UNIVERSITY OF NAIROBI

SYNTHESIS AND ANTIGLYCEMIC ACTIVITY OF *IN SILICO* DESIGNED ANALOGUES OF ALLYLPROPYLDISULPHIDE

BY

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I56/81011/2015

A Thesis Submitted in Partial Fulfillment of the Requirements for Award of the Degree of Master of Science in Chemistry of the University of Nairobi

2018

DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for research. Where other people's work or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

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ACKNOWLEDGEMENT

I would first like to thank my thesis advisor and research main supervisor, Dr. Albert Ndakala of the College of Biological and Physical Sciences, School of Physical Sciences, Department of Chemistry at the University of Nairobi. His office was always open whenever I ran into a trouble spot or had a question about my research or writing. He consistently allowed this study to be my own work, but steered me in the right direction whenever he thought I needed it.

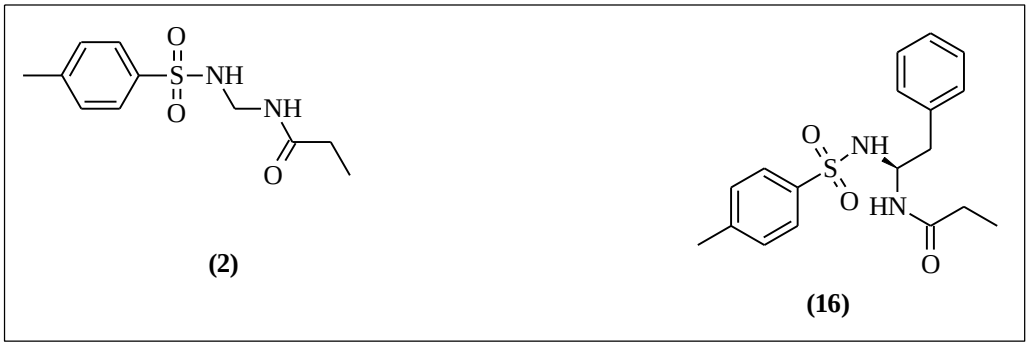
I would also like to thank the experts who were involved in the validation survey for this research project: Prof. Faith Okalebo (University of Nairobi, School of Pharmacy) for supervision of the bioassay component of this study, Dr. Solomon Derese (University of Nairobi, Department of chemistry) for supervision of the computation studies in this research, Dr. Pitchou Ngoy (University of Rhodes, South Africa) for assistance in the nuclear magnetic resonance spectroscopy of the analogues, Mr. Martin Murigi (Jomo Kenyatta University of Agriculture and Technology, Juja) for assistance in mass spectromety, Mr. Juma Siminyu (University of Nairobi, Pharmacology laboratory) for assistance in bioassay and Ms. Alice Mutua (University of Nairobi, Department of Chemistry) for assistance with infrared spectroscopy. Without their passionate participation and input, the validation survey could not have been successfully conducted.

Finally, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of research and writing this thesis. This accomplishment would not have been possible without them. Thank you.

ABSTRACT

Diabetes is a major global health challenge. The disease has a high prevalence mainly due to change in lifestyle associated with improved economic growth. Sulfonylurea monotherapy, sulfonylurea-biguanide combination therapy and insulin administration are some of the common treatments for type 2 *Diabetes mellitus*. Some of these drugs have side effects and are expensive, especially to patients in developing countries. Consequently, there is need to identify alternative diabetes drugs inspired by nature that are cheaper with reduced side effects. Although nature continues to provide antiglycemic compounds such as allylpropyldisulphide (ADPS) from onions, their activity is often limited for direct pharmaceutical use. This study exploited the positive attributes of computer-aided drug design in the process of drug discovery to optimize *N*-({[4-methylphenyl)sulfonyl]amino}methyl)propanamide **(2)**, an analog of APDS, as a potential antiglycemic agent. Ligands were modeled to complement and bind to the target allosteric site of the insulin-degrading enzyme (IDE). Genetic Optimization for Ligand Docking (GOLD); a Cambridge crystallography and data center software application was used to bind ligands to the target and data analyzed in XLSTAT. Moreover, toxicological evaluation was carried out in data warrior; an OSIRIS property explorer tool. The computational study identified *N*-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide **(2)** and *N*-(1-{[(4-methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide **(16)** as attractive compounds for further analysis. The two compounds were synthesized and evaluated *In vivo* for

antiglycemic activity. Although both compounds exhibited antiglycemic activity, compound **2** was the most potent with maximum blood glucose suppression of 59% observed after an hour while compound **16** suppressed blood glucose by a maximum of 45% after 30 minutes of oral dextrose administration. The antiglycemic activity of compound **2** was comparable to metformin (a standard diabetes drug) where it suppressed blood glucose by 59% after one hour and metformin suppressed the same by 62% after two hours. From the antiglycemic screens, it is recommended that compound **2** be further optimized *In silico* to identify more potent antiglycemic compounds.



(2) **(16)**

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LIST OF ABBREVIATIONS

3-AR: 3-Adreno Receptor

ANOVA: Analysis of Variance

APDS: Allylpropyldisulphide

ATP: Adenosine Triphosphate

BE: Binding Energy

bwt: Body weight

CADD: Computer Aided Drug Design

CCDC: Cambridge Crystallographic and Data Center

CPU: Central Processing Unit

DPP-IV: Dipeptidyl Peptidase IV

ED_{50}: Effective dose for 50% of the population

F- analog: Phenylalanyl APDS analog

GA: Genetic Algorithm

G-analog: Glycyl APDS analog

GOLD: Genetic Optimization for Ligand Docking

GSK-3: Glycogen Synthase Kinase-3

IDE: Insulin Degrading Enzyme

LBDD: Ligand Based Drug Design

LD_{50}: Lethal dose for 50% of the population

L-DOPA: L-3,4-Dihydroxyphenylalanine

mp: Melting Point

PDB: Protein Data Bank

PDHK: Pyruvate Dehydrogenase Kinase

PPAR: Peroxisome Proliferate Activated γ-Receptor

PTP-1B: Protein-tyrosine Phosphatase 1B

SAR: Structure Activity Relationships

SBDD: Structure Based Drug Design

SUR1: Sulfonylurea Receptor 1

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Diabetes mellitus is a condition in which the body is unable to produce or respond to insulin (World Health Organization, 2014). This causes abnormal carbohydrate metabolism characterized by elevated blood sugar. The disease may be congenital or acquired and is of three major types. Type 1 *diabetes* occurs when the body is unable to produce insulin. In type 2 *diabetes*, insulin production is normal however, the body is unable to utilize the insulin it produces due to desensitization of insulin receptors on body cells. Gestational diabetes occurs when insulin is less effective during pregnancy (World Health Organization, 2013).

The disease is a major global health challenge with a constantly rising prevalence. For example, an estimated population of 180 million people suffered diabetes in 1980 and 422 million people in 2014 with half of the mortality being directly caused by high blood sugar levels (World Health Organization, 2016). Increased prevalence is mainly due to change in lifestyle as a result of economic growth and influence of technology that has made more people increasingly physically inactive.

Currently, pharmaceutical research incorporates computer aided drug design (CADD) in medicinal chemistry. This has positively impacted the identification and development of

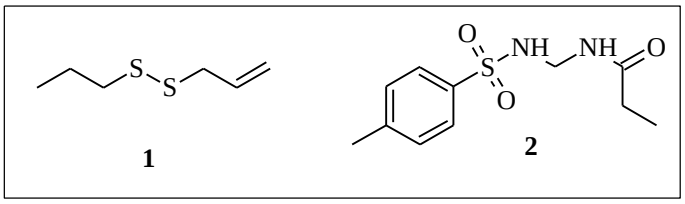
potential therapeutic agents (Hughes *et al.* 2011). Computer aided drug design refers to the use of computer applications to design ligands that are complementary to the target receptor in terms of shape and charge that may mimic a therapeutic effect (Reynolds *et al.* 2010).

Hileman, (2006) reported that the cost of pharmaceutical research is estimated at US$ 880 million and approximately takes 14 years from initial steps of drug design and discovery to successful marketing. CADD links different fields of pharmaceutical chemistry such as computational chemistry and structural biology with the sole aim of discovering novel therapeutic agents within the shortest time possible (Taft *et al.,* 2008).

There are two strategies in CADD; Ligand based drug design (LBDD) and structure based drug design (SBDD). SBDD involves deriving novel therapeutic agents based on the molecular composition of the target. This may involve, for example, using a computer application to screen diverse ligands to the crystal structure of a known enzyme receptor for fitness on the binding pocket of the target and selecting the best ligand. LBDD, on the other hand, is achieved by selecting a series of ligands known to exhibit good activity and screening them using diverse software in an attempt to deduce functional groups on the molecules that are responsible for activity e.g. hydrophilic groups, steric groups etc. (Guner, 2000). For the best results, both strategies are employed.

This study combined both CADD strategies. LBDD was implemented first by comparing a novel class of antiglycemic sulphonamides to commercially available sulphonylureas such as azidoiodoglibenclamide, glibenclamide, and glimperide. A large portion of the

LBDD protocol was implemented during the hit to lead optimization process where ligands were modeled virtually based on allylpropyldisulphide (APDS) (**1**) to identify a novel compound/lead (**2**) (Swaleh *et al.,* 2013), that was targeted for synthesis and lead optimization in this study.



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1.2 Statement of the Problem

Currently, type 2 diabetes has a high prevalence throughout the world; this is mainly due to lifestyle changes. In addition, drugs used in management of diabetes are expensive especially to patients in developing countries. Since the drugs currently used have serious side effects, there is need to develop new drugs with mild side effects. It is worth noting that natural compounds are a good source of such drugs.

Experiments have demonstrated that APDS (**1**), the antiglycemic ingredient in onions, interferes with insulin catabolism leading to increased insulin levels and decreased blood glucose levels (Augusti *et al.*, 1996). However, the hypoglycemic effect of APDS is short lived (Augusti *et al.,* 1996), thus there was need to modify its structure to obtain a desirable molecule of enhanced antiglycemic effect. Through *in silico* modification, APDS (**1**) was modified to enhance the hypoglycemic effect leading to the virtual development of *N-({[4-methylphenyl)sulfonyl]amino}methyl)propanamide* (**2**) as a lead compound (Swaleh *et al.,* 2013).

In this study, the lead molecule was, not only, synthesized to assess its antiglycemic activity *in vivo* but, further virtual screening was also done to optimize its antiglycemic effect.

1.3 Objectives

1.3.1 General Objective

The general objective of this study was to develop antiglycemic analogues of allylpropyldisulphide (APDS).

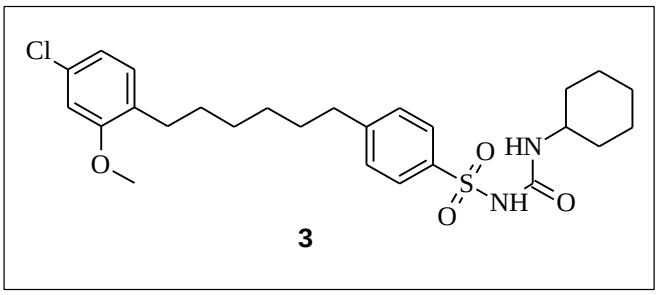
1.3.2 Specific Objectives

The specific objectives of this study were to:-

i. Identify *in silico* APDS analogs that allosterically modulate the insulin degrading enzyme (IDE) for synthesis.
ii. Characterize the synthesized APDS analogs.
iii. Assess the antiglycemic activity of the synthesized APDS analog *in vivo*.

1.4 Justification and Significance of the Study

A previous study documents the antiglycemic effects of APDS (**1**) in onions (Andallu. *et al*., 2001). However the hypoglycemic effect was only short-lived (Augusti *et al.,* 1996). As a result, APDS (**1**) was modified *in silico* to enhance its hypoglycemic effect (Swaleh. *et al.,* 2013). It is worth noting that *In silico* modification of drugs has been widely employed in the pharmaceutical world to identify more potent derivatives. A good example is the variety of sulphonylureas employed in the management of type 2 *diabetes mellitus,* these include; glibenclamide (**3**).



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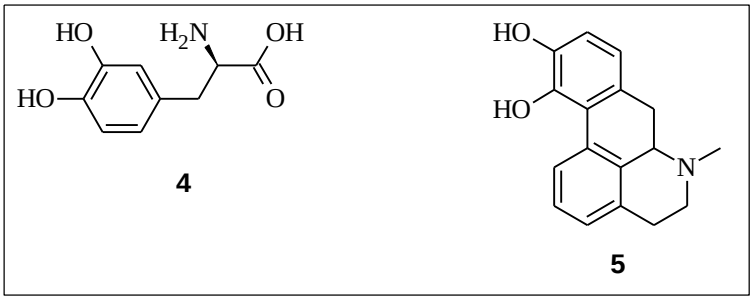
Although *in silico* studies are favorable, practical synthesis and bioassay is required to verify the antiglycemic effect *in vivo*.

Recognizing that amino acids are natural compounds with a potential to interact with biosystems, this study focused on virtual lead optimization of **2** by attaching side chains of various amino acids to the lead compound. For example, it is recognized that the amino acids interact with the polypeptide chain of the allosteric site on the insulin degrading enzyme (IDE) (Camberos *et al*., 2001), the receptor that is targeted in this study. Furthermore, several commercial drugs such as L-DOPA (**4**) and its virtually derived synthetic derivative, apomorphine (**5**), have amino acid side chains.



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CHAPTER TWO

LITERATURE REVIEW

2.1 Diabetes

Diabetes mellitus is a disease where patients have high sugar content in blood due to metabolic disorder. This disease may be acquired or inherited. There are three types of diabetes; type 1, type 2 and gestational diabetes (Sarwar *et al.*, 2010). Type 1 *diabetes mellitus* is caused when the pancreas doesn't produce sufficient insulin and is managed by intravenous insulin administration (WHO, 1999). Type 2 *diabetes mellitus* is caused by desensitization of insulin receptors on body cells even though insulin production is normal (World Health Organization, 2013).

It should be noted that type 1 is a lifetime disease and patients are prescribed management drugs rather than healing drugs. The focus of this study is type 2 *diabetes mellitus*, a chronic disorder that occurs when the body is unable to utilize the insulin it produces; this is due to desensitization of insulin receptors on body cells. The major clinical manifestations of type 2 diabetes is a constant increase of blood sugar which leads to increased viscosity of blood and reduced circulation within small capillaries which presents as diabetes blindness. The peripheral nerves are greatly affected and this leads to numbness of the extremities. In addition to this, wound healing is slow. The disease also presents itself in the form of increased frequency of short calls that eventually lead to dehydration (Baynes and Thorpe, 1999).

Onset of type 2 *diabetes mellitus* is late and is associated with obesity, genetic susceptibility, physical inactivity, dietary patterns and increased stress. More often insulin secretion is normal but the glucose levels in blood rise because the body cells are resistant to the effects of insulin. Thus large amounts of insulin are required in order to reduce excess glucose from blood and to meet energy needs of the body. In type 2 *diabetes mellitus*, some glucose enters cells, thus, the body cells do not need to derive energy from other sources e.g. ketones therefore, ketosis is not common (Baynes and Thorpe., 1999). Diagnosis of this condition is through determination of blood sugar levels.

Type 2 *diabetes mellitus* is managed through diet and adequate exercise. Drug therapy is prescribed when diet and exercise fail to balance blood glucose levels to normal levels. It is important to monitor blood glucose levels so as to ensure that drug therapy is working for the patient. Several drugs have been developed to manage the disease however; these drugs are expensive to populations in the developing world. This disease is characterized by sudden polyuria, polydipsia, weight loss, fatigue, constant hunger and change in vision.

Type 2 *diabetes mellitus* has the highest prevalence mainly caused by excess body weight and physical inactivity. As a result blood glucose levels increase and if not managed it leads to damage of body tissues resulting in kidney failure, blindness and amputation (World Health Organization, 2013). This is as a result of a combination of neuropathy and decreased blood flow in blood capillaries. Diabetic adults are at risk of heart attacks and stroke (Sarwar *et al.,* 2010). Historically, type 2 *diabetes mellitus* was exclusively

diagnosed in adults but currently there is an increase in diagnosis in young populations. Preventive measures have been taken so as to delay onset of the disease. These include; body weight control, physical activity, eating healthy diets while avoiding saturated fatty acids and sugars (processed sugars) in addition to avoiding tobacco use (World Health Organization, 2013).

Gestational diabetes occurs when insulin is less effective to lower blood glucose levels during pregnancy, a condition that normalizes once the pregnancy is due. Therefore expectant mothers are not subjected to drug therapy (World Health Organization, 2013).

Early diagnosis of diabetes is affordable and the public is urged to get tested early so as to avoid complications that arise from late diagnosis (World Health Organization, 2013).

2.2 Diabetes Management

The main goal of diabetes management is to restore carbohydrate metabolism to normalcy (Qaseem *et al.*, 2007). There are general methods of managing the condition in addition to the use of chemotherapy.

2.2.1 General Methods of Diabetes Management

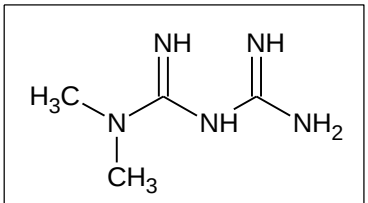
Management strategies for diabetes are dynamic because diabetes constantly damages different body tissues. Initial management of Type 2 *Diabetes mellitus* is through diet; low carbohydrate diet and physical exercises but when these strategies fail, therapy is prescribed (Tuomilehto *et al.*, 2001).

Another approach is the use of bariatric surgery which results in sudden weight loss. This procedure results in reduction of the stomach size which is achieved by using a gastric

band to section the stomach to form a small pouch or by resecting and re-routing the small intestines to a small pouch created in the stomach (gastric bypass surgery) or by removing a portion of the stomach (sleeve gastrectomy). This surgery has demonstrated rapid lowering of blood glucose, leading to patients to use less to none of the diabetes therapies (Robinson, 2009).

2.2.2 Diabetes Chemotherapy

The major types of diabetic drugs include; sulfonylureas, metformin, thiozolidinediones, and alphaglucosidase inhibitors. Metformin (**6**), a biguanide drug works by decreasing glucose production which consequently leads to an increase in insulin sensitivity (The American Society of Health-System Pharmacists, 2016). It is the initial drug administered for type 2 diabetes management (Fischer, 2010).



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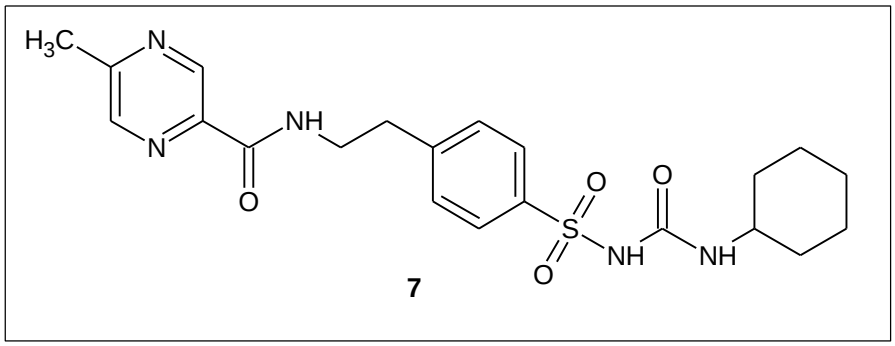
The sulfonylurea drugs stimulate the beta cells of the pancreas to produce more insulin which results in internalization of glucose by cells. Sulfonylureas such as glucotrol/glipizide (**7**) are administered orally or intravenously.



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The absorption of glipizide (**7**); a second generation sulfonylurea drug, occurs in the gastrointestinal tract (GIT). Glipizide (**7**) blocks the potassium channel on the pancreatic beta cells which results in depolarization that causes calcium influx thereby influencing the release of insulin (Urbanova, 2015). Glibenclamide, a sulfonylurea binds to sulfonylurea receptor 1 (SUR1) in pancreatic beta cells causing insulin release.

Glibenclamide (**3**) is commonly administered as a combination drug with metformin (**6**) (Ahmadi *et al*., 2014). Sulfonylureas have high potency which allows them to be administered in low doses. For example, glimperide is administered once a day (Easton *et al.*, 2006).

Thiazolidinediones activate the peroxisome proliferate activated γ-receptor (PPAR) which increase sensitivity of skeletal muscles to insulin and decrease glucose production by the liver. Their major side effect is fluid retention (Inzucchi *et al*., 2012).

α-Glucosidase inhibitors inhibit α-glucosidase enzymes responsible for catabolism of sugars in the ileum. This leads to reduction of glucose absorption thus overall decreased blood glucose. There is risk of hypoglycemia when using these drugs (White and

Campbell, 2008). Frequent monitoring of blood glucose levels is important to ensure that drug therapy is working for the patient (Sanders, 2002).

The increased awareness on the safety of the current drugs employed in management of type 2 *diabetes mellitus* has inspired researchers to develop new strategies to manage the disease. Computer aided drug design has been widely exploited in developing therapies for diverse diseases, including diabetes (Takenaka, 2001).

2.3 Computer Aided Drug Design in Diabetes Drug Discovery

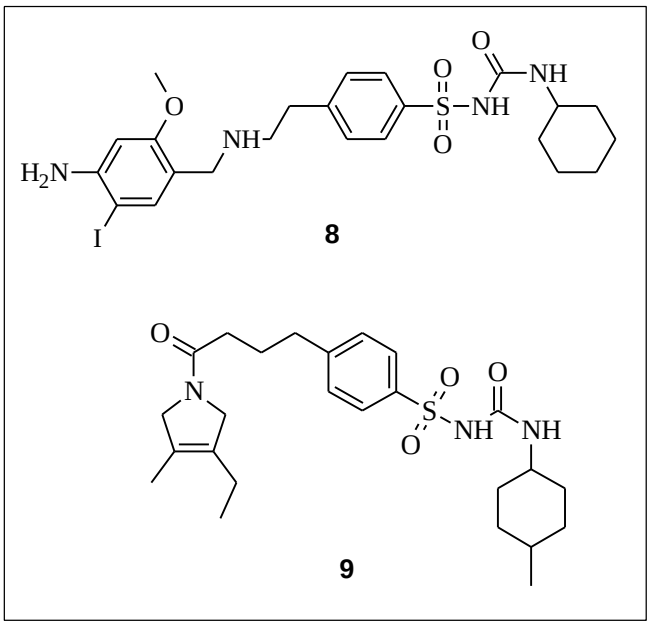
Computer aided drug design involves the use of special software and libraries (compound and receptor libraries) and requires specialized computational skills in chemistry and molecular biology (Dixon *et al.,* 2006). It is widely used in research and development of new drugs through ligand modeling, structure activity relationships (SAR) and pharmacokinetic studies (Hughes *et al.,* 2011). The integration of *in silico* and experimental (synthesis and bioassay) techniques in pharmaceutical research has broadened the understanding of intermolecular and intramolecular interactions (Weigelt, 2010). The *In silico* approach requires the selection of a good target once the pathology of a disease is understood in addition to selection of bioactive ligand(s). It also involves use of molecular modifications popular in medicinal chemistry (Semeghini *et al.,* 2011).

The main advantage of this approach in drug discovery is the short time taken and the reduction in cost relative to pre – *In silico* era (Reynolds *et al.,* 2010). Moreover, computer aided drug design aids in selecting the most promising lead molecules in addition to lead optimization and virtual toxicity studies.

Computer aided drug design strategies are divided into two; structure-based drug design (SBDD) and Ligand-based drug design (LBDD) (Drwal and Griffith, 2013). These techniques have been widely employed in the discovery of novel bioactive compounds

2.3.1 Structure-Based Drug Design

Structure based drug design (SBDD) is the most popular protocol and involves the use of three-dimensional information of biological targets obtained from the protein data banks to identify novel molecules that complement the binding site (Mandal *et al.,* 2009). Molecular docking and virtual screening are key elements of SBDD where ligands are evaluated based on binding energy (BE) and molecular interactions (root mean square deviations) (Kalyaanamoorthy and Chen, 2011). Availability of three-dimensional structures of receptors thus allows for the computational analysis of the nature of the binding site (topology). Topological studies facilitate modeling complementary ligands to the target (Mandal *et al.,* 2009). These (novel) ligands are analyzed based on their affinity to the target. Ligands with high affinity towards validated targets infer that such ligand-target interactions interfere with cellular processes that may lead to desired therapeutic effects (Urwyler, 2011). An exemplification of the applications of SBDD was the development of sulfonylureas from SUR-1 receptors to manage type 2 *diabetes mellitus* (Vaibhav and Prasad, 2013). These sulfonylureas include; azidoiodoglibenclamide (**8**) and glimperide (**9**)**.** Their characteristic potency allows them to be administered in low doses.



2.3.2 Ligand Based Drug Design

Ligand based drug design involves the use of a series of molecules known to exhibit good activity and virtually analyze them in specialized software in a bid to determine functional groups responsible for the activity exhibited by these molecules (Tropsha, 2010). For example, the sulfonylurea moiety is the functional group responsible for the antiglycemic activity exhibited by sulfonylureas (Seino, 2012). An investigation focused on sulphonylureas for treatment of diabetes without regard to the target receptor would therefore be an LBDD approach. However, a combination of both SBDD and LBDD is commonly employed for best results.

2.4 Current Diabetes Drug Targets

Extensive research has been conducted on possible diabetes targets in an attempt to develop new and more effective antiglycemic therapies. The targets that are favorable for *In silico* analysis include; peroxisome proliferate activated receptor (PPAR), 3-Adreno

receptor (3-AR), glycogen synthase kinase-3 (GSK-3), cannabinoid receptors, pyruvate dehydrogenase kinase (PDHK), fructose biphosphatases and dipeptidyl peptidase IV (DPP-IV) (Elchebly *et al.*, 1999).

There are several potential drug targets in the insulin signaling pathway. For example, the binding of insulin and other activating ligands activate the insulin receptor, while the phosphorylation of the GSK-3 receptor causes inhibition of glycogen synthesis and negative signaling of insulin. Moreover, binding of ligands to protein-tyrosine phosphatase 1B (PTP-1B) receptor causes dephosphorylation of the insulin receptor and inactivates the insulin receptor (David, 2001). Binding of agonists to 3-AR receptor increases insulin activity and fat metabolism (DeSouza and Buky, 2001). Binding of sulfonylureas to the SUR-1 receptor inhibits the potassium channel on pancreatic beta cells resulting in cell membrane depolarization causing calcium influx thereby influencing the release of insulin (Urbanova, 2015). Other targets such as the insulin degrading enzyme (IDE) in the insulin signaling pathway are attracting increased attention in diabetes research.

2.5 Insulin Degrading Enzyme: A Diabetes Target for Allylpropyldisulphide

The insulin-degrading enzyme (also known as insulysin/ insulin protease) catabolises insulin in the liver. This enzyme belongs to the M16A metalloprotease group which are known to cleave multiple short polypeptides that vary by far in sequence. The enzyme is recognized by its ability to catabolise the beta chain of insulin (Mirsky *et al*., 1949). IDE activity is regulated allosterically. For example, when short peptides bind to the allosteric site, IDE is rendered inactive (Swaleh *et al.,* 2013).

IDE is a validated and an attractive diabetes drug target that can be exploited in *Diabetes mellitus* management. The crystal structure (3UTV and 2YB3) for IDE can be retrieved from the protein data bank (PDB). The IDE crystal stucture reveals defined *N* and *C* terminal units which form a proteolytic chamber containing a zinc binding site. IDE exists in two conformations; an open conformation, where substrates freely access the active site and a closed conformation, in which the active site is blocked. Studies by (Easton *et al.*, 2006) on aqueous onion (*Allium cepa*) extracts revealed its hypoglycemic effects on *Diabetes mellitus* induced rats. This hypogycemic activity was attributed to the presence of allylpropyldisulphide (**1**) in onions which allosterically modulates IDE resulting in inactivation (Ozougwu, 2011). This leads to high insulin concentration in blood and hence lowering the blood glucose levels (Affholter *et.al.*,1988). However, The hypoglycemic effect of APDS (**1**) is short lived (Ozougwu, 2011).

Previous *in silico* structural modification of APDS to enhance its hypoglycemic effects identified a promising lead molecule; *(N-({[4-methylphenyl)sulfonyl]amino}methyl)propanamide)* (**2**) (Swaleh *et al.*, 2013). The lead displayed relatively good chemical properties and had no toxic effects during ADMET analysis (dry lab analysis). Although *in silico* studies were favorable, practical synthesis and bioassay were required to verify the antiglycemic effect *in vivo*. Moreover, there is need for lead optimization so as to maximize the drug-likeliness of the lead compound (**2**). In this study, derivatives of **2** were synthesized, characterized and subjected to *in vivo* assay to assess the *in silico* antiglycemic effect.

CHAPTER THREE

MATERIALS AND METHODS

3.1 General Experimentation and Instrumentation

This study had three components; computational studies, synthesis and antiglycemic assay. Computational studies employed specialized software (*CCDC Gold, Osiris property explorer, Medchem Designer* ^{TM}*, XLSTAT and Chemsketch*^{TM}) to guide virtual selection of promising molecules for synthesis and bioassay.

All reactions were done in oven dried glass apparatus. The starting reagents, catalysts and solvents were purchased from Sigma-Aldrich and used without further purification. The reaction products were subjected to appropriate purification techniques such as column chromatography and re-crystallization. The solvents for column chromatography and recrystallization were distilled in glass apparatus before use.

Column chromatography was done on Merck silica gel 60 (70-230 mesh) as stationary phase. Analytical TLC on Merck silica gel 60 F_{254} pre-coated aluminium plates was used to monitor the progress of reactions, the profile of eluent fractions from the columns and the purity of the compounds. The chromatographic spots were visualized under UV light at 254 or 366 nm.

The ^{1}H and ^{13}C NMR spectra were recorded on 300 MHz Bruker Avance spectrometers using tetramethylsilane (TMS) as the internal standard. For chemical bond connectivity, the Homonuclear Correlation Spectroscopy (COSY) and Heteronuclear Multiple Bond Connectivity (HMBC) spectra were acquired and processed using standard Bruker®

software. Melting points of the compounds were determined on a melting point apparatus.

The Mass spectra were recorded in Shimadzu GCMS-QP2010 SE, EI. CTC alalytics auto-sampler to determine the total mass of the synthesized APDS analogues and infrared spectra were recorded in Shimadzu IRAffinitty-1S Fourier transform infrared spectrophotometer and spectra displayed by lab solutions IR software.

3.2 Computational Studies

The computational studies were anchored on a computational study that had identified (*N*-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide) as a lead compound for further study against IDE (Swaleh *et al*., 2013). Molecular modeling was used to optimize the lead compound to identify more *in silico* potent derivatives. Considering that the lead compound was earmarked for synthesis from glycine; an amino acid, lead optimization was guided by changing amino acid side chains using the lead molecule to generate a database of ligands of structure diversity.

The model molecules (ligands) were constructed in ChemSketch^{TM} and saved as mol files. A 3-D structure of the target receptor (IDE) was retrieved from the protein data bank (PDB) (Nicholas *et al*., 2012). The software Gold ^{TM} was used to visualize and dock the ligands to the target. During docking, the binding energies of the ligands to the target were determined and compared against the lead compound to shortlist the candidate molecules for synthesis. To achieve this goal, the active site of the enzyme (IDE) was the first to be prepared.

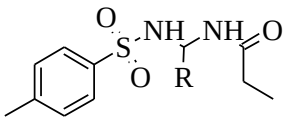
3.2.1 Active Site Preparation

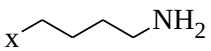
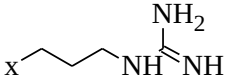
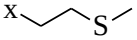
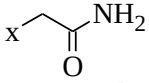
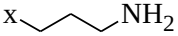
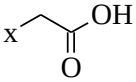
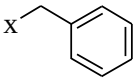
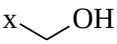
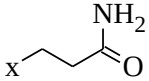
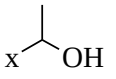
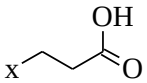
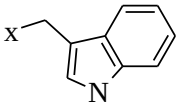
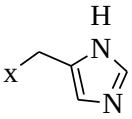
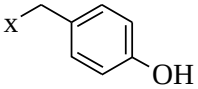
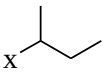
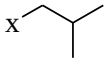
The active site was prepared by first retrieving a 3-D structure of the target (IDE) from PDB; (Nicholas *et al*., 2012) PDB code 3TUV. 3TUV was processed in GOLD software for docking to take place. Hermes, an application from the GOLD suite was launched. Under the GOLD tab, 'setup and run a dock' option was selected and 3TUV loaded. Under the 3TUV tab; 'protein' option was highlighted to make sure that 3TUV box was selected. All 'hydrogens' were deleted. All water molecules were deleted except the ones in the binding site so as to mimic conditions *in vivo* and consume less CPU during docking. Under the amino acids tab, a list of all amino acid residues and water molecules present at the binding pocket is generated. It is important to note that the list is relative to the default radius of the binding pocket. Under 'global options,' the 'define binding site' option was selected followed by selecting the 'one or more ligands' option. Under the ligand option, ligand B (ATP) was selected. This defines the binding pocket to be the one that binds ligand B. under the 'select atoms within' option, the binding site was defined as 6Å (default value) around ligand B and 'detect cavity restrict atom' selected. After the preparation of the active site, the candidate ligands were modeled to assess their *in silico* interaction with the active pocket.

3.2.2 Ligand Modelling

Considering that the lead compound was planned for synthesis from glycine; an amino acid, the lead optimization process was guided by changing the amino acid side chains at position 'R' (Table 1) in the lead molecule to generate numerous analogues.

Table 1: The APDS ligands in the Database



Ligand	R-group	Ligand	R-group
Alanine APDS	x-CH_3 Ala-analog	Lysine APDS	 Lys-analog
Arginine APDS	 Arg-analog	Methionine APDS	 Met-analog
Asparagine APDS	 Asn-analog	Ornithine APDS	 Orn-analog
Aspartic acid APDS	 Asp-analog	Phenylalanine APDS	 Phe-analog
Cysteine APDS	 Cys-analog	Serine APDS	 Ser-analog
Glutamine APDS	 Gln-analog	Threonine APDS	 Thr-analog
Glutamic acid APDS	 Glu-analog	Tryptophan APDS	 Trp-analog
Histidine APDS	 His-analog	Tyrosine APDS	 Tyr-analog
Isoleucine APDS	 Ile-analog	Valine APDS	 Val-analog
Leucine APDS	 Leu-analog	Glycine APDS (lead compound)	x-H Gly-analog

The model molecules (ligands) were constructed in ChemSketch™ and saved as .mol files in preparation for development of a database of ligands selective to the target site.

3.2.3 Database Development

Database development is an important step prior to molecular docking because it tests the selectivity of the target and rank 'test ligands' in this case modeled ligands (Corbeil *et al.*, 2012). A database was developed by scaffold searching Cambridge crystallographic and data center (CCDC) libraries i.e. mercury 3.7 and mogul 1.7.1. Using 'nucleoside phosphate' as search query (Figure 1), 19 ligands were retrieved from the CCDC libraries.

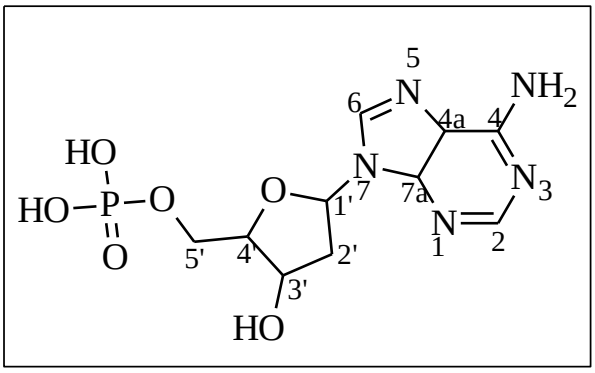


Figure 1: Nucleoside phosphate scaffold

The nucleoside phosphate functional group is the active motif that is known to bind to the target because ATP is a natural allosteric inhibitor (Nicholas *et al.*, 2012). Using the 'sulphonamide functional group' as search query, 38 additional ligands were retrieved from the CCDC libraries. Moreover, 4 additional antidiabetic drugs; metformin and sulphonylureas (azidoiodoglibenclamide, glibenclamide and glimperide) were added to the database. At the end of this process, the database comprised of 82 ligands. Database

development is essential prior to docking so as to establish diversity of target characteristics essential during analysis.

