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Bioassay-guided isolation and identification of anti-inflammatory compounds in Sclerochloa Dura

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Abstract

Sclerochloa dura was known to have anti-inflammatory properties and the aim of this thesis is to isolate and identify compounds that are present in fractions and has high inhibition level against arachidonic acid (AA) when tested in bioassay.

The methanolic extracts of the plant were stored as fractions and contained several bioactive compounds. They were subjected to separation through various chromatographic methods and structures of major isolated compounds were elucidated using NMR and ESI-MS-TOF.

The five compounds identified in the fractions are genistein-4'-O-glucoside, luteolin-4'-O-glucoside, chrysoeriol-7-O-β-glucoside, isovitexin and (2E)-3-(4-Hydroxy-3-methoxyphenyl)-N-propylacrylamide.

Acknowledgement

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List of Symbols and Abbreviations

1D One-dimensional

2D Two-dimensional

AA Arachidonic acid

ACN Acetonitrile

COSY (H, H)-correlated spectroscopy

COX Cyclooxygenase

DAD Diode array detector

DEPT Distortionless enhancement polarization transfer

DMSO Dimethyl sulfoxide

EI Electron impact

ELSD Evaporative light scattering detector

ESI Electron spray ionization

FA Formic acid

HMBC Heteronuclear multiple bond correlation

HPLC High performance liquid chromatography

HSQC Heteronuclear single quantum coherence

IPR Isopropanol

LOX Lipoxygenase

MeOH Methanol

MPLC Medium pressure liquid chromatography

MS Mass spectrometry

NMR Nuclear magnetic resonance

NOE Nuclear overhauser effect

NOESY Nuclear overhauser effect spectroscopy

PLA2 Phospholipase A2 enzyme

ROESY Rotating-frame overhauser effect spectroscopy

RP Reverse phase

TFA tetrafluoroacetic acid

TOF Time-of-flight mass spectroscopy

TOCSY Total correlation spectroscopy

UV Ultraviolet-visible spectroscopy

UPLC Ultra performance liquid chromatography

1. Introduction

1.1 Inflammation

Inflammation is part of a complex biological response of body tissues towards harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective defence mechanism involving immune cells, blood vessels, and molecular mediators. The damaged cells release chemicals, including histamine, bradykinin, and prostaglandins which induces blood vessels to leak fluid into the tissues thus causing the swelling of tissues [1]. As a result, this helps to isolate foreign substances from further contact with body tissues [2]. A series of complex inflammatory reactions result in allodynia (central pain sensitization due to increased response of neurons) and hyperalgesia (an increased sensitivity to pain due to damage to peripheral nerves) [3].

For centuries, humans have tried to alleviate pain with plant-based medicines for ailments such as rheumatoid arthritis, asthma and many others [4]. In modern times, researchers have tried many experiments that mimic the internal inflammation mechanism in order to test various bioactive compounds that inhibit inflammation [5].

Natural products extracted from *Sclerochloa dura* were tested on human cells to determine its anti-inflammatory activities. The cell type, human fibroblastlike synoviocyte cell line SW982, was selected due to its ability to activate phospholipase A2 (PLA2) enzyme thus triggering the release of arachidonic acid (AA) upon stimulation with interleukin-1b; a cytokine protein. Published results have shown that the extracts were able to reduce the activity of PLA2 enzyme and as a result it minimises the concentration of AA [6]. This is an important discovery because AA is a causative agent that mediates or modulates the cascading effect of a series of complex inflammatory reactions [7].

The compounds found in *S. dura* could potentially target the PLA2 enzyme to reduce or eliminate inflammation, by inhibition of AA release, thus labelled with anti-inflammatory properties. Whilst there have been six compounds isolated and identified with anti-inflammatory properties, it is possible that there could be potentially more

bioactive compounds present in the plant that also contributes to the aforementioned property.

1.2 Compounds with anti-inflammatory properties in *S. dura*

Sclerochloa dura of the genus *Sclerochloa* are flowering spikelet species with its name derived from Greek word skleros (hard) and chloa (grass) and hence the common name of hardgrass. It can be found native in Europe and Asia; present as introduced species to parts North America and Australia. It reaches up to 16cm in height with wide and flat leaf blades and grows on dry, sandy or gravelly soil [8].

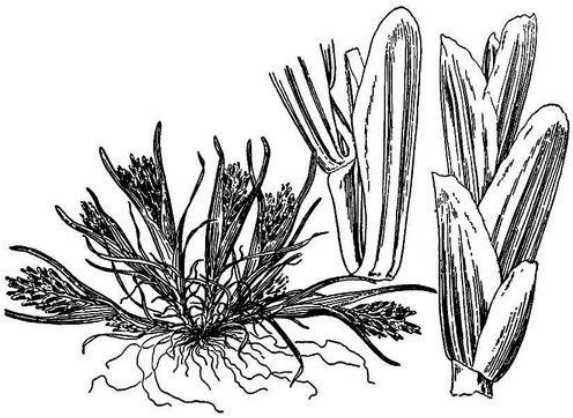


Figure 1 *Sclerochloa dura sourced from USDA NRCS PLANTS Database*

This plant was used in traditional treatment of menstrual disorders such as menorrhagia or excessive bleeding and pain; taken in form of tea to mitigate the problem [6]. This palliative effect is attributed to some of the compounds that were identified in the plant and were confirmed to have anti-inflammatory properties. These compounds are as shown below; -

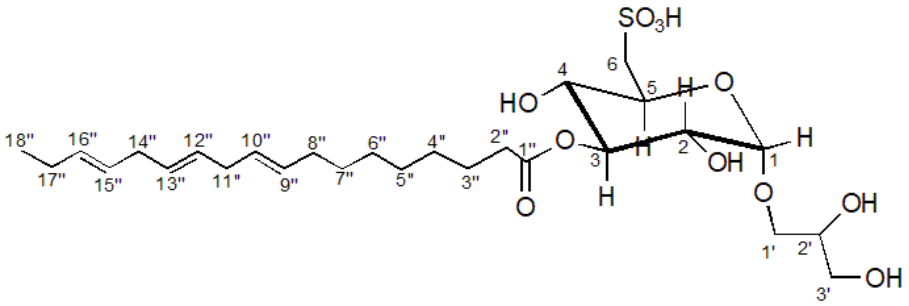
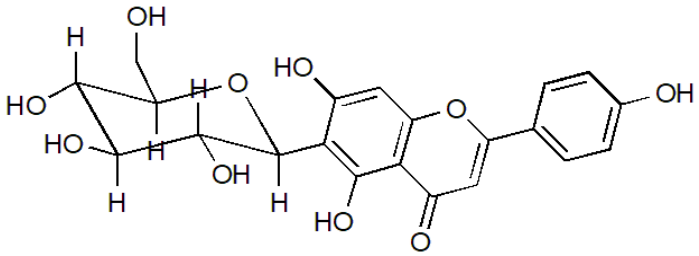


***Figure 2**: 1-O-(3-O-linolenoyl-6-deoxy-6-sulfo-α-D-glucopyranosyl)-glycerol*



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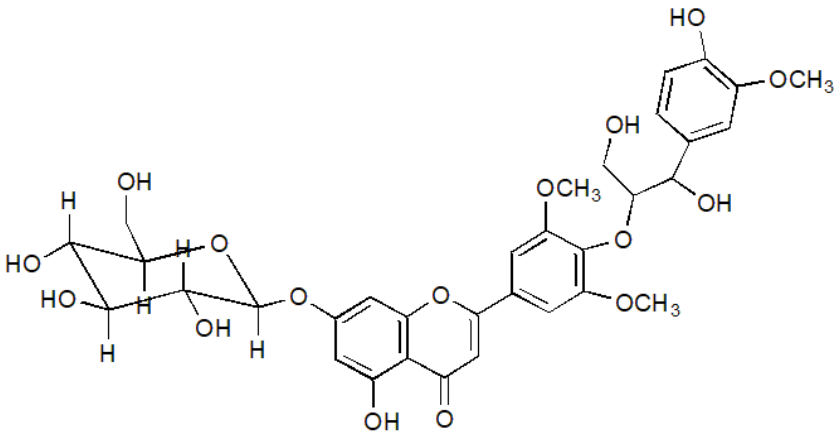


***Figure 4**: tricin 4'-O-(erythro-β-guaiacylglyceryl) ether 7-O-β-glucopyranoside*

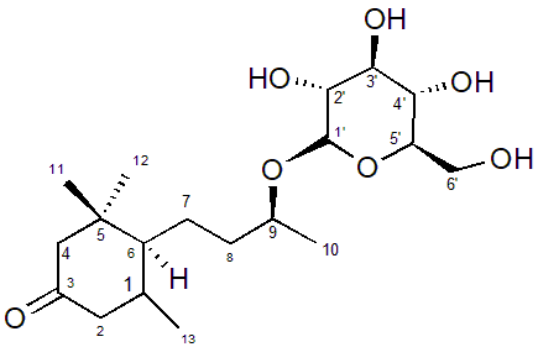


Figure 5: *Byzantionoside B*

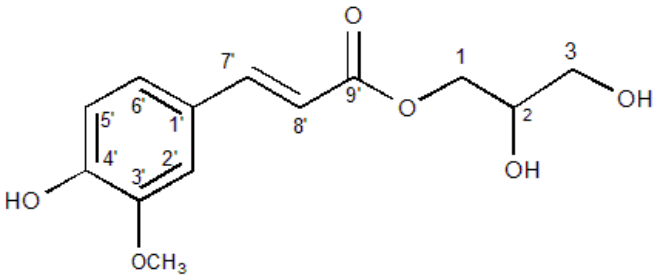


Figure 6*: 1-O-feruloyl glycerol*

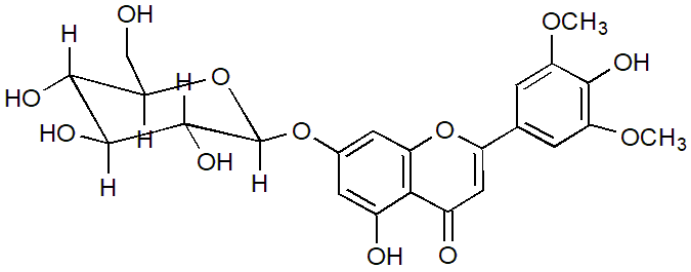


Figure 7*: tricin 7-glucoside*

The aim of this research is to expand the library of compounds found in the plant, other than those shown above, that exhibits similar anti-inflammatory properties. As part of the continuation of the previous research done on this plant, the samples collected from the plant have been stored in cold storage and some of its fraction pools were biotested for its AA inhibition. This thesis will focus on isolating and structural elucidating compounds, according to bioassay results from those fraction pools and

its AA inhibitory activity through various chromatographic methods, such as analytical and semi preparative HPLC, UPLC-ESI-TOF MS and identification using NMR.

2. Theory

2.1. Medicinal plants

Herbal or botanical medicine refers to the use of plant material to treat injuries or illnesses. Plant parts such as seeds, leaves, stems, bark, roots, flowers and extracts of plants, are main ingredients in herbal medicine since ancient times [4]. Such medicines are usually administered either raw, topical applications, in pills and capsules or as teas and tinctures. In early medical history, plants were consumed either raw or combined with hot water as soup or tea [9].

The use of plant materials in traditional medicine can be traced back to prehistoric time [4]. However the first medical history that was ever recorded was found to be during the Mesopotamian era in 2600 B.C [10]. Plants with shapes or form representing a human body were often used to treat injuries or illnesses, one example would be the eyebright plant. It was used in eye treatment due to its resemblance to the human eyes [10].

Throughout the years, the use of medicinal plants has evolved, as seen during the early 16th century where a Swiss physician Paracelsus, who considered himself to be an alchemist, began applying chemistry when making herbal medicines by combining herbs with mineral ores. He focused on the processes such as solution, evaporation, precipitation, and distillation, thus purifying the plant extracts. One of the finest examples would be the extraction of opium, which he purified and dissolved in alcohol, forming laudanum, the first ever painkiller [11].

It was during the early 19th century that modern chemistry started to become a widespread practice. This happened when a German apothecary apprentice Sertürner isolated morphine from opium, pioneering the methods of medicinal preparation by viewing plants as mediums containing active ingredients or chemical based entities [12]. Henceforth began the steps of isolation and chemical identification of pharmacologically active compounds from crude plant materials.

From extractions of active ingredients in plants to synthesizing it in the laboratory, modern chemistry is rigorously applied in almost all drug formulation steps. In many cases, naturally occurring compounds are used as a template in synthesizing pharmacologically active compounds that retain or increase its effectiveness compared to the original structure [13].

2.2. Natural products found in plants

Natural products are defined as chemical substances that are formed by living organisms. Four main classes of natural products are namely carbohydrates, lipids, proteins, and nucleic acids. These compounds are essential to the metabolism and reproduction of the organisms. When biochemical reactions occur within an organism, metabolites involved in those reactions are sorted either as primary or secondary metabolites. Primary metabolites play a central role in the development of cellular functions within an organism and can be found in large quantities with broad distribution in all living things [14, 15].

In contrast, secondary metabolites are organic compounds that are produced in the organism but do not involve directly in cell growth, reproduction, and development of the organism. Only some primary metabolites such as α-amino acids, acetyl coenzyme A, mevalonic acid and intermediates of shikimic acid pathway are involved in the biosynthesis of secondary metabolites [16].

While there is an estimate of over 300,000 secondary metabolites existing in living organisms, the presumed main function of these chemical compounds is to protect and increase its survival chances regulating the interactions between plants, microorganisms, insects, and animals [15, 17]. One such example would be the presence of nicotine, a secondary metabolite, in tobacco (*Nicotiana tabacum)* plant. A study showed that when the nicotine expressing gene is silenced and the content of nicotine in plant is subdued to within 3% - 4% of the normal level, the Tobacco Hornworm (*Manduca sexta)* insect feeds more on those plants. Hence, it was deduced that nicotine protects tobacco plants from natural predators [18].

While the term natural products is defined for any substances produced in organisms, it is commonly used for organic compounds found in nature that exhibits biological effects on other organisms and for the purpose of this thesis, it refers to secondary metabolites [15, 19].

Despite the diversity of secondary metabolites, it can be classified into six categories due to its structural characteristics formed during biosynthesis of these compounds. They mainly consist of polyketide and fatty acid, terpenoid and steroids, phenylpropanoid, alkaloids, specialised amino acids and peptides and specialised carbohydrates [13, 15, 19]. Polyketides such as the antibiotic erythromycin A and fatty acids like arachidonic acid are formed by stepwise condensation of ethanoate units, biosynthesised from acyl precursors such as acetyl coenzyme A [15, 17].

Terpenoids and steroids are structures derived from isopentenyl diphosphate and whilst terpenoids do not have related skeletal structures, steroids have cyclopentaperhydrophenanthrene backbone which are modified terpenoids biosynthesised from triterpene lanosterol. Terpenoid is further broken down to six smaller classes which are monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), and carotenoids (C_{40}). Essential oils such as geranium oil are monoterpenoids and cholesterol is an example for a steroid. Saponins are derivatives of triterpenoids when sugar moieties are attached to it [15, 17].

Alkaloids such as nicotine from tobacco (*Nicotiana tabacum)* plant and morphine from opium poppy (*Papaver somniferum)* are derived biosynthetically from amino acids and contains nitrogen in their structures. Although amino acids are classified as primary metabolites, some are exceptions and are present in low concentrations. Penicillin is an antibiotic derived from such amino acids. The same applies to other kinds of sugars that have limited occurrence but are attached to natural products, thus forming glycosides [17].

Phenylpropanoids are naturally occurring aromatic compounds biosynthesised from precursors phenylalanine and tyrosine amino acids via shikimate or polyketide pathways. This class of natural product is biosynthesised to form various types of polyphenols such as phenolic acids, flavonoids, lignans among many other as seen in Figure 8 [20].

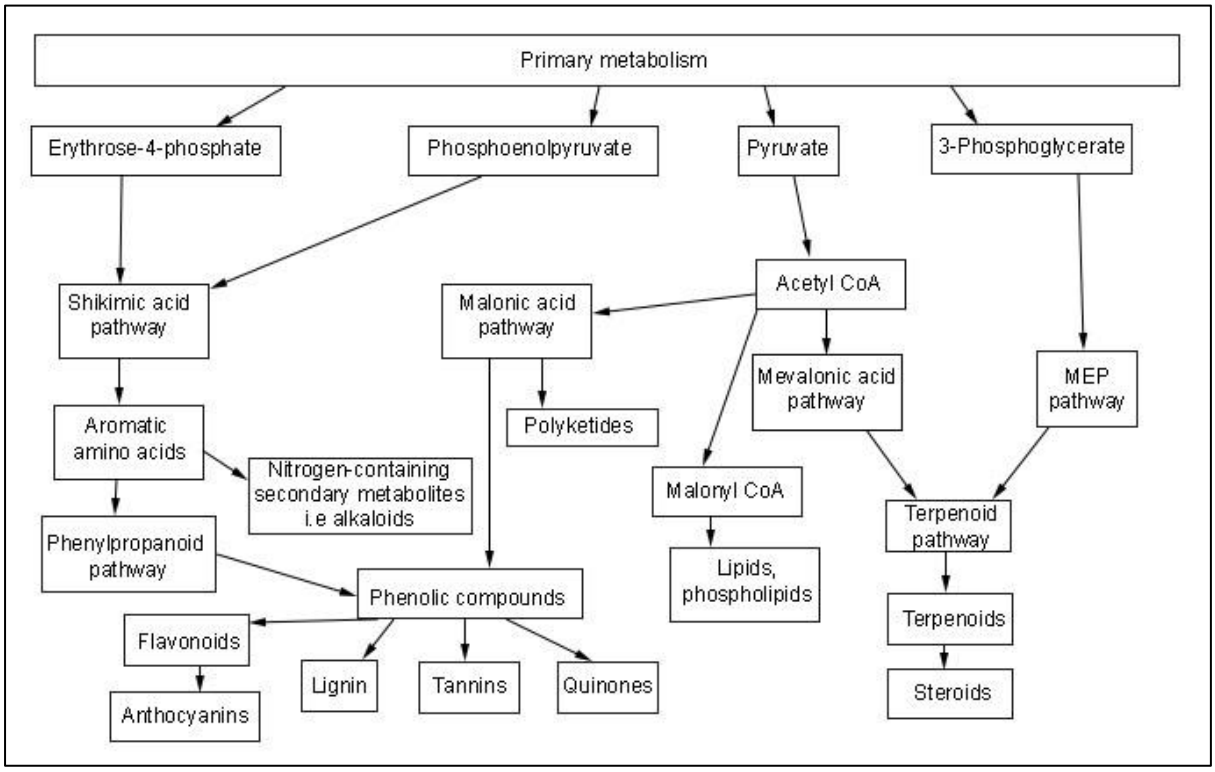


Figure 8 Simplified overview of biosynthesis of secondary metabolite pathway (modified version)[14, 21]

While there are many more derivatives from these main classes of natural products, this thesis will focus solely on flavonoids due to the nature of chemical compounds found in methanolic extract of *S.dura.*

2.2.1. Flavonoids

Flavonoids are polyphenolic compounds, part of the main class of natural products that are found in plants. The major groups of flavonoids are flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavones. They are characterized by a

15-carbon skeleton, arranged in C6-C3-C6 manner with differences depending on substituents and unsaturation [22-24].

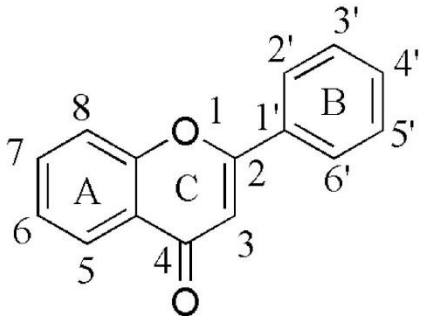


Figure 9 2-phenyl-chromone is the basic structure of flavonoid [24]

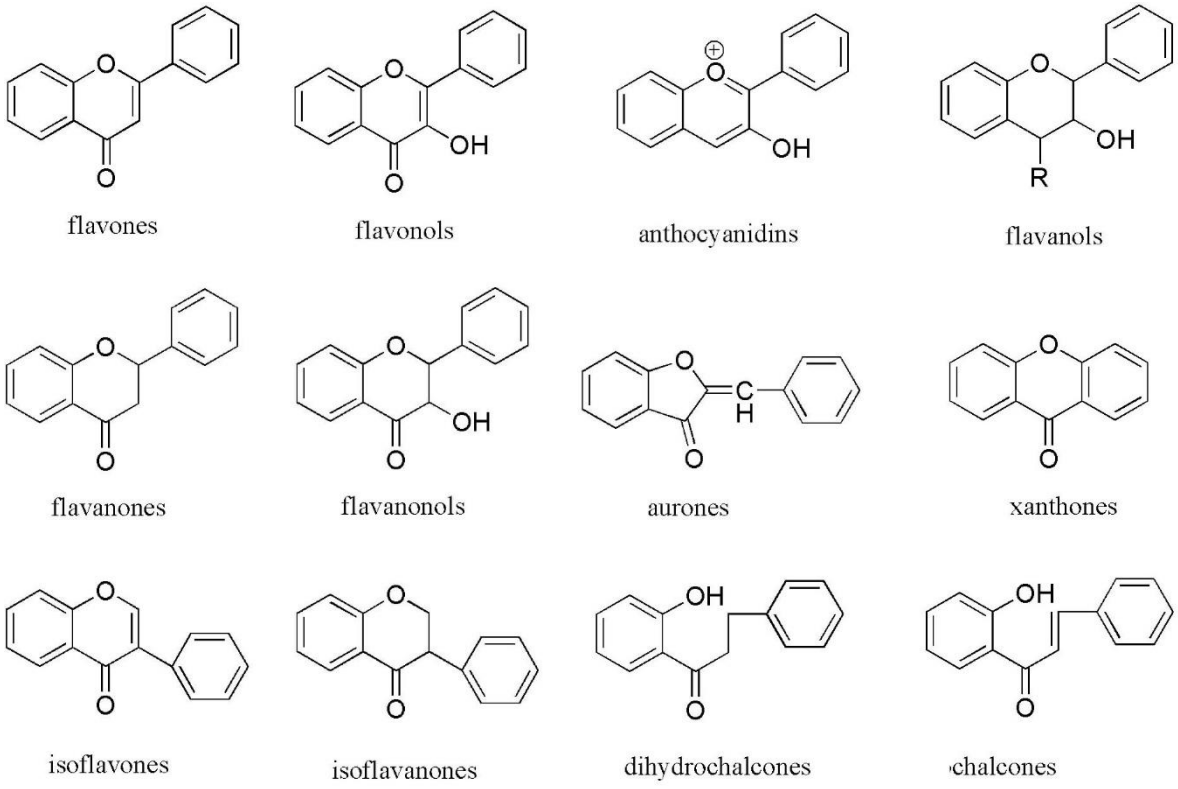


Figure 10 Structural backbone of general flavonoid classes [24]

While flavones and flavonols are found stored as aglycones (free form), most flavonoids are present as O- or C- glycosides. They are an O- glycoside when the sugar is connected to the aglycone through an oxygen-carbon bond while C-glycosides have the sugar bound to the flavonoid structure through carbon-carbon bond. Glycosidic flavonoids tend to be more stable than free flavonoids and they are

usually more polar than aglycones, as the sugar moiety increases solubility in water [23].

The chemical properties of a flavonoid depend on the arrangement of the substituents in the structure [25]. Studies have often linked the antioxidant properties of flavonoids with anti-inflammatory effects, among many other biological activities [25-27].

Flavonols such as quercetin (11) affects the enzymes cyclooxygenase (COX) and lipoxygenase (LOX) pathways, which are agents in inflammatory reaction in the body. Rutin (12), also a type of flavonol, was reported to have the ability to inhibit COX enzyme, thus regulating the production of prostaglandin [28].

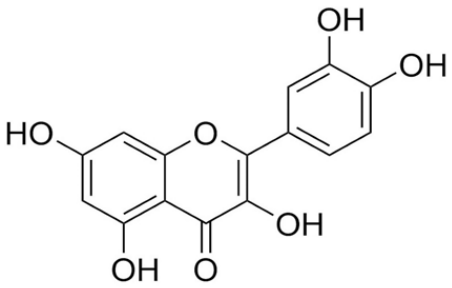


Figure 11 Quercetin

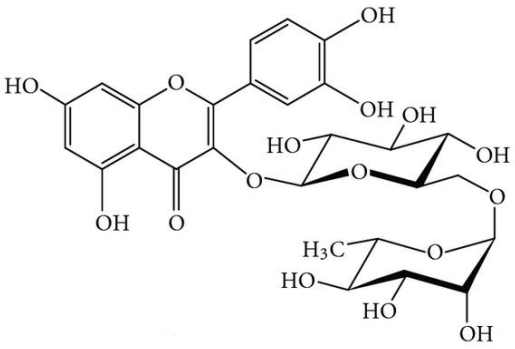


Figure 12 Rutin

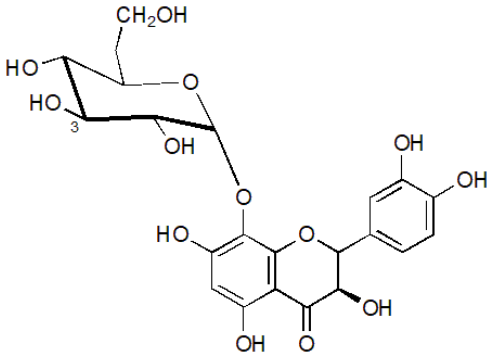


Figure 13 Gossypin

Flavonoids are more potent anti-inflammatory agents when in glycosidic form as compared to their corresponding aglycones especially with the presence of a hydroxyl group at position 3; the anti-oedematous activity of flavonoids increases significantly. As a result, flavonoids such as gossypin (13) showed selective inhibitions on LOX product formation [29].

2.2.2. Isoflavonoids

Flavanones that undergo oxidative ring rearrangement of the aromatic ring forms isoflavonoids and they are mostly found in legumes such as soy [15].

