Design, synthesis, and evaluation of pentapeptides that inhibit the function of heat shock protein 70

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Design, synthesis, and evaluation of pentapeptides that inhibit the function of heat shock protein 70

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Thesis/Dissertation Sheet

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Abstract 350 words maximum: (PLEASE TYPE)

Protein-protein interactions (PPIs) regulate the cell's protein-folding machinery, which relies on a multiprotein complex formed with heat shock protein 70(Hsp70). Hsp70 is a molecular chaperone responsible for partially folding nascent peptides and refolding misfolded proteins, whereupon it transfers the partially folded proteins to Hsp90. The transfer process is mediated by the co-chaperone heat shock organising protein (HOP). HOP binds to Hsp70 via its tetratricopeptide repeat 1 (TPR1) domain. Hence, the PPI between HOP and Hsp70 is critical for the Hsp70's function. This interaction is particularly important in cancer cells, where Hsp70 is overexpressed to fold the rapidly produced proteins and facilitate cancer growth.

This thesis describes the de-novo design, synthesis and biological evaluation of molecules aimed to regulate the interaction between Hsp70 and HOP. Peptides were designed based on the sequence of HOP's TPR1 domain. The aim was to mimics the HOP interaction with Hsp70 and inhibits Hsp70's function by modulating Hsp70-HOP interaction. The author synthesized seven molecules in this series. Using solid phase peptide synthesis, the seven peptides were purified by HPLC and verified by LCMS, ^{1}H NMR and 2D NMR. These compounds were then tested in a binding assay and a functional luciferase refolding assay. A structurally unique Hsp70 inhibitor, C1, was identified as lead molecule, which is the first molecule to directly regulate PPI and inhibit protein folding events.

The author then synthesized tagged version of C1 and tested in a protein pulldown assay against Hsp70 to assess its ability to bind to Hsp70. To identify the domain binding site, a pulldown assay was run using the C1-Tag against Hsp70's substrate binding domain (SBD). The author demonstrated that C1 bound to Hsp70 at the SBD. Finally, a structure-activity relationship study on C1 was carried out by producing molecules to perform an alanine scan. C1 is a pentapeptide, and each amino acid residue was substituted for alanine in the backbone. Of the five derivatives produced, the author synthesized three.

This project is demonstrating a proof of a successful approach for designing new small molecules that will modulate PPIs and interfere in large dynamic protein complexes.

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°C	Degrees Celsius
Å	Ångström; 10^{-10} m
ADP	Adenosine diphosphate
Ala, A	Alanine
ATP	Adenosine triphosphate
Boc	Di-tert-butyl dicarbonate
D	Doublet
D2O	Deuterium oxidexxxi
Da	Dalton
DCM, CH_2Cl_2	Dichloromethane
dd	Doublet of doublet
DIC	N,N′-diisopropylcarbodiimide
DIPEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DMTMM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4- methylmorpholinium

	chloride
DPBS	Dulbecco‘s Phosphate Buffer Saline
eq.	Equivalents
Fmoc	Fluorenomethoxycarbonyl
g	Grams
Gln, Q	Glutamine
Glu, E	Glutamic acid
Gly, G	Glycine
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HCl	Hydrochloric acid
His,	H Histidine
HOAt	1-hydroxy-7-azabenzotriazole
HOP	Heat shock organizing protein
HPLC	High performance liquid chromatography
hr	Hour
Hs	Heat shock
Hsp	Heat shock protein
kDa	KiloDalton

L	Liter
LC/MS	Liquid chromatography mass spectrometry
Leu, L	Leucine
LP	Linear protected
Lys, K	Lysine
m	Multiplet
M	Molar
m/z	Mass-to-charge ratio
MeOH	Methanol
Met, M	Methionine
mg	Milligrams
min	Minute
mL	Milliliter
mmol	Millimole
MW	Molecular weight
NaCl	Sodium chloride
nM	Nanomolar
OMe	Methyl ester
PEG	Polyethylene glycol
Phe, F	Phenylalanine

Ppm	Parts per million
Pro, P	Proline
PVDF	Polyvinylidene fluoride
q	Quartet
RP-HPLC	Reversed phase-high performance liquid chromatography
Rt	Room temperature
s	Singlet
SAR	Structure-activity relationship
Ser, S	Serine
SPPS	Solid-phase peptide synthesis
t	Triplet
Tag	PEG biotin
TBST	Tris buffered saline with 0.1% Tween-20
TBTU	O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate
t-Bu	tert-Butyl
TFA	Trifluoroacetic acid
TPR	Tetratricopeptide repeat
UV	Ultraviolet
Val, V	Valine

v:v	Volume to volume
µL	Microlitres
µM	Micromolar
µm	Micrometre
Δ	Difference
α	Alpha
β	Beta
γ	Gamma
δ	Delta

Acknowledgements

Firstly, I would like to express my sincere appreciation to my supervisor A/Professor Shelli McAlpine. Thank you for always support me. You have been an outstanding inspiration to me. Without your guidance, motivation and support, none of this would have been possible. Thank you from the bottom of my heart.

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Yuantao Huo

September 2018

Abstract of the thesis

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By

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Master of Science by Research in Chemistry

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School of Chemistry, Faculty of Science

The University of New South Wales

Australia

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Chapter 1: Introduction

1.1 Heat Shock Proteins (Hsps)

Heat shock proteins (Hsps) are a family of proteins that have been conserved throughout evolution and they are present in a diversity of species ranging from protozoa to humans. Members of the Hsp family, are classified according to their molecular weight in kilodaltons: Hsp90, Hsp70, Hsp40, and the small Hsp family (typically 20 to 27 kDa) (**Figure 1.1**).[1]

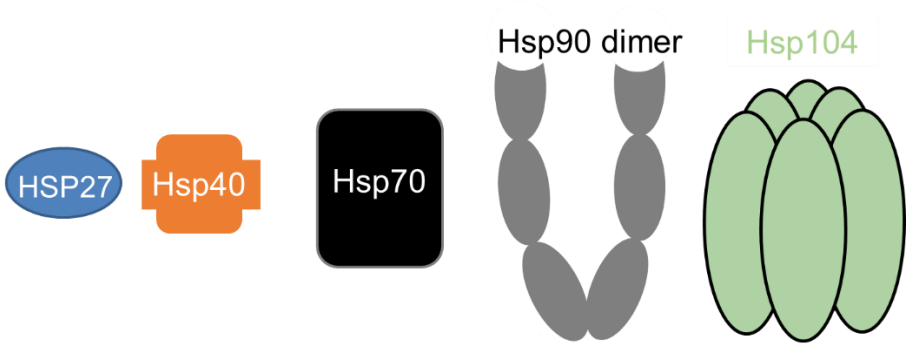


Figure 1.1: A schematic representation of the members of the heat shock protein family

Hsps were first discovered in the mid-1960s, and were identified in cells exposed to heat shock, where the levels of Hsps increase when cells are heated or under stress.[2] All normal cells contain basal levels of Hsps, and these basal levels perform the primary cellular function of folding of proteins, often referred to as client proteins, into their correct three-dimensional conformation.[3] Hsps are also referred to as molecular chaperones, and they perform a cytoprotective mechanism, where cells upregulate the expression of Hsps in response to physical and chemical stresses including heat shock, nutrient deprivation and oxidative conditions,[4] in order to ensure that under these stress conditions, the proteins are not being misfolded.[5] If misfolded proteins accumulate, they

aggregate and become insoluble, which leads to cell death.[6] Thus, Hsps are upregulated under stressor conditions in order to ensure aggregated client proteins are refolded or degraded. This function protects the cell from becoming overwhelmed with aggregated proteins, and can stop the cell from subsequently triggering apoptosis under these stressor conditions.[7]

The intracellular function of Hsps is not only restricted to protein refolding, other roles include: transport of functional proteins into specific subcellular compartments assembly of protein-protein complexes, and cell cycle control.[8] The Hsps are also central to regulating protein folding events, where these folding events are involved in many diseases, including cancer.[9] Hsps are overexpressed in cancer cells, as these cells are under high levels of stress due to rapid cell division and limited nutrients. The increased expression of Hsps are observed in many tumor types compared to adjacent normal tissues.[10] The rapid production of proteins, and increased cell division in cancer cells requires excess Hsps in order to ensure prompt refolding of mutant client proteins and overexpressed nascent proteins, all of which are involved in most aspects of tumor cell progression.[11] As such, the excess Hsps allow tumor cells to tolerate mutations that would otherwise lead to cell apoptosis.[12]

1.2 Heat shock protein 70 (Hsp70)

1.21 The functions of Hsp70

Heat Shock Protein 70 (Hsp70) is one of the most important members of the Hsp family.[13] In humans, Hsp70 usually exists as a monomeric protein under physiological conditions and is found in multiple parts of the cell including: the cytosol, nuclei,

endoplasmic reticulum, mitochondria,[14] as well as on the cell membrane and on the outer membrane of the cell.[15] Depending on its localisation, Hsp70 mediates different cellular functions.[16] Extracellular Hsp70 stays in a soluble form, complexed with antigenic peptides, and moderates the immune responses in the body.[17] Hsp70 could also be released from intact cells after stress in a membrane-associated form, which could act as a signal activator for other cells.[18] Intracellular Hsp70 guards cells against damages caused by multiple types of stress,[19] and it is involved in the folding of newly synthesised polypeptides, refolding of misfolded proteins, as well as the delivery of partially folded proteins to other chaperones, in order to complete the folding process.[20]

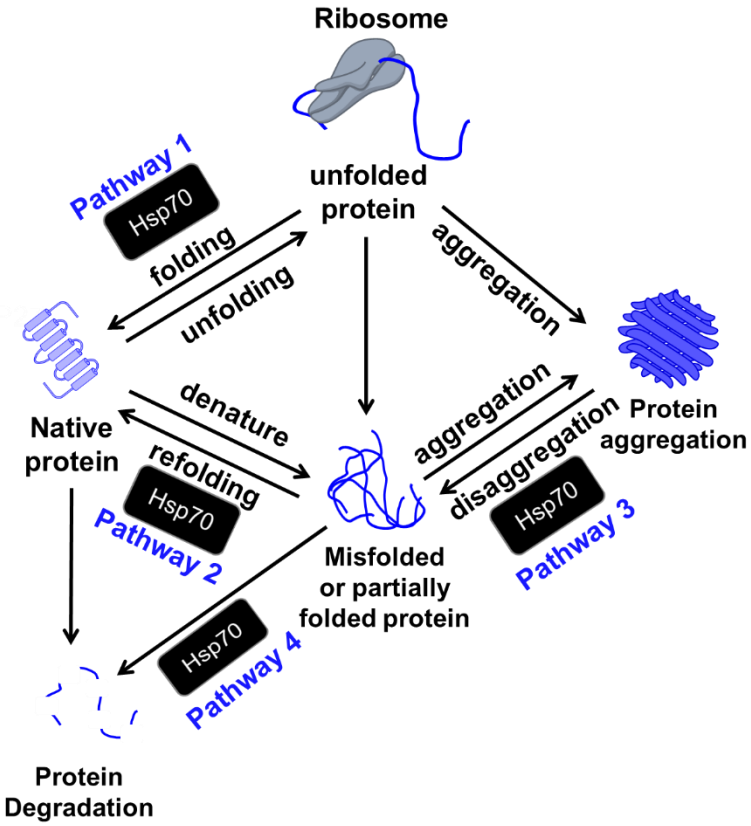


Figure 1.2: The proteostasis network and Hsp70 chaperone pathways

Hsp70 interacts with several members from heat shock protein family to form a

chaperone network and control cellular protein homeostasis (**Figure 1.2**).[22] In the pathway 2 and 3, Hsp70 interact with small heat shock proteins like Hsp27 to refold the misfolded or aggregated proteins.[23] In the pathway 4, Hsp70 delivers the misfolded protein to it, then Hsp104 uses the energy from ATP hydrolysis to drive protein degradation.[24] The Hsp60 and Hsp70 chaperone systems play a central role in the steady state of mitochondrial protein level.[25] Hsp40 could regulate the ATPase activity of Hsp70 and also delivery the unfolded proteins to it.[26]

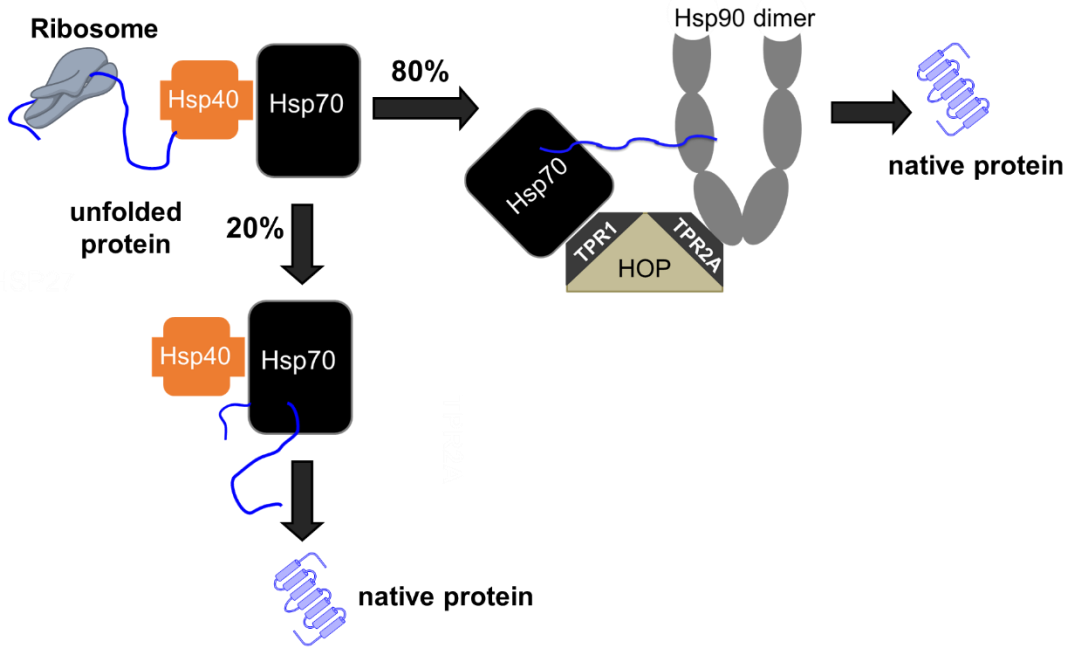


Figure 1.3: The protein folding function of Hsp70. The figure was modified from Hartl. [21]. Hsp70 stabilized unfolded protein on ribosomes and in initiating folding alone with Hsp40 that acts downstream in completing folding 20% of nascent peptides. 80 % of Substrates were transfer from Hsp70 to Hsp90 by the coupling protein HOP.

The most important interaction between Hsps is the assembly of Hsp70 and Hsp90, which is critical for the folding the newly formed peptides to their native state (**Figure 1.3**).[27] All proteins that are released from the ribosome are firstly folded by Hsp70, and 80% of these are passed onto Hsp90 for the final folding process (**Figure 1.3**).[21] These

folding processes are facilitated by secondary proteins or co-chaperones that bind to Hsp70 and regulate it's function.[28]

1.22 Hsp70 as a promising target

Hsp70 is critical to the normal function of cells and has emerged as a highly attractive therapeutic target because it is involved in several diseases including viral infections[9], neurodegenerative diseases[29] , especially in cancer.[14] Hsp70 is over-expressed in many cancer cell lines.[14] In tumour cells, uncontrollable proliferation leads to a decline in regulatory processes in the cell, and increase in mutations that result in the accumulation of misfolded proteins and their aggregation, which normally would trigger cell death.[6] In order to survive, the stress associated with the high levels of unfolded and aggregated proteins trigger cancer cells to over-express Hsp70. Hsp70 folds nascent peptides and refolds misfolded proteins that are critical for cancer proliferation and survival. Although other members of the Hsp family including Hsp27, Hsp40 and Hsp90 are also over-expressed in cancer cells, Hsp70 is the central chaperone directing these processes. Hsp70 cooperates with each of these Hsps and refolds misfolded proteins in order to regulate oncogenic client proteins that are involved in multiple cancer cell survival pathways.[19, 30-32]

Separate to its chaperone function, Hsp70 also directly interacts with proteins involved in the apoptosis pathways and inhibits apoptosis in cancer cells. Therefore tumour cells that over-express Hsp70 are resistant to cell death and chemotherapy.[33] Hsp70 knock down studies, using siRNA against Hsp70, showed that when Hsp70 levels were decreased in cancer cells, there was a corresponding decrease in tumour growth rate and resistance to chemotherapy, while apoptosis levels were increased.[34] Thus,

decreasing Hsp70 function decreases tumour growth, and chemotherapeutic resistance, while increasing cell death. Inhibition of Hsp70 in normal cell lines did not result in obvious apoptosis.[35] The high impact of depleting Hsp70 on cancer cells, while low impact on normal cells makes Hsp70 an excellent and highly promising anti-cancer approach over traditional chemotherapeutic drugs.[36]

1.22 The structure of Hsp70

Hsp70 contains two major structural domains: The nucleotide binding domain (NBD) and the substrate binding domain (SBD) (**Figure 1.4**) [37, 38]. The NBD is 44 kDa in size and is involved in binding to and hydrolyzing ATP; this supplies energy needed for Hsp70 to change its conformation and fold proteins.[39] Hsp40 and some nucleotide exchange factors (NEFs) directly bind to the NBD and assist Hsp70 in carrying out its chaperone function. NEFs are proteins that stimulate the exchange of ADP for ATP bound to Hsp70, which is necessary for the functional cycle of Hsp70.[40] The 28 kDa SBD contains the substrate binding pocket and a C-terminal lid (CTL).[38] The CTL is approximately 3 kDa, with the rest of the SBD binding pocket comprised of the remaining 25 kDa.

Several types of co-chaperones bind to Hsp70, with TPR-containing co-chaperones being perhaps the most important..[41] The tetratrico-peptide repeat (TPR) domains contain at least two TPR motifs, which are ~34 amino acids repeated and form two anti-parallel α-helices.[42] The TPR-containing co-chaperones include HOP and C-terminus of Hsc70-interacting protein (CHIP), which directly interact with C-terminal lid of Hsp70.[43] The major function of the TPR-containing co-chaperones is to tripper Hsp70 to perform a specific function that is designated by the specific TPR-containing co-chaperone that is forming a complex (**Figure 1.4**).[44]

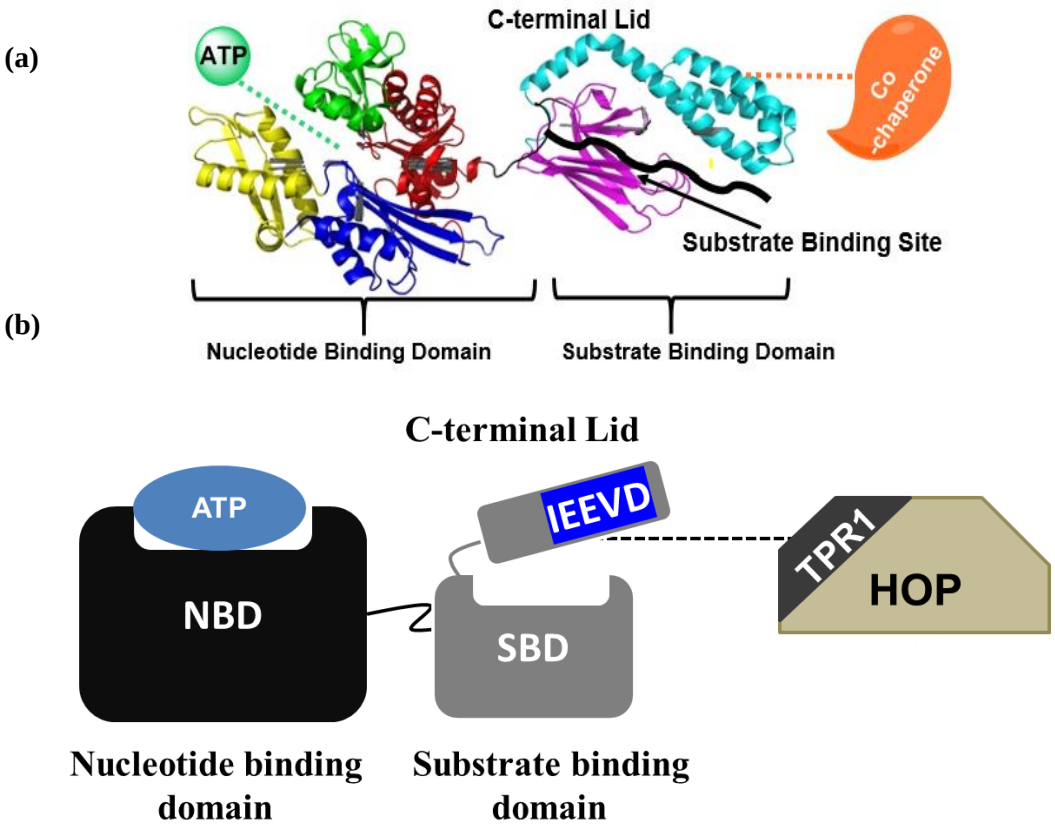


Figure 1.4*:* (a)The Figure was adapted from Bertelsen et al.[37] The crystal structure of the E.coli Hsp70 protein (Dank). **(b)** A schematic representation the structure of Hsp70 and the interactions of Hsp70 with HOP

HOP is the TPR co-chaperone that binds to Hsp70 and regulates its protein folding function (**Figure 1.4**).[45] Hsp70 binds to HOP via its C-terminal IEEVD motif,[46] which is the last five amino acids located on the CTL. IEEVD stands for the single letter code of the following residues: Isoleucine – Glutamic acid – Glutamic acid – Valine – Aspartic acid. This IEEVD motif is highly conserved among all Hsp70 isoforms (**Figure 1.4b**).[44]

1.3 Hsp70 and HOP interaction

1.31 HOP and TPR1

HOP is one of the most well-studied TPR-containing co-chaperones of Hsp70. HOP, also known as Stress-induced Phosphoprotein 1 (STIP1) is 60 KDa in size and contains

477 amino acids in total. As one of the major co-chaperone of heat shock protein family, HOP is composed of three distinct tetratrico-peptide repeat (TPR) domains TPR1, TPR2A and TPR2B **(Figure 1.5)**.[43]

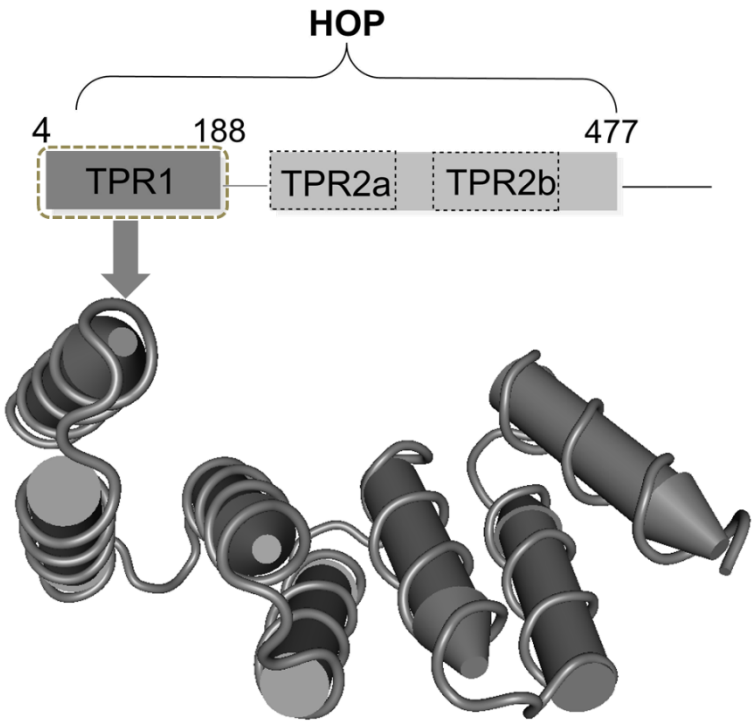


Figure 1.5 Schematic of the HOP and the crystal structure of the TPR1 domain

Each TPR domain contains at least three tandem-repeat TPR motifs. A TPR motif is a ~34 amino acid sequence that forms two anti-parallel α-helices, and a TPR domain is where 3 of these motifs, ie. 3 x 34 amino acids = 6~7 helices, are repeated to form the domain.[42] There is sequence diversity between TPR motifs within a domain.[47] The TPR motif was first reported in yeast (1990) as a protein-protein interaction module involved in cell cycle.[48] The TPR motifs sit at regular angles and form a groove with a large surface area that allows proteins to bind to that region.[43] The TPR1 domain from HOP, contains 7 helices that consists of 3 TPR motifs and forms a cradle-shaped binding pocket **(Figure 1.5)**.[49]

1.32 Hsp70-HOP interaction

HOP acts as a scaffolding protein, mediating the interaction of the molecular chaperones Hsp70 and Hsp90.[50] Specifically, the TPR1 domain of HOP binds to the C-terminal motif of Hsp70 and the TPR2A domain binds to the Hsp90 **(Figure 1.6).** Through the direct interaction at the C-terminus of Hsp70 and Hsp90, HOP is able to bind to Hsp70 and Hsp90 simultaneously allowing Hsp70 to transfer proteins to Hsp90 for completion of the protein folding complex (**Figure 1.6**). This transfer process is not only responsible for folding the newly formed peptides to their native state but also for refolding damaged proteins during cellular stress and in cancer. [27]

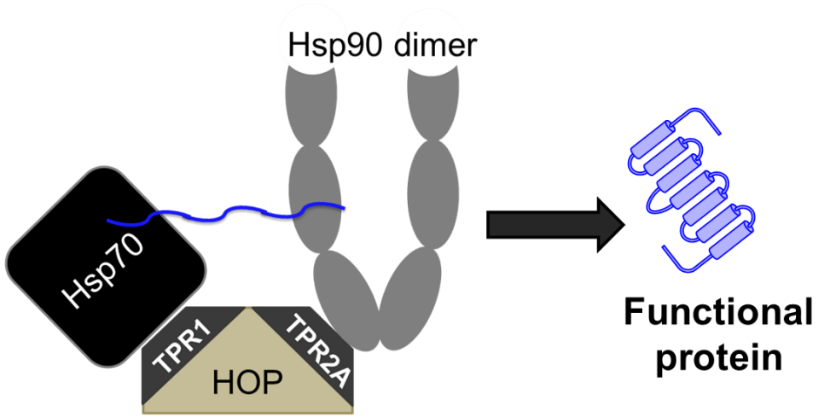


Figure 1.6 The Hsp70-HOP-Hsp90 complex facilitates the transfer of unfolded proteins from Hsp70 to Hsp90 for folding. The C-terminus of Hsp70 binds to the TPR1 domain of HOP and the C-terminal domain of Hsp90 binds to the TPR2A domain.

Under the physiological condition, the binding between Hsp70 and HOP is relatively weak in order to allow for the dynamic assemble of the Hsp70-HOP-Hsp90 complex. ATP hydrolysis and the conformational changes of Hsp70 is critical for assembly and disassembly of Hsp70-HOP-Hsp90 complex. When Hsp70 is bound to ATP in the open conformation (**Figure 1.7**), the binding affinity between Hsp70 and HOP is low (Kd = 1.3 μM).[49] When the ATP is hydrolysed to ADP, the C-terminal lid of Hsp70 closes and the client protein is locked in the substrate binding domain, where this complex of Hsp70-

substrate binds more tightly to HOP than without the substrate . When ADP is released from Hsp70, and ATP binds again, Hsp70 forms an open conformation and releases the client protein to Hsp90 [21, 51] During the changing of Hsp70's different conformations, the association and dissociation of Hsp70-HOP-Hsp90 complex also dynamically switches over.[49] In order for oncogenic proteins to be utilised by cancer cells, they must be correctly folded in to their native state, which requires Hsp70 to pick them up and transfer them via the Hsp70, Hsp90 and HOP complex (**Figure 1.6-7**).[52] Thus, the Hsp70-HOP interaction is likely a good target to inhibit, as this interaction specifically enables cancer cell growth.

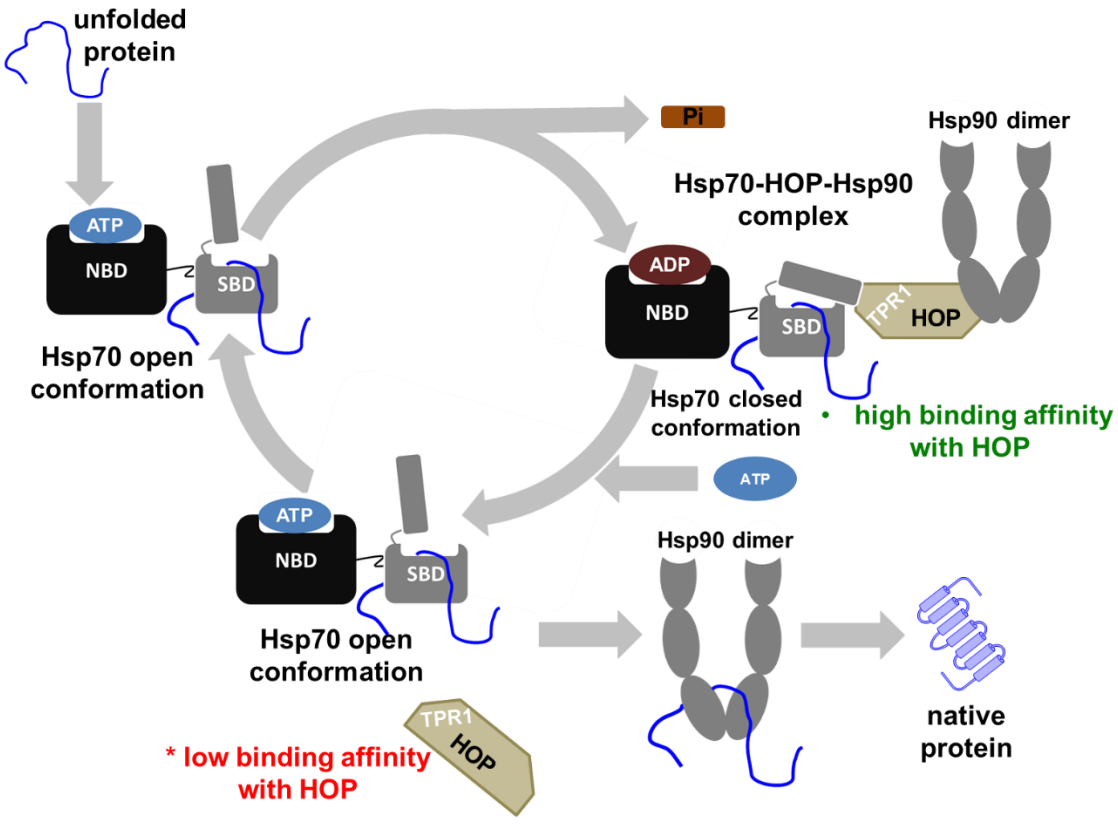


Figure 1.7 The functional cycle and conformational changes of Hsp70

1.4 A novel strategy for inhibiting function of Hsp70

1.41 Hsp70's Inhibitors

Currently, there are more than 20 molecules that have been reported to inhibit Hsp70. More than half of them interact with the NBD, seven interact with the SBD and the remaining compounds' binding sites are unknown.[53, 54] Although Hsp70 is a promising target for several serious diseases such as cancer, to date there have been no Hsp70 inhibitors that have gone through clinical trials.[55-57] The lack of Hsp70 inhibitors in the clinic is related to the fact that all Hsp70 inhibitors reported to date are either non-selective and target multiple proteins in the cell, or are highly toxic, likely the result of a non-specific mechanism. Thus, there is a need to develop highly selective Hsp70 inhibitors.

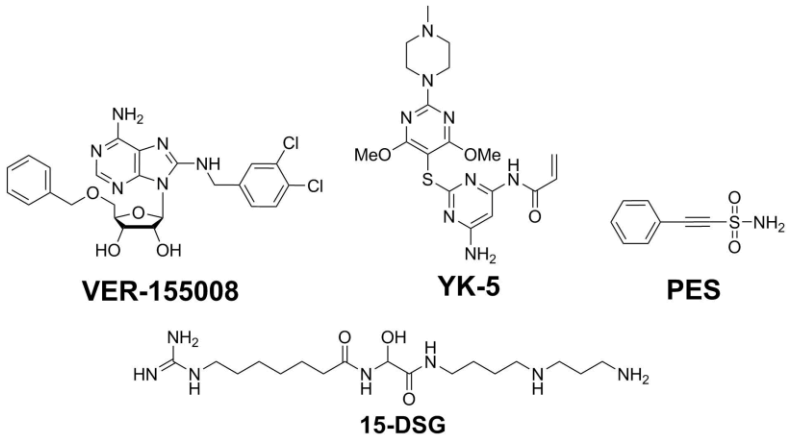


Figure 1.8: Structures of published Hsp70 inhibitors. VER-155008 is an ATP analogue targeting the NBD of Hsp70. YK-5 binds to the NBD and blocks interactions of Hsp70 with HOP. PES is an allosteric inhibitor that targets the SBD of Hsp70. 15-DSG interacts to the C-terminus of Hsp70 and impact with ATPase activity.

Most published molecules act on the NBD of Hsp70. These molecules bind to an allosteric site on NBD or the ATP binding site of Hsp70 and inhibit protein-folding function of Hsp70.[58-61] VER -155008 is one of the most well studied Hsp70 inhibitors (**Figure 1.8**)., and is an ATP analogue that acts as competitive inhibitor for the ATP

binding site, and therefore inhibits the essential ATPase activity of Hsp70 and blocks ATP/ADP nucleotide binding/exchange.[37] VER -155008 prevents proliferation in multiple cancer cell lines,[60] but has a low bioavailability as it is rapidly degraded *in vivo*.[44] This molecule also binds to Hsp90 and produces a heat shock response in the cells, making it unsuitable as a cancer therapeutic.[62] The heat shock response (HSR), is a classic cellular response when stress threatens the cell, this response produces an increase in multiple proteins that protect the cell and induce resistance, making chemotherapeutics that produce this effect problematic[63,64]

YK-5 is another well studied inhibitor (**Figure 1.8**) that binds to the NBD of Hsp70. Evidence indicates that YK-5 allosterically disrupts the interaction between Hsp70 and HOP, which prevents their interaction with Hsp90 and subsequently leads to apoptosis.[65] Although it could bind to Hsp70 in the biochemical setting assay, it also binds to other proteins producing a non-specific effect in a cellular setting. It is a good example of inhibiting protein's function through protein-protein interaction, but poor selectivity has limited its pharmaceutical development.[66]

2-phenylethynesulfonamide (PES), as well as its derivative PES-Cl (**Figure 1.8**) bind to the SBD of Hsp70, however its small size already suggests there will be a lack of selectivity for Hsp70. In a biochemical setting, PES allosterically reduces the degree of interaction between HSP70 and its co-chaperones Hsp40, C-terminus of Hsp70 Interacting Protein (CHIP) and BAG-1. [65, 67] PES also induces apoptosis in cancer cells and it is suggested this is a result of its ability to inhibit Hsp70 function, although this has not been proven.[68]

Only one compound, 15-deoxyspergualin (15-DSG) binds to the EEVD region at C-terminus of Hsp70 and inhibits its ATPase activity (**Figure 1.8**).[69] Despite binding to the C-terminus of Hsp70, its impact on TPR co-chaperones has not been investigated.

However, 15-DSG also binds to other cellular targets including Hsp90, suggesting that it acts via multiple mechanisms in the cell.[39] It is ineffective when tested in the animal models because has a very low bioavailability and is unstable in aqueous solution.[41] Thus, modulating Hsp70's C-terminus has not yet been accomplished effectively despite the promising phenotype observed when using siRNA knockdown studies against Hsp70.

1.42 Targeting PPIs as a promising approach

Protein-protein interactions (PPIs) control the association and dissociation of multi-protein complexes and co-regulate the dynamic signalling pathways that govern basic activities of cells and allows them to function accurately.[37, 70-75] Modulating PPIs is becoming a highly selective and specific approach for both drug discovery and for developing a mechanistic understanding of cellular processes.

Despite the advances made in understanding of pathology about cancer, it remains a leading cause of death, with more than 7 million deaths per year worldwide. The poor selectivity of normal drugs used in chemotherapy results in high toxicity to normal tissues and this toxicity triggers cell protection mechanisms including the heat shock response, which leads to drug resistance. Hence, new cancer therapies should aim to inhibit specific proteins that are involved in vital pathways relevant to cancer.[76] PPIs play a key role in individual pathways, and as such selectively targeting a specific PPI is a promising therapeutic approach for cancer, likely to eliminate off-target effects and lower chemotherapeutic resistance.[77]

Developing molecules that modulate PPIs has been a significant challenge and there has been little success relative to the high production of molecules regulating ATP binding events.[70, 73, 77] There are several well-described challenges to targeting PPIs. First,

proteins typically interact with other proteins via a relatively large contact surface area (1150–10000Å), which is much larger than the contact area between small molecules and proteins (100–600Å).[77] PPIs usually lack clear grooves or deep binding pockets at their interaction surface, rather a series of weak interactions across a large surface results in a high binding specificity and affinity.[70] Given these challenges, appropriate design strategies for molecules that can block these large shallow surfaces are limited. High throughput evaluation methods using small molecule libraries are unable to identify druggable molecules that effectively target PPIs.[78] New strategies are required to produce molecules that will block these surfaces, and one such approach is explored in this thesis, targeting the Hsp70-HOP interface.

1.43 Peptides Drug Development

Roughly 100 peptide therapeutics are evaluated in clinical trials each year. The global peptide drug market is predicted to increase from $21 billion in 2015 to $46.6 billion in 2024,[78] with the increase in revenue forecast to come from the development of new peptide drugs.[79] Peptides are uniquely suited for inhibiting protein-protein interactions.[79] Compared with small molecules or antibody therapies, peptide-based drugs have many advantages and their effectiveness is attributed to several characteristics: 1) stereochemistry every 3 atoms on the backbone, which produces unique 3D structures and offers high selectivity against a specific protein surface[80] 2) peptides have low toxicity since they are not accumulated in the body and they are biocompatible lowering the possibility of an elicit immune response compared to antibody therapies.[81] [78], [82] 3) peptides typically have a large surface area, which is ideal for blocking proteins from interacting.[83] 4) Peptides are also relatively straightforward to synthesize and modify, with some variants able to penetrate tumor cells easily.[84]

Cyclic peptides present significantly improvement of binding affinity relative to their linear counterparts because they have all the advantages of linear peptides but also have restricted conformational flexibility. The restricted movement means that they have decreased initial entropy and upon binding they will have a higher binding affinity relative to a linear peptide with the same structure. However, the disadvantage is that cyclic peptides may be restricted into a single conformation that cannot bind to the protein surface, making it ineffective, when the linear peptide can still successfully bind. Cyclic peptides no longer have free N and C terminus, making them less chemically inert and better cell membrane permeability properties compared to their linear counterparts.[85] However, because linear molecules have more flexibility they have greater potential to mold themselves to the targeted shallow protein surface, and adopt a suitable conformation. In summary, peptides are good drug-like candidates, therapeutic agents and biochemical tools. [86]

1.44 Peptides synthesis

Many strategies and reagents are used to synthesize peptide molecules. Solid phase peptide synthesis (SPPS) was used in this project. SPPS is a method of synthesizing peptide that uses solid supports such as polystyrene. SPPS allows the peptides to be created in a rapid, cheap way and it is easy to remove by-products and excess reagents.[87]

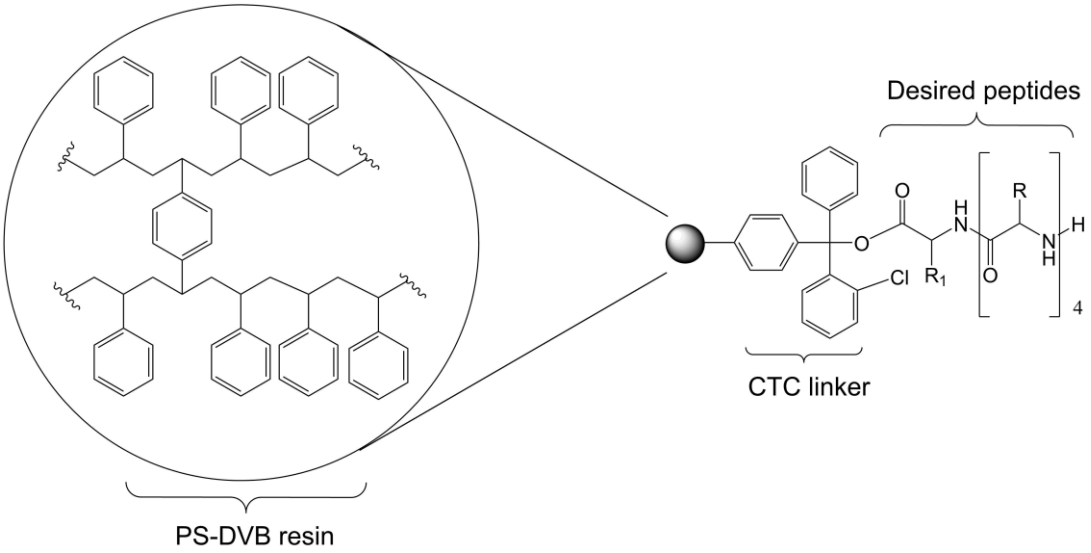


Figure 1.9 PS-1% DVB resin with a CTC linker and attached desired peptide

SPPS generate the peptide chain by a series of sequential coupling reactions started at the C-terminus of the peptide. A 2-chlorotrityl-chloride(CTC) linked with 1 % DVB crosslinked resin was used and gave this synthesis a solid support (**Figure 1.9**). This PS-DVB resin is now the most commonly used support for SPPS. The CTC linker allows peptides to be cleaved using mild condition. (50% trifluoroethanol in CH_2Cl_2), which does not affect protecting groups used on sidechains. Thus, SPPS performed in this thesis utilized CTC resin as linker to link the resin and the first amino acid residue and further to generate desired peptides (**Figure 1.9**)

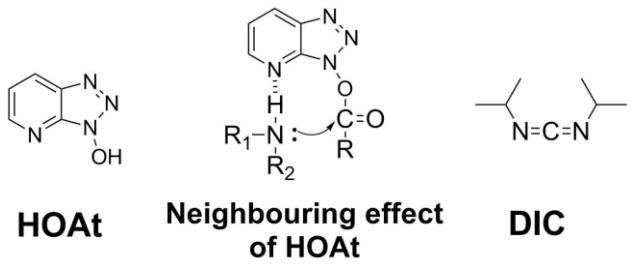
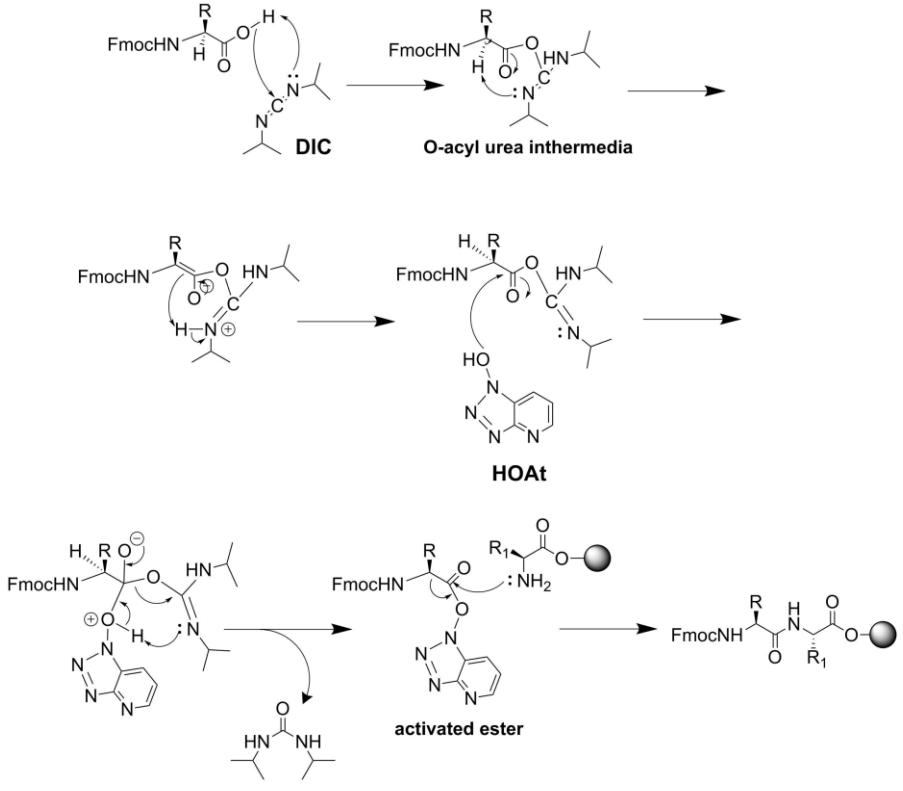


Figure 1.10: Structure of coupling agents, HOAt and DIC.

There are a number of standard coupling agents used for solid-phase peptide synthesis. This synthesis used 1-hydroxy-7-azabenzotriazole (HOAt) as a primary coupling agent (**Figure 1.10**), because it is very effective and prevents racemisation from occurring. High coupling efficiency of HOAt is due to the neighbouring group effect of the pyridine nitrogen located at the aromatic system, which could activates the amine to couple to the acid. Diisopropylcarbodiimide (DIC) was also used to activate the carboxylic acid of the Fmoc-protected amino acids. Adding HOAt to DIC produces a benzotriazoyl ester, which is less reactive than the O-acylisourea intermediate for acid activation. Thus, using HOAt with the DIC, reduces racemization of the protected amino acid (**Scheme 1.1**). Deprotection of the amine in Fmoc-protected amino acids was done in the presence of a nucleophilic base, in this case 20 % piperidine in DMF.



Scheme 1. 1: Mechanism of coupling reaction using DIC and HOAt

After sequences finished, peptides were cleaved from resin under mild condition (50% trifluoroethanol in CH_2Cl_2). The linear peptide then can be cyclised by joining its 'head' and 'tail' terminus to produce a macrocycle (**Figure 1.11**). In this project, protecting groups have been kept to ensure only head to tail macrocycles are generated.

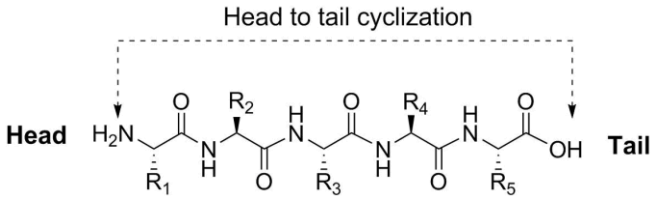


Figure 1.11: Macrocyclization strategies

Cyclisation of peptides shorter than 5 amino acids can be difficult as the peptide must undergo a conformational folding with high entropic cost.[88] The McAlpine group has well established procedures for cyclisation of short chain peptides,[64] using coupling agents O-(7-Azabenzotriazol-1-yl)-N,N,N',N' -tetramethyluronoium hexafluorphosphate (HATU), O-(Benzotriazol-1-yl) -N,N,N',N'- tetram- ethyluronium tetrafluoroborate (TBTU), and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (**Figure 1.12**).[89] The N,N -Diisopropylethylamine (DIPEA) was added as base. The reaction was running under very dilute conditions in CH_2Cl_2.

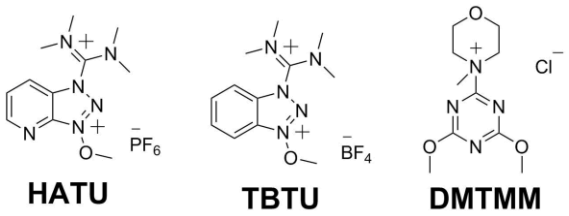


Figure 1. 12: Structure of coupling agents, HATU, TBTU and DMTMM

1.45 The TPR mimics

Work done by Kawakami and co-workers in 2011, showed that the interaction between Hsp90 and HOP could be inhibited using a peptide sequence derived from HOP that binds to Hsp90.[90] This was an excellent demonstration of concept that a sequence from HOP can be used to inhibit PPIs between the chaperone Hsp90 and the co-chaperone HOP. Buckton et al, extended this work and made smaller peptides (5 – 8 amino acids in length) based on Kawakami's sequence.[91] The most potent inhibitor generated was a five amino acid cyclic peptide, which disrupted the interaction between Hsp90 and the co-chaperone CYP40 **(Figure 1.13)**.[91] This work showed that a small peptide containing only five amino acids still contains bio-activity from larger molecule and could be used to disrupt the binding of co-chaperones to chaperones and inhibit their functions.

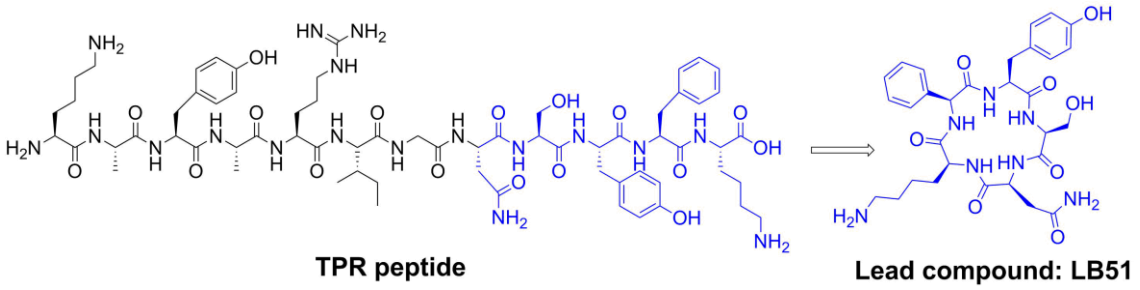


Figure 1.13 : A cyclic pentapeptide was synthesised base on the TPR peptide made by Kawakami and Co,[90] and identified as the hit.[91]

1.5 Project aim

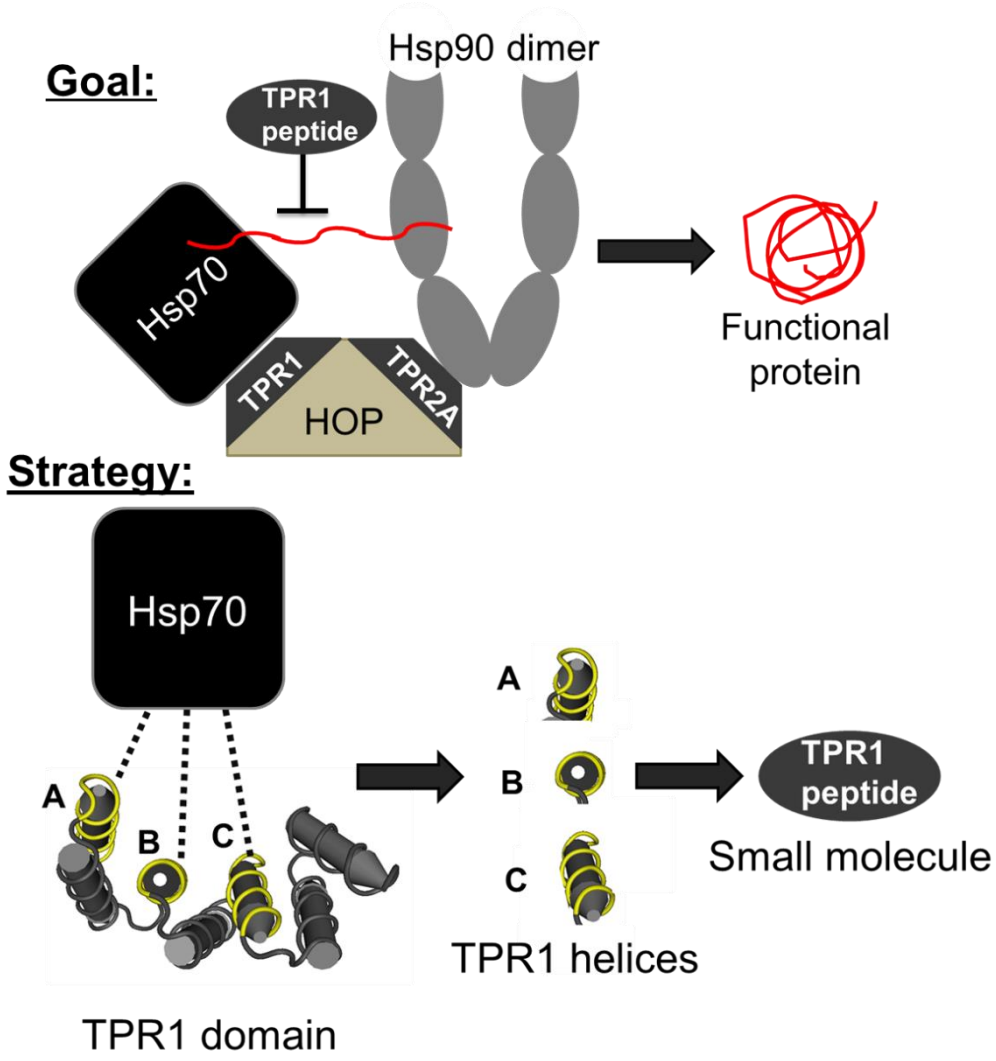


Figure 1.14: Design and strategy. A small molecule designed from the TPR1 domain of HOP can be used to prevent the folding function of Hsp70. Hsp70 interacts with three of the seven helices in the TPR1 domain of HOP, these three helices were used as a design scaffold for the first generation Hsp70 inhibitors.

In this project, the author implemented Buckton's strategy on designing Hsp70 modulators.[91] Based on X-ray crystallography,[92] three helices from the HOP TPR1 domain interacted with Hsp70: Helices A, B and C (**Figure 1.14**). With this knowledge, the author and colleagues designed molecules starting from these three helices in the TPR1 domain, with the aim of producing an inhibitor that would block the transfer of

partially folded protein from Hsp70 to Hsp90. This thesis outlines the synthesis of molecules that were based on these three helices and their subsequent evaluation in biochemical binding assays and functional assays in order to assess the compounds' ability to modulate the PPI between Hsp70 and HOP.

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Chapter 2: Synthesis and biological evaluation of Hsp70 inhibitors

2.1 Introduction

2.1.1 Background

Peptides and peptidomimetics have been evolutionarily designed to modulate protein-protein interactions (PPI).[1] Designing peptides to mimic the sequence of one protein domain that binds to another protein has been utilized successfully by several labs, and has produced molecules that impact a protein's function.[2-5]

As discussed in the introduction, Kawakami identified a 12-amino acid TPR peptide (**Figure 2.1**) that mimicked the TPR2A domain of the co-chaperone HOP and bound to Hsp90.[5-8] The McAlpine group utilised this strategy to design and synthesise a library of peptides that effectively bound to Hsp90.[9, 10] McAlpine and co-workers generated derivatives of the TPR peptide (**Figure 2.1**), and developed a successful derivative that incorporated the last 5 residues of the TPR peptide.[9, 10] The most effective molecule produced via this strategy was LB51, a cyclic structure that inhibited the Hsp90-co-chaperone interaction *in vitro*. LB51 had an IC_{50} of ~ 4 μM against the Hsp90 and co-chaperone CYP40 binding interaction and higher cell membrane permeability than the parent TPR peptide, making it good lead structure (**Figure 2.1**).[9]

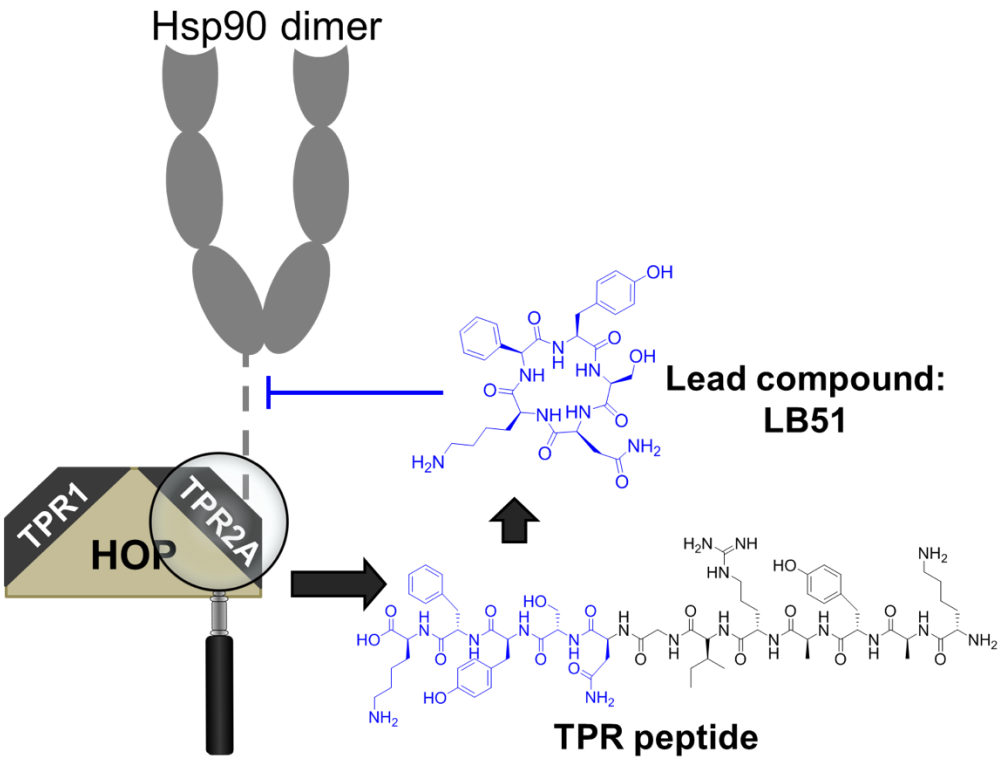


Figure 2. 1: a cyclic pentapeptide was synthesised based on the TPR peptide made by Kawakami and Co,[6] and identified as the hit.[9] , which could directly block co-chaperone access to Hsp90's C-terminus.[9]

The similarity of the interaction between Hsp70 and HOP versus Hsp90 and HOP, suggested that this design strategy may successfully produce Hsp70 inhibitors. This thesis describes the author's work on the first mimics of the HOP TRP1 domain that was designed to bind to Hsp70 (**Figure 2.2**). Based on the crystal structure of HOP's TRP1 domain, three out of seven helices, A, B, and C, bind directly to Hsp70's C-terminus.[11] Hsp70 binds to HOP via its C-terminal IEEVD motif,[12] which is the last five amino acids located on the C-terminus of Hsp70. The complex formed between Hsp70, HOP and Hsp90 could be inhibited if the binding site between Hsp70 and HOP was inhibited. This strategy should block the transfer of protein between Hsp70 and Hsp90 and stop the final folding event. Ultimately this strategy should lead to accumulation of unfolded protein

and cell death.

