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INVESTIGATIONS OF SECONDARY METABOLITES FROM FUNGI: ISOLATION,
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Abstract

Trichomoniasis, caused by *Trichomonas vaginalis* remains a worldwide threat, afflicting an astonishing 3.9% of the world's population. This parasite is recognized as a cause of vaginitis and can result in serious health consequences including infertility, premature delivery, and neonatal death. Currently nitroimidazoles (metronidazole and tinidazole) has been the treatment of choice for decades. However, nitroimidazole resistance has been detected up to 9.6% of clinical *T. vaginalis* isolates, which makes the spread of drug resistance a realistic concern. *Mycoplasma genitalium* is a sexually transmitted pathogenic bacterium that resides in the genitourinary tract in humans. It has been implicated in urogenital infections of both men and women, which include preterm birth, cervicitis and more importantly recognized as an independent etiologic agent of acute and persistent male NGU (non-gonococcal urethritis). The rapidly increasing resistance to the recommended first and second line antibiotics (macrolides and quinolones) has necessitated the need to explore novel drug targets in this pathogen. Our previous studies based on identifying fungal derived bioactive natural products against *T. vaginalis* led to the discovery of decalin-linked tetramic acid containing metabolites. Interestingly, this series of tetramic acids also exhibits potent activity against *M. genitalium*. An exploratory structure-activity study of phomasetin (**1**) was undertaken to identify the structural features that drive the bioactivity. Further, tetramic acid of **1** and pyrrolocin A (**2**) was derivatized using 'click' chemistry to modify the bioactivity. The semisynthetic analogues of **1** and **2** hold promise to improve treatment options against *T. vaginalis* and *M. genitalium* through the creation of compounds that exhibit improved activity and selectivity.

The other approach to explore novel drug targets in *M. genitalium* includes identifying bioactive natural products from fungi. To address this, we have screened samples from a fungal natural product extract library assembled through the University of Oklahoma Citizen Science Soil Collection Program. Analysis of one of the bioactive extracts using bioassay guided fractionation led to the purification of the compound PF-1140 along with a new and several other known pyridones. The *N*-hydroxy pyridones are generally regarded as siderophores with high binding affinity for iron (III) under physiological conditions. Results from UV-vis absorption spectroscopy-based titration experiments revealed that PF-1140 is capable of undergoing complexation with several metals including Fe^{3+} , Co^{2+} , Ni^{2+} and Cu^{2+} . The results suggest that metal chelation may play an important role in the potent inhibition of *M. genitalium* survival through disruption of metal homeostasis.

Chromosome segregation is an essential cellular process that has a potential to yield numerous targets for drug development. This pathway is presently under-utilized partially due to the difficulties in the development of robust reporter assays suitable for high throughput screening. In bacteria, chromosome segregation is mediated by two partially redundant systems, condensins and ParAB. These two systems are synthetically lethal in *Pseudomonas aeruginosa*, giving rise to a straightforward screening assay based on cell viability. Using this assay, we screened a library of fungal extracts for potential inhibitors of the ParAB pathway as judged by their enhanced activity on condensin-deficient cells. We found such activity in extracts of *Humicola variabilis* and fractionated it to several novel [sterigmatocystin NPDG 1, sterigmatocystin NPDG 2, sterigmatocystin NPDG 3, sterigmatocystin NPDG 4 and sterigmatocystin NPDG 5] and two known analogues including sterigmatocystin (6) and deoxysterigmatocystin (7). The analog sterigmatocystin

NPDG **1** induced defects in chromosome segregation which manifested themselves as increased frequencies of anucleate cells in drug-treated cells and a delay in cell division. Overproduction of Par A and Par B altered the susceptibility of bacteria to sterigmatocystin, indicating that the drug directly interacts with the proteins. Sterigmatocystin analogs varied in their potency revealing the importance of the terminal benzoic ring of the molecule, and the structural basis for this variability was suggested by molecular docking studies. The data describe the first known inhibitor of ParAB and expand the known spectrum of activities of sterigmatocystin to include bacterial chromosome segregation.

Chapter 1: An introduction to fungal natural products

1.1 Natural products in history

Nature has demonstrated the ability to produce a plethora of biologically active compounds, referred to as natural products. Natural products can be isolated from plants, marine organisms, microorganisms, vertebrates and invertebrates.¹ Throughout the ages, humans have relied on natural products as a vital source of active ingredients in therapeutic agents.² The earliest records of natural products dating as far back as 2600 BC, depicted the utilization of oils from *Cupressus sempervirens* and *Commiphora sp.* (myrrh) for medicinal purposes.³ Many civilizations including Egyptians, Greek, Chinese, Tibetans, Indians, Mayan, and Aztecs used plant-based drugs ranging from gargles, pills, infusions to ointments, which are still commonly used today.⁴ In addition to plant sources, fungi were used in Japanese, Korean and in particular Chinese traditional medicine including *Ophiocordyceps sinensis* (caterpillar fungus), *Grifola frondose* (maitake), *Calvatia craniiformis* (brain puffball) and *Ganoderma lucidum* (Lingzhi mushroom).⁵ These terrestrial fungi have been long used to treat a variety of ailments including coughs, colds, parasitic infections, and health-promoting benefits including detoxification, improving immunity and, anti-inflammatory.⁶

Moreover, the early Greek records (~ 300 BC) reported the natural scientists and physicians (-Theophrastus and Dioscorides) who dealt with collection and storage of medicinal herbs.^{7,8} During the dark and middle ages, the monasteries in England, Ireland, France and Germany preserved this western knowledge about medicinal values and resources.⁸ In the meantime, Arabs expanded the uses of their own resources while preserving the Greco-

Roman knowledge and combining with Chinese, Indian herbs unfamiliar to the Greco-Roman world.⁹ It was the Arabs who were the first to privately own pharmacies in 8th century.⁷ Further, the Persian pharmacist, physician and philosopher, Avicenna remarkably contributed much to the sciences of pharmacy and medicine through his work such as *Canon medicinae*.¹⁰

1.2 Natural products from plants and marine origin

The birth of the scientific field of pharmacognosy began with the isolation of plant-derived metabolite morphine from *Papaver somniferum* by Friedrich Wilhelm Adam Sertürner in 1816.¹¹ This discovery was followed by the isolation of other secondary metabolites including strychnine in 1818,¹² colchicine and quinine in 1820,^{13, 14} and salicin in 1828 (Figure 1.1)¹⁵ These compounds came to be known as natural products or secondary metabolites that are considered to provide specialized survival benefits to the producing organism through a variety of mechanisms including self-defense against competing organisms, metal transporting agents, inter-cellular communication and other interactions with organisms in their environment.^{16 17} Plant derived bioactive metabolites continue to provide novel and important leads against various pharmacological targets including cancer, Alzheimer's, HIV/AIDS and malaria.

Some example of modern pharmaceuticals derived from nature include most widely used breast cancer drug, paclitaxel, which was isolated from the bark of *Taxus brevifolia* (Pacific Yew).¹⁸ Another example includes the discovery of vinca alkaloids (vinblastine, vincristine, vinorelbine) from *Catharanthus roseus* with activity to inhibit malignant angiogenesis by disrupting microtubule dynamics.¹⁹ Other examples of antitumor

compounds currently in clinical trials include ingenol 3-O-angelate, a derivative of the polyhydroxy diterpenoid ingenol isolated from the sap of *Euphorbia peplus*.²⁰ Combretastain A-4 phosphate, a stilbene derivative from *Combretum caffrum* acts as anti-angiogenic agent causing vascular shutdowns in tumors and is currently in phase II clinical trials.² The class of compounds known as calanolides isolated from *Calophyllum sp.* were reported to be active against HIV-1.²¹ Calanolide A, calanolide B along with prostratin from *Homalanthus nutans*, have now progressed into clinical and preclinical development in the drug discovery pipeline.^{21 22} Artemisinin and its derivative artemotil were first isolated from the plant *Artemisia annua* and are both approved as antimalarial drugs.²³ Further, other derivatives of artemisinin are in various stages of clinical development as antimalarial drugs.²⁴ (Figure 1.2)

Though plants have proven to be an excellent source for bioactive natural products, the marine environment has a clear track record in offering novel structural entities.²⁵ The harsh marine conditions favored the production of a variety of compounds bearing unique structural features which contributed to the development of present drugs.²⁵ Some examples include ziconotide, a peptide discovered from a cone snail and approved in 2004 for the treatment of pain.²⁶ Plitidepsin was isolated from Mediterranean tunicate *Aplidium albicans* and is effective in treating various cancers, including melanoma, bladder, non-hodgkin lymphoma and acute lymphostatic leukemia and is currently in clinical trials.²⁷ Trabectedin was isolated from acidian *Ecteinascidin turbinata* and became the first marine anticancer drug to be approved.²⁷ Halichondrin B was isolated from marine sponge *Halichondria okadai* and its derivative eribulin was approved for the treatment of breast cancer.²⁸ (Figure 1.3)

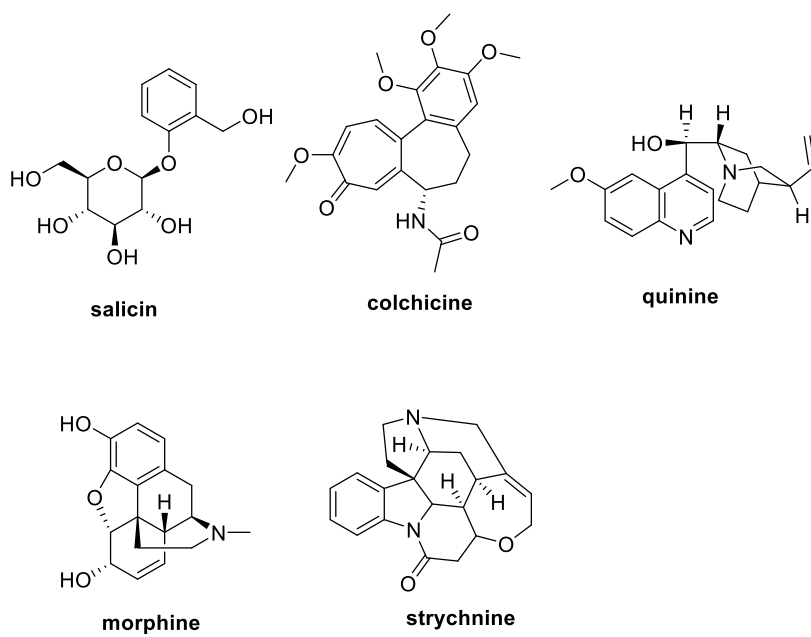


Figure 1.1. Natural products isolated from plants

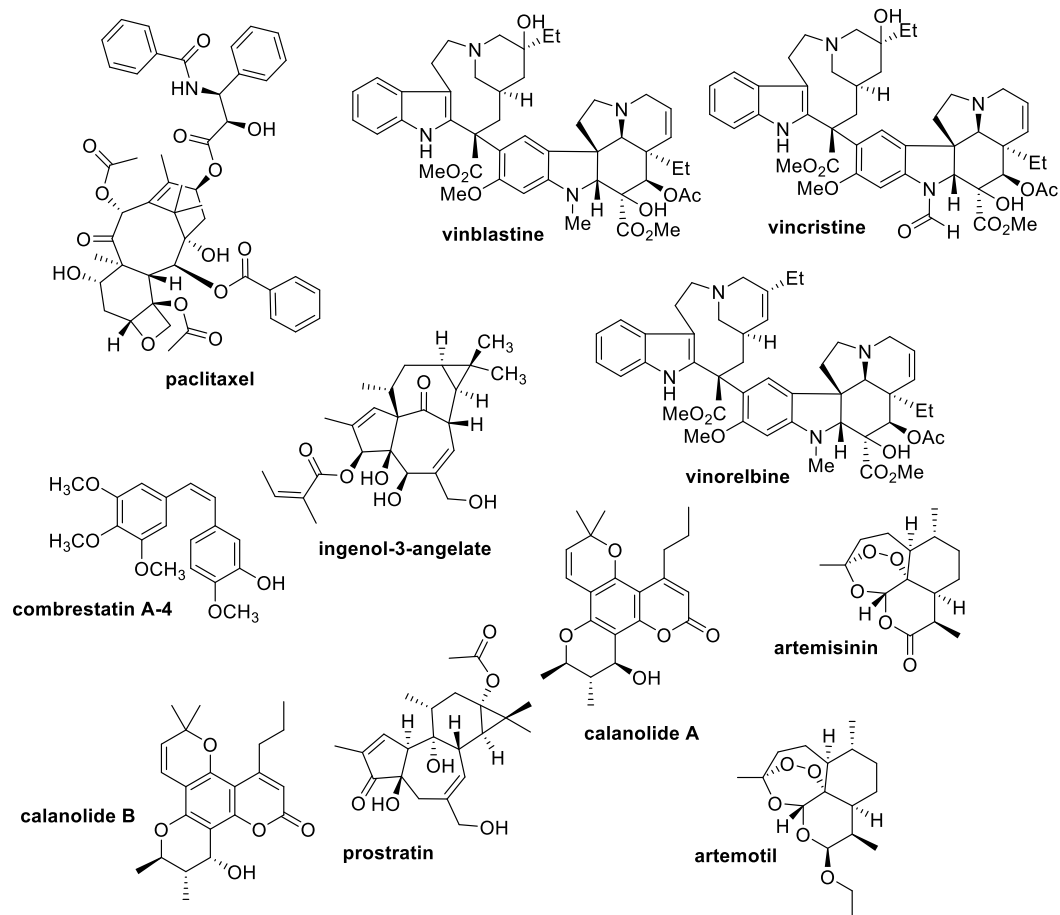


Figure 1.2. Examples of natural products with therapeutic values isolated from plants

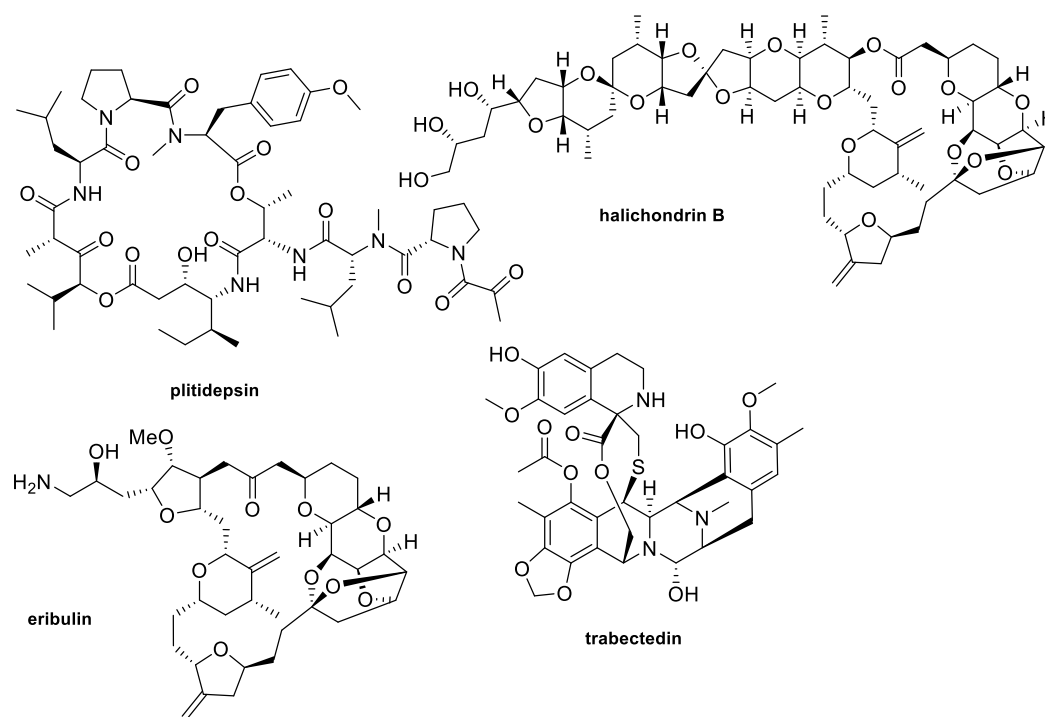


Figure 1.3. Examples of marine derived natural products with therapeutic values

1.3 Natural products in modern pharmaceuticals

Natural products continue to produce a significant portion of clinical candidates and medicinal compounds in modern times. Natural products, natural product derivatives, natural product mimics, or synthetic compounds with natural product derived pharmacophores have made up to 50% of the drugs approved in the United States between 1981 to 2014.²⁹ Further, it is estimated that natural products and natural product-inspired compounds are approximately 10-50% of small molecule drugs approved each year.²⁹ Although natural products play a vital role in drug discovery, many pharmaceutical companies have either substantially reduced or terminated their natural product research and focused on developing synthesis-driven drug discovery programs. The reasons behind this shift are the development of advanced techniques such as high throughput screening

(HTS), combinatorial chemistry and metagenomics.³⁰ Further, due to the structural complexity, not all natural products can be completely synthesized as the process would be inefficient and expensive.³¹ Despite this shift in focus, new and successful pharmaceutical and biotechnology industries are once again turning to natural products to harness new useful entities with the help of advanced drug discovery and medicinal chemistry knowledge. These efforts lead to the discovery of 58% of antibacterial and 32% of new anticancer chemical entities between 1981-2014. The well-known examples include anticancer drugs such as paclitaxel, vinblastine, topotecan and antifungal drugs such as amphotericin B.²⁹

1.4 Fungi as a source of natural products

The fungal kingdom is a well-known and prolific source of potential therapeutic compounds. According to the report in 2002, the microbes were responsible for the production of approximately 22,500 bioactive secondary metabolites with an account of 38% are from fungal natural products derived secondary metabolites.³² This provides evidence for the significant contribution offered by fungi in the discovery of bioactive secondary metabolites.

The serendipitous discovery of penicillin from *Penicillium notatum* by Alexander Fleming in 1929 was the beginning of fungal natural products which helped to save millions of lives over the years.³³ Since then, there was a worldwide effort to discover antibiotics from fungi. These attempts lead to the discovery of other classes of β -lactam antibiotics such as cephalosporins, carbapenems, monobactams and norcardicins.³⁴ Among these antibiotics, penicillins (e.g., ampicillin, amoxicillin), and cephalosporins (e.g., cefuroxime, cefalotin)

dominated 65% of the world antibiotic market.³⁵ In addition to the outstanding discovery of antibiotics, another impressive fungal natural products includes cyclosporine, an immunosuppressant used in the treatment of rheumatoid arthritis, and to prevent rejection in organ transplant.³⁶ Further, fungal natural products gained fame for their use as antifungal agents such as echinocandins³⁷ and griseofulvin³⁸. Another interesting fungal derived compound class is known as statins, (e.g., lovastatin) which are used to treat cardiovascular diseases.³⁹ (Figure 1.4)

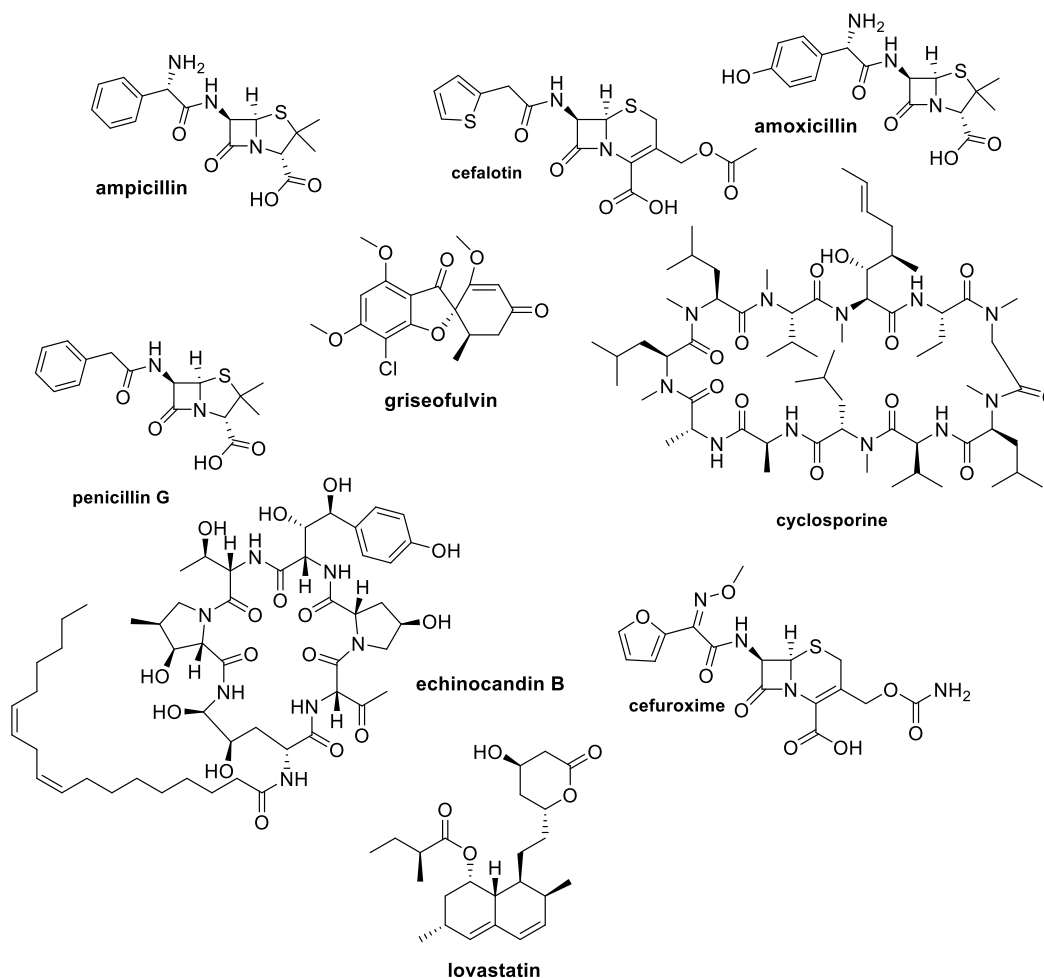


Figure 1.4. Examples of fungal derived natural products with therapeutic values

Presently, there seems to be a limited number of anti-viral natural products derived from fungi. Ganoderic acid β , isolated from the fruiting bodies of *Ganoderma lucidum*, displayed significant anti-HIV-1 protease activity with an IC_{50} of 20 μM .⁴⁰ Although to date no fungal natural products have been clinically approved as anticancer therapeutics, plenty of compounds have been reported with anti-cancer properties. For example, ophiobolin A is a potent inducer of glioblastoma cell death by induction of paraptosis.⁴¹ Fusarisetin A isolated from *Fusarium sp.* is a potent cancer migration inhibitor for various types of cancers.⁴² The indole fungal metabolite tryprostatin A isolated from *Aspergillus fumigatus*, is an inhibitor of mitogen activated protein kinase dependent microtubule assembly at the mitotic phase.⁴³ Fungal natural products have also been investigated for anti-malarial activity (e.g., pycnidione, codinaeopsin)^{44,45} and anti-tuberculosis activity. (e.g., plectasin)⁴⁶ (Figure 1.5)

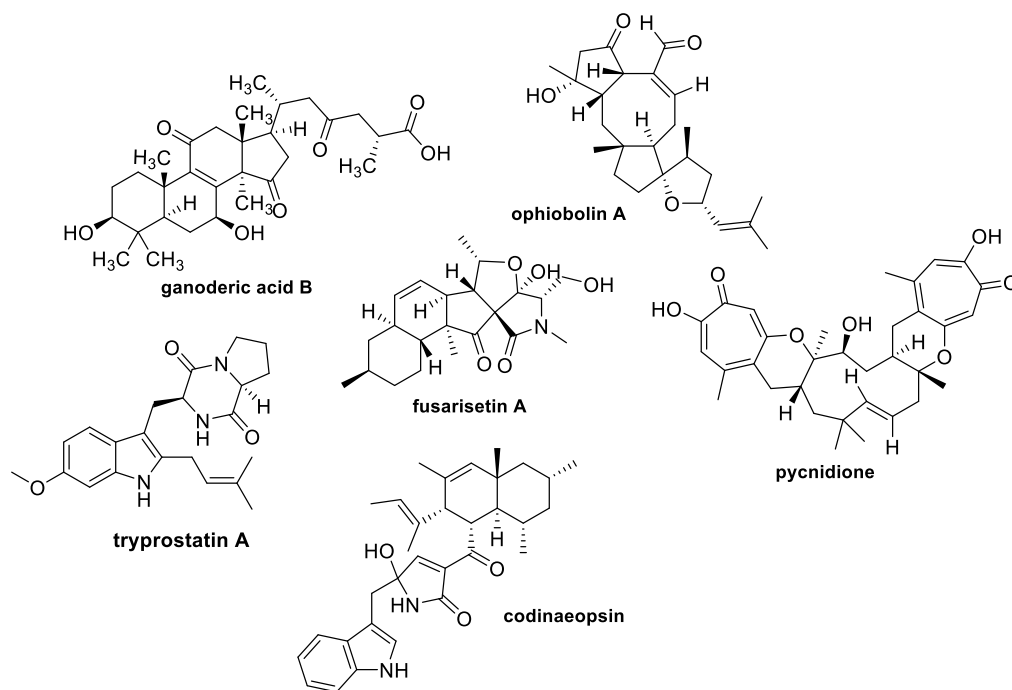


Figure 1.5. Examples of bioactive natural products isolated from fungi

Apart from the chemically and functionally diverse fungal natural products that have been discovered so far, the extent of fungal diversity on earth is even more remarkable. The fungal kingdom is comprised of an estimation of 2.2-3.8 million species on earth.⁴⁷ However, only 5% have been identified so far leaving a wide field of untapped potential of novel metabolites producers.⁴⁸ Further, recent advances in synthetic biology and DNA sequencing have expanded the possibilities by providing new opportunities in the field of fungal natural products. These include manipulating the natural products regulatory gene clusters, re-engineering of the biosynthetic enzymes and heterologous expression of gene clusters.⁴⁹ As the need for new drugs and drug leads is still growing with emerging and existing diseases, fungal natural product-based drug discovery will continue to be a great source for finding novel chemical entities. By leveraging the medicinal chemistry knowledge, biotechnology, molecular biology tools combined with large fungal diversity, fungal natural products will continue to play a vital role in natural product discovery for many years to come.

1.5 Fungal derived hydroxamic acids and metal binding

Metals are fundamental elements of all forms of life. They are essential for the maintenance of life and the absence can cause growth disorders, malfunction, and, death.⁵⁰ Metals are required in macro and microelements in several structural and functional roles. For example, metal cofactors serve structural and catalytic roles in biological processes, including DNA replication, transcription, precursor biosynthesis, and responses to oxidative stress.⁵¹ Most organisms require the first-row transition metals manganese, iron, cobalt, nickel, copper, and zinc.⁵²

Iron is the most abundant element on earth and contributes to 80% of the earth's core.⁵⁰ Iron is the most widely used transition metal in biological systems. In bacterial pathogens, iron is required for pentose phosphate pathway, cofactor biosynthesis and DNA replication and transcription.⁵³ Humans require iron for synthesis of its oxygen transport proteins including hemoglobin, myoglobin and for the formation of heme enzymes and iron containing enzymes.⁵⁴

Siderophores are low molecular weight, exceptionally high iron-binding affinity molecules secreted by microorganisms (bacteria and fungi) to overcome iron limitation in the host environment.⁵⁵ Siderophores are mostly hexadentate, in which the binding units vary between hydroxamates, catecholates, α -hydroxy carboxylates and a mixture of these functional groups.⁵⁶ The hydroxamate siderophores are produced mainly by fungi and some strains of bacteria.⁵⁵

More than 135 hydroxamate metal chelators have been isolated and reported in recent years and most of them are peptides with a variety of structural modifications. The first known chelator ferrichrome was isolated from smut fungus *Ustilago sphaerogana* in 1952.⁵⁷ Some of the small molecule hydroxamic acid examples include; pyridoxatin, fusaricide, cordypyridones A and B, leporin B, PF-1140 and akanthomycin. (Figure 1.6) Fusaricide was isolated from *Fusarium sp.* and was found active against *Candida albicans* (MIC = 16 $\mu\text{g/mL}^{-1}$) and *Penicillium chrysogenum* (MIC = 8 $\mu\text{g/mL}^{-1}$).⁵⁸ Further, fusaricide was found to actively decrease the cellular labile iron pool in human non-small cell lung cancer (NSCLC) NCI-H460 cells and induce apoptosis by exhibiting potential anticancer effects.⁵⁹ Leporin B was isolated from *Aspergillus leporis* and found to be an inducer of the hexokinase II gene (HKII) with an IC_{50} of 10 μM .⁶⁰ Cordypyridone A and B were

isolated from entomopathogenic fungal strain *Cordyceps nipponica* and the potent antimalarial activity against *Plasmodium falciparum* was reported as $IC_{50} = 0.066 \mu\text{g/mL}^{-1}$ for cordypyridone A and $IC_{50} = 0.037 \mu\text{g/mL}^{-1}$ for cordypyridone B.⁶¹ The entomopathogenic fungus *Akanthomyces gracilis* was found to produce akanthomycin which inhibited the growth of *Staphylococcus aureus* at 250 ng.⁶² Further, the tris-chelate iron complex of pyridoxatin was isolated from mycelium of the fungus *Tolypocladium terricola* and was found to be cytotoxic (IC_{50} 1.24 – 1.83 μM) to human cancer cells by induction of G_0/G_1 cell cycle arrest and apoptosis.⁶³

The hydroxamic acid metal chelators are undoubtedly an attractive option for therapeutic applications due to their high chelating efficacy and metal sequestering capacity under physiological conditions.

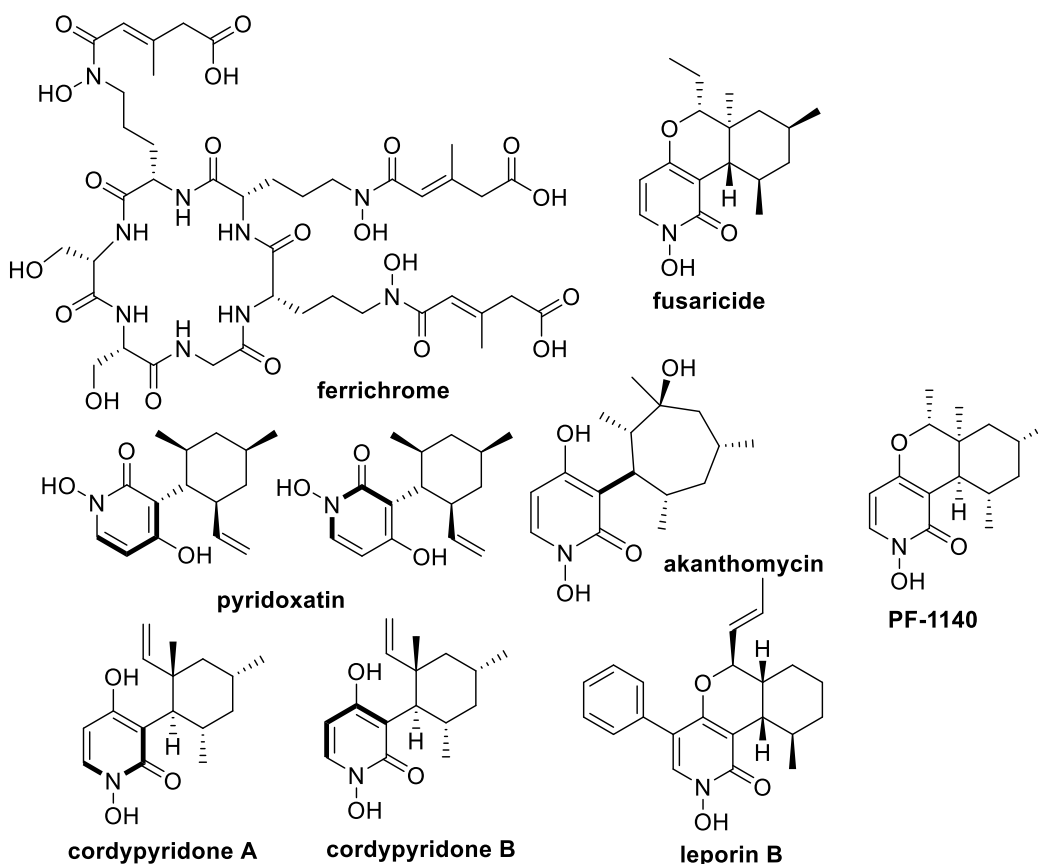


Figure 1.6. Examples of 4-hydroxy pyridones isolated from fungi

1.6 Derivatization of natural products

Natural products play a vital role in identifying lead compounds for drug development often with great potency when tested against specific targets.⁶⁴ Derivatization of natural product drug leads has long been used to generate compounds with improved pharmacological properties. These properties include solubility, bioavailability, stability, exposure, and metabolism.⁶⁵ For example, β -lactam antibiotics derivatized by semi-synthesis have a broader spectrum of activities (e.g., amoxicillin) and greater resistance to penicillinase (e.g., methicillin).⁶⁶ (Figure 1.7) Further, the chemotherapeutic drug paclitaxel and semi-synthetic analogues (e.g., docetaxel, cabazitaxel) (Figure 1.7) are

clinically approved for the treatment of additional cancers including breast, non-small cell lung, ovarian and head and neck cancers.⁶⁷ Similarly, the fungal polyketide natural product lovastatin is semi-synthetically derivatized to obtain more potent simvastatin as a cholesterol-lowering statin drug.⁶⁸

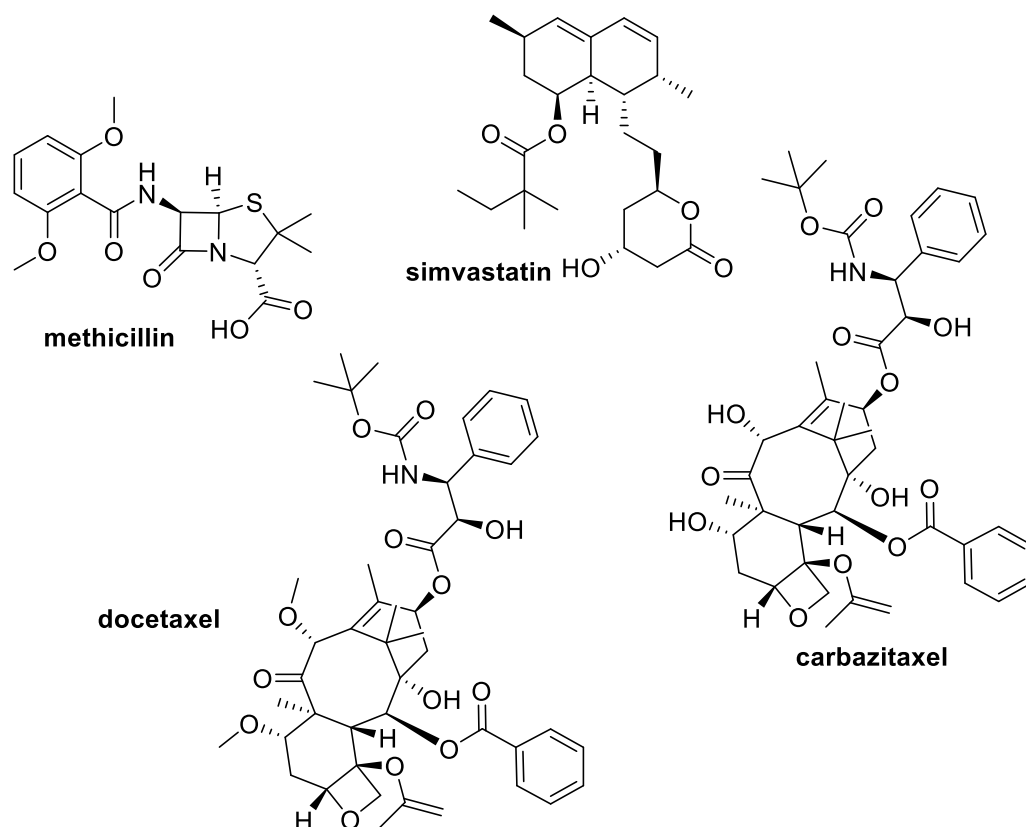


Figure 1.7. Semi-synthetically modified drug candidates

Semi-synthetic drug candidates are generally produced by chemically transforming the starting materials obtained from natural sources to final products. However, due to structural complexity of many natural products even relatively simple chemical modifications are challenging.⁶⁵ Total synthesis of many important natural products has been achieved and this can lead to a wide range of analogues with structural diversity.⁶⁹ The chemical modifications advance the understanding of structure-activity relationships

(SAR) and pharmacologically required entities. On the contrary, the complexity of the bioactive molecules can make these processes time-consuming and extremely low yields with difficulties in scaling up at the early stages of drug development process.⁷⁰

Recently, combinatorial biosynthesis technologies including genetics and natural product biosynthesis have been developed for generation of new natural product scaffolds.⁷¹ Even though these methods have huge potential, they are generally time-consuming and unreliable compared to chemical methods, with each modification of the natural product scaffold requiring a new genetic construct. This led to the loss of biosynthetic efficiency and poor yields of the expected derivatives.⁶⁵

However, utilizing the strengths of each approach have succeeded in derivatizing natural products. Combining semi-synthesis, total chemical synthesis and combinatorial biosynthesis has great promise in introducing greater diversity into natural products.⁷² For example, biosynthesis and biochemical engineering of stereochemically complex natural products and rapid generation of analogues along with non-biological functional groups via synthetic chemistry approaches.⁷³

1.7 Prospects for natural product discovery and future opportunities

In the past decade, the pharmaceutical industry has largely abandoned the pursuit of discovering new bioactive natural product scaffolds due to their discovery model (e.g., higher profitability)³⁰ The economic value of natural products may be less until their primary function and utility is exposed and established. Further, natural products research requires extensive analytical skills, broad knowledge, and in-depth experience, which takes

time to accrue. Despite that, natural products continue to serve an inspiration with about 50% of clinically approved drugs are natural products based.²⁹

Natural products are undoubtedly capable of production of various precious lead compounds ranging from simple chemical structures to highly complex structures. Currently, most drug discovery processes are primarily focused on isolation, purification, screening and identifying novel drug candidates.⁷⁴ However, large scale extraction or biotechnology production is required to move potential lead compounds forward and make them clinically feasible.² Further, to be competitive with other advanced drug discovery techniques, natural product research should increase the efficiency of screening, isolation, robotic separation, and purification.² Moreover, pharmacokinetic and pharmacodynamic investigations, drug stability and formulation studies are attractive strategies to draw the attention for the compounds of interest and move them forward.⁷⁵

With the advent of new hyphenated spectroscopy technologies such as HPLC-MS, HPLC-NMR and HPLC-NMR-MS, further means of rapid compound identification and dereplication are now possible.⁷⁶ Further, combination of metabolomics technologies with natural product discovery processes is also beneficial in multiple levels.⁷⁷ The objective of metabolomics is to construct unbiased observations with highly reproducible analytical tools followed by data analysis to locate correlations between all available data.⁷⁸ This emerging field combines leading analytical platforms such as mass spectrometry hyphenated with gas chromatography (GC), liquid chromatography (LC) and NMR spectroscopy.⁷⁹

The advancements in analytical instrumentation and sophisticated hyphenation of separation techniques combined with multifaceted identification tools undoubtedly will now be used to advance the discovery of natural products to identify potential novel bioactive compounds to combat against existing and forthcoming diseases for many years to come.

Chapter 2: Chapter Overview

2.1 Hypothesis

Trichomonas vaginalis and *Mycoplasma genitalium* are causative agents of sexually transmitted diseases.^{80,81} Trichomoniasis is caused by the parasite *T. vaginalis*, while the pathogenic bacterium *M. genitalium* cause cervicitis, endometritis, pelvic inflammatory diseases in men and women.⁸² Current clinical treatment options for trichomoniasis are limited to antiprotozoal nitroimidazoles including metronidazole and tinidazole.⁸³ The commonly used antibiotics in treating *M. genitalium* infection are macrolides, quinolones and tetracyclines.⁸⁴ Even though these drugs offer appreciably high levels (>90%) of clinical efficacy, concerns have arisen due to several noted side effects (e.g., carcinogenic, and mutagenic properties) and resistance.⁸¹ Further, a limited number of prior studies had focused in identifying new leads from natural products to combat against *T. vaginalis* and *M. genitalium*.^{85,86} Therefore, we carried out an extensive search of bioactive secondary metabolites produced by fungi, which led to the identification of several tetramic acids with a remarkable activity.⁸⁷ The derivatization efforts of tetramic acids to improve the activity and selectivity against these pathogens contributed to build the hypothesis of my research.

In addition to the tetramic acids, our primary screening and analysis of bioactive fungal extracts against *M. genitalium* led to the identification of hydroxamic acids. The structural features, bioactivity and semi-synthetic modifications collectively contributed to build the hypothesis which led to the investigation of chelation of metal ions.

Another aspect of my research was to isolate and identify fungal derived bioactive compounds targeting chromosome segregation pathway of the pathogen, *Pseudomonas*

aeruginosa. Chromosome segregation during cell division is an essential cellular process that has a potential to yield numerous targets for drug development.⁸⁸ Up to now, very few inhibitors of chromosome segregation have been reported and the hypothesis of my research was developed based on the discovery of selective antibiotics.

When examined collectively with the aim of discovering new fungal natural products for drug discovery, the hypothesis guiding my research was: **Identifying fungal derived bioactive natural products against *P. aeruginosa* and *M. genitalium* and utilizing semi-synthesis to improve the activity against *T. vaginalis* and *M. genitalium*.** This hypothesis was tested via the following specific aims:

1. Derivatization of tetramic acids including phomasetin and pyrrolocin A via semi-synthesis to improve the activity and selectivity against *T. vaginalis* and *M. genitalium*
2. Isolation and identification of hydroxamic acids from *Penicillium* sp. with activity against *M. genitalium*.
3. Development of reporter assays suitable for high throughput screening assay for selective inhibition of chromosome segregation in *Pseudomonas aeruginosa* and isolation of sterigmatocystin derivatives from fungal metabolites and structure elucidation.

2.2 Chapter 3. PF-1140 as a broad-spectrum metal chelator with activity against pathogenic bacterium *Mycoplasma genitalium*

Chapter 3 will discuss the investigation of bioactive compounds against a sexually transmitted bacteria *Mycoplasma genitalium*. Screening of our fungal extract library

resulted in the identification of *Penicillium sp.* isolate that exhibited selective inhibition of *M. genitalium*. Further studies of the extract led to the identification of *N*-hydroxy pyridone, PF-1140. Several other semi-synthetic analogues of PF-1140 along with other pyridones isolated from the fungal extract were tested to confirm the importance of the bioactive metabolite. Further, studies of the bioactive metabolite led to identify new insights of the structural features that contribute to activity of these compounds.

2.3 Chapter 4. Isolation of novel sterigmatocystin analogues as chromosome segregation inhibitors in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria commonly found in the environment, particularly in fresh water.⁸⁹ *P. aeruginosa* can cause a wide array of community-acquired infections like pneumonia, otitis externa, puncture wounds leading to osteomyelitis, and folliculitis.⁹⁰ It is commonly an opportunistic pathogen and common in individuals in an immunocompromised state such as cystic fibrosis, burns, cancer, bronchiectasis, AIDS, organ transplant, uncontrolled diabetes mellitus, and ICU admissions.⁹¹ Individuals with invasive devices such as indwelling catheters or endotracheal tubes are also at risk due to the organism's unique ability of biofilm formation.⁹² Due to extreme versatility, antibiotic resistance, and a wide range of dynamic defenses, *P. aeruginosa* is an extremely challenging organism to treat with modern-day medicine.⁹³

Chromosome segregation during cell division is an essential cellular process in the cell cycle. In *P. aeruginosa* chromosome segregation is mediated by two partially redundant systems including condensins and ParAB.^{94,95} The screening assay based on cell viability

was developed based on these two systems that are synthetically lethal in *P. aeruginosa*. This assay was used to screen our fungal extract library for potential inhibitors of the ParAB pathway. The activity was found in the fungal extract of *Humicola variabilis*. Further fractionation led to identify novel sterigmatocystin analogue along with several new and known sterigmatocystins. The new analogue induced defects in chromosome segregation which resulted increased frequencies of anucleate cells. Several confirmation assays were performed which led to the conclusion of formation of toxic product with drug target which is responsible for the observed activity.

2.4 Chapter 5. Semi-synthetic derivatization studies of the fungal metabolite phomasetin with activity against *Trichomonas vaginalis* and *Mycoplasma genitalium*

In chapter 5, I discuss the derivatization studies of the fungal metabolites phomasetin and pyrrolocin A to improve the activity against *T. vaginalis* and *M. genitalium*. Previous investigations on bioactive secondary metabolites against *T. vaginalis* resulted a series of tetramic acids which include trichosetin ($EC_{50} = 2.5 \mu M$), equisetin ($EC_{50} = 3.0 \mu M$), beauversetin ($EC_{50} = 0.8 \mu M$), phomasetin ($EC_{50} = 0.35 \mu M$) and pyrrolocin A ($EC_{50} = 0.06 \mu M$).⁸⁷ The structure activity relationship (SAR) suggested that modifications to the *trans*-decalin system may provide additional opportunities for generating new analogues that offer even further improvements in potency and selectivity. Another interesting and intriguing feature of these compounds is the ability to undergo tautomerization due to the tetramate moiety. Further, out of all the tetramic acids in the series phomasetin and pyrrolocin A were isolated in higher yields. Therefore, phomasetin was chemically

modified to improve the activity and selectivity. Mainly the chemical modifications were carried out at the olefinic side chain and tetramate moiety. The terminal alkyne generated at the tetramate moiety was converted to a triazole using ‘click’ chemistry and the triazole moiety was modified with different substituents. The chemical modifications of bioactive phomasetin were applied to pyrrolocin A to expand the library. This chapter encloses the chemical modifications and bioactivity of the semi-synthesized compounds against *T. vaginalis* and *M. genitalium*.

Chapter 3. Metal ion binding properties of PF-1140 with activity against pathogenic bacterium *Mycoplasma genitalium*

This chapter was adapted from a manuscript which is currently being prepared for submission in 2023. The authors are Thilini Peramuna, Karen Wendt, Gwendolyn E. Wood, and Robert H. Cichewicz.

The work presented in this chapter was conducted as follows: Thilini Peramuna performed extraction, purification, structure elucidation and semi-synthesis of compounds 1-6, Karen Wendt performed the isolation of fungal isolates and large-scale fermentations, Gwendolyn E. Wood conducted the assay development and bioassay.

3.1 Introduction

Mycoplasma genitalium is a sexually transmitted pathogenic bacterium that causes urogenital infections in men and women.^{96, 97, 98, 84} Those infected by *M. genitalium* exhibit an increased risk of cervical shedding, and coinfection with other STIs such as HIV, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*.^{82, 86} Several studies have reported substantially high levels of prevalence and persistence of *M. genitalium* infections in the US general population.^{99, 100, 101} The US centers for disease control and prevention guidelines for the treatment of *M. genitalium* infections call for azithromycin (1 g single dose) as first-line therapy and moxifloxacin as second-line therapy in cases involving azithromycin-resistant infection.¹⁰² While these drugs continue to show efficacy in the clinic, the rapid spread of resistance against these antibiotics has continued to increase across the globe.^{103, 104} The intrinsic resistance of *M. genitalium* to beta-lactam antibiotics,

and cephalosporins due to the lack of a cell wall means that alternative treatment options for managing *M. genitalium* infections are severely limited.¹⁰⁵ This has triggered the search for new bioactive compounds and cell targets to control this pathogen.

Metals are essential to all forms of life. In bacteria, metal cofactors serve several structural and catalytic roles in biological processes including DNA replication, transcription, precursor biosynthesis and responses to oxidative stress.⁵² Therefore, metal acquisition systems are considered essential components for the host to compete for limited resources.¹⁰⁶ In *M. genitalium* metal transporter systems and regulatory pathways controlling metal homeostasis have been reported.¹⁰⁷ An intriguing feature associated with *N*-hydroxy pyridone moiety is its remarkable affinity for iron (III) under physiological conditions.^{108, 109} The *N*-hydroxy pyridones such as fusaricide, akantomycin, leporin B, cordypyridones A and B have been isolated from several fungal sources.^{108, 110, 109, 61, 111} Diverse bioactivities are attributed to these compounds, including antifungal, antibacterial, insecticidal, anticancer, and cytotoxic activities.⁵⁸ In this report, our collaborative team explored the effects of *N*-hydroxy pyridones on Mycoplasma survival to determine whether such compounds might serve as valuable chemical tools for inhibiting this pathogenic organism.

3.2 Results and Discussion

3.2.1 Structure characterization of purified natural products

During the screening of 1,012 fungal-derived extracts, several samples were identified that inhibited *M. genitalium* including a notably potent extract from the fungal isolate coded IL02284 PFAT-2. The fungus was identified as a *Penicillium sp.* based on BLASTN

analysis of its ITS sequence data (Genbank accession number OP009585). Bioassay guided fractionation was undertaken to determine the active components in the sample.

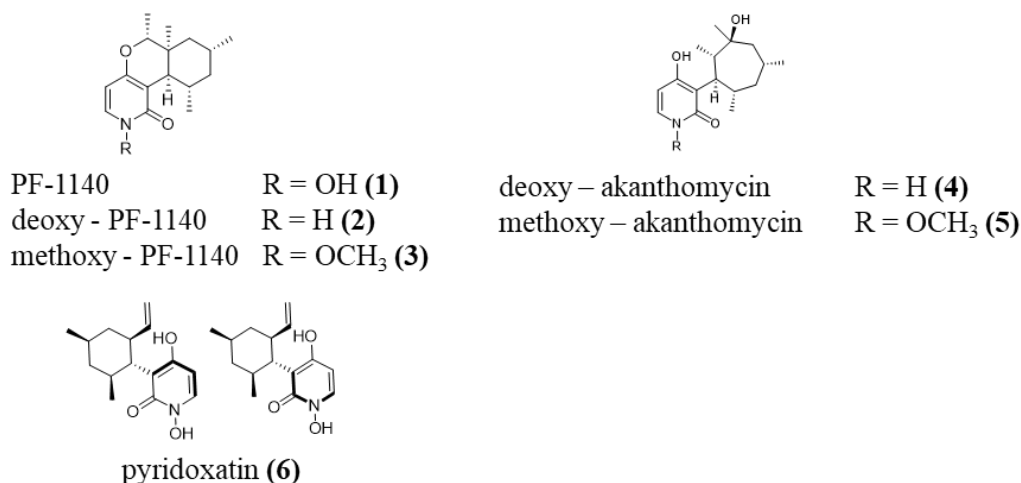


Figure 3.1. Structures of fungal derived natural products and semi-synthesized natural products used in this study

Compound **1** was obtained as white colorless amorphous solid, and its molecular formula (C₁₆H₂₃NO₃) was determined by interpretation of its HRESIMS data. The ¹H and ¹³C NMR spectra were used to establish that the proton and carbon resonances generated for **1** were identical to those reported for fungal metabolite PF-1140.¹¹² Further analysis of a single crystal prepared from natural product **1** using X-ray spectroscopy revealed, this particular crystalline form of the sample consisted of racemate. Those findings differed from the results obtained following chiral chromatographic analysis of the PF-1140 sample, which suggested that the natural product occurred as a scalemate. (3:1 mixture of enantiomers; Figure A-21, appendix A). These results from the chiral HPLC aligned with the specific rotation value we observed for our sample of **1** {[α]_D²⁰ -65 (c 0.26, MeOH)}, which differed from the reported value {[α]_D²⁰ -124 (c 0.26, MeOH)}¹¹² using what was presented to have been enantiomerically pure material.

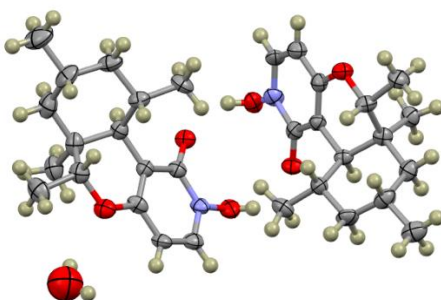


Figure 3.2. Enantiomeric mixture of **1** as determined by single crystal X-ray diffraction analysis

Compound **4** was purified as a colorless solid and the HRESIMS data were consistent with the molecular formula $C_{16}H_{25}NO_3$. The ^{13}C and 1H spectra of **4** each contained two sets of overlapping signals, which were attributed to atropisomerism, as previously reported.¹¹¹ A comparison of ^{13}C and 1H spectra for **4** with those described for deoxyakanthomycin led us to deduce that compounds were the same.¹¹²

Compound **5** was obtained as a colorless solid and was assigned the molecular formula $C_{17}H_{27}NO_4$ based on interpretation of HRESIMS data. The 1H and ^{13}C NMR spectra for compound **5** exhibited a similar doubling of most of the resonances, which was indicative of atropisomers. Similar to the previous metabolites discussed, attempts to purify single atropisomeric species failed with the newly purified samples quickly reestablishing 1:1 mixture of atropisomers. Therefore, structure determination was performed using the compounds as a mixture. Examination of 1H , ^{13}C , COSY, HSQC and HMBC correlation data led to establish that the seven-membered carbocyclic ring connected to 2-pyridone moiety was closely related to the previously known akanthomycin.¹¹² The presence of methoxy signal at δ 3.95 (s) showed that **5** differed from **4** by the replacement of the *N*-H group of **4** by *N*-OCH₃.

Table 3.1. ^{13}C (400 MHz) and ^1H (500 MHz) NMR data for **5** in $\text{CD}_3\text{OD}-d_4$

Position	5a, δ_{C}	5a, δ_{H} (J in Hz)	5b, δ_{C}	5b, δ_{H} (J in Hz)
1				
2	160.05		160.95	
3	117.75		118.04	
4	162.27		162.50	
5	100.71	6.04, d (8.0)	101.89	5.96, d (8.0)
6	133.89	7.63, d (8.0)	133.89	7.60, d (8.0)
7	44.65	2.77, dd (6.0, 10.5)	43.35	3.03, dd (5.0, 11.0)
8	47.10	2.14, m	47.99	2.05, m
9	76.73		77.16	
10a	51.29	1.64, m	50.34	1.66, m
10b		1.63, m		1.62, m
11	28.87	2.05, m	29.48	2.05, m
12a	50.42	1.76, m	51.29	1.72, m
12b		1.00, m		1.02, m
13	36.76	2.65, m	37.25	2.45, m
14	16.40	0.83, d (7.0)	17.59	0.88, d (7.0)
15	27.94	1.09, s	28.70	1.16, s
16	25.29	0.92, d (6.5)	25.49	0.92, d (6.5)
17	23.09	0.60, d (6.5)	23.22	0.60, d (6.5)
18	65.24	3.95, s	65.24	3.95, s

3.2.2 Structure-activity relationship of purified natural products and semi-synthesized compounds

Compound **1** exhibited pronounced inhibitory activity against *M. genitalium* (MIC_{90} 0.01 $\mu\text{g/mL}$), as well as show strong inhibition against and clinical isolates of *M. genitalium* including G37 (IC_{50} 0.106 μM), Sea-1 (IC_{50} 0.068 μM) and Sea-2 (IC_{50} 0.098 μM). However, both deoxy-akanthomycin (**4**) and methoxyakanthomycin (**5**) were inactive (MIC_{90} 25 $\mu\text{g/mL}$) under the same test conditions. The lack of activity of **4** and **5** suggest the *N*-OH functionality played a key role in generating the antibacterial activity. To further investigate the importance of the *N*-OH pyridone moiety of **1**, *N*-deoxy PF-1140 (**2**) and *N*- methoxy PF-1140 (**3**) were prepared via semi-synthesis. The multi-fold loss of activity

(MIC₉₀ 25 µg/mL) resulting from both structural modifications aligned with our hypothesis that *N*-OH in PF-1140 plays a key role in the activity in *M. genitalium*. To further investigate the *N*-OH functionality of **1**, we tested pyridoxatin (**6**) from the library of purified natural products from the University of Oklahoma, Natural Products Discovery Group.¹¹³ The lack of activity for **6** (MIC₉₀ 25 µg/mL) suggested that the chemical structure of **1** along with the pyridone moiety is also essential in exhibiting the bioactivity.

Table 3.2. Bioassay results of fungal derived natural products and semi-synthesized natural products used in this study (**1-6**)

Compound	MIC ₉₀ (µg/mL)
PF-1140 (1)	0.01
deoxy-PF-1140 (2)	25
methoxy-PF-1140 (3)	25
deoxy-akanthomycin (4)	25
methoxy-akanthomycin (5)	>25
pyridoxatin (6)	25

Table 3.3. Bioassay results of **1** against the clinical isolates of *M. genitalium*

Compound	G37 (IC ₅₀ /µM)	Sea-1(IC ₅₀ /µM)	Sea-2(IC ₅₀ /µM)
PF-1140 (1)	0.106 +/- 0.013	0.068 +/- 0.003	0.098 +/- 0.012

3.2.3 Spectroscopic analysis of metal ion binding properties of PF-1140

Based on the published reports of the metal-binding capabilities of hydroxamic acids, such as that observed in **1**, we hypothesized that this structural feature may play a role in metal binding in our bioassay system. Further fueling our interest in this natural product **1** was the recent finding in the metal acquisition systems of *M. genitalium*. *M. genitalium* preferentially accumulated zinc, iron, and copper likely through constitutive metal transporters. Further, Fur (Ferric uptake regulator) mutant *M. genitalium* contained ~10

fold more nickel than the wild type strain.¹¹⁴ These results revealed the different mechanisms use by *M. genitalium* to obtain essential nutrients and usage of nickel to replace iron content within the host. Therefore, a versatile metal chelator may be helpful to restrict the access of essential metal ions.

To test the metal chelating activity of **1**, several biologically relevant metal ions (Mg^{2+} , Ca^{2+} , Zn^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Mn^{2+}) were selected and subjected to binding studies using UV-vis absorbance spectroscopy.¹¹⁵ (Figure 3.3) The formation of metal-organic complexes upon incubation of **1** with various metal ions in a series of molar equivalents (ME) led to the formation of new chromophores. The UV absorption corresponding to the newly formed chromophore resulted the appearance of novel absorption band/bands in the UV spectrum.¹¹⁵ The gradual increment of metal ions resulted in formation of more metal bound complexes which led to increased intensities of absorption at a particular wavelength. The UV-vis spectra of **1** incubated with a series of (0.5 MEs to 25 MEs) CoCl_2 at neutral pH resulted in an increased relative absorbance at ~ 540 nm. Following incubation with 0.5 MEs to 1 MEs of NiCl_2 resulted no visible changes in each absorbance. The subsequent addition of NiCl_2 from 5 ME to 25 ME resulted an increased absorbance at ~ 410 nm and ~ 750 nm, which resembled the presence of increased concentrations of PF-1140 nickel complex. The copper PF-1140 complex formation was recognized due to the appearance of new UV-vis absorption band at 820 nm at 0.75 ME. The gradual increment of UV-vis absorbance at 820 nm indicated the conversion of **1** to more copper-bound form. Incubation with 0.3 ME of FeCl_3 resulted UV-vis absorbance at 490 nm and 510 nm. Fe (III) and PF-1140 complex formation at very low molar equivalents of FeCl_3 resembled the high binding affinity of **1** for Fe (III) comparative to the other metal

ions tested. The appearance of novel absorption bands has not observed with subsequent incubation of MnCl_2 , CaCl_2 , MgCl_2 and ZnCl_2 at neutral pH even at high molar equivalents. These data suggested that compound **1** did not bind with Mn^{2+} , Ca^{2+} , Mg^{2+} and Zn^{2+} under the test conditions. In contrast, the data obtained from this assay confirmed that **1** chelates Co^{2+} , Ni^{2+} , Cu^{2+} and Fe^{3+} at neutral pH. This led to the conclusion that **1** may chelate with essential metal ions that are acquired by *M. genitalium* within the host.

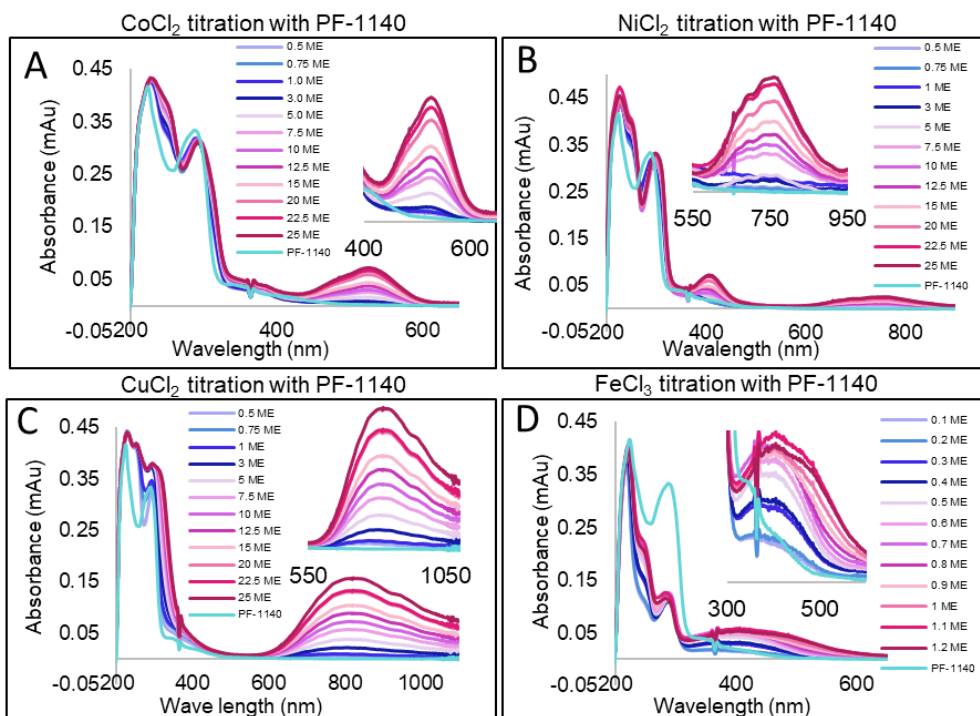


Figure 3.3. UV-vis absorbance spectra of **1** with various metal ions

3.2.4 Conclusions

Employing bioassay guided fractionation, we identified the *N*-hydroxy pyridone metabolite **1** is a potent inhibitor of *M. genitalium*. Additionally, derivatives of **1**, including *N*-deoxy and *N*-methoxy were semi-synthesized to further explore structural requirements affecting the activity. The potential chelating capabilities of hydroxamic acid moiety in **1** with different types of metal ions were tested based on UV-vis based metal binding assay. The

chelation capability provides valuable insights concerning the essential metal ions required by *M. genitalium*. The data suggest that **1** forms complexes with iron, cobalt, nickel, and copper. This provides evidence that **1** could affect metal homeostasis in *M. genitalium*. Further, these results may justify the recent finding of the survival of this pathogen under iron starved conditions that the metal iron chelator **1** may chelate with several essential metal ions simultaneously, and the defensive strategies conducted by substituting other metal ions to compensate iron requirement may have been prevented.

3.3 Materials and Methods

3.3.1 General Experimental Procedures

Optical rotation measurements were obtained on a Rudolph Research Autopol III automatic polarimeter. UV-vis spectra were obtained using a Hewlett-Packard 8453 spectrophotometer. NMR data were collected on Varian 400 and 500 MHz NMR spectrometers. HRESIMS data were obtained on an Agilent 6538 high-mass-resolution QTOF mass spectrometer. LC-ESI-MS data were obtained on a Shimadzu LCMS-2020 system (ESI quadrupole) coupled to a photodiode array detector with a Phenomenex Kinetex column (2.6 μm , C₁₈ column, 100Å, 75 x 3.0 mm). ECD spectra were collected on a JASCO J-715 circular dichroism spectrometer. Vacuum column chromatography was performed using silica gel (VWR, 40-60 μm , 6Å) and HP20_{ss} gel (Sorbtech). Preparative HPLC was conducted using Shimadzu system equipped with SCL-10A VP pumps and a system controller using a Gemini 5 μm C18 column (210 Å, 250 x 21.2 mm) with a flow rate of 10 mL/min. The semipreparative HPLC were carried out on a Waters system (1525 binary pumps and Waters 2998 photodiode array detectors) using a Gemini 5 μm C₁₈ (250

x 10 mm, 110 Å), and biphenyl column (250 x 10 mm, 110 Å) with a flow rate of 4 mL/min. All solvents were of ACS grade or better.

3.3.2 Fungal Isolate and Fermentation

The *Penicillium* sp. isolate was obtained from a soil sample collected near Oregon, Illinois. The fungus was identified via ITS sequencing (Genbank accession number OP009585). The mycelium was homogenized in a BulletBlender (MidSci #BB24M) with zirconium oxide beads in TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) and the resulting lysate was subjected to PCR. The ITS region (i.e., ITS1, 5.8S, and ITS2 regions) was amplified and sent for sequencing. The resulting sequence was compared to fungal sequences in Genbank, which led to greater than 99% identity matches to isolates described as *Penicillium* sp. Sequence data was deposited in GenBank (accession number OP009585). The fungus was grown in mycobags (Unicorn Bags, Plano, TX) with a monolayer of Cheerios breakfast cereal supplemented with a 0.3% sucrose and 0.005% chloramphenicol solution. Cultures were grown for four weeks at room temperature.

3.3.3 Extraction and Purification

The *Penicillium* sp. grown on Cheerios breakfast cereal was homogenized and extracted with 2L of EtOAc (x3). The EtOAc extract (15 g) was subjected to silica gel vacuum liquid chromatography with step-gradient elution consisting of 1:1 hexanes/DCM, DCM, 10:1 DCM/MeOH and MeOH yielding four fractions. The fraction 10:1 DCM/MeOH (4 g) was further fractionated by HP20ss vacuum liquid chromatography using MeOH-H₂O step-gradient (30:70, 50:50, 70:30, 90:10, and 100:0) and washed with CH₂Cl₂/MeOH (50:50) to yield six fractions. Bioassay analysis of these fractions revealed that the 90% MeOH fraction contained the bioactive material. The 90% MeOH fraction was further fractionated

by preparative HPLC (C18, gradient elution with 50-100% MeOH in H₂O over 20 min using a 10 mL/min flow rate), followed by semipreparative HPLC [Biphenyl, isocratic MeCN-H₂O (55:45), flow rate 4 mL/min] to obtain **1** (80.0 mg), **4** (2.5 mg) and **5** (1.8 mg).

3.3.4 Culture of organisms

Mycoplasma genitalium strain G37C, a single-colony filter-cloned isolate of the G37 type strain was used for these experiments (Iverson-Cabral 2007). *M. genitalium* was routinely grown in SP-4 broth (Tully 1977) containing 100 U/ml penicillin, at 37 °C with 5% CO₂ in tissue culture flasks. *M. genitalium* strains Sea-1 and Sea-2 were isolated in Seattle, WA from men with urethritis (Iverson-Cabral 2015).

3.3.5 *M. genitalium* assay

MIC values for *M. genitalium* were determined in microbroth dilution assays (Hamasuna 2009). *M. genitalium* was grown to log phase, scraped, and frozen in multiple aliquots at -80 °C. Frozen stocks (0.2 ml) were inoculated into 2 ml SP-4 broth in 35 mm petri dishes and incubated for 48 hours to obtain metabolically active, log phase *M. genitalium*. Adherent bacteria were scraped into the culture supernatant, filtered (0.45 mm) then diluted in fresh SP-4 broth to a final concentration of 10⁵-10⁶ CFU per ml. Two-fold serial dilutions of test compounds were prepared in flat bottom, 96-well plates in 0.1 ml SP-4, 0.1 ml of the inoculum was added, and plates were incubated for approximately 7 days monitoring color change of the phenol red indicator from red to orange (due to fermentation of glucose, indicating growth). The most dilute well with no color change was considered the MIC which inhibited growth by at least 90% (confirmed by microscopy). DMSO alone at concentrations corresponding to the highest drug concentration did not inhibit *M.*

genitalium growth in any experiments. Precise IC₅₀ values were determined by quantitative PCR. After assessing MIC endpoints by color change, 1/10 volume (20:1) of Triton lysis solution (10% Triton X-100, 100 mM Tris HCl pH 8, 10 mM EDTA) was added to each well and the plates were incubated at 95 °C for 30 minutes. Lysates were diluted 1:10 in TE, then the number of *M. genitalium* genomes quantified using a TaqMan qPCR method (Jensen 2004, Wood 2017). PCRs were performed in triplicate and compared to a quadruplicate standard curve of known genome concentrations. The drug concentration resulting in a 50% reduction in *M. genitalium* genomes relative to untreated wells was calculated using a four-parameter logistic regression model.

3.3.6 X-ray Crystallographic Analysis of Compound 1

Crystals of compound **1** were obtained from MeOH. A colourless, plate-shaped crystal of dimensions 0.068 x 0.302 x 0.420 mm was selected for structural analysis. Intensity data for this compound were collected using a D8 Quest k-geometry diffractometer with a Bruker Photon II cmos area detector and an Incoatec Ims microfocus Mo Ka source ($\lambda = 0.71073 \text{ \AA}$). The sample was cooled to 100K. Cell parameters were determined from a least-squares fit of 9931 peaks in the range $2.42 < 2\theta < 23.97^\circ$. A total of 81326 data were measured in the range $2.201 < 2\theta < 25.026^\circ$ using ϕ and ω oscillation frames. The data were corrected for absorption by the empirical method giving minimum and maximum transmission factors of 0.7296 and 0.8620. The data were merged to form a set of 10886 independent data with $R(\text{int}) = 0.1179$ and a coverage of 99.9 %.

The orthorhombic space group $P2_12_12_1$ was determined by systematic absences and statistical tests and verified by subsequent refinement. The structure was solved by direct methods and refined by full-matrix least-squares methods on F^2 . The positions of

hydrogens bonded to carbons were initially determined by geometry and were refined using a riding model. Hydrogens bonded to nitrogens and oxygens were located on a difference map, and their positions were refined independently, with geometry restraints. Hydrogens on the water were best located to form hydrogen bonds, and their positions were finally refined with a riding model. Non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atom displacement parameters were set to 1.2 (1.5 for methyl) times the isotropic equivalent displacement parameters of the bonded atoms. A total of 744 parameters were refined against 16 restraints and 10886 data to give $wR(F^2) = 0.2126$ and $S = 1.065$ for weights of $w = 1/[s^2(F^2) + (0.0600 P)^2 + 14.9200 P]$, where $P = [F_o^2 + 2F_c^2]/3$. The final $R(F)$ was 0.0687 for the 8049 observed, $[F > 4s(F)]$, data. The largest shift/s.u. was 0.001 in the final refinement cycle. The final difference map had maxima and minima of 0.395 and -0.362 e/Å³, respectively. The X-ray crystallographic data for **6** have been deposited at the Cambridge Crystallographic Data Centre under accession number 22013. Copies of these data can be obtained from free of charge from the CCDC via www.ccdc.cam.ac.uk

3.3.7 UV-vis based metal binding assay

All metal salt solutions and metallo-organic solutions were freshly prepared in acid-washed glassware. Stock solutions of 50 mM CoCl₂, NiCl₂, CuSO₄, MnCl₂, FeCl₃, MgCl₂, CaCl₂, ZnCl₂ and 10 mM PF-1140 (**6**) solutions were prepared in HPLC grade MeOH. Incubations were performed at a volume of 1 mL in 2 mL glass vials. To each vial 50 µL of **1** (final concentration 0.5 mM), 5 µL to 250 µL of metal ion solutions (final concentration 0.25 mM – 12.5 mM) were added and final volume was adjusted using MeOH. The Fe³⁺ solutions were prepared by adding 1 µL – 12 µL of 50 mM stock solution (final

concentration 0.05 mM – 0.6 mM). Solutions were incubated at room temperature for 12 hours and the absorbance was measured using UV-vis spectrophotometer. The Fe^{3+} solutions were diluted 20-fold into MeOH prior measuring the absorbance.

3.3.8 Semi-synthesis of *N*-deoxy PF-1140 (2)

Compound **1** (10 mg) was dissolved in 0.5 mL ethanol and catalytic amount of palladium was added. The mixture was purged with H_2 for 5h at room temperature. The product was purified by semipreparative HPLC using a Phenomenex Gemini 5 μm C_{18} 250 mm $\times$ 10 mm column eluted with 50% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ to afford compound **1** (4.0 mg, 40% yield). Compound **1**: white powder, HRESIMS m/z 262.1814 $[\text{M}+\text{H}]^+$ (cald for $\text{C}_{16}\text{H}_{24}\text{NO}_2$ 262.1807)

3.3.9 Semi-synthesis of *N*-methoxy PF-1140 (3)

Compound **1** (10 mg) was dissolved in 0.5 mL acetone and NaH (2.5 mg, 3 equiv) was added in two portions. The reaction mixture was stirred at room temperature for 12 hours with the addition of CH_3I (10 μL , 3 equiv). The product was purified by semipreparative HPLC using a Phenomenex Gemini 5 μm C_{18} 250 mm $\times$ 10 mm column eluted with 70% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ to afford compound **3** (7.5 mg, 75% yield).

PF-1140 (1)

White, amorphous powder, $[\alpha]_{\text{D}}^{25}$ -65 (c 0.26, MeOH), HRESIMS $[\text{M} + \text{H}]^+ m/z$ 278.1764 (cald for $\text{C}_{16}\text{H}_{24}\text{NO}_3$, 278.1756)

Methoxy-PF-1140 (3)

White, amorphous powder, $[\alpha]_D^{25}$ -20 (c 0.26, MeOH), ^1H and ^{13}C NMR, see Table 3.2 in materials and methods; HRESIMS $[\text{M} + \text{Na}]^+$ m/z 314.1741 (calcd for $\text{C}_{17}\text{H}_{25}\text{NNaO}_3$, 314.1732)

Methoxy-akanthomycin (5)

White, amorphous powder, $[\alpha]_D^{25}$ -8.8 (c 0.34, MeOH), ^1H and ^{13}C NMR, see Tables 3.1; HRESIMS $[\text{M} + \text{H}]^+$ m/z 332.1838 (calcd for $\text{C}_{17}\text{H}_{27}\text{NNaO}_4$, 332.1838)

Chapter 4. Isolation of novel sterigmatocystin analogues as chromosome segregation inhibitors in *Pseudomonas aeruginosa*

This chapter was adapted from a manuscript which is currently being prepared for submission in 2023. The authors are Thilini Peramuna, Karen Wendt, Hang Zhao, Zoya M. Petrushenko, Sidra Ajmal, Kaushika Premachandra, Robert H. Cichewicz and Valentin V. Rybenkov

The work presented in this chapter was conducted as follows: Thilini Peramuna performed extraction, purification, structure elucidation of compounds 1-7, Karen Wendt performed the isolation of fungal isolates and large-scale fermentations, Hang Zhao, Zoya M. Petrushenko, Sidra Ajmal and Kaushika Premachandra conducted the assay development, bioassay, and modeling.

4.1 Introduction

Chromosome segregation during cell division is a hallmark event in the life of every cell.^{88, 116} This process of chromosome segregation is differentially organized in bacteria and eukaryotic cells.¹¹⁶ In particular, chromosome replication and segregation are restricted to distinct stages of the cell cycle in eukaryotic cells but occur concurrently in bacteria.¹¹⁷ These and many other distinctions create a room of opportunity for the design of selective antibiotics targeting the chromosome segregation pathway.¹¹⁸ Up to now, however, very few inhibitors of chromosome segregation have been reported and none have progressed to clinical applications.¹¹⁹

Two systems cooperate in chromosome segregation of *P. aeruginosa* in a partially redundant manner including condensins, SMC and MksB and the partitioning protein ParAB.¹²⁰ The primary function of SMC and MksB is in global chromosome folding whereas ParAB contributes to its segregation.¹²¹ Any two of these three proteins can be deleted with only a modest loss of fitness which is referred to ‘synthetic lethality’. This leads to a straightforward screening assay based on cell viability.¹²¹

4.2 Results and Discussion

4.2.1 Development of an assay for detecting *P. aeruginosa* inhibitors

We used the synthetic lethality of condensins and ParB to develop a screening assay for inhibitors of the ParAB pathway. The assay is based on the increased susceptibility of cells deficient in SMC and MksB. During screening, we sought compounds that have a lower minimal inhibitory concentration, MIC, in SMC MksB mutant cells than in the parental strain (Figure 4.1). The rationale here was that the compounds that inhibit chromosome segregation would be synergistic with genetic mutations impair the pathway. In particular, inhibitors of the ParAB pathway would be potentiated in the SMC MksB mutant.

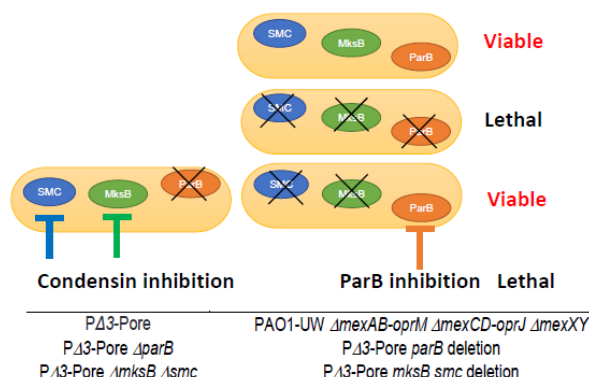


Figure 4.1. Hit screen strategy using synthetic lethality of condensins and Par B in *P. aeruginosa*

Using this assay, we screened a library of fungal extracts collected through Citizen Science Soil Collection Programme. We found an antibacterial activity that was potentiated by the SMC MksB mutation in an extract from Oklahoma soil sample collection OK07084 TV8-2 and authenticated it as a *Humicola* sp. Bioassay guided fractionation on the extract led to the isolation of five new sterigmatocystin derivatives [sterigmatocystin NPDG **1** (**1**), sterigmatocystin NPDG **2** (**2**), sterigmatocystin NPDG **3** (**3**), sterigmatocystin NPDG **4** (**4**) and sterigmatocystin NPDG **5** (**5**)] and two known analogues including sterigmatocystin (**6**) and deoxysterigmatocystin (**7**).

4.2.2 Testing purified natural products

Two *P. aeruginosa* strains were screened side by side. The parental strain, PΔ3Pore (WT), is deficient in three main efflux transporters MexAB-OprM, MexXY and MexCD, and harbors a “pore”, a truncated, plugless version of FhuA porin. The combination of these mutations makes bacteria highly susceptible to diverse chemicals due to the inability to preclude their penetration into the cell. The use of this trait increases the hit rate of the screen. The mutant strain was also deficient for SMC and MksB (*smc mksB*). An extract from *Humicola* sp. displayed a 2-fold lower MIC in *smc mksB* than WT cells. The lowest MIC values were found for compounds **1** (50 µg/mL) and **2** (200 µg/mL). (Table. 4.1). The other compounds were inactive.

Table 4.1. MIC and IC₅₀ of **1** and **2** against three permeable *P. aeruginosa* strains

	MIC, µg/ml			IC ₅₀ , µg/ml		
	PΔ3-Pore WT	PΔ3-Pore ΔparB	PΔ3-Pore ΔmksB Δsmc	PΔ3-Pore WT	PΔ3-Pore ΔparB	PΔ3-Pore ΔmksB Δsmc
1	100	100	50	62±12	41±13	32±2
2	>400	>400	200	263.6	191.7	135.8

4.2.3 Structure characterization of purified natural products

Chemical investigation on the fungal extract led to the isolation of five new sterigmatocystin derivatives **1-5** and two known compounds, **6** and **7**. (Figure 4.2) Compound **6** was identified as sterigmatocystin and compound **7** was identified as deoxysterigmatocystin by comparing the ¹H and ¹³C NMR data published in the literature.^{122, 123}

Compound **1** was obtained as a pale-yellow solid. The HRESIMS data (*m/z* 325.0703 [M+H]⁺, calcd. 325.0712), together with interpretation of ¹H and ¹³C NMR data led to its proposed molecular formula C₁₈H₁₂O₆. The ¹H and ¹³C NMR data of **1** were almost identical to those of compound **6** except the different coupling patterns and coupling constants of H-3, H-5, and H-6 (Table 4.2). Three resonances of aromatic protons appeared at δ 7.38 (d, *J* = 2.5 Hz, H-3), 7.18 (d, *J* = 8.5 Hz, 2.5 Hz, H-5) and 7.43 (d, *J* = 8.5, H-6) suggesting that OH at C-3 in compound **6** was at C-4 in compound **1**. The OH at C-4 was confirmed based on the HMBC correlations from H-3 to C-4 and C-5 and H-5 to C-3, C-4 and C-6.