A study was conducted to understand the effects of isoflavonoids on inhibition of AA. Published data showed that isoflavonoids daidzein (14), genistein, biochanin A and formononetin showed significant results on the suppression of arachidonic acid release. RAW 264.7 cell line were tested on and based on previous studies, the blocking of phosphorylation of $cPLA_2$ tend to completely inhibit AA release in these cells. It was found that isoflavones in aglycone form had higher inhibitory effects compared to when it's in glycosylated form. The decreased activity was attributed to the higher molecular weight, hydrophilicity, and steric hindrance by sugar moiety [30].

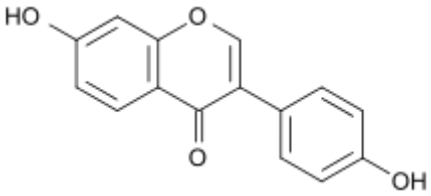


Figure 14 Diadzein

Sophoricoside (15), an isoflavone O-glycoside that was found in *Sophora japonica L*., was isolated and tested for anti-inflammatory properties. It was identified as a selective inhibitor of COX-2 activity and it also showed significant reduction of carrageenan-induced paw edema, when experimented on mice [31].

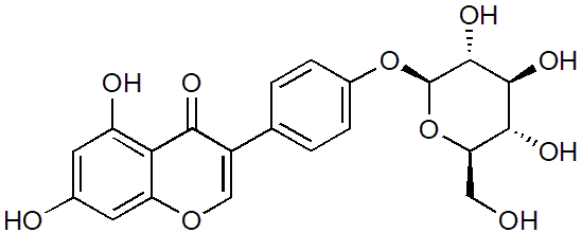


Figure 15 Sophoricoside

Flavonoids extraction process depends on the chemical properties of target compounds. If in aglycone form, it is sparingly soluble in water and more soluble in organic solvents, such as acetonitrile or methanol, whereas glycosides are freely soluble in polar solvents and in water.

2.3. Isolation of natural products

To evaluate physical and biological properties of a natural product, it needs to be in pure form for structural elucidation and biotesting. Through extraction and purification, compounds of similar chemical properties can be isolated. There are two phases involved in obtaining pure natural product compounds. Phase I is extraction of natural product; performed in a previous research on *S. dura,* explained under experimental section.

Phase 2 is isolation of natural product; the goal is to separate natural products from other co-existing compounds. The fractions obtained from the previous step were tested in this thesis as a continuation of that research project, focusing mostly on Phase 2.

2.3.1. Separation techniques

Isolation of compounds can be achieved through chromatographic separation. Injected sample mixture is continuously distributed between stationary phase and mobile phase due to the different equilibrium distribution coefficient of diverse materials that forms the stationary phase and the mobile phase [32]. Difference in physical and chemical properties ensure that the concentrations of compounds are not the same in the two phases [33]. In a liquid-solid chromatographic separation, some of the common types of stationary phases utilised are silica gel and bonded phase silica gel among many others [22].

Silica gel is made up of porous siloxane and with crosslinked structure of -Si-O-Si-, forming a polyporous material represented by the formula $SiO_2.xH_2O$. Furthermore, the number of silanol groups and water content determines its absorptivity. Whilst there are more options for mobile phase when running normal phase chromatography, reverse phase chromatography is generally selected for separation of natural products with relatively high polarity [22].

Meanwhile, bonded phase silica gel is formed by chemically bonding groups such as octadecyl silane, dihydroxypropyl or cyano groups to porous silica gels. It provides different selectivity for the separation of compounds and its widely used for reverse phase chromatography. Methanol, water, isopropyl alcohol and acetonitrile are some of the standard eluents used with bonded phase silica gels [22].

Natural products have many different attributes and no single type of column is sufficient in complete separation and isolation of natural products. Due to such various properties, a RP-HPLC is often used to separate more polar analytes from their less polar counterparts. In this case, the target of the separation method is to isolate natural

products exhibiting high anti-inflammatory properties, to which isolation is subjected to the compound's polarity. In gradient mode, samples are generally injected with high ratio of polar solvent to non-polar solvent for mobile phase and run for a relatively long period of time, approaching high concentrations of non-polar solvent by the end of the run [33].

2.3.2. Analytical HPLC

Analytical Reverse Phase High Performance Liquid Chromatography (RP-HPLC) is a robust method with qualitative and quantitative nature for the separation of non-volatile and polar compounds. It is usually connected to an ultraviolet (UV) detector as it is the most simple and common method of detection. While some natural products do not possess UV chromophores, most compounds have absorption bands between 200nm to 500nm. There are three types of UV detector commercially available, namely fixed wavelength, multiple wavelength, and photodiode array (DAD). DAD has an added advantage of which it can provide complete UV spectra of constituents during separation. It is a favoured detector when separating flavonoids and other chromophores as it detects natural products with distinct absorbing wavelengths. Conversely, there are some natural products that do not contain chromophore and thus cannot be detected within the UV-visible range [33].

Another detector that is frequently used in tandem with an UV detector is the evaporative light scattering detector (ELSD). It can detect non-chromophores and has higher sensitivity. Analytes are nebulised using N_2 gas flow, forming aerosol which is transferred along a heated drift tube where the sample is evaporated along with solvents. A light beam is then directed to the remaining solid particles, which results in scattering of incident light, detected by a photodiode or a photomultiplier.

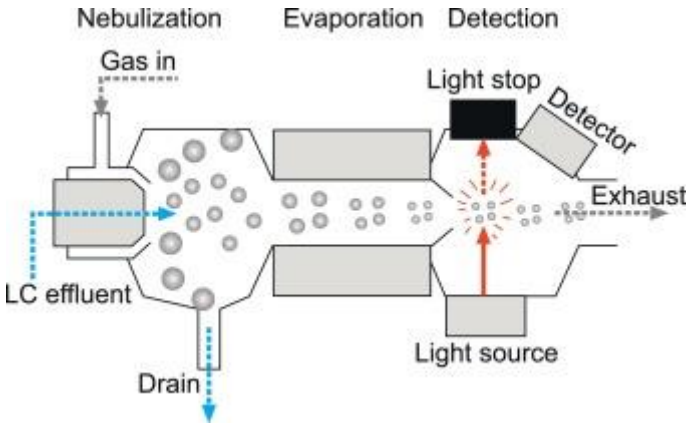


Figure 16 A scheme showing how eluents are detected using Evaporative Light Scattering Detector [34]

Due to non-volatile properties of certain samples, high temperature and high gas flow rate is required to ensure that the detector is sensitive enough for detecting such compounds. Unlike the UV detector which is concentration dependent, ELSD is not influenced by mass of compounds but rather it relies on direct light scattering of dried particles to provide signals [34]. As such, the generated result does not depend on physiochemical or spectral properties of the sample and compounds that are not UV active can be quantified when compared to a standard. It is commonly used for the detection of weak chromophores, such as in saponins and terpenes, both in aglycone and glycosidic form [33, 35] .

2.3.3. Semi-preparative HPLC

Isolation and purification of compounds after baseline separation in analytical RP-HPLC is achieved using semi-preparative RP-HPLC. Once conditions on analytical scale were established, scaling up is required for sample fractionation. There are differences between analytical and semi-preparative HPLC as each instrument has different functionalities. While it is common to increase the size of analytical conditions, such as amount of solvent used or injection volume when running experiments on the semi-preparative, it is not always on a one-to-one scale.

When benzoic acid, benzene and naphthalene were prepared for analytical and semi-preparative HPLC, the amount of sample injected in semi-preparative was 20 times larger compared to analytical scale. Both maintained identical column packing but

increased flowrate, from 0.8mL/min to 15mL/min and increased internal diameter of column, from 4.6mm to 20mm. The elution chromatogram were shown to be identical and it was attributed to the effect of increased flowrate setting, resulting in almost the same component band concentration being retained in the detection cell for the same time period [36].

Table 1: Comparison between analytical, semi-preparative and preparative HPLC [36].

Scale	**Function**	**Column I.D. range (mm)**	**Column length (mm)**	**Maximum load**	**Flow rate (mL/min)**
Analytical	Compound identification and separation	3 - 8	250	20 mg	0.5 – 3
Semi-preparative	Collection of small amount of purified compound (<0.5g)	10 - 20	250	300 mg	3 -10
Preparative	Collection of large amount of purified compound (>0.5g)	30 - 50	250-1000	2 g	10 - 100

Gradient elution is generally employed in analytical instrument to achieve the best baseline separation whereas isocratic elution method is applied for both semi-preparative and preparative instrument. This is because, isocratic method improves productivity and is more cost effective when running on a larger scale [37].

Although the condition scales are much bigger in semi- preparative column compared to analytical column, the principle remains the same. On the other hand, the effects of scaling up are broader peaks and lowered purity. It is imperative that the type of column used for both analytical and semi-preparative column has the same line of packing material to retain. The most common detector used on a semi-preparative instrument is the DAD-UV [38].

2.4 Structural elucidation of natural products

2.4.1 Mass spectrometry

Mass spectroscopy is one of the top analytical techniques, frequently used for compound identification as it can provide both qualitative (in terms of structural characterization) and quantitative (concentration or molecular mass information) analyses on analyte molecules. Analytes are introduced into an ionization source of the mass spectrometer where they get ionized forming either positive of negative ions. Generated ions are separated by its mass to charge ratio (m/z) and subsequent ion detection sheds a light on the molecular mass or concentration of analyte compounds [39, 40].

A mass spectrometer consists of the following units: Ion Source, Ion Analyser and Detector. Samples are introduced into the ionization source, a mechanical device that employs ionization method such as electron impact (EI) or electrospray ionization (ESI) among many other [40].

An Electron beam from tungsten filament is used to knock an electron from analyte to form an ion. As it is a hard ionization technique, it very common for this type of ionization method to generate a large amount of fragment ions. It is particularly useful for compounds that are non-polar and low molecular weight. However, its ability to detect large molecules is significantly poor and as a result not usable in qualitative analysis of natural products [40].

Electrospray Ionization (ESI), on the other hand, is a favoured technique when analyzing large compounds with high molecular weight as it produces no fragments upon ionization, allowing the mass spectrometric analysis for structural identification and chemical composition [41]. In ESI, the sample is injected through a capillary that has an applied voltage at the tip of the capillary, causing a dispersion of sample into an aerosol of charged liquid droplets. Because of solvent evaporation and constant flow of the nebulizing gas N_2, the size of the droplets reduces which then releases the charged analytes from sample. ESI has two modes, namely positive and negative

whereby the charge of sample depends on the positive or negative potential of the ion source [41].

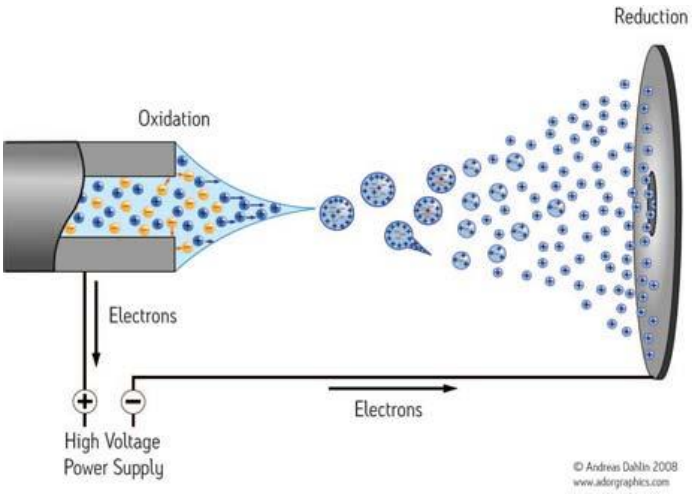


Figure 17 Scheme of electrospray ionization process, obtained from Waters [42]

An ion analyser separates ions based on its m/z ratio. The typical types of ion analyser are namely linear quadrupole ion trap (LIT), time-of-flight analyser (TOF), triple quadrupole analyser (QqQ) , magnetic sector mass analyser, electrostatic sector mass analyser and ion cyclotron resonance (ICR). LIT and QqQ provide low resolution mass (up to two decimal places) and is suitable for research involving synthesis of organic compounds, whereas TOF provides high resolution mass value (up to four decimal places). TOF is an ideal ion analyser for non-targeted analyses where unknown compounds can be identified with high accuracy evidently suitable for analytes that contain biological samples or samples with natural products [41, 43].

Coupled with chromatographic instruments, such as Gas Chromatograph (GC) or HPLC, the hyphenated system of GC-MS or HPLC-MS has become a technique capable of analysing the polarity and volatility of analytes (GC and LC) and molecular masses of analytes (MS) [39, 41]. In HPLC-MS, polarity of analyte combined with its molecular mass generates more information about the compounds present in the sample [43].

In natural product research, a combination of ESI-TOF-MS techniques is a powerful tool for the identification of unknown natural products. ESI is a soft ionization method whereby it does not result in many fragmentation patterns, but in adduct form, retaining its original molecular structure upon detection. It also utilises very little amount of sample, making it an ideal solution for analytes with very low concentrations [41].

Table 2: Common adducts formed during MS ionization process [44]

ESI positive mode	**ESI negative mode**
$[M+H]^+$	$[M-H]^-$
$[M+NH_4]^+$	$[M-CH_3]^-$
$[M+Na]^+$	$[M-H_2O]^-$
$[M+ CH_3]^+$	$[M+Cl]^-$

Although ESI does not form fragment ions, while the charged species is travelling through the analyser, it may form some fragments. In the case of natural products such as flavonoids, the glycosylated forms are fragmented and hence provide structural information of the type of sugar attached to the compound [23].

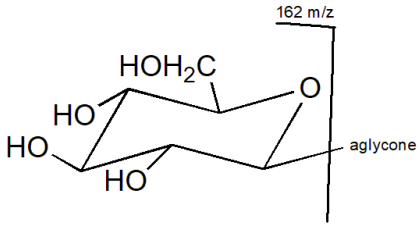


Figure 18 Characteristic loss of sugar moiety in glycosylated natural products

In summary, the combined use of LC-ESI-TOF-MS enables the identification of natural products. Databases such as Metlin and Chemspider are useful for searching for compounds with the same mass in samples generated from ESI method. That coupled with information such as ppm error, Hydrogen Deficiency Index (HDI) and isotopic pattern can be used as references to further narrow down the chemical composition of natural product. The chemical composition obtained from such libraries about the compound acts as a guide when elucidating structure from NMR data.

2.4.2. NMR spectrometry

Nuclear Magnetic Resonance (NMR) Spectroscopy is one of the most powerful research technique when elucidating plant metabolite structures and is also applicable in the profiling of novel compounds [23]. Structural information is obtained from NMR when nuclei, placed in an external magnetic field, absorbs electromagnetic (EM) radiation in radiofrequency region and re-emits excess energy in form of EM waves. The inherent magnetic field of an atom in a molecule changes the resonance frequency, depending on the magnet field strength, providing details about the electronic state and gives rise to bonding patterns. The determination of structure and relative stereochemistry of novel compounds requires data from 1D- and 2D-NMR experiments coupled with prior data from LC-MS as reference on molecular composition [45].

Large amount of sample is often required in NMR experiments (0.1-2.5mM concentration), but with smaller NMR probes, larger magnets, pulse programs, updated software and use of cryoprobes for higher sensitivity in signal detection, less amount is required to obtain a reasonably good spectrum [23] [46].

2.4.2.1 **1D**-NMR Experiments

Proton NMR or 1H-NMR experiment provides information about the chemical shift, relative number of protons, multiplicity pattern and spin-spin coupling constants (*J*), giving insight about their chemical environment and consequently the structure. For compounds such as flavonoids, it also provides information about the glycosidic bonds, number of sugar moiety and anomeric configuration of monosaccharides [47].

Carbon-13 NMR (or ^{13}C-NMR) experiment depends on the abundance of the carbon isotope ^{13}C, recorded with proton broad-band decoupling to remove coupling with attached protons. This in turn increases signal intensity of neighbouring carbon atom due to NOE, differentiating carbons that are attached to protons and quaternary carbons. On the other hand, due to the low abundance of carbon 13 isotopes occurring

naturally, the sensitivity is much lower compared to proton NMR experiment [23, 45]. Distinguishing primary, secondary, tertiary, and quaternary carbon becomes possible by APT (Attached Proton Test) and DEPT (Distortionless Enhancement by Polarization Transfer) experiments. The spectra from these experiments are known as “edited” because peak intensity and phase is modified with respect to regular ^{13}C spectrum [45].

2.4.2.2 **2D**-NMR Experiments

Two-dimensional NMR experiments such as COSY, HSQC, HMBC, NOESY and HSQC-TOCSY provide informative data that allow determination of structure and relative stereochemistry of isolated natural product. ^{1}H-^{1}H COSY shows neighbouring protons coupling within the molecule. HSQC establishes single bond correlation between protons and its attached carbon ($^{1}J_{CH}$), whereas HMBC complements HSQC by giving information about long range interactions between carbon and neighbouring protons by two or three and sometimes four bonds away. It is essential to pick the correct sets of experiments to address assignment on quaternary carbons found in natural products, such as flavonoids. NOESY is an experiment that is based on the Nuclear Overhauser Effect (NOE) that changes the intensity of an NMR signal from a nucleus when the neighbouring nucleus is saturated. Such correlations are mainly used to establish the stereochemistry of the molecule. HSQC-TOCSY experiment helps to identify protons that belong to the same coupling system, which is useful when the molecule has similar structural sub-units [45, 48].

NMR-1D and 2D experiments have been used to elucidate flavonoid structures from *Sophora japonica [31]*, Indian spice *Mammea longifolia*, identification of novel flavonoid from *Ononis vaginalis* and many others [47].

3. Results and discussion

Fractions exhibiting high inhibitory levels against arachidonic acid were investigated for their chemical constituents. Fractions E and F have been subjected to a series of experiments to identify, isolate and elucidate those compounds. For the purpose of this thesis, all fractions from previous study on *Sclerochloa dura* are labelled as crude fractions

3.1. Method development

All fractions previously collected and stored are termed as crude fractions. The fractions were selected based on previous findings about its bioactivity (extraction method and inhibition level are attached in appendix). Fractions that were exhibiting high inhibitory levels against arachidonic acid were subjected to a series of experiments to identify number of compounds, isolate and elucidate those compounds. A method was developed to increase baseline separation among the similar polarity of compounds by using reverse phase high performance liquid chromatography (RP-HPLC) before confirming its chemical composition through high resolution mass spectroscopy (HRMS). Compounds were then isolated through semi preparative HPLC and run on NMR to identify and characterize them.

Isolation and purification of crude fractions were guided by bioassay inhibition of arachidonic acid release. Fractions labelled as E and F were selected due to its high inhibition level of above 80%, with 79 fractions selected for HPLC separation. (list attached in appendix)

3.2. Method development using chromatographic separation

Separation of flavonoids can be difficult due to the similarities in their structure. As such, optimization of chromatographic condition is pertinent to achieving baseline separation, allowing isolation of compounds in later stage. There were many factors

that were tested to obtain the ideal conditions for fraction separation. Method was developed by testing various conditions, such as types of reverse phased columns, types of solvents, temperature, flow rate and elution method [47, 49].

In selection of suitable solvents, acetonitrile-water or methanol- water are common binary solvent systems used in RP-HPLC, as they are compatible in gradient mode and UV detection. On occasions, solvent such as isopropanol can be an alternative to acetonitrile [47, 50]. Given that polyphenolic compounds have easily ionizable hydroxy groups, acid modifiers such as tetrafluoroacetic acid (TFA) or formic acid (FA) are added in small quantities to supress such effect, producing sharper peaks with less tailing in chromatograms [47, 51, 52].

From then on, various methods were employed to achieve baseline separation which are tabulated in the following order;

Table 3: Parameters tested in method development

System	Phase A	Phase B	Flow Rate (mL min^{-1})
A	Water	Acetonitrile/methanol (50/50)	1.00
B	Water	Acetonitrile/methanol (40/60)	1.00
C	Water	Acetonitrile/isopropanol (30/70)	0.50
D	Water	Acetonitrile/isopropanol (50/50)	0.70
E	Water + 0.1% v/v FA	Acetonitrile + 0.1% v/v FA	0.65

The selected fractions were dissolved in 1ml of solvent containing equal parts of Phase A and Phase B solvent mixtures. System E was selected for further HPLC analysis as it showed best baseline separation with sharp peaks and minimal tailing.

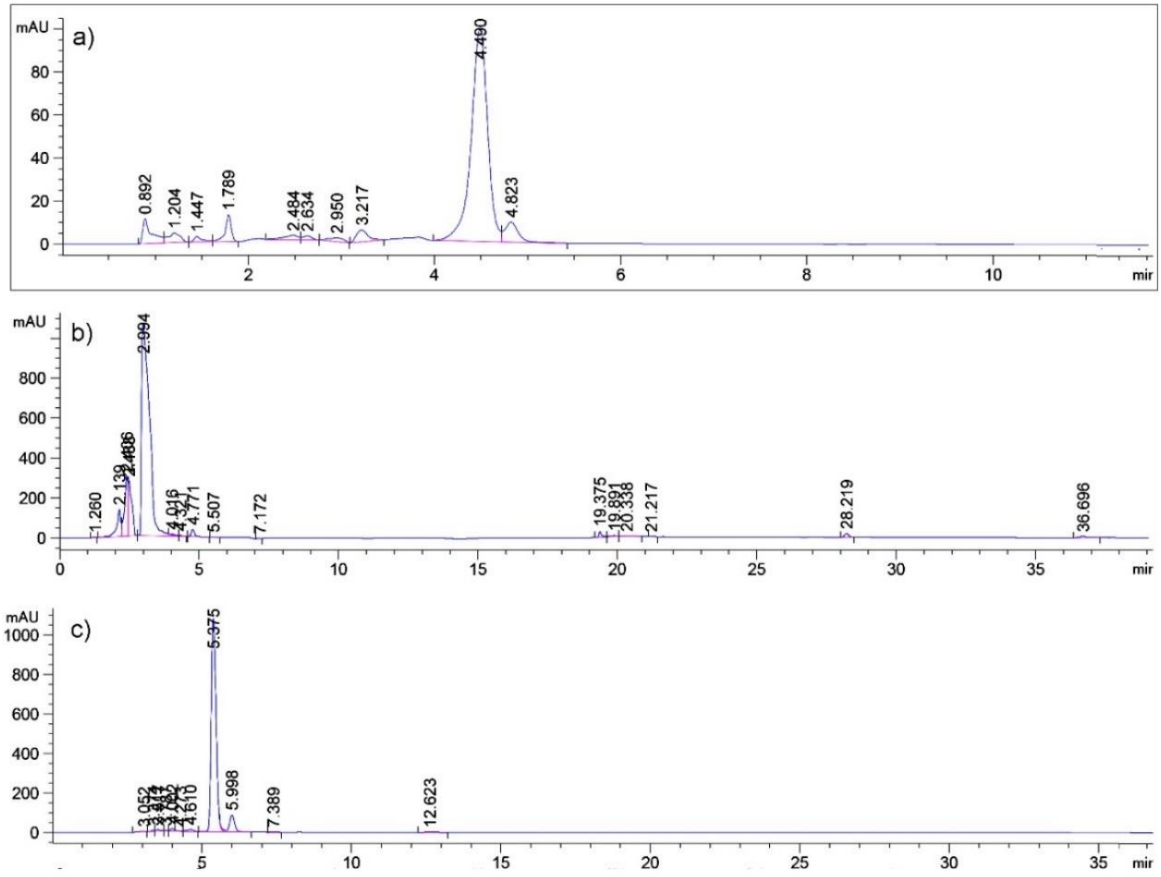


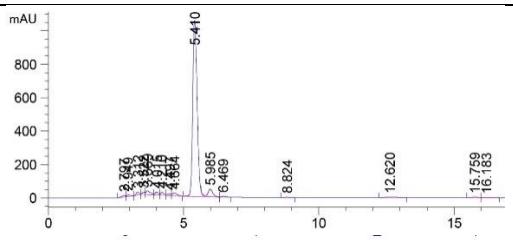
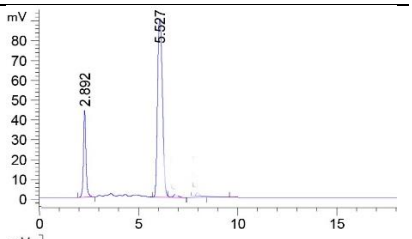
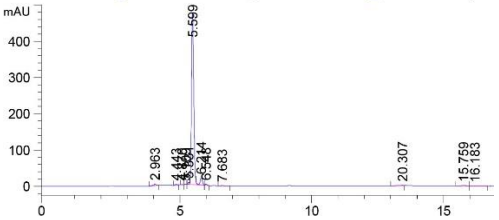
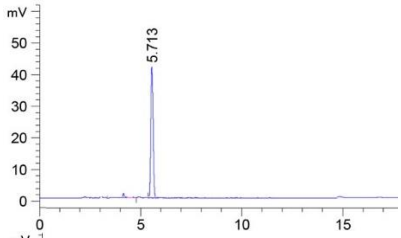
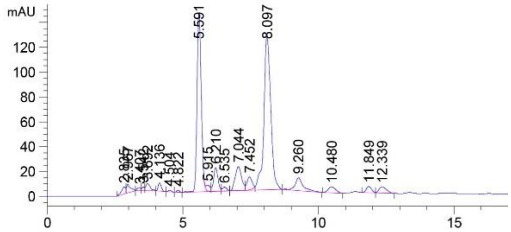
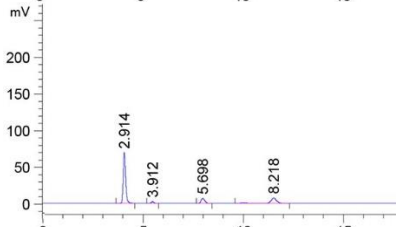
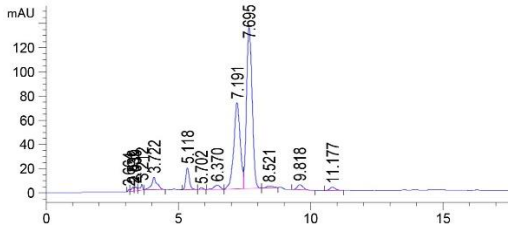
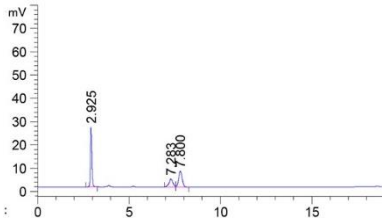
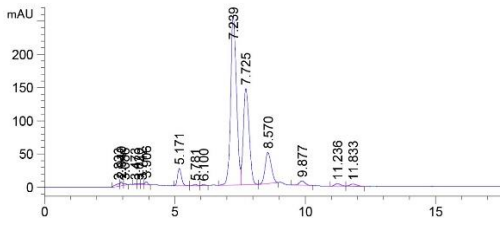
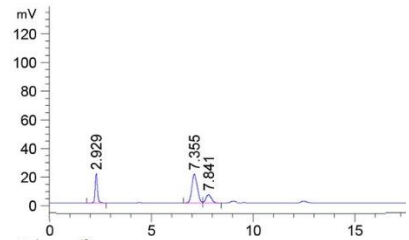
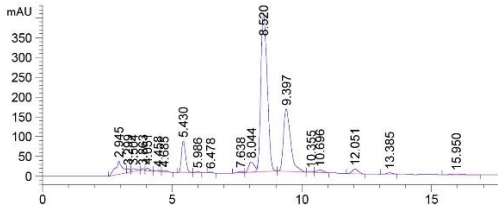
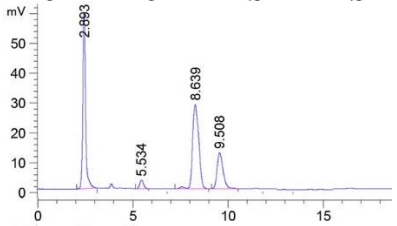
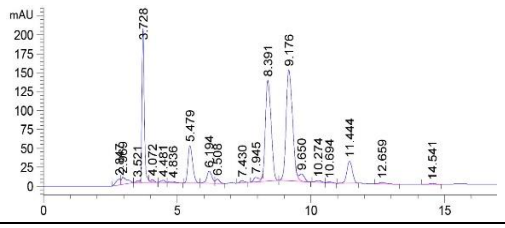
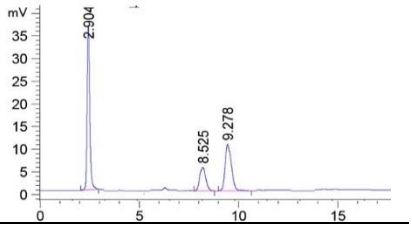
Figure 19 a) Fraction E35 at 260nm using System A, b) Fraction E36 at 260nm using System D and c) Fraction E38 detected at 260nm using System E.

