10 min, 10% to 45% B over 30 min) to afford peptide macrocycle **28** (2.0 mg, 78% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **28** from peptide **12** following propargylation (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **28** (Rt = 8.78 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{28}H_{44}N_8O_7$ $[M+H]^+$ 605.34; found 605.29.

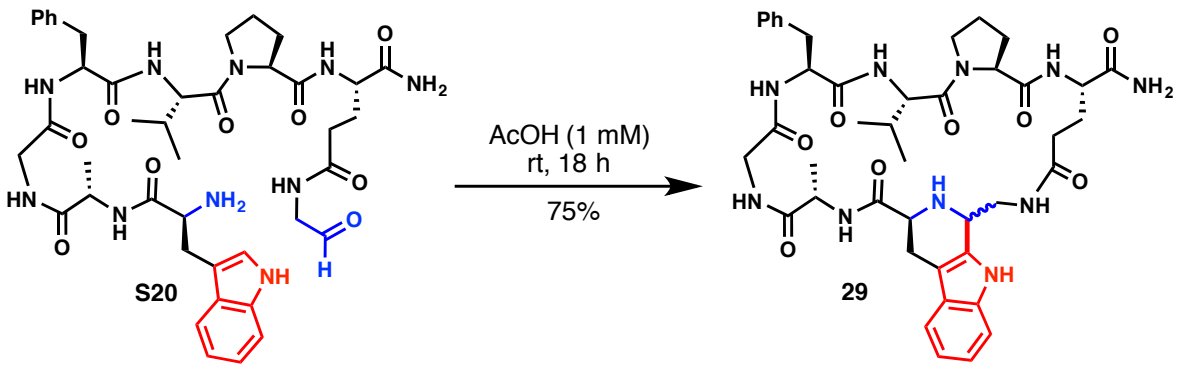


28

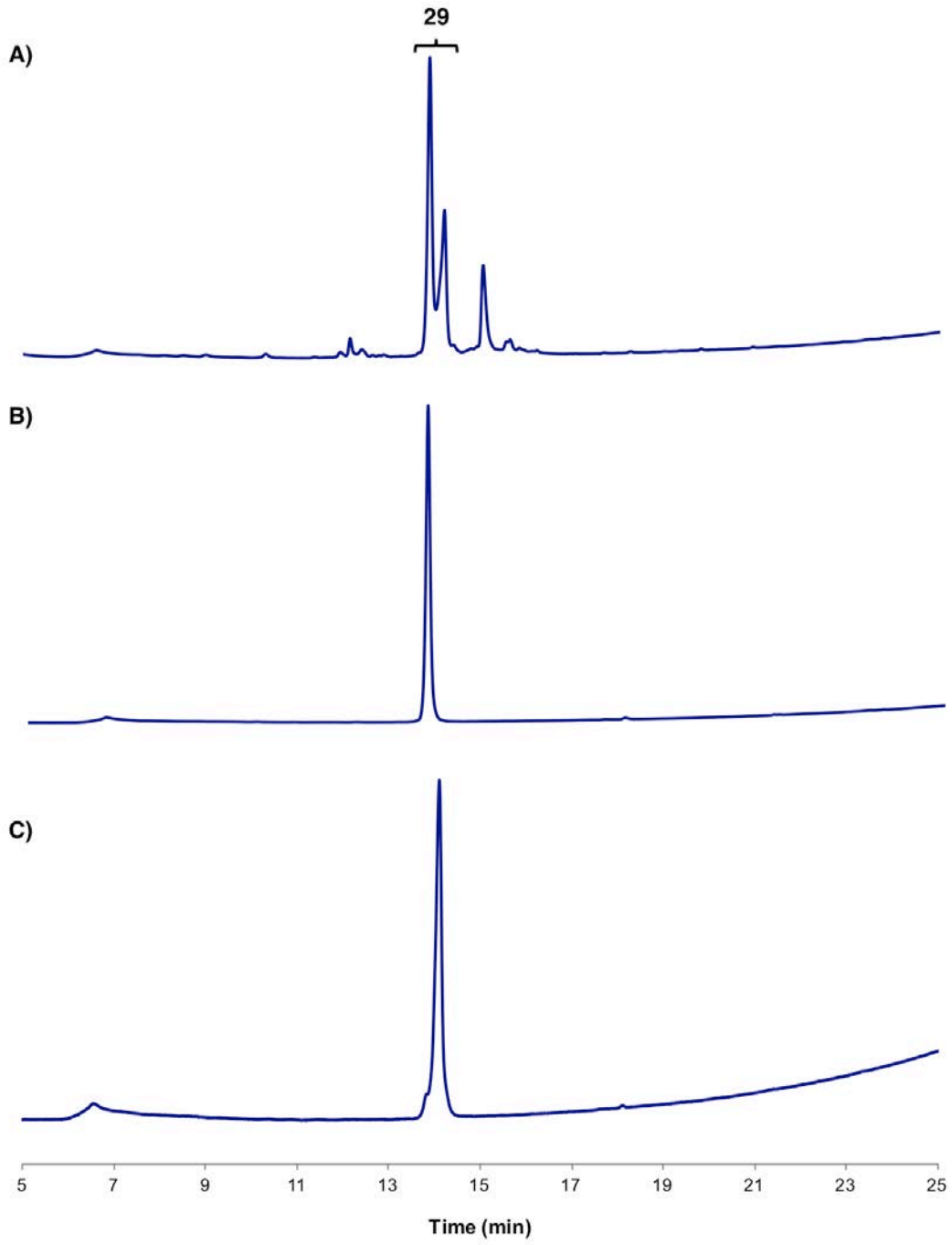
^{1}H, DMSO-d$_6$, 600 MHz

Macrocyclization Data for Pictet-Spengler

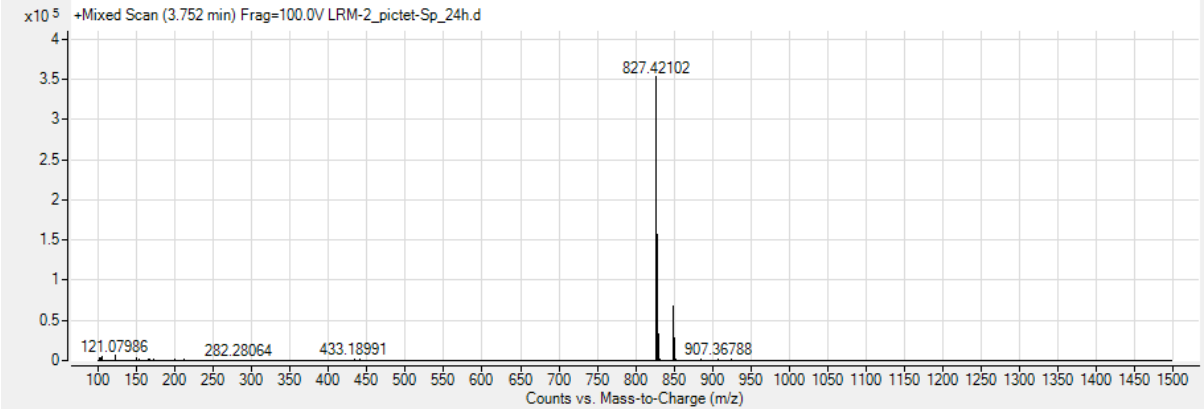
Peptide 29



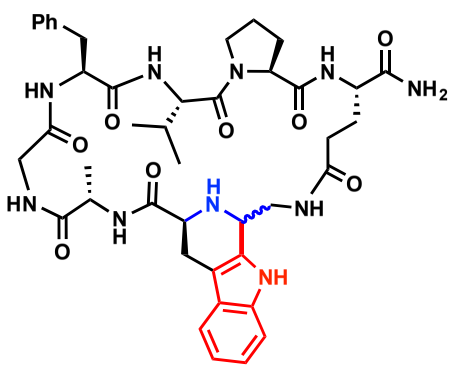
Peptide **29** was prepared from linear amino aldehyde **S20** using a Pictet-Spengler macrocyclization approach. Peptide **S20** (3.0 mg, 3.6 μmol) was dissolved in AcOH (1 mM) and stirred at rt. At t = 18 h, the reaction was purified by preparative reverse-phase HPLC (5% B for 5 min, 5% B to 25% B over 5 min, then 25% to 65% B over 30 min) to afford peptide macrocycle **29** (2.2 mg, 75% combined yield) as a partially separable mixture of diastereomers and as white solids following lyophilization.



A) Crude Pictet-Spengler reaction to form diastereomeric macrocycles **29** (0 to 100% B over 25 min, λ = 230 nm). **B)** Purified **29**, major diastereomer (Rt = 13.7 min, 0 to 100% B over 25 min, λ = 230 nm). **C)** Purified **29**, minor diastereomer (Rt = 14.1 min, 0 to 100% B over 25 min, λ = 230 nm). Note: some mixed fractions were also obtained upon HPLC purification.

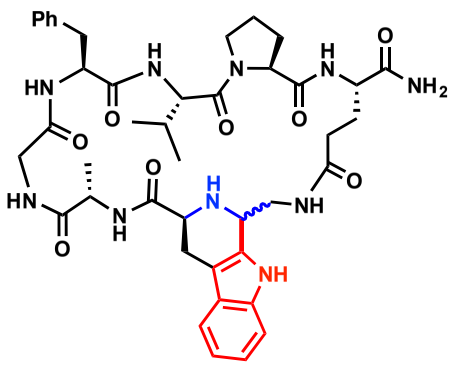


LRMS (ESI-TOF): calc'd for $C_{42}H_{55}N_{10}O_8$ $[M+H]^+$ 827.42; found 827.42.



29 (major diastereomer)

^{1}H, D_2O, 600 MHz

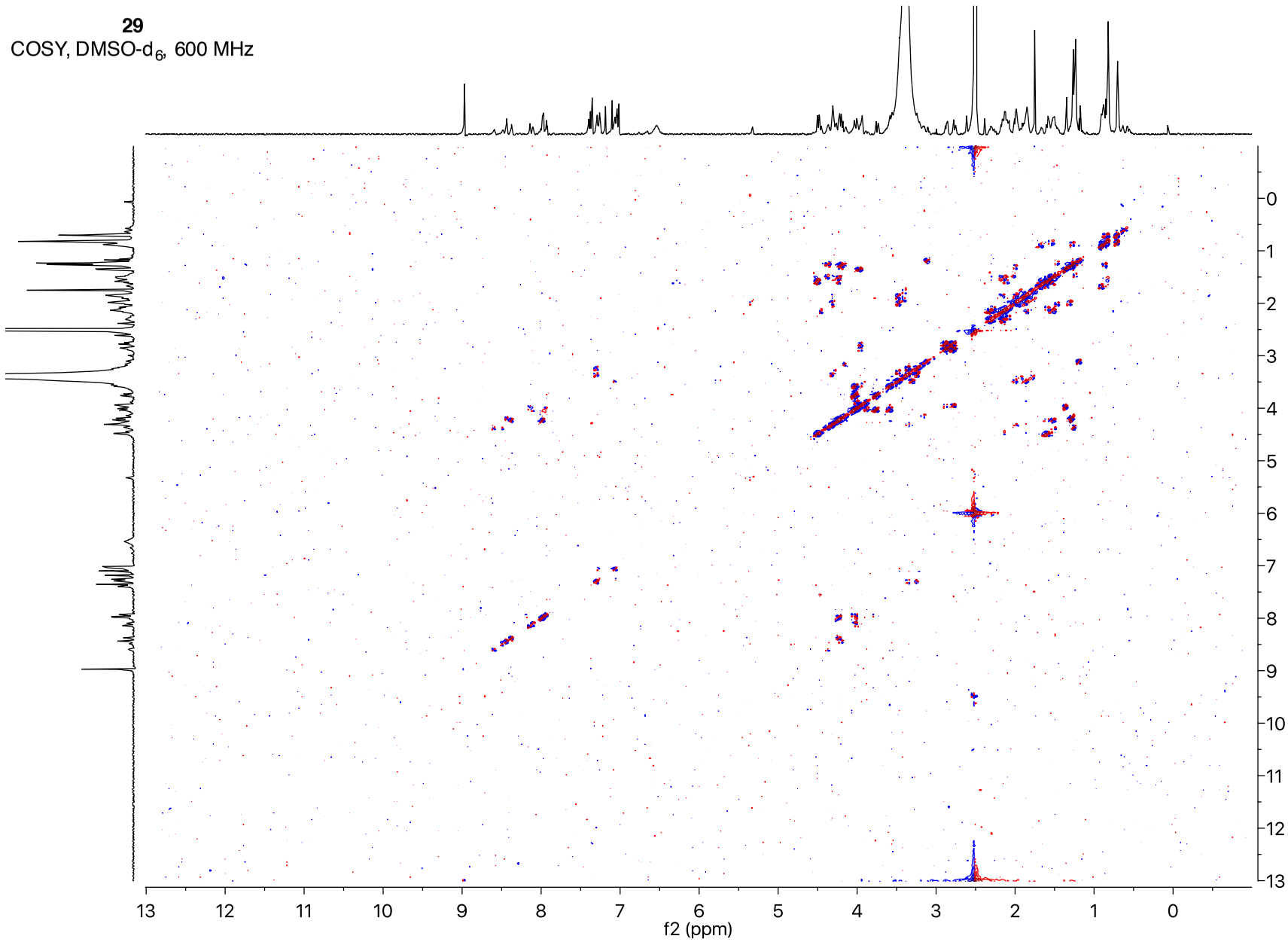


29 (minor diastereomer)

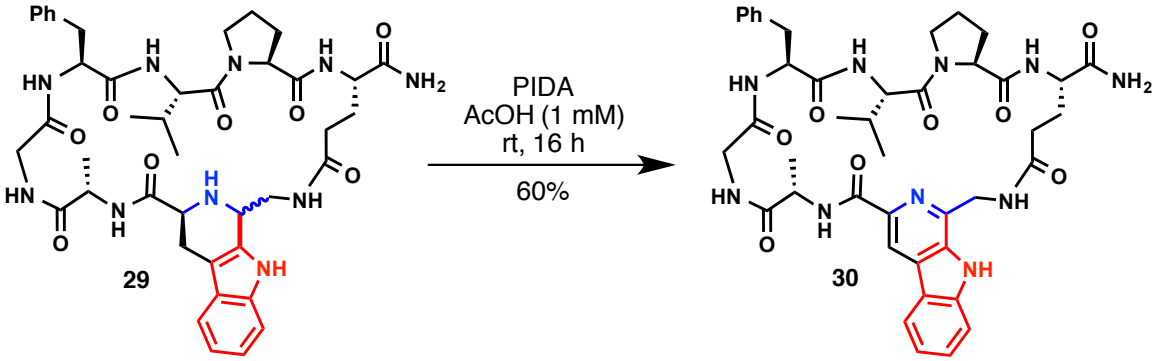
^{1}H, D_2O, 600 MHz

29

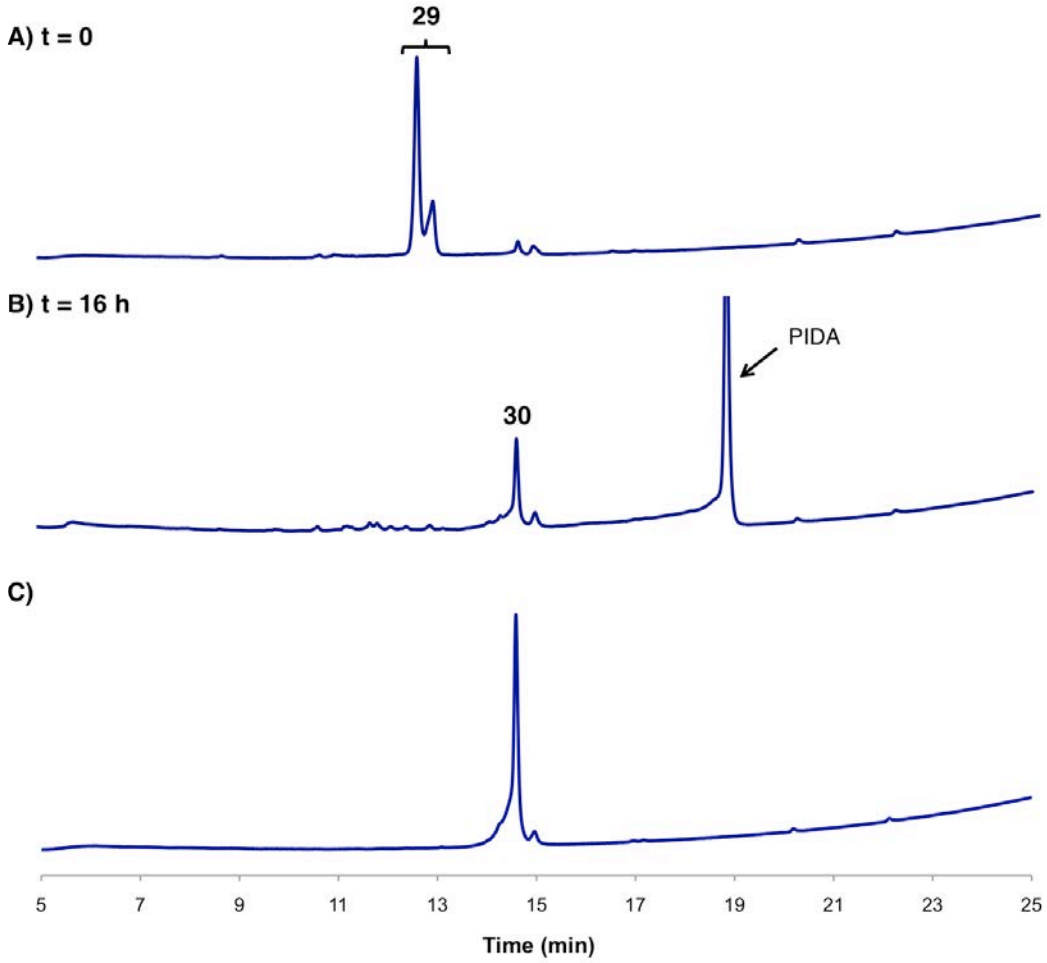
COSY, DMSO-d_6, 600 MHz



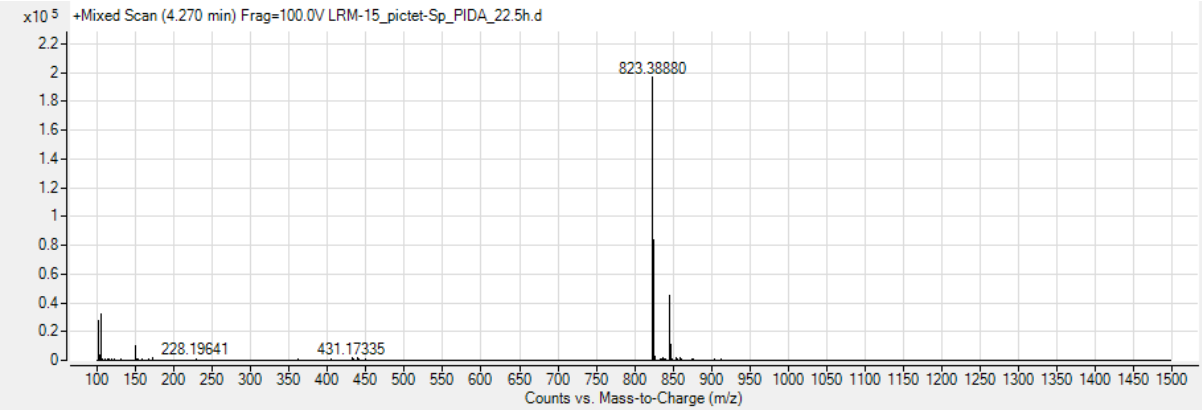
Peptide 30



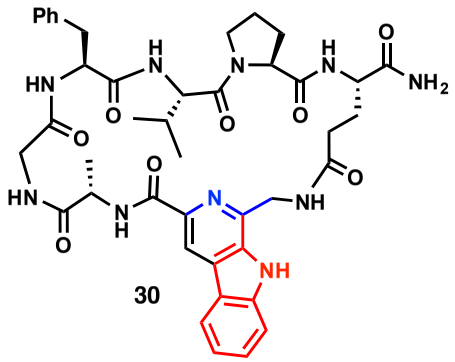
Peptide **30** was prepared by oxidation of diastereomeric tetrahydro-β-carboline **29**. Peptide **29** (2.0 mg, 2.4 μmol) was dissolved in AcOH (2.4 mL, 1 mM) and treated with phenyliodine diacetate (PIDA) (1.6 mg, 2 eq.). The reaction was stirred at rt for 16 h, at which point LC-MS analysis indicated complete consumption of the starting peptide. The reaction was concentrated to ~1 mL under a stream of nitrogen, diluted in a mixture of water and MeCN, and purified by preparative reverse-phase HPLC (25% B for 5 min, then 25% to 75% B over 25 min) to afford peptide macrocycle **30** (1.2 mg, 60% yield) as a white solid following lyophilization.



A) Reaction at t = 0 depicting diastereomeric tetrahydro-β-carboline **29** (5 to 100% B over 25 min, λ = 230 nm). **B)** Crude oxidation to afford β-carboline **30** (5 to 100% B over 25 min, λ = 230 nm). **C)** Purified β-carboline oxidation product **30** (Rt = 14.6 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{42}H_{51}N_{10}O_8$ $[M+H]^+$ 823.39; found 823.39.

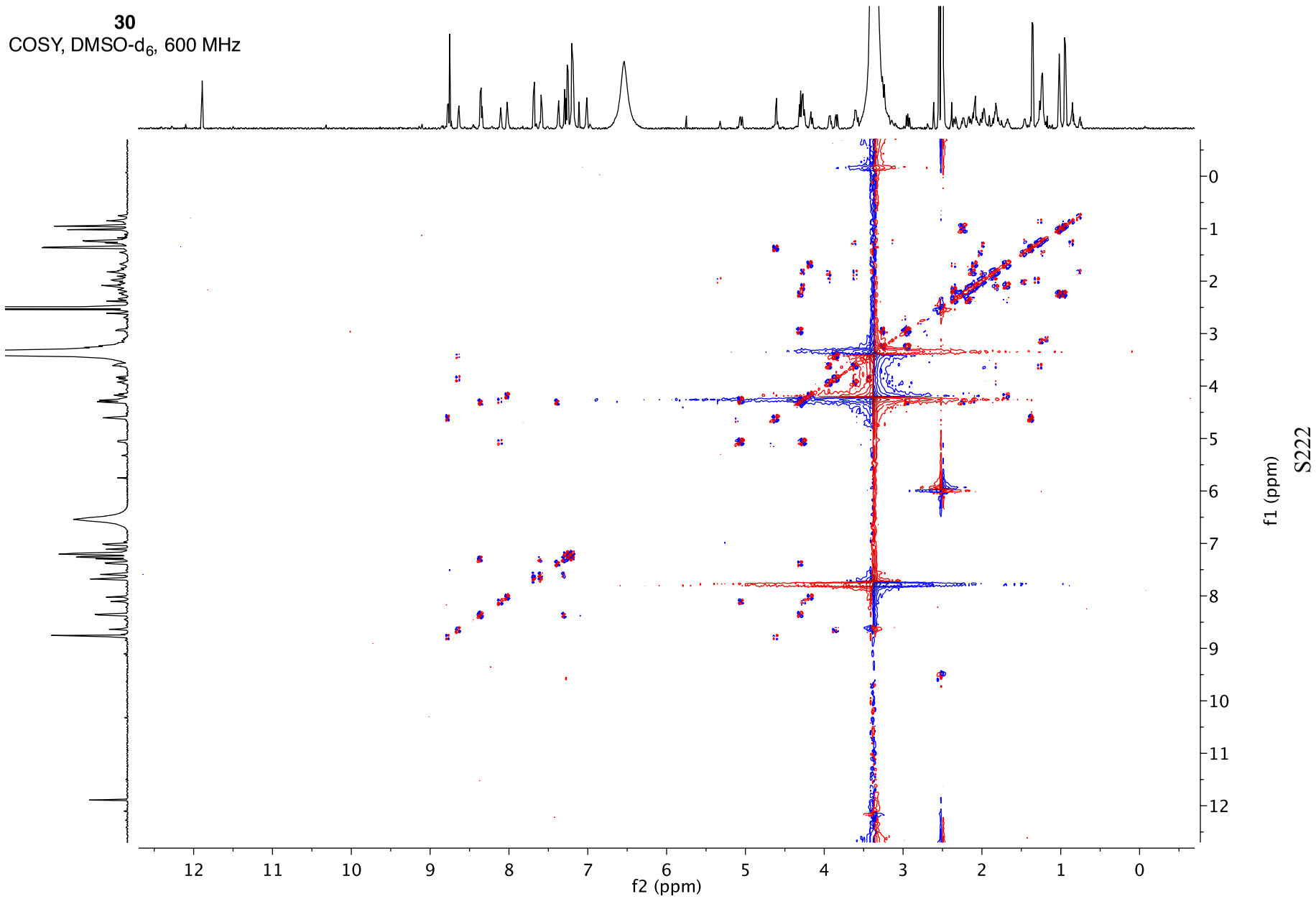


30

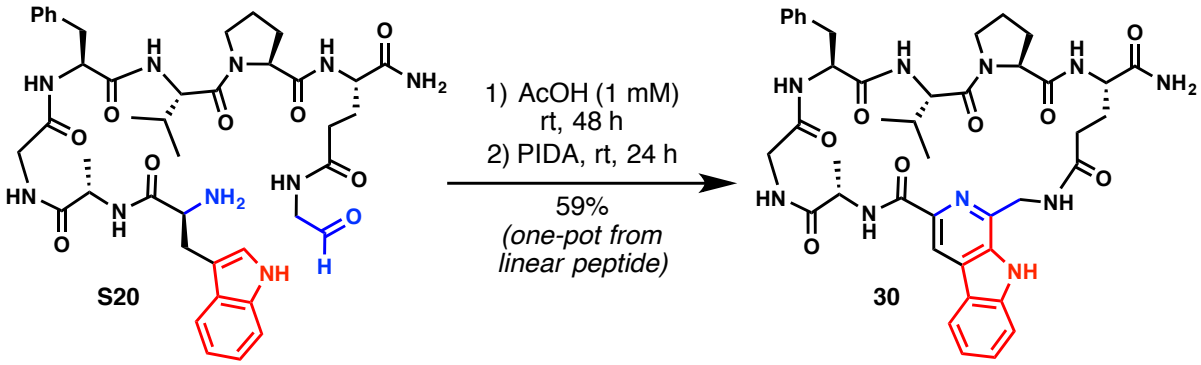
^{1}H, DMSO-d$_6$, 600 MHz

30

COSY, DMSO-d_6, 600 MHz

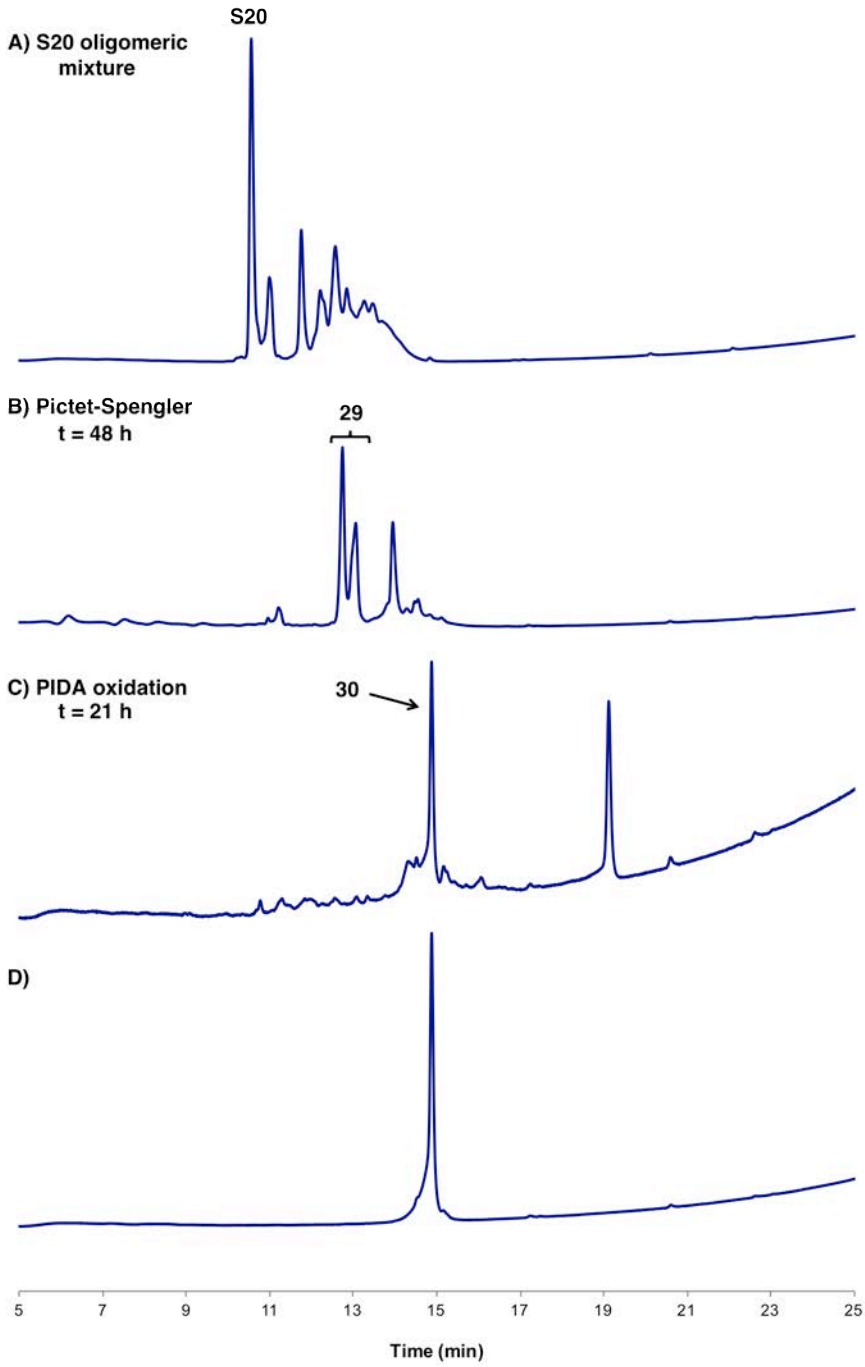


Peptide 30 (one-pot protocol)

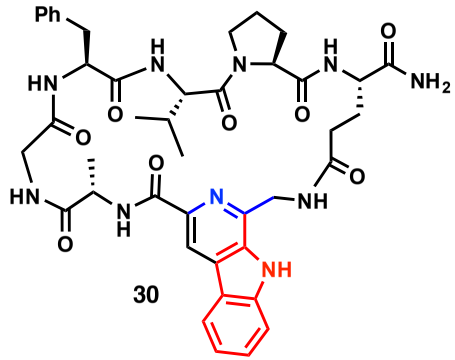


Peptide **30** was prepared in a one-pot fashion from linear amino aldehyde **S20** (2.0 mg, 2.4 μmol). Peptide **S20** was dissolved in AcOH (2.4 mL, 1 mM) and stirred at rt. At t = 48 h, phenyliodine diacetate (PIDA) (1.6 mg, 2 eq.) was added and the reaction was monitored for an additional 24 h before LC-MS indicated complete consumption of the tetrahydro-β-carboline. The crude mixture was concentrated to ~1 mL under a stream of nitrogen, diluted in a mixture of water and MeCN, and purified by preparative reverse-phase HPLC (25% B for 5 min, then 25% to 75% B over 25 min) to afford peptide macrocycle **30** (1.1 mg, 59% yield) as a white solid following lyophilization.

Note: The one-pot oxidation protocol was performed on starting peptide aldehyde **S20** *that had been stored as a lyophilized solid at 4 °C for a period of 4 months. Over this time period, substantial oligomerization of the peptide aldehyde was observed (see HPLC traces below). Nonetheless, upon addition of AcOH (final peptide concentration of 1 mM) Pictet-Spengler macrocyclization led to clean formation of the monomeric macrocycle* **30**. *The inherent reversibility of the amino aldehyde oligomerization allows the reaction to proceed efficiently despite the presence of a complex mixture of starting peptide.*



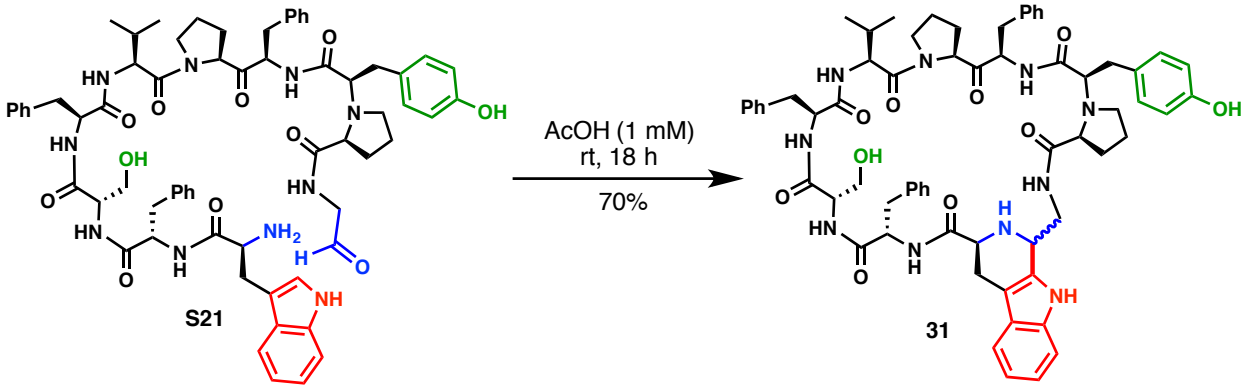
A) Peptide amino aldehyde **S20** after storage at 4 ^{o}C for 4 months (oligomeric mixture) (5 to 100% B over 25 min, λ = 230 nm). **B)** Crude Pictet-Spengler reaction to form diastereomeric macrocycles **29**. **C)** Crude oxidation following one-pot addition of PIDA. **D)** Purified β-carboline oxidation product **30** (Rt = 14.9 min, 5 to 100% B over 25 min, λ = 230 nm).



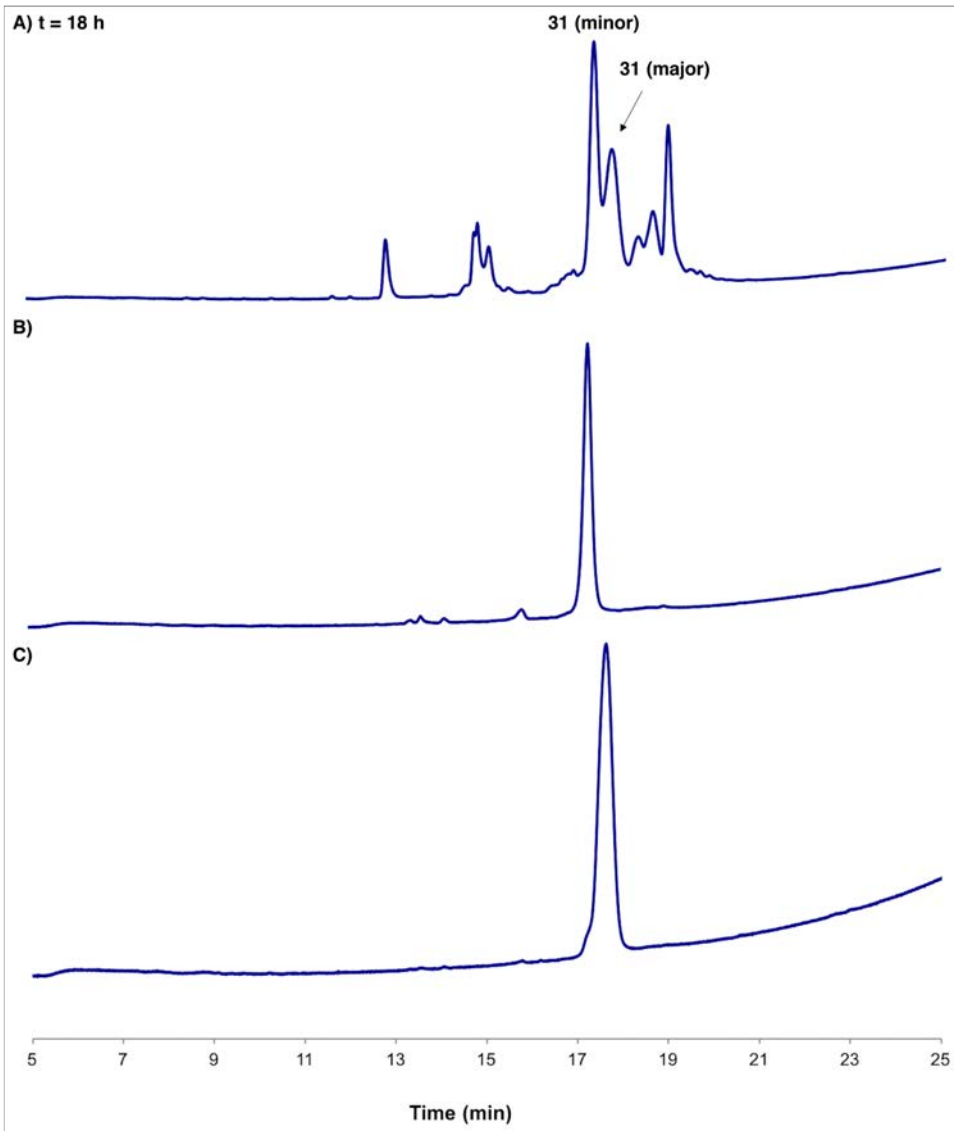
30

^{1}H, DMSO-d_6, 600 MHz

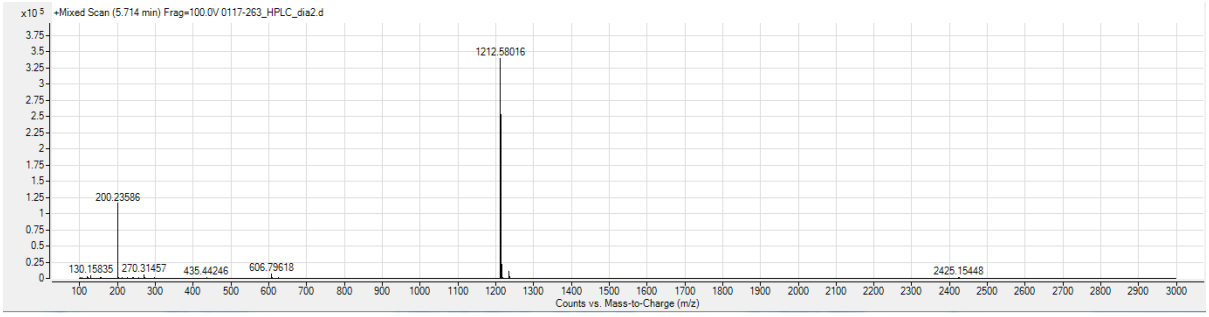
Peptide 31



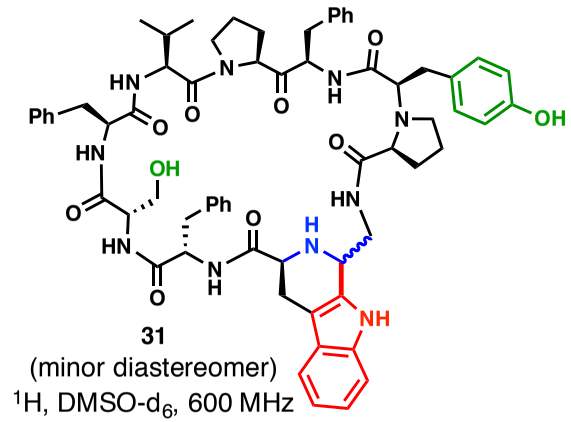
Peptide **31** was prepared from linear amino aldehyde **S21** using a Pictet-Spengler macrocyclization approach. Peptide **S21** (5.1 mg, 4.1 μmol) was dissolved in AcOH (1 mM) and stirred at rt. At t = 18 h, the reaction was purified by preparative reverse-phase HPLC (40% B for 10 min, 40% to 80% B over 30 min) to afford peptide macrocycle **31** (3.5 mg, 70% combined yield) as a partially separable mixture of diastereomers and as white solids following lyophilization.