Compound **2** was isolated as a pale-yellow solid, and its molecular formula ($C_{18}H_{12}O_6$) was ascertained from HRESIMS data (m/z 325.0702 $[M+H]^+$, calcd. 325.0712) and interpretation of 1H and ^{13}C data. Comparison of 1H and ^{13}C data of compound **2** to compound **1** revealed that compound **2** had a similar chemical structure as that of **1**. The only structural difference between compounds **1** and **2** was the differences in coupling patterns and coupling constants of H-3, H-4 and H-5 (Table 4.2). Three resonances of aromatic protons appeared at δ 7.49 (d, $J = 8.0$ Hz, 1.5 Hz, H-3), 7.18 (t, $J = 8.0$ Hz, H-4) and 7.24 (d, $J = 8.0$ Hz, 1.5 Hz, H-5) suggesting that OH at C-2 in compound **1** was at C-6 in compound **2**. Correlations of H-3/H-4/H-5 in the COSY spectrum were also observed. The OH at C-6 was confirmed based on HMBC correlations from H-5 to C-6, C-4 and C-3.

Compound **3** was isolated as a pale-yellow solid and its HRESIMS data m/z 339.0853 ($[M+H]^+$, calcd for $C_{19}H_{15}O_6$, 339.0869) was consistent with the molecular formula $C_{19}H_{14}O_6$ based on the interpretation of 1H and ^{13}C data. The analysis of 1H and ^{13}C data of compound **3** to compound **2** revealed the structural differences in the aromatic protons in compound **3**. Three resonances of aromatic protons appeared at δ 7.56 (d, $J = 9.0$ Hz, H-3), 7.37 (dd, $J = 9.0$ Hz, 3.0 Hz, H-4) and 7.47 (d, $J = 3.5$ Hz, H-6) in compound **3**. The methoxyl group at C-5 in compound **3** was assigned based on HMBC correlations from H-19 to C-5.

Compound **4** was obtained as a pale-yellow solid. The HRESIMS data was found to be m/z 355.0799 $[M+H]^+$. (calcd. 355.0818). The interpretation of 1H and ^{13}C data led to its molecular formula as $C_{19}H_{14}O_6$. The analysis of 1H and ^{13}C data of compound **4** to compound **3** revealed key structural differences including the presence of OH at C-6 and

changes in the aromatic protons in compound **4**. In compound **4** two resonances of aromatic protons appeared at δ 7.39 (s) for H-3 and at 6.86 (s) for H-5. The methoxyl group in compound **4** was assigned at C-4 based on the HMBC correlation from H-19 to C-4.

Compound **5** was obtained as pale-yellow solid, and the molecular formula was found to be $C_{19}H_{14}O_6$ based on HRESIMS data m/z 355.0799 ($[M+H]^+$, calcd. 355.0818) and interpretation of 1H and ^{13}C data. Comparison of 1H and ^{13}C data of compound **5** to compound **4** revealed that compound **5** had a similar chemical structure as that of **4**. The only structural difference between compounds **4** and **5** was the differences in coupling patterns and coupling constants of H-4 and H-5 (Table 4.4). In compound **5** two resonances of aromatic protons appeared at δ 6.97 (d, $J = 9.0$ Hz) for H-4 and δ 7.43 (d, $J = 9.0$ Hz) for H-5. The methoxyl group at C-6 in compound **5** was assigned based on HMBC correlations from H-19 to C-6.

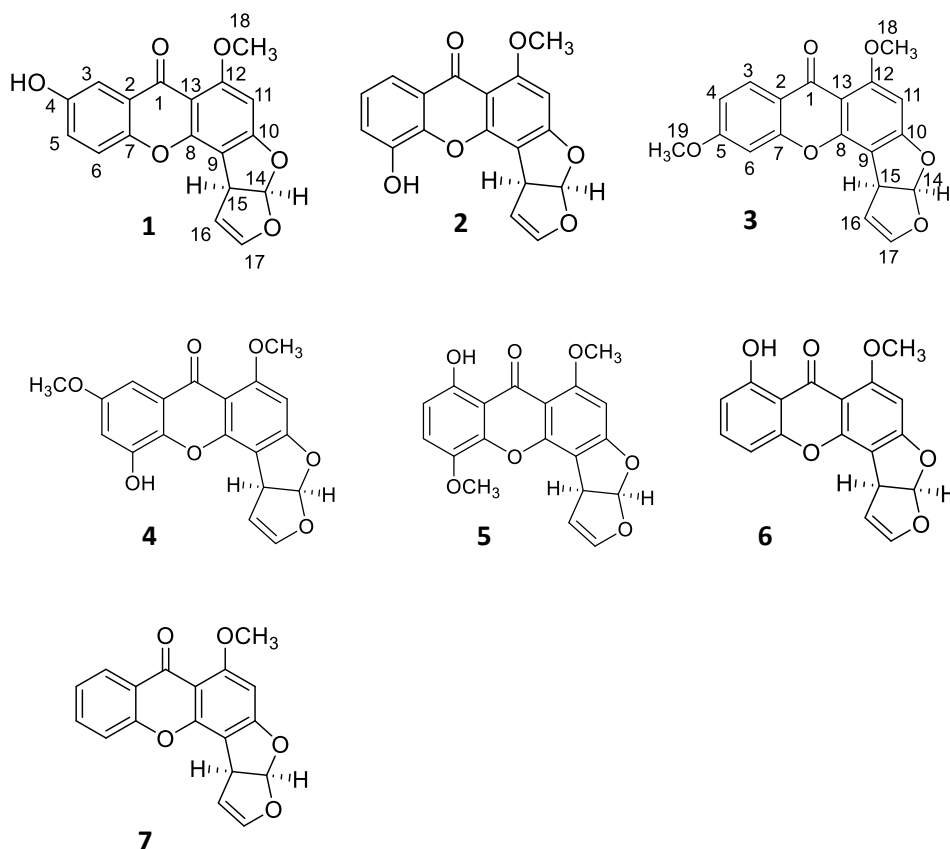


Figure 4.2. Structures of fungal metabolites **1-7**

The relative configurations of compounds **1-5** were determined by comparing the coupling constants with the reported values. Large coupling constants between H-14 and H-15 of **1** (Table 4.4), **2**, **3**, **4** and **5** ($J = 7.0$) suggested *cis* configuration ($14R^*$, $15S^*$) between these two protons as sterigmatocystin.¹²²

The optical rotation of **1** $[\alpha]^{20}_D -98$ (c 0.1, MeOH), **2** $[\alpha]^{20}_D -110$ (c 0.1, MeOH), **3** $[\alpha]^{20}_D -134$ (c 0.1, MeOH), **4** $[\alpha]^{20}_D -64$ (c 0.1, MeOH), **5** $[\alpha]^{20}_D -96$ (c 0.1, MeOH), **6** $[\alpha]^{20}_D -55$ (c 0.1, MeOH) and **7** $[\alpha]^{20}_D -76$ (c 0.1, MeOH) were found levorotary, which supported these metabolites shared the same absolute configuration. Taking into account these data, as well

as their shared biosynthetic origins, we propose that compound **1-5** bear 14*R*, 15*S* configurations.

Table 4.2. ¹H (500 MHz) and ¹³C (400 MHz) NMR data for compounds **1** and **2** in DMSO-*d*₆

	1		2	
No	δ^{C}	δ^{H} (<i>J</i> in Hz)	δ^{C}	δ^{H} (<i>J</i> in Hz)
1	173.3		173.5	
2	123.1		123.6	
3	109.1	7.38, d (2.5)	115.4	7.49, dd (8.0, 1.5)
4	153.4		123.9	7.18, t (8.0)
5	122.7	7.18, dd (8.5, 3.0)	119.6	7.24, dd (8.0, 1.5)
6	118.5	7.43, d (9.0)	145.8	
7	153.8		143.6	
8	153.4		153.2	
9	106.2		106.6	
10	162.9		163.1	
11	90.4	6.63, s	90.7	6.67, s
12	162.5		162.5	
13	106.1		106.3	
14	113.0	6.94, brs	113.1	6.96, d (7.0)
15	47.4	4.84, brs	47.5	4.92, dt (7.0, 2.0)
16	102.7	5.53, brs	102.8	5.49, t (2.5)
17	145.4	6.73, brs	145.3	6.73, t (2.5)
18	56.5	3.85, s	56.5	3.86, s

Table 4.3. ^1H (500 MHz) and ^{13}C (400 MHz) NMR data for compounds **3** and **4** in $\text{DMSO-}d_6$

	3		4	
No	δC	δH (<i>J</i> in Hz)	δC	δH (<i>J</i> in Hz)
1	173.1		172.5	
2	122.8		113.9	
3	118.9	7.56, d (9.0)	105.7	7.39, s
4	122.7	7.37, dd (9.0, 3.0)	146.1	
5	155.7		102.4	6.86, s
6	106.5	7.47, d (3.5)	150.1	
7	148.6		153.8	
8	153.4		153.4	
9	106.3		106.2	
10	163.0		162.2	
11	90.6	6.68, s	90.5	
12	162.6		162.2	
13	106.1		106.2	
14	113.1	6.96, d (7.0)	112.9	6.93, d (7.0)
15	47.4	4.87, dt (7.0, 2.5)	47.4	4.85, d (7.0)
16	102.7	5.56, t (2.5)	102.7	5.53, t (2.0)
17	145.5	6.74, t (2.5)	145.4	6.72, t (2.0)
18	56.6	3.87, s	56.5	3.84, s
19	55.6	3.85, s	55.7	3.84, s

Table 4.4. ^1H (500 MHz) NMR data for compounds **1** in CDCl_3 and ^1H (500 MHz) and ^{13}C (400 MHz) NMR data for compound **5** in $\text{DMSO-}d_6$

	1	5	
No	δH (<i>J</i> in Hz)	δC	δH (<i>J</i> in Hz)
1		181.4	
2		108.8	
3	8.09, brs	148.6	
4		120.3	6.97, d (9.0)
5	7.24, dd (6.5, 3.0)	105.2	7.43, d (9.0)
6	7.33, d (9.0)	142.9	
7		150.8	
8		154.0	
9		104.9	

10		164.8	
11	6.42, s	91.2	6.70, s
12		163.4	
13		106.7	
14	6.83, d (7.0)	113.7	6.96, d (7.0)
15	4.83, dt (7.0, 2.5)	47.6	4.85, dt (7.0, 2.5)
16	5.47, t (3.0)	103.1	5.53, t (2.5)
17	6.50, t (3.0)	146.0	6.74, t (2.5)
18		57.2	
19		56.9	

4.2.4 Compound 1 induces chromosome segregation and cell division defects

The ability of **1** to impair chromosome segregation was evaluated by inspecting chromosome morphology using fluorescence microscopy. The exposure of exponentially growing cells to sublethal concentrations of compound **1** led to a high frequency of anucleate cells at 0.25x and 0.5x MIC of **1** (Fig. 4.3AB). Thus, the drug indeed inhibits chromosome segregation in *P. aeruginosa*. This activity was enhanced by the deletion of condensins ($p < 0.05$) but not *parB* (Figure. 4.3B), indicating that the drug acts primarily on the ParAB pathway.

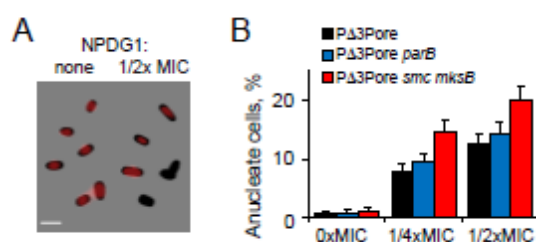


Figure 4.3. Micrograph of *P. aeruginosa* incubated with **1** and quantification graphs

4.2.5 Molecular Docking Studies

The genetic studies described above suggested that **1** directly inhibits ParA or ParB proteins. To elucidate a plausible molecular mechanism of this interaction, we performed computer assisted docking of **1** to the proteins. Although multiple crystal structures of ParA and ParB are available, none are for the *P. aeruginosa* proteins. Therefore, we generated the structure of the ParAB complex using Alphafold 2. The predicted structure is shown in Figure 5A together with a DNA-bound ParB from *Caulobacter crescentus* (PMID: 32698006), which are virtually superimposable in globular regions.

Molecular docking was then performed using Schrodinger software suite. The highest-ranking binding site for **1** was predicted at a junction between the DNA-binding and N-terminal domains (Fig. 4.4A). The largely hydrophobic binding pocket could accommodate all sterigmatocystin analogs. In contrast to the other compounds, however, **1** was predicted to form two hydrogen bonds, which extended simultaneously to Tyr152 of ParA and the Leu15 backbone of ParB (Fig. 4.4B). Notably, compound **7** was the only inactive compound predicted to produce two hydrogen bonds, but these involved the same Tyr152 of ParA and a residue on a different surface of ParB, Gln154 (Fig. 4.4B). Thus, the bioactive analog of sterigmatocystin (**1**) appears to form a unique complex at the interface of ParA and ParB.

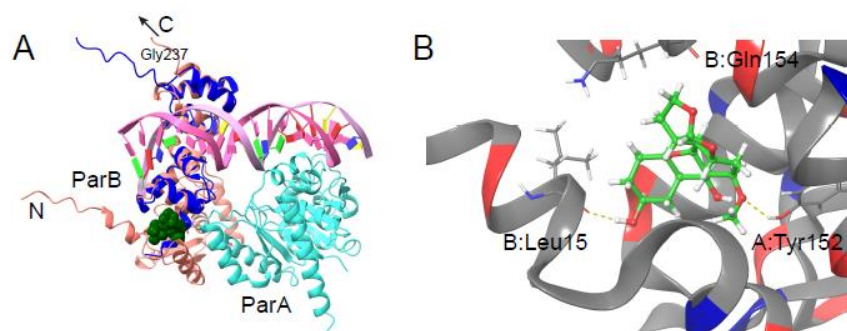


Figure 4.4. Molecular docking of **1** to ParAB protein

4.3 Materials and Methods

4.3.1 General Experimental Procedures

4.3.2 Strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 1 and Table 2. The P43-Pore strain was constructed by sequential in-frame deletion of three efflux pumps from PAO1-UW¹²⁴. An IPTG-inducible *fhuA* gene was then inserted into this strain using plasmid pGK-LAC-*fhuA* $\Delta C / \Delta 4L$ ¹²⁵ to yield P43-Pore. Deletions of *parB*, *smc* and *mksB* in this strain were generated as previously described.^{124, 126} Complementation plasmid pUCP-SMC, pUCP-MksB were constructed and described as before.¹²⁷ To construct ParB complementation plasmid, *lacI^q* gene and T7 early promoter were amplified by PCR from plasmid pEX-LacI-MksB¹²⁷ and incorporate between SacI and XbaI restriction sites of pUCP22, generating pUCP-LacI plasmid. pUCP-LacI-ParB were constructed by incorporation of *parB* gene between XbaI and HindIII restriction sites of pUCP-LacI plasmid.

4.3.3 Microbiology methods

Luria-Bertani (LB) broth (10 g of Bacto tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7.0) was used for bacterial growth. The parental strain P43-Pore and its single *parB*, double *smc mksB* deletion strains were inoculated overnight at 37°C. Cells were subcultured at 1:100 into a fresh 30-ml volume of LB medium and grown at 37°C to an optical density at 600 nm (OD600) of 0.3. The cells culture was then supplemented with 0.1 mM isopropyl β -d-1-thiogalactopyranoside (IPTG), to induce expression of “Pore” protein. Fungal extracts were dissolved in dimethylsulfoxide (DMSO) at 10 mg/ml. The minimal inhibitory concentrations (MIC) of fungal extracts were determined using a 2-fold broth dilution method¹²⁵ with or without IPTG as appropriate. The OD600 was measured using a Tecan Spark 10 M microplate reader. The growth curves were then fit to a Hill equation using Matlab to determine the half-maximal inhibitory (IC₅₀) concentration. The average and standard deviation (SD) of MIC and IC₅₀ were calculated from at least two independent measurements.

The anucleate cell formation was quantified as before with modifications.¹²⁷ Briefly, the bacteria were incubated overnight at 37°C in LB medium with shaking, subcultured into a fresh LB medium, supplied with the extract and further incubated overnight. 300 μ l cells were then fixed in final 70% ice cold ethanol, incubated on ice for at least 10 min, rinsed with phosphate buffered saline (PBS), stained with 100 nM DAPI and 1x SyproOrange, deposited onto 1% agarose pad microscope slides, and observed using fluorescence microscopy. (Olympus BX50 equipped with a BX-FLA mercury light source and a 100 $\times$ 1.43-numerical-aperture oil immersion objective). Phase-contrast and fluorescent images were collected using a ORCA-Fusion Digital CMOS camera and analyzed by Photoshop.

Optical rotation measurements were made using a Rudolph Research Autopol III automatic polarimeter. NMR data were collected on Varian 400 and 500 MHz NMR spectrometers. Accurate mass (HRESIMS) data were collected on an Agilent 6546 high mass resolution QTOF mass spectrometer. LC-ESI-MS data were obtained on a Shimadzu LC-MS 2020 system (ESI quadrupole) coupled to a photodiode array detector with a Phenomenex Kinetex column (2.6 μm C₁₈ column, 100 Å, 75 x 3.0 mm). Vacuum column chromatography was performed over silica gel (VWR, 40-60 μm , 6Å) and HP20ss gel (Sorbtech). The preparative HPLC system was equipped with Shimadzu SCL-10A VP pumps and a system controller using a Gemini 5 μm C₁₈ column (210 Å, 250 x 21.2 mm) with a flow rate of 10 mL/min. The semipreparative HPLC system utilized Waters 1525 binary pumps with Waters 2998 photodiode array detectors and Gemini 5 μm C₁₈ (110 Å, 250 x 10 mm) and biphenyl column (110 Å, 250 x 10 mm) with a flow rate of 4 mL/min. All solvents used were of ACS grade or better.

Table 4.5. Strains used in this study

Strain	Relevant genotype or description	Source or reference
DH5 α	<i>supE44</i> Δ <i>lacU169</i> <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Lab stock
SM10 (λ pir)	<i>thi</i> <i>thr</i> <i>leu</i> <i>tonA</i> <i>lacY</i> <i>supE</i> <i>recA::RP4-2-</i> Tc::Mu Km λ pir	Lab stock
PAO1-UW	<i>lacIq+</i> Δ (<i>lacZ</i>)M15+ <i>tetA+</i> <i>tetR+</i>	ATCC 47085
P Δ 3-Pore	PAO1-UW Δ <i>mexAB-oprM</i> Δ <i>mexCD-oprJ</i> Δ <i>mexXY</i>	This study
P Δ 3-Pore Δ <i>parB</i>	P Δ 3-Pore <i>parB</i> deletion	This study
P Δ 3-Pore Δ <i>mksB</i> Δ <i>smc</i>	P Δ 3-Pore <i>mksB</i> <i>smc</i> deletion	This study

Table 4.6. Plasmids used in this study

Plasmid	Description	Source or reference
pEX18AP- Δ <i>mexAB-oprM</i>	<i>mexAB-oprM</i> deletion vector	124
pEX18AP- Δ <i>mexCD-oprJ</i>	<i>mexCD-oprJ</i> deletion vector	124
pEX18AP- Δ <i>mexXY</i>	<i>mexXY</i> deletion vector	124
pGK-LAC- <i>fhuA</i> Δ C/ Δ 4L	FhuA insertion vector	125
pEXG2- Δ <i>parB</i>	<i>parB</i> deletion vector	124
pEX18Ap- Δ <i>smc</i>	<i>smc</i> deletion vector	126
pEX18Ap- Δ <i>mksB</i>	<i>mksB</i> deletion vector	126
pUCP22	Shuttle vector	
pUCP-SMC	SMC expression vector	127
pUCP-MksB	MksB expression vector	127
pUCP-LacI	pUCP22 with inducible promoter	This study
pUCP-LacI-ParB	ParB expression vector	This study

4.3.4 Fungal Isolates and Fermentation

The *Humicola* sp. isolate was obtained from a soil sample collected near Oklahoma City, OK. The fungus was identified by homogenizing the mycelium in a BulletBlender (MidSci #BB24M) with zirconium oxide beads in TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). The lysate was subjected to PCR with the ITS region (i.e., ITS1, 5.8S, and ITS2 regions) was amplified and sent for sequencing. The resulting sequence was compared to fungal sequences in Genbank, which led to greater than 99% identity matches to isolates

described as *Humicola* sp. Sequence data was deposited in GenBank (accession number OP007127).

The fungus was grown in mycobags (Unicorn Bags, Plano, TX) with a monolayer of Cheerios breakfast cereal supplemented with a 0.3% sucrose and 0.005% chloramphenicol solution. Cultures were grown for four weeks at room temperature.

4.3.5 Extraction and Purification

Fungal biomass was extracted with 2 L of EtOAc (x3) at room temperature. The EtOAc extract (12 g) was subjected to silica gel vacuum liquid chromatography with step-gradient elution consisting of 1:1 hexanes/DCM, DCM, 10:1 DCM/MeOH and MeOH to yield four fractions. The 10:1 DCM/MeOH fraction (3 g) was further fractionated by HP20ss vacuum-liquid chromatography eluted with a MeOH-H₂O step-gradient (30:70, 50:50, 70:30, 90:10, and 100:0) and washed with CH₂Cl₂-MeOH (50:50). Bioassay analysis of the resulting fractions revealed that 90% MeOH fraction contained the bioactive material. The bioactive fraction was further fractionated by C₁₈ preparative HPLC (250 x 21.2 mm, 5 μ m) with a MeOH-H₂O gradient (40:60 to 50:50) followed by semipreparative HPLC using a biphenyl column (isocratic CH₃CN-H₂O, 60:40) to obtain **1** (11.0 mg), **2** (3.0 mg), **3** (1.3 mg), **4** (2.0 mg), **5** (10.4 mg), **6** (7.8 mg), and **7** (1.8 mg).

Compound 1

Pale yellow solid, $[\alpha]_D^{20}$ -98 (c 0.1, MeOH); ¹H and ¹³C NMR, see Table 4.3; HRESIMS [M+H]⁺ m/z 325.0703 (calcd for C₁₈H₁₃O₆, 325.0712)

Compound 2

Pale yellow solid, $[\alpha]^{20}_{\text{D}} -110$ (c 0.1, MeOH); ^1H and ^{13}C NMR, see Table 4.3; HRESIMS
 $[\text{M}+\text{H}]^+ m/z$ 325.0702 (calcd for $\text{C}_{18}\text{H}_{13}\text{O}_6$, 325.0712)

Compound 3

Pale yellow solid, $[\alpha]^{20}_{\text{D}} -134$ (c 0.1, MeOH); ^1H and ^{13}C NMR, see Table 4.4; HRESIMS
 $[\text{M}+\text{H}]^+ m/z$ 339.0853 (calcd for $\text{C}_{19}\text{H}_{15}\text{O}_6$, 339.0869)

Compound 4

Pale yellow solid, $[\alpha]^{20}_{\text{D}} -64$ (c 0.1, MeOH); ^1H and ^{13}C NMR, see Table 4.4; HRESIMS
 $[\text{M}+\text{H}]^+ m/z$ 355.0799 (calcd for $\text{C}_{19}\text{H}_{15}\text{O}_7$, 355.0818)

Compound 5

Pale yellow solid, $[\alpha]^{20}_{\text{D}} -96$ (c 0.1, MeOH); ^1H and ^{13}C NMR, see Table 4.5; HRESIMS
 $[\text{M}+\text{H}]^+ m/z$ 355.0799 (calcd for $\text{C}_{19}\text{H}_{15}\text{O}_7$, 355.0818)

Chapter 5. Semisynthetic derivatization studies of the fungal metabolite phomasetin with activity against the human parasites *Trichomonas vaginalis* and *Mycoplasma genitalium*

This chapter was adapted from a manuscript which is currently being prepared for submission in 2023. The authors are Thilini Peramuna, Ziwei Hu, Karen Wendt, Gwendolyn E. Wood, Robert H. Cichewicz, and Adam S. Duerfeldt

*The work presented in this chapter was conducted as follows: Thilini Peramuna performed extraction, purification, structure elucidation of compounds **1-6f'** and semi-synthesis of compounds **4a-6f'**, Ziwei Hu performed semi-synthesis of compounds **1a-3b**. Karen Wendt performed the isolation of fungal isolates large-scale fermentations and *T. vaginalis* assay, Gwendolyn E. Wood conducted the *M. genitalium* assay development and bioassay.*

5.1 Introduction

Trichomoniasis is the most prevalent sexually transmitted parasitic disease in the world. The disease is caused by the flagellated protozoan *Trichomonas vaginalis*.⁸³ It is estimated that 5-7 million new cases of trichomoniasis are reported in the United States and >200 million cases in the world every year.¹²⁸ Those who are infected with trichomoniasis exhibit an increased risk of developing bacterial vaginosis, candidiasis, HIV infection, cervical and prostate cancer.¹²⁹ Trichomoniasis has also been associated with adverse pregnancy outcomes including low birth weight, preterm delivery and premature rupture of placental membranes.⁸¹ Current clinical treatments for *T. vaginalis* infection are limited to nitroimidazoles.⁸⁵ Although administration of nitroimidazoles offer high level of clinical

efficacy in the majority of cases, treatment failures have been reported in 5-17% of infections.¹³⁰ Further, possible carcinogenic and mutagenic properties has been associated with nitroimidazoles apart from several side effects including vomiting and nausea.^{131 132} Considering the safety concerns, high rate of infection recurrence, and reduction in drug susceptibility, there is a need for new disease management options with a distinct mechanism of action for treating *T. vaginalis* infections.

Mycoplasma genitalium is a sexually transmitted pathogenic bacterium that causes nongonococcal urethritis (NGU), cervicitis, endometritis, and pelvic inflammatory disease (PID) in men and women.⁸⁰ Those who are infected by the bacterium exhibit an increased risk for HIV infection, cervical shedding, as well as certain adverse pregnancy outcomes including preterm birth and spontaneous abortion.¹³³ The prevalence of *M. genitalium* infection is ranging from 7-19.2% in the US.⁹⁹ The treatment of *M. genitalium* infection is challenging as certain antibiotics including penicillins and cephalosporins are not usable due to lack of cell wall.⁹⁶ The commonly used antibiotics for treating this infection are macrolides, tetracycline, and quinolones.¹³⁴ The CDC (Centers for Disease Control and Prevention) guideline for the treatment of *M. genitalium* infection indicates azithromycin as a first-line and moxifloxacin as a second-line therapeutics.¹³⁵ However, the rapid emergence of antimicrobial resistance to the recommended first and second line antibiotics has become a concern.¹⁰³ The occurrence of multi-drug resistant strains has reached more than 50% for macrolides and 10% for quinolones.¹⁰³ The global rise in *M. genitalium* resistance has triggered the need to explore novel drug targets and designing new antimicrobial agents to circumvent this deadly infection.

Derivatization of bio active natural products is an attractive strategy to improve activity, selectivity, potency, solubility, and other physiochemical properties.⁶⁵ Further, it provides synthetic handles for further derivatization toward the production of semisynthetic variants.¹³⁶ Our previous report based on screening fungal natural product library identified a group of promising tetramic-acid-containing fungal secondary metabolites as inhibitors of *T. vaginalis*.⁸⁷ The compounds including phomasetin (**1**), pyrrolocin A (**2**) and beauversetin were identified as the most potent molecules against *T. vaginalis*. The tetramic acids including equisetin, beauversetin, **1** and **2** showed a remarkable inhibitory activity against *M. genitalium*.

Considering the potency and selectivity for *T. vaginalis* and *M. genitalium* we selected **1** to construct a semi-synthetic library to explore further possibilities in improving activity and selectivity. However, as we have not yet discovered the target of the tetramic acids in any of these pathogens, systematic modification of **1** was challenging. Therefore, chemical modifications were carried out at the olefinic side chain, decalin ring and mainly at the tetramic acid using ‘click’ chemistry. This report focuses on our efforts in chemically modifying **1** and the activity and selectivity towards test pathogens.

5.2 Results and Discussion

The improved activity of **2** (EC₅₀ 0.06 µM) compared to **1** (EC₅₀ 1.8 µM) suggested that elongation and oxidation of the olefinic side chain improved the activity. (Figure 5.1)

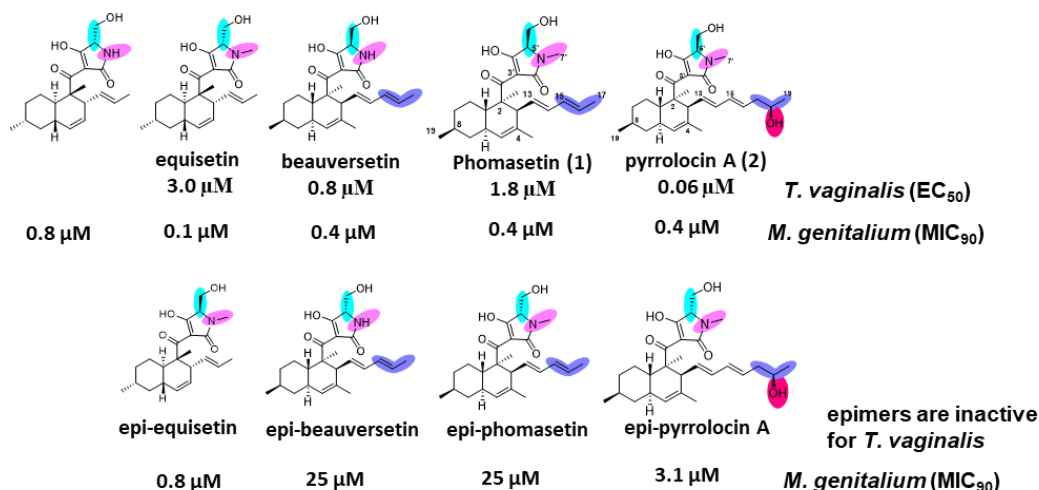


Figure 5.1. Series of tetramic acids with activity against *T. vaginalis* and *M. genitalium*

Based on this observation the initial set of chemical modifications were carried out on the olefinic side chain of **1**. (Figure 5.2)

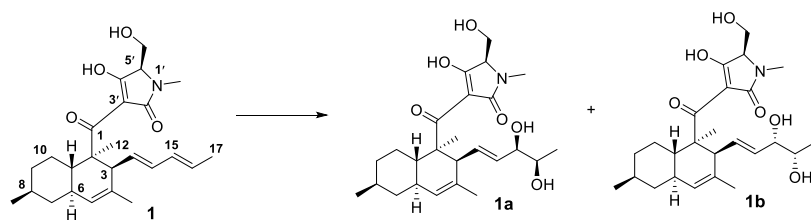


Figure 5.2. Oxidation [NMO (1.5 eq), OsO₄ (0.1 eq) (H₂O/THF, 2h)] of **1**

Table 5.1. Bioactivity of **1a** and **1b** against *T. vaginalis* and *M. genitalium*

ID	<i>T. vaginalis</i> EC ₅₀ (μM)	<i>M. genitalium</i> MIC ₉₀ (μg/mL)	SI <i>M. genitalium</i>
1a	>10	>50	ND
1b	>10	12.5	ND

The oxidation of **1** yielded two products (**1a** and **1b**) of diols at C-15 and C-16. The relative configurations of **1a** and **1b** were confirmed by COSY, HSQC, HMBC and ROSEY correlations. The absolute configurations of diols were determined using the modified Mosher method. The absolute configurations of C-15 and C-16 stereocenters in **1a** were

determined as 15*R* and 16*R*. The absolute configurations of C-15 and C-16 stereocenters in **1b** were determined as 15*S* and 16*S*. According to the bioassay results both compounds lost the potency against test pathogens regardless of the stereochemistry.

We next evaluated the significance of the olefinic side chain towards the potency in both pathogens. The olefinic side chain was reduced using hydrogenation reaction. Interestingly the hydrogenation of **1** yielded four products including **2a**, **2b**, **2c** and **2d**. (Figure 5.3)

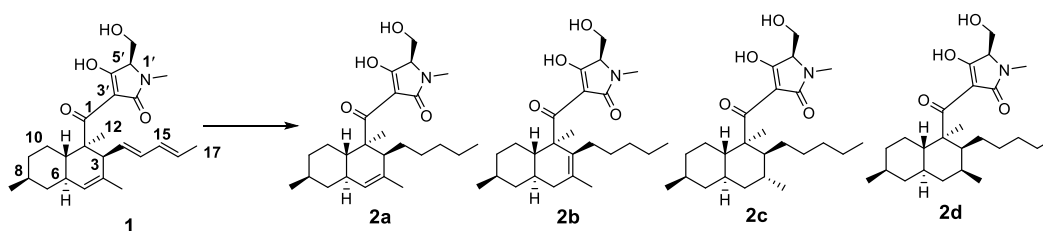


Figure 5.3. Hydrogenation [H₂, Pd/C (EtOH, 4h)] of **1**

Table 5.2. Bioactivity of **2a** – **2d** against *T. vaginalis* and *M. genitalium*

ID	<i>T. vaginalis</i> EC ₅₀ (μM)	<i>M. genitalium</i> MIC ₉₀ (μg/mL)	SI <i>M. genitalium</i>
2a	0.71	1.6	17
2b	>10	12.5	13
2c	2.7	1.6	15
2d	2.1	0.2	18

In compound **2a**, hydrogenation has taken place at C-13, 14, 15 and 16 in the olefinic side chain. In contrast, in compound **2b** a migration of C-5 double bond to C-3 in the decalin ring along with the reduction at C-13, 14, 15 and 16 had taken place. A complete reduction of C-4 double bonds was observed in compounds **2c** and **2d** which led to the formation of a new stereocenter at C-4. The relative configuration of the chemical structures was confirmed using COSY, ROSEY and HMBC data. The absolute configuration of the decalin moiety in **1** was reported as 2*R*, 3*S*, 6*R*, 8*S* and 11*S*.¹³⁷ The absolute configurations

of **2c** and **2d** were deduced based on key ^1H - ^1H ROSEY correlations. In **2d** based on key ^1H - ^1H ROSEY correlation between H-4 $\leftrightarrow$ H-3eq the absolute configuration of C-4 was deduced as 4*S*. No ROSEY correlation was observed in **2c** which led to the assignment of absolute configuration of C-4 as 4*R*.

Migration of C-5 double bond to C-3 in **2b** led to reduced activity in both pathogens. In contrast compound **2a** was active against *T. vaginalis* with an EC_{50} of 0.71 μM . Compounds **2c** and **2d** showed similar activity (EC_{50} $\sim$ 2 μM) against *T. vaginalis*. Compounds **2a** and **2c** had same activity (MIC_{90} 1.6 $\mu\text{g/mL}$) against *M. genitalium* and in contrast 4*S* configuration at C-4 in **2d** led to the increased potency (MIC_{90} 0.2 $\mu\text{g/mL}$). The selectivity index (SI) of all the analogues against *M. genitalium* were calculated based on the concentration of each analogue that inhibited 90% of *M. genitalium* growth versus the concentration of each analogue that inhibited 50% alamar blue reduction in vero cells. The analogues **2a**, **2c** and **2d** showed selectivity indices of 17, 15 and 18 respectively.

A significant feature in all the tetramic acids tested in this study was the formation of tautomers. Therefore, next we turned our focus towards investigating the tetramic acid. The reaction of **1** with $\text{NaBH}(\text{OAc})_3$ led to the formation of **3a** and **3b**. (Figure 5.4)

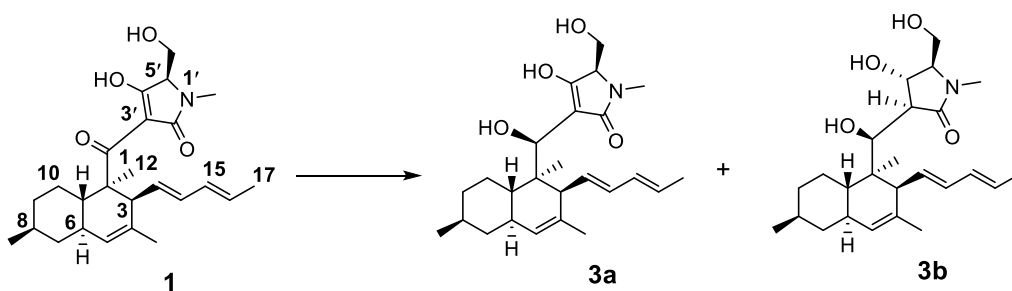


Figure 5.4. Reduction of **1** using $\text{NaBH}(\text{OAc})_3$ (1.5 eq), EtOH (4 h)

Table 5.3. Bioactivity of **3a** and **3b** against *T. vaginalis* and *M. genitalium*

ID	<i>T. vaginalis</i> EC ₅₀ (μM)	<i>M. genitalium</i> MIC ₉₀ (μg/mL)	SI <i>M. genitalium</i>
3a	>10	3.1	<2
3b	>10	12.5	ND

A new stereocenter was generated at C-1 in compounds **3a** and **3b**. The relative configurations of **3a** and **3b** were confirmed using ¹H, ¹³C, COSY, HMBC and ROSEY NMR data. The absolute configuration of the alcohol at C-1 in **3a** was determined using modified Mosher method and was assigned as *R*. The absolute configurations of alcohols at C-1 and C-4' in **3b** were determined using modified Mosher method and were assigned as 1*S* and 4'*S*. On the basis of the ¹H-¹H ROSEY correlations in **3b** between H-1 ↔ H-3' and H-3' ↔ H-5', the absolute configuration of C-3' was deduced as *S*. Both compounds exhibited decreased potency (EC₅₀ >10 μM) against *T. vaginalis*. In contrast, compound **3a** was active (MIC₉₀ 3.1 μg/mL) against *M. genitalium* suggesting that alcohol at C-1(1*R*) improved the activity. However, the SI (<2) of **3a** for *M. genitalium* has drastically decreased. Compound **3b** lost the potency against *M. genitalium*.

Next, we decided to focus on modifying the primary alcohol at C-6' in the tetramic acid. First, we modified 6'-OH by substituting an ester and several ethers. (Figure 5.5)

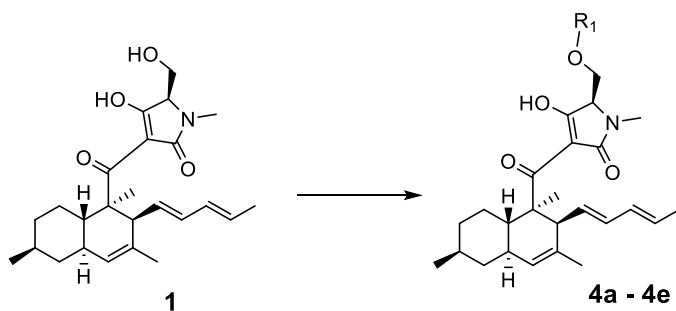
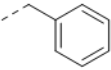
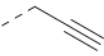


Figure 5.5. Esterification ($(\text{CH}_3\text{CO})_2\text{O}$ (5eq), pyridine (5 h) and etherification of **1** using K_2CO_3 (10 eq) R_1Br (5 eq), (12 h)

The esterification of **1** (**4a**) was achieved using acetic anhydride. Different ether analogs of **1** were semi-synthesized by substituting benzene (**4b**), methyl (**4c**), 2-methyl butene (**4d**) and 1-propyne (**4e**).

The esterification and etherification of **1** led to a complete loss in activity ($\text{EC}_{50} > 10 \mu\text{M}$) against *T. vaginalis*. The loss of activity ($\text{MIC}_{90} 12.5 \mu\text{g/mL}$) was observed in **4a** and **4b** against *M. genitalium*. However, **4c**, **4d** and **4e** showed activity against *M. genitalium* with highest activity in **4c** ($\text{MIC}_{90} 1.6 \mu\text{g/mL}$) with a SI of 12.

Table 5.4. Bioactivity of **4a** – **4e** against *T. vaginalis* and *M. genitalium*

ID	R ₁	<i>T. vaginalis</i> EC ₅₀ (μM)	<i>M. genitalium</i> MIC90 (μg/mL)	SI <i>M. genitalium</i>
4a		>10	12.5	ND
4b		>10	12.5	ND
4c		>10	1.6	12
4d		>10	3.1	5
4e		>10	6.3	ND

The copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction, known as ‘click chemistry’ involves stepwise carbon – nitrogen bond formation via a copper acetylide intermediate to give exclusively 1,4-disubstituted 1,2,3-triazoles.¹³⁸ ‘Click chemistry’ has become a powerful technique for the rapid construction of pharmacologically active compounds.¹³⁹ The 1,2,3 -triazole ring is chemically stable and relatively resistant to metabolic degradation.¹⁴⁰ Therefore, we deployed a click-chemistry based linking strategy to couple a variety of substituents using the terminal alkyne at the tetramic acid to derive triazole linked phomasetin series. During the derivatization we observed formation of two products with 1:1 ratio and the comparison of NMR chemical shifts suggested the formation of C-5'epimer at the tetramic acid as an additional product. (Figure 5.6)

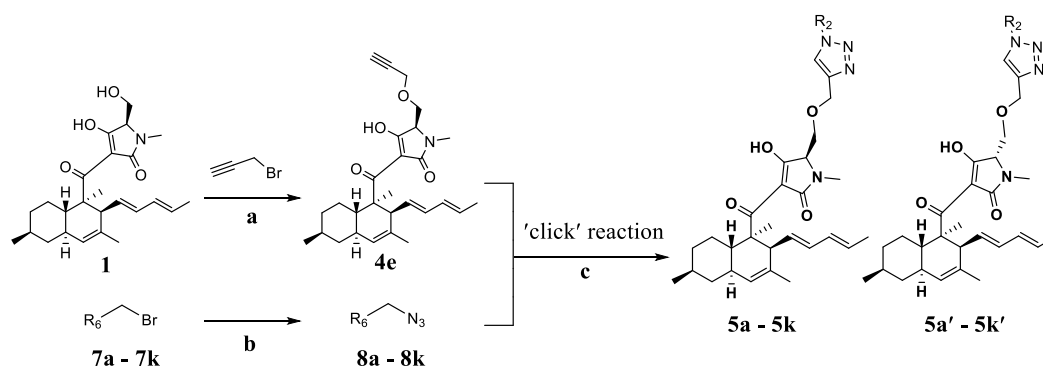
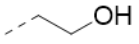
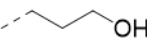
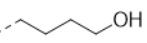
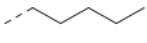
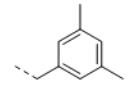
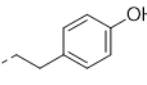
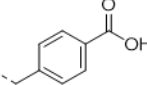


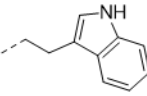
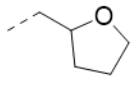
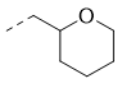
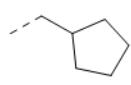
Figure 5.6. General scheme for click chemistry products with **1**. Reaction conditions (a) K₂CO₃, DMF, 12h (b) NaN₃, K₂CO₃, H₂O, 80°C, 12 h (c) CuSO₄·5H₂O, sodium ascorbate, ACN/H₂O (1:1)

First, we linked primary hydroxy group with different chain lengths which led to the formation of products including **5a/5a'**, **5b/5b'** and **5c/5c'**. (Table 5.5) The compounds **5a**, **5b** and **5c** were identified as the corresponding triazole analog of **1**. The compounds **5a'**, **5b'**, and **5c'** were identified as 5' epimers of the corresponding triazole analogues of **1**. Interestingly, **5a'** and **5c** showed potency against *M. genitalium* with MIC₉₀ of 3.1 µg/mL and 6.3 µg/mL respectively. The SI of **5a'** for *M. genitalium* was 17. The other compounds showed no activity against either pathogen. Next, triazole linked hydrocarbon chain analogues (**5d**, **5d'**) were semi-synthesized. However, both analogues were inactive against the test pathogens. The dimethyl substituted aryl ring was installed to yield **5e** and **5e'** analogues. However, both analogues showed no activity. Next, we decided to link hydrogen bond donor and acceptor groups including phenol (**5f** and **5f'**) and benzoic acid (**5g** and **5g'**) groups. Interestingly, **5f** (MIC₉₀ 3.1 µg/mL) and **5f'** (MIC₉₀ 1.6 µg/mL) showed activity against *M. genitalium* while **5g** (EC₅₀ 2.2 µM) and **5g'** (EC₅₀ 7.5 µM) showed activity against *T. vaginalis*. A high value of SI (24) was obtained for **5f'** compared to the SI of **5f** (12) for *M. genitalium*. The bicyclic molecule indole has recognized for its

diverse therapeutic activity in medicinal chemistry.¹⁴¹ The indole is used in drug discovery of medicinal drugs such as antimicrobials, anticancer agents, anti-proliferative agents, and anti-inflammatory agents.^{142, 143, 144} Therefore, we used indole to obtain the analogues containing triazole linked indole (**5h** and **5h'**). Both analogues were active against *M. genitalium* with MIC₉₀ of 3.1 µg/mL for **5h** and MIC₉₀ of 6.3 µg/mL for **5h'**. The SI of **5h** was 13 and the SI of **5h'** was 3.8. Incorporating heterocycles is an attractive strategy in medicinal chemistry.^{145, 146} Further, their confined geometries add rigidity to a molecule's structure in maintaining the spatial orientation of the pharmacophoric groups.^{147, 148} Therefore, tetrahydrofuran (**5i**, **5i'**) and tetrahydropyran (**5j**, **5j'**) heterocycles were incorporated to **1** via triazole link. The tetrahydrofuran analogue (**5i**) was potent (EC₅₀ 5.4 µM) against *T. vaginalis* and tetrahydropyran analogues (**5j**, **5j'**) showed equipotent (MIC₉₀ 3.1 µg/mL) against *M. genitalium* with an SI of 3.1. The tetrahydrofuran was substituted by cyclopentane (**5k**, **5k'**) to test the importance of oxygen in tetrahydrofuran. Compounds **5k** and **5k'** showed activity against *M. genitalium* with MIC₉₀ of 6.3 µg/mL and 3.1 µg/mL respectively. The SI of **5k** and **5k'** were obtained as 6.3 and 3.1 respectively.

Table 5.5. Bioactivity of **5a-5k'** against *T. vaginalis* and *M. genitalium*

ID	R ₂	<i>T. vaginalis</i> EC ₅₀ (μM)	<i>M. genitalium</i> MIC90 (μg/mL)	SI <i>M. genitalium</i>
5a		>10	12.5	ND
5a'		>10	3.1	17
5b		>10	12.5	ND
5b'		>10	12.5	ND
5c		>10	6.3	ND
5c'		>10	12.5	ND
5d		>10	12.5	ND
5d'		>10	12.5	ND
5e		>10	12.5	ND
5e'		>10	12.5	ND
5f		>10	3.1	12
5f'		>10	1.6	24
5g		2.2	12.5	ND
5g'		7.5	>12.5	ND

ID	R ₂	<i>T. vaginalis</i> EC ₅₀ (μM)	<i>M. genitalium</i> MIC90 (μg/mL)	SI <i>M. genitalium</i>
5h		>10	3.1	13
5h'		>10	6.3	3.8
5i		5.4	12.5	ND
5i'		>10	12.5	ND
5j		>10	3.1	3.1
5j'		>10	3.1	3.1
5k		>10	6.3	6.3
5k'		>10	3.1	3.1

When examined collectively, the modifications at 6'-OH in tetramate ring of **1** through triazole linked substituents led to the conclusion that primary alcohol, phenol, benzoic acid and tetrahydropyran improved the activity. Thus, we decided to incorporate those groups to **2** via click chemistry. (Figure 5.8)

Since the click chemistry reaction required a terminal alkyne, first we semi-synthesized the terminal alkyne (**6a**) at C-5. This reaction yielded a disubstituted terminal alkyne analogue (**6b**) of **2** as a minor product. (Figure 5.7) Both **6a** and **6b** were tested against the pathogens. The mono-substituted analogue (**6a**) showed activity against both pathogens including MIC₉₀ of 3.1 μg/mL for *M. genitalium* and EC₅₀ of 3.9 μM for *T. vaginalis*. The disubstituted

alkyne (**6b**) was active (MIC₉₀ 3.1 µg/mL) against *M. genitalium*. The SI of **6a** was 23 and the SI of **6b** was 18 for *M. genitalium*.

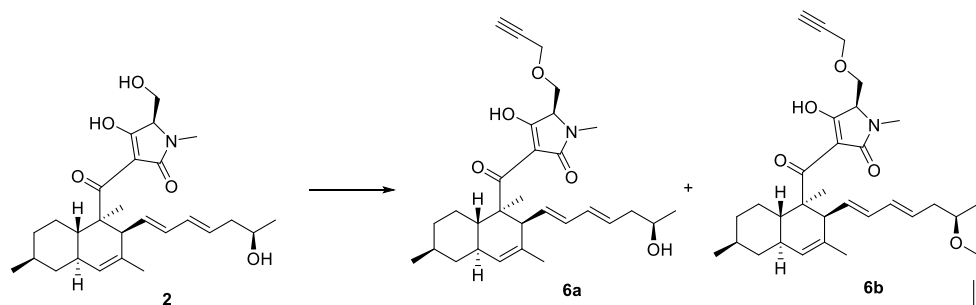


Figure 5.7. Etherification of **2** using NaH (6 eq) propargyl bromide (4 eq), (12 h)

Table 5.6. Bioactivity of **6a** and **6b** against *T. vaginalis* and *M. genitalium*

ID	<i>T. vaginalis</i> EC ₅₀ (µM)	<i>M. genitalium</i> MIC ₉₀ (µg/mL)	SI <i>M. genitalium</i>
6a	3.9	3.1	23
6b	>10	3.1	18

During semi-synthesis of triazole analogues we obtained the 5' epimer of **2** as an additional product as expected. (Figure 5.8) The primary alcohol linked via triazole yielded **6c** and **6c'**. The analogue **6c** was active against *M. genitalium* with MIC₉₀ of 6.3 µg/mL and a SI of 10. The phenolic and benzoic acid derivatives of **2** were obtained (**6d** and **6e**) with their respective 5' epimer (**6d'** and **6e'**). Interestingly **6d** was active against *T. vaginalis* (EC₅₀ = 3.3 µM), and **6d'** was active (MIC₉₀ 6.3 µg/mL) against *M. genitalium* with SI of 20. The antitrichomonal activity was observed for tetrahydropyran analogue **6f** with an EC₅₀ of 6.8 µM. (Table 5.7)

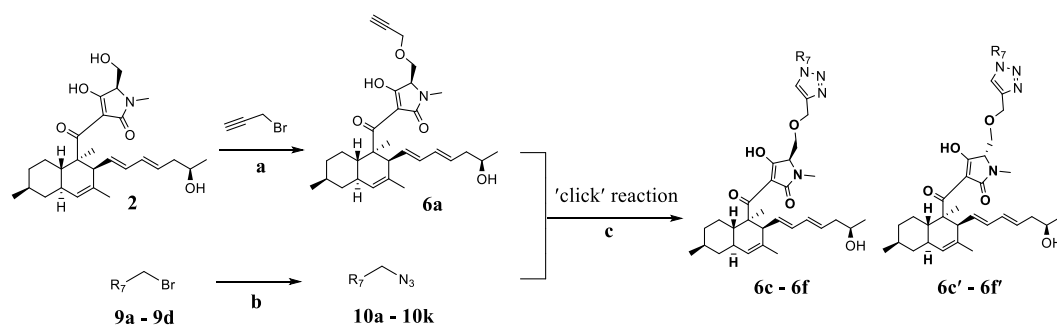


Figure 5.8. General scheme for click chemistry products with **2**. Reaction conditions (a) NaH, DMF, 12h (b) NaN₃, K₂CO₃, H₂O, 80 °C, 12 h (c) CuSO₄·5H₂O, sodium ascorbate, ACN/H₂O (1:1)

Table 5.7. Bioactivity of **6c-6f'** against *T. vaginalis* and *M. genitalium*

ID	R ₇	<i>T. vaginalis</i> EC ₅₀ (μM)	<i>M. genitalium</i> MIC90 (μg/mL)	SI <i>M. genitalium</i>
6c		>10	6.3	10
6c'		>10	>25	ND
6d		3.3	>25	ND
6d'		>10	6.3	20
6e		>10	25	ND
6e'		>10	>25	ND
6f		6.8	12.5	ND
6f'		>10	12.5	ND

In summary, fungal derived tetramic acid **1** was semi-synthetically modified to investigate the opportunities in improving potency and selectivity against *T. vaginalis* and *M. genitalium*. The systematic modifications at different moieties of **1** revealed evidence in improving activity and selectivity. (Figure 5.9) In general, the olefin at C-4 in the decalin ring was important. The oxidation at C-15 and C-16 in the olefinic side chain had no effect in driving the activity. The reduction at olefinic side chain improved the activity. The alcohol at C-4' did not improve the activity.

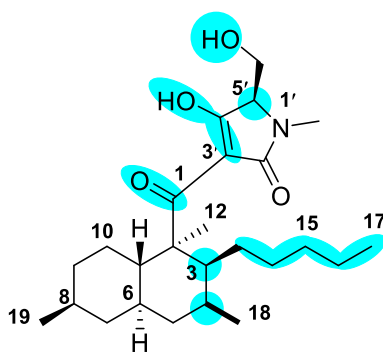


Figure 5.9. Significant structural features of **1** in exhibiting the potency against *T. vaginalis* and *M. genitalium*

Click chemistry was used to obtain more bioactive molecules against both pathogens. The triazole linked benzoic acid analogue (**5g**) and tetrahydrofuran analogue (**5i**) were identified as the most potent (EC_{50} 2.2 μ M and 5.4 μ M) compounds from the phomasetin series against *T. vaginalis*. The phenol analogue of **2** was the most active (EC_{50} 3.3 μ M) compound from the pyrrolocin A series against *T. vaginalis*. The triazole linked phenol analogue (**5f'**) of 5' epi-phomasetin was the most active and selective (MIC_{90} 1.6 μ g/mL) against *M. genitalium* with a SI of 24.

In future, chemical modifications at the decalin system may provide additional opportunities for generating new analogues that offer even further improvements in

potency and selectivity in both pathogens. Further, chemical investigation of methyl at N-CH₃, C-2, C-4 and C-8 may be beneficial in improving activity and selectivity.

5.3 Materials and Methods

5.3.1 General Experimental Procedures

NMR data were collected on Varian 400 and 500 MHz NMR spectrometers using DMSO-*d*₆ and CD₃CN-*d*₃ as solvents. HRESIMS data were obtained on an Agilent 6538 high-mass-resolution QTOF mass spectrometer. LC-ESI-MS data were obtained on a Shimadzu LCMS-2020 system (ESI quadrupole) coupled to a photodiode array detector with a Phenomenex Kinetex column (2.6 μm, C₁₈ column, 100Å, 75 x 3.0 mm). Vacuum column chromatography was performed using silica gel (VWR, 40-60 μm, 6Å) and HP20ss gel (Sorbtech). Preparative HPLC was conducted using Shimadzu system equipped with SCL-10A VP pumps and a system controller using a Gemini 5 μm C₁₈ column (210 Å, 250 x 21.2 mm) with a flow rate of 10 mL/min. The semipreparative HPLC were carried out on a Waters system (1525 binary pumps and Waters 2998 photodiode array detectors) using a Gemini 5μm C₁₈ (250 x 10 mm, 110 Å) with a flow rate of 4 mL/min. All solvents were of ACS grade or better.

5.3.2 Fungal Isolates and Fermentation

The *Phoma* sp. isolate (CA9310 TV8-3) was isolated from a soil sample collected near Castaic, CA, USA, while the *Penicillium* sp. isolate (KY6863 TV8-3) was obtained from a soil sample collected near Raceland, KY, USA. The fungal isolates were previously identified via ITS sequencing.⁸⁷ Fungi were cultured in mycobags (Unicorn Bags, Plano, TX, USA) on a solid state medium composed of a monolayer of Cheerios supplemented

with a 0.3% sucrose solution and 0.005% chloramphenicol, at room temperature for 4 weeks.

5.3.3 Extraction and Purification

The *Phoma* sp. grown on Cheerios breakfast cereal in nine large mycobags was homogenized and extracted with 5L of EtOAc (x3). The EtOAc soluble material (70 g) was subjected to silica gel vacuum liquid chromatography with step-gradient elution consisting of 1:1 Hexanes/DCM, DCM, 10:1 DCM/MeOH and MeOH yielding four fractions. The fraction 10:1 DCM/MeOH (32 g) was further fractionated by HP20ss vacuum liquid chromatography using MeOH-H₂O step-gradient (30:70, 50:50, 70:30, 90:10, and 100:0) and washed with CH₂Cl₂/MeOH (50:50) to yield six fractions. The 90% MeOH fraction contained **1** and the epimer and the fraction was further fractionated by preparative HPLC [C18, gradient elution with 40-50% MeCN in H₂O (containing 0.1% HCOOH)] over 20 min using a 10 mL/min flow rate to obtain 800 mg of **1**.

The *Penicillium* sp. grown on three large mycobags of Cheerios breakfast cereal was homogenized, extracted, and partitioned with EtOAc. The EtOAc soluble material was (15 g) subjected to silica gel vacuum liquid chromatography with elution steps of 1:1 hexanes/DCM, DCM, 10:1 DCM/MeOH, and MeOH, yielding 4 fractions. The 10:1 DCM/MeOH fraction (7 g) was subjected to HP20ss vacuum liquid chromatography and eluted with a step gradient of MeOH in H₂O (30%, 50%, 70%, 90%, and 100%) and 1:1 DCM/MeOH. Yielding 6 fractions. The 90% MeOH fraction contained **2** and the epimer. The 90% fraction was further fractionated by preparative HPLC [C18, isocratic elution with 60% MeCN in H₂O (containing 0.1% HCOOH)] over 20 min using a 10 mL/min flow rate to obtain 120 mg of **2**.

5.3.4 Culture of organisms

Trichomonas vaginalis Donne (PRA-98) was purchased from the American Type Culture Collection (ATCC, Bethesda, MD). Culture medium was Keister's Modified TYI-S33 medium (adjusted to pH 6.0 and without Diamond's vitamin solution).⁸⁷ Cultures were grown in a 37 °C incubator in sealed tubes to maintain anaerobicity.

Mycoplasma genitalium strain G37C, a single-colony filter-cloned isolate of the G37 type strain was used for these experiments (Iverson-Cabral 2007). *M. genitalium* was routinely grown in SP-4 broth (Tully 1977) containing 100 U/mL penicillin, at 37 °C with 5% CO₂ in tissue culture flasks.

5.3.5 *T. vaginalis* assay

Trichomonads were seeded at 40,000 trichomonads per well into 96 well plates. Pure compounds were dissolved in DMSO and added to the wells such that the concentration of DMSO never exceeded 0.5%. Trichomonads were exposed to atmospheric conditions during the assay setup for less than 30 minutes. BD GasPak EZ Anaerobe sachets (Becton Dickinson and Company, NJ, USA) were used according to manufacturer instructions during the 18 hr assay incubation period. Fixing solution (PBS with 1% glutaraldehyde, 5 µM propidium iodide, and 5 µM acridine orange) was added to all wells, shook on an orbital shaker to disperse cell clumps, and returned to the incubator for 3 hr. The plates were then imaged using the PerkinElmer Operetta and analyzed using the Harmony 3.5.1 software package. EC₅₀s were calculated in R (R Development Core Team. R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing;

Vienna, Austria, 2019) using the ‘drc’ package (Ritz, C., Baty, F., Streibig, J. C., Gerhard, D. (2015), Dose-Response Analysis Using R, PLOS ONE, 10(12).

5.3.6 *M. genitalium* assay

MIC values for *M. genitalium* were determined in microbroth dilution assays (Hamasuna 2009). *M. genitalium* was grown to log phase, scraped, and frozen in multiple aliquots at -80 °C. Frozen stocks (0.2 ml) were inoculated into 2 ml SP-4 broth in 35 mm petri dishes and incubated for 48 hours to obtain metabolically active, log phase *M. genitalium*. Adherent bacteria were scraped into the culture supernatant, filtered (0.45 µm) then diluted in fresh SP-4 broth to a final concentration of 10⁵-10⁶ CFU per ml. Two-fold serial dilutions of test compounds were prepared in flat bottom, 96-well plates in 0.1 ml SP-4, 0.1 ml of the inoculum was added, and plates were incubated for approximately 7 days monitoring color change of the phenol red indicator from red to orange (due to fermentation of glucose, indicating growth). The most dilute well with no color change was considered the MIC which inhibited growth by at least 90% (confirmed by microscopy). DMSO alone at concentrations corresponding to the highest drug concentration did not inhibit *M. genitalium* growth in any experiments.

5.3.7 Synthesis of **1a** and **1b**

Compound **1** (10 mg, 1 equiv) was dissolved in THF/H₂O (v:v 1:0.5) and OsO₄ (0.1 equiv) and NMO (2 equiv) were added. The reaction mixture was stirred at 0 °C for 2h. The products were purified by semipreparative HPLC using Gemini 5 µm C₁₈ column (isocratic, 70% MeCN in water) to yield compound **1a** and **1b**

5.3.8 Synthesis of 2a, 2b, 2c, 2d

Compound **1** (10 mg) was dissolved in 1 mL EtOH and catalytic amount of palladium was added. The mixture was purged with H₂ for 8h at room temperature. The products were purified over a semipreparative HPLC using a Gemini 5 µm C₁₈ column (isocratic, 65% MeCN in water) to yield compound **2a – 2d**

5.3.9 Synthesis of 3a, 3b

Compound **1** (10 mg) was dissolved in 1 mL EtOH and NaBH(OAc)₃ (10 equiv) was added. The mixture was stirred for 2.5 h at room temperature. The products were purified by semipreparative HPLC using Gemini 5 µm C₁₈ column (isocratic, 60% MeCN in water) to yield compound **3a** and **3b**

5.3.10 Synthesis of 4a

Compound **1** (10 mg) was mixed with Ac₂O (1 mL) in DCM (2 mL) followed by the addition of 4 Å molecular sieves (50 mg). The reaction mixture was stirred at 40 °C overnight, and the solvent was removed *in vacuo*. The residue was purified by semipreparative HPLC using Gemini 5 µm C₁₈ column (isocratic, 80% MeCN in water) to yield compound **4a**

5.3.11 Synthesis of 4b

Compound **1** was dissolved in 1 mL DMF and K₂CO₃ (10 equiv) and benzyl bromide (5 equiv) was added. The mixture was stirred overnight at room temperature. The products were purified by semipreparative HPLC using Gemini 5 µm C₁₈ column (isocratic, 70% MeCN in water) to yield compound **4b**

5.3.12 Synthesis of **4c**

Compound **1** was dissolved in 1 mL DMF and K₂CO₃ (10 equiv) and methyl iodide (5 equiv) was added. The mixture was stirred overnight at room temperature. The products were purified by semipreparative HPLC using Gemini 5 μm C₁₈ column (isocratic, 65% MeCN in water) to yield compound **4c**

5.3.13 Synthesis of **4d**

Compound **1** was dissolved in 1 mL DMF and K₂CO₃ (10 equiv) and 1-bromo-2-methyl propane (5 equiv) was added. The mixture was stirred overnight at room temperature. The products were purified by semipreparative HPLC using Gemini 5 μm C₁₈ column (isocratic, 75% MeCN in water) to yield compound **4d**

5.3.14 Synthesis of **4e**

Compound **1** was dissolved in 1 mL DMF and K₂CO₃ (10 equiv) and propargyl bromide (5 equiv) was added. The mixture was stirred overnight at room temperature. The products were purified by semipreparative HPLC using Gemini 5 μm C₁₈ column (isocratic, 70% MeCN in water) to yield compound **4e**

5.3.15 Synthesis of compounds **5a-5k**

To a solution of corresponding reactant **7a-7k** (5 equiv) in water (10 mL) was added K₂CO₃ (10 equiv) and sodium azide (10 equiv). The reaction mixture was stirred at 80 °C for 12 h, then cooled to room temperature and extracted by DCM (10 mL × 3). The organic layer was partitioned, combined, washed with brine (15 mL), dried over MgSO₄, and concentrated to afford crude product **8a – 8k**. The residue **8a – 8k** was then dissolved in a

mixture of ACN/H₂O (v:v 4:1, 1 mL). Compound **1** (1 equiv), sodium ascorbate (5 equiv), and CuSO₄·5H₂O (3 equiv) were added into the mixture. The reaction mixture was stirred at 35 °C overnight. The products were purified by semipreparative HPLC using Gemini 5 µm C₁₈ column (isocratic, 70-75% MeCN) to afford the products **5a – 5k** and **5a'- 5k'**

5.3.16 Synthesis of **6a**

Compound **2** was dissolved in 1 mL DMF and NaH (6 equiv) and propargyl bromide (4 equiv) was added. The mixture was stirred overnight at room temperature. The products were purified by semipreparative HPLC using Gemini 5 µm C₁₈ column (isocratic, 75% MeCN in water) to yield compound **6a**

5.3.17 Synthesis of compounds **6c-6f**

To a solution of corresponding reactant **9a-9d** (5 equiv) in water (10 mL) was added K₂CO₃ (10 equiv) and sodium azide (10 equiv). The reaction mixture was stirred at 80 °C for 12 h, then cooled to room temperature and extracted by DCM (10 mL × 3). The organic layer was partitioned, combined, washed with brine (15 mL), dried over MgSO₄, and concentrated to afford crude product **10a – 10d**. The crude residue **10a – 10d** was then dissolved in a mixture of ACN/H₂O (v:v 4:1, 1 mL). Compound **1** (1 equiv), sodium ascorbate (5 equiv), and CuSO₄·5H₂O (3 equiv) were added into the mixture. The reaction mixture was stirred at 35 °C overnight. The products were purified by semipreparative HPLC using Gemini 5 µm C₁₈ column (isocratic, 70-75% MeCN) to afford the products **6c – 6f** and **6c'- 6f'**

Compound **1a**: pale yellow solid (3.5 mg, 35% yield); ¹H and ¹³C NMR, see tables 5.03; HRESIMS *m/z* 446.2548 [M-H]⁻ (calcd for C₂₅H₃₆NO₆, 446.2543)

Compound **1b**: pale yellow solid (3.7 mg, 37% yield); ^1H and ^{13}C NMR, see tables 5.03;
HRESIMS m/z 446.2547 $[\text{M}-\text{H}]^-$ (calcd for $\text{C}_{25}\text{H}_{36}\text{NO}_6$, 446.2543)

Compound **2a**: white solid (1.5 mg, 15% yield); ^1H and ^{13}C NMR, see tables 5.01;
HRESIMS m/z 440.2779 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{39}\text{NNaO}_4$, 440.2777)

Compound **2b**: white solid (2.0 mg, 20% yield); ^1H and ^{13}C NMR, see tables 5.01;
HRESIMS m/z 440.2778 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{39}\text{NNaO}_4$, 440.2777)

Compound **2c**: white solid (2.2 mg, 22% yield); ^1H and ^{13}C NMR, see tables 5.02;
HRESIMS m/z 442.2932 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{41}\text{NNaO}_4$, 442.2933)

Compound **2d**: white solid (1.6 mg, 16% yield); ^1H and ^{13}C NMR, see tables 5.02;
HRESIMS m/z 442.2932 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{41}\text{NNaO}_4$, 442.2936)

Compound **3a**: pale yellow solid (3.4 mg, 34% yield); ^1H and ^{13}C NMR, see tables 5.04;
HRESIMS m/z 438.2617 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{37}\text{NNaO}_4$, 438.2620)

Compound **3b**: pale yellow solid (3.2 mg, 32% yield); ^1H and ^{13}C NMR, see tables 5.04;
HRESIMS m/z 440.2775 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{39}\text{NNaO}_4$, 440.2777)

Compound **4a**: pale yellow solid (2.1 mg, 21% yield); ^1H and ^{13}C NMR, see tables 5.05;
HRESIMS m/z 478.2573 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{27}\text{H}_{37}\text{NNaO}_5$, 478.2569)

Compound **4b**: pale yellow solid (6.3 mg, 63% yield); ^1H and ^{13}C NMR, see tables 5.05;
HRESIMS m/z 526.2941 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{32}\text{H}_{41}\text{NNaO}_4$, 526.2933)

Compound **4c**: pale yellow solid (4.5 mg, 45% yield); ^1H and ^{13}C NMR, see tables 5.06;
HRESIMS m/z 450.2621 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{26}\text{H}_{37}\text{NNaO}_4$, 450.2620)

Compound **4d**: pale yellow solid (2.0 mg, 20% yield); ^1H and ^{13}C NMR, see tables 5.06;
HRESIMS m/z 504.3092 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{30}\text{H}_{43}\text{NNaO}_4$, 504.3090)

Compound **4e**: pale yellow solid (8.3 mg, 83% yield); ^1H and ^{13}C NMR, see tables 5.07;
HRESIMS m/z 474.2622 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{28}\text{H}_{37}\text{NNaO}_4$, 474.2620)

Compound **5a**: pale yellow solid (6.6 mg, 66% yield); ^1H and ^{13}C NMR, see tables 5.08;
HRESIMS m/z 561.3052 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{32}\text{H}_{42}\text{N}_4\text{NaO}_5$, 561.3053)

Compound **5a'**: pale yellow solid (3.2 mg, 32% yield); ^1H and ^{13}C NMR, see tables 5.08;
HRESIMS m/z 561.3052 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{32}\text{H}_{42}\text{N}_4\text{NaO}_5$, 561.3053)

Compound **5b**: pale yellow solid (3.7 mg, 37% yield); ^1H and ^{13}C NMR, see tables 5.09;
HRESIMS m/z 575.3210 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{31}\text{H}_{44}\text{N}_4\text{NaO}_5$, 575.3209)

Compound **5b'**: pale yellow solid (3.9 mg, 39% yield); ^1H and ^{13}C NMR, see tables 5.09;
HRESIMS m/z 575.3209 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{31}\text{H}_{44}\text{N}_4\text{NaO}_5$, 575.3209)

Compound **5c**: pale yellow solid (4.8 mg, 48% yield); ^1H and ^{13}C NMR, see tables 5.10;
HRESIMS m/z 589.3366 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{32}\text{H}_{46}\text{N}_4\text{NaO}_5$, 589.3366)

Compound **5c'**: pale yellow solid (5.0 mg, 50% yield); ^1H and ^{13}C NMR, see tables 5.10;
HRESIMS m/z 589.3367 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{32}\text{H}_{46}\text{N}_4\text{NaO}_5$, 589.3366)

Compound **5d**: pale yellow solid (5.3 mg, 53% yield); ^1H and ^{13}C NMR, see tables 5.11;
HRESIMS m/z 587.3573 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{33}\text{H}_{48}\text{N}_4\text{NaO}_4$, 587.3573)

Compound **5d'**: pale yellow solid (4.2 mg, 42% yield); ^1H and ^{13}C NMR, see tables 5.11;
HRESIMS m/z 587.3574 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{33}\text{H}_{48}\text{N}_4\text{NaO}_4$, 587.3573)

Compound **5e**: pale yellow solid (2.1 mg, 21% yield); ^1H and ^{13}C NMR, see tables 5.12;
HRESIMS m/z 635.3573 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{37}\text{H}_{48}\text{N}_4\text{NaO}_4$, 635.3573)

Compound **5e'**: pale yellow solid (1.7 mg, 17% yield); ^1H and ^{13}C NMR, see tables 5.12;
HRESIMS m/z 635.3577 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{37}\text{H}_{48}\text{N}_4\text{NaO}_4$, 635.3573)

Compound **5f**: pale yellow solid (3.9 mg, 39% yield); ^1H and ^{13}C NMR, see tables 5.13;
HRESIMS m/z 637.3367 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{36}\text{H}_{46}\text{N}_4\text{NaO}_5$, 637.3366)

Compound **5f'**: pale yellow solid (4.2 mg, 42% yield); ^1H and ^{13}C NMR, see tables 5.13;
HRESIMS m/z 637.3364 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{36}\text{H}_{46}\text{N}_4\text{NaO}_5$, 637.3366)

Compound **5g**: pale yellow solid (2.4 mg, 24% yield); ^1H and ^{13}C NMR, see tables 5.14;
HRESIMS m/z 651.3155 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{36}\text{H}_{44}\text{N}_4\text{NaO}_6$, 651.3159)

Compound **5g'**: pale yellow solid (2.8 mg, 28% yield); ^1H and ^{13}C NMR, see tables 5.14;
HRESIMS m/z 651.3159 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{36}\text{H}_{44}\text{N}_4\text{NaO}_6$, 651.3159)

Compound **5h**: pale yellow solid (5.0 mg, 50% yield); ^1H and ^{13}C NMR, see tables 5.15;
HRESIMS m/z 660.3526 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{38}\text{H}_{47}\text{N}_5\text{NaO}_4$, 660.3526)

Compound **5h'**: pale yellow solid (4.8 mg, 48% yield); ^1H and ^{13}C NMR, see tables 5.15;
HRESIMS m/z 660.3525 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{38}\text{H}_{47}\text{N}_5\text{NaO}_4$, 660.3526)

Compound **5i**: pale yellow solid (5.4 mg, 54% yield); ^1H and ^{13}C NMR, see tables 5.16;
HRESIMS m/z 601.3366 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{33}\text{H}_{46}\text{N}_4\text{NaO}_5$, 601.3366)

Compound **5i'**: pale yellow solid (4.5 mg, 54% yield); ^1H and ^{13}C NMR, see tables 5.16;
HRESIMS m/z 601.3368 $[\text{M}+\text{Na}]^+$ (cald for $\text{C}_{33}\text{H}_{46}\text{N}_4\text{NaO}_5$, 601.3366)

Compound **5j**: pale yellow solid (5.8 mg, 58% yield); ^1H and ^{13}C NMR, see tables 5.17;
HRESIMS m/z 615.3523 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{34}\text{H}_{48}\text{N}_4\text{NaO}_5$, 615.3522)

Compound **5j'**: pale yellow solid (3.8 mg, 38% yield); ^1H and ^{13}C NMR, see tables 5.17;
HRESIMS m/z 615.3522 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{34}\text{H}_{48}\text{N}_4\text{NaO}_5$, 615.3522)

Compound **5k**: pale yellow solid (4.8 mg, 48% yield); ^1H and ^{13}C NMR, see tables 5.18;
HRESIMS m/z 599.3574 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{34}\text{H}_{48}\text{N}_4\text{NaO}_4$, 599.3573)

Compound **5k'**: pale yellow solid (4.6 mg, 46% yield); ^1H and ^{13}C NMR, see tables 5.18;
HRESIMS m/z 599.3574 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{34}\text{H}_{48}\text{N}_4\text{NaO}_4$, 599.3573)

Compound **6a**: pale yellow solid (6.4 mg, 64% yield); ^1H and ^{13}C NMR, see tables 5.19;
HRESIMS m/z 518.2884 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{41}\text{NNaO}_5$, 518.2882)

Compound **6b**: pale yellow solid (4.1 mg, 41% yield); ^1H and ^{13}C NMR, see tables 5.19;
HRESIMS m/z 556.3038 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{33}\text{H}_{43}\text{NNaO}_5$, 556.3039)

Compound **6c**: pale yellow solid (1.5 mg, 15% yield); ^1H and ^{13}C NMR, see tables 5.20;
HRESIMS m/z 605.3316 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{33}\text{H}_{46}\text{N}_4\text{NaO}_6$, 605.3315)

Compound **6c'**: pale yellow solid (0.5 mg, 5% yield); ^1H and ^{13}C NMR, see tables 5.20;
HRESIMS m/z 605.3315 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{33}\text{H}_{46}\text{N}_4\text{NaO}_6$, 605.3315)

Compound **6d**: pale yellow solid (5.2 mg, 52% yield); ^1H and ^{13}C NMR, see tables 5.21;
HRESIMS m/z 681.3631 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{38}\text{H}_{50}\text{N}_4\text{NaO}_6$, 681.3628)

Compound **6d'**: pale yellow solid (2.0 mg, 20% yield); ^1H and ^{13}C NMR, see tables 5.21;
HRESIMS m/z 681.3628 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{38}\text{H}_{50}\text{N}_4\text{NaO}_6$, 681.3628)

Compound **6e**: pale yellow solid (6.0 mg, 60% yield); ^1H and ^{13}C NMR, see tables 5.22;
HRESIMS m/z 695.3420 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{38}\text{H}_{48}\text{N}_4\text{NaO}_7$, 695.3421)

Compound **6e'**: pale yellow solid (4.0 mg, 40% yield); ^1H and ^{13}C NMR, see tables 5.22;
HRESIMS m/z 695.3422 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{38}\text{H}_{48}\text{N}_4\text{NaO}_7$, 695.3421)

Compound **6f**: pale yellow solid (5.1 mg, 51% yield); ^1H and ^{13}C NMR, see tables 5.23;
HRESIMS m/z 659.3785 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{36}\text{H}_{52}\text{N}_4\text{NaO}_6$, 659.3783)

Compound **6f'**: pale yellow solid (4.7 mg, 47% yield); ^1H and ^{13}C NMR, see tables 5.23;
HRESIMS m/z 659.3785 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{36}\text{H}_{52}\text{N}_4\text{NaO}_6$, 659.3785)

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Appendix A: Supporting Data for Chapter 3

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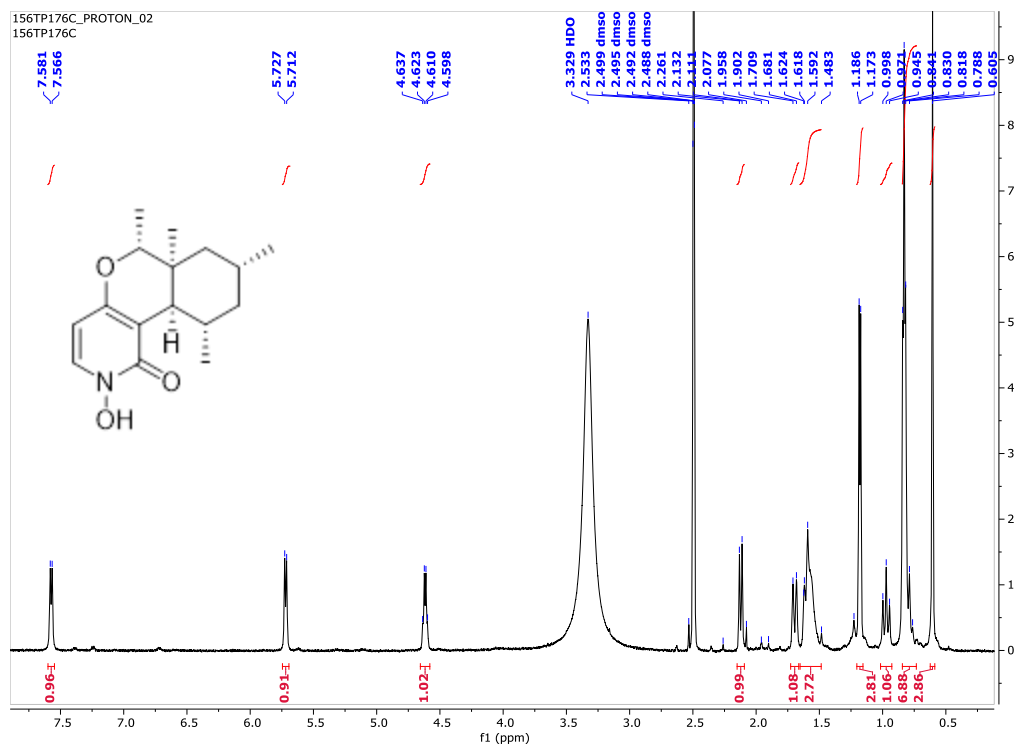


