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UNIVERSITY OF CALIFORNIA,
IRVINE

Design and Synthesis of Macrocyclic Peptides for Studying the Supramolecular Assemblies of β-Sheets

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Chemistry

by

Matthew Thomas Klun

Thesis Committee:
Professor James S. Nowick, Chair
Professor Elizabeth R. Jarvo
Professor Gregory A. Weiss

2015

Dedication

To my family:

Thomas Klun

Margaret Klun

Jeffrey Klun

And

Kristin Mead

Thank you for all of your love and support!

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Acknowledgments

I would like to extend my thanks and most sincere gratitude toward my parents. My father nurtured my passion for science and taught me to follow a firm code of ethics. My mother taught me to set goals, pursue them tenaciously, but not to take myself so seriously.

I need to thank Dr. Scott Bur and Dr. Brian O'Brien from Gustavus Adolphus College for inspiring me to pursue a career in chemistry. With their teaching of the core classes, and especially their elective courses, they showed me how exciting chemistry is and how creative one can be in the field. I also have to thank Dr. Steve Miller for being my mentor and my first research advisor. He not only helped me to establish myself as a research scientist, but he also challenged me to put myself out of my comfort zone and establish collaborative efforts.

My time at UCI was amazing and molded me into the scientist I am today. This work would have been impossible without Phil Dennison and John Greaves for the upkeep of the NMR and mass spectrometry facilities, respectively. I would also like to thank Dr. Weiss and Dr. Jarvo for their support and for being on my thesis committee. I wouldn't have been nearly as successful without my fellow group members. In particular, I want to thank Johnny Pham and Ryan Spencer; their mentorship and leadership was invaluable. I also have to thank Kevin Chen. His patience, passion, and stalwart spirit always kept me driving forward, even during the darkest of times.

I must thank my betrothed, Kristin Mead. She is not only the best proofreader I could have ever asked for, she is my best friend. She made a lot of sacrifices, and moved out to California with me during my last year of graduate school after being long-distance for three years. I'm so lucky that you're my companion.

Lastly, I want to thank James Nowick for showing me what it means to be a true scientist. I always admired his passion for sharing knowledge with the world, by being the leader of chemistry outreach, and being a pioneer for the free online lecture series for UCI. He opened my eyes to the beauty of NMR spectroscopy and revealed the importance of honest and thorough data analysis.

Abstract of the Thesis

Design and Synthesis of Macrocyclic Peptides for Studying The Supermolecular
Assemblies of β-Sheets

By

Matthew Thomas Klun

Master of Science in Chemistry

University of California, Irvine, 2015

Professor James S. Nowick, Chair

This thesis describes my work in improving macrocyclic peptides as model systems used
for studying β-sheet assemblies. Chapter 1 details a strategy for synthesizing macrocyclic
peptides bearing N-terminal substituents. This synthetic strategy was applied to a macrocyclic
peptide with two natural sequences of amyloid-β (Aβ) developed by Nowick group member Dr.
Spencer. Two peptides with extended N-termini were synthesized and isolated, one with glycine
as a proof-of-concept and one with glutamine to extend the natural sequence of Aβ.

In Chapter 2, a system modeled from a macrocyclic peptide developed by former Nowick
group member Dr. Khakshoor was explored. Part 1 describes the design of a pair of hetero-
oligomeric peptides which take advantage of favorable aromatic interactions between
phenylalanine and pentafluorophenylalanine. The formation of new aromatic interactions are
observed, however these interactions are not as strong as predicted. In part 2, a sequential peptide
design was performed, exploring which amino acids create a stable hydrophobic core to improve
the oligomerization propensity and stability of Dr. Khakshoor's original model. It was
determined that increasing the hydrophobicity at the N-terminus and in the aromatic core of the
peptide increased the stability of the peptide oligomer by an order of magnitude.

Chapter 1

Synthesis of Macrocyclic β-Sheet Peptides Bearing
N-Terminal Peptide Substituents

Preface

Chapter 1 introduces a synthetic strategy for synthesizing a new class of β-sheet macrocycles bearing N-terminal peptide substituents for studying amyloidogenic diseases. The synthetic strategy not only allows for further elucidation of peptide sequences, but could also be utilized to introduce other functionalities to the peptide macrocycles such as linkers or fluorescent tags.

Peptide macrocycles are composed of two β-strands linked together with ornithine as β-hairpin mimics. In a normal macrocyclic peptide synthesis, a linear peptide is synthesized on resin with Boc-Orn(Fmoc)-OH capping the two sequences.[1] Previously, macrocyclic peptides with N-terminal substituents have been introduced by replacing one of the Boc-Orn(Fmoc)-OH with Cbz-Orn(Fmoc)-OH. Unfortunately, the deprotection of the Cbz protecting group requires the peptide to be cleaved from the resin and the subsequent purification steps decrease the yield significantly.[2, 3]

This new method for adding new peptides to the N-terminus of the peptide includes synthesizing the first strand of the peptide macrocycle, capping the sequence with Fmoc-Orn(Dde)-OH. This allows N-terminal substituents to be added to the peptide while still on the resin, allowing for excess reagent to be reacted with the elongating peptides. The bottom strand of the peptide is then synthesized on resin, simplifying the purification process, decreasing the amount of time required to synthesize peptides, and increasing the overall yield.

The efficacy of this strategy was demonstrated by the synthesis of two macrocyclic peptides with the sequence of Aβ$_{17-36}$ within the macrocycle, and either glycine or glutamine

added to the N-terminus of the macrocycle, with yields comparable to a normal peptide synthesis.[4] These peptides are based on the scaffold developed by Dr. Spencer.

Introduction

Peptide aggregation through intermolecular β-sheet interactions is a common feature of many neurological diseases including Alzheimer's disease.[5] In Alzheimer's disease, the 40-42 amino acid-long peptide amyloid β (Aβ) is in a complex equilibrium. These disordered peptides interact with each other and eventually form β-sheet hydrogen bonding interactions, which are further stabilized through side-chain interactions. These β-sheets then further assemble to create toxic, oligomeric species, and eventually form infinitely repeating patterns known as fibrils.[6-8] Studying peptides that are able to make controlled, soluble oligomeric species can improve our understanding of the toxicity of amyloidogenic disease peptides.

The Nowick group has developed a method for studying soluble β-sheet oligomers using peptide macrocycles, and has been particularly successful in studying Aβ.[9-15] These peptide macrocycles contain two β-strands with peptide sequences 5-9 amino acids long. One of these two β-strands have a specialized N-methylated peptide, or artificial amino acid to block infinite β-sheet aggregation.[4, 16, 17] Lastly, the top and bottom strands of the macrocycle are held together and capped by ornithine which allow the two β-strands to hydrogen bond to each other.[19]

Typically, peptide macrocycles in the Nowick lab are straightforward to synthesize (Figure 1). First, Boc-Orn(Fmoc)-OH is loaded on 2-chlorotrityl resin, and the first β-strand is created by automated solid-phase peptide synthesis, as described by Dr. Spencer. The sequence is capped with a second Boc-Orn(Fmoc)-OH.[4] The synthesis is completed by coupling the second β-strand off of ornithine, and deprotecting the terminal peptide. The linear peptide is then cleaved from the resin using a 20% solution of hexafluoroisopropanol in dichloromethane and dried under vacuum. The linear peptides are then cyclized with 4 equivalents of HOBT and HBTU coupling reagents in dimethylformamide (DMF) overnight. After removing DMF under
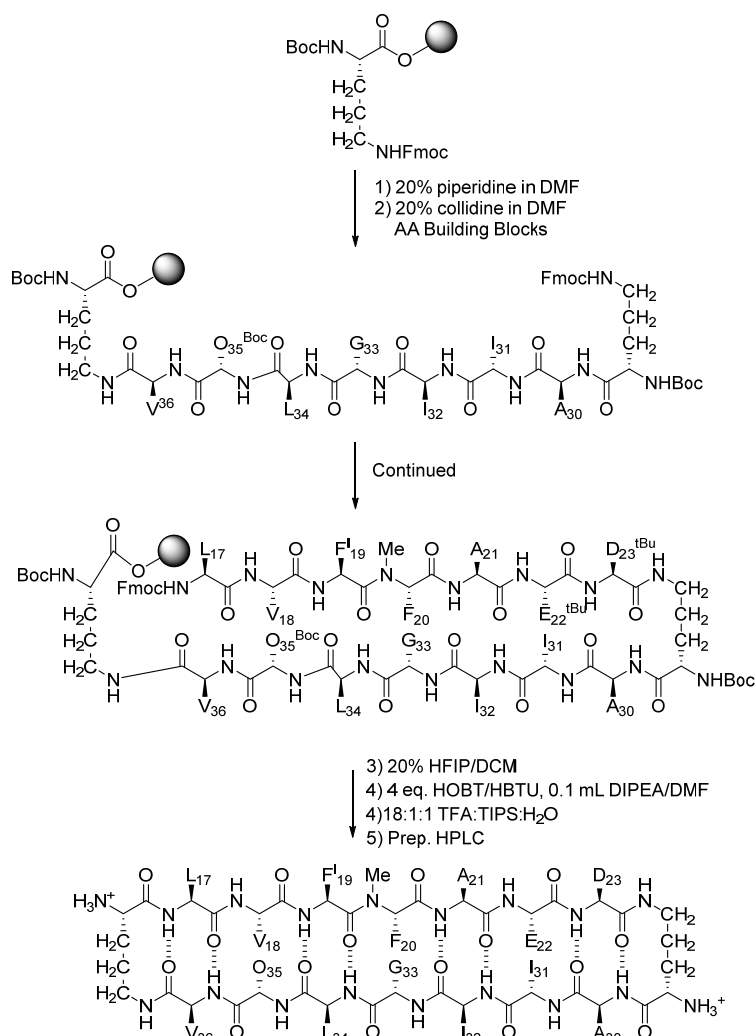
Figure 1. Reaction scheme for synthesizing peptide macrocycles.

vacuum, the peptides are globally deprotected with a 18:1:1 solution of trifluoroacetic acid (TFA):triisopropylsilane(TIPS):H_2O. The cyclized peptide is lastly purified via preparative HPLC, and lyophilized. Typical peptide yields are 15-25% based on 0.1 mM loading, yielding roughly 20-30 mg of purified material.[1]

Recently, Nowick group member Dr. Spencer developed peptide **1**, containing two heptapeptide sequences of Aβ (Figure 2). The top strand contains Aβ$_{17-23}$, with an N-methyl group on Phe$_{20}$ in order to block infinite β-sheet aggregation, and Phe$_{19}$ was replaced with iodo-phenylalnine for experimental phasing with X-ray crystallography.[4] The bottom strand contains Aβ$_{30-36}$ with Met$_{35}$ replaced with ornithine to make the peptide more soluble. Crystallographic studies of peptide **1** reveal a trimeric structure. This trimeric model was applied to natural Aβ$_{17-36}$ in a computational Replica-Exchange Molecular Dynamics study (REMD), and was found to be a stable soluble oligomer (Figure 3).
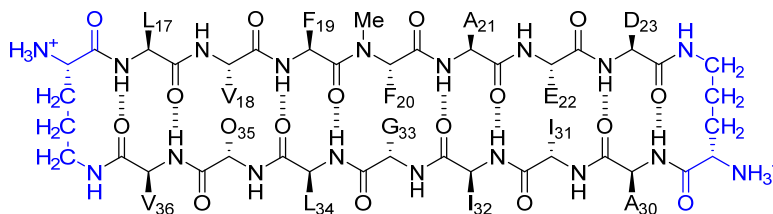
Figure 2. Peptide **1** with ornithine turns highlighted in blue. The top strand contains the sequence Aβ$_{17-23}$, F$_{20}$ N-methylated. The bottom strand contains the sequence Aβ$_{30-36}$ M$_{35}$O.
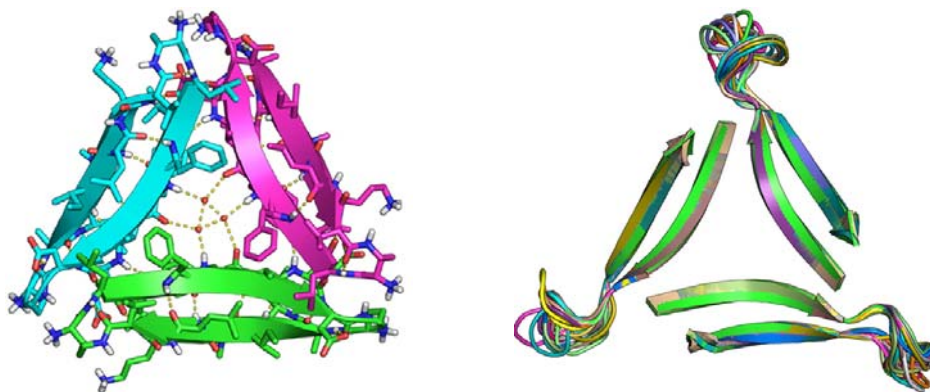
Figure 3. (Left) Crystallographic structure of peptide **1**, showing the oligomeric trimer. (Right) A cartoon representation of 20 low-energy conformers of Aβ$_{17-36}$ from REMD studies. Both figures are from Dr. Spencer's JACS 2014 paper.

It appears that this trimeric peptide model can accommodate more of the natural peptide sequence. Instead of increasing the size of the macrocycle, I decided to add peptides to the N-terminus of one of the strands. The macrocyclic model induces β-sheet interactions on the inside of the peptide, and peptides which are connected to the outside of the macrocycle could either extend the β-sheet, or introduce a new domain. This introduces the potential for the creation of more natural Aβ-models.

Previously in the Nowick group, peptides decorated with N-terminal peptide substituents were synthesized by capping the β-strands with Cbz-Orn(Fmoc)-OH replacing Boc-Orn-Fmoc-OH (Figure 4).[2, 3] With this method, in order to add N-terminal substituents, the linear peptide needs to be cleaved from the resin and cyclized. The Cbz protecting groups are then deprotected by Pd/C under a hydrogen atmosphere. This method is successful in creating the desired peptides, however the peptide yield suffers significantly compared to a normal synthesis, with 9% yield, based off of a 0.2 mmol loading yielding 24 mg of material.[2]

Results and Discussion

Designing macrocycles with N-terminal substituents: I proposed a new method to synthesize peptides with N-terminal substituents by incorporating other orthogonally protected ornithines. This allows the substituents to be added to the N-terminus of a β-strand while the peptide is still attached to the resin. I attempted the synthesis of these peptides with three different versions of ornithine with the α-amine protected with Fmoc for the elongation of the N-terminus. After the addition of the substituents to the N-terminus of the peptides, the δ-amine of ornithine would be deprotected in order synthesize the second β-strand of the peptide macrocycle. There were two requirements for the protecting group on the δ-amine: The protecting group needs to be unaffected by acid and base deprotection common in normal peptide synthesis. The protecting

group also must be facile to remove while the peptide is still on the resin. I attempted the synthesis with Alloc, ivDde, and Dde protecting groups on the δ-amine of ornithine (Figure 4).
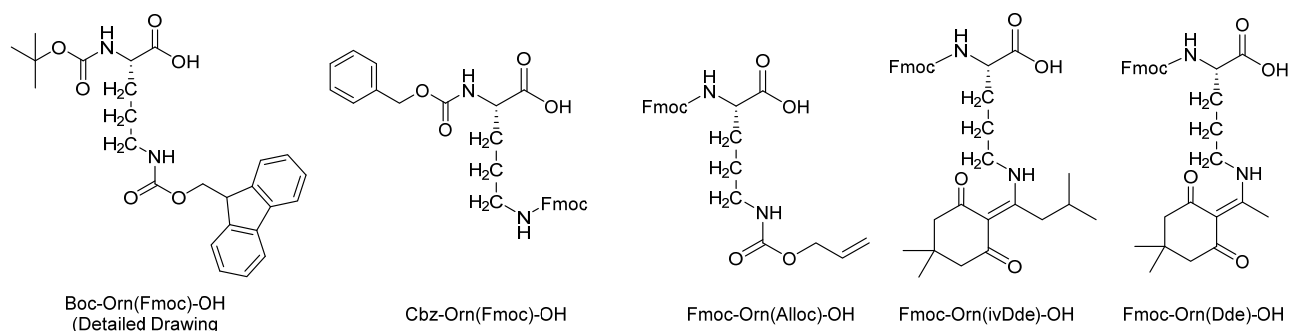
Figure 4. Structures of the various ornithine building blocks used in the synthesis of macrocyclic peptides. Fmoc-Orn(Alloc)-OH, Fmoc-Orn(ivDde)-OH and Fmoc-Orn(Dde)-OH were studied in this work.

Alloc as the protecting group: The first δ-amine protecting group of ornithine I tested was Alloc. The deprotection cocktail of Alloc requires $Pd(PPh_4)_4$ with phenylsilane as a cation scavenger to prevent addition of the allyl group to the free amine. While this normally would be an effective, inexpensive choice for a deprotecting group, I was attempting to design a synthetic scheme which could be universally applicable. Currently iodophenylalanine and bromophenylalanine are often incorporated into peptide macrocycles for solving X-ray crystallographic phasing. The palladium reagent is extremely reactive with these aryl halides. According to mass spectrometric studies, the deprotection of Alloc in peptides containing iodophenylalanine is sluggish and the iodine is removed, making X-ray crystallographic phasing more difficult (Figure 5).
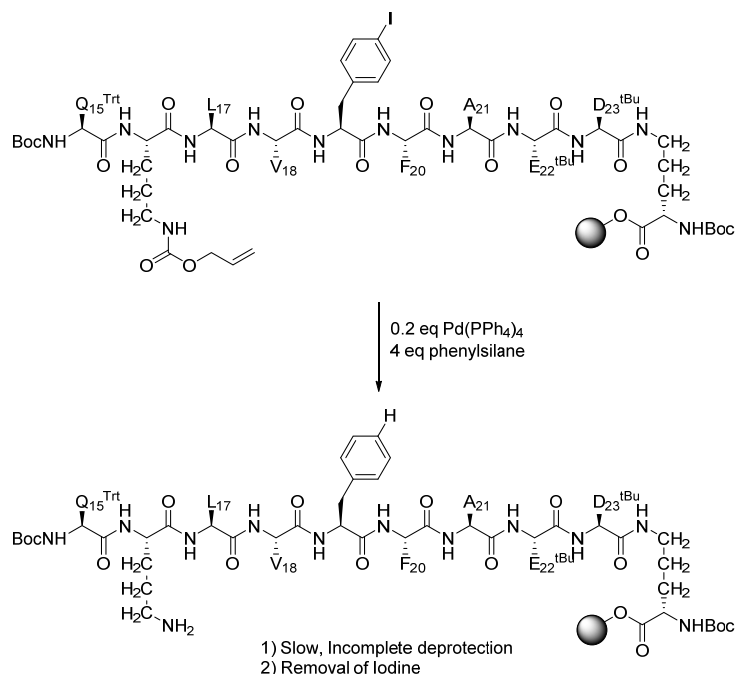
Figure 5. The removal of iodine from iodophenylalanine with the Alloc deprotection cocktail.

ivDde as the protecting group: The second set of protecting groups that I attempted to use were Dde derivatives. The deprotection protocol is fairly mild with dilute hydrazine in DMF. I attempted the synthesis of peptides with N-terminal substituents with ivDde-protected ornithine, since literature stated that the ivDde was more stable to the conditions of deprotection in normal peptide synthesis.[18] Unfortunately, when I attempted to remove ivDde from the peptide, I found that the typical deprotection protocol of 2% hydrazine in DMF was extremely slow and incomplete. When the concentration of hydrazine was increased to 10%, I observed peptide deletions as well as an unidentified addition to the peptide.

Dde as the protecting group: In the end, I was able to synthesize two peptides with N-terminal substituents based off peptide **1** using Fmoc-Orn(Dde)-OH. Peptide **2** was synthesized with glycine added to the N-terminus of Aβ$_{17-23}$ to test my concept with minimal perturbation to the overall structure of the macrocycle. Peptide **3** was synthesized with glutamine added to the N-terminus to extend the natural sequence to Aβ$_{15-23}$ with the ornithine replacing Lys$_{16}$ (Figure 6).
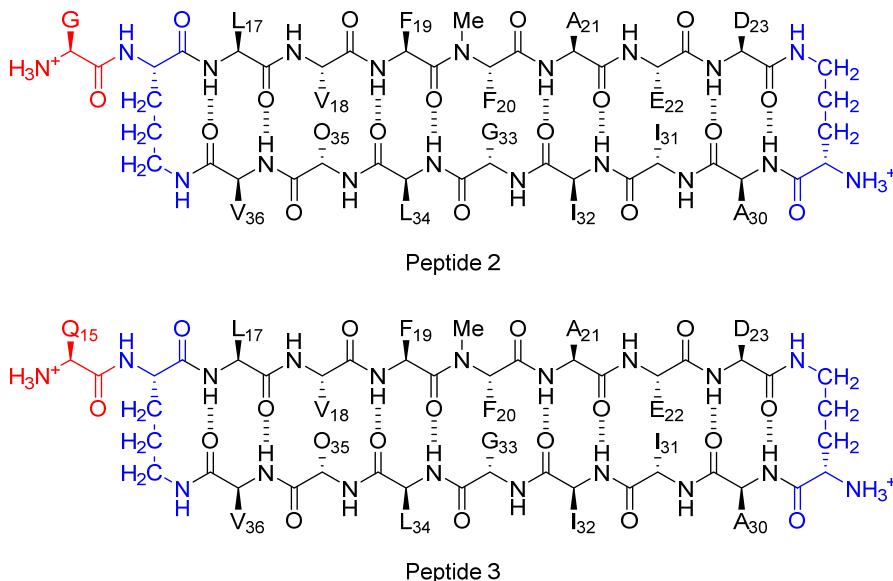
Peptide 2

Peptide 3

Figure 6. Peptides **2** and **3** with ornithine turns highlighted in blue and the extended peptide attached to the macrocycles highlighted in red. The top strand with Aβ$_{15-23}$, F$_{20}$ N-methylated, K$_{16}$O and the bottom strand with Aβ$_{30-36}$ M$_{35}$O.

*Preventing epimerization***:** An important aspect of the design of these peptides is that the ornithine attached to the resin must be Boc-Orn-(Fmoc)-OH. The α-amine of the ornithine attached to the resin is responsible for the cyclization of the peptide. If any amino acids are attached to this C-terminal ornithine, the peptide is susceptible to epimerization during the cyclization step (Figure 7).
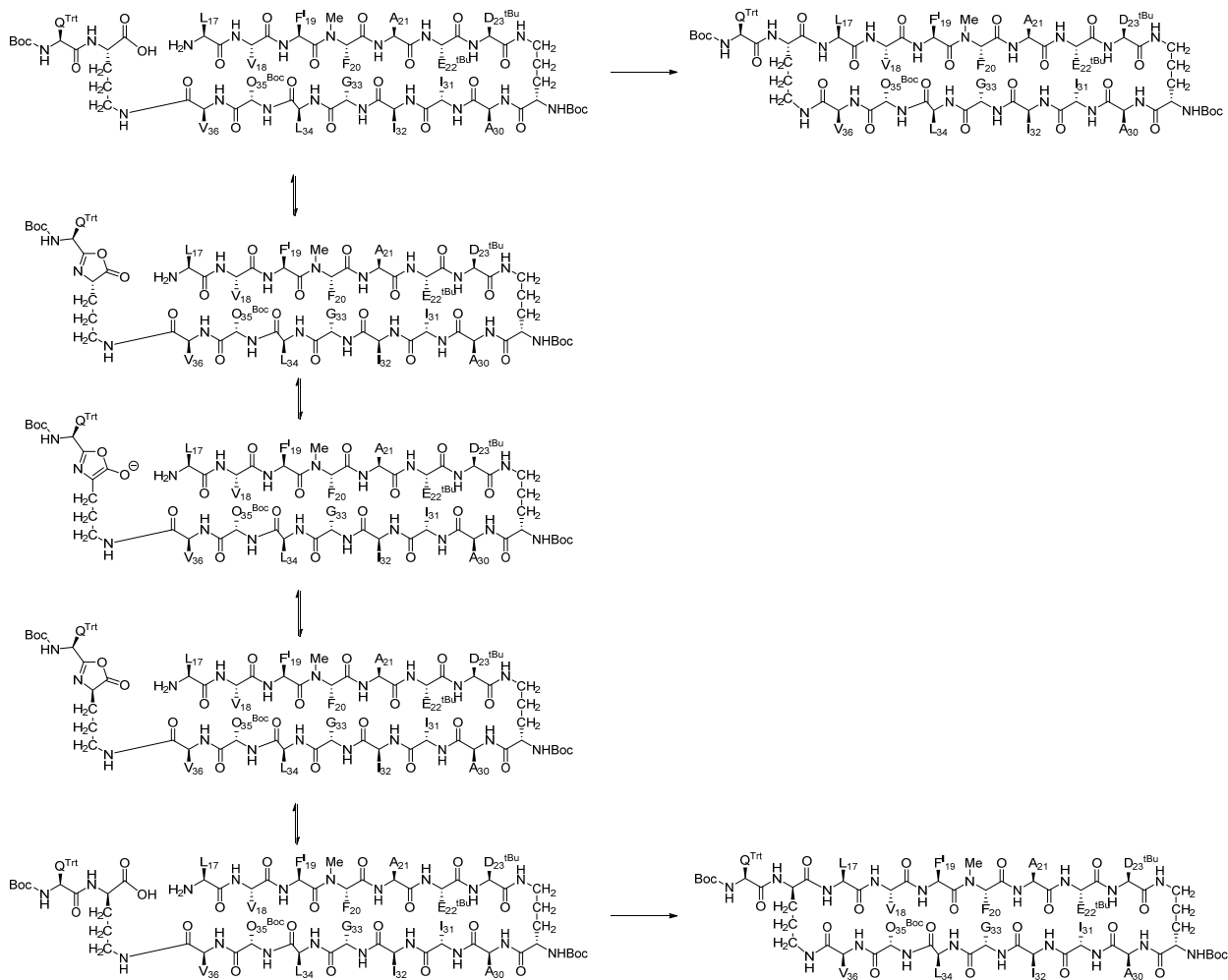
Figure 7. Possible epimerization reaction with N-terminal substituents on the C-terminal ornithine.

Final synthetic strategy

Initially the synthesis of a peptide macrocycle with N-terminal substituents is similar to a normal macrocyclic peptide (Figure 8). First, Boc-Orn(Fmoc)-OH is attached to 2-chlorotrityl resin. Solid-phase synthesis is then carried out to construct the first heptapeptide β-strand of the macrocycle. Fmoc-Orn(Dde)-OH is then added, replacing the incorporation of a second Boc-Orn(Fmoc)-OH. For the synthesis of peptide **3**, the Fmoc protecting group is then removed with piperidine and Boc-Gln(Trt)-OH is coupled to the N-terminus.
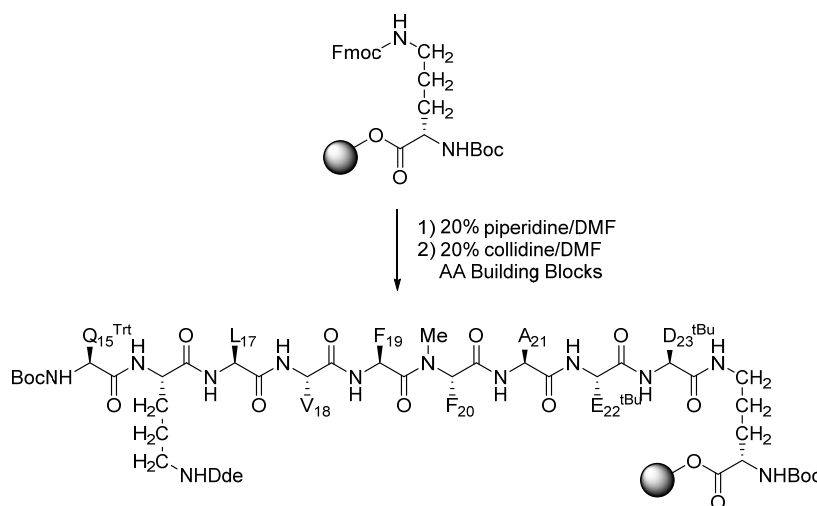
Figure 8. Synthesis of first β-strand of peptide **3**, using Fmoc-Orn(Dde)-OH.

After the sequence of the N-terminal portion added to the first β-strand, the resin is removed from the automated synthesizer, and the Dde group is deprotected with a 2% solution of hydrazine in DMF.[19] The Dde protecting group is completely removed after 20 minutes of shaking. After rinsing the resin with dry DMF, the second strand of the peptide is coupled to the δ-amine of ornithine, deprotecting the terminal peptide. The rest of the synthesis follows normal macrocyclic peptide synthesis (Figure 9). The yields of the peptides are comparable, 20% yield based on 0.07 mmol loading, yielding 24 mg purified peptide.
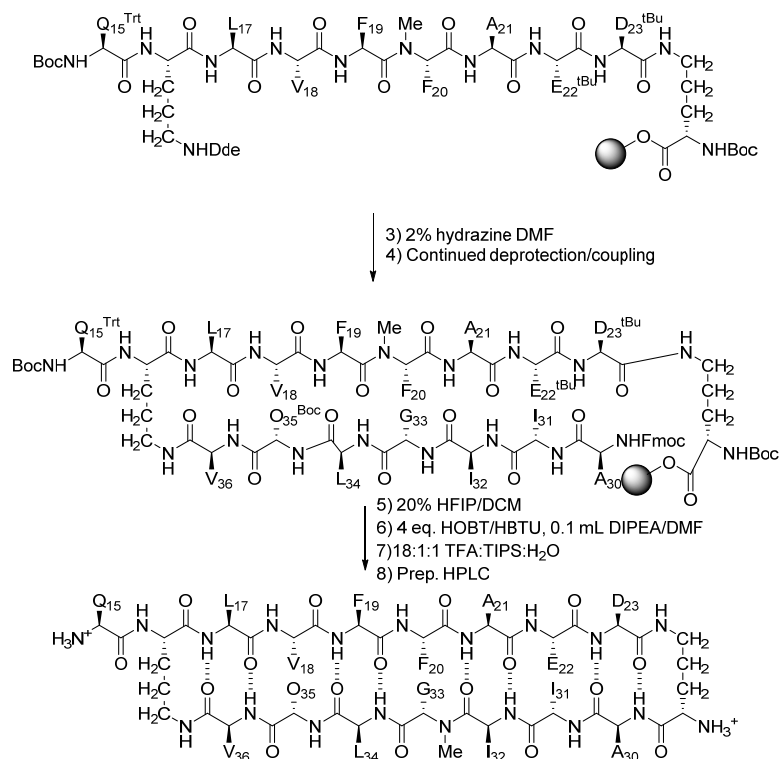
Figure 9. The completion of the synthesis of macrocyclic peptide **3**.

Preventing pyroglutamation of N-terminal Glutamine: An unforeseen challenge for synthesizing peptide **3** was pyroglutamation, which is facile under the acidic conditions of global deprotection. Typically, the peptide is deprotected in 18:1:1 TFA:TIPS:H_2O for 1 hour. The TFA is often removed *in vacuo* aided by a heated water bath. The temperature and time required for removal of TFA aids in the formation of the undesired pyroglutamate product (Figure 11).
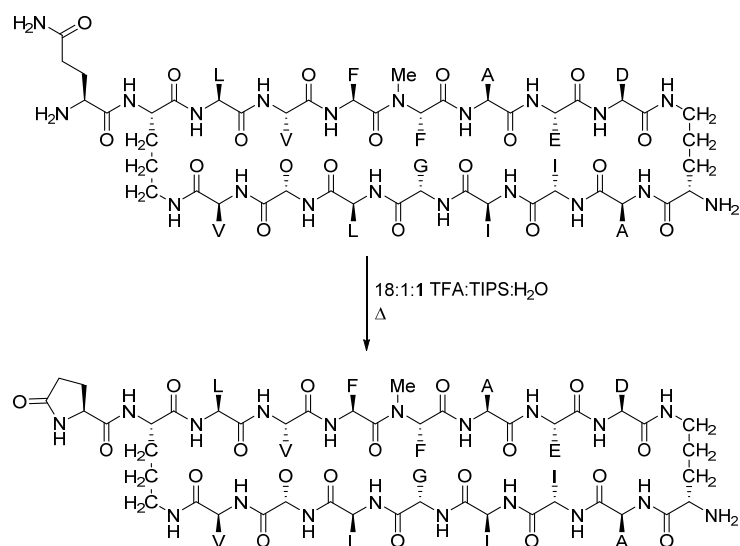
Figure 10. Reaction for the formation of the pyroglutamate product of peptide **3**.

I found that the best method to prevent pyroglutamation is to reduce the global deprotection time and to precipitate the peptide in ether.[20] During the global deprotection, 250 mL dry ether is cooled in an ice bath. After a half-hour in the deprotection cocktail, the peptide solution is added dropwise to the cold ether. The reaction flask is rinsed with ice-cold ether and added to the bulk. The solution is then placed in a freezer overnight to settle the precipitate and to further precipitate out the peptide. After this extended cooling period, the ether and precipitate are agitated to slurry, and the precipitate is centrifuged, disposing the supernatant ether. Afterwards, the precipitated peptide is dried under vacuum, and purified via preparative HPLC.

*Alternative N-methylation***:** I also attempted the synthesis of peptides **5** and **6,** with N-methylation on the bottom strand (Aβ_{30-36}) of the macrocycle. These peptides were insoluble in water (Figure 12). This could be due to infinite aggregation of the peptide along the unblocked nonapeptide top strand, which is a result of the amyloidogenicity of the sequence. I believe that X-ray crystallographic studies of these peptides could reveal intriguing oligomeric assemblies of natural Aβ, but methods for isolating these peptides would need to be developed further.
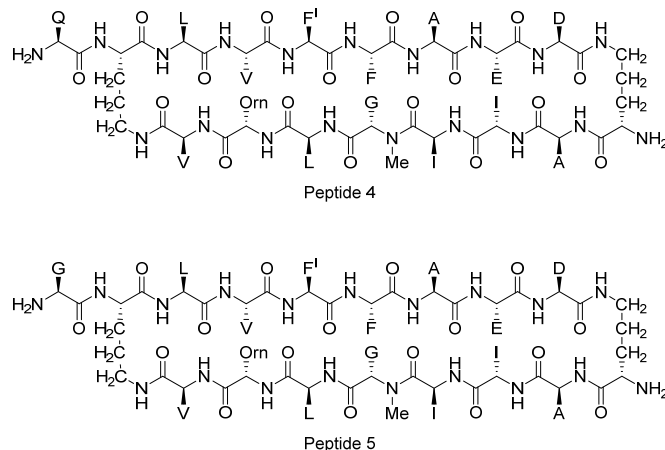
Peptide 4

Peptide 5

Figure 11. Peptides **4** and **5** with the N-methylated peptide on the lower strand, these peptides were not readily soluble in water, making it difficult to purify.

Conclusion

I determined a new method for adding one amino acid to the N-terminus of macrocyclic peptides to create potentially more natural peptide mimics. The synthetic strategy, featuring the use of Fmoc-Orn(Dde)-OH, can be applied to any of the existing peptide macrocycle systems to extend the peptide sequence and functionality. I designed and synthesized two peptides based on Dr. Spencer's crystallographic β-sheet macrocycle, extending the sequence with the addition of glycine and glutamine with high purity and yield using this synthetic route. An immediate application of these macrocycles is to observe oligomers of these β-sheet macrocycles using X-ray crystallography. Another application of this synthetic scheme is to add non-peptide N-terminal substituents, such as a fluorophores for microscopy experiments or peptide linkers.

At the moment, I have not determined how stable the Dde protecting group is to additional deprotections with piperidine for creating even longer peptide tails to attach to the N-terminus. If this proves to be problematic, Alloc can be used as the protecting group. If it is required, alternative strategies for solving the phasing in X-ray crystallography would be needed. One method could be designing peptides so that the iodinated substituents would be added to the peptide after Alloc deprotection.

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Experimental Section of Chapter 1

Isolation of Peptides **2** and **3**

After deprotecting peptides **2** and **3**, the peptides were dissolved in 10 mL of DI H$_2$O. The solutions were then centrifuged for 5 minutes at 5,000 rpm. The liquid portion was decanted from the precipitate, and then filtered through a 0.45 micron filter, and purified by preparative RP-HPLC on a C-18 column at ambient temperature. The peptide was eluted with acetonitrile and water containing 0.1% TFA. The peptides were purified in a series of two linear gradients, one from 5%-30% acetonitrile in water over 25 minutes, and the second from 30%-60% over 1 hour. The purified fractions were combined and lyophilized. Peptide **2** yielded 20 mg peptide, 17% recovery based on 0.07 mmol loading. Peptide **3** yielded 24 mg peptide, 20% recovery based on 0.07 mmol peptide loading.