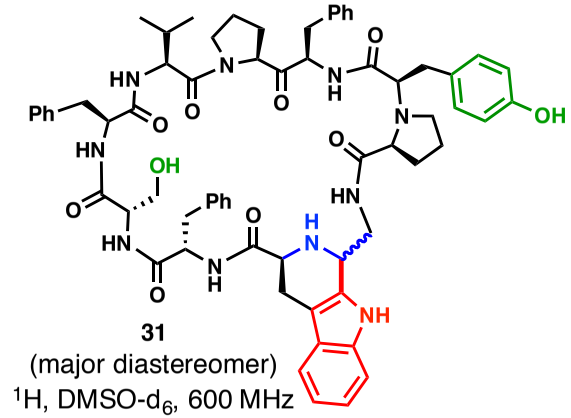
A) Crude Pictet-Spengler reaction to form diastereomeric macrocycles **31** (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified **31**, minor diastereomer (Rt = 17.3 min, 5 to 100% B over 25 min, λ = 210 nm). **C)** Purified **31**, major diastereomer (Rt = 17.6 min, 5 to 100% B over 25 min, λ = 210 nm). Note: some mixed fractions were also obtained upon HPLC purification.



LRMS (ESI-TOF): calc'd for $C_{26}H_{40}N_5O_5$ $[M+H]^+$ 1212.59; found 1212.59.



31
(minor diastereomer)
^{1}H, DMSO-d$_6$, 600 MHz

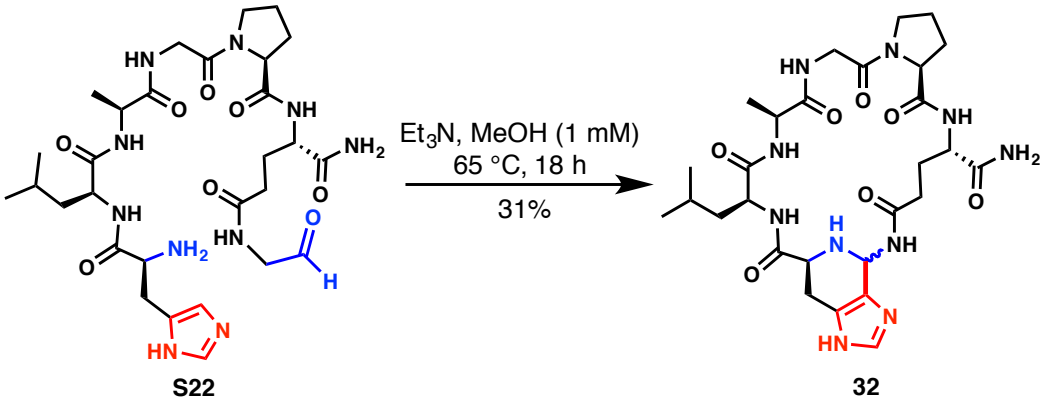


31

(major diastereomer)

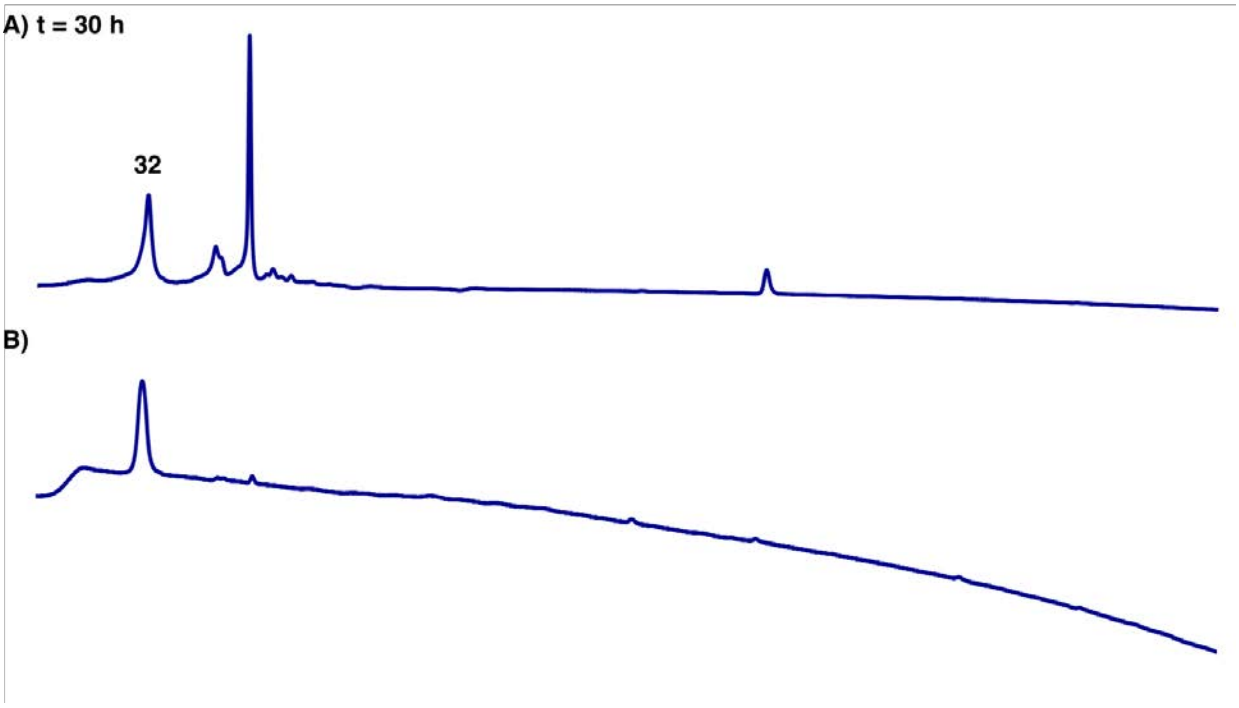
^{1}H, DMSO-d$_6$, 600 MHz

Peptide 32

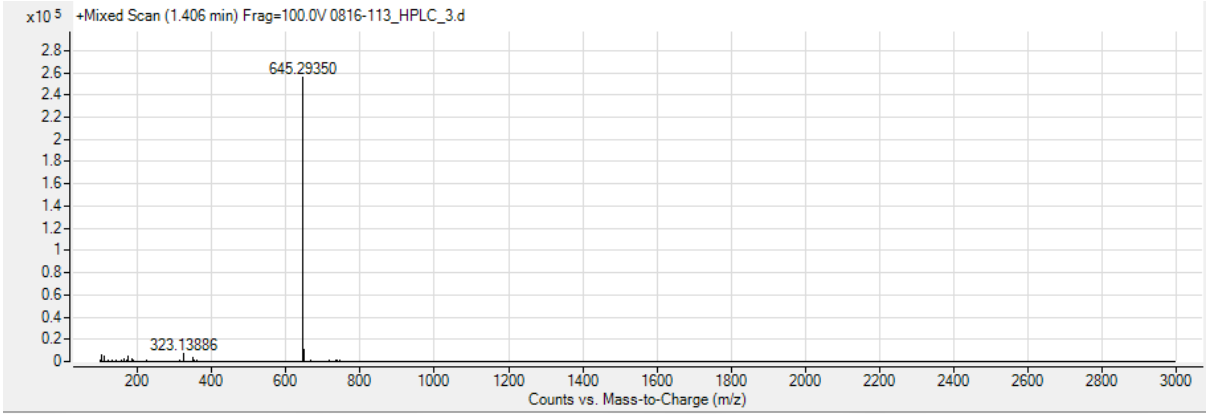


S22 32

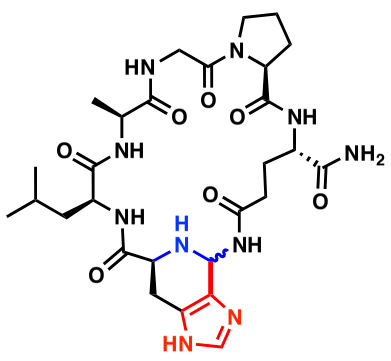
Peptide **32** was prepared according to a modified literature report.[10] To a solution of purified peptide aldehyde **S22** (6.8 mg, 10.3 μmol) in MeOH (1 mM) was added Et_3N (2 equiv.), and the resulting solution was heated to 65 °C and monitored by LC-MS analysis. At t = 30 h, the reaction was purified by preparative reverse-phase HPLC (5% B for 5 min, 5% to 40% B over 30 min) to afford peptide macrocycle **32** (2.1 mg, 31% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **32** from amino aldehyde **S22** following Pictet-Spengler macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **32** (Rt = 7.00 min, 5 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{29}H_{44}N_{10}O_7$ $[M+H]^+$ 645.35; found 645.29, $[M+2H]^{2+}$ 323.18; found 323.14.

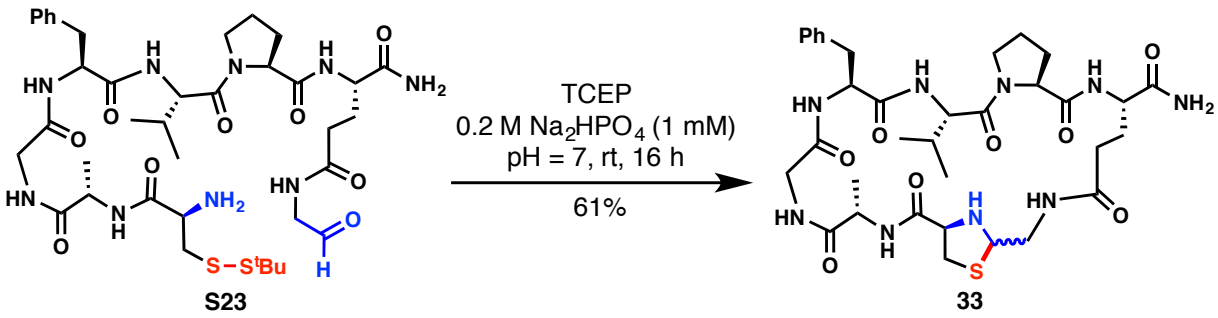


32

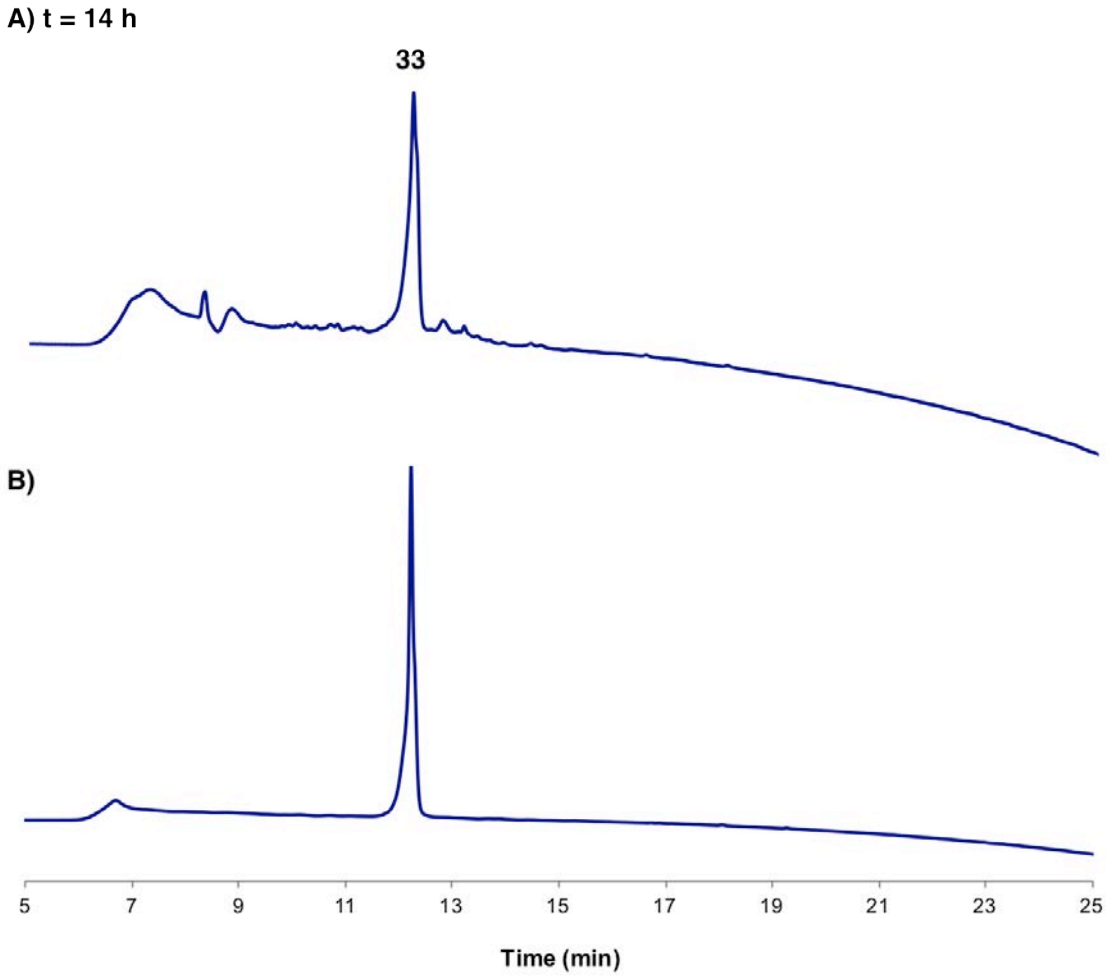
^{1}H, DMSO-d$_{6}$, 600 MHz

Characterization Data for Thiazolidine Formation

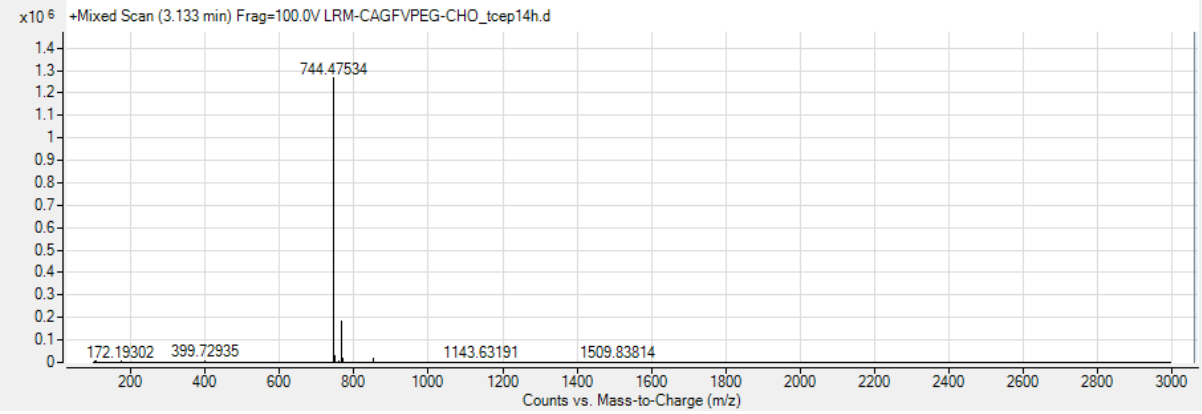
Peptide 33



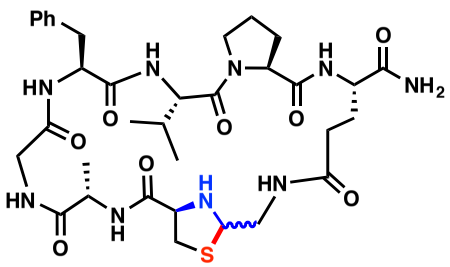
Peptide **33** was prepared according to general procedure 5 from S*t*Bu-disulfide protected linear amino aldehyde **S23** (4.5 mg, 5.3 μmol). At t = 16 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, 20% to 50% B over 25 min) to afford peptide macrocycle **33** (2.4 mg, 61% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **33** from amino aldehyde **S23** following thiazolidine macrocyclization (0 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **33** (Rt = 12.2 min, 0 to 100% B over 25 min, λ = 210 nm).

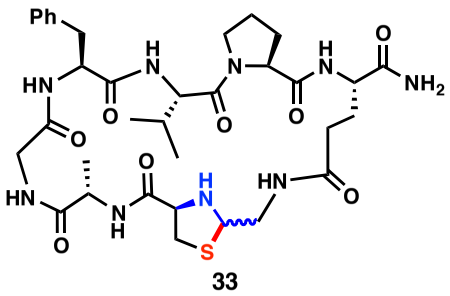


LRMS (ESI-TOF): calc'd for $C_{34}H_{50}N_9O_8S$ $[M+H]^+$ 744.35; found 744.48.



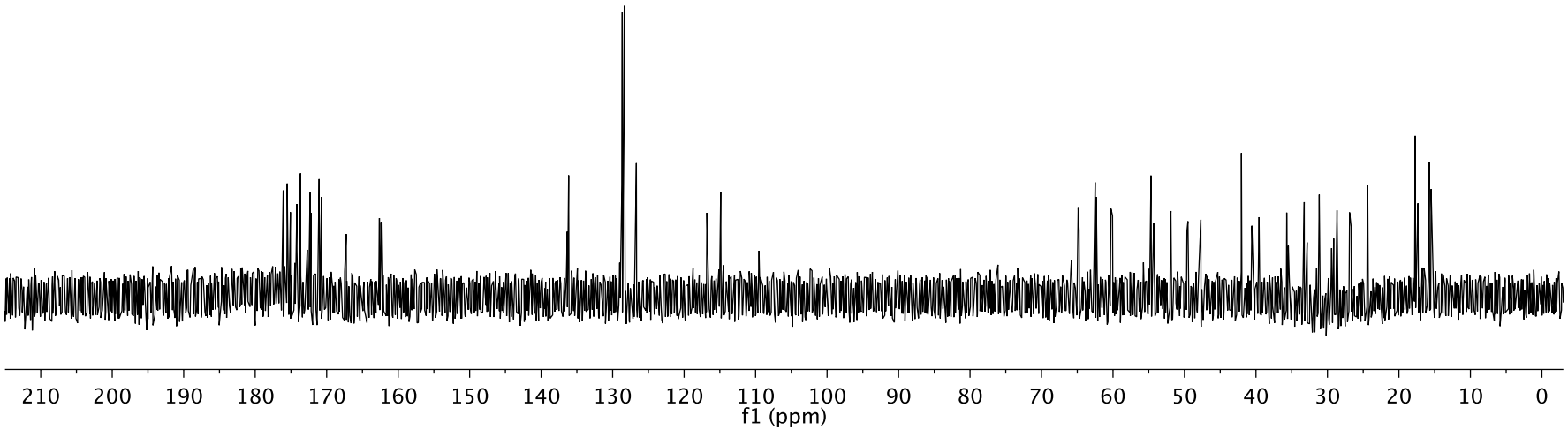
33

^{1}H, D_2O, 600 MHz



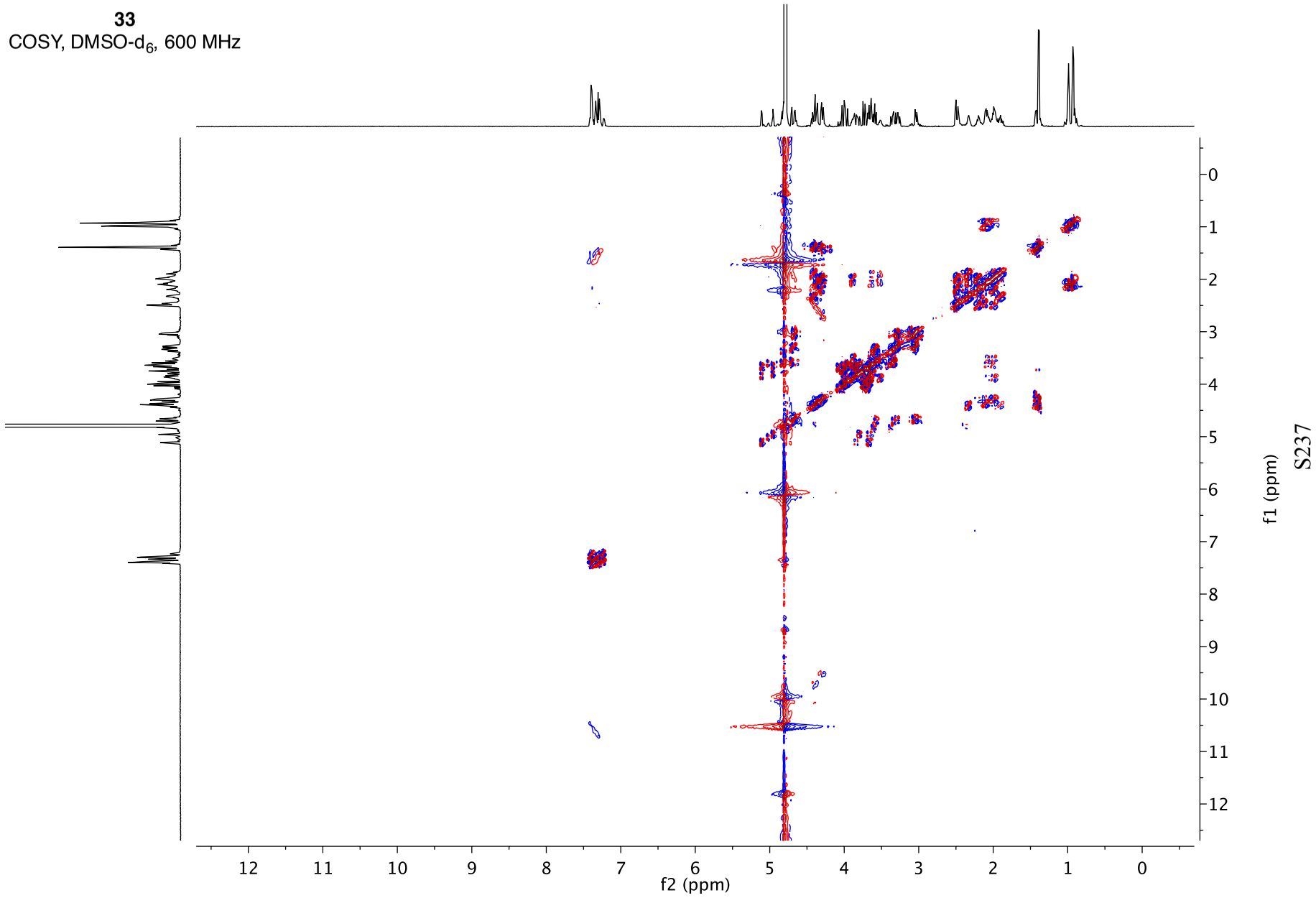
33

^{13}C, D_2O, 151 MHz

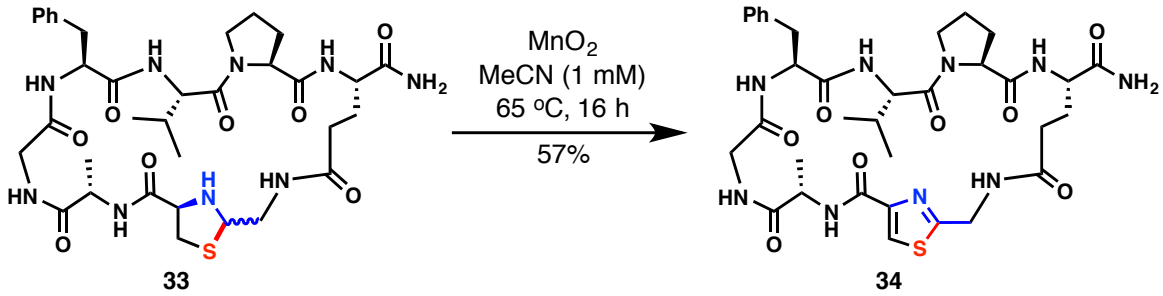


33

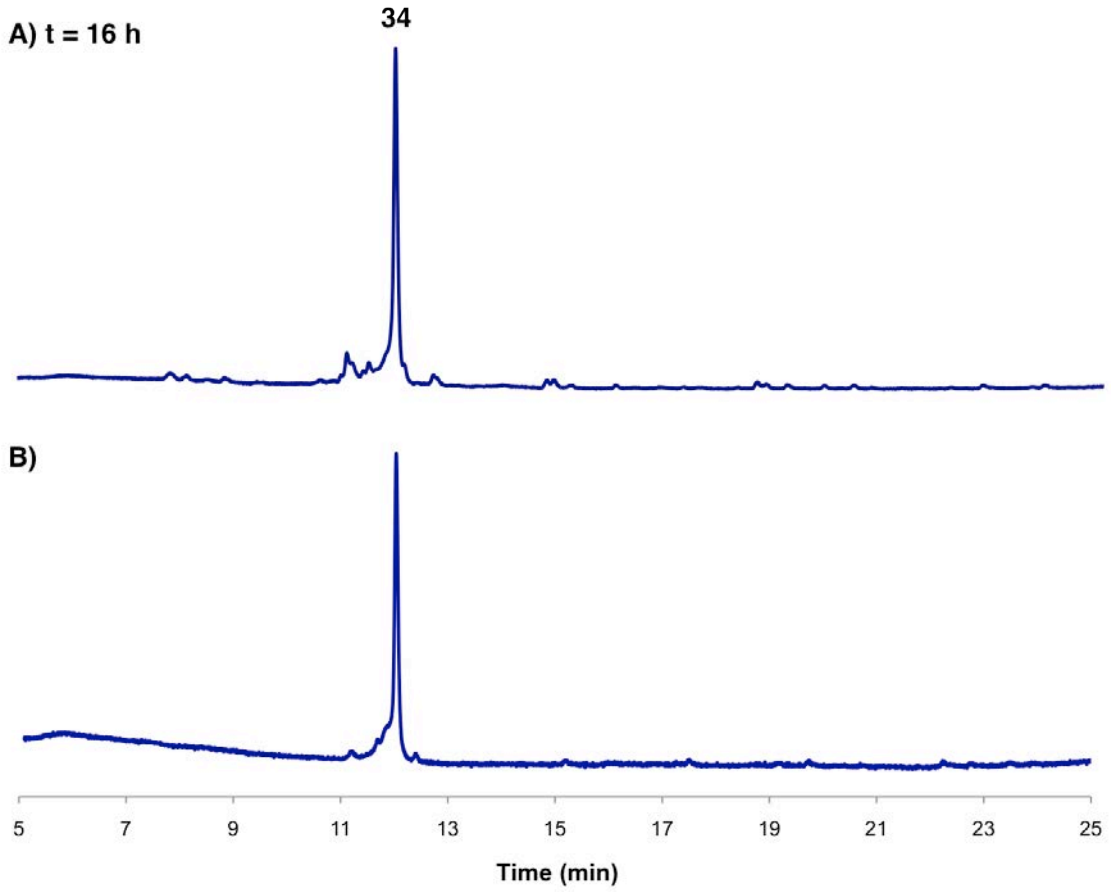
COSY, DMSO-d_6, 600 MHz



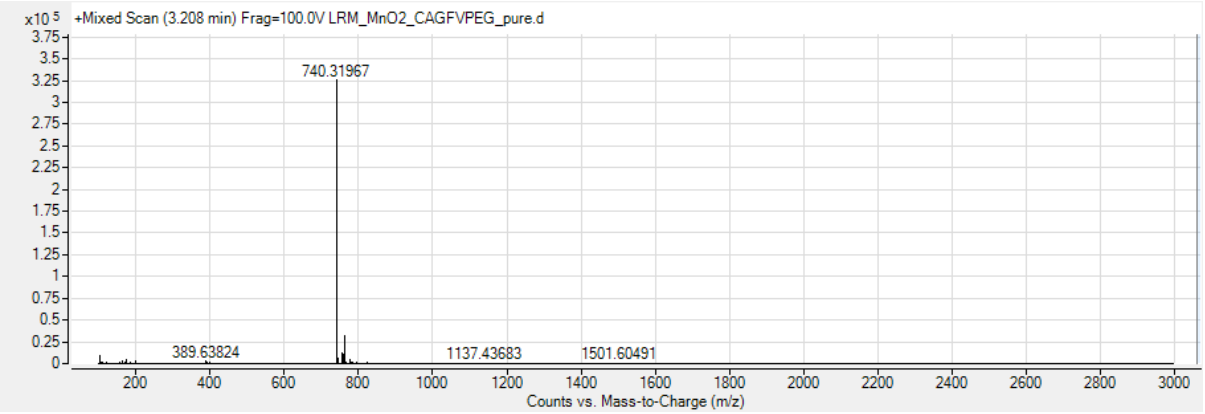
Peptide 34



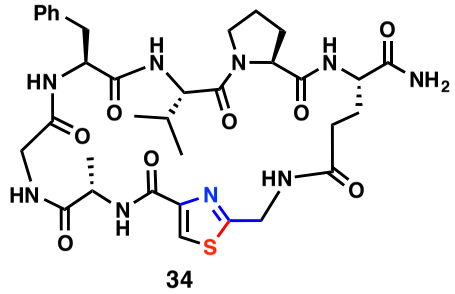
Peptide **34** was prepared from diastereomeric thiazolidines **33** upon oxidation with MnO_2. Peptide **33** (2.0 mg, 2.7 μmol) was dissolved in MeCN (1 mM concentration) and treated with 50 eq. of MnO_2. The reaction was heated at 65 ^{o}C and monitored by LC-MS. At t = 16 h, the reaction was filtered and purified by preparative reverse-phase HPLC (20% B for 5 min, 20% to 50% B over 30 min) to afford peptide thiazole **34** (1.1 mg, 57% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **34** from thiazolidine **33** following oxidation (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **34** (Rt = 12.0 min, 5 to 100% B over 25 min, λ = 230 nm).

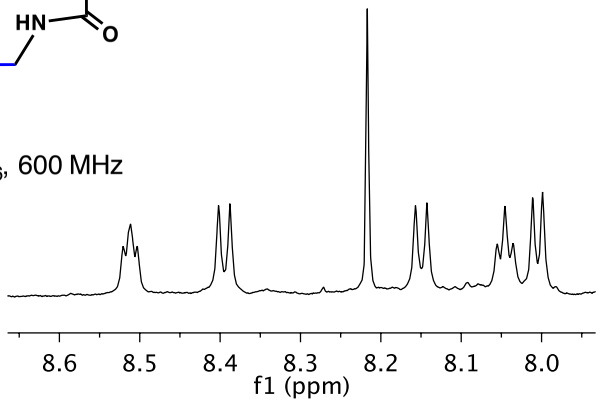


LRMS (ESI-TOF): calc'd for $C_{34}H_{46}N_9O_8S$ $[M+H]^+$ 740.32; found 740.32.