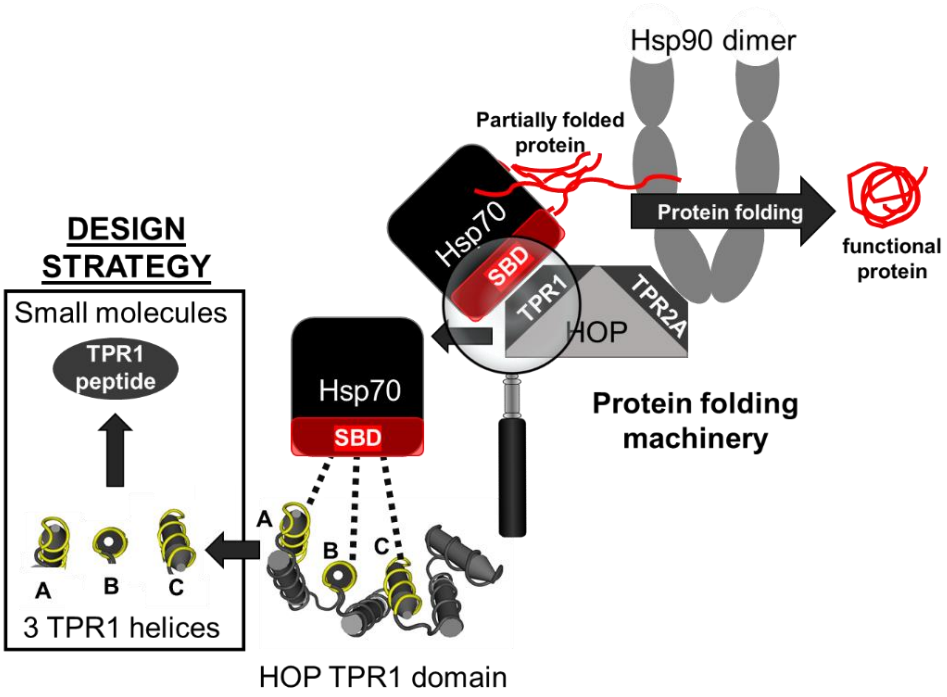
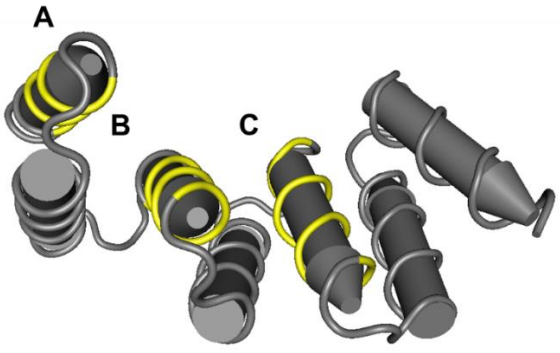


Figure 2. 2: The substrate binding domain (SBD) of Hsp70 interacts with three of the seven helices in the TPR1 domain of HOP (A, B, and C), where this figure of the HOP TPR1 domain is based on a crystal structure from *Scheufler and co-workers.*[13] These three helices were used to design the Hsp70 modulators.

Sequences from helices A, B, and C were used as scaffolds for designing the Hsp70 inhibitors because these sequences contained residues that, based on the crystal structure, interacted with Hsp70 via electrostatic, hydrophobic, or hydrogen bonding interactions (**Figure 2.3**).[13]

(a)



(b)

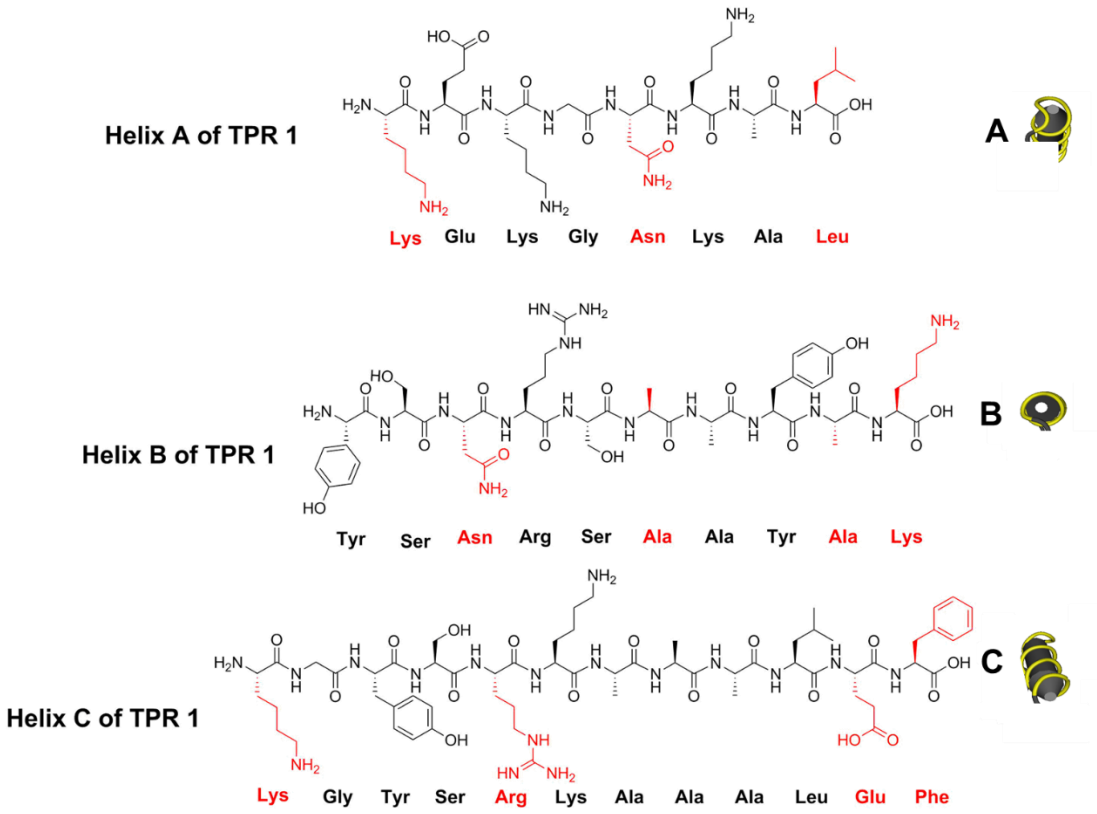


Figure 2. 3: Critical peptide sequences from the three TPR1 helices (A,B and C) of HOP that bind to Hsp70. The residues in red, from electrostatic, hydrogen bonding or hydrophobic interactions with the C-terminus of Hsp70.[13]

2.1.2 Compound Design Strategy

The sequences of helices A, B, and C are 8, 10, and 12 amino acids in length respectively, and where these 3 peptide regions contain the key residues involved in binding with Hsp70 (**Figure 2.4**). However, these peptides are too long to be considered as lead structures in developing new small molecule drugs. Thus, we opted to utilize a similar strategy that was successful for Hsp90.[9, 14] The most effective inhibitor of Hsp90 was a pentapeptide, **LB51**. Thus, 5 amino acid peptides were used as the starting point for designing small molecule inhibitors based on helix sequences of A, B and C (**Figure 2.3**). Linear sequences were produced as those mimicked the linear helix, however, when

producing Hsp90 inhibitors, the most successful were cyclic variants. Thus, both linear and cyclic molecules were generated (**Figure 2.4** and **Figure 2.5**).

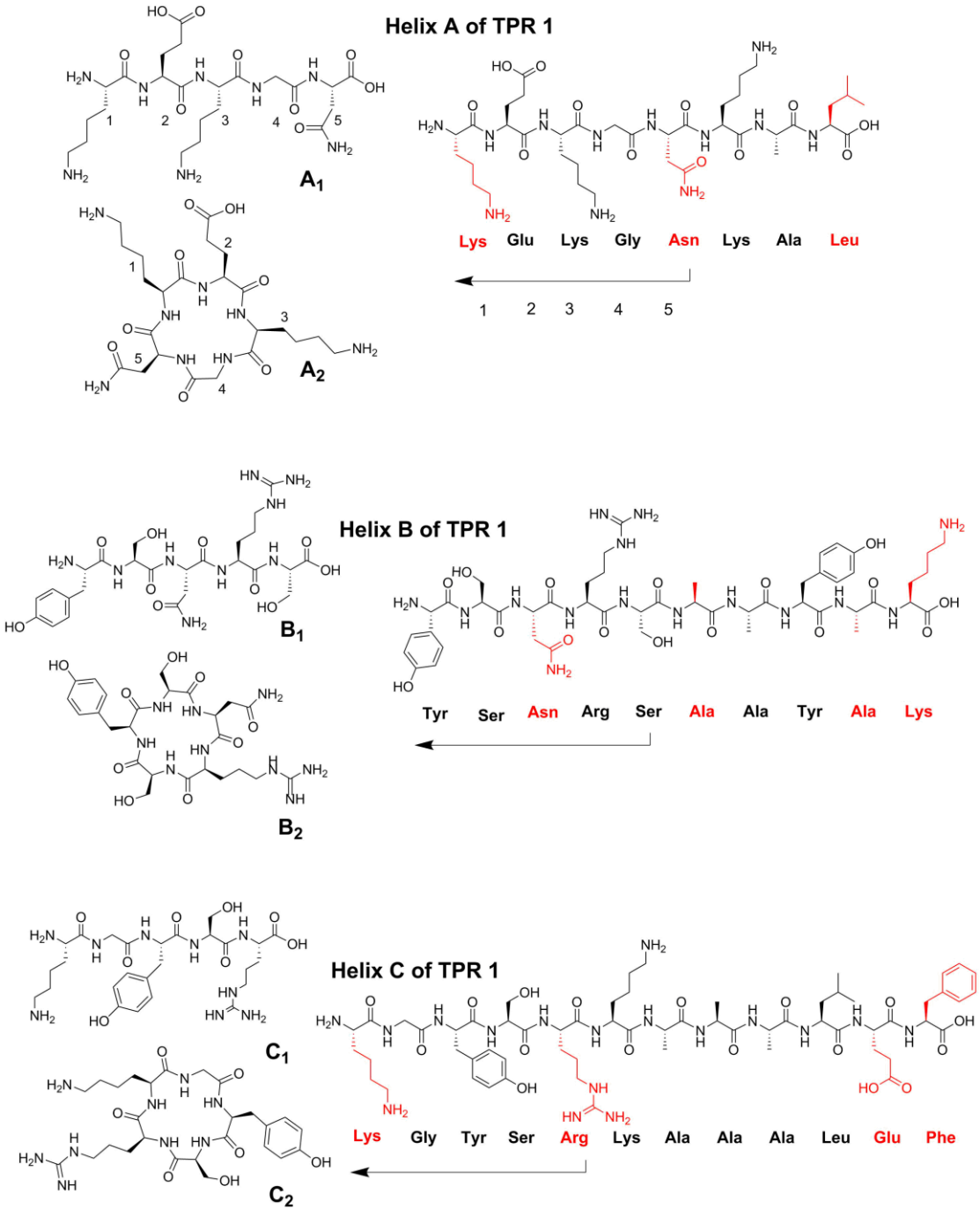


Figure 2. 4: Structure of analogues that were designed based on the N-terminus of helices A, B and C. Final structures produced were molecules ***A1, A2, B1, B2,*** **C1** *and* ***C2***.

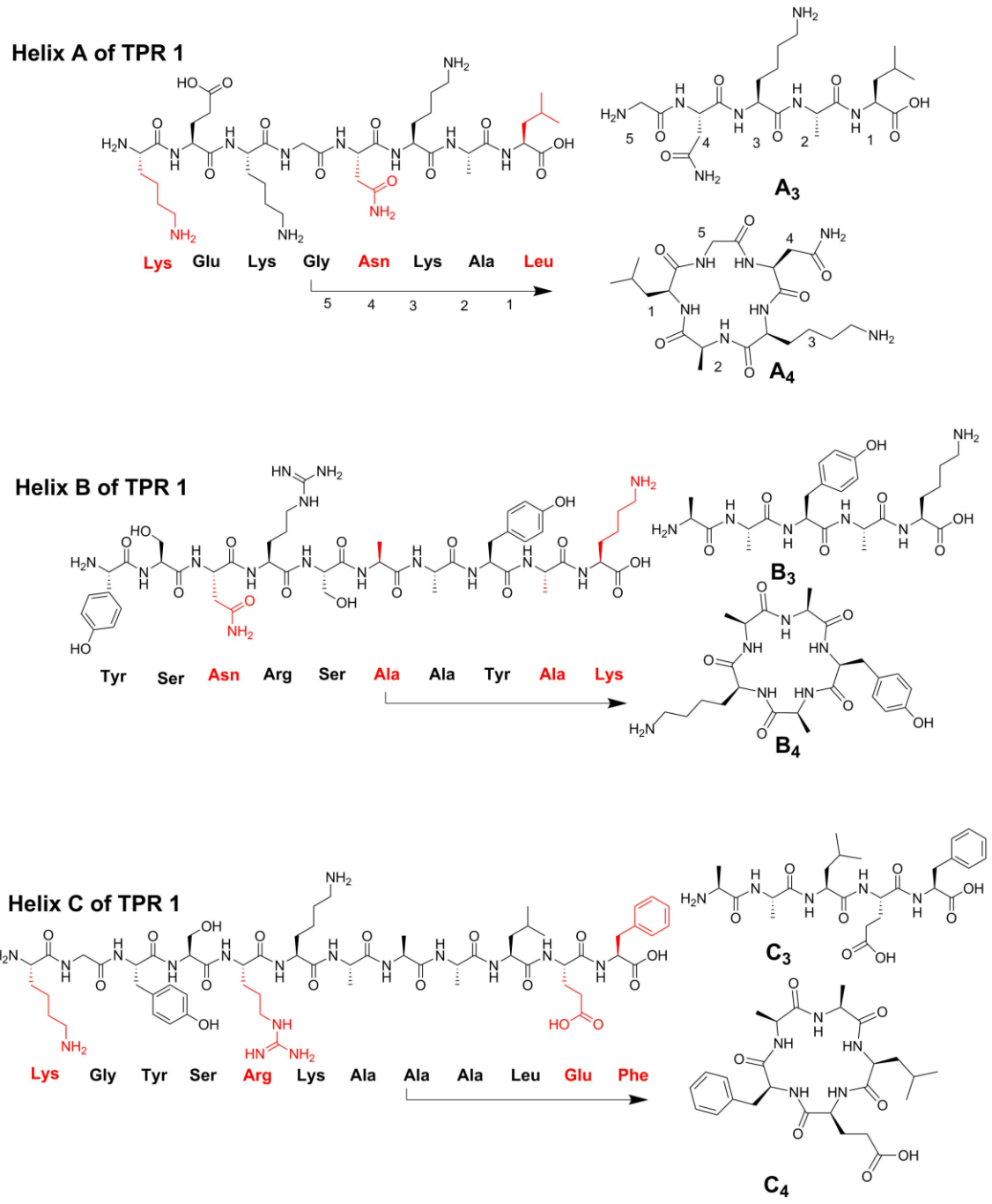


Figure 2. 5: Structure of analogues that were designed based on the C-terminus of helices A, B and C. Final structures produced were molecules A3, A4, B3, B4, **C3** and **C4**.

In addition to the successful precedent of cyclic peptides being successful at inhibiting the Hsp90 and HOP interaction, cyclic and linear pentapeptides have unique advantages when binding to a protein.[9, 15] Cyclic molecules can bind more tightly than linear molecules because their conformational rigidity results in a lower entropic cost

compared to the same sequence of a linear peptide. However, linear molecules are flexible and therefore they have greater binding opportunities because they can adopt the most effective conformation when binding to the protein target. The linear peptides **A1, B1** and **C1** and their cyclic variants, **A2, B2,** and **C2**, were designed based on the five amino acids from the N-terminus of helices A, B and C (***Figure 2.4***). The linear peptides A3, B3, and **C3** along with their cyclic analogues, **A4, B4**, and **C4**, were synthesized based on the sequence of the C-terminus of helices A, B and C (**Figure 2.5**).

2.2 Results and Discussions

2.2.1 Compound Synthesis

In the first generation of Hsp70 inhibitors, the author synthesised the seven compounds shown in black, where the five compounds shown in grey were synthesised by my colleague Samantha Zaiter **(Figure 2.6).**

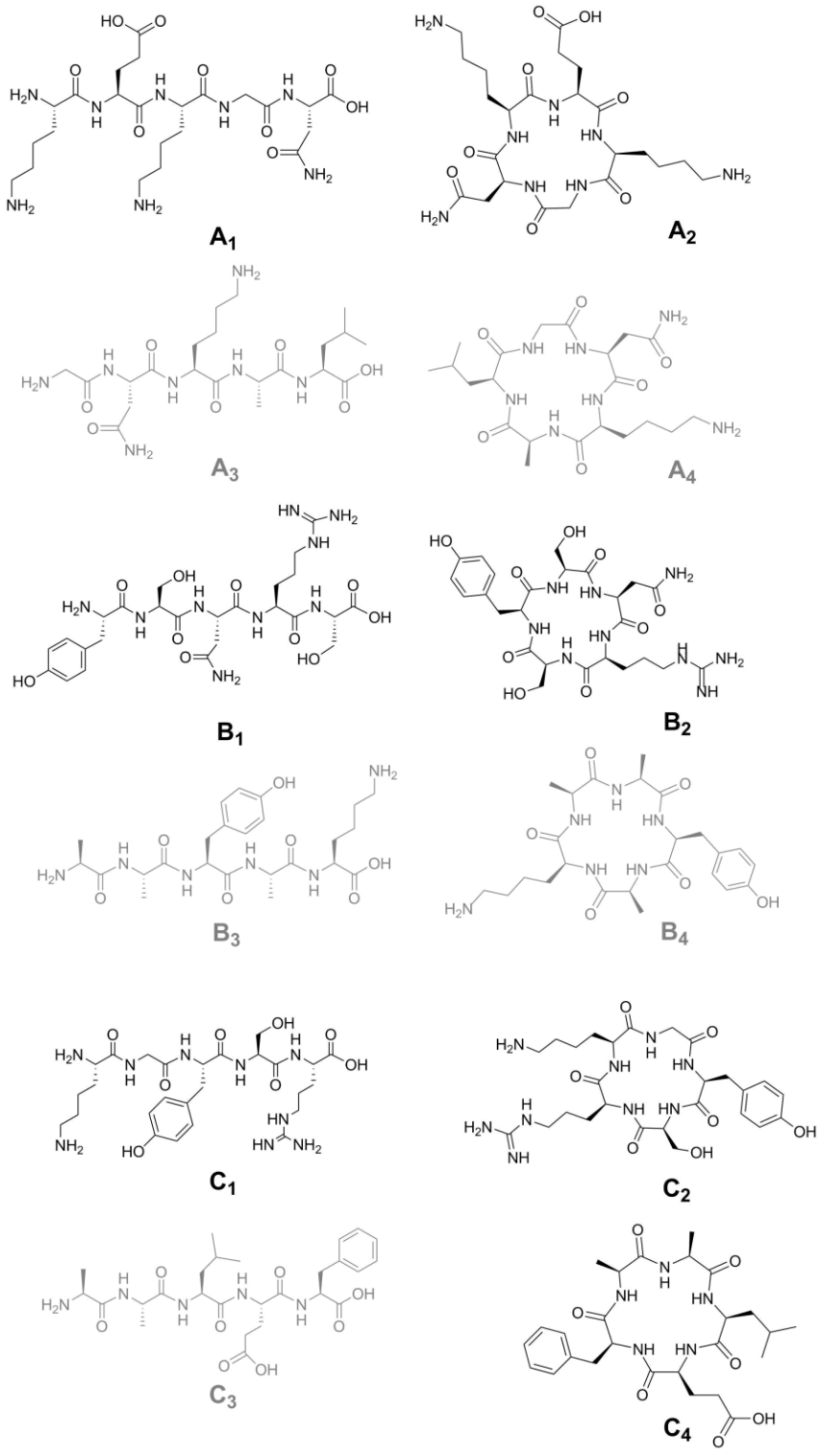
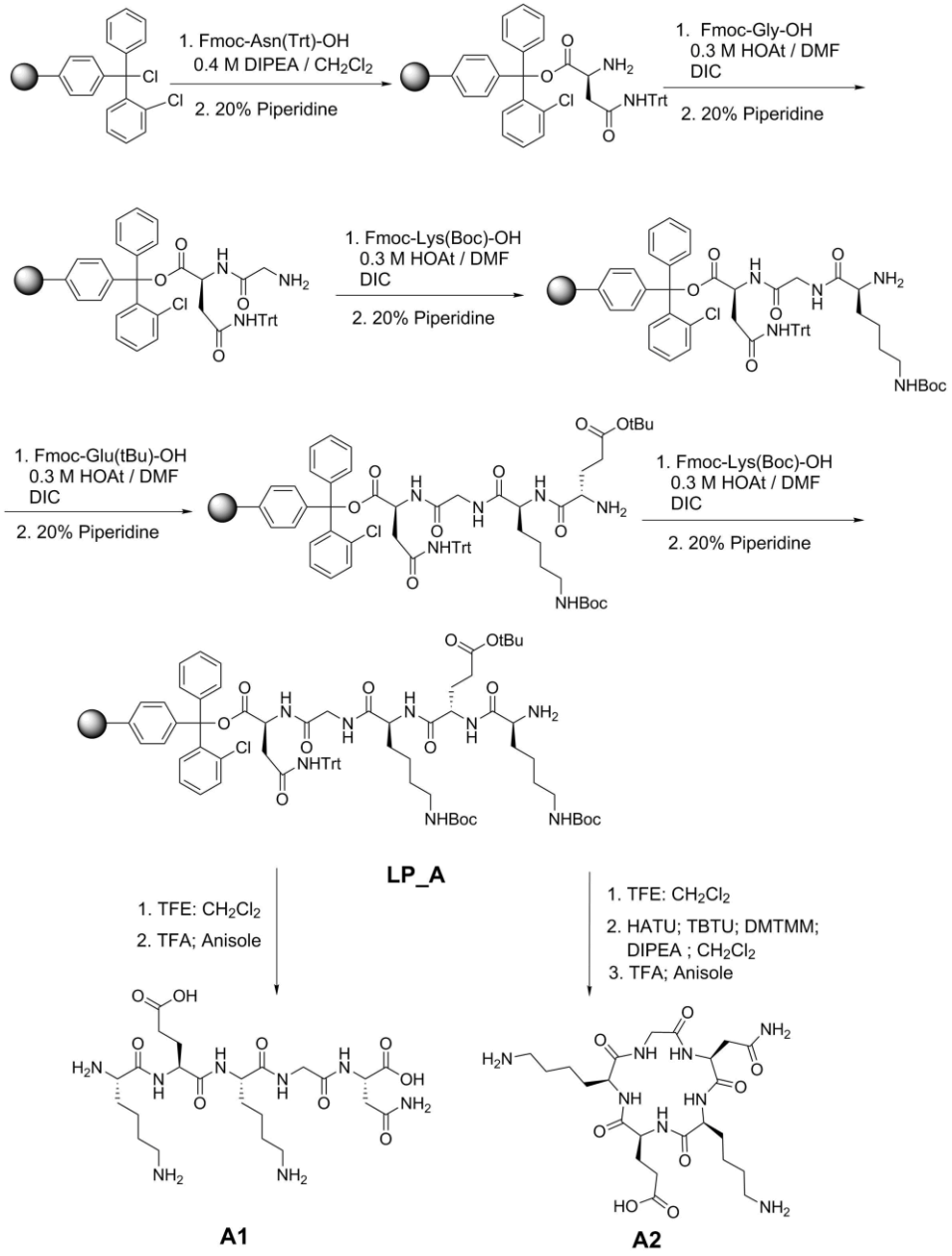


Figure 2. 6: The library of 1st generation Hsp70 C-terminus inhibitors. Structures in black (**A1, A2**, **B1, B2, C1, C2** and **C4**) were completed by the author.

2.2.1.1 Synthesis of A1 and A2

The synthesis of the first generation TPR1 mimics (**Figure 2.6**) was achieved utilizing solid phase peptide synthesis (SPPS). Synthesis of compounds **A1** and **A2** are described (**Scheme 2.1**) SPPS was performed in a 60 mL polypropylene solid-phase extraction cartridge with a 20 μm polyethylene frit. Agitation of the reaction tube was performed by a tube shaker.

Before loading, 2-chlorotrityl chloride (2-ClTrt) resin was swelled in dichloromethane for 30 minutes. The first amino acid, Fmoc-protected asparagine was dissolved and added to the drained resin using a solution of 0.1 M diisopropyl ethyl amine (DIPEA) in dichloromethane. A sample of resin was removed, and the resin loading was determined to be 0.565 mmol/g. The Fmoc protecting group was removed by adding a solution of 20 % piperidine in dimethyl formamide (DMF) to the resin for 10 minutes, draining and then adding fresh solution for another 10 minutes. In order to confirm the Fmoc removal, a ninhydrin test was used, and upon the indication that it was positive, the peptide bound resin was taken onto the next reaction. Following the deprotection, the coupling of Fmoc protected glycine was achieved using both the coupling agents 1-hydroxy-7-azabenzotriazole (HOAt) and diisopropylcarbodiimide (DIC). The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected lysine, Fmoc-protected glutamate, and Fmoc-protected lysine, which finally generated the desired protected linear peptide A (**LP_A)**.



Scheme 2. 1 The synthesis scheme for **A1** and **A2.**

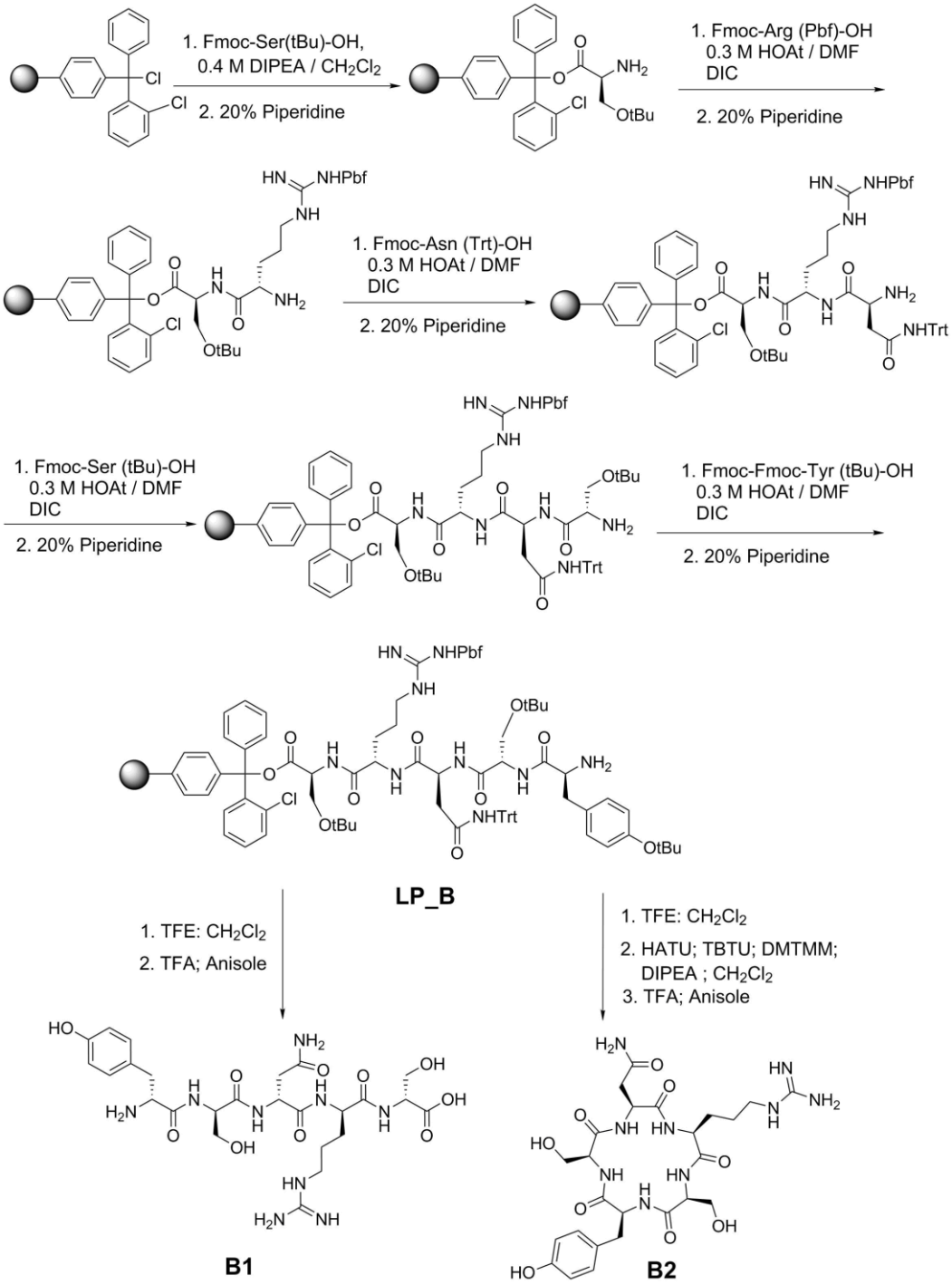
LP_A was cleaved from the resin under mildly acidic conditions over 24 hours using 50% (v/v) trifluoroethanol (TFE) in dichloromethane. LCMS was performed in order to monitor the reaction progress. After complete cleavage the resin slurry was filtered under vacuum, and the filtrate containing the cleaved linear peptide was collected. The solvent was removed under reduced pressure to afford the **LP_A** as a fine off-white powder

precipitate. The **LP_A** was then split into two groups, where the first batch was globally deprotected using trifluoracetic acid (TFA) and anisole (as a carbocation scavenger) in order to produce the deprotected linear peptide, **A1**. The crude **A1** was purified using flash chromatography and high-performance liquid chromatography (HPLC). The mobile consisted of milli-Q water with 0.1% (v/v) formic acid, and HPLC grade acetonitrile with 0.1% (v/v) formic acid. From every injection, all obvious peaks were collected as individual fractions and checked by LC/MS. All fractions with identical traces, as indicated in LC/MS, were combined and lyophilised.

The second batch of **LP_A** was cyclised for under dilute conditions (0.001 M) in anhydrous dichloromethane, using DIPEA and a mixture cocktail composed of the following coupling agents: 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholin-4-ium chloride (DMTMM), 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and O-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyl-uronium tetrafluoroborate (TBTU). **LP_A** and coupling agents were dissolved separately anhydrous dichloromethane in order to make each a solution of 0.001 M. The dissolved peptide was injected into coupling agent cocktail solution dropwise. The reaction mixture was stirred at room temperature for at least 4 hours. Then the protected **A2** was globally deprotected using TFA and anisole to produce the crude deprotected **A2**. The crude mixture was then purified using HPLC under the same condition as used on **A1**. Both **A1** (11% yield) and **A2** (15% yield) were characterized for purity and structure using LC-MS, ^{1}HNMR, and 2D NMR.

2.2.1.2 Synthesis of B1 and B2

Scheme 2.2 shows the synthesis of compounds **B1** and **B2** in detail. Before loading, 2-ClTrt resin was swelled in dichloromethane for 30 minutes. The first amino acid, Fmoc-protected serine was dissolved and added to the drained resin using a solution of 0.1 M DIPEA in dichloromethane. A sample of resin was removed, and the resin loading was determined to be 0.63 mmol/g. The Fmoc protecting group was removed by adding a solution of 20 % piperidine in DMF to the resin for 10 minutes, draining and then adding fresh solution for another 10 minutes. In order to confirm the Fmoc removal, a ninhydrin test was used and upon the indication that it was positive, the peptide bound resin was taken onto the next reaction. Following the deprotection, the coupling of Fmoc protected arginine was achieved using both the coupling agents HOAt and DIC. The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected asparagine, Fmoc-protected serine and Fmoc-protected tyrosine, which finally generated the desired protected linear peptide, **LP_B**



Scheme 2. 2*:* The synthesis scheme for **B1** and **B2**.

LP_B was cleaved from the resin under mildly acidic conditions over 24 hours using 50% (v/v) TFE in dichloromethane. LCMS was performed in order to monitor the reaction progress. The resin slurry was filtered under vacuum, and the filtrate containing

the cleaved linear peptide was collected. The solvent was removed under reduced pressure to afford the **LP_B** as a fine off-white powder precipitate. The **LP_B** was then split into two groups, where the first batch was globally deprotected using TFA and anisole (as a carbocation scavenger) in order to produce the deprotected linear peptide, **B1**. The crude **B1** was purified using HPLC. The mobile consisted of milli-Q water with 0.1% (v/v) formic acid, and HPLC grade acetonitrile with 0.1% (v/v) formic acid. From every injection, all obvious peaks were collected as individual fractions and checked by LC/MS. All fractions with identical traces as indicated in LC/MS were combined and lyophilised.

The second batch of **LP_B** was cyclised for under dilute conditions (0.001 M) in anhydrous dichloromethane, using DIPEA and a mixture cocktail composed of the following coupling agents: DMTMM, HATU, and TBTU. **LP_B** and coupling agents were dissolved separately in order to make each solution of 0.001 M. The dissolved peptide was injected into coupling agent cocktail solution dropwise. The reaction mixture was stirred at room temperature for at least 4 hours. Then the protected **B2** was globally deprotected using TFA and anisole to produce the crude deprotected **B2**. The crude mixture was then purified using HPLC under the same conditions used on **B1.** Both **B1** (13 % yield)and **B2** (17 % yield) were characterized for purity and structure using LC-MS, ^{1}HNMR, and 2D NMR.

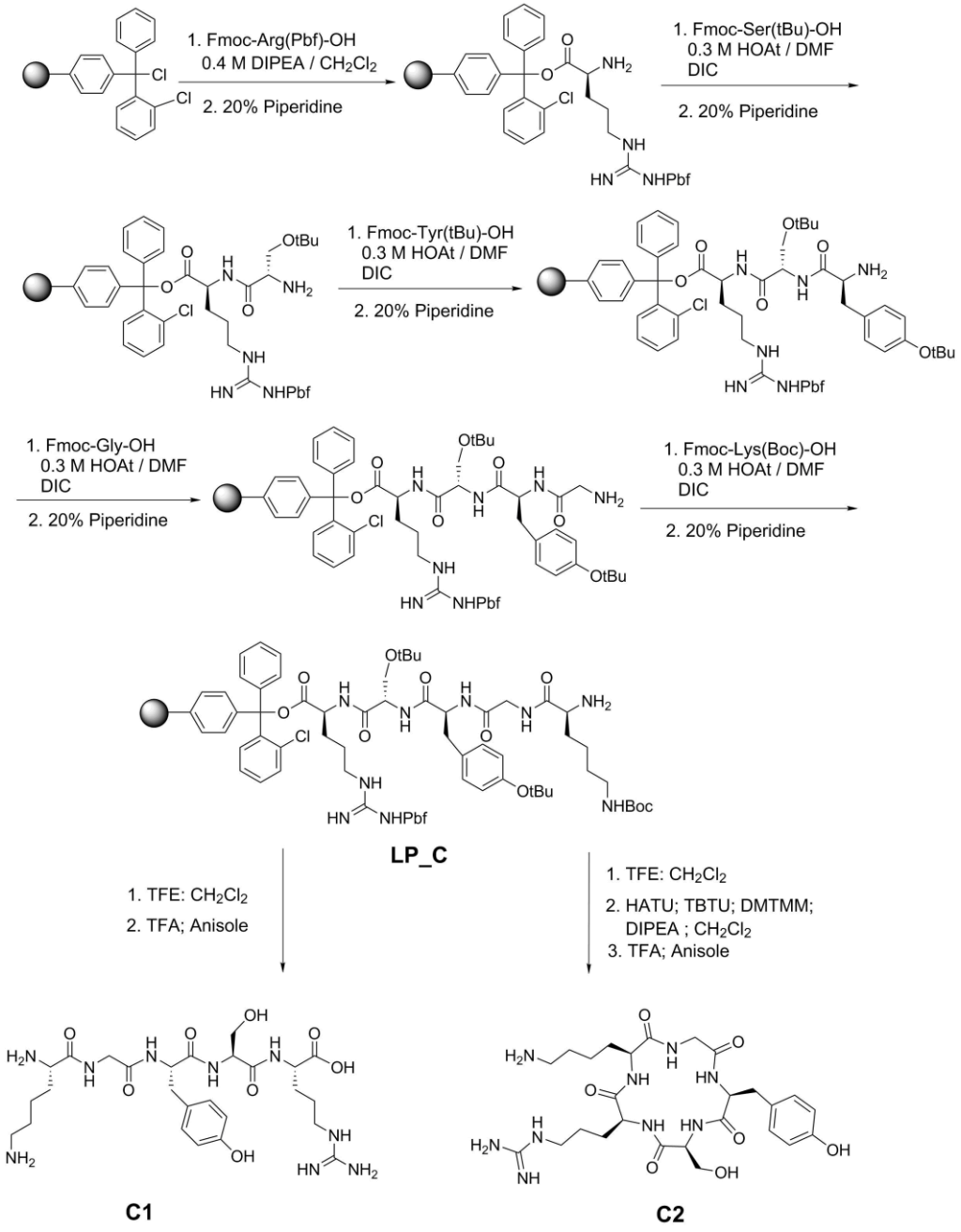
2.2.1.3 Synthesis of C1 and C2

The synthesis of compounds **C1** and **C2** are described (**Scheme 2.3**). Before loading, 2-ClTrt resin was swelled in dichloromethane for 30 minutes. The first amino acid, Fmoc-protected arginine was dissolved and added to the drained resin using a solution of 0.1 M DIPEA in dichloromethane. A sample of resin was removed, and the resin loading was

determined to be 0.39 mmol/g. The Fmoc protecting group was removed by adding a solution of 20 % piperidine in dimethyl formamide (DMF) to the resin for 10 minutes, draining and then adding fresh solution for another 10 minutes. In order to confirm the Fmoc removal, a ninhydrin test was used, and upon the indication that it was positive, the peptide bound resin was taken onto the next reaction.

Following the deprotection, the coupling of Fmoc protected serine was achieved using both the coupling agents HOAt and DIC. The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected asparagine, Fmoc-protected serine and Fmoc-protected tyrosine, which generated the desired protected linear peptide, **LP_C**.

LP_C was cleaved from the resin under mildly acidic conditions over 24 hours using 50% (v/v) TFE in dichloromethane. LCMS was performed in order to monitor the reaction progress. The resin slurry was filtered under vacuum, and the filtrate containing the cleaved linear peptide was collected. The solvent was removed under reduced pressure to afford the **LP_C** as a fine off-white powder precipitate. The **LP_C** was then split into two groups, where the first batch was globally deprotected using TFA and anisole (as a carbocation scavenger) in order to produce the deprotected linear peptide, **C1**. The crude **C1** was purified using HPLC. The mobile consisted of milli-Q water with 0.1% (v/v) formic acid, and HPLC grade acetonitrile with 0.1% (v/v) formic acid. From every injection, all obvious peaks were collected as individual fractions and checked by LC/MS. All fractions with identical traces as indicated in LC/MS were combined and lyophilized.



Scheme 2. 3: The synthesis scheme for **C1** and **C2**.

The second batch of **LP_C** was cyclised for under dilute conditions (0.001 M) in anhydrous dichloromethane, using DIPEA and a mixture cocktail composed of the following coupling agents: DMTMM, HATU, and TBTU. **LP_C** and coupling agents were dissolved separately in order to make each a solution of 0.001 M. The dissolved peptide was injected into coupling agent cocktail solution dropwise. The reaction mixture

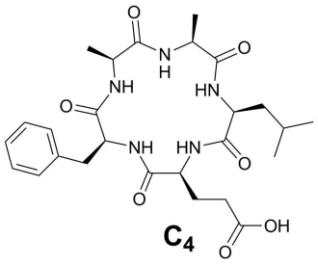
was stirred at room temperature for at least 4 hours. Then the protected **C2** was globally deprotected using TFA and anisole to produce the crude deprotected **C2**. The crude mixture of **C2** was purified using HPLC.

C2 was the most challenging molecule challenging to purify, because it was highly polar and was not retained on the **C1**8 column used to purify the compound. **C2** always eluted with the solvent front, which caused co-elution with an impurity. **C2** required a second purification via HPLC, using a modified method that had a much slower gradient and a very low loading of the peptide onto the column. This allowed **C2** to be separated from the impurity, and the same method was used to successfully purify the other hydrophilic compounds. Finally, Both **C1** (7% yield) and **C2** (4% yield) were fully characterized using LC-MS, ^{1}HNMR, and 2D NMR.

2.2.1.4 Synthesis of C4

While I was synthesizing compounds based on the N-terminus of these three helices, my colleague, Samantha Zaiter, was synthesizing compounds based on the C-terminus (**A3, A4, B3, B4, C3,** and **C4**). Technical issues arose during her synthesis of **C4 (Figure 2.7a)**. The compound appeared pure by LCMS, however, impurities were very observed in NMR. The ^{1}H NMR spectra had the following additional peaks: ^{1}H NMR (600 MHz, D_2O) δ 3.946(s, 17H), 3.886-3.869(m, 12H), 3.745-3.728(m, 12H) **(Figure 2.7b).** Based on the HSQC and COSY spectra, the additional peaks do not connect with the backbone of **C4**, nor were they a solvent impurity. The impurity was not observed in the UV spectrum or the total ion count of the LCMS or the HPLC trace and therefore it was not possible to separate it via HPLC. The synthesis of **C4** was replicated and monitored by both LC-MS and NMR at each step for the impurity, and it was only upon cyclization that the impurity was observed by ^{1}H NMR.

(a)



(b)

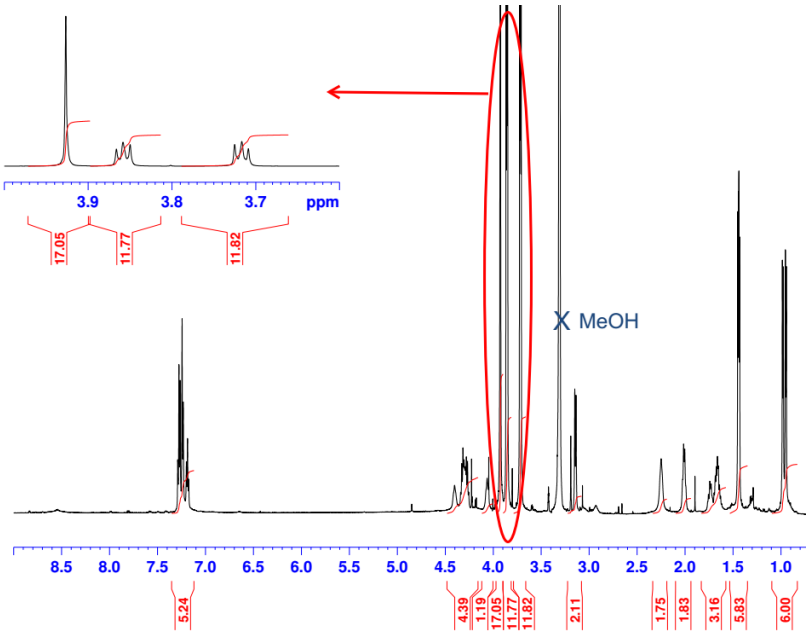


Figure 2. 7: Structure and ^{1}H NMR spectrum of **C4**. The highlight peaks belong to impurity

Cyclisation reactions are run using anhydrous solvent and a mixture of coupling agents in order to enhance the formation of the amide bond required for macrocyclization. The McAlpine group typically uses three different coupling reagents for solution phase macrocyclization but usually in less than 1 equivalent each: HATU, TBTU, and DMTMM (**Figure 2.8)**.[16] This method, reported by the McAlpine lab suggested that different conformations of the linear peptide were in solution and the individual conformations were activated by different coupling agents[17].

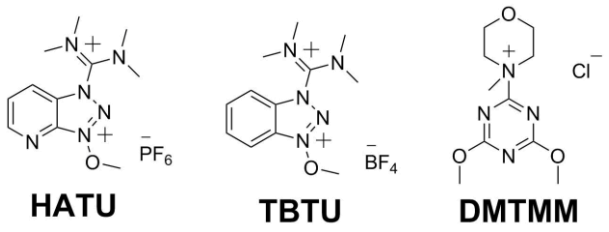
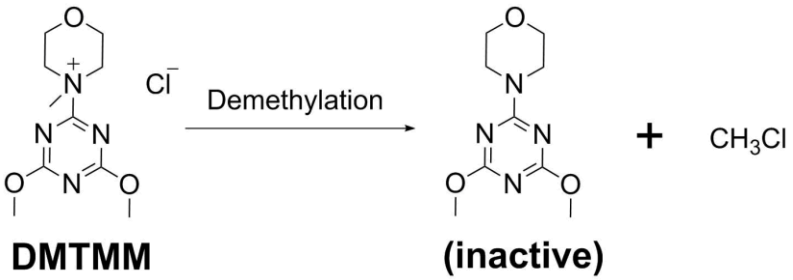


Figure 2. 8: Structure of HATU, TBTU and DMTMM.

All three reagents appeared to provide benefits, and generally the by-products could be easily removed.[18] However, under anhydrous conditions, DMTMM can be demethylated and yield methyl chloride and non-reactive 2-(morpholi-4-yl)-4,6-dimethoxy-1,3,5-triazine (**Scheme 2. 4).**[18] This process gives the exact NMR spectra as the impurity observed in **C4** (**Figure 2.7b).**

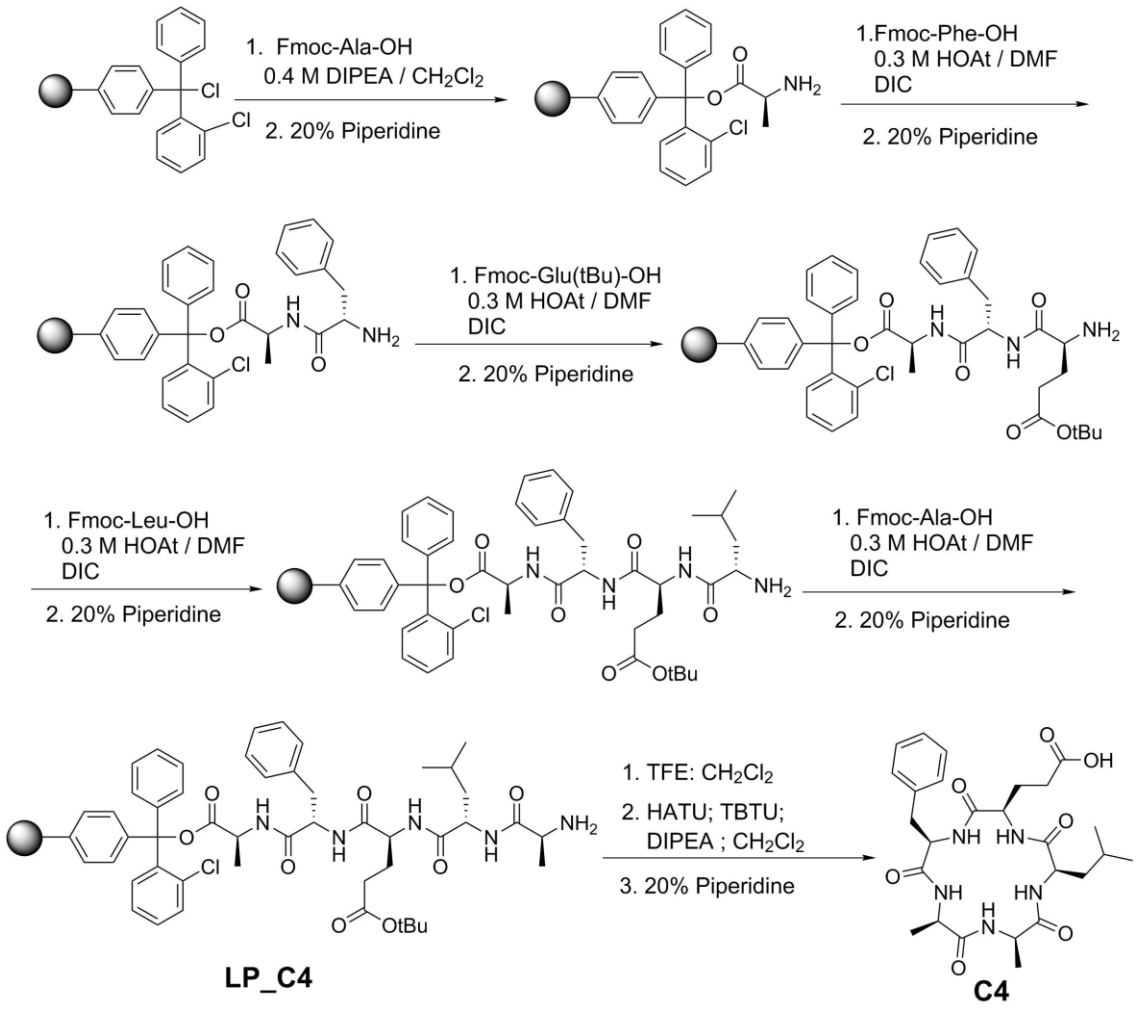


Scheme 2. 4: Decomposed of DMTMM to an inactive molecule.

To test this hypothesis, the synthesis of **C4** was carried out again. **Scheme 2. 5** shows the synthesis of compounds **C4** in detail. Before loading, 2-ClTrt resin was swelled in dichloromethane for 30 minutes. Then the first amino acid, Fmoc-protected alanine was dissolved and added to the drain resin using a solution of 0.1 M DIPEA in dichloromethane. A sample of resin was removed, and the resin loading was determined

to be 0.63 mmol/g. Fix using same language I used The Fmoc protecting group was removed using 20 % piperidine in DMF for 2 times 10 minutes. To confirm the Fmoc removal, a positive ninhydrin test was used. Following the deprotection, the coupling of Fmoc protected phenylalanine was achieved using both the coupling agents HOAt and DIC. The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected glutamate, Fmoc-protected leucine and Fmoc-protected alanine, and finally generate the desired protected linear peptide, **LP_C4**.

LP_C4 was cleaved from the resin under mildly acidic conditions using 50% (v/v) TFE in dichloromethane for 24 hours. LCMS was performed to monitor the reaction progress. The slurry was filtered under vacuum, and the filtrate containing the cleaved linear peptide was collected. The solvent was removed under reduced pressure to afford the LP_**C4** to precipitation as a fine off-white powder.



Scheme 2. 5: The synthesis scheme for **C4**

LP_C4 was then cyclised without DMTMM. The reaction was carried out under dilute conditions (0.001 M) in anhydrous dichloromethane, using DIPEA and a mixture cocktail composed of the following coupling agents: HATU, and TBTU. **LP_C4** and coupling agents were dissolved separately in order to make each a solution of 0.001 M. The dissolved peptide was injected into coupling agent cocktail solution dropwise. Although the cyclisation took a longer time to go to completion, the impurity was not present in the crude cyclised product. Then the protected **C4** was globally deprotected

using TFA and anisole to produce the crude deprotected **C4.** Pure **C4** was obtained after a single round of HPLC purification, as confirmed by ^{1}H NMR in the final yield of 12% (**Figure 2.9**). Interestingly, **C4** was the only compound that had the impurity associated with DMTMM, all other compounds used DMTMM during the cyclization without producing the impurity.

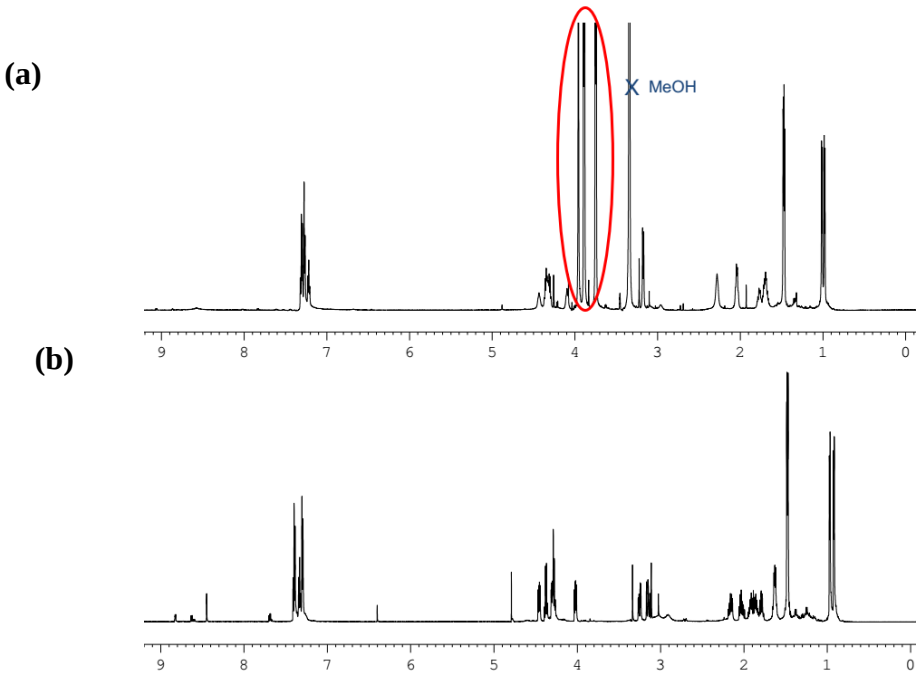


Figure 2. 9: ^{1}H NMR spectrum of **C4**. a) The highlighted peaks belong to impurity when the compound was first synthesized. b) spectrum belongs to **C4** when the compound was synthesized without DMTMM. This spectrum lacks impurity peaks.

2.2.2 Binding assay

2.2.2.1 Compound Screen

With these twelve TPR1 peptides in hand, the author performed an *in vitro* protein binding assay in order to evaluate the compound's impact on the Hsp70–HOP interaction. This assay involved a pulldown technique, followed by western blot quantification, which is a well-established and "gold-standard" method used to determine the physical interaction between two proteins (**Figure 2. 10**).[19] All the compounds were screened at 5 and 50 μM against 200 nM Hsp70 and 100 nM HOP.

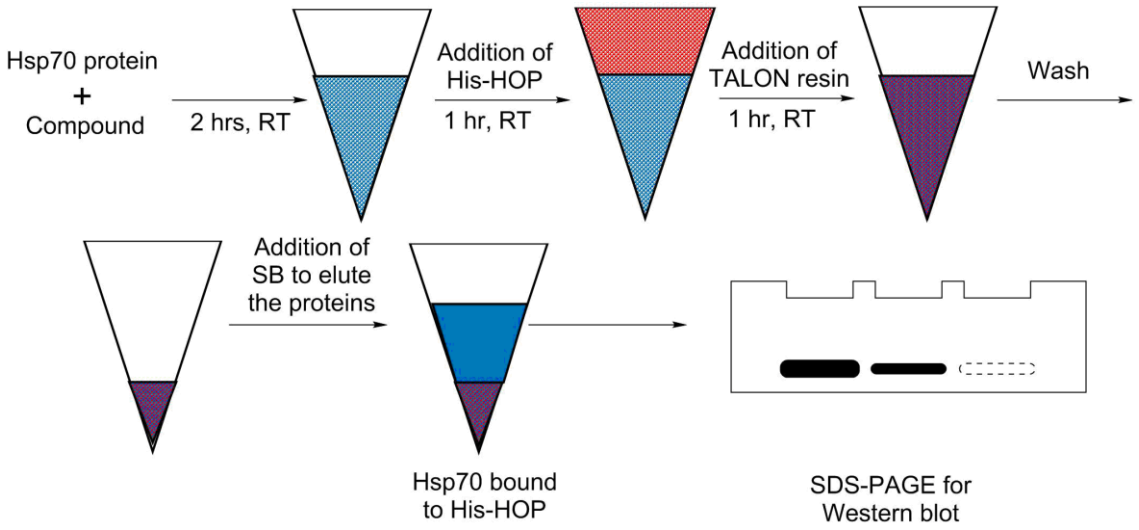


Figure 2. 10 : Schematic for pulldown assay

In the pulldown binding assay, Hsp70 was incubated with each compound in binding buffer for two hours. The control comprised of 1% DMSO in place of the compounds, due to all compounds were dissolved in DMSO and for each reaction 1% of DMSO were added. His-tagged HOP was added to each reaction, and incubating for another hour at

room temperature, allowing it to bind to Hsp70. Talon-metal affinity resin to immobilise the His-tagged proteins, was added to all of the reactions and incubated for additional one hour. All of above steps were carried out in a buffer containing 1% Triton-X-100 at room temperature with gentle agitation to ensure that all binding events were specific, and not a function of random aggregation effects. The resin was then isolated and washed 5 times with washing buffer to remove non-specific binding.

The resin-bound proteins were then eluted by boiling in sample buffer, which contains 0.5 M DTT and 10% SDS to cleave the protein from resin and also denature the protein. The supernatant was loaded on a 4-8 % SDS-PAGE gel to allow the HOP and Hsp70 bound to HOP to be separated using gel electrophoresis. Subsequent transfer to PVDF membrane and detection using antibodies via western blot gave bands that could be quantified for both proteins. Detection and quantitation of Hsp70 bound to HOP in the presence and absence of each compound, relative to the DMSO control showed how effectively the compounds impacted the binding interaction (**Figure 2.10)**.