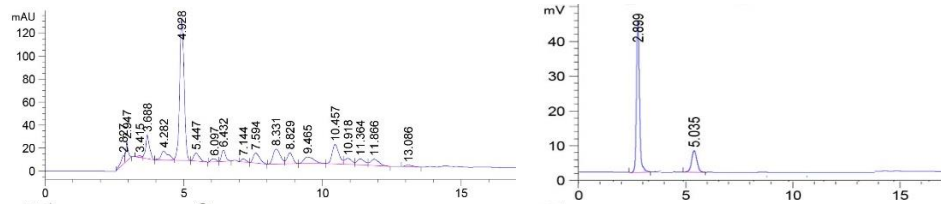
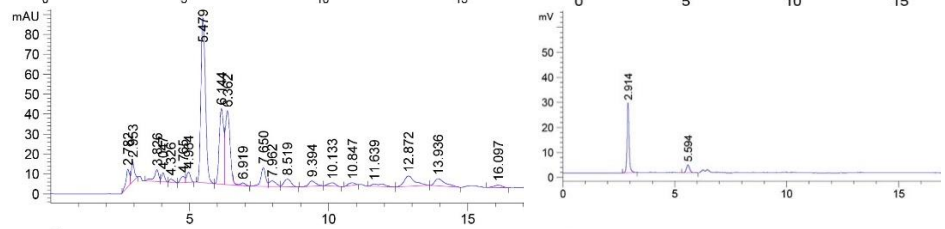
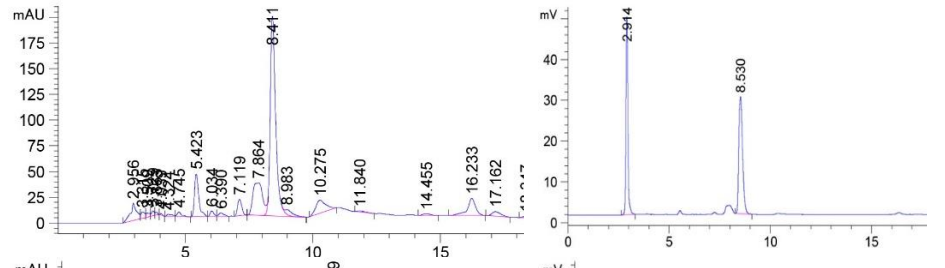
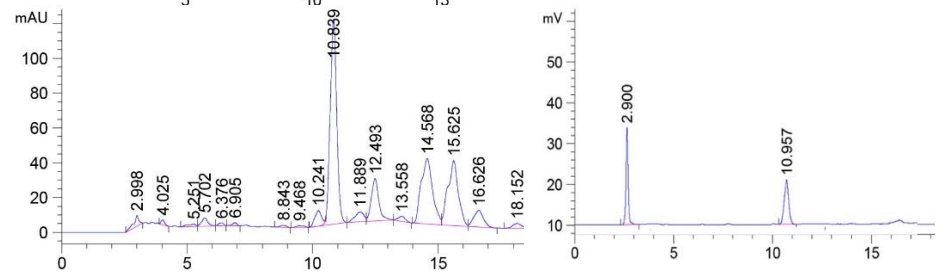
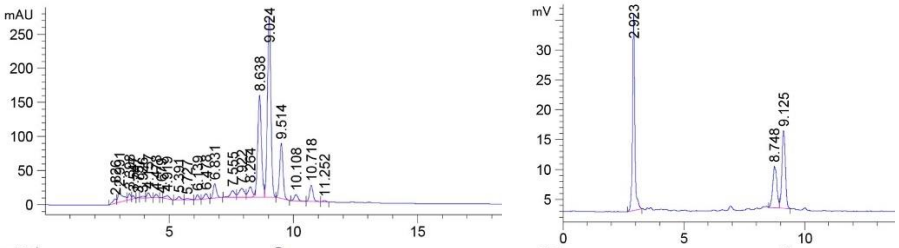
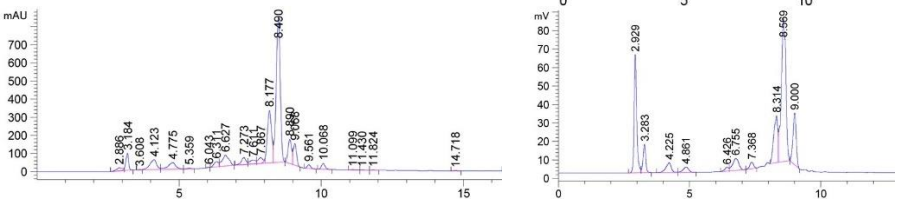
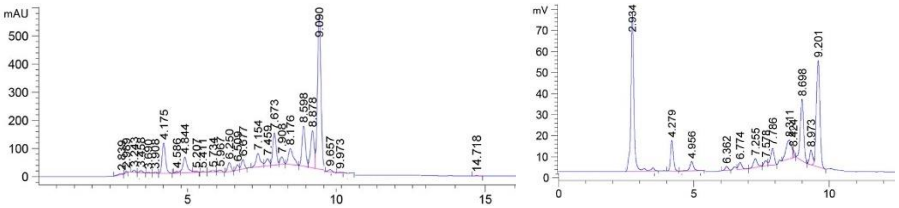
3.3. HPLC Analysis of Bioactive Fractions

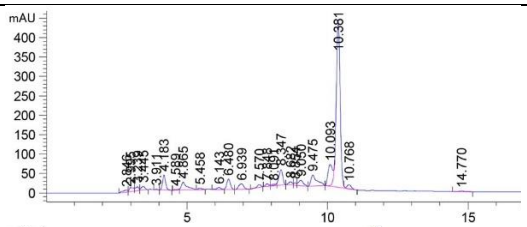
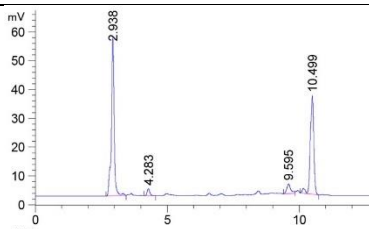
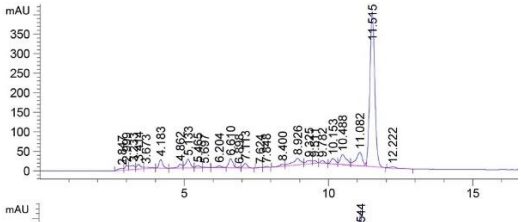
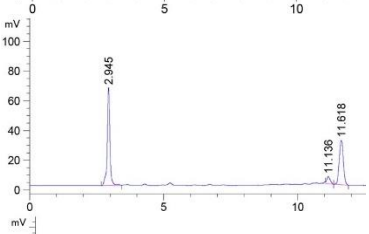
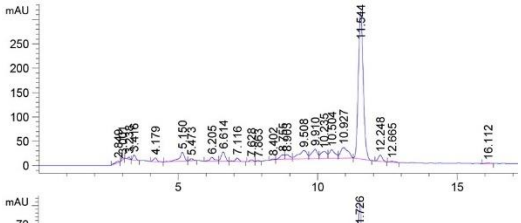
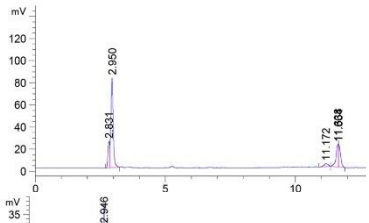
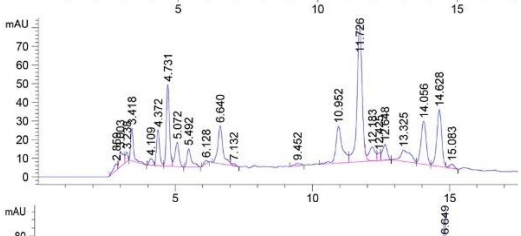
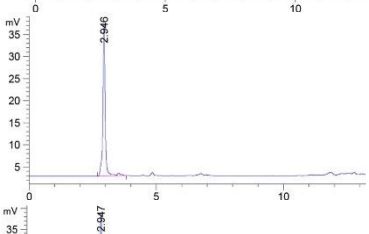
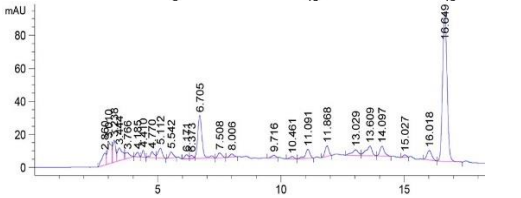
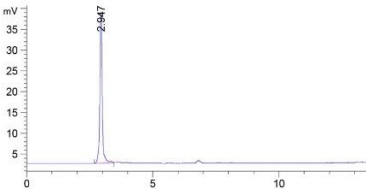
The crude fractions were analysed first using Pursuit 5 C18 column, which was later replaced by Zorbax Bonus RP C18 column, as it was found to give sharp peaks with better baseline separation. All fractions were initially subjected to system E conditions, but as the fraction labelling increases, the fractions experienced longer eluting time. As a result, two different gradient runs were employed on fraction sets E35 -F53 and from F58-F87. This was done during final stages of method development to increase separation efficiency.

Selected fractions with recorded mass of more than 5mg, high inhibition level (>80%) and high absorbance values (>70 mAU) are tabulated below.

Table 4: Qualitative analysis on fractions from *Sclerochloa dura*

Fraction	Inhibition Level (%) [6]	Chromatogram (λ=280nm)	ELSD Signal
E36	119		
E46	-		
F11	92		
F13	136		
F15	115		
F18	124		
F19	133		

F22	102	
F24	103	
F29	102	
F53	138	
F58	151	
F59	149	
F63	123	

F64	137		
F65	132		
F66	111		
F75	108		
F80	113		

The peak eluting around t_R = 2 minutes in the ELSD signal is the unretained solvent peak (t_o) as UV detectors do not see the solvent peak due to their design [53].

3.4. Determination of chemical composition using UPLC-MS

Once baseline separation is achieved, the selected samples were tested on LC-MS under the similar parameters as analytical HPLC to identify the chemical composition of the major peaks found in those fractions.

LC-MS system was used in analysis of *Filipendula ulmaria L*., a herbal plant, because of its ability to simultaneously monitor retention time of separated peaks and the chemical composition of said peaks during analysis [54-56].

Also, for the identification of flavonoids in extracts of the roots of Astragali Radix, commonly used in traditional Chinese medicine, the fragmentation pattern obtained from MS data in ESI positive mode was consistent with the identified structures. [49]

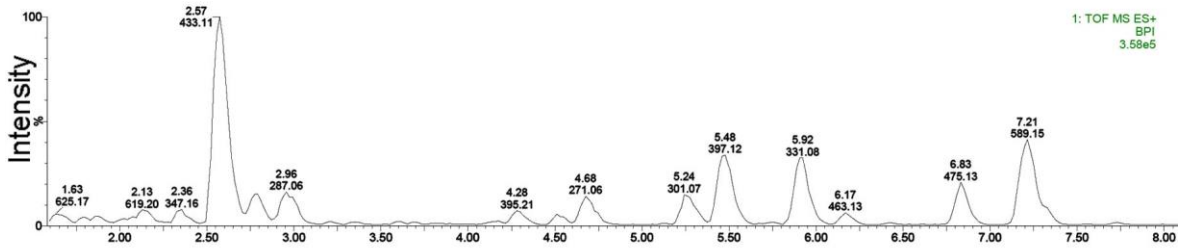


Figure 20 UPLC-MS result of fraction F22 shows that the retention time at 2.57 minutes is the same as the peak in analytical RP-HPLC at t_R 4.93 minutes.

Based on the data obtained from UPLC-MS, the results are compared to that from analytical HPLC to ascertain the molecular mass and chemical composition to the major peaks found in individual fractions.

Table 5: Comparing major eluents' retention time between RP-HPLC and UPLC-MS

Fraction	Number of peaks	Retention Time, min (HPLC)	Retention Time, min (UPLC-MS)	$[M+H]^+$ (m/z)	Parent Ion (m/z)
E36	1	5.41	2.57	433.11	
E46	1	5.60	2.57	433.11	
F11	2	5.59	2.57	433.11	
		8.10	4.53	287.06	
F13	2	7.19	10.10	287.06	449.11
		7.70	10.23	433.11	
F15	2	7.24	10.10	287.06	449.11
		7.73	10.23	433.11	
F18	3	5.43	8.63	433.11	
		8.52	10.21	271.06	433.12
		9.40	10.45	463.13	
F19	3	5.49	8.63	433.11	
		8.39	10.23	271.06	433.11
		9.18	10.45	463.13	
F22	1	4.93	2.57	433.11	

F24	3	5.48	2.41	433.11
		6.14	3.14	801.23
		6.36	3.37	801.23
F29	1	8.41	4.93	493.14
F53	1	10.84	6.33	689.21
F58	2	8.64	7.99	411.19
		9.02	8.56	441.20
F59	2	8.18	7.99	411.19
		8.49	8.54	441.20
F63	1	9.09	10.64	455.22
F64	1	10.38	10.64	455.22
F66	1	11.54	10.66	455.22
F75	1	11.73	5.94	331.08
F80	1	16.65	5.22	529.34
			5.41	527.32

From the table above, spectrum generated by ESI positive mode provides information about the molecule due to low fragmentation. Analyses on MS data show a loss of 162 m/z from parent ion, which can be attributed to the loss of glucose moiety, a characteristic of O-glycoside polyphenols [57, 58].

By comparing the retention time between HPLC and that of UPLC-MS, the theoretical mass can be assigned to major peaks, thus enabling the isolation of different compounds. The fractions that has same m/z values, but at two drastically different retention times provides insight that those fractions contain isomeric molecules.

3.5. Isolation of compounds with Semi-Preparative HPLC

Upon assigning peaks to possible chemical compositions, fractions were subjected to semi-preparative HPLC using isocratic elution method. Because of differences in flow rate, type and diameter of column, the chromatograms showed shifts in elution time. However, the isolated compounds were verified by performing the identical isocratic

elution on those compounds with the analytical HPLC using the same column type which is the Pursuit 5 C18 column.

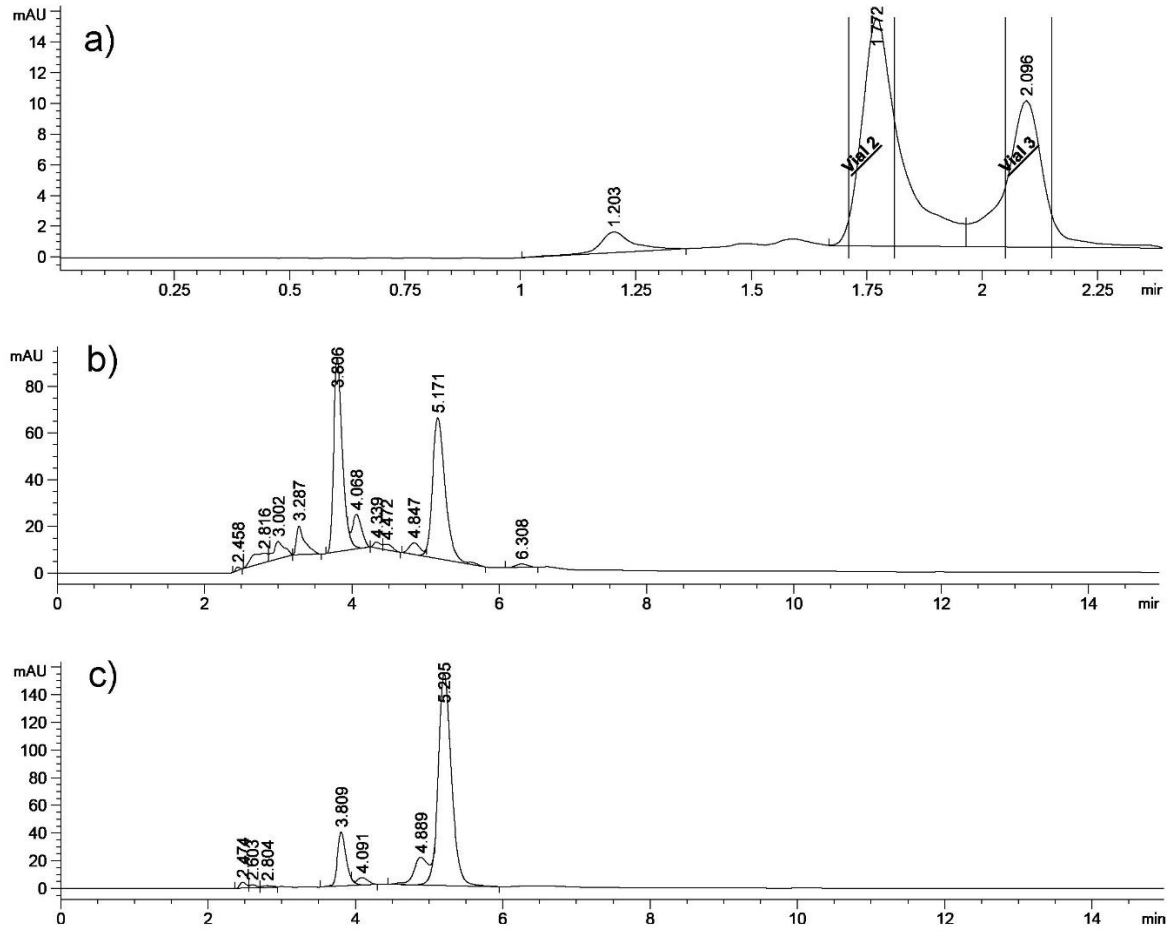


Figure 21 a) Isocratic elution method using semi preparative HPLC of crude F11 b) Isocratic elution of F11 using analytical HPLC c) Chromatogram of isolated fraction F11-02 using the same elution method as b)

Repeated injections of crude fraction into semi-preparative HPLC concentrated the amount of a compound of interest. The sub-fractions were then run on NMR for complete identification and characterization.

3.6. Structural Elucidation of bioactive compounds in *S. dura*

The most abundant class of substances found in these fractions are isoflavonoids. The compounds are characterised by the aromatic structures seen in Figure 10, providing relatively clear signals with minimal interferences from 6.0ppm to around 8.00ppm.

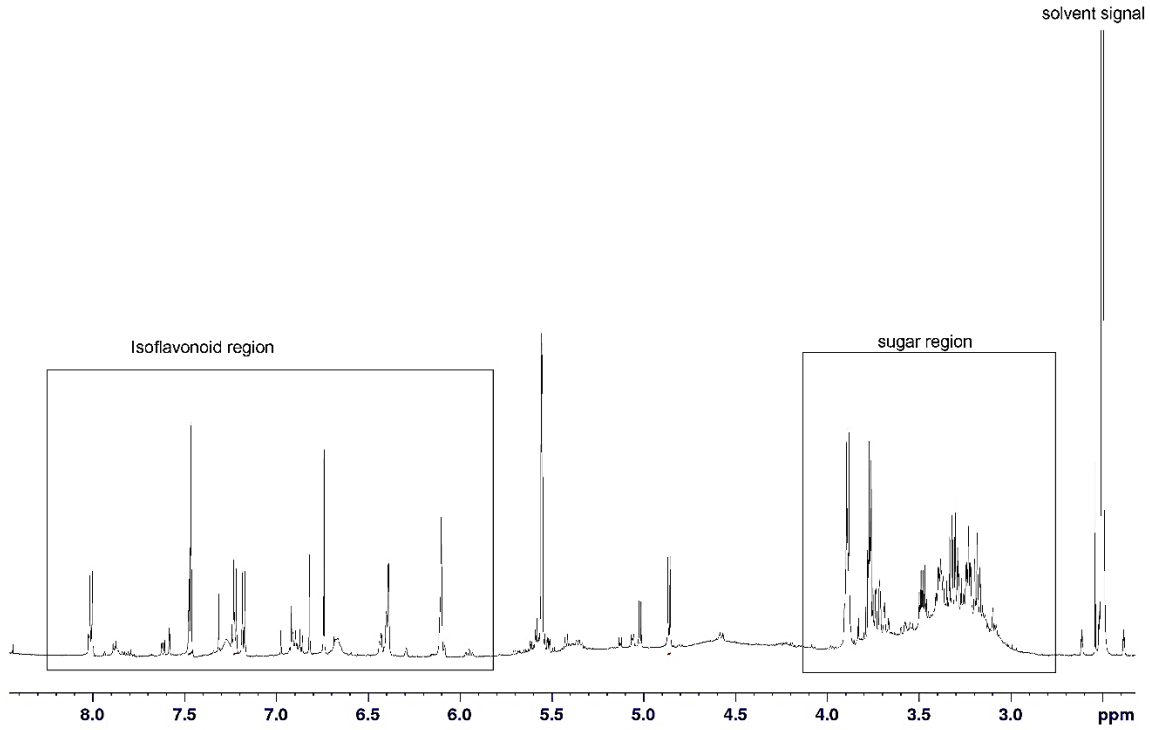


Figure 22 ^{1}H NMR spectrum of isoflavonoid

1D and 2D NMR experiments were ran on crude fractions and isolated sub-fractions. Due to the low masses (<2.5mg) obtained from purification step, signals from crude and purified compounds were compared for reliable signal assignment. From the five compounds elucidated below, three have complete characterization and are newly discovered in this plant while one was previously identified in *S.dura* as isovitexin and the other has only partial characterization due to complexities in overlapping signals.

Compound 1

Compound 1 is identified as genistein-4′-O-glucoside and it was obtained by semi-preparative HPLC run on pool F13-F16. The molecular formula of the compound was established as $C_{21}H_{20}O_{10}$ by HRMS (ESI positive mode) (m/z 433.1133) and theoretical mass is 432.3773. The molecular mass suggested that compound 1 is an isomer to isovitexin because although they possess same molecular formula, the elution times of both compounds in analytical HPLC were as far as 2 minutes apart. The structure was confirmed using ^{1}H NMR, ^{13}C NMR, HSQC, COSY, HMBC and HSQC-TOCSY data and is in agreement with literatures [59, 60].

Complete assignment of ^{1}H and ^{13}C NMR signals of the sugar portion is accomplished using HSQC and HMBC experiments together to identify the coupling constant of anomeric proton doublet at δ_H 5.02 (J = 7.52Hz), confirming the arrangement as β-glucopyranose. However, there is an uncertainty to the coupling constants for the rest of the protons present in the sugar molecule due to overlapping signals of residual water. The glycosylation site at C-4' of the aglycone of compound 1 was also determined by HMBC experiment, which showed long-range correlation between the anomeric proton and carbon resonance at δ_C 160.10 (C-4') and can be seen in Figure 20. NMR and MS spectra of compound 1 are attached as appendices.

Compound 2

Compound 3 was isolated from the same pool as compound 1, but the concentration of compound 3 was higher in F11, F12 and F13 compared to compound 1. Molecular formula of compound 3 is determined as $C_{21}H_{20}O_{11}$ which was deduced from HRMS (ESI positive mode) with measured mass of 449.1080 m/z. MS data also showed that compound 3 is present in both glycosylated and its aglycone form as there is an abundance of compound with mass 287.0614 (ESI positive mode), with loss of 162 m/z, a typical characteristic of a pyranose moiety. The structure is confirmed from NMR experiments both in 1D and 2D (COSY, HSQC, HMBC, HSQC-TOCSY and NOESY) and it is identified as luteolin-4'-O-glucoside.

HMBC experiment of this compound verified the attachment of glucose moiety to C-4', as there is strong correlation between anomeric proton at δ_H 4.85 (J = 7.26Hz) and δ_C 148.5 (C-4'). NOESY experiment support this information as there is signal between anomeric proton and proton from the aromatic region at δ_H 7.22 (J = 8.56Hz).

Compound 3

This compound is identified as chrysoeriol-7-O-β-glucoside and it was obtained by semi-preparative HPLC run on pool F18 and F19. The molecular formula of the compound was established as $C_{22}H_{22}O_{11}$ by HRMS (neutral mass) (m/z 462.4035) and

theoretical mass is 462.1166. The structure of this compound was confirmed by extensive 1D and 2D NMR measurements (COSY, HSQC and HMBC).