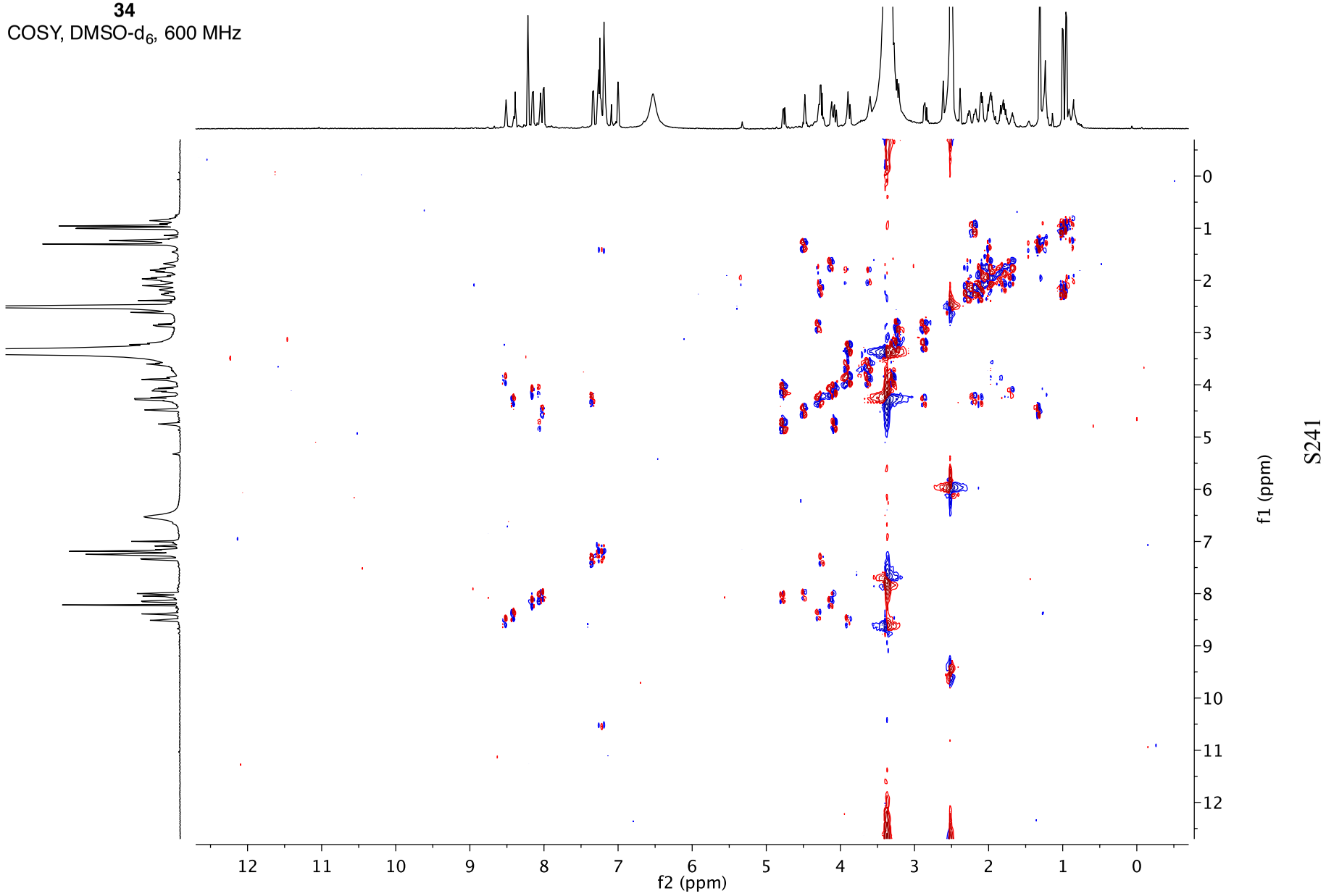
34

^{1}H, DMSO-d$_6$, 600 MHz

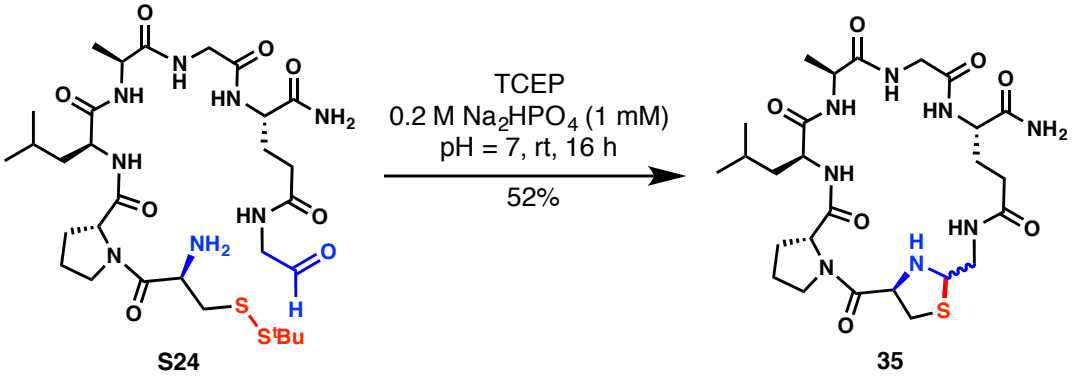


34

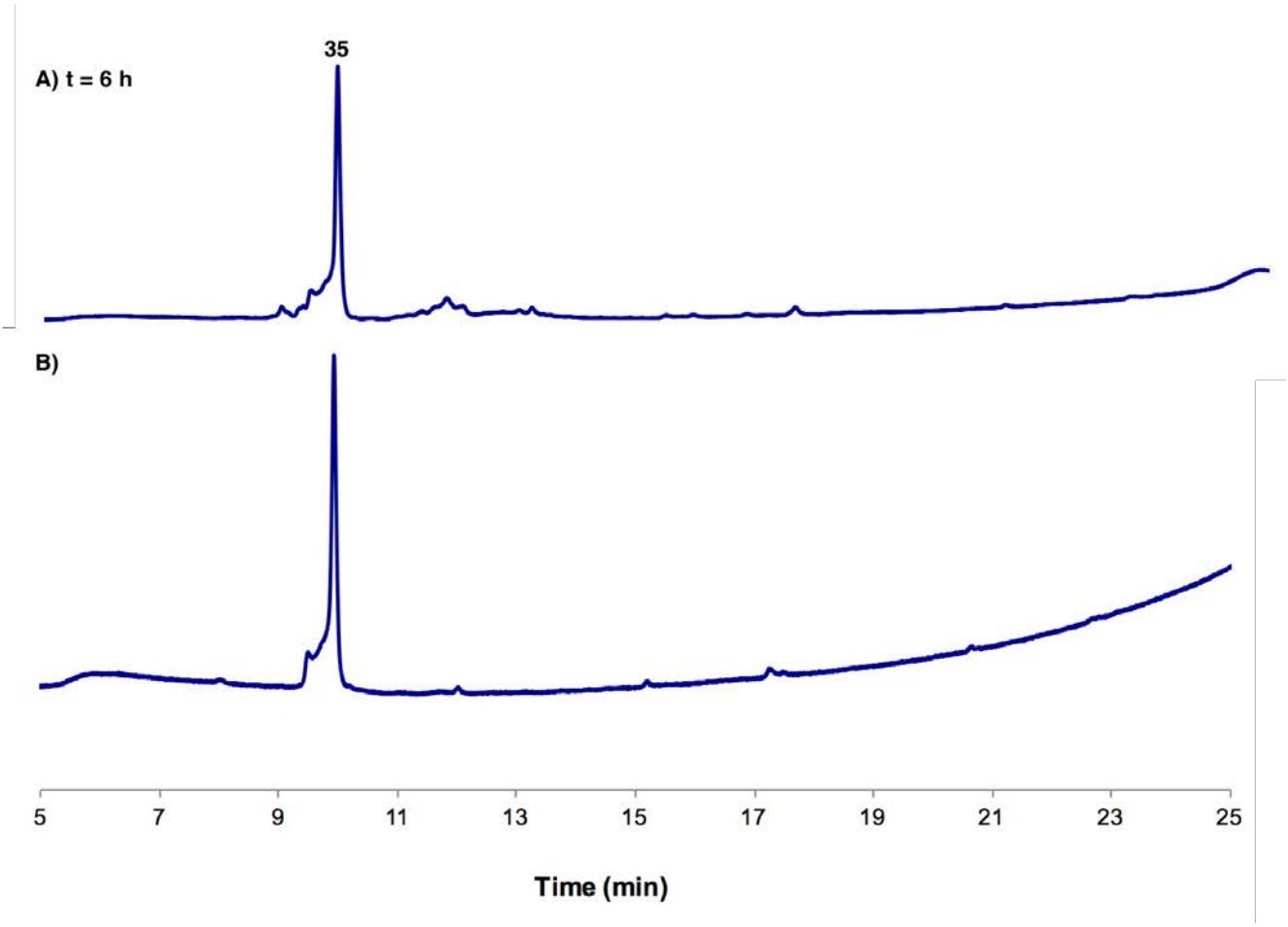
COSY, DMSO-d_6, 600 MHz



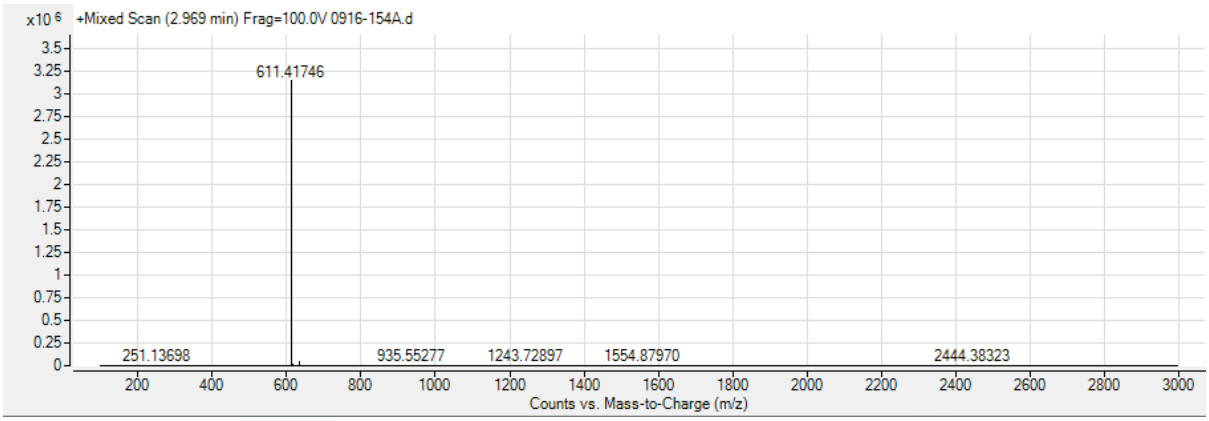
Peptide 35



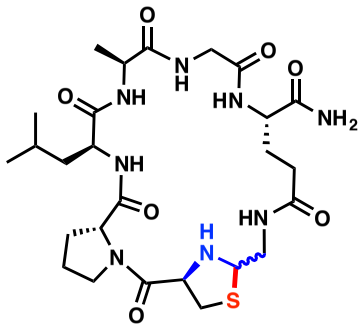
Peptide **35** was prepared according to general procedure 5 from S*t*Bu-disulfide protected linear amino aldehyde **S24** (10.0 mg, 14 μmol). At t = 6 h, the reaction was purified by preparative reverse-phase HPLC (10% B for 10 min, 10% to 45% B over 35 min) to afford peptide macrocycle **35** (4.4 mg, 52% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **35** from amino aldehyde **S24** following thiazolidine formation (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **35** (Rt = 12.64 min, 5 to 100% B over 25 min, λ = 230 nm).

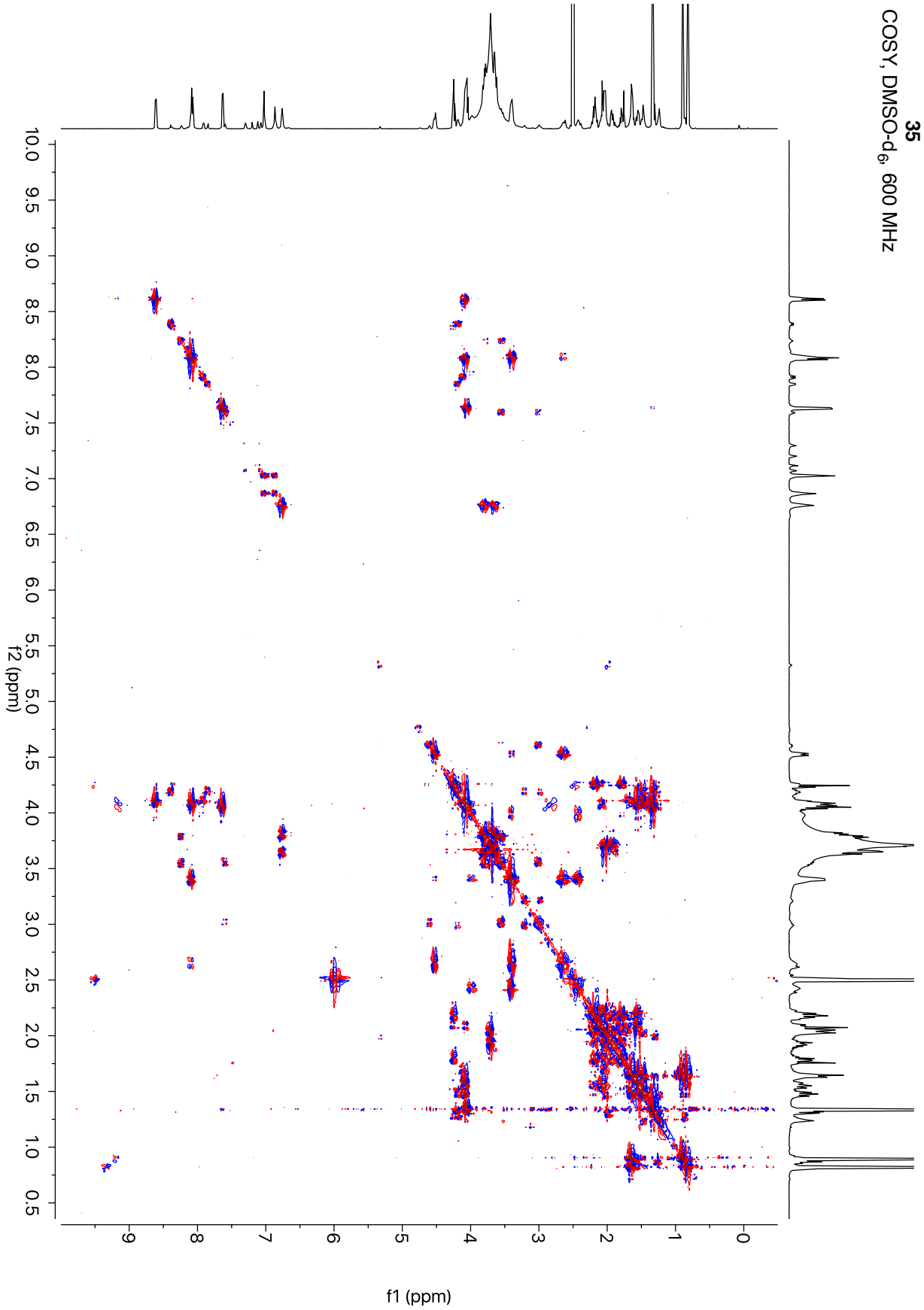


LRMS (ESI-TOF): calc'd for $C_{26}H_{42}N_8O_7S$ $[M+H]^+$ 611.30; found 611.42.



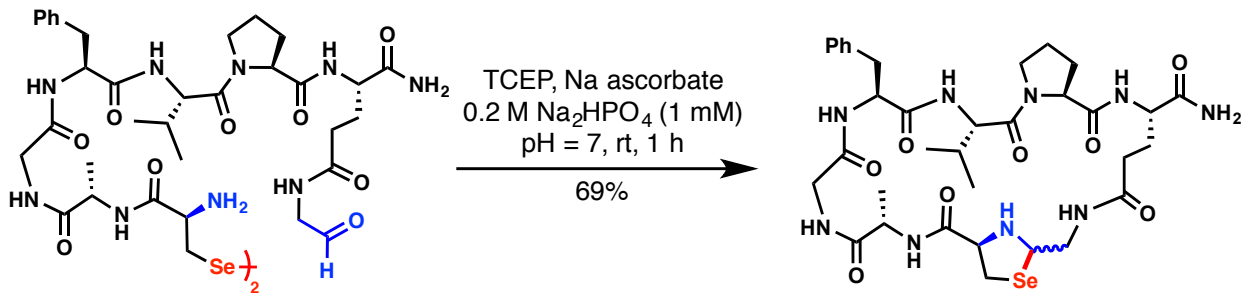
35

^{1}H, DMSO-d$_{6}$, 600 MHz


35
COSY, DMSO-d6, 600 MHz
f2 (ppm)
f1 (ppm)

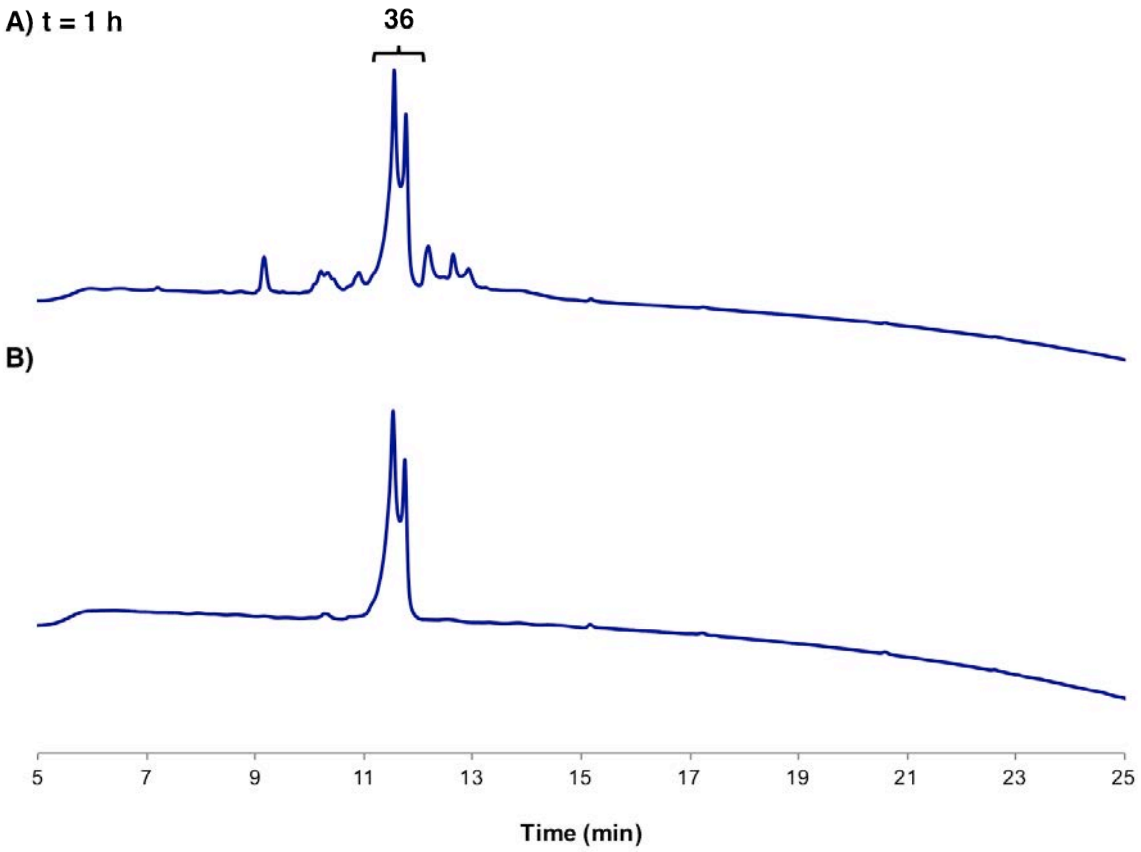
Characterization Data for Selenazolidine Formation

Peptide 36

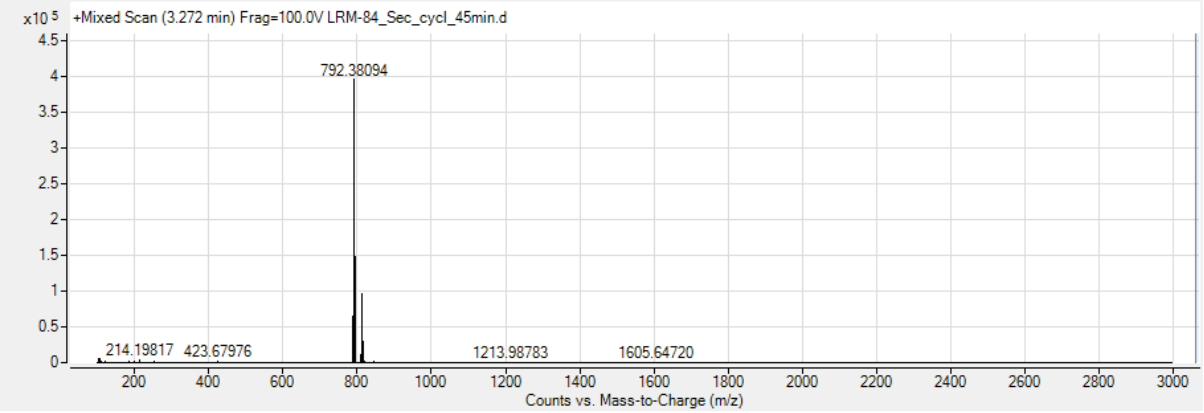


S25 **36**

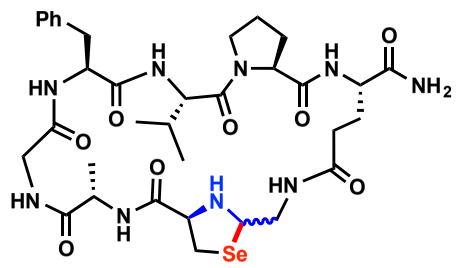
Peptide **36** was prepared according to general procedure 6 from linear amino aldehyde selenocystine dimer **S25** (3.7 mg, 2.3 μmol). At t = 1 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, 20% to 60% B over 25 min) to afford peptide macrocycle **36** (2.5 mg, 69% yield) as a mixture of diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **36** from amino aldehyde **S25** following selenazolidine macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **36** (Rt = 11.5, 11.7 min, 0 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{34}H_{50}N_9O_8Se$ $[M+H]^+$ 792.29; found 792.38.

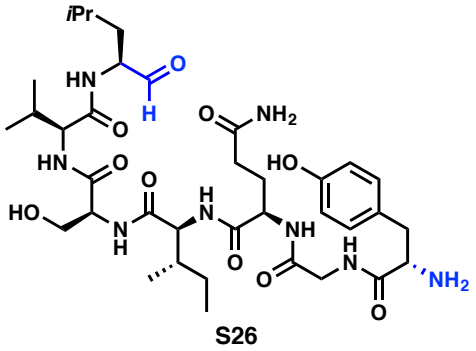


36

^{1}H, D_2O, 600 MHz

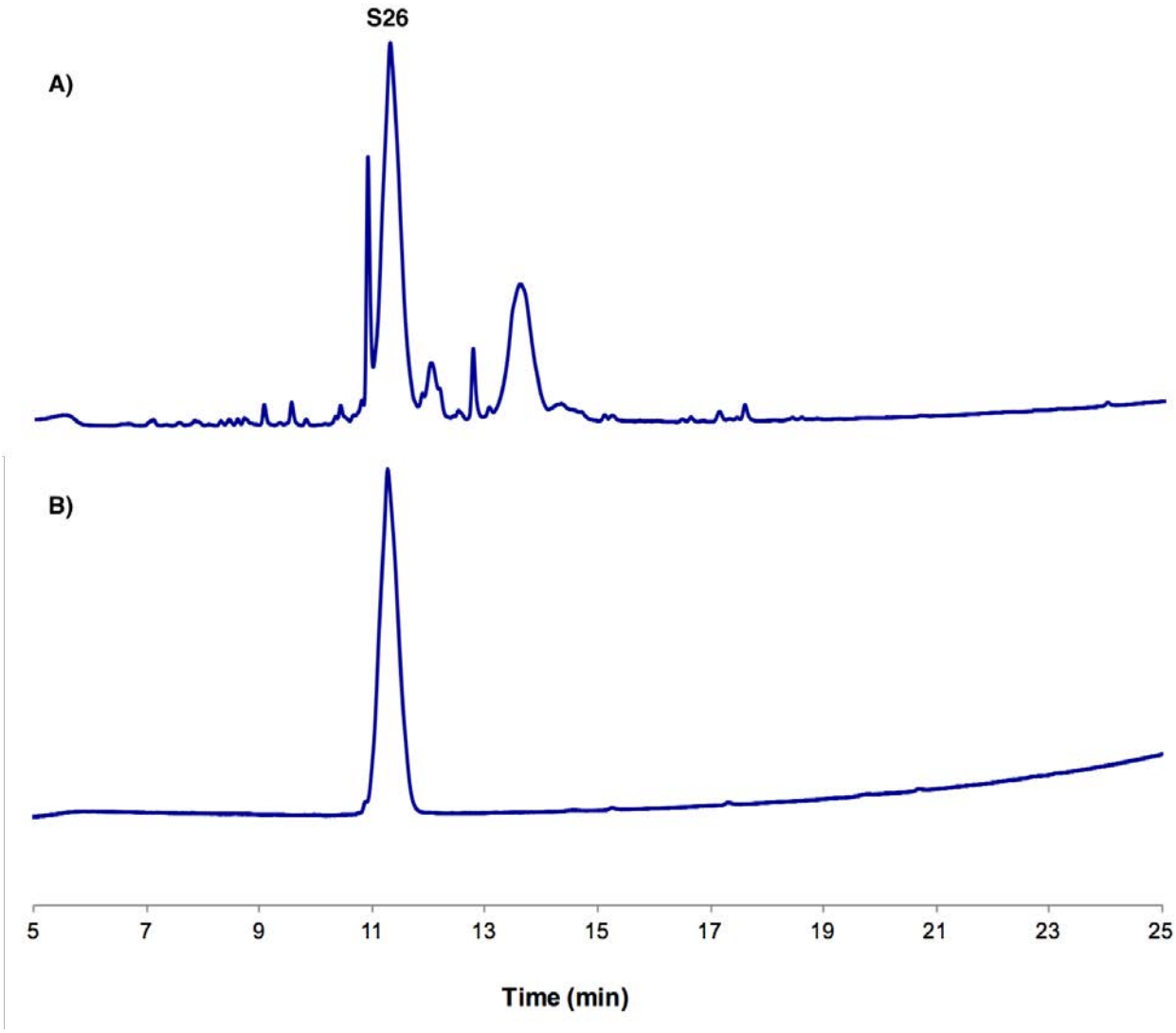
Characterization Data for Scytonemide A Analogues

Peptide S26

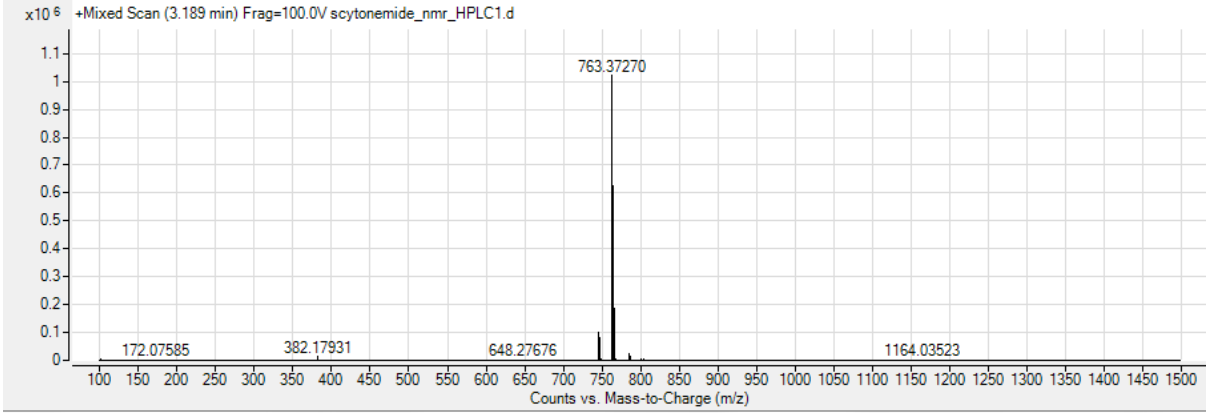


S26

Amino aldehyde **S26** was prepared on a 40 umol scale according to general procedure 2a. Fmoc-Ile-CHO was loaded onto Rink TG resin and the peptide elongated under standard Fmoc-SPPS protocols. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (15% B for 5 min, then 15% B to 55% B over 25 min) to afford peptide **S26** (22.5 mg, 73% yield based on the original resin loading) as a white solid following lyophilization.

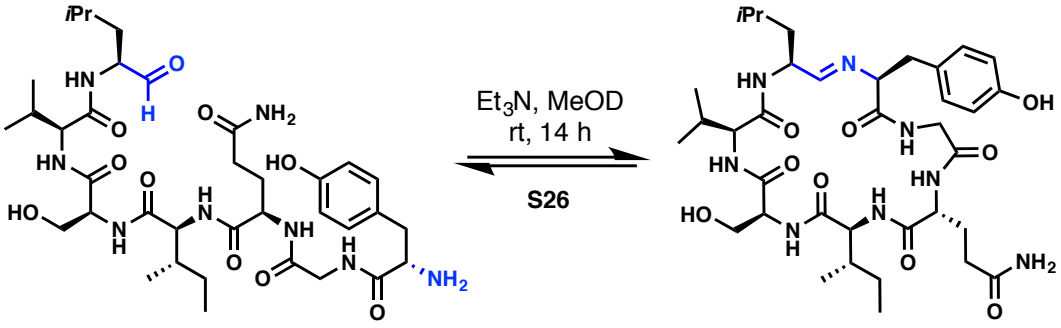


Purified peptide **S26** (Rt = 11.27 min, 5 to 100% B over 25 min, λ = 230 nm).

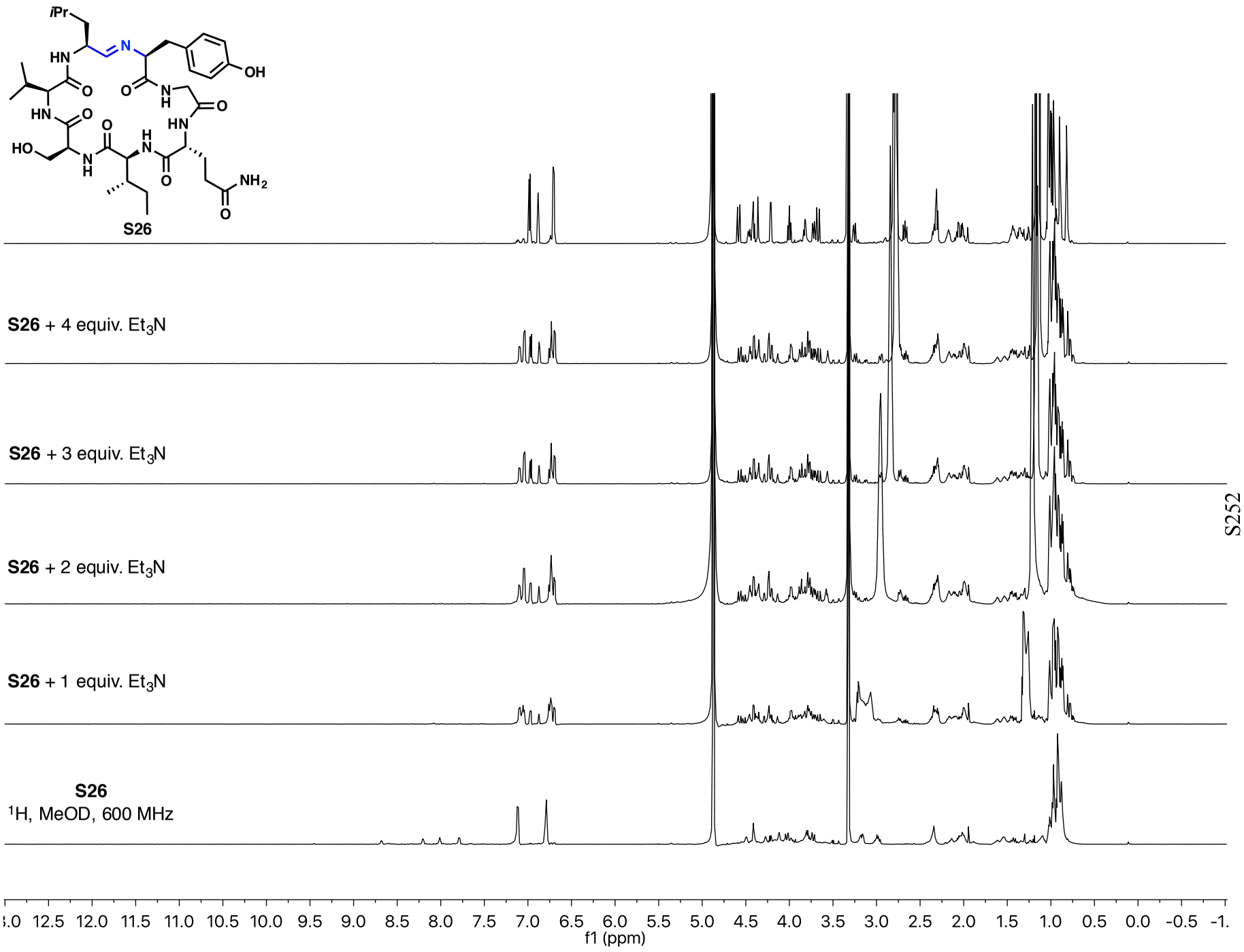


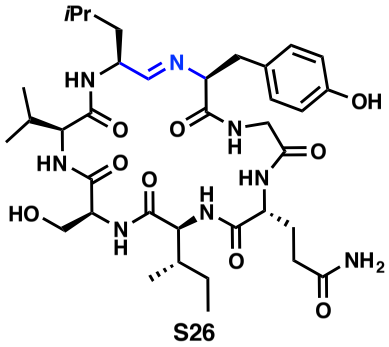
LRMS (ESI-TOF): calc'd for $C_{33}H_{44}N_5O_{10}S$ $[M+H]^+$ 763.43; found 763.37.

Equilibration Studies



Aldehyde-imine equilibration of peptide **S26** (5.0 mg, 6.5 μmol) was induced by first dissolving the peptide in MeOD (1 mM). Et_3N (4.0 equiv. total) was then added, equivalent-wise (5 min between each addition), to the resulting solution. After the final addition, the solution was allowed to stir at room temperature for 14 hours. The compound was characterized without further purification.

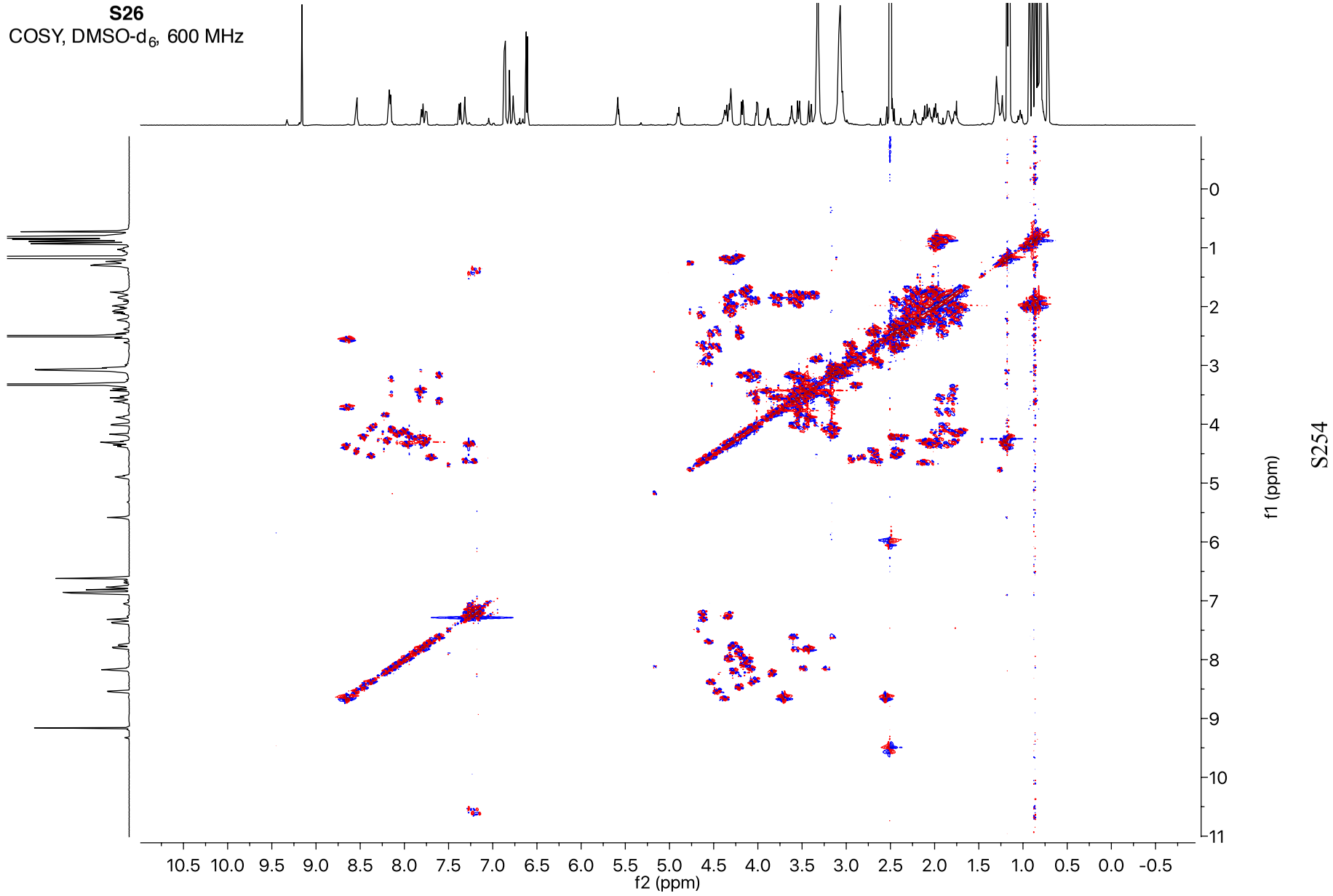

S26
S26 + 4 equiv. Et3N
S26 + 3 equiv. Et3N
S26 + 2 equiv. Et3N
S26 + 1 equiv. Et3N
S26
1H, MeOD, 600 MHz
f1 (ppm)