ESI-MS of the first β-strand of peptide **3** protected with Dde

ESI-MS of peptide **3**, with the first β-strand synthesized, after ivDde deprotection with 10% hydrazine in DMF after 6 hours

Chemical Formula: $C_{56}H_{82}N_{13}O_{15}I$
Exact Mass: 1321.5

mklun87dde4-1 12 (0.220) Cm (3:26)

04-Sep-2014
21:13:17
TOF MS ES+
8.86e3

[M-Gln+42+H]⁺ 1236.45
1237.48
1238.47

[M+H]⁺ 1322.52
1323.53
1324.54

[M+ivDde-Gln+42+H]⁺ 1442.59
1443.60
1444.58
1458.49
1474.46

[M+ivDde+H]⁺ 1528.63
1530.68
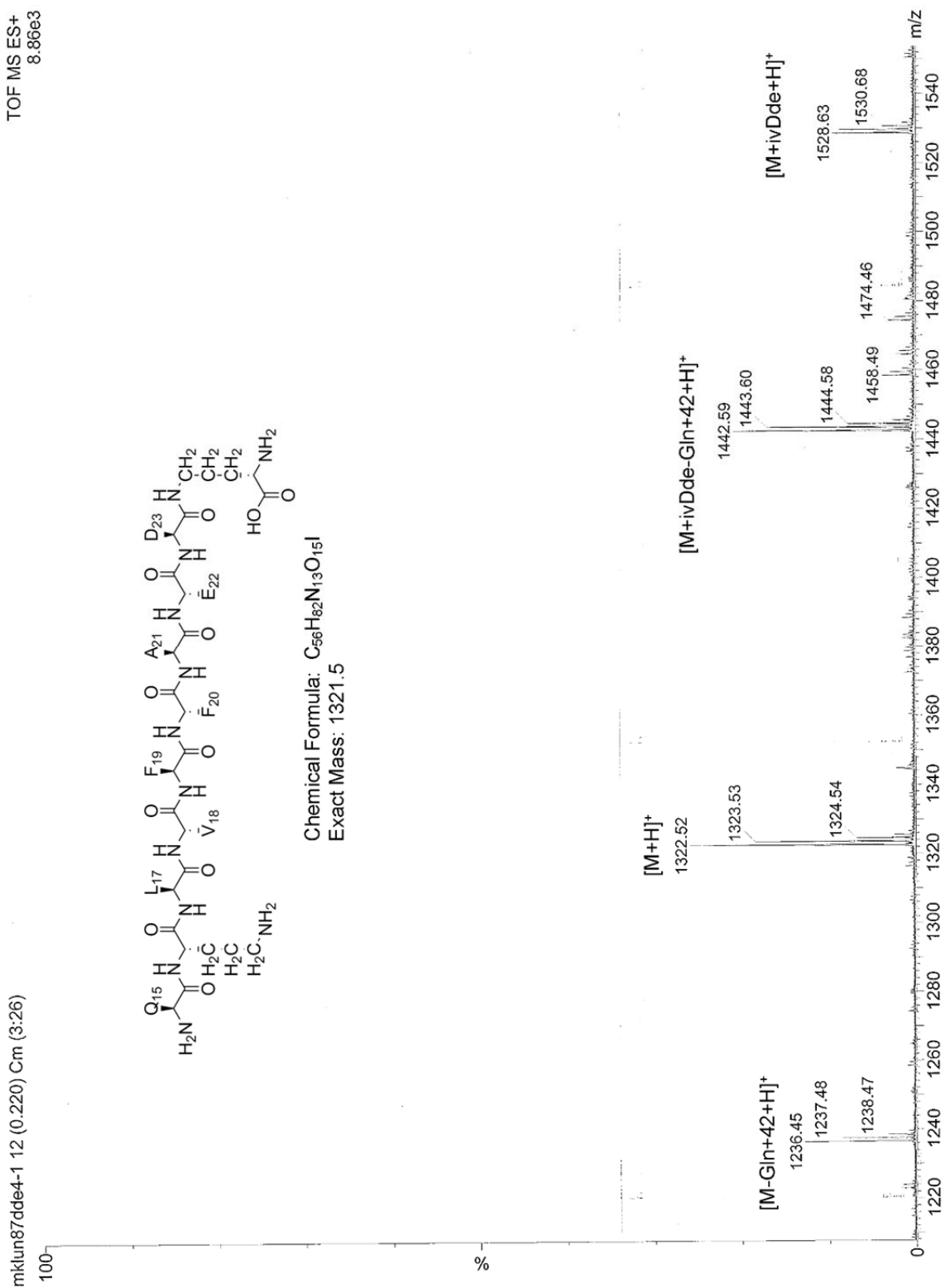
ESI-MS of peptide **3**, with the first β-strand synthesized, after Dde deprotection with 2% hydrazine in DMF after 30 minutes
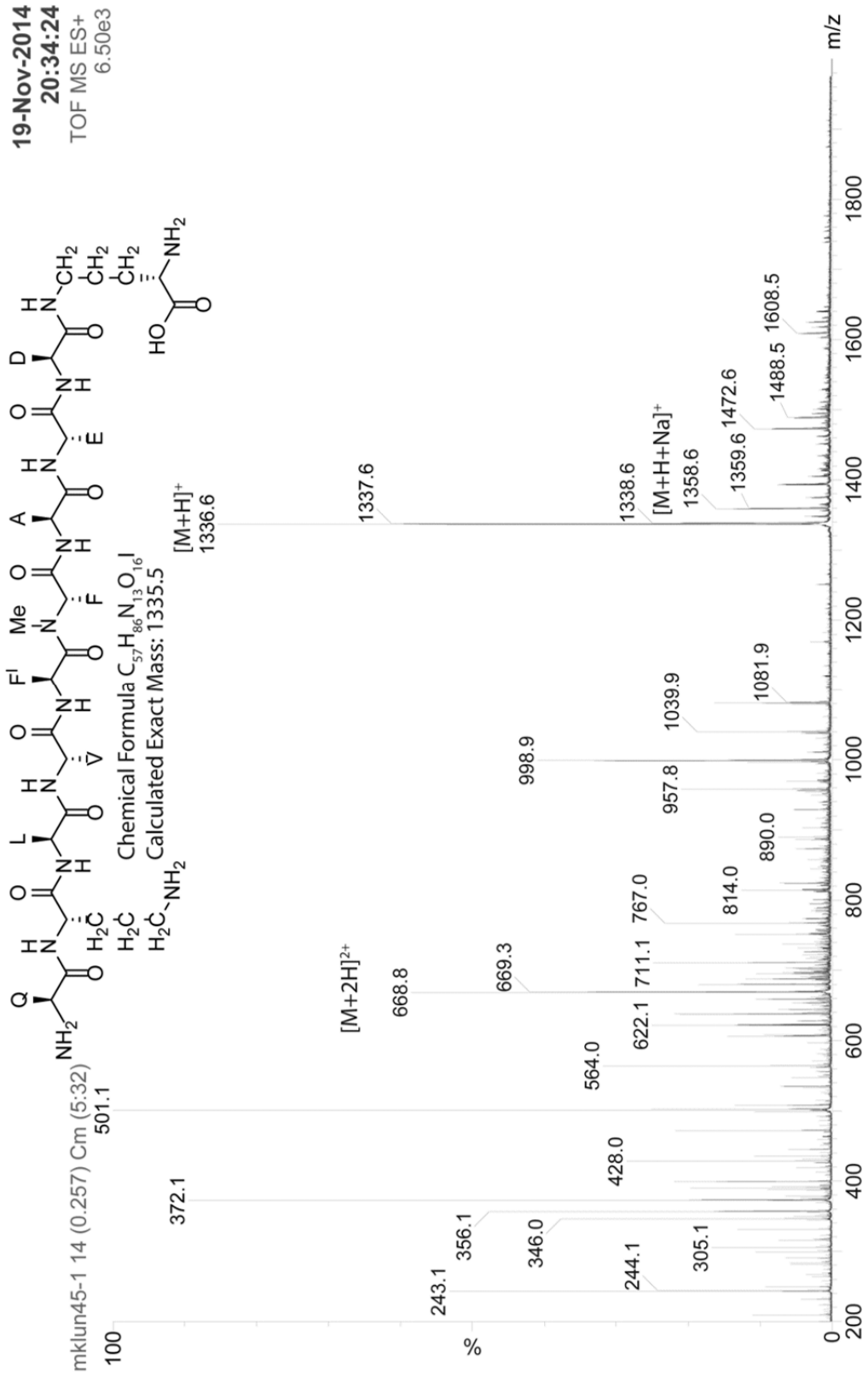
ESI-MS of the corresponding linear intermediate of peptide **3**

20-Nov-2014
10:02:58
TOF MS ES+
5.46e3

Chemical Formula: $C_{90}H_{146}N_{21}O_{23}I$
Calculated Mass: 2015.9

$[M+2H]^{2+}$
1009.5

mklun46-1 17 (0.312) Cm (5:31)
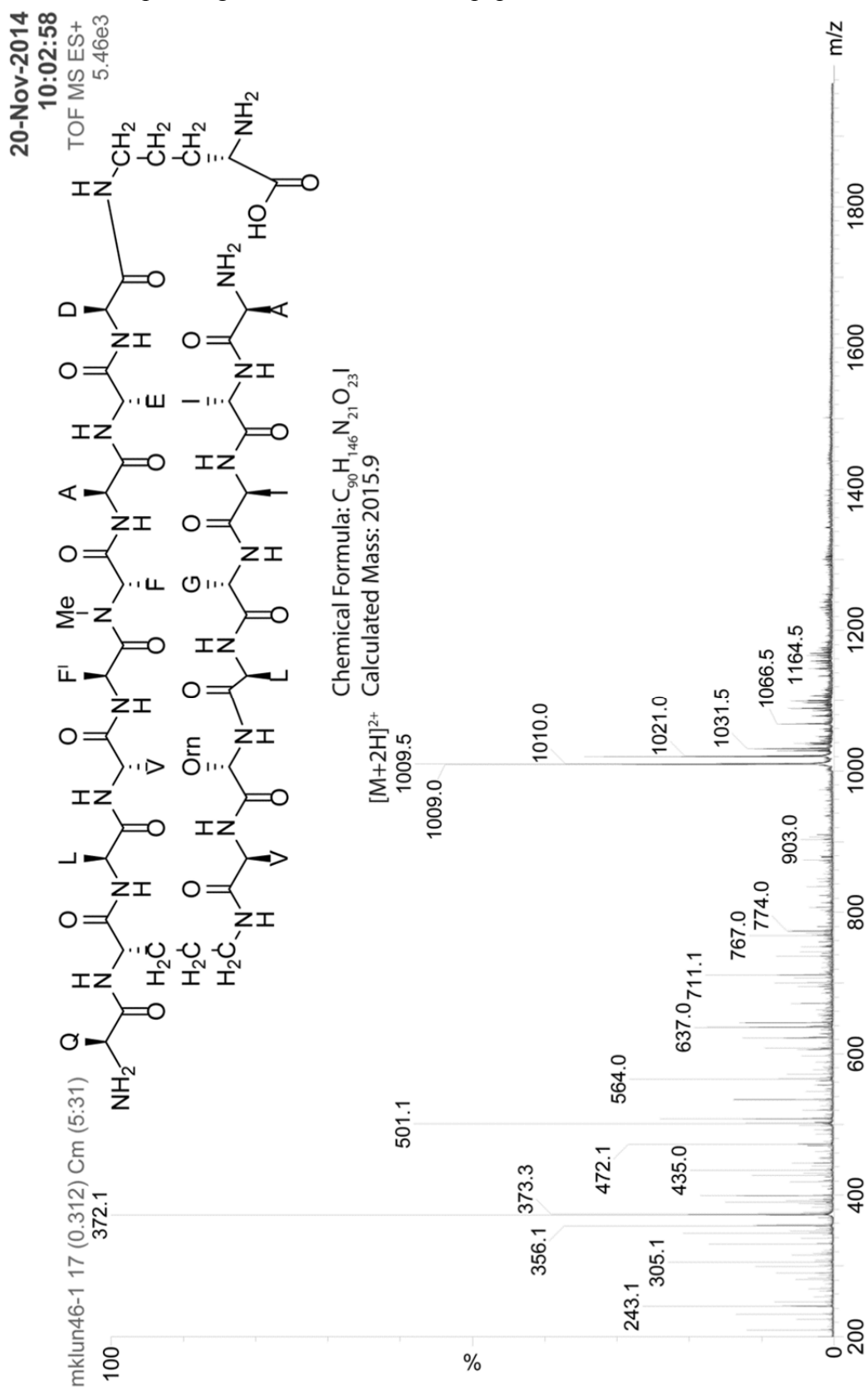
ESI-MS of the pyroglutamate of peptide **3**
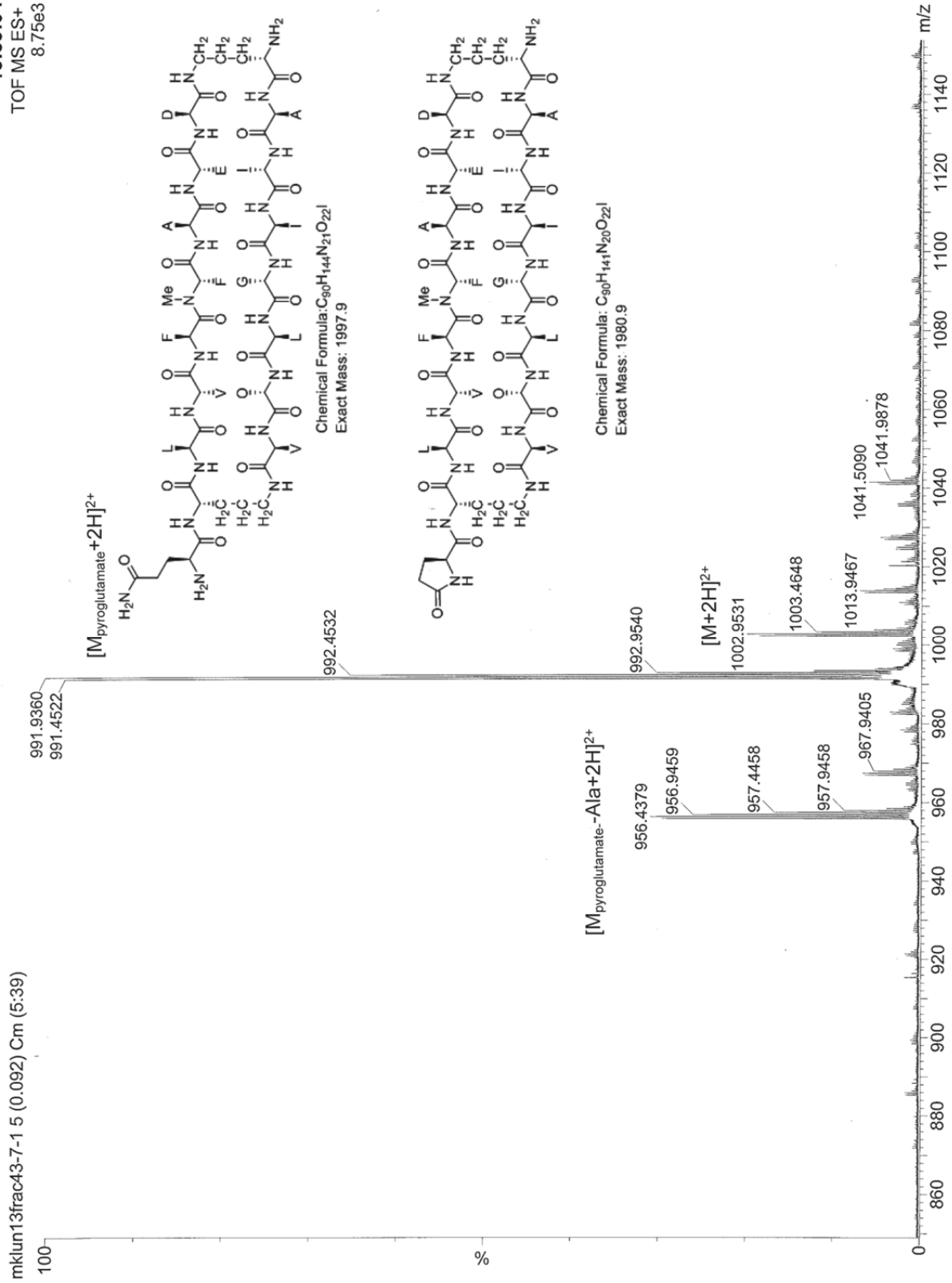
ESI-MS of the cyclized, purified peptide **3**

23-Nov-2014
10:29:54
TOF MS ES+
5.70e3
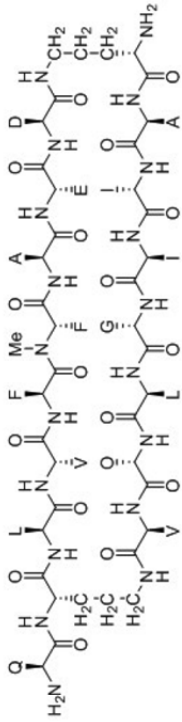
Chemical Formula: $C_{90}H_{144}N_{21}O_{22}I$
Exact Mass: 1997.9

mklun48-1 8 (0.147) Cm (7:17)