Figure A.1. ^1H NMR (500 MHz, DMSO) of **1**

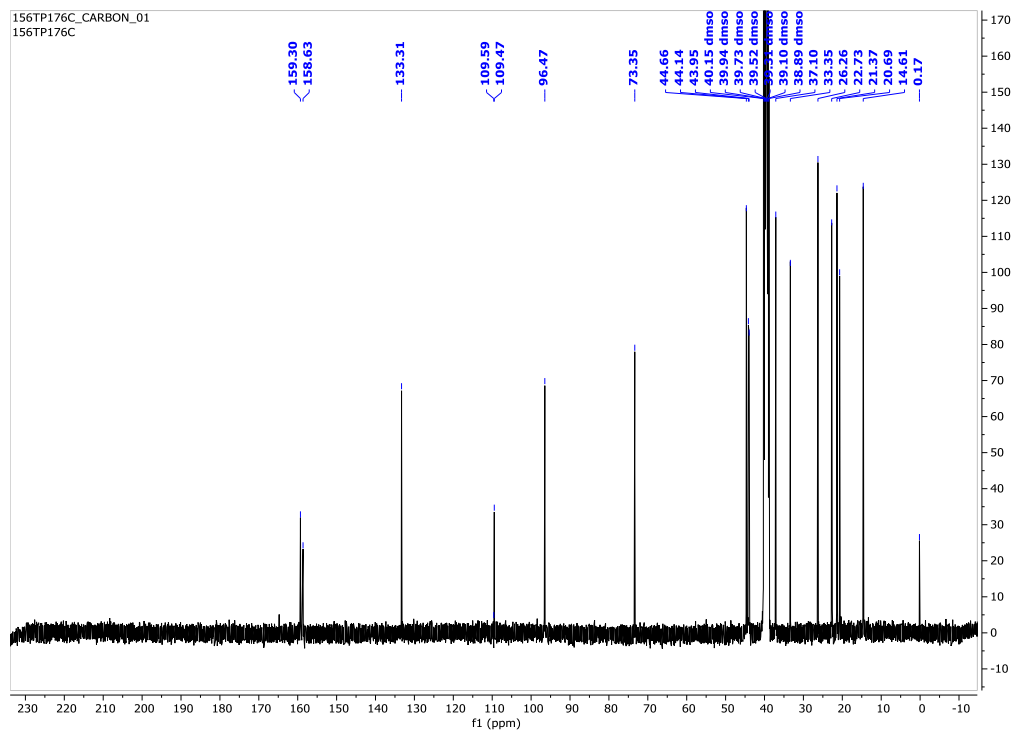


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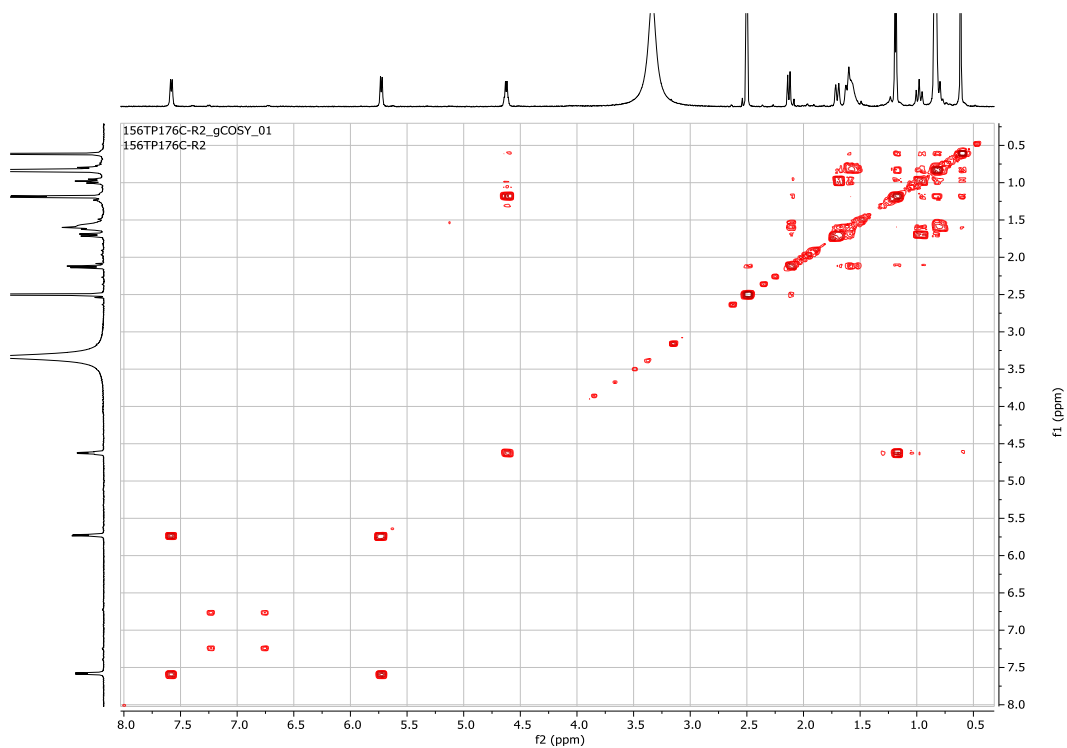


Figure A.3. COSY (500 MHz, DMSO) spectrum of compound **1**

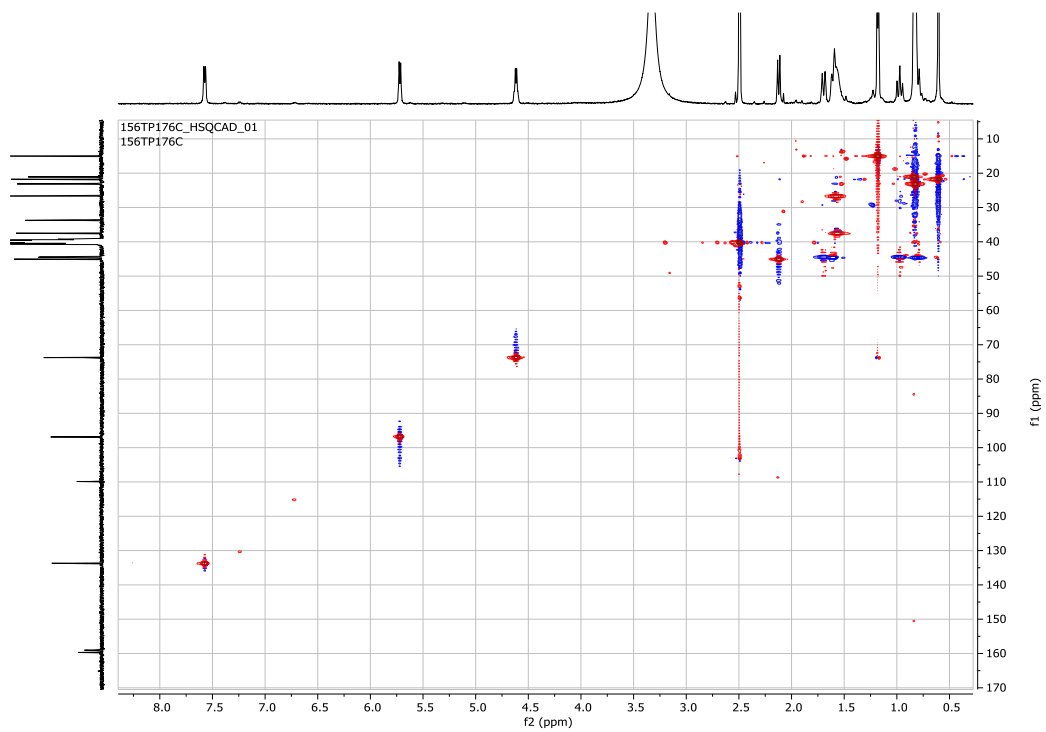


Figure A.4. HSQC (500 MHz, DMSO) spectrum of compound **1**

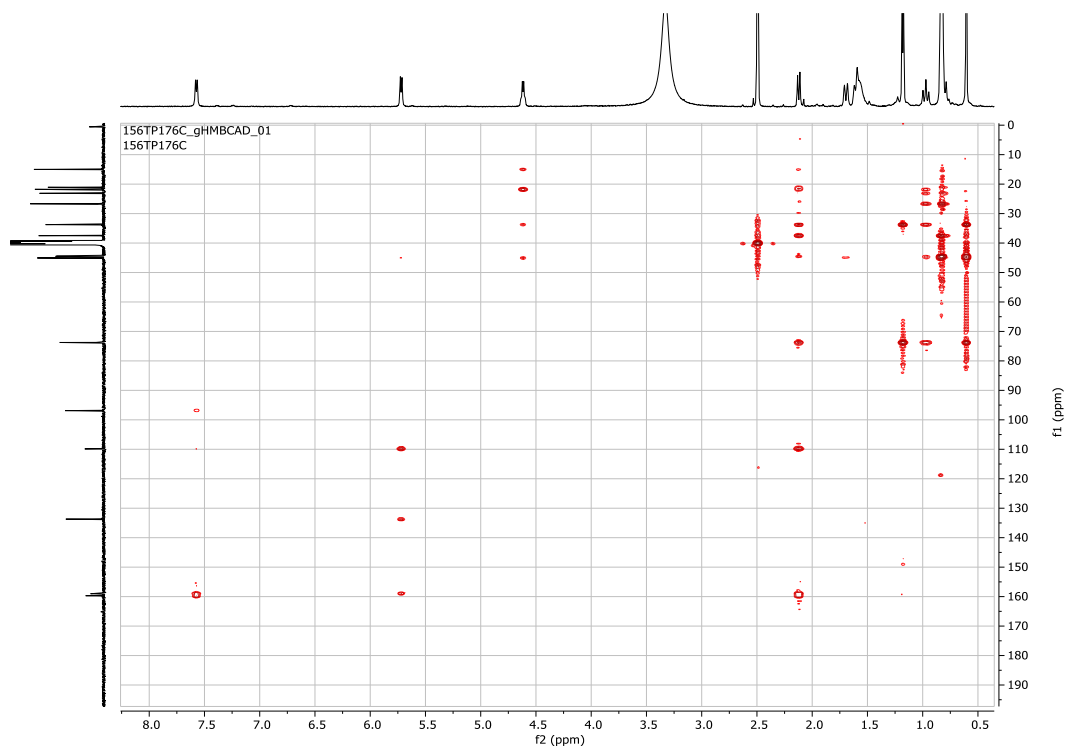


Figure A.5. HMBC (500 MHz, DMSO) spectrum of compound **1**

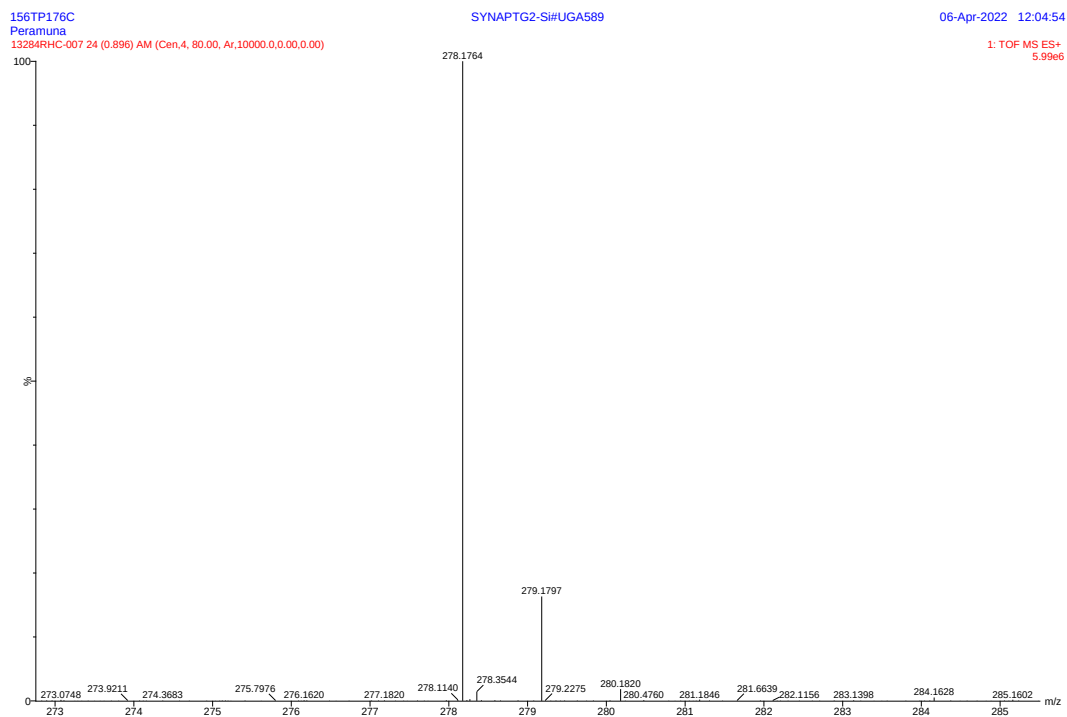


Figure A.6. HRESIMS spectrum of compound **1**

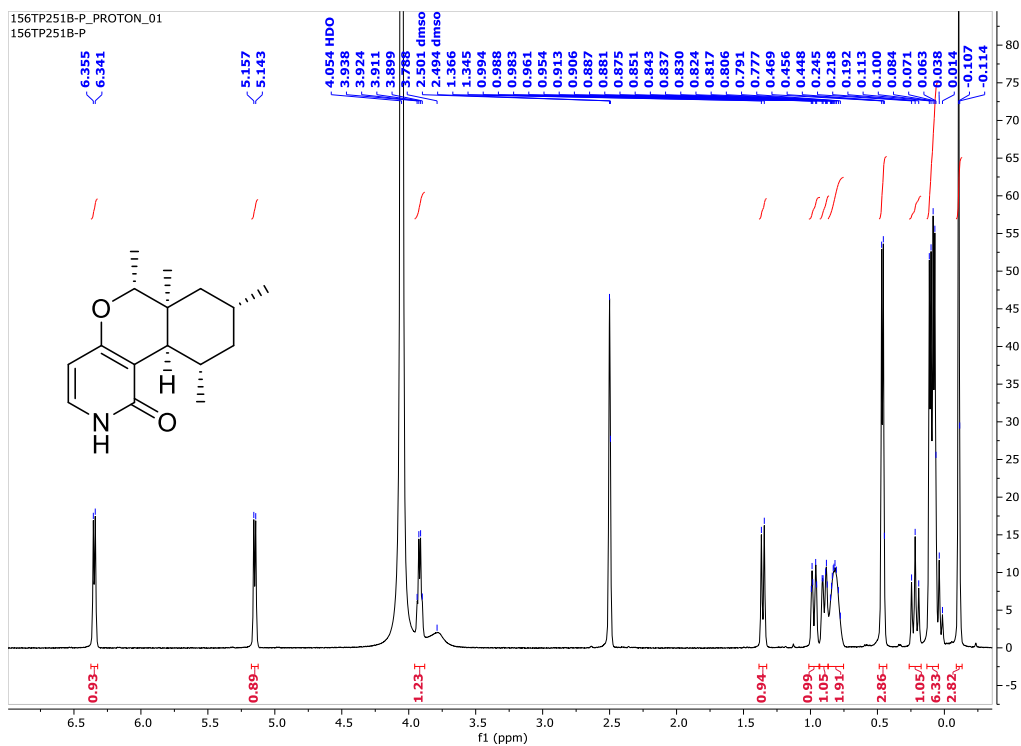
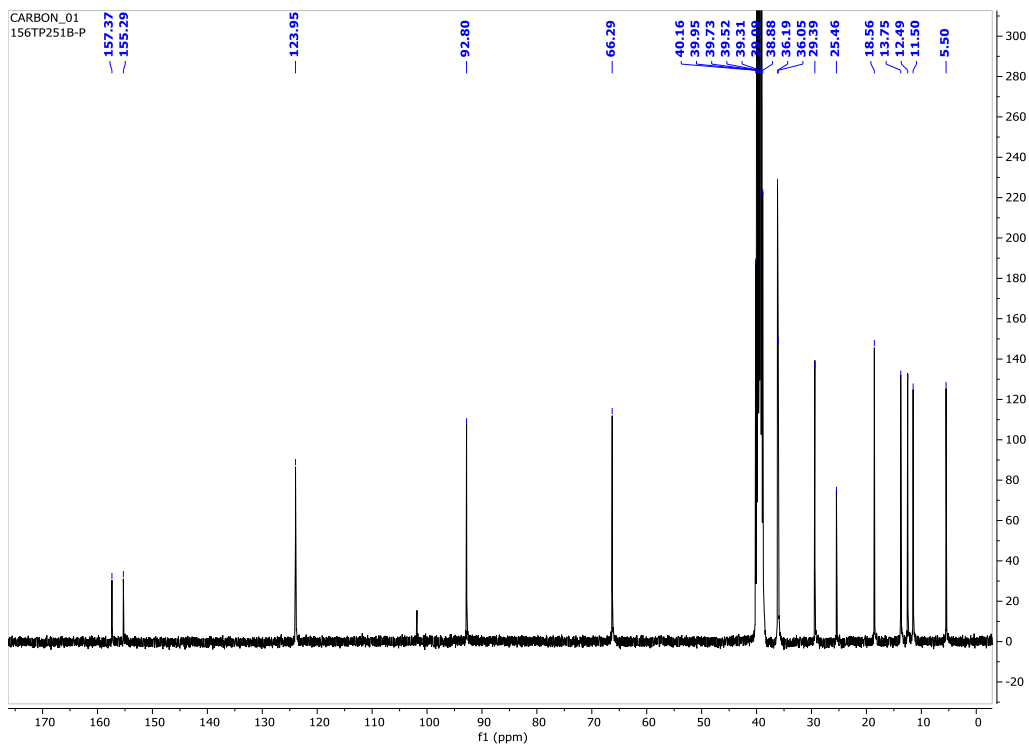


Figure A.7. ^1H NMR (500 MHz, DMSO) of **2**



Figure

A.8. ^{13}C NMR (400 MHz, DMSO) of **2**

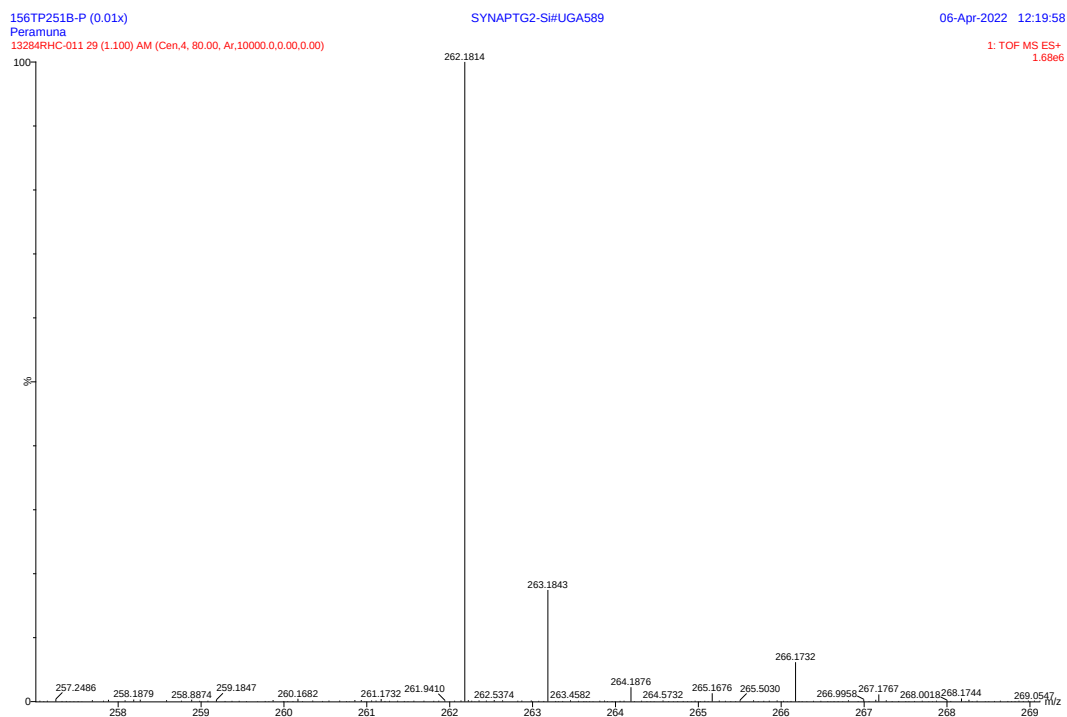


Figure A.9. HRESIMS spectrum of compound 2

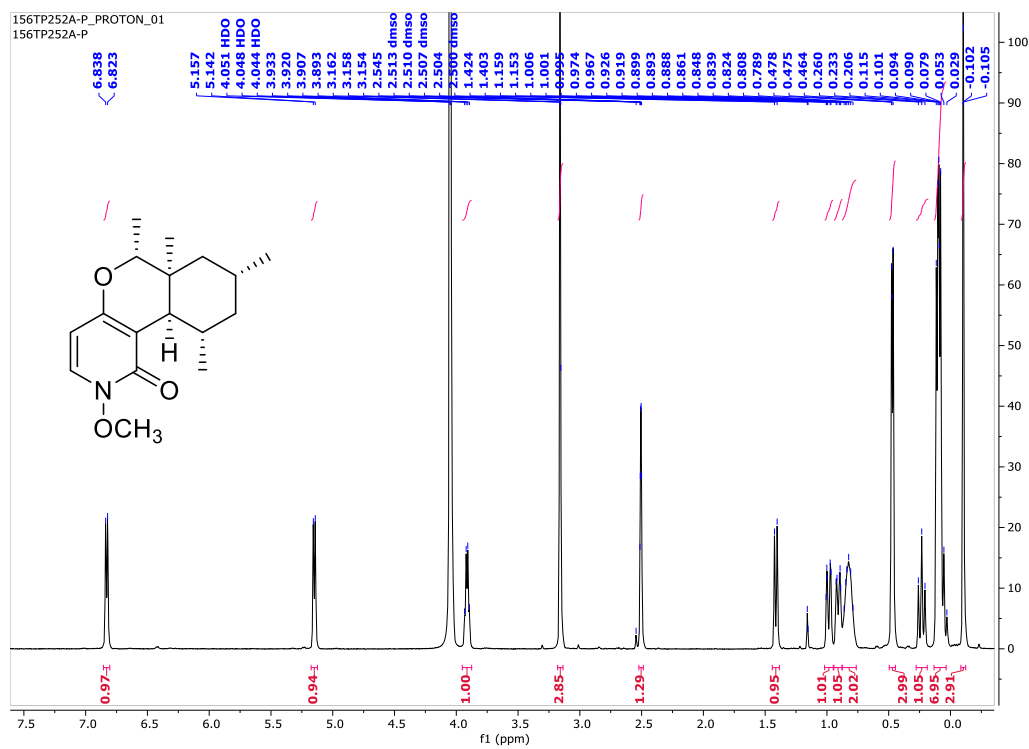


Figure A.10. ^1H NMR (500 MHz, DMSO) of 3

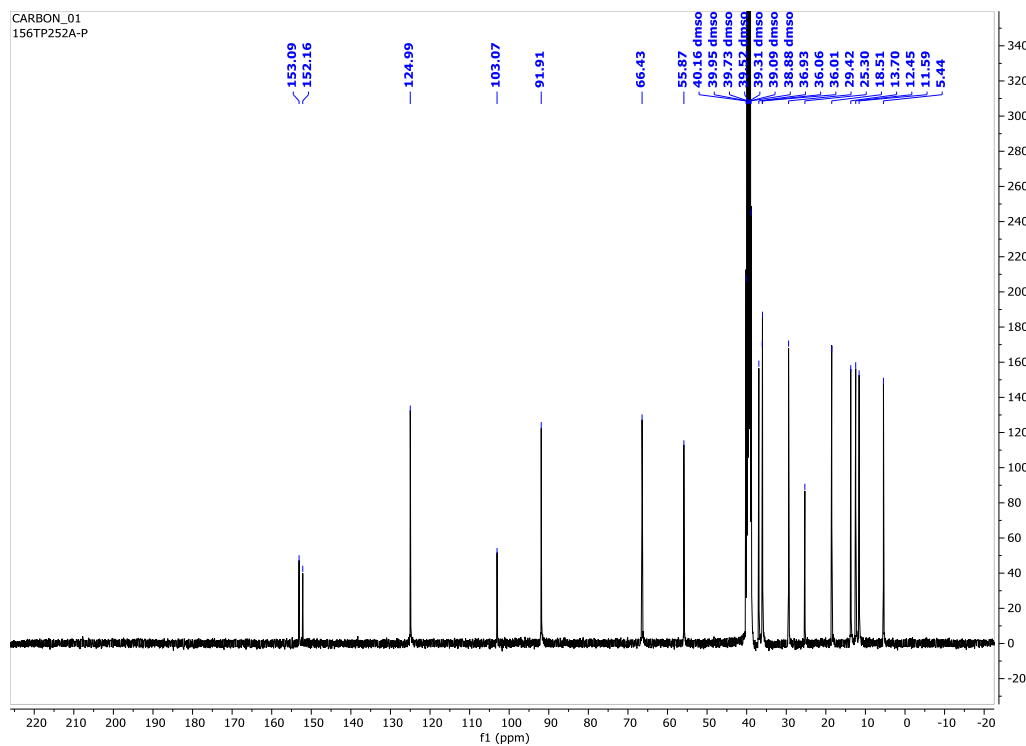


Figure A.11. ^1H NMR (400 MHz, DMSO) of **3**

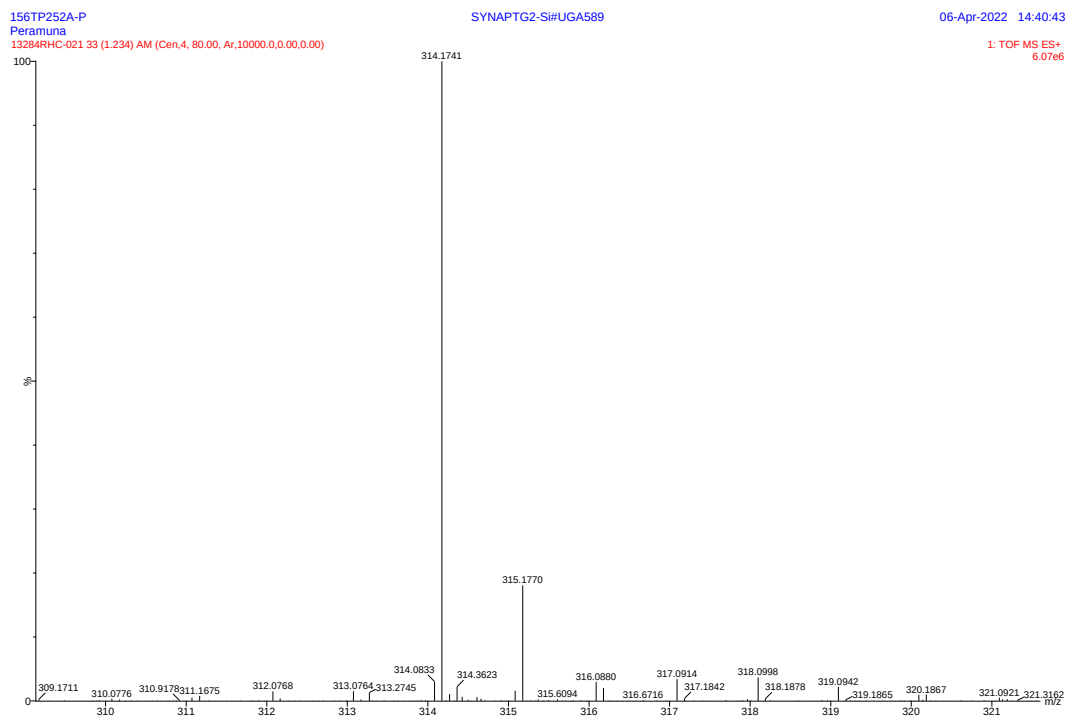


Figure A.12. HRMS spectrum of compound **3**

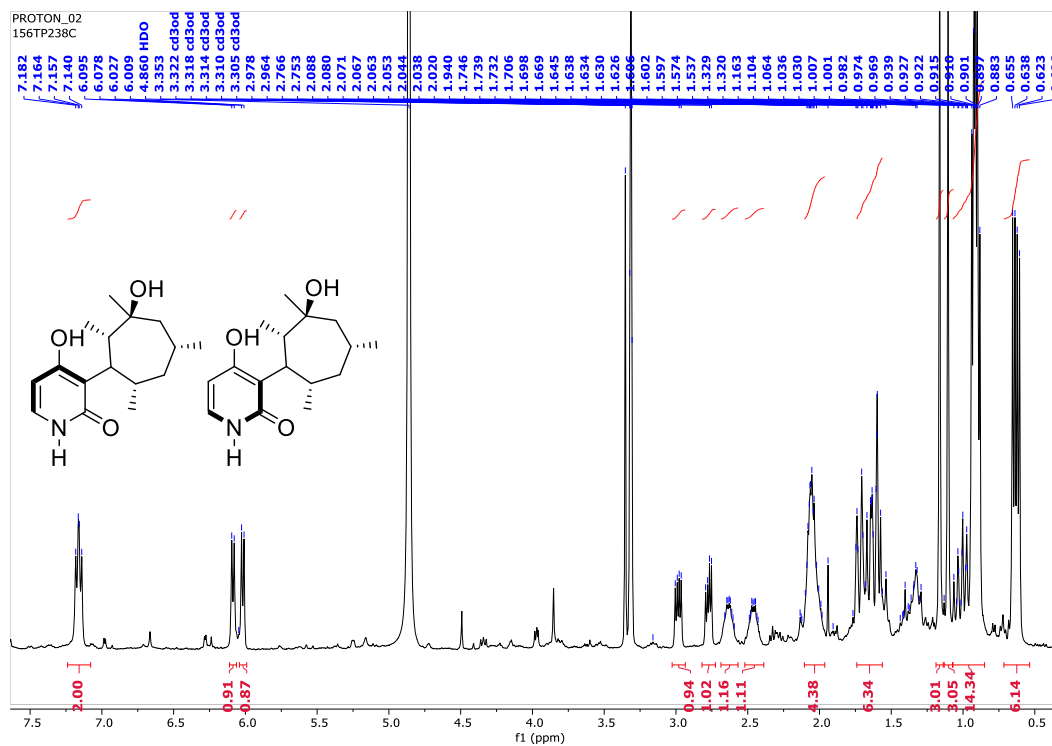


Figure A.13. ^1H NMR (400 MHz, MeOH-d_4) of **4**

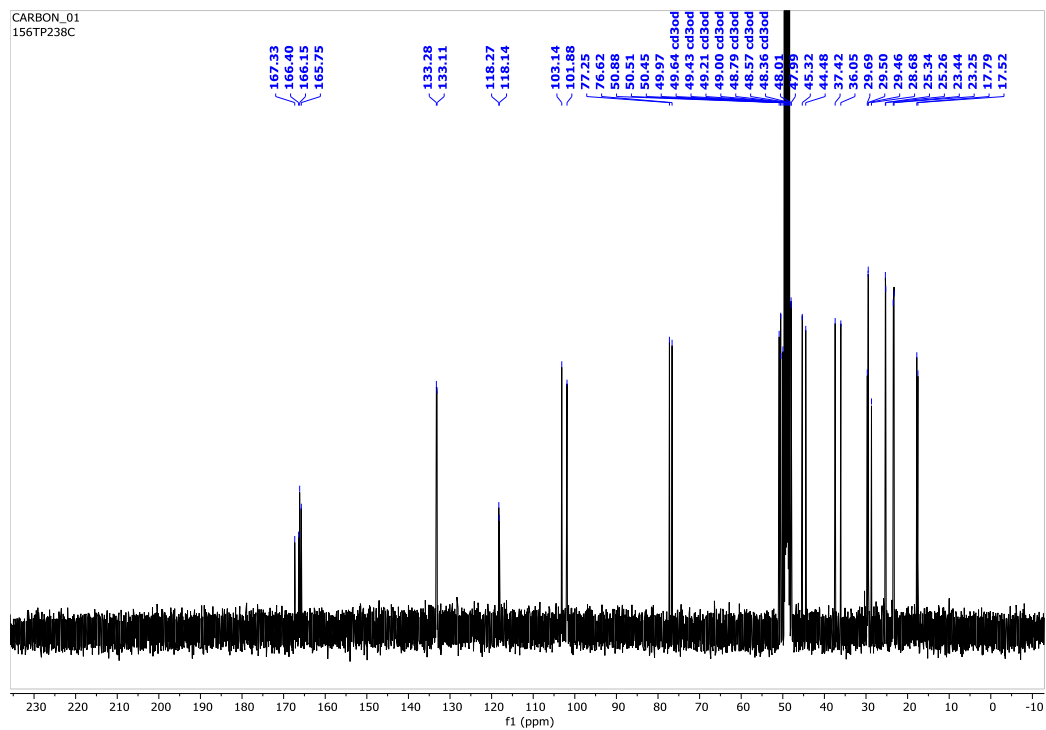


Figure A.14. ^{13}C NMR (400 MHz, MeOH-d_4) of **4**

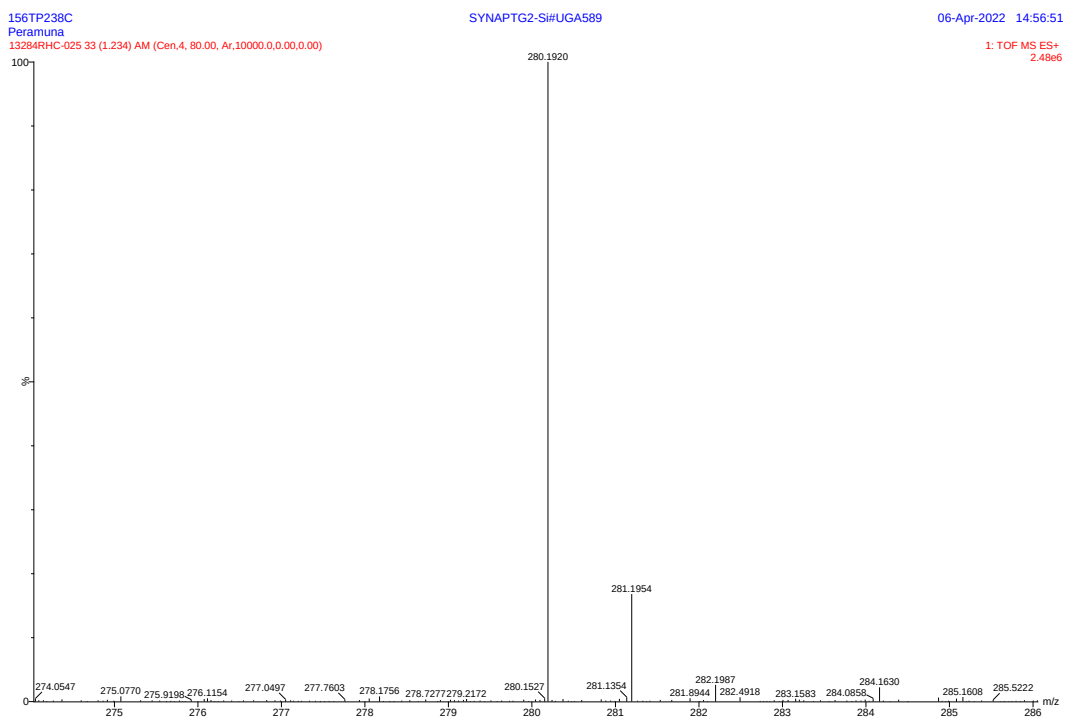


Figure A.15. HRESIMS spectrum of compound **4**

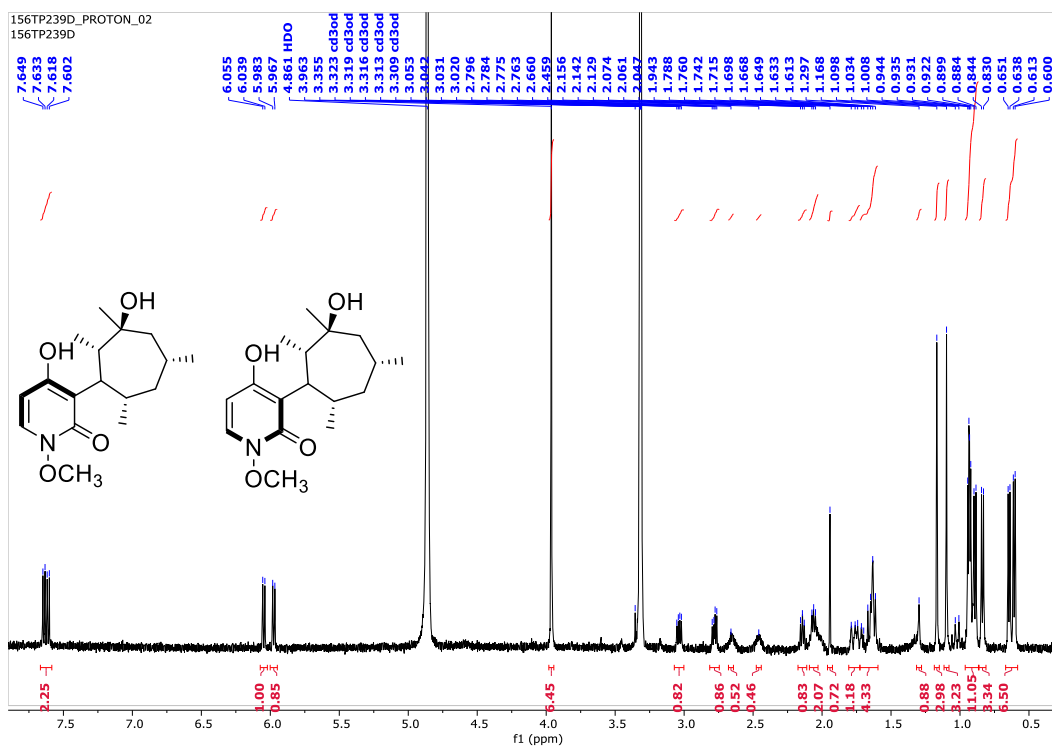


Figure A.16. ^1H NMR (500 MHz, MeOH-d_4) of **5**

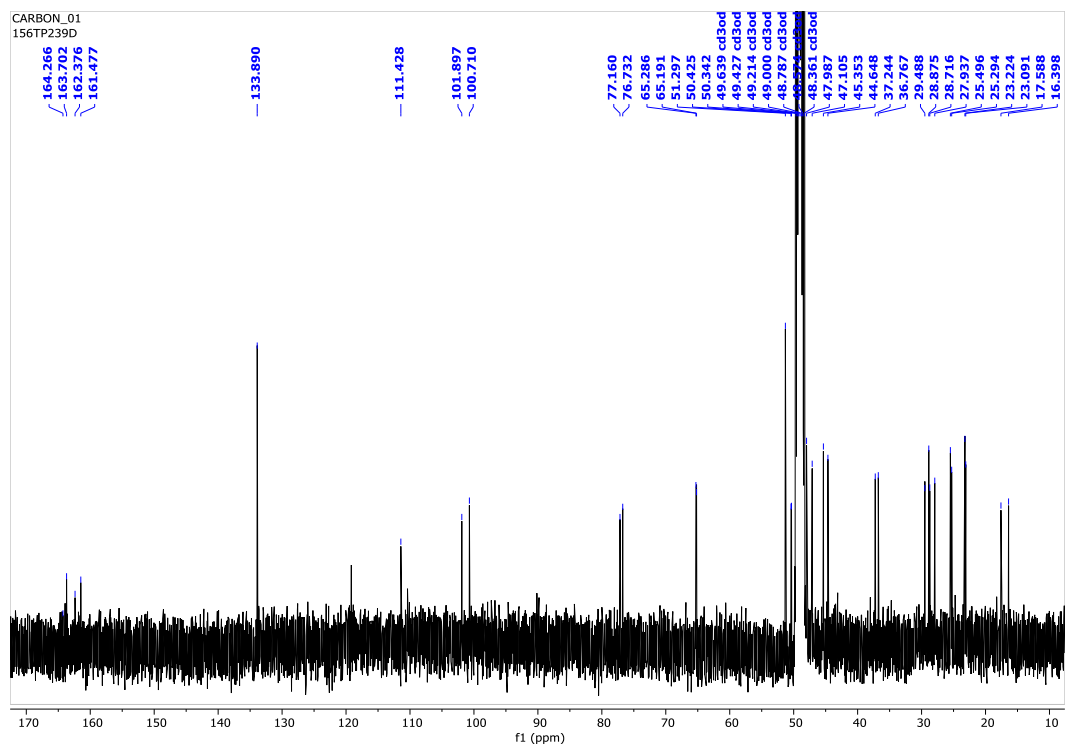


Figure A.17. ^{13}C NMR (400 MHz, $\text{MeOH-}d_4$) of **5**

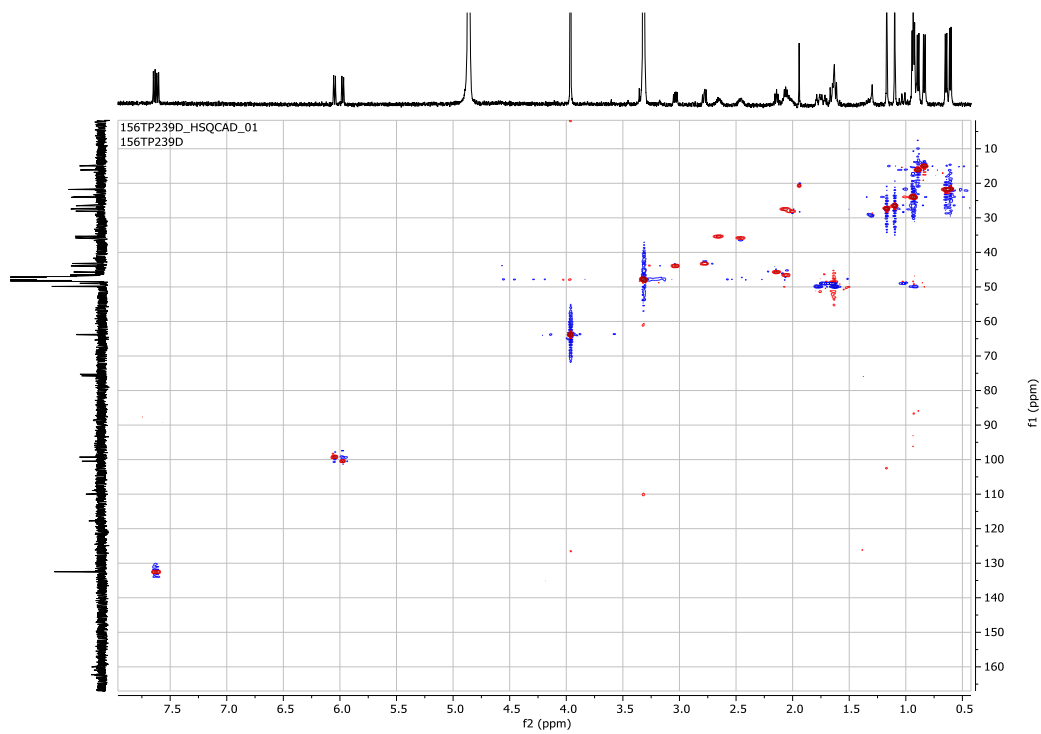


Figure A.18. HSQC (500 MHz, $\text{MeOH-}d_4$) spectrum of compound **5**

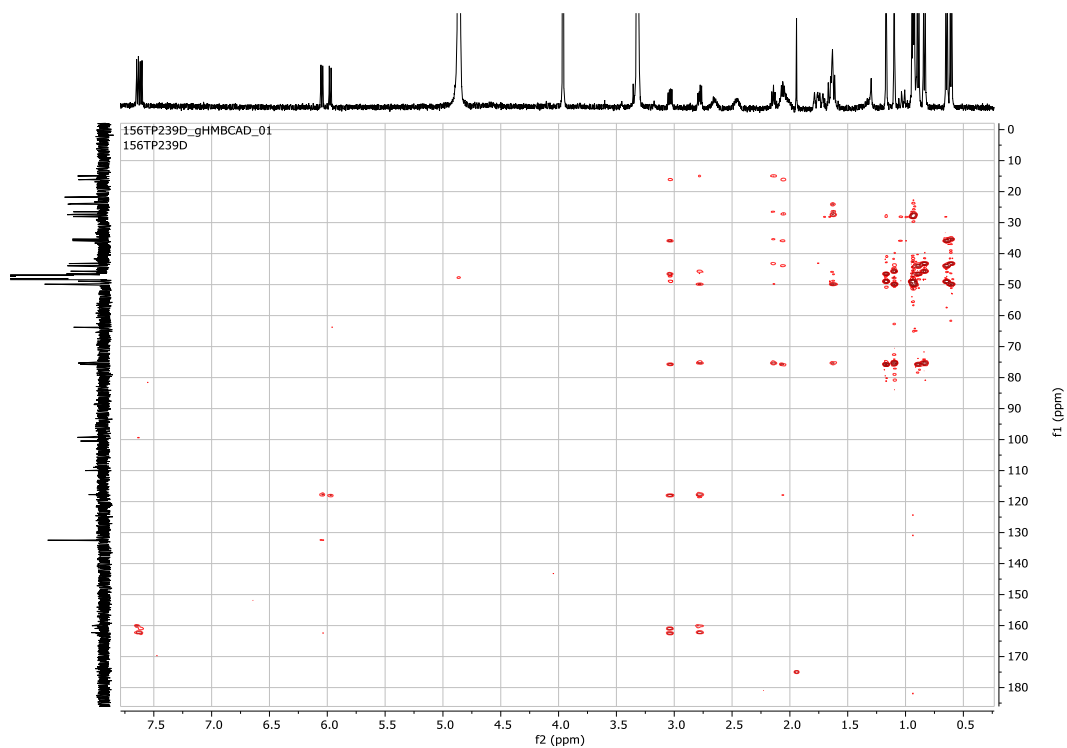


Figure A.19. HMBC (500 MHz, MeOH- d_4) spectrum of compound **5**

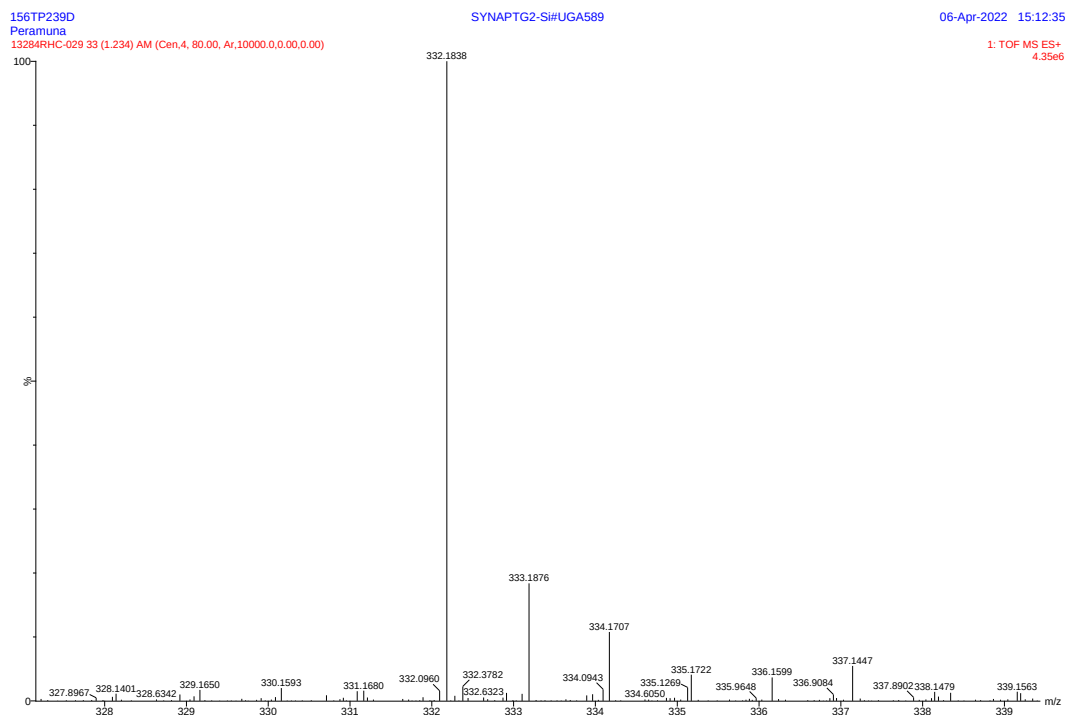


Figure A.20. HRESIMS spectrum of compound **5**

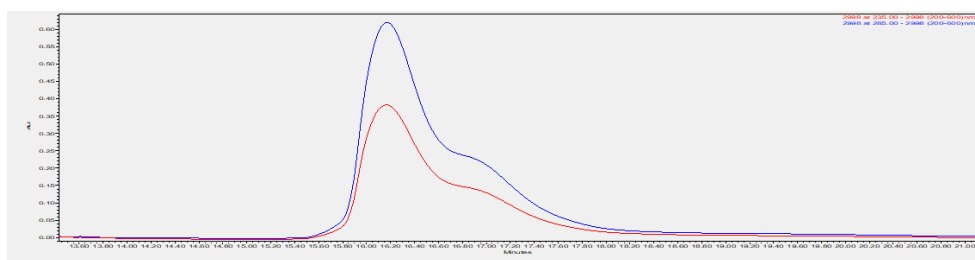


Figure A.21. Chiral HPLC analysis of compound **1**: HPLC conditions: Lux cellulose-2 analytical column, 50% MeCN, 235 nm, 285 nm.

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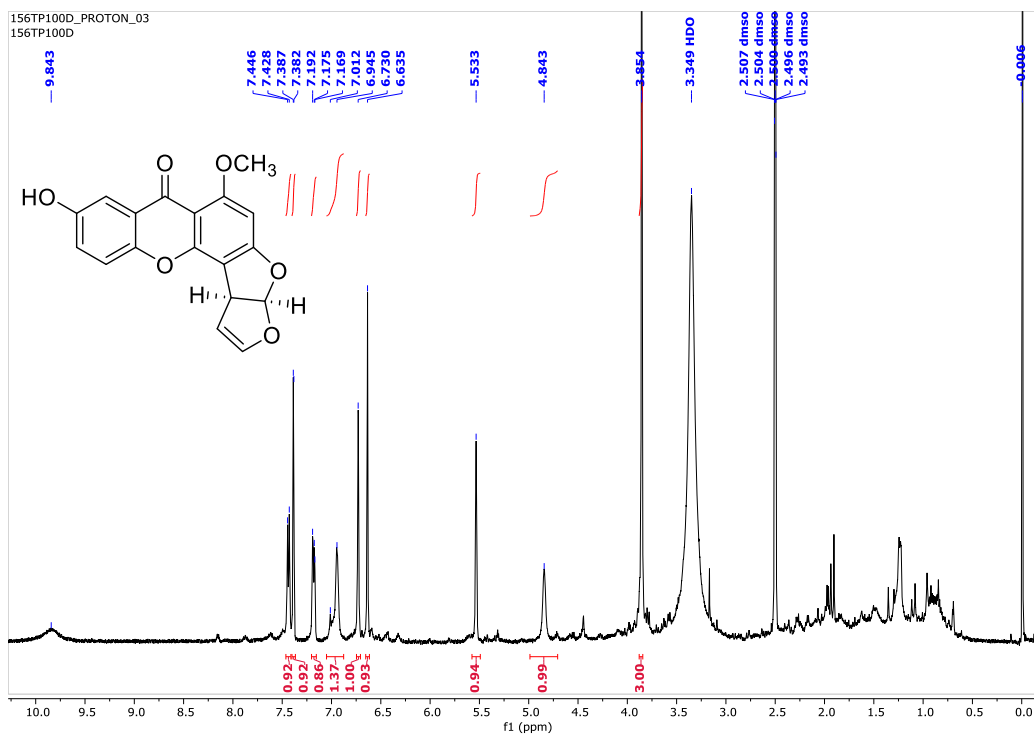