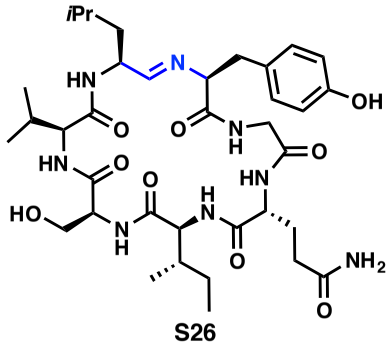


S26

^{1}H, DMSO-d$_6$, 600 MHz

S26
COSY, DMSO-d_6, 600 MHz



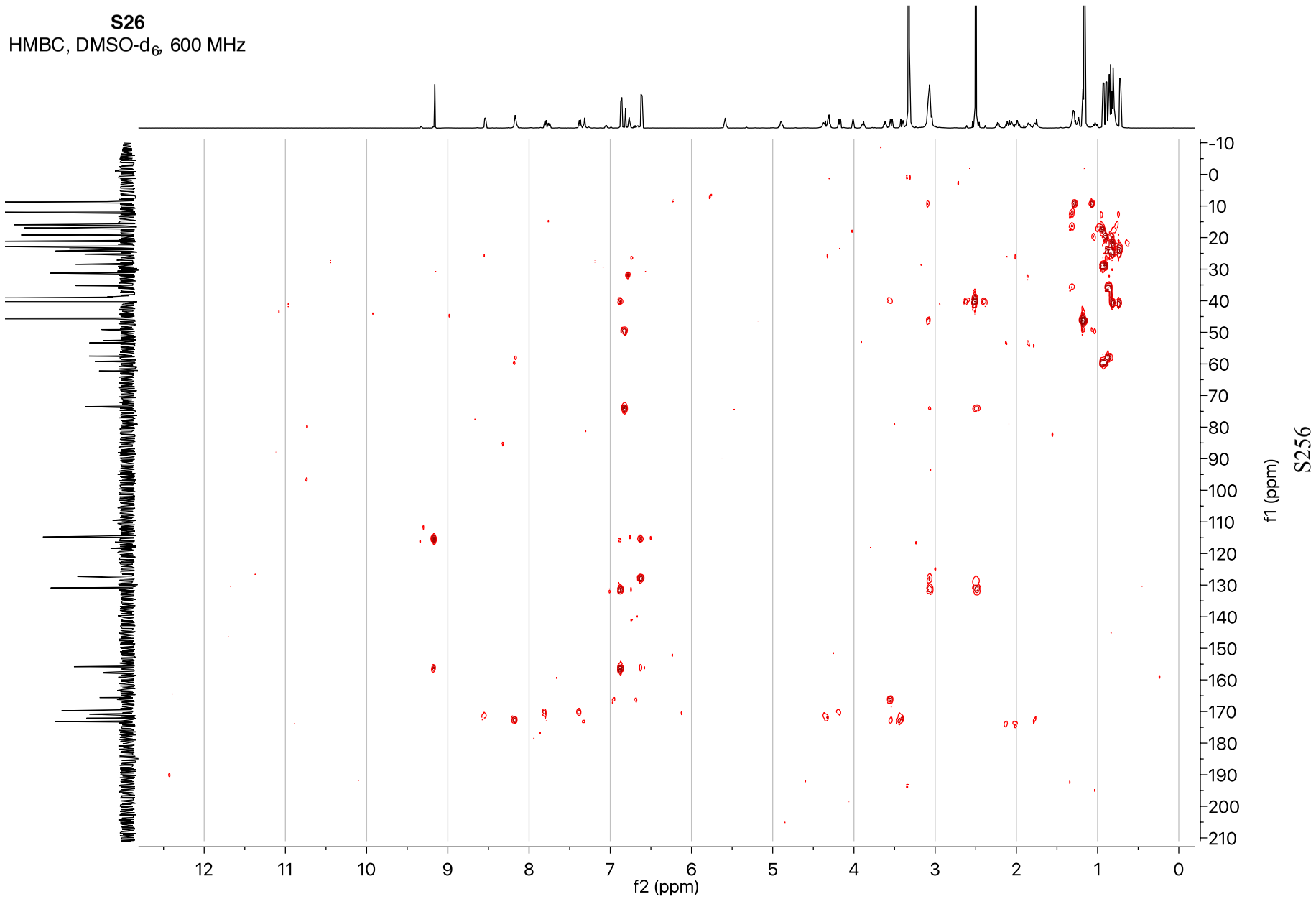


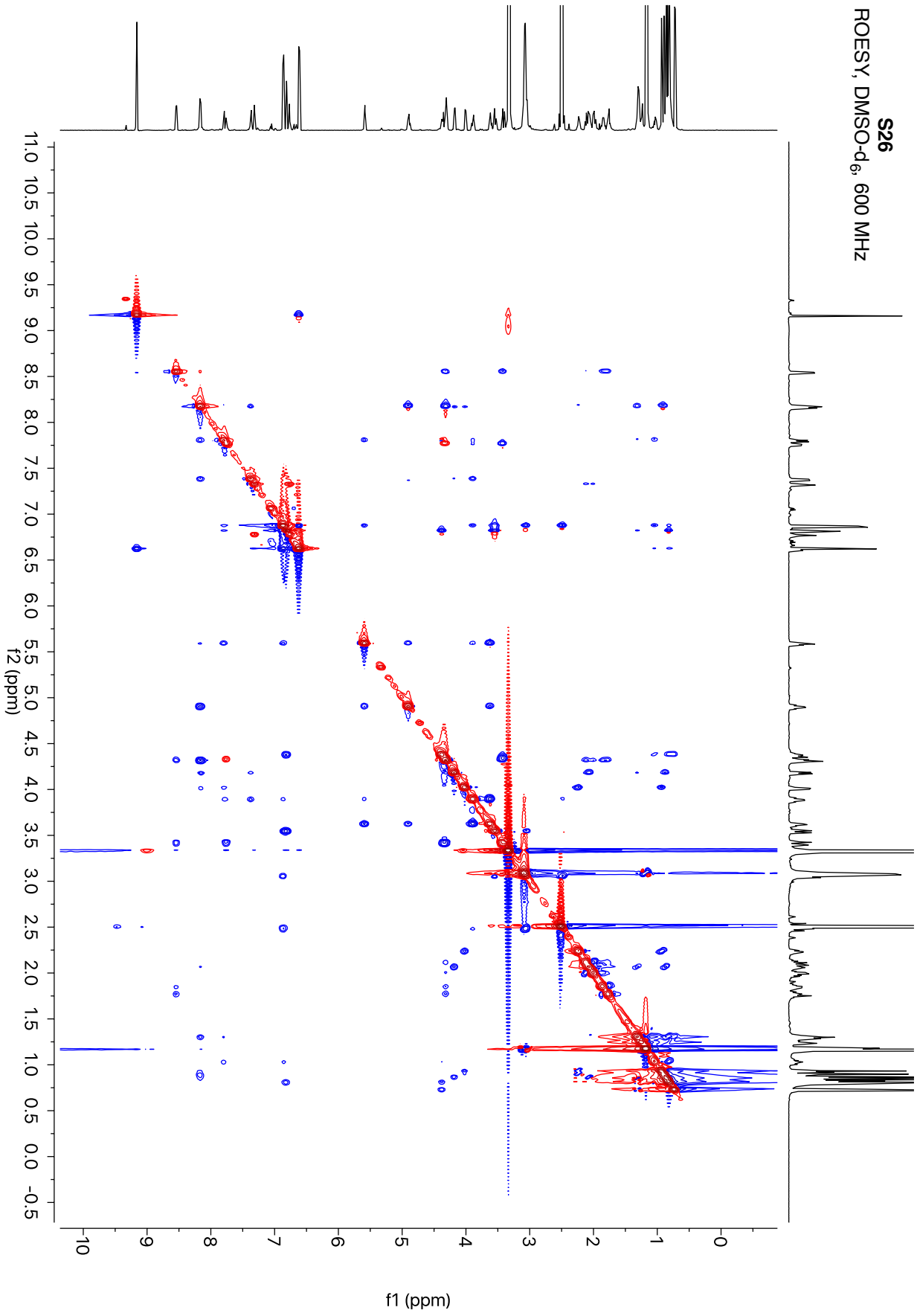
S26

^{13}C, DMSO-d_6, 151 MHz

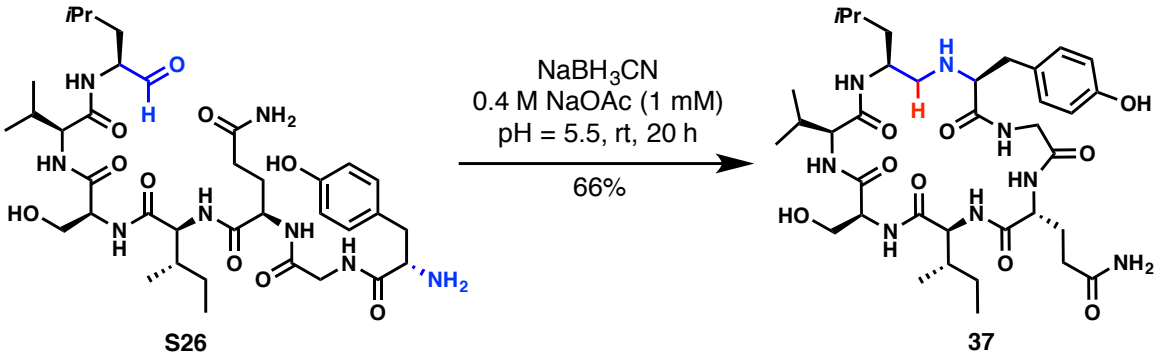
S26

HMBC, DMSO-d_6, 600 MHz

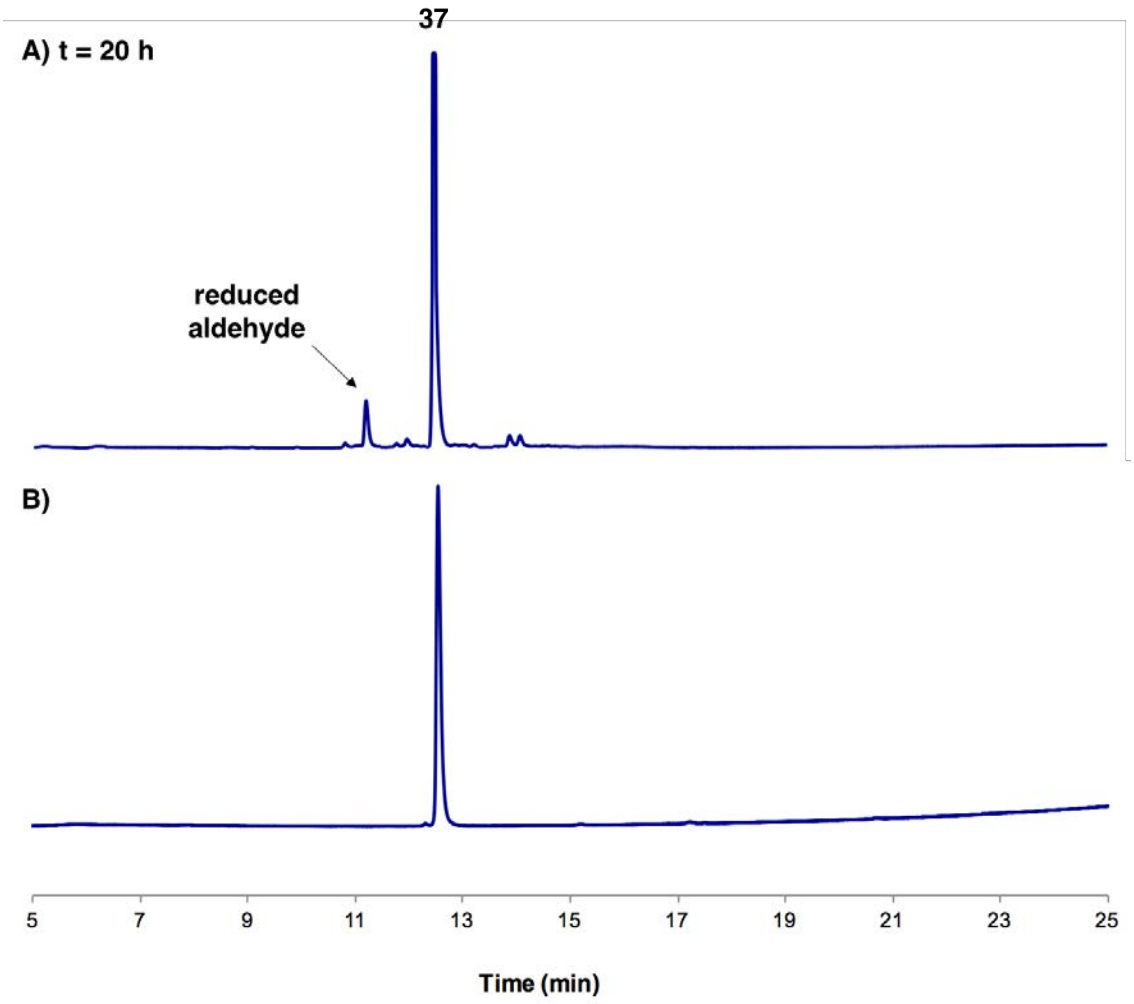



S26
ROESY, DMSO-d6, 600 MHz
f2 (ppm)
f1 (ppm)

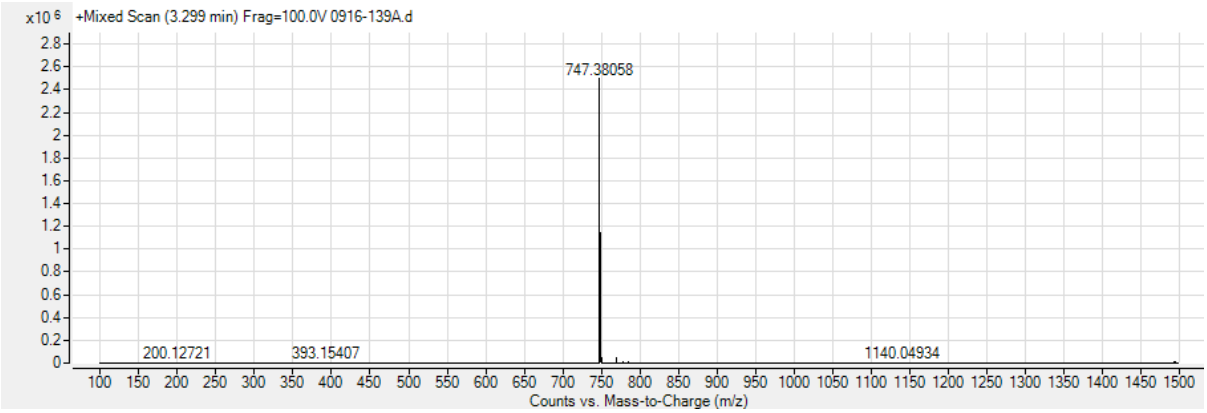
Peptide 37



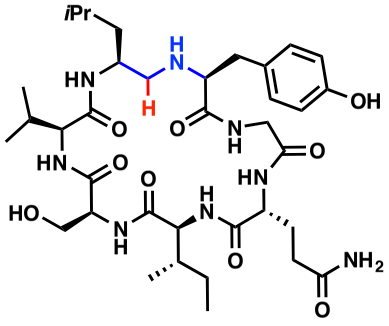
Peptide **37** was prepared according to general procedure 4 from linear amino aldehyde **S26** (5.7 mg, 7.5 μmol). At t = 20 h, the reaction was purified by preparative reverse-phase HPLC (20% B for 5 min, 20% to 60% B over 30 min) to afford peptide macrocycle **37** (3.7 mg, 66% yield) as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **37** from amino aldehyde **S26** following reductive amination (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **37** (Rt = 12.54 min, 5 to 100% B over 25 min, λ = 230 nm).

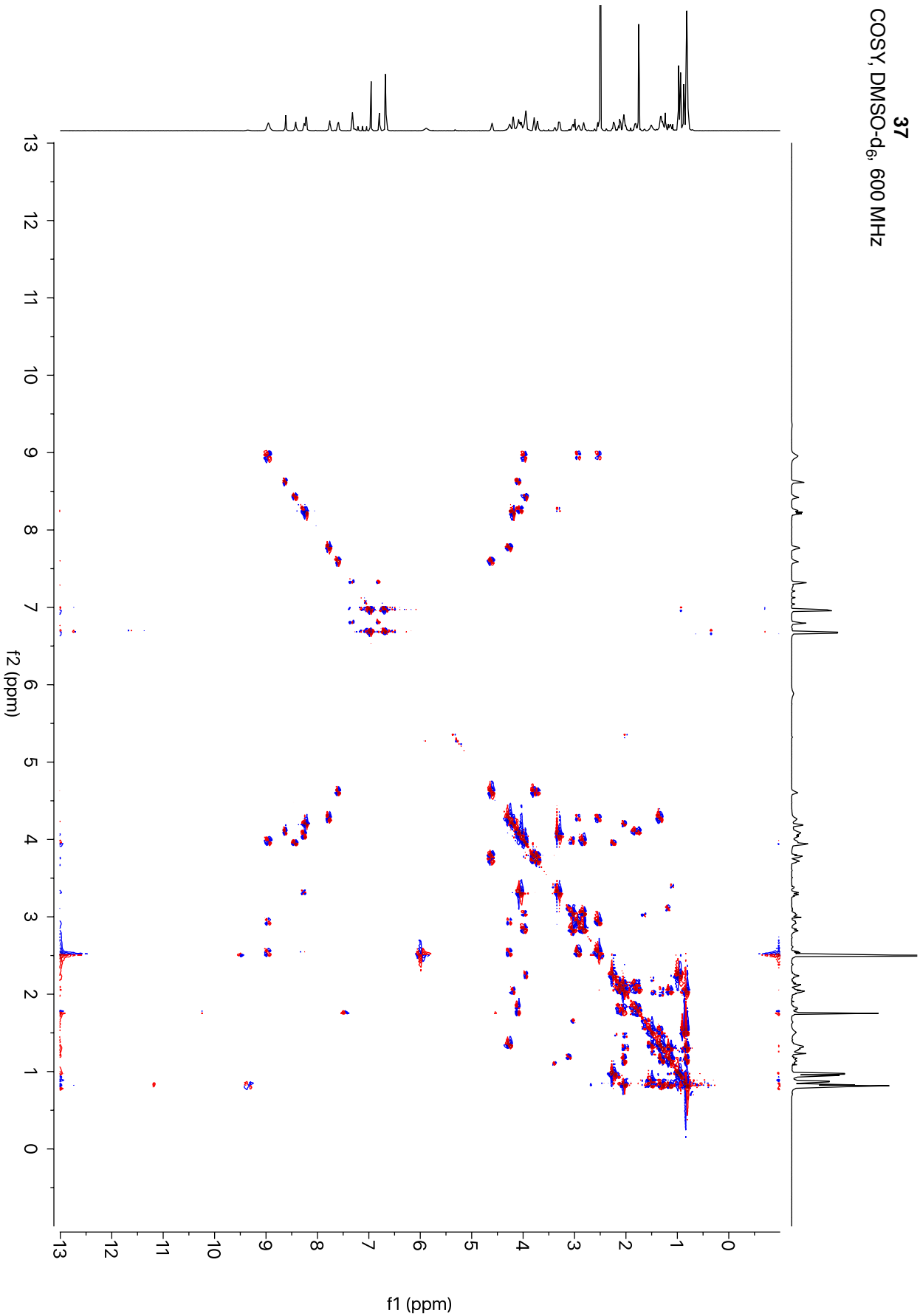


LRMS (ESI-TOF): calc'd for $C_{36}H_{58}N_8O_9$ $[M+H]^+$ 747.44; found 747.38.



37

^{1}H, DMSO-d_6, 600 MHz

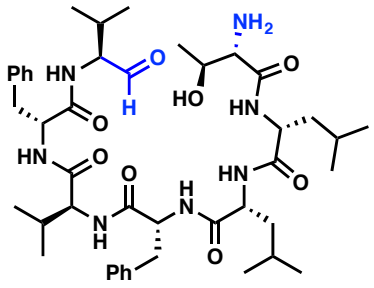

37
COSY, DMSO-d6, 600 MHz
f2 (ppm)
f1 (ppm)

Scytonemide Analogue Notes:

- Attempts to purify the natural product proved fruitless, as the cyclic imine reverted to the linear form on removal of the Et_3N.
- Qualitative analysis suggests that under most conditions explored herein, the equilibrium favors the open-chain peptide aldehyde; as such, competitive nucleophilic addition to the aldehyde dominated during attempts at a Strecker macrocyclization, resulting in almost exclusive formation of the corresponding linear cyanohydrin.

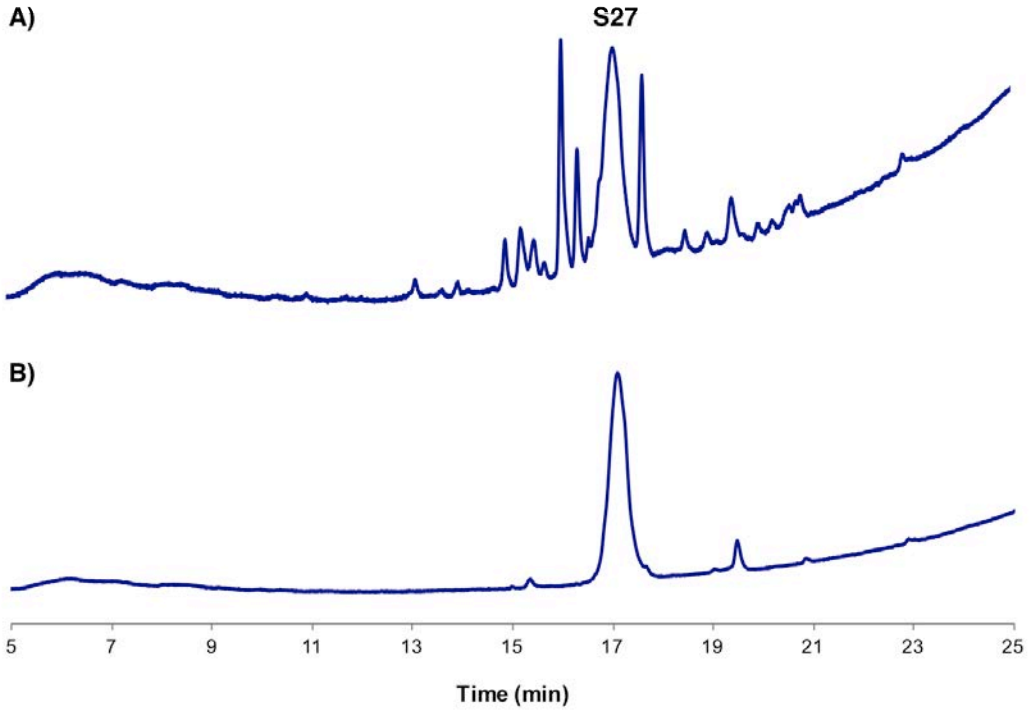
Characterization Data for Koranimine Analogues

Peptide S27



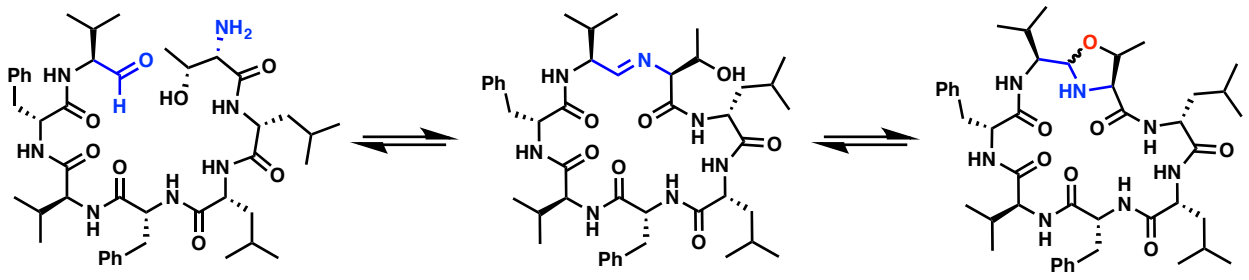
S27

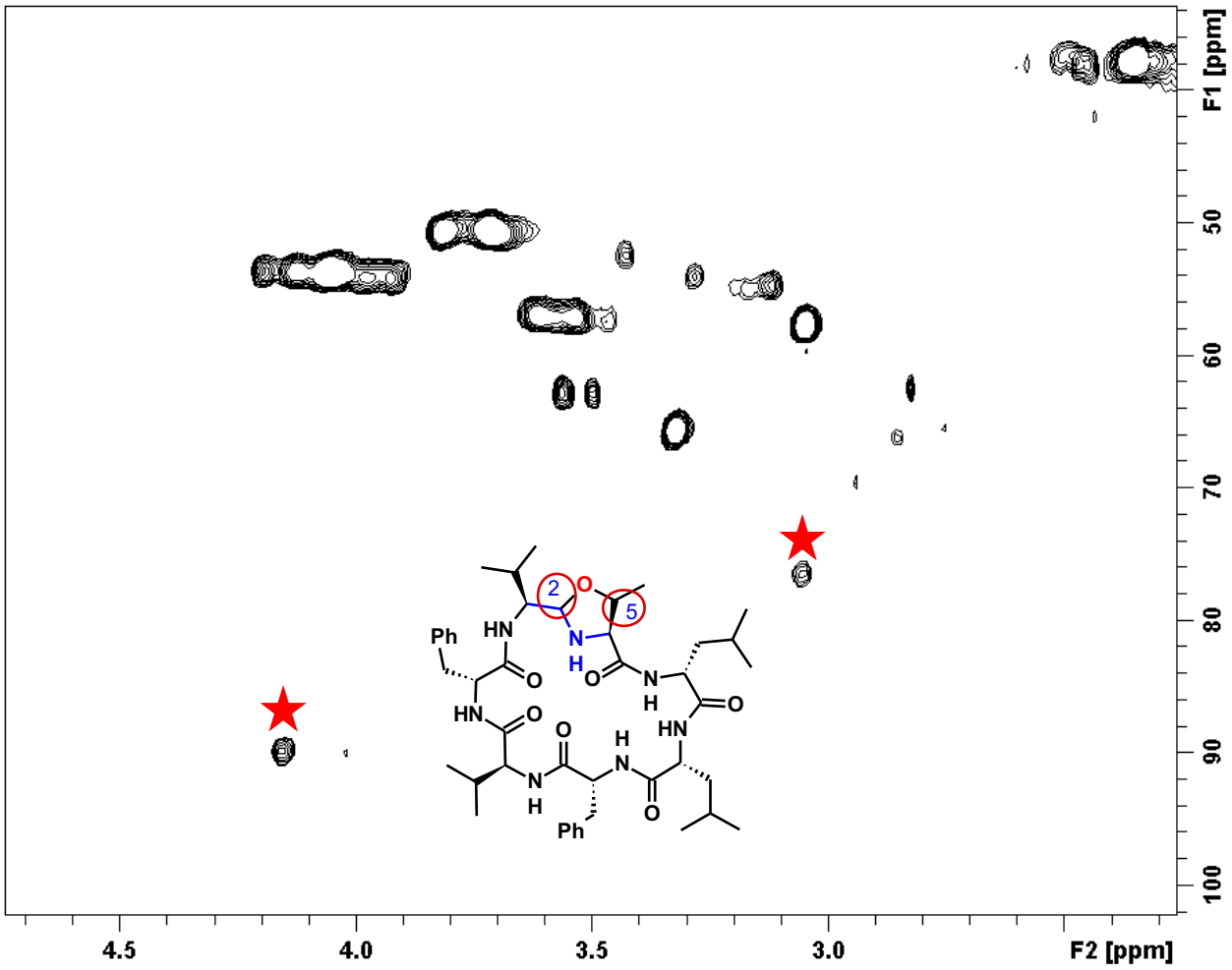
Amino aldehyde **S27** was prepared on a 50 μmol scale according to general procedure 2a. Fmoc-Val-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (40% B for 5 min, then 40% B to 80% B over 30 min) to afford peptide **S27** (14.4 mg, 35% yield based on the original resin loading) as a white solid following lyophilization.



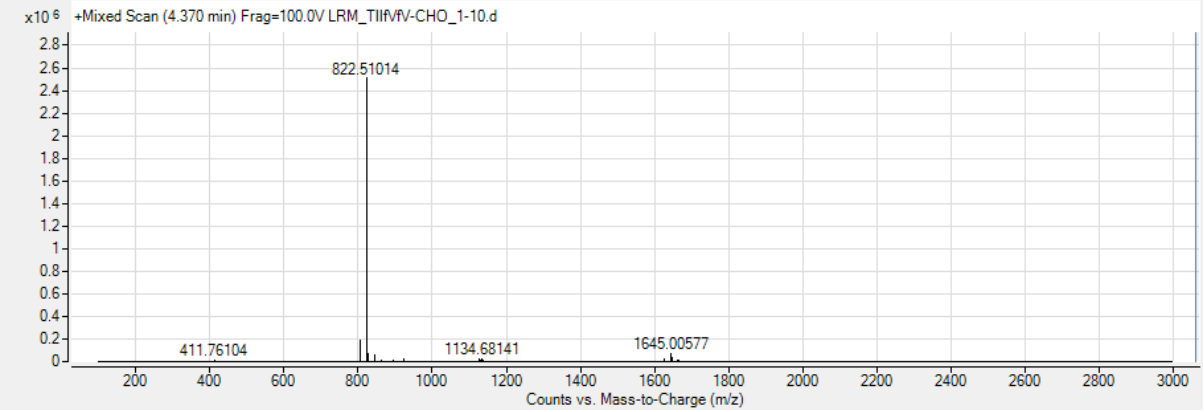
A) Crude analytical HPLC trace of amino aldehyde **S27** following Fmoc-SPPS and acidic cleavage from the resin (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **S27** (Rt = 16.9 min, 5 to 100% B over 25 min, λ = 230 nm).

A note on the structure of koranimine: LC-MS analysis of purified **S27** indicated a mixture of aldehyde ($[M+H]^+$ = 822.51) and spontaneous formation of a condensation product (imine/oxazolidine - see details below). NMR analysis in DMSO and upon treatment with Et_3N in MeOD (conditions which promoted the macrocyclization of Scytonemide A) revealed a complex product mixture in which the characteristic imine peak ($\delta \approx 6.8$ ppm) was not observed. This is consistent with the isolation paper, in which characteristic imine signals were not observed in the ^1H or ^{13}C NMR spectra of isolated koranimine. A lack of sufficient material from the isolation also precluded detailed NMR analysis of the peptide.[11] We hypothesize that koranimine may also be present as an equilibrium mixture of diastereomeric cyclic oxazolidines formed intramolecularly upon attack of the N-terminal Thr β-alcohol onto the macrocyclic imine. This ring-chain tautomerism has been well-characterized for oxazolidines.[12–14] In the context of macrocyclic peptides, such an intramolecular cyclization event is reminiscent of the spontaneous thiazolidine formation in the natural product lugdunin.[15] ^1H–^{13}C HSQC NMR data (see below, in 99.9% DMSO-d_6) provides preliminary evidence of the formation of cyclic oxazolidine.

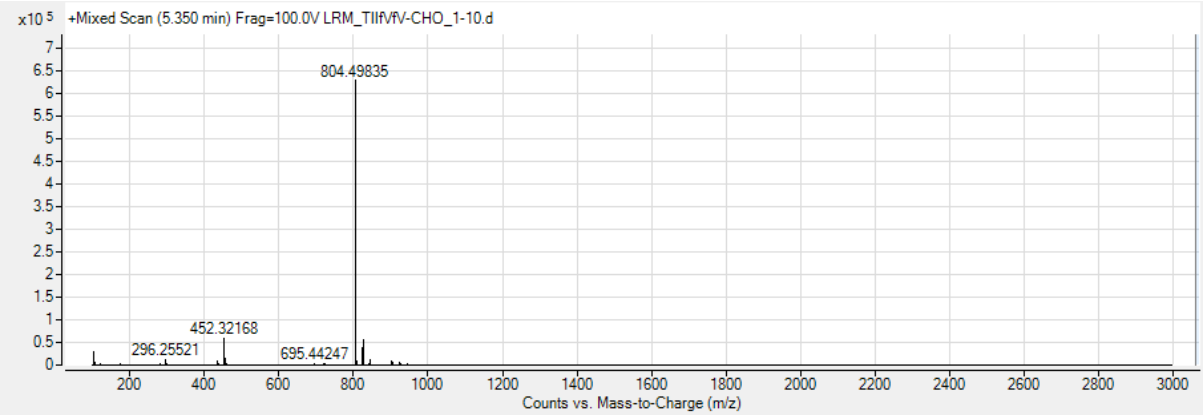




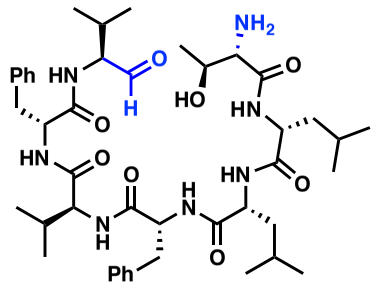
Predicted and observed C2 and C5 carbon chemical shifts of ca. 90 and 76 ppm, respectively.



LRMS (ESI-TOF): calc'd for $C_{44}H_{68}N_7O_8$ $[M+H]^+$ 822.51; found 822.51 (aldehyde form).

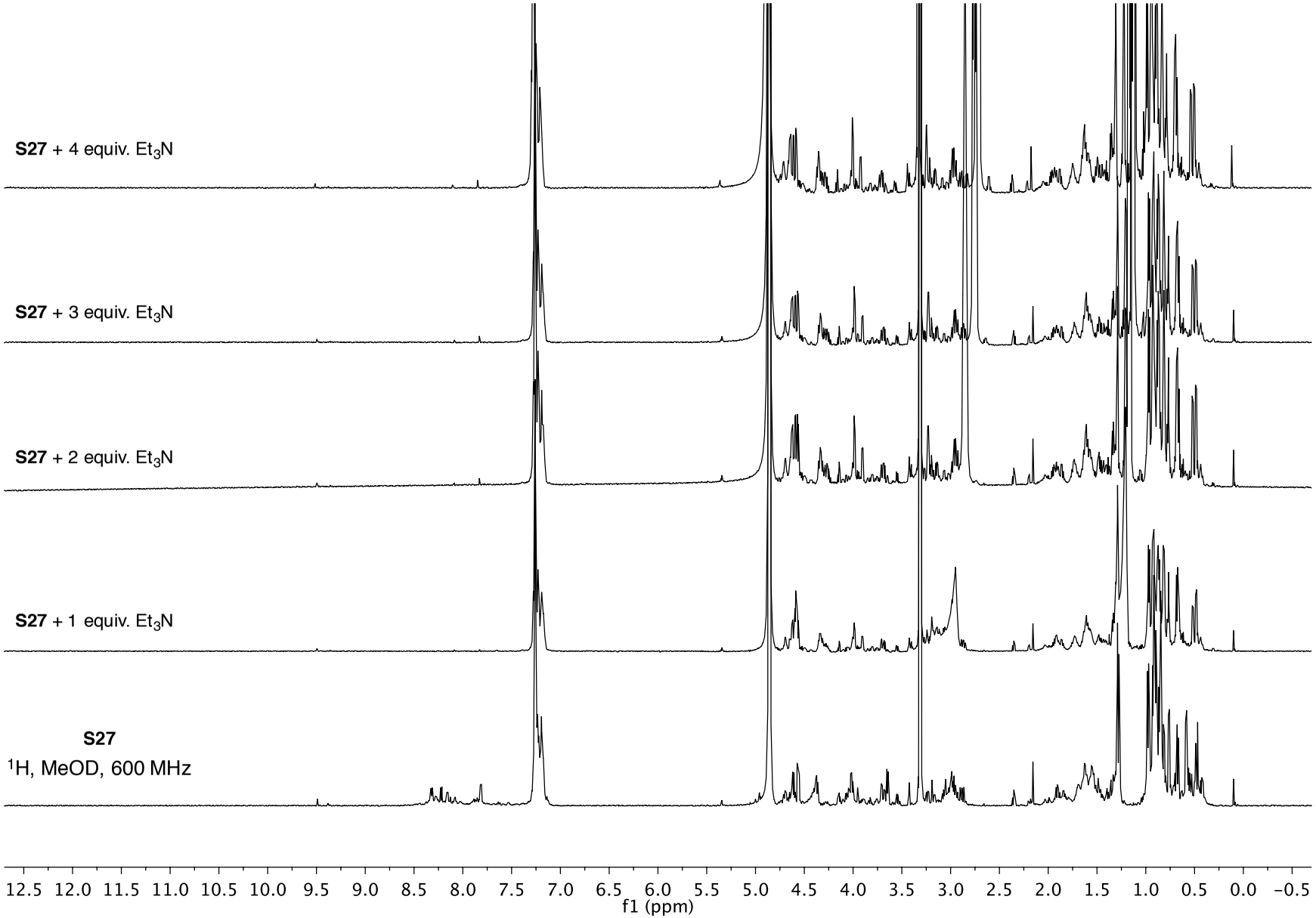


LRMS (ESI-TOF): calc'd for $C_{44}H_{66}N_7O_7$ $[M+H]^+$ 804.50; found 804.50 (imine/oxazolidine form).

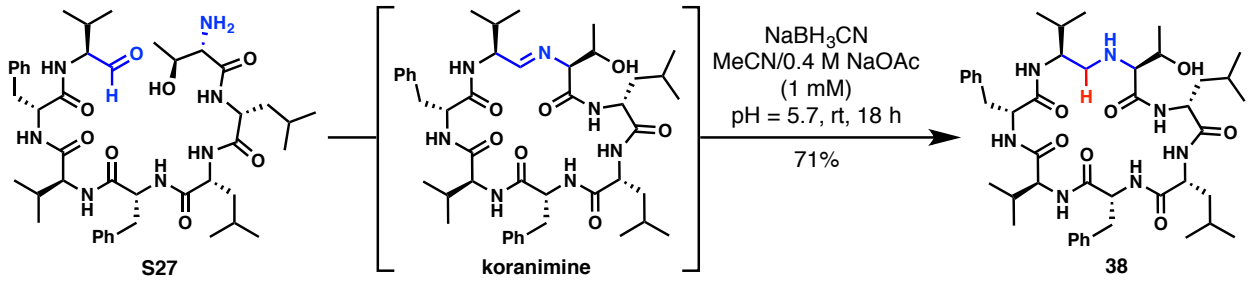


S27

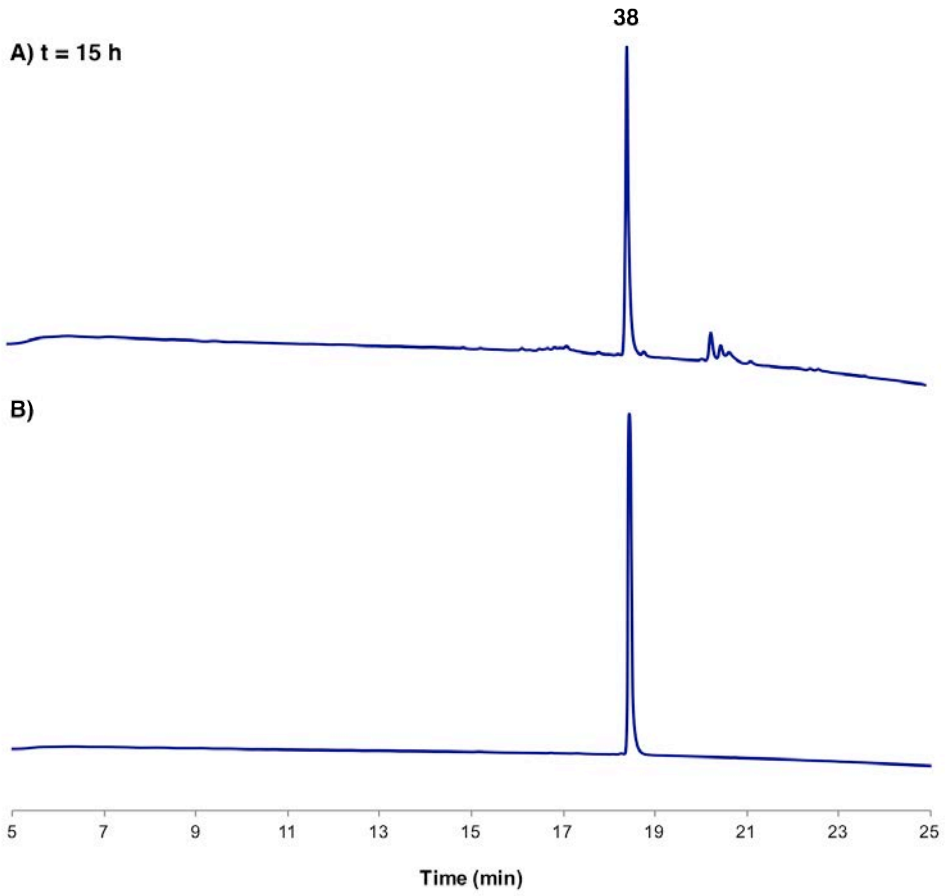
^{1}H, DMSO-d_6, 600 MHz


S27 + 4 equiv. Et_3N
S27 + 3 equiv. Et_3N
S27 + 2 equiv. Et_3N
S27 + 1 equiv. Et_3N
S27
^1H, MeOD, 600 MHz
12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

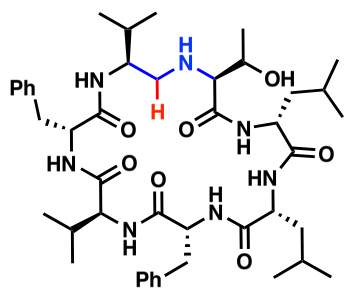
Peptide 38



Peptide **38** was prepared according to general procedure 4 from linear amino aldehyde **S27** (7.0 mg, 9.8 µmol). The peptide was solubilized in a 1:1 mixture of MeCN and 0.4 M NaOAc buffer (pH = 5.7). At t = 18 h, the reaction was purified by preparative reverse-phase HPLC (45% B for 5 min, then 45% to 80% B over 30 min) to afford peptide macrocycle **38** (3.1 mg, 71% yield) as a white solid following lyophilization.

