UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Design and Synthesis of Macrocyclic Peptides with Anti-Cancer Potency

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Chemistry

by

Chun-Chieh Lin

Committee in Charge:

University of California, San Diego

Professor Pieter Dorrestein
Professor Jerry Yang

San Diego State University

Professor Mikael Bergdahl, Chair
Professor Shelli R. McAlpine, Co-Chair
Professor Kathleen McGuire

2013

The Dissertation of Chun-Chieh Lin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

San Diego State University

2013

DEDICATION

To my dear dad, mom and beloved little Sun

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LIST OF ABBREVIATIONS

μM	Micromolar
CTC	Chlorotrityl chloride
nM	Nanomolar
t-Bu	*tert*-Butyl
HOAt	1-Hydroxybenzotriazole
HOBt	1-Hydroxybenzotriazole
DIC	*N*,*N*′-Diisopropylcarbodiimide
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
Fmoc	9-Fluorenylmethyl chloroformate
IPA	Ispropyl alcohol
TFE	*2,2,2*-Trifluoroethanol
v/v	Volume to volume
NaH	Sodium hydride
BnBr	Benzyl bromide
DAST	Diethylaminosulfur trifluoride
°C	Degree Celcius
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
$BrCCl_3$	Bromotricchloromethane
K_2CO_3	Potassium carbonate
SAR	Structure-activity relationship

SanA	Sansalvamide A
NCI	National Cancer Institute
GI	Growth inhibition
Boc	*tert*-butoxycarbonyl
OMe	Methyl ester
OEt	Ethyl ester
TBTU	O-(Benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate
HATU	2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate
DEPBT	3-(Diethoxy-phosphoryloxy)-3H-benzo[d][1,2,3] triazin-4-one
HODhbt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzo-triazine
DMTMM	4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride
T3P	2,4,6-Tripropyl-1,3,5,2,4,6-trioxatriphosphorinane-2,4,6-trioxide
PyBroP	Bromotri(pyrrolidino)phosphonium hexafluorophosphate
PPh_3	Triphenylphosphine
FDPP	Pentafluorophenyl diphenyl phosphinate
PS-DVB	Polystyrene-divinylbenzene
DCM	Dichloromethane
ACN	Acetonitrile

DMF	*N*,*N*-Dimethylmethanamide
Bn	Benzyl
Cbz	Carboxybenzyl
LiOH	Lithium hydroxide
MeOH	Methanol
HBr	Hydrobromic acid
HCl	Hydrochloroic acid
NMR	Nuclear magnetic resonance
LC/MS	Liquid chromatography/mass spectrometry
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
DIPEA	*N*,*N*-Diisopropylethylamine
mg	Milligram
g	Gram
mL	Milliliter
L	Liter
mm	Millimeter
Na_2SO_4	Sodium sulfate
Ala	Alanine
Cys	Cysteine
Gly	Glycine
Ser	Serine

Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Phe	Phenylalanine
Thr	Threonine
Val	Valine
EtOAc	Ethyl acetate
Hex	Hexane
Na_2SO_4	Sodium sulfate
TFA	Trifluoroacetic acid
min	Minute
h	Hour
M	Molar
LP	Linear pentapeptide
DDLP	Double deprotected linear pentapeptide
THF	Tetrahydrofuran
mmol	Millimole
Å	Ångström; 10^{-10} m
HSP90	Heat shock protein 90
kDa	Kilodalton
TMSD	Trimethylsilyldiazomethane
LR	Lawesson's reagent

DME	1,2-Dimethoxyethane
$KHCO_3$	Potassium bicarbonate
TEA	Triethylamine
OEt	Ethyl ester
PEG	Polyethylene glycol
EtOH	Ethanol
rt	Room temperature
IC_{50}	half maximal inhibitory concentration
DI	Deionized
DDI	Double deionized

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Chapter 2, in part, is a reprint of the material as it appears in "Design and synthesis of Hsp90 inhibitors: Exploring the SAR of Sansalvamide A derivatives" *Bioorganic Medical Chemistry* **2010**, 18, 6822-6856 Sellers, R. P.; Alexander, L. D.; Johnson, V. A.; Lin, C. –C,; Savage, J.; Corral, R.; Moss, J.; Slugocki, T. S.; Singh, E. K.; Davis, M. R.; Ravula, S.; Spicer, J. E.; Oelrich, J. L.; Thornquist, A.; Pan, C. –M.; McAlpine, S. R. The dissertation author was the primary investigator and author of this paper.

Chapter 3, in part, is a reprint of material as it appears in "Total Synthesis and Biological Activity of Natural Product Urukthapelstatin A" *Organic Letters*, **2013**, 15, 3574-3577. Lin, C. –C.; Tantisantisom, W.; McAlpine, S. R. The dissertation author was the primary investigator and author of this paper.

VITA

1996-2000	Bachelor of Science, Chemistry Fu Jen Catholic University, Taiwan
2000-2003	Master of Science, Biological Science and Technology National Chiao Tung University, Taiwan
2008-2013	Doctor of Philosophy, Chemistry University of California, San Diego San Diego State University

PUBLICATIONS

1. **Lin, C.-C.**; Tantisantisom W.; McAlpine, S. R. "Total Synthesis and Biological Activity of Natural Product Urukthapelstatin A" *Organic Letters*, **2013**, 15, 3574-3577.

2. Kim, S. J.; **Lin, C.-C.**; Pan, C.-M.; Rananaware, D. P.; Ramsey, D. M.; McAlpine, S. R. "A structure-activity relationship study on multiheterocyclic molecules: two linked thiazoles are required for cytotoxic activity" *MedChemComm* **2013**, 4, 406-410.

3. Pan, C.-M.; **Lin, C.-C.**; Kim, S. J.; Sellers, R. P.; McAlpine, S. R. "Progress toward the synthesis of Urukthaplestatin A and two analogues" *Tetrahedron Letters*, **2012**, 53, 4065-4069.

4. Sellers, R. P.; Alexander, L. D.; Johnson, V. A.; **Lin, C.-C.**; Savage, J.; Corral, R.; Moss, J.; Slugocki, T. S.; Singh, E. K.; Davis, M. R.; Ravula, S.; Spicer, J. E.; Thornquist, A.; Pan, C.-M.; McAlpine, S. R. "Design and synthesis of Hsp90 inhibitors: Exploring the SAR of Sansalvamide A derivatives" *Bioorganic and Medicinal Chemistry* **2010**, 18, 6822-6856.

5. Pan, P.-S.; Vasko, R. V.; Lapera, S.; Johnson, V. A.; Sellers, R. P.; **Lin, C.-C.**; Pan, C.-M.; Davis, M. R.; Ardi, V. C.; McAlpine, S. R. "A comprehensive study of Sansalvamide a derivatives: the structure-activity relationships of 78 derivatives in two pancreatic cancer cell lines" *Bioorganic and Medicinal Chemistry* **2009**, 17, 5806-5825.

FIELDS OF STUDY

Major Field: Organic Synthesis

Studies in Chemistry
Professor Shelli R. McAlpine

ABSTRACT OF THE DISSERTATION

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Chapter 1

The background of peptides as therapeutics and anti-cancer agents is discussed in this chapter. The development for peptide synthesis via both solution and solid phase is introduced as well as the macrocyclization strategies.

Chapter 2

Sansalvamide A (SanA) exhibits anti-cancer potency in the micromolar range against multiple cancer cell lines. Structure-activity relationship (SAR) and the mechanistic studies of SanA have been investigated in our lab. This chapter focuses on the design and synthesis of SanA derivatives. Five SanA derivatives were designed based on the lead compounds in SanA project. The synthesis of these derivatives was accomplished via both solution and solid phase peptide synthesis. Studies for SAR and mechanism of action are also discussed in this chapter.

Chapter 3

The novel macrocyclic peptide Urukthapelstatin A (Ustat A) possesses a unique scaffold featuring five directly-linked azoles. Ustat A exhibits nanomolar cytotoxicity against cancer cells yet its mechanism of action remains undefined. This chapter focuses on the synthesis of Ustat A and its derivatives. The design of Ustat A derivatives is depicted and the synthestic approaches are illustrated. In addition, several strategies for cyclizing the Ustat A macrocycle are discussed. Finally, the cytotoxicity of synthetic Ustat A and its fragments is also demonstrated.

Chapter 1-Introduction

1.1 Peptide as therapeutics

Peptides are made up of amino acids connected via amide bonds. There are diverse sources of naturally-occurring medicinally active peptides including animals, plants, and microorganisms. These medicinal peptide natural products possess pharmaceutical activities including anti-microbial, anti-viral, anti-inflammatory and anti-cancer activities,[1] providing novel scaffolds for the development of therapeutics.[2]

Naturally-occurring amino acids (L), D-amino acids and unnatural amino acids have provided a diverse source for synthesizing medically active peptides. Modifying amino acid side chains within a peptide allows chemists to adjust the properties of that peptide to fit the desired profile for drug design. In addition, peptide metabolites are non-toxic, and are quickly removed from the human body.[3] These advantages make peptides a good source for effective therapeutics.

Linear peptides exhibit a high degree of conformational flexibility, which allows non-specific binding with biological molecules. Linear peptides are also susceptible to enzymatic degradation due to their flexibility and the unprotected termini.[4] Alternatively, cyclic peptides have rigidity and limited conformations that selectively bind to their biological targets.[5] Cyclic peptides are resistant to proteases due to the change of lacking the amino acid ring, which results in long half-lives in cells.[5b, 6]

One of the most well-known cyclic peptide drugs is Vancomycin (Vancocin™, **Figure 1.1**). It was first isolated from the soil bacterium *Amycolatopsis oreientalis* in 1956.[1a] Vancomycin is a tricyclic glycopeptide that exhibits potent antibiotic activity

against Gram-positive bacteria and was approved by FDA in 1958 to treat Gram-positive bacterial infection.[7] The biological mechanism of Vancomycin involves the inhibition of bacterial cell wall synthesis, resulting in growth inhibition.[8] Vancomycin has been regarded as a drug of last resort against bacterial infections due to its antibiotic activity against drug-resistant bacteria.[8]

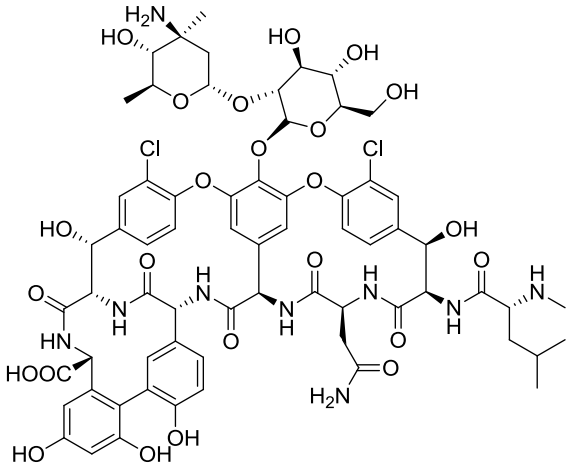


Figure 1.1 Structure of Vancomycin

Another example of cyclic peptide drugs is cyclosporine A (trademarked as Neroal, Gengraf, Pulminiq, Restasis, and Sandimmune, **Figure 1.2**). This naturally-occurring cyclic undecapeptide was discovered in 1970 from the metabolite of *Tolypocladium inflatum*.[9] The pharmaceutical application of cyclosporine A was discovered in 1978 when its highly potent immunosuppressant activity was observed.[10] Its mechanism of action involves blocking signal transduction required for activating T cells.[11] It was approved by the FDA in 1983 as a drug[12] and has been used for preventing the immuno rejection caused from organ transplant.

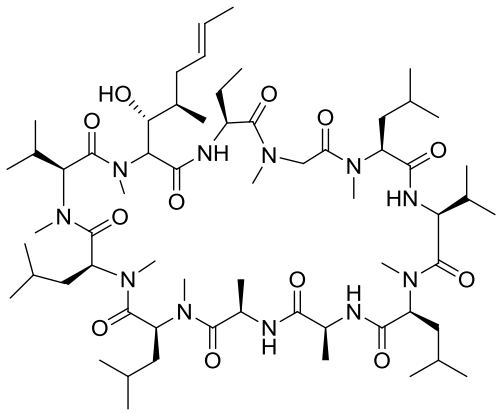


Figure 1.2 Structure of Cyclosporine A

In addition to cyclic peptide natural products, synthetic cyclic peptides are also an important source for therapeutics. Cyclic peptides could be designed for specific binding toward a biological target. For example, integrin $\alpha_V\beta_3$ is not present in normal cells[13] and is a pivotal protein that is involved in angiogenesis and metastasis in tumor cells. This protein specifically binds to the Arg-Gly-Asp (RGD) tripeptide motif.[14] Inhibition of integrin $\alpha_V\beta_3$ by a RGD-containing cyclic peptide demonstrated growth inhibition of tumor cells and induced tumor regression.[15] Thus, several antagonists against $\alpha_V\beta_3$ have been developed.[16] One of the most potent antagonists, the cyclic pentapeptide *cyclo*[Arg-Gly-Asp-D-Phe-Val] (**Figure 1.3**), was designed and synthesized by the Kessler group.[17] This cyclic pentapeptide exhibited a strong inhibition of tumor cell adhesion against several tumor cell lines (mean IC_{50} = 0.43 μM). In addition, radiolabeled derivatives of this cyclic peptide have also been used as probes and studied for tumor targeting.[18]

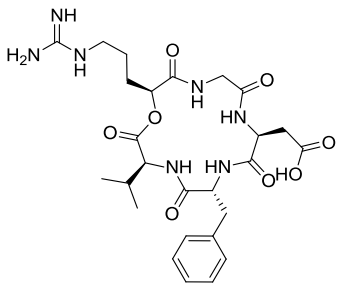


Figure 1.3 Structure of Cyclo[Arg-Gly-Asp-D-Phe-Val]

1.2 Peptides as anti-cancer agent

Cancer is a disease caused by cellular mutations, which result in unlimited cell proliferation, division and increased resistance of apoptosis.[19] The uncontrolled cell cycle results in the formation of malignant tumors that can invade adjacent tissues and spread to other parts of body via the blood and lymph systems (metastasis). Cancer will cause death if the spread is out of control. The acquired capabilities of cancer are further defined with 6 features: (I) sustaining proliferative signaling, (II) evading growth suppressors, (III) activating invasion and metastasis, (IV) enabling replicative immortality, (V) inducing angiogenesis, (VI) resisting cell death, and 2 more emerging hallmarks: (VII) reprogramming of energy metabolism and (VIII) evading immune destruction.[20]

According to the American Cancer Society (ACS), from 2006 to 2013 a total of new cancer cases increased from 1.4 million to almost 1.7 million in the U.S.[21] These statistics indicate the growing rate of cancer incidence. In addition, the overall cost of cancer care in 2010 was estimated to be $125 billion dollars and is predicted to increase to $158 billion dollars per year by 2020.[22]

There are three main categories of cancer treatments: surgery, radiation, and chemotherapy.[23] Chemotherapy uses a cytotoxic agent to kill cancerous cells. Conventional chemotherapeutic agents include Fluorouracil,[24] Cisplatin,[25] and Paclitaxel[26] (**Figure 1.4**). Fluorouracil has been used to treat cancer over 40 years. It is a pyrimidine analog and irreversibly inhibits thymidylate synthease, resulting in the termination of DNA synthesis, causing cell apoptosis. Cisplatin is a platinum-containing anticancer agent that causes DNA damage and induces cell apoptosis. Paclitaxel, originally isolated from the plant *Taxus brevifolia*, is a mitotic inhibitor that stabilizes the microtubules, resulting in the termination of mitosis as well as the consequent cell growth. However, conventional chemotherapy has some disadvantages including drug resistance and lack of specificity.[27] Thus, developing new selective anti-cancer agents is essential and peptides serve as a major inspiration for selectively modulating proteins.[2]

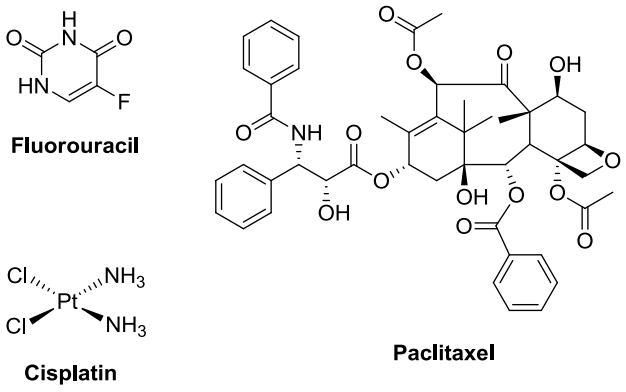


Figure 1.4 Structure of conventional chemotherapeutic agents

Peptides, compared to small organic molecules, have several advantages including low pharmacotoxicity,[28] rapid synthesis and modification, and tumor-

penetrating ability.[29] In addition, the resistance of peptides against enzymatic proteolysis can be improved by incorporating D-amino acids into the original peptide sequence[30] or cyclizing the parent linear peptides.[11, 30c, 31] There are currently 400-600 or more of peptide candidates in clinically and preclinical development.[32] From 2000 to 2008, peptide new chemical entities (NCEs) that entered clinical trials were studied in a wide spectrum of indications such as metabolic or cardiovascular diseases, and cancer was the most studied indication; indicating the rising focus on peptides as anti-cancer agents.[33]

Degarelix (**Figure 1.5**, Firmagon®, Ferring) is an example of anti-cancer peptide drugs. It was approved by U.S. FDA for the treatment of prostate cancer in 2009.[34] The structure of degarelix is a linear decapeptide and the mechanism of action involves the inhibition of the hormones required for cell growth, resulting in reduced growth of cancer cells.[34-35]

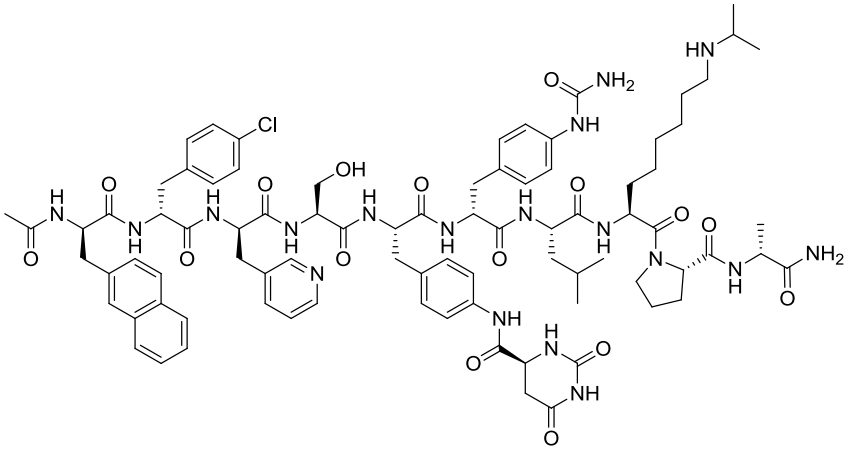


Figure 1.5 Structure of Degarelix

Cyclic peptides have been also evaluated as cancer therapeutics. For example, Cilengitide (**Figure 1.6 (a),** Merck-Serono), classified as an integrin inhibitor, is a cyclic

pentapeptide and currently in phase III clinical trials to treat glioblastoma and squamous cell cancer.[36] ADH-1 (**Figure 1.6, (b)**, Exherin^{TM}, Adherex) also a cyclic pentapeptide, is an N-cadherin antagonist that inhibits the growth of cancer cells. It is now being evaluated in clinical trials phase I for treating advance solid tumors.[37]

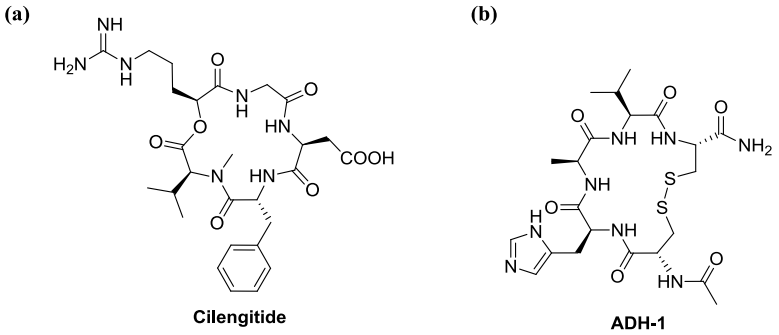
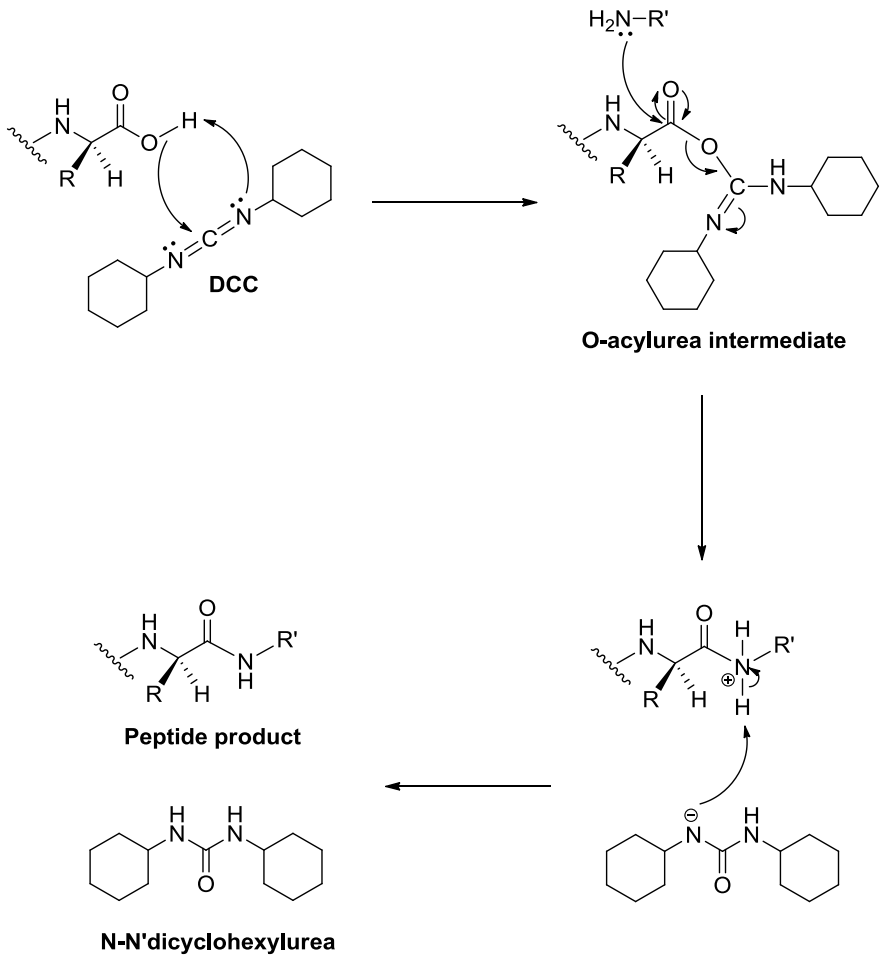


Figure 1.6 Structures of (a) Cilengitide and (b) ADH-1

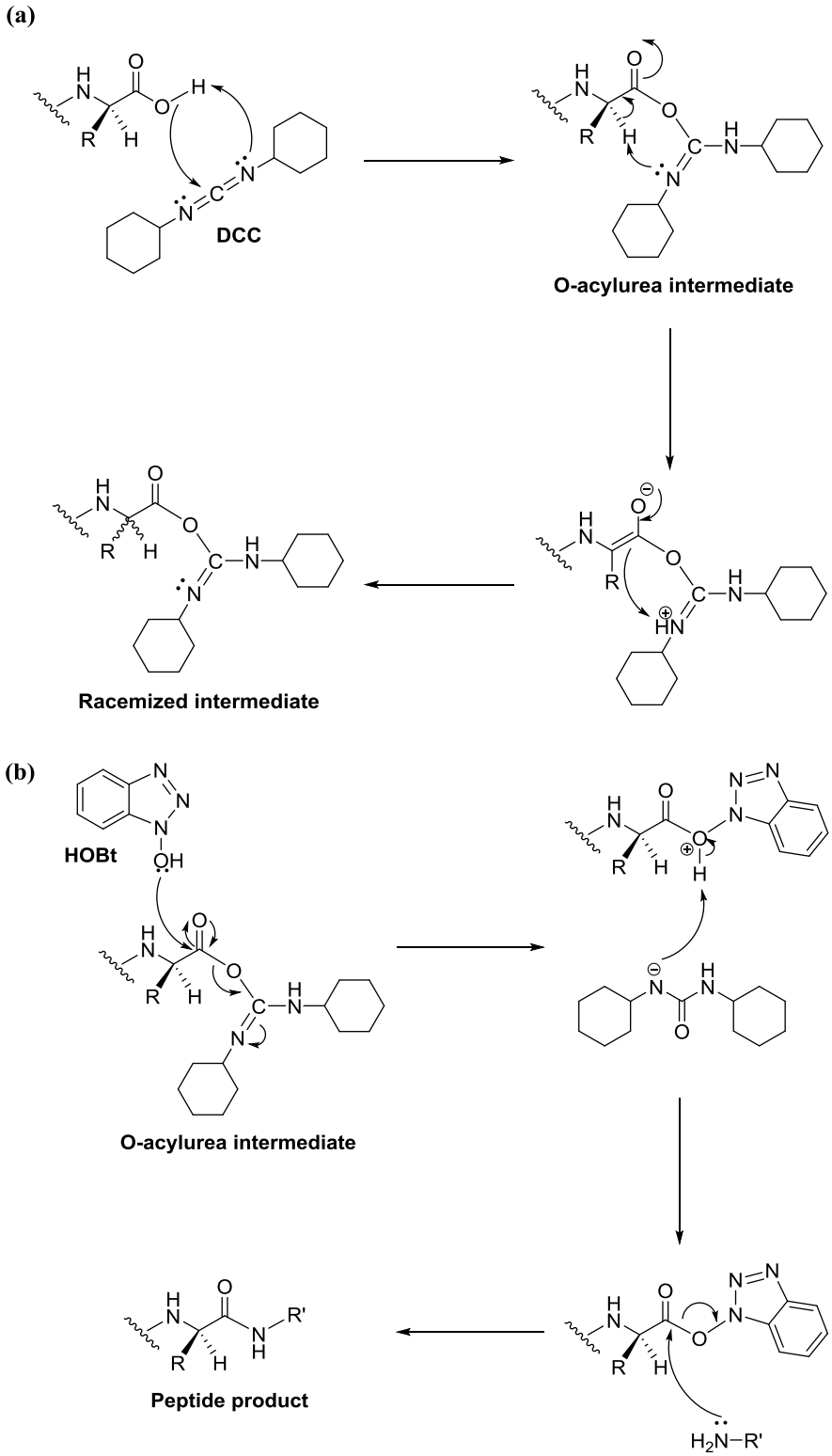
1.3 Solution phase peptide synthesis

The synthesis of peptides involves amide bond formation between an unprotected carboxylic acid and an unprotected amine. However, the reaction is not spontaneous under room temperature and only occurs at high temperatures,[38] which typically causes damages to the substrates. To facilitate amide bond formation, chemicals called coupling reagents are usually utilized. In general, coupling reagents activate the carboxylic acid group by converting the –OH of the acid into a good leaving group, which encourages the nucleophilic attack from the amine. Dicyclohexylcarbodiimide (DCC) was the first coupling reagent and has been used for over a half century.[39] DCC activates the carboxylic acid into an O-acylurea intermediate, which reacts with an amine to form an amide bond (**Scheme 1.1**).



Scheme 1.1 Mechanism of DCC-catalyzed peptide coupling

The main issue with coupling, however, is racemization at the α-carbon. Racemization can sometimes occur after the O-acylurea intermediate is formed (**Scheme 1.2 (a)**).[40] To address this problem, the coupling additive 1-hydroxybenzotriazole (HOBt) has been employed to convert the O-acylurea intermediate into the more active OBt ester, which reduces racemization (**Scheme 1.2 (b)**).[41]



Scheme 1.2 Mechanism of (a) DCC-induced racemization. (b) peptide coupling with HOBt

In 1993, a new coupling agent, 1-hydroxy-7-azabenzotriazole (HOAt), was reported by Carpino group. HOAt gave high yields (87% yield vs. incompletion in 7 h) and low racemization (DL-isomer%: <2% vs. ~4%).[42] The high coupling yields are attributed to neighboring group effects of the pyridine nitrogen, which induce the amine to quickly couple to the acid (**Figure 1.7**).[42] To date, numerous coupling reagents based on HOBt or HOAt have been reported.[43]

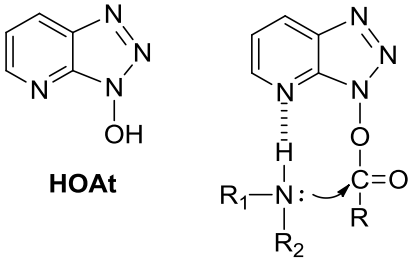


Figure 1.7 Neighboring group effect of HOAt

During my PhD research, I utilized TBTU, HATU, DEPBT for peptide coupling in solution phase. (**Figure 1.8**) TBTU is a HOBt-based coupling reagent that is in aminium salt form. It is economically effective, efficiently induces coupling reactions, and is almost racemization free (d.e. =97%).[44] HATU, also an aminium salt, is a HOAt-based coupling reagent and therefore is a better coupling reagent than TBTU in terms of yield, reaction rate, and reduced racemization levels.[45] DEPBT is derived from HODhbt (structurally related to HOBt) and exhibits high efficiency on peptide coupling with very little racemization.[46] In addition, DMTMM, PyBroP and T3P® (**Figure 1.8**) are also used in our lab for particular reaction conditions such as coupling with hindered amines or peptide macrocyclization.[43a]

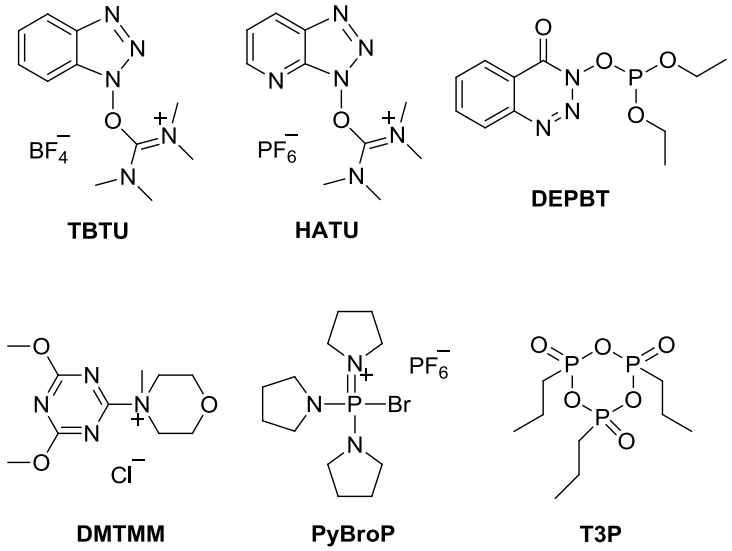


Figure 1.8 Coupling reagents that I used

1.4 Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) is a method that builds peptide chains on solid support via the iterative addition of amino acids. The concept of SPPS was first introduced by Merrifield in 1963.[47] The advantages of SPPS include rapid reactions, simple reaction procedures and easy purification compared to solution phase synthesis.[47-48] The solid support used by Merrifield group for SPPS is polystyrene-divinylbenzene crosslinked resin (PS-DVB), which possess several important features such as chemical inertness, inexpensive cost, and easy availability. To date, polystyrene-based resin has become the most common resin for SPPS.

I used the PS-DVB resin (**Figure 1.9**) with various preloaded amino acids for SPPS. The preloaded amino acids are anchored to the resin via 2-chlorotrityl chloride (CTC) linker. CTC linker was first employed by Barlos group in 1991.[49] It has been considered as a primary linker for SPPS due to several advantages including the mild

conditions for peptide cleavage (trifluoroethanol or 1% trifluoroacetic acid),[50] reducing the racemization of the preloaded amino acid,[49] and minimizing the formation of the undesired diketopiperazine.[51] During the process of SPPS, Fmoc-protected amino acids are used for peptide elongation because mild deprotection conditions (20% piperidine in DMF) between coupling of amino acids are used. Diisopropylcarbodiimide (DIC) and HOBt are employed as the coupling reagent and additive. Once the desired length of peptide chain has achieved, 2,2,2-trifluoroethanol (TFE) is introduced to cleave the peptide chain from the resin, resulting in a free C-terminal carboxylic acid on the peptide.

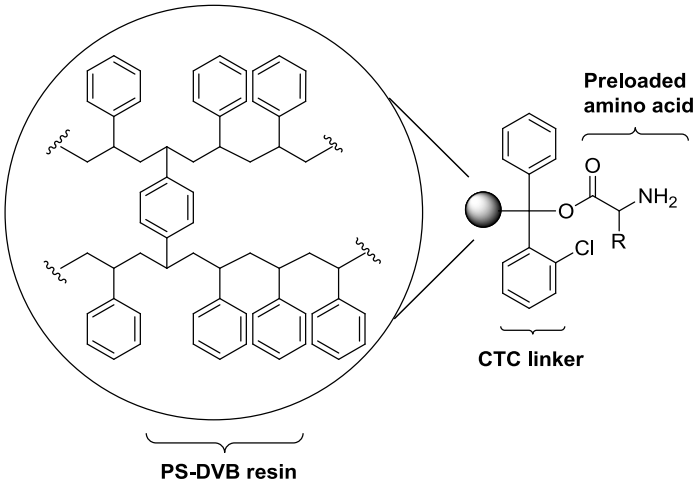
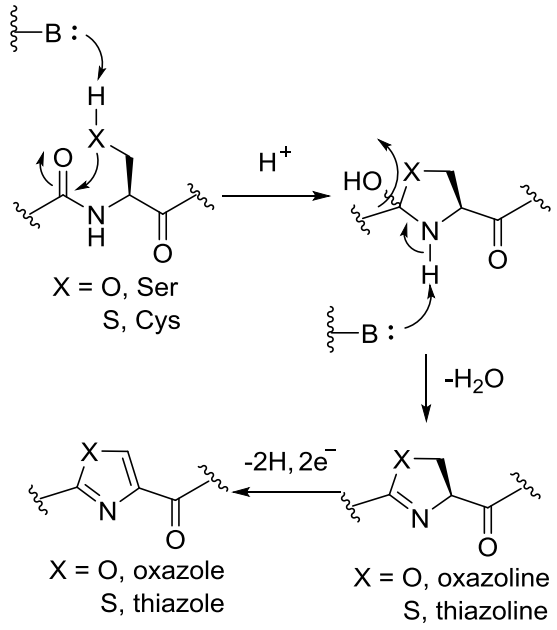


Figure 1.9 PS-DVB resin that I used for SPPS

1.5 Heterocycle synthesis

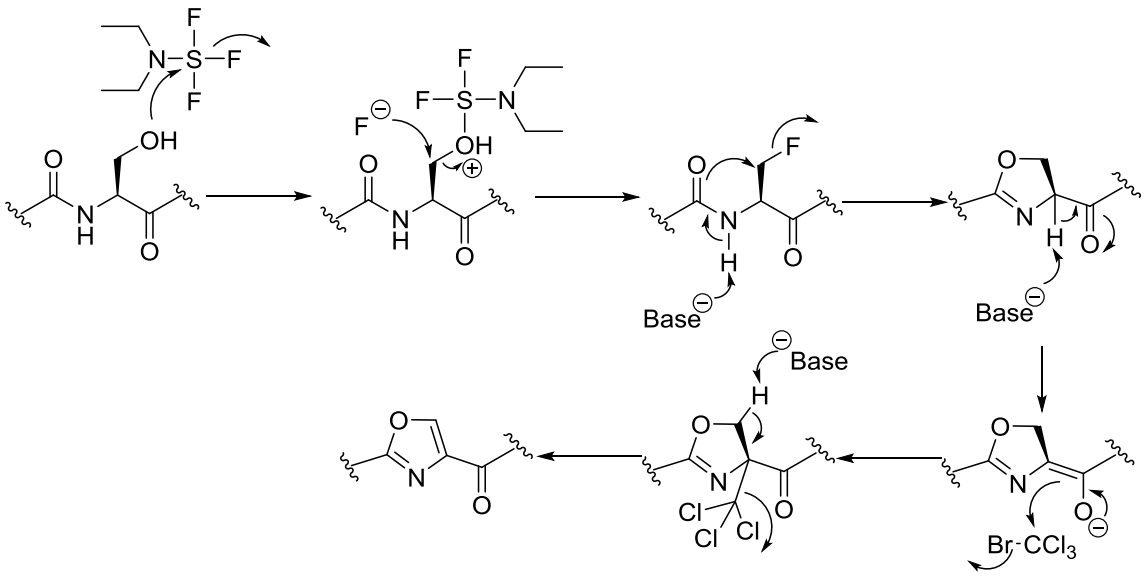
Heterocycles such as oxazoles and thiazoles are commonly observed in several biological active peptide natural products. These heterocycles not only provide the resistance of peptide backbone against proteolysis but act as key moieties for the antibiotic or anti-cancer activities.[52] In nature, these heterocycles are synthesized via the enzymatic post-translational modifications featuring the side chain cyclodehydration on serine or cysteine, followed by the dehydrogenation of the azoline ring[53] (**Scheme 1.3**).



Scheme 1.3 Biosynthesis of oxazole and thiazole

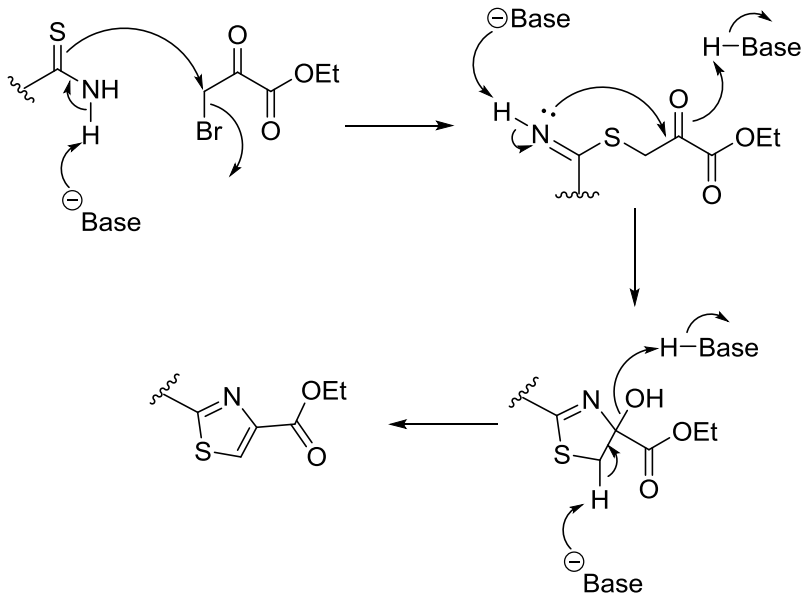
Organic chemists have been developed the synthetic strategies for oxazole/thiazole synthesis.[54] Oxazoles could be synthesized from serine peptide side chains via a step-wise cyclodehydroation/oxidation procedure utilizing either diethylaminosulfur trifluoride (DAST) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or a Swern oxidation and then triphenylphosphine (PPh_3). The major method for oxazole

synthesis is the Wipf-Willams protocol[54c] (**Scheme 1.4**). The β-hydroxy amide was first converted to the oxazoline using DAST under basic condition. The consequential addition of DBU and bromotrichloromethane generated the oxazole ring.



Scheme 1.4 Mechanism of oxazole synthesis

Similar to the oxazole synthesis, thiazoles could be generated via a thiazoline formation and then a base induced oxidation.[55] Another strategy for synthesizing thiazoles is a Hantzsch thiazole synthesis involving a directly condensation between a thioamide and an α-bromo ketone.[54e, 54f] Shown in **Scheme 1.5**, the intermolecular cyclization between the thioamide and ethyl bromopyruvate is initialized by the base to generate the hydroxythiazoline intermediate, which is then dehydrated to form the thiazole ring. During my PhD, I employed the Wipf-Willams protocol and the Hantzsch condition to synthesize oxazoles and thiazoles, respectively.



Scheme 1.5 Mechanism of thiazole synthesis

1.6 Strategies for peptide macrocyclization

The synthetic strategies for peptide cyclization are generally categorized in four different types according to the location of ring closure:[56] (I) head-to-tail (N-terminus to C-terminus), (II) head-to-side chain, (III) side chain-to-tail, and (IV) side chain-to-side chain (**Figure 1.10**). The side chain cyclization approaches (II-IV) typically include macrocyclizaiton via the formation of a disulfide bridge, a macrolactam, or a macrolactone.[57] For example, a disulfide bridge can be formed between two cysteines. A macrolactone can be obtained via the condensation between an aspartic acid and a lysine. Macrocyclization in a head-to-tail fashion is the most common strategy to synthesize cyclic peptides; however, the ring size greatly affects the success of this method. Cyclization of small precursors like tripeptides or tetrapeptides is usually difficult, while

it is straightforward to synthesize larger cyclicpeptides containing seven amino acids or more.

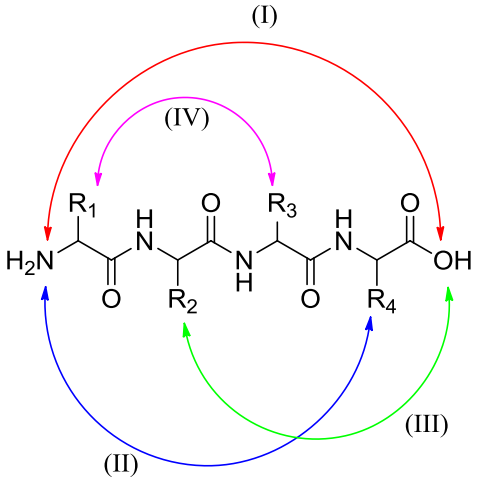


Figure 1.10 Four different ways for peptide macrocyclization

Selection of the ring closure site plays a key role in peptide macrocyclization. A poor choice for closing the ring causes slow reaction rates, low yields, and undesired side reactions including racemization at the C-terminus residue or dimerization.[57] For instance, cyclization at a bulky residue such as *N*-methyl or a β-branched amino acid typically results in a poor reaction yield, while cyclizing between a L- and a D-amino acid has a fast cyclization rate.[58] In addition, the achievement of peptide macrocyclization also depends on the conformation of linear peptide precursor, which governs the spatial proximity of two termini. Studies showed that cyclization of all L- or all D-peptide sequences are challenging due to their extended conformation, which orients the two termini apart from each other.[56] Incorporation of "turn inducers" such as Gly, Pro, or an *N*-alkyl residue in the peptide sequence will orient the peptide into an appropriate

conformation and facilitate macrocyclization.[59] During my PhD, I synthesized macrocyclic peptides using the head-to-tail strategy and the synthetic procedures of these molecules are depicted in the following chapters.

1.7 References

1. (a) Corey, E. J. C.; Kürti, L.; Czako, B., Molecules and Medicine. *John Wiley & sons, Inc., New Jersey* **2007**, 138; (b) Belofsky, G. N.; Jensen, P. R.; Fenical, W., Sansalvamide: A new cytotoxic cyclic depsipeptide produced by a marine fungus of the genus Fusarium. *Tetrahedron Letters* **1999,** *40* (15), 2913-2916; (c) Tamilarasu, N. H. L.; Rana, T. M., Design, Synthesis, and Biological Activity of a Cyclic Peptide: An Inhibitor of HIV tat-TAR Interaction in Human Cells. *Bioorg. Med. Chem. Lett.* **2000,** *10*, 971-974.

2. (a) Smith, A. B.; Razler, T. M.; Ciavarri, J. P.; Hirose, T.; Ishikawa, T.; Meis, R. M., A Second-Generation Total Synthesis of (+)-Phorboxazole A. *The Journal of Organic Chemistry* **2008,** *73* (4), 1192-1200; (b) Heller, M.; Sukopp, M.; Tsomaia, N.; John, M.; Mierke, D. F.; Reif, B.; Kessler, H., The Conformation of cyclo(−d-Pro−Ala4−) as a Model for Cyclic Pentapeptides of the dL4 Type. *Journal of the American Chemical Society* **2006,** *128* (42), 13806-13814; (c) Chatterjee, J.; Mierke, D.; Kessler, H., N-Methylated Cyclic Pentaalanine Peptides as Template Structures. *Journal of the American Chemical Society* **2006,** *128* (47), 15164-15172; (d) Tyndall, J. D. A.; Fairlie, D. P., Macrocycles Mimic The Extended Peptide Conformation Recognized By Aspartic, Serine, Cysteine and Metallo Proteases. *Current Medicinal Chemistry* **2001,** *8* (8), 893-907.

3. Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatisky, M., Synthetic therapeutic peptides: science and market. *Drug Discovery Today* **2010,** *15* (1–2), 40-56.

4. Hubbard, S. J., The structural aspects of limited proteolysis of native proteins. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1998,** *1382* (2), 191-206.

5. (a) Amidon, G. L.; Lee, H. J., Absorption of Peptide and Peptidomimetic Drugs. *Annual Review of Pharmacology and Toxicology* **1994,** *34* (1), 321-341; (b) Liu, S.; Gu, W.; Lo, D.; Ding, X.-Z.; Ujiki, M.; Adrian, T. E.; Soff, G. A.; Silverman, R. B., N-Methylsansalvamide A Peptide Analogues. Potent New Antitumor Agents. *Journal of Medicinal Chemistry* **2005,** *48* (10), 3630-3638.

6. Nicolaou, K. C.; Chen, J. S.; Dalby, S. M., From nature to the laboratory and into the clinic. *Bioorganic & Medicinal Chemistry* **2009,** *17* (6), 2290-2303.

7. Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C., Glycopeptide and Lipoglycopeptide Antibiotics. *Chemical Reviews* **2005,** *105* (2), 425-448.

8. Levine, D. P., Vancomycin: A History. *Clinical Infectious Diseases* **2006,** *42*, S5-S12.

9. Svarstad, H.; Bugge, H.; Dhillion, S., From Norway to Novartis: cyclosporin from Tolypocladium inflatum in an open access bioprospecting regime. *Biodiversity & Conservation* **2000,** *9* (11), 1521-1541.

10. Linden, P. K., History of Solid Organ Transplantation and Organ Donation. *Critical care clinics* **2009,** *25* (1), 165-184.

11. Haubner, R.; Gratias, R.; Diefenbach, B.; Goodman, S. L.; Jonczyk, A.; Kessler, H., Structural and Functional Aspects of RGD-Containing Cyclic Pentapeptides as Highly Potent and Selective Integrin αVβ3 Antagonists. *Journal of the American Chemical Society* **1996,** *118* (32), 7461-7472.

12. Katsara, M.; Tselios, T.; Deraos, S.; Deraos, G.; Matsoukas, M.-T.; Lazoura, E.; Matsoukas, J.; Apostolopoulos, V., Round and Round we Go: Cyclic Peptides in Disease. *Current Medicinal Chemistry* **2006,** *13* (19), 2221-2232.

13. (a) Hynes, R. O., Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **1992,** *69*, 11-25; (b) Hynes, R. O., A reevaluation of integrins as regulators of angiogenesis. *Nat. Med.* **2002,** *8*, 918-921; (c) Brooks, P. C.; Clark, R. A.; Cheresh, D. A., Requirement of vascular integrin αVβ3 for angiogenesis. *Science* **1994,** *264*, 569-571.

14. Xiong, J.-P.; Stehle, T.; Zhang, R.; Joachimiak, A.; Frech, M.; Goodman, S. L.; Arnaout, M. A., Crystal Structure of the Extracellular Segment of Integrin αVβ3 in Complex with an Arg-Gly-Asp Ligand. *Science* **2002,** *296*, 151-155.

15. Brooks, P. C.; Montgomery, A. M. P.; Rosenfeld, M.; Reisfeld, R. A.; Hu, T.; Klier, G.; Cheresh, D. A., Integrin αvβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **1994,** *79*, 1157-1164.

16. (a) Haubner, R.; Finsinger, D.; Kessler, H., Stereoisomeric Peptide Libraries and Peptidomimetics for Designing Selective Inhibitors of the αvβ3 Integrin for a New Cancer Therapy. *Angewandte Chemie International Edition in English* **1997,** *36* (13-14), 1374-1389; (b) Hood, J. D.; Bednarski, M.; Frausto, R.; Guccione, S.; Reisfeld, R. A.; Xiang, R.; Cheresh, D. A., Tumor Regression by Targeted Gene Delivery to the Neovasculature. *Science* **2002,** *296*, 2404-2407.

17. Gurrath, M.; MÜLler, G.; Kessler, H.; Aumailley, M.; Timpl, R., Conformation/activity studies of rationally designed potent anti-adhesive RGD peptides. *European Journal of Biochemistry* **1992,** *210* (3), 911-921.

18. Haubner, R.; Wester, H.-J.; Reuning, U.; Senekowitsch-Schmidtke, R.; Diefenbach, B.; Kessler, H.; Stöcklin, G.; Schwaiger, M., Radiolabeled αvβ3 Integrin Antagonists: A New Class of Tracers for Tumor Targeting. *The Journal of Nuclear Medicine* **1999,** *40*, 1061-1071.

19. Singh, E. K.; Sellers, R. P.; Alexander, L. D.; McAlpine, S. R., Conformational based design of macrocycles as antitumor agents. *Current Opinion in Drug Discovery & Development* **2008,** *11*, 544-552.

20. Hanahan, D.; Weinberg, Robert A., Hallmarks of Cancer: The Next Generation. *Cell* **2011,** *144* (5), 646-674.

21. (a) Jemal, A.; Siegel, R.; Ward, E.; Murray, T.; Xu, J.; Smigal, C.; Thun, M. J., Cancer Statistics, 2006. *CA: A Cancer Journal for Clinicians* **2006,** *56* (2), 106-130; (b) Siegel, R.; Naishadham, D.; Jemal, A., Cancer statistics, 2013. *CA: A Cancer Journal for Clinicians* **2013,** *63* (1), 11-30.

22. Mariotto, A. B.; Robin Yabroff, K.; Shao, Y.; Feuer, E. J.; Brown, M. L., Projections of the Cost of Cancer Care in the United States: 2010–2020. *Journal of the National Cancer Institute* **2011,** *103* (2), 117-128.

23. American Cancer Society. *Cancer Facts and Figures 2013* **2013**.

24. Longley, D. B.; Harkin, D. P.; Johnston, P. G., 5-Fluorouracil: mechanisms of action and clinical strategies. *Nature Reviews Cancer* **2003,** *3*, 330-338.

25. Einhorn, L. H., Treatment of testicular cancer: a new and improved model. *Journal of Clinical Oncology* **1990,** *8* (11), 1777-81.

26. Jordan, M. A.; Wilson, L., Microtubules as a target for anticancer drugs. *Nature Reviews Cancer* **2004,** *4*, 253-265.

27. Kakde, D.; Jain, D.; Shrivastava, V.; Kakde, R.; Patil, A. T., Cancer Therapeutics-Opportunities, Challenges and Advances in Drug Delivery. *Journal of Applied Pharmaceutical Science* **2011,** *1* (9), 1-10.

28. Demmer, O.; Frank, A. O.; Kessler, H., Design of Cyclic Peptides. In *Peptide and Protein Design for Biopharmaceutical Applications*, John Wiley & Sons, Ltd: 2009; pp 133-176.

29. (a) Thayer, A. M., IMPROVING PEPTIDES. *Chemical & Engineering News Archive* **2011,** *89* (22), 13-20; (b) Borghouts, C.; Kunz, C.; Groner, B., Current strategies for the development of peptide-based anti-cancer therapeutics. *Journal of Peptide Science* **2005,** *11* (11), 713-726.

30. (a) Miller, S. M.; Simon, R. J.; Ng, S.; Zuckermann, R. N.; Kerr, J. M.; Moos, W. H., Comparison of the proteolytic susceptibilities of homologous L-amino acid, D-amino acid, and N-substituted glycine peptide and peptoid oligomers. *Drug Development Research* **1995,** *35* (1), 20-32; (b) Tugyi, R.; Uray, K.; Iván, D.; Fellinger, E.; Perkins, A.; Hudecz, F., Partial d-amino acid substitution: Improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102* (2), 413-418; (c) Molhoek, E. M.; van Dijk, A.; Veldhuizen, E. J. A.; Haagsman, H. P.; Bikker, F. J., Improved proteolytic stability of chicken cathelicidin-2 derived peptides by d-amino acid substitutions and cyclization. *Peptides* **2011,** *32* (5), 875-880.

31. Nguyen, L. T.; Chau, J. K.; Perry, N. A.; de Boer, L.; Zaat, S. A. J.; Vogel, H. J., Serum Stabilities of Short Tryptophan- and Arginine-Rich Antimicrobial Peptide Analogs. *PLoS ONE* **2010,** *5* (9), e12684.

32. Sun, L., Peptide-Based Drug Development. *Modern Chemistry & Applications* **2013,** *1* (1), 1000e103.

33. Reichert, J. M.; Pechon, P.; Tartar, A.; Dunn, M. K., Development trends for peptide therapeutics. The Peptide Therapeutics Foundation: 2010.

34. Steinberg, M., Degarelix: A gonadotropin-releasing hormone antagonist for the management of prostate cancer. *Clinical Therapeutics* **2009,** *31, Part 2* (0), 2312-2331.

35. Liu, K. K. C.; Sakya, S. M.; O'Donnell, C. J.; Flick, A. C.; Li, J., Synthetic approaches to the 2009 new drugs. *Bioorganic & Medicinal Chemistry* **2011,** *19* (3), 1136-1154.

36. Brown, K. C., Peptidic Tumor Targeting Agents: The Road from Phage Display Peptide Selections to Clinical Applications. *Current Pharmaceutical Design* **2010,** *16* (9), 1040-1054.

37. Yarom, N.; Stewart, D.; Malik, R.; Wells, J.; Avruch, L.; J. Jonker, D., Phase I Clinical Trial of Exherin (ADH-1) in Patients with Advanced Solid Tumors. *Current Clinical Pharmacology* **2013** *8*(1), 81-88.

38. Jursic, B. S.; Zdravkovski, Z., A Simple Preparation of Amides from Acids and Amines by Heating of Their Mixture. *Synthetic Communications* **1993,** *23* (19), 2761-2770.

39. Sheehan, J. C.; Hess, G. P., A New Method of Forming Peptide Bonds. *Journal of the American Chemical Society* **1955,** *77* (4), 1067-1068.

40. Anderson, G. W.; Callahan, F. M., RACEMIZATION BY THE DICYCLOHEXYLCARBODIIMIDE METHOD OF PEPTIDE SYNTHESIS. *Journal of the American Chemical Society* **1958,** *80* (11), 2902-2903.

41. (a) König, W.; Geiger, R., A New Method for Synthesis of Peptides: Activation of the Carboxyl Group with Dicyclohexylcarbodiirnide using 1 -Hydroxybenzotriazoles as Additives. *Chemische Berichte* **1970,** *103* (3), 788-798; (b) König, W.; Geiger, R., Racemization in Peptide Syntheses. *Chemische Berichte* **1970,** *103* (7), 2024-2033.

42. Carpino, L. A., 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. *Journal of the American Chemical Society* **1993,** *115* (10), 4397-4398.

43. (a) Valeur, E.; Bradley, M., Amide bond formation: beyond the myth of coupling reagents. *Chemical Society Reviews* **2009,** *38* (2), 606-631; (b) Joullie, M. M.; Lassen, K. M., Evolution of amide bond formation. *Arkivoc* **2010,** *8*, 189-250; (c) Al-Warhi, T. I.; Al-Hazimi, H. M. A.; El-Faham, A., Recent development in peptide coupling reagents. *Journal of Saudi Chemical Society* **2012,** *16* (2), 97-116.

44. Ivanov, A. S.; Zhalnina, A. A.; Shishkov, S. V., A convergent approach to synthesis of bortezomib: the use of TBTU suppresses racemization in the fragment condensation. *Tetrahedron* **2009,** *65* (34), 7105-7108.

45. Tsuda, Y.; Okada, Y., Solution-Phase Peptide Synthesis. In *Amino Acids, Peptides and Proteins in Organic Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA: 2010; pp 201-251.

46. Li, H.; Jiang, X.; Ye, Y.-h.; Fan, C.; Romoff, T.; Goodman, M., 3-(Diethoxyphosphoryloxy)-1,2,3- benzotriazin-4(3H)-one (DEPBT): A New Coupling Reagent with Remarkable Resistance to Racemization. *Organic Letters* **1999,** *1* (1), 91-94.

47. Merrifield, R. B., Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *Journal of the American Chemical Society* **1963,** *85* (14), 2149-2154.

48. (a) Merrifield, R. B., Solid Phase Peptide Synthesis. II. The Synthesis of Bradykinin. *Journal of the American Chemical Society* **1964,** *86* (2), 304-305; (b) Merrifield, R. B., Solid-Phase Peptide Synthesis. III. An Improved Synthesis of Bradykinin*. *Biochemistry* **1964,** *3* (9), 1385-1390; (c) Merrifield, R. B., Solid Phase Peptide Synthesis. IV. The Synthesis of Methionyl-lysyl-bradykinin1. *The Journal of Organic Chemistry* **1964,** *29* (10), 3100-3102.

49. Chatzi, K. B. O.; Gatos, D.; Stavropoulos, G., 2-Chlorotrityl chloride resin. *International Journal of Peptide and Protein Research* **1991,** *37* (6), 513-520.

50. Bollhagen, R.; Schmiedberger, M.; Barlos, K.; Grell, E., A new reagent for the cleavage of fully protected peptides synthesised on 2-chlorotrityl chloride resin. *Journal of the Chemical Society, Chemical Communications* **1994,** *0* (22), 2559-2560.

51. (a) Rovero, P.; Viganò, S.; Pegoraro, S.; Quartara, L., Synthesis of the bradykinin B1 antagonist [desArg10]HOE 140 on 2-chlorotrityl resin. *Lett Pept Sci* **1996,** *2* (6), 319-323; (b) Chiva, C.; Vilaseca, M.; Giralt, E.; Albericio, F., An HPLC-ESMS study on the solid-phase assembly of C-terminal proline peptides. *Journal of Peptide Science* **1999,** *5* (3), 131-140.

52. Kelly, W. L., Biosynthesis: Ringing in a new view. *Nature Chemical Biology* **2012,** *8* (6), 505-507.

53. Sinha Roy, R.; M. Gehring, A.; C. Milne, J.; J. Belshaw, P.; T. Walsh, C., Thiazole and oxazole peptides: biosynthesis and molecular machinery. *Natural Product Reports* **1999,** *16* (2), 249-263.

54. (a) Wipf, P.; Miller, C. P., A new synthesis of highly functionalized oxazoles. *The Journal of Organic Chemistry* **1993,** *58* (14), 3604-3606; (b) Yeh, V. S. C., Recent advances in the total syntheses of oxazole-containing natural products. *Tetrahedron* **2004,** *60* (52), 11995-12042; (c) Phillips, A. J.; Uto, Y.; Wipf, P.; Reno, M. J.; Williams, D. R., Synthesis of Functionalized Oxazolines and Oxazoles with DAST and Deoxo-Fluor. *Organic Letters* **2000,** *2* (8), 1165-1168; (d) Deeley, J.; Bertram, A.; Pattenden, G., Novel polyoxazole-based cyclopeptides from Streptomyces sp. Total synthesis of the cyclopeptide YM-216391 and synthetic studies towards telomestatin. *Organic & Biomolecular Chemistry* **2008,** *6* (11), 1994-2010; (e) Aguilar, E.; Meyers, A. I., Reinvestigation of a modified Hantzsch thiazole synthesis. *Tetrahedron Letters* **1994,** *35* (16), 2473-2476; (f) Merritt, E. A.; Bagley, M. C., Holzapfel-Meyers-Nicolaou Modification of the Hantzsch Thiazole Synthesis. *Synthesis* **2007,** *2007* (EFirst), 3535-3541.

55. Boden, C. D. J.; Pattenden, G.; Ye, T., The Synthesis of Optically Active Thiazoline and Thiazole Derived Peptides from N-Protected α-Amino Acids. *Synlett* **1995,** *1995* (05), 417-419.

56. Goodman, M.; Toniolo, C.; Moroder, L.; Felix, A., Houben-Weyl Methods in Organic Chemistry, Volume E22, - Synthesis of Peptides and Peptidomimetics, Volumes 1-5 (Workbench Edition). Thieme Medical Publishers Inc.: 2004; pp 461-472.

57. Jiang, S.; Li, Z.; Ding, K.; Roller, P. P., Recent Progress of Synthetic Studies to Peptide and Peptidomimetic Cyclization. *Current Organic Chemistry* **2008,** *12* (17), 1502-1542.

58. Brady, S. F.; Varga, S. L.; Freidinger, R. M.; Schwenk, D. A.; Mendlowski, M.; Holly, F. W.; Veber, D. F., Practical synthesis of cyclic peptides, with an example of dependence of cyclization yield upon linear sequence. *The Journal of Organic Chemistry* **1979,** *44* (18), 3101-3105.

59. Kopple, K. D., Synthesis of cyclic peptides. *Journal of Pharmaceutical Sciences* **1972,** *61* (9), 1345-1356.

Chapter 2-Sansalvamide A

2.1 Background of Sansalvamide A

Sansalvamide A (SanA) is a natural product (**Figure 2.1**) that was isolated by the Fenical group in 1999. SanA came from from a marine fungus *Fusarium* sp. that was located on the coast of Little San Salvador Island, Bahamas.[1] The structure of SanA natural product was elucidated as a macrocyclic depsipeptide that contained two L-leucines (Leu), L-phenylalanine (Phe), L-valnine (Val), and a leucic acid (OLeu).

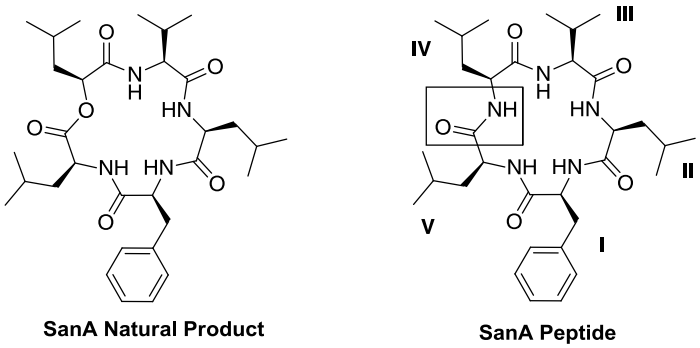


Figure 2.1 Structures of SanA natural product and SanA peptide

Fenical reported that the SanA natural product demonstrated cytotoxic activity against the colon cancer cell-line HCT-116 with an *in vitro* IC_{50} value of 16.7 μM.[1] The linear form of SanA natural product was also isolated but it had no cytotoxicity in the assay. Thus, the cyclization of the peptide sequence is critical for cytotoxicity. Evaluation of SanA's activity against the National Cancer Institute (NCI) 60 cell-line panel generated a mean IC_{50} of 46.7 μM. It is noteworthy that SanA exhibited greater potency against the colon cancer cell-line COLO 205 and melanoma cell-line SK-MEL-2 with IC_{50} values of 6.0 μM and 10.1 μM, respectively than the average.[1] These cytotoxicity

values are comparable to mitomycin C, a FDA-approved antitumor agent with IC_{50} = 15.8 μM for the same two cell-lines.[1] Given the cyclic scaffold and promising cytotoxicity values, SanA is an important lead molecule in the development of novel anti-cancer agents.

The first total synthesis of SanA natural product was accomplished by Silverman and coworkers in 2000 via solid phase synthesis using a phenylalanine-bound polymer resin.[2] In 2002, the Silverman group investigated the importance of the lactone linkage within the SanA natural product by synthesizing the first SanA analog, where the lactone linkage between two leucines was replaced by an amide bond (**Figure 2.1**, SanA Peptide). Not surprisingly, the SanA peptide was found to be 10 times more potent against HCT-116 than the natural product depsipeptide.[3] Thus, the amide is more favorable than the ester for SanA's anti-cancer activity.[3]

Given that the SanA peptide is more biologically active than the natural product depsipeptide, our lab has synthesized SanA peptide derivatives. Substituting numerous amino acids around the macrocycle at each position (**Figure 2.1**, Position I-V of SanA Peptide) provided Structure-Activity Relationship (SAR) information on SanA. Synthesizing PEG-biotinylated SanA derivatives allowed us to determine the biological target. During my PhD, I have synthesized five SanA peptide derivatives to contribute our SanA project. Their role in understanding the SAR and determining their biological target are described in the following sections.

2.2 Design of Sansalvamide A derivatives

A common modification used when investigating the SAR of peptides is to exchange L-amino acid for the corresponding D-amino acid.[4] Incorporating D-amino acids into the peptide sequence alters the macrocycle's conformation and frequently changes its biological activity.[4, 5] In addition, incorporating D-amino acids also prolongs the half-life of the peptide since it is less susceptible to enzymatic degradation than the corresponding L-amino acid peptide.[6] During our initial stage of the SanA project, derivatives were synthesized in which each L-amino acid was sequentially substituted by the corresponding D-amino acid.[7] Performing a D-amino acid substitution on the SanA peptide scaffold revealed a potent SanA derivative (**1**, **Figure 2.2**). Compound **1** contains a D-Val at position III, and as shown; it possesses better anti-cancer activity against PL45 (pancreatic cancer cell line) and HCT-15 (colon cancer cell line) than SanA peptide. This observation indicated that the D-configuration at position III is important for cytotoxicity of this scaffold.

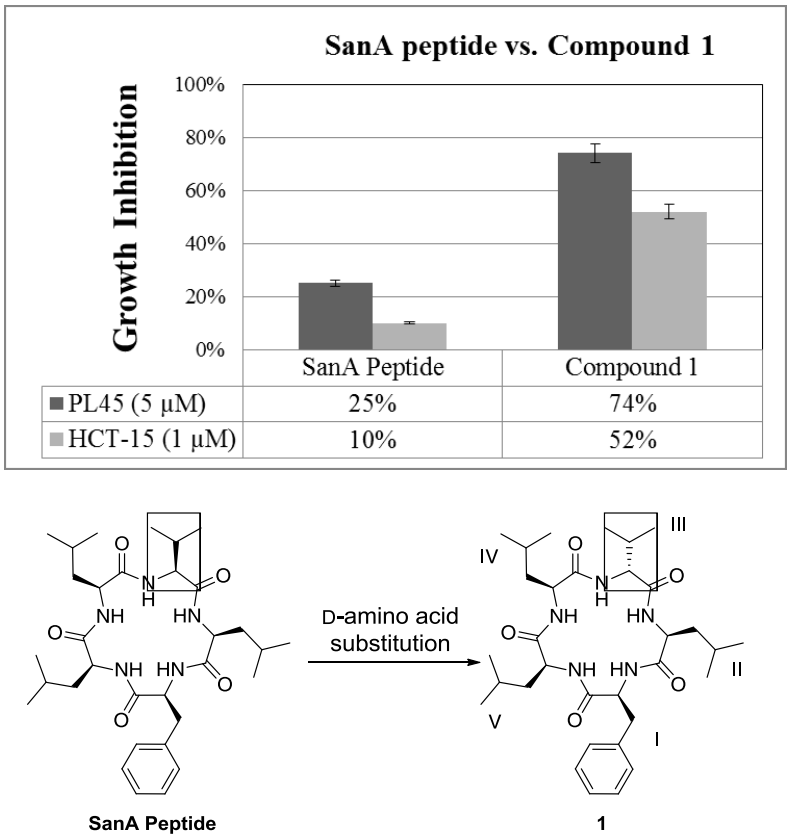


Figure 2.2 Cytotoxicity of SanA peptide and compound 1 in various cell lines. Margin of error is ±5%

Another common modification made on peptides when investigating the SAR is *N*-methylation. Many naturally occurring biologically active cyclic peptides contain one or more *N*-methyl amino acids within their peptide backbone.[8] In fact, *N*-methylation has been used as a technique to increase the potency of peptides for years.[7a, 9] Research shows that *N*-methylation locks the 3D structure of the cyclic peptide into a limited number of conformations.[10] A peptide locked in a bioactive conformation will have an increased binding affinity toward its biological target, which will result in improved potency over the non-*N*-methylated analog.[11] In addition to exploring *N*-methylation and D-amino

acids, several modifications, including the incorporation of unnatural polar and nonpolar amino acids were used to study the SAR of SanA.[7, 12] From the our initial SAR studies[7e, 12b], several features were found in a lead molecule, Compound **2**, and these features were determined to be essential for potency: two consecutive D-amino acids, one *N*-methyl amino acid, and a minimum of two phenyl groups. Compound **2** (**Figure 2.3**) meets these criteria as it contains two consecutive D-amino acids at position IV and V, *N*-methyl Val at position III, and two phenyl groups. As a result, it has 80% and 78% growth inhibition against the pancreatic cancer cell lines PL45 and BxPc3, respectively.

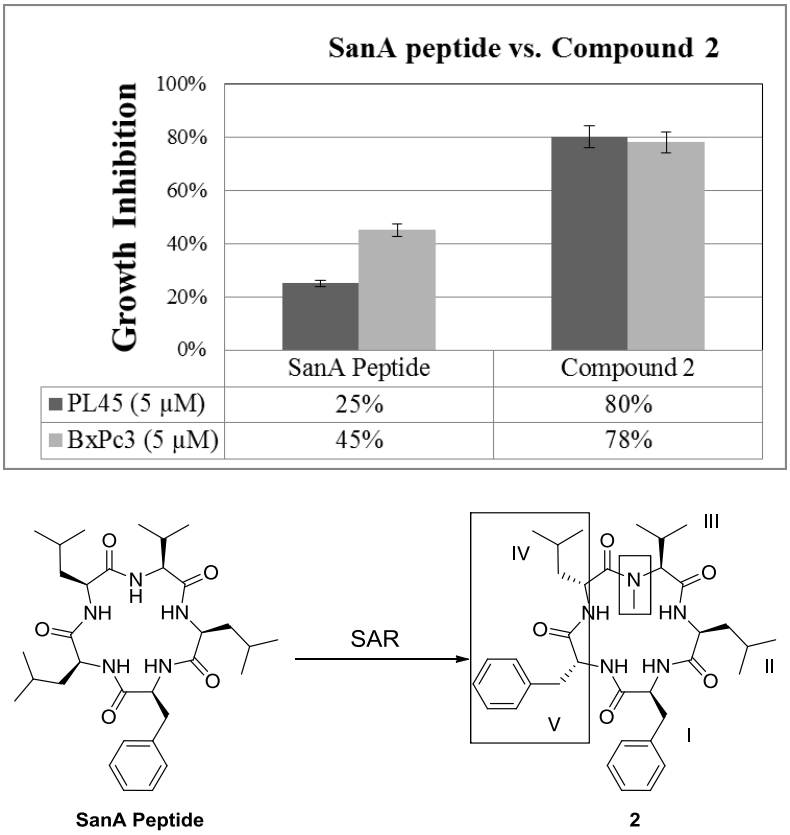


Figure 2.3 Cytotoxicity of SanA peptide and compound 2 in various cell lines. Margin of error is ±5%

Through SAR studies, it was revealed that modifications on position IV resulted in the least impact on cytotoxicity.[13] My colleagues then investigated SanA's mechanism of action using pull down assays with biotinylated SanA derivatives. Thus, position IV was chosen as the site to attach a biotin tag in order to optimize the binding affinity toward a potential target protein. Shown in **Figure 2.4**, Leu at Position IV of SanA peptide was exchanged to L-Lys. Lysine was then attached to PEG-biotin to complete the synthesis of biotinylated SanA peptide (compound **3**).

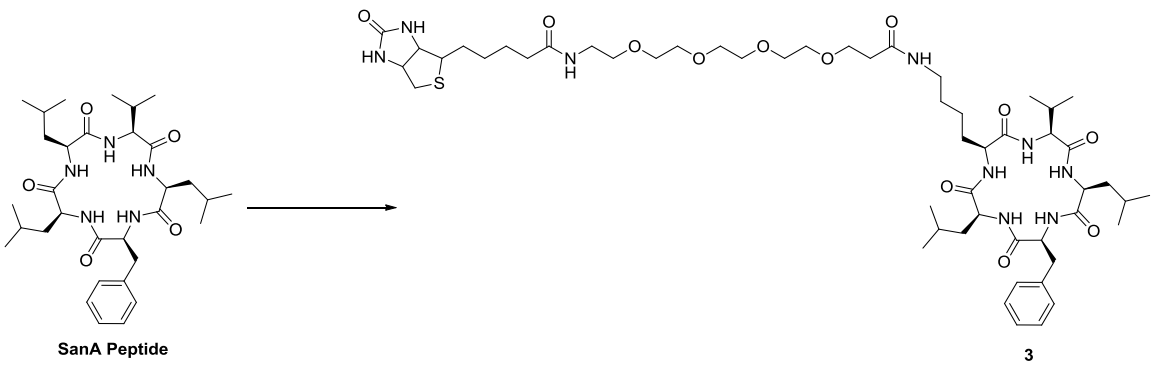
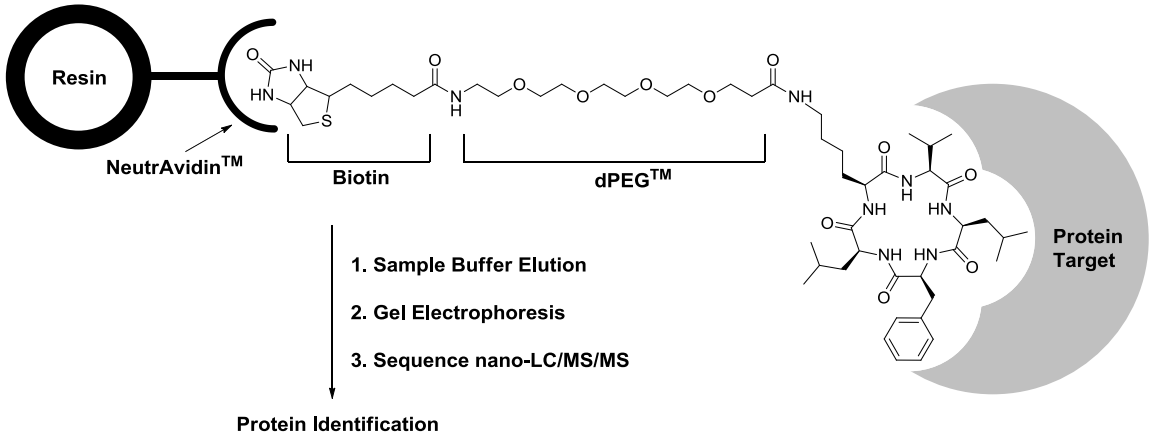


Figure 2.4 Structure of compound 3

To determine if SanA peptide's biological target was a protein, compound **3** was incubated with cell lysate. Immobilization of the SanA complex using NeutrAvidin® Agarose Resins occurred by biotin binding to the bead. Washing removed non-specific proteins that were sticking to the beads. The proteins bound specifically to SanA were purified via gel electrophoresis and identified by nano-LC/MS/MS (**Figure 2.5**, **(a)**). The result showed that compound **3** pulled down five proteins: Heat shock protein 90 (HSP90), keratin, α-tubulin, β-tubulin, and actin (**Figure 2.5**, **(b)**). HSP90 is a well-known oncogenic protein. Keratin, tubulin, and actin are usually pulled down with

HSP90 as they are associated with HSP90 in the cell.[13] A comparison of lane 2 and negative controls (lane 3 and 4) showed that the binding between HSP90 and compound **3** was specific and was not due to hydrophobic effects. Additional biochemical and cell based assays allowed us to prove that HSP90 was the protein target of SanA.[14]

(a)



(b)

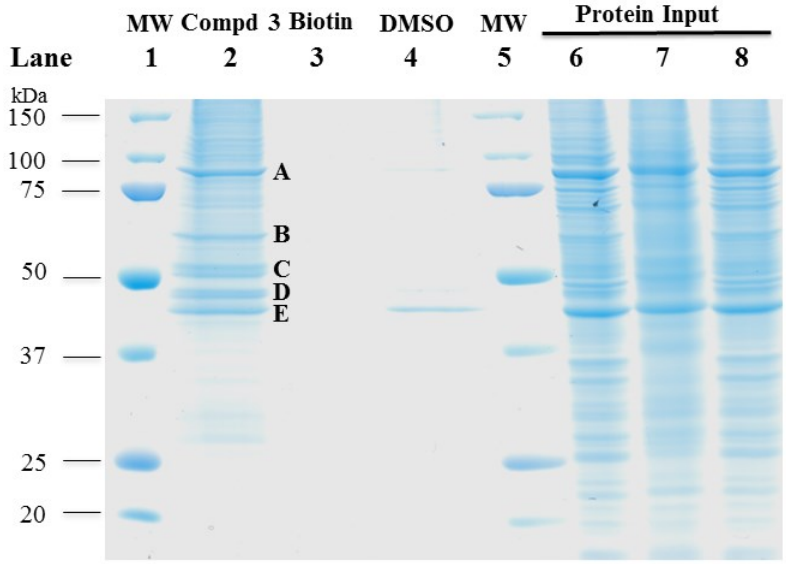


Figure 2.5 (a) Schematic diagram of pull-down assay (b) Bands isolated in the pull-down assay using HCT-116 colon cancer cell lysate. Lanes: 1 and 5, MW marker (kDa); 2, Compound 3; 3, negative control (PEG-biotin linker); 4, DMSO control; and 6-8, protein input for lanes 2-4, respectively. Pulled down protein A: HSP90, B: Keratin, C: α-tubulin, D: β-tubulin, E: actin.

Pull down assays were then run using potent SanA derivatives: compounds **1** and **2**, in order to determine if their target protein was HSP90. I contributed to this project by synthesizing compounds **4**, **5**, and **6** (**Figure 2.6**). Compounds **4** and **5** were derived from the incorporation of a biotin tag on **1** at positions IV and V, respectively. Moving the biotin tag from IV to V allowed us to determine the optimal tag position that minimizes the disruption of the SanA-protein interaction. A lysine residue was incorporated into compound **2** at position IV to produce compound **6**. Unlike compound **1**, we chose not to place a tag at V on compound **2** because we would have to replace the Phe, and Phe is important to **2**'s activity.

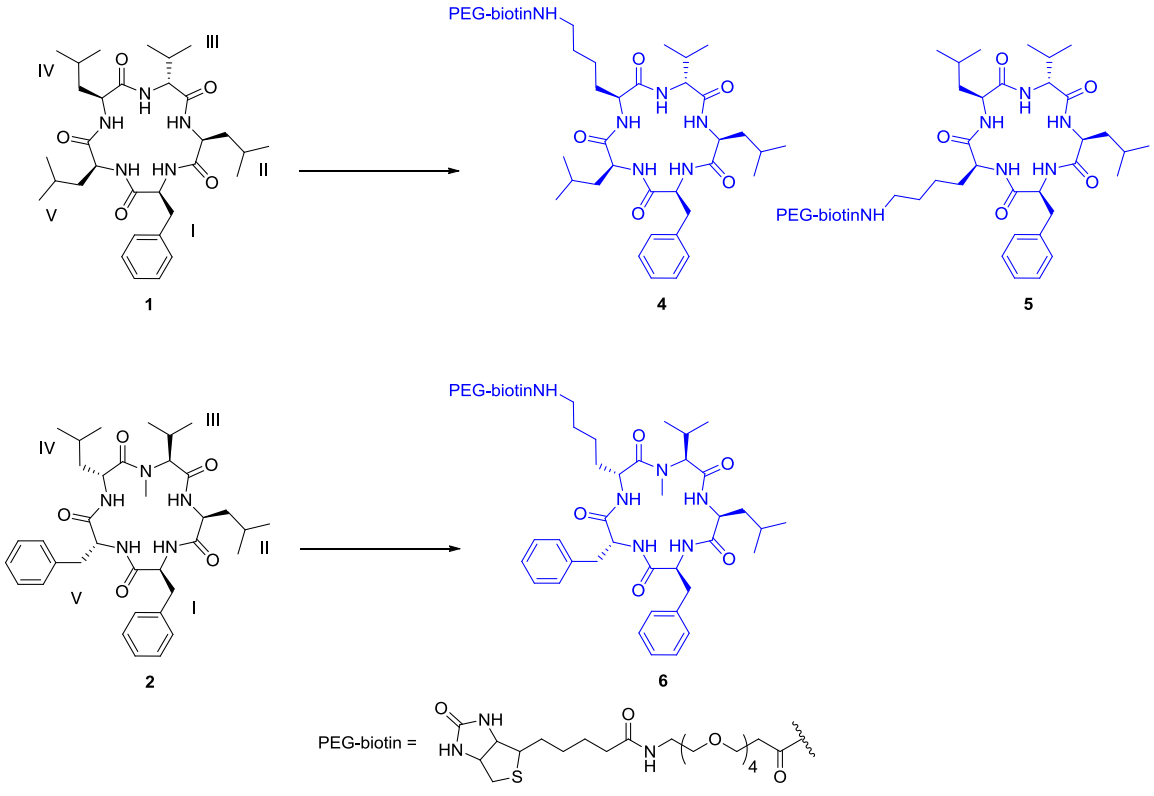


Figure 2.6 Structure of biotinylated SanA derivatives

In addition to the investigation of SanA's biological target, our lab designed a new generation of SanA derivatives as a part of our SAR study. Using computational modeling, we designed compounds **7** to **10** *de novo* based on the lead derivative compound **2** (**Figure 2.7**). A benzoxyl group was incorporated on the β-carbon of the Phe at position I. Modification of the stereochemistry at the α- and β-carbon of the hydroxyl phenylalanine produced four SanA derivatives. I was responsible for synthesizing two of the SanA derivatives, compounds **9** and **10** (shown in blue).

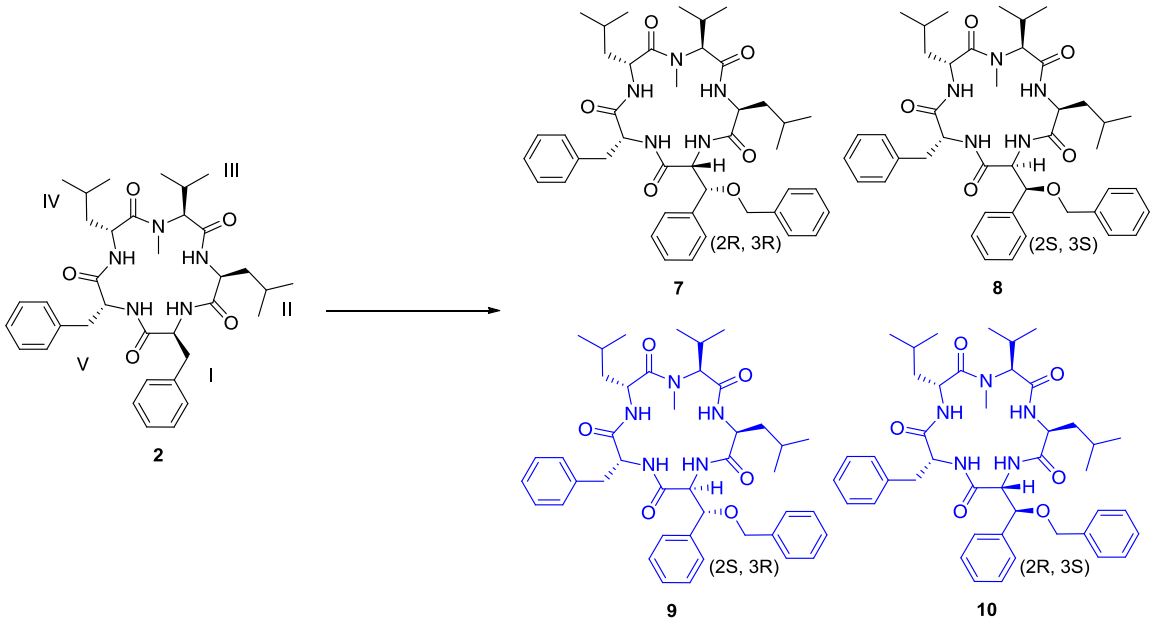


Figure 2.7 Structure of *de novo* designed SanA derivatives

2.3 Retrosynthetic approach of Sansalvamide A derivatives

The retrosynthetic approach of SanA derivatives is summarized in **Figure 2.8**. The SanA macrocycle was furnished from a double-deprotected linear precursor using a head-to-tail peptide coupling. The linear precursor was generated via either solution

phase or solid phase peptide synthesis. Both solution and solid phase syntheses employed a step-wise linear approach to build the pentapeptide. When the solution phase synthetic strategy was employed, the linear precursor was synthesized starting from a C-protected amino acid. Amino acids were sequentially coupled to generate the dipeptide, tripeptide, tetrapeptide and, finally, linear pentapeptide. In solid phase peptide synthesis (SPPS), the initial amino acid was a free amine and it was bound via its acid to the resin. Repeatedly coupling from the single amino acid bound resin furnished the pentapeptide. The final linear precursor was obtained by cleaving the pentapeptide from the resin.