3.2.4 Docking

GOLD, a CCDC docking application was used to dock the database and score ligands in terms of fitness in the active site.

Docking was performed in GOLD by launching Hermes. The GOLD setup wizard was launched by selecting ‘GOLD’ from the main menu option. In the ‘select proteins to use’ window, 3TUV was launched by hitting the ‘Load protein’ button. In the GOLD wizard, ‘Next’ button was selected to proceed to ‘protein setup’ and the 3TUV tab adjacent to the ‘Global options’ tab selected. The protein file was processed by adding ‘hydrogens.’ These were added to the protein to ensure ionization and tautomeric states were defined unambiguously. ‘Waters' were deleted except the ones at the binding pocket. This is because water molecules play key roles in protein-ligand recognition. They either mediate hydrogen bonding in the protein-ligand complex or are displaced by the ligand on binding.

3TUV.pbd was the raw PDB file and the original protein-ligand complex. To dock a ligand to the active site, the co-crystalized ligand (in this case ATP/ligand B) must be removed. In the protein setup window, ‘Delete ligands’ option beneath the ‘extract/delete waters’ option was selected. This window enabled extraction of a ligand from the protein so that a new ligand can be docked. Underneath the ‘extract and reload’ header, ‘B’ was selected and the ‘extract’ button selected. A new window popped-up and prompted the extracted ligand to be saved; saved as ligand B.Mol2.

Back to the global options tab where ‘next’ was selected and this led to ‘define the binding site’ step as previously discussed. ‘Next’ button was selected bringing the ‘configuration template’ dialogue. Configuration templates are important because they load recommended settings for different types of docking protocols with reference to the target (IDE). Since IDE inactivates insulin (a protein) by cleavage, it bears a protease function thus the protease configuration template was selected.

All ligands from the database developed in Section 3.2.3 were loaded. This was done by selecting the ‘add’ button at the bottom of the GOLD wizard. Navigating to the respective folder containing database ligands, the ‘Ctrl + A’ command was employed to select all ligands and were loaded by selecting the ‘open’ button.

The number of docks to be performed per ligand was specified under genetic algorithms (GA) without altering the default settings with a value of 10. The ‘next’ button was selected to proceed to ‘choose a fitness function’ window.

During a dock run, solutions found by GOLD were scored according to fitness functions. Goldscore is the most accurate fitness function therefore selected. Additional options were available by clicking on the ‘more>>’ button. The ‘allow early termination’ was switched on. This instructs GOLD to terminate the dock if at any point the best three solutions fall within 1.5 Å RMSD of each other. The ‘next’ button was selected to proceed to ‘GA search option’ window.

Gold optimizes the fitness score using GA. GA is controlled by a number of parameters divided into 3 speeds; slow, medium and fast. Slow is the most accurate parameter and it equates 100,000 operations thus selected.

The ‘next’ button was selected to proceed to the finish window. At this point, the docking setup was complete and this was affirmed by the appearance of ‘run GOLD’ button. However, the ‘advanced’ button was selected to proceed to the standard GOLD interface. Selecting the ‘output options’ under global settings; in the ‘file format options’ window ‘same as input’ button was selected to activate the ‘output format’ adjacent. The output directory was a desktop file where all docking solutions were saved. The ‘save ligand rank (.rnk) files, ‘save ligand log files’ and ‘save initialized ligand files’ boxes were selected to instruct GOLD to retain output files listing fitness-function rankings and ligand log files. The ‘information in file’ tab and the ‘selecting solutions’ tabs was selected retaining the default settings.

The ‘docking run’ was initiated by clicking the ‘run GOLD’ button at the bottom of the GOLD interface. A finish GOLD configuration window popped-up containing three ‘save files’ options. By rechecking the ‘GOLD conf file’ and ‘protein’ tick boxes are activated and file names, the ‘save’ button was selected to initiate docking.

3.2.5 Toxicity Studies

The best ranked ligands in the docking studies were subjected to virtual toxicological evaluation to access their safety. This was done using data warrior; an OSIRIS property explorer application. Data warrior was launched from the desktop. A new working area was created by selecting ‘new’ from the ‘file’ tab. A structure window popped up, under

'column type' section 'select structure' was selected and the 'okay' button clicked. A 'view window' appeared which was double clicked to activate the 'OSIRIS structure editor' where ligands were drawn. Once a ligand was drawn, the 'okay' button was clicked. The 'add compound properties' option under the 'chemistry tab' was selected. A new window; 'calculate compound properties' appeared and under the 'druglikeliness tab,' ClogP, ClogS, Druglikeliness boxes were selected. Under the 'LE, TOX, Shape tab,' mutagenic, tumorigenic, reproductive effect and irritant boxes were selected and the 'okay' button clicked. This process identified *N*-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide and *N*-(1-{[(4-methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide as the synthetically strategic molecules for bioassay.

3.3 Synthetic Procedures

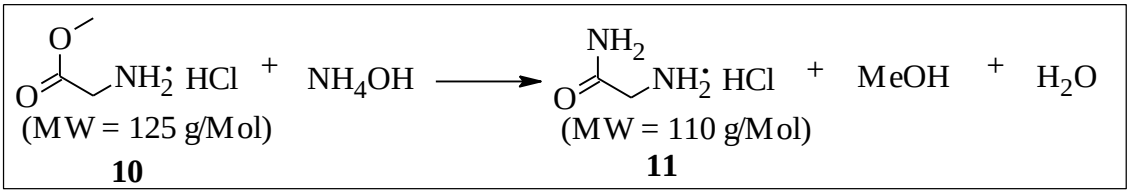
The two APDS analogs from glycine (*N*-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide) and phenylalanine (*N*-(1-{[(4-methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide) analogs were synthesized based on the synthetic procedures specified below.

3.3.1 Preparation of N-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide (2)

N-({[(4-Methylphenyl)sulfonyl]amino}methyl)propanamide (**2**), also referred to as the glycyl analog, was synthesized from glycine methyl ester via four steps as described below.

3.3.1.1 Synthesis of 2-aminoacetamideHCl (11)

Scheme 1: Synthesis of 2-aminoacetamideHCl

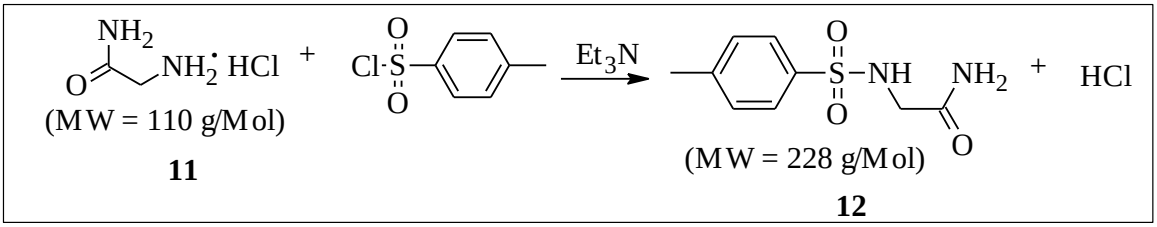


2-Aminoacetamide (**11**) was synthesized using a modification of a procedure developed by Labrecque *et al.* (2000). The glycine methyl ester hydrochloride (**10**) (1.5 g, 12.0 mmol) was treated with a saturated solution of ammonia (4 mL). The mixture was stirred at room temperature for 3 hours and then concentrated. The residue was dissolved in H_2O (5 mL) and then extracted with ether (3 x 30 mL). The combined ether layer was dried with Na_2SO_4, filtered and then treated with $HCl_{(g)}$ to precipitate a white powder **11** (1.02 g, 77% yield) which was filtered and left to dry.

R_f = 0.35 (30% EtOAc: Hexane), melting point (mp) above 250^0C; **Appendix 3E** (cm^{-1}) 3700-2450 (NH_2), 2349, 1666 (C=O), 1485, 1404, 1323, 1092, 1033, 899, 833; **Appendix 3A** (DMSO-d_6, 300MHz): δ_H 3.72 (2H, *s*, H-2): **Appendix 3B** (DMSO-d_6, 75 MHz): δ_C 167.3 (-C=0) and 49.3 (C-2).

3.3.1.2 Synthesis of 2-{[(4-methylphenyl)sulfonyl]amino}acetamide (12)

Scheme 2: Synthesis of 2-{[(4-methylphenyl)sulfonyl]amino}acetamide

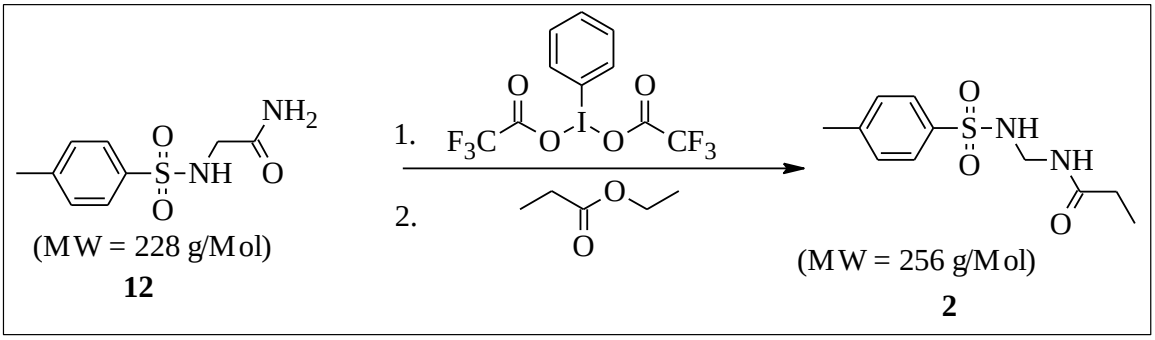


p-TsCl (3.93 g, 20.61 mmol) was added in several portions at 0°C within 20 min to a mixture of **11** (1.48 g, 13.39 mmol) and Et_3N (17 mL) in dry CH_2Cl_2 (30 mL). The reaction mixture was stirred at room temperature for 48 hours before being washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and purified by column chromatography while monitoring by thin layer chromatography. The eluent was concentrated to provide a solid which was recrystallized in a mixture of 3:7 ethyl acetate/hexane to obtain 2-{[(4-methylphenyl)sulfonyl]amino}acetamide (**12**) (Zhang *et al*., 2009) (0.5 g, 17% yield) as glassy white crystals.

R_f = 0.45 (30% EtOAc: Hexane), mp (162-163 ^{0}C), **Appendix 4E** (cm^{-1}) 3356 (NH_2), 3260, 1597 (C=O), 1528, 1385 (SO_2), 1300, 1153, 1096, 1018, 899, 814, 664; **Appendix 4A** (DMSO-d_6, 300MHz): δ_H 2.32 (3H, *s*), 7.31 (2H, *m*, H-3), 7.70 (2H, *m*, H-4) and 4.2 (2H, *s*, H-2$^{'}$): **Appendix 4B** (DMSO-d_6, 75 MHz): δ_C 21.3 (-CH_3), 144.3 (C-4), 129.7 (C-3), 127.5 (C-2), 136.9 (C-1), 171.1 (C-1$^{'}$) and 49.3 (C-2').

3.3.1.3 Synthesis of N-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide (2)

Scheme 3: Synthesis of N-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide



A procedure of Monica *et al.* (2003) was modified by adding 1,1-bis(trifluoroacetoxy)iodobenzene (2.83 g, 6.58 mmol) to a solution of **12** (1.5 g, 6.57 mmol) in CH_3CN (4 mL) and H_2O (1 mL). The mixture was heated at 60^0C for 30 min. The mixture was acidified using 2M HCl, refluxed for 1 hour and extracted with ether (10 mL) to remove side products of 1,1-bis(trifluoroacetoxy)iodobenzene.

The water layer was basified and treated with ethylpropanoate (30 mL). The mixture was refluxed at 50^0C for 5 hours and then concentrated to provide a residue which was purified by column chromatography to provide *N*-({[(4-Methylphenyl)sulfonyl]amino}methyl)propanamide (**2**) (1.4 g, 83% yield) as white crystals.

R_f = 0.4 (30% MeOH: DCM), mp above 247-250^0C; **Appendix 5E** (cm^{-1}) 3394 (NH_2), 2989, 2692, 1674 (C=O), 1473, 1396, 1176, 1126, 1064, 1033, 833, 798, 721, and 683; **Appendix 5F** (M/z) 257 (M+1); **Appendix 5A** (DMSO-d_6, 300MHz): δ_H 2.32 (2H, *s*), 7.31 (2H, *m*, H-3), 7.70 (2H, *m*, H-4), 4.73 (2H, *s*, H-6), 2.23 (2H, *q*, *J* = 7.29, H-2') and

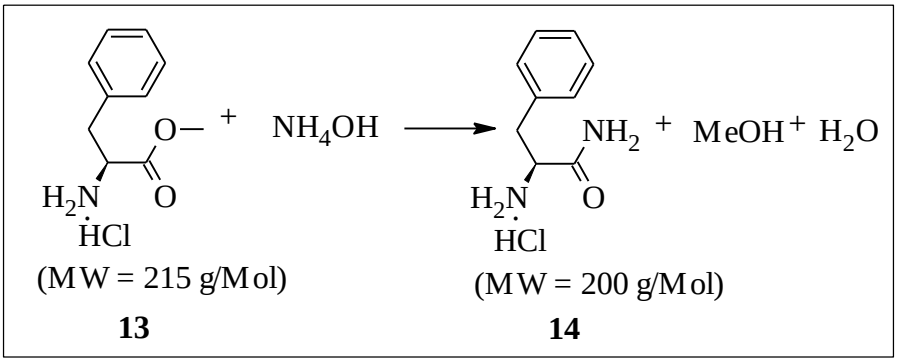
1.04 (3H, *t*, *J* = 7.29Hz, H-3') : **Appendix 5B** (DMSO-d_6, 75 MHz): δ_C 21.3 (-CH_3), 144.3 (C-4), 129.7 (C-3), 127.5 (C-2), 135.1 (C-1), 66.2 (C-6), 171.0 (-C=O), 30.2 (C-2') and 9.4 (C-3').

3.3.2 Preparation of N-(1-{[(4-methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide (16)

N-(1-{[(4-Methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide (**16**), herein also referred to as the phenylalanyl analog was synthesized based on the procedures described below.

3.3.2.1 Synthesis of 2-amino-3-phenylpropanamideHCl (14)

Scheme 4: Synthesis of 2-amino-3-phenylpropanamideHCl



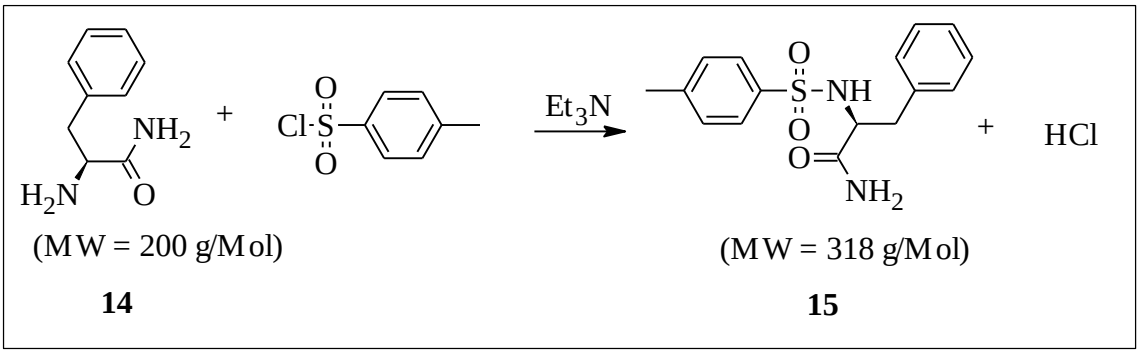
2-Amino-3-phenylpropanamide (**14**) was synthesized using a modification of a procedure developed by Labrecque *et al.* (2000). Methyl 2-amino-3-phenylpropanoate (**13**) (1.5 g, 6.98 mmol) was treated with a saturated solution of ammonia (4 mL). The mixture was stirred at room temperature for 3 hours and then concentrated. The residue was dissolved in H_2O (5 mL) and then extracted with ether (3 x 30 mL). The combined ether layer was

dried with Na_2SO_4, filtered and then treated with $HCl_{(g)}$ to precipitate a white powder **14** (1.25 g, 89% yield) which was filtered and left to dry.

R_f = 0.37 (30% EtOAc: Hexane), mp above 250^0C; **Appendix 7E** (cm^{-1}) 3360 (NH2), 3260 (NH), 2873 (NH_2), 1685 (C=O); **Appendix 7A** (DMSO-d_6, 300MHz): δ_H 3.63 (1H, *t*, *J* = 7.60 Hz, H-2), 3.05 (2H, *d*, *J* = 7.60 Hz, H-3), 7.21 (2H, *m*, H-7), 7.36 (2H, *m*, H-5), and 7.32 (2H, *m*, H-6) : **Appendix 7B (**DMSO-d_6, 75MHz): δ_C 177.9 (-C=O), 54.1 (C-2), 39.2 (C-3), 135.7 (C-4), 129.1 (C-5), 128.7 (C-6) and 128.9 (C-7).

3.3.2.2 Synthesis of 2-{[(4-methylphenyl)sulfonyl]amino}-3-phenylpropanamide (15)

Scheme 5: Synthesis of 2-{[(4-methylphenyl)sulfonyl]amino}-3-phenylpropanamide



(MW = 200 g/Mol) (MW = 318 g/Mol)

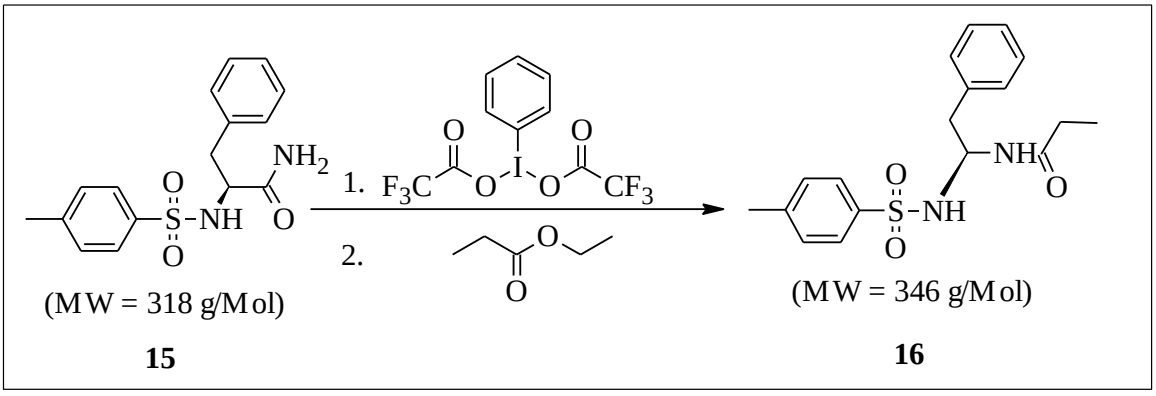
14 **15**

p-TsCl (2.12 g, 11.11 mmol) was added in several portions at 0°C within 20 min to a mixture of **14** (1.48 g, 7.43 mmol) and Et_3N (17 mL) in dry CH_2Cl_2 (50 mL). The reaction mixture was stirred at room temperature for 24 hours before being washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and purified by column chromatography (30 % EtOAc: Hexane) while monitoring by thin layer chromatography (Zhang *et al*., 2009) which was recrystallized in a mixture of hexane and ethyl acetate to provide **15** (1.13 g, 48% yield) as glassy white crystals.

R_f = 0.45 (30% EtOAc: Hexane), mp 147-150^{0}C; **Appendix 8E** (cm^{-1}) 3417 (NH_2), 3248, 3325, 3209, 3086, 3032, 2920, 2870, 2739, 1670 (C=O), 1639, 1539, 1446, 1323, 1157 (SO_2), 1092, 1042, 945, 814, 748, 702, 664; **Appendix 8A** (DMSO-d_6, 300MHz): δ_H 7.70 (2H, *m*, H-2), 7.31 (2H, *m*, H-3), 2.32 (3H, *s*, H-5), 5.09 (1H, *t*, *J* = 6.78 Hz, H-5), 2.92 (2H, *d*, *J* = 6.78 Hz, H-3'), 7.25 (2H, *m*, H-2"), 7.28 (2H, *m*, H-3") and 7.20 (1H, *m*, H-4") : **Appendix 8B (**DMSO-d_6, 75MHz): δ_C 137.9 (C-1), 127.5 (C-2), 129.7 (C-3), 144.3 (C-4), 21.3 (-CH_3), 174.8 (-C=O), 56.8 (C-2'), 37.2 (C-3'), 135.7 (C-1"), 129.1 (C-2"), 128.7 (C-3") and 128.9 (C-4").

3.3.2.3 Synthesis of N-(1-{[(4-methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide (16)

Scheme 6: Synthesis of N-(1-{[(4-methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide



A procedure of Monica *et al.* (2003) was modified by adding 1,1-bis(trifluoroacetoxy)iodobenzene (3.04 g, 7.07 mmol) to a solution of **15** (1.5g, 4.71 mmol) in CH_3CN (4 mL) and H_2O (1 mL). The mixture was heated at 60^{0}C for 30 min.

The mixture was acidified using 2M HCl, refluxed for 1 hour and extracted with ether (10mL) to remove side products of 1,1-bis(trifluoroacetoxy)iodobenzene.

The water layer was basified and treated with ethyl propanoate (30 mL). The mixture was refluxed at 50^0C for 5 hours and then concentrated to provide a residue which was purified by column chromatography to provide *N*-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide **16** (1.4 g, 86% yield) as white crystals.

R_f = 0.52 (30% MeOH: DCM), mp 235-237^{0}C; **Appendix 9E** (cm^{-1}) 3371 (NH_2), 2989, 2684, 2492, 1674 (C=O), 1473, 1396, 1199, 1130, 1026, 833, 798, 721; **Appendix 9F** (M/z) 347 (M+1 peak); **Appendix 9A (**DMSO-d_6, 300MHz): δ_H 7.70 (2H, *m*, H-2), 7.31 (2H, *m*, H-3), 2.32 (3H, *s*, H-5), 5.19 (1H, *t*, *J* = 6.63, H-1'), 3.10 (2H, *d*, *J* = 6.63), 2.18 (2H, *q*, *J* = 7.30 Hz, H-2"'), 1.04 (3H, *t*, *J* = 7.30 Hz, H-3"'), 7.31 (2H, *m*, H-2"), 7.25 (2H, *m*, H-3") and 7.22 (1H, *m*, H-4") : **Appendix 9B** (DMSO-d_6, 75MHz): δ_C 135.1 (C-1), 127.5 (C-2), 129.7 (C-3), 144.3 (C-4), 21.3 (-CH_3), 79.5 (C-1'), 30.5 (C-2'), 136.5 (C-1"), 128.6 (C-2"), 128.7 (C-3"), 128.9 (C-4"), 170.6 (-C=O), 30.2 (C-2"') and 9.4 (C-3"').

3.4 Bioassay Procedures

The antiglycemic activity of the synthesized glycine and phenylalanine APDS analogues were assessed in mice at the School of Pharmacy, University of Nairobi. The screening results were subjected to statistical analysis to determine their significance.

3.4.1 Collection and Authentication of Materials

Dextrose (grade: A.R) was obtained from Lobachemie laboratory reagents and fine chemicals, Mumbai-India. Metformin (Glucphage, 500mg) was obtained from Megalife Pharmacy situated in Ruai along Kangundo Road, Nairobi on 18th April 2018. Distilled water and absolute ethanol were obtained from the University of Nairobi store in the School of Pharmacy. Swiss albino mice were obtained from the animal house of the Department of Pharmacology and Pharmacognosy, School of Pharmacy, University of Nairobi. Glucometer (GLUKOR GM-500, SN-MM015G051) was obtained from HuBBDIC CO.LTD, Dong-Korea.

3.4.2 Breeding of Mice

Swiss albino mice of either sex were bred in plastic cages in groups of six with wood shavings as bedding. The mice were fed with mice pellets from Unga Kenya Limited and had free access to water. Breeding occurred in a 12 hour dark-light cycle with room temperatures maintained at 20-25^{0}C. The animals were allowed to acclimatize in the laboratory for a week before screening.

3.4.3 Ethical Considerations

The principles of ethical experimentation involving laboratory animals were adhered to. These principles are; reduction, refinement and replacement (Hau *et al*., 2016). The principle of reduction was achieved by use of minimal animals per group to obtain valid scientific outcome (Barthold *et al*, 2011). The principle of refinement was achieved by minimizing pain and suffering to the animals (Olfert and McWilliam, 1993).

3.4.4 Solubilization and Suspension of Test Compounds

Two grams of test compounds (G-analog (**2**) and F-analog (**16**)) were dissolved in 10ml of ethanol and this solution was diluted to obtain 500 $mgKg^{-1}$ and 1000 $mgKg^{-1}$ of each test material respectively. Metformin tablet sourced from Megalife Pharmacy, batch no.F71282.90 C04/2014 was crushed and suspended in water to obtain a suspension of 100 mg/mL. Dextrose solution was prepared to obtain a final concentration of 0.5 mg/ml.

3.4.5 Oral Glucose Tolerance Test

The mice were fasted for 24 hours and divided into six groups each with four mice. One hour before the test, all the mice were given 50% dextrose solution by gavage. Each mouse received 5 mg/Kg of dextrose solution. An hour after dextrose administration, glucose levels were measured using a glucometer (HuBBDIC CO.LTD SN-MM015G051). 5 µL of blood for glucose testing was obtained using the tail snip method; the tip of the tail was swabbed with ethanol and a small snip obtained using a pair of scissors to get the blood sample for testing.

After obtaining the baseline of the sample, two control groups were treated with 3.01mL of metformin (500 $mgKg^{-1}$) and 3.3ml of saline (45 $mgKg^{-1}$). Four test groups were treated with glycyl APDS analog (**2**) at 500 $mgKg^{-1}$ and 1000 $mgKg^{-1}$ and phenylalanyl APDS analog (**16**) at 500 $mgKg^{-1}$ and 1000 $mgKg^{-1}$. The APDS analogs and the controls were administered by gavage.

After drug administration, glucose levels were measured at 0, 30, 60, 90, 120 and 180 minutes. During analysis, the behavioral responses of the animals were also observed.

Equation 1 describes the general formula that was used for calculating the volumes to be administered to each mouse per group.

$$\text{volume} = \frac{\text{body weight (Kg)} \times \text{Dose (mg/Kg)}}{\text{Concentration (mg/mL)}} \quad \text{----------(Equation}$$

3.5 Biostatistical Analysis

For each group, the mean glucose levels and standard deviation of the mean was computed. Glucose levels were expressed as a % of the baseline value calculated from equation 2.

$$\text{Mean \% reduction in blood glucose level} \left[\frac{\text{Mean}_{\text{saline}} - \text{Mean}_{\text{drug}}}{\text{Mean}_{\text{saline}}} \right] 100\% \quad \text{----(Equation 2)}$$

Oneway analysis of variance (oneway ANOVA) was conducted to determine whether there was a difference in blood glucose levels per group at different time points (Howell, 2002). The post-hoc Tukey's multicomparison test was conducted at various time points to compare antiglycemic compounds and the negative control (Marusteri and Bacarea, 2010). Graphs were plotted to display changes in glucose levels. For inferential data analysis, the level of significance was $\alpha = 0.05$ (Benjamini and Hochberg, 1995).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Outline of the Study

This study was carried out in three stages; computational studies to identify allylpropyldisulphide (APDS) analogs for synthesis, synthesis of the identified APDS analogs and finally, the antiglycemic evaluation of the synthesized analogs *in vivo*. Results from each stage are discussed in this chapter.

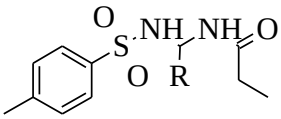
4.2 *In Silico* Selection of APDS Analogues for Synthesis

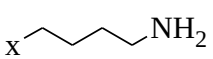
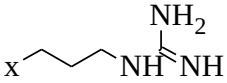
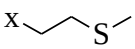
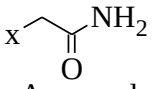
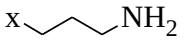
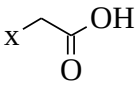
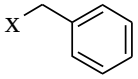
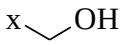
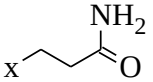
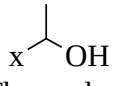
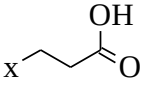
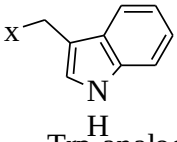
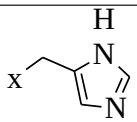
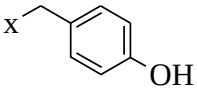
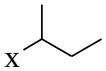
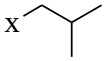
The computational study assessed the interaction of a database comprising of ligands including analogues (Table 2) of APDS, a molecule present in onions and exhibits a short-lived antilycemic activity (Ozougwu, 2011), against the allosteric site of the insulin degrading enzyme (IDE). To achieve this goal, various specialized electronic tools were employed; *Chemsketch* was used to draw APDS analogs, *CCDC GOLD* was used for docking the database inclusive of APDS analogs, *XLSTAT* was used to verify the active hits and *data warrior* was used to assess their virtual toxicity in predicting the safety of the active ligands prior to synthesis.

4.2.1 Features of the In-Silico Database

The *In-silico* database of ligands for virtual screening against the insulin degrading enzyme (IDE) contained a total of 78 ligands comprising of, among others, 20 APDS analogs (Table 2) with motifs derived from side chains of various amino acids.

Table 2: Structures of the APDS analogues in the database



APDS Analog	R-group	APDS Analog	R-group
Alanine APDS	 Ala-analog	Lysine APDS	 Lys-analog
Arginine APDS	 Arg-analog	Methionine APDS	 Met-analog
Asparagine APDS	 Asn-analog	Ornithine APDS	 Orn-analog
Aspartic acid APDS	 Asp-analog	Phenylalanine APDS	 Phe-analog
Cysteine APDS	 Cys-analog	Serine APDS	 Ser-analog
Glutamine APDS	 Gln-analog	Threonine APDS	 Thr-analog
Glutamic acid APDS	 Glu-analog	Tryptophan APDS	 Trp-analog
Histidine APDS	 His-analog	Tyrosine APDS	 Tyr-analog
Isoleucine APDS	 Ile-analog	Valine APDS	 Val-analog
Leucine APDS	 Leu-analog	Glycine APDS (lead compound)	x-H Gly-analog

The ligand database (Table 3) also contains 19 nucleosides, 35 sulphonamides retrieved from *CCDC* libraries, three type 2 diabetes drugs and ATP (a natural ligand of IDE).

Table 3: The other ligands in the *In-silico* database

20 APDS analogs	19 Nucleosides	35 Sulphonamides		Natural ligand
Asp-analog	ADENTP	BINNAP	DIKDAD	ATP
Cys-analog	ADENTP02	BIYSEH03	DIKDEH	3 Commercial drugs
Gln-analog	ARCMPH	BIYSEH04	DIKDIL	Azidoiodoglibenclamide (**8**)
His-analog	AZURPH	BIYSEH05	NODWIO	Glibenclamide (**3**)
Ile-analog	COXZUM	BIYSEH06	NUSHOY	Glimperide (**9**)
Leu-analog	COYPOX	BIYSEH07	SOHVOC	
Lys-analog	EMETEW	BIYSEH08	SOHXIY	
Met-analog	IWIXAO	BIYSEH09	SOHXIY01	
Ala-analog	JAGHUU	BIYSEH10	SOHXUK	
Arg-analog	KOGPUS	CEKLAH	TIGNAA	
Asn-analog	LIRQUY	CEKLEL	TIGNEE	
Orn-analog	MEMLAR	CEKLIP	TUFNUF	
Phe-analog	NAXLI	CEKLOV	TUFPAN	
Ser-analog	NUFJUV	CEKLUB	XIFRAH	
Thr-analog	NUFKAC	CEKMAI	XIFREL	
Trp-analog	RBADPM10	CEKMEM	ZOJJUD	
Tyr-analog	THPPTH	DIKCIK		
Val-analog	TPYOP10	DIKCOQ		
Gly-analog	TUBJEF	DIKCUW		
Glu-analog				

The database was docked to the allosteric site of the insulin degrading enzyme (IDE) to identify the ligands with the best binding interactions.

4.2.2 Binding Interactions of the Database to the Insulin Degrading Enzyme

The insulin degrading enzyme (IDE) is a protease that catabolizes insulin, a hormone which regulates the glucose in blood and therefore important in diabetes therapy (Yonezawa *et al*., 1986). Recognizing that ligands that interact with IDE will slow down the catabolism of insulin, the database was docked on IDE using the software *GOLD*

(Genetic Optimization for Ligand Docking), an algorithm tool for docking ligands to protein targets, to identify the best ligands.

The 3D file (3TUV) of IDE retrieved from the protein data bank (Nicholas *et al*., 2012) contains ATP, a natural ligand that allosterically modulates IDE (Ciesla *et al.*, 2011). The binding pocket (Figure 3a) of IDE readily binds (Figure 2b) to the natural ligand ATP to IDE using 7 hydrogen bonds with an overall H-bond length of 2.48Å (Table 4).

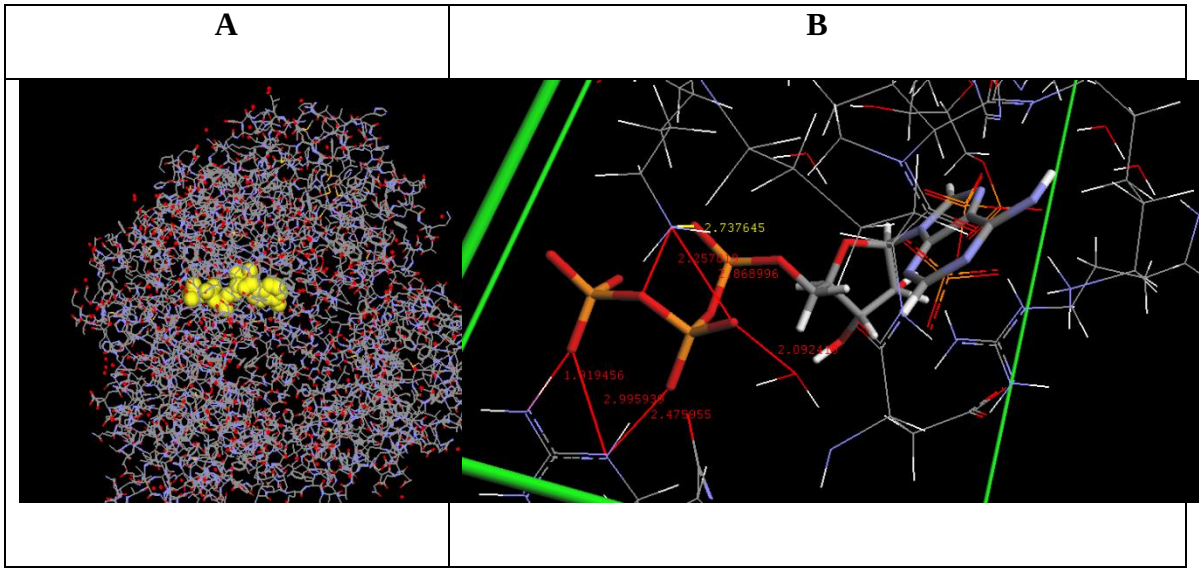


Figure 2: 3D view of IDE highlighting the binding pocket in yellow (a) Zoomed 3D binding view of ATP in IDE allosteric site

The interactions (Table 4) of ATP and the target is of an electrostatic nature with ATP situated adjacent to the alpha helix twist interacting with the amino acid residues arginine at position 893 and lysine at position 906. A water molecule also plays a role in the interaction with ATP.