Figure B.1. ^1H NMR (500 MHz, DMSO) of **1**

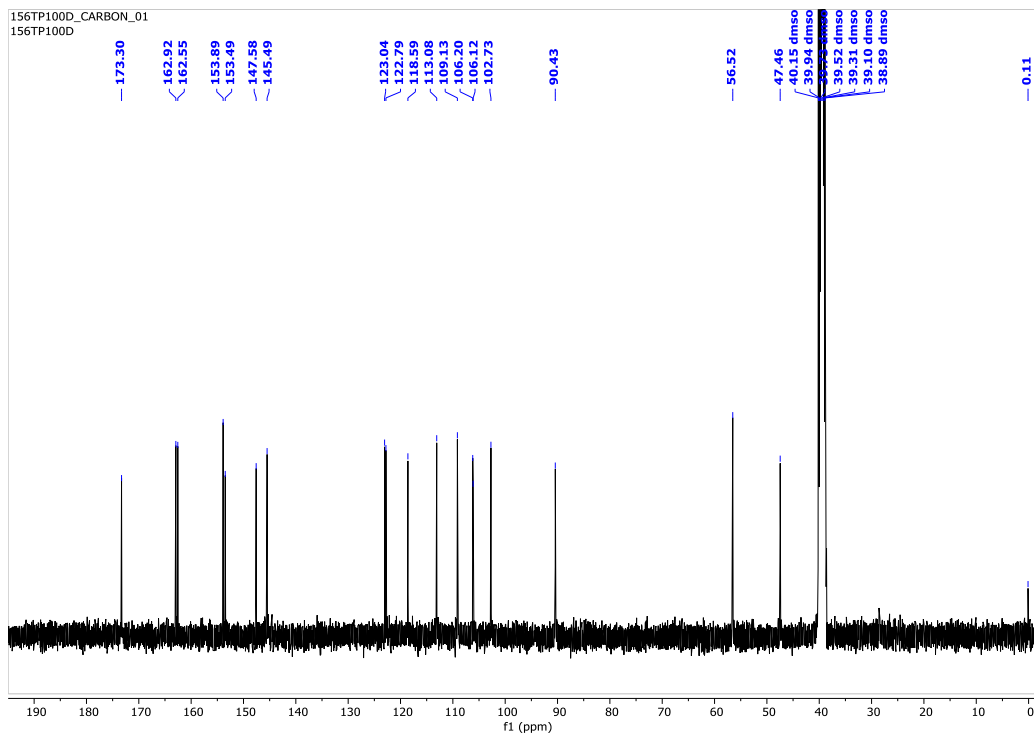


Figure B.2. ^{13}C NMR (400 MHz, DMSO) of **1**

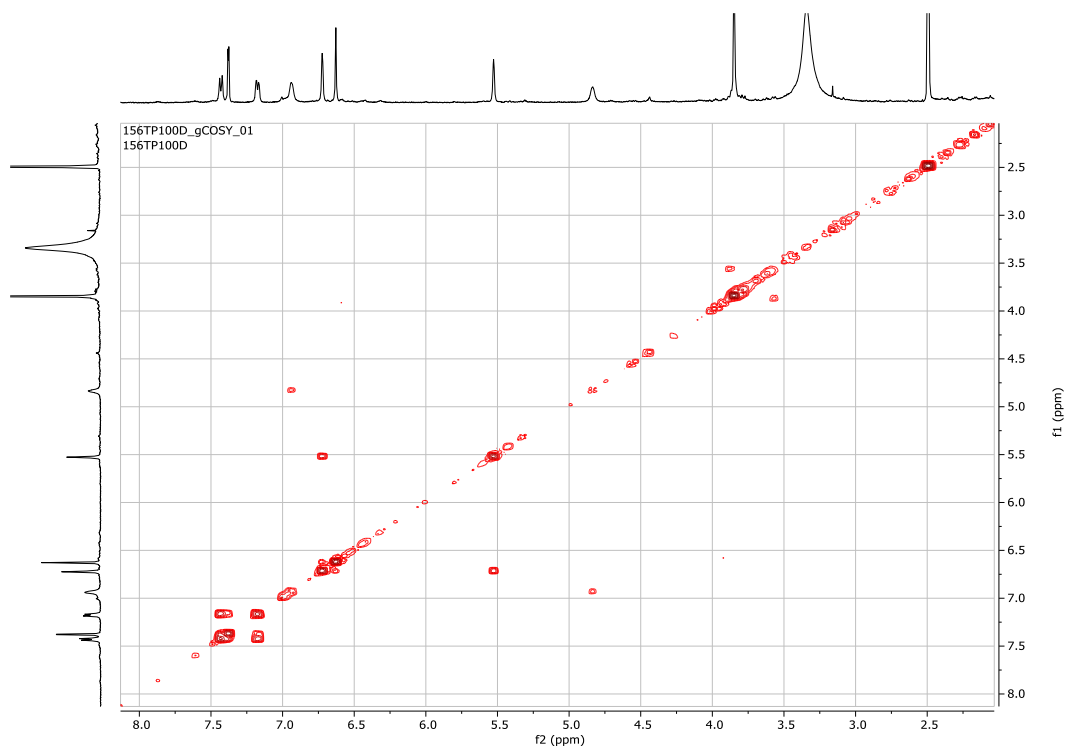


Figure B.3. COSY (500 MHz, DMSO) spectrum of compound **1**

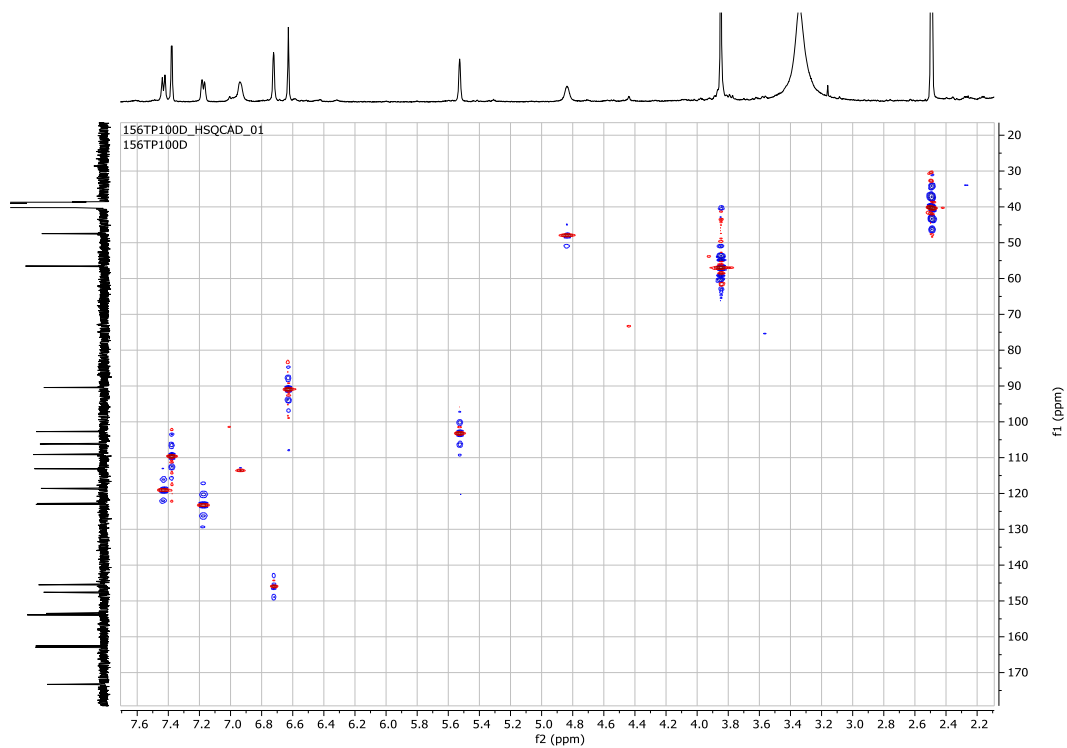


Figure B.4. HSQC (500 MHz, DMSO) spectrum of compound **1**

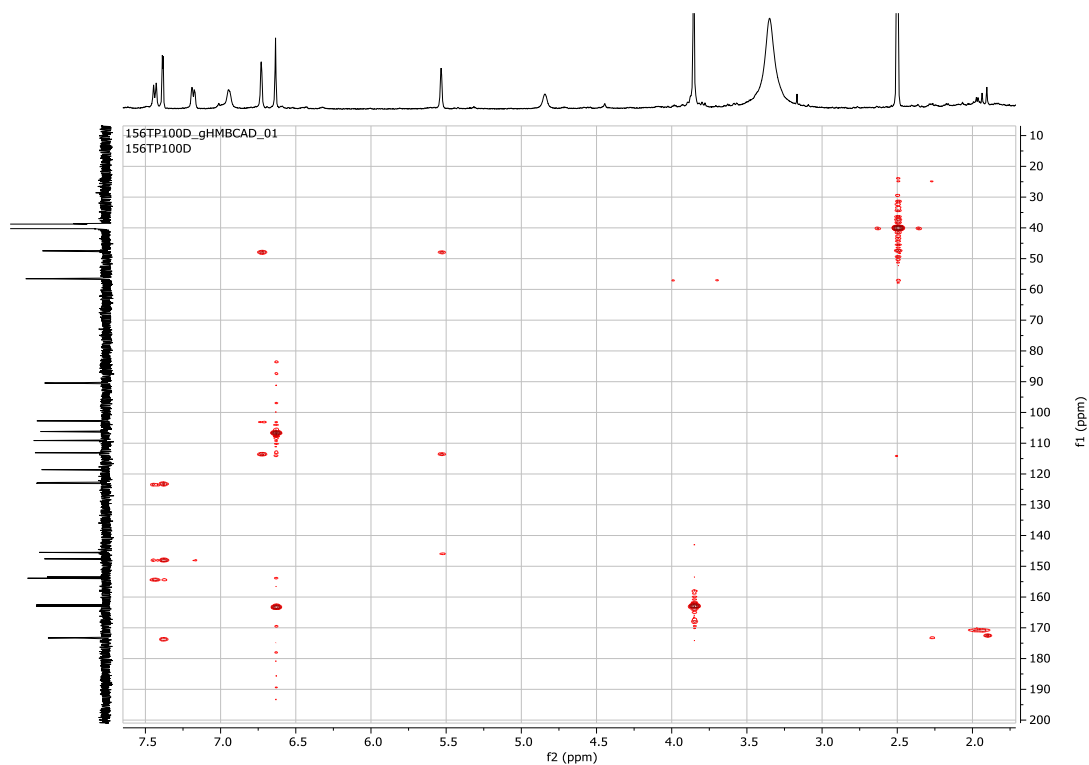


Figure B.5. HMBC (500 MHz, DMSO) spectrum of compound **1**

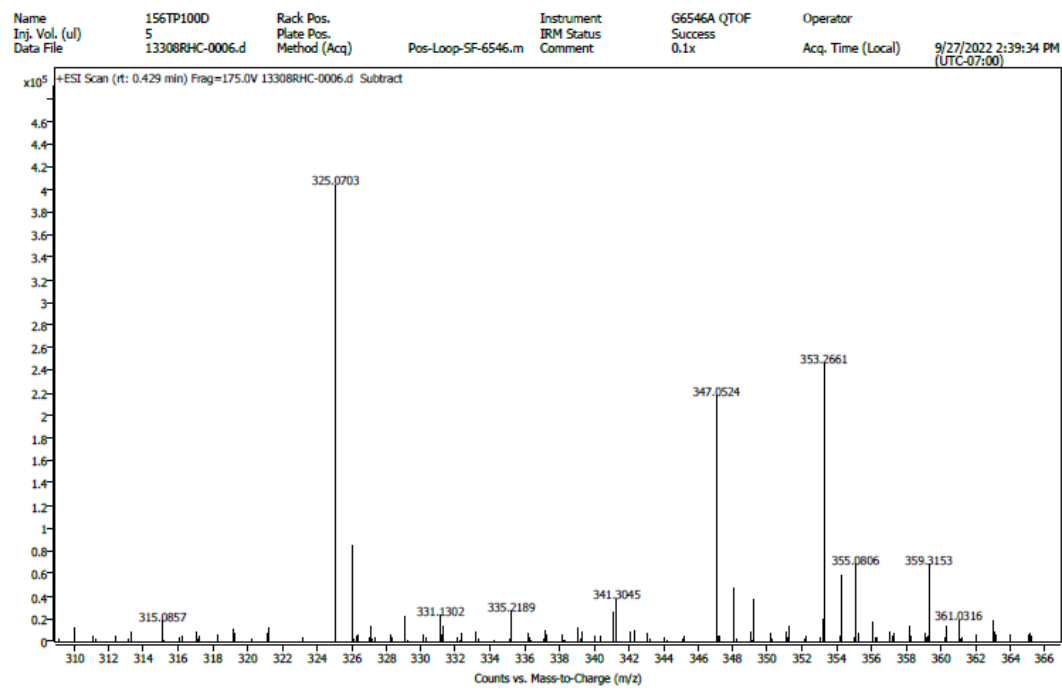


Figure B.6. HRESIMS spectrum of compound **1**

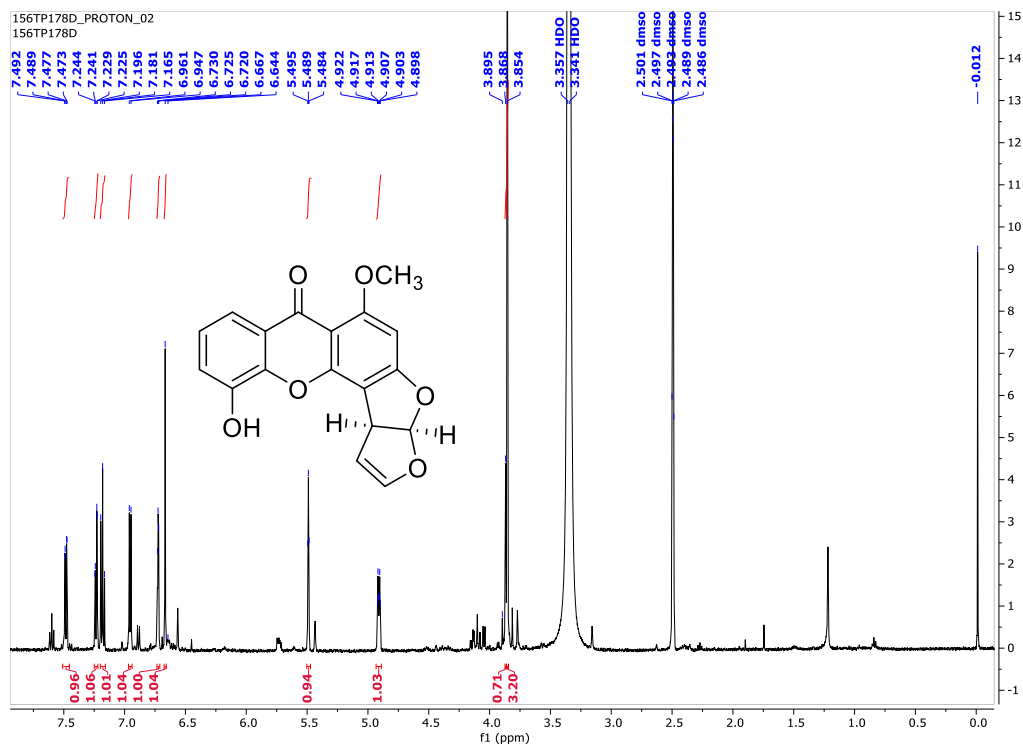


Figure B.7. ^1H NMR (500 MHz, DMSO) of 2

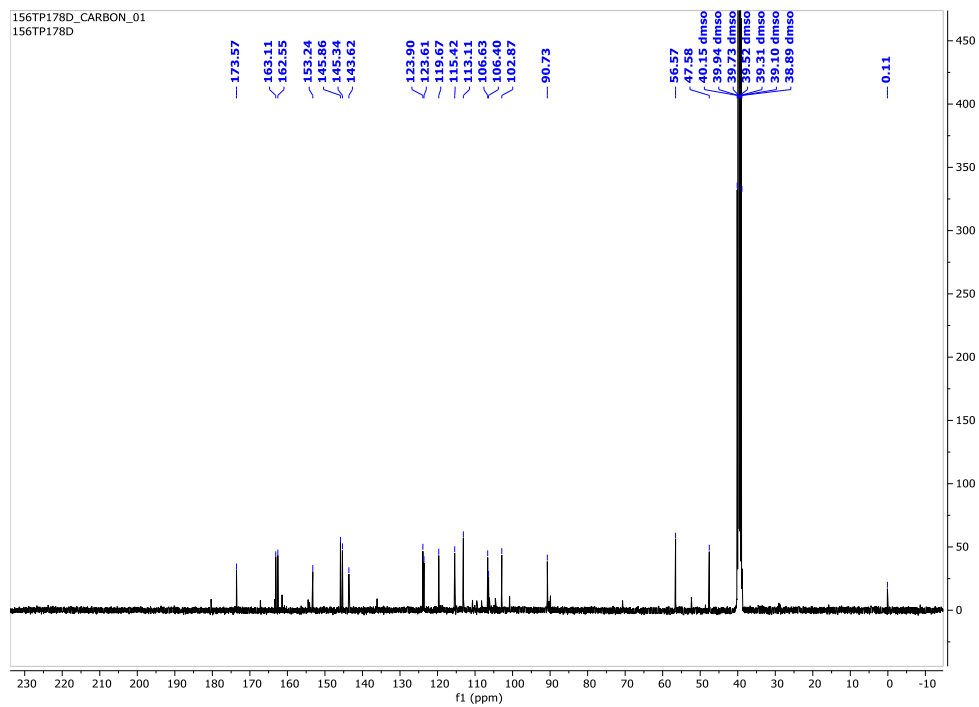


Figure B.8. ^{13}C NMR (400 MHz, DMSO) of 2

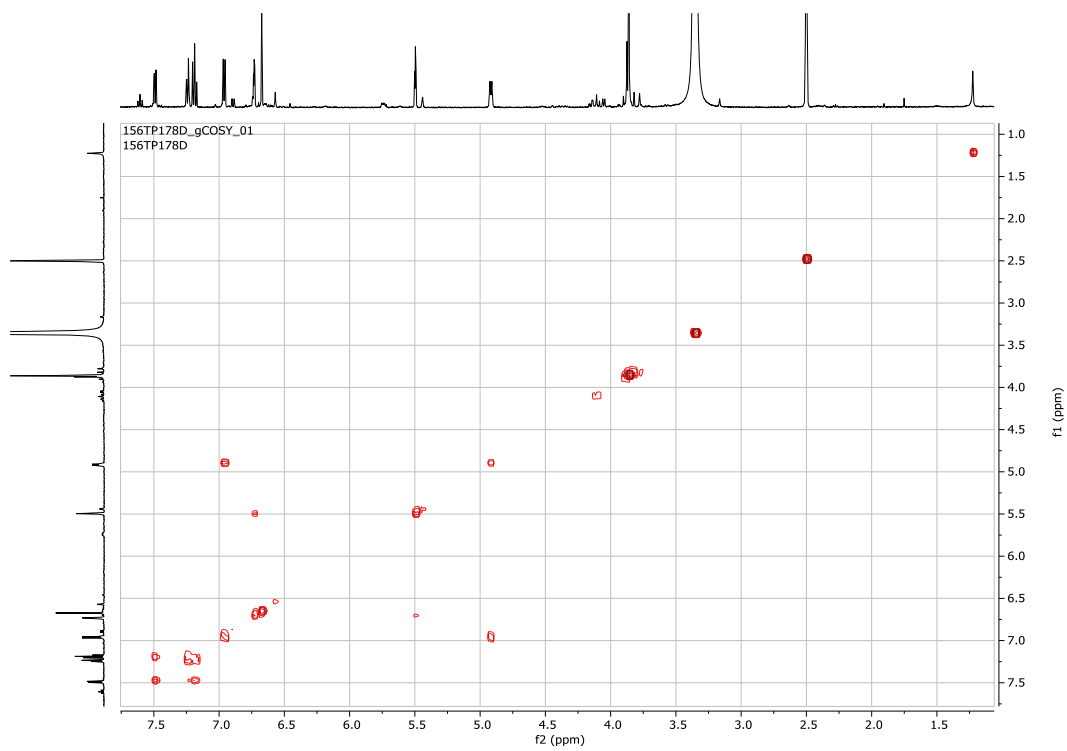


Figure B.9. COSY (500 MHz, DMSO) spectrum of compound **2**

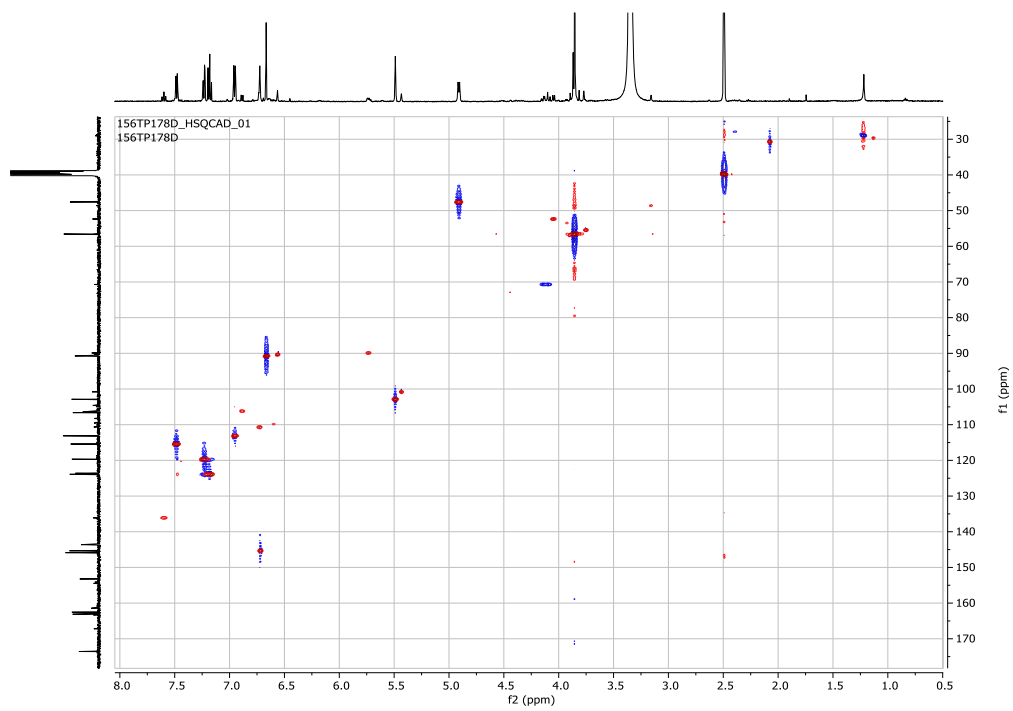


Figure B.10. HSQC (500 MHz, DMSO) spectrum of compound **2**

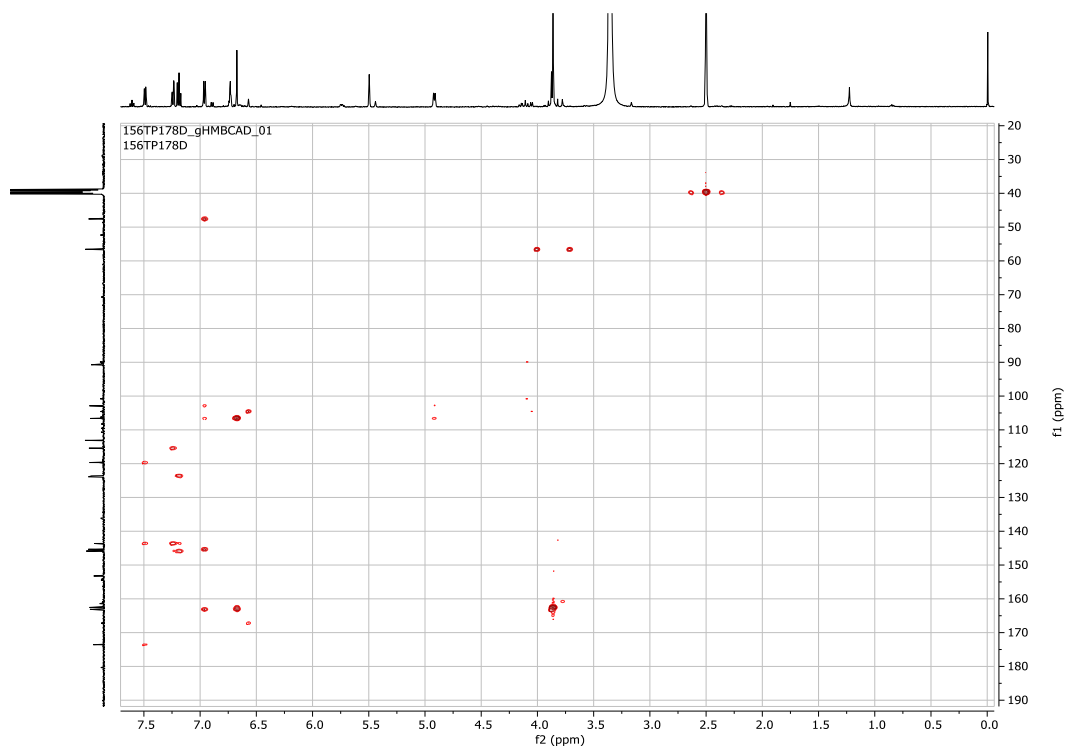


Figure B.11. HMBC (500 MHz, DMSO) spectrum of compound **2**

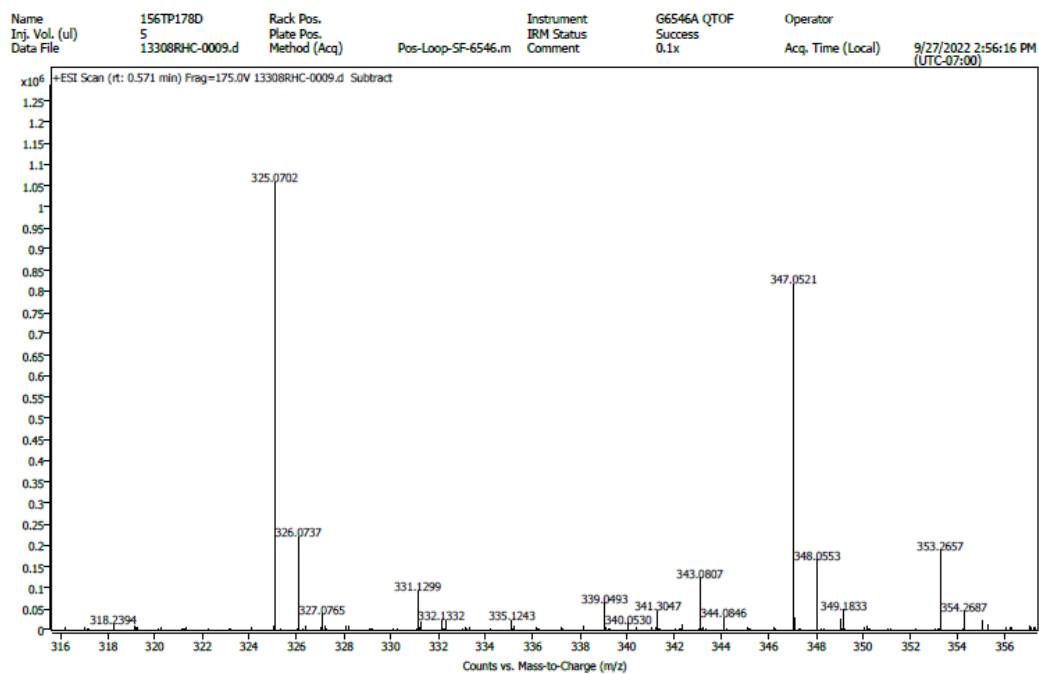


Figure B.12. HRESIMS spectrum of compound **2**

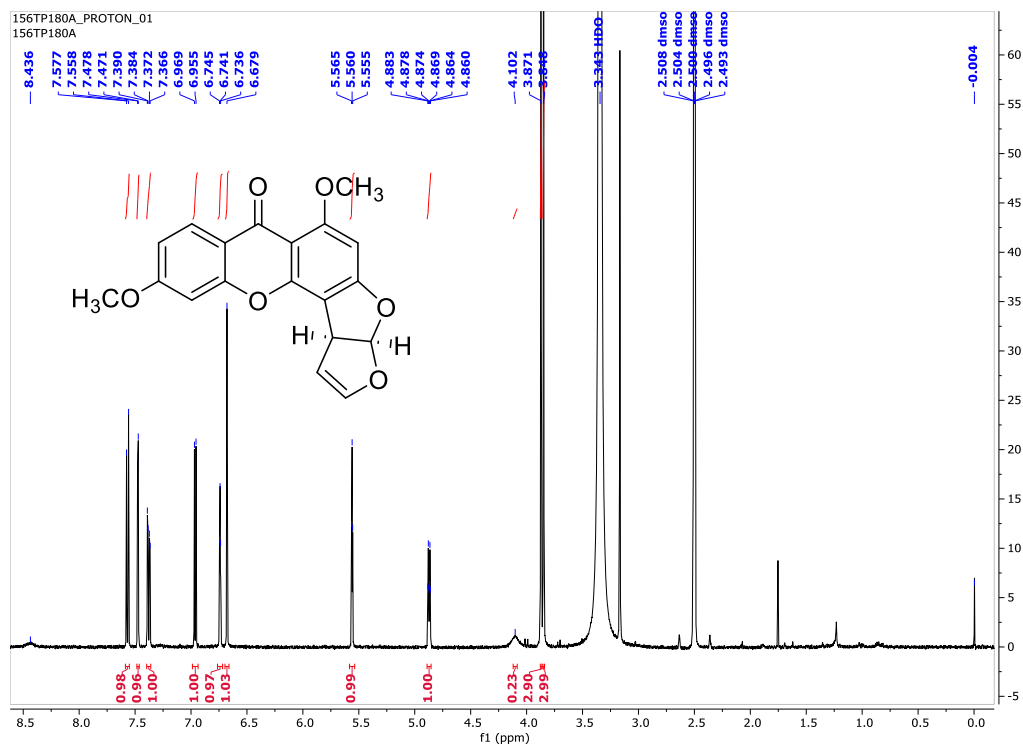


Figure B.13. ^1H NMR (500 MHz, DMSO) of **3**

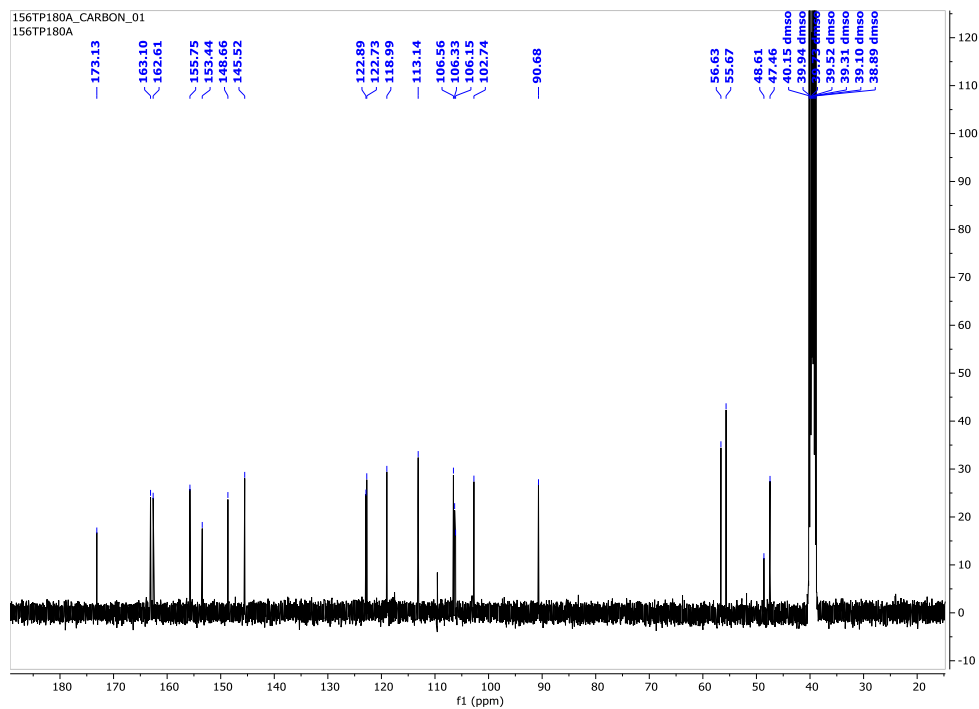


Figure B.14. ^{13}C NMR (400 MHz, DMSO) of **3**

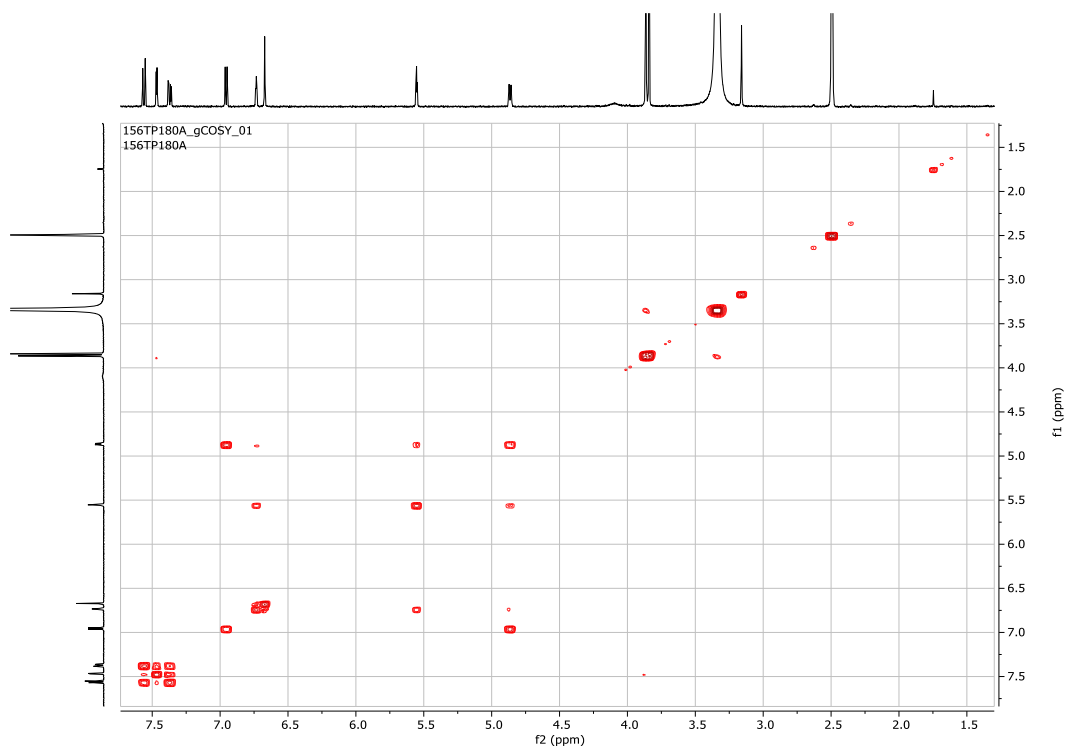


Figure B.15. COSY (500 MHz, DMSO) spectrum of compound **3**

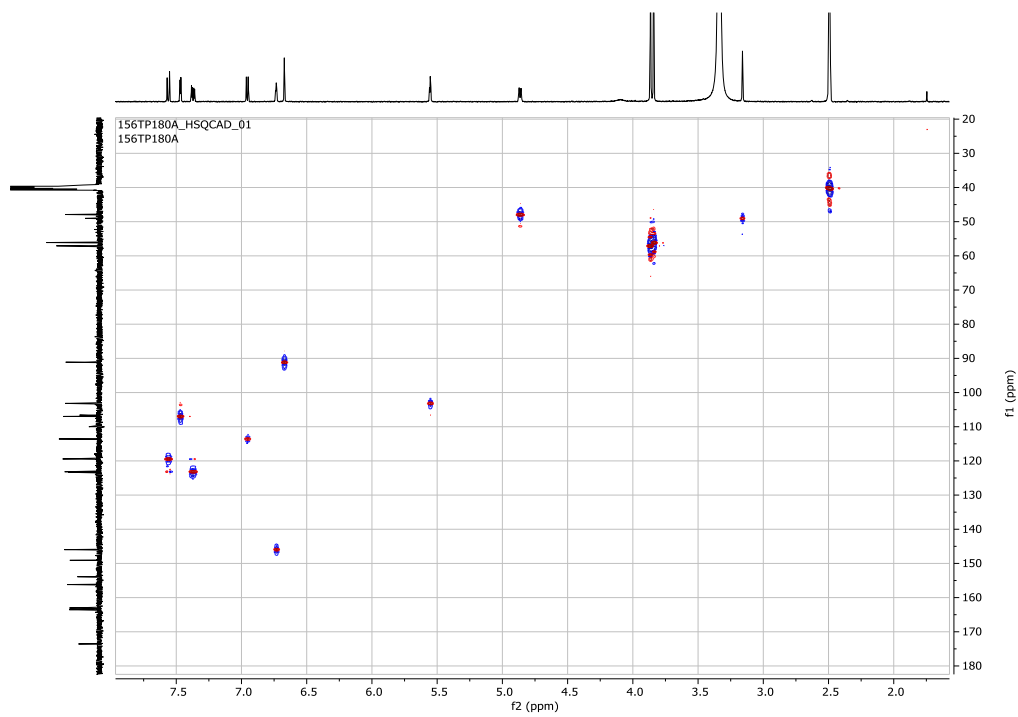


Figure B.16. HSQC (500 MHz, DMSO) spectrum of compound **3**

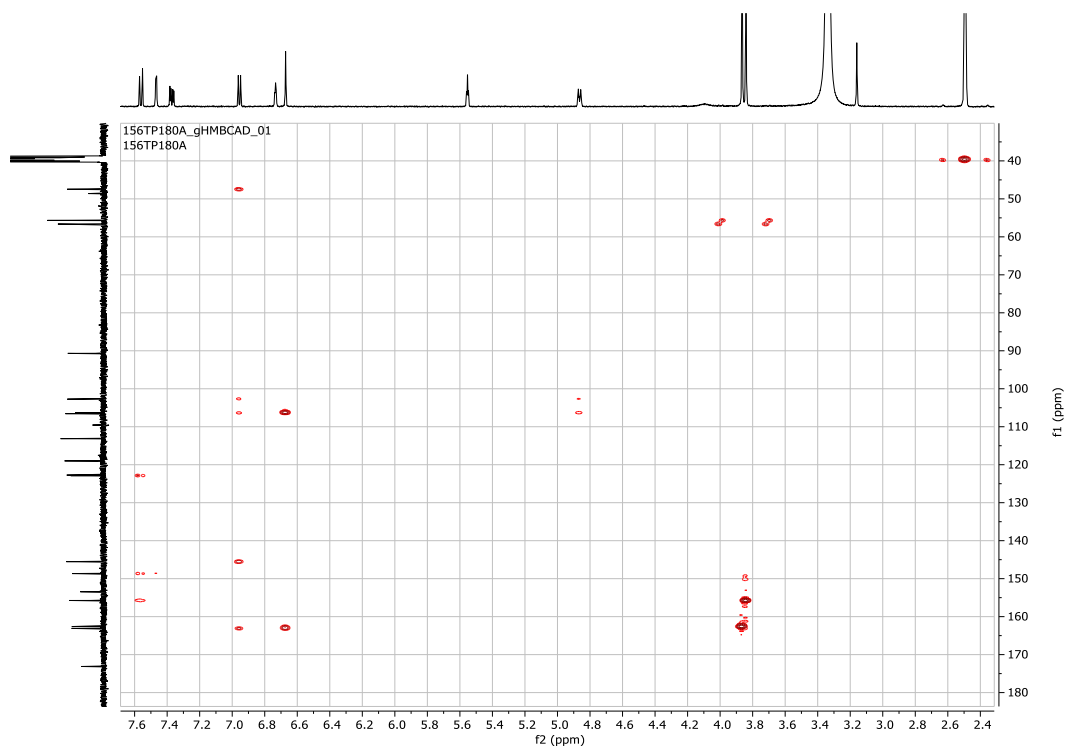


Figure B.17. HMBC (500 MHz, DMSO) spectrum of compound **3**

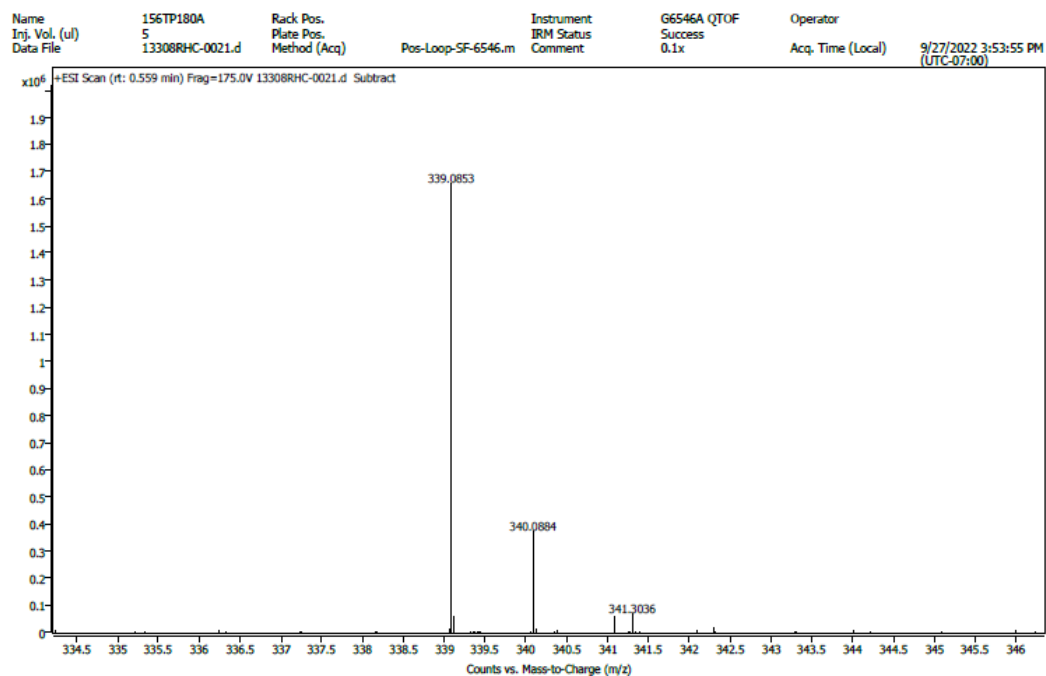


Figure B.18. HRESIMS spectrum of compound **3**

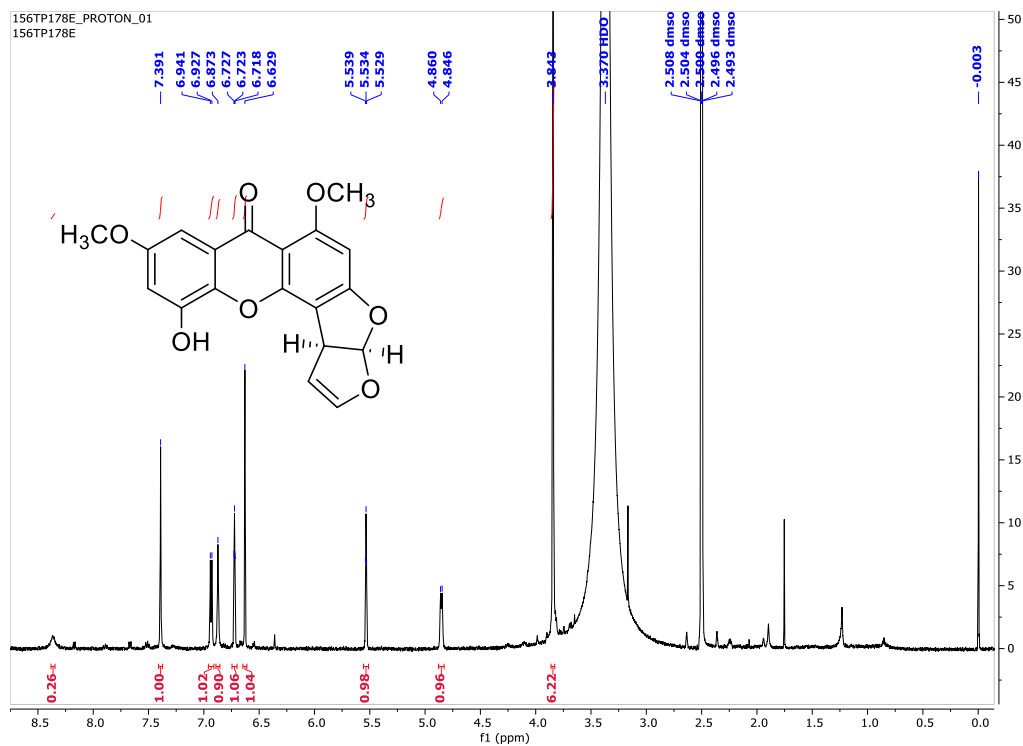


Figure B.19. ^1H NMR (500 MHz, DMSO) of **4**

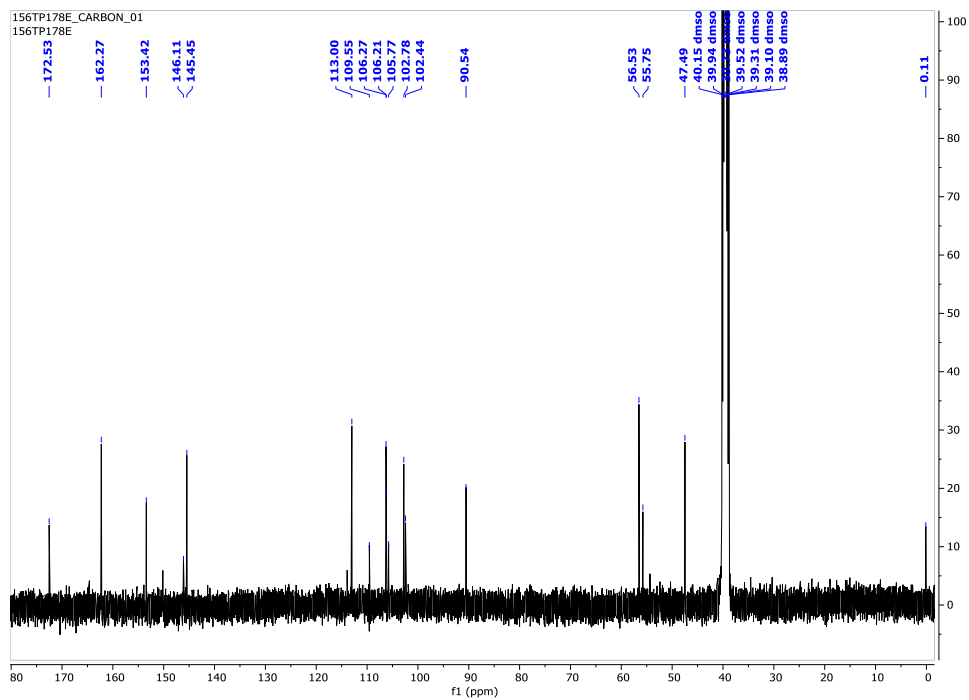


Figure B.20. ^{13}C NMR (400 MHz, DMSO) of **4**

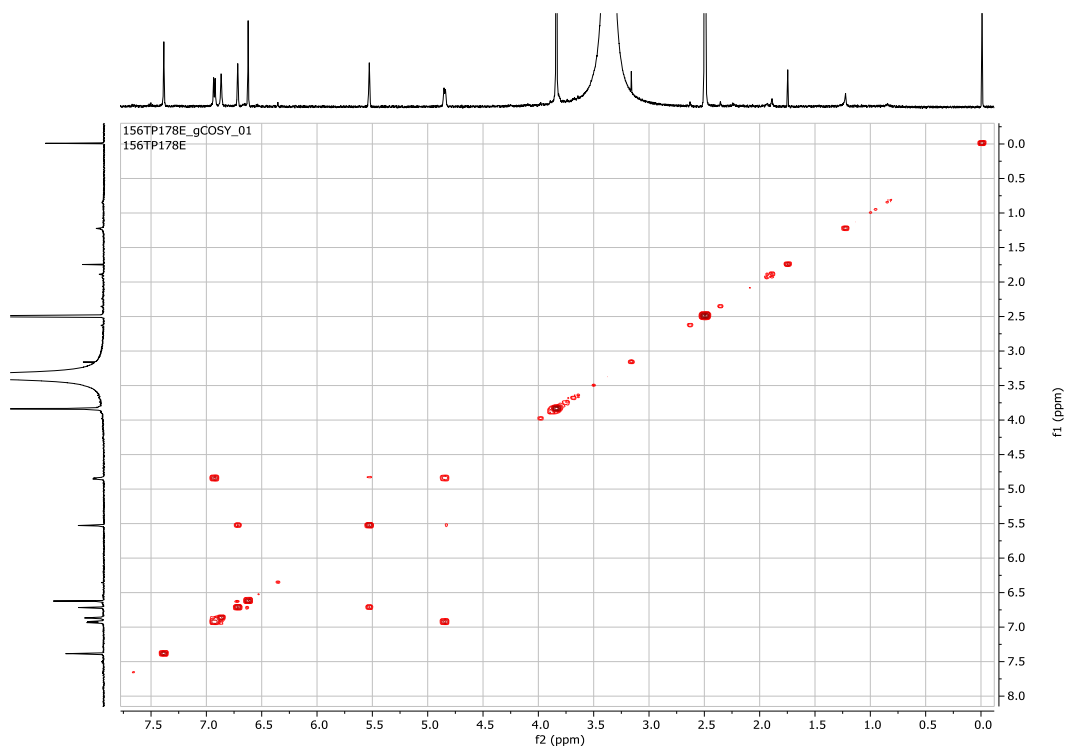


Figure B.21. COSY (500 MHz, DMSO) spectrum of compound **4**

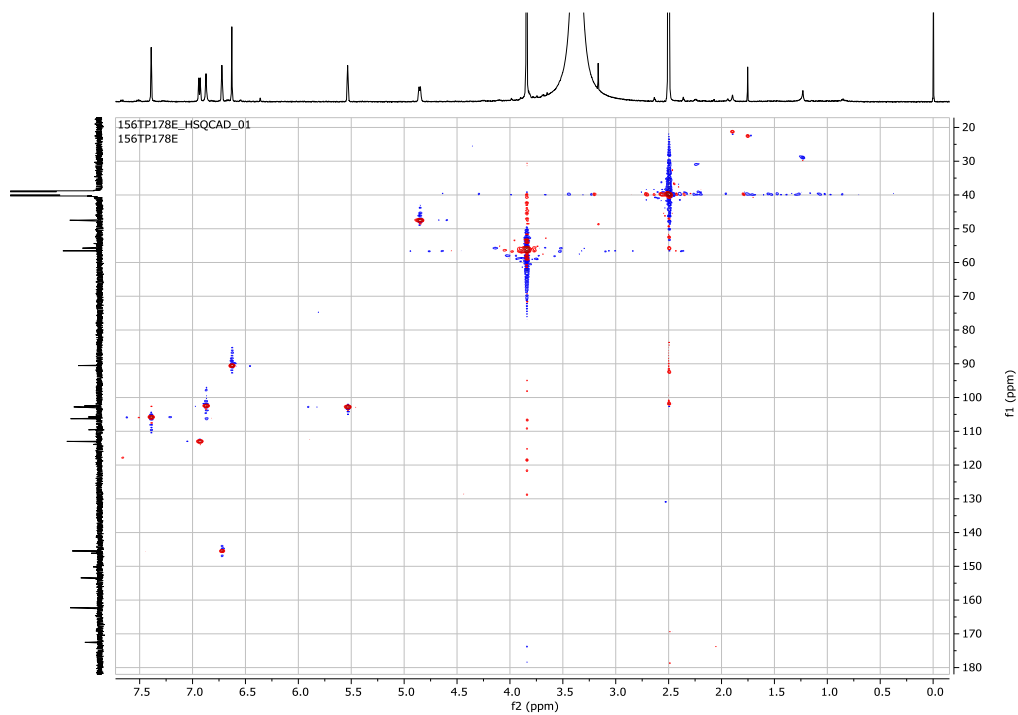


Figure B.22. HSQC (500 MHz, DMSO) spectrum of compound **4**

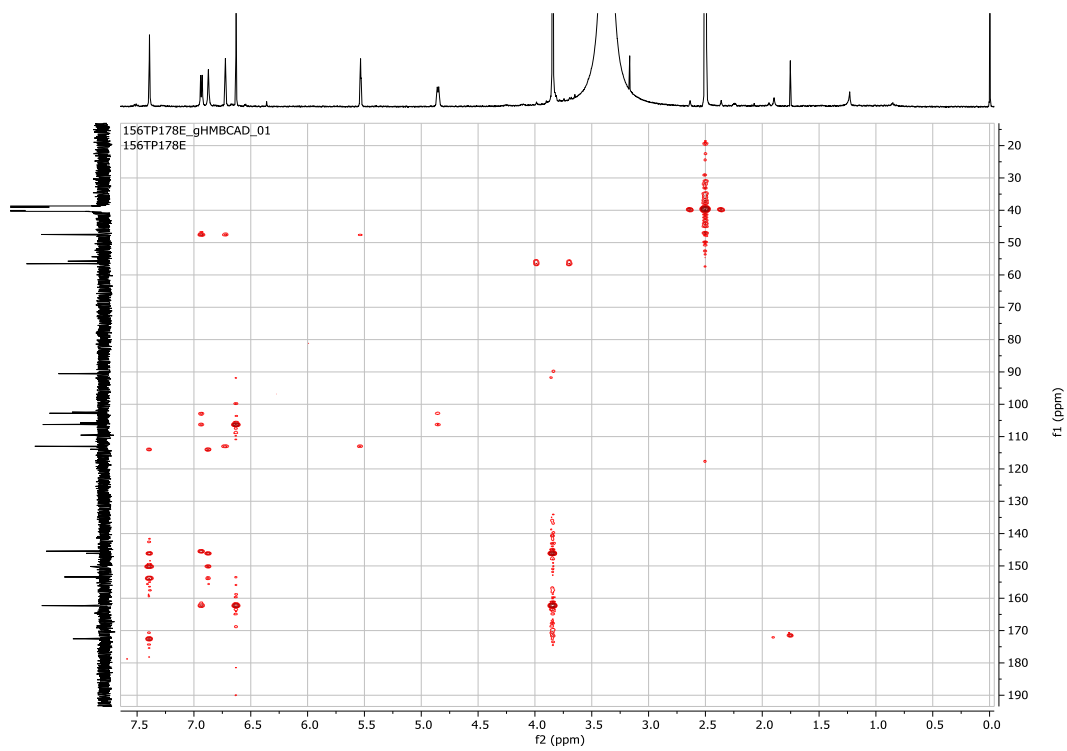


Figure B.23. HMBC (500 MHz, DMSO) spectrum of compound **4**

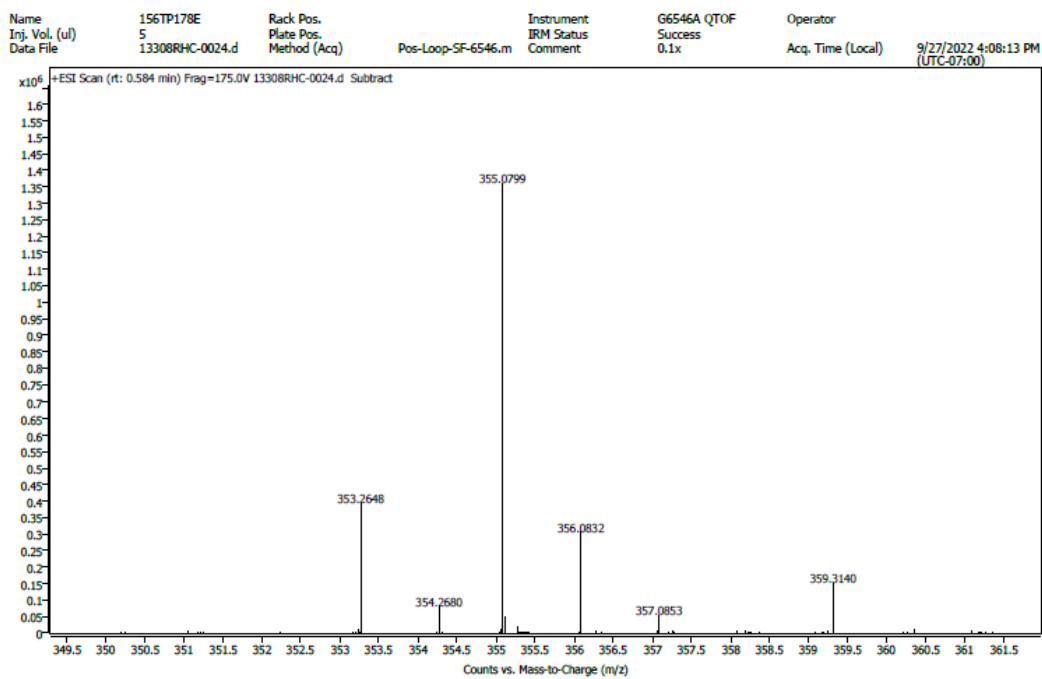


Figure B.24. HRESIMS spectrum of compound **4**

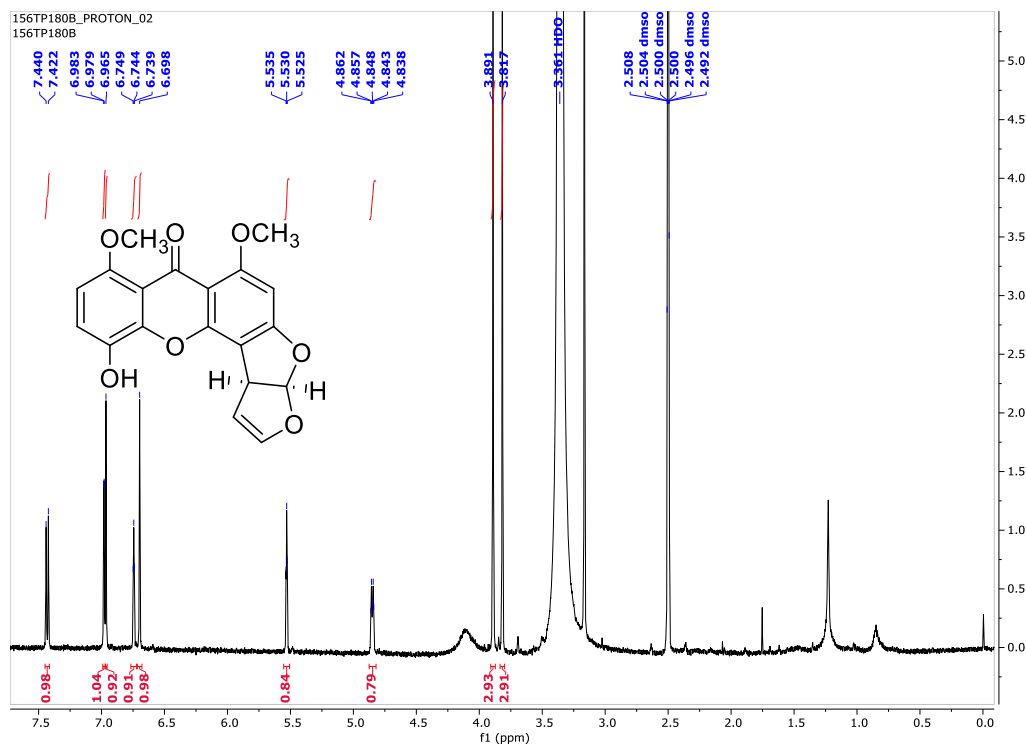


Figure B.25. ^1H NMR (500 MHz, DMSO) of 5

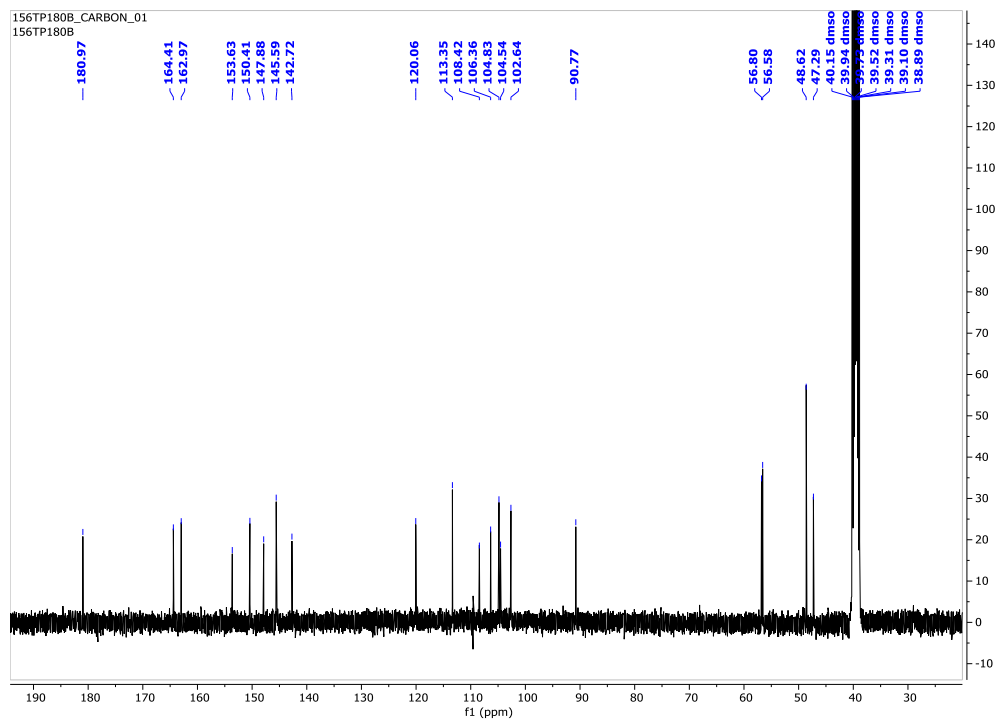


Figure B.26. ^{13}C NMR (400 MHz, DMSO) of 5

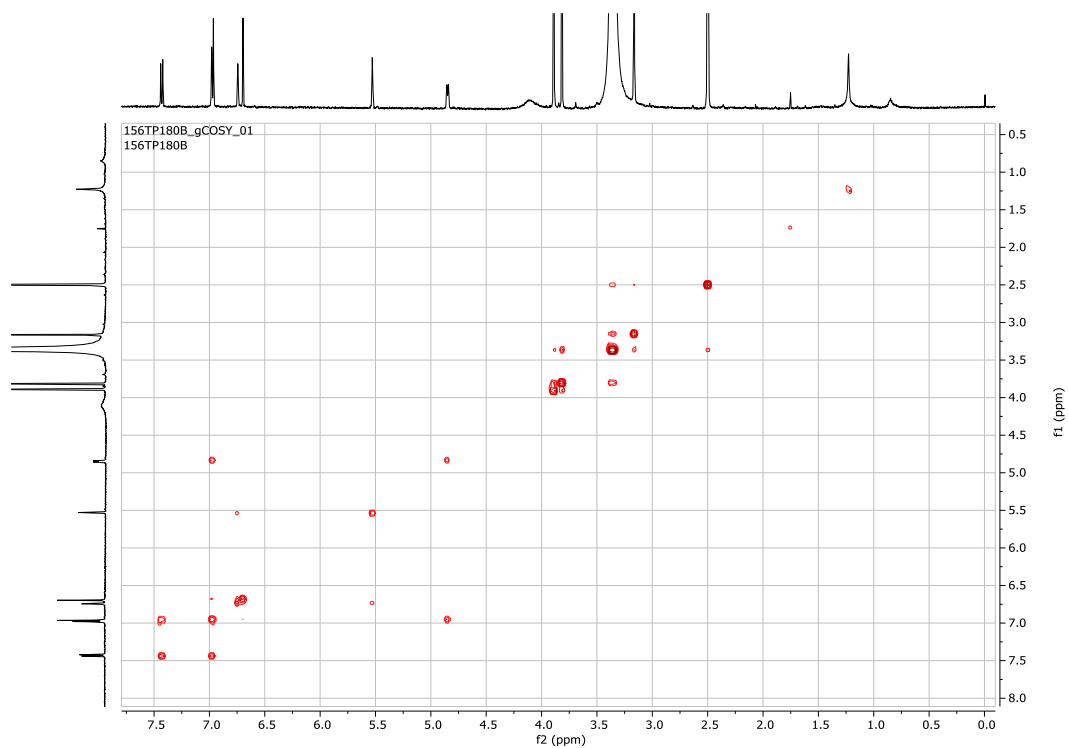


Figure B.27. COSY (500 MHz, DMSO) spectrum of compound **5**

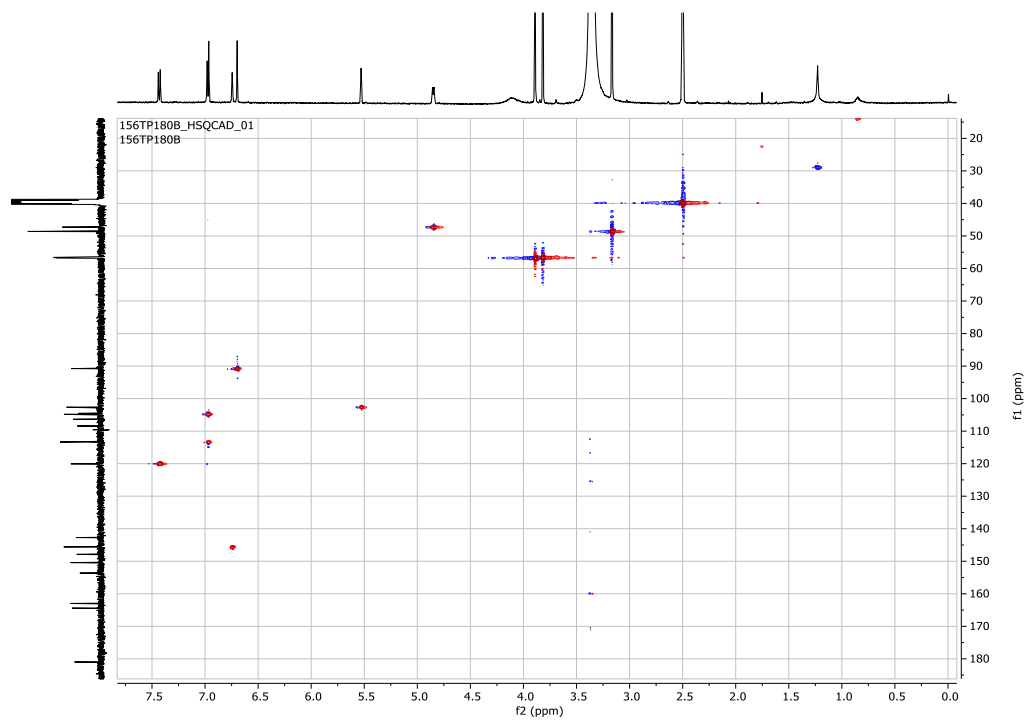


Figure B.28. HSQC (500 MHz, DMSO) spectrum of compound **5**

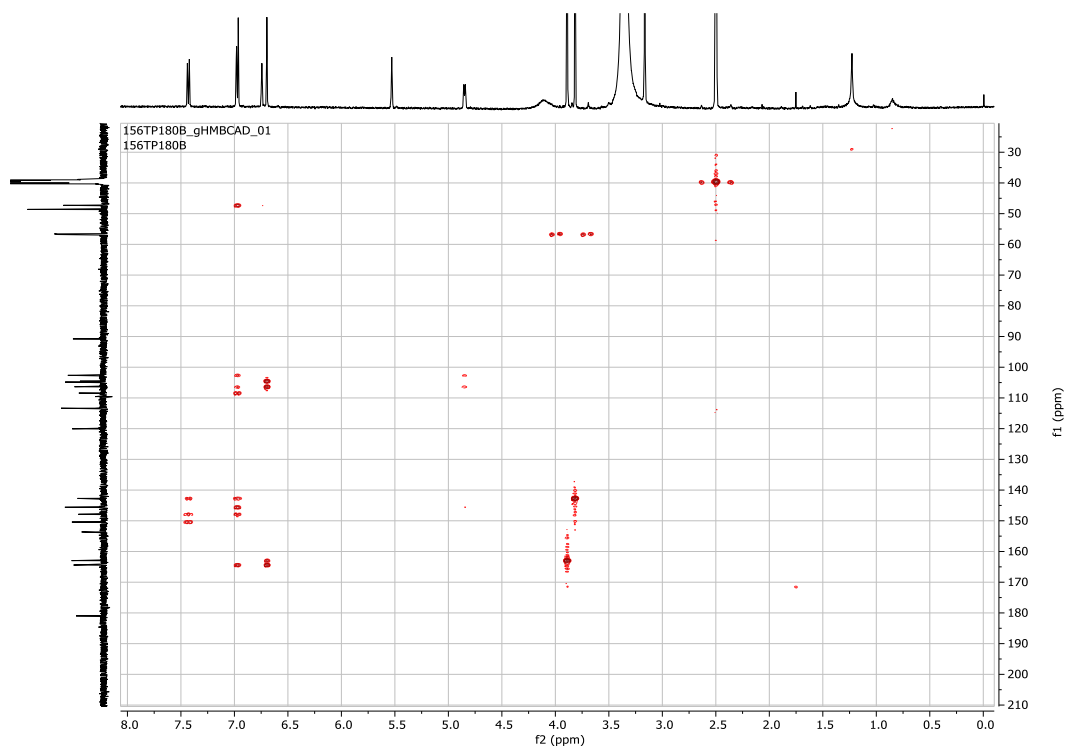


Figure B.29. HMBC (500 MHz, DMSO) spectrum of compound 5

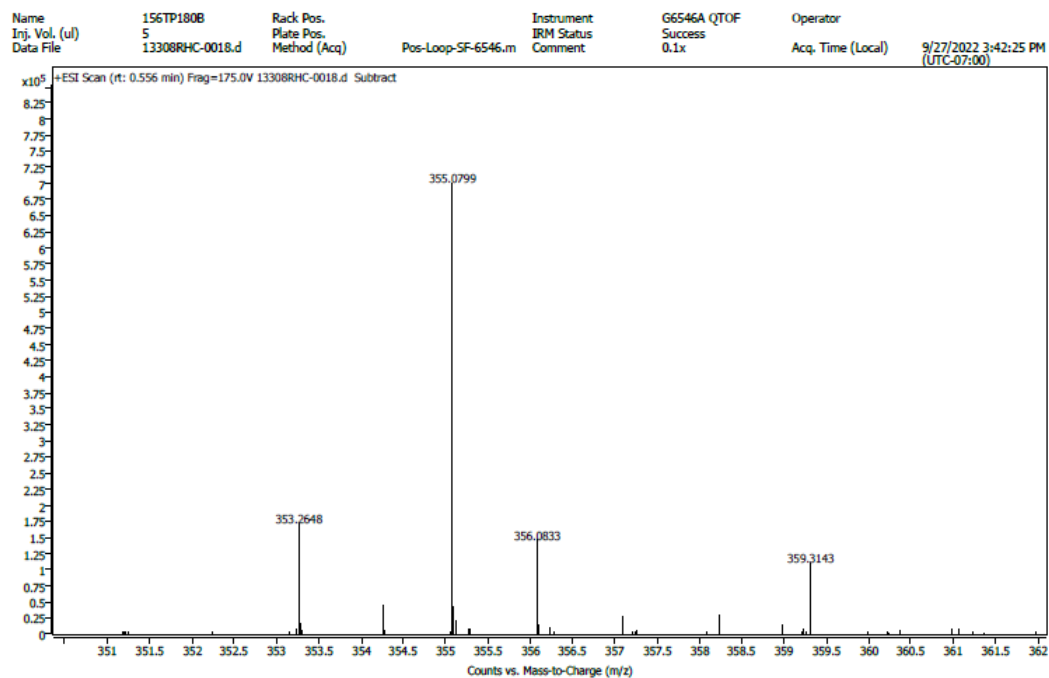


Figure B.30. HRESIMS spectrum of compound 5

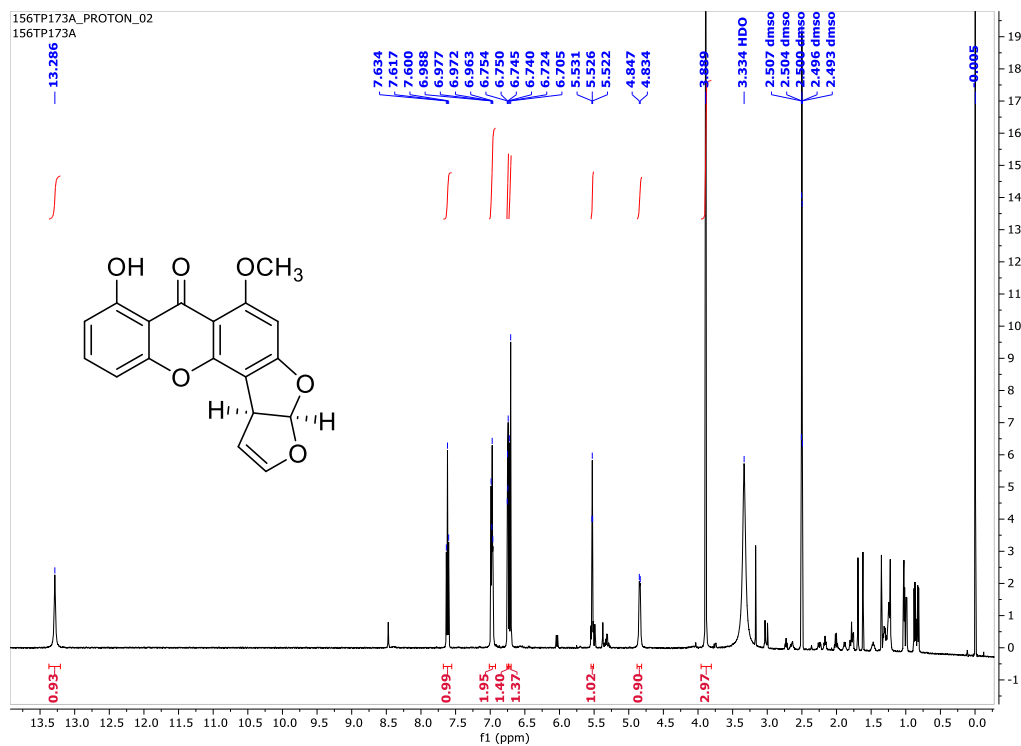


Figure B.31. ^1H NMR (500 MHz, DMSO) of **6**

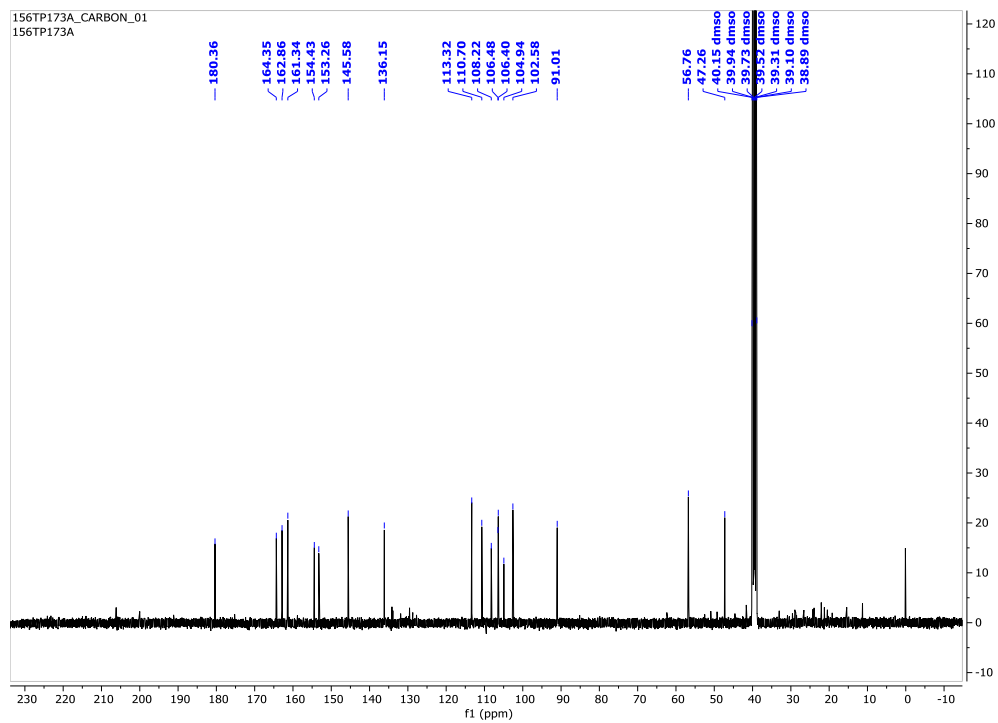


Figure B.32. ^{13}C NMR (400 MHz, DMSO) of **6**

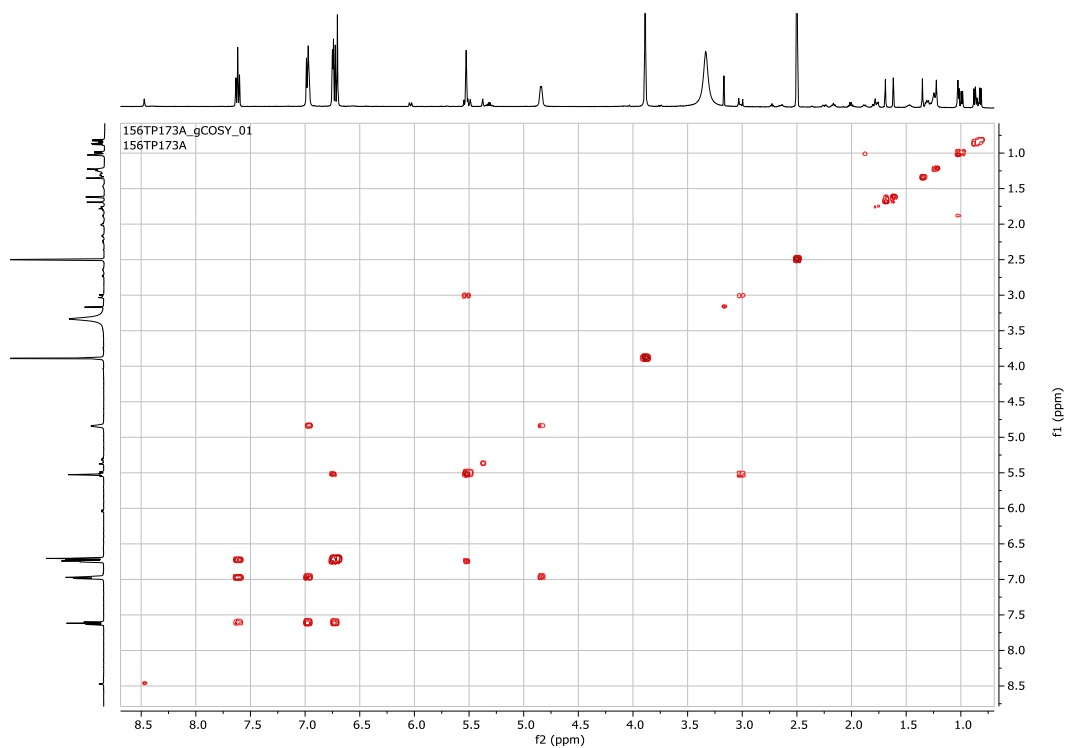


Figure B.33. COSY (500 MHz, DMSO) spectrum of compound **6**

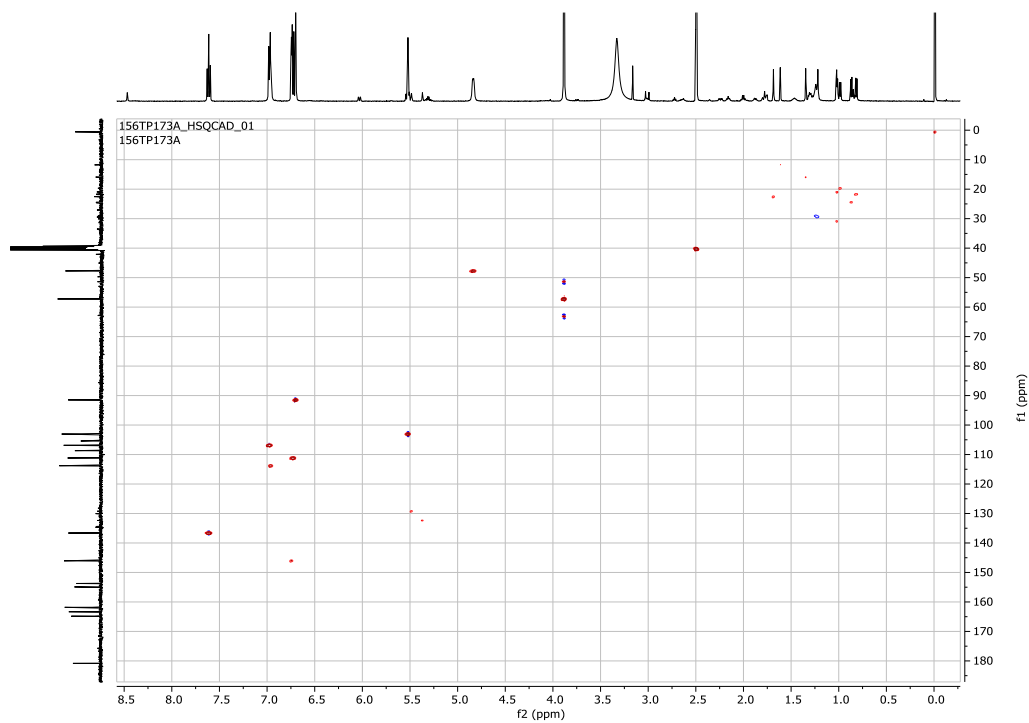


Figure B.34. HSQC (500 MHz, DMSO) spectrum of compound **6**

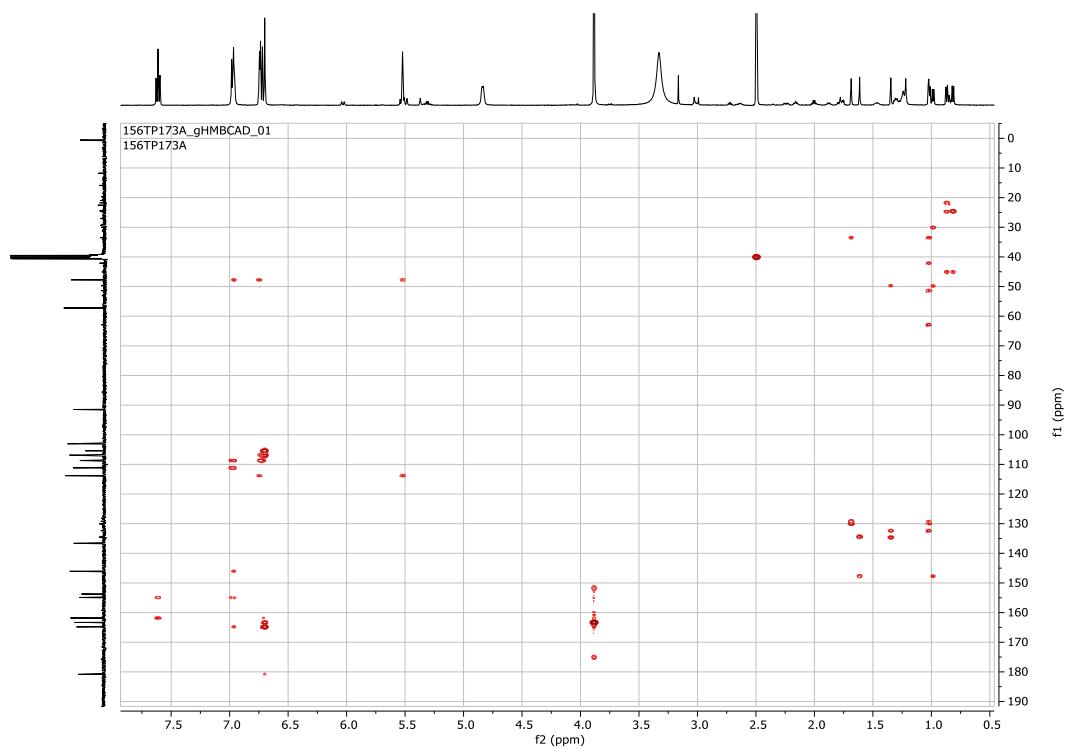


Figure B.35. HMBC (500 MHz, DMSO) spectrum of compound **6**

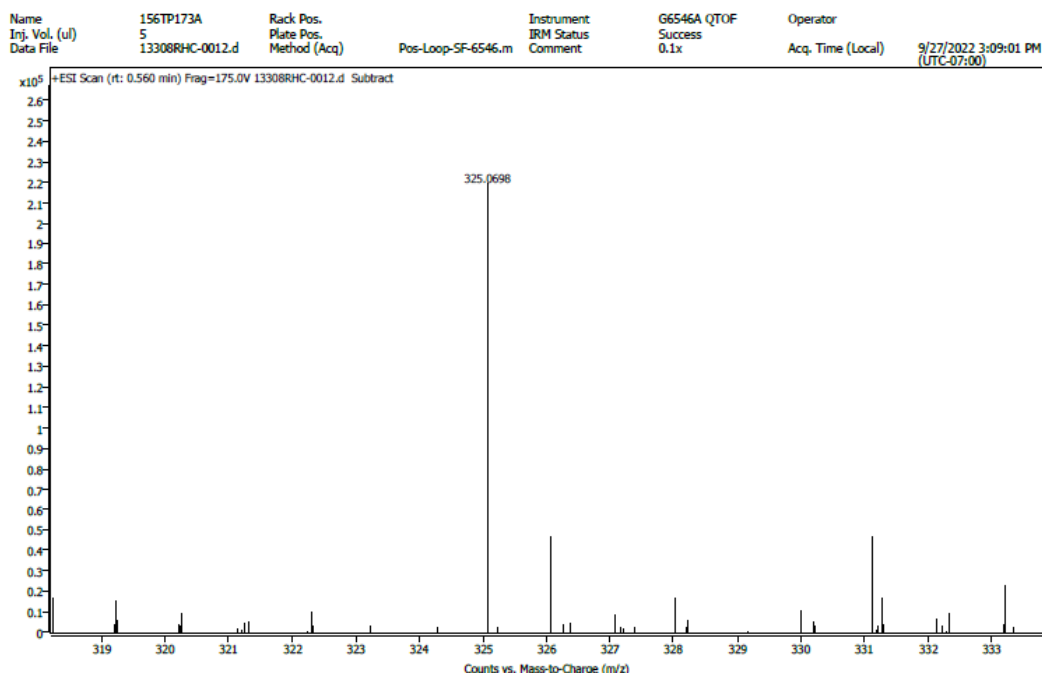


Figure B.36. HRESIMS spectrum of compound **6**

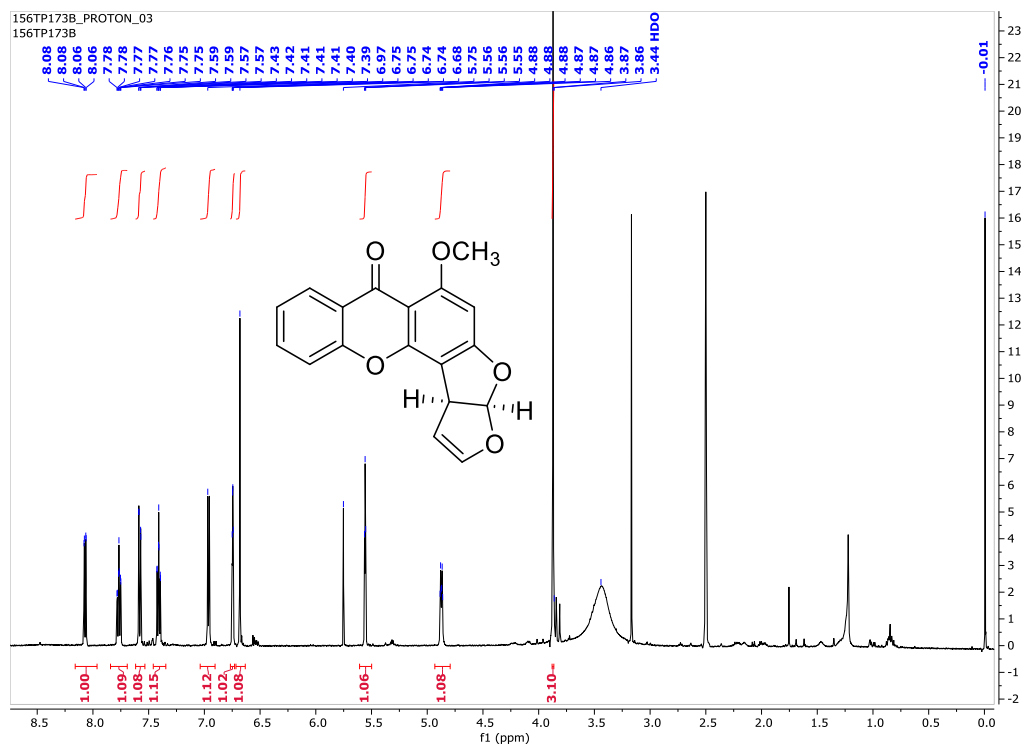


Figure B.37. ^1H NMR (500 MHz, DMSO) of 7

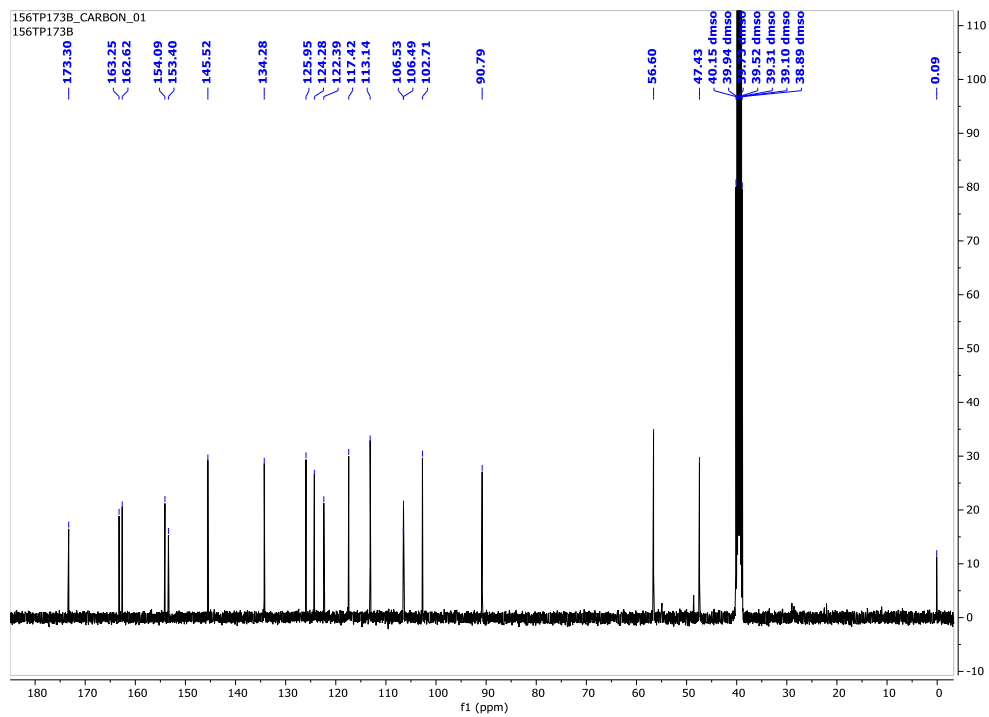


Figure B.38. ^{13}C NMR (400 MHz, DMSO) of 7

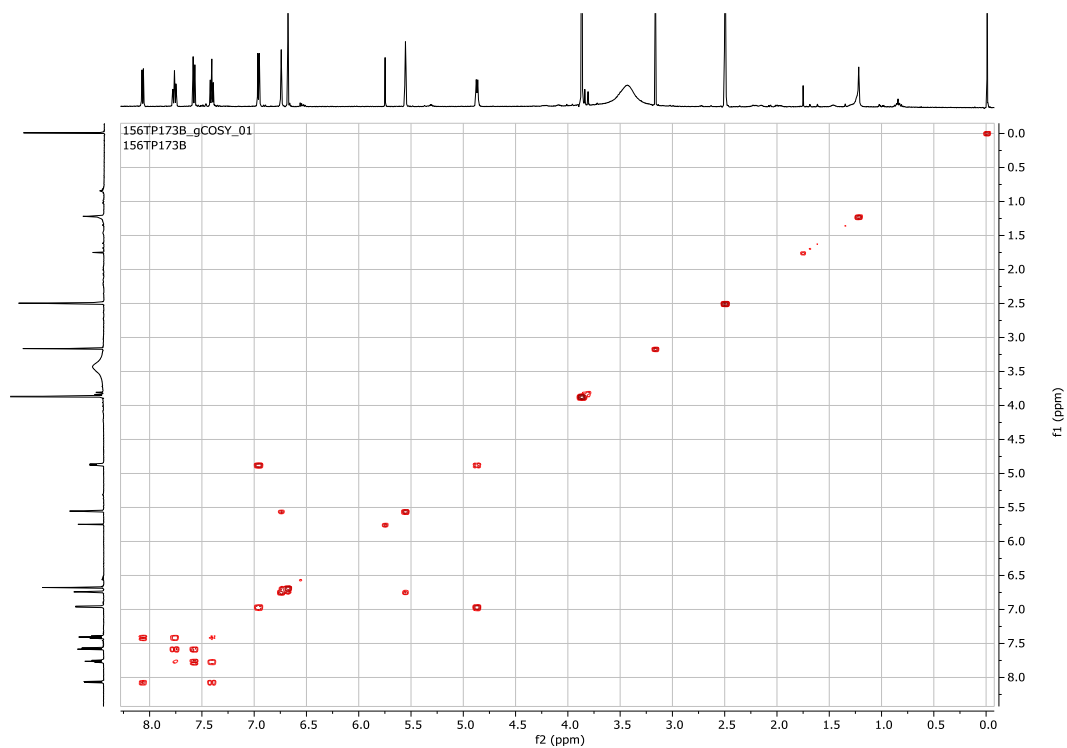


Figure B.39. COSY (500 MHz, DMSO) spectrum of compound **7**

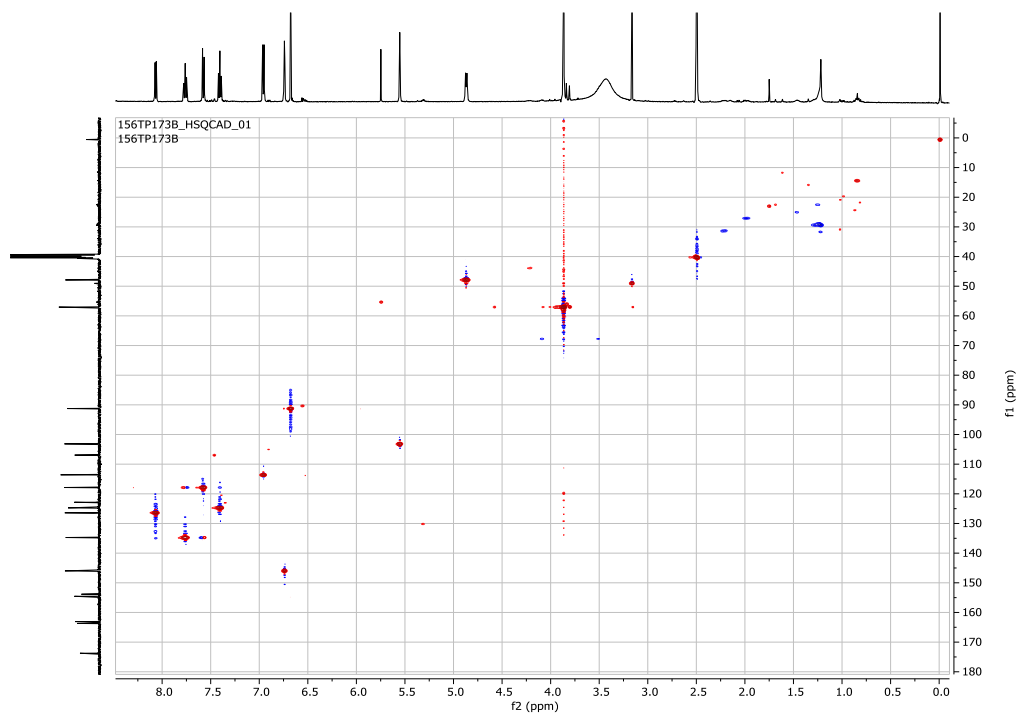


Figure B.40. HSQC (500 MHz, DMSO) spectrum of compound **7**

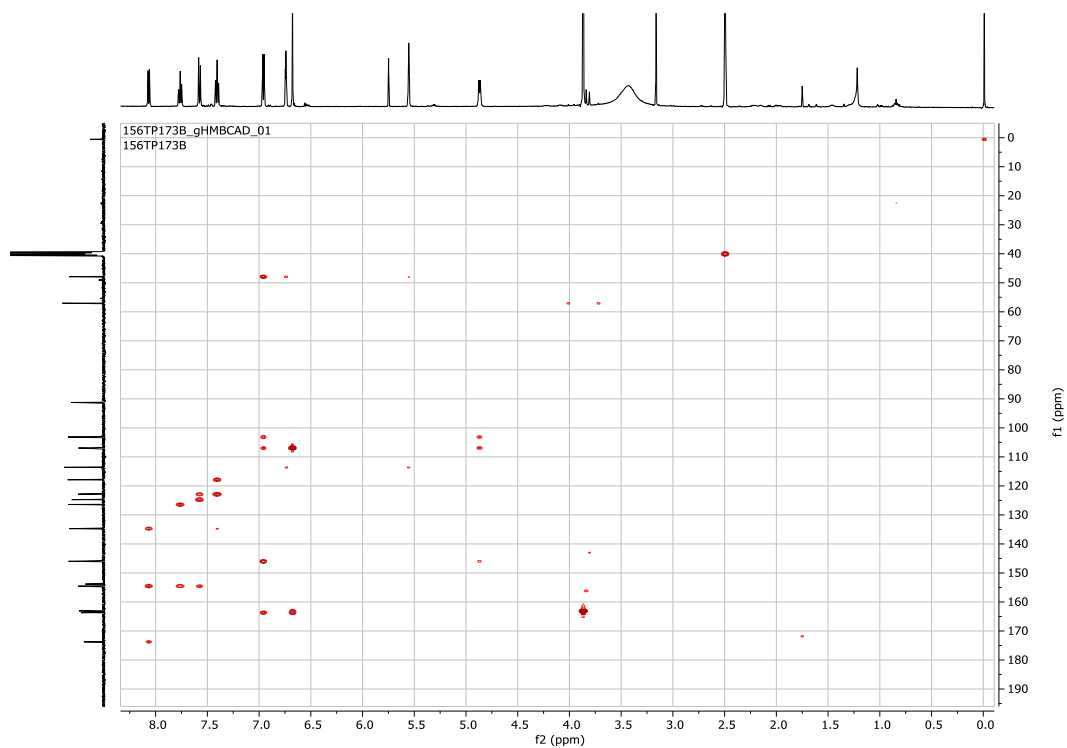


Figure B.41. HMBC (500 MHz, DMSO) spectrum of compound **7**

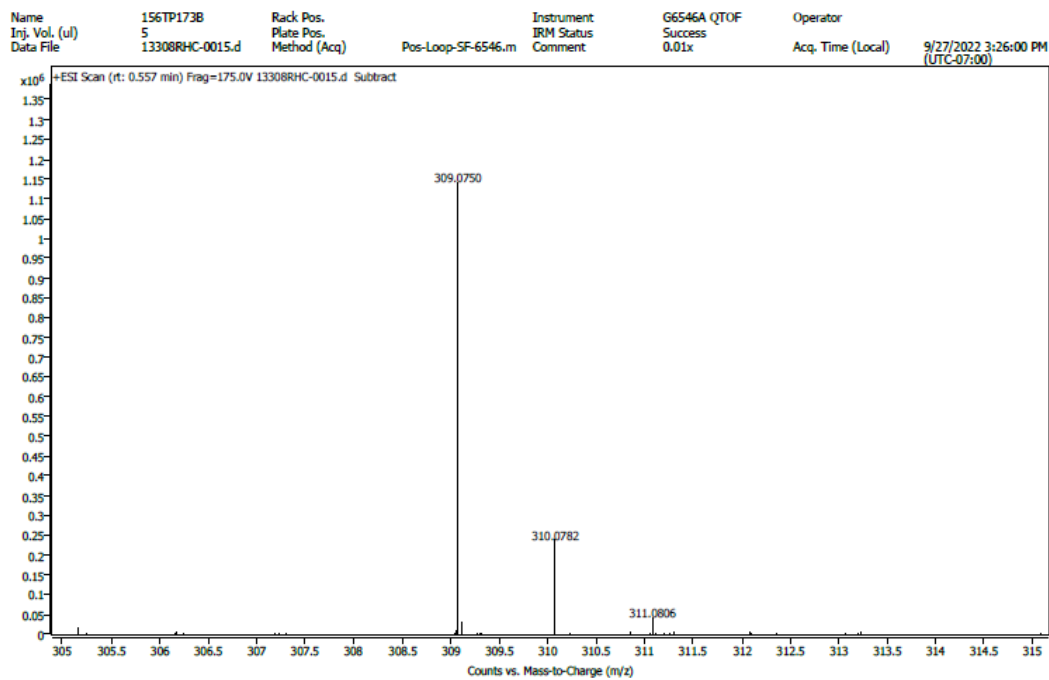


Figure B.42. HRESIMS spectrum of compound **7**

Appendix C: Supporting Data for Chapter 5

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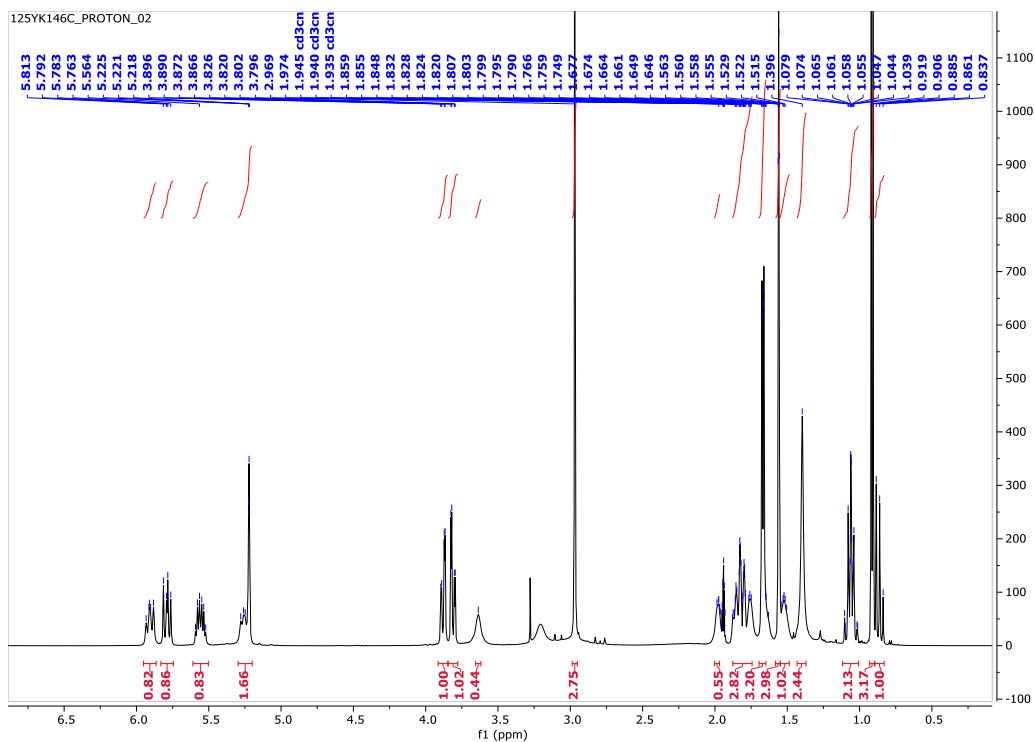


Figure C.1. ^1H NMR (500 MHz, $\text{CD}_3\text{CN}-d_3$) of **1**

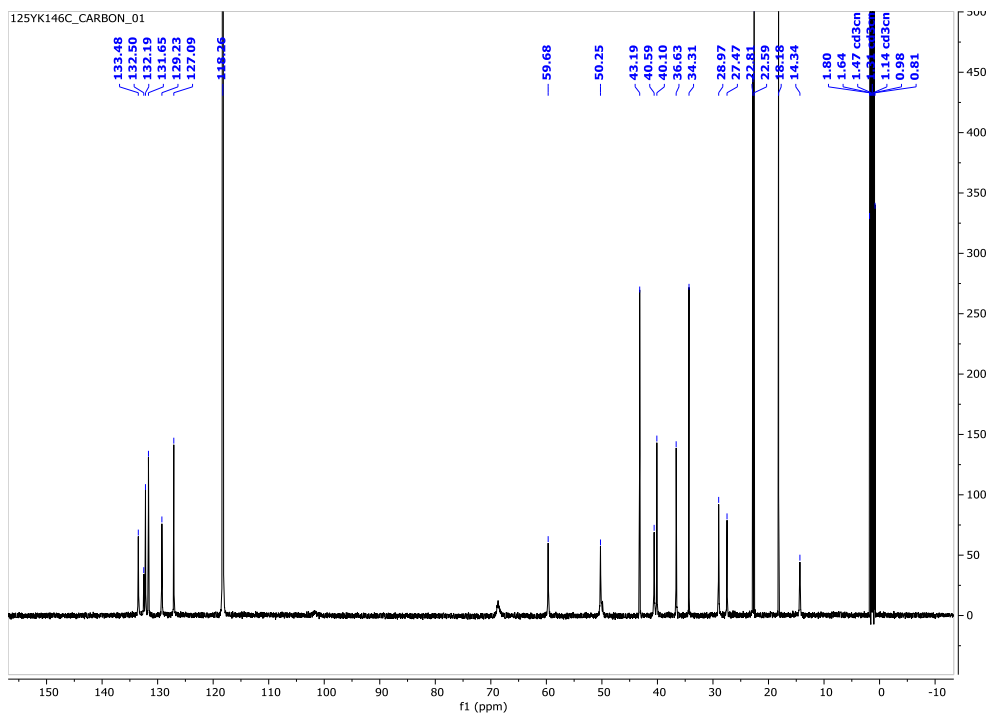


Figure C.2. ^{13}C NMR (400 MHz, $\text{CD}_3\text{CN}-d_3$) of **1**

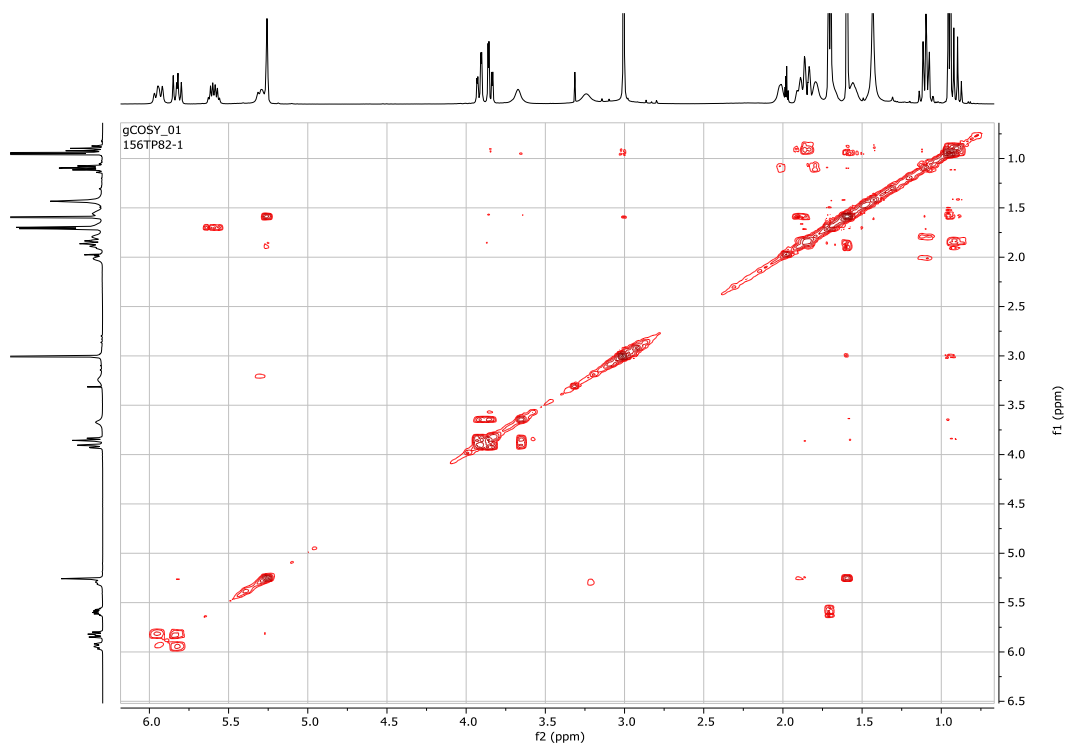


Figure C.3. COSY (500 MHz, $\text{CD}_3\text{CN}-d_3$) spectrum of compound **1**