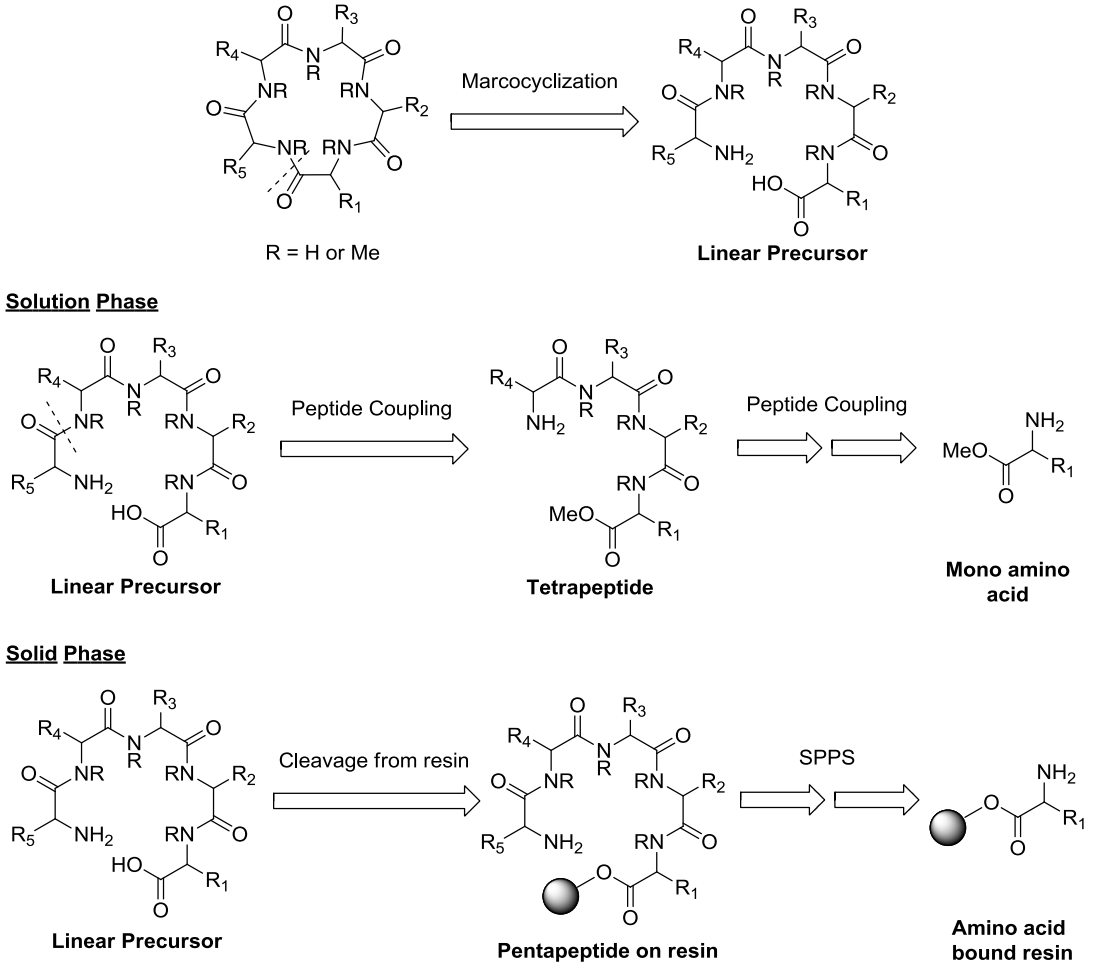


Figure 2.8 Retrosynthetic approach of Sansalvamide A macrocycle

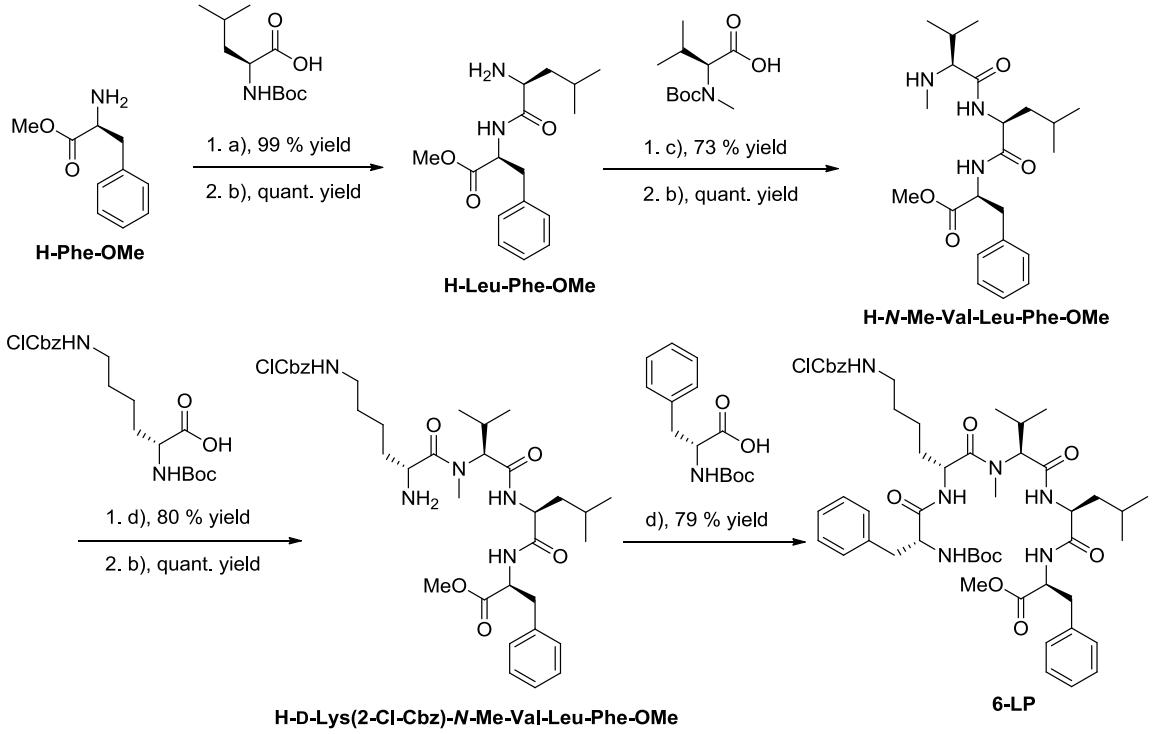
2.4 Synthesis of Sansalvamide A derivatives

2.4.1 Synthesis of linear precursor via solution phase

2.4.1.1 Synthesis of protected linear pentapeptide

I synthesized the linear pentapeptide for **6** using solution phase (**Scheme 2.1**, **6-LP**). Utilizing free acid Boc-Leu-OH (1.0 equivalent), free amine H-Phe-OMe (1.1 equivalents), coupling reagent 2(1-H-benzotriazole-1-yl)-1,1,3 tetramethyluronium tetrafluoroborate (TBTU, 1.2 equivalents), and base diisopropylethylamine (DIPEA, 4.0 equivalents), I synthesized the dipeptide H-Phe-Leu-OMe via coupling reaction. Dissolving the reagents in anhydrous methylene chloride (DCM, 0.1M) under argon, the reaction was monitored via thin-layer chromatography (TLC). Upon completion (typically 60-90 min), the reaction mixture was diluted with DCM and washed with aqueous HCl (10% v/v) to remove base, followed by a basic pH wash using saturated aqueous sodium bicarbonate ($NaHCO_3$) to remove excess coupling agent. The organic layer was collected, dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated *in vacuo*. The crude product was purified by a flash column chromatography using silica gel as stationary phase and ethyl acetate/hexane as a solvent system for mobile phase. Pure dipeptide Boc-Leu-Phe-OMe was afforded in 99% yield. The purity and structure the dipeptide were confirmed by ^1H NMR spectroscopy. Deprotection of the amine deprotection on the dipeptide was accomplished using trifluoroacetic acid (TFA) and DCM (TFA: DCM = 1:4, 0.1 M). Anisole (2 equivalents) was added to the reaction. The reaction was run at room temperature and monitored by TLC. Upon completion, the solution was concentrated *in vacuo* to yield the free amine

dipeptide (H-Leu-Phe-OMe) in quantitative yield. The free amine was subjected to the next coupling reaction without purification.



Conditions: a) TBTU (1.2 eq.), DIPEA (4.0 eq.), DCM (0.1 M); b) anisole (2.0 eq.), TFA:DCM (1:4, 0.1 M); c) HATU (1.2 eq.), DIPEA (4.0 eq.), DCM (0.1 M); d) TBTU and HATU (overall 1.2 eq.), DIPEA (7.0 eq.), DCM (0.1 M)

Scheme 2.1 Synthesis of linear pentapeptide for compound 6

Synthesizing the tripeptide Boc-*N*-Me-Val-Leu-Phe-OMe was accomplished using free acid Boc-*N*-Me-Val-OH (1.0 equivalent), free amine H-Leu-Phe-OMe (1.1 equivalents), coupling reagent 2-(H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 1.2 equivalents), and base DIPEA (4.0 equivalents) in anhydrous DCM (0.1 M). Monitoring the reaction by TLC showed it was complete in 1 h, whereupon it was subjected to an acid-base extraction using 10% (v/v) $HCl_{(aq.)}$ and then saturated $NaHCO_{3(aq)}$. The organic layer was collected, dried, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography to yield the desired

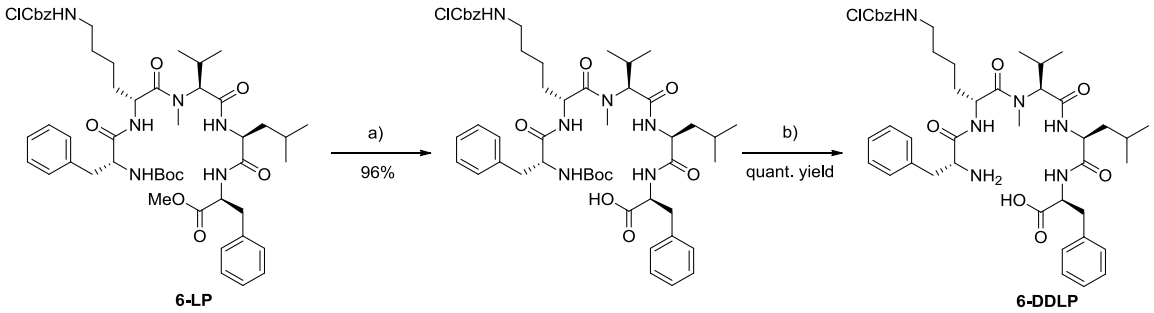
tripeptide Boc-*N*-Me-Val-Leu-Phe-OMe in 73% yield. The structure and purity of tripeptide were confirmed via ^{1}H NMR spectroscopy. The Boc protecting group of the tripeptide was removed using a mixture of trifluoroacetic acid (TFA) and DCM (TFA:DCM = 1:4, 0.1 M) and anisole (2.0 equivalents). The reaction was monitored by TLC every 15 min. Upon completion, the reaction was concentrated *in vacuo* to remove excess of TFA producing the free amine H-*N*-Me-Val-Leu-Phe-OMe in quantitative yield.

The tripeptide free amine (1.1 equivalents) was then coupled with Boc-D-Lys(2-Cl-Cbz)-OH (1.0 equivalent) using TBTU (0.4 equivalent), HATU (0.8 equivalent), DIPEA (7.0 equivalents) in anhydrous DCM (0.1 M) to generate the tetratpeptide Boc-D-Lys(2-Cl-Cbz)-*N*-Me-Val-Leu-Phe-OMe in 80% yield. Removal of Boc protecting group from the tetrapeptide using TFA/DCM mixture (1:4, 0.1 M) and anisole (2.0 equivalents) afforded the free amine tetrapeptide. Finally, coupling the free amine (1.1 equivalents) with Boc-D-Phe-OH (1.0 equivalent) utilizing TBTU (0.4 equvalent), HATU (0.8 equivalent), DIPEA (7 equivalents) in anhydrous DCM (0.1 M) furnished the desired pentapeptide (Boc-D-Phe-D-Lys(2-Cl-Cbz)-*N*-Me-Val-Leu-Phe-OMe, **6-LP**) in 79% yield via a flash column chromatography.

2.4.1.2 Synthesis of double deprotected linear precursor

A step-wise acid and amine deprotection were performed on **6-LP** to generate **6-DDLP**, the double deprotected linear peptide, which was then ready for the macrocyclization step. Using lithium hydroxide (LiOH, 8.0 equvalents) in MeOH (0.1 M), acid-deprotection of **6-LP** was run at room temperature for 12 h and monitored by

LC/MS (**Scheme 2.2**). Upon completion, the reaction was purified by an acid wash to furnish the free acid linear pentapeptide (Boc-D-Phe-D-Lys(2-Cl-Cbz)-*N*-Me-Val-Leu-Phe-OH) in 96% yield. The Boc group on the free acid linear pentapeptide was then removed using a mixture of TFA and DCM (1:4, 0.1 M) and anisole (2.0 equvalents). Reaction completion was monitored by TLC and LC/MS, and upon completion, the solution was concentrated *in vacuo* to give the double deprotected linear pentapeptide for **6** (**6-DDLP**) in quantitative yield.



Conditions: a) LiOH (8.0 eq), MeOH (0.1 M); b) anisole (2.0 eq.), TFA:DCM (1:4, 0.1 M)

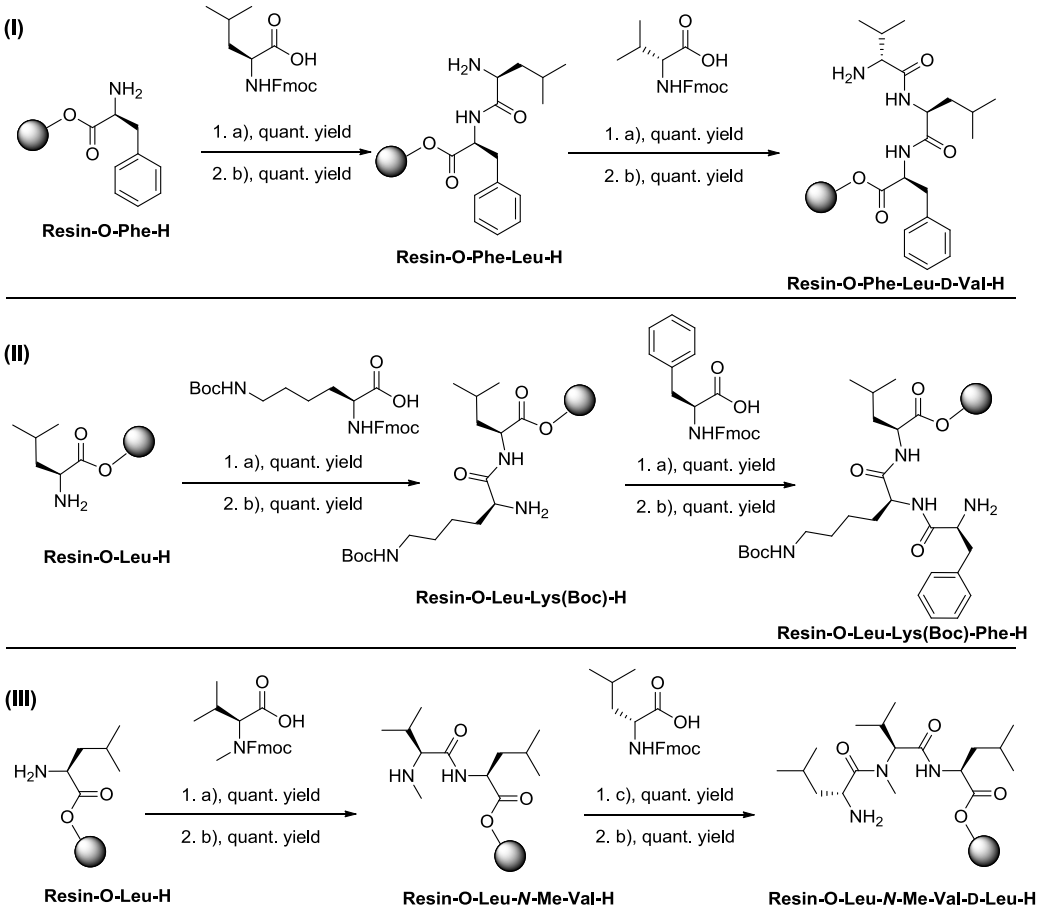
Scheme 2.2 Synthesis of double deprotected linear precursor for compound 6

2.4.2 Synthesis of linear precursor via solid phase

2.4.2.1 Synthesis of resin-bound linear pentapeptides

Generating the linear pentapeptide for **4**, **5**, **9**, and **10** was rapidliy accomplished using solid phase peptide synthesis (SPPS), and commercially available chlorotrityl chloride (CTC) resin. The carboxyl terminus of amino acid was covalently attached to CTC resin while its free amino terminus was coupled with the appropriate amino acid. Synthesis of the dipeptide for **4** utilized pre-loaded Phe resin (1.0 equivalent), Fmoc-Leu-OH (3.0 equivalents), coupling reagent 1-hydroxybenzotriazole (HOBt, 3.0 equivalents)

and diisopropylcarbodiimide (DIC, 6.0 equivalents) (**Scheme 2.3**). The pre-loaded Phe-resin was first placed in a fritted polypropylene cartridge, swollen using N, N-dimethylformamide (DMF) for 30 min, and then drained using a vacuumed vessel. Fmoc-Leu-OH and HOBt were dissolved in DMF to a concentration of 0.2 M. The DMF solution was added to the cartridge containing the swollen resin, and was followed by DIC, which initiated the coupling reaction. The suspension in the cartridge was mixed and was then allowed to shake for 2 h on an automatic shaker. The reaction suspension was drained to give the Fmoc protected dipeptide bound resin (Resin-O-Phe-Leu-Fmoc). To confirm the reaction completion, a ninhydrin test was employed. The ninhydrin solution stays yellow in the presence of a protected amine and changes from yellow to blue in the presence of a free amine. A yellow result confirmed the completed formation of Resin-O-Phe-Leu-Fmoc. The Fmoc protecting group on dipeptide Resin-O-Phe-Leu-Fmoc was removed using 20% piperidine in DMF via the following steps: DMF wash (3 x 1 min), 20% piperidine in DMF wash (1 x 5 min and 1 x 10min), DMF wash (2 x 1 min), isopropanol (IPA) wash (1 x 1 min), DMF wash (1 x 1 min), IPA wash (1 x 1 wash), and DMF wash (3 x 1 min). A ninhydrin test gave a blue color, confirming the removal of the Fmoc group.



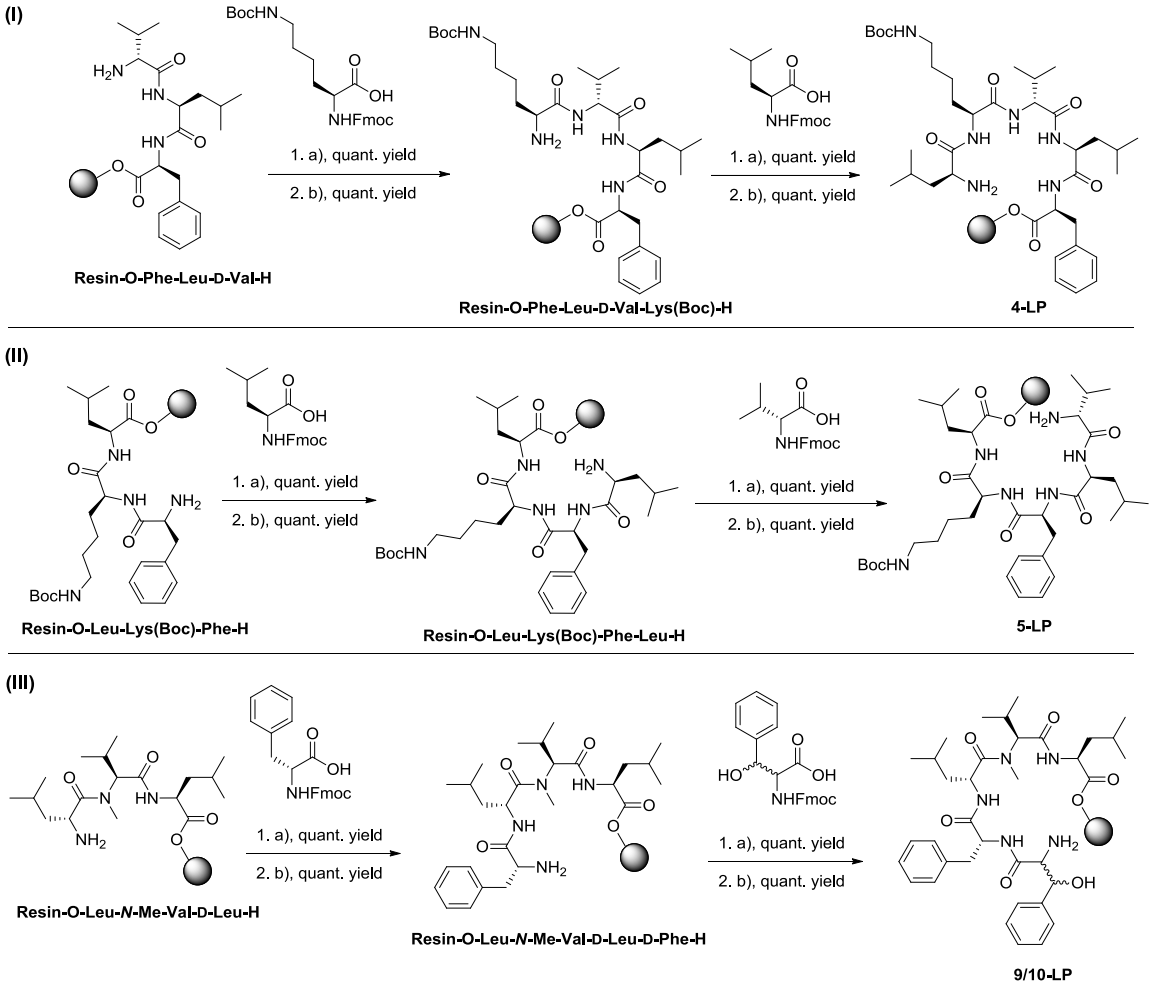
Conditions: a) HOBt (3.0 eq.), DIC (6.0 eq.), DMF (0.2 M); b) 20% piperidine/DMF; c) HOAt (3.0 eq.), DIC (6.0 eq.), DMF (0.2 M).

Scheme 2.3 Synthesis of resin-bound tripeptides. (I) for 4; (II) for 5; (III) for 9 and 10

The resulting free amine dipeptide Resin-O-Phe-Leu-H underwent the next coupling reaction to furnish the Fmoc-protected tripeptide Resin-O-Phe-Leu-D-Val-Fmoc, using Fmoc-D-Val-OH (3.0 equivalents), coupling reagent 1-hydroxybenzotriazole (HOBt, 3.0 equivalents) and diisopropylcarbodiimide (DIC, 6.0 equivalents). The Fmoc group on the tripeptide was removed using 20% piperidine in DMF and the resulting free amine Resin-O-Phe-Leu-D-Val-H was coupled to the next amino acid. The same coupling and deprotecting procedures were employed to synthesize free amine tripeptides for **5**

(**Scheme 2.3, II**), **9** and **10** (**Scheme 2.3, III**). Due to the steric hindrance of the secondary amino terminus, the coupling of Resin-O-Leu-*N*-Me-Val-H and Fmoc-D-Leu-OH (for compound **9/10**) utilized the more reactive coupling reagent 1-hydroxy-7-azabenzotriazole (HOAt) instead of HOBt. The coupling reaction was allowed to run overnight, followed by Fmoc removal using 20% piperidine to furnish the free amine tripeptide Resin-O-Leu-*N*-Me-Val-D-Leu-H (**Scheme 2.3, III**).

The free amine tripeptide Resin-O-Phe-Leu-D-Val-H was continued to couple with Fmoc-Lys(Boc)-OH (3.0 equivalents) using HOBt (3.0 equivalents) and DIC (6.0 equivalents) in DMF (0.2 M) to yield the protected tetra peptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-Fmoc (**Scheme 2.4, I**), which was subjected to the piperidine deprotection procedure. The protected pentapeptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-Leu-Fmoc was afforded via the coupling of the free amine tetrapeptide with Fmoc-Leu-OH (3.0 equivalents) using HOBt (3.0 equivalents) and DIC (6.0 equivalents) in DMF (0.2 M). Finally, an amine deprotection on the protected pentapeptide using 20% piperidine prepared the free amine pentapeptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-Leu-H (**4-LP**). The same procedure was utilized to generate the free amine pentapeptides. Fmoc-Leu-OH and Fmoc-D-Val-OH were used for the synthesis of Resin-O-Leu-Lys(Boc)-Phe-Leu-D-Val-H (**Scheme 2.4**, **II**, **5-LP**) while Fmoc-D-Phe-OH and (2S, 3R)/ (2R, 3S)-racemic-Fmoc-β-hydroxy-Phe-OH were incorporated for the synthesis of Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S, 3R)/(2R, 3S)-racemic-β-OH-Phe-H (**Scheme 2.4**, **III**, **9/10-LP**).

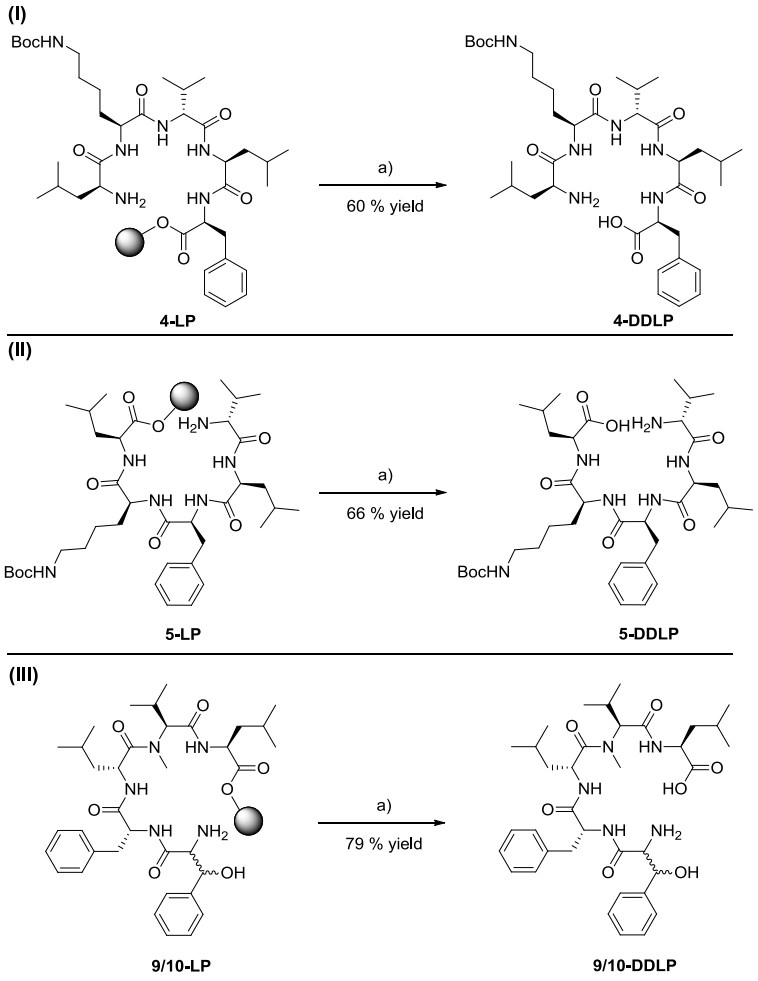


Conditions: a) HOBt (3.0 eq.), DIC (6.0 eq.), DMF (0.2 M); b) 20% piperidine/DMF.
Scheme 2.4 Synthesis of resin-bound pentapeptides. (I) for 4; (II) for 5; (III) for 9 and 10.

2.4.2.2 Cleavage of linear pentapeptides from resin

The free amine pentapeptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-Leu-H (**4-LP**) underwent a cleaving process using a mixture of 2,2,2-trifluoroethanol (TFE) in DCM (TFE:DCM = 1:1, v/v) (**Scheme 2.5**). The peptide-bound resin was stirred in the mixture for 24 h, followed by a filtration using a Buchner funnel to separate the resin and the cleaved pentapeptide solution. The solution was then concentrated *in vacuo* to furnish the

desired double deprotected pentapeptide HO-Phe-Leu-D-Val-Lys(Boc)-Leu-H (**4-DDLP**, 60% yield) ready for cyclization. The same procedure was utilized to afford **5-DDLP** and **9/10-DDLP** in 66% and 79% yield, respectively.



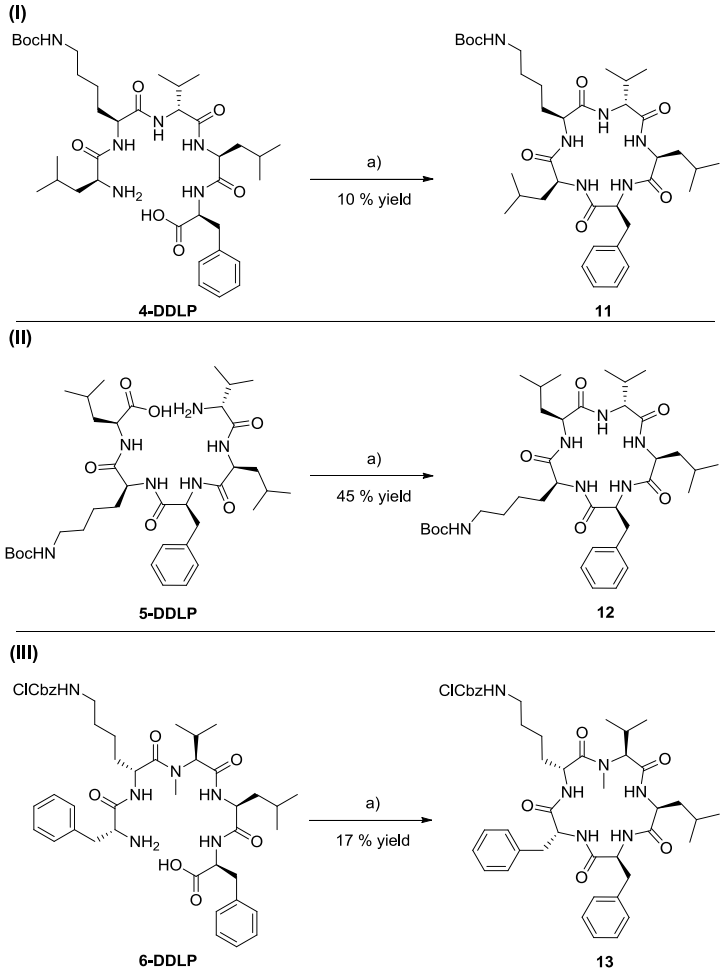
Conditions: a) 50%TFE in DCM (1:1, v/v).

Scheme 2.5 Cleavage of linear pentapeptides from resin. (I) for 4; (II) for 5; (III) for 9 and 10.

2.4.3 Macrocyclization of Sansalvamide A derivatives

2.4.3.1 *In situ* Macrocyclization

Macrocyclization of **4-DDLP**, **5-DDLP** and **6-DDLP** is summarized in **Scheme 2.6**. Cyclizing the **DDLP**s using a combination of coupling reagents under dilute conditions generated their respective macrocyclic compounds (**Scheme 2.6**, **11**, **12**, and **13**). Compound **11** was synthesized from **4-DDLP** (1.0 equivalent) using coupling reagents TBTU (0.7 equivalent), HATU (0.7 equivalent), and 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4-(3H)-one (DEPBT, 0.7 equivalent) with DIPEA (8.0 equivalents) in a mixture of anhydrous DCM and ACN (1:1, 0.007 M). The reaction was monitored by TLC and LC/MS for approximately 4 h, whereupon it was complete. The reaction mixture was then subjected to an acid-base wash to give the crude product and was purified via a flash column chromatography to afford pure **11** (cyclo-Phe-Leu-D-Val-Lys(Boc)-Leu) in 10% yield. The structure and purity of **11** was confirmed by LC/MS and ^{1}H NMR. The same procedure was employed to generate **12** (cyclo-Phe-Leu-D-Val-Leu-Lys(Boc)) in 45% yield and **13** (cyclo-Phe-Leu-*N*-Me-Val-D-Leu-D-Phe) in 17% yield.



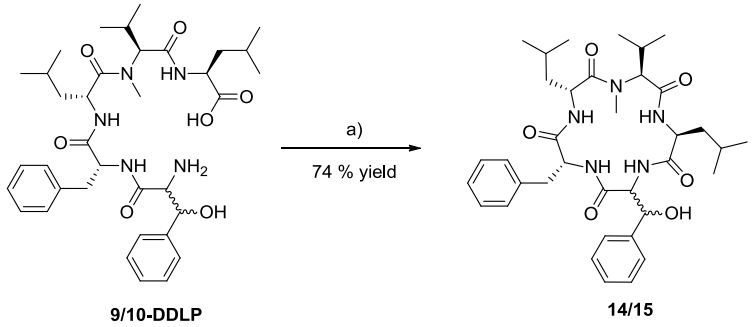
Condition: a) TBTU (0.7 eq.), HATU (0.7 eq.), DEPBT (0.7 eq.), DIPEA (8.0 eq.), DCM:ACN (1:1, 0.007 M).

Scheme 2.6 *In situ* Macrocyclization of SanA derivatives

2.4.3.2 Macrocyclization using the syringe pump

Low yields from cyclizing 4, 5, and 6 were from the formation of dimerized or oligomerized **DDLP**s.[15] Utilizing a syringe pump decreased the formation of unwanted dimer or oligomer during the cyclization of **9**/**10-DDLP** (0.0007 M). Shown in **Scheme 2.7**, the macrocyclization of **9/10-DDLP** was performed with coupling reagents TBTU, HATU, and DEPBT (0.7 equivalent each), base DIPEA (8.0 equivalents) in anhydrous

DCM (0.0007 M) utilizing a syringe pump. First, the coupling reagents were added to an argon-purged round bottom flask, followed by the addition of 75% of the total volume of anhydrous DCM required for an overall 0.0007 M concentration. DIPEA was then added into the bulk DCM solution. Next, **9/10-DDLP** was dissolved in the remaining amount of DCM and placed into a syringe. The **DDLP** solution was added drop-wise, over 4 h, to the bulk DCM solution using the syringe pump. After the addition of all DDLP, the reaction was allowed to run overnight and was monitored via LC/MS. Upon completion, the reaction mixture was subjected to an acid-base wash. The resulting crude product was then purified via a flash column chromatography to furnish the racemic compound **14/15** (*cyclo*-(2S,3R)/(2R, 3S)-*racemic*-β-hydroxy-Phe-Leu-*N*-Me-Val-D-Leu-D-Phe) in 74% yield. The purity and structure of **14/15** was confirmed via LCMS and ^{1}H NMR spectroscopy.



Condition: a) TBTU (0.7 eq.), HATU (0.7 eq.), DEPBT (0.7 eq.), DIPEA (8.0 eq.), DCM (0.0007 M).

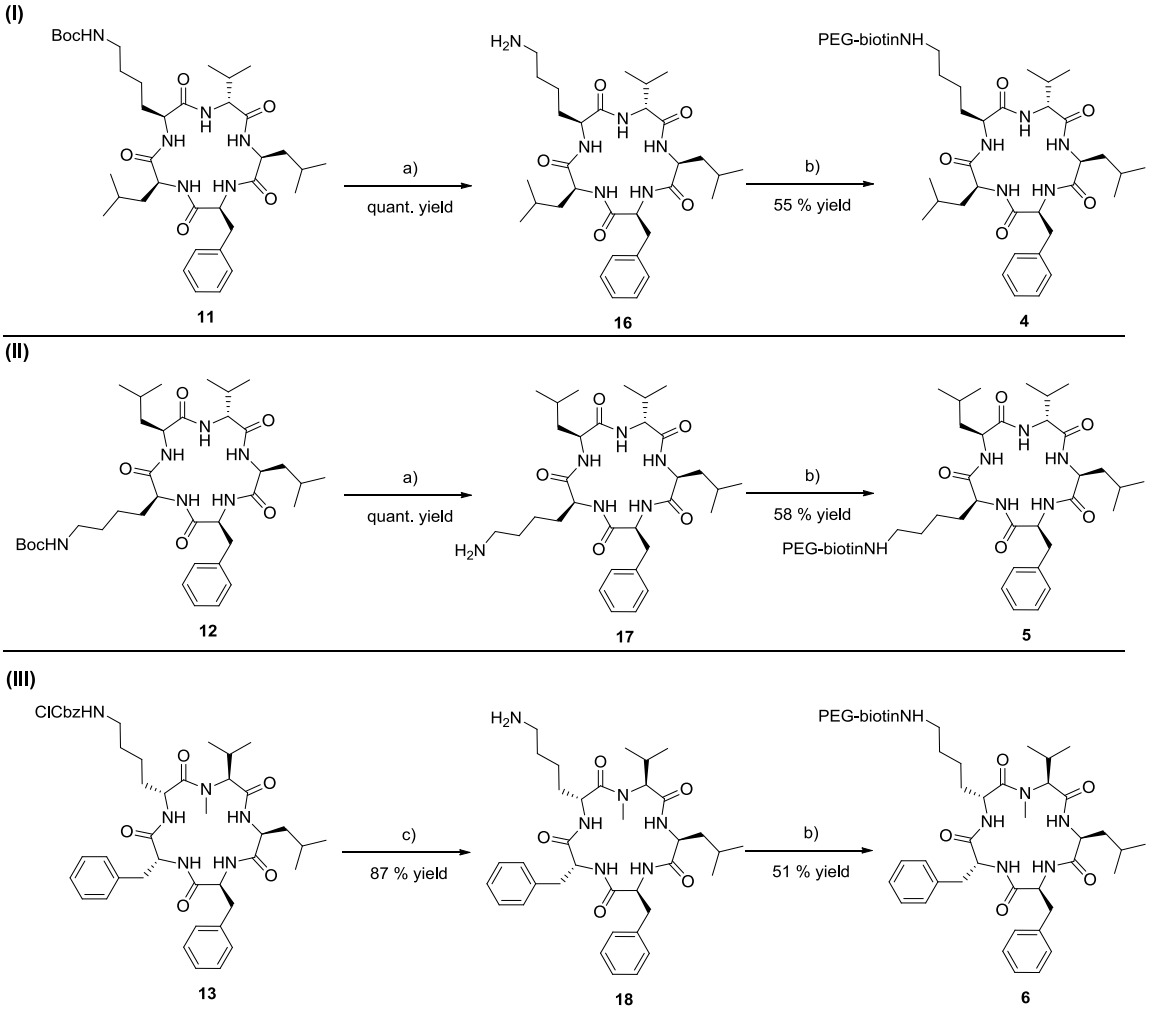
Scheme 2.7 Macrocyclization of SanA derivatives using synringe pump

2.4.4 Modification of cyclized Sansalvamide A derivatives

2.4.4.1 Synthesis of biotinylated Sansalvamide A derivatives

The Boc groups on Compound **11** and **12** were removed using a mixture of TFA/DCM (1:4, 0.1 M) and anisole (2.0 equivalents) to yield the free amines at the lysine residues of compound **16** and **17** in quantitative yield (**Scheme 2.8**, **I** and **II**). Biotinylation of compound **16** using *N*-hydroxysuccinimidyl-d-biotin-15-amino-4,7,10,13,-tetraoxapentadecylate (NHS-dPEG4-biotin, 1.4 equivalents), and base DIPEA (4.0 equivalents) in DCM (0.1 M) produced biotinylated compound **4** under argon (**Scheme 2.8, I**). Compound **16** and NHS-dPEG4-biotin were dissolved in DCM to a concentration of 0.1 M and DIPEA was then added drop-wise. The reaction was stirred for 1 h and monitored by LC/MS. Upon completion, the reaction mixture was diluted with DCM, followed by a neutral extraction using deionized water. The organic layer was collected, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting crude reaction was purified via RP-HPLC to yield the biotinylated compound **4** in 55% yield. The same procedure was applied on the synthesis of compound **5** with 58% yield (**Scheme 2.8**, **II**).

The amine deprotection of compound **13** was carried out via removal of the Cbz group using hydrogen bromide (HBr), generating the free lysine residue of **18** (**Scheme 2.8**, **III**). Compound **13** was dissolved in HBr (33% in glacial acetic acid, w/w) to a 0.1 M concentration. The reaction was run for 2 h and monitored via LCMS every 30 min. Upon completion, the reaction mixture was purified via dimethyl ether extraction and cotton filtration to furnish compound **18** in 87% yield. Finally, the biotinylation of **18** generated compound **6** in 51% yield (**Scheme 2.8**, **III**).

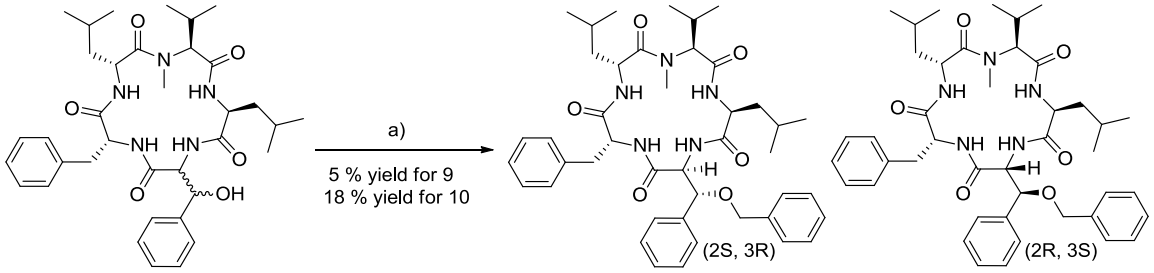


Condition: a) anisole (2.0 eq.), TFA:DCM (1:4, 0.1 M); b) NHS-dPEG4-biotin (1.2 eq.), DIPEA (8.0 eq.), DCM (0.1 M); c) 33% HBr in glacial acetic acid (0.1 M)
Scheme 2.8 Synthesis of biotinylated SanA derivatives

2.4.4.2 Synthesis of benzylated Sansalvamide A derivatives

Shown in **Scheme 2.9**, benzylation of the racemic compound **14/15** was performed utilizing sodium hydride (NaH, 1.1 equivalents), and benzyl bromide (BnBr, 2.0 equivalents) in a mixture of THF/DMF (1:1, 0.1 M). The starting material containing the two diastereomers and NaH were dissolved in THF/DMF to a concentration of 0.1 M. After 10 min, BnBr was added drop-wise to the reaction mixture. Monitoring the reaction

via LC/MS indicated it was complete in 5 h. Upon completion, the reaction mixture was diluted with DCM and extracted with deionized water to remove excess reagents. The organic layer was collected, dried and concentrated *in vacuo*. The crude product was purified to give the diastereomers **9/10**, which were purified and separated via RP-HPLC to furnish compound **9** ((2S, 3R)-β-benzoxy-Leu-*N*-Me-Val-D-Leu-D-Phe, 5% yield) and **10** ((2R, 3S)-β-benzoxy-Leu-*N*-Me-Val-D-Leu-D-Phe, 18% yield). The structures of the two diastereomers were verified via ROESY and ^{1}H NMR, while the purity was verified by ^{1}H NMR and LCMS.



14/15 **9** **10**

Condition: a) 60% NaH (1.1 eq.), BnBr (2.0 eq.), THF:DMF (1:1, 0.1 M)
Scheme 2.9 Synthesis of benzylated SanA derivatives

2.5 Biological assay of Sansalvamide A derivatives

2.5.1 Pull down assay of biotinylated Sansalvamide A derivatives

The protein target of biotinylated SanA derivatives (compounds **4**, **5** and **6**) that I synthesized were evaluated utilizing pull down assays. These assays were completed by my colleague Worawan Tantisantisom. Briefly, the biotinylated compound was incubated with cell lysate of human colon cancer cell line HCT-116, followed by the addition of NeutrAvadin-bound beads to immobilize the SanA-target protein(s) complex. The beads were washed several times to remove non-specifically bound proteins. The target protein(s) was eluted from the beads by sample buffer, purified via a gel electrophoresis and finally, visualized using a Coomassie blue stain.

Compounds **4** and **5**, both derived from the lead compound **1**, contain a biotin tag at different positions (position IV for **4** and position V for **5**) in order to evaluate the optimal position for biotin tagging. As shown in **Figure 2.9**, both compound **4** and **5** pulled down several protein bands including HSP90. Comparison of the HSP90 band in lane 2 to the negative control (lane 3, biotin linker only; lane 4, isopropanol only) indicated the interaction between HSP90 and compound **4** and **5** is specific.

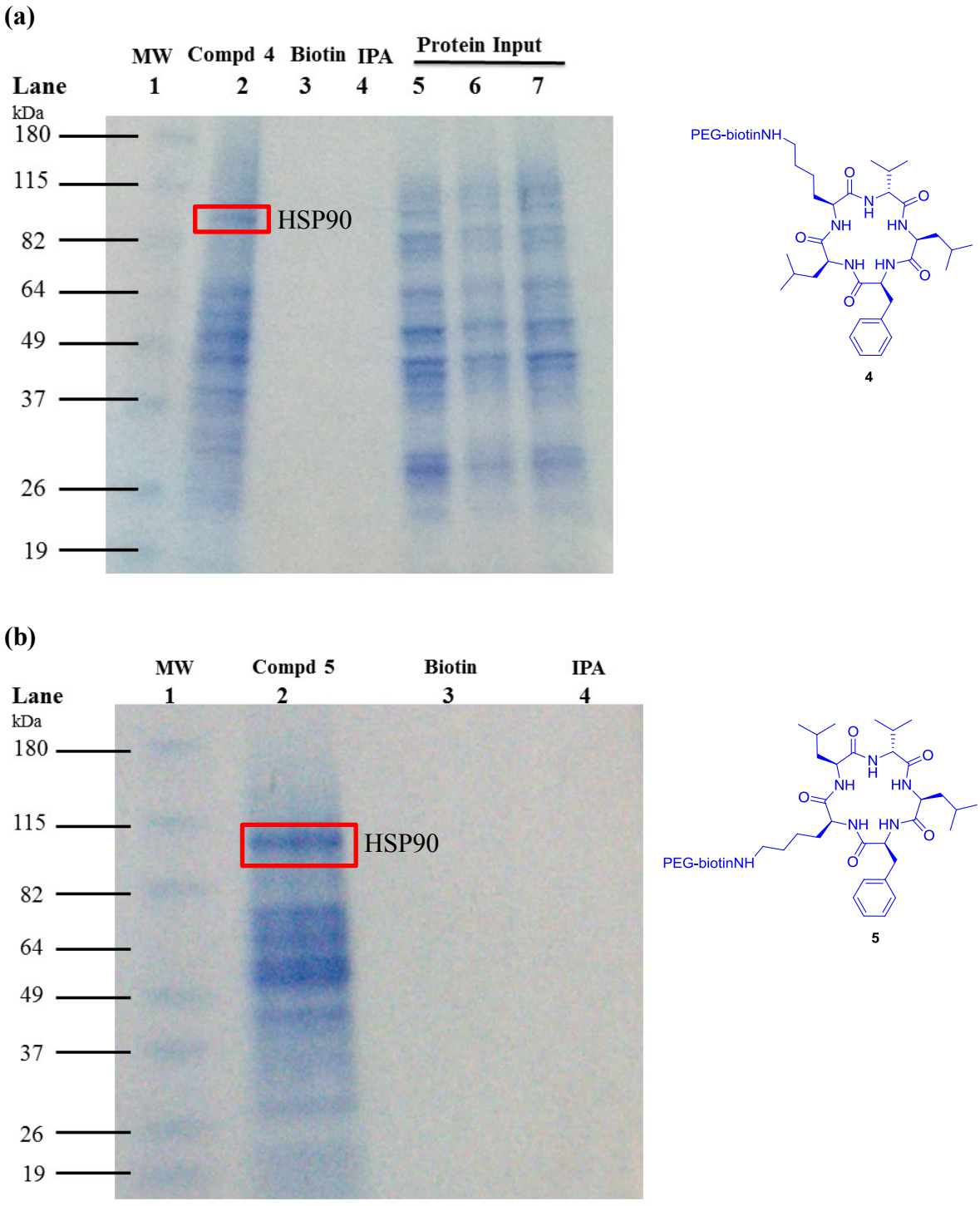


Figure 2.9 Bands isolated in the pull-down assay using HCT-116 colon cancer cell lysate. (a)Lanes: 1, MW marker (kDa); 2, Compound 4; 3, negative control (PEG-biotin linker); 4, IPA control; and 5-7, protein input for lanes 2-4, respectively. (b)Lanes: 1, MW marker (kDa); 2, Compound 5; 3, negative control (PEG-biotin linker); 4, IPA control. Pulled down HSP90 is boxed.

Compound **6**, the biotinlyated derivative of the lead compound **2**, was also studied via pull down assay. As shown in **Figure 2.10**, a protein band between 90-95 kDa indicated that compound **6** also pulled down HSP90. The comparison between lane 2 to negative controls (lanes 3 and 4) indicated that the interaction between HSP90 and compound **6** is specific.

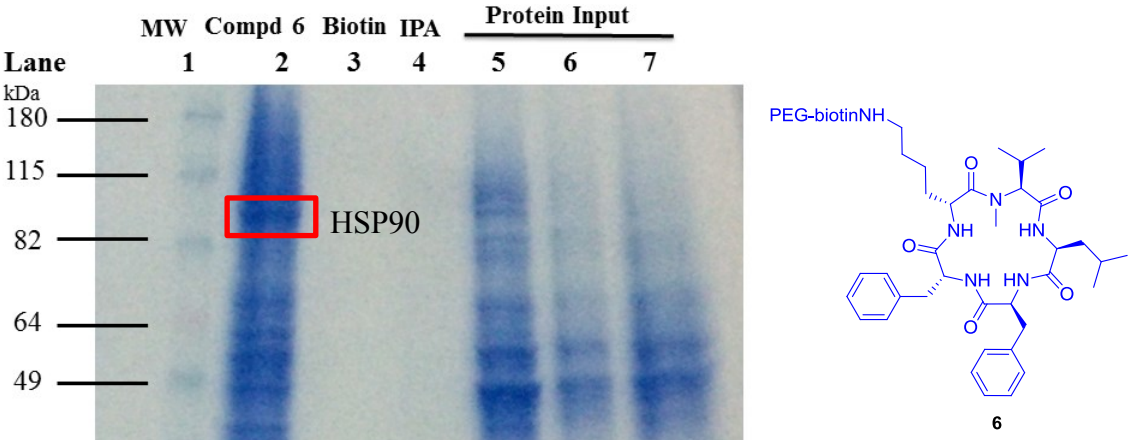


Figure 2.10 Bands isolated in the pull-down assay using HCT-116 colon cancer cell lysate. Lanes: 1, MW marker (kDa); 2, Compound 6; 3, negative control (PEG-biotin linker); 4, IPA control; and 5-7, protein input for lanes 2-4, respectively. Pulled down HSP90 is boxed.

2.5.2 Cytotoxicity assay of benzylated Sansalvamide A derivatives

The cytotoxicity of SanA derivatives was tested via ^{3}H-labeled thymidine uptake assays, which were completed by my colleague Leslie Alexander. Carcinogenic cells were cultured in 96 well plates for overnight incubation, followed by the addition of SanA derivatives (10 μM in DMSO) or DMSO control. After incubation for 54 h, 1 μCi of ^{3}H-labeled thymidine was added to each well, where the added ^{3}H-labeled thymidine was incorporated into the DNA of live cells. The cells were harvested after an additional 18 h of incubation, at which time the amount of incorporated ^{3}H-labeled thymidine was quantified via a scintillation counter. The amount of ^{3}H-labeled thymidine incorporation is proportional to the amount of cell proliferation. Low levels of ^{3}H-labeled thymidine incorporation, as compared to the DMSO control, indicate lower cell proliferation and greater compound cytotoxicity. On the contrary, higher amounts of thymidine incorporation demonstrated that the treated cells are continuing to go through cell cycle and the compound exhibits little cytotoxicity. All experiments were completed at least three times.

Compounds **7**-**10**, as well as the parent compounds SanA peptide and compound **2**, were tested for their cytotoxicity against the drug-resistant human colon cancer cell line HCT-116. As shown in **Figure 2.11**, SanA peptide only exhibited 35% growth inhibition while **2** showed a higher cytotoxicity (74%). The observation follows the trend for potent SanA derivatives: an *N*-methyl, two D-amino acids and multiple phenyl groups are essential for cytotoxicity. Compounds **7**-**10**, designed *de novo* based on **2**, contain an *N*-methyl, two consecutive D-amino acid, and three phenyl groups within their core structures. Three compounds (compounds **8**-**10**) demonstrated decreased potency with

limited effects on cell growth inhibition compared to the parent compound **2,** and only **7** exhibited improved potency over **2** (74% for **2** vs. 94% for **7**).

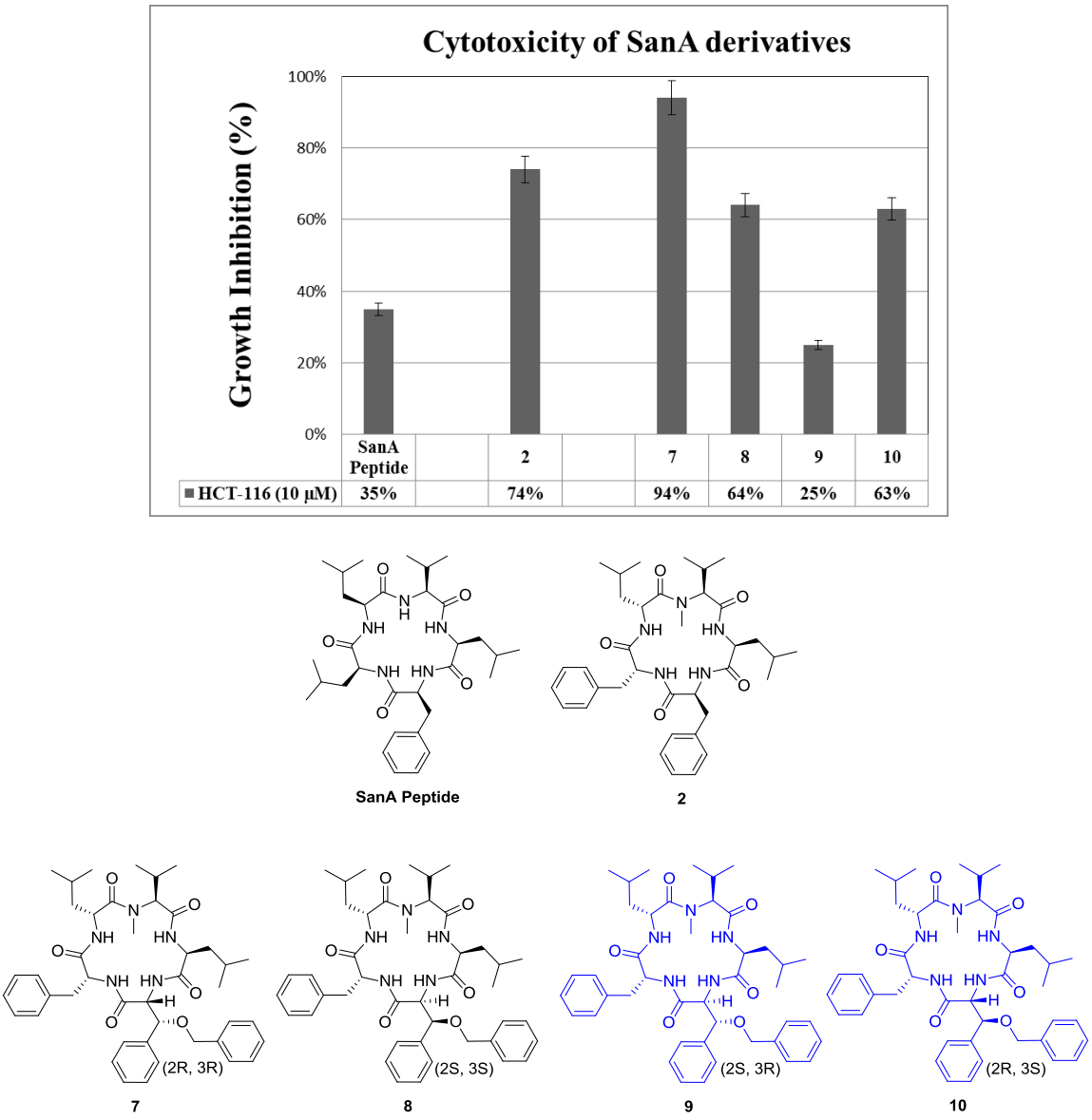
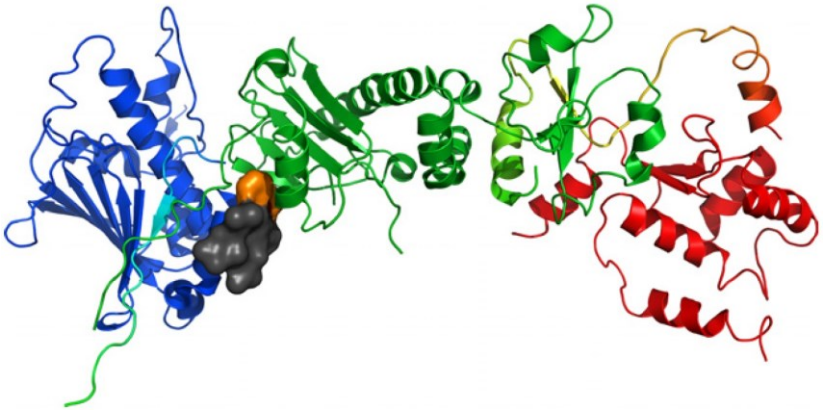


Figure 2.11 Cytotoxicity of SanA derivatives against colon cancer cell line HCT-116. Margin of error is ±5%

The only structural difference between these four compounds (**7-10**) is the configuration at the α- and β-carbon of the benzyl protected phenylserine at position I. Compound **7** contains a (*R, R*) configuration and exhibited high growth inhibition while the other three diastereomers contain the (*S, S*), (*S, R*), and (*R, S*) configurations and showed lower levels of growth inhibition compared to **7**. We reasoned that the growth inhibition values could be attributed the binding affinity between HSP90 and these series of SanA derivatives. Thus, a computer-based docking experiment was performed by my colleague Jeremiah Savage using the well-established program Autodock to predict the binding between HSP90 and compounds **7** and **8**. As shown in **Figure 2.12**, the compounds bind to HSP90 in different orientations. Compound **7** shows a conformation where the benzoxy Phe side chain is oriented coplanar to the macrocycle. This allows the side chain inserts into the binding pocket on HSP90. In contrast, the benzoxy Phe side chain of **8** is perpendicular to the macrocyle, which prevents its insertion into the binding pocket and results in a very different binding orientation. This observation suggests that, in this series of compounds, the 3-D shape greatly alters the binding orientation and is the primary reason for the variation in potency. The (*R, R*) configuration is oriented in a favorable 3-D structure for binding to HSP90 and explains its outstanding potency.

(a)



(b)

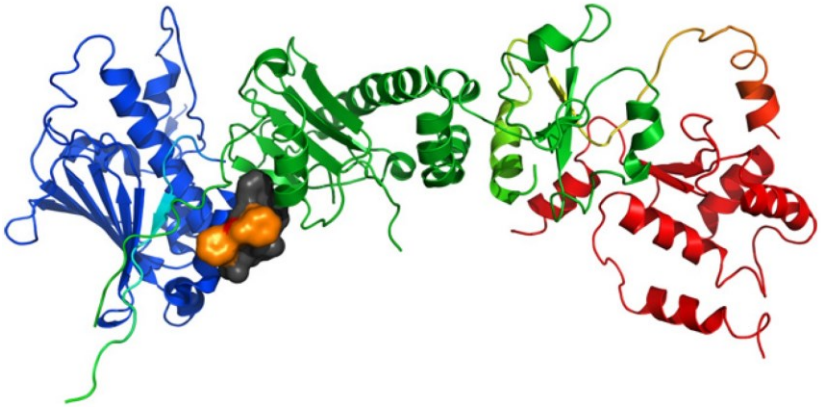


Figure 2.12 Predicted binding of HSP90 and SanA derivatives. (a) Compound 7 binds to HSP90. (b) Compound 8 binds to HSP90.
The benzoxy Phe side chain is colored in orange. Blue, green and red represent the N, middle and C terminal domains of HSP90, respectively.

2.6 Conclusion

Using both solution and solid phase peptide synthesis, I have successfully synthesized five SanA derivatives. The biotinylated SanA derivatives were furnished by attaching a PEG-biotin and their protein target was identified. Specifically compounds **4-6**, containing a PEG-biotin at position IV or V, were tested in pull down assays for the studies of their biological target(s). The results of pull down assays show that all biotinylated compounds pulled down HSP90, which supports our initial mechanism of

action studies. Furthermore, the pull down results of compound **5** indicated that position V could be the better position to place a biotin tag. Compounds **7**-**10**, all designed *de novo* from **2**, contain necessary moieties for potent SanA derivatives and all inhibited growth of HCT-116 cells, although **7** was the optimal structure. The SAR and computer docking results showed the impact of 3-D shape on binding to HSP90. Other supporting bioassays such as HSP90 competitive binding assays and HSP90 client protein binding assays are currently being completed by my colleagues. These assays are investigating the mechanism by which **7** inhibits HSP90. The results of these future studies will provide the McAlpine lab with greater insight for the development of novel anti-cancer agents.

Chapter 2, in part, is a reprint of the material as it appears in "Design and synthesis of Hsp90 inhibitors: Exploring the SAR of Sansalvamide A derivatives" *Bioorganic Medical Chemistry* **2010**, 18, 6822-6856 Sellers, R. P.; Alexander, L. D.; Johnson, V. A.; Lin, C. –C,; Savage, J.; Corral, R.; Moss, J.; Slugocki, T. S.; Singh, E. K.; Davis, M. R.; Ravula, S.; Spicer, J. E.; Oelrich, J. L.; Thornquist, A.; Pan, C. –M.; McAlpine, S. R. The dissertation author was the primary investigator and author of this paper.

2.7 Reference

1. Belofsky, G. N.; Jensen, P. R.; Fenical, W., Sansalvamide: A new cytotoxic cyclic depsipeptide produced by a marine fungus of the genus Fusarium. *Tetrahedron Letters* **1999,** *40* (15), 2913-2916.

2. Lee, Y.; Silverman, R. B., Rapid, High-Yield, Solid-Phase Synthesis of the Antitumor Antibiotic Sansalvamide A Using a Side-Chain-Tethered Phenylalanine Building Block. *Organic Letters* **2000,** *2* (23), 3743-3746.

3. Gu, W.; Liu, S.; Silverman, R. B., Solid-Phase, Pd-Catalyzed Silicon-Aryl Carbon Bond Formation. Synthesis of Sansalvamide A Peptide. *Organic Letters* **2002,** *4* (23), 4171-4174.

4. (a) Peeters, T. L.; Macielag, M. J.; Depoortere, I.; Konteatis, Z. D.; Florance, J. R.; Lessor, R. A.; Galdes, A., d-Amino acid and alanine scans of the bioactive portion of porcine motilin. *Peptides* **1992,** *13* (6), 1103-1107; (b) Grieco, P.; Balse, P. M.; Weinberg, D.; MacNeil, T.; Hruby, V. J., d-Amino Acid Scan of γ-Melanocyte-Stimulating Hormone: Importance of Trp8 on Human MC3 Receptor Selectivity. *Journal of Medicinal Chemistry* **2000,** *43* (26), 4998-5002.

5. Aumailley, M.; Gurrath, M.; Müller, G.; Calvete, J.; Timpl, R.; Kessler, H., Arg-Gly-Asp constrained within cyclic pentapoptides Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1. *FEBS Letters* **1991,** *291* (1), 50-54.

6. Tugyi, R.; Uray, K.; Iván, D.; Fellinger, E.; Perkins, A.; Hudecz, F., Partial d-amino acid substitution: Improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102* (2), 413-418.

7. (a) Carroll, C. L.; Johnston, J. V. C.; Kekec, A.; Brown, J. D.; Parry, E.; Cajica, J.; Medina, I.; Cook, K. M.; Corral, R.; Pan, P.-S.; McAlpine, S. R., Synthesis and Cytotoxicity of Novel Sansalvamide A Derivatives. *Organic Letters* **2005,** *7* (16), 3481-3484; (b) Styers, T. J.; Kekec, A.; Rodriguez, R.; Brown, J. D.; Cajica, J.; Pan, P.-S.; Parry, E.; Carroll, C. L.; Medina, I.; Corral, R.; Lapera, S.; Otrubova, K.; Pan, C.-M.; McGuire, K. L.; McAlpine, S. R., Synthesis of Sansalvamide A derivatives and their cytotoxicity in the MSS colon cancer cell line HT-29. *Bioorganic & Medicinal Chemistry* **2006,** *14* (16), 5625-5631; (c) Rodriguez, R. A.; Pan, P.-S.; Pan, C.-M.; Ravula, S.; Lapera, S.; Singh, E. K.; Styers, T. J.; Brown, J. D.; Cajica, J.; Parry, E.; Otrubova, K.; McAlpine, S. R., Synthesis of Second-Generation Sansalvamide A Derivatives: Novel Templates as Potential Antitumor Agents. *The Journal of Organic Chemistry* **2007,** *72* (6), 1980-2002; (d) Pan, P.-S.; McGuire, K. L.; McAlpine, S. R., Identification of Sansalvamide a analog potent against pancreatic cancer cell lines. *Bioorganic & Medicinal Chemistry Letters* **2007,** *17* (18), 5072-5077; (e) Otrubova, K.; Lushington, G.; Vander Velde, D.; McGuire, K. L.; McAlpine, S. R., Comprehensive Study of Sansalvamide A Derivatives and their Structure–Activity Relationships against Drug-Resistant Colon Cancer Cell Lines. *Journal of Medicinal Chemistry* **2008,** *51* (3), 530-544.

8. (a) Cohen, D. J.; Loertscher, R.; Rubin, M. F.; Tilney, N. L.; Carpenter, C. B.; Strom, T. B., Cyclosporine: A New Immunosuppressive Agent for Organ Transplantation. *Annals of Internal Medicine* **1984,** *101* (5), 667-682; (b) Whyte, A. C.; Joshi, B. K.; Gloer, J. B.; Wicklow, D. T.; Dowd, P. F., New Cyclic Peptide and Bisindolyl Benzenoid Metabolites from the Sclerotia of Aspergillus sclerotiorum. *Journal of Natural Products* **2000,** *63* (7), 1006-1009; (c) Reshef, V.; Carmeli, S., Schizopeptin

791, a New Anabeanopeptin-like Cyclic Peptide from the Cyanobacterium Schizothrix sp. *Journal of Natural Products* **2002,** *65* (8), 1187-1189.

9. (a) Liu, S.; Gu, W.; Lo, D.; Ding, X.-Z.; Ujiki, M.; Adrian, T. E.; Soff, G. A.; Silverman, R. B., N-Methylsansalvamide A Peptide Analogues. Potent New Antitumor Agents. *Journal of Medicinal Chemistry* **2005,** *48* (10), 3630-3638; (b) Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H., N-Methylation of Peptides: A New Perspective in Medicinal Chemistry. *Accounts of Chemical Research* **2008,** *41* (10), 1331-1342; (c) Chatterjee, J.; Laufer, B.; Kessler, H., Synthesis of N-methylated cyclic peptides. *Nat. Protocols* **2012,** *7* (3), 432-444.

10. (a) Chatterjee, J.; Mierke, D.; Kessler, H., N-Methylated Cyclic Pentaalanine Peptides as Template Structures. *Journal of the American Chemical Society* **2006,** *128* (47), 15164-15172; (b) Chatterjee, J.; Rechenmacher, F.; Kessler, H., N-Methylation of Peptides and Proteins: An Important Element for Modulating Biological Functions. *Angewandte Chemie International Edition* **2013,** *52* (1), 254-269.

11. Doedens, L.; Opperer, F.; Cai, M.; Beck, J. G.; Dedek, M.; Palmer, E.; Hruby, V. J.; Kessler, H., Multiple N-Methylation of MT-II Backbone Amide Bonds Leads to Melanocortin Receptor Subtype hMC1R Selectivity: Pharmacological and Conformational Studies. *Journal of the American Chemical Society* **2010,** *132* (23), 8115-8128.

12. (a) Sellers, R. P.; Alexander, L. D.; Johnson, V. A.; Lin, C.-C.; Savage, J.; Corral, R.; Moss, J.; Slugocki, T. S.; Singh, E. K.; Davis, M. R.; Ravula, S.; Spicer, J. E.; Oelrich, J. L.; Thornquist, A.; Pan, C.-M.; McAlpine, S. R., Design and synthesis of Hsp90 inhibitors: Exploring the SAR of Sansalvamide A derivatives. *Bioorganic & Medicinal Chemistry* **2010,** *18* (18), 6822-6856; (b) Pan, P.-S.; Vasko, R. C.; Lapera, S. A.; Johnson, V. A.; Sellers, R. P.; Lin, C.-C.; Pan, C.-M.; Davis, M. R.; Ardi, V. C.; McAlpine, S. R., A comprehensive study of Sansalvamide A derivatives: The structure–activity relationships of 78 derivatives in two pancreatic cancer cell lines. *Bioorganic & Medicinal Chemistry* **2009,** *17* (16), 5806-5825; (c) Otrubova, K.; McGuire, K. L.; McAlpine, S. R., Scaffold Targeting Drug-Resistant Colon Cancers. *Journal of Medicinal Chemistry* **2007,** *50* (9), 1999-2002; (d) Styers, T. J.; Rodriguez, R.; Pan, P.-S.; McAlpine, S. R., High-yielding macrocyclization conditions used in the synthesis of novel Sansalvamide A derivatives. *Tetrahedron Letters* **2006,** *47* (4), 515-517.

13. Vasko, R. C.; Rodriguez, R. A.; Cunningham, C. N.; Ardi, V. C.; Agard, D. A.; McAlpine, S. R., Mechanistic Studies of Sansalvamide A-Amide: An Allosteric Modulator of Hsp90. *ACS Medicinal Chemistry Letters* **2010,** *1* (1), 4-8.

14. (a) Ramsey, D. M.; McConnell, J. R.; Alexander, L. D.; Tanaka, K. W.; Vera, C. M.; McAlpine, S. R., An Hsp90 modulator that exhibits a unique mechanistic profile. *Bioorganic & Medicinal Chemistry Letters* **2012,** *22* (9), 3287-3290; (b) Ardi, V. C.; Alexander, L. D.; Johnson, V. A.; McAlpine, S. R., Macrocycles That Inhibit the Binding

between Heat Shock Protein 90 and TPR-Containing Proteins. *ACS Chemical Biology* **2011,** *6* (12), 1357-1366; (c) Alexander, L. D.; Partridge, J. R.; Agard, D. A.; McAlpine, S. R., A small molecule that preferentially binds the closed conformation of Hsp90. *Bioorganic & Medicinal Chemistry Letters* **2011,** *21* (23), 7068-7071; (d) Kunicki, J. B.; Petersen, M. N.; Alexander, L. D.; Ardi, V. C.; McConnell, J. R.; McAlpine, S. R., Synthesis and evaluation of biotinylated sansalvamide A analogs and their modulation of Hsp90. *Bioorganic & Medicinal Chemistry Letters* **2011,** *21* (16), 4716-4719.

15. Jiang, S.; Li, Z.; Ding, K.; Roller, P. P., Recent Progress of Synthetic Studies to Peptide and Peptidomimetic Cyclization. *Current Organic Chemistry* **2008,** *12* (17), 1502-1542.

Chapter 3-Urukthapelstatin A

3.1 Background of Urukthapelstatin A

Numerous natural products containing oxazoles, thiazoles, and the saturated congeners such as oxazoline and thiazoline have shown promising biological activities.[1] For example, Ulapualide A (**Figure 3.1, (a)**), an oxazole-containing macrolide isolated in 1986 from eggmasses of the nudibranch *Hexabranchus sanguineus*, has an outstanding anti-cancer activity against L1210 leukemia cell line (IC_{50} = 11-34 nM).[2] Dendroamide A, a cyclic hexapeptide contains two thiazoles and one methyloxazole, was isolated in 1996 from the alga *Stigonema dendroideum* Fremy. It exhibits multidrug-resistance (MDR) reversing activity against MCF-7/ADR cancer cell line (**Figure 3.1 (b)**).[3]

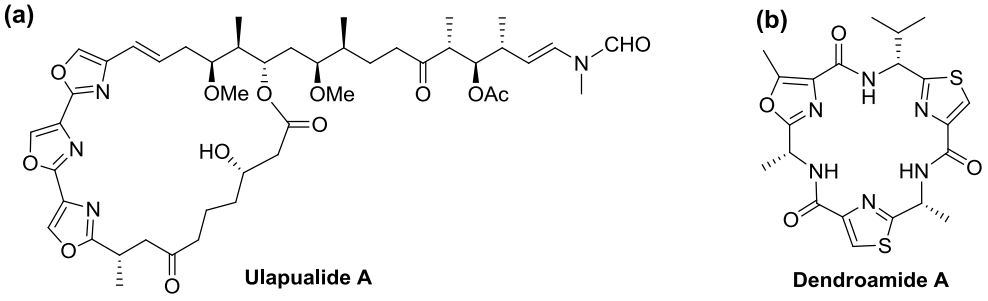


Figure 3.1 Structures of (a) Ulapualide A and (b) Dendroamide A

In 2007, Urukthapelstatin A (Ustat A, **Figure 3.2**), an oxazole- and thiazole-containing macrocyclic peptide, was isolated by Matsuo *et al.* from the cultured mycelia of marine-derived *Thermoactinomycetaceae* bacterium *Mechercharimyces asporophorigenens* YM11-542.[4] This natural product (Ustat A) possesses a promising cytotoxic potency against several human cancer cell lines with a mean GI_{50} of 15.5 nM.

Specifically, it exhibited effective potency against these cancer cell lines: MCF-7 (breast), HCT-116 (drug-resistant, colon), A549 (lung), DMS114 (lung) and NCIH460 (lung) with GI_{50}'s between 3.5-5.2 nM. Given its highly cytotoxic effect, the mechanism of Ustat A was explored inorder to evaluate its potential as an anticancer therapy. Testing Ustat A in histone deacetylase (HDAC), farnesyl transferase (FPTase), proteasome, and telomerase assays showed that no significant inhibition. These mechanistic studies indicate that Ustat A may have a unique mechanism of action that is currently unknown. In the structural aspect, Ustat A is composed of the heterocyclic region containing five consecutive azoles, the peptide chain with D-*allo*-Isoleucine, L-Valine, and an enamide moiety (**Figure 3.2**). To date, neither synthesis nor mechanism of action has been reported. I set out to accomplish the synthesis of Ustat A natural product, as well as undertake a mechanistic evaluation. In this chapter, several convergent synthetic strategies are discussed with the goal of synthesizing Ustat A. In addition, investigation of Ustat A cyclic molecule and fragments biological activity are explored.

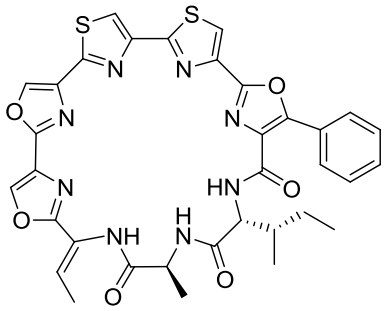


Figure 3.2 Structure of Urukthapelstatin A natural product

3.2 Structural features of Ustat A and structural-related natural products

Ustat A macrocyle (**Figure 3.2**) bears two directly-linked oxazoles, two directly-linked thiazoles, one phenyloxazole and a peptide region with an enamide moiety. The important structural features of Ustat A include a direct linkage between azoles and an enamide moiety. The direct azole linkage is found in several natural products that are current candidates for drug development[5] including Diazoamide A, Cystothiazole A (**Figure 3.3**), Leucamide A and Microcin B17 (**Figure 3.4**). Diazoamide A was isolated from the Philippine ascidian *Diazona angulata* by the Fenical group in 1991.[6] It contains a bis-oxazole moiety[7] and exhitbits an outstanding anti-cancer potency against HCT-116 cancer cell line (GI_{50} < 19 nM). Cystothiazole A is a bis-thiazole antibiotics isolated from the myxobacterium *Cystobacter fuscus* in 1998.[8] This molecule showed promising cytotoxicities against HCT-116 and K562 cancer cell lines with GI_{50} = 0.3 μM and 0.25 μM, respectively.

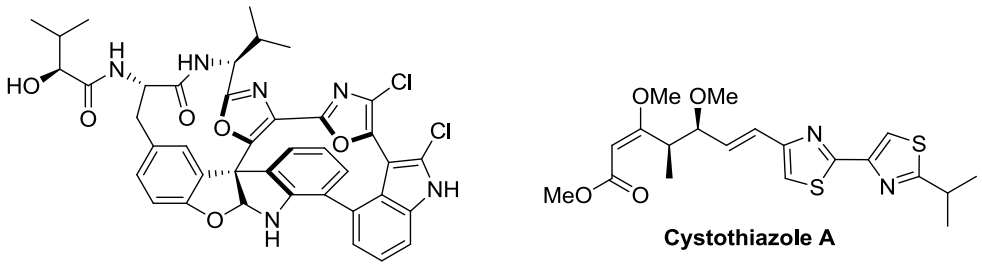


Figure 3.3 Structures of Diazoleamide A and Cystothiazole A

Leucamide A and Microcin B17 are two peptides that possess the mixed bis-azole tandem pairs (**Figure 3.4**). Leucamide A is a cyclic heptapeptide with a subunit

consisting of a methyloxazole and a thiazole.[9] This cyclic peptide was found cytotoxic against several tumor cell lines including HM02, HepG2 and Huh7 with GI_{50} values at low micromolar range. Microcin B17 contains two oxazole-thiazole subunits and exhibits a bactericidal activity against gram-negative bacteria via the inhibition of DNA replication.[10] These molecules have shown promising biological activities and indicated the importance of the directly-linked azole moiety.

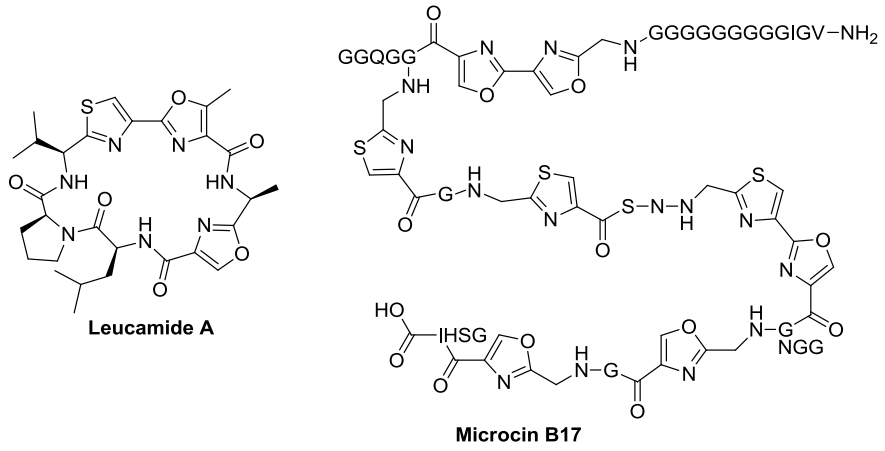


Figure 3.4 Structures of Leucamide A and Microcin B17

Next to the linked azoles of Ustat A is the peptide region with an enamide moiety, which is an important subunit in bioactive natural products and pharmaceutical drug lead molecules.[11] The structure-activity relationship (SAR) study of Salicylihalamide A has demonstrated the importance of the enamide moiety. Salicylihalamide A[12] is an enamide-containing macrolide isolated from the sponge *Haliclona* sp. The De Brabander group synthesized Salicylihalamide A analogues (**Figure 3.5**) and tested them in the Vacuolar ATPase inhibition assay. The analogue I, in which the enamide is intact, showed no significant change on the IC_{50} value. However, the removal of the enamide in analogue II

dramatically reduced its biological activity (**Figure 3.5**). Thus, these structural features have made Ustat A an attractive target for total synthesis and SAR study. Only 3 natural products share structural homology with Ustat A. The reported synthetic strategies for these molecules provide insights on the most effective synthetic route of Ustat A and they are discussed in the following paragraphs.

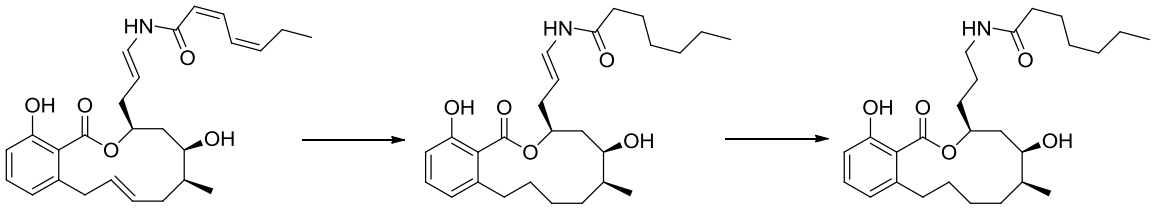


Figure 3.5 Structures of Salicylihalamide A and analogues

3.2.1 YM-216391

YM-216391 was first isolated from *Streptomyces nobilis* and the structure was proposed by Hayata *et al*. in 1999 (**Figure 3.6**).[13] It contains a peptide region and five tandem heterocycles including three consecutive oxazoles, one thiazole and one phenyloxazole. The peptide region is composed of a Gly, a D-Val and an Ile. In 2005, the first total synthesis of YM-216391 has been reported by Pattenden *et al*. based on the purposed structure.[14] As shown in **Figure 3.6**, YM-216391 was furnished by installing the thiazole via a thiazoline formation and then oxidation. The macrocycle of YM-216391 was afforded via continuous peptide coupling and macrolactamization between the D-Val-Ile dipeptide and the thioamide-containing tetraoxazole fragment. Peptide coupling between the trisoxazole and phenyloxazole species would generate the

tetraoxazole fragment. The preparation of trisoxazole species involved a peptide coupling and an oxazole formation from two mono oxazole subunits.

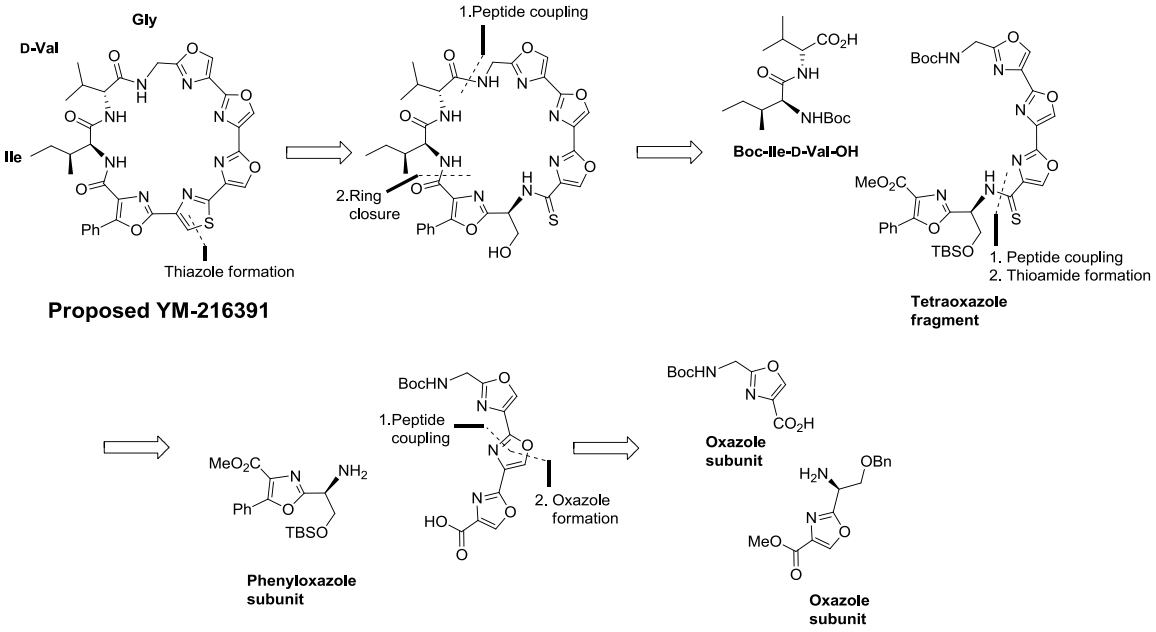


Figure 3.6 Proposed structure and synthetic strategy of YM-216391

Also in 2005, Sohda *et al* published the full report on the biological activities and the revised structural elucidation (**Figure 3.7**).[15] The inhibitory assay of YM-216391 against human cervical cancer HeLa S3 cells showed low nanomolar growth inhibition (IC_{50} = 14 nM). Further investigation on the profile of its anticancer potency showed that it exhibited cytotoxic potency against 39 human cancer cell lines with mean GI_{50} value of 37 nM. Interestingly, the peptide region was elucidated by Sohna *et al.* as a Gly-Val-D-*allo*-Ile sequence and the optical rotation was opposite to that of the synthetic YM-216391($[\alpha]_D$ = +48^o vs. $[\alpha]_D$ = -56^o). Although the synthetic product was proven to be the

enantiomer of the natural product[16], the synthetic strategy established by the Pattenden group still provided an approachable route for this class of molecules.

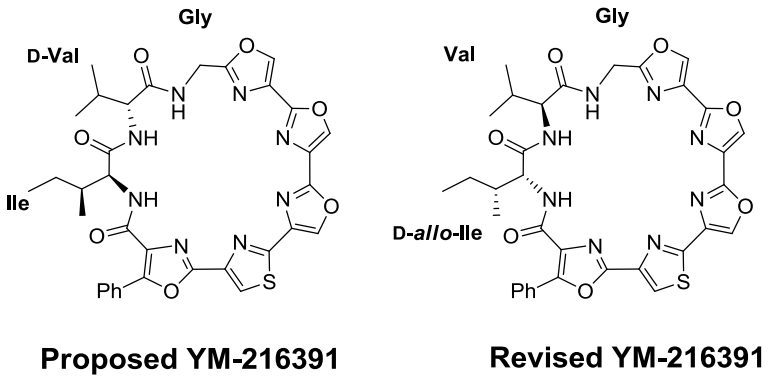


Figure 3.7 Proposed and revised structures of YM-216391

3.2.2 IB-01211

IB-01211(**Figure 3.8**) was isolated in 2005 from the marine microorganism *Thermoactinomyces genus* strain ES7-008[17].The natural product mechercharmycin A was also isolated in 2005 from marine-derived bacterium *Thermoactinomyces* sp. YM3-251 with the same structure as IB-01211[18]. The macrocycle of IB-01211 is almost identical to Ustat A and YM-216391 and contains four oxazoles, one thiazole, and a tripeptide unit.

In 2007, the first total synthesis of IB-01211 was published by Hernandez *et al*.[19] The key step feathered a macrocyclization through an intramolecular Hantzsch thiazole formation on the linear precursor to install the thiazole (**Figure 3.8**). The linear precursor was assembled in a convergent manner from three building blocks: the D-*allo*-Ile-Val dipeptide, the bromoketal bis-oxazoles, and the bis-oxazole thioamide.

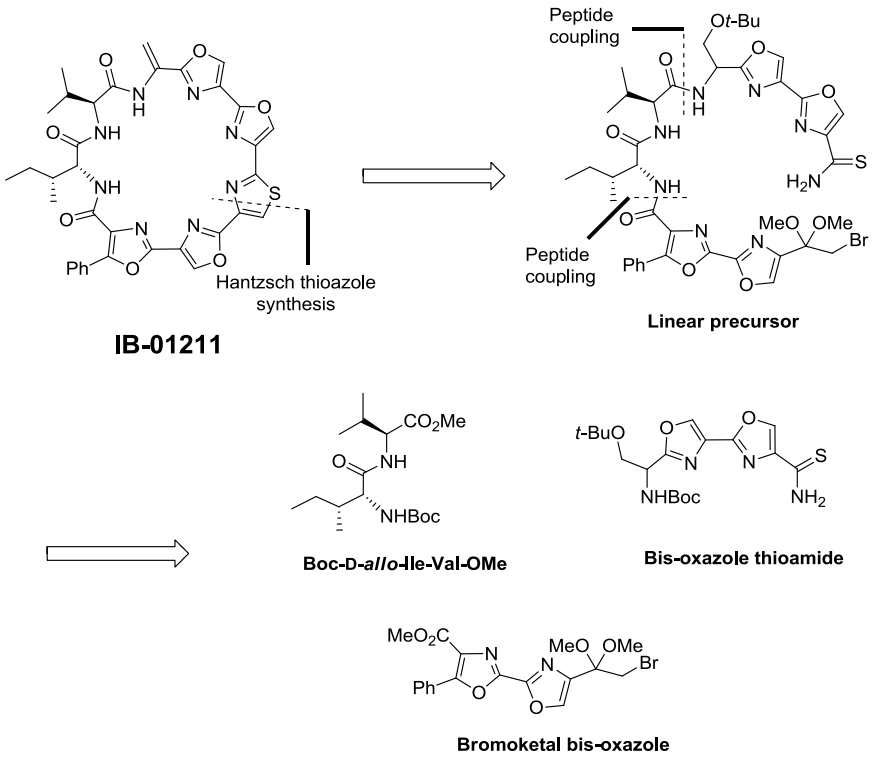


Figure 3.8 Structure and synthetic strategy of IB-01211

Upon finding the effective synthetic route of IB-01211, structure-activity relationship (SAR) study was also conducted by the same group in 2008.[20] The structure of IB-01211 was modified in three ways (**Figure 3.9**): a) methyl substituents were introduced on the polyoxazoles, b) the exocyclic double bond was replaced by a methylene group, and c) PEG residues were attached to the marcocycle. The anti-cancer potency of natural product and derivatives were evaluated against three human cancer cell lines (A-549, HT-29, and MDA-MB-231). The result showed that the natural product exhibited potent cytotoxicity with GI_{50} value of 30-90 nM. However, none of the derivatives showed comparable cytotoxicity to that of natural product, which indicated that a) either substituting methyl group on the polyoxazoles, or PEG residues on the

macrocycle, is not facilitating anticancer activity; and b) the exocyclic double bond may be crucial. Finally, the mechanistic study showed that IB-01211 was able to induce cancer cell apoptosis through cell cycle arrest at G2 phase.