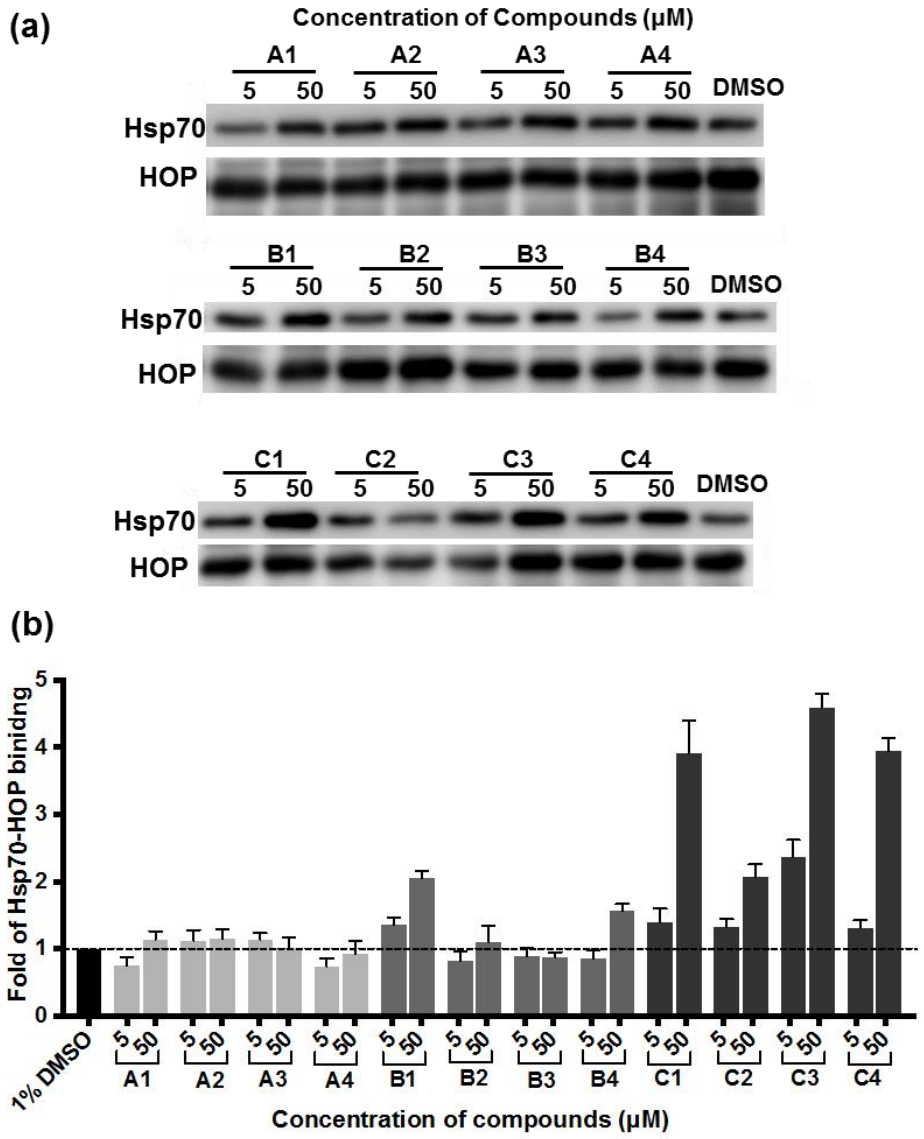


Figure 2. 11: The activity of the 12 TPR1 peptides on the binding interaction between Hsp70 and HOP. (**a**) Representative western blot images of Hsp70 from one replicate of each compound series (**A1-4, B1-4, C1-4**) with a representative image of HOP shown. (**b**) The fold of Hsp70 bound to HOP, relative to the control (1% DMSO). The data represent the mean ± SEM, from at least three independent experiments.

By comparing their efficacy to the DMSO control, compounds that showed a similar ratio of Hsp70 bound to HOP would be considered inactive. All of the compounds that were designed from Helix A showed no activity in disrupting the Hsp70-HOP interaction (**Figure 2.11).** Compounds from the B series showed some binding disruptions, where

B2 and **B3** were inactive, but **B1** and **B4** showed a small increase in the amount of Hsp70 bound to HOP. Unexpectedly these compounds behaved as enhancers of the interaction between Hsp70 and HOP **(Figure 2.11).** Compounds based on Helix C, were very active enhancers, where **C1**, **C3** and **C4** were the most effective. At 50 µM, **C1, C3** and **C4** showed a minimum of 4-fold increase in the Hsp70-HOP interaction relative to the control. Based on the results of the compound screen, **C1, C3** and **C4** were chosen as potential leads for the future evaluation, while **C2** was used as negative controls (**Figure 2. 12**).

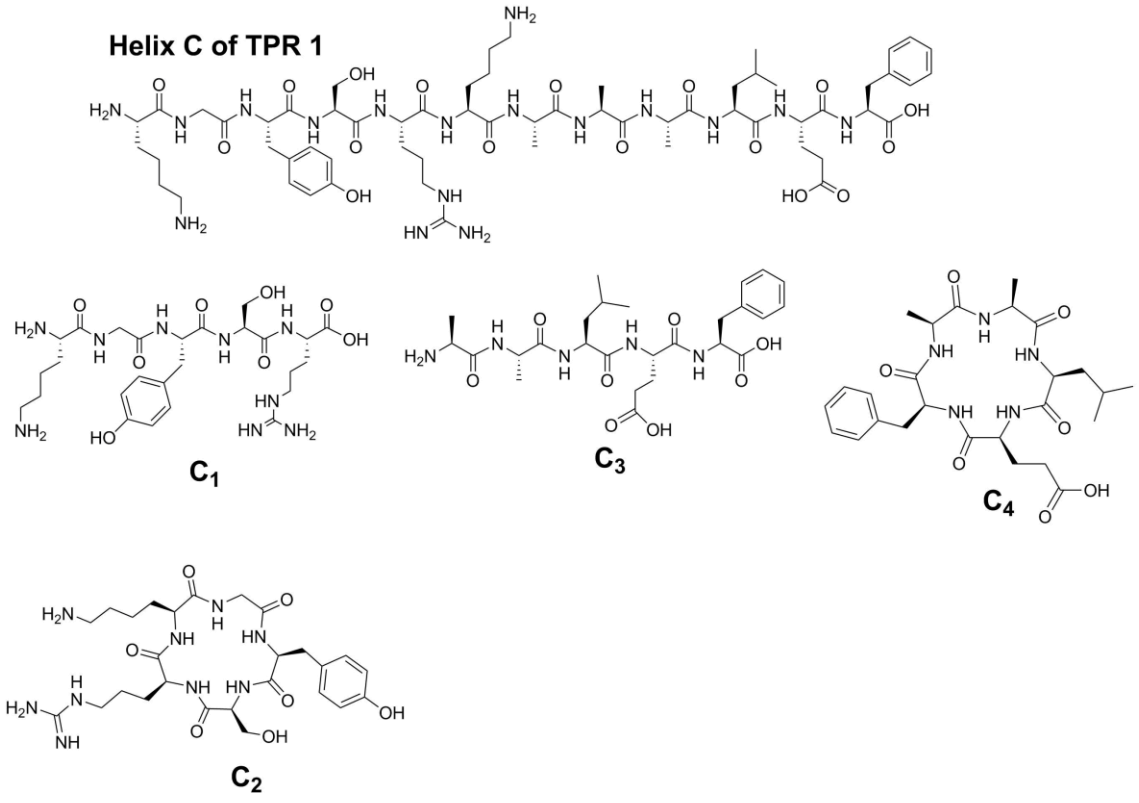


Figure 2. 12 Structure of Helix C, and all C-series compounds C2 was used as negative control.

2.2.2.2 Concentration dependent assay

These four compounds were tested in the binding assays at multiple concentrations

to determine if their activity was concentration dependant. The author tested compounds **C1-C4** at five concentrations ranging from 0.5 to 50 μM, using the same conditions described in the binding assay (**Figure 2.10**). Each compound was incubated with Hsp70 at different concentrations, followed by the addition of His-tagged HOP. The control comprised of 1% DMSO in place of the compounds. All reactions were conducted in a buffer containing 1% Triton-X-100 as well. TALON resin was used to immobilise His-tagged protein. Detection and quantitation of Hsp70 bound to HOP in the presence of each compound, relative to the DMSO control.

These concentration studies showed a clear trend: compounds **C1, C3,** and **C4** all increased the amount of Hsp70 that bound to HOP in a concentration dependent manner (**Figure 2.13**). Despite being the cyclic variant of **C1**, and comprised of the same exact 5 amino acids, **C2** was not active at any concentration. **C2** was therefore be considered as a negative control for all future studies because it was sequentially identical to **C1** but was inactive. These data indicate that the cyclic peptide **C2** is locked in the wrong conformation for binding (**Figure 2.13**). The results from the concentration dependent assay match the trends observed from the initial compound screen, which clearly showed that compounds **C1, C3,** and **C4** could effectively stabilise and enhance the Hsp70-HOP interaction. These results were intriguing because peptides were derived from helices of HOP's TPR1 domain and we had anticipated that the mimics we synthesized would competitively bind to Hsp70 and prevent HOP from forming an interaction. Instead, these molecules promoted the binding interaction. Therefore, it was important to understand how this enhanced binding affinity translated to a functional assay that involved Hsp70 and HOP.

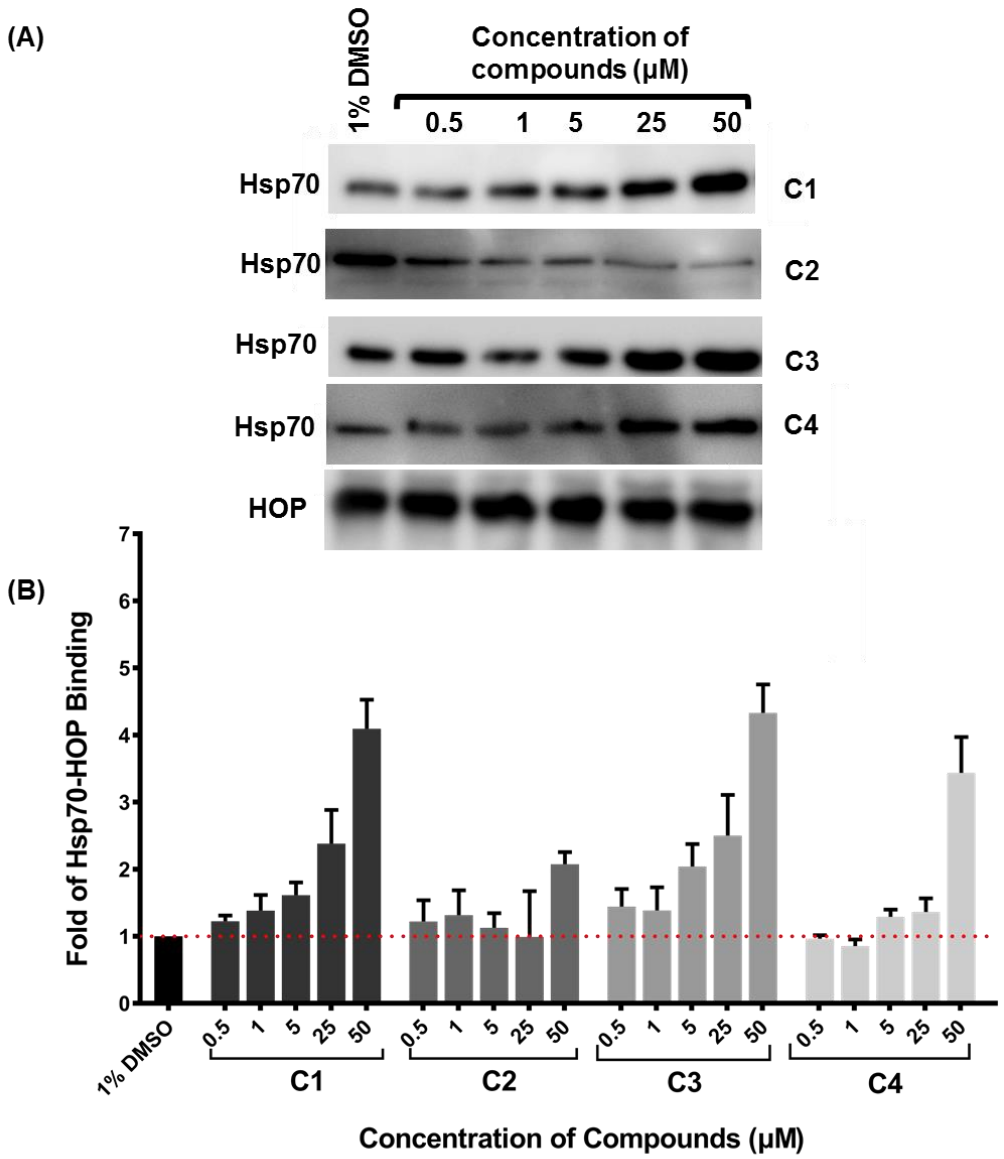


Figure 2. 13: Concentration dependent assay of the two most active compounds (**C1-4**) (**A**) Representative western blot images of Hsp70 from one replicate of each compound series (**C1-4**) with a representative image of HOP shown. (**B**) The fold of Hsp70 bound to HOP, relative to the control (1% DMSO). The data represent the mean ± SEM, from at least three independent experiments.

2.2.3 Luciferase Refolding Assay

Compounds **C1, C3** and **C4** all stabilized the Hsp70-HOP complex, and therefore it was possible they would enhance the protein-folding function of Hsp70. Enhancing Hsp70's protein folding function would potentially allow nascent or misfolded proteins

to be refolded more effectively into their native state in the presence of compounds. As a result, these molecules could be used for some degenerative diseases, such as Alzheimer's or Parkinson's disease.[20] In both diseases, proteins are denatured and misfolded protein, and there is a decrease in the protein folding machinery that is regulated by Hsp70. Another hypothesis is that these three peptides may lock Hsp70 and HOP together, halting their dynamic assembly and disassembly. Trapping the proteins into a single conformation would lead to their inability to fold proteins, and the molecules would be essentially protein-folding inhibitors. As a result, the cellular would aggregate, and precipitate out under physiological conditions, resulting in cell death, an optimal outcome for a cancer cell.

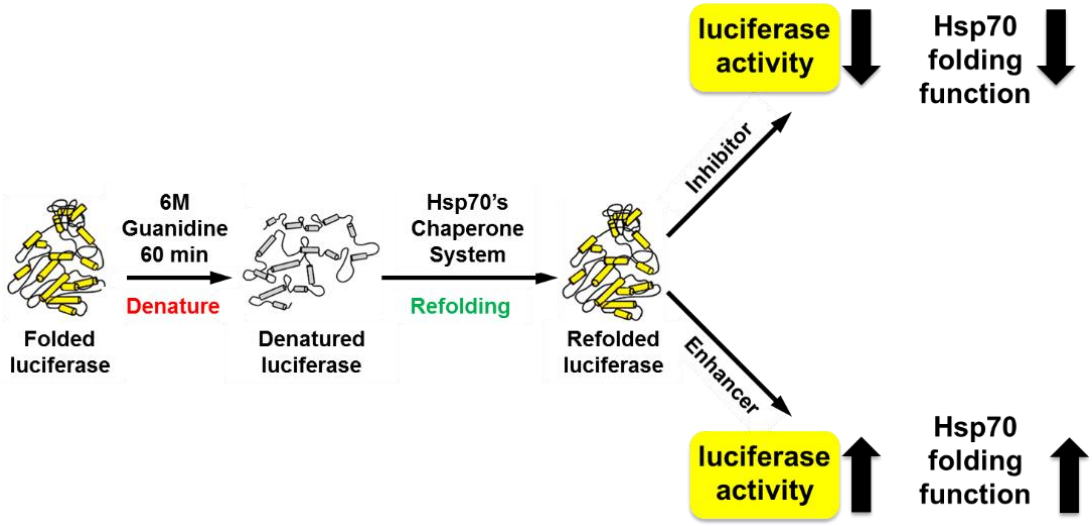


Figure 2. 14: Schematic depiction of a luciferase refolding assay.

Evaluating how the stabilisation of Hsp70-HOP by the C-series (**C1-C4**) impacted protein folding was accomplished using a luciferase re-folding assay (**Figure 2.14**). The luciferase refolding assay was adapted from established literature methods[21] The whole

chaperone refolding system was replaced from bacteria protein to recombinant human protein in order to keep all proteins consistent with binding assay. The ratio of Hsp70/Hsp40/GrpE constant at 1.0:0.2:0.1 was kept. Although Hsp70 /Hsp40/GrpE could accomplish very efficient refolding, With the addition of Hsp90 and HOP, the refolding of denatured luciferase protein was dramatically increased that is ~40% higher when using the same concentration of Luciferase (**Figure 2.15**). This result indicated that luciferase protein could be transferred from Hsp70 to Hsp90 via a HOP binding, and therefore more luciferase could be refolded and produce higher luminescent signal.

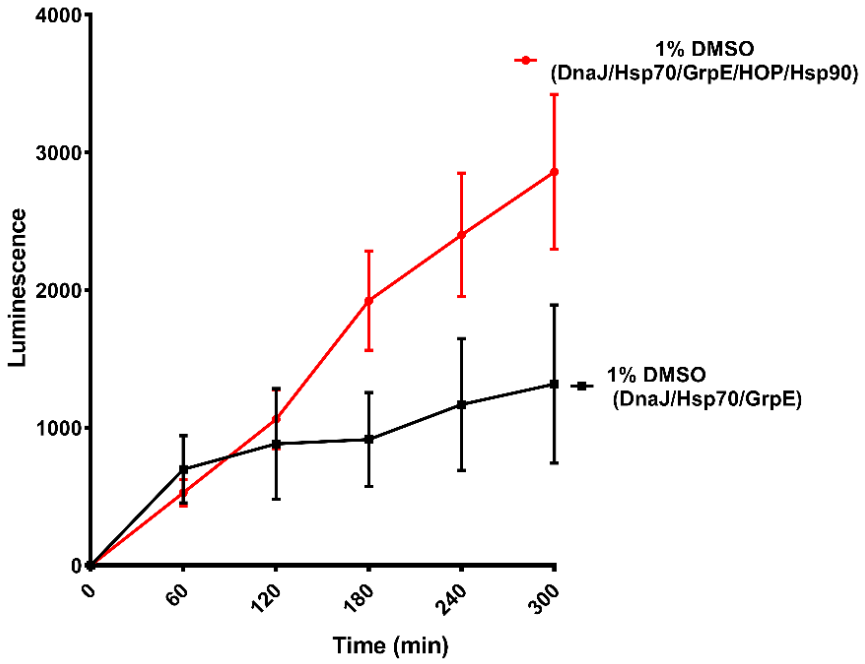


Figure 2. 15: Impact of Hsp90 and HOP on Luciferase refolding. Compared the signal produced by luciferase in the presence of DNAJ, Hsp90, HOP, Hsp70, GrpE versus DNAJ, Hsp70, and GrpE, the experiment that has Hsp90 and HOP produces a signal that is ~40% higher when using the same concentration of Luciferase.

All compounds from the C series were screened in the luciferase assay. To start with, luciferase was denatured by 6 M Guanidine for 60 mins at room temperature. Native luciferase was used as a control and kept at room temperature for 60 mins as well. Both

the native and denatured luciferase were then diluted 1 in 40 using diluting buffer on ice for 20 minutes.

The compounds (50 µM) were pre-incubated with 0.5 µM Hsp70 in refolding buffer for 30 minutes on ice, followed by the addition of 50 nM Hsp90, 50 nM HOP, 50 nM GrpE, 100 nM DnaJ, and denatured luciferase. Following the addition of denatured luciferase, the reactions were incubated at room temperature. 2 µL aliquots of each reaction were taken at various time points and were combined with 23 µL of Bright-Glo reagent in a white 384-well polystyrene microplate. The luminescence of the respective wells was measured using a Tecan F200 Pro multimode plate reader. The final data was plotted as the % of luciferase refolded over time relative to the maximum luminescence signal from DMSO at 5 hours. The luminescence of these wells was measured, and the raw luminescence values were plotted.

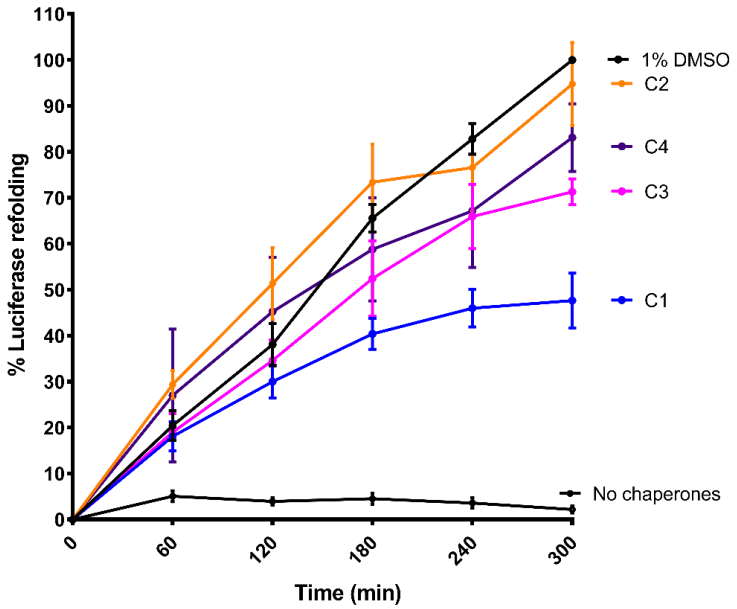


Figure 2. 16: impact of compounds **C1, C2, C3** and **C4** on luciferase refolding. Each treatment was made relative to the raw luminescence value of 1% DMSO at the 5-hour period as this represented 100% refolding.

Despite the stabilization effect that **C1, C3** and **C4** had on the binding event between Hsp70 and HOP, **C4** showed comparable activity to DMSO and **C2**, the negative control (**Figure 2.16**). **C3** was slightly active in this assay and inhibited luciferase refolding by ~30%. **C1** was the most active in the re-folding assay and at 50 µM, **C1** inhibited luciferase refolding by ~60% (**Figure 2.16**).

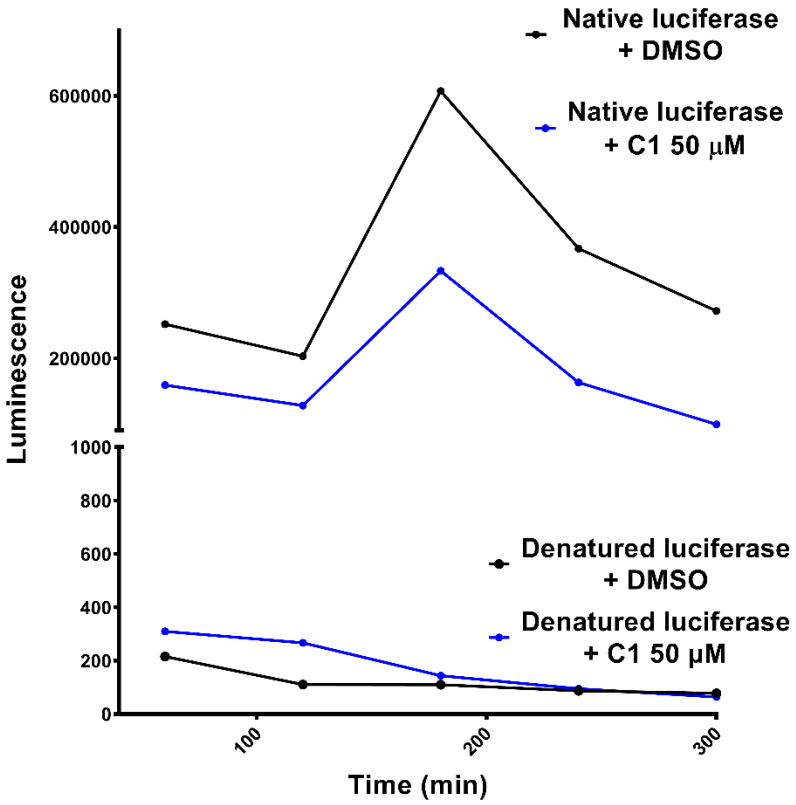


Figure 2. 17: Control experiment to determine if compound **C** interact with either denatured or native luciferase. **C1** at 50 µM were incubated with native or denatured luciferase on ice for hour in the absence of chaperones, then the luminescence of each treatment was measured. Data were plotted as raw luminesce for each treatment.

The **C1** were evaluated with native and denatured luciferase in the absence of chaperones and measured with luminescence over time (**Figure 2.17**). This control

experiment was important in order to ensure that the molecule is inhibiting the refolding process and not acting as a quenching agent. Based on the data in Figure (**Figure 2.17**), **C1** didn't impact native or denatured luciferase in the absence of chaperones, indicating that the influence of **C1** on the denatured luciferase refolding was via their regulation of the chaperone system. Hence, the decrease in luciferase protein folding with the present of **C1** is related to the stabilization of the Hsp70-HOP interaction and not a luminescence quenching effect.

In order to confirm that **C1** was actively inhibiting the luciferase refolding event, the author evaluated **C1** in a concentration dependent assay (**Figure 2.20**). Even at concentrations as low as 10 µM, **C1** impacted the ability of the Hsp70 chaperone machinery to fold luciferase demonstrating that the protein folding machinery of the Hsp70 was responding to the effects of **C1** treatment (**Figure 2.20**).

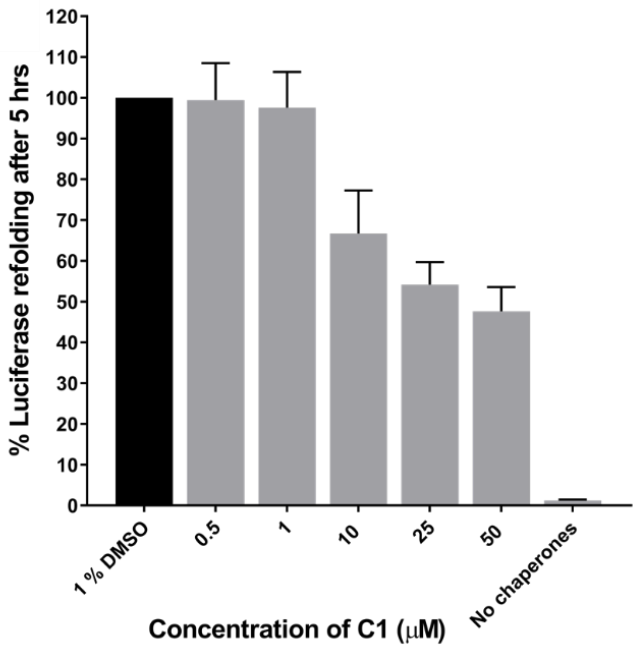


Figure 2. 18: The impact of **C1** at multiple concentrations on luciferase refolding after 5 hours. The graphs represent the mean ± SEM, from at least three independent experiments.

The binding assay results (**Figure 2.11 & 2.13**) and luciferase data **(Figures 2.16 & 2.18**) indicate that **C1**, and to a lesser extent **C3**, effectively impacted the Hsp70-HOP interaction and inhibited the Hsp70 chaperone folding event. However, it was not yet clear if these molecules produced the effects by binding to Hsp70 or via binding to HOP. Synthesising tagged variants of these two compounds would allow the author to evaluate whether the compounds pulled out Hsp70 or HOP or both proteins.

2.2.4 Tagged Compounds Pulldown and Competitive Assay

The PEG4-Biotin was chosen as the tag to label **C1**. Biotin is a small natural vitamin that binds with high affinity to avidin proteins. Biotinylated molecules typically retain biological activity because the biotin group is relatively small (**Figure 2.19**). The resin the author used was Neutravidin, which is a chemically modified version of avidin with higher capacity and minimal nonspecific binding. The bound formation between biotin and the resin is rapid and stable. PEG spacer arm increases solubility of the peptide and also prevents the labelled peptide aggregating with each other. The extra-long linker arm reduces steric hindrance, which could maintain the binding activity of **C1** in the tagged compounds.

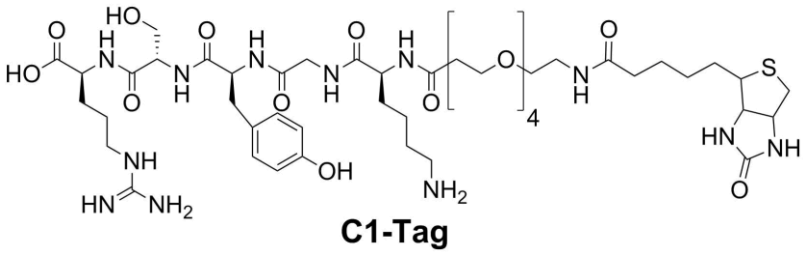
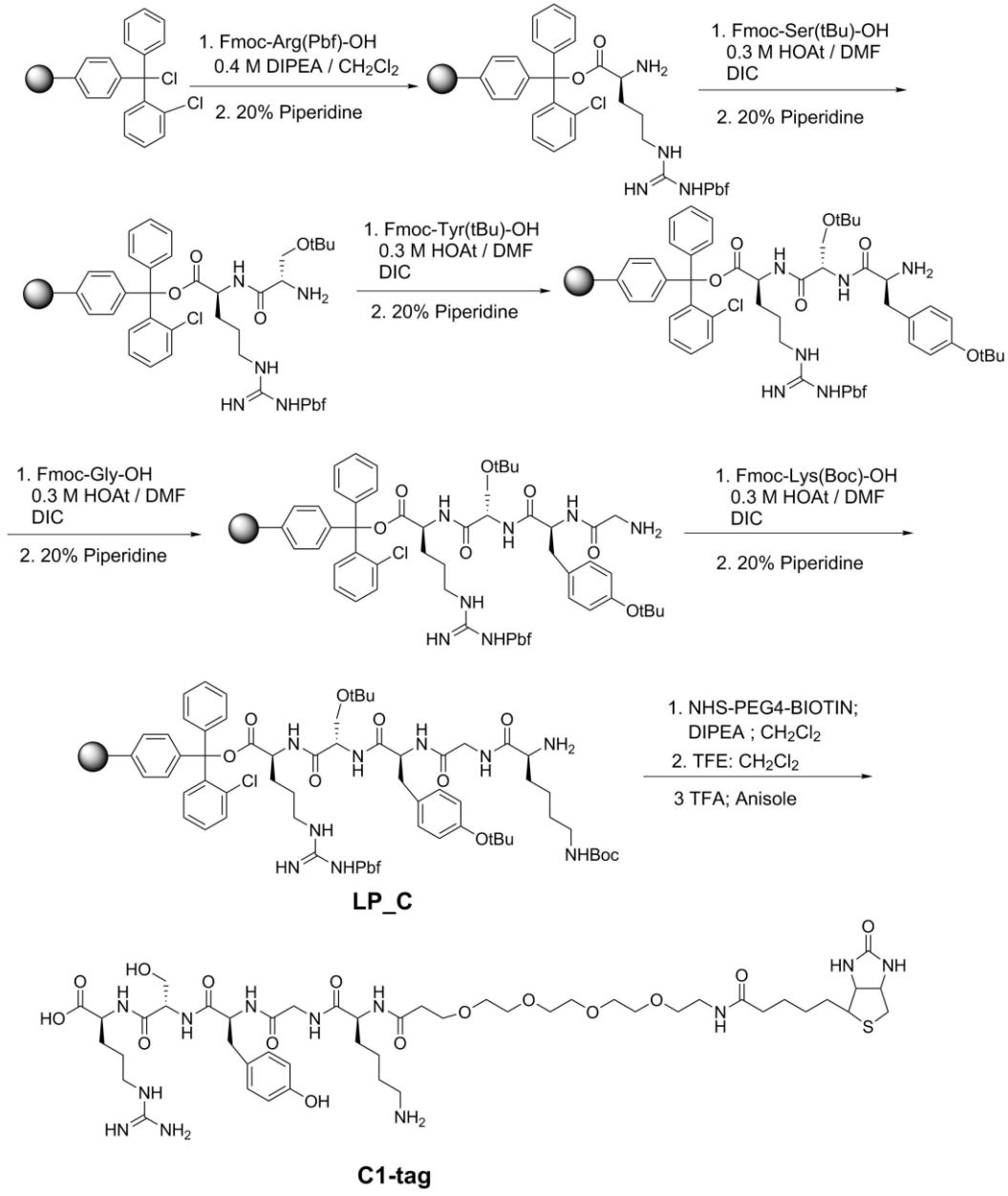


Figure 2. 19: Structure of **C1-Tag**

2.2.4.1 Synthesis of C1-Tag

Scheme 2.6 shows the synthesis of compounds **C1-Tag** in detail. The synthesis of **C1-Tag** (**Figure 2.19**) was achieved utilising SPPS. Before loading, 2-ClTrt resin was swelled in dichloromethane for 30 minutes. Then the first amino acid, Fmoc-protected arginine was dissolved and added to the drain resin using a solution of 0.1 M DIPEA in dichloromethane. A sample of resin was removed, and the resin loading was determined to be 0.73 mmol/g. The Fmoc protecting group was removed adding a solution of 20 % piperidine in 20 % piperidine in DMF for 10 minutes, draining and then adding fresh solution for another 10 minutes. To confirm the Fmoc removal, a positive ninhydrin test was used. Following the deprotection, the coupling of Fmoc protected serine was achieved using both the coupling agents HOAt and DIC. The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected asparagine, Fmoc-protected serine and Fmoc-protected tyrosine, and finally generate the desired protected linear peptide, **LP_C**.



Scheme 2. 6: The synthesis scheme for **C1-Tag**

The resin-bound **LP_C** was then dried in vacuo overnight. All biotin-coupling reactions were carried out under nitrogen in a final concentration of 0.05 M in anhydrous DCM (**Scheme 2.6**). The EZ-Link NHS-PEG4-Biotin was coupled to the resin bound linear peptide using DIPEA. N-hydroxysuccinimide (NHS) group is the most commonly used biotinylation reagents. In weakly basic buffers, like DIPEA. NHS-biotin reagents

react efficiently with primary amino groups (-NH2) by nucleophilic attack, forming an amide bond and releasing the NHS group. The mixture was stirred at room temperature for 2 hours and reactions were monitored by negative ninhydrin test and LCMS. The Tagged peptide was then cleaved from the resin, deprotected and purified in a similar manner to **C1** and final compounds were characterised using LC/MS, 1H NMR and 2D NMR in the yield of 15% (**Scheme 5). C3-Tag** (**Figure 2.20**) was synthesised by my colleague Samantha Zaiter following the same synthetic route as **C1-Tag**.

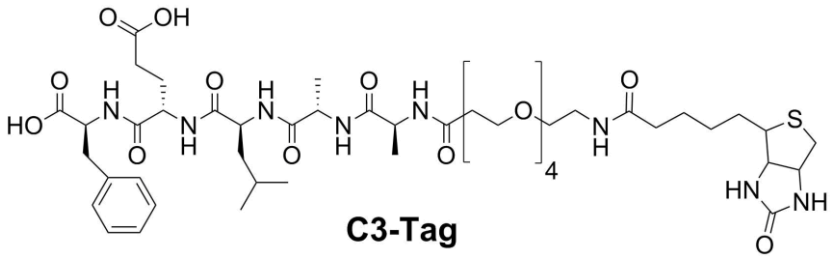


Figure 2. 20: Structure of **C3-Tag**

2.2.4.2 Protein pulldown assay

With **C1-Tag** and **C3-Tag** in hand, a pulldown assay was carried out to determine whether these compounds could directly interact with Hsp70 or HOP. All pulldown experiments were run using similar conditions. Biotin-Tagged compounds were dissolved in 100% DMSO and were diluted to a final concentration of 1% DMSO in each reaction. The Tagged compound at various concentrations (0 – 200 µM) were incubated with the protein in binding buffer at a final volume of 100 µL for 1 hour at room temperature. The proteins were then added at a final concentration of 200 nM to each reaction. Pulldown of the biotin tagged compounds was achieved by incubating each reaction with Neutravidin® for an hour. The Neutravidin® Agarose Resins were pre-blocked with 1.3% w/v of Bovine Serum Albumin (BSA) in binding buffer for 1 hour at room temperature.

Following the incubation with Neutravidin®, the supernatant was removed from each reaction, and the resin was washed six times with wash buffer. Finally, the beads were boiled with 5 x Laemmli sample buffer. The supernatant of each sample was loaded onto an 8% Tris-Glycine gel following the Gel electrophoresis procedure. Proteins were transferred onto a PVDF membrane and imaged following the Western blotting procedure. Hsp70 protein was detected using Hsp70 primary antibody, followed by HRP-conjugated secondary antibody. HOP protein was detected using HOP primary anti-body. Then the membrane was incubated with the HRP-conjugated secondary antibody as well. The relative amount of protein pulled down were analysed via Image J and transformed to a fold of protein pulled down relative to 1% DMSO control.

For the Hsp70 pulldown assay, as the amount of **C3-Tag** was increased, there was not a corresponding increase in Hsp70 bound to the molecule (**Figure 2.21**), showing that **C3** didn't directly bond to Hsp70. In contrast, as the amount of **C1-Tag** increased, more Hsp70 was pulled out, establishing that **C1** bound to Hsp70 (**Figure 2.21**) in a concentration dependent manner. Given the fact that the **C3-Tag** didn't show any activity, the Tagged variant of **C3** could be treated as a perfect negative control which demonstrated that the Peg4-Biotin is not forming any non-selective interactions with Hsp70, proving that the bound Hsp70 was due to the interaction between compound **C1** and Hsp70 (**Figure 2.21**). A pulldown experiment with the Tagged compounds and HOP, showed that **C1-Tag** was also able to pull out HOP, and **C3-Tag** was treated as a negative control in this assay and all only pulled out background levels of HOP (**Figure 2.22**).

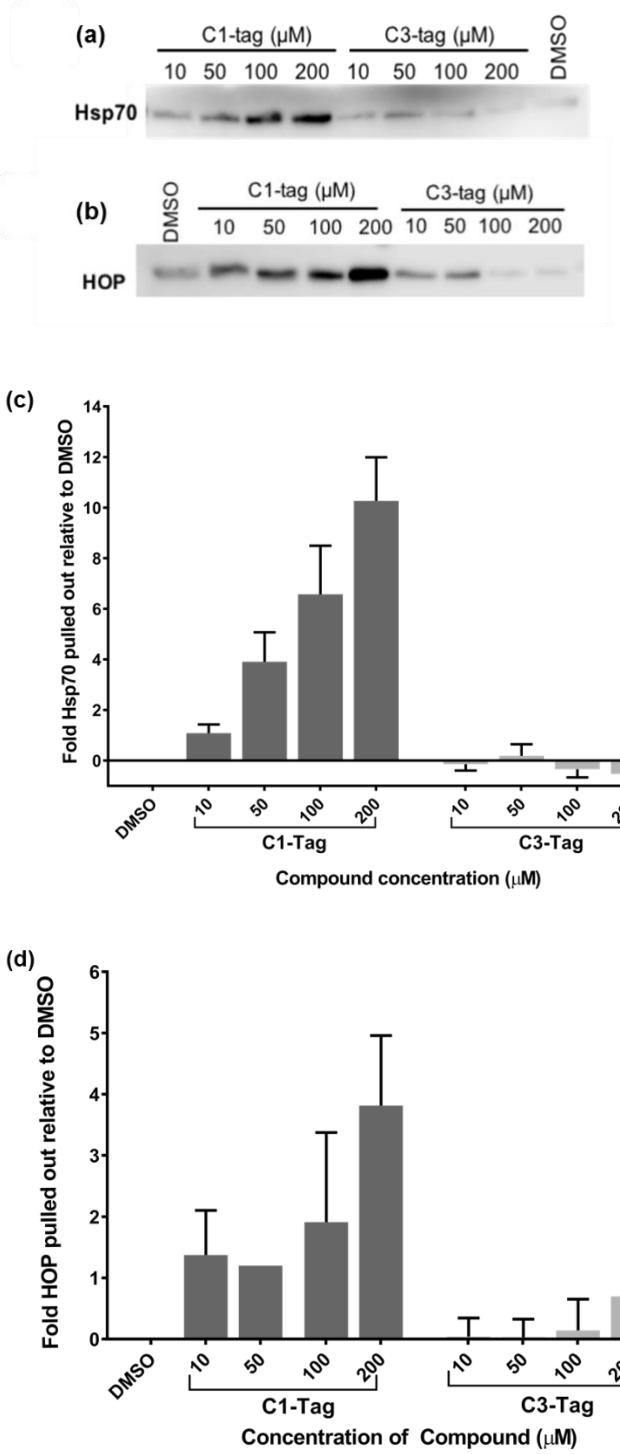


Figure 2. 21: (**a**) A western blot image of the pulldown assay with Hsp70 in the presence of **C1-Tag** and **C3-Tag** at increasing concentrations. The DMSO control is 1% DMSO in place of tagged compound. (**b**) A western blot image of the pulldown assay with HOP in the presence of **C1-Tag** and **C3-Tag** at increasing concentrations. (**c**) Graph showing the average fold Hsp70 pulled down using **C1-Tag** and **C3-Tag** compared to DMSO. (**d**) Graph showing the average fold HOP pulled down using **C1-Tag** and **C3-Tag** compared to DMSO. All treatments were subtracted from the raw signal of DMSO and were subsequently divided by the raw signal of DMSO control. The data represent the mean ± SEM, from at least two independent experiments

A direct comparison between the fold of Hsp70 versus HOP pulled down by **C1-Tag** demonstrates that **C1** is forming a tight interaction with Hsp70, and a weak interaction with HOP (**Figure 2.22**). These data account for the observed trends described above, where **C1**'s ability to stabilise the interaction between Hsp70 and HOP comes from a strong interaction with Hsp70 and a moderate interaction with HOP. The strong interaction with Hsp70 are likely electrostatic interactions between basic residues on **C1** (Arg and Lys) forming ionic bonds with the EEVD, which is the acidic region of the SBD on Hsp70. Hydrophobic interactions have less strength and are likely responsible for forming weak binding connections with HOP, hence the stabilisation observed.

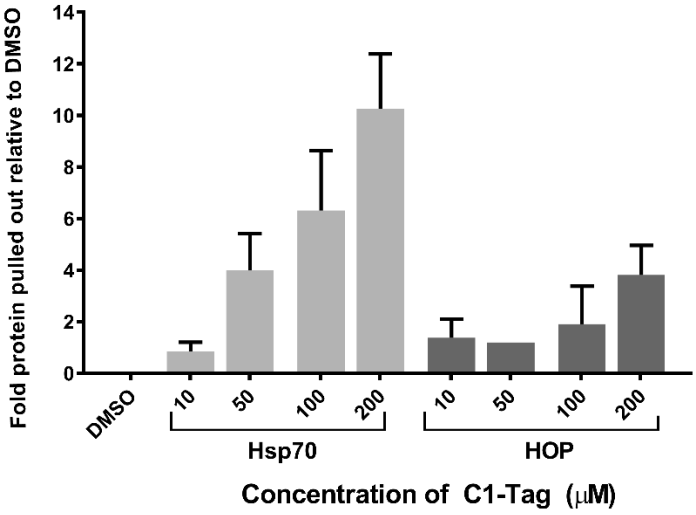


Figure 2. 22: HOP and Hsp70 pulled down using **C1-Tag.** All treatments were subtracted from the raw signal of DMSO and were subsequently divided by the raw signal of DMSO control. The data represent the mean ± SEM, from at least three independent experiments.

2.2.4.3 Competitive binding assay

In order to verify these results, a competitive binding assay was performed using **C1**

-Tag, tagged-free **C1** and Hsp70 (**Figure 2.24**). This assay was carried out under the identical conditions performed in the pulldown assay mentioned before. After **C1-Tag** was incubated with Hsp70, increasing amounts of **C1** ranging from 10 to 200 µM were added in order to displace **C1-Tag** and competitively bind to Hsp70.

Consistent with a molecule that binds to Hsp70, as more **C1** was added and bound to Hsp70, there was a corresponding decrease in the amount of Hsp70 being pulled out by **C1-Tag** because there was less Hsp70 available (**Figure 2.25)**. These data suggest that **C1-Tag** is binding to the same place as **C1**.

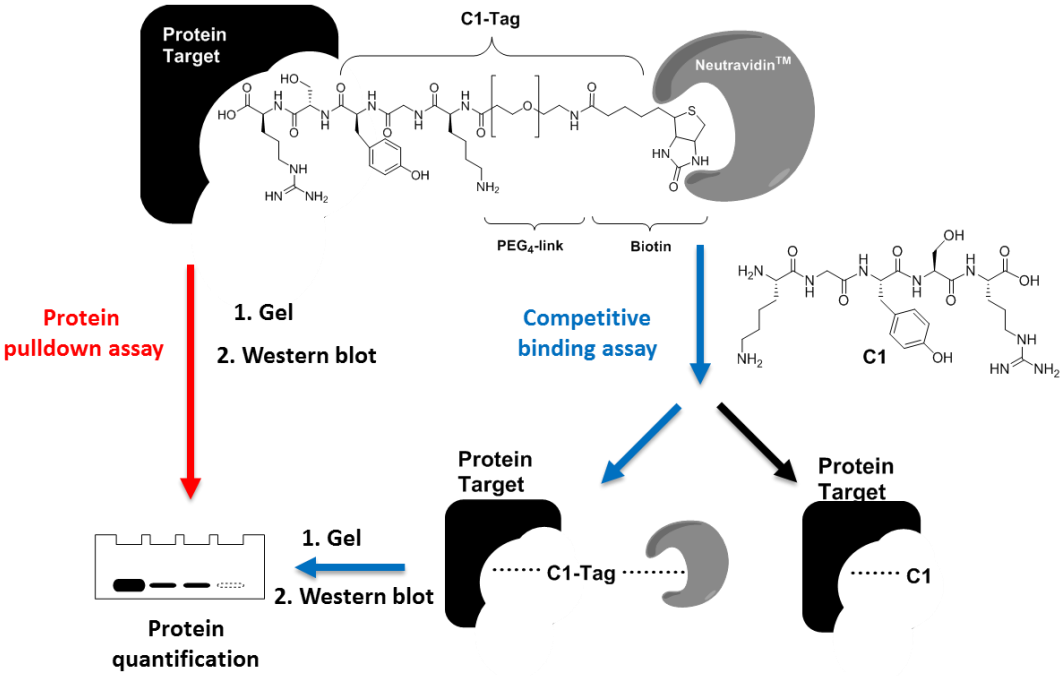


Figure 2. 23: Schematic depiction of protein pulldown and competitive binding assay

Based on the pulldown data (**Figure 2.21, 2,22)** and competitive binding assay (**Figure 2.23),** it appears that **C1** and **C3** stabilise the Hsp70-HOP interaction via different mechanisms. **C1** stabilises the Hsp70-HOP interaction via binding to both Hsp70 and

HOP, albert stronger with Hsp70 than HOP**.** In contrast, only little amount of Hsp70 or HOP was pulled out by **C3-Tag** compared to **C1-Tag** (**Figure 2.23**) indicating that **C3** has little affinity for Hsp70 and HOP in isolated systems, therefore it is likely that **C3** needs both Hsp70 and HOP present in the system.

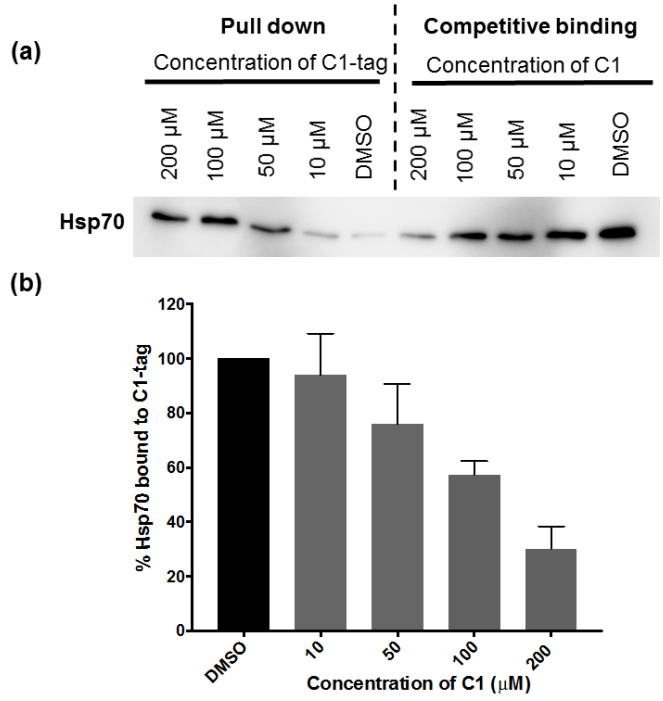


Figure 2. 24: (a)A western blot image of the pulldown of **Hsp70** with **C1-Tag** (left side), here 1% DMSO control is used as a control in place of **C1-Tag**. The competitive binding of **C1** to Hsp70 (right) is shown, the DMSO control replaces **C1** and represents 100% binding. **(b)** Competitive binding data plotted as the mean ± SEM, n = 3.

2.2.5 Domain Expression and Pulldown assay

Given the results we got, **C1** could selectively bond to Hsp70 and effectively enhance the interaction between Hsp70 and HOP, so the question will be does **C1** bind to the same site as HOP does on Hsp70, or **C1** bind to different spots and play allosteric modulation on Hsp70? To answer this question, figuring out the exact binding site of compound **C1** on Hsp70 is required. To explore that , different domains of Hs70 will be expressed in

bacteria and tested with compounds in binding assay.

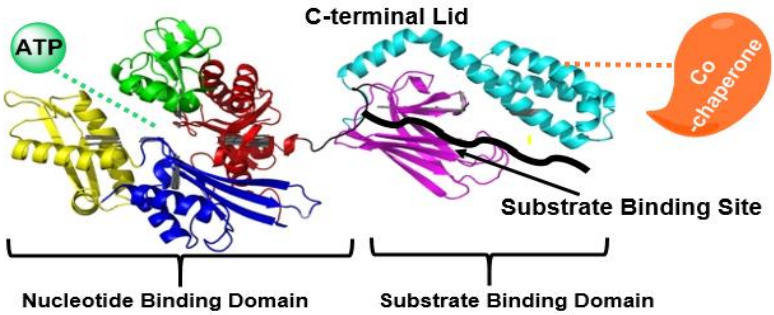


Figure 2. 25: The crystal structure of the E.coli Hsp70 protein

Hsp70 consists of two different domains, N-terminal nucleotide binding domain (NBD), and C-terminal substrate binding domain (SBD) ***(*Figure 2.25*)***.[22] HOP binds to this heat shock protein through the direct interaction between its TPR1 domain and IEEVD (Isoleucine, Glutamic acid, Glutamic acid, Valine, Aspartic acid) region on Hsp70's C-terminal lid ***(*Figure 2.26*)***.

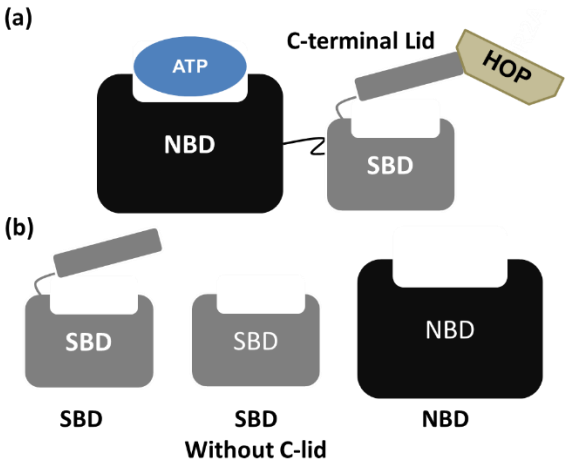


Figure 2. 26: (**a**) Structure of Hsp70, the binding sites of ATP on NBD and **HOP** on C-terminal lid. (**b**) different domains of Hsp70, the full-length SBD was expressed and purified. The future work will be the expression of other two type of domains.

2.2.5.1 Domain expression

SBD of human Hsp72 (**Figure 2.26**) was expressed and purified according to published schemes[23] and the protocols from Jason E. Gestwicki. The pMCSG7 plasmid, which carried the gene of Hsc70 391-646 residues and N-terminal 6xHis tag, was designed and produced by Jason E. Gestwicki's lab. The domain was expressed in BL21(DE3) cells **(Figure 2. 27)**.

Proteins were transformed into Escherichia coli (*E.coli*) strain BL21(DE3) for expression. The *E.coli* cells were made chemically competent using $CaCl_2$, then the plasmid coding the SBD was transformed into the bacteria using heat shock. The transformed cells were streaked on an agar plate supplemented with ampicillin. A single colony of transformed cells was cultured on a small scale (200 mL) in LB media supplemented with ampicillin at a final concentration of 100 µg/mL. Then 200 mL culture was scaled up to 15 L using a 19 L New Brunswick ^{TM} Bioflo 415 fermenter. The growth of the large-scale culture was monitored via UV-VIS spectroscopy, where the optical density of the culture at 600 nm (OD_{600}) was montired overtime, till a value of 0.6-0.8 was reached indicating that the bacteria where at their optimal growth phase. Once the bacteria were at their optimal growth phase, isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 200 µM was added to induce the expression of the SBD. The IPTG induction was carried out at 30 ^{o}C for 16 hrs. Then the cells were harvested from the fermenter and the cells were centrifuged at 10,000 g for 30 minutes at 4 ^{0}C, to afford 200 g of wet cells containing the SBD protein.

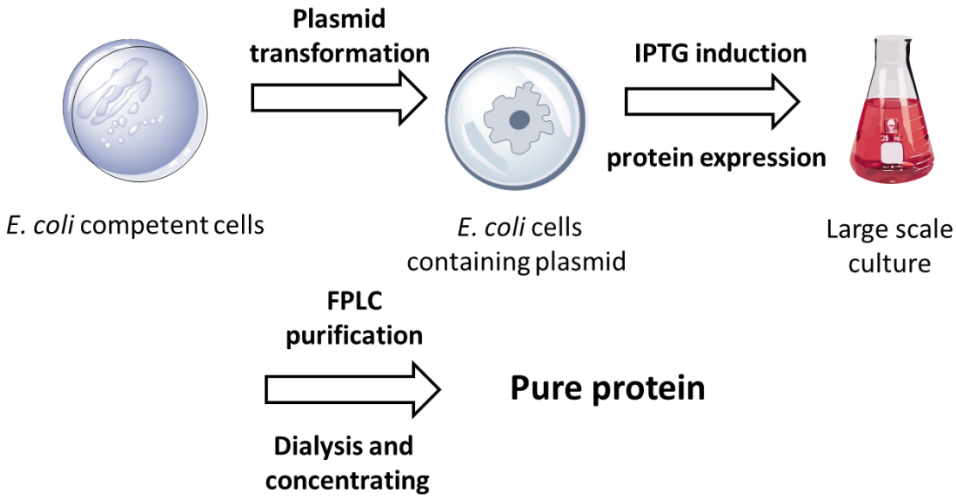


Figure 2. 27: Schematic depiction of domain expression

Bacterial cell pellets containing His-tagged SBD were suspended in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, and lysed using a ultrasonic homogenizer sonicator. Crude lysate was clarified by centrifugation at 10,000 x g for 30 minutes. The supernatant, which was the cell lysate was collected, filtered and loaded onto a 5mL Ni-NTA Superflow cartridge. The His-Tagged SBD was then eluted via a linear gradient up to 300mM imidazole. The SBD was purified via two rounds of FPLC with Ni-NTA affinity purification. The fractions were concentrated, and the protein was dialysed against ddH_2O overnight, followed by buffer exchange with 25mM Tris-HCl, 5mM $MgCl_2$ and 10mM KCl buffer.

2.2.5.2 Domain pulldown assay

With the pure SBD in hand, the domain pulldown experiment was run using similar conditions as protein pulldown assay (**Figure 2.23**). **C1-Tag** and **C3-Tag** were dissolved in 100% DMSO and were diluted to a final concentration of 1% DMSO in each reaction. The tagged compounds at various concentrations (0 – 200 µM) were incubated with the protein in binding buffer at a final volume of 100 µL for 1 hour at room temperature. The proteins were then added at a final concentration of 5 µM to each reaction. Pulldown of the biotin tagged compounds was achieved by incubating each reaction with Neutravidin® for an hour. The Neutravidin® Agarose Resins were pre-blocked with 1.3% w/v of Bovine Serum Albumin (BSA) in binding buffer for 1 hour at room temperature. Following the incubation with Neutravidin®, the supernatant was removed from each reaction, and the resin was washed six times with wash buffer. Finally, protein was eluted from the resin and the supernatant of each sample was analysed using SDS-PAGE gel electrophoresis. Proteins were transferred onto a PVDF membrane. The SBD was detected using anti-6X His-tag antibody followed by a chemiluminescent detection using Horse Raddish Peroxidase (HPR) conjugated secondary anti-body. The relative amount of protein pulled down were analysed via Image J and transformed to a fold of protein pulled down relative to 1% DMSO control.

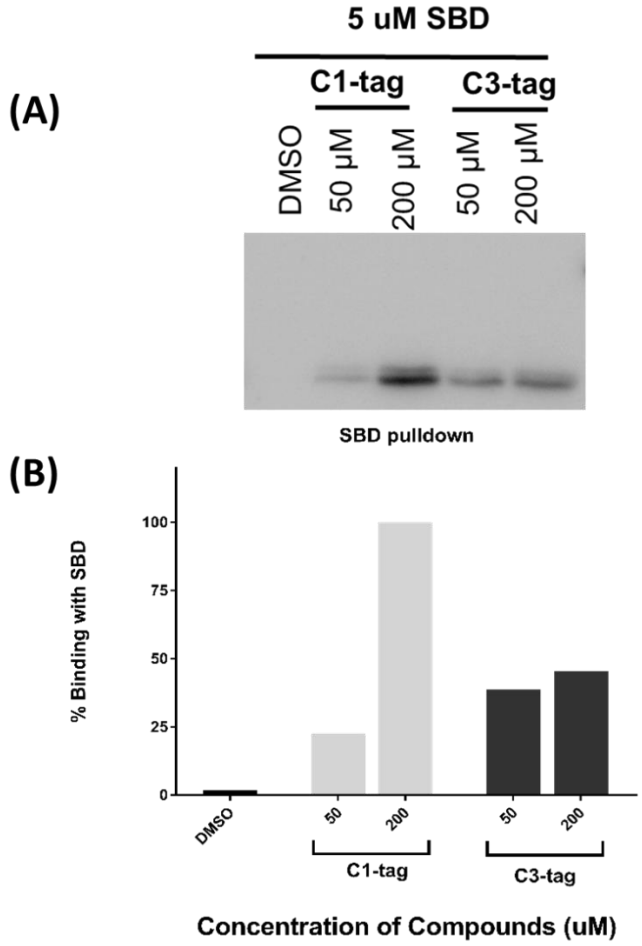


Figure 2. 28: (**A**) A western blot image of the pulldown assay with SBD in the presence of **C1-Tag** and **C3-Tag** at increasing concentrations. (**B**) Graph showing the average fold Hsp70 pulled down using **C1-Tag** and **C3-Tag** compared to DMSO.