$[M+2H]^{2+}$
1000.5

$[M+3H]^{3+}$
667.0

$[2M+3H]^{3+}$
1333.7

$[3M+4H]^{4+}$
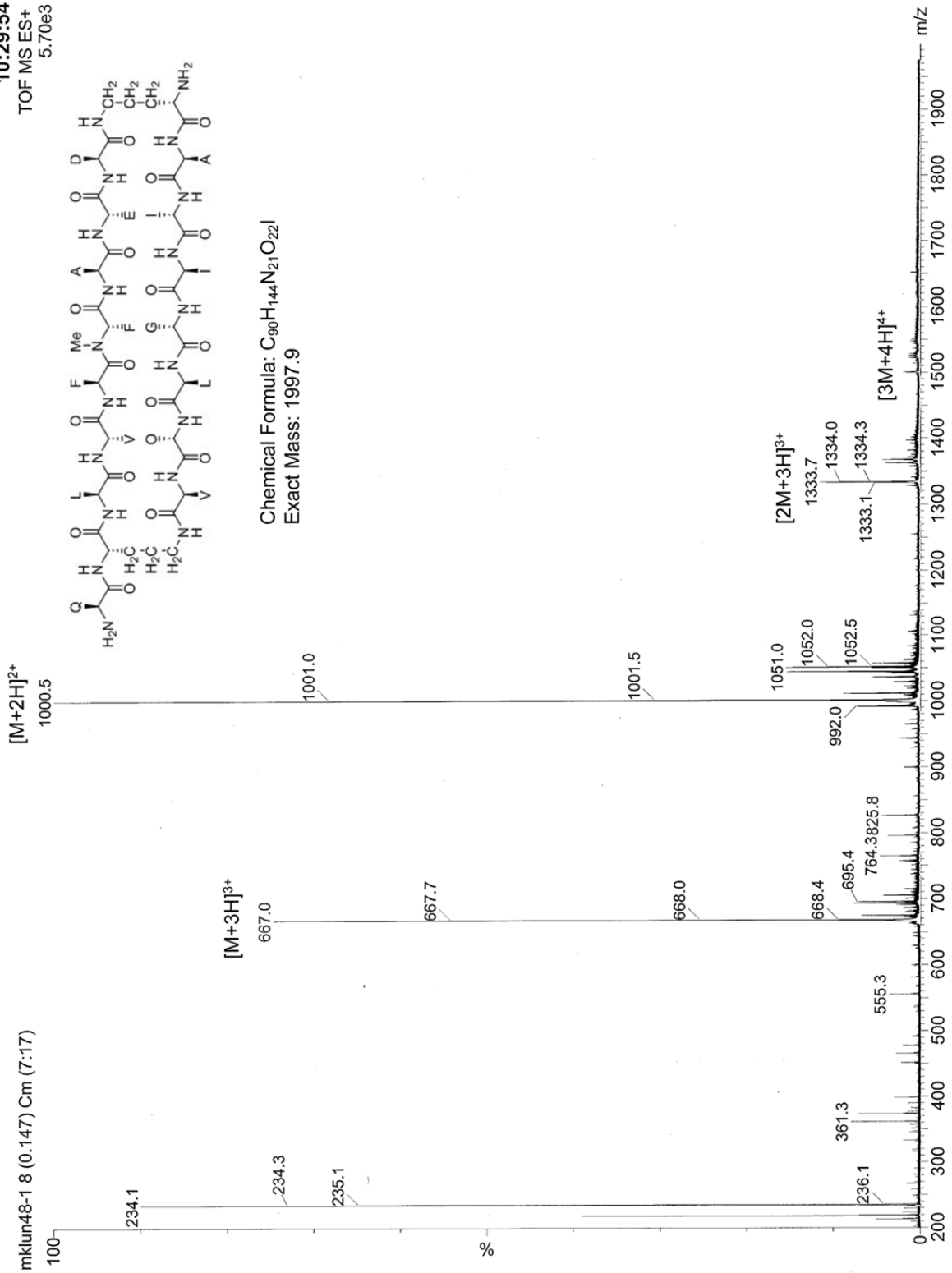
Analytical RP-HPLC of peptide **3**

```
Data File D:\NOWICK HPLC USERS\MATT\MTK-II-GLN-TOP-POOL.D
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======================================================================
Acq. Operator   : KELSEY
Acq. Instrument : Agilent HPLC                  Location : Vial 61
Injection Date  : 11/23/2014 2:45:37 PM
                                                Inj Volume : 20.0 µl
Acq. Method     : D:\NOWICK HPLC USERS\ZHENGJ\METHODS\RKS 5-100 OVER 20 MIN AT 214 AT 1 ML.M
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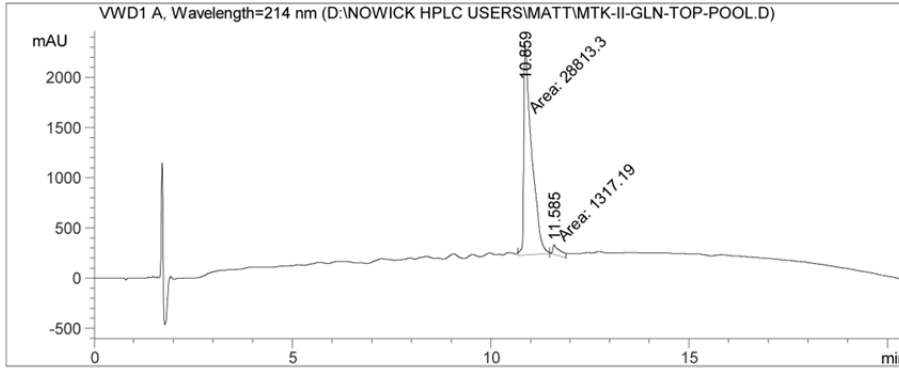


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                    Area Percent Report
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Sorted By            :        Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs



Signal 1: VWD1 A, Wavelength=214 nm

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  2  11.585 MM T  0.1906 1317.18738   99.59083    4.3716

Totals :                  3.01305e4  2198.58692


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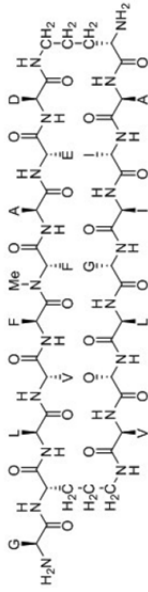
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Exact Mass: 1926.9

mklun-top-gly-pool-1 27 (0.495) Cm (6:28)

$[M+2H]^{2+}$
964.5

$[M+3H]^{3+}$
643.3

$[2M+3H]^{3+}$
1286.3

$[3M+4H]^{4+}$
1287.3

$[4M+5H]^{5+}$
1447.0

$[M+H]^+$
1929.0

965.5

975.5

976.5

977.0
987.0
987.5

1285.7
1286.7
1287.0
1294.0

644.0

651.0
651.3
656.3

207.1
252.1
373.3
435.1

%
100

0
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 m/z

HPLC of peptide **2**

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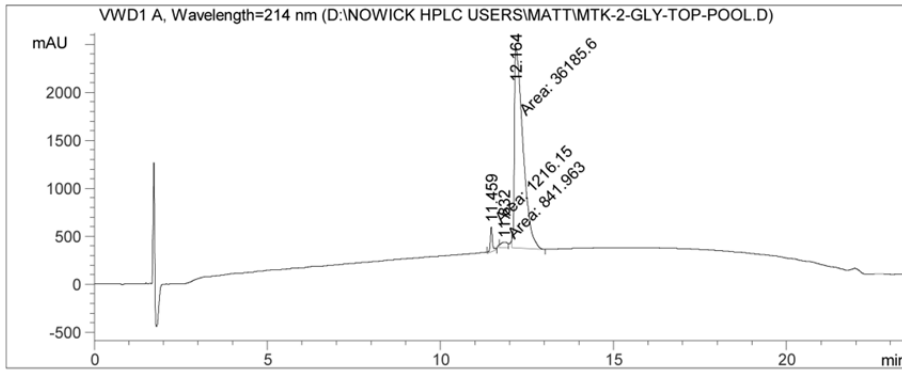
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Sorted By             :       Signal
Multiplier:                 :       1.0000
Dilution:                   :       1.0000
Use Multiplier & Dilution Factor with ISTDs



Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area       Height     Area
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   3  12.164 MM T  0.2877 3.61856e4  2096.28516  94.6184

Totals :                   3.82437e4  2420.66226


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Chapter 2

Exploration of Macrocyclic Peptides Which Oligomerize Through an Aromatic Hydrophobic Interface

Preface

Chapter 2 explores peptides which oligomerize through a hydrophobic, aromatic interface based on peptide **6** developed by former Nowick group member Dr. Khakshoor. Part 1 describes a pair of peptides which have the ability to heterodimerize through the interaction using fluorinated peptides. Previously, fluorinated amino acids have been used to engineer peptides to assemble in α-helix systems. Waters and Cheng developed a series of peptides which have stabilized folding through favorable aromatic interactions with pentafluorophenylalanine (Pff) and Phe.[1, 2] Gao developed a pair of peptides which create hetero-oligomers directed through this same aromatic Pff/Phe interaction.[3]

I designed two peptides based on Peptide **6**, which was found to assemble as a tetramer, interacting through Tyr and Phe in a hydrophobic core.[4, 5] The goal was to create heterodimeric assemblies in a similar fashion to the peptides designed by Gao: one peptide with two Pff in the hydrophobic core (peptide **9**), and one peptide with two Phe in the hydrophobic core (peptide **8**). Peptides **8** and **9** were synthesized and analyzed via NMR to determine their assembly in D_2O. Separately, both peptides were able to tetramerize in the same fashion as Peptide **6**. When the peptides were mixed however, the ^{1}H NMR spectrum revealed a complicated equilibrium. While the formation of a new heterodimeric system was revealed using TOCSY and ROESY NMR techniques, the peaks corresponding to the heterodimeric species are small in comparison to the rest of the peaks. At this time, it isn't clear that this interaction is there due to the lack of favorability, or if it exists simply due to random interactions. The initial model of Dr. Khakshoor's original crystallographic structure does not have a face-face π-stacking interaction,

and the geometry of the Phe/Pff interaction may not have been favorable in this case. Perhaps heterooligomerization of peptides through these fluorinated side chains interactions could be a powerful strategy if it becomes apparent that β-sheet peptide macrocycles interact in the appropriate π-stacking orientation.

In part 2, I attempted to optimize the formation of the tetramer of Dr. Khakshoor's macrocyclic peptide system.[4, 5] Sequentially, each amino acid residue was altered in order to explore which positions were responsible for creating the hydrophobic core. The ability for these peptides to form stable tetramers was studied with concentration-dependent [1]H NMR, and DOSY. The concentration-dependent [1]H NMR revealed that increasing the hydrophilicity of residues 2 and 6 removed the ability for the peptide to form tetramers. Increasing the hydrophobicity of the aromatic and the N-terminal amino acids increased the propensity of the peptides to create tetramers. Increasing the hydrophobicity of the C-terminal amino-acid overall destabilized the oligomers. DOSY revealed that the peptide aggregates were tetramers. As a result, VSFTFTS (Peptide **14**) was determined to be the optimized sequence. Not only did peptide **14** form a tetramer at submillimolar concentrations, it also had a rich and well-defined NOESY spectrum which agreed with the tetramer model developed by Dr. Khakshoor.

Chapter 2 - Part 1

Design and Identification of Heterotetramerizing Peptides Through Aromatic Interactions

Introduction

There are several diseases which are caused by the oligomerization of peptides through β-sheet interactions. These include Alzheimer's disease, Parkinson's disease, type 2 diabetes, senile amyloidosis, and prion diseases.[6-10] One way to explore how these β-sheet peptides assemble is to design peptides which oligomerize in a controlled fashion.

Fluorinated amino acids have been introduced to enhance peptide assemblies due to their unique hydrophobicity and electronic characteristics. For example, pentafluoropheynylalanine (Pff) not only is more hydrophobic than Phe, it has been found to favorably interact with Phe (Figure 12). Waters and Cheng developed a series of peptides which have stabilized folding through favorable aromatic interactions with Pff and Phe.[1, 2] Gao developed a pair of peptides which create hetero-oligomers directed through this same aromatic Pff/Phe interaction.[3]
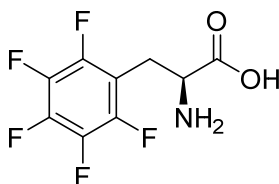
Figure 12. The chemical structure of pentafluorophenylalanine (Pff).

The Nowick group used the β-sheet interaction of the crystallographic dimer of protein NuG2 to design a similar chemical model system that dimerizes through β-sheet interactions.[11] Former Nowick group member, Dr. Omid Khakshoor, developed macrocyclic β-sheet peptide **6** in order to mimic the NuG2 β-sheet interaction (Figure 13).[4] The macrocycle contains the heptapeptide sequence of the dimerization interface of NuG2 replacing Thr with Ser at R_2 and R_7 to identify hydrogen bonding interactions more easily on the top strand. The 'bottom-strand'

contains two Hao units flanking lysine. The Hao units are tripeptide mimics which facilitate hydrogen bonding within the macrocycle. β-Sheet hydrogen-bonding within the ring orients the 'top-strand' peptide sequence to facilitate further β-sheet interactions. At the same time, Hao blocks the formation of hydrogen-bonding interactions with the sterically bulky methoxy group.[12] During this study, TOCSY and NOESY NMR revealed that that peptide **6** dimerized through antiparallel β-sheet interactions. Ultra-centrifugation and DOSY studies concluded that peptide **6** formed a tetramer, consisting of a dimer of dimers. Dr. Khakshoor later performed X-ray diffraction crystallography with peptide **7**, which has a 4-bromophenylalanine (FBr) within the peptide sequence to solve crystallographic phasing.[5]
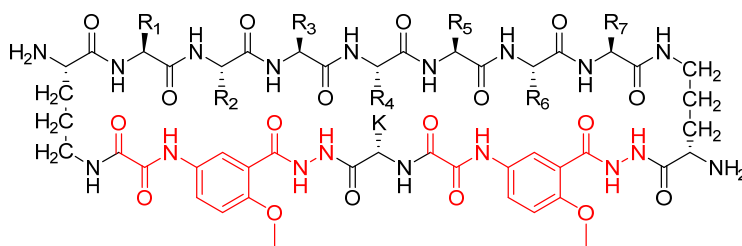
Figure 13. Macrocyclic β-sheet peptide motif designed by Dr. Khakshoor. The Hao units are highlighted in red.

Peptide	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Wild Type	Thr	Thr	Phe	Thr	Tyr	Thr	Thr
Peptide **6**	Thr	Ser	Phe	Thr	Tyr	Thr	Ser
Peptide **7**	Thr	Tyr	FBr	Phe	Tyr	FBr	Ser

Table 1: Peptide sequences of Dr. Khakshoor's studies. Peptide **6** was the original sequence and peptide **7** was used in X-ray diffraction crystallographic studies with 4-bromophenylalanine (FBr).[4, 5]

A crystal structure confirmed the anti-parallel dimeric interactions found in peptide **6**, and showed further assembly into a trimer of dimers with Phe_3 and Tyr_5 pointed toward the core of the trimer (Figure 14). The structure for the tetramer of peptide **6** was modeled from the crystal structure of peptide **7**. In this predicted structure, the inter-dimer interactions were between Phe and Tyr, forming the hydrophobic core of the tetramer (Figure 15).[5]

31
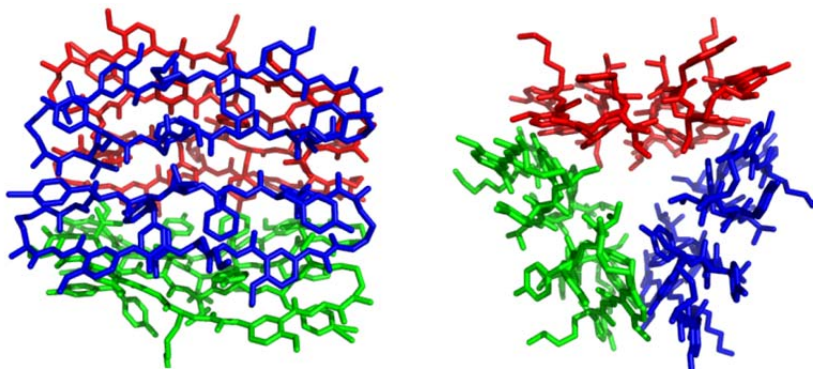
Figure 14. The X-ray crystallographic structure for peptide **7**, PDB: 3NI3. (Left) From the top, highlighting the hydrogen-bonding β-sheet dimers. (Right) From the side, highlighting the orientation of the trimer of dimers.
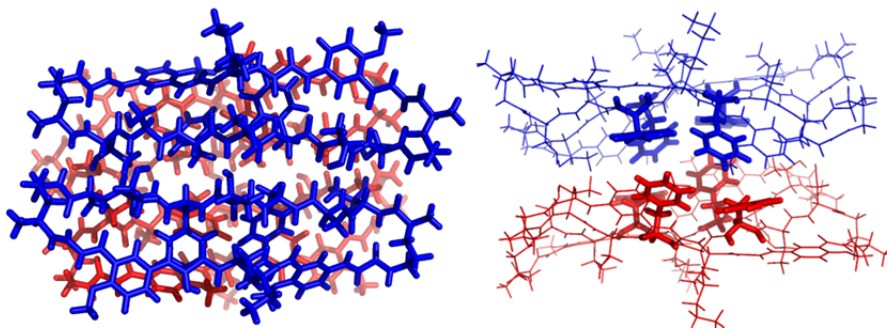
Figure 15. The calculated structures for the tetrameric species of Peptide **6**, based on the trimer of dimers found in peptide **7**. (A) From the top showing the dimeric interaction (B) From the side, highlighting the positions of the aromatic residues Phe_3 and Tyr_5 within the core.

Peptide Design: I designed two peptides based on Dr. Khakshoor's tetrameric peptide **6**, altering only positions 3 and 5 (Figure 16). I designed peptides **8** and **9** to heterodimerize, controlled by the interaction of Pff and Phe (Figure 17). Peptide **8** was designed to be a single mutant with two Phe and peptide **9** was a double mutant with two Pff. I explored the structures and interaction geometries of these peptides using various NMR techniques including concentration dependent ^{1}H NMR studies, TOCSY, ROESY, and DOSY.

Peptide	R_3	R_5
Peptide **6**	Phe	Tyr
Peptide **8**	Phe	Phe
Peptide **9**	Pff	Pff

Figure 16. Design of peptides **8** and **9**, altering the aromatic amino acids R_3 and R_5.
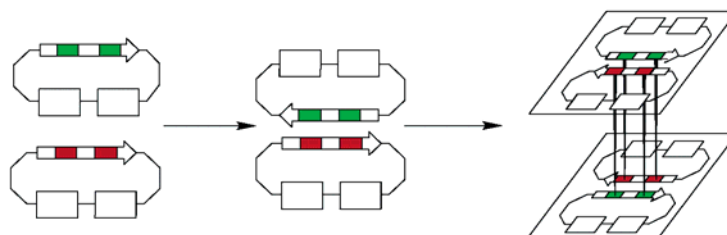
Figure 17. Cartoon of the predicted heterotetramer of peptides **8** and **9** through aromatic interactions. The red bands represent Phe, and the green bands represent Pff.

Synthesis of Macrocyclic β-Sheets: I synthesized the cyclic peptides by the method outlined by Dr. Khakshoor.[4] The corresponding protected linear peptide was synthesized, cleaved from the resin with protected side chains, cyclized, deprotected, and purified by HPLC. Dr. Khakshoor's protocol indicated deprotection with 18:1:1 TFA:TIPS:H_2O solution. It was observed in mass spectrometry that after deprotection, the serines often became TFA esters, which could be removed via solvolysis by dissolving in MeOH and bringing to reflux. Dr. Khakshoor reported that this step would only take an hour. I found that peptide **9** required eight hours in MeOH to remove these TFA esters.

Peptide Mixing NMR Experiments: Peptides **8** and **9** were mixed 1:1 at 0.4 mM and 2.25 mM to observe the formation of a heterodimeric species. The initial concentration of 0.4 mM was chosen because at this concentration, both peptides **8** and **9** had monomeric species present. The mixture of peptides **8** and **9** at a concentration of 0.4 mM each revealed the formation of a new species in the aromatic region and in the alpha proton region. Also, the defining peaks of peptide **9** broadened severely while peptide **8** still had well-defined monomeric species present in the mixture (Figure 18).
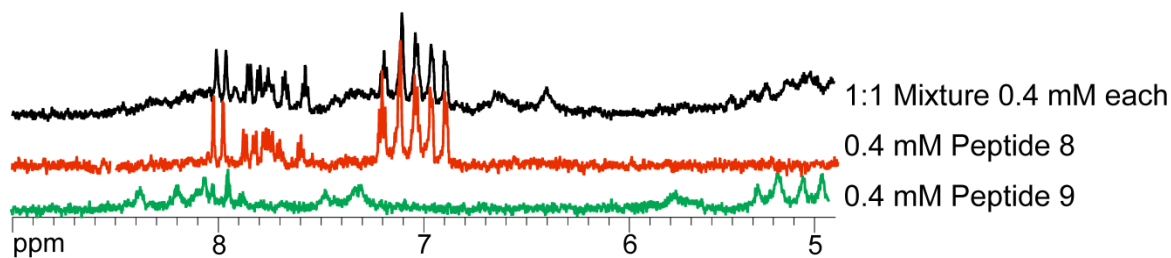
Figure 18. [1]H NMR spectrum of peptides **8** and **9** mixed at a concentration of 0.4 mM each. The [1]H spectra of peptides **8** (red) and **9** (green) are shown below for comparison.

The concentration of 2.25 mM in these mixing experiments was chosen because aggregate species predominated in both peptides. The [1]H NMR of the mixture appeared as the superposition of peptides **8** and **9** with little variation except for the emergence of some new peaks of NMR upfield from the aromatic region (Figure 19). TOCSY, ROESY, and DOSY spectroscopy were required to elucidate the identity of new peaks.
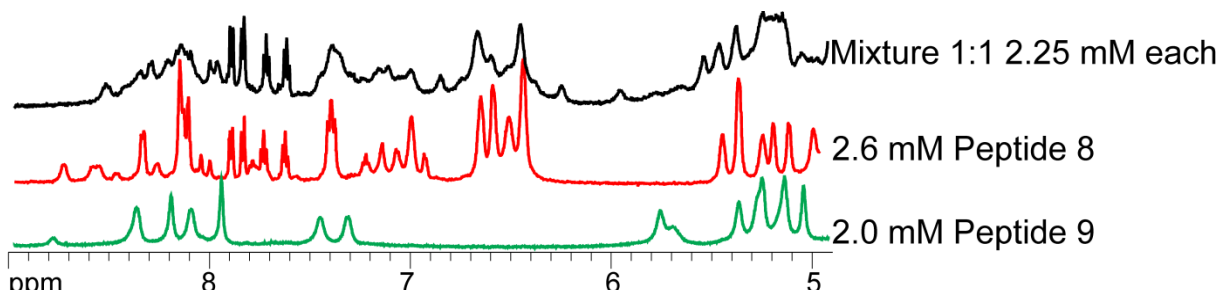
Figure 19. [1]H NMR spectrum of peptides **8** and **9** mixed at a concentration of 2.25 mM each. The [1]H spectra of peptides **8** (red) and **9** (green) are shown below for comparison.

The TOCSY spectrum of the 1:1 mixture with 2.25 mM each was compared to the TOCSY spectra of peptides **8** and **9** separately. The same comparison was made for the ROESY spectrum of the same mixture (Figure 20). If the crosspeaks of the peptides **8** and **9** overlapped the mixed spectra entirely, it would mean that all of the peaks present were just superpositions of the two spectra combined and no new species would have formed. The TOCSY spectrum revealed three new peaks upfield from the aromatic region which did not overlap with the superpositioned spectra. The ROESY spectrum of the mixture revealed new proton interactions in the alpha region, possibly indicating a new hydrogen-bonded species (Figure 21).
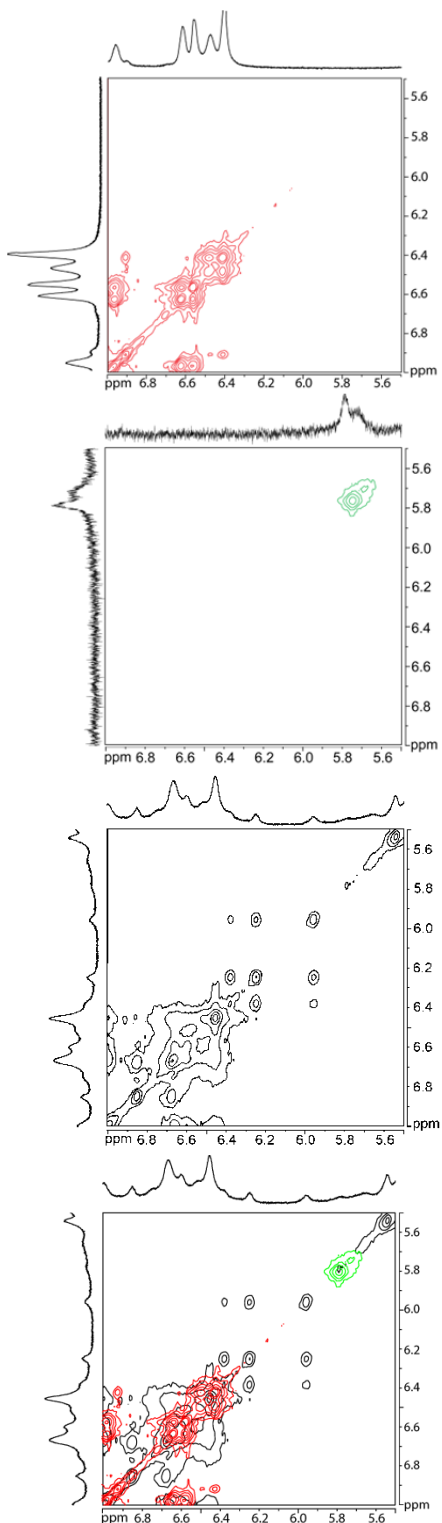
Figure 20. (Red) TOCSY of peptide **8** by itself. (Green) TOCSY of peptide **9** by itself. (Black) TOCSY of peptides **8** and **9** mixed together, and the superposition of the three spectra, highlighting newly formed peaks from the formation of a hetero-oligomerizing species.

Figure 21. (Red) ROESY of Peptide **8** by itself. (Green) ROESY of peptide **9** by itself. (Black) ROESY of peptides **8** and **9** mixed together, and the superposition of the three spectra, highlighting newly formed peaks from the formation of a hetero-oligomerizing species.

Discussion

Mixing studies: The mixing experiments from both concentrations show the formation of new peaks. The majority of the mixed peptide spectrum was the superposition of peptide **8**. ROESY and TOCSY NMR spectroscopy revealed the formati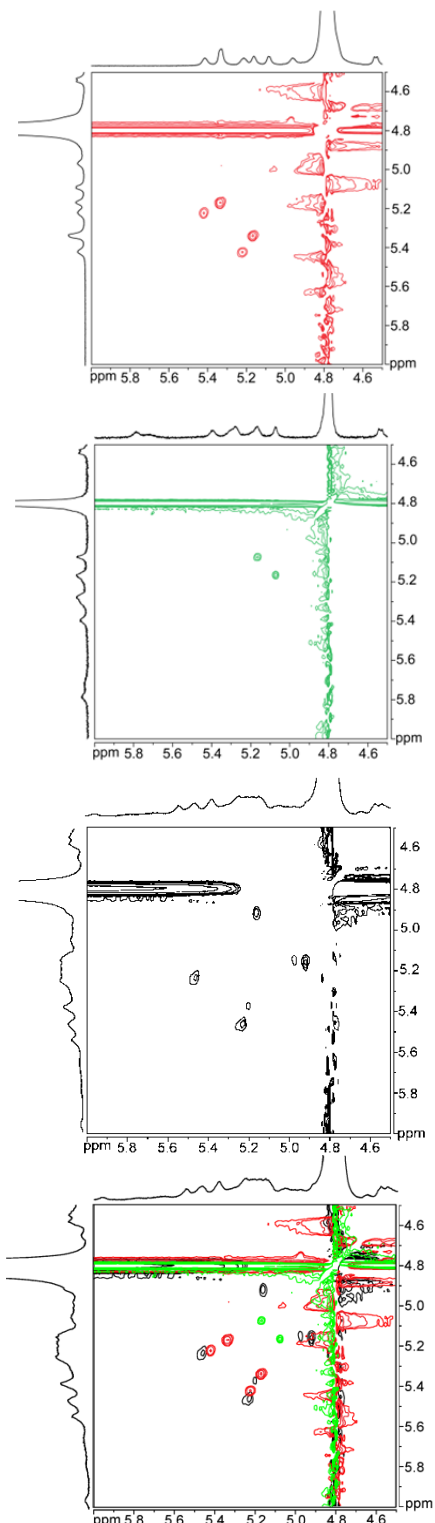on of new aggregate species when peptides **8** and **9** were mixed together. ROESY exposed the formation of new α-proton interactions, which possibly reveals new anti-parallel β-sheet interactions. The TOCSY revealed that a new species corresponding to upfield-shifted ortho-, meta-, and para- positions on Phe, possibly due to a new face-face interaction with Pff.

Conclusions

These macrocyclic NuG2 mimics formed consistent anti-parallel β-sheets, and the aggregation was controlled by aromatic residue interactions. Peptide **9**, with its hydrophobic aromatic Pff residues had a much higher propensity for aggregation than peptides **6** and **8**. Mixing peptides **8** and **9** revealed the formation of new heterodimeric interactions, possibly through Pff and Phe. However, the new peaks arising from these new interactions are small in comparison to original peptide peaks from peptides **8** and **9** separately. This could possibly be due to the fact that, in order for Pff and Phe to interact favorably, they must have a face-face stacking interaction. The oligomer model described by Dr. Khakshoor (Figure 15) shows Phe and Tyr interacting in a t-shaped orientation. In order for the appropriate Pff/Phe face-face interaction to occur, peptides **8** and **9** possibly would need to position themselves in an unfavorable orientation.[1-3] If there is another β-sheet peptide which dimerizes through aromatic face-face interactions, this Phe/Pff strategy would more than likely be effective in creating a heterodimer.

Chapter 2 - Part 2

Optimization of a Tetrameric Peptide Using a Sequential Design

Introduction

In part 1, the purpose was to create heterodimeric peptide pairs through the hydrophobic interface. This part highlights efforts to design peptides which form heterodimeric peptide interactions through the hydrophilic face of the β-strands. In order to achieve this, I needed to design a peptide sequence which created a stabilized tetramer species through the hydrophobic face. The peptides were designed in a stepwise, rational manner, testing to see which amino acids were responsible for the stability of the tetramer (Figure 22). The various mutants were tested with concentration dependent [1]H NMR, TOCSY, ROESY, and DOSY for folding, interactions, and aggregate size. These peptides were compared to peptide **6** as the baseline, and peptide **9** since it created oligomers at submillimolar concentrations.

Peptide Design:

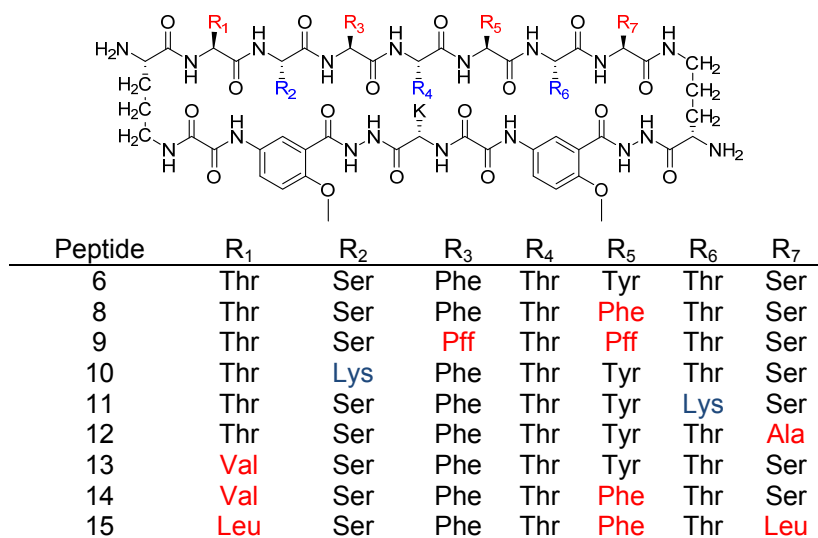| Peptide	R₁	R₂	R₃	R₄	R₅	R₆	R₇
6	Thr	Ser	Phe	Thr	Tyr	Thr	Ser
8	Thr	Ser	Phe	Thr	Phe	Thr	Ser
9	Thr	Ser	Pff	Thr	Pff	Thr	Ser
10	Thr	Lys	Phe	Thr	Tyr	Thr	Ser
11	Thr	Ser	Phe	Thr	Tyr	Lys	Ser
12	Thr	Ser	Phe	Thr	Tyr	Thr	Ala
13	Val	Ser	Phe	Thr	Tyr	Thr	Ser
14	Val	Ser	Phe	Thr	Phe	Thr	Ser
15	Leu	Ser	Phe	Thr	Phe	Thr	Leu

Figure 22. Peptide sequences used for determining the patterns for creating low-concentration aggregates. Residues marked in blue highlight mutants affecting the hydrophilic outer residues while residues colored red designate peptides affecting the hydrophobic core according to Dr. Khakshoor's tetramer model.

The peptides in this study were designed to determine which amino acids influence the formation of a hydrophobic core. Peptides **8** and **9** were initially studied for heterodimeric experiments described earlier; however they provided insight into the importance of a hydrophobic, aromatic interior. Peptides **10** and **11** were designed to discourage the exchange among hydrophobic core systems by introducing lysine residues to the hydrophilic face of the peptide. Peptide **12** was designed to test the role of the C-terminus for developing a hydrophobic pocket. Peptide **13** was designed to test the role of the N-terminus for developing a hydrophobic pocket. Peptides **14** and **15** were designed to combine the most favorable factors for increasing peptide aggregation and improving peptide folding.

β-sheet formation of the peptides: The difference in chemical shift between the two diastereotopic protons on the δ-carbon of the ornithine turns is a defining feature of a β-sheet macrocycle. This chemical shift arises since the pro-S proton is closer in space to ornithine's alpha proton and carbonyl than the pro-R proton when the peptide is folded (Table 2).[13]

The α-proton shifts were compared to published random coil values.[14] If the α-proton shifts of the non-ornithine peptides were different than the random coil, the peptide had some organized folding pattern. Only peptides that were able to fold into aggregate species at concentrations within the concentration dependent [1]H NMR were analyzed in this fashion, peptides **10**, **11**, and **12** were omitted because it would require an extremely high concentration of peptide in order to analyze the aggregates properly. The other peptides in this study had α-protons shifted downfield to similar degrees, revealing the formation of organized β-sheet oligomers (Table 2).[6]

Peptide	Δ δ$^{\delta Orn}$	Average Δ-α proton
6	0.69	0.79
8	0.67	0.76
9	0.65	0.84
13	0.65	0.84
14	0.64	0.73
15	0.61	0.81

Table 2. Key NMR statistics revealing peptides abilities to form β-sheet oligomers. The average magnetic anisotropy between the two diasteromeric protons on the delta carbon of delta-ornithine reveal the formation of an internal β-sheet. The average change in chemical shift of alpha protons from random coil values show the formation of organized hydrophobic cores.

ROESY/NOESY: All studied peptides exhibited strong intramolecular NOEs between the α-protons pointing in toward the ring and the internal Hao protons. Nearly all observable aggregating peptides formed in-register anti-parallel β-sheet interactions determined by NOEs from the α-protons pointing outside of the ring (Figure 23). The in-register anti-parallel β-sheet formation was not observed for peptide **9**.
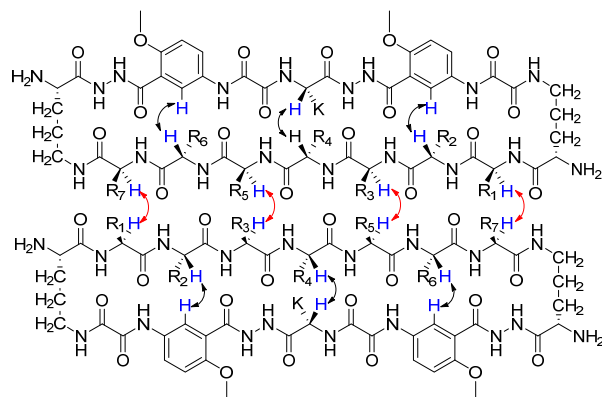
Figure 23. Key NOEs present in the peptides. The black arrows represent defining NOEs present in all aggregating peptides. The red arrows are intermolecular NOEs, present in all peptides except for peptide **9**.

Concentration Dependent ^{1}H NMR studies: Peptide aggregation is concentration dependent and the chemical equilibria between aggregates and monomeric species can be observed by comparing NMR at increasing concentrations of peptide. The formation of an aggregate species

is marked by the simultaneous formation of oligomeric peaks and disappearance of monomeric peaks (Figure 24). The methods for concentration dependent ^{1}H NMR experiments are described in the experimental section. The mole fractions of the aggregates were calculated by dividing the integrals of the incoming oligomer peaks by the integrations of oligomeric and monomeric resonances corresponding to the same protons determined by ROESY. A peptide which has a high propensity to oligomerize will have a higher mole fraction of oligomers at lower concentrations. The lysine mutants (peptides **10** and **11**) did not fold into aggregate species and a mole fraction plot could not be created.
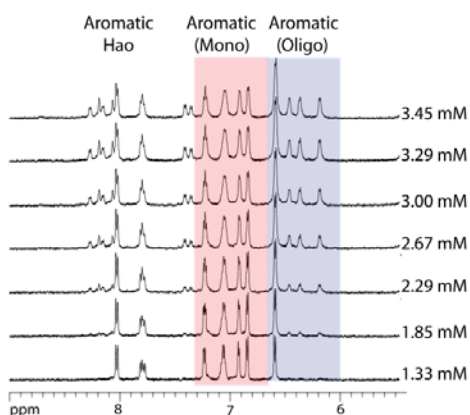
Figure 24. Concentration dependent ^{1}H NMR study of peptide 6, highlighting the incoming oligomeric peaks (blue) and receding monomeric peaks (red). Spectra recorded on the Avance 600 at 295 K.

The mole ratio of aggregate species compared to total peptide concentration reveals the relative propensities of each peptide to form aggregate species (Figure 25). The aggregation propensities of the peptides were compared by their OC_{50}, the concentration at which 50% of the peptide in solution is associated as an oligomer (Table 3).

Figure 25. The concentration dependence of oligomer formation of the peptides. The mole fraction of aggregate species compared to total peptide concentration reveals the relative propensities of each peptide to form aggregate species.

Peptide	6	8	9	12	13	14	15
OC_{50} (mM)	>4	0.91	0.13	>>4	1.08	0.33	0.13

Table 3. The OC_{50} of the peptides, the concentration of peptide at which the mole fraction of oligomers is 50%.
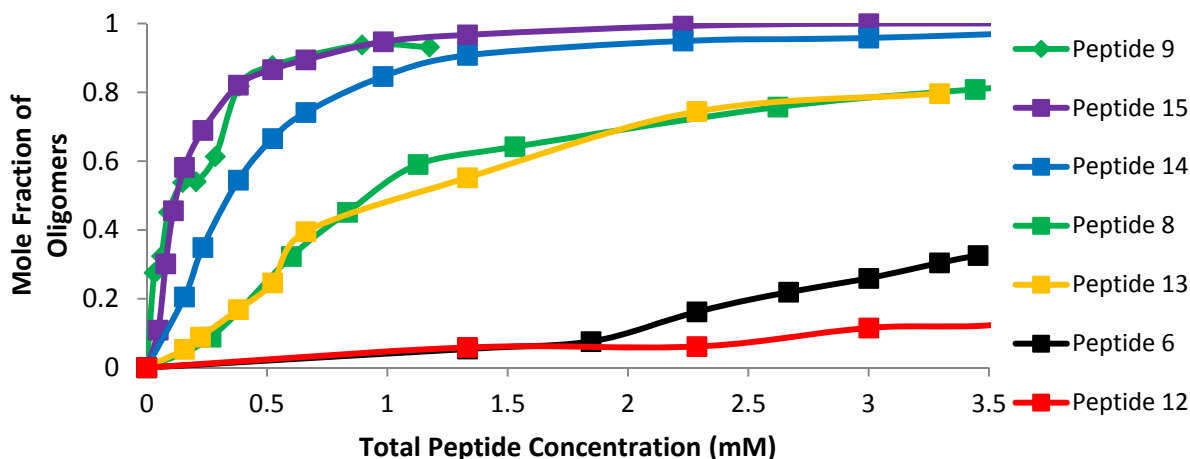
The mole fraction plots of the various mutants were compared to peptide **6** as the baseline and peptide **9**, which has the highest propensity to form aggregates. Peptide **12** reveals that increasing the hydrophobicity of the C-terminus destabilizes oligomer formation, and has an OC_{50} higher than peptide **6**. Peptides **8** and **13** had increased aggregation propensity significantly and comparably. This revealed that increasing the hydrophobicity of the N-terminus and internal aromatic core were important for creating the hydrophobic core. Peptide **14** reveals an OC_{50} an order of magnitude lower than peptide **6**. Peptide **15** had a comparable OC_{50} to peptide **9**, which had the lowest OC_{50}.

Although peptides **14** and **15** have similar aggregation propensities, there were several key differences in the ^{1}H NMR spectra which could reveal major differences in peptide aggregation behavior. The ^{1}H NMR spectrum of peptide **15** (Figure 26) is extremely broad, whereas the spectrum for peptide **14** (Figure 27) has more defined peaking structure. The

increased peak broadness could imply these aggregate species are in rapid exchange with higher

order, soluble aggregate species.
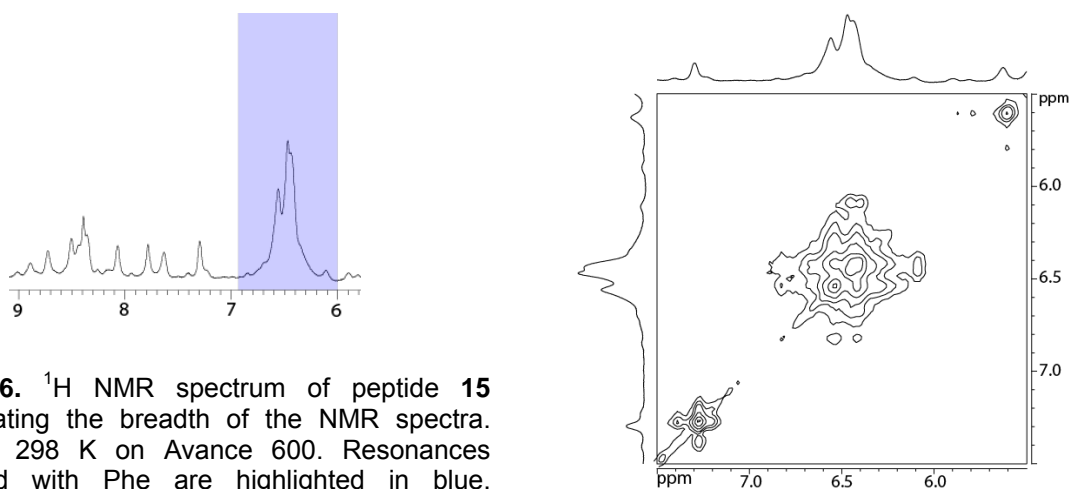
Figure 26. [1]H NMR spectrum of peptide **15** demonstrating the breadth of the NMR spectra. 3.92 mM 298 K on Avance 600. Resonances associated with Phe are highlighted in blue. TOCSY spectrum reveal Phe crosspeaks.
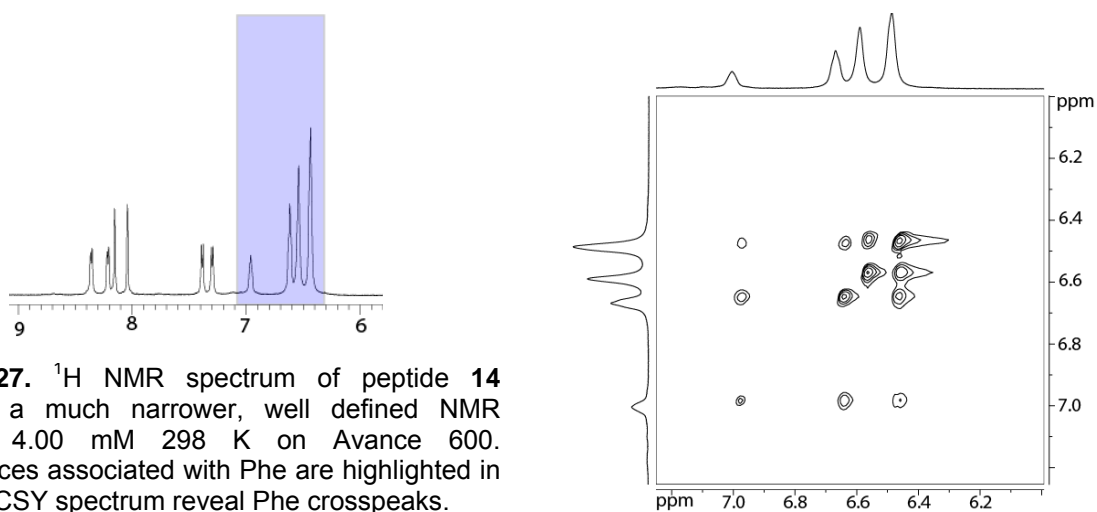

Figure 27. [1]H NMR spectrum of peptide **14** showing a much narrower, well defined NMR spectra. 4.00 mM 298 K on Avance 600. Resonances associated with Phe are highlighted in blue. TOCSY spectrum reveal Phe crosspeaks.

DOSY studies: The relative sizes of the aggregate species can be determined by diffusion ordered spectroscopy (DOSY). DOSY spectroscopy implements a pulse field gradient in order to measure diffusion constants, determining the relative sizes of aggregates.[15] The non-oligomerizing lysine mutants (peptides **10** and **11**) were studied and it was found that the diffusion coefficient corresponded to the diffusion constant of the monomeric species of peptide **6** with a diffusion constant of $1.95 * 10^{-9}$ m^2/s. This diffusion constant was used to determine the size of oligomeric species for peptides **14** and **15**, since the concentration to study the monomeric peptide was too dilute to perform DOSY studies. The diffusion coefficients for these species were $1.23 * 10^{-9}$ m^2/s and $1.14 * 10^{-9}$ m^2/s. The ratios of these values corresponded to tetrameric species, with 0.58 and 0.63 respectively, revealing a tetrameric oligomer for both peptides (Table 4).

Peptide	Aggregate D (m^2/s)	Monomer D (m^2/s)	Ratio
6	1.20E-9	1.95 E-9	0.61
9	1.21E-9	1.95 E-9	0.61
14	1.23E-9	1.95 E-9	0.63
15	1.14 E-9	1.95 E-9	0.58

Table 4. Diffusion constants for aggregate and monomeric species of the peptides obtained from DOSY studies.

Peptide 14

Of all of the peptides studied, peptide **14** was the optimized version of Dr. Khakshoor's original peptide macrocycle. According to the experiments, the peptides formed an in-register anti-parallel dimer which formed a tetrameric oligomer. This peptide also had an extremely rich NOESY spectrum and correlates well to the tetramer model developed by Dr. Khakshoor. Major crosspeaks are highlighted in the experimental section (Figure 28). While the concentration of the NOESY NMR from Dr. Khakshoor's spectrum of peptide **6** was 8.5 mM, peptide **14** formed the same oligomer at submillimolar concentrations.
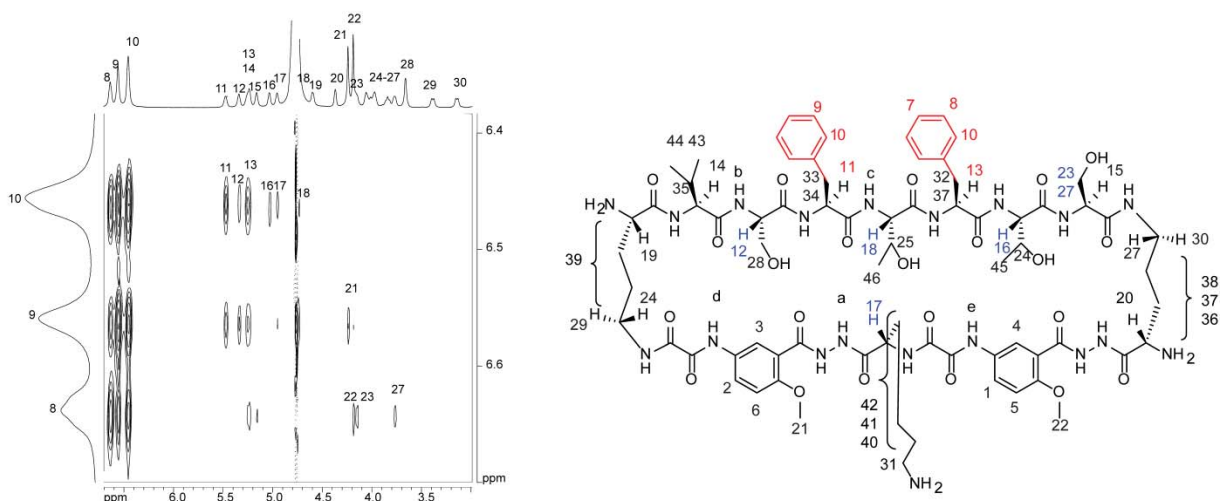
Figure 28. (Left) NOESY spectrum of peptide **14** highlighting phenylalanine on the F1 dimension and the α- and β-proton region on the F2 dimension, highlighting the formation of a hydrophobic core. (Right) Peptide **14** with phenylalanine is highlighted in red, and the proton crosspeaks highlighted in the NOESY spectrum revealing the formation of the hydrophobic core.

Conclusions

Macrocycle and intermolecular β-sheet folding: The chemical shift difference between δ-protons in $^{\delta}$Orn, downfield-shifting of the α-protons, and intramolecular NOEs all revealed that the aggregating macrocyclic peptides had similar folding abilities. An excess of hydrophobic residues affected the change of α proton shifts significantly, however the NMR spectra of peptides **9** and **15** were extremely broad. Intermolecular NOEs showed that all peptides organized themselves into in-register anti-parallel β-sheets.

Concentration dependent ^{1}H NMR studies: The concentration dependent ^{1}H NMR studies revealed the parameters for improving the oligomerization of these macrocyclic heptapeptides. In comparison to peptide **6**, aromatic, hydrophobic residues at Phe_3 and Tyr_5 were important for developing strong oligomeric species. Increasing hydrophilicity at Ser_2 and Ser_6 resulted in peptides that were unable to fold or oligomerize. Hydrophobic residues at the N-terminus were more important than residues at the C-terminus. Increasing hydrophobicity at the C-terminus reduced the propensity for oligomerization.

These studies revealed lead candidates for developing controlled oligomers. Peptides **14** and **15** formed soluble oligomers at submillimolar concentrations. According to DOSY studies, these two peptides created tetrameric species. However, analysis of the NMR spectra reveals that the oligomerization of peptide **15** is not well controlled. The broadness of the spectra could indicate rapid exchange among the tetramer and higher-order aggregate species and the interactions between Phe residues have been significantly reduced, revealing that the hydrophobic core was not stable. Peptide **14** on the other hand, was especially well behaved with a higher resolution ^{1}H NMR spectrum and a well-defined NOESY. The hydrophobic core of peptide **14** was determined to be the optimized peptide used in later studies to design peptides which could oligomerize through the hydrophilic face of the β-sheet. Unfortunately, as demonstrated by peptides **10** and **11**, increasing the hydrophilicity of Ser_2 and Ser_6 destabilize peptide oligomers.

References

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NMR spectroscopic studies

All NMR spectroscopic studies were carried out in D_2O. All peptides were gravimetrically measured and dissolved in "100%" D2O (Cambridge Isotope Laboratories, DLM-4) and incubated for 6 hours. Molecular weights were calculated with the free amines as a TFA salt. 1D [1]H, 2D ROESY and TOCSY NMR spectra were conducted on an Avance 600 MHz NMR spectrometer at 298 K.

Concentration dependent [1]H NMR experiments were performed by creating an 8.00 mM stock of the peptide. Aliquots of this concentrated peptide stock were added to an NMR tube with 450 μL D_2O and an NMR spectrum was taken with each addition.

2D TOCSY spectra were collected with 150 ms spin-lock mixing time. 2D ROESY spectra were collected with 300 ms spin-lock mixing time. 2D TOCSY and ROESY spectra were executed with 2048 points in the f2 domain and 512 points in the f1 domain and processed to a 1024 x 1024 matrix using the QSINE processing parameters. NMR spectra were processed using Bruker XWinNMR software. 2D TOCSY, ROESY and NOESY spectra were collected with 2048 points in the f2 dimension and 512 points in the f1 dimension and processed to a 1024 x 1024 matrix using QSINE processing parameters. Spectra were processed using Bruker XWinNMR software.
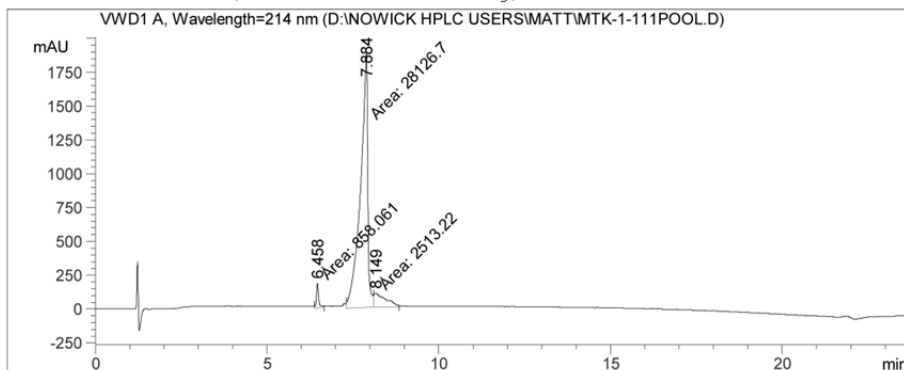
Analytical RP-HPLC of Peptide **6**

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Analysis Method : D:\NOWICK HPLC USERS\NT\5-100 OVER 20MIN AT 214NM.M
Last changed    : 9/17/2014 8:03:44 PM by TA
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================================================================

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Dilution:                  :        1.0000
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Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area       Height      Area
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----|-------|----|-------|----------|----------|--------|
   1   6.458 MM T  0.0776  858.06128   184.29547   2.7242
   2   7.884 MM T  0.2463 2.81267e4   1903.00269  89.2968
   3   8.149 MM T  0.4133 2513.22095   101.34061   7.9790

Totals :                  3.14980e4   2188.63877


================================================================
                    *** End of Report ***
================================================================
```

ESI–MS of Peptide **6**
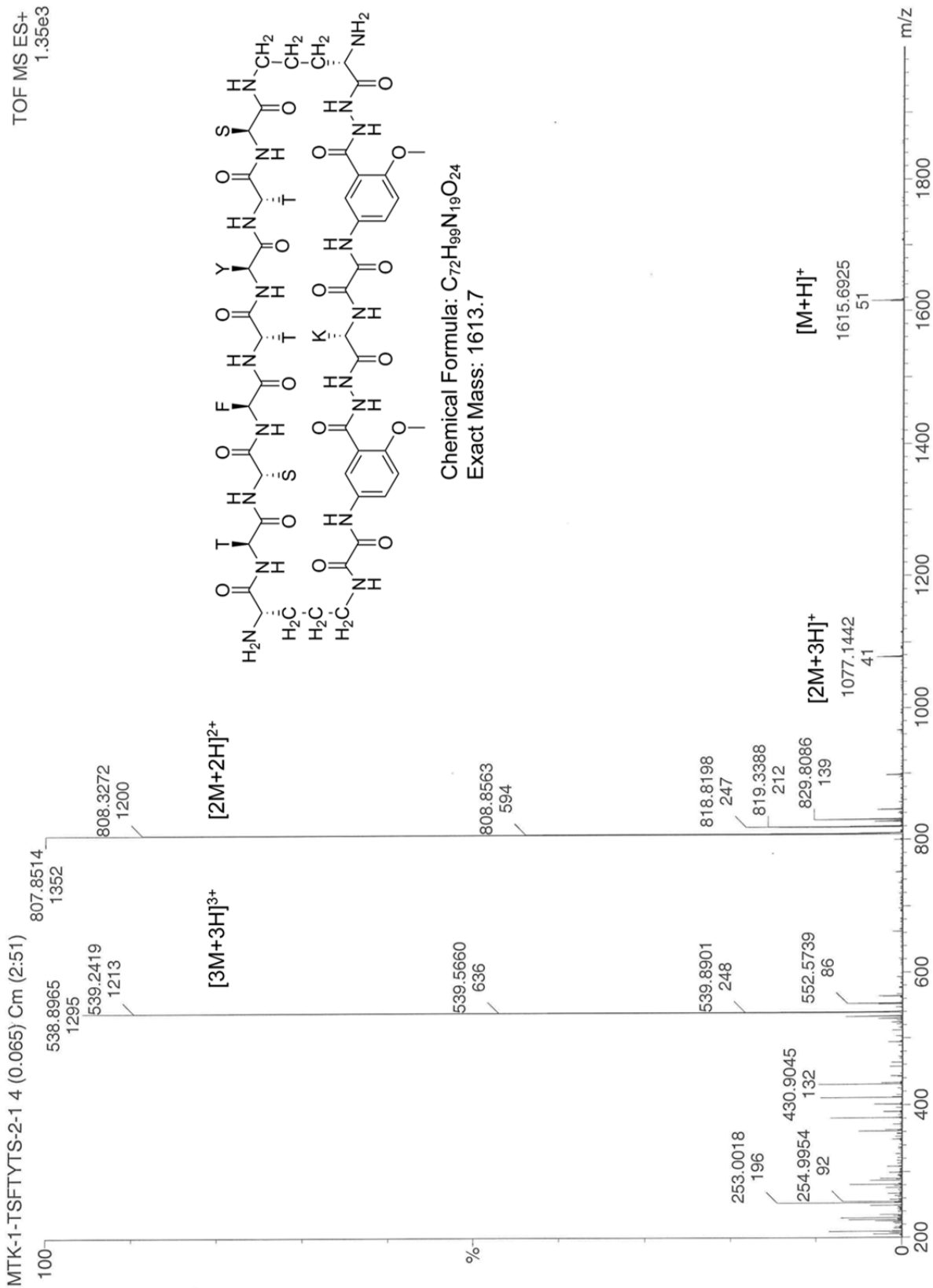
Chemical Formula: C₇₂H₉₉N₁₉O₂₄
Exact Mass: 1613.7

ESI–MS of Peptide **6**

TOF MS ES+
1.35e3

MTK-1-TSFTYTS-2-1 4 (0.065) Cm (2:51)

Chemical Formula: $C_{72}H_{99}N_{19}O_{24}$
Exact Mass: 1613.7

$[M+H]^+$
1615.6925
51

$[2M+3H]^+$
1077.1442
41

$[2M+2H]^{2+}$
808.3272
1200

$[3M+3H]^{3+}$
539.2419
1213

807.8514
1352

538.8965
1295

808.8563
594

539.5660
636

818.8198
247

819.3388
212

829.8086
139

539.8901
248

552.5739
86

430.9045
132

253.0018
196

254.9954
92

m/z

100

%

0

200   400   600   800   1000   1200   1400   1600   1800

1D ^{1}H NMR Spectrum of Peptide **6**
4.00 mM in D$_2$O, 600 MHz, 298 K
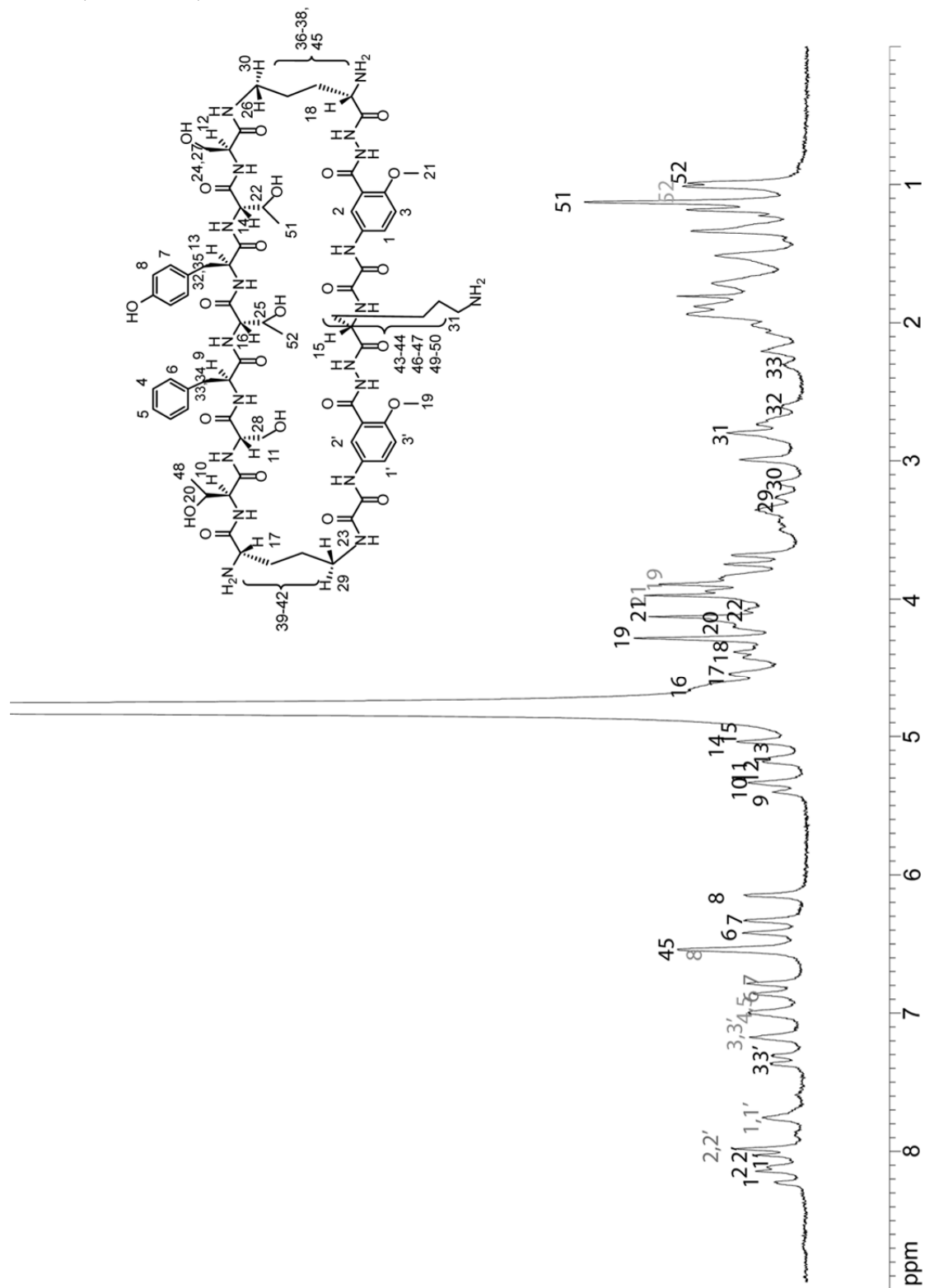
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D$_2$O, 600 MHz, 298 K
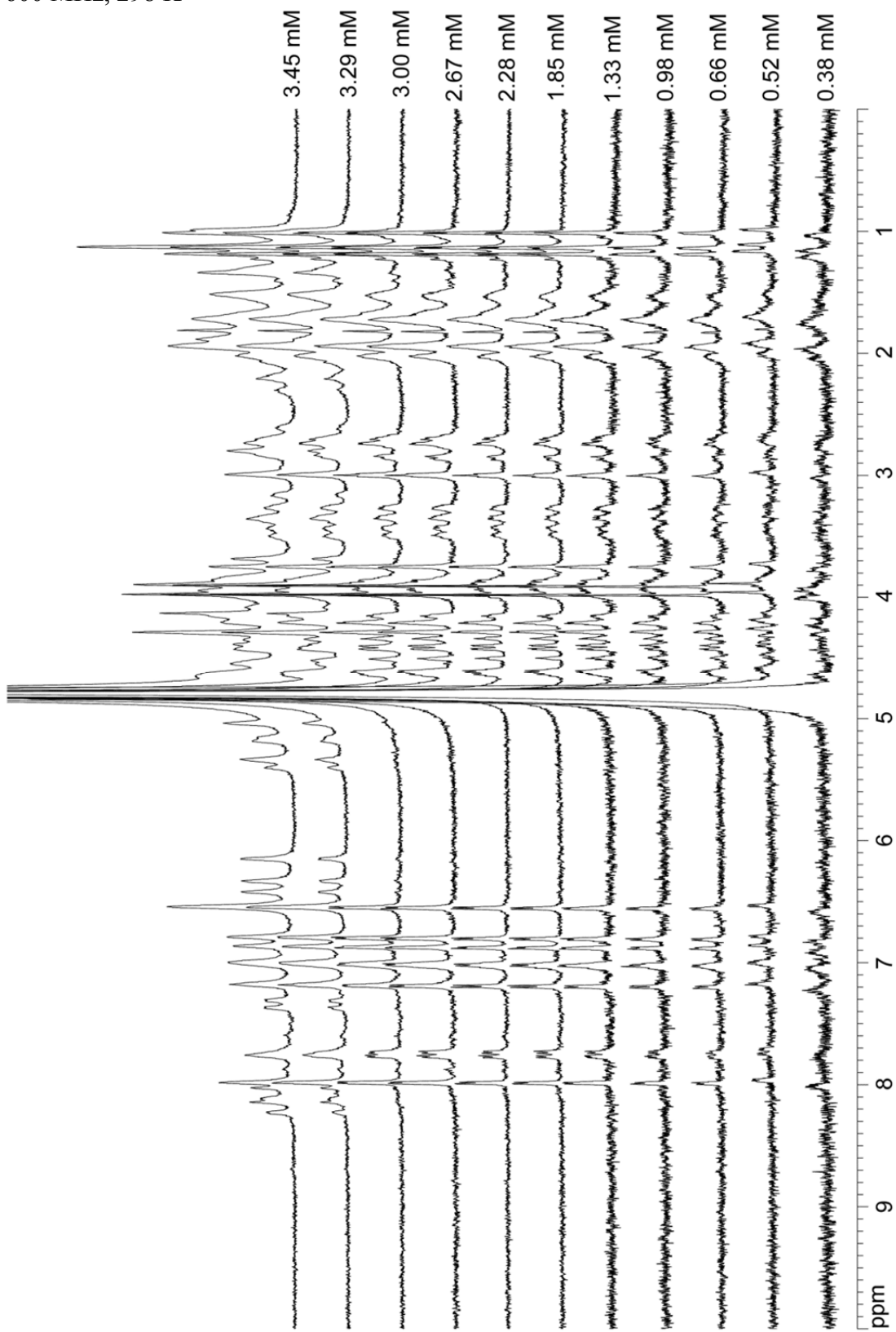
3.45 mM
3.29 mM
3.00 mM
2.67 mM
2.28 mM
1.85 mM
1.33 mM
0.98 mM
0.66 mM
0.52 mM
0.38 mM