Table 4: Interactions of ATP with the binding pocket.

Interactions	Site of interaction	Bond length
Between O (ATP) and N (893 ARG)	Alpha helix	1.92Å
Between O (ATP and N (893 ARG)	Alpha helix	3.0Å
Between O (ATP) and N (893 ARG)	Alpha helix	2.48Å
Between O (ATP) and N (906 LYS)	Alpha helix	2.26Å
Between O (ATP) and 1124 H_2O		2.09Å
Between O (ATP) and N (906 LYS)	Alpha helix	2.87Å
Between O (ATP) and N (906 LYS)	Alpha helix	2.74 Å

The ligands in the database were docked using *GOLD* against the prepared IDE allosteric site and the ligands scored in terms of fitness. Figure 3 illustrates a docking-interaction of the Leu-APDS analog with the binding pocket on IDE.

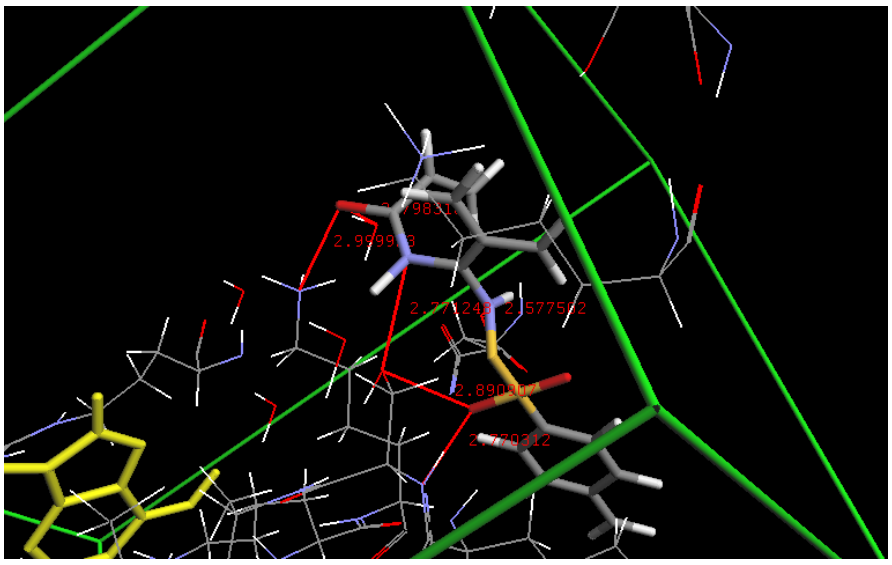


Figure 3: LEU-APDS analog docked against IDE allosteric site

GOLD was used to predict the conformation of the ligands to the binding site based on the binding energy and root mean square deviation (RMSD) of the virtual interaction. Since binding energy is the amount of energy required for a stable ligand target interaction (Ortiz *et al.*, 1995), a stable interaction with therapeutic benefits is implied

when the binding energy is low (Wang and Wade., 2002). RMSD refers to the distance between a ligand and a target and is measured in Å. For an interaction to be considered favorable, the RMSD should be less than 1.5Å for a therapeutic effect to be effective (Yusuf *et al.*, 2008).

Considering that binding energy and root mean square deviation (RMSD) are a function of the protein target (IDE) to the respective ligand, the scores were converted into their respective z-scores using Equation (3) to rank the interactions; (Ebalunode *et al*., 2012).

$$Z = \frac{x - m}{d}$$ ----------(Equation 3)

In equation (3), 'x' is the best score of a ligand per dock, 'μ' is the mean and 'δ' is the respective standard deviation of a ligand. Statistically, a z-score determines how far a score deviates from the mean (Edward and Fawcet, 2014).

The docking report of the APDS analogs and their respective z-scores is summarized in Table 5. A comprehensive report for all ligands in the database is in appendix 1.

Table 5: Summary of the docking report for APDS analogs

Ligand	Best binding energies of the ligands (KJ/mol)										z-score
ALA-APDS	46.00	45.80	45.31	45.24	46.54	40.91	45.61	45.43	45.23	45.71	-0.58
ARG-APDS	59.11	48.63	54.02	45.15	46.30	54.63	52.00	49.49	57.69	40.52	1.64
ASN-APDS	54.34	50.85	47.42	49.27	52.01	49.07	53.86	51.65			1.65
ASP-APDS	55.81	54.45	54.18								0.95
CYS-APDS	53.58	51.04	52.60								1.05
GLN-APDS	56.37	45.37	54.58	43.20	54.26	41.17	47.34	54.42	50.65		1.53
GLU-APDS	49.23	54.06	46.35	40.35	53.80	49.28	54.20				1.75
GLY-APDS	47.36	47.09	47.51	45.81							1.15
HIS-APDS	63.40	54.39	46.40	45.68	44.43	43.31					1.50
ILE-APDS	40.99	46.53	48.60	47.92	47.17	47.33	46.02	42.06	47.39		-0.12
LEU-APDS	52.31	41.14	50.35	44.29	52.55	46.06	51.36				1.41
LYS-APDS	51.43	52.26	51.03	49.45	45.01	41.16	49.94	49.33	39.04	44.46	0.64
MET-APDS	48.65	54.23	54.01	38.78	47.35	54.52					1.80
ORN-APDS	45.65	38.18	44.38	42.44	48.25	45.90	51.70	51.80	42.29	40.45	0.44
PHE-APDS	57.91	52.82	53.62	51.74	45.18	48.06	48.40	42.30	48.75	47.51	1.36
SER-APDS	56.70	58.24	57.85								0.88
THR-APDS	52.10	51.21	51.11								1.64
TRP-APDS	57.85	60.05	61.31	57.04	54.47	55.91	56.74	60.78			1.53
TYR-APDS	60.93	46.63	46.23	44.03	54.90	55.07	44.02	43.19	47.13	47.97	2
VAL-APDS	46.20	46.12	42.19	45.68	45.68	45.66	42.54	46.06	46.13	40.81	-0.57

The most promising APDS analogs ranked based on the Z-score are summarized in Table 6.

Table 6: Ranking of APDS analogs after Z-score evaluation

Rank	Ligand	Binding Energy (KJ/mol)	RMSD (Å)	z-score
1	Tyr-APDS	-18.440	1.28	2.0
2	Met-APDS	-20.748	1.46	1.8
3	Glu-APDS	-25.713	0.79	1.75
4	Asn-APDS	-16.730	3.55	1.65
5	Arg-APDS	-37.60	1.22	1.64
6	Thr-APDS	-19.256	1.21	1.64
7	Trp-APDS	-19.256	1.21	1.53
8	Gln-APDS	-18.486	1.61	1.53
9	His-APDS	-27.020	1.99	1.50
10	Leu-APDS	-64.465	1.24	1.41
11	Phe-APDS	-25.650	0.80	1.36
12	Gly-APDS	-14.439	2.30	1.15
13	Cys-APDS	-16.10	1.52	1.05
14	ATP	+3.374	1.64	1.38
15	Azidoiodoglibenclamide (**8**)	-64.450	0.91	-0.74
16	Glibenclamide (**3**)	-74.450	1.99	0.30
17	Glimperide (**9**)	-117.288	1.40	-0.10

In shortlisting the APDS analogs, the z-score of ATP of 1.38 was used as the threshold value because ATP is a natural ligand that complexes with IDE to modulate its activity thereby exhibiting antiglycemic effects. 10 APDS analogs out of the 20 modeled analogs surpassed the threshold value. Ligands with z-scores less than that of the primary model molecule, glycyl-analog, were rejected.

4.2.3 Virtual Bioactive Evaluation of the Interactions

The shortlisted ligands based on the z-score were subjected to virtual bioactive evaluation and scored against various parameters (Table 7) to identify ideal APDS analogs for further consideration.

Table 7: Virtual bioactivity scores of shortlisted ligands

APDS analog	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Tyr-analog	0.12	-0.19	-0.36	-0.33	**0.26**	-0.01
Met-analog	-0.16	-0.14	-0.73	-0.74	0.12	-0.03
Glu-analog	0.16	-0.23	-0.45	-0.39	**0.39**	0.14
Asn-analog	-0.05	-0.31	-0.41	-0.64	**0.20**	-0.06
Arg-analog	**0.39**	0.03	-0.29	-0.84	**0.72**	0.18
Thr-analog	-0.08	-0.30	-0.60	-0.62	0.11	-0.04
Trp-analog	**0.26**	-0.13	-0.17	-0.44	**0.26**	0.03
Gln-analog	0.07	-0.03	-0.32	-0.59	**0.33**	0.03
His-analog	**0.31**	-0.05	-0.15	-1.00	0.39	0.25
Leu-analog	-0.07	-0.33	-0.55	-0.67	0.12	-0.08
Phe-analog	0.09	-0.24	-0.24	-0.42	**0.26**	-0.07
Gly-analog	-0.31	-0.49	-0.77	-0.94	-0.34	-0.16

Among the APDS analogues evaluated, only the tyrosyl, asparagyl, glutamyl and phenylalanyl analogs exhibited activity at the active site as determined from the significantly positive scores for attributes evaluated. The tryptophanyl, arginyl and histidyl APDS analogs exhibited non-specific activity to the target. Although, the tyrosyl, asparagyl, glutamyl and glutaminyl APDS analogs exhibited specific activity to the target, these were excluded from further consideration for being too challenging to access synthetically. The phenylalanyl APDS analog also exhibited activity specific to the target.

The interactions (Figure 5) of the phenylalanyl-ADPS analog with the target have an average bond length of 2.81Å. These interactions are summarized in Table 8.

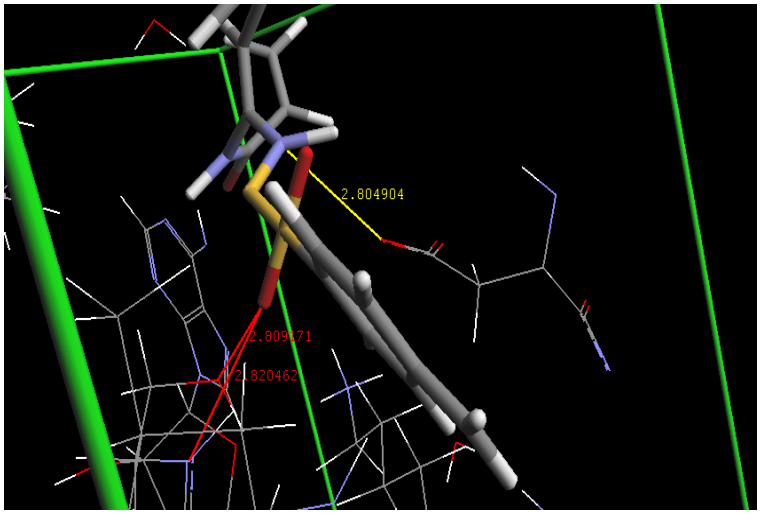


Figure 4: 3D binding view of the phenylalanyl-APDS analog

Table 8: Interactions of the phenylalanyl-APDS analog with the binding pocket

No.	Interaction	Site of interaction	Bond length
1	Between O (phe-analog) and O (901 SER)	Alpha helix	2.81Å
2	Between O (phe-analog) and N (901 SER)	Alpha helix	2.82Å
3	Between N (phe-analog) and O (426 ASP)	Coil	2.80Å

From the summary, it is apparent that the interaction of the phenylalanyl-APDS analog and the target is of a mixed nature; the phenylalanyl-APDS analog occupies a pocket with mixed interactions; 2 electrostatic interactions with a nitrogen atom of serine at position 901 and an oxygen atom of aspartic acid at position 426. It has a hydrogen bonding interaction with the hydroxyl group of serine at position 901.

Since the glycyl APDS analog was the primary model compound, it was selected by default for synthesis. Figure 5 illustrates the interaction of the glycyl-ADPS analog with

the target with an average bond length of 2.80Å. Table 9 summarizes the interactions of the glycyl-ADPS analog with the binding pocket.

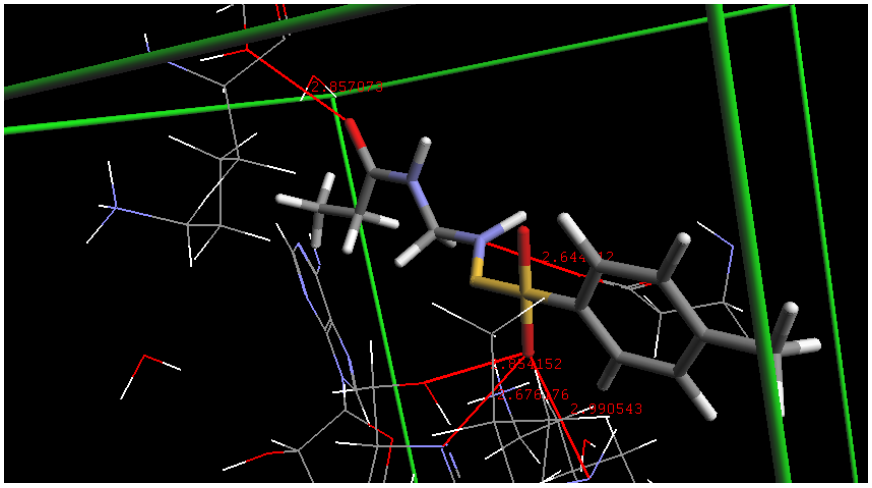


Figure 5: 3D binding view of the glycyl-APDS analog

Table 9: Interaction of the glycyl-APDS analog with the binding pocket

No.	Interaction	Site of interaction	Bond length
1	Between O (gly-analog) and N (900 LEU)	Alpha helix	2.99Å
2	Between O (gly-analog) and N (901 SER)	Alpha helix	2.68Å
3	Between O (gly-analog) and O (901 ARG)	Alpha helix	2.85Å
4	Between N (gly-analog) and O (426 ASP)	Coil	2.64Å
5	Between O (gly-analog) and O (617 SER)	Beta strand	2.86Å

Just like the phenylalanyl analog, the interactions of the glycyl-APDS analog and the target is of a mixed nature; the glycyl-APDS analog is in a pocket with 3 electrostatic interactions with leucine at position 900 and serine at position 901. 3 hydrogen bond interactions with the oxygen atom of serine at position 901, the oxygen atom of aspartic acid at position 426 and finally with oxygen atom of serine at position 617. Since mixed interactions provide a more stable protein-ligand interaction therefore, glycyl APDS analog was also considered an attractive ligand for further evaluation.

Consequently, the glycyl and the phenylalanyl APDS analogs were selected for virtual toxicity evaluation to determine their safety.

4.2.4 In Silico Toxicological Evaluation of Selected Ligands

The glycine and phenylalanine analogs were subjected to virtual toxicity evaluation in *data warrior*. The parameters that were assessed and scored are summarized in Table 10.

Table 10: Virtual toxicity of selected APDS analogs

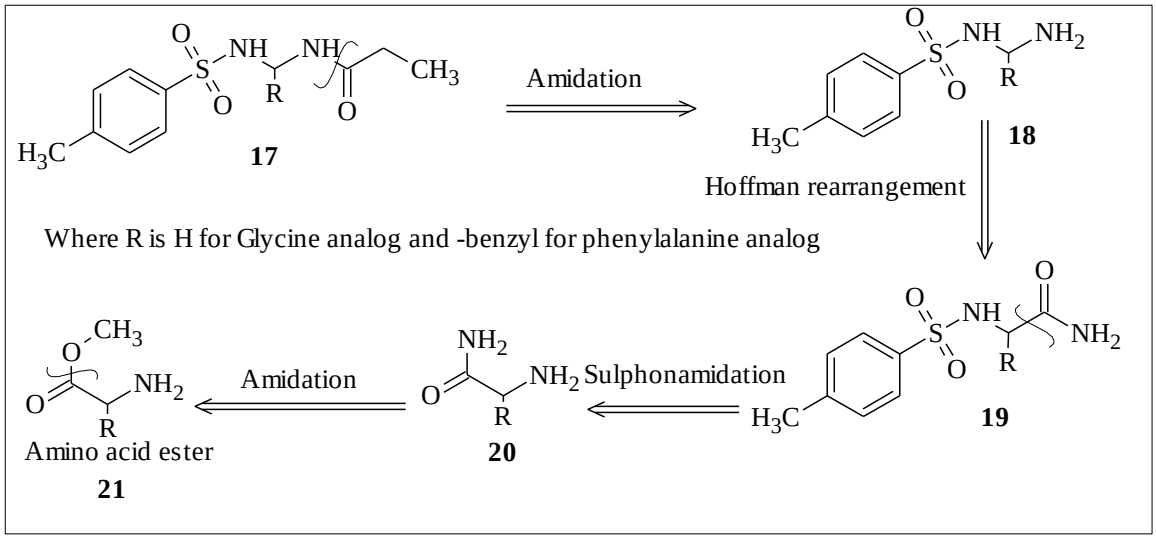
APDS analog	Druglikeness score	Mutagenic	Tumorigenic	Reproductive effect	Irritant
Phe-analog	-4.5124	None	None	None	None
Gly-analog	-2.4883	None	None	None	None

Both analogs were found to be non-toxic *in silico* and the negative druglikeness scores infer that these analogs could be of therapeutic interest and therefore worthy of synthesis.

4.3 Synthesis of the Selected APDS Analogs

Synthesis of the glycyl and phenylalanyl analogs of APDS was based on the retrosynthetic pathway in Scheme 1.

Scheme 7: Retrosynthetic pathway for the selected APDS analogs

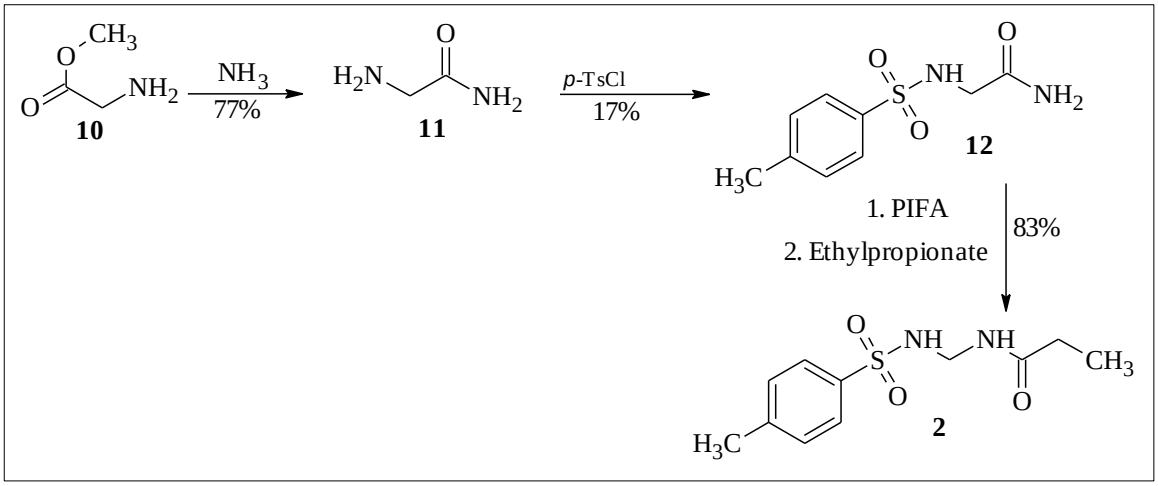


The synthesis was approached based on the recognition that amidation of an amine (**18**) derived from Hoffmann degradation of the primary amide (**19**) would incorporate a polar, electron deficient imidate terminal of the target APDS analog (**17**) that would strongly interact with the binding pocket of IDE (Aubé *et al*., 1993). The primary amide (**19**) could readily be accessed from *N*-sulphonamidation of primary amide (**20**) of an appropriate amino acid. Based on this realization, the synthesis of the glycyl and phenylalanyl APDS analogs is discussed below.

4.3.1 Synthesis of the Glycyl APDS Analog (2)

The glycyl-analog was synthesized as outlined in Scheme 2.

Scheme 8: Synthetic scheme for the glycyl-APDS analog



The starting material of the synthesis was glycine methyl ester (**10**), which was treated with ammonia solution to provide primary amide **11** in 77% yield. The confirmation of the transformation was inferred from the ^{1}H NMR (Table 11) of **10** and **11**, which both showed two germinal protons. The absence of methoxy protons resonating at δ_H 3.69 (3H, *s*) in **10** and the corresponding carbonyl in **11** affirmed the success of the amidation. This was further supported by the HMBC spectra of **11** which exhibited ^{2}J correlations of H-2 with C-2 (δ_H 49.3) and C-1 (δ_H 167.3) (Appendix 3D).

Table 11: NMR chemical shifts for **10** and **11**

Position	**10**			**11**		
	δ_H (ppm)	HMBC	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	HMBC
1			169.1		167.3	
1-OMe	3.69 (3H, *s*)		169.1			
2	3.56 (2H, *s*)	C-2, C-1	41.1	3.72 (2H, *s*)	49.3	C-2, C-1

Sulphonamidation of **11** was accomplished using *p*-tosylchloride in the presence of Et_3N to provide **12** in 17% yield. The success of the sulphonamidation reaction was confirmed from the ^{1}H NMR data (Table 12) of **12**, which exhibited addition of the tosyl group as

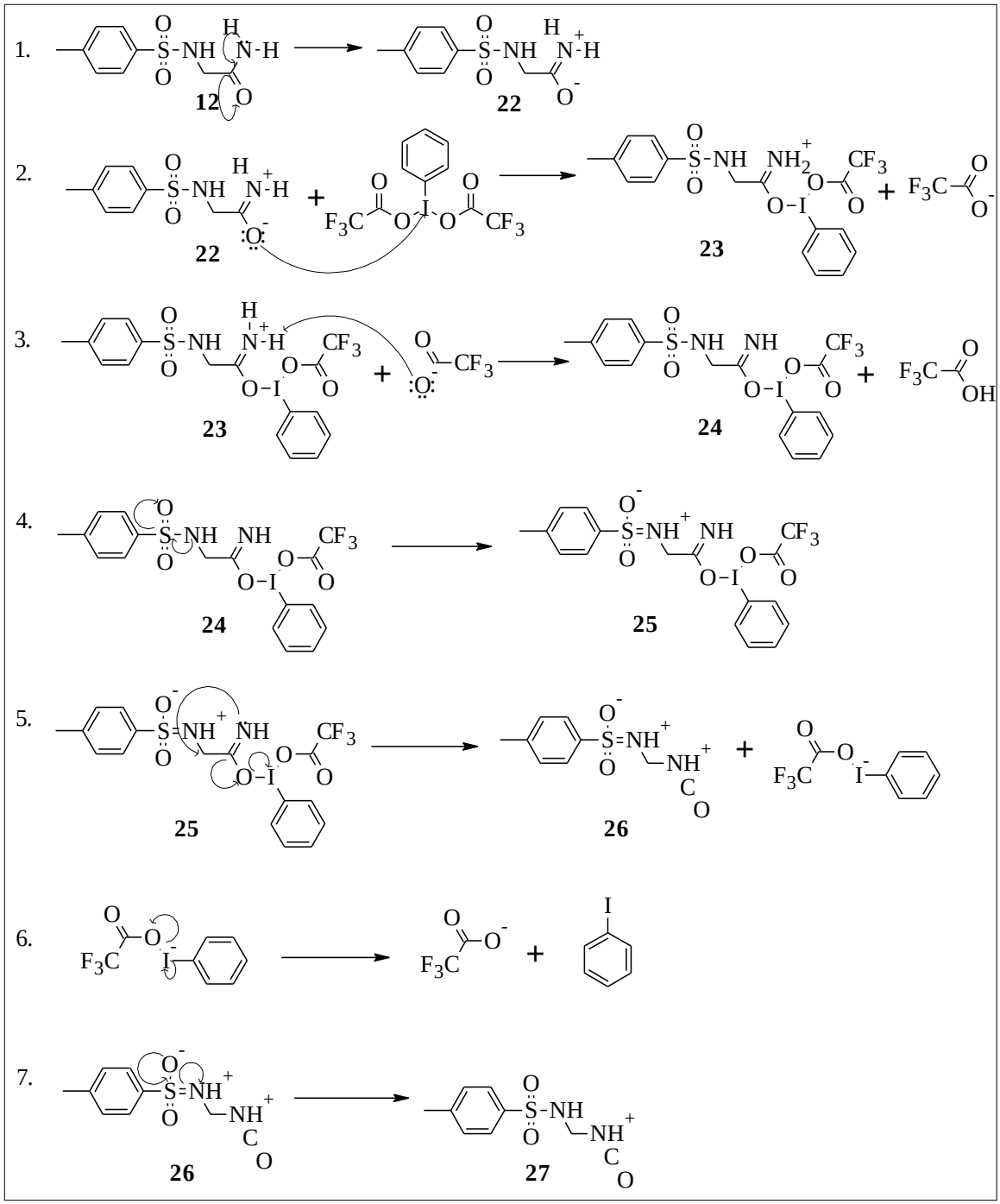
apparent from the methyl protons resonating at δ_H 2.32 (3H, *s*) and aromatic protons resonating at δ_H 7.31 (2H, *m*) and 7.70 (2H, *m*). This was further supported by the HMBC spectra which exhibited ^2J and ^3J correlations (Appendix 4D).

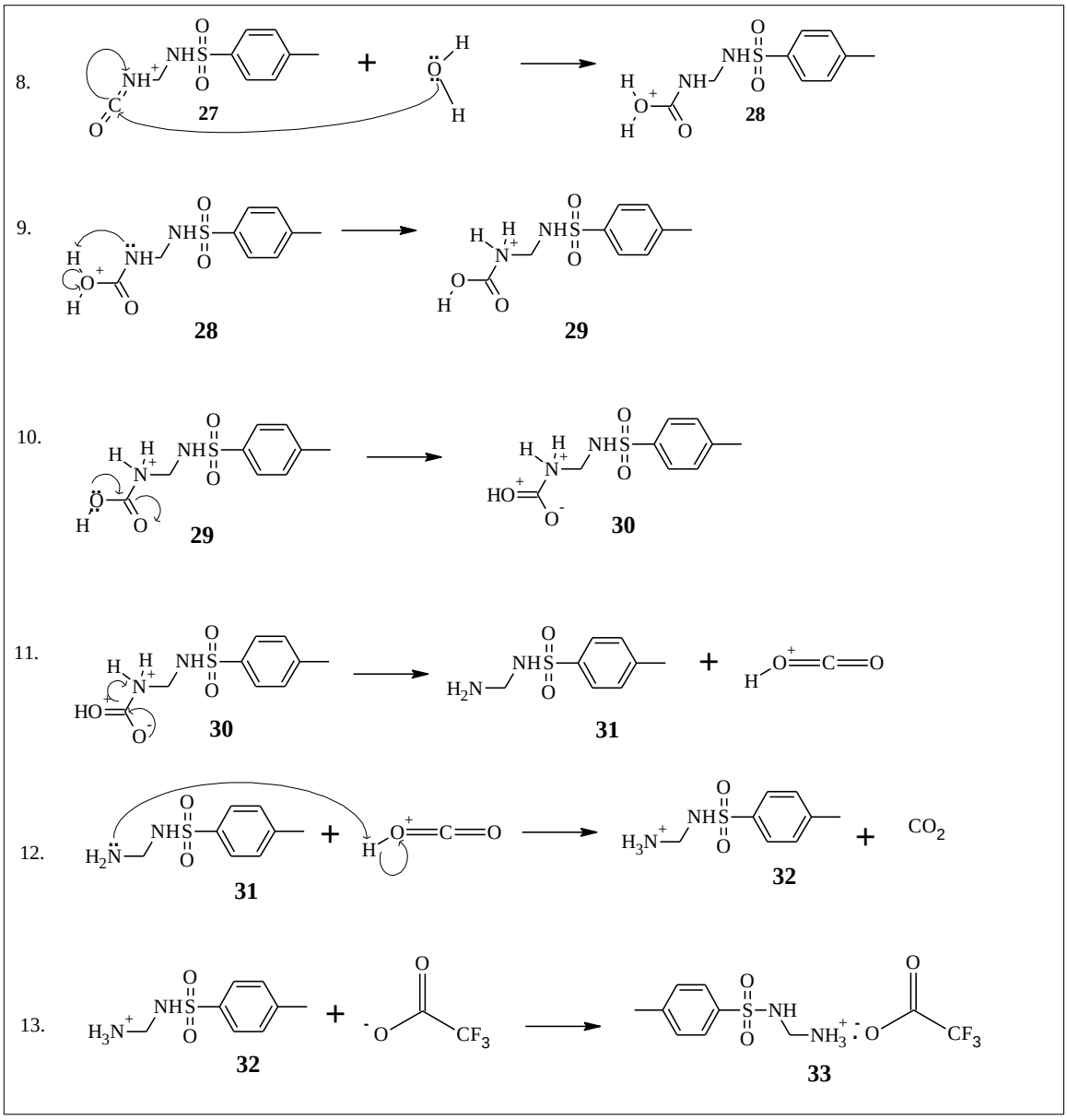
Table 12: NMR chemical shifts for **11** and **12**

Pos.	**11**			**12**		
	δ_H (ppm)	δ_C (ppm)	HMBC	δ_H (ppm)	δ_C (ppm)	HMBC
1		167.3			171.1	
1’					136.9	
2	3.72 (2H, *s*)	49.3	C-2, C-1	4.21 (2H, *s*)	49.3	C-2, C-1
2’				7.70 (2H, *m*)	127.5	C-2’, C-1’, C-3’, C-4’
3’				7.31 (2H, *m*)	129.7	C-3’, C-2’, C-1’, C-4’, CH_3-4’
4’					144.3	
4’-CH_3				2.32 (3H, *s*)	21.3	CH_3-4’, C-4’, C-3’

The Hoffmann rearrangement of **12** catalyzed with phenyliodinebis(trifluoroacetate) provided an amine salt. This reaction mechanism is illustrated in Scheme 3.

Scheme 9: Reaction mechanism of the Hoffmann rearrangement of 12





Since the amine salt (**33**) presented a challenge in isolation by chromatography, subsequent amidation was done in a one-pot reaction. This was accomplished under reflux using ethyl propionate to provide the glycine APDS analog **2** in 83%. The ^{1}H NMR data (Table 13) of **2** was characteristic of the expected product as supported by the presence of two protons resonating at δ_H 4.73 (2H, *s*) in comparison to the starting material **10** where the same protons resonated at δ_H 4.20 (2H, *s*). This could only have

been possible if the Hoffmann rearrangement reaction took place before the amidation. Moreover, two additional carbons were observed in the ^{13}C NMR; δ_C 30.2 (C-2) and 9.4 (C-3). The structure was further supported by HMBC which exhibited ^{2}J and ^{3}J correlations (Appendix 5D).

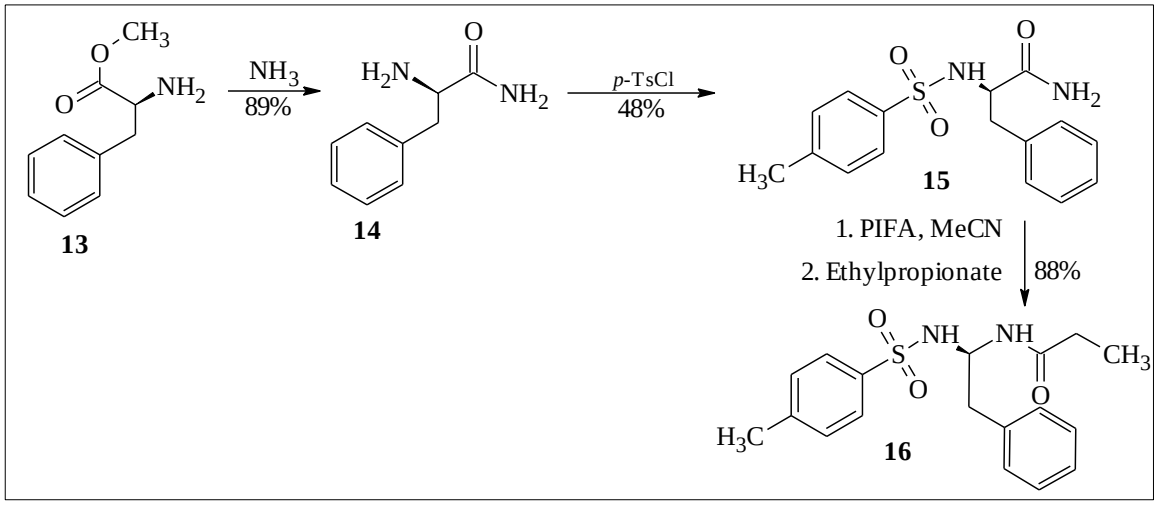
Table 13: NMR chemical shifts for **12** and **2**

Pos.	**12**			**2**		
	δ_H (ppm)	δ_C (ppm)	HMBC	δ_H (ppm)	δ_C (ppm)	HMBC
1		171.1			171.1	
1’		136.9		4.73 (2H, *s*)	66.2	C-1’, C-1
1”					135.1	
2	4.21 (2H, *s*)	49.3	C-2, C-1	2.23 (2H, *q*, *J* = 7.29Hz)	30.2	C-2, C-1, C-3
2’	7.70 (2H, *m*)	127.5	C-2’, C-1’, C-3’, C-4’			
2”				7.70 (2H, *m*)	127.5	C-2”, C-1”, C-3”, C-4”
3				1.04 (3H, *t*, *J* = 7.29Hz)	9.4	C-3, C-2, C-1
3’	7.31 (2H, *m*)	129.7	C-3’, C-2’, C-1’, C-4’, CH_3-4’			
3”				7.31 (2H, *m*)	129.7	C-3”, C-2”, C-1”, C-4”, CH_3-4”
4’		144.3				
4”					144.3	
4’-CH_3	2.32 (3H, *s*)	21.3	CH_3-4’, C-4’, C-3’			
4”-CH_3				2.32 (3H, *s*)	21.3	CH_3-4”, C-4”, C-3”

4.3.2 Synthesis of the Phenylalanyl APDS Analog (16)

The phenylalanyl-analog was synthesized as outlined in Scheme 4.

Scheme 10: Synthetic scheme for the phenylalanyl-APDS analog



The starting material for the synthesis was L-phenylalanine methyl ester (**13**), which was treated with ammonia solution to provide primary amide **14** in 89% yield. The confirmation of the transformation was inferred from the ^{1}H NMR data (Table 14) of **13** and **14**, which showed the absence of methoxy protons resonating at δ_H 3.71 (3H, *s*) in **13** and the corresponding carbonyl in **14** affirmed the success of the amidation. This was further supported by the HMBC spectra of **14** which exhibited ^{3}J and ^{2}J correlations of H-2 with C-2 (δ_H 54.1), C-2 (δ_H 177.9) and C-1' (δ_H 135.7) (Appendix 7D).

