ISOLATION AND CHARACTERIZATION OF NEW COMPOUNDS FROM *Strychnos henningsii* AND THEIR ANTI-MALARIAL ACTIVITIES

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NOVEMBER, 2013

DECLARATION

DECLARATION BY THE STUDENT

I declare that the research project presented here is my original work, and has not been presented in any institution for award of any degree.

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DECLARATION BY THE SUPERVISORS

This thesis has been submitted for examination with our approval as university supervisors

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DEDICATION

This piece of research is heartily dedicated to my wife, Josephine and son, Emmanuel Kithome for the strength, prayers and encouragement both have given me during this study period. This pushed me to carry on even when it was hard sometimes to go on.

ABSTRACT

Traditional medicines play an important role in the management of common fever associated with malarial symptoms, particularly in the rural Africa. However, their potential use as sources of medicines has not been fully exploited or even identified to know the active compounds. The present study was carried out to confirm the medicinal value of *Strychnos henningsii* in treating malaria as it has been traditionally used by the Kamba herbalist of Machakos, and Makueni and Embu Counties. The herbalists assisted in the identification, collection and use of this plant .the dried leaves were extracted with organic solvents of different polarities to yield crude extracts. The combined crude extract was subjected to chromatographic separation technique to yield pure crystalline compounds. Their structures were determined by the employment of spectroscopic technique especially proton NMR and ^{13}C which yielded nine compounds of which seven turned out to be new and unique indole alkaloids. The other two their structures could not be determined because the amount were too small for structural determination. The crude extract was tested for larvicidal activity against mosquito larvae and bioassay against *Plasmodium falciparum* that showed high activity.

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ACKNOWLEDGEMENT

I am greatly indebted to Prof. P. K. Ndalut who has been my research supervisor; under him I have learned so much on working with alkaloids in this project.

I would also like to appreciate the traditional healers who helped me in identifying the local name(s) of the plant used for treatment of malaria in the region.

I would also like to thank staff (both teaching and technical) of Chemistry and Biochemistry department of The University of Eldoret for their support during this period of study.

Last and not least, I thank God Almighty, my Savior, who provided for me and gave me good health during this long period of study.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is a parasitic disease that involves high fevers, shaking chills, flu-like symptoms, and anemia [1]. Malaria is a major public health problem in more than 90 countries, inhabited by more than 2.4 billion people - 40% of the world's population [2]. Globally, 247 million clinical episodes of malaria areestimated to occur every year, of which 86% are in sub-Saharan Africa. The disease is estimated to kill a child every 30 seconds and to cause up to 600million new infections worldwide annually [3]. Half of the world's population is at risk of malaria and an estimated 243 million cases led to nearly 863 000 deaths in 2008 [4]. Those at high risk are expectant mothers and children below 5 years.The advent of long-lasting insecticidal nets and artemisinin-based combination therapy, plus a revival of support for indoor residual spraying of insecticide, presents a new opportunity for large-scale malaria control. The World malaria report 2009 describes the global distribution of cases and deaths, how WHO-recommended control strategies have been adopted and implemented in endemic countries, sources of funding for malaria control, and recent evidence that prevention and treatment can alleviate the burden of disease.

Malaria is a major killer disease in Africa and a primary cause of death and poverty, undermining development in some of the poorest countries in the world. There were an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing

nearly a million deaths, mostly of children under 5 years [5]. 109 countries were endemic for malaria in 2008, 45 within the WHO African region.

Poor reproductive health accounts for up to 18% of the globalburden of disease and 32% of the total burden of disease for women of reproductive age [10]. According to the report, maternalrelated conditions contribute the largest share and are actually thenumber one cause of death, followed by malaria and HIV/AIDS indeveloping countries.

The Kenya National Health Sector is striving to address this problemthrough the implementation of the Strategic Plan-II which recognizesthe role played by traditional healthcare providers such as Traditional Birth Attendants (TBAs). Indigenous knowledge is an integral part of life in rural thirdworld communities. Rather than ignore it, this knowledge should beexplored, strengthened through research and scientific evidence documented and disseminated especially to healthcare providersso that they can become informed about the actual properties of herbs used during pregnancy [11].

Literature shows that herbal remedy use during pregnancy iscommon in developing and even developed countries. An Australiansurvey of three hundred women attending antenatal clinicsuggested that 12% had taken herbal medicine during their pregnancy ((11)) while in the USA, a survey of 200pregnantwomen demonstrated that 15% used home remedies (ginger,chamomile, and cola) in an attempt to relieve morning sickness.

According to WHO estimates, 80% of rural poorin developing countries rely solely on herbal medicine as theironly form of remedy for a variety of health issues. Supportingevidence has been presented by several other authors, furthermore, suggested that thisbroad use of traditional medicine could be attributable to accessibilityand

affordability of the herbs. In these ruralcommunities,sick persons will consult regular physicians only as a last resort [12].

Though the majority of the cases and approximately 90% of the malaria deaths are found in Sub-Saharan Africa, the disease is growing in Asia and Latin America.

1.2 Life cycle of the malaria parasite

The malaria parasite develops both in humans and in the female Anopheles mosquitoes. The size and genetic complexity of the parasite mean that each infection presents thousands of antigens to the human immune system. The parasite also changes through several life stages even while in the human host, presenting different antigens (proteins) at different stages of its life cycle. [13] Understanding which of these can be a useful target for vaccine development has been complicated. In addition, the parasite has developed a series of strategies that allow it to confuse, hide, and misdirect the human immune system.

1.3 The causes of human malaria

The four malaria species that produce human disease are *P. vivax, P. falciparum, P. malariae* and *P. ovale*. *Plasmodium falciparum*, the most lethal strain, is the most prevalent species throughout the tropics and subtropics [14].

Malaria is usually transmitted when a person is bitten by an infected female Anopheles mosquito. Only Anopheles mosquitoes transmit malaria and to do so the mosquito must have been infected by having drawn blood from a person already infected with malaria.

Malaria is a serious disease, which can be fatal. Malaria is caused by a parasite and the clinical symptoms of the disease include fever and flu-like illness, such as chills, headache, muscle aches, and tiredness. These symptoms may be accompanied by nausea, vomiting, and diarrhea. Malaria can also cause anemia and jaundice (yellow coloring of the skin and eyes) because of the loss of red blood cells.

Infection with one type of malaria, *Plasmodium falciparum*, if not promptly treated, may cause kidney failure, seizures, mental confusion, coma, and death.

Although malaria can be fatal, illness and death can be prevented with proper diagnosis and treatment.

The existence of malaria as an enemy of humankind certainly predates written history [15]. For thousands of years malaria has been a deadly scourge, and it remains one today. From American president John Adams who nearly succumbed to malaria in Amsterdam while on a diplomatic mission, back down the timeline to the early Chinese, Indians, Greeks, and Romans, malaria has not spared its victims, rich or poor.

For thousands of years, traditional herbal remedies have been used to treat malaria; [16] the historian Herodotus (484–425 BC) wrote that the builders of the Egyptian pyramids were given large amount of garlic [17], likely to protect them against malaria. Sneferu, the founder of the Fourth dynasty of Egypt, reigning from around 2613 BC to 2589 BC, used bed nets as protection against mosquitoes. In her day (69–30 BC), Cleopatra VII, the last Pharaoh of Ancient Egypt, also slept under a mosquito net. Malaria became widely recognized in ancient Greece by the 4th century BC, and is implicated in the decline of many city-state populations. Hippocrates (460–370 BC), the "father of medicine", related

the presence of intermittent fevers with climatic and environmental conditions and classified the fever according to periodicity: *Tritaiospyretos* / *Febristertiana*, and *Tetrataiospyretos* / *Febrisquartana* (every fourth day).

It wasn't until the 19th century that information about the true cause of malaria became known. Yet despite this knowledge, malaria still ravages Sub-Saharan Africa, South-East Asia and Latin America, taking as itsvictims mainly young children and pregnant women.

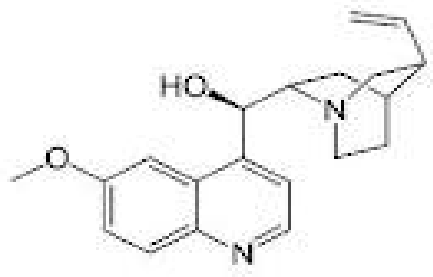
However, without certain discoveries leading to a better understanding of the biology of malaria, organizations like Medicines for Malaria Venture (MMV) would not be in the position to seek new solutions to treating this deadly disease, creating a glimmer of hope that one day malaria will be truly just another page in the history books.

1.4 Background on malaria treatment

Two important currently used antimalarial drugs are derived from plants whose medicinal values had been noted for centuries: [18] artemisinin from the Qinghao plant (*Artemisia annua L*, China, 4th century) and quinine from the cinchona tree (South America, 17th century).

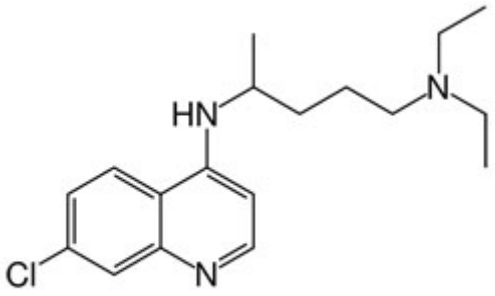
1.5Malaria treatment milestones

1.5.1 Quinine((*R*)-(6-methoxyquinolin-4-yl)((2*S*,4*S*,8*R*)- 8-vinylquinuclidin-2-yl)methanol) [19]



Quinine comes from the bark of a tree native to South America. According to legend it was first brought to Europe by a Countess who had been treated with it in Peru in the 1600s. The bark was named *Cinchona* in 1742 by Linnaeus. In 1820, two French chemists isolated quinine from the cinchona bark and quinine became a treatment of reference for intermittent fever throughout the world. It is a natural white crystallinealkaloid having antipyretic (fever-reducing), antimalarial, analgesic (painkilling), anti-inflammatory properties and a bitter taste [20]. It is a stereoisomer of quinidine which, unlike quinine, is an anti-arrhythmic. Quinine contains two major fused-ring systems: the aromaticquinoline and the bicyclicquinuclidine.Quinine remains an important and effective treatment for malaria today, despite sporadic observations of quinine resistance.

1.5.2 Chloroquine

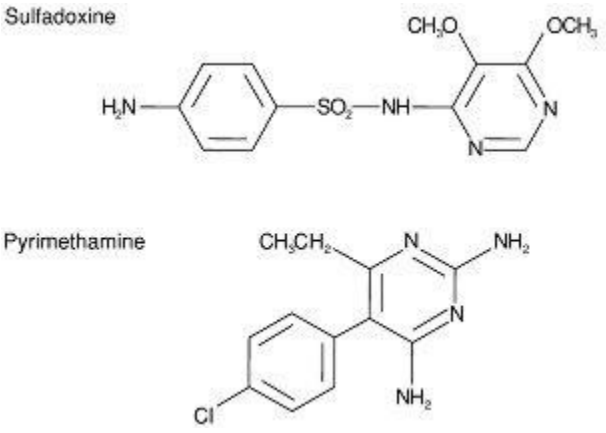


Chloroquine

Research by German scientists to discover a substitute for quinine led to the synthesis in

1934 of Resochin (chloroquine) and Sontochin (3-methyl-chloroquine) ((21)). These compounds belonged to a new class of antimalarials, the four-amino quinolines. Following the war, chloroquine and dichlorodiphenyltrichloroethane (DDT) emerged as the two principal weapons in WHO's global eradication malaria campaign. Subsequently, chloroquine resistant *P. falciparum* probably arose in four separate locations starting with the Thai-Cambodian border around 1957; in Venezuela and parts of Colombia around 1960; in Papua New Guinea in the mid-1970s and in Africa starting in 1978 in Kenya and Tanzania and spreading by 1983 to Sudan, Uganda, Zambia and Malawi.

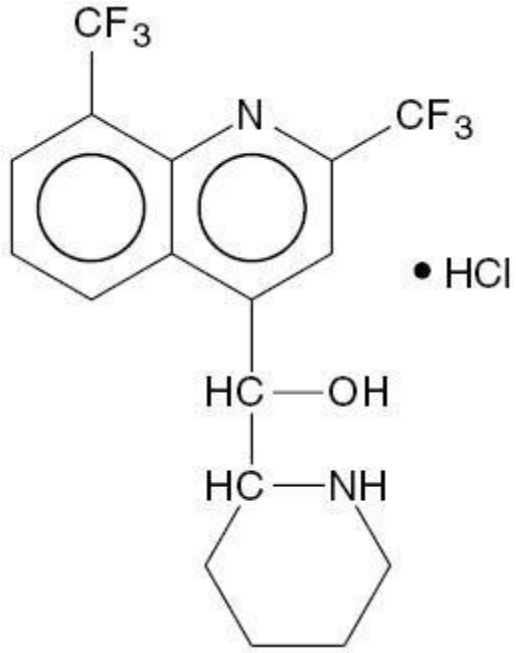
1.5.3 Sulfadoxine/Pyrimethamine(4-Amino-*N*-(5,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide)



A pyrimidine derivative, proguanil, also emerged from the antimalarial pipeline during World War II. Proguanil's success in treating humans led to further study of its chemical class and to the development of pyrimethamine. Resistance to the two monotherapies appeared quickly (within one year in the case of proguanil). Sulfones and sulfonamides were then combined with proguanil or pyrimethamine in hopes of increasing efficacy and forestalling or preventing resistance. By 1953, *P. falciparum* resistance had already been

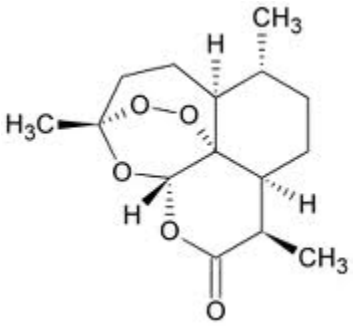
noted in Tanzania. When Sulfadoxine/Pyrimethamine (SP) was introduced in Thailand in 1967, resistance appeared that same year and resistance spread quickly throughout South-East Asia. Resistance to SP in Africa remained low until the late 1990s but since then it has spread rapidly.

1.5.4 Mefloquine([(*R**,*S**)-2,8-bis(trifluoromethyl)quinolin-4-yl]-(2-piperidyl)methanol)



The development of mefloquine was a collaborative achievement of the US Army Medical Research and Development Command, WHO/TDR and Hoffman-La Roche, Inc. Mefloquine's efficacy in preventing falciparum malaria when taken regularly was shown in 1974 and its potential as a successful treatment agent was shown soon after. Resistance to mefloquine began to appear in Asia in 1985, around the time the drug became generally available.

1.5.5 Artemisinin



Artemisinin was isolated by Chinese scientists in 1972 from *Artemisia annua* (sweet wormwood), better known to Chinese herbalists for more than 2000 years as Qinghao ((22)). In the early 1970s, initial testing by Chinese scientists of *Qinghao* extracts in mice infected with malaria showed it to be as effective as chloroquine and quinine in clearing the parasite. Mao Tse Tung's scientists then began testing in humans and in 1979 published their findings in the Chinese Medical Journal.

Artemisinin and other artemether-group drugs are currently the main line of defense against drug resistant malaria in many parts of South-East Asia. To date there have been no reported cases of resistance to artemisinin.

Artemisinin is today a very potent and effective anti-malarial drug, especially when used in combination with other malaria medicines.

Use of artemisinin-based combination therapies (ACTs) hasincreased compared to 2006 but remains very low in most Africancountries; in 11 of 13 countries surveyed during 2007–2008, fewer than 15% of children under 5 years of age with fever had received an ACT.((23))

For over 1,500 years Chinese herbalists have used leaves from the *Artemisia annua* shrub (sweet wormwood) to treat malaria. However, it is only in the late 1960s that its anti-malarial ingredient, artemisinin, was identified and extracted. Today, artemisinin is considered the treatment of choice for uncomplicated falciparum malaria, as prescribed by the World Health Organization (WHO) in 2001.

Artemissia annua grows in temperate regions and in tropical areas at higher altitudes. As a treatment for malaria, it has traditionally been grown in Asia, predominantly China and Vietnam, where it was originally harvested from the wild. Due to the rapid increase in demand and the relatively low dry-leaf and artemisinin yield from wild varieties, most *A. annua* is now commercially grown on small holdings and larger plantations. This has resulted in the development of new high-yielding varieties, producing between 1 and 4 tonnes per hectare of dry leaf and up to 1.2% artemisinin content.

Commercial cultivation of *A.annua* has now spread to other parts of Asia, including India, and also to Africa, especially East Africa. Production 'trials' of the plant, and seed development, are also being conducted in Europe, Australia, and Brazil.

The extraction of artemisinin from the plant has traditionally used N-Hexane, a cost-effective solvent. However, N-Hexane is poisonous and flammable, raising potential safety and environmental concerns. This has lead to the search for new, safer solvents to enable the extraction of artemisinin, including supercritical CO_2, Ethanol, HFCs (hydrofluorocarbons) and Ionic Liquids.

Following its extraction and purification from the plant, artemisinin is processed synthetically under Good Manufacturing Practice (GMP) guidelines into a range of

derivatives, including dihydroartemisinin (DHA), artesunate, arteether, and artemether. It is in one of these forms that it is combined with other suitable anti-malarials e.g., amodiaquine, piperaquineetc, to produce ACTs (artemisinin-based combination therapies).

In early 2006, due to global concerns about the need to safeguard the efficacy of this antimalarial, the WHO urged the malaria community to stop the production and use of artemisininmonotherapies and switch to ACTs.

The use of drug combinations, including non-artemisinin-based and artemisinin-based combination therapy (ACT), is a novel strategy that enhances therapeutic efficacy and delays the emergence of multidrug-resistant *Plasmodium falciparum* ((24)).

1.6 The future - Synthetic drugs

It is a triumph for malaria treatment, but bad news for farmers. A synthetic version of the world's most effective antimalarial drug, artemisinin, can now be made in just three weeks rather than 18 months. The advance could help to stem the rise of drug-resistant malaria.Despite artemisinin's success, the malaria parasite is developing resistance to it. One way to delay resistance is by offering artemisinins combined with other drugs. However, the World Health Organization says 25 countries still allow artemisinin to be sold on its own, with 28 companies manufacturing it.

1.7 Problem statement

Malaria exerts high death toll in Africa especially to children below 5 years. In Kenya, Malaria is one of the leading causes of morbidity and mortality as it kills an estimated 34,000 children less than five years every year. 77% of Kenya's population lives in areas where the disease is transmitted.

Malaria has caused a heavy hospital burden to many in developing countries and many people spend many man hours treating the disease or looking for treatment of the same and as such many populations in sub-Saharan Africa cannot afford the available conventional medicine. In Kenya, the disease is responsible for 30% of out-patient visits (requiring more than eight million out-patient treatments at health facilities each year) and 15% of all hospital admissions. About 3.5 million children are at risk of infection and developing severe malaria.

Drug resistant malaria has become one of the most important problems in malaria control in recent years. Therefore there is need to develop and extract new compounds with activity against *Plasmodium spp*. for treatment and applications in combination therapies.

Many indigenous communities have used herbs in treatment of life threatening ailments including malaria and this is knowledge which is disappearing drastically. Hence the need to research and document such knowledge and plants used for the treatments.

1.8 Justification

The Kamba community from Eastern Province has for many years used the leaves of *Strychnos henningsii* to treat malaria and related ailments like fever, shivering and common cold. This traditional knowledge has been practiced for generations by community wherever they have been. Unfortunately, lately with the introduction of civilization in the 19th century, such traditional knowledge on treatment is rapidly disappearing. Therefore there is urgent need for modern scientific validation to make it relevant to modern healthcare systems in this country.

Furthermore the increasing rate of drug resistance to malaria is an incentive to look for alternative method of malaria management and treatment.

1.9 Objectives

The main objective is to scientifically validate the traditional knowledge of anti-malarial drug use of *Strychnos henningsii*. Specific objective is to extract crude extract , isolate active compounds and determine their structure. Secondly to confirm the bioactivity of the isolated active compounds. a crude leave extract from the leaves of *Strychnos henningsii* to test for activity against *Plasmodium falciparum*. To further isolate and estimate percentage and physical properties of pure compounds by column chromatography and structural analysis (characterization) of the isolated pure compounds using Nuclear Magnetic Resonance (NMR).

To carry out bio-assay of the crude extract of *Strychnoshenningsii* and a combined therapy bio-assay of the crude extract of *Strychnoshenningsii* with *Artemisiaannua* crude extract (which is the current drug of choice) and test whether the combination gives a better activity against *Plasmodium falciparum* as compared with the singular crudes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Species description and distribution

Strychnos is a genus of flowering plants, belonging to family Loganiaceae. The genus is panotropical and comprises of about 200 species of trees and lianas, distributed around the world's tropics ((25)).They are subdivided into three groups: one in Asia with 44 species, one in America with about 70 species and one in Africa with 75 species. Although investigations into the genus have been going on for a long time, the African members suffered a long period of neglect ((26)). The genus has been associated with poisonous plants such as*Strychnostoxifera* as known by indigenous Indians of South America which was used for hunting monkeys and fighting their enemies. Some of the species of this genus that research has been conducted include: *S. toxifera, S.nux-vomica, S. staudtii, S. rubiginosa, S. diplotricha, S.myrtoides, S. spinosa, S. longicaudata, S. ngouniensis, S. variabilis,* e.t.c. Majority of these plants have alkaloids that have been isolated displaying activity against common pathogens.

Botanic description

Strychnos henningsii is a small erect, much-branched tree, 2-12 m tall with a clean green-reddish stem. Bark peeling, crown compact with dark green, glossy foliage ((27)). Twigs are represented with pale ashy or straw colored waxy skin splitting lengthwise. Lenticels are very few and inconspicuous. Leaves opposite, sub-sessile, ovate, 2.5-6.5 cm long and

0.8-4.5 cm wide, margins entire, leaf tips acuminate. Leaves strongly 3-5 nerved from base, cuneate or rarely sub-cordate at base. Floral cymes borne on flat clusters in the leaf axils, 2-2.5 mm long, 4 mm wide when open, scented, yellowish-green in colour turning orange with age. Fruit up to 1.9 cm long and 6-11 mm broad, oblong or roundish, 1-2 seeded (coffee-like); red, brown or orange when ripe. The genus *Strychnos* has about 190 species mainly found in the tropics.

An ethnobotanical survey was conducted on the medicinal plants of Eastern Cape of South Africa ((28)). The decoction of the roots leaves and barks of *Strychnos henningsii*and *Leonoti sleonorus*of the families *Loganiaceae* and *Lamiaceae* taken orally for a long period (6-12 months), depending on the severity of theailments, are widely used for the management of diabetes mellitus ((29)).

Research conducted on medicinal plants used for treatment of common ailments in lower eastern Kenya identified *S. henningsii* as one of the key plant used for management of diabetes, arthritis, stomach ulcers and malaria ((30)).

Functional uses

In East Africa *S. henningsii* is used in the preparation of fatty-meat and milk soups ((31)). Timber: Its valued timber is brown to dark grey, heavy, hard, durable and termite-resistant. Wood used for fencing, hut poles, and tool handles. Poison: The bark contains a poisonous bitter alkaloid causing paralysis. Despite this the plant still has significant medicinal uses. Medicine: *S. henningsii* is used in African traditional medicine to treat various ailments including rheumatism, syphilis, gastrointestinal disorders (purgative) and snake bites. The ground bark is a mouth antiseptic and applied onto wounds in cattle

and horses to hasten healing. Some of the applications can be explained partially by the presence of reticuline-like alkaloids. *S. henningsii* has potential in the development of newantinociceptive(reducing sensitivity to painful stimuli) and antispasmodic(drug or aherb that suppresses muscle spasms)drugs.

Common uses:

The tree has been used by some communities in diverse ways. These include:

a)Erosion control: The species is important in protecting soils from water erosion in highlandareas.

b)Shade or shelter: *S. henningsii* is an important shade tree.

c)Ornamental: The physical attributes of *S. henningsii*, shiny foliage, pleasant shade and fragrant flowers make it a suitable choice for gardening.

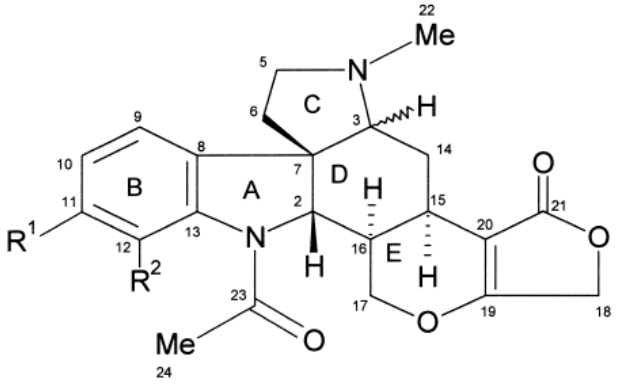
d)Boundary or barrier or support: *S. henningsii* is used as a live fence and its poles are good for fencing.

Related research from close plant types

A good number of species of *Strychnos* plants have exhibited activity against *Plasmodium spp* ((32)). The *in vitro* anti-malarial activities of 46 alkaloids and extracts from *Strychnos* species have been evaluated. Two types of quasidimeric alkaloids exhibit high and selective activities against *Plasmodium*. Strychnopentamine and isostrychnopentamine have been found to be active against chloroquine-sensitive and -resistant strains (50% inhibitory concentration [IC_{50}] ≈ 0.15 μM) ((33)), while dihydrousambarensine exhibited a 30-fold higher activity against the chloroquine-

resistant strain (IC_{50} = 0.03 μM) than it did against the chloroquine-sensitive strain. The common species of these include *Strychnos usambarensis* and *Strychnos madagascariensis.*

*Strychnos madagscarians*is one of the plants in the family whose compounds are now on clinical trials. The following compounds have been isolated from *Strychnos diplotricha and Strychnos myrtoides* ((34)).

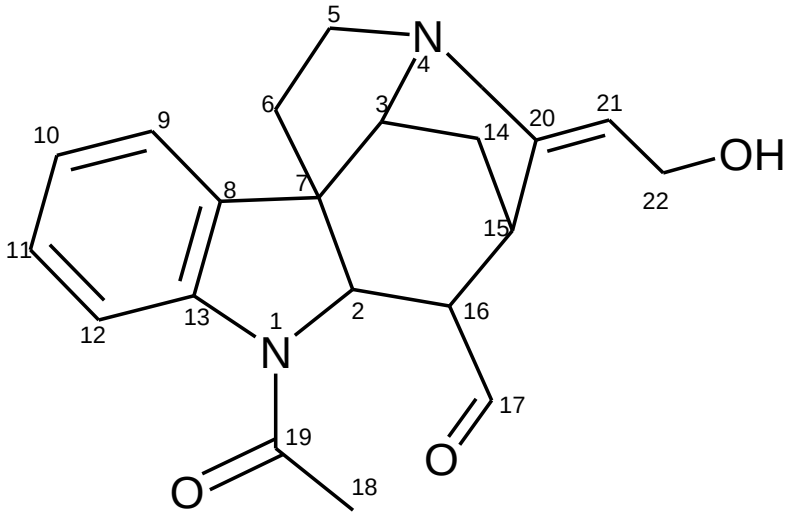


	R^1	R^2	H-3	
1	OMe	H	α	3-*epi*-myrtoidine
2	H	H	α	11-demethoxy-3-*epi*-myrtoidine
3	H	OH	α	11-demethoxy-12-hydroxy-3-*epi*-myrtoidine
4	OMe	H	β	myrtoidine
5	H	H	β	11-demethoxymyrtoidine

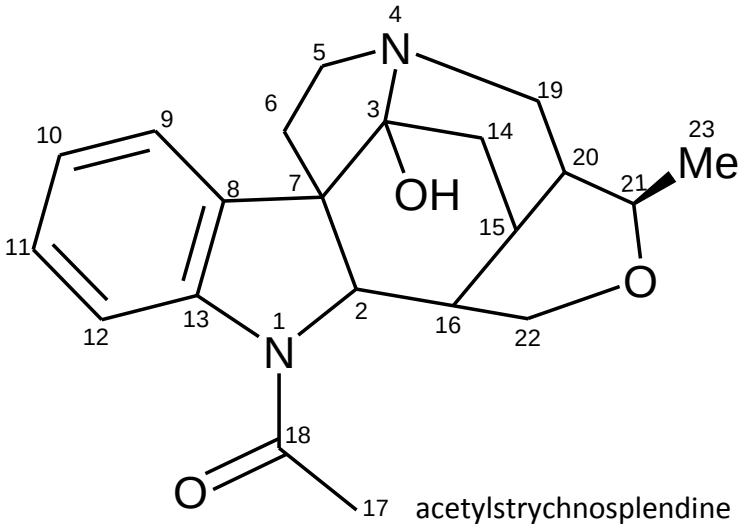
2.5 Scientific research done on *Strychnos henningsii*

The *Strychnos* family has been researched extensively and *S. henningsii* has not escaped. From *S. henningsii*, four alkaloids have been isolated and reported from the root bark. These are diaboline, holstiine, N(a)-acetylstrychnosplendine and N(a)-acetyl-11-methoxystrychnosplendine ((35)). Their structures are shown below:

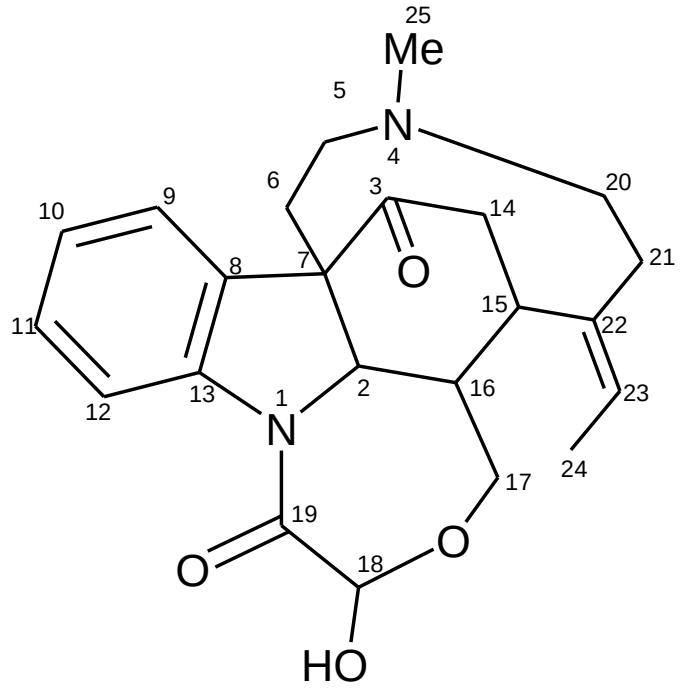
Diaboline



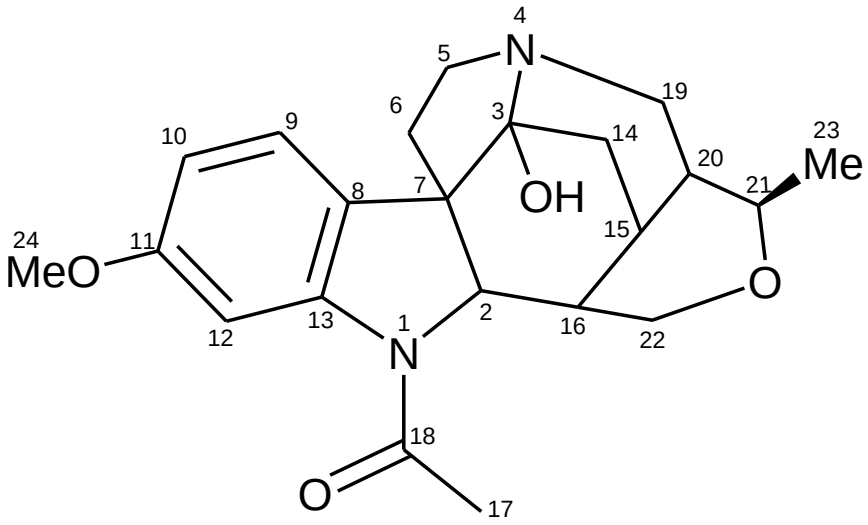
Holstiine



N(a)- acetylstrychnosplendine



N(a)-acetyl-methoxystrychnosplendine



However, leave extracts have not been reported.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Requirements

The following are some of the solvents obtained from Kobian Chemicals Ltd. (Kenya)

- n-hexane General Purpose Reagent (GPR) 99%
- Ethyl acetate – General Purpose Reagent (GPR) 99%
- Chloroform – General Purpose Reagent (GPR) 99%
- Deuterated chloroform – General Purpose Reagent (GPR) 99%
- Methanol – General Purpose Reagent (GPR) 95%

All the solvents were double distilled to improve on their purity.

Also obtained from Kobian Chemicals included:

- Silica gel of particles size 0.063-0.2 mm
- TLC plates of the type Algram^{R} GUV_{254}
- Whatman R filter papers

There were reagents also obtained from KEMRI – Nairobi:

- 12, 96 – well microculture test plates
- Micropipettes, ^{3}H – hypoxanthine label, RPMI-1640, N_2 CO_2 and O_2 gases, Gas box
- 96 – well test plate harvester integrated with a microbeta reader counter
- Concentrated sulphuric acid of battery acid grade.

Anopheles gambiae eggs were obtained from International Centre for Insect Physiology and Ecology (I.C.I.P.E) Nairobi.

Kitchen yeast used for larval food was bought from *Tuskys* supermarket in Eldoret.

Sample vials were obtained from Chemistry and Biochemistry Department, University of Eldoret.

Water was distilled at University of Eldoret.

The BuciiR-114 rotavapor of the Chemistry Lab III was used.

Bruker WM. 200MHz NMR machine of The University of Nairobi was used.

3.2 Experimental: sample collection

Following a survey on the ethno-medical use of the plant in the Ukambani and Mbeere regions (Machakos and Embu Counties), in the Eastern Province of Kenya through interviews with traditional health practitioners (THPs), several *S. henningsii* leave samples were collected for the study. A good amount of the samples were collected in Yatta district, of Machakos County. The plantwas botanically authenticated by a taxonomist and voucher specimens deposited at the East African Herbarium, National Museums of Kenya (NMK). Only the leaves were collected for the study since it was the only part used ethno-botanically by the communities. This was done in August 2007.

3.3 Preparation and extraction of plant materials

The leaves were air-dried under shadefor 14 days, and then ground using a kitchen blender obtained from the Department of Botany. Approximately 120g of the ground powder was soaked in 2.5 liters of methanol. The residue was then resoaked in 5% water/methanol to extract any other remaining compounds from the ground plant material. The two portions of the crude extract were then filtered using a cotton wool into

a larger beaker where they were concentrated under reduced pressure temperature range of 30°C and 50°C using a Buchii rotavapor. The crude extract weighed 42 g.

3.4 Isolation of the compounds from the crude extracts

A preliminary chromatographic analysis was carried out to roughly ascertain the number of compounds present in the crude extract. This was also done to approximate the polarity of the compounds from the crude extracts. This was achieved by carrying out a thin layer chromatography (TLC) profile analyses with careful varying of polarities of various solvents (hexane, dichloromethane, ethyl acetate, acetone and methanol). Sephadex was used to partition the crude extracts with 1:1 dichloromethane/methanol to remove the chlorophyll from the crude extracts. The active compounds known from the *Strychnos* species are alkaloids. Therefore a special procedure for the separation of alkaloids from the other compounds was done. This involved running/partitioning of the crude extracts into a column with the least polar solvents, such as, hexane ((36)). The remaining crude extract was then partitioned between 0.1M tartaric acid and ethyl acetate. This was two-step procedure whereby the first partition was done to obtain neutral and weakly basic alkaloids which are contained in the ethyl acetate layer. The aqueous layer was then neutralized with NH_4OH and again extracted with ethyl acetate. The organic layer now contained strongly basic alkaloids while the aqueous layer had quaternary ammonium ions.

The crude extract mixture of alkaloids were then isolated by column chromatography. The crude alkaloid extract weighed 14.5 grams.

3.5 Column chromatography

This is a gravity column which was mounted by filling a 4 cm internal diameter and 37 cm length with dichloromethane (DCM). Prepared slurry of silica gel was then added in the column. To achieve a bubble free column the gel was allowed to settle under gravity with occasional tapping of the column with a rubber cork for the present bubbles to elute to the top of column. This was then left overnight to settle before mounting the mixture of alkaloids.

3.6 Elution

A dry sample of the crude extract weighing 10 grams was mixed with silica gel in the ratio of 1:1 in methanol. Then the mixture was placed in a rotavaping flask then concentrated to dryness under reduced pressure using a rotary evaporator (Buchi R-114) to avoid decomposition of the compounds. This was then introduced into the already packed column.