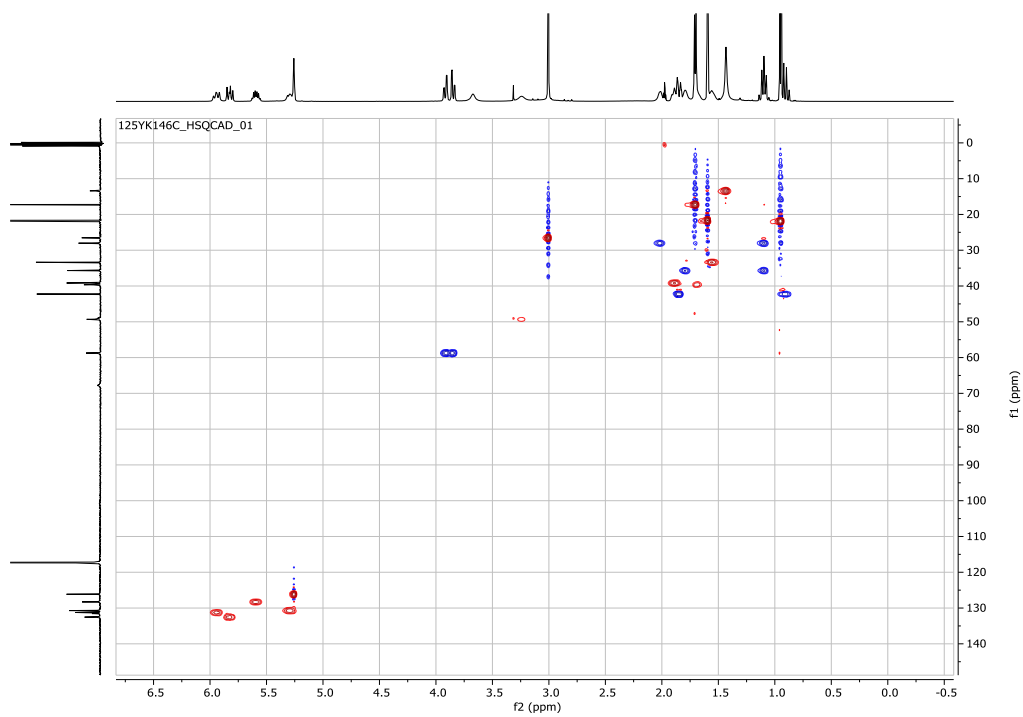


Figure C.4. HSQC (500 MHz, $\text{CD}_3\text{CN}-d_3$) spectrum of compound **1**

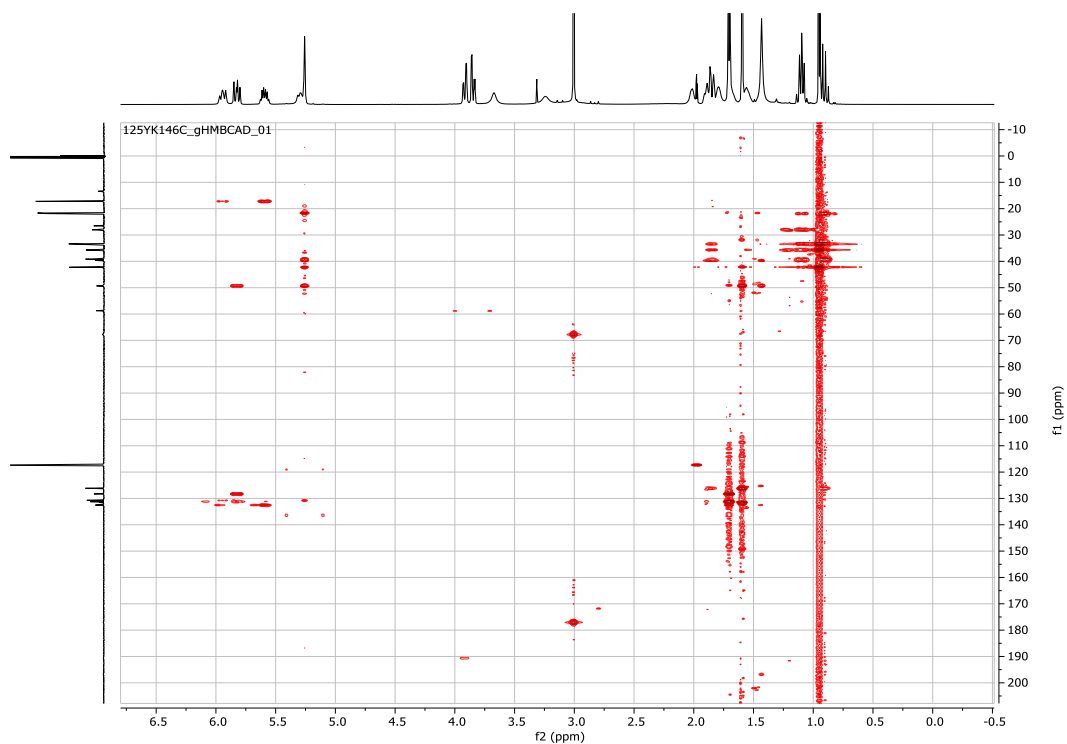


Figure C.5. HMBC (500 MHz, $\text{CD}_3\text{CN}-d_3$) spectrum of compound **1**