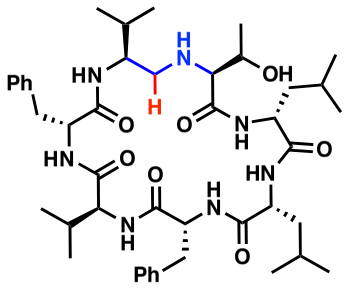


A) Crude analytical HPLC trace of the formation of **38** from amino aldehyde **S27** following reductive amination (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **38** (Rt = 18.4 min, 5 to 100% B over 25 min, λ = 230 nm).



38

^{1}H, DMSO-d$_6$, 600 MHz

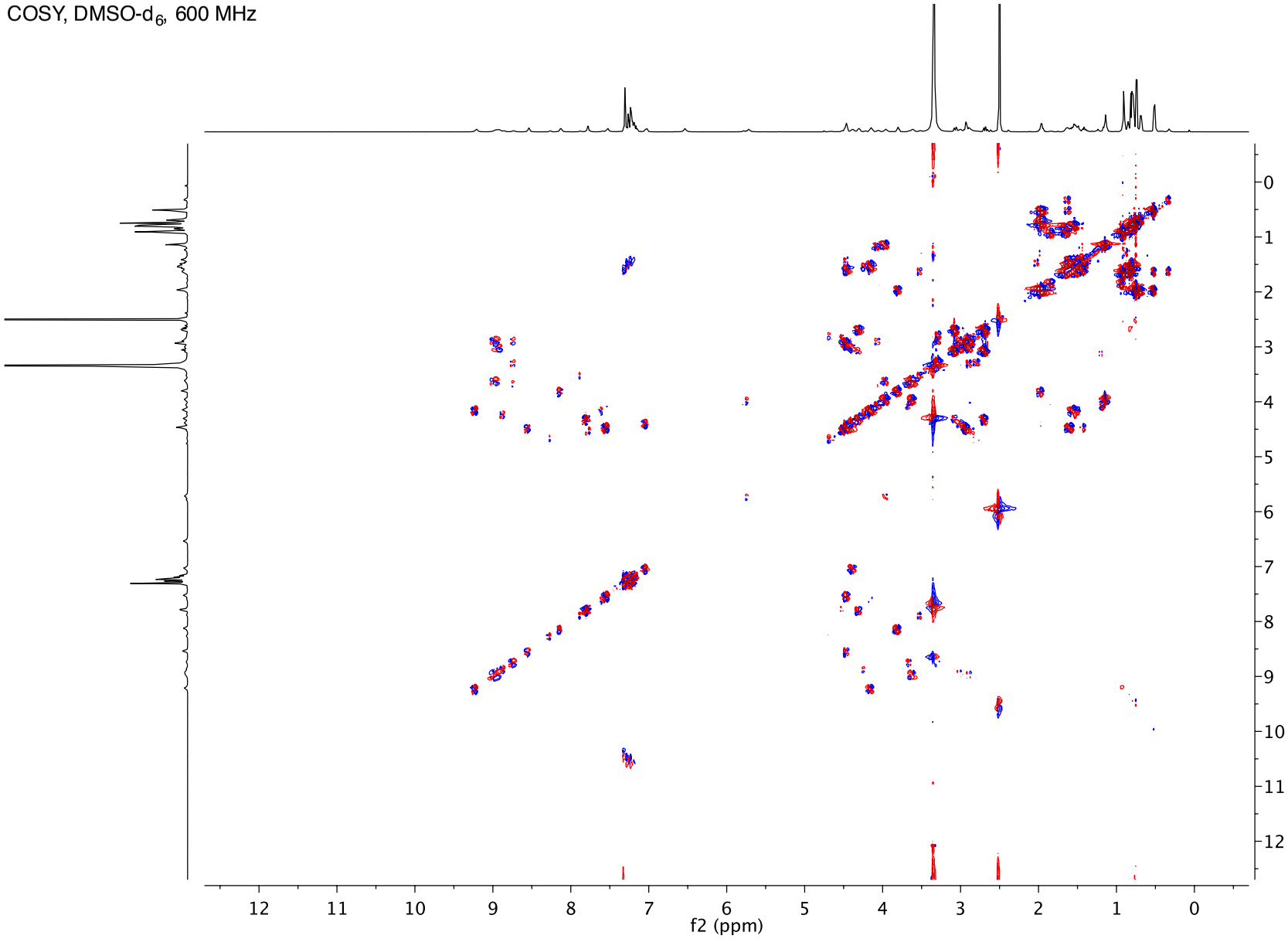


38

^{13}C, DMSO-d$_6$, 151 MHz

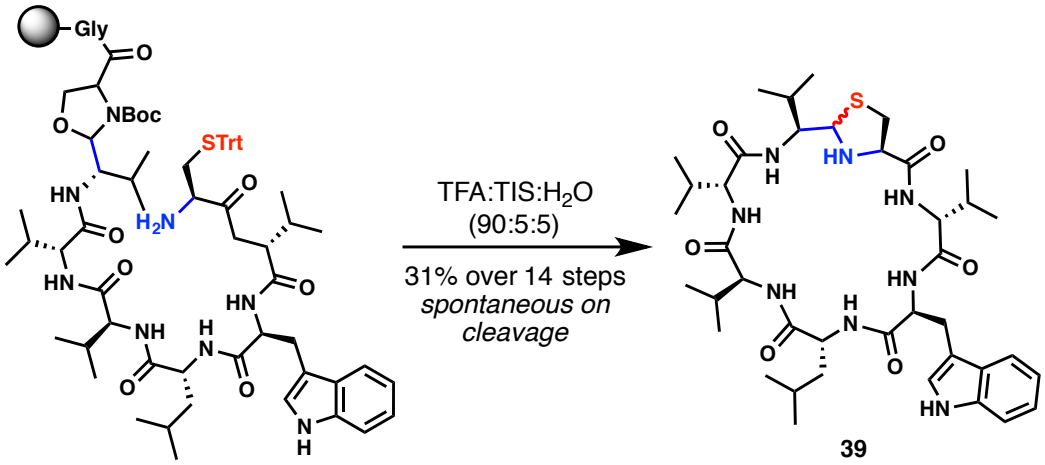
38

COSY, DMSO-d_6, 600 MHz

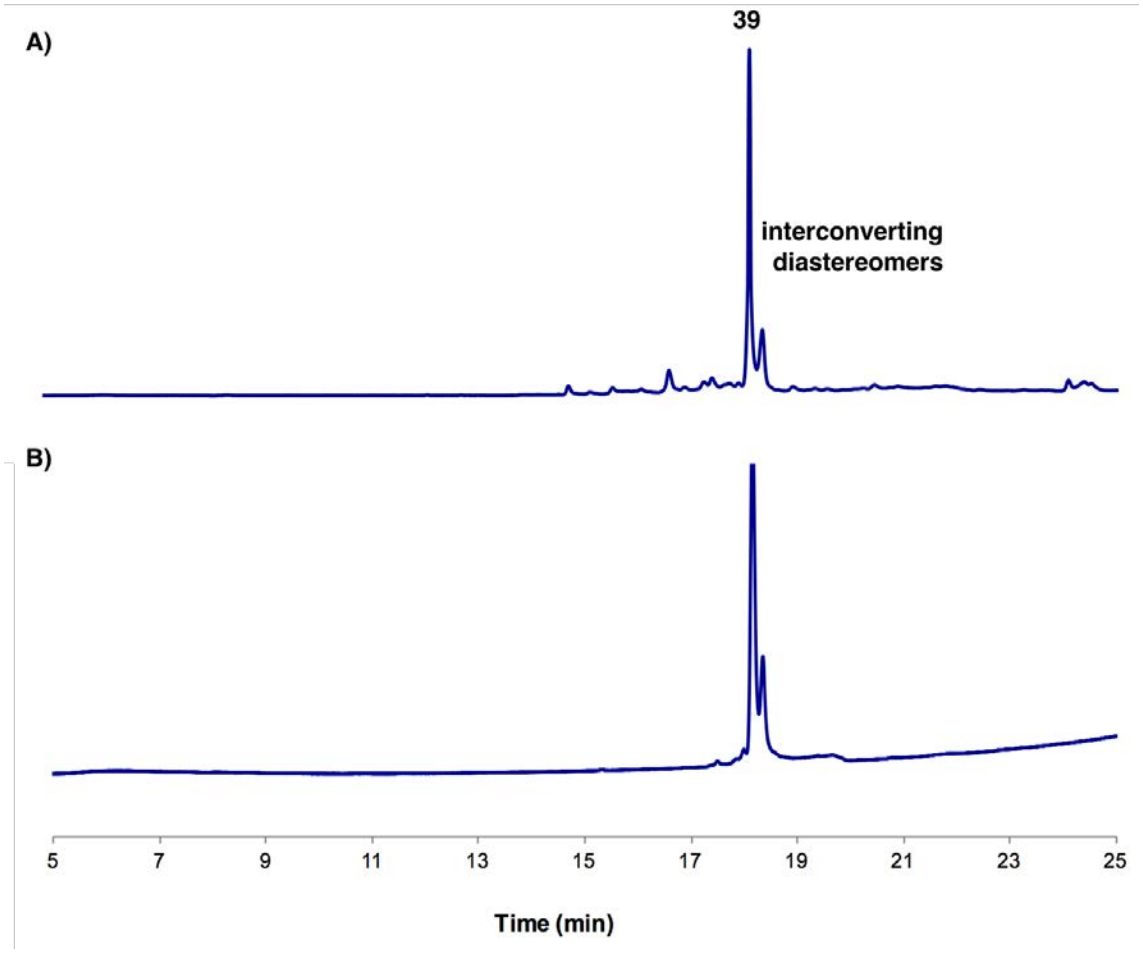


Characterization Data for Lugdunin Analogues

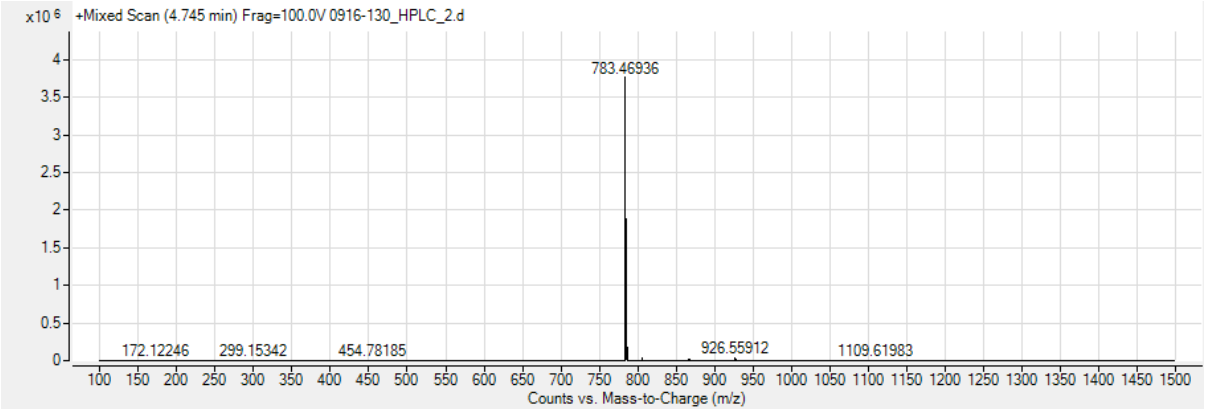
Peptide 39



Amino aldehyde **39** was prepared on a 14.5 μmol scale according to general procedure 2a. Fmoc-Leu-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS protocols. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (50% B for 5 min, then 50% B to 100% B over 30 min) to afford lugdunin **39** (3.4 mg, 31% yield based on the original resin loading) as a mixture of interconverting diastereomers and as a white solid following lyophilization.

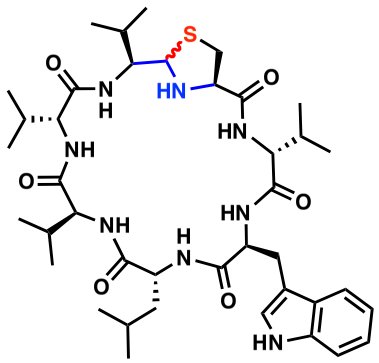


A) Crude analytical HPLC trace of the formation of **39** following cleavage from resin (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **39** (Rt = 18.2, 18.4 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{40}H_{62}N_8O_6S$ $[M+H]^+$ 783.46; found 783.46.

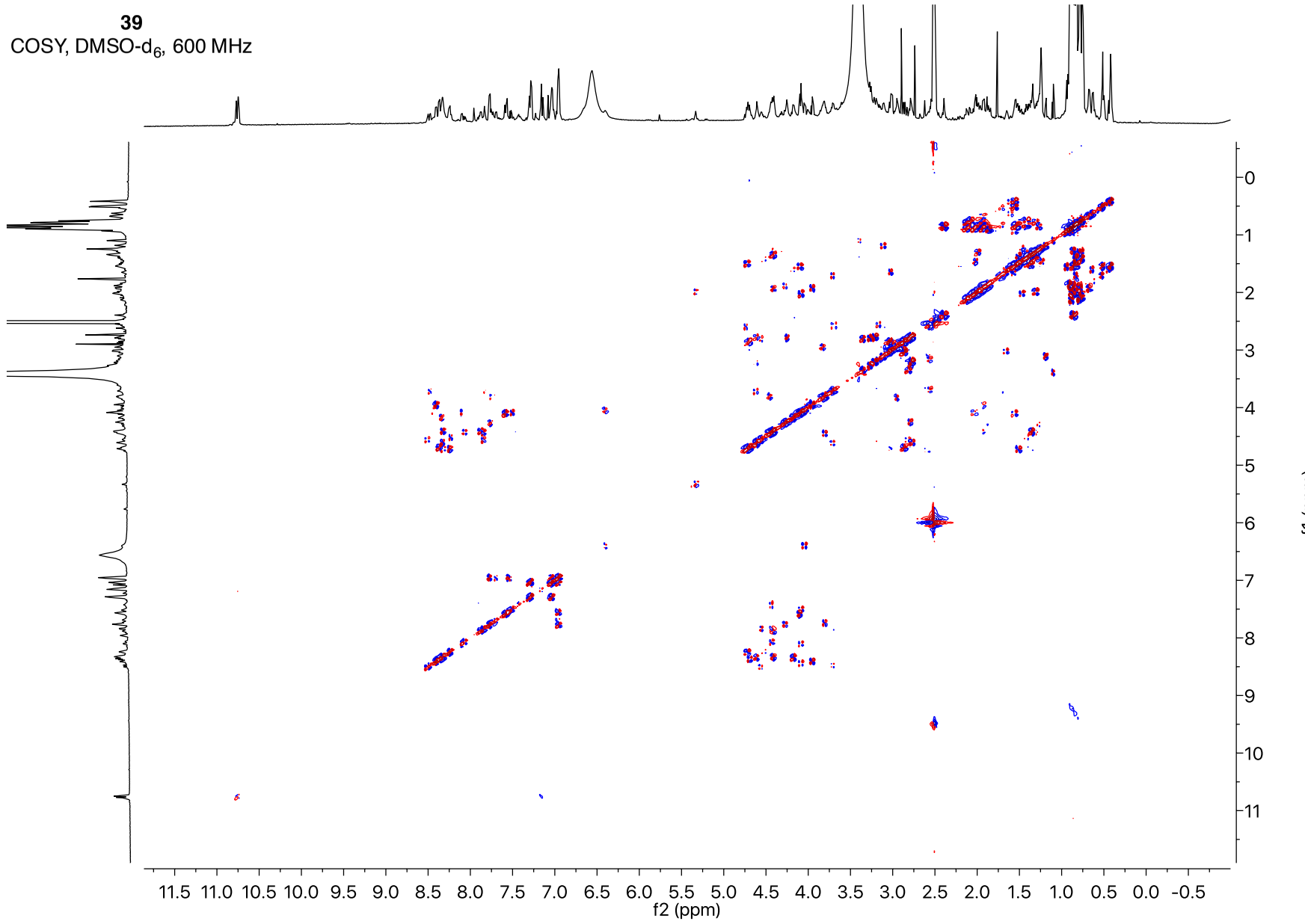
The recorded NMR spectra for synthetic lugdunin (**39**) are in agreement with literature data.[15]



39

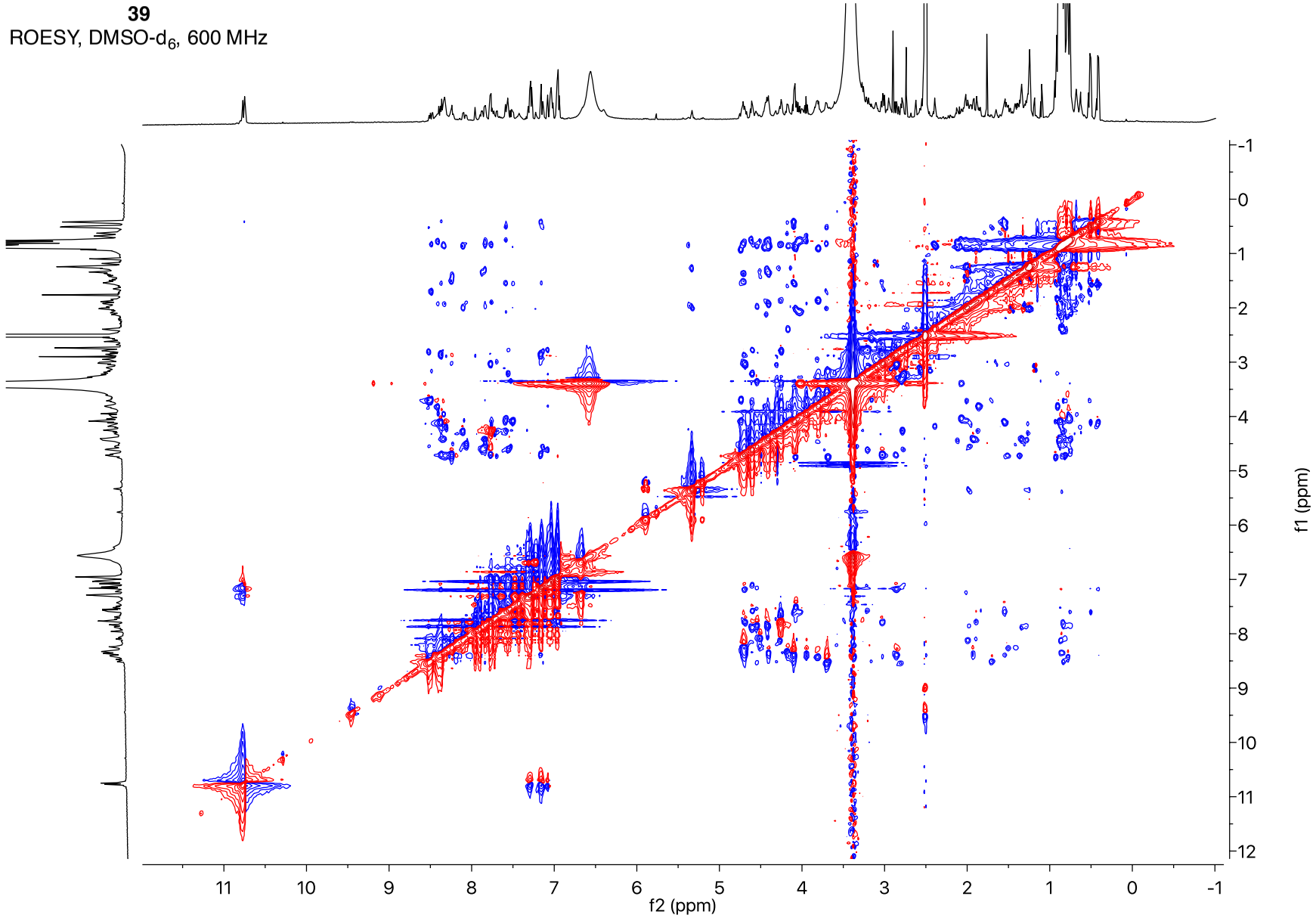
^{1}H, DMSO-d$_{6}$, 600 MHz

39
COSY, DMSO-d_6, 600 MHz

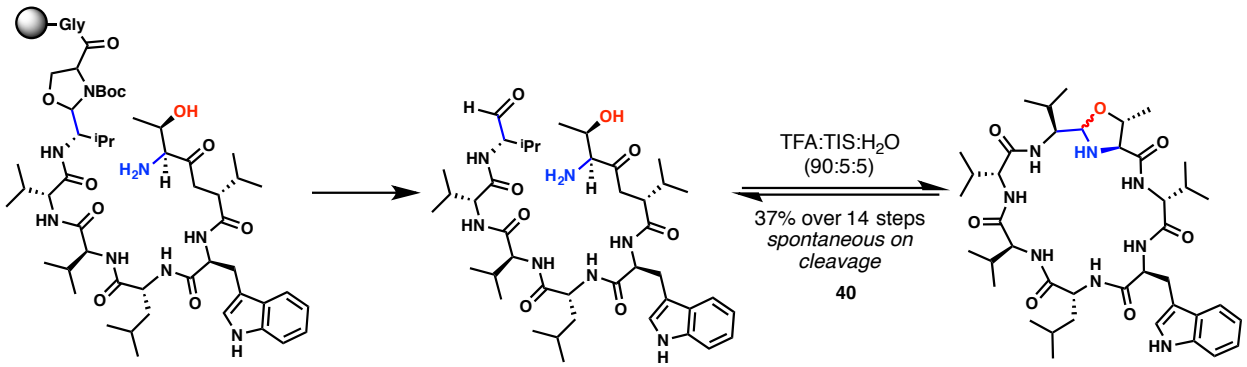


39

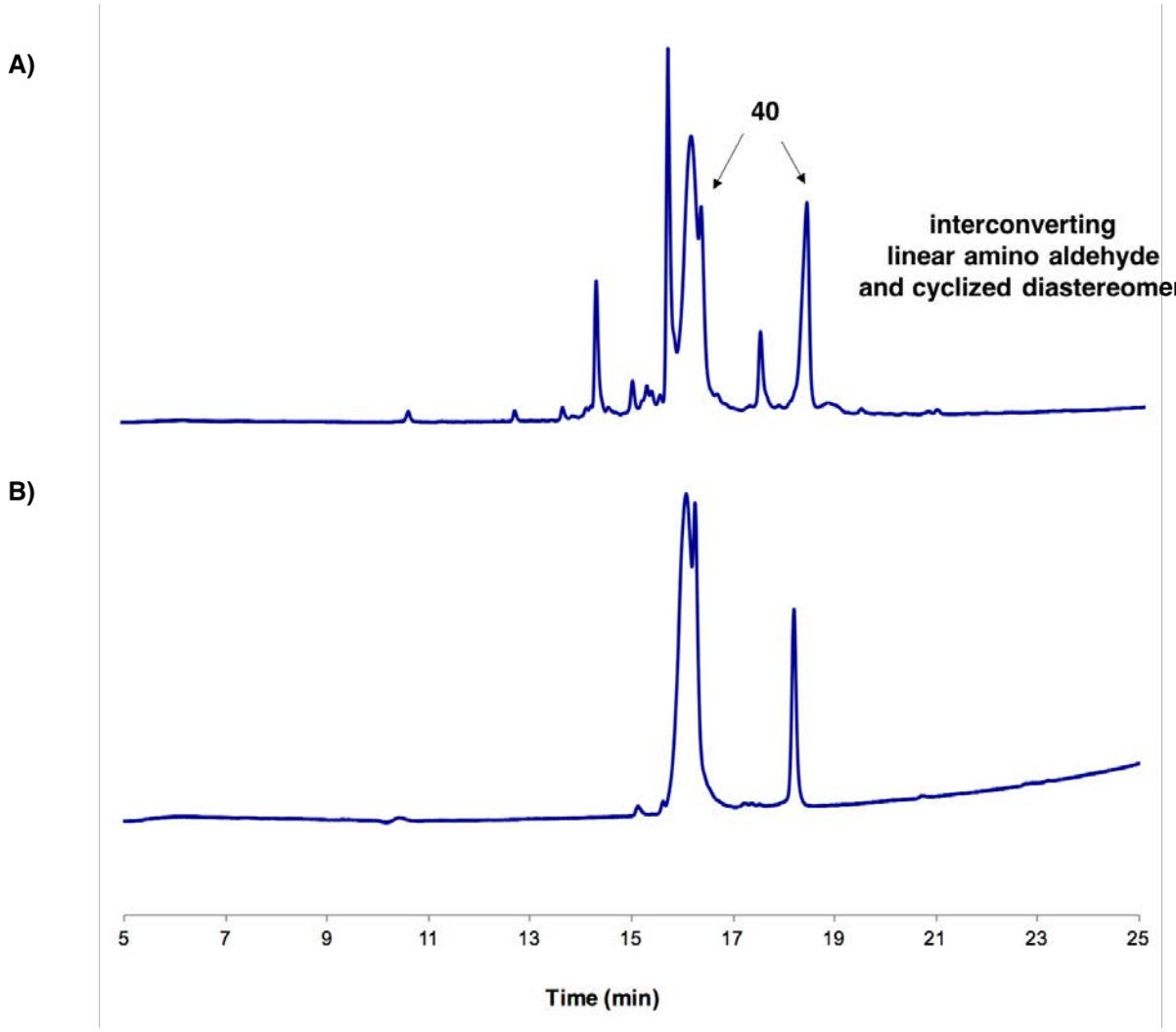
ROESY, DMSO-d_6, 600 MHz



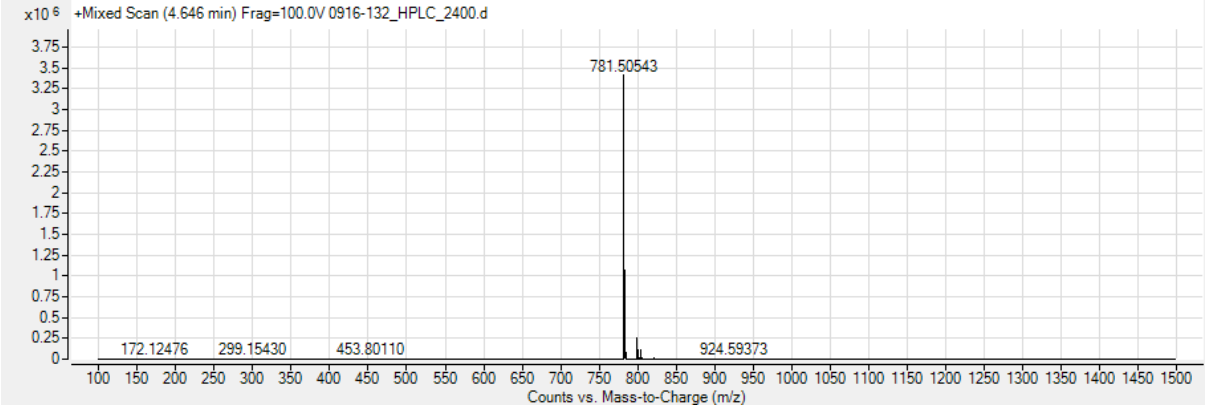
Peptide 40



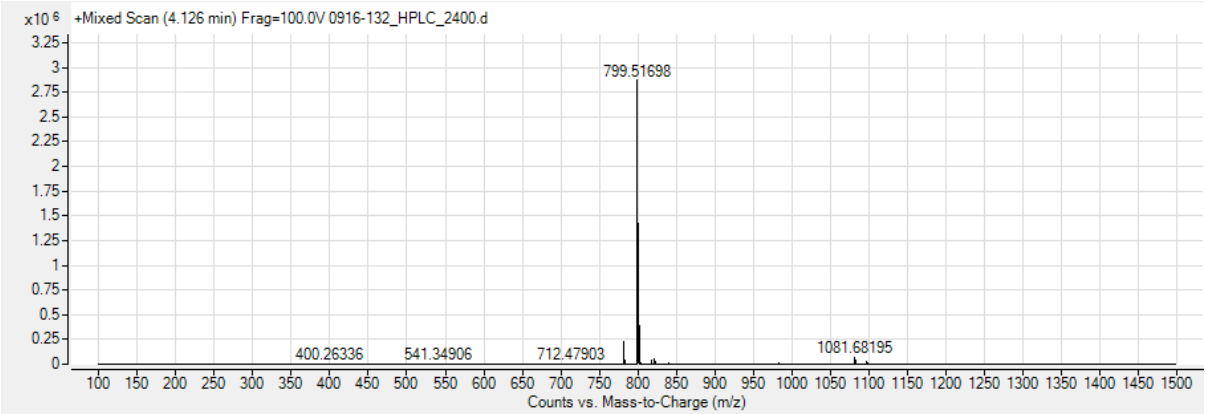
Amino aldehyde **40** was prepared on a 14.5 μmol scale according to general procedure 2a. Fmoc-Leu-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS protocols. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (50% B for 5 min, then 50% B to 100% B over 30 min) to afford Thr-lugdunin **40** (4.1 mg, 37% yield based on the original resin loading) as an interconverting mixture the linear amino aldehyde and the cyclized diastereomers, and as a white solid following lyophilization.



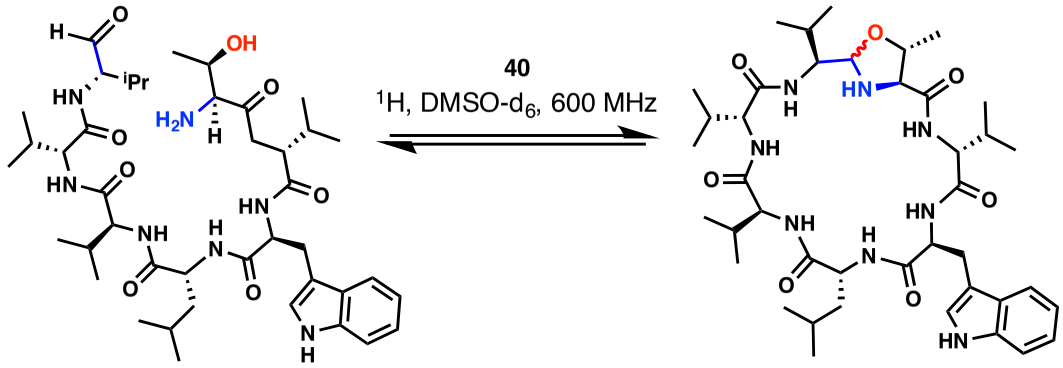
A) Crude analytical HPLC trace of the formation of **40** following cleavage from resin (5 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **40** (Rt = 16.07, 16.25, 18.20 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{41}H_{64}N_8O_7$ $[M+H]^+$ 781.50; found 781.51 (cyclized).



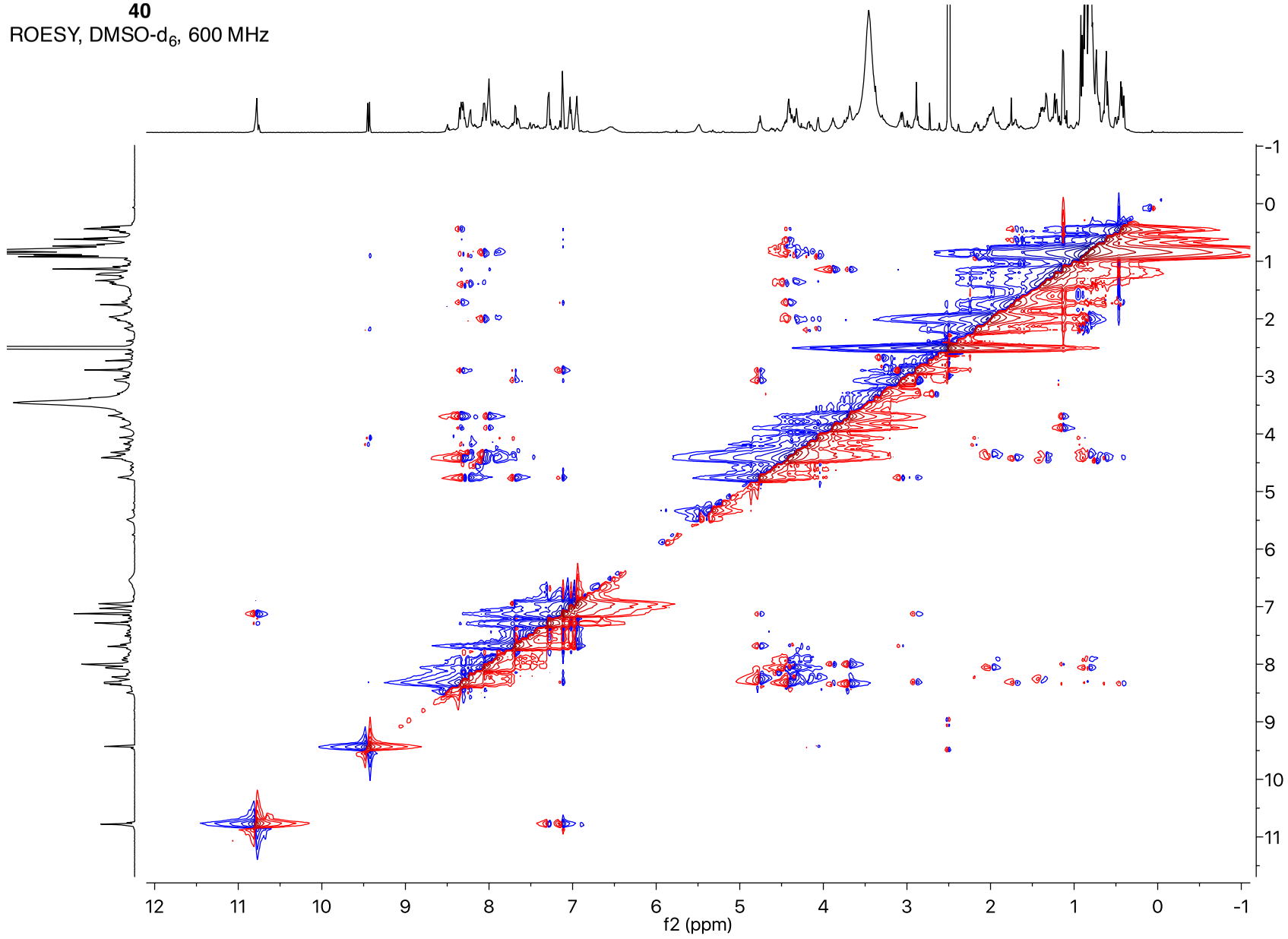
LRMS (ESI-TOF): calc'd for $C_{41}H_{64}N_8O_7$ $[M+H]^+$ 799.51; found 799.52 (linear).


40
^{1}H, DMSO-d$_6$, 600 MHz

12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

40

ROESY, DMSO-d_6, 600 MHz

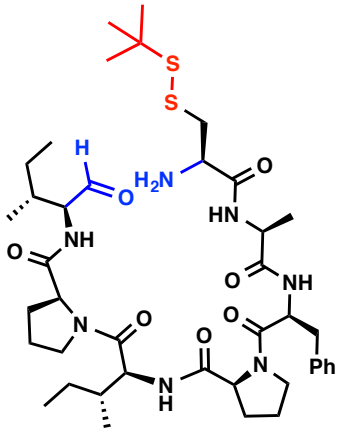


Lugdunin Analogue Notes:

- As noted in the original report,[15] lugdunin (**39**) was isolated as a mixture of interconverting, inseparable diastereomers. A substantial (and inseparable) amount of the linear peptide aldehyde was observed in the case of **40**, while no aldehyde was apparent by analytical HPLC or LC-MS analysis in the case of **39.**
- Complete removal of the standard side-chain Fmoc-Trp(Boc)-OH protecting group proved difficult, so the unprotected Fmoc-Trp-OH was incorporated instead. In order to avoid potential acetylation of the free indole nitrogen, all subsequent capping steps were shortened to one 30-second treatment of 10% Ac_2O in pyridine.