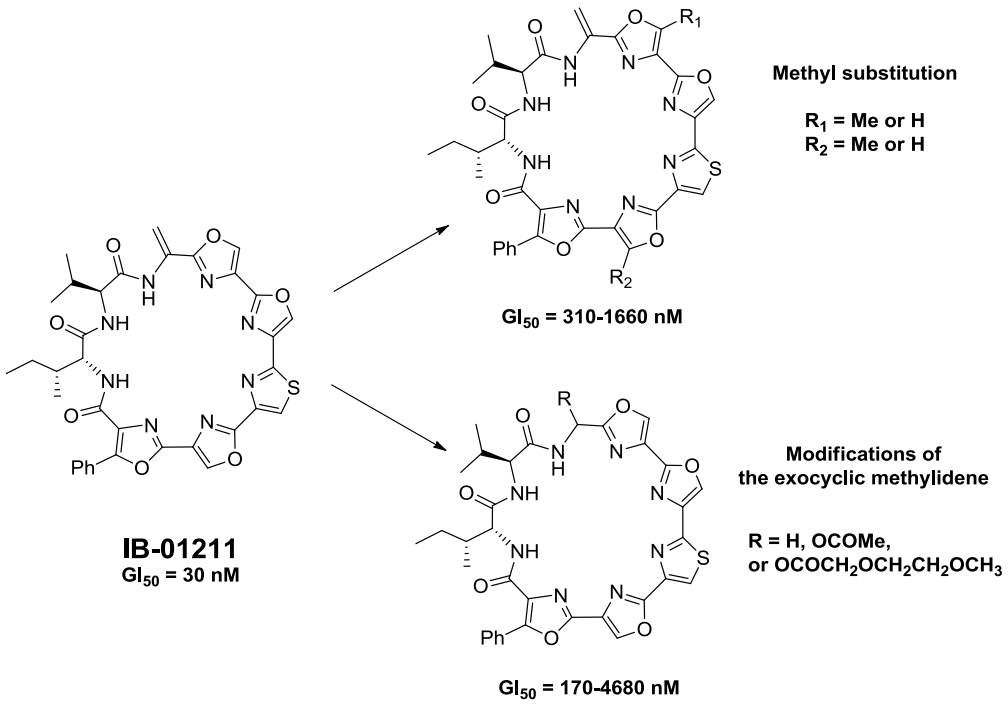
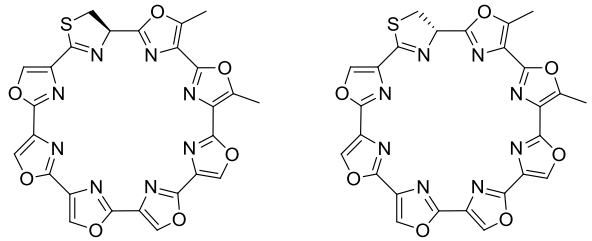


Figure 3.9 SAR of IB-01211 (Tested against A-549 cancer cell line)

3.2.3 Telomestatin

Telomestatin (**Figure 3.10**) is a novel macrocycle that was isolated from *Streptomyces anulatus* 3533-SV4 by Shin-ya *et al.* in 2001.[21] Telomestatin is a potent anti-cancer agent with IC_{50} = 5 nM. Telomestatin binds the telomere and stabilizes the antiparallel G-quadruplex form. Stabilizing the quadruplex inhibits the telomerase from elongating the telomere, which eventually inhibits the growth of cancer cells.



(R)-Telomestatin
Natural product

(S)-Telomestatin

Figure 3.10 Structures of Telomerstatins

The structure of Telomestatin contains eight directly-linked heterocycles, including five oxazoles, two methyloxazoles, and one thiazoline. The first total synthesis of Telomestatin was completed by Doi *et al.* in 2006 and the absolute configuration on the thiazoline C-sp^3 was determined to be (R).[22]

The natural product was afforded by the sequential formation of the last oxazole and the thiazoline on the cyclic precursor (**Figure 3.11)**. The cyclic precursor was synthesized by coupling two trisoxazole fragments, followed by macrocyclization. The two trisoxazole fragments were assembled fromamino acid-based building blocks. The SAR study of telomestatin analogs was reported by the same group in 2011.[23] The results showed that the (S)-isomer (**Figure 3.10**) was 4 fold more potent than the natural product.

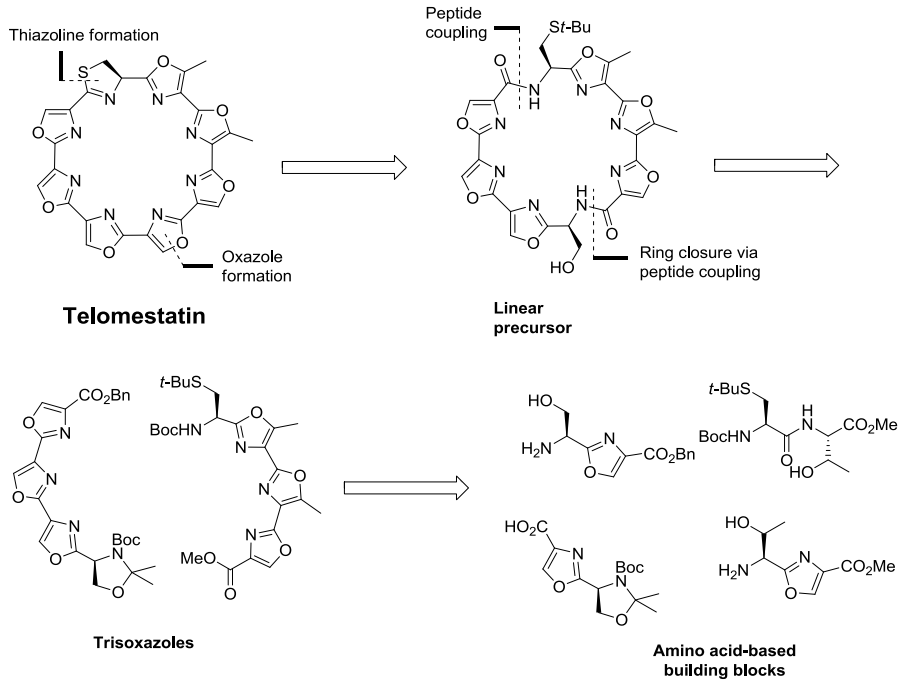


Figure 3.11 Synthetic strategy of Telomerstatin

From the synthetic studies of theses natural products and their derivatives, we see that the convergent synthetic approach was employed for all molecules. Indeed, with azole-containing subunits, one can build up complex molecules in an efficient manner using a convergent strategy. Thus, we will apply the convergent approach to the synthesis of Ustat A natural product.

3.3 Rational Design of Ustat A Derivatives

The goal of Ustat A project is to develop an efficient synthetic route to synthesize the Ustat A natural product, and then construct a library of derivatives in order to study their SAR. The Hernandez group designed a series of IB-01211 derivatives by adding methyl to the oxazole moiety, removing the exocyclic double bond, or substituting the exocyclic double bond with several different PEG substituents. The results indicated that

the exocyclic double bond is essential for the anti-cancer properties of the molecule. In contrast, the methylated oxazole moiety and PEG substituents have not improved the anticancer potency (**Figure 3.9**). Interestingly, during their SAR study of IB-01211, the Hernandez group did not modify the type of heterocycles, and thus heterocyclic replacement is a novel approach for investigating structure-activity relationships. Thus, in the preliminary design of Ustat A derivatives, we modified the heterocyclic region in order to find novel mechanistic insights.

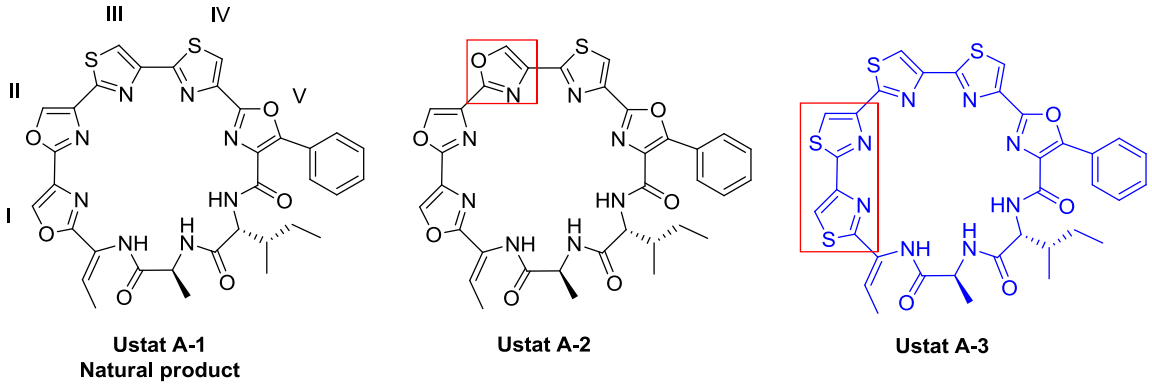


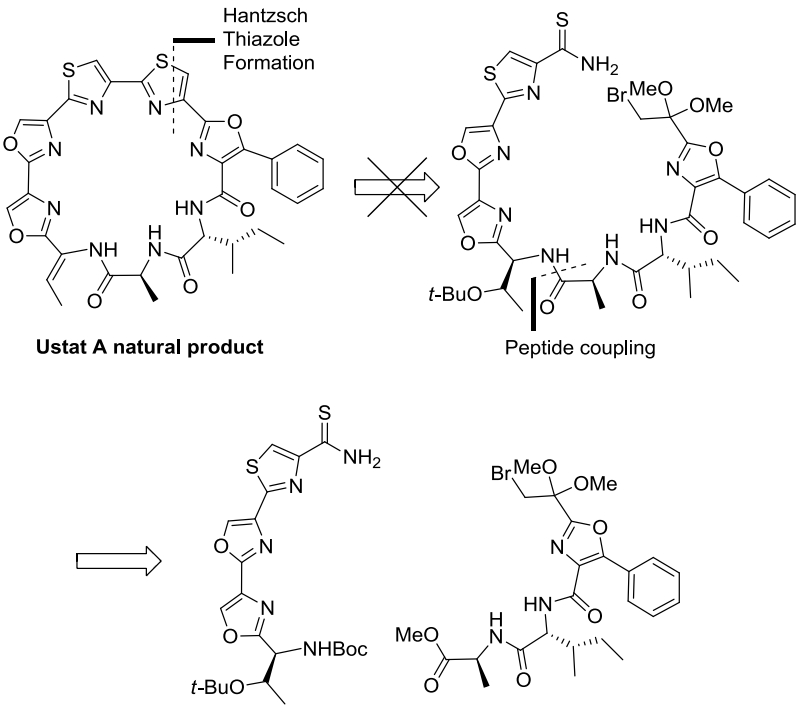
Figure 3.12 Structures of Urukthapelstatin A and two derivatives.

In this project, I was responsible for Ustat A-3 (blue colored in **Figure 3.12**) while my colleagues were working on the synthesis of Ustat A natural product and Ustat A-2. As shown in **Figure 3.12**, we retained the enamide moiety and the peptide region in the two derivatives whereas the heterocycle region has been modified by changing the numbers of oxazoles and thiazoles. The thiazole at position III was replaced by an oxazole in Ustat A-2, generating a molecule that has four oxazoles. My compound Ustat A-3 has two thiazoles introduced at positions I and II generating with four thiazoles

overall. Since oxazole-based molecules are less flexible than thiazole-based ones,[24] Ustat A-2 would be significantly more rigid than Ustat A-3. The rigidity will impact the macrocyclic conformation and change the interaction between the molecule and its biological target. In addition, modification to the oxazole/thiazole ration varies the number of hydrogen bond as an oxazole has two H-bond acceptors while a thiazole has only one. These modifications will allow us to understand how the anticancer potency of Ustat A can be tuned by the physical shape or the electronic effects within heterocycle region. Subsequent modification of the peptide region would then be evaluated. Our ultimate goal is to conduct synthesis and a SAR study of Ustat A in order to understand its mechanism of action.

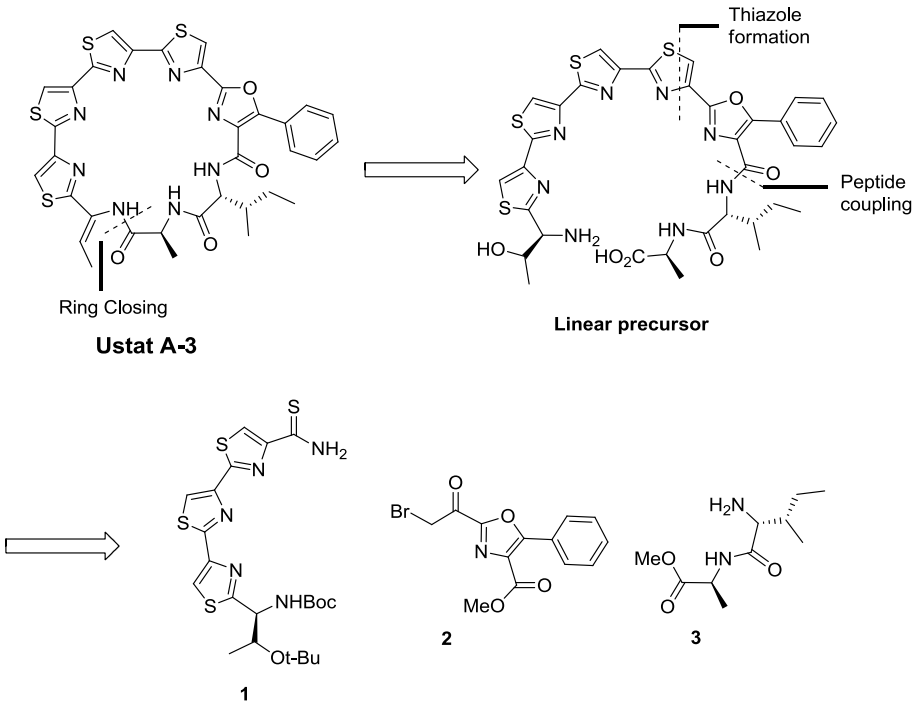
3.4 Retrosynthetic Approach of Ustat A-3

Hernandez *et al*. had reported a macrocyclization strategy via Hantzsch thiazole synthesis.[19] This approach seemed ideal since it could install several synthetic units simultaneously. Thus, Ustat A natural product was designed to close using Hantzsch macrocyclization. The linear precursor was synthesized from two fragments via peptide coupling (**Scheme. 3.1**). In the preparation of macrocyclization, bromoketal-containing linear precursor was subjected to hydrolysis of the bromoketal group. However, the decomposition of linear precursor was observed during the hydrolysis step. Therefore, a revised retrosynthetic approach was proposed for the synthesis of Ustat A and two derivatives. Note: upon closer evaluation of Hernandez data, there was no data showing that the macrocyle had been formed, suggesting this route was never succeeded.



Scheme 3.1 Initial Synthetic Strategy of Ustat A natural product

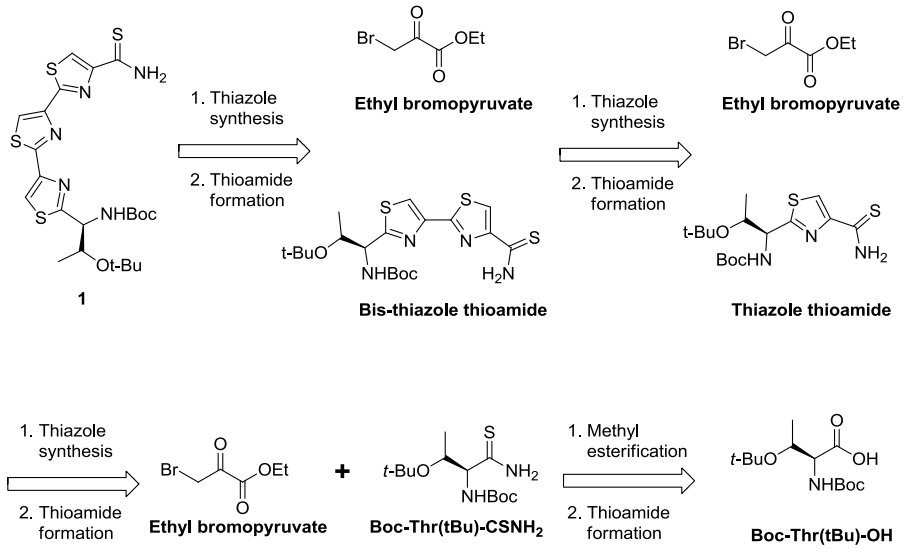
In the revised retrosynthetic approach, Ustat A-3 was constructed from three fragments (**Scheme 3.2**). The double deportected linear precursor would be cyclized using peptide-coupling macrocyclization between Thr and Ala, followed by the *Z*-enamide installation at the hydroxyl side chain of Thr residue. The linear precursor was generated by performing a Hantzsch reaction between fragments **1** and **2**. Deprotection of acid on **2** and peptide coupling with **3** yields the acyclic molecule. Commercially available amino acids and reagents would be used for the synthesis of these three fragments.



Scheme 3.2 Revised Retroynthetic Approach for Ustat A-3

3.4.1 Retrosynthesis of Ustat-3 Fragment 1

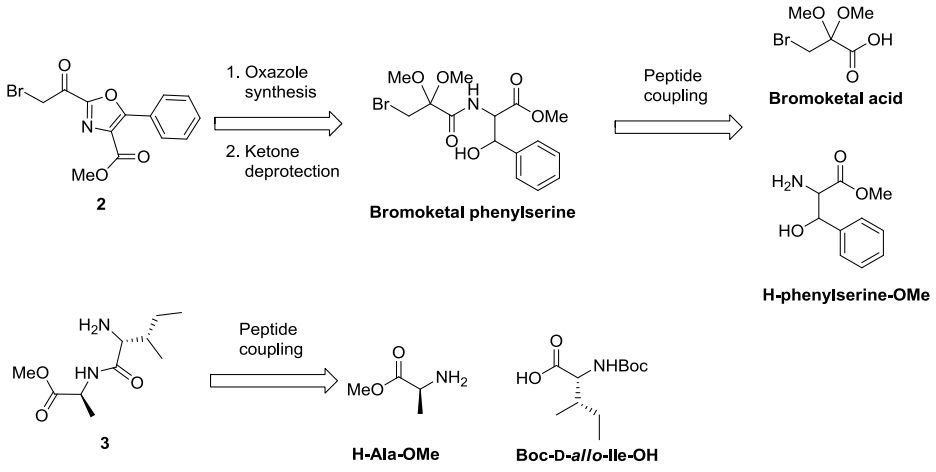
Fragment **1** was generated from commercially available chemicals using sequential Hantzsch reactions with a thioamide and an ethyl bromopyruvate (**Scheme 3.3**). The bis-thiazole thioamide was furnished from the thiazole-thioamide and ethyl bromopyruvate via the Hantzsch thiazole synthesis, followed by the two-step thioamide formation at the resulting ethyl ester moiety. Following the same synthetic method, the thiazole-thioamide was yielded from Boc-Thr(*t*-Bu)-thioamide, which was simply prepared from the free acid Boc-Thr(*t*-Bu)-OH.



Scheme 3.3 Retrosynthesis of Ustat-3 Fragment 1

3.4.2 Retrosynthesis of Ustat-3 Fragment 2 and 3

Fragment **2** was constructed from the bromoketal phenylserine that was cyclized and oxidized to give the phenyloxazole (**Scheme 3.4**). An amide bond coupling between the bromoketal acid and the free amine on phenylserine afforded the desired pseudopeptide. Fragment **3** was synthesized using peptide coupling between two amino acids: Boc-D-*allo*-Ile-OH and H-Ala-OMe.

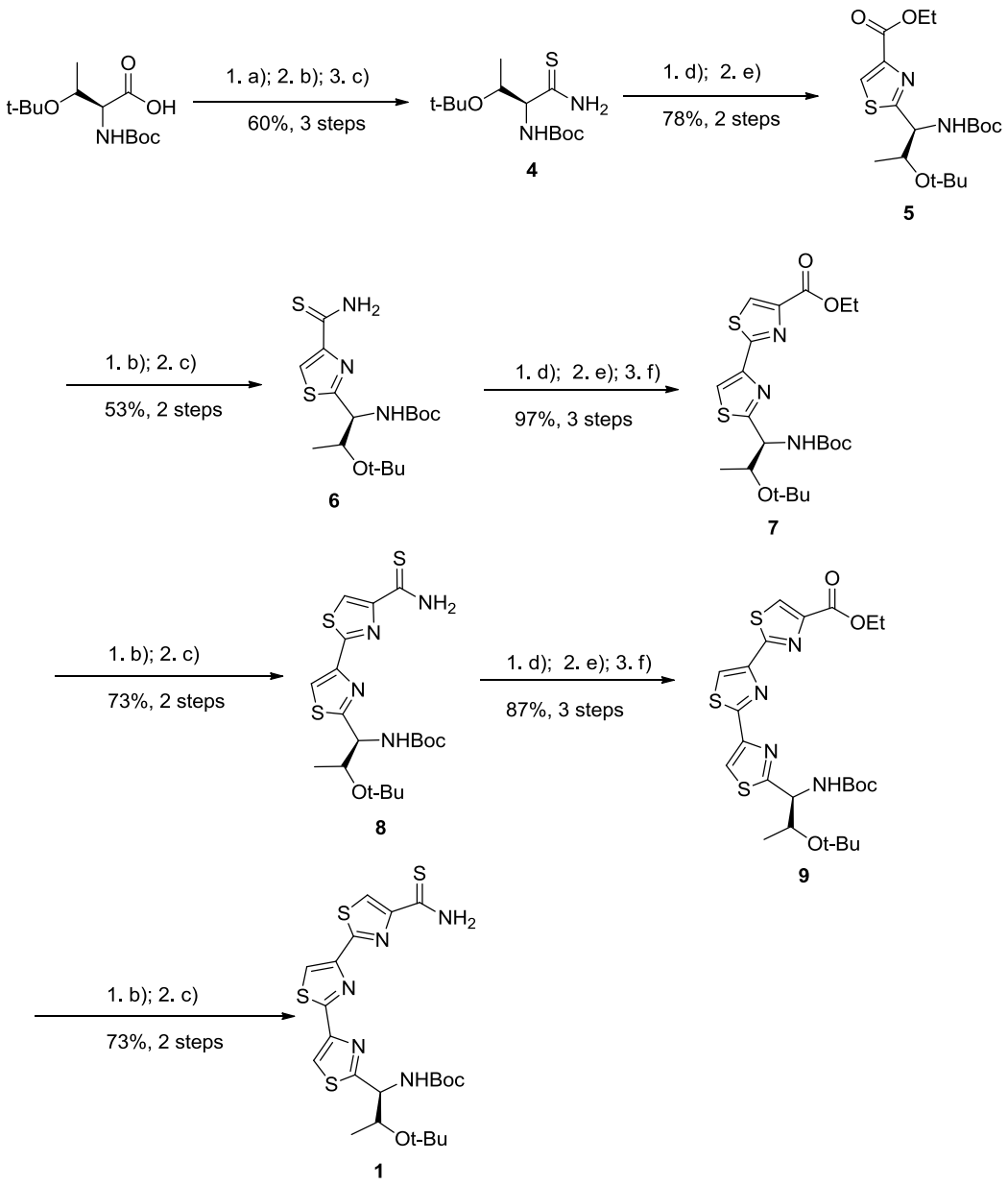


Scheme 3.4 Retrosynthesis of Ustat A-3 Fragment 2 and 3

3.5 Synthesis of Ustat A-3

3.5.1 Synthesis of Fragment 1 for Ustat A-3

The construction of Ustat A-3 began with the synthesis of Fragment **1**.Since the successful synthesis of Fragment 1 relies on a high yielding thiazole synthesis we evaluated several methods in the literatures.[25-26] We believe the optimal Hantzsch reaction conditions for our molecule were those developed by the Nicolaou group.[27] Their method utilized Lawesson's reagent, which converts amides to thioamides. Synthesis of Fragment **1** was started by converting the commercially available threonine free acid Boc-Thr(*t*-Bu)-OH to the methyl ester Boc-Thr(*t*-Bu)-OMe using the methylating agent Trimethylsilyl diazomethane (TMSD) (**Scheme 3.5**). Boc-Thr(*t*-Bu)-OH was dissolved in a mixture of anhydrous benzene and methanol (3:1) in a round-bottom flask to make a 0.1 M solution. The methylating agent TMSD (2.0 M in diethyl ether) was added drop-wise into the reaction mixture until the mixture became slightly yellow. The reaction was stirred under argon and monitored by thin layer chromatography (TLC). Upon completion, the solvent was evaporated *in vacuo* and the resulting crude methyl ester Boc-Thr(*t*-Bu)-OMe was confirmed by ^{1}H NMR spectroscopy and then taken on to the next reaction without further purification.



Conditions:
a) TMSD, Benzene/MeOH (3:1, 0.1 M). b) NH_4OH/MeOH (9:1, 0.1 M), ultrasound. c) Lawesson's reagent. d) $KHCO_3$ (8.0 eq.), $BrCH_2COCO_2Et$ (3.0 eq.), DME (0.05 M), 16 h, rt. e) pyridine (9.0 eq.) 5 min, 0 ^oC, TFAA (4.0 eq.), 3 h, 0 ^oC to rt then TEA (2.0 eq.), 1 h, rt, DME (0.05 M). f) NaOEt (1.2 eq.), EtOH (0.05 M), 0 ^oC, 1 h.

Scheme 3.5 Synthesis of Ustat A-3 Fragment 1

The ester Boc-Thr(*t*-Bu)-OMe was then converted to the corresponding amide using ammonium hydroxide (**Scheme 3.5**). The ester was dissolved in a mixture of ammonium hydroxide aqueous solution and methanol (9:1) to make a 0.1 M solution. The reaction mixture was ultrasonicated until homogeneous, stirred under room temperature, and monitored via TLC. Upon completion, the solvent was removed *in vacuo* to afford the desired amide Boc-Thr(*t*-Bu)-NH_2 in quantitative yield. The resulting amide was verified by ^{1}H NMR spectroscopy and used for next transformation without further purification.

The amide was then converted to a thioamide using Lawesson's reagent (LR). The amide and LR (0.8 equivalent) were dissolved in anhydrous tetrafuran (THF) to give final concentration of 0.05 M, and the reaction mixture was heated to reflux under argon. After TLC indicated reaction completion, the solution was concentrated *in vacuo*, and the residue was purified via flash column chromatography under gradient condition of ethyl acetate (EA) and hexane (Hex) as the eluting solvent system. The pure thioamide **4** (Boc-Thr(*t*-Bu)-SNH_2) was furnished in a 60% overall yield for three steps. The structure and purity of **4** were confirmed via ^{1}H NMR spectroscopy.

The thioamide **4** was subjected to the modified Hantzsch thioazole synthesis, which involves reaction of a thioamide and an α-halogenated ketone under basic conditions, generate a hydroxyl thiazoline intermediate. Subsequent dehydration of the thiazoline affords the desired thiazole. The thioamide **4** and potassium bicarbonate ($KHCO_3$, 8.0 equivalents) was dissolved in anhydrous 1,2-dimethoxyethane (DME) to a concentration of 0.05 M. The slurry solution was allowed to stir for 5 min. Ethyl bromopyruvate (3.0 equivalents) was then added drop-wise into the reaction mixture. The

reaction was stirred for 16 h at room temperature and monitored by TLC. Upon completion, the solvent was removed *in vacuo*. The residue was re-dissolved in chloroform and extracted with deionized water and brine. The organic layer was collected, dried over sodium sulfate, filtered, and concentrated *in vacuo* to afford the crude thiazoline. The thiazoline intermediate was dissolved in anhydrous DME to a concentration of 0.05 M, stirred and cooled to 0 ^{o}C. Pyridine (9.0 equivalents) was then added drop-wise to the stirred solution. After stirring for 5 min, trifluoroacetic anhydride (TFAA, 4.0 equivalents) was added drop-wise to the reaction mixture. The mixture was stirred for an additional 3 h at 0 ^{o}C then warmed up to room temperature. Finally, triethylamine (TEA, 2.0 equivalents) was added to the solution, which was then stirred for an additional 1 h and monitored by TLC. Upon completion, the solution was concentrated *in vacuo*, and the residue was re-dissolved in chloroform, subjected to an acid-base extraction, and purified via flash column chromatography to yield the pure thiazole **5** (Boc-Thr(*t*-Bu)-thiazole-OEt) in 78% yield over two steps.

In order to convert the ethyl ester moiety of **5** to thioamide, the thiazole **5** was first subjected to amide conversion using a mixture of ammonium hydroxide and methanol (**Scheme 3.5**). The reaction mixture was ultrasonicated until homogeneous, stirred under room temperature and monitored via TLC. Upon completion, the solvent was evaporated *in vacuo* and the resulting amide Boc-Thr(*t*-Bu)-thiazole-NH_2 was afforded in quantitative yield without further purification.

The amide Boc-Thr(*t*-Bu)-thiazole-NH_2 was then converted to thioamide using Lawesson's reagent (LR). In the attempt of improving reaction yield, the reaction was carried out in anhydrous DME instead of THF. The amide and LR (0.8 equivalents) were

dissolved in DME, and the reaction mixture was heated to 60 ^{o}C under argon. After TLC confirmation of completion, the solvent was removed *in vacuo*, and the crude product was purified via flash column chromatography. The pure thioamide **6** (Boc-Thr(*t*-Bu)-thiazole-SNH_2) was furnished in a moderate 53% yield over two steps.

The thioamide **6** was carried on to the synthesis of second thiazole via the same Hantzsch thiazole synthesis conditions to generate the dithiazole **7** (Boc-Thr(*t*-Bu)-dithiazole-OEt) (**Scheme 3.5**). However, after flash column purification, the trifluoroacetamide byproduct (**Figure 3.13**, trifluoroacetyl-**7**) was obtained along with the pure dithiazole **7**. To remove the trifluoroacetyl group, the mixture product was dissolved in ethanol and cooled to 0 ^{o}C. Sodium ethoxide (1.2 equivalents) was added and the reaction mixture was allowed to stir for 1 h. Reaction completion was confirmed by TLC; the mixture was concentrated, subjected to an acid-base extraction, and purified by flash column chromatography to furnish the pure dithiazole **7** in an excellent 97% yield over three steps.

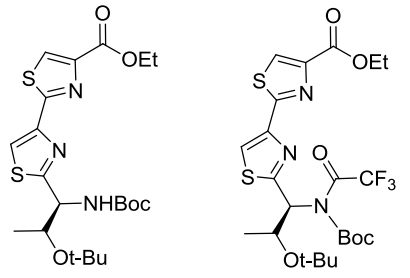


Figure 3.13 Strutures of thiazole 7 and its trifluoroacetyl byproduct

The ethyl ester moiety in dithiazole **7** was converted to an amide using ammonium hydroxide. For the next thiomide formation, both starting material and reagent contain aromatic rings, therefore I used benzene in order to improve the reaction

yield. The amide Boc-Thr(*t*-Bu)-dithiazole-NH_2 and LR (0.8 equivalent) were dissolved in anhydrous benzene. The reaction mixture was heated to 60 ^oC and monitored by TLC. Upon completion, the mixture was concentrated *in vacuo*, subjected to a flash column chromatography to furnish the pure thioamide **8** (Boc-Thr(*t*-Bu)-dithiazole-SNH_2) in 73% yield.

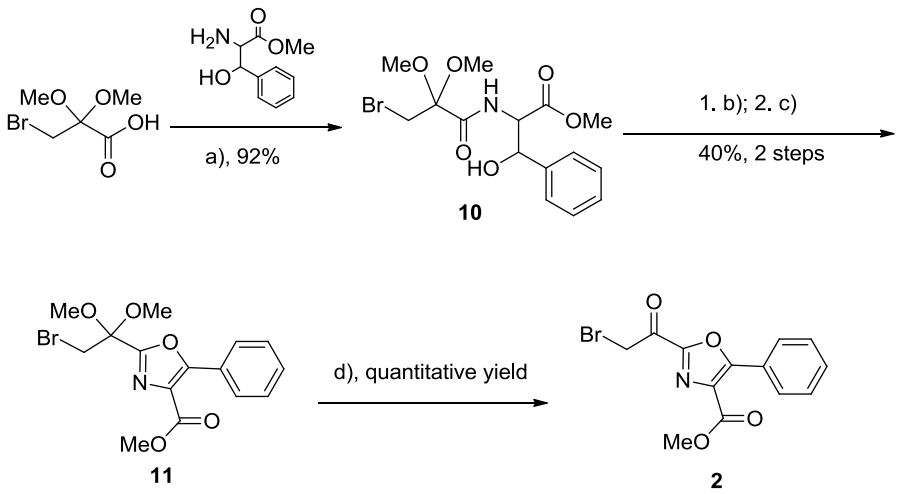
The third thiazole was prepared by reacting the thioamide **8** with ethyl bromopyruvate under basic conditions, followed by the dehydration of the thiazoline intermediate using pyridine and TFAA (**Scheme 3.5**). The undesired trifluoroacetamide byproduct was also observed with the tristhiazole **9** (Boc-Thr(*t*-Bu)-tristhiazole-OEt) in this transformation. After solvolysis of the trifluoroacetamide byproduct using sodium ethoxide, the pure tristhiazole **9** was afforded in 87% yield via flash column chromatography over three steps.

Finally, the tristhiazole **9** underwent amide conversion with ammonium hydroxide and then thioamide formation using LR (0.8 equivalent) in benzene to deliver the desired tristhiazole thioamide Fragment **1** (Boc-Thr(*t*-Bu)-tristhiazole-OEt) in 73% yield over two steps.

3.5.2 Synthesis of Fragment 2 for Ustat A-3

The synthesis of fragment **2** started by reacting the free amine (2R, 3S)/(2S, 3R)-racemic H-*β*-hydroxyl-Phe-OMe (1.1 equivalents) with the pre-made free acid 3-bromo-2,2-dimethoxypropionic acid[28] (1.0 equivalent) via our standard peptide coupling conditions to deliver the racemic pseudopeptide **10** (Bromoketal-*β*-hydroxyl-Phe-OMe) (**Scheme 3.6**). The free amine, free acid, coupling reagent TBTU (1.1 equivalents) and

base DIPEA (4.0 equivalents) were dissolved in anhydrous dichloromethane (DCM) at 0.1 M. The reaction mixture was stirred under argon and monitored by TLC. Upon completion, the reaction solution was diluted with DCM, extracted with 10% (v/v) $HCl_{(aq)}$ to remove the excess base, followed by basic extraction with saturated solution of sodium bicarbonate ($NaHCO_3$). The organic layer was dried over sodium sulfate, filtered, and concentrated *in vacuo*. The resulting crude product was purified via flash column chromatography using EA-Hex as the eluting solvent system to furnish the pure pseudopeptide **10** in 92% yield. The structure and purity were confirmed using ^{1}H NMR spectroscopy.



Conditions:
a) TBTU (1.1 eq.), DIPEA (4.0 eq.), DCM (0.1 M). b) DAST (1.1 eq.), DCM (0.1 M), 30 min, -78 ^oC then pyridine (2.0 eq.), 1 h, -78 ^oC to rt. c) DBU (2.0 eq.), DCM (0.1 M), 10 min, -47 ^oC, $BrCCl_3$ (2.0 eq.), 12 h, -47 ^oC to rt. d) Formic acid (0.1 M), 20 min, 60 ^oC.

Scheme 3.6 Synthesis of Ustat A-3 Fragment 2

In the synthesis of oxazole-containing molecules, several methods have been reviewed[29]. One common and efficient method is the two-step protocol applied on a *β*-hydroxyl amide involving cyclodehydration/oxidation using DAST/DBU. In general,

diethylaminosulfur trifluoride (DAST) is an efficient fluorinating agent, which converts the hydroxyl group of serine to fluorine. Next, a base, usually potassium carbonate (K_2CO_3), is used for the cyclodehydration, which affords the oxazoline intermediate in the first step. Bromotrichloromethane ($BrCCl_3$) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) are introduced in the second step, oxidizing the oxazoline into the desired oxazole. However, different base in the first step has to be carefully chosen to form the oxazoline for the synthesis of phenyloxazole. Previous studies and attempting[19] showed that β-elimination is favored over cyclodehydration when using K_2CO_3 with the β-hydroxyl phenylalanine moiety. Several bases were used in order to address this problem and pyridine was found to give the optimal reaction yield. Thus, for the synthesis of phenyloxazole **11**, the DAST/pyridine protocol was used during the cyclodehydration step and $BrCCl_3$/DBU was introduced in the second step. The pure pseudopeptide **10** (1.0 equivalent) was dissolved in anhydrous DCM (0.1 M) at -78 ^oC under argon. DAST (1.1 equivalents) was added drop-wise to the cooled DCM solution and the reaction mixture was stirred for 30 min. Pyridine (2.0 equivalents) was introduced to the reaction to induce cyclodehydration. The reaction was allowed to warm to room temperature, and stirred for an additional 1 h. Upon completion, the formation of the oxazoline was confirmed by TLC and LC/MS, The reaction mixture was then diluted with DCM, extracted with saturated $NaHCO_{3(aq)}$, and then purified via flash column chromatography. The resulting pure phenyloxazoline intermediate was then moved on to the oxidation step.

The pure phenyloxazoline was dissolved in DCM (0.1 M) at -47 ^oC under argon. DBU (2.0 equivalents) was added drop-wise to the DCM solution, which was then stirred for 10 min. Next, $BrCCl_3$ (2.0 equivalents) was added into the mixture. The reaction was

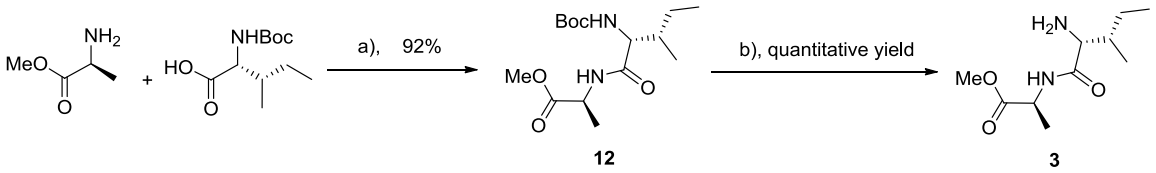
allowed to warm to room temperature when running for an additional 12 h. Upon completion, the crude product was purified via flash column chromatography to afford the phenyloxazole **11** (bromoketal phenyloxazole-OMe) in 40% yield over two steps.

Finally, Fragment **2** (**Scheme 3.6**) was furnished via acid-induced ketone deprotection. The phenyloxazole **11** was dissolved in formic acid (0.1 M) and stirred at 60 ^{o}C for 20 min. Upon completion, the reaction was extracted by gently adding saturated $NaHCO_{3(aq)}$ solution, and the resulting bromoketo phenyloxazole (Fragment **2**) was taken on to the Hantzsch thiazole synthesis without further purification.

3.5.3 Synthesis of Fragment 3 for Ustat A-3

The synthesis of Fragment **3** started with the peptide coupling between a free amine H-Ala-OMe (1.1 equivalents) and a free acid Boc-D-*allo*-Ile-OH (1.0 equivalent) (**Scheme 3.7**). The starting materials, coupling reagent TBTU (1.1 equivalents), and DIPEA (4.0 equivalents) were dissolved in anhydrous DCM (0.1 M). The reaction was stirred under argon and monitored by TLC. Upon completion, the crude product was purified via flash column chromatography to provide the pure dipeptide **12** (Boc-D-*allo*-Ile-Ala-OMe) in 92% yield.

The Boc protecting group of the dipeptide **12** was removed to yield Fragment **3**. The dipeptide was dissolved in a mixture of TFA/DCM (1:4, 0.1 M) with the presence of anisole (2.0 equivalents) and the reaction was monitored by TLC. Once the reaction was completed, the solution was concentrated *in vacuo* to yield Fragment **3**, which was taken on to the next step without further purification.



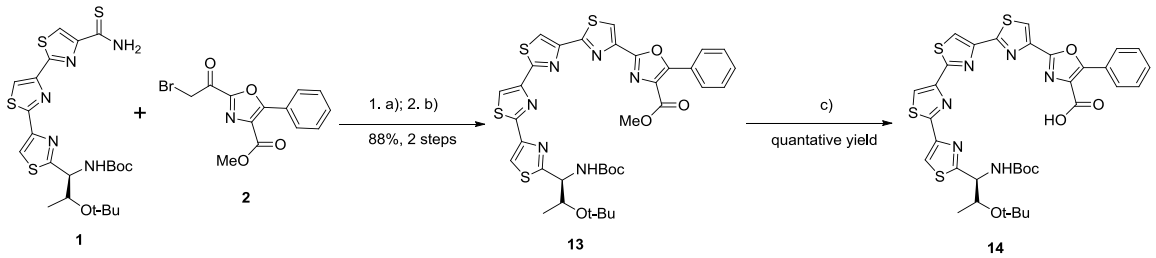
Conditions:
a) TBTU (1.1 eq.), DIPEA (4.0 eq.), DCM (0.1 M). b) TFA/DCM (1:4, 0.1 M), anisole (2.0 eq.)

Scheme 3.7 Synthesis of Ustat A-3 Fragment 3

3.5.4 Synthesis of Linear Precursor for Ustat A-3

With Fragments **1** and **2** in hand, the heterocycle fragment **13** (Boc-Thr(*t*-Bu)-tetrathiazole-phenyloxazole-OMe) was synthesized via the Hantzsch thiazole synthesis (**Scheme 3.8**). Fragment **1** (1.0 equivalent) and $KHCO_3$ (8.0 equivalents) were dissolved in anhydrous DME. The reaction mixture was stirred for 10 min and the pre-dissolved DME Fragment **2** was added drop-wise to make a final reaction concentration of 0.05 M. The reaction was run for 16 h at room temperature. Upon completion via TLC and LC/MS confirmation, the solvent was removed *in vacuo*. The residue was re-dissolved in chloroform and then extracted with brine and deionized water. The resulting thiazoline intermediate was re-dissolved in anhydrous DME to a concentration of 0.05 M, stirred and cooled to 0 ^{o}C. Pyridine (9.0 equivalents) was then added drop-wise to the stirred solution. After stirring for 5 min, TFAA (4.0 equivalents) was added to the reaction mixture drop-wise and stirred for an additional 3 h at 0 ^{o}C then the reaction was warmed up to room temperature. Finally, TEA (2.0 equivalents) was added to the solution, which was then stirred for an additional 1 h and monitored by TLC. Upon completion, the solution was concentrated *in vacuo*, and the residue was re-dissolved in chloroform,

subjected to an acid-base extraction, and purified via flash column chromatography to yield the pure heterocycle compound **13** in 88% yield over two steps.



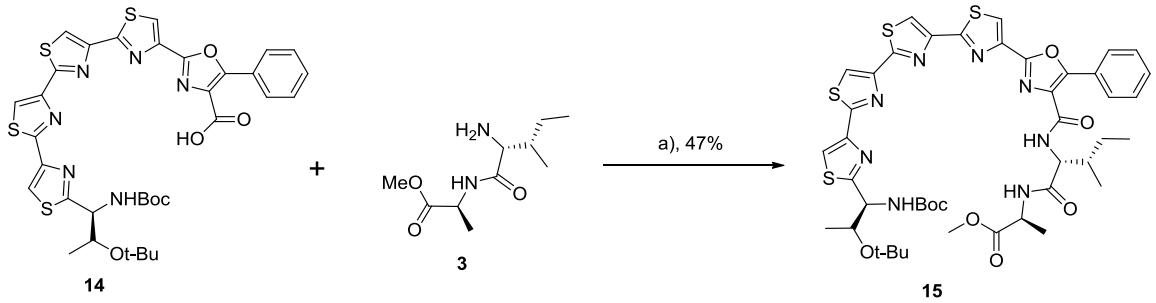
Conditions:
a) $KHCO_3$ (8.0 eq.), DME (0.05 M), 16 h, rt; b) pyridine (9.0 eq.), 5 min, 0 ^oC, TFAA (4.0 eq.), 3 h, 0 ^oC to rt then TEA (2.0 eq.) 1 h, rt, DME (0.05 M). c) LiOH (8.0 eq.), H_2O (cat.), MeOH (0.1 M)

Scheme 3.8 Synthesis of Ustat A-3 Heterocycle Fragment 13 and 14

Acid deprotection of the heterocycle fragment **13** was carried out by adding lithium hydroxide (LiOH, 8.0 equivalents) and dissolving the mixture in methanol (0.1 M) with a catalytic amount of water. The reaction was run for 8 h; upon completion, the solvent was evaporated *in vacuo* and the residue was diluted with DCM and extracted with 10% (v/v) $HCl_{(aq)}$ to give the resulting free acid **14** (Boc-Thr(*t*-Bu)-tetrathiazole-phenyloxazole-OH) in quantitative yield.

The protected linear precursor **15** (Boc-Thr(*t*-Bu)-tetrathiazole-phenyloxazole-D-*allo*-Ile-Ala-OMe) was synthesized by coupling the free acid **14** (1.0 equivalent) with the free amine fragment **3** (1.1 equivalents) using our standard peptide coupling procedure (**Scheme 3.9**). The starting materials, coupling reagent TBTU (1.1 equivalents) and DIPEA (4.0 equivalents) were dissolved in anhydrous DCM (0.1 M). The reaction was

stirred, and monitored via TLC and LC/MS. Upon completion, the reaction mixture was subjected to an acid-base extraction, followed by purification via flash column chromatography. Pure linear precursor **15** was furnished in 47%; the structure and purity was confirmed via ^{1}H NMR, LC/MS.

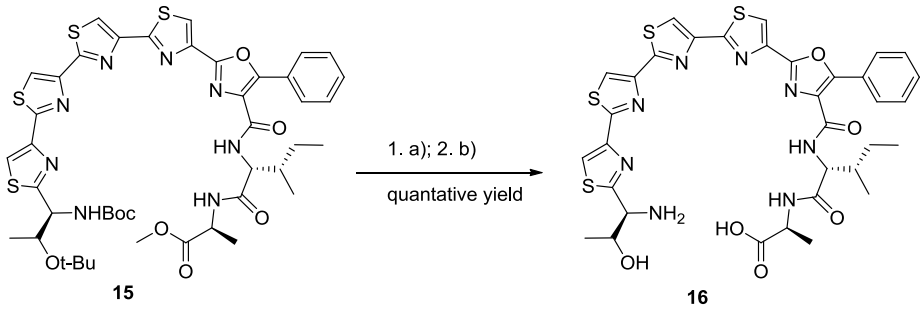


Conditions:
a) TBTU (1.1 eq.), DIPEA (4.0 eq.), DCM (0.1 M)

Scheme 3.9 Synthesis of Ustat A-3 Linear Precursor 15

3.5.5 Macrocyclization of Ustat A-3

Macrocyclization of Ustat A-3 begun with the sequential acid and amine deprotection of the linear precursor **15**. The acid was deprotected by dissolving the linear precursor **15** and LiOH (8.0 equivalents) in methanol (0.1 M) with a catalytic amount of water. The reaction was run for over 12 h and monitored by LC/MS. Upon completion, the solvent was evaporated *in vacuo* and the residue was re-dissolved in DCM, followed by an acid extraction using aqueous HCl (10% v/v) solution. The resulting Boc protected free acid (Boc-Thr(*t*-Bu)-tetrathiazole-phenyloxazole-D-*allo*-Ile-Ala-OH) was subjected to the amine deprotection without further purification (**Scheme 3.10**).



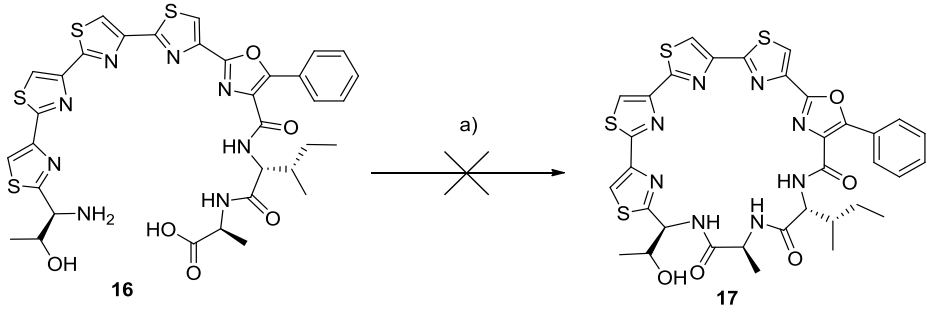
Conditions:
a) LiOH (8.0 eq.), H_2O (cat.), MeOH (0.1 M). b) TFA/DCM (1:1, 0.1 M), anisole (2.0 eq.).

Scheme 3.10 Double Deprotection of Linear Precursor for Ustat A-3

The simultaneous deprotection of secondary alcohol and amine was carried out by dissolving the linear precursor free acid in a mixture of TFA/DCM (1:1, 0.1 M) with anisole (2.0 equivalents). Upon completion, the crude mixture was concentrated *in vacuo* to yield the double deprotected linear precursor **16** (H-Thr-tetrathiazole-phenyloxazole-D-*allo*-Ile-Ala-OH), which was used without further purification.

Finally, macrocyclization of Ustat A-3 was carried out at low concentration of the peptide by activating the carboxylic acid moiety using the coupling reagent pentafluorophenyl diphenylphosphinate (FDPP) (**Scheme 3.11**). The double deprotected linear precursor **16** was dissolved in a mixture of anhydrous DMF/DCM (1:4, 0.8 mM), along with FDPP (1.5 equivalents) and DIPEA (7.0 equivalents). The reaction mixture was then stirred overnight under argon. In the first check point, LC/MS showed the disappearance of starting material, however, the desired cyclic product **17** was not observed. To facilitate reaction completion, additional amounts of FDPP and DIPEA were added to the mixture. Unfortunately, the main peaks appeared on LC/MS were

identified as the coupling reagent. The cyclized product was never observed in LC/MS, despite the reaction running for 3 days.



Condition:
a) FDPP (1.5 eq.), DIPEA (7.0 eq.), DMF/DCM (1:4, 0.8 mM), 3 d.

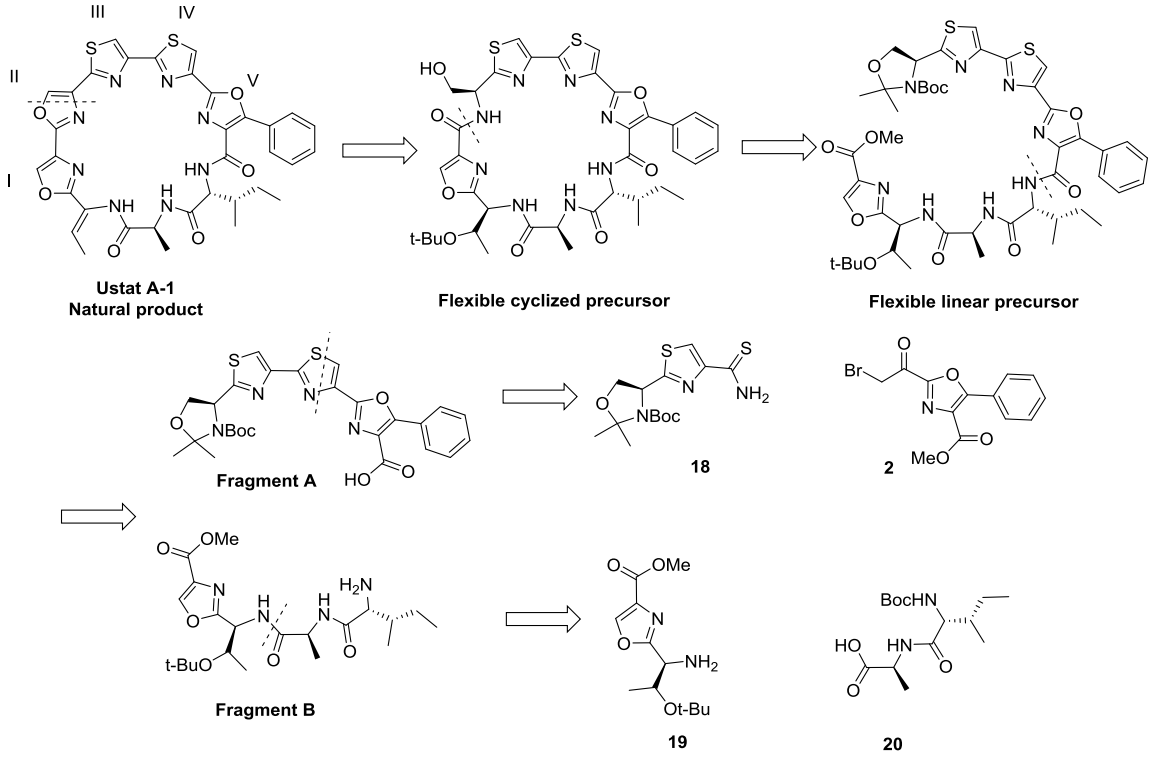
Scheme 3.11 Macrocyclization attempt of Ustat A-3

In addition to the cyclization attempt of Ustat A-3, the cyclization of Ustat A-1 and Ustat A-2 were also performed in our lab, where my colleagues used multiple coupling reagents (TBTU, HATU, and DEPBT) under highly dilute conditions. Unfortunately, the peaks of coupling reagents were observed and no trace of the cyclized molecule was found in LC/MS. These unsuccessful attempts to cyclize Ustat A and its derivatives underscored the necessity to re-design the synthetic strategy.

3.6 Retrosynthetic Approach of Ustat A-1

Based on the previous failed attempts, closing the ring between Thr and Ala residues of the linear precursor was no longer an option. The ring closing failure was likely due to poor flexibility of the heterocycle region, which hinders the two termini on the precursor from reaching each other to close. Thus, a new synthetic strategy in closing the ring was pursued and it was initiated on the synthesis of the Ustat A natural product (Ustat A-1). Our goal was to reduce the molecule's rigidity as a linear precursor by

forming the middle heterocycle after cyclization (**Scheme 3.12**). Ustat A natural product could be obtained via the oxazole formation on the position II of the cyclic precursor. The cyclic precursor could be synthesized by cyclizing the linear precursor, which could be formed by coupling the two heterocycle-containing fragments: Fragment A and Fragment B. Finally, Fragment A will be generated via the Hantzsch thiazole synthesis between the thioamide **18** and a pre-made bromoketone **2.** Fragment B could be derived from the oxazole **19** and the dipeptide **20** via peptide coupling.



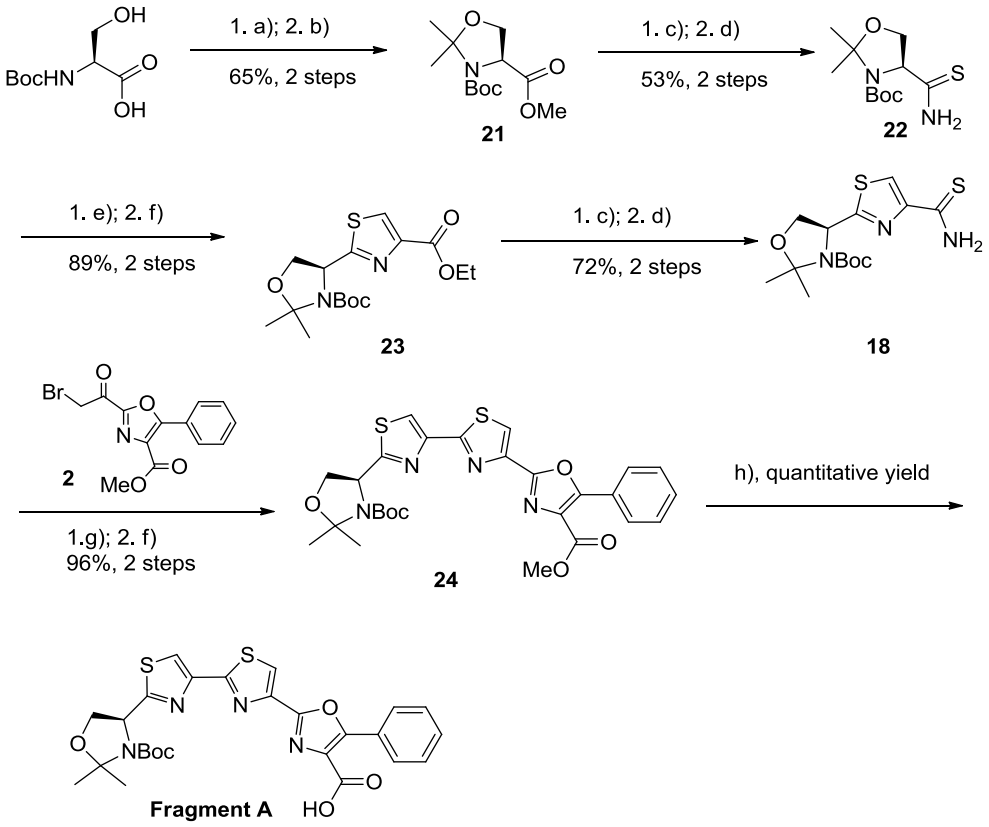
Scheme 3.12 Altered Synthetic Strategy of Ustat A-1 (natural product)

3.7 Synthesis of Ustat A-1

3.7.1 Synthesis of Fragment A for Ustat A-1

The main structural elements of Fragment A include one serine-derived dimethyloxazolidine, two thiazoles, and one phenyloxazole. The thiazole will be synthesized via the Hantzsch thiaozle synthesis and the phenyloxazole will be generated from Fragment **2** of Ustat A-3 (**Scheme 3.6**). The synthesis of Fragment A began with the construction of thiazole in the thioamide **18**. As shown in **Scheme 3.13**, Boc-Ser-OH was protected using dimethoxypropane (DMP) and pyridinium *p*-toluenesulfonate (PPTS) to form Boc-oxazolidine-OH, followed by esterification using TMSD to install the methyl ester. Boc-Ser-OH was dissolved in anhydrous THF in a round-bottom flask to make a solution of 0.17 M. DMP (10.0 equivalents) and PPTS (0.3 equivalent) was added, and the reaction was stirred, heated to reflux for 12 h, and then cooled to room temperature. Upon completion and confirmation by TLC and LC/MS, the reaction mixture was concentrated *in vacuo*. The residue was partitioned between EA and water. The aqueous layer was re-extracted with EA and the collected organic layer was dried over sodium sulfate, filtered, and concentrated *in vacuo.* The resulting crude oxazolidine free acid (Boc-oxazolidine-OH) was confirmed by ^{1}H NMR spectroscopy and taken on to the esterification without further purification. The crude Boc-oxazolidine-OH was dissolved in a mixture of anhydrous benzene and methanol (3:1, 0.1 M). TMSD (2.0 M in diethyl ether) was drop-wise added into the reaction mixture until the solution became slightly yellow. Upon completion, the solvent was removed *in vacuo* and the resulting crude methyl ester **21** (Boc-oxazolidine-OMe) was subjected to a flash column purification.

The pure ester **21** was furnished in 65% over two steps and the structure was confirmed by ^{1}H NMR spectroscopy.



Condition:
a) DMP (10.0 eq.), PPTS (0.3 eq.), THF (0.17 M), reflux, 12 h. b) TMSD, Benzene: MeOH (3:1, 0.1 M). c) NH_4OH : MeOH (1:1, 0.05 M). (d) Lawesson's Reagent (0.8 eq.), THF (0.1 M), reflux, 12 h. e) $KHCO_3$ (8.0 eq.), $BrCH_2COCO_2Et$ (3.0 eq.), DME (0.05 M), 16 h, rt. f) pyridine (9.0 eq.), 5 min, 0 ^{o}C, TFAA (4.0 eq.), 3 h, 0 ^{o}C to rt then TEA (2.0 eq.), 1 h, rt, DME (0.05 M). g) $KHCO_3$ (8.0 eq.), DME (0.05 M), 16 h, rt. h) LiOH (8.0 eq.), MeOH (0.1 M).

Scheme 3.13 Synthesis of Ustat A-1 Fragment A

The ester **21** was then subjected to a two-step thioamide conversion using ammonium hydroxide and then LR. It was dissolved in a mixture of ammonium hydroxide aqueous solution and methanol (1:1, 0.05 M). The solution was stirred and

monitored via TLC. Upon completion, the solution was concentrated *in vacuo* to afford the desired amide (Boc-oxazolidine-NH_2) as confirmed by ^{1}H NMR. The amide was used for next step without further purification.

The amide and LR (0.8 equivalent) were dissolved in anhydrous THF to the concentration of 0.1 M, and the reaction mixture was heated to reflux under argon. Upon confirmation of a new spot by TLC and disappearance of starting material, the solvent was evaporated *in vacuo*, and the residue was purified via flash column chromatography. The pure thioamide **22** (Boc-oxazolidine-SNH_2) was furnished in 53% over two steps. The structure and purity were confirmed via ^{1}H NMR.

Next, the thioamide **22** was converted to a thioazole using the Hantzsch procedure (**Scheme 3.13**). The thioamide **22** and potassium bicarbonate ($KHCO_3$, 8.0 equivalents) were dissolved in anhydrous DME (0.05 M). The slurry solution was stirred for 5 min, followed by the drop-wise addition of ethyl bromopyruvate (3.0 equivalents). The reaction was run for 16 h at room temperature and monitored by TLC. Upon disappearance of starting material, the solvent was evaporated *in vacuo*. The residue was partitioned in chloroform and deionized water. The combined organic layer was dried over sodium sulfate, filtered, and concentrated *in vacuo* to afford the thiazoline intermediate. The crude intermediate was dissolved in anhydrous DME (0.05 M), stirred and cooled to 0 ^{o}C. Pyridine (9.0 equivalents) was then added drop-wise to the stirred solution. After stirring for 5 min, TFAA (4.0 equivalents) was added drop-wise to the DME solution and stirred for an additional 3 h at 0 ^{o}C then warmed up to room temperature. Finally, 2 equivalents of TEA were added to the reaction mixture, which was then stirred for an additional 1 h and monitored by TLC. Upon completion, the

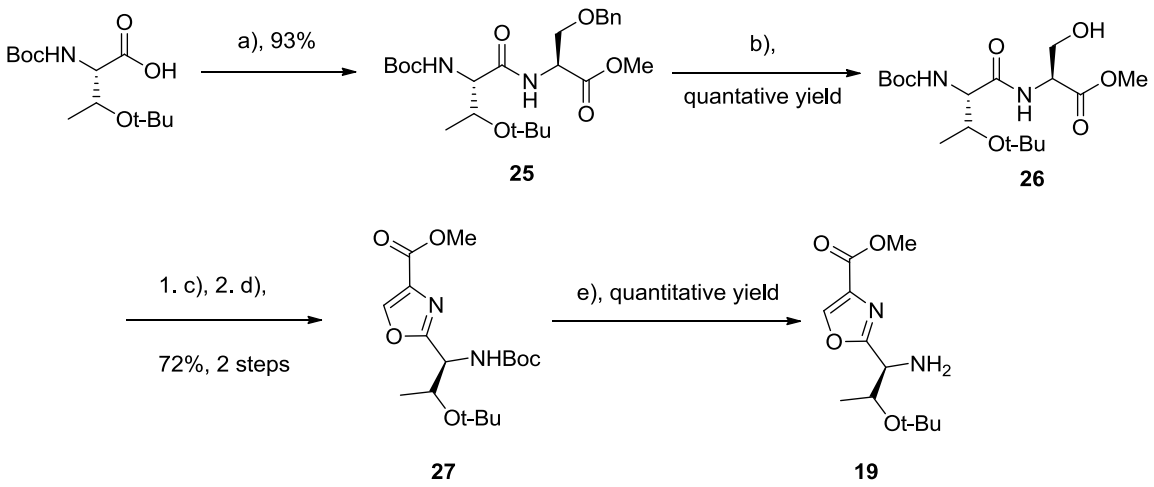
solution was concentrated *in vacuo*. The residue was diluted with chloroform and subjected to an acid-base wash. The combined organic layer was concentrated *in vacuo* and then purified via flash column chromatography to yield the pure thiazole **23** (Boc-oxazolidine-thiazole-OEt) in 89% yield over two steps.

The thiazole of **23** was then converted to the thioamide via the condition using ammonium hydroxide then LR (0.8 equivalent). The pure thioamide **18** (Boc-oxazolidine-thiazole-SNH_2) was furnished in 72% yield over two steps. With the building subunits **18** and **2** in hand, the synthesis of fragment A was initiated by the Hantzsch thiazole synthesis using one equivalent of **18** and three equivalents of **2** to furnish the trisazole **24** in an excellent 96% yield over two steps. Acid deprotection was completed using LiOH (8.0 equivalents) in methanol (0.1 M) to afford the desired Fragment A (Boc-oxazolidine-dithiazole-phenyloxazole-OH) in quantitative yield.

3.7.2 Synthesis of Fragment B for Ustat A-1

The synthesis of Fragment B began with the formation of the oxazle **19** (Boc-Thr(*t*-Bu)-oxazole-OMe), which was derived from a protected threonine free acid (**Scheme 3.14**). Boc-Thr(*t*-Bu)-OH (1.0 equivalents), H-Ser(Bn)-OMe (1.2 equivalents), and TBTU (1.2 equivalents) were dissolved in anhydrous DCM (0.1 M) and DIPEA (4.0 equivalents) was added slowly to the DCM solution. The reaction mixture was stirred for 30 min and monitored by TLC. Upon disappearance of starting material, the solution was concentrated in vacuo , subjected to an acid-base wash, and purified by flash column chromatography to give the pure dipeptide **25** (Boc-Thr(*t*-Bu)-Ser(Bn)-OMe) in 93% yield.

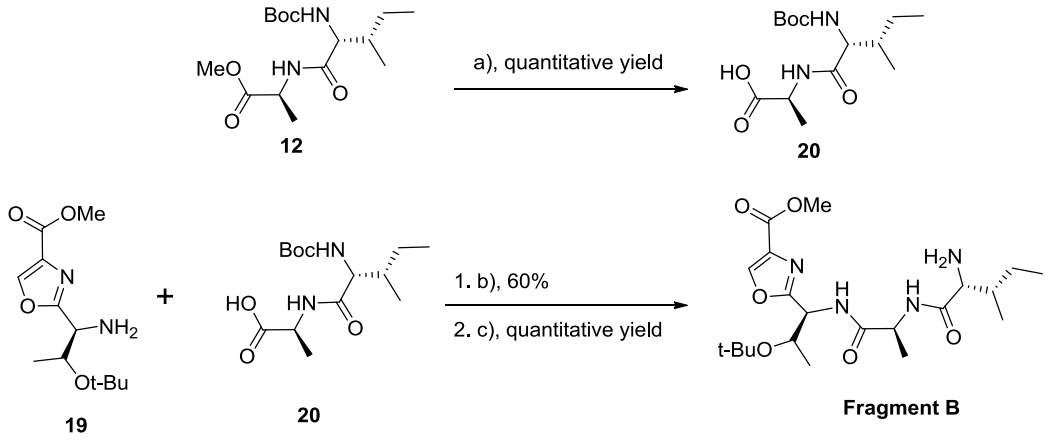
In order to prepare the hydroxyl moiety for the next oxazole formation, dipeptide **25** was subjected to hydrogenlysis, thereby removing the benzyl group using palladium black (10% w/w) in absolute ethanol (0.1 M). After purging with hydrogen for 3 h, the reaction was run for an additional 12 h. When completed, the used palladium black was filtered by Celite® and the filtrate was concentrated to furnish the β-hydroxyl containing dipeptide **26** (Boc-Thr(*t*-Bu)-Ser-OMe) in quantitative yield. The DAST protocol was then applied on the dipeptide **26** to generate the oxazoline intermediate. Oxidation of the oxazoline with $BrCCl_3$furnished the oxazole **27** (Boc-Thr(*t*-Bu)-oxazole-OMe) in 72% yield over two steps. Finally, selective removal of Boc group[30] was performed on **27** yielding the desired free amine **19** quantitatively.



Conditions:
a) NH_2-Ser(Bn)-OMe (1.2 eq.), TBTU (1.2 eq.), DIPEA (4 eq.), DCM (0.1 M). b) H_2, Pd (10% w/w), EtOH (0.1 M). c) DAST (1.1 eq.), DCM (0.1 M), 30 min, -78 ^oC then K_2CO_3, 1 h, -78 ^oC to rt. d) DBU (2.0 eq.) DCM (0.1 M), 10 min, -78 ^oC, $BrCCl_3$ (2.0 eq.), 12 h, -78 ^oC to rt. e) 4M HCl/dioxane

Scheme 3.14 Synthesis of the oxazole moiety for Ustat A-1 Fragment B

The pre-made dipeptide **12** (**Scheme 3.7**) was acid deprotected using LiOH (8.0 equivalents) and methanol (0.1 M) generating the free acid **20** (Boc-D-*allo*-Ile-Ala-OH). The free amine **19** was then coupled with the free acid **20** to furnish the Boc-protected Fragment B (Boc-D-*allo*-Ile-Ala-Thr(*t*-Bu)-oxazole-OMe) in a 60% yield. **S**elective Boc removal was performed to generate the free amine Fragment B in quantitative yield.



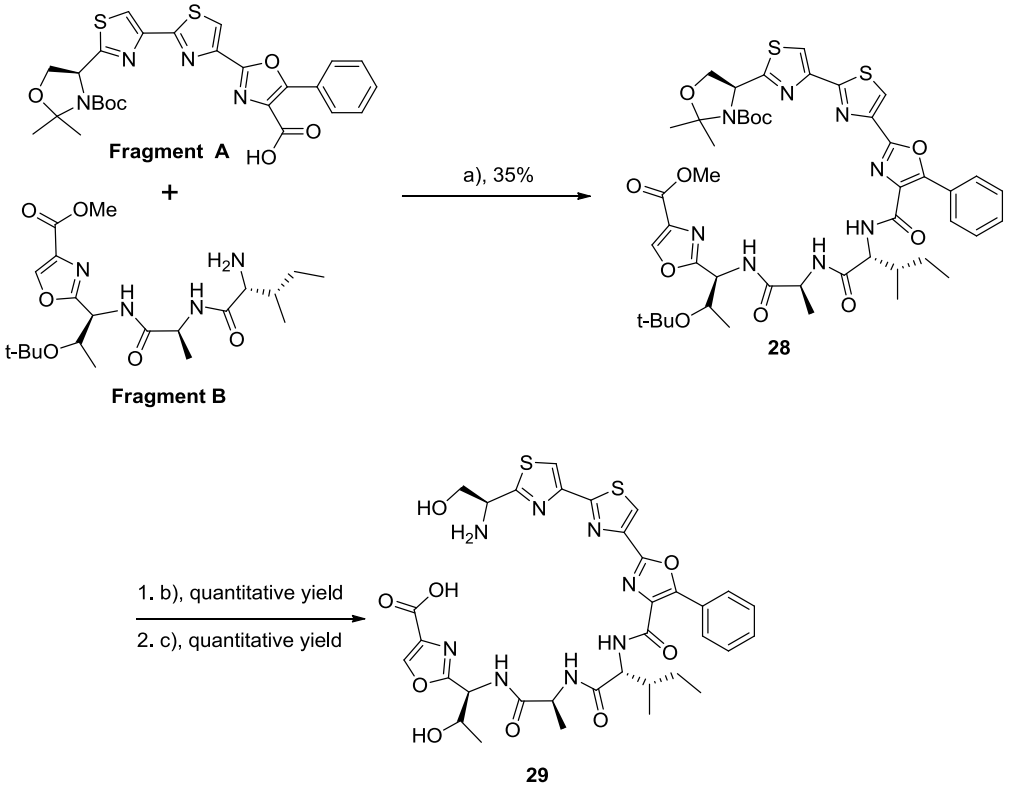
Conditions:
(a) LiOH (8.0 eq.), MeOH (0.1 M). b) HATU (0.4 eq.), DMTMM (0.8 eq.), DIPEA (4.0 eq.), DCM (0.05 M). c) 4M HCl/dioxane

Scheme 3.15 Synthesis of Ustat A-1 Fragment B

3.7.3 Synthesis of Linear Precursors for Ustat A-1

The linear precursor **28** (**Scheme 3.16**) was synthesized by peptide coupling between the free acid fragment A (1.0 equivalent) and the free amine fragment B (1.2 equivalents) using HATU, DMTMM, PyBrOP (0.6 equivalent of each coupling reagents), and DIPEA (4.0 equivalents) in an anhydrous DCM solution (0.025 M). Upon completion, as confirmed via LC/MS, the reaction mixture was extracted via an acid-base wash, followed by a flash column chromatography, yielding the linear precursor **28** (Boc-

oxazolidine-dithiazole-phenyloxazole-D-*allo*-Ile-Ala-Thr(*t*-Bu)-oxazole-OMe) in 35% yield. Next, the methyl ester of **28** was hydrolyzed using LiOH (8.0 equivalents) in methanol (0.1 M), followed by the amine deprotection using 4M HCl/dioxzane to selectively remove the Boc group of **28**. Unfortunately, instead of yielding the desired product with the intact *tert*-butyl group, the linear precursor **29** with a free Thr residue was found during this step.

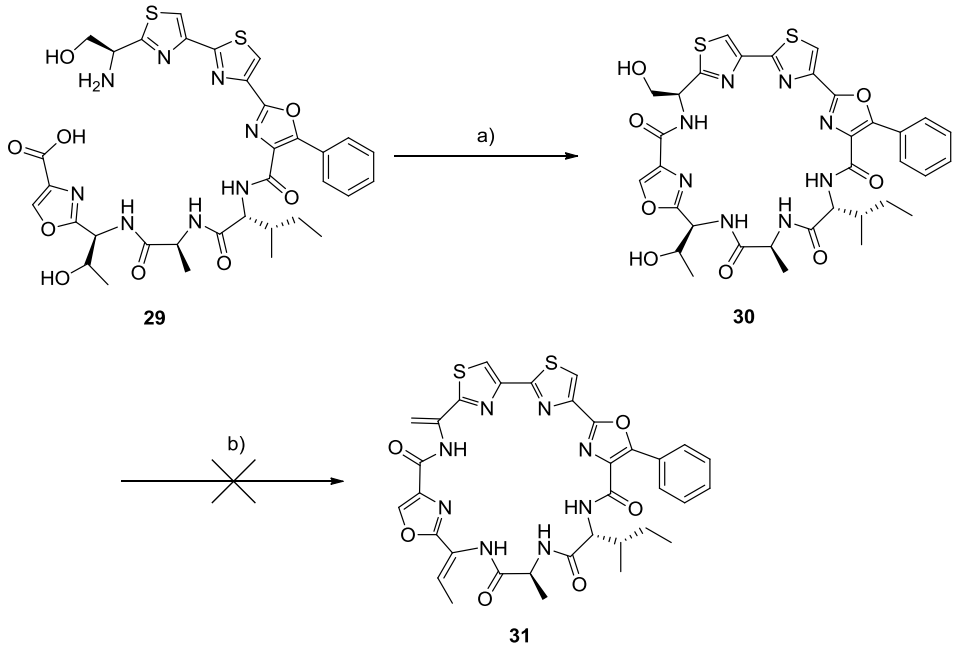


Conditions:
a) HATU (0.6 eq.), DMTMM (0.6 eq.), DIPEA (4.0 eq.), DCM (0.025 M). b) LiOH (8.0 eq.), MeOH (0.1 M). c) 4M HCL/dixoane.

Scheme 3.16 Synthesis of double deprotected linear precursor for Ustat A-1

3.7.4 Macrocyclization of Ustat A-1

The double deprotected linear precursor **29** was then subjected to macrocyclization under highly dilute conditions (**Scheme 3.17**). Combining **29**, coupling reagents (HATU, DMTMM, and T3P), DMAP (0.2 equivalent) and DIPEA (8.0 equivalents) in a mixture of anhydrous DCM/DMF solution (1:1) generating a 2 mM solution. The reaction was run for approximately 12 h under nitrogen and the mass of cyclized compound **30** was observed by LC/MS. Upon completion, as confirmed by LC/MS, the solvent was removed *in vacuo*. The residue was diluted with DCM and then extracted via an acid-base wash to furnish the crude cyclized compound **30**.



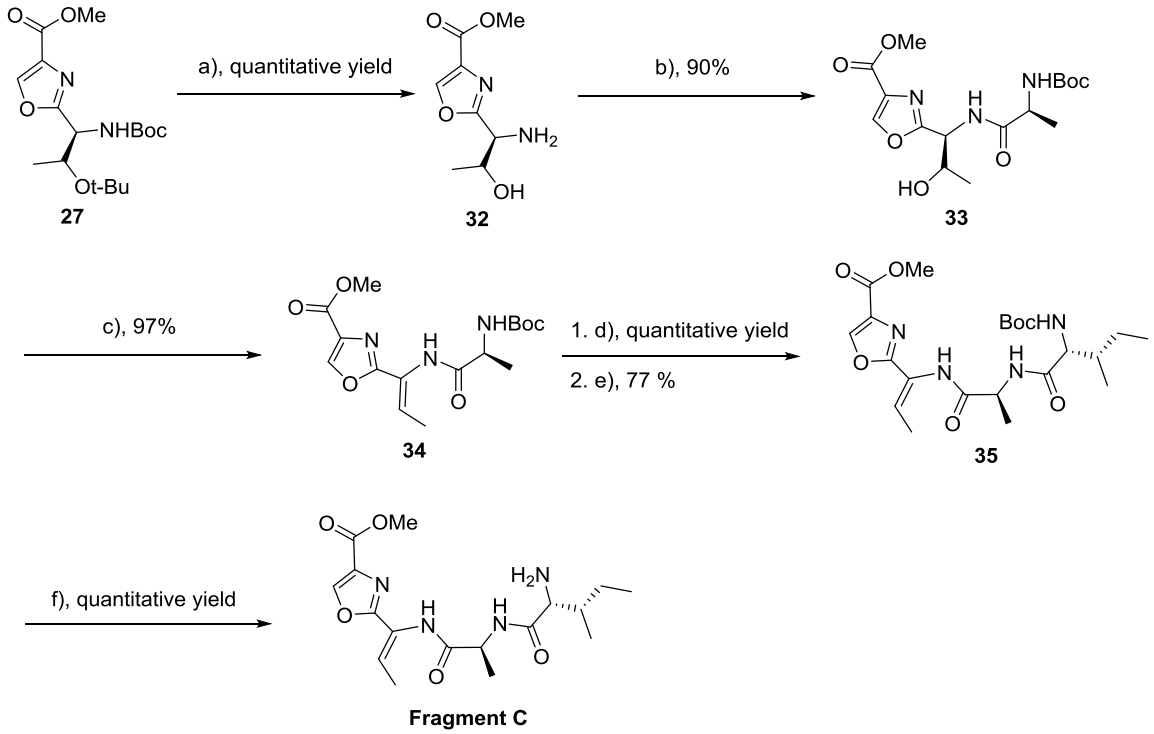
Conditions:
a) HATU (0.6 eq.), DMTMM (0.6 eq.), T3P (0.8 eq.), DMAP (0.2 eq.) DIPEA (8.0 eq.), DCM/DMF (1:1, 2 mM). e) MsCl (8.0 eq.), DBU (10.0 eq.), DCM (8 mM), 0 ^{o}C to rt.

Scheme 3.17 Macrocyclization of Ustat A-1

At this stage, the two hydroxyl groups in **30** are problematic because the DAST/DBU protocol is likely to fluorinate both hydroxyl groups. To address this issue, we planned to form the exocyclic double bond first using MsCl/DBU, then form the final oxazole.[22] Thus, the crude cyclized compound **30** was subjected to MsCl/DBU elimination. Unfortunately, the mass of target compound **31** was only observed in trace amount with the major product was a mixture of mono-chlorinated molecules. Thus, we needed a new synthetic strategy that avoided having two hydroxyl groups on the macrocycle.

3.7.5 Synthesis of Fragment C

There are two possible options to avoid having two hydroxyl groups within the macrocycle: 1) replace the *t*-Bu group with a robust protecting group (TBDPS); or 2) form the enamide moiety prior to the macrocyclization. We chose to explore option 2) first (**Scheme 3.18**). Fragment C was synthesized to replace Fragment B in the Ustat A synthetic route. The synthesis of Fragment C began with the amine deprotection of the oxazole molecule **27** using a mixture of TFA/DCM (1:1, 0.1 M) and anisole (2.0 equivalents) to furnish the free amine **32** in quantitative yield. Peptide coupling of the free amine **32** and Alanine free acid delivered the pseudopeptide **33** (Boc-Ala-Thr-oxazole-OMe) (**Scheme 3.18**). The free amine **32** (1.1 equivalents) was coupled with the free acid (1.0 equivalent) using DMTMM (2.0 equivalents) and DIPEA (8.0 equivalents) in anhydrous DCM solution (0.1 M). The pure pseudopeptide **33** was afforded in 90% yield via flash column purification.



Conditions:
a) 50% TFA/DCM (0.1 M), anisole (2.0 eq.). b) BocNH-Ala-OH (1.0 eq.), DMTMM (2.0 eq.), DIPEA (8.0 eq.), DCM (0.1 M). c) MsCl (8.0 eq.), TEA (10.0 eq.), DCM (0.05 M). d) 20% TFA/DCM (0.1 M), anisole (2.0 eq.). e) BocNH-D-*allo*-Ile-OH (1.0 eq.), DMTMM (1.5 eq.), DIPEA (8.0 eq.), DCM (0.05 M). f) 4M HCl/dioxane

Scheme 3.18 Synthesis of Ustat A-1 Fragment C

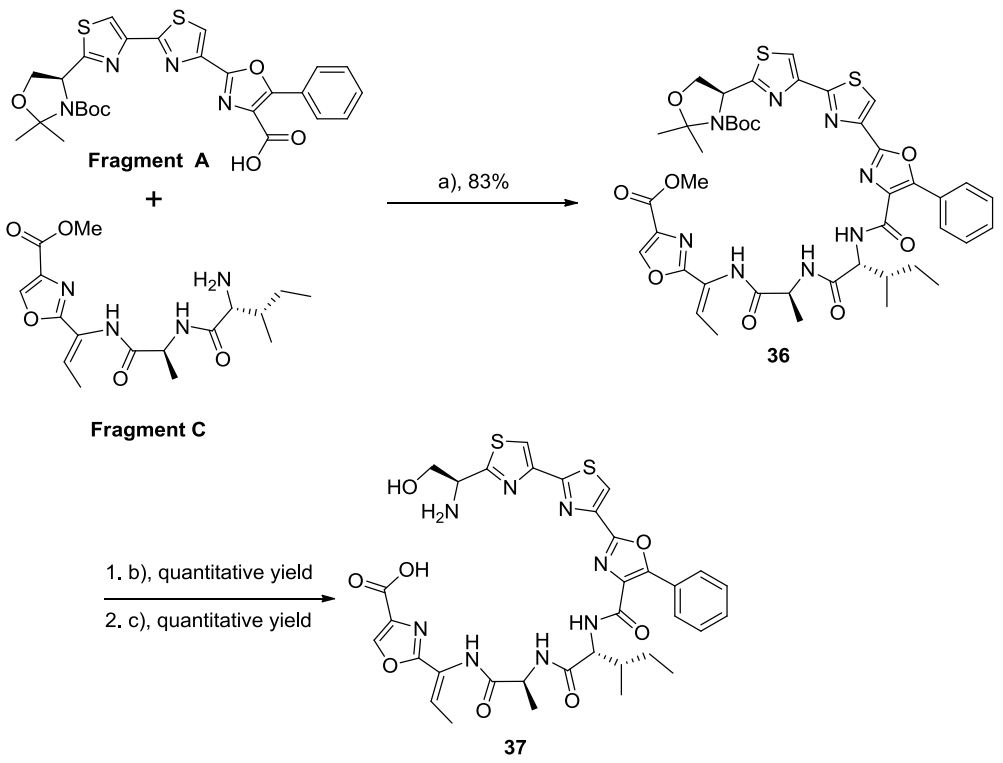
The pseudopeptide **33** was dissolved in anhydrous DCM (0.05 M) under nitrogen and triethylamine (TEA, 10.0 equivalents) was slowly added to the solution. After stirring for 5 min, MsCl (8.0 equivalents) was added into the solution. When TLC showed no starting material remained, the reaction mixture was extracted with deionized water and the aqueous layer was extracted twice with DCM. The combined organic layers were concentrated *in vacuo* and the residue was re-dissolved in anhydrous DCM (0.05 M) under nitrogen. TEA (10.0 equivalents) was added to the DCM solution, which was then

stirred for 8 h. Upon completion, the solution was extracted with deionized water, followed by a flash column chromatography to furnish the pure pseudopeptide **34** (Boc-Ala-enamide-oxazole-OMe) in 97% yield. The structure and conformation were confirmed by ^{1}H NMR and NOESY.

Amine deprotection of **34** using 20% TFA produced the free amine H-Ala-enamide-oxazole-OMe in quantitative yield (**Scheme 3.18**). The resulting free amine was coupled with the free acid Boc-D-allo-Ile-OH using DMTMM (1.5 equivalents) and DIPEA (8.0 equivalents) to afford the pseudopeptide **35** in 77% yield. Finally, amine deprotection using 4M HCl generated the desire Fragment C in quantitative yield.

3.7.6 Synthesis of Linear Precursors from Fragment C

The linear precursor **36** was produced via peptide coupling between the free acid fragment A (1.0 equivalent) and the free amine fragment C (1.1 equivalent) using HATU, DMTMM, PyBrOP (0.7 equivalent of each coupling reagents), and DIPEA (8.0 equivalents) in an anhydrous DCM solution (0.01 M) (**Scheme 3.19**). Upon completion, which was confirmed by LC/MS, the reaction mixture was extracted via an acid-base wash, purified via a flash column chromatography to yield the linear precursor **36** (Boc-oxazolidine-dithiazole-phenyloxazole-D-*allo*-Ile-Ala-enamide-oxazole-OMe) in 83% yield. The linear precursor **36** was subjected to acid deprotection using LiOH in methanol to hydrolyze the methyl ester. Subsequent amine deprotection using 4M HCl/dioxzane produced the double deprotected linear precursor **37** in quantitative yield. The identity of compound **37** was confirmed using LC/MS.



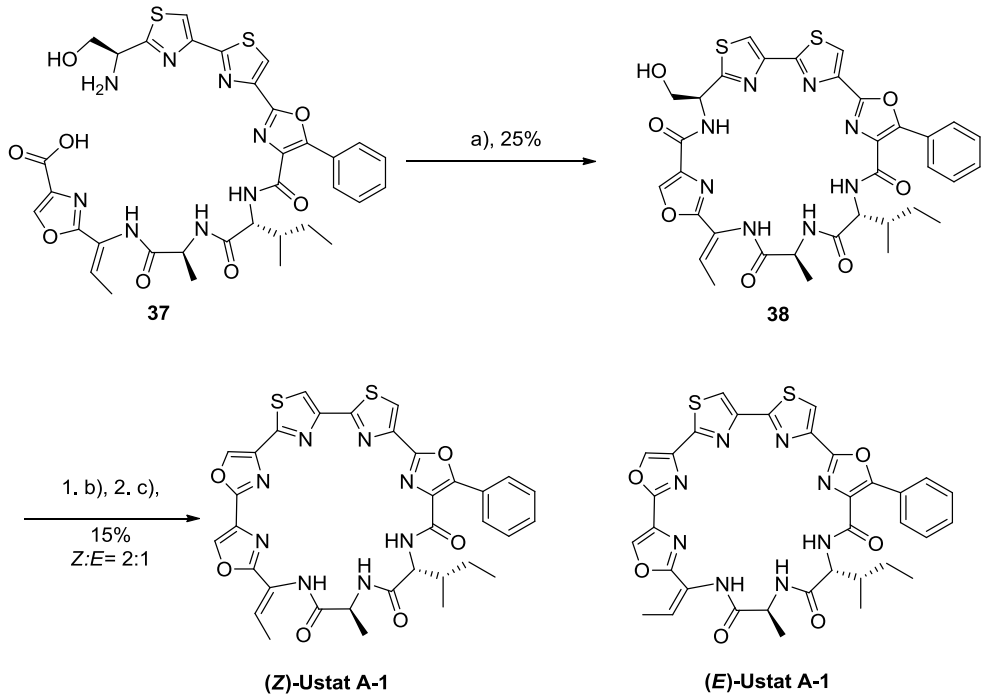
Conditions:
a) HATU (0.7 eq.), DMTMM (0.7 eq.), PyBroP (0.7 eq.), DIPEA (8.0 eq.), DCM (0.01 M). b) LiOH (8.0 eq.), MeOH (0.1 M); c) 4M HCL/dixoane

Scheme 3.19 Synthesis of Linear Precursors from Fragment C

3.7.7 Synthesis of Ustat A-1

The cyclic precursor **38** was macrocyclized from **37** via peptide coupling condition using a cocktail of coupling reagents (**Scheme 3.20**). The linear precursor **37** was dissolved in half the total volume of anhydrous DCM/DMF (1:1) for an overall concentration of 1.7 mM. Coupling agents HATU, DMTMM, T3P (0.7 equivalent of each), and DIPEA (8.0 equivalents) were dissolved in a round bottom flask with the remaining half of the total volume. The solution of **37** was added to the reaction flask containing multiple coupling reagents via a syringe pump at a rate of 0.5 mL/min. The

mixture was stirred overnight and monitored by LC/MS. Upon completion, the reaction mixture was subjected to an acid-base extraction, followed by a HPLC purification to generate the cyclic precursor **38** in 25% yield.



37 **38** **(*Z*)-Ustat A-1** **(*E*)-Ustat A-1**

Conditions:
a) HATU (0.7 eq.), DMTMM (0.7 eq.), T3P (0.7 eq.), DIPEA (8.0 eq.), DCM/DMF (1:1, 1.7 mM). b) DAST (20.0 eq.), THF (6 mM), 30 min, -78 ^{o}C then pyridine (20.0 eq.), 12 h, -78 ^{o}C to rt. c) DBU (10.0 eq.), DCM (0.01 M), -78 ^{o}C, $BrCCl_3$ (10.0 eq), 12 h, -78 ^{o}C to rt.