Evidence that **C1** bound to the SBD was produced by pulling down the SBD protein in a concentration dependent manner using **C1-Tag** (**Figure 2. 28**). At 200 µM **C1-Tag** pulled down Hsp70's SBD more effectively compared to **C3-Tag**. Based on these data, the SBD is a likely binding for **C1** on Hsp70. Given that **C1** is able to bind to the SBD of Hsp70 it directly stabilising the Hsp70-HOP interaction, and this leads to an inhibition of Hsp70 's folding function. Therefore, from the library of 12 TPR1 peptides, **C1** was identified as the lead molecule in the series. SAR was then conducted on **C1** to develop a more potent analogue and to identify which residues are critical for the activity of the peptide.

2.2.6 Analogues of C1: Alanine Scan

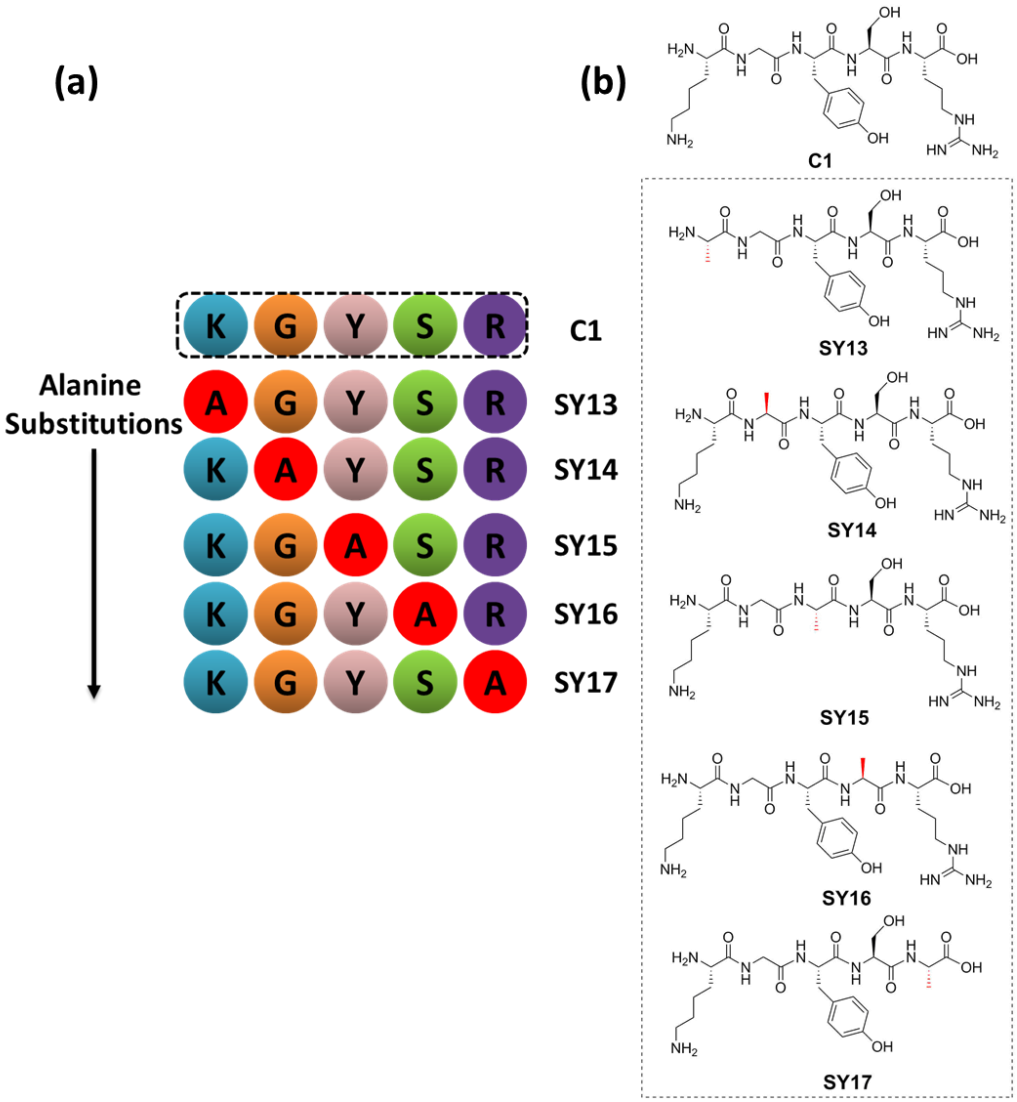


Figure 2. 29: **C1** and its alanine analogues **(a)** Sequence of **C1** and its alanine analogues. (**K**: Lysine, **G**: Glycine, **Y**: Tyrosine, **S**: Serine, **R**: Arginine, **A**, Alanine). **(b)** Structure of **C1** and all alanine compounds (**SY13-17**). The alanine in each compound is highlight in red.

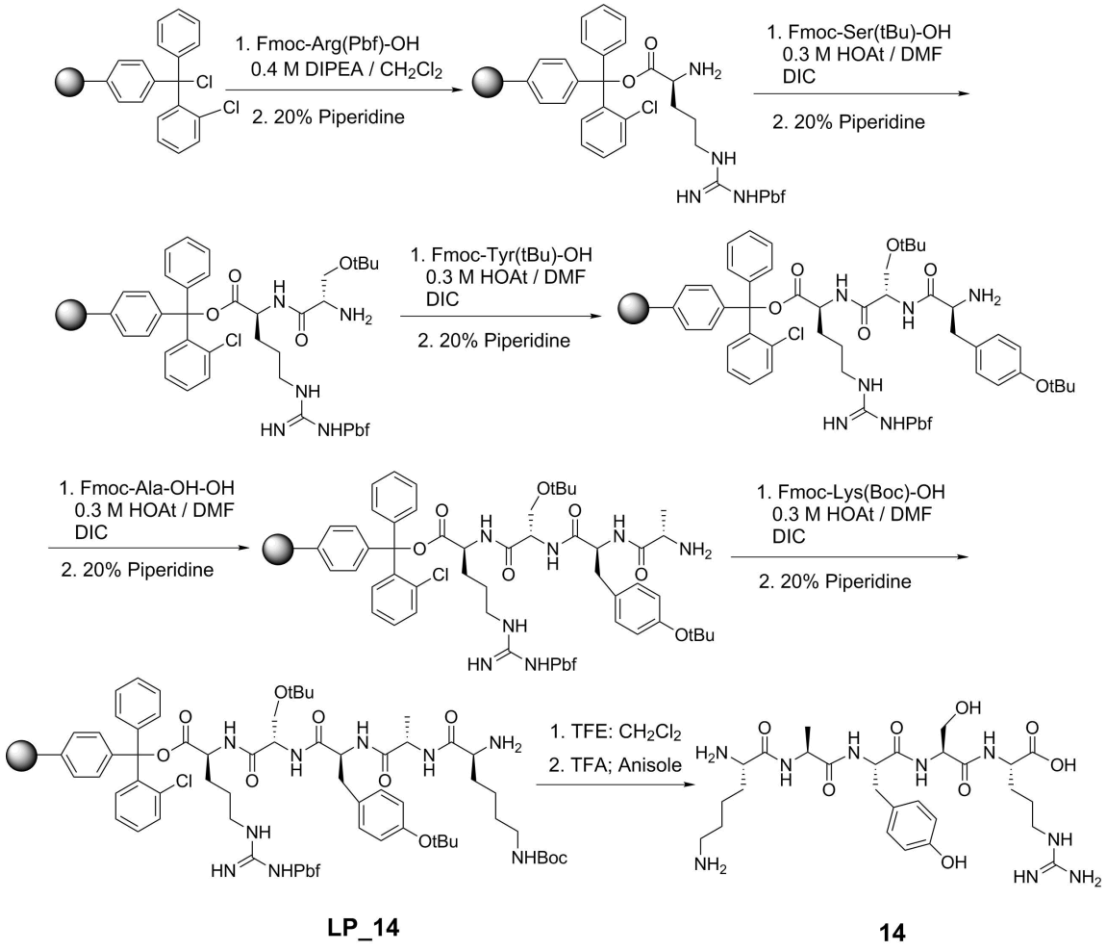
An alanine scan was designed and carried out to determine which residue of **C1** is more significant for the binding with Hsp70 (**Figure 2.21)**. An aniline scan involves substituting each residue on **C1** with alanine, as this amino acid has methyl side chain that is a small, non-bulky and chemically inert.[24] Furthermore, substitution with an alanine has minimal impacts on the conformation of the backbone of a peptide, in

comparison to glycine, which is the simplest amino acid, but introduces conformational flexibility into the peptide backbone.[25] The alanine scan library comprised of 5 peptides (**Figure 2.29**), 3 compounds (**SY14 , 15 and 16**) were synthesised by the author, the compounds **SY13** and **SY17** were synthesised by colleagues Alex Valois and Xandria Ong respectively (**Figure 2.29).**

2.2.6.1 Synthesis of SY14

The synthesis of all of these alanine scanning compounds (**Figure 2.29**) was achieved utilizing SPPS. **Scheme 2.7** shows the synthesis of **SY14** in detail. SPPS was performed in a 60 mL polypropylene solid-phase extraction cartridge with a 20 μm polyethylene frit. Agitation of the reaction tube was performed by a tube shaker. Before loading, 2-chlorotrityl chloride (2-ClTrt) resin was swelled in dichloromethane for 30 minutes. Then the first amino acid, Fmoc-protected arginine was dissolved and added to the drain resin using a solution of 0.1 M diisopropyl ethyl amine (DIPEA) in dichloromethane. A sample of resin was removed, and the resin loading was determined to be 0.47 mmol/g. The Fmoc protecting group was removed using 20 % piperidine in dimethyl formamide (DMF) to the resin for 10 minutes, draining and then adding fresh solution for another 10 minutes. To confirm the Fmoc removal, a positive ninhydrin test was used. Following the deprotection, the coupling of Fmoc protected serine was achieved using both the coupling agents 1-Hydroxy-7-azabenzotriazole (HOAt) and diisopropylcarbodiimide (DIC). The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected tyrosine, Fmoc-protected alanine and Fmoc-protected lysine, and finally generate the desired protected linear

peptide, **LP_14**.



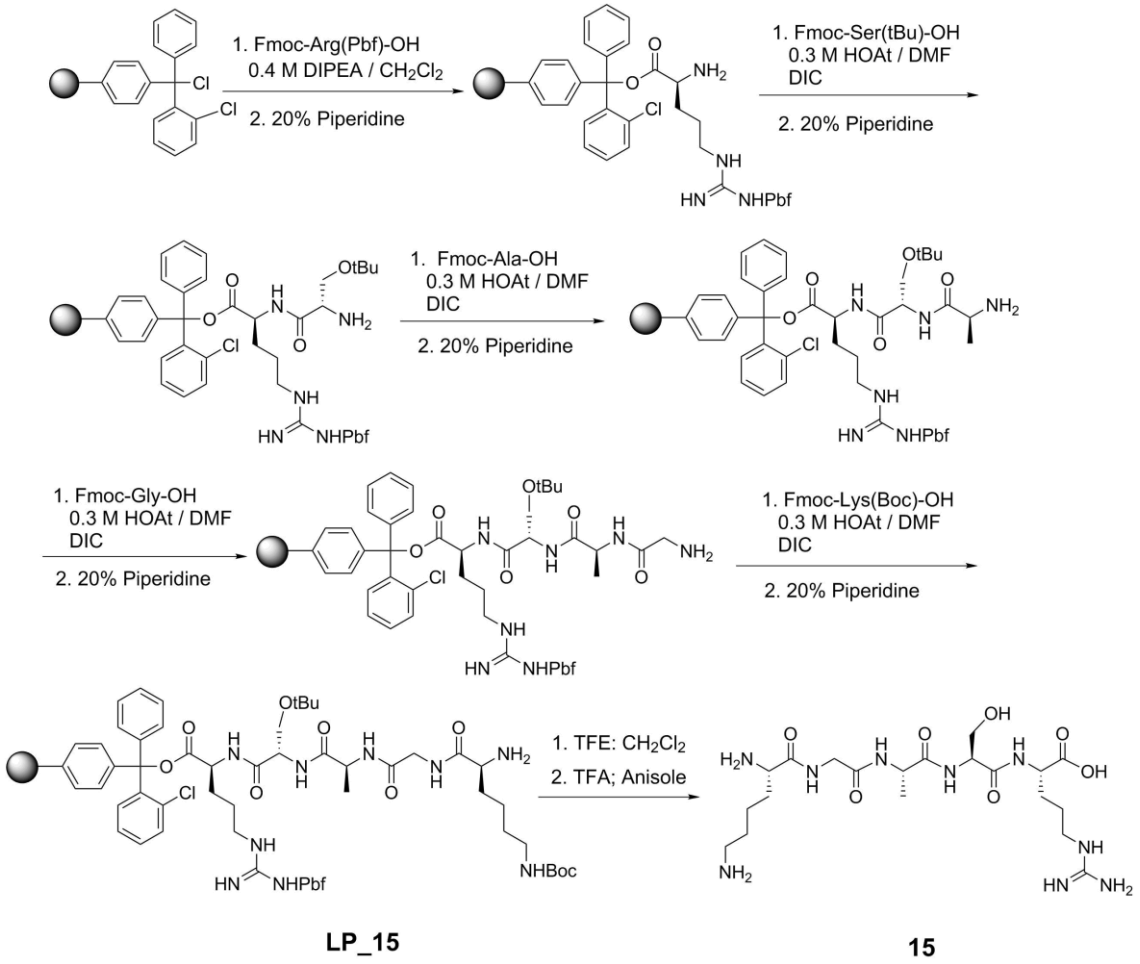
Scheme 2. 7: The synthesis scheme for **SY14**.

LP_14 was cleaved from the resin under mildly acidic conditions using 50% (v/v) trifluoroethanol (TFE) in dichloromethane for 24 hours. LCMS was performed to monitor the reaction progress. The slurry was filtered under vacuum, and the filtrate containing the cleaved linear peptide was collected. The solvent was removed under reduced pressure to afford the **LP_14** to precipitation as a fine off-white powder. The **LP_14** was then globally deprotected using TFA and anisole (as a carbocation scavenger) in order to produce the deprotected linear peptide, **SY14**. The crude **SY14** was purified using high-performance liquid chromatography (HPLC). The mobile consisted of milli-Q water with

0.1% (v/v) formic acid, and HPLC grade acetonitrile with 0.1% (v/v) formic acid. From every injection, all obvious peaks were collected as individual fraction and checked by LC/MS. All fractions with identical traces as indicated in LC/MS were combined and lyophilised. The final product was characterized using LC-MS, ^{1}HNMR, and 2D NMR in the yields of 16%.

2.2.6.2 Synthesis of SY15

Scheme 2.8 shows the synthesis of **SY15** in detail. SPPS was performed in a 60 mL polypropylene solid-phase extraction cartridge with a 20 μm polyethylene frit. Agitation of the reaction tube was performed by a tube shaker. Before loading, 2-chlorotrityl chloride (2-ClTrt) resin was swelled in dichloromethane for 30 minutes. Then the first amino acid, Fmoc-protected arginine was dissolved and added to the drain resin using a solution of 0.1 M diisopropyl ethyl amine (DIPEA) in dichloromethane. A sample of resin was removed, and the resin loading was determined to be 0.49 mmol/g. The Fmoc protecting group was removed using 20 % piperidine in dimethyl formamide (DMF) for 2 times 10 minutes. To confirm the Fmoc removal, a positive ninhydrin test was used. Following the deprotection, the coupling of Fmoc protected serine was achieved using both the coupling agents HOAt and DIC. The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected alanine, Fmoc-protected glycine and Fmoc-protected lysine, and finally generate the desired protected linear peptide, **LP_15**.



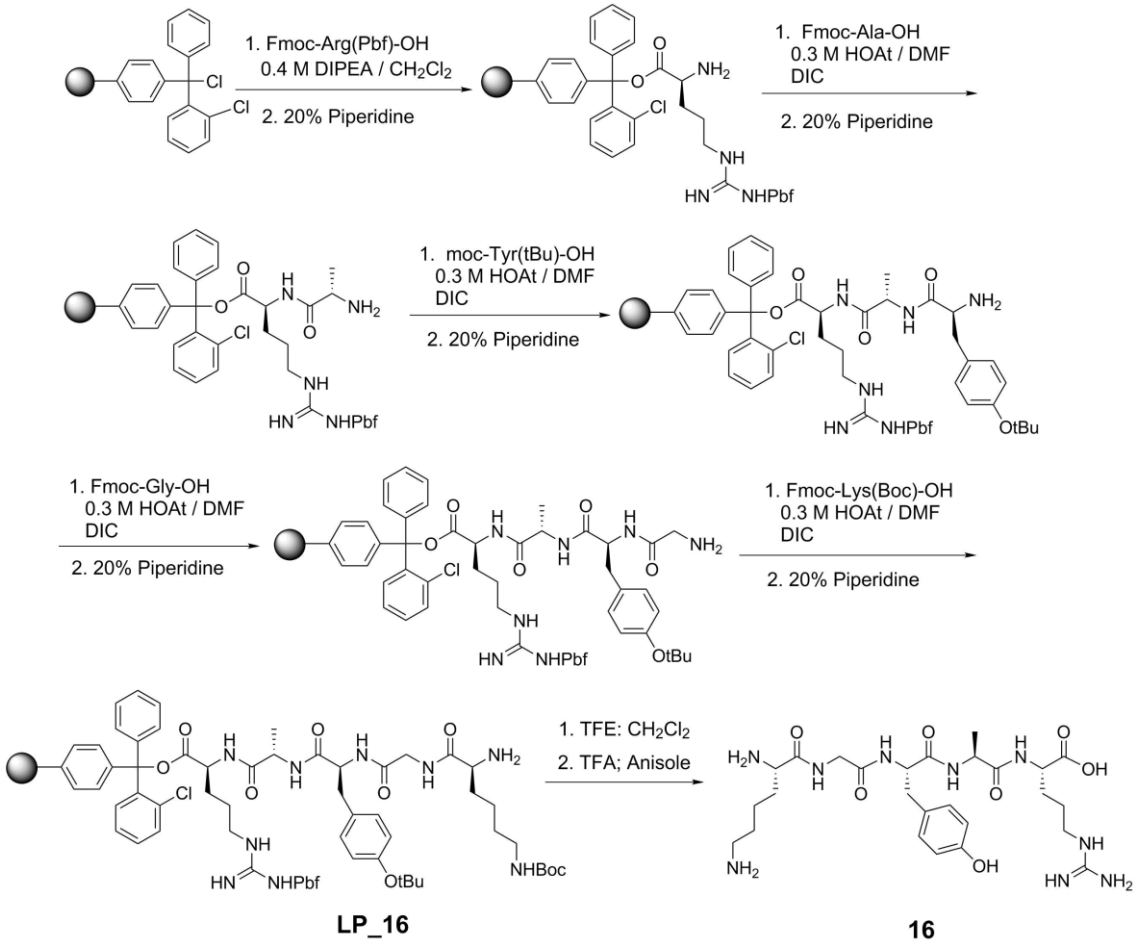
Scheme 2. 8: The synthesis scheme for **SY15**.

LP_15 was cleaved from the resin under mildly acidic conditions using 50% (v/v) trifluoroethanol (TFE) in dichloromethane for 24 hours. LCMS was performed to monitor the reaction progress. The slurry was filtered under vacuum, and the filtrate containing the cleaved linear peptide was collected. The solvent was removed under reduced pressure to afford the **LP_15** to precipitation as a fine off-white powder. The **LP_15** was then globally deprotected using TFA and anisole (as a carbocation scavenger) in order to produce the deprotected linear peptide, **SY15**. The crude **SY15** was purified using high-performance liquid chromatography (HPLC). The mobile consisted of milli-Q water with 0.1% (v/v) formic acid, and HPLC grade acetonitrile with 0.1% (v/v) formic acid. From

every injection, all obvious peaks were collected as individual fraction and checked by LC/MS. All fractions with identical traces as indicated in LC/MS were combined and lyophilised. The final product was characterized using LC-MS, ^{1}HNMR, and 2D NMR in the yields of 12%.

2.2.6.3 Synthesis of SY16

Scheme 2.9 shows the synthesis of **SY16** in detail. SPPS was performed in a 60 mL polypropylene solid-phase extraction cartridge with a 20 μm polyethylene frit. Agitation of the reaction tube was performed by a tube shaker. Before loading, 2-chlorotrityl chloride (2-ClTrt) resin was swelled in dichloromethane for 30 minutes. Then the first amino acid, Fmoc-protected arginine was dissolved and added to the drain resin using a solution of 0.1 M diisopropyl ethyl amine (DIPEA) in dichloromethane. A sample of resin was removed, and the resin loading was determined to be 0.49 mmol/g. The Fmoc protecting group was removed using 20 % piperidine in dimethyl formamide (DMF) for 2 times 10 minutes. To confirm the Fmoc removal, a positive ninhydrin test was used. Following the deprotection, the coupling of Fmoc protected alanine was achieved using both the coupling agents HOAt and DIC. The reaction vessel was agitated on the shaker for at least 2 hours at room temperature. The coupling reaction was confirmed using negative ninhydrin test, followed by amine deprotection using 20% piperidine N,N-dimethylformamide solution. This same procedure was used to sequentially couple Fmoc-protected tyrosine, Fmoc-protected glycine and Fmoc-protected lysine, and finally generate the desired protected linear peptide, **LP_16**.



Scheme 2. 9: The synthesis scheme for **SY16.**

LP_16 was cleaved from the resin under mildly acidic conditions using 50% (v/v) trifluoroethanol (TFE) in dichloromethane for 24 hours. LCMS was performed to monitor the reaction progress. The slurry was filtered under vacuum, and the filtrate containing the cleaved linear peptide was collected. The solvent was removed under reduced pressure to afford the **LP_16** to precipitation as a fine off-white powder. The **LP_16** was then globally deprotected using TFA and anisole (as a carbocation scavenger) in order to produce the deprotected linear peptide, **SY16**. The crude **SY16** was purified using high-performance liquid chromatography (HPLC). The mobile consisted of milli-Q water with 0.1% (v/v) formic acid, and HPLC grade acetonitrile with 0.1% (v/v) formic acid. From every injection, all obvious peaks were collected as individual fraction and checked by

LC/MS. All fractions with identical traces as indicated in LC/MS were combined and lyophilised. The final product was characterized using LC-MS, ^{1}HNMR, and 2D NMR in the yields of 11%. With these 5 molecules in hand, binding assays and functional assay are currently ongoing in order to evaluate the molecules bioactivity. The inactivity of a molecule with alanine replacing a specific residue will inform us which residue is critical in order for **C1** to bind to Hsp70.

2.3 Conclusions

Effectively modulating protein-protein interactions is a significant challenge. This chapter has outlined the design, synthesis and evaluation of new Hsp70 modulators. These molecules are the first in class that inhibit Hsp70's function by stabilising a Hsp70-HOP binding event. Based on the sequences located within HOP's TPR1 domain, the author designed a library of molecules that was derived from Helices in this domain. During my master's thesis project, I synthesized six linear peptides (**A1, B1, C1, SY14, SY15**, and **SY16**), four cyclic pentapeptides (**A2, B2, C2** and **C4**) and a biotin-labelled compound (**C1-Tag**). After screening the first series of compounds, I successfully identified a hit, **C1**, which directly bound to Hsp70 and effectively eliminated its protein folding function by stabilising the Hsp70-HOP complex.

Previous work had suggested cyclic peptides might be more effective as inhibitors, but in this case, the cyclic peptides were not as effective in modulating the Hsp70-HOP interaction compared to their linear counterparts. Specifically comparing C2, which is the cyclic version of **C1**, the cyclisation induced a dramatic decrease in binding activity between Hsp70 and HOP compared to its linear analogue **C1**. These data suggest that the residues in **C2** are fixed in an undesirable conformation, which results in the loss of

activity. Compounds **C3** and C4 are both active in the binding activity yet they are not effective in the protein folding functional assay. These data suggested that they molecules bind to Hsp70 or HOP, but in such a way that still allows Hsp70 to function.

Analysis of **C3**'s ability to bind to the protein, indicated that the molecule did not bind to Hsp70 or HOP alone but required the presence of both proteins to bind effectively. These data suggest that a specific conformation of Hsp70 is needed in order for **C3** to find a suitable binding site. This conformation is only induced once HOP has bound to Hsp70. Studies examining this hypothesis are ongoing.

The conformational changes of Hsp70 is the key factor for the function of this chaperone. Complete Hsp70 activity requires the functional cycle of Hsp70, which is driven by ATP hydrolysis.[26] The ATP bound state of Hsp70 forms a closed C-terminus conformation and binds to client proteins through the substrate binding domain (SBD) (**Figure 2.30**).[27] The ADP bound state of Hsp70 is an open conformation for the release of client proteins and transferring them to Hsp90. During the changing of Hsp70's different conformations, the association and dissociation of Hsp70-HOP-Hsp90 complex also dynamically switches over.[28] My hypothesis on how **C1, C3** and **C4** effectively enhance binding between Hsp70 and HOP is the following: conformational flexibility of Hsp70 is restricted to a single conformation during the binding assay, where Hsp70 is bound to HOP. The HOP bound state of Hsp70 is forced to expose some hidden residues onto the surface, which allow **C3** to bind to the protein. The binding event between **C3** and Hsp70 then increases the binding affinity between Hsp70 and HOP.

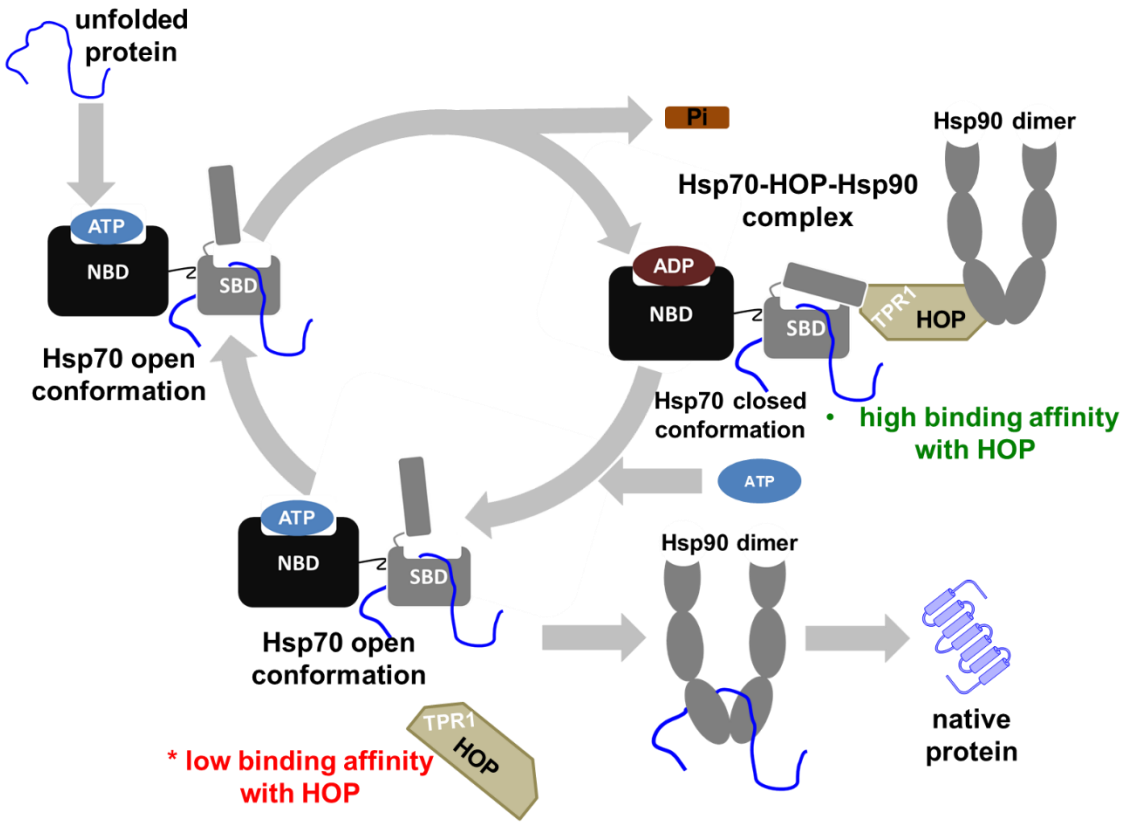


Figure 2. 30: The functional cycle and conformational changes of Hsp70

My hypothesis of how **C1** enhances binding between Hsp70 and HOP but inhibits protein folding the binding between Hsp70 and HOP is the following: Unlike the binding assay, the protein folding assay requires the dynamic assembly of the Hsp70-HOP-Hsp90 complex. The initial binding affinity between Hsp70 and HOP is low (Kd = 1.3 μM) compared to binding between Hsp90 and HOP (Kd = 90 nM). [29] [30] Thus, in the protein folding assay, only a small amount of Hsp70 (~10%) is bound to Hop, making **C3** and **C4** ineffective.

However, unlike **C3**, the pulldown data suggests that **C1** has a binding site on Hsp70 that is available when Hsp70 is not bound to HOP. Thus, I propose that **C1** binds to Hsp70, facilitates HOP binding, and stabilizes the complex making it harder for the dynamic disassembly to occur. This stabilized binding complex between Hsp70-**C1**-HOP

inhibits the protein folding process Hsp70 and HOP must have a dynamic relationship, where they can behave separately or as a complex if needed (**Figure 2.31**). This stabilized binding event that is driven by **C1** is likely responsible for halting the dynamic assembly between Hsp70-HOP, which impacts the binding and release of client proteins such as luciferase, and consequently block the entire Hsp70 folding cycle.

This stabilised binding mechanism is similar to the mechanism by which the drug Taxol performs. Taxol, [31] an effective chemotherapy medication, triggers the apoptosis of cancer cells by suppressing the dynamic assembly of mitotic spindles. By stabilising the microtubule polymer, Taxol blocks the proliferation and division of tumours and induces the cancer autolysis.[32] Thus, compound **C1** has proven to bind to Hsp70, while inhibiting its protein folding function. Understanding the key residues that form the interaction between Hsp70 and **C1** is the next step of the project and is ongoing using the alanine scan molecules. Performing additional mechanistic assays including binding affinity for specific domains and evaluating these compounds in cells will offer a deeper understanding of how these molecules impact the cellular role of Hsp70.

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Chapter 3: Methods and experimental

3.1 General Remarks

All chemicals were purchased from commercial suppliers (Chem-Impex International and Sigma Aldrich) and used without further purification. All moisture sensitive reactions were performed using anhydrous solvents under nitrogen gas. Removal of solvent was carried out under reduced pressure using a Buchi R-210 rotary evaporator.

Semi-preparative HPLC for purification was performed using a GRACE VisionHT C18 column (5 μm, 22 x 150 mm) or a Phenomenex Aeris XB-C18 column (5 μm, 21.2 x 150 mm) on a Shimadzu Prominence High Performance LCMS 2010EV system. The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B) at a flow rate of 5 mL/min, starting at 95% or 98% Mobile Phase A and 5% or 2% Mobile Phase B.

LC/MS analyses were performed using a Phenomenex Aeris XB-C18 column (3.6 μm, 2.1 x 100 mm) on either a Shimadzu LCMS 2020, Shimadzu LCMS 8030 or LCQ Deca XP Plus (Thermo Finnigan). The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B) at a flow rate of 0.2 mL/min, starting at 95% Mobile Phase A and 5% Mobile Phase B.

1H and 13C NMR spectra were obtained on Bruker Avance III 600 MHz. Multiplicity of NMR signals were represented by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublet. Assignment of

resonances for each residue was accomplished using 1H, HSQC, HMBC and COSY spectra.

3.2 General Synthetic Procedures

Solid-Phase Peptide Synthesis

Stepwise SPPS was performed in a polypropylene solid-phase extraction cartridge fitted with a 20 μm polyethylene frit purchased from Applied Separations (Allentown, PA).

Resin Loading

The resin was weighed, transferred to the cartridge and swelled in CH_2Cl_2 for 1 hour prior to the resin loading reaction. The appropriate Fmoc-protected amino acid was dissolved in the minimum amount of 0.4M DIPEA in CH_2Cl_2. The swelled resin was then drained, and the dissolved amino acid was added to resin in the cartridge. The suspension was agitated for a minimum of 6 hours at room temperature. The resin was washed 3 times with CH_2Cl_2, 3 times with DMF and 3 times with CH_2Cl_2. The resin was then dried *in vacuo* overnight. A ~ 5 mg sample of resin was used to determine the amino acid loading. 20% piperidine in DMF was added to the sample to cleave the Fmoc protecting group. The resin was filtered away and the remaining solution was diluted 1 in 20 and the UV absorbance measured at 301 nm using a Cary 50 Bio UV-Vis instrument. DMF was used as a blank and samples were measured in a 1 mL quartz cuvette. The resin loading was then determined using the following formula:

Resin loading (mmol/g)

= (Abs x cleavage volume x dilution factor)/(extinction coefficient x cuvette width x resin mass)

= (Abs x 1 mL x 20)/(7800 mL $mmol^{-1}$ cm^{-1} x 1 cm x resin mass in g)

= (Abs x 20 mL)/(7800 mL $mmol^{-1}$ x resin mass in g)

Coupling Reaction

Prior to each coupling reaction, the resin was swelled in DMF for 0.5 – 1 hr, then the DMF was drained. Couplings were performed in DMF at a concentration of 0.3 M. Fmoc-protected amino acid (2 equiv.) and either 1-hydroxybenzotriazole hydrate (HOBt) or 1-hydroxy-7-azabenzotriazole HOAt (2 equiv.) were mixed with the resin. N, N′-diisopropylcarbodiimide (DIC) (4 equiv.) was then added to activate the reaction. Coupling reaction was run for a minimum of 2 hours while shaking (Labquake tube shaker, Thermo Fisher Scientific) at room temperature. A negative ninhydrin test was used to confirm reaction completion. Once completed, the reaction mixture was drained, and the resin was subjected to *Fmoc Removal*.

Fmoc Removal

The Fmoc protecting group was removed using the following washes: DMF (3 x 1 min), 20% piperidine in DMF (1 x 5 min), 20% piperidine in DMF (1 x 10 min), DMF (2 x 1 min),

*i*PrOH (1 x 1 min), DMF (1 x 1 min), *i*PrOH (1 x 1 min) and DMF (3 x 1 min). The resin was then ready for the next coupling reaction.

On-resin Biotinylation

Once the desired peptide was generated, the final Fmoc protecting group was removed following *Fmoc Removal* procedure with the following additional washes by CH_2Cl_2.The resin-bound peptide was then dried *in vacuo* overnight. All biotin-coupling reactions were carried out under nitrogen in a final concentration of 0.05 M anhydrous CH_2Cl_2. The resin bound peptide (1.0 equivalent) was stirred in 50% of the final CH_2Cl_2 volume and then DIPEA (8 equivalent) was added. The EZ-Link NHS-PEG4-Biotin (1.5 equivalents) was dissolved in the remaining CH_2Cl_2 volume and was added to the reaction mixture. The solution was stirred at room temperature and reactions were monitored by negative ninhydrin test and LCMS. Once completed, the reaction mixture was drained, and the resin was subjected to *Resin Cleavage of Linear Peptide*

Resin Cleavage of Linear Peptide

Once the desired peptide was generated, the final Fmoc protecting group was removed following *Fmoc Removal* procedure with the following additional washes: DMF (3 x 1 min), *i*PrOH (3 x 1 min) and MeOH (3 x 1 min). The resin-bound peptide was then dried *in vacuo* overnight. The resin was then cleaved from the linear peptide using TFE and CH_2Cl_2 (1:1 v/v) at a concentration of 10 mL/g resin. The reaction mixture was stirred at room temperature for 24 hours before filtering the resin. The filtrate was concentrated and washed at least 10 times with CH_2Cl_2 to remove residual entrapped TFE. The product was then dried *in vacuo* overnight to produce the linear peptide.

Macrocyclisation

Macrocyclisation of the linear peptide was achieved using a cocktail of 3 coupling reagents: HATU (1 eq.), TBTU (0.8 equiv.) and DMTMM (0.8 equiv.). A special case occurred where macrocyclisation was achieved using only HATU (1 equiv.) and TBTU

(1 equiv.). DMTMM was removed as the methylmorpholinium by-product from the cyclisation formed a stable complex with the peptide and this by-product was inseparable by HPLC and was only detected via NMR. The reaction was performed under nitrogen and in dilute conditions using anhydrous solvents at concentration of 0.001 M. The linear peptide and coupling reagents were dissolved separately in CH_2Cl_2, where 20% of the final volume was used to dissolve the linear peptide and the other 80% dissolved the coupling reagents. DIPEA (4 equiv.) was added to each solution. The linear peptide solution was then added drop-wise to the coupling reagents solution *via* a syringe pump over approximately 2 hours. The reaction was stirred overnight and monitored *via* LC/MS. (Note: if the reaction failed to reach completion after stirring overnight, additional HATU (1 equiv.) was added and the reaction was monitored using LC/MS.) Upon completion, the reaction mixture was evaporated and the dry solid was redissolved in CH_2Cl_2 and extracted 3 times with milli-Q water. The aqueous layers were combined and extracted 3 times with fresh CH_2Cl_2. All organic layers were combined and dried over Na_2SO_4, filtered and evaporated under reduced pressure before the compound was dried *in vacuo* overnight.

Side Chain Deprotection

Amino acid side chain protecting groups were removed using TFA in CH_2Cl_2 (9:1 v/v) with anisole (at least 2 equiv. per protecting group). Anisole was added to the peptide, whilst stirring, followed by the TFA solution at a concentration of 4 mL/g compound. The reaction was left stirring at room temperature for at least 6 hours. The reaction was monitored using LC/MS and once complete the reaction solution was dried under a stream of nitrogen before redissolving in CH_2Cl_2 and evaporating multiple times to remove

residual entrapped TFA. The product was precipitated using diethyl ether, collected *via* centrifugation and lyophilised to produce the crude linear and cyclic peptide.

3.3 Biology Methods

3.3.1 Protein binding assay

All test compounds were dissolved in 100% DMSO and were diluted to a final concentration of 1% DMSO in each reaction. Each reaction consisted of Human native Hsp72 (ENZO, ADI-NSP-555) at a final concentration of 200 nM, the compound at various concentrations (0 – 100 µM) and binding buffer (150 mM NaCl, 20 mM Tris HCl, Triton X-100 (1% v/v), pH 7.4) at a final volume of 100 µL. The reactions were rocked at room temperature for 2 hours, then His-tagged HOP (StressMarq Biosciences, SPR-302-C) at a final concentration of 100 nM was added to each reaction, followed by an hour incubation. Protein pull-down was achieved by incubating each reaction with Talon-Metal Affinity Resin (Clontech, 635501), for an hour. The supernatant was removed, and the resin was washed six times with wash buffer (300 mM NaCl, 20 mM Tris HCl, 1% Triton X-100). Finally, the beads were boiled with 5 x Laemmli sample buffer (10% SDS, 30% glycerol, 0.5 M DTT, 0.05% bromophenol blue, 250 mM Tris HCl, pH 6.8). The supernatant of each sample was loaded onto an 8% Tris-Glycine gel. The gel was made using the SureCast Gel Handcast system (Invitrogen, HC1000S) according to the manufacturer's protocol. The proteins were separated using SDS-PAGE gel electrophoresis.

Proteins were transferred onto a PVDF membrane which was blocked with 5% non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 1 hr at 4 0C. The

membrane was then incubated with Hsp70 primary antibody (ENZO, ADI-SPA-811F, 1:6000 dilution) in 2.5% non-fat milk (in TBS-T) at 4 °C overnight. After wash with cold TBS-T, the membrane was incubated with the respective HRP-conjugated secondary antibody (abcam, ab6721, 1:1000 diltuion) at 4 °C for 30 min, followed by five washes with cold TBS-T. Immunoblotting was performed using West Pico chemiluminescent substrate (Thermo scientific, 34580) and the images were captured by ImageQuant LAS 4010 digital imaging system (GE Healthcare). Following the detection of Hsp70, the membrane was then washed with cold TBS-T five times. Then the membrane was incubated with HOP primary antibody (abcam, ab126724,1:15000 dilution) in 2.5% non-fat milk (in TBS-T) at 4 °C overnight. The previous steps were repeated to detect HOP on the PVDF membrane. The respective ratio of Hsp70 to HOP was analysed via Image J and transformed to a fold Hsp70 bound to HOP. Each experiment was completed at least n=3.

3.3.2 Protein pulldown assay

Biotin-tagged compounds were dissolved in 100% DMSO and were diluted to a final concentration of 1% DMSO in each reaction. Each reaction consisted of Recombinant Human Hsp70 protein (ab48997) at a final concentration of 200 nM, the tagged compound at various concentrations (0 – 200 µM) and binding buffer (150 mM NaCl, 20 mM Tris HCl, Triton X-100 (1% v/v), pH 7.4) at a final volume of 100 µL. The reactions were rocked at room temperature for 1 hour. Protein pull-down was achieved by incubating each reaction with NeutrAvidin® Agarose Resins (Thermo Scientific, 29201 or 29204), for an hour. The NeutrAvidin® Agarose Resins were pre-blocked with 1.3% w/v of Bovine Serum Albumin (Sigma, A2153) in binding buffer for 1 hour at room temperature. Following the incubation with NeutrAvidin®, the supernatant was removed

from each reaction, and the resin was washed six times with wash buffer (300 mM NaCl, 20 mM Tris HCl, 1% Triton X-100). Finally, the beads were boiled with 5 x Laemmli sample buffer (10% SDS, 30% glycerol, 0.5 M DTT, 0.05% bromophenol blue, 250 mM Tris HCl, pH 6.8). The supernatant of each sample was loaded onto an 8% Tris-Glycine gel. The gel was made using the SureCast Gel Handcast system (Invitrogen, HC1000S) according to the manufacturer's protocol. The proteins were separated using SDS-PAGE gel electrophoresis.

Proteins were transferred onto a PVDF membrane which was blocked with 5% non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 1 hr at 4 0C. The membrane was then incubated with Hsp70 primary antibody (ENZO, ADI-SPA-811F, 1:4000 dilution) in 2.5% non-fat milk (in TBS-T) at 4 °C overnight. After wash with cold TBS-T, the membrane was incubated with the respective HRP-conjugated secondary antibody (abcam, ab6721, 1:1000 diltuion) at 4 °C for 30 min, followed by five washes with cold TBS-T. Immunoblotting was performed using West Pico chemiluminescent substrate (Thermo scientific, 34580) and the images were captured by ImageQuant LAS 4010 digital imaging system (GE Healthcare). The relative amount of Hsp70 was analysed via Image J and transformed to a fold of Hsp70 pulled down relative to 1% DMSO control.

3.3.3 Competitive binding assay

All test compounds and biotin-tagged compounds were dissolved in 100% DMSO and were diluted to a final concentration of 1% DMSO in each reaction. Each reaction consisted of Recombinant Human Hsp70 protein (ab48997) at a final concentration of 200 nM, the tagged compound at finial concentration of 200 µM and binding buffer (150

mM NaCl, 20 mM Tris HCl, Triton X-100 (1% v/v), pH 7.4) at a final volume of 100 µL. The reactions were rocked at room temperature for 1 hour. Then the untagged compound was added to each reaction at various concentrations (0 – 200 µM) and incubated for 1 hour. Protein pull-down was achieved by incubating each reaction with NeutrAvidin® Agarose Resins (Thermo Scientific 29201 or 29204), for an hour. The NeutrAvidin® Agarose Resin was pre-blocked with 1.3% of Bovine Serum Albumins (Sigma, A2153) for 1 hour. Following the incubation with NeutrAvidin®, the supernatant was removed, and the resin was washed six times with wash buffer (300 mM NaCl, 20 mM Tris HCl, 1% Triton X-100). Finally, the beads were boiled with 5 x Laemmli sample buffer (10% SDS, 30% glycerol, 0.5 M DTT, 0.05% bromophenol blue, 250 mM Tris HCl, pH 6.8). The supernatant of each sample was loaded onto an 8% Tris-Glycine gel. The gel was made using the SureCast Gel Handcast system (Invitrogen, HC1000S) according to the manufacturer's protocol. The proteins were separated using SDS-PAGE gel electrophoresis.

Proteins were transferred onto a PVDF membrane which was blocked with 5% non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 1 hr at 4 0C. The membrane was then incubated with Hsp70 primary antibody (ENZO, ADI-SPA-811F, 1:4000 dilution) in 2.5% non-fat milk (in TBS-T) at 4 °C overnight. After wash with cold TBS-T, the membrane was incubated with the respective HRP-conjugated secondary antibody (abcam, ab6721, 1:1000 diltuion) at 4 °C for 30 min, followed by five washes with cold TBS-T. Immunoblotting was performed using West Pico chemiluminescent substrate (Thermo scientific, 34580) and the images were captured by ImageQuant LAS 4010 digital imaging system (GE Healthcare). The relative amount of Hsp70 was

analysed via Image J and transformed to a precent of Hsp70 bound to the tagged molecule relative to DMSO control. Each experiment was completed at least n=3.

3.3.4 Luciferase renaturation assay

The luciferase refolding assay was adapted from literature methods[1]. Luciferase (QuantiLum recombinant luciferase, Promega, E1701) was denatured in 2x denaturing buffer (25 mM HEPES (pH 7.2), 50 mM potassium acetate, 5 mM DTT and 6 M Guanidine HCl) at a concentration of 0.5 mg/mL for 60 mins at room temperature. Native luciferase was used as a control and was incubated in 1 x diluting buffer (25 mM HEPES (pH 7.6), 50 mM potassium acetate, 5 mM DTT) at a concentration of 0.5 mg/mL for 60 mins at room temperature. Both the native and denatured luciferase were then diluted 1 in 40 using 1 x diluting buffer on ice for 20 minutes.

Human native Hsp72 (ENZO, ADI-NSP-555) at a final concentration of 0.5 µM was incubated with each compound at 50 µM in 1 x refolding buffer (28 mM HEPES (pH 7.6) 120 mM potassium acetate, 12 mM magnesium acetate, 2.2 mM DTT, 0.25 mM ATP, 8.8 mM creatine phosphate, 35 U/mL creatine kinase), for 2 hours on ice. Then each reaction was made up to a final volume of 50 µL by adding the following proteins at these final concentrations: 100 nM DnaJ (abcam, ab91598), 50 nM GrpE (abcam, ab63839), 50 nM Hsp90 (ENZO ADI-SPP-770-F), 50 nM HOP (StressMarq Biosciences, SPR-302-C) and finally 2 µL of denatured luciferase. Two control reactions were set up and consisted of 2 µL of either native or denatured luciferase, 1% DMSO and refolding buffer to a final volume of 50 µL. The reactions were incubated at room temperature. 2 µL aliquots of each reaction were taken at various time points and were combined with 23 µL of Bright-Glo reagent (Promega, E2610) in a white 384-well polystyrene microplate

(PerkinElmer, 6007290). The luminescence of the respective wells was measured using a Tecan F200 Pro multimode plate reader. The final data was plotted as the % of luciferase refolded over time relative to the maximum luminescence signal from DMSO at 5 hours. To determine whether the compounds interacted with native or denatured luciferase the following reactions were set-up: 2 µL of native or denatured luciferase, compound at various concentrations (1, 5, 25 and 50 µM) and 1 x refolding buffer to final volume of 50 µL. The reactions were incubated for 20 minutes on ice. 2 µL aliquots of each reaction was combined with 23 µL of Bright-Glo reagent (Promega, E2610) in a white 384-well polystyrene microplate. The luminescence of these wells was measured and the raw luminescence values were plotted.

3.3.5 Reference

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3.4 Experimental Procedures

3.4.1 Experimental Procedures for A1

Resin- O-Asn(Trt)-NH_2

The resin bound amino acid Resin-O-Asn(Trt)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1.1 g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. 1.44 g Fmoc-Asn(Trt)-OH (2.42 mmol, 2 equiv.) that was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) was added to the swelled resin. The reaction was agitated at room temperature for 8 hours. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Asn(Trt)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.565 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Asn(Trt)-NH_2.

Resin-O-Asn(Trt)-Gly-NH_2

Resin-O-Asn(Trt)-Gly-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Leu-NH_2 (0.57 mmol, 1 equiv.), 0.37 g Fmoc-Gly-OH (1.24 mmol, 2 equiv.), 2.1 mL HOAt (1.24 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 0.42 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Asn(Trt)-Gly-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Asn(Trt)-Gly-NH_2.

Resin-O-Asn(Trt)-Gly-Lys(Boc)-NH_2

Resin-O-Asn(Trt)-Gly-Lys(Boc)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Asn(Trt)-Gly-NH_2 from the previous reaction, 0.69 g Fmoc-Lys(Boc)-OH (1.24 mmol, 2 equiv.), 2.1 mL HOAt (1.24 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 0.42 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Asn(Trt)-Gly-Lys(Boc)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Asn(Trt)-Gly-Lys(Boc)-NH_2.

Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-NH_2

Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Asn(Trt)-Gly-Lys(Boc)-NH_2 from the previous reaction, 0.63 g Fmoc-Glu(tBu)-OH (1.49 mmol, 2.4 equiv.), 2.1 mL HOAt (1.24 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 0.42 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 3 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-NH_2.

Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NH_2

Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-NH_2

from the previous reaction, 0.69 g Fmoc-Lys(Boc)-OH (1.24 mmol, 2 equiv.), 2.1 mL HOAt (1.24 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 0.42 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was allowed to run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NH_2.

HO-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NH_2

The protected linear peptide HO-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 7.0 mL of trifluoroethanol and 7.0 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale-yellow solid (627 mg, 90% overall).

HO-Asn-Gly-Lys-Glu-Lys-NH_2 (Compound A1)

The deprotected linear peptide was generated following the *Global Deprotection* procedure using 0.15 g HO-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NH_2 (0.14 mmol, 1 equiv.), 0.8 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.12 mL anisole (1.11 mmol, 8 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude linear peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (6 mg, 11% overall)

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{23}H_{43}N_8O_9^+$, 575.30; found 575.40

^{1}H NMR (600 MHz, D_2O): δ 4.54 (dd, *J* = 4.75, 8.80 Hz, 1H, αH Asn), 4.37 (dd, *J* = 4.99, 9.12 Hz, 1H, αH Glu), 4.28 (t, *J* = 7.11 Hz, 1H, αH Lys), 3.13 (m, 3H, αH Gly, αH Lys), 3.08 -3.00 (m, 4H, εH Lys), 2.87-2.64 (m 2H, βH Asn), 2.49 – 2.21 (m, 2H, γH Glu), 2.22 – 1.79 (m, 6H, βH Glu, βH Lys), 1.87-1.62 (m, 4H, δH Lys), 1.54-1.49 (m, 4H, γH Lys).

3.4.2 Experimental Procedures for A2

***cyclo*-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)**

The protected cyclic peptide *cyclo*-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc) was synthesised following the *Macrocyclisation* procedure using 0.3 g of HO-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc)-NH_2 (0.28 mmol, 1.0 equiv.), 0.11 g HATU (0.28 mmol, 1 equiv.), 0.07 g TBTU (0.22 mmol, 0.8 equiv.), 0.06 g DMTMM (0.2 mmol, 0.8 equiv.), 0.40 mL DIPEA (2.4 mmol, 8 equiv.) in anhydrous CH_2Cl_2 (280 mL, 0.001 M), The reaction was then stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Macrocyclisation* procedure and dried *in vacuo* to produce the crude, protected, cyclic peptide *cyclo*-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc).

***cyclo*-Asn-Gly-Lys-Glu-Lys (Compound A2)**

The deprotected cyclic peptide *cyclo*-Asn-Gly-Lys-Glu-Lys was synthesised following the *Global Deprotection* procedure using 0.2 g *cyclo*-Asn(Trt)-Gly-Lys(Boc)-Glu(tBu)-Lys(Boc) (0.19 mmol, 1 equiv.), 0.8 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v)

and 0.16 mL anisole (1.5 mmol, 8 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred for 5 hours and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude cyclic peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (10.2 mg, 15% overall)

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{23}H_{41}N_8O_8^+$, 557.31; found 557.40

^{1}H NMR (600 MHz, D_2O): δ 4.71 (t, *J* = 6.50 Hz, αH Asn), 4.37 (dd, *J* = 5.2, 9.95 Hz, 1H, αH Lys), 4.3 (dd, *J* = 5.08, 10.05 Hz, 1H, αH Lys), 4.17 (t, *J* = 7.38 Hz, 2H, αH Glu), 4.12 – 3.65 (m, 2H, αH Gly), 3.12 -2.94 (m, 4H, εH Lys), 2.93-2.84 (m 2H, βH Asn), 2.42 (t, *J* = 7.28 Hz, 2H, γH Glu), 2.21 – 2.04 (m, 2H, βH Glu), 2.03 - 1.99 (m, 8H, δH Lys, , βH Lys), 1.60-1.33 (m, 4H, γH Lys).

3.4.3 Experimental Procedures for B1

Resin-O-Ser(tBu)-NH_2

The resin bound amino acid Resin-O-Ser(tBu)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1.0 g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. 0.84 g Fmoc-Ser(tBu)-OH (2.2 mmol, 2 equiv.) that was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) was added to the swelled resin. The reaction was agitated at room temperature overnight. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-

Ser(tBu)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.63 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Ser(tBu)-NH_2.

Resin-O-Ser(tBu)-Arg(Pbf)-NH_2

Resin-O-Ser(tBu)-Arg(Pbf)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using 1.0 g Resin-O-Ser(tBu)-Arg(Pbf)-NH_2 (0.72 mmol, 1 equiv.), 0.82 g Fmoc-Arg(Pbf)-OH (1.3 mmol, 2 equiv.), 2.1mL HOAt (1.3 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 2.5 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ser(tBu)-Arg(Pbf)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ser(tBu)-Arg(Pbf)-NH_2.

Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-NH_2

Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Ser(tBu)-Arg(Pbf)-Ala-NH_2 from the previous reaction, 0.75 g Fmoc-Tyr(tBu)-OH (1.3 mmol, 2 equiv.), 2.1mL HOAt (1.3 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 2.5 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-NH_2.

Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-NH_2

Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-NH_2 from the previous reaction, 0.49 g Fmoc-Ser(tBu)-OH (1.3 mmol, 2 equiv.), 2.1mL HOAt (1.3 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 2.5 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-NH_2.

Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NH_2

Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-NH_2 from the previous reaction, 0.58 g Fmoc-Tyr(tBu)-OH (1.3 mmol, 2 equiv.), 2.1mL HOAt (1.3 mmol, 2 equiv.), 0.42 mL DIC (2.5 mmol, 4 equiv.) and 2.5 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was allowed to run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NH_2.

HO-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NH_2

The protected linear peptide HO-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved

from the resin using a solution of 13.0 mL of trifluoroethanol and 13.0 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale-yellow solid (600 mg, 88% overall).

HO-Ser-Arg-Asn-Ser-Tyr-NH_2 (Compound B1)

The deprotected linear peptide HO-Ser-Arg-Asn-Ser-Tyr-NH_2 was generated following the *Global Deprotection* procedure using 0.08 g HO-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NH_2 (0.06 mmol, 1 equiv.), 1 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.5 mL anisole (0.05 mmol, 8 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude linear peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (18.3 mg, 13% overall).

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{25}H_{40}N_9O_{10}^+$, 626.29; found 626.35

^{1}H NMR (600 MHz, D_2O): δ 7.27 – 6.84 (m, 4H, Tyr), 4.73 (dd, *J* = 5.2, 9.95 Hz,1H, αH Asn), 4.52 (t, *J* = 5.93 Hz, 1H, αH Lys), 4.44 (dd, *J* = 5.63, 6.65 Hz, 1H, αH Arg), 4.31 (t, *J* = 4.87 Hz, 1H, αH Ser), 4.28 (t, *J* = 7.8 Hz, 1H, αH Ser), 3.97 – 3.73 (m, 4H, βH Ser), 3.34 – 3.05 (m, 4H, βH Tyr, δH Arg), 2.95 – 2.74 (m, 2H, βH Asn), 2.02 - 1.72 (m, 2H, βH Arg), 1.70-1.58 (m, 2H, γH Arg).

3.4.4 Experimental Procedures for B2

***cyclo*-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)**

The protected cyclic peptide *cyclo*-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu) was synthesised following the *Macrocyclisation* procedure using 0.3 g HO-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)-NH_2 (0.23 mmol, 1 equiv.), 0.089 g HATU (0.23 mmol, 1 equiv.), 0.06 g TBTU (0.19 mmol, 0.8 equiv.), 0.52 g DMTMM (0.19 mmol, 1 equiv.), 0.32 mL DIPEA (2.4 mmol, 8 equiv.) in anhydrous CH_2Cl_2 (233 mL, 0.001 M), The reaction was then stirred for 5 hours and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Macrocyclisation* procedure and dried *in vacuo* to produce the crude, protected, cyclic peptide *cyclo*-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu).

***cyclo*-Ser-Arg-Asn-Ser-Tyr (Compound B2)**

The deprotected cyclic peptide *cyclo*-Ser-Arg-Asn-Ser-Tyr was synthesised following the *Global Deprotection* procedure using 0.3 g *cyclo*-Ser(tBu)-Arg(Pbf)-Asn(Trt)-Ser(tBu)-Tyr(tBu)(0.18 mmol, 1 equiv.), 1.2 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.2 mL anisole (2.0 mmol, 10 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred for 4 hours and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude cyclic peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (19 mg, 17% overall).

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{25}H_{38}N_9O_9^+$, 608.28; found 608.30

^{1}H NMR (600 MHz, D_2O): δ 7.35 – 6.72 (m, 4H, Tyr), 4.57 (t, *J* = 6.93 Hz,1H, αH Asn), 4.52 – 4.42 (m, 1H, αH Lys), 4.41 – 4.31 (m, *2*H, αH Ser), 4.26 (q, *J* = 5.13 Hz, 1H, αH

Arg), 3.96 – 3.67 (m, 4H, βH Ser), 3.34 – 3.05 (m, 4H, βH Tyr, δH Arg), 3.04 – 2.84 (m, 2H, βH Asn), 2.06 - 1.82 (m, 2H, βH Arg), 1.78-1.58 (m, 2H, γH Arg).