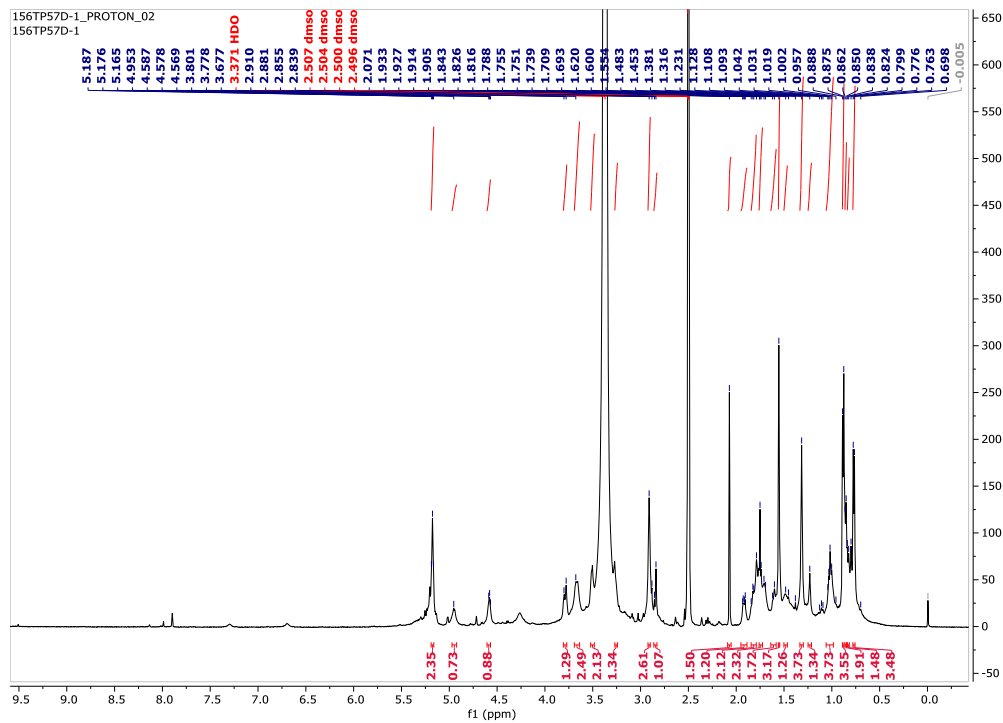


Figure C.6. ^1H NMR (500 MHz, DMSO) of **1a**

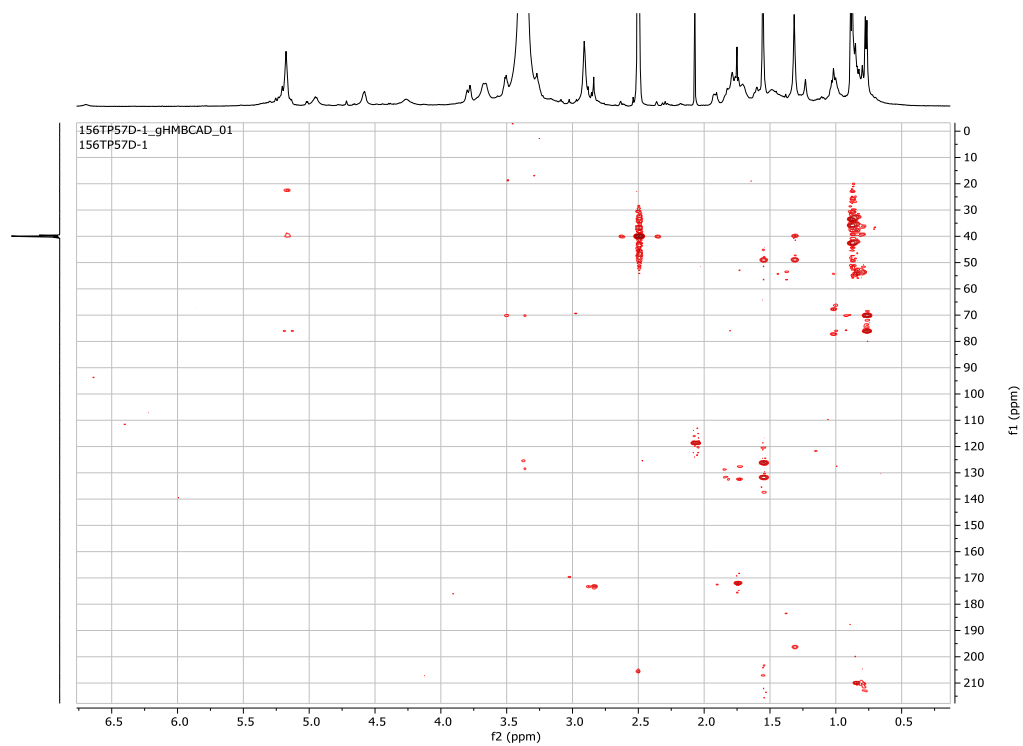


Figure C.7. HMBC (500 MHz, DMSO) spectrum of **1a**

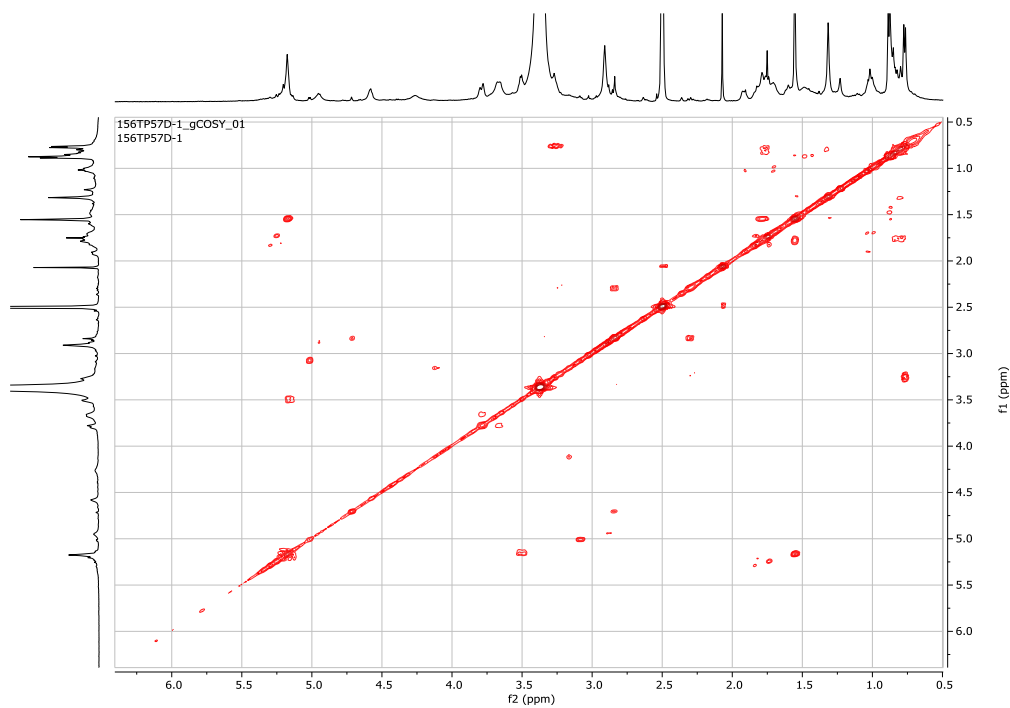


Figure C.8. COSY (500 MHz, DMSO) spectrum of **1a**

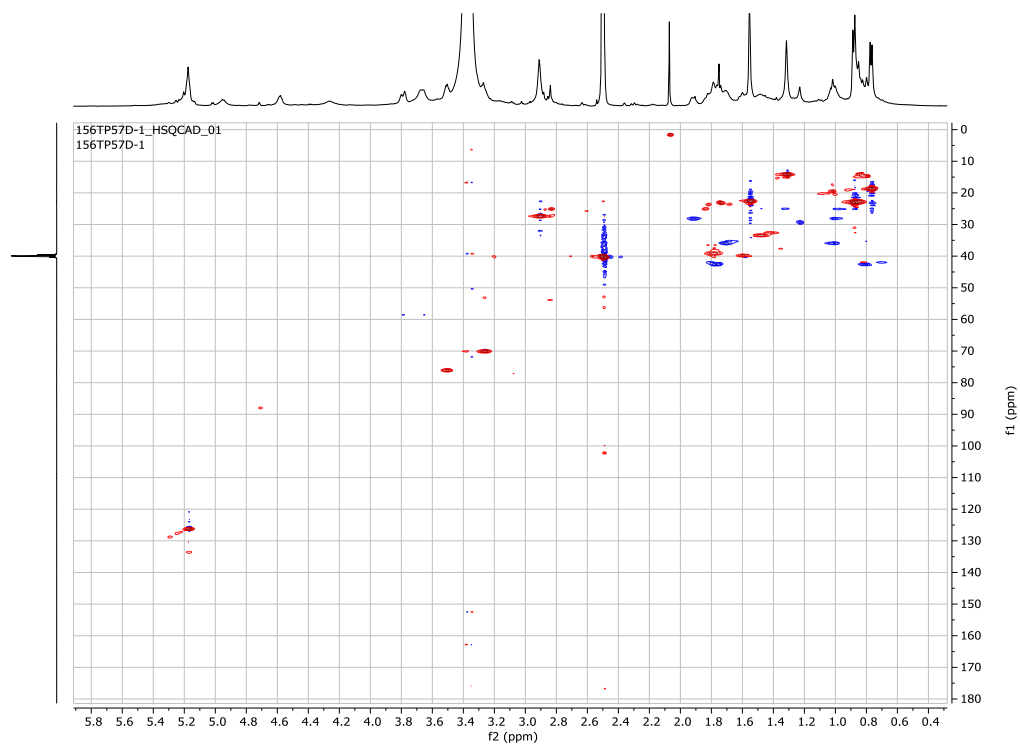


Figure C.9. HSQC (500 MHz, DMSO) spectrum of **1a**

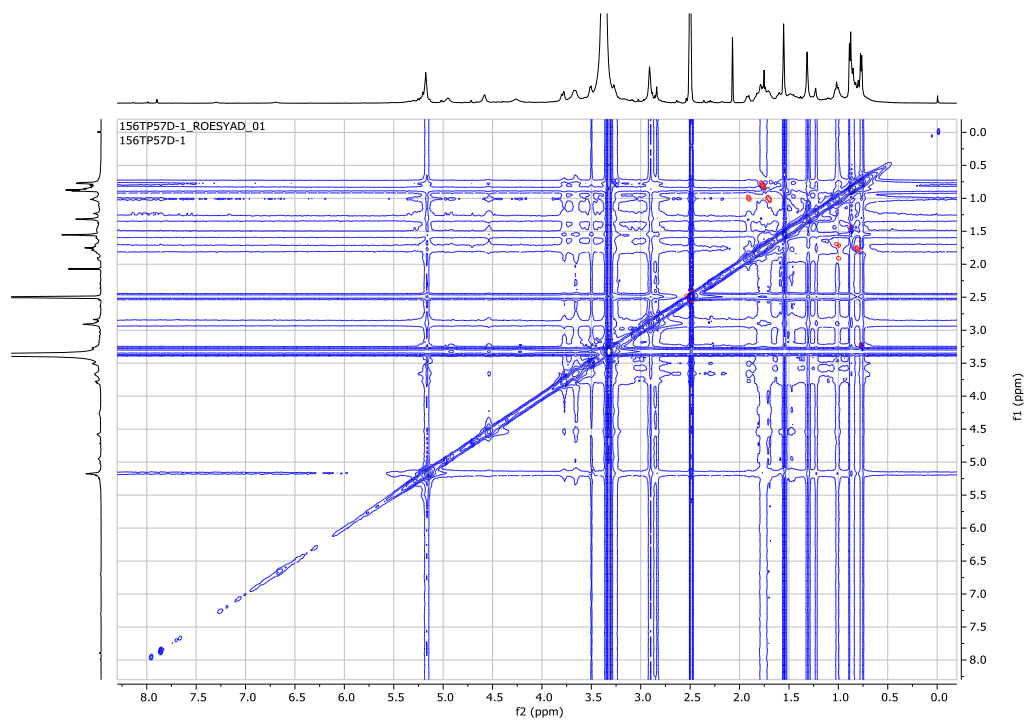


Figure C.10. ROESY spectrum (500 MHz, DMSO) of compound **1a**

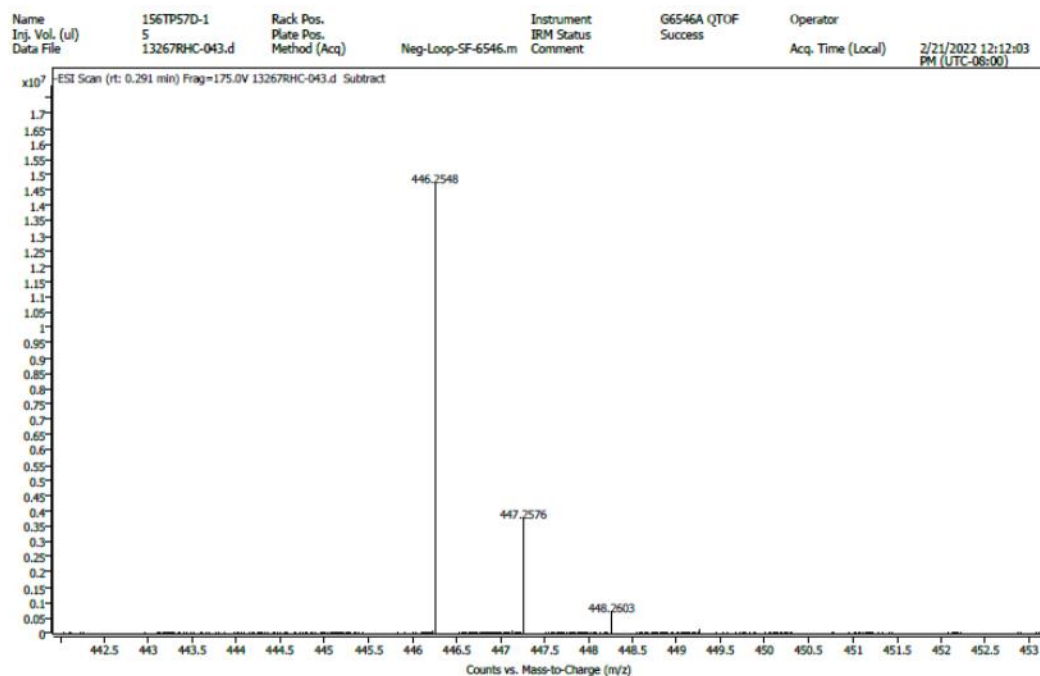


Figure C.11. HRESIMS spectrum of compound **1a**

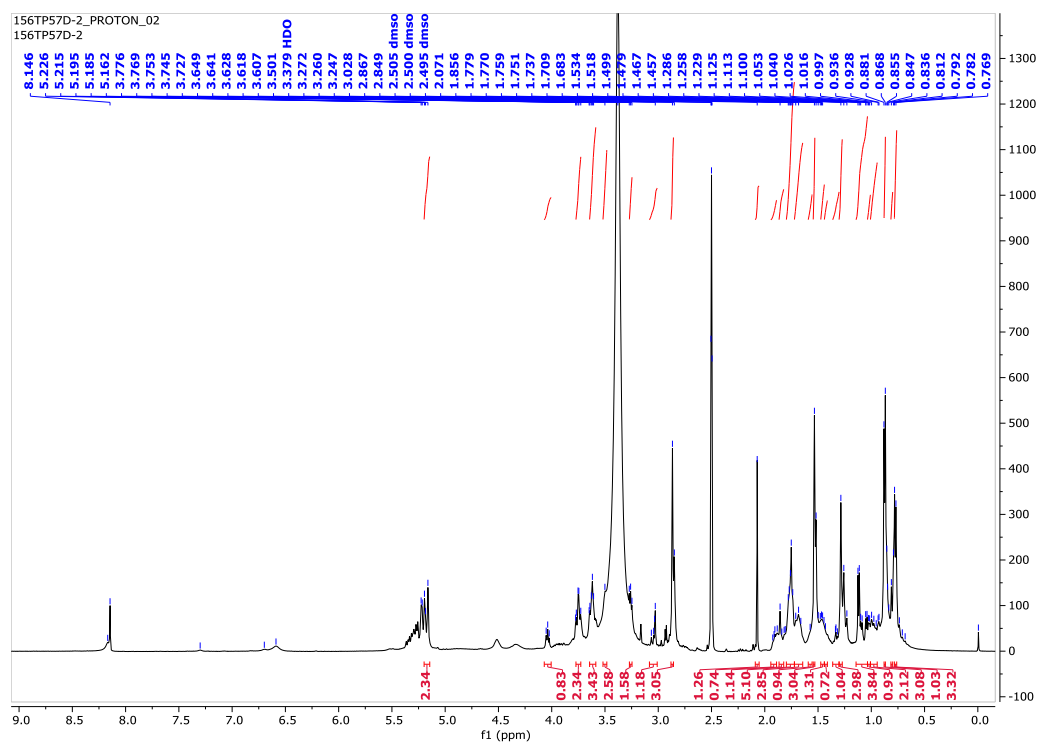


Figure C.12. ^1H NMR (500 MHz, DMSO) of **1b**

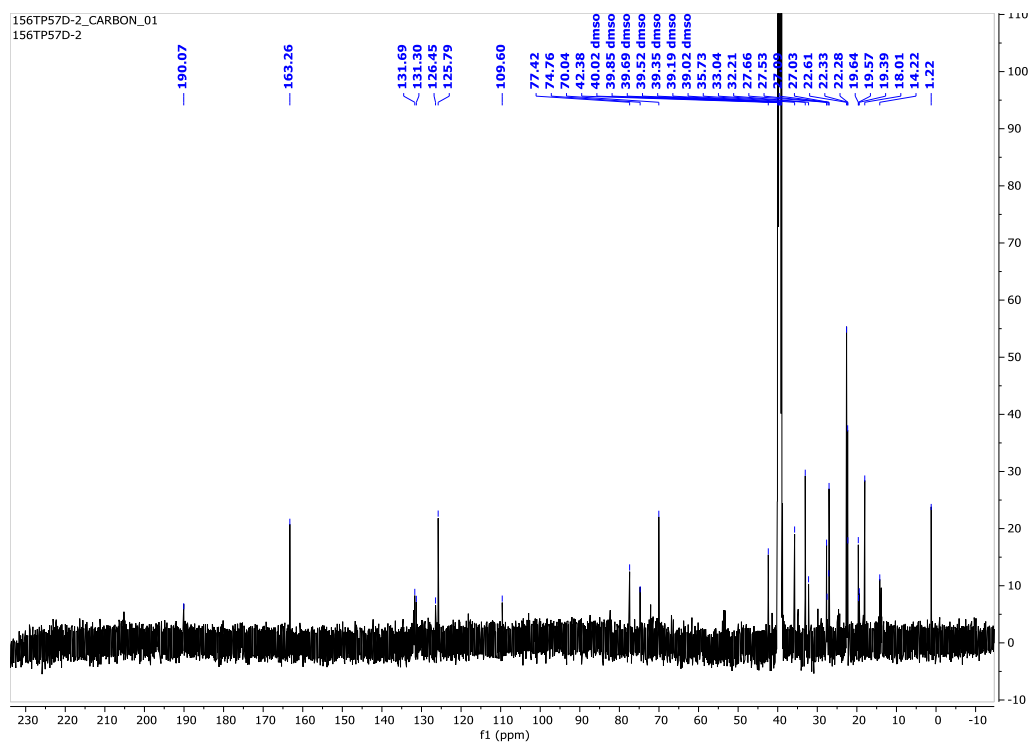


Figure C.13. ^{13}C NMR (400 MHz, DMSO) of **1b**

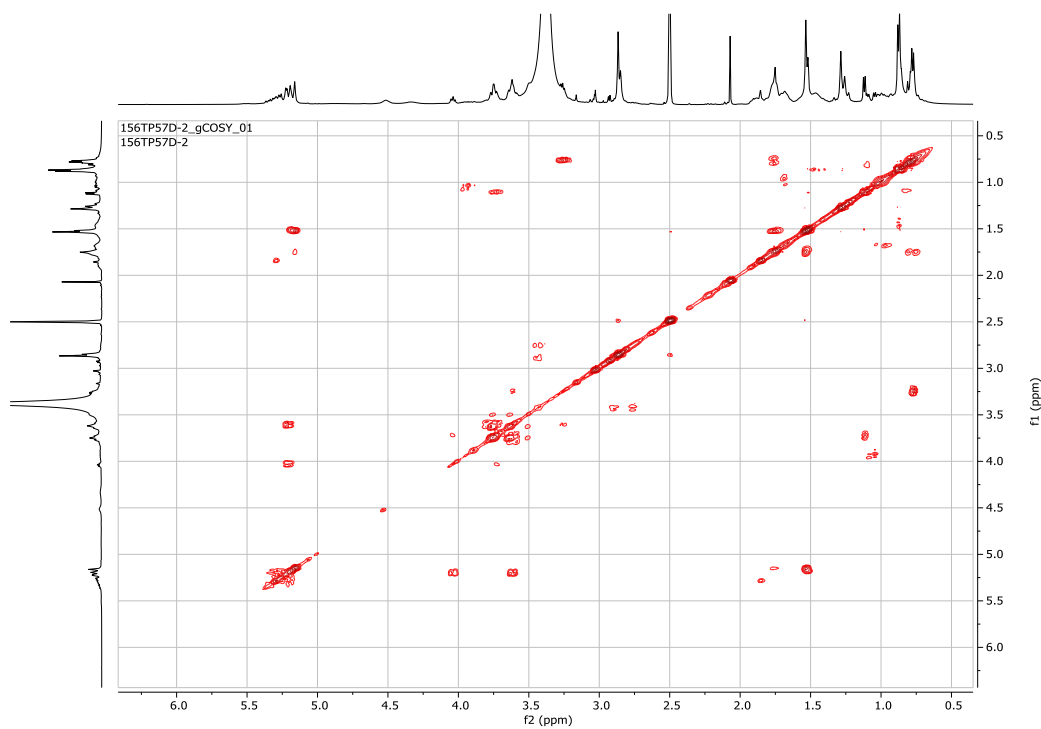


Figure C.14. COSY (500 MHz, DMSO) spectrum of **1b**

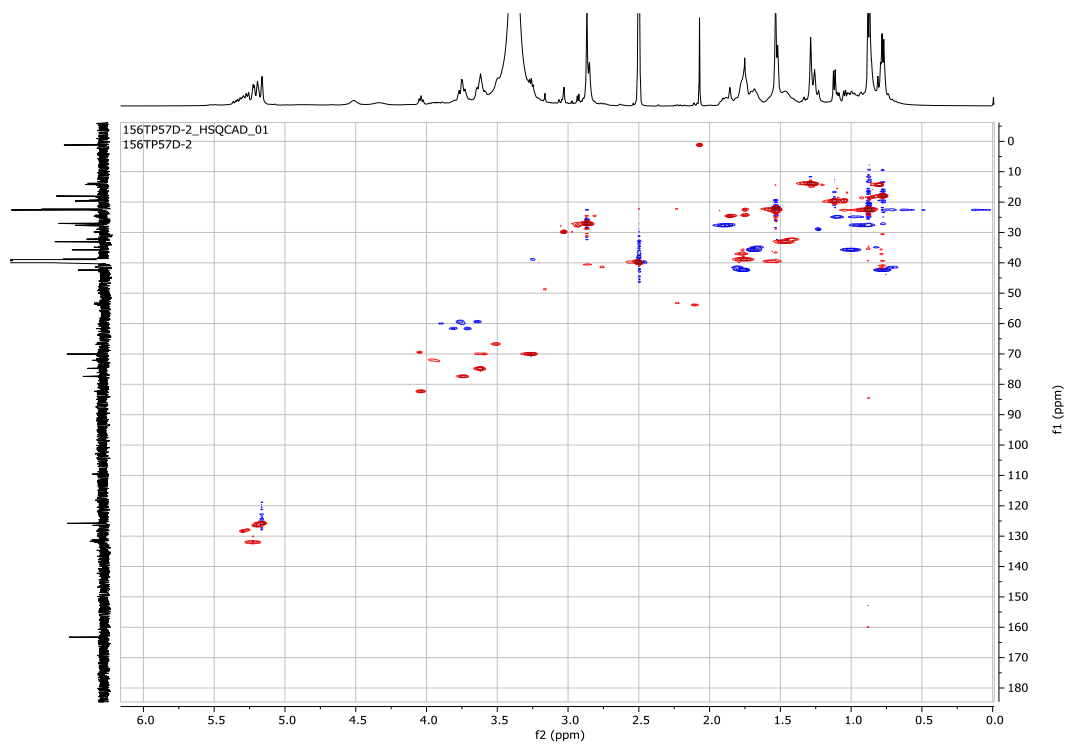


Figure C.15. HSQC (500 MHz, DMSO) spectrum of **1b**

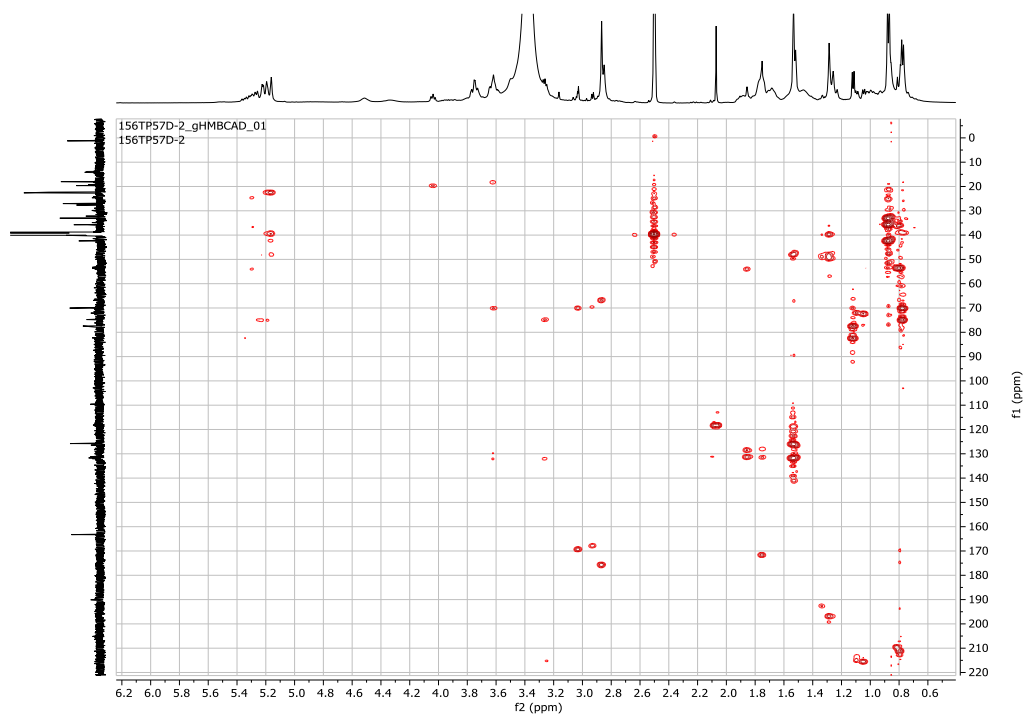


Figure C.16. HMBC (500 MHz, DMSO) spectrum of **1b**

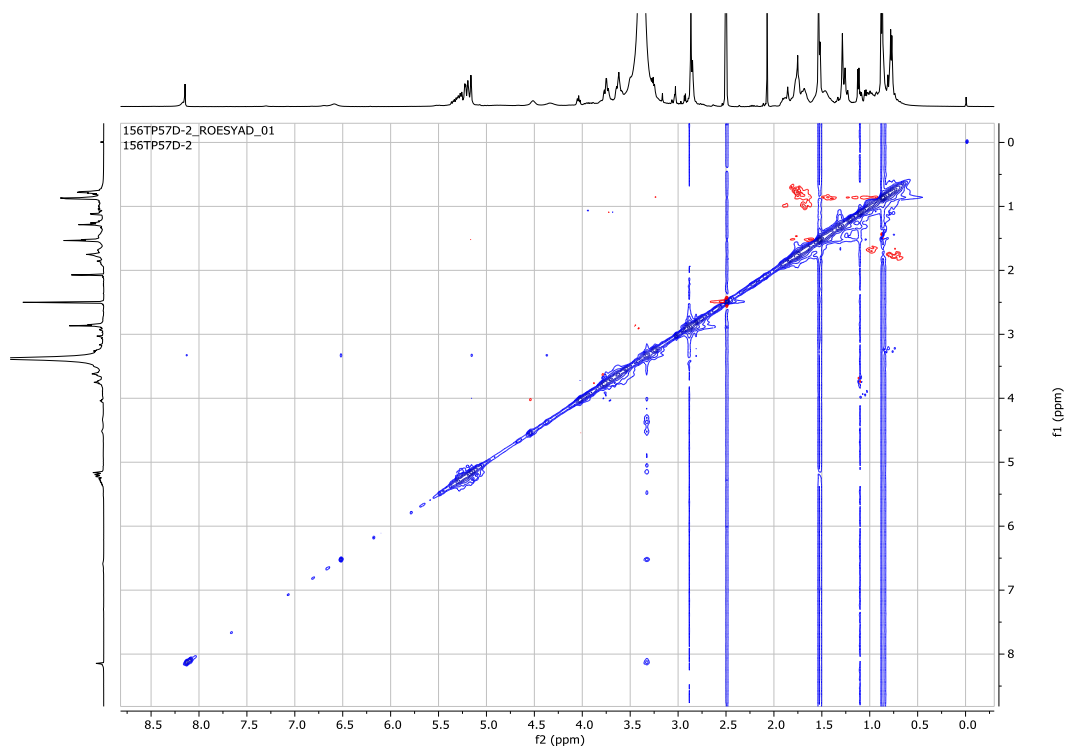


Figure C.17. ROESY spectrum (500 MHz, DMSO)) of compound **1b**

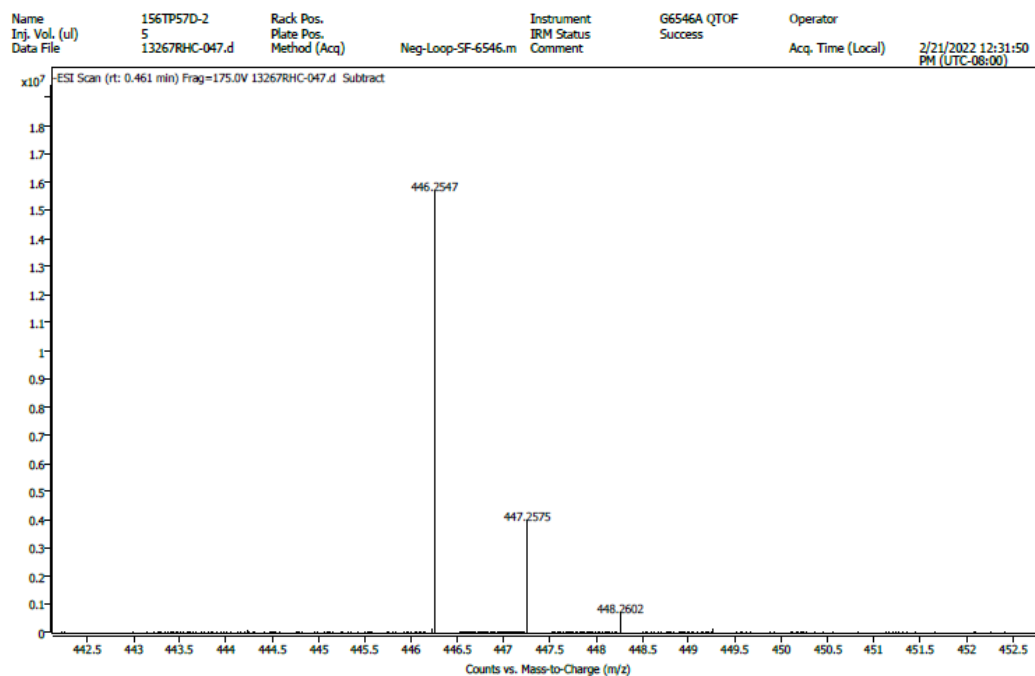


Figure C.18. HRESIMS spectrum of compound **1b**

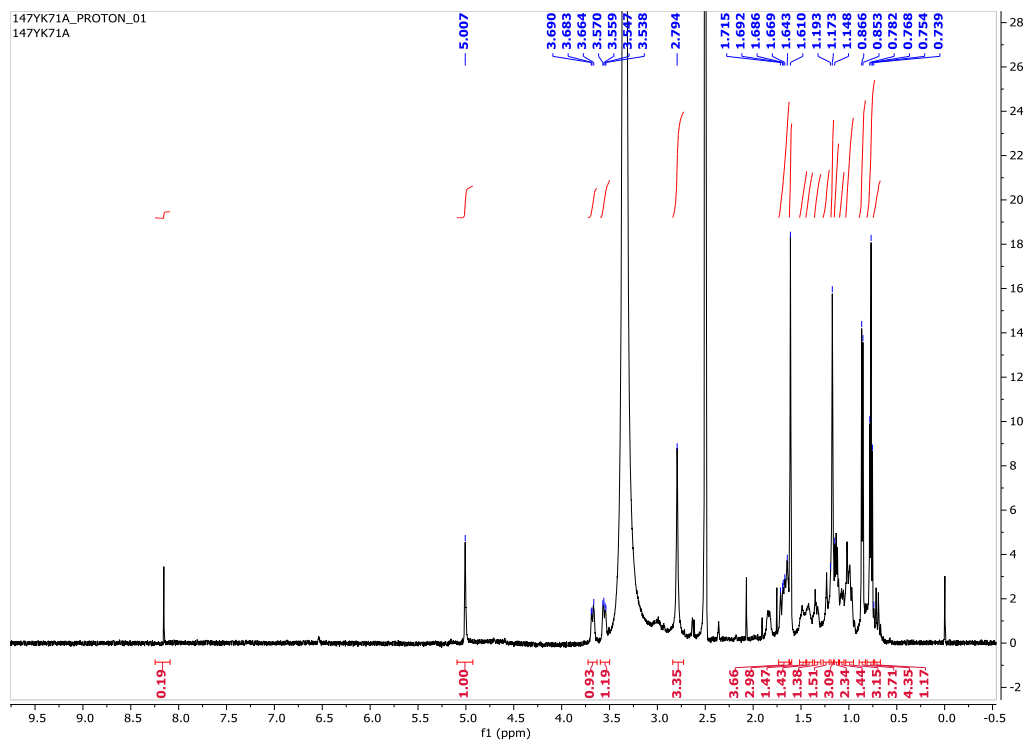


Figure C.19. ^1H NMR (500 MHz, DMSO) of

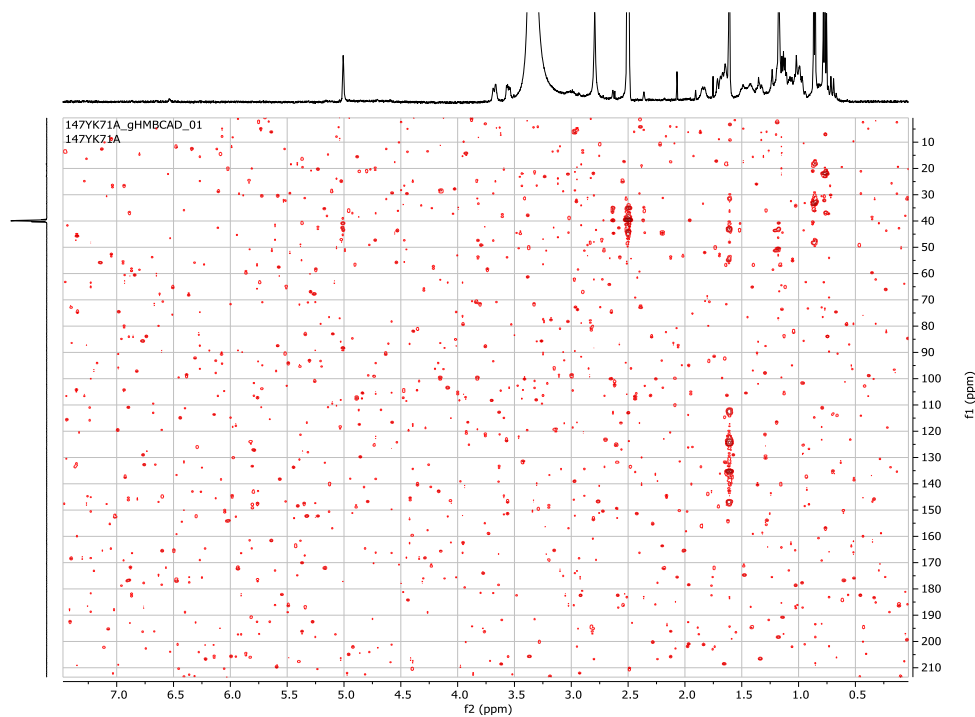


Figure C.20. HMBC (500 MHz, DMSO) spectrum of compound **2a**

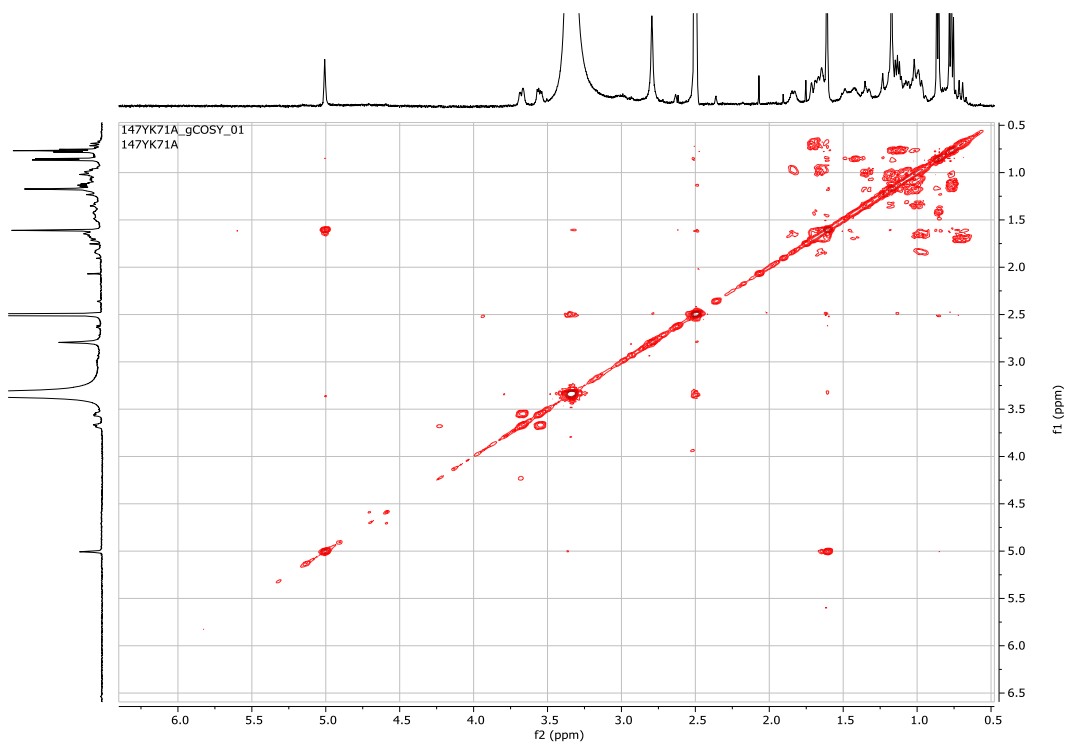


Figure C.21. COSY (500 MHz, DMSO) spectrum of compound **2a**

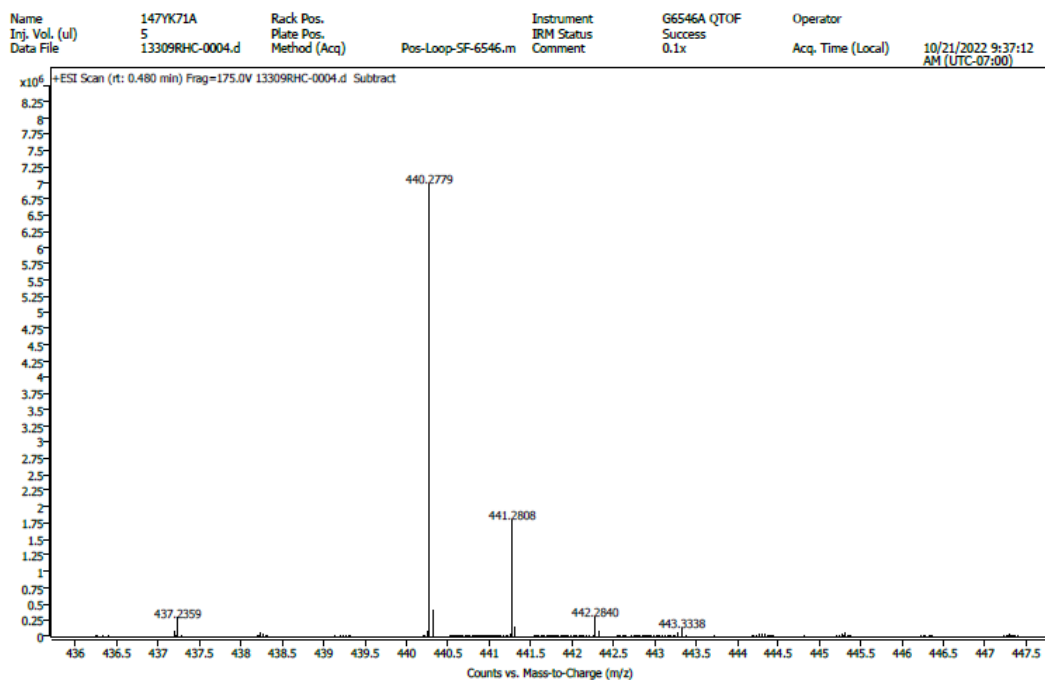


Figure C.22. HRESIMS spectrum of compound **2a**

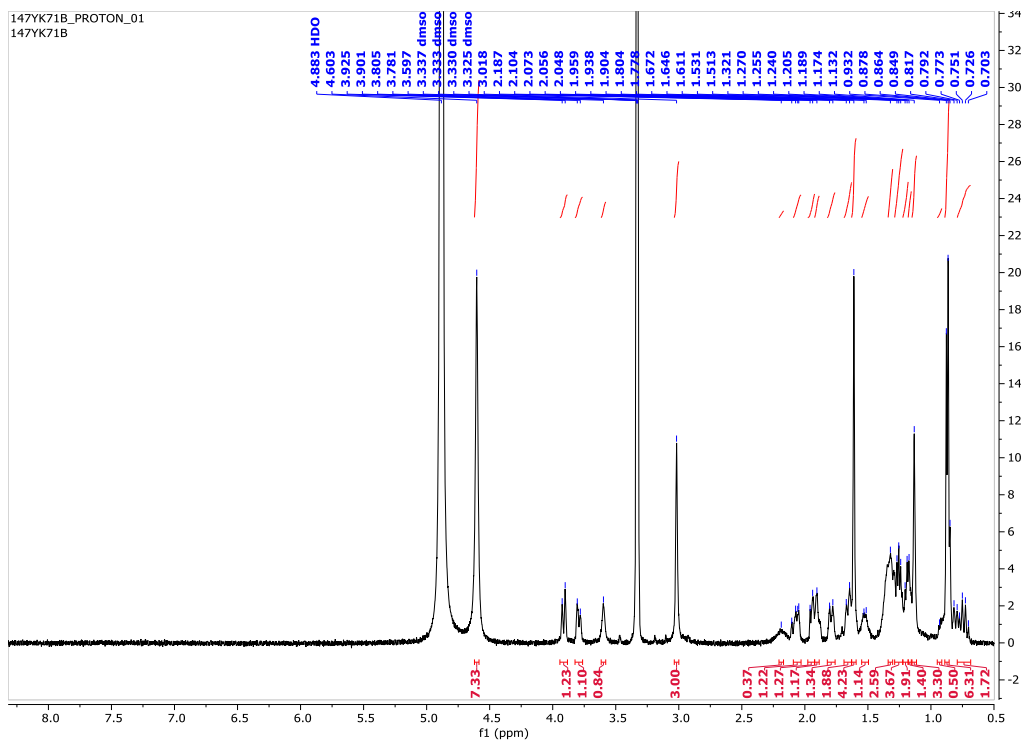


Figure C.23. ^1H NMR (500 MHz, DMSO) of **2b**

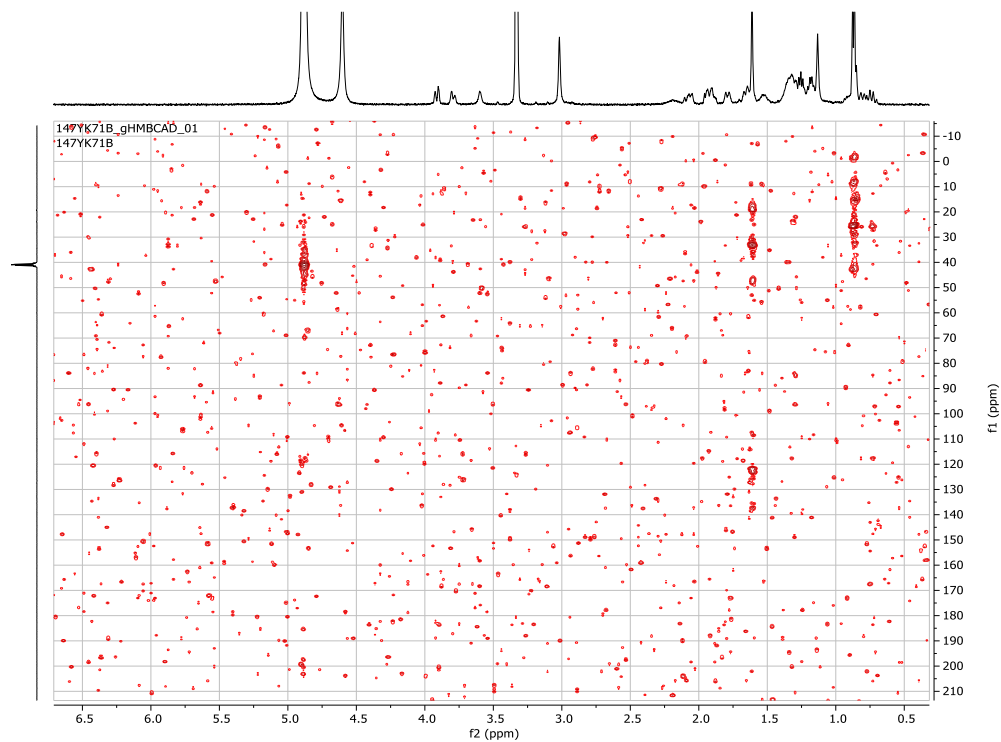


Figure C.24. HMBC (500 MHz, DMSO) spectrum of compound **2b**

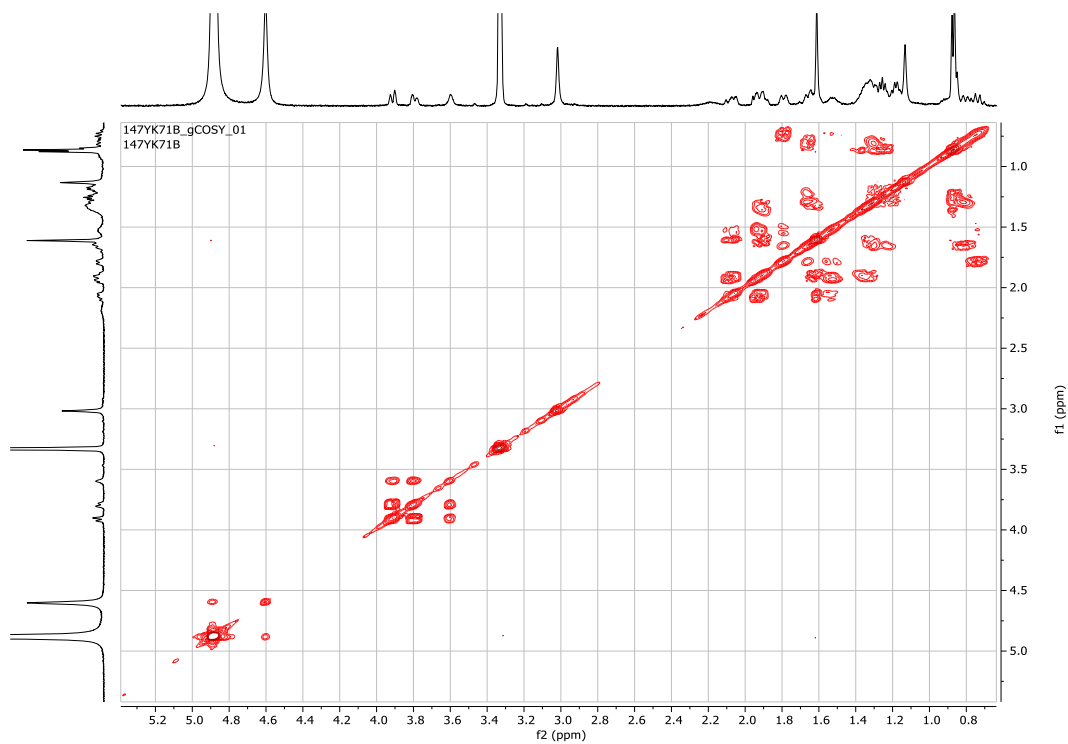


Figure C.25. COSY (500 MHz, DMSO) spectrum of compound **2b**

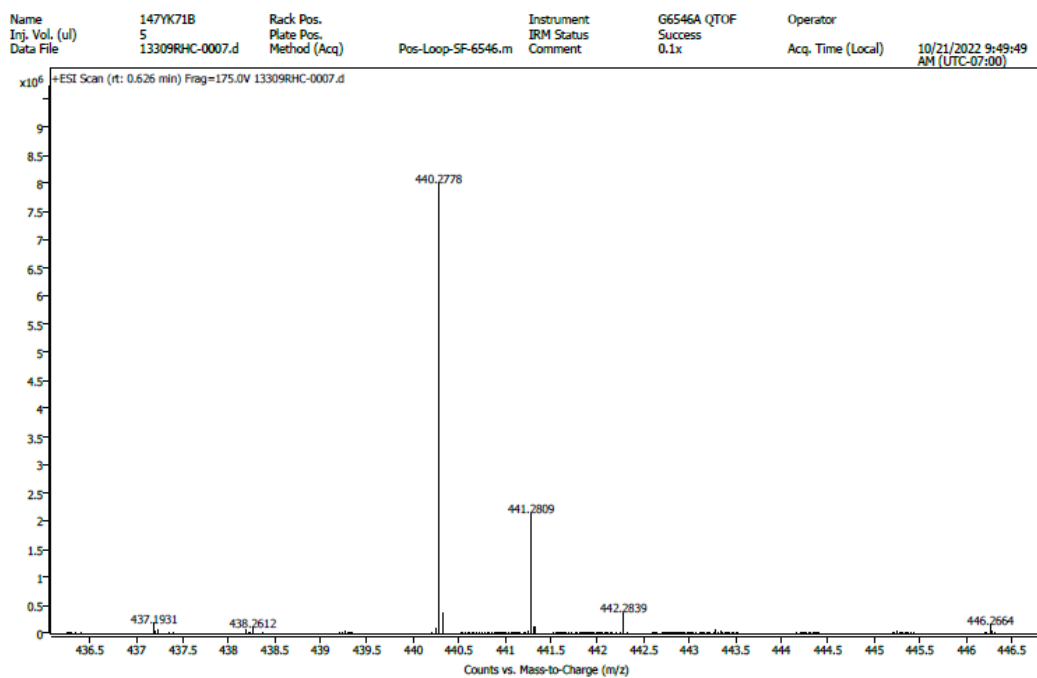


Figure C.26. HRESIMS spectrum of compound **2b**

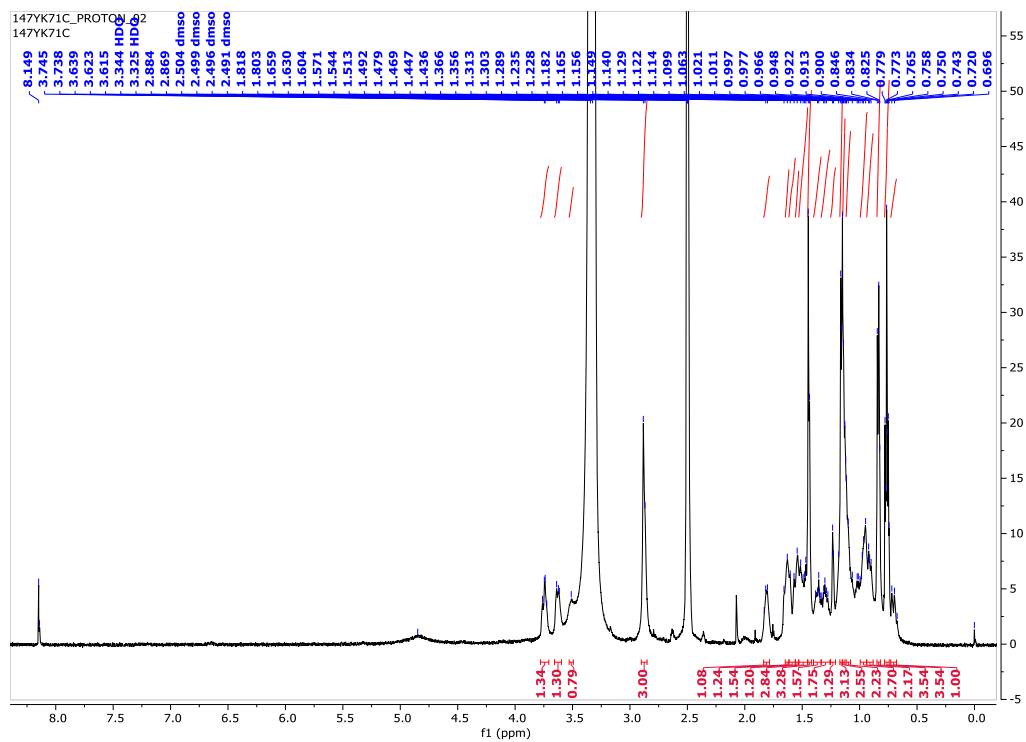


Figure C.27. ^1H NMR (500 MHz, DMSO) of 2c

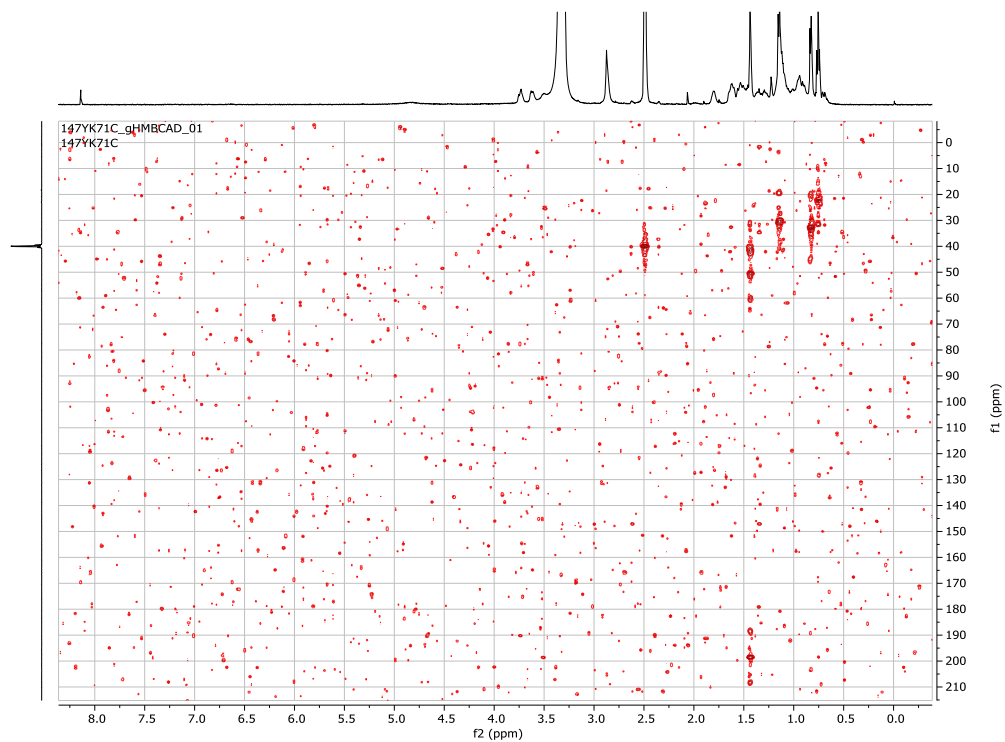


Figure C.28. HMBC (500 MHz, DMSO) spectrum of 2c

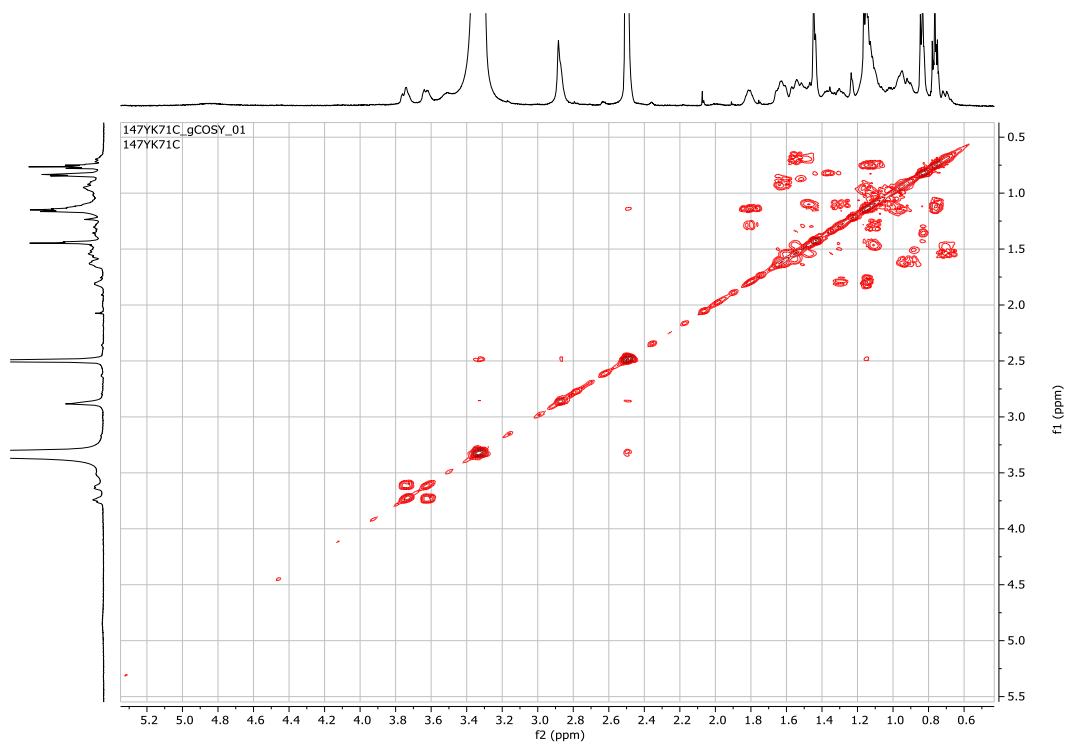


Figure C.29. COSY (500 MHz, DMSO) spectrum of **2c**

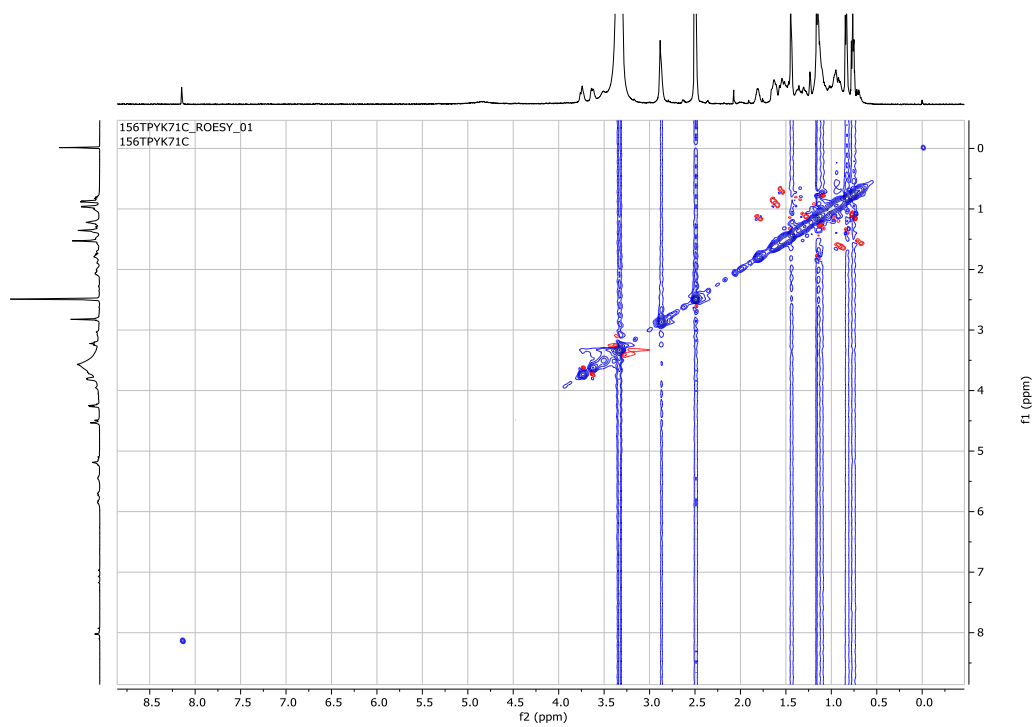


Figure C.30. ROESY spectrum (500 MHz, DMSO) of compound **2c**

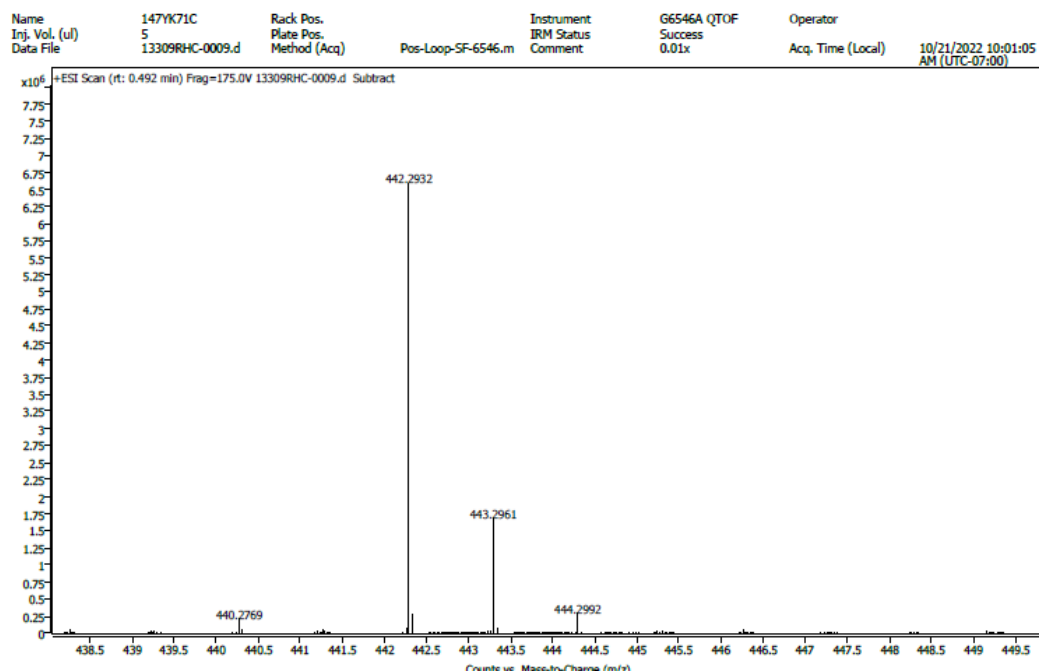


Figure C.31. HRESIMS spectrum of compound **2c**

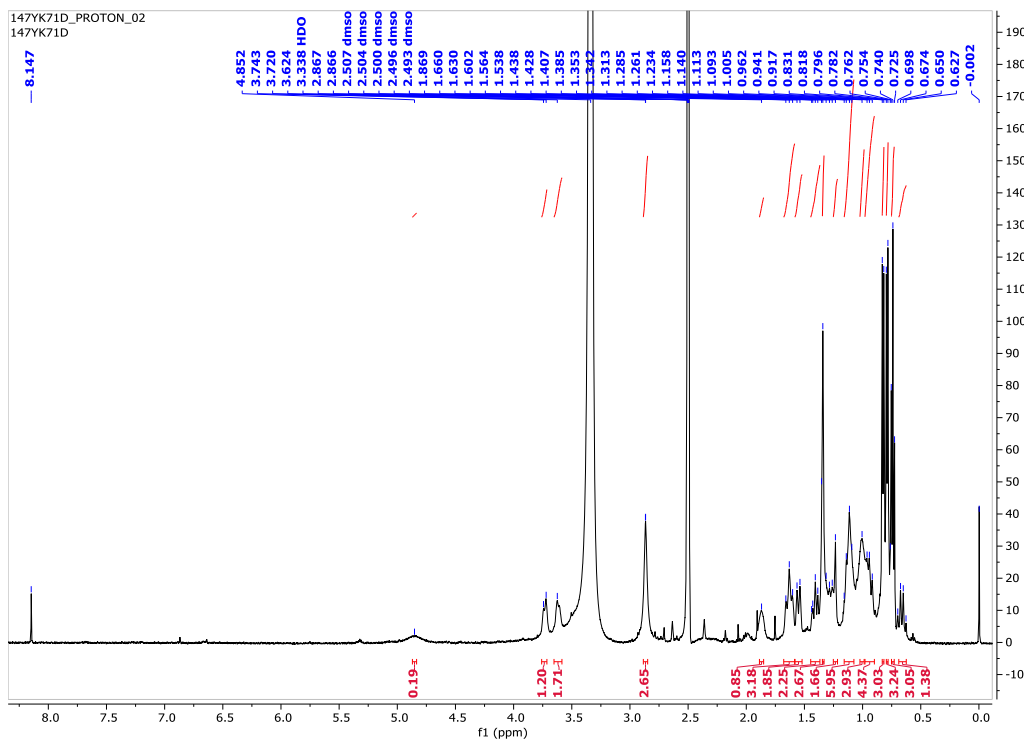


Figure C.32. ^1H NMR (500 MHz, DMSO) of **2d**

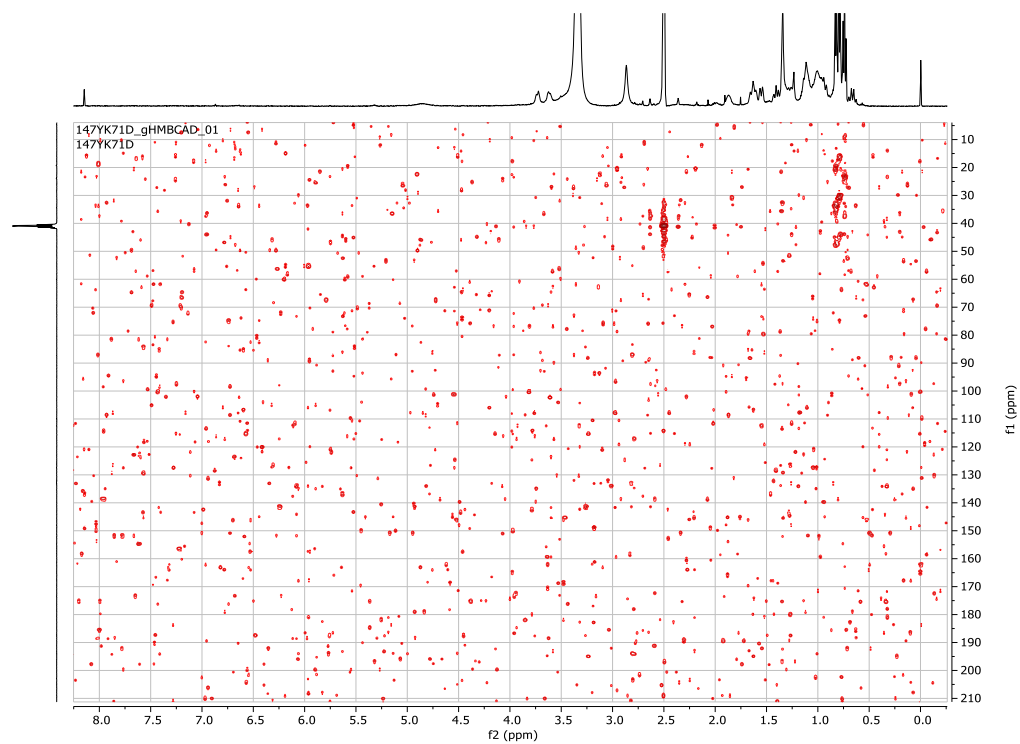


Figure C.33. HMBC (500 MHz, DMSO) spectrum of **2d**

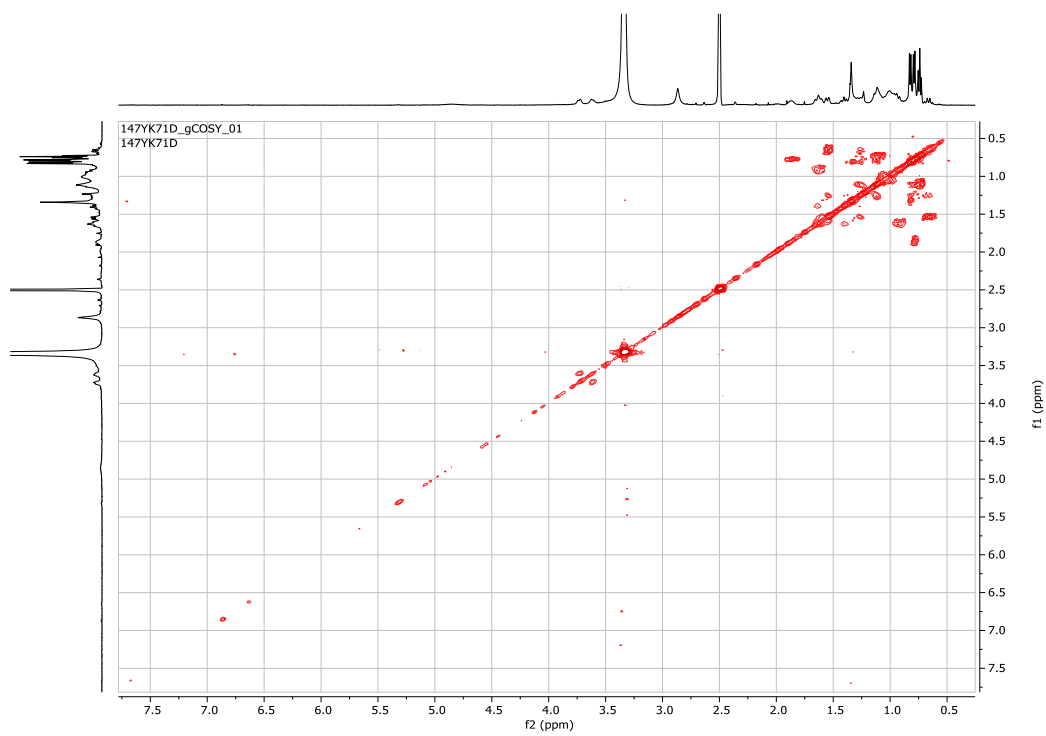


Figure C.34. COSY (500 MHz, DMSO) spectrum of **2d**

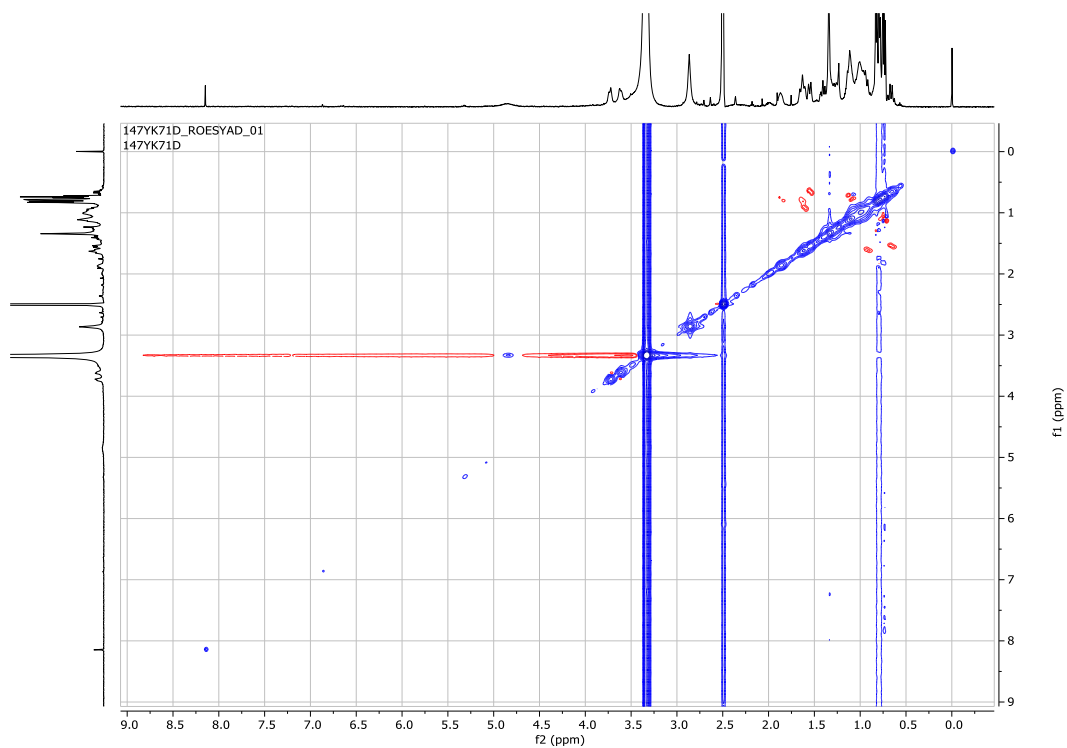


Figure C.35. ROESY spectrum (500 MHz, DMSO)) of compound **2d**

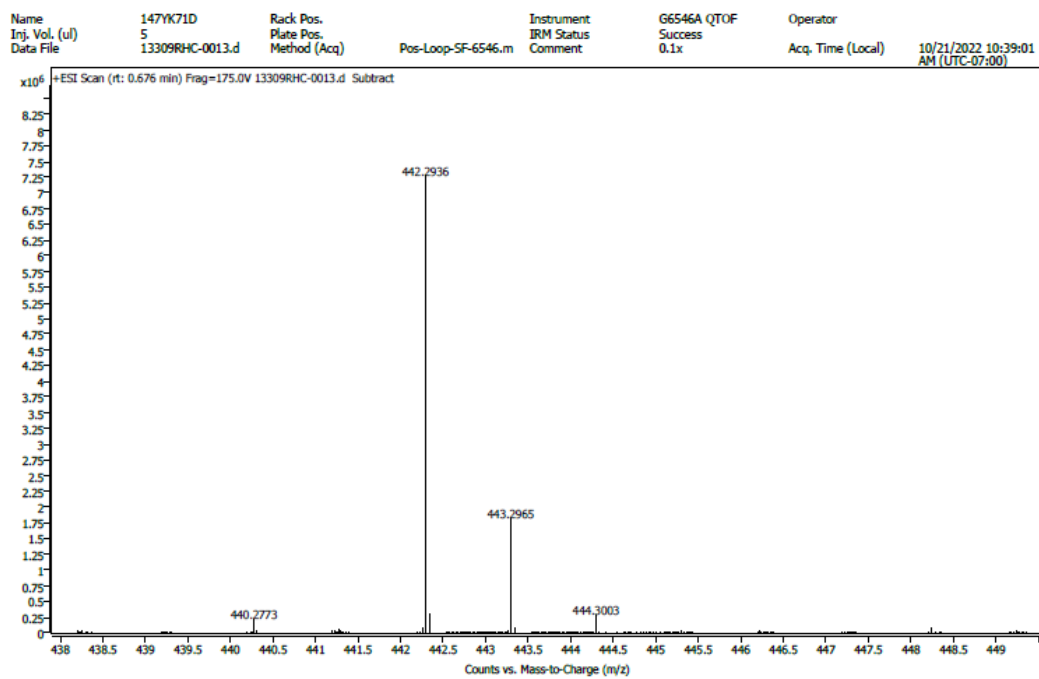


Figure C.36. HRESIMS spectrum of compound **2d**

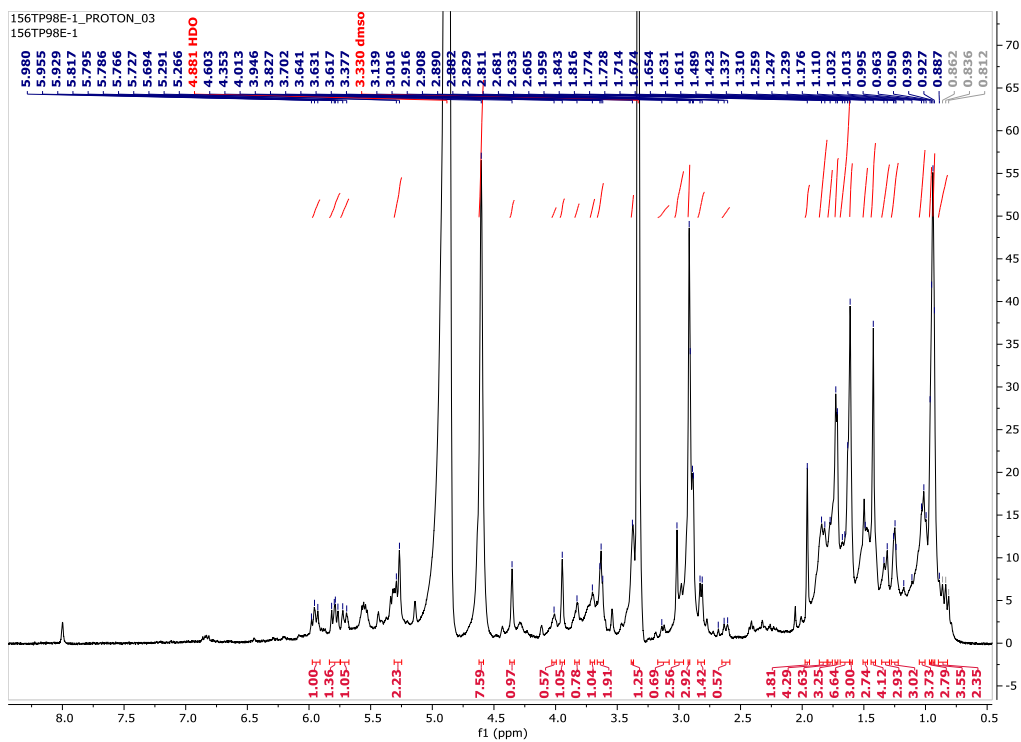


Figure C.37. ^1H NMR (500 MHz, DMSO) of 3a

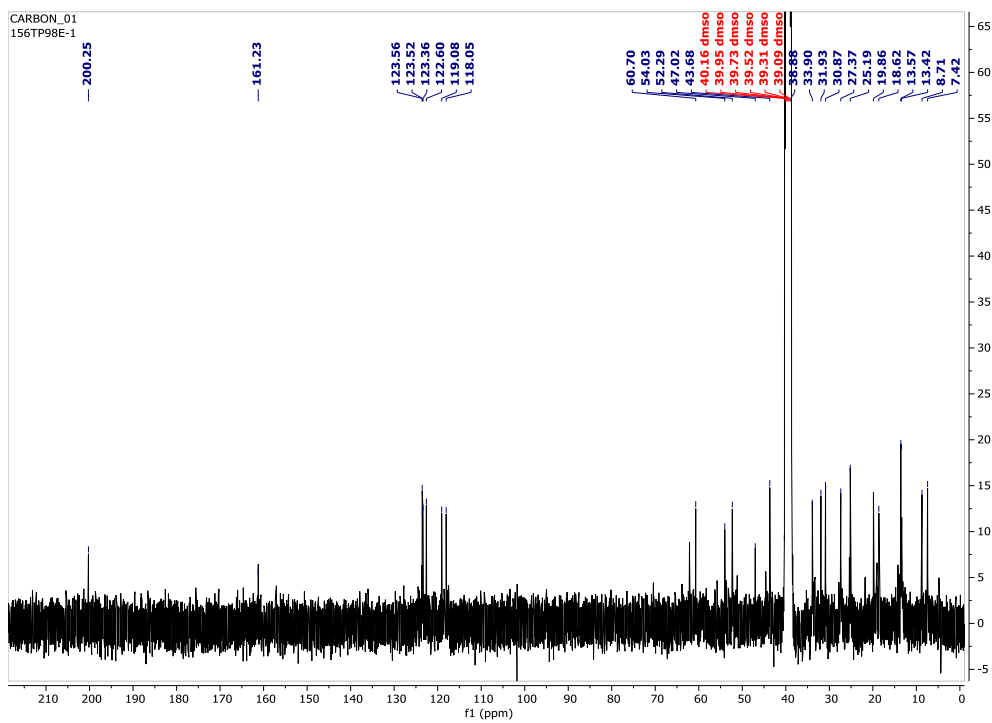


Figure C.38. ^{13}C NMR (400 MHz, DMSO) of 3a

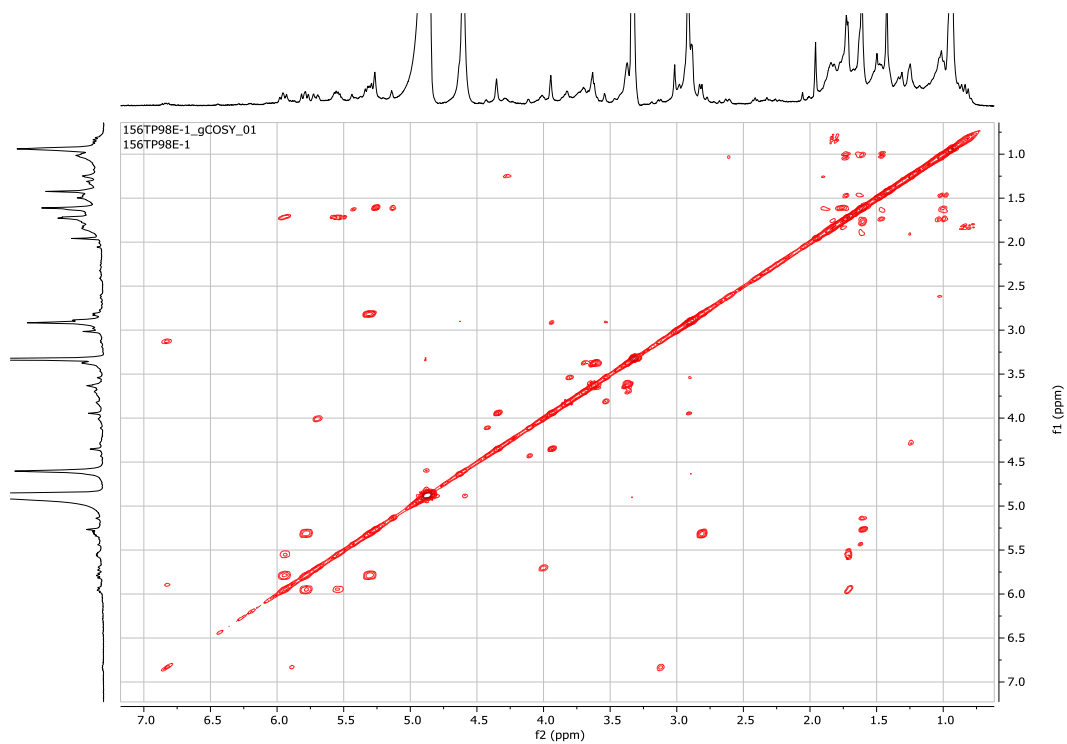


Figure C.39. COSY (500 MHz, DMSO) spectrum of **3a**

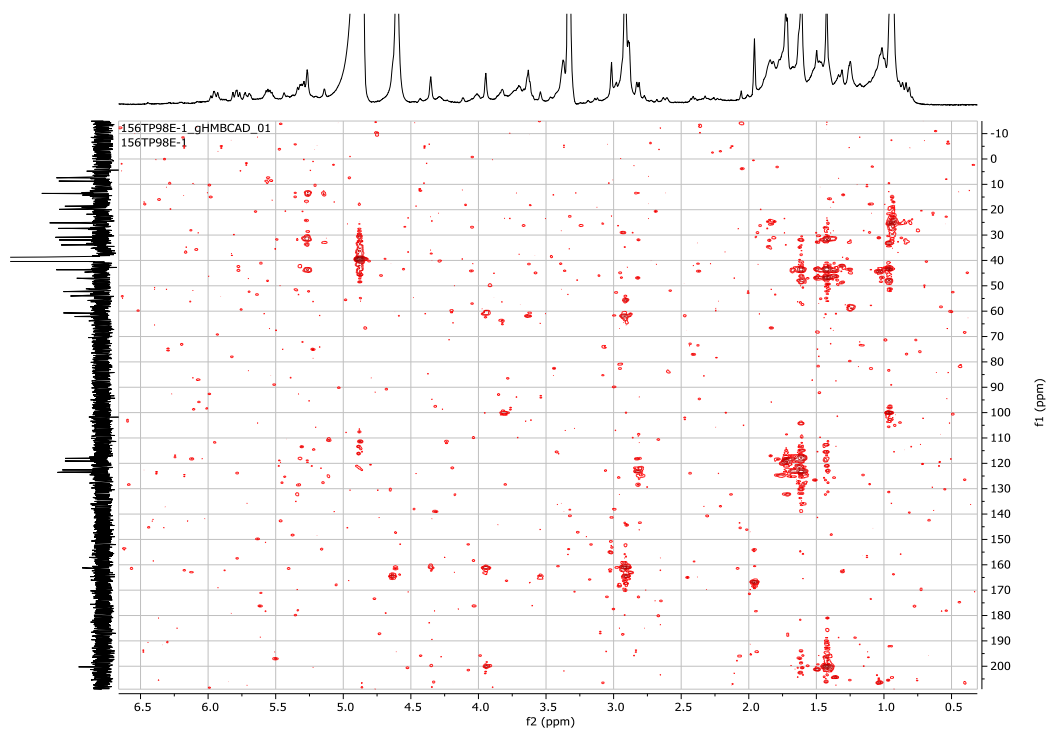


Figure C.40. HMBC (500 MHz, DMSO) spectrum of **3a**

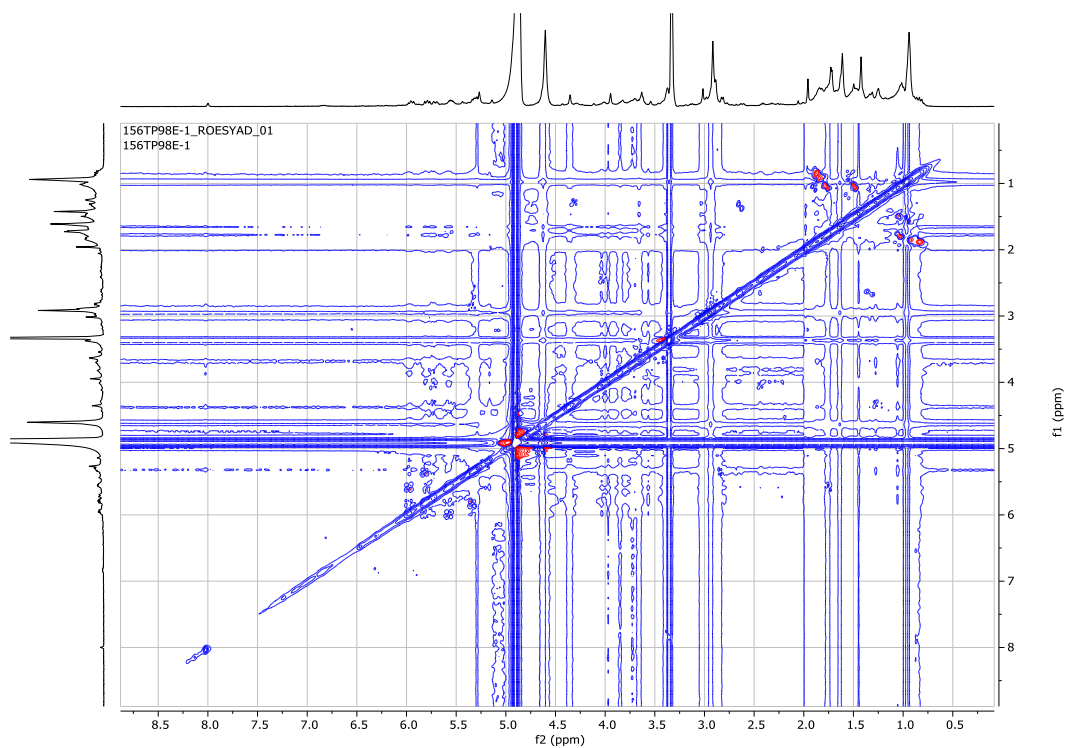