Table 14: NMR chemical shifts for **13** and **14**

Pos.	**13**			**14**		
	δ_H (ppm)	δ_C (ppm)	HMBC	δ_H (ppm)	δ_C (ppm)	HMBC
1		174.5			177.9	
1-OMe	3.71 (3H, *s*)	174.5	C-1, C-2			
1’		135.7			135.7	
2	3.65 (1H, *t*, *J* = 7.59Hz)	55.9	C-2, C-1, C-3, C-1’	3.63 (1H, *t*, *J* = 7.6Hz)	54.1	C-2, C-1, C-3, C-1’
2’	7.29 (2H, *m*)	129.1	C-2’, C-1’, C-3, C-3’, C-4’	7.36 (2H, *s*)	129.1	C-2’, C-1’, C-3, C-3’, C-4’
3	3.04 (2H, *d*, *J* = 7.59Hz)	37.4	C-3, C-2, C-1, C-1’, C-2’	3.05 (2H, *d*, *J* = 7.6Hz)	39.2	C-3, C-2, C-1, C-1’, C-2’
3’	3.56 (2H, *m*)	128.7	C-3’, C-2’, C-1’, C-4’	7.32 (2H, *s*)	128.7	C-3’, C-2’, C-1’, C-4’
4’	3.56 (1H, *m*)	128.9	C-4’, C-3’, C-2’	7.21 (1H, *s*)	128.9	C-4’, C-3’, C-2’

Sulphonamidation of **14** was accomplished using *p*-tosylchloride in the presence of Et_3N to provide **15** in 48% yield. The success of the sulfonamidation was confirmed from the ^{1}H NMR data (Table 15) of **15**, which exhibited addition of the tosyl group as apparent from the methyl protons resonating at δ_H 2.32 (3H, *s*) and aromatic protons resonating at δ_H 7.31 (2H, *m*) and 7.70 (2H, *m*). This was further supported by the HMBC spectra which exhibited ^2J and ^3J correlations (Appendix 8D).

Table 15: NMR chemical shifts for **14** and **15**

Pos.	**14**			**15**		
	δ_H (ppm)	δ_C (ppm)	HMBC	δ_H (ppm)	δ_C (ppm)	HMBC
1		177.9			174.8	
1’		135.7			135.7	
1’’					137.9	
2	3.63 (1H, *t*, *J* =7.60Hz)	54.1	C-2, C-3, C-1’, C-1	5.09 (1H, *t*, *J* =7.60Hz)	56.8	C-2, C-3, C-1’, C-1
2’	7.36 (2H, *m*)	129.1	C-2’, C-1’, C-3, C-3’, C-4’	7.25 (2H, *m*)	129.1	C-2’, C-1’, C-3, C-3’, C-4’
2’’				7.70 (2H, *m*)	127.5	C-2’’, C-1’’, C-3’’, C-4’’
3	3.05 (2H, *d*, *J* =7.60Hz)	39.2	C-3, C-2, C-1, C-1’, C-2’	2.92 (2H, *d*, *J* =7.60Hz)	37.2	C-3, C-2, C-1, C-1’, C-2’
3’	7.32 (2H, *m*)	128.7	C-3’, C-4’, C-2’, C-1’	7.28 (2H, *m*)	128.7	C-3’, C-4’, C-2’, C-1’
3’’				7.31 (2H, *m*)	129.7	C-3’’, C-4’’, CH_3-4’’, C-2’’, C-1’’
4’	7.21 (1H, *m*)	128.9	C-4’, C-3’, C-2’	7.20 (2H, *m*)	128.9	C-4’, C-3’, C-2’
4’’					144.3	
4’’-CH_3				2.32 (3H, *s*)	21.3	CH_3-4’’, C-4, C-3

Since the amine salt presented a challenge in isolation by chromatography, subsequent amidation was done in a one-pot reaction. This was accomplished under reflux using ethyl propionate to provide the phenylalanyl APDS analog **16** in 88%. The ^{1}H NMR data (Table 16) of **16** was characteristic of the expected product as supported by the presence of two protons resonating at δ_H 2.18 (2H, *q*, *J* =7.30 Hz) and 1.04 (3H, *t*, *J* =7.30 Hz) in comparison to the starting material **15** where the same protons resonated at δ_H 5.09 (1H, *t*,

J = 7.60 Hz) and 2.92 (2H, *d*, *J* = 7.60 Hz). This could only have been possible if the Hoffmann rearrangement reaction took place before the amidation. Moreover, two additional carbons were observed in the ^{13}C NMR; δ_C 30.2 (C-2) and 9.4 (C-3). The structure was further supported by HMBC which exhibited ^{2}J and ^{3}J correlations (Appendix 9D).

Table 16: NMR chemical shifts for **15** and **16**

Pos.	**15**			**16**		
	δ_H (ppm)	δ_C (ppm)	HMBC	δ_H (ppm)	δ_C (ppm)	HMBC
1	174.8				170.6	
1’		135.7		5.19 (1H, *t*, *J* =6.63Hz)	79.2	C-1’, C-1, C-2’, C-1”
1”		137.9			136.5	
1”’					135.1	
2	5.09 (1H, *t*, *J* =7.60Hz)	56.8	C-2, C-3, C-1’, C-1	2.18 (2H, *q*, *J* =7.30Hz)	30.2	C-2, C-1, C-3
2’	7.25 (2H, *m*)	129.1	C-2’, C-1’, C-3, C-3’, C-4’	3.10 (2H, *d*, *J* =6.63Hz)	30.5	C-2’, C-1’, C-1”, C-2”
2”	7.70 (2H, *m*)	127.5	C-2’’, C-1’’, C-3’’, C-4’’	7.31 (2H, *m*)	128.6	C-2”, C-1”, C-2’, C-3”, C-4”
2”’				7.70 (2H, *m*)	127.5	C-2”’, C-1”’, C-3”’, C-4”’
3	2.92 (2H, *d*, *J* =7.60Hz)	37.2	C-3, C-2, C-1, C-1’, C-2’	1.04 (3H, *t*, *J* =7.30Hz)	9.4	C-3, C-2, C-1
3’	7.28 (2H, *m*)	128.7	C-3’, C-4’, C-2’, C-1’			
3”	7.31 (2H, *m*)	129.7	C-3’’, C-4’’, CH_3-4’’, C-2’’, C-1’’	7.25 (2H, *m*)	128.7	C-3”,C-2”, C-1”, C-4”
3”’				7.30 (2H, *m*)	129.7	C-3”’, C-4”’, CH_3-4”’, C-2”’, C-1”’
4’	7.20 (2H, *m*)	128.9	C-4’, C-3’, C-2’			
4”			144.3	7.22 (1H, *m*)	128.9	C-4”, C-3”, C-2”
4”-CH_3	2.32 (3H, *s*)	21.3	CH_3-4”, C-4”, C-3”			
4”’					144.3	
4”’-CH_3				2.32 (3H, *s*)	21.3	CH_3-4”’, C-4”’, C-3”’

With the synthesis of the glycine and phenylalanine APDS complete, they were then subjected to bioassay to access their antiglycemic potential.

4.4 Antiglycemic Activity

The synthesized APDS analogues were evaluated for antiglycemic activity by assessing the blood glucose suppression *In vivo* by oral glucose tolerance test (OGTT). Fasted rats (test groups) were treated with different doses of the synthesized APDS analogs and the results compared to two control groups; metformin group (positive control) and saline group (negative control). The results of these screens are discussed below.

4.4.1 Blood Glucose Suppression

Blood glucose levels of the test compounds (glycyl APDS analog and phenylalanyl APDS analog herein referred to as G and F respectively) and controls at different dosages are presented in Table 17.

Table 17: Blood glucose levels per group as a function of time

Group	Time (minutes)						
	-60	0	30	60	90	120	180
	Blood glucose levels (mg/dL) (mean ± SD)						
Metformin (50mg/k)	136.3±2.5	124.8±22.7	148±12.8	138±15.4	131±4.9	122.5±11.4	124.8±4.1
Saline (45mg/k)	102.8±8.2	115.3±7.2	189±53.8	181±21.5	187±18.4	184±14.3	145.3±27.9
$G_{(500mg/k)}$	118±2.9	103.3±7.6	153±27.1	122±9.4	136±20.9	145.5±18.4	136.5±6.0
$F_{(500mg/k)}$	118.8±11.2	110.3±6.3	144±16.5	144±26.0	147±14.4	153.8±10.1	151.8±10.1
$G_{(1000mg/k)}$	121.8±6.9	117.8±25.4	159±39.5	134±1.4	126±8.5	140.5±6.4	137.5±7.8
$F_{(1000mg/k)}$	105.5±9.1	102.3±8.7	174±64.0	121±13.8	130±9.2	143±27.6	112.7±16.4
Results from oneway ANOVA							
F-value		1.32	0.71	5.69	8.86	6.2	3.07
P-value		0.3018	0.6223	**0.0039**	**0.0004**	**0.0026**	**0.0418**

Table 17 displays averages of the screen results with respective deviations from all the 6 groups of mice. Metformin was the positive control group and saline the negative control group. Time -60 minutes (one hour before oral administration of glucose) reflect fasting blood glucose levels prior to respective group treatments, the treatments were allowed to distribute in the body tissues of the mice for an hour. Time 0 minutes is represents blood glucose readings one hour after administration of group treatments before oral administration of glucose by gavage. Time 30 minutes reflect blood glucose levels after administration of glucose, reason for significant increase in blood glucose levels across all the groups. At time 60 minutes, glucose levels in each group varied according to treatment, the antiglycemic effect of these treatments was observed at this time point. The effect of the test compounds was observed at an interval of 30 minutes up to a total of 180 minutes from time 0. Blood glucose readings started to drop as the treatments exhibited antiglycemic activity and rose as the treatments ceased to be effective. Sampling was carried out at intervals of 30 minutes.

Results from oneway ANOVA illustrate the point in time where significant difference in blood glucose levels between the groups was observed. There is no significant difference in glucose levels at baseline time (at time 0) to 30 minutes after drug administration, while there was a significant difference ($P \leq 0.05$) between group means between 60-180 minutes with the most significant difference being observed between 60-90 minutes.

Data from the oneway ANOVA (McHugh, 2011) was further subjected to Post-Hoc Tukey's multi-comparison test analysis to determine how statistically significant the

group means were different and between which groups the difference was observed (pairwise comparison) (Marusteri and Bacarea, 2010).

Significant difference was determined by comparing a pair of group means shown in Table 18.

Table 18: Summary of pairwise comparison of the test groups

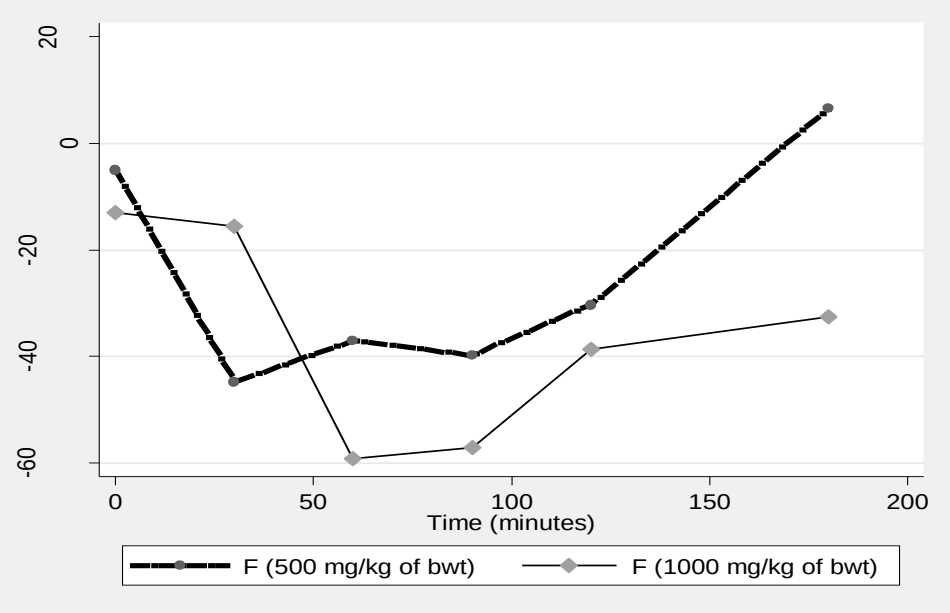
Time	Test groups		
	Mean reduction in glucose(upper and lower confidence intervals)		
	Metformin-500mg	G-500mg	F-500mg
0 min	+9.5 (-24.69, 43.69)	-12.0 (-46.19, 22.19)	-5.0 (-39.19, 29.19)
30 min	-40.8 (-131.87, 50.37)	-35.8 (-126.87, 55.37)	-44.8 (-135.87, 46.31)
60 min	-42.3 (-83.17, -1.32)*	-58.8 (-99.67, -17.82)**	-37.0 (-77.93, 3.93)
90 min	-56.3 (-90.05, -22.44)**	-50.3 (-84.55, -16.94)**	-39.8 (-73.56, -5.94)
120 min	-61.5 (-98.48, -24.52)**	-38.5 (-75.47, -1.52)*	-30.3 (-67.23, 6.73)
180 min	-20.5 (-55.06, 14.06)	-8.8 (-43.31, 25.81)	+6.5(-28.06, 41.06)

In Table 18, the interval in the glucose levels is coded with ()*, such that a P value lying in the range (0.001-0.01) was coded with ()** to infer 99.99% confidence and a P value lying in the range (0.01-0.05) was coded with ()* to infer 95% confidence.

After 30 minutes, both APDS analogs exhibited antiglycemic activity though the difference was not statistically significant in comparison with saline. After 60 minutes, there was clear reduction of blood glucose levels for the test compounds with a P value that indicates that difference was statistically significant.

After 90 minutes, the difference in the groups between the test compounds and saline (negative control) was not statistically significant. After 120 minutes, the effects of the test compounds had faded and wore out after 180 minutes.

Blood glucose suppression observed in the four groups of mice treated with the two APDS analogs at two dosages are presented in graphs 1 and 2.

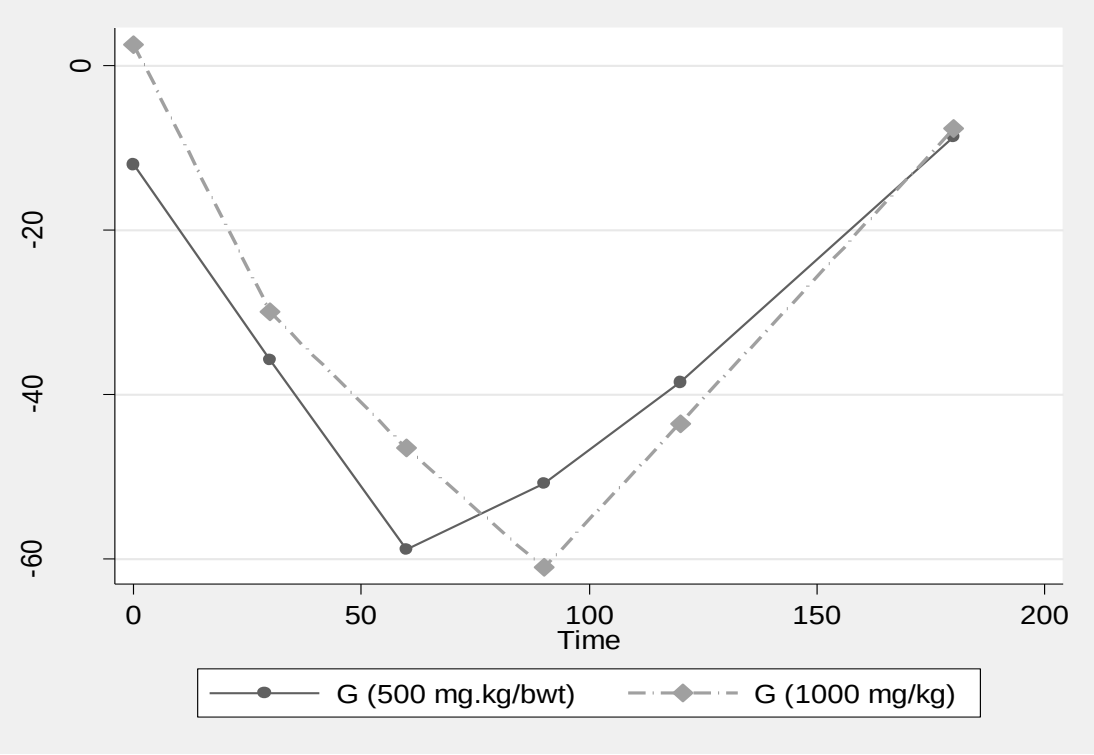


(confidence intervals were omitted to improve clarity)

Graph 1: Evaluation of dose dependent relationship between F-500 and F-1000 mg/kg bwt

Graph 1 was extrapolated by converting the phenylalanyl analogue data in Table 17 using Equation 2 (Appendix 10). These calculations help to determine whether F was effective reducing blood glucose and this reduction was calculated and expressed as a %.

Graph1 illustrates reduction in blood glucose where maximum effect for the 1000 mg/Kg occurred between 60 to 90 minutes while that of the 500 mg/Kg dose occurred between 0 to 30 minutes. At both doses, the higher dose caused a greater reduction of 59% while the lower dose reduced blood glucose levels by 45%.

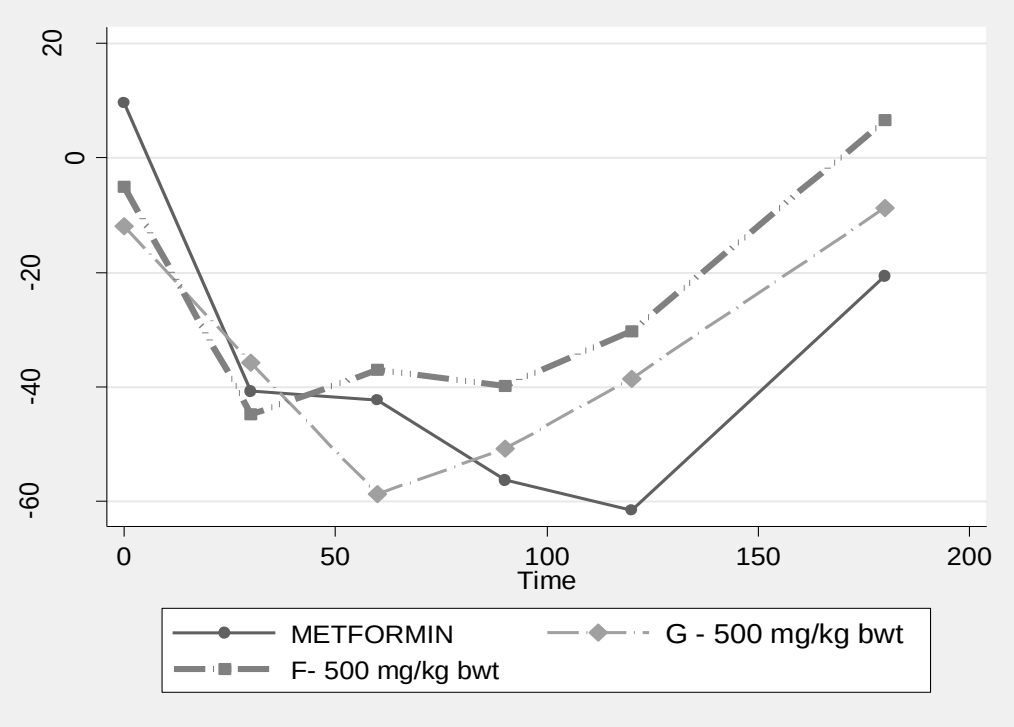


Graph 2: Evaluation of dose dependent relationship between G-500 and G-1000 mg/kg bwt

Graph 2 was extrapolated by converting the glycyl APDS analogue data in Table 17 using Equation 2 (Appendix 10). These calculations help to determine whether G was effective reducing blood glucose and this reduction was calculated and expressed as a %.

The 1000 mg/Kg dose of the G-analog suppressed blood glucose levels by 61% after 90 minutes and the 500mg/Kg dose suppressed the same by 59% after one hour. However, it was noted that half the mice treated with the higher dose died 30 minutes after sample administration. The animals had severe convulsions which indicated severe hypoglycemic reaction. As a result of the deaths recorded, it was difficult to compare the effects of two doses of the glycyl analog. Consequently, subsequent discussions are based on the results of the 500 $mgKg^{-1}$ dosage.

A comparison of blood glucose reduction by metformin, F-analog and G-analog at 500 $mgKg^{-1}$ (Graph 3) showed that it was metformin that exerted its maximal effects at 120 minutes.



Graph 3: Comparison of the potency of metformin, G and F at a dose of 500 mg/kg bwt

Graph 3 was extrapolated from the data on all APDS and metformin at 500 mg/Kg in Table 17 using Equation 2 (Appendix 10). Considering that the control (metformin), a standard type diabetes drug is obtained over the counter at 500mg/Kg it was fair to compare it with the analogs at a similar dose. These calculations help to determine whether G was effective reducing blood glucose and this reduction was calculated as a %.

The maximal hypoglycemic effects of G (glycly analog) occurred at about 60 minutes where blood glucose was reduced by 59%. Metformin reduced blood glucose by 62%,

this is comparable to that of G. However, metformin suppressed blood glucose for two hours. The F (phenylalanyl analog) was least efficacious with maximal effect at 30 minutes where blood glucose levels reduced by 45%.

The blood glucose data for the F and G analogs were analyzed by One-way analysis of variance (oneway ANOVA) to determine if there is a significant difference with time. It involved comparing (Table 18) P-values from oneway ANOVA the level of significance (α=0.05) such that P values greater than 0.05 would indicate no statistically significant difference between group means at the given time point, while P values less than 0.05 inferred that the difference between group means at the given time point they were statistically significant.

From the foregoing discussion, it is apparent that the glycyl APDS analog (**2**) exhibited better antiglycemic effects compared to the phenylalanyl analog (**16**) but was weaker than that of the standard drug (metformin) in the long run.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

The main objective of this study was to develop APDS analogs and evaluate their antiglycemic activity. Summarized below are the conclusions and recommendations of the study.

5.1 Conclusions

From the *in silico* studies of 20 APDS analogs modelled around the side chains of amino acids, two analogs (the glycyl APDS analog (**2**) and the phenylalanyl APDS analog (**16**) were selected for synthesis after being identified *in silico* as the most promising APDS analogs to exhibit antiglycemic effects against the insulin degrading enzyme (IDE).

The glycyl APDS analog (**2**) and the phenylalanyl APDS analog (**16**) were successfully synthesized and characterized using various spectroscopic techniques such as nuclear magnetic resonance, infrared spectroscopy and mass spectrometry.

From the glucose tolerance evaluation of mice administered with the two APDS analogs in mice, the glycyl APDS analog (**2**) exhibited better antiglycemic effects compared to the phenylalanyl analog (**16**) where **2** reduced blood glucose by 59% after one hour and **16** was suppressed blood glucose by 45% after 30 minutes. However, the effects were short-lived compared to the standard drug (metformin) which suppressed glucose by 62% after two hours.

5.2 Recommendations

Based on the outcome of the study, I recommend the following:

1. Since the glycyl APDS analog (**2**) was observed in the computational modelling studies to interact with all three protein strands (alpha chain, coil and beta strand) in the binding pocket, but had a shorter antiglycemic lifetime compared to metformin, further optimization of **2** by SBDD should be carried out to identify an analog of prolonged antiglycemic activity *in vivo*.
2. Although the study identified **2** as the more potent APDS analog that exhibited antiglycemic effects *in vivo*, the effective dose (ED_{50}) and lethal dose (LD_{50}) were not established in this study. Therefore, a dose-dependent response study on **2** needs to be undertaken to determine its efficacy and toxicity.

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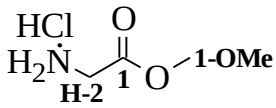
APPENDICES

Appendix 1: Summary of the docking report for APDS analogs

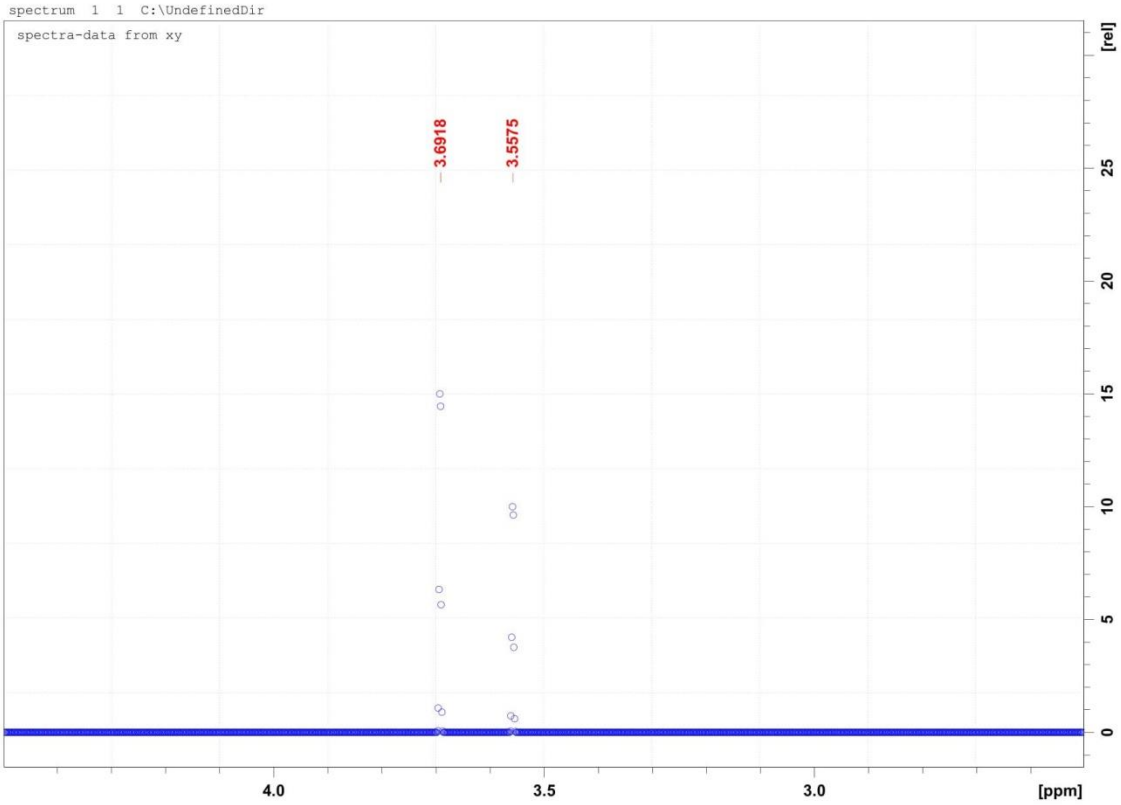
Ligand	Best binding energies of the ligands										z-score
ADENTP	0	0	0	0	0	0	0	0	0	0	0
ADENTP02	0	0	0	0	0	0	0	0	0	0	0
EMETEW	0	0	0	0	0	0	0	0	0	0	0
ARCMPH	0	0	0	0	0	0	0	0	0	0	0
AZURPH	0	0	0	0	0	0	0	0	0	0	0
COXZUM	36.96	45.45	47.38	49.73	45.10	43.84	46.14	48.25	53.19	49.59	1.46
COYBAU	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
COYPOX	64.28	63.50	62.75	68.26	65.66	67.55	65.16	66.14	62.56	67.35	1.04
IWIXAO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
JAGHUU	60.25	29.83	58.58	59.88	66.17	52.85	59.92	60.74	51.85	51.32	1.34
KOGPUS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
LIRQUY	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
MEMLAR	73.71	49.36	50.51	53.21	62.70	40.88	60.61	43.61	69.93	58.92	1.58
MOLVIT	0	0	0	0	0	0	0	0	0	0	0
NAXLII	0	0	0	0	0	0	0	0	0	0	0
NAXLUU	0	0	0	0	0	0	0	0	0	0	0
NUFJUV	0	0	0	0	0	0	0	0	0	0	0
NUFKAC	0	0	0	0	0	0	0	0	0	0	0
RBADPM10	0	0	0	0	0	0	0	0	0	0	0
THPPTH	0	0	0	0	0	0	0	0	0	0	0
TPYPOP10	65.22	63.58	63.60	58.02	64.63	64.70	60.31	64.44	64.25	65.15	1.01
TUBJEF	53.90	50.00	44.75	55.13	67.83	55.65	40.97	50.31	52.52	43.42	1.09
BINNAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
BIYSEH	39.60	55.57	39.74	44.79	55.99	54.52	46.34				1.34
BIYSEH03	47.39	46.99	45.19	44.81	45.95	45.58	48.37				1.11
BIYSEH04	48.68	46.55	45.36	47.00	50.30	44.24	46.52	45.91	45.78	49.03	1.23
BIYSEH05	47.45	48.17	47.78								0.84
BIYSEH06	45.70	45.55	46.60	47.58	47.16						0.98
BIYSEH07	45.96	49.34	45.04	43.45	39.27	48.25	42.10	48.54	49.08		0.77
BIYSEH08	52.68	46.79	50.64	43.32	47.33	48.82	48.14	48.57	49.53	49.08	1.26
BIYSEH09	45.92	52.08	47.42	48.54	48.05	46.30	48.12	44.21	44.58	47.36	1.35
BIYSEH10	46.53	47.11	47.26								0.84
CEKLAH	62.47	60.65	63.79	52.78	50.30	51.75	48.20	46.60	61.56	62.57	1.26

CEKLEL	47.78	51.23	44.34	45.80	45.57	46.87	47.40	49.04	46.57	45.59	1.12
CEKLIP	58.20	50.94	64.62	56.55	61.15	49.98	46.19	42.91	37.85	60.84	1.45
CEKLOV	43.72	49.09	46.40	38.01	51.06	50.73	44.41	49.46	49.56	40.69	1.29
CEKLUB	50.18	45.39	48.24	50.50	44.61	47.06	49.68	42.02	47.78	48.78	1.19
CEKMAI	40.05	57.68	46.35	52.83	51.03	43.64	52.01	56.10	46.46	53.19	1.39
CEKMEM	38.99	45.00	51.04	40.93	42.83	41.26	39.89	54.03	49.05	49.43	1.67
DIKCIK	43.92	45.20	50.93	45.41	44.24	47.29	51.10	45.04	43.68	44.66	1.39
DIKCOQ	54.98	53.51	60.44	52.16	50.61	56.84	52.55	53.21	57.53	44.33	1.29
DIKCUW	59.31	60.27	57.98	49.52	44.79	51.01	58.66	58.17	61.73	15.52	1.19
DIKDAD	45.12	51.89	46.37	46.13	44.79	46.14	45.94	48.65	45.19	45.77	1.42
DIKDEH	44.55	49.84	46.48	56.72	35.76	42.40	54.03	46.00	47.46	42.42	1.66
DIKDIL	61.46	64.70	73.19	59.06	59.32	66.37	56.43	72.49	47.13	59.57	1.32
NODWIO	70.50	63.97	66.04	47.81	73.10	62.70	48.95	57.86	59.85	59.30	1.35
NUSHOY	52.27	52.46	47.67	44.45	55.67	52.37	54.03	44.29	45.45	47.96	1.21
SOHVOC	53.73	48.24	54.39	53.77	52.80	52.97	43.91	44.10	52.99	42.47	1.2
SOHXIY	48.48	44.60	45.68	44.67	52.36	43.19	41.38	56.62	38.17	44.96	1.78
SOHXIY01	48.66	43.52	37.92	46.24	48.08	38.41	47.86	39.59	41.26	50.15	1.47
SOHXUK	44.11	45.26	48.04	44.00	40.02	46.18	46.05	47.92	57.53	46.65	1.83
Azidoiodoglibenc (**8**)	48.73	52.30	58.46	46.32	49.92	41.73	51.32	54.42	36.54	47.56	1.54
Glibenclamide (**3**)	54.62	50.25	56.04	50.92	50.39	53.11	66.09	52.85	55.18	47.14	1.58
Glipizide (**9**)	59.03	63.56	54.29	53.02	40.86	52.28	51.46	48.93	44.15	51.77	1.55
TIGNAA	39.45	42.19	42.29	42.06							1.7
TIGNEE	49.06	41.66	41.38	41.76	42.15	41.54	44.83	47.97	44.64	51.36	1.6
TUFNUF	44.01	45.06	37.73	38.87	61.78	37.36	43.51	46.48	43.23	61.28	1.91
TUFPAN	64.34	63.03	68.37	53.85	57.65	59.55	57.66	62.24	61.99	49.57	1.06
XIFRAH	54.22	52.56	54.41	54.06	58.89	56.91	61.99	35.73	65.66	63.05	1.33
XIFREL	62.42	67.79	61.94	66.71	55.95	61.67	72.71	63.32	68.16		1.2
ZOJJUD	48.75	64.03	61.78	45.32	58.11	54.83	47.58	52.85	58.94	51.86	1.39
ATP	67.80	74.61	64.25	79.85	67.30	70.65	82.37	72.81	73.15	68.21	1.2

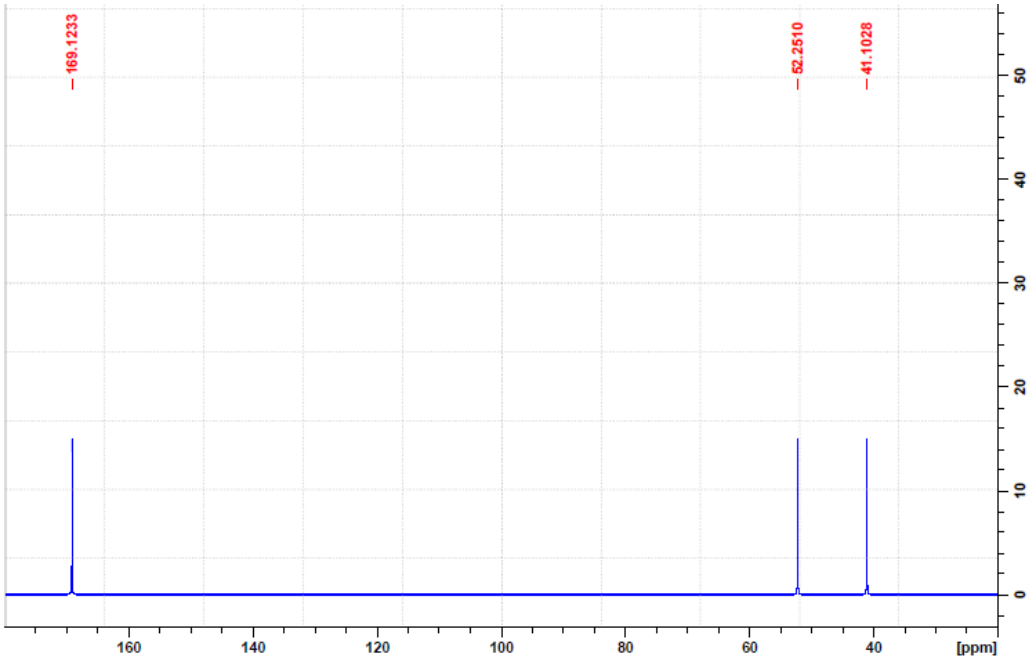
Appendix 2: Spectral data for Glycine methylesterHCl **(10)**