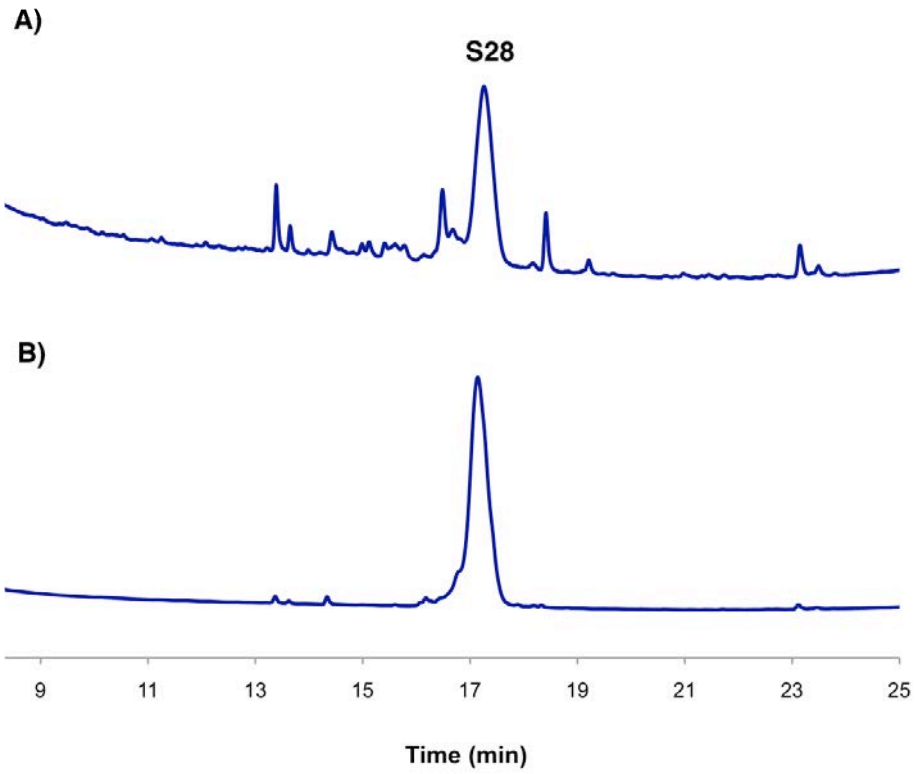
Characterization Data for Sanguinamide A and Analogues

Peptide S28

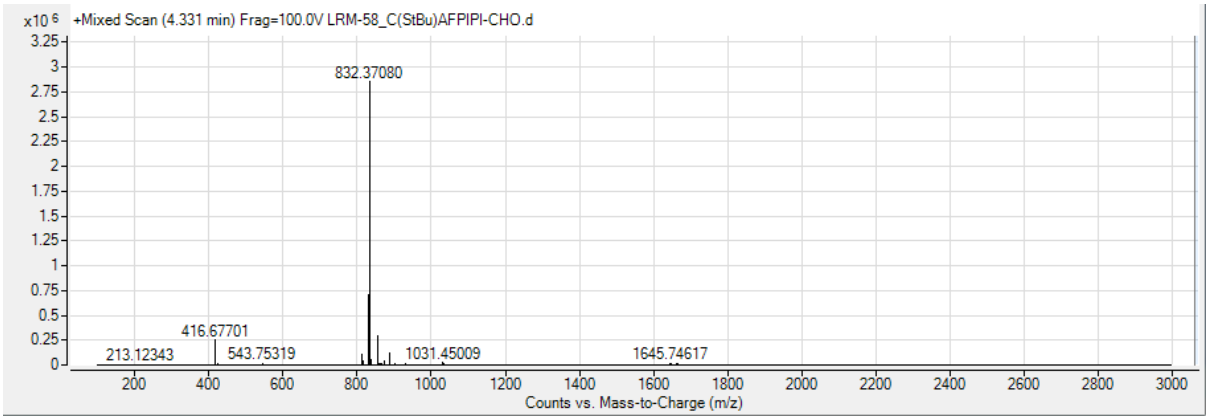


S28

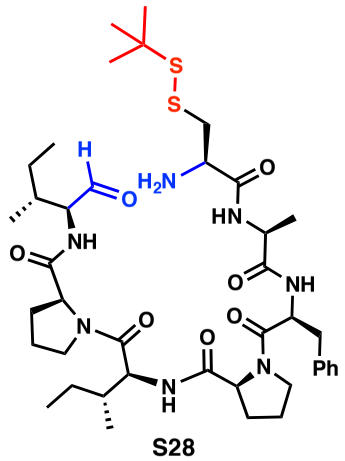
Amino aldehyde **S28** was prepared on a 50 μmol scale according to general procedure 2a. Fmoc-Ile-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (30% B for 5 min, then 30% B to 80% B over 35 min) to afford peptide **S28** (26.6 mg, 64% yield based on the original resin loading) as a white solid following lyophilization.



A) Crude analytical HPLC trace of amino aldehyde **S28** following Fmoc-SPPS and acidic cleavage from the resin (0 to 100% B over 25 min, λ = 230 nm). **B)** Purified peptide **S28** (Rt = 17.1 min, 0 to 100% B over 25 min, λ = 230 nm).



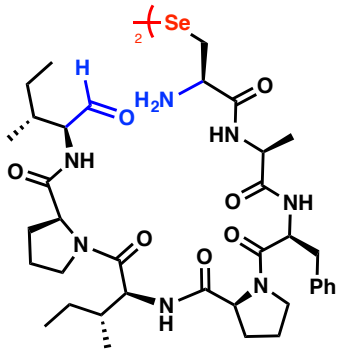
LRMS (ESI-TOF): calc'd for $C_{41}H_{66}N_7O_7S_2$ $[M+H]^+$ 832.45; found 832.37.



S28

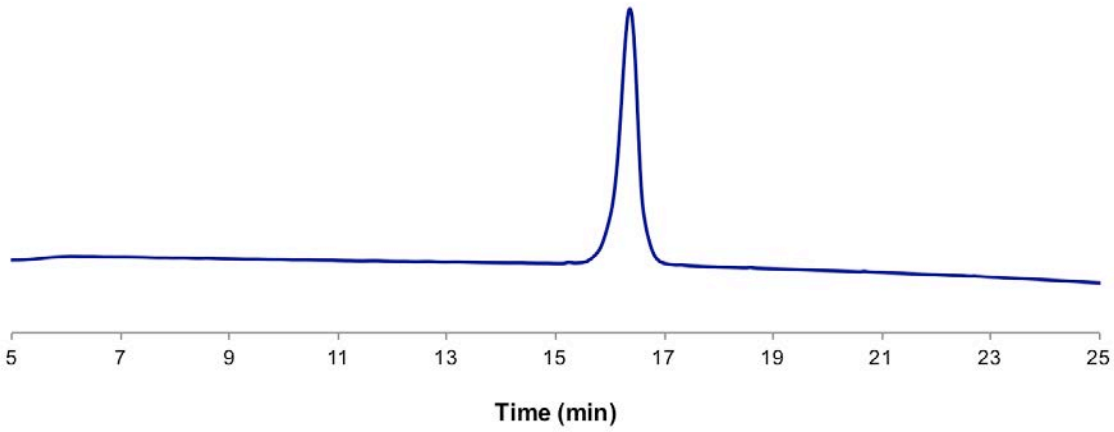
^{1}H, MeOD, 600 MHz

Peptide S29

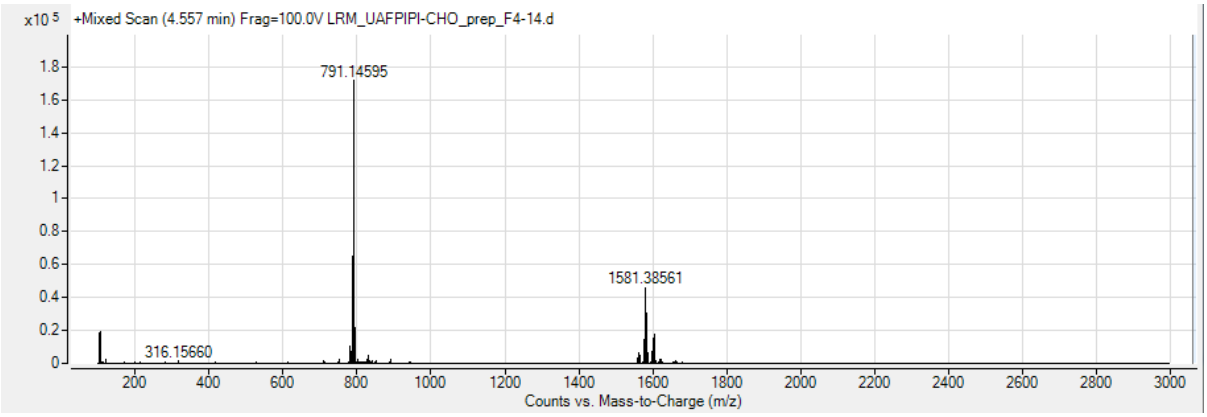


S29

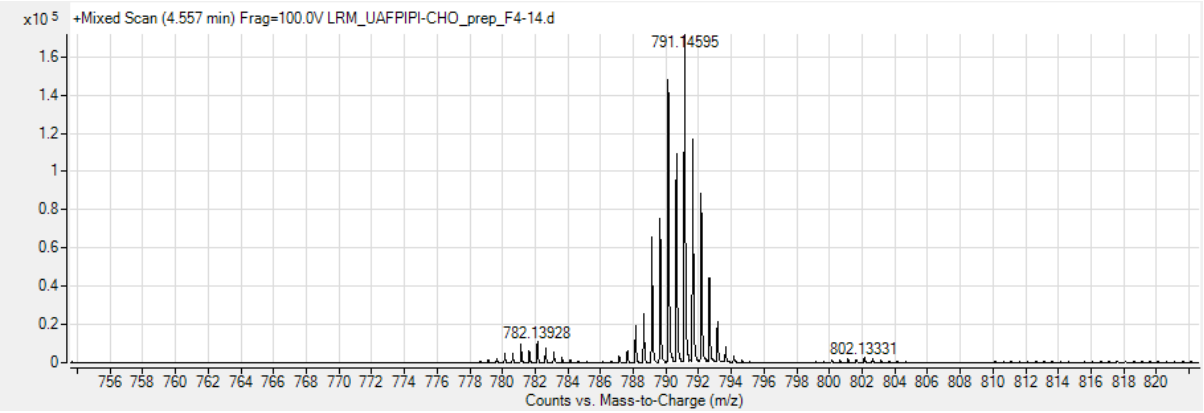
Amino aldehyde **S29** was prepared on a 40 μmol scale according to general procedure 2a. Fmoc-Ile-CHO was loaded onto Rink TG resin and the peptide elongated using standard Fmoc-SPPS. Following cleavage from the resin and ether precipitation, the crude peptide was purified by preparative reverse-phase HPLC (25% B for 5 min, then 25% B to 70% B over 30 min) to afford peptide **S29** (6.9 mg, 22% yield based on the original resin loading) as a white solid following lyophilization.



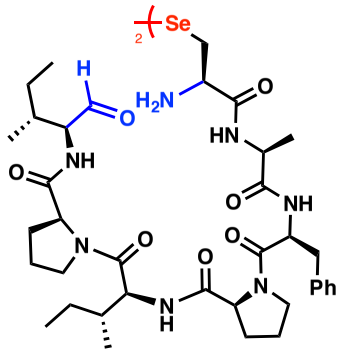
Purified peptide **S29** (Rt = 16.3 min, 5 to 100% B over 25 min, λ = 210 nm).



LRMS (ESI-TOF): calc'd for $C_{74}H_{113}N_{14}O_{14}Se_2$ $[M+H]^+$ 1581.69; found 1581.39, $[M+2H]^{2+}$ 791.33; found 791.15.



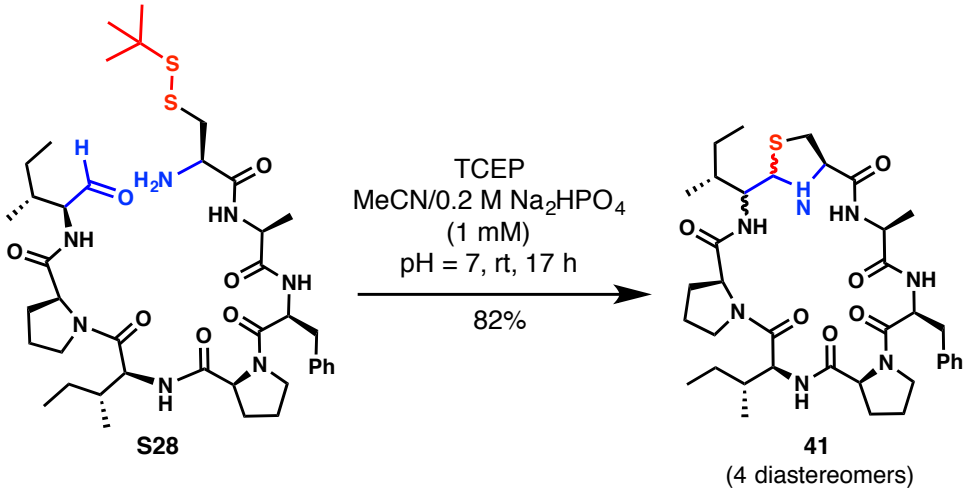
LRMS (ESI-TOF): Zoom-in of LRMS spectrum showing the isotopic distribution characteristic of a diselenide.



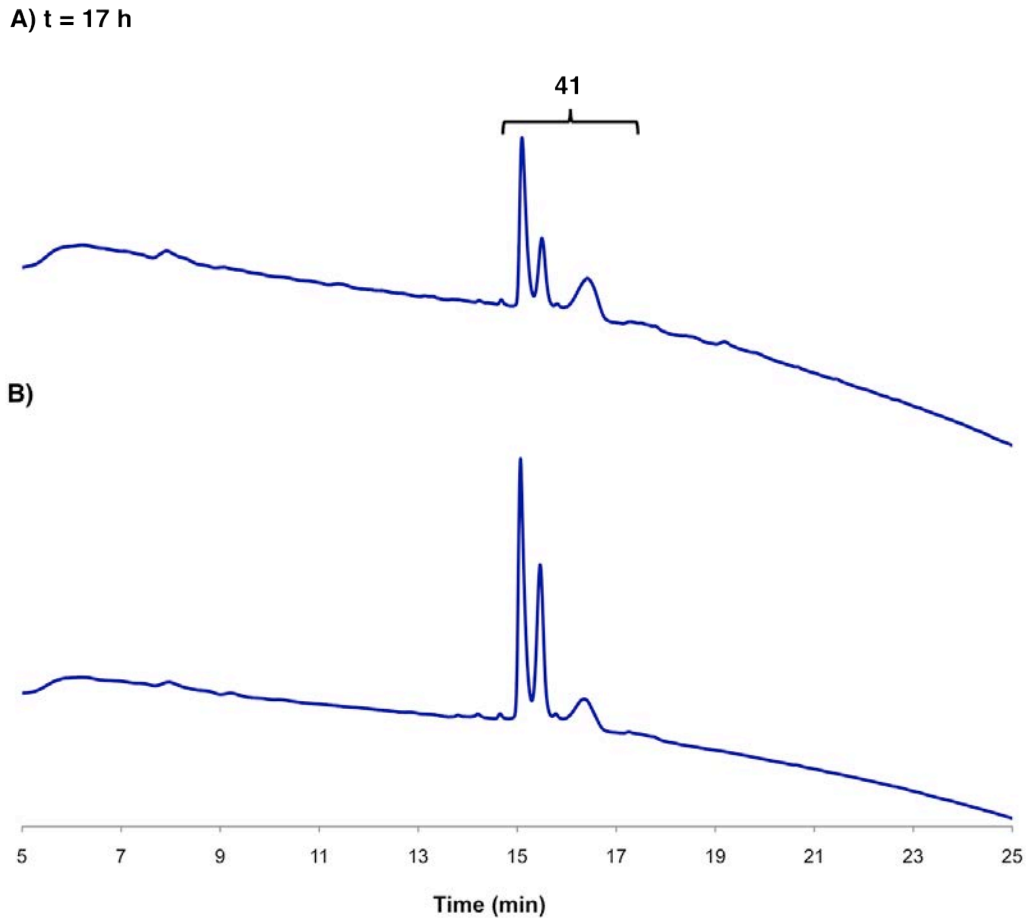
S29

^{1}H, DMSO-d_6, 600 MHz

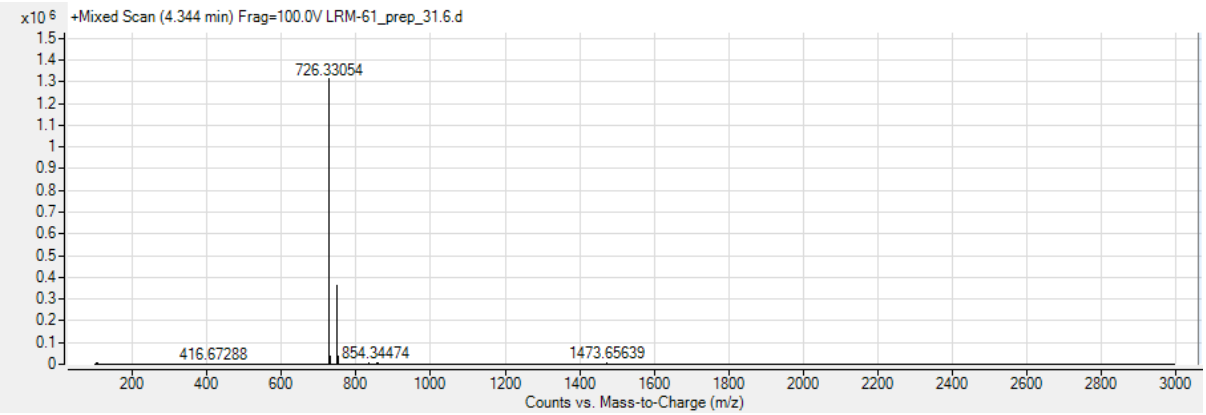
Peptide 41



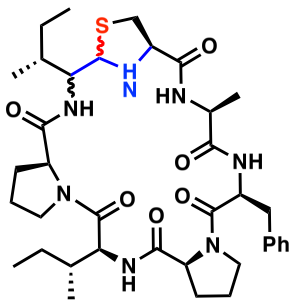
Peptide **41** was prepared according to general procedure 5 from S*t*Bu-disulfide protected linear amino aldehyde **S28** (6.4 mg, 7.7 μmol). The peptide aldehyde was solubilized in a mixture of MeCN (3 mL) and 0.2 M Na_2HPO_4 (4.7 mL, pH = 7) and treated with TCEP. At t = 17 h, the reaction was purified by preparative reverse-phase HPLC (35% B for 5 min, 35% to 70% B over 30 min) to afford peptide macrocycle **41** (4.6 mg, 82% yield) as a mixture of four diastereomers and as a white solid following lyophilization.



A) Crude analytical HPLC trace of the formation of **41** from amino aldehyde **S28** following thiazolidine macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified diastereomeric peptide **41** (Rt = 15.1, 15.4, 16.3 min, 5 to 100% B over 25 min, λ = 210 nm).



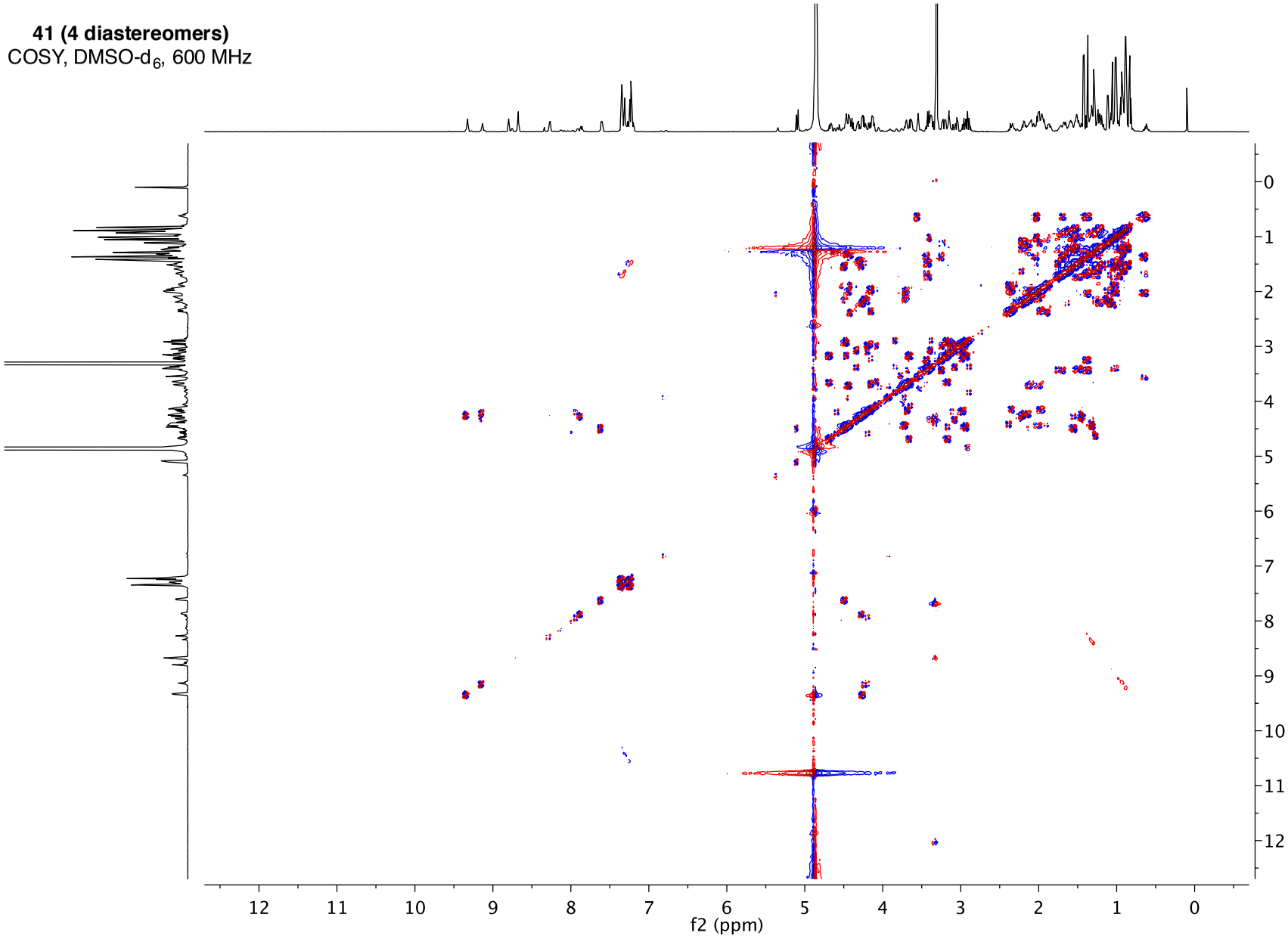
LRMS (ESI-TOF): calc'd for $C_{37}H_{56}N_7O_6S$ $[M+H]^+$ 726.40; found 726.33.



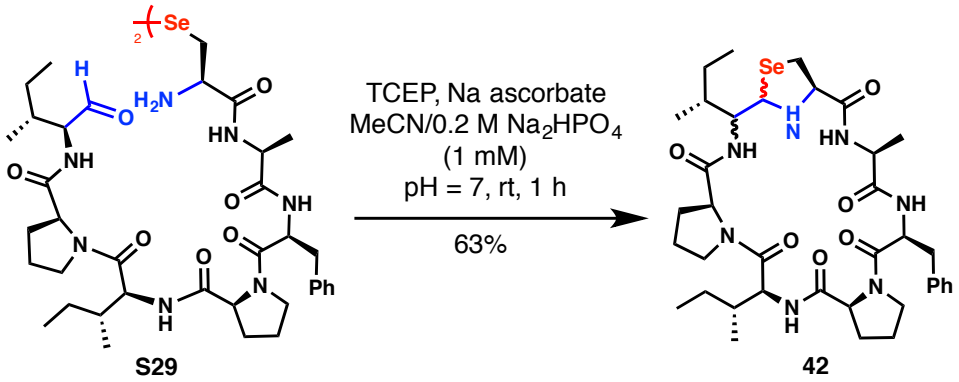
41 (4 diastereomers)

^{1}H, MeOD, 600 MHz

41 (4 diastereomers)
COSY, DMSO-d_6, 600 MHz

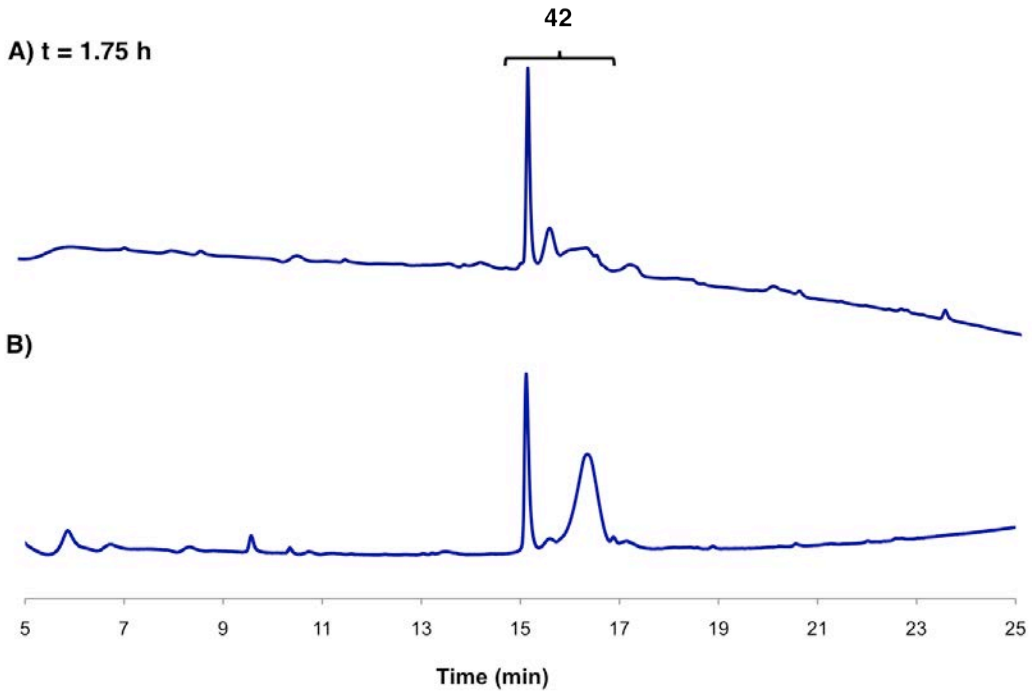


Peptide 42

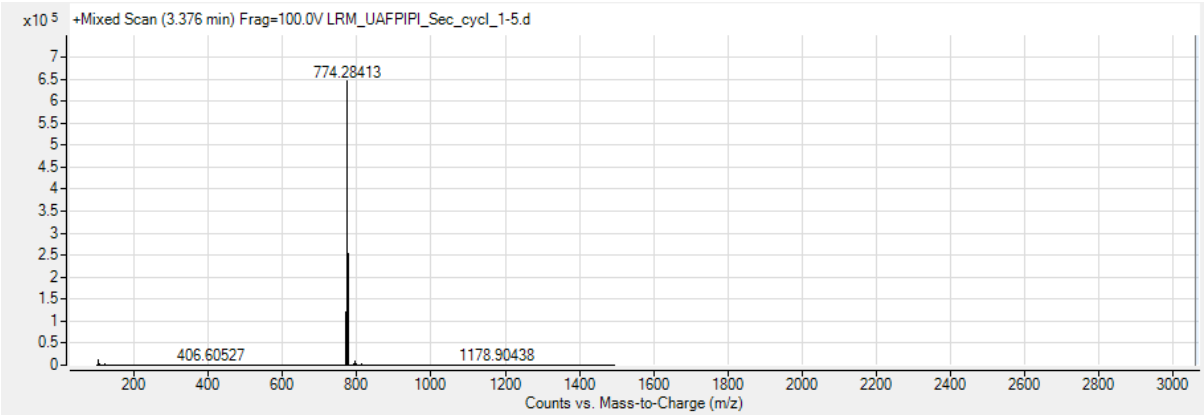


S29 42

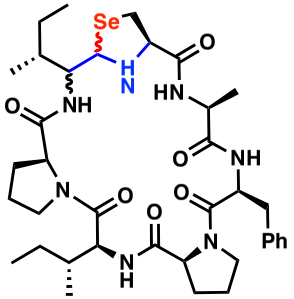
Peptide **42** was prepared according to general procedure 6 from linear amino aldehyde selenocystine dimer **S29** (5.7 mg, 3.6 μmol). The peptide aldehyde was solubilized in a mixture of MeCN (2 mL) and 0.2 M Na_2HPO_4 (5.2 mL, pH = 7) and treated with TCEP and sodium ascorbate. At t = 2 h, the reaction was purified by preparative reverse-phase HPLC (20% B to 35% B over 5 min, 35% to 80% B over 25 min) to afford peptide macrocycle **42** (3.5 mg, 63% yield) as a mixture of four diastereomers and as a white solid following lyophilization. *Note: The ratio of diastereomers fluctuates in different buffers and solvent systems (e.g. the formic acid eluent used on the LC-MS and TFA buffer used for analytical HPLC give rise to different ratios of the diastereomeric products).*



A) Crude analytical HPLC trace of the formation of **42** from amino aldehyde **S29** following selenazolidine macrocyclization (5 to 100% B over 25 min, λ = 210 nm). **B)** Purified peptide **42** as a mixture of diastereomers (5 to 100% B over 25 min, λ = 230 nm). Note: the purified sample was taken from a mixture of DMSO following NMR analysis.



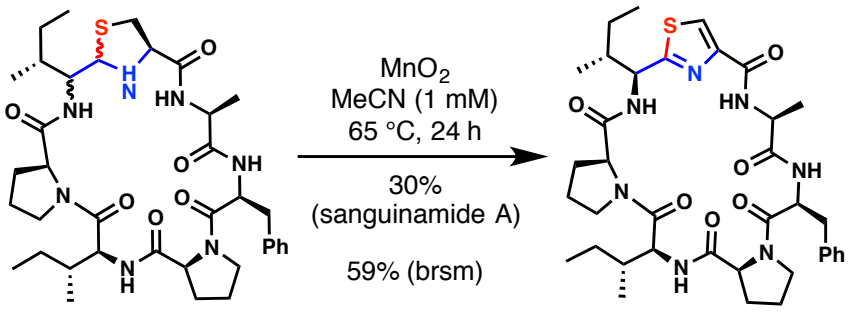
LRMS (ESI-TOF): calc'd for $C_{37}H_{56}N_7O_6Se$ $[M+H]^+$ 774.35; found 774.28.



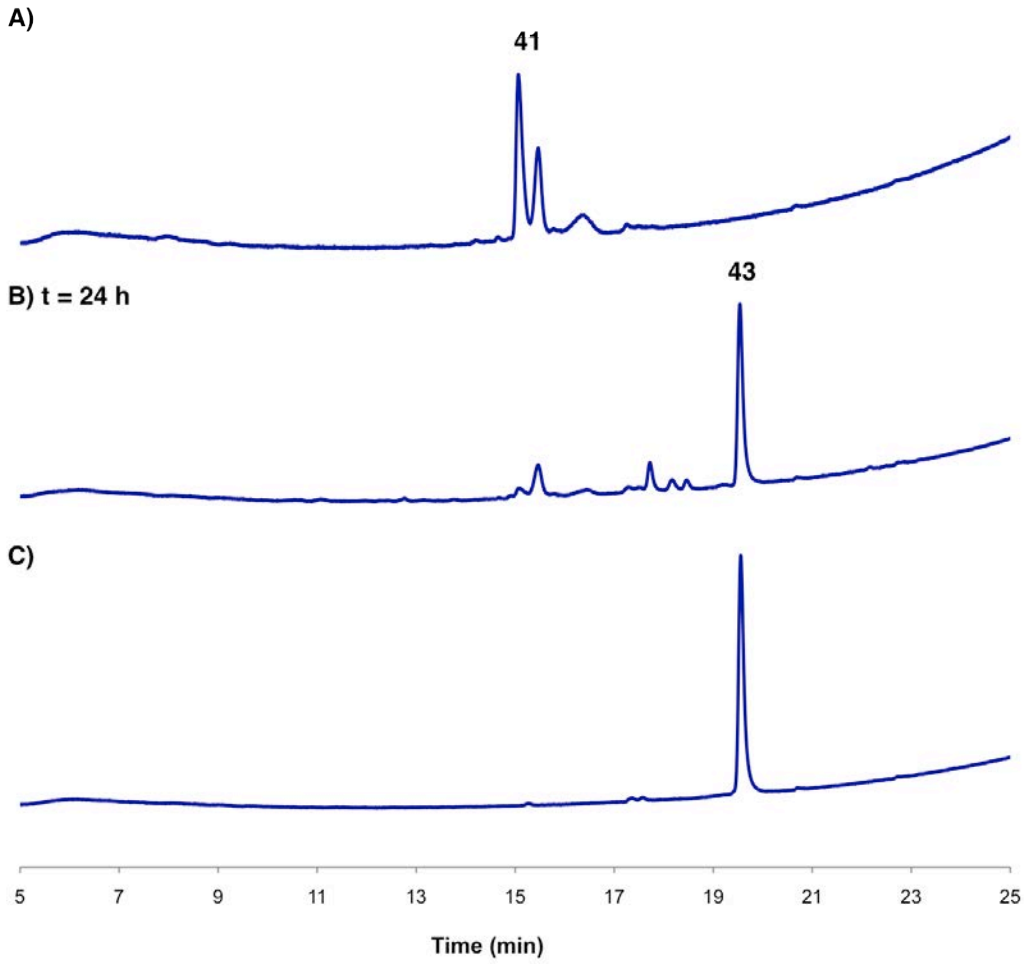
42 (4 diastereomers)

^{1}H, DMSO-d$_{6}$, 600 MHz

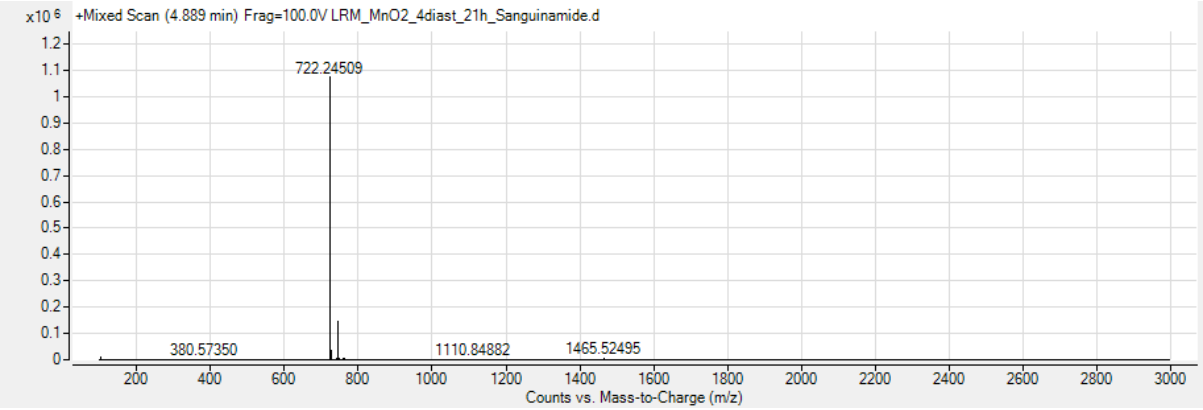
Peptide 43 (Sanguinamide A)



Peptide **43** was prepared from diastereomeric thiazolidines **41** upon oxidation with MnO_2. Peptide **41** (3.4 mg, 4.7 μmol) was dissolved in MeCN (1 mM concentration) and treated with MnO_2 (50 eq.). The resulting mixture was heated at 65 ^{o}C and monitored by LC-MS. At t = 24 h, the reaction was filtered and purified by preparative reverse-phase HPLC (45% B for 5 min, 45% to 80% B over 30 min) to afford Sanguinamide A (**43**) (1.0 mg, 30% yield) and recovered thiazolidine starting material **41** (1.0 mg, 29%) as white solids following lyophilization.