Coupling constant of anomeric proton at δ_H 5.12 (J = 7.26 Hz) shows that it is also a β-glucopyranoside and it is uncertain on the proton shifts at C2" and C3" due to the overlapping of those signals. COSY experiment showed couplings between anomeric proton and the two protons between δ_H 3.22 and 3.23. The location of methoxy group at C-3' and hydroxy group at C-4' was confirmed by correlation signal in HMBC spectrum with surrounding protons in the same spin system. Main correlations of HMBC and COSY can be seen in Table 3.5.

Due to low concentration of sample (<2.0mg), the quaternary carbon at C-7 showed no visible correlation with anomeric proton albeit the faint correlation between proton at C-6 at δ_H 6.08 (br s) and C-7 at δ_C 162.4.

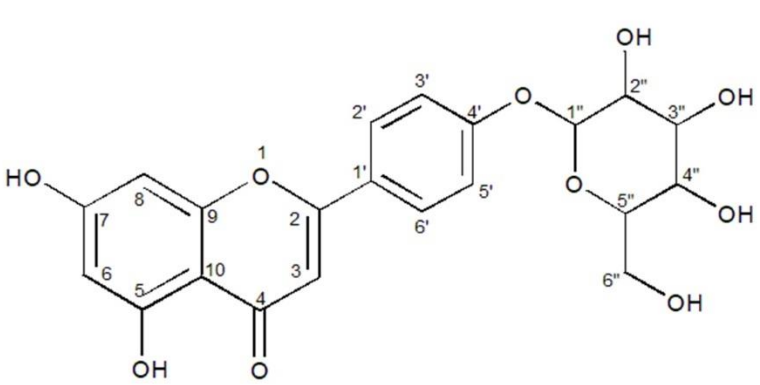
Compound 4

This compound was found in F63, F64, F65 and F66 as the main compound. HRMS (ESI positive mode) showed mass of compound to be 455.2177 m/z, showing chemical composition of $C_{25}H_{31}N_2O_6$ which is possibly a dimer, in $[2M+H-CH_4]^+$ adduct form. The structure is confirmed using NMR as the compound found in the fraction pool F63-F66 has the molecular formula of $C_{13}H_{17}NO_3$ with theoretical mass of 235.27. Compound is identified as 3-(4-Hydroxy-3-methoxyphenyl)-N-propylacrylamide, supported by 1D and 2D (COSY, HSQC, HMBC, NOESY) NMR experiments.

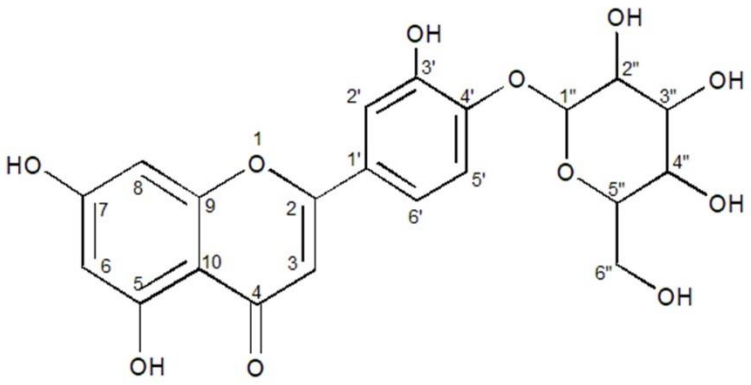
The location of methoxy group at C-3' and hydroxy group at C-4' was confirmed by analysis of HMBC spectrum. Furthermore, the configuration of the double bond present between C-1 and C-2 is E was established when the coupling constant for the protons attached to those carbons is measured at 15.75Hz, indicative of E-configuration. The presence of amide was detected in the broad singlet peak at δ_H 9.13 and the alkyl chain connected to it was confirmed by detecting the correlation signals between C-3 and C-5 in HMBC.

Isovitexin

This compound was already discovered in the previous research on this plant in fraction F07 and it was also found abundant in fraction pool E. The compound was dissolved in DMSO-d_6 instead of deuterated methanol, thus showing slightly different shifts from previously reported data [6]. As such, ^{1}H and ^{13}C assignments are tabulated in Table 5, and the retention time of this compound with molecular mass obtained from MS are provided as additional information in Table 3.3.



1



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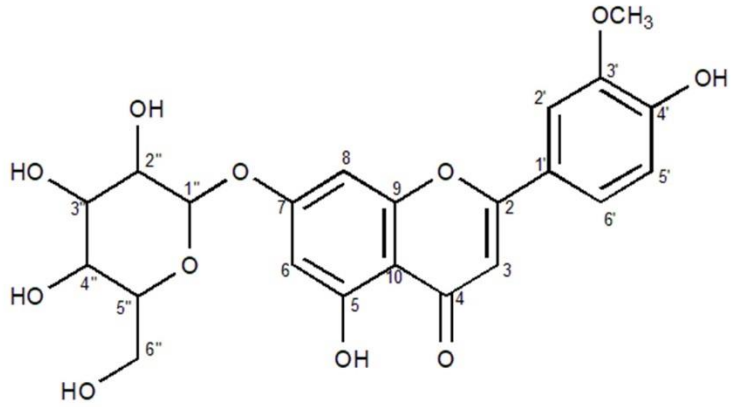

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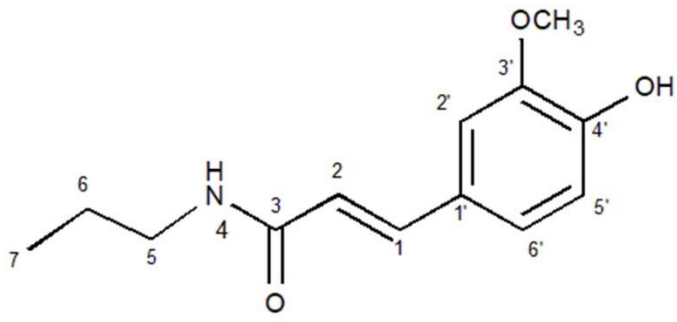


4

Figure 23 Compounds found in E and F fractions from methanolic extract of S.Dura 1) GenIstein-4'-O-glucoside 2) Luteolin-4'-O-glucoside 3) Chrysoeriol-7-O-β-glucoside and 4) (2E)-3-(4-Hydroxy-3-methoxyphenyl)-N-propylacrylamide

Table 6: NMR Spectroscopic data (600MHz and 800 MHz, DMSO-d_6) of compounds 1-4 and isovitexin

	1		2		3		4		isovitexin	
Position	**δ_H (J in Hz)**	**δ_C**	**δ_H (J in Hz)**	**δ_C**	**δ_H (J in Hz)**	**δ_C**	**δ_H (J in Hz)**	**δ_C**	**δ_H (J in Hz)**	**δ_C**
1							7.30 (d, 1H, J = 15.75)	138.7		
2		162.5		162.5		162.4	6.44 (d, 1H, J = 15.75)	119.1		162.9
3	6.81 (s, 1H)	103.7	6.69 (s, 1H)	103.6	6.96 (s, 1H)	104.7		165.3	6.62 (s, 1H)	102.3
4		181.3		181.1		181.2	9.13 (br s, 1H, NH)			181.3
5		161.3		161.3		167.8	3.15 (q, 2H, J = 6.65)	38.5		160.8
6	6.10 (br s, 1H)	99.6	6.05 (br s, 1H)	99.8	6.08 (br s, 1H)	99.7	1.50 (m, 2H)	24.5		109.3
7		162.7		162.4[b]		162.4[b]	0.86 (m, 3H)	14.0		167.1
8	6.39 (br s, 1H)	94.4	6.33 (br s, 1H)	94.5	6.41 (br s,1H)	94.7			6.38 (br s, 1H)	94.1
9		157.6		157.6		157.6				156.6
10		102.8		102.4		102.0				102.1
1’		124.9		125.1		126.0		126.4		120.8
2’	8.01 (d, 1H, J = 8.56)	128.0	7.45 (br s, 1H)[a]	113.6	7.31 (br s,1H)	104.8	7.10 (d, 1H, J = 1.89)	110.7	7.86 (d, 1H, J = 8.77)	128.2
3’	7.18 (d, 1H, J = 8.56)	116.6		147.4		152.9		148.2	6.89 (d, 1H, J = 8.77)	116.4
4’		160.1		148.5		161.3		147.8		161.8
5’	8.01 (d, 1H, J = 8.56)	116.6	7.22 (d, 1H, J = 8.56)	116.2	6.90 (dd, 1H, J = 1.81, 8.77)	116.0	6.79 (d, 1H, J = 8.06)	115.6	7.86 (d, 1H, J = 8.77)	128.2
6’	7.18 (d, 1H, J = 8.56)	128.0	7.44 (d, 1H, J = 8.56)	117.9	7.87 (m, 1H)	128.2	6.97 (dd, 1H, J = 1.89, 8.06)	121.4	6.89 (d, 1H, J = 8.77)	116.4

[a]overlapping signals [b] tentative assignments

Position	1		2		3		4		isovitexin	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
glucose										
1”	5.02 (d, 1H, J = 7.44)	99.9	4.85 (d, 1H, J = 7.26)	101.4	5.12 (d, 1H, J = 7.25)	102.0			4.61 (d, 1H, J = 9.84)	73.3
2”	3.27 (m, 1H)	73.2	3.32 (m, 1H)	73.3	3.22 (m, 1H)[c]	76.6			4.15 (t, 1H, J = 9.26)	70.2
3”	3.18 (m, 1H)	69.6	3.37 (m, 1H)	77.3	3.23 (m, 1H)[c]	74.2			3.21 (m, 1H)	79.2
4”	3.38 (m, 1H)	77.2	3.18 (m, 1H)	69.8	3.14 (m, 1H)	69.9			3.15 (m, 1H)	70.8
5”	3.30 (m, 1H)	76.5	3.31 (m, 1H)	75.9	3.07 (m, 1H)	77.4			3.17 (m, 1H)	81.5
6”	3.48, 3.71 (2H)	60.6	3.49, 3.73 (2H)	60.7	3.40, 3.59 (2H)	60.8			3.42 (dd, 1H, J = 5.75,11.67) 3.71 (dd, 1H, J = 1.70,11.67)	61.6
3’-OCH_3					3.88 (s, 3H)	56.7	3.79 (s, 3H)	55.5		

[c]interchangeable assignments

Table 7: Key COSY, HMBC and NOESY correlations of compound 1-4

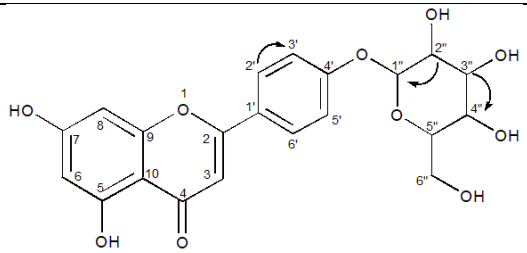
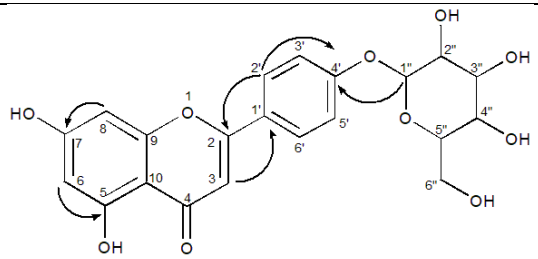
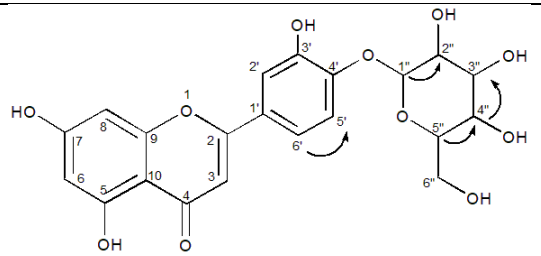
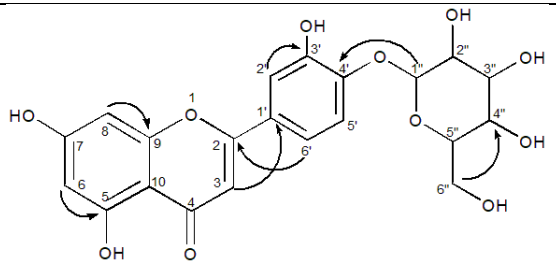
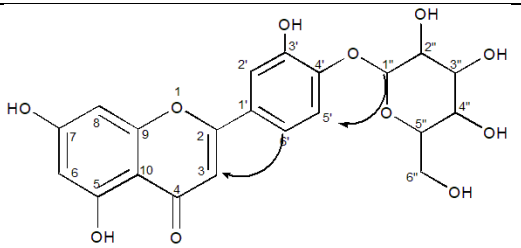
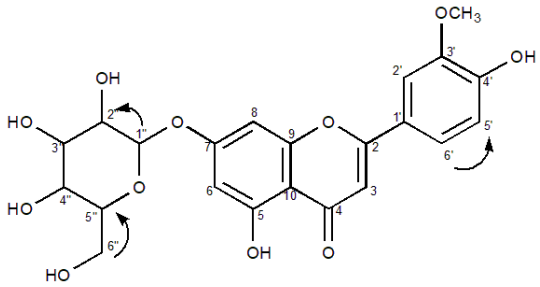
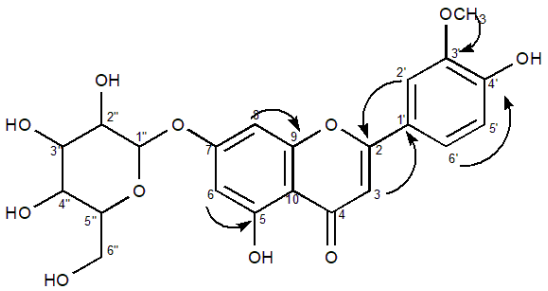
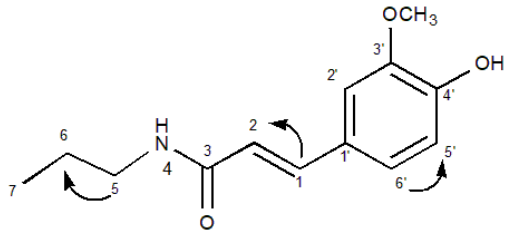
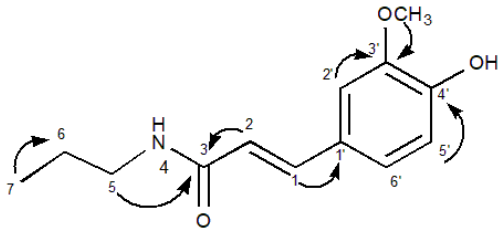
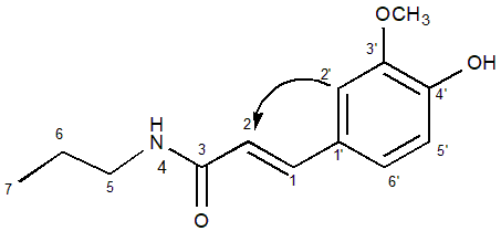
Compound	COSY	HMBC	NOESY
1			

Table 7 continuation

Compound	COSY	HMBC	NOESY
2			
3			
4			

4. Conclusion

The goal of this project has been achieved by finding three isoflavonoids that have not been found in this plant before and identifying a potentially new compound, (2E)-3-(4-Hydroxy-3-methoxyphenyl)-N-propylacrylamide.

Method development have proved to be successful by establishing the separation conditions to identify the retention time of unknown compounds and its molecular mass through HPLC and UPLC-ESI-TOF-MS.

Structural elucidation of compounds 1 to 4 were completed via a series of 1D and 2D experiments in NMR. Compound 1 is identified as genIstein-4'-O-glucoside, compound 2 is luteolin-4'-O-glucoside, compound 3 is chrysoeriol-7-O-β-glucoside and compound 4 is (2E)-3-(4-Hydroxy-3-methoxyphenyl)-N-propylacrylamide.

The retention time of isovitexin from the developed method is identified and can be used as reference for further analysis.

5. Future works

There are more compounds in other fractions that shows high bioactivity but due to the low mass, other forms of identification such as X-ray crystallography can be carried out to identify those structures.

Purified compounds 1 to 4 should be tested for various kinds of biological activity to understand its individual anti-inflammatory properties. HPLC chromatograms have shown that there are many other different compounds present in the fractions and tests should be carried out to isolate and elucidate them. It could potentially lead to discovery of new drug candidate.

6. Experimental

6.1. General

Solvents and chemicals were supplied by Sigma-Aldrich, VWR and Merck. Plant extraction was previously obtained by the following method; The plant's aerial parts were crushed and extracted through water reflux which was then filtered and concentrated using rotary evaporator. Samples were freeze-dried to obtain solid samples after which multistage fractionation was performed using Medium Pressure Liquid Chromatography (MPLC). Samples from methanolic extract (**D**) were biotested and further extraction was conducted, separating to fraction pools labelled as **E, F** and **G** after preliminary bioassay results showed high inhibition activity [6]. Fractions were stored in cold storage prior to this thesis.

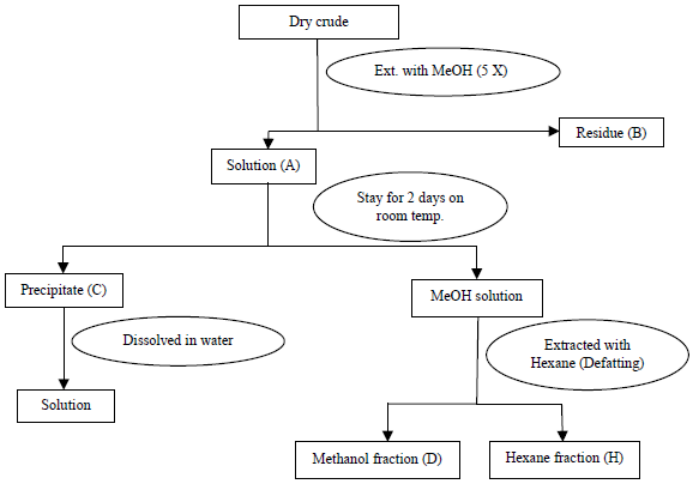


Figure 24 Extraction of bioactive compounds from S.dura [6]

6.2. Analyses

6.2.1. HPLC and Semi Preparative HPLC

Analytical HPLC was carried out using an Agilent 1260 series equipped with software OpenLAB CDS Agilent Chemstation for LC, 3D Systems, Rev C.01.07 [27]. Detection was made by a 1260 Infinity ELSD (Agilent) and UV-DAD detector, detecting at 260, 280, 360 nm with reference wavelength of 540nm. All separation was done at 25°C at 0.65 mL min^{-1} and

nebulizer and evaporator of ELSD was operated at 40°C with nitrogen gas flow at 1.4 SLM. Stationary phase used was Agilent Zorbax Bonus-RP 4.6x250mm column, 5μm particle size for method development and Agilent Pursuit 5 C18 4.6x150mm column, 5μm particle size was used for separation and isolation of compounds. Elution methods are specified in Table 3.

Fractions in Table 4 were subjected to two different elution methods; gradient elution from high water concentration to low water concentration was performed on fractions E35-F53 in the following manner; 80% -70% A from 0-15 minutes, 70% -0% A from 15-20 minutes and recalibrated back 0% -80% A from 20 – 25 minutes. Meanwhile, for fractions F58-F87, the following elution method was conducted; 75% -45% A from 0-15 minutes, 45% -0% A from 15-20 minutes and recalibrated back 0% -75% A from 20 – 25 minutes.

Semi preparative HPLC was performed using Agilent Infinity 1260 preparative HPLC system using UV-DAD at 280nm and 360nm with stationary phase Agilent Pursuit 5 C18 21.2 x 150mm column, 5μm particle size. Samples were collected under the following conditions; injection volume of 10μL, column temperature at 25°C and flow rate of 20 mL min^{-1}, eluent A: Water + 0.1%FA and eluent B: ACN + 0.1%FA; isocratic elution at 60A:40B and injection volume of 20μl. Fraction collector used is from the instrument.

Mobile phases components were water obtained from Millipore Elix 5, formic acid and acetonitrile from VWR (USA).

6.2.2. Preparation of standard solution

Standard solution was prepared for method development. 6.0mg of chlorogenic acid, 4.4mg of pyrocatechol, 2.8mg of 4-hydroxy-3-methoxycinnamic acid and 2.2mg of (-)-epicatechin was dissolved in 1.5 mL of 4:1 water and acetonitrile solution.

6.2.3. Mass Spectrometry

ESI-MS spectra were recorded on a Synapt G2-Si (Waters) mass spectrometer with an TOF analyzer and an ESI source; positive and negative ions were detected. UPLC-MS was

performed using ACQUITY UPLCr HSS T3 1.8µm column at 0.3 mL min^{-1} with formic acid added in water and acetonitrile as mobile phases. Data was analysed using MassLynx V4.1 (Waters). All samples were diluted 100x using water and acetonitrile with ratio of 4:1 from samples used in HPLC.

6.2.4. NMR Spectrometry

^{1}H NMR and ^{13}C NMR data were recorded using a NMR Bruker AVANCE™ 600MHz spectrometer and Bruker NMR Ascend™ 800MHz spectrometer with a 5 mm triple-resonance cryo probe equipped with a z-gradient and measured temperature at 298K. Samples were placed in 3 mm Samplejet NMR tubes due to low sample mass range from 5mg to 0.5mg and the samples were dissolved in the DMSO-d6. Chemical shifts are reported in ppm and solvent signal is used as reference. Full characterization was done using the pulse sequences from Bruker library which are ^{1}H and ^{13}C for 1D NMR and edited HSQC, HMBC, ^{1}H-^{1}H COSY, NOESY and HSQC-TOCSY for 2D-NMR.

The data was analysed with Bruker TopSpin 3.5pl7 with the following abbreviations used to describe the 1H signal splitting pattern: s (singlet), d(doublet), dd (doublets of doublets), m(multiplets) and br s (broad singlet).

6.3. Fraction recovery

Sample waste were collected after each run for recovery. A FreeZone® 1 Liter Benchtop freeze dryer (Labconco) operated at -35°C and at 8mbar was used to freeze dry sample for approximately 18h to remove most of the water and formic acid.

Smart evaporator (BioChromato) at 25°C with constant nitrogen gas flow is used to further dry samples to completely remove water from the samples. It was also used to remove DMSO-d6 solvent from NMR samples.

7. References

1. Patton, K.T. and G.A. Thibodeau, *The Human Body in Health & Disease - E-Book*. 2017: Elsevier Health Sciences.

2. Henochowicz, S.I., *Immune response*, in *A.D.A.M Medical Encyclopedia [Internet]*. 2016: Atlanta.

3. Zhang, J.-M. and J. An, *Cytokines, Inflammation and Pain.* International anesthesiology clinics, 2007. **45**(2): p. 27-37.

4. Heinrich, M., et al., *Fundamentals of Pharmacognosy and Phytotherapy E-Book*. 2017: Elsevier Health Sciences.

5. Butler, M.S., *The Role of Natural Product Chemistry in Drug Discovery.* Journal of Natural Products, 2004. **67**(12): p. 2141-2153.

6. Bukhari, S.M., *Isolation and structure elucidation of anti-inflammatory compounds from Sclerochloa dura*, in *Department of Chemistry*. 2013, Norwegian University of Science and Technology: Trondheim,Norway. p. 123.

7. Samuelsson, B.I., *Arachidonic acid metabolism: role in inflammation.* Zeitschrift Fur Rheumatologie, 1991. **50**(1): p. 3-6.

8. Hitchcock, A.S.C., Agnes, *Manual of the Grasses of the United States*. 1951, Washington D.C., USA: U.S Department of Agriculture.

9. Benzie, I.F.F. and S. Wachtel-Galor, *Herbal Medicine: Biomolecular and Clinical Aspects, Second Edition*. 2011: CRC Press.

10. Rooney, A., *The History of Medicine*. 2012: Rosen Publishing Group.

11. Edwards, A.S. *Paracelcus, the man who brought chemistry to medicine*. 2012 28.12.2017 [cited 2018; Available from: https://www.aaas.org/blog/scientia/paracelsus-man-who-brought-chemistry-medicine.

12. Roy Upton, R.H., *Chapter 3 - Traditional Herbal Medicine, Pharmacognosy, and Pharmacopoeial Standards: A Discussion at the Crossroads A2 - Mukherjee, Pulok K*, in *Evidence-Based Validation of Herbal Medicine*. 2015, Elsevier: Boston. p. 45-85.

13. Bohlin, G.S.L., *Drugs of Natural Origin: A Treatise of Pharmacognosy*. 7 ed. 2015: Swedish Pharmaceutical Society.

14. Cseke, L.J., et al., *Natural Products from Plants, Second Edition*. 2016: CRC Press.

15. Hanson, J.R., *Natural Products: The Secondary Metabolites*. 2003: Royal Society of Chemistry.

16. Herbert, R.B., *The Biosynthesis of Secondary Metabolites*. 1989: Springer Netherlands.

17. McMurry, J.E., *Organic Chemistry with Biological Applications*. 2014: Cengage Learning.

18. Steppuhn, A., et al., *Nicotine's Defensive Function in Nature.* PLOS Biology, 2004. **2**(8): p. e217.

19. Gray, S.D.S.Z.L.A.I., *Natural Products Isolation*. 2005: Springer Science & Business Media. 515.

20. Korkina, L., et al., *Plant phenylpropanoids as emerging anti-inflammatory agents.* Mini reviews in medicinal chemistry, 2011. **11**(10): p. 823-835.

21. Schmidt, D.D., et al., *Specificity in Ecological Interactions. Attack from the Same Lepidopteran Herbivore Results in Species-Specific Transcriptional Responses in Two Solanaceous Host Plants.* Plant Physiology, 2005. **138**(3): p. 1763-1773.

22. Rensheng Xu, Y.Y., Weimin Zhao, *Introduction to Natural Products Chemistry*. 2011: CRC Press.

23. J, P.P.F.J.G.C., *Structural Analysis of Flavonoids and Related Compounds - A Review of Spectroscopic Applications*. 2012: INTECH Open Access Publisher.