Elution was carried out with opening of the tap with a concentration of 100% dichloromethane and slowly varying the polarity with methanol upto 10% methanol in dichloromethane. Finally the column was washed with 95% methanol.

The eluent was collected in 60ml test tubes. These were pooled together with similar compounds and concentrated *in vacuo*. The fractions containing pure compounds were allowed to crystallize. Purity of the collected fractions was checked using a thin layer chromatography (TLC) plate. Pure fractions gave a single distinct spot on TLC.

3.7 *In vitro* anti-plasmodial assay

The *in vitro* semi-automated micro-dilution assay technique that measures the ability of theextracts to inhibit the incorporation of [G-3H] hypoxanthine into the malaria parasite was used(Amersham International, Burkinghamshire, UK).

Laboratory adapted *P. falciparum* cultures of ENT 30 (CQ-resistant isolate, originally from Entosopukia, Kenya) and NF 54 (chloroquine-resistant strain)

Two strains of *Plasmodium falciparum*: were used in the study. Parasite cultivation was carriedout using previously described procedures ((37)).

Test samples were prepared by dissolving aqueous extracts in distilled sterilized water while themethanolic extracts were dissolved in 100% DMSO (dimethylsulphoxide) and diluted to lower the concentration ofDMSO to ≤1%. Stock solutions (1µg/ml) of chloroquine and mefloquine were prepared for use as reference drugs. The semi-automated micro-dilution technique was adapted in assessing *invitro* anti-plasmodial activity. Briefly, 96-well flat-bottom microculture plates were pre-coated with test solutions in duplicate.

Test samples were prepared by dissolving 104 cells/well in 24-well plates and incubated at37ºC for 2 days. The culture medium was replaced by fresh MEM containing test extracts atdifferent concentrations, and incubated further for another 2 days. Cells for each sample were detached by trypsinization in triplicate wells, and the number of viable cells determined by atryphan blue exclusion test. A haemocy-tometer was used to aid in counting viable cells.

3.8 Reading the results using microbeta counter

Cassettes containing the filter mats were loaded into microbeta counter (maximum of sixteen cassettes) at a time ((38)).

This estimated radioactivity incorporated into each parasite nucleic acid from each well in a liquid scintillation counter. Values obtained were inversely proportional to the percentage inhibition of the parasite by the plant extract or drug sample. The concentrations of the drug samples that inhibit the growth of over 50% of the parasite (IC_{50}) indicated the biological activity in the drug test sample. This drug concentration is given by the equation:

$$IC_{50} = \frac{Anti\log\left(\log X_1 + \left(\log Y_{50} - \log Y_1\right)\left(\log X_2 - \log X_1\right)\right)}{\left(\log Y_2 - \log Y_1\right)}$$

Where Y_{50} is the cpm value midway between parasitisized and non-parasitisized control cultures and X_1,Y_1, X_2 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints ((39)).

CHAPTER FOUR

RESULTS

4.1 Column chromatography

The column showed clear separation of 9 pure compounds labeled: PKM 092, PKM 094, PKM 095, PKM 096, PKM 097, PKM 099, PKM 103, PKM 104 and PKM 107.

The compounds had the following percentages of separation and weights using the two solvents (Dichloromethane and methanol):

a) Compound PKM 092; 1%MeOH/DCM (16mg)

b) Compound PKM 094; 1%MeOH/DCM (11mg)

c) Compound PKM 095; 2%MeOH/DCM (4mg)

d) Compound PKM 096; 3%MeOH/DCM (9mg)

e) Compound PKM 097; 4%MeOH?DCM (15mg)

f) Compound PKM 099; 0.5%MeOH/DCM (8mg)

g) Compound PKM 103; 1%MeOH/DCM (3mg)

h) Compound PKM 104; 1%MeOH/DCM (12mg)

i) Compound PKM 107; 3%MeOH/DCM (18mg)

4.2 Physical properties

The compounds had similar properties in that when left for the solvents to evaporate, they crystallized to form powder like residues at the bottom of the test tubes. Other detailed physical properties of isolates from *Strychnoshenningsii* are shown in the table below:

Table 4. 1: Physical properties of the crude and pure compounds

Compound	**Purity**	**Physical state**
Crude	Impure	Slurry
PKM 092	Pure	Powdered white
PKM 094	Pure	Powder almost colourless
PKM 095	Pure	Nearly purple crystals
PKM 096	Pure	Brown powder
PKM 097	Pure	Dark brown powder
PKM 099	Pure	Crystalline powder
PKM 103	Pure	White powder
PKM 104	Pure	White powder
PKM 107	Pure	Brown powder

4.3 *In vitro* anti-plasmodial activity

Results of *in vitro* anti-plasmodial assay of methanolic and aqueous extracts of *Strychnos henningsii* leaves against *Plasmodium falciparum* D6 and W2 strains are summarized in table below.

Table 4. 2: Results of in vitro anti-plasmodial activity

Test samples	**IC_{50} Values**	**Comment**
Crude extract from *Artemisia annua*	3.2 ±1.63	Very active
Crude extract from *Strychnoshenningsii*	7.39 ± 2.65	Moderately active
Crude extracts from *Strychnoshenningsii* and *Artemisia annua*	4.52 ±1.70	Active

4.5 Structure elucidation of compounds

The NMR was done at 400 MHz and using the proton NMR and carbon NMR the following structures were proposed for the compounds.

a) PKM 092

This analysis will be quoted from NMR data at the appendix from pages 68 to 73. The data was analyzed comparatively with that of N-(a)-acetylstrychnosplendine as both data shows great similarity. The proton data for compound PKM 092 showed aromatic protons with absorptions of 7.3, 7.2, 7.1 and 6.9. Cyclic protons have absorption between 3.9 to 2.3. Also there are protons attached to electron withdrawing atoms like oxygen and shows peaks of 5.6, 3.9, 2.9, 3.5 and 3.6. The proton data suggested an indole structure which is consistent with alkaloids from *Strychnoshenningsii*. These were then compared with the ^{13}C spectra. The data showed 27 carbons in the compound and were given atom numbers as tabulated in table 4.3. C2 with 64.83 is in the third ring in the indole block. C3 is in the cyclo hexane and lower absorption suggests a de-shielded atom. C5 with 51.3 appears in the region of C-NR2. C8, C9, C10, C11, C12 and C13 all appear in the aromatic region. C14, C15 and C16 suggested carbons in a cyclic ring. C17 suggest a C-C bond but neighboring an electron withdrawing atom. C18 is highly de-shielded and suggested intra-molecular interactions with neighboring atoms. C19is a characteristic peak of the carbon attached to the carbonyl in the indole ring of the *Strychnos* alkaloids. C20, C21, C23 and C27suggested cyclic carbons attached to electron withdrawing atom. C22, C24, C25 and C26 have absorptions in cyclic region. C28 suggested a methyl group

as well as C29 but attached to a Nitrogen atom. With the above suggestions, the atoms were tabulated as shown below:

Table 4. 3:Tabulation of the carbon atoms and corresponding hydrogen atoms for compound labeled PKM 092

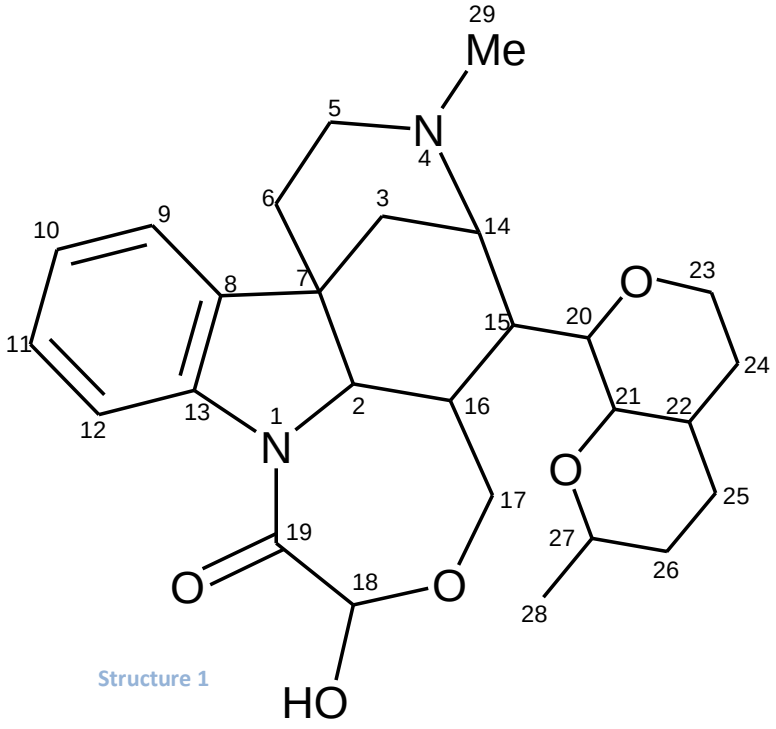
Atom	δ_C	δ_H	Atom	δ_C	δ_H
2	**64.83**	**3.9**	**17**	**59.45**	**3.5, 3.2**
3	**37.50**	**1.9, 1.6**	**18**	**92.84**	**5.6**
5	**51.3**	**2.3, 2.2**	**19**	**170.99**	
6	**37.50**	**1.9, 1.6**	**20**	**106.10**	**3.9**
7	**46.95**		**21**	**110.34**	**2.9**
8	**127.85**		**22**	**47.88**	**1.9**
9	**129.06**	**7.3**	**23**	**72.31**	**3.6, 3.5**
10	**125.15**	**7.2**	**24**	**47.19**	**1.7, 1.4**
11	**121.56**	**7.1**	**25**	**24.41**	**1.7, 1.4**
12	**119.48**	**6.9**	**26**	**28.28**	**1.7, 1.4**
13	**129.47**		**27**	**105.30**	**3.8**
14	**55.64**	**2.6**	**28**	**21.94**	**1.2**
15	**52.44**	**2.0**	**29**	**47.17**	**2.3**
16	**27.14**	**2.4**			

The above tabulated Carbon atoms were compared with their corresponding Carbon atoms from data published of N-(a)-acetylstrychnosplendine and tabulated as shown below:

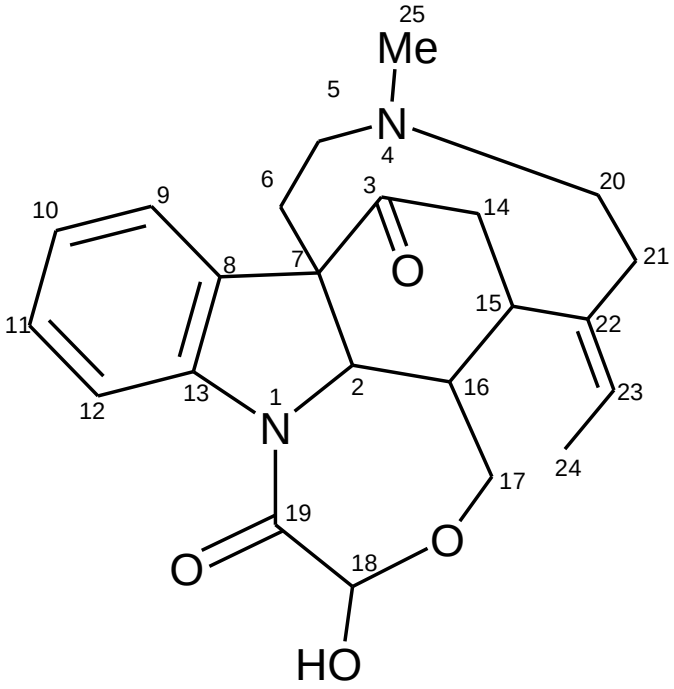
Table 4. 4Comparison of tabulated carbon atoms of PKM 092 and N(a)-strychnosplendine

Atom	PKM 092 δ_C	N(a)-δ_C	Atom	PKM 092 δ_C	N(a)-δ_C
2	**64.83**	**60.3**	**17**	**59.45**	**55.9**
3	**37.50**	**45.0**	**18**	**92.84**	**106.1**
5	**51.3**	**57.0**	**19**	**170.99**	**169.6**
6	**37.50**	**32.6**	**20**	**106.10**	**63.3**
7	**46.95**	**60.3**	**21**	**110.34**	
8	**127.85**	**131.2**	**22**	**47.88**	**55.9**
9	**129.06**	**127.8**	**23**	**72.31**	**77.9**
10	**125.15**	**109.6**	**24**	**47.19**	**40.7**
11	**121.56**	**158.1**	**25**	**24.41**	**20.3**
12	**119.48**	**104.5**	**26**	**28.28**	
13	**129.47**	**142.8**	**27**	**105.30**	
14	**55.64**	**33.7**	**28**	**21.94**	
15	**52.44**	**48.2**	**29**	**47.17**	
16	**27.14**	**38.2**			

With the above comparisons and analysis, the following structure of compound labeled PKM 092 was suggested. N(a)-acetylstrychnosplendine has been shown to correlate the close similarity between the two compounds isolated.



Structure 1



N(a)-acetylstrychnosplendine

b) PKM 094

This analysis will be quoted from NMR spectra in the appendix on pages 74 to 78. The raw NMR data was preliminary scanned and compared with that of the four already isolated compounds from *Strychnoshenningsii*. The closest is Holstiine. Therefore the following analysis and allocation of the atoms was done closely relating to that of Holstiine. The protons showed presence of aromatic protons in ranges of 7.8, 7.5, 7.3 and 6.9. Next were protons attached to electronegative atoms with ranges from 5.7 to 5.6. Cyclic protons occurred between 4.0 and 3.3. The other recorded protons are aliphatic protons. The ^{13}C data was then used to validate the already suggested proton regions. Comparing with the data for Holstiine, the carbon atoms were allocated numbers. C2 and C3 suggested cyclic carbons but C3 has higher absorption probably due to presence of attached to an electronegative atom. C5 and C6 suggested carbon atoms attached to a cyclic ring but the higher absorption for C5 suggests intramolecular interactions not experienced by C6. C8, C9, C10, C11, C12 and C13 suggested aromatic carbons. C7, C14 and C15 have absorption suggesting quarternary carbons. C16 and C17 suggested the other carbons completing the third cyclic ring very common in indole alkaloids. C17 with a higher absorption suggests it is attached next to electron withdrawing atom. C18 is highly de-shielded and suggested intra-molecular interactions with neighboring atoms. C19 is a characteristic peak of the carbon attached to the carbonyl in the indole ring of the *Strychnos* alkaloids. C20 and C21 suggested cyclic carbons attached to oxygen atoms. C22 has absorption of a methyl group attached to a cyclic ring. C23 appears in the carboxylic ring in NMR data. The analysis above gave the following table of Carbon atoms and their corresponding protons for compound PKM 094.

Table 4. 5Tabulation of the carbon atoms and corresponding hydrogen atoms for compound labeled PKM 094

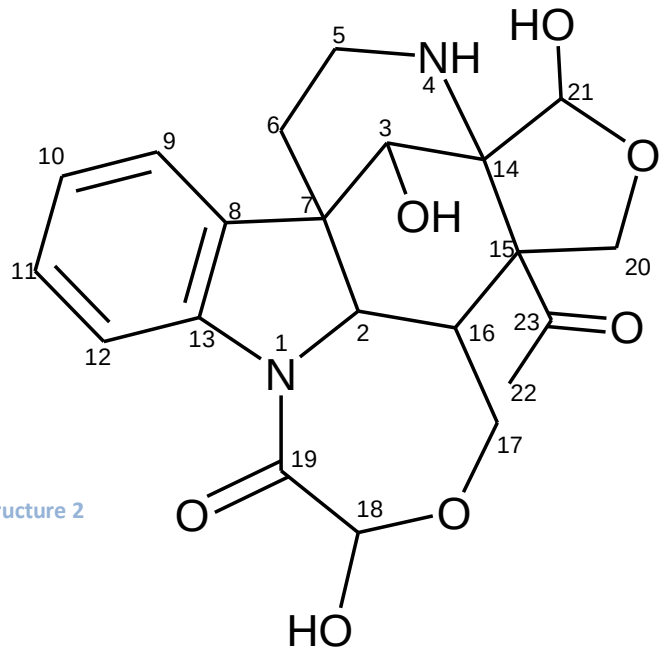
Atom	δ_C	δ_H	Atom	δ_C	δ_H
2	47.16	3.9	14	70.66	
3	70.10	3.8	15	53.30	
5	46.95	2.8; 2.7	16	24.90	2.5
6	37.58	1.8; 1.6	17	47.16	3.5; 3.3
7	48.10		18	92.85	5.6
8	138.90		19	170.98	
9	128.65	7.8	20	64.86	4.0; 3.8
10	125.10	7.5	21	104.99	5.7
11	128.40	7.3	22	28.32	2.0
12	119.46	6.9	23	183.44	
13	140.48				

The data for ^{13}C was closely related with that of Holstiine and tabulated below for comparison to help in proposing a structure that would capture the information analysed above.

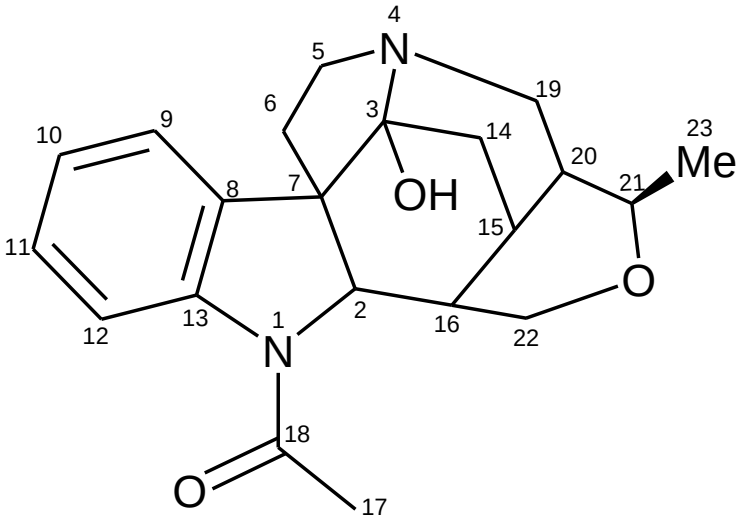
Table 4. 6Comparison of tabulated carbon atoms of PKM 094 and Holstiine

Atom	PKM 094 δ_C	Holstineδ_C	Atom	PKM 094 δ_C	Holstineδ_C
2	47.16	42.1	14	70.66	134.0
3	70.10	61.3	15	53.30	58.8
5	46.95	53.1	16	24.90	27.8
6	37.58	38.6	17	47.16	48.5
7	48.10	53.1	18	92.85	
8	138.90	138.9	19	170.98	169.6
9	128.65	126.8	20	64.86	
10	125.10	124.1	21	104.99	121.3
11	128.40	126.2	22	28.32	21.0
12	119.46	121.3	23	183.44	204.1
13	140.48	141.8			

With the above NMR data analysis and comparisons with Holstiine, the following structure of compound labeled PKM 094 was suggested. Below also is the structure of Holstiine used to compare.



Structure 2



Holstiine

c) PKM 096

The NMR data discussed here appears in appendix of pages 79 to 84. This particular compound was found to have NMR similarities with N(a)-acetylstrychnosplendine and therefore the following analysis were discussed using the already isolated compound as a reference. Preliminary scanning showed presence of aromatic protons with absorptions of 7.9, 7.5, 7.3 and 6.9. Absorptions at 5.6 and 4.6 suggested protons attached or near electron withdrawing atoms. Cyclic protons had absorptions between 3.6 and 2.0. The other peaks showed aliphatic protons. The data was compared with ^{13}C NMR data which was compared with data for N(a)-acetylstrychnosplendine as they had shown close data similarity. The data showed compound 096 had 19 carbon atoms. The labelling of the carbon atoms was done. C2 and C3 suggested cyclic carbons but the higher absorption of C2 further suggests presence of electron withdrawing atoms. C5 and C6 suggests atoms attached to the third ring of the indole ring. C8, C9, C10, C11, C12 and C13 suggest carbon atoms in the aromatic region. C14, C15 and C16 have peaks suggesting the rest of the cyclic ring. C14 with higher absorption suggested attachment to an electron withdrawing atom. C17 and C18 have peaks in the cyclic but substituted region. C18 is highly de-shielded and suggested intra-molecular interactions with neighboring atoms. C19 is a characteristic peak of the carbon attached to the carbonyl in the indole ring of the *Strychnos* alkaloids. C20 peak suggests a cyclic carbon attached to high electron withdrawing atom like oxygen which by withdrawing the electrons it de-shields the carbon atom leading to such peak. C21 suggested a methyl peak and attached to a nitrogen. The analysis of the NMR data is summarized in the table below:

Table 4. 7Tabulation of the carbon atoms and corresponding hydrogen atoms for compound labeled PKM 096

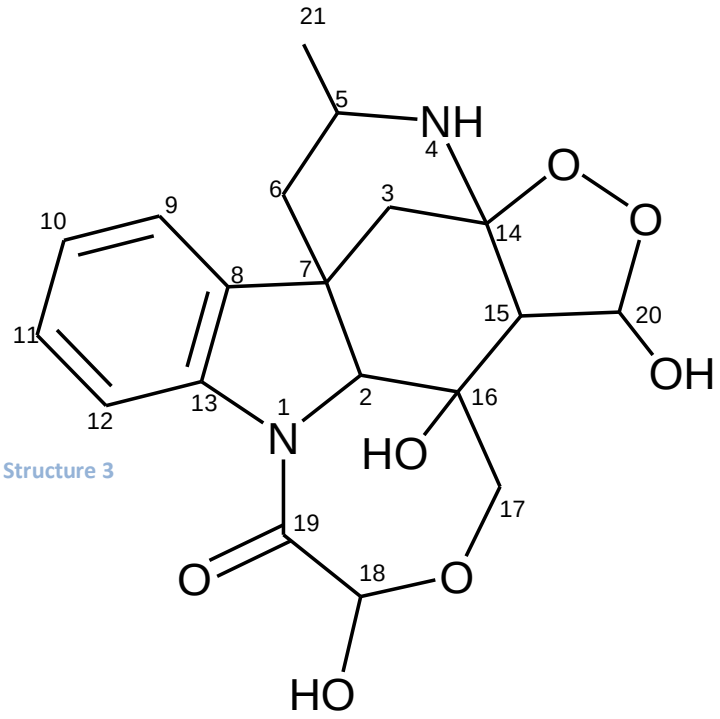
Atom	δ_C	δ_H	Atom	δ_C	δ_H
2	48.24	3.9	13	140.8	
3	37.45	2.0; 1.7	14	72.31	
5	46.96	2.8	15	48.02	1.9
6	47.17	1.6	16	47.38	
7	28.24		17	70.11	3.6; 3.4
8	133.9		18	92.81	5.6
9	129.29	7.9	19	170.99	
10	125.14	7.5	20	92.27	4.6
11	121.53	7.3	21	21.94	1.2
12	119.49	6.9			

The compared NMR data with the already known compound N(a)-acetylstrychnosplendine was also tabulated to help in suggesting a close structure for compound PKM 096 as shown below:

Table 4. 8 Comparison of tabulated carbon atoms of PKM 096 and N(a)-acetylstrychnosplendine

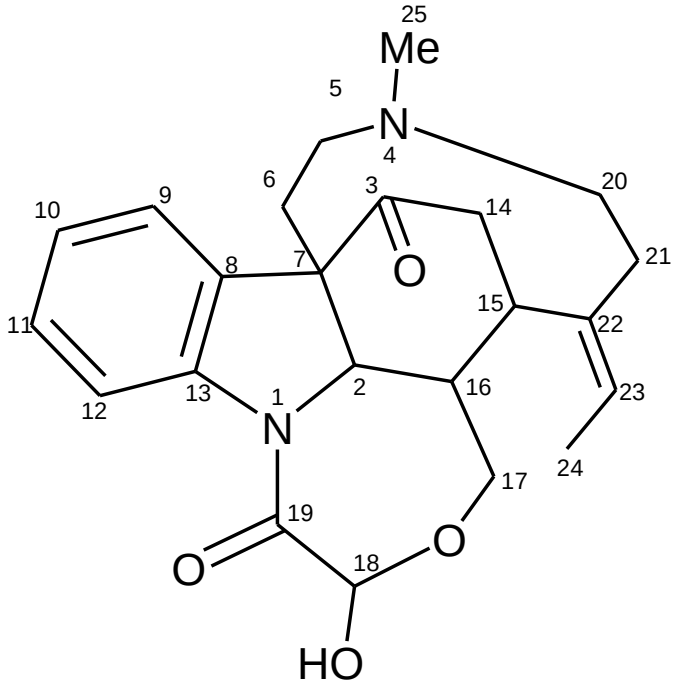
Atom	PKM 096 δ_C	N(a)-δ_C	Atom	PKM 096 δ_C	N(a)-δ_C
2	48.24	60.3	13	140.8	142.8
3	37.45	34.5	14	72.31	77.9
5	46.96	45.0	15	48.02	43.5
6	47.17	32.6	16	47.38	40.7
7	28.24	60.3	17	70.11	63.3
8	133.9	131.2	18	92.81	
9	129.29	127.8	19	170.99	169.6
10	125.14	109.6	20	92.27	
11	121.53	158.1	21	21.94	20.3
12	119.49	104.5			

With the above NMR data for compound 096 which was then compared with N(a)-acetylstrychnosplendine, the following structure was suggested.



Structure 3

N(a)-acetylstrychnosplendine



d) PKM 097

The NMR data discussed for compound PKM 097 is on the appendix on pages 85 to 94. The NMR data was prescreened and compared with an already isolated compound. Diaboline showed the closest similarity and was used for the analysis and discussion of the NMR data to suggest a structure for compound labelled PKM 097. The proton NMR showed presence of aromatic protons with absorptions of 7.9, 7.5, 7.4 and 7.2. A peak of 6.1 suggested a proton attached to a saturated carbon next to an electron withdrawing atom. A peak at 5.7 suggested a proton attached to an SP hybridized carbon with electron withdrawing atom. Cyclic protons were in the range of 4.4 to 2.0. The lower recorded peaks suggested aliphatic protons. The ^{13}C NMR data of compound 097 showed it had 35 carbons. With close analysis from the atoms were allocated numbers as discussed here. C2 and C3 suggested those at the third cyclic ring of indole alkaloids. C2 with higher absorption peak suggests they had different hybridization with C3. C5 and C6 had peaks known for atoms attached to the third indole ring. C8, C9, C10, C11, C12 and C13 had peaks in the aromatic region which is a characteristic of the first indole ring. C14 C15 and C16 peaks suggested the other carbon atoms in the third ring. The higher absorption of C14 can be attributed to the neighboring ring with electron withdrawing atom. C17 peak suggested a cyclic carbon atom de-shielded and hence higher absorption. C18 is suggesting a highly de-shielded and suggested intra-molecular interactions with neighboring atoms. C19 is a characteristic peak of the carbon attached to the carbonyl in the indole ring of the *Strychnos* alkaloids. C20 peak suggested an amide attached to a cyclic carbon. C21, C22, C25, C28 and C29 were allocated the numbers as to peaks suggesting carbon either attached to oxygen and are cyclic or saturated carbon atoms.

C23, C26, C27, C31, C32, C33, C34 and C35 are carbon atoms appearing in the cyclic atoms and less substituted. The peak ranges from 72 to 52 and the difference depended on the presence of either electron withdrawing or donating atoms. C24, C30, C36 and C37 have peaks suggesting methyl groups attached either to amides or extension from cyclic rings. The information discussed on the NMR data was then tabulated for ^{13}C against corresponding protons in the table below:

Table 4. 9Tabulation of the carbon atoms and corresponding hydrogen atoms for compound labeled PKM 097

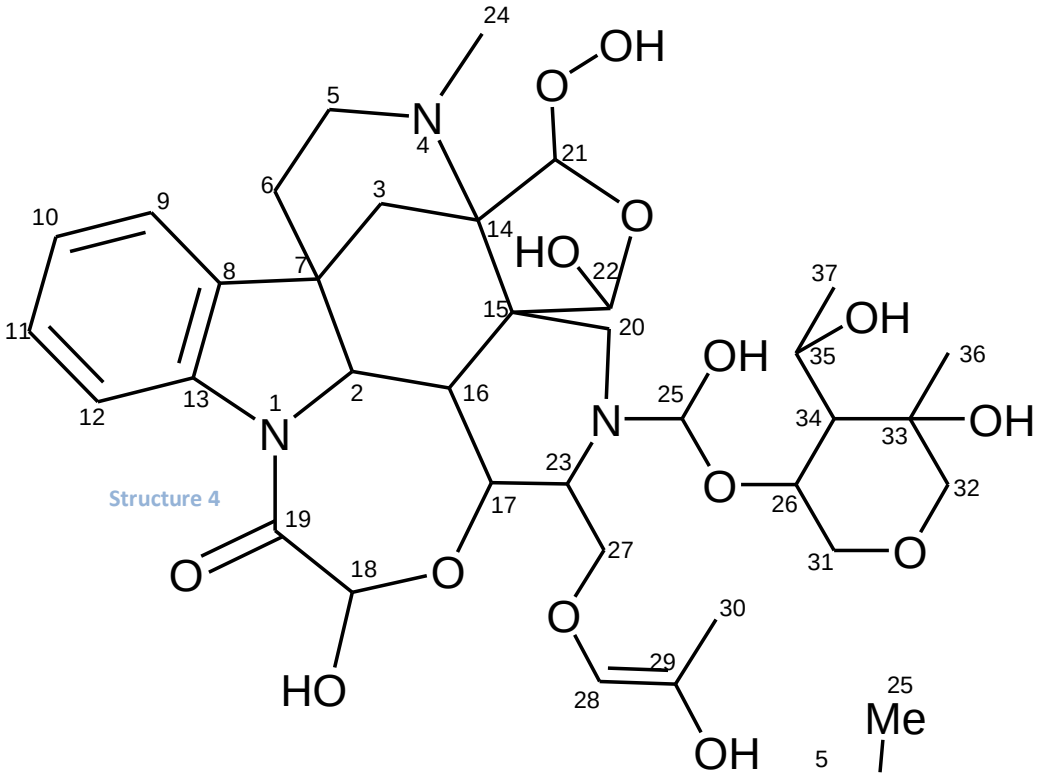
Atom	δ_C	δ_H	Atom	δ_C	δ_H
2	51.35	3.9	20	28.27	2.3; 2.0
3	27.15	1.8; 1.5	21	106.15	5.5
5	46.99	2.3; 2.2	22	105.29	5.6
6	37.48	1.9; 1.6	23	55.68	2.9
7	47.20		24	37.43	2.2
8	133.75		25	110.30	5.7
9	129.59	7.9	26	51.44	2.9
10	127.87	7.5	27	52.45	4.4; 3.9
11	129.20	7.4	28	119.48	6.1
12	125.14	7.2	29	122.45	
13	139.97		30	21.64	1.7
14	64.79		31	70.66	3.9; 3.6
15	47.20		32	72.70	3.8; 3.6
16	27.15	2.4	33	52.93	
17	59.20	2.8	34	54.63	2.0
18	92.83	5.6	35	70.14	3.9
19	170.97		36	22.00	1.3
			37	21.64	1.2

A further tabulation of 13C for compound PKM 097 and Diaboline was done which helped in proposing a structure for compound PKM 097.

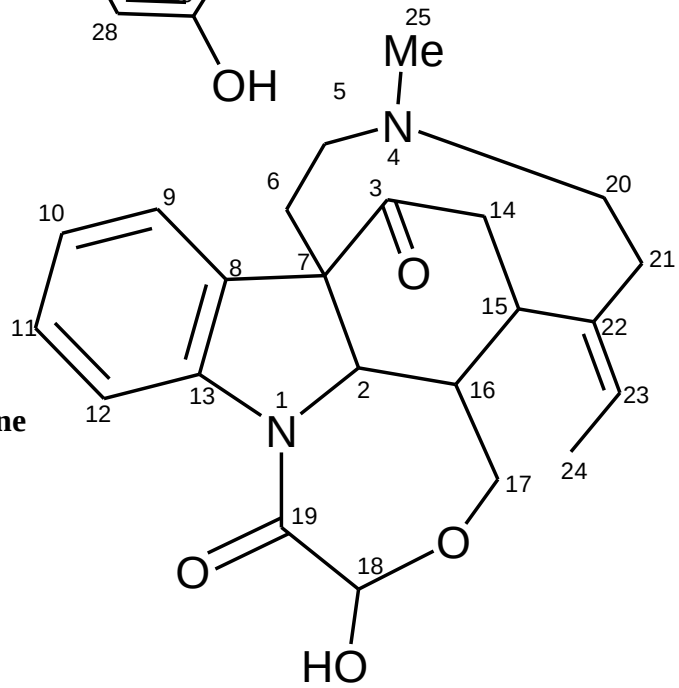
Table 4. 10Comparison of tabulated carbon atoms of PKM 097 and Diaboline

Atom	**PKM 097δ_C**	**Diabolinδ_C**	**Atom**	**PKM 097 δ_C**	**DiabolinδC**
2	51.35	58.2	17	59.20	58.2
3	27.15	98.7	18	92.83	21.0
5	46.99	45.0	19	170.97	169.6
6	37.48	32.6	20	28.27	40.7
7	47.20	60.3	21	106.15	77.9
8	133.75	138.9	22	105.29	63.3
9	129.59	126.8	23	55.68	
10	127.87	124.1	24	37.43	
11	129.20	126.2	25	110.30	
12	125.14	121.3	26	51.44	
13	139.97	141.8	27	52.45	
14	64.79	58.2	28	119.48	
15	47.20	48.5	29	122.45	
16	27.15	26.0	30	21.64	

With the above NMR data from compound PKM 097 and Diaboline, the following structure of compound labeled PKM 097 was suggested.



N(a)-acetylstrychnosplendine



e) PKM 099

The NMR data discussed in this compound is in the appendix pages 95 to 102. A prescreening of the NMR data revealed that it was easy to relate with Diaboline, a compound earlier isolated and characterized in *Strychnoshenningsii*. The proton NMR data showed presence of aromatic protons with peaks of 7.9, 7.6, 7.3 and 7.2. A peak at 5.2 suggested a proton affected by an electron withdrawing atom. Cyclic protons were n the peaks between 4.1 and 2.4. The other protons were aliphatic. The ^{13}C NMR data was closely related with that of Diabolineand showed 26 carbon atoms which were allocated numbers. C2 and C3 suggested cyclic atoms of the third ring characteristic of indole ring of *Strychnos*alkaloids. C5 and C6 suggested peaks of carbon atoms attached to the third ring of the indole ring. C8, C9 C10 C11, C12 and C13 have peaks suggesting they are aromatic carbons. C14 C15 and C16 have peaks suggesting the other atoms of the third ring of the indole ring. C17 suggested a cyclic carbon but the high absorption is expected for the highly substituted fourth ring. C18 is suggesting a highly de-shielded and suggested intra-molecular interactions with neighboring atoms. C19 is a characteristic peak of the carbon attached to the carbonyl in the indole ring of the *Strychnos* alkaloids. C20, C21 and C22 have absorptions suggesting cyclic carbon atoms and highly de-shielded. C23 and C27 suggest methyl groups attached to amide group. C24 absorption peak suggested a cyclic carbon attached to two electron withdrawing atoms. C25 NMR data suggested an aliphatic carbon atom and de-shielded. C26 suggested a methyl group. C28 had a characteristic methoxy absorption peak. The analysis was broken down into the table below for all the carbon atoms with their corresponding protons.