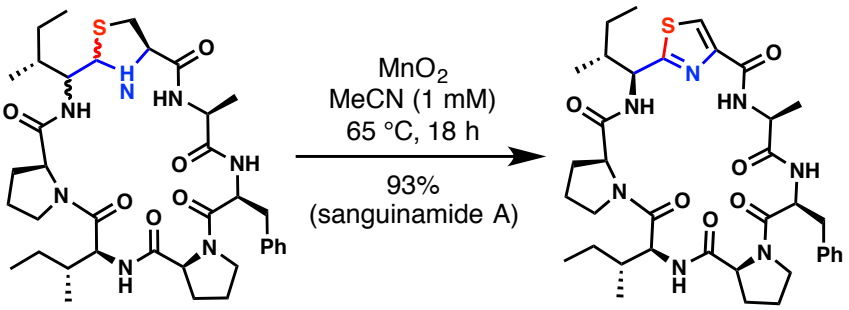


A) Starting thiazolidine **41** as a mixture of diastereomers (5 to 100% B over 25 min, λ = 230 nm). **B)** Crude analytical HPLC trace of the formation of **43** following oxidation with MnO_2 (5 to 100% B over 25 min, λ = 230 nm). **C)** Purified peptide **43** (Rt = 19.6 min, 5 to 100% B over 25 min, λ = 230 nm).



LRMS (ESI-TOF): calc'd for $C_{37}H_{52}N_7O_6S$ $[M+H]^+$ 722.37; found 722.25.

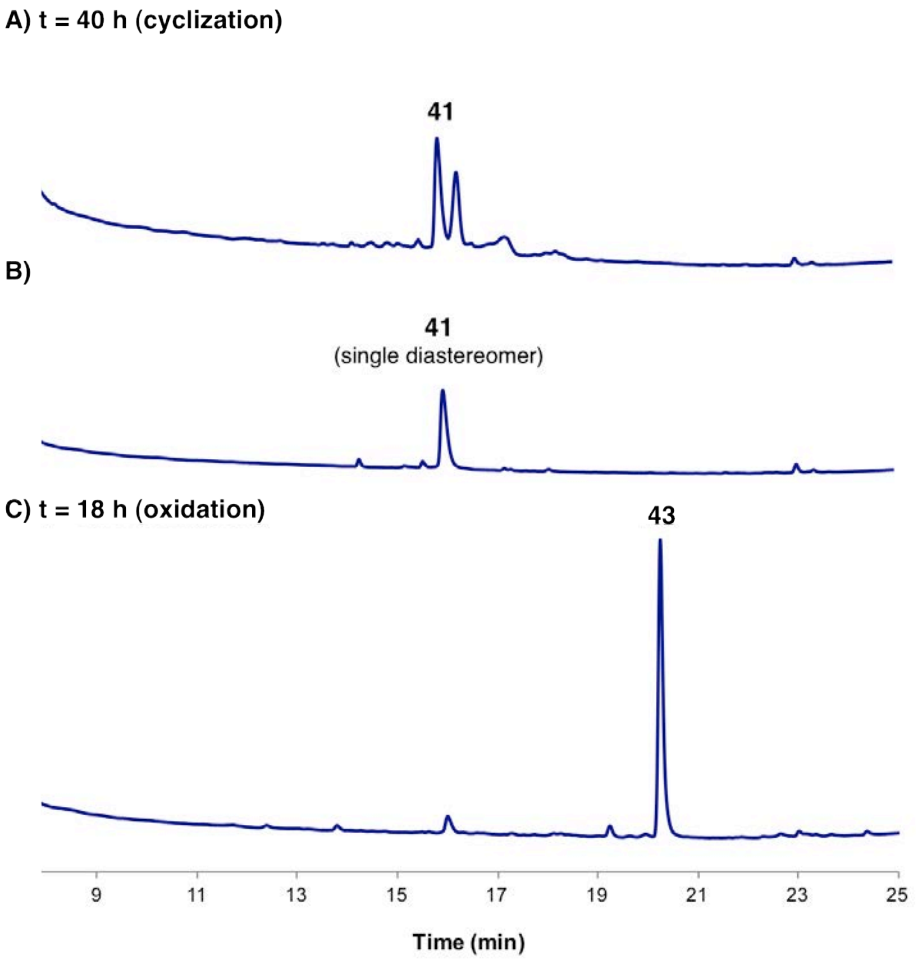
*Note: Sanguinamide A (**43**) could also be prepared from a single isolated thiazolidine diastereomer (unknown configuration at the thiazolidine). The oxidation step proceeded in 93% yield to afford Sanguinamide A:*



41
(single diastereomer, unknown configuration at the thiazolidine)

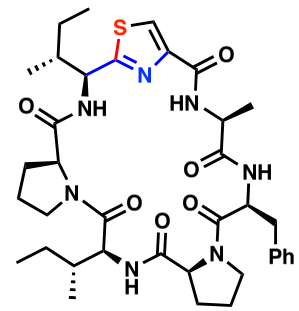
43

Thiazolidine **41** was prepared according to general procedure 5 and purified by preparative reverse-phase HPLC. A single diastereomer of **41** (2.6 mg, 3.6 μmol, unknown configuration at the thiazolidine) was isolated and subjected to oxidation with MnO_2 as described above. After 18 h at 65 ^{o}C, LC-MS analysis indicated consumption of the thiazolidine. The reaction was filtered and purified by preparative reverse-phase HPLC (45% B for 5 min, 45% to 85% B over 30 min) to afford Sanguinamide A (**43**) (2.4 mg, 93% yield) as a white solid following lyophilization.



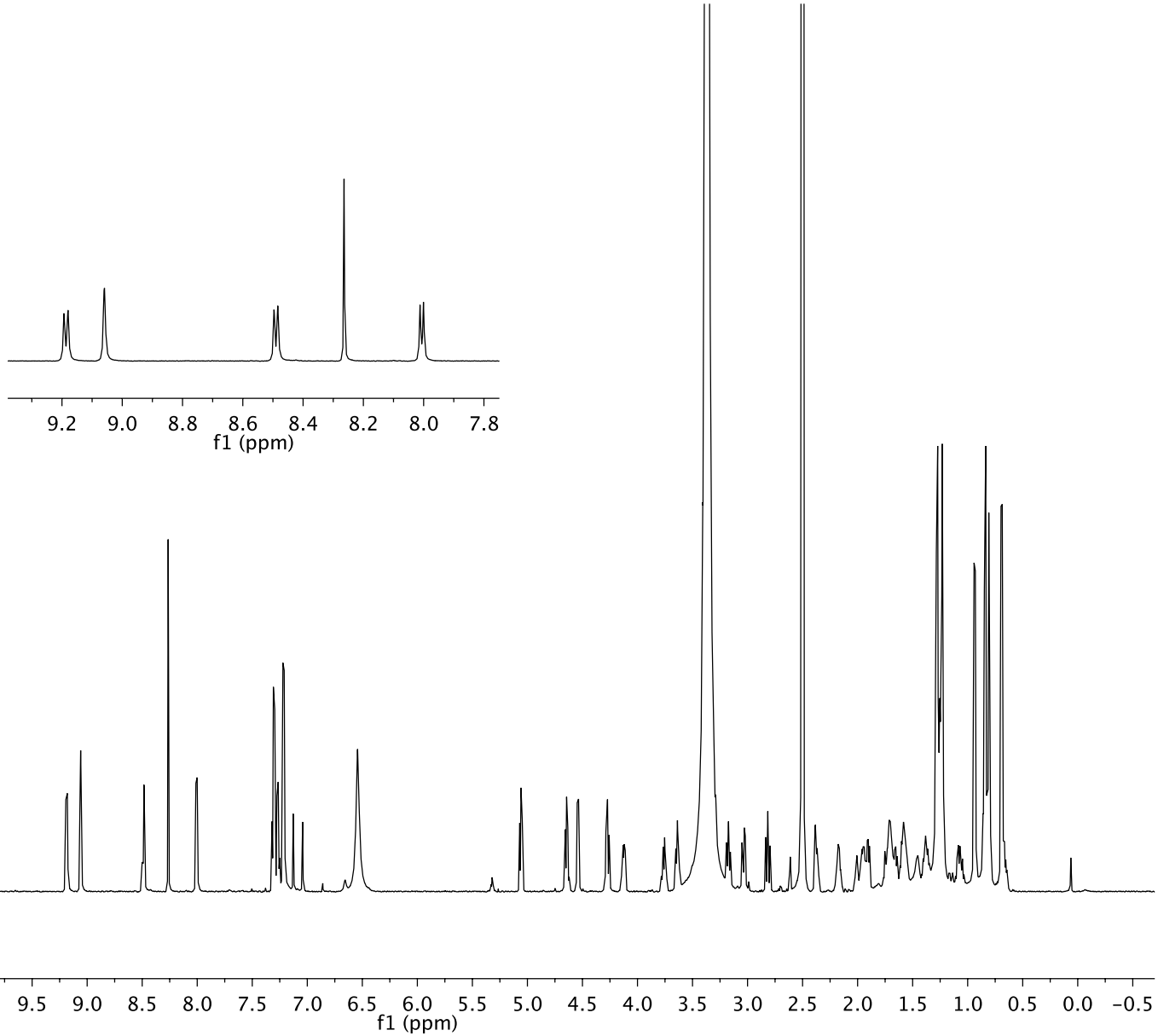
A) Crude thiazolidine **41** as a mixture of diastereomers (0 to 100% B over 25 min, λ = 230 nm). **B)** A single, purified diastereomer of macrocyclic thiazolidine **41** (Rt = 16.0 min, 0 to 100% B over 25 min, λ = 230 nm). **C)** Crude analytical HPLC trace (following filtration) of the formation of **43** upon oxidation with MnO_2 (0 to 100% B over 25 min, λ = 230 nm).

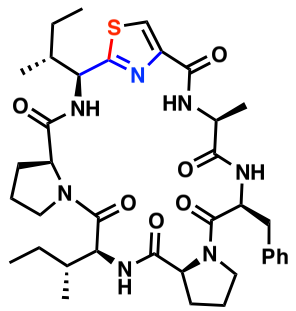
The recorded NMR spectra for synthetic sanguinamide A (**43**) are in agreement with literature data.[16]



43

^{1}H, DMSO-d$_{6}$, 600 MHz



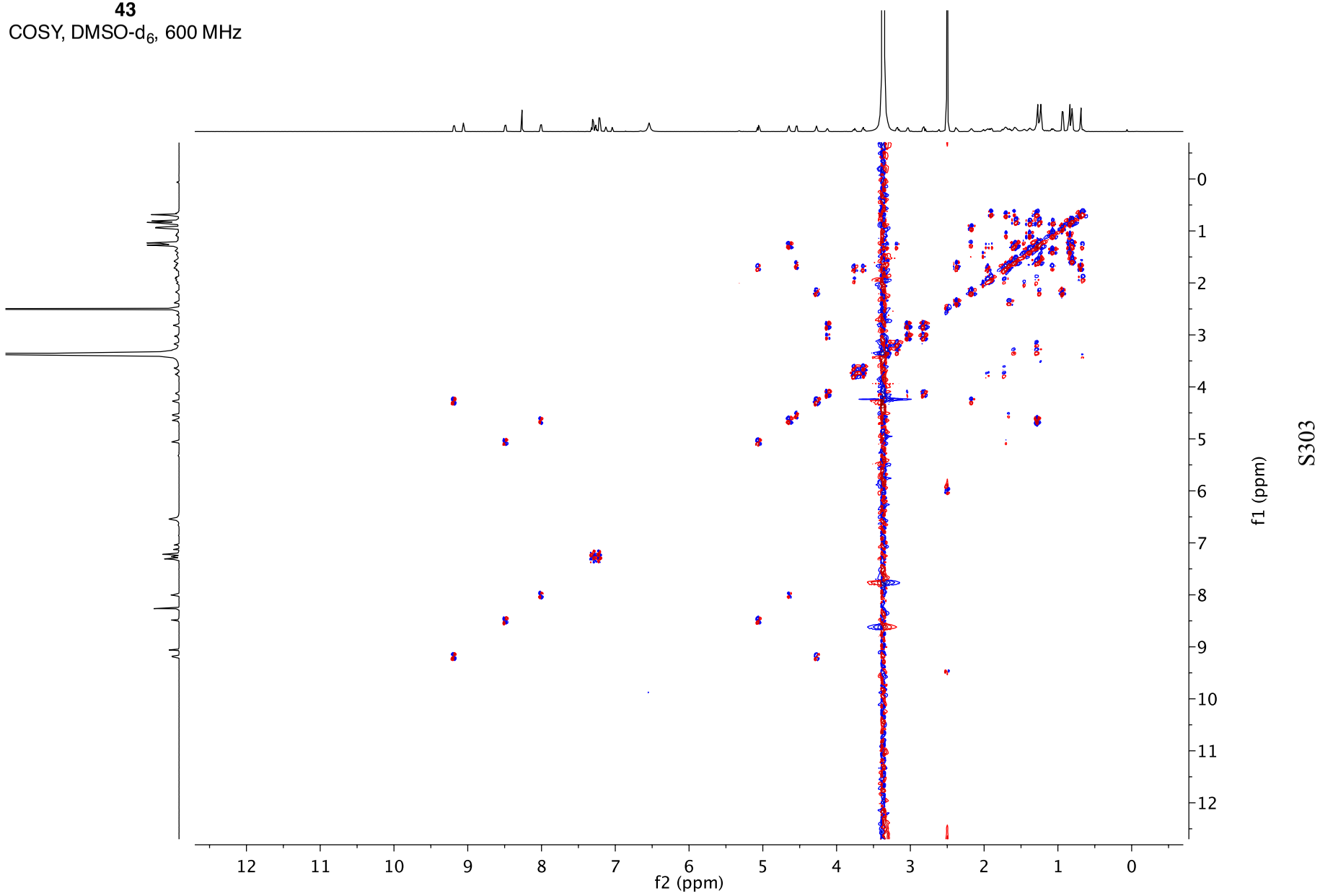


43

^{13}C, DMSO-d_6, 151 MHz

43

COSY, DMSO-d_6, 600 MHz



Circular Dichroism

All far-UV CD spectra were acquired at room temperature in 30% (v/v) acetonitrile-d3 using a JASCO J-815 spectropolarimeter. A quartz cuvette with a pathlength of 1 mm was used for all experiments. All spectra are the averages of four repeats, recorded over a range from 250 to 190 nm with each scan recorded at intervals of 0.5 nm and an averaging time of 3 s.

Amide Temperature Coefficient Measurements by NMR

The temperature dependence of amide chemical shifts is well established and has been widely used to assess the solvent shielding properties of amide protons.[17–19] Accordingly, amide proton temperature coefficients were extracted from 1D and TOCSY spectra acquired on a Bruker Avance III 600 MHz spectrometer. Spectra were measured at 282, 291, 300 and 309 K and referenced to acetonitrile at 1.968 ppm. Assignments of the amide protons were made using conventional 2D NMR approaches.

Temperature coefficients are categorized as the following; $\Delta\delta_{HN}/\Delta T$ with values less than -4.6 ppb/K indicate water-exposed NHs. Intermediate values from –4.6 to –3.0 ppb/K indicate intermediate shielding and potentially weak or strained hydrogen bonding. Whereas $\Delta\delta_{HN}/\Delta T$ values greater than –3.0 ppb/K place NHs in the highly shielded and potentially strongly hydrogen bound category.[20–22] This information is summarized in the **Table S1**.

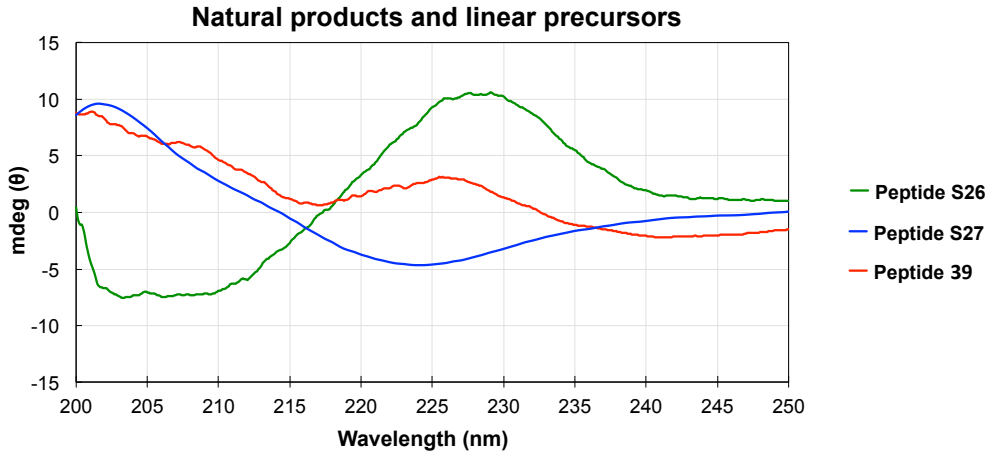
TOCSY spectra of peptides

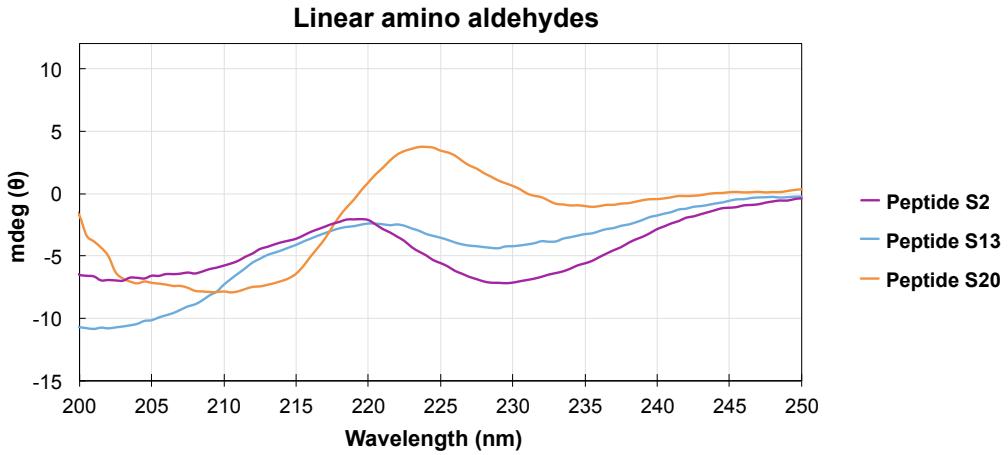
The TOCSY spectra presented in this supplementary information were acquired at 291 K in 30% (v/v) acetonitrile-d3 with a mixing time of 80 ms.

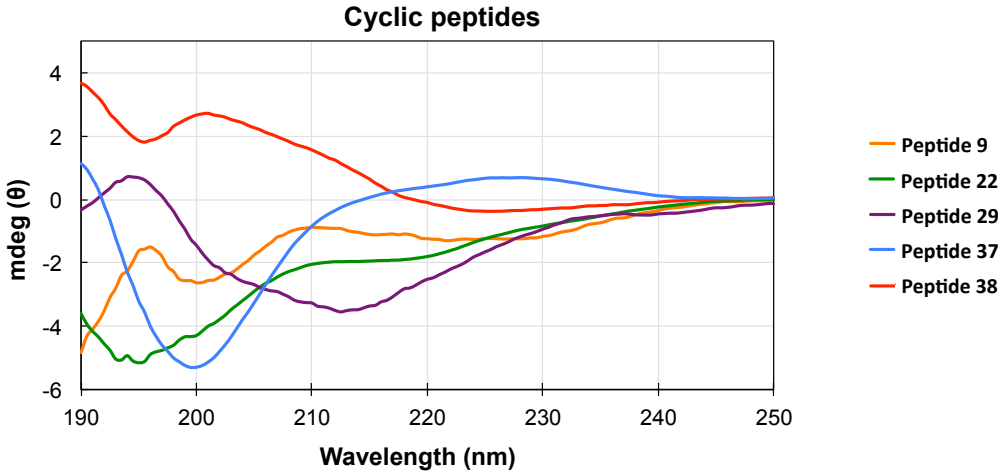
N-H Shift (ΔδHN/ΔT)	Structural Implications
> –3.0 ppb K^{-1}	strong shielding or H-bonding
< –4.6 ppb K^{-1}	solvent exposed
< –3 and > –4.6 ppb K^{-1}	weak/strained shielding or H-bonding

Table S1. Amide proton chemical shift values, $\Delta\delta_{HN}/\Delta T$, and their corresponding properties.

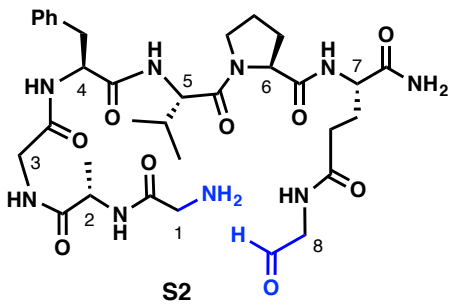
Circular Dichroism Studies







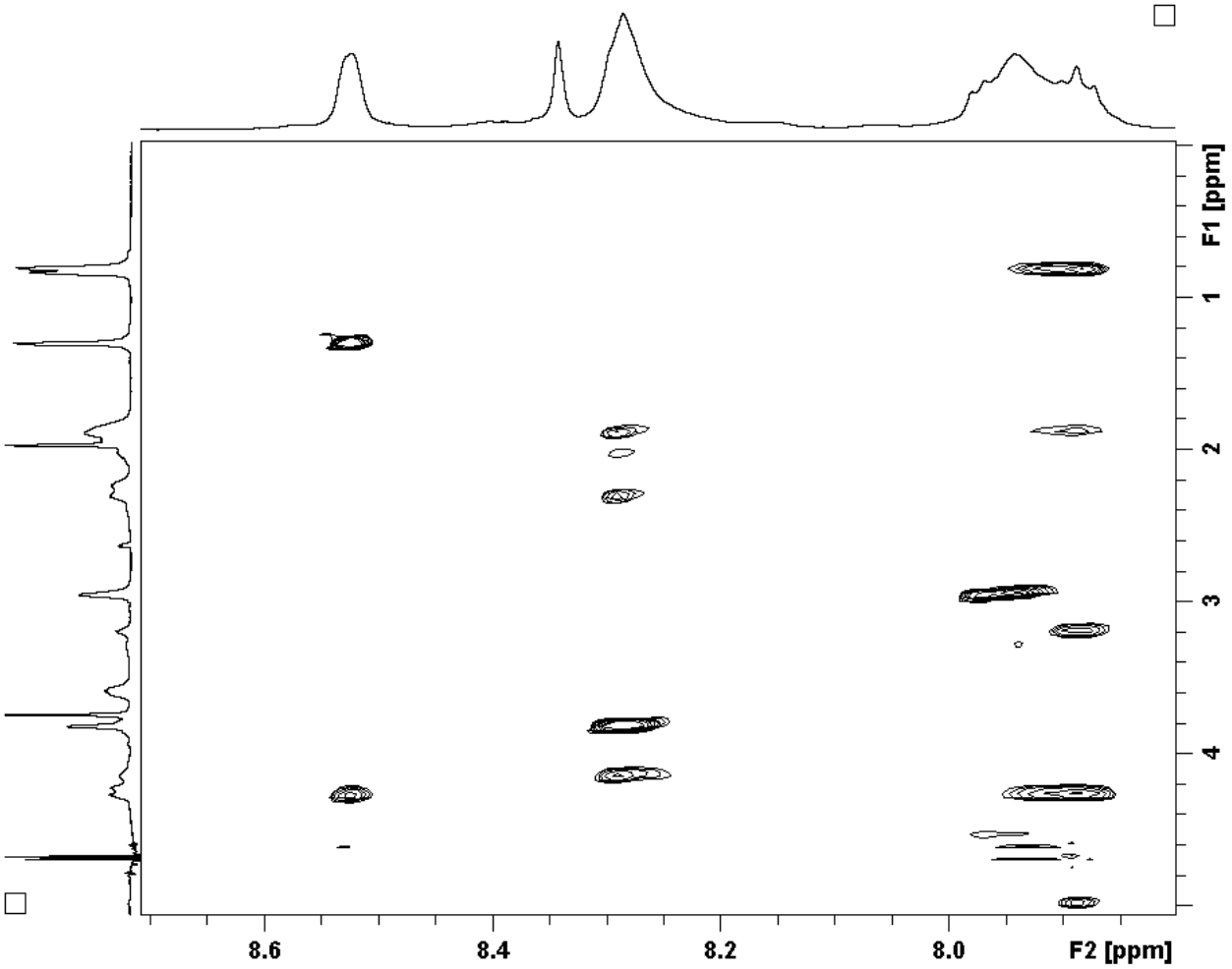
Variable Temperature NMR Studies

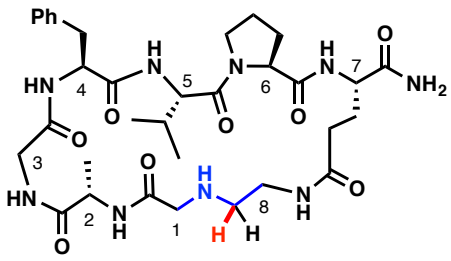


S2

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Gly1	
Ala2	−6.10
Gly3	−6.87
Phe4	−5.70
Val5	−6.81
Pro6	
Glu7	−8.25
Glu7b	−8.04
Gly8	

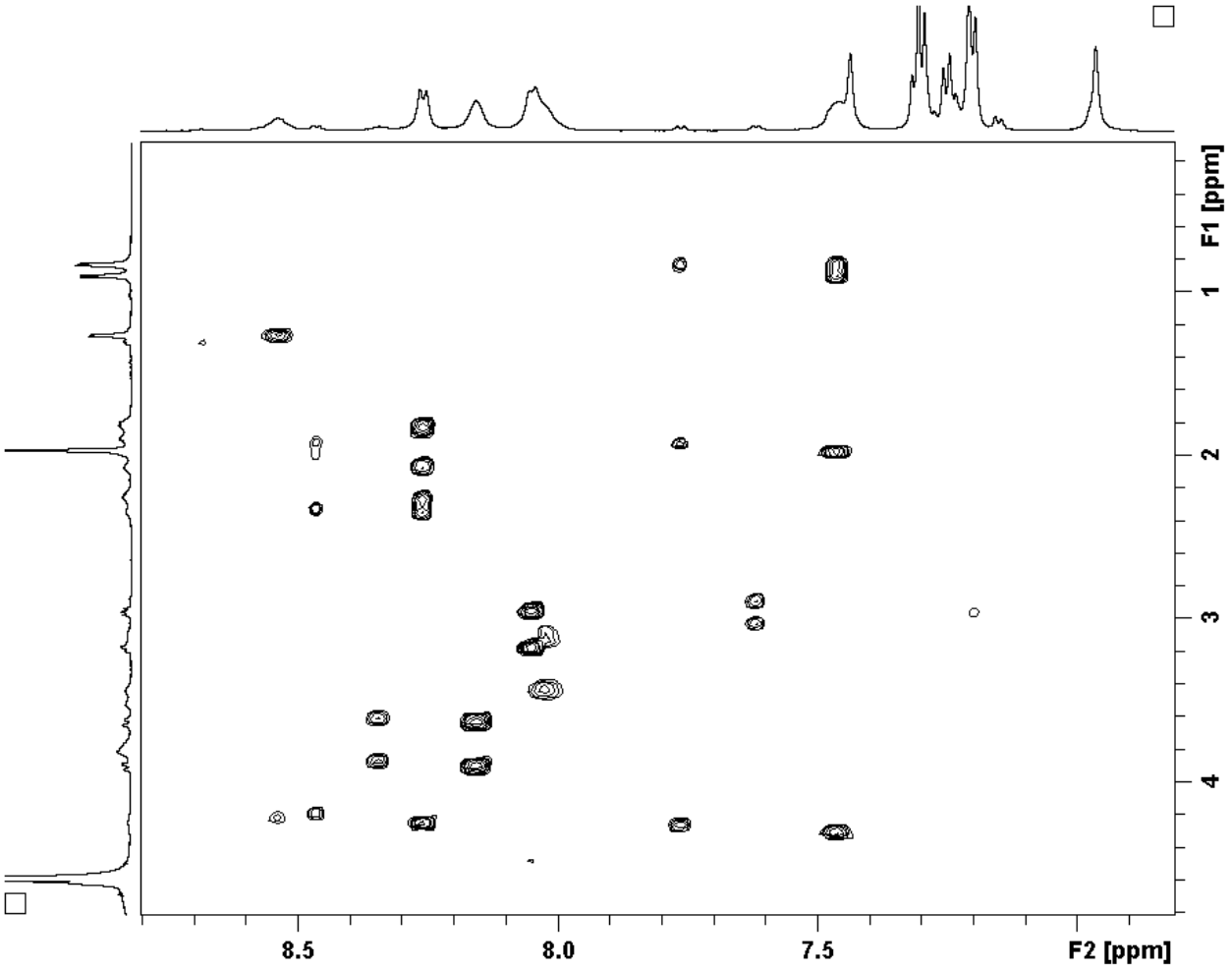


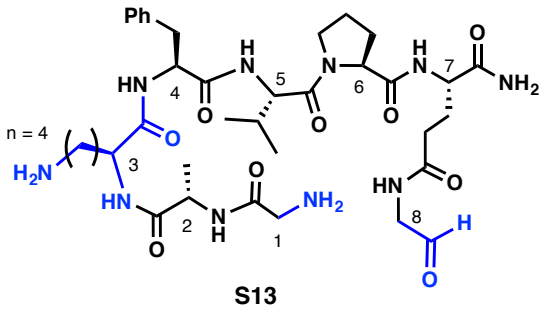


9

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Gly1	–5.36
Ala2	–5.88
Gly3	–5.80
Phe4	–6.09
Val5	–4.58
Pro6	
Glu7	–6.72
Gly8	–5.88
Val5 minor	–4.83
Phe4 minor	–3.56

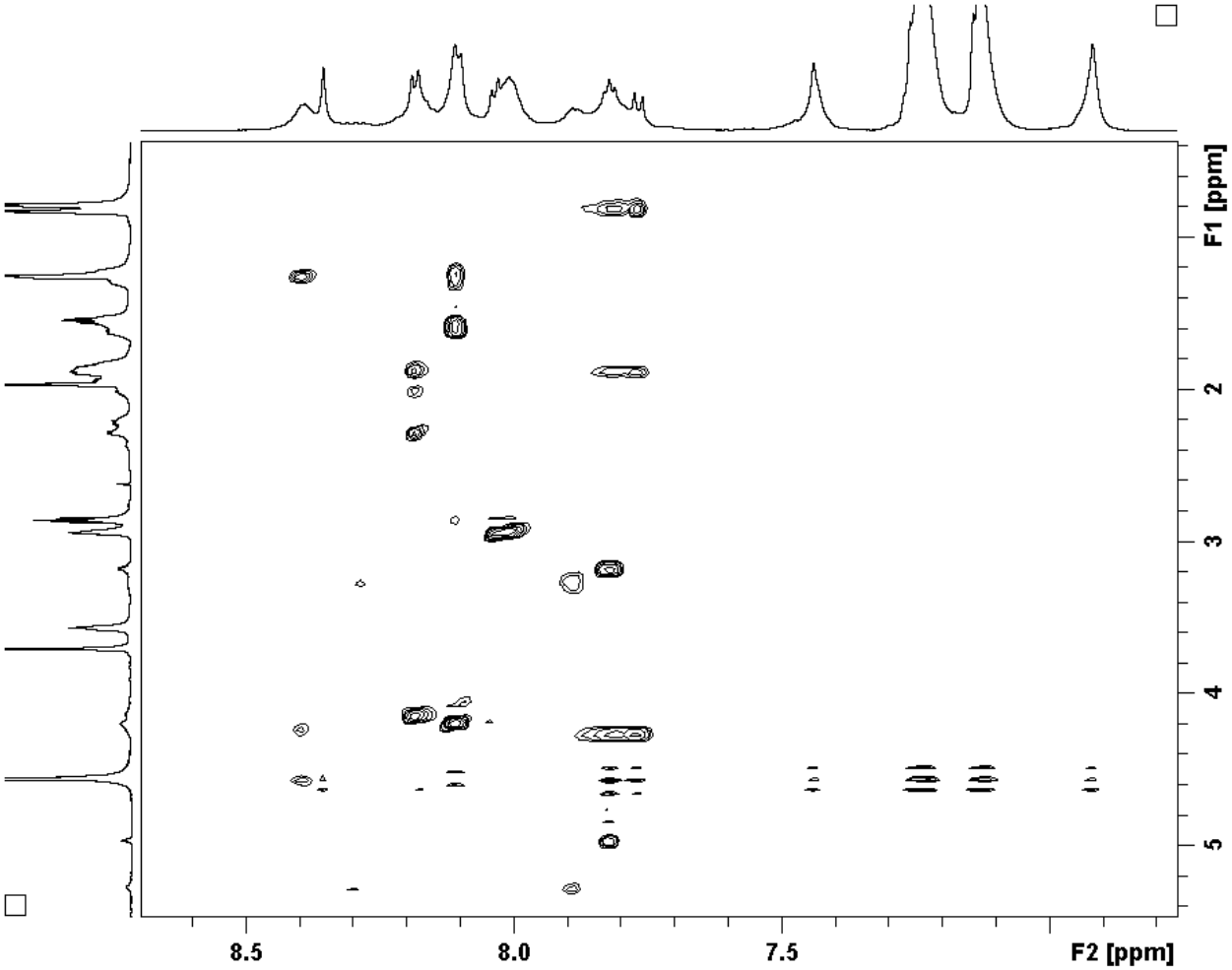


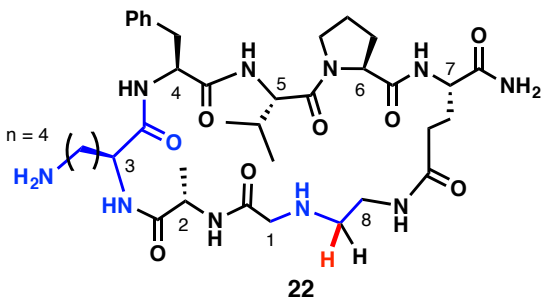


S13

Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Gly1	−6.47
Ala2	−5.59
Lys3	−6.69
Phe4	−6.65
Val5	−9.32
Pro6	
Glu7	−7.13
Gly8	−6.35

TOCSY, 30% (v/v) MeCN-d$_3$, 600 MHz

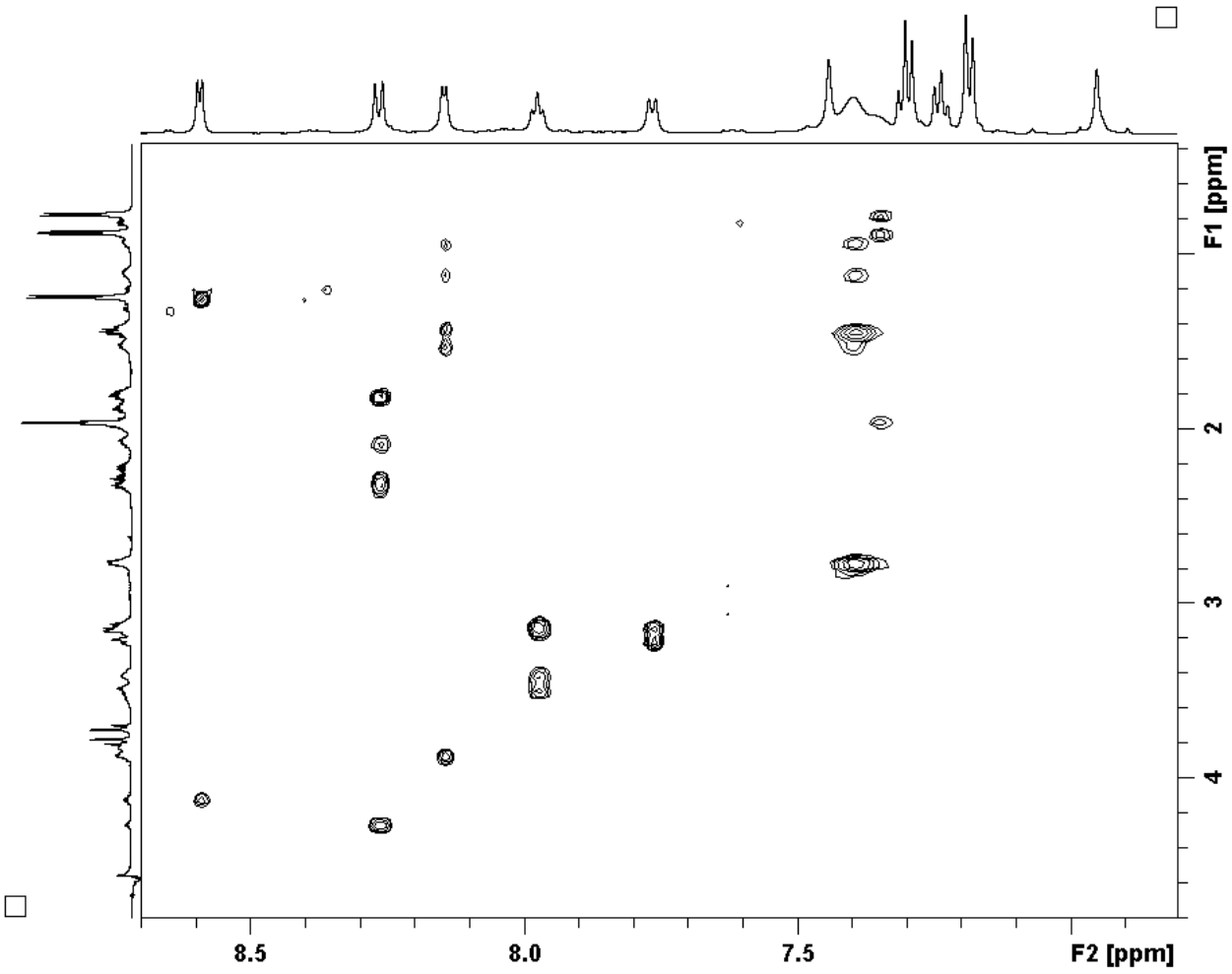


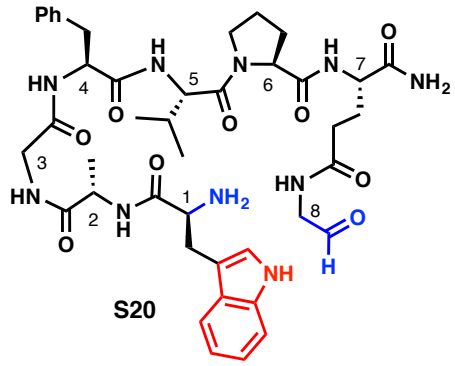


22

Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Gly1	–4.84
Ala2	–5.61
Lys3	–5.80
Phe4	–8.01
Val5	–2.92
Pro6	
Glu7	–7.02
Gly8	–4.90