24. Birt, D.F. and E. Jeffery, *Flavonoids.* Advances in Nutrition, 2013. **4**(5): p. 576-577.

25. Dengada, A.H., *Isolation and Structural Elucidation of Compounds from Natural Products*. 2014, Virginia Tech.

26. Rahman, A., *Studies in Natural Products Chemistry*. 2012: Elsevier.

27. Lameira, J., et al., *A combined X-ray and theoretical study of flavonoid compounds with anti-inflammatory activity.* Journal of Molecular Structure: THEOCHEM, 2008. **862**(1): p. 16-20.

28. Guardia, T., et al., *Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat.* Il Farmaco, 2001. **56**(9): p. 683-687.

29. Ferrándiz, M.L. and M.J. Alcaraz, *Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids.* Agents and Actions, 1991. **32**(3): p. 283-288.

30. Jun, M., et al., *Suppression of arachidonic acid metabolism and nitric oxide formation by kudzu isoflavones in murine macrophages.* Molecular nutrition & food research, 2005. **49**(12): p. 1154-1159.

31. Kim, B.H., et al., *Anti-inflammatory action of legume isoflavonoid sophoricoside through inhibition on cyclooxygenase-2 activity.* Planta medica, 2003. **69**(05): p. 474-476.

32. Poole, C.F., *The Essence of Chromatography*. 2003: Elsevier.

33. Wolfender, J.-L., *HPLC in natural product analysis: the detection issue.* Planta medica 2009. **75**(7): p. 719-734.

34. Magnusson, L.-E., D.S. Risley, and J.A. Koropchak, *Aerosol-based detectors for liquid chromatography.* Journal of Chromatography A, 2015. **1421**: p. 68-81.

35. Adnani, N., C.R. Michel, and T.S. Bugni, *Universal quantification of structurally diverse natural products using an evaporative light scattering detector.* Journal of natural products, 2012. **75**(4): p. 802-806.

36. *Scaling up*. [cited 2018 30/05/2018]; Available from: https://www.shimadzu.com/an/hplc/support/prep/prep3.html#2.

37. Jablonski, J.-A.M.C., Rui, *Using Isocratic Preparative Liquid Chromatography as a Quick First-Step Purification for Natural Product Isolation*. 2013, Waters: Milford, MA, U.S.A.

38. Elyashberg, M.E., A.J. Williams, and K.A. Blinov, *Chapter 9 Application of Computer-assisted Structure Elucidation (CASE) Methods and NMR Prediction to Natural Products*, in *Modern NMR Approaches to the Structure Elucidation of Natural Products: Volume 1: Instrumentation and Software*. 2016, The Royal Society of Chemistry. p. 187-243.

39. Ho, C.S., et al., *Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications.* The Clinical Biochemist Reviews, 2003. **24**(1): p. 3-12.

40. Siuzdak, G., *An Introduction to Mass Spectrometry Ionization: An Excerpt from The Expanding Role of Mass Spectrometry in Biotechnology, 2nd ed.; MCC Press: San Diego, 2005.* JALA: Journal of the Association for Laboratory Automation, 2004. **9**(2): p. 50-63.

41. Banerjee, S. and S. Mazumdar, *Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte.* International Journal of Analytical Chemistry, 2012. **2012**: p. 40.

42. Waters. *Common Ionization*. 2018; Available from: http://www.waters.com/waters/en_NO/Common-Ionization/nav.htm?cid=10073251&locale=en_NO.

43. Allwood, J.W. and R. Goodacre, *An introduction to liquid chromatography–mass spectrometry instrumentation applied in plant metabolomic analyses.* Phytochemical Analysis, 2010. **21**(1): p. 33-47.

44. Waters. *Background Ion List*. Available from: https://www.waters.com/webassets/cms/support/docs/bkgrnd_ion_mstr_list.pdf.

45. Richards, S.A. and J.C. Hollerton, *Essential Practical NMR for Organic Chemistry*. 2010: Wiley.

46. Krishna, N.R. and L. Berliner, *Modern Techniques in Protein NMR*. 1999: Springer US.

47. Andersen, O.M. and K.R. Markham, *Flavonoids: Chemistry, Biochemistry and Applications*. 2005: CRC Press.

48. Reynolds, W.F. and R.G. Enríquez, *Choosing the Best Pulse Sequences, Acquisition Parameters, Postacquisition Processing Strategies, and Probes for Natural Product Structure Elucidation by NMR Spectroscopy.* Journal of Natural Products, 2002. **65**(2): p. 221-244.

49. Lv, Y.-W., et al., *Identification and Determination of Flavonoids in Astragali Radix by High Performance Liquid Chromatography Coupled with DAD and ESI-MS Detection.* Molecules, 2011. **16**(3): p. 2293.

50. Desai, A.M., et al., *Acetonitrile shortage: use of isopropanol as an alternative elution system for ultra/high performance liquid chromatography.* Analytical methods : advancing methods and applications, 2011. **3**(1): p. 56-58.

51. Li, T., et al., *Development and Validation of RP-HPLC Method for the Simultaneous Quantification of Seven Flavonoids in Pericarpium Citri reticulatae.* Food Analytical Methods, 2014. **7**(1): p. 89-99.

52. Wang, L., et al., *An analysis method for flavan-3-ols using high performance liquid chromatography coupled with a fluorescence detector.* Journal of Food and Drug Analysis, 2017. **25**(3): p. 478-487.

53. *Liquid Chromatography Problem Solving and Troubleshooting.* Journal of Chromatographic Science, 1995. **33**(5): p. 278-278.

54. Pemp, E., G. Reznicek, and L. Krenn, *Fast quantification of flavonoids in Filipendulae ulmariae flos by HPLC/ESI-MS using a nonporous stationary phase.* Journal of Analytical Chemistry, 2007. **62**(7): p. 669-673.

55. de Villiers, A., P. Venter, and H. Pasch, *Recent advances and trends in the liquid-chromatography–mass spectrometry analysis of flavonoids.* Journal of Chromatography A, 2016. **1430**: p. 16-78.

56. Rak, G., P. Fodor, and L. Abrankó, *Three-step HPLC–ESI-MS/MS procedure for screening and identifying non-target flavonoid derivatives.* International Journal of Mass Spectrometry, 2010. **290**(1): p. 32-38.

57. Mittal, A., et al., *Nontargeted identification of the phenolic and other compounds of Saraca asoca by high performance liquid chromatography-positive electrospray*

ionization and quadrupole time-of-flight mass spectrometry. ISRN pharmaceutics, 2013. **2013**.

58. Kumar, B.R., *Application of HPLC and ESI-MS techniques in the analysis of phenolic acids and flavonoids from green leafy vegetables (GLVs).* Journal of Pharmaceutical Analysis, 2017. **7**(6): p. 349-364.

59. Agrawal, P.K., *Carbon-13 NMR of Flavonoids*. 2013: Elsevier Science.

60. Parajuli, P., et al., *Biosynthesis of natural and non-natural genistein glycosides.* RSC Advances, 2017. **7**(26): p. 16217-16231.

Appendix

List of methanolic extracts of *S.dura* fractions tested in HPLC-UV-ELSD

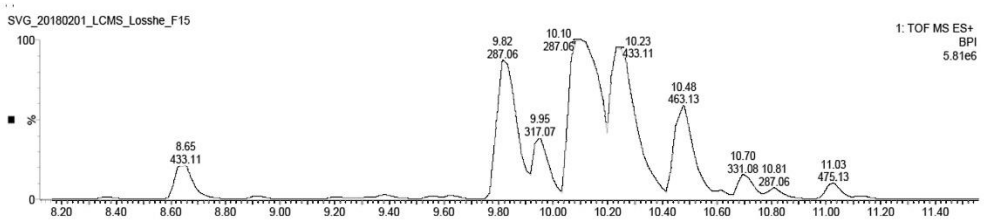
No	Sample ID	Fraction name	Amount (mg)	Inhibition level (%)
1	AD122175-16	C-1626-E-07	19.9	-
2	AD122175-17	C-1626-E-08	18.1	1, (-17)
3	AD122175-18	C-1626-E-09	40.6	4, (-13)
4	AD122175-44	C-1626-E-35	40.6	122, 109
5	AD122175-45	C-1626-E-36	42.6	119, 64
6	AD122175-46	C-1626-E-37	42.6	82, 34
7	AD122175-47	C-1626-E-38	41.9	50, 10
8	AD122175-48	C-1626-E-39	46.7	36, 7
9	AD122175-55	C-1626-E-46	70.6	-
10	AD122175-57	C-1626-E-48	68.6	12, 1
11	AD122175-58	C-1626-E-49	68.3	24, 7
12	AD122175-59	C-1626-E-50	77.2	28, 14
13	AD122175-64	C-1626-E-55	32.4	-
14	AD122175-77	C-1626-E-68	5.6	-
15	AD122175-92	C-1626-F-03	20.4	65
16	AD122175-97	C-1626-F-08	22.9	55
17	AD122175-98	C-1626-F-09	19.9	108
18	AD122175-99	C-1626-F-10	13.9	103
19	AD122175-100	C-1626-F-11	12.5	92
20	AD122175-101	C-1626-F-12	12.6	127
21	AD122175-102	C-1626-F-13	11.3	136
22	AD122175-103	C-1626-F-14	10.9	124
23	AD122175-104	C-1626-F-15	10.9	115
24	AD122175-105	C-1626-F-16	11.7	115
25	AD122175-106	C-1626-F-17	11.8	110
26	AD122175-107	C-1626-F-18	12.6	124
27	AD122175-108	C-1626-F-19	10.8	133
28	AD122175-109	C-1626-F-20	9.1	129
29	AD122175-110	C-1626-F-21	9.1	100

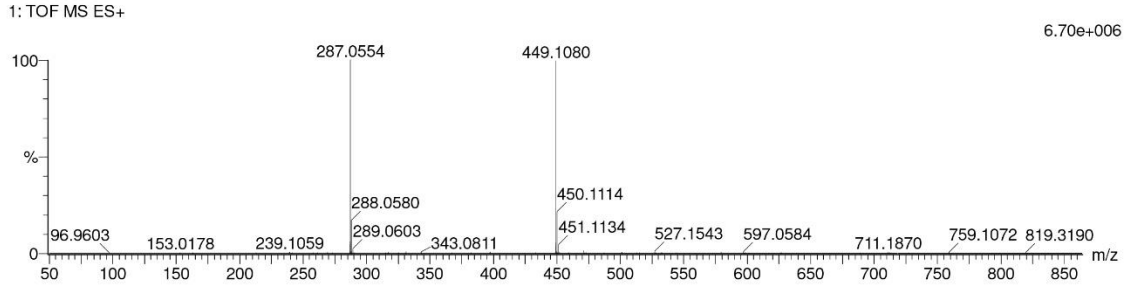
30	AD122175-111	C-1626-F-22	8.2	102
31	AD122175-112	C-1626-F-23	7.3	90
32	AD122175-113	C-1626-F-24	6.8	103
33	AD122175-114	C-1626-F-25	7.0	91
34	AD122175-115	C-1626-F-26	6.3	91
35	AD122175-116	C-1626-F-27	6.3	91
36	AD122175-117	C-1626-F-28	5.8	96
37	AD122175-118	C-1626-F-29	6.1	102
38	AD122175-119	C-1626-F-30	6.2	96
39	AD122175-120	C-1626-F-31	6.7	80
40	AD122175-121	C-1626-F-32	8.0	84
41	AD122175-123	C-1626-F-34	7.3	60
42	AD122175-124	C-1626-F-35	6.7	70
43	AD122175-125	C-1626-F-36	5.9	58
44	AD122175-126	C-1626-F-37	5.4	43
45	AD122175-127	C-1626-F-38	6.0	42
46	AD122175-128	C-1626-F-39	6.8	75
47	AD122175-129	C-1626-F-40	7.1	92
48	AD122175-134	C-1626-F-45	5.9	109
49	AD122175-135	C-1626-F-46	5.6	109
50	AD122175-136	C-1626-F-47	5.3	103
51	AD122175-137	C-1626-F-48	5.8	109
52	AD122175-138	C-1626-F-49	6.2	114
53	AD122175-139	C-1626-F-50	6.2	110
54	AD122175-140	C-1626-F-51	7.9	134
55	AD122175-141	C-1626-F-52	9.1	137
56	AD122175-142	C-1626-F-53	8.1	138
57	AD122175-143	C-1626-F-54	7.4	137
58	AD122175-147	C-1626-F-58	9.8	151
59	AD122175-148	C-1626-F-59	11.1	149
60	DATAMIS_ALYTCN-149	C-1626-F-60	0.3mg/ml	144
61	DATAMIS_ALYTCN-150	C-1626-F-61	0.3mg/ml	129
62	DATAMIS_ALYTCN-151	C-1626-F-62	0.3mg/ml	124

63	DATAMIS_ALYTCN-152	C-1626-F-63	0.3mg/ml	123
64	DATAMIS_ALYTCN-153	C-1626-F-64	0.3mg/ml	137
65	DATAMIS_ALYTCN-154	C-1626-F-65	0.3mg/ml	132
66	DATAMIS_ALYTCN-155	C-1626-F-66	0.3mg/ml	111
67	DATAMIS_ALYTCN-156	C-1626-F-67	0.3mg/ml	99
68	DATAMIS_ALYTCN-157	C-1626-F-68	0.3mg/ml	87
69	DATAMIS_ALYTCN-158	C-1626-F-69	0.3mg/ml	100
70	DATAMIS_ALYTCN-159	C-1626-F-70	0.3mg/ml	120
71	DATAMIS_ALYTCN-160	C-1626-F-71	0.3mg/ml	101
72	DATAMIS_ALYTCN-161	C-1626-F-72	0.3mg/ml	107
73	DATAMIS_ALYTCN-162	C-1626-F-73	0.3mg/ml	110
74	DATAMIS_ALYTCN-163	C-1626-F-74	0.3mg/ml	111
75	DATAMIS_ALYTCN-164	C-1626-F-75	0.3mg/ml	108
76	DATAMIS_ALYTCN-165	C-1626-F-76	0.3mg/ml	108
77	DATAMIS_ALYTCN-166	C-1626-F-77	0.3mg/ml	104
78	DATAMIS_ALYTCN-167	C-1626-F-78	0.3mg/ml	113
79	DATAMIS_ALYTCN-168	C-1626-F-79	0.3mg/ml	109
80	DATAMIS_ALYTCN-169	C-1626-F-80	0.3mg/ml	113
81	DATAMIS_ALYTCN-170	C-1626-F-81	0.3mg/ml	116
82	DATAMIS_ALYTCN-171	C-1626-F-82	0.3mg/ml	121
83	DATAMIS_ALYTCN-172	C-1626-F-83	0.3mg/ml	118
84	DATAMIS_ALYTCN-173	C-1626-F-84	0.3mg/ml	98
85	DATAMIS_ALYTCN-174	C-1626-F-85	0.3mg/ml	71
86	DATAMIS_ALYTCN-175	C-1626-F-86	0.3mg/ml	87
87	DATAMIS_ALYTCN-176	C-1626-F-87	0.3mg/ml	84

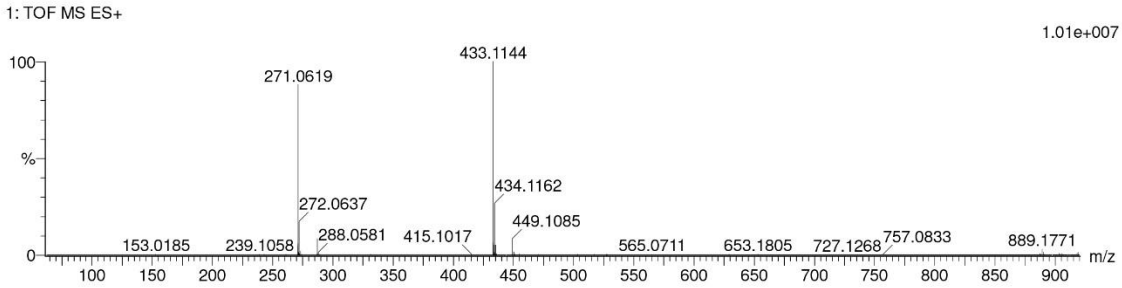
UPLC-ESI-TOF-MS data

F15



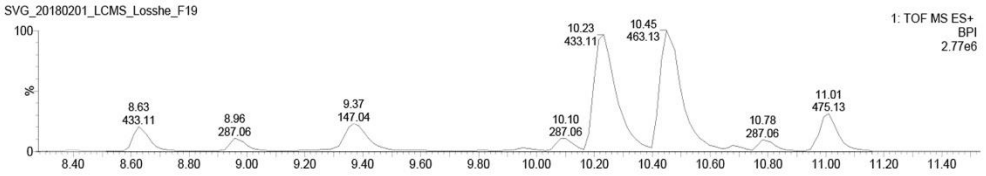


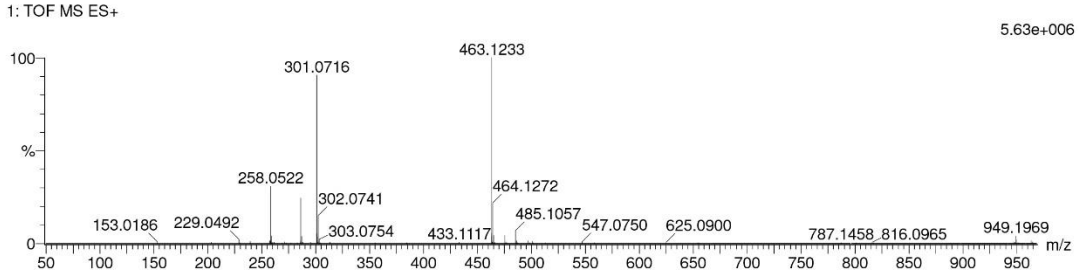
Minimum:				-1.5				
Maximum:		5.0	20.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
449.1080	449.1060	2.0	4.5	8.5	530.4	0.004	99.60	C19 H22 O11 Na
	449.1084	-0.4	-0.9	11.5	536.0	5.530	0.40	C21 H21 O11
	449.1001	7.9	17.6	17.5	543.3	12.908	0.00	C26 H18 O6 Na
	449.1025	5.5	12.2	20.5	543.6	13.146	0.00	C28 H17 O6
	449.1154	-7.4	-16.5	21.5	545.5	15.040	0.00	C30 H18 O3 Na



Minimum:				-1.5				
Maximum:		5.0	20.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
433.1144	433.1135	0.9	2.1	11.5	630.9	0.196	82.20	C21 H21 O10
	433.1111	3.3	7.6	8.5	633.2	2.469	8.47	C19 H22 O10 Na
	433.1204	-6.0	-13.9	21.5	635.1	4.399	1.23	C30 H18 O2 Na
	433.1076	6.8	15.7	20.5	633.3	2.545	7.85	C28 H17 O5
	433.1229	-8.5	-19.6	24.5	636.7	5.957	0.26	C32 H17 O2

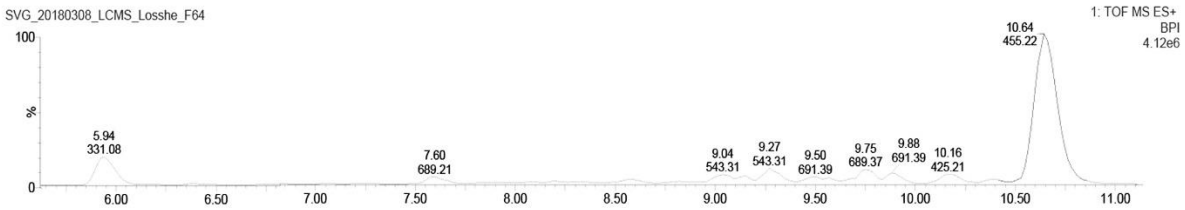
F19

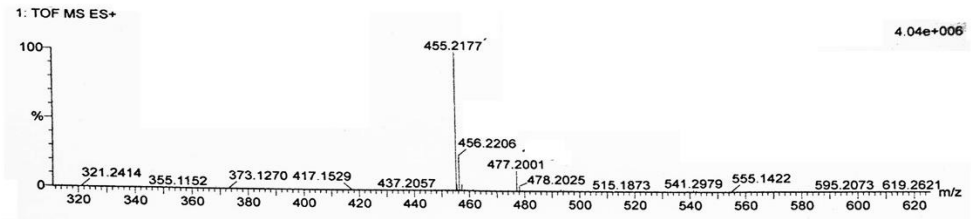




Minimum:				-1.5				
Maximum:		5.0	20.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
463.1233	463.1216	1.7	3.7	8.5	573.5	0.021	97.96	C20 H24 O11 Na
	463.1240	-0.7	-1.5	11.5	577.4	3.898	2.03	C22 H23 O11
	463.1158	7.5	16.2	17.5	583.5	10.023	0.00	C27 H20 O6 Na
	463.1182	5.1	11.0	20.5	583.6	10.099	0.00	C29 H19 O6
	463.1310	-7.7	-16.6	21.5	585.4	11.918	0.00	C31 H20 O3 Na

F64

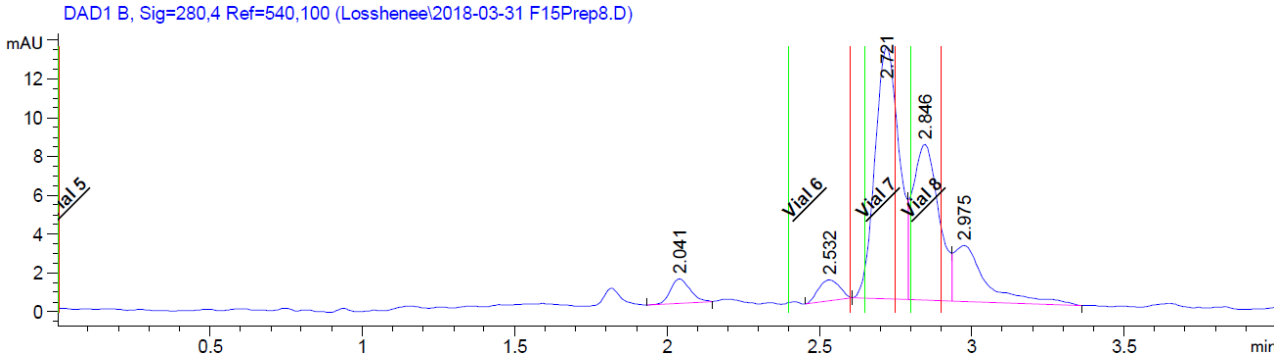




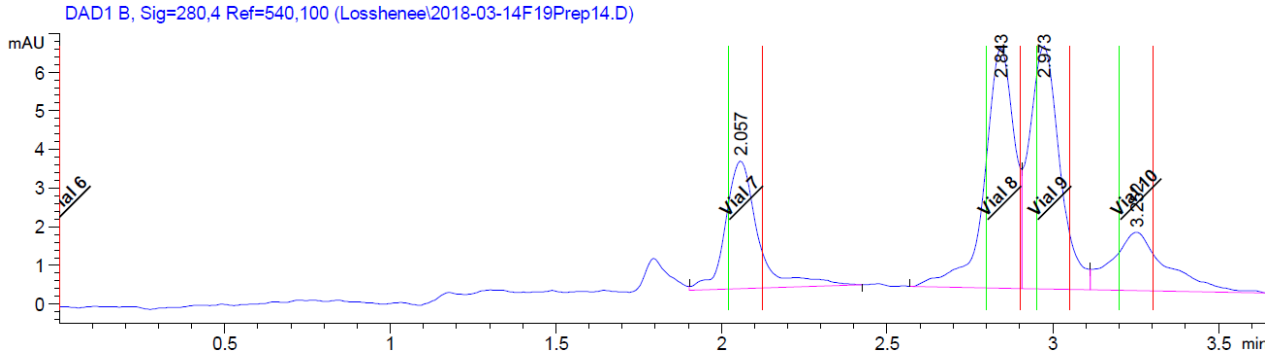
Minimum:				-1.5				
Maximum:		5.0	20.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
455.2177	455.2155	2.2	4.8	12.5	523.4	0.463	62.95	C21 H27 N8 O4
	455.2268	-9.1	-20.0	12.5	524.7	1.784	16.80	C20 H27 N10 O3
	455.2158	1.9	4.2	8.5	525.2	2.281	10.22	C23 H32 N2 O6 Na
	455.2142	3.5	7.7	7.5	526.3	3.447	3.18	C20 H31 N4 O8
	455.2131	4.6	10.1	9.5	526.4	3.547	2.88	C19 H28 N8 O4 Na
	455.2171	0.6	1.3	13.5	527.3	4.397	1.23	C24 H28 N6 O2 Na
	455.2145	3.2	7.0	14.5	527.9	5.033	0.65	C20 H24 N12 Na
	455.2254	-7.7	-16.9	7.5	528.2	5.305	0.50	C19 H31 N6 O7
	455.2182	-0.5	-1.1	11.5	528.3	5.430	0.44	C25 H31 N2 O6 •
	455.2169	0.8	1.8	17.5	528.7	5.836	0.29	C22 H23 N12
	455.2244	-6.7	-14.7	9.5	529.2	6.290	0.19	C18 H28 N10 O3 Na
	455.2257	-8.0	-17.6	3.5	529.2	6.320	0.18	C21 H36 O9 Na
	455.2195	-1.8	-4.0	16.5	529.5	6.648	0.13	C26 H27 N6 O2
	455.2118	5.9	13.0	4.5	530.1	7.248	0.07	C18 H32 N4 O8 Na
	455.2128	4.9	10.8	13.5	530.3	7.391	0.06	C17 H23 N14 O2
	455.2230	-5.3	-11.6	4.5	530.5	7.618	0.05	C17 H32 N6 O7 Na
	455.2129	4.8	10.5	2.5	530.7	7.791	0.04	C19 H35 O12
	455.2198	-2.1	-4.6	12.5	530.8	7.914	0.04	C28 H32 O4 Na •
	455.2241	-6.4	-14.1	2.5	531.3	8.412	0.02	C18 H35 N2 O11
	455.2115	6.2	13.6	8.5	531.7	8.772	0.02	C16 H27 N10 O6
	455.2212	-3.5	-7.7	17.5	531.7	8.791	0.02	C29 H28 N4 Na