Table 4. 11Tabulation of the carbon atoms and corresponding hydrogen atoms for compound labeled PKM 099

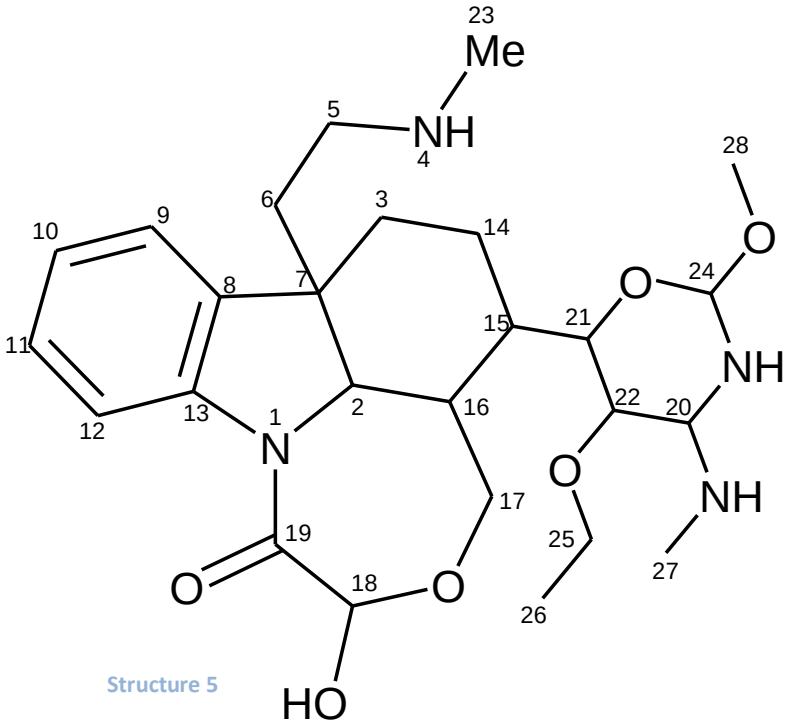
Atom	δ_C	δ_H	Atom	δ_C	δ_H
2	55.69	4.1	16	27.15	2.4
3	28.18	1.8; 1.6	17	59.77	3.5; 3.2
5	47.19	2.5	18	92.77	5.6
6	37.31	1.6	19	170.98	
7	46.98		20	70.67	4.0
8	129.94		21	64.75	3.1
9	127.99	7.9	22	72.30	3.6
10	122.47	7.6	23	24.24	2.4
11	125.16	7.3	24	119.51	5.2
12	121.97	7.2	25	54.60	3.4
13	130.42		26	21.97	1.2
14	21.69	1.8; 1.5	27	24.24	2.4
15	28.30	1.7	28	52.94	3.2

Diaboline was used to analyze the data and compare with that of compound 099 as shown in the table below:

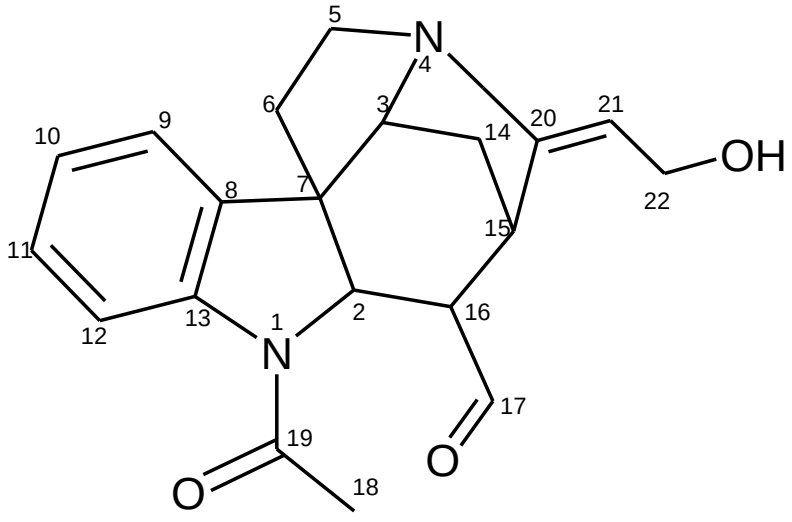
Table 4. 12Comparison of tabulated carbon atoms of PKM 099 and Diaboline

Atom	PKM 099 δ_C	Diabolineδ_C	Atom	PKM 099 δ_C	Diabolineδ_C
2	55.69	58.2	16	27.15	38.0
3	28.18	98.7	17	59.77	58.2
5	47.19	45.0	18	92.77	21.0
6	37.31	32.6	19	170.98	169.6
7	46.98	60.3	20	70.67	43.5
8	129.94	138.9	21	64.75	40.7
9	127.99	126.8	22	72.30	77.9
10	122.47	124.1	23	24.24	20.3
11	125.16	126.2	24	119.51	63.3
12	121.97	121.3	25	54.60	
13	130.42	141.8	26	21.97	
14	21.69	33.7	27	24.24	
15	28.30	26.0	28	52.94	

With the above NMR data of compound 099 and comparison with Diaboline, the following structure of compound labeled PKM 099 was suggested.



Structure 5



f) PKM 104

The NMR data discussed in this compound is in the appendix pages 103 to 110. A prescreening of the NMR data revealed that it was easy to relate with N(a)-acetylstrychnosplendine. The proton NMR showed aromatic protons with absorptions of 7.9, 7.5, 7.3 and 6.9. Some other absorptions at 5.7, 5.5 and 5.6 suggested protons attached to carbons which are highly de-shielded. Cyclic protons have peaks between 3.9 and 2.3. The other lower absorptions seen in the data suggested aliphatic protons. The ^{13}C NMR data was analyzed with close association with N(a)-acetylstrychnosplendine. The data showed compound 104 had 26 carbon atoms. C2 and C3 suggested cyclic peaks characteristic of carbon atoms in the third ring of indole alkaloids. C5 and C6 suggested carbon attached to the third ring. C5 has higher absorption suggesting it is attached to the amide group. C8, C9, C10, C11, C12 and C13 have peaks suggesting aromatic carbons. C14, C15, C16 and C17 absorptions peaks suggested cyclic carbon atoms. The varying absorptions suggests further the various groups attached to the carbons. C18 is suggesting a highly de-shielded and suggested intra-molecular interactions with neighboring atoms. C19 is a characteristic peak of the carbon attached to the carbonyl in the indole ring of the *Strychnos* alkaloids. C20 and C27 have absorptions suggesting cyclic carbon atoms with two protons that are not chemical equivalents. C21, C22, and C28 have peaks that suggested cyclic carbon atoms which are directly attached to Oxygen atoms which de-shielded the atoms leading to high absorption peaks. C23 and C24 had peaks suggesting carbon atoms attached to amide groups. C25 and C26 are chemical equivalent carbon atoms, their absorption suggested cyclic carbon atoms each attached to an electron

withdrawing atom. The above analysis were tabulated in the table below to show the carbon atom and the corresponding protons.

Table 4. 13Tabulation of the carbon atoms and corresponding hydrogen atoms for compound labeled PKM 104

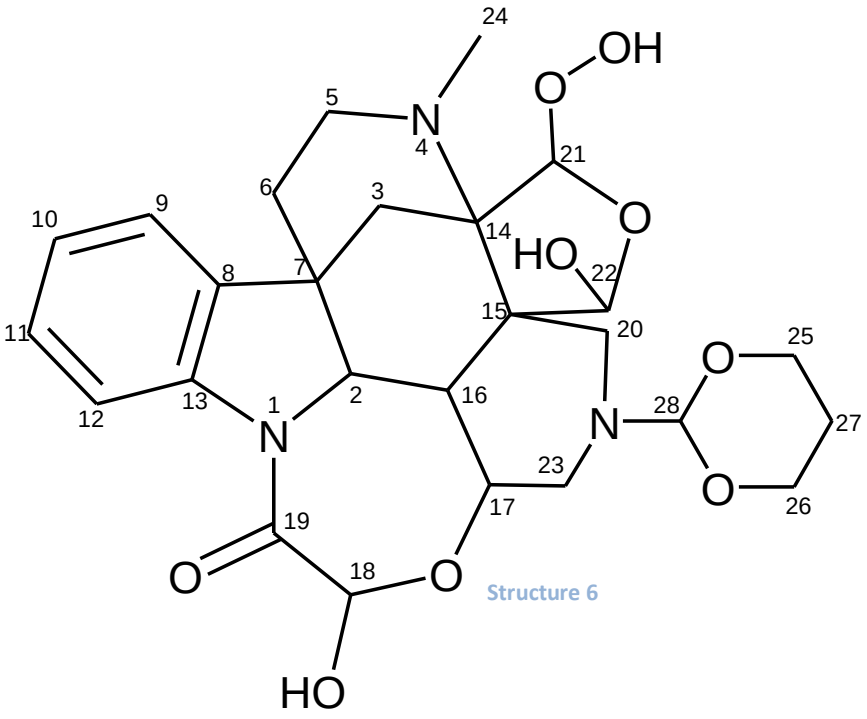
Atom	δ_C	δ_H	Atom	δ_C	δ_H
2	59.48	3.9	16	52.40	3.0
3	24.23	18.; 1.6	17	48.45	2.5; 2.3
5	51.29	2.3; 2.1	18	92.82	5.6
6	37.46	1.9; 1.6	19	171.01	
7	46.97		20	28.25	2.4; 2.0
8	129.19		21	106.12	5.7
9	127.87	7.9	22	104.99	5.5
10	125.15	7.5	23	48.25	2.5; 2.2
11	121.55	7.3	24	37.46	2.3
12	121.57	6.9	25	64.82	3.8; 3.7
13	129.60		26	64.82	3.8; 3.7
14	54.64		27	21.96	1.7
15	47.18		28	119.49	5.9

N(a)-acetylstrychnosplendine was used to help understand the NMR data of compound 104 and their ^{13}C NMR was tabulated below to show the similarities:

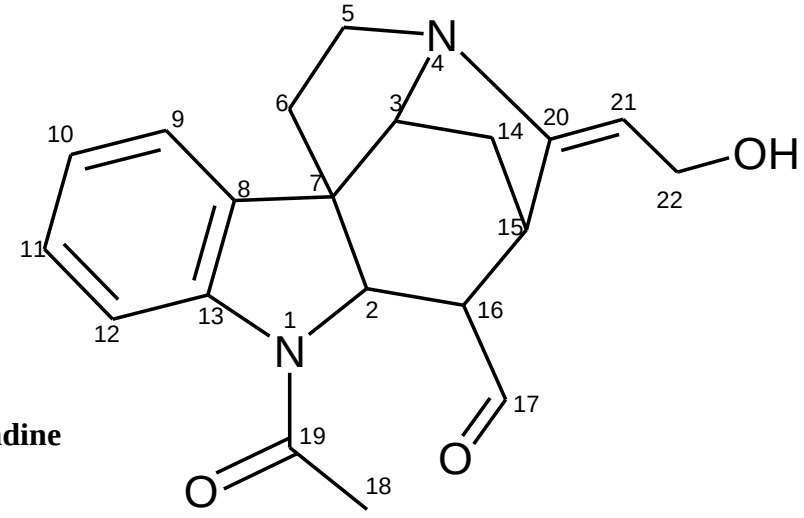
Table 4. 14 Comparison of tabulated carbon atoms of PKM 104 and N(a)-acetylstrychnosplendine

Atom	PKM 104 δ_C	N(a)-δ_C	Atom	PKM 104 δ_C	N(a)-δ_C
2	59.48	60.3	16	52.40	38.2
3	24.23	98.7	17	48.45	21.0
5	51.29	45.0	18	92.82	43.5
6	37.46	32.6	19	171.01	169.6
7	46.97	60.3	20	28.25	40.7
8	129.19	131.2	21	106.12	77.9
9	127.87	127.8	22	104.99	63.3
10	125.15	109.6	23	48.25	20.3
11	121.55	158.1	24	37.46	55.9
12	121.57	104.5	25	64.82	
13	129.60	142.8	26	64.82	
14	54.64	33.7	27	21.96	
15	47.18	40.7	28	119.49	

With the above NMR data of compound 104 and with close comparison with N(a)-acetylstrychnosplendine, the following structure of compound labeled PKM 104 was suggested.



Structure 6



N(a)-acetylstrychnosplendine

g) PKM 107

The NMR data discussed in this compound is in the appendix pages 111 to 118. A prescreening of the NMR data revealed that it was easy to relate with N(a)-acetylstrychnosplendine. The proton NMR showed aromatic protons with absorptions of 7.9, 7.5, 7.2 and 6.8. Protons with 6.0 suggested attached to carbon with electron withdrawing atoms. Cyclic protons had absorptions between 5.6 and 2.3, the others were aliphatic protons. ^{13}C NMR was used to confirm what the proton analysis and showed the compound had 22 carbon atoms. The compound also had close spectra data with N(a)-acetylstrychnosplendine and carbon atoms were numbered. C2 and C3 suggested the cyclic carbons. C2 is SP hybridized and hence gave a higher absorption than C3. C5 and C6 suggested peaks for carbons attached to the third indole ring. C8, C9, C10, C11, C12 and C13 have absorption peaks at the aromatic region. C14 and C15 suggested quaternary cyclic carbon atoms. C16 suggested a cyclic carbon. C17 suggested an absorption for cyclic carbon attached to oxygen atom. C18 is suggesting a highly de-shielded and suggested intra-molecular interactions with neighboring atoms. C19 is a characteristic peak of the carbon attached to the carbonyl in the indole ring of the Strychnos alkaloids. C20 and C23 suggested cyclic carbon atoms attached to an amide group. C21 suggested aliphatic carbon attached to an electron withdrawing atom. C22 absorption peak is a cyclic carbon atom attached to an electronegative compound. C24 suggested a methyl group attached to an amide group. The NMR data for compound 107 was analysed and tabulated for all carbon atoms with their corresponding protons as shown in the table below:

Table 4. 15Tabulation of the carbon atoms and corresponding hydrogen atoms for compound labeled PKM 107

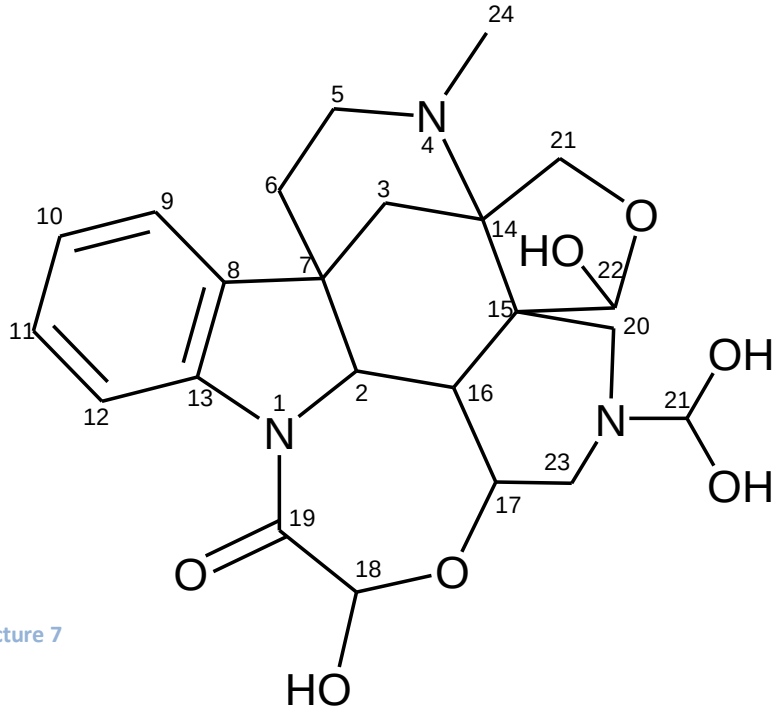
Atom	δ_C	δ_H	Atom	δ_C	δ_H
2	73.57	3.8	14	52.44	
3	28.30	1.8; 1.6	15	51.27	
5	47.19	2.9; 2.1	16	27.13	2.3
6	27.13	1.8; 1.6	17	54.64	3.0
7	46.97		18	92.85	6.0
8	128.78		19	170.98	
9	121.88	7.9	20	37.55	2.3; 2.3
10	127.80	7.5	21	110.31	6.0
11	121.55	7.2	22	106.11	5.6
12	125.12	6.8	23	24.48	2.4; 2.2
13	129.12		24	21.97	2.35

N(a)-acetylstrychnsplendine used to allocate atoms with compound 107 was tabulated below:

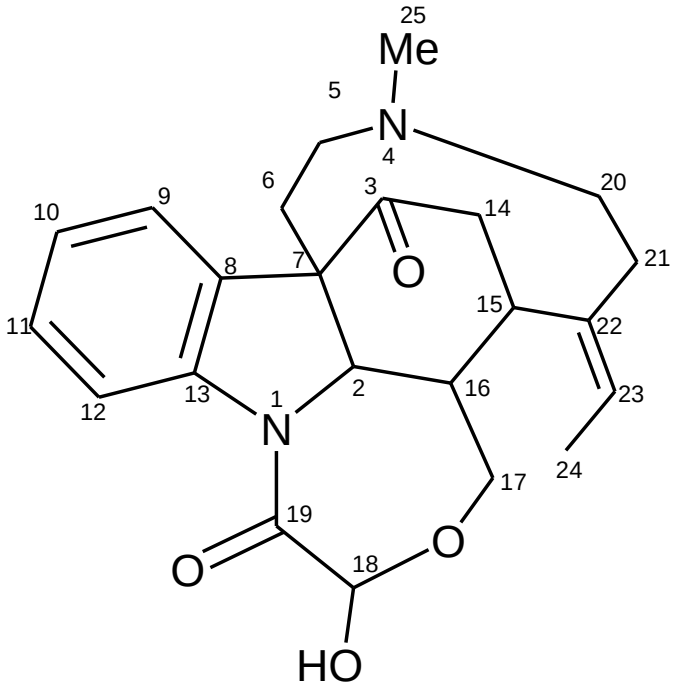
Table 4. 16Comparison of tabulated carbon atoms of PKM 107 and N(a)-acetylstrychnosplendine

Atom	PKM 107 δ_C	N(a)-δ_C	Atom	PKM 107 δ_C	N(a)-δ_C
2	**73.57**	**3.8**	**14**	**52.44**	
3	**28.30**	**1.8; 1.6**	**15**	**51.27**	
5	**47.19**	**2.9; 2.1**	**16**	**27.13**	**2.3**
6	**27.13**	**1.8; 1.6**	**17**	**54.64**	**3.0**
7	**46.97**		**18**	**92.85**	**6.0**
8	**128.78**		**19**	**170.98**	
9	**121.88**	**7.9**	**20**	**37.55**	**2.3; 2.3**
10	**127.80**	**7.5**	**21**	**110.31**	**6.0**
11	**121.55**	**7.2**	**22**	**106.11**	**5.6**
12	**125.12**	**6.8**	**23**	**24.48**	**2.4; 2.2**
13	**129.12**		**24**	**21.97**	**2.35**

With the above NMR data for compound 107 and with comparison of N(a)-acetylstrychnosplendine, the following structure of compound labeled PKM 107 wassuggested.



Structure 7



N(a)-acetylstrychnosplendine

CHAPTER FIVE

DISCUSSION

Strychnos henningsii has been hardly studied and only one publication is available. Four compounds had been isolated by ICIPE group. Based on the structures determined by the group we were able to propose new structures based on the three parent compounds namely Diaboline, Holstiine and N(a)-acetylstrychnosplendine. The pure compounds were analyzed using proton NMR and ^{13}C and their data was closely comparable with the four already isolated compounds from *Strychnos henningsii*. The NMR data also confirmed the presence of carbons and protons characteristic of indole alkaloids as had been earlier proposed by the ICIPE group. Based on this information we were able to propose seven compounds with new structures. The other two are yet to be characterized. Lastly we have demonstrated that traditional knowledge as practiced by the Kamba community in the treatment of malarial and related ailments in the use of *Strychnos henningsii* leaves is valid.

CHAPTER SIX

RECOMMENDATIONS

- Further structural analysis on these new compounds need further confirmation using other spectroscopic techniques. It is expected that synthesis of these new compounds are needed because the natural source are very minute.
- A better formulation method for producing a better anti-malarial drug is needed.
- Further studies need to be carried out to establish if there is any toxicity of the compounds found in the plant.
- There needs to be developed a medicinal botanical garden for biodiversity conservation and medicinal uses.

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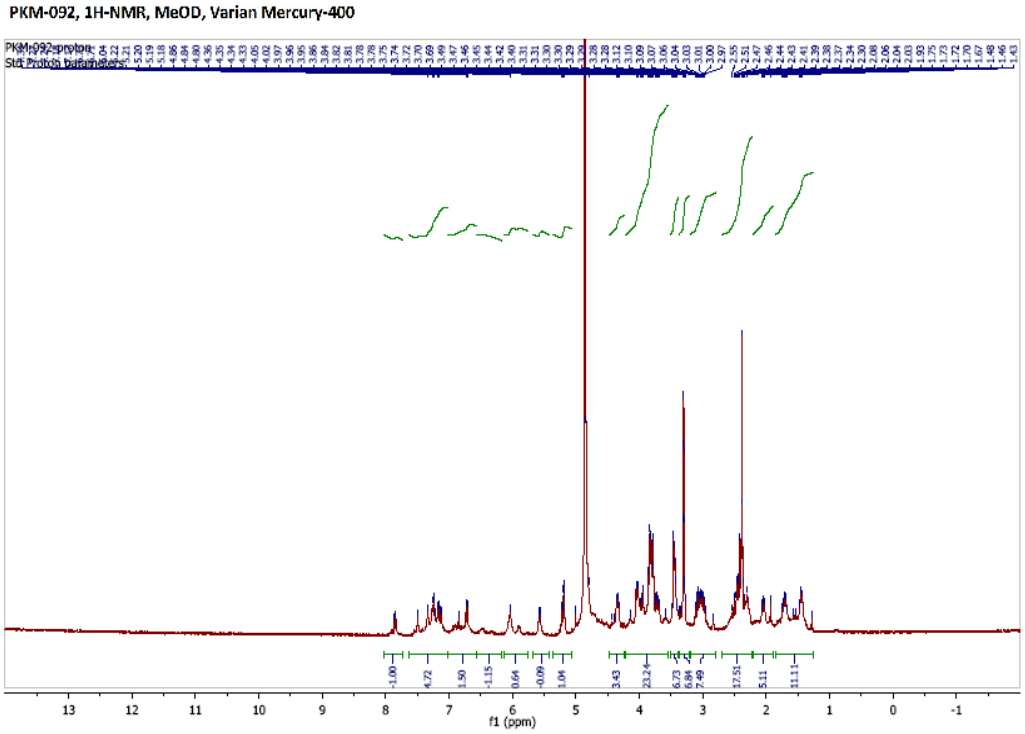
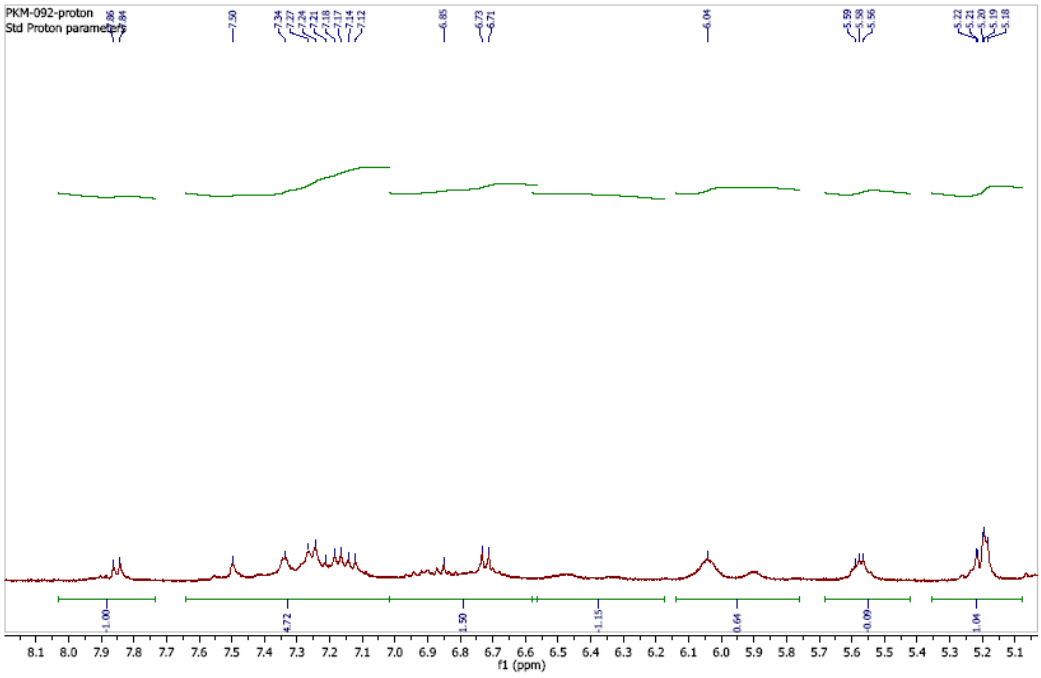
APPENDIX I

a). PKM 092



PKM-092, 1H-NMR, MeOD, Varian Mercury-400



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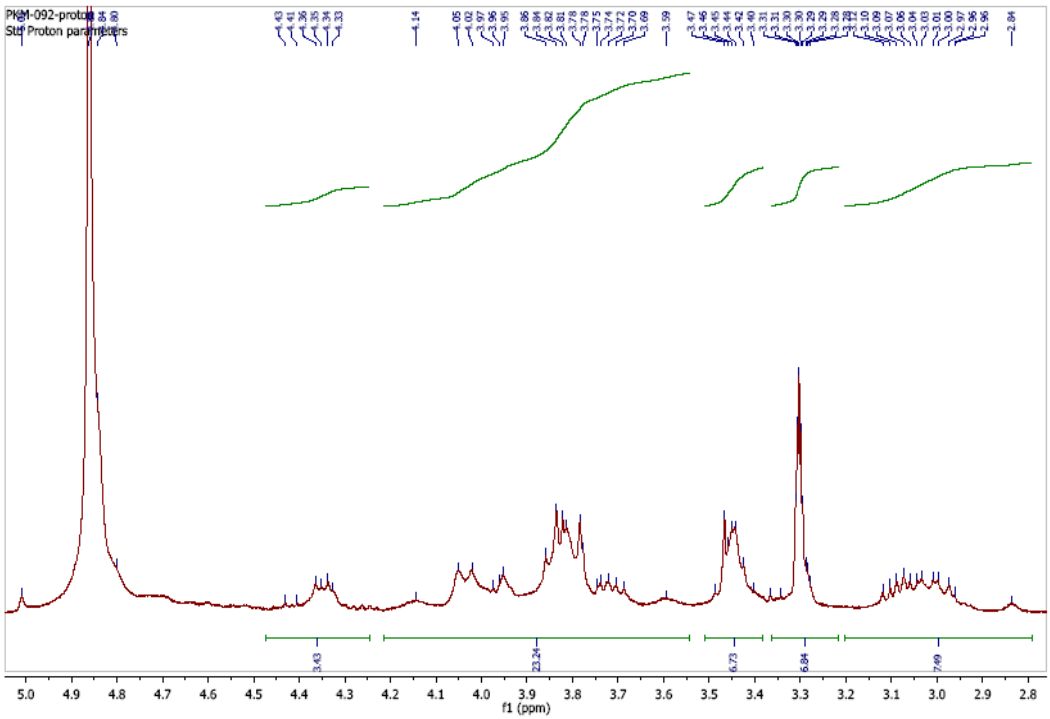

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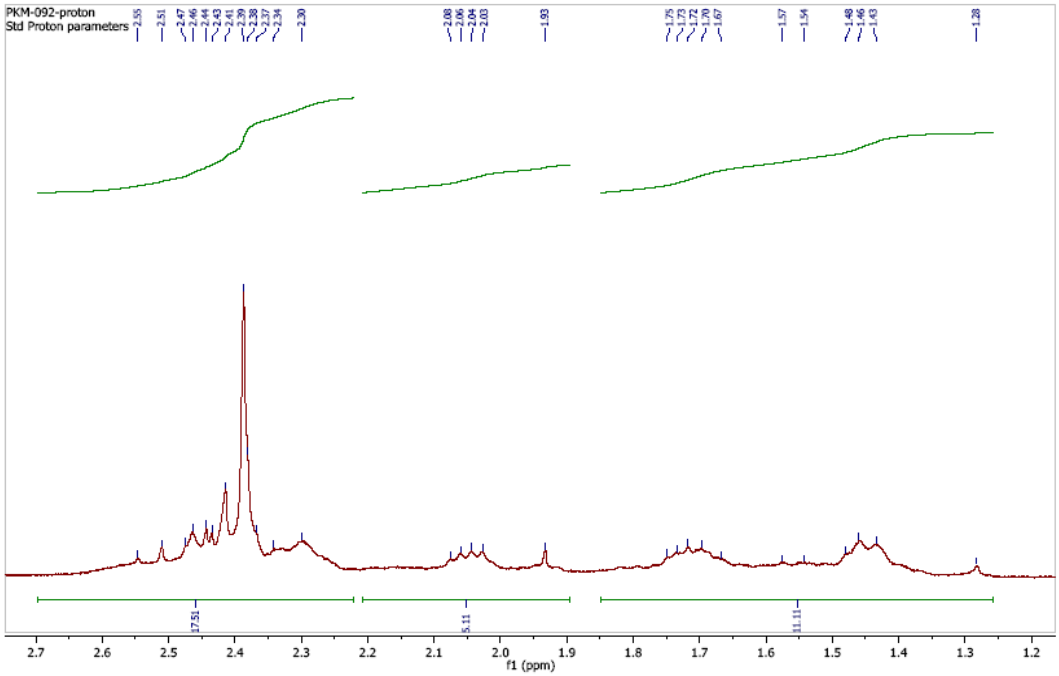
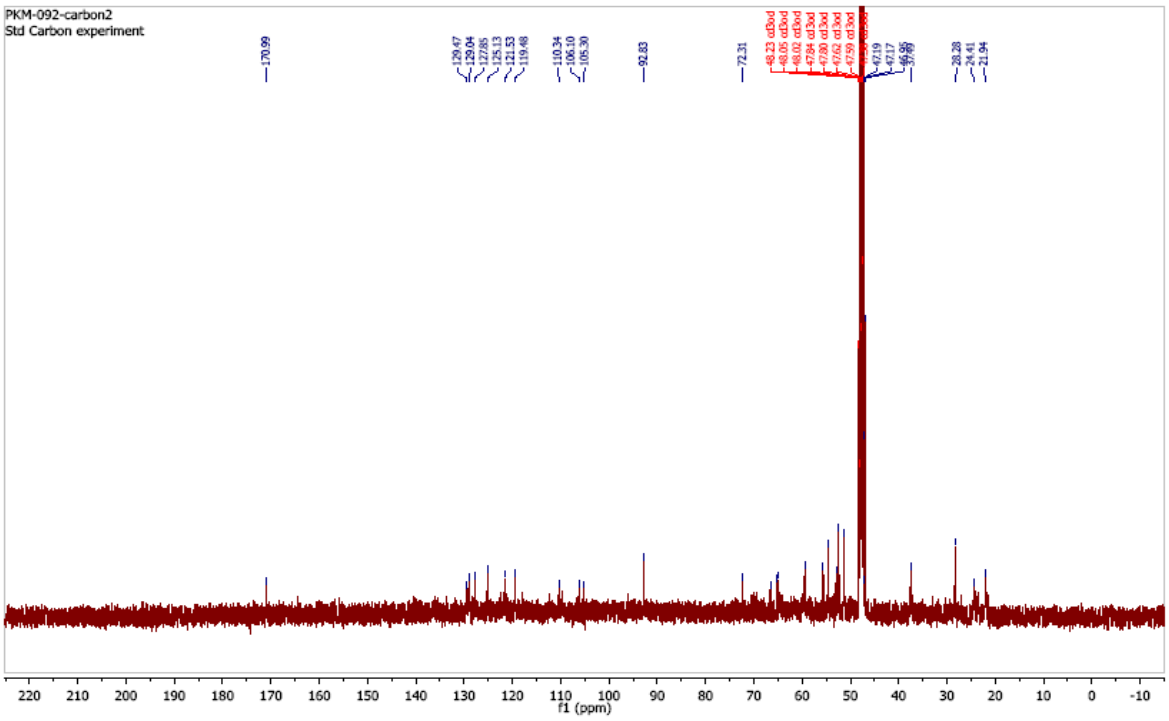
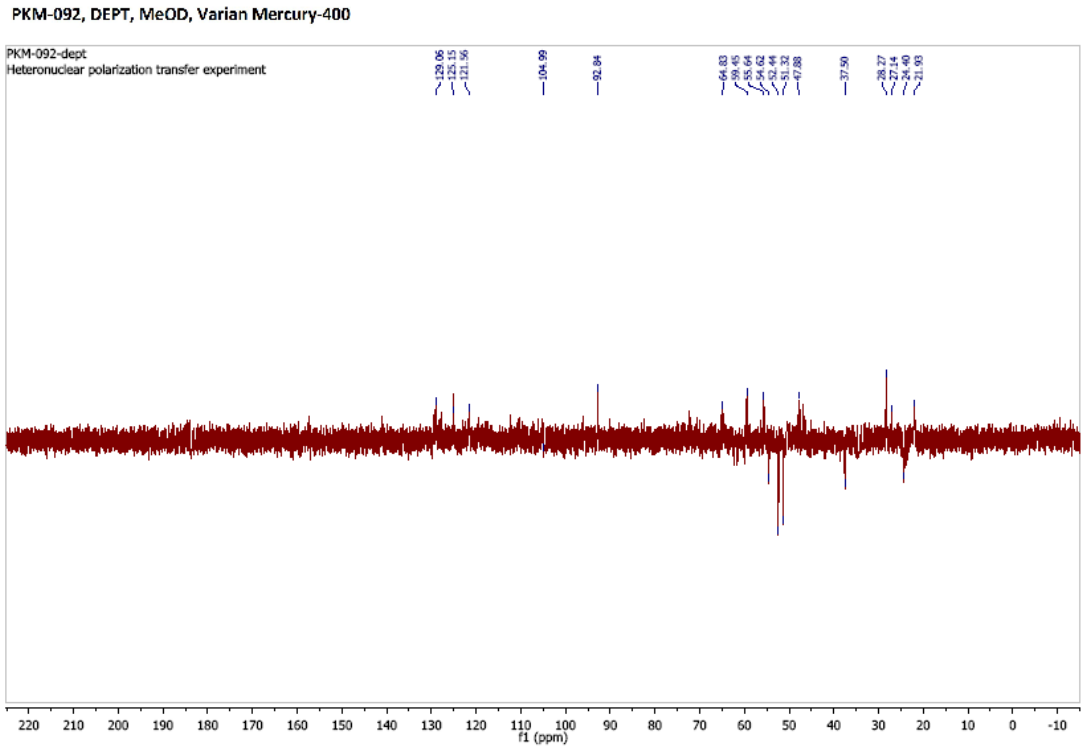