3.4.5 Experimental Procedures for C1

Resin-O-Arg(Pbf)-NH_2

The resin bound amino acid Resin-O-Arg(Pbf)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1.0 g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. 1.5 g Fmoc-Arg(Pbf)-OH (2.3 mmol, 2 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) and was added to the swelled resin. The reaction was agitated at room temperature overnight. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Arg(Pbf)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.39 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Arg(Pbf)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using 1.0 g Resin-O- Arg(Pbf)-NH_2 (0.39 mmol, 1 equiv.), 0.32 g Fmoc-Ser(tBu)-OH (0.83 mmol, 2 equiv.), 1.4 HOAt (0.83 mmol, 2 equiv.), 0.28 mL DIC (1.7 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-

NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 from the previous reaction, 0.38 g Fmoc-Tyr(tBu)-OH (0.83 mmol, 2 equiv.), 1.4 HOAt (0.83 mmol, 2 equiv.), 0.28 mL DIC (1.7 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NHFmoc**.** The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2 from the previous reaction, 0.25 g Fmoc-Gly -OH (0.83 mmol, 2 equiv.), 1.4 HOAt (0.83 mmol, 2 equiv.), 0.28 mL DIC (1.7 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly -NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly -NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly -NH_2 from the previous reaction, 0.39 g Fmoc-Lys(Boc)-OH (0.83 mmol, 2 equiv.), 1.4 HOAt (0.83 mmol, 2 equiv.), 0.28 mL DIC (1.7 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2.

HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2

The protected linear peptide HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 13.0 mL of trifluoroethanol and 13.0 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale yellow solid (324 mg, 72% overall).

HO-Arg-Ser-Tyr-Gly-Lys-NH_2 (Compound C1)

The deprotected linear peptide HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2 was generated following the *Global Deprotection* procedure using 0.06 g of Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2 (0.05 mmol, 1 equiv.), 0.55 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.05 mL anisole (0.4 mmol, 8 equiv.) to simultaneously cleave the linear peptide from the resin and remove side chain protection groups. The reaction mixture was stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global*

Deprotection procedure to afford the crude linear peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (8 mg, 7% overall)

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{26}H_{44}N_9O_8^+$, 610.33; found 610.30

^{1}H NMR (600 MHz, D_2O): δ 7.15 (d, *J* = 8.91 Hz, 2H, Tyr), 6.846 (d, *J* = 8.54 Hz, 2H, Tyr), 4,63 (t, J = 6.9 Hz, 1H, αH Tyr), 4.435 (t, J = 5.66 HZ, 1H, αH Ser), 4.2056 (dd, J = 5.26, 7.67 Hz, 1H, αH Arg), 4.09 – 3.88 (m, 3H, αH Lys, 2βH Ser), 3.86-3.71 (m, 2H, αH Gly), 3.25-2.92 (m, 6H, 2βH Tyr, 2εH Lys, 2δH Arg), 2.01-1.84 (m, 2H, βH Lys), 1.83 – 1.38 (m, 8H, 2γH 2δH Lys, 2βH 2γH Arg)

3.4.6 Experimental Procedures for C1-Tag

Resin-O-Arg(Pbf)-NH_2

The resin bound amino acid Resin-O-Arg(Pbf)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (0.504 g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained.0.719 g Fmoc-Arg(Pbf)-OH (1.1 mmol, 2 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) and was added to the swelled resin. The reaction was agitated at room temperature overnight. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Arg(Pbf)-NHFmoc. A sample of resin was removed, and the resin loading was

determined to be 0.73 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Arg(Pbf)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using 0.504 g Resin-O- Arg(Pbf)-NH_2 (0.73 mmol, 1 equiv.), 0.28 g Fmoc-Ser(tBu)-OH (0.73 mmol, 2 equiv.), 1.3 mL HOAt (0.73 mmol, 2 equiv.), 0.25 mL DIC (1.5 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 from the previous reaction, 0.34 g Fmoc-Tyr(tBu)-OH (0.73 mmol, 2 equiv.), 1.3 mL HOAt (0.73 mmol, 2 equiv.), 0.25 mL DIC (1.5 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NHFmoc**.** The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2 from the previous reaction, 0.22 g Fmoc-Gly -OH (0.73 mmol, 2 equiv.), 1.3 mL HOAt (0.73 mmol, 2 equiv.), 0.25 mL DIC (1.5 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly -NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly -NH_2 from the previous reaction, 0.34 g Fmoc-Lys(Boc)-OH (0.73 mmol, 2 equiv.), 1.3 mL HOAt (0.73 mmol, 2 equiv.), 0.25 mL DIC (1.5 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH-PEG4-Biotin

The protected peptide peptide *resin*-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH-PEG4-Biotin was synthesised following the *On-resin Biotinylation* procedure using 0.73 g *resin*-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2 (0.1 mmol, 1 equiv.), 0.09 g EZ-

Link NHS- PEG4-Biotin (0.15 mmol, 1.5 equiv.), 0.15 mL DIPEA (0.8 mmol, 8 equiv.) in anhydrous CH_2Cl_2 (0.68 mL, 0.15 M), The reaction was then stirred for 4 hours and monitored *via* negative ninhydrin test and LCMS. Upon completion, the reaction mixture was drained, and the resin was subjected to *Resin Cleavage of Linear Peptide*

HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH-PEG4-Biotin

The protected linear peptide HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH-PEG4-Biotin was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 5 mL of trifluoroethanol and 5 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale yellow solid (0.12 g, 72% overall).

HO-Arg-Ser-Tyr-Gly-Lys-NH-PEG4-Biotin (Compound C1-Tag) The deprotected linear peptide HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH-PEG4-Biotin was generated following the *Global Deprotection* procedure using 0.12 g of Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH-PEG4-Biotin (0.09 mmol, 1 equiv.), 0.55 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.05 mL anisole (0.72 mmol, 8 equiv.) to simultaneously cleave the linear peptide from the resin and remove side chain protection groups. The reaction mixture was stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude linear peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (14 mg, 15% overall)

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{26}H_{44}N_9O_8^+$, 1083.54; found 1084

^{1}H NMR (600 MHz, D_2O): δ 7.15 (d, *J* = 8.11 Hz, 2H, Tyr), 6.846 (d, *J* = 8.14 Hz, 2H, Tyr), 4,63 4.58(m, 2H, αH TyrαH Ser), 4.45-4.39 (m, 2H, αH Arg, αH Lys), 4.3-4.162 (m, 2H, αH Gly), 3.958-3.213 (m, 4H, 2δH Arg, 2βH Ser), 3.80-3.73 (m, 2H, 2εH Lys), 3.724-3.526 (m, 14H, 2βH Tyr), 3.42-3.35 (m, 2H,), 3.35-3.27 (m, 1H), 3.27-3.152 (m, 2H), 3.15-3.06 (m, 2H), 3.05-2.813 (m, 4H), 2.81-2.69 (m, 1H), 2.69-2.46 (m, 2H), 2.30-2.22 (m, 2H), 1.97-1.215 (m, 14H, βH Lys, 2γH 2δH Lys, 2βH 2γH Arg)

3.4.7 Experimental Procedures for C2

***cyclo*-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)**

The protected cyclic peptide *cyclo*-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc) was synthesised following the *Macrocyclisation* procedure using 0.2 g HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc)-NH_2 (0.19 mmol, 1 equiv.), 0.071 g HATU (0.19 mmol, 1 equiv.), 0.048 g TBTU (0.15 mmol, 0.8 equiv.), 0.41 g DMTMM (0.15 mmol, 1 equiv.), 0.19 mL DIPEA (1.5 mmol, 8 equiv.) in anhydrous CH_2Cl_2 (186 mL, 0.001 M), The reaction was then stirred for 5 hours and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Macrocyclisation* procedure and dried *in vacuo* to produce the crude, protected, cyclic peptide *cyclo*-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-Lys(Boc).

***cyclo*-Arg-Ser-Tyr-Gly-Lys (Compound C2)**

The deprotected cyclic peptide *cyclo*-Arg-Ser-Tyr-Gly-Lys was synthesised following the *Global Deprotection* procedure using 0.15 g *cyclo*- Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Gly-

Lys(Boc) (0.14 mmol, 1 equiv.), 1.2 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.12 mL anisole (1.1 mmol, 10 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred for 4 hours and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude cyclic peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (8 mg, 4% overall).

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{26}H_{42}N_9O_7^+$, 592.32; found 592.35.

^{1}H NMR (600 MHz, D_2O): δ 7.22 -6.80 (m, *4*H, Tyr), 4,63 – 4.05 (m, 4H, αH Tyr, αH Ser, αH Arg, αH Lys), 2.1- (m, 4H, 2βH Ser, αH Gly), 3.35-2.6 (m, 6H, 2βH Tyr, 2εH Lys, 2δH Arg), 2.1 -1.3 (m, 10H, βH Lys, 2γH 2δH Lys, 2βH 2γH Arg)

3.4.8 Experimental Procedures for C4

Resin-O-Ala-NH_2

The resin bound amino acid Resin-O-Ala-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1.02 g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. 1.05 g Fmoc-Ala-OH (3.4 mmol, 3 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) and was added to the swelled resin. The reaction was agitated at room temperature overnight. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Ala-NHFmoc. A sample of resin was removed, and the resin loading was determined to be

0.63 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Ala-NH_2.

Resin-O-Ala-Phe-NH_2

Resin-O-Ala-Phe-NH_2 was synthesized according to the *Coupling Reaction* procedure, using 1.0 g Resin-O-Phe-NH_2 (0.63 mmol, 1 equiv.), 0.59 g Fmoc-Phe-OH (1.3 mmol, 2 equiv.), 2.15mL HOAt (1.3 mmol, 2 equiv.), 0.43 mL DIC (2.6 mmol, 4 equiv.) and 0.4 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ala-Phe-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ala-Phe-NH_2.

Resin-O-Ala-Phe-Glu(OtBu)-NH_2

Resin-O-Ala-Phe-Glu(OtBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Ala-Phe-NH_2 from the previous reaction, 0.55 g Fmoc-Glu(OtBu)-OH (1.3 mmol, 2 equiv.), 2.15mL HOAt (1.3 mmol, 2 equiv.), 0.43 mL DIC (2.6 mmol, 4 equiv.) and 0.4 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ala-Phe-Glu(OtBu)-NHFmoc**.** The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ala-Phe-Glu(OtBu)-NH_2.

Resin-O-Ala-Phe-Glu(OtBu)-Leu-NH_2

Resin-O-Ala-Phe-Glu(OtBu)-Leu-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Ala-Phe-Glu(OtBu)-NH_2 from the previous reaction,

0.46 g Fmoc-Leu-OH (1.3 mmol, 2 equiv.), 2.15mL HOAt (1.3 mmol, 2 equiv.), 0.43 mL DIC (2.6 mmol, 4 equiv.) and 0.4 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ala-Phe-Glu(OtBu)-Leu-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ala-Phe-Glu(OtBu)-Leu-NH_2.

Resin-O-Ala-Phe-Glu(OtBu)-Leu-Ala-NH_2

Resin-O-Ala-Phe-Glu(OtBu)-Leu-Ala-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Ala-Phe-Glu(OtBu)-Leu-NH_2 from the previous reaction, 0.40 g Fmoc-Ala-OH (1.3 mmol, 2 equiv.), 2.15mL HOAt (1.3 mmol, 2 equiv.), 0.43 mL DIC (2.6 mmol, 4 equiv.) and 0.4 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Ala-Phe-Glu(OtBu)-Leu-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Ala-Phe-Glu(OtBu)-Leu-Ala-NH_2.

HO-Ala-Phe-Glu(OtBu)-Leu-Ala-NH_2

The protected linear peptide HO-Ala-Phe-Glu(OtBu)-Leu-Ala-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 13.0 mL of trifluoroethanol and 13.0 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale-yellow solid.

***cyclo*-Ala-Phe-Glu(OtBu)-Leu-Ala**

The protected cyclic peptide *cyclo*-Ala-Phe-Glu(OtBu)-Leu-Ala was synthesised following the *Macrocyclisation* procedure using 0.09 g HO-Ala-Phe-Glu(OtBu)-Leu-Ala (0.15 mmol, 1 equiv.), 0.056 g HATU (0.15 mmol, 1 equiv.), 0.04 g TBTU (0.12 mmol, 0.8 equiv.), 0.15 mL DIPEA (1.2 mmol, 8 equiv.) in anhydrous CH_2Cl_2 (149 mL, 0.001 M), The reaction was then stirred for 5 hours and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Macrocyclisation* procedure and dried *in vacuo* to produce the crude, protected, cyclic peptide *cyclo*-Ala-Phe-Glu(OtBu)-Leu-Ala

***cyclo*-Ala-Phe-Glu-Leu-Ala (Compound C4)**

The deprotected cyclic peptide *cyclo*-Ala-Phe-Glu-Leu-Ala was synthesised following the *Global Deprotection* procedure using 0.08 g *cyclo*-Ala-Phe-Glu-Leu-Ala (0.14 mmol, 1 equiv.), 0.32 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.12 mL anisole (1.1 mmol, 8 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred for 4 hours and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude cyclic peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (7mg, 13% overall).

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{26}H_{38}N_5O_7^+$, 532.28; found 532.30

^{1}H NMR (600 MHz, D_2O): δ 7.57 – 7.06 (m, 5H, Phe), 4.51 – 4.32 (m, 2H, αH Ala, αH Leu), 4.32- 4.23 (m, 2H, αH Phe, αH Glu), 4.04 – 3.98 (m, 1H, αH Ala), 3.28 – 3.10 (m, 2H, βH Phe), 2.20 – 1.98 (m, 2H, γH Glu), 1.96 – 1.80 (m, 2H, βH Glu), 1.82 – 1.76 (m,

1H, γH Leu), 1.68 – 1.58 (t, *J* = 7.03 Hz, 2H, βH Leu), 1.51 – 1.43 (m, 6H, βH Ala), 0.97 (d, *J* = 6.11 Hz, 3H, $(CH_3)_2$ Leu), 0.92 (d, *J* = 5.98 Hz, 3H, $(CH_3)_2$ Leu).

3.4.9 Experimental Procedures for SY14

Resin-O-Arg(Pbf)-NH_2

The resin bound amino acid Resin-O-Arg(Pbf)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (0.497 g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. 0.71g Fmoc-Arg(Pbf)-OH (1.1 mmol, 2 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) and was added to the swelled resin. The reaction was agitated at room temperature overnight. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Arg(Pbf)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.47 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Arg(Pbf)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using 0.497 g Resin-O- Arg(Pbf)-NH_2 (0.47 mmol, 1 equiv.), 0.18 g Fmoc-Ser(tBu)-OH (0.23 mmol, 2 equiv.), 0.28 mL HOAt (0.47 mmol, 2 equiv.), 0.16 mL DIC (0.9 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-

Ser(tBu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 from the previous reaction, 0.22 g Fmoc-Tyr(tBu)-OH (0.46 mmol, 2 equiv.), 0.28 mL HOAt (0.47 mmol, 2 equiv.), 0.16 mL DIC (0.9 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NHFmoc**.** The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-NH_2 from the previous reaction, 015g Fmoc-Ala -OH (0.47 mmol, 2 equiv.), 0.28 mL HOAt (0.47 mmol, 2 equiv.), 0.16 mL DIC (0.9 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala -NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala -NH_2 from the previous reaction, 0.22 g Fmoc-Lys(Boc)-OH (0.47 mmol, 2 equiv.), 0.28 mL HOAt (0.47 mmol, 2 equiv.), 0.16 mL DIC (0.9 mmol, 4 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NH_2.

HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NH_2 (Compound LP-14)

The protected linear peptide HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 13.0 mL of trifluoroethanol and 13.0 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale-yellow solid (188 mg, 84% overall).

HO-Arg-Ser-Tyr-Ala-Lys-NH_2 (Compound SY14)

The deprotected linear peptide HO-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NH_2 was generated following the *Global Deprotection* procedure using 0.1 g of Resin-O-Arg(Pbf)-Ser(tBu)-Tyr(tBu)-Ala-Lys(Boc)-NH_2 (0.09 mmol, 1 equiv.), 0.55 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.08 mL anisole (0.76 mmol, 8 equiv.) to simultaneously cleave the linear peptide from the resin and remove side chain protection groups. The reaction mixture was stirred overnight and monitored *via* LC/MS. Upon

completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude linear peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (12 mg, 16% overall)

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{27}H_{46}N_9O_8^+$, 624.33; found 624

^{1}H NMR (600 MHz, D_2O): δ 7.16 (d, *J* = 8.5 Hz, 2H, Tyr), 6.846 (d, *J* = 8.54 Hz, 2H, Tyr), 4,63 (t, J = 6.9 Hz, 1H, αH Tyr), 4.435-4.34 (m, 2H, αH Ser, αH Ala), 4.2056 (dd, J = 5.26, 7.67 Hz, 1H, αH Arg), 4.09 – 3.88 (m, 3H, αH Lys, 2βH Ser), 3.25-2.92 (m, 6H, 2βH Tyr, 2εH Lys, 2δH Arg), 2.01-1.78 (m, 2H, βH Lys), 1.83 – 1.376 (m, 8H, 2γH 2δH Lys, 2βH 2γH Arg),1.3681-1.3565 (m, 3H, 3βH Ala)

3.4.10 Experimental Procedures for SY15

Resin-O-Arg(Pbf)-NH_2

The resin bound amino acid Resin-O-Arg(Pbf)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1.08 g, 1.188 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. 1.5 g Fmoc-Arg(Pbf)-OH (2.3 mmol, 2 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) and was added to the swelled resin. The reaction was agitated at room temperature overnight. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Arg(Pbf)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.49 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Arg(Pbf)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using 0.5 g Resin-O- Arg(Pbf)-NH_2 (0.24 mmol, 1 equiv.), 0.2 g Fmoc-Ser(tBu)-OH (0.49 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2 equiv.), 0.16 mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Ala-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Ala-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-NH_2 from the previous reaction, 0.15 g Fmoc-Ala-OH (0.49 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2 equiv.), 0.16 mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Ala-NHFmoc**.** The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Ala-NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Ala-NH_2 from the previous reaction, 0.16 g Fmoc-Gly -OH (0.49 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2

equiv.), 0.16 mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly -NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly -NH_2.

Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NH_2

Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly -NH_2 from the previous reaction, 0.23 g Fmoc-Lys(Boc)-OH (049 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2 equiv.), 0.16 mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NH_2.

HO-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NH_2

The protected linear peptide HO-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 13.0 mL of trifluoroethanol and 13.0 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale yellow solid (167 mg, 78% overall).

HO-Arg-Ser-Ala-Gly-Lys-NH_2 (Compound SY15)

The deprotected linear peptide HO-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NH_2 was generated following the *Global Deprotection* procedure using 0.1 g of Resin-O-Arg(Pbf)-Ser(tBu)-Ala-Gly-Lys(Boc)-NH_2 (0.05 mmol, 1 equiv.), 0.55 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.05 mL anisole (0.4 mmol, 8 equiv.) to simultaneously cleave the linear peptide from the resin and remove side chain protection groups. The reaction mixture was stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude linear peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (16 mg, 12 % overall)

LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{20}H_{40}N_9O_7^+$, 518.3.33; found 518

^{1}H NMR (600 MHz, D_2O): δ 4.435 (t, J = 5.66 HZ, 1H, αH Ser), 4,42 – 4.37 (m, 2H, αH Ala), 4.2056 (dd, J = 5.26, 7.67 Hz, 1H, αH Arg), 4.09 – 3.88 (m, 3H, αH Lys, 2βH Ser), 3.86-3.71 (m, 2H, αH Gly), 3.25-2.92 (m, 4H, , 2εH Lys, 2δH Arg), 2.01-1.84 (m, 2H, βH Lys), 1.83 – 1.45 (m, 8H, 2γH 2δH Lys, 2βH 2γH Arg), 1,43 (d, J = 7.19 Hz, 3H, 3βH Ala)

3.4.11 Experimental Procedures for SY16

Resin-O-Arg(Pbf)-NH_2

The resin bound amino acid Resin-O-Arg(Pbf)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1.08 g, 1.188 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. 1.5 g Fmoc-Arg(Pbf)-OH (2.3 mmol, 2 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.40 M) and was added to the swelled resin. The reaction was agitated at room temperature overnight. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Arg(Pbf)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.49 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Arg(Pbf)-NH_2.

Resin-O-Arg(Pbf)-Ala-NH_2

Resin-O-Arg(Pbf)-Ala-NH_2 was synthesized according to the *Coupling Reaction* procedure, using 1.0 g Resin-O- Arg(Pbf)-NH_2 (0.39 mmol, 1 equiv.), 0.16 g Fmoc- Ala-OH (0.49 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2 equiv.), 0.16 mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 5 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ala-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ala-NH_2.

Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-NH_2

Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ala-NH_2 from the previous reaction, 0.22 g Fmoc-Tyr(tBu)-OH (0.49 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2 equiv.), 0.16

mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight, and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-NHFmoc**.** The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-NH_2.

Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly-NH_2

Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-NH_2 from the previous reaction, 0.15 g Fmoc-Gly -OH (0.49 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2 equiv.), 0.16 mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly -NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly -NH_2.

Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NH_2

Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly -NH_2 from the previous reaction, 0.23 g Fmoc-Lys(Boc)-OH (0.49 mmol, 2 equiv.), 0.82 mL HOAt (0.49 mmol, 2 equiv.), 0.16 mL DIC (0.98 mmol, 4 equiv.) and 1.18 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run overnight and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NHFmoc. The Fmoc group was

then removed following the *Fmoc Removal* procedure to afford Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NH_2.

HO-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NH_2

The protected linear peptide HO-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 13.0 mL of trifluoroethanol and 13.0 mL of CH_2Cl_2. The resin containing solution was filtered and dried *in vacuo* to yield the protected linear pentapeptide as a pale-yellow solid (189 mg, 82% overall).

HO-Arg-Ala-Tyr-Gly-Lys-NH_2 (Compound SY16)

The deprotected linear peptide HO-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NH_2 was generated following the *Global Deprotection* procedure using 0.1 g of Resin-O-Arg(Pbf)-Ala-Tyr(tBu)-Gly-Lys(Boc)-NH_2 (0.05 mmol, 1 equiv.), 0.55 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.05 mL anisole (0.4 mmol, 8 equiv.) to simultaneously cleave the linear peptide from the resin and remove side chain protection groups. The reaction mixture was stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude linear peptide. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (8 mg, 11% overall)

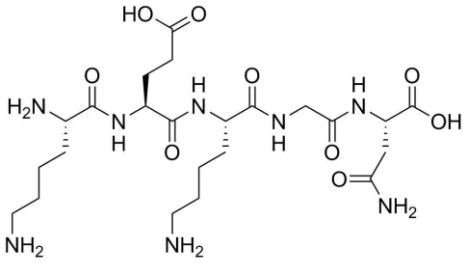
LC/MS (ESI) *m/z*: $[M+H]^+$ calculated for $C_{26}H_{44}N_9O_8^+$, 594.33; found 594

^{1}H NMR (600 MHz, D_2O): δ 7.15 (d, *J* = 8.55 Hz, 2H, Tyr), 6.846 (d, *J* = 8.54 Hz, 2H, Tyr), 4,63 (t, J = 6.9 Hz, 1H, αH Tyr), 4.32 (q, J = 7 HZ, 1H, αH Ala), 4.2056 (dd, J =

5.26, 7.67 Hz, 1H, αH Arg), 4.09 – 3.88 (m, 1H, αH Lys), 3.86-3.71 (m, 2H, αH Gly), 3.25-2.92 (m, 6H, 2βH Tyr, 2εH Lys, 2δH Arg), 2.01-1.84 (m, 2H, βH Lys), 1.83 – 1.41 (m, 8H, 2γH 2δH Lys, 2βH 2γH Arg), 1,37 (d, J = 7.14 Hz, 3H, 3βH Ala)

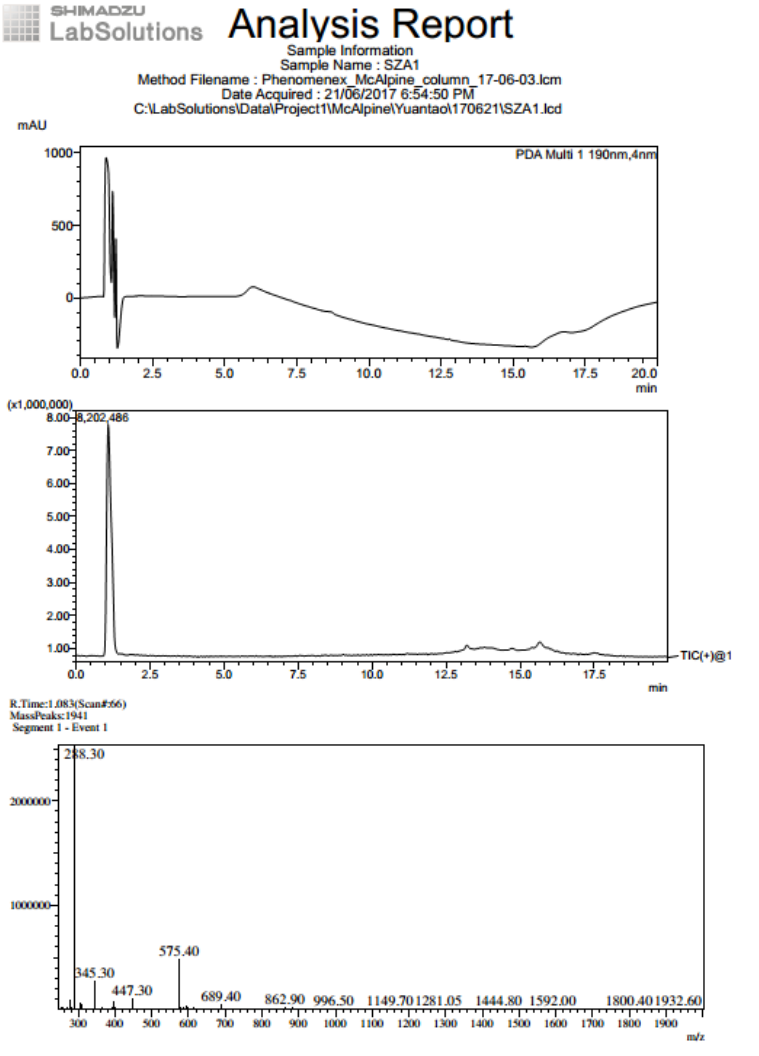
Chapter 4: Appendix

4.1.1 LC/MS of A1



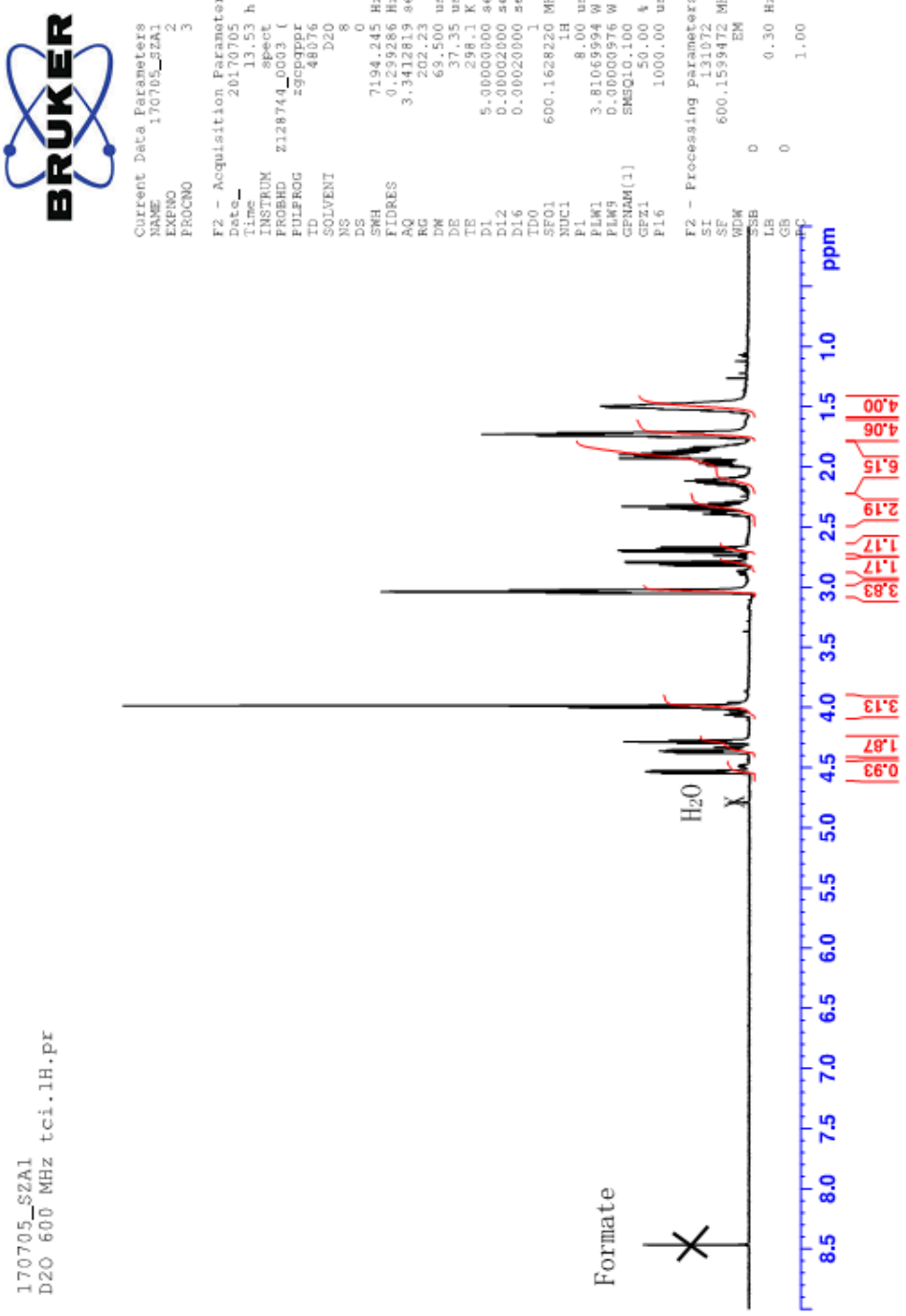
Chemical Formula: $C_{23}H_{42}N_8O_9$
Exact Mass: 574.31
Molecular Weight: 574.64

15/11/2017 2:31:46 PM Page 1 / 1

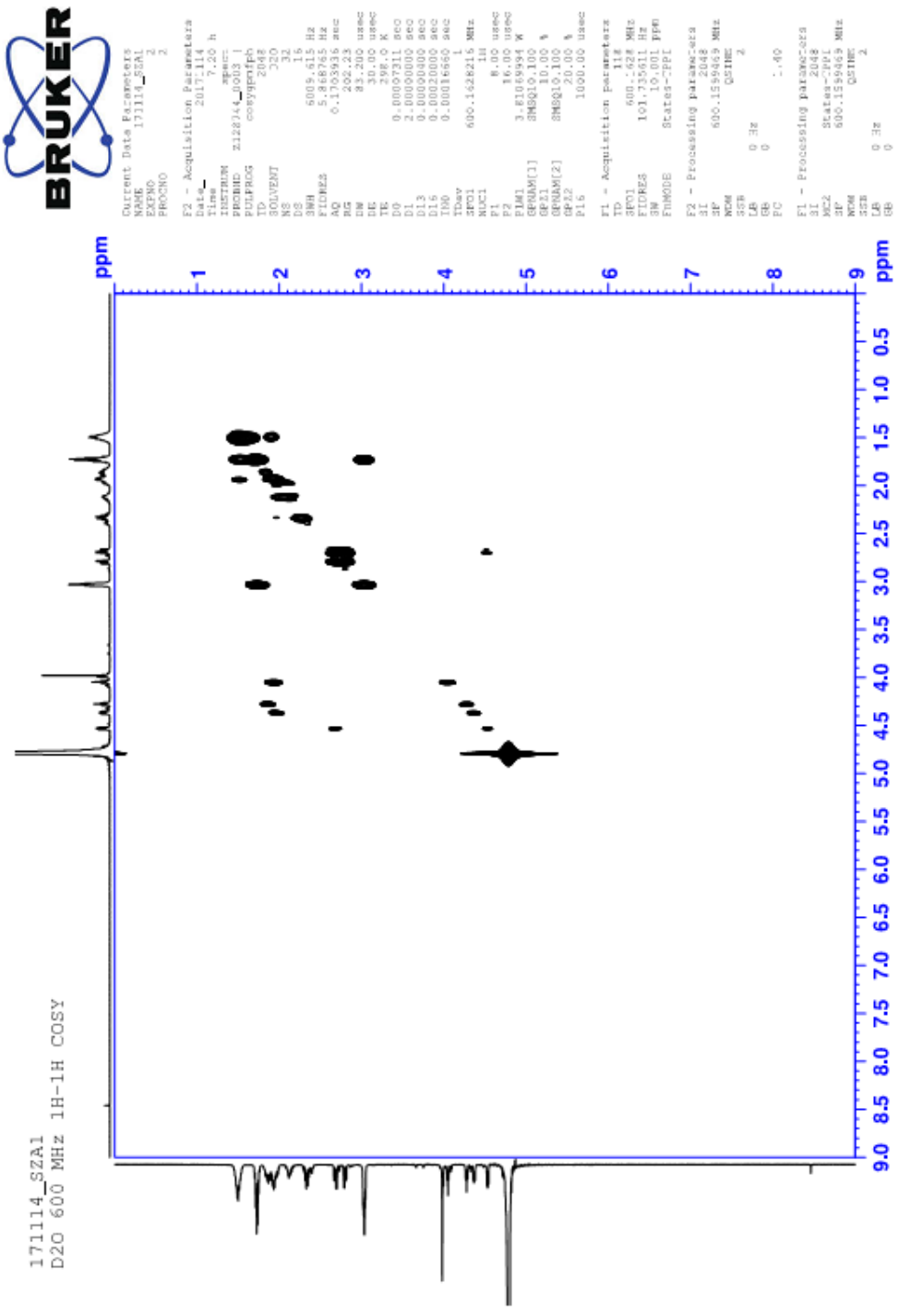


C:\LabSolutions\Data\Project1\McAlpine\Yuantao\170621\SZA1.lcd

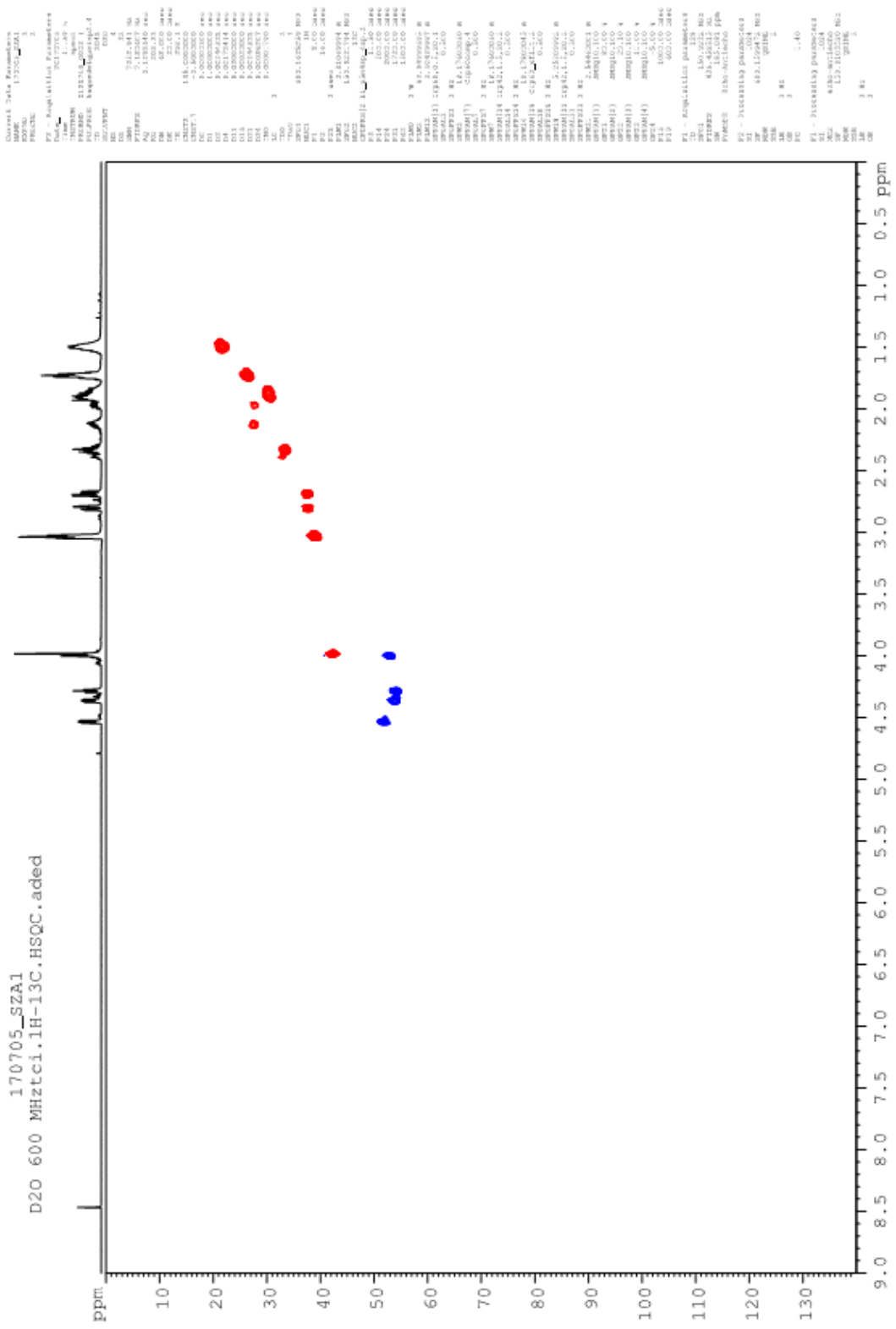
4.1.2 ^{1}H NMR of A1



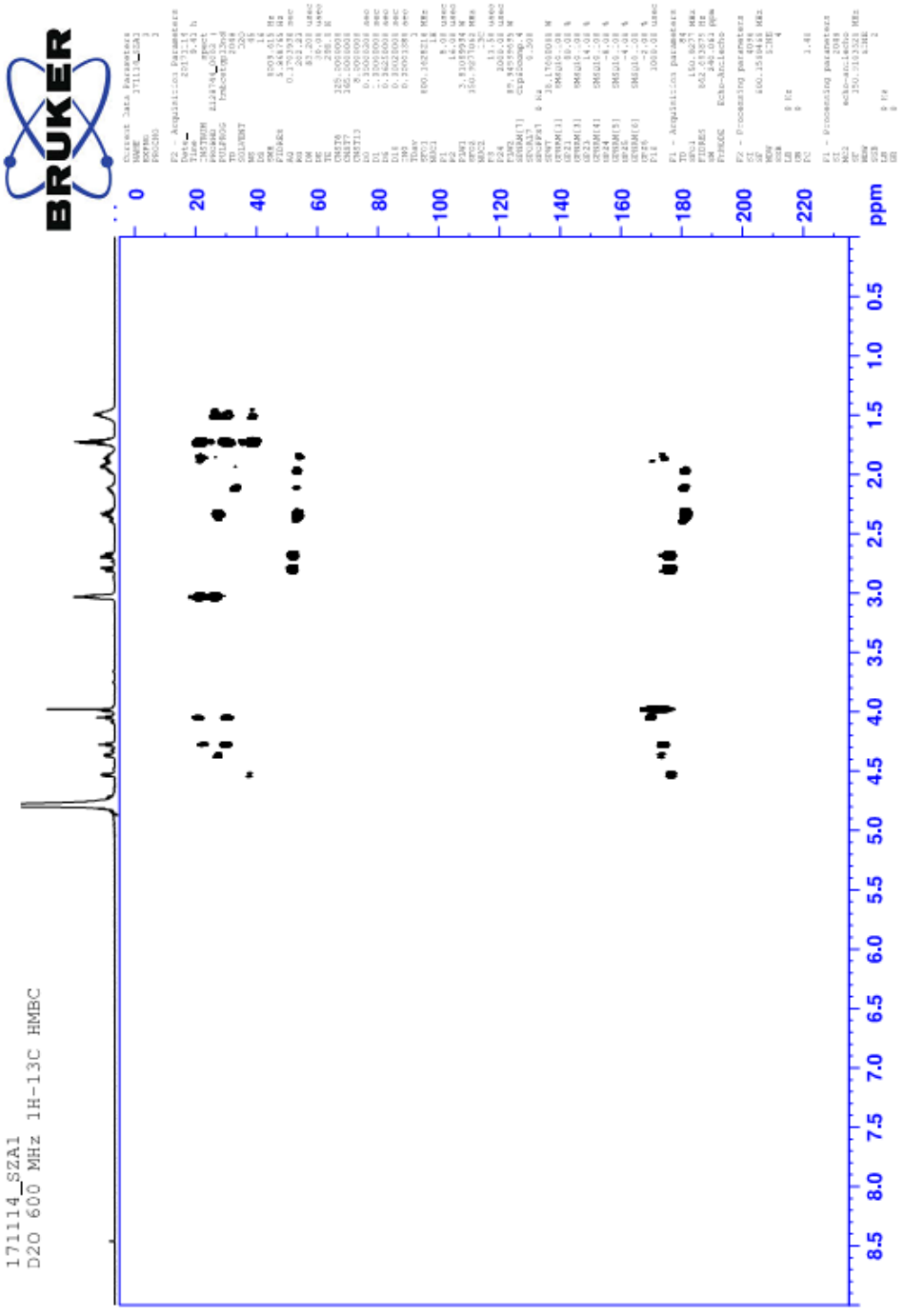
4.1.3 ^{1}H-^{1}H COSY of A1



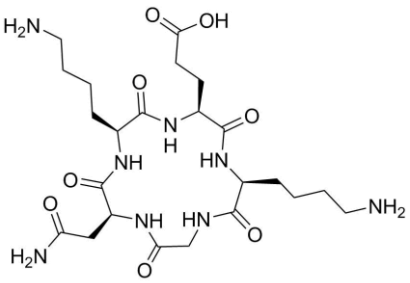
4.1.4 ^{1}H-^{13}C HSQC of A1



4.1.5 ^{1}H-^{13}C HMBC of A1



4.2.1 LC/MS of A2



Chemical Formula: C23H40N8O8
Exact Mass: 556.30
Molecular Weight: 556.62

15/11/2017 2:28:53 PM Page 1 / 1

SHIMADZU LabSolutions **Analysis Report**

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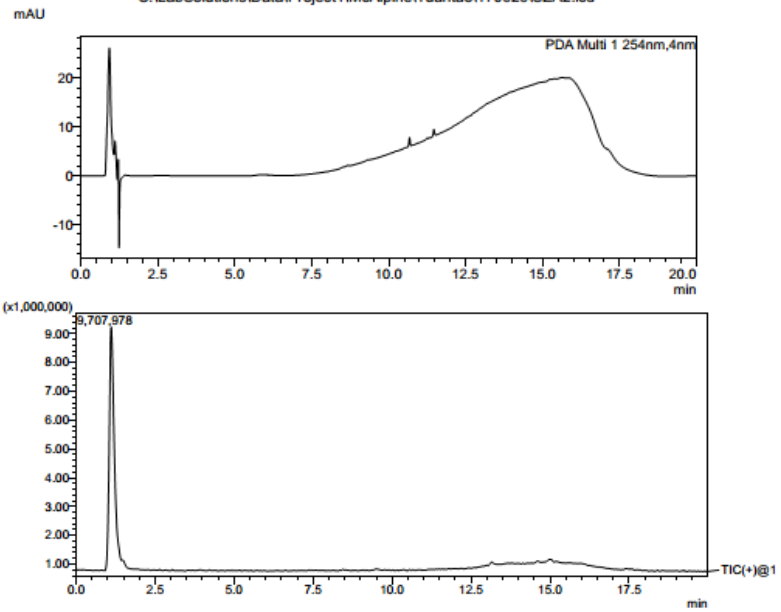

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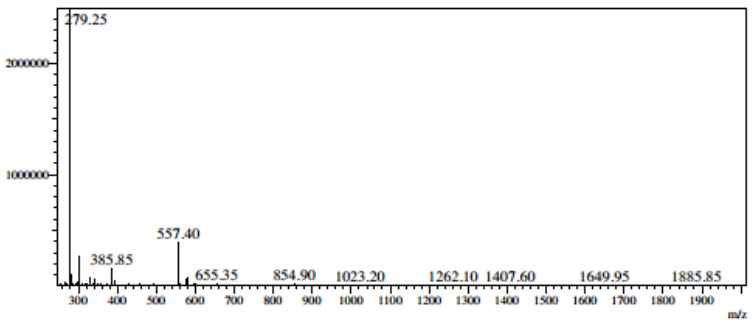

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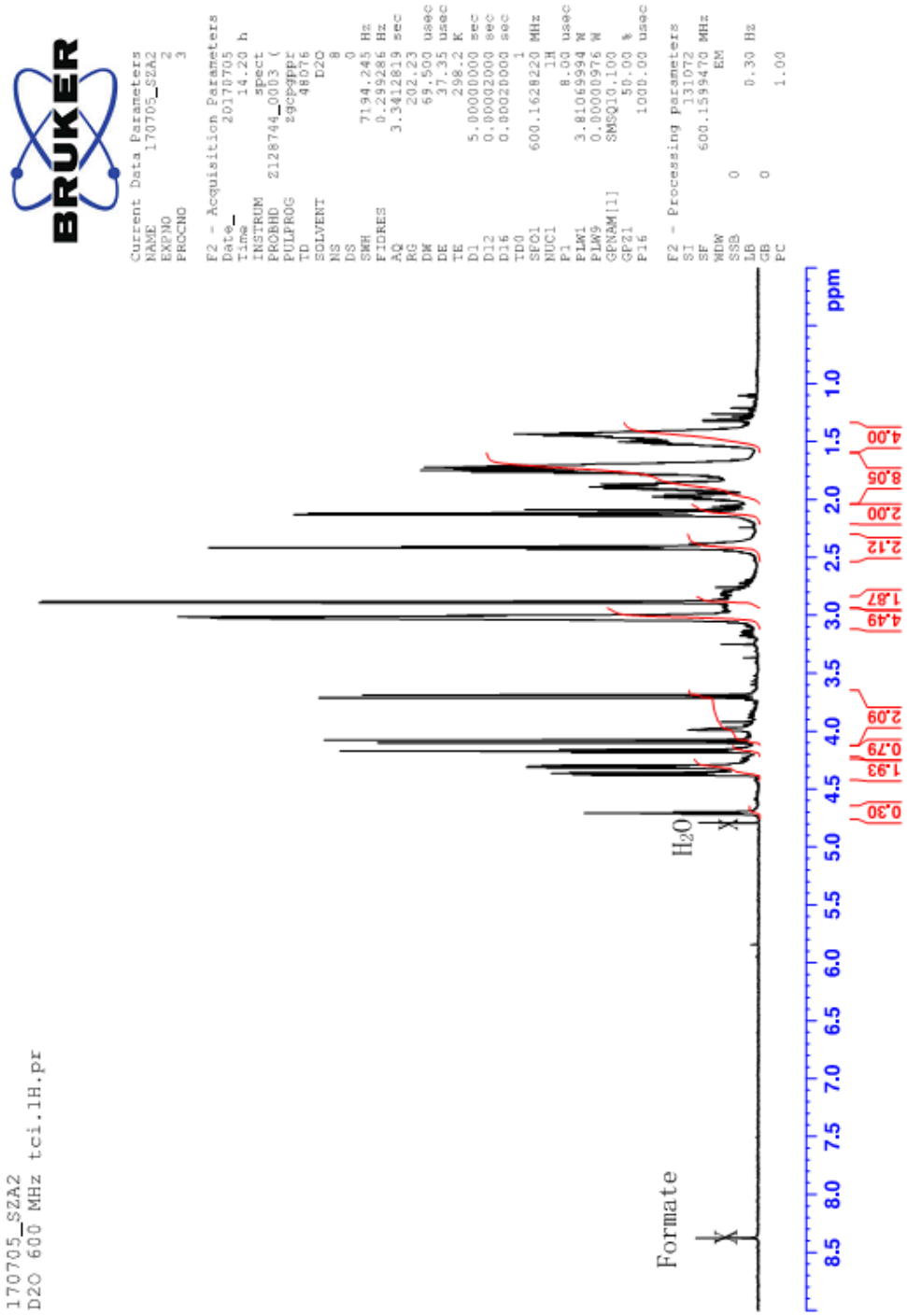
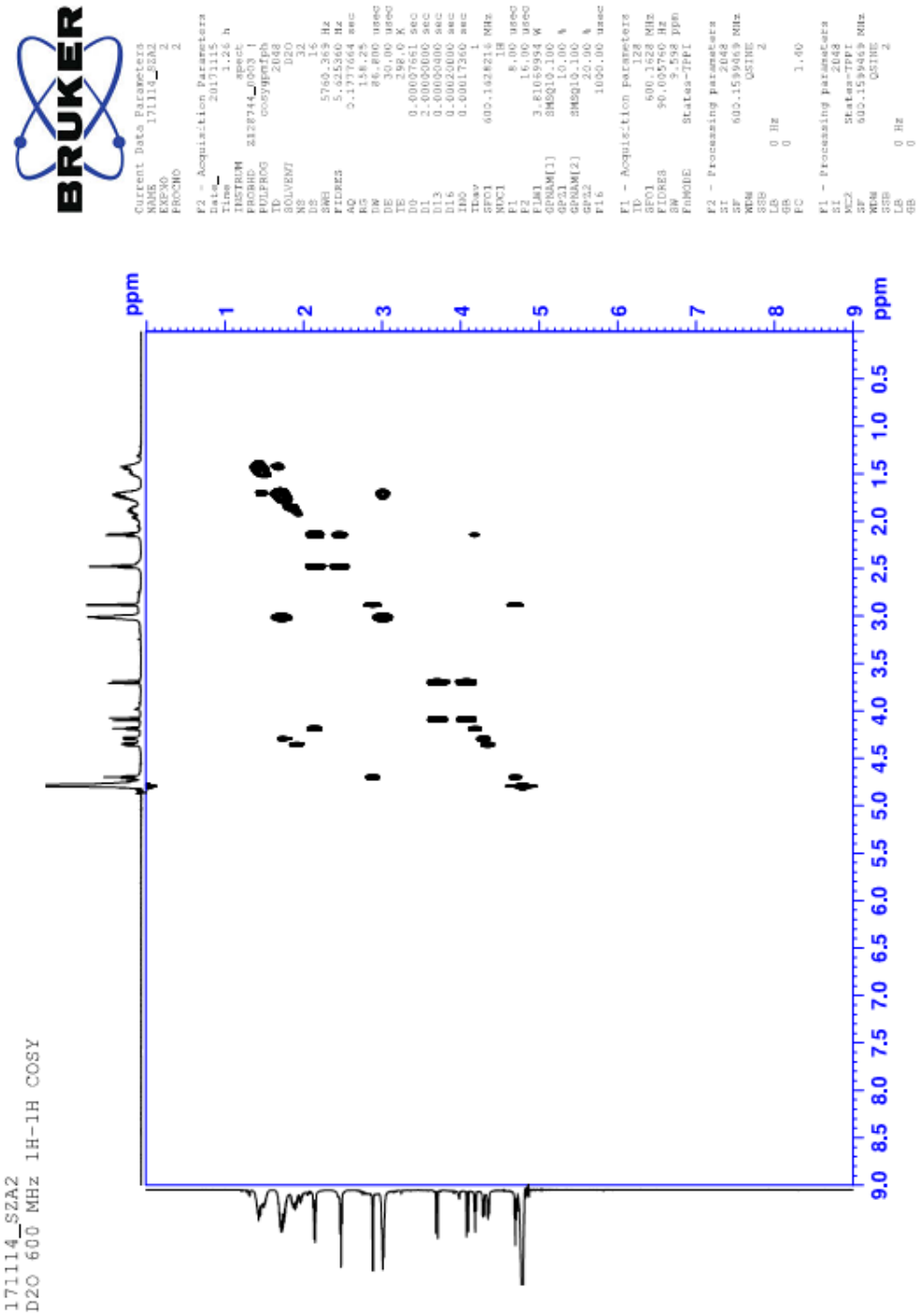
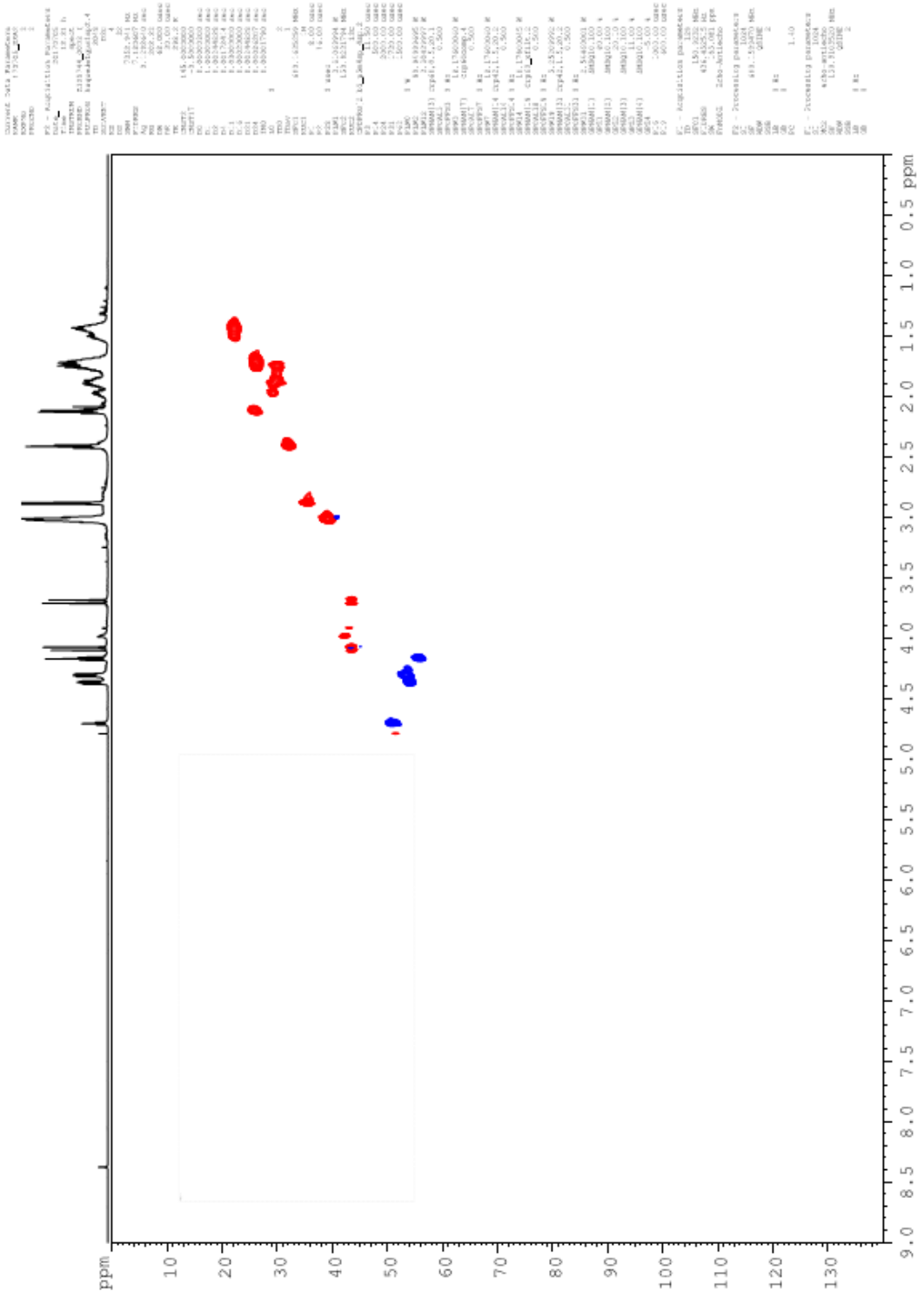
4.2.2 ^{1}H NMR of A2