Figure C.41. ROESY spectrum (500 MHz, DMSO)) of compound **3a**

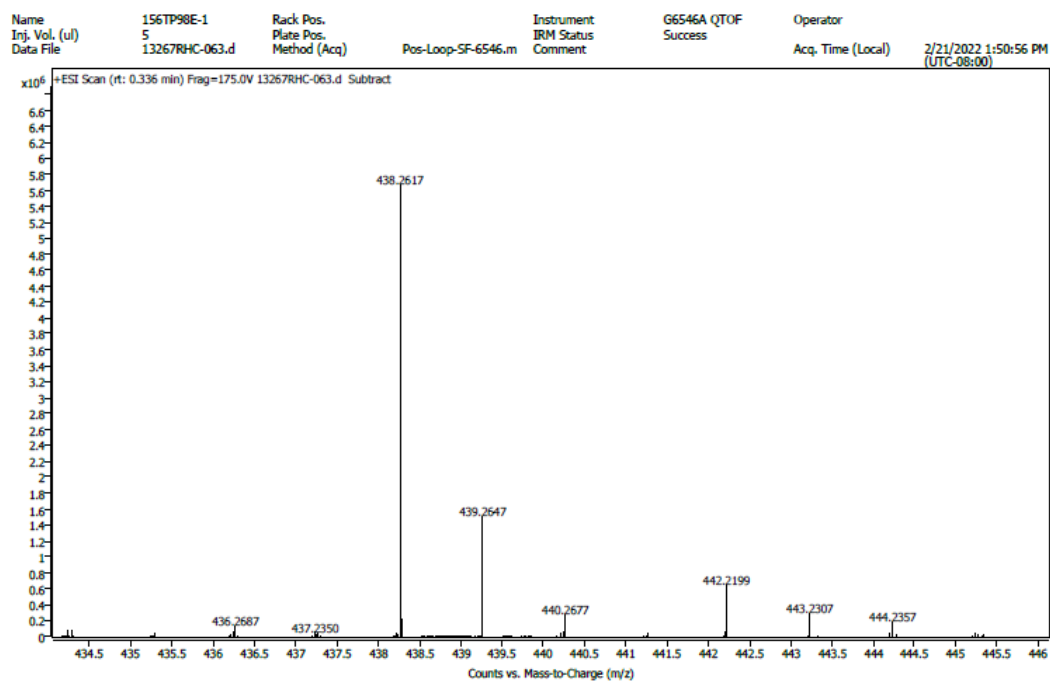


Figure C.42. HRESIMS spectrum of compound **3a**

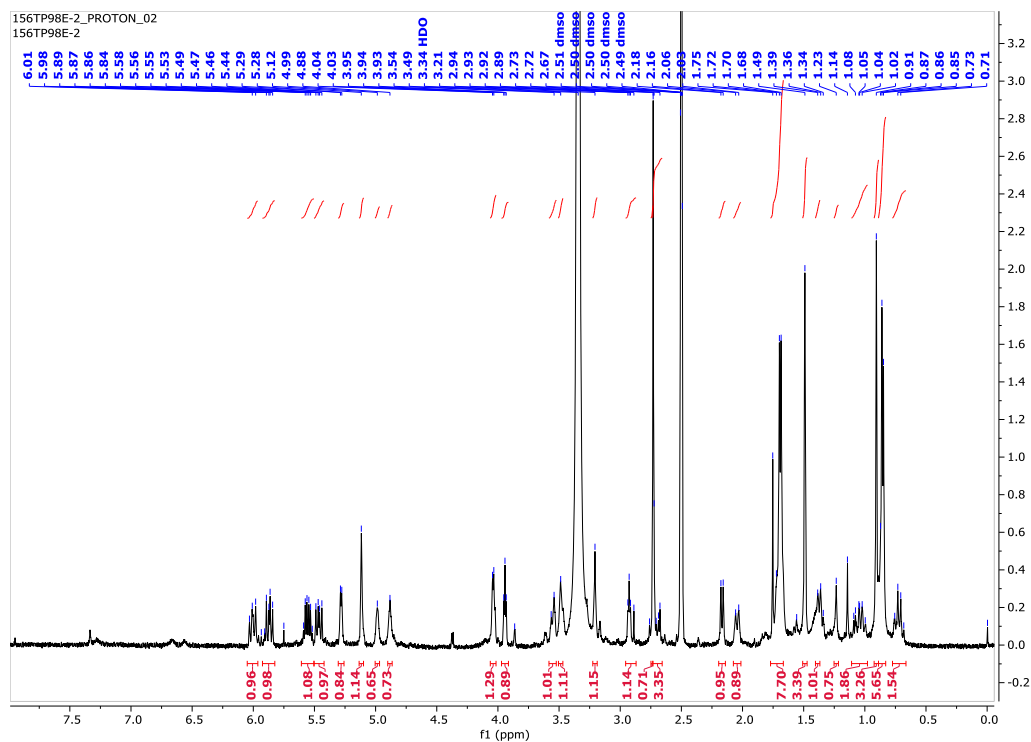


Figure C.43. ^1H NMR (500 MHz, DMSO) of **3b**

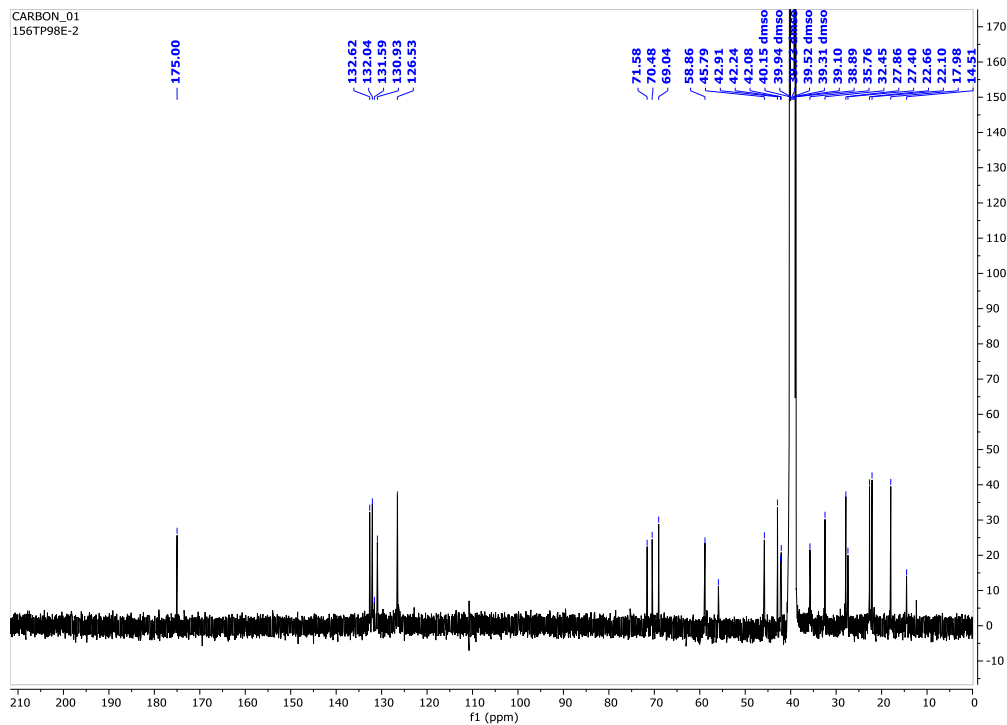


Figure C.44. ^{13}C NMR (400 MHz, DMSO) of **3b**

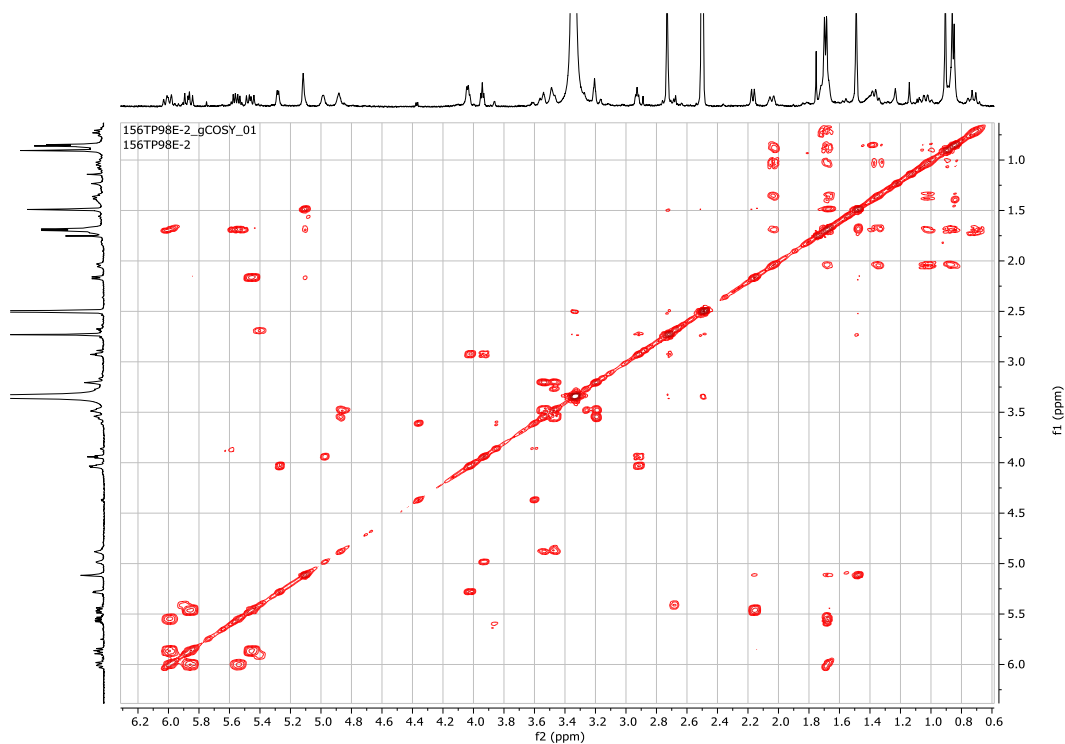


Figure C.45. COSY (500 MHz, DMSO) spectrum of **3b**

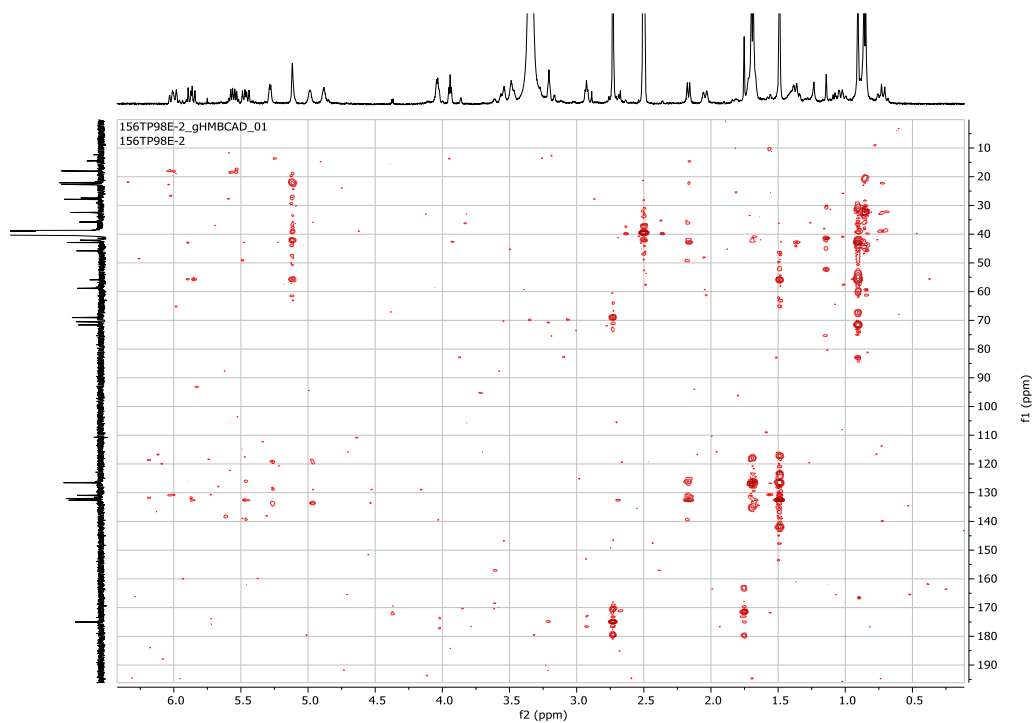


Figure C.46. HMBC (500 MHz, DMSO) spectrum of **3b**

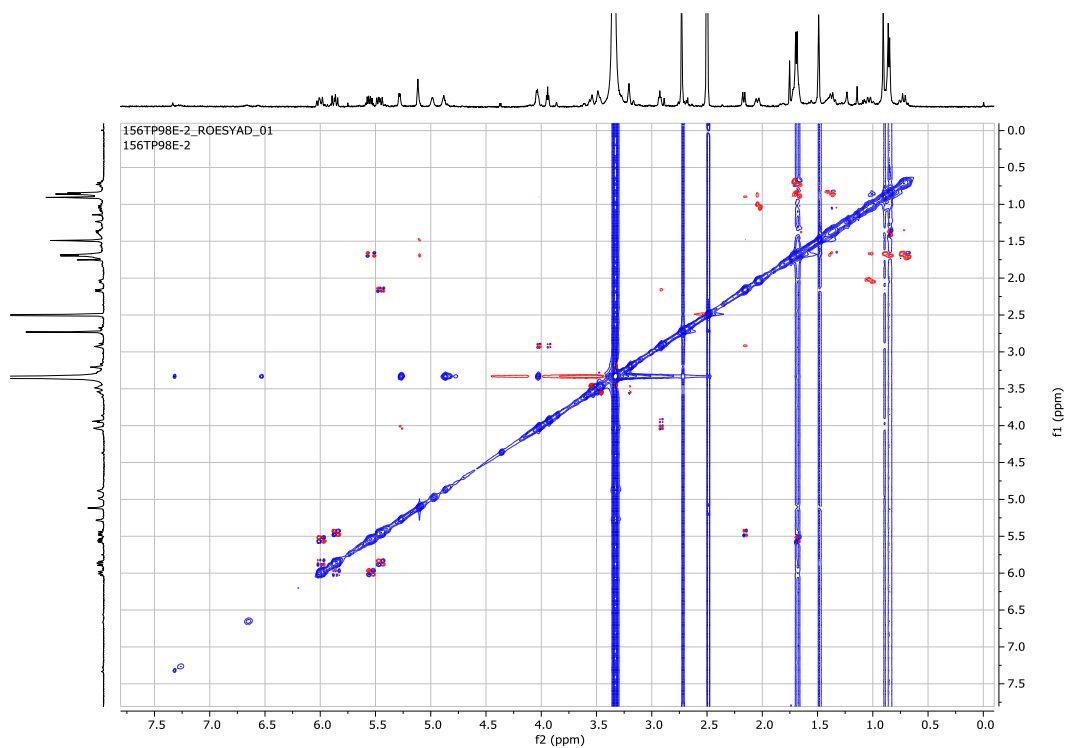


Figure C.47. ROESY spectrum (500 MHz, DMSO)) of compound **3a**

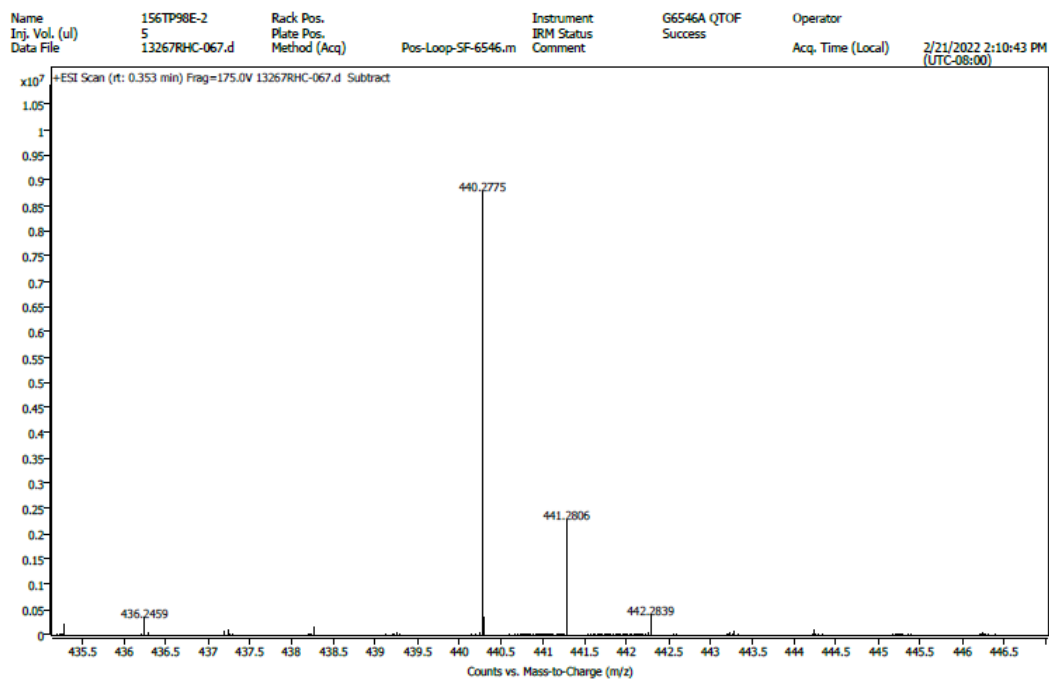


Figure C.48. HRESIMS spectrum of compound **3b**

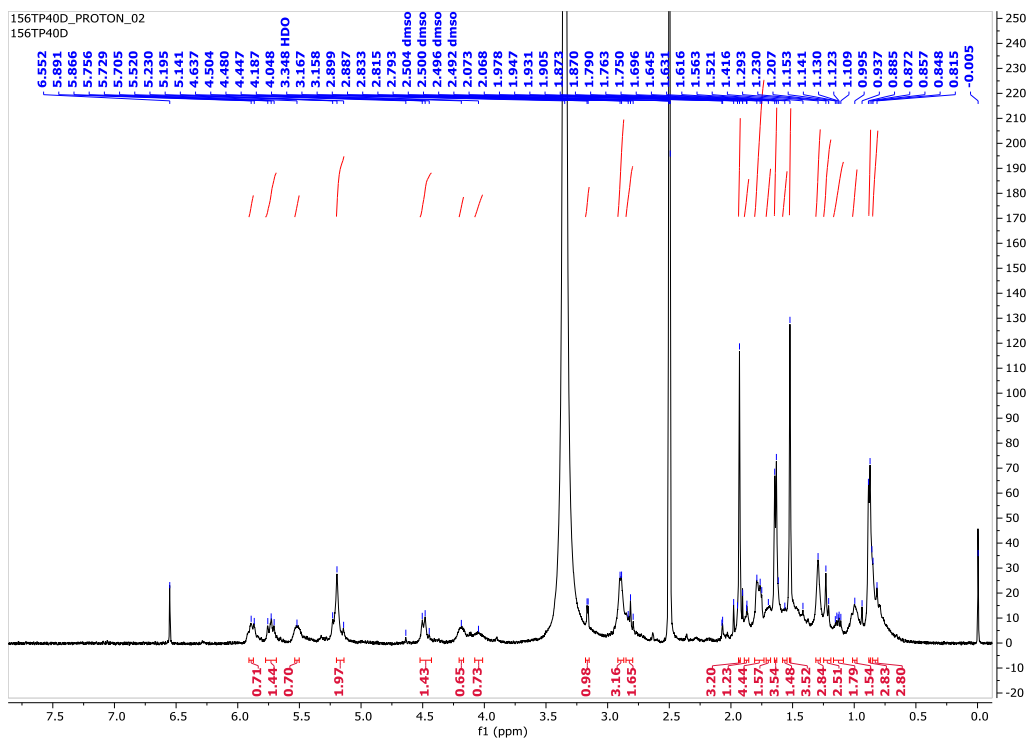


Figure C.49. ^1H NMR (500 MHz, DMSO) of 4a

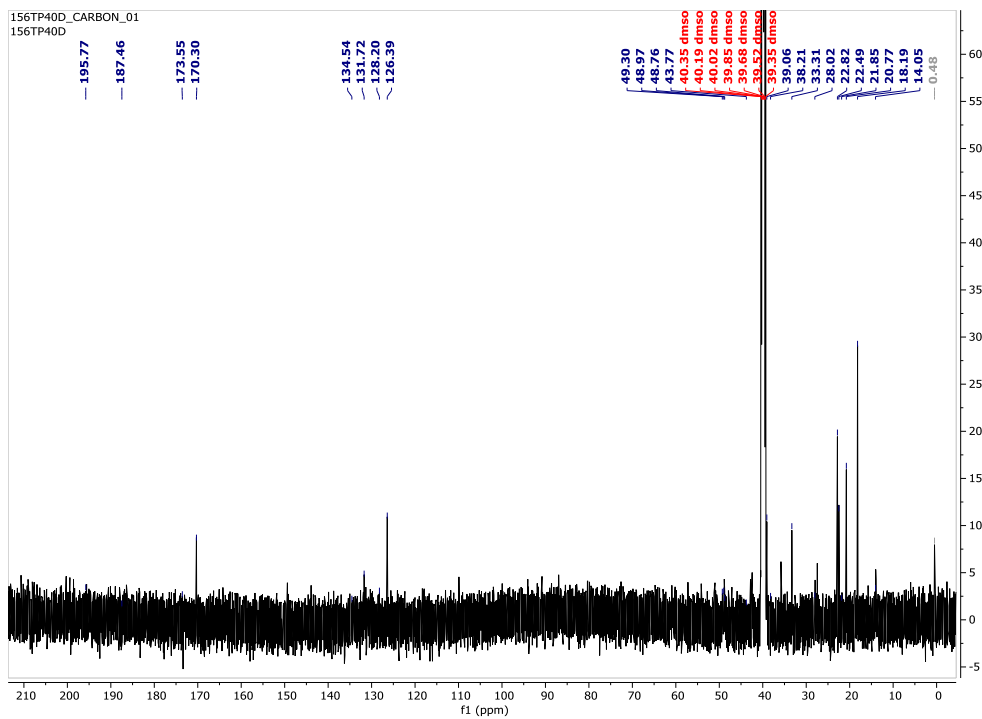


Figure C.50. ^{13}C NMR (400 MHz, DMSO) of 4a

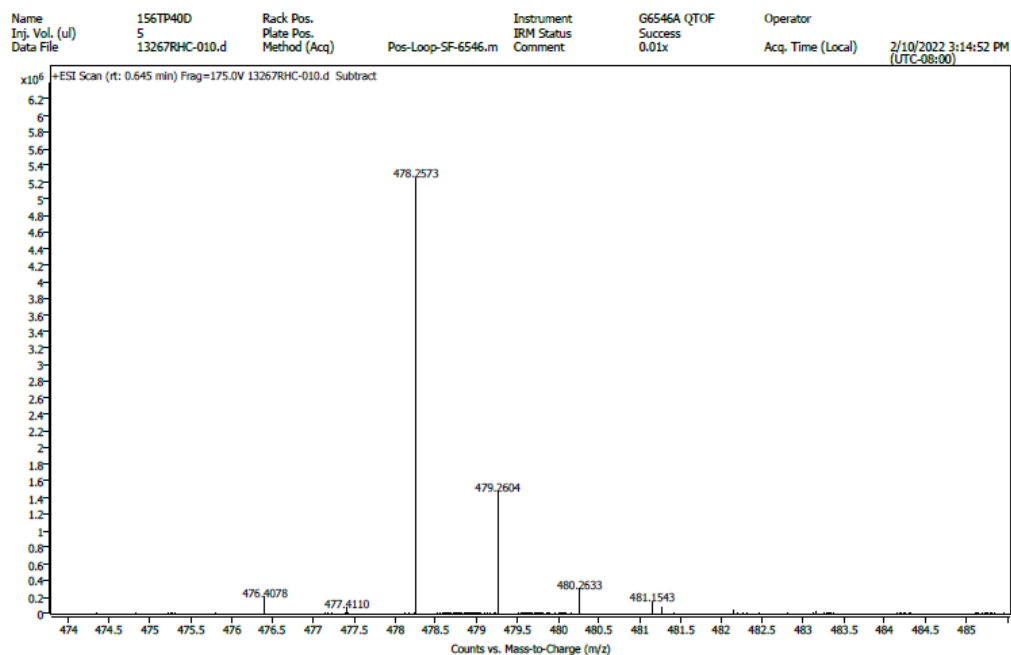


Figure C.51. HRESIMS spectrum of compound **4a**

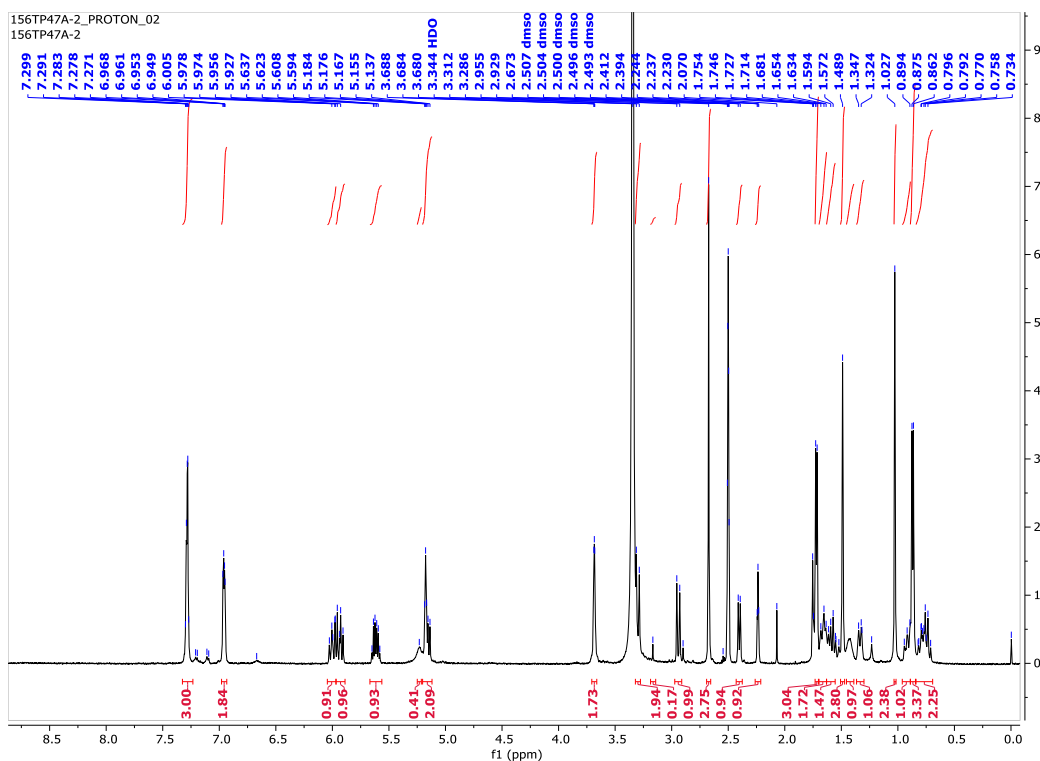


Figure C.52. ^1H NMR (500 MHz, DMSO) of **4b**

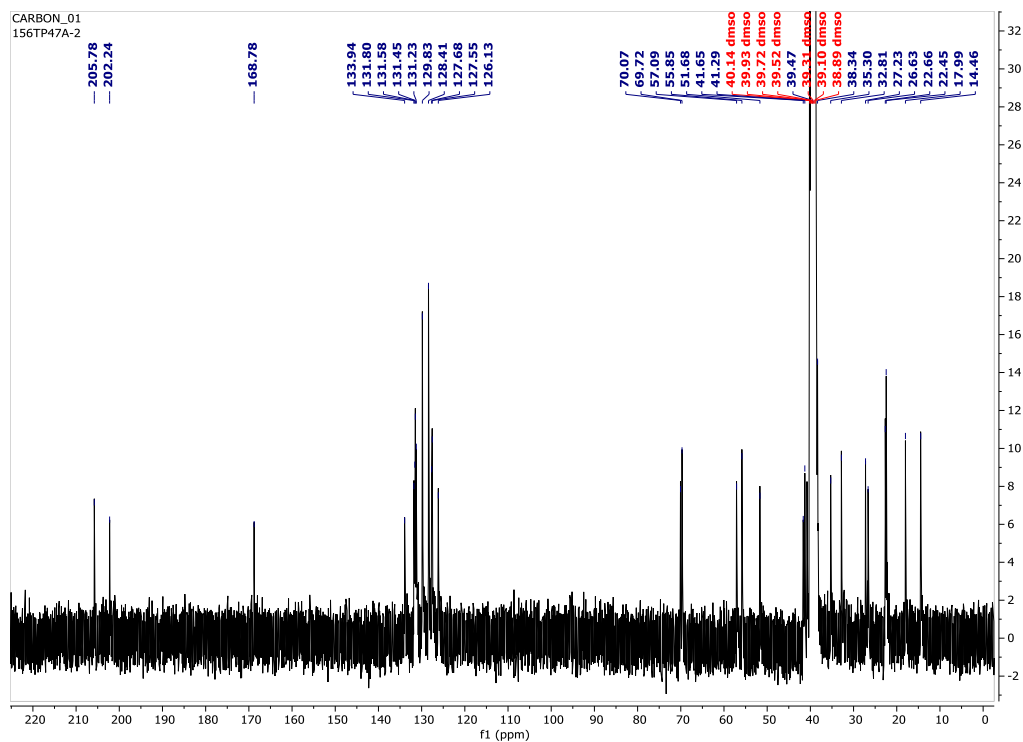


Figure C.53. ^{13}C NMR (400 MHz, DMSO) of **4b**

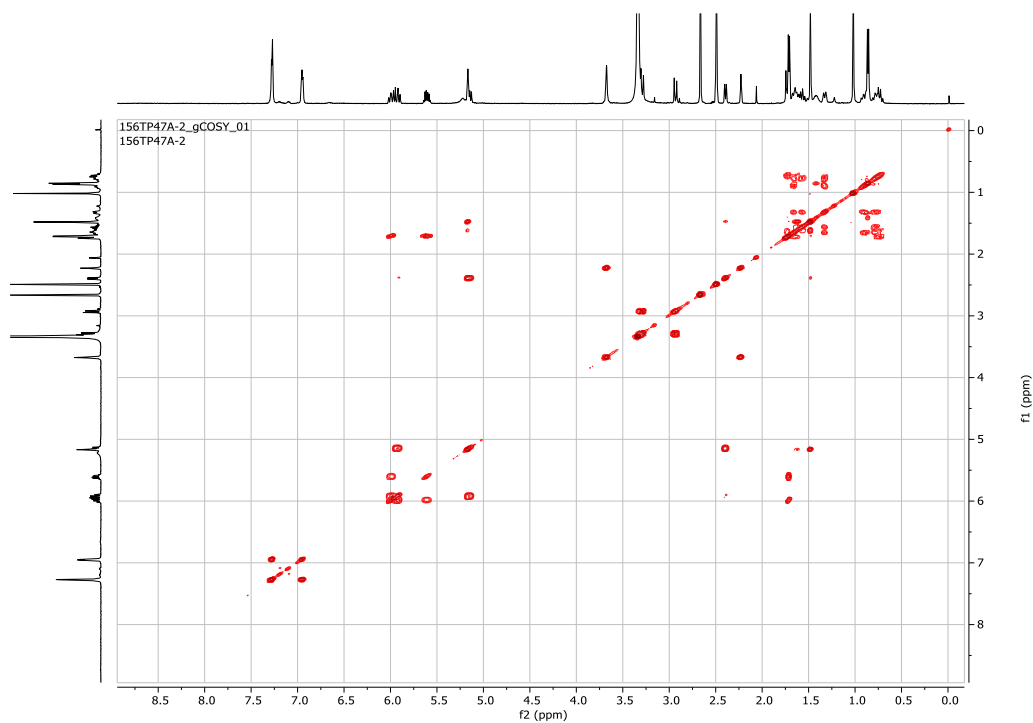


Figure C.54. COSY (500 MHz, DMSO) spectrum of **4b**

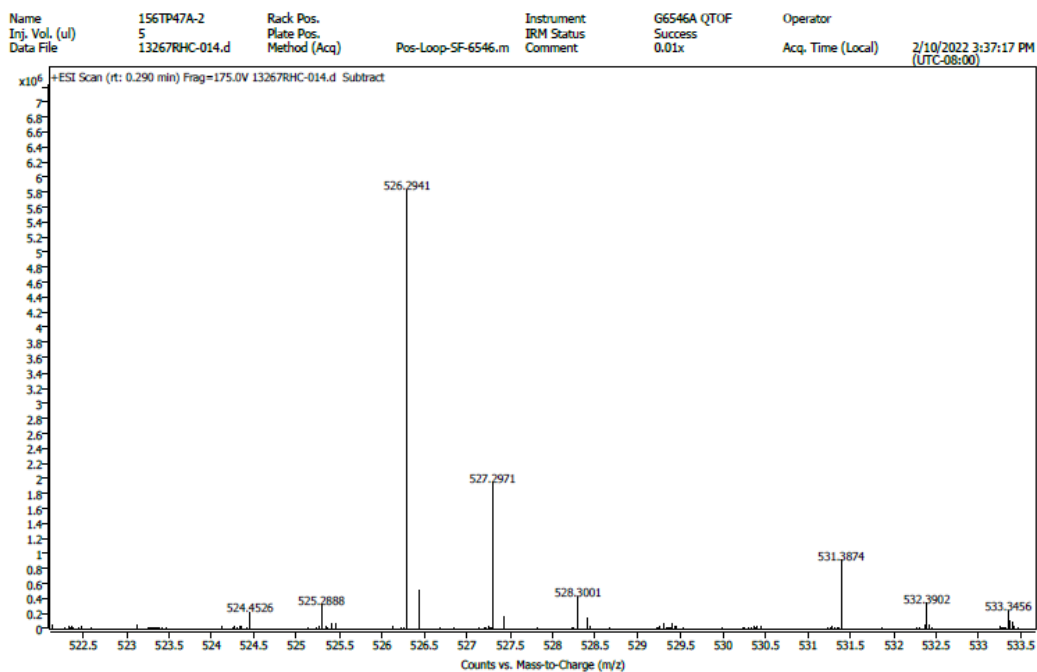


Figure C.55. HRESIMS spectrum of compound **4b**

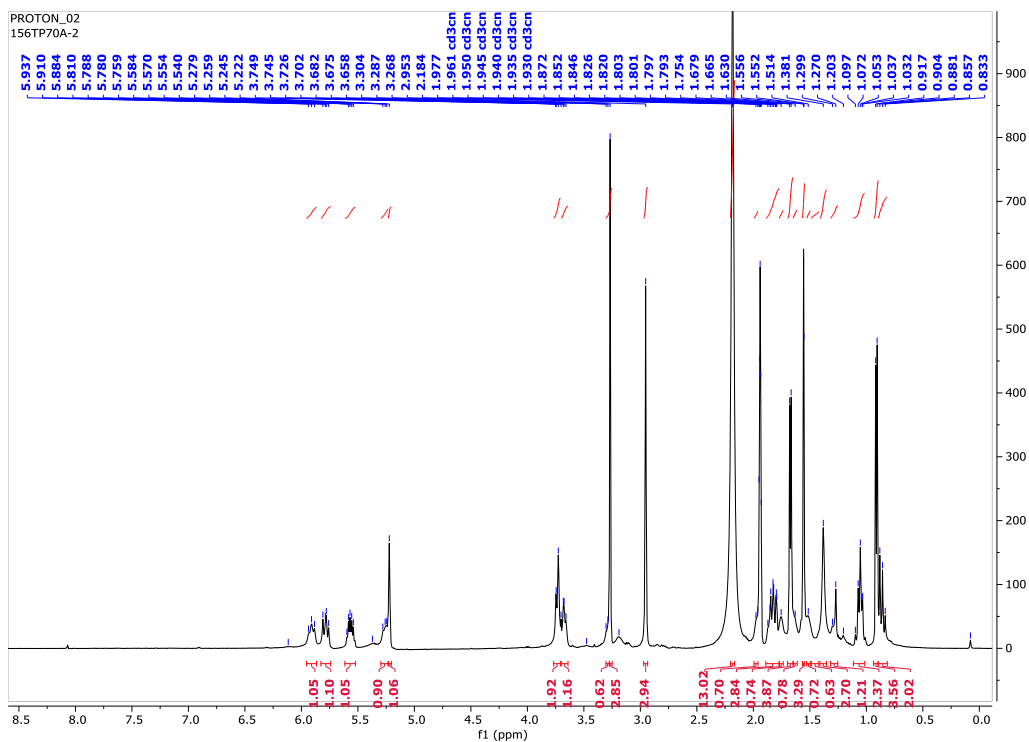


Figure C.56. ^1H NMR (500 MHz, $\text{CD}_3\text{CN}-d_3$) of **4c**

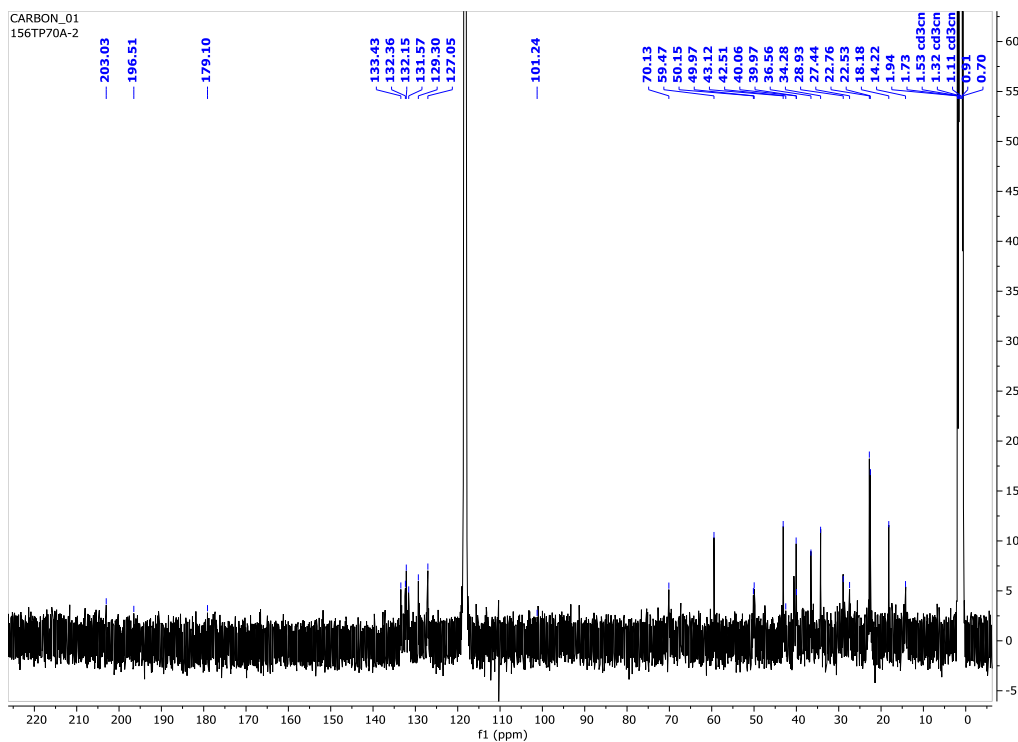


Figure C.57. ^{13}C NMR (400 MHz, $\text{CD}_3\text{CN}-d_3$) of **4c**

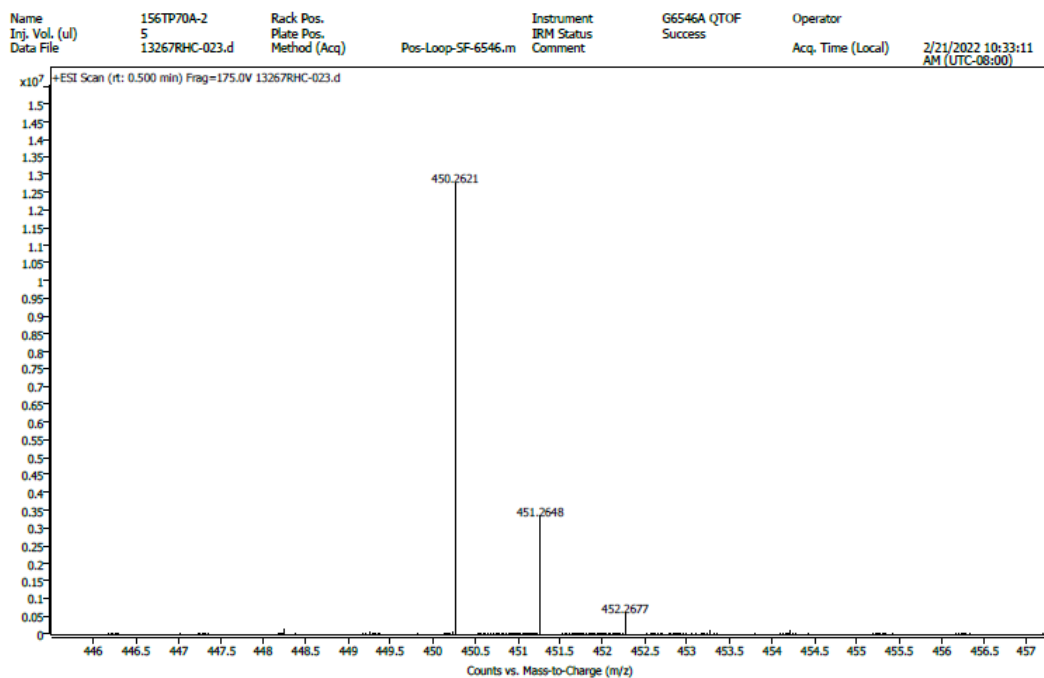


Figure C.58. HRESIMS spectrum of compound **4c**

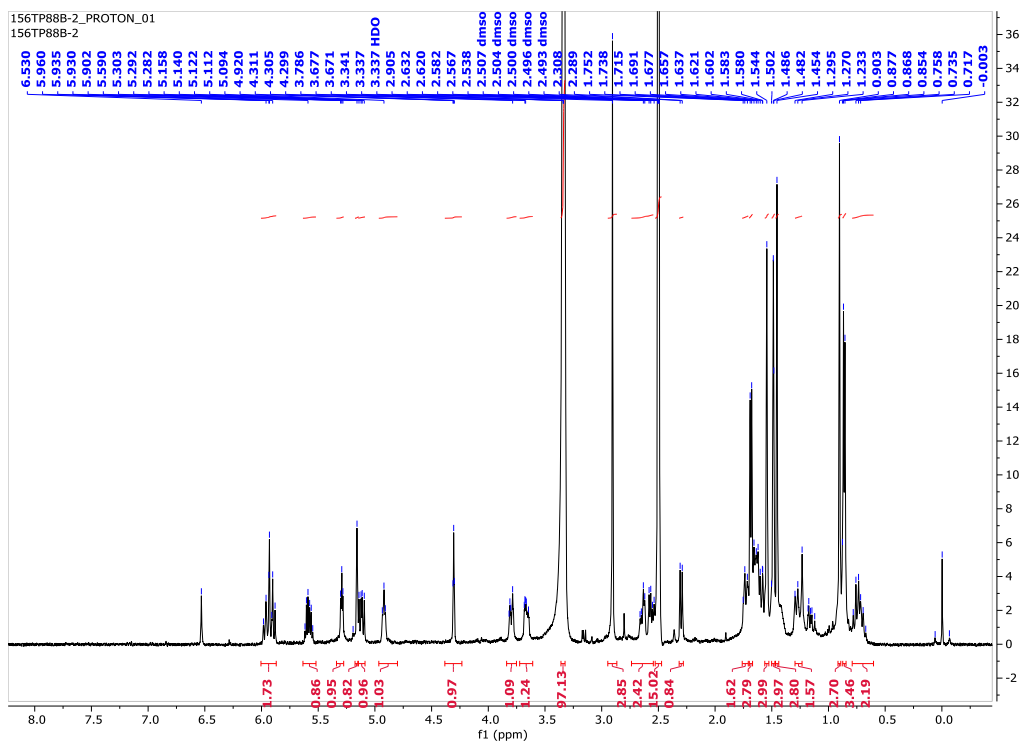


Figure C.59. ^1H NMR (500 MHz, DMSO) of **4d**

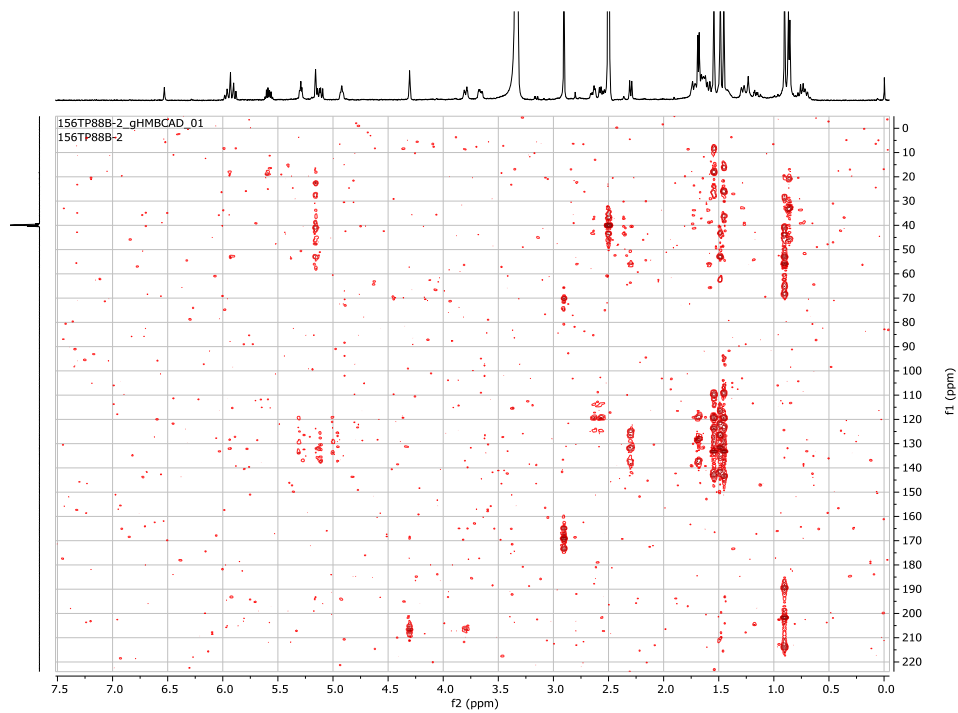


Figure C.60. HMBC (500 MHz, DMSO) spectrum of **4d**

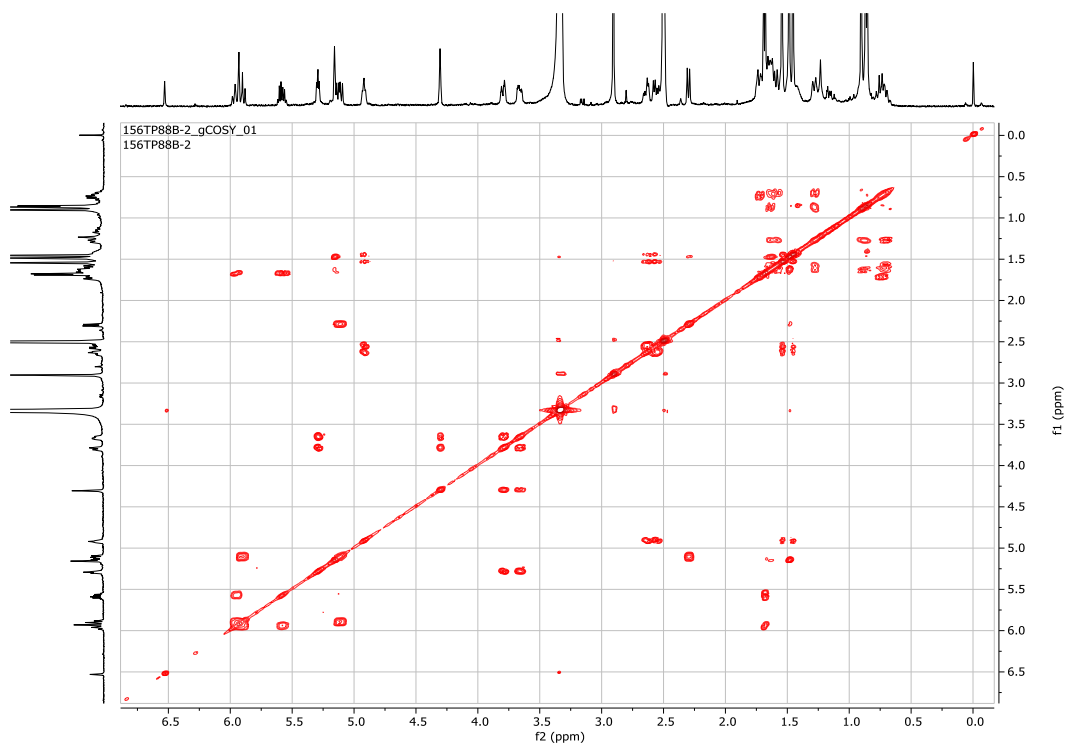


Figure C.61. COSY (500 MHz, DMSO) spectrum of **4d**

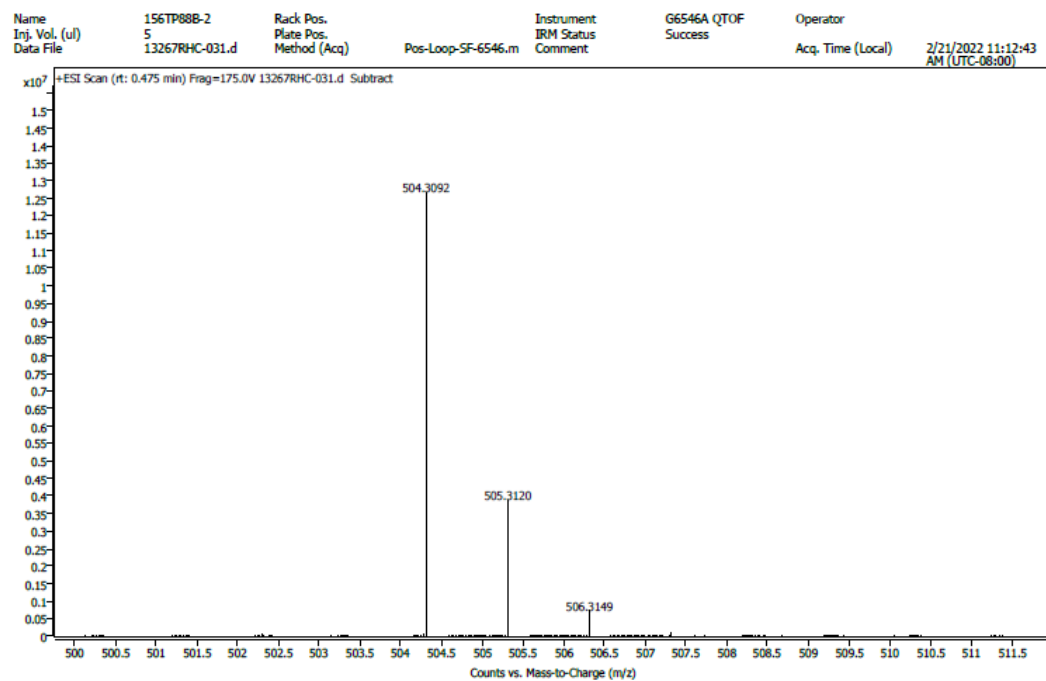


Figure C.62. HRESIMS spectrum of compound **4d**

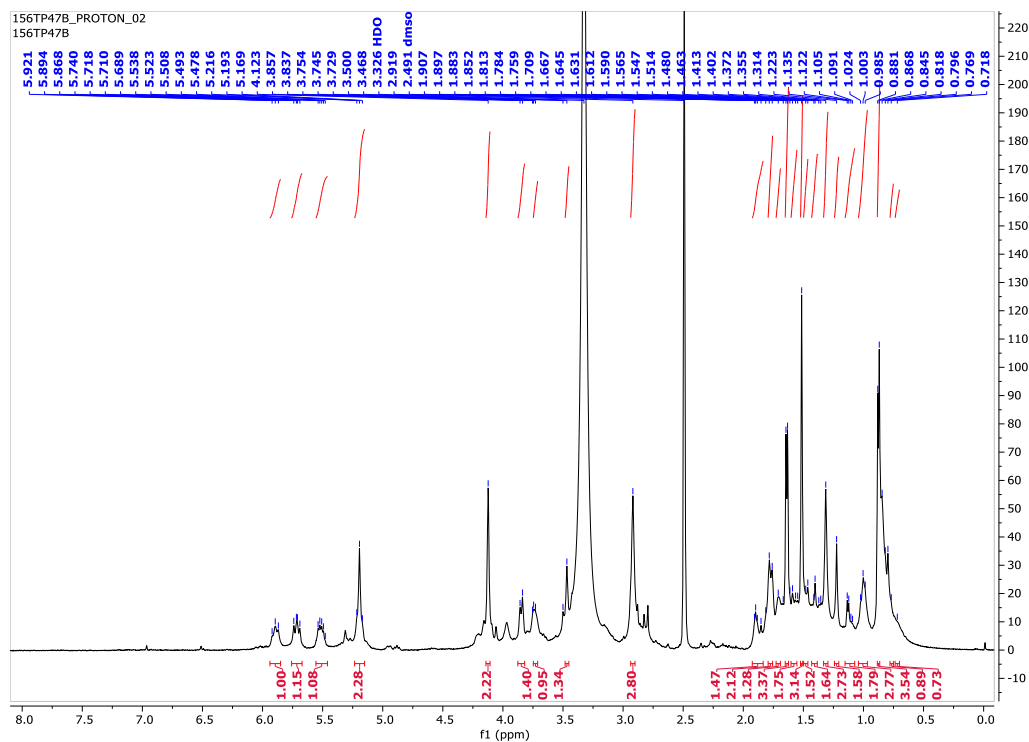


Figure C.63. ^1H NMR (500 MHz, DMSO) of **4e**

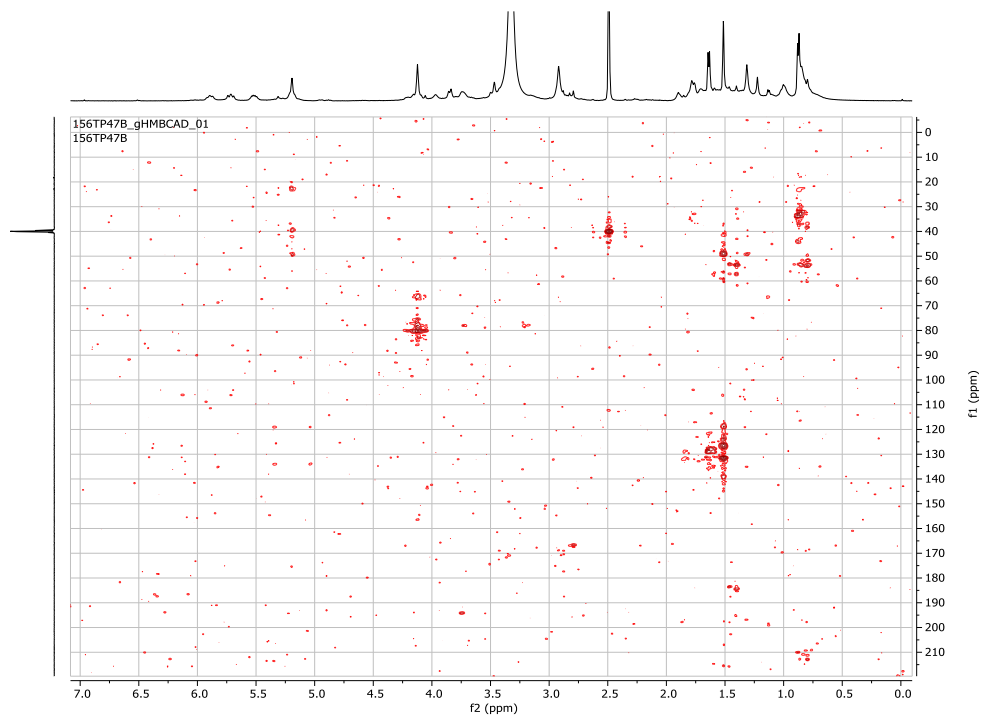


Figure C.64. HMBC (500 MHz, DMSO) spectrum of **4e**

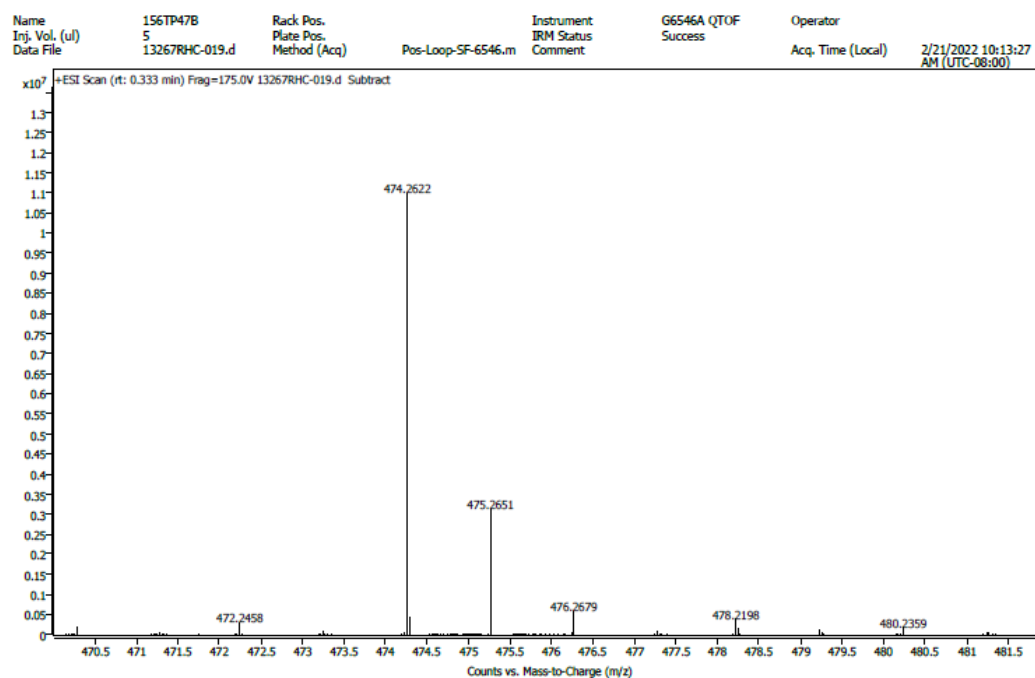


Figure C.65. HRESIMS spectrum of compound **4e**

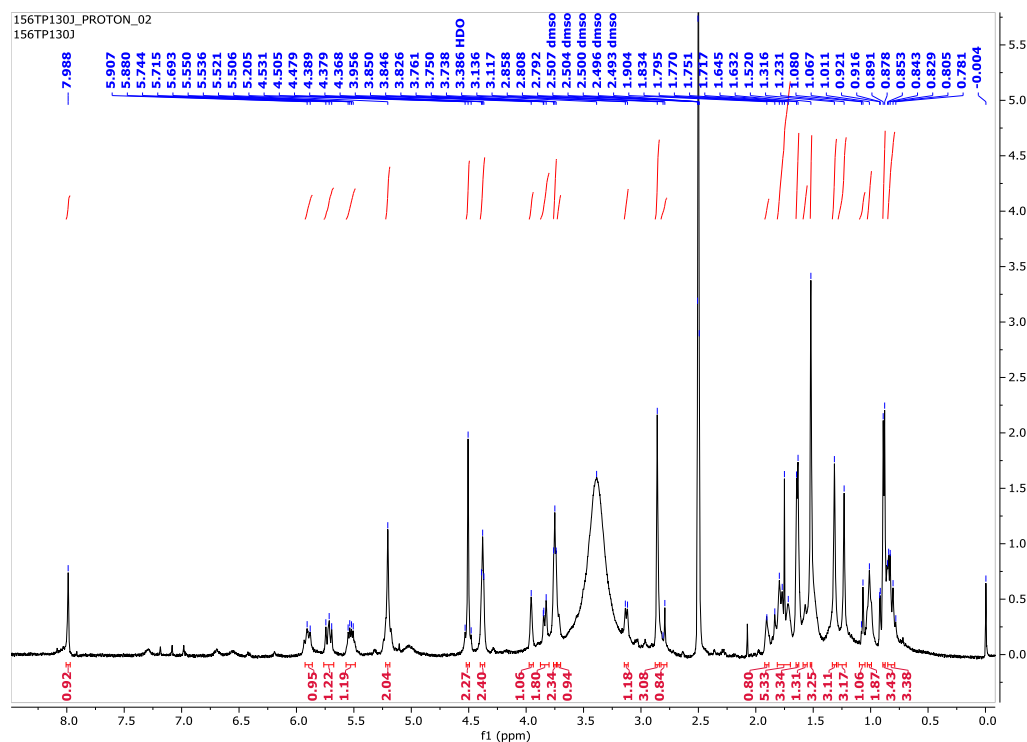


Figure C.66. ^1H NMR (500 MHz, DMSO) of **5a**

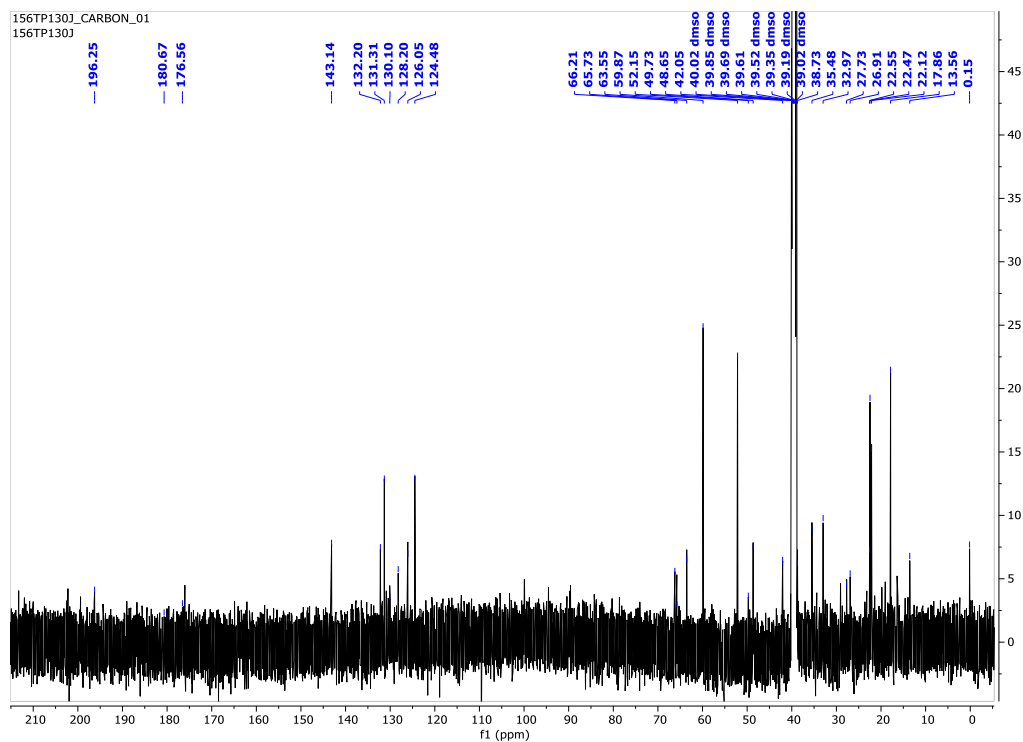


Figure C.67. ^{13}C NMR (400 MHz, DMSO) of **5a**

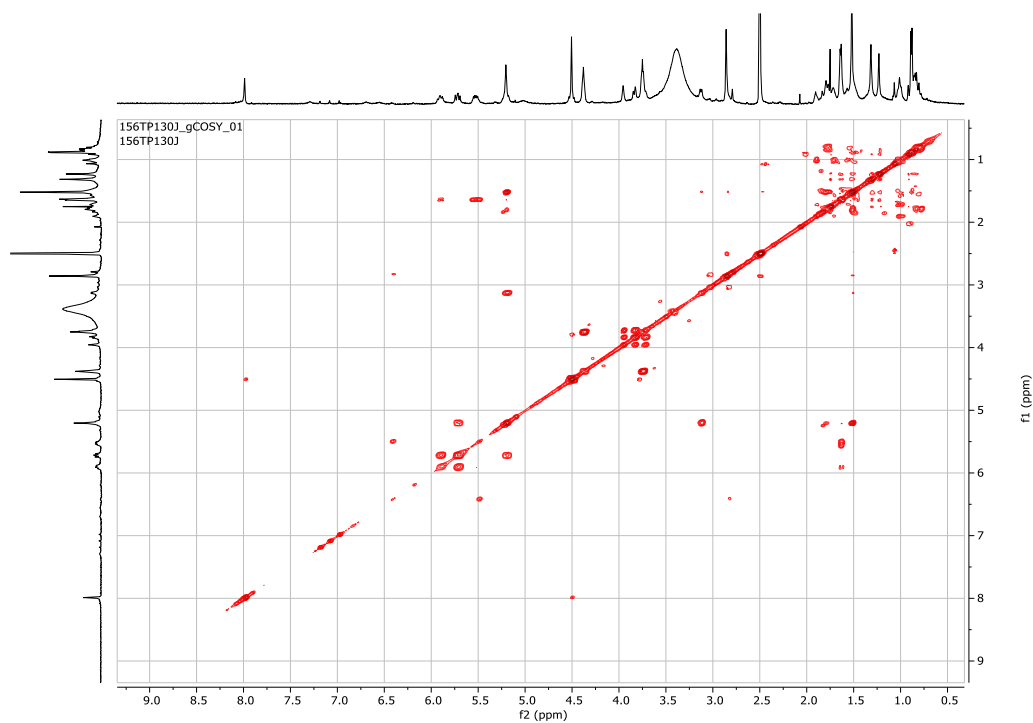


Figure C.68. COSY (500 MHz, DMSO) spectrum of **5a**

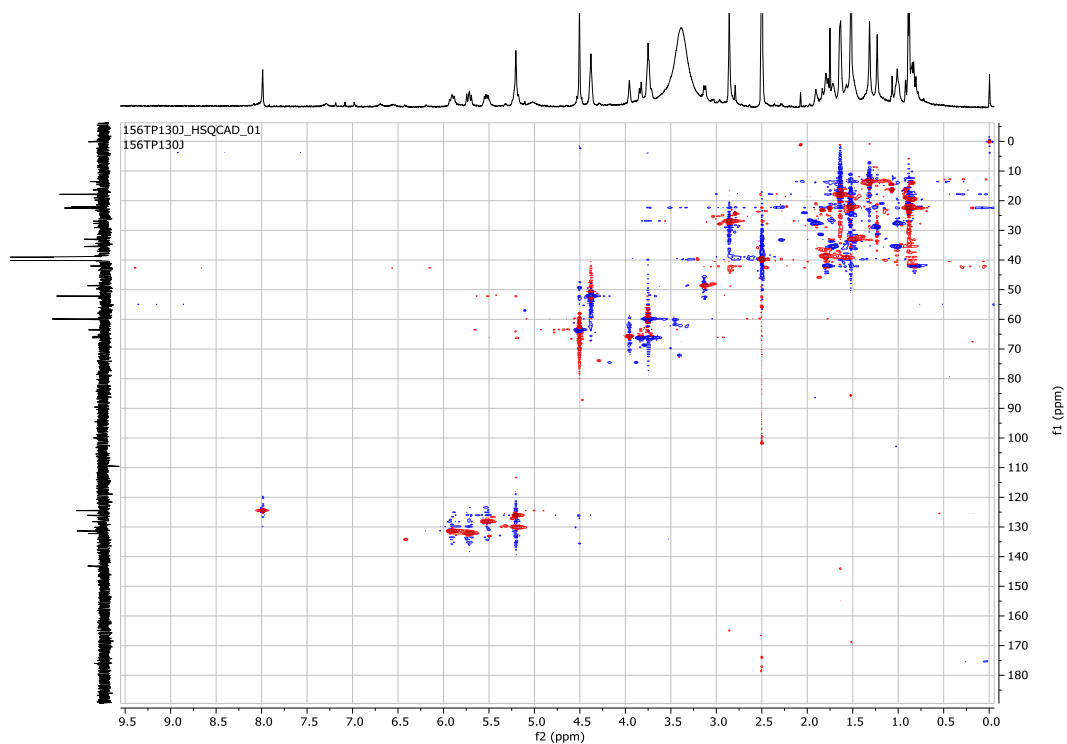


Figure C.69. HSQC (500 MHz, DMSO) spectrum of **5a**

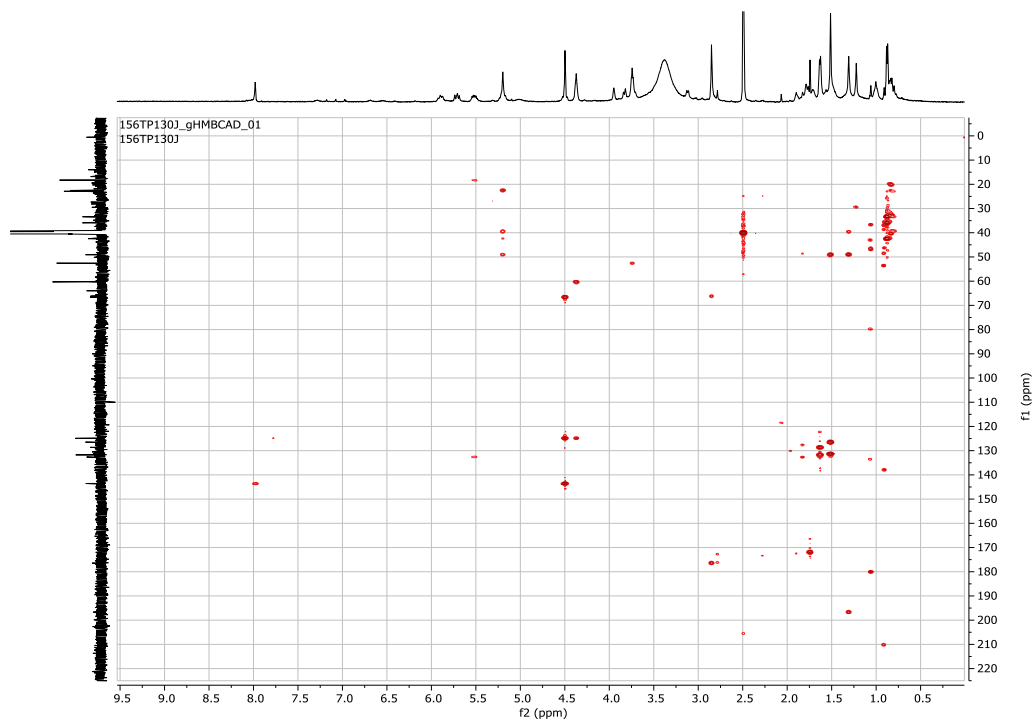


Figure C.70. HMBC (500 MHz, DMSO) spectrum of **5a**

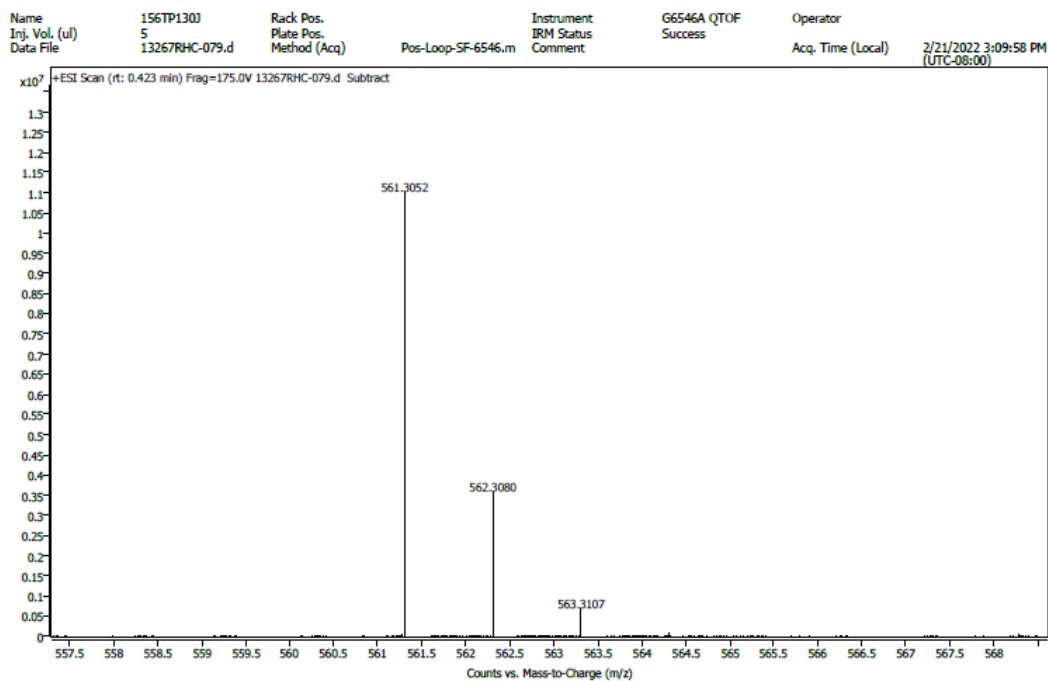


Figure C.71. HRESIMS spectrum of compound **5a**

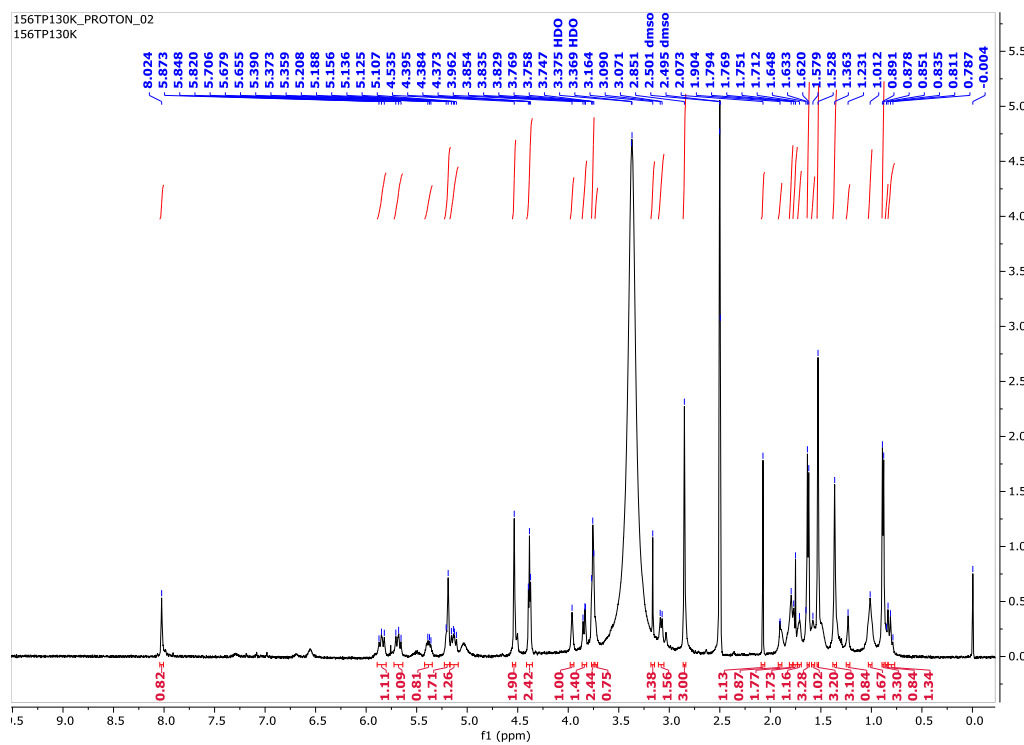


Figure C.72. ^1H NMR (500 MHz, DMSO) of **5a'**

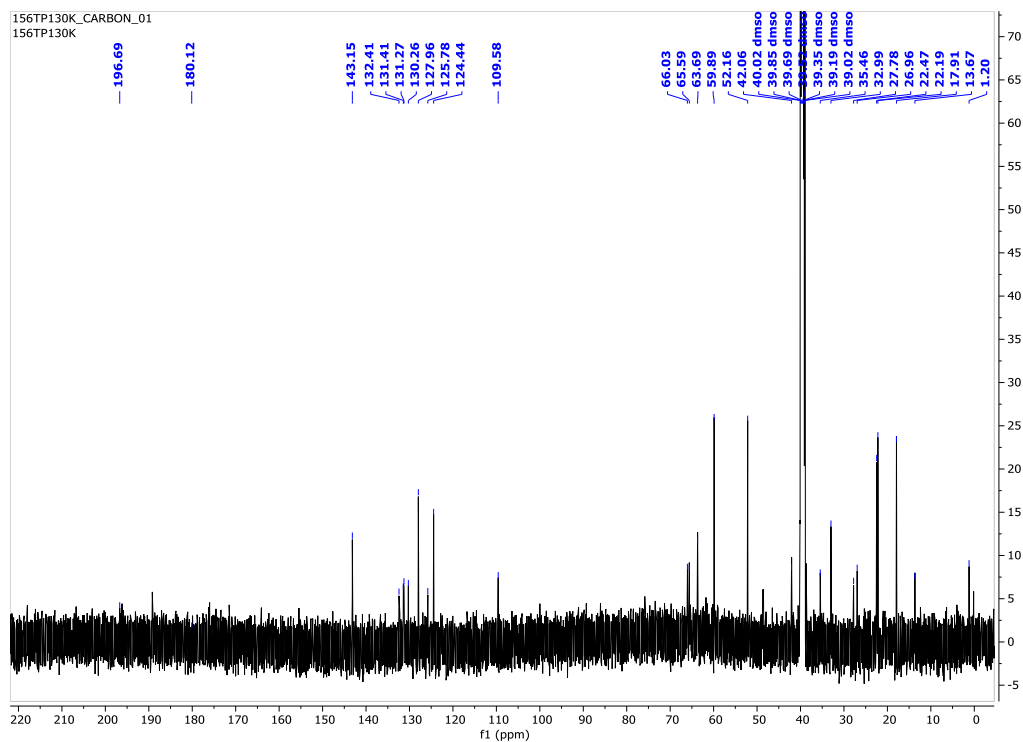


Figure C.73. ^{13}C NMR (400 MHz, DMSO) of **5a'**

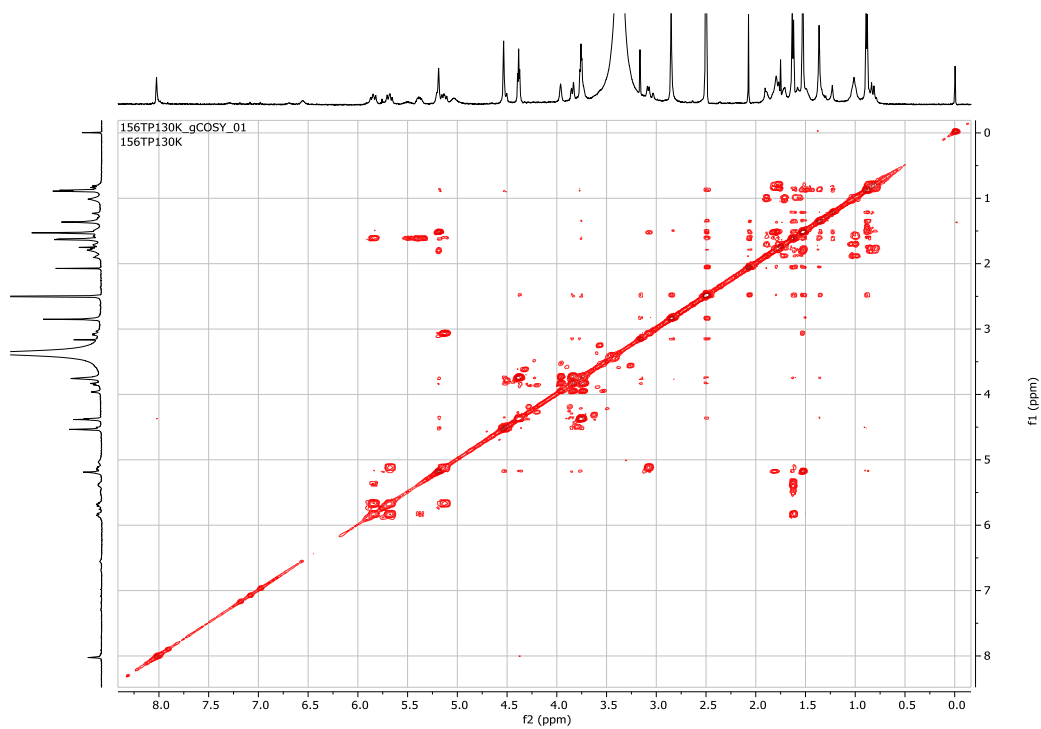


Figure C.74. COSY (500 MHz, DMSO) spectrum of **5a'**

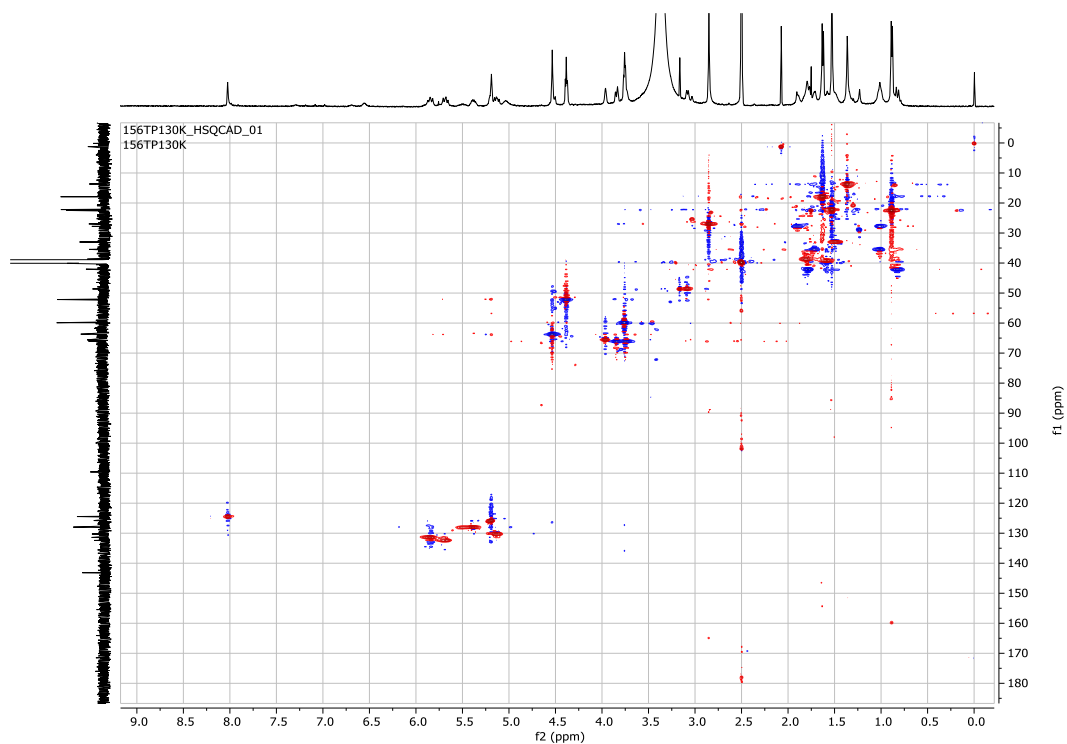


Figure C.75. HSQC (500 MHz, DMSO) spectrum of 5a'

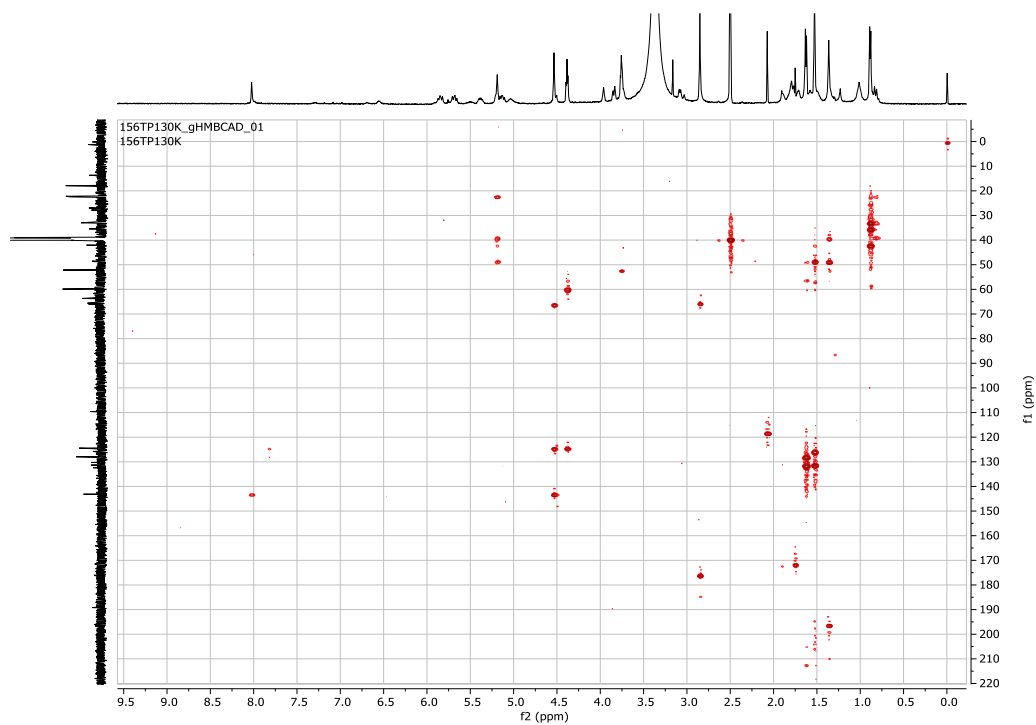


Figure C.76. HMBC (500 MHz, DMSO) spectrum of 5a'