Semi-preparative HPLC data

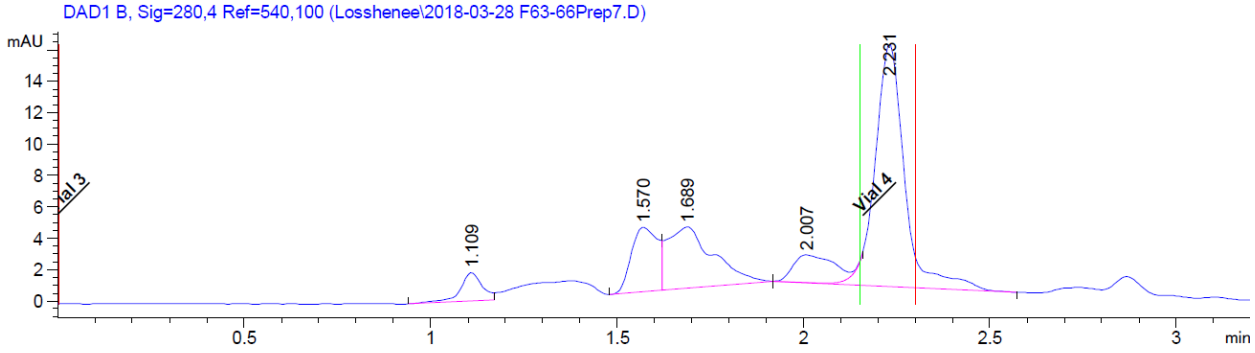
F15



F19



F63 - F66



NMR data

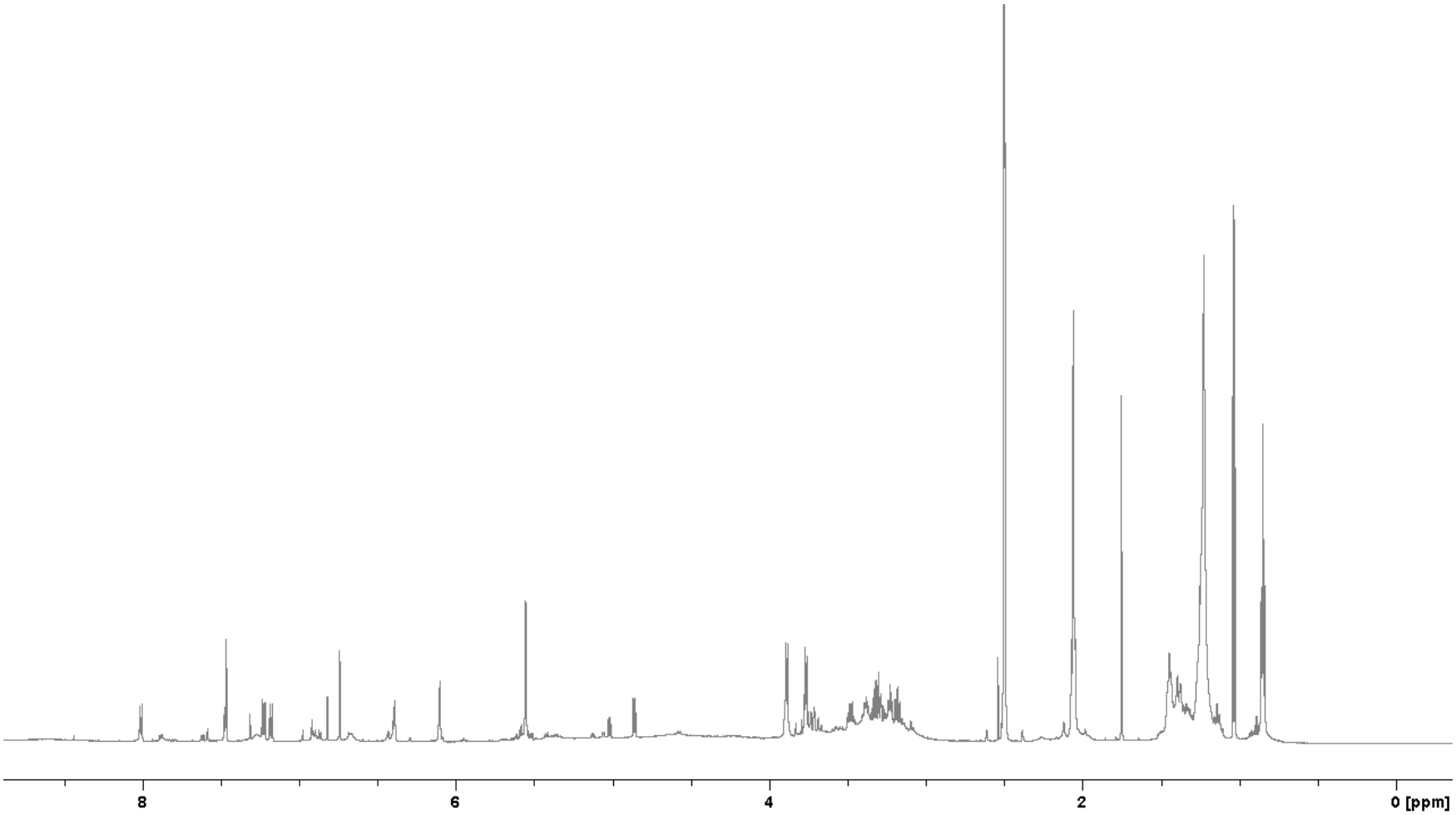


Figure 25 ^{1}H NMR spectrum of compound 1 in DMSO-d_6

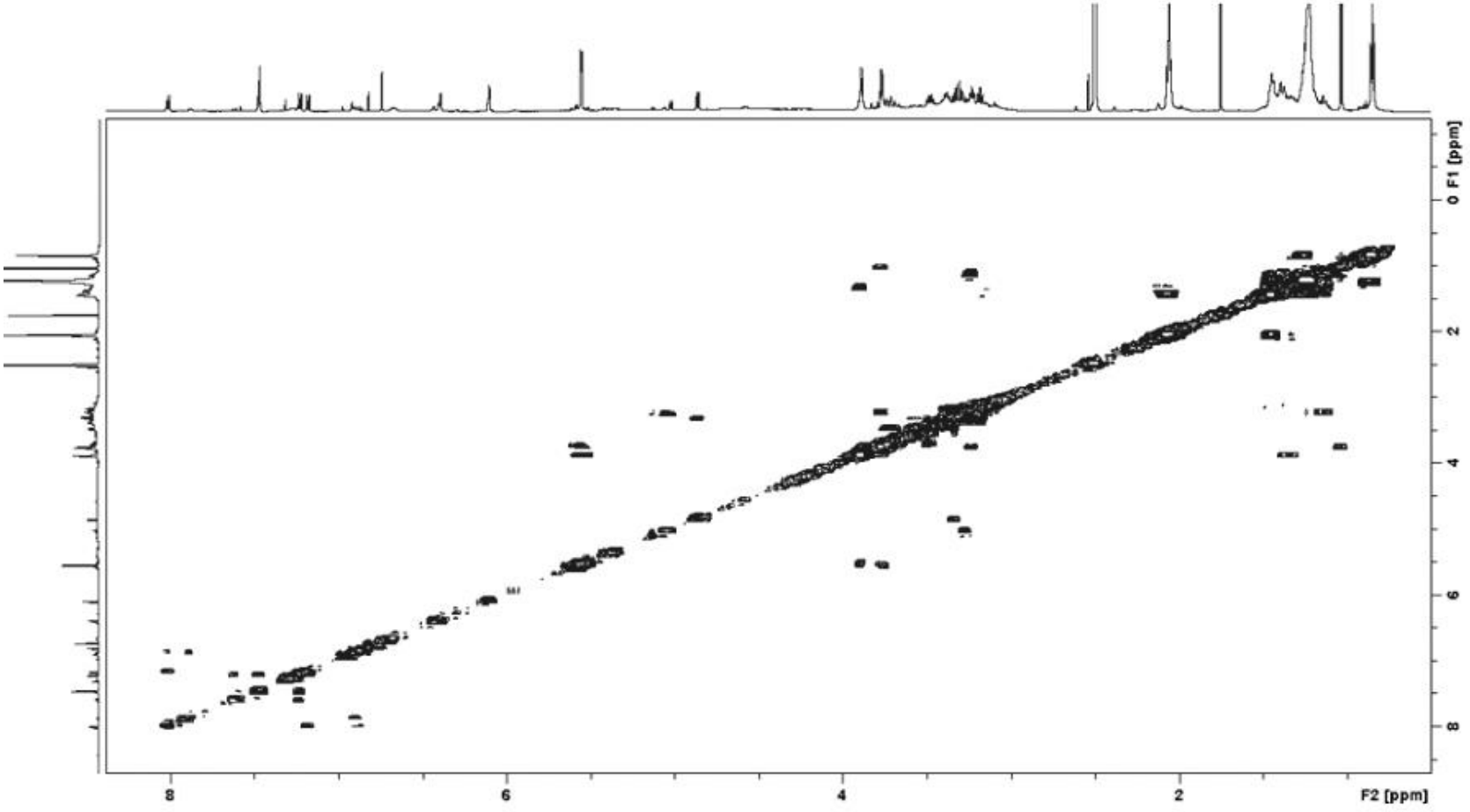


Figure 26 COSY spectrum of compound 1 in DMSO-d_6

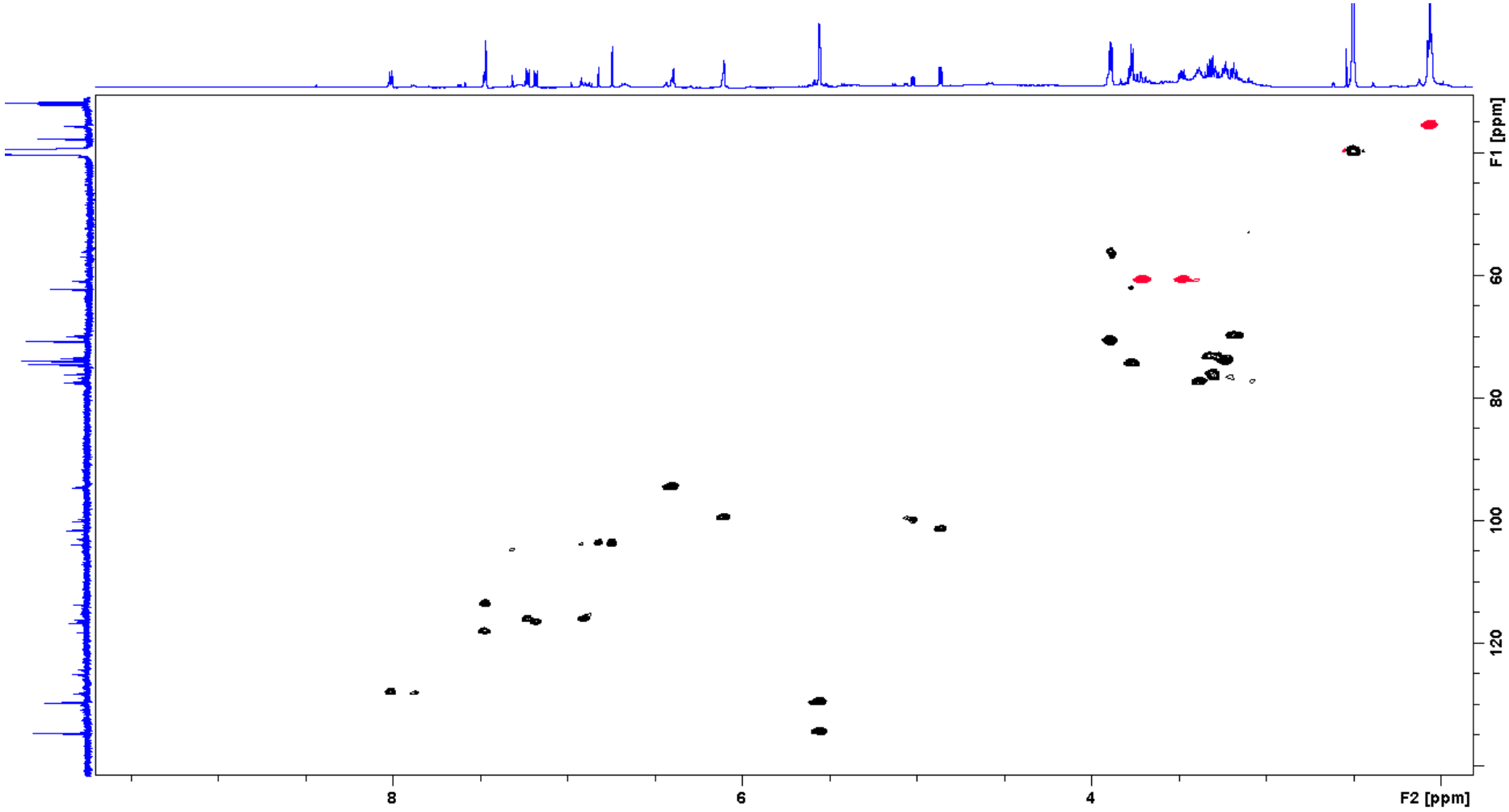


Figure 27 Edited HSQC spectrum of compound 1 in DMSO-d_6

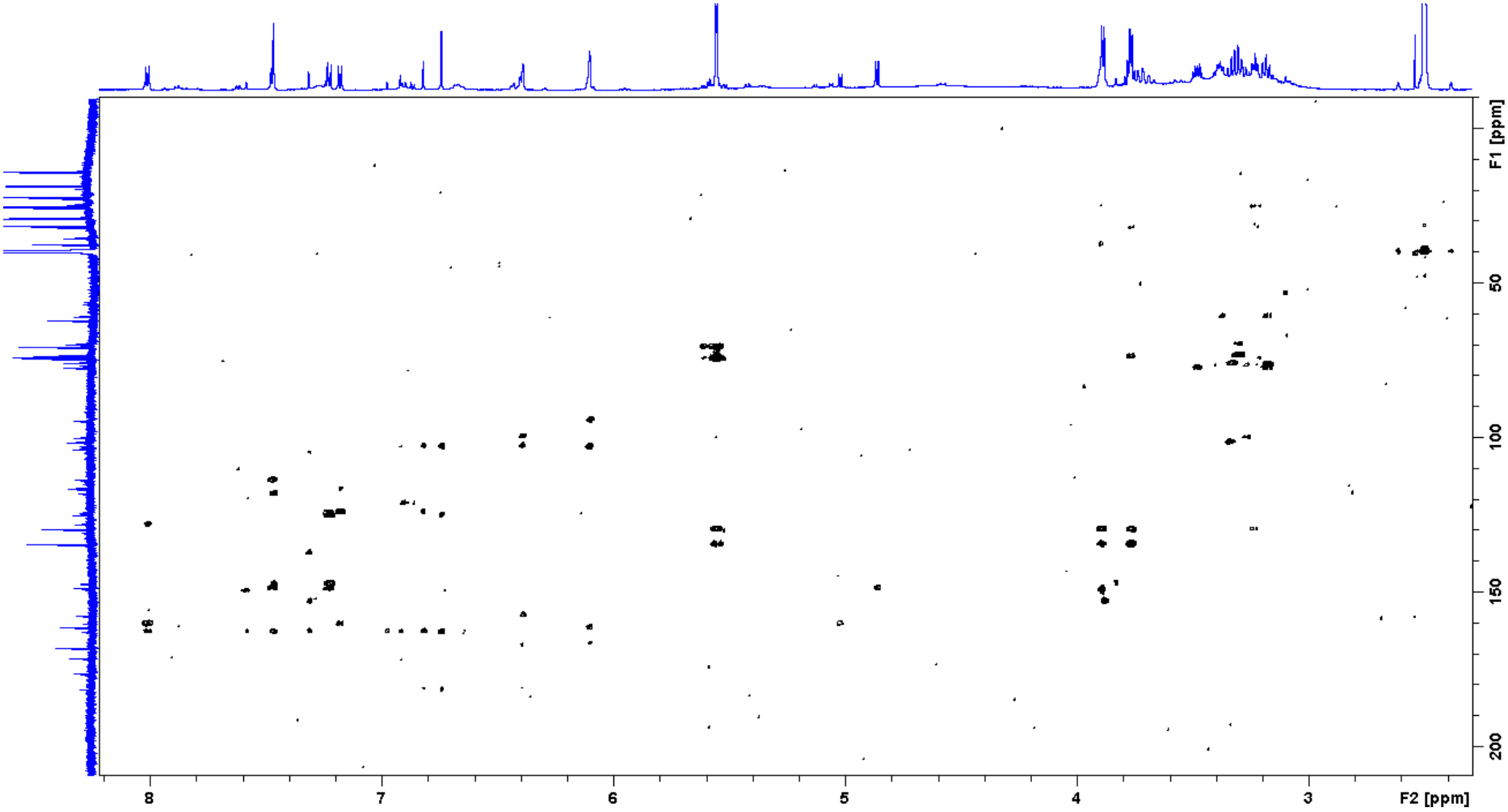


Figure 28 HMBC spectrum of compound 1 in DMSO-d_6

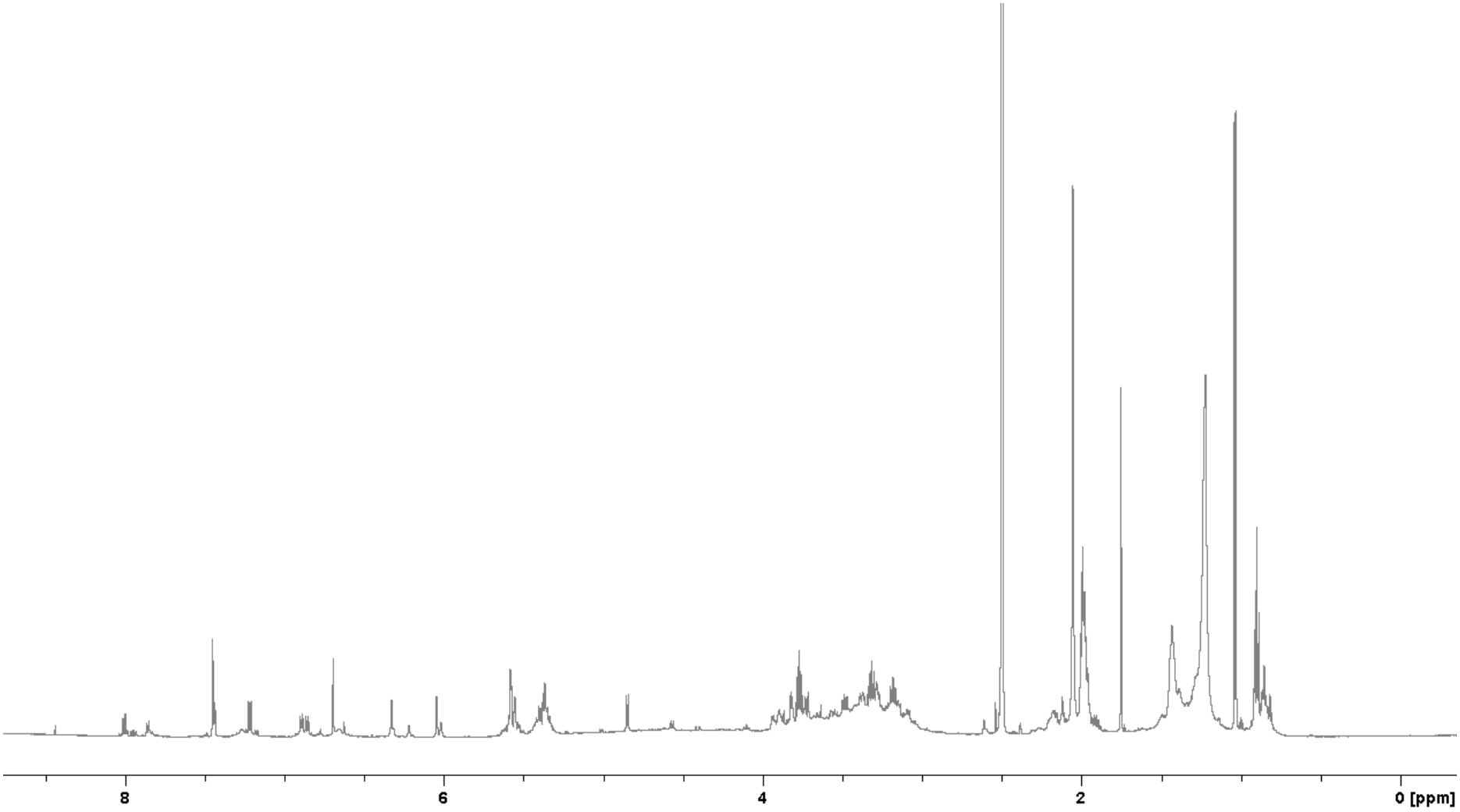


Figure 29 ^{1}H NMR spectrum of compound 2 in DMSO-d_6

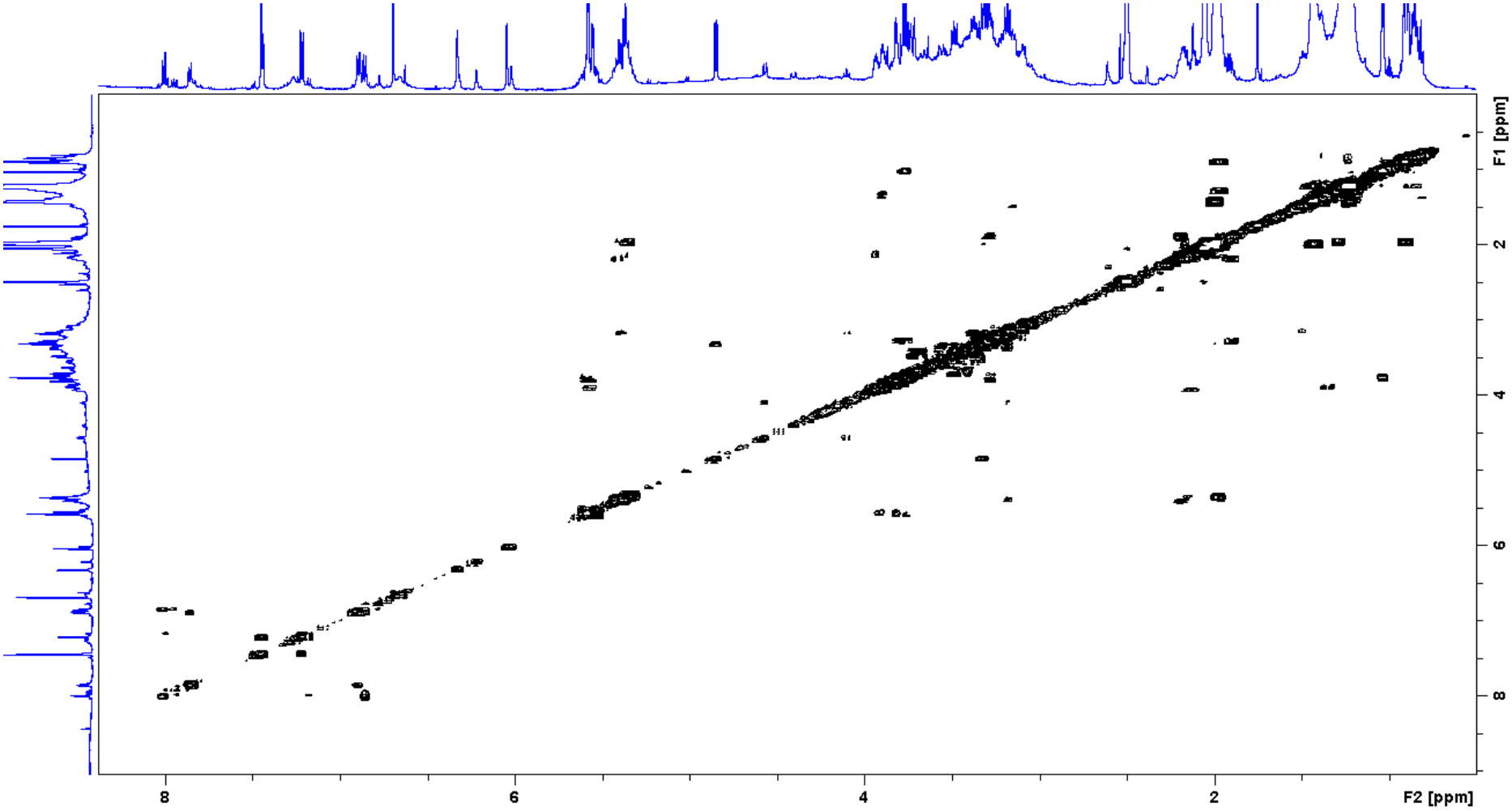


Figure 30 COSY spectrum of compound 2 in DMSO-d_6