4.2.3 ^{1}H-^{1}H COSY of A2



4.2.4 ^{1}H-^{13}C HSQC of A2



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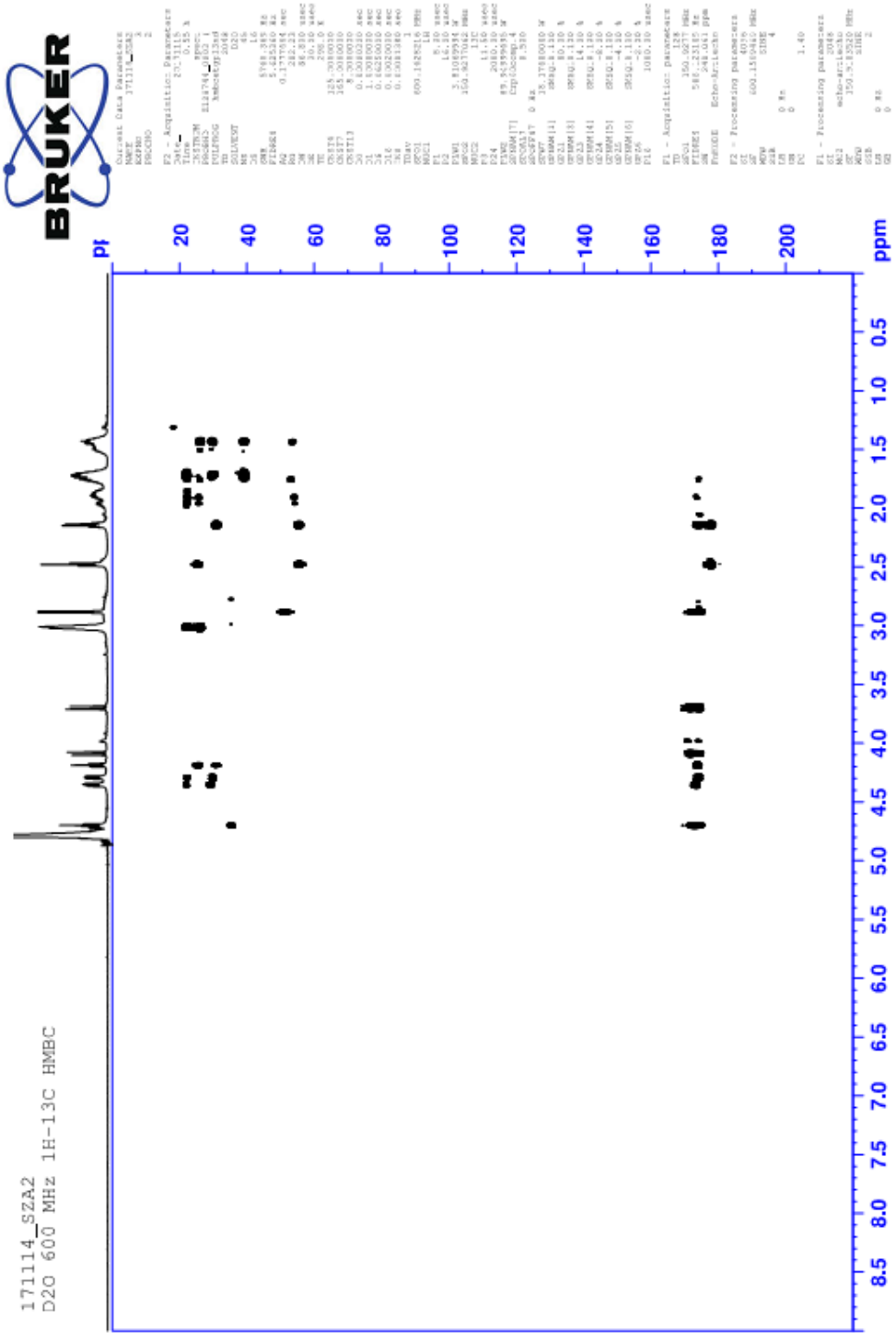
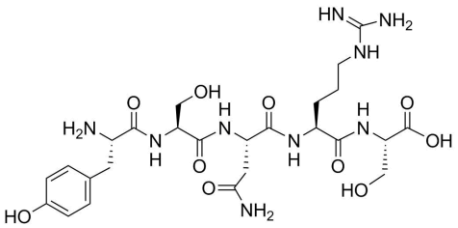


4.3.1 LC/MS of B1



Chemical Formula: $C_{25}H_{39}N_9O_{10}$
Exact Mass: 625.28
Molecular Weight: 625.64

15/11/2017 2:35:03 PM Page 1 / 1

SHIMADZU LabSolutions

Analysis Report

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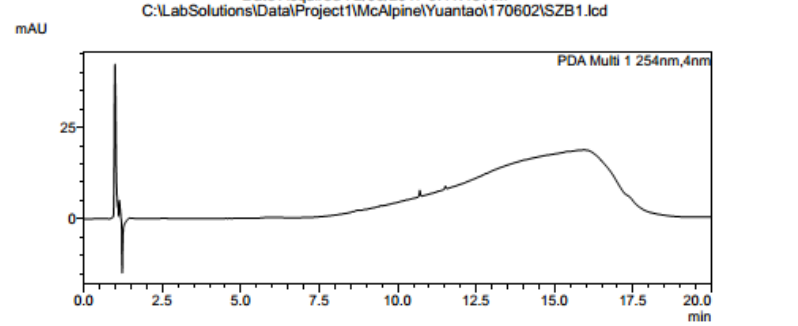

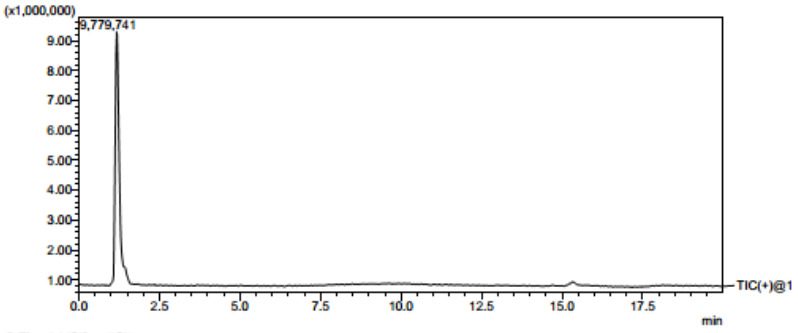

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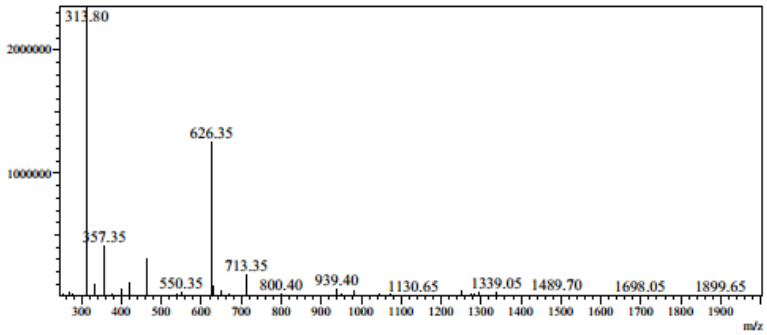

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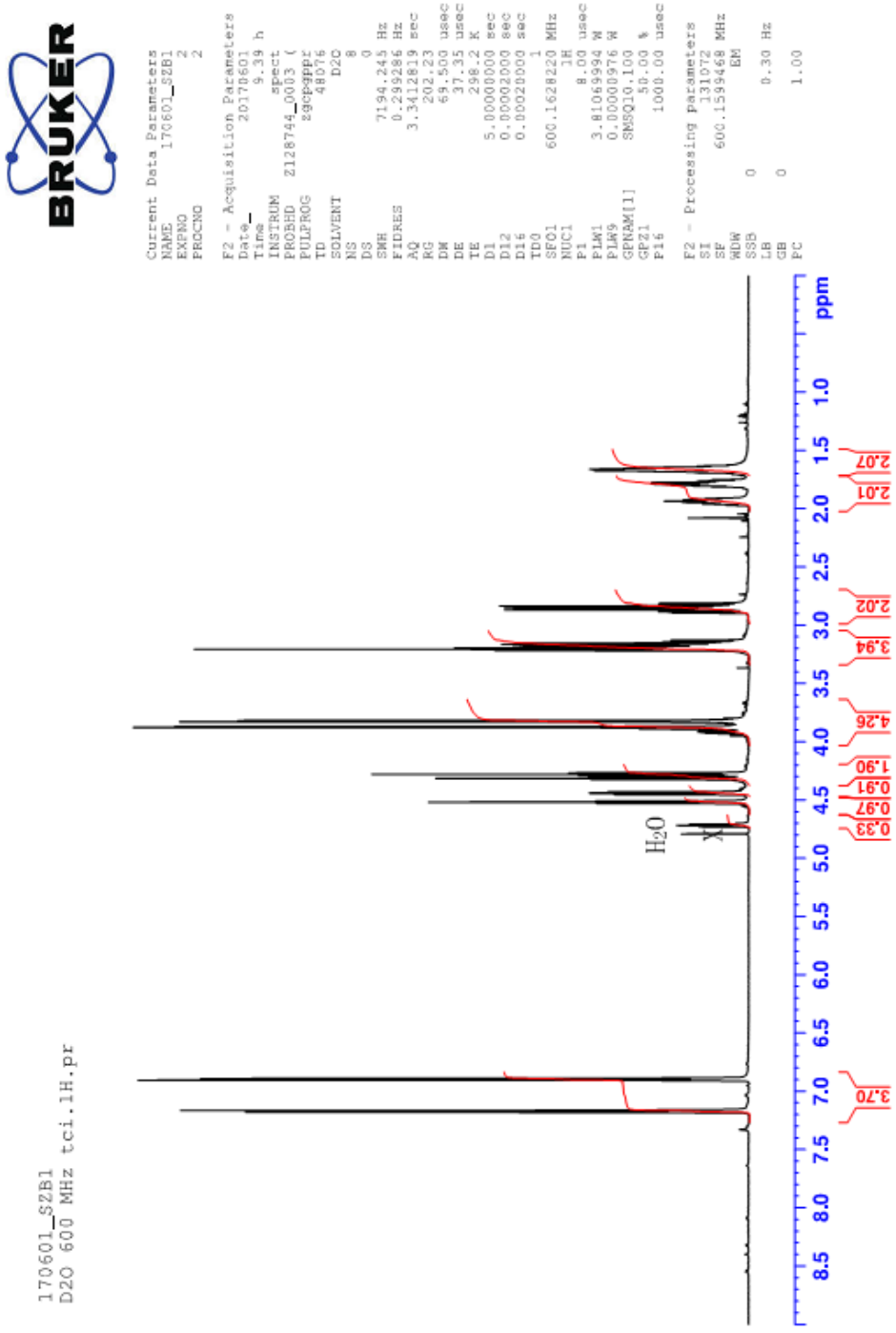
4.3.2 ^{1}H NMR of B1



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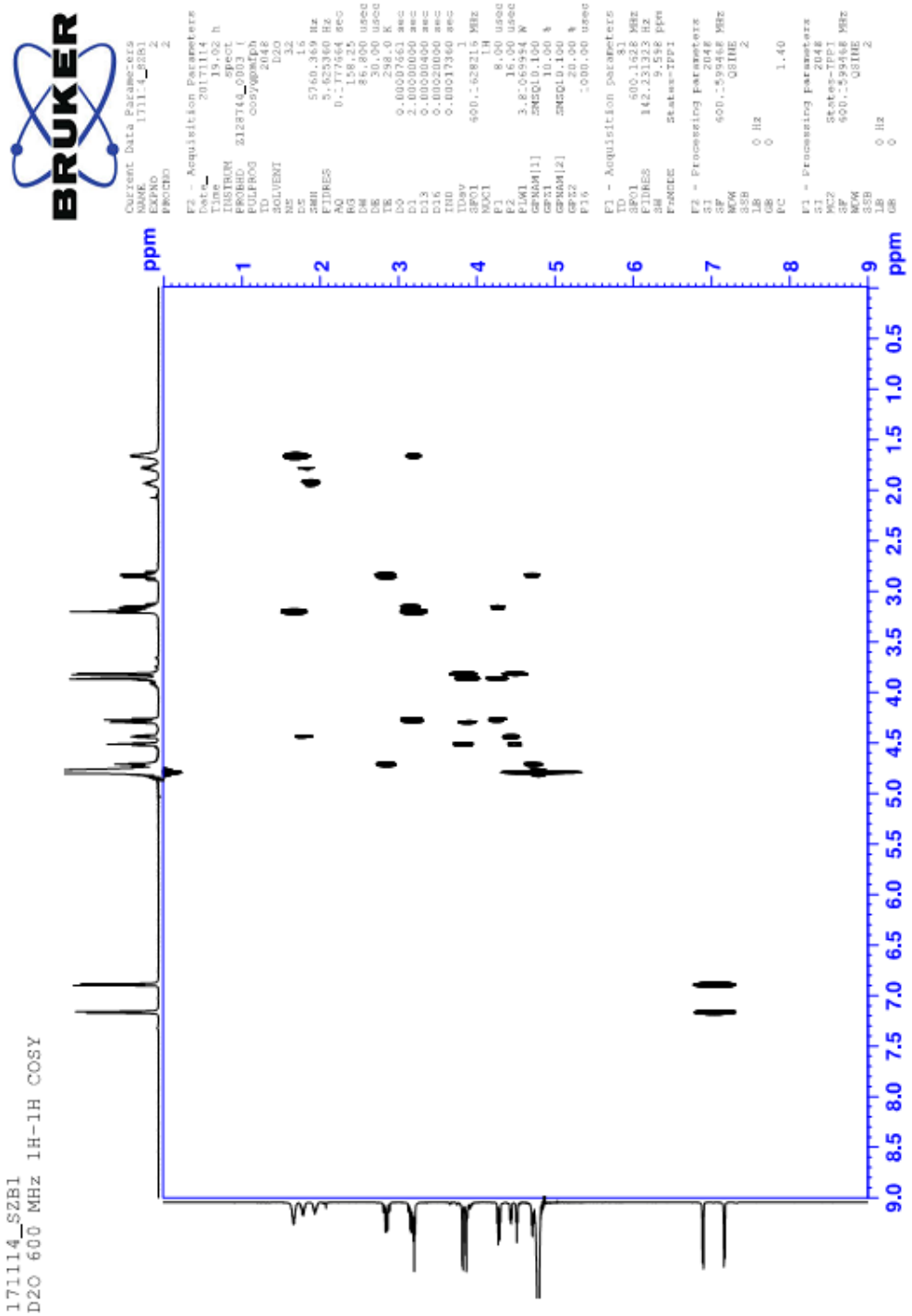
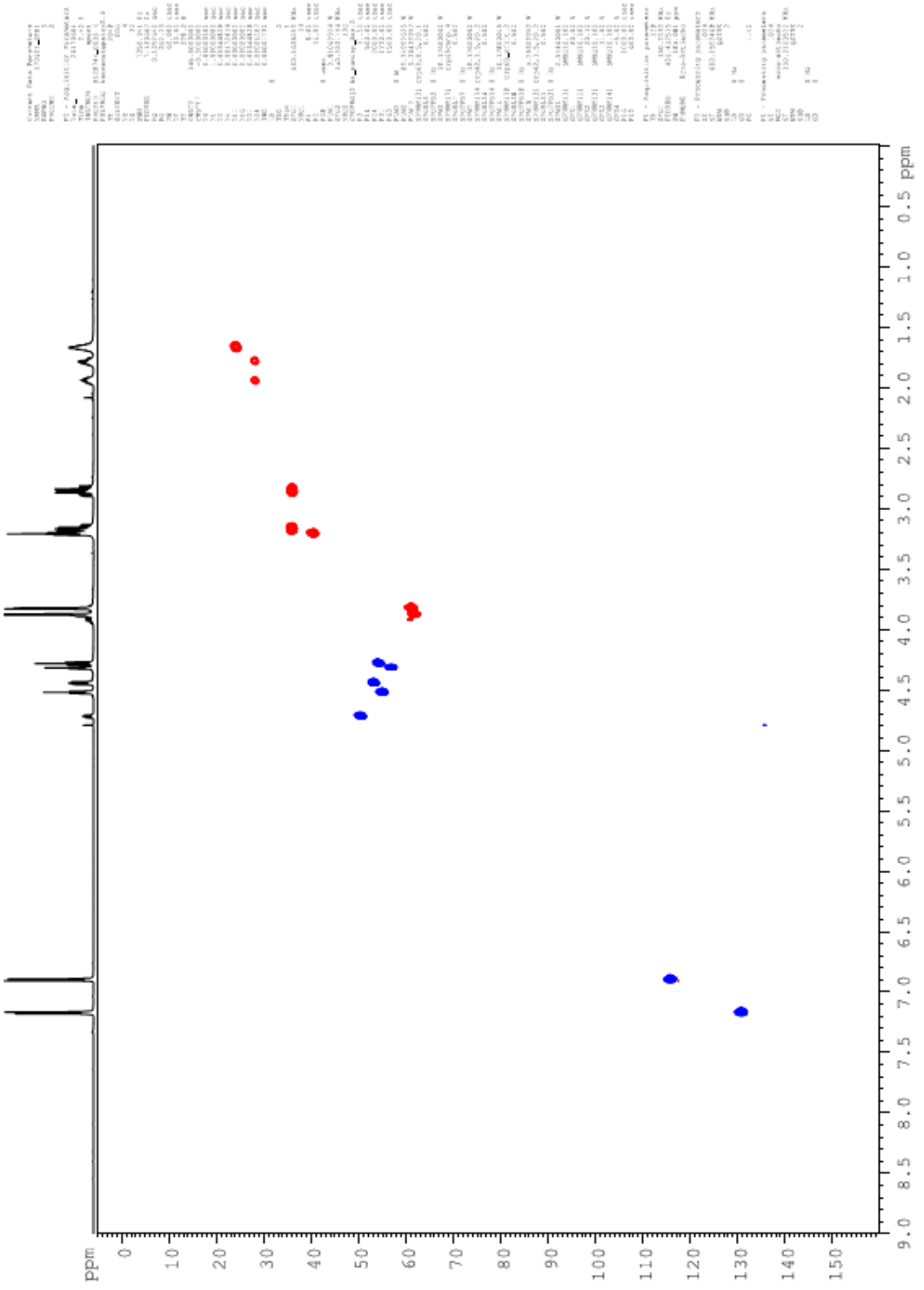
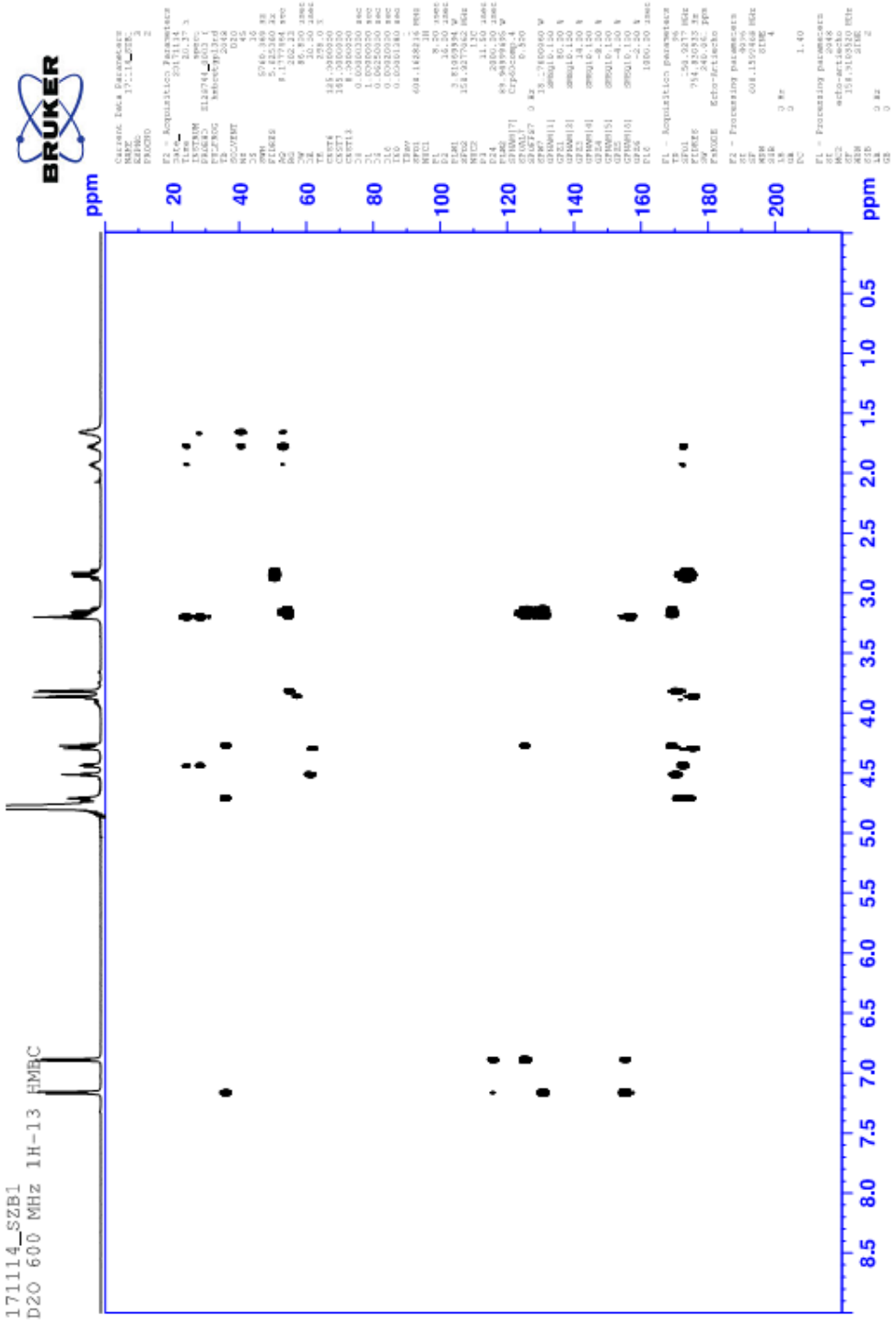


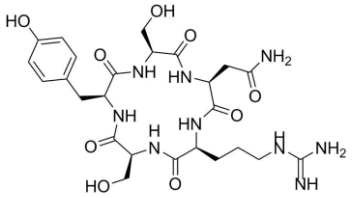
4.3.4 ^{1}H-^{13}C HSQC of B1



4.3.5 ^{1}H-^{13}C HMBC of B1



4.4.1 LC/MS of B2



Chemical Formula: $C_{25}H_{37}N_9O_9$
Exact Mass: 607.27
Molecular Weight: 607.63

15/11/2017 2:38:40 PM Page 1 / 1

SHIMADZU LabSolutions **Analysis Report**

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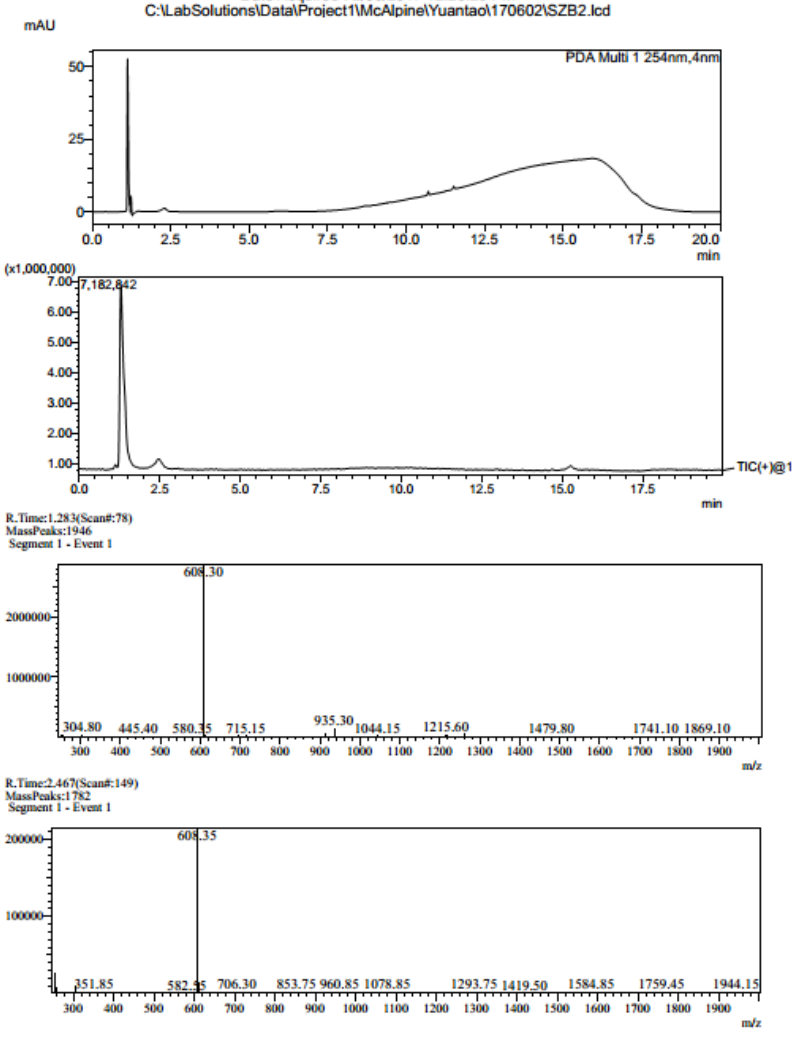

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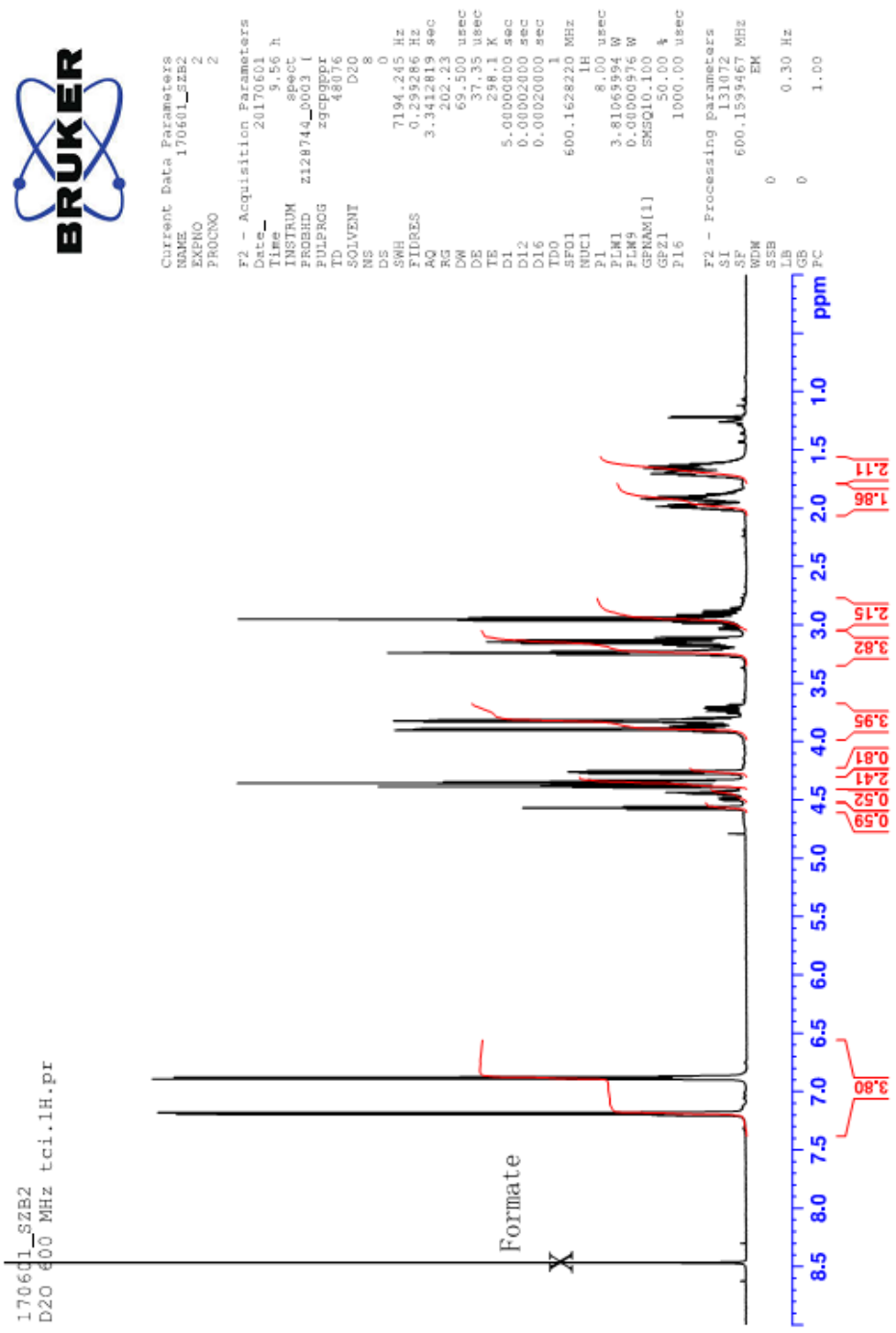
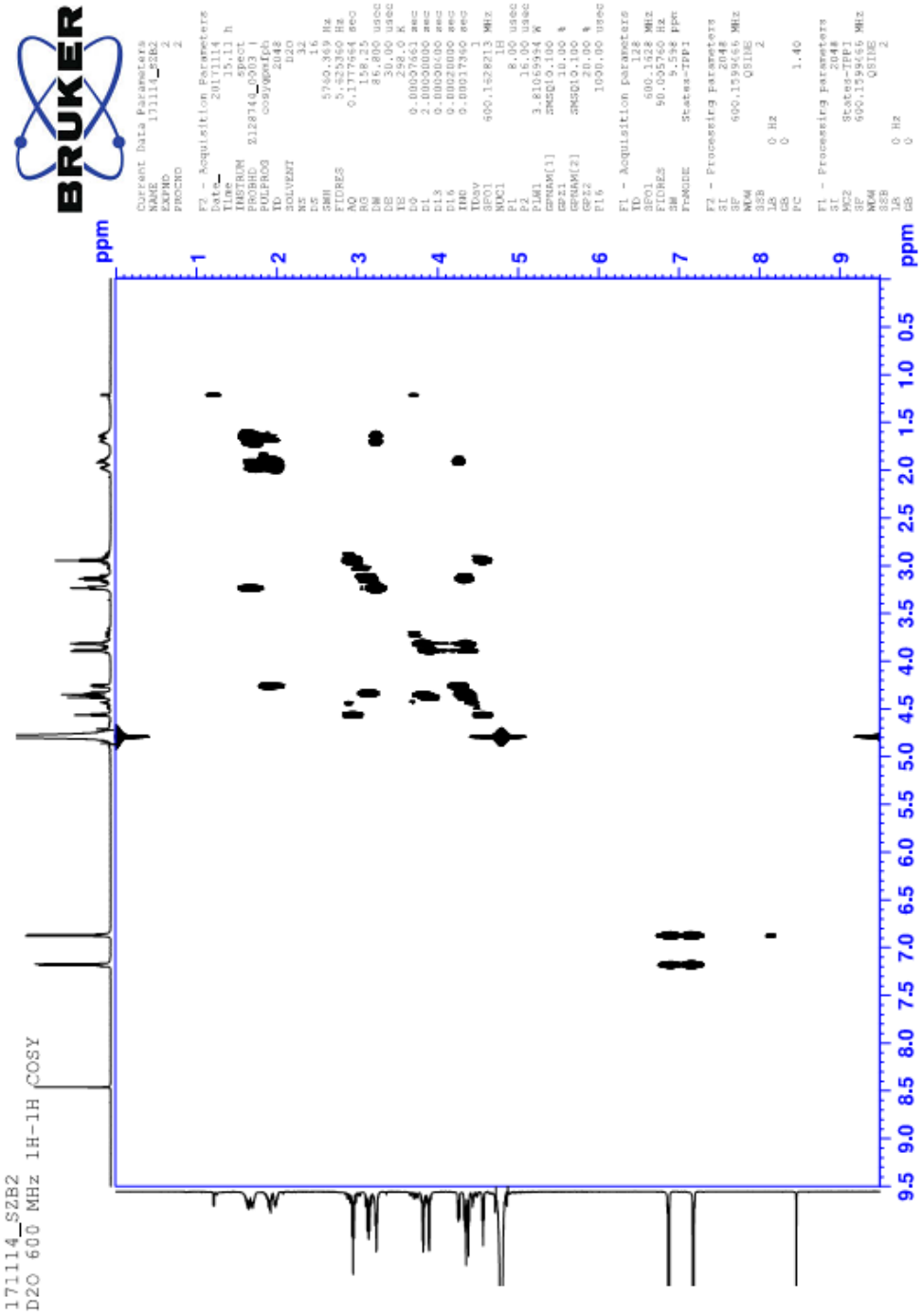
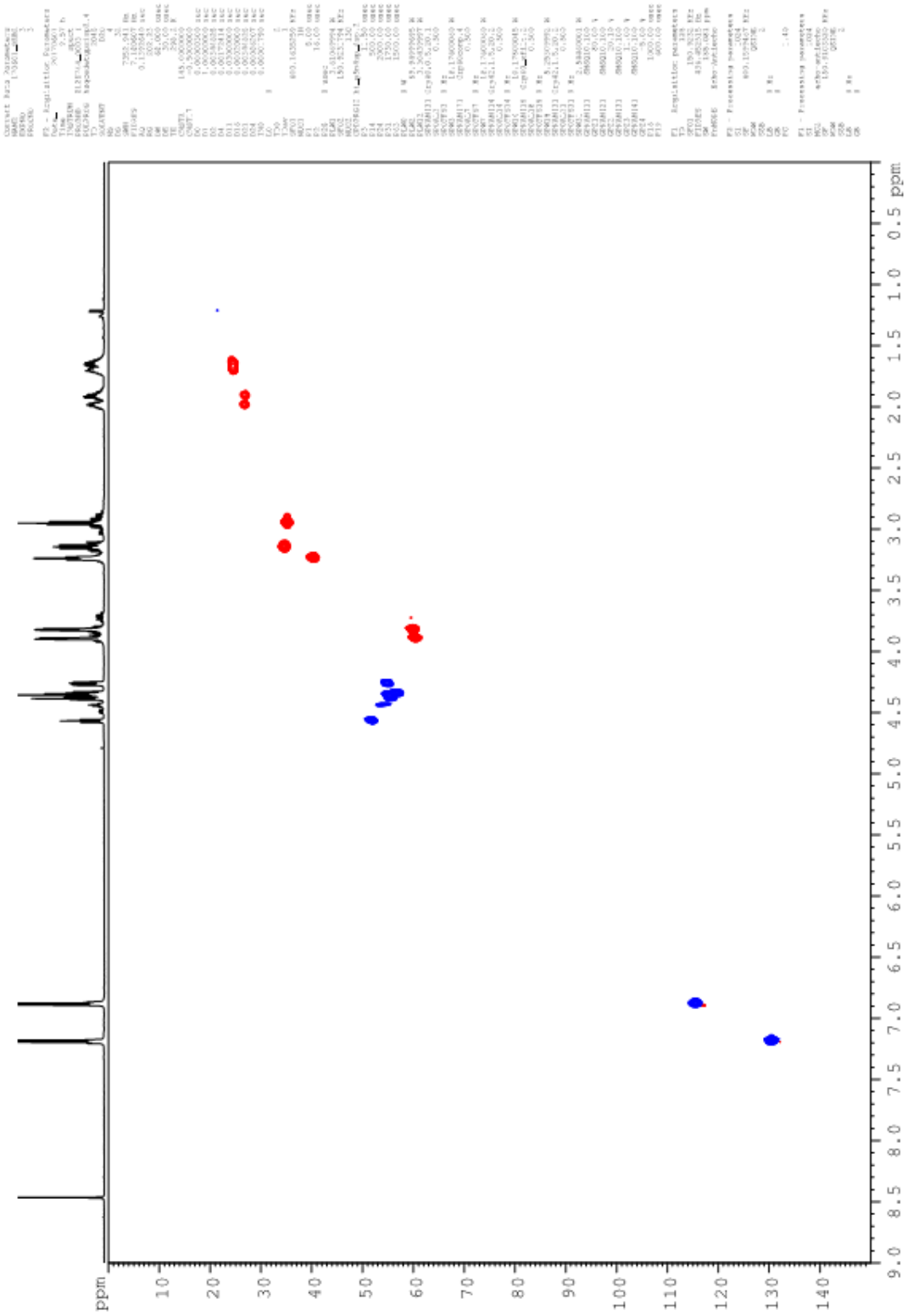
4.4.2 ^{1}H NMR of B2



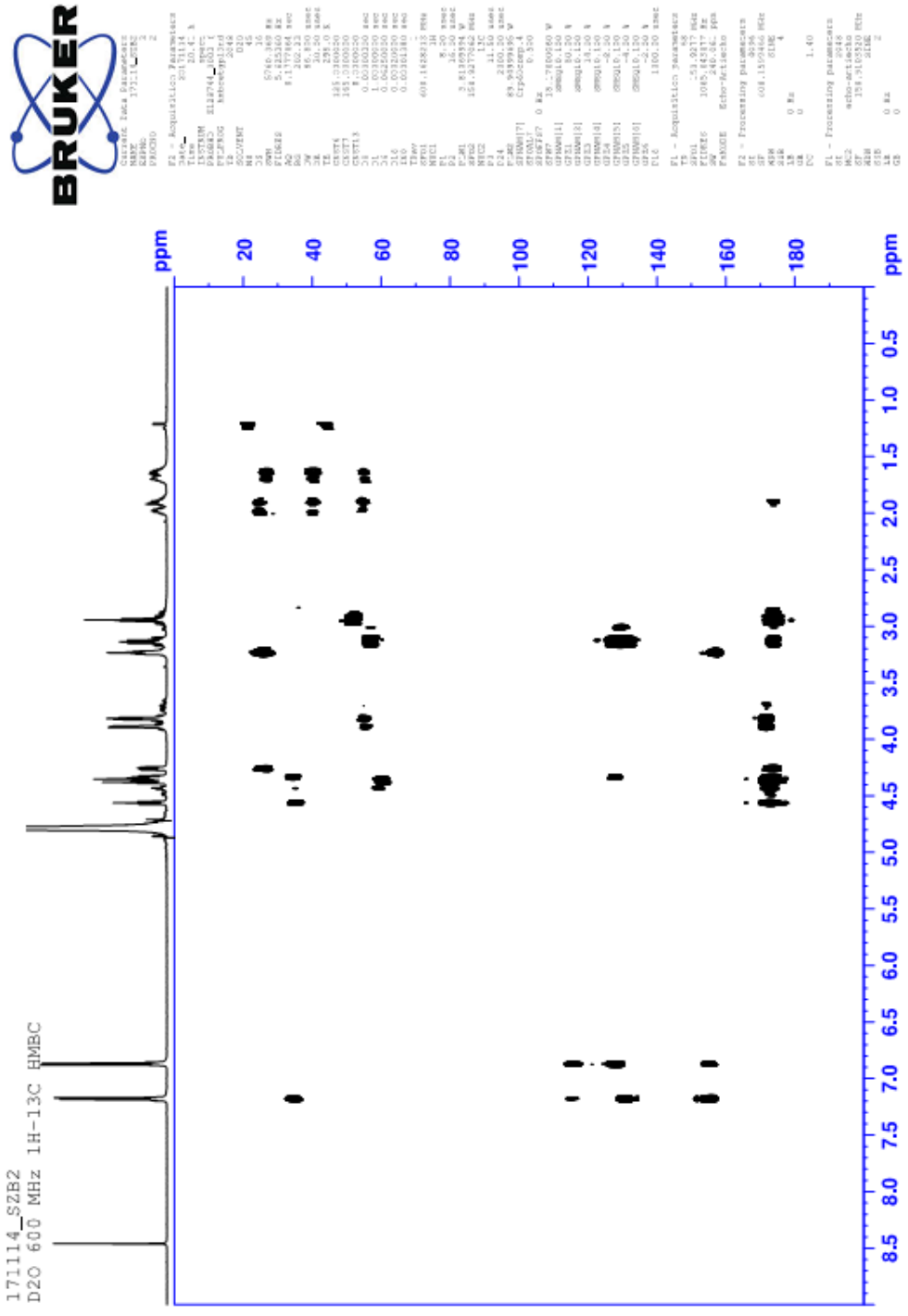
4.4.3 ^{1}H-^{1}H COSY of B2



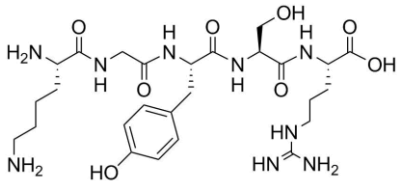
4.4.4 ^{1}H-^{13}C HSQC of B2



4.4.5 ^{1}H-^{13}C HMBC of B2



4.5.1 LC/MS of C1



Chemical Formula: $C_{26}H_{43}N_9O_8$
Exact Mass: 609.32
Molecular Weight: 609.69

15/11/2017 2:41:09 PM Page 1 / 1

SHIMADZU LabSolutions

Analysis Report

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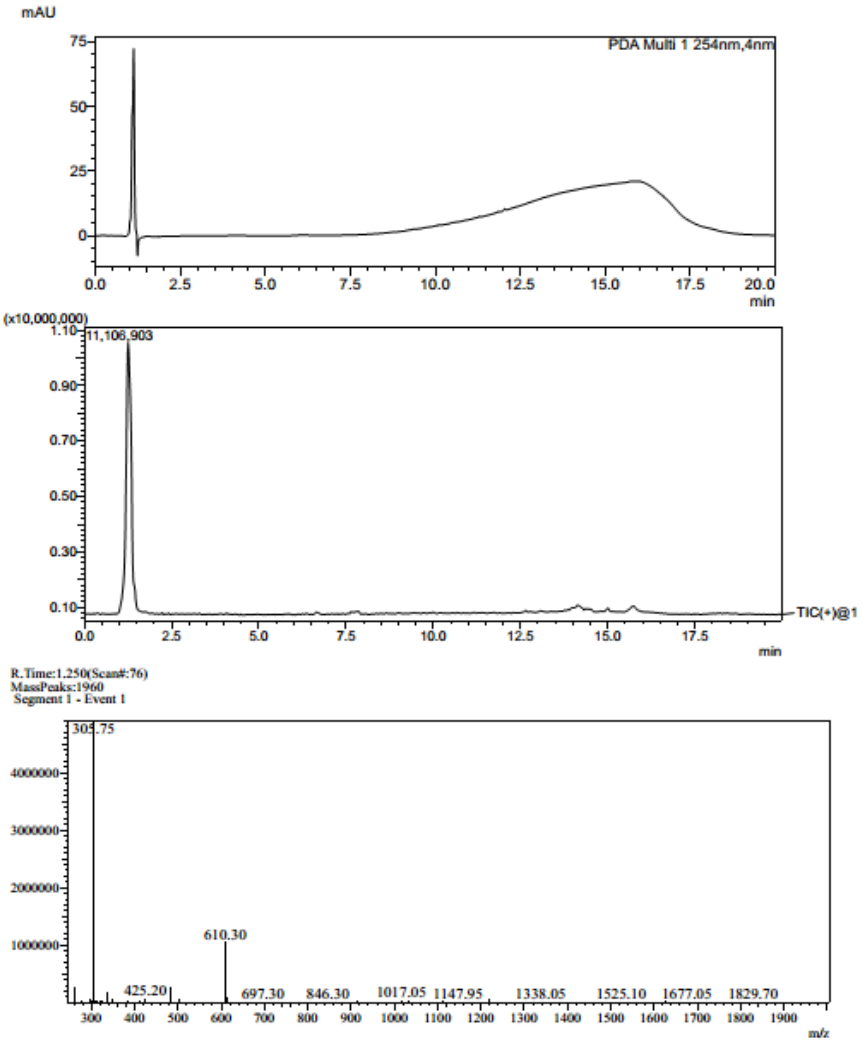

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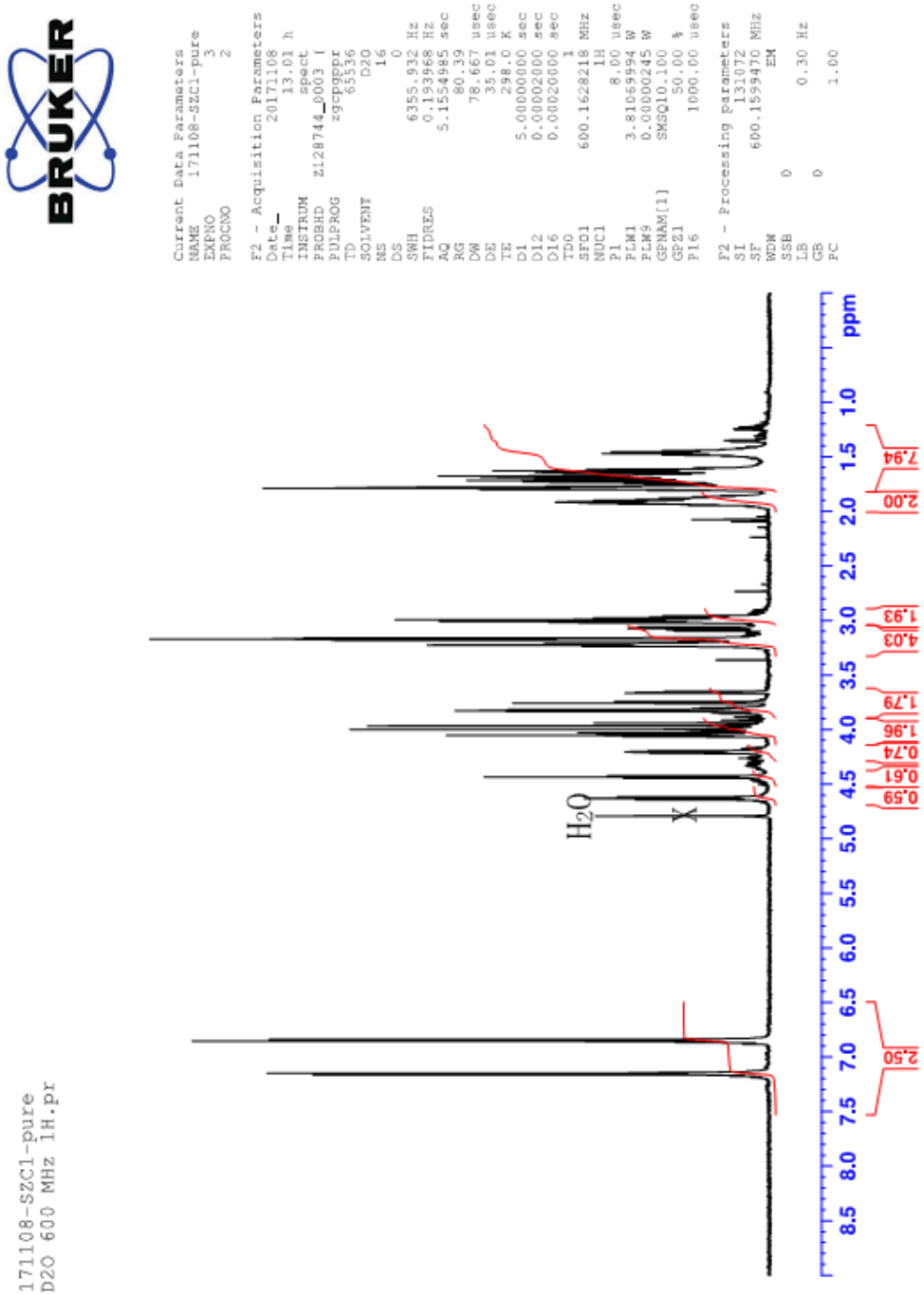
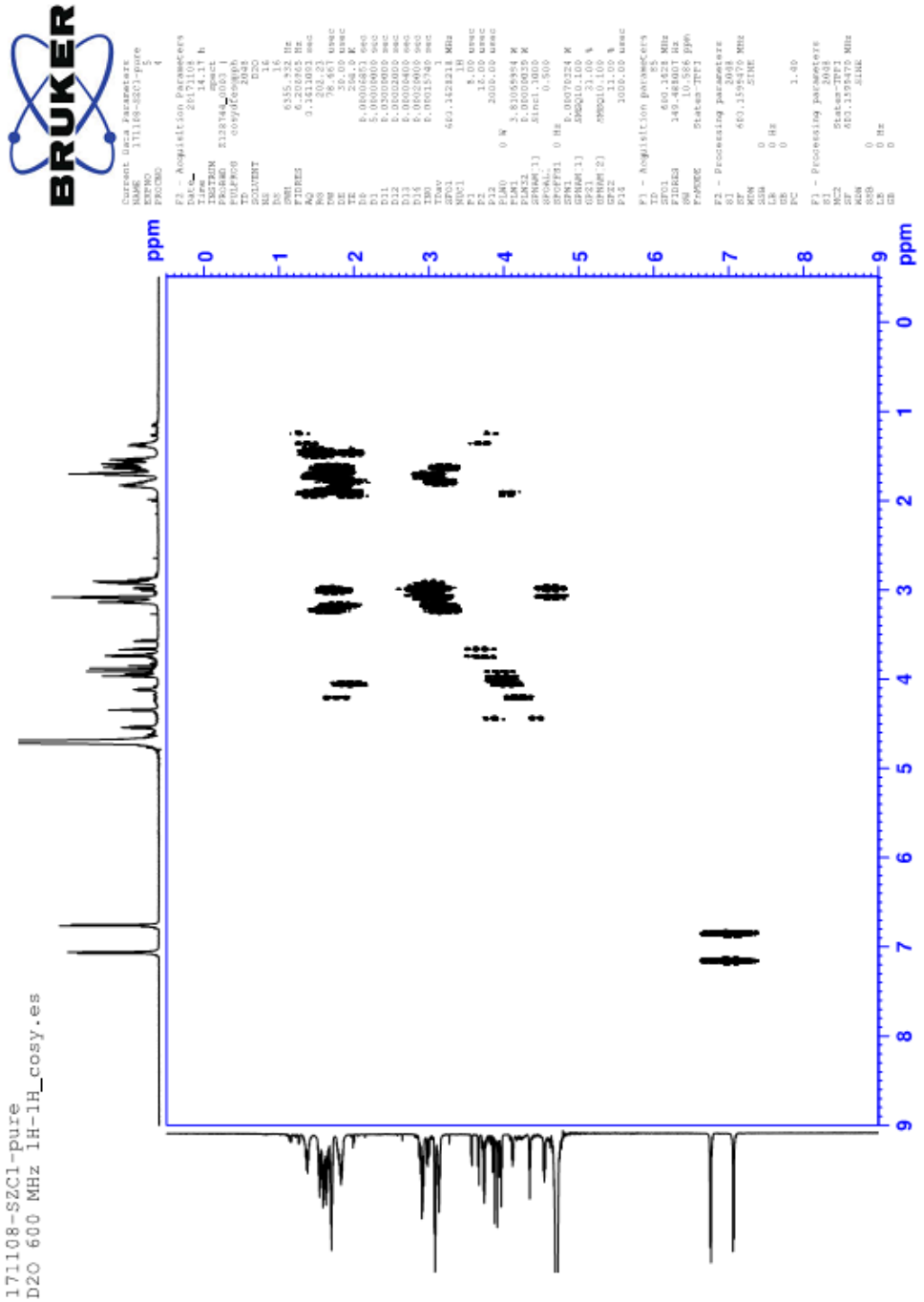
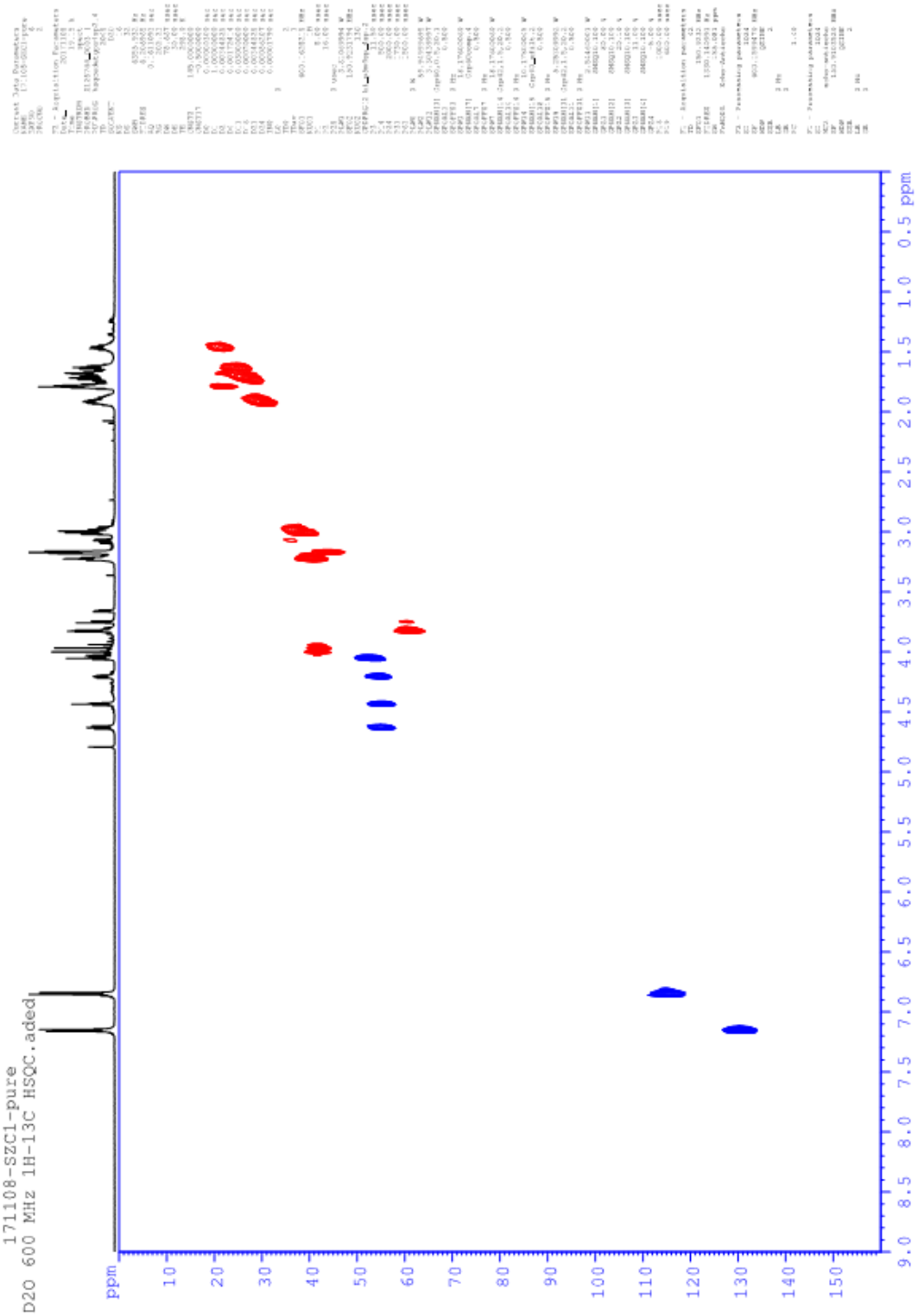
4.5.2 ^{1}H NMR of C1



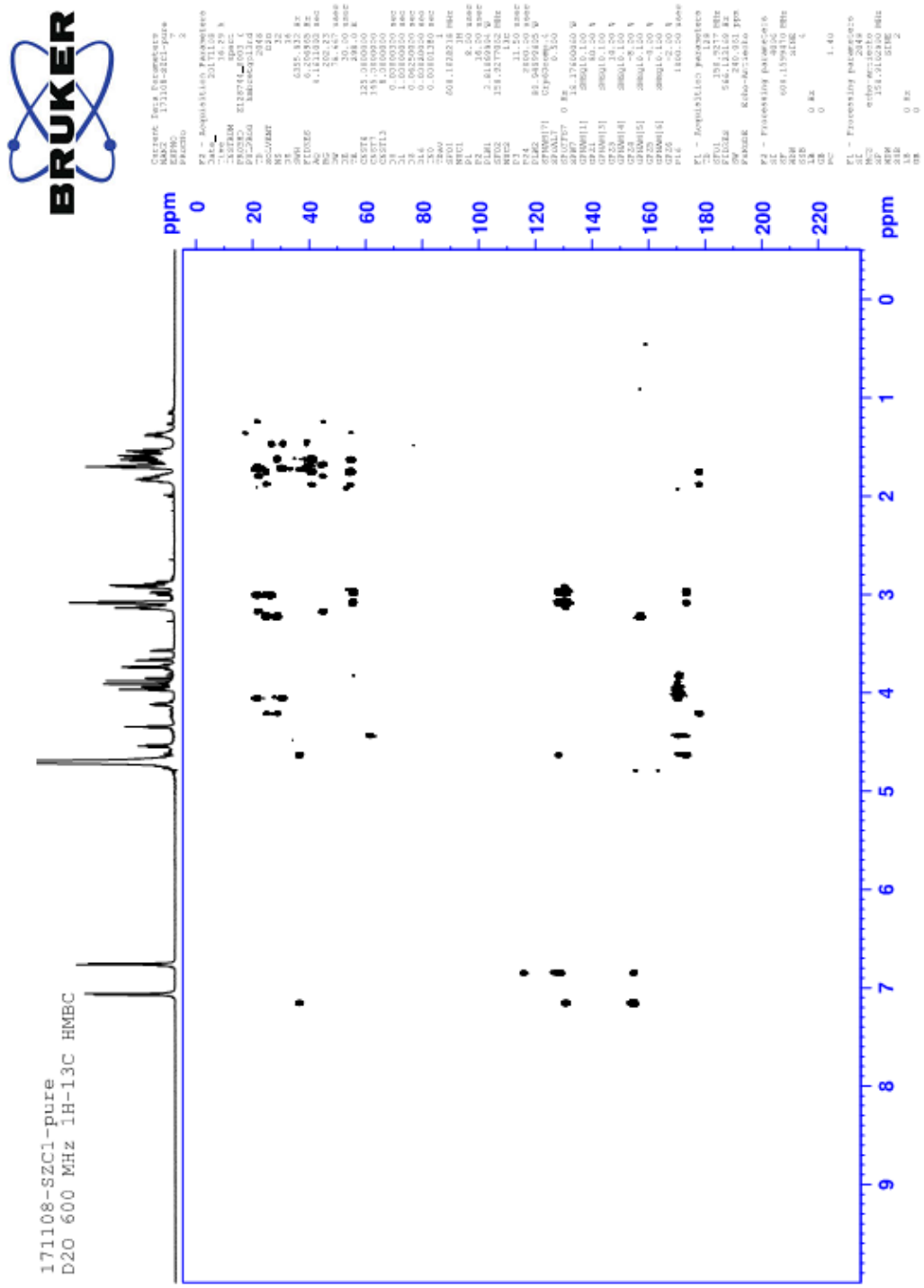
4.5.3 ^{1}H-^{1}H COSY of C1



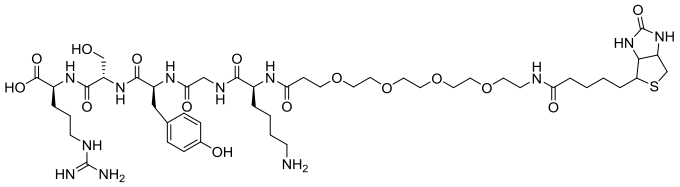
4.5.4 ^{1}H-^{13}C HSQC of C1



4.5.5 ^{1}H-^{13}C HMBC of C1



4.6.1 LC/MS of C1-Tag



Chemical Formula: $C_{47}H_{78}N_{12}O_{15}S$
Exact Mass: 1082.54
Molecular Weight: 1083.27

SHIMADZU
LabSolutions

Analysis Report

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Batch Filename	: 18-01-21.lcb		
Vial #	: 1-40	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 21/01/2018 10:30:08 PM	Acquired by	: System Administrator
Date Processed	: 21/01/2018 10:50:09 PM	Processed by	: System Administrator