2D TOCSY Spectrum of Peptide **6**
4.00 mM in D₂O, 600 MHz, 298K
150-ms spin-locking mixing time
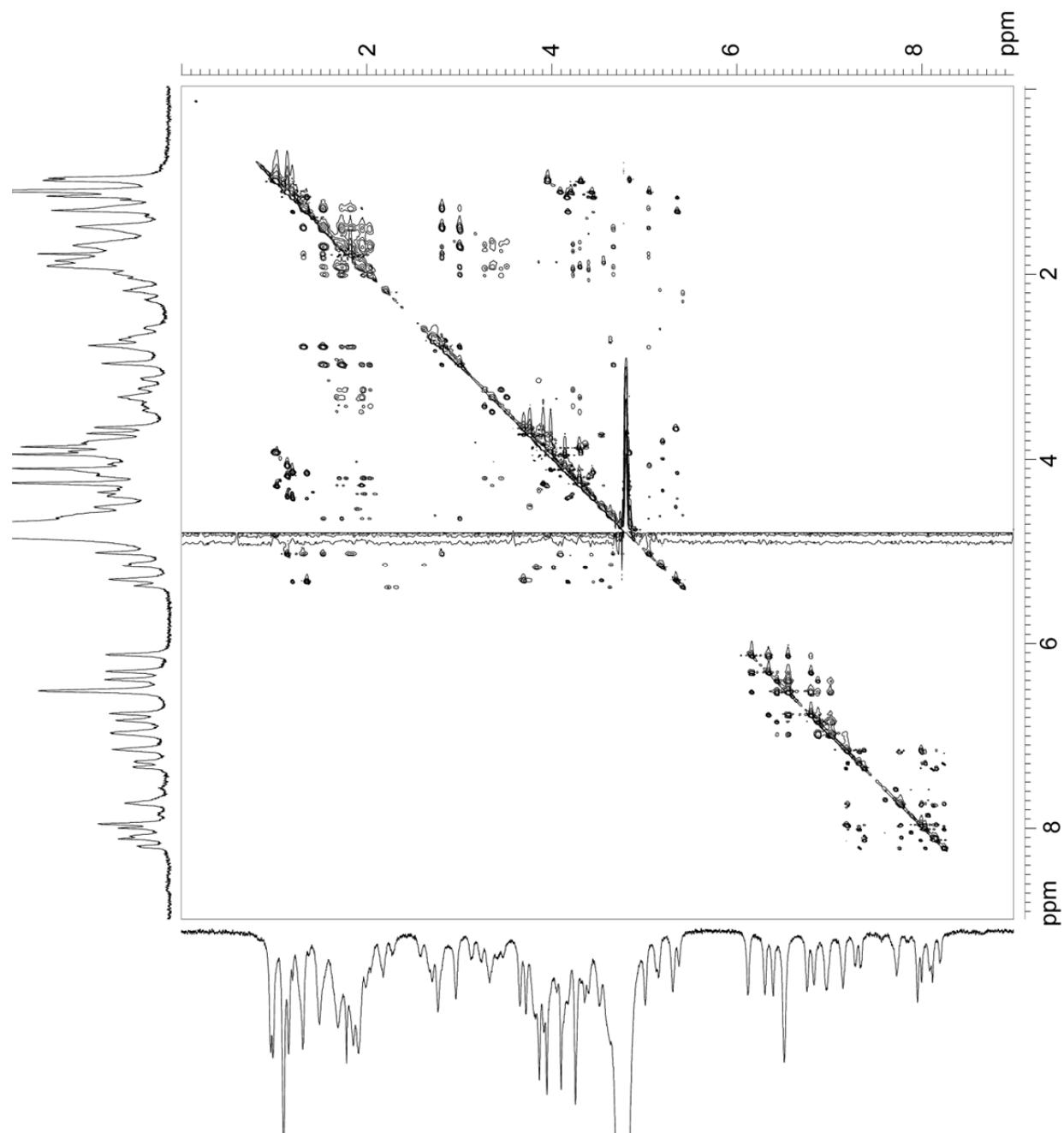
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300-ms spin-locking mixing time
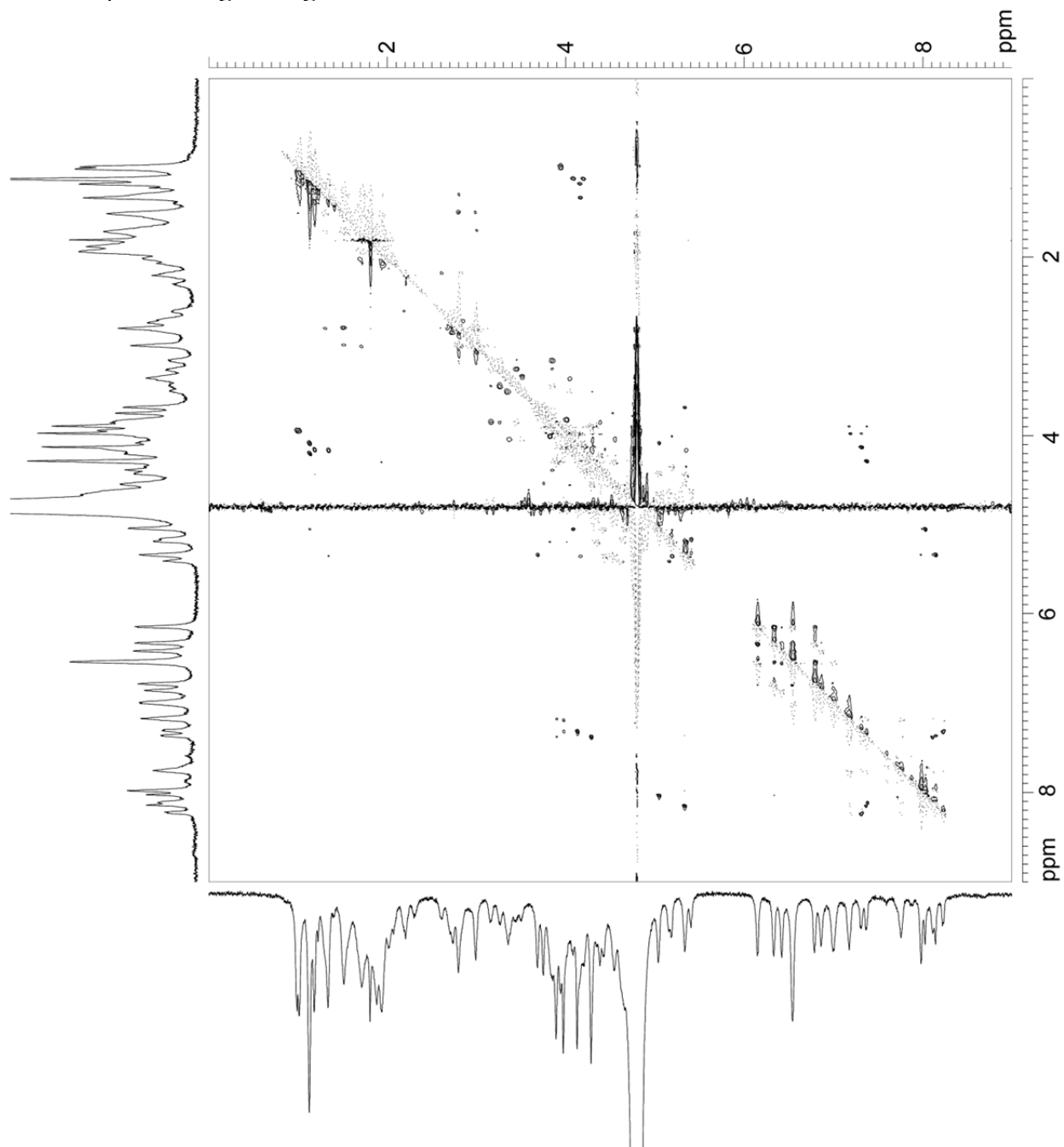
DOSY Spectrum of Peptide **6**
4.00 mM in D$_2$O, 600 MHz, 298 K

Analytical RP-HPLC of Peptide **8**

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                                                  Inj Volume : 20.0 µl
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Analysis Method : D:\NOWICK HPLC USERS\NT\5-100 OVER 20MIN AT 214NM.M
Last changed    : 9/17/2014 8:03:44 PM by TA
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Sample Info     :
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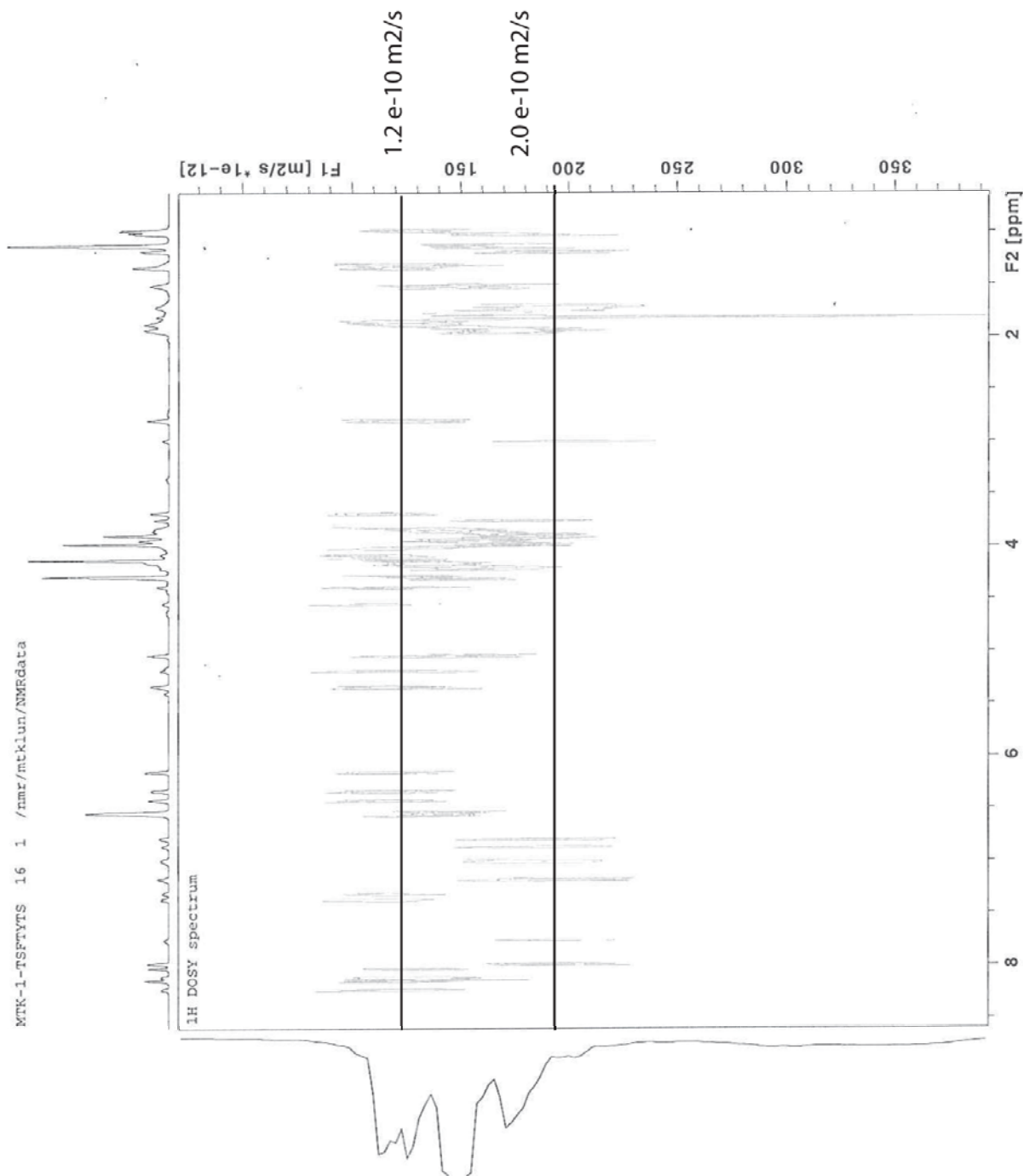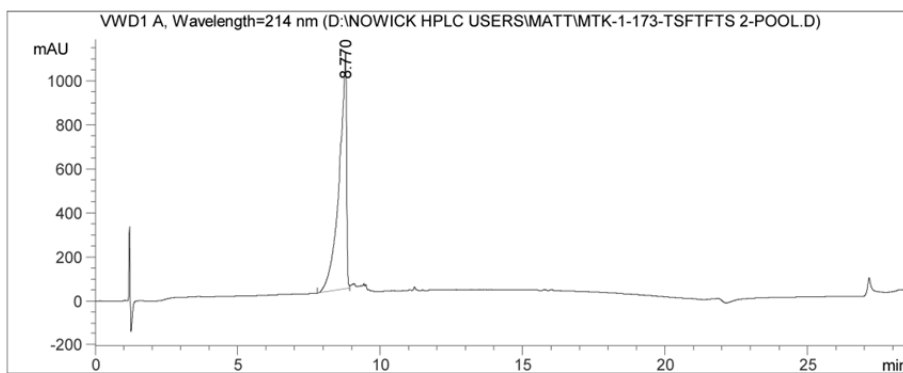

VWD1 A, Wavelength=214 nm (D:\NOWICK HPLC USERS\MATT\MTK-1-173-TSFTFTS 2-POOL.D)

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Dilution:                   :      1.0000
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Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area        Height      Area
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Totals :                 1.90851e4  1063.73206


========================================================================
                      *** End of Report ***
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ESI–MS of Peptide **8**

TOF MS ES+
4.17e3

Chemical Formula: $C_{72}H_{99}N_{19}O_{23}$
Exact Mass: 1597.7 m/z

[M+H]⁺
1599.6556
127

[M+2H]²⁺

[M+3H]³⁺

MTK-1-69-2-1 7 (0.115) Sm (SG, 2x3.00); Cm (2:57)

799.8478
4175

800.3477
3677

800.8346
1797

801.3348
619

810.8282
479

821.8167
273

533.5551
2186

533.8989
1976

534.2321
1006

534.5652
364

207.0728
2585

229.0543
918

261.0835
503

310.1421
381

100

%

0

200   400   600   800   1000   1200   1400   1600   1800

m/z
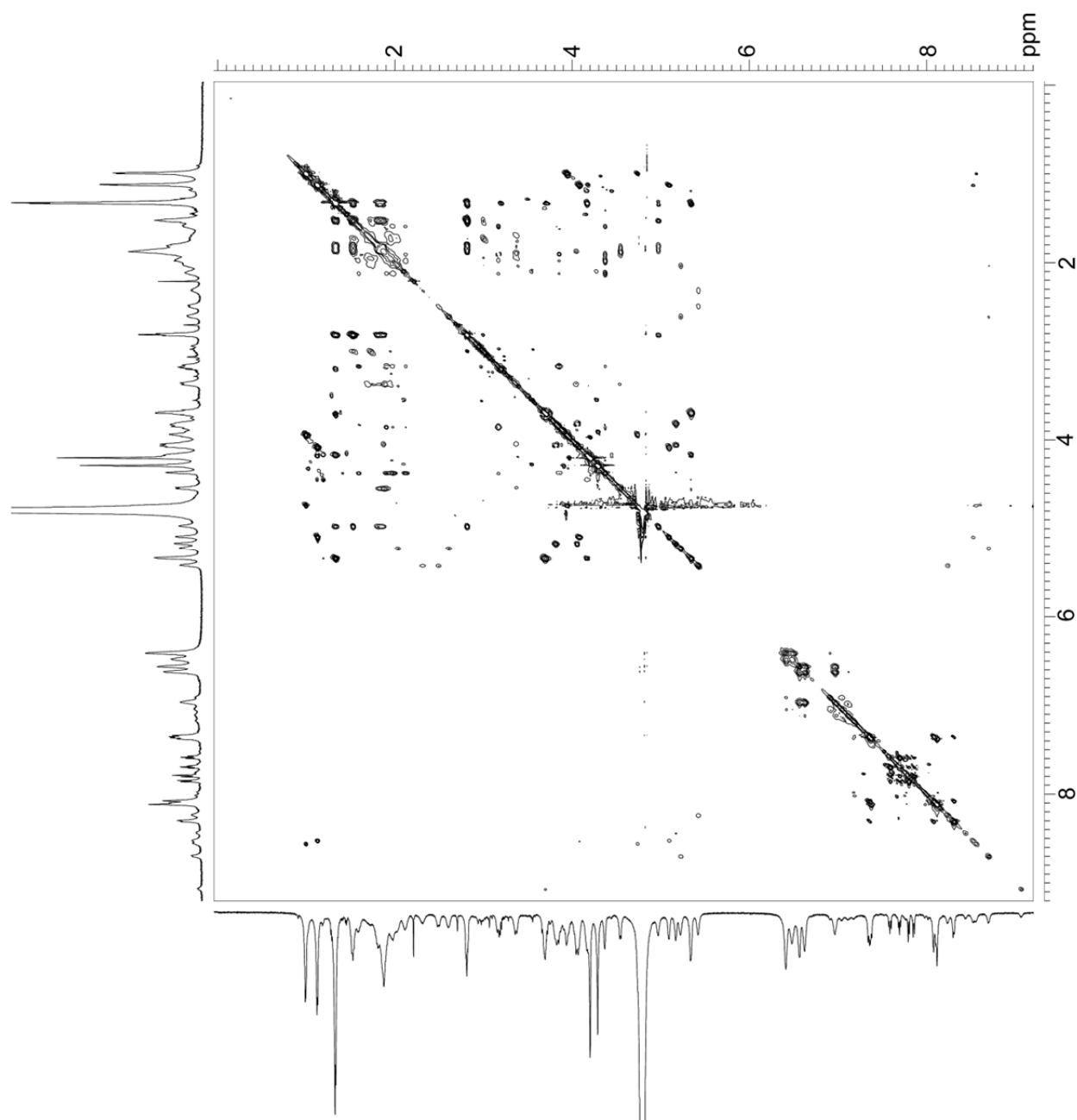
1D ^{1}H NMR Spectrum of Peptide **8**

4.58 mM in D$_2$O, 600 MHz, 298 K
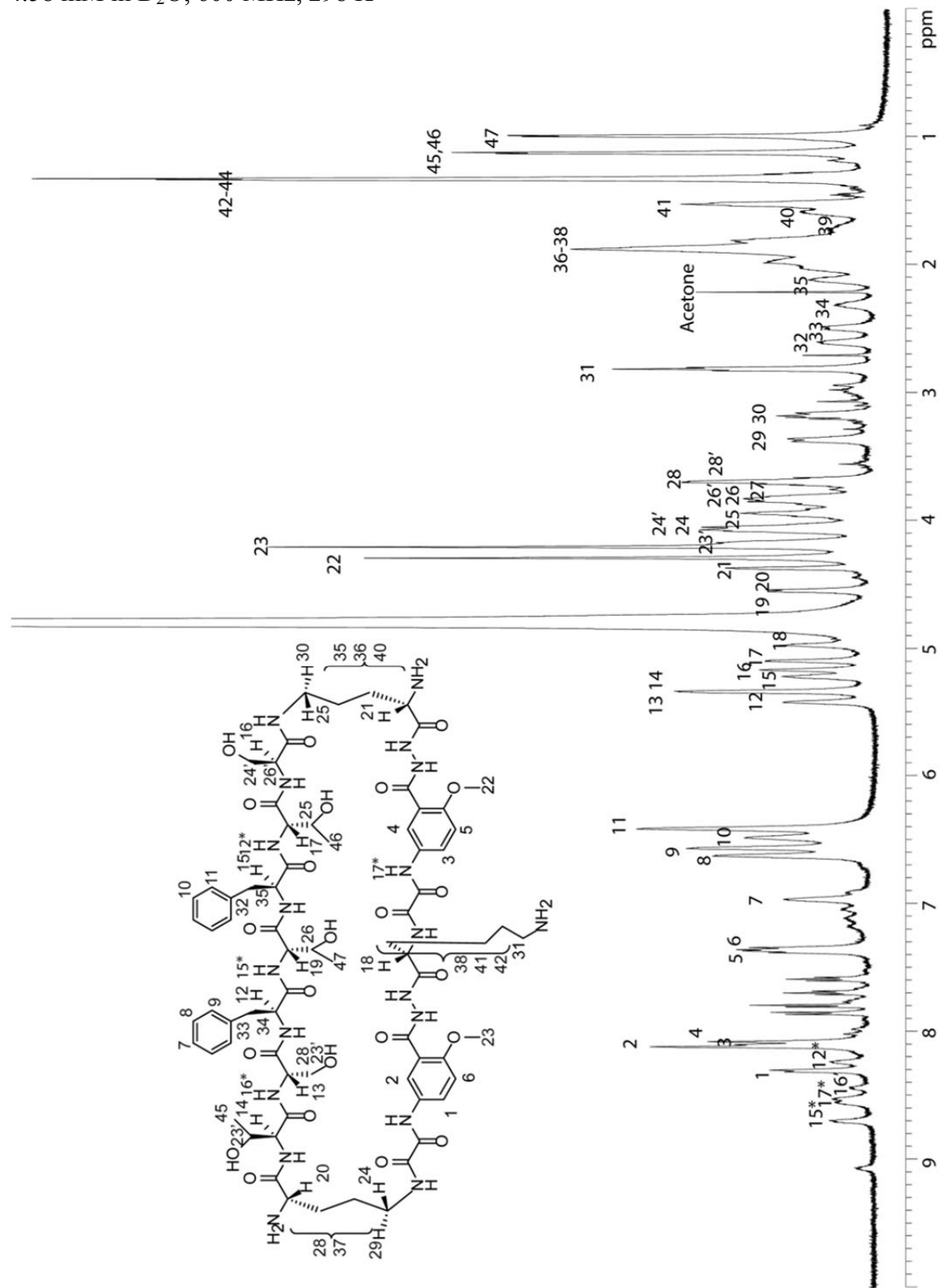
Concentration Dependent 1D ^{1}H NMR Spectra of Peptide **8**
D$_2$O, 600 MHz, 290 K
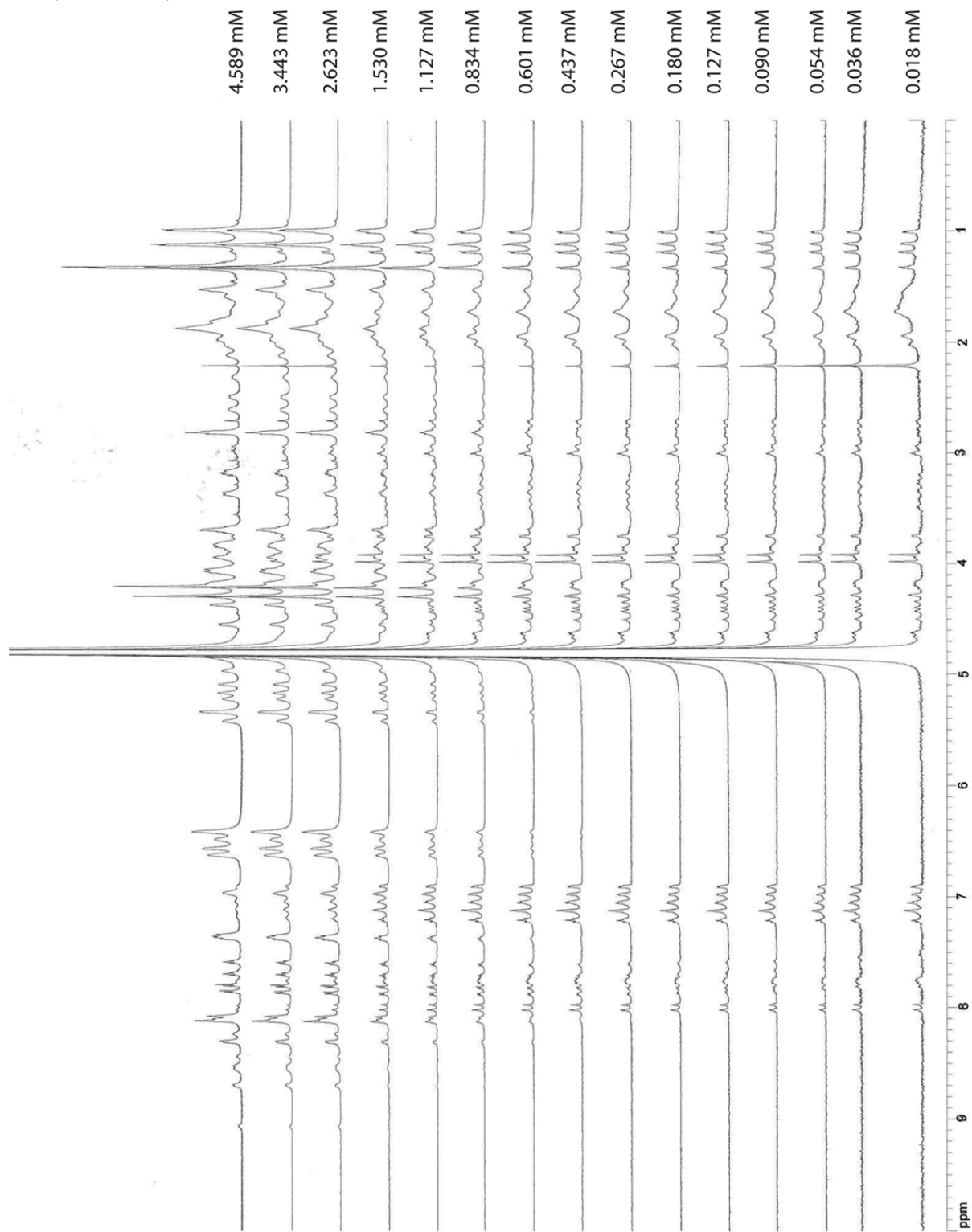
4.589 mM
3.443 mM
2.623 mM
1.530 mM
1.127 mM
0.834 mM
0.601 mM
0.437 mM
0.267 mM
0.180 mM
0.127 mM
0.090 mM
0.054 mM
0.036 mM
0.018 mM

2D TOCSY Spectrum of Peptide **8**
4.58 mM in D₂O, 600 MHz, 298 K
150-ms spin-locking mixing time

2D ROESY Spectrum of Peptide **8**
4.58 mM in D₂O, 600 MHz, 298K
300-ms spin-locking mixing time

DOSY Spectrum of Peptide **8**
4.58 mM in D$_2$O, 600 MHz, 298K
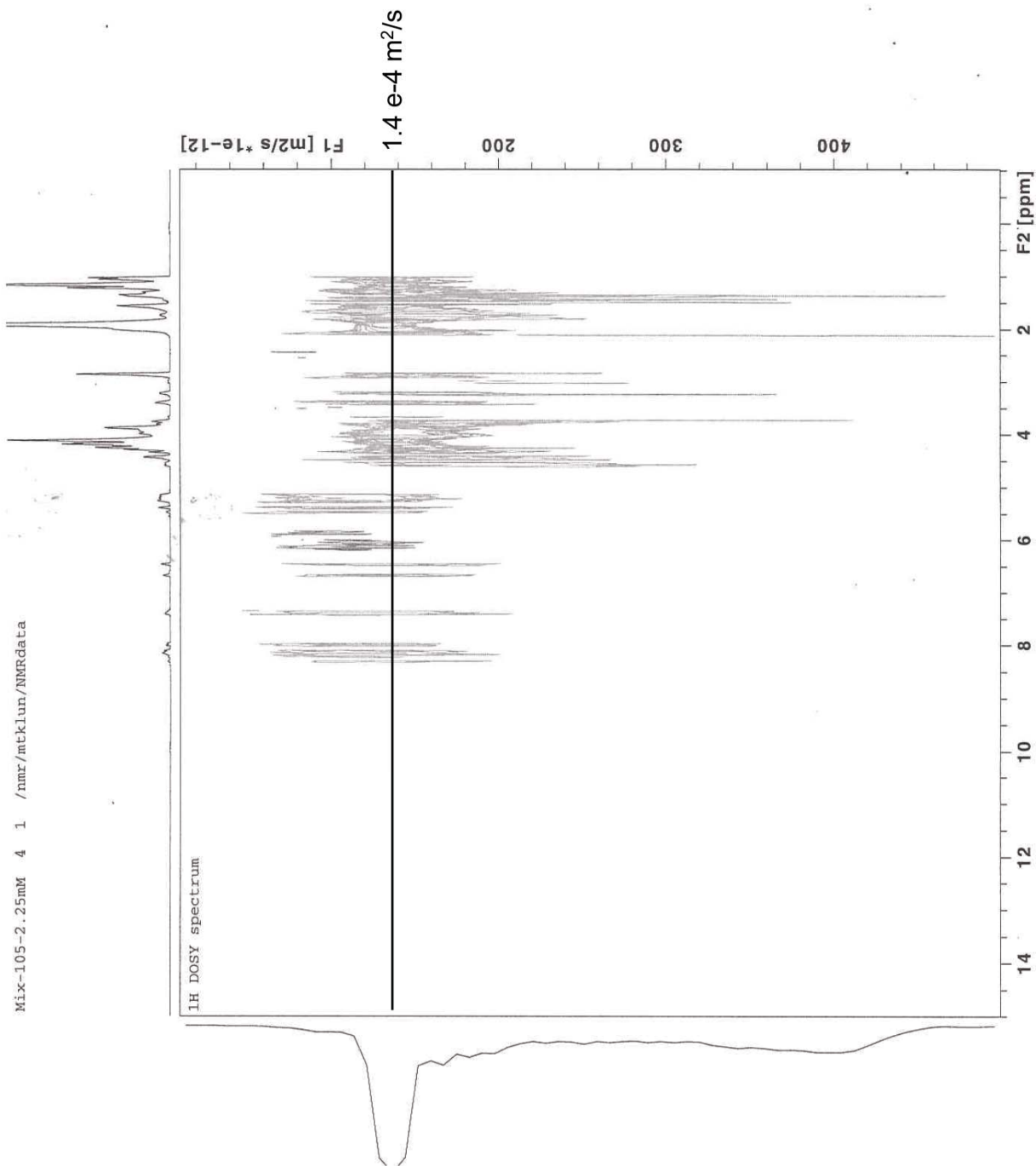
Analytical RP-HPLC of Peptide 9

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                                                 Inj Volume : 20.0 µl
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                  (modified after loading)
Analysis Method : D:\NOWICK HPLC USERS\NT\5-100 OVER 20MIN AT 214NM.M
Last changed    : 9/17/2014 8:03:44 PM by TA
                  (modified after loading)
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VWD1 A, Wavelength=214 nm (D:\NOWICK HPLC USERS\MATT\MTK-1-TS5T5TS-POOL.D)

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Dilution:                   :      1.0000
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Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area       Height      Area
 #   [min]         [min] mAU   *s   [mAU   ]        %
----|-------|----|-------|----------|----------|--------|
  1  11.747 VV    0.3339 5.65215e4  2122.91577 100.0000

Totals :                  5.65215e4  2122.91577


========================================================================
                      *** End of Report ***
========================================================================
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ESI-MS of Peptide **9**

Chemical Formula: $C_{72}H_{89}N_{19}O_{23}F_{10}$
Exact Mass: 1777.6 m/z

MTK-1-TS5T5TS-1 18 (0.299) Cm (2:60)

TOF MS ES+
6.27e3

1D ^{1}H NMR Spectrum of Peptide **9**
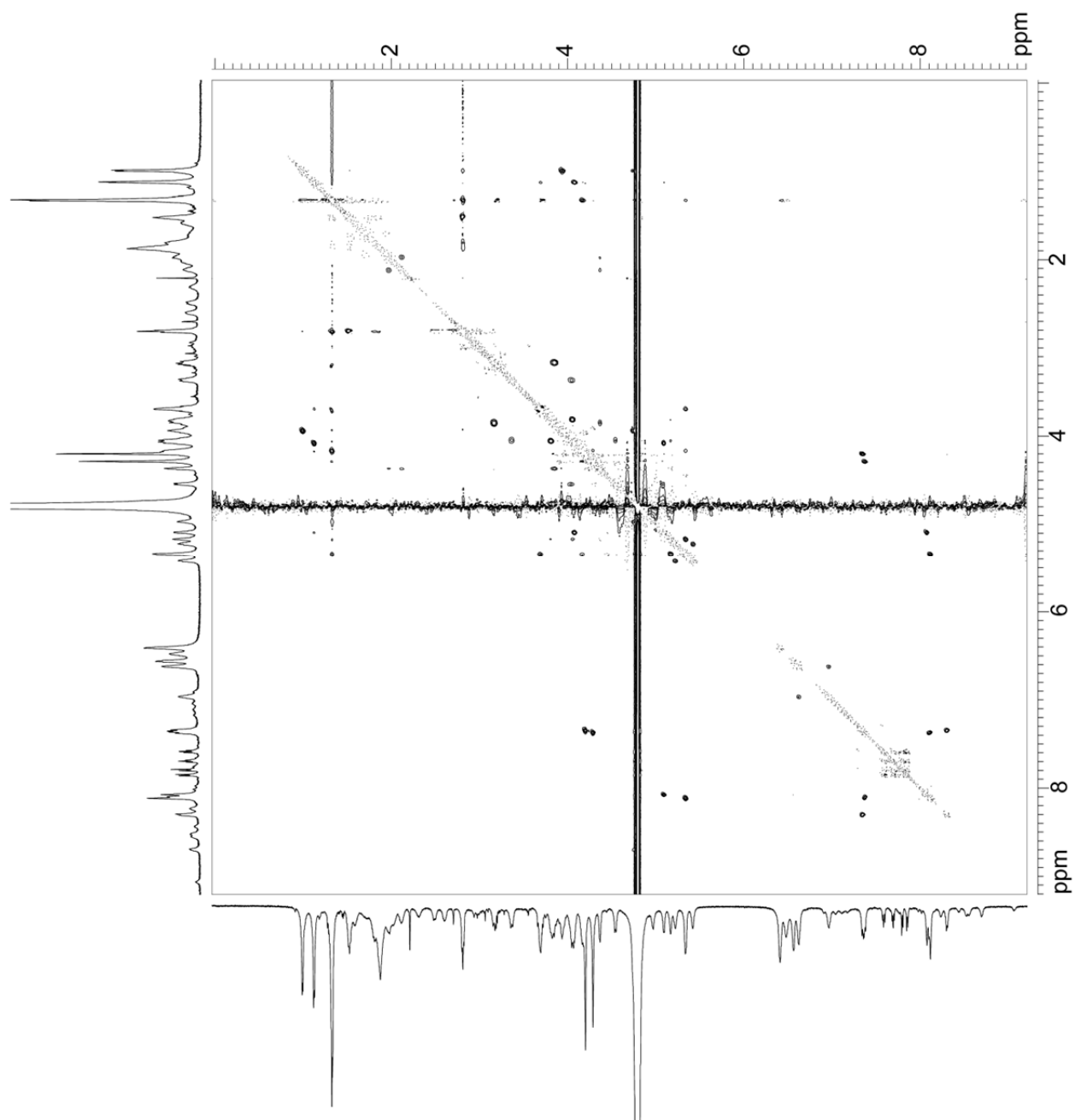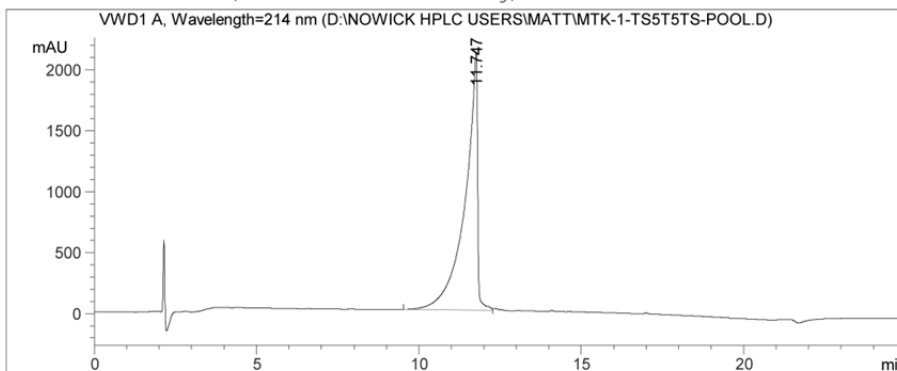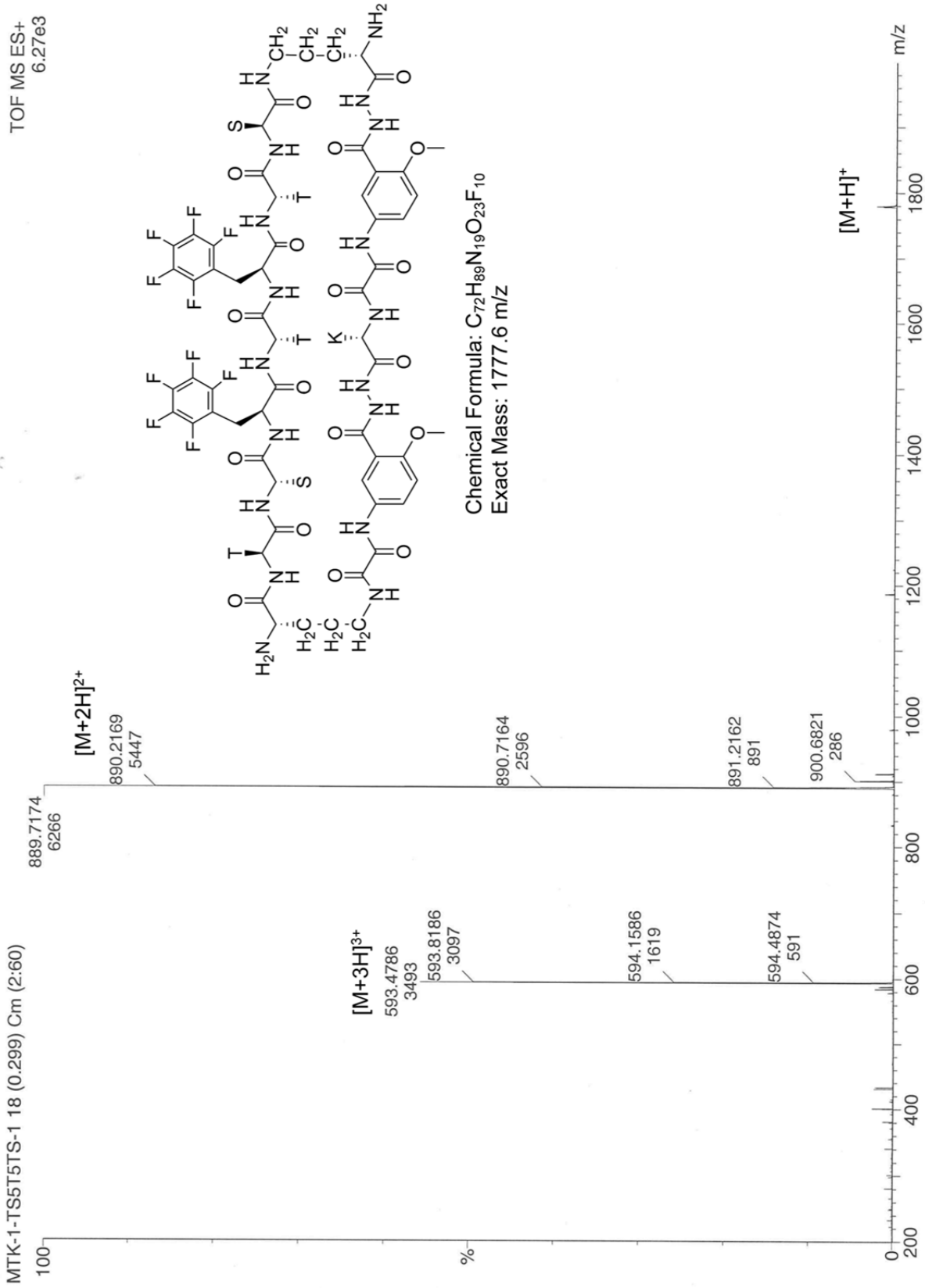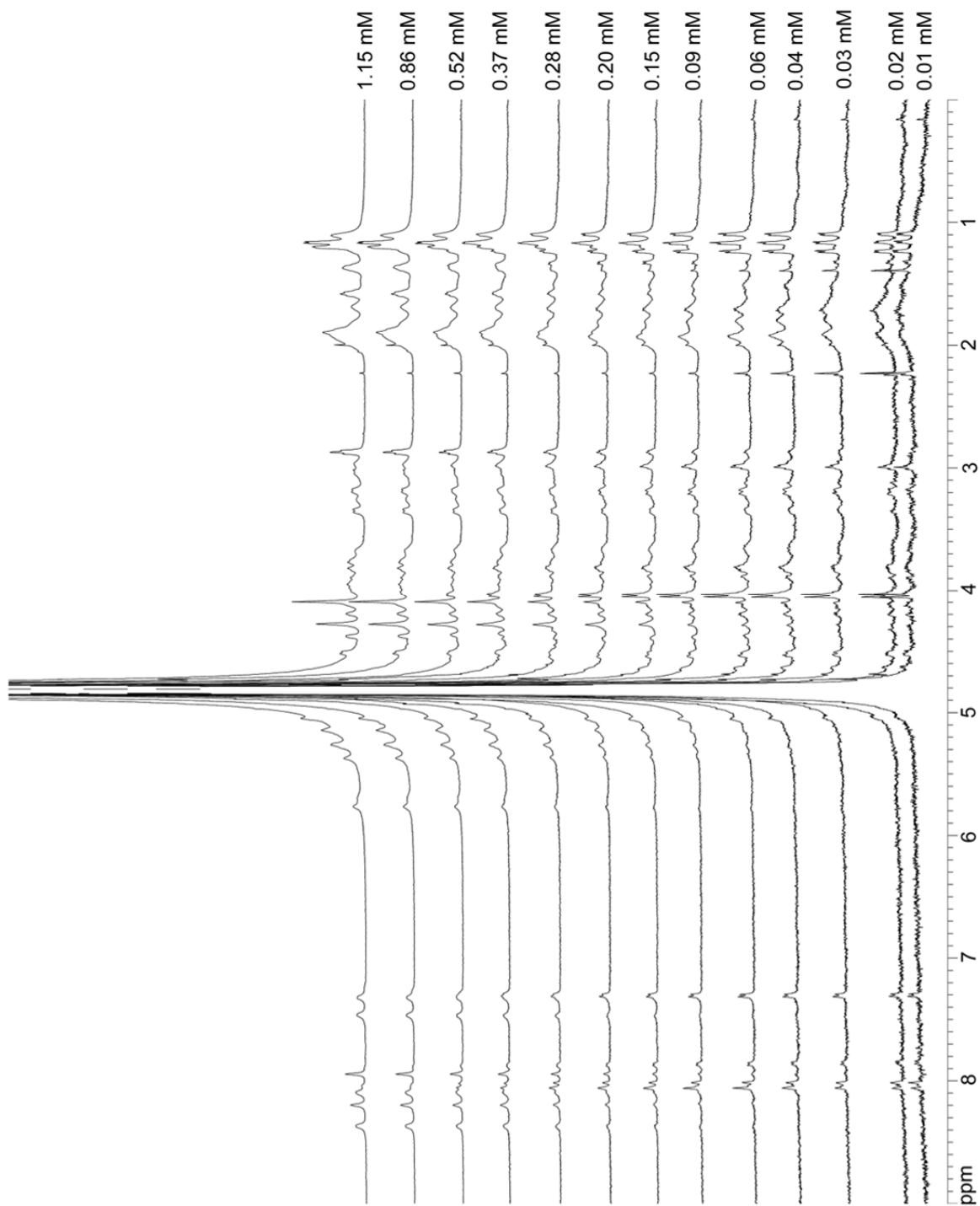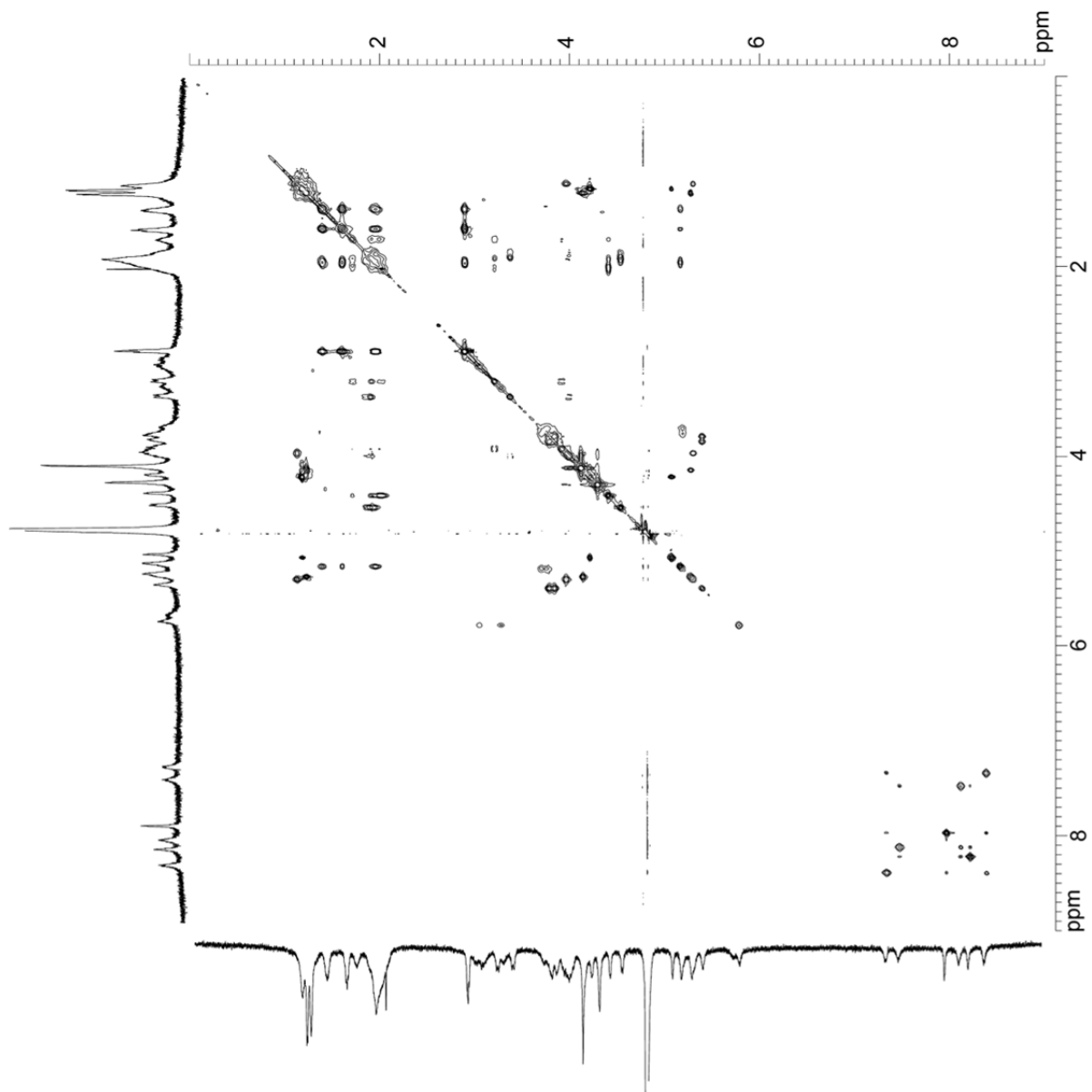1.15 mM in D$_2$O, 600 MHz, 298K
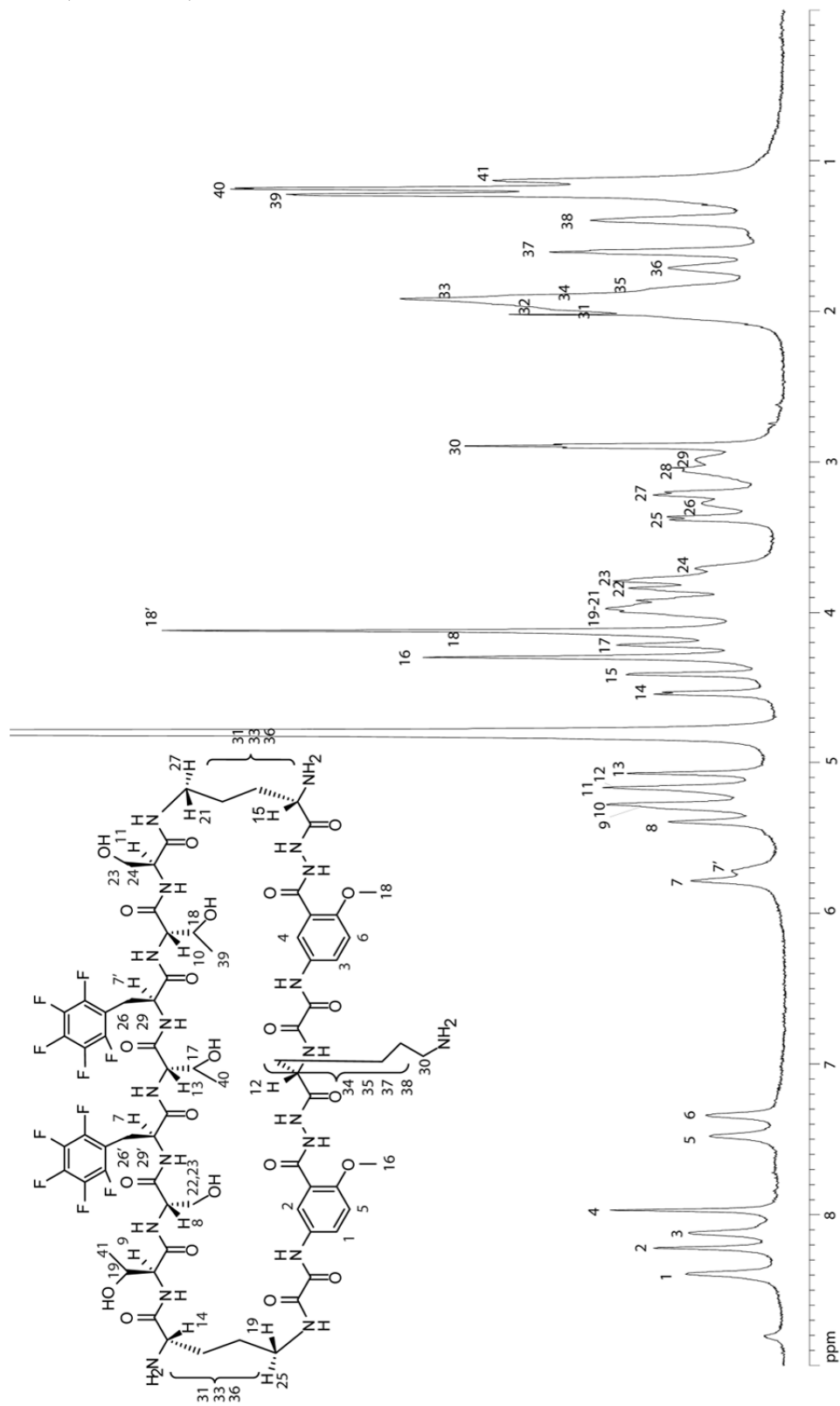
Concentration Dependent 1D ^{1}H NMR Spectra of Peptide **9**
D$_2$O, 600 MHz, 298K