PKM-092, 13C-NMR, MeOD, Varian Mercury-400




PKM-092, DEPT, MeOD, Varian Mercury-400
PKM-092-dept
Heteronuclear polarization transfer experiment
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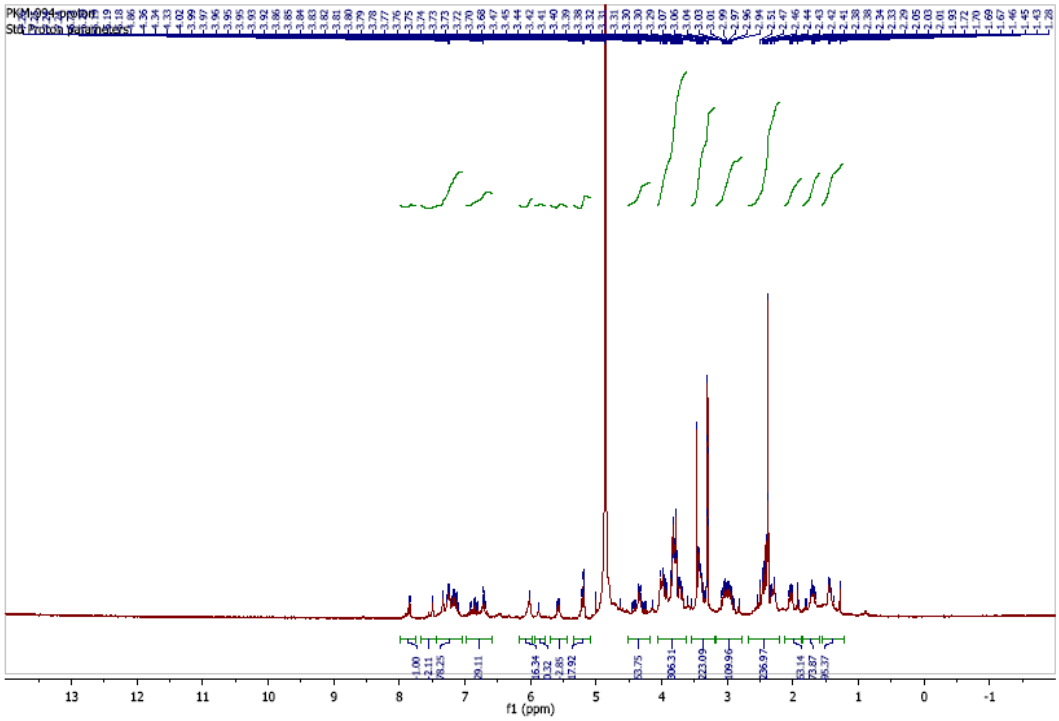
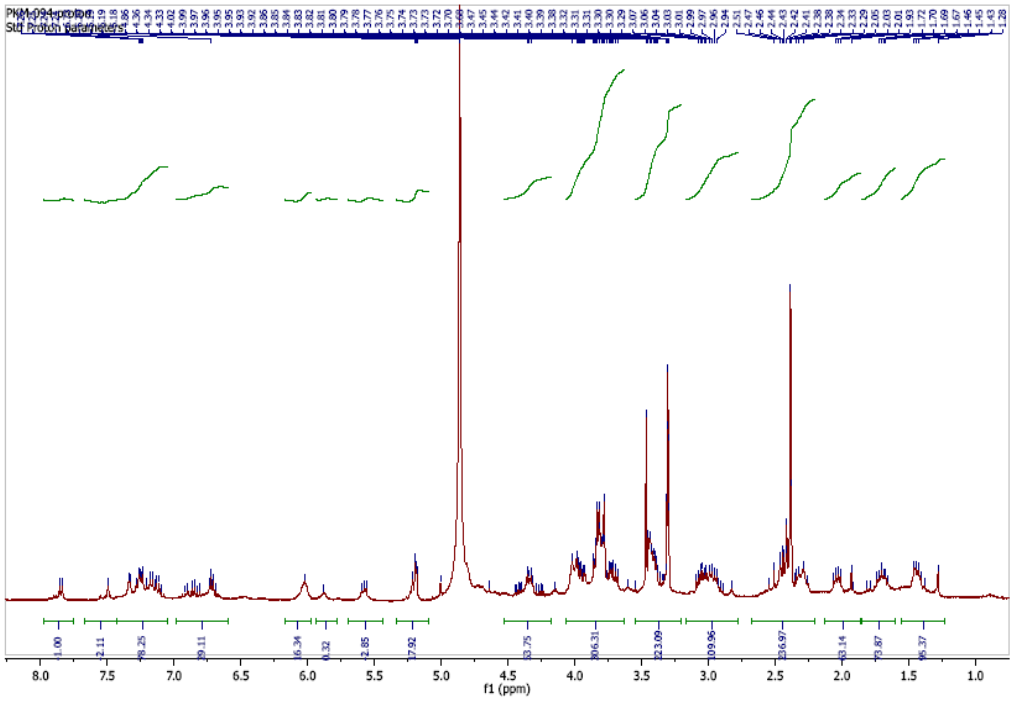
125.15
121.56
104.99
92.84
64.83
59.45
55.64
54.62
52.44
51.32
47.88
37.59
28.27
27.14
24.40
21.93
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

b) PKM 094

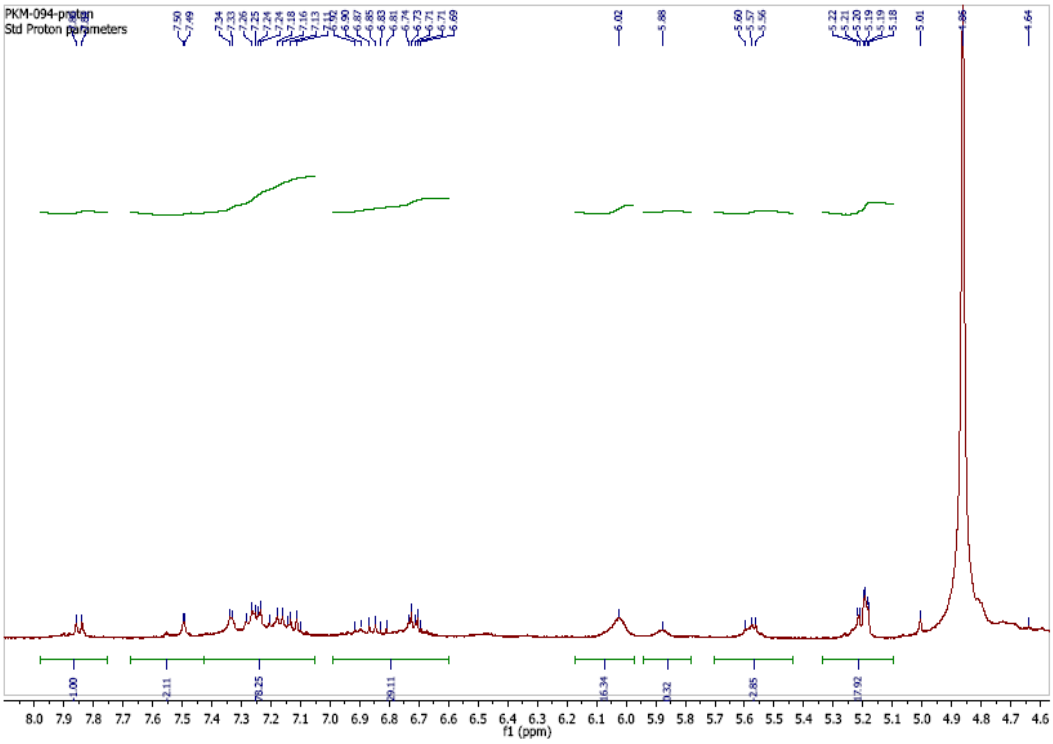
PKM-094, 1H-NMR, MeOD, Varian Mercury-400



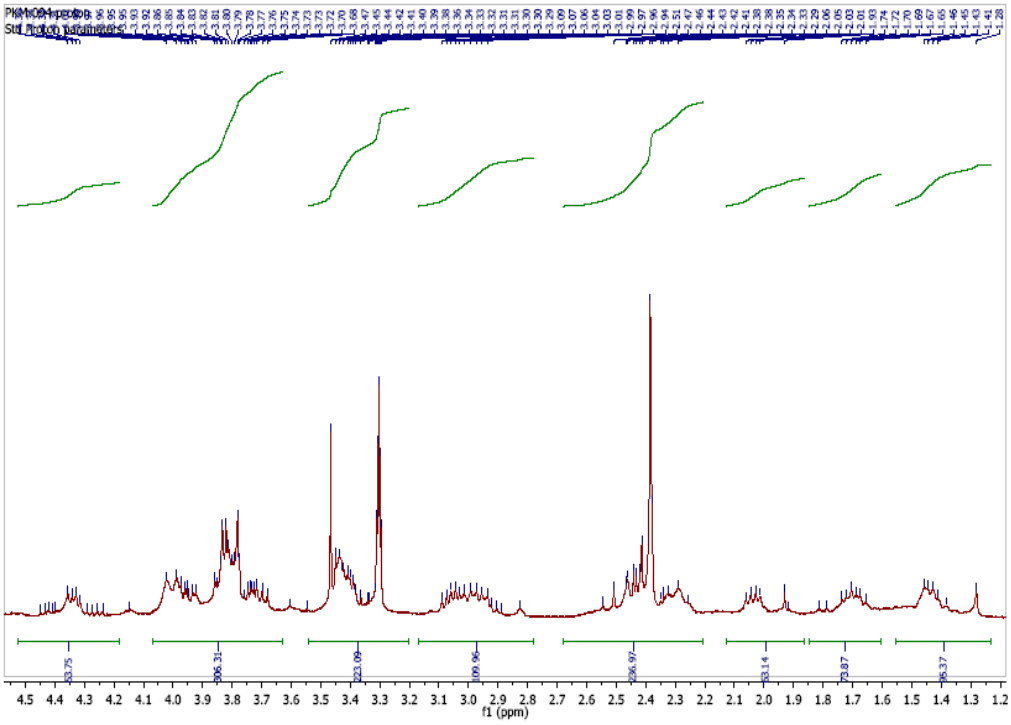
PKM-094, 1H-NMR, MeOD, Varian Mercury-400



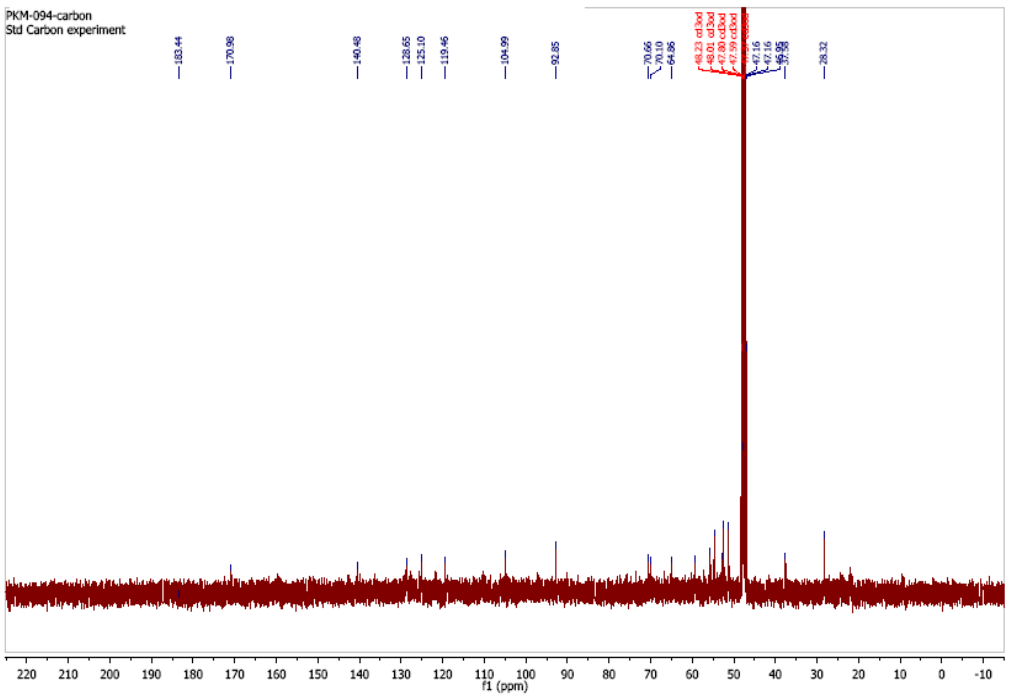
PKM-094, 1H-NMR, MeOD, Varian Mercury-400



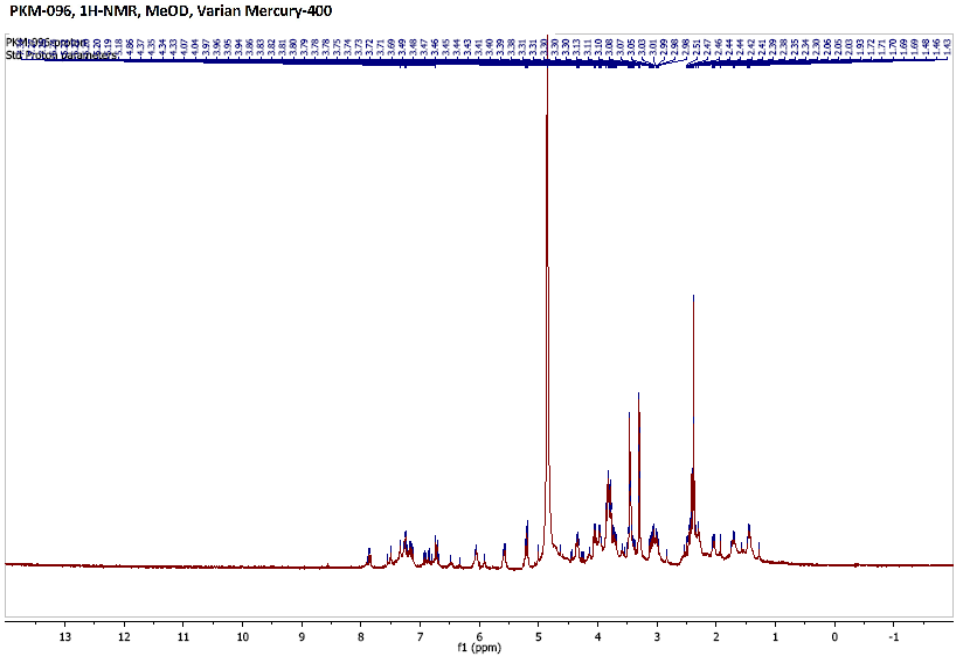
PKM-094, 1H-NMR, MeOD, Varian Mercury-400



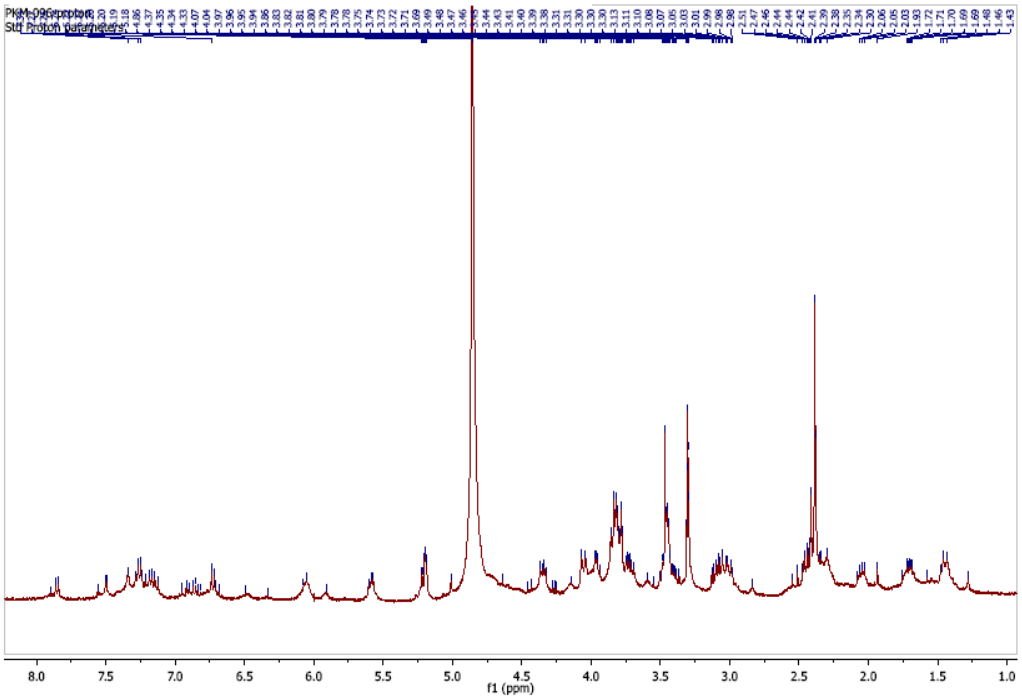
PKM-094, 13C-NMR, MeOD, Varian Mercury-400



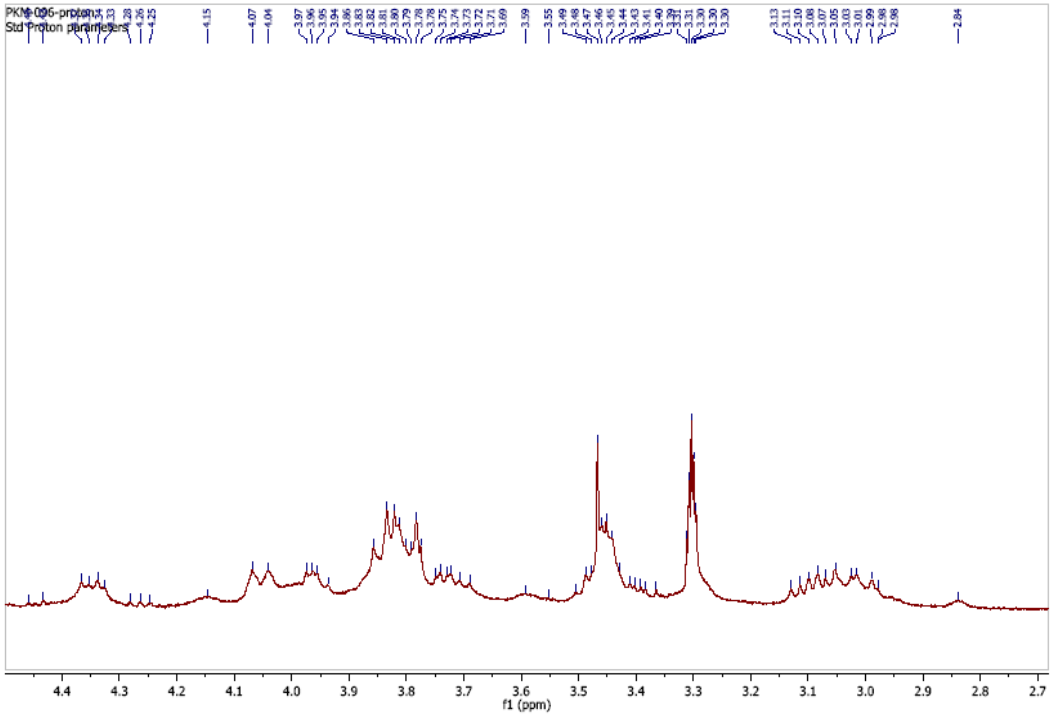
c) PKM 096


PKM-096, 1H-NMR, MeOD, Varian Mercury-400
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
f1 (ppm)

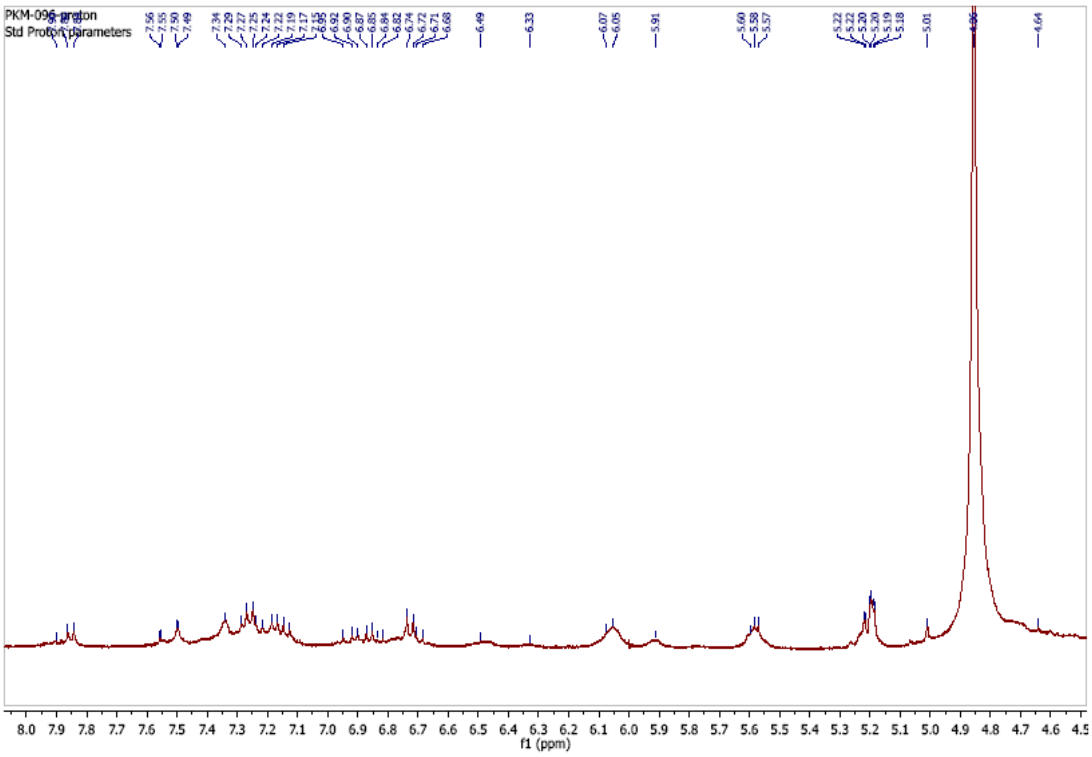
PKM-096, 1H-NMR, MeOD, Varian Mercury-400



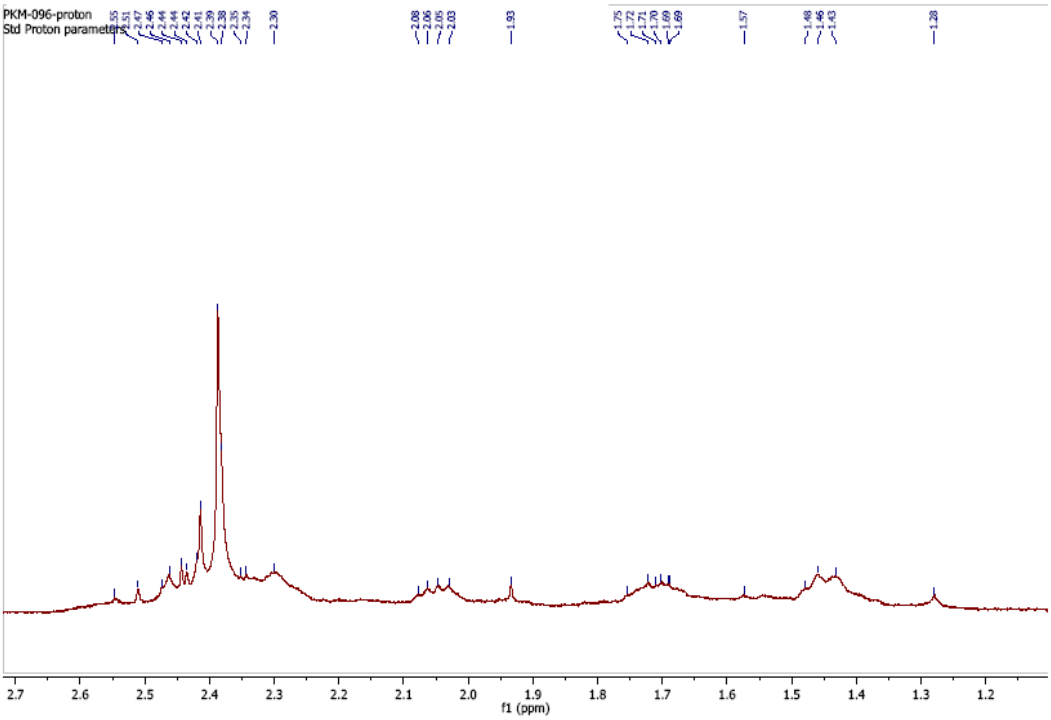
PKM-096, 1H-NMR, MeOD, Varian Mercury-400



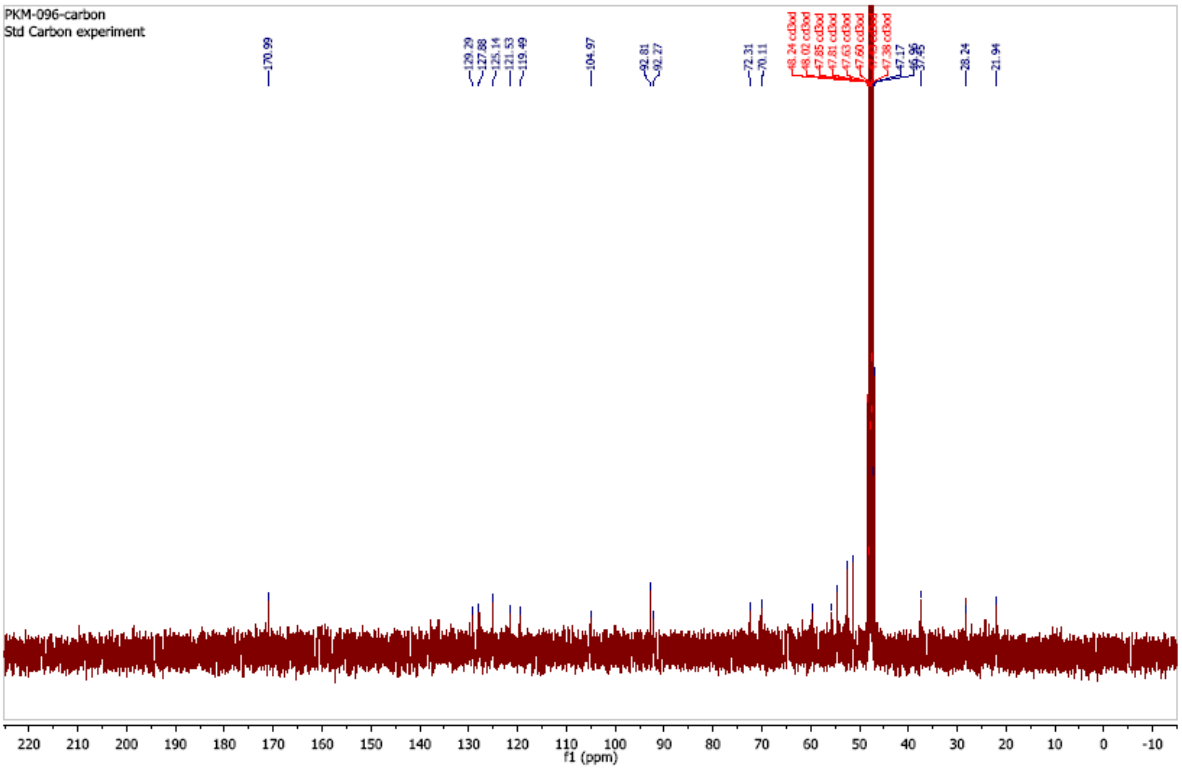
PKM-096, 1H-NMR, MeOD, Varian Mercury-400



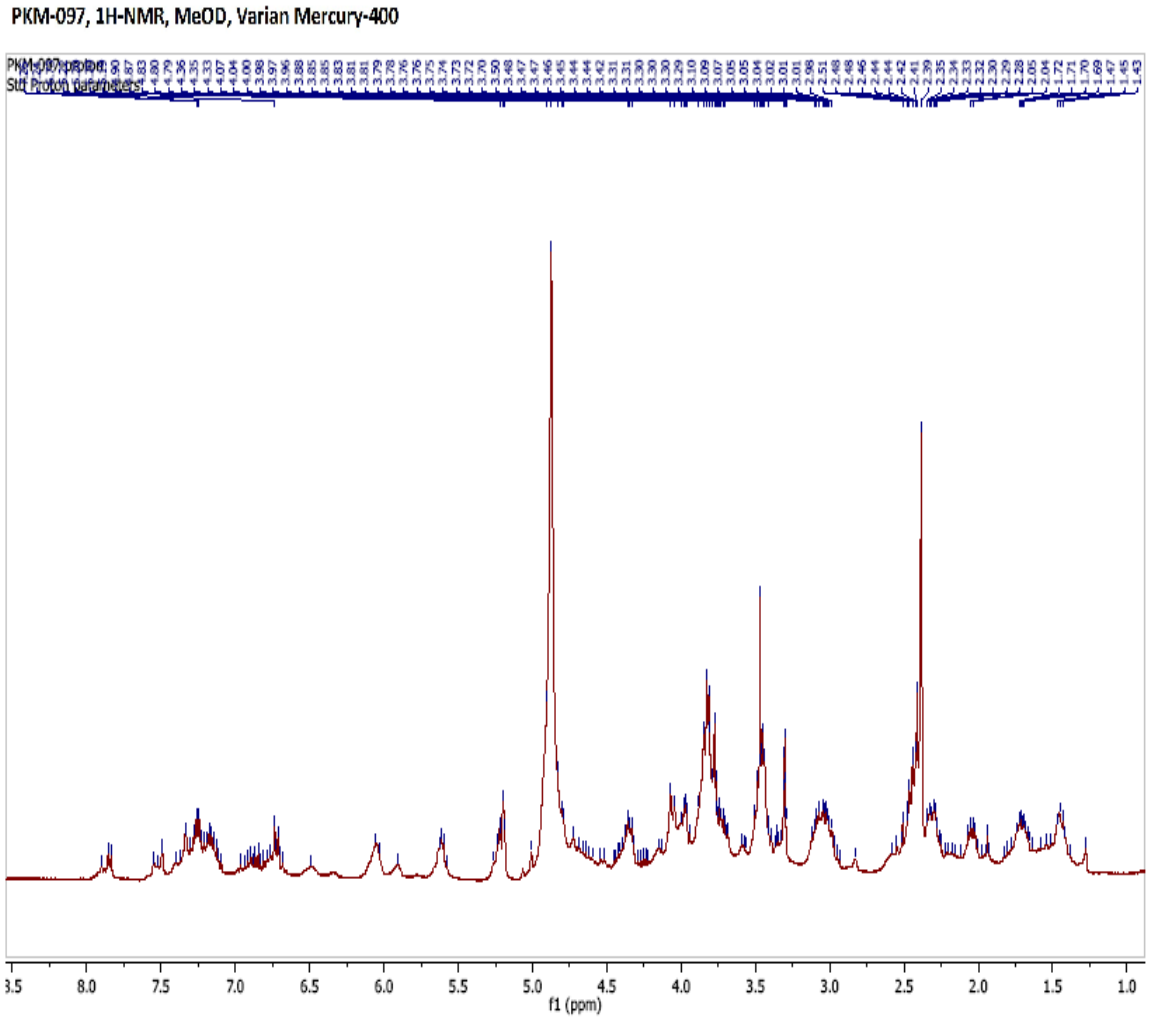
PKM-096, 1H-NMR, MeOD, Varian Mercury-400



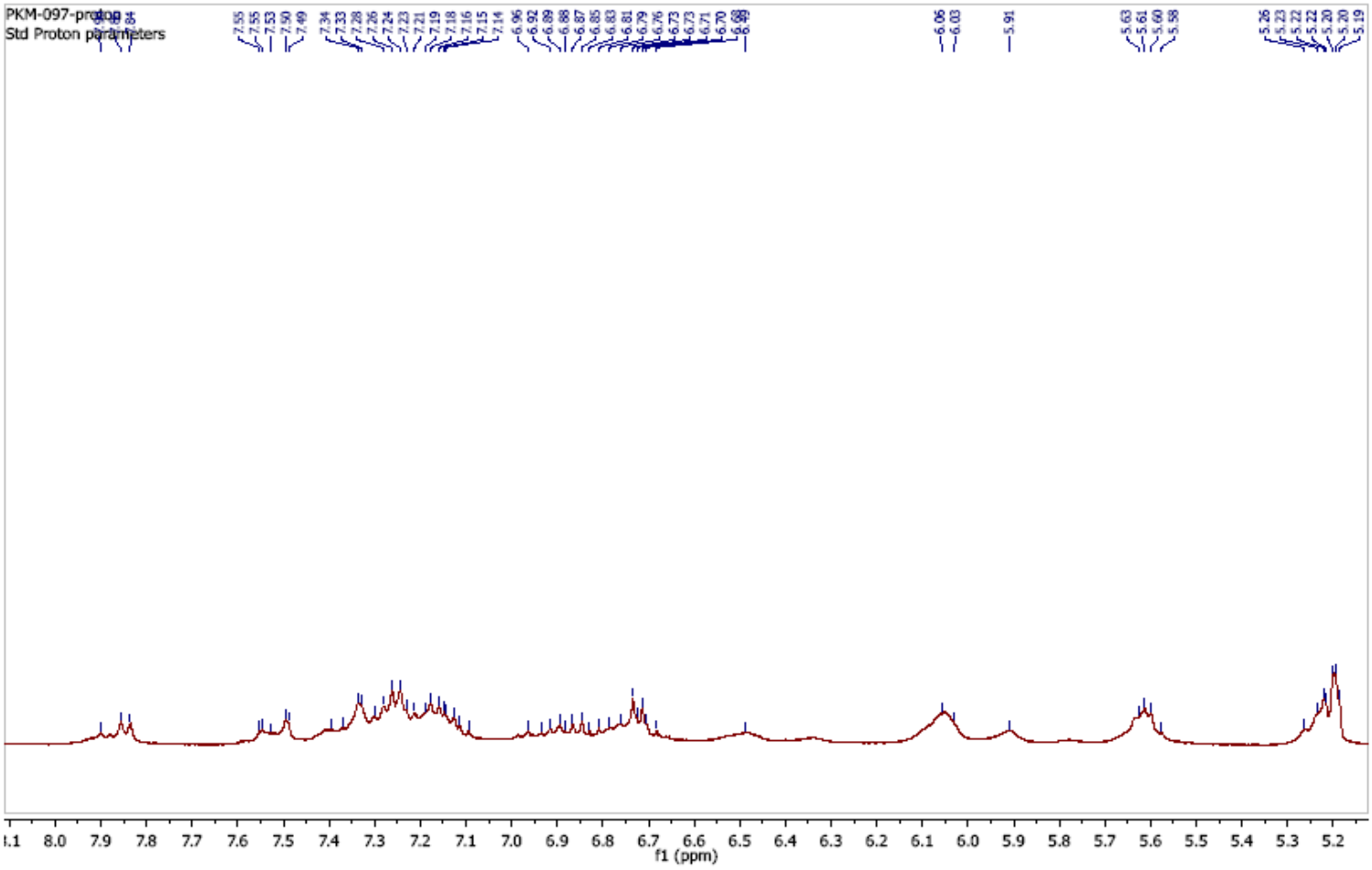
PKM-096, 13C-NMR, MeOD, Varian Mercury-400



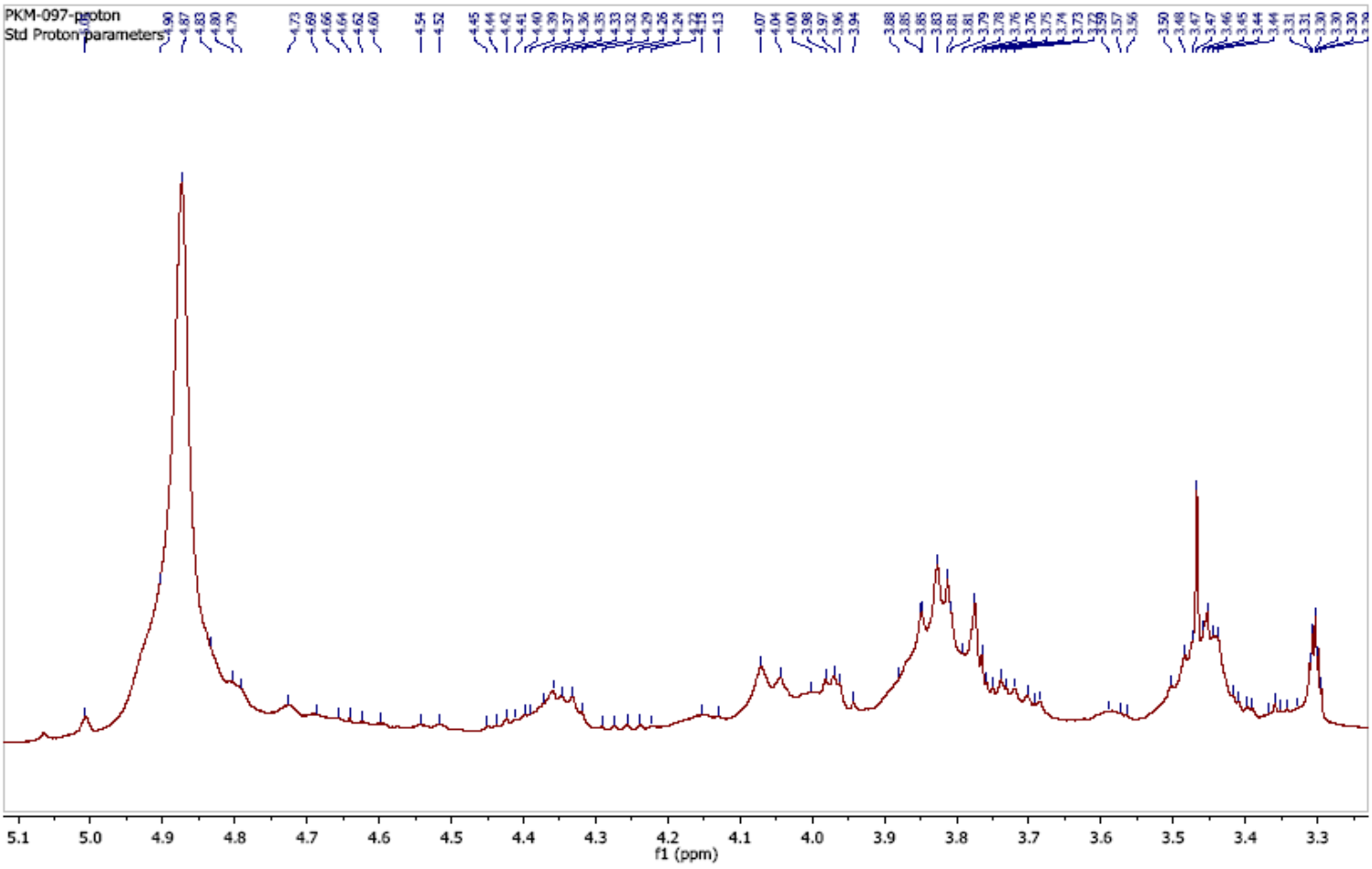
d) PKM 097



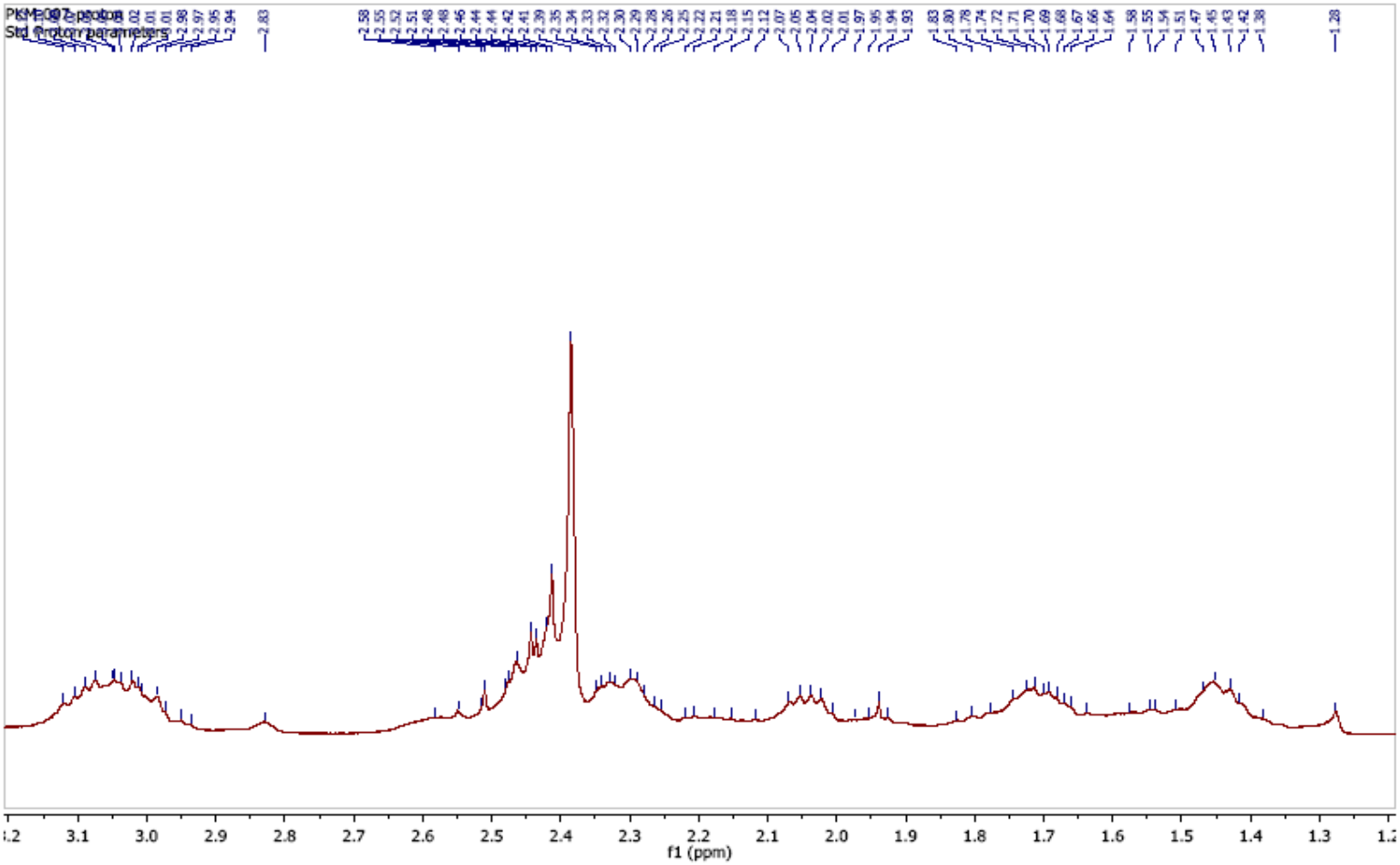
PKM-097, 1H-NMR, MeOD, Varian Mercury-400