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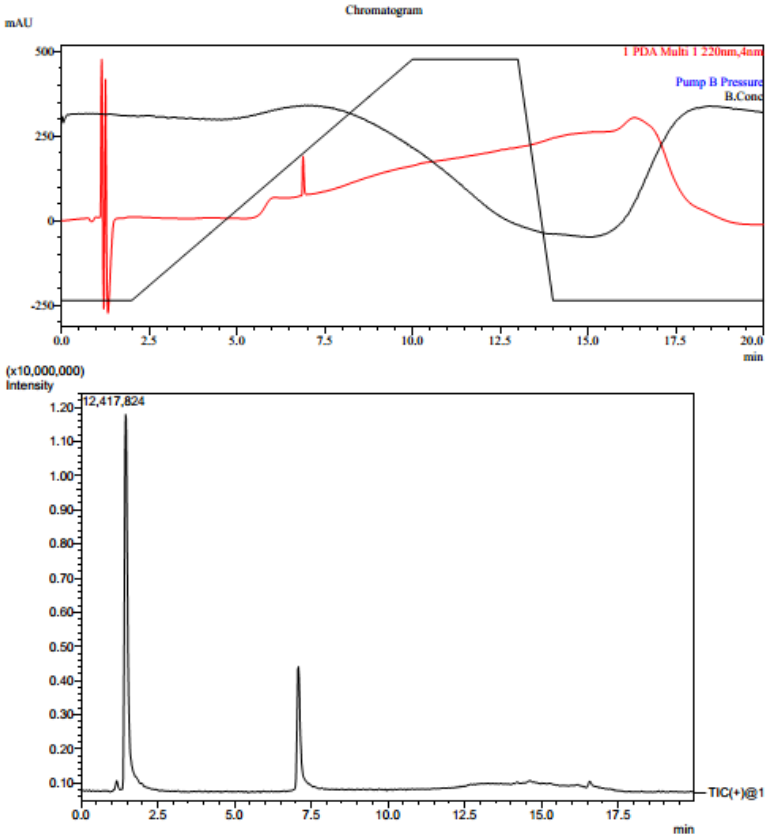

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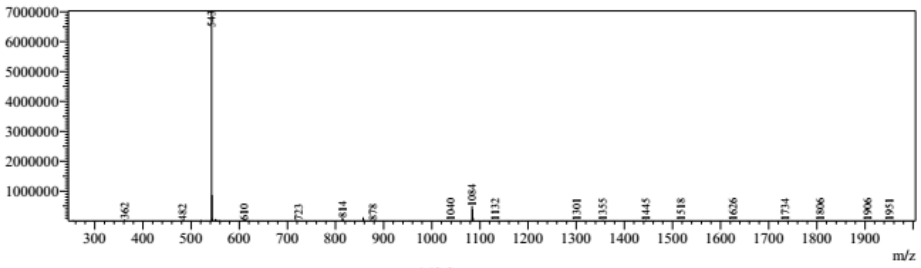

MS Spectrum

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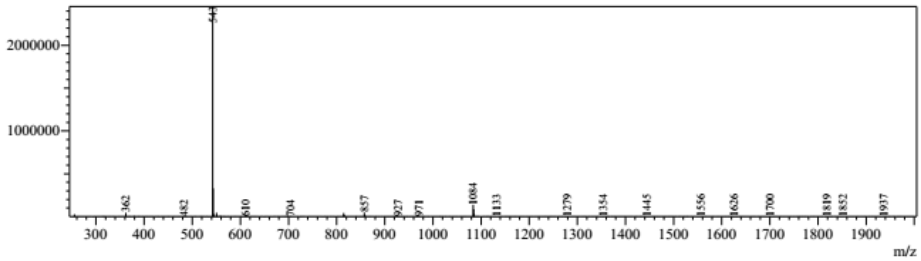
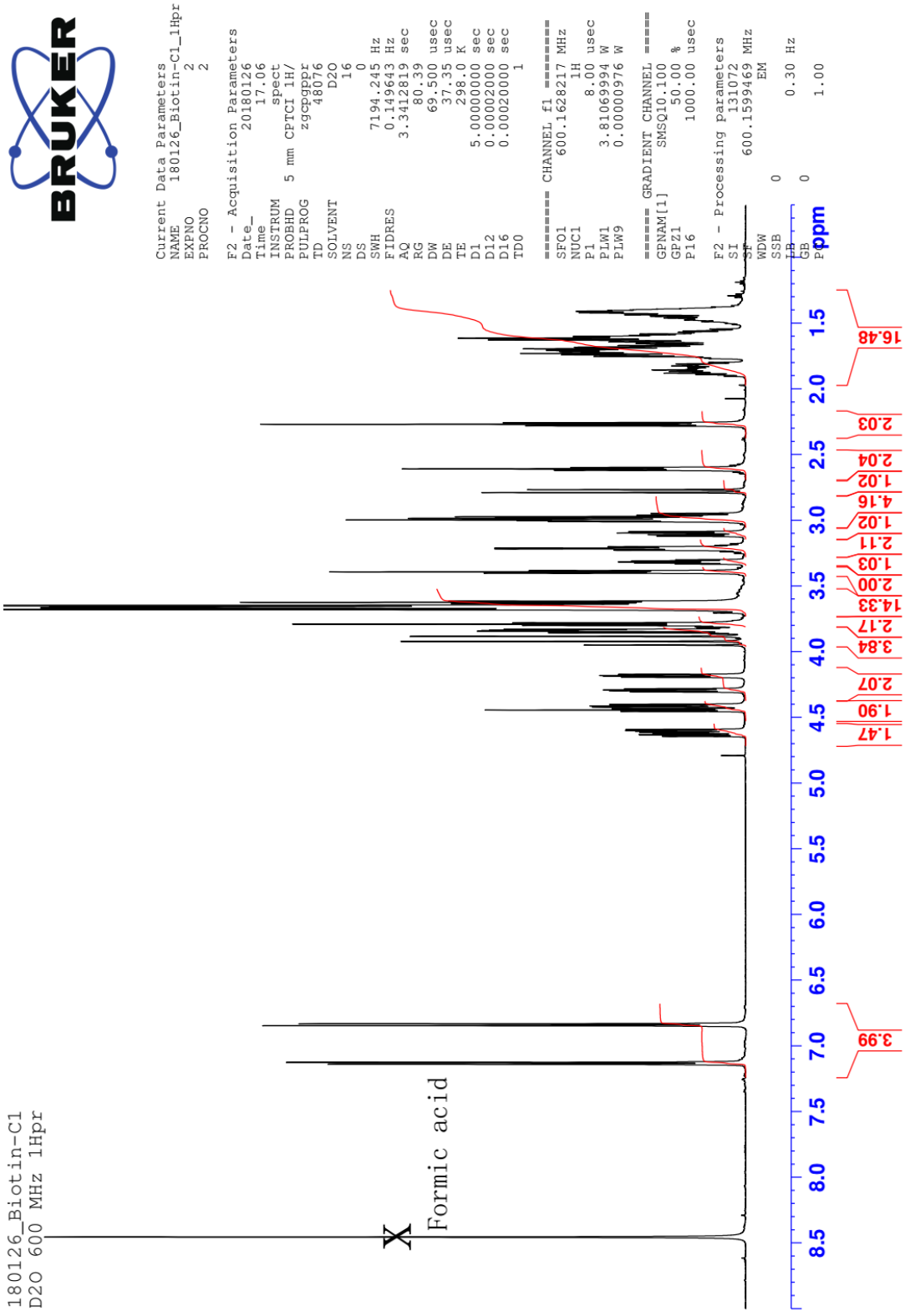
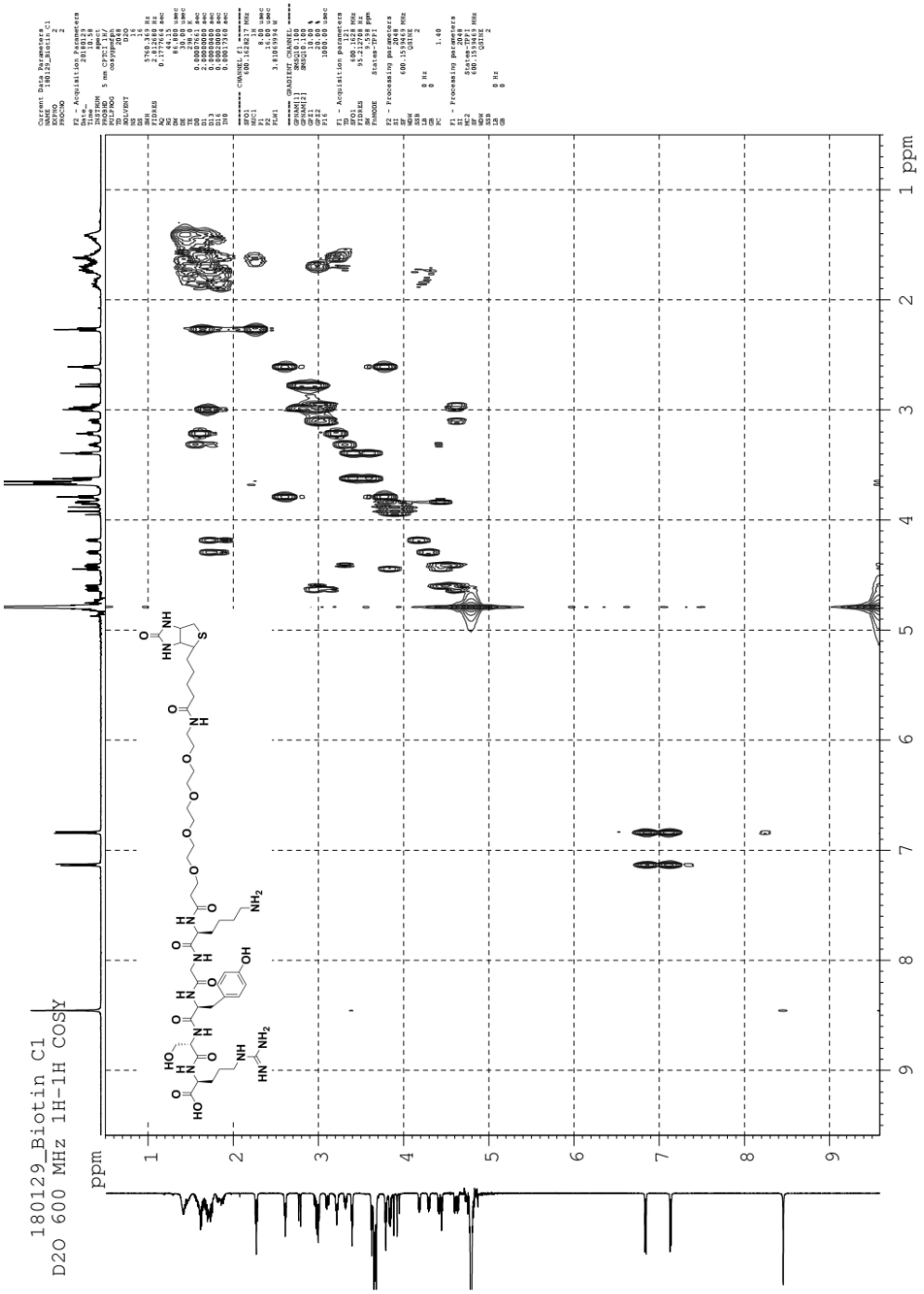


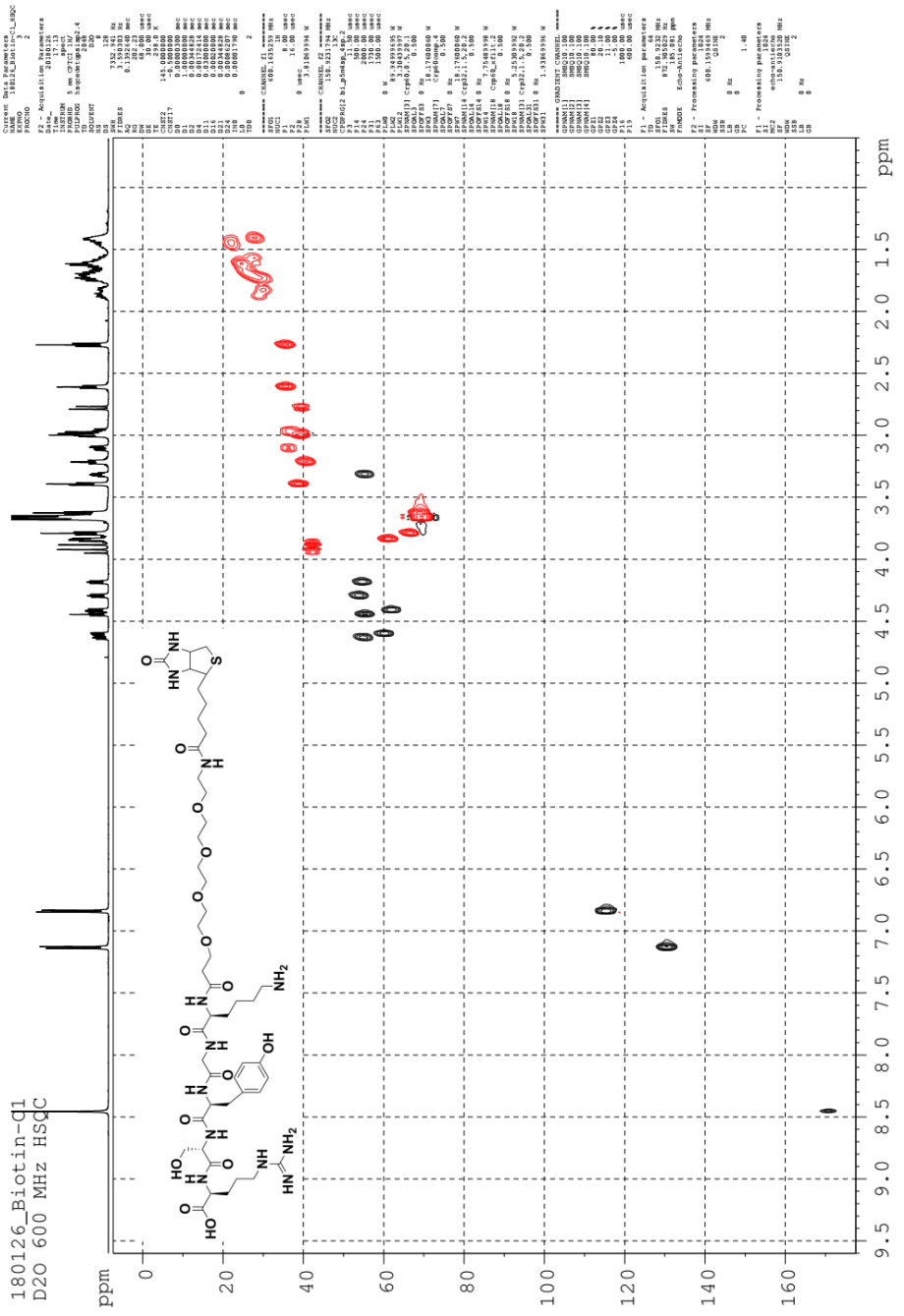
4.6.2 ^{1}H NMR of C1-Tag



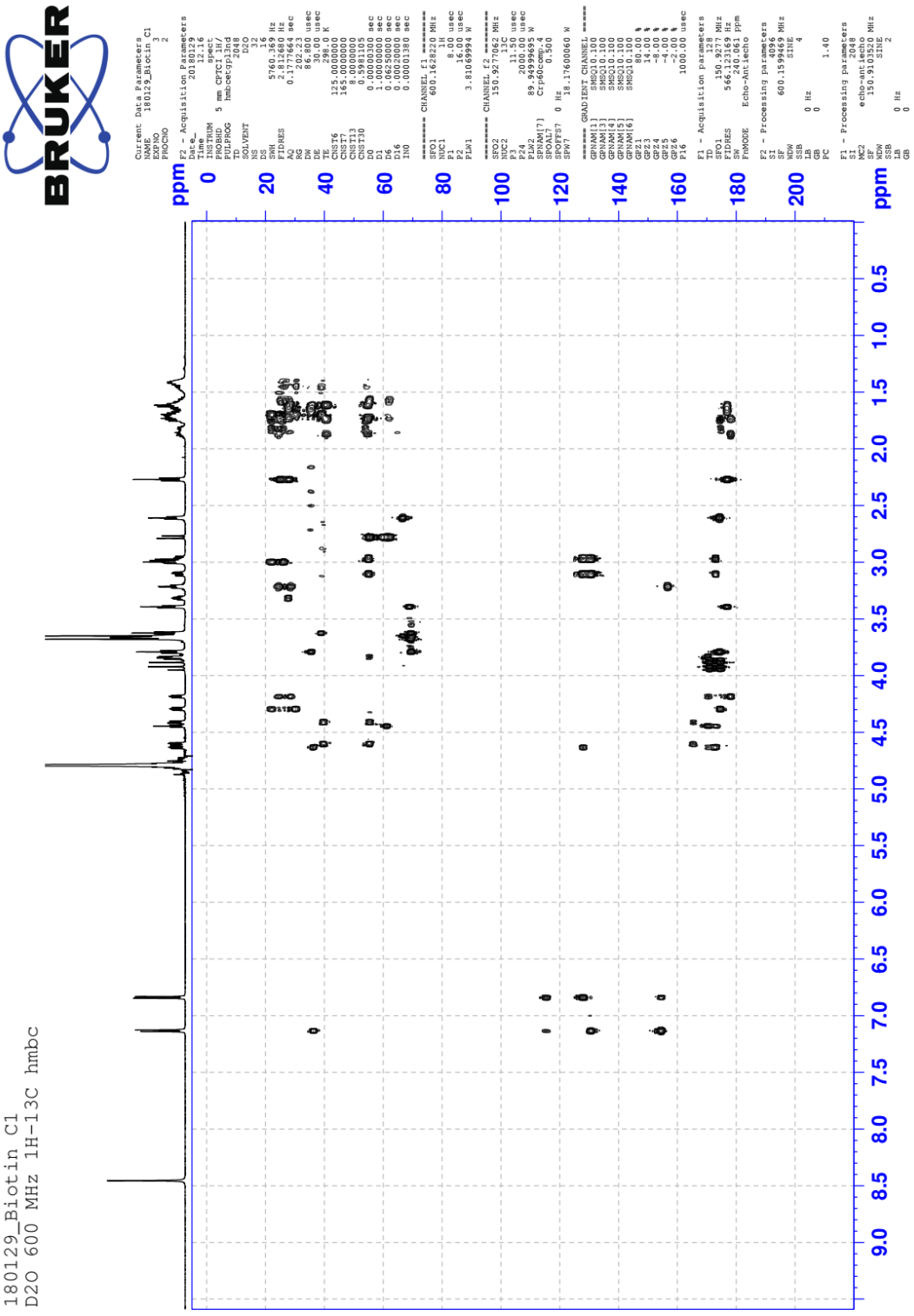
4.6.3 ^{1}H-^{1}H COSY of C1-Tag



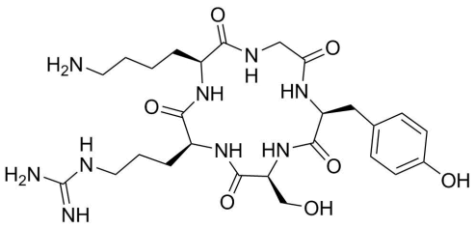
4.6.4 ^{1}H-^{13}C HSQC of C1-Tag



4.6.5 ^{1}H-^{13}C HMBC of C1-Tag



4.7.1 LC/MS of C2



Chemical Formula: $C_{26}H_{41}N_9O_7$
Exact Mass: 591.31
Molecular Weight: 591.67

15/11/2017 2:33:28 PM Page 1 / 1

SHIMADZU LabSolutions **Analysis Report**

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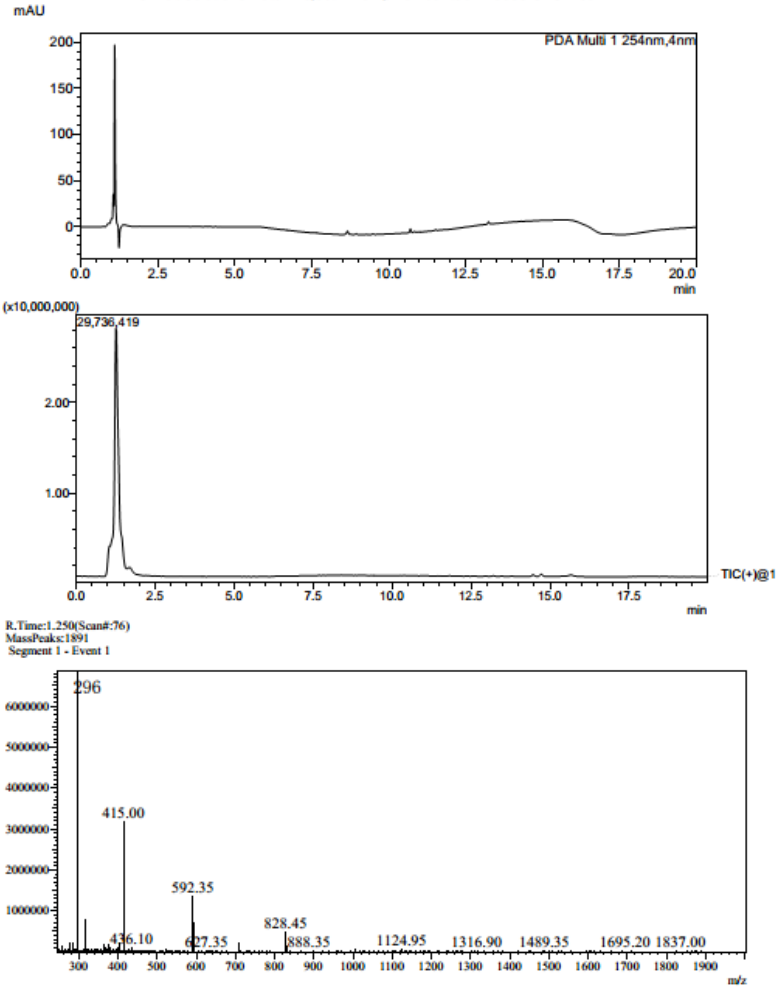
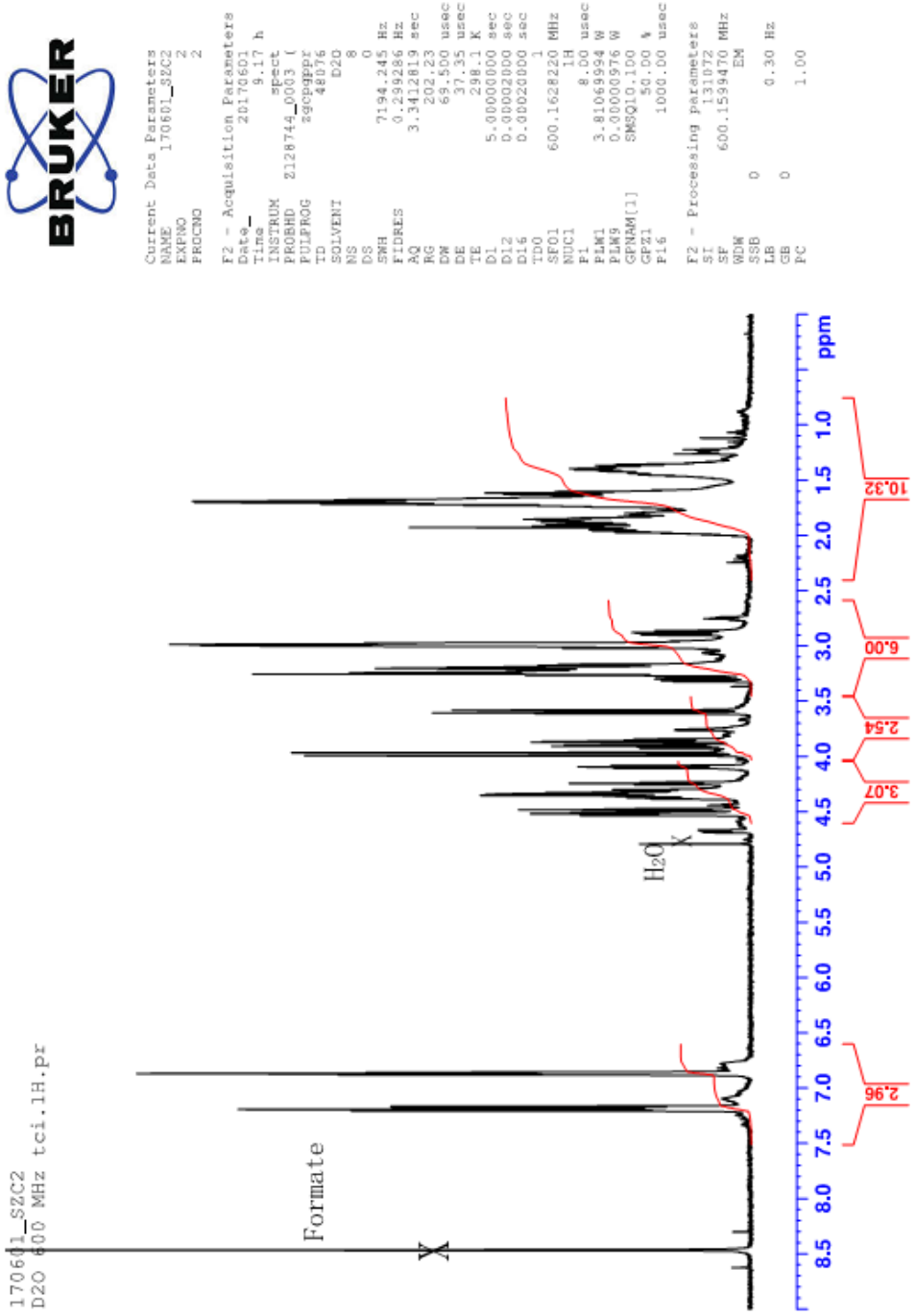
Sample Name : SZC2
Method Filename : Phenomenex_McAlpine_column_17-06-03.lcm
Date Acquired : 5/06/2017 4:25:09 PM
C:\LabSolutions\Data\Project1\McAlpine\Yuantao\170605\SZC2.lcd



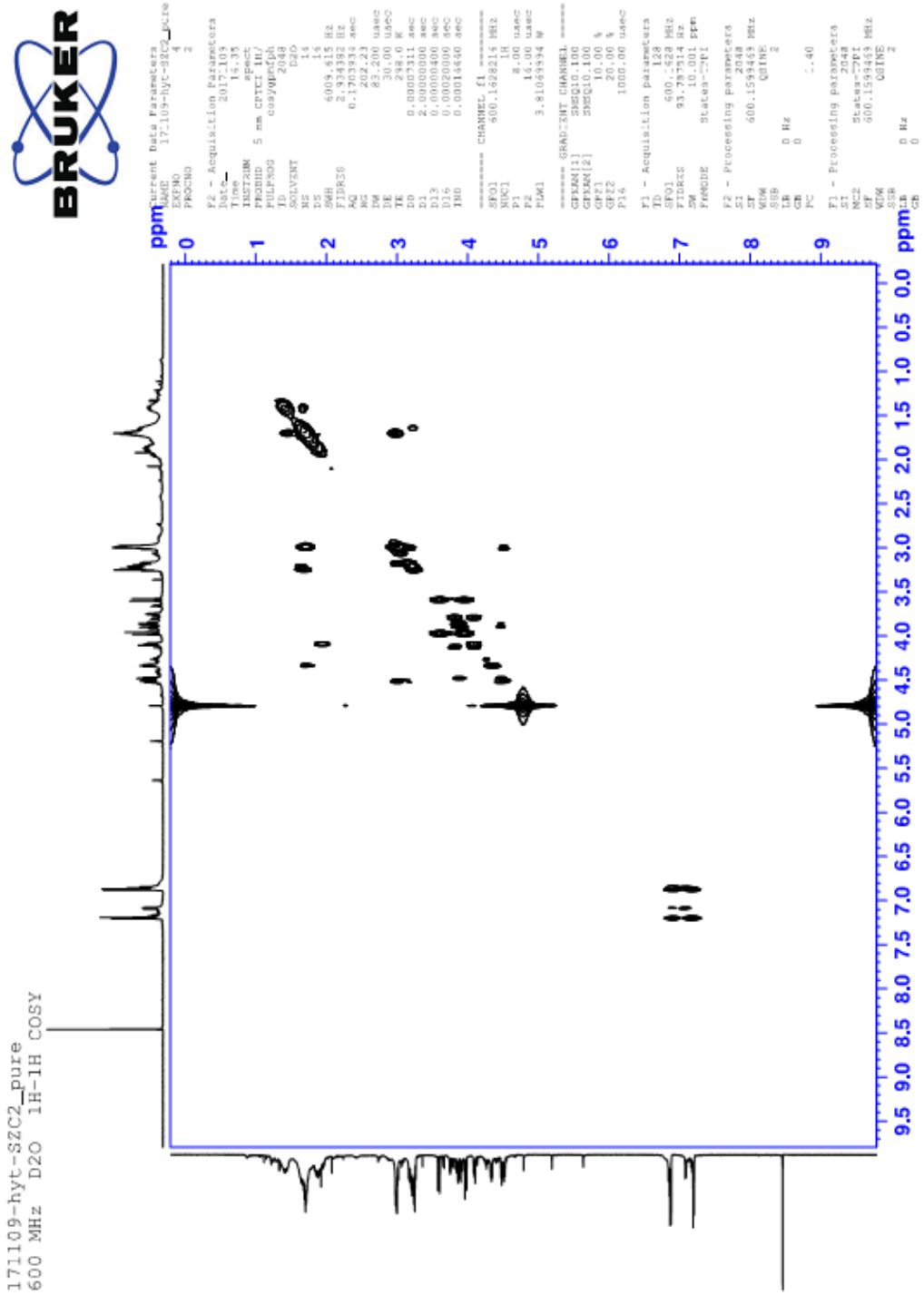
R.Time:1.250(Scan#:76)
MassPeaks:1891
Segment 1 - Event 1

C:\LabSolutions\Data\Project1\McAlpine\Yuantao\170605\SZC2.lcd

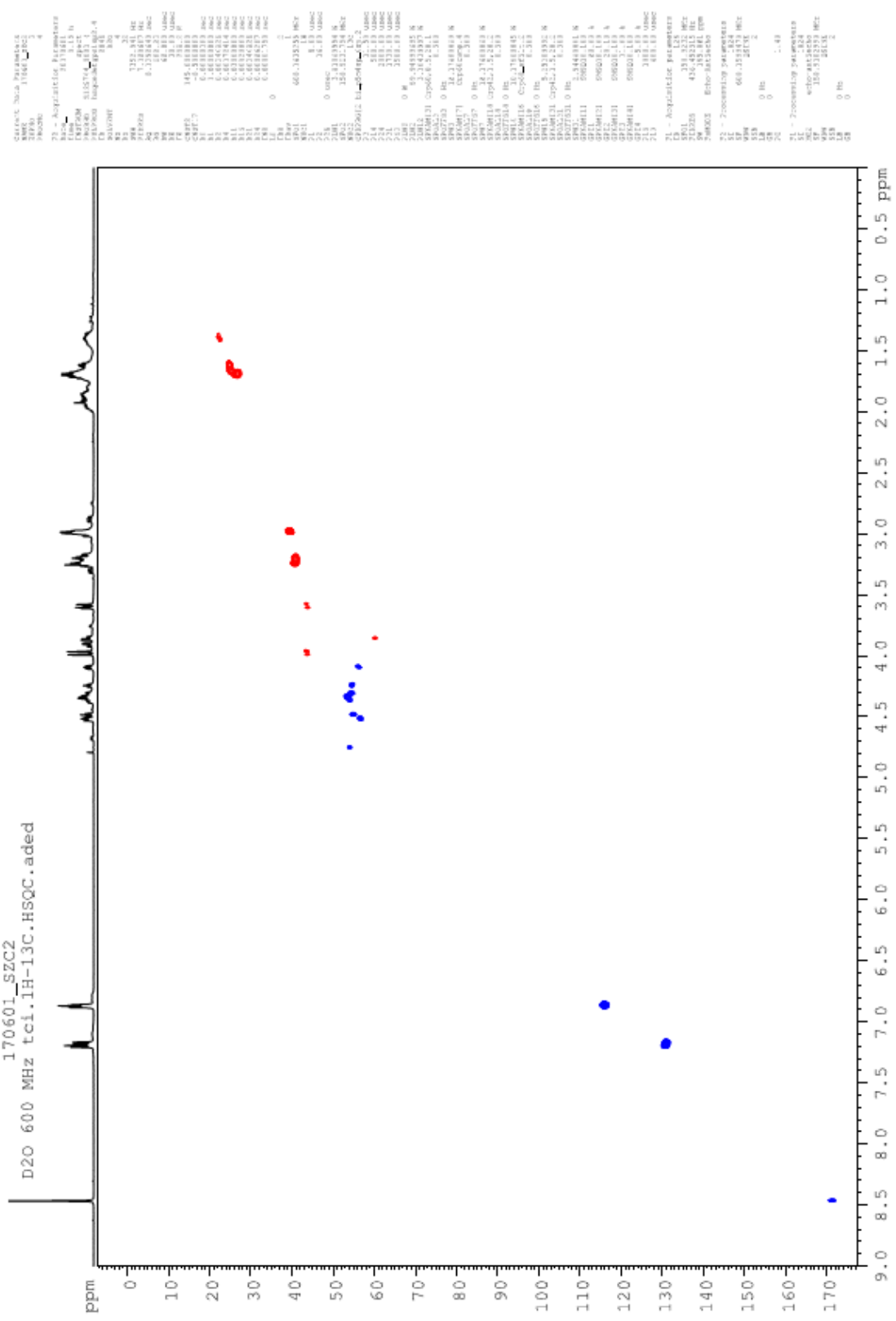
4.7.2 ^{1}H NMR of C2



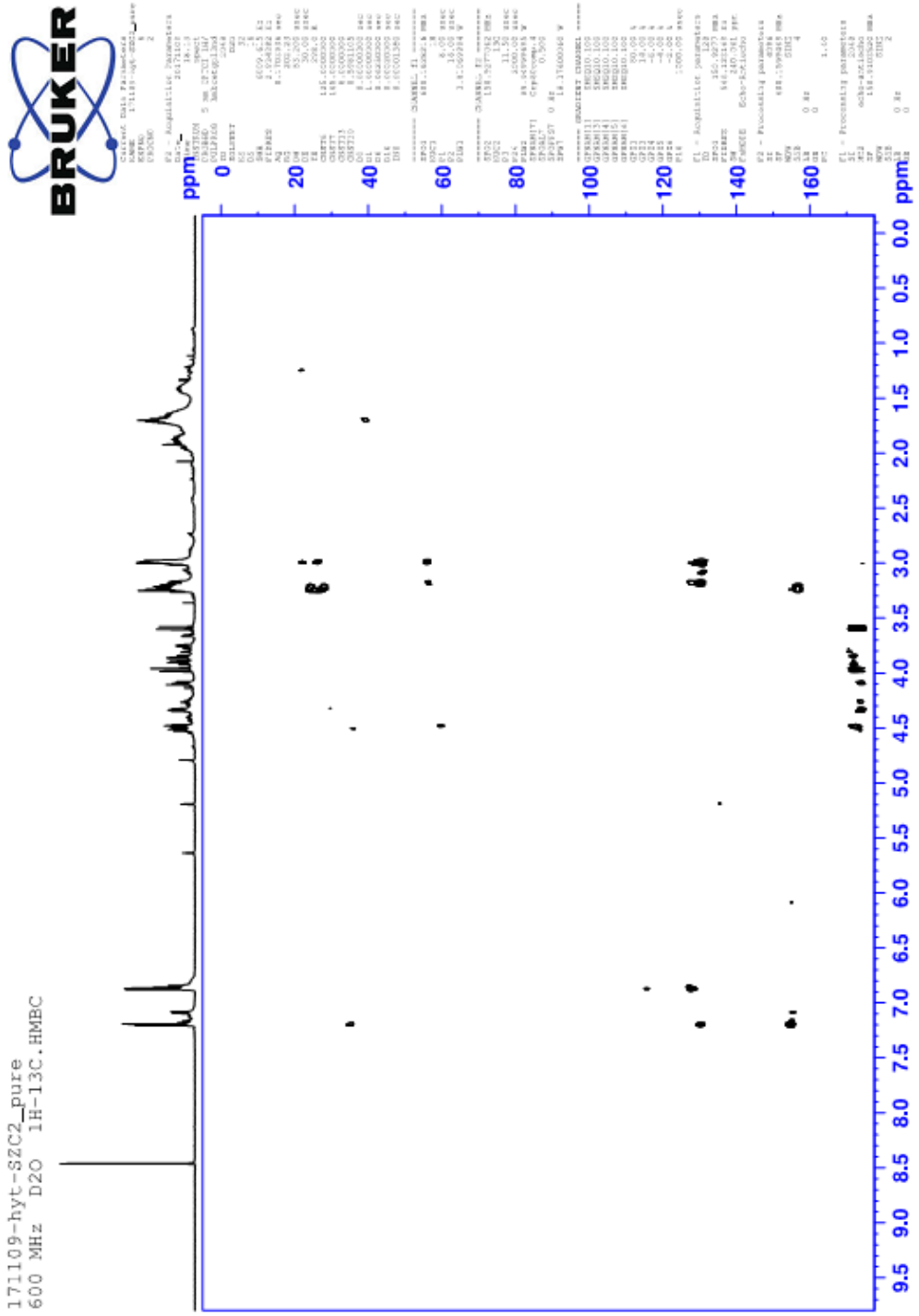
4.7.3 ^{1}H-^{1}H COSY of C2



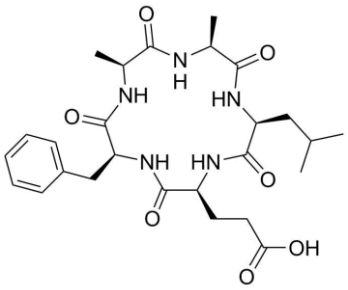
4.7.4 ^{1}H-^{13}C HSQC of C2



4.7.5 ^{1}H-^{13}C HMBC of C2



4.8.1 LC/MS of C4



Chemical Formula: $C_{26}H_{37}N_5O_7$
Exact Mass: 531.27
Molecular Weight: 531.61

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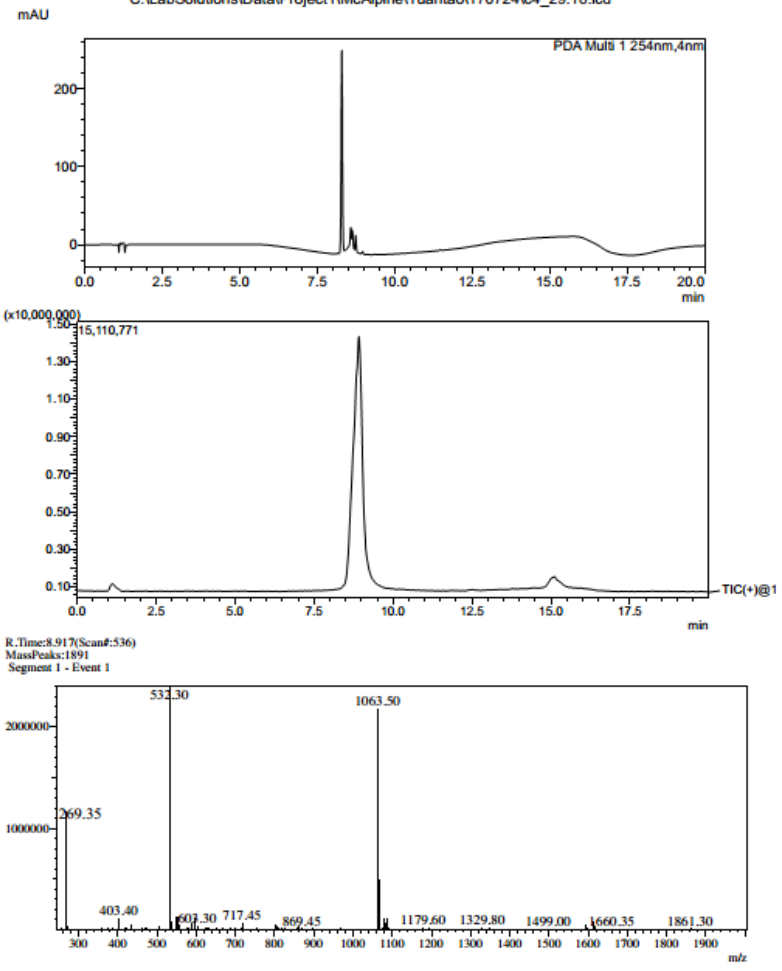
Analysis Report

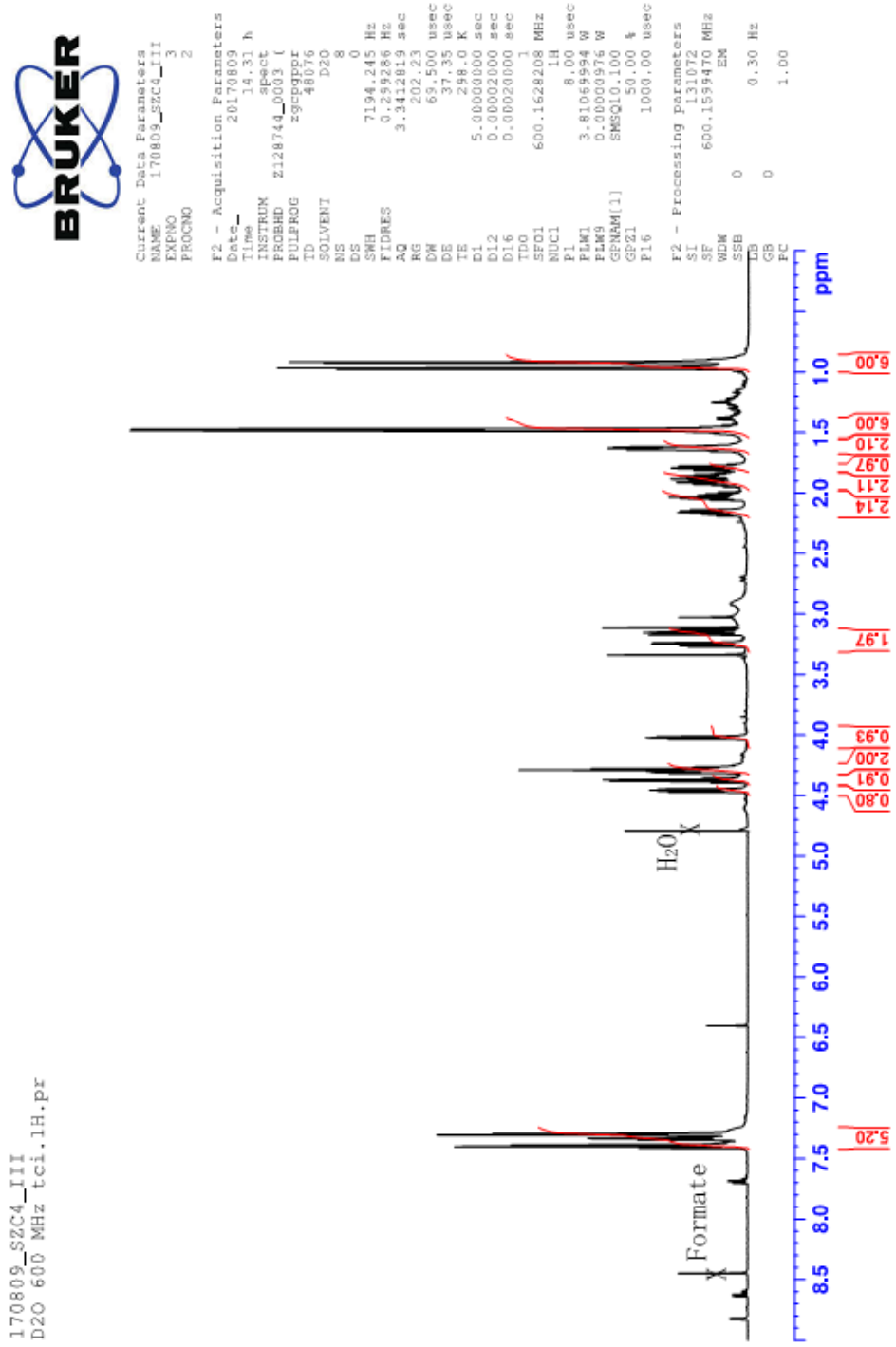
Sample Information
Sample Name : c4_29.16
Method Filename : Phenomenex_McAlpine_column_17-06-03.lcm
Date Acquired : 24/07/2017 8:21:15 PM
C:\LabSolutions\Data\Project1\McAlpine\Yuantao\170724\c4_29.16.lcd



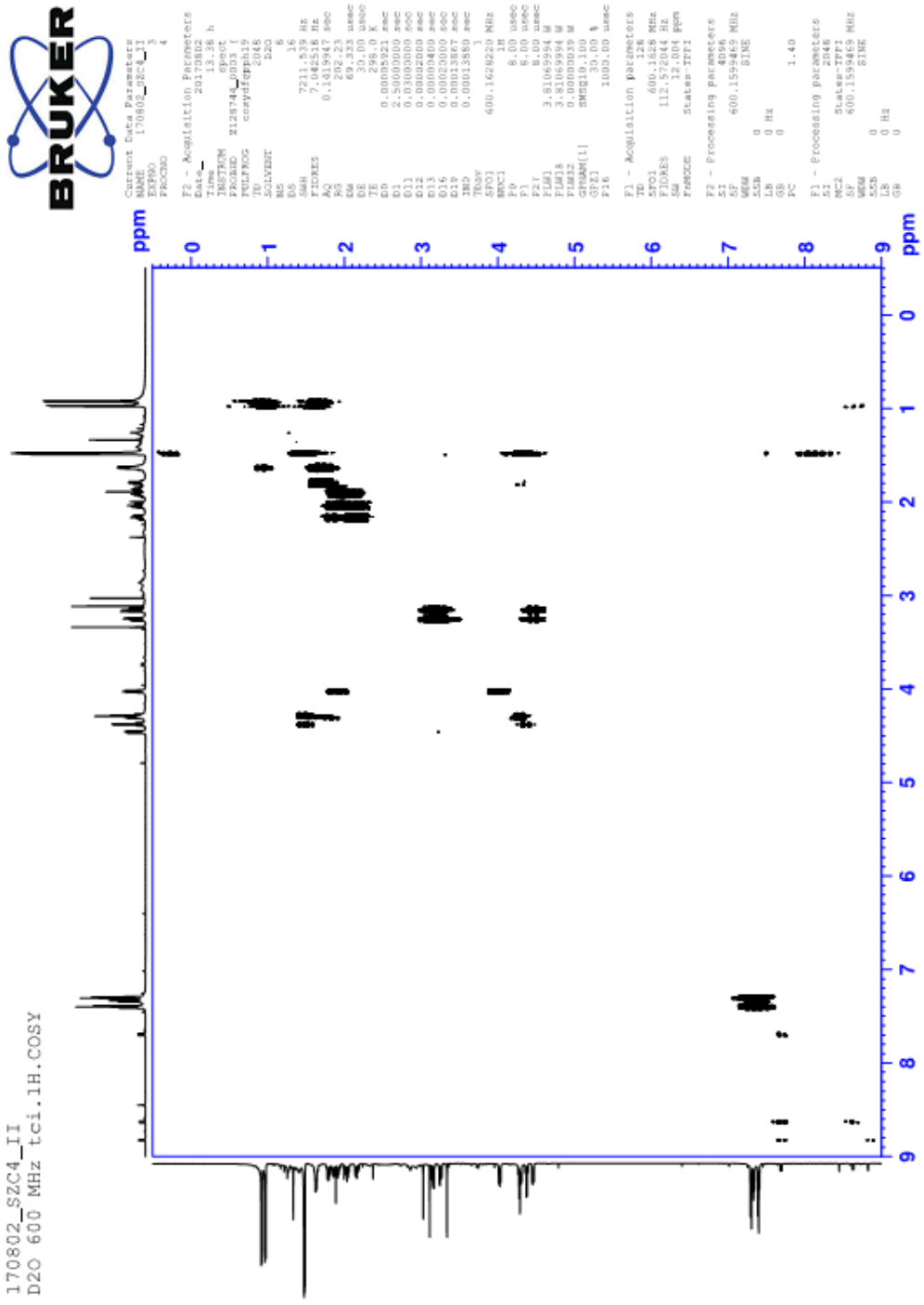
R.Time:8.917(Scan#:536)
MassPeaks:1891
Segment 1 - Event 1

C:\LabSolutions\Data\Project1\McAlpine\Yuantao\170724\c4_29.16.lcd

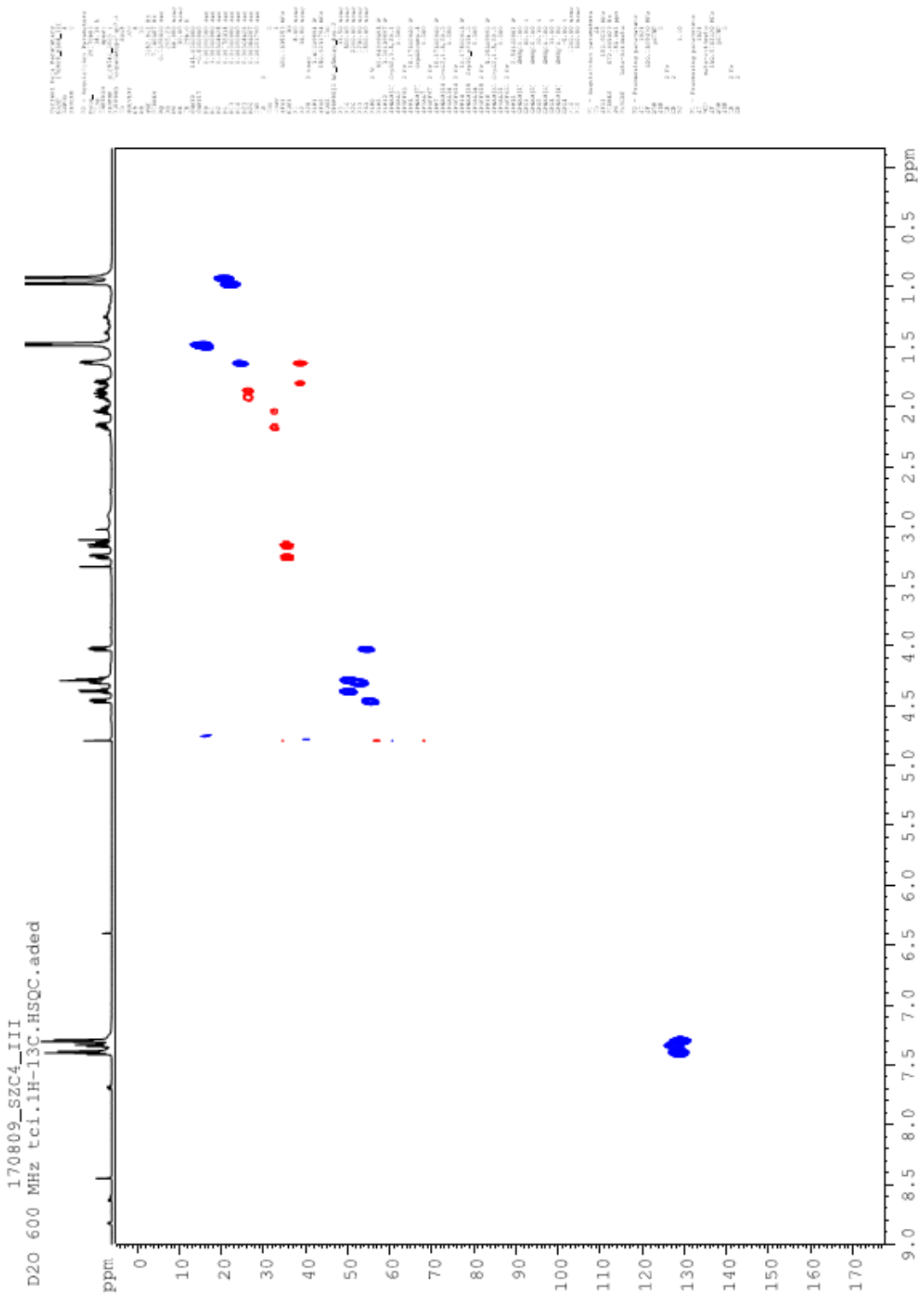
4.8.2 ^{1}H NMR of C4



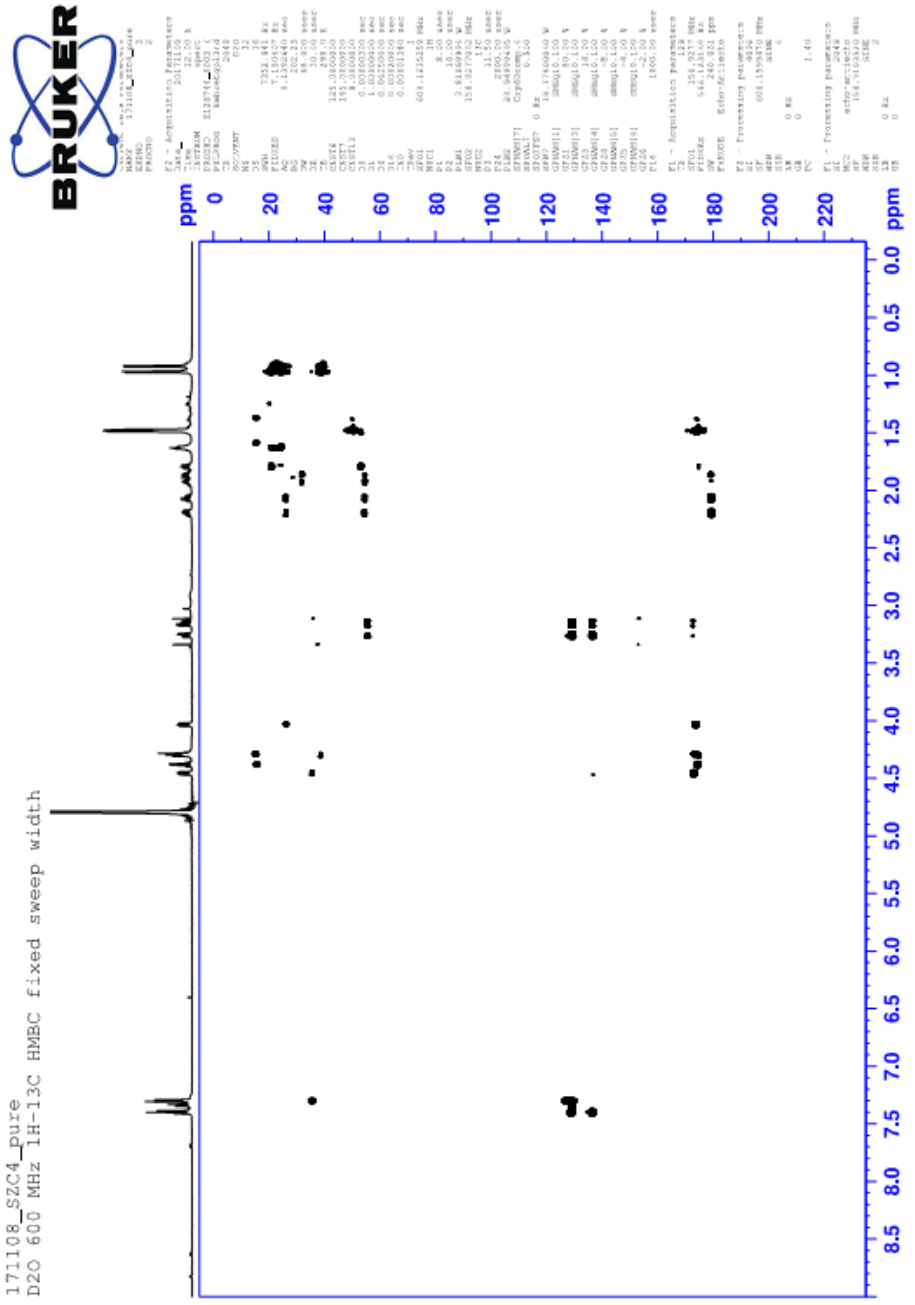
4.8.3 ^{1}H-^{1}H COSY of C4



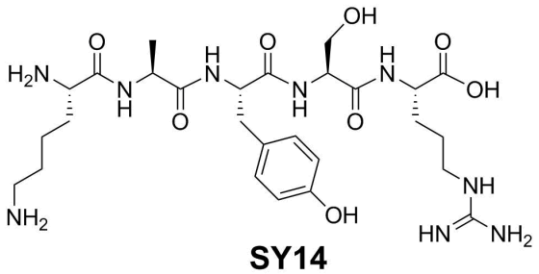
4.8.4 ^{1}H-^{13}C HSQC of C4



4.8.5 ^{1}H-^{13}C HMBC of C4



4.9.1 LC/MS of SY14



SY14

Chemical Formula: $C_{27}H_{45}N_9O_8$
Exact Mass: 623.34

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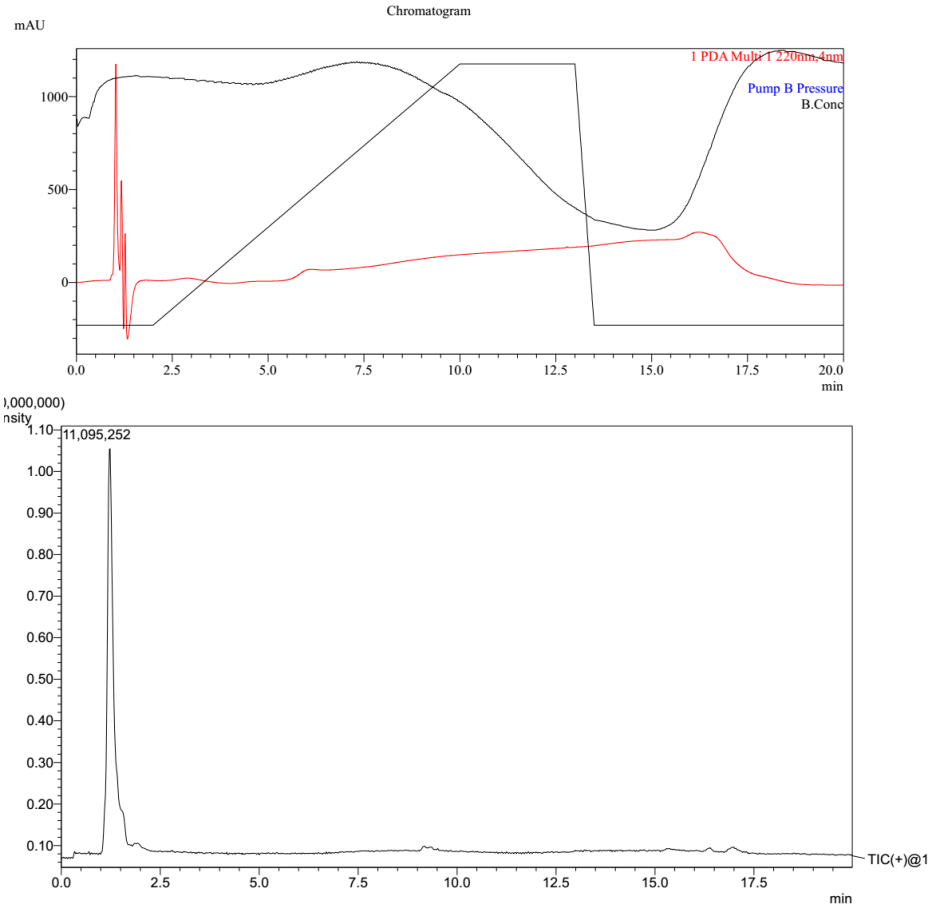
Analysis Report