2D TOCSY Spectrum of Peptide **9**
1.15 mM in D₂O, 600 MHz, 298K
150-ms spin-locking mixing time

2D ROESY Spectrum of Peptide **9**
1.15 mM in D₂O, 600 MHz, 298K
300-ms spin-locking mixing time
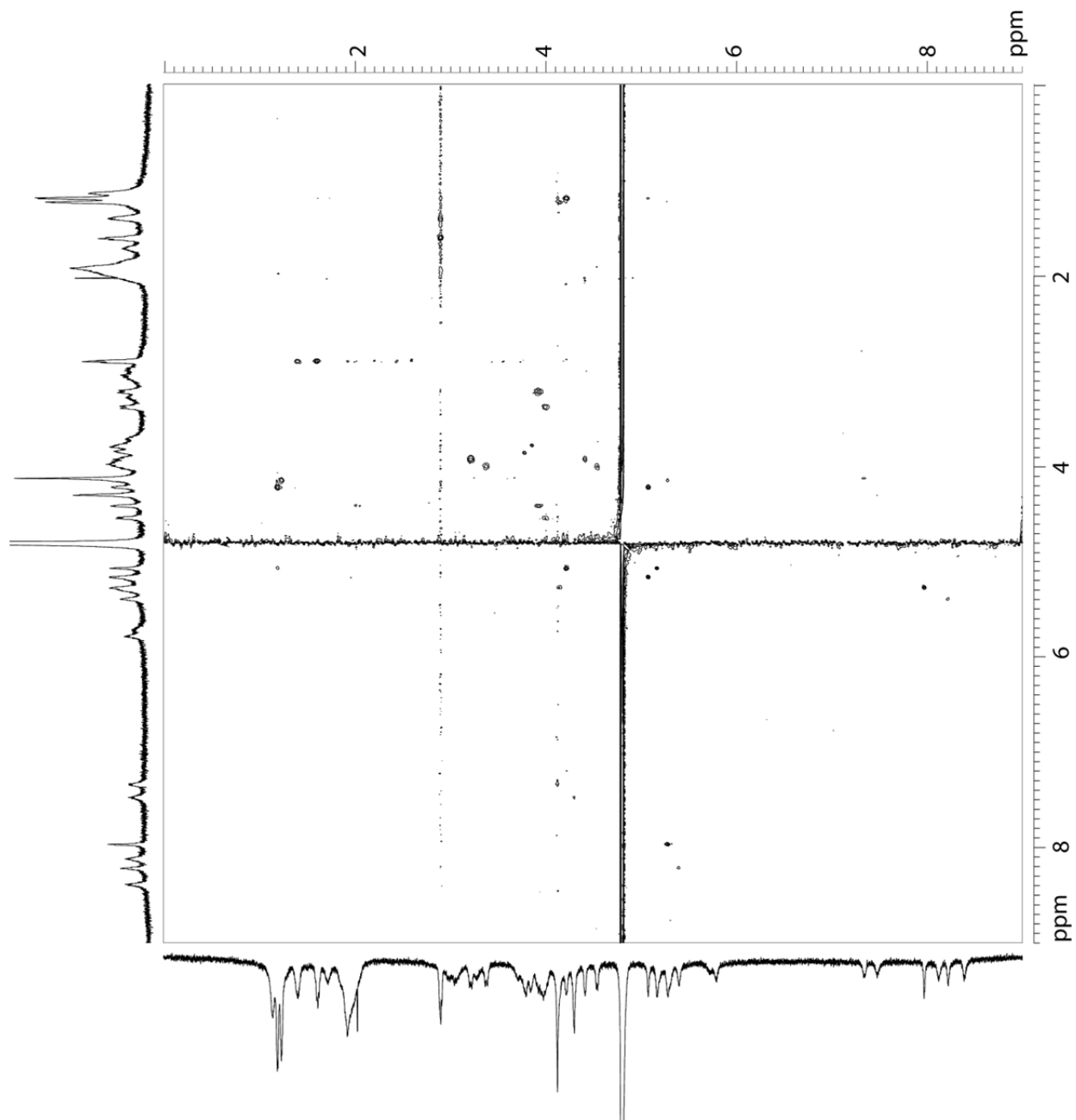
DOSY Spectrum of Peptide **9**
1.15 mM in D₂O, 600 MHz, 298K

1D ^{1}H NMR Spectra of peptides **8** (red, 0.4 mM), peptide **9** (green, 0.4 mM), and both peptides mixed in D$_2$O (black, 0.4 mM each), 600 MHz, 298 K
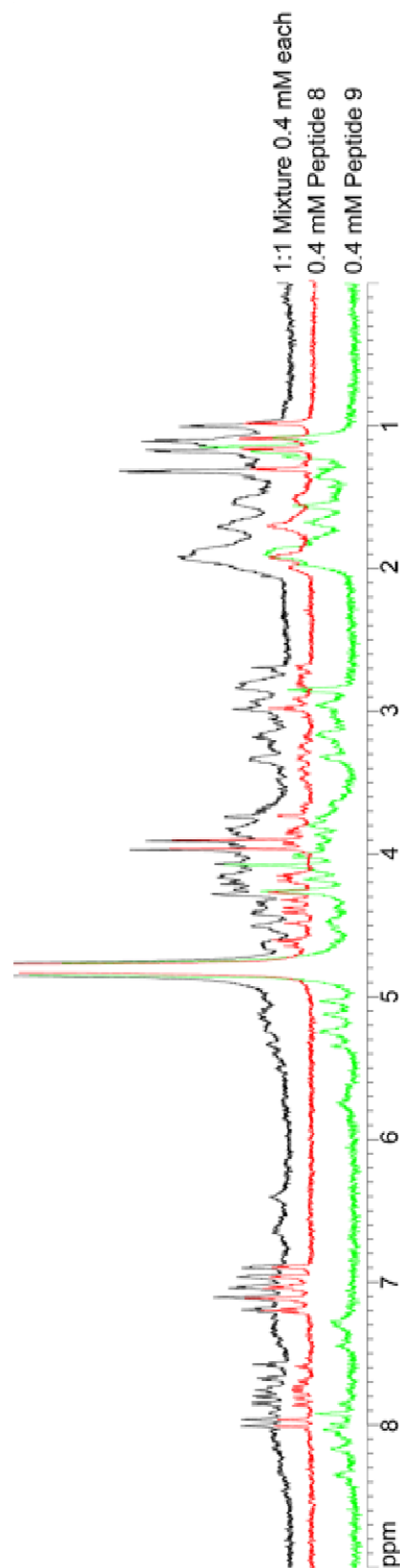
1D ^{1}H NMR spectra of peptides **8** (red, 2.5 mM), peptide **9** (green, 2.0 mM), and both peptides mixed in D$_2$O (black, 2.25 mM each), 600 MHz, 298 K
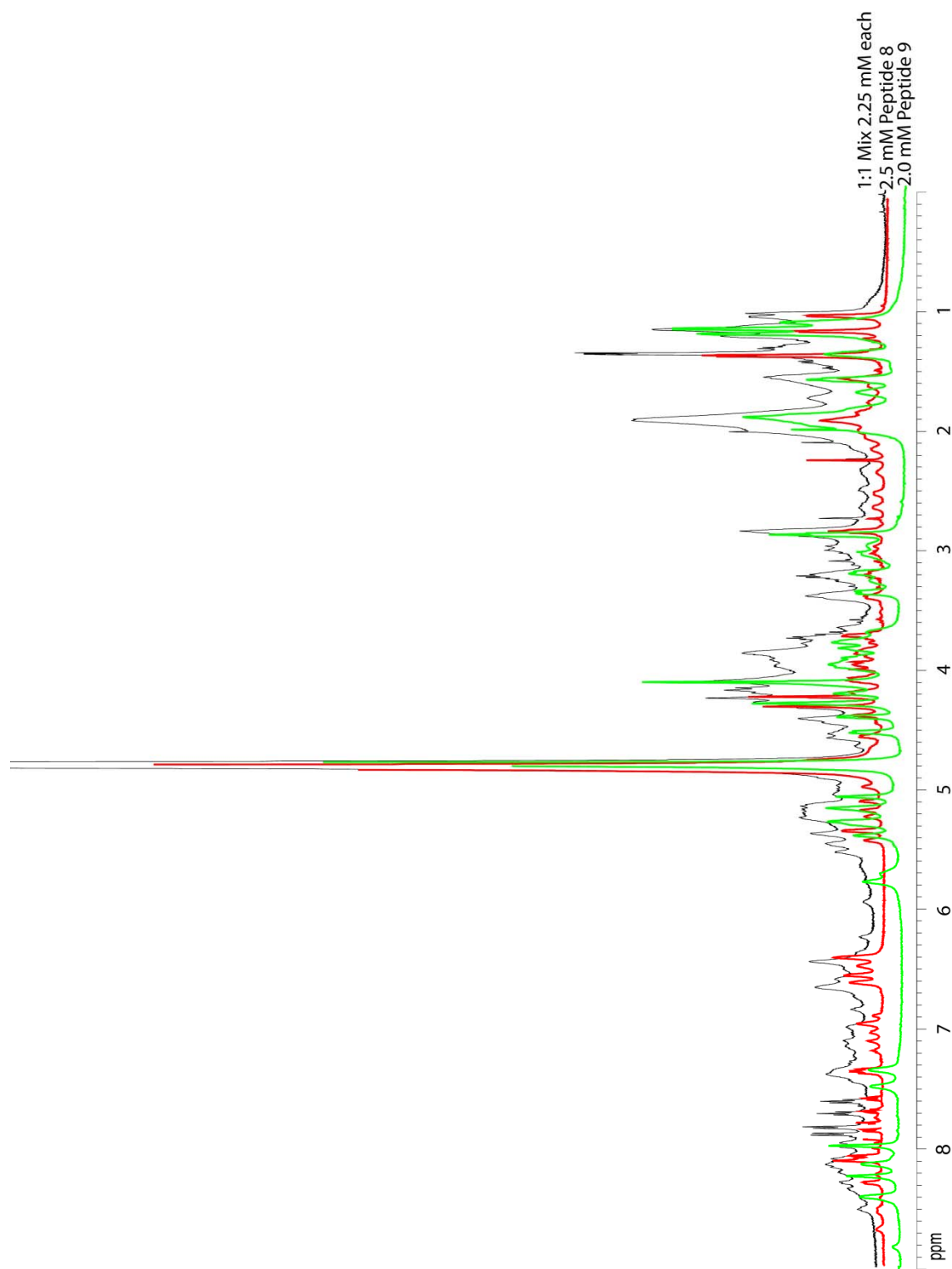
2D TOCSY Spectrum of Peptides **8** and **9** Mixed
2.25 mM each in D₂O, 600 MHz, 298 K
150-ms spin-locking mixing time

2D TOCSY Spectra of peptides **8** (red, 2.5 mM), peptide **9** (green, 2.0 mM), and both peptides mixed in D$_2$O (black, 2.25 mM each), 600 MHz, 298 K

150-ms spin-locking mixing time

2D ROESY spectrum of peptides **8** and **9** mixed
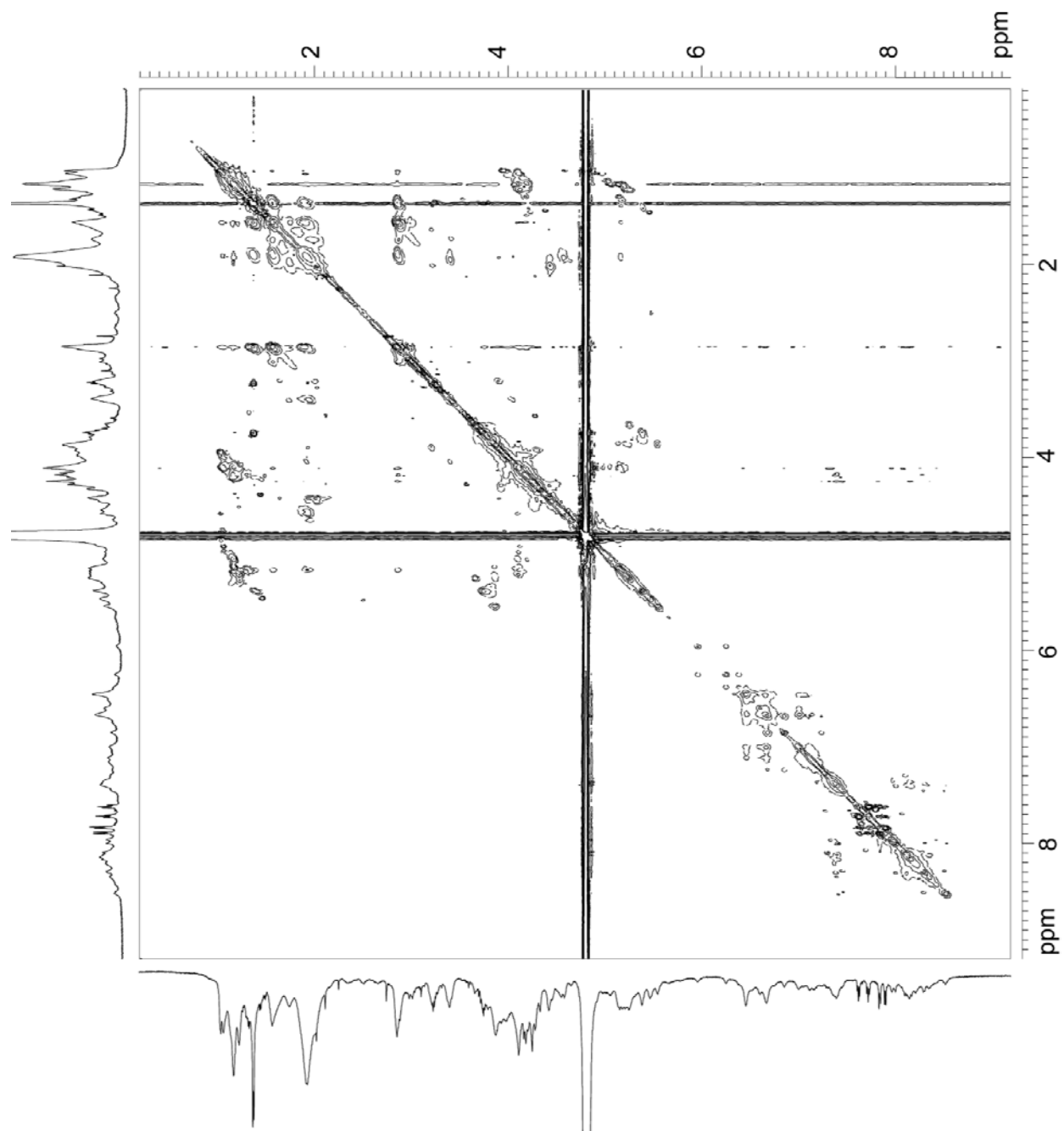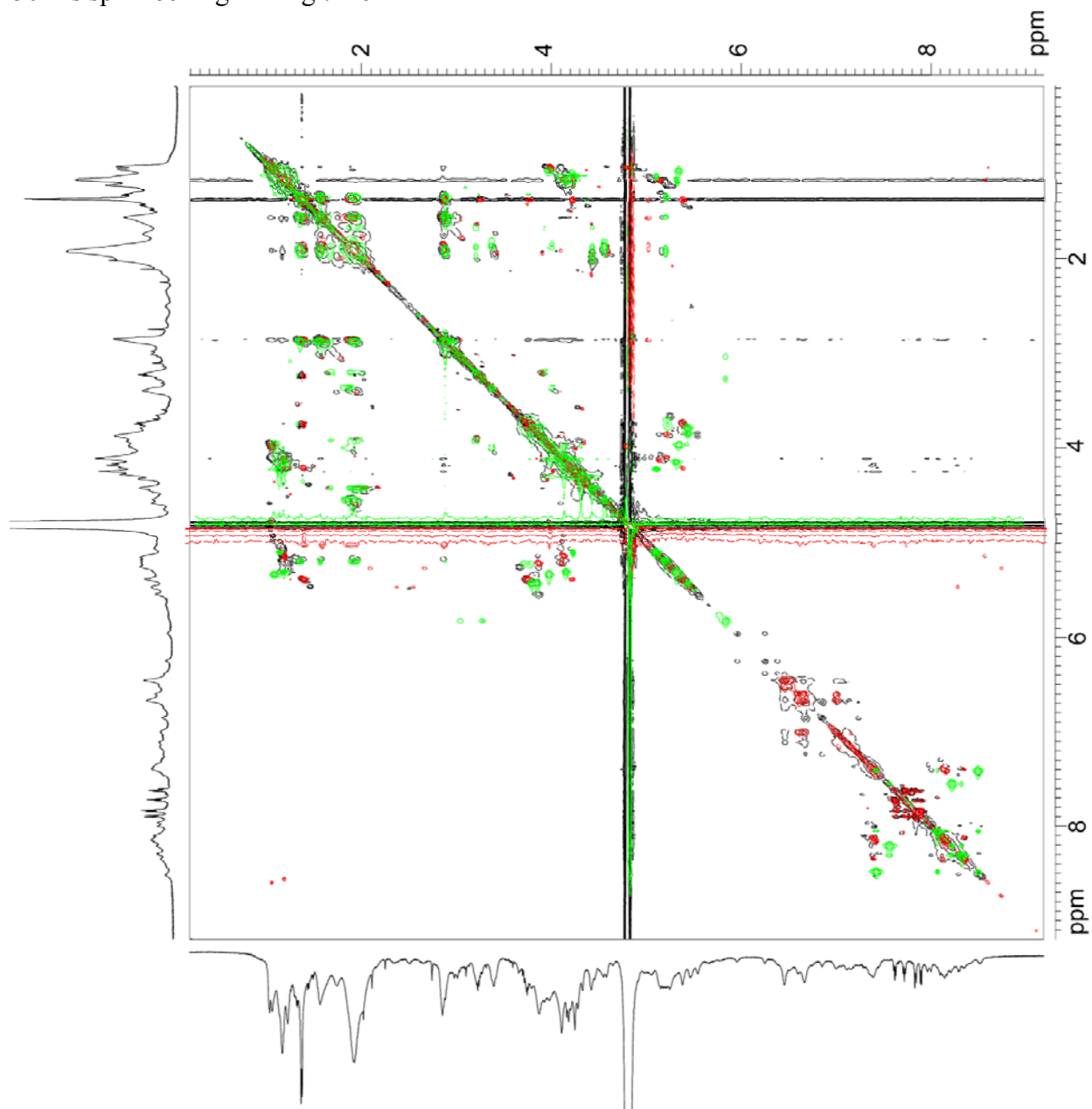2.25 mM each in D$_2$O, 600 MHz, 298 K
300-ms spin-locking mixing time

2D ROESY Spectra of peptides **8** (red, 2.5 mM), peptide **9** (green, 2.0 mM), and both peptides mixed in D_2O (black, 2.25 mM each), 600 MHz, 298 K
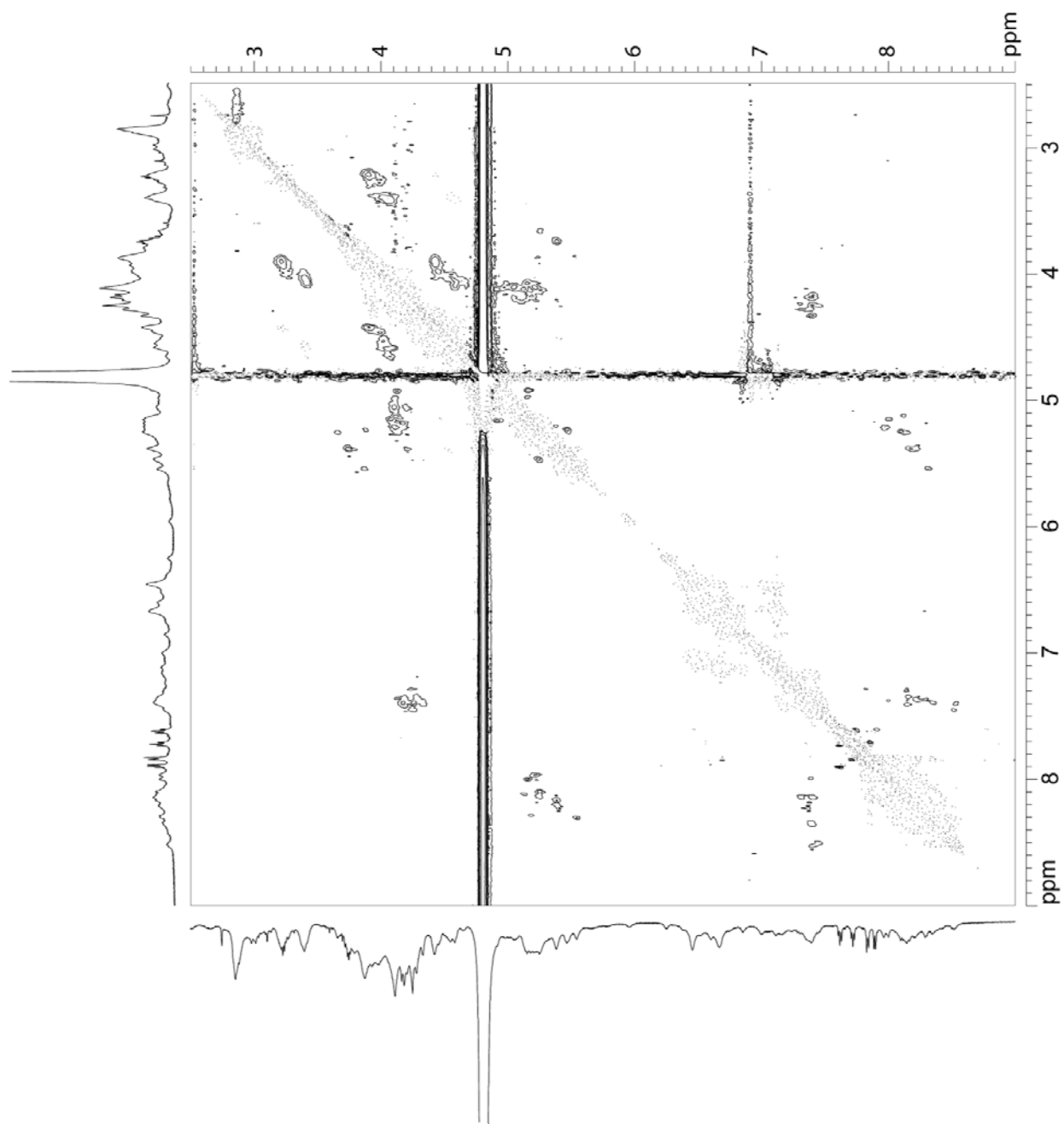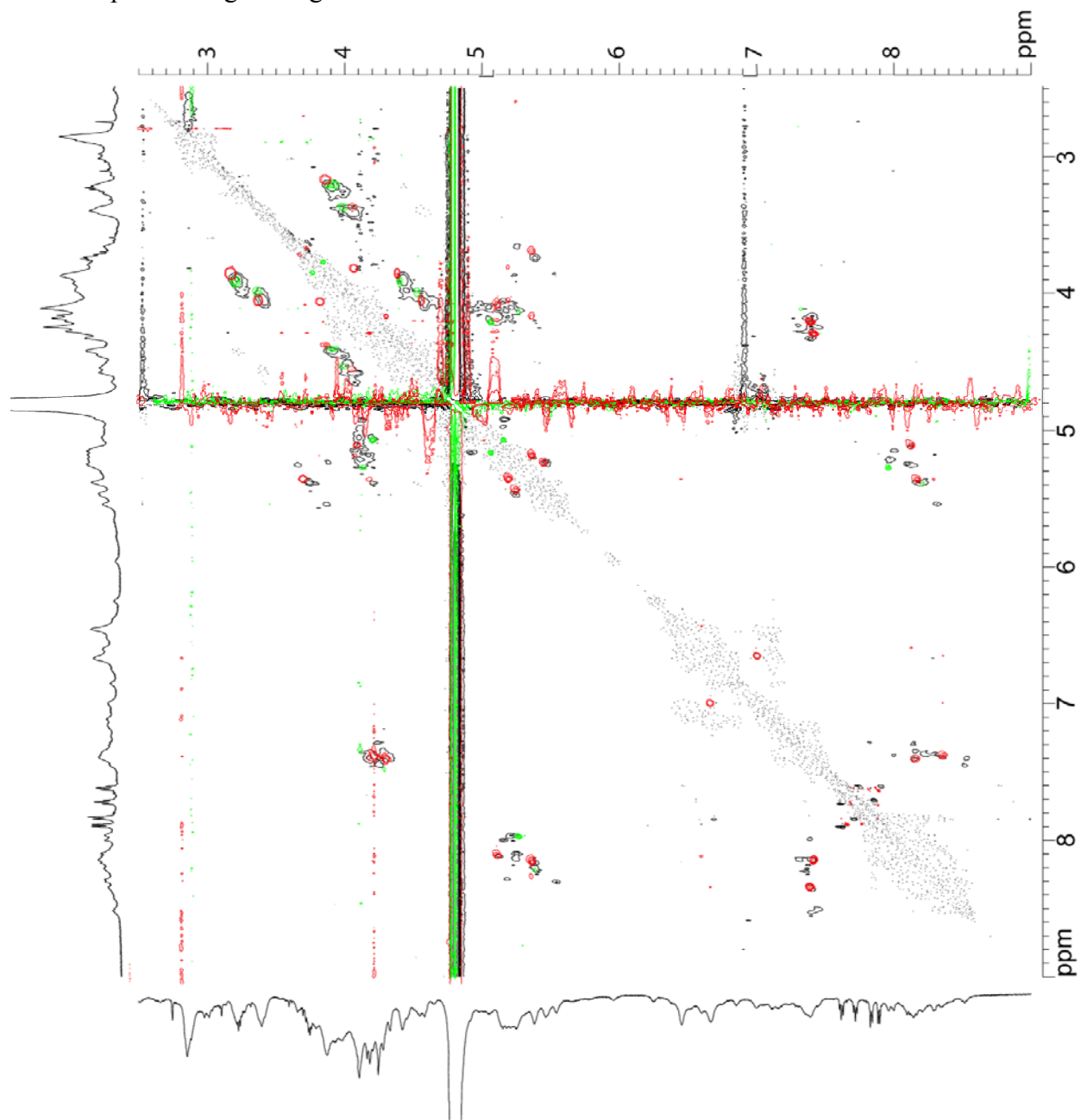300-ms spin-locking mixing time

Analytical RP-HPLC of Peptide **10**

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======================================================================
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Analysis Method : D:\NOWICK HPLC USERS\ZHENGJ\METHODS\5-100 OVER 20 MIN AT 214 AT 1 ML.M
Last changed    : 5/4/2013 7:11:01 AM by Ryan
                  (modified after loading)
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======================================================================


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Dilution:                   :       1.0000
Sample Amount:              :       20.00000 [ng/ul]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs



Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area       Height     Area
  #   [min]        [min]   mAU   *s    [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   7.187 BV   0.1302 9914.84570 1053.63867  79.2774
   2   7.490 VV   0.2088 2591.67725  183.06549  20.7226

Totals :                 1.25065e4   1236.70416
```

ESI-MS of Peptide **10**

MTK-1-TKFTYTS-1 6 (0.110) Cm (3:38)

17-Sep-2014
11:09:44

TOF MS ES+
3.50e4

Chemical Formula: $C_{75}H_{106}N_{20}O_{23}$
Exact Mass:1654.7
Calculated m/z
$[M+H]^{+}$:1655.7
$[2M+3H+TFA]^{3+}$:1142.24
$[2M+3H]^{3+}$:1104.2
$[M+2H]^{2+}$:828.4
$[M+3H]^{3+}$:552.6

$[M+2H]^{2+}$ 828.36

828.87

$[M+3H]^{3+}$ 552.57

552.91

553.26

553.60

553.94

560.26

$[2M+3H]^{3+}$ 829.92

827.41

840.41

839.40

850.90

$[2M+3H+TFA]^{3+}$ 1142.54

1104.55

1104.21

1143.21

1143.55

1143.89

$[M+H]^{+}$ 1656.84

245.12

249.01

335.12

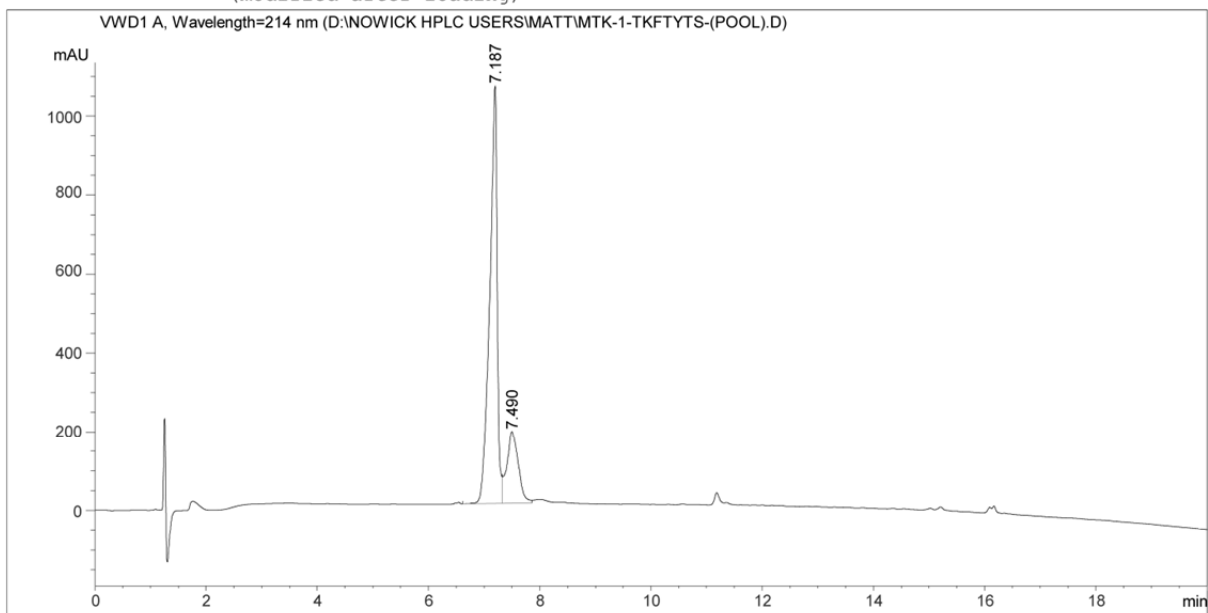1D ^{1}H NMR Spectrum of Peptide **10**
4.00 mM in D$_2$O, 600 MHz, 298 K
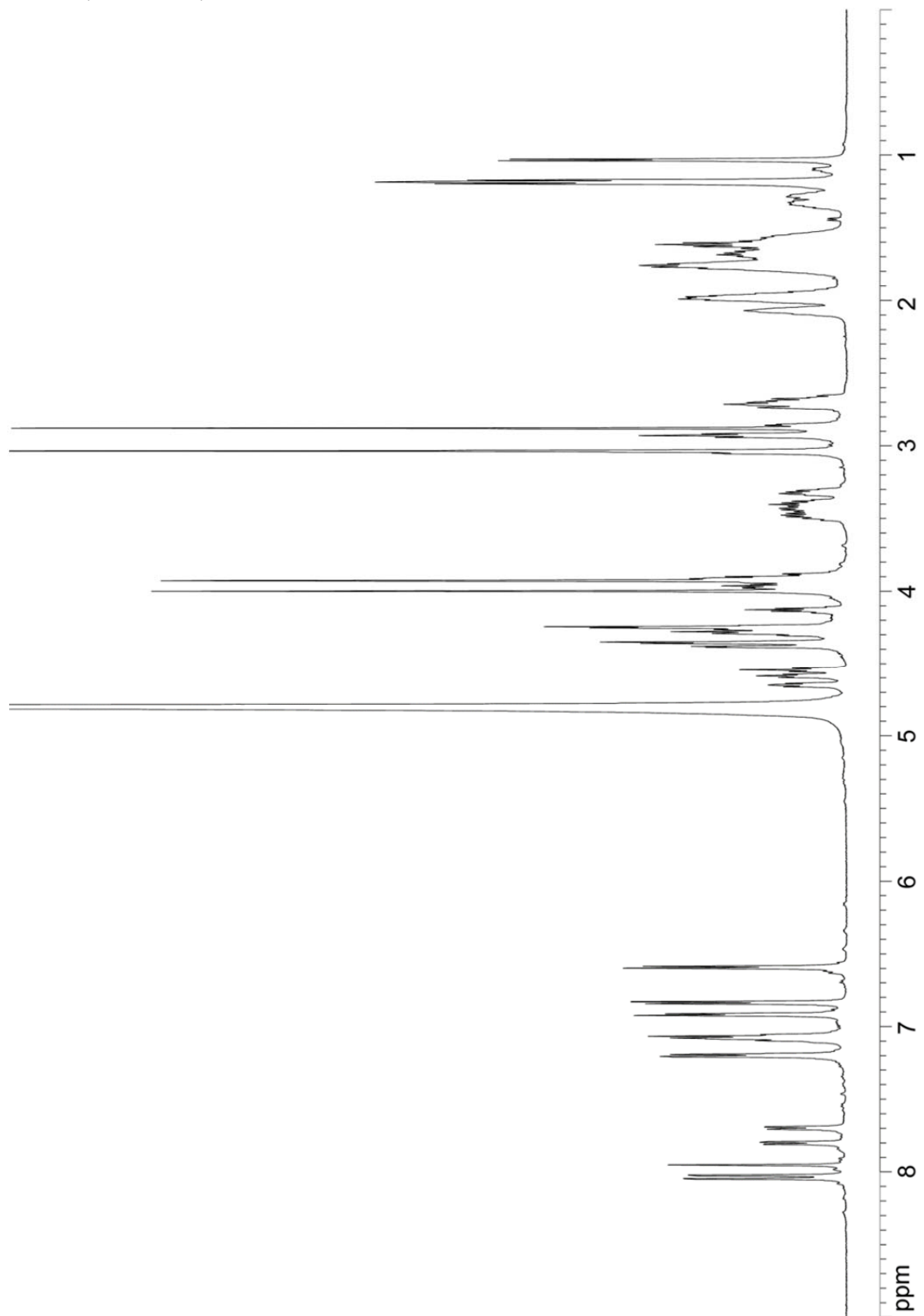
Analytical RP-HPLC of Peptide **11**