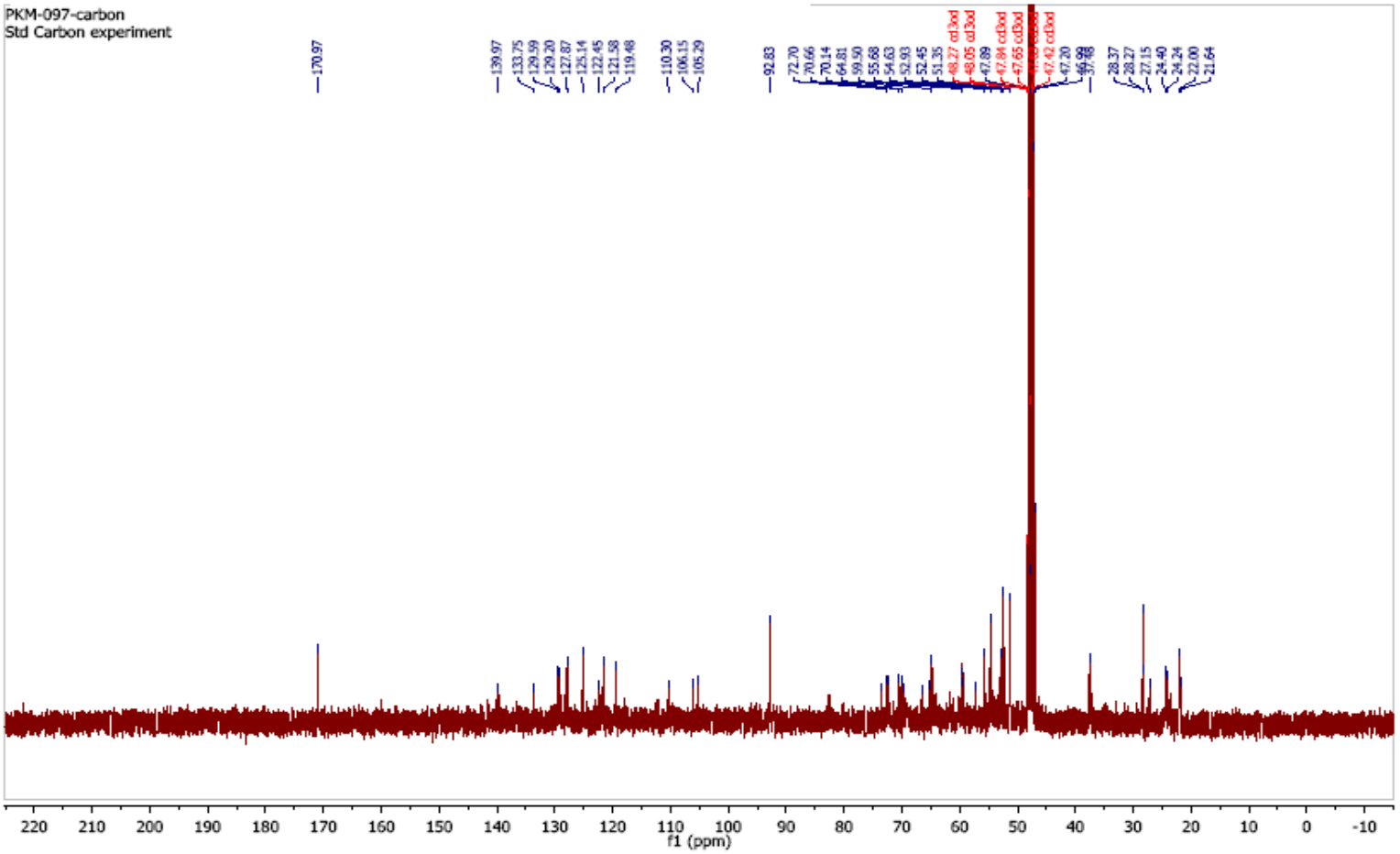
PKM-097, 1H-NMR, MeOD, Varian Mercury-400



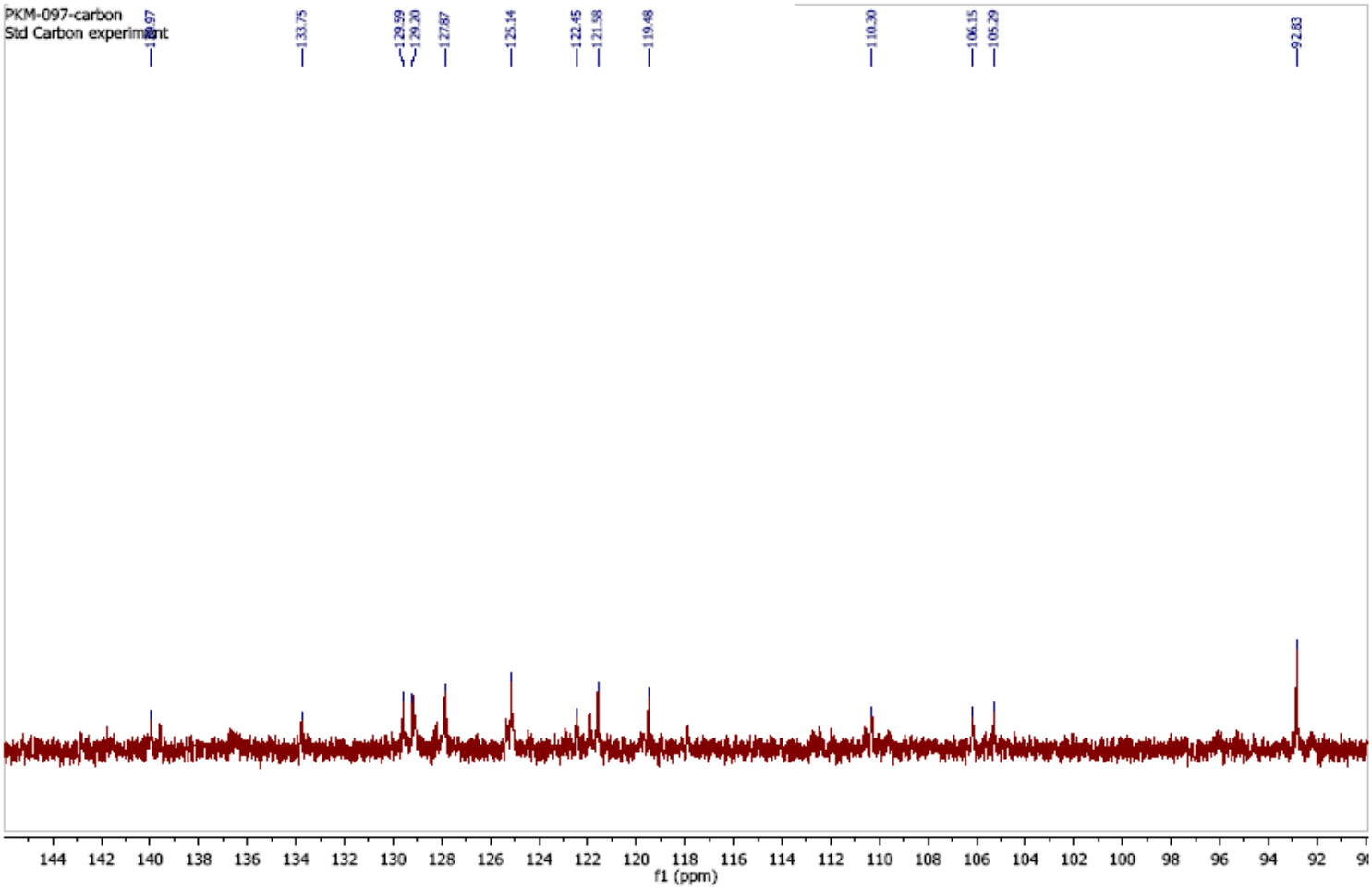
PKM-097, 1H-NMR, MeOD, Varian Mercury-400



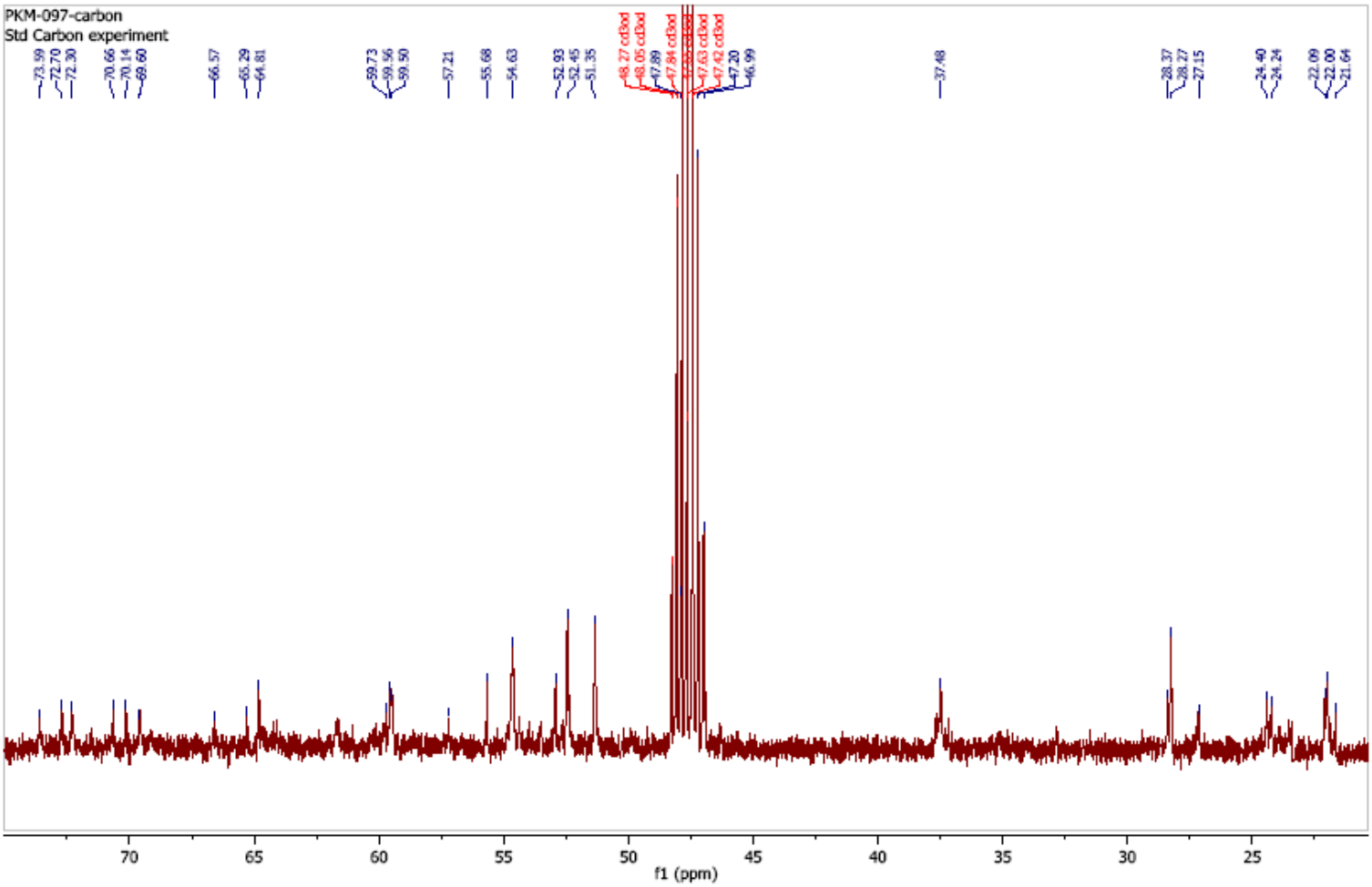
PKM-097, 13C-NMR, MeOD, Varian Mercury-400



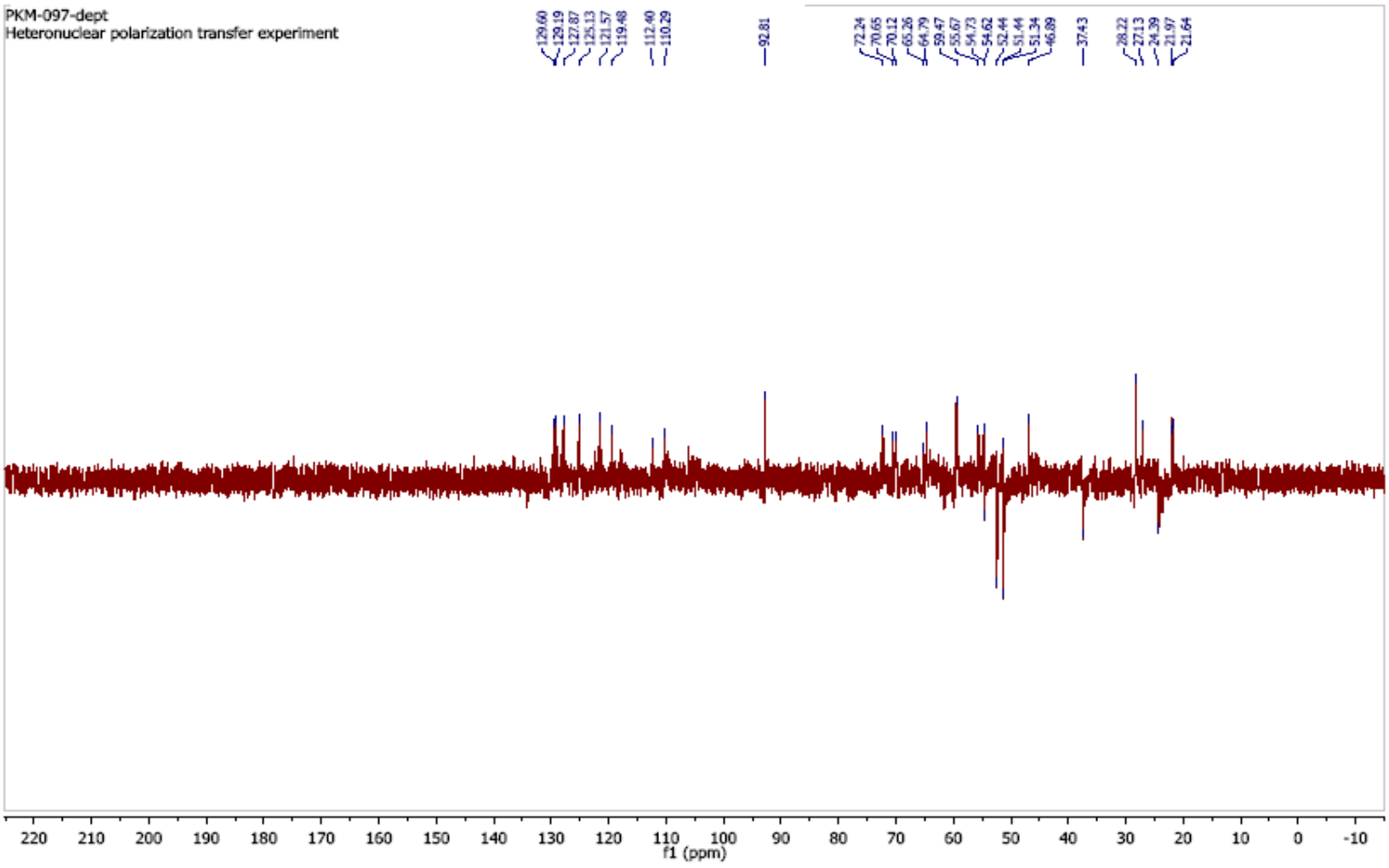
PKM-097, 13C-NMR, MeOD, Varian Mercury-400



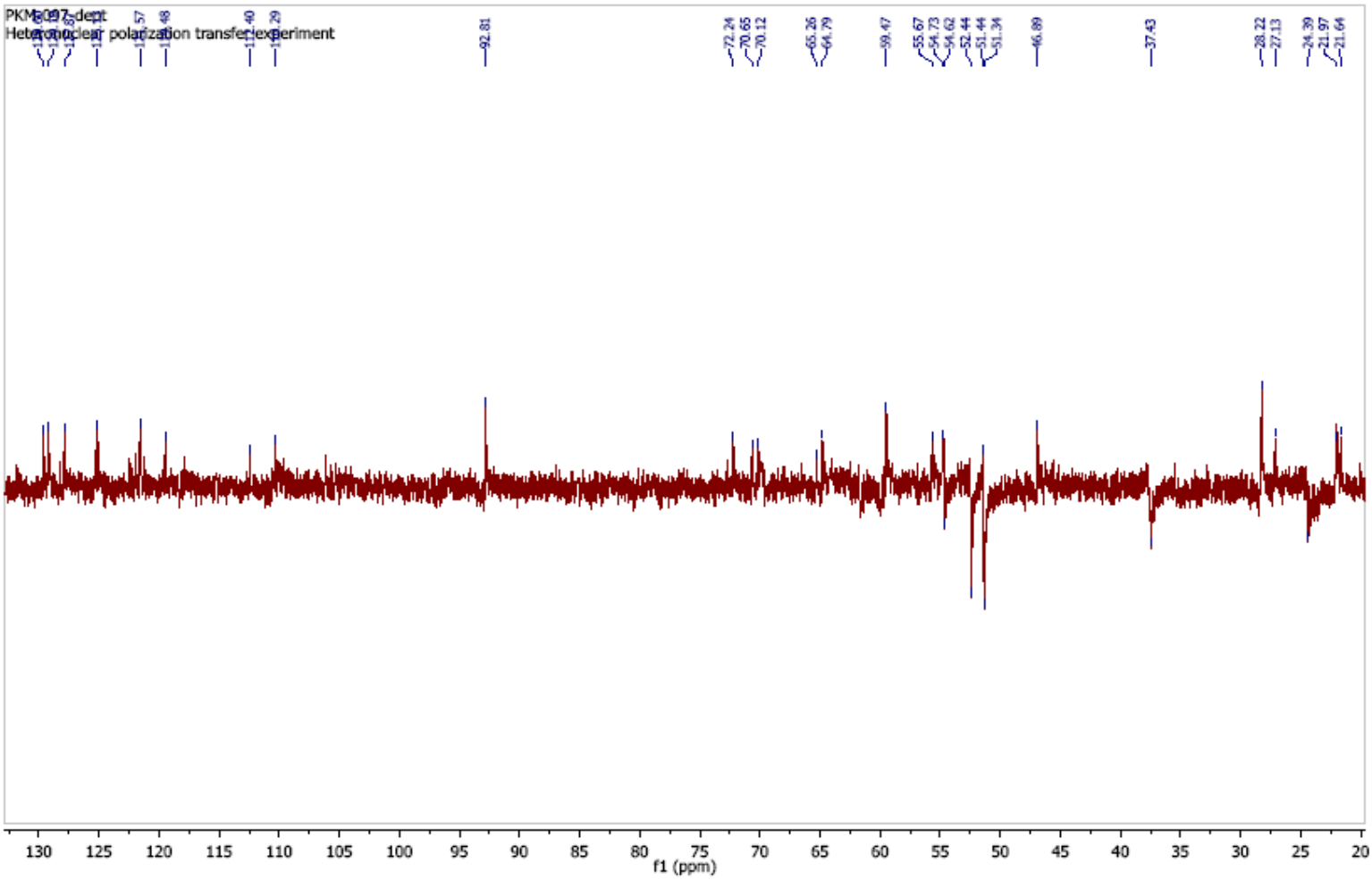
PKM-097, 13C-NMR, MeOD, Varian Mercury-400



PKM-097, DEPT-135, MeOD, Varian Mercury-400

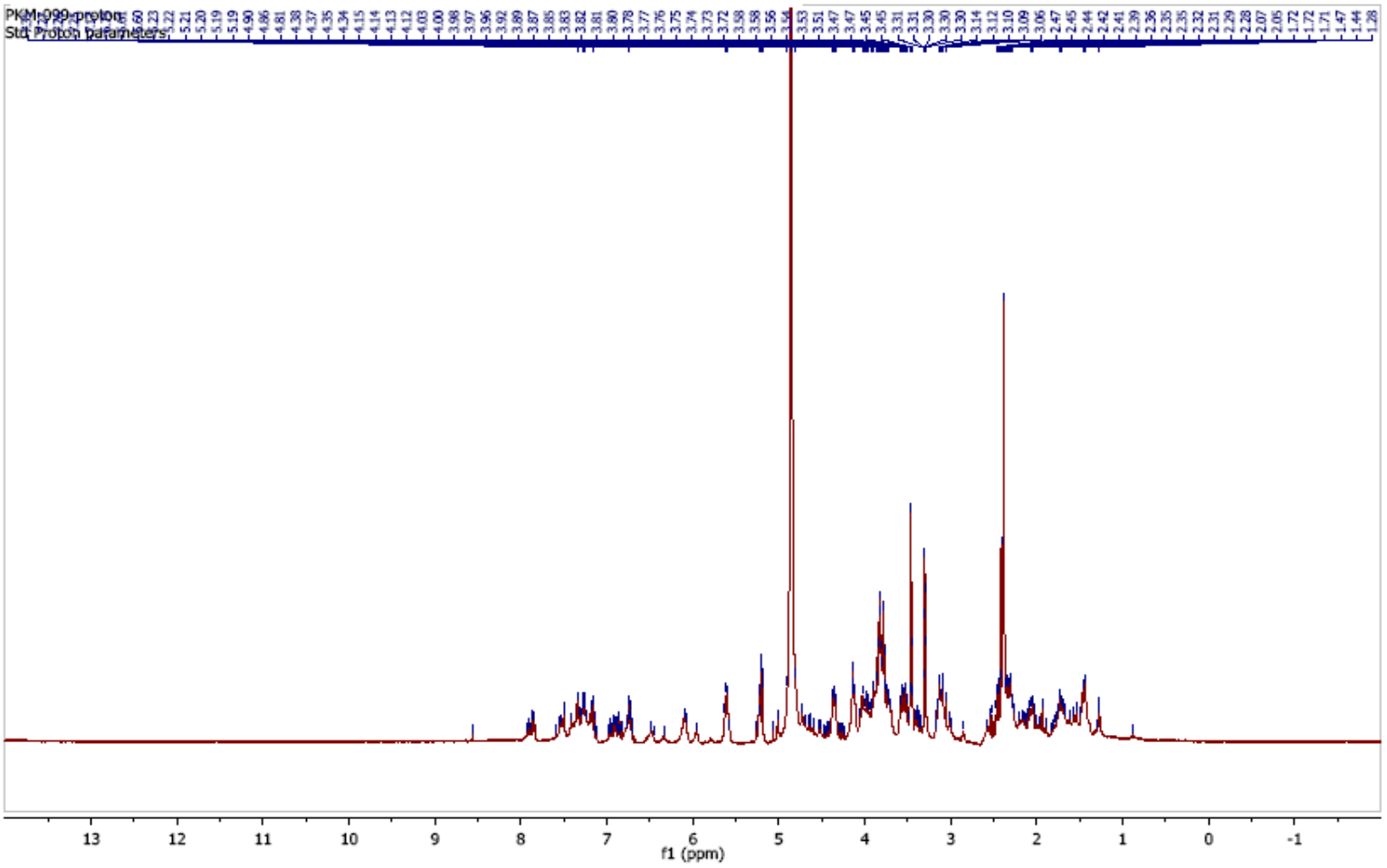


PKM-097, DEPT-135, MeOD, Varian Mercury-400

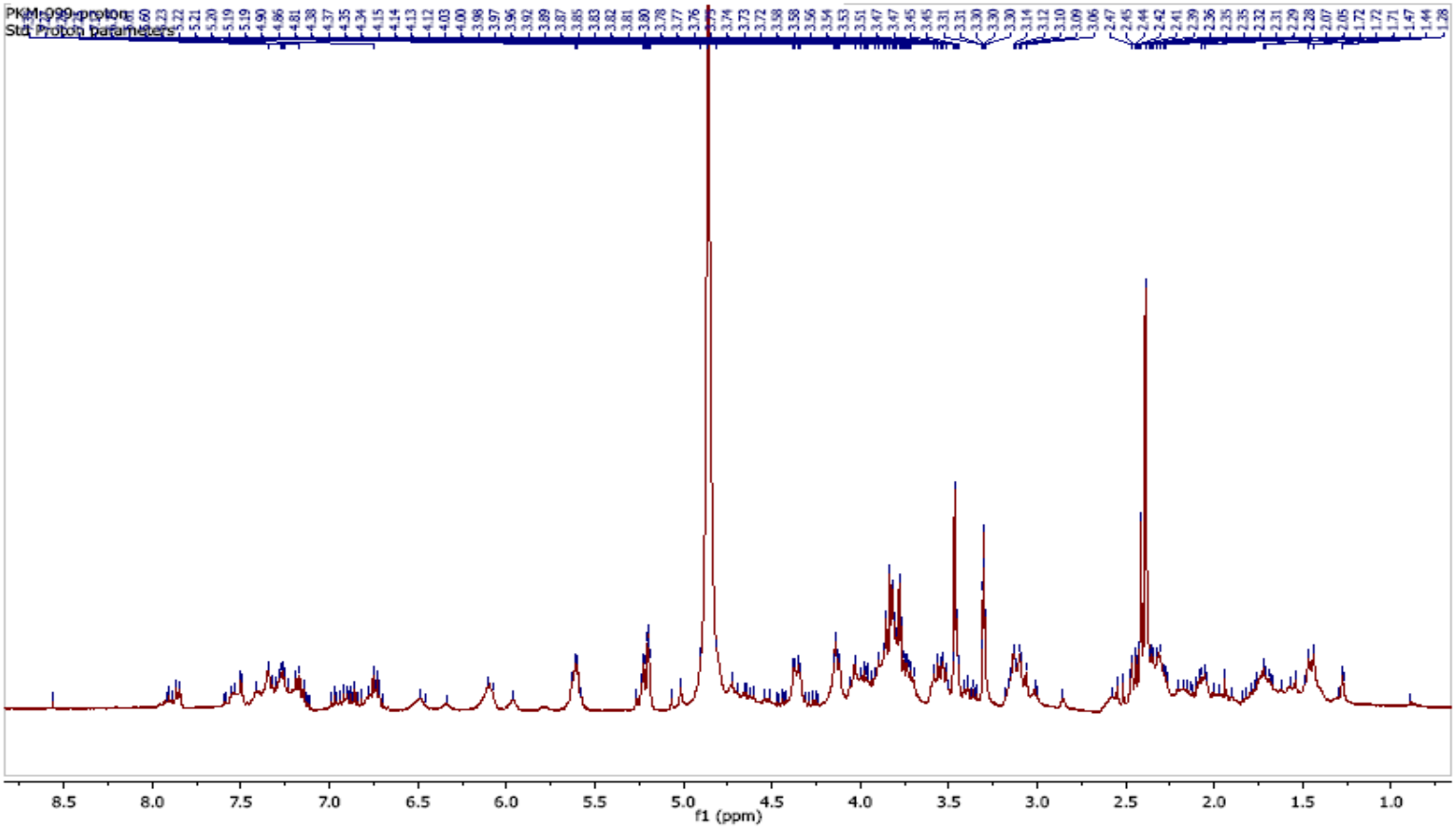


e) PKM 099

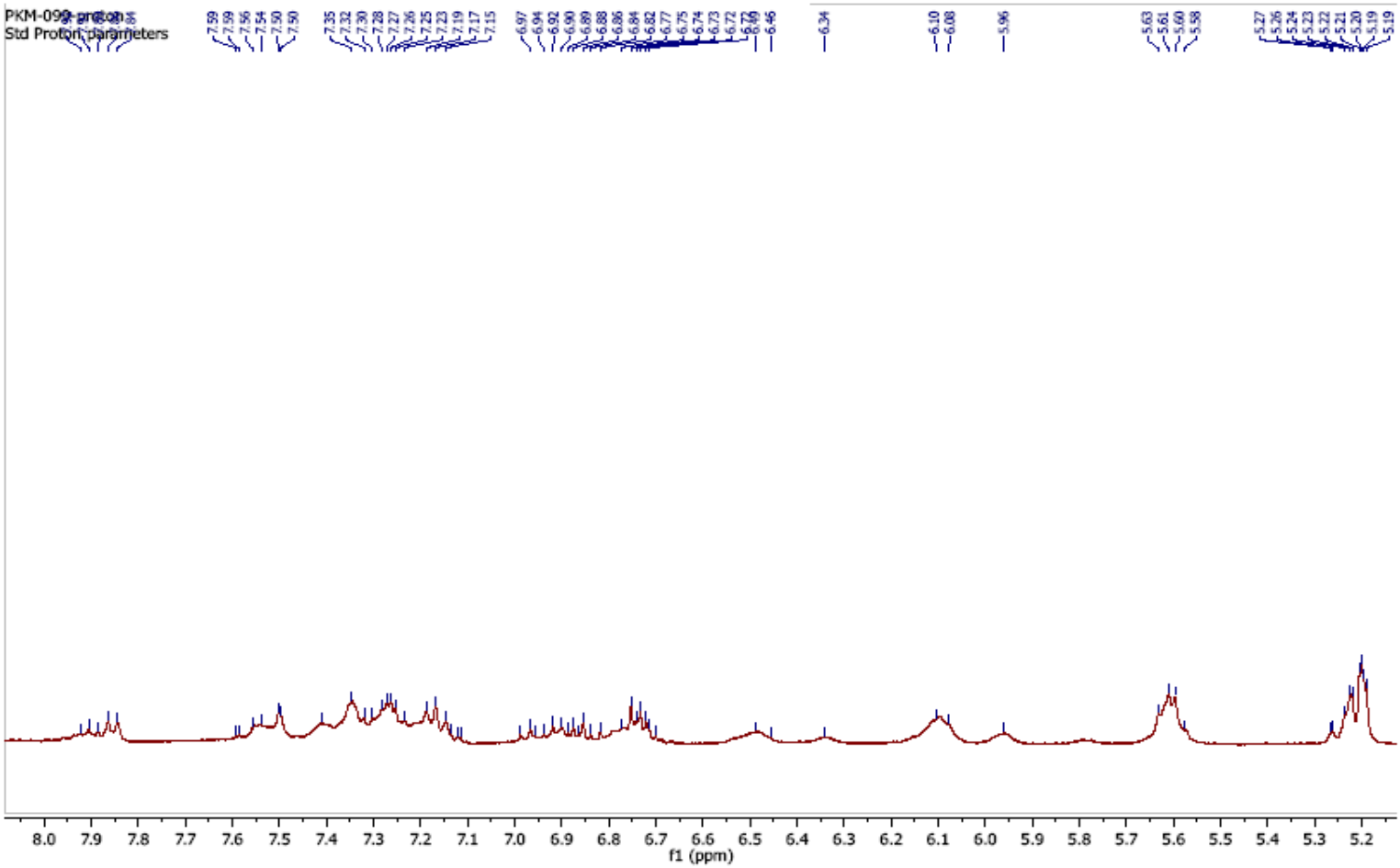
PKM-099, 1H-NMR, MeOD, Varian Mercury-400



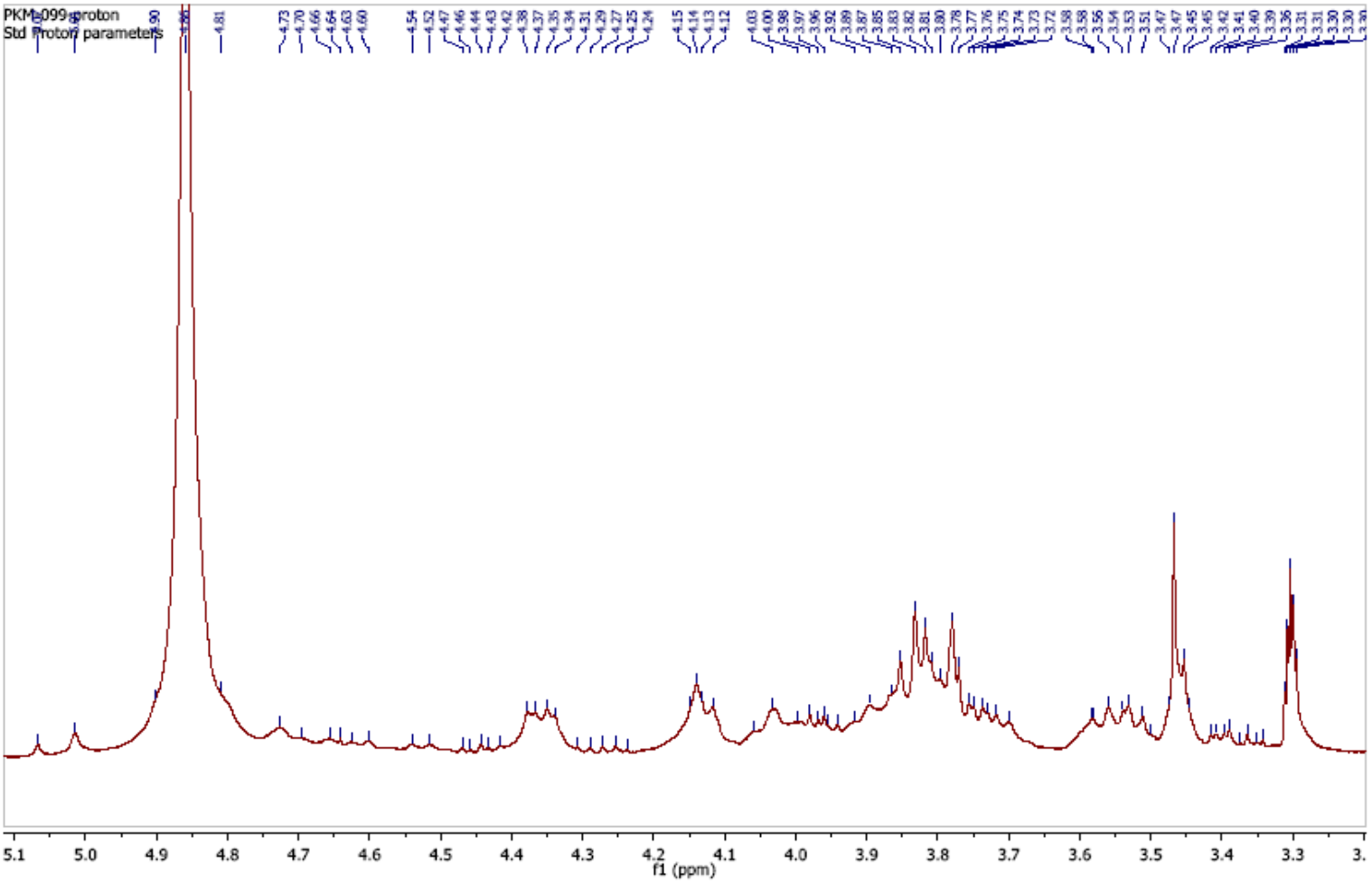
PKM-099, 1H-NMR, MeOD, Varian Mercury-400