Scheme 3.20 Synthesis of Ustat A-1

The cyclic precursor **38** was subjected to the DAST/DBU protocol using DAST (20.0 equivalents) and pyridine (20.0 equivalents) in THF (6 mM) to from the oxazoline intermediate, followed by the oxidation using $BrCCl_3$ (10.0 equivalents) and DBU (10.0 equivalents) to yield the oxazole at position II (**Scheme 3.20**). The crude product was purified via HPLC and the pure Ustat A molecule was furnished in 15% yield.

Interestingly, the ^{1}H NMR of the purified Ustat A showed two sets of signals, which indicated there are two molecules in the NMR sample. After careful examination based on the ^{1}H NMR and 2D NMR (HSQC, HMBC, NOESY), it was determined that there were *E*/*Z* isomers of Ustat A with the *E*: *Z* ratio of 1:2 around the enamide. Prior to the formation of the natural product, the *Z*-enamide was the only product observed. Thus, I believe the produced HF during the fluorination step induced the isomerization.

3.8 Biological activity of Ustat A and its fragments

Screening natural product-derived fragments has served an important purpose in fragment-based drug discovery.[31] Previous work has successfully shown that the structure of fragments can be modified to generate drug candidates that have optimized taeget-binding affinity.[32] Thus, fragments of Ustat A were evaluated for their biological activity and this study would provide insights into the minimum size required for biological activity.

During the synthesis toward Ustat A natural product and derivatives, the heterocycle-containing fragments were evaluated for their anticancer potency against Human colon cancer cell line HCT-116 using Cell Counting Kit-8 (CCK8) assay. The biological assays were done by my colleagues Dimple Rananaware and Worawan Tantisantisom. The colon cancer cells were treated with an overall 40 μM concentration of Ustat A-3 fragments or media control for a period of 72 h. CCK8 reagent was then added to the compound treated cells. The water soluble reagent was reduced by the dehydrogenase in living cells to produce an orange colored formazan dye. The amount of generated orange formazan is proportional to the amount of viable cells. Utilizing a

microplate reader to measure the intensity of orange color at 450 nm, the cell viability was determined colorimetrically.

The cytotoxicity of Ustat A fragments is summarized in **Figure 3.14**. We observed that incorporating oxazoles into fragments (i.e. **27, 39** and **40**) did not increase the anti-cancer potency.

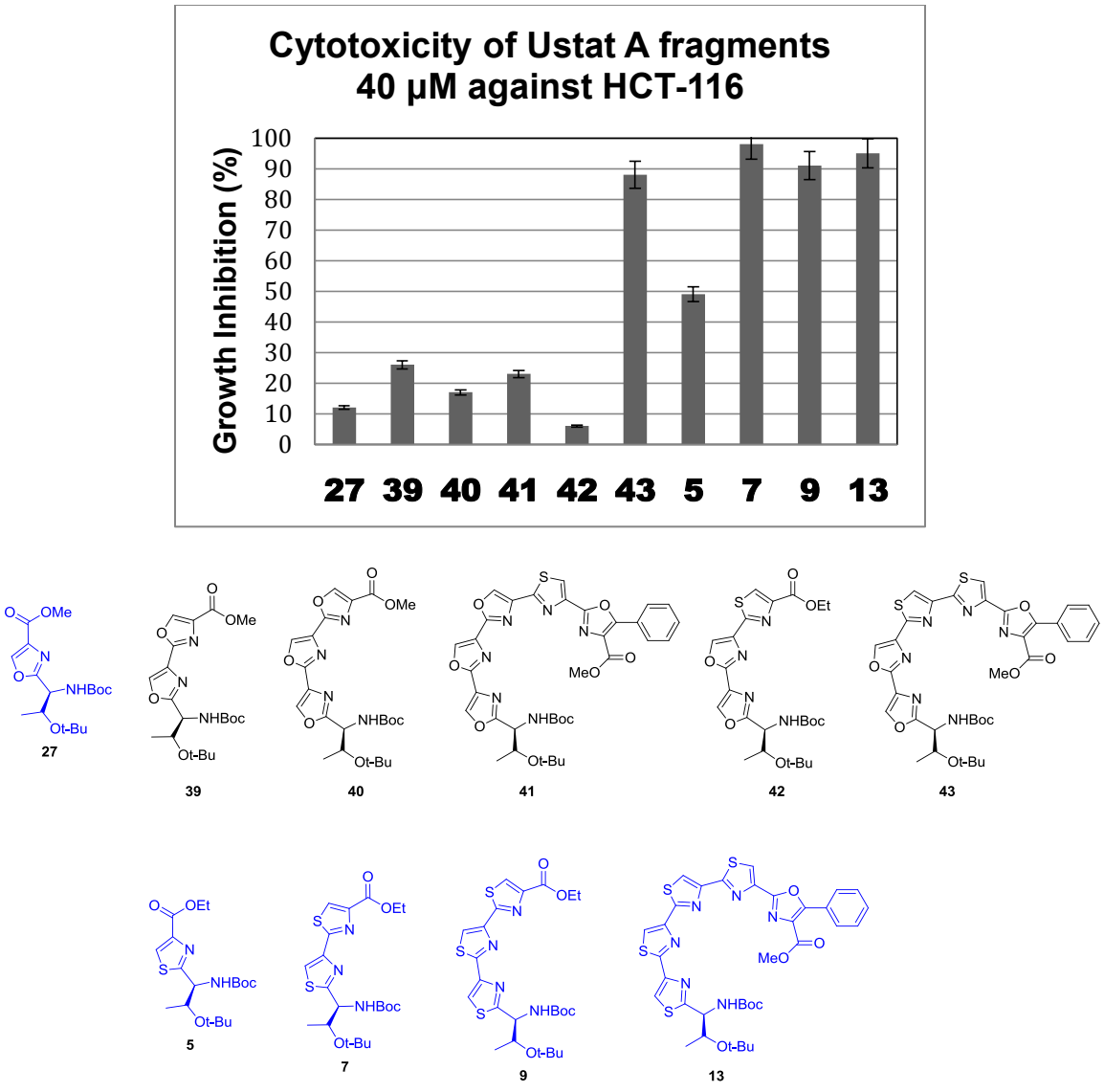


Figure 3.14 Growth Inhibition of Ustat A heterocycle-containing fragments against drug-resist colon cancer cell line HCT-116 at 40 μM (The blue colored molecules were synthesized by me)

On the contrary, incorporating thiazoles into fragments (i.e. **5, 7** and **9**) dramatically enhanced the cytotoxicity. In addition, only when fragments that contain two or more directly-linked thiazoles exhibited greater than 50% growth inhibition (**43**, **7**, **9**, and **13**). These observations indicated that multiple directly-linked thiazoles are essential for cytotoxicity.

We evaluated the IC_{50} values (**Table 3.1**) of non-potent compound **27** and the potent fragments **7**, **9**, **13** and **43** (defined as ≥50% cytotoxicity against HCT-116 at 40 μM). The penta-azoles, compounds **13** and **43,** exhibited the highest potency among the potent compounds. This phenomenon indicated that five consecutive heterocycles containing multiple directly-linked thiazoles are optimal for maximum cytotoxicity against the HCT-116 cancer cell line.

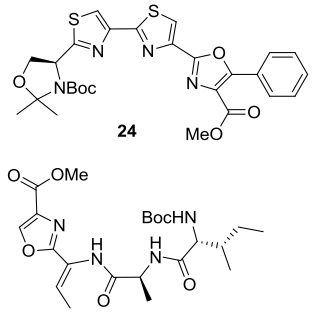
Table 3.1 IC_{50} values of potent Ustat A heterocycle-containing fragments against drug-resist colon cancer cell line HCT-116 (100 μM is the highest test concentration)

Fragments	**27**	**7**	**9**	**13**	**43**
IC_{50} (μM)	>100	19.4	26.5	18.7	18.2

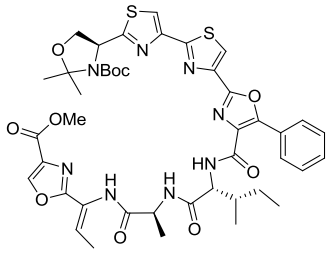
In addition, we applied the established evaluating model to test the anti-cancer potency of the fragments derived from the successful Ustat A synthetic route. Compounds **24**, **35**, **36**, **38** and Ustat A were tested for growth inhibition against HCT-116 cancer cell line. The cytotoxicity values were summarized in **Table 3.2**. Interestingly, compound **24** showed an 8 μM GI_{50} value, whereas compounds **35** and **36** exhibited no cytotoxicity. This result indicates that the upper hemisphere of Ustat A is essential for the anti-cancer potency. It also matches the previous observation that

multiple directly-linked thiazoles are essential for cytotoxicity. Despite containing directly-linked thiazoles within the structure, compound **38** exhibited less potency than **2**. This observation suggests that the conformation of the macrocycle affects its potency. Finally, Ustat A exhibited low nanomolar cytotoxicity, which is similar to the number in the published report.

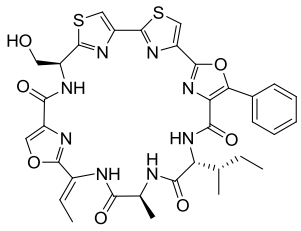
Table 3.2 IC_{50} values of Ustat A derived compounds against drug-resist colon cancer cell line HCT-116 (*% growth inhibition at 40 μM)



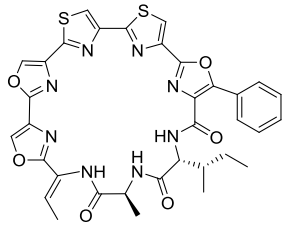
35



36



38



Ustat A (E/Z *mixture*)

Compounds	**24**	**35**	**36**	**38**	**Ustat A**
%GI*	99	10	0	100	100
IC_{50} (μM)	8	N/A	N/A	30	0.02

3.9 Conclusion

On the journey toward the synthesis of Ustat A-3, the convergent synthetic strategy was employed to generate the linear precursor of Ustat A-3 from three small fragments: Fragment **1**, **2**, and **3**. Fragment **1** was synthesized from the commercially available chemicals: Boc-Thr(*t*-Bu)-OH and ethyl bromopyruvate. So were Fragment **2**, and **3** from small starting materials. I was able to efficiently transform these starting materials to three consecutive thiazoles using Hantzsch thiazole synthesis conditions. Although synthesis of the phenyloxazole was tricky, I was also able to make Fragment **2**. The unsuccessful cyclization attempt for Ustat A-3 indicated that the rigidity of the linear precursor stops macrocyclization, and therefore the ring closure site must be chosen carefully. I re-designed the synthetic route and was able to synthesize Ustat A-1, the natural product. The linear precursor of Ustat A natural product was assembled from two fragments and the macrocycle was formed by peptide coupling between the oxazole at position I and the thiazole at position III. Although *E/Z* isomerization was observed in the end of synthesis, it was the first practicable synthetic route for the Ustat A natural product. Finally, Ustat A fragments were tested for cytotoxicity against colon cancer cell line HCT-116. The results showed that the penta-azole fragments (with at least two directly-linked thiazole in the structure) could optimize the anti-cancer potency. The cytotoxicity assay of Ustat A natural product indicated that the anti-cancer potency is resulted from the upper hemisphere of Ustat A and also affected by the conformation of the macrocycle. In addition, the configuration of the enamide seemed to have little effect on the potency. The new synthetic route to address the *E/Z* isomerization is currently underway and the mechanistic study of Usata A will be investigated in the near future.

Chapter 3, in part, is a reprint of material as it appears in "Total Synthesis and Biological Activity of Natural Product Urukthapelstatin A" *Organic Letters*, **2013**, 15, 3574-3577. Lin, C. –C.; Tantisantisom, W.; McAlpine, S. R. The dissertation author was the primary investigator and author of this paper.

3.10 References

1. (a) Jin, Z., Muscarine, imidazole, oxazole, and thiazole alkaloids. *Natural Product Reports* **2003,** *20* (6), 584-605; (b) Blunt, J. W.; Copp, B. R.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R., Marine natural products. *Natural Product Reports* **2005,** *22* (1), 15-61; (c) Jin, Z., Muscarine, imidazole, oxazole and thiazole alkaloids. *Natural Product Reports* **2009,** *26* (3), 382-445; (d) Turner, C.; Liang, H., Recent Advances in the Assembly of Tri-substituted Oxazoles. *Current Organic Chemistry* **2011,** *15* (16), 2846-2870; (e) Jin, Z., Muscarine, imidazole, oxazole, and thiazole alkaloids. *Natural Product Reports* **2011,** *28* (6), 1143-1191.

2. Roesener, J. A.; Scheuer, P. J., Ulapualide A and B, extraordinary antitumor macrolides from nudibranch eggmasses. *Journal of the American Chemical Society* **1986,** *108* (4), 846-847.

3. Ogino, J.; Moore, R. E.; Patterson, G. M. L.; Smith, C. D., Dendroamides, New Cyclic Hexapeptides from a Blue-Green Alga. Multidrug-Resistance Reversing Activity of Dendroamide A. *Journal of Natural Products* **1996,** *59* (6), 581-586.

4. (a) Matsuo, Y.; Kanoh, K.; Yamori, T.; Kasai, H.; Katsuta, A.; Adachi, K.; Shin-ya, K.; Shizuri, Y., Urukthapelstatin A, a Novel Cytotoxic Substance from Marine-derived Mechercharimyces asporophorigenens YM11-542. *J. Antibiot.* **2007,** *60* (4), 251-255; (b) Matsuo, Y.; Kanoh, K.; Imagawa, H.; Adachi, K.; Nishizawa, M.; Shizuri, Y., Urukthapelstatin A, a Novel Cytotoxic Substance from Marine-derived Mechercharimyces asporophorigenens YM11-542. *J. Antibiot.* **2007,** *60* (4), 256-260.

5. Riego, E.; Hernández, D.; Albericio, F.; Álvarez, M., Directly Linked Polyazoles: Important Moieties in Natural Products. *Synthesis* **2005,** *2005* (12), 1907-1922.

6. Lindquist, N.; Fenical, W.; Van Duyne, G. D.; Clardy, J., Isolation and structure determination of diazonamides A and B, unusual cytotoxic metabolites from the marine ascidian Diazona chinensis. *Journal of the American Chemical Society* **1991,** *113* (6), 2303-2304.

7. Knowles, R. R.; Carpenter, J.; Blakey, S. B.; Kayano, A.; Mangion, I. K.; Sinz, C. J.; MacMillan, D. W. C., Total synthesis of diazonamide A. *Chemical Science* **2011,** *2* (2), 308-311.

8. Ojika M, S. Y., Tsukamoto A, Sakagami Y, Fudou R, Yoshimura T, Yamanaka S., *J. Antibiot.* **1998,** *51*, 275-281.

9. Kehraus, S.; König, G. M.; Wright, A. D.; Woerheide, G., Leucamide A: A New Cytotoxic Heptapeptide from the Australian Sponge Leucetta microraphis. *The Journal of Organic Chemistry* **2002,** *67* (14), 4989-4992.

10. Bayer, A.; Freund, S.; Nicholson, G.; Jung, G., Posttranslational Backbone Modifications in the Ribosomal Biosynthesis of the Glycine-Rich Antibiotic Microcin B17. *Angewandte Chemie International Edition in English* **1993,** *32* (9), 1336-1339.

11. (a) Vidal, J. P.; Escale, R.; Girard, J. P.; Rossi, J. C.; Chantraine, J. M.; Aumelas, A., Lituarines A, B, and C: a new class of macrocyclic lactones from the New Caledonian sea pen Lituaria australasiae. *The Journal of Organic Chemistry* **1992,** *57* (22), 5857-5860; (b) Kunze, B.; Jansen, R.; Hofle, G.; Reichenbach, H., Crocacin, a new electron transport inhibitor from *Chondromyces crocatus* (myxobacteria). Production, isolation, physico-chemical and biological properties. *J. Antibiot.* **1994,** *47* (8), 881; (c) Sugie, Y.; Dekker, K. A.; Hirai, H.; Ichiba, T.; Ishiguro, M.; Shiomi, Y.; Sugiura, A.; Brennan, L.; Duignan, J.; Huang, L. H.; Sutcliffe, J.; Kojima, Y., CJ-15,801, a novel antibiotic from a fungus, *Seimatosporium* sp. *J. Antibiot.* **2001,** *54* (12), 1060.

12. Erickson, K. L.; Beutler, J. A.; Cardellina, J. H.; Boyd, M. R., Salicylihalamides A and B, Novel Cytotoxic Macrolides from the Marine Sponge Haliclona sp. *The Journal of Organic Chemistry* **1997,** *62* (23), 8188-8192.

13. Hayata, K.; Takebayashi, Y.; Nagai, K.; Hiramoto, M., *Jpn. Kokai Tokkyo Koho* **1999**, JP11180997-A.

14. Deeley, J.; Pattenden, G., Synthesis and establishment of stereochemistry of the unusual polyoxazole-thiazole based cyclopeptide YM-216391 isolated from Streptomyces nobilis. *Chemical Communications* **2005,** *0* (6), 797-799.

15. (a) Sohda, K.-y.; Nagai, K.; Yamori, T.; Suzuki, K.-i.; Tanaka, A., YM-216391, a Novel Cytotoxic Cyclic Peptide from *Streptomyces nobilis*. *J. Antibiot.* **2005,** *58* (1), 27-31; (b) Sohda, K.-y.; Hiramoto, M.; Suzumura, K.-i.; Takebayashi, Y.; Suzuki, K.-i.; Tanaka, A., YM-216391, a Novel Cytotoxic Cyclic Peptide from *Streptomyces nobilis*. *J. Antibiot.* **2005,** *58* (1), 32-36.

16. Deeley, J.; Bertram, A.; Pattenden, G., Novel polyoxazole-based cyclopeptides from Streptomyces sp. Total synthesis of the cyclopeptide YM-216391 and synthetic

studies towards telomestatin. *Organic & Biomolecular Chemistry* **2008,** *6* (11), 1994-2010.

17. Romero, F.; Malet, L.; Canedo, M. L.; Cuevas, C.; Reyes, J., *WO 2005/000880 A2* **2005**.

18. Kanoh, K.; Matsuo, Y.; Adachi, K.; Imagawa, H.; Nishizawa, M.; Shizuri, Y., Mechercharmycins A and B, Cytotoxic Substances from Marine-derived *Thermoactinomyces sp.* YM3-251. *J. Antibiot.* **2005,** *58* (4), 289-292.

19. Hernández, D.; Vilar, G.; Riego, E.; Cañedo, L. M.; Cuevas, C.; Albericio, F.; Álvarez, M., Synthesis of IB-01211, a Cyclic Peptide Containing 2,4-Concatenated Thia- and Oxazoles, via Hantzsch Macrocyclization†. *Organic Letters* **2007,** *9* (5), 809-811.

20. Hernández, D.; Altuna, M.; Cuevas, C.; Aligué, R.; Albericio, F.; Álvarez, M., Synthesis and Antitumor Activity of Mechercharmycin A Analogues. *Journal of Medicinal Chemistry* **2008,** *51* (18), 5722-5730.

21. Shin-ya, K.; Wierzba, K.; Matsuo, K.-i.; Ohtani, T.; Yamada, Y.; Furihata, K.; Hayakawa, Y.; Seto, H., Telomestatin, a Novel Telomerase Inhibitor from Streptomyces anulatus. *Journal of the American Chemical Society* **2001,** *123* (6), 1262-1263.

22. Doi, T.; Yoshida, M.; Shin-ya, K.; Takahashi, T., Total Synthesis of (R)-Telomestatin. *Organic Letters* **2006,** *8* (18), 4165-4167.

23. Doi, T.; Shibata, K.; Yoshida, M.; Takagi, M.; Tera, M.; Nagasawa, K.; Shin-ya, K.; Takahashi, T., (S)-Stereoisomer of telomestatin as a potent G-quadruplex binder and telomerase inhibitor. *Organic & Biomolecular Chemistry* **2011,** *9* (2), 387-393.

24. Haberhauer, G.; Drosdow, E.; Oeser, T.; Rominger, F., Structural investigation of westiellamide analogues. *Tetrahedron* **2008,** *64* (8), 1853-1859.

25. (a) Aguilar, E.; Meyers, A. I., Reinvestigation of a modified Hantzsch thiazole synthesis. *Tetrahedron Letters* **1994,** *35* (16), 2473-2476; (b) Boden, C. D. J.; Pattenden, G.; Ye, T., The Synthesis of Optically Active Thiazoline and Thiazole Derived Peptides from N-Protected α-Amino Acids. *Synlett* **1995,** *1995* (05), 417-419; (c) Merritt, E. A.; Bagley, M. C., Holzapfel-Meyers-Nicolaou Modification of the Hantzsch Thiazole Synthesis. *Synthesis* **2007,** *2007* (EFirst), 3535-3541.

26. Ozturk, T.; Ertas, E.; Mert, O., Use of Lawesson's Reagent in Organic Syntheses. *Chemical Reviews* **2007,** *107* (11), 5210-5278.

27. (a) Nicolaou, K. C.; Safina, B. S.; Zak, M.; Lee, S. H.; Nevalainen, M.; Bella, M.; Estrada, A. A.; Funke, C.; Zécri, F. J.; Bulat, S., Total Synthesis of Thiostrepton. Retrosynthetic Analysis and Construction of Key Building Blocks. *Journal of the*

American Chemical Society **2005,** *127* (31), 11159-11175; (b) Nicolaou, K. C.; Zak, M.; Safina, B. S.; Estrada, A. A.; Lee, S. H.; Nevalainen, M., Total Synthesis of Thiostrepton. Assembly of Key Building Blocks and Completion of the Synthesis. *Journal of the American Chemical Society* **2005,** *127* (31), 11176-11183.

28. Yan, Y.-L.; Cohen, S. M., Efficient Synthesis of 5-Amido-3-hydroxy-4-pyrones as Inhibitors of Matrix Metalloproteinases. *Organic Letters* **2007,** *9* (13), 2517-2520.

29. (a) Turchi, I. J., Oxazole chemistry. A review of recent advances. *Industrial & Engineering Chemistry Product Research and Development* **1981,** *20* (1), 32-76; (b) Phillips, A. J.; Uto, Y.; Wipf, P.; Reno, M. J.; Williams, D. R., Synthesis of Functionalized Oxazolines and Oxazoles with DAST and Deoxo-Fluor. *Organic Letters* **2000,** *2* (8), 1165-1168; (c) Yeh, V. S. C., Recent advances in the total syntheses of oxazole-containing natural products. *Tetrahedron* **2004,** *60* (52), 11995-12042.

30. Han, G.; Tamaki, M.; Hruby, V. J., Fast, efficient and selective deprotection of the tert-butoxycarbonyl (Boc) group using HCl/dioxane (4 m). *The Journal of Peptide Research* **2001,** *58* (4), 338-341.

31. (a) Rees, D. C.; Congreve, M.; Murray, C. W.; Carr, R., Fragment-based lead discovery. *Nat Rev Drug Discov* **2004,** *3* (8), 660-672; (b) Erlanson, D., Introduction to Fragment-Based Drug Discovery. In *Fragment-Based Drug Discovery and X-Ray Crystallography*, Davies, T. G.; Hyvönen, M., Eds. Springer Berlin Heidelberg: 2012; Vol. 317, pp 1-32; (c) Over, B.; Wetzel, S.; Grütter, C.; Nakai, Y.; Renner, S.; Rauh, D.; Waldmann, H., Natural-product-derived fragments for fragment-based ligand discovery. *Nat Chem* **2013,** *5* (1), 21-28.

32. (a) Quada Jr, J. C.; Boturyn, D.; Hecht, S. M., Photoactivated DNA cleavage by compounds structurally related to the bithiazole moiety of bleomycin. *Bioorganic & Medicinal Chemistry* **2001,** *9* (9), 2303-2314; (b) Thomas, C. J.; McCormick, M. M.; Vialas, C.; Tao, Z.-F.; Leitheiser, C. J.; Rishel, M. J.; Wu, X.; Hecht, S. M., Alteration of the Selectivity of DNA Cleavage by a Deglycobleomycin Analogue Containing a Trithiazole Moiety. *Journal of the American Chemical Society* **2002,** *124* (15), 3875-3884; (c) Kita, M.; Oka, H.; Usui, A.; Ishitsuka, T.; Mogi, Y.; Watanabe, H.; Kigoshi, H., Synthesis and biological activities of the tris-oxazole macrolactone analogs of mycalolides. *Tetrahedron* **2012,** *68* (42), 8753-8760.

Chapter 4-Experimental Methods

4.1 General remarks

All chemicals were purchased from commercial suppliers (Novabiochem, Sigma-Aldrich, Acros Organics, Peptide International, Chem-Implex International, GL-Biochem, and Fisher scientific) and used as received without further purification. All moisture and air sensitive reactions were performed with anhydrous solvents under argon or nitrogen unless otherwise stated. All reactions were monitored by thin-layer chromatography (TLC) and/or liquid chromatography-mass spectrometry (LC/MS). TLC was carried out on silica gel plates 250 μm Whatman® (4861-820) using UV light (λ = 254 nm) as visualizing method. The developing agents for TLC include potassium permanganate (general purpose), bromocresol green (for detection of carboxylic acids) and ninhydrin (for detection of amines).

SiliCycle SiliaFlash silica gel (60 Å, particle size 40-63 μm) and Davisil® silica gel (60 Å, particle size 40-63 μm) were used for flash column chromatography. ^{1}H and ^{13}C NMR spectra obtained at SDSU were recorded at 30 ^{o}C on a 600-MHz Varian NMR-S, 400-MHz Varian NMR-S, and 200-MHz Varian NMR-S. NMR spectra collected at UNSW were obtained at 25 ^{o}C on Bruker Avance III 300 MHz and 600 MHz with BBFO z-gradient probe. Multiplicity of NMR signals was designated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad, dd = doublet of doublet.

High-resolution mass spectrometry (HRMS) analyses were recorded on a Thermo LTQ Orbitrap XL ESI/APCI with UPLC system at the Bioanalytical Mass Spectrometry Facility in Mark Wainwright Analytical Centre at the University of New South Wales.

LC/MS analyses at SDSU were performed on an Agilent 1200 Series HPLC (Zorbax Agilent SB-C18 column, 3.5µm, 2.1 x 30mm) attached to an Agilent 62440A mass spectrometer operating in the positive electrospray ionization (ESI+) mode. The mobile phase was made of double deionized (DDI) water with 0.1% (v/v) formic acid (solvent A) and HPLC grade acetonitrile with 0.1% (v/v) formic acid (solvent B). The gradient elution was as follows: flow rate 1.0 mL/min; initial 80% solvent A, 20% solvent B; at 4.5 min 10% solvent A, 90% solvent B hold 0.1 min; at 7 min 85% solvent A, 15% solvent B.

Semi-preparative HPLC purifications at SDSU were done on a Waters Flex Inject system (Phenomenex Symmetry C18 column, 3.5µm, 4.6 x 75mm) with a Dual λ Absorbance Detector (Waters 2487). The mobile phase was prepared by HPLC grade acetonitrile with 0.1% (v/v) trifluoroacetic acid (solvent A) and DDI water with 0.1% (v/v) trifluoroacetic acid. The gradient elution was as follows: flow rate 2.0 mL/min; initial 70% solvent A, 30% solvent B; at 30 min 100% solvent B, hold for 15 min; at 48 min 70% solvent A, 30% solvent B, hold for 2 min.

LC/MS data at UNSW were obtained on Shimadzu Prominence High-performance LCMS 2010EV system (Waters Symmetry® C18 column, 3.5µm, 4.6x75mm) connected to a Shimadzu LCMS 2010EV mass spectrometer running in the positive eletrospray ionization (ESI+) mode unless mentioned otherwise. The Mobile phase was composed of DDI water with 0.1% (v/v) formic acid (solvent A), and HPLC

grade acetonitrile with 0.1% (v/v) formic acid (solvent B). The gradient elution was as follows: flow rate 0.5 mL/min; initial 70% solvent A, 30% solvent B; at 4 min 100% solvent B; at 12 min 70% solvent A, 30% solvent B.

Semi-preparative HPLC purifications were carried out on a Shimadzu Prominence High-performance LCMS 2010EV system (Phenomenex® Jupiter C18 column, 4μm, 250x10mm). The Mobile phase was prepared by DDI water with 0.1% (v/v) formic acid (solvent A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (solvent B). The gradient elution as follows: flow rate 2mL/min; initial 70% solvent A, 30% solvent B hold for 35 min; at 35 min 100% solvent B hold for 13min; at 48 min 70% solvent A, 30% solvent B hold for 2 min.

4.2 General experimental procedures

4.2.1. General peptide synthesis (for solution phase)

The free amine (1.1 equivalents), the free acid (1.0 equivalent), and the coupling reagent (1.2-2.2 equivalents) were weighed into a round bottom flask. The flask was purged with argon or nitrogen and then sealed with a rubber septum. The starting materials were dissolved in anhydrous solvent to a 0.1 M concentration. The base DIPEA (4.0-8.0 equivalents) was added to the reaction flask. An additional amount of anhydrous solvent was added if insolubility of the materials was observed. The reaction mixture was stirred at room temperature for 1 to 2 h and monitored by TLC every 30 min. An additional amount of coupling reagent was added to the reaction flask if the reaction was not done in 4 h. Upon completion, the reaction mixture was diluted with DCM and an acidic extraction was done with (10% (v/v) $HCl_{(aq)}$) to remove excess free amine and

base. A subsequent basic extraction was done using saturated aqueous $NaHCO_3$ solution to remove excess coupling reagent and side products. The organic layers were combined, dried over anhydrous Na_2SO_4, filtered through a bückner funnel, and concentrated *in vauco*. The resulting crude product was purified via a flash column chromatography on silica gel using an ethyl acetate/hexane gradient solvent system to yield desired peptide products. The pure peptide product was then verified via NMR and LC/MS.

4.2.2 Boc removal

The Boc protecting group was removed via dissolving the protected peptide in DCM (80% for 0.1 M overall concentration) with anisole (2.0 equivalents), followed by the addition of TFA (20% for 0.1 M overall concentration). The reaction was run at room temperature under open atmosphere and monitored via TLC every 15 min. Upon completion, the reaction solution was co-evaporated with DCM for 5 times to remove excess TFA and then concentrated *in vacuo*. The obtained crude product was used without further purification.

4.2.3 Methyl ester hydrolysis

The methyl ester group was hydrolyzed via dissolving the protected peptide to a 0.1 M solution in MeOH, followed by the addition of LiOH (8.0 equivalents). The reaction was stirred at room temperature under open atmosphere and monitored via TLC every 2 h. A catalytic amount of deionized waster was added if the reaction was not complete in 8 h. Upon completion, the solvent was removed *in vacuo* and the crude residue was diluted with DCM. Acidic extractions were performed (10% (v/v) $HCl_{(aq.)}$) twice to remove LiOH. The organic layer was then dried and concentrated *in vacuo*. The resulting crude product was subjected to next reaction without further purification.

4.2.4 Peptide macrocyclization

The macrocyclization of double deprotected linear peptide (DDLP) was perforemed using a combination of coupling reagents (generally TBTU, HATU, DEPBT, 1.8-2.4 equivalents overall). The DDLP and coupling reagents were weighed into a round bottom flask and sealed with a rubber septum. The starting materials were dissolved in anhydrous solvent(s) for an overall concentration of 0.001-0.007 M under argon or nitrogen. DIPEA (8.0 equivalents) was then added to the reaction solution. The reaction was monitored via TLC and LC/MS every 2 h. If the reaction was not complete within 6 h, additional coupling reagents were added and the reaction was allowed to run overnight. Upon completion as confirmed by LC/MS, the crude mixture was extracted with acidic aqueous solution (10% (v/v) $HCl_{(aq.)}$) twice and then saturated $NaHCO_3$ solution for 3 times. The aqueous layer was re-extraccted with ethyl acetate. The organic layers were collected, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting crude product was purified via a flash column chromatography on silica gel using ethyl acetate/hexane as a gradient system. If needed, reverse-phase HPLC was utilized to further purify the macrocycles using a gradient of acetonitrile and DDI water (both with 0.1% TFA) as a gradient system.

4.2.5 Syringe pump macrocyclization

The coupling reagents (generally TBTU, HATU, DEPBT, 1.8-2.4 equivalents total) were weighed into a round bottom flask and dissolved in 50%-75% of the volume of anhydrous solvent that results in a 1-7 mM overall concentration. The DDLPs were dissolved in the remaining volume of the anhydrous solvent and the solution was transferred to a 30 mL syringe. DIPEA (8.0 equivalents) was then added to the solution

of coupling reagents. The DDLP solution was added to the round bottom flask utilizing a syringe pump at a rate of 30 mL/h. The reaction was monitored via TLC and LC/MS every hour. The macrocyclization was usually completed in 6 h. In some cases additional coupling reagents were added and the reaction was allowed to run overnight. Upon completion, the reaction mixture was extracted with 10% (v/v) $HCl_{(aq.)}$ twice and then saturated $NaHCO_3$ solution for 3 times. After further extraction of the aqueous layer using ethyl acetate, the organic layers were collected, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography using an ethyl acetate/hexane gradient system. When necessary, reverse-phase HPLC was utilized for further purification using a gradient of acetonitrile and DDI water (both with 0.1% TFA) as a gradient system.

4.2.6 Cbz removal

Under open atmosphere, the cyclic peptide with a Lys(Cbz) was dissolved in HBr (33% in glacial acetic acid) to a 0.1 M concentration. The reaction was run at room temperature for approximately 2 h and monitored via LC/MS. Upon completion, the reaction mixture was washed with diethyl ether several times and the product was concentrated *in vacuo*.

4.2.7 Peg-Biotin attachment

The NHS-PEG4-Biotin (1.2 equivalents) and the free lysine-containing cyclic peptide were weighed into a sealed round bottom flask and purged with argon. Anhydrous DCM was added to make a 0.1 M solution and DIPEA (8.0 equivalents) was added to trigger the coupling. The reaction was run at room temperature and monitored

via LC/MS every 30 min. Upon completion, the solvent was removed *in vacuo* and the residue was purified via HPLC to yield the desired biotin-tagged cyclic peptide.

4.2.8 Benzylation

Under an atmosphere of argon, the free hydroxyl-containing cyclic peptide was dissolved in anhydrous solvent to make a 0.1 M solution. NaH (60% in mineral oil, 1.1 equivalents) was added to the solution. BnBr (2.0 equivalents) was added slowly to the reaction mixture. The reaction was monitored via LC/MS every hour. Upon completion, the reaction mixture was diluted with DCM and extracted by DI water. The organic layer was collected, dried, and concentrated *in vacuo*. The crude product was purified by a flash column chromatography on silica gel, followed by the final purification via HPLC.

4.2.9 Hydrogenolysis

The benzyl protected compound and a catalytic amount of palladium black (10%) were weighed into a dry round bottom flask. The flask was then sealed with a rubber septum and vaccumed through a niddle for 2 min. Ethanol was added to make a solution of 0.1 M concentration. Hydrogen was bubbled through the reaction solution using a balloon (at least 3 times). The reaction was allowed to stir overnight and checked via TLC. If the reaction was not done after one night, additional hydrogen gas was purged for 3 times and the reaction was run for an additional night. Upon complete disappearance of starting material, the reaction was filtered over Celite® to remove the catalyst palladium. The filtrate was then concentrated *in vacuo* and the obtained material was directly taken on to the next reaction. When necessary, it was purified via flash column chromatography.

4.2.10 Oxazole synthesis

A) Oxazoline formation

The free serine peptide was dissolved in anhydrous DCM to a 0.1 M concentration and purged with argon or nitrogen. The solution was then cooled to -78 ^{o}C and fluorinating agent DAST (1.1 equivalents) was added drop-wise at a rate of 0.1 mL/min. The reaction was continued to stir for 30 min at -78 ^{o}C, followed by the addition of the base K_2CO_3 (2.0 equivalents) in one portion. After stirring for an additional 30 min, the reaction was allowed to warm to room temperature over 1 h. Upon verification of completion via TLC, the reaction mixture was diluted with DCM and extracted with saturated $NaHCO_3$ aqueous solution. After a further extraction of aqueous layer with DCM, the organic layers were collected, dried over Na_2SO_4 and concentrated *in vacuo*. The resulting crude oxazoline was taken on to the oxidation step without further purification.

Note: When generating phenyl oxazoline, pyridine was used to replace K_2CO_3. The crude phenyl oxazoline was purified via flash column chromatography prior to the oxidation step.

B) Oxidation

The oxazoline was dissolved in anhydrous DCM to a 0.1 M concentration and then cooled to -47 ^{o}C. DBU (2.0 equivalents) was added drop-wise (0.1 mL/min) to the DCM solution and stirred for 10 min. $BrCCl_3$ (2.0 equivalents) was added drop-wise at a rate of 0.1 mL/min to the reacting solution and stirred at -47 ^{o}C for an additional 2 h. The reaction was allowed to warm to room temperature and stirred for an additional 12 h.

Upon completion as confirmed by TLC, the reaction solution was diluted with DCM and extracted with 10% $HCl_{(aq.)}$ then with saturated sodium bicarbonate. After back extraction of the aqueous layers with ethyl acetate, the organic layers were collected, dried and concentrated *in vacuo*. The crude product was purified via a flash column chromatography on silica gel using ethyl acetate/hexane as a gradient system to yield the pure oxazole.

4.2.11 Amide conversion

The ester was weighed in a round bottom flask and dissolved in a mixture of ammonium hydroxide and methanol (1:1~3:1) to a 0.1 M concentration. The reaction was allowed to stir for 12 h and monitored via TLC. Upon completion, the reaction solution was concentrated *in vacuo*. The yielding amide was then used for the next reaction without further purification.

4.2.12 Thioamide conversion

The amide (1.0 equivalent) and Lawesson's reagent (0.8 equivalent) was weighed and placed to a round bottom flask. The flask was then sealed with an oven-dried reflux condenser and purged with argon or nitrogen. The starting materials were dissolved with anhydrous solvent to a 0.05 M concentration. The reaction mixture was heated and refluxed for 12 h and monitored via TLC. Upon completion, the reaction was cooled to room temperature and concentrated *in vacuo*. The residue was purified via a flash column chromatography on silica gel using ethyl acetate/hexane as a gradient system. The purified thioamide was verified via ^{1}H NMR.

4.2.13 Thiazole synthesis

A) Hydroxythiazoline formation

The thioamide (1.0 equivalent) and potassium bicarbonate ($KHCO_3$, 8.0 equivalents) were weighed and dissolved in anhydrous DME to a 0.05 M concentration. The reaction solution was stirred at room temperature for 15 min prior to the addition of the bromoketone reactant (3.0 equivalents). The reaction was then stirred for an additional 12 h and monitored via TLC. Upon the complete disappearance of the thioamide, the solvent was removed *in vacuo* and the residue was partitioned between ethyl acetate and DI water. The aqueous layer was re-extracted with ethyl acetate twice and the organic layers were then combined, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude hydroxythiazoline was carried on to the dehydration step without further purification.

B) Dehydration

The crude hydroxythiazoline (1.0 equivalent) was dissolved in anhydrous DME to a 0.05 M concentration and the solution was cooled to 0 ^{o}C in an ice bath. Pyridine (9.0 equivalents) was added drop-wise to the solution and the reaction mixture was stirred for 10 min. TFAA (4.0 equivalents) was added drop-wise at a rate of 0.1 mL/min and the reaction mixture was continued to stir at 0 ^{o}C for 3 h and then warmed to room temperature. TEA (2.0 equivalents) was gentlely added to the reaction solution, which was further stirred for an hour and monitored by TLC. Upon completion, the solvent was removed *in vacuo* and the residue was partitioned between chloroform and 10% $HCl_{(aq)}$ to remove excess base. The organic layer was collected and further extracted with saturated

$NaHCO_3$ aqueous solution. The aqueous layer was re-extracted with ethyl acetate twice and the organic layers were combined, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting crude was purified via a flash column chromatography on silica gel utilizing an ethyl acetate/hexane gradient system to yield the pure thiazole product.

Note: The by-product thiazole trifluoroacetate may be observed after the reaction is completed. Exposure of the trifluoroacetate to 1.2 equivalents of NaOEt in 0.05 M ethanol solution at 0°C for 1 h gave the desired thiazole ethyl ester.

4.2.14 Solid phase peptide synthesis (SPPS)

Peptide Coupling

The resin used for SPPS were pre-loaded CTC resin with an approximately 0.5 mmol/g loading scale. The resin was weighed, transferred to a fritted polypropylene cartridge and swollen in DMF for 30 min prior to peptide coupling. After DMF was drained, a 0.2 M DMF solution containing Fmoc protected amino acid (3.0 equivalents) and HOBt (3.0 equivalents) was poured into the cartridge in one portion and mixed with the resin. DIC (6.0 equivalents) was then added to initiate the coupling process. The cartridge was allowed to shake for a minimum of 2 h on a shaker (Labquake tube shaker, Thermo Fisher Scientific) and checked for completion via a ninhydrin test. Upon completion, the reaction solution was drained and the resin was carried on to Fmoc removal.

Note: When performing the peptide coupling between a Fmoc amino acid and an *N*-methyl amino terminus, HOBt was replaced by HOAt and the reaction was allowed to run overnight.

Fmoc removal

After the peptide coupling was complete, the resin was treated with the following steps to remve the Fmoc protecting group: DMF wash (3 x 1 min), 20% Piperdine/DMF wash (1 x 5 min and 1 x 10 min), DMF wash (2 x 1 min), IPA wash (1 x 1 min), DMF wash (1 x 1 min), IPA wash (1 x 1 min), and DMF wash (3 x 1 min). The completion of Fmoc removal was then verify via a ninhydrin test. The resin was then subjected to the next peptide coupling.

Once the desired peptide length was achived, the peptide-bound resin was treated as followed for the final Fmoc removal: DMF wash (3 x 1 min), 20% Piperdine/DMF (1 x 5 min and 1 x 10 min), DMF wash (3 x 1 min), IPA wash (3 x 1 min), and MeOH wash (3 x 1 min). The completion of Fmoc removal was then verified via a ninhydrin test. The resin was then dried *in vacuo* overnight.

Cleaving the assembled peptide from resin

The dried resin was weighed into a round bottom flask and a mixed solution of TFE/DCM (1:1, v/v) was added to the flask (10 mL solution for 1 g of dried resin). The flask was sealed with a rubber septum and stirred for 24 h. The suspension was filtered through a Büchner funnel and the filtered resin was further washed with additional DCM to fully extract the cleaved peptide. The filtrate was concentrated and dried *in vacuo* overnight. The resulting solid was then re-dissolved in DCM, co-evaporated with DCM several times and dried *in vacuo* overnight to completely remove residual TFE. The purity of resulting peptide was verified via LC/MS.

4.3 Sansalvamide A derivatives

4.3.1 Experimental methods for compound 4

4.3.1.1 Resin-O-Phe-Leu-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The dipeptide Resin-O-Phe-Leu-Fmoc was synthesized using 509 mg (0.33 mmol, 1.0 equivalent) of Resin-O-Phe-NH_2, 346 mg (0.99 mmol, 3.0 equivalents) of Fmoc-Leu-OH, 150 mg of HOBt (0.99 mmol, 3.0 equivalents), 0.3 mL of DIC (1.98 mmol, 6.0 equivalents) and 1.6 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound dipeptide.

4.3.1.2 Resin-O-Phe-Leu-NH_2

The dipeptide Resin-O-Phe-Leu-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.1.3 Resin-O-Phe-Leu-D-Val-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The tripeptide Resin-O-Phe-Leu-D-Val-Fmoc was synthesized using the Resin-O-Phe-Leu-NH_2 prepared from previous step, 331 mg (0.99 mmol, 3.0 equivalents) of Fmoc-D-Val-OH, 150 mg of HOBt (0.99 mmol, 3.0 equivalents), 0.3 mL of DIC (1.98 mmol, 6.0 equivalents) and 1.6 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound tripeptide.

4.3.1.4 Resin-O-Phe-Leu-D-Val-NH_2

The tripeptide Resin-O-Phe-Leu-D-Val-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.1.5 Resin-O-Phe-Leu-D-Val-Lys(Boc)-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The tetrapeptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-Fmoc was synthesized using the Resin-O-Phe-Leu-D-Val-NH_2 prepared from previous step, 452 mg of Fmoc-Lys(Boc)-OH (0.99 mmol, 3.0 equivalents), 150 mg of HOBt (0.99 mmol, 3.0 equivalents), 0.3 mL of DIC (1.98 mmol, 6.0 equivalents) and 1.6 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound tetrapeptide.

4.3.1.6 Resin-O-Phe-Leu-D-Val-Lys(Boc)-NH_2

The tetrapeptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.1.7 Resin-O-Phe-Leu-D-Val-Lys(Boc)-Leu-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The pentapeptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-Leu-Fmoc was synthesized using the Resin-O-Phe-Leu-D-Val-Lys(Boc)-NH_2 prepared from previous step, 346 mg of Fmoc-Leu-OH (0.99 mmol, 3.0 equivalents), 150 mg of HOBt (0.99 mmol, 3.0 equivalents), 0.3 mL of DIC (1.98 mmol, 6.0 equivalents) and 1.6 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound pentapeptide.

4.3.1.8 Resin-O-Phe-Leu-D-Val-Lys(Boc)-Leu-NH_2

The pentapeptide Resin-O-Phe-Leu-D-Val-Lys(Boc)-Leu-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.1.9 HO-Phe-Leu-D-Val-Lys(Boc)-Leu-NH_2

Following the ***Cleaving the assembled peptide from resin*** procedure for SPPS: The double deprotected linear pentapeptide (DDLP) HO-Phe-Leu-D-Val-Lys(Boc)-Leu-NH_2 was synthesized using the resin-bound peptide prepared from previous step, 5 mL of TFE and 5 mL of DCM. The resulting slurry was filtered and dried *in vacuo* to yield HO-Phe-Leu-D-Val-Lys(Boc)-Leu-NH_2 as a pale yellow solid (138 mg, overall 60%).

LC/MS (ESI): *m/z* calculated $C_{36}H_{62}N_6O_8$ $[M+H^+]$ = 719.92, found 719.8

4.3.1.10 *cyclo*-Phe-Leu-D-Val-Lys(Boc)-Leu (compound 11)

Compound **11** was synthesized using 138 mg of the DDLP from previous step (0.19 mmol, 1.0 equivalent), 43 mg of TBTU (0.13 mmol, 0.7 equivalent), 51 mg of HATU (0.13 mmol, 0.7 equivalent), 40 mg of DEPBT (0.13 mmol, 0.7 equivalent), 0.2 mL DIPEA (1.14 mmol, 6.0 equivalents) and 28 mL of anhydrous DCM (0.007 M) following the ***peptide cyclization*** procedure. All starting materials were weighed into a round bottom flask and dissolved in 28 mL of anhydrous DCM. The reaction was then allowed to stir at room temperature and monitored via LCMS. Upon completion, the reaction mixture was diluted with DCM (overall volume = 100 mL) and extracted twice with 10% (v/v) $HCl_{(aq.)}$. The organic layer was re-extracted with a saturated $NaHCO_3$ aqueous solution (100 mL x 3). The basic aqueous layer was back-washed with ethyl

acetate (100 mL). The organic layers were collected, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting residue was purified via a flash column chromatography on silica gel using an ethyl acetate-hexane gradient system to yield compound **11** as a light yellow solid (13 mg, 10% yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 8.61 (m, 2H), 8.32-8.09 (m, 1H), 7.69-7.49 (m, 1H), 7.38-7.09 (m, 5H), 4.63 (m, 1H), 4.41 (m, 1H), 4.32-4.13 (m, 1H), 3.93-3.75 (m, 1H), 3.75-3.58 (m, 1H), 3.17-2.93 (m, 3H), 2.11-1.88 (m, 2H), 1.75-1.45 (m, 6H), 1.45-1.16 (m, 15H), 1.07-0.72 (m, 17H).

LC/MS (ESI): *m/z* calculated $C_{37}H_{60}N_6O_7$ [M+H$^+$] = 701.9, found 702.6

4.3.1.11 *cyclo*-Phe-Leu-D-Val-Lys-Leu (compound 16)

The Boc protecting group of compound **11** (10.2 mg, 1.45 x 10^{-5} mol) was removed via the ***Boc removal*** procedure. Boc removal was verified via ^{1}H NMR and LC/MS to yield compound **16** (9.5 mg, quantitative yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.27-7.00 (m, 5H), 4.57-4.35 (m, 2H), 4.29 (m, 1H), 4.15 (m, 1H), 3.77 (d, *J* = 10.4 Hz, 1H), 3.65-3.45 (m, 2H), 3.01-2.86 (m, 2H), 2.86-2.72 (m, 2H), 2.15-1.77 (m, 3H), 1.77-1.31 (m, 16H), 1.11-0.95 (m, 1H), 0.95-0.50 (m, 17H).

LC/MS (ESI): *m/z* calculated $C_{32}H_{52}N_6O_5$ [M+H$^+$] = 601.8, found 601.7

4.3.1.12 *cyclo*-Phe-Leu-D-Val-Lys(PEG-Biotin)-Leu (compound 4)

Compound **4** was synthesized following the ***Peg-Biotin attachment*** procedure. The reaction was performed utilizing 7 mg of compound **16** (1.16 x 10^{-5} mol, 1.0 equivalent), 9.6 mg of NHS-dPEG$_4$-biotin (1.63 x 10^{-5} mol, 1.4 equivalents), 8 µL of DIPEA (4.65 x 10^{-5} mol, 4.0 equivalents) and 0.12 mL of DCM (0.1 M). The crude

product was purified via HPLC to yield compound **4** as a white solid (6.8 mg, 55% yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.37-7.08 (m, 5H), 4.60 (m, 1H), 4.54-4.44 (m, 2H), 4.39 (m, 1H), 4.31 (m, 2H), 4.25 (dd, *J* = 10.2, 5.2 Hz, 1H), 3.87-3.47 (m, 16H), 3.41-3.34 (m, 3H), 3.27-3.10 (m, 2H), 2.93 (m, 3H), 2.58 (t, *J* = 6.2 Hz, 2H), 2.43 (t, *J* = 6.3 Hz, 2H), 2.22 (m, 7H), 2.01 (m, 3H), 1.80-1.42 (m, 12H), 1.06-0.65 (m, 17H).

LC/MS (ESI): *m/z* calculated $C_{53}H_{87}N_9O_{12}S$ [M+Na^+] = 1097.4, found 1097.3

4.3.2 Experimental methods for compound 5

4.3.2.1 Resin-O-Leu-Lys(Boc)-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The dipeptide Resin-O-Leu-Lys(Boc)-Fmoc was synthesized using 1.0 g (0.81 mmol, 1.0 equivalent) of Resin-O-Leu-NH_2, 1.1 g (2.43 mmol, 3.0 equivalents) of Fmoc-Lys(Boc)-OH, 372 mg of HOBt (2.43 mmol, 3.0 equivalents), 0.75 mL of DIC (4.86 mmol, 6.0 equivalents) and 4.0 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound dipeptide.

4.3.2.2 Resin-O-Leu-Lys(Boc)-NH_2

The dipeptide Resin-O-Leu-Lys(Boc)-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.2.3 Resin-O-Leu-Lys(Boc)-Phe-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The tripeptide Resin-O-Leu-Lys(Boc)-Phe-Fmoc was synthesized using the Resin-O-Leu-Lys(Boc)-NH_2 prepared from previous step, 942 mg (2.43 mmol, 3.0 equivalents) of Fmoc-Phe-OH, 372 mg of HOBt (2.43 mmol, 3.0 equivalents), 0.75 mL of DIC (4.86 mmol, 6.0 equivalents) and 4.0 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound tripeptide.

4.3.2.4 Resin-O-Leu-Lys(Boc)-Phe-NH_2

The tripeptide Resin-O-Leu-Lys(Boc)-Phe-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.2.5 Resin-O-Leu-Lys(Boc)-Phe-Leu-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The tetrapeptide Resin-O-Leu-Lys(Boc)-Phe-Leu-Fmoc was synthesized using the Resin-O-Leu-Lys(Boc)-Phe-NH_2 prepared from previous step, 859 mg (2.43 mmol, 3.0 equivalents) of Fmoc-Leu-OH, 372 mg of HOBt (2.43 mmol, 3.0 equivalents), 0.75 mL of DIC (4.86 mmol, 6.0 equivalents) and 4.0 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound tetrapeptide.

4.3.2.6 Resin-O-Leu-Lys(Boc)-Phe-Leu-NH_2

The tetrapeptide Resin-O-Leu-Lys(Boc)-Phe-Leu-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.2.7 Resin-O-Leu-Lys(Boc)-Phe-Leu-D-Val-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The pentapeptide Resin-O-Leu-Lys(Boc)-Phe-Leu-D-Val-Fmoc was synthesized using the Resin-O-Leu-Lys(Boc)-Phe-Leu-NH_2 prepared from previous step, 825 mg (2.43 mmol, 3.0 equivalents) of Fmoc-D-Val-OH, 372 mg of HOBt (2.43 mmol, 3.0 equivalents), 0.75 mL of DIC (4.86 mmol, 6.0 equivalents) and 4.0 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound pentapeptide.

4.3.2.8 Resin-O-Leu-Lys(Boc)-Phe-Leu-D-Val-NH_2

The pentapeptide Resin-O-Leu-Lys(Boc)-Phe-Leu-D-Val-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.2.9 HO-Leu-Lys(Boc)-Phe-Leu-D-Val-NH_2

Following the ***Cleaving the assembled peptide from resin*** procedure for SPPS: The double deprotected linear pentapeptide (DDLP) HO-Leu-Lys(Boc)-Phe-Leu-D-Val-NH_2 was synthesized using the resin-bound peptide prepared from previous step, 7 mL of TFE and 7 mL of DCM. The resulting slurry was filtered and dried *in vacuo* to yield HO-Leu-Lys(Boc)-Phe-Leu-D-Val-NH_2 as a pale yellow solid (386 mg, overall 66%).

LC/MS (ESI): *m/z* calculated $C_{36}H_{62}N_6O_8$ $[M+H^+]$ = 719.92, found 719.9

4.3.2.10 *cyclo*-Leu-Lys(Boc)-Phe-Leu-D-Val (compound 12)

Compound **12** was synthesized using 232 mg of the DDLP from previous step (0.33 mmol, 1.0 equivalent), 76 mg of TBTU (0.23 mmol, 0.7 equivalent), 86 mg of HATU (0.23 mmol, 0.7 equivalent), 68 mg of DEPBT (0.23 mmol, 0.7 equivalent), 0.33 mL DIPEA (1.9 mmol, 6.0 equivalents) and a mixture of anhydrous DCM/ACN (1:1, total volume = 46 mL, 0.007 M) following the ***peptide cyclization*** procedure. All starting materials were weighed into a round bottom flask and dissolved in the anhydrous solvent. The reaction was then allowed to stir at room temperature and monitored via LCMS. Upon completion, the reaction mixture was diluted with DCM (overall volume = 100 mL) and extracted twice with 10% (v/v) $HCl_{(aq.)}$. The organic layer was re-extracted with a saturated $NaHCO_3$ aqueous solution (100 mL x 3). The basic aqueous layer was back-washed with ethyl acetate (100 mL). The organic layers were collected, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting residue was purified via a flash column chromatography on silica gel using an ethyl acetate-hexane gradient system to yield compound **12** as a light yellow solid (101 mg, 45% yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.33-7.17 (m, 5H), 4.64-4.54 (m, 1H), 4.50 (t, *J* = 7.4 Hz, 1H), 4.23 (dd, *J* = 10.1, 5.5 Hz, 1H), 3.82 (dd, *J* = 10.5, 1.1 Hz, 1H), 3.54 (dd, *J* = 9.8, 6.3 Hz, 1H), 3.11 (dd, *J* = 13.2, 8.4 Hz, 1H), 3.05-2.91 (m, 2H), 2.10-1.93 (m, 2H), 1.82 (m, 1H), 1.62 (m, 2H), 1.57-1.51 (m, 2H), 1.51-1.27 (m, 15H), 1.10 (m, 2H), 1.04-0.88 (m, 15H), 0.88-0.83 (m, 2H).

LC/MS (ESI): *m/z* calculated $C_{37}H_{60}N_6O_7$ [M+H$^+$] = 701.9, found 702.1

4.3.2.11 *cyclo*-Leu-Lys-Phe-Leu-D-Val (compound 17)

The Boc protecting group of compound **12** (80.2 mg, 1.14 x 10^{-4} mol) was removed via the ***Boc removal*** procedure. Boc removal was verified via ^{1}H NMR and LC/MS to yield compound **17** (68.7 mg, quantitative yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 8.64 (m, 2H), 8.05 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.70-7.63 (m, 1H), 7.33-7.17 (m, 5H), 4.72-4.61 (m, 1H), 4.52 (m, 1H), 4.27-4.16 (m, 1H), 3.86-3.77 (m, 1H), 3.67 (m, 1H), 3.17 (m, 1H), 3.08-2.97 (m, 2H), 2.89-2.79 (m, 3H), 2.02 (m, 2H), 1.92-1.79 (m, 1H), 1.68-1.50 (m, 5H), 1.50-1.35 (m, 2H), 1.35-1.15 (m, 3H), 1.06-0.81 (m, 17H).

LC/MS (ESI): *m/z* calculated $C_{32}H_{52}N_6O_5$ $[M+H^+]$ = 601.8, found 601.7

4.3.2.12 *cyclo*-Leu-Lys(PEG-Biotin)-Phe-Leu-D-Val (compound 5)

Compound **5** was synthesized following the ***Peg-Biotin attachment*** procedure. The reaction was performed utilizing 51 mg of compound **17** (8.5 x 10^{-5} mol, 1.0 equivalent), 70 mg of NHS-dPEG$_4$-biotin (1.19 x 10^{-4} mol, 1.4 equivalents), 0.06 mL of DIPEA (3.4 x 10^{-4} mol, 4.0 equivalents) and 0.85 mL of DCM (0.1 M). The crude product was purified via HPLC to yield compound **5** as a white solid (53 mg, 58% yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.96 (br, 1H), 7.86 (br, 1H), 7.39-7.13 (m, 5H), 4.60 (t, *J* = 7.8 Hz, 1H), 4.50 (m, 2H), 4.31 (m, 1H), 4.27-4.19 (m, 1H), 3.87-3.66 (m, 6H), 3.66-3.57 (m, 7H), 3.54 (m, 3H), 3.36 (m, 3H), 3.27-3.16 (m, 5H), 3.16-3.08 (m, 2H), 3.04-2.85 (m, 3H), 2.78-2.71 (m, 1H), 2.62-2.47 (m, 1H), 2.43 (t, *J* = 6.2 Hz, 2H), 2.22 (t, *J* = 6.2 Hz, 2H), 2.12-1.94 (m, 2H), 1.91-1.70 (m, 2H), 1.63 (m, 3H), 1.57-1.51 (m, 1H), 1.51-1.40 (m, 6H), 1.14 (m, 2H), 1.05-0.83 (m, 17H).

LC/MS (ESI): *m/z* calculated $C_{53}H_{87}N_9O_{12}S$ $[M+H^+]$ = 1075.4, found 1075.4

4.3.3 Experimental methods for compound 6

4.3.3.1 MeO-Phe-Leu-NHBoc

The dipeptide MeO-Phe-Leu-NHBoc was synthesized following the ***general peptide synthesis*** procedure. The reaction was performed using 951 mg (4.4 mmol, 1.1 equivalents) of MeO-Phe-NH_2, 1.0 g (4.0 mmol, 1.0 equivalent) of HO-Leu-NHBoc, 1.55 g of TBTU (4.8 mmol, 1.2 equivalents), 2.8 mL of DIPEA (0.016 mol, 4.0 equivalents) and 40 mL of DCM (0.1 M). The crude reaction was purified by column chromatography (silica gel, EtOAc/Hex) to yield the dipeptide (1.56 g, 99% yield).

R_f = 0.8 (EtOAc/Hex = 1:1)

^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.45-7.00 (m, 5H), 6.49 (d, *J* = 8.0 Hz, 1H), 4.86 (m, 2H), 4.13 (m, 2H), 3.72 (s, 3H), 3.13 (t, *J* = 5.5 Hz, 2H), 1.45 (s, 9H), 1.09-0.79 (m, 6H).

4.3.3.2 MeO-Phe-Leu-NH_2

The dipeptide MeO-Phe-Leu-NH_2 was synthesized following the ***Boc removal*** procedure. The reaction was performed by dissolving 685 mg of MeO-Phe-Leu-NHBoc in 14 mL of DCM, followed by adding 0.38 mL (3.5 mmol, 2.0 equivalents) of anisole and then 3.5 mL of TFA. The reaction mixture was concentrated *in vacuo* with DCM (250 mL x 5) and taken on to the next reaction without further purification (510 mg, quantitative yield) as a slightly yellow oil.

4.3.3.3 MeO-Phe-Leu-*N*-Me-Val-NHBoc

Following the ***general peptide synthesis*** procedure: The tripeptide MeO-Phe-Leu-*N*-Me-Val-NHBoc was synthesized using 510 mg of MeO-Phe-Leu-NH_2 (1.75 mmol, 1.1

equivalents), 367 mg of HO-*N*-Me-Val-NHBoc (1.59 mmol, 1.0 equivalents), 724 mg of HATU (1.9 mmol, 1.2 equivalents), 1.1 mL of DIPEA (6.35 mmol, 4.0 equivalents) and 16 mL of DCM (0.1 M). The crude reaction was purified by column chromatography (silica gel, EA/Hex) to yield the tripeptide (585 mg, 73% yield).

R_f = 0.6 (EtOAc/Hex = 1:1)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.3-7.1 (m, 5H), 6.5 (br, 1H), 6.3 (br, 1H), 4.8 (m, 1H), 4.4 (m, 1H), 4.0 (d, *J* = 8.0 Hz, 1H), 3.7 (s, 3H), 3.1 (m, 2H), 2.8 (s, 3H), 2.3 (m, 2H), 1.8-1.6 (m, 1H), 1.5 (s, 9H), 1.1-0.9 (m, 12H)

4.3.3.4 MeO-Phe-Leu-*N*-Me-Val-NH_2

Following the ***Boc removal*** procedure: MeO-Phe-Leu-*N*-Me-Val-NH_2 was synthesized by dissolving 585 mg of MeO-Phe-Leu-NHBoc in 10 mL of DCM, followed by adding 0.25 mL (2.3 mmol, 2.0 equivalents) of anisole and then 2.3 mL of TFA. The reaction mixture was concentrated *in vacuo* with DCM (250 mL x 5) and taken on to the next reaction without further purification (510 mg, quantitative yield) as a light brown oil.

4.3.3.5 MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-NHBoc

The tetrapeptide MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-NHBoc was synthesized following the ***general peptide synthesis*** procedure. The reaction was performed using 469 mg (1.16 mmol, 1.1 equivalents) of MeO-Phe-Leu-*N*-Me-Val-NH_2, 436 mg (1.05 mmol, 1.0 equivalent) of HO-D-Lys(2-Cl-Cbz)-NHBoc, 135 mg of TBTU (0.42 mmol, 0.4 equivalent), 320 mg of HATU (0.84 mmol, 0.8 equivalent), 0.73 mL of DIPEA (4.2 mmol, 4.0 equivalents) and 11 mL of DCM (0.1 M). The crude reaction was

purified by column chromatography (silica gel, EA/Hex) to yield the tetrapeptide (675 mg, 80% yield).

R_f = 0.35 (EtOAc/Hex = 1:1)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.4-7.1 (m, 9H), 6.5 (d, *J* = 7.6 Hz, 1H), 6.4 (d, *J* = 7.6 Hz, 1H), 5.3 (d, *J* = 8.1 Hz, 1H), 5.2 (s, 2H), 4.9 (m, 1H), 4.8 (m, 1H), 4.6 (m, 1H), 4.4 (d, *J* = 11.0 Hz, 1H), 3.7 (s, 3H), 3.2 (m, 2H), 3.0 (s, 3H), 2.3 (m, 2H), 1.6 (m, 9H), 1.4 (s, 9H), 1.0-0.9 (m, 12H).

4.3.3.6 MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-NH_2

The tetrapeptide MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-NH_2 was synthesized following the ***Boc removal*** procedure. The reaction was performed by dissolving 650 mg of MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-NHBoc in 6.5 mL of DCM, followed by adding 0.18 mL (1.6 mmol, 2.0 equivalents) of anisole and then 1.6 mL of TFA. The reaction mixture was concentrated *in vacuo* with DCM (250 mL x 5) and taken on to the next reaction without further purification (570 mg, quantitative yield) as a slightly yellow oil.

4.3.3.7 MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-D-Phe-NHBoc

Following the ***general peptide synthesis*** procedure: The pentapeptide MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-D-Phe-NHBoc was synthesized using 570 mg of MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-NH_2 (0.81 mmol, 1.1 equivalents), 195 mg of HO-D-Phe-NHBoc (0.74 mmol, 1.0 equivalent), 95 mg of TBTU (0.3 mmol, 0.4 equivalent), 224 mg of HATU (0.6 mmol, 0.8 equivalent), 0.5 mL of DIPEA (2.9 mmol, 4.0 equivalents) and 8.1 mL of DCM (0.1 M). The crude reaction was purified by column chromatography (silica gel, EtOAc/Hex) to yield the pentapeptide (550 mg, 79% yield).

R_f = 0.3 (EtOAc/Hex = 1:1)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.5-7.1 (m, 14H), 6.9 (br, 1H), 6.5 (d, *J* = 7.7 Hz, 1H), 6.4 (d, *J* = 7.7 Hz, 1H), 5.2 (m, 2H), 5.0 (br, 1H), 4.9-4.8 (m, 2H), 4.4-4.3 (m, 2H), 3.7 (s, 3H), 3.2-3.1 (m, 4H), 3.0 (s, 3H), 2.3 (m, 2H), 1.7-1.3 (m, 9H), 1.4 (s, 9H), 0.9-0.7 (m, 12H).

4.3.3.8 HO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-D-Phe-NH_2

The methyl ester of the pentapeptide MeO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-D-Phe-NHBoc was synthesized following the ***Methyl ester hydrolysis*** procedure. The reaction was performed using 540 mg of the pentapeptide (0.57 mmol, 1.0 equivalent), 191 mg of LiOH monohydrate (4.56 mmol, 8.0 equivalents) and 5.7 mL of MeOH (0.1 M). The pentapeptide was dissolved in MeOH and LiOH was added to the reaction solution. The reaction was run for 12 h. Upon completion, the solvent was removed *in vacuo* and the residue was diluted with 100 mL DCM and then extracted with 100 mL of 10% (v/v) $HCl_{(aq.)}$. The acidic aqueous layer was then further extracted with DCM (100 mL x 3). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield the free acid HO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-D-Phe-NHBoc as a slightly yellow solid (511 mg, 96% yield). The free acid was taken on to Boc removal reaction without any further purification.

Following the ***Boc removal*** procedure: the DDLP HO-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-D-Phe-NH_2 was synthesized by dissolving 511 mg of the free acid in 4.4 mL of DCM, followed by adding 0.11 mL (1.1 mmol, 2.0 equivalents) of anisole and then 1.1 mL of TFA. Upon completion, the reaction mixture was concentrated *in vacuo*

with DCM (250 mL x 5) and taken on to the next reaction without further purification (456 mg, quantitative yield).

4.3.3.9 *cyclo*-Phe-Leu-*N*-Me-Val-D-Lys(2-Cl-Cbz)-D-Phe (compound 13)

Compound **13** was synthesized using 228 mg of the DDLP from previous step (0.27 mmol, 1.0 equivalent), 44 mg of TBTU (0.14 mmol, 0.5 equivalent), 62 mg of HATU (0.16 mmol, 0.6 equivalent), 49 mg of DEPBT (0.16 mmol, 0.6 equivalent), 0.38 mL DIPEA (2.2 mmol, 8.0 equivalents) and 40 mL of anhydrous DCM (0.007 M) following the ***peptide cyclization*** procedure. All starting materials were weighed into a round bottom flask and dissolved in the anhydrous solvent. The reaction was then allowed to stir at room temperature and monitored via LCMS. Upon completion, the reaction mixture was diluted with DCM (overall volume = 100 mL) and extracted twice with 10% (v/v) $HCl_{(aq.)}$. The organic layer was re-extracted with a saturated $NaHCO_3$ aqueous solution (100 mL x 3). The basic aqueous layer was back-washed with ethyl acetate (100 mL). The organic layers were collected, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting residue was purified via a flash column chromatography on silica gel using an ethyl acetate-hexane gradient system to yield compound **13** as a light yellow solid (37.5 mg, 17% yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.4-7.0 (m, 14H), 5.2 (br, 2H), 5.1 (d, *J* = 10.6 Hz, 1H), 4.7 (m, 1H), 4.5 (m, 1H), 4.3-4.1 (m, 3H), 3.1-2.7 (m, 4H), 2.9 (s, 3H), 2.3-2.1 (m, 2H), 1.8 (m, 1H), 1.6-1.2 (m, 8H), 1.0-0.8 (m, 12H).

LC/MS (ESI): *m/z* calculated $C_{44}H_{57}N_6O_7Cl$ [$M+H^+$] = 818.4, found 818.2

4.3.3.10 *cyclo*-Phe-Leu-*N*-Me-Val-D-Lys-D-Phe (compound 18)

The Cbz protecting group of compound **13** (26 mg, 3.2 x 10^{-5} mol) was removed via the ***Cbz removal*** procedure using 0.32 mL of HBr. Cbz removal was verified via ^{1}H NMR and LC/MS to yield compound **18** (18 mg, 87% yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.3-7.0 (m, 10H), 5.0 (d, *J* = 10.6 Hz, 1H), 4.7 (m, 1H), 4.5 (m, 1H), 4.3 (m, 2H), 4.2 (m, 1H), 2.9 (s, 3H), 2.9-2.8 (m, 4H), 2.3-2.1 (m, 2H), 1.9-1.7 (m, 1H), 1.7-1.2 (m, 8H), 1.0-0.8 (m, 12H).

LC/MS (ESI): *m/z* calculated $C_{36}H_{52}N_6O_5$ [M+H^+] = 649.8, found 649.6

4.3.3.11 *cyclo*-Phe-Leu-*N*-Me-Val-D-Lys(PEG-Biotin)-D-Phe (compound 6)

Compound **6** was synthesized following the ***Peg-Biotin attachment*** procedure. The reaction was performed utilizing 16 mg of compound **18** (2.5 x 10^{-5} mol, 1.0 equivalent), 21 mg of NHS-dPEG$_4$-biotin (3.5 x 10^{-5} mol, 1.4 equivalents), 0.017 mL of DIPEA (1.0 x 10^{-4} mol, 4.0 equivalents) and 0.25 mL of DCM (0.1 M). The crude product was purified via HPLC to yield compound **6** as a white solid (14 mg, 51% yield).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.41-6.97 (m, 10H), 4.68 (m, 1H), 4.58-4.43 (m, 2H), 4.42-4.23 (m, 2H), 3.86-3.43 (m, 16H), 3.23 (m, 3H), 3.03-2.78 (m, 3H), 2.67 (s, 3H), 2.58 (m, 1H), 2.46-2.35 (m, 1H), 2.21 (m, 2H), 1.86-1.42 (m, 12H), 1.34-1.02 (m, 6H), 1.03-0.80 (m, 17H).

LC/MS (ESI): *m/z* calculated $C_{57}H_{87}N_9O_{12}S$ [M+H^+] = 1123.4, found 1123.2

4.3.4 Experimental methods for compound 9 and 10

4.3.4.1 Resin-O-Leu-*N*-Me-Val-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The dipeptide Resin-O-Leu-*N*-Me-Val-Fmoc was synthesized using 2.0 g (1.64 mmol, 1.0 equivalent) of Resin-O-

Leu-NH_2, 1.74 g (4.92 mmol, 3.0 equivalents) of Fmoc-*N*-Me-Val-OH, 743 mg of HOBt (4.92 mmol, 3.0 equivalents), 1.48 mL of DIC (9.84 mmol, 6.0 equivalents) and 8.0 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound dipeptide.

4.3.4.2 Resin-O-Leu-*N*-Me-Val-NH_2

The dipeptide Resin-O-Leu-*N*-Me-Val-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.4.3 Resin-O-Leu-*N*-Me-Val-D-Leu-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The tripeptide Resin-O-Leu-*N*-Me-Val-D-Leu-Fmoc was synthesized using the Resin-O-Leu-*N*-Me-Val-NH_2 prepared from previous step, 1.74 g (4.92 mmol, 3.0 equivalents) of Fmoc-D-Leu-OH, 670 mg of HOAt (4.92 mmol, 3.0 equivalents), 1.48 mL of DIC (9.84 mmol, 6.0 equivalents) and 8.0 mL of DMF (0.2 M). The reaction was run overnight and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound tripeptide.

4.3.4.4 Resin-O-Leu-*N*-Me-Val-D-Leu-NH_2

The tripeptide Resin-O-Leu-*N*-Me-Val-D-Leu-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.4.5 Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The tetrapeptide Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-Fmoc was synthesized using the Resin-O-Leu-*N*-Me-Val-D-Leu-NH_2 prepared from previous step, 1.9 g (4.92 mmol, 3.0 equivalents) of Fmoc-D-Phe-OH, 743 mg of HOBt (4.92 mmol, 3.0 equivalents), 1.48 mL of DIC (9.84 mmol, 6.0 equivalents) and 8.0 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound tetrapeptide.

4.3.4.6 Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-NH_2

The tetrapeptide Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.4.7 Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-racemic-β-OH-Phe-Fmoc

Following the ***peptide coupling*** procedure for SPPS: The pentapeptide Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S, 3R)/(2R, 3S)-β-OH-Phe-Fmoc was synthesized using the Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-NH_2 prepared from previous step, 1.98 g (4.92 mmol, 3.0 equivalents) of Fmoc-(2S, 3R)/(2R, 3S)-β-OH-Phe-OH, 743 mg of HOBt (4.92 mmol, 3.0 equivalents), 1.48 mL of DIC (9.84 mmol, 6.0 equivalents) and 8.0 mL of DMF (0.2 M). The reaction was run for 2 h and a negative ninhydrin test was performed to verify the reaction completion. The reaction mixture was drained to give the Fmoc-protected resin-bound pentapeptide.

4.3.4.8 Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-racemic-β-OH-Phe-NH_2

The pentapeptide Resin-O-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S, 3R)/(2R, 3S)-β-OH-Phe-NH_2 was synthesized following the ***Fmoc removal*** procedure for SPPS. A positive ninhydrin test was performed to verify the completion of Fmoc removal.

4.3.4.9 HO-Leu-*N*-Me-Val-D-Leu-D-Phe-racemic-β-OH-Phe-NH_2

Following the ***Cleaving the assembled peptide from resin*** procedure for SPPS: The double deprotected linear pentapeptide (DDLP) HO-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S, 3R)/(2R, 3S)-β-OH-Phe-NH_2 was synthesized using the resin-bound peptide prepared from previous step, 14 mL of TFE and 14 mL of DCM. The resulting slurry was filtered and dried *in vacuo* to yield HO-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S, 3R)/(2R, 3S)-β-OH-Phe-NH_2 as a pale yellow solid (860 mg, overall 79% yield).

LC/MS (ESI): *m/z* calculated $C_{36}H_{53}N_5O_7$ $[M+H^+]$ = 668.8, found 668.8

4.3.4.10 *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-racemic-β-OH-Phe (compound 14/15)

Compound **14/15** was synthesized using 160 mg of the DDLP from previous step (0.23 mmol, 1.0 equivalent), 52 mg of TBTU (0.14 mmol, 0.6 equivalent), 44 mg of HATU (0.14 mmol, 0.6 equivalent), 41 mg of DEPBT (0.14 mmol, 0.6 equivalent), 0.32 mL DIPEA (1.84 mmol, 8.0 equivalents) and anhydrous DCM (total volume = 460 mL, 0.0005 M) following the ***syringe pump cyclization*** procedure. All coupling reagents were weighed into a round bottom flask and dissolved in 340 mL of DCM. The DDLP was dissolved in the remaining DCM and added drop-wise to the flask via a syringe pump at a rate of 30 mL/h. Upon addition of all of the peptide solution, the reaction was then allowed to stir overnight at room temperature and monitored via LCMS. Upon completion, the solvent was removed *in vacuo* and the residue was diluted with DCM

(overall volume = 100 mL) and extracted twice with 10% (v/v) $HCl_{(aq.)}$. The organic layer was re-extracted with a saturated $NaHCO_3$ aqueous solution (100 mL x 3). The basic aqueous layer was back-washed with ethyl acetate (100 mL). The organic layers were collected, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting residue was purified via a flash column chromatography on silica gel using an ethyl acetate-hexane gradient system to yield the racemic compound **14/15** as a light yellow solid (110 mg, 74% yield).

LC/MS (ESI): *m/z* calculated $C_{36}H_{51}N_5O_6$ $[M+H^+]$ = 650.8, found 651.7

4.3.4.11 *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-β-benzoxy-Phe (compound 9 and 10)

Compound **9** and **10** were synthesized following the ***benzylation*** procedure using a mixture of compound **14**/**15** (110 mg, 0.17 mmol, 1.0 equivalent), 7.5 mg of NaH (60% in mineral oil, 0.19 mmol, 1.1 equivalents), 0.04 mL of BnBr (0.34 mmol, 2.0 equivalents) and 1.7 mL of anhydrous THF/DMF (1:1, 0.1 M). Upon completion via the confirmation of LCMS, the reaction mixture was extracted with DI water and DCM. The organic layer was collected, dried and concentrated *in vacuo*. The residue was subjected to a flash column chromatography for a preliminary purification. Further purification of resulting crude product (12 mg) was performed via RP-HPLC to yield compound **9** (0.6 mg, 5% yield) and **10** (2.2 mg, 18% yield).

Compound **9**

^1H NMR (400 MHz, CD_3OD): δ = 8.0 (m, 1H), 7.7 (m, 1H), 7.6 (m, 1H), 7.5-7.2 (m, 15H), 7.0 (d, 1H), 6.7 (d, 1H), 4.5 (m, 1H), 4.4 (m, 1H), 4.3 (m, 1H), 4.2 (m, 1H), 4.1 (m, 1H), 3.6-3.5 (m, 5H), 3.0 (m, 1H), 2.4-2.2 (m, 2H), 2.0 (m, 2H), 1.7-1.5 (m, 4H), 1.4-1.2 (m, 9H), 1.1-0.8 (m, 9H).

LC/MS (ESI): *m/z* calculated $C_{43}H_{57}N_5O_6$ [2M+Na^+] = 1502.9, found 1503.7

Compound **10**

^{1}H NMR (400 MHz, CD_3OD): δ = 7.3-6.8 (m, 15H), 4.7 (m, 1H), 4.6 (m, 1H), 4.5 (m, 1H), 4.4 (m, 1H), 4.2 (m, 1H), 4.1 (t, *J* = 8.2 Hz, 1H), 3.6 (m, 1H), 2.9-2.7 (m, 5H), 2.3-2.2 (m, 2H), 2.0 (m, 1H), 1.7-1.5 (m, 4H), 1.4-1.2 (m, 5H), 1.1-0.8 (m, 18H).

LC/MS (ESI): *m/z* calculated $C_{43}H_{57}N_5O_6$ [2M+Na^+] = 1502.9, found 1502.7

4.4 Urukthapelstatin A

4.4.1 Experimental methods for Ustat A-3 Fragment 1

4.4.1.1 Boc-Thr(tBu)-$CSNH_2$ (compound 4)

To a round bottom flask was added Boc-Thr(tBu)-OH (1.5 g, 5.45 mmol), benzene (40.8 mL) and MeOH (13.6 mL). TMSD (2M solution in diethyl ether) was added drop-wise to the reaction solution until it turned slightly yellow. The mixture was

stirred at room temperature for 1 h and checked via TLC. Upon disappearance of the starting material, the solvent was removed *in vacuo* and the residue was co-evaporated with DCM (100 mL x 5). The obtained crude ester was subject to the ***amide conversion*** procedure, followed by the ***thioamide conversion*** procedure to yield the desired thioamide **4** as a yellow oil (980 mg, 60% yield).

R_f = 0.6 (EtOAc: Hex = 0.35:0.65)

^{1}H NMR: ($CDCl_3$, 400 MHz): δ = 8.46 (br, 1H), 7.67 (br, 1H), 6.05 (br, 1H), 4.40-4.25 (m, 1H), 4.24-4.15 (m, 1H) 1.46 (s, 9H), 1.27 (s, 9H), 1.10 ppm (d, *J* = 5.7 Hz, 3H). HRMS(ESI): calcd for $C_{13}H_{26}N_2O_3SNa^+$ [M + Na^+] 313.1562, found 313.1550.

4.4.1.2 Boc-Thr(tBu)-Thiazole-OEt (compound 5)

Following the ***thiazole synthesis*** procedure: compound **5** was synthesized using using 832 mg of thioamide **4**, 2.3g of $KHCO_3$, and 1.2 mL of ethyl bromopyruvate for the first step; 2.1 mL of pyridine, 1.6 mL of TFAA and 0.8 mL of TEA for the second step. (yellow oil, 860 mg, 78% yield).

R_f = 0.37 (EtOAc: Hex = 0.2:0.8)

^{1}H NMR: ($CDCl_3$, 400 MHz) δ = 8.05 (s, 1H), 5.80 (d, *J* = 7.9 Hz, 1H), 4.95 (d, *J* = 7.9 Hz, 1H), 4.42 (q, *J* = 7.0 Hz, 2H), 4.34 (d, *J* = 5.6 Hz, 1H), 1.48 (s, 9H), 1.39 (t, *J* = 6.9 Hz, 3H), 1.19 (d, *J* = 6.4 Hz, 3H), 0.96 ppm (s, 9H). HRMS(ESI): calcd for $C_{18}H_{30}N_2O_5SNa^+$ [M + Na^+] 409.1773, found 409.1760.

4.4.1.3 Boc-Thr(tBu)-Thiazole-$CSNH_2$ (compound 6)

Compound **6** was synthesized following the ***amide conversion*** procedure, followed by the ***thioamide conversion*** procedure using 867 mg of thiazole **5** and 544 mg

of Lawesson's reagent in 50 mL of anhydrous DME at 60°C for 5 h (bright yellow oil, 440 mg, 53% yield).

R_f = 0.6 (EtOAc: Hex = 0.35:0.65)

^{1}H NMR: ($CDCl_3$, 400 MHz): δ = 8.59 (br, 1H), 8.36 (s, 1H), 7.52 (br, 1H), 5.69 (d, *J* = 6.9 Hz, 1H), 4.87 (d, *J* = 6.9 Hz, 1H), 4.23 (br, 1H) 1.49 (s, 9H), 1.22 (d, *J* = 6.7 Hz, 3H), 0.99 ppm (s, 3H) ; HRMS(ESI): calcd for $C_{16}H_{27}N_3O_3S_2Na^+$ [M + Na^+] 396.1392, found 396.1378.

4.4.1.4 Boc-Thr(tBu)-Dithiazole-OEt (compound 7)

Following the ***thiazole synthesis*** procedure: Compound **7** was synthesized utilizing 832 mg of thioamide **6**, 940 mg of $KHCO_3$, and 0.5 mL of ethyl bromopyruvate for the first step; 0.86 mL of pyridine, 0.66 mL of TFAA and 0.33 mL of TEA for the second step. Finally, 86 mg of NaOEt was used to yield the desired thiazole **7** as yellow oil (545 mg, 97% yield).

R_f = 0.6 (EtOAc: Hex = 0.35:0.65)

^{1}H NMR: ($CDCl_3$, 400 MHz) δ = 8.17 (s, 1H), 8.05 (s, 1H), 5.76 (d, *J* = 8.0 Hz, 1H), 4.94 (d, *J* = 9.2 Hz, 1H), 4.46 (q, *J* = 6.9 Hz, 2H), 4.35 (d, *J* = 6.3 Hz, 1H), 1.54 (s, 9H), 1.44 (t, *J* = 7.5 Hz, 3H), 1.25 (d, *J* = 6.7 Hz, 3H), 0.99 ppm (s, 9H). HRMS(ESI): calcd for $C_{21}H_{31}N_3O_5S_2Na^+$ [M + Na^+] 492.1603, found 492.1588.

4.4.1.5 Boc-Thr(tBu)-Dithiazole-$CSNH_2$ (compound 8)

Compound **8** was synthesized following the ***amide conversion*** procedure, followed by the ***thioamide conversion*** procedure using 538 mg of thiazole **7** and 320 mg of Lawesson's reagent in 46 mL of anhydrous benzene at 60°C for 5 h (bright yellow oil, 382 mg, 53% yield).

R_f = 0.6 (EtOAc: Hex = 0.35:0.65)

^{1}H NMR: ($CDCl_3$, 400 MHz): δ = 8.69 (br, 1H), 8.47 (s, 1H), 7.86 (s, 1H), 7.54 (br, 1H), 5.76 (d, *J* = 7.5 Hz, 1H), 4.95 (d, *J* = 8.1 Hz, 1H), 4.37 (d, *J* = 7.5 Hz, 1H) 1.52 (s, 9H), 1.26 (d, *J* = 7.6 Hz, 3H), 0.99 (s, 3H) ppm. HRMS(ESI): calcd for $C_{19}H_{28}N_4O_3S_3Na^+$ [M + Na^+] 479.1222, found 479.1465.

4.4.1.6 Boc-Thr(tBu)-Trithiazole-OEt (compound 9)

Following the ***thiazole synthesis*** procedure: Compound **9** was synthesized utilizing 382 mg of thioamide **8**, 670 mg of $KHCO_3$, and 0.35 mL of ethyl bromopyruvate for the first step; 0.61 mL of pyridine, 0.47 mL of TFAA and 0.24 mL of TEA for the second step. Finally, 68 mg of NaOEt was used to yield the desired thiazole **9** as a slightly yellow solid (400 mg, 87% yield).

R_f = 0.5 (EtOAc: Hex = 0.35:0.65)

^{1}H NMR: ($CDCl_3$, 400 MHz) δ = 8.20 (s, 1H), 8.16 (s, 1H), 7.97 (s, 1H), 5.78 (d, *J* = 7.9 Hz, 1H), 4.96 (d, *J* = 7.9 Hz, 1H), 4.47 (q, *J* = 7.4 Hz, 2H), 4.38 (d, *J* = 4.6 Hz, 1H), 1.52 (s, 9H), 1.45 (t, *J* = 7.3 Hz, 3H), 1.26 (d, *J* = 7.0 Hz, 3H), 1.01 ppm (s, 9H). HRMS(ESI): calcd for $C_{24}H_{32}N_4O_5S_3Na^+$ [M + Na^+] 575.1433, found 575.1416.

4.4.1.7 Boc-Thr(tBu)-Trithiazole-$CSNH_2$ (Fragment 1)

Fragment **1** was synthesized following the ***amide conversion*** procedure, followed by the ***thioamide conversion*** procedure using 400 mg of thiazole **9** and 250 mg of Lawesson's reagent in 30 mL of anhydrous benzene refluxing for 5 h (bright yellow oil, 286 mg, 73% yield).

R_f = 0.45 (EtOAc: Hex = 0.35:0.65)

^{1}H NMR: ($CDCl_3$, 400 MHz): δ = 8.72 (br, 1H), 8.51 (s, 1H), 7.96 (s, 1H), 7.99 (s, 1H), 7.59 (br, 1H), 5.78 (d, *J* = 8.4 Hz, 1H), 4.96 (d, *J* = 7.8 Hz, 1H), 4.38 (d, *J* = 6.6 Hz, 1H) 1.52 (s, 9H), 1.26 (d, *J* = 6.4 Hz, 3H), 1.00 ppm (s, 3H) HRMS(ESI): calcd for $C_{22}H_{29}N_5O_3S_4Na^+$ [M + Na^+] 562.1051, found 562.1041.

4.4.2 Experimental methods for Ustat A-3 Fragment 2

4.4.2.1 *Racemic* Bromoketal-*β*-hydroxyl-Phe-OMe (compound 10)

Following the ***general peptide synthesis*** procedure: Compound **10** was synthesized using 508 mg of 3-bromo-2,2-dimethoxypropionic acid, 546.6 mg of (2R, 3S)/(2S, 3R)-*racemic* NH_2-β-hydroxyl-Phe-OMe, 1.66 mL of DIPEA and 918 mg of TBTU (slightly yellow oil, 852 mg, 92% yield).