```
Data File D:\NOWICK HPLC USERS\MATT\MTK-1-TSFTYKS-POOL.D
Sample Name: MTK-1-TSFTYKS-2-7pool

=======================================================================
Acq. Operator   : Kim
Acq. Instrument : Agilent HPLC                     Location : Vial 71
Injection Date  : 1/16/2013 5:08:28 PM
                                                Inj Volume : 20.0 µl
Acq. Method     : D:\NOWICK HPLC USERS\KVL\5-100 OVER 20MIN AT 214NM.M
Last changed    : 1/16/2013 1:12:24 PM by Kim
                  (modified after loading)
Analysis Method : D:\NOWICK HPLC USERS\NT\5-100 OVER 20MIN AT 214NM.M
Last changed    : 9/17/2014 8:03:44 PM by TA
                  (modified after loading)
```



```
=======================================================================
                      Area Percent Report
=======================================================================

Sorted By             :        Signal
Multiplier:           :         1.0000
Dilution:             :         1.0000
Sample Amount:        :        20.00000  [ng/ul]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area       Height      Area
  #   [min]        [min]   mAU   *s     [mAU   ]       %
----|-------|----|-------|----------|----------|--------|
  1   7.332 VV    0.1343 2.63468e4  2698.39868 100.0000

Totals :                  2.63468e4  2698.39868


=======================================================================
                      *** End of Report ***
```
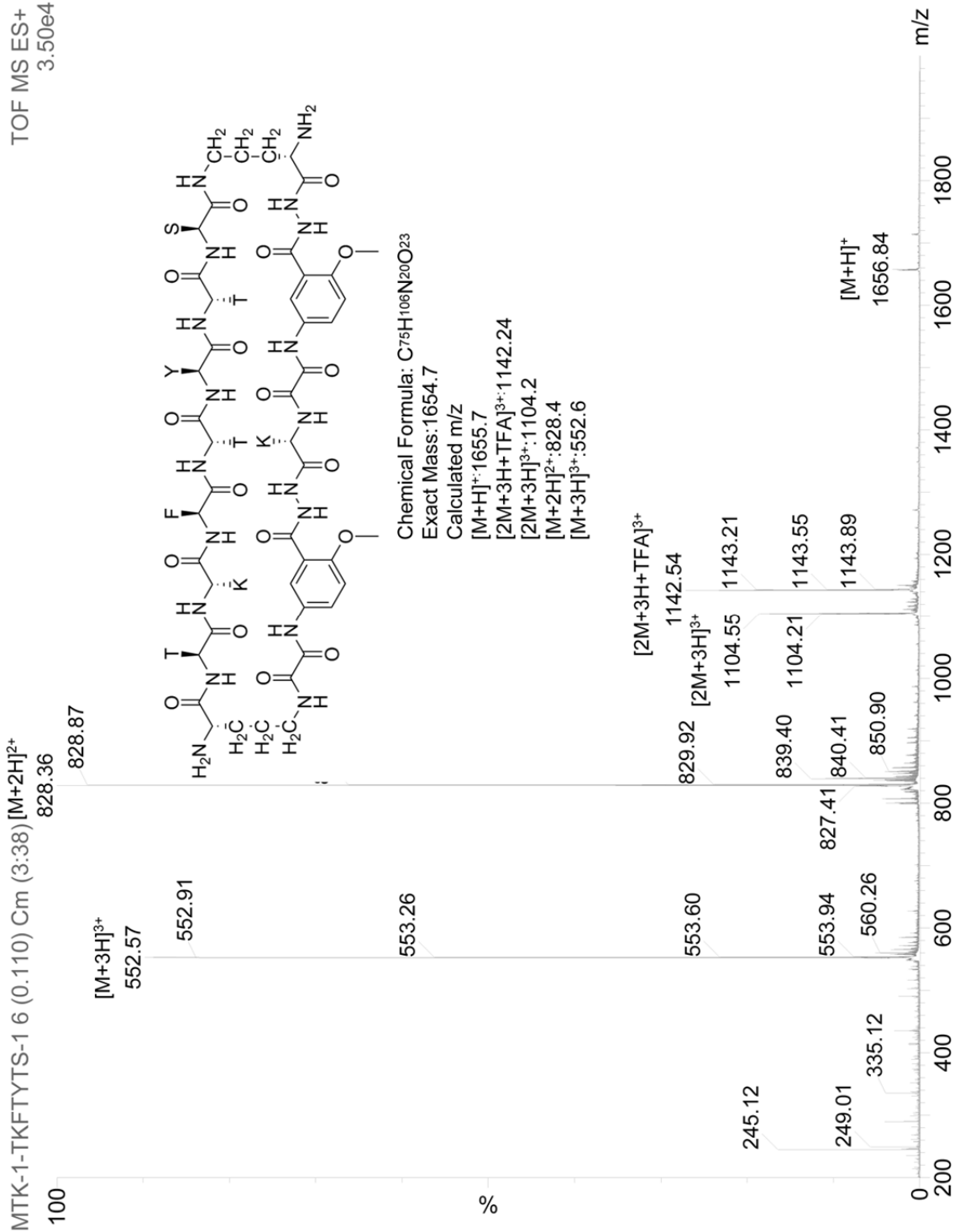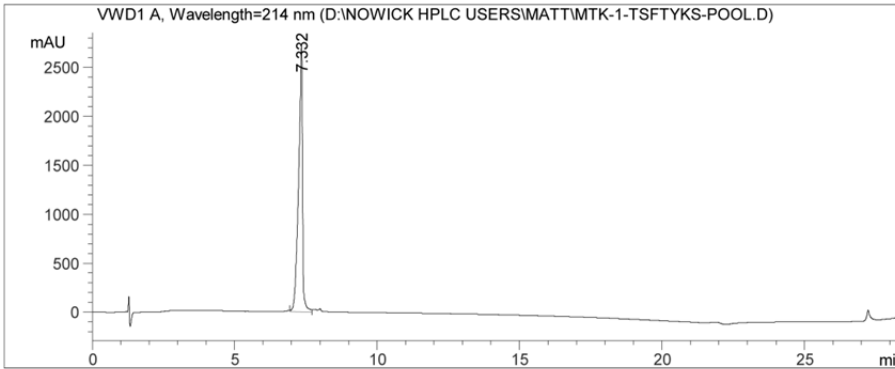
ESI-MS of Peptide **11**

17-Sep-2014
10:37:21
TOF MS ES+
2.47e4

MTK-1-TSFTYKS-1 6 (0.110) Cm (5:28) [M+2H]²⁺

Chemical formula: $C_{74}H_{104}N_{20}O_{23}$
Exact mass:1640.75
Calculated m/z
[M+H]⁺: 1641.76
[2M+3H+TFA]³⁺:1132.80
[2M+3H]³⁺: 1094.84
[M+2H]²⁺: 821.38
[M+3H]³⁺: 547.92

[M+3H]³⁺
547.89
548.24
548.58
548.92
549.25
553.91
799.87

821.35
821.85
822.39
822.90
823.40
897.38

[2M+3H+TFA]³⁺
1133.18
1132.86 1133.86
1134.20
1134.54

[2M+3H]³⁺
1095.20

[M+H]⁺

245.12
249.01
435.21

100

%

0
200   400   600   800   1000   1200   1400   1600   1800   m/z
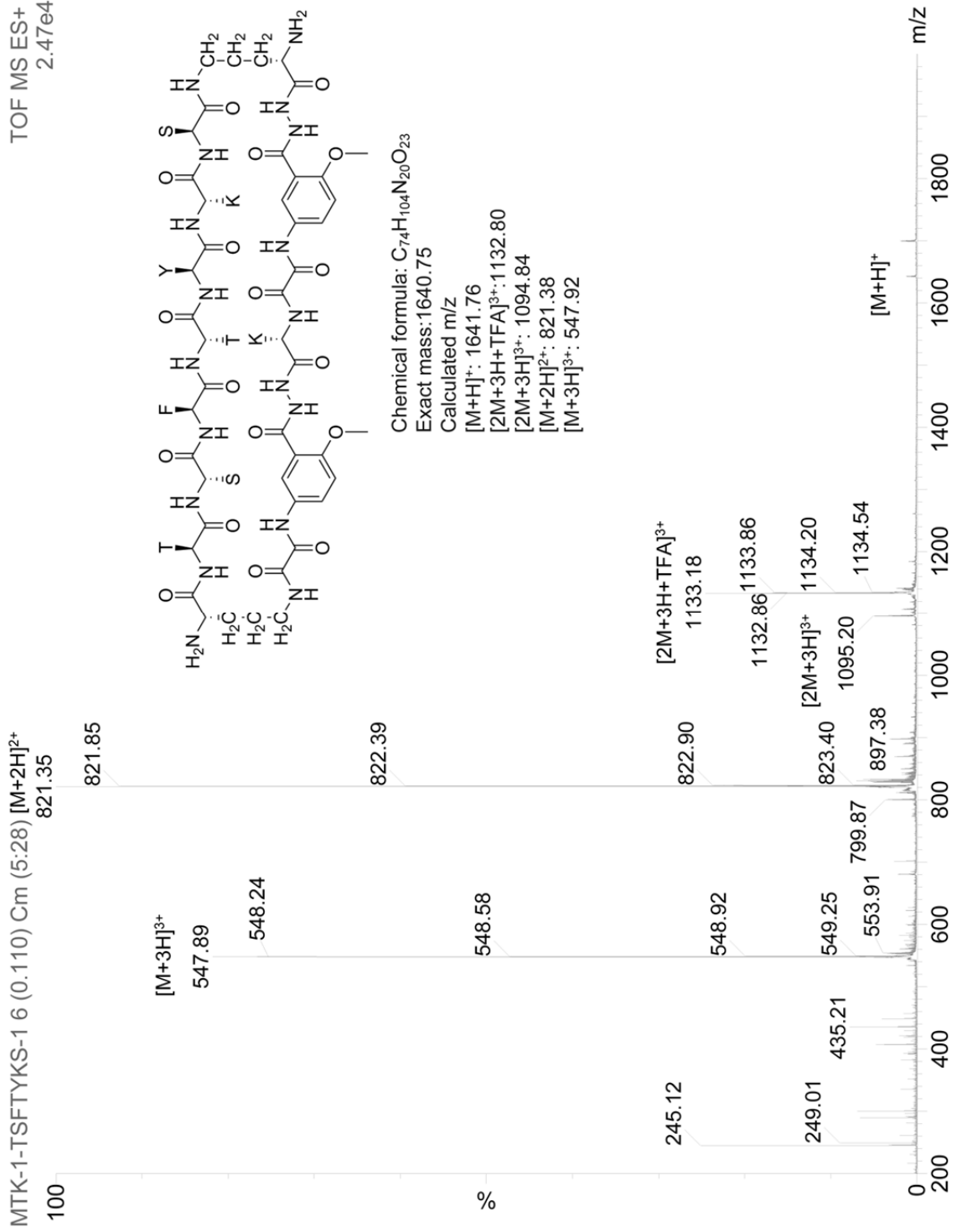
1D ^{1}H NMR Spectrum of Peptide **11**
4.00 mM in D$_2$O, 600 MHz, 298 K
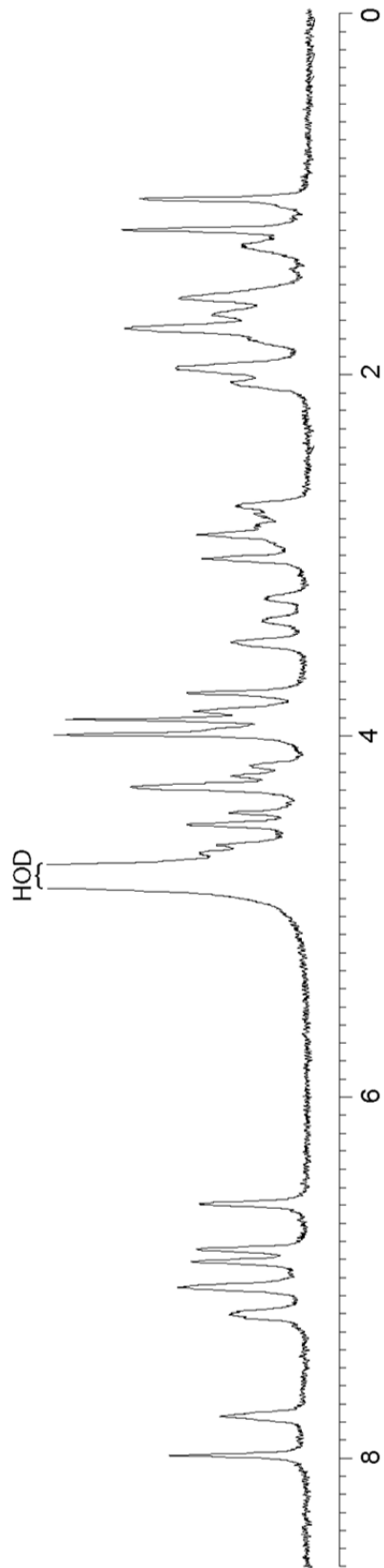
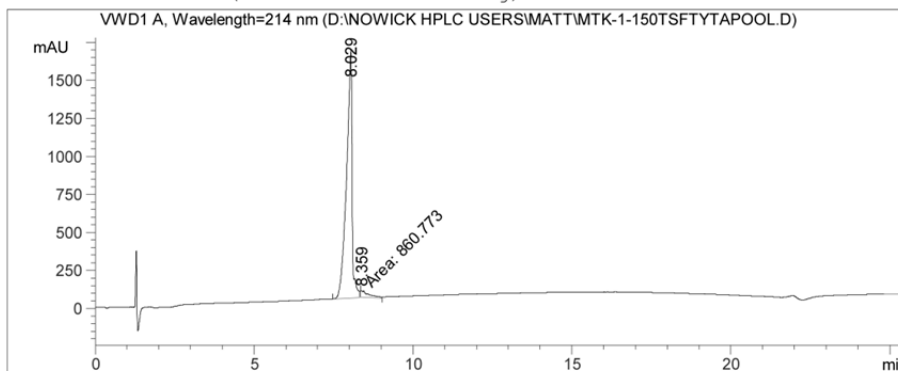DOSY Spectrum of Peptide **11**
4.00 mM in D$_2$O, 600 MHz, 298 K
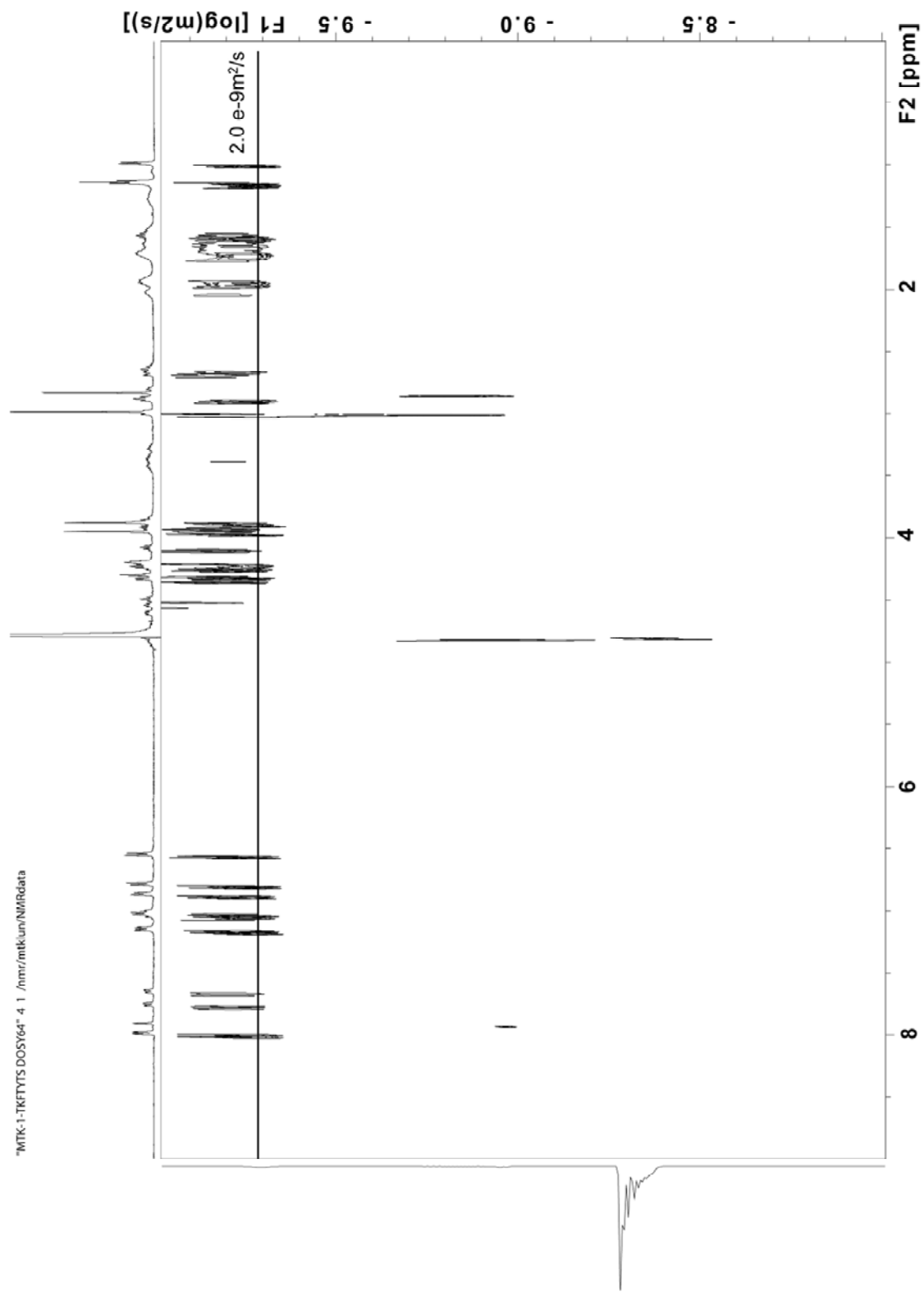
Analytical RP-HPLC of Peptide **12**

```
Data File D:\NOWICK HPLC USERS\MATT\MTK-1-150TSFTYTAPOOL.D
Sample Name: MTK-1-150TSFTYTAPool

===================================================================
Acq. Operator   : Kim
Acq. Instrument : Agilent HPLC                Location : Vial 82
Injection Date  : 1/9/2013 5:23:05 PM
                                              Inj Volume : 20.0 µl
Acq. Method     : D:\NOWICK HPLC USERS\KVL\5-100 OVER 20MIN AT 214NM.M
Last changed    : 1/8/2013 10:34:42 PM by Kim
                    (modified after loading)
Analysis Method : D:\NOWICK HPLC USERS\NT\5-100 OVER 20MIN AT 214NM.M
Last changed    : 9/17/2014 8:03:44 PM by TA
                    (modified after loading)
```


VWD1 A, Wavelength=214 nm (D:\NOWICK HPLC USERS\MATT\MTK-1-150TSFTYTAPOOL.D)

```
===================================================================
                      Area Percent Report
===================================================================


Sorted By              :       Signal
Multiplier:                 :       1.0000
Dilution:                   :       1.0000
Sample Amount:              :     20.00000  [ng/ul]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs



Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area        Height      Area
 #   [min]         [min]  mAU   *s    [mAU   ]        %
----|-------|----|-------|----------|----------|--------|
  1   8.029 BV   0.1668 1.99270e4  1620.43945  95.8592
  2   8.359 MM T 0.3189  860.77283   44.99155   4.1408

Totals :                 2.07877e4  1665.43100


===================================================================
                      *** End of Report ***
===================================================================
```
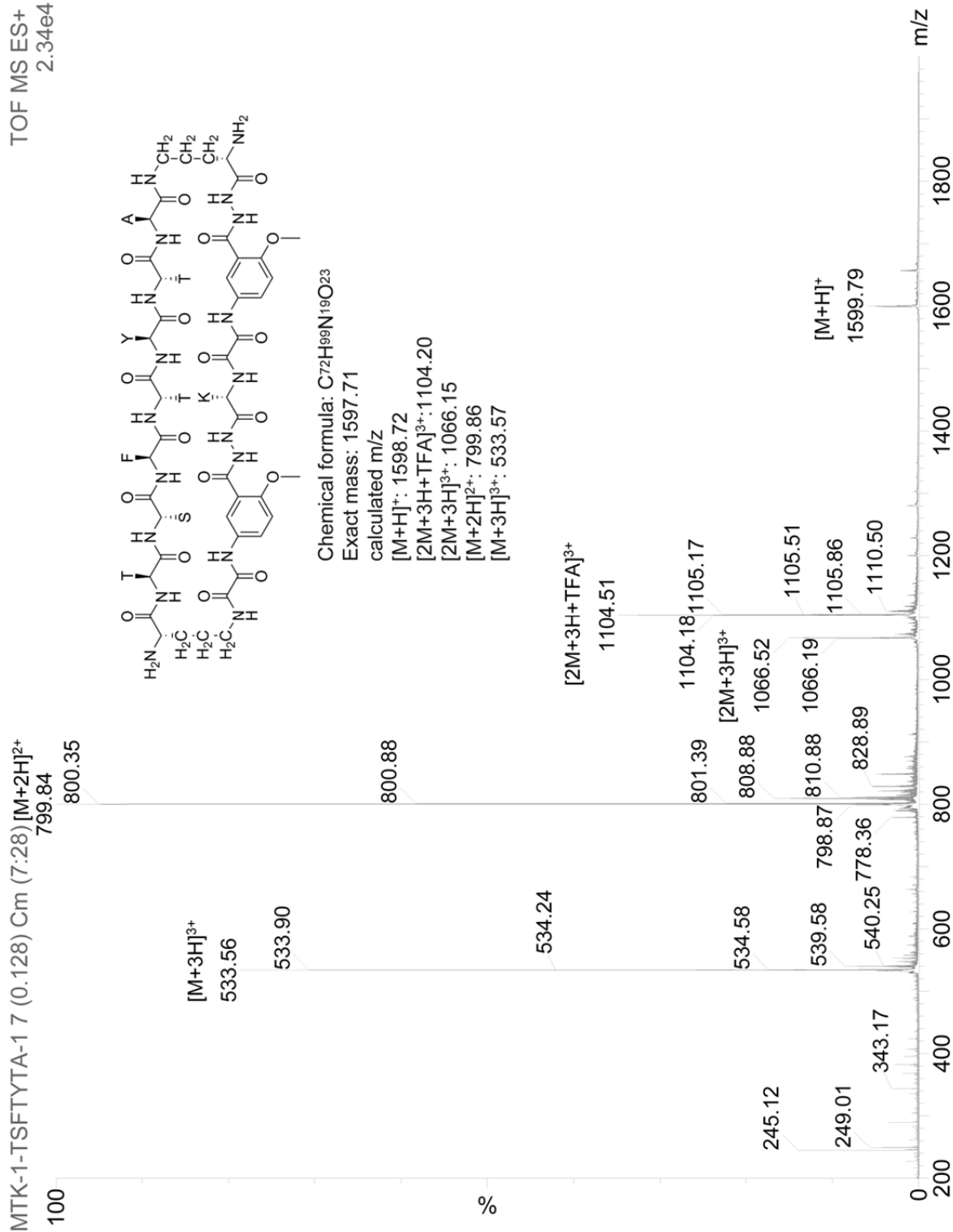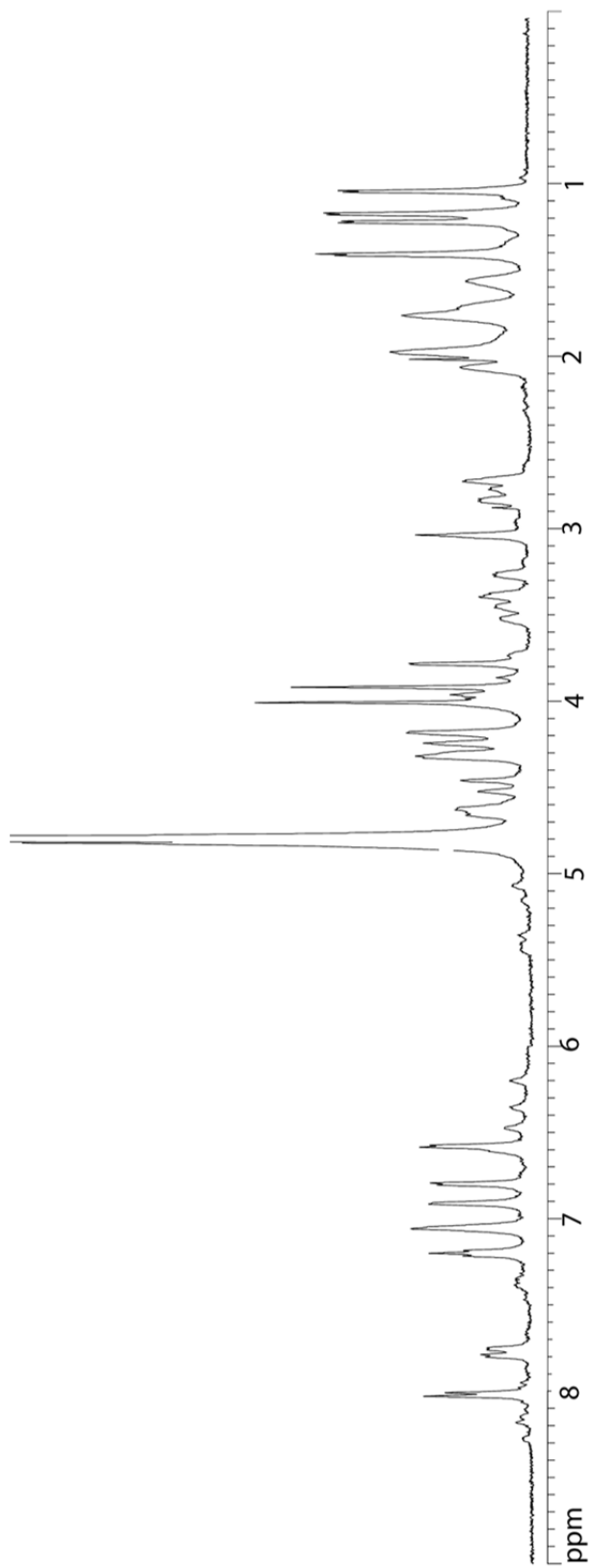
MTK-1-TSFTYTA-1 7 (0.128) Cm (7:28) [M+2H]²⁺

17-Sep-2014
11:15:48
TOF MS ES+
2.34e4

Chemical formula: C₇₂H₉₉N₁₉O₂₃
Exact mass: 1597.71
calculated m/z
[M+H]⁺: 1598.72
[2M+3H+TFA]³⁺:1104.20
[2M+3H]³⁺: 1066.15
[M+2H]²⁺: 799.86
[M+3H]³⁺: 533.57

1D ^{1}H NMR Spectrum of Peptide **12**
3.95 mM in D$_2$O, 600 MHz, 298 K

Concentration Dependent 1D ^{1}H NMR Spectra of Peptide **12**
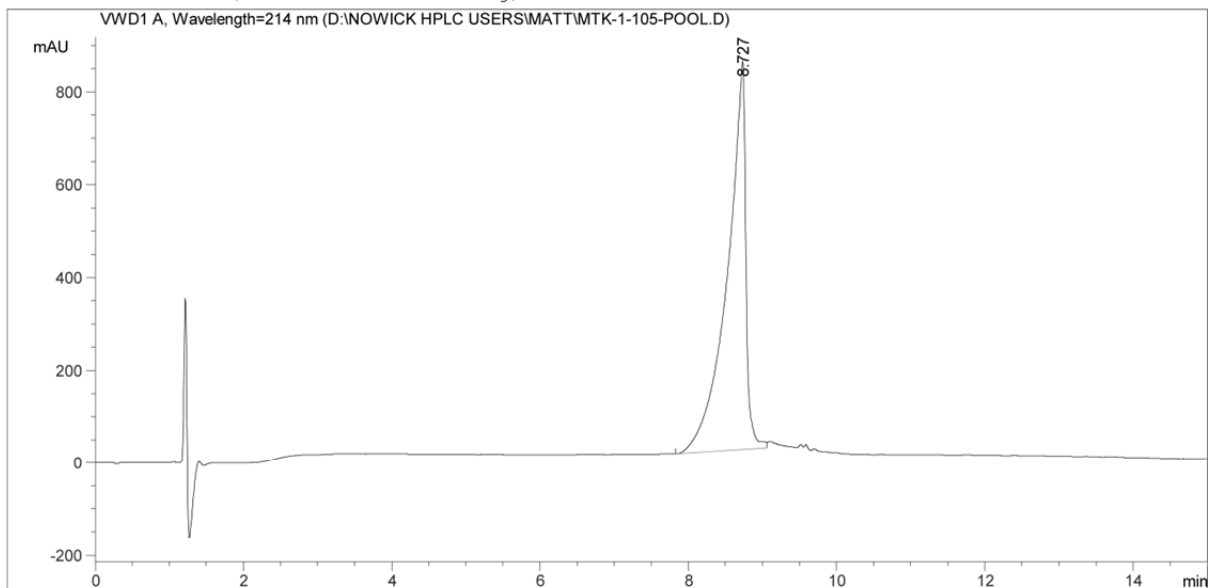D$_2$O, 600 MHz, 298 K
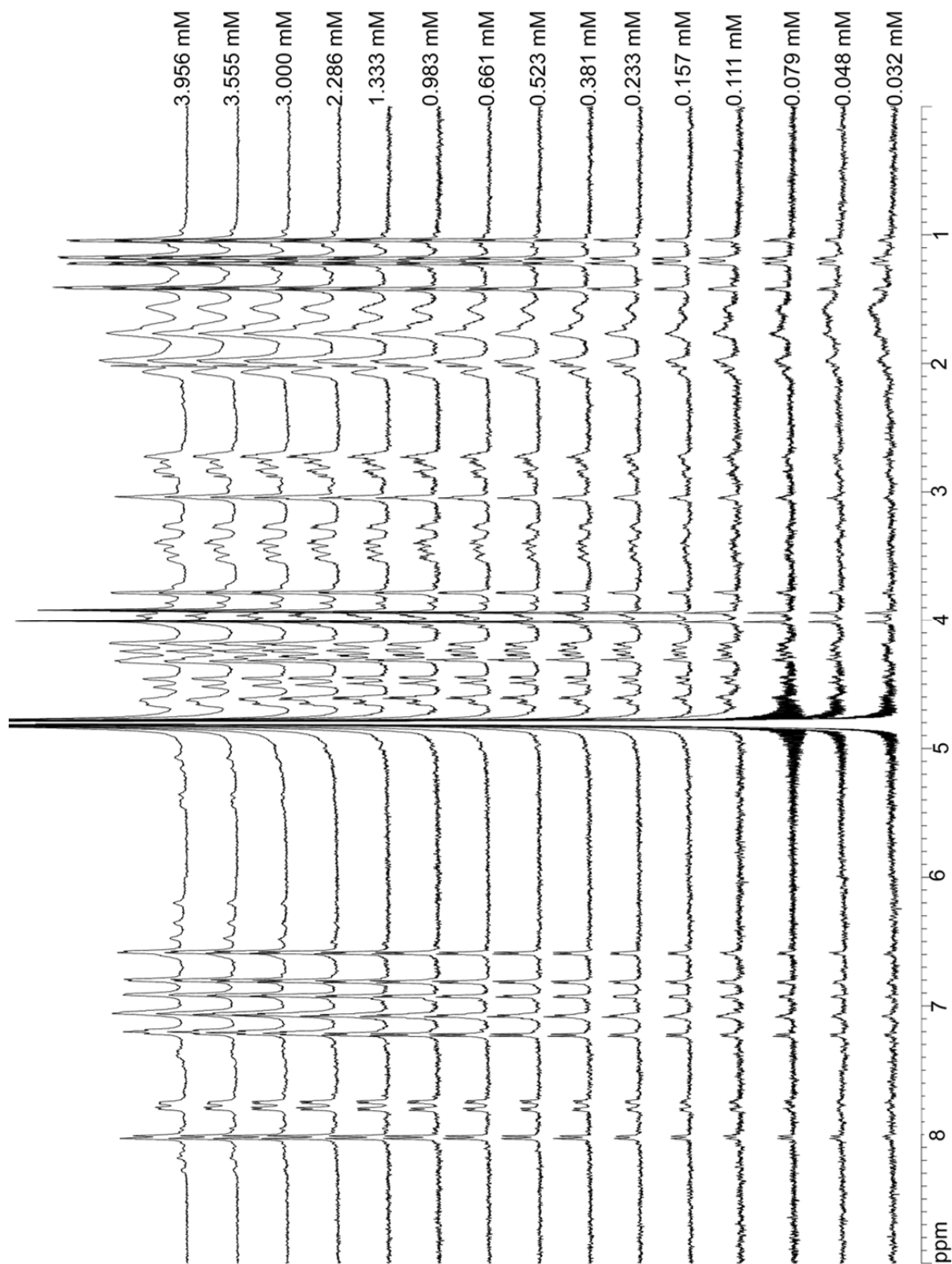
Analytical RP-HPLC of Peptide 13

```
Data File D:\NOWICK HPLC USERS\MATT\MTK-1-105-POOL.D
Sample Name: MTK-1-105-pool

=======================================================================
Acq. Operator   : MATT
Acq. Instrument : Agilent HPLC                    Location : Vial 41
Injection Date  : 10/2/2012 12:44:57 PM
                                                  Inj Volume : 20.0 µl
Acq. Method     : D:\NOWICK HPLC USERS\ZHENGJ\METHODS\5-100 OVER 20 MIN AT 214 AT 1 ML.M
Last changed    : 9/8/2012 4:48:50 PM by mATT
Analysis Method : D:\NOWICK HPLC USERS\ARYA\METHODS\5-100 OVER 15 MIN AT 214 ARYA.M
Last changed    : 10/27/2012 11:39:40 PM by MATT
                  (modified after loading)
```



```
=======================================================================
                      Area Percent Report
=======================================================================

Sorted By            :        Signal
Multiplier:                   :       1.0000
Dilution:                     :       1.0000
Sample Amount:                :       20.00000  [ng/ul]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area       Height      Area
  #   [min]        [min]  mAU   *s   [mAU   ]       %
----|-------|----|-------|----------|----------|--------|
  1   8.727 BV   0.2202 1.44441e4   837.07318 100.0000