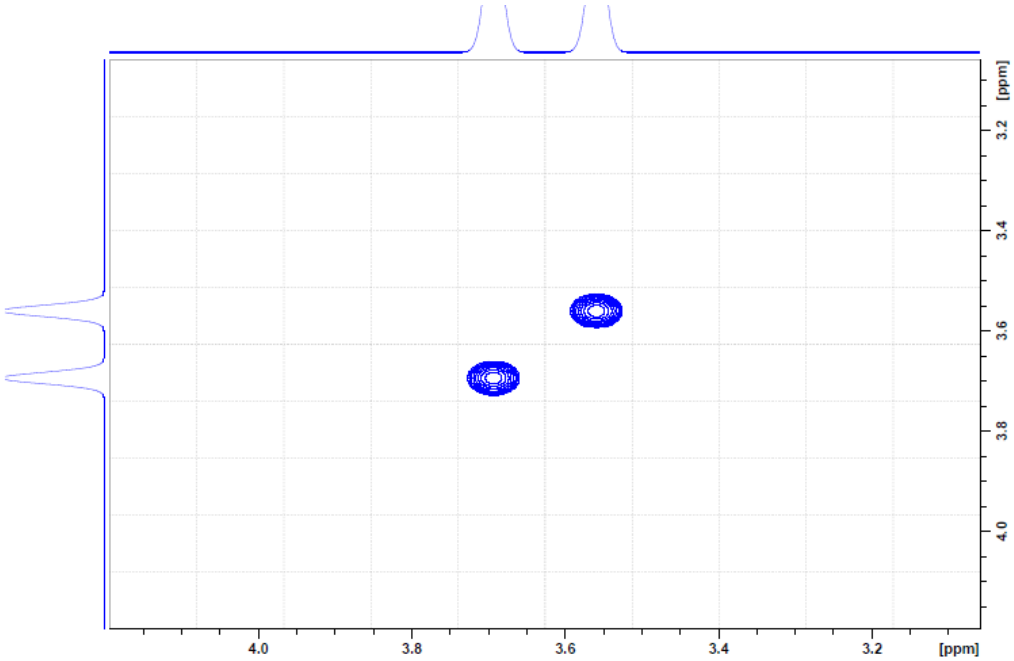
Appendix 2A: ^{1}H-NMR of 10



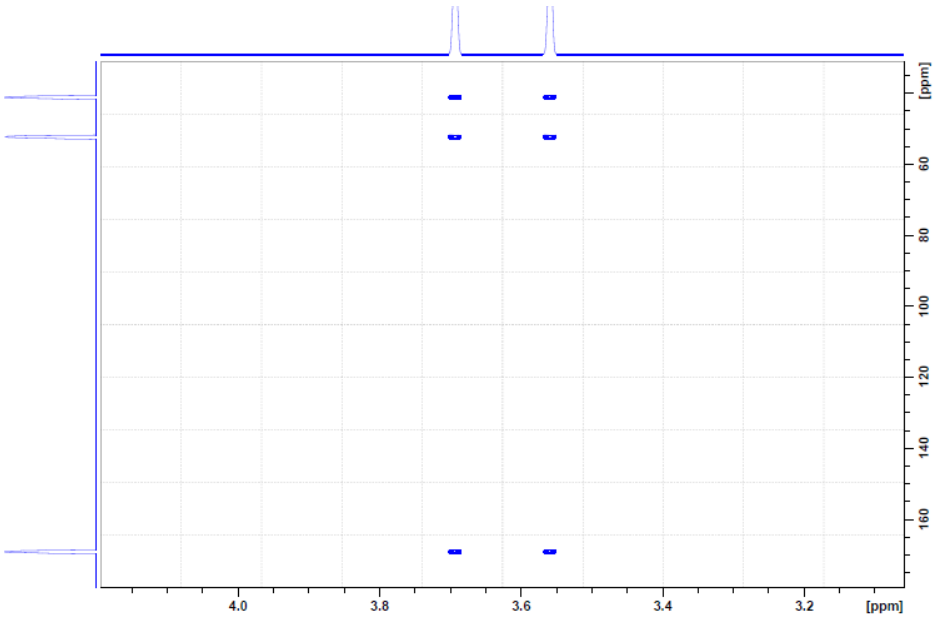
Appendix 2B: ^{13}C-NMR of 10



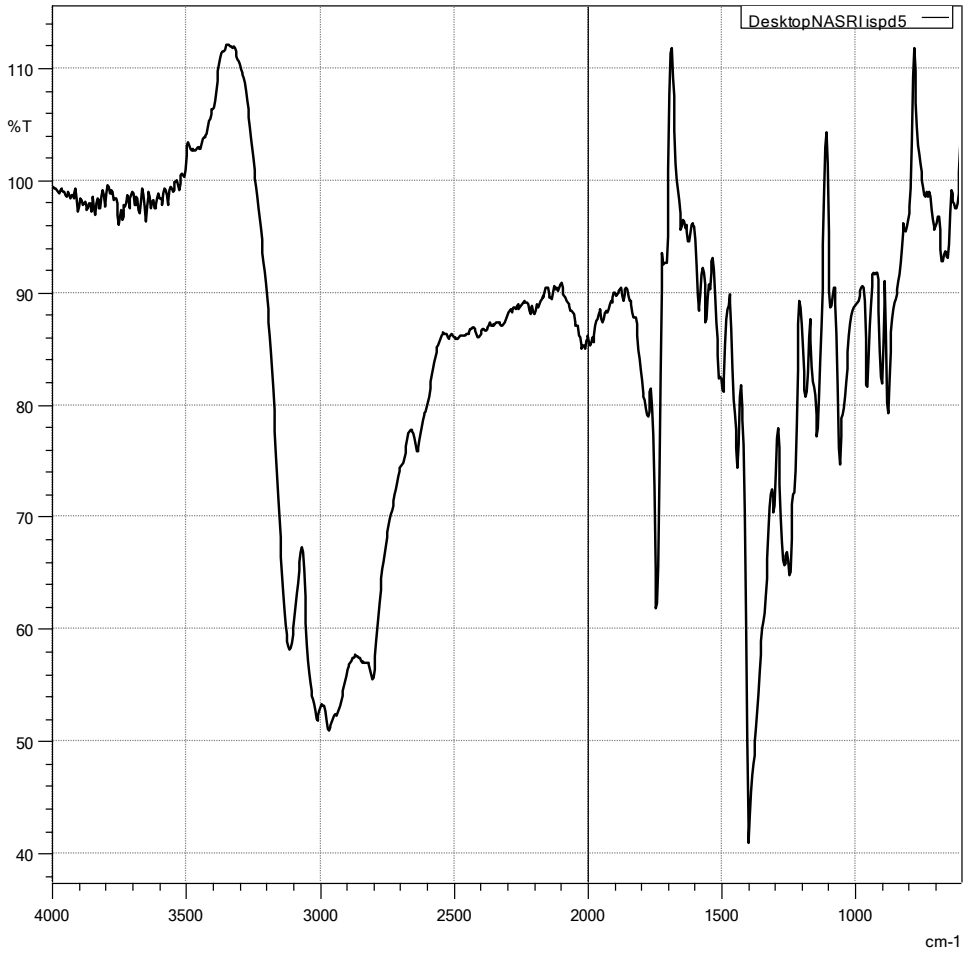
Appendix 2C: COSY of 10



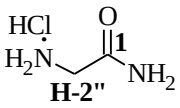
Appendix 2D: HMBC of 10



Appendix 2E: IR of 10

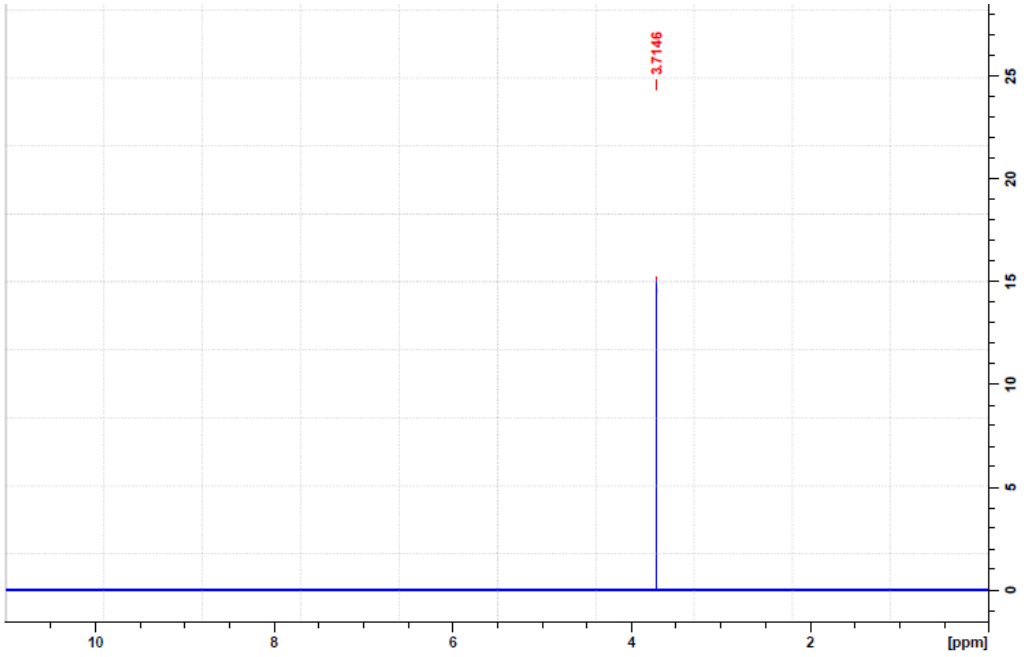


Appendix 3: Spectral data for 2-aminoacetamide HCl **(11)**

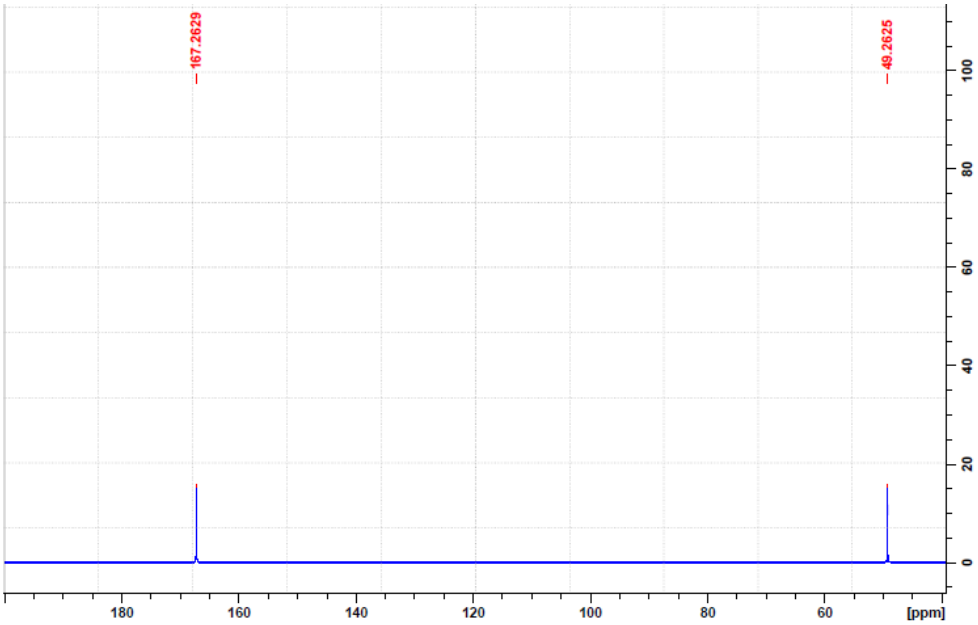


H-2"

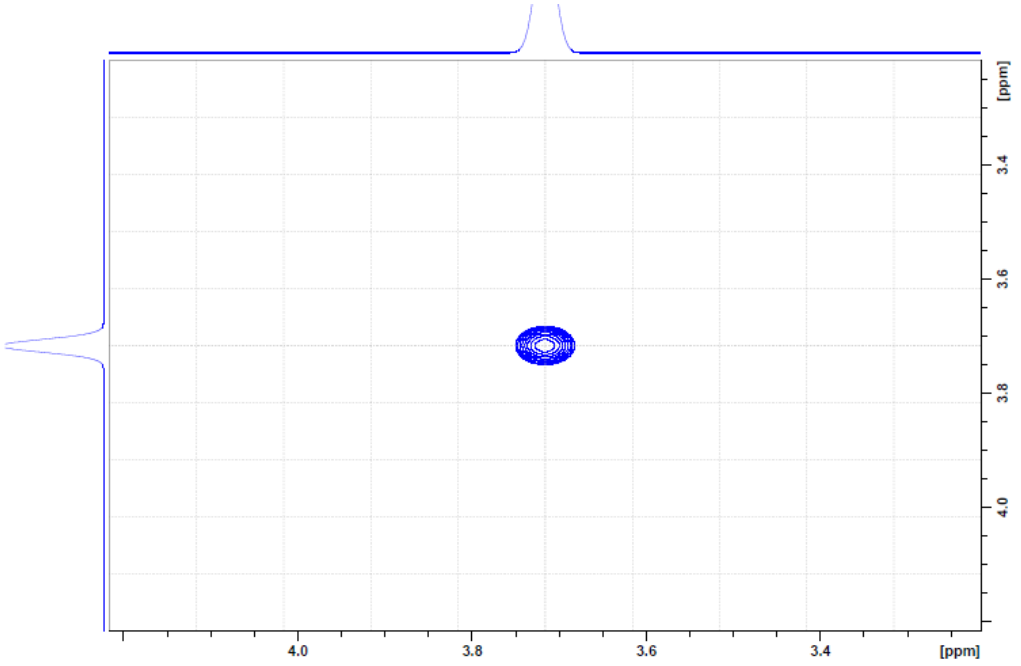
Appendix 3A: ^{1}H-NMR of 11



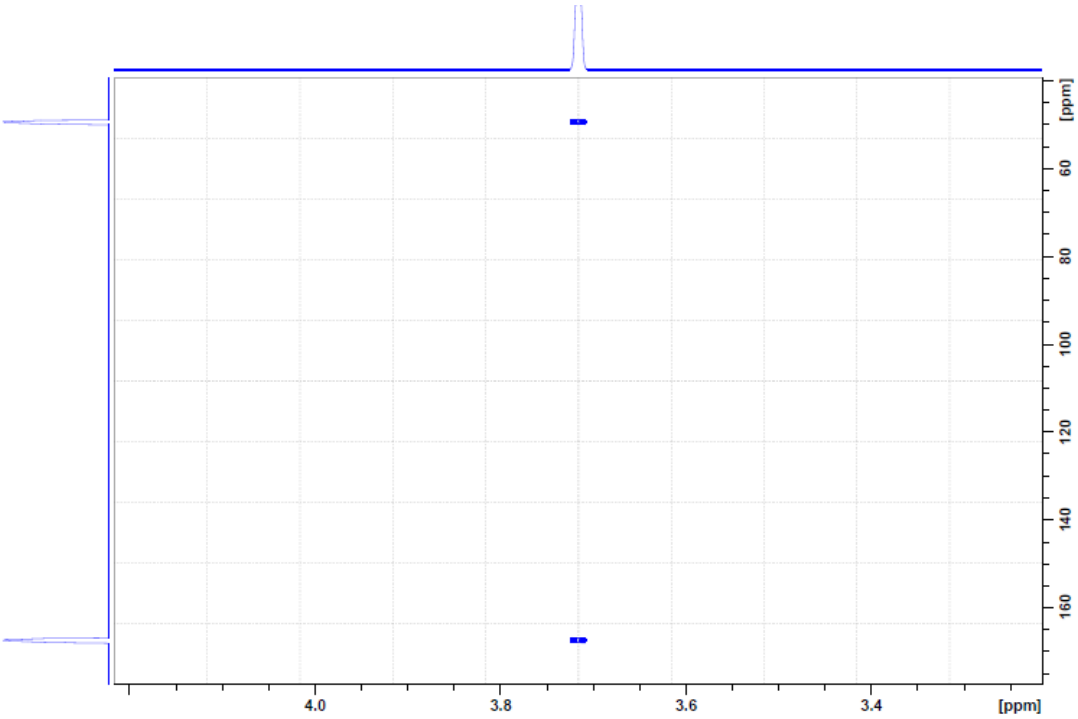
Appendix 3B: ^{13}C-NMR of 11



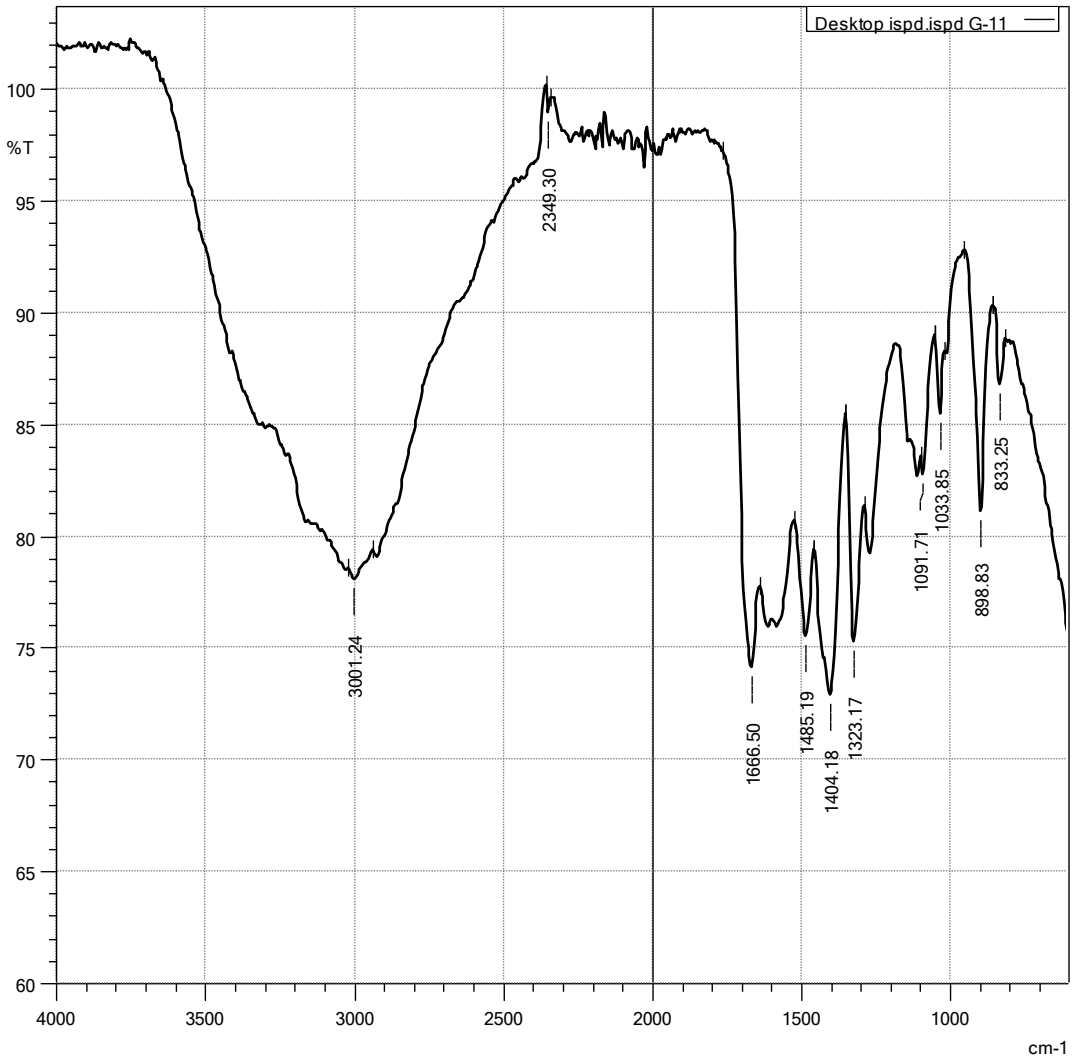
Appendix 3C: COSY of 11



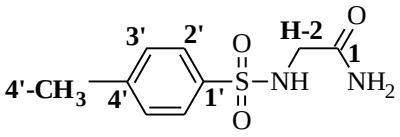
Appendix 3D: HMBC of 11



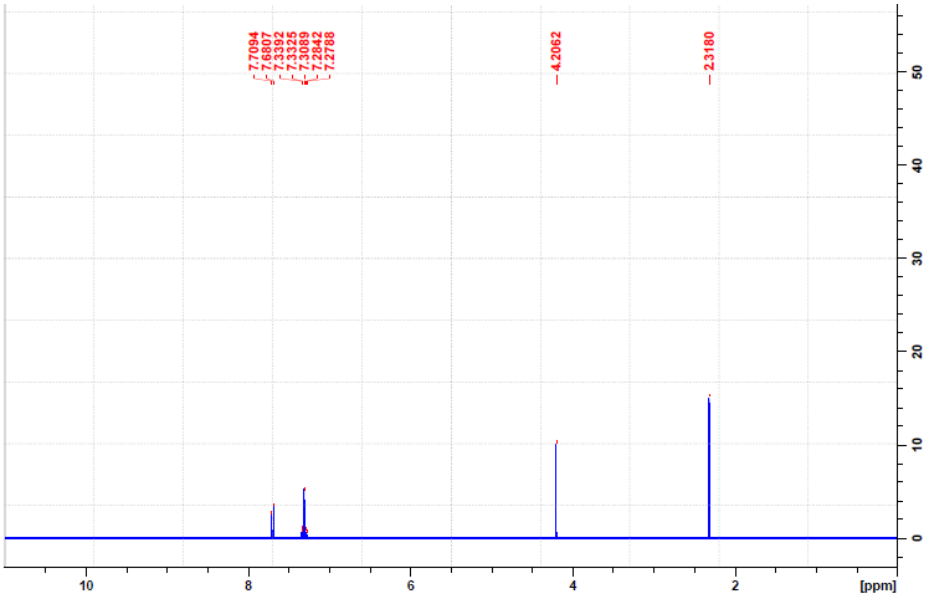
Appendix 3E: IR of 11



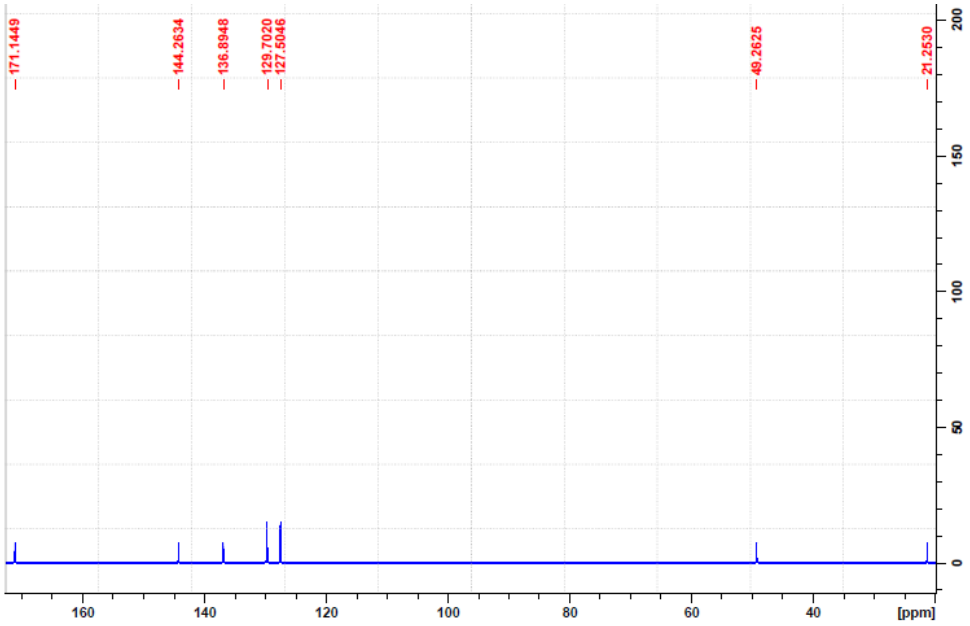
Appendix 4: Spectral data for 2-{[(4-methylphenyl)sulfonyl]amino}acetamide **(12)**



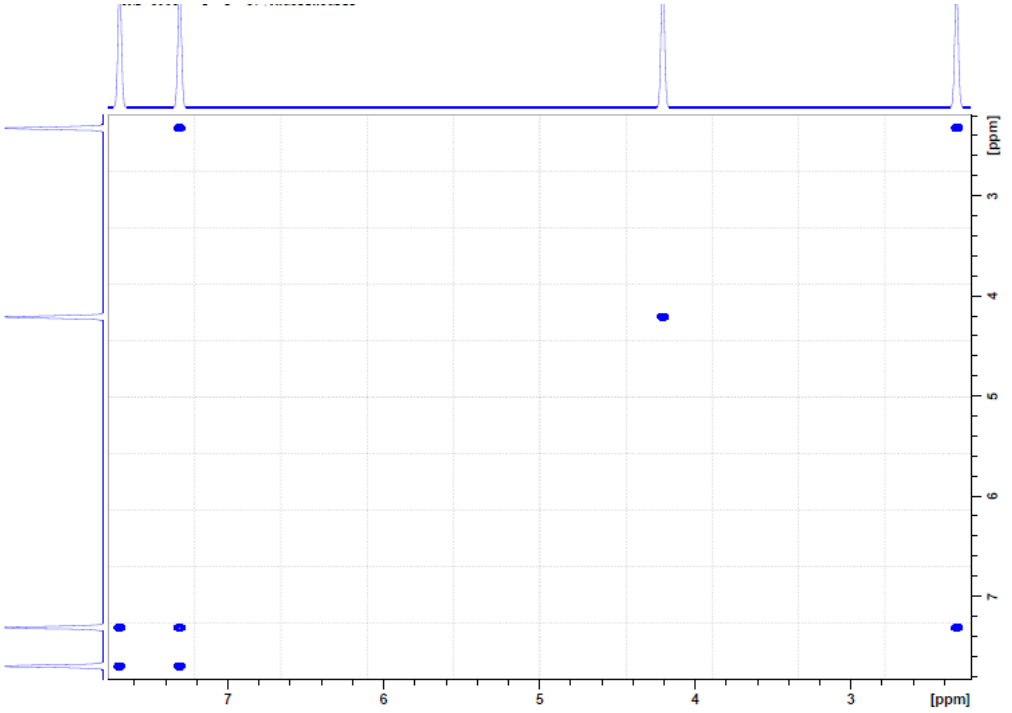
Appendix 4A: ^{1}H-NMR of 12



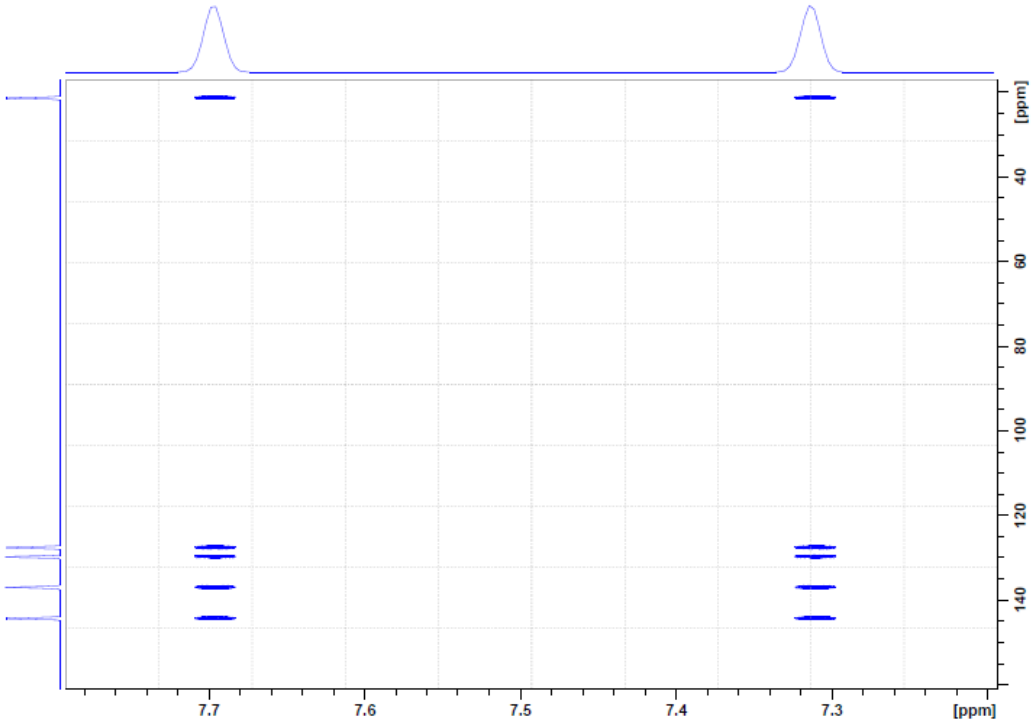
Appendix 4B: ^{13}C-NMR of 12



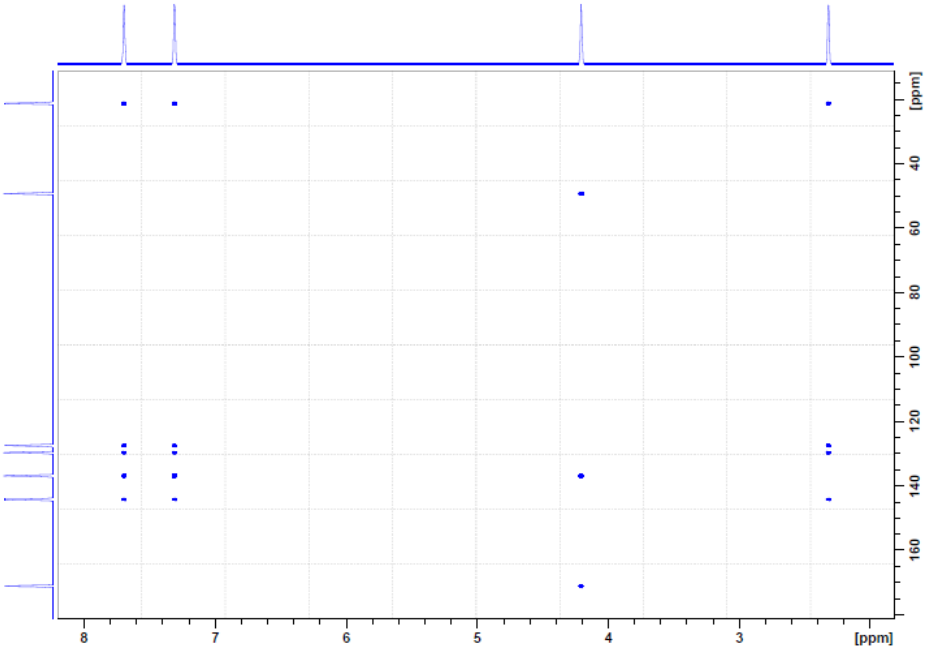
Appendix 4C(i): COSY of 12



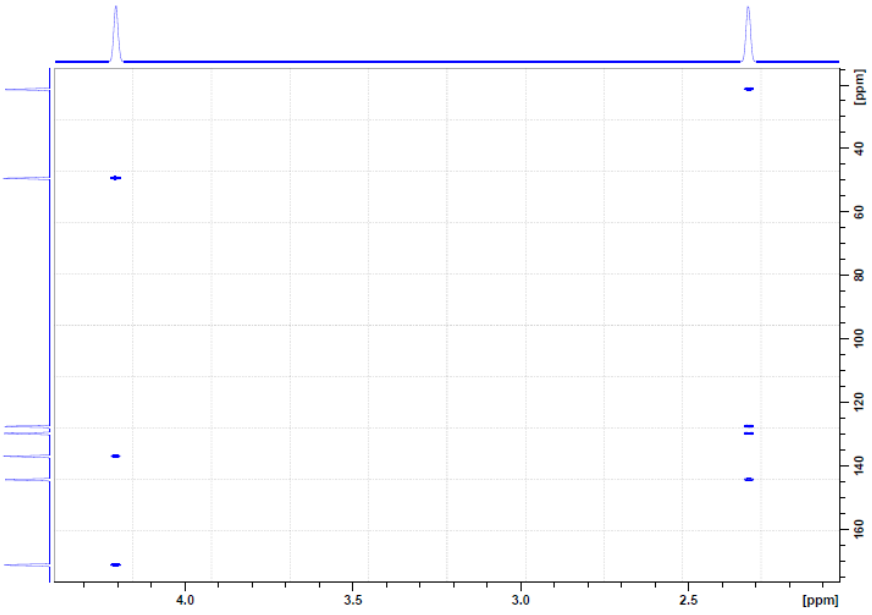
Appendix 4C(ii): COSY of 12



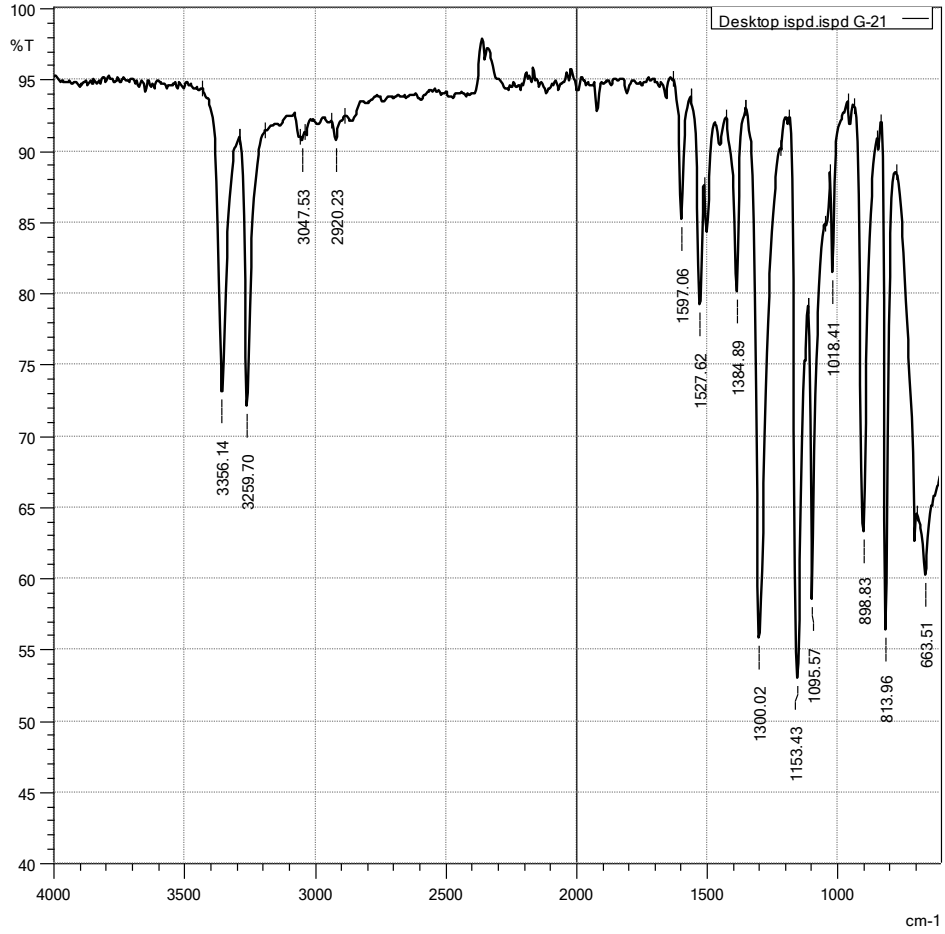
Appendix 4D(i) HMBC of 12



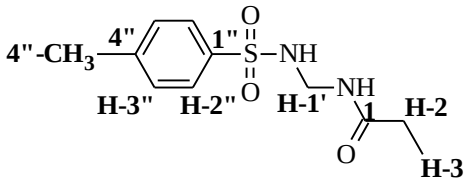
Appendix 4D(ii): HMBC of 12



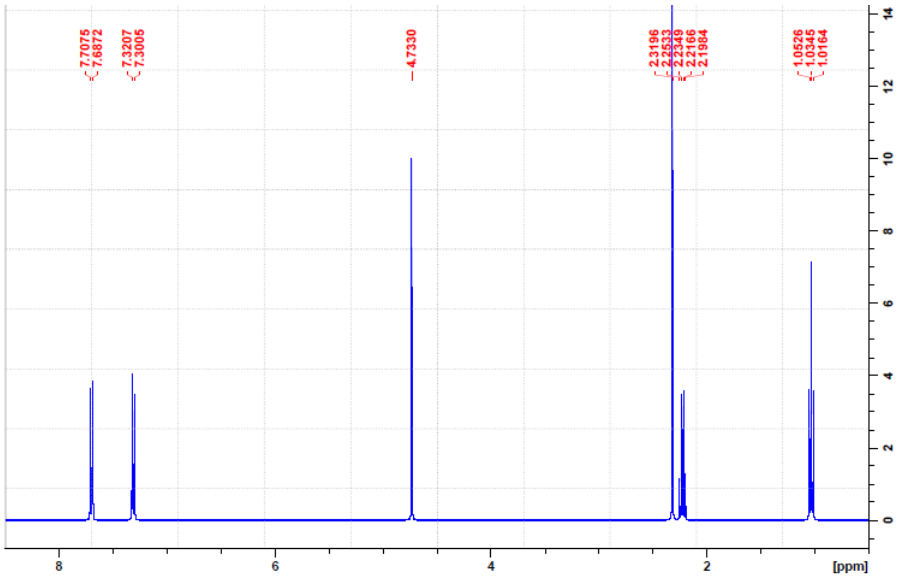
Appendix 4E: IR of 12



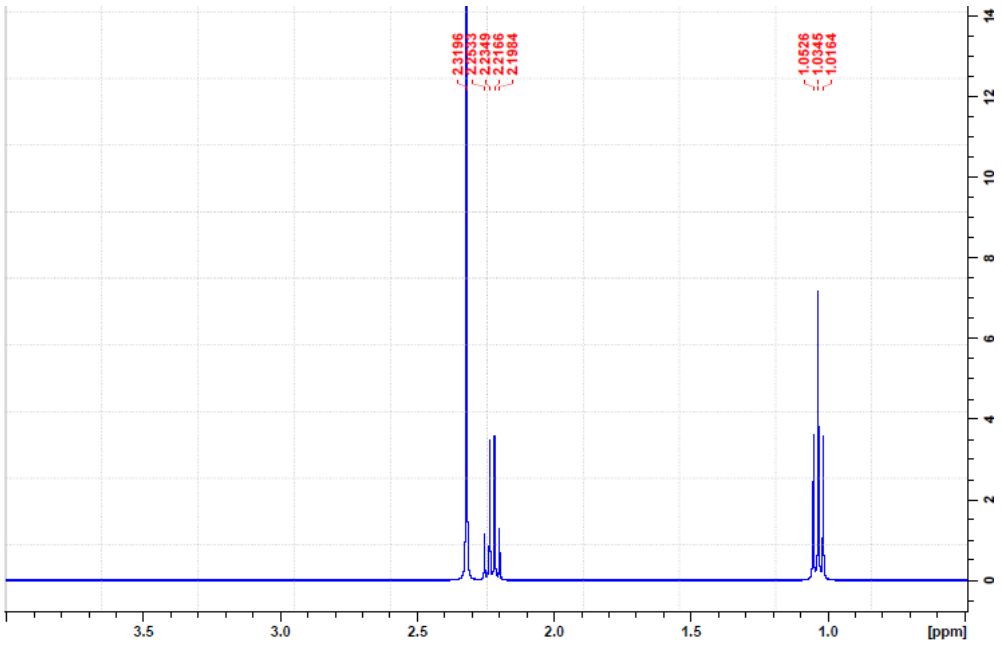
Appendix 5: Spectral data for *N*-({[(4-methylphenyl)sulfonyl]amino}methyl)propanamide **(2)**