R_f = 0.5 (EtOAc:Hex = 1:1)

^{1}H NMR: ($CDCl_3$, 400 MHz): δ = 7.50 (d, *J* = 8.9 Hz, 1H), 7.37-7.14 (m, 5H), 5.36 (t, *J* = 3.4 Hz,1H), 4.86 (dd, *J* = 9.2, 3.1 Hz, 1H), 3.71 (s, 3H), 3.44 (s, 2H), 3.22 (s, 3H), 2.92 (s, 3H), 2.62 ppm (d, *J* = 4.0 Hz, 1H). ^{13}C NMR: ($CDCl_3$, 400 MHz) δ = 170.38, 167.44, 139.45, 128.38, 127.91, 125.78, 100.28, 72.95, 58.02, 52.59, 50.18, 49.97, 29.59 ppm ; HRMS(ESI): calcd for $C_{15}H_{20}BrNO_6Na^+$ [M + Na^+] 412.0372, found 412.0361.

4.4.2.2 Bromoketal-Phenyloxazole-OMe (compound 11)

Following the ***oxazole synthesis*** procedure: Compound **11** was synthesized utilizing 852 mg of the *racemic* dipeptide **10**, 0.75 mL of DAST, 0.35 mL of pyridine for the first step and 0.31 mL of DBU and 0.2 mL of $BrCCl_3$ for the second step (colorless oil, 334.6 mg, 40% yield).

R_f =0.6 (EtOAc:Hex = 1:1)

^{1}H NMR: ($CDCl_3$, 400 MHz): δ = 8.06-8.00 (m, 2H), 7.45-7.38 (m, 3H), 3.87 (s, 3H), 3.82 (s, 2H), 3.30 ppm (s, 6H). ^{13}C NMR: ($CDCl_3$, 400 MHz) δ = 162.38, 157.99, 156.11, 130.59, 128.57, 128.42, 126.99, 126.51, 99.12, 52.28, 52.20, 52.13, 50.20, 50.16, 50.13, 31.68, 31.59, 31.51 ppm ; HRMS(ESI): calcd for $C_{15}H_{16}BrNO_5Na^+$ [M + Na^+] 392.0110, found 392.0101.

4.4.2.3 Bromoketo-Phenyloxazole-OMe (Fragment 2)

Fragment **2** was synthesized using 624 mg of the phenyloxazole **11** in 18 mL of formic acid at 60^{o}C for 20 min. Upon completion, confirmed by LC/MS, the reaction was diluted with DCM and washed with saturated $NaHCO_3$ solution. The organic layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The crude fragment **2** was taken on without further purification.

4.4.3 Experimental methods for Ustat A-3 Fragment 3

4.4.3.1 Boc-D-*allo*-Ile-Ala-OMe (compound 12)

Following the ***general peptide synthesis*** procedure: Compound **12** was synthesized utilizing 1.0 g of Boc-D-*allo*-Ile-OH (4.32 mmol), 664 mg of H-Ala-OMe (4.76 mmol), 3.0 mL of DIPEA (0.02 mmol) and 1.53g of TBTU (4.76 mmol) in 43.5 mL of anhydrous DCM. The reaction mixture was stirred at room temperature for 45 min. Upon completion, confirmed via TLC, the reaction was washed with 10% aqueous HCl solution (50 mL x 2), and saturated aqueous $NaHCO_3$ solution (50 mL x 2). The collected organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The obtained crude residue was purified by flash column chromatography (silica gel, EtOAc/Hex) to yield the desired dipeptide compound **12** as a white solid (1.25 g, 92%).

R_f = 0.5 (EtOAc:Hex = 1:1)

^{1}H NMR: ($CDCl_3$, 400 MHz): δ = 6.53 (d, *J* = 6.5 Hz, 1H), 4.92 (br, 1H), 4.61 (m, 1H), 4.14 (br, 1H), 3.77 (s, 3H), 2.10-1.95 (m, 1H), 1.52-1.37 (m, 1H), 1.47 (s, 9H), 1.42 (d, *J* = 7.1 Hz, 3H) 1.31-1.16 (m, 1H), 0.95 (t, *J* = 7.3 Hz, 3H), 0.87 ppm (d, *J* = 7.1 Hz, 3H). ^{13}C NMR: ($CDCl_3$, 400 MHz) δ = 173.20, 171.39, 155.79, 79.89, 57.96, 52.32, 47.89, 37.10, 28.21, 26.23, 18.14, 14.09, 11.60 ppm ; HRMS(ESI): calcd for $C_{15}H_{28}N_2O_5Na^+$ [M + Na^+] 339.1896, found 339.1884.

4.4.3.2 H-D-*allo*-Ile-Ala-OMe (Fragment 3)

Fragment **3** was synthesized following the ***Boc removal*** procedure utilizing 1.35 g of the dipeptide **12**. The crude fragment **3** was then taken on without further purification.

4.4.4 Experimental methods for the assembly of Ustat A-3 fragments

4.4.4.1 Boc-Thr(tBu)-Tetrathiazole-Phenyloxazole-OMe (compound 13)

Following the ***thiazole synthesis*** procedure: Compound **13** was synthesized utilizing 286 mg of Fragment **1**, 424 mg of $KHCO_3$, and 528 mg of Fragment **2** for the first step; 0.4 mL of pyridine, 0.3 mL of TFAA and 0.2 mL of TEA for the second step (pale yellow solid, 360 mg, 88% yield).

R_f: 0.25 (EtOAc:Hex = 2:3)

^{1}H NMR: ($CDCl_3$, 400 MHz) δ = 8.23 (s, 1H), 8.22-8.19 (m, 2H), 8.11 (s, 1H), 8.0(s, 1H), 7.58-7.46 (m, 3H), 5.79 (d, *J* = 8.7 Hz, 1H), 4.97 (d, *J* = 7.9 Hz, 1H), 4.38 (d, *J* = 6.3 Hz, 1H), 4.01(s, 3H), 1.52 (s, 9H), 1.27 (d, *J* = 5.8 Hz, 3 H), 1.01 ppm (s, 9H) ; HRMS(ESI): calcd for $C_{35}H_{36}N_6O_9S_4Na^+$ [M + Na^+] 787.1477, found 787.0550.

4.4.4.2 Boc-Thr(tBu)-Tetrathiazole-Phenyloxazole-D-*allo*-Ile-Ala-OMe (compound 15)

Compound **15** was synthesized using 360 mg of **13** and 158 mg of LiOH following the ***methyl ester hydrolysis*** procedure. The resulting free acid was then coupled with 170 mg of fragment **3** using 0.33 mL of DIPEA, and 183 mg of TBTU following the ***general peptide synthesis*** procedure (pale yellow solid, 209 mg, 47%)

R_f =0.65 (EtOAc:Hex = 1:1).

^{1}H NMR: ($CDCl_3$, 400 MHz) δ = 8.45-8.35 (m, 2H), 8.28 (s, 1H), 8.15 (s, 1H), 8.12 (s, 1H), 8.01 (s, 1H), 7.84 (d, *J* = 9.3 Hz, 2H), 7.56-7.43 (m, 3H), 6.60 (d, *J* = 6.5 Hz, 1H), 5.79 (d, *J* = 9.3 Hz, 1H), 4.97 (d, *J* = 9.3 Hz, 1H), 4.72-4.56 (m, 2H), 4.39 (d, *J* = 8.3 Hz, 1H), 3.75 (s, 3H), 2.05-2.00 (m, 1H), 1.53 (s, 9H), 1.45 (d, *J* = 7.3 Hz, 3H), 1.07 (d, *J* = 7.0 Hz, 3H), 1.02 (s, 9H), 0.99 (t, *J* = 7.3 Hz, 3H), 0.89-0.85 ppm (m, 5H); HRMS(ESI-TOF): calcd for $C_{44}H_{52}N_8O_8S_4Na^+$ [M + Na^+] 971.2683, found 971.2689.

4.4.4.3 H-Thr-Tetrathiazole-Phenyloxazole-D-*allo*-Ile-Ala-OH (compound 16)

Following the ***methyl ester hydrolysis*** procedure: The methyl ester of **15** was hydrolyzed using 100 mg of **15** (0.1 mmol), 35 mg of LiOH (0.8 mmol) and 60 mL of MeOH with a catalytic amount of DI water. The resulting free acid was then subjected to the **Boc removal** procedure using 0.5 mL of TFA (50% in DCM) and 0.02 mL of anisole (0.16 mmol) to simultaneously remove both Boc and tert-butyl groups. The obtained double deproteccted linear precursor **16** was then taken on to the next step without further purification (82 mg, quantitative yield)

4.4.4.4 *cyclo*-Thr-Tetrathiazole-Phenyloxazole-D-*allo*-Ile-Ala (compound 17)

Following the ***syringe pmup cyclization*** procedure: 79 mg of **16** (0.084 mmol, 1.0 equivalent) was cyclized using 49 mg of FDPP (0.126 mmol, 1.5 equivalents), 0.11 mL DIPEA (0.588 mmol, 7.0 equivalents) in a mixture of anhydrous DCM/DMF (85 mL/ 21

mL, total concentration = 0.8 mM). The coupling reagent FDPP and the base DIPEA were placed in a round bottom flask and dissolved in 53 mL of the mixed solvent. The DDLP was dissolved in the remaining solvent and added drop-wise to the flask via a syringe pump at a rate of 30 mL/h. Upon addition of all of the peptide solution, the reaction was then allowed to stir overnight at room temperature and monitored via LCMS. The disappearance of the starting material was observed via LC/MS yet there was no sign of the desired product. Thus, the reaction was allowed to run for two more days. Upon the decomposition of the main LC/MS peak, the solvent was removed *in vacuo* and the residue was diluted with DCM (overall volume = 100 mL) and extracted twice with 10% (v/v) $HCl_{(aq.)}$. The organic layer was re-extracted with a saturated $NaHCO_3$ aqueous solution (100 mL x 3). The basic aqueous layer was back-washed with ethyl acetate (100 mL). The organic layers were collected, dried over Na_2SO_4, filtered, and concentrated *in vacuo*.

4.4.5 Experimental methods for Ustat A-1 fragment A

4.4.5.1 Boc-Oxazolidine-OMe (compound 21)

Boc-Ser-OH (1.23 g, 6.0 mmol) was dissolved in THF (40 mL) and 2,2-dimethoxypropane (1.74 mL, 60.0 mmol) and PPTS (450 mg, 1.8 mol) was added. The solution was heated at reflux for 16 h then cooled and concentrated *in vacuo*. The residue was partitioned between EtOAc (100 mL) and DI water (100 mL). The aqueous layer was

further extracted by EtOAC (50 mL x 2). The organic layers were combined, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was then dissolved in a mixture of benzene/MeOH (3:1, total volume = 60 mL). TMSD (2M solution in hexane) was added drop-wise until slightly yellow. The mixture was stirred at room temperature for 1 h. Upon completion, the solvent was removed and the residue was co-evaporated with DCM (100 mL × 5) *in vacuo*. The obtained crude ester was purified via flash column chromatography on silica gel to yield the pure ester **21** (colorless oil, 1.02g, 65% yield). R_f = 0.58 (EtOAc:Hex = 1:3)

^{1}H NMR: ($CDCl_3$, 300 MHz) δ= 4.44 (dd, *J* =6.8 Hz, 2.6 Hz, 0.4H), 4.33 (dd, *J* = 7.0 Hz, 3.1 Hz, 0.6H), 4.15-4.05 (m, 1H), 4.03-3.95 (m, 1H), 3.71 (s, 3H), 1.62 (s, 1.65H), 1.59 (s, 1.35H), 1.49 (s, 1.72H), 1.45 (s, 4.68H), 1.37 (s, 5.6H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 171.7, 171.3, 152.1, 151.2, 95.0, 94.4, 80.8, 80.3, 66.2, 66.0, 59.2, 59.1, 52.4, 52.2, 28.3, 28.2, 26.0, 25.1, 24.9, 24.3. HRMS(ESI): calcd for $C_{12}H_{21}NO_5Na^+$ $[M + Na]^+$= 282.1318, found 282.1313.

4.4.5.2 Boc-Oxazolidine-CSNH$_2$ (compound 22)

Following the ***amide conversion*** procedure, followed by the ***thioamide conversion*** procedure: Compound **21** (0.94 g, 3.6 mmol) was dissolved in a mixture of NH_4OH/MeOH (2:1, overall 120 mL) and stirred for 12 h. Upon completion, the solvent was evaporated *in vacuo*, and the resulting amide was dissolved in THF (36 mL). Lawesson's reagent (1.17 g, 2.9 mmol) was added to the THF solution and the mixture was heated at reflux for 16 h. Upon completion, the solvent was removed and the crude product was purified via flash column chromatography on silica gel to afford the desired thioamide **22** (colorless oil, 500 mg, 53% yield).

R_f =0.63 (EtOAc:Hex = 1:1)

^{1}H NMR: ($CDCl_3$, 300 MHz) δ = 7.99 (br, 1H), 7.59 (br, 1H), 4.78-4.73 (m, 1H), 4.24 (br, 2H), 1.67 (s, 3H), 1.51 (s, 3H), 1.44 (s, 9H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 207.0, 152.5, 95.4, 81.8, 69.0, 67.1, 28.3, 27.2, 22.9. HRMS(ESI): calcd for $C_{11}H_{20}N_2O_3SNa^+$ $[M + Na]^+$= 283.1093, found 283.1080

4.4.5.3 Boc-Oxazolidine-Thiazole-OEt (compound 23)

Following the ***thiazole synthesis*** procedure: Compound **23** was synthesized utilizing 500 mg of thioamide **22**, 1.54 g of $KHCO_3$, and 0.8 mL of ethyl bromopyruvate for the first step; 1.4 mL of pyridine, 1.07 mL of TFAA and 0.53 mL of TEA for the second step. The crude product was purified via a flash column chromatography to yield the desired thiazole **23** as a slightly yellow oil (611 mg, 89% yield).

R_f =0.73 (EtOAc:Hex = 1:1)

^{1}H NMR: ($CDCl_3$, 300 MHz) δ= 8.10 (s, 1H), 5.37 (br, 0.3H), 5.27 (d, J =5.5 Hz, 0.7H), 4.42 (q, J =7.2 Hz, 2H), 4.36-4.23 (m, 2H), 1.80 (s, 2H), 1.74 (s, 1H), 1.58 (s, 3H), 1.52 (s, 3H), 1.40 (t, J =7.0 Hz, 3H), 1.32 (s, 6H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 175.3, 161.4, 151.5, 147.2, 127.1, 95.3, 94.8, 81.5, 81.0, 69.4, 69.0, 61.5, 60.0, 28.2, 27.3, 26.6, 24.0, 22.8, 14.4, 13.9. HRMS(ESI): calcd for $C_{16}H_{24}N_2O_5SNa^+$ $[M + Na]^+$= 379.1304, found 379.1301.

4.4.5.4 Boc-Oxazolidine-Thiazole-$CSNH_2$ (compound 18)

Compound **18** was synthesized following the ***amide conversion*** procedure, followed by the ***thioamide conversion*** procedure. The thiazole **23** (575 mg, 1.6 mmol) was dissolved in a mixture of NH_4OH/MeOH (3:1, overall 64 mL) and stirred overnight. Upon completion, the solvent was evaporated *in vacuo*, and the resulting amide was

dissolved in benzene (32 mL). Lawesson's reagent (520 mg, 1.3 mmol) was added to the solution and the mixture was heated at reflux for 16 h. Upon completion, the solvent was removed and the crude product was purified via flash column chromatography on silica gel to afford the desired thioamide **18** (light yellow oil, 398 mg, 72% yield).

R_f =0.3 (EtOAc:Hex = 3:7).

^{1}H NMR: ($CDCl_3$, 300 MHz) δ= 8.60 (br, 1H), 8.40 (s, 1H), 7.82 (br, 1H), 5.30 (br, 0.4H), 5.16 (d, *J* =5.5 Hz, 0.6H), 4.36-4.26 (m, 1H), 4.26-4.14 (m, 1H), 1.78 (s, 1.6H), 1.73 (s, 1.4H), 1.60 (s, 3H), 1.52 (s, 3H), 1.35 (s, 6H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 190.6, 173.6, 172.8, 152.8, 152.4, 151.3, 127.6, 127.6, 95.3, 94.9, 81.7, 81.1, 69.0, 68.4, 59.2, 29.7, 28.3, 27.2, 26.4, 24.2, 23.1. HRMS(ESI): calcd for $C_{14}H_{21}N_3O_3S_2Na^+$ [M + Na]$^+$= 366.0922, found 366.0914.

4.4.5.5 Boc-Oxazolidine-Dithiazole-Phenyloxazole-OMe (compound 24)

Compound **24** was synthesized following the ***thiazole synthesis*** procedure. To a solution of **23** (364 mg, 1.06 mmol) in DME (21 mL) was added $KHCO_3$ (850 mg, 8.49 mmol) and the resulting suspension was stirred at room temperature for 10 min. The solution of bromoketo phenyloxazole **2** (987 mg, 3.04 mmol) in DME (21 mL) was added drop-wise to the suspension and the mixture was stirred overnight. Upon completion, the solvent was removed *in vacuo* and the residue was redissolved in ethyl acetate, extracted with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude thiazoline intermediate was redissolved in DME (42 mL) and pyridine (0.77 mL, 9.55 mmol) was slowly added at 0 °C. After 5 min, TFAA (0.6 mL, 4.24 mmol) was added and the reaction was stirred for an additional 3 h at 0 °C. The reaction mixture was allowed to warm to room temperature, followed by slowly addition of TEA (0.3 mL, 2.12 mmol)

and the reaction was stirred for 20 min. Upon completion, the solvent was then evaporated and the residue was dissolved in EtOAc (100 mL), washed with HCl solution (pH = 1, 100 mL x 2), saturated aqueous $NaHCO_3$ solution (100 mL x 2). The organic layer was dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by flash column chromatography to yield compound **24** as white solid (583 mg, 96% yield).

R_f = 0.5 (EtOAc:Hex = 1:1).

^{1}H NMR: ($CDCl_3$, 300 MHz) δ= 8.38-7.89 (m, 4H), 7.88-7.42 (m, 3H), 5.38 (br, 0.4H), 5.27 (d, *J* =5.1 Hz, 0.6H), 4.40-4.20 (m, 2H), 3.98 (s, 3H), 1.83 (s, 1.7H), 1.77 (s, 1.3H), 1.62 (s, 3H), 1.54 (s, 3H), 1.34 (s, 6H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 174.6, 173.9, 163.9, 162.5, 155.4, 152.4, 151.4, 148.2, 143.4, 130.5, 130.2, 129.0, 128.9, 128.6, 128.5, 127.8, 126.7, 121.7, 117.4, 95.3, 94.8, 81.4, 80.9, 69.1, 68.7, 59.3, 59.2, 53.6, 52.4, 29.7, 28.2, 27.2, 26.4, 24.2, 23.1. HRMS(ESI): calcd for $C_{27}H_{28}N_4O_6S_2Na^+$ $[M + Na]^+$= 591.1348, found 591.1343.

4.4.5.6 Boc-Oxazolidine-Dithiazole-Phenyloxazole-OH (Fragment A)

Fragment **A** was synthesized following the ***methyl ester hydrolysis*** procedure utilizing 146 mg (0.26 mmol) of **24**, 86 mg (2.06 mmol) of $LiOH{\cdot}H_2O$ and 2.6 mL MeOH. The obtained free acid Fragment **A** was taken on without further purification or characterization (142 mg, quantitative yield).

4.4.6 Experimental methods for Ustat A-1 fragment B

4.4.6.1 Boc-Thr(tBu)-Ser(Bn)-OMe (compound 25)

Following the ***general peptide synthesis*** procedure: The dipeptide **25** was synthesized using 1.37 g of Boc-Thr(tBu)-OH, 1.25 g of free amine NH_2-Ser(Bn)-OMe, 3.5 mL of DIPEA, and 1.92 g of TBTU (colorless oil, 2.2g, 93% yield).

R_f = 0.74 (EtOAc:Hex = 1:3)

^{1}H NMR: ($CDCl_3$, 300 MHz): δ = 7.96 (d, *J* = 5.4 Hz, 1H), 7.34-7.21 (m, 5H), 5.60 (br, 1H), 4.70-4.67 (m, 1H), 4.49 (q, *J* = 19.2 Hz, 2H), 4.14-4.10 (m, 2H), 3.82 (dd, *J* = 5.7, 2.4 Hz, 1H), 3.74 (s, 3H), 3.67 (dd, *J* = 2.4, 2.6 Hz, 1H), 1.45 (s, 9H), 1.27 (s, 9H), 1.12 ppm (d, *J* = 4.8 Hz, 3H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 170.60, 170.16, 155.67, 137.40, 128.34, 127.79, 127.66, 127.60, 79.92, 75.13, 73.20, 69.37, 67.08, 58.43, 52.94, 52.38, 28.29, 28.15, 17.22 ppm ; HRMS(ESI): calcd for $C_{24}H_{38}N_2O_7Na^+$ [M + Na^+] 489.2577, found 489.2561.

4.4.6.2 Boc-Thr(tBu)-Ser-OMe (compound 26)

The dipeptide **26** was synthesized following the ***hydrogenolysis*** procedure using 1.1 g of **25** and 10 mg of Pd black in ethanol. The obtained dipeptide was taken on without further purification or characterization (870 mg, quantitative yield).

4.4.6.3 Boc-Thr(tBu)-Oxazole-OMe (compound 27)

The oxazole **27** was synthesized following the ***oxazole synthesis*** procedure using 1.05 g of **26**, 0.6 mL of DAST and 770 mg of K_2CO_3 for the first step, 0.84 mL of DBU and 0.55 mL of $BrCCl_3$ for the second step (white solid, 717 mg, 72% yield).

R_f =0.74 (EtOAc:Hex = 1:1)

^{1}H NMR: ($CDCl_3$, 300 MHz) δ = 8.21 (s, 1H), 5.58 (d, *J* = 6.3 Hz, 1H), 4.85 (d, *J* = 6.9 Hz, 1H), 4.12 (d, *J* = 6.5 Hz, 1H), 3.89 (s, 3H), 1.48 (s, 9H), 1.21 (d, *J* = 4.8 Hz, 3H),

0.92 ppm (s, 9H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 164.56, 161.58, 155.78, 143.72, 133.34, 79.92, 74.35, 68.22, 55.34, 52.13, 28.81, 28.78, 20.13 ppm ; HRMS(ESI): calcd for $C_{17}H_{28}N_2O_6Na^+$ [M + Na^+] 379.1845, found 379.1832.

4.4.6.4 H-Thr(tBu)-Oxazole-OMe (compound 19)

To a 50 mL round bottom flask was added the oxazole **27** (260 mg, 0.85 mmol) and 5 mL of 1,4-dioxane. 17 mL of 4 M HCl was then added to the solution and the reaction mixture was cooled to 0 ^oC. The reaction was stirred at 0 ^oC for 10 min and then at room temperature for an additional 1 h. Upon completion, confirmed via TLC, the reaction mixture was concentrated *in vacuo* to remove excess acid and the residue was taken on without further purification or characterization (218 mg, quantitative yield).

4.4.6.5 Boc-D-*allo*-Ile-Ala-OH (compound 20)

Following the ***methyl ester hydrolysis*** procedure: Compound **20** was synthesized using 224 mg of the dipeptide **12** (0.71 mmol), 238 mg of LiOH (5.67 mmol) and 7.1 mL of MeOH. The resulting free acid was then subjected to the next step without further purification or characterization (214 mg, quantitative yield).

4.4.6.6 H-D-*allo*-Ile-Ala-Thr(tBu)-Oxazole-OMe (Fragment B)

To a 100 mL round bottom flask was added the free amine **19** (218 mg, 0.85 mmol), the free acid **20** (214 mg, 0.71 mmol), DMTMM (157 mg, 0.57 mmol), HATU (108 mg, 0.28 mmol) and purged with nitrogen. DCM (15.0 mL) was added to the flask and DIPEA (0.5 mL, 2.83 mmol) was added drop-wise to the mixture. The reaction was run for 2 h at room temperature. Upon completion, confirmed via TLC, the reaction mixture was diluted with DCM (40 mL), extracted with 10% HCl solution (50 mL x 2),

then saturated aqueous $NaHCO_3$ solution (50 mL x 2). The collected organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography (silica gel, EtOAc/Hex) to yield the desired dipeptide Boc-D-*allo*-Ile-Ala-Thr(tBu)-Oxazole-OMe as light brown solid (227 mg, 60%). The dipeptide (203 mg, 0.42 mmol) was then dissolved in 5 mL of 1,4-dioxane. 10 mL of 4 M HCl was then added to the solution and the reaction mixture was cooled to 0 ^{o}C. The reaction was stirred at 0 ^{o}C for 10 min and then at room temperature for an additional 1 h. Upon completion, confirmed via TLC, the reaction mixture was concentrated *in vacuo*. The obtained free amine fragment **B** was taken on without further purification or characterization (165 mg, quantitative yield).

4.4.7 Experimental methods for Ustat A-1 fragment C

4.4.7.1 H-Thr-Oxazole-OMe (compound 32)

The oxazole **19** (300 mg, 0.84 mmol) was dissolved in 50% TFA (TFA:DCM = 1:1, total volume = 8.4 mL) and anisole (0.22 mL, 2 mmol) was added to the mixture. The reaction was stirred for 1.5 h at room temperature. Upon completion, the solution was concentrated *in vacuo* with DCM (100 mL x 5) to yield compound **32** as a light brown oil (168 mg, quantitative yield). The free amine **32** was taken on without further purification or characterization.

4.4.7.2 Boc-Ala-Thr-Oxazole-OMe (compound 33)

Following the ***general peptide synthesis*** procedure: Compound **33** was synthesized using **32** (168 mg, 0.84 mmol), Boc-Ala-OH (145 mg, 0.77 mmol), DMTMM (423 mg, 1.53 mmol), DIPEA (1.07 mL, 6.12 mmol) and 7.7 mL of DCM. The reaction was run for 3 h at room temperature. Upon completion, confirmed via TLC, the

reaction mixture was diluted with DCM (40 mL), extracted with 10% HCl solution (50 mL x 2) and then saturated aqueous $NaHCO_3$ solution (50 mL x 2). The collected organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography (silica gel, EtOAc/hexanes) to yield compound **33** as light brown solid (323 mg, 90% yield).

R_f =0.3 (EtOAc:Hex = 3:1)

^{1}H NMR: ($CDCl_3$, 300 MHz): δ = 8.17 (s, 1H), 7.39 (d, *J* =6.78 Hz, 1H), 5.37 (br, 1H), 5.21 (d, *J* =7.15 Hz, 1H), 4.43 (br, 1H), 4.26 (br, 1H), 3.88 (s, 3H), 3.78-3.54 (br, 1H), 1.42 (s, 9H), 1.38 (d, *J* =6.2 Hz, 3H), 1.24 (d, *J* =5.1 Hz, 3H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 173.4, 163.7, 161.3, 155.6, 144.2, 132.9, 80.2, 68.2, 55.9, 52.5, 52.3, 50.3, 29.7, 19.3, 18.1. HRMS(ESI): calcd for $C_{16}H_{25}N_3O_7Na^+$ $[M + Na]^+$= 394.1591, found 394.1591

4.4.7.3 Boc-Ala-(*Z*)-Enamide-Oxazole-OMe (compound 34)

Compound **33** (150 mg, 0.4 mmol) was dissolved in 8.0 mL of DCM and then TEA (0.44 mL, 4.0 mmol) was added drop-wise to the solution. After 10 min, MsCl (0.25 mL, 3.2 mmol) was added drop-wise and the reaction was stirred for an additional 1 h. Upon completion, confirmed via TLC, the reaction mixture was diluted with DCM (40 mL) and extracted by DI water (50 mL x 2). The organic layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was dissolved in DCM (8 mL) and TEA (0.44 mL, 4.0 mmol) was added to the solution. The reaction was monitored by LC/MS and upon completion, the reaction mixture was diluted with DCM (40 mL) and extracted by DI water (50 mL x 2). The organic layer was collected, dried and concentrated. The

crude product was purified via a flash column chromatography on silica gel to furnish compound **34** (139 mg, 97% yield) as a white solid.

R_f =0.58 (EtOAc:Hex = 3:1).

^{1}H NMR: (DMF-d_7, 300 MHz) δ = 9.54 (s, 1H), 8.77 (s, 1H), 6.95 (d, *J* =7.3 Hz, 1H), 6.59 (q, *J* =7.2 Hz, 1H), 4.35 (p, *J* =7.2 Hz, 1H), 3.88 (s, 3H), 1.84 (d, *J* =7.1 Hz, 3H), 1.45 (d, *J* =6.9 Hz, 3H), 1.44 (s, 9H). ^{13}C NMR: (DMF-d_7, 75 MHz) δ = 172.2, 160.8, 155.6, 145.0, 133.5, 128.1, 124.1, 78.3, 51.5, 50.5, 27.9, 18.0, 13.1. HRMS(ESI): calcd for $C_{16}H_{23}N_3O_6Na^+$ $[M + Na]^+$= 376.1485, found 376.1486.

4.4.7.4 Boc-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OMe (compound 35)

The Boc protecting group of compound **34** (122 mg, 0.35 mmol) was removed following the ***Boc removal*** procedure using 20% TFA (TFA:DCM = 1:4, overall 3.45 mL) and anisole (0.075 mL, 0.69 mmol). The obtained free amine was coupled to Boc-D-*allo*-Ile-OH (75 mg, 0.31 mmol) following the ***general peptide synthesis*** procedure using DMTMM (174 mg, 0.63 mmol), DIPEA (0.44 mL, 2.5 mmol) and 6.3 mL of DCM. The reaction was monitored by LC/MS. Upon completion, the reaction mixture was diluted with DCM (40 mL) and extracted by DI water (50 mL x 2). The organic layer was collected, dried and concentrated. The crude product was purified via a flash column chromatography on silica gel to furnish compound **35** (114 mg, 77% yield) as a white solid.

R_f = 0.35 (EtOAc:Hex = 3:1)

^{1}H NMR: ($CDCl_3$, 300 MHz) δ = 8.19 (br, 1H), 8.12 (s, 1H), 7.06 (d, *J* =7.2 Hz, 1H), 6.70 (q, *J* =7.2 Hz, 1H), 5.41 (d, *J* =6.6 Hz, 1H), 4.74 (p, *J* =7.3 Hz, 1H), 4.06 (dd, *J* =8.8 Hz, 6.0 Hz, 1H), 3.90 (s, 3H), 2.01 (m, 1H), 1.82 (d, *J* =7.2 Hz, 3H), 1.47 (d, *J* =7.0 Hz,

3H), 1.46 (m, 1H), 1.37 (s, 9H), 1.23 (m, 1H), 0.94 (t, *J* =7.4 Hz, 3H), 0.92 (d, *J* =6.8 Hz, 3H). ^{13}C NMR: ($CDCl_3$, 150 MHz) δ = 172.6, 170.8, 161.6, 160.4, 156.4, 143.7, 133.7, 130.3, 122.5, 80.1, 58.7, 52.2, 48.8, 36.4, 31.9, 29.7, 29.6, 28.2, 26.3, 17.4, 14.6, 14.1, 11.6. HRMS(ESI): calcd for $C_{22}H_{34}N_4O_7Na^+$ $[M + Na]^+$= 489.2325, found 489.2326.

4.4.7.5 H-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OMe (Fragment C)

To a 5 mL round-bottom flask was added **35** (22 mg, 0.047 mmol), and HCl (4M in dioxane, 0.5 mL). The reaction was stirred for 20 min at room temperature. Upon completion, HCl was removed *in vacuo* to yield fragment **C** (17.2 mg, quantitative yield) as a light brown solid. The free amine was taken on without further purification.

4.4.8 Experimental methods for the assembly of Ustat A-1 fragments

4.4.8.1 Boc-Oxazolidine-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-Thr(tBu)-Oxazole-OMe (compound 28)

Following the ***general peptide synthesis*** procedure: Compound **28** was synthesized using fragment **A** (142 mg, 0.26 mmol), fragment **B** (165 mg, 0.38 mmol), DMTMM (43 mg, 0.15 mmol), HATU (60 mg, 0.15 mmol), PyBroP (72 mg, 0.15 mmol), DIPEA (0.2 mL, 1.02 mmol) and 10.3 mL of DCM. The reaction was run for 4 h at room temperature. Upon completion, confirmed via LC/MS, the reaction mixture was diluted with DCM (40 mL), extracted with 10% HCl solution (50 mL x 2) and then saturated aqueous $NaHCO_3$ solution (50 mL x 2). The collected organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography (silica gel, EtOAc/hexanes) to yield compound **28** as light brown solid (87 mg, 35% yield).

R_f = 0.28 (EtOAc:Hex = 3:2)

^{1}H NMR: ($CDCl_3$, 300 MHz) δ= 8.39 (s, 1H) 8.37 (s, 1H), 8.25-8.16 (m, 2H), 8.13 (s, 1H), 7.89 (m, 1H), 7.57-7.41(m, 3H), 7.12-6.93 (m, 1H), 6.88 (d, *J* = 7.4 Hz, 0.6H), 6.77 (d, *J* = 7.3 Hz, 0.4H), 5.41 (br, 0.4H), 5.27 (br, 0.6H), 5.16 (d, *J* = 9.0 Hz, 1H), 4.84-4.55 (m, 1H), 4.46-4.10 (m, 3H), 3.92 (s, 3H), 2.28-2.14 (m, 1H), 1.86 (s, 2H), 1.80 (s, 1H), 1.64 (d, *J* = 3.6 Hz, 3H), 1.61-1.52 (m, 3H), 1.52-1.43 (m, 6H), 1.37 (s, 6H), 1.33-1.26 (m, 2H), 1.23 (d, *J* = 6.3 Hz, 2H), 1.17 (d, *J* = 6.3 Hz, 1H), 1.07 (d, *J* = 6.8 Hz, 2H), 0.99 (d, *J* = 2.8 Hz, 8H), 0.96 (s, 3H). HRMS(ESI): calcd for $C_{47}H_{60}N_8O_{11}S_2Na^+$ $[M + Na]^+$= 999.3721, found 999.3699.

4.4.8.2 H-Ser-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-Thr-Oxazole-OH (compound 29)

The methyl ester of **28** was hydrolyzed following the ***methyl ester hydrolysis*** procedure using **28** (75 mg, 7.63 x 10^{-5} mol), LiOH (27 mg, 6.4 x 10^{-4} mol), 2 mL of MeOH and 2 mL of DCM. After work-up, the obtained free acid was dissolved in 5 mL of 1,4-dioxane. 17 mL of 4 M HCl was then added to the solution and the reaction mixture was cooled to 0 ^oC. The reaction was stirred at 0 ^oC for 10 min and then at room temperature for an additional 30 min. Upon completion, confirmed via TLC, the reaction mixture was concentrated *in vacuo* to remove excess acid and the residue was taken on without further purification or characterization (59 mg, quantitative yield).

4.4.8.3 *cyclo*-Ser-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-Thr-Oxazole (compound 30)

Following the ***syringe pump cyclization*** procedure: Compound **30** was synthesized using 59 mg of **29** from previous step (7.63 x 10^{-5} mol, 1.0 equivalent), 13 mg of DMTMM (4.58 x 10^{-5} mol, 0.6 equivalent), 18 mg of HATU (4.58 x 10^{-5} mol, 0.6

equivalent), 18 μL of T3P (50% in EtOAc, 6.11 x 10^{-5} mol, 0.8 equivalent), 2 mg of DMAP (1.53 x 10^{-5} mol, 0.2 equivalent), 0.1 mL DIPEA (6.11 x 10^{-4} mol, 8.0 equivalents) and anhydrous DCM/DMF (DCM:DMF = 1:1, total volume = 40 mL, 2 mM). All coupling reagents were weighed into a round bottom flask and dissolved in 20 mL of the mixed solvent. Compound **29** was dissolved in the remaining solvent and added drop-wise to the flask via a syringe pump at a rate of 30 mL/h. Upon addition of all of the solution, the reaction was then allowed to stir overnight at room temperature and monitored via LCMS. Upon completion, the solvent was removed *in vacuo* and the residue was diluted with ethyl acetate (overall volume = 100 mL) and extracted twice with 10% (v/v) $HCl_{(aq.)}$. The organic layer was re-extracted with a saturated $NaHCO_3$ aqueous solution (100 mL x 3). The basic aqueous layer was back-washed with ethyl acetate (100 mL). The organic layers were collected, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was taken on to the next step without further purification.

4.4.8.4 *cyclo*-Enamide-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole (compound 31)

Compound **31** was synthesized using 60 mg of crude **29** from previous step (8.03 x 10^{-5} mol, 1.0 equivalent), 0.12 mL of DBU (8.03 x 10^{-4} mol, 10.0 equivalents), 0.05 mL of MsCl (6.42 x 10^{-4} mol, 8.0 equivalents) and 10 mL of DCM (8 mM). Compound **29** was dissolved in DCM and the solution was cooled to 0 ^{o}C. DBU was then added drop-wise to the solution. After 10 min, MsCl was added drop-wise and the reaction was allowed to stir overnight. Upon the disappearance of starting material, confirmed via

LC/MS, the reaction mixture was diluted with DCM (40 mL) and extracted by 10% HCl (50 mL x 2). The organic layer was collected, dried and concentrated *in vacuo*.

4.4.8.5 Boc-Oxazolidine-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OMe (compound 36)

Following the ***general peptide synthesis*** procedure: Compound **36** was synthesized using 43 mg (0.078 mmol) of fragment A, 32 mg (0.087 mmol) of fragment C, 22.0 mg (0.047 mmol) of PyBroP, 16.0 mg (0.055 mmol) of DMTMM, 21.0 mg (0.055 mmol) of HATU, 0.11 mL (0.62 mmol) of DIPEA dissolved in 7.84 mL DCM. The reaction was stirred overnight and monitored by LC/MS. Upon completion, the reaction mixture was diluted with DCM (50 mL) and extracted by 10% HCl solution (50 mL x 2), then saturated aqueous $NaHCO_3$ solution (50 mL x 2). The organic layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified via a flash column chromatography on silica gel to yield compound **36** (59 mg, 83% yield) as a white solid.

R_f = 0.4 (EtOAc: Hex = 3:1)

^{1}H NMR: ($CDCl_3$, 300 MHz) δ = 8.37 (br, 1H), 8.30-8.22 (m, 2H), 8.20 (br, 1H), 8.07 (s, 1H), 8.04 (br, 1H), 7.98 (d, *J* =7.8 Hz, 1H), 7.56, (br, 1H), 7.43-7.34 (m, 3H), 6.64 (q, *J* =7.2 Hz, 1H), 5.40 (br, 0.4H), 5.29 (d, *J* =5.2 Hz, 0.6H), 4.76 (p, *J* =7.3 Hz, 1H), 4.53 (dd, *J* =8.2 Hz, 6.5 Hz, 1H), 4.45-4.23 (m, 2H), 3.83 (s, 3H), 2.15 (m, 1H), 1.85 (s, 1.8H), 1.79 (s, 1.2H), 1.72 (d, *J* =7.2 Hz, 3H), 1.64 (s, 3H), 1.57 (s, 4.8H), 1.53 (s, 2.2H), 1.36 (s, 6H) 1.30-1.27 (br, 1H), 1.08 (d, *J* =6.8 Hz, 3H), 0.95 (t, *J* =7.3 Hz, 3H). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ = 174.9, 171.8, 171.1, 161.9, 161.4, 160.5, 154.2, 153.0, 148.0, 143.5, 133.5, 131.3, 130.2, 129.6, 128.5, 128.3, 126.7, 122.3, 121.7, 117.7, 95.3, 81.0,

69.1, 59.3, 58.3, 52.2, 49.2, 38.9, 38.6, 36.6, 28.3, 27.2, 26.4, 26.2, 23.1, 17.6, 15.1, 14.1, 13.9, 11.5. HRMS(ESI): calcd for $C_{43}H_{50}N_8O_{10}S_2Na^+$ $[M + Na]^+$= 925.2989, found 925.2989.

4.4.8.6 H-Ser-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OH (compound 37)

The methyl ester of **36** (59 mg, 0.065 mmol) was hydrolyzed following the ***methyl ester hydrolysis*** procedure using MeOH (1.3 mL) and LiOH (27.4 mg, 0.65 mmol). The reaction was run overnight at room temperature. Upon completion, confirmed via LC/MS, the solvent was removed *in vacuo*. The residue was diluted in DCM (50 mL) and extracted with 10% HCl aqueous solution (50 mL x 2). The organic layer was collected, dried over Na_2SO_4, and concentrated *in vacuo* to afford the free acid intermediate. The free acid was then dissolved in HCl (4M in dioxane, 0.8 mL) and stirred for 20 min. Upon completion, confirmed via TLC, HCl was removed *in vacuo* and the resulting double deprotected linear precursor **37** (48.8 mg, quantitative yield) was used without further purification or characterization.

4.4.8.7 *cyclo*-Ser-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole (compound 38)

Following the ***syringe pump macrocyclization*** procedure: The precursor **37** (20 mg, 0.027 mmol) was dissolved in a mixture of DCM (4 mL), DMF (2 mL), and DIPEA (0.1 mL). The mixture was then placed into a syringe. To a 25 mL round-bottom flask was added HATU (13 mg, 0.034 mmol), DMTMM (7 mg, 0.025 mmol), T3P (5 µL, 0.018 mmol), DIPEA (0.1 mL), and DCM (10 mL). The mixture of **37** was injected to the flask at the rate of 0.1mL/min. The reaction was stirred overnight and monitored by

LC/MS. Upon completion, the solvent was removed *in vacuo*. The residue was dissolved in DCM (50 mL), extracted by 10% HCl solution (50 mL x 2), then saturated aqueous $NaHCO_3$ solution (50 mL x 2). The organic layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was subjected to reversed-phase HPLC purification to furnish the macrocyclic compound **38** (4.3 mg, 25%) as a white solid.

^{1}H NMR: (CD_3OD, 600 MHz) δ = 8.47, (s, 1H), 8.45 (br, 1H), 8.39 (s, 1H), 8.32-8.29 (m, 2H), 8.28 (s, 1H), 7.57-7.45 (m, 3H), 6.73 (q, *J* =7.2 Hz, 1H), 5.72 (t, *J* =6.2 Hz, 1H), 5.03 (d, *J* =3.6 Hz, 1H), 4.58 (br, 1H), 4.46 (q, *J* =7.4 Hz, 1H), 4.01-3.92 (m, 2H), 2.31 (m, 1H), 1.84 (d, *J* =7.2 Hz, 3H), 1.67 (d, *J* =7.4 Hz, 3H), 1.48-1.41 (m, 1H), 1.22-1.17 (m, 1H), 0.99 (t, *J* =7.3 Hz, 3H), 0.88 (d, *J* =6.8 Hz, 3H). ^{13}C NMR: (CD_3OD, 150 MHz) δ = 174.6, 174.2, 171.2, 164.7, 163.6, 162.5, 161.3, 156.1, 154.4, 149.4, 144.2, 143.6, 137.6, 132.5, 131.7, 131.2, 129.8, 129.5, 128.3, 124.0, 123.6, 122.8, 65.7, 57.1, 53.4, 52.7, 39.8, 27.8, 17.7, 15.1, 14.5, 12.5. HRMS(ESI): calcd for $C_{34}H_{34}N_8O_7S_2Na^+$ [M + Na]$^+$= 753.1890, found 753.1891.

4.4.8.8 *cyclo*-Dioxazole-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide (Ustat-1)

Following the ***oxazole synthesis*** procedure: The cyclic precursor **38** (10 mg, 13.7 μmol) was dissolved in a mixture of DCM (0.5 mL) and THF (1.0 mL) then cooled to -78^{o}C. DAST (20 μL, 0.12 mmol) was added to the cooled mixture and stirred for 30 min. Pyridine (20 μL, 0.24 mmol) was added to the mixture and the reaction was allowed to warm to room temperature while running overnight. Upon completion, confirmed via LC/MS, the reaction mixture was diluted with DCM (20 mL) and extracted with saturated

aqueous $NaHCO_3$ solution (20 mL x 2). The organic layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was dissolved in DCM (1 mL) and cooled to -78°C. DBU (20 μL, 0.13 mmol) was added to the cooled mixture and stirred for 20 min. $BrCCl_3$ (15 μL, 0.15 mmol) was added to the mixture and the reaction was run overnight and allowed to warm to room temperature. Upon completion, confirem via LC/MS, the reaction was diluted with DCM (20 mL) and extracted with 10% HCl solution (20 mL x 2), then saturated aqueous $NaHCO_3$ solution (20 mL x 2). The organic layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was subjected to reversed-phase HPLC purification to furnish a 2:1 inseparable mixture of (*Z*)-Ustat-1 with its (*E*)-isomer (*Z*:*E*= 2:1, ratio determined by ^{1}H NMR integrations of protons at position 9, overall 1.50 mg, 15% yield) as a white solid.

(*Z*)-Ustat-1

^{1}H NMR: ($CDCl_3$, 600 MHz): δ = 8.65 (d, *J* =11.0 Hz, 1H), 8.45 (d, *J* =7.7 Hz, 2H), 8.27 (s, 1H), 8.14 (s, 1H), 8.11 (s, 1H), 7.96 (d, *J* =6.6 Hz, 1H), 7.94 (s, 1H), 7.75 (s, 1H), 7.51 (t, *J* =7.5 Hz, 3H), 6.56 (q, *J* =7.2 Hz, 1H), 5.06 (dd, *J* =11.2 Hz, 4.7 Hz, 1H), 4.60 (p, *J* =7.0 Hz, 1H), 2.26 (m, 1H), 1.94 (d, *J* =7.1 Hz, 3H), 1.49 (m, 1H), 1.48 (d, *J* =7.2 Hz, 3H), 1.26 (m, 1H), 1.08 (d, *J* =7.1 Hz, 3H), 0.91 (t, *J* =7.5 Hz, 3H). ^{13}C NMR: ($CDCl_3$, 150 MHz, obtained from HSQC and HMBC) δ = 171.7, 161.4, 160.3, 157.7, 156.6, 153.1, 148.4, 142.6, 138.3, 136.6, 130.3, 130.2, 128.6, 128.6, 128.1, 128.1, 127.8, 126.9,

123.2, 118.4, 117.7, 57.0, 50.0, 37.9, 26.6, 16.1, 14.8, 14.1, 11.7. HRMS(ESI): calcd for $C_{34}H_{30}N_8O_6S_2Na^+$ $[M + Na]^+$= 733.1628, found 733.1609.

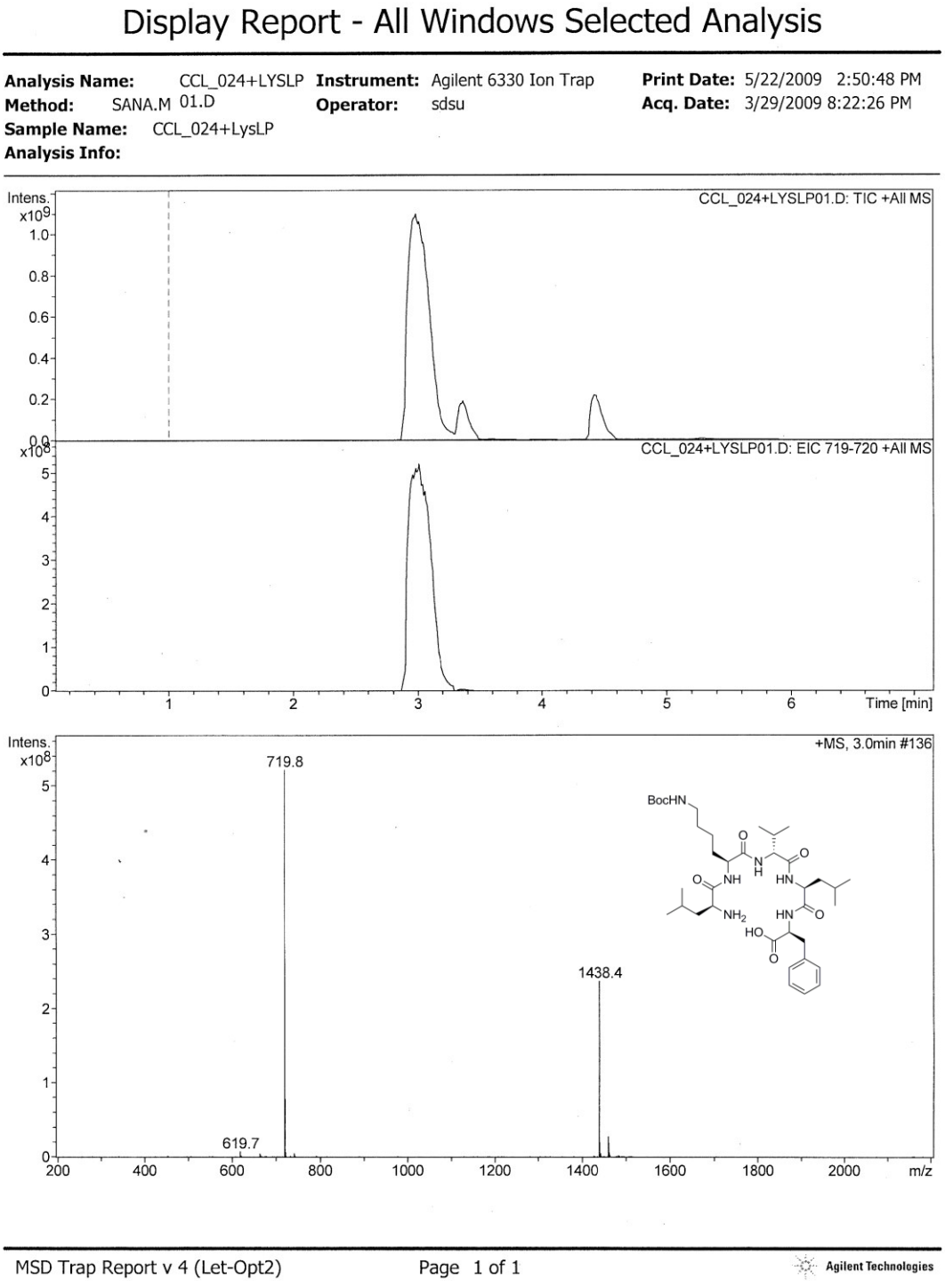
(*E*)-isomer

^{1}H NMR: ($CDCl_3$, 600 MHz): δ = 8.67 (d, *J* =11.5 Hz, 1H), 8.45 (d, *J* =7.7 Hz, 2H), 8.36 (s, 1H), 8.24 (s, 1H), 8.16 (s, 1H), 8.09 (s, 1H), 7.93 (s, 1H), 7.76 (s, 1H), 7.46 (t, *J* =6.8 Hz, 3H), 6.62 (q, *J* =7.6 Hz, 1H), 5.04 (dd, *J* =12.0 Hz, 4.8 Hz, 1H), 4.46 (p, *J* =6.8 Hz, 1H), 2.26 (m, 1H), 2.18 (d, *J* =7.7 Hz, 3H), 1.45 (d, *J* =7.3 Hz, 3H), 1.31 (m, 1H), 1.26 (m, 1H), 1.10 (d, *J* =7.9 Hz, 3H), 0.88 (t, *J* =7.4 Hz, 3H). ^{13}C NMR: ($CDCl_3$, 150 MHz, obtained from HSQC and HMBC) δ = 172.3, 161.4, 157.7, 153.1, 148.4, 142.6, 138.6, 136.6, 130.2, 130.2, 128.6, 128.6, 128.1, 128.1, 126.9, 126.9, 121.9, 118.4, 117.7, 57.0, 51.0, 37.9, 29.7, 16.3, 14.8, 14.2, 13.7. HRMS(ESI): calcd for $C_{34}H_{30}N_8O_6S_2Na^+$ $[M + Na]^+$= 733.1628, found 733.1609.

Appendices

Appendix A – Supporting spectra for Chapter 2

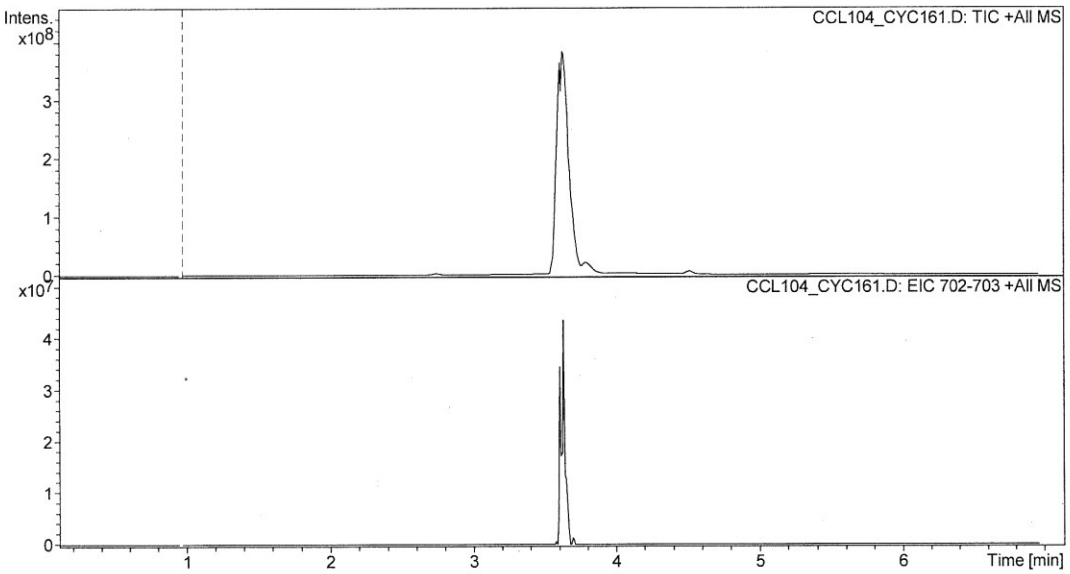
Supporting Spectra for Compound 4

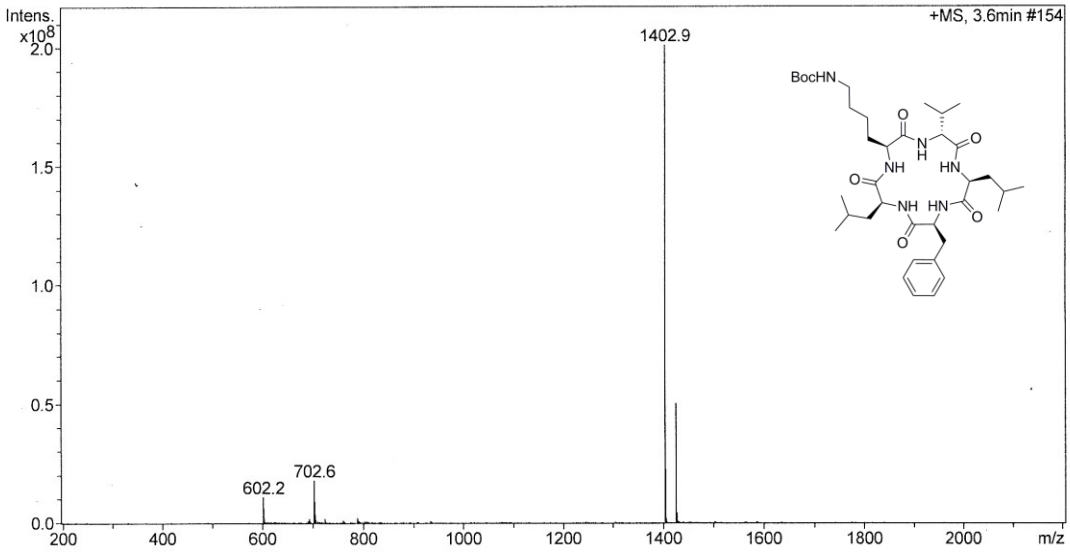


LC/MS HO-Phe-Leu-D-Val-Lys(Boc)-Leu-H

Display Report - All Windows Selected Analysis

Analysis Name: CCL104_CYC161. **Instrument:** Agilent 6330 Ion Trap **Print Date:** 5/19/2009 6:53:19 PM
Method: SANA.M D **Operator:** sdsu **Acq. Date:** 5/12/2009 12:02:52 PM
Sample Name: CCL104_cyc161
Analysis Info:





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LCMS *cyclo*-Phe-Leu-D-Val-Lys(Boc)-Leu

Display Report - All Windows Selected Analysis

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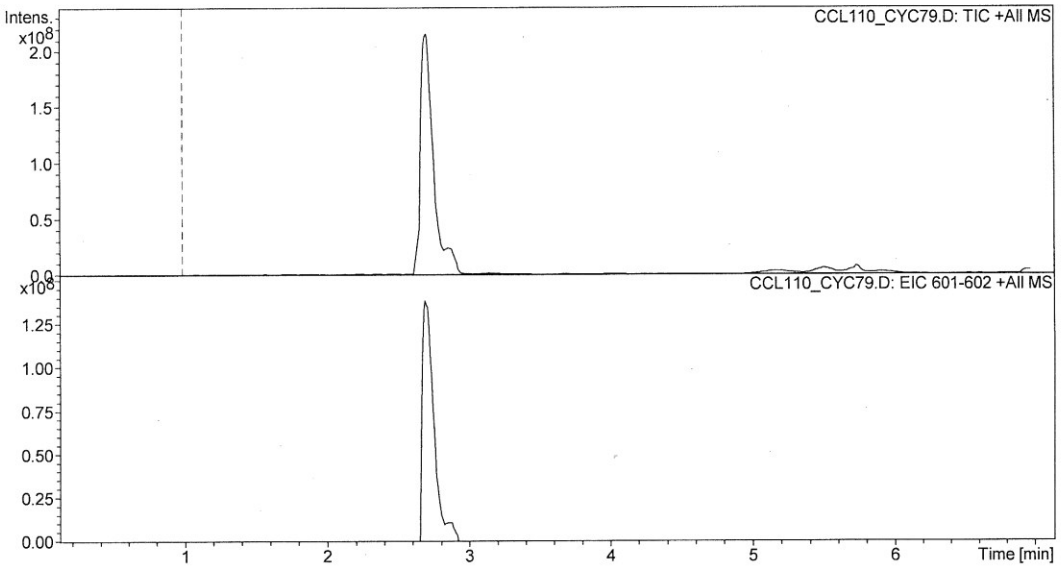
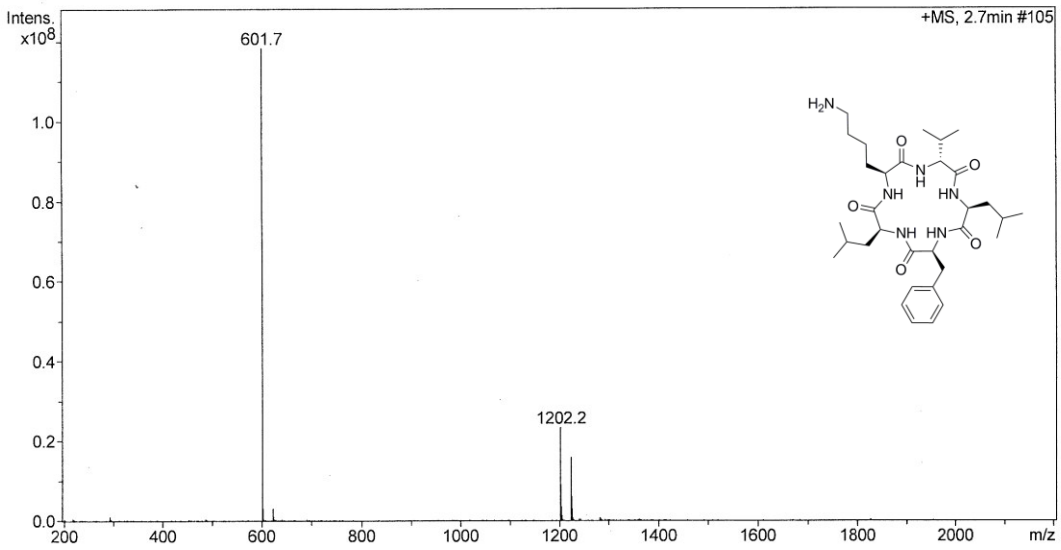
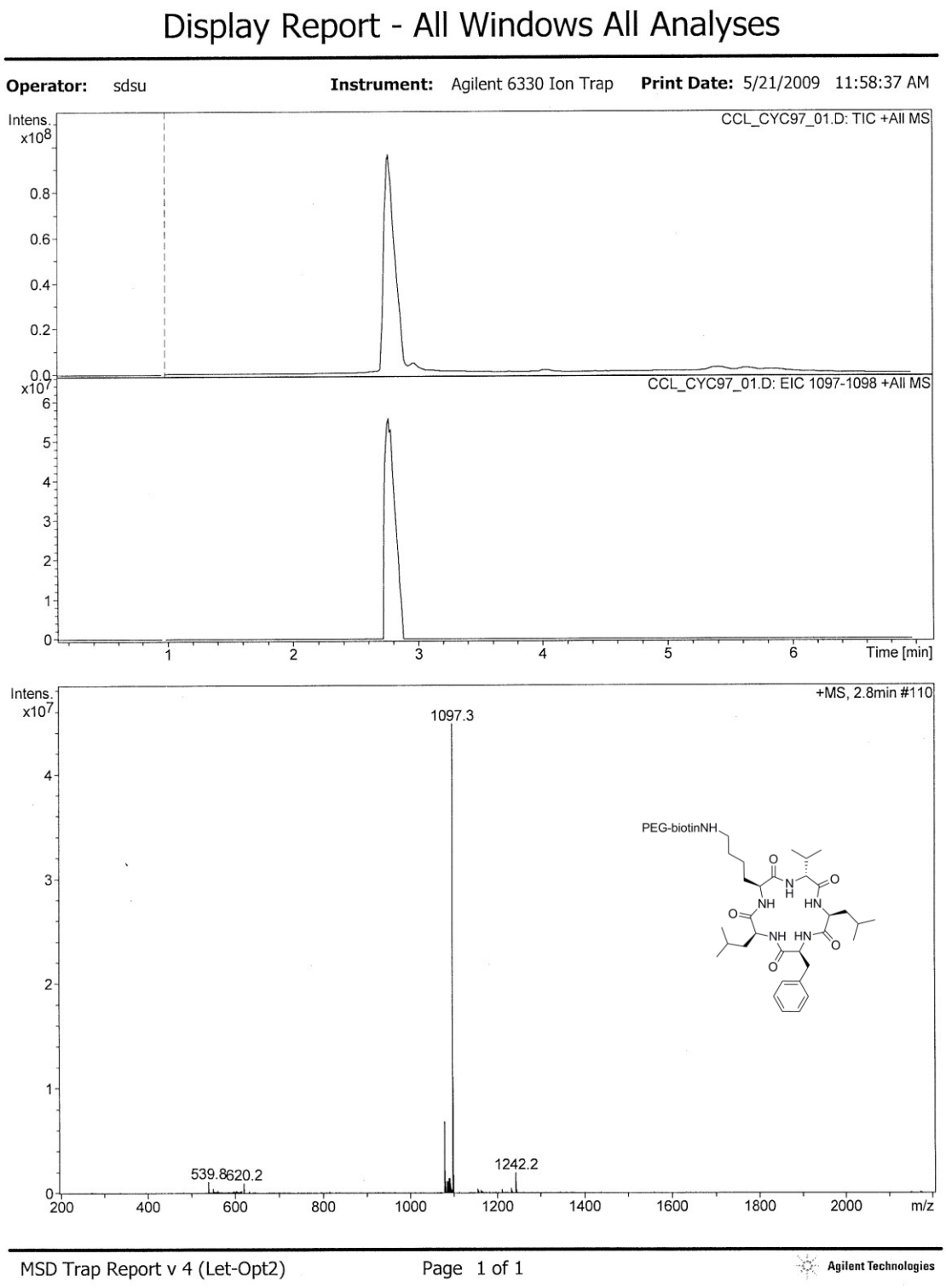
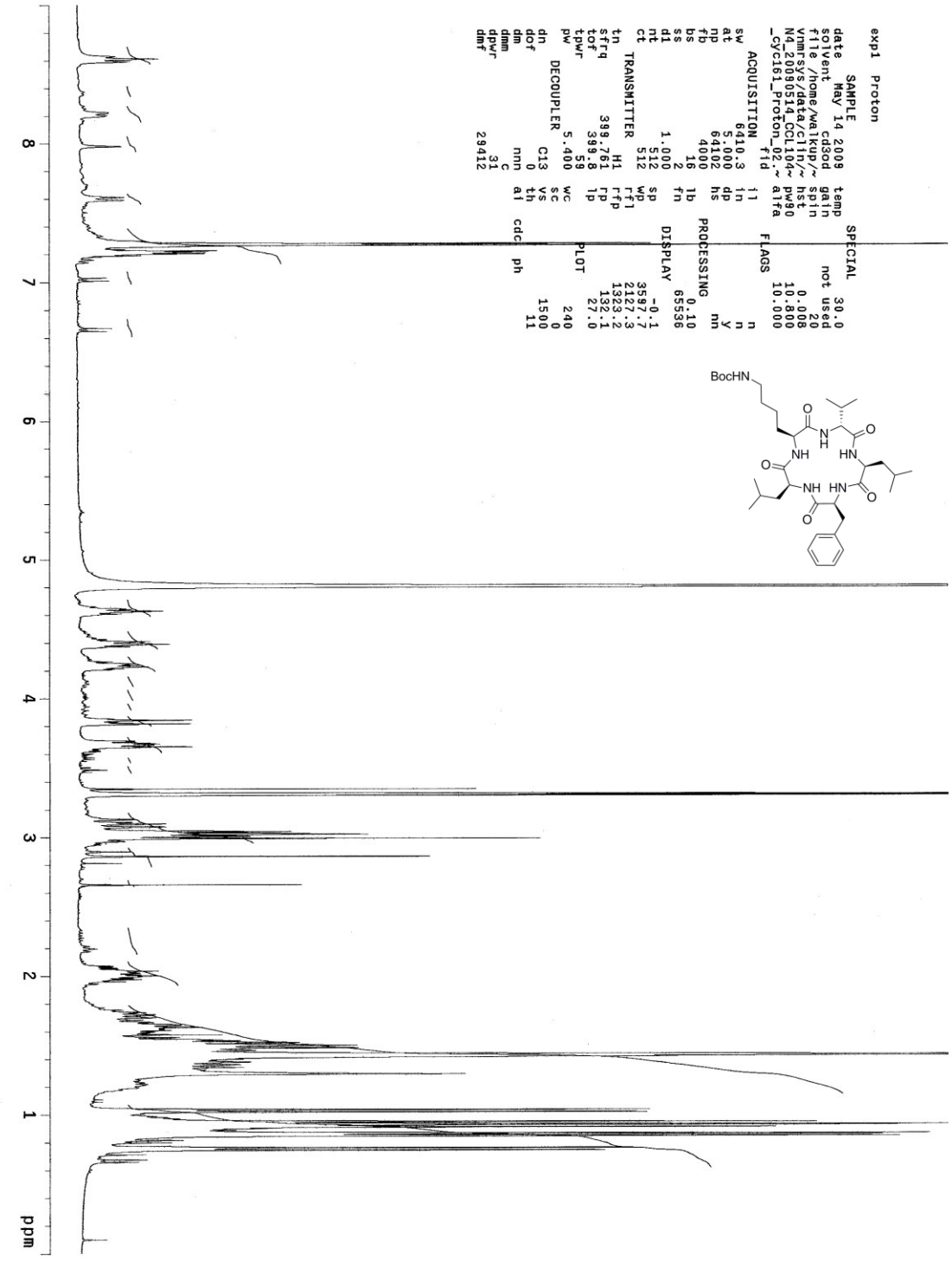




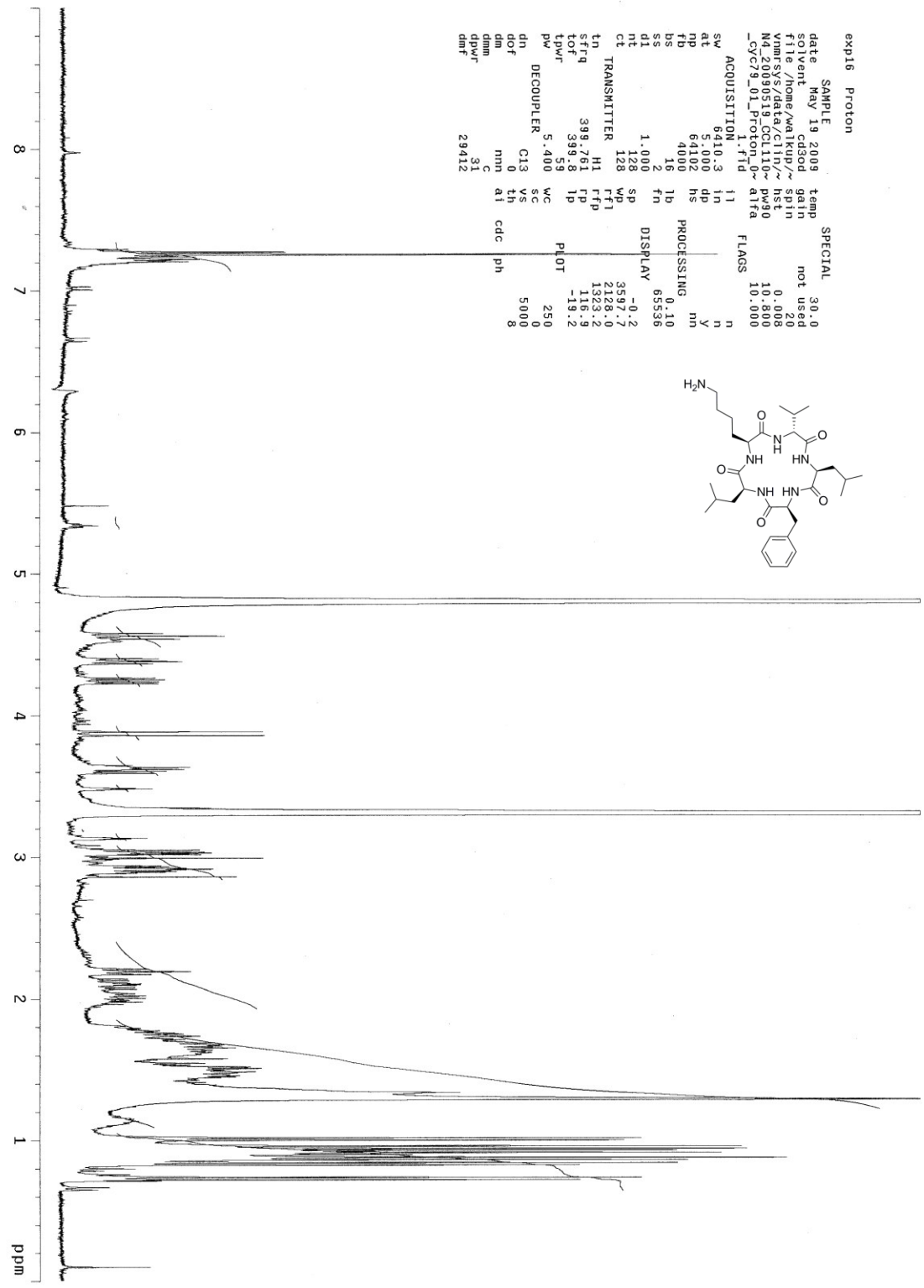
LCMS *cyclo*-Phe-Leu-D-Val-Lys-Leu



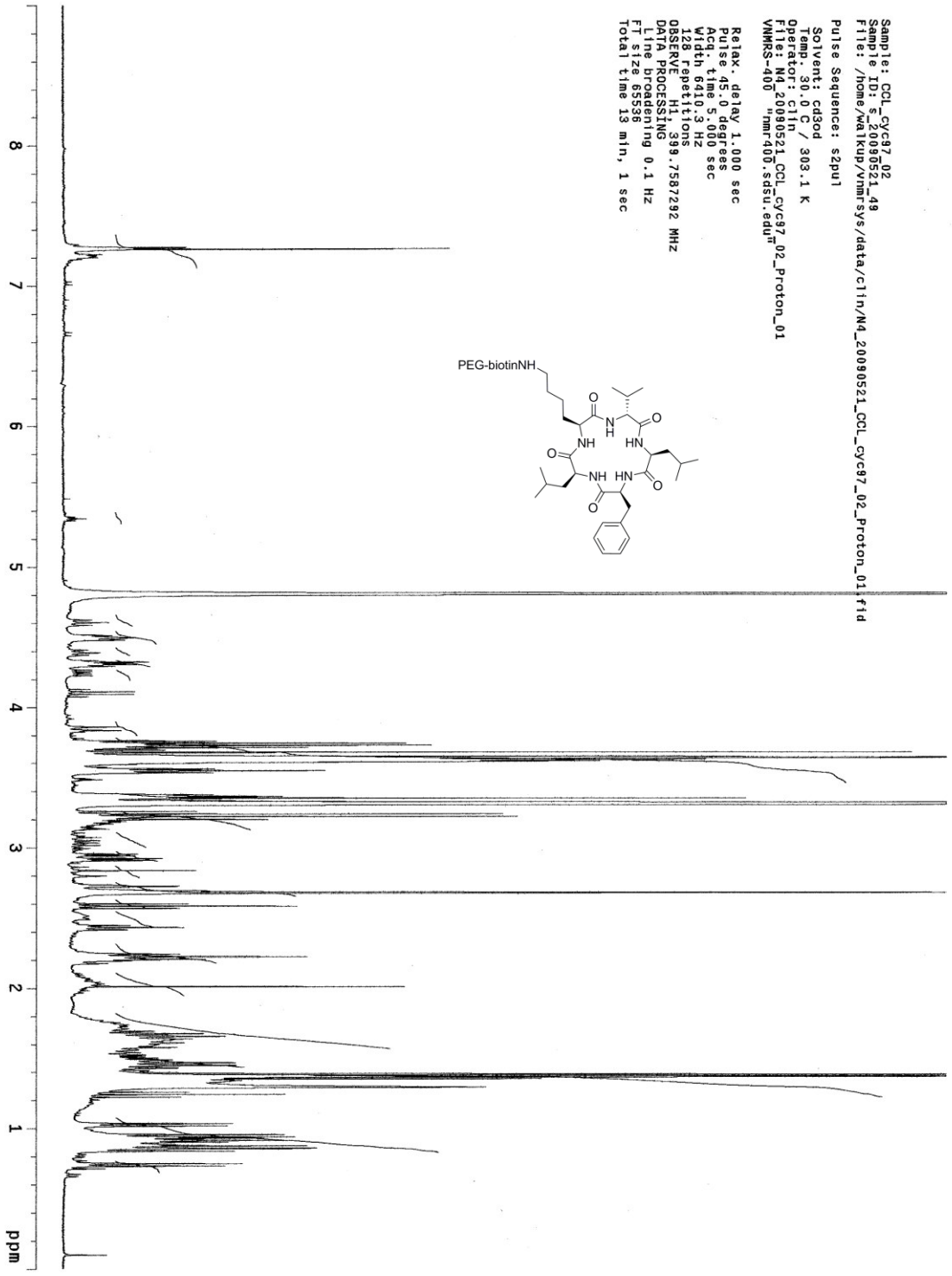
LCMS *cyclo*-Phe-Leu-D-Val-Lys(PEG-Biotin)-Leu (Compound 4)



^{1}H NMR *cyclo*-Phe-Leu-D-Val-Lys(Boc)-Leu

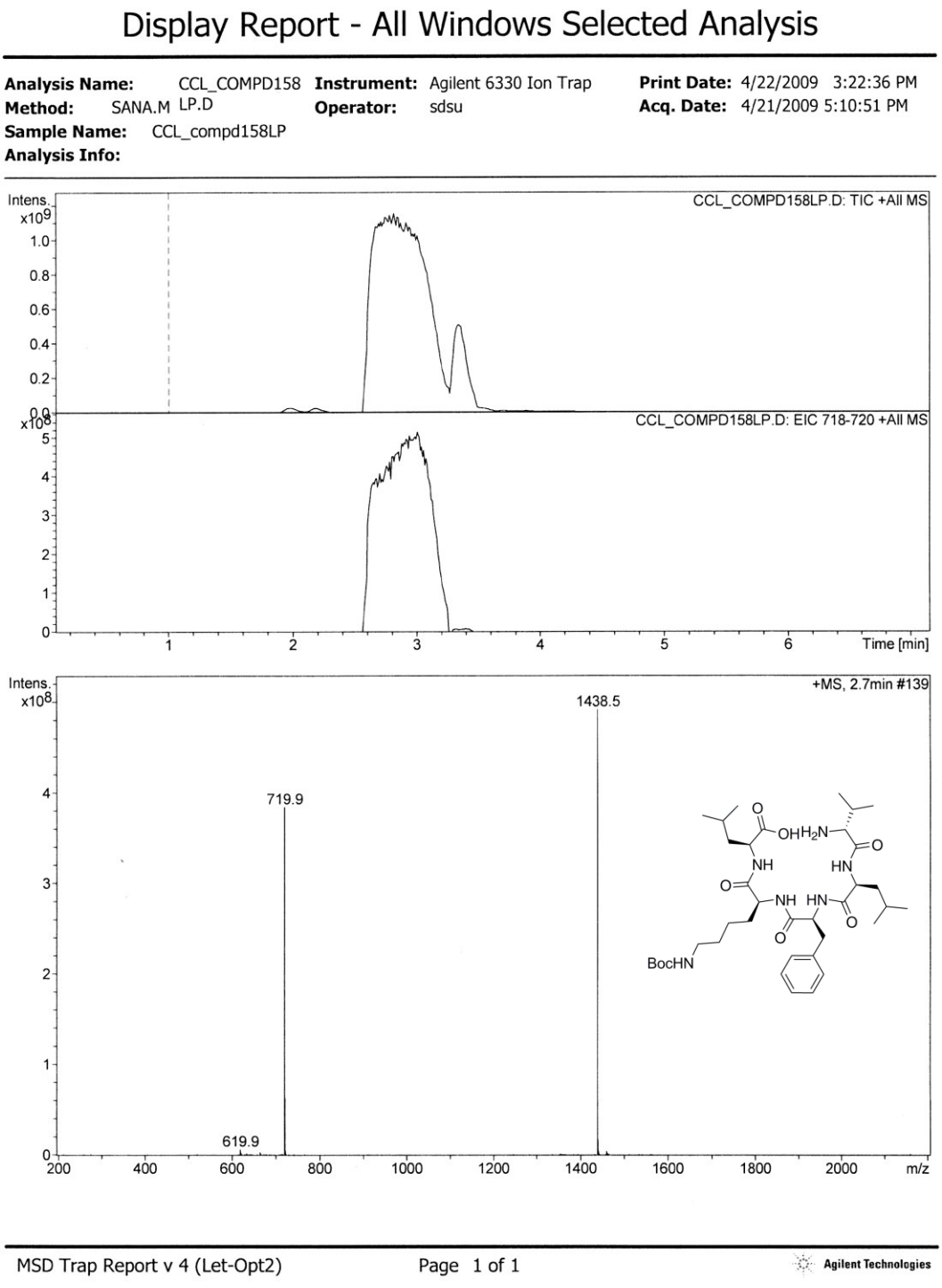


^{1}H NMR *cyclo*-Phe-Leu-D-Val-Lys-Leu



^{1}H NMR *cyclo*-Phe-Leu-D-Val-Lys(PEG-Biotin)-Leu (Compound 4)

Supporting Spectra for Compound 5



LCMS HO-Leu-Lys(Boc)-Phe-Leu-D-Val-H

Display Report - All Windows Selected Analysis

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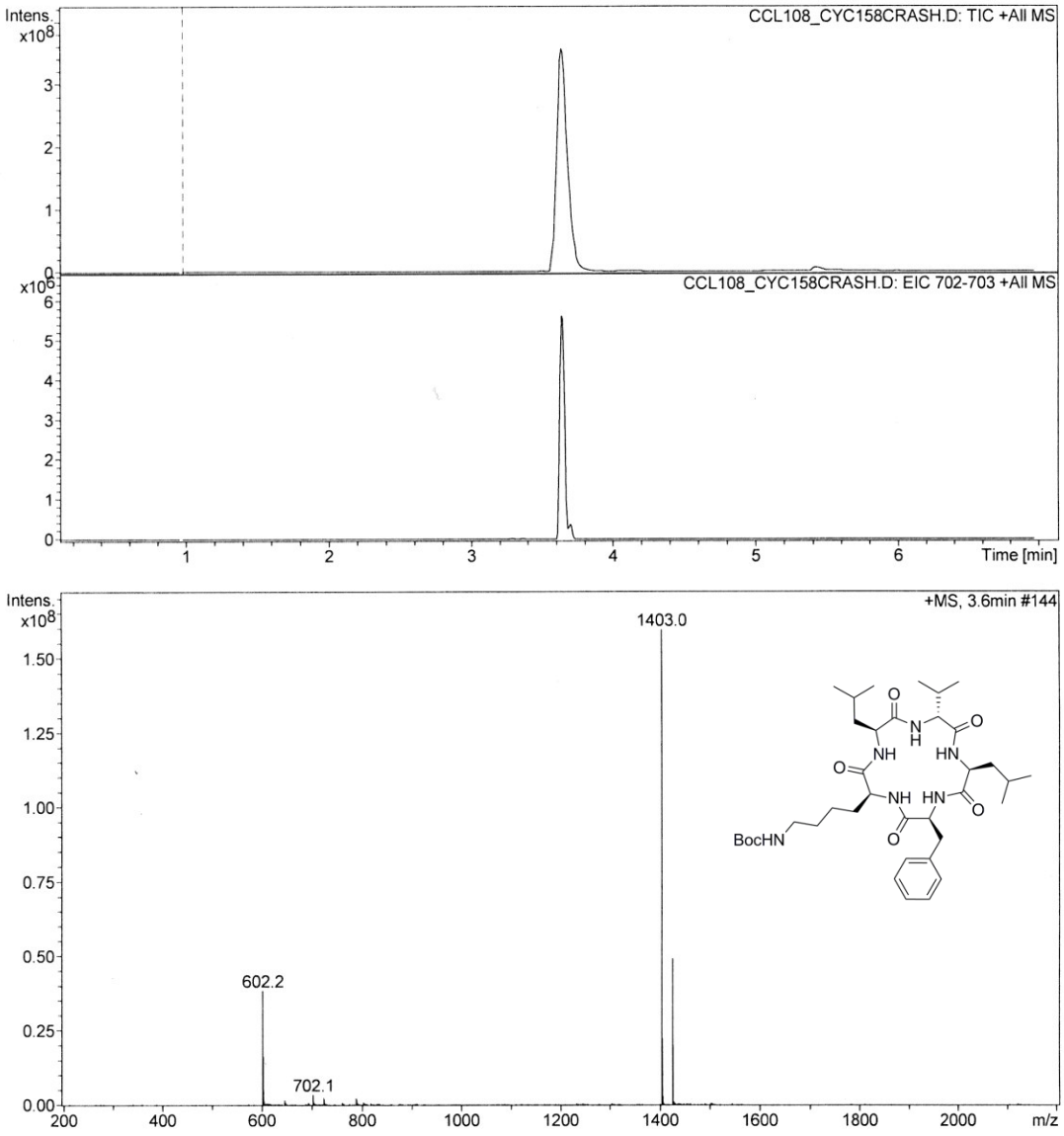
Analysis Info:



LCMS *cyclo*-Leu-Lys(Boc)-Phe-Leu-D-Val

Display Report - All Windows Selected Analysis

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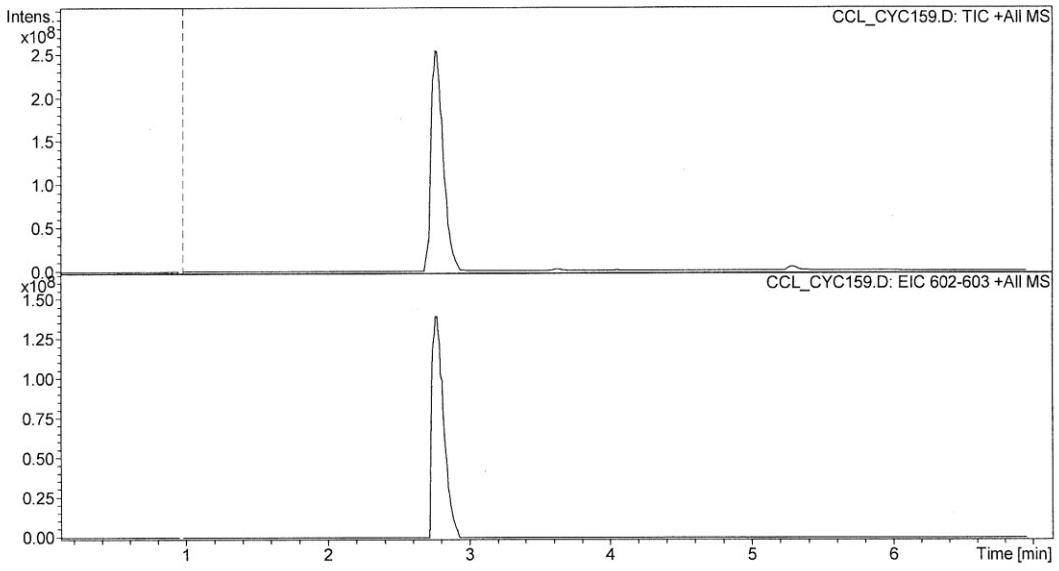
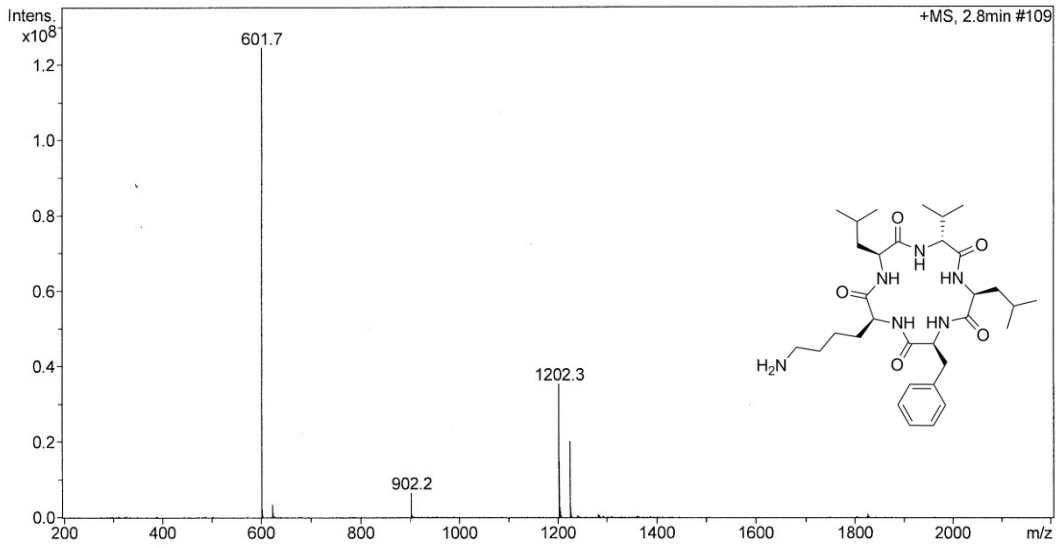
Sample Name: CCL_cyc159
Analysis Info:





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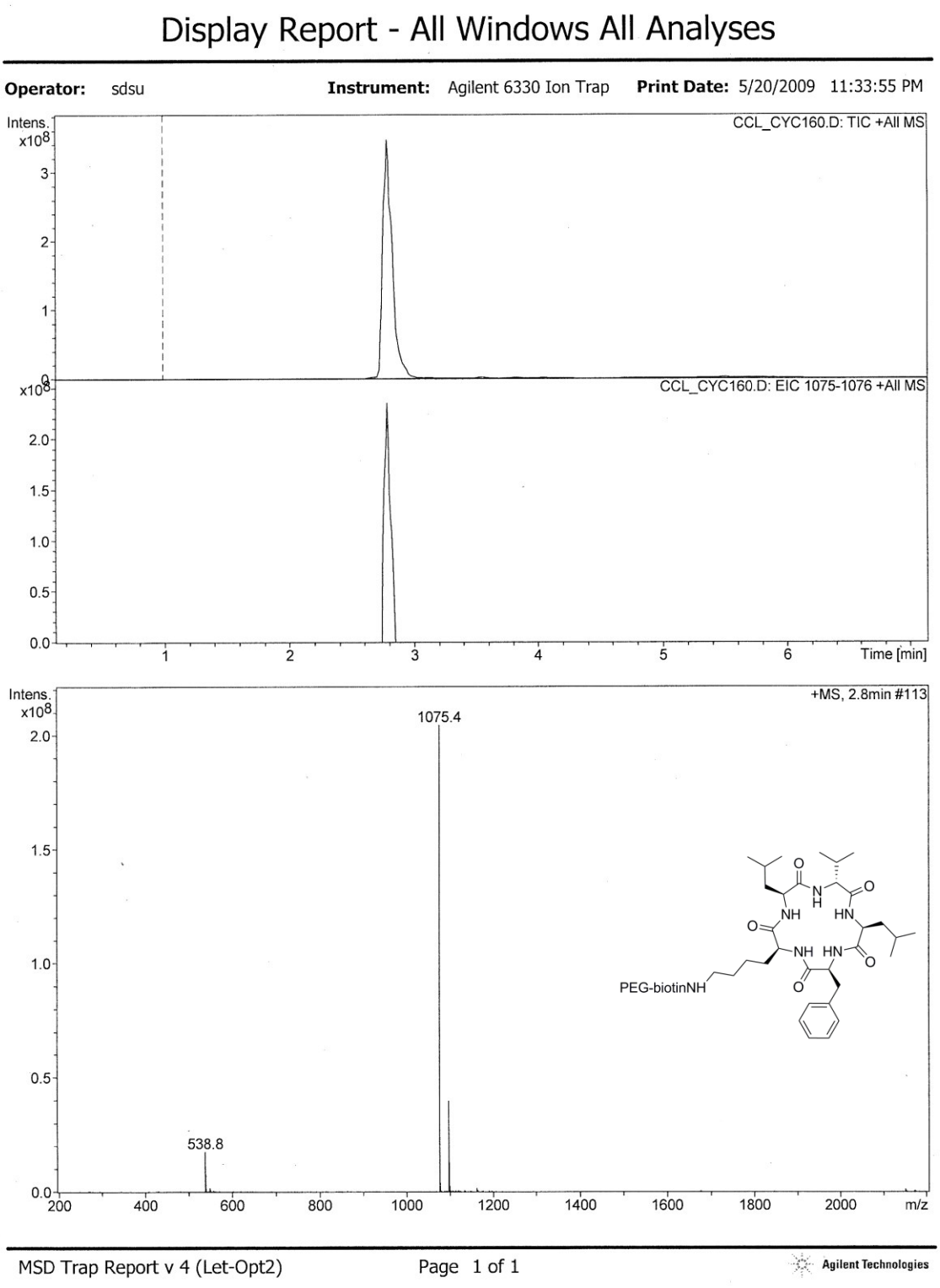
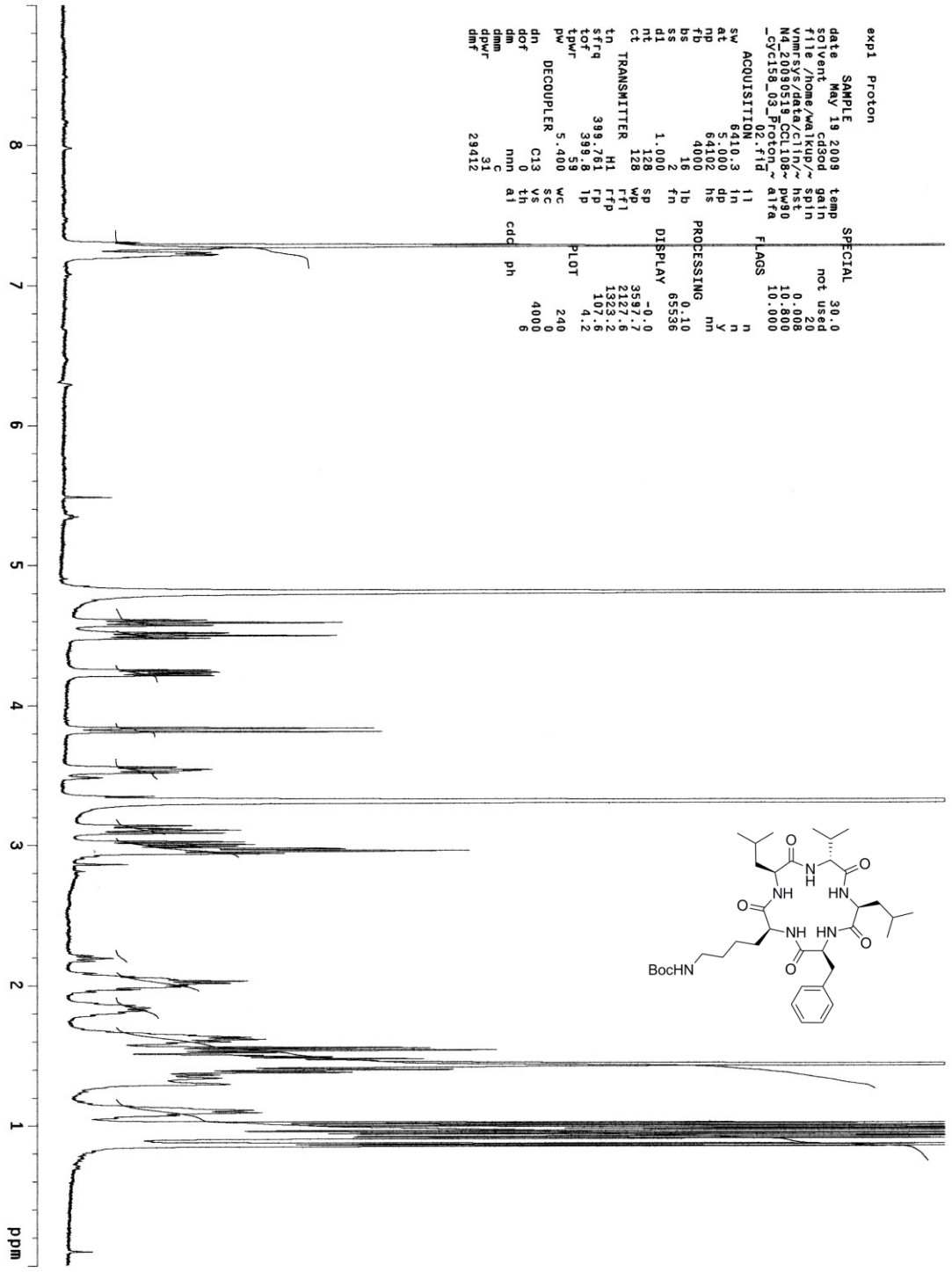
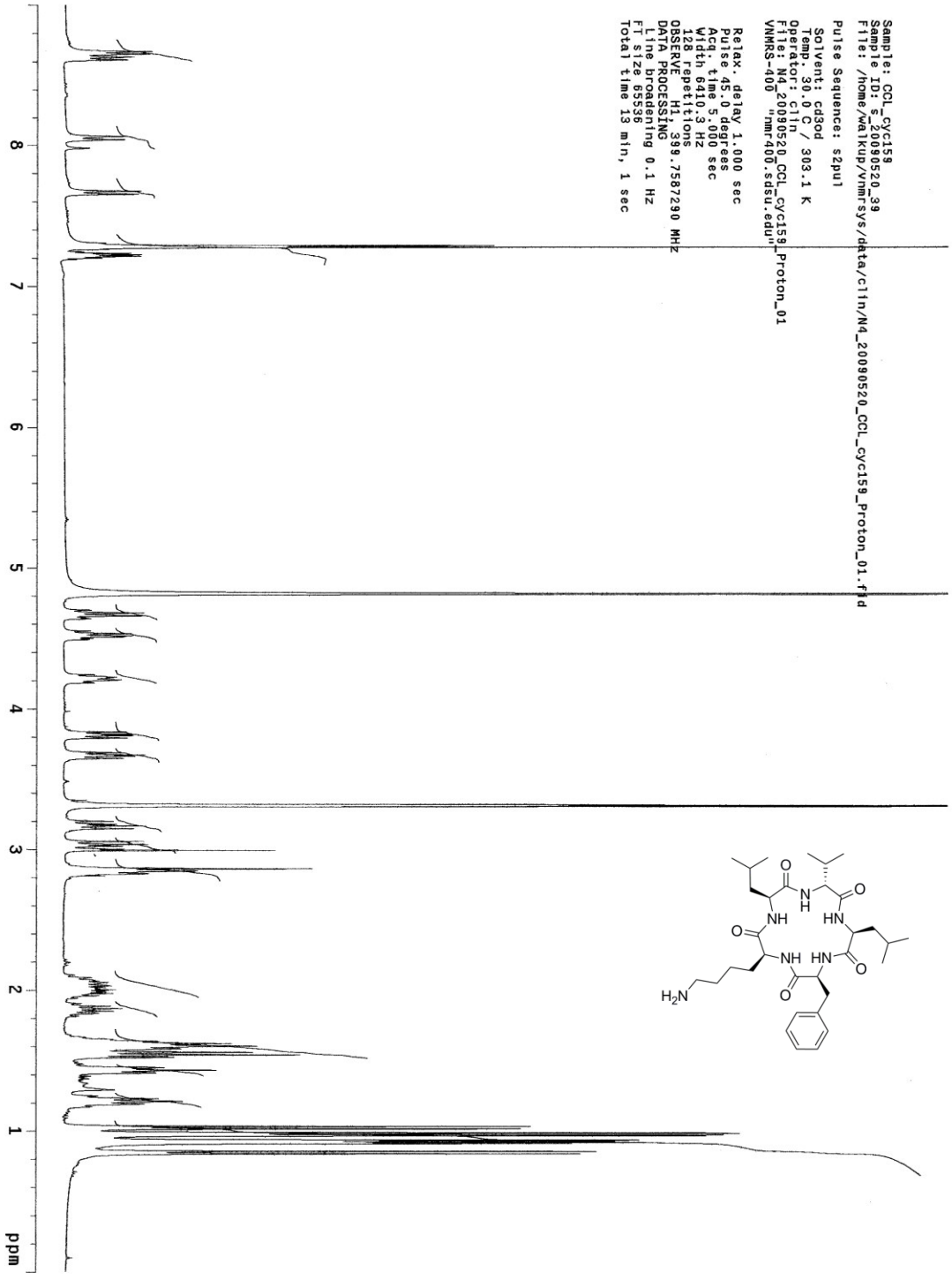
LCMS *cyclo*-Leu-Lys-Phe-Leu-D-Val



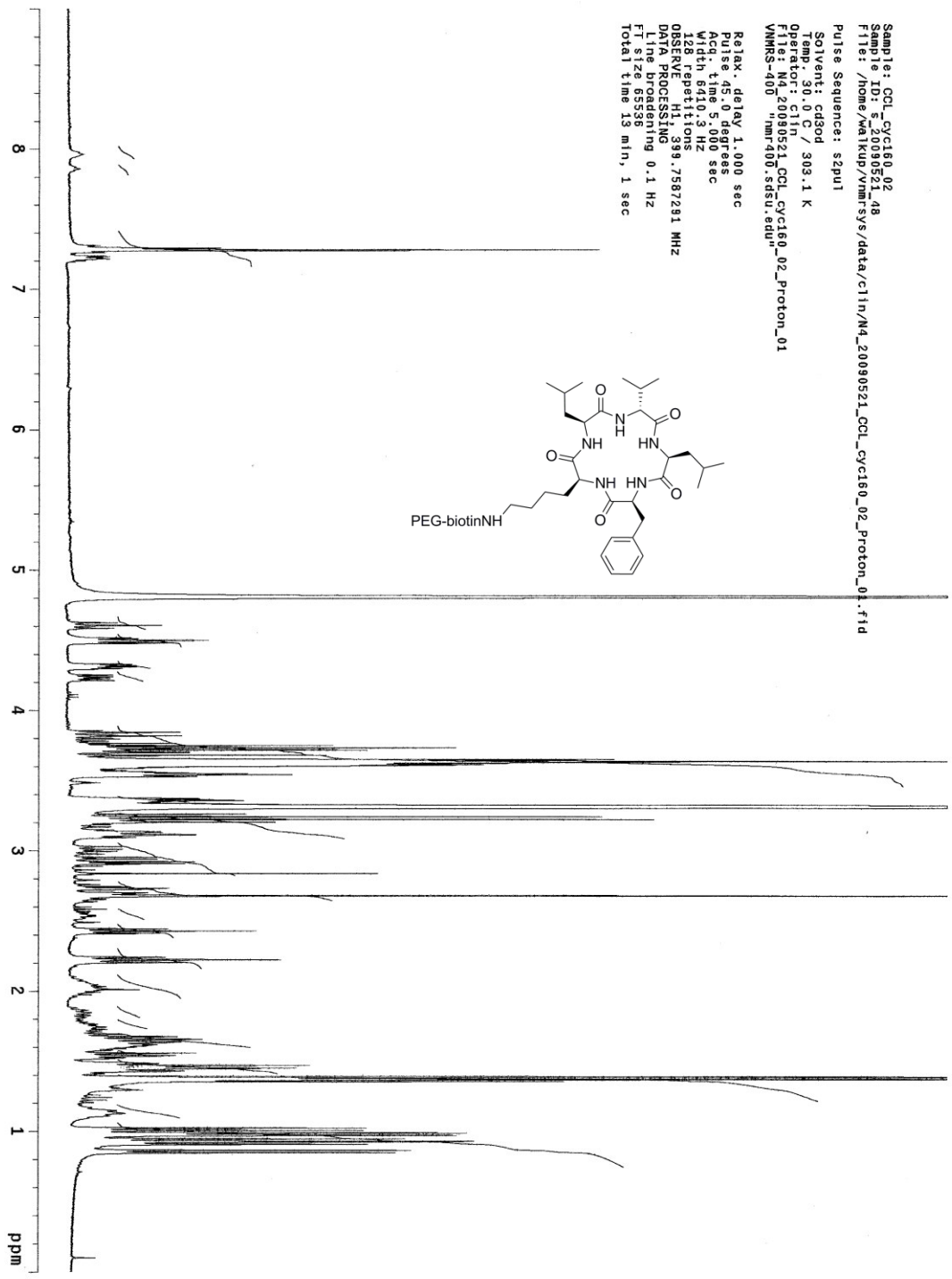
LCMS *cyclo*-Leu-Lys(PEG-Biotin)-Phe-Leu-D-Val (Compound 5)



^{1}H NMR *cyclo*-Leu-Lys(Boc)-Phe-Leu-D-Val

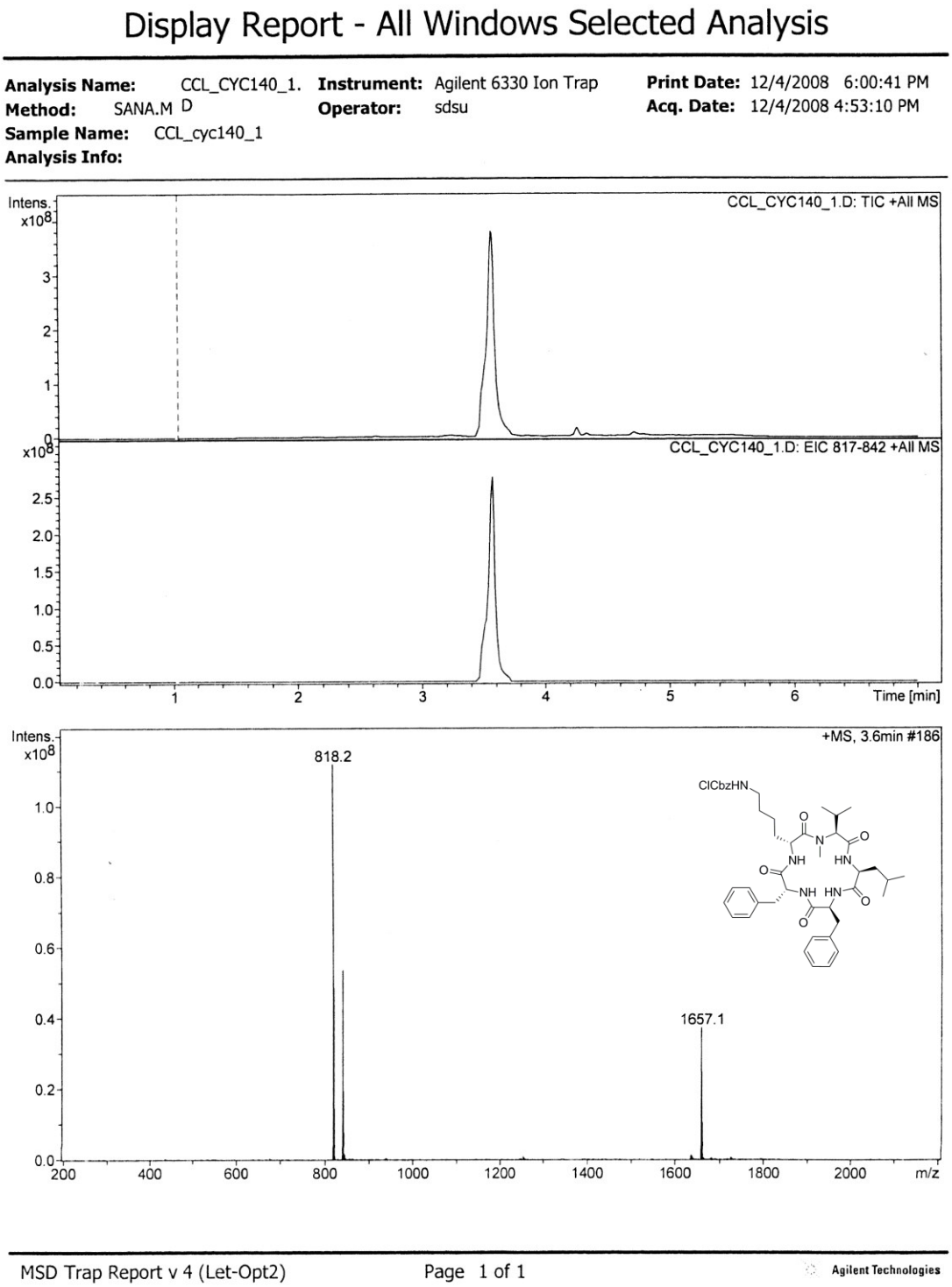


^{1}H NMR *cyclo*-Leu-Lys-Phe-Leu-D-Val



^{1}H NMR *cyclo*-Leu-Lys(PEG-Biotin)-Phe-Leu-D-Val (Compound 5)

Supporting Spectra for Compound 6



LCMS *cyclo*-D-Phe-D-Lys(2-Cl-Cbz)-*N*-Me-Val-Leu-Phe

Display Report - All Windows Selected Analysis

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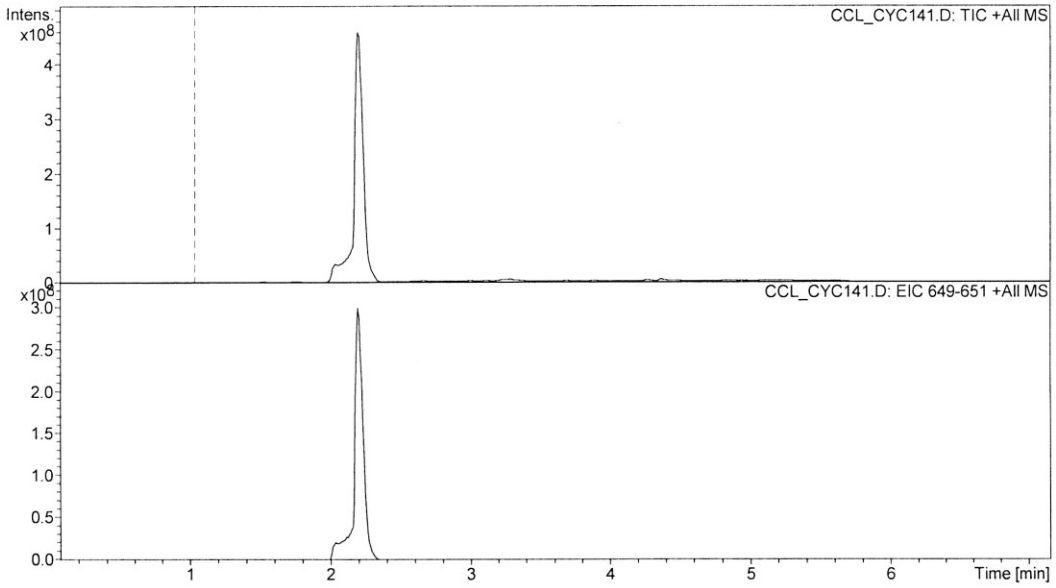
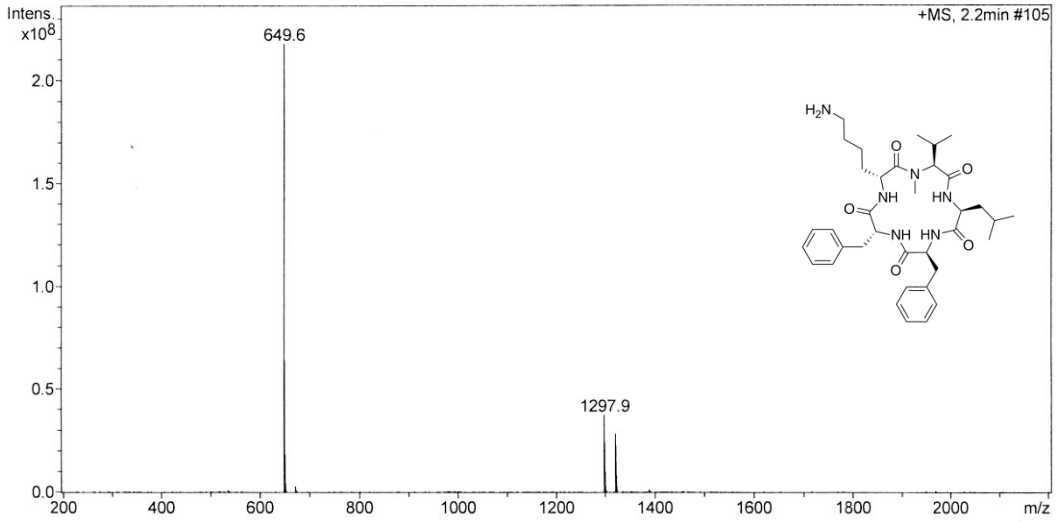
Sample Name: CCL_cyc141
Analysis Info:





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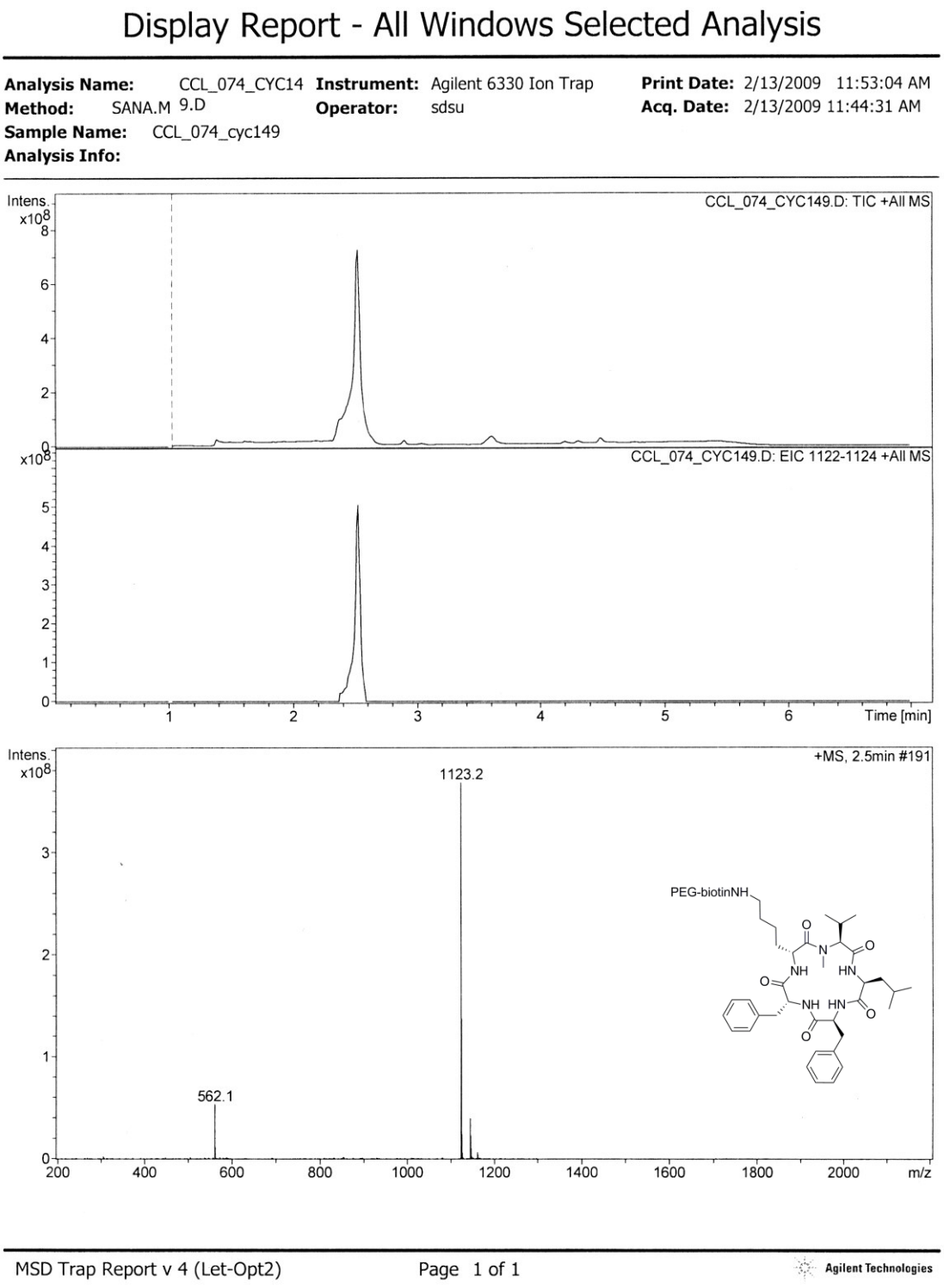
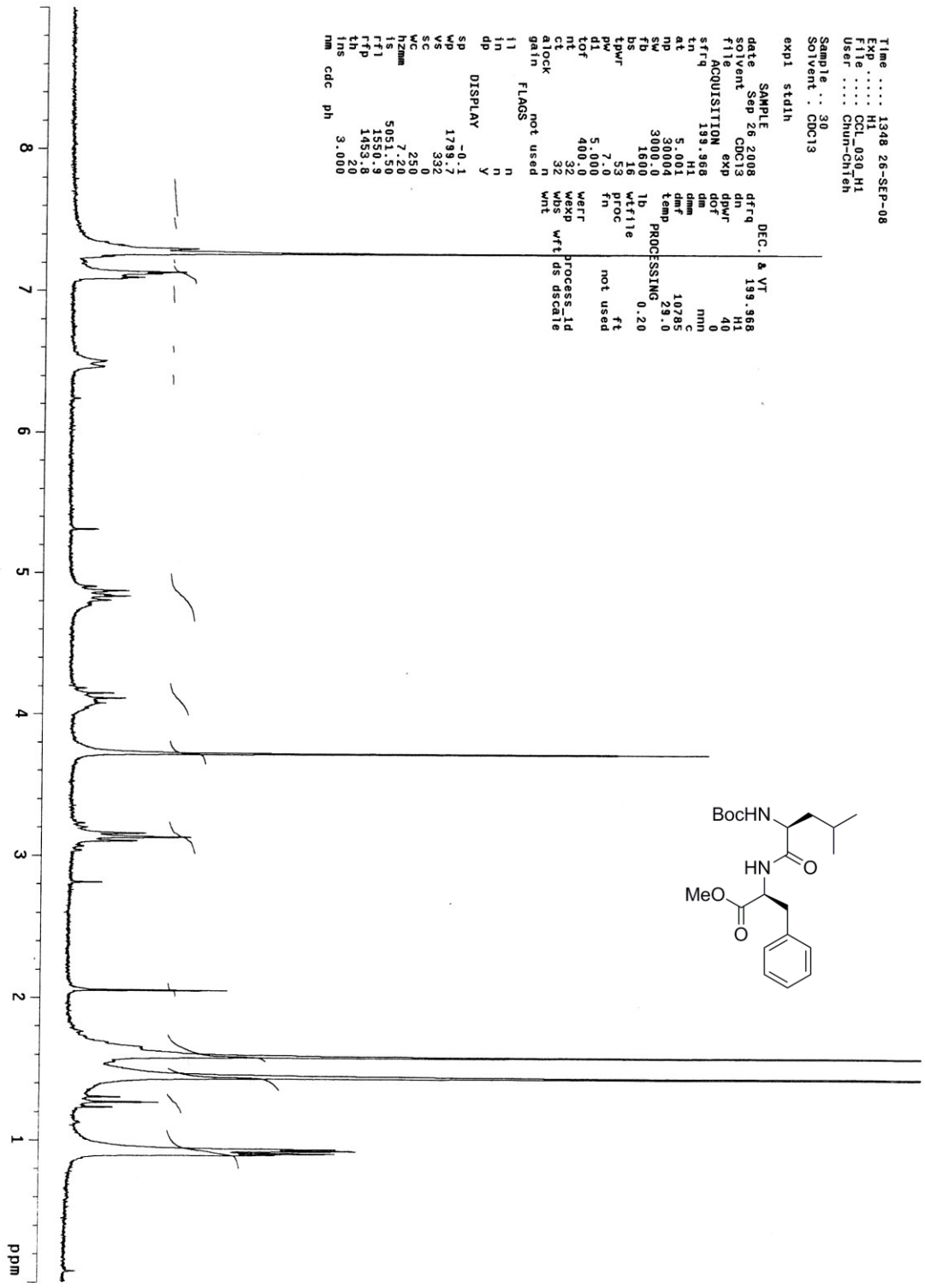
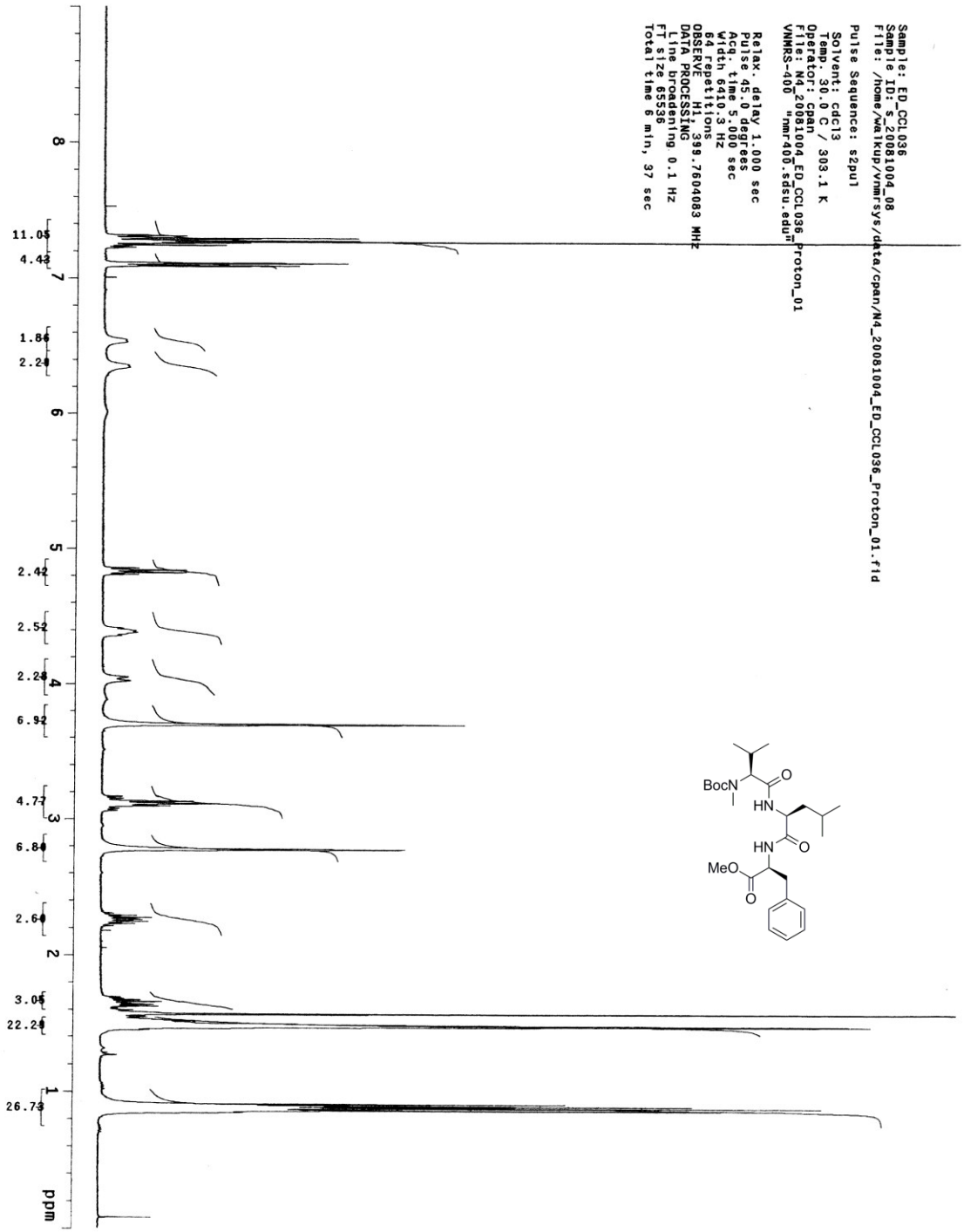
LCMS *cyclo*-D-Phe-D-Lys-*N*-Me-Val-Leu-Phe



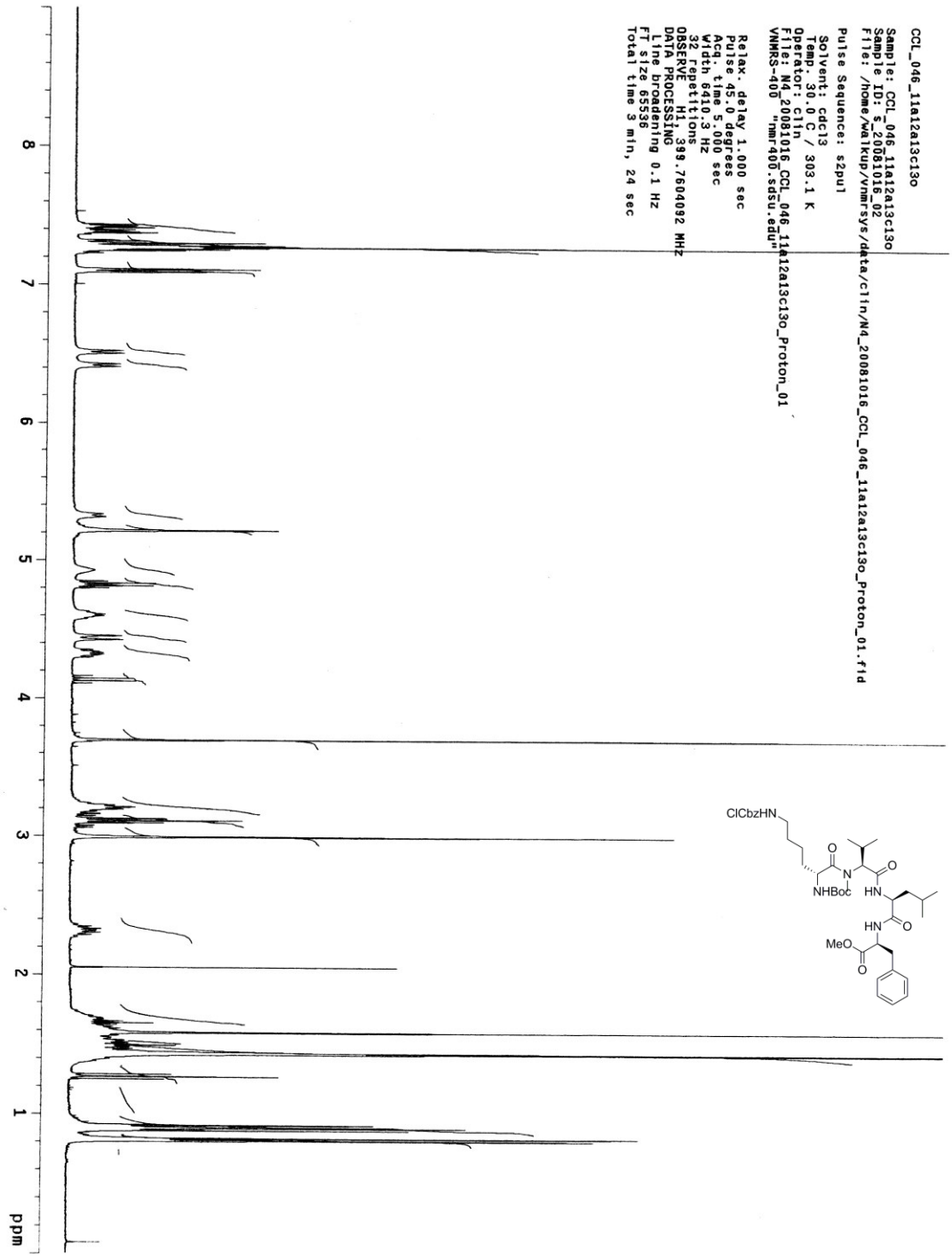
LCMS *cyclo*-D-Phe-D-Lys(PEG-Biotin)-*N*-Me-Val-Leu-Phe (Compound 6)



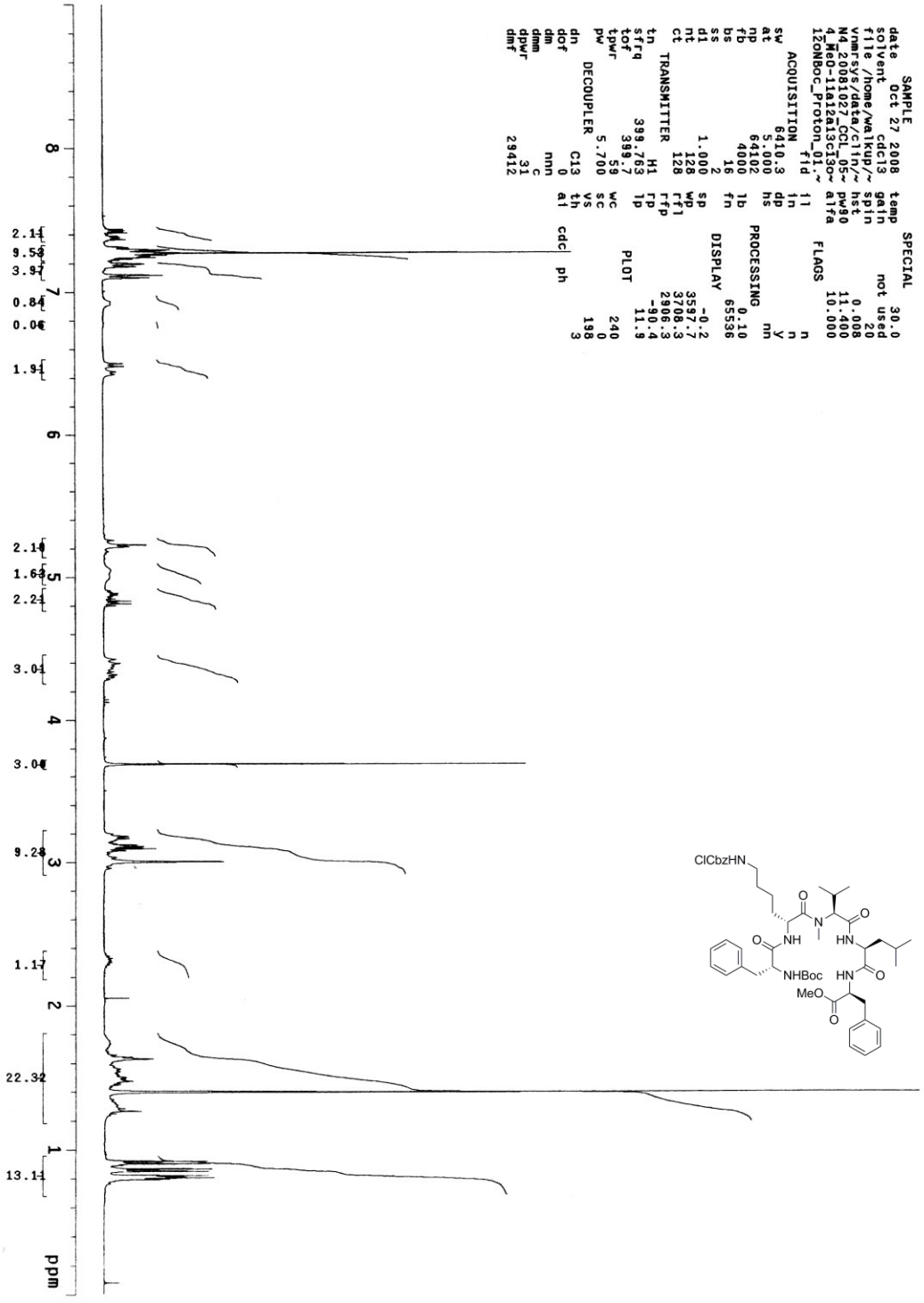
^{1}H NMR Boc-Leu-Phe-OMe



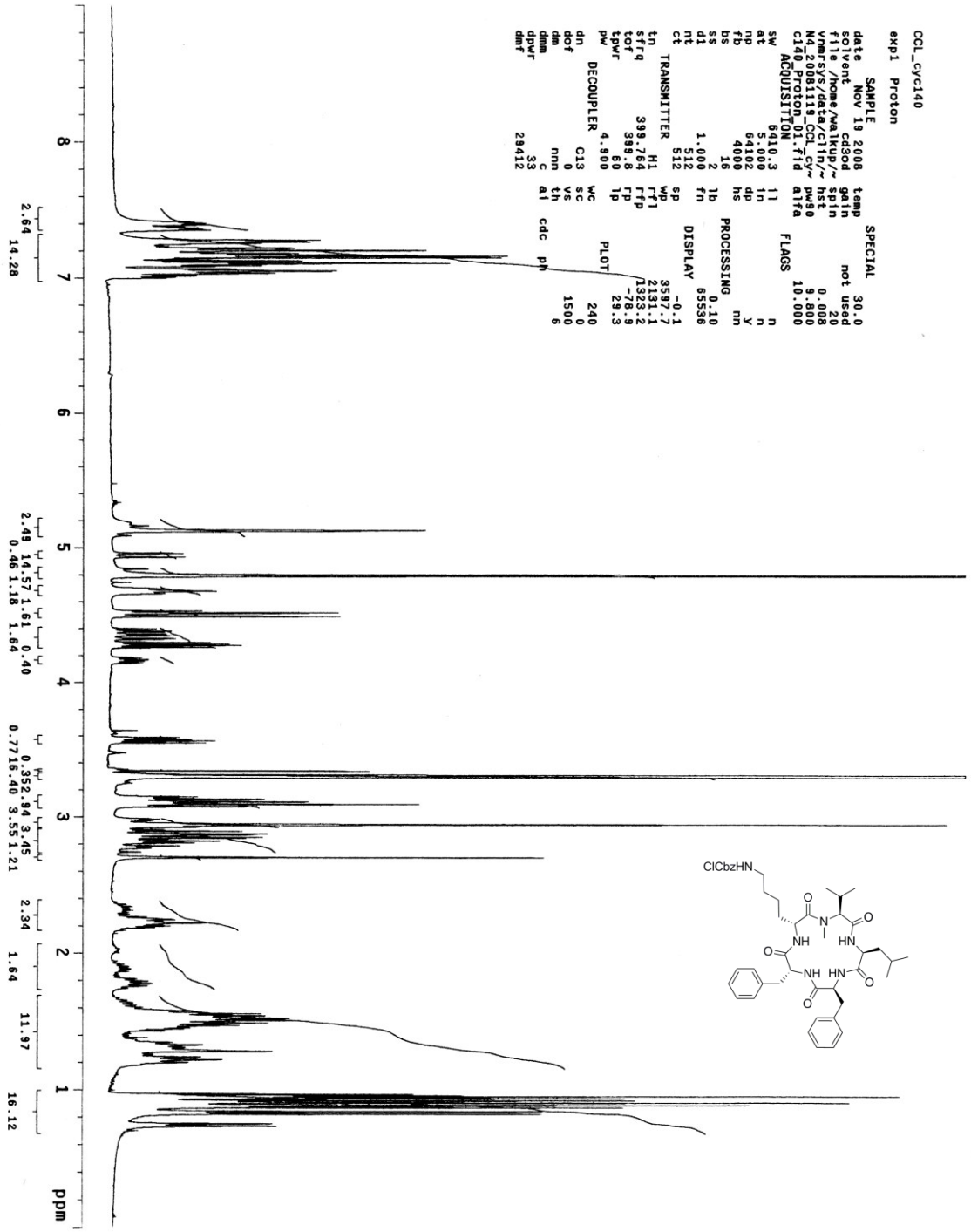
^{1}H NMR Boc-*N*-Me-Val-Leu-Phe-OMe



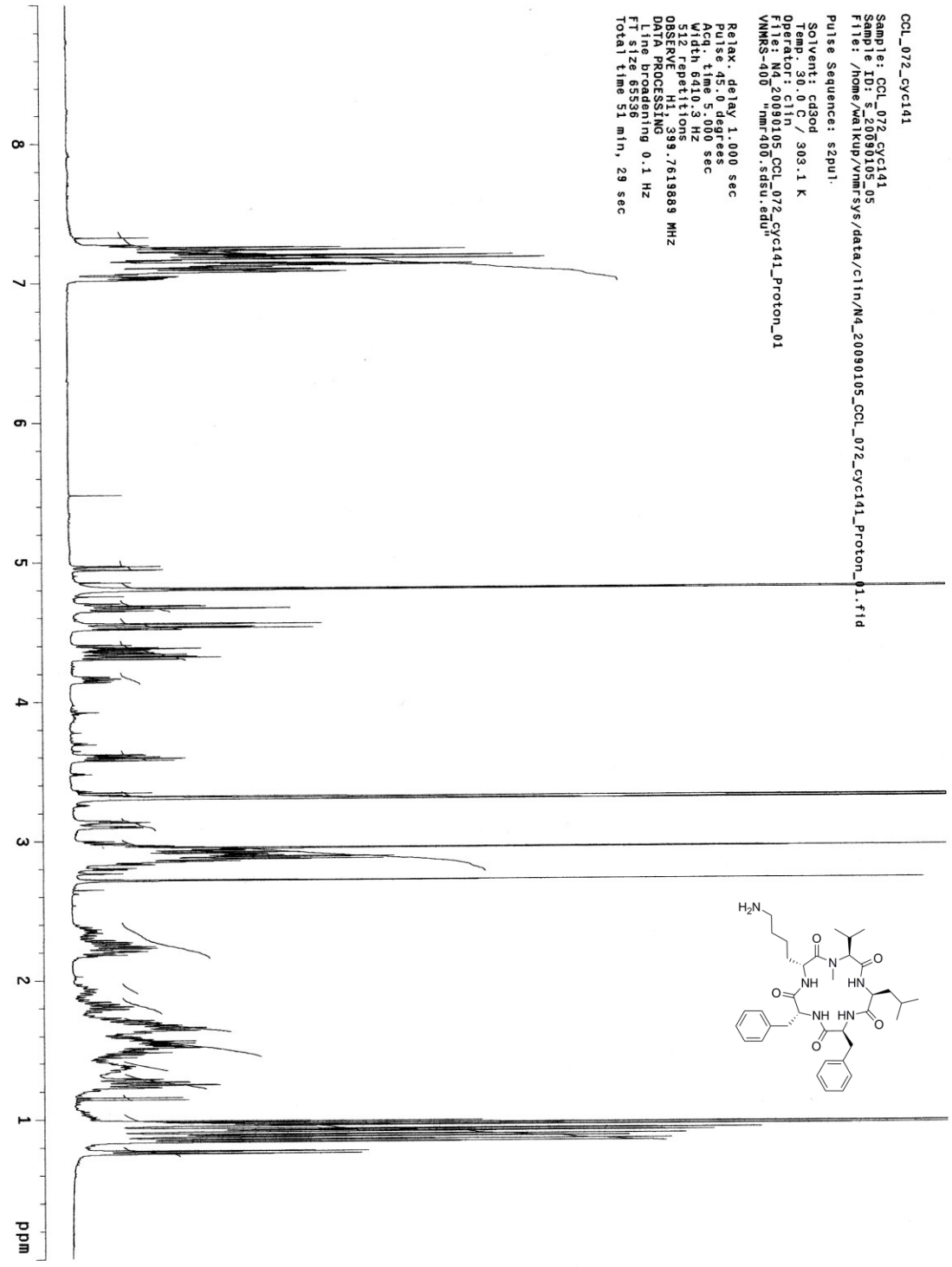
^{1}H NMR Boc-D-Lys(2-Cl-Cbz)-*N*-Me-Val-Leu-Phe-OMe



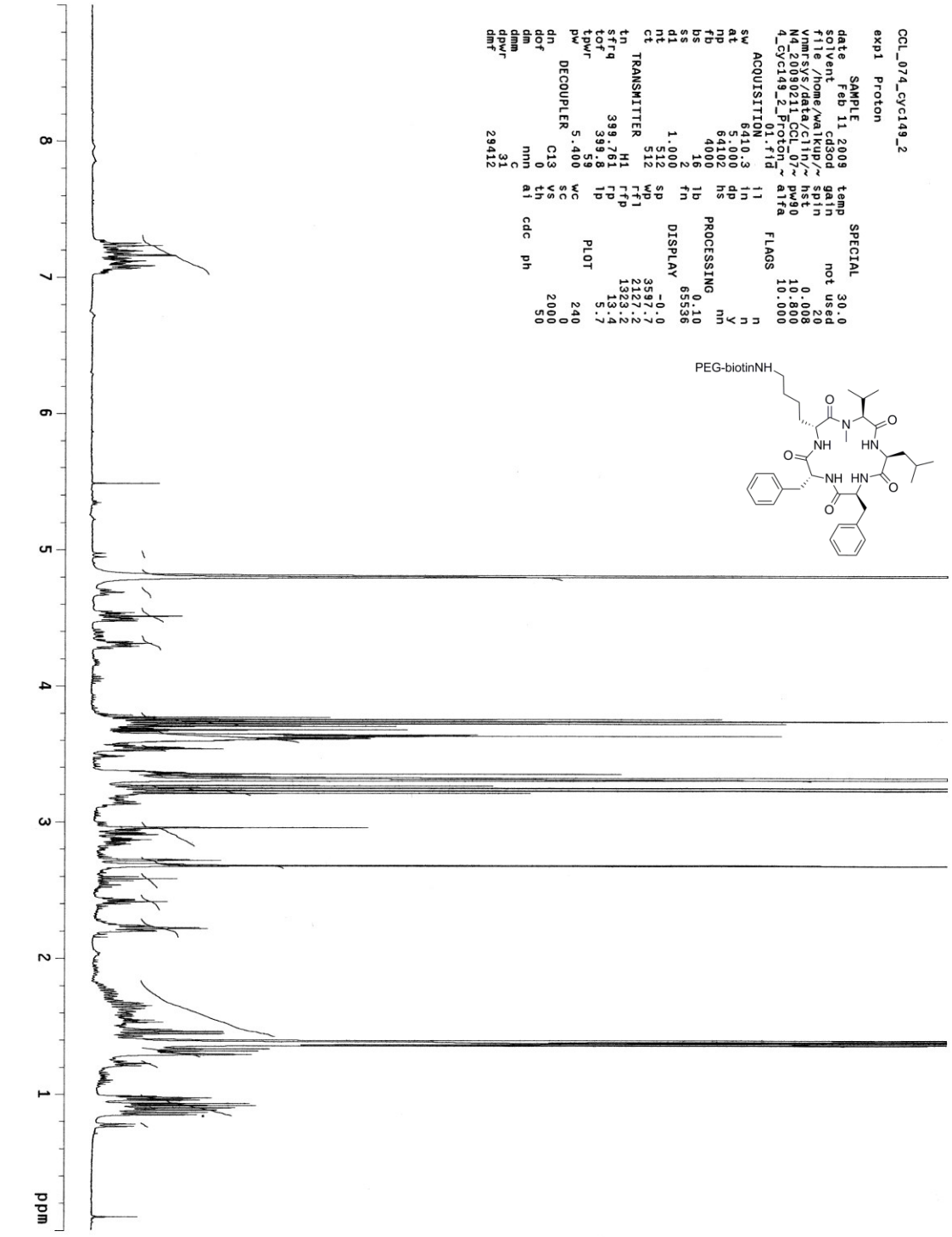
^{1}H NMR Boc-D-Phe-D-Lys(2-Cl-Cbz)-*N*-Me-Val-Leu-Phe-OMe



^{1}H NMR *cyclo*-D-Phe-D-Lys(2-Cl-Cbz)-*N*-Me-Val-Leu-Phe

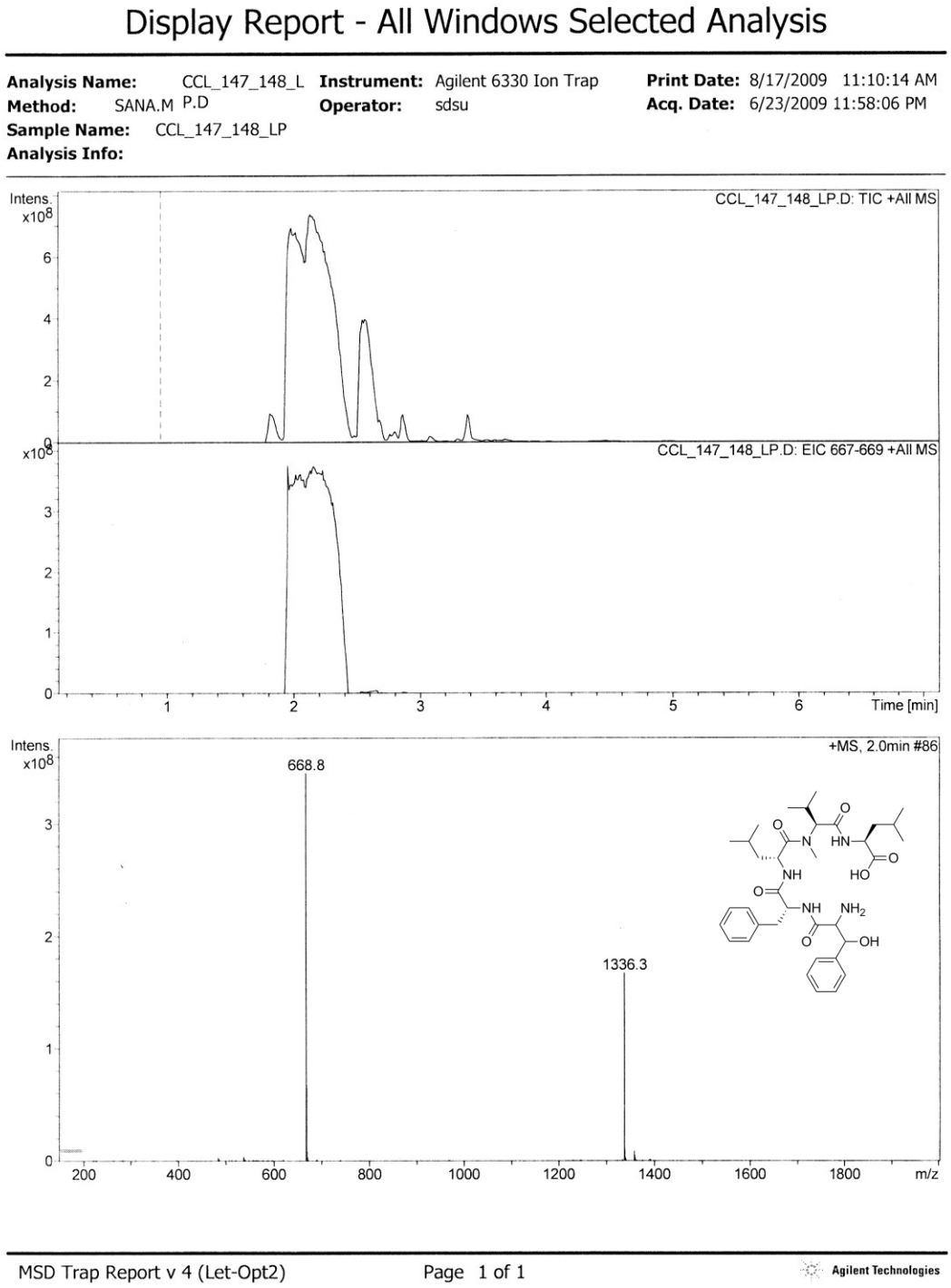


^{1}H NMR *cyclo*-D-Phe-D-Lys-*N*-Me-Val-Leu-Phe



^{1}H NMR *cyclo*-D-Phe-D-Lys(PEG-Biotin)-*N*-Me-Val-Leu-Phe (Compound 6)

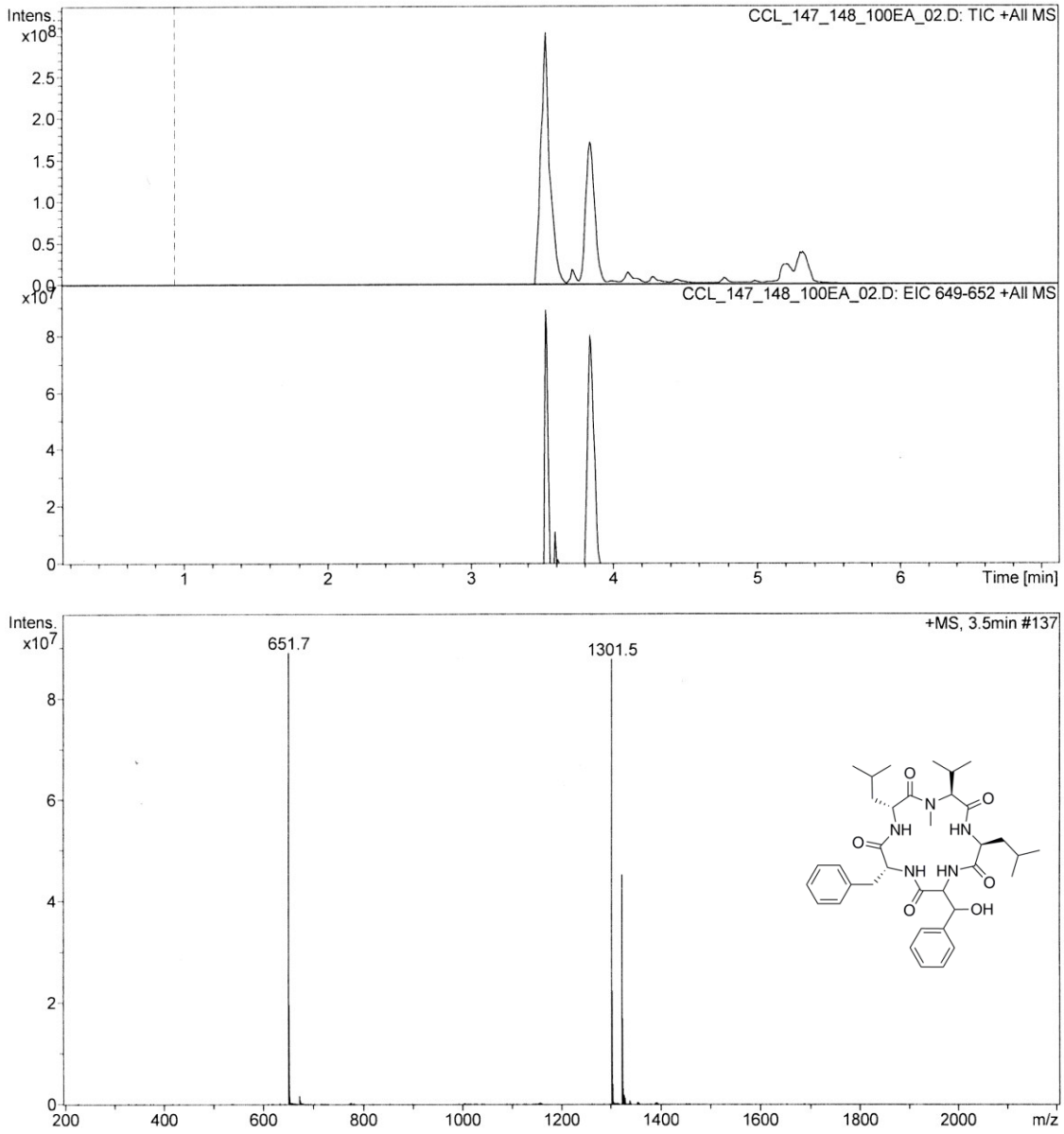
Supporting Spectra for Compound 9/10



LCMS HO-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S,3R)/(2R,3S)-racemic-β-OH-Phe-H

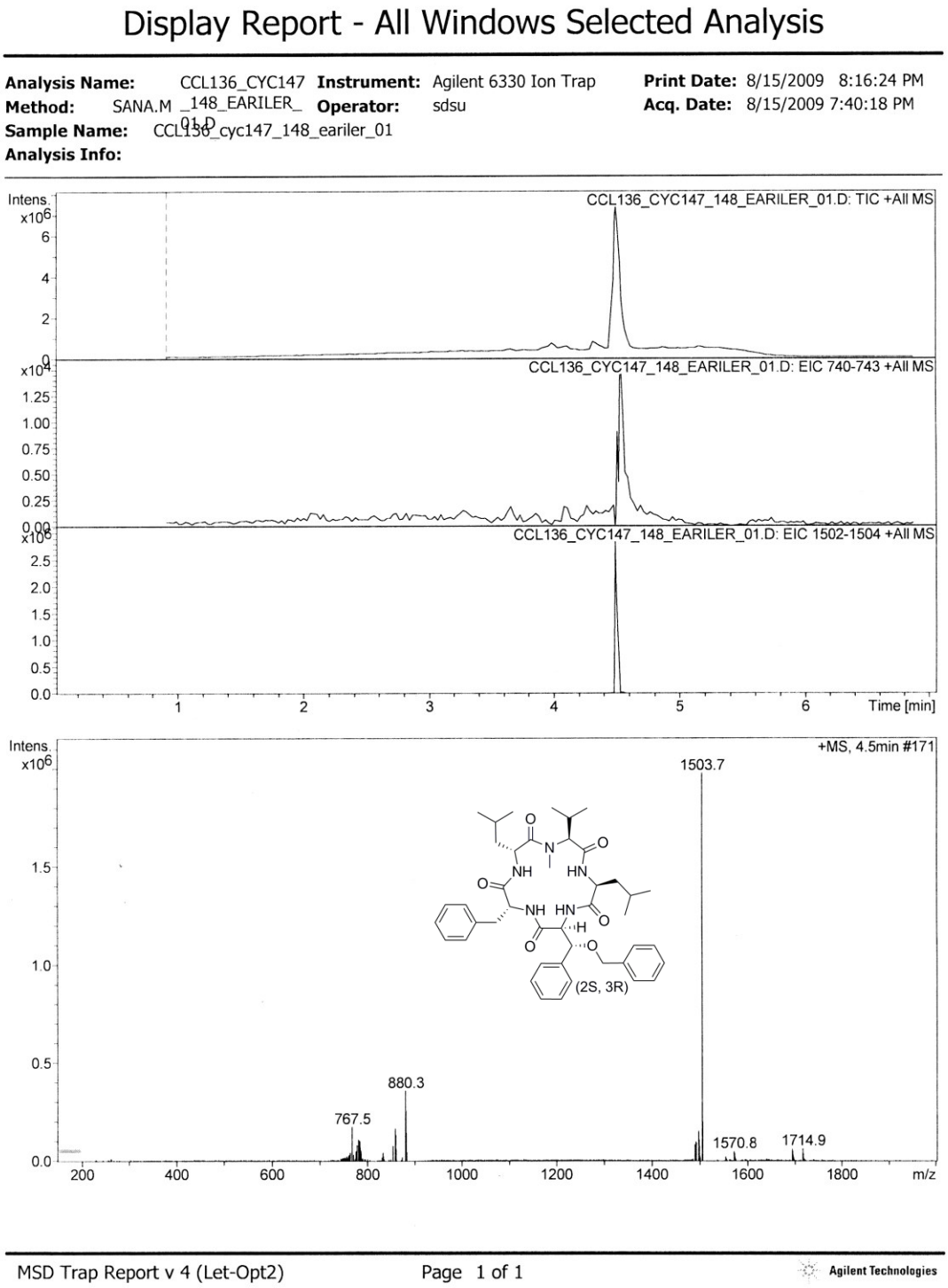
Display Report - All Windows Selected Analysis

Analysis Name: CCL_147_148_1 **Instrument:** Agilent 6330 Ion Trap **Print Date:** 7/6/2009 2:53:07 PM
Method: SANA.M 00EA_02.D **Operator:** sdsu **Acq. Date:** 7/5/2009 9:39:45 PM
Sample Name: CCL_147_148_100EA_02
Analysis Info:



MSD Trap Report v 4 (Let-Opt2) Page 1 of 1 Agilent Technologies

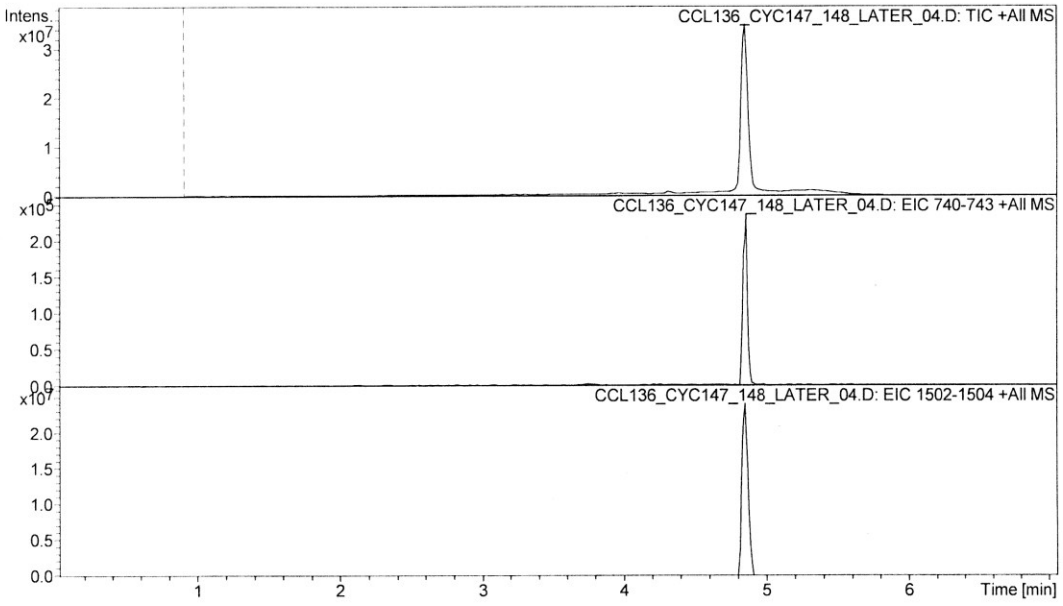
LCMS *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S,3R)/(2R,3S)-racemic-β-OH-Phe

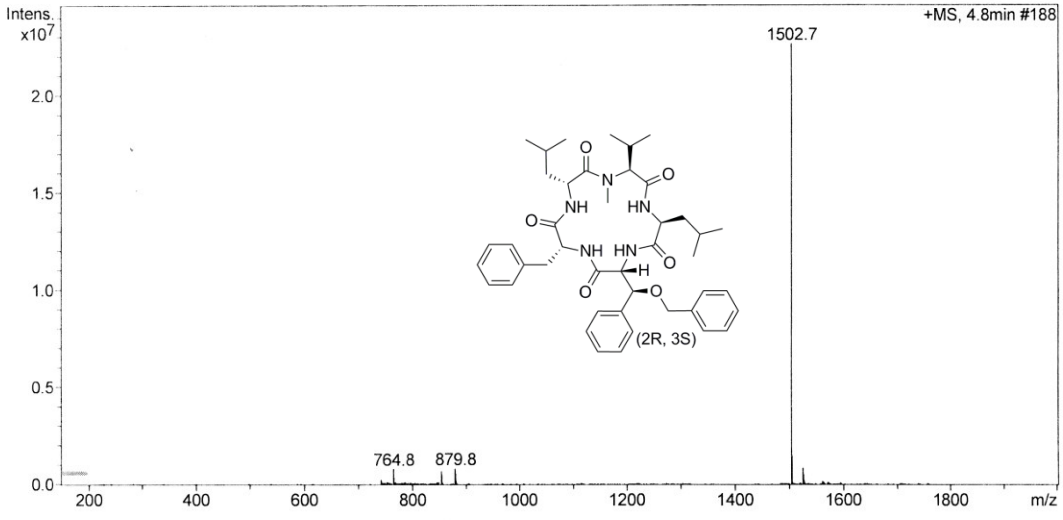


LCMS *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S,3R)-β-benzoxy-Phe (compound 9)

Display Report - All Windows Selected Analysis

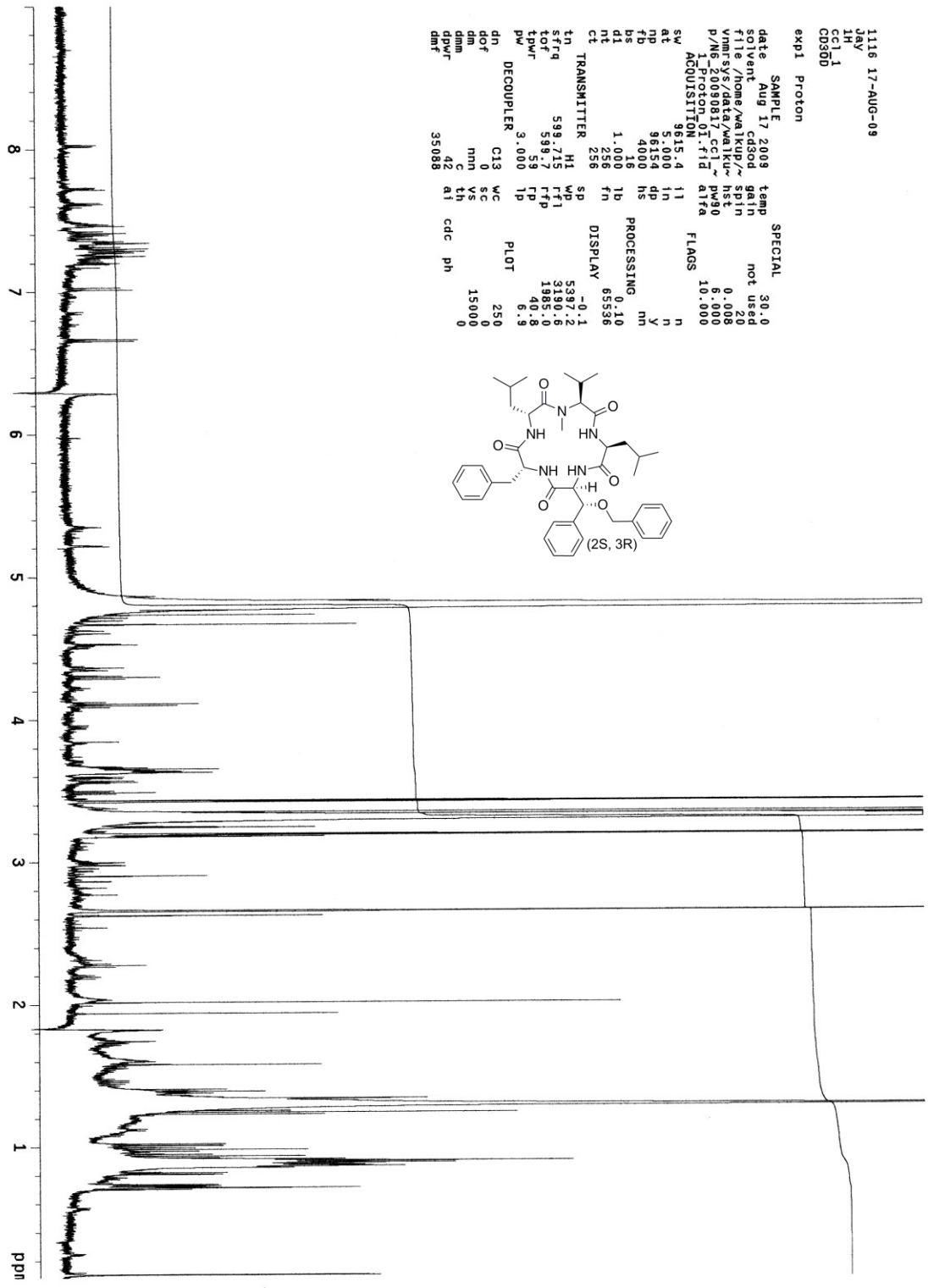
Analysis Name: CCL136_CYC147_148_LATER_04.D **Instrument:** Agilent 6330 Ion Trap **Print Date:** 8/15/2009 9:30:37 PM
Method: SANA.M **Operator:** sdsu **Acq. Date:** 8/15/2009 9:22:08 PM
Sample Name: CCL136_cyc147_148_later_04
Analysis Info:



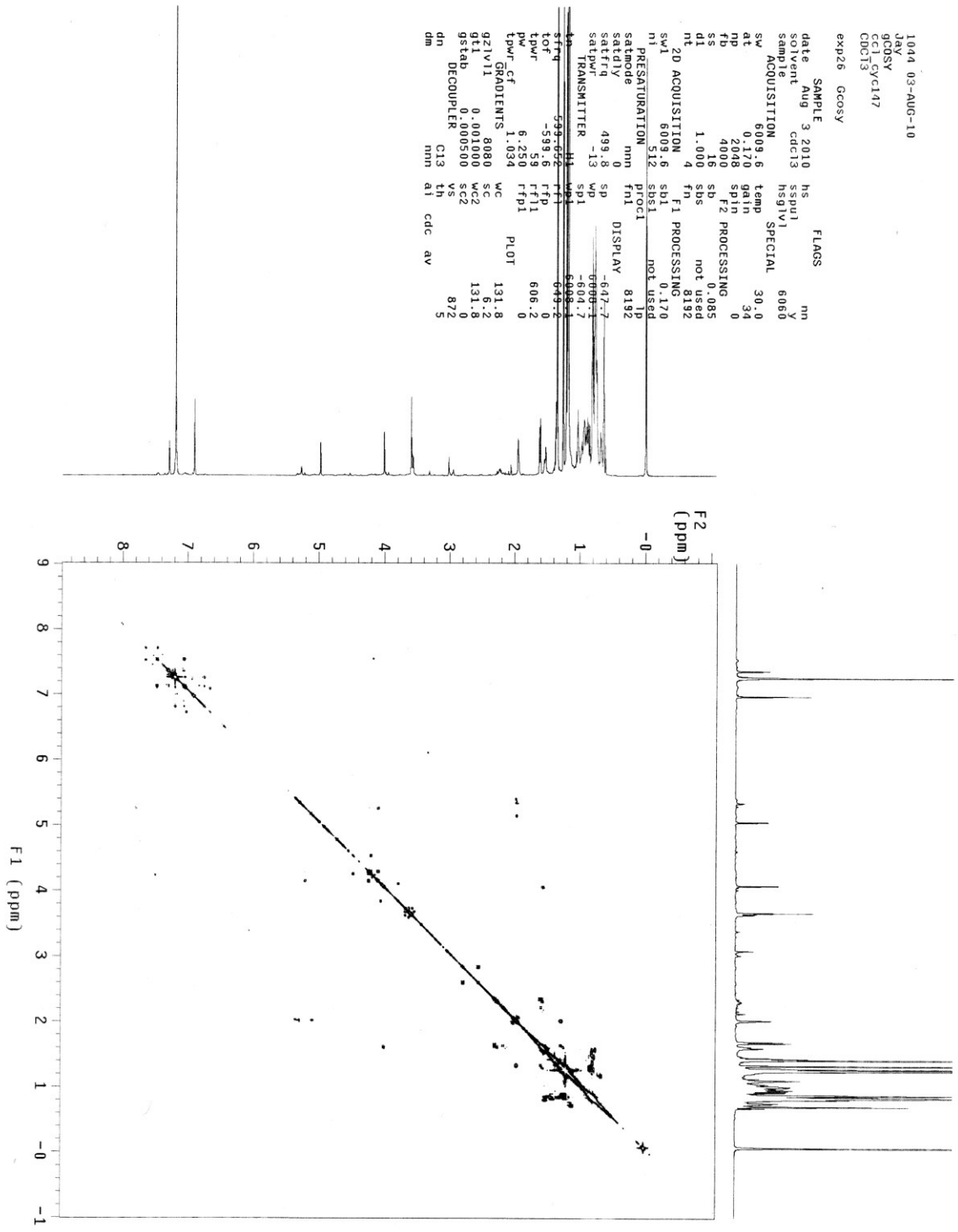


MSD Trap Report v 4 (Let-Opt2) Page 1 of 1 Agilent Technologies

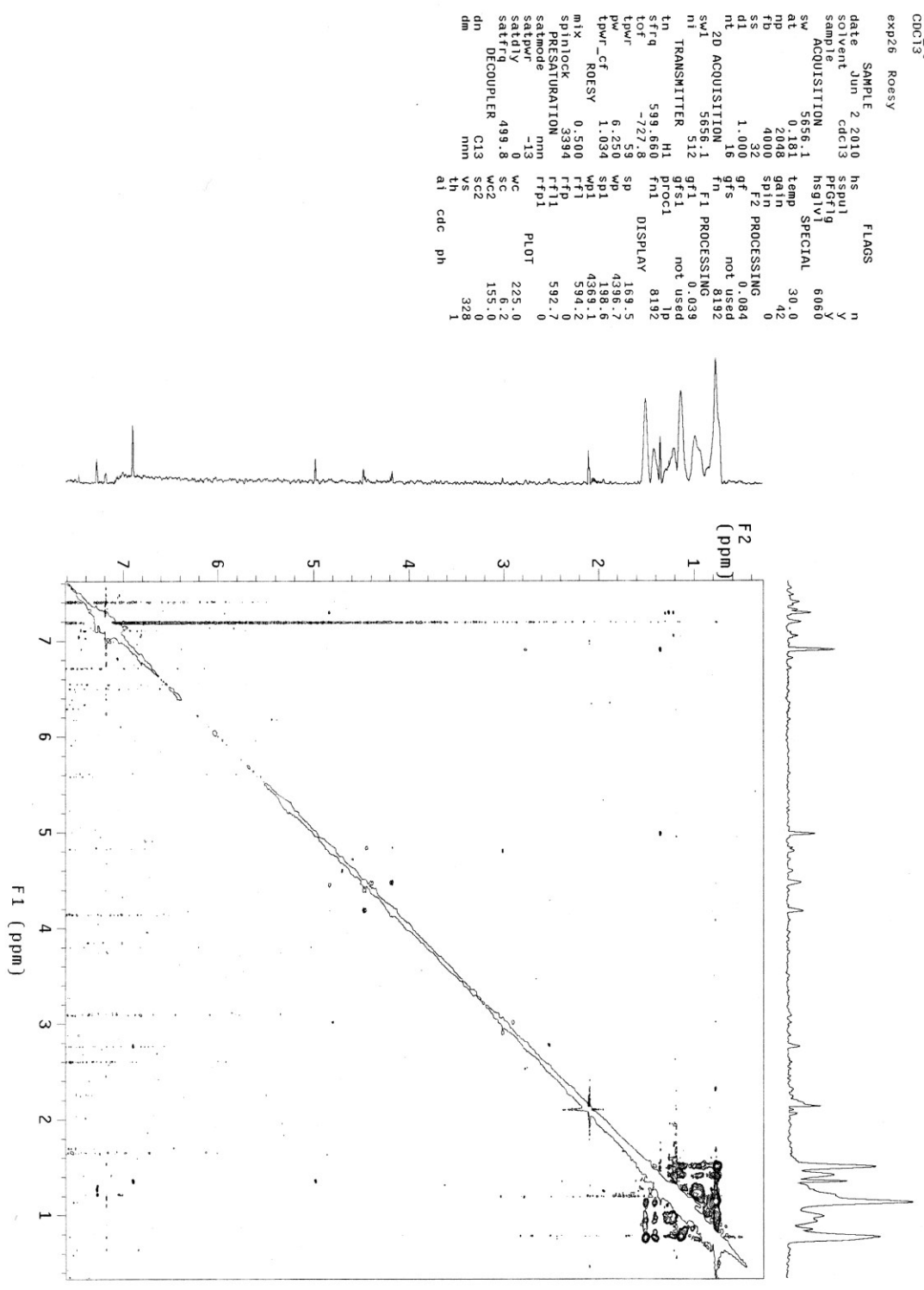
LCMS *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2R,3S)-β-benzoxy-Phe (compound 10)



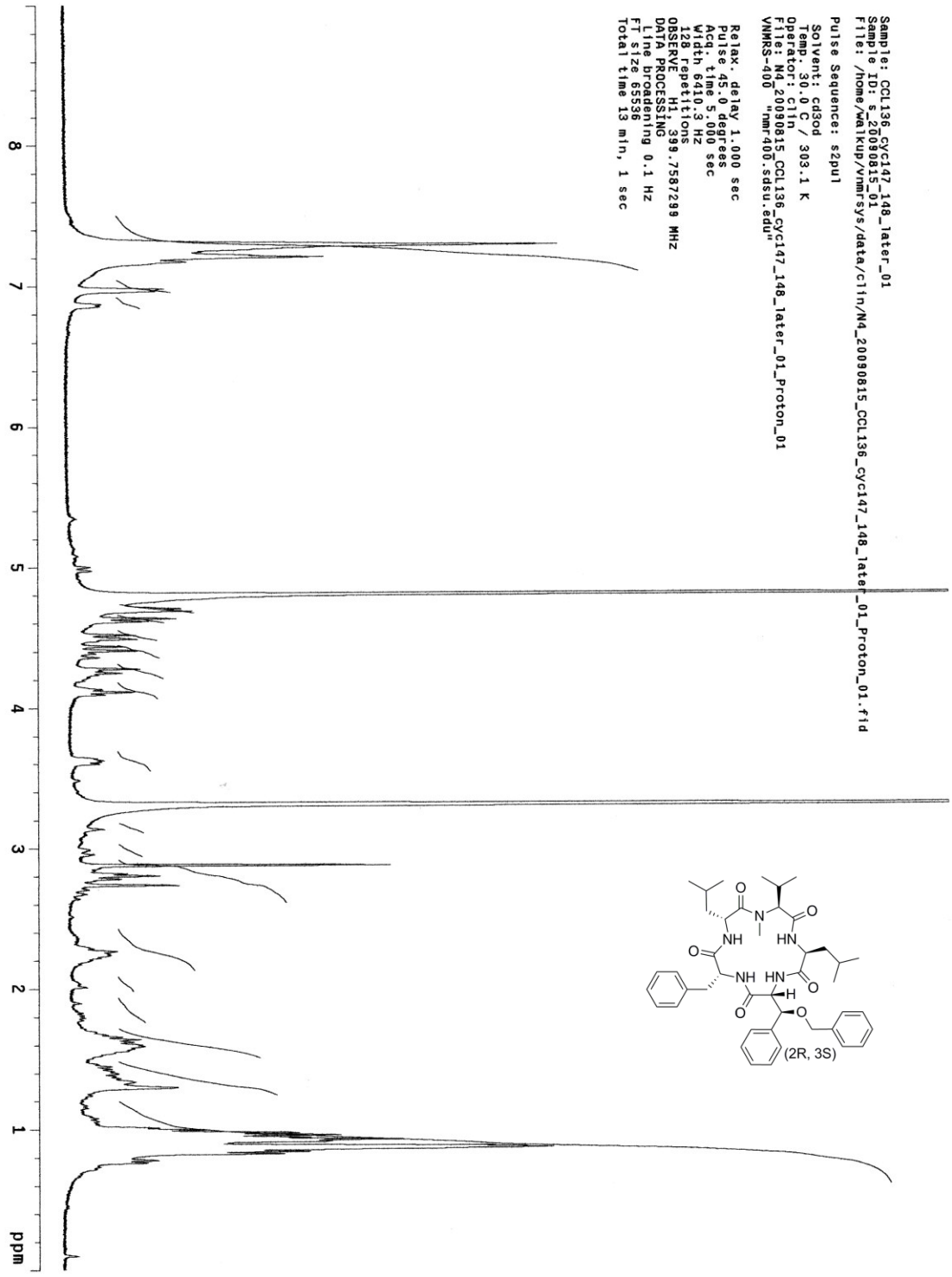
^{1}H NMR *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S,3R)-β-benzoxy-Phe (compound 9)



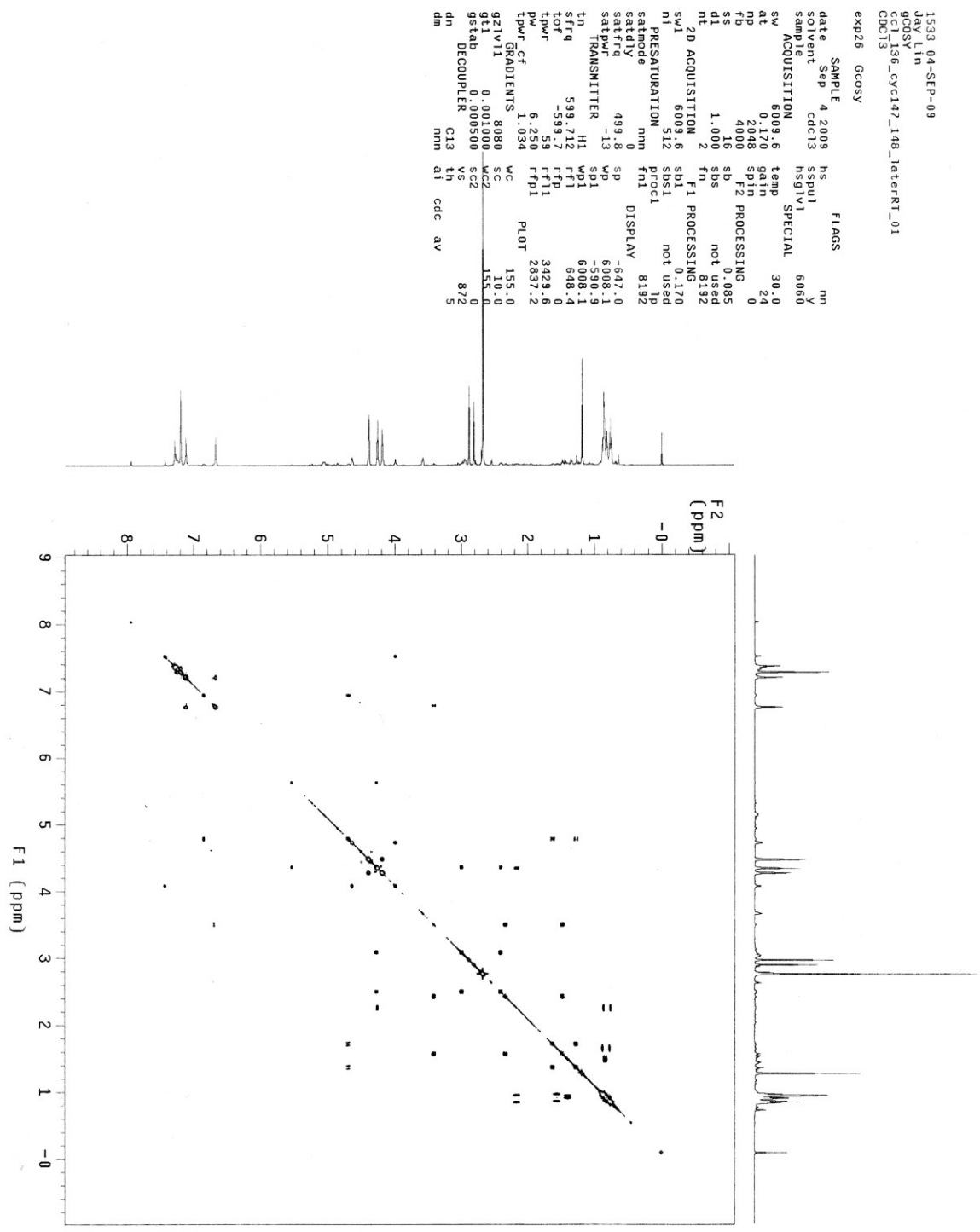
COSY *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S,3R)-β-benzoxy-Phe (compound 9)