Totals :                 1.44441e4   837.07318
```
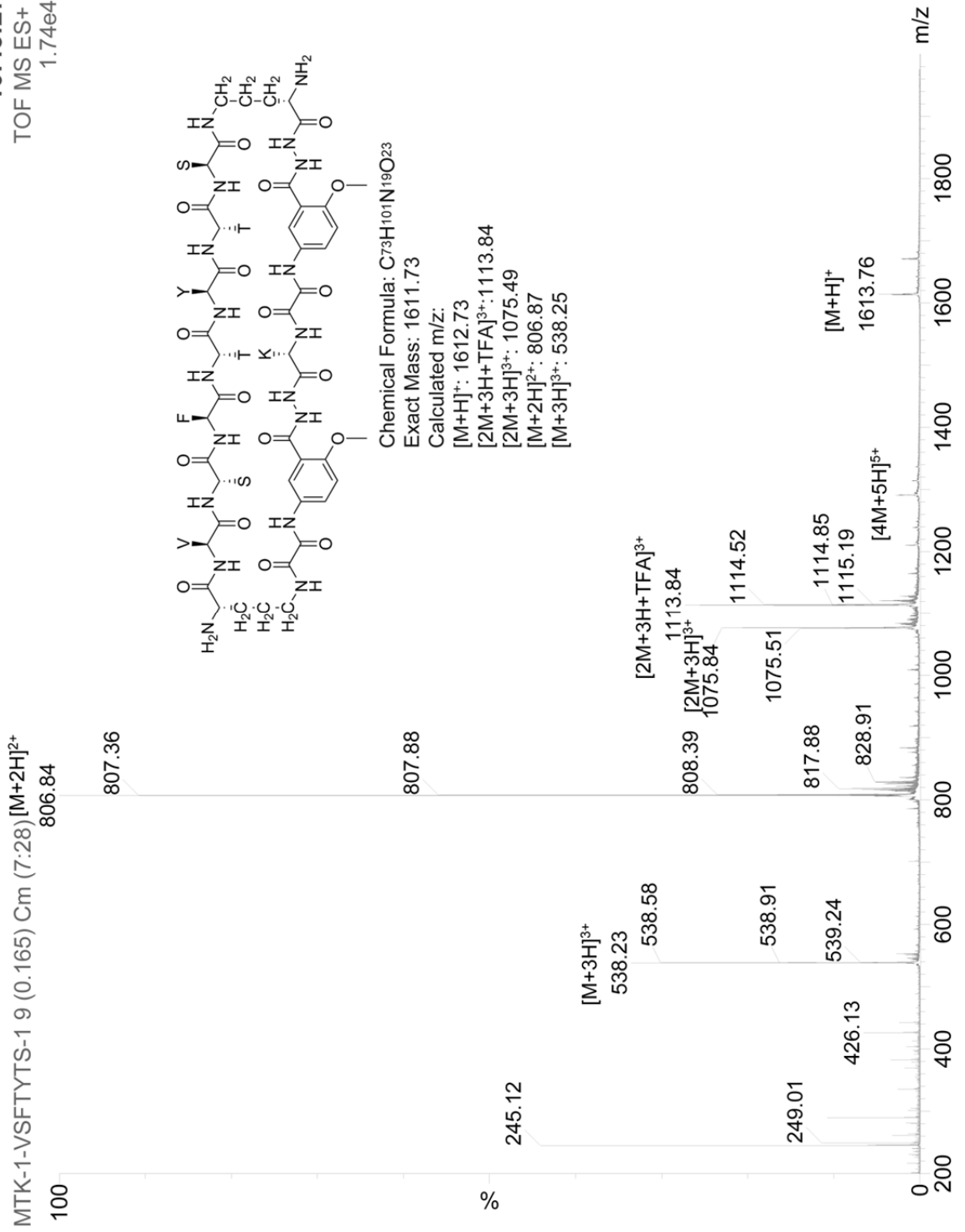
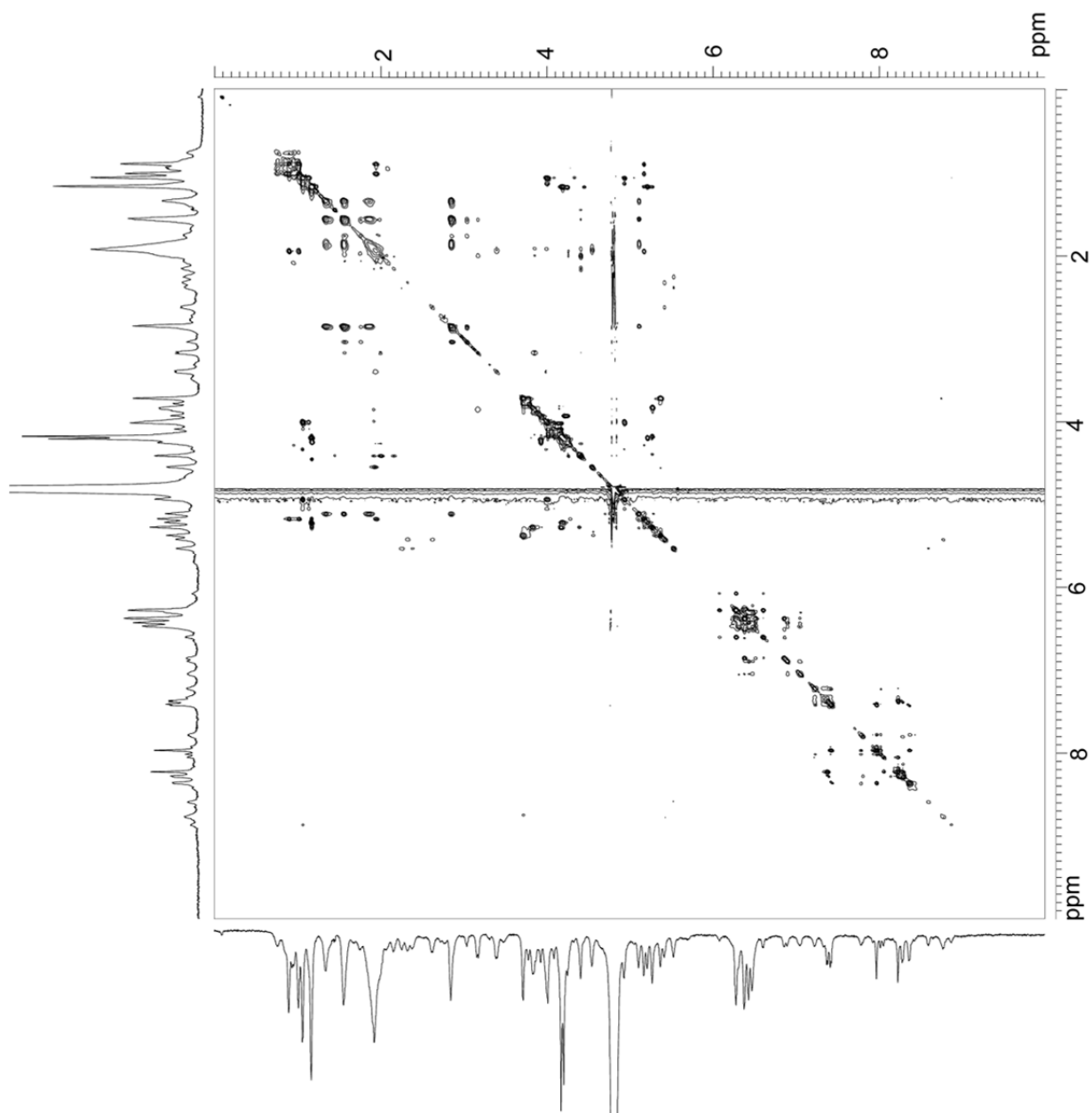ESI-MS of Peptide **13**

17-Sep-2014
10:45:27
TOF MS ES+
1.74e4

MTK-1-VSFTYTS-1 9 (0.165) Cm (7:28)

Chemical Formula: $C_{73}H_{101}N_{19}O_{23}$
Exact Mass: 1611.73
Calculated m/z:
$[M+H]^+$: 1612.73
$[2M+3H+TFA]^{3+}$:1113.84
$[2M+3H]^{3+}$: 1075.49
$[M+2H]^{2+}$: 806.87
$[M+3H]^{3+}$: 538.25

$[M+2H]^{2+}$
806.84

807.36

807.88

$[2M+3H+TFA]^{3+}$
1113.84

$[2M+3H]^{3+}$
1075.84

808.39

817.88

828.91

1075.51

1114.52

1114.85

1115.19

$[4M+5H]^{5+}$

$[M+H]^+$
1613.76

$[M+3H]^{3+}$
538.23

538.58

538.91

539.24

426.13

245.12

249.01

100

%

0

200   400   600   800   1000   1200   1400   1600   1800

m/z

1D ^{1}H NMR Spectrum of Peptide **13**
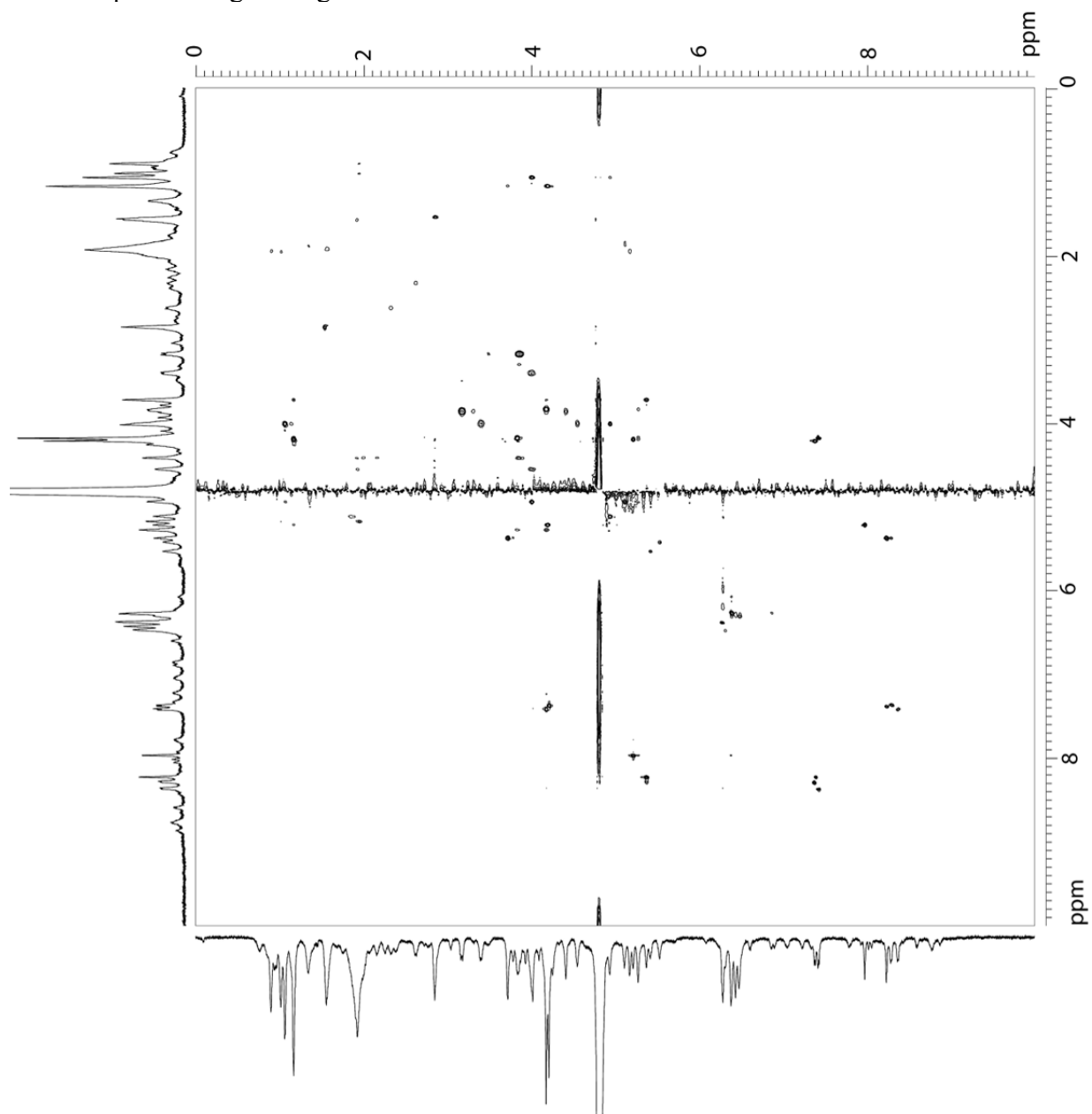3.52 mM in D$_2$O, 600 MHz, 298 K
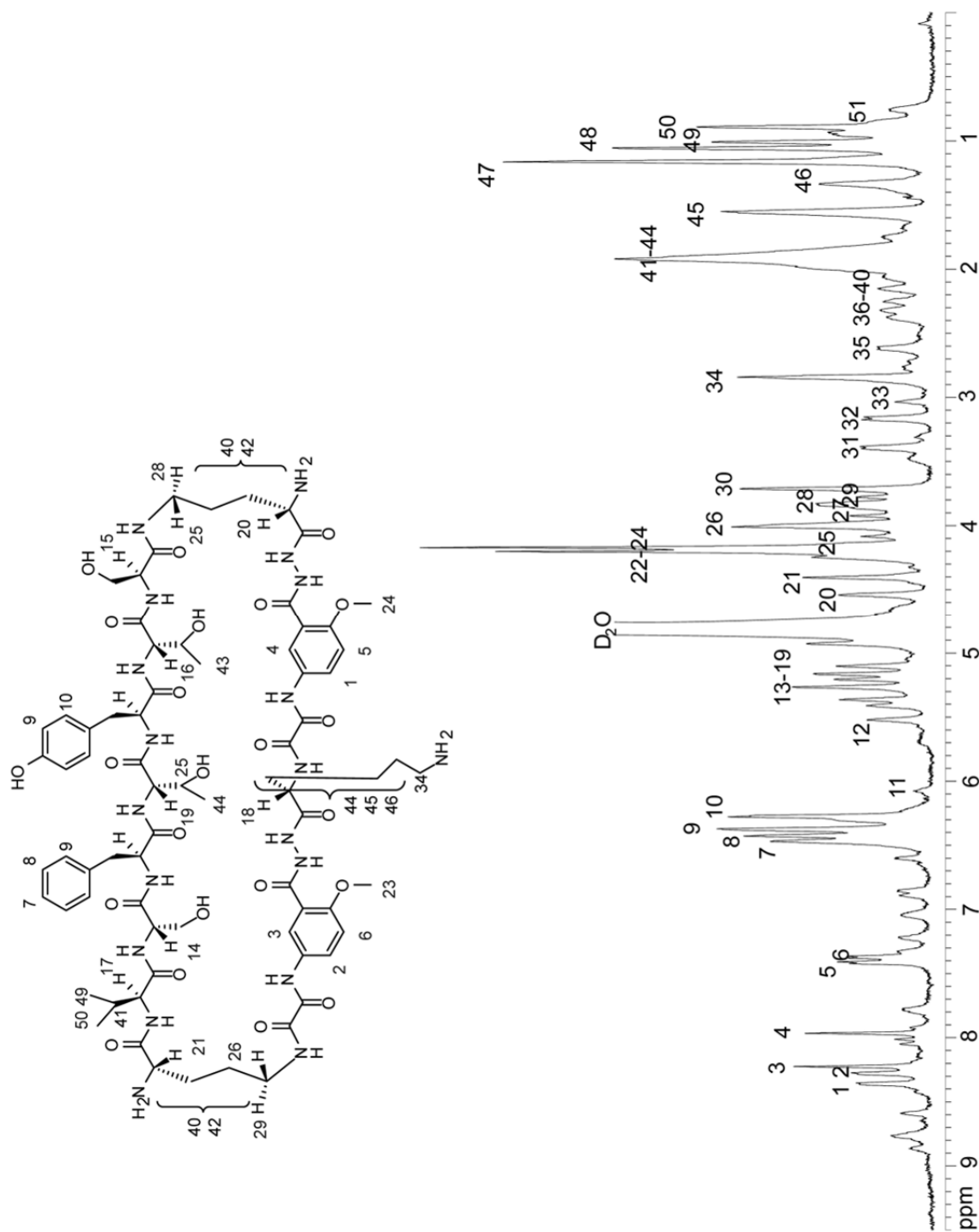
Concentration Dependent 1D ^{1}H NMR Spectra of Peptide **13**
D$_2$O, 600 MHz, 298 K
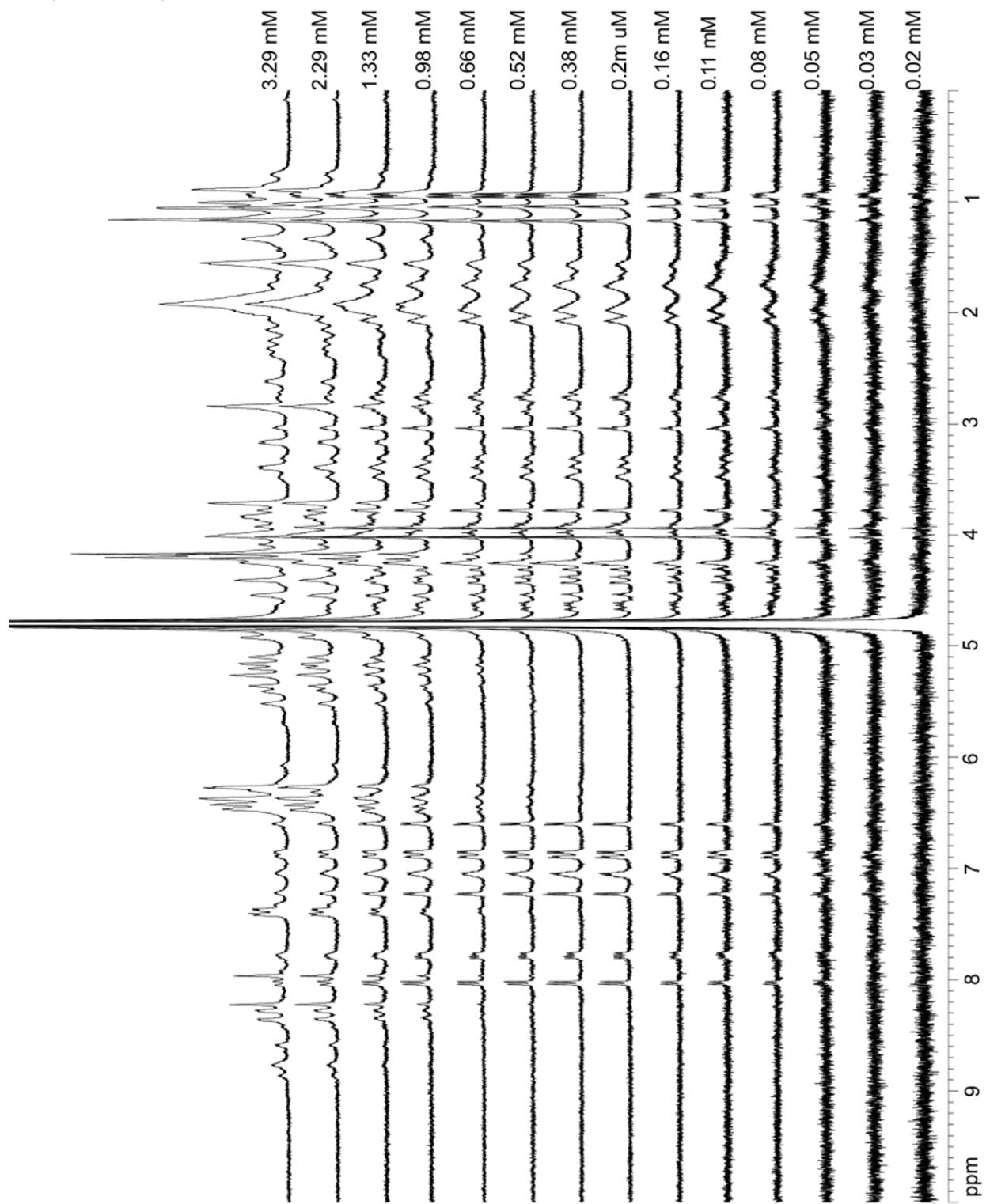
2D TOCSY Spectrum of Peptide **13**
3.52 mM in D₂O, 600 MHz, 298 K
150-ms spin-locking mixing time

2D ROESY Spectrum of Peptide **13**
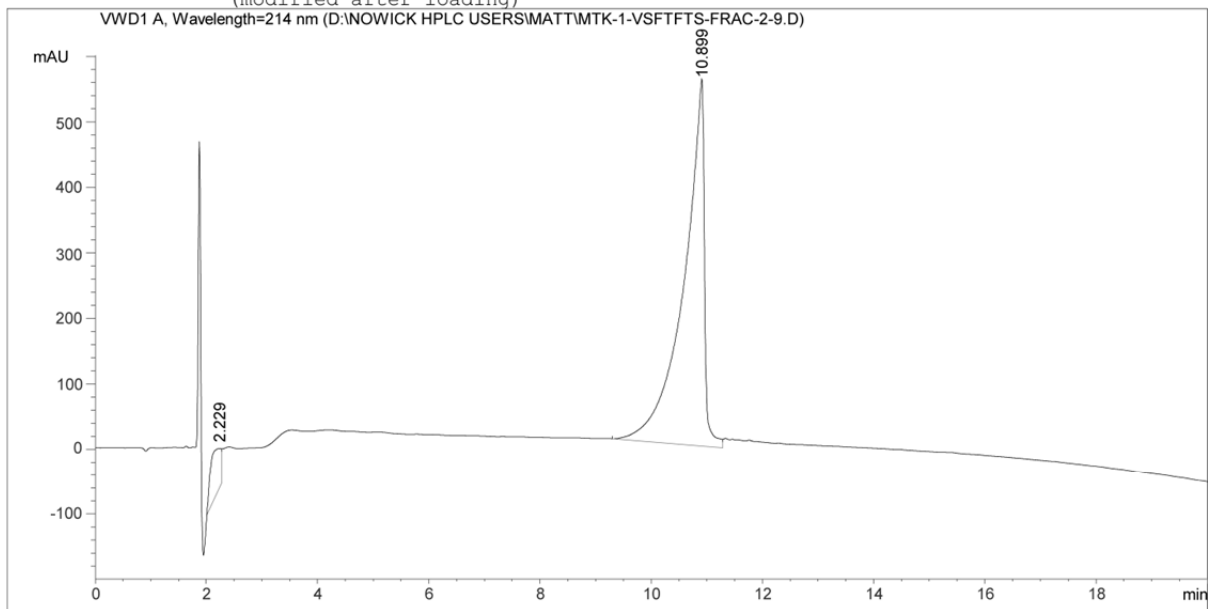3.52 mM in D₂O, 600 MHz, 298 K
300-ms spin-locking mixing time

Analytical RP-HPLC of Peptide **14**

```
Data File D:\NOWICK HPLC USERS\MATT\MTK-1-VSFTFTS-FRAC-2-9.D
Sample Name: MTK-1-VSFTFTS-Frac-2-9

========================================================================
Acq. Operator   : Johnny
Acq. Instrument : Agilent HPLC                      Location : Vial 76
Injection Date  : 2/16/2013 2:34:35 AM
                                             Inj Volume : 20.0 µl
Acq. Method     : D:\NOWICK HPLC USERS\ZHENGJ\METHODS\5-100 OVER 20 MIN AT 214 AT 1 ML.M
Last changed    : 1/7/2013 12:34:37 AM by Kim
Analysis Method : D:\NOWICK HPLC USERS\ZHENGJ\METHODS\5-100 OVER 20 MIN AT 214 AT 1 ML.M
Last changed    : 5/4/2013 7:11:01 AM by Ryan
                  (modified after loading)
```



```
========================================================================
                        Area Percent Report
========================================================================

Sorted By              :       Signal
Multiplier:                    :       1.0000
Dilution:                      :       1.0000
Sample Amount:                 :       20.00000  [ng/ul]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area       Height      Area
 #   [min]        [min]   mAU   *s     [mAU  ]       %
----|-------|----|-------|----------|----------|--------|
 1   2.229 BV   0.2391  950.42828    61.29357   6.2405
 2  10.899 BV   0.3201 1.42795e4    561.34326  93.7595

Totals :                1.52300e4    622.63683
```
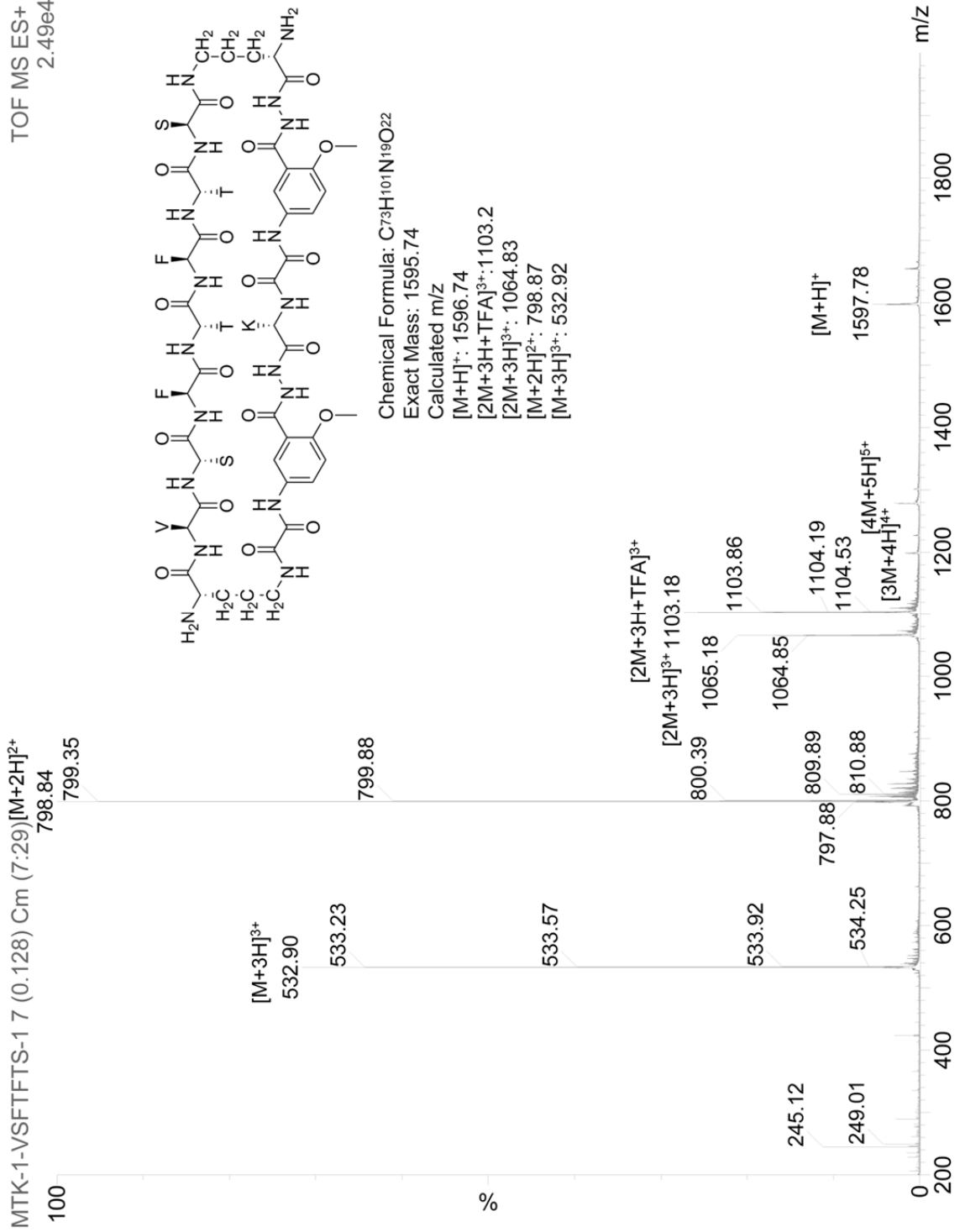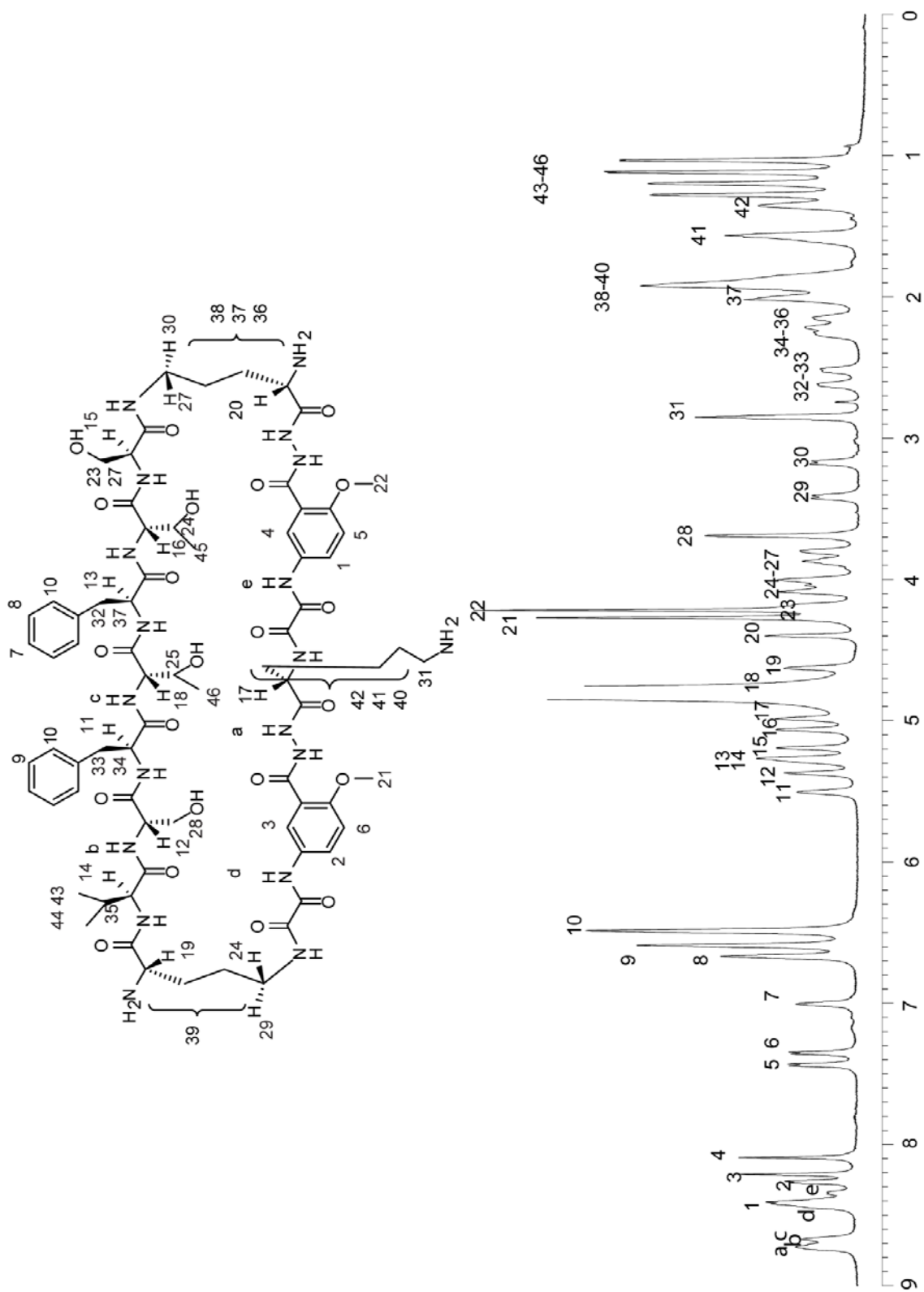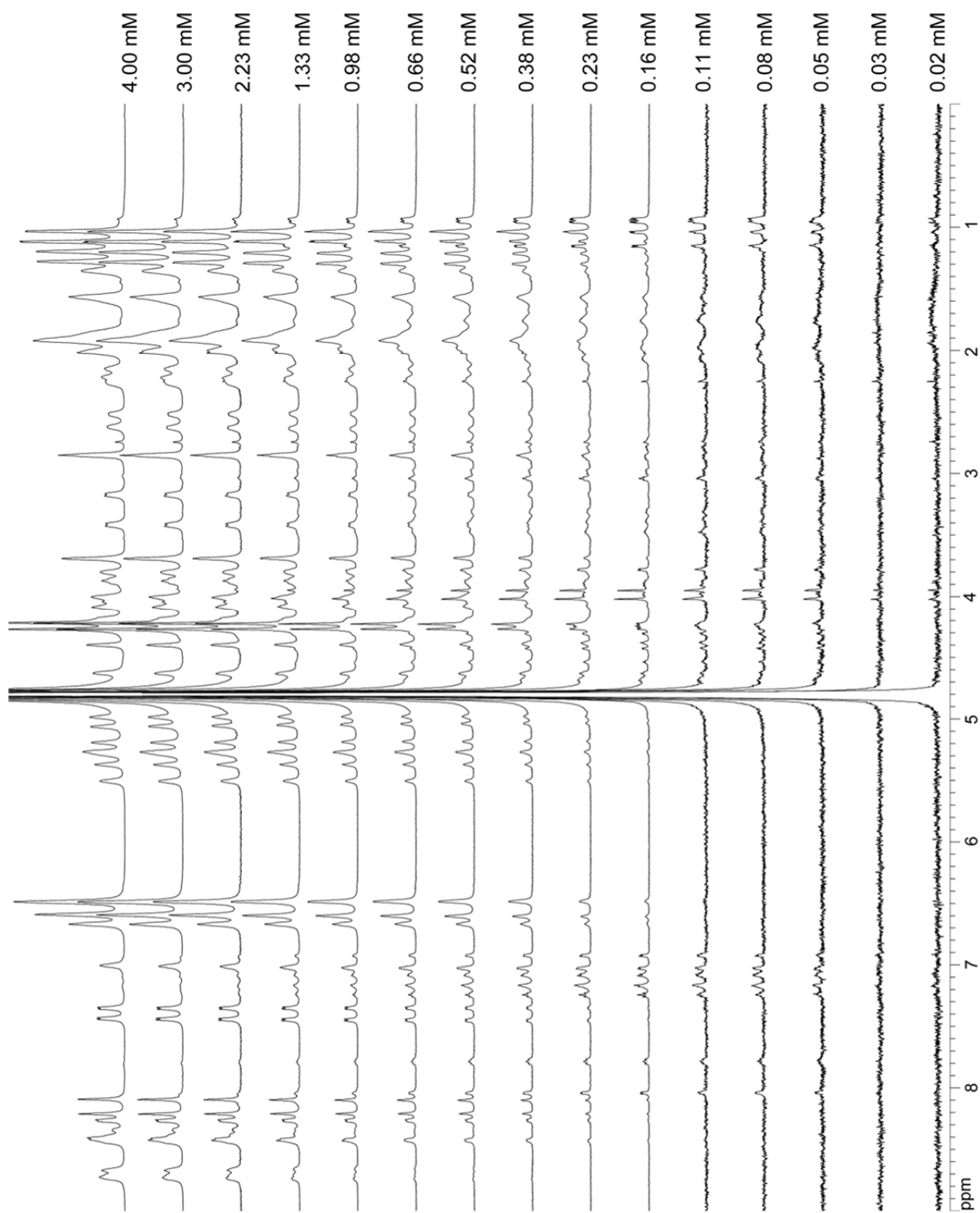
17-Sep-2014
10:53:34
TOF MS ES+
2.49e4

MTK-1-VSFTFTS-17 (0.128) Cm (7:29)

Chemical Formula: C₇₃H₁₀₁N₁₉O₂₂
Exact Mass: 1595.74
Calculated m/z
[M+H]⁺: 1596.74
[2M+3H+TFA]³⁺:1103.2
[2M+3H]³⁺: 1064.83
[M+2H]²⁺: 798.87
[M+3H]³⁺: 532.92

[M+3H]³⁺
532.90

533.23

533.57

533.92

534.25

245.12

249.01

[M+2H]²⁺
798.84

799.35

799.88

797.88

800.39

809.89

810.88

[2M+3H+TFA]³⁺ 1103.18

[2M+3H]³⁺ 1065.18

1064.85

1103.86

1104.19

1104.53

[4M+5H]⁵⁺

[3M+4H]⁴⁺

[M+H]⁺
1597.78

[M+H]⁺

m/z

100

%

0

200   400   600   800   1000   1200   1400   1600   1800

1D ^{1}H NMR Spectrum of Peptide **14**
4.00 mM in D$_2$O, 600 MHz, 298K

Concentration Dependent ^{1}H NMR Spectra of Peptide **14**
D$_2$O, 600 MHz, 298K

4.00 mM
3.00 mM
2.23 mM
1.33 mM
0.98 mM
0.66 mM
0.52 mM
0.38 mM
0.23 mM
0.16 mM
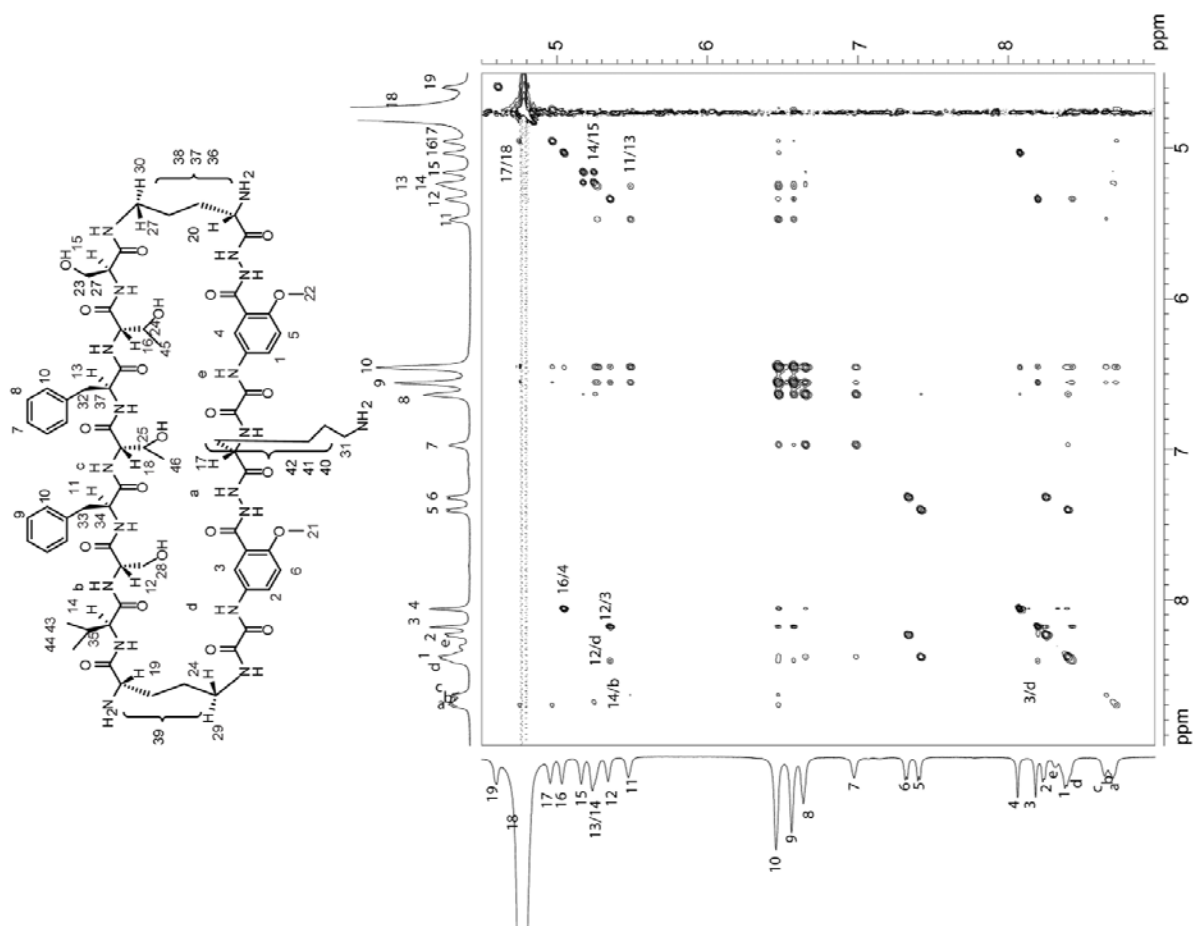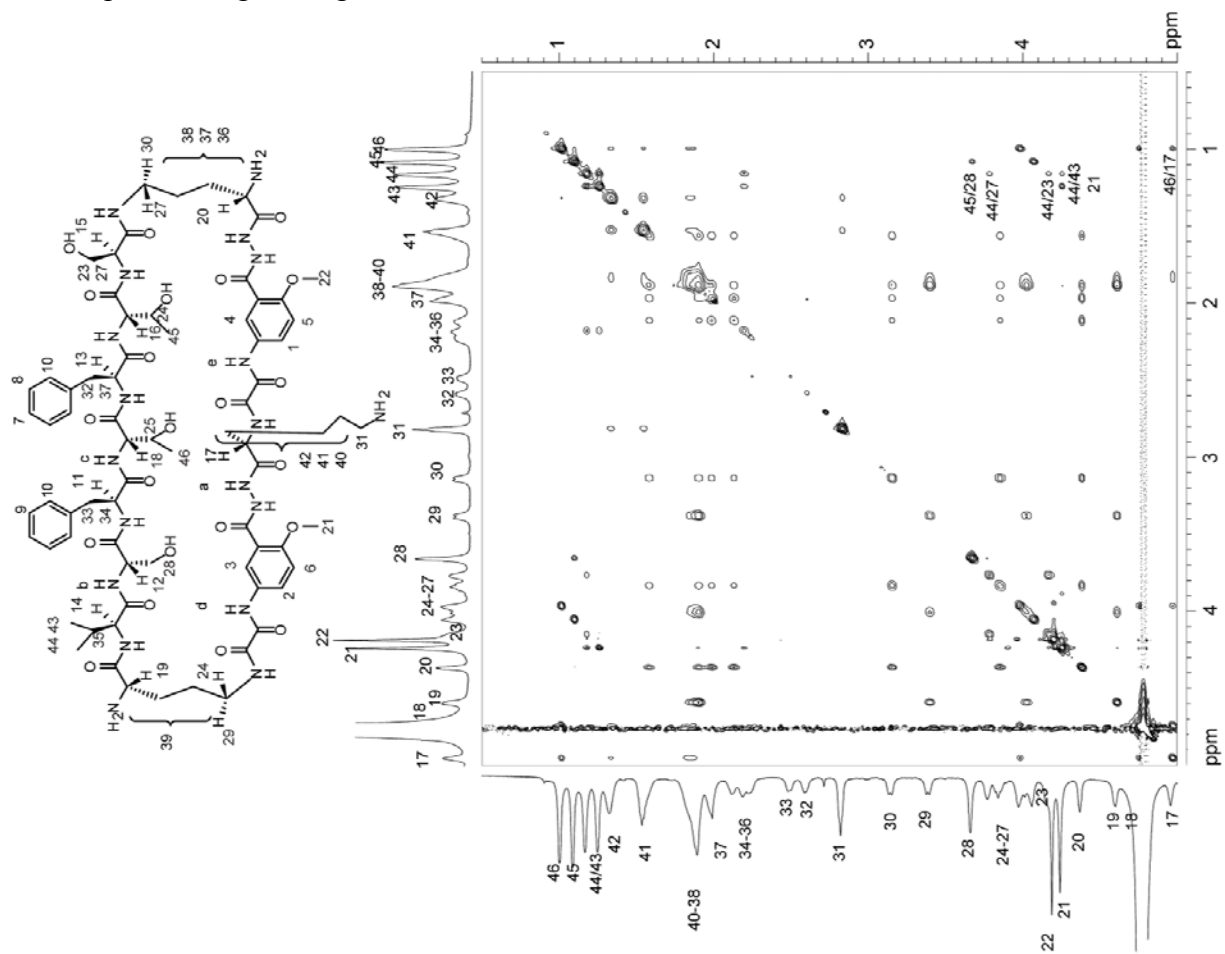0.11 mM
0.08 mM
0.05 mM
0.03 mM
0.02 mM

2D TOCSY Spectrum of Peptide **14**
4.00 mM in D₂O, 600 MHz, 298K
150-ms spin-locking mixing time
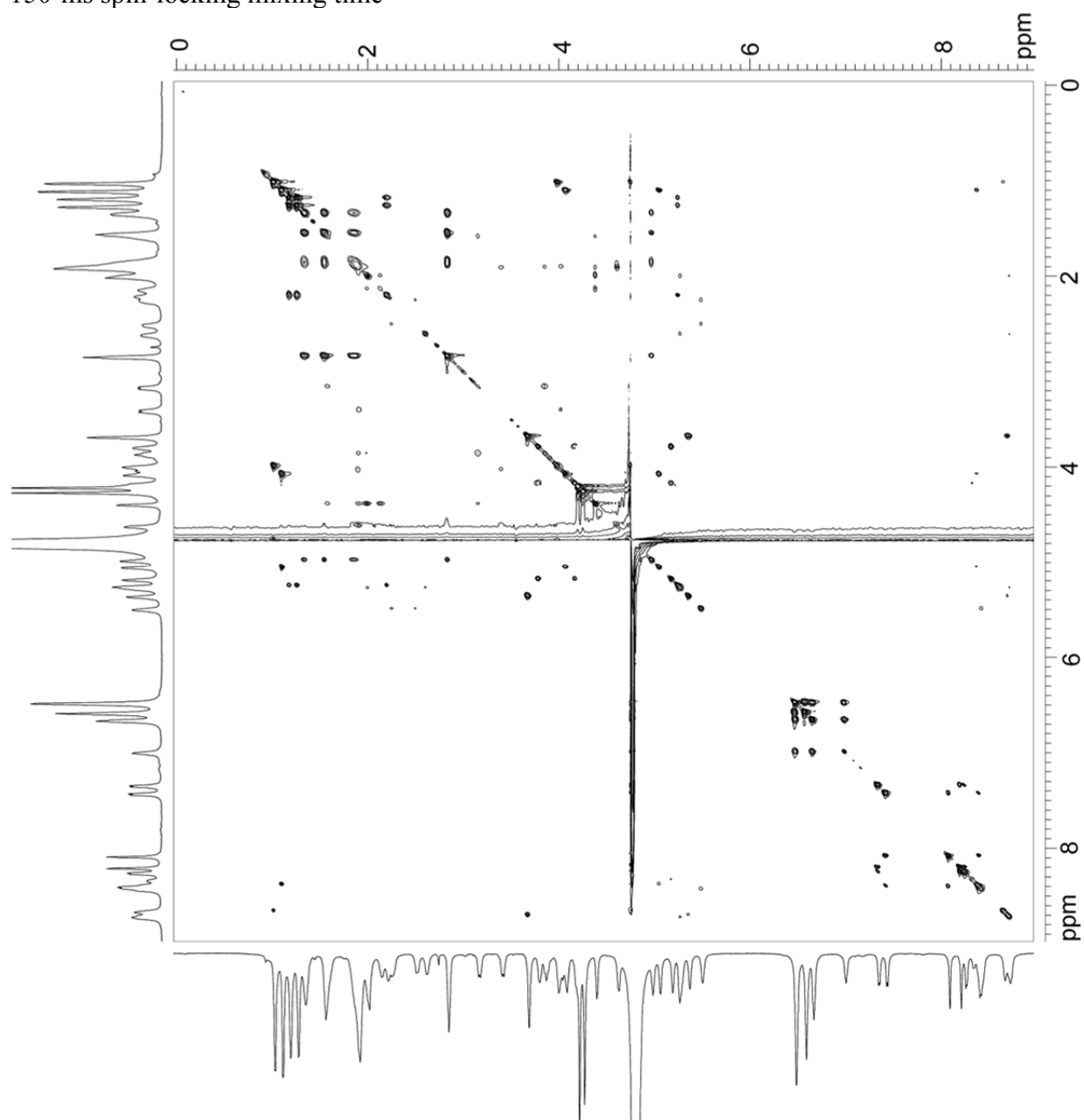
2D NOESY Spectrum of Peptide **14**
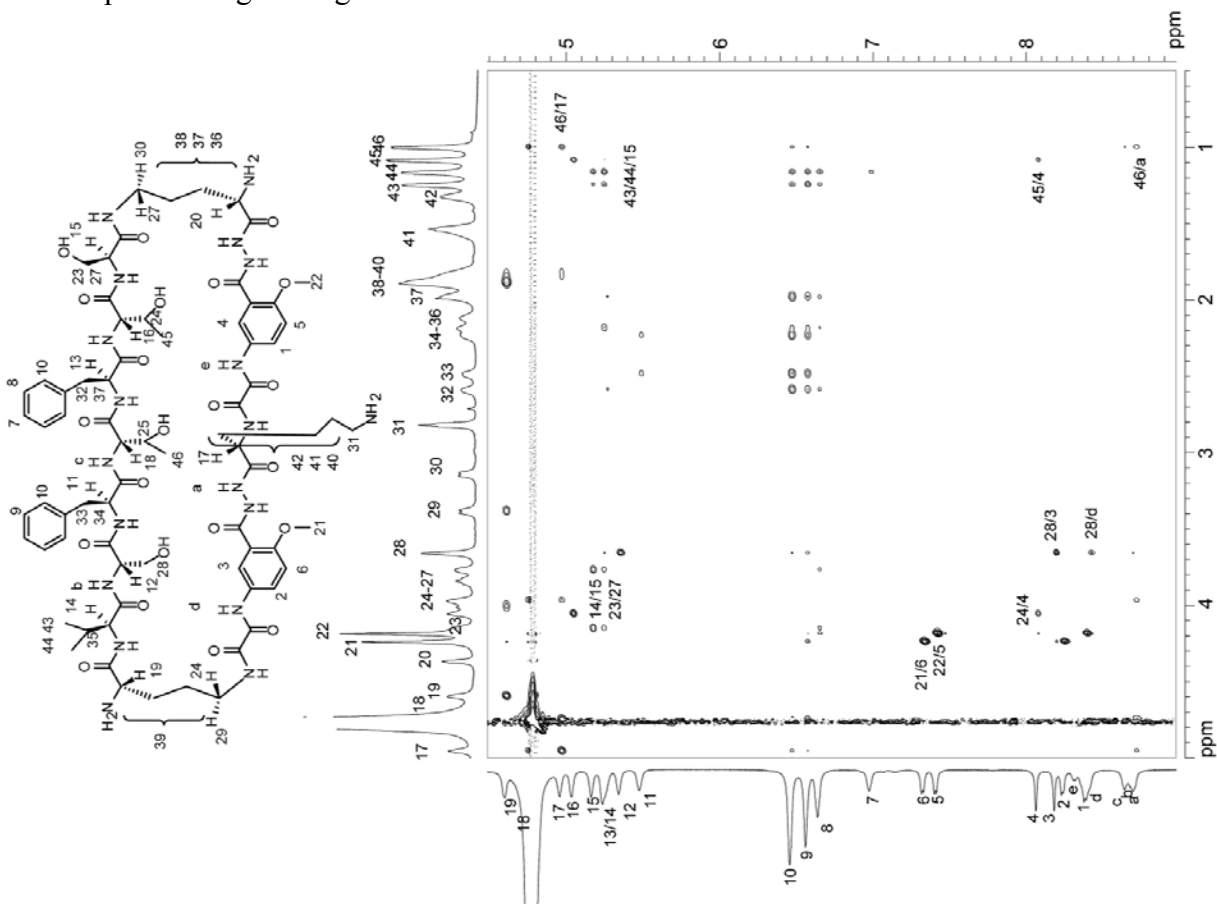4.00 mM in D₂O, 600 MHz, 298K
200-ms spin-locking mixing time
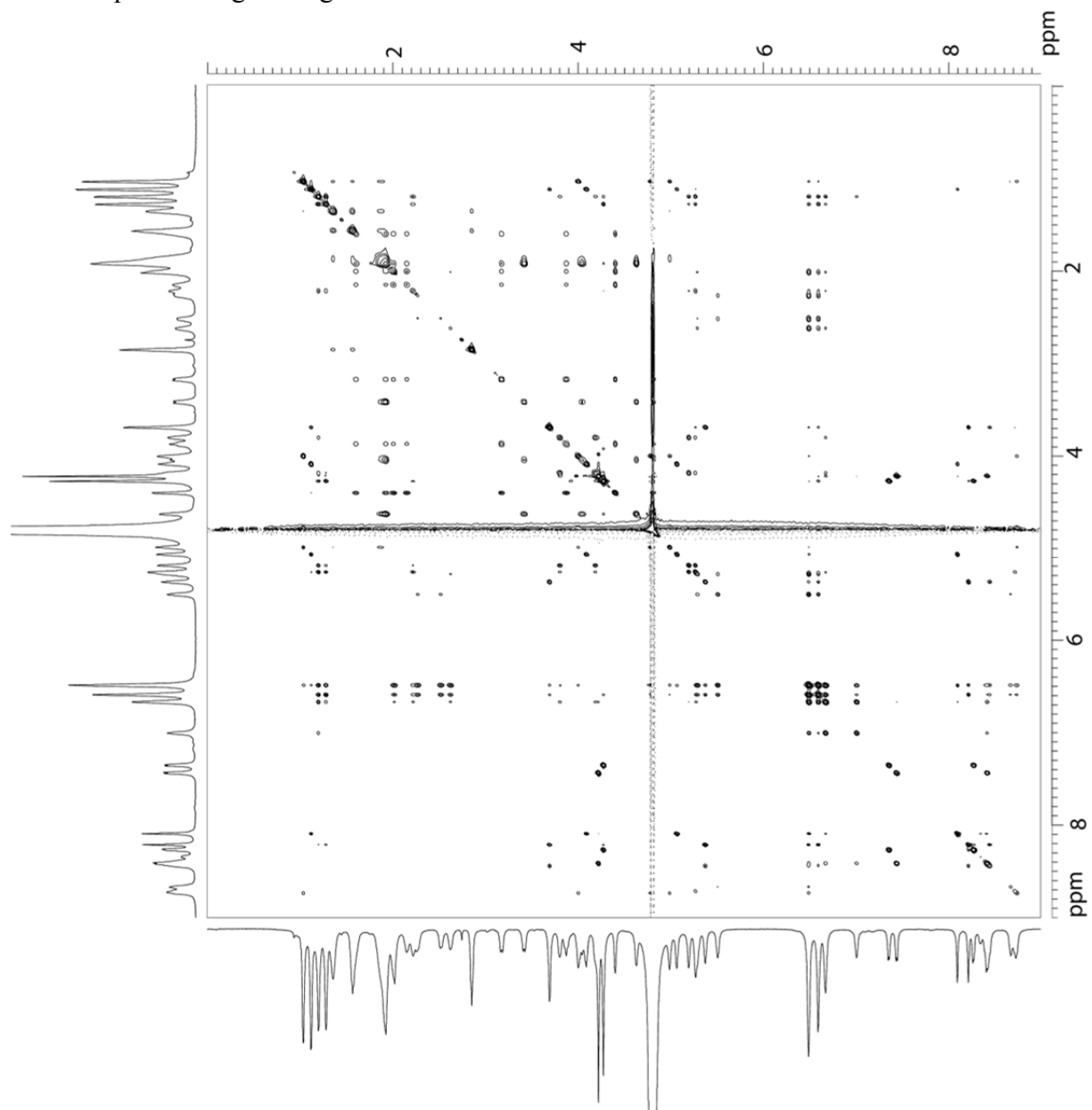
Expansion 1 of 2D NOESY Spectrum of Peptide **14**
4.00 mM in D₂O, 600 MHz, 298K
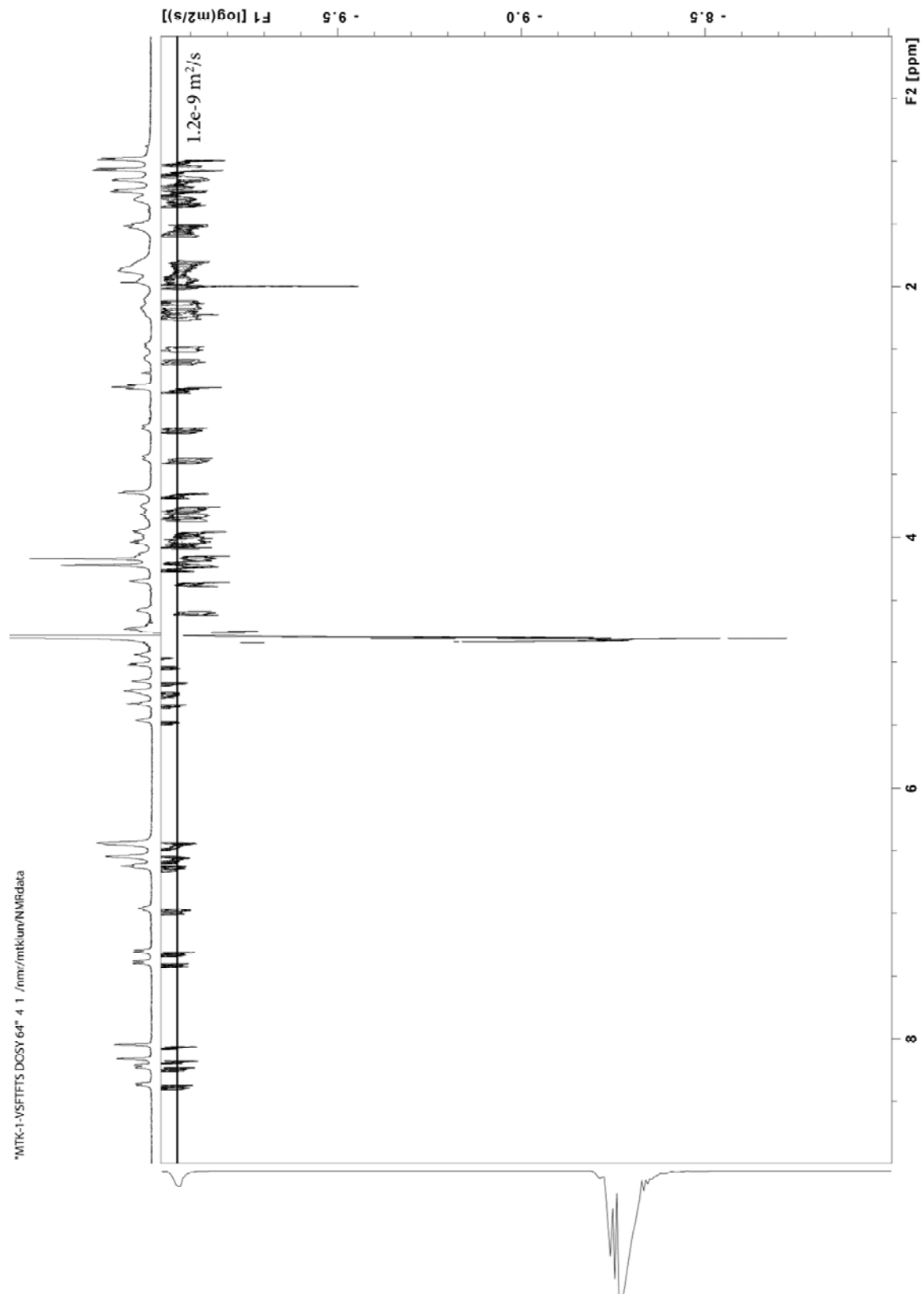200-ms spin-locking mixing time

Expansion 2 of 2D NOESY Spectrum of Peptide **14**
4.00 mM in D$_2$O, 600 MHz, 298K
200-ms spin-locking mixing time

Expansion 3 of 2D NOESY Spectrum of Peptide **14**
4.00 mM in D₂O, 600 MHz, 298K
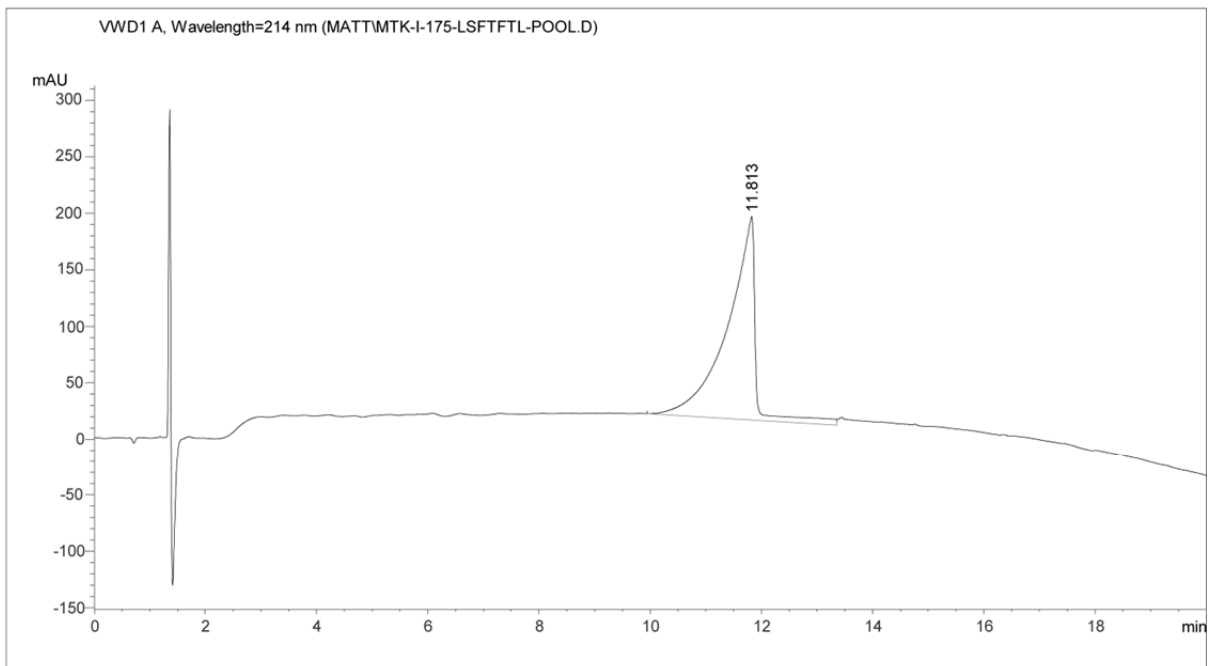200-ms spin-locking mixing time

DOSY Spectrum of Peptide **14**
4.00 mM in D₂O, 600 MHz, 298K

Analytical RP-HPLC of Peptide **15**