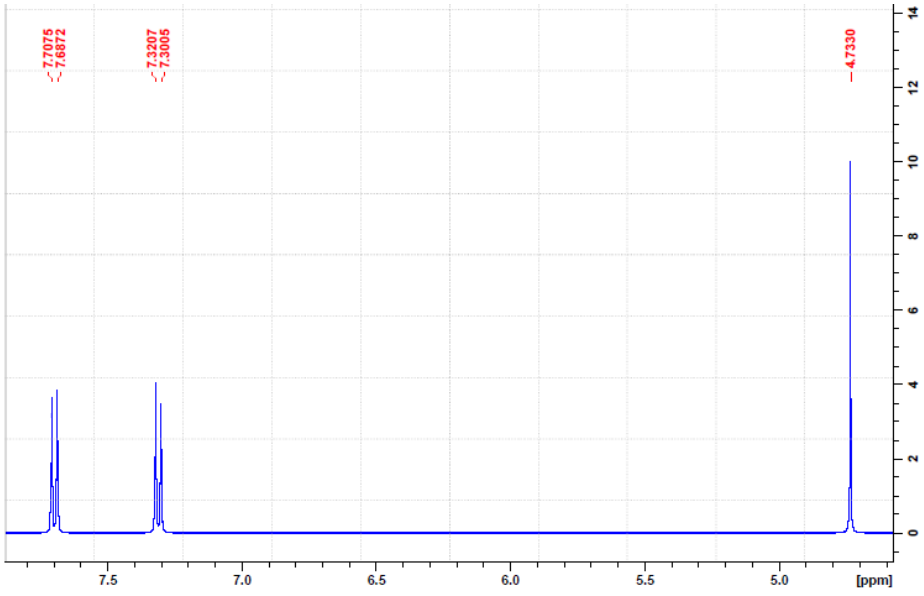
Appendix 5A(i): ^{1}H-NMR of 2



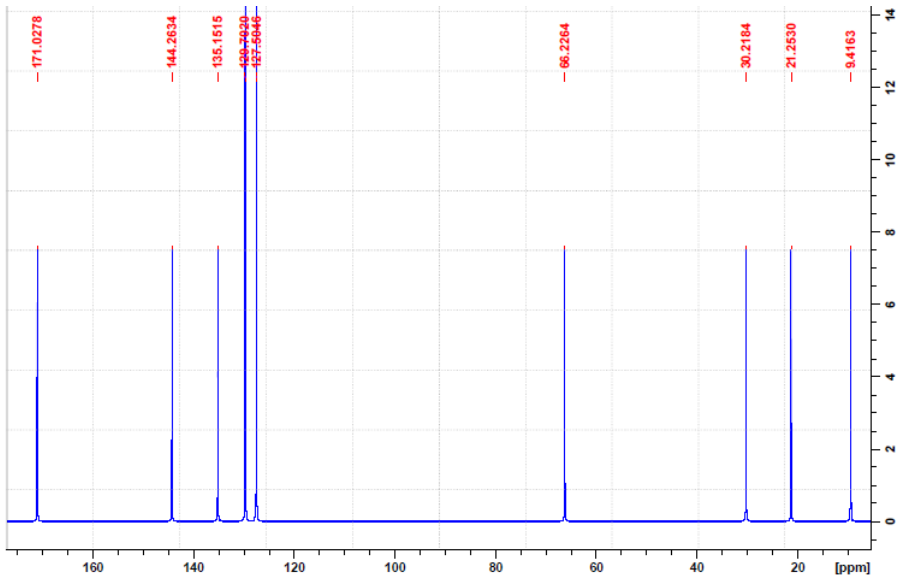
Appendix 5A(ii): HNMR of 2



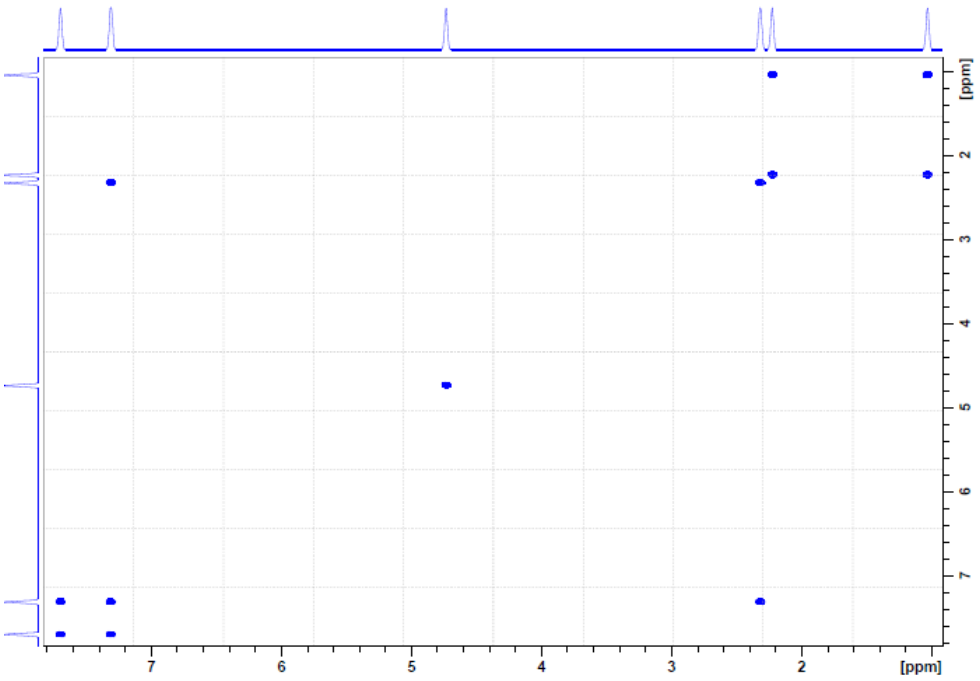
Appendix 5A(iii): HNMR of 2



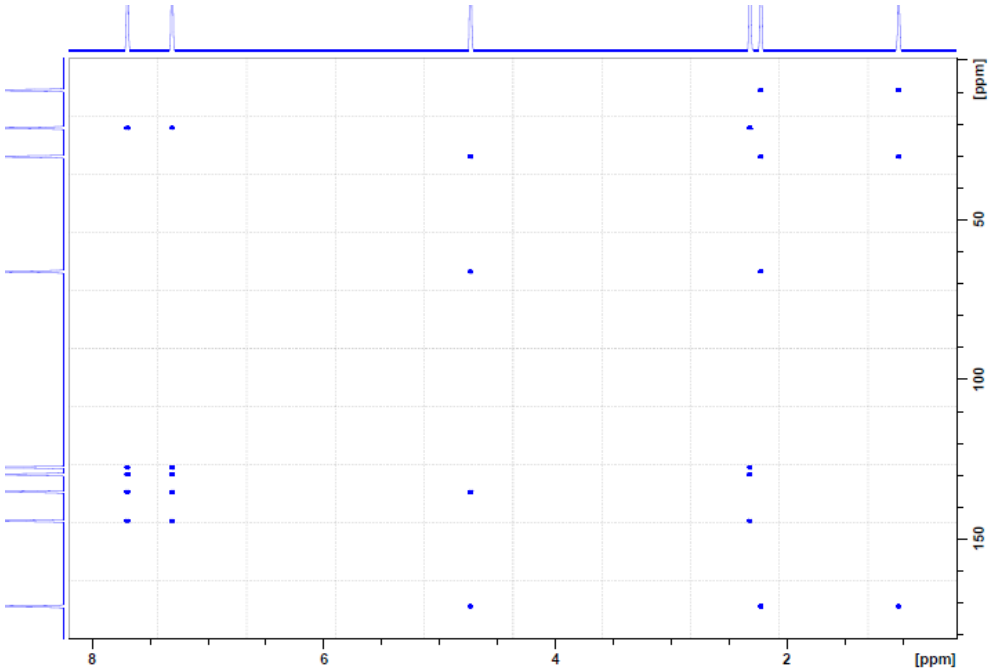
Appendix 5B: ^{13}C-NMR of 2



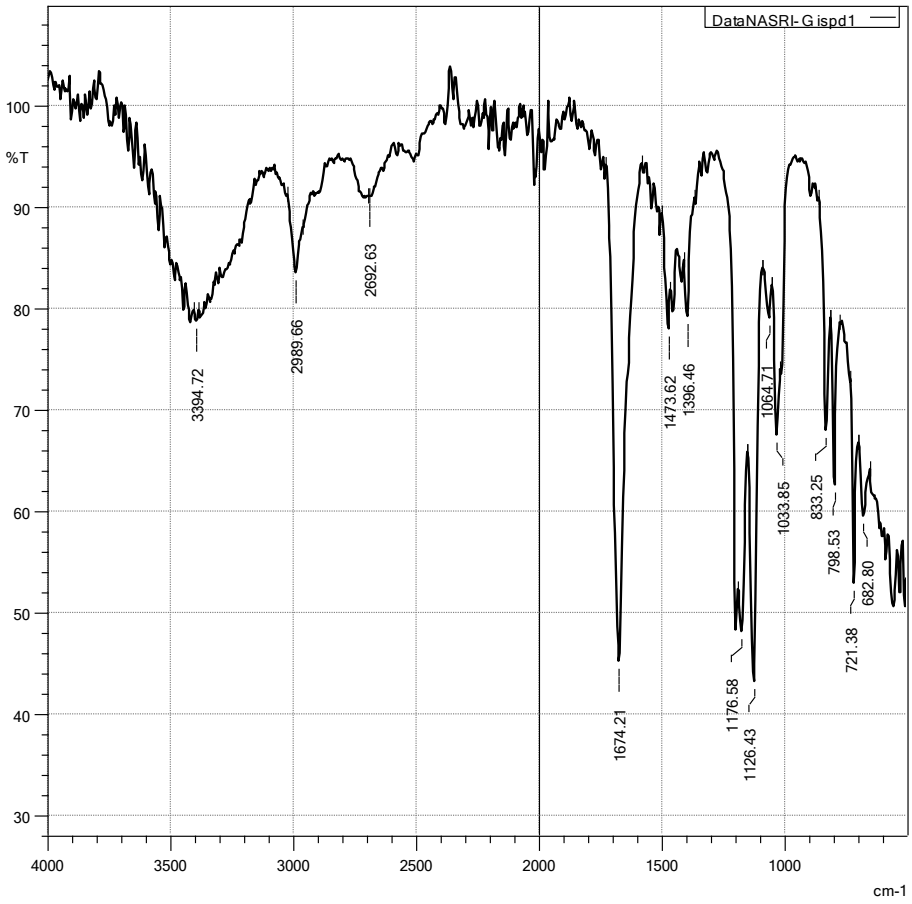
Appendix 5C: COSY of 2



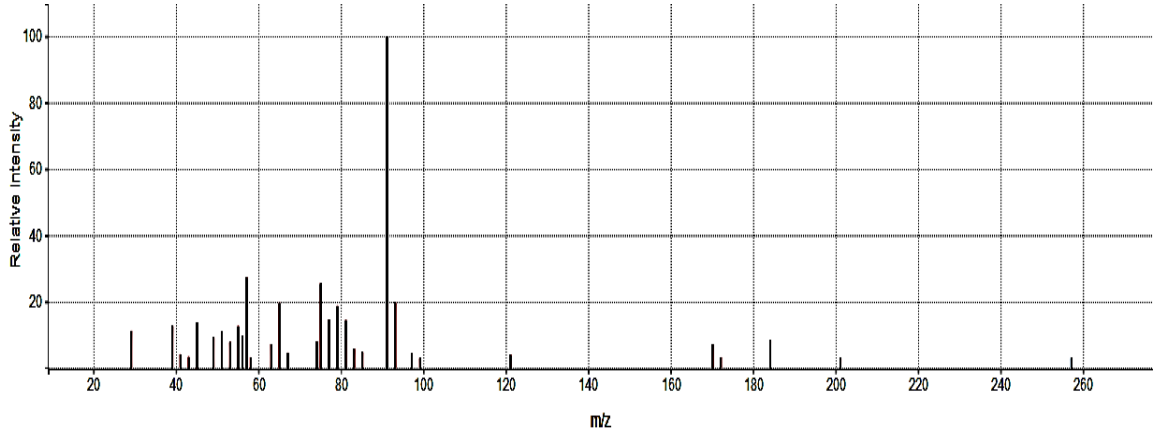
Appendix 5D: HMBC of 2



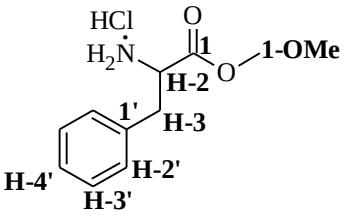
Appendix 5E: IR of 2



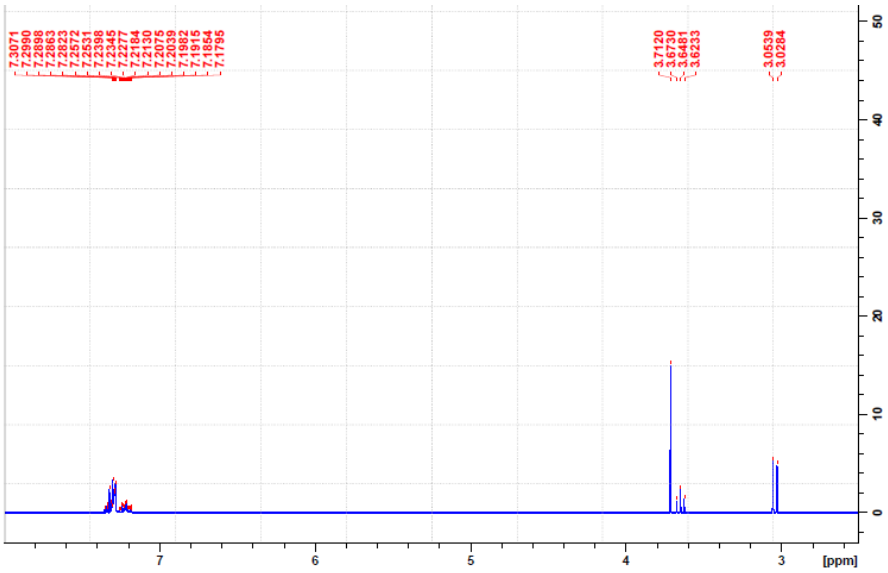
Appendix 5F: MS of 2



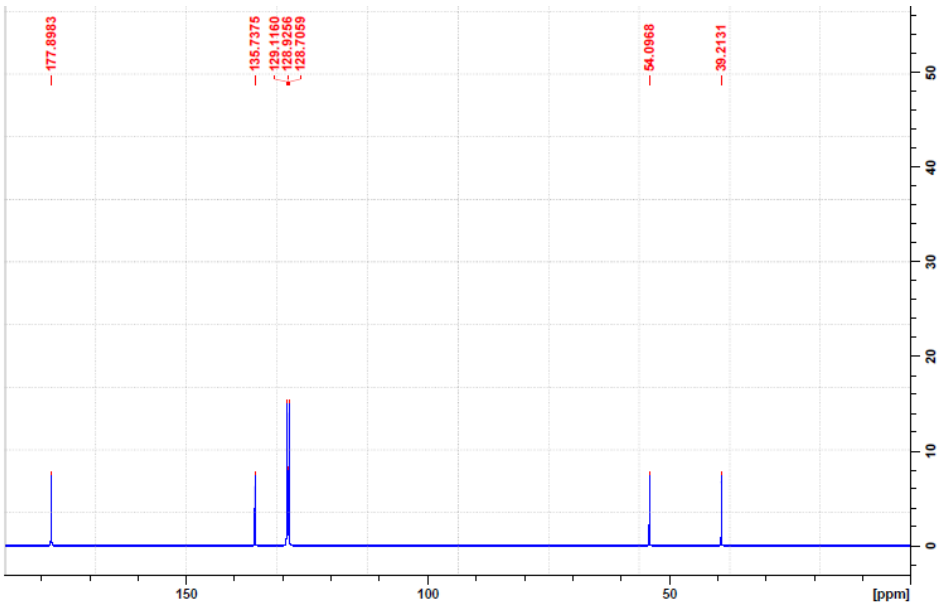
Appendix 6: Spectral data for L-phenylalaninemethylester HCl **(13)**



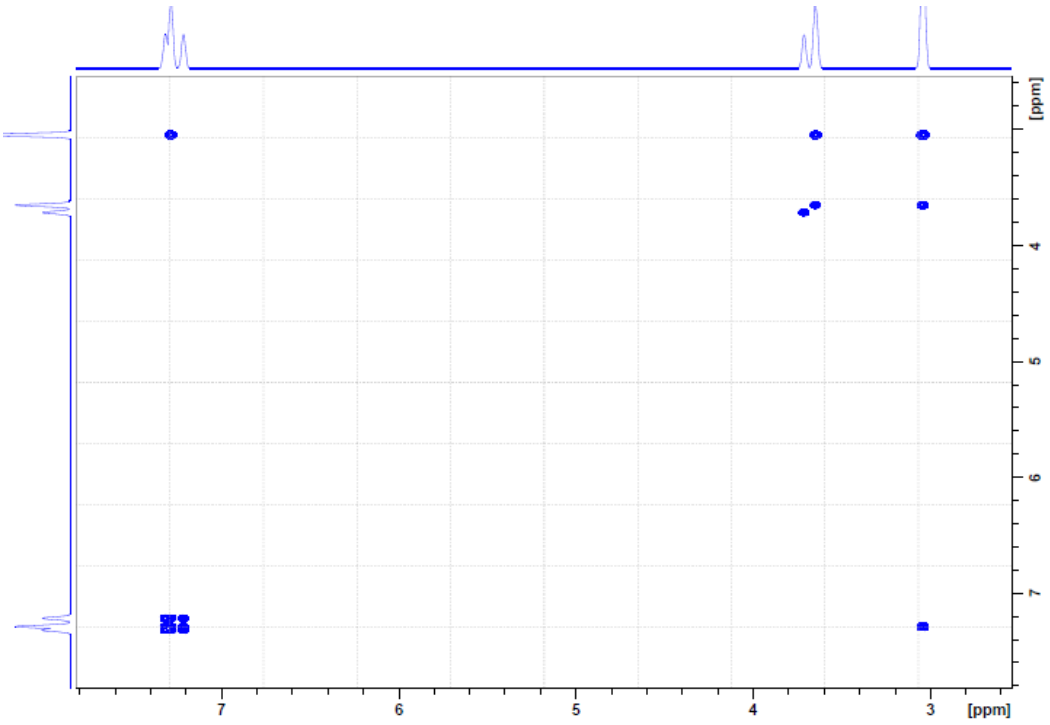
Appendix 6A: ^{1}H-NMR of 13



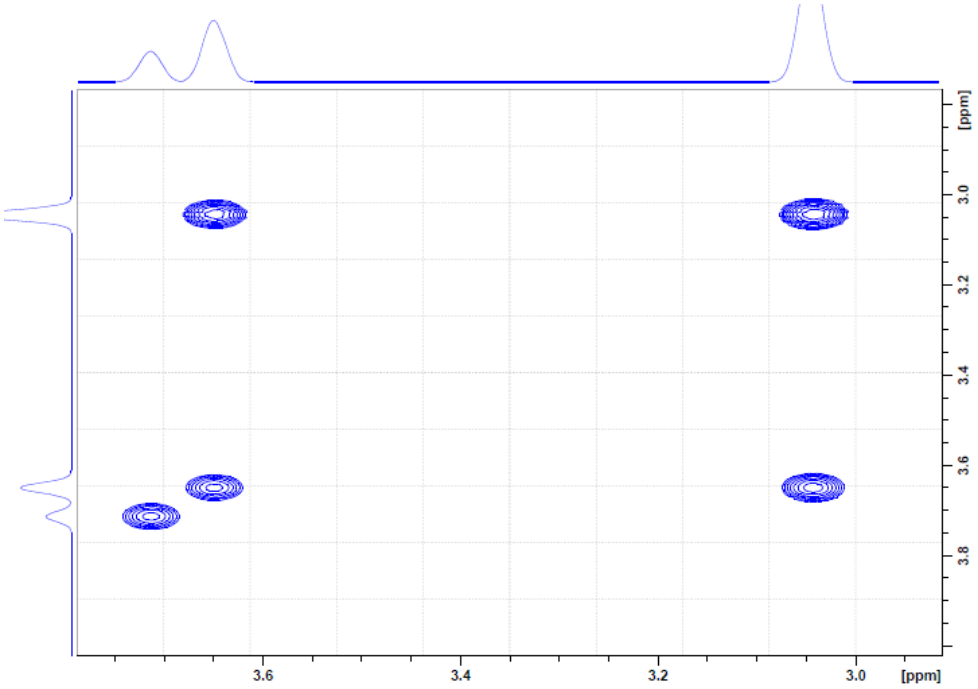
Appendix 6B: ^{13}C-NMR of 13



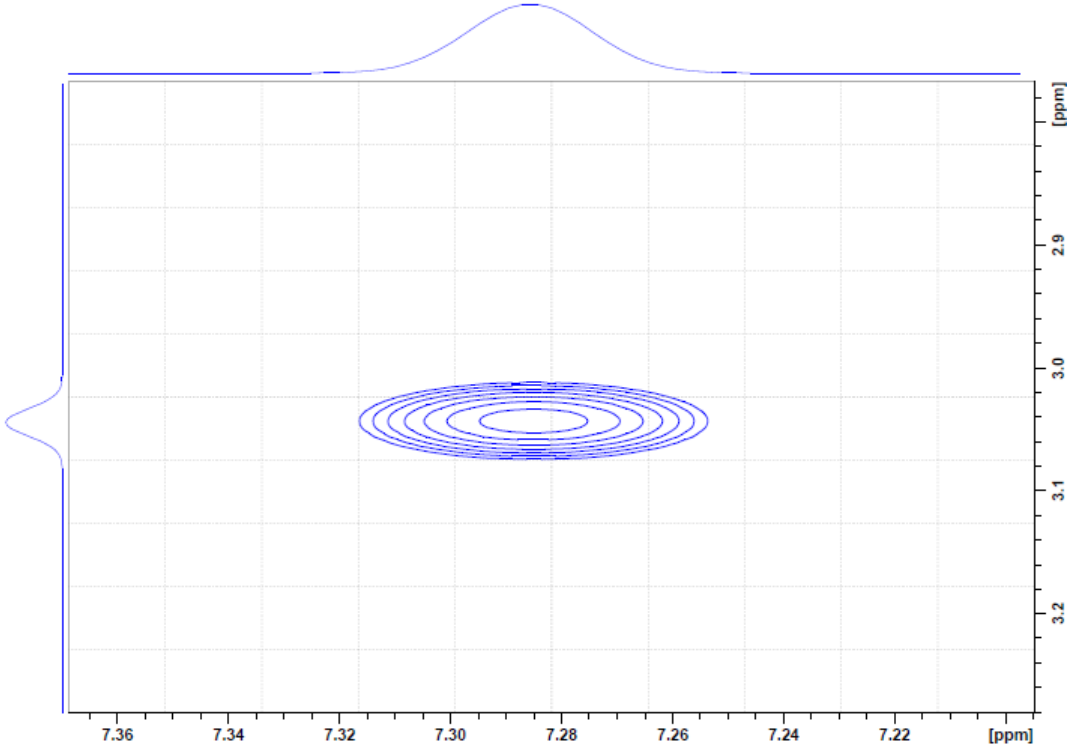
Appendix 6(i): COSY of 13



Appendix 6C(ii): COSY of 13

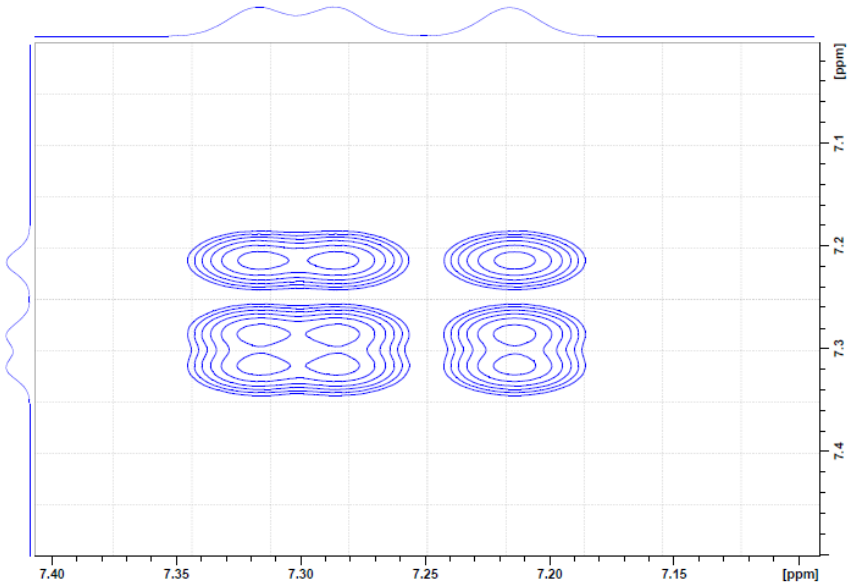


Appendix 6C(iii): COSY of 13

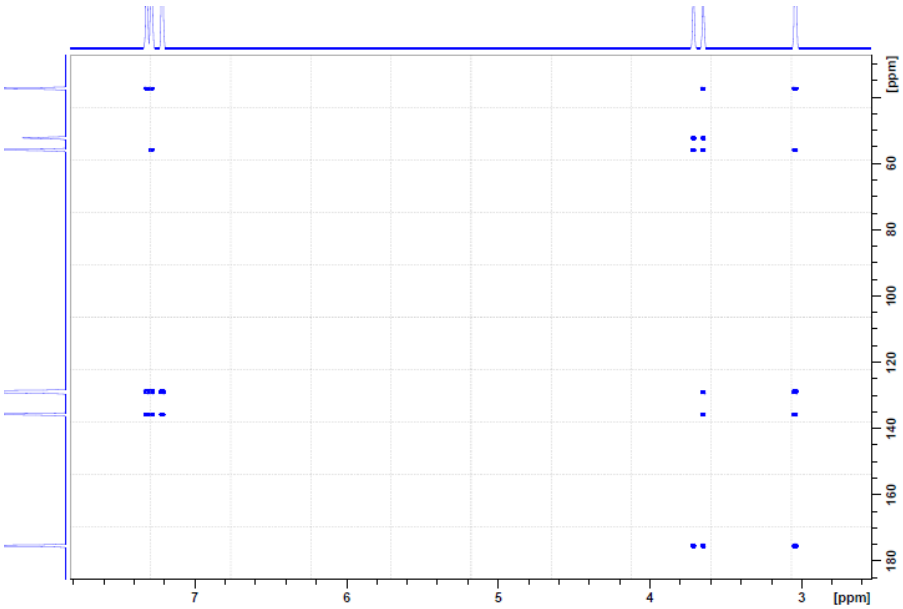


a

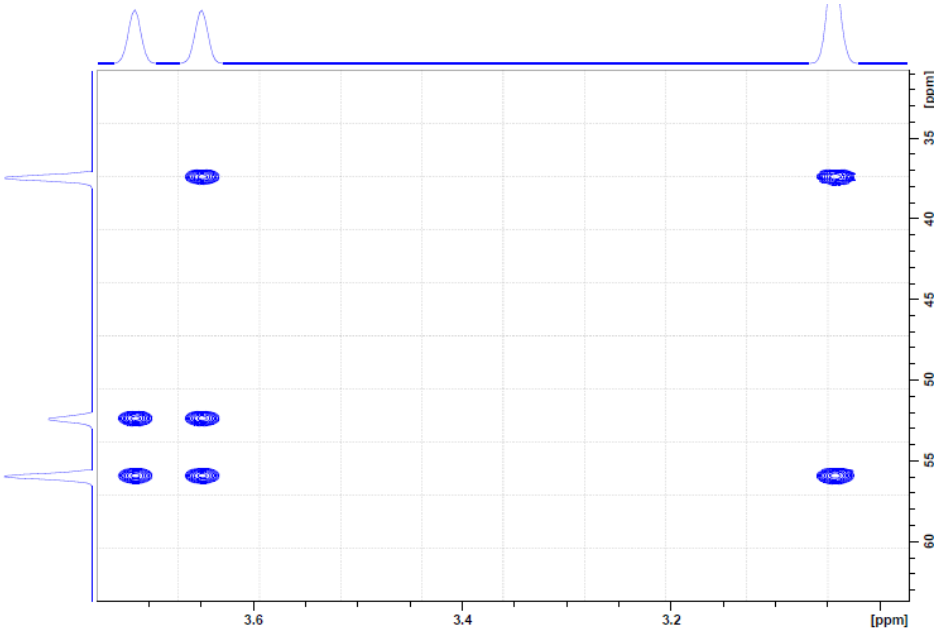
Appendix 6C(iv): COSY of 13



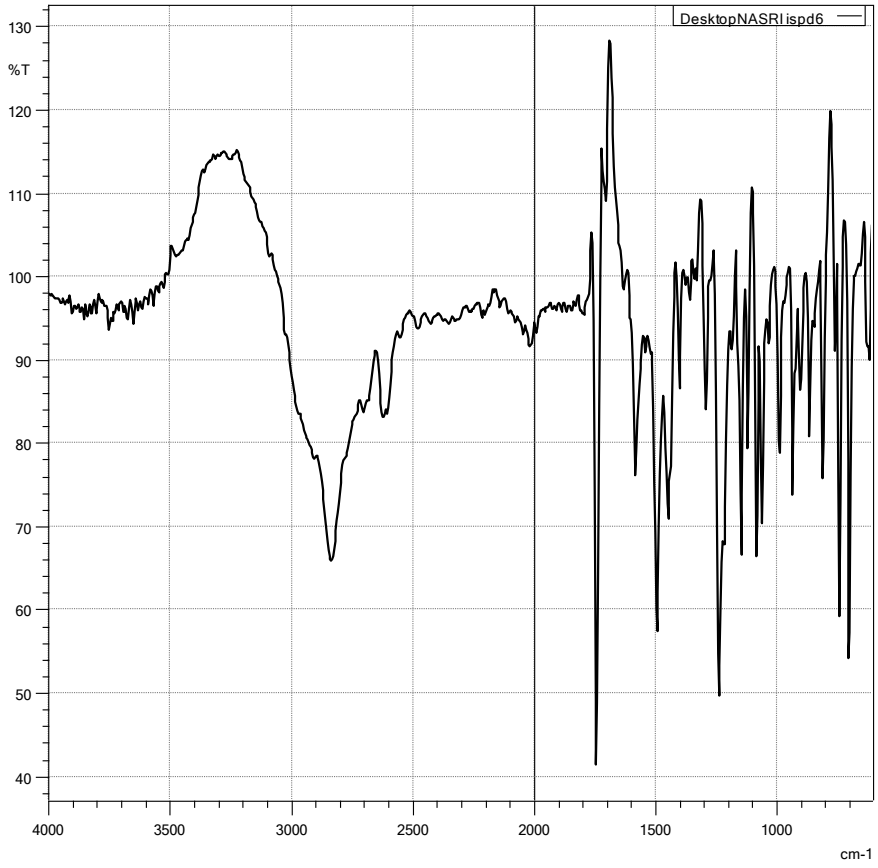
Appendix 6D(i): HMBC of 13



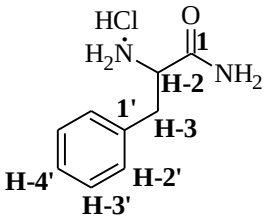
Appendix 6D(ii): HMBC of 13



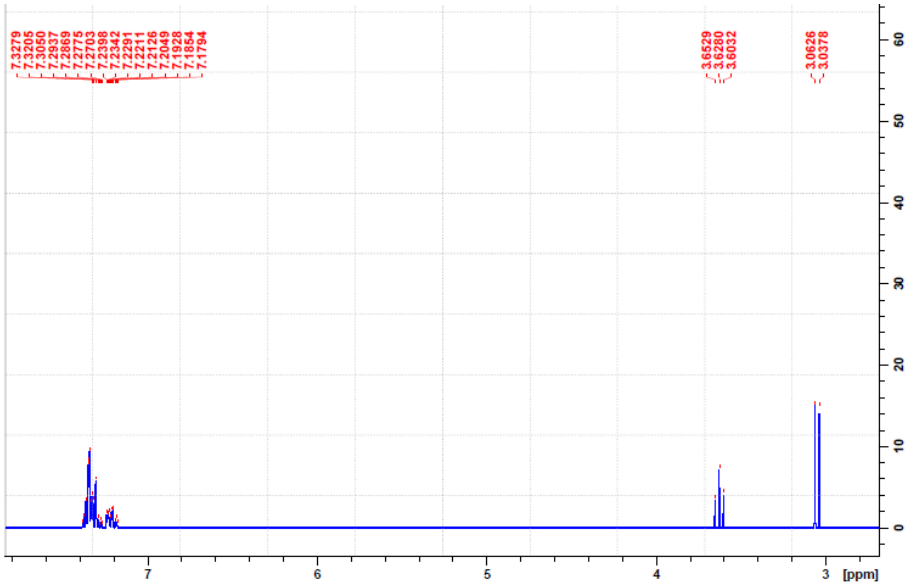
Appendix 6E: IR of 13



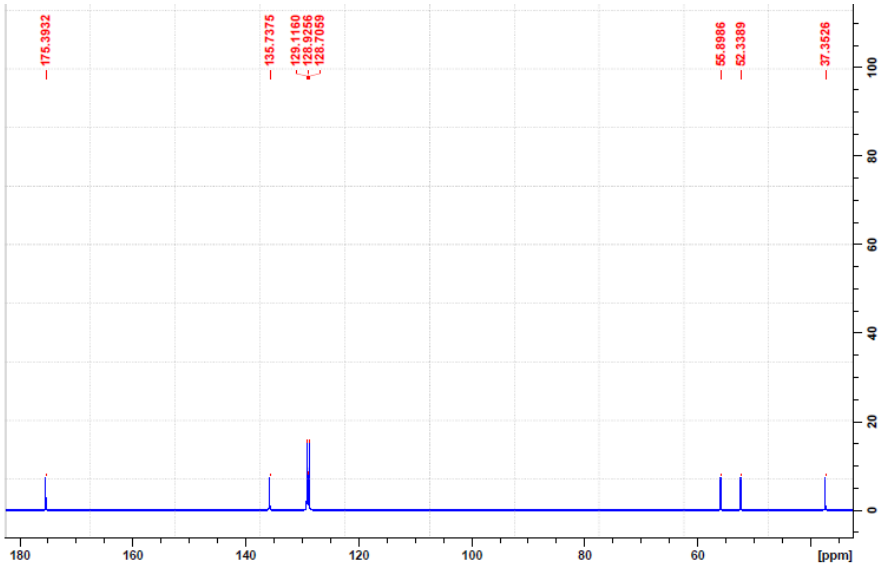
Appendix 7: Spectral data for 2-amino-3-phenylpropanamide HCl **(14)**