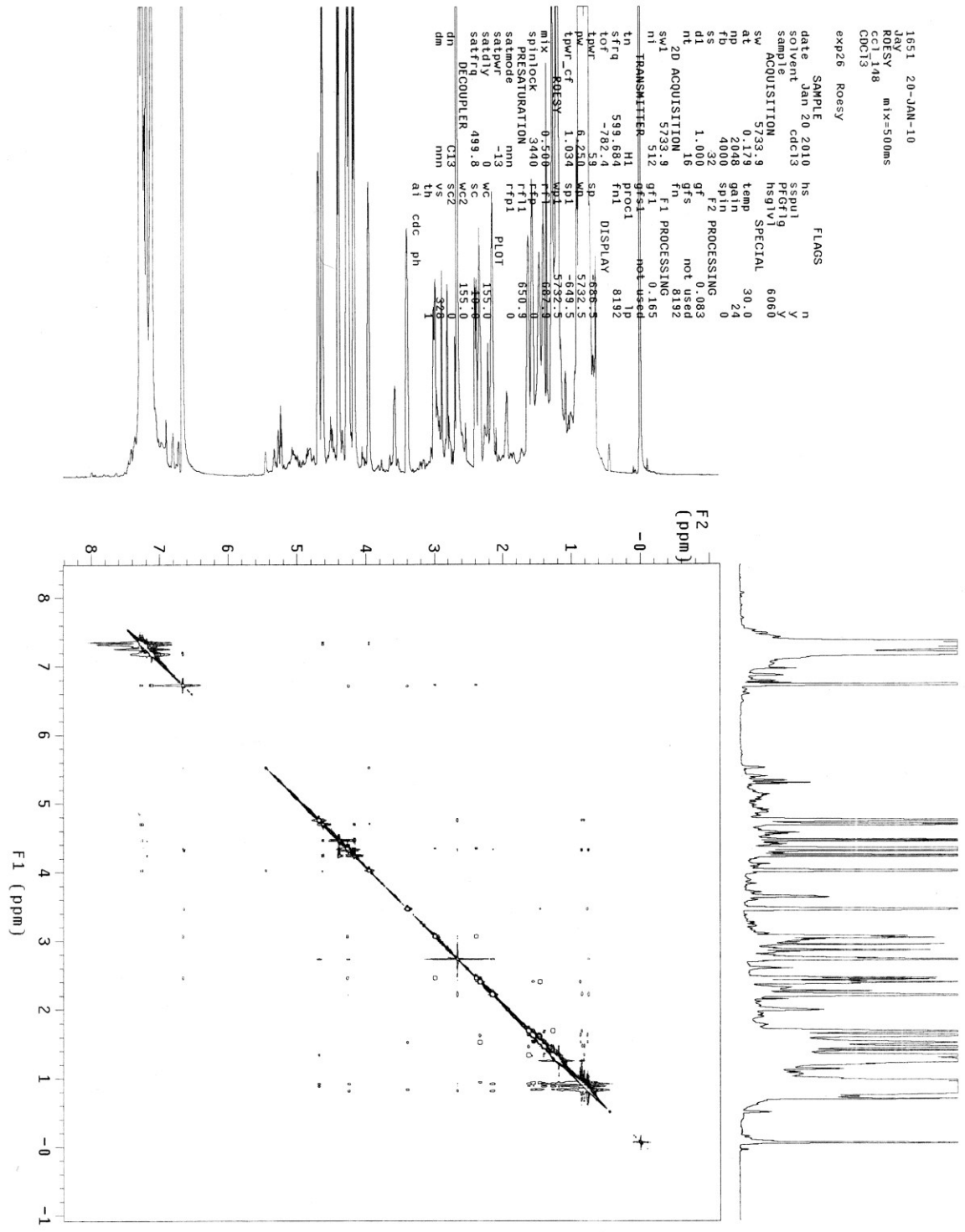
ROESY *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2S,3R)-β-benzoxy-Phe (compound 9)



^{1}H NMR *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2R,3S)-β-benzoxy-Phe (compound 10)



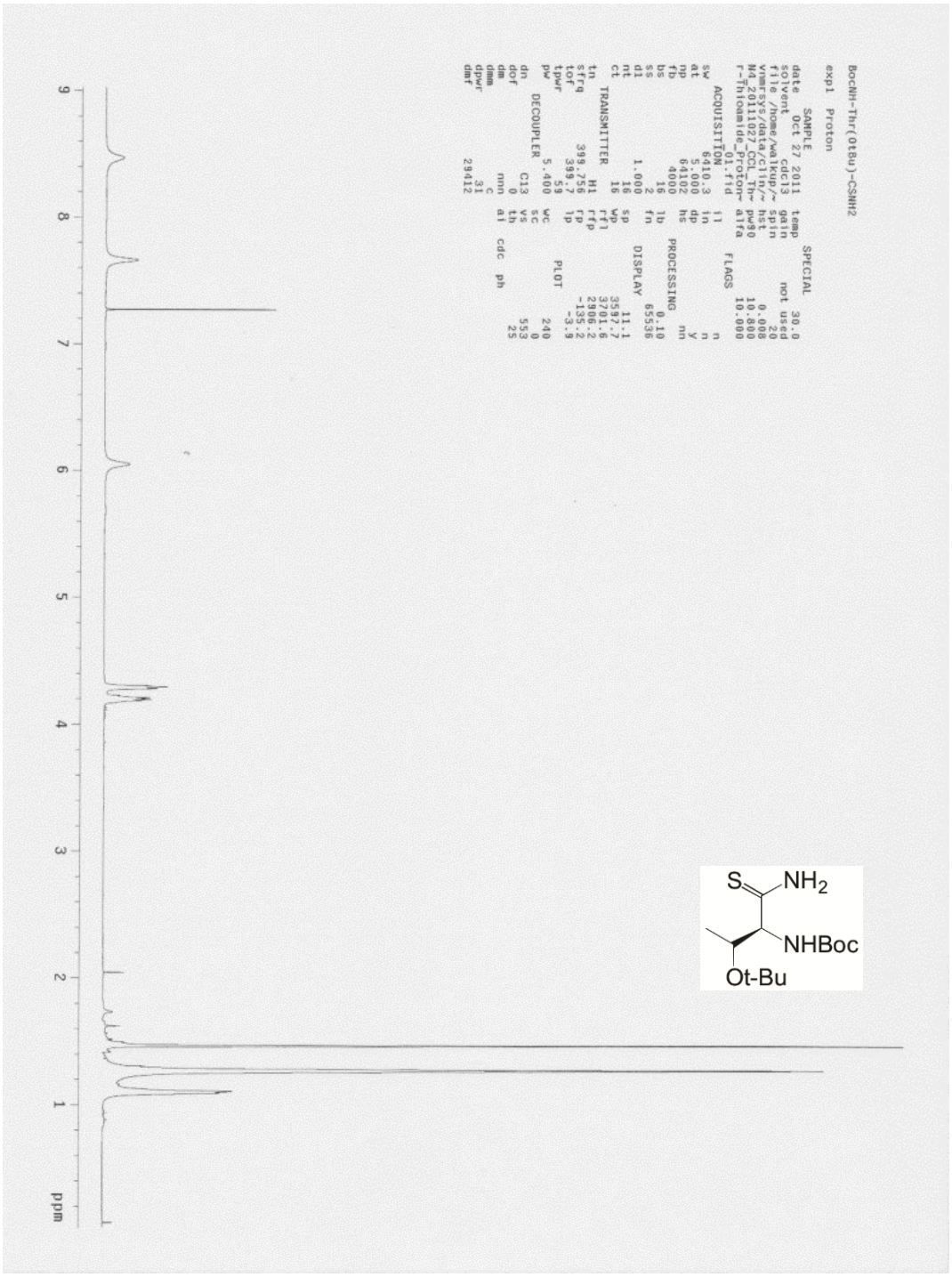
COSY *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2R,3S)-β-benzoxy-Phe (compound 10)



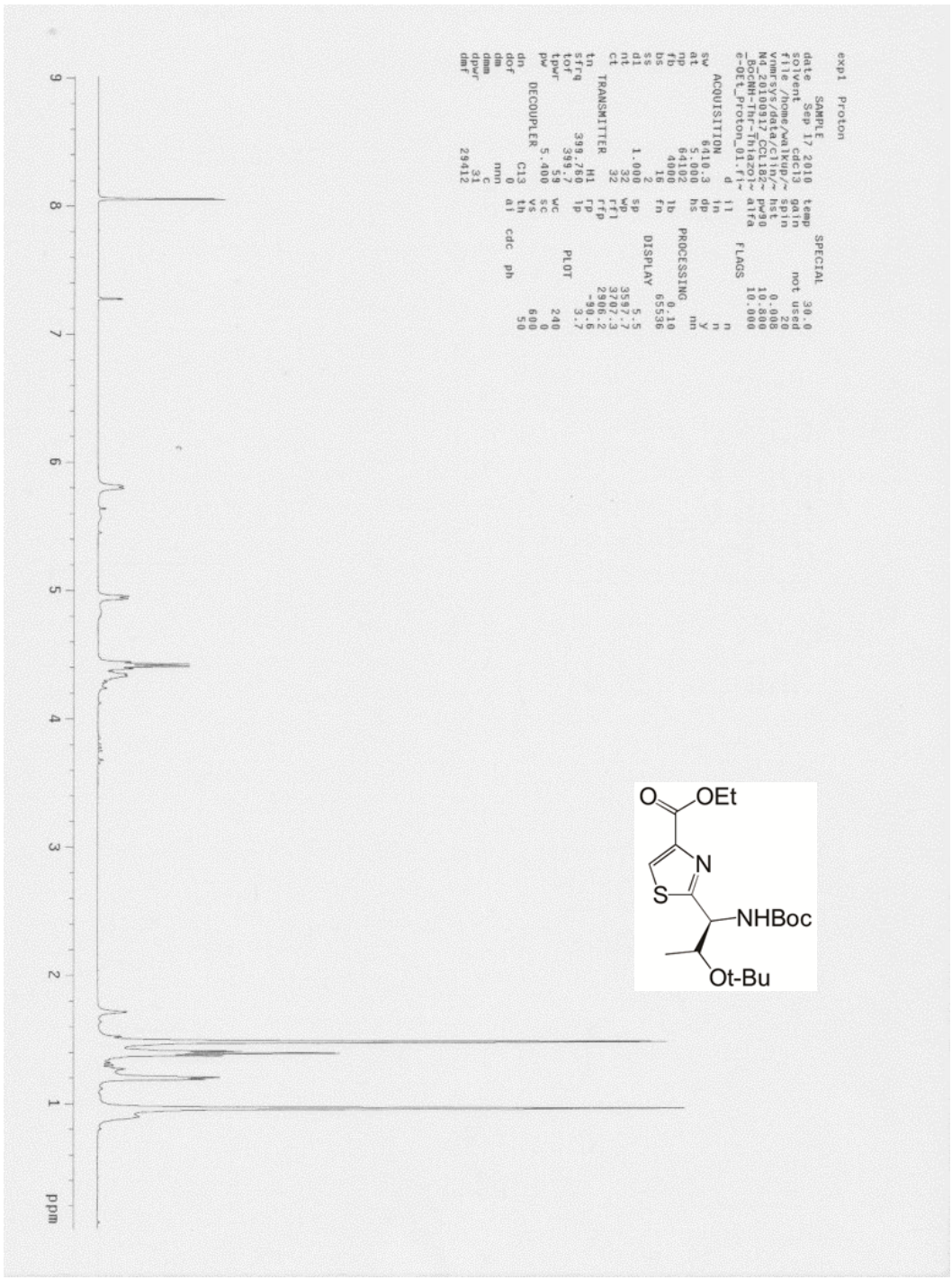
ROESY *cyclo*-Leu-*N*-Me-Val-D-Leu-D-Phe-(2R,3S)-β-benzoxy-Phe (compound 10)

Appendix B – Supporting spectra for Chapter 3

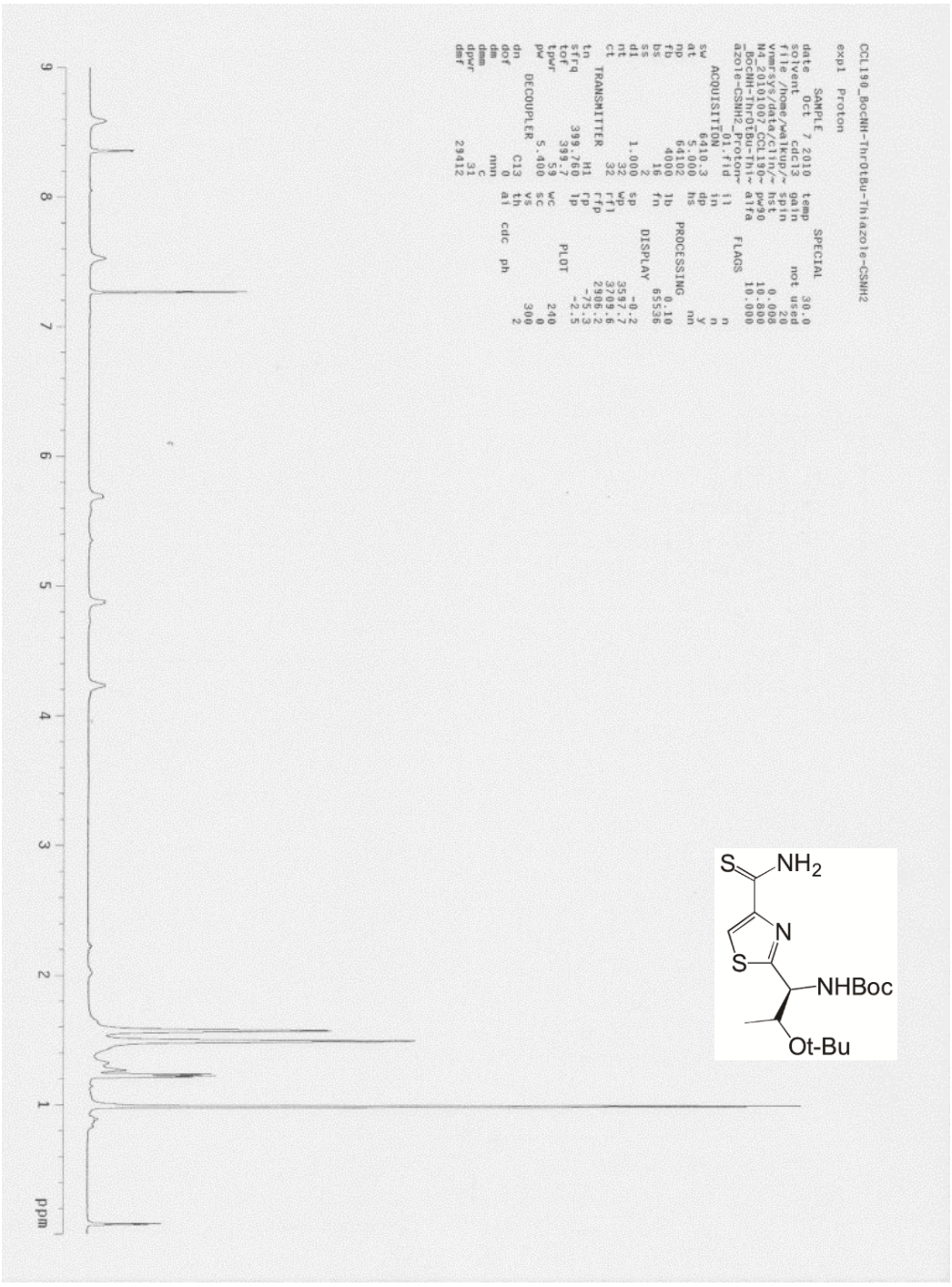
Supporting Spectra for Ustat A-3



^{1}H NMR Boc-Thr(*t*-Bu)-SNH$_2$



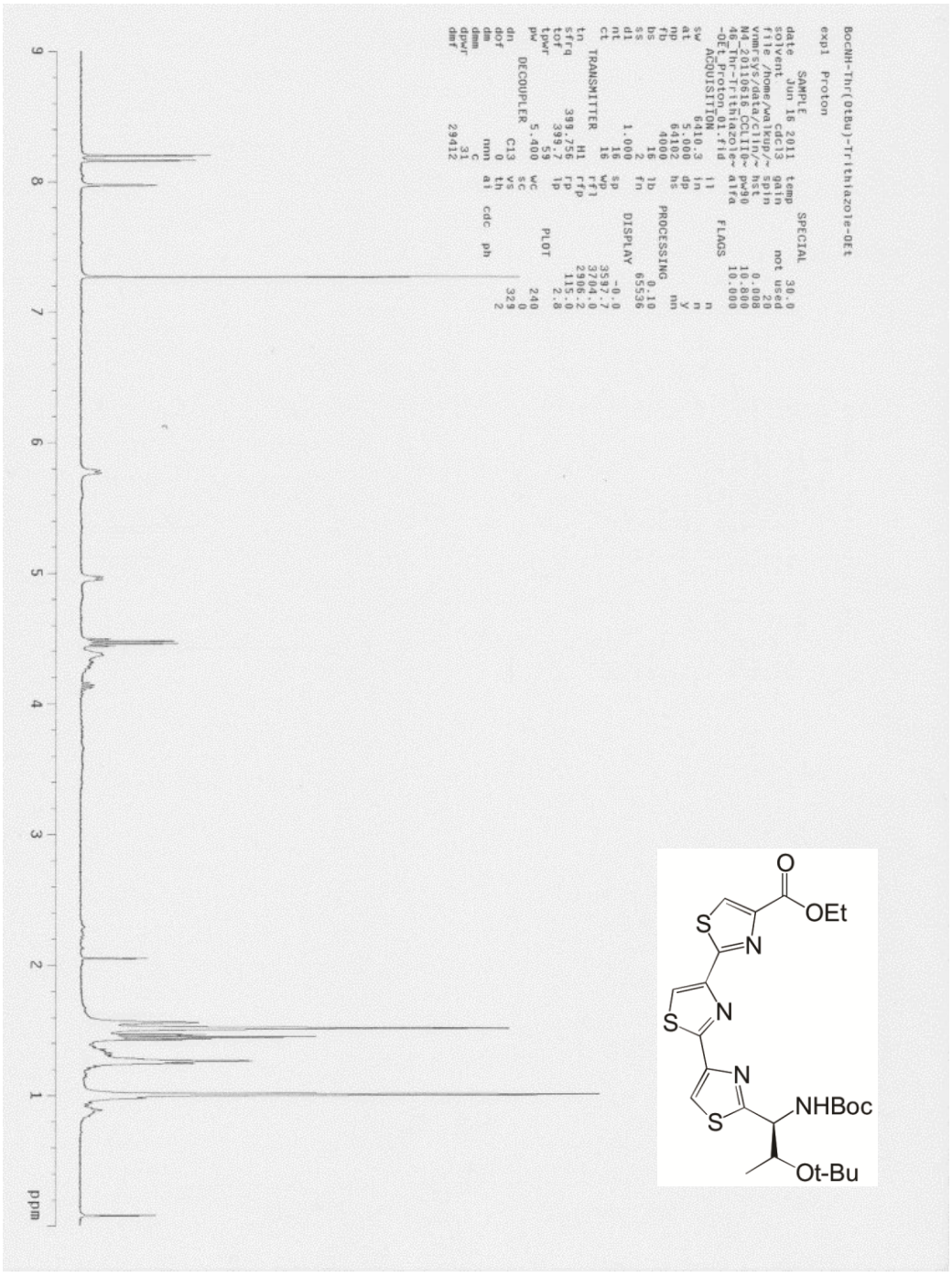
^{1}H NMR Boc-Thr(*t*-Bu)-Thiazole-OEt



^{1}H NMR Boc-Thr(*t*-Bu)-Thiazole-SNH$_2$

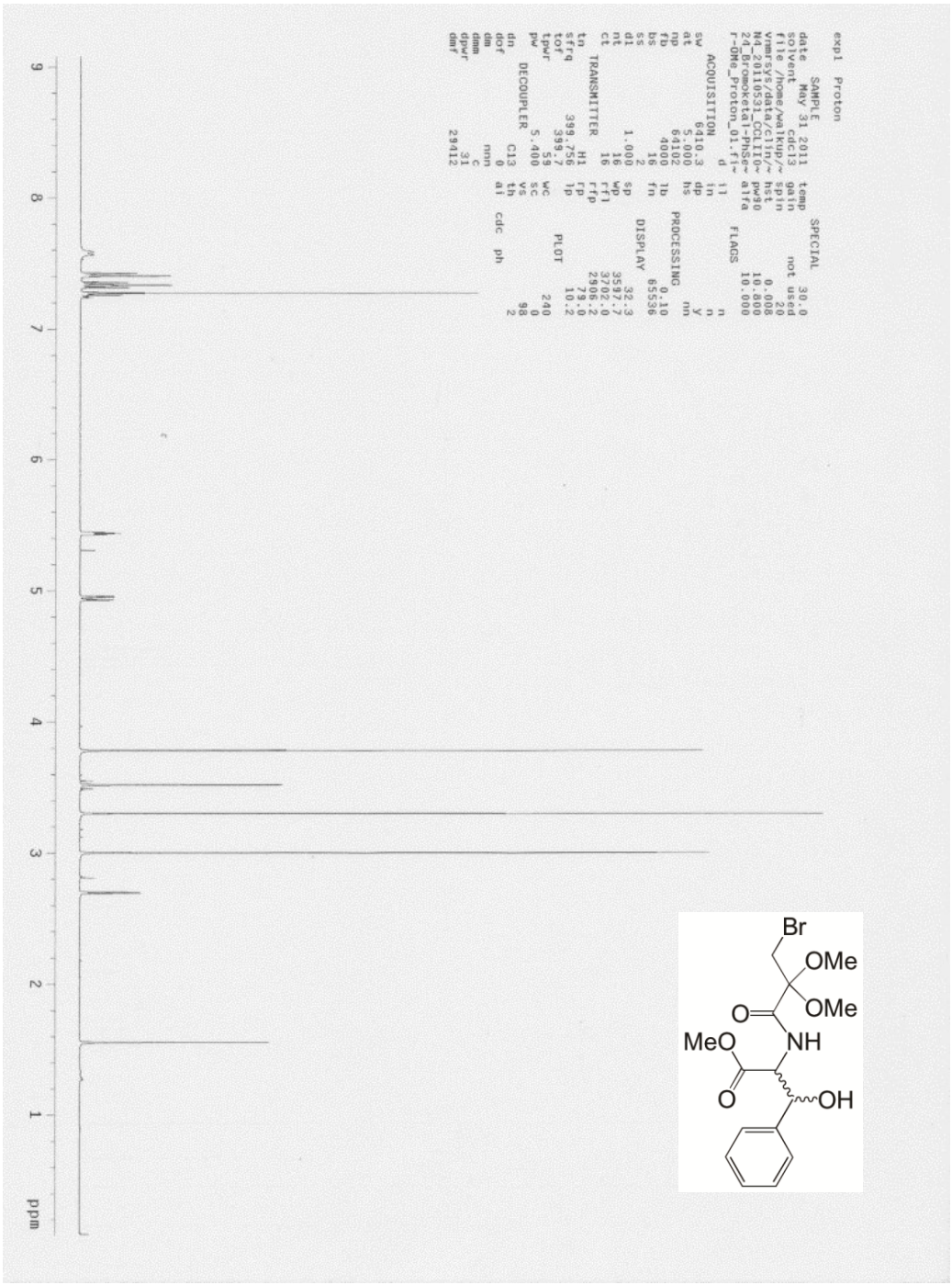
^{1}H NMR Boc-Thr(*t*-Bu)-Dithiazole-OEt

^{1}H NMR Boc-Thr(*t*-Bu)-Dithiazole-SNH$_2$

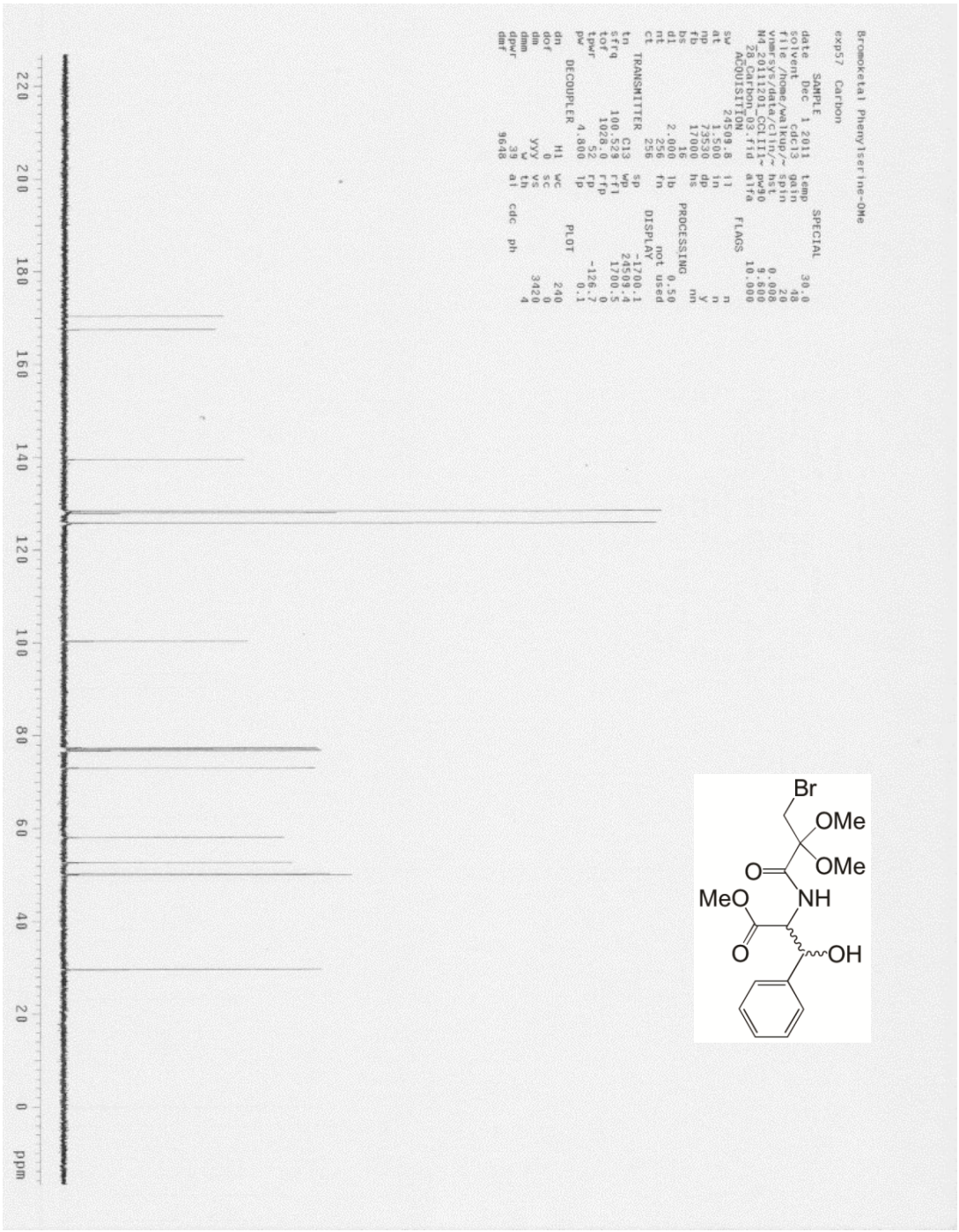


^{1}H NMR Boc-Thr(*t*-Bu)-Trithiazole-OEt

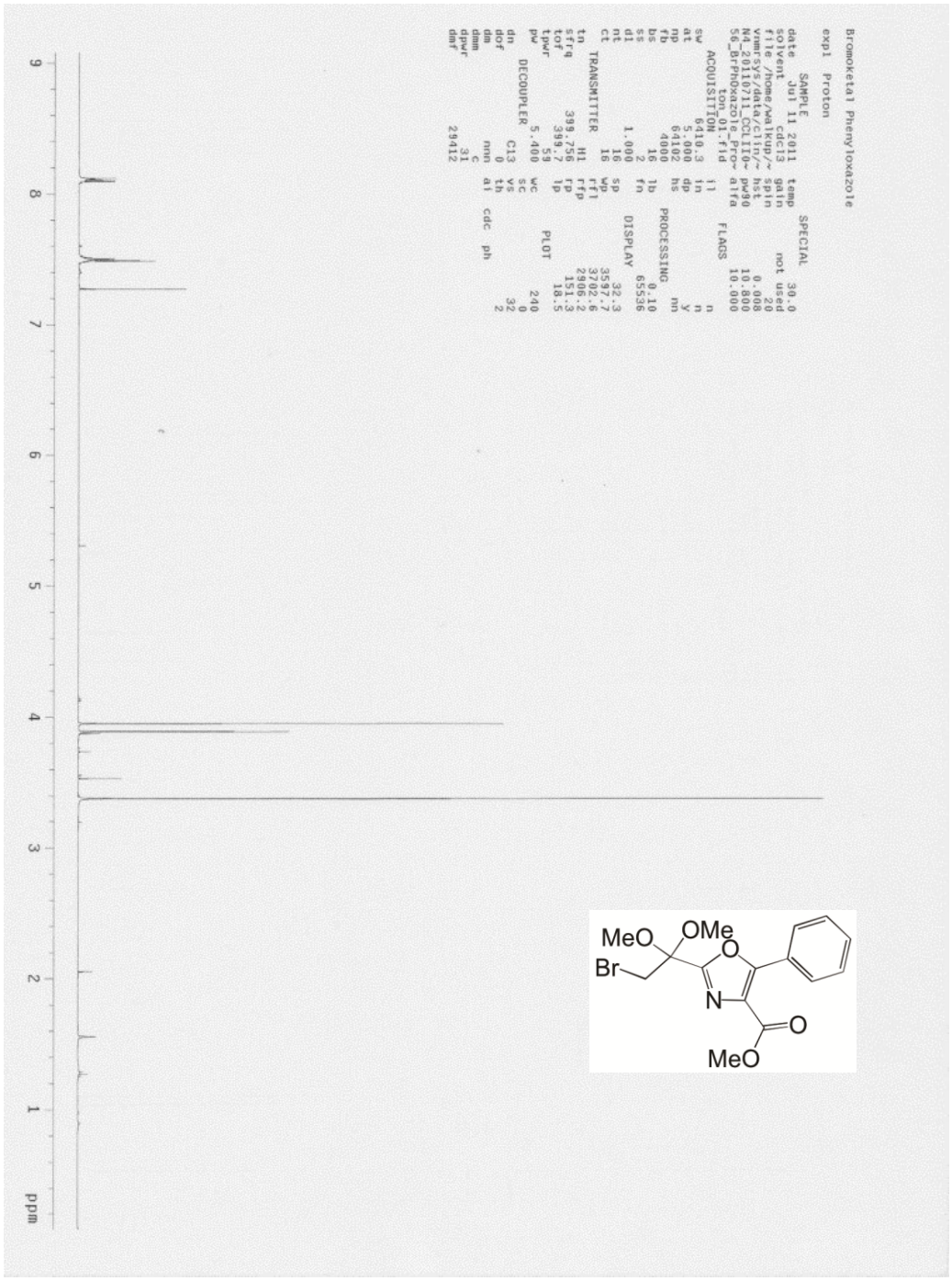
^{1}H NMR Boc-Thr(*t*-Bu)-Trithiazole-SNH$_2$ (fragment 1)



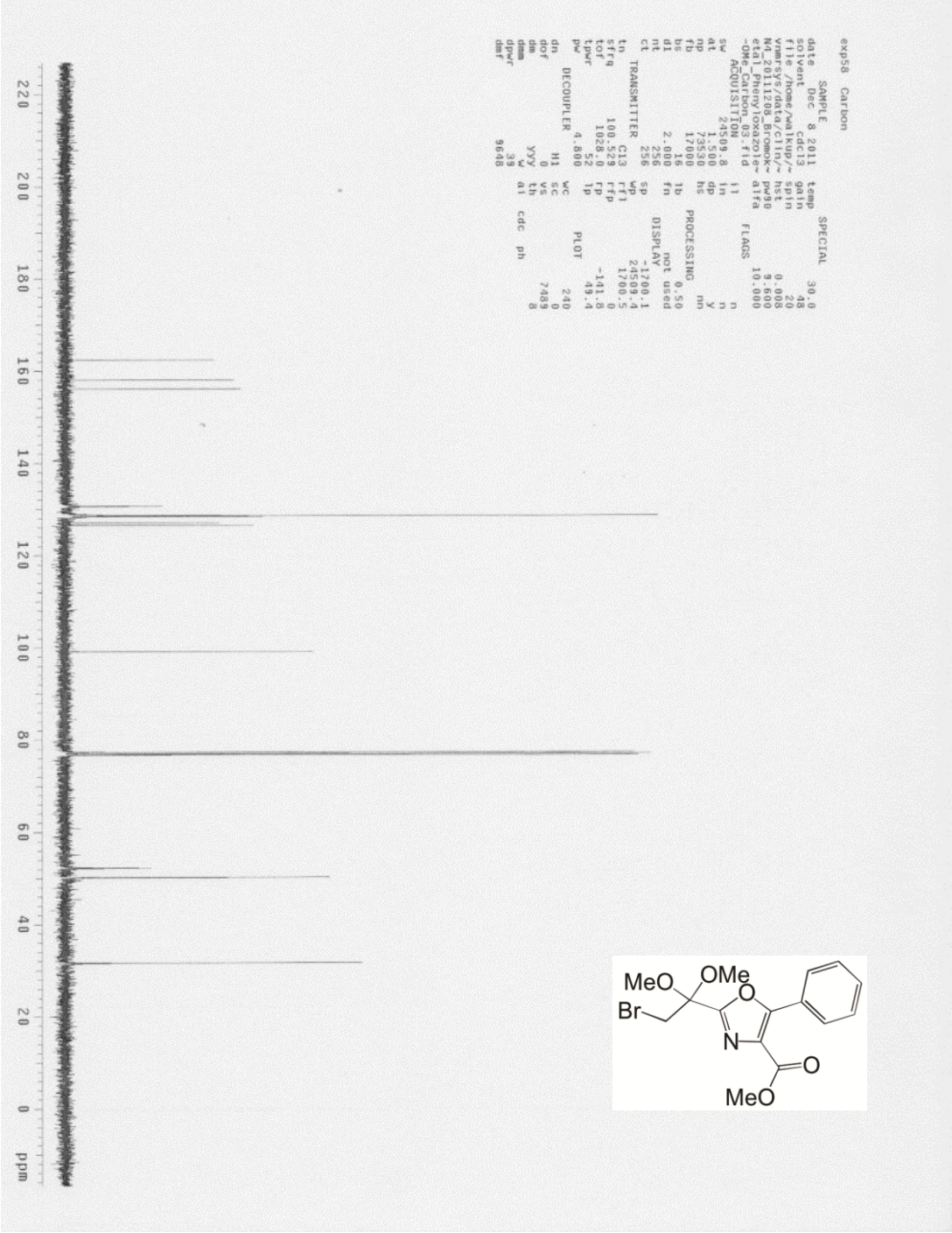
^{1}H NMR Bromoketal-*β*-hydroxyl-Phe-OMe



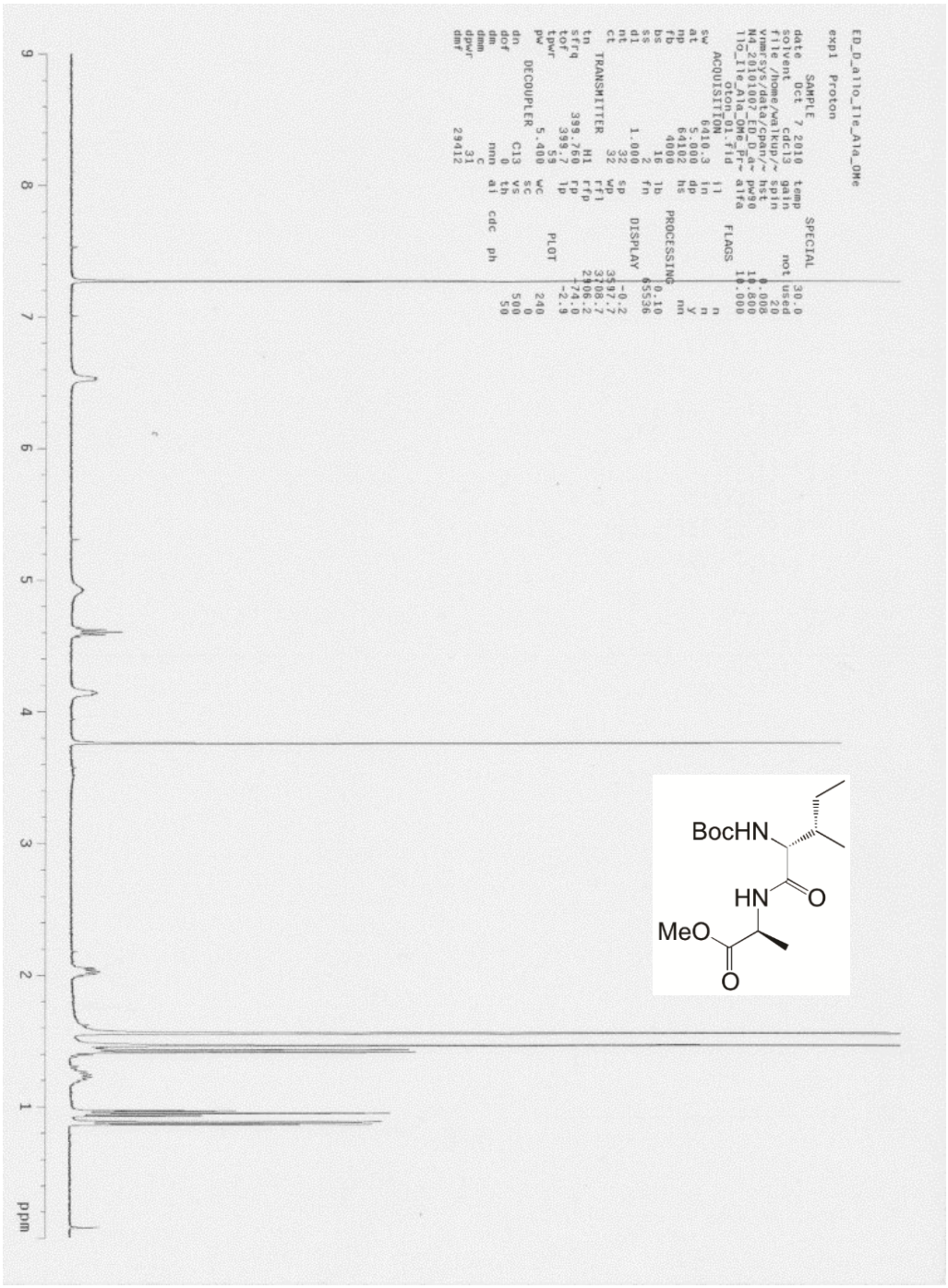
^{13}C NMR Bromoketal-*β*-hydroxyl-Phe-OMe



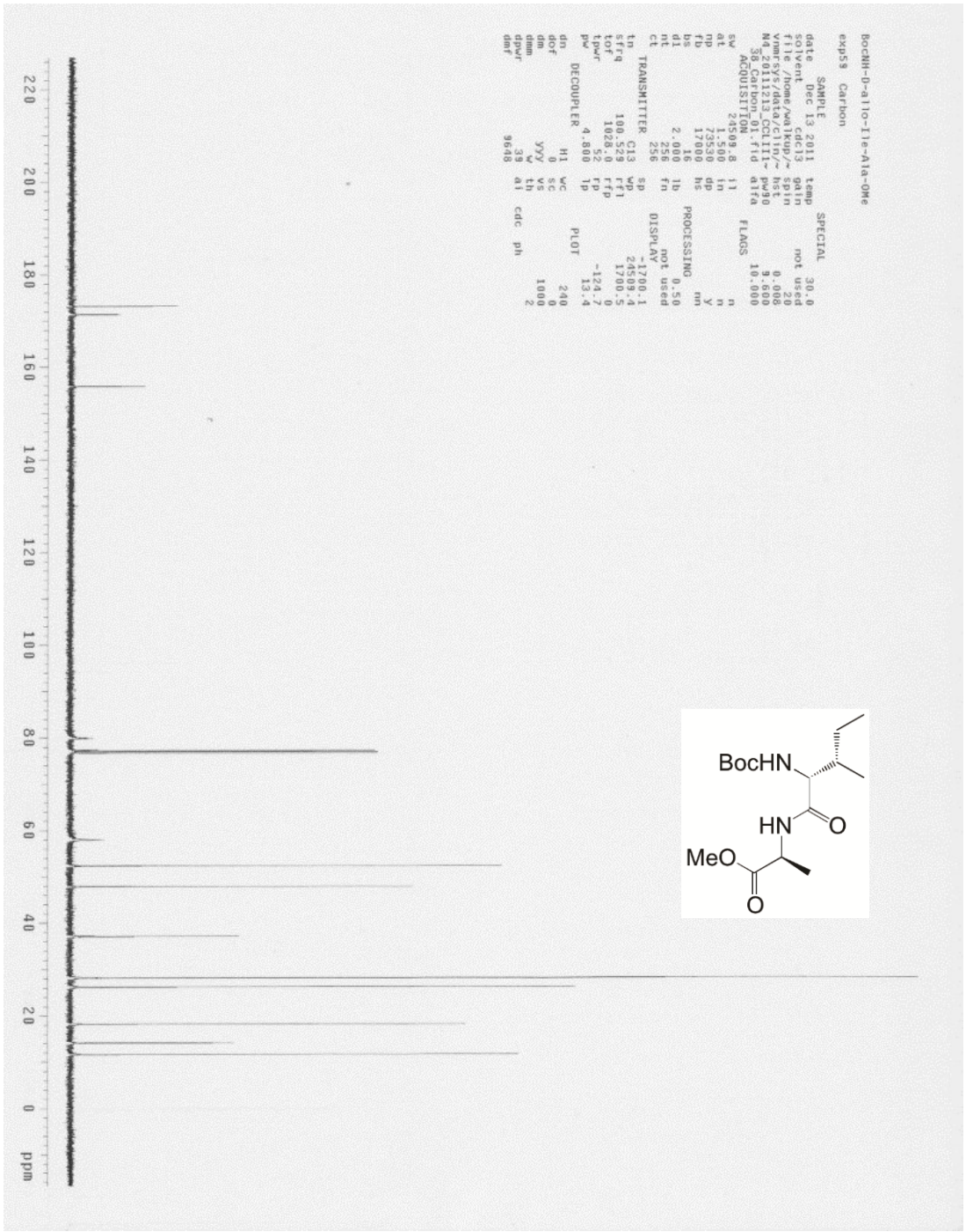
^{1}H NMR Bromoketal Phenyloxazole-OMe



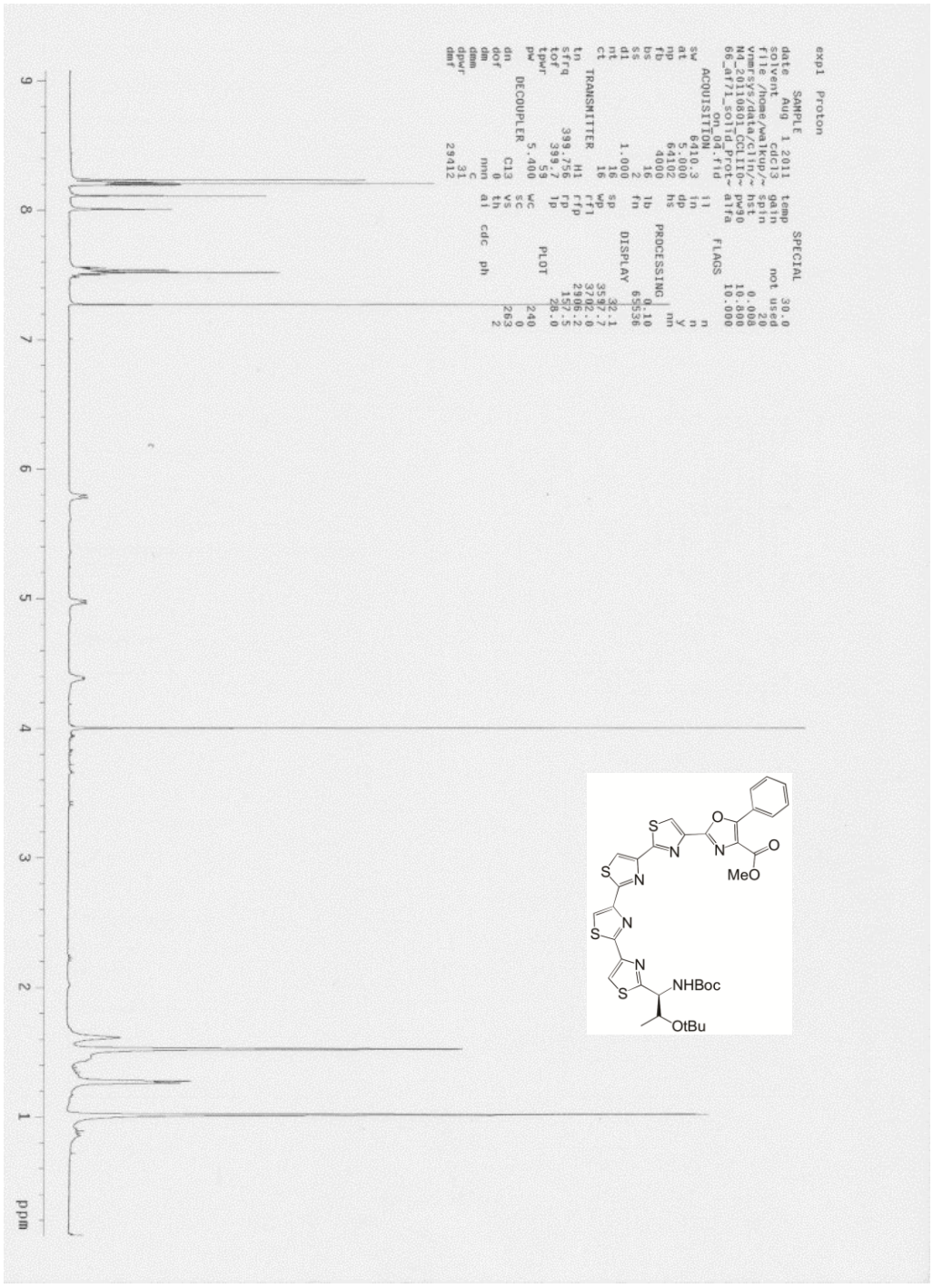
^{13}C NMR Bromoketal Phenyloxazole-OMe



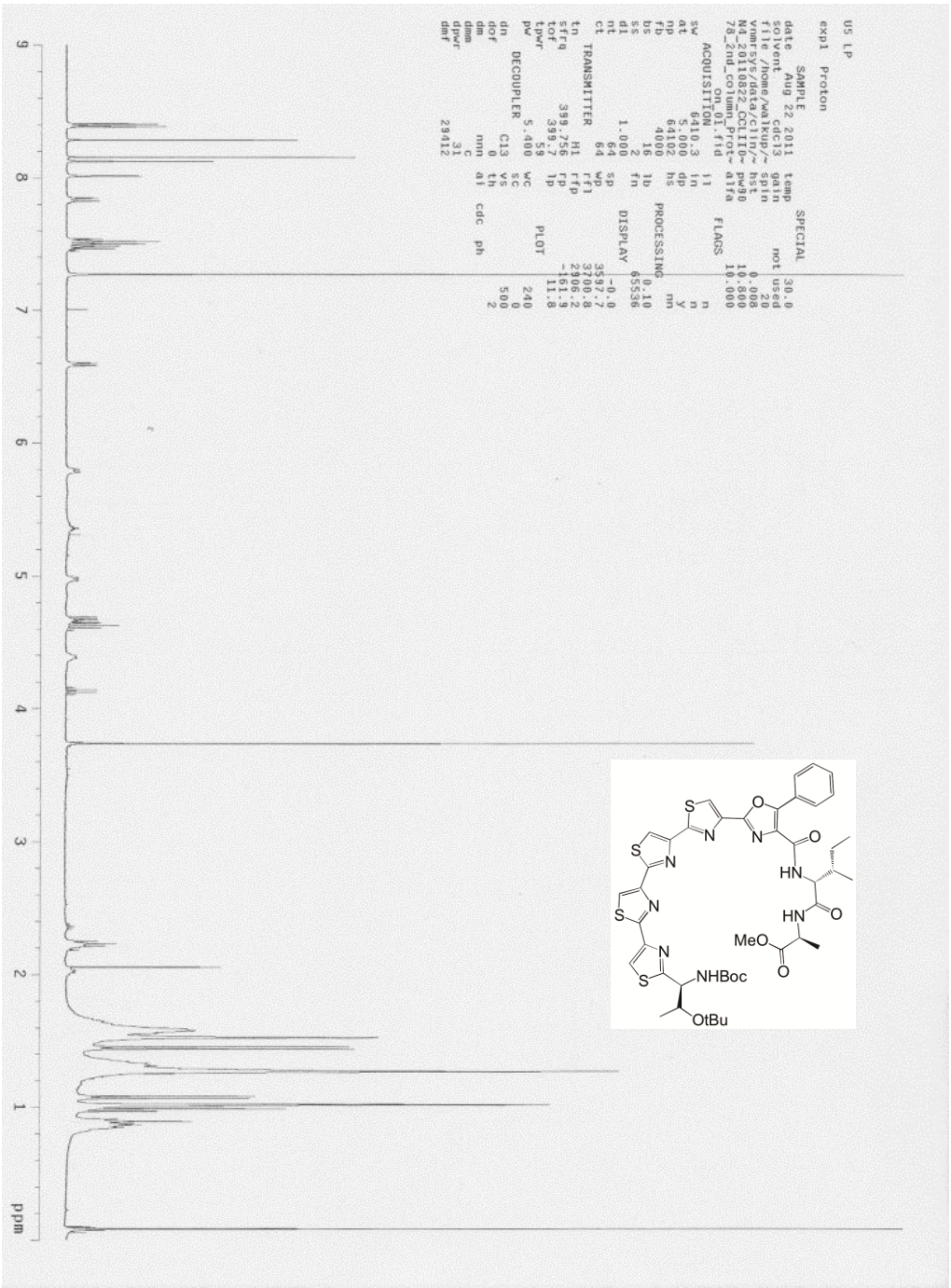
^{1}H NMR Boc-D-*allo*-Ile-Ala-OMe



^{13}C NMR Boc-D-*allo*-Ile-Ala-OMe



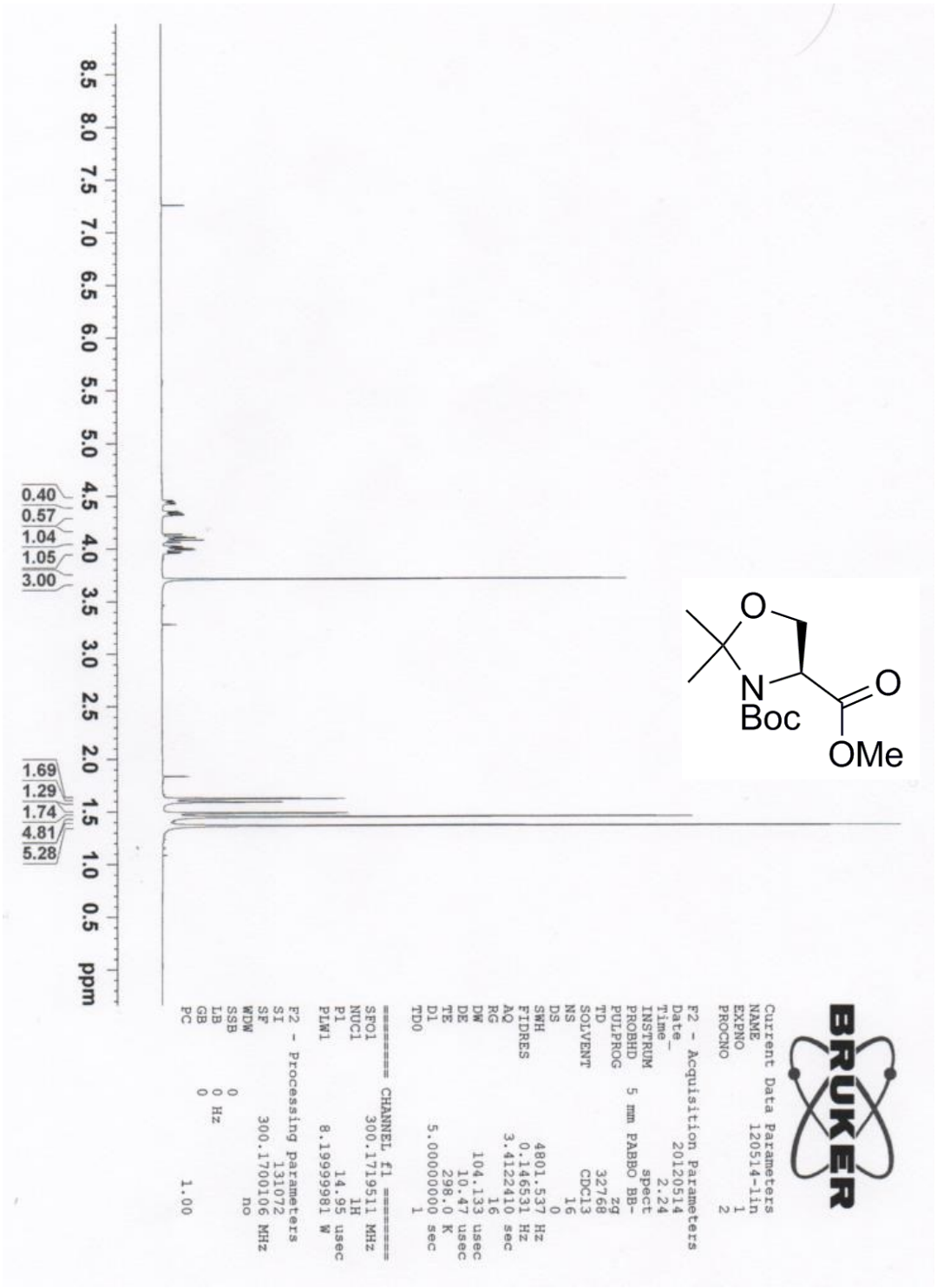
^{1}H NMR Boc-Thr(*t*-Bu)-Tetrathiazole-Phenyloxazole-OMe (compound 13)



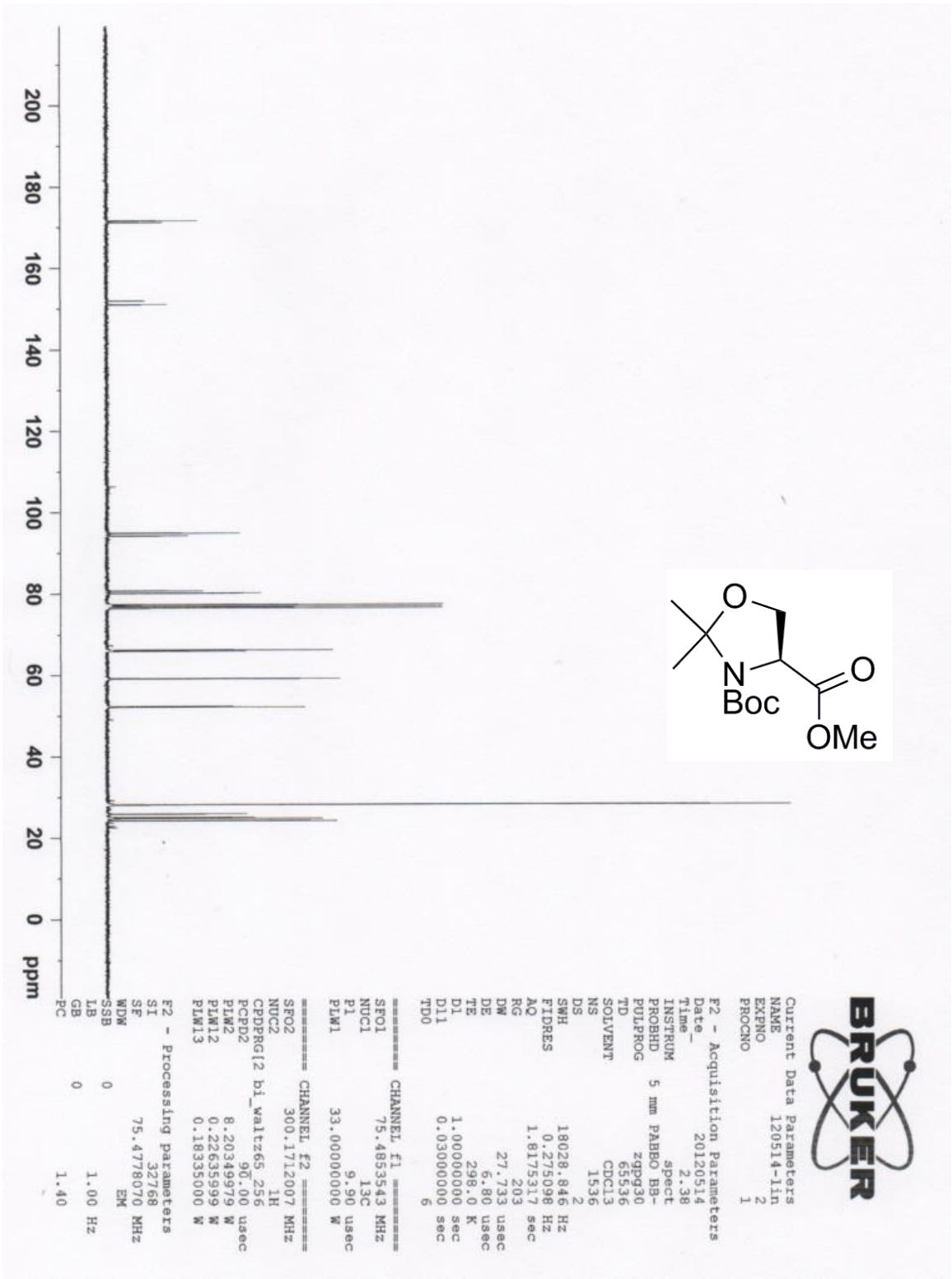
^{1}H NMR Boc-Thr(*t*-Bu)-Tetrathiazole-Phenyloxazole-D-*allo*-Ile-Ala-OMe

(compound 15)

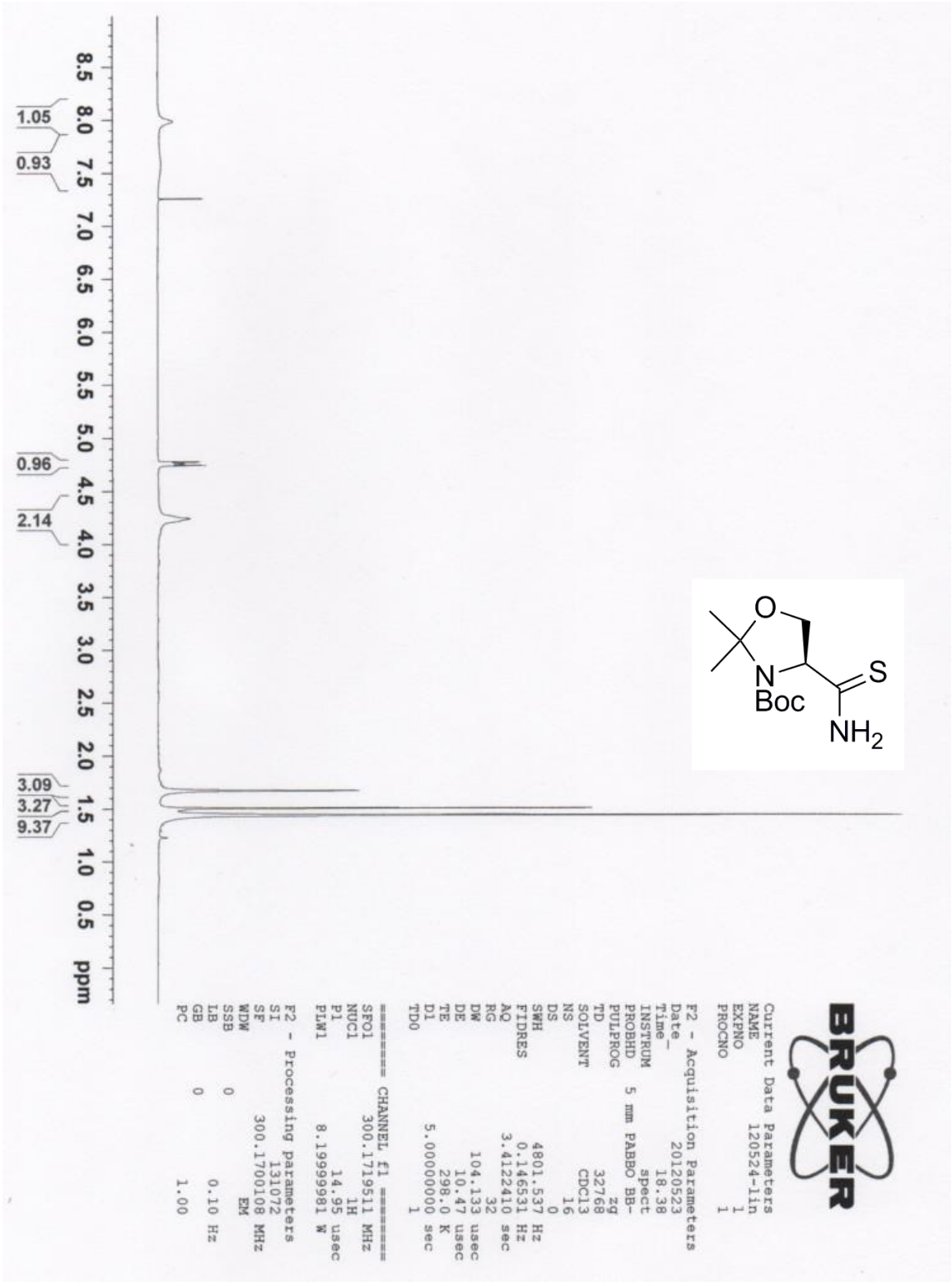
Supporting Spectra for Ustat A-1



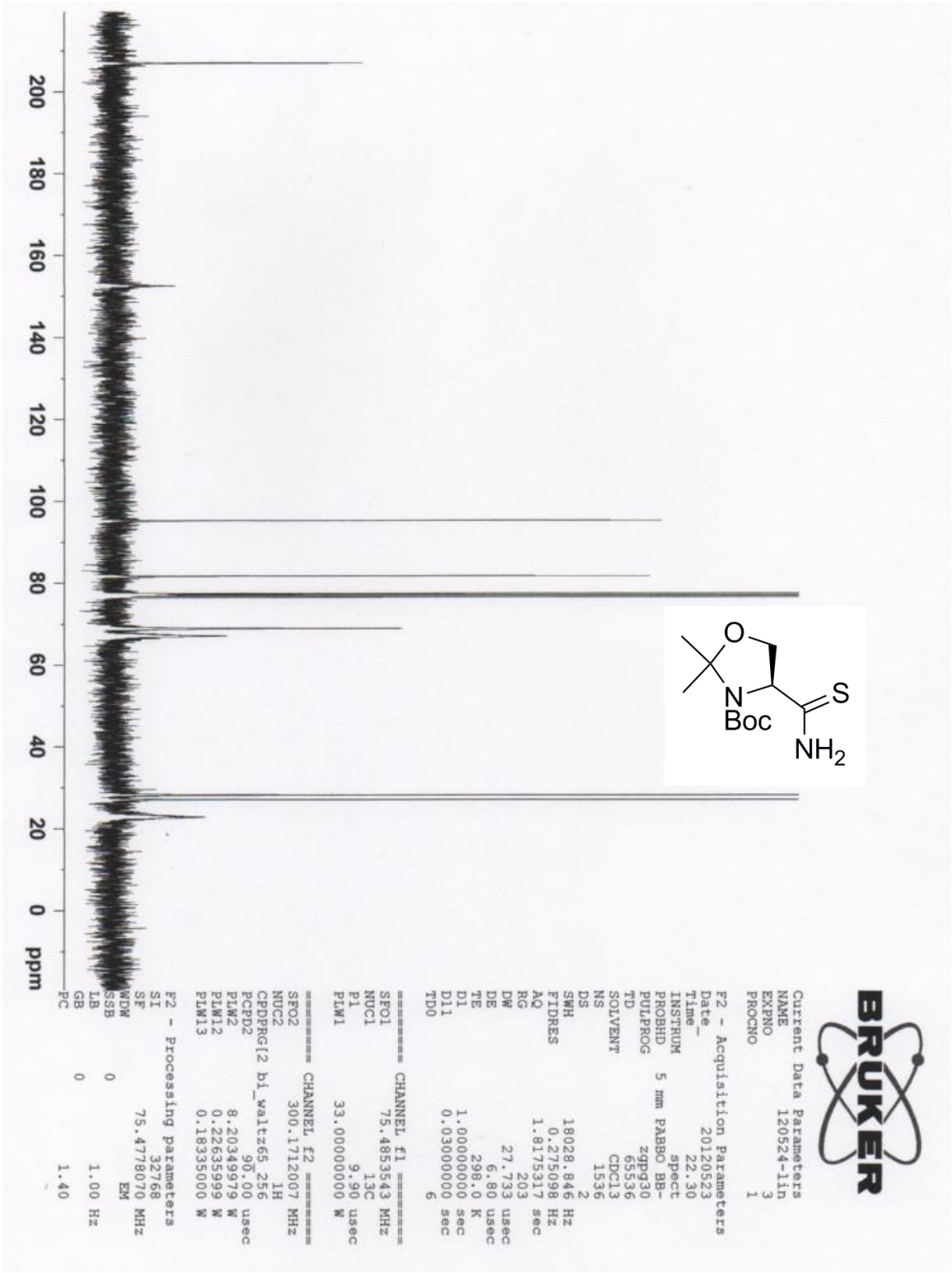
^{1}H NMR Boc-Oxazolidine-OMe



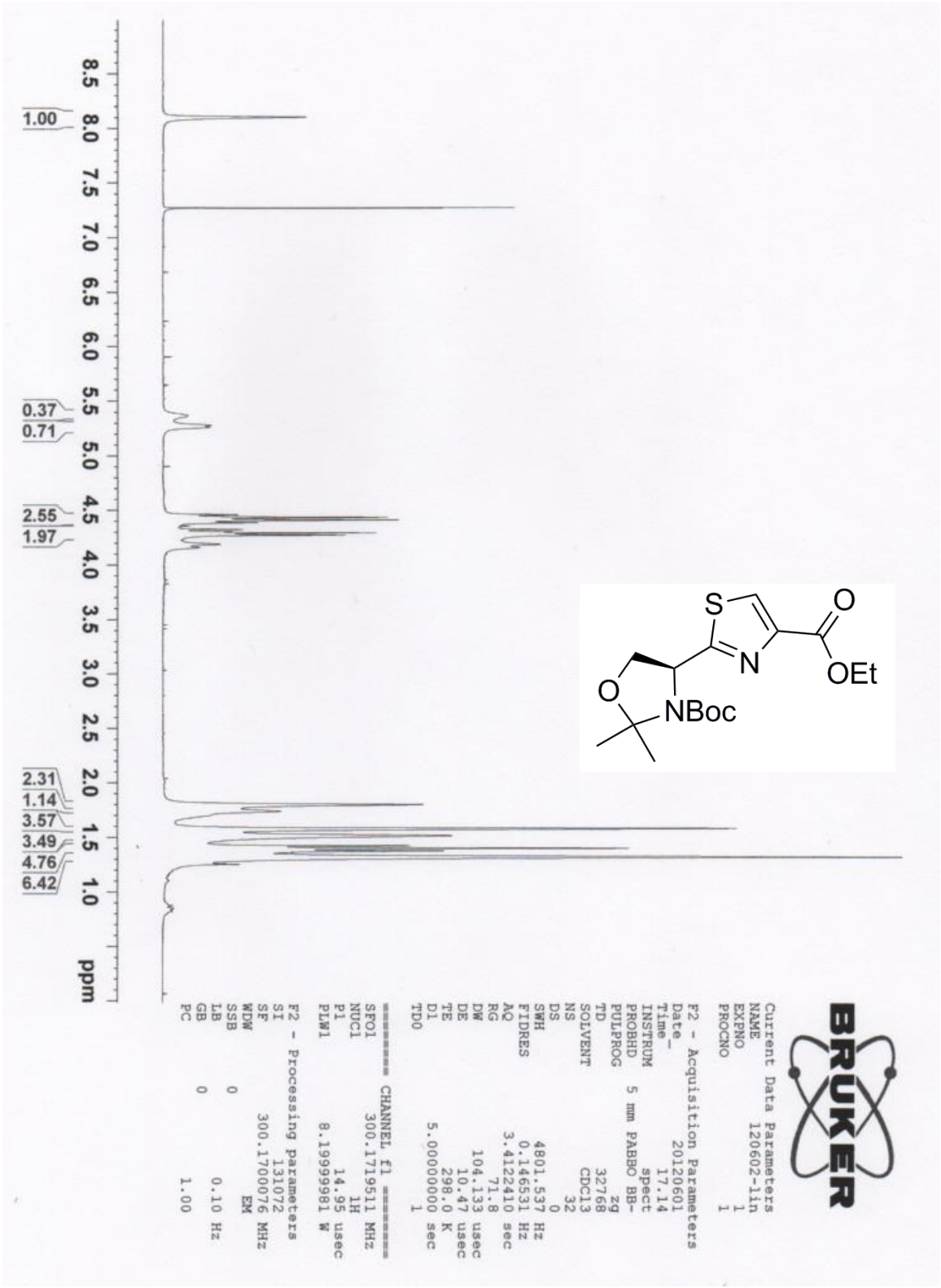
^{13}C NMR Boc-Oxazolidine-OMe



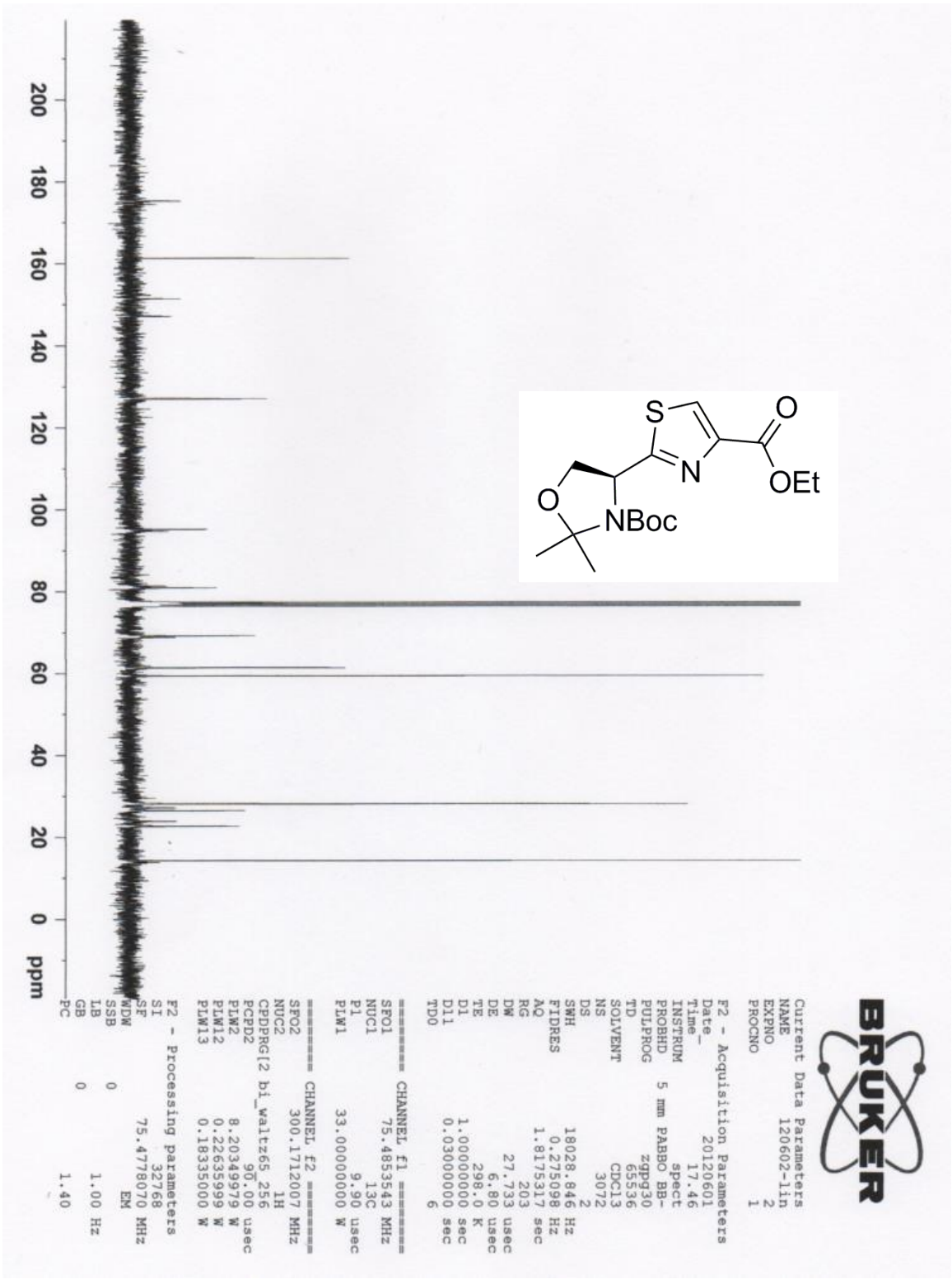
^{1}H NMR Boc-Oxazolidine-CSNH$_2$



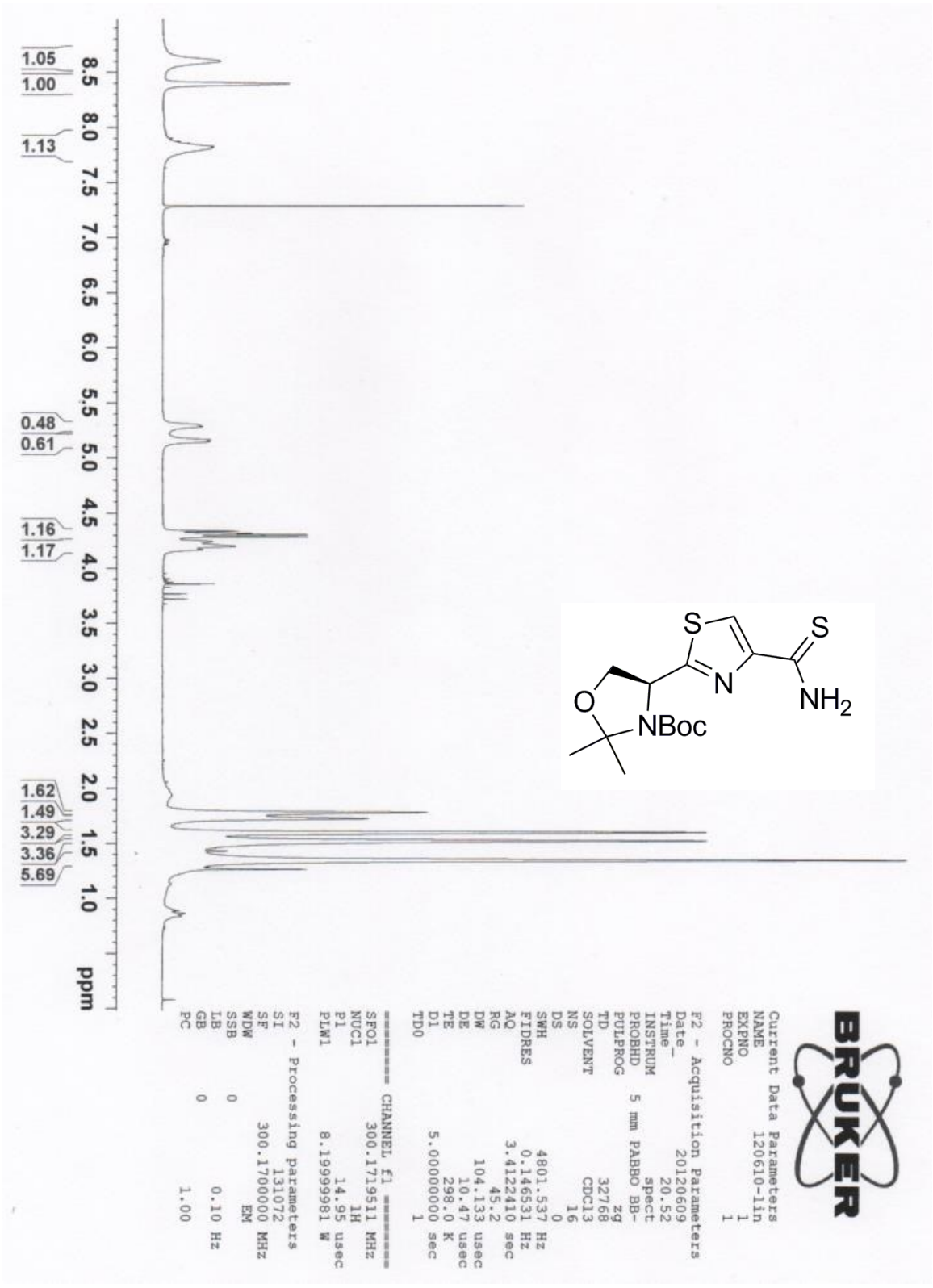
^{13}C NMR Boc-Oxazolidine-CSNH$_2$



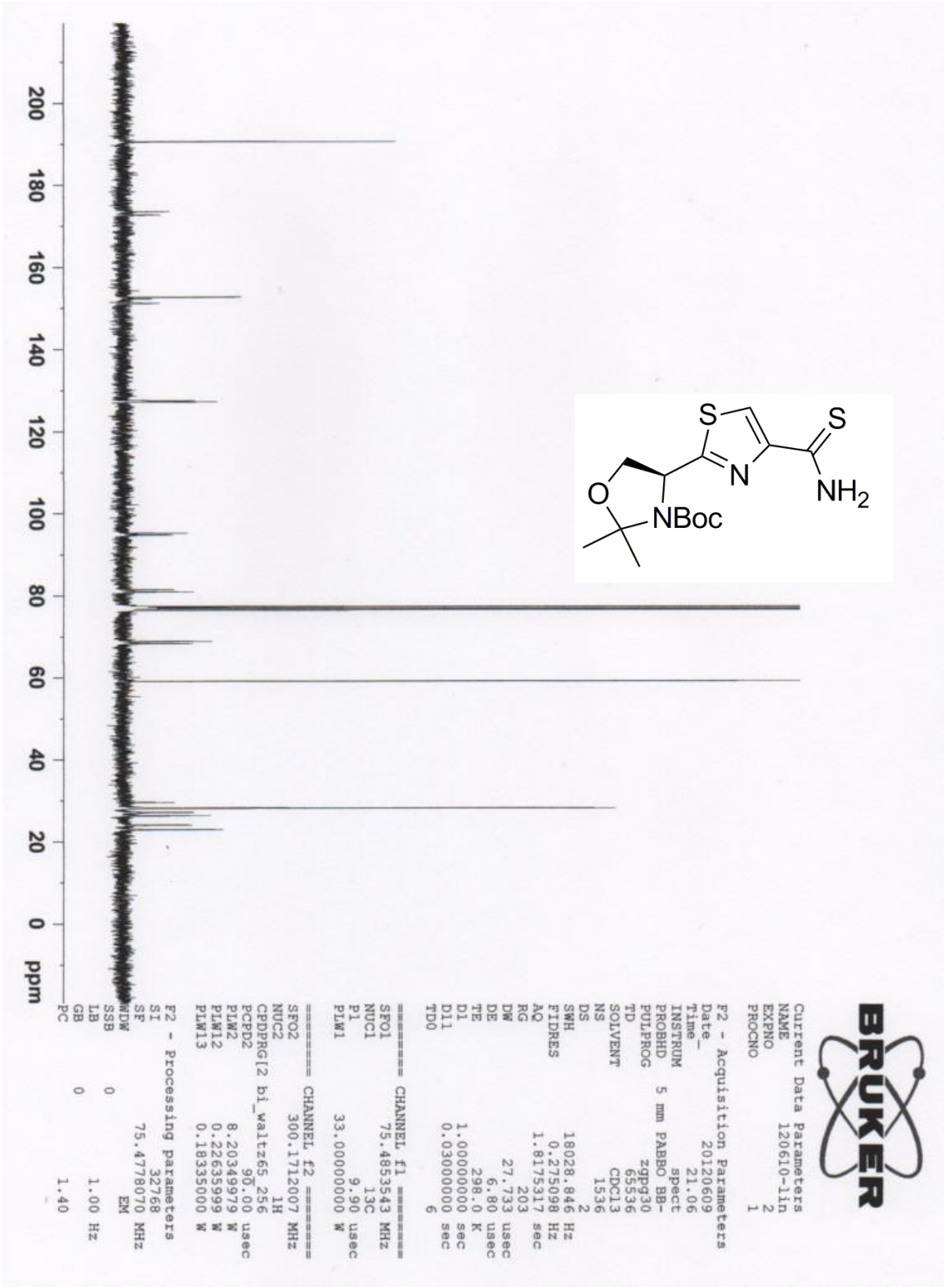
^{1}H NMR Boc-Oxazolidine-Thiazole-OEt



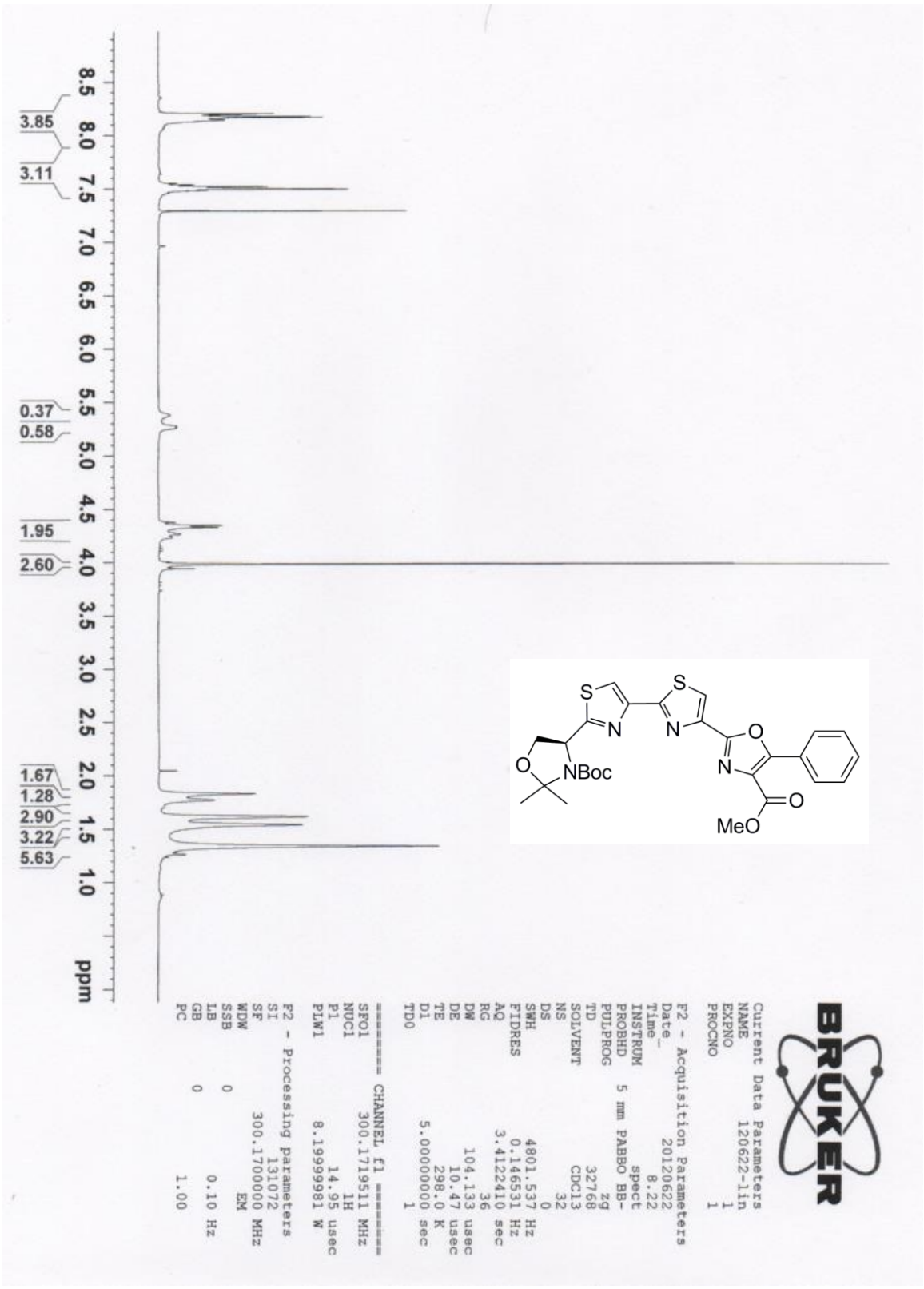
^{13}C NMR Boc-Oxazolidine-Thiazole-OEt



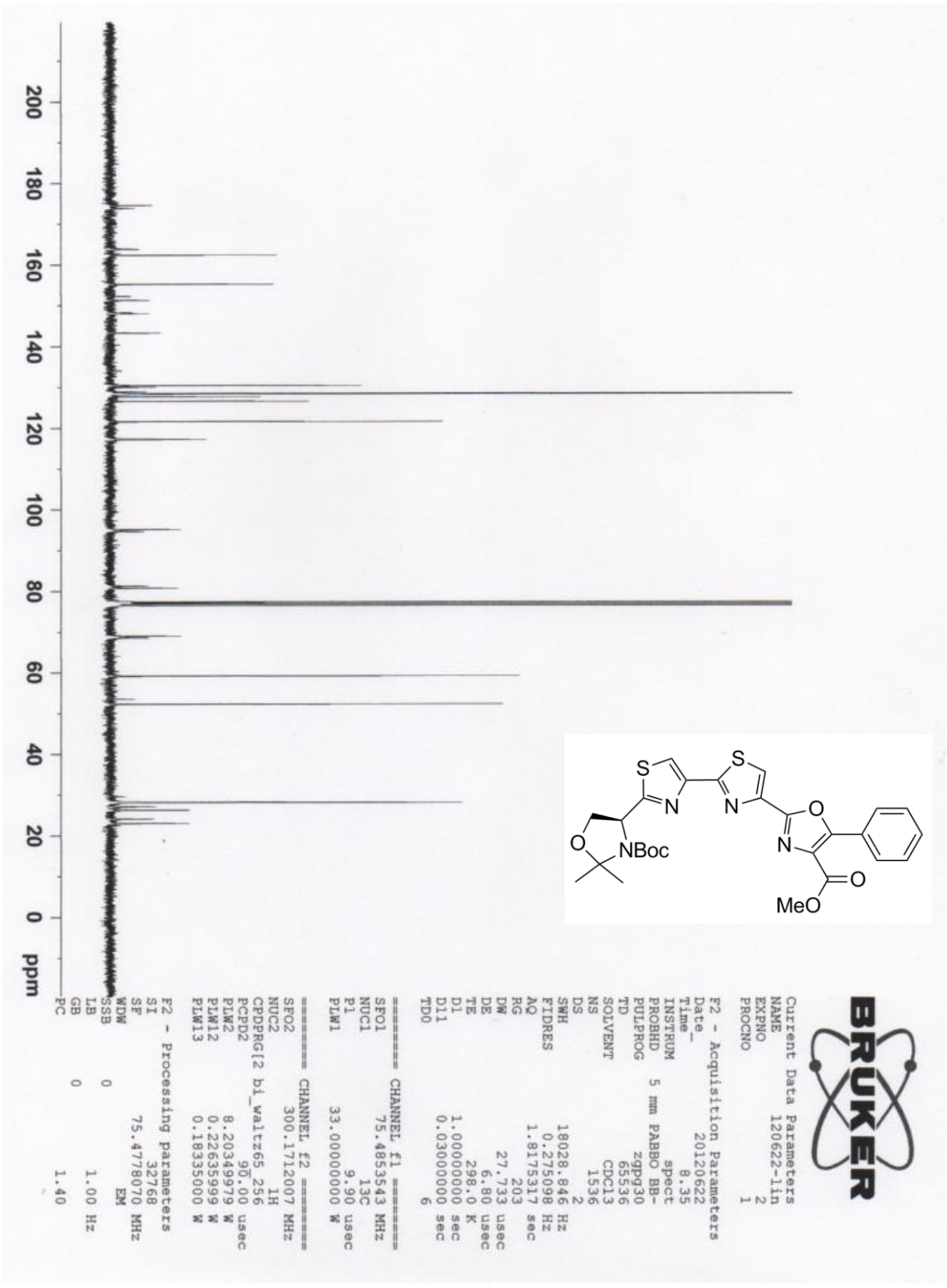
^{1}H NMR Boc-Oxazolidine-Thiazole-CSNH$_2$