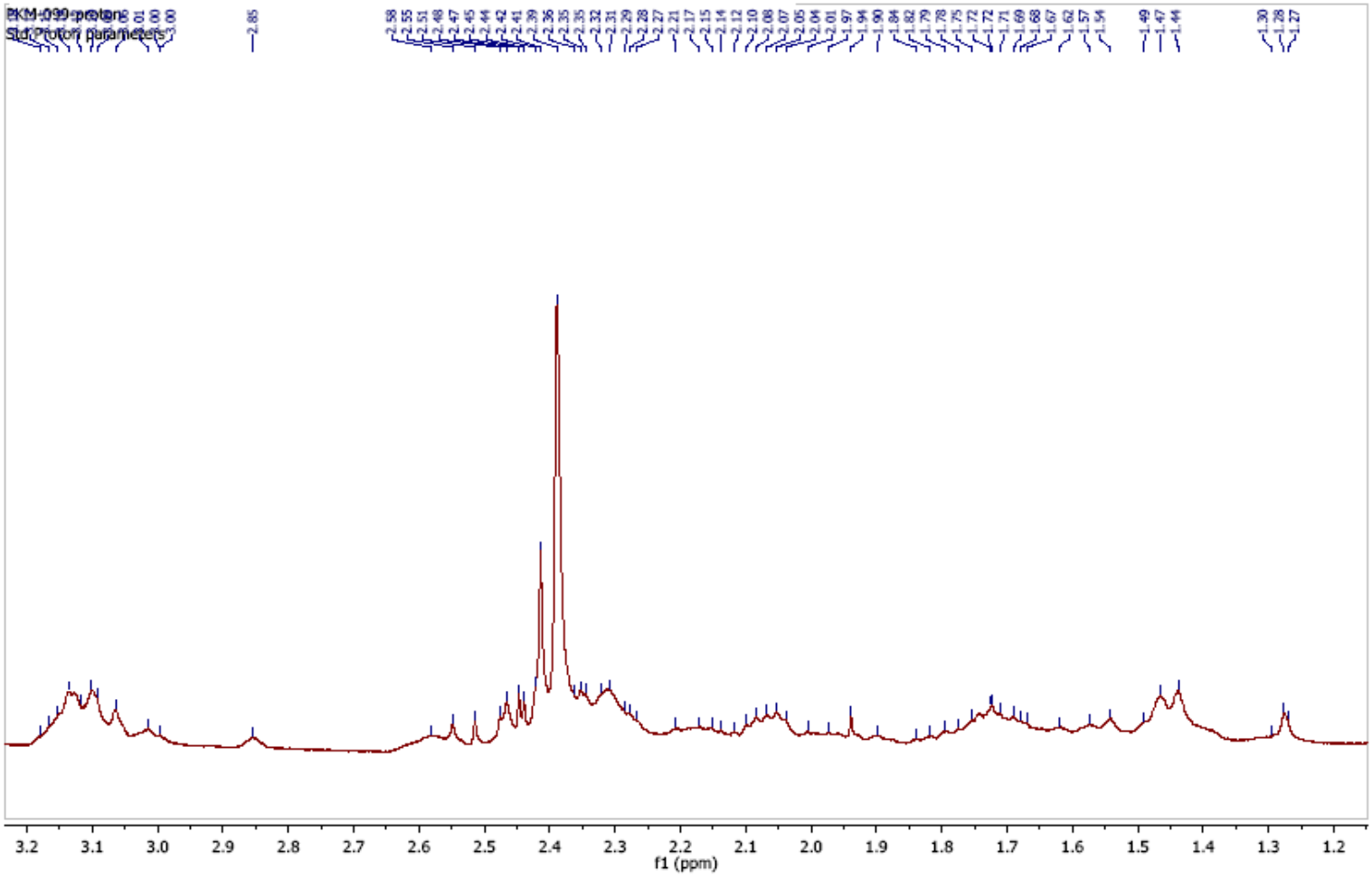
PKM-099, 1H-NMR, MeOD, Varian Mercury-400



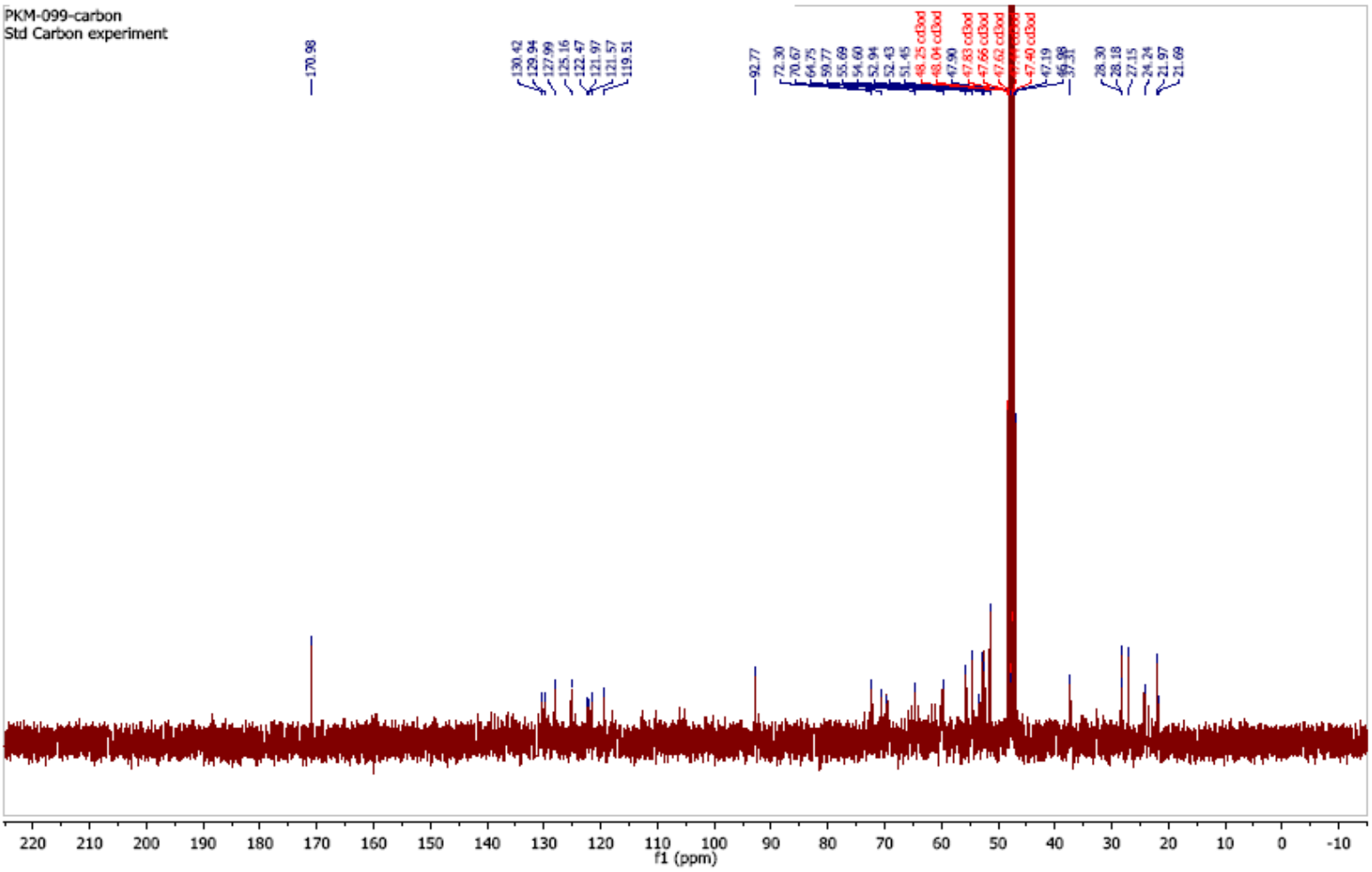
PKM-099, 1H-NMR, MeOD, Varian Mercury-400



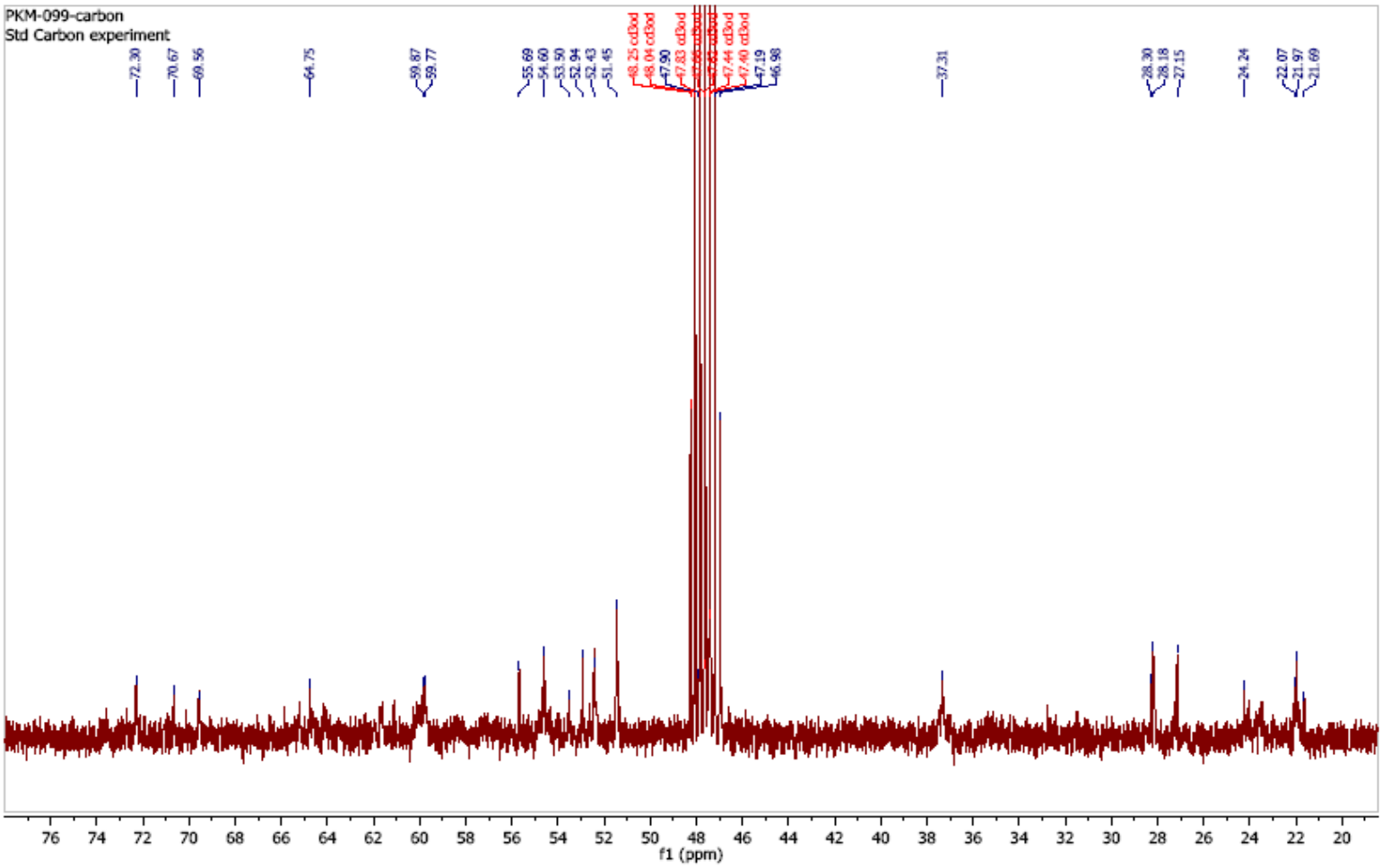
PKM-099, 1H-NMR, MeOD, Varian Mercury-400



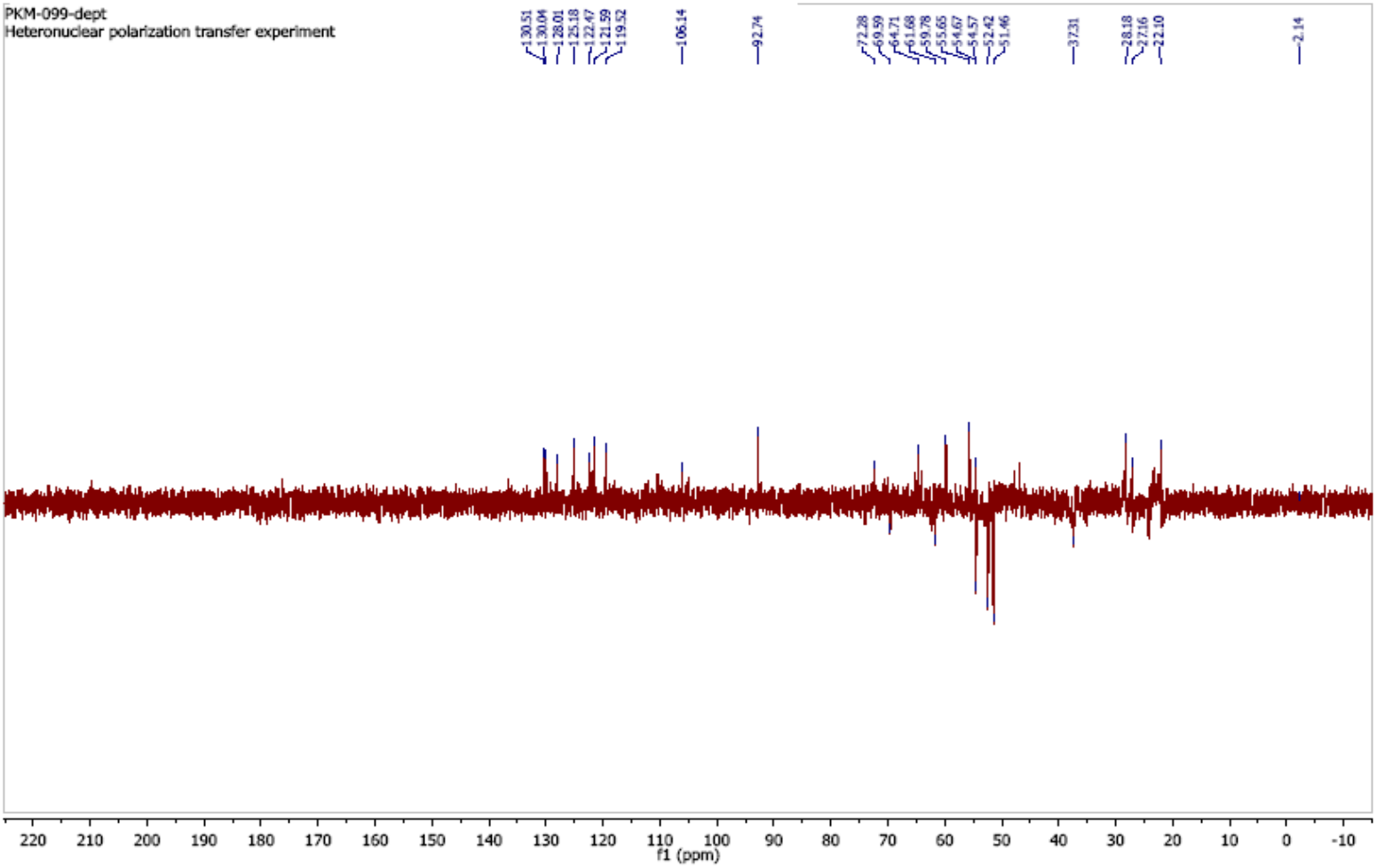
PKM-099, 13C-NMR, MeOD, Varian Mercury-400



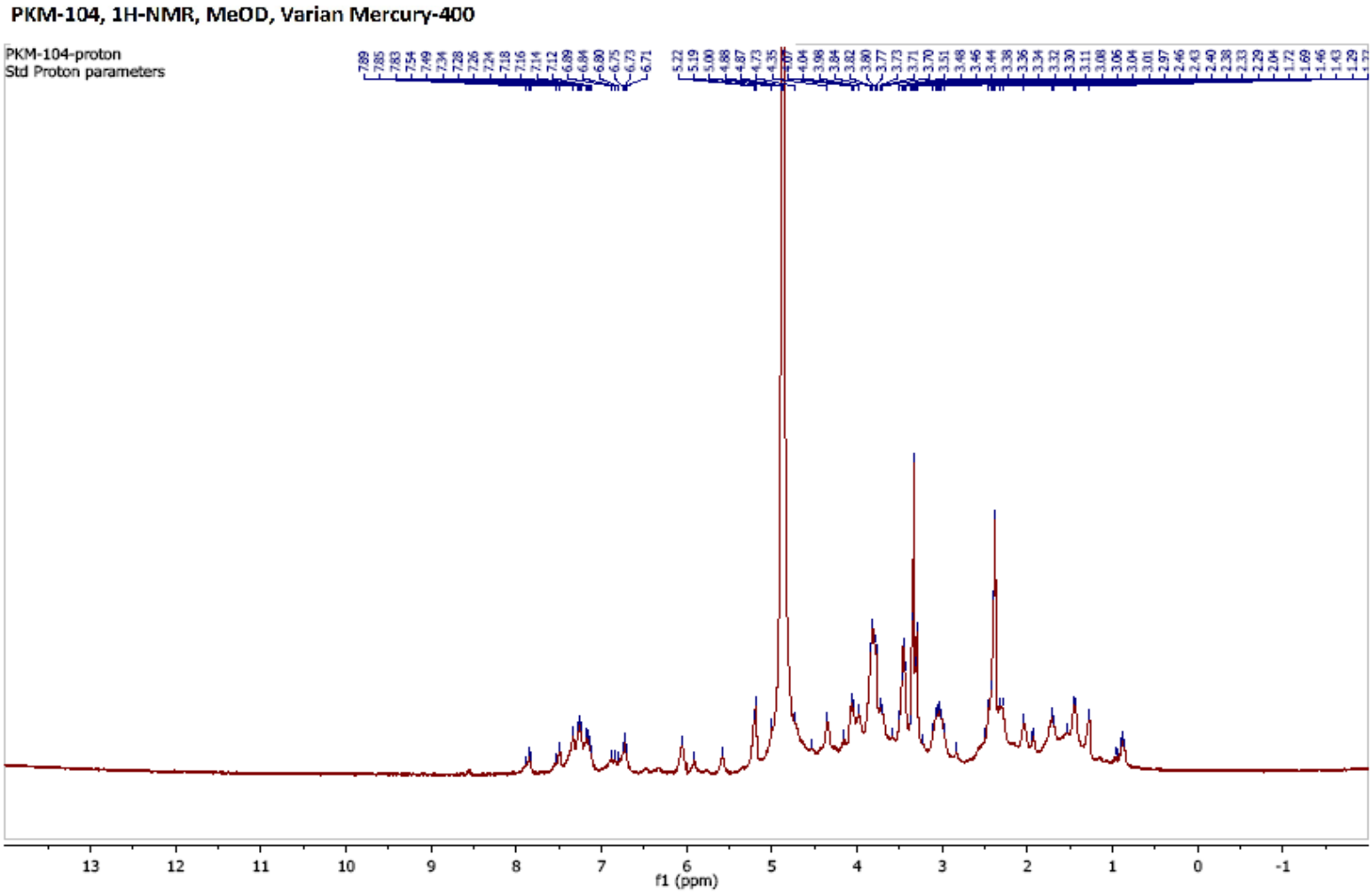
PKM-099, 13C-NMR, MeOD, Varian Mercury-400



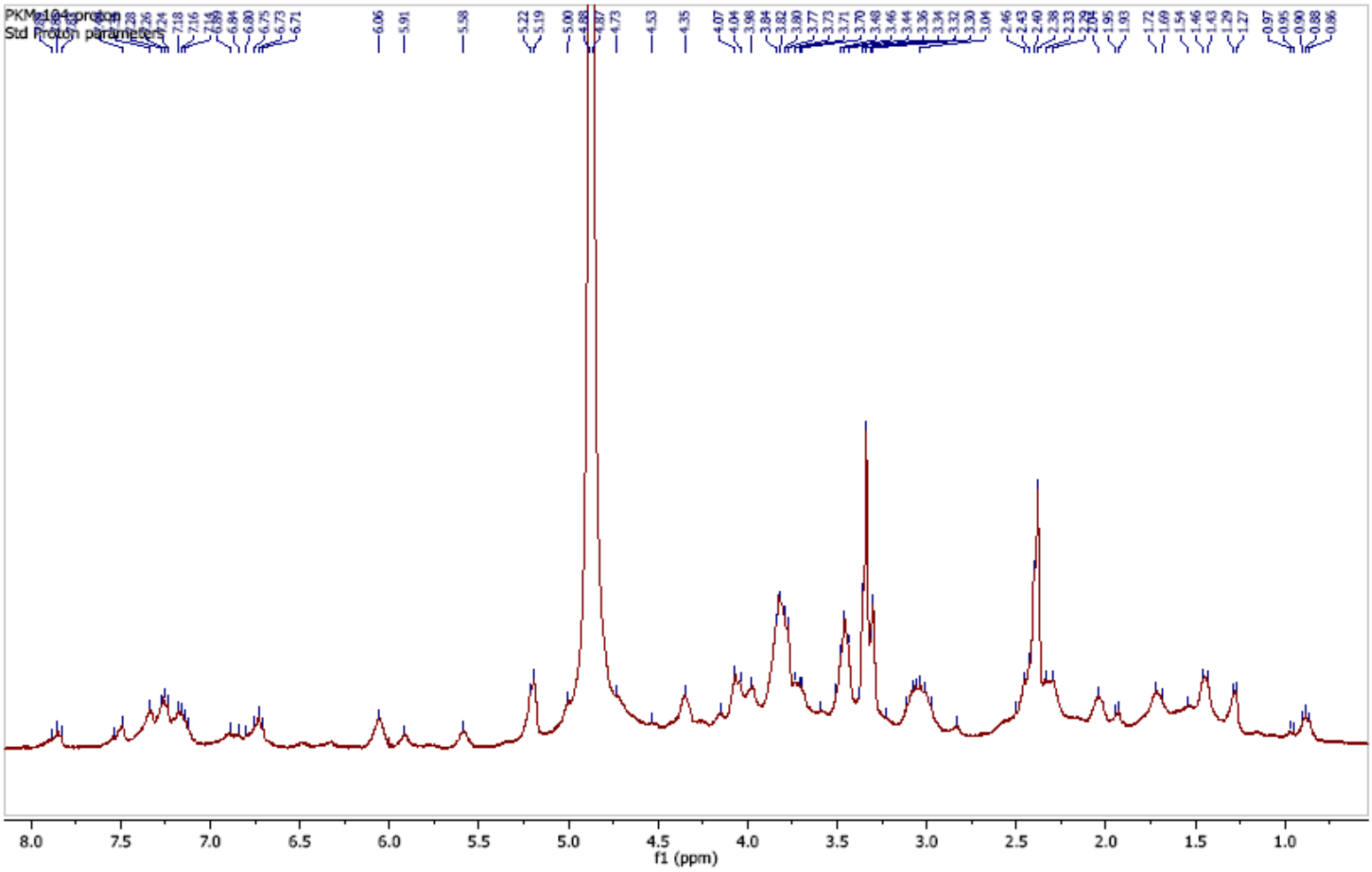
PKM-099, DEPT, MeOD, Varian Mercury-400



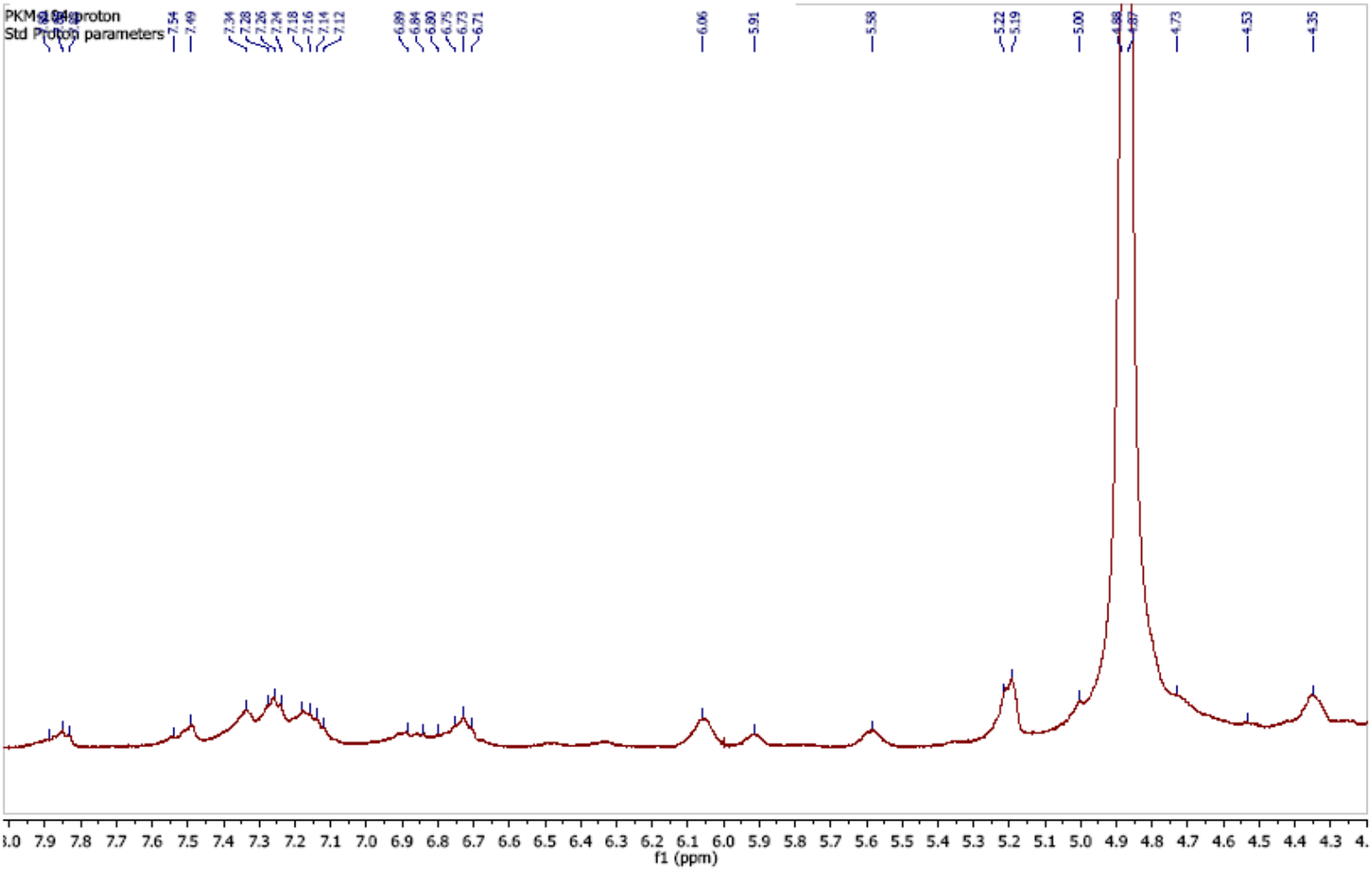
f) PKM 104


PKM-104, 1H-NMR, MeOD, Varian Mercury-400
PKM-104-proton
Std Proton parameters
f1 (ppm)

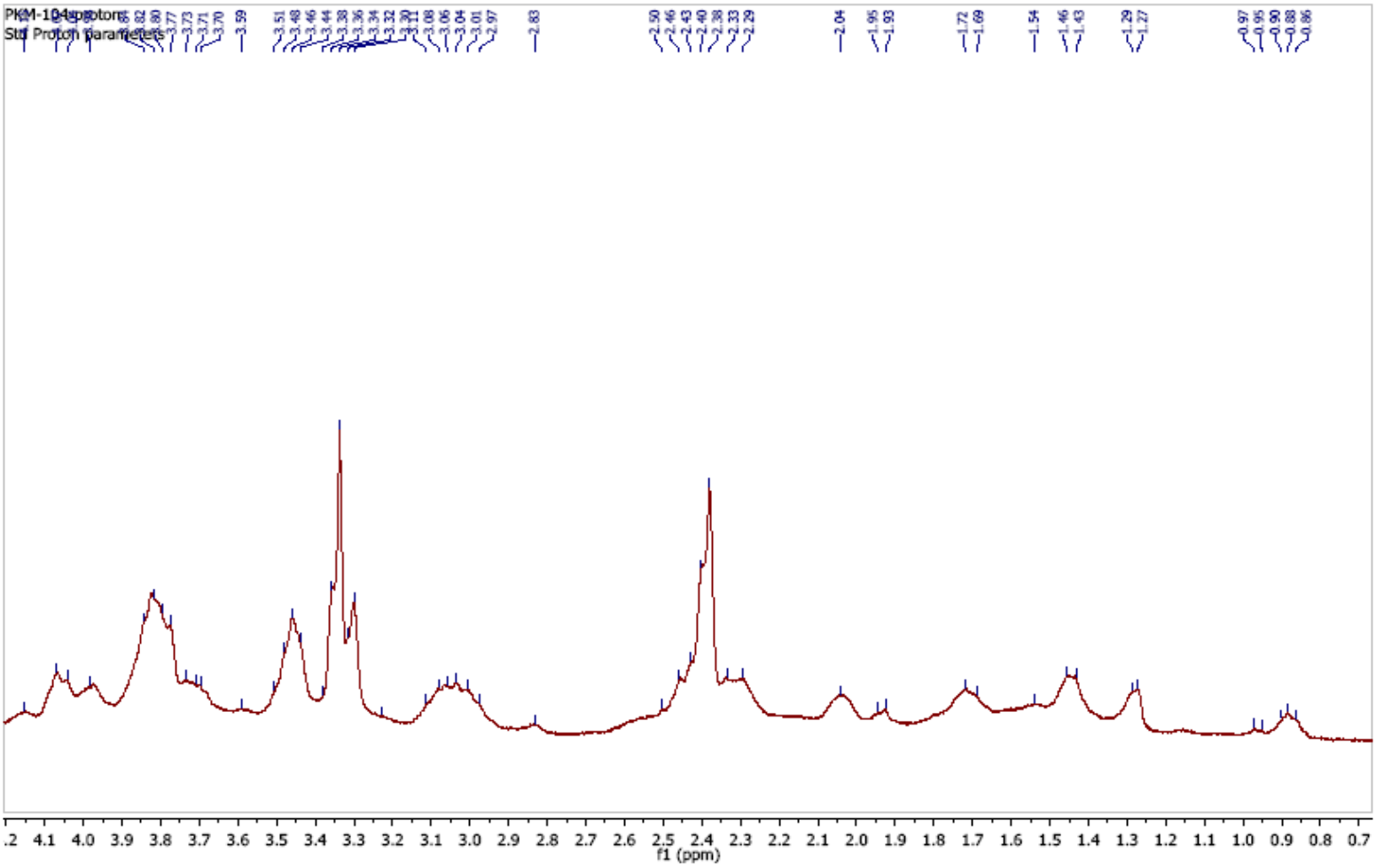
PKM-104, 1H-NMR, MeOD, Varian Mercury-400



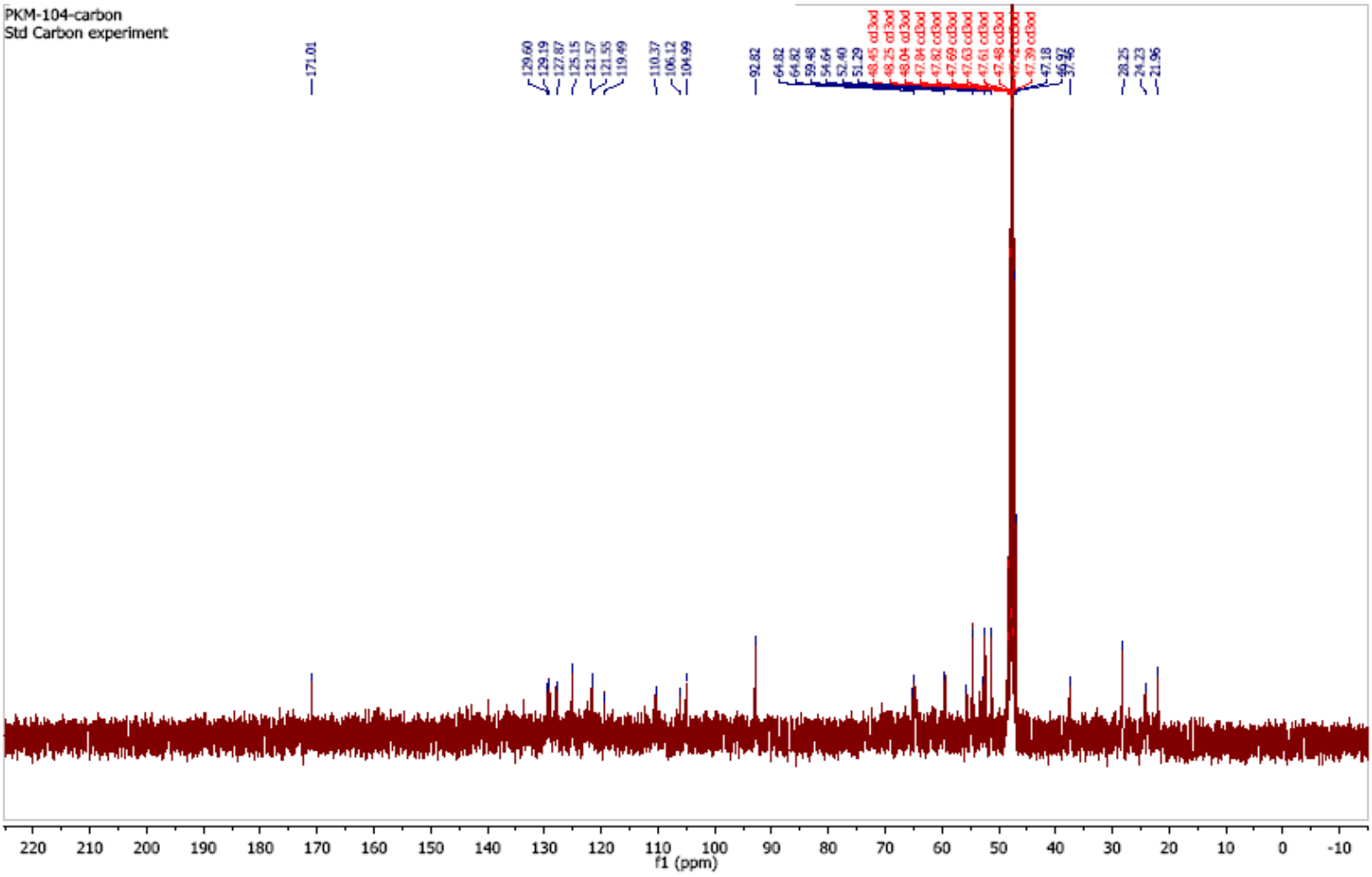
PKM-104, 1H-NMR, MeOD, Varian Mercury-400