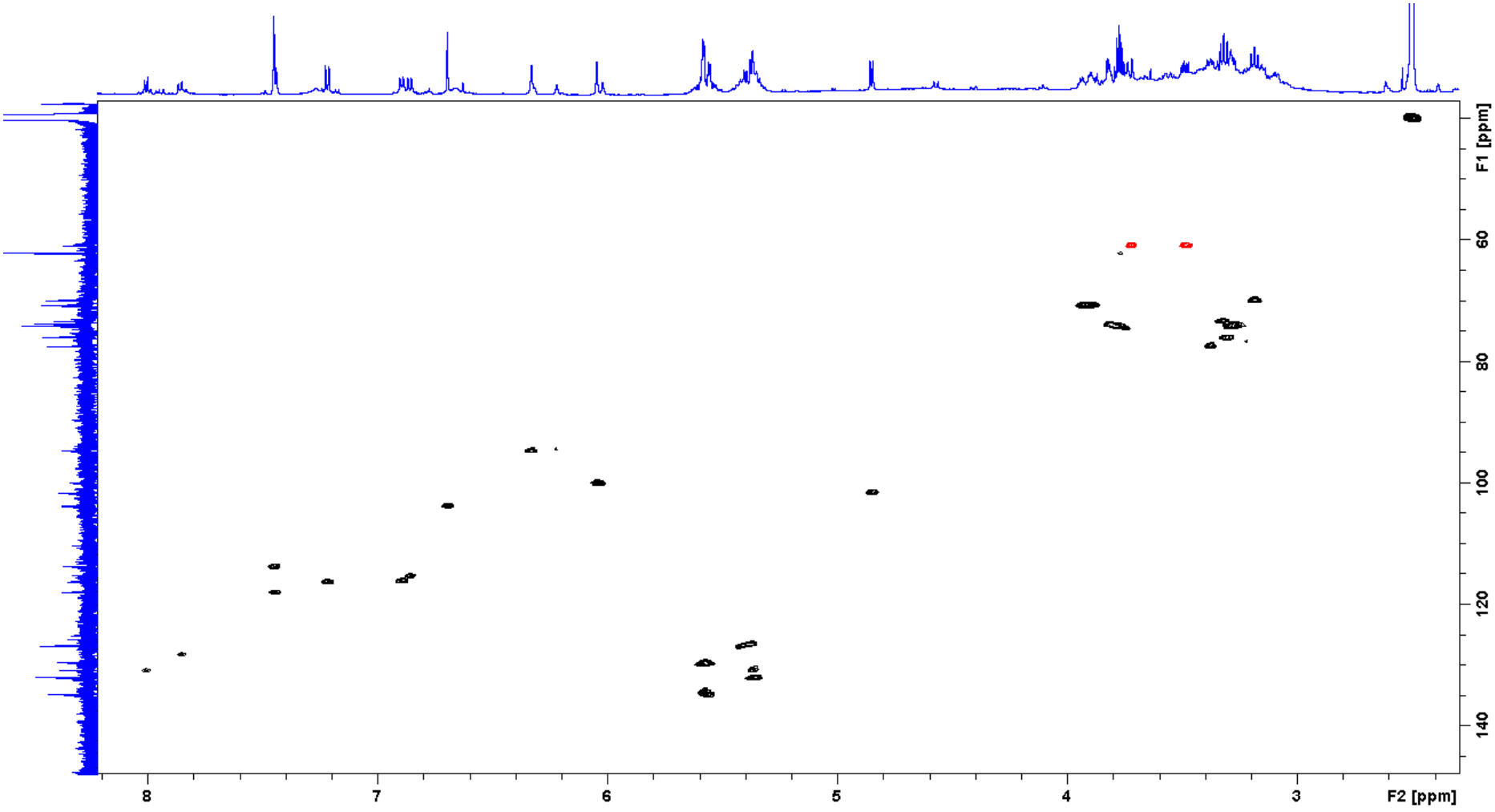


Figure 31 Edited HSQC spectrum of compound 2 in DMSO-d_6

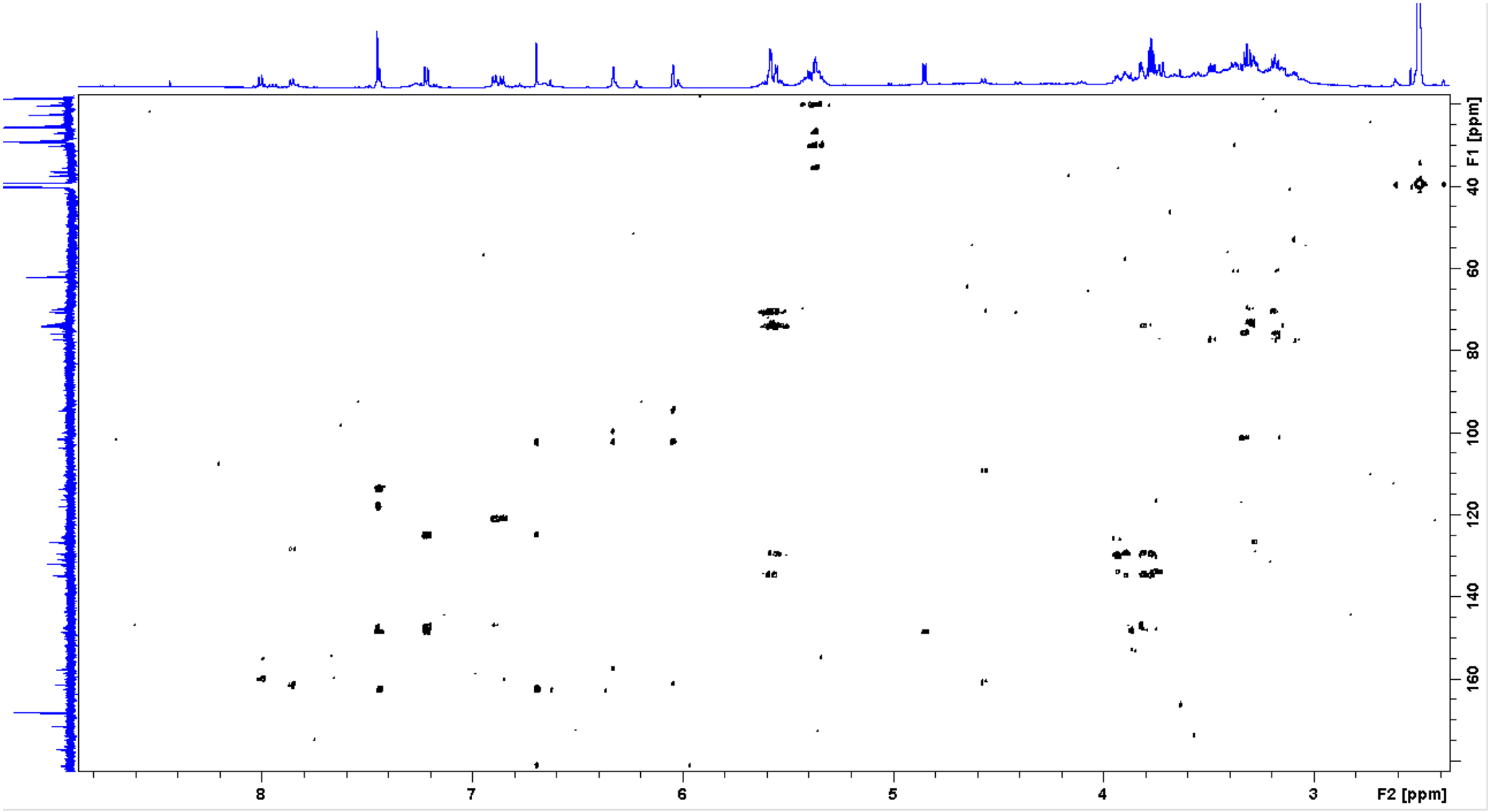


Figure 32 HMBC spectrum of compound 2 in DMSO-d_6

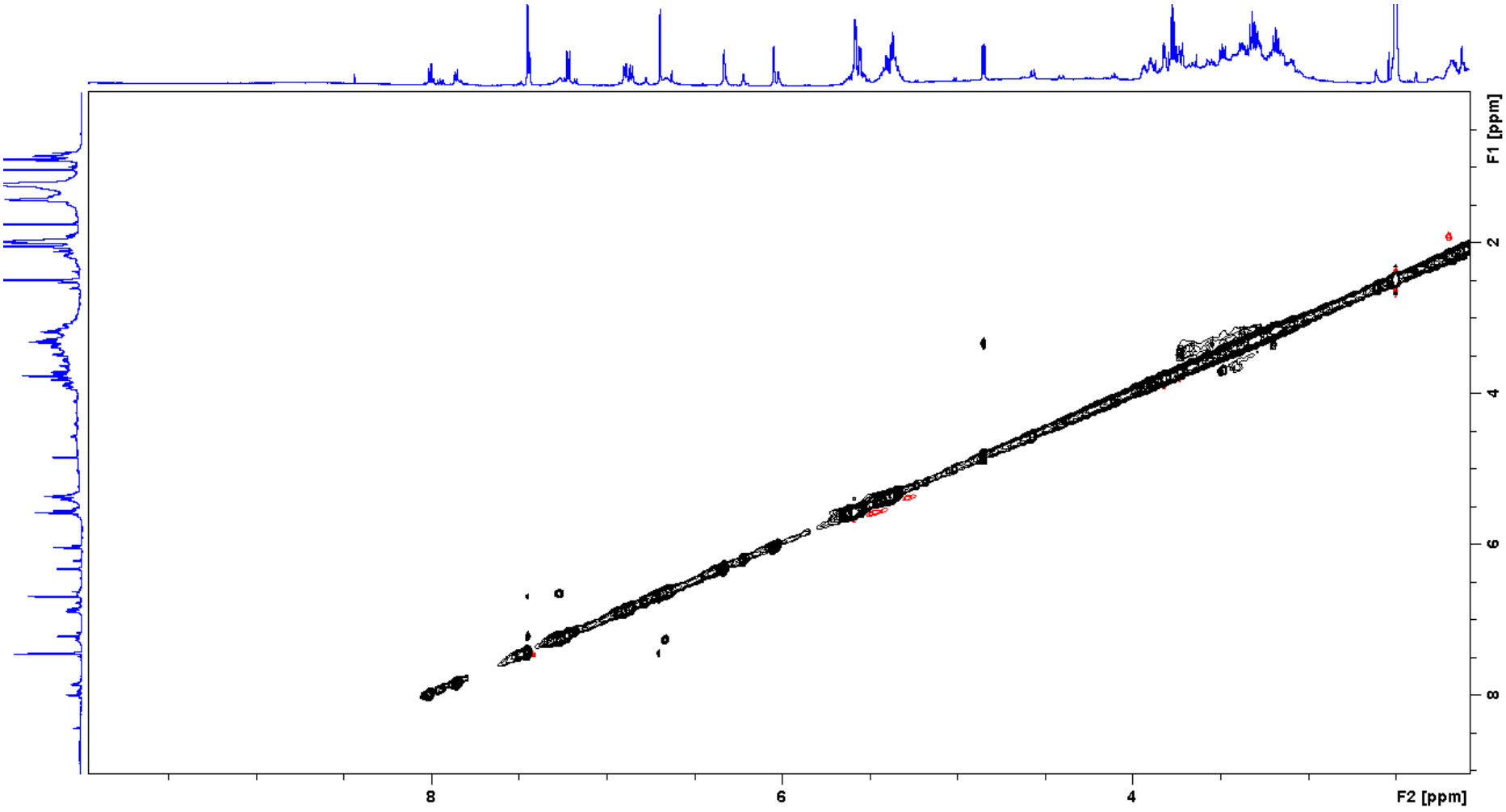


Figure 33 NOESY spectrum of compound 2 in DMSO-d_6

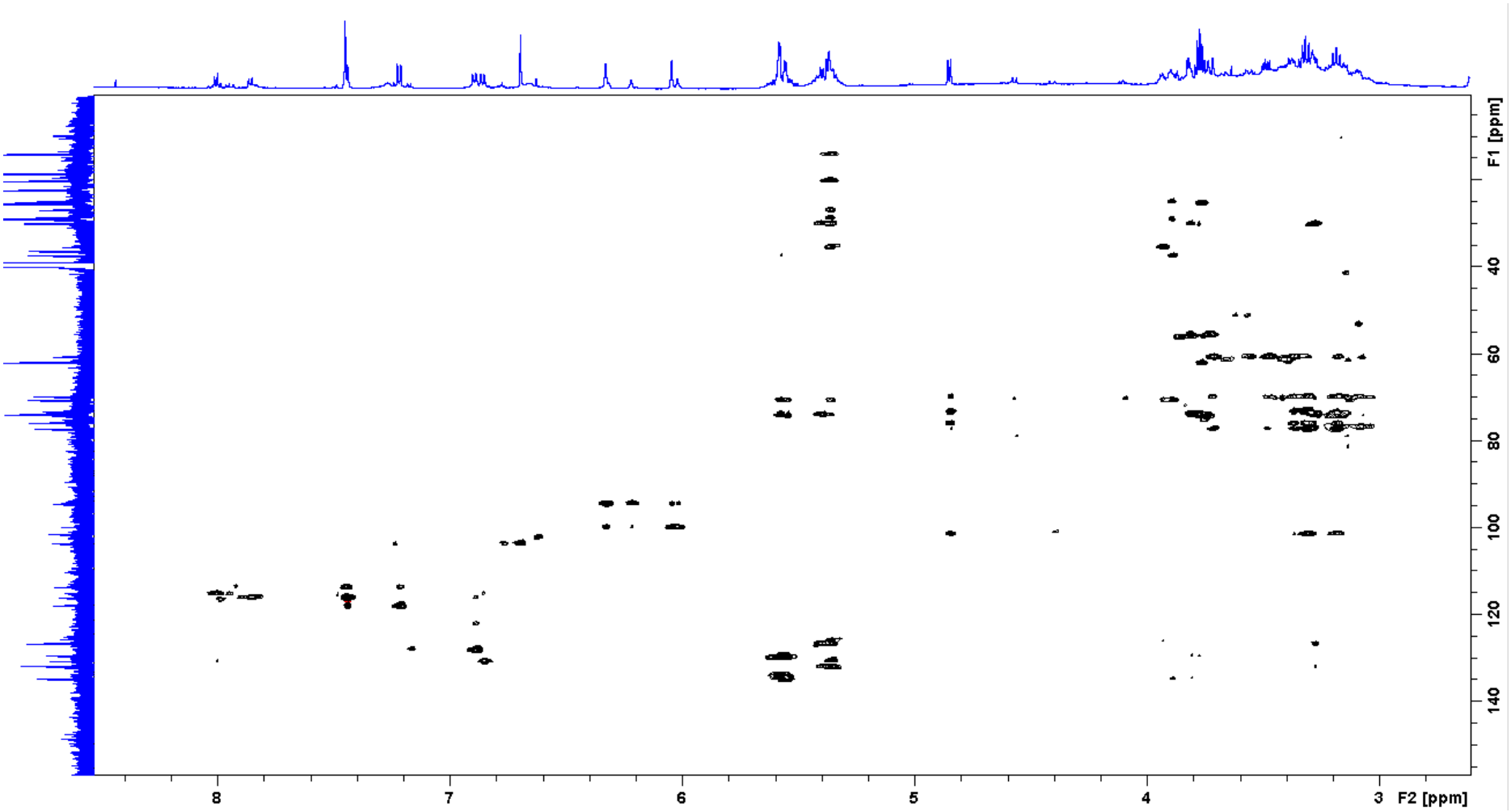


Figure 34 HSQC-TOCSY spectrum of compound 2 in DMSO-d_6

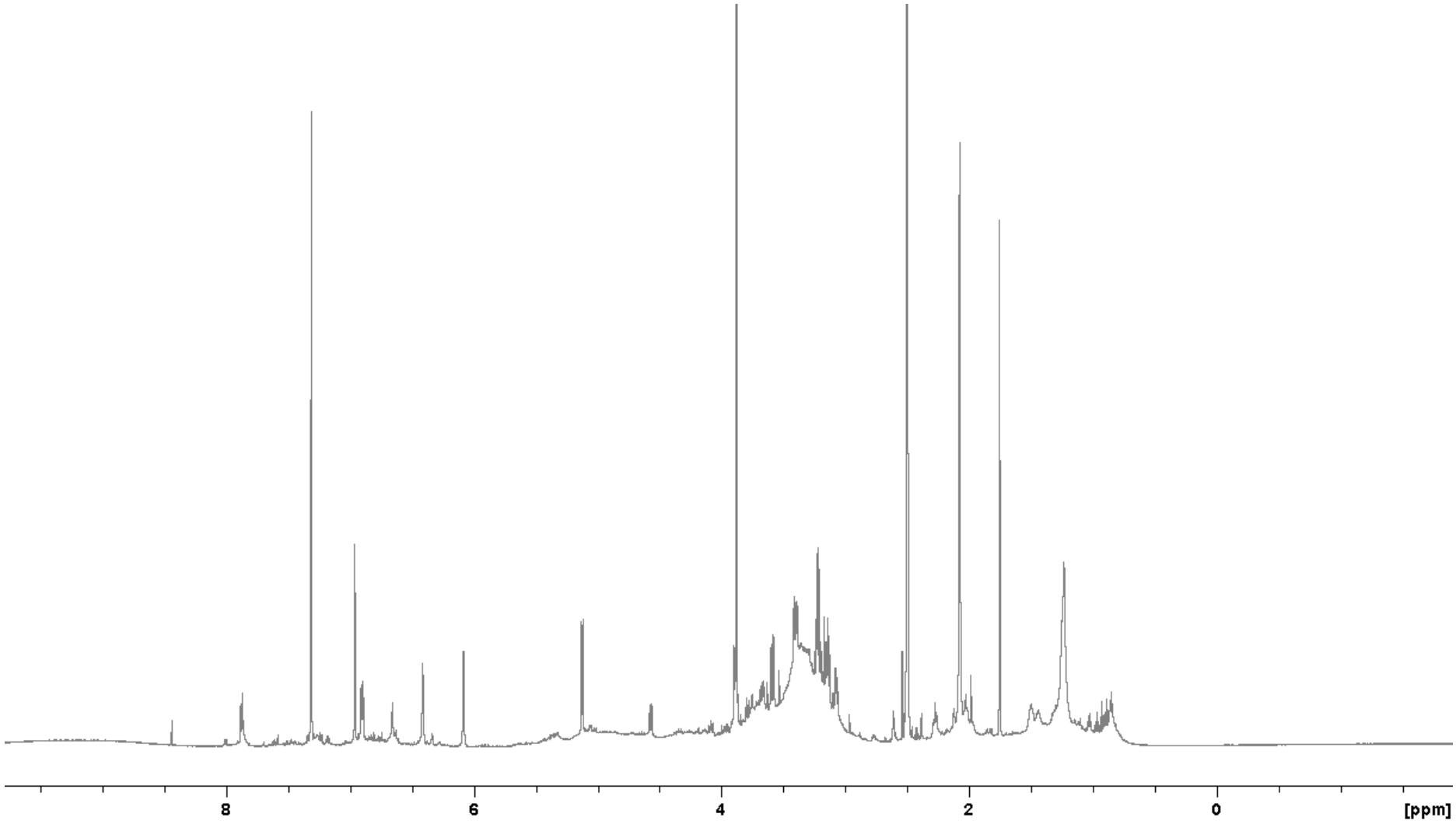


Figure 35 ^{1}H NMR spectrum of compound 3 in DMSO-d_6

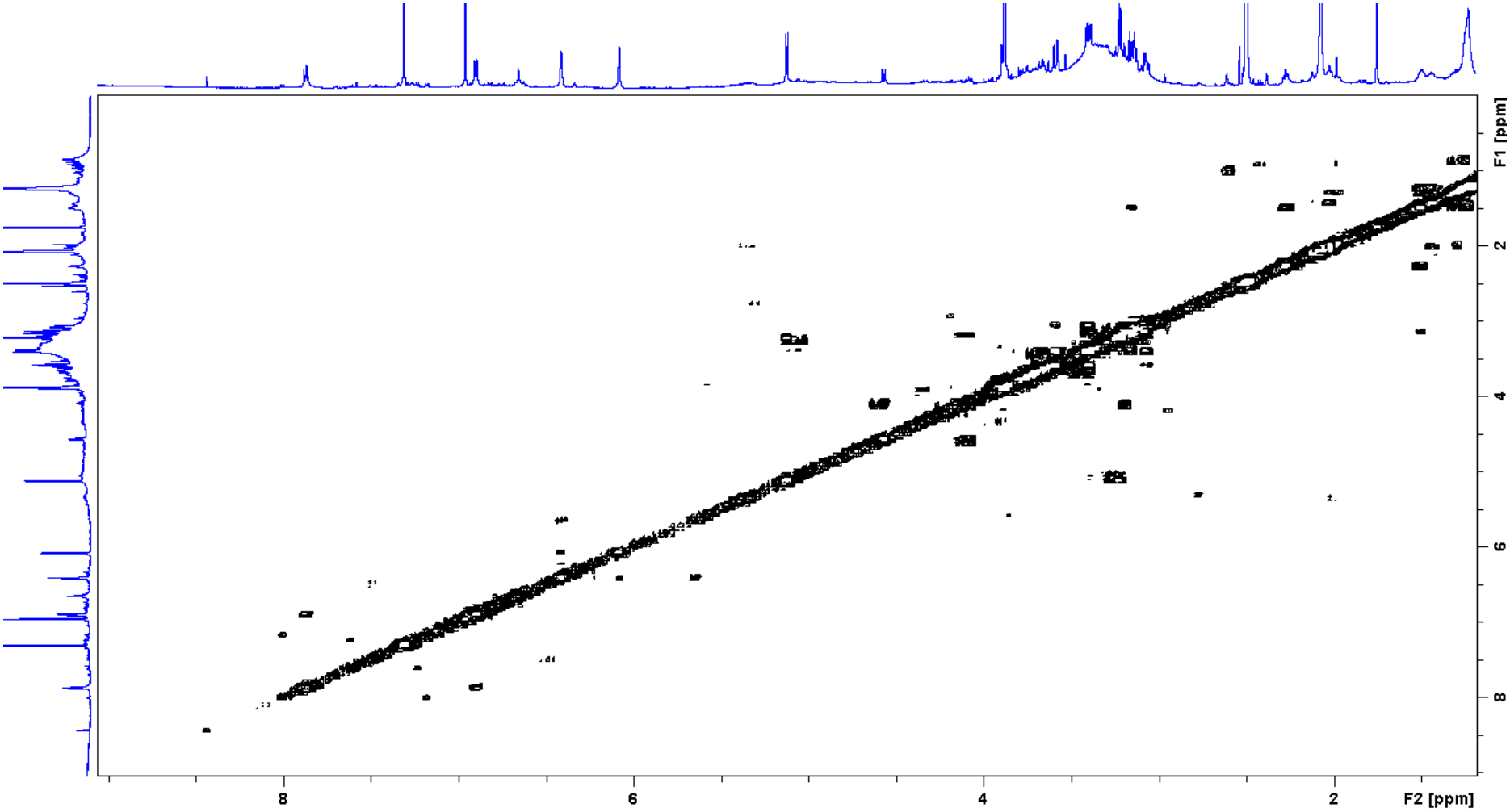


Figure 36 COSY spectrum of compound 3 in DMSO-d_6

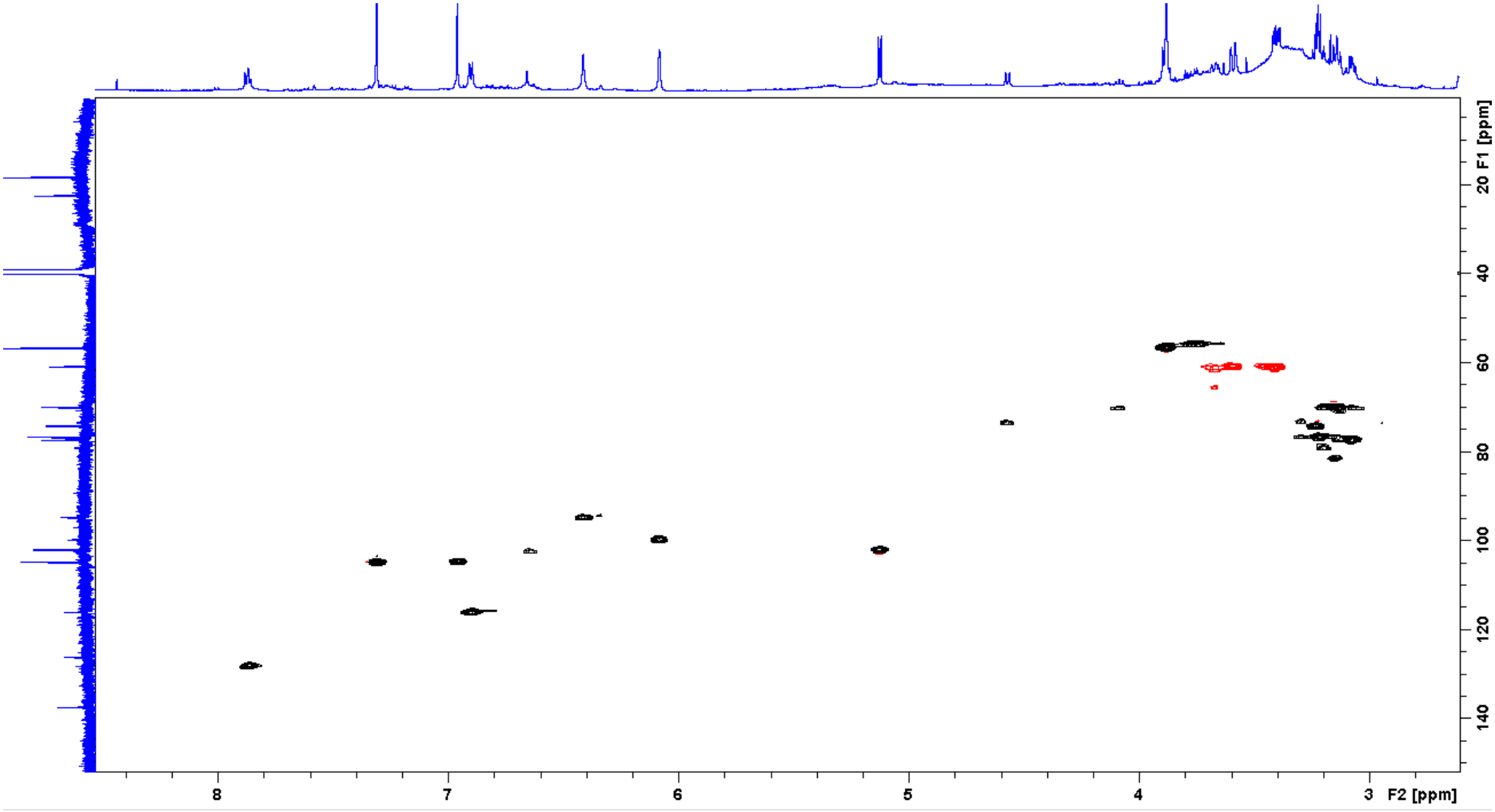


Figure 37 Edited HSQC spectrum of compound 3 in DMSO-d_6