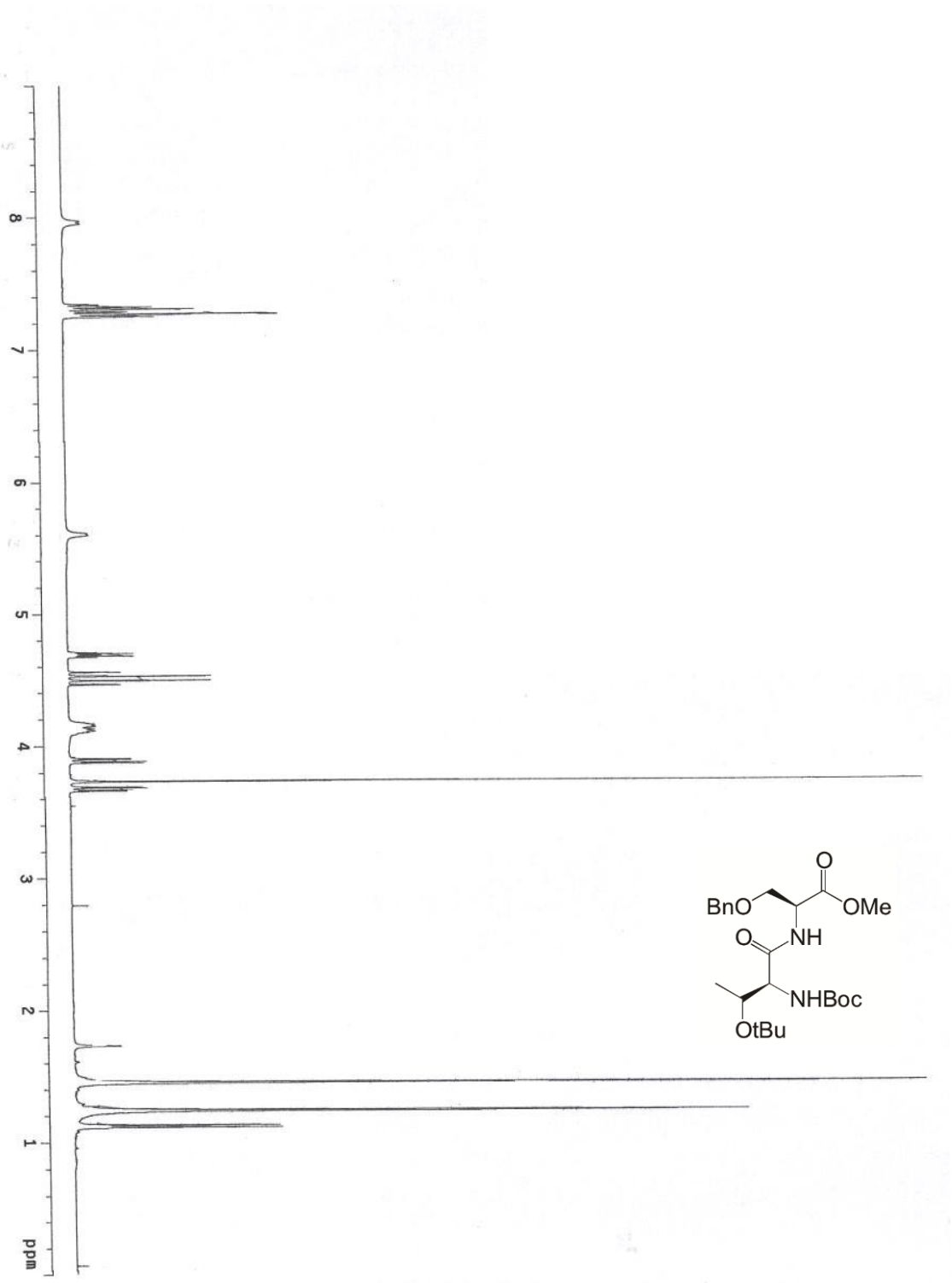
^{13}C NMR Boc-Oxazolidine-Thiazole-CSNH$_2$



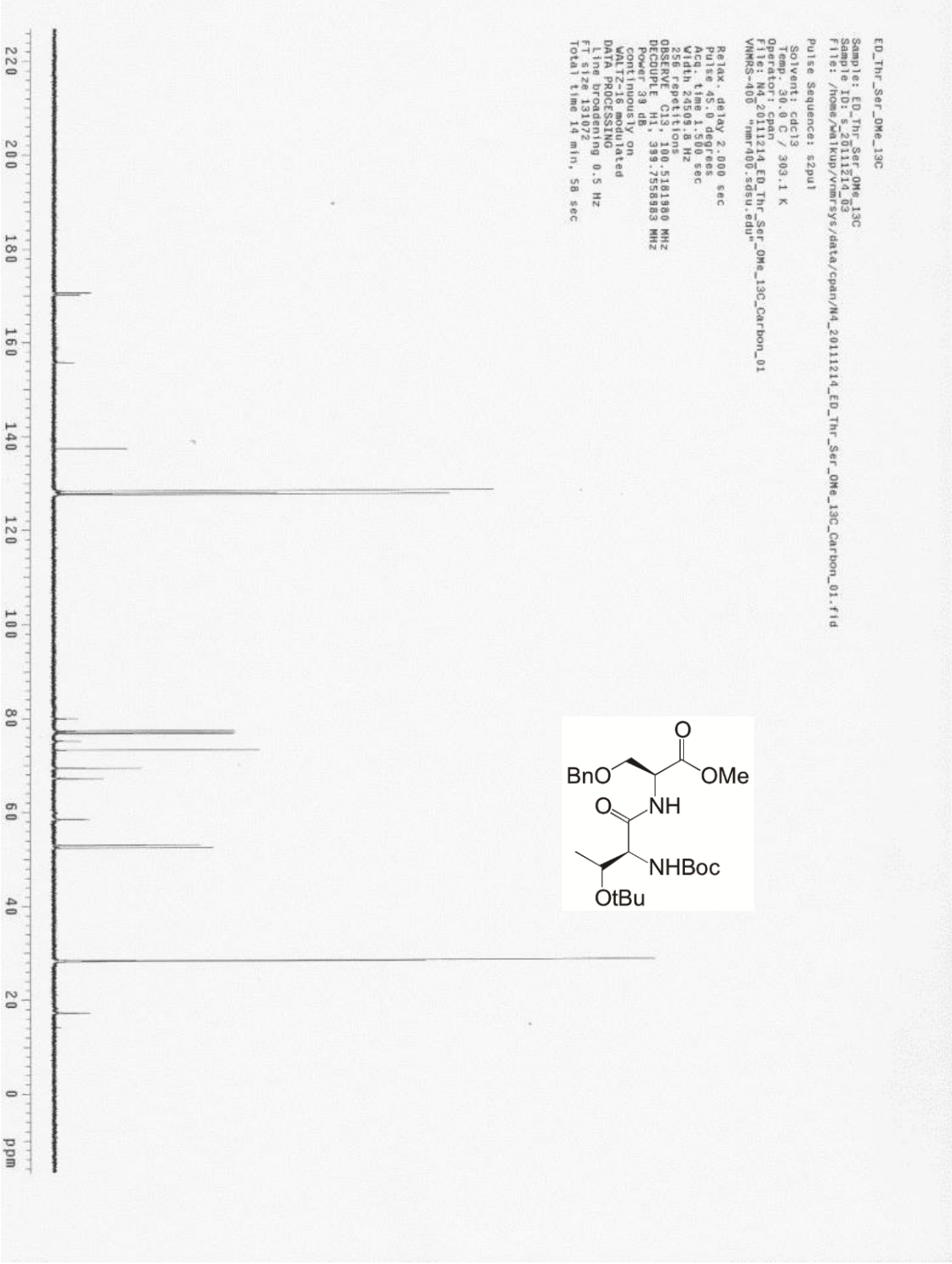
^{1}H NMR Boc-Oxazolidine-Dithiazole-Phenyloxazole-OMe



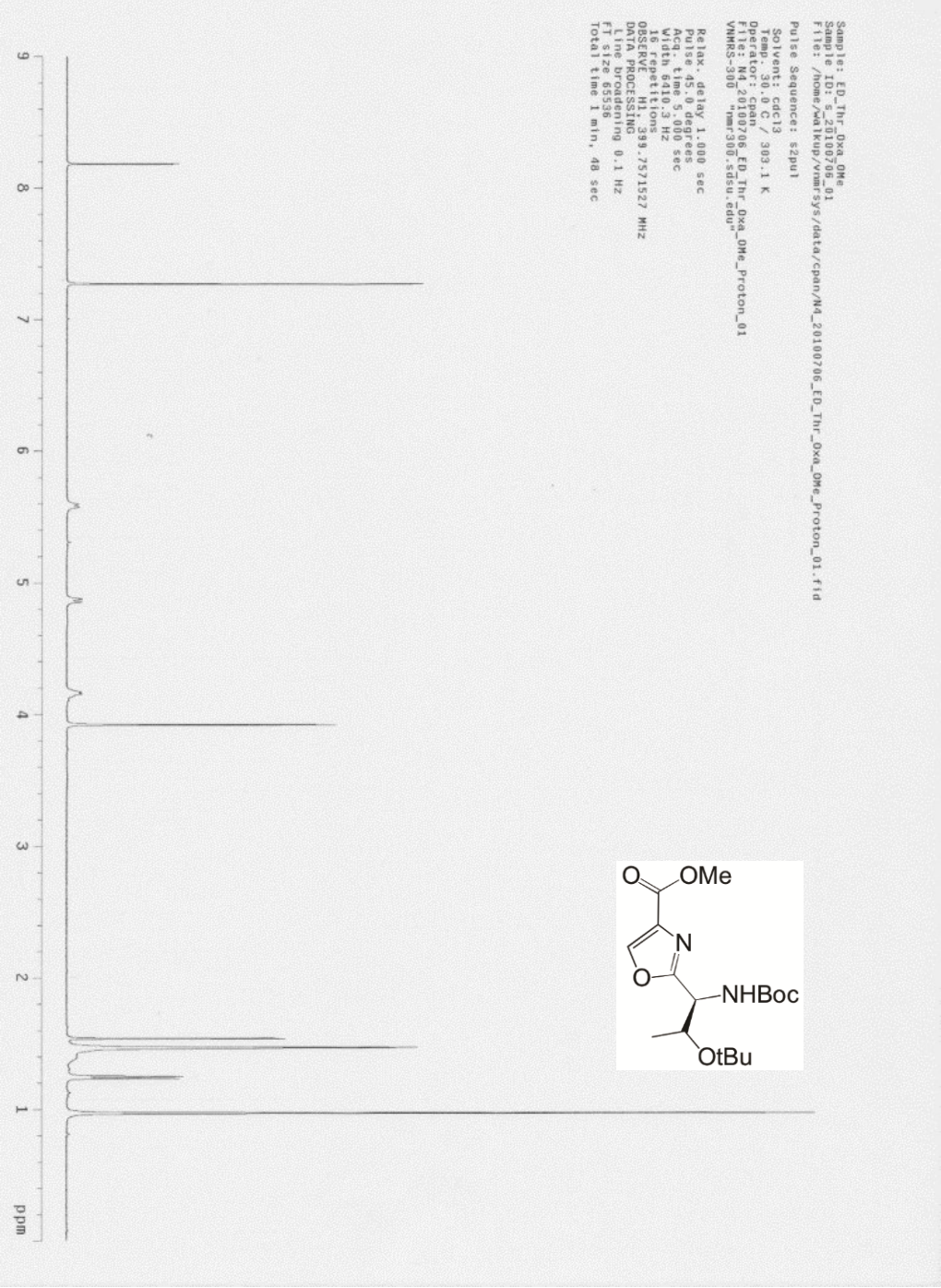
^{13}C NMR Boc-Oxazolidine-Dithiazole-Phenyloxazole-OMe



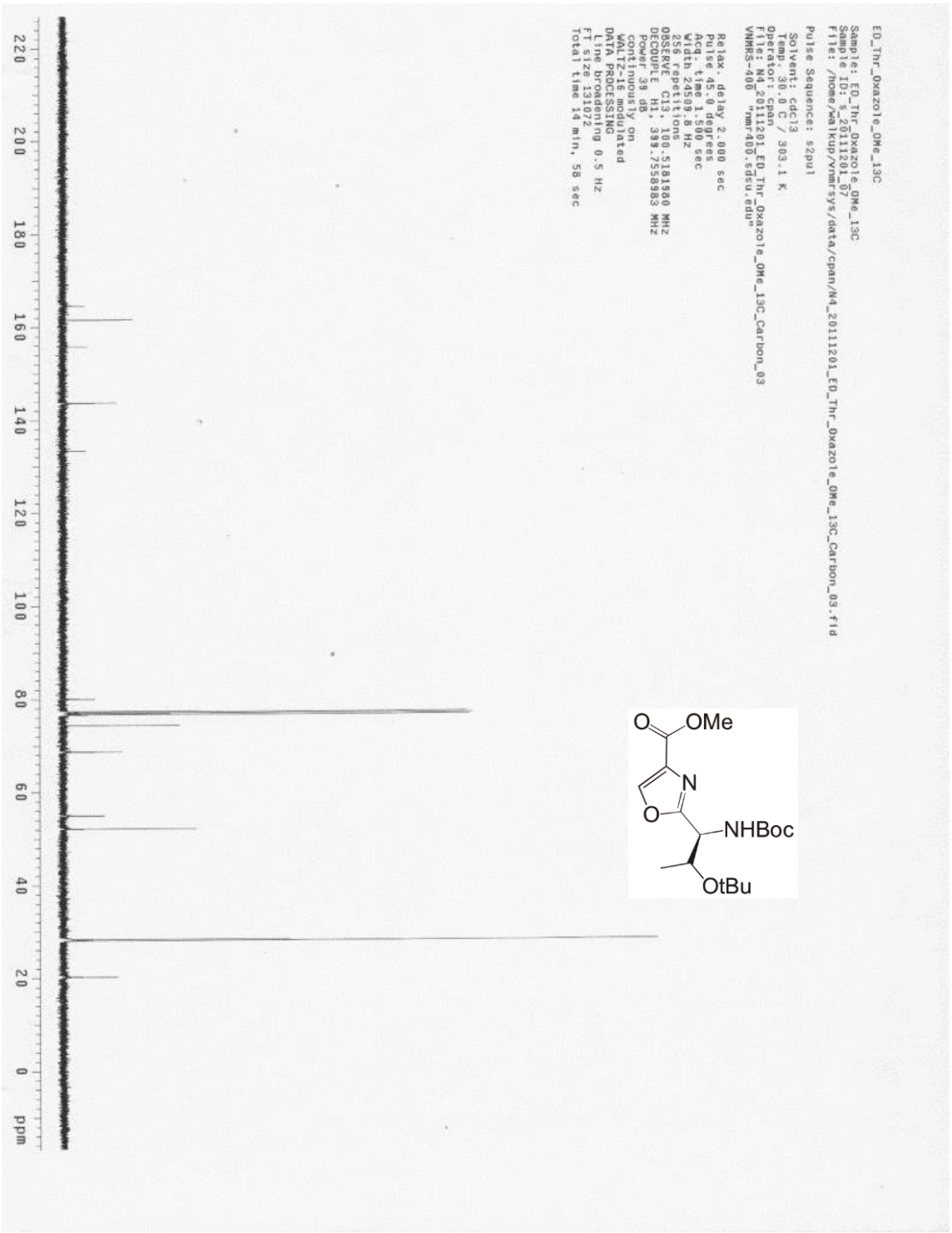
^{1}H NMR Boc-Thr(tBu)-Ser(Bn)-OMe



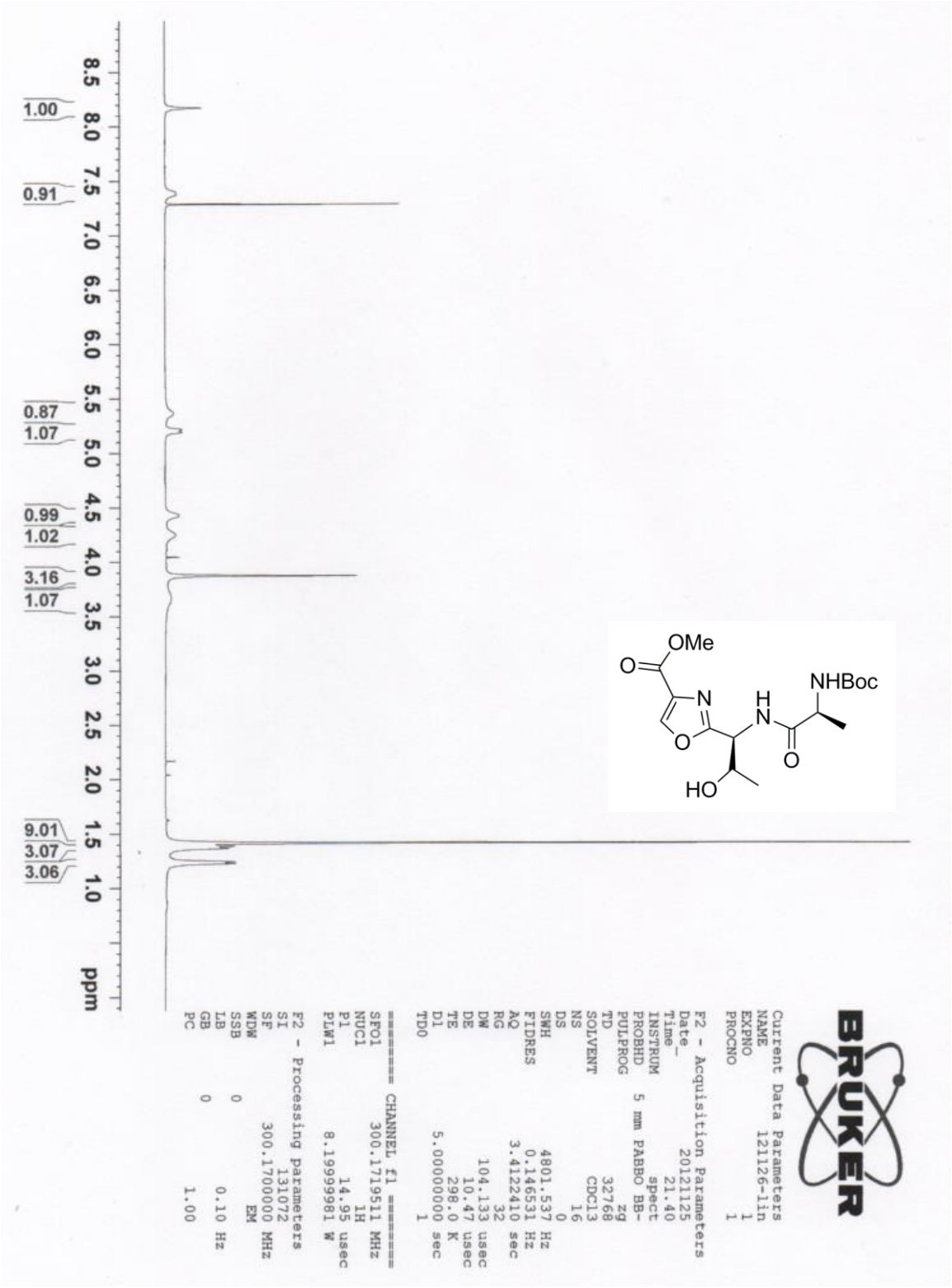
^{13}C NMR Boc-Thr(tBu)-Ser(Bn)-OMe



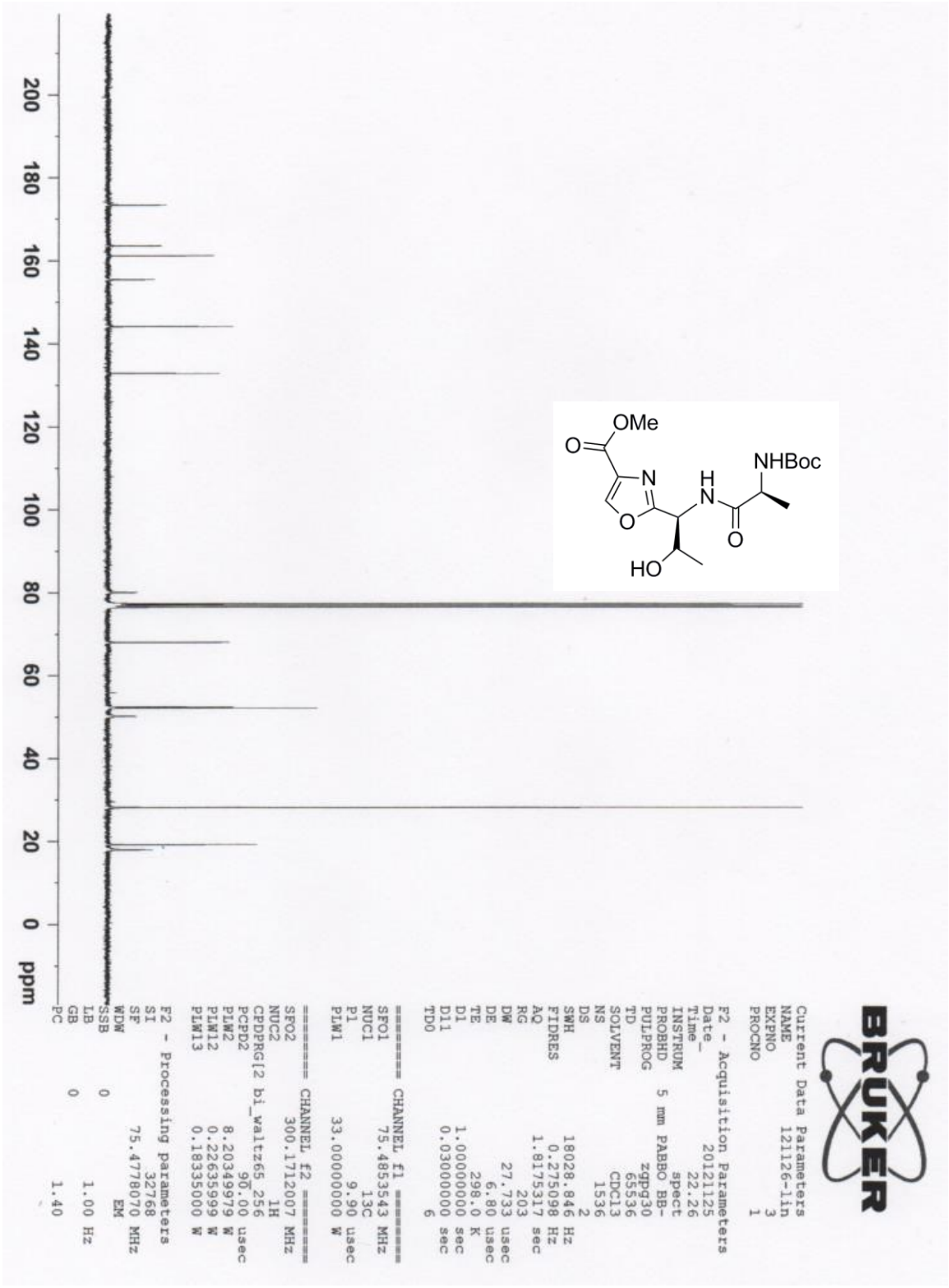
[1]H NMR Boc-Thr(tBu)-Oxazole-OMe



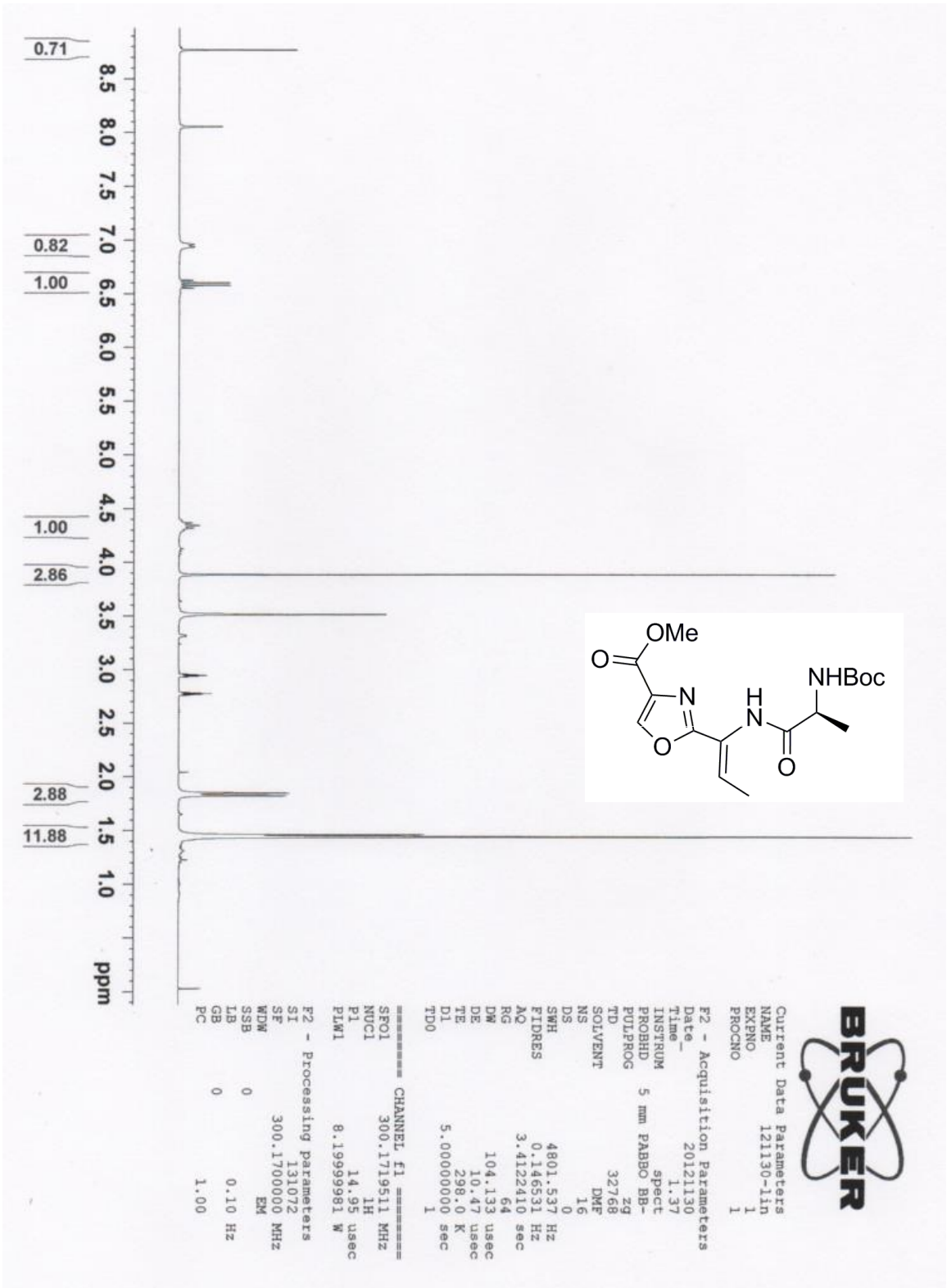
^{13}C NMR Boc-Thr(tBu)-Oxazole-OMe



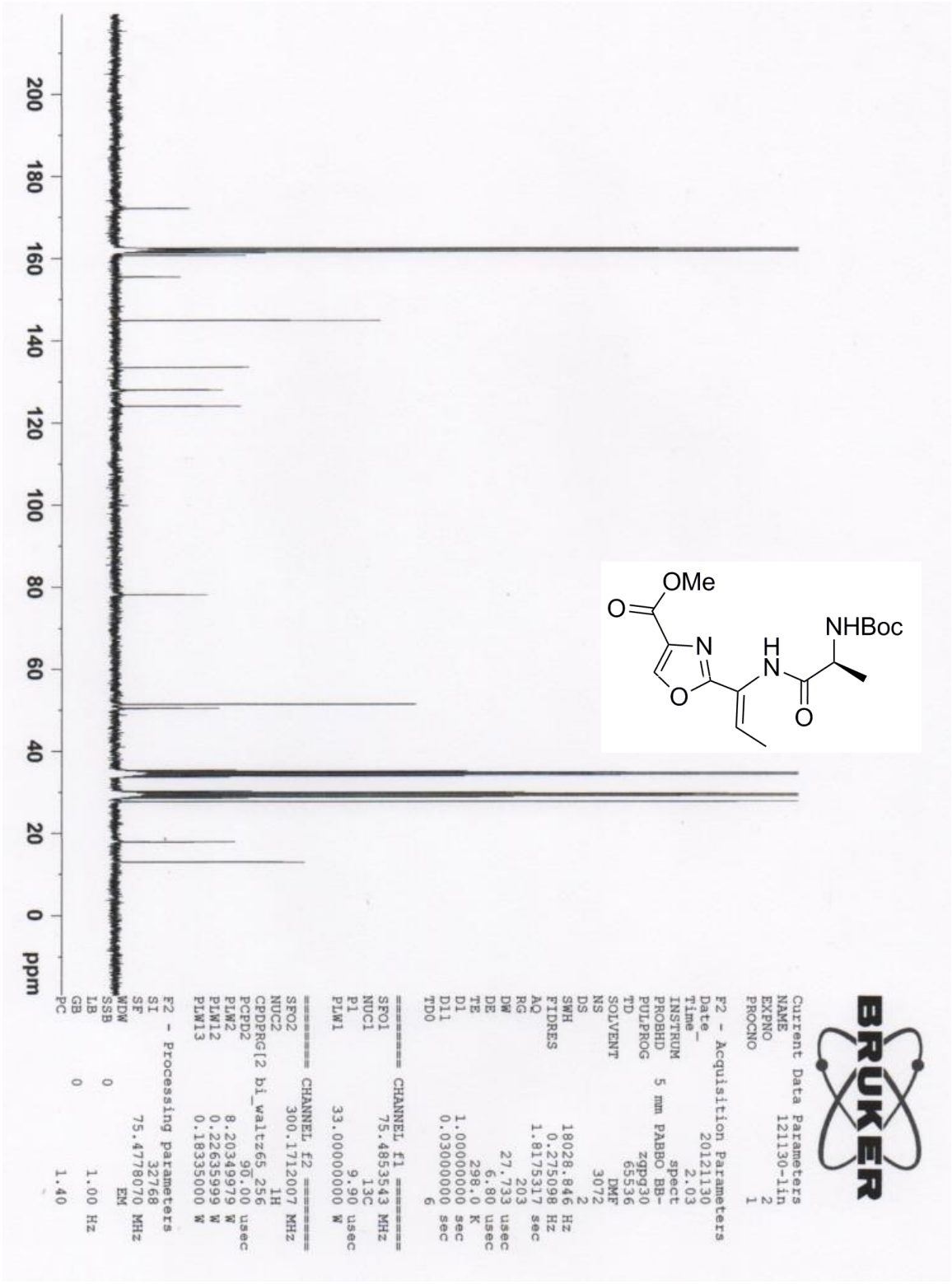
^{1}H NMR Boc-Ala-Thr-Oxazole-OMe



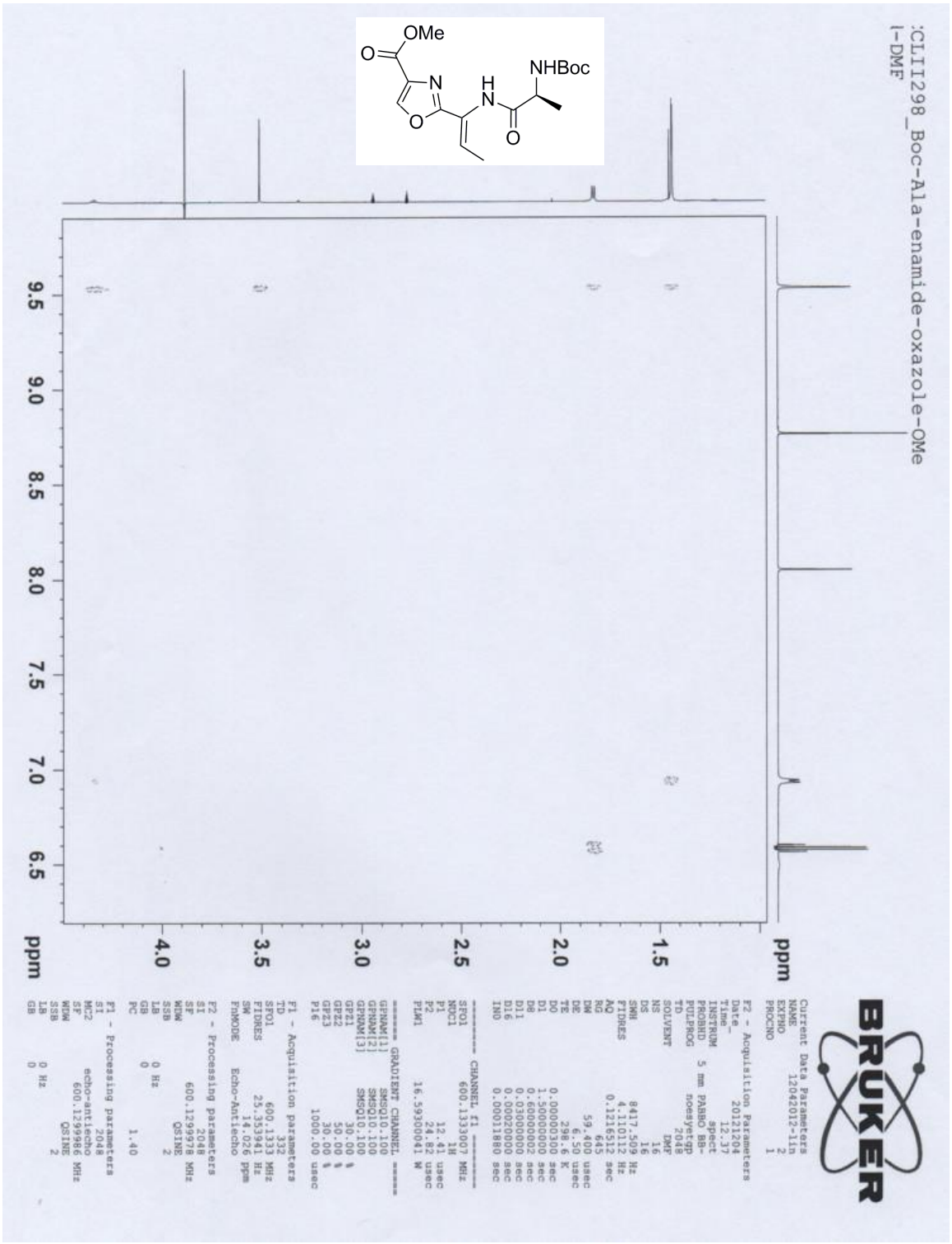
^{13}C NMR Boc-Ala-Thr-Oxazole-OMe



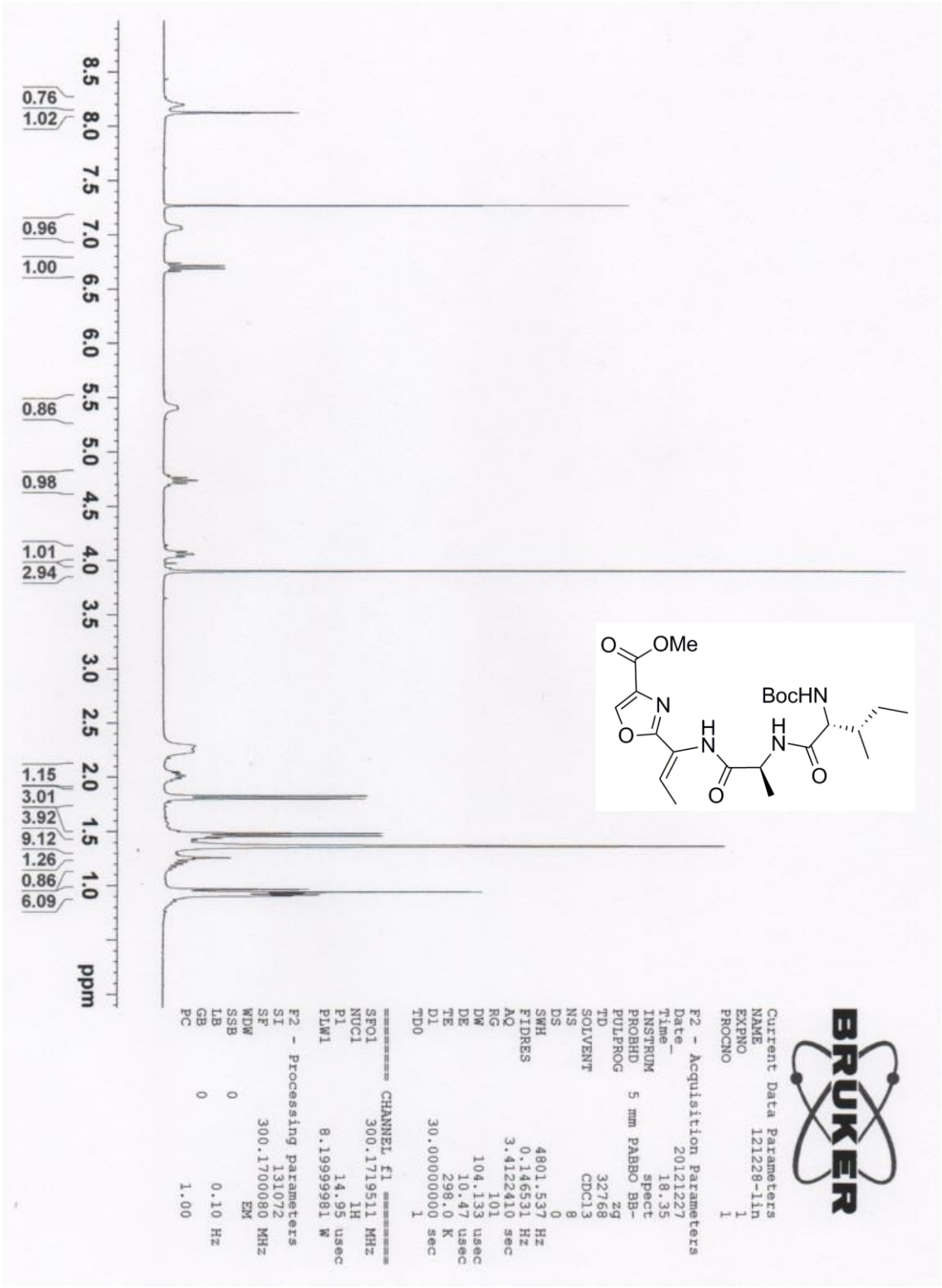
^{1}H NMR Boc-Ala-(*Z*)-Enamide-Oxazole-OMe



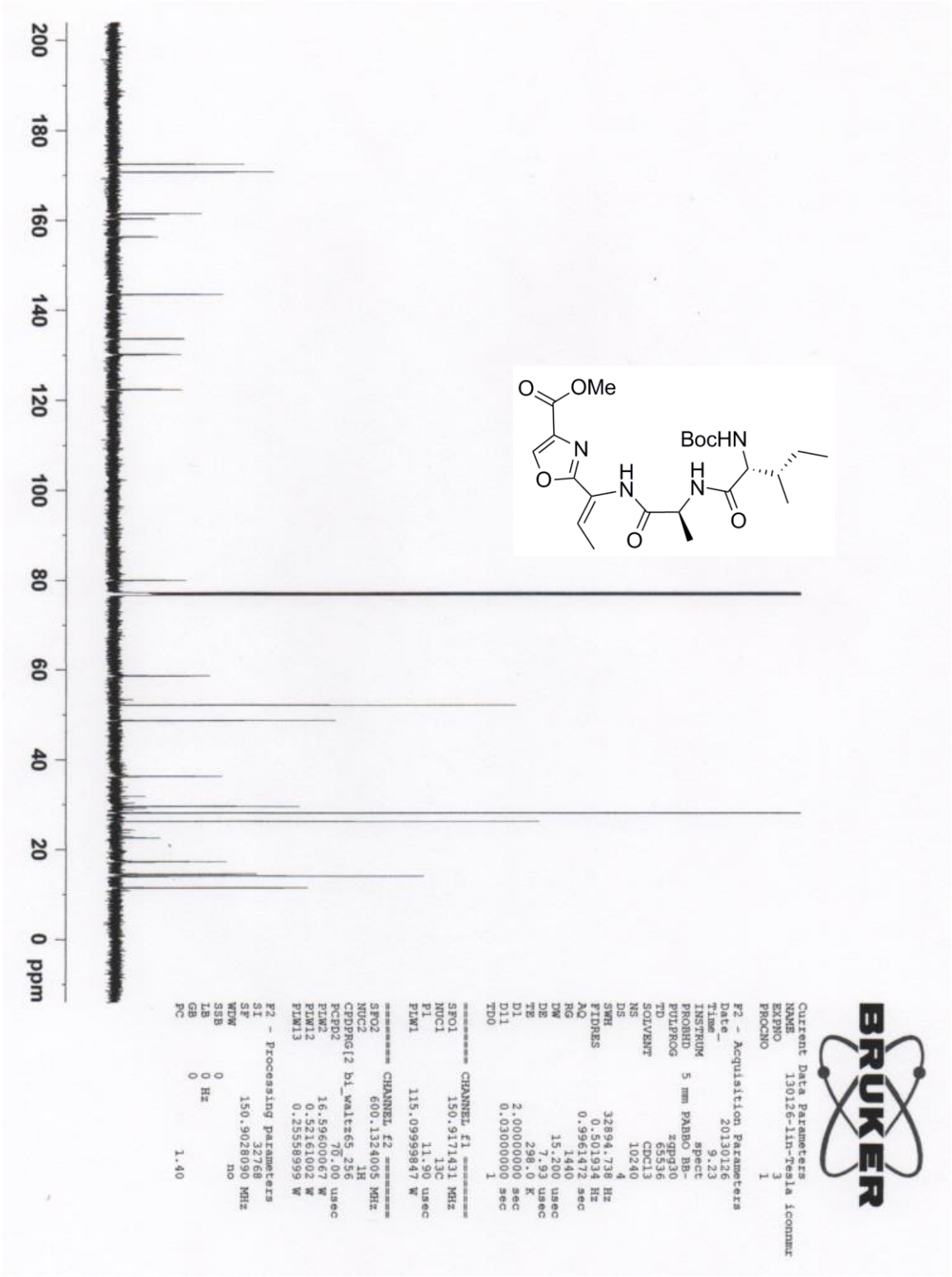
^{13}C NMR Boc-Ala-(*Z*)-Enamide-Oxazole-OMe



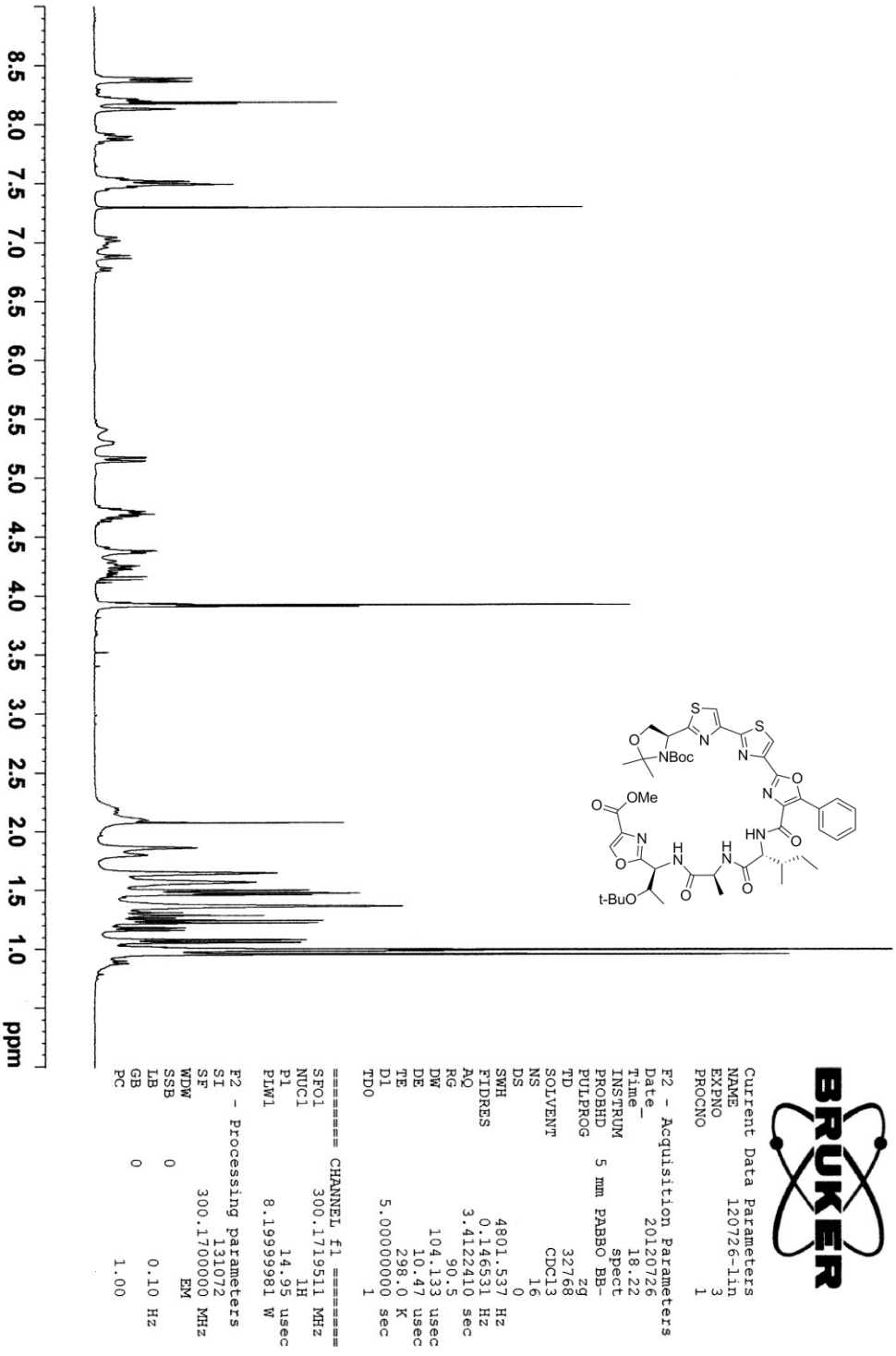
NOESY Boc-Ala-(*Z*)-Enamide-Oxazole-OMe



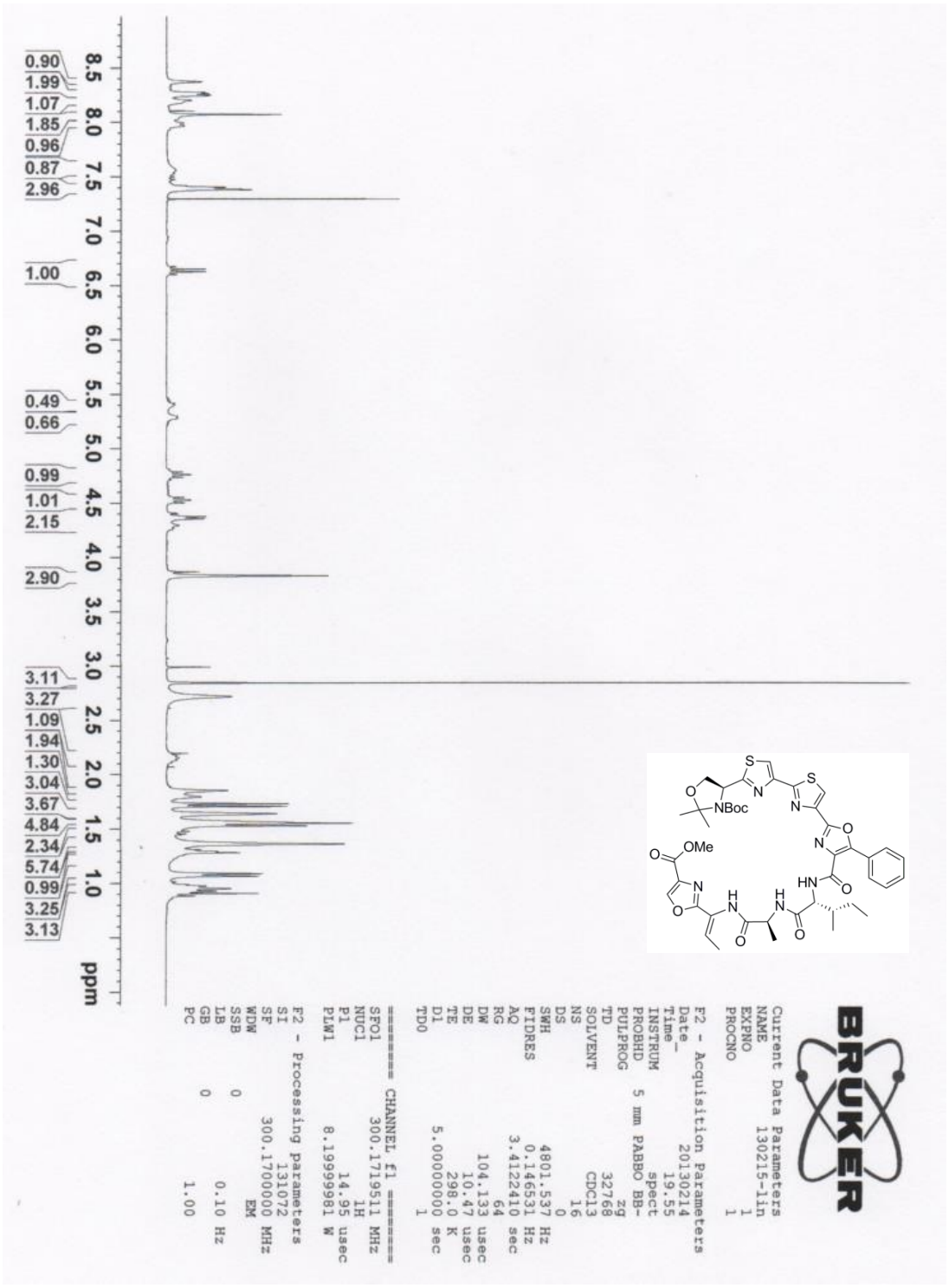
^{1}H NMR Boc-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OMe



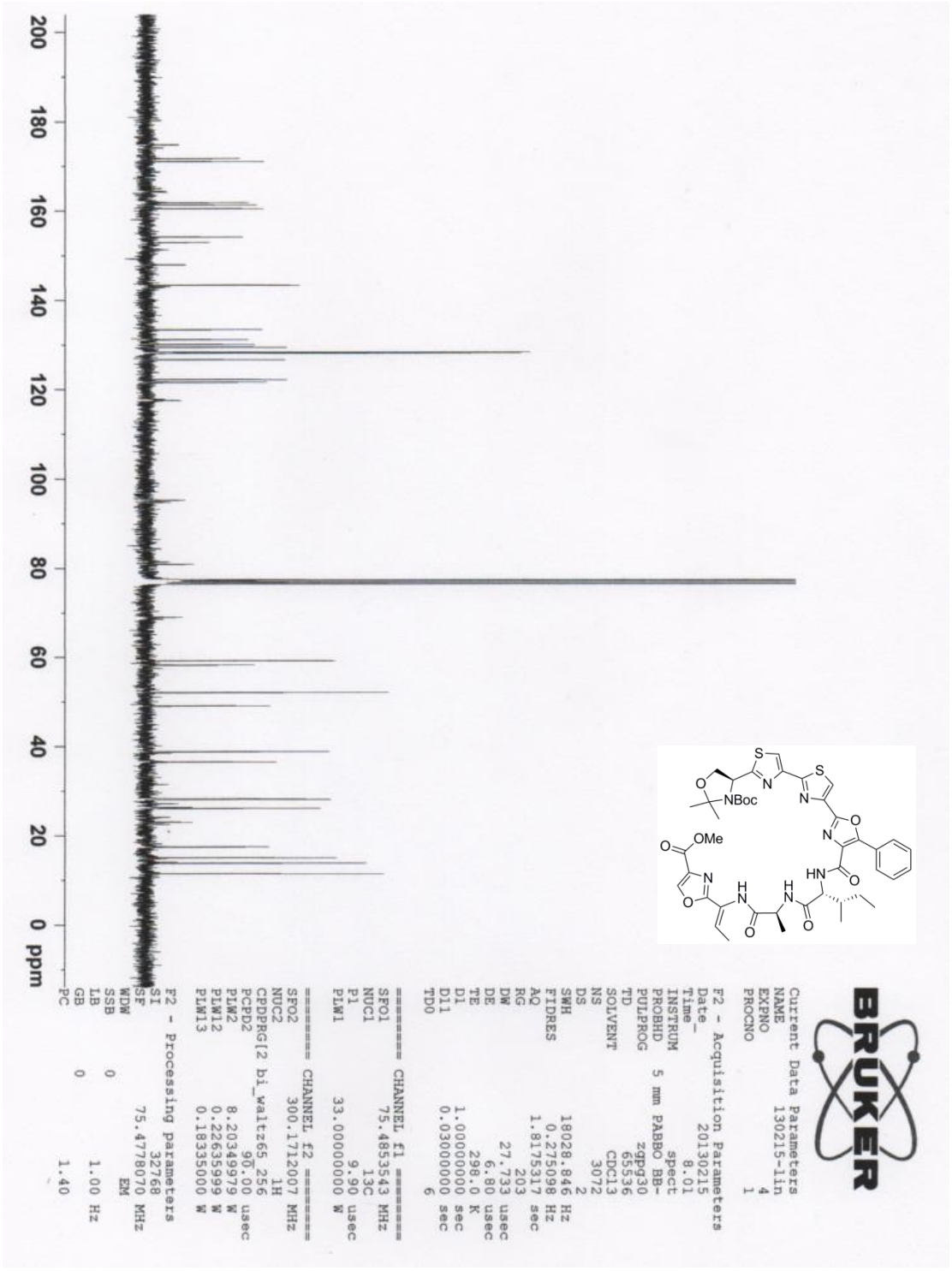
^{13}C NMR Boc-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OMe



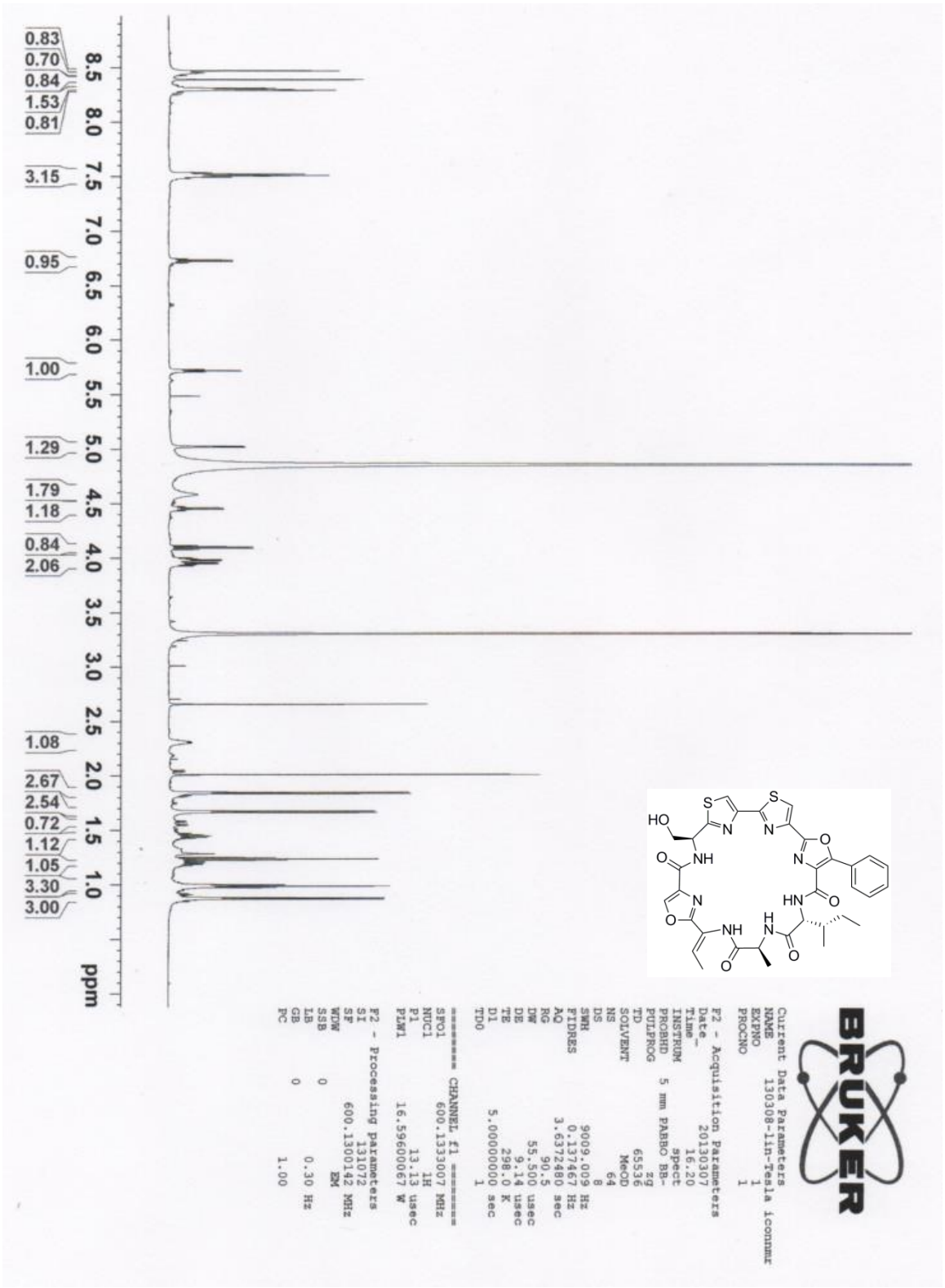
^{1}H NMR Boc-Oxazolidine-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-Thr(tBu)-Oxazole-OMe



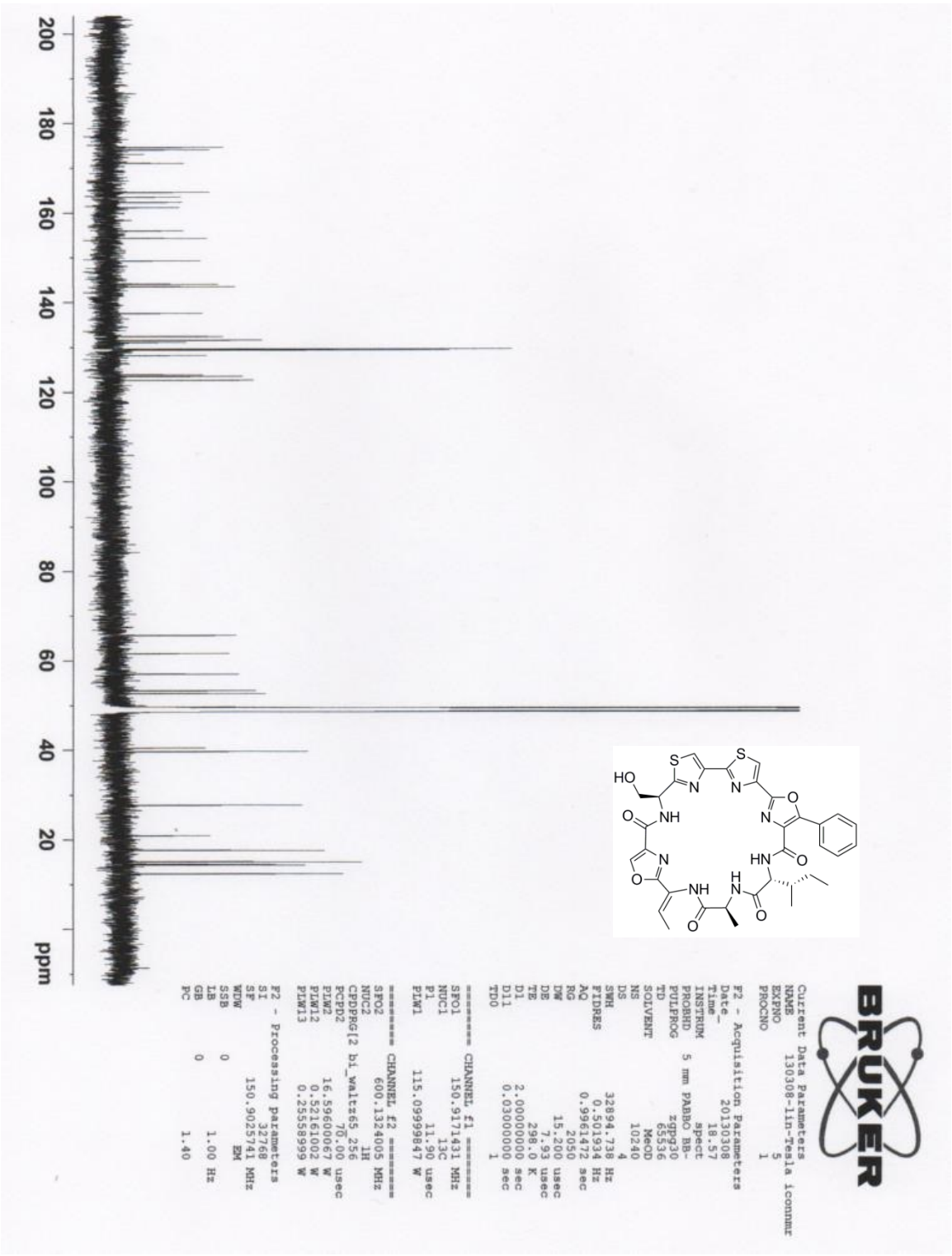
^{1}H NMR Boc-Oxazolidine-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OMe



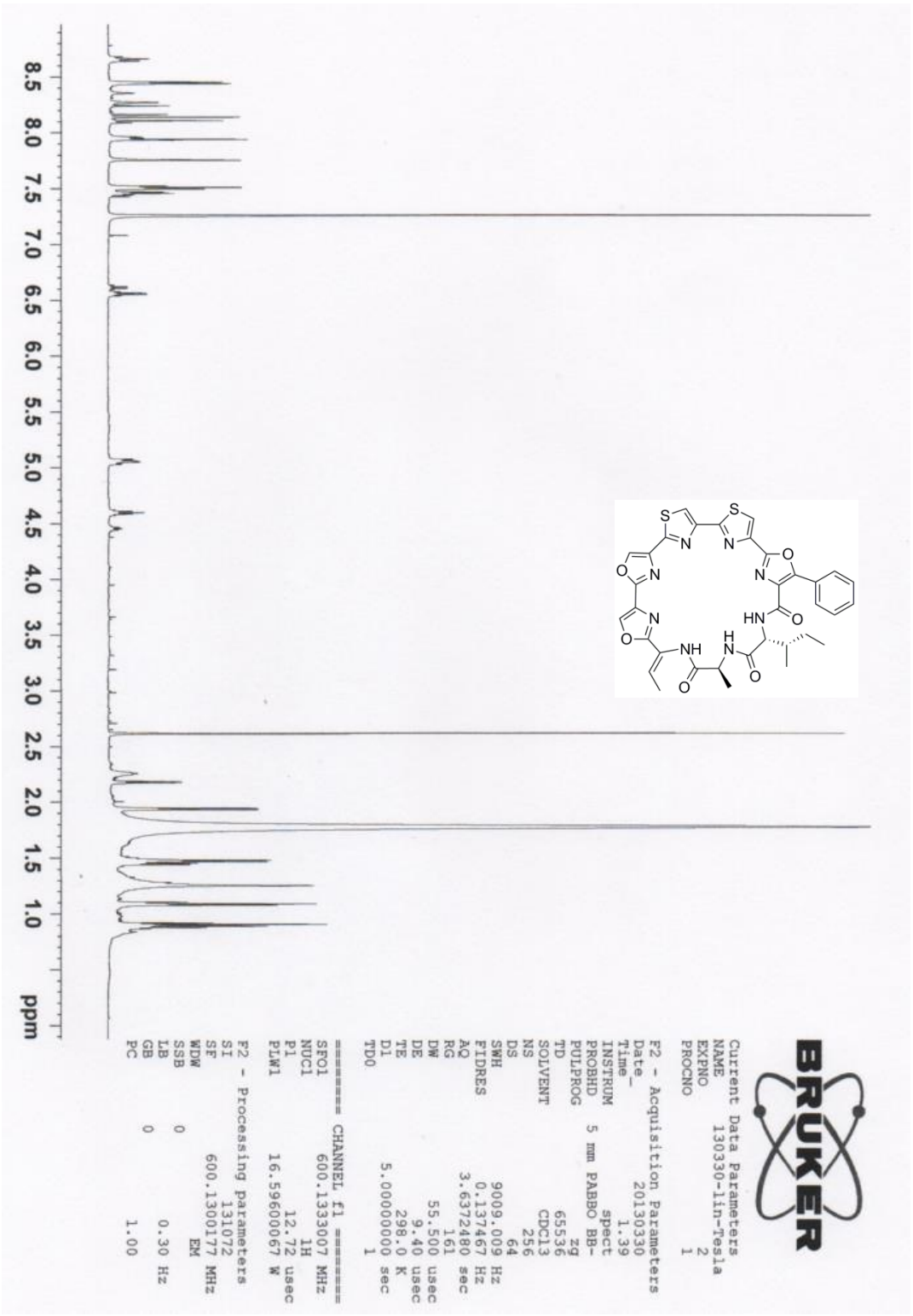
^{13}C NMR Boc-Oxazolidine-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole-OMe



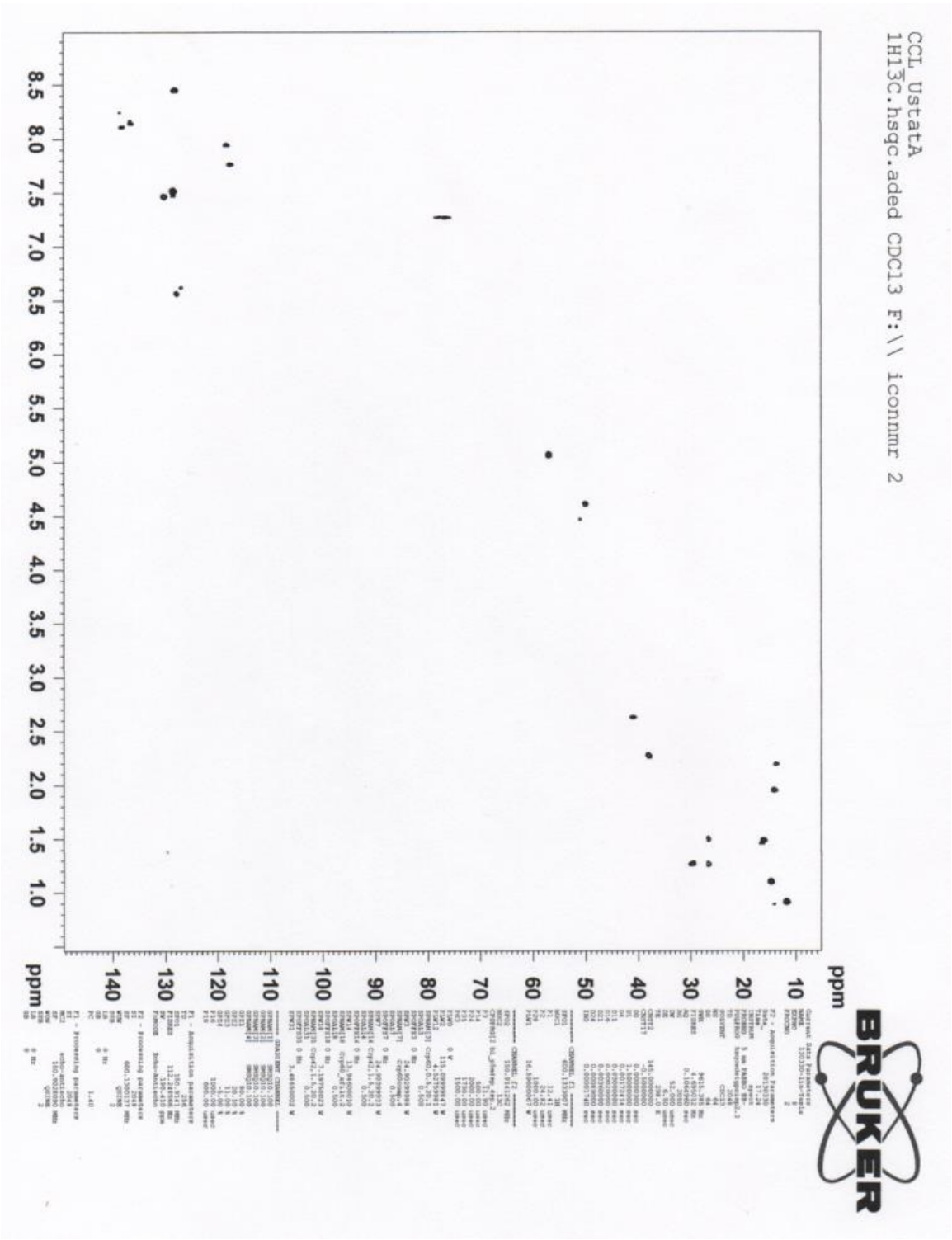
^{1}H NMR *cyclo*-Ser-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole



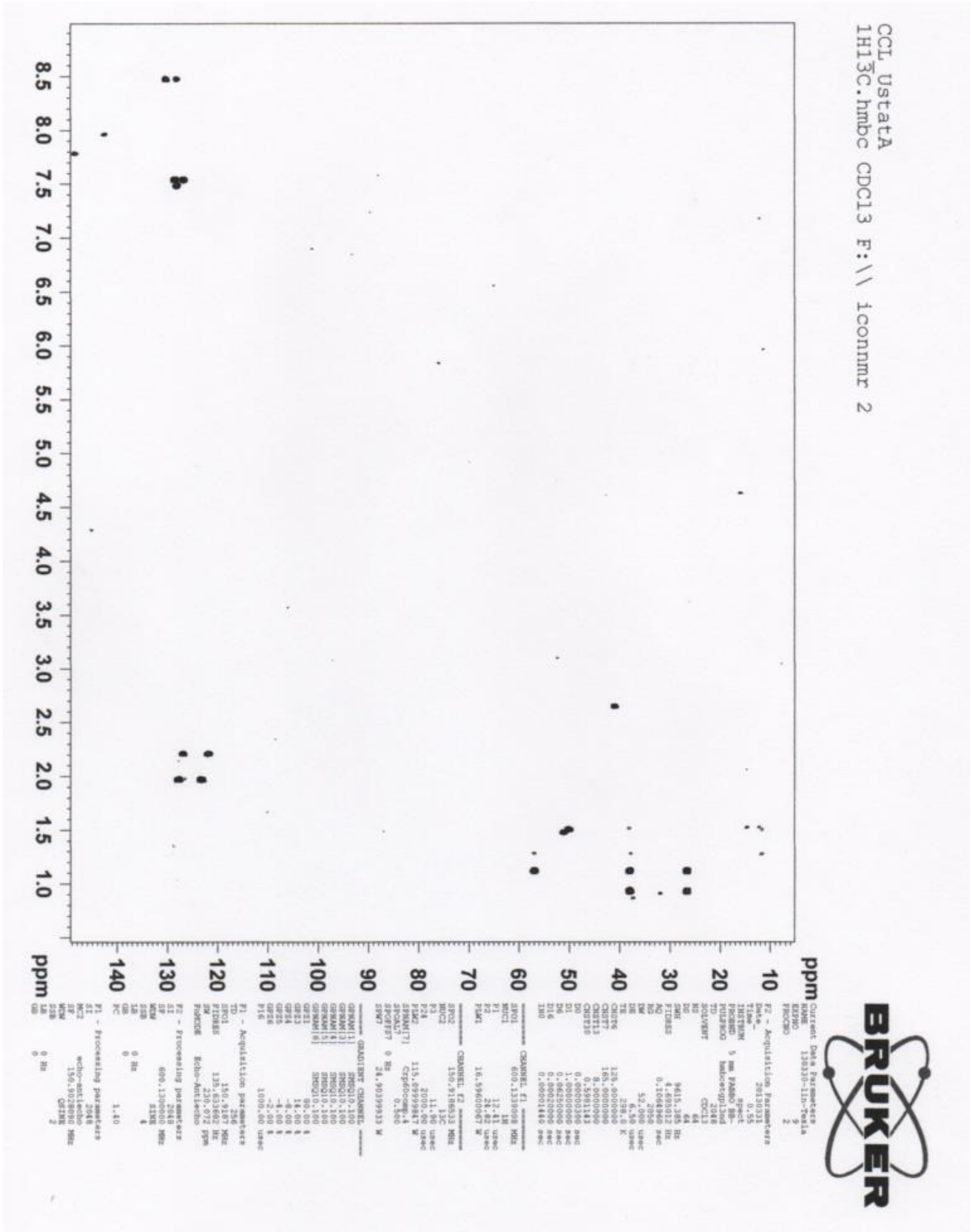
^{13}C NMR *cyclo*-Ser-Dithiazole-Phenyloxazole-D-*allo*-Ile-Ala-(*Z*)-Enamide-Oxazole



^{1}H NMR Ustat A-1 (*E*/*Z* mixture)



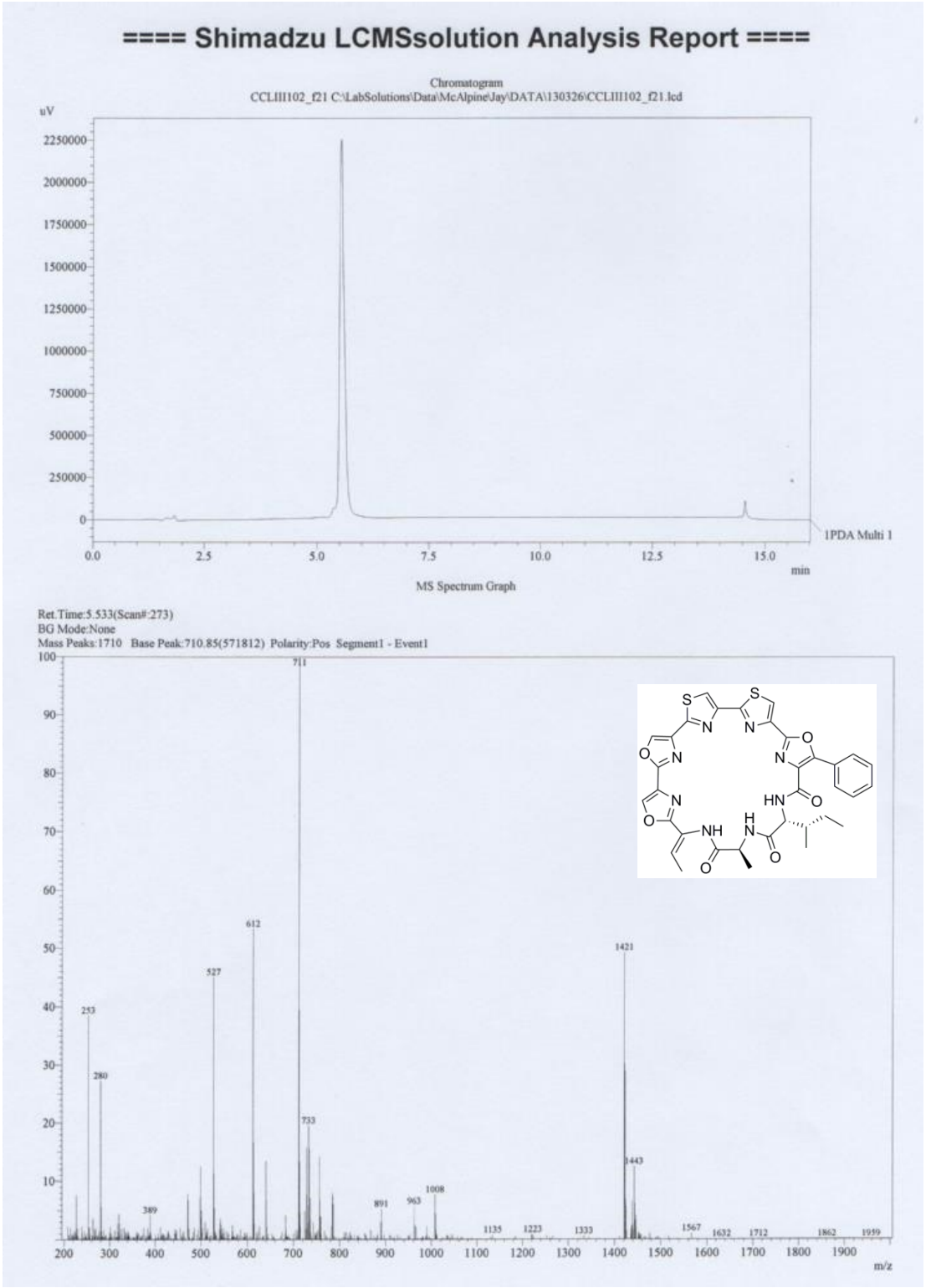
HSQC Ustat A-1 (*E*/*Z* mixture)



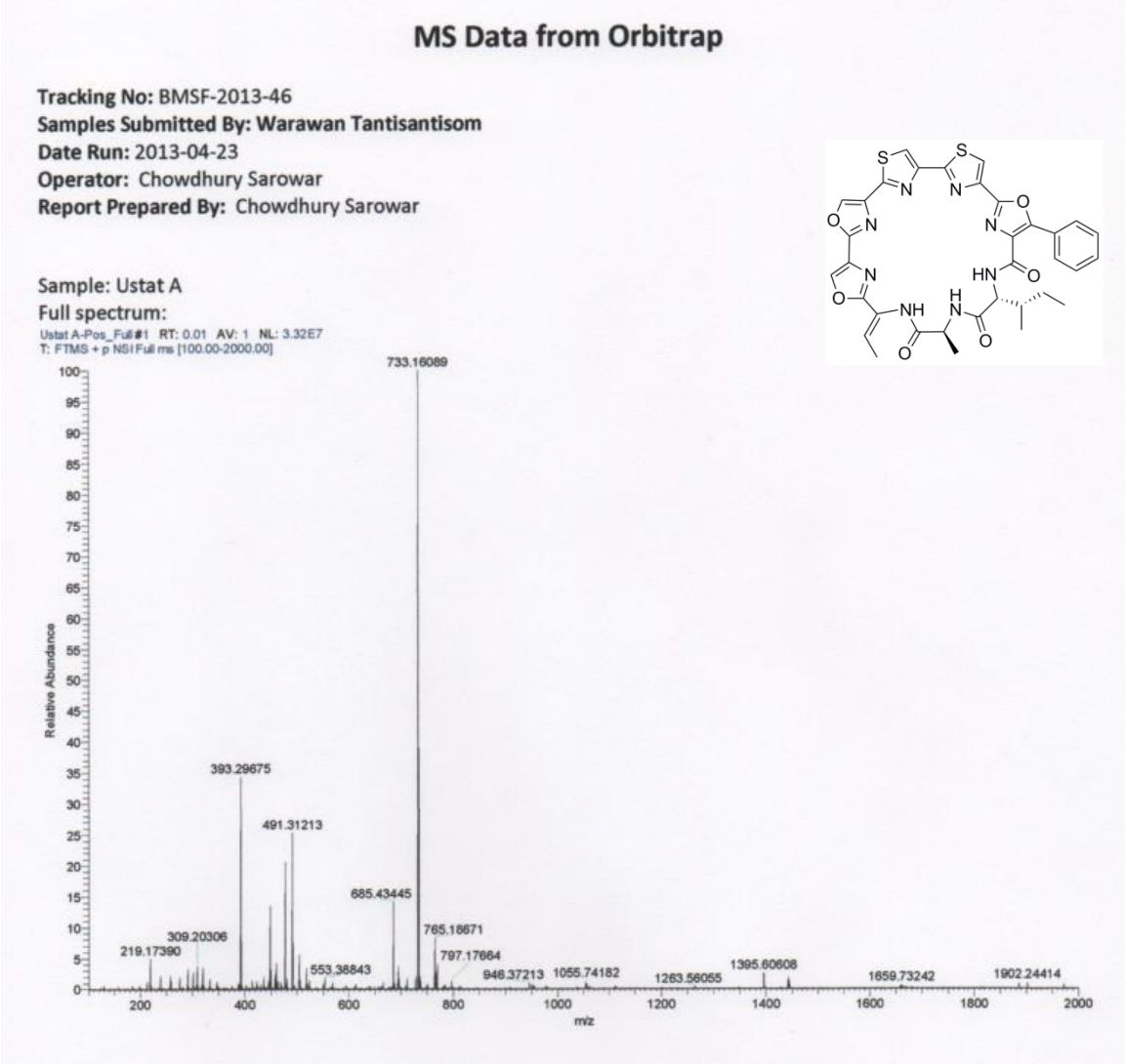
HMBC Ustat A-1 (*E*/*Z* mixture)

NOESY, X axis: 1.9-2.2 ppm, Y axis: 6.1-8.5 ppm

Ustat A-1 (*E*/*Z* mixture)



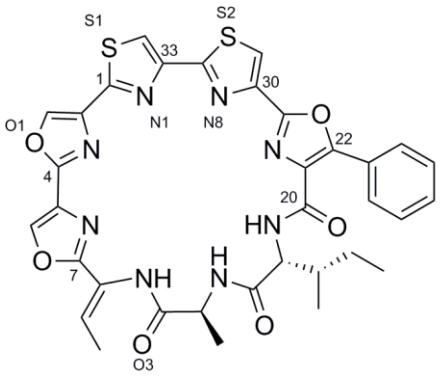
LCMS Ustat A-1 (*E*/*Z* mixture)



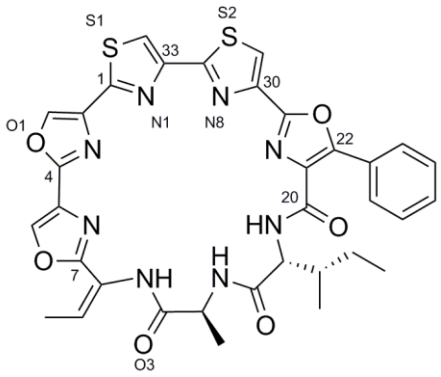
HRMS Ustat A-1 (*E*/*Z* mixture)

Appendix C – Tables of NMR Chemical Shifts for Ustat A-1

^{1}H NMR*



(*Z*)-**Ustat A-1**

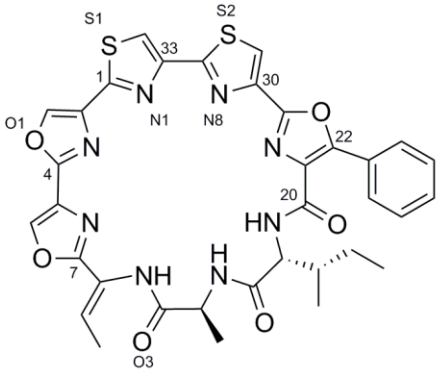


(*E*)-**Ustat A-1**

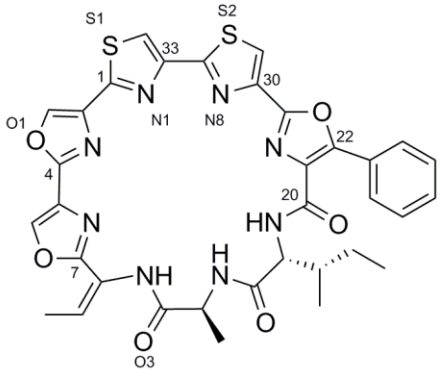
Position	Natural Product	(*Z*)-**Ustat A-1**	(*E*)-**Ustat A-1**	Position	Natural Product	(*Z*)-**Ustat A-1**	(*E*)-**Ustat A-1**
1				20			
2				21			
3	9.07, s	8.14, s	8.16, s	22			
4				23			
5				24	8.34, d, 7.5	8.45, d, 7.7	8.45, d, 7.7
6	8.88, s	8.11, s	8.24, s	25	7.55, dd, 6.8, 7.5	7.51, t, 7.5	7.46, t, 6.8
7				26	7.51, dd, 6.8, 6.8	7.51, t, 7.5	7.46, t, 6.8
8				27	7.55, dd, 6.8, 7.5	7.51, t, 7.5	7.46, t, 6.8
9	6.62, q, 7.5	6.56, q, 7.2	6.62, q, 7.6	28	8.34, d, 7.5	8.45, d, 7.7	8.45, d, 7.7
10	1.82, d, 7.5	1.94, d, 7.1	2.18, d, 7.7	29			
11				30			
12	4.75, dq, 6.8, 7.5	4.60, p, 7.0	4.46, p, 6.8	31	8.69, s	7.94, s	7.93, s
13	1.50, d, 6.8	1.48, d, 7.2	1.45, d, 7.3	32			
14				33			
15	4.52, dd, 8.3, 9.0	5.06, dd, 11.2, 4.7	5.04, dd, 12.0, 4.8	34	8.59, s	7.75, s	7.76, s
16	2.06, m	2.26, m	2.26, m	N4-H	9.49, br s	8.27, br s	8.36, br s
17	0.85, d, 6.8	1.08, d, 7.1	1.10, d, 7.9	N5-H	8.75, br d, 7.5	7.96, br d, 6.6	8.09, br
18	1.09, m, 1.46, m	1.49, m, 1.26, m	1.26, m, 1.31, m	N6-H	9.00, br	8.65, br d, 11.0	8.67, br d, 11.5
19	0.86, t, 7.5	0.91, t, 7.5	0.88, t, 7.4				

*Data for natural product from: Matsuo, Y.; Kanoh, K.; Imagawa, H.; Adachi, K.; Nishizawa, M.; Shizuri, Y. *J. Antibiot.* **2007**, *60*, 256-260.

^{13}C NMR*



(*Z*)-**Ustat A-1**



(*E*)-**Ustat A-1**

Position	Natural Product	(*Z*)-**Ustat A-1**	(*E*)-**Ustat A-1**	Position	Natural Product	(*Z*)-**Ustat A-1**	(*E*)-**Ustat A-1**
1	157.26	157.7	157.7	18	25.24	26.6	29.7
2	135.50			19	11.09	11.7	14.2
3	139.43	136.6	136.6	20	159.95		
4	155.11	156.6		21	130.52	130.2	130.2
5	129.56			22	151.00	153.1	153.1
6	140.12	138.3	138.6	23	126.64	126.9	126.9
7	159.65	160.3		24	127.78	128.1	128.1
8	123.70	123.2	121.9	25	128.30	128.6	128.6
9	128.78	127.8	126.9	26	129.84	130.3	130.2
10	13.38	14.1	13.7	27	128.30	128.6	128.6
11	170.30	171.7	172.3	28	127.78	128.1	128.1
12	48.13	50.0	51.0	29	154.02		
13	19.80	16.1	16.3	30	141.93	142.6	142.6
14	169.45			31	123.05	118.4	118.4
15	58.45	57.0	57.0	32	161.08	161.4	161.4
16	36.92	37.9	37.9	33	147.79	148.4	148.4
17	15.09	14.8	14.8	34	121.06	117.7	117.7

*Data for natural product from: Matsuo, Y.; Kanoh, K.; Imagawa, H.; Adachi, K.; Nishizawa, M.; Shizuri, Y. *J. Antibiot.* **2007**, *60*, 256-260.