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

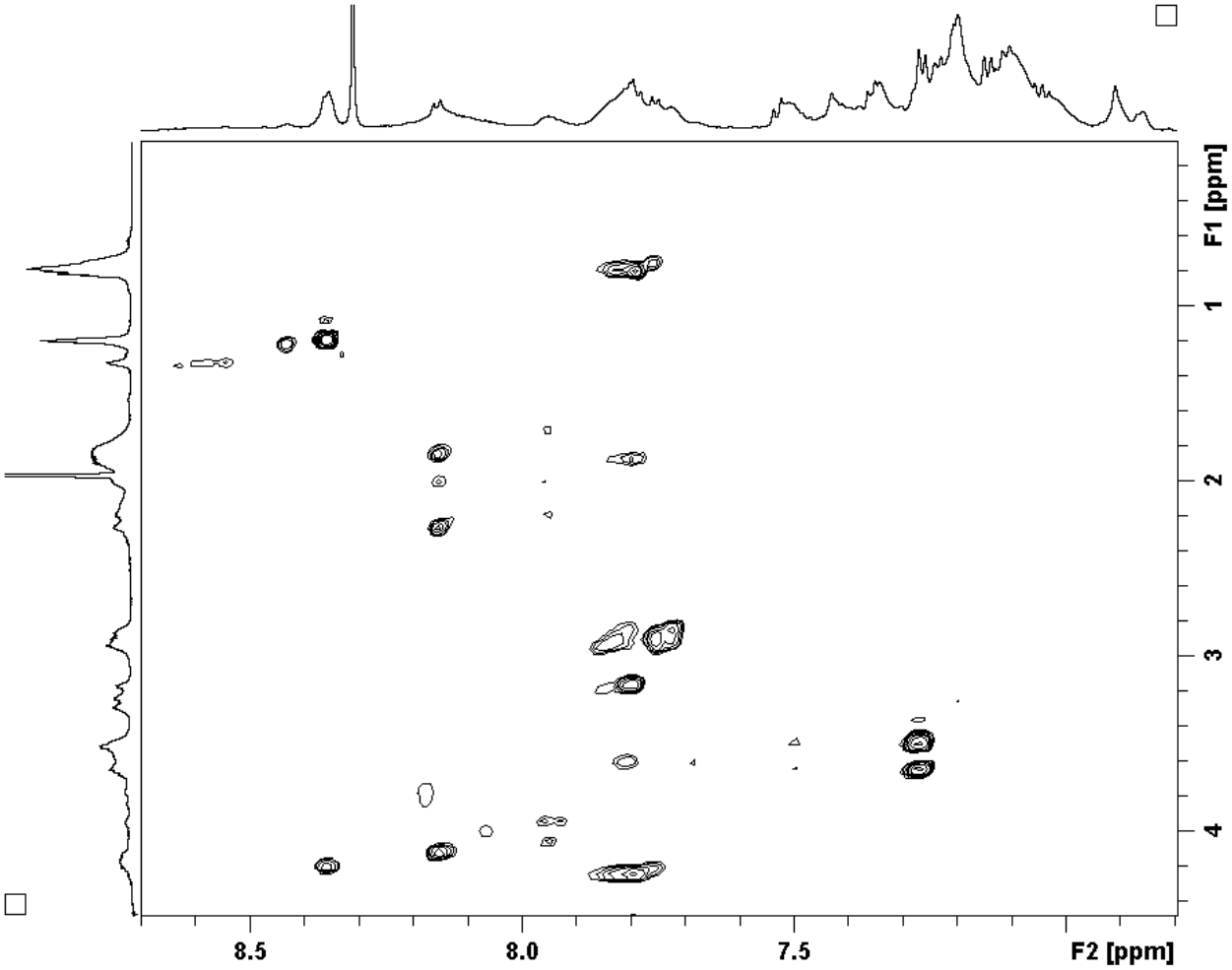


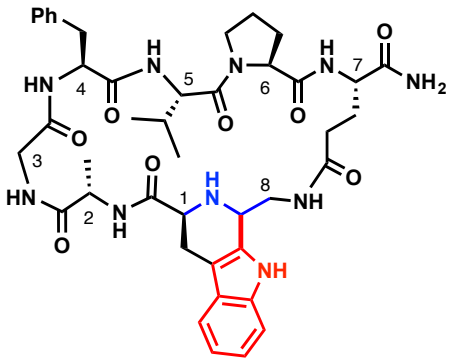


S20

Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Trp1	−6.49
Ala2	−6.71
Gly3	−5.87
Phe4	−1.97
Val5	−5.98
Pro6	
Glu7	−7.28
Gly8	−6.31

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

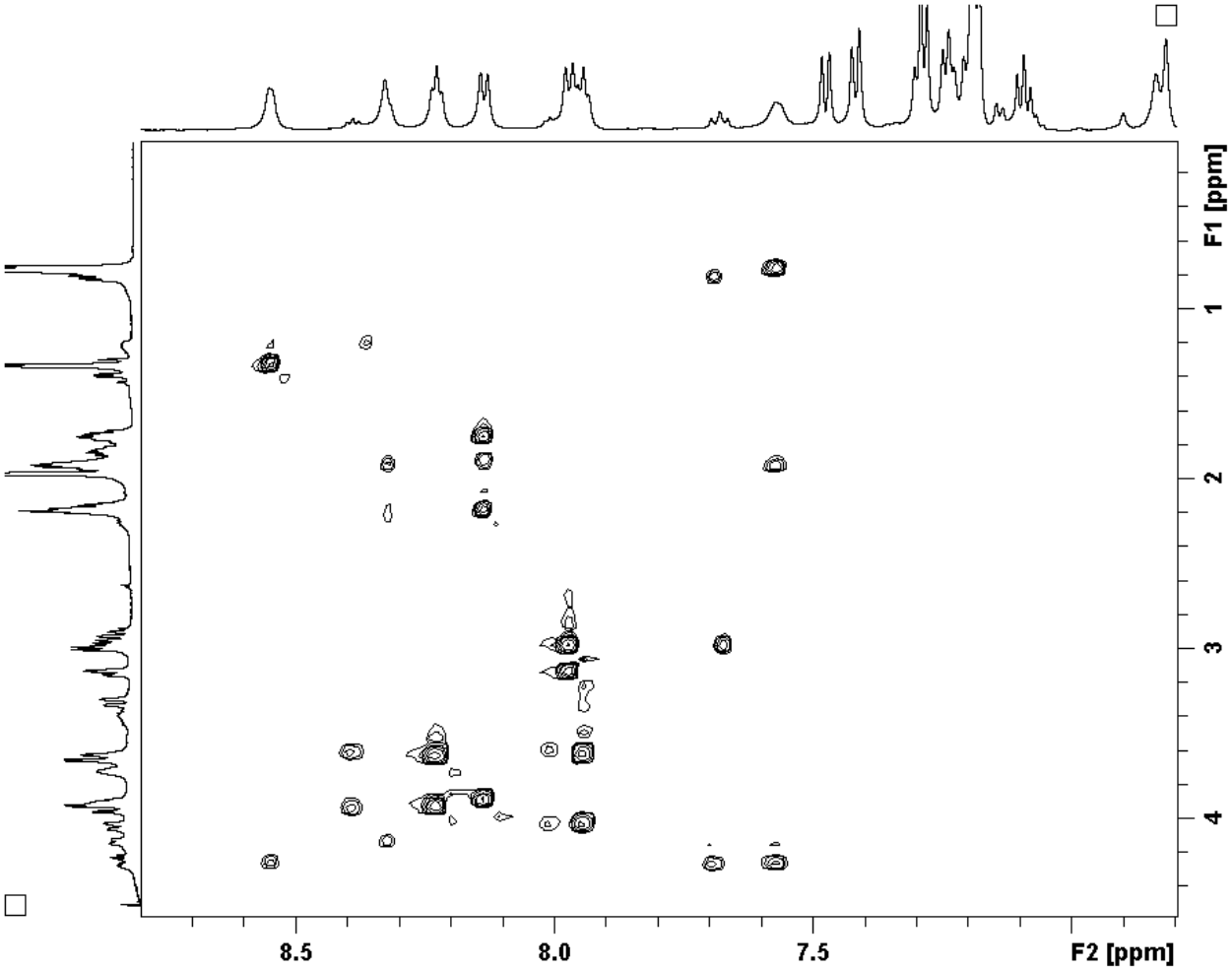


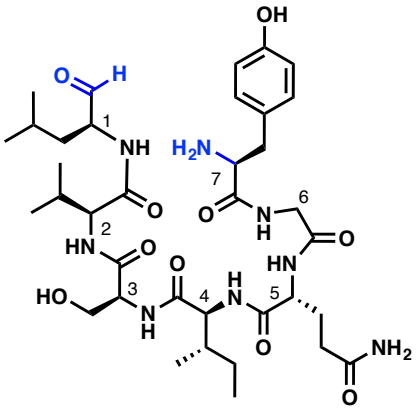


Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Trp1	−5.34
Ala2	−6.17
Gly3	−4.90
Phe4	−4.94
Val5	−5.49
Pro6	
Glu7	−5.98
Gly8	−5.81

29 (major diastereomer)

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

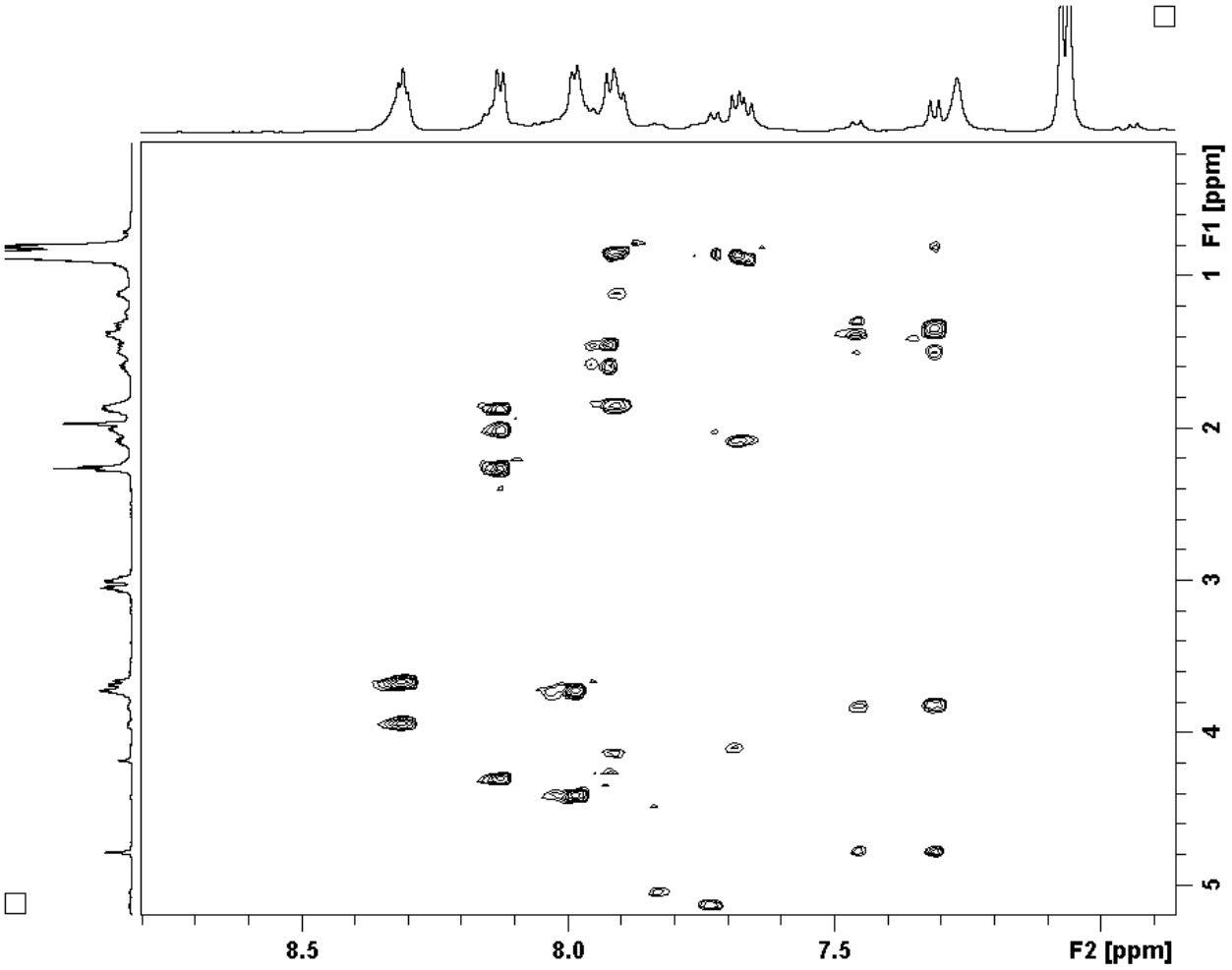


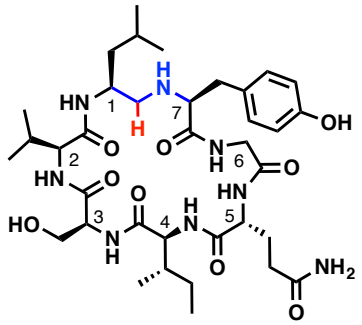


Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Leu1	−4.96
Val2	−4.72
Ser3	−4.89
Ile4	−4.61
Gln5	−5.03
Gly6	−5.21
Tyr7	−4.62
Val2, minor	−4.44
Tyr7, minor	−5.07

S26 (scytonemide A aldehyde)

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

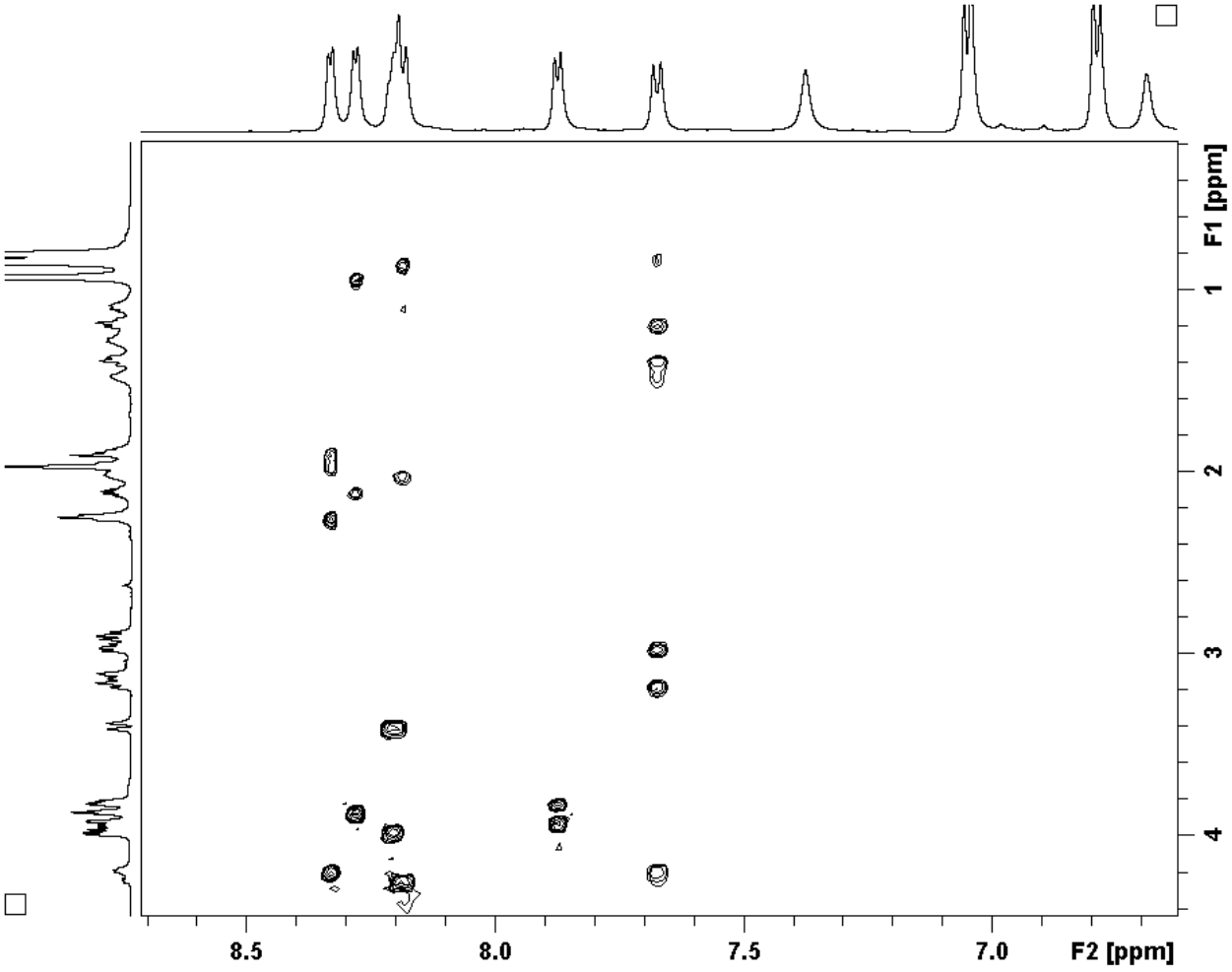


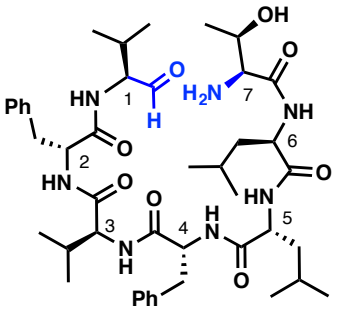


37

Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Leu1	–4.29
Val2	–6.30
Ser3	–2.99
Ile4	–7.59
Gln5	–5.65
Gly6	–3.92
Tyr7	–4.29

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

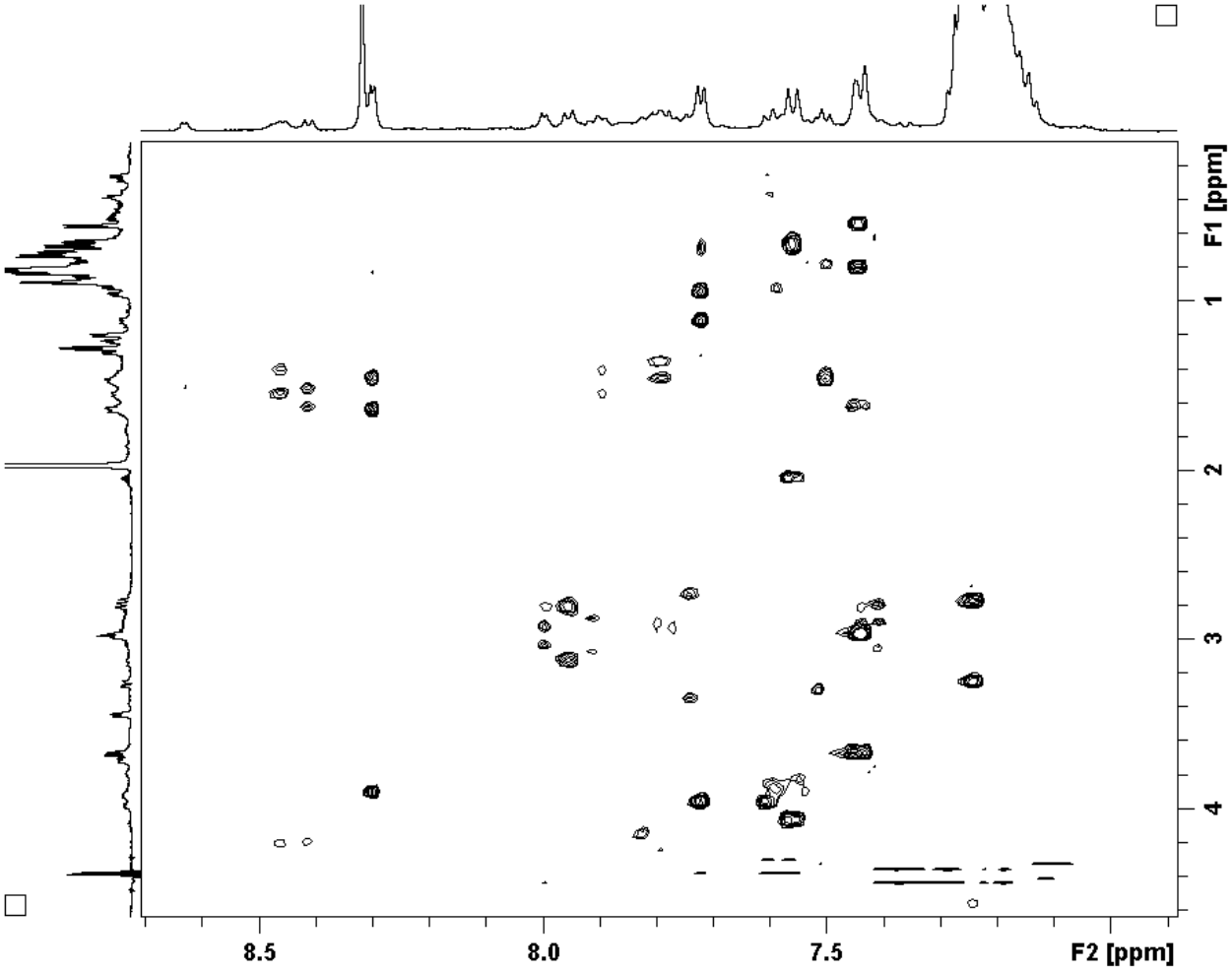


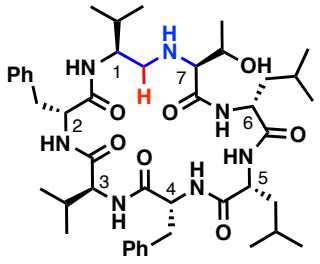


Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Val1	−3.89
Phe2	−2.47
Val3	−2.43
Phe4	−4.25
Leu5	−4.73
Leu6	−5.72
NH2	−6.04

S27 (koranimine aldehyde)

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

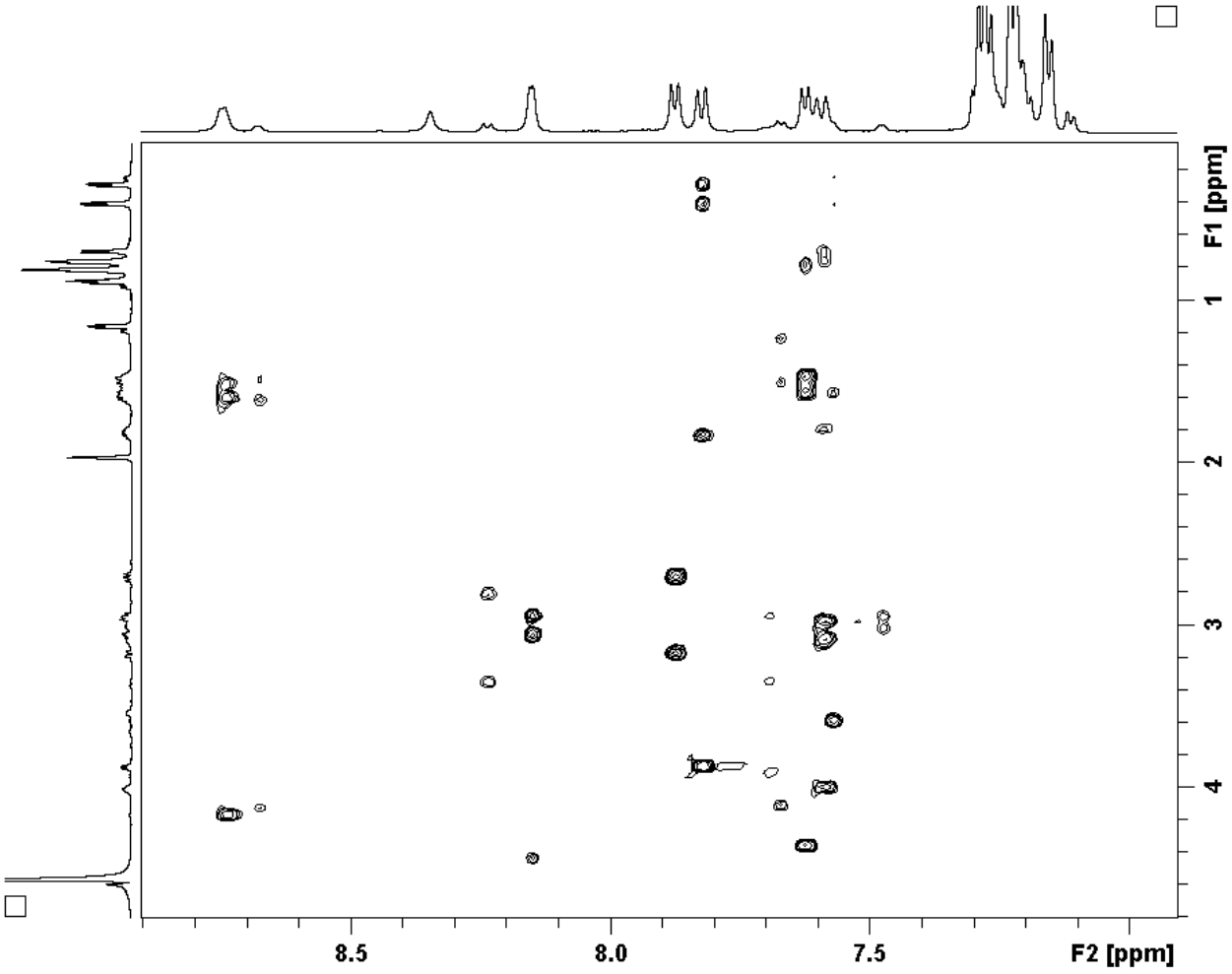


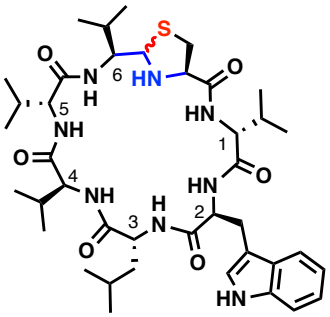


38

Amino Acid	ΔδHN/ΔT (ppb K^{-1})
Val1	−4.40
Phe2	−1.51
Val3	−8.67
Phe4	−9.70
Leu5	−3.85
Leu6	−8.02
Val-OH7	−4.36

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

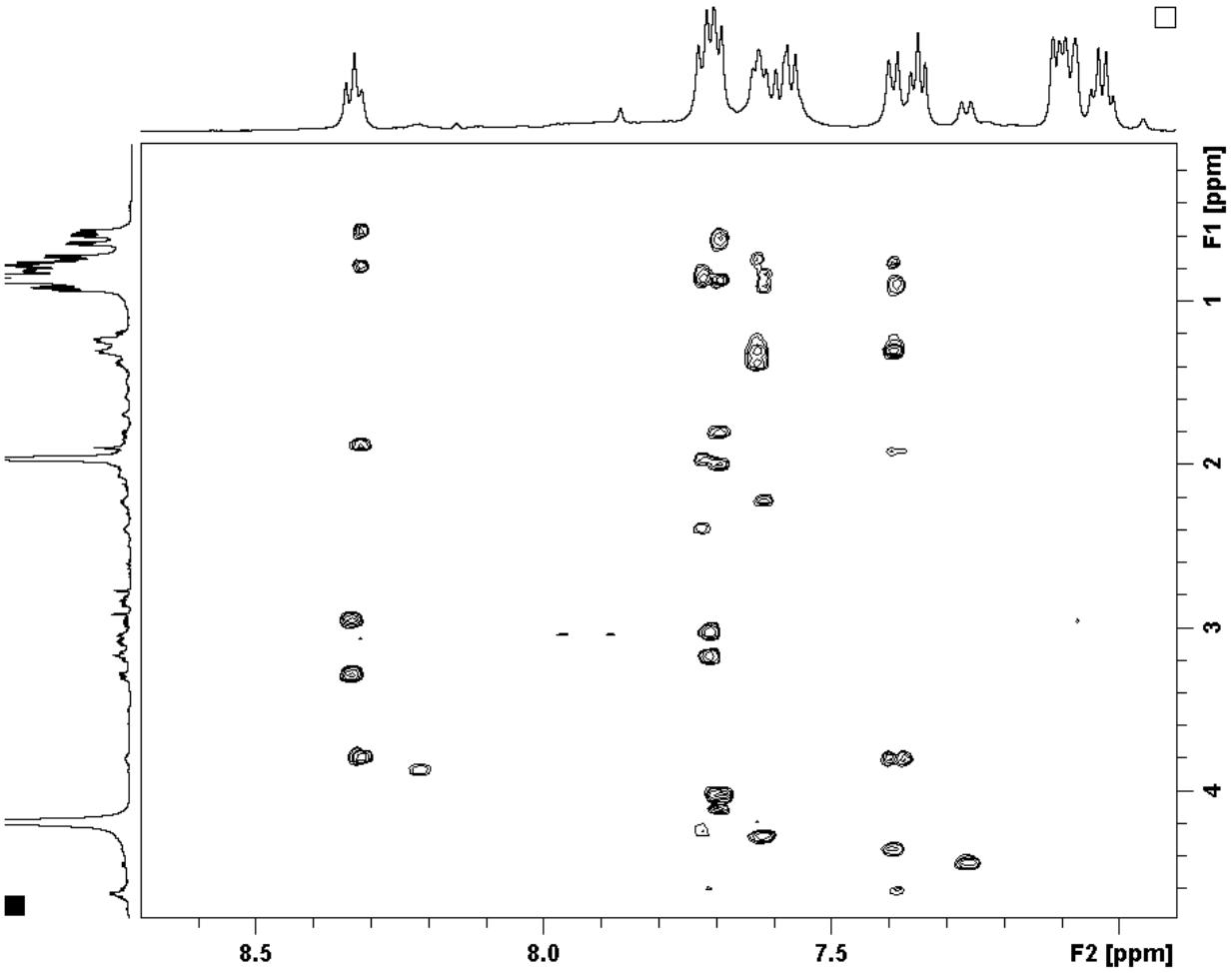




39 (lugdunin)

TOCSY, 30% (v/v) MeCN-d_3, 600 MHz

Peak, (Residue)	ΔδHN/ΔT (ppb K^{-1})
1 (Leu3 iso 1)	−2.90
2 (Trp2 iso 1)	−5.24
3 (Thia iso 1)	−5.32
4 (Trp2 iso 2)	−5.31
5 (Leu3 iso 2)	−2.28
6 (Val)	−2.35
7 (Val1 iso 1)	−5.47
8 (Val1 iso 2)	−5.00
9 (Val)	−5.07
10 (Thia iso 2)	−2.97
11	−5.39



Troubleshooting and Frequently Asked Questions

A. Aldehyde Synthesis

Question 1: How were the peptide sequences chosen?

Answer: While many of the substrate structures were inspired by natural products, a number of the peptides were designed through the use of the *Random Peptide Sequence* tool provided by The Sequence Manipulation Suite. See **Random Peptide Sequence Generator** (p. S9) for more information.

Question 2: How do you prepare peptide aldehydes?

Answer: Although there are multiple ways to prepare peptide aldehydes for the imine-mediated macrocyclization strategy, we prefer direct incorporation of a masked aldehyde (e.g. aminoacetaldehyde dimethyl acetal—see general protocol 1) onto the solid-phase or anchoring of a pre-formed Fmoc-protected amino aldehyde onto "Rink TG" resin (see general protocol 2). Both strategies are described in the general methods. An alternative approach includes the use of a Weinreb amide functionalized resin. However, as this method requires cleavage of the peptide from the resin using a strong reductant ($LiAlH_4$), some sensitive functional groups may pose a problem. Both general methods 1 and 2, on the other hand, allow for release of the target aldehyde upon acidic cleavage from the resin. Although not employed in this study, backbone amide linker (BAL) technologies may also be readily employed for the preparation of C-terminally modified peptides, including peptide aldehydes.[23]

Question 3: Do you see epimerization of C-terminal aldehydes with α-chirality?

Answer: Epimerization of chiral aldehydes is an important factor to consider. Loading amino aldehydes via an oxazolidine linkage onto TG-resin in some cases may lead to epimerization. We observed a small amount of epimerization in the preparation of aldehyde **S12**, a pentapeptide bearing a D-Leu aldehyde at the C-terminus. Nevertheless, this minor component was separable by HPLC, thus providing homogeneous material based on NMR analysis of the purified amino aldehyde. Generally, when dealing with aldehydes with α-chirality, care should always be taken when purifying and analyzing the crude peptide.

Furthermore, the purified material should be evaluated by NMR prior to use in a macrocyclization reaction.

Question 4: How do you store the aldehydes?

Answer: We generally store the aldehydes as lyophilized solids at –20 °C. They are generally stable, but over time will start to oligomerize. This process is not necessarily deleterious to the outcome of a macrocyclization reaction—the reversibility of oligomer formation allows re-equilibration under the dilute reaction conditions to afford good yields of the desired monomeric macrocycle. For a direct example of this reversible phenomenon, refer to the one-pot Pictet-Spengler-oxidation approach to afford peptide **30**. In this example, application of an aldehyde that had been stored at 4 °C for four months provided nearly identical yields in a Pictet-Spengler macrocyclization reaction as a freshly prepared aldehyde. When using an aldehyde that has oligomerized, we suggest pre-equilibrating the reaction mixture by stirring at 1 mM concentration in the desired reaction solvent prior to the addition of an external nucleophile (e.g. KCN or $NaBH_3CN$).

B. General Macrocyclization Questions

Question 1: How do you monitor the reactions?

Answer: The progress of reactions is most efficiently monitored using LC-MS or analytical HPLC. A small sample of the reaction mixture is diluted in water or MeOH and injected directly into the instrument.

Question 2: How do you purify the crude reaction mixture?

Answer: Preparative reverse-phase HPLC is the most efficient way to purify the peptide macrocycles. Following elution from the column, pure fractions are combined and concentrated on a lyophilizer to afford the target peptide as a fluffy white solid.

Question 3: What are the major byproducts observed?

Answer: By-product formation generally depends on the type of macrocyclization reaction:

- Strecker macrocyclization: Addition of KCN to the aldehyde to form a cyanohydrin will

often occur in peptides that are not conformationally pre-disposed to cyclization. The reversibility of this addition, however, often allows for the funneling of material to the target α-aminonitrile Strecker product.

- Reductive amination: Reduction of the aldehyde to the corresponding alcohol is the most commonly observed by-product. The extent of aldehyde reduction is highly pH dependent, so various buffer and pH conditions (NaOAc buffers, pH = 3.5–7.5 and/or phosphate buffers pH = 6–8) may be screened.
- Thiazolidine cyclizations: In the case of peptides bearing StBu-disulfide protected Cys residues, incomplete reduction of the starting disulfide may cause the reaction to stall. Adding more reductant (TCEP) and keeping the solution degassed can prevent unwanted disulfide formation and help drive the reaction.
- Selenazolidine cyclization: Selenocysteine can deselenize in the presence of TCEP. Although we add sodium ascorbate to the cyclization mixture to prevent this process, potential cleavage of the C–Se bond should be monitored.

A general by-product that may result from any of the above imine-macrocyclizations is oligomerization (formation of dimers, trimers, etc.). This problem can be remedied by varying the reaction concentration, and specifically, running the reaction at higher dilution. Another possible by-product is erosion of α-chirality in the case of non-Gly C-terminal aldehydes. Since these reactions are mediated by a thermodynamic (equilibrium) process, it is possible that the C-terminal residue may epimerize prior to or during the cyclization event. By observing the effects of the cyclization reaction on the α-center of the peptide aldehyde, valuable information on the structure and predisposition of a peptide to cyclize can be gained.

Question 4: What effect does pH have on the reaction?

Answer: The pH can have a substantial effect on the nature of the aldehyde-imine equilibrium. As discussed above, pH effects may be employed to reduce by-product formation (e.g. aldehyde reduction in the reductive amination reaction). Give the profound effects observed with pH adjustments, we suggest having on-hand a number of potential buffer systems of varying pH, e.g. aqueous NaOAc buffers at various pH values between 3.5 and 7.5, and Na_2HPO_4 buffers between pH 6 and 8. Generally speaking, these buffers can be used interchangeably in any of the

macrocyclization reactions (with the exception of the Pictet-Spengler reactions) and can therefore be used as a tool for optimization.

Question 5: Do any special precautions need to be taken when setting up the reactions?
Answer: The macrocyclizations proceed in open-air flasks and most often under aqueous conditions. As such, they are generally very operationally simple.

References

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