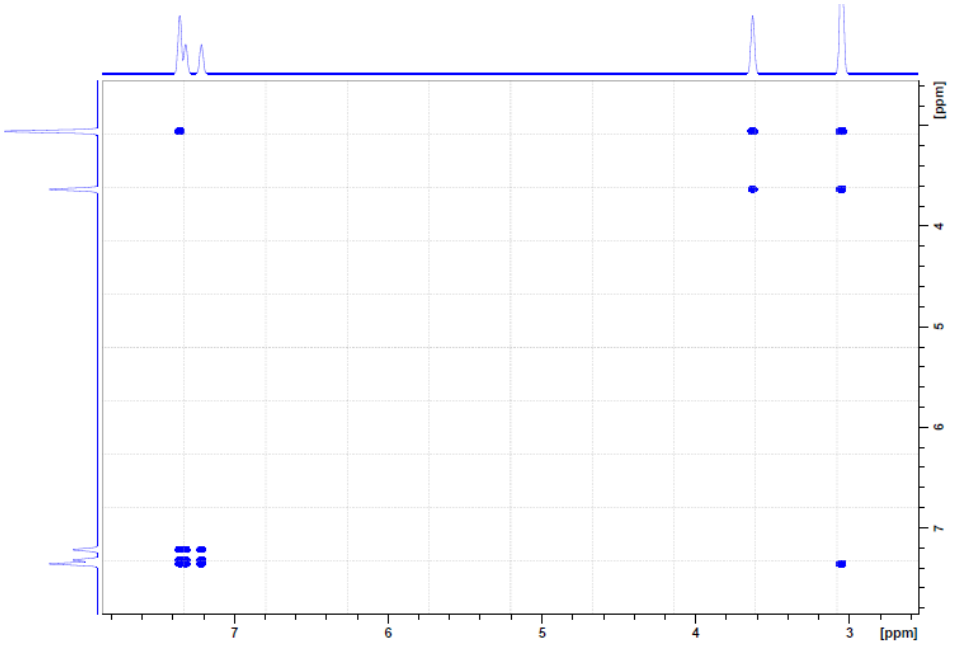
Appendix 7A: ^{1}H-NMR of 14



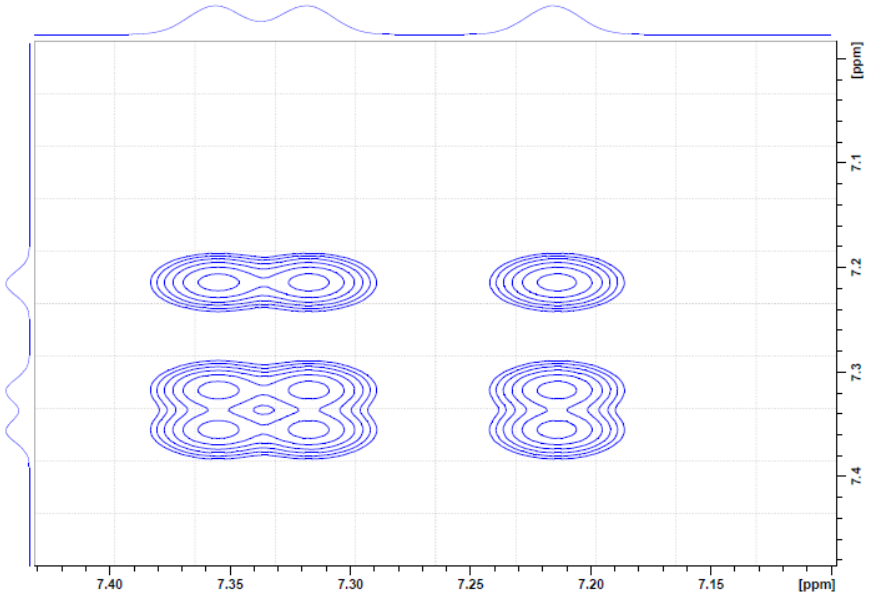
Appendix 7B: ^{13}C-NMR of 14



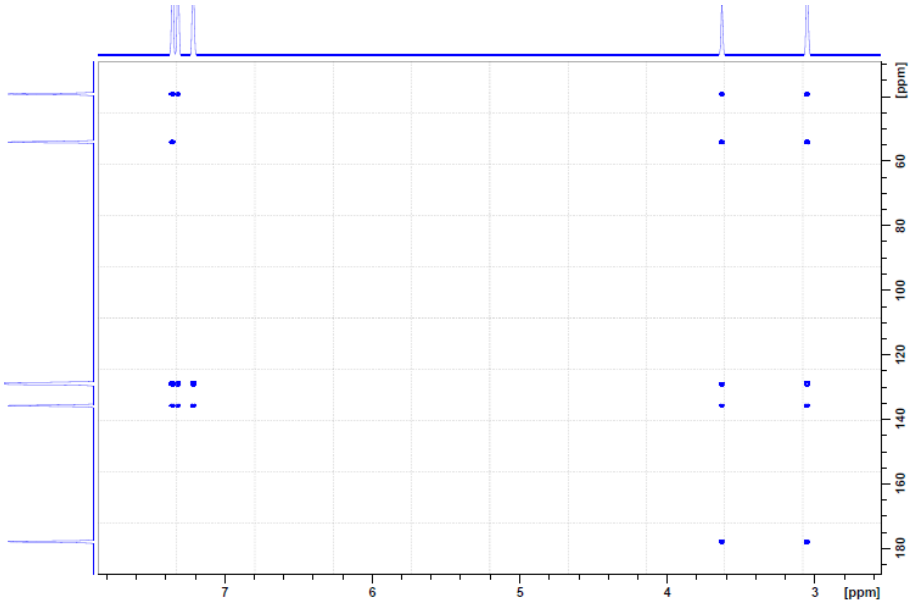
Appendix 7C(i): COSY of 14



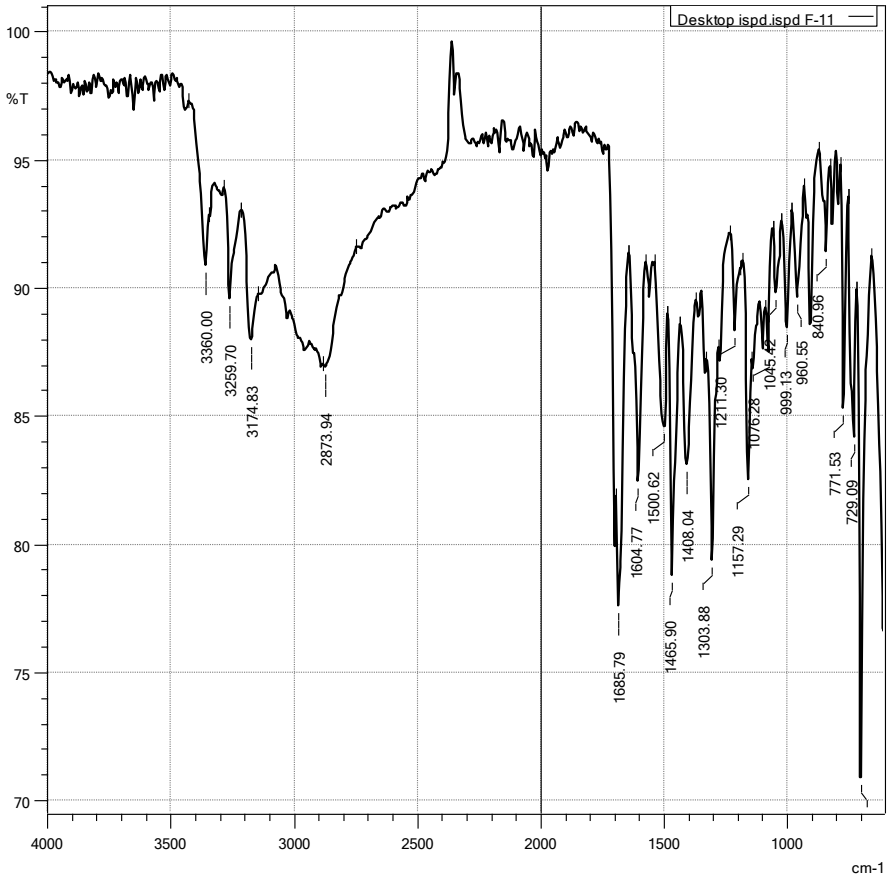
Appendix 7C(ii): COSY of 14



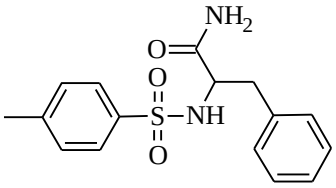
Appendix 7D: HMBC of 14



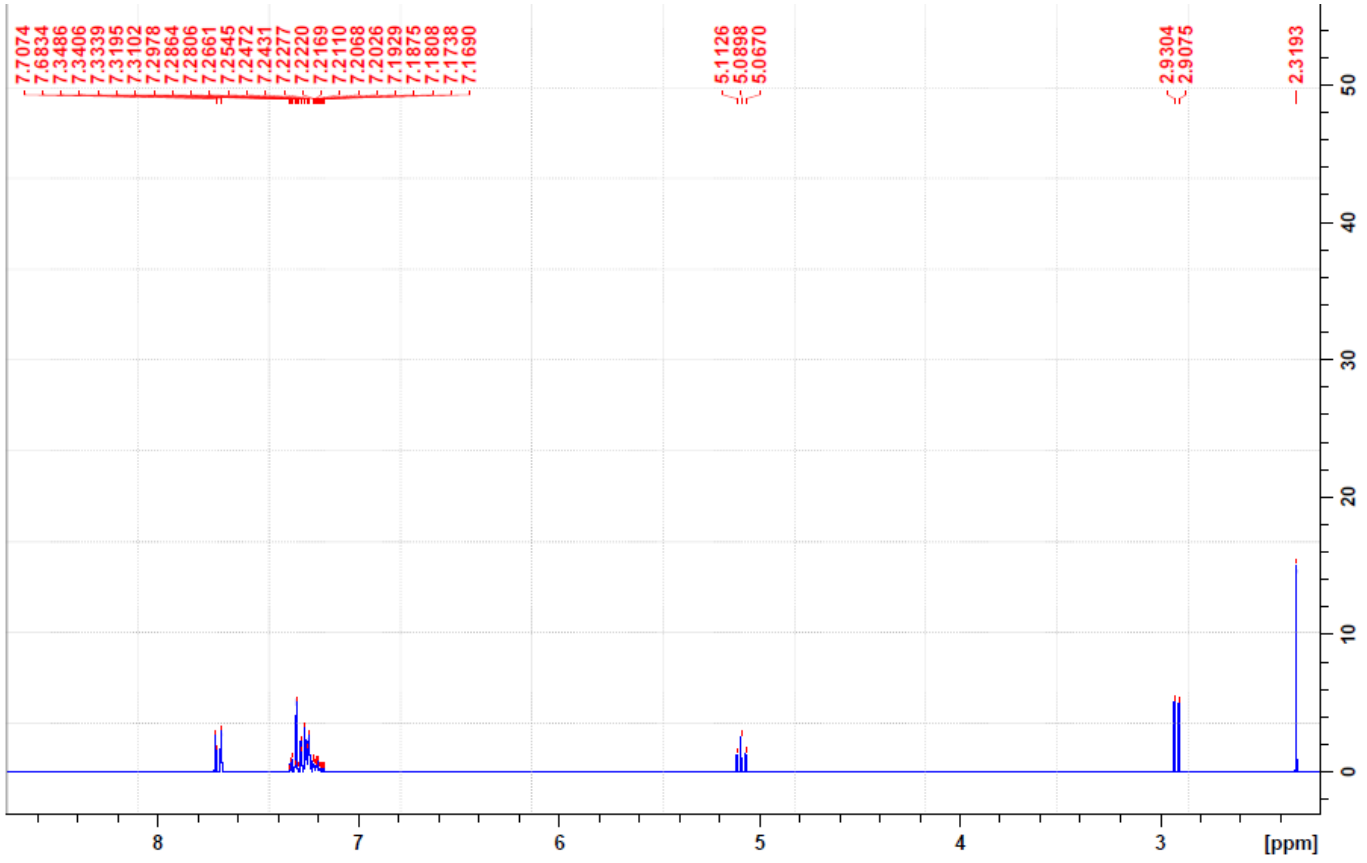
Appendix 7E: IR of 14



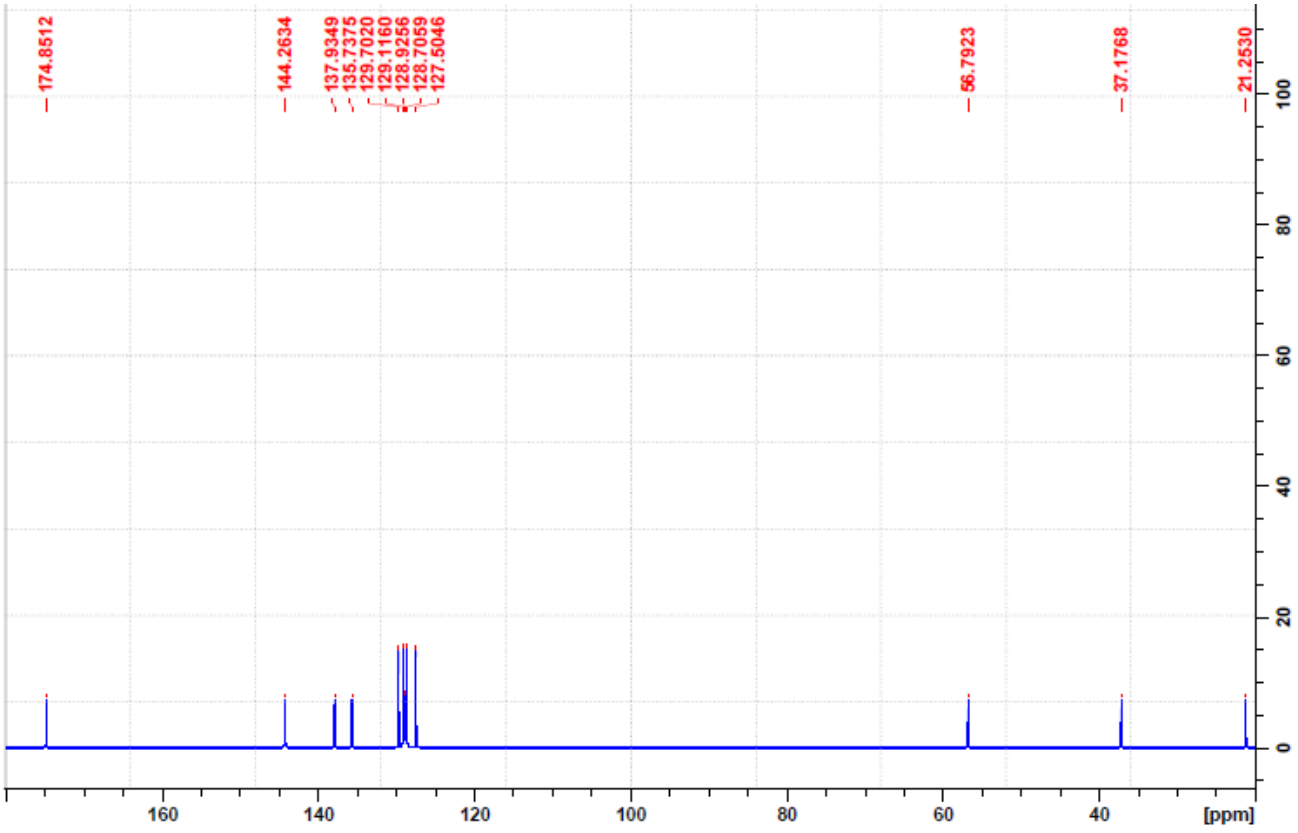
Appendix 8: Spectral data for 2-{[(4-methylphenyl)sulfonyl]amino}-3-phenylpropanamide **(15)**