<Sample Information>

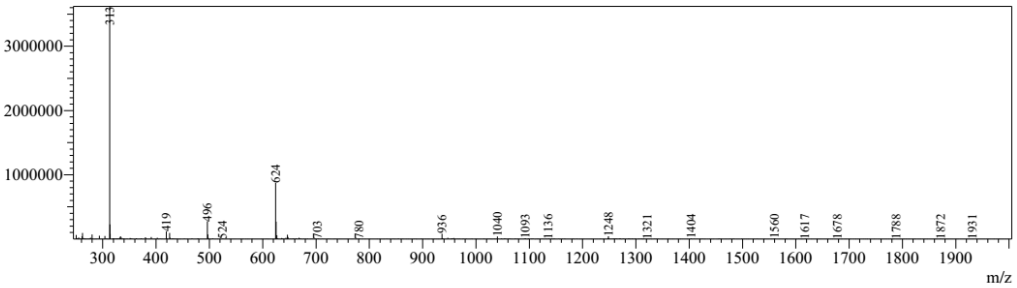
Sample Name	: SY14		
Sample ID	:		
Data Filename	: SY14.lcd		
Method Filename	: Phenomenex_McAlpine_column_17-06-03.lcm		
Batch Filename	: 18-01-30.lcb		
Vial #	: 1-90	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 31/01/2018 12:20:07 AM	Acquired by	: System Administrator
Date Processed	: 31/01/2018 12:40:10 AM	Processed by	: System Administrator

<Chromatogram>



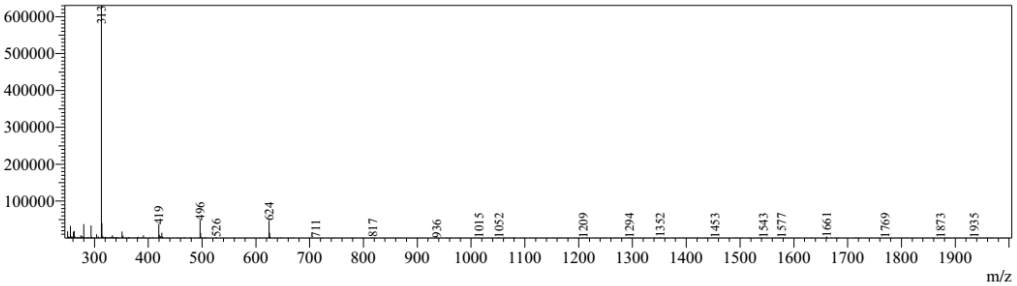
MS Spectrum

R.Time:1.267(Scan#:77)
MassPeaks:1933
Segment 1 - Event 1

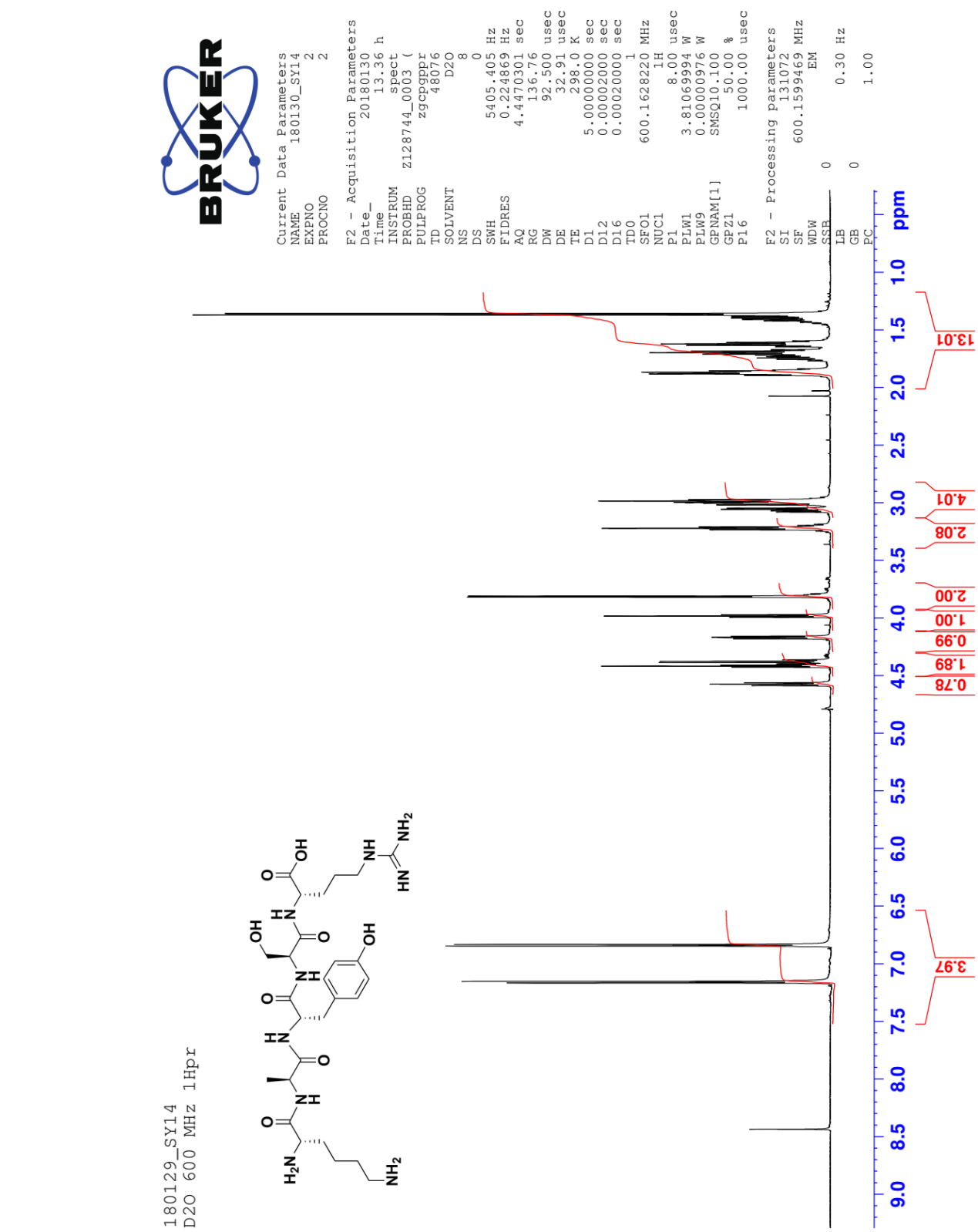


MS Spectrum

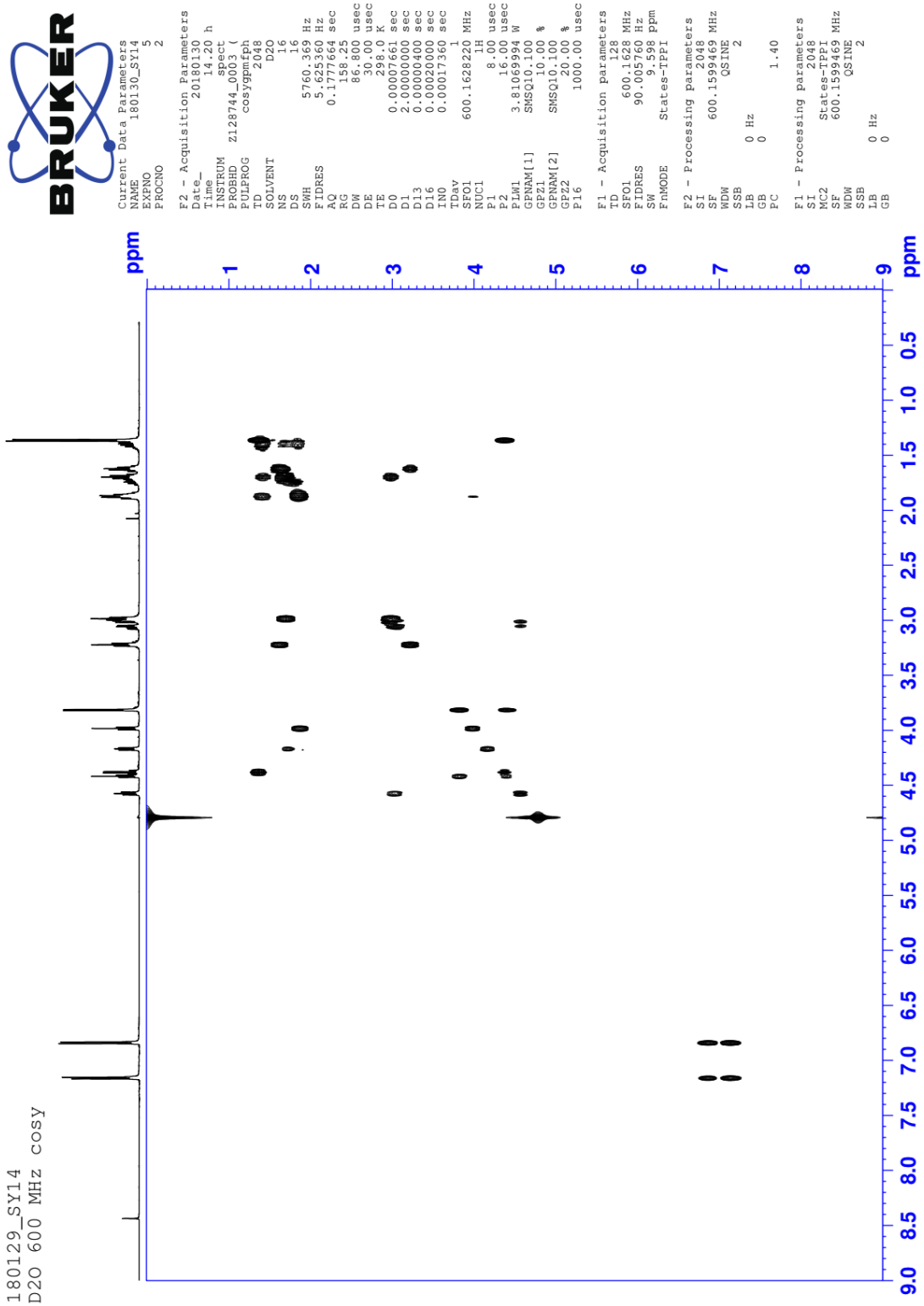
R.Time:1.467(Scan#:89)
MassPeaks:1824
Segment 1 - Event 1



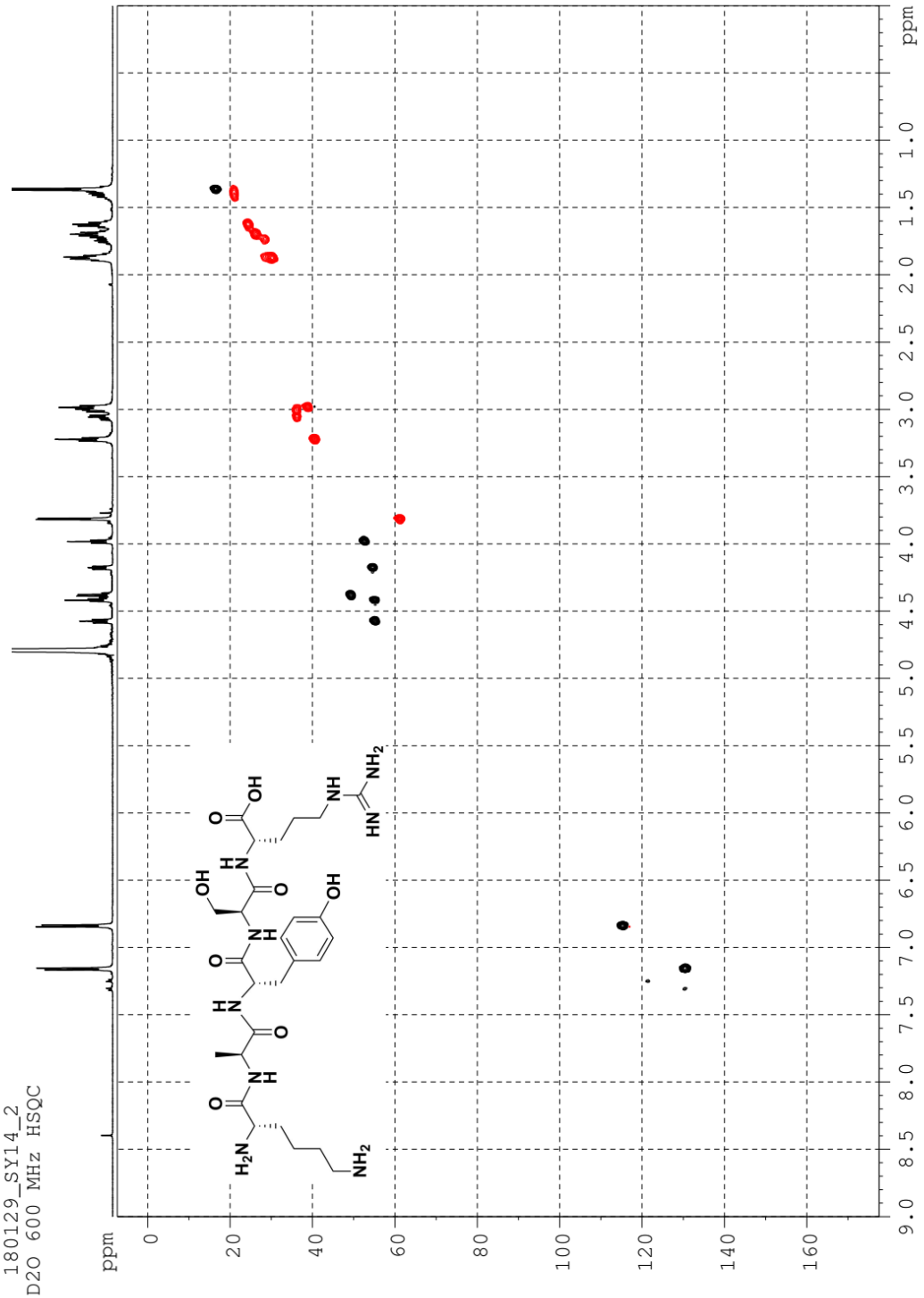
4.9.2 ^{1}H NMR of SY14



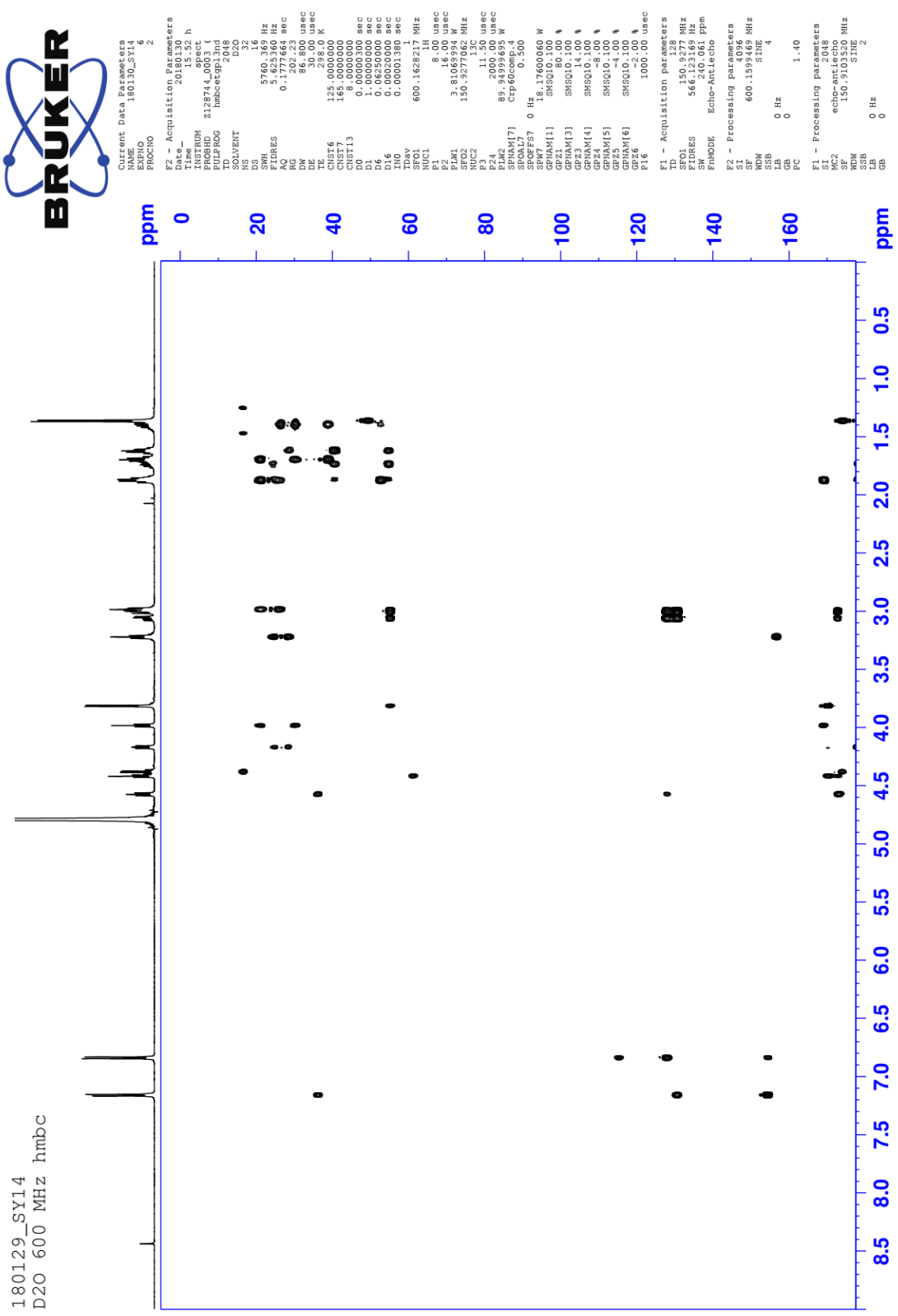
4.9.3 ^{1}H-^{1}H COSY of SY14



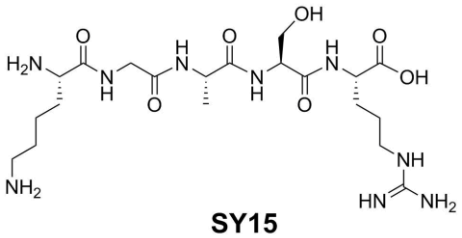
4.9.4 ^{1}H-^{13}C HSQC of SY14



4.9.5 ^{1}H-^{13}C HMBC of SY14



4.10.1 LC/MS of SY15



SY15

Chemical Formula: $C_{20}H_{39}N_9O_7$
Exact Mass: 517.30

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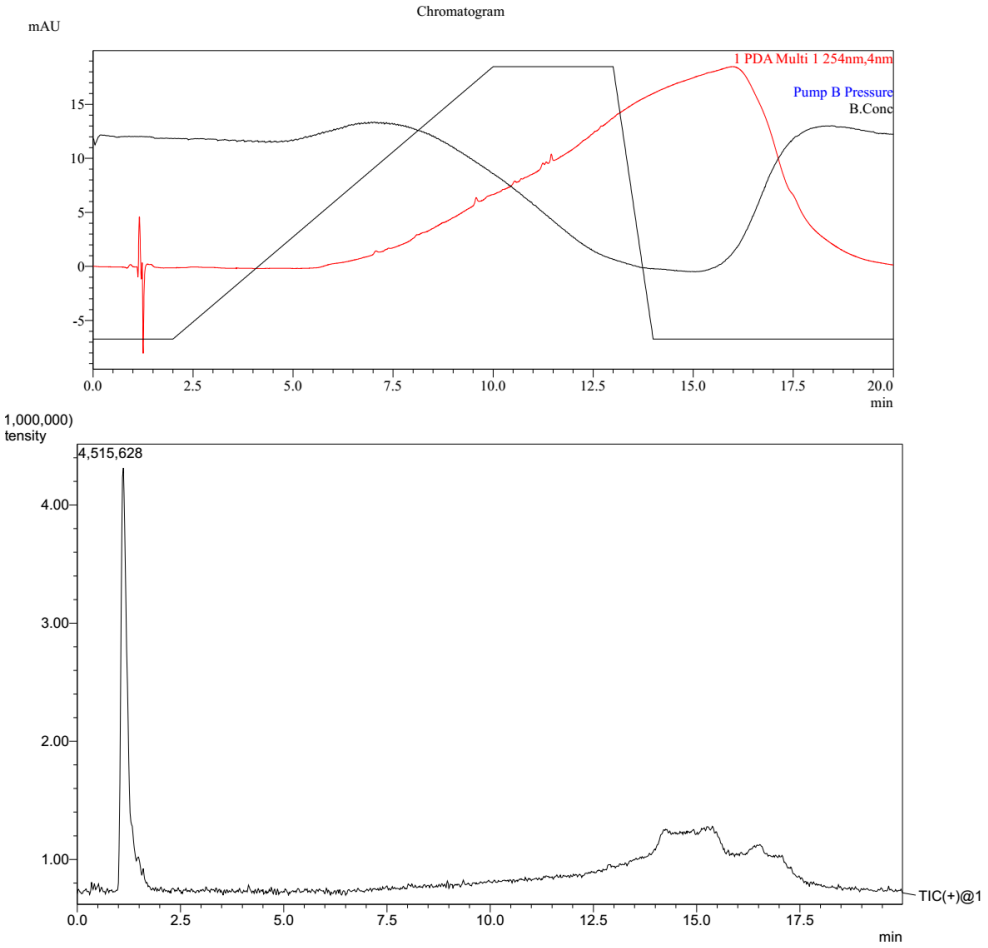
Analysis Report

<Sample Information>

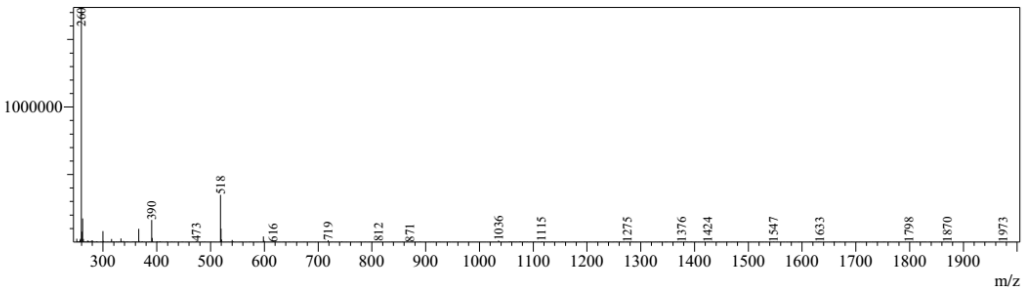
Sample Name	: SY15_13.6		
Sample ID	:		
Data Filename	: SY15_13.6.lcd		
Method Filename	: Phenomenex_McAlpine_column_1.lcm		
Batch Filename	: 18-02-10.lcb		
Vial #	: 1-37	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 11/02/2018 7:25:25 AM	Acquired by	: System Administrator
Date Processed	: 11/02/2018 7:45:28 AM	Processed by	: System Administrator

<Chromatogram>



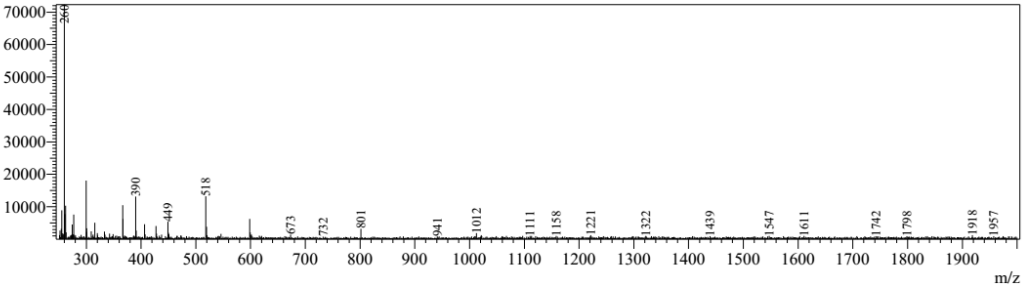
MS Spectrum

R.Time:1.100(Scan#:67)
MassPeaks:1853
Segment 1 - Event 1

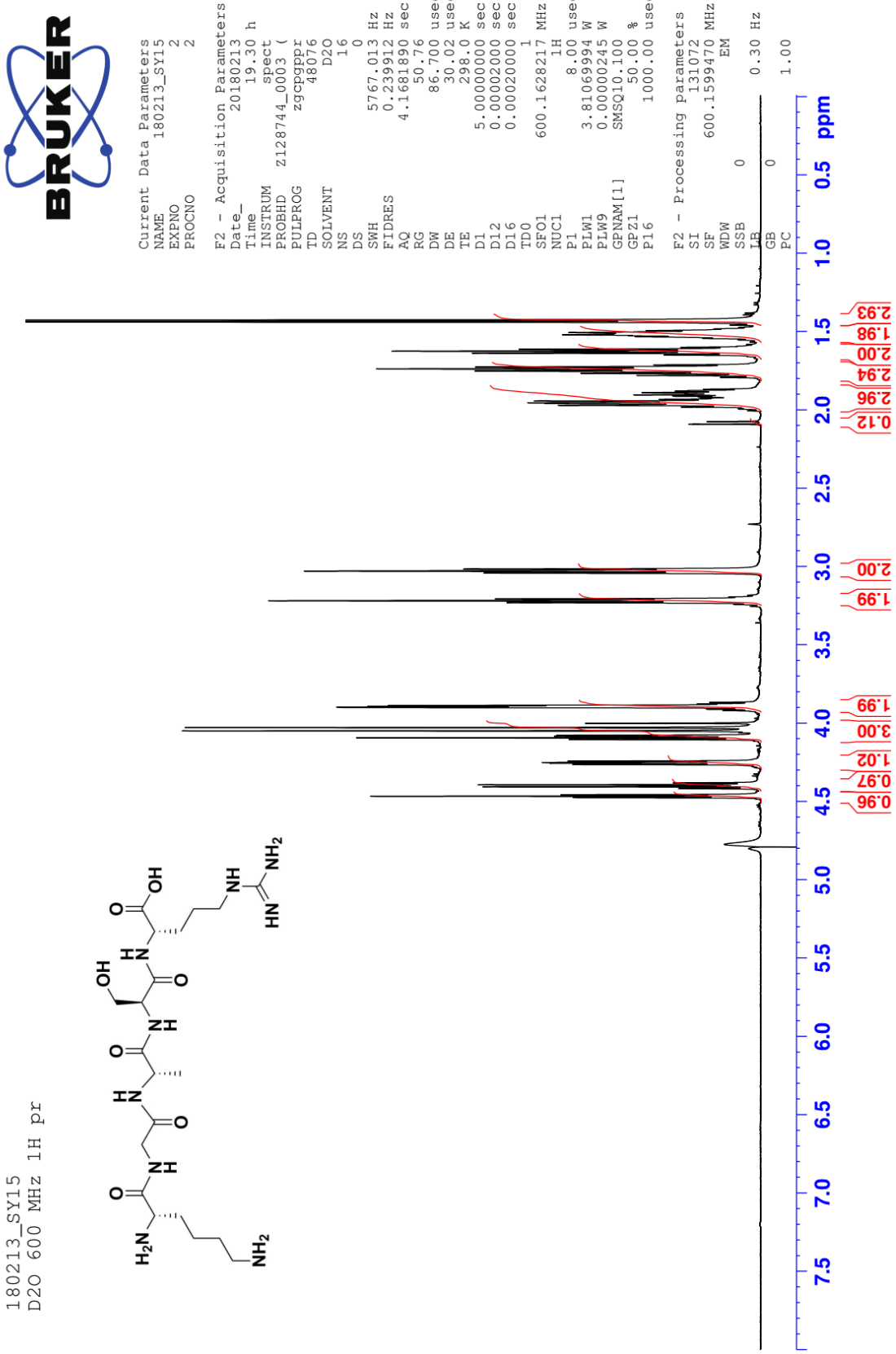


MS Spectrum

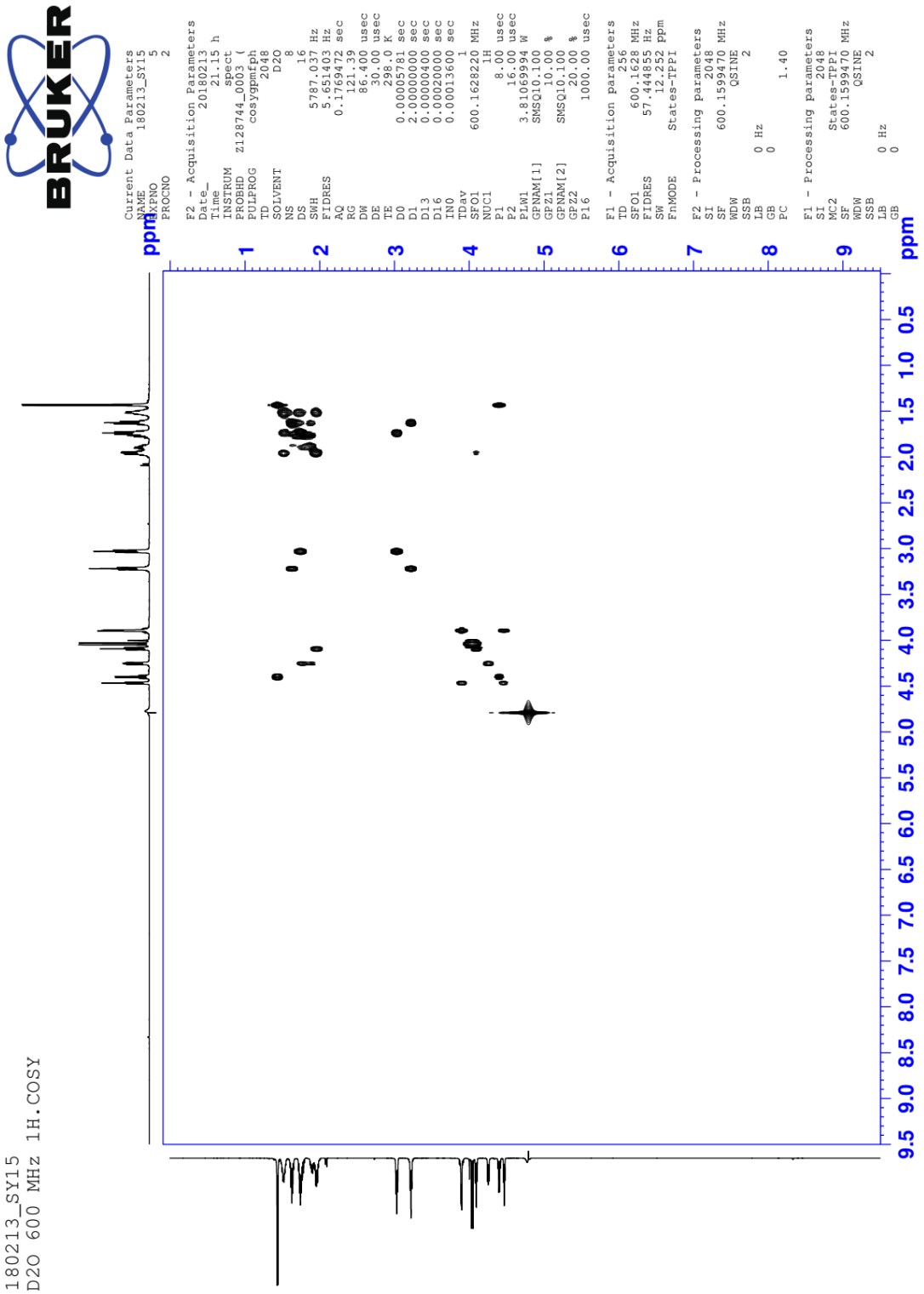
R.Time:1.450(Scan#:88)
MassPeaks:1812
Segment 1 - Event 1



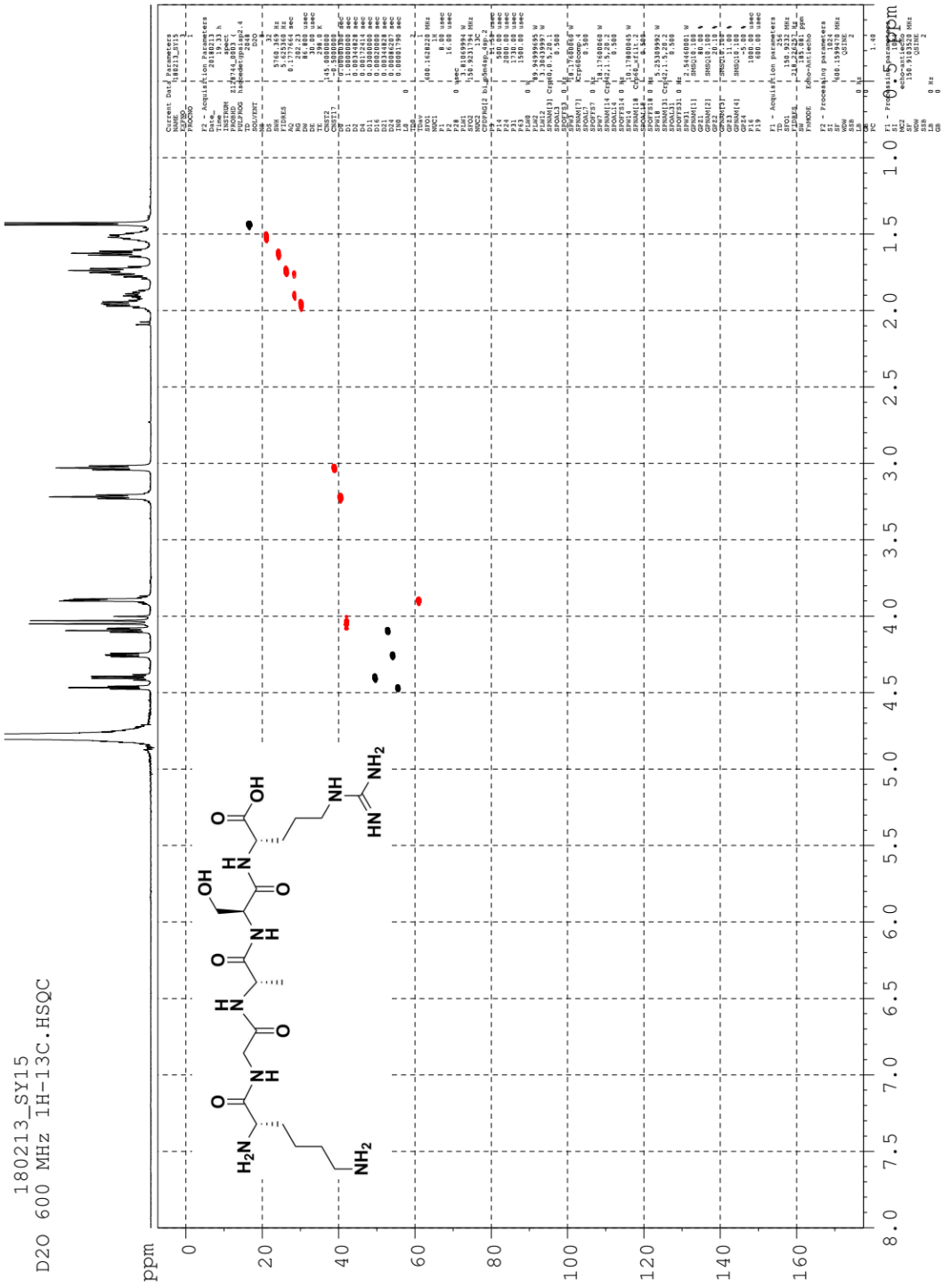
4.10.2 ^{1}H NMR of SY15



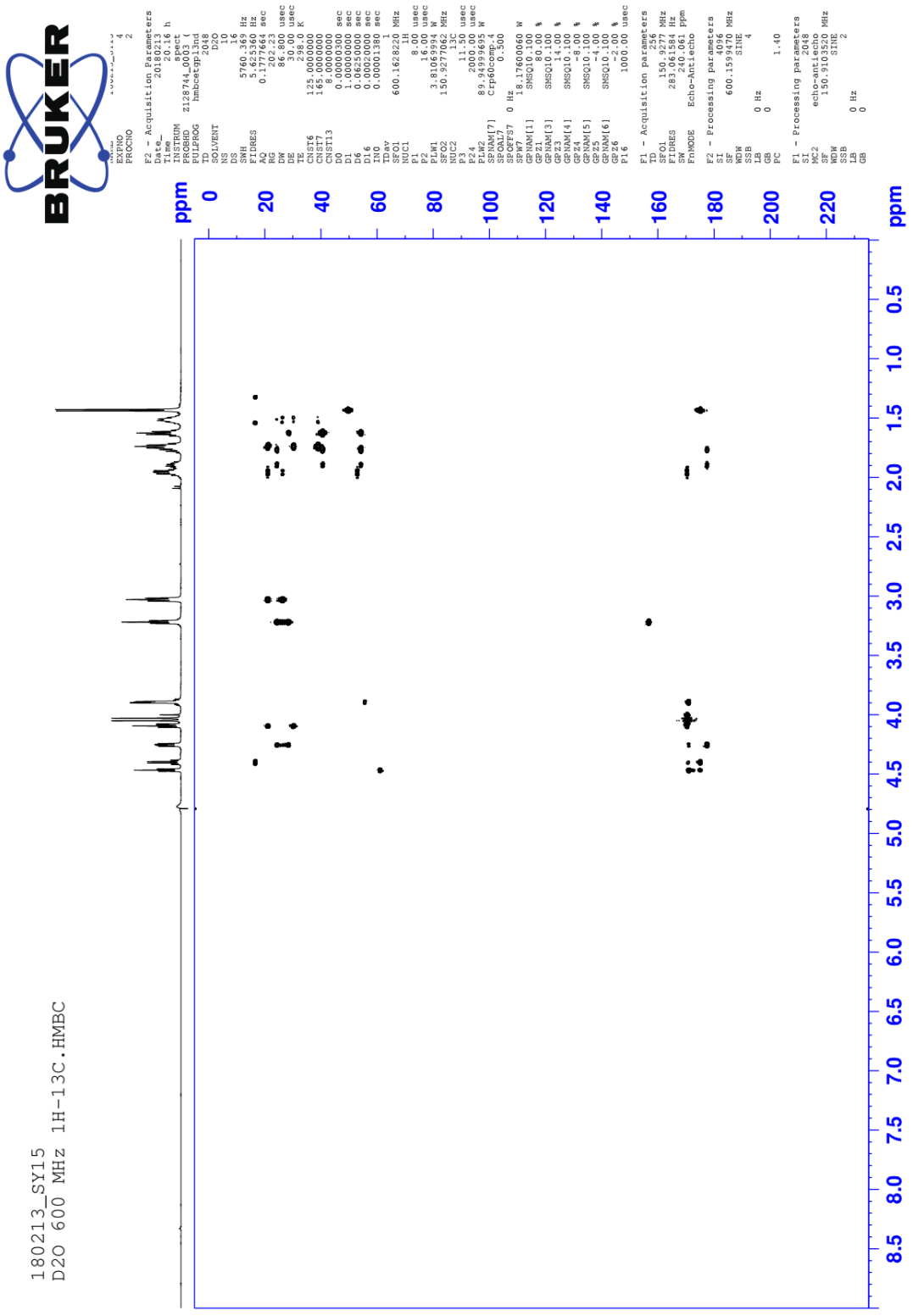
4.10.3 ^{1}H-^{1}H COSY of SY15



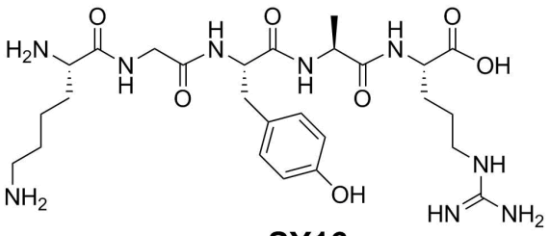
4.10.4 ^{1}H-^{13}C HSQC of SY15



4.10.5 ^{1}H-^{13}C HMBC of SY15



4.11.1 LC/MS of SY16



SY16

Chemical Formula: $C_{26}H_{43}N_9O_7$

Exact Mass: 593.33

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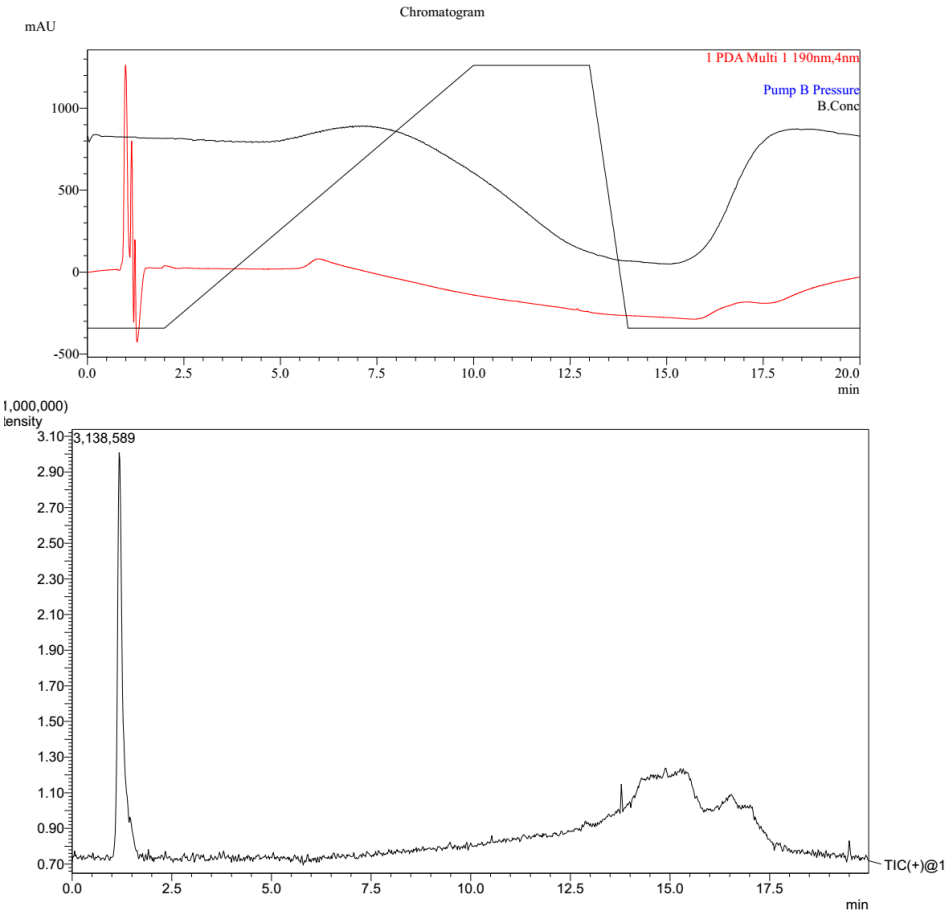
Analysis Report

<Sample Information>

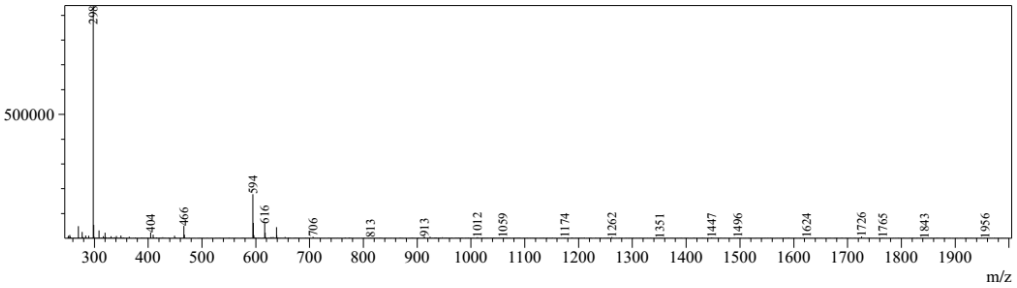
Sample Name	: SY16		
Sample ID	:		
Data Filename	: SY16.lcd		
Method Filename	: Phenomenex_McAlpine_column_1.lcm		
Batch Filename	: 18-02-10.lcb		
Vial #	: 1-90	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 11/02/2018 9:49:38 AM	Acquired by	: System Administrator
Date Processed	: 11/02/2018 10:09:41 AM	Processed by	: System Administrator

<Chromatogram>



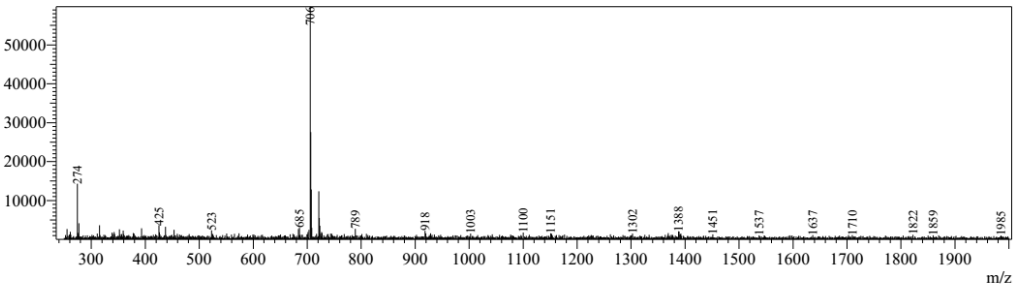
MS Spectrum

R.Time:1.200(Scan#:73)
MassPeaks:1858
Segment 1 - Event 1

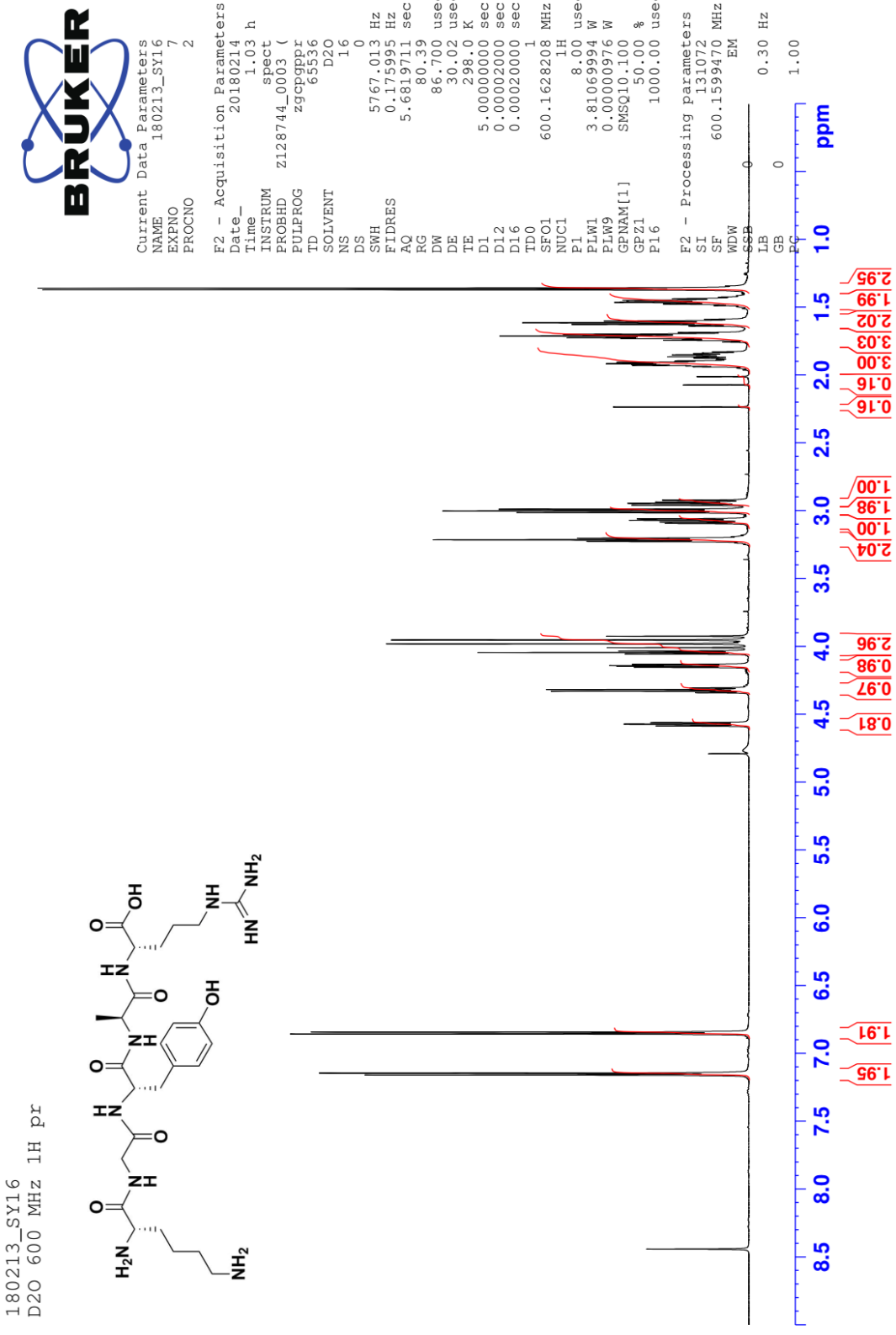


MS Spectrum

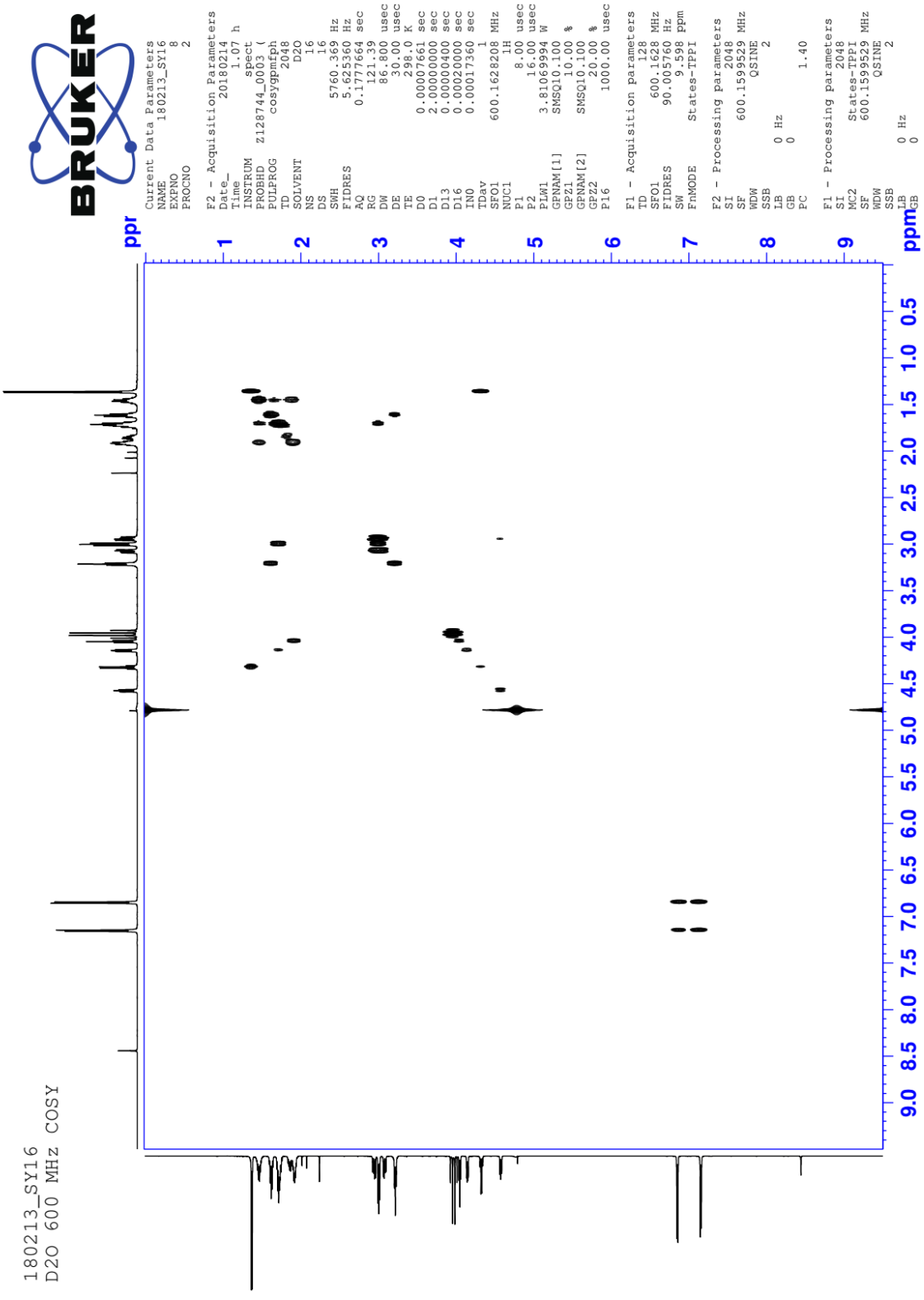
R.Time:15.000(Scan#:901)
MassPeaks:1849
Segment 1 - Event 1



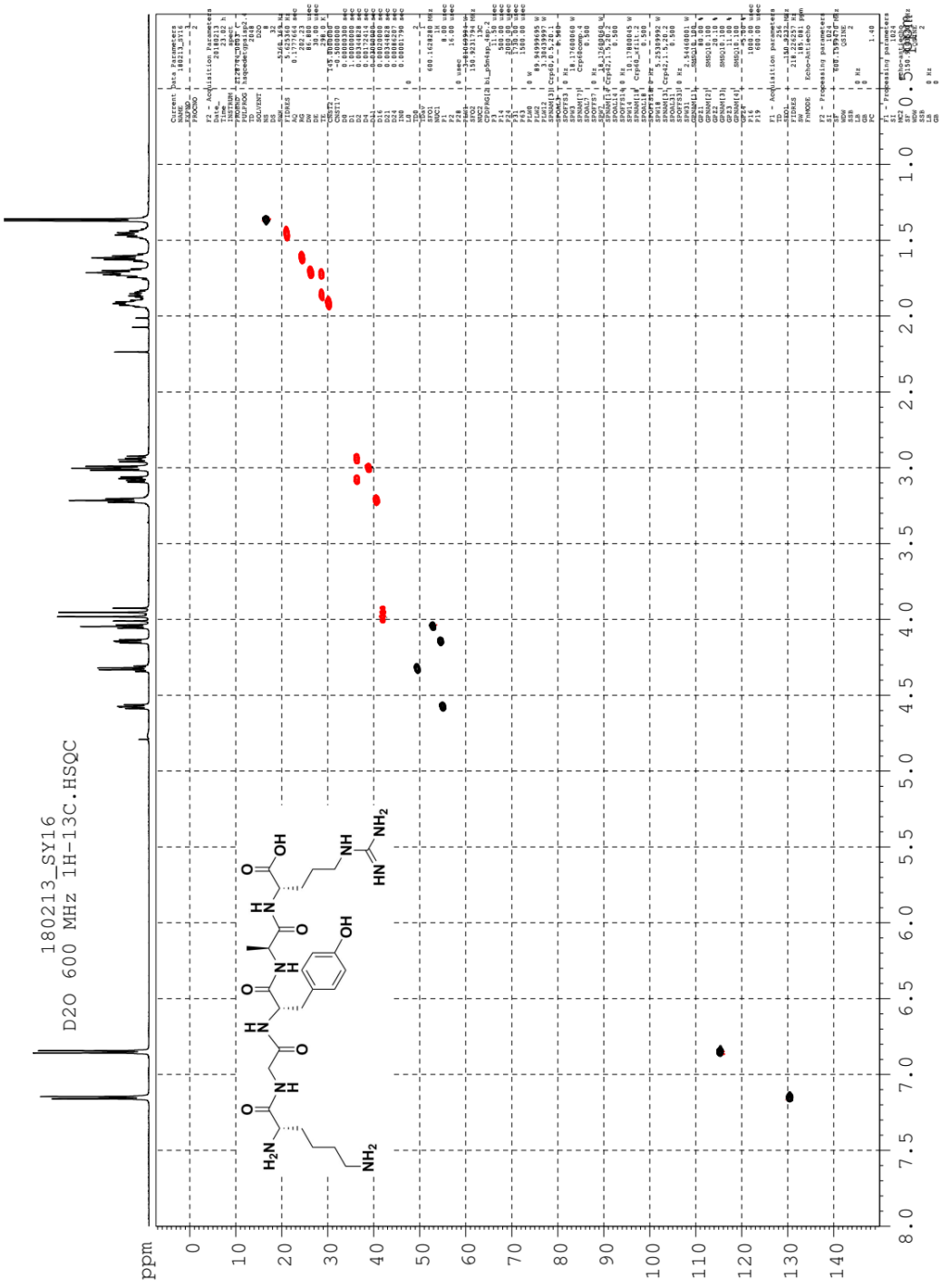
4.11.2 ^{1}H NMR of SY16



4.11.3 ^{1}H-^{1}H COSY of SY16



4.11.4 ^{1}H-^{13}C HSQC of SY16



4.11.5 ^{1}H-^{13}C HMBC of SY16