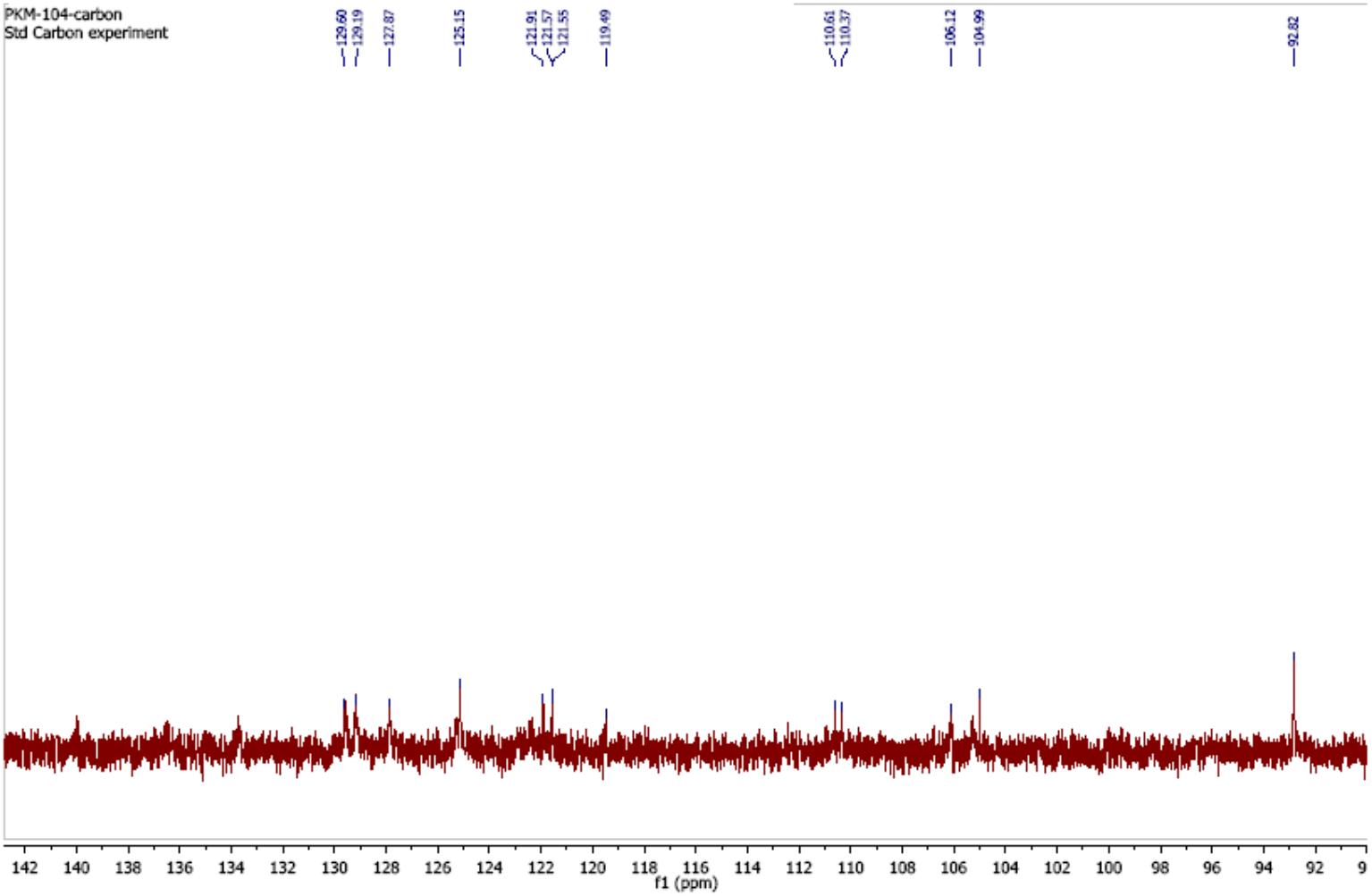
PKM-104, 1H-NMR, MeOD, Varian Mercury-400



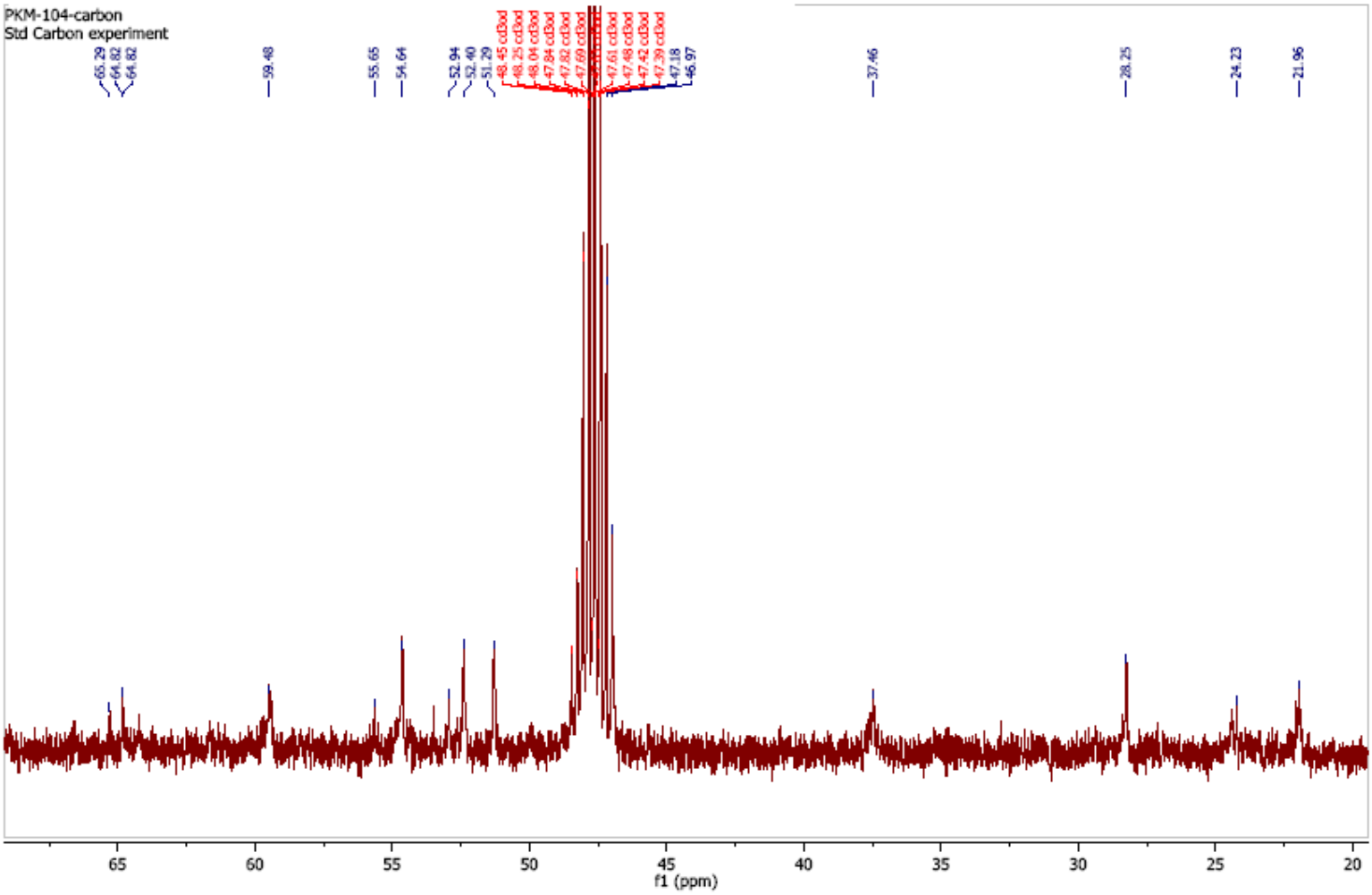
PKM-104, 13C-NMR, MeOD, Varian Mercury-400



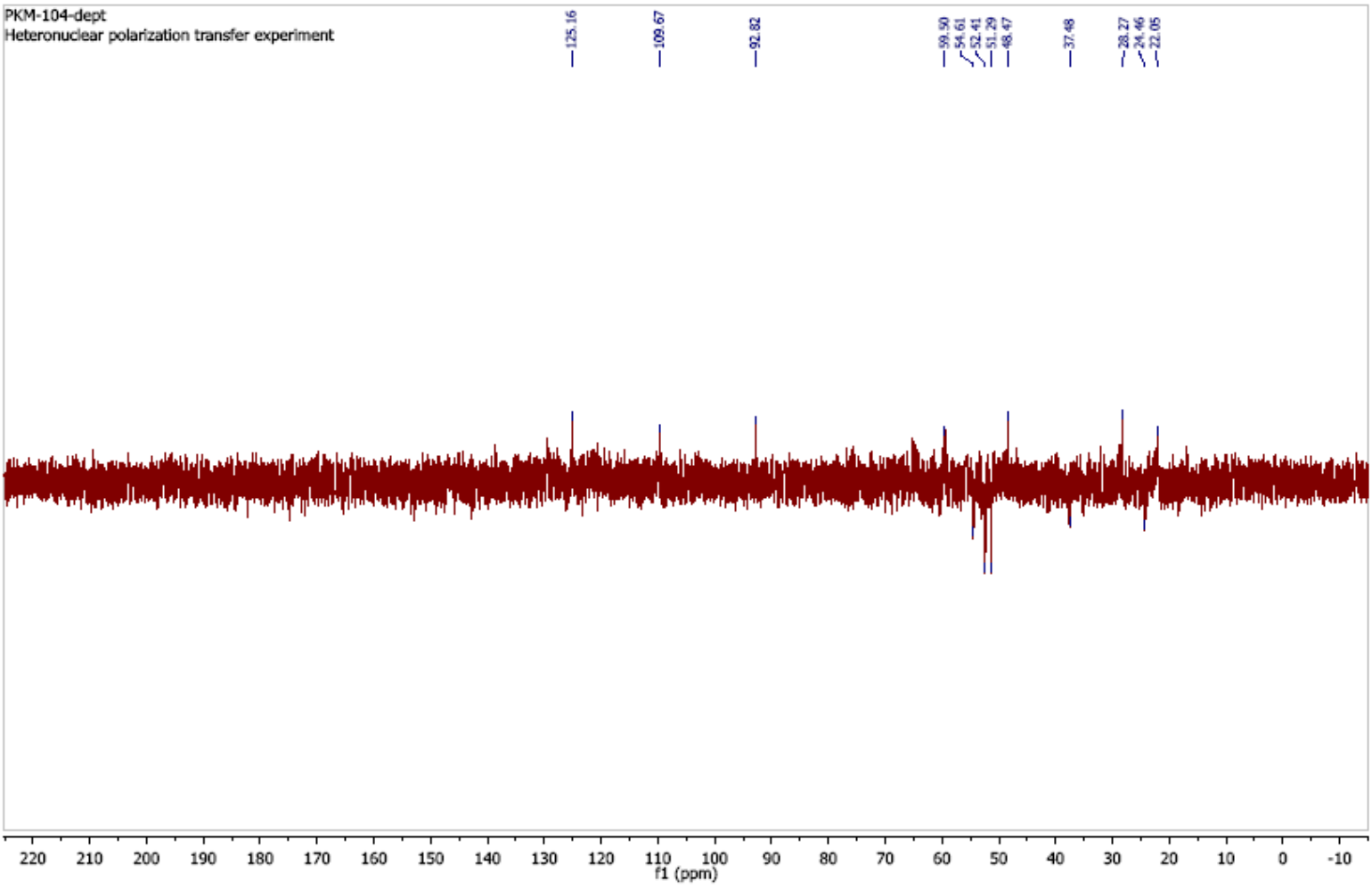
PKM-104, 13C-NMR, MeOD, Varian Mercury-400



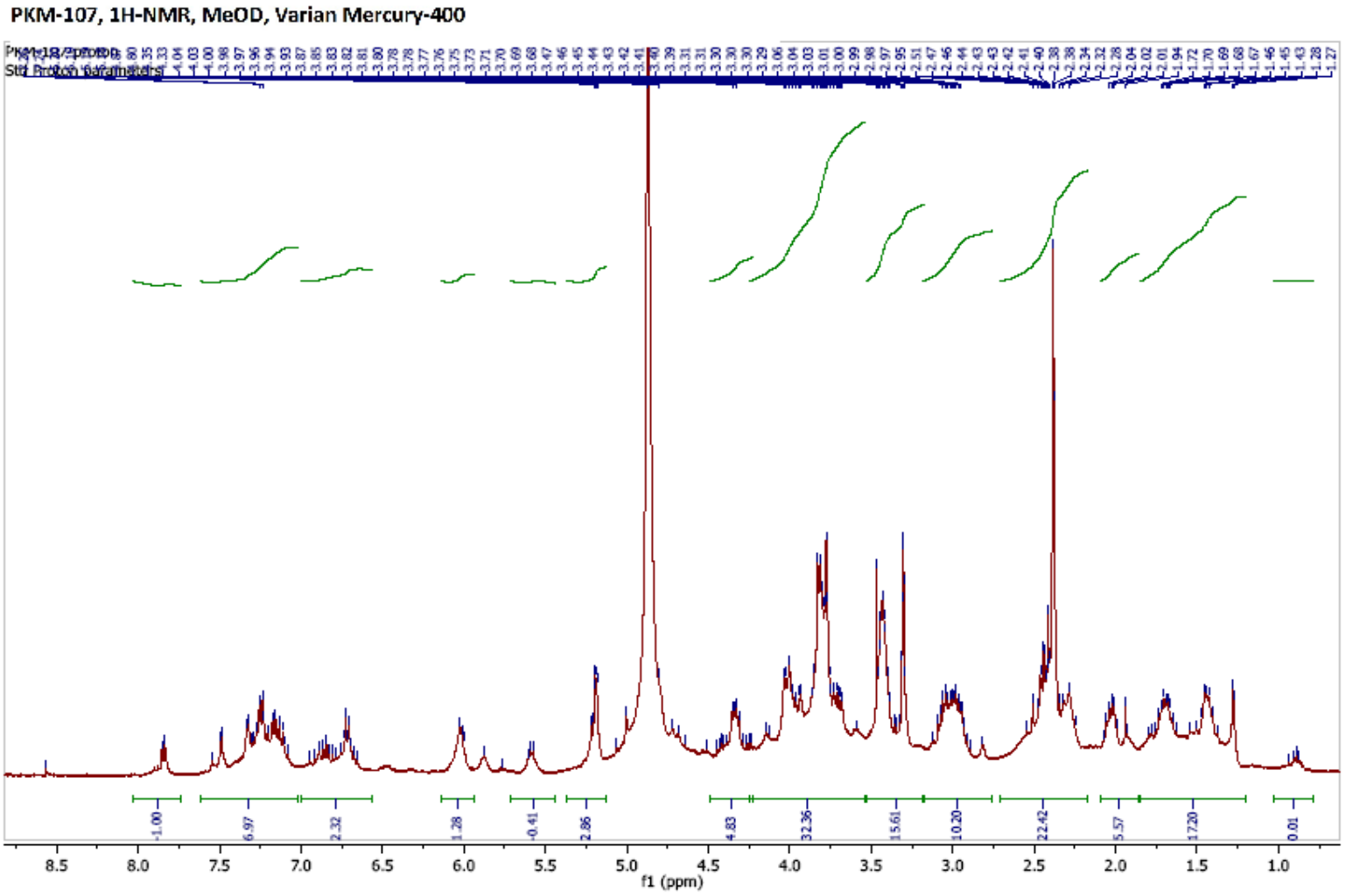
PKM-104, 13C-NMR, MeOD, Varian Mercury-400



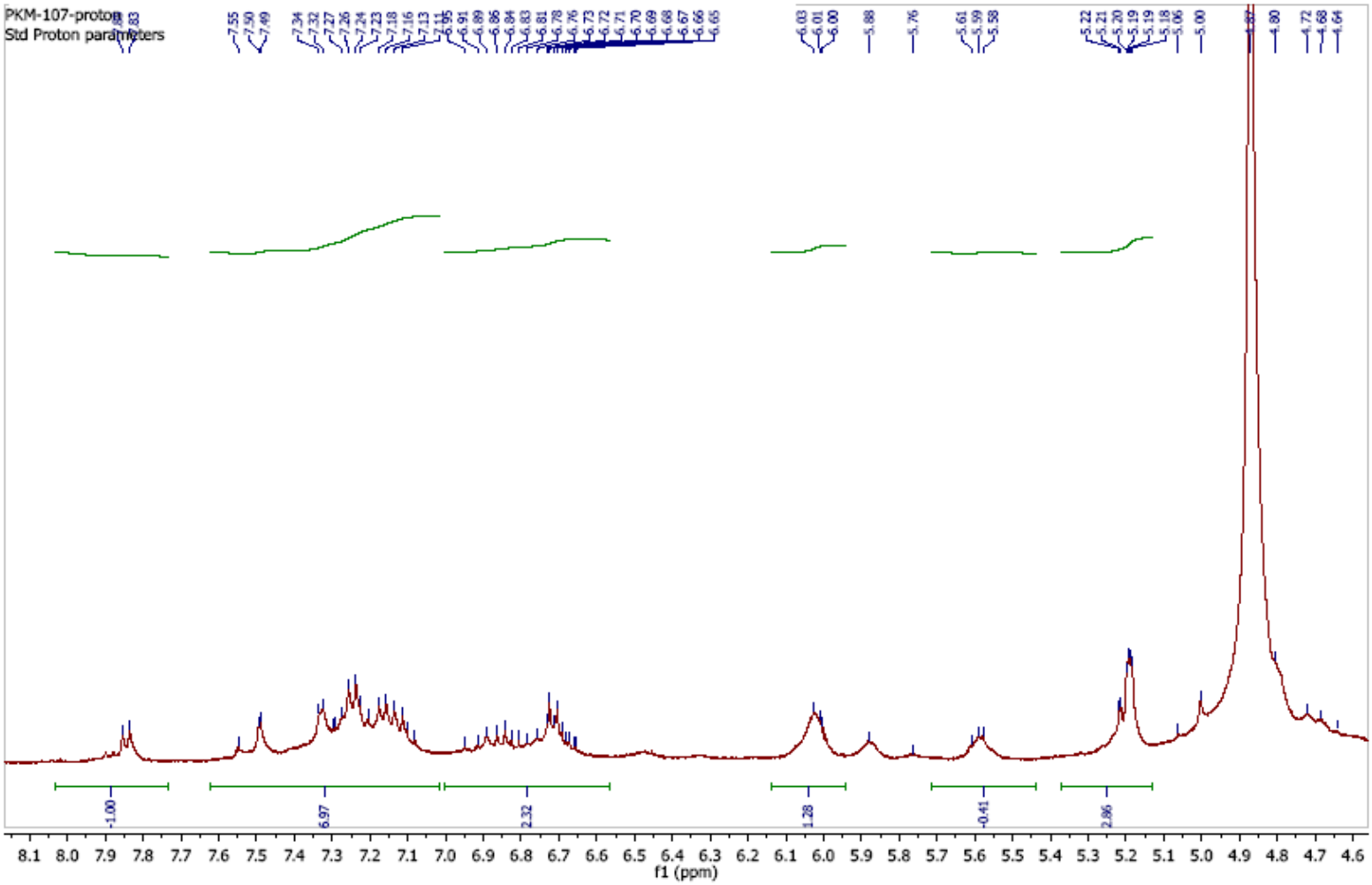
PKM-104, DEPT-135, MeOD, Varian Mercury-400



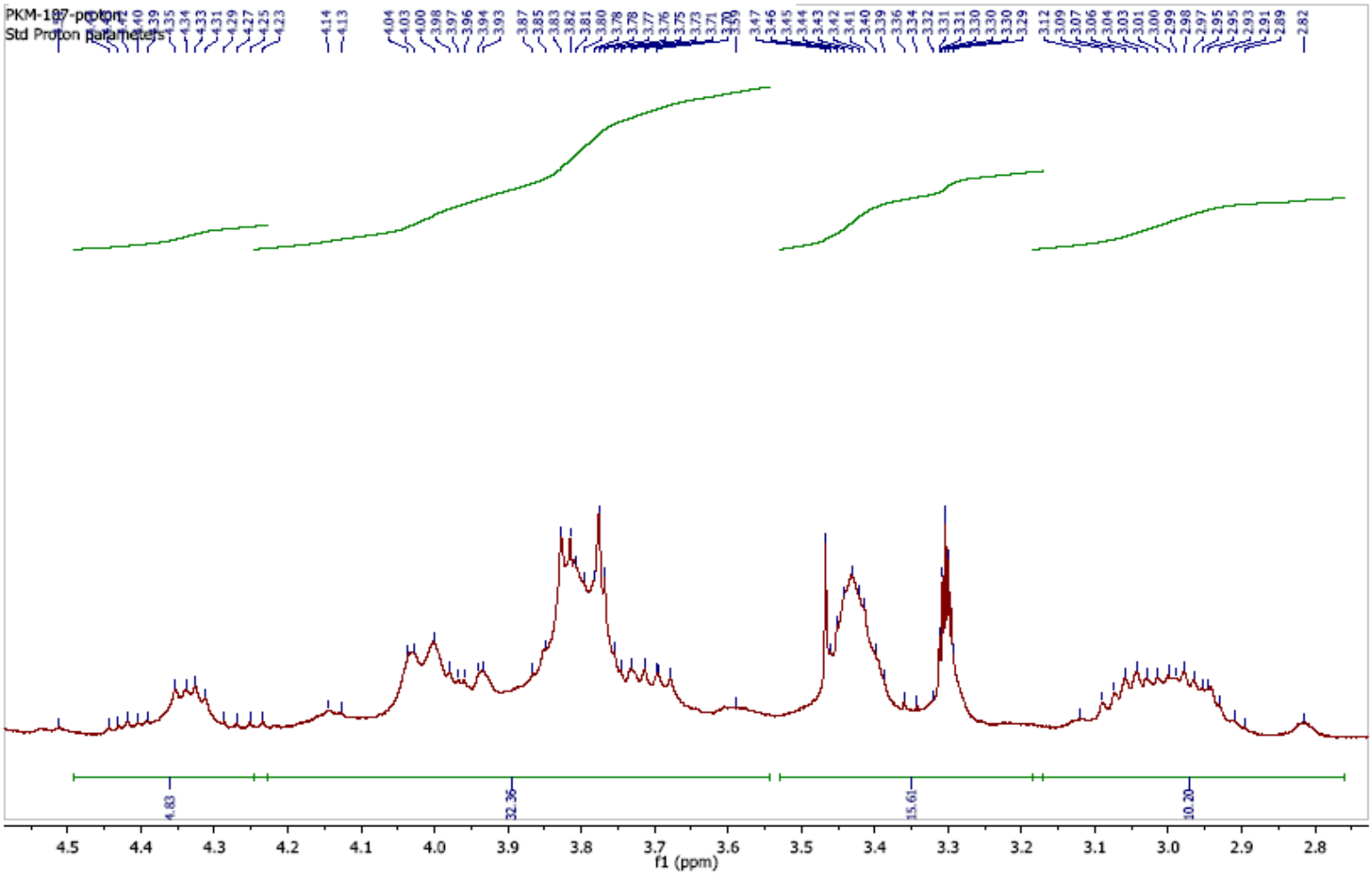
g) PKM 107


PKM-107, 1H-NMR, MeOD, Varian Mercury-400
1.00
6.97
2.32
1.28
0.41
2.86
4.83
32.36
15.61
10.20
22.42
5.57
17.20
0.01
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)

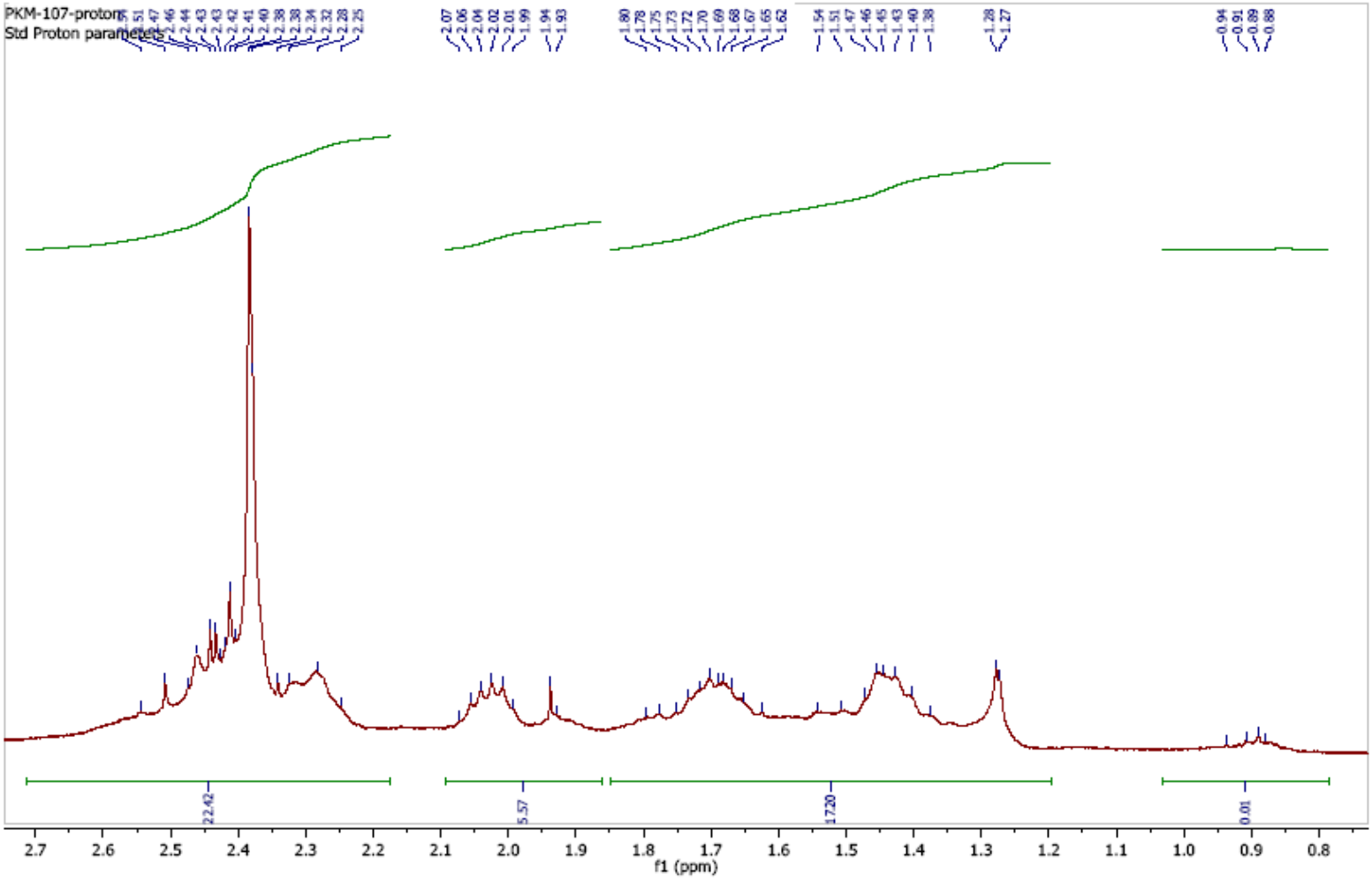
PKM-107, 1H-NMR, MeOD, Varian Mercury-400



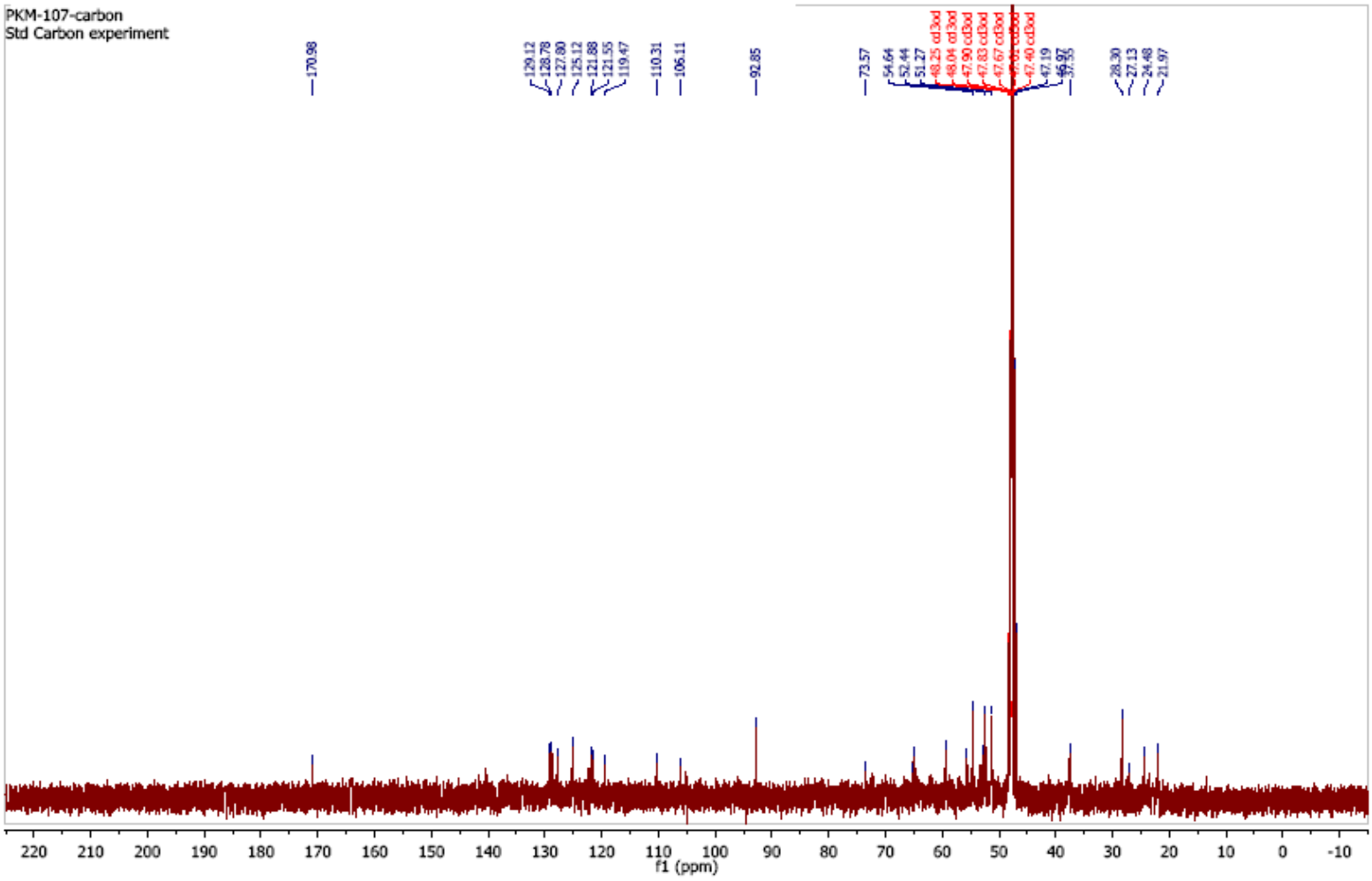
PKM-107, 1H-NMR, MeOD, Varian Mercury-400



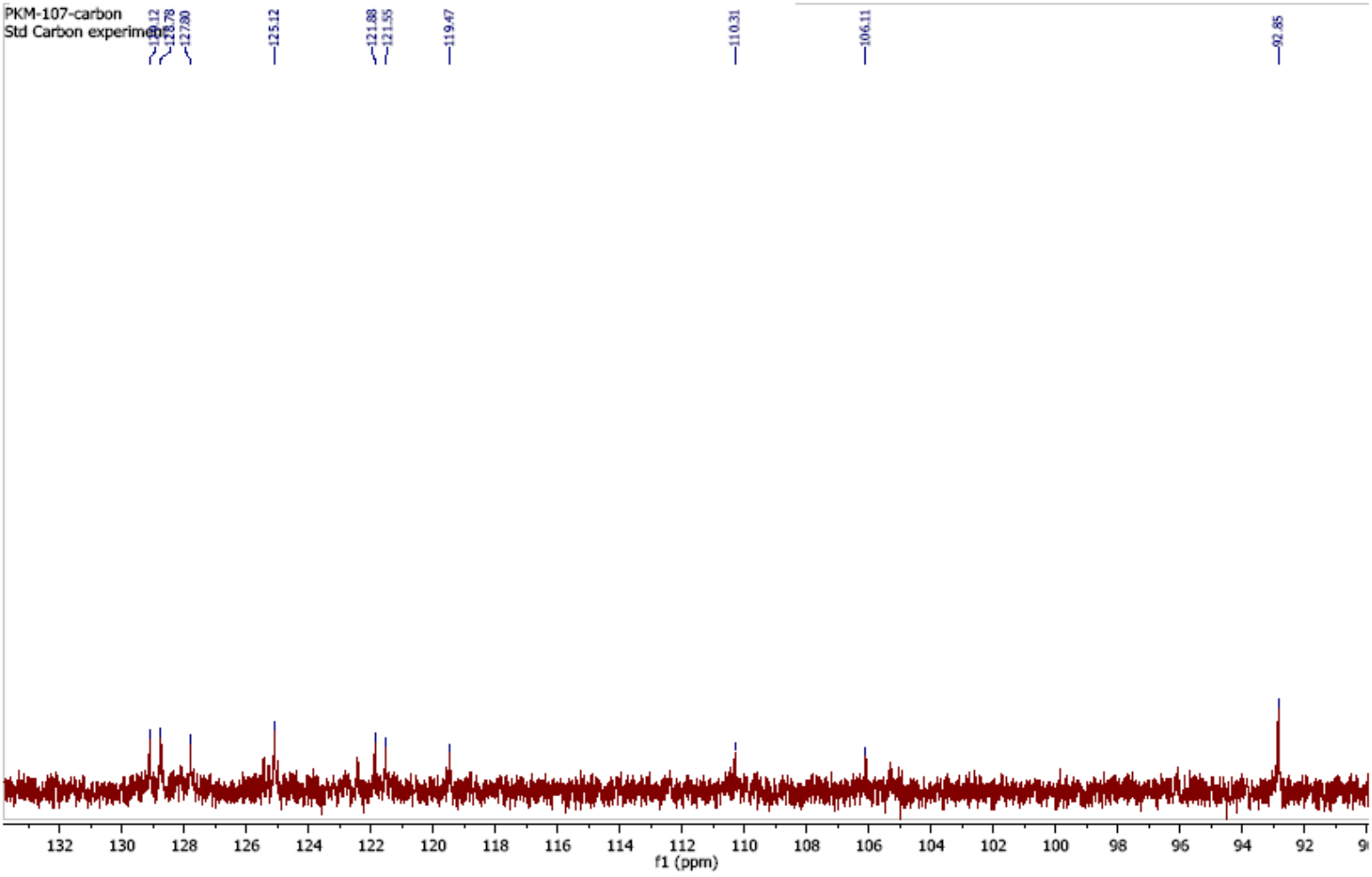
PKM-107, 1H-NMR, MeOD, Varian Mercury-400



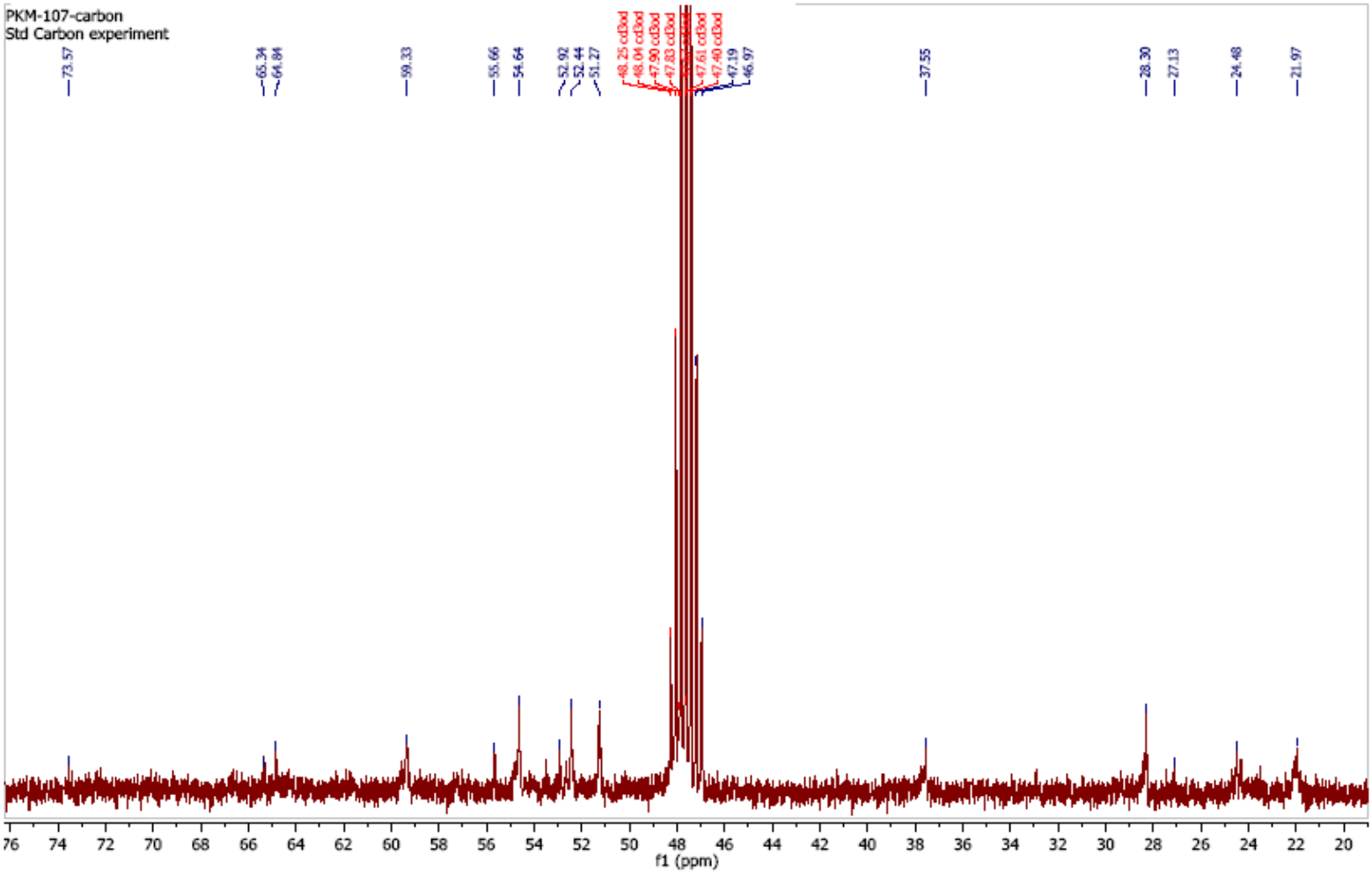
PKM-107, 13C-NMR, MeOD, Varian Mercury-400