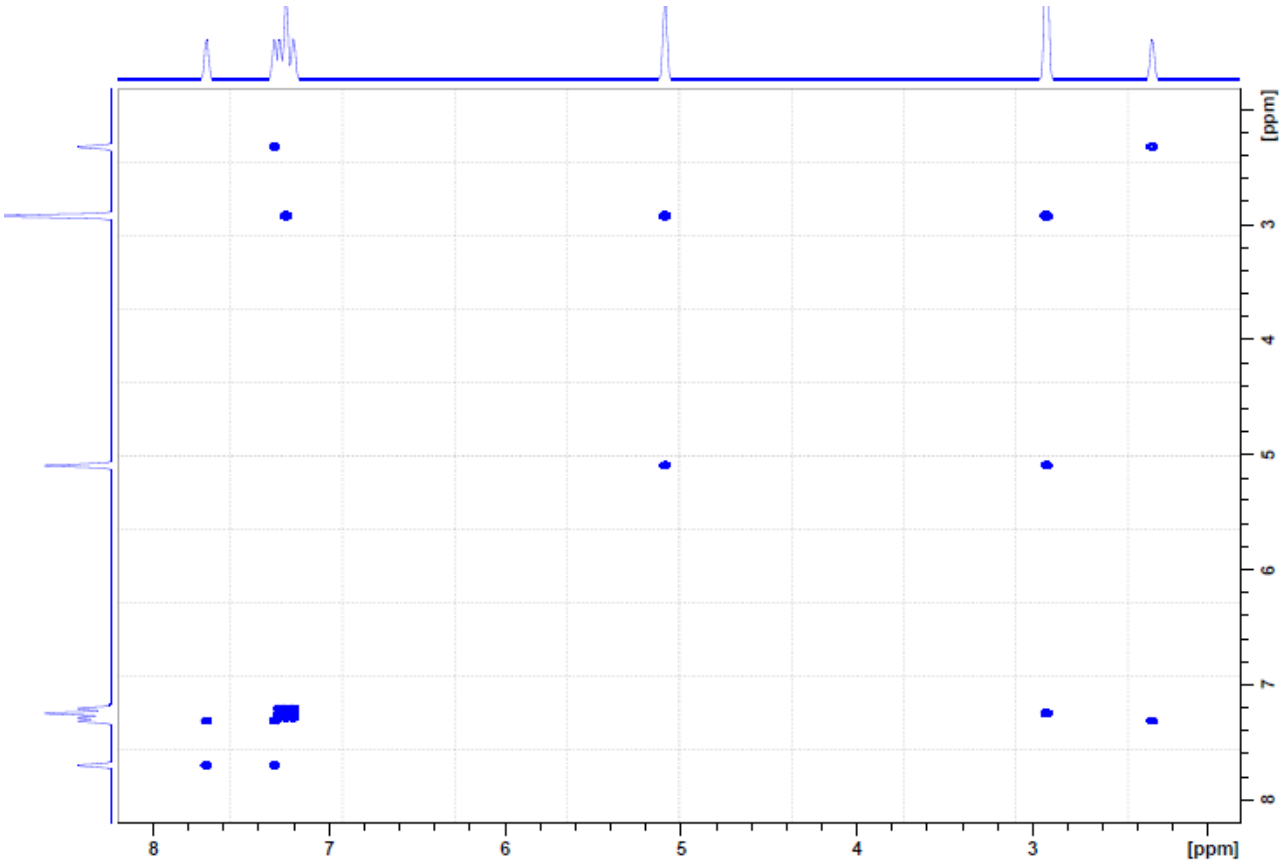
Appendix 8A: ^{1}H-NMR of 15



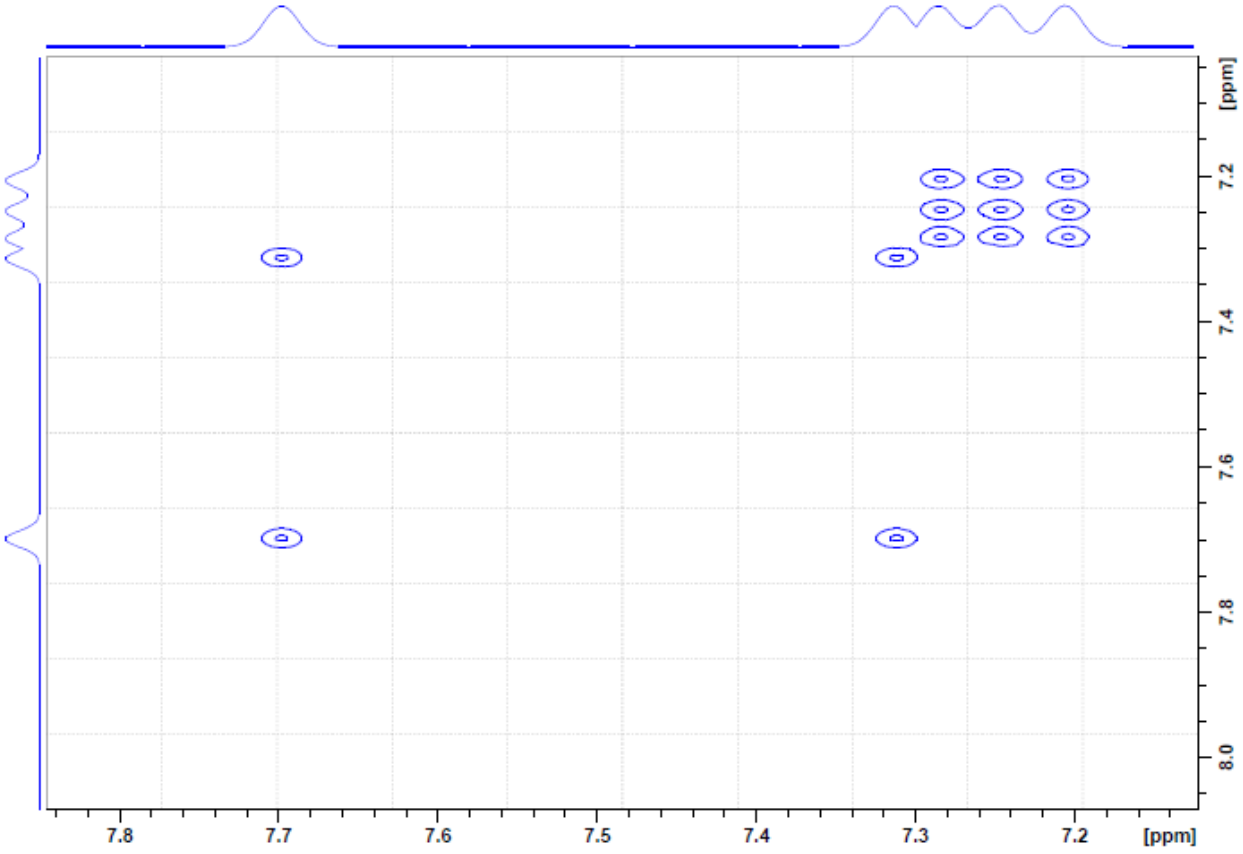
Appendix 8B: ^{13}C-NMR of 15



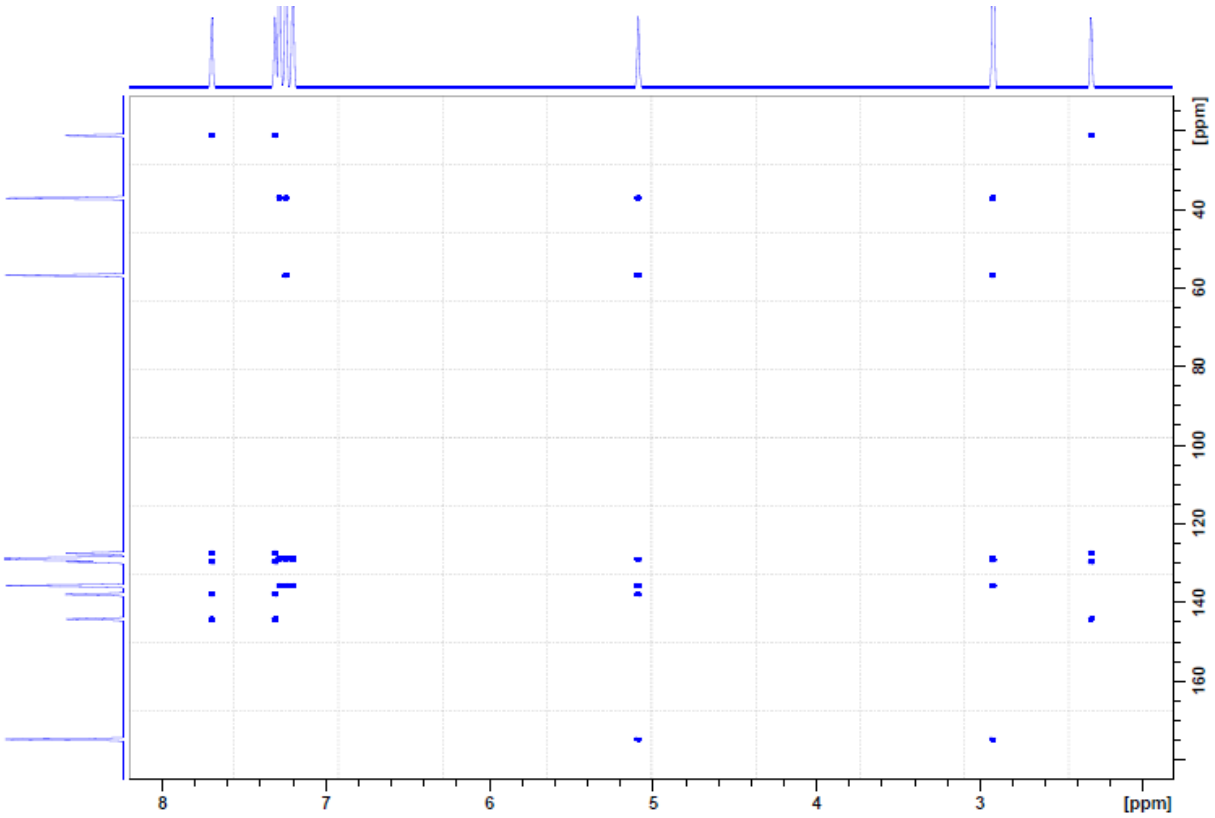
Appendix 8C(i): COSY of 15



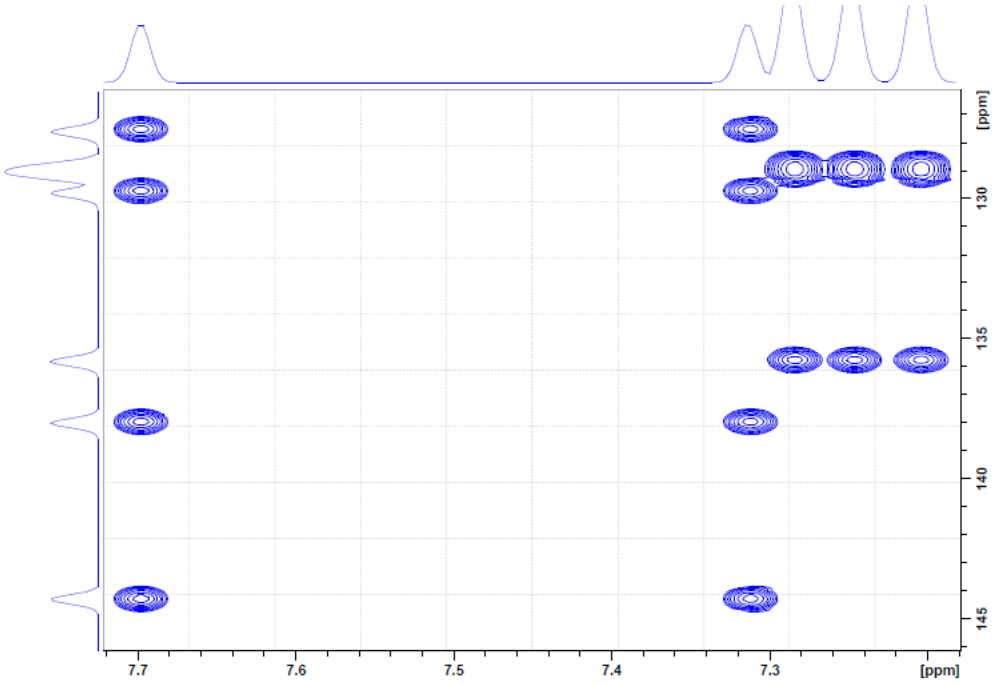
Appendix 8C(ii): COSY of 15



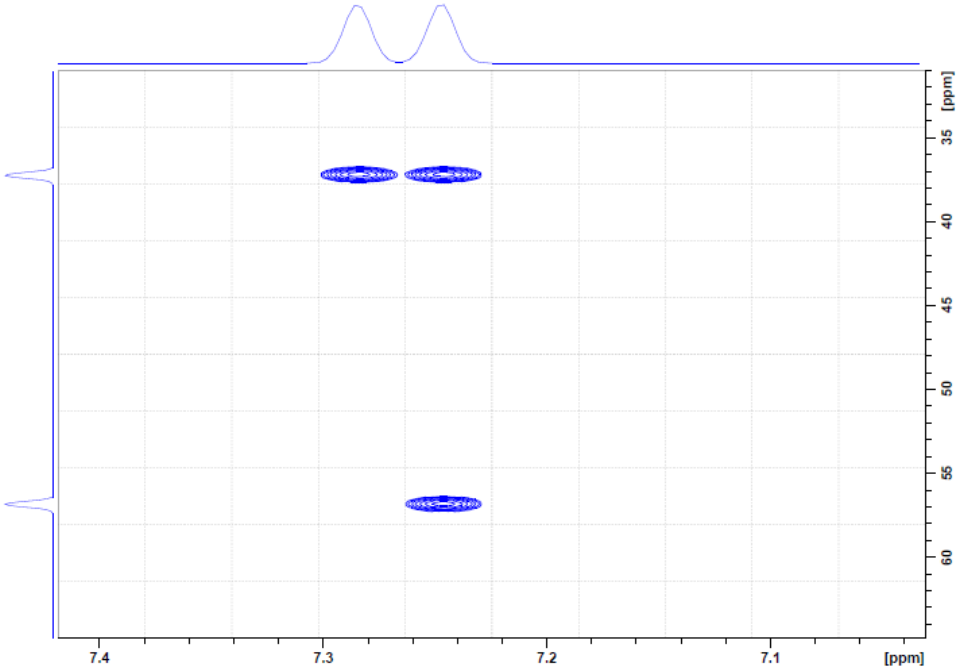
Appendix 8D(i): HMBC of 15



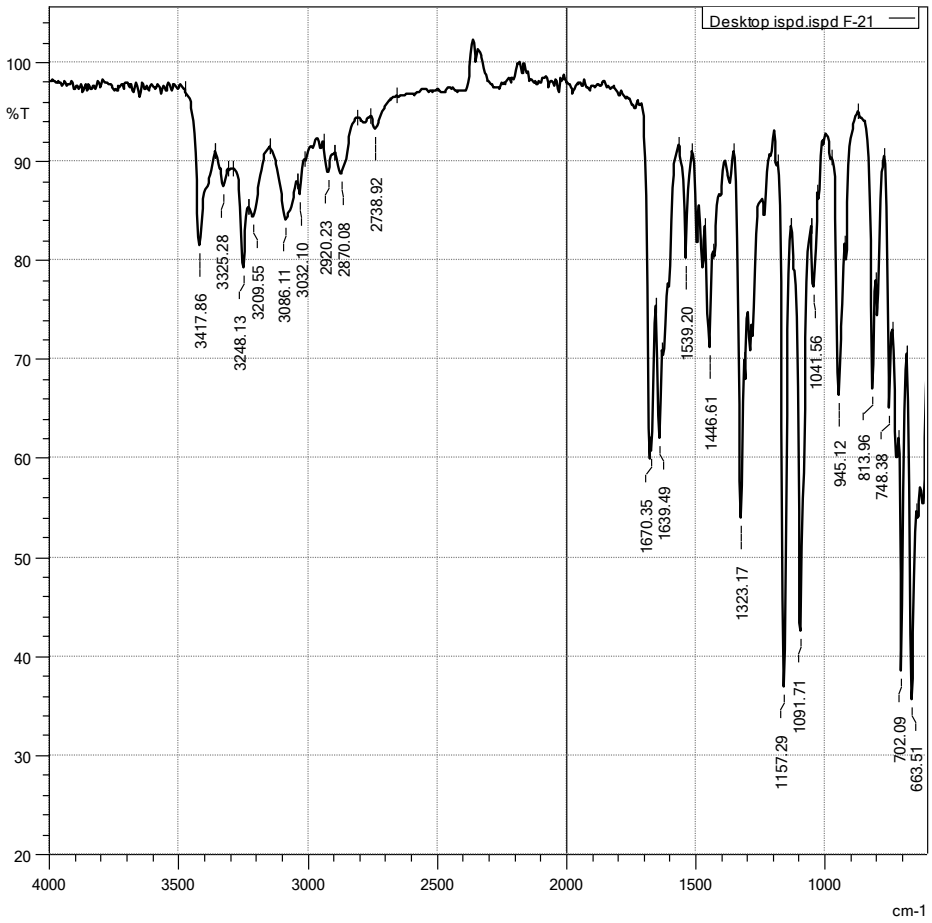
Appendix 8D(ii): HMBC of 15



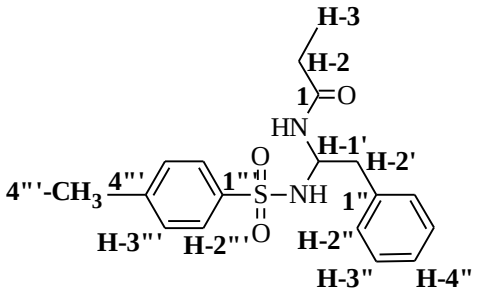
Appendix 8D(iii): HMBC of 15



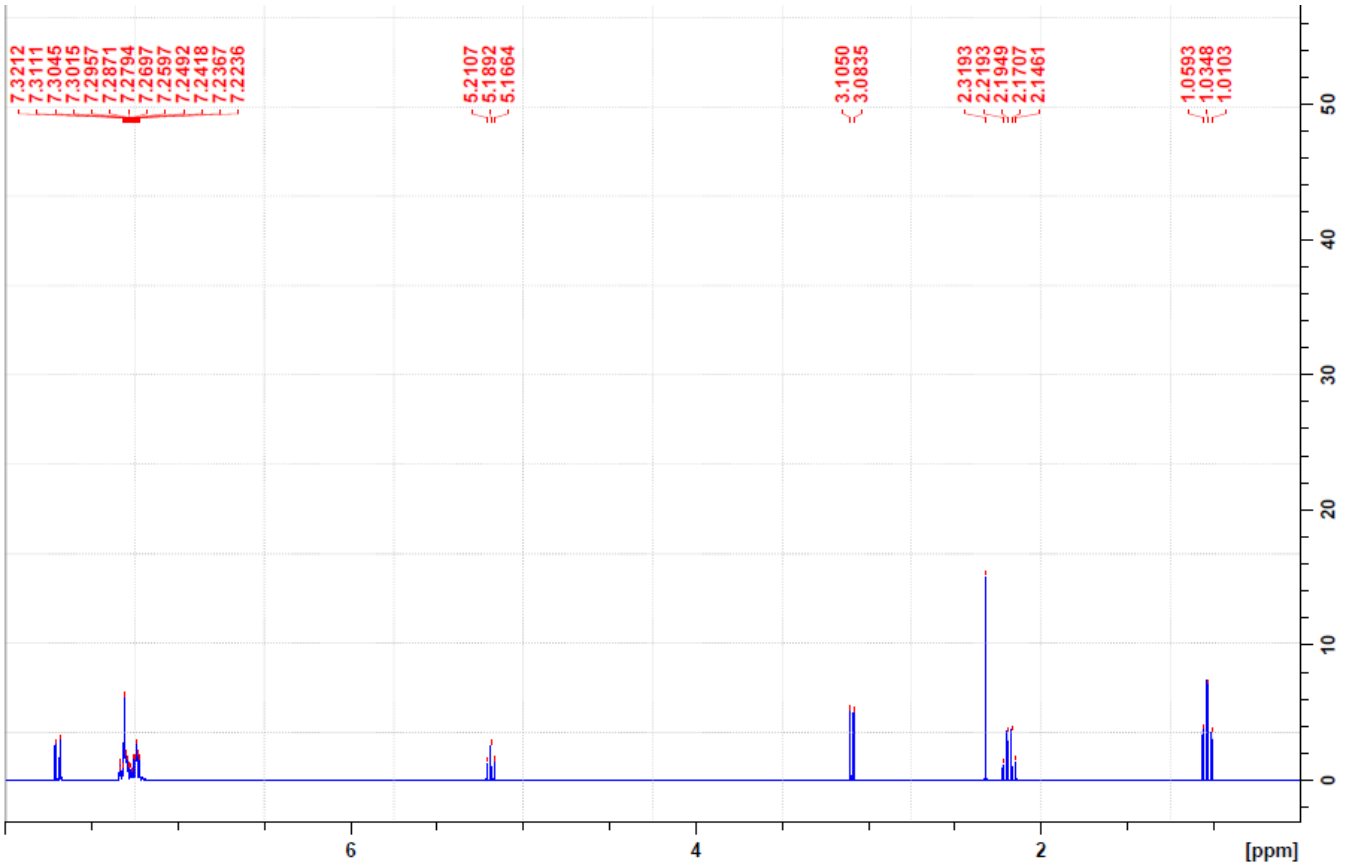
Appendix 8E: IR of 15



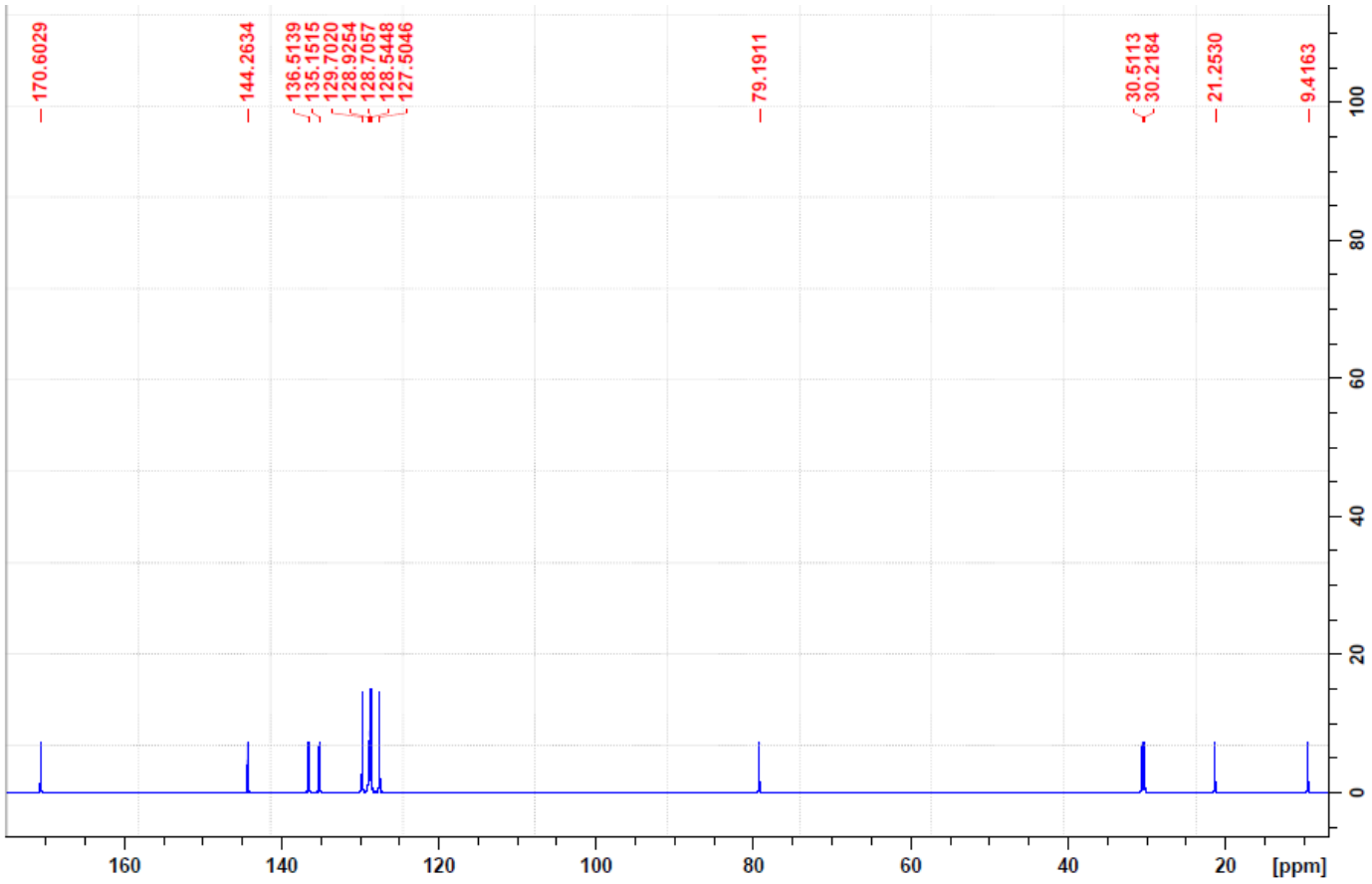
Appendix 9: Spectral data for *N*-(1-{[(4-methylphenyl)sulfonyl]amino}-2-phenylethyl)propanamide **(16)**



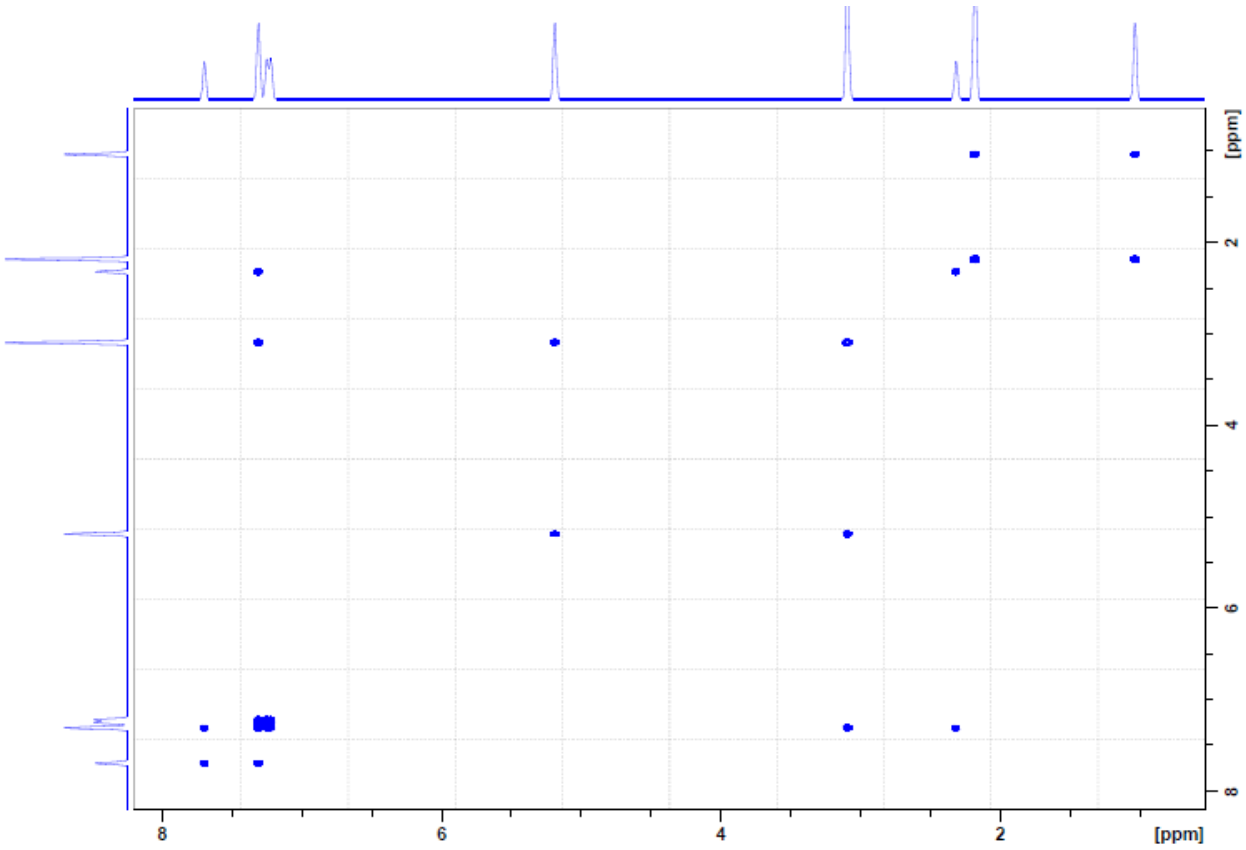
Appendix 9A: ^{1}H-NMR of 16



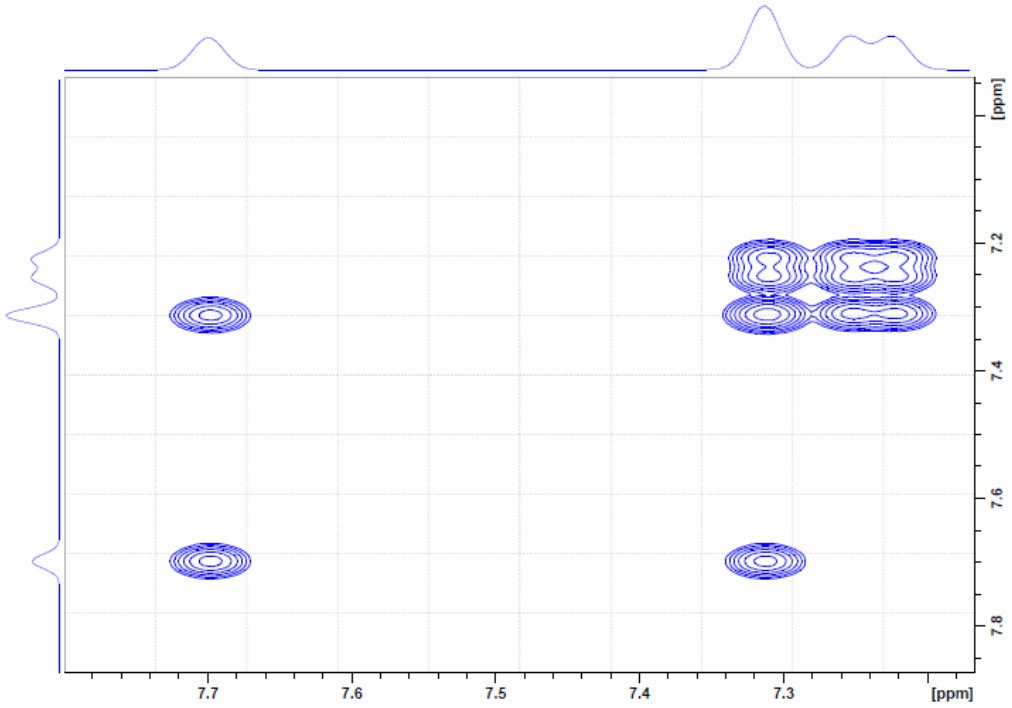
Appendix 9B: ^{13}C-NMR of 16



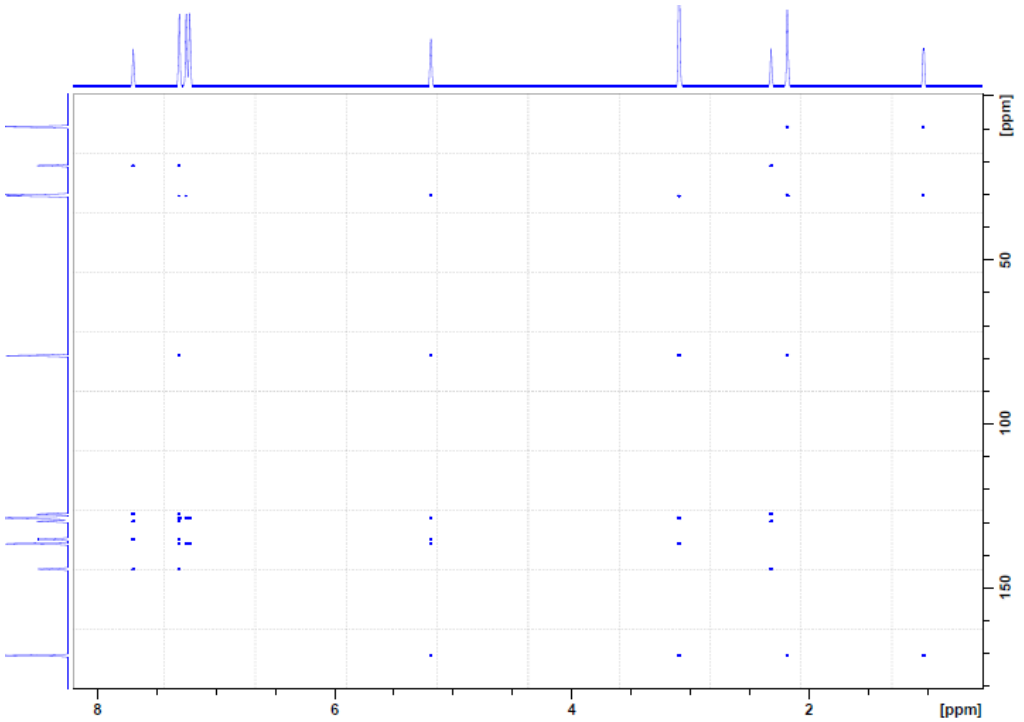
Appendix 9C(i): COSY of 16



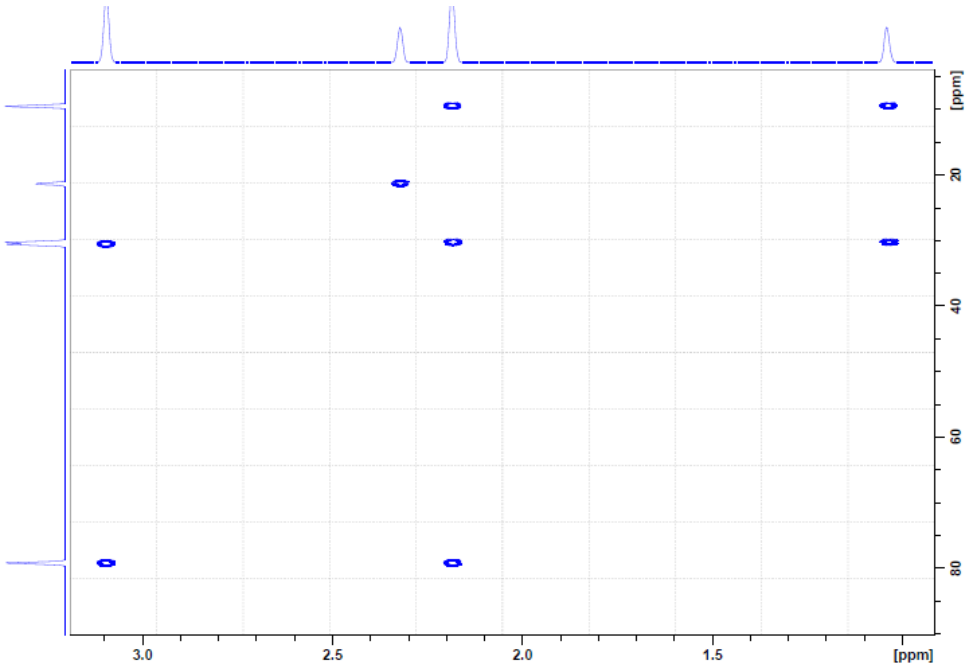
Appendix 9C(ii): COSY of 16



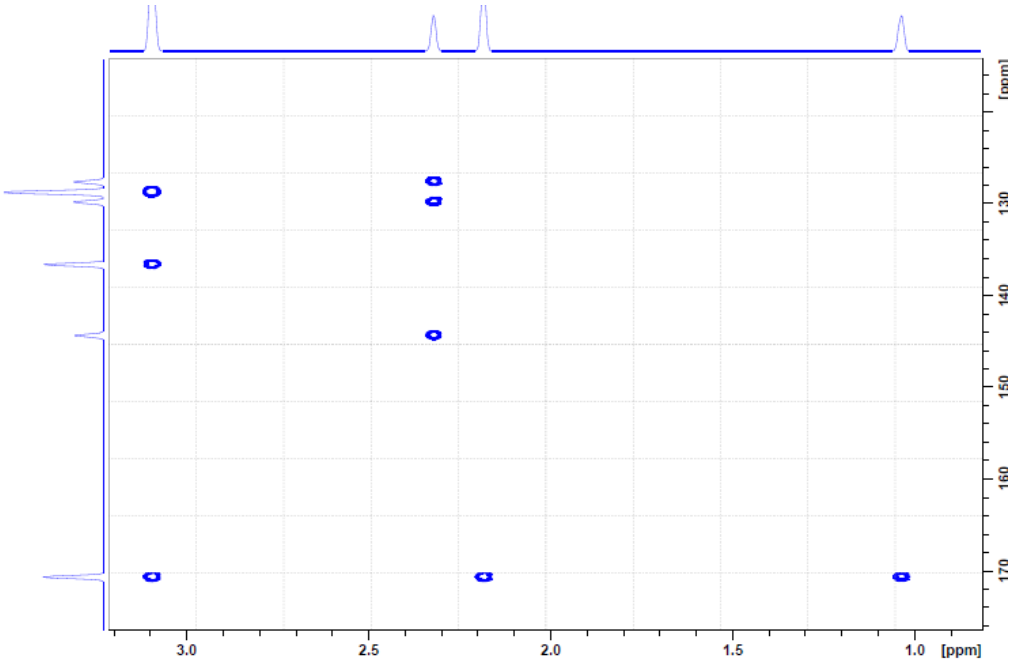
Appendix 9D(i): HMBC of 16



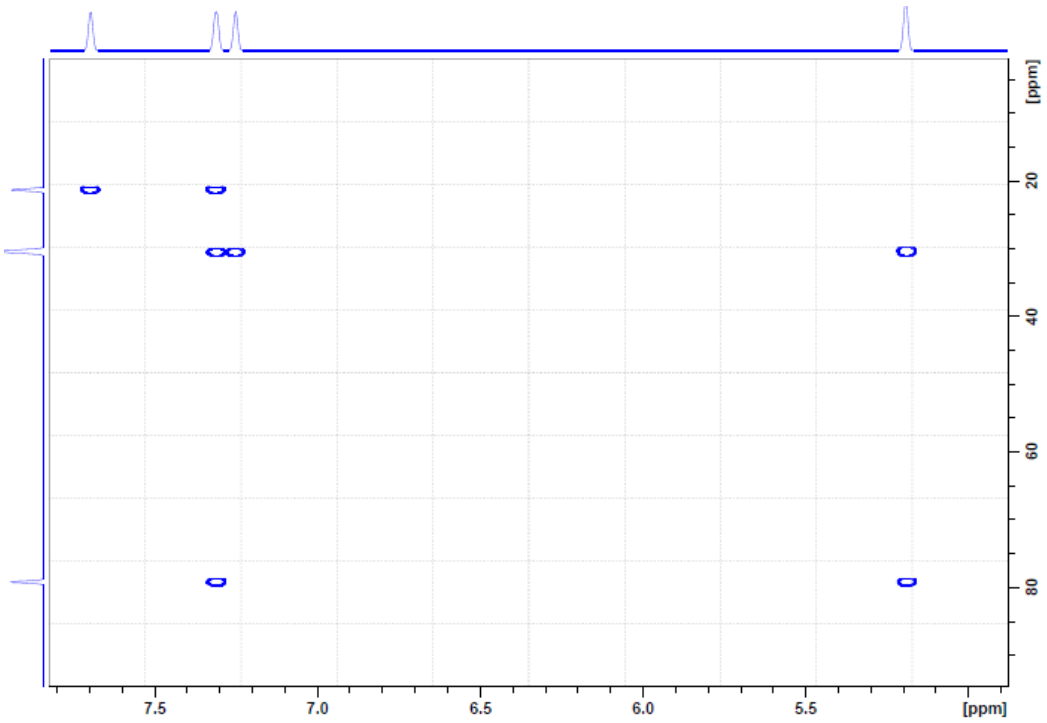
Appendix 9D(ii): HMBC of 16



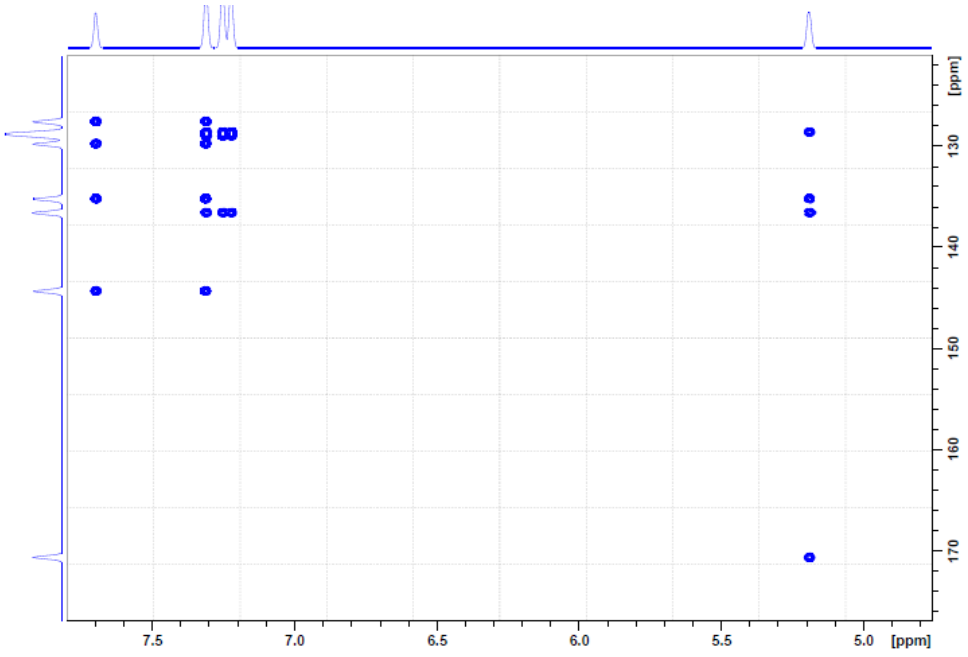
Appendix 9D(iii): HMBC of 16



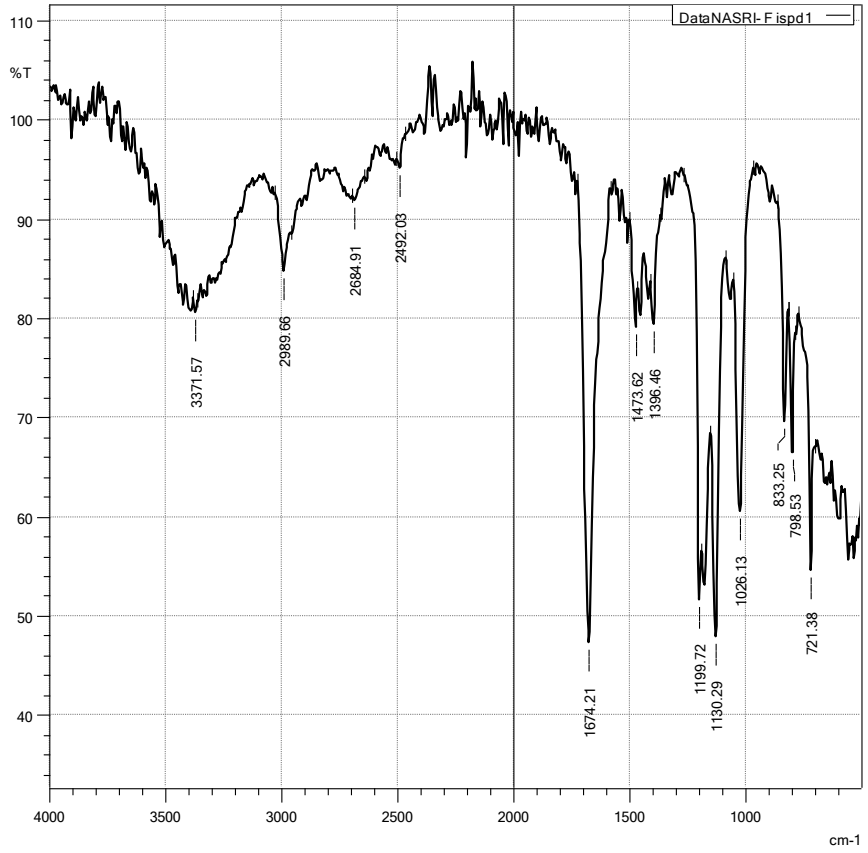
Appendix 9D(iv): HMBC of 16



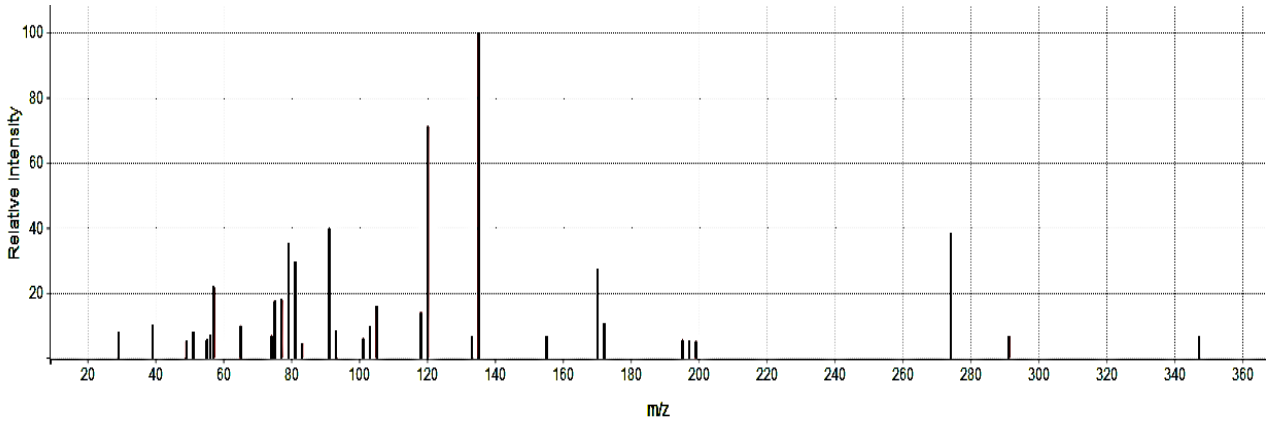
Appendix 9D(v): HMBC of 16



Appendix 9E: IR of 16



Appendix 9F: MS of 16



Appendix 10: Summary of % blood glucose reduction

group	diff_0	diff_30	diff_60	diff_90	diff_120	diff_180
metformin-500mg	9.5	-40.75	-42.25	-56.25	-61.5	-20.5
G-500mg	-12	-35.75	-58.75	-50.75	-38.5	-8.75
G-1000mh	2.5	-30	-46.5	-61	-43.5	-7.75
F-500mg	-5	-44.75	-37	-39.75	-30.25	6.5
F-1000mg	-13	-15.5	-59.17	-57	-38.67	-32.58

Appendix 11: Summary of pairwise comparison of the test groups

pairwise comparison of glucose levels at 0 minutes					
test group	comparator	mean difference	p-value	lci	uci
METFORMIN-500mg	SALINE	9.5	0.946	-24.690	43.690
G-500mg	SALINE	-12	0.869	-46.190	22.190
F-500 mg	SALINE	-5	0.997	-39.190	29.190
G-1000mg	SALINE	2.5	1	-31.690	36.690
F-1000 mg	SALINE	-13	0.827	-47.190	21.190
G-500mg	METFORMIN	-21.5	0.381	-55.690	12.690
F-500 mg	METFORMIN	-14.5	0.756	-48.690	19.690
G-1000mg	METFORMIN	-7	0.985	-41.190	27.190
F-1000 mg	METFORMIN	-22.5	0.334	-56.690	11.690
F-500 mg	G-500mg	7	0.985	-27.190	41.190
G-1000mg	G-500mg	14.5	0.756	-19.690	48.690
F-1000mg	G-500mg	-1	1	-35.190	33.190
G-1000mg	F-500mg	7.5	0.98	-26.690	41.690
F-1000mg	F-500mg	-8	0.973	-42.190	26.190
F-1000mg	G-100mg	-15.5	0.703	-49.690	18.690

pairwise comparison of glucose levels after 30 minutes					
test group	comparator	mean difference	p-value	lci	uci
METFORMIN	SALINE	-40.75	0.709	-131.871	50.371
G-500mg	SALINE	-35.75	0.804	-126.871	55.371
F-500mg	SALINE	-44.75	0.627	-135.871	46.371

G-1000mg	SALINE	-30	0.92	-128.422	68.422
F-1000mg	SALINE	-15.5	0.993	-106.621	75.621
G-500mg	METFORMIN	5	1	-86.121	96.121
F-500mg	METFORMIN	-4	1	-95.121	87.121
G-1000mg	METFORMIN	10.75	0.999	-87.672	109.172
F-1000mg	METFORMIN	25.25	0.945	-65.871	116.371
F-500mg	G-500mg	-9	0.999	-100.121	82.121
G-1000mg	G-500mg	5.75	1	-92.672	104.172
F-1000mg	G-500mg	20.25	0.978	-70.871	111.371
G-1000mg	F-500mg	14.75	0.996	-83.672	113.172
F-1000mg	F-500mg	29.25	0.902	-61.871	120.371
F-1000mg	G-100mg	14.5	0.997	-83.922	112.922

pairwise comparison of glucose levels after 60 minutes					
test group	comparator	mean difference	p-value	lci	uci
METFORMIN	SALINE	-42.25	**0.041**	-83.176	-1.324
G-500mg	SALINE	-58.75	**0.003**	-99.676	-17.824
F-500mg	SALINE	-37	0.088	-77.926	3.926
G-1000mg	SALINE	-46.5	0.077	-96.624	3.624
F-1000mg	SALINE	-59.16667	**0.006**	-103.372	-14.962
G-500mg	METFORMIN	-16.5	0.776	-57.426	24.426
F-500mg	METFORMIN	5.25	0.998	-35.676	46.176
G-1000mg	METFORMIN	-4.25	1	-54.374	45.874
F-1000mg	METFORMIN	-16.91667	0.809	-61.121	27.288
F-500mg	G-500mg	21.75	0.536	-19.176	62.676
G-1000mg	G-500mg	12.25	0.964	-37.874	62.374
F-1000mg	G-500mg	-0.4166667	1	-44.621	43.788
G-1000mg	F-500mg	-9.5	0.988	-59.624	40.624
F-1000mg	F-500mg	-22.16667	0.593	-66.371	22.038
F-1000mg	G-100mg	-12.66667	0.967	-65.501	40.168

pairwise comparison of glucose levels after 90 minutes					
test group	comparator	mean difference	p-value	lci	uci
METFORMIN	SALINE	-56.25	**0.001**	-90.057	-22.443
G-500mg	SALINE	-50.75	**0.002**	-84.557	-16.943

F-500mg	SALINE	-39.75	0.017	-73.557	-5.943
G-1000mg	SALINE	-61	**0.003**	-102.405	-19.595
F-1000mg	SALINE	-57	**0.002**	-93.516	-20.484
G-500mg	METFORMIN	5.5	0.994	-28.307	39.307
F-500mg	METFORMIN	16.5	0.619	-17.307	50.307
G-1000mg	METFORMIN	-4.75	0.999	-46.155	36.655
F-1000mg	METFORMIN	-0.75	1	-37.266	35.766
F-500mg	G-500mg	11	0.891	-22.807	44.807
G-1000mg	G-500mg	-10.25	0.962	-51.655	31.155
F-1000mg	G-500mg	-6.25	0.993	-42.766	30.266
G-1000mg	F-500mg	-21.25	0.571	-62.655	20.155
F-1000mg	F-500mg	-17.25	0.649	-53.766	19.266
F-1000mg	G-100mg	4	1	-39.645	47.645

pairwise comparison of glucose levels after 120 minutes					
test group	comparator	mean difference	p-value	lci	uci
METFORMIN	SALINE	-61.5	**0.001**	-98.477	-24.523
G-500mg	SALINE	-38.5	**0.039**	-75.477	-1.523
F-500mg	SALINE	-30.25	0.143	-67.227	6.727
G-1000mg	SALINE	-43.5	0.063	-88.787	1.787
F-1000mg	SALINE	-38.66667	0.06	-78.606	1.273
G-500mg	METFORMIN	23	0.376	-13.977	59.977
F-500mg	METFORMIN	31.25	0.123	-5.727	68.227
G-1000mg	METFORMIN	18	0.785	-27.287	63.287
F-1000mg	METFORMIN	22.83333	0.462	-17.106	62.773
F-500mg	G-500mg	8.25	0.976	-28.727	45.227
G-1000mg	G-500mg	-5	0.999	-50.287	40.287
F-1000mg	G-500mg	-0.1666667	1	-40.106	39.773
G-1000mg	F-500mg	-13.25	0.926	-58.537	32.037
F-1000mg	F-500mg	-8.416667	0.981	-48.356	31.523
F-1000mg	G-100mg	4.833333	0.999	-42.904	52.570

pairwise comparison of glucose levels at 180 min					
test group	comparator	mean difference	p-value	lci	uci
METFORMIN	SALINE	-20.5	0.424	-55.060	14.060
G-500mg	SALINE	-8.75	0.959	-43.310	25.810
F-500mg	SALINE	6.5	0.989	-28.060	41.060
G-1000mg	SALINE	-7.75	0.99	-50.077	34.577

F-1000mg	SALINE	-32.58333	0.105	-69.912	4.745
G-500mg	METFORMIN	11.75	0.872	-22.810	46.310
F-500mg	METFORMIN	27	0.174	-7.559	61.560
G-1000mg	METFORMIN	12.75	0.918	-29.577	55.077
F-1000mg	METFORMIN	-12.08333	0.893	-49.412	25.245
F-500mg	G-500mg	15.25	0.708	-19.310	49.810
G-1000mg	G-500mg	1	1	-41.327	43.327
F-1000mg	G-500mg	-23.83333	0.35	-61.162	13.495
G-1000mg	F-500mg	-14.25	0.876	-56.577	28.077
F-1000mg	F-500mg	-39.08333	**0.038**	-76.412	-1.755
F-1000mg	G-100mg	-24.83333	0.489	-69.449	19.783

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