```
Data File C:\CHEM32\1\DATA\MATT\MTK-I-175-LSFTFTL-POOL.D
Sample Name: MTK-I-175-LSFTFTL.D

=====================================================================
Acq. Operator   : Kim
Acq. Instrument : Agilent HPLC                    Location : Vial 79
Injection Date  : 3/3/2013 12:56:00 AM
                                            Inj Volume : 10.0 µl
Acq. Method     : D:\NOWICK HPLC USERS\KVL\5-100 OVER 20MIN AT 214NM.M
Last changed    : 12/8/2012 6:14:14 PM by Kim
Analysis Method : D:\NOWICK HPLC USERS\ZHENGJ\METHODS\5-100 OVER 20 MIN AT 214 AT 1 ML.M
Last changed    : 5/4/2013 7:11:01 AM by Ryan
                  (modified after loading)
```


VWD1 A, Wavelength=214 nm (MATT\MTK-I-175-LSFTFTL-POOL.D)

```
=====================================================================
                         Area Percent Report
=====================================================================

     Sorted By             :       Signal
     Multiplier:           :        1.0000
     Dilution:             :        1.0000
     Sample Amount:        :       20.00000  [ng/ul]   (not used in calc.)
     Use Multiplier & Dilution Factor with ISTDs


     Signal 1: VWD1 A, Wavelength=214 nm

     Peak RetTime Type  Width     Area       Height      Area
      #   [min]        [min]  mAU   *s   [mAU   ]       %
     ----|-------|----|-------|----------|----------|--------|
        1  11.813 BV   0.4437 6304.28516  179.92001  85.4590
        2  27.321 BB   0.1111 1072.68958  138.00522  14.5410
```
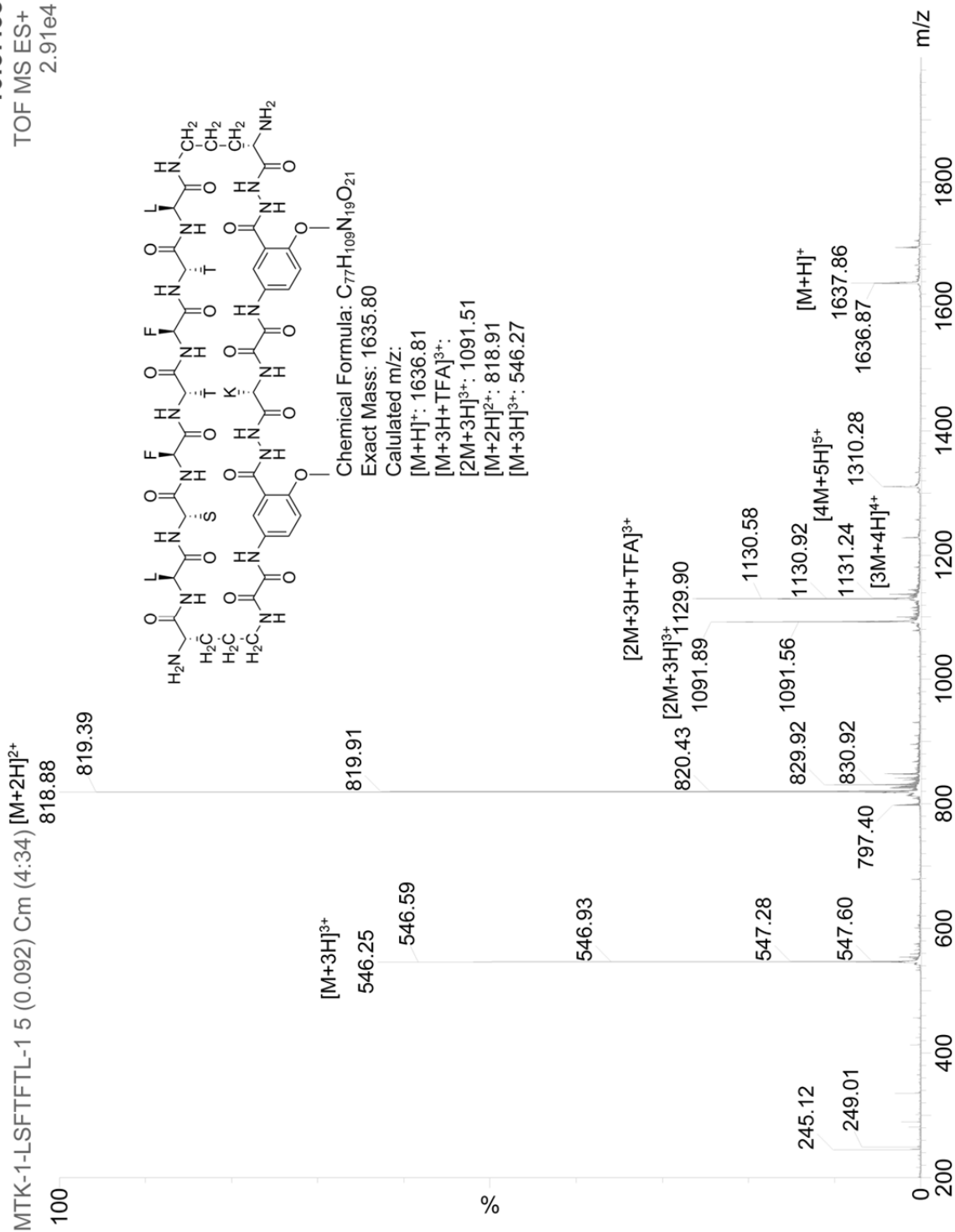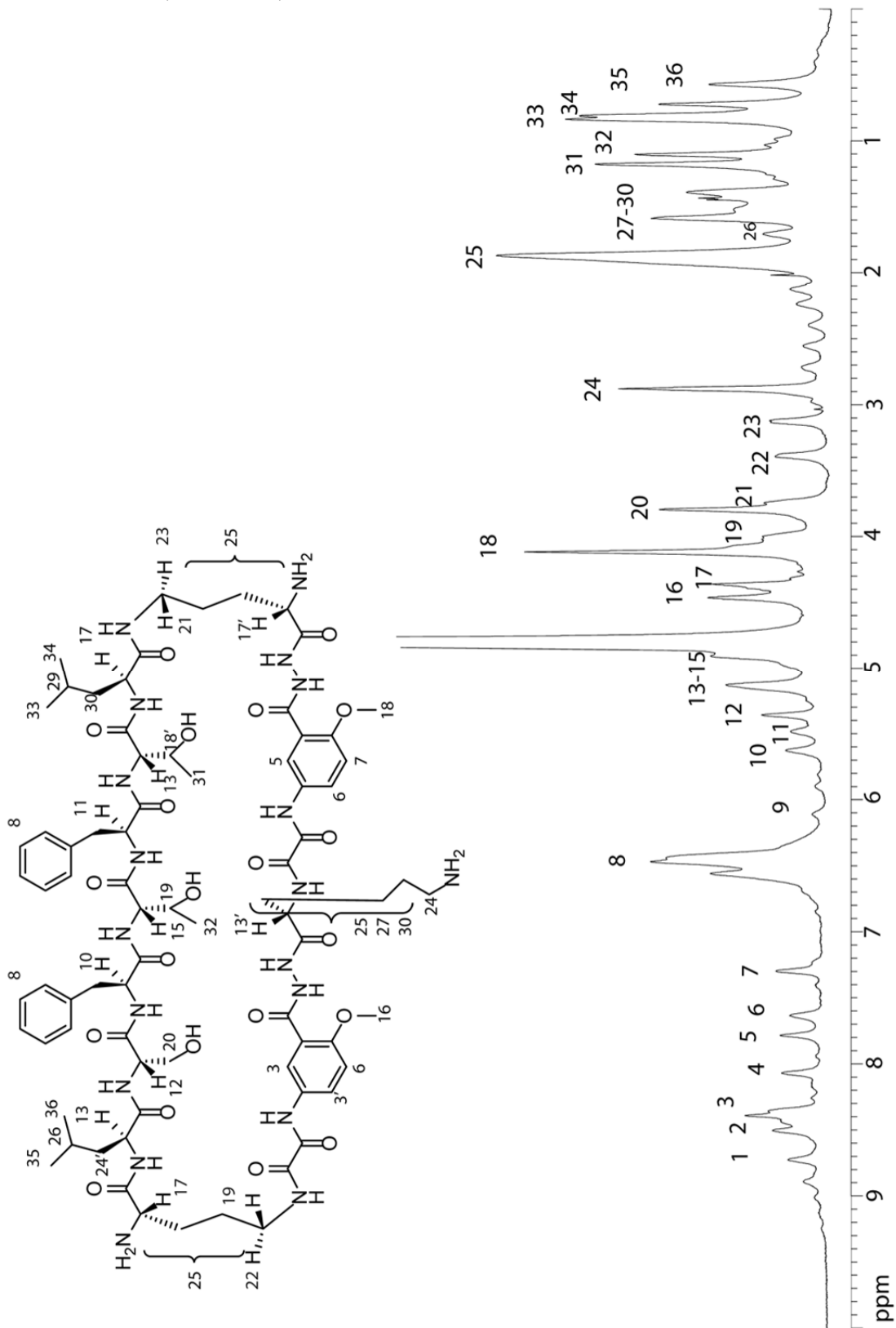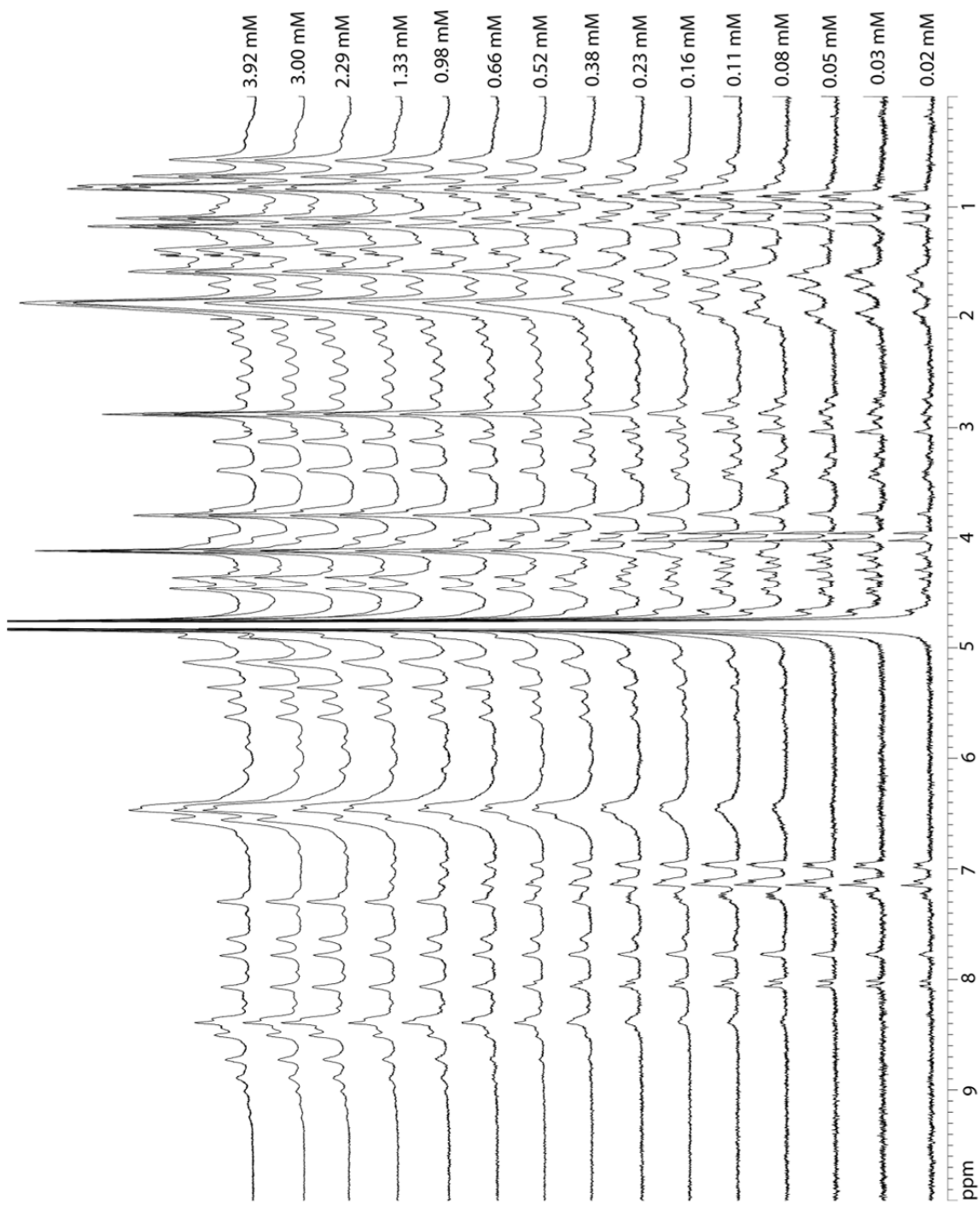
ESI-MS of Peptide **15**

17-Sep-2014
10:57:35
TOF MS ES+
2.91e4

MTK-1-LSFTFTL-1 5 (0.092) Cm (4:34)

Chemical Formula: C$_{77}$H$_{109}$N$_{19}$O$_{21}$
Exact Mass: 1635.80
Caluated m/z:
[M+H]$^+$: 1636.81
[M+3H+TFA]$^{3+}$:.
[2M+3H]$^{3+}$: 1091.51
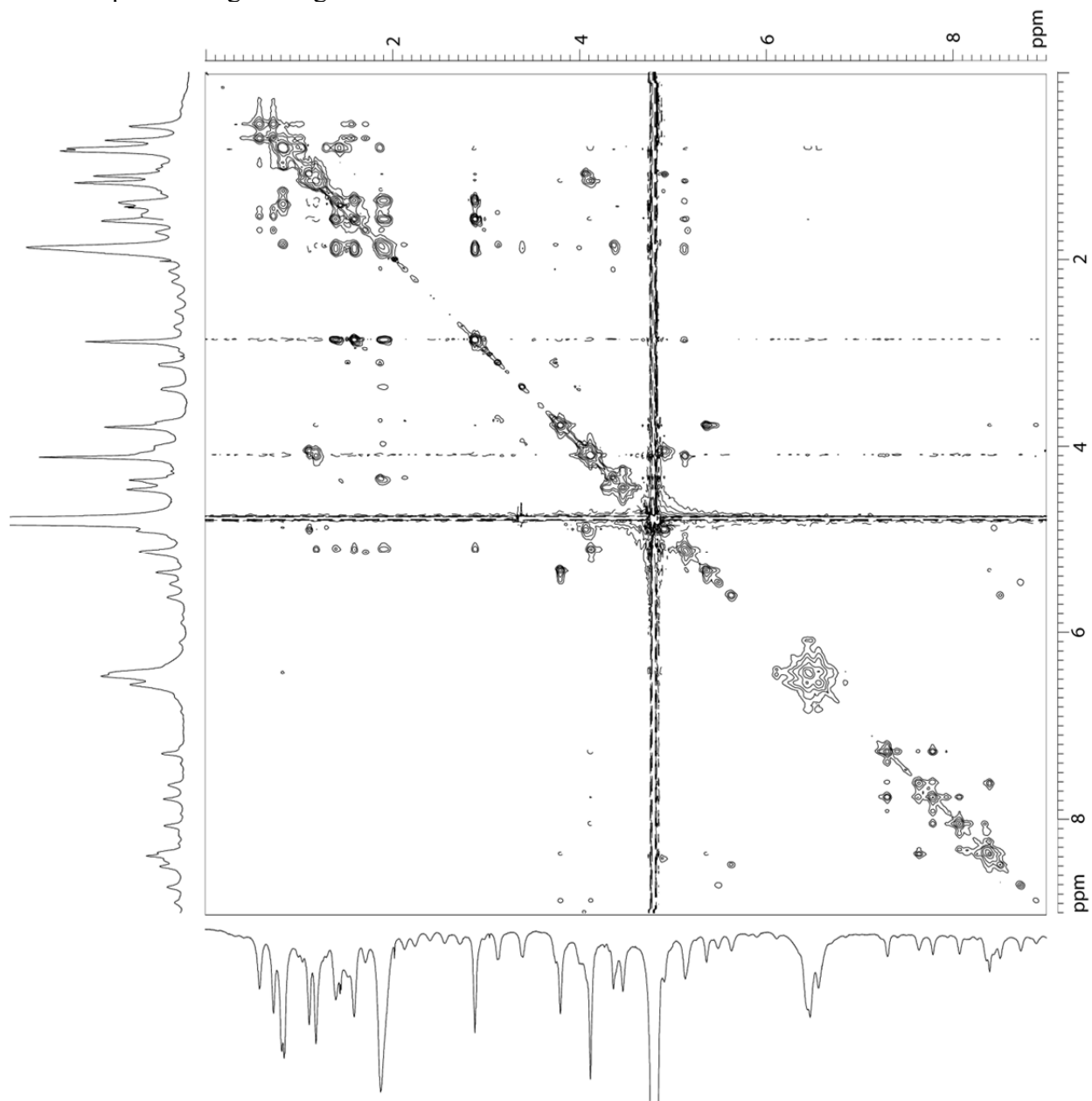[M+2H]$^{2+}$: 818.91
[M+3H]$^{3+}$: 546.27

1D ^{1}H NMR Spectrum of Peptide **15**
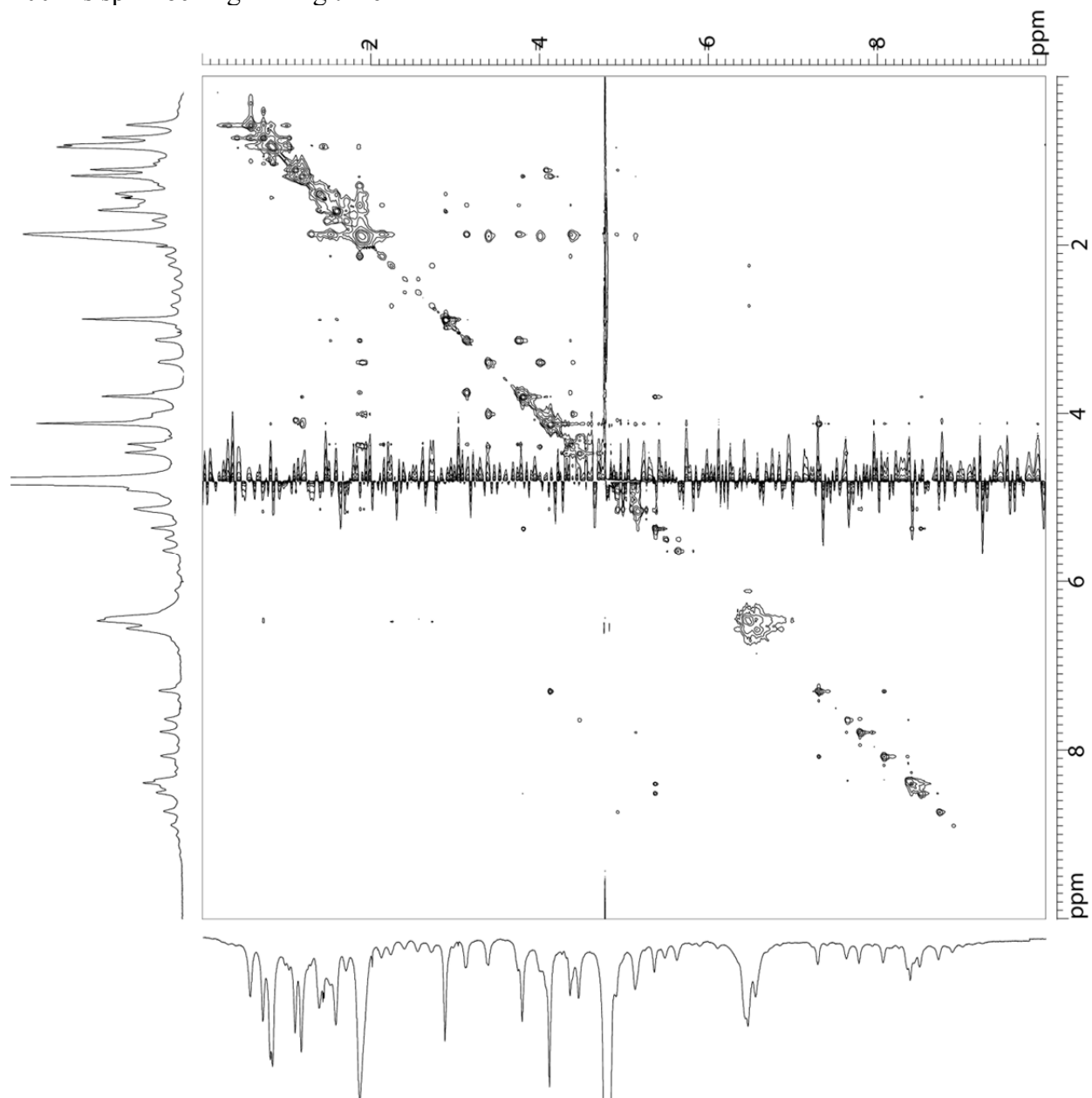3.92 mM in D$_2$O, 600 MHz, 298K

Concentration Dependent 1D ^{1}H NMR Spectra of Peptide **15**
D$_2$O, 600 MHz, 298K

2D TOCSY Spectrum of Peptide **15**
3.92 mM in D₂O, 600 MHz, 298K
150-ms spin-locking mixing time

2D ROESY Spectrum of Peptide **15**
3.92 mM in D₂O, 600 MHz, 298K
200-ms spin-locking mixing time

DOSY Spectrum of Peptide **15**
3.92 mM in D$_2$O, 600 MHz, 298K