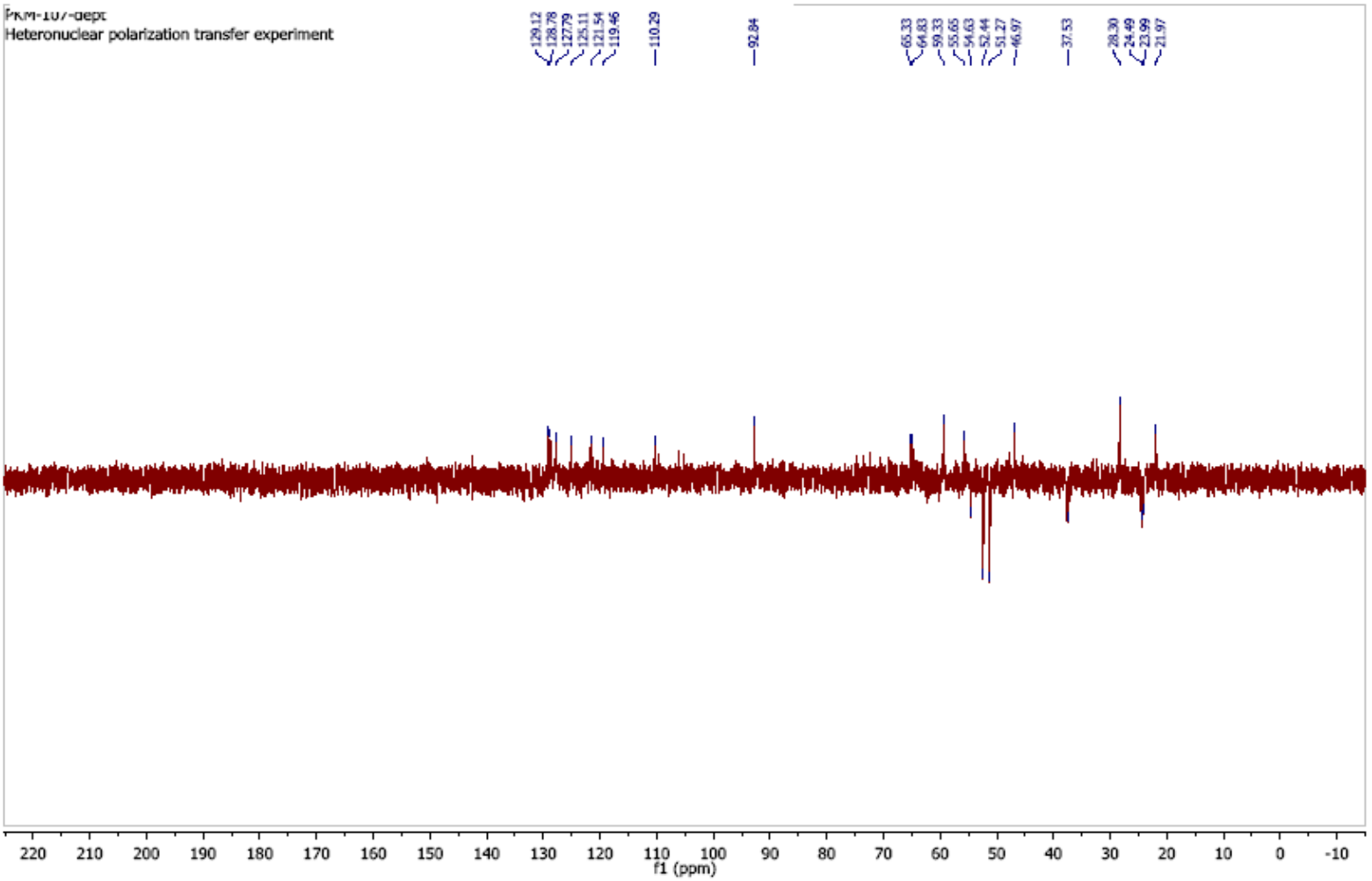
PKM-107, 13C-NMR, MeOD, Varian Mercury-400



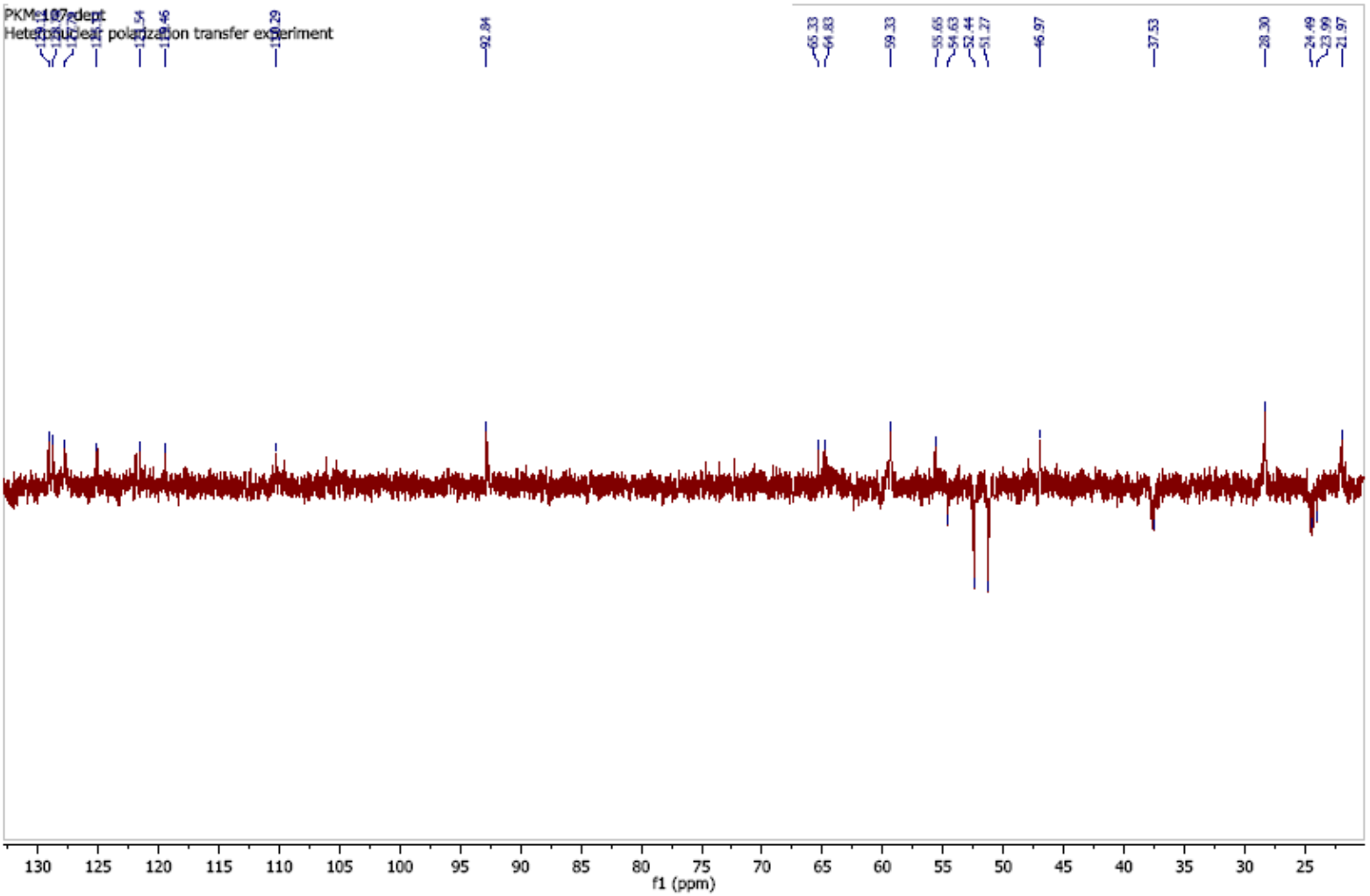
PKM-107, 13C-NMR, MeOD, Varian Mercury-400



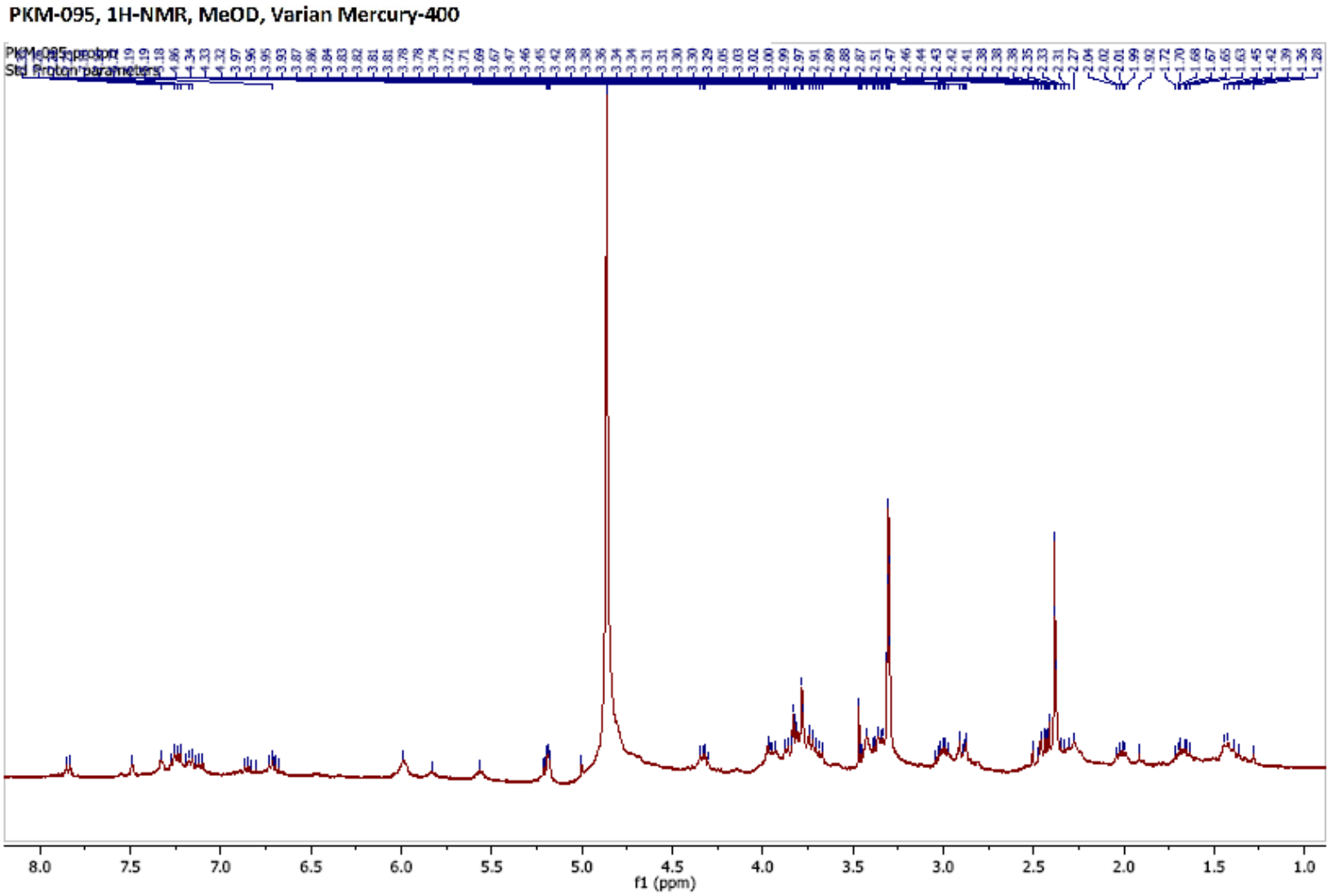
PKM-107, DEPT, MeOD, Varian Mercury-400



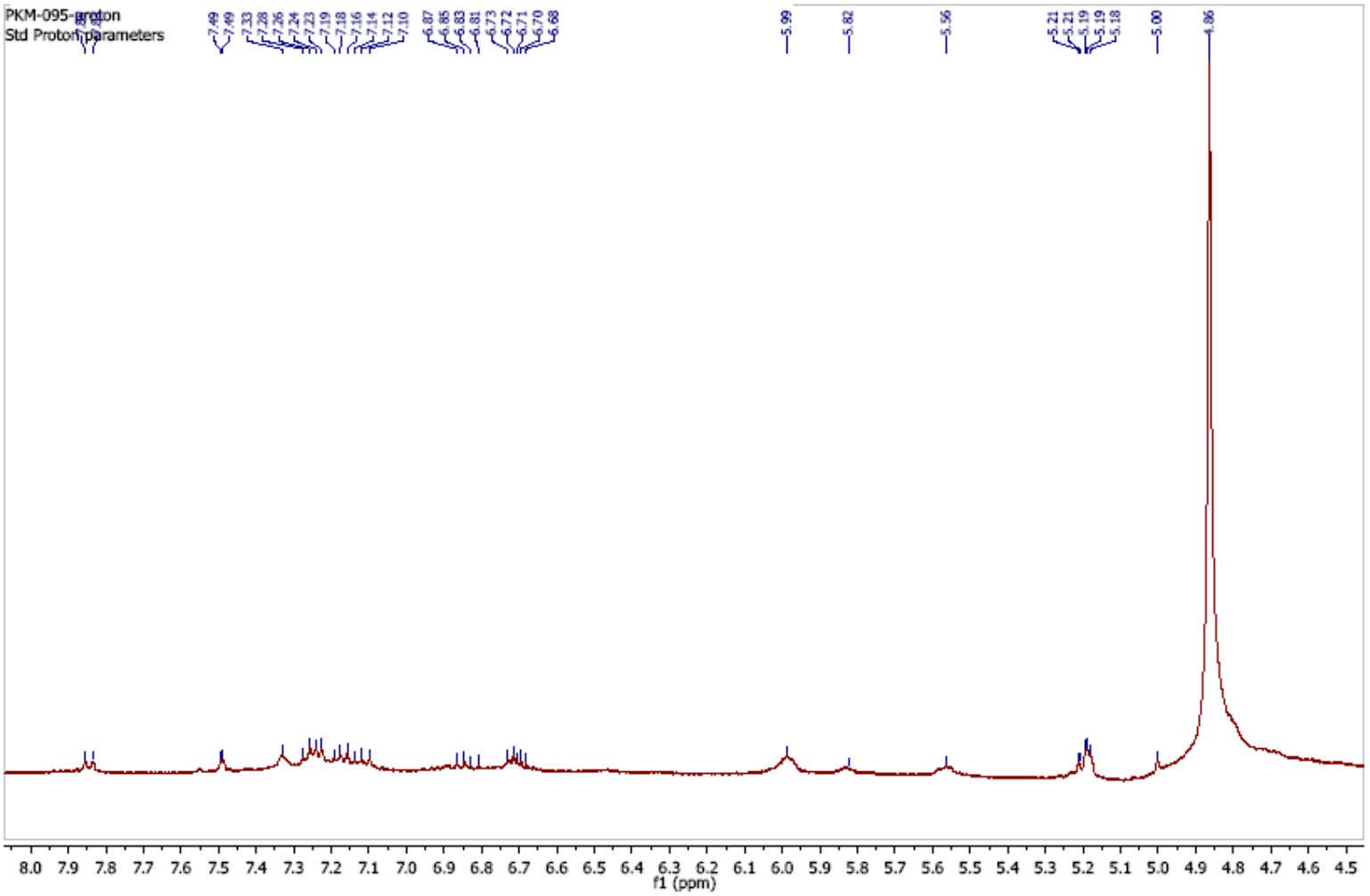
PKM-107, DEPT, MeOD, Varian Mercury-400



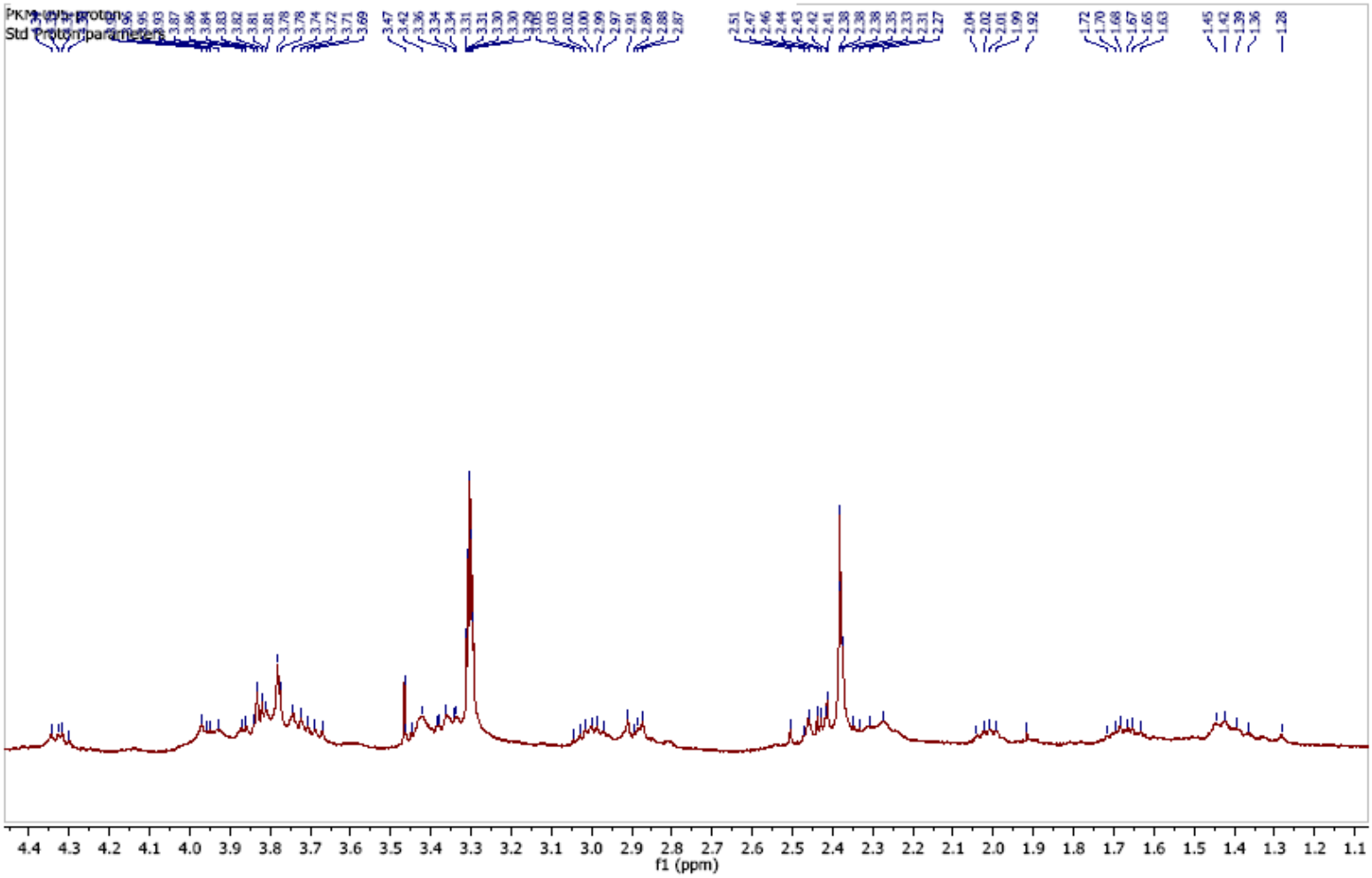
h) PKM 095


PKM-095, 1H-NMR, MeOD, Varian Mercury-400
PKM-095-proton
Std Proton parameters
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)

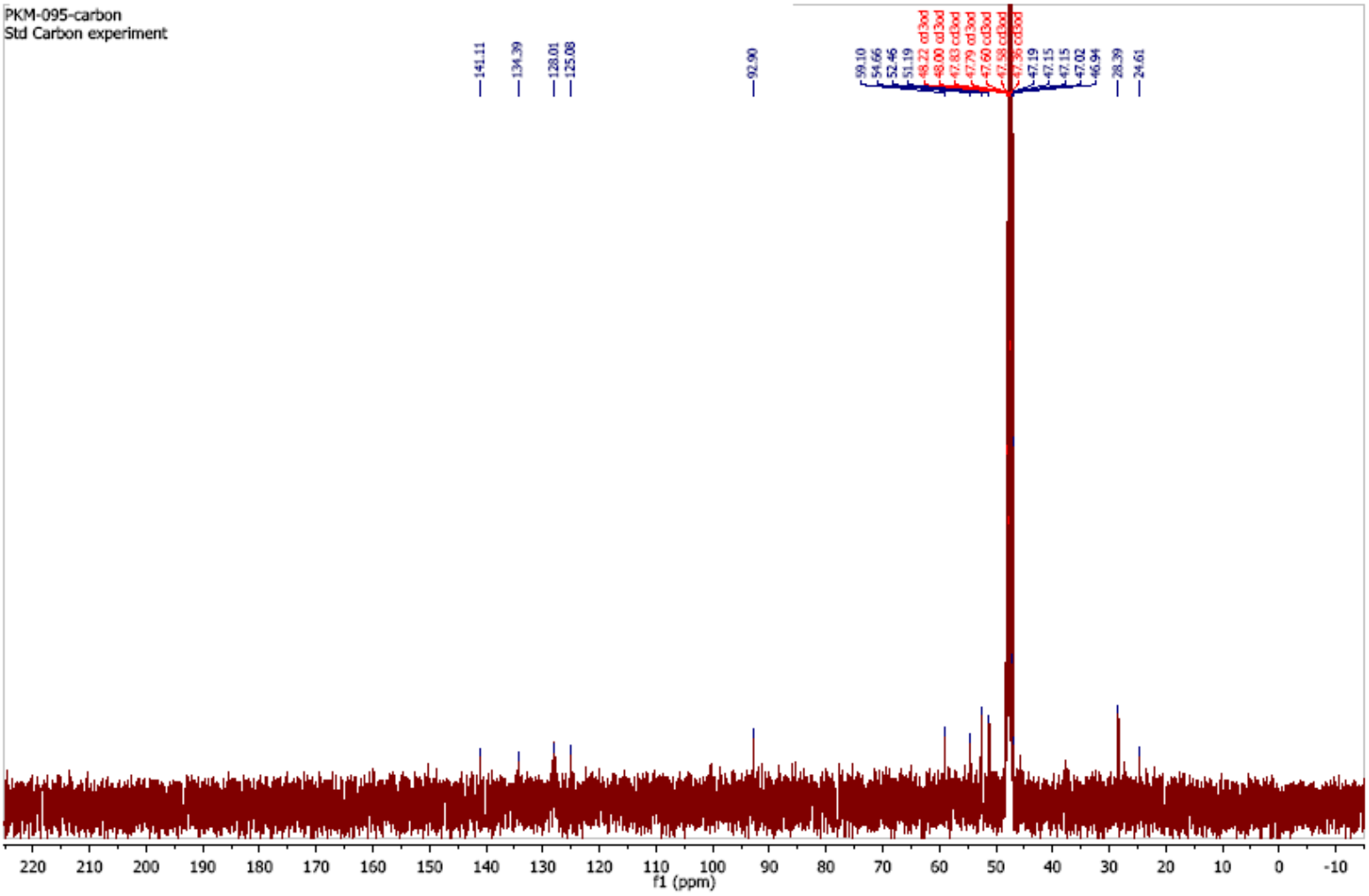
PKM-095, 1H-NMR, MeOD, Varian Mercury-400



PKM-095, 1H-NMR, MeOD, Varian Mercury-400

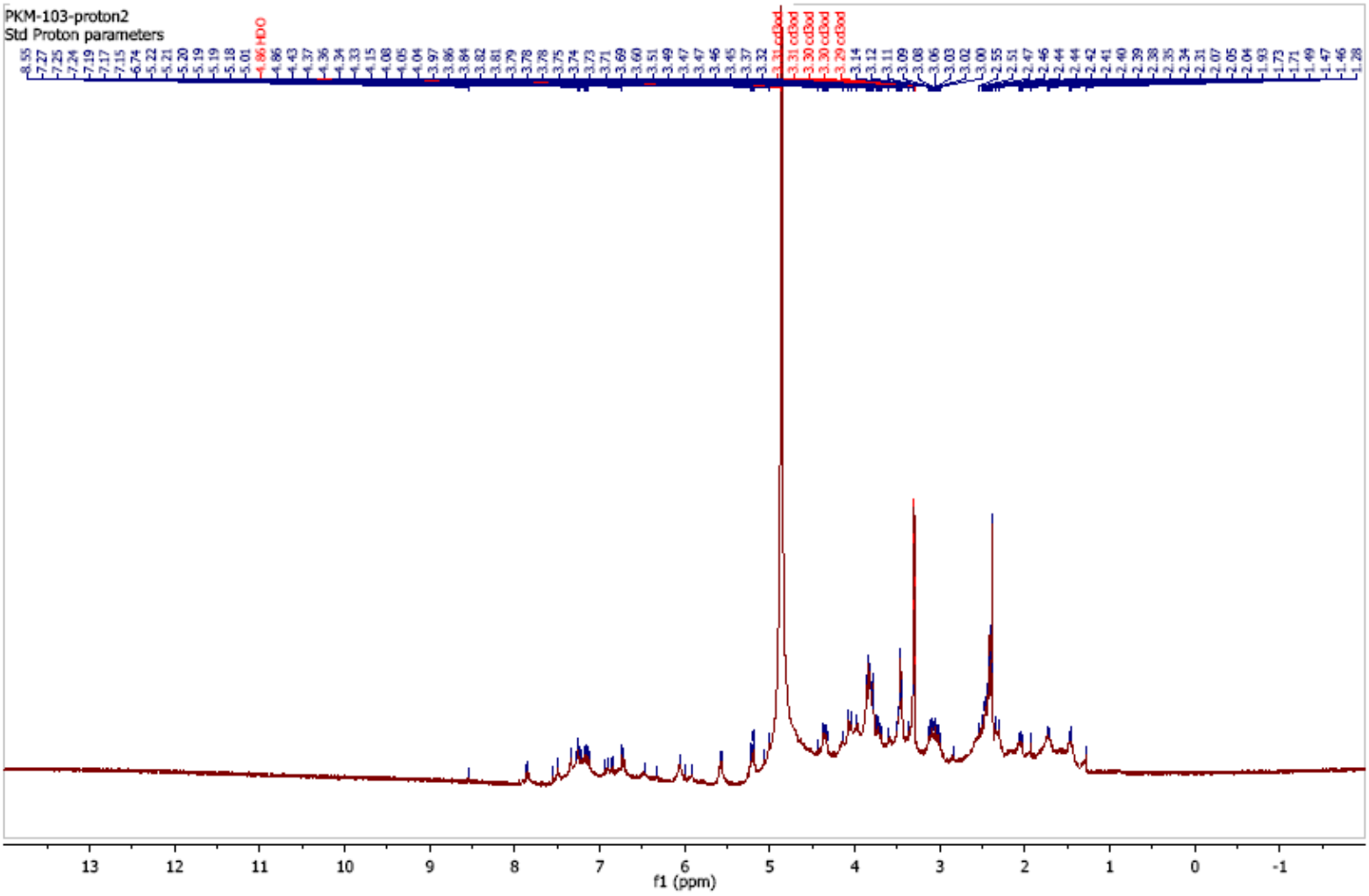


PKM-095, 13C-NMR, MeOD, Varian Mercury-400

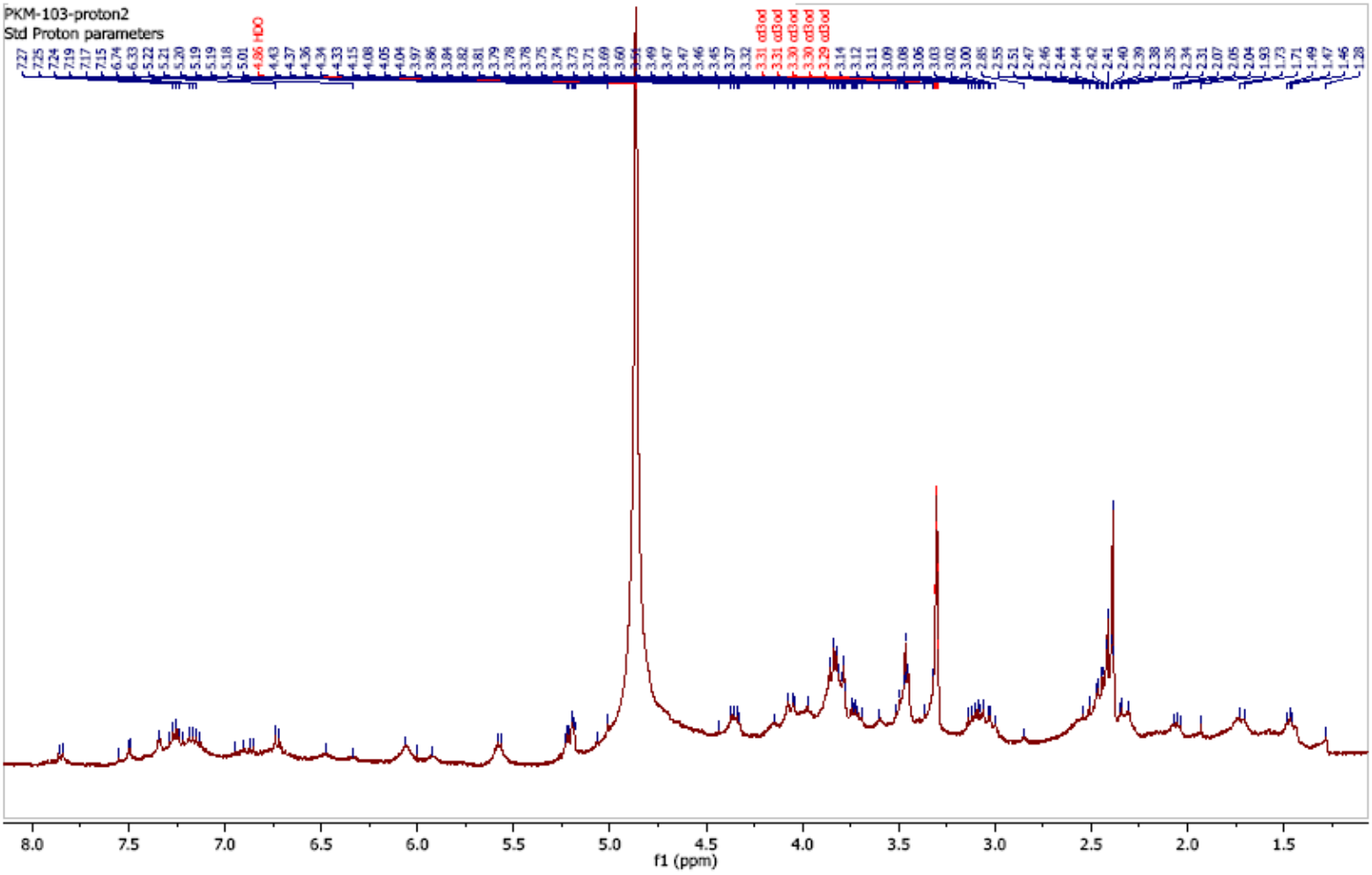


i) PKM 103

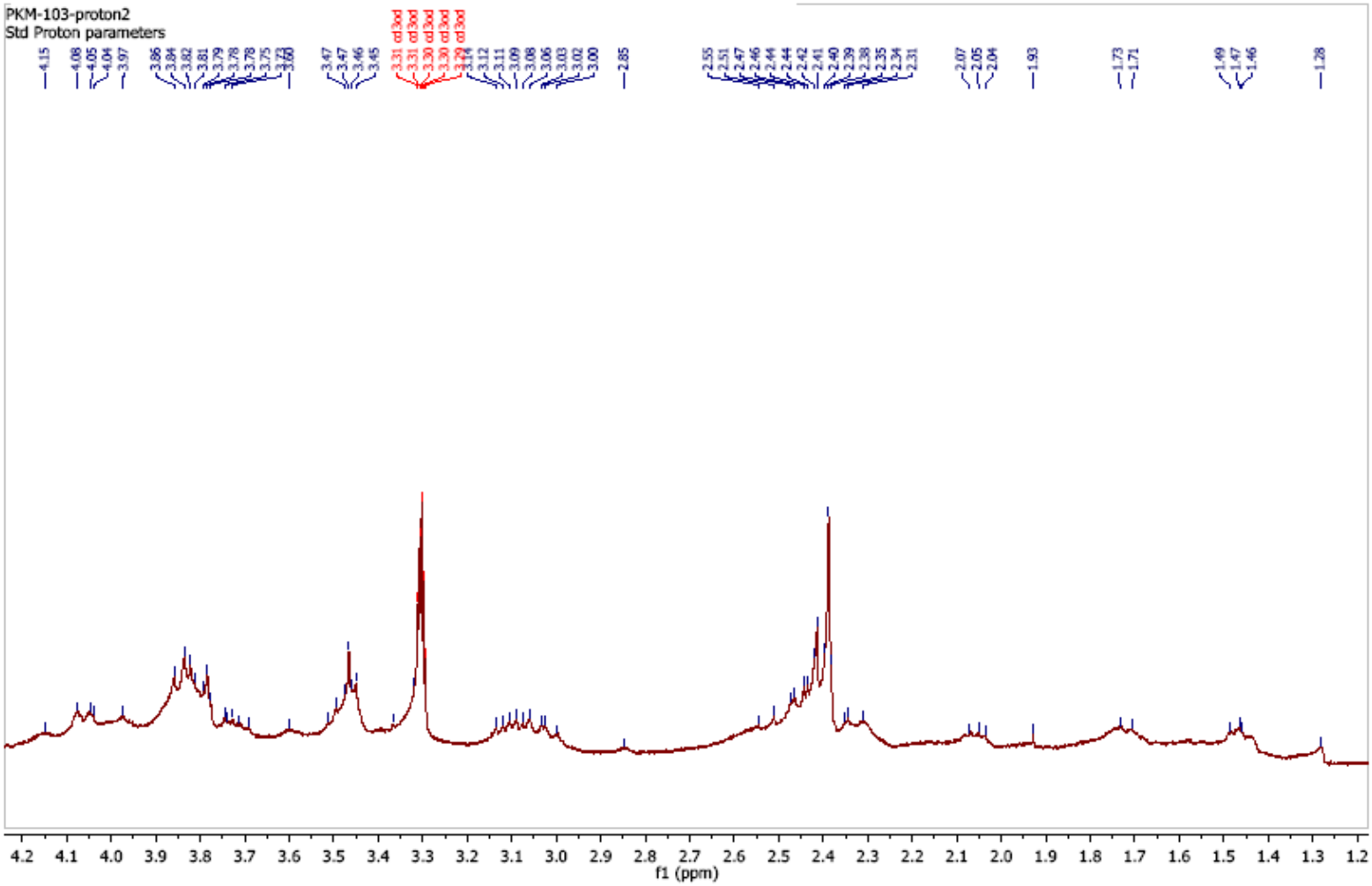
PKM-103, 1H-NMR, MeOD, Varian Mercury-400



PKM-103, 1H-NMR, MeOD, Varian Mercury-400



PKM-103, 1H-NMR, MeOD, Varian Mercury-400



PKM-103, 13C-NMR, MeOD, Varian Mercury-400