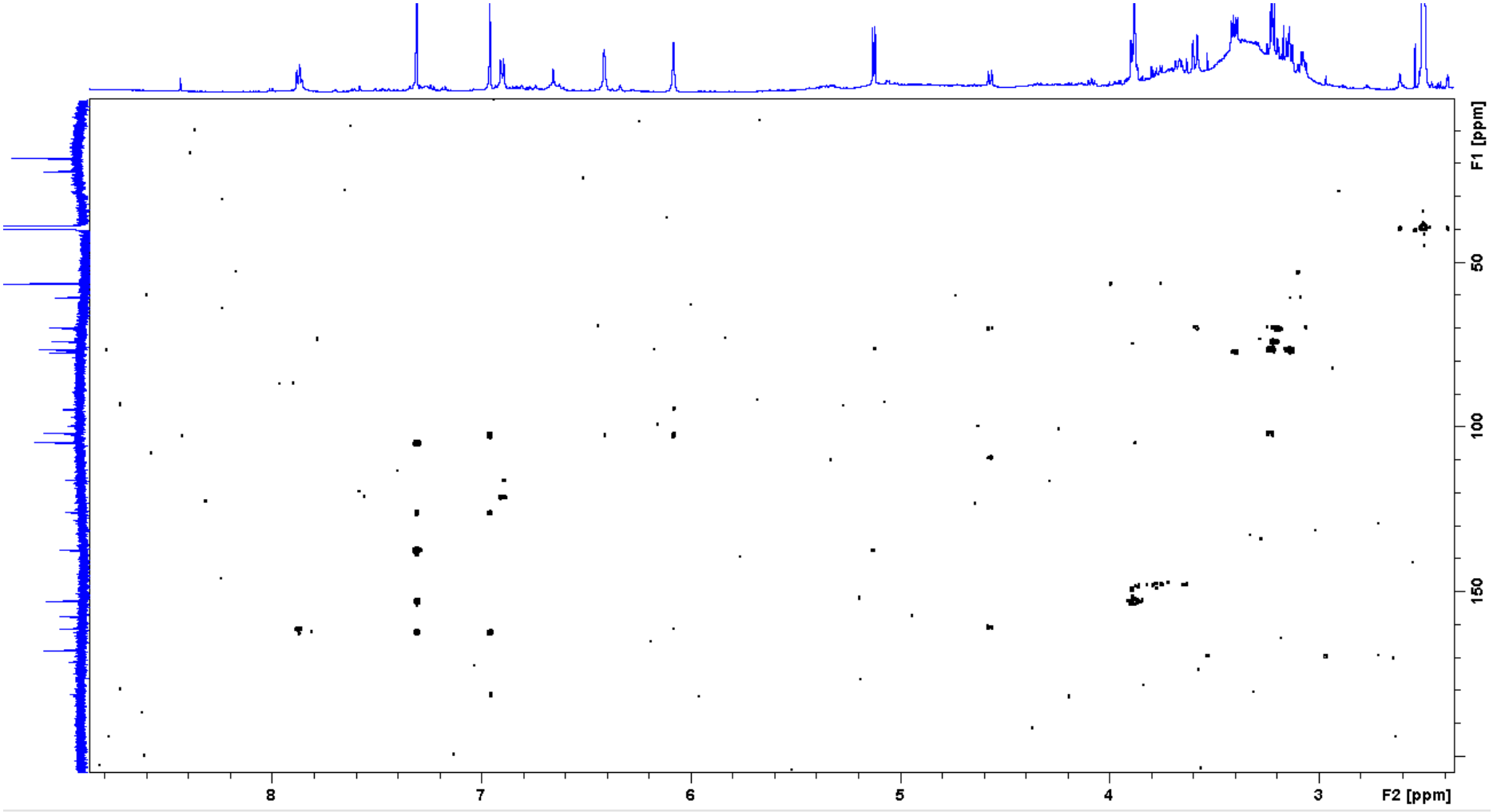


Figure 38 HMBC spectrum of compound 3 in DMSO-d_6

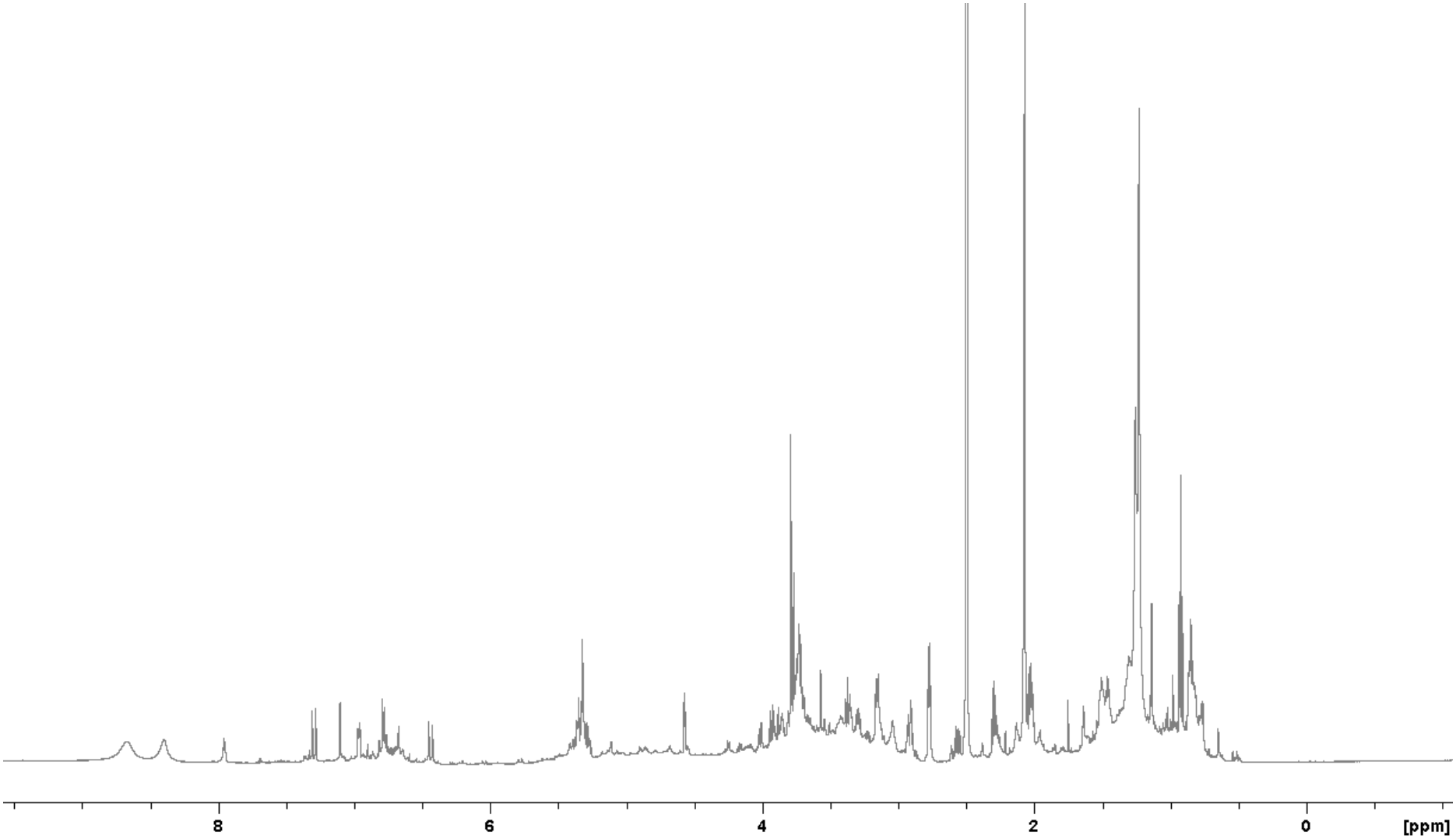


Figure 39 ^{1}H NMR spectrum of compound 4 in DMSO-d_6

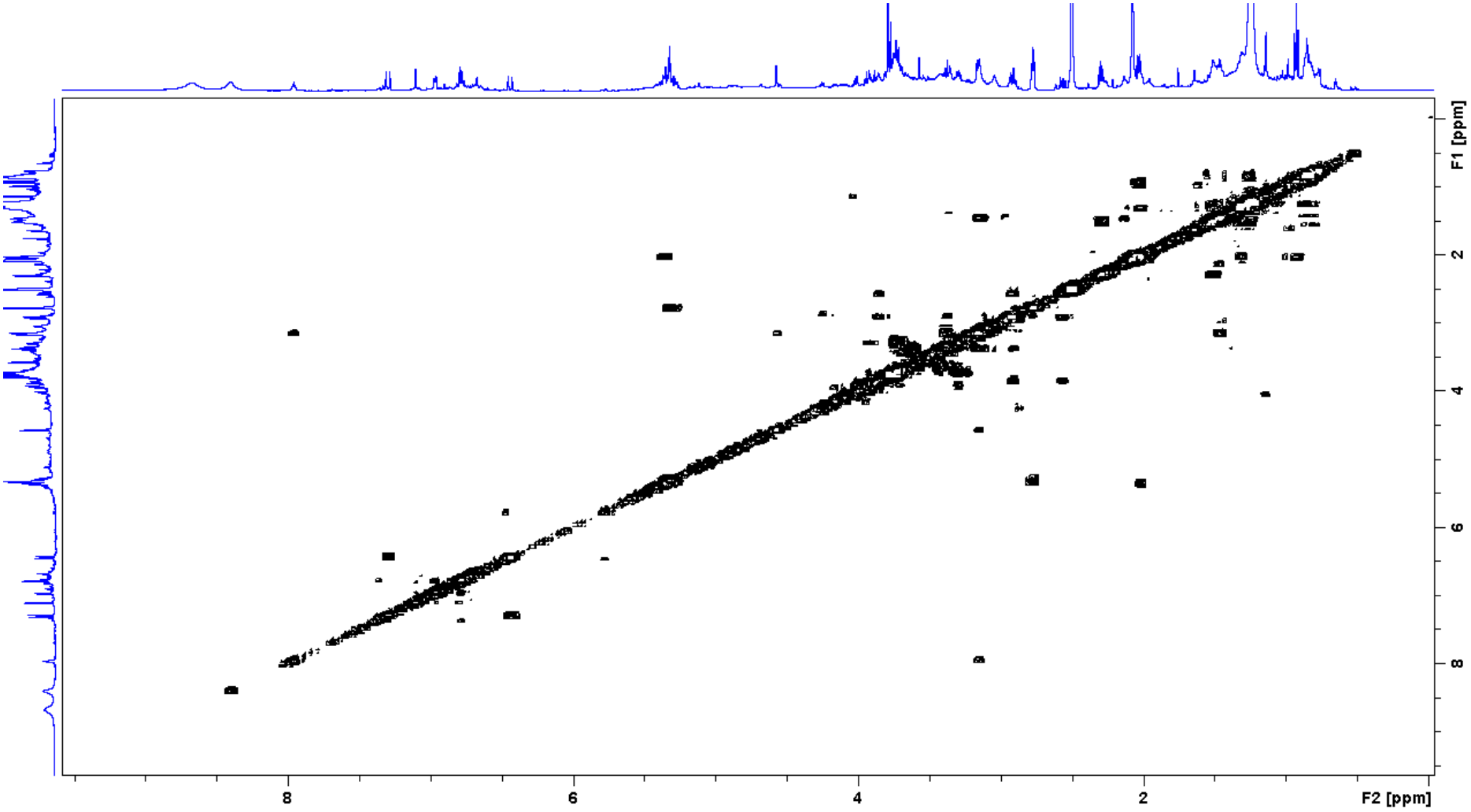


Figure 40 COSY spectrum of compound 4 in DMSO-d_6

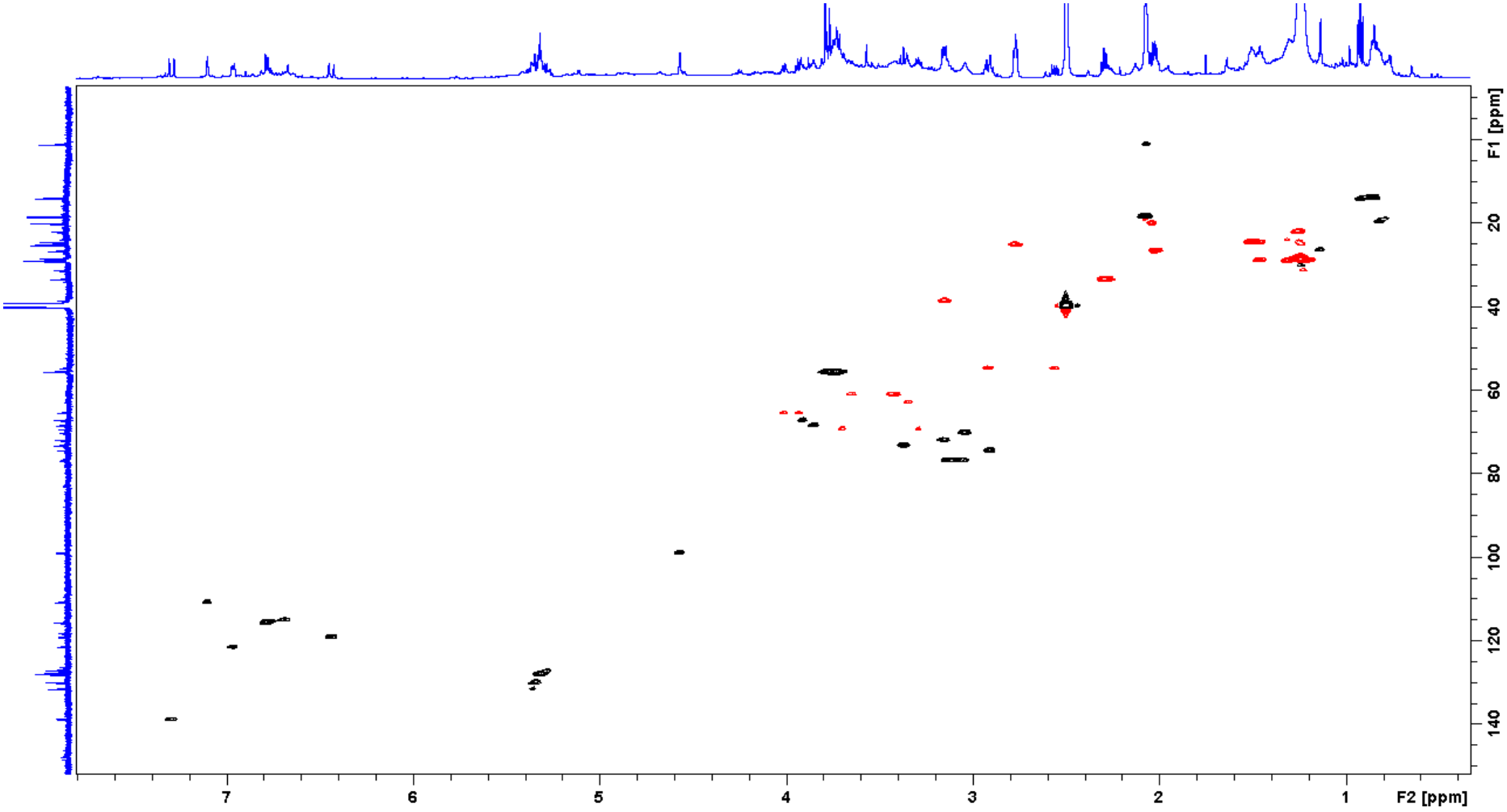


Figure 41 Edited HSQC spectrum of compound 4 in DMSO-d_6

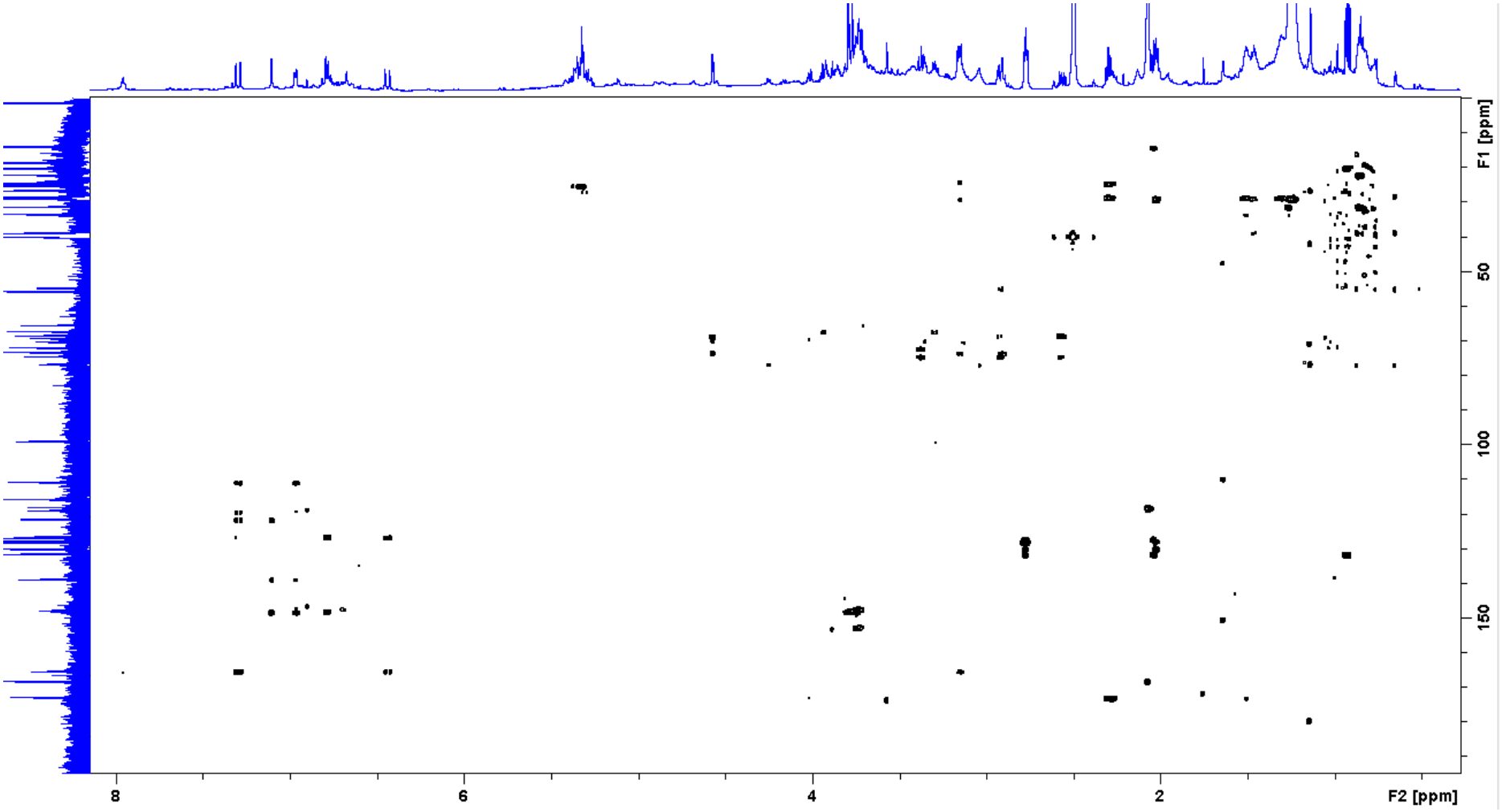


Figure 42 HMBC spectrum of compound 4 in DMSO-d_6

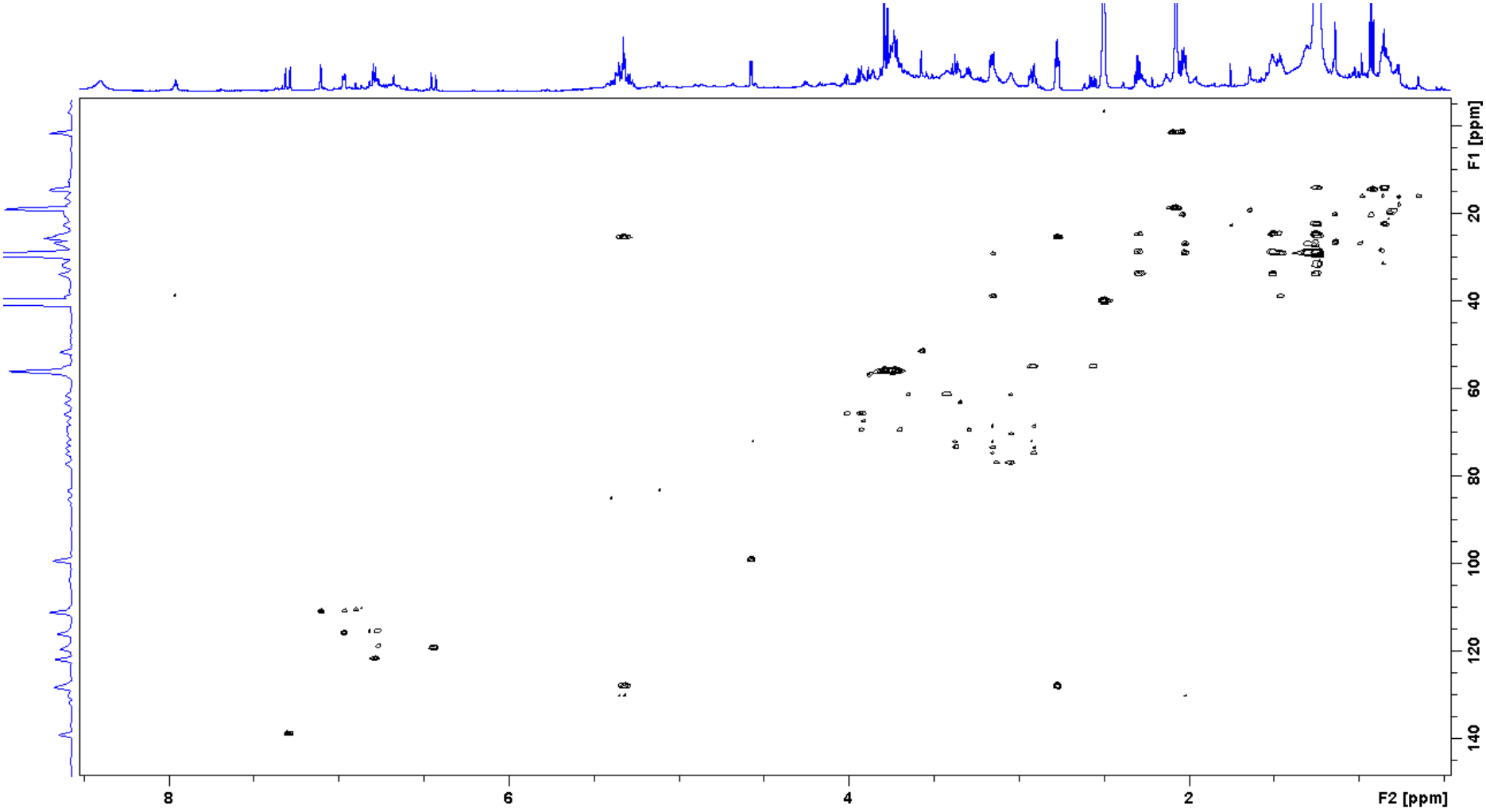


Figure 43 HSQC-TOCSY spectrum of compound 4 in DMSO-d_6

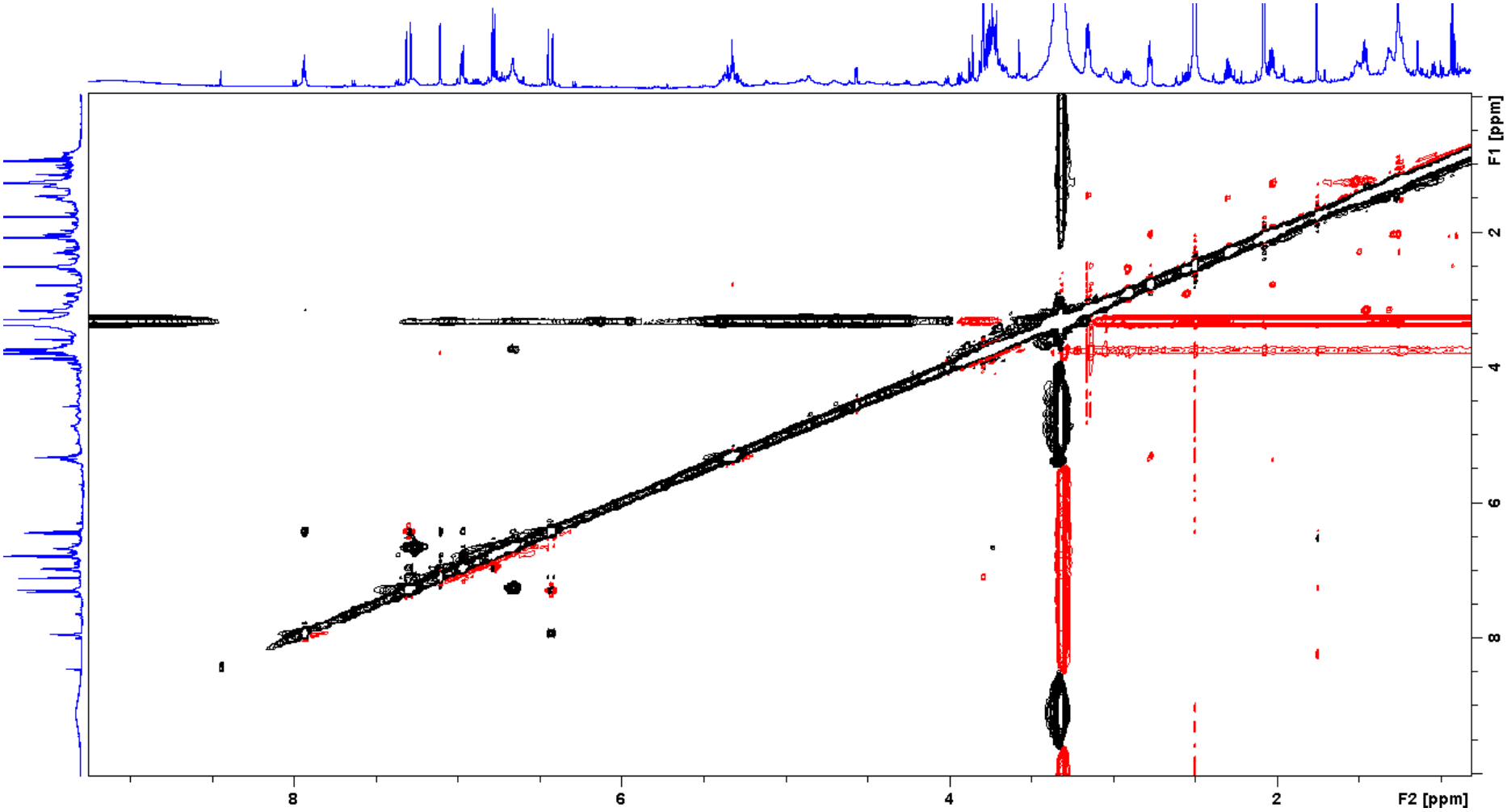


Figure 44 NOESY spectrum of compound 4 in DMSO-d_6