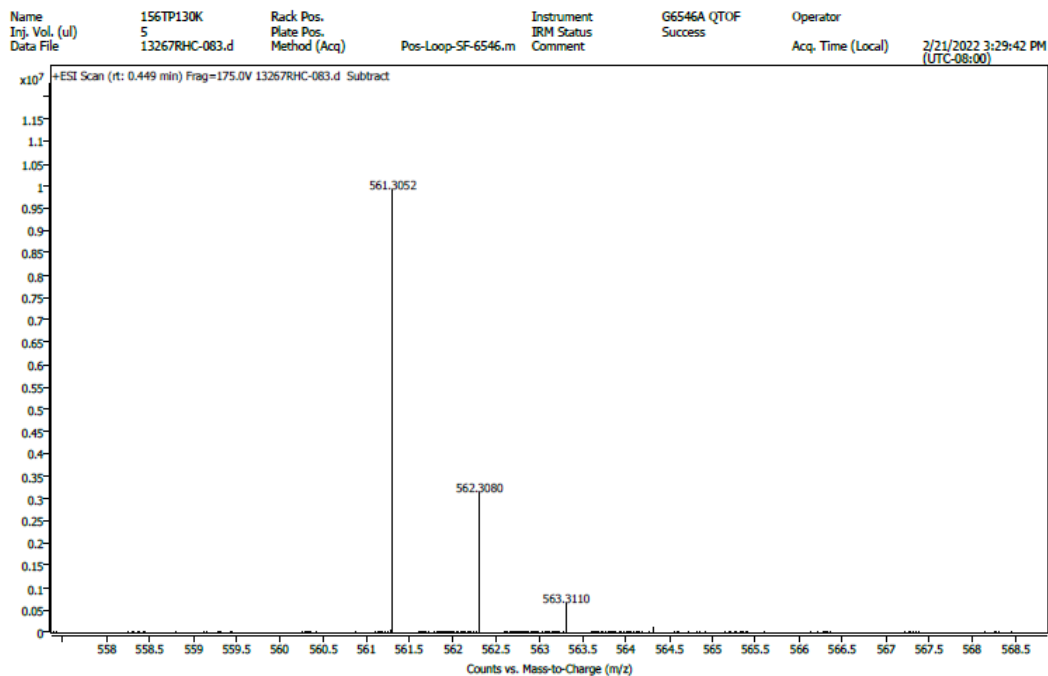


Figure C.77. HRESIMS spectrum of compound **5a'**

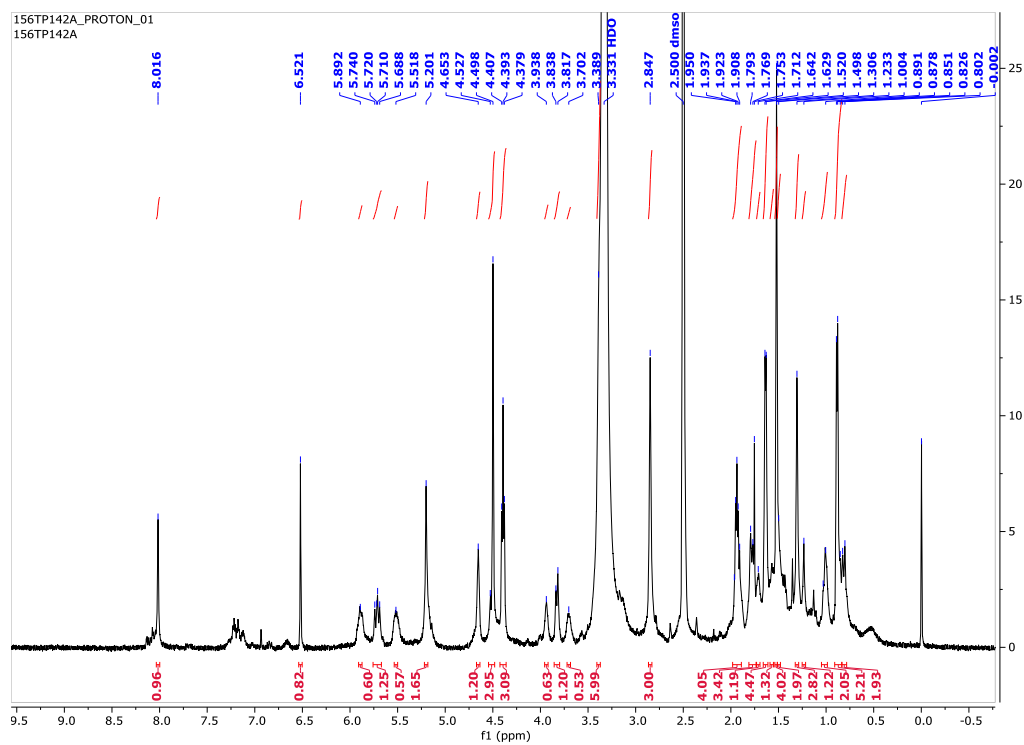


Figure C.78. ¹H NMR (500 MHz, DMSO) of **5b**

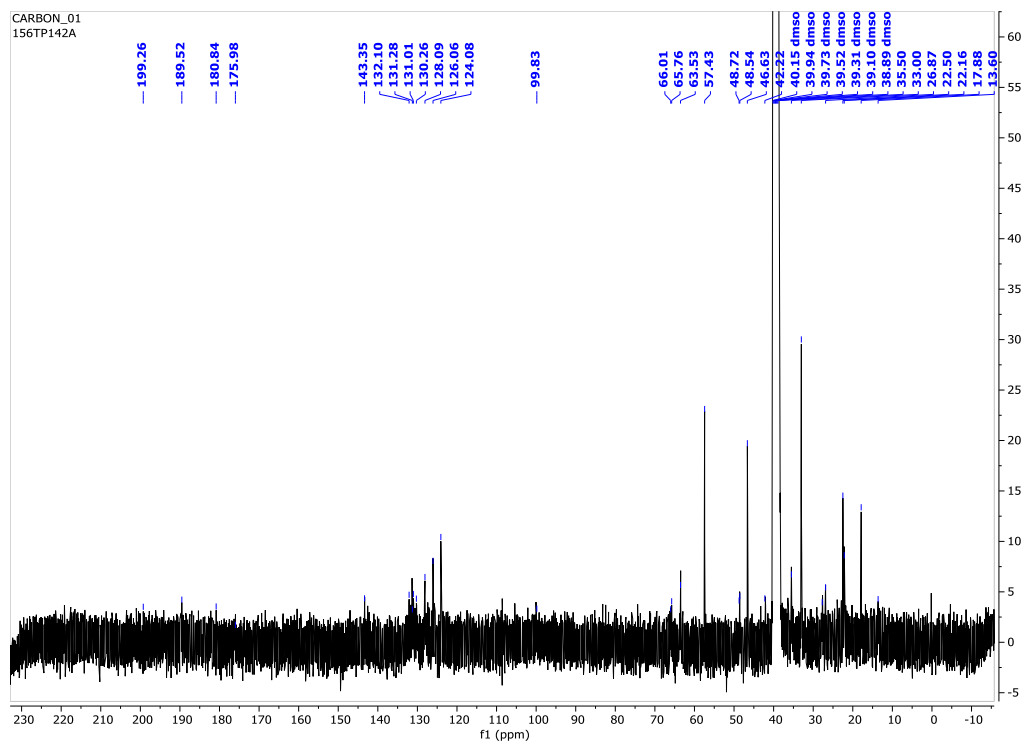


Figure C.79. ^{13}C NMR (400 MHz, DMSO) of **5b**

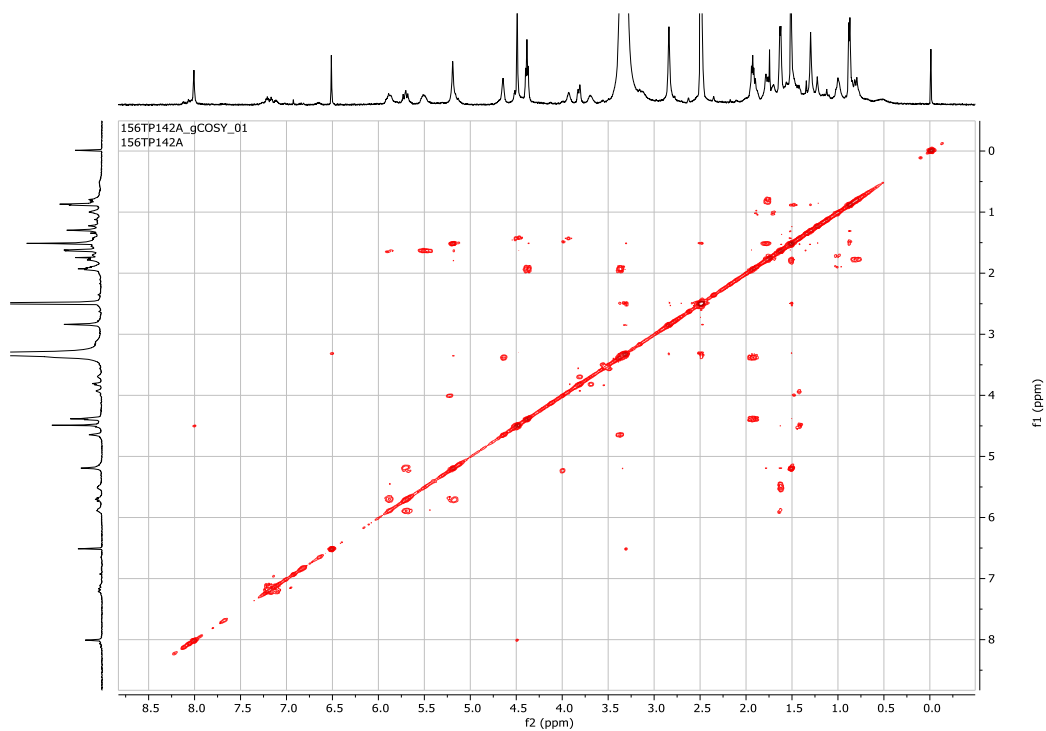


Figure C.80. COSY (500 MHz, DMSO) spectrum of **5b**

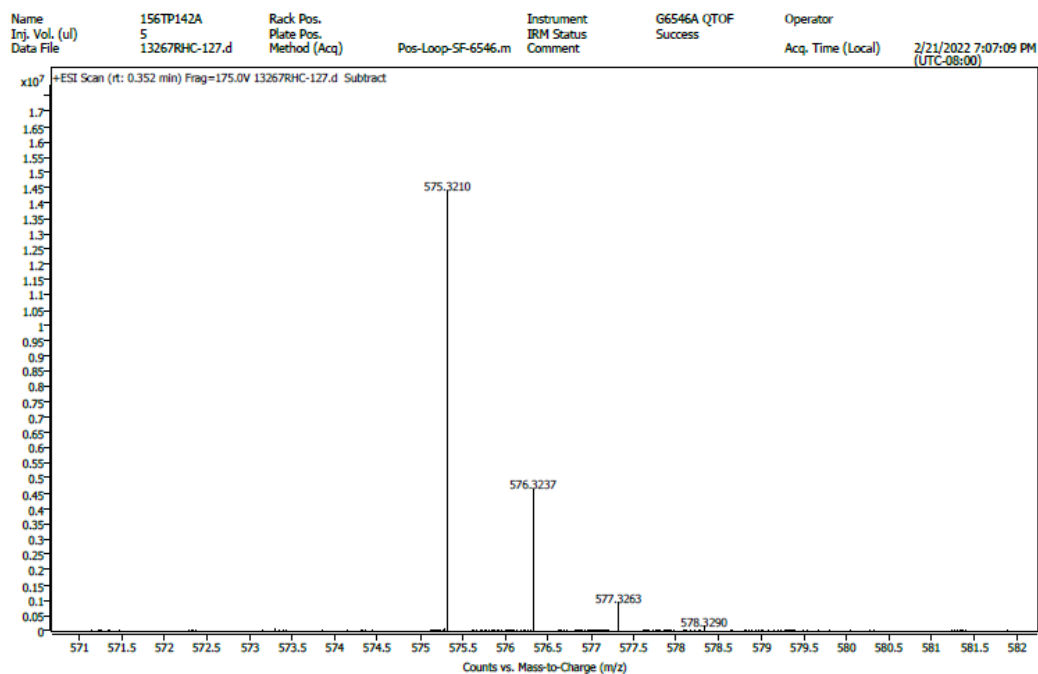


Figure C.81. HRESIMS spectrum of compound **5b**

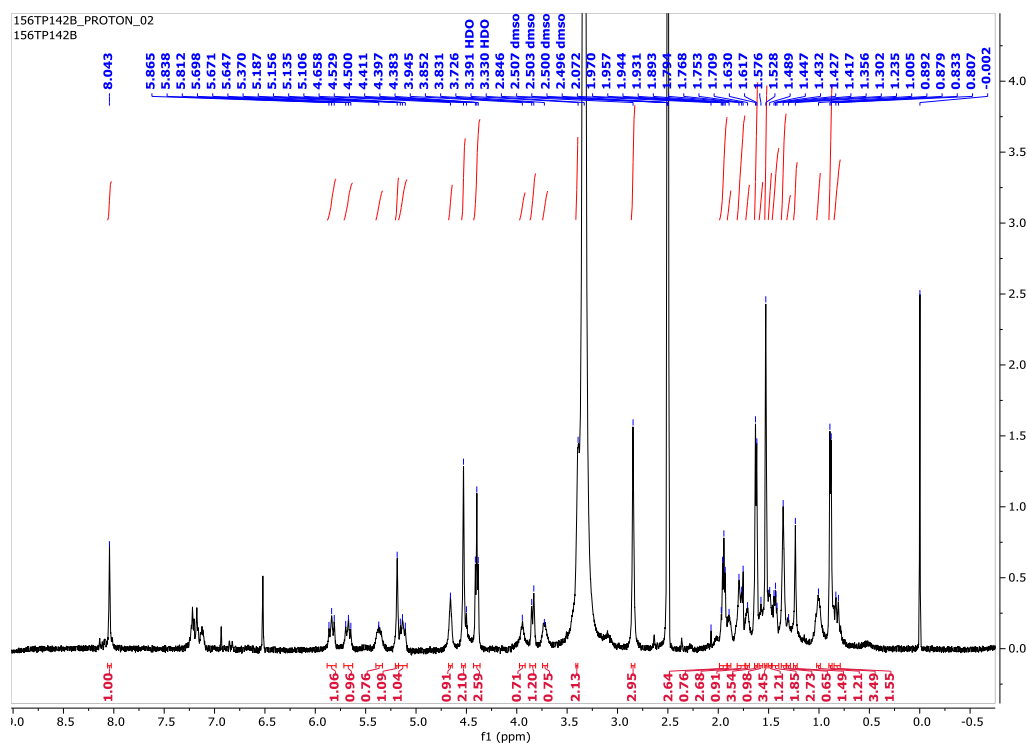


Figure C.82. ^1H NMR (500 MHz, DMSO) of **5b'**

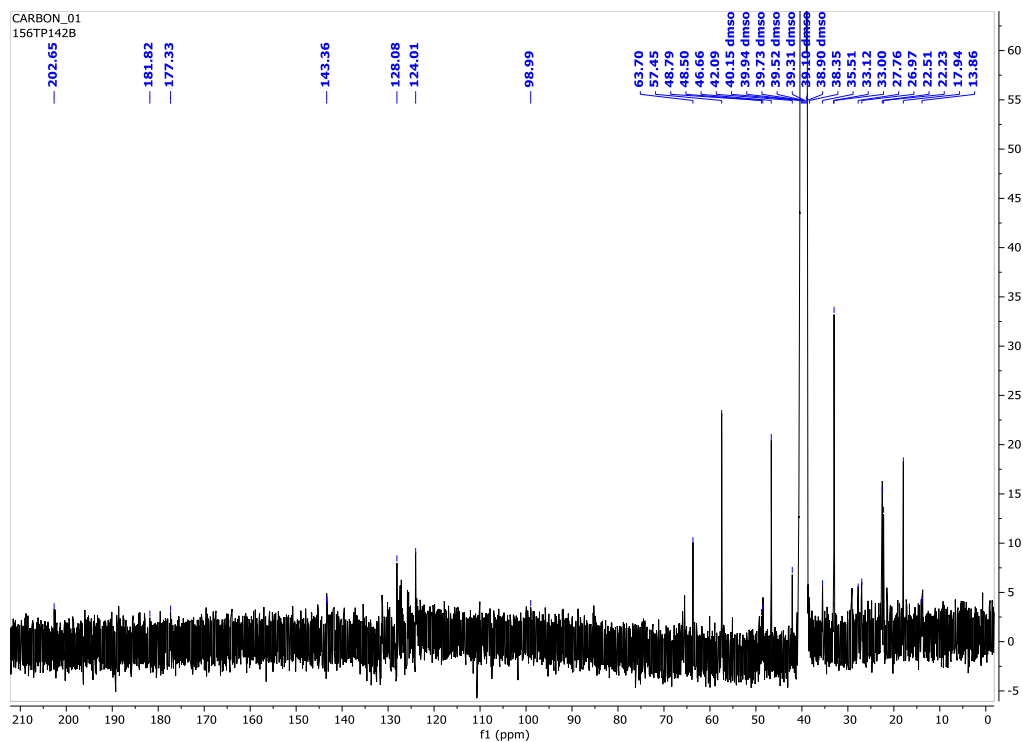


Figure C.83. ^{13}C NMR (400 MHz, DMSO) of **5b'**

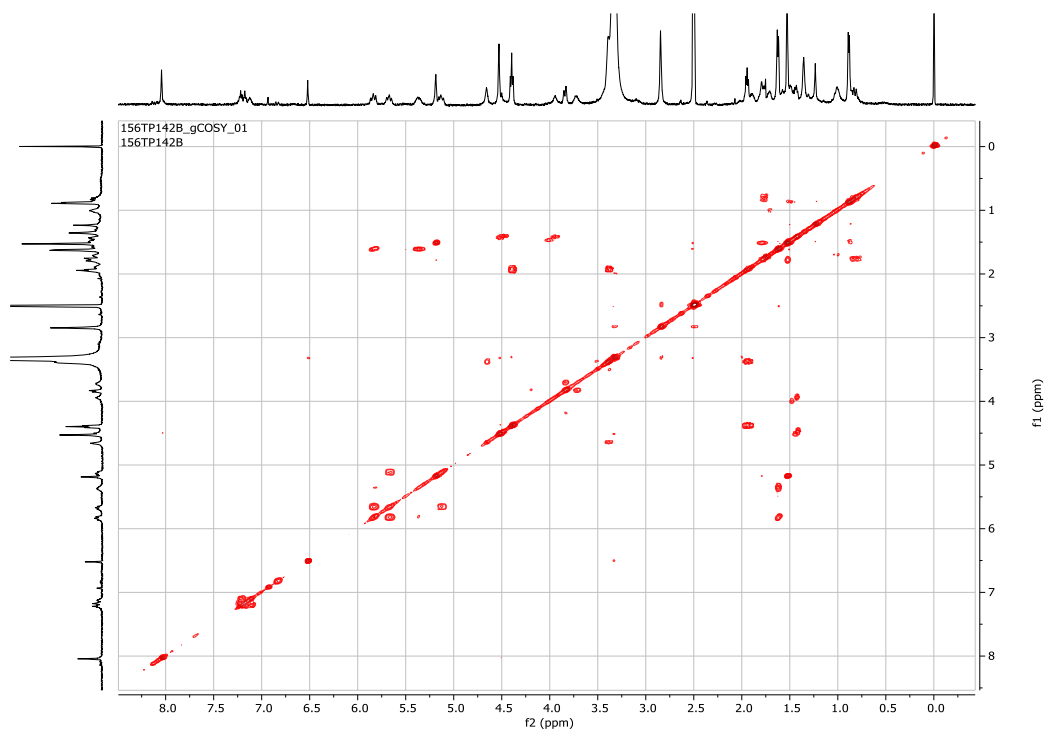


Figure C.84. COSY (500 MHz, DMSO) spectrum of **5b'**

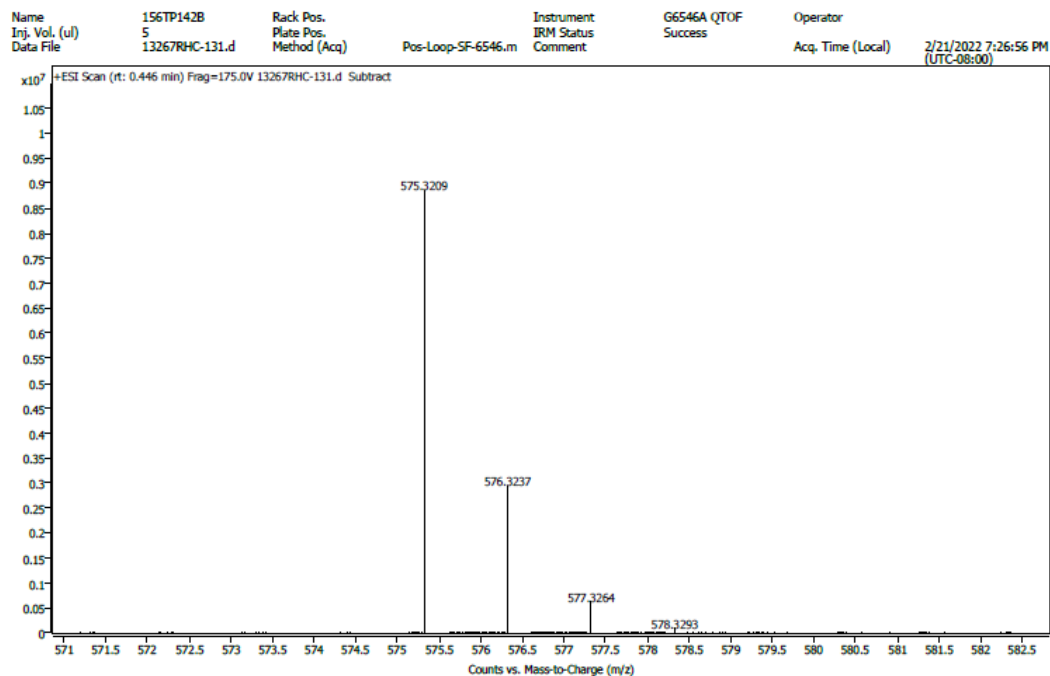


Figure C.85. HRESIMS spectrum of compound **5b'**

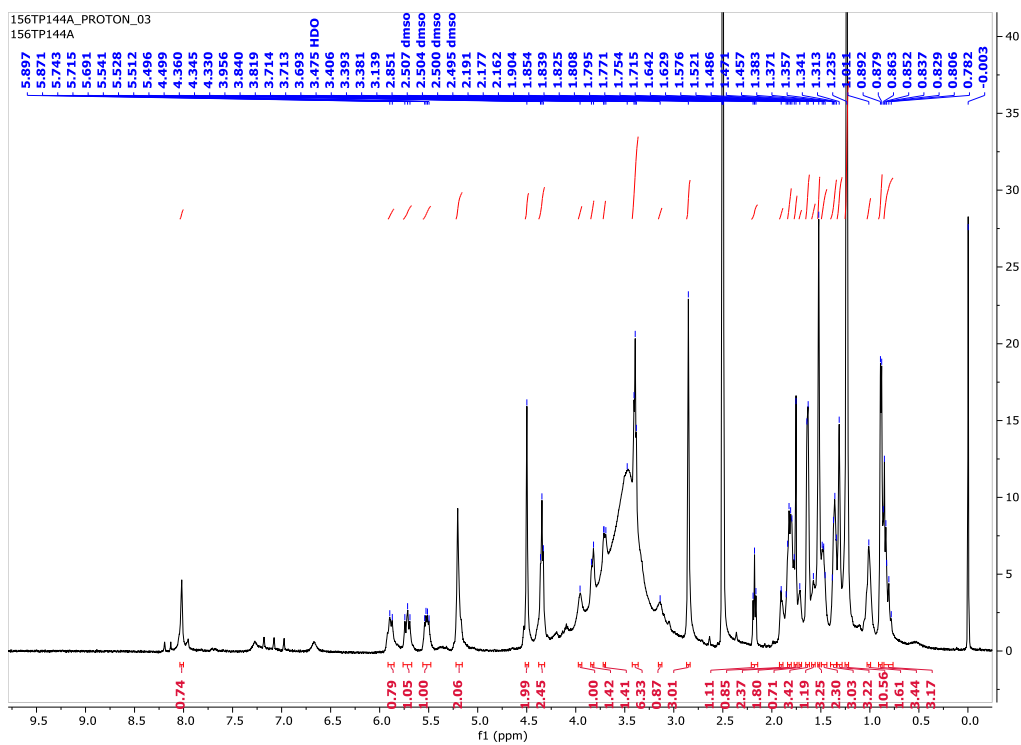


Figure C.86. ¹H NMR (500 MHz, DMSO) of **5c**

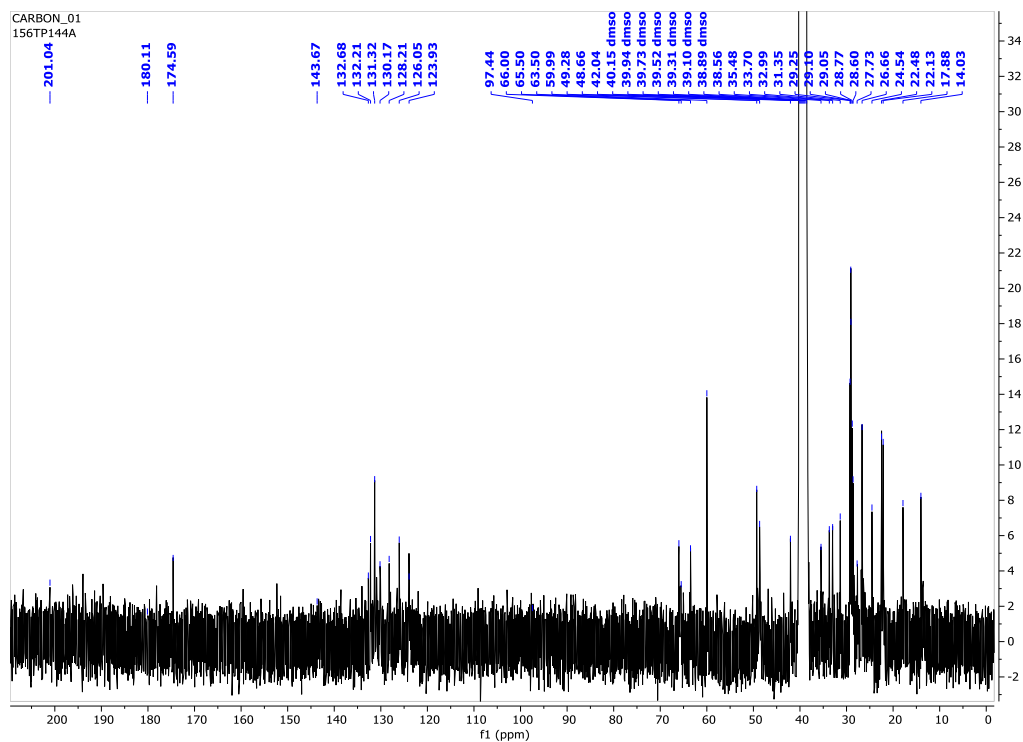


Figure C.87. ^{13}C NMR (400 MHz, DMSO) of **5c**

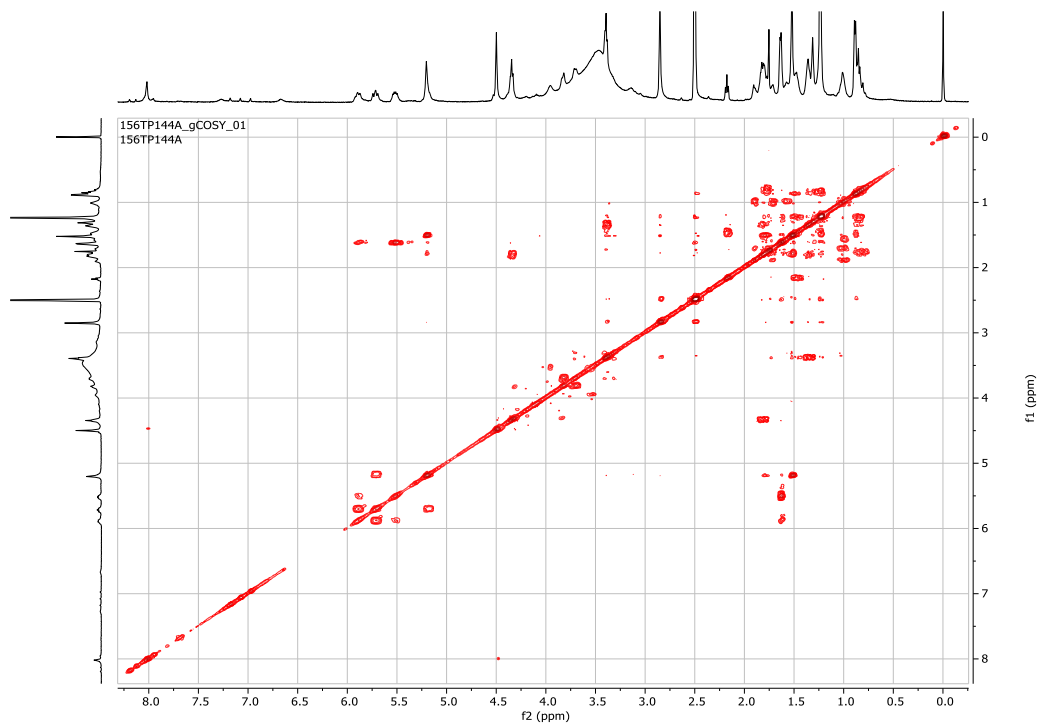


Figure C.88. COSY (500 MHz, DMSO) spectrum of **5c**

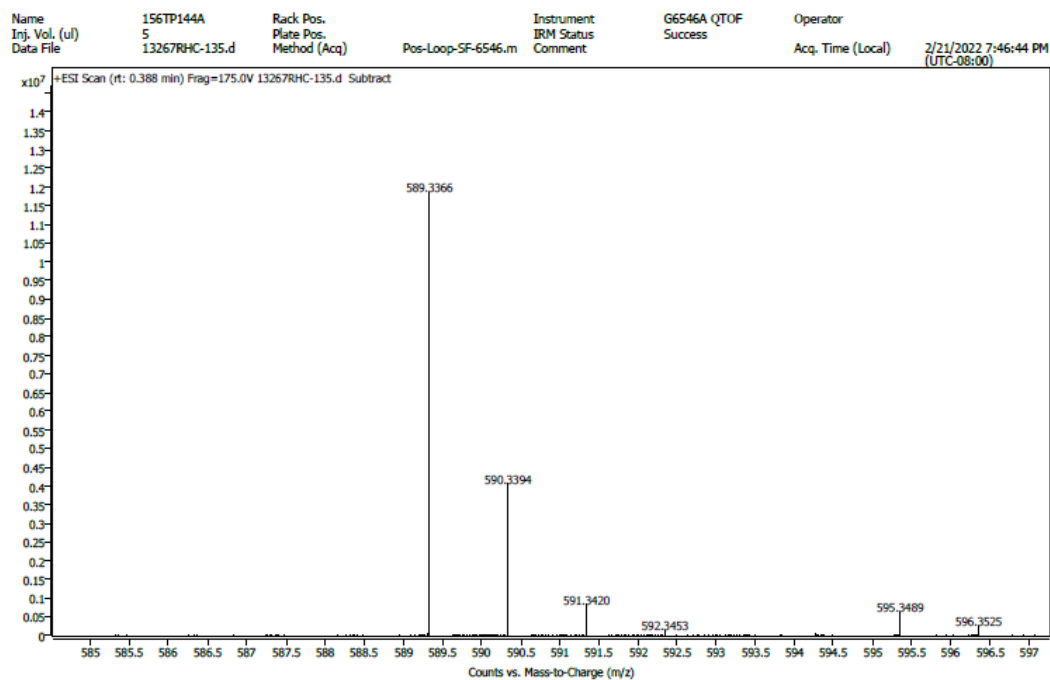


Figure C.89. HRESIMS spectrum of compound **5c**

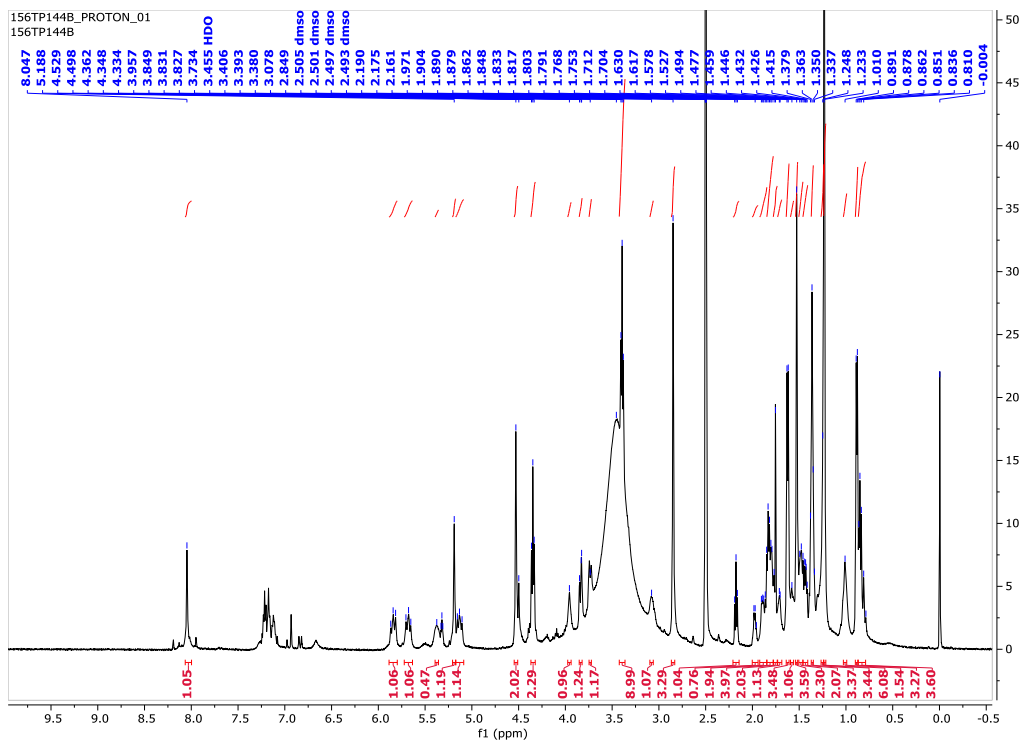


Figure C.90. ^1H NMR (500 MHz, DMSO) of **5c'**

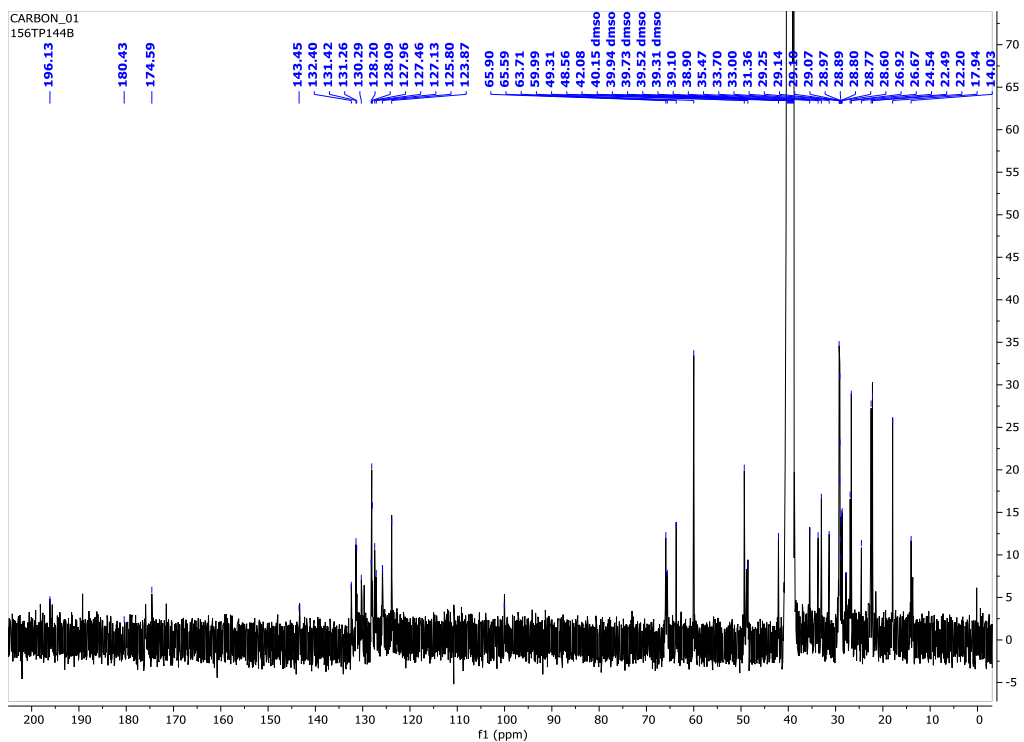


Figure C.91. ^{13}C NMR (400 MHz, DMSO) of **5c'**

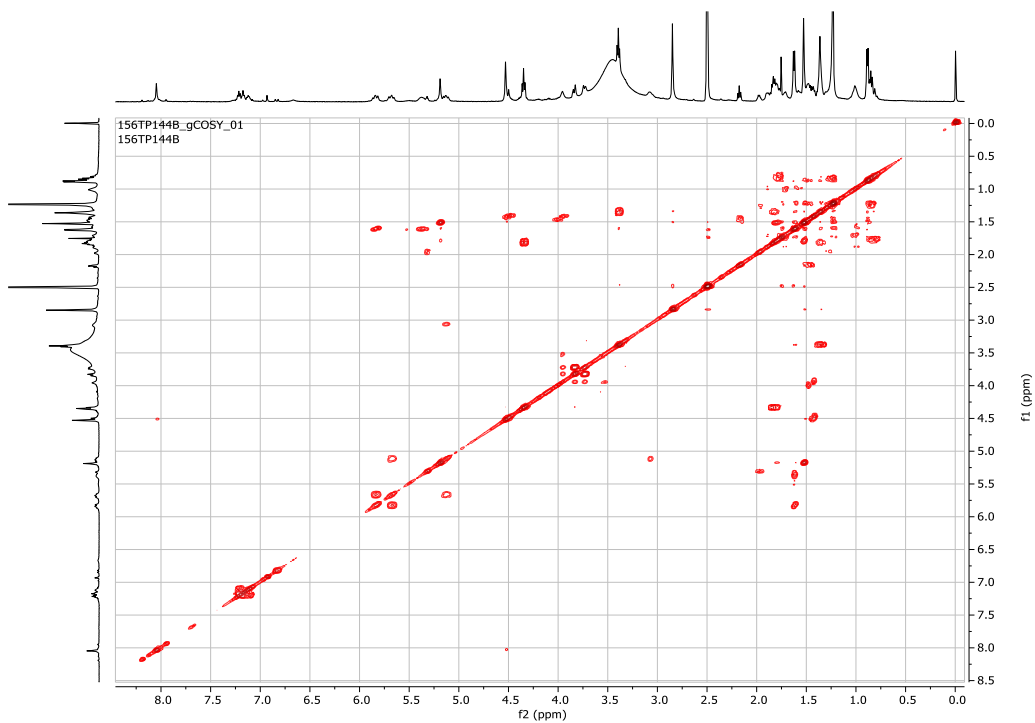


Figure C.92. COSY (500 MHz, DMSO) spectrum of **5c'**

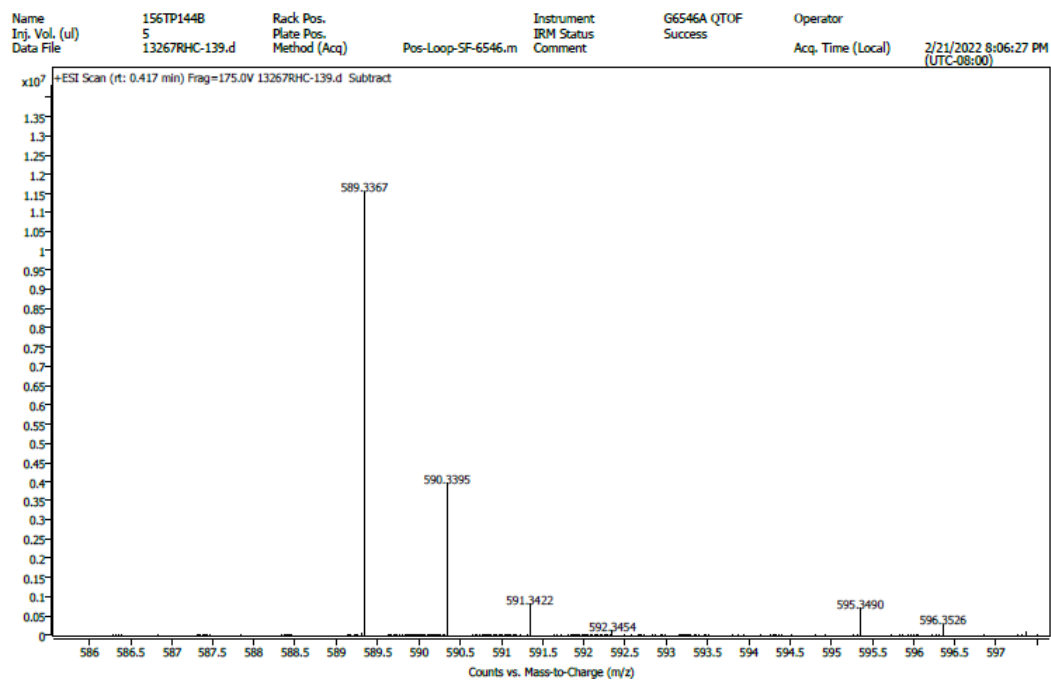


Figure C.93. HRESIMS spectrum of compound **5c'**

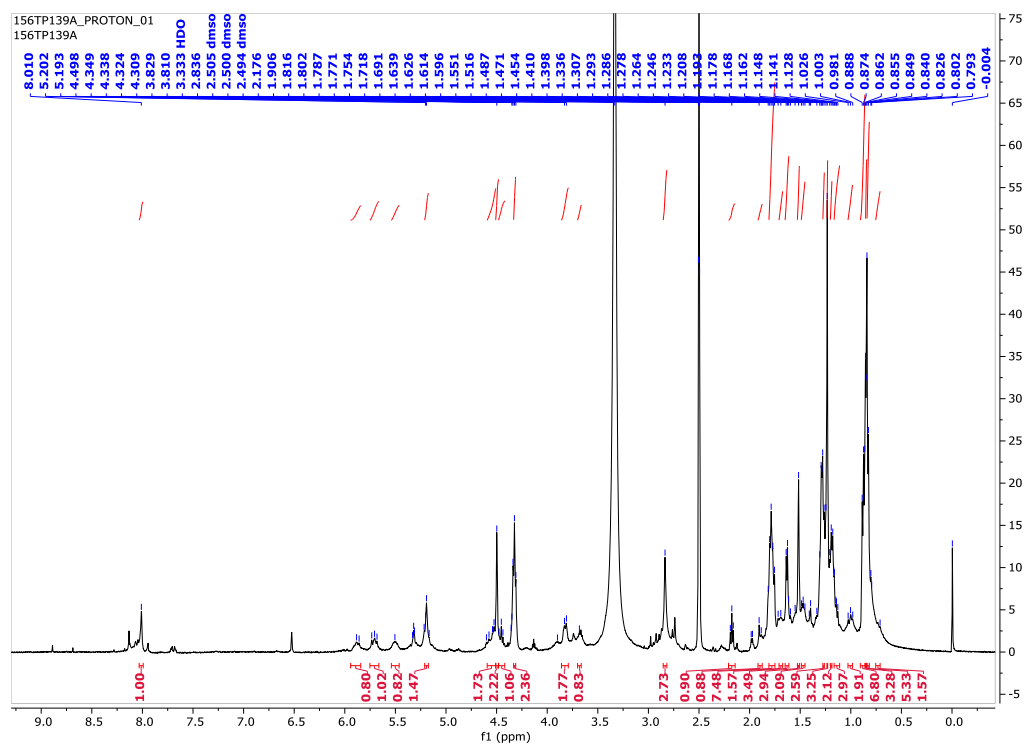


Figure C.94. ^1H NMR (500 MHz, DMSO) of **5d**

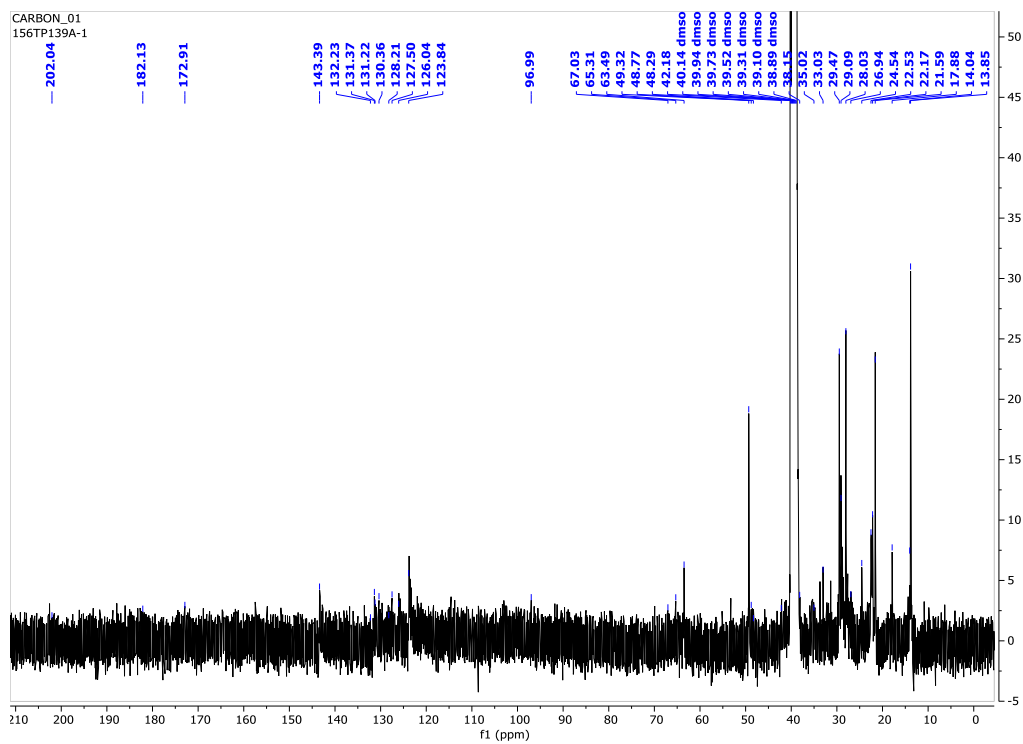


Figure C.95. ^{13}C NMR (400 MHz, DMSO) of **5d**

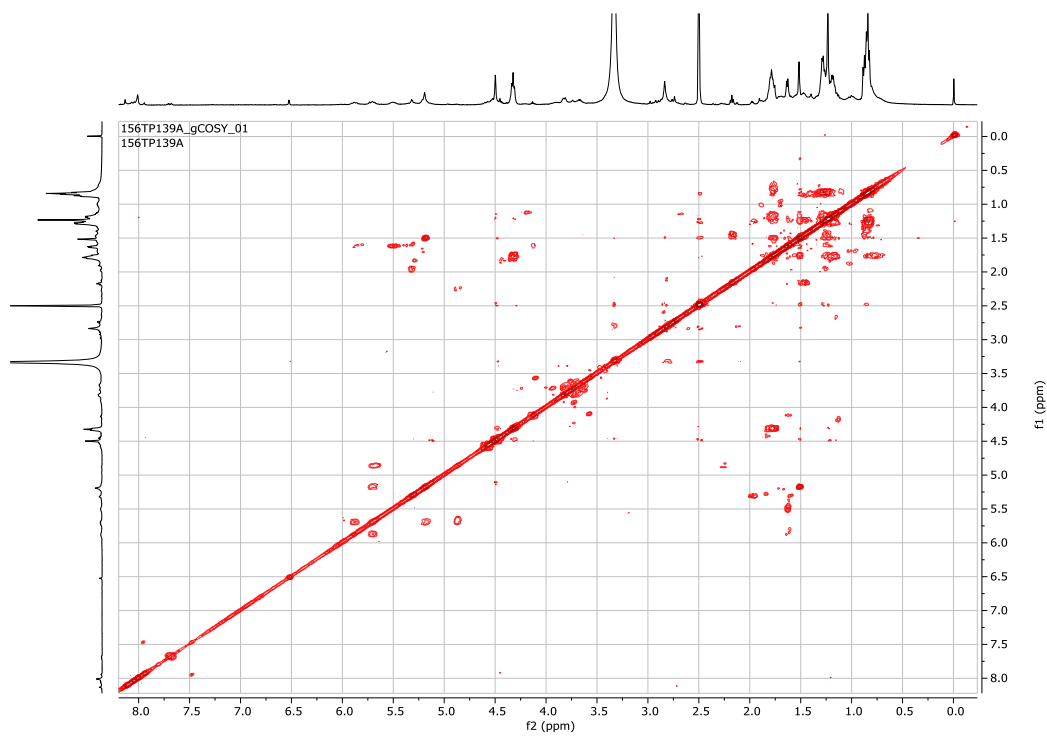


Figure C.96. COSY (500 MHz, DMSO) spectrum of **5d**

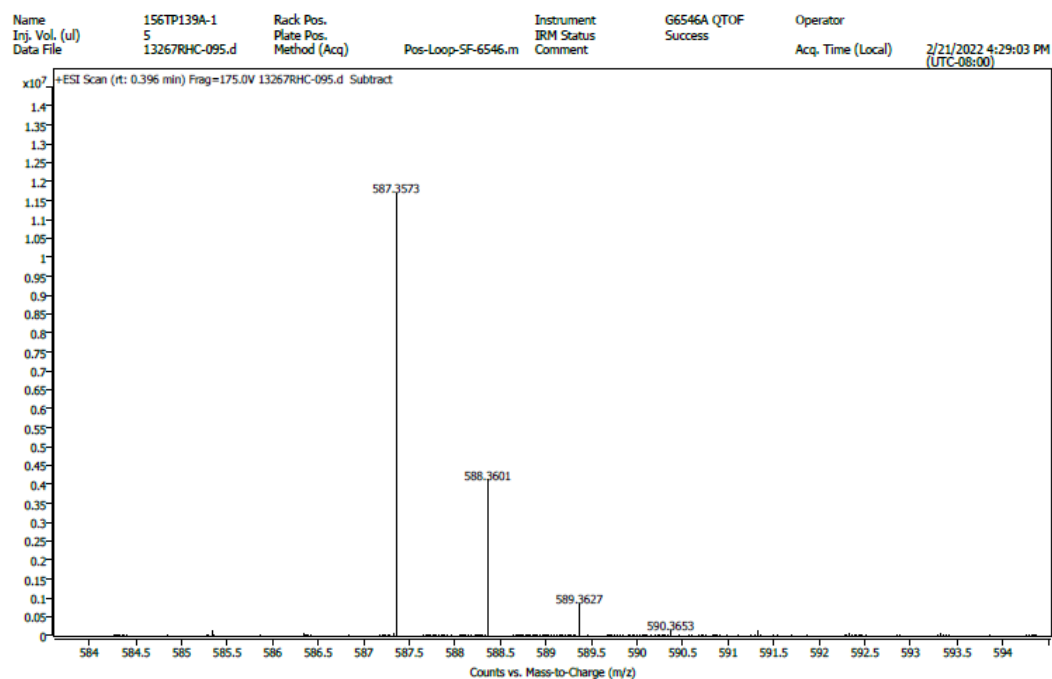


Figure C.97. HRESIMS spectrum of compound **5d**

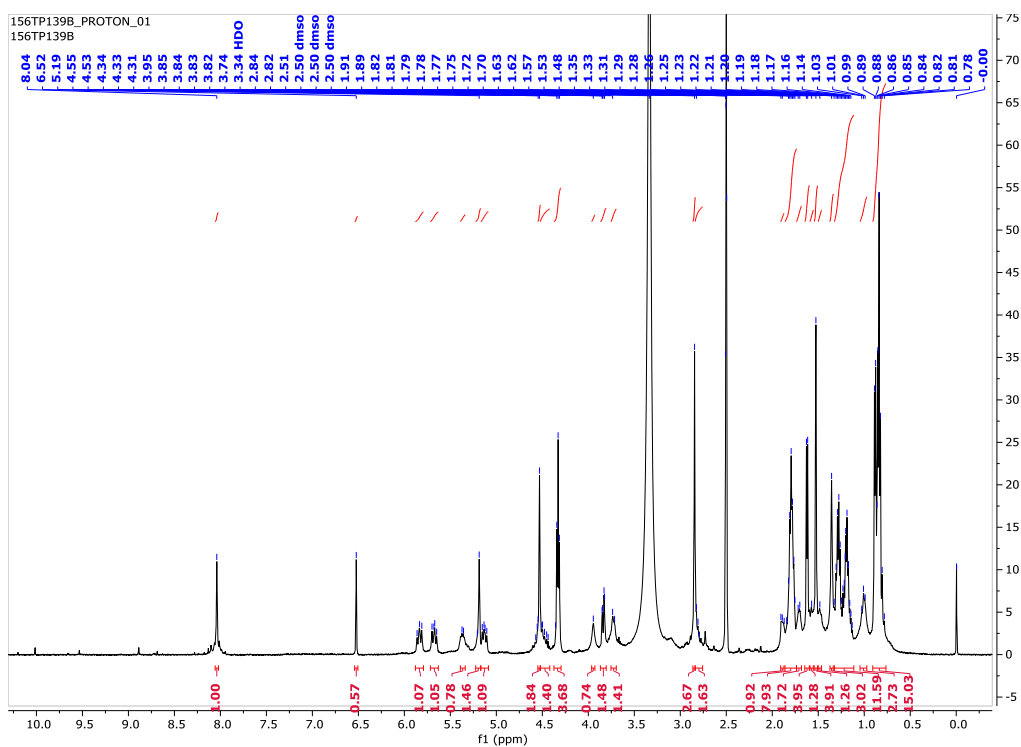


Figure C.98. ^1H NMR (500 MHz, DMSO) of **5d'**

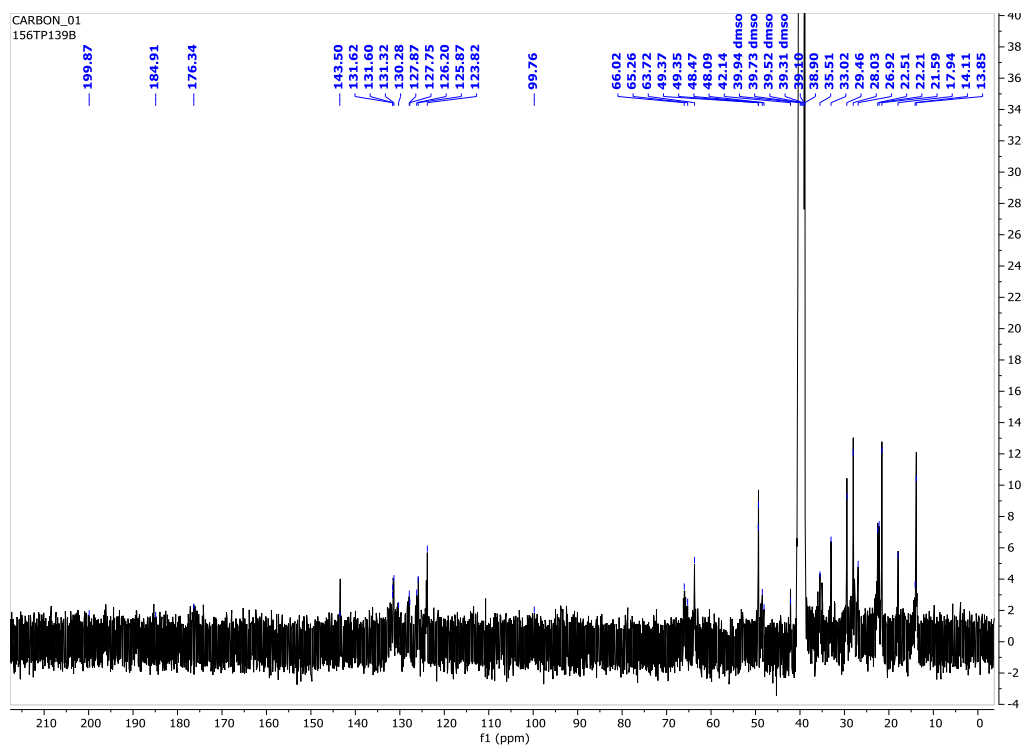


Figure C.99. ^{13}C NMR (400 MHz, DMSO) of **5d'**

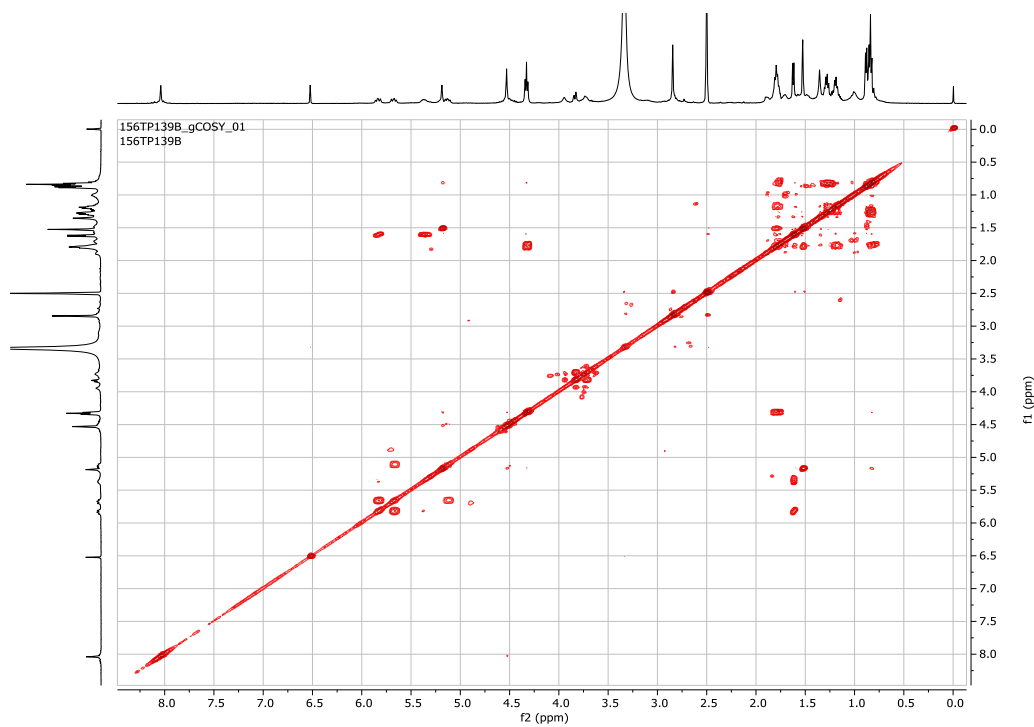


Figure C.100. COSY (500 MHz, DMSO) spectrum of **5d'**

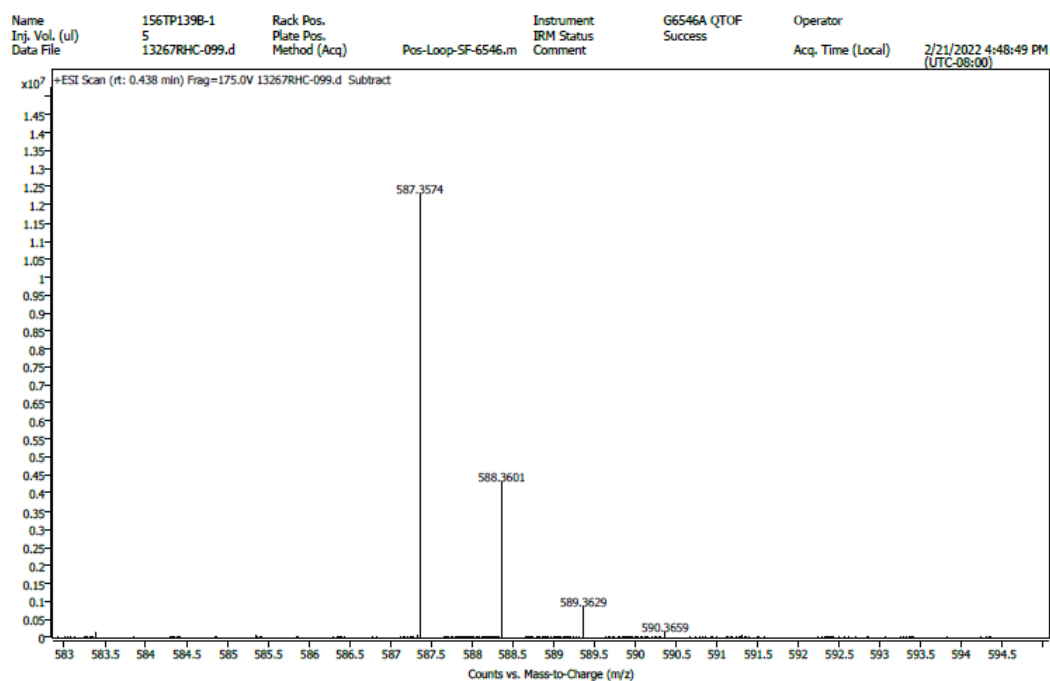


Figure C.101. HRESIMS spectrum of compound **5d'**

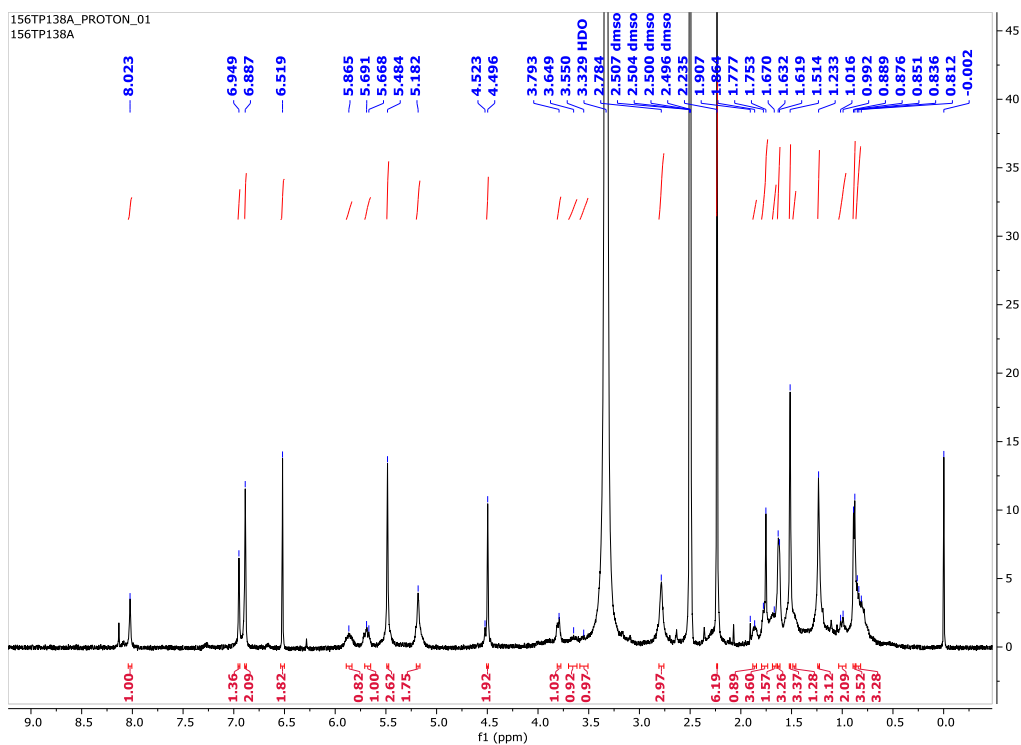


Figure C.102. ^1H NMR (500 MHz, DMSO) of **5e**

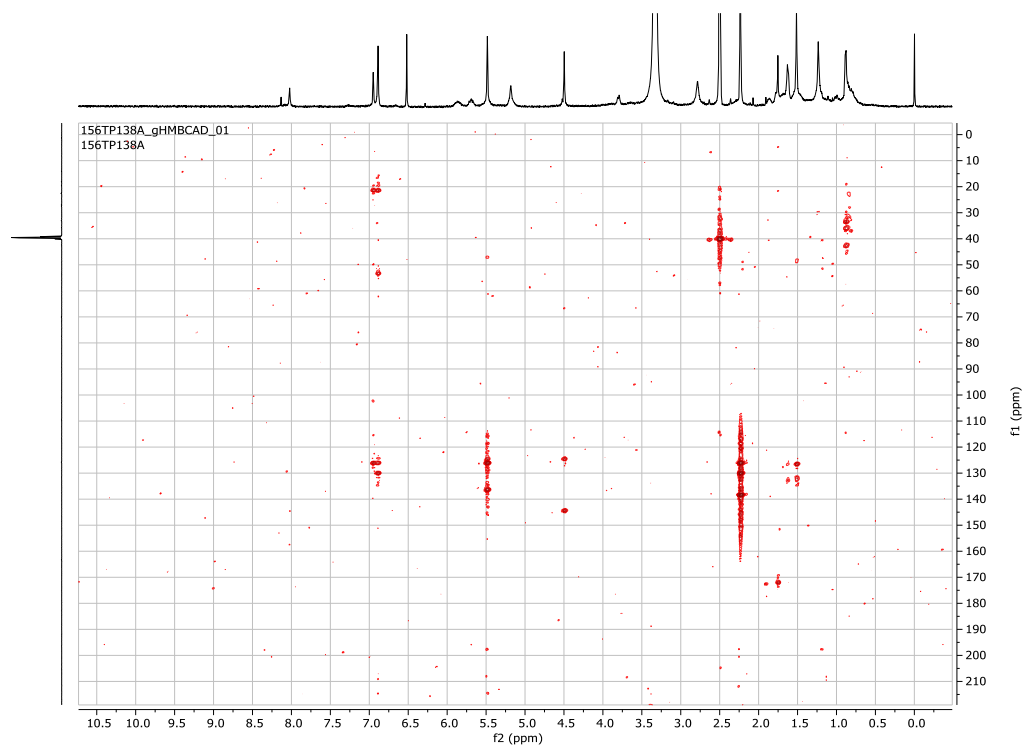


Figure C.103. HMBC (500 MHz, DMSO) spectrum of **5e**

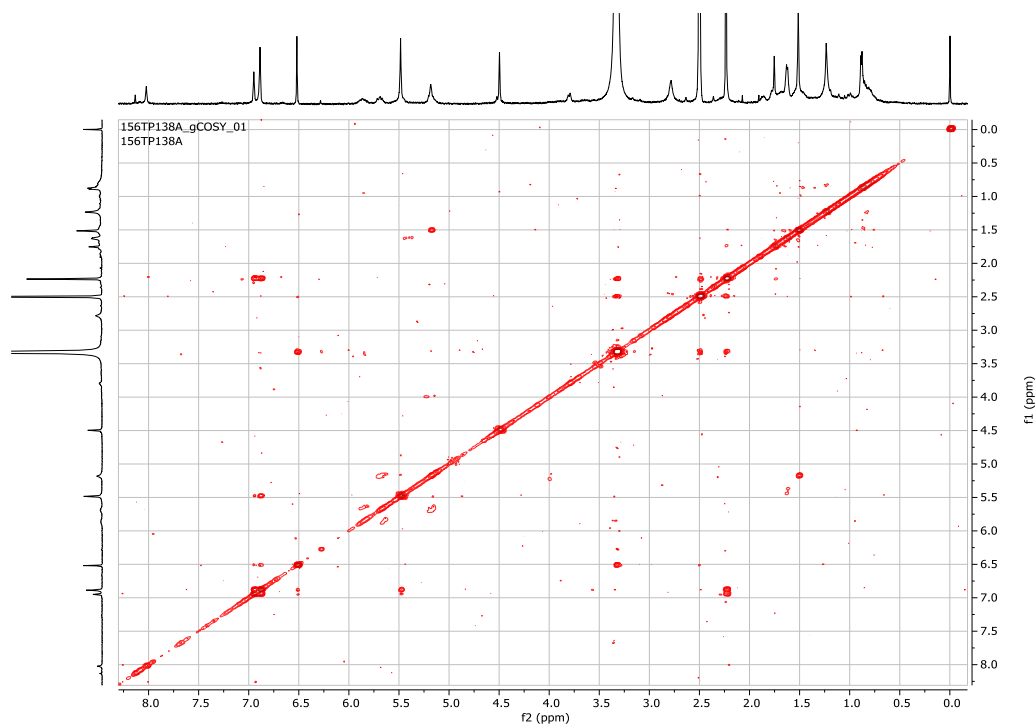


Figure C.104. COSY (500 MHz, DMSO) spectrum of **5e**

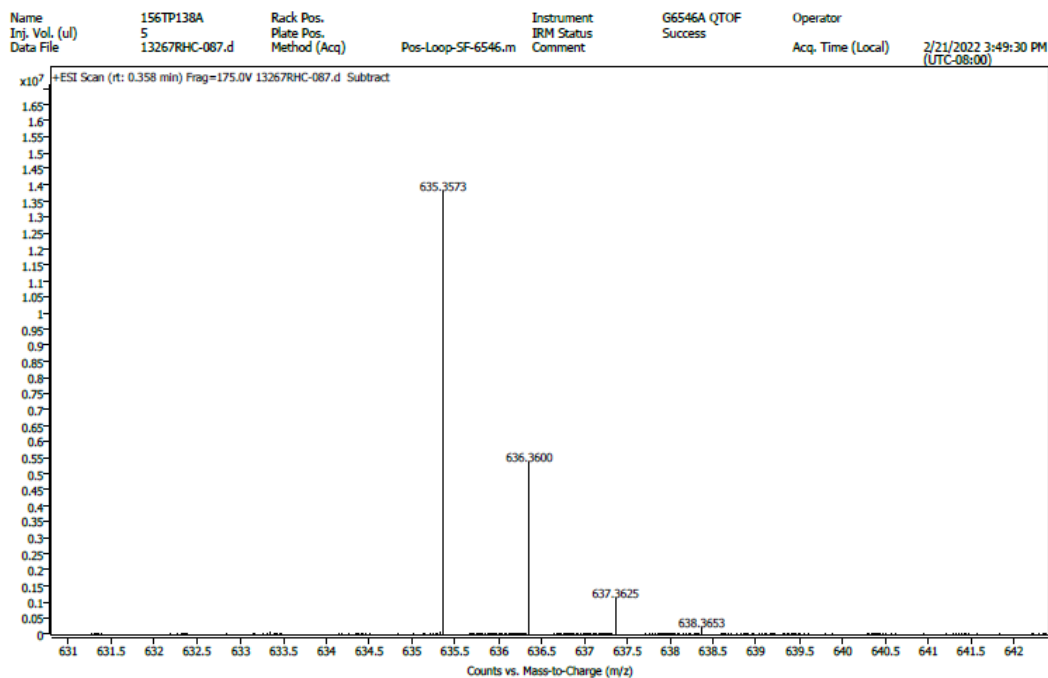


Figure C.105. HRESIMS spectrum of compound **5e**

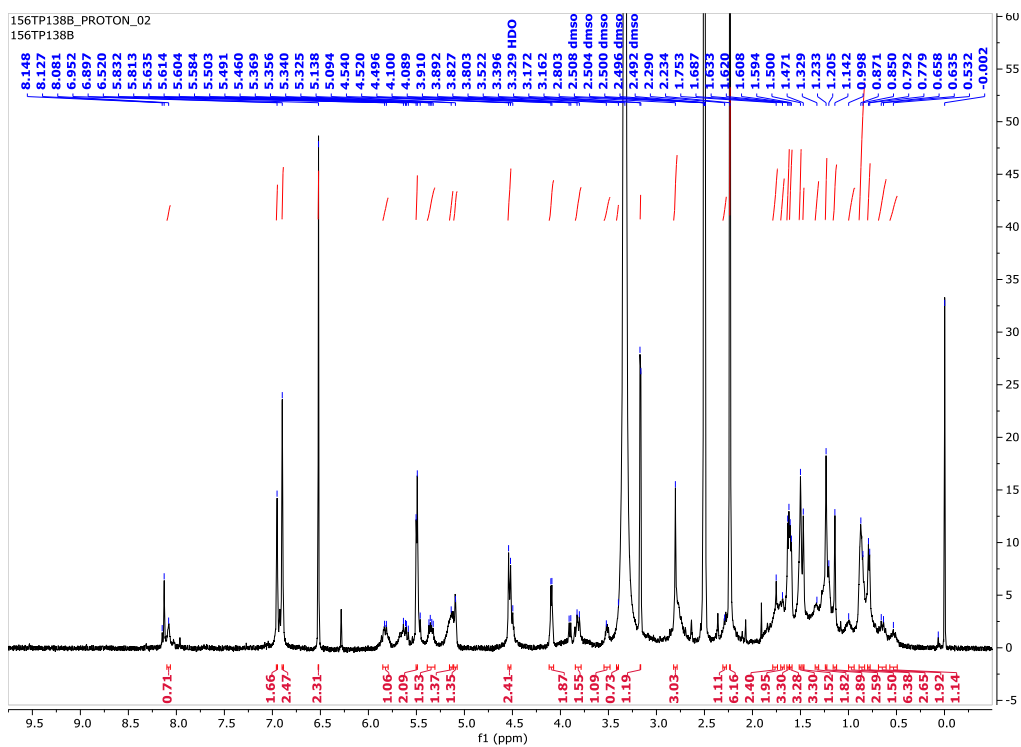


Figure C.106. ^1H NMR (500 MHz, DMSO) of **5e'**

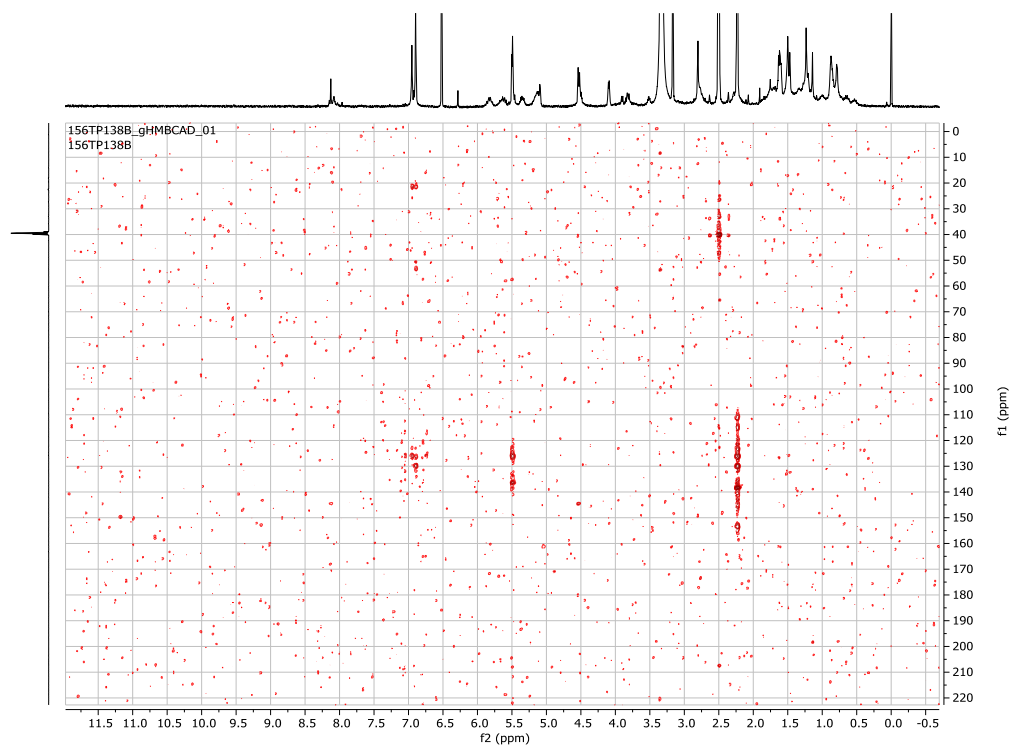


Figure C.107. HMBC (500 MHz, DMSO) spectrum of **5e'**

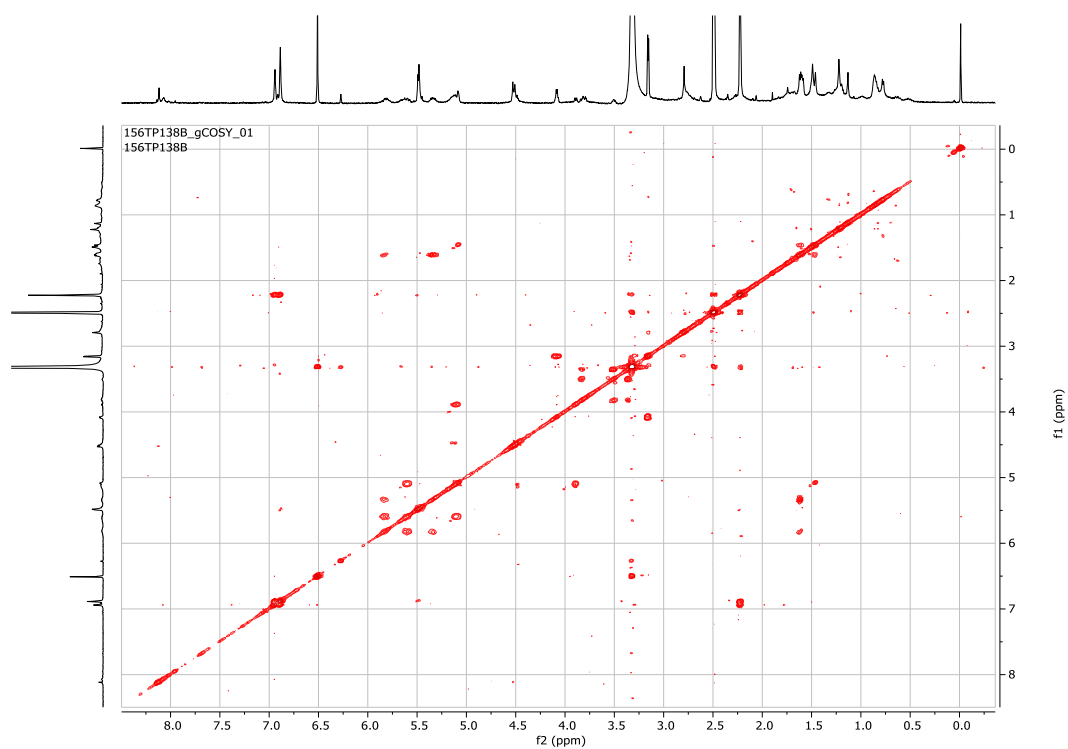


Figure C.108. COSY (500 MHz, DMSO) spectrum of **5e'**

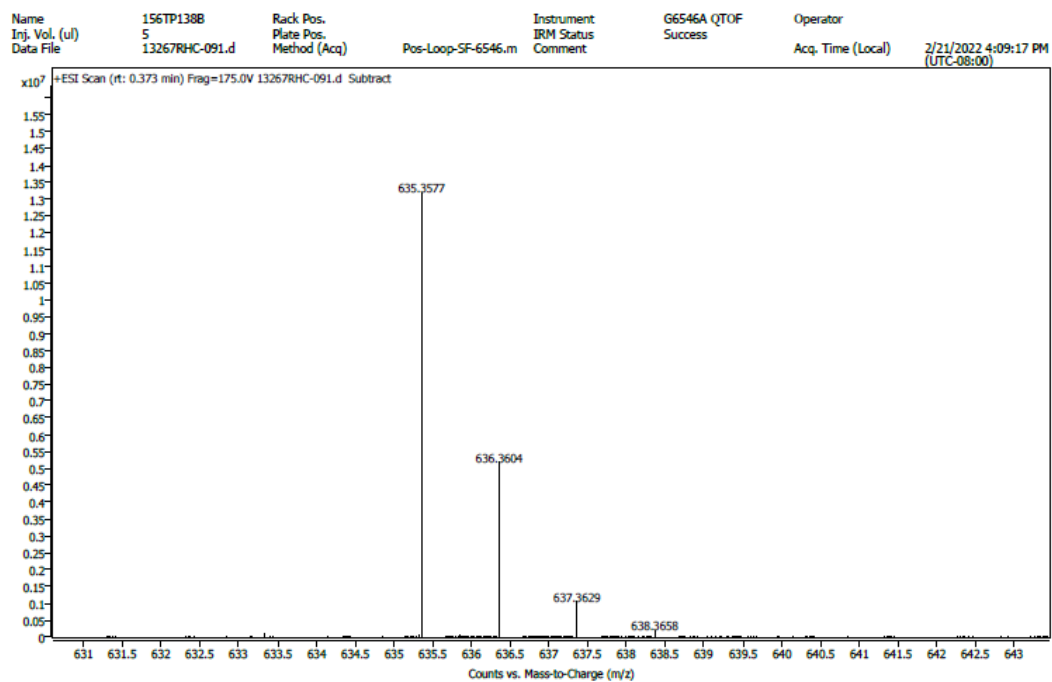


Figure C.109. HRESIMS spectrum of compound **5e'**

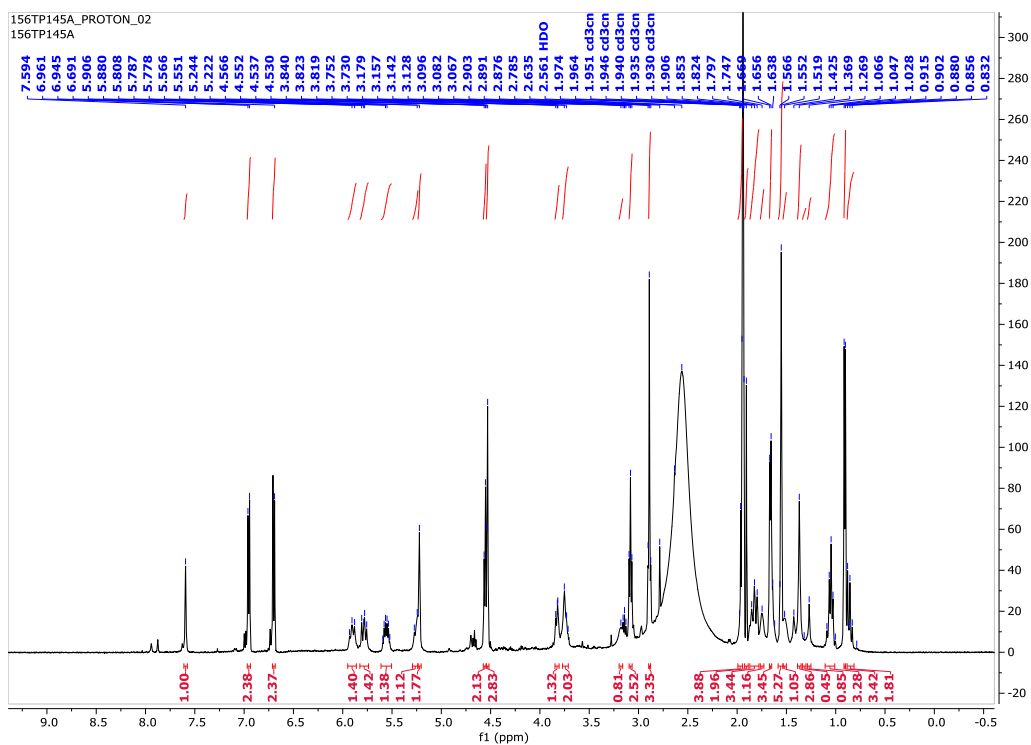


Figure C.110. ^1H NMR (500 MHz, $\text{CD}_3\text{CN}-d_3$) of **5f**

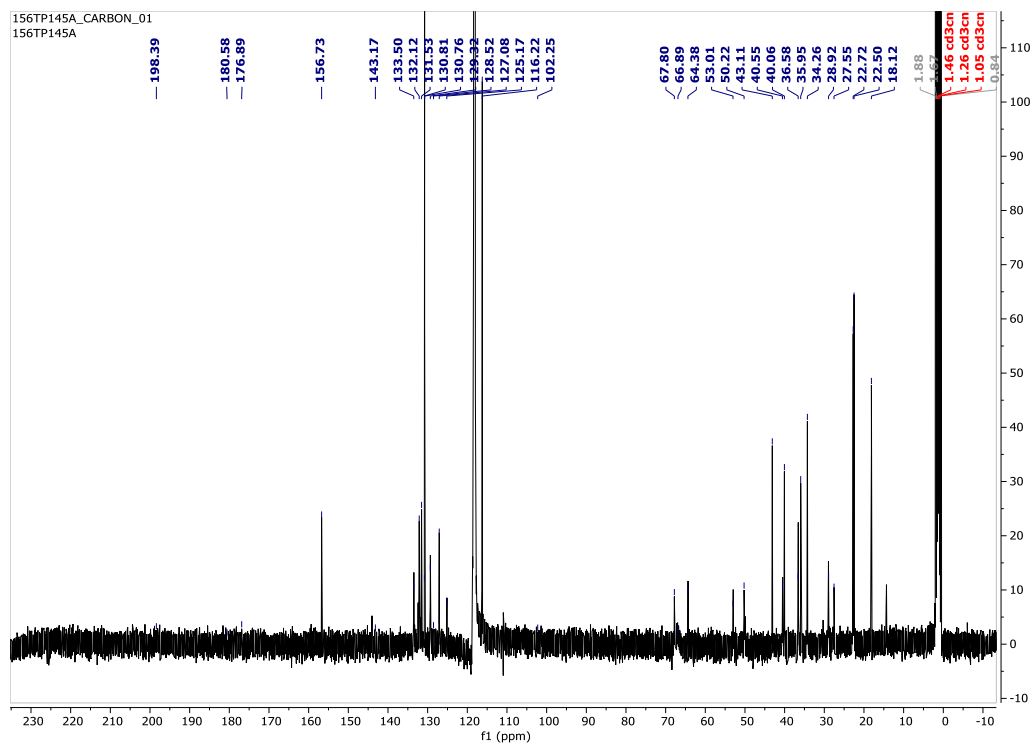


Figure C.111. ^{13}C NMR (400 MHz, $\text{CD}_3\text{CN}-d_3$) of **5f**

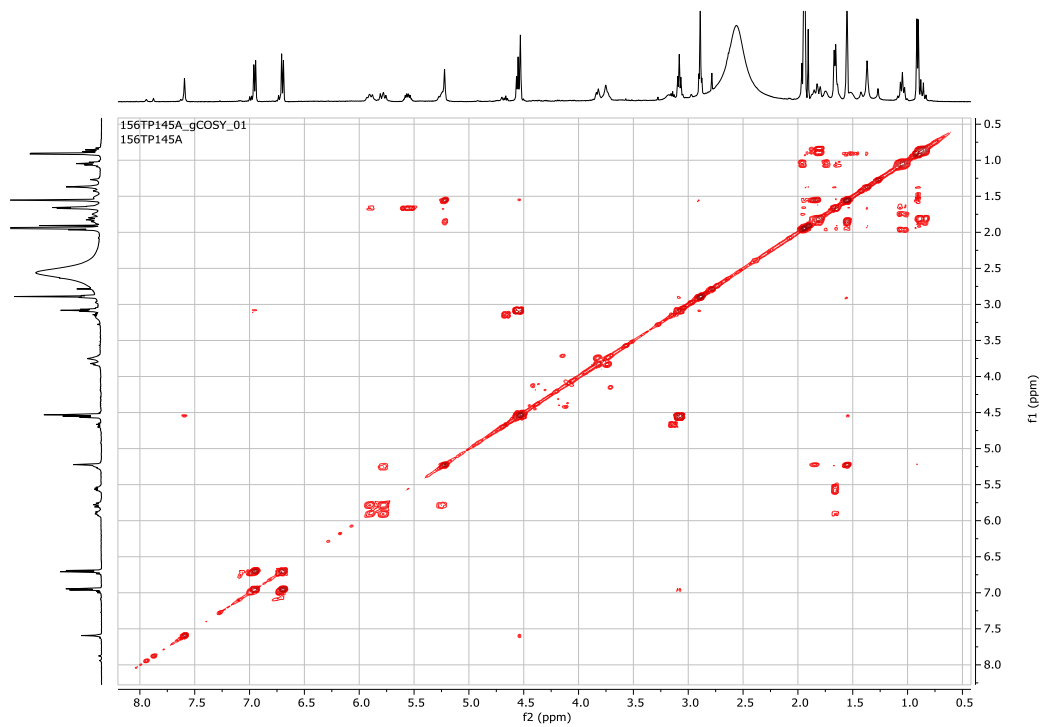


Figure C.112. COSY (500 MHz, $\text{CD}_3\text{CN}-d_3$) spectrum of **5f**

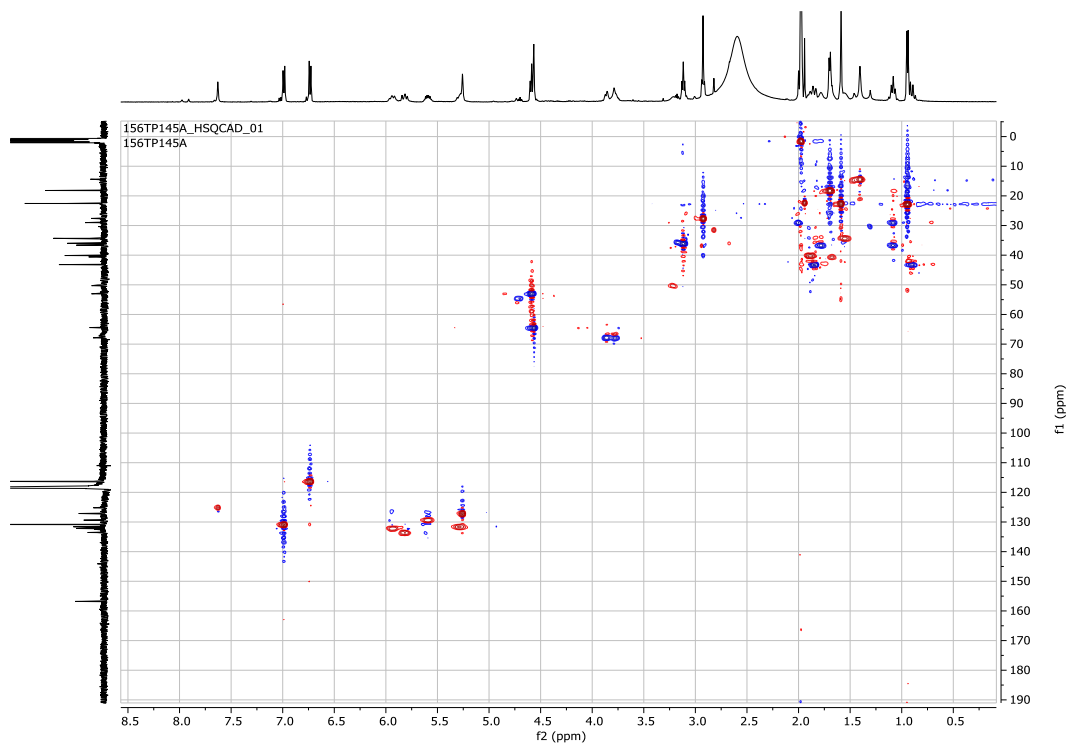


Figure C.113. HSQC (500 MHz, $\text{CD}_3\text{CN}-d_3$) spectrum of **5f**

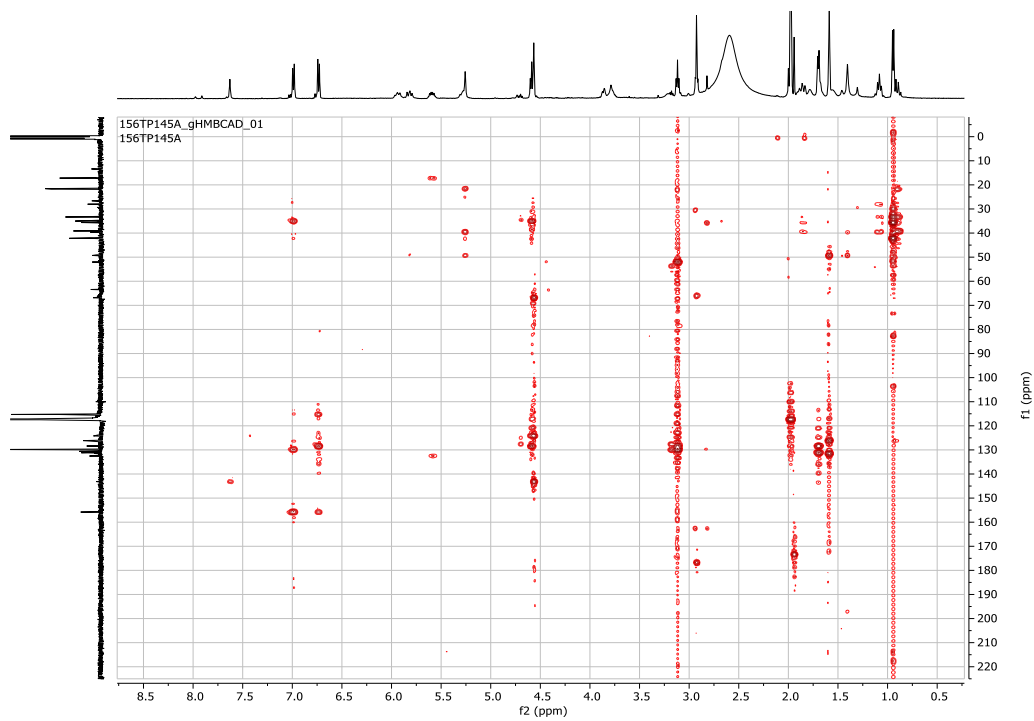


Figure C.114. HMBC (500 MHz, $\text{CD}_3\text{CN}-d_3$) spectrum of **5f**

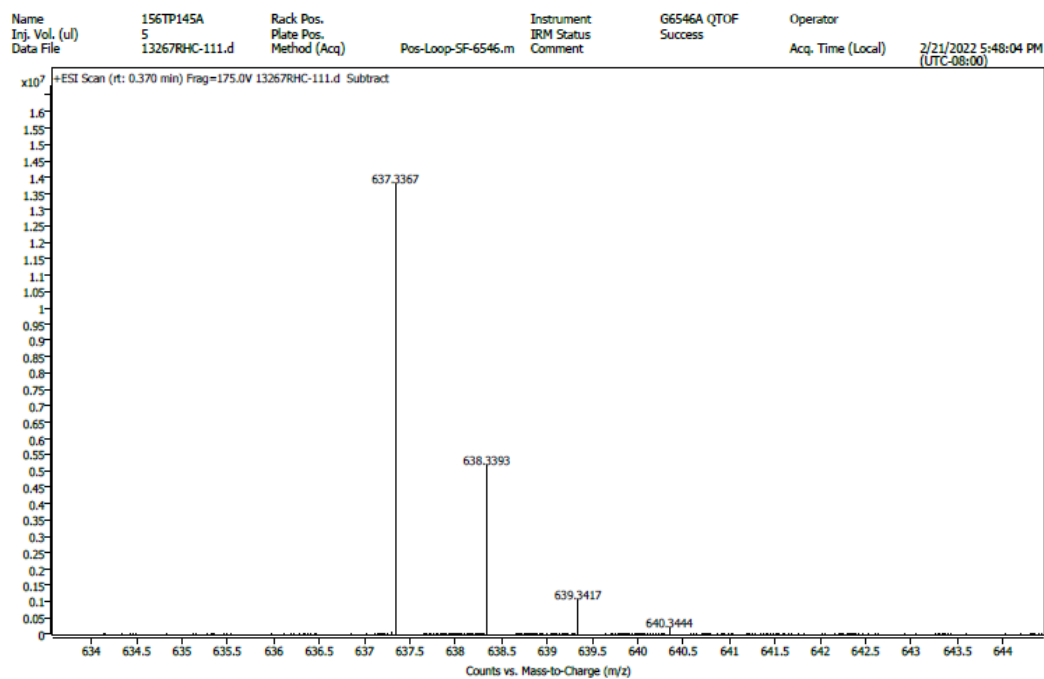


Figure C.115. HRESIMS spectrum of compound **5f**

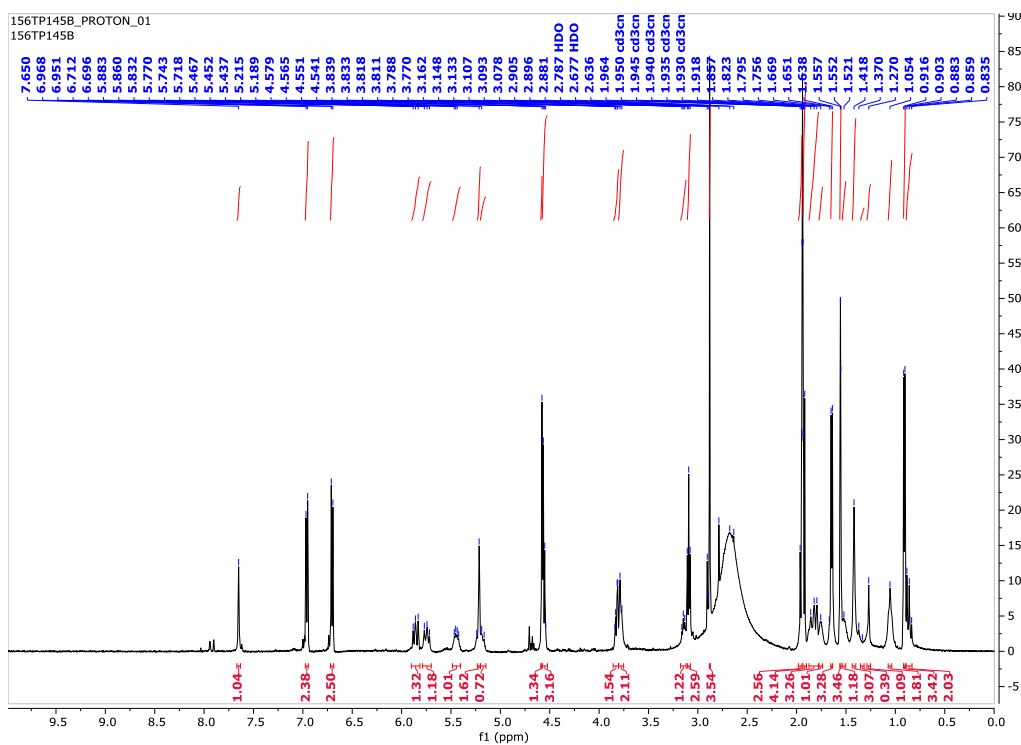


Figure C.116. ^1H NMR (500 MHz, $\text{CD}_3\text{CN}-d_3$) of **5f**

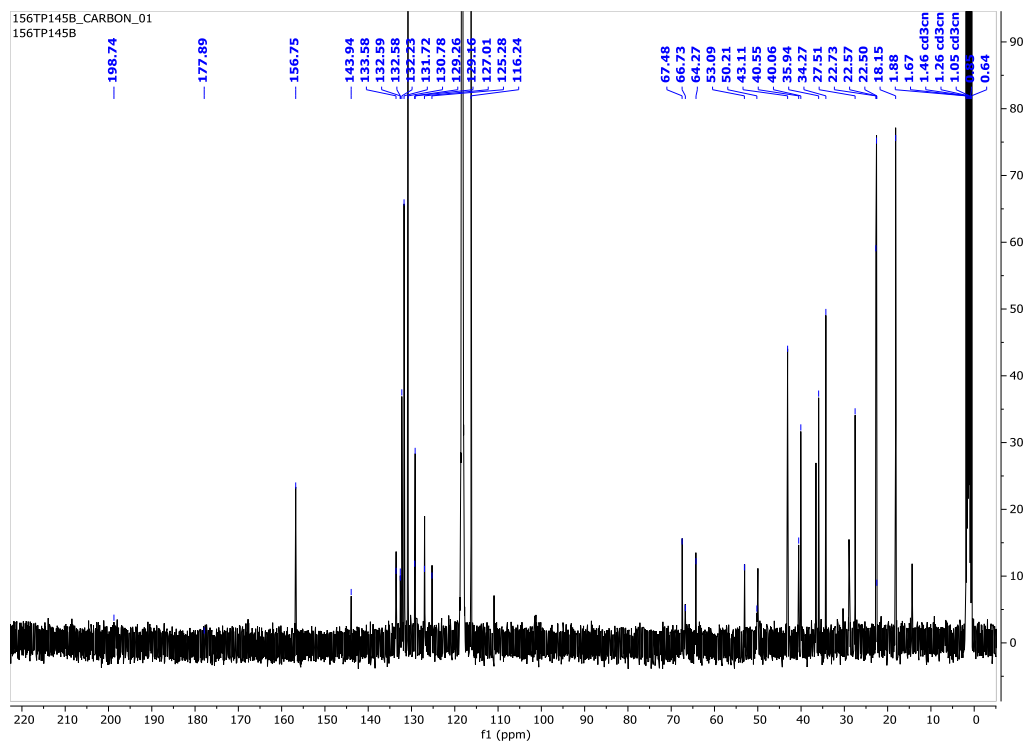


Figure C.117. ^{13}C NMR (400 MHz, $\text{CD}_3\text{CN}-d_3$) of **5f'**

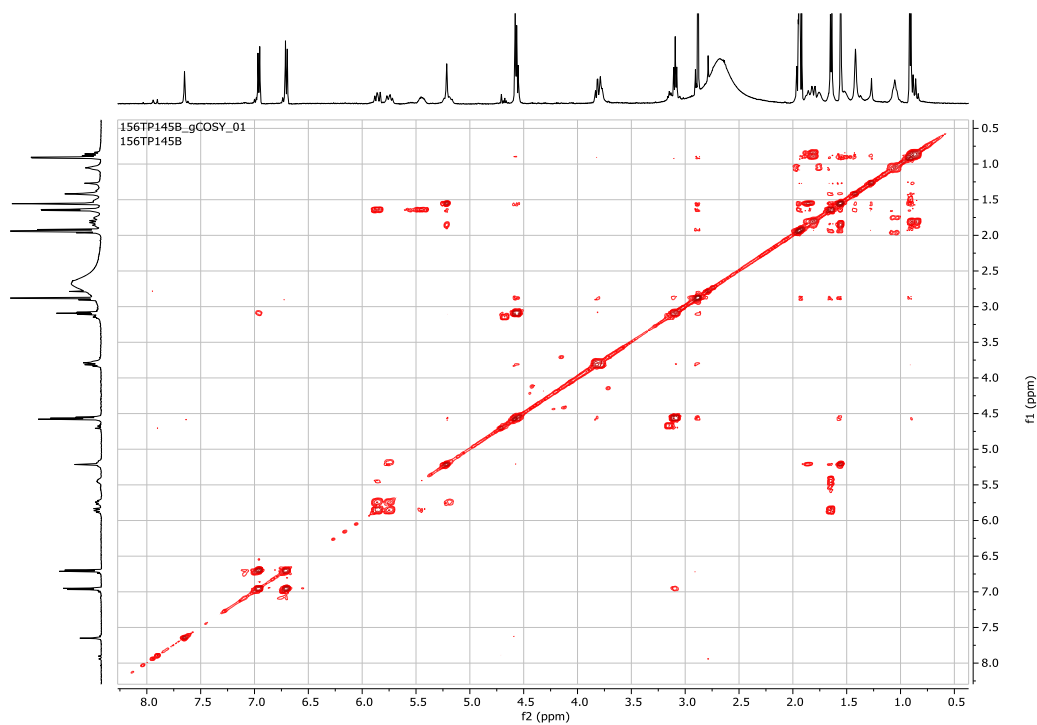


Figure C.118. COSY (500 MHz, $\text{CD}_3\text{CN}-d_3$) spectrum of **5f'**

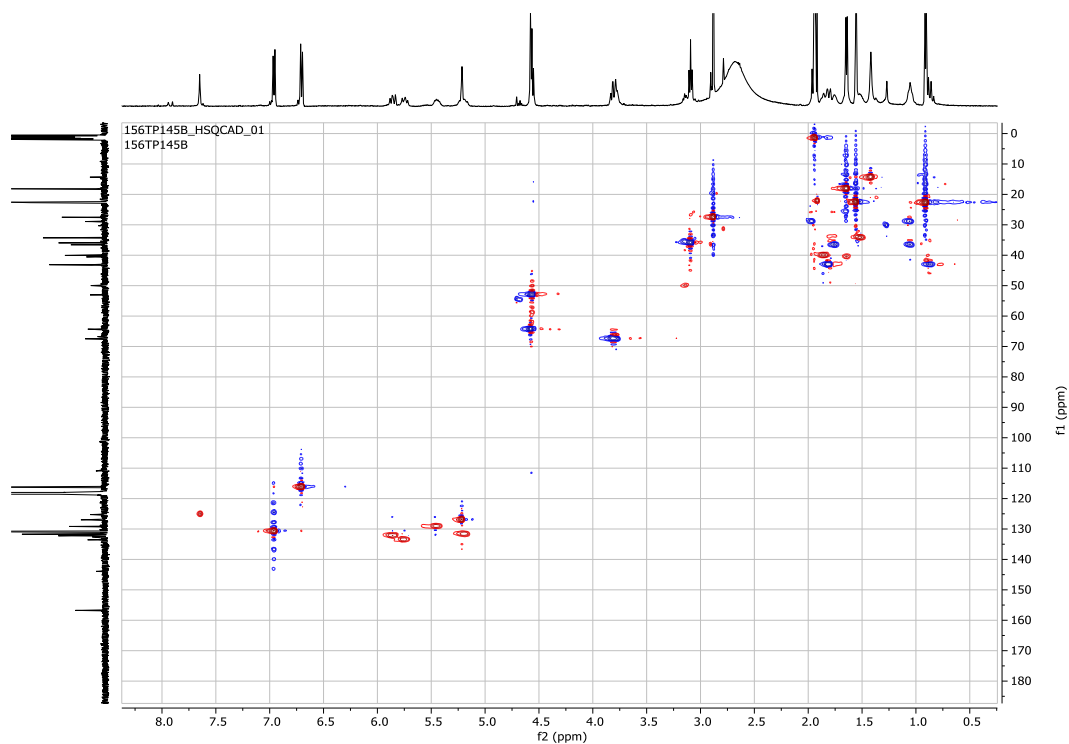


Figure C.119. HSQC (500 MHz, CD₃CN-*d*₃) spectrum of 5f'

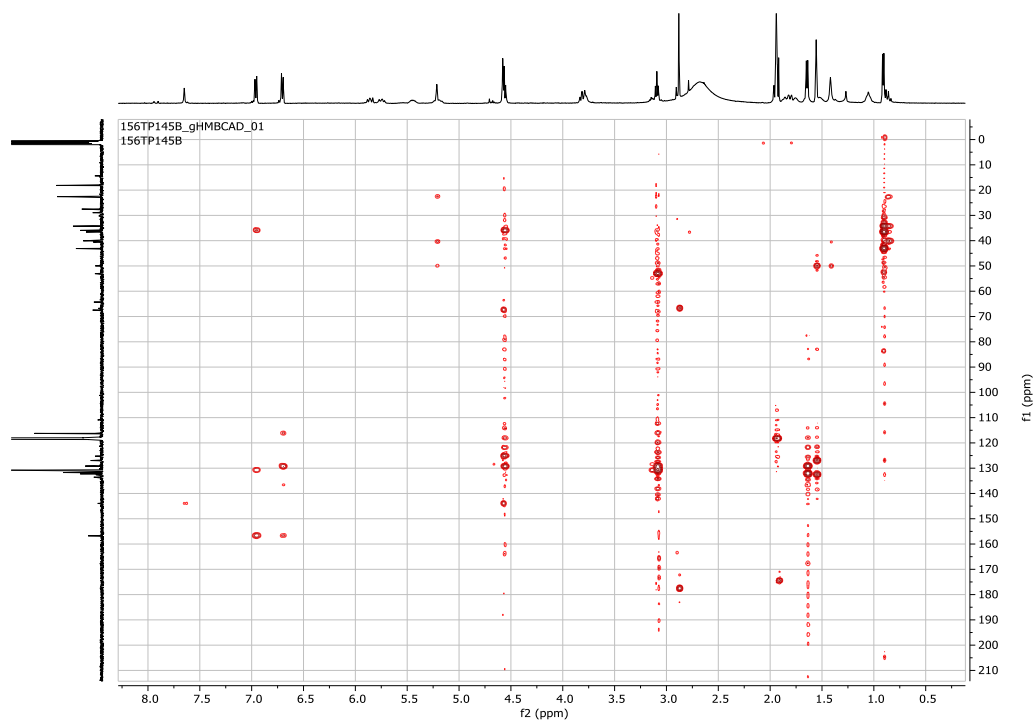


Figure C.120. HMBC (500 MHz, CD₃CN-*d*₃) spectrum of 5f'

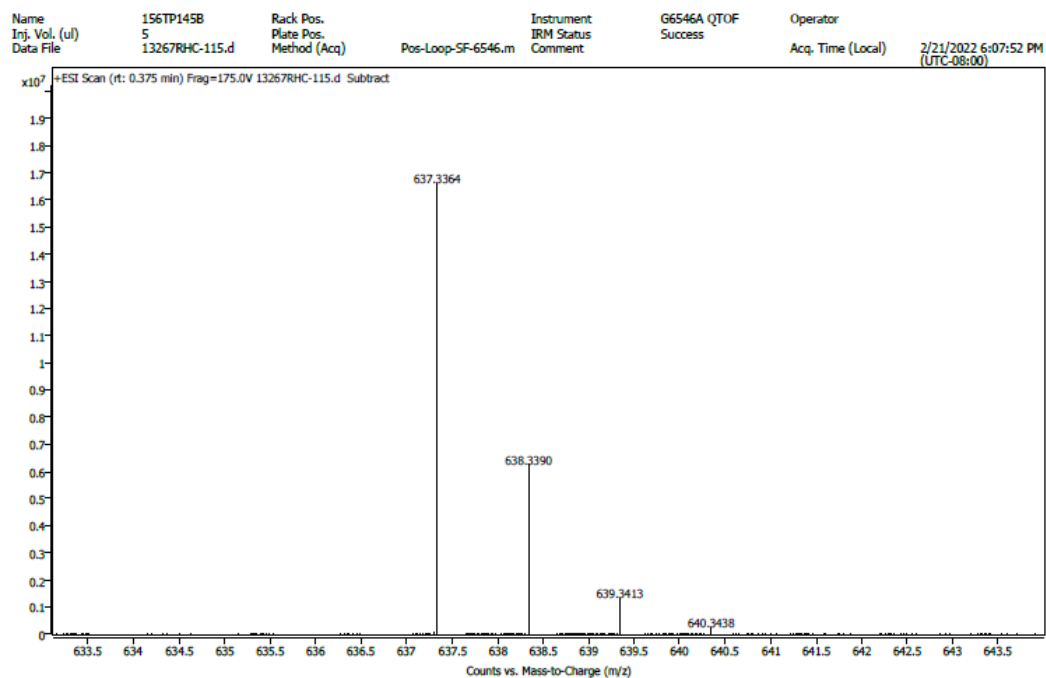


Figure C.121. HRESIMS spectrum of compound **5f'**

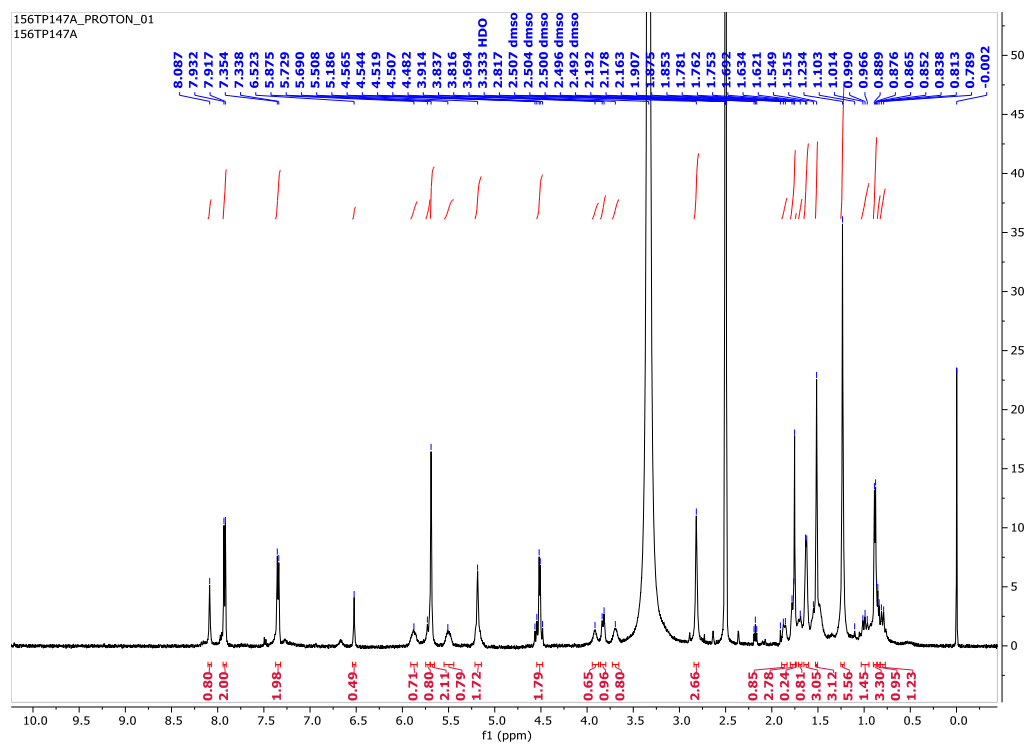


Figure C.122. ¹H NMR (500 MHz, DMSO) of **5g**

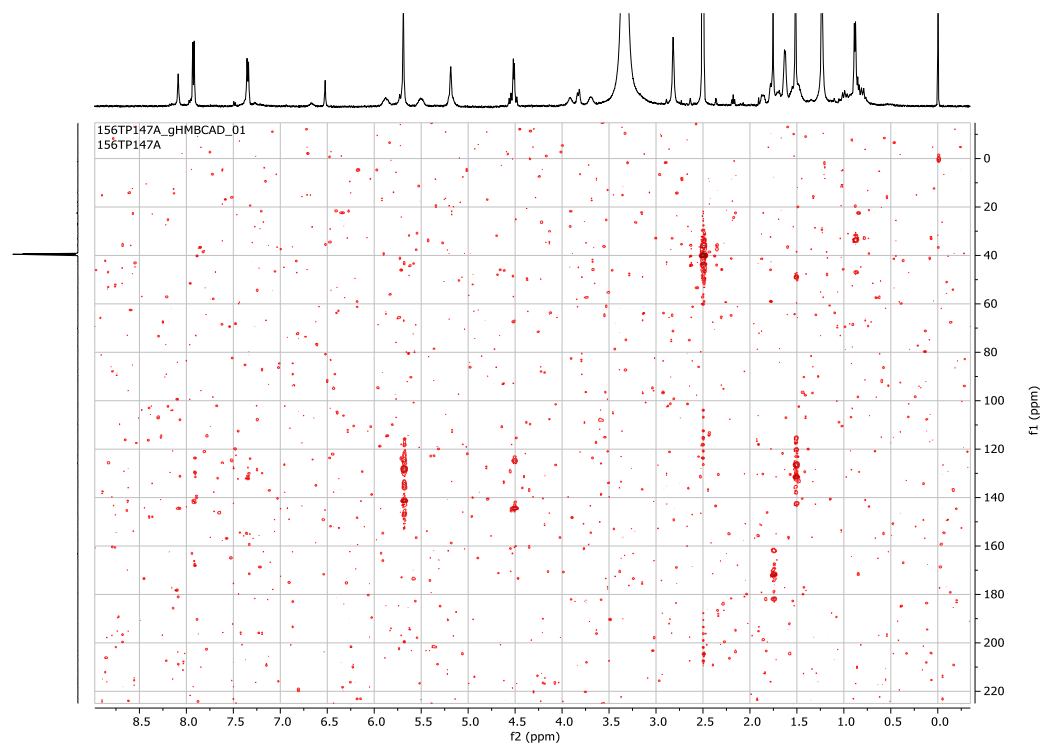


Figure C.123. HMBC (500 MHz, DMSO) spectrum of **5g**

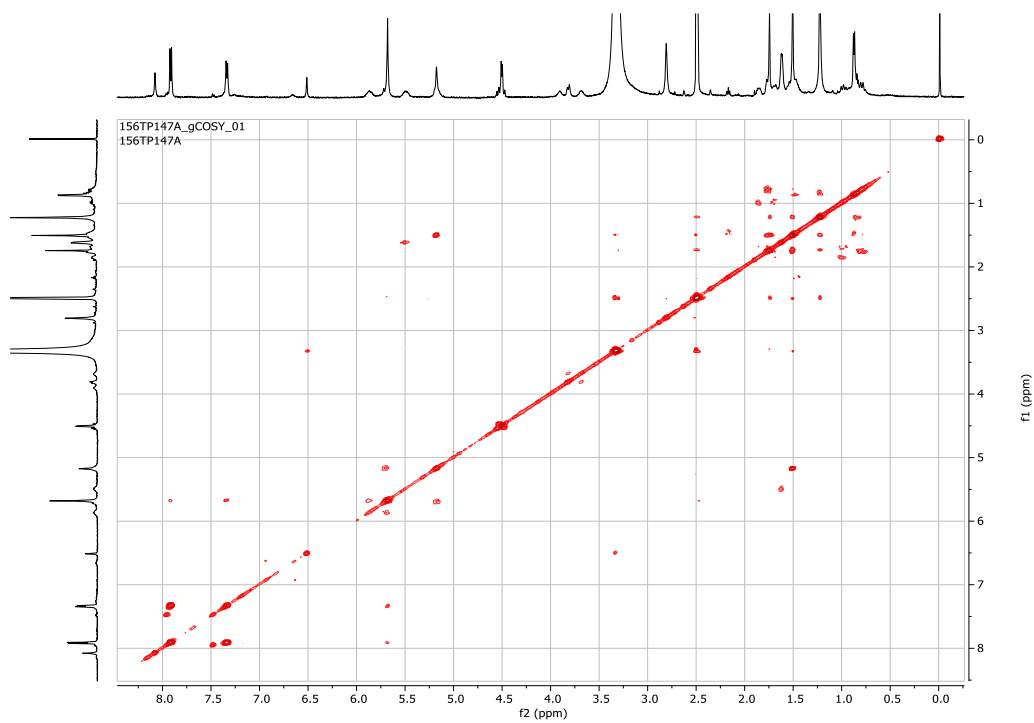


Figure C.124. COSY (500 MHz, DMSO) spectrum of **5g**

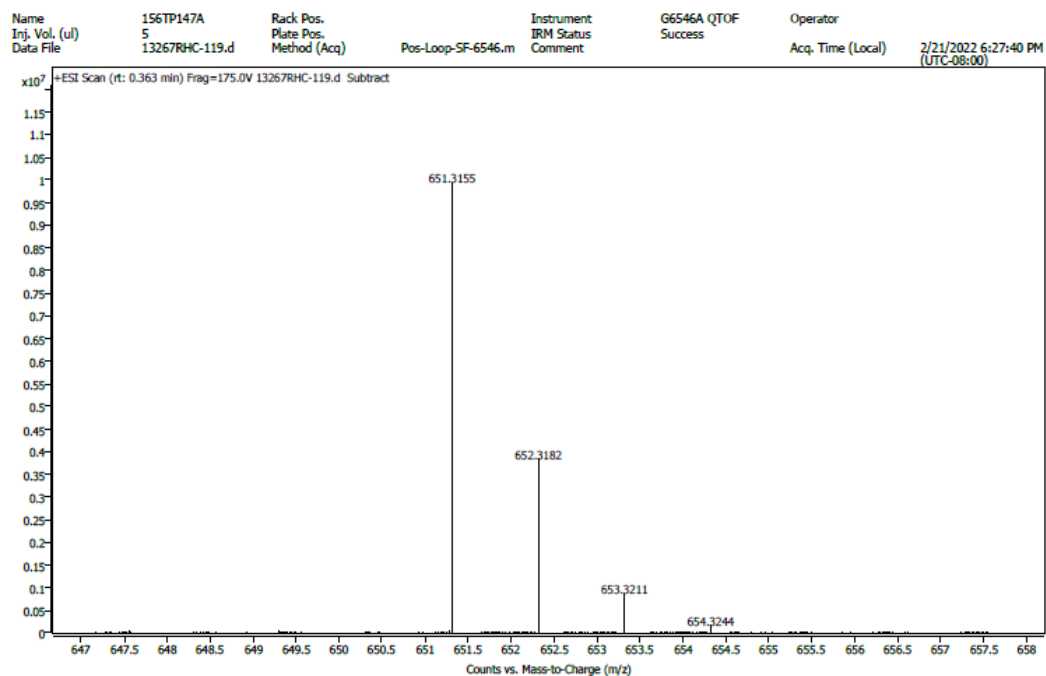


Figure C.125. HRESIMS spectrum of compound **5g**

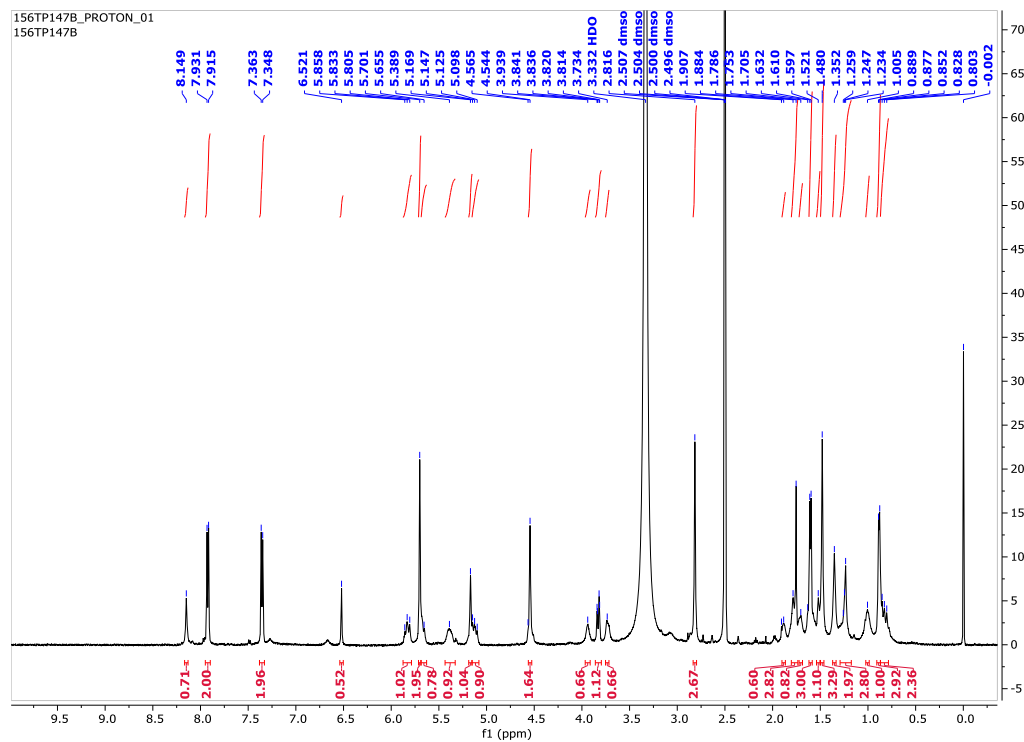


Figure C.126. ¹H NMR (500 MHz, DMSO) of **5g'**

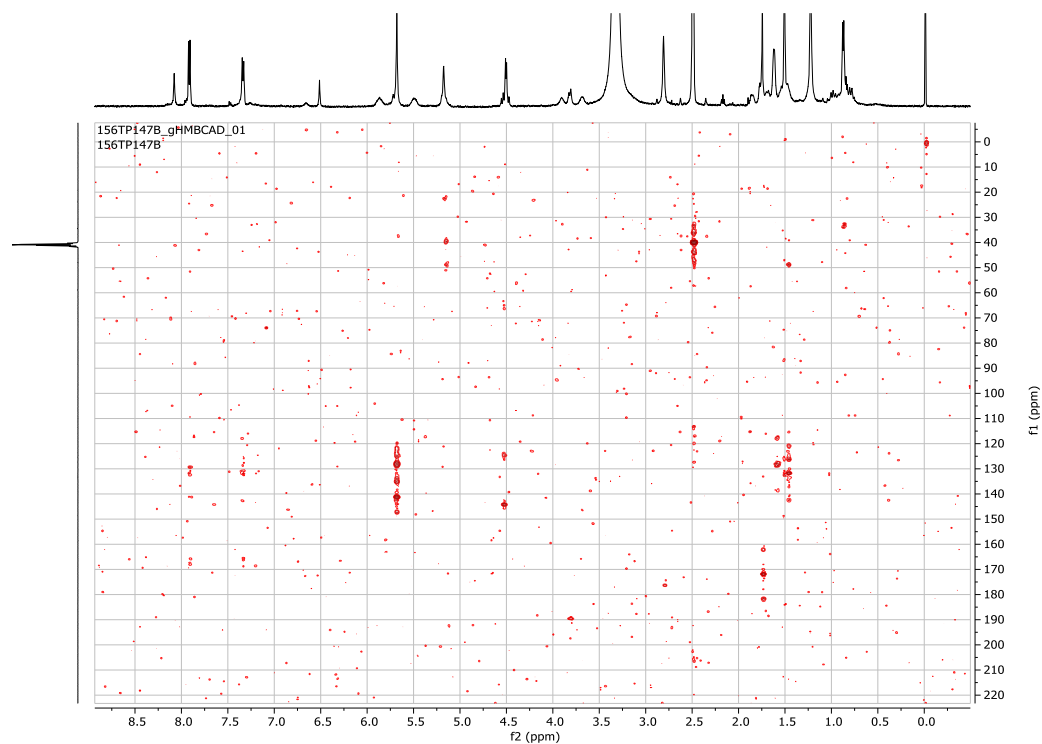


Figure C.127. HMBC (500 MHz, DMSO) spectrum of **5g'**

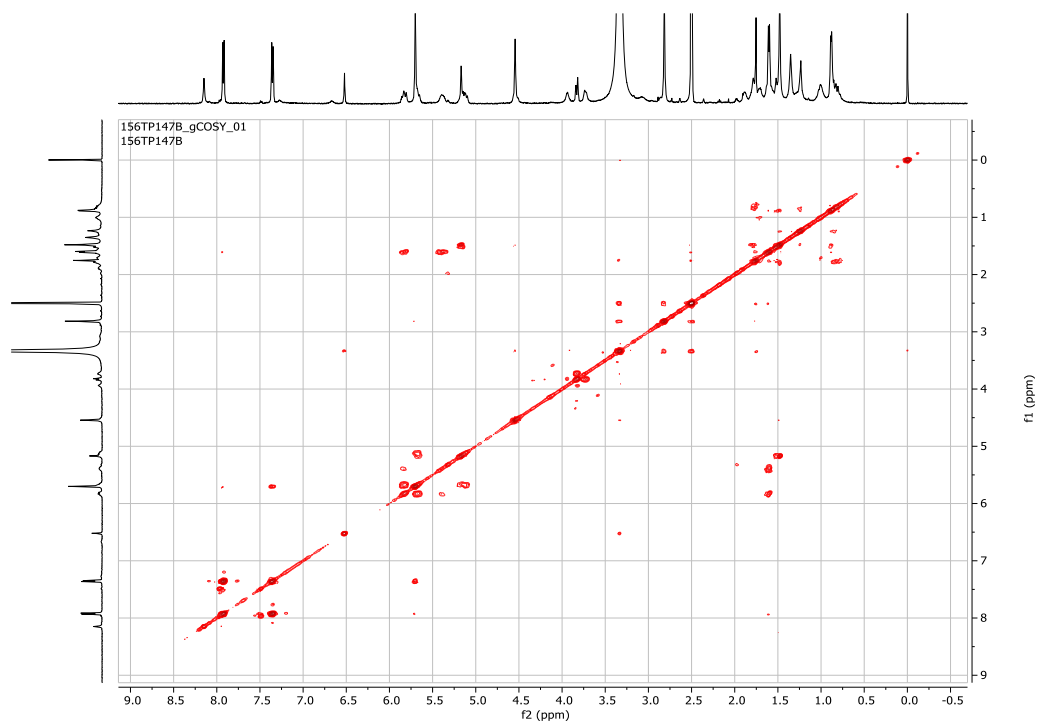


Figure C.128. COSY (500 MHz, DMSO) spectrum of **5g'**

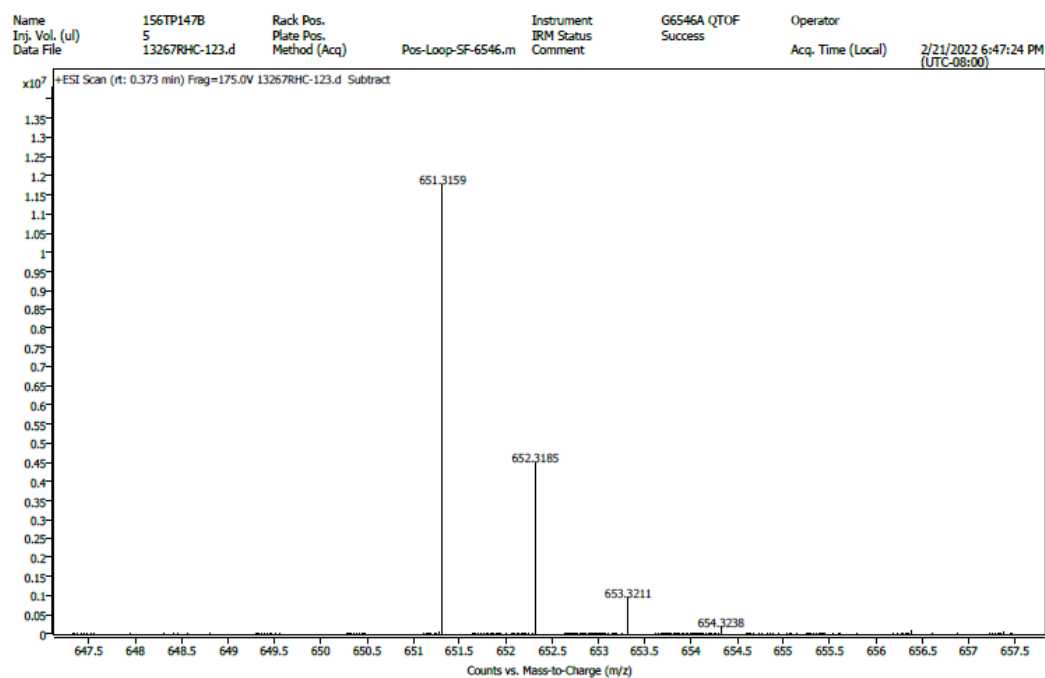


Figure C.129. HRESIMS spectrum of compound **5g'**

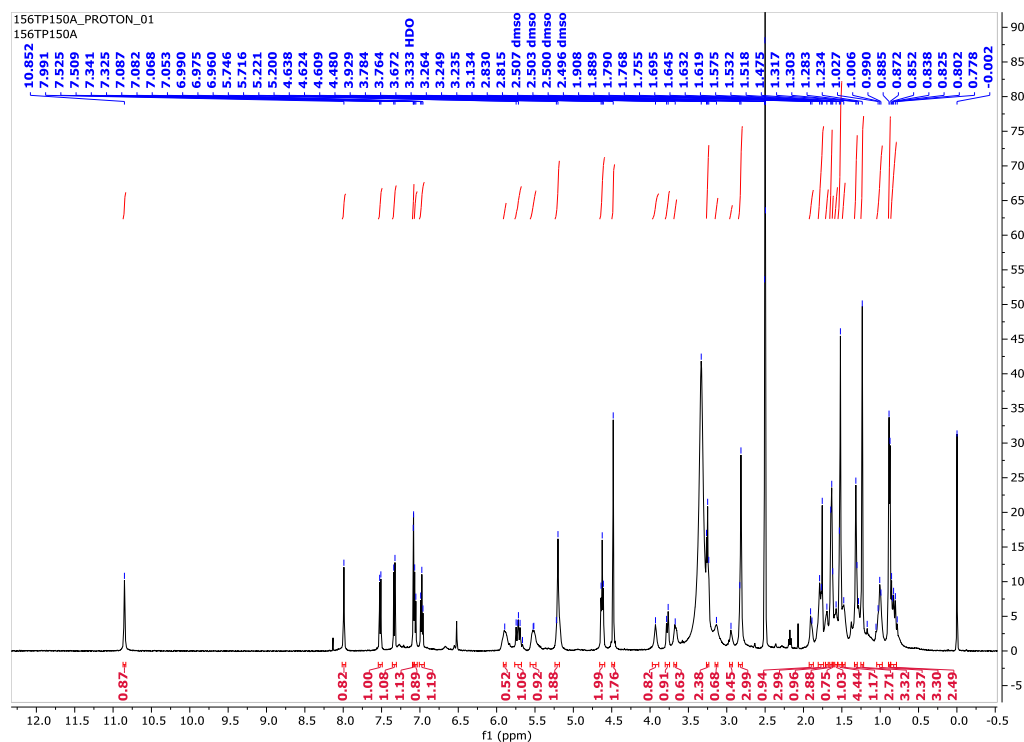


Figure C.130. ^1H NMR (500 MHz, DMSO) of **5h**

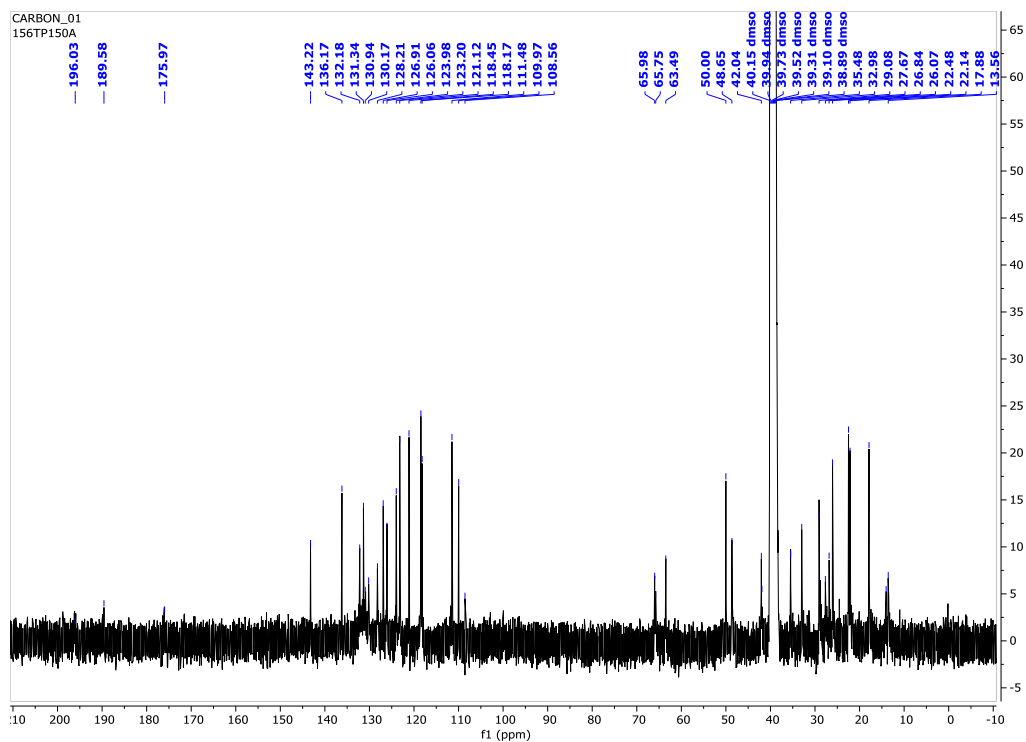


Figure C.131. ^{13}C NMR (400 MHz, DMSO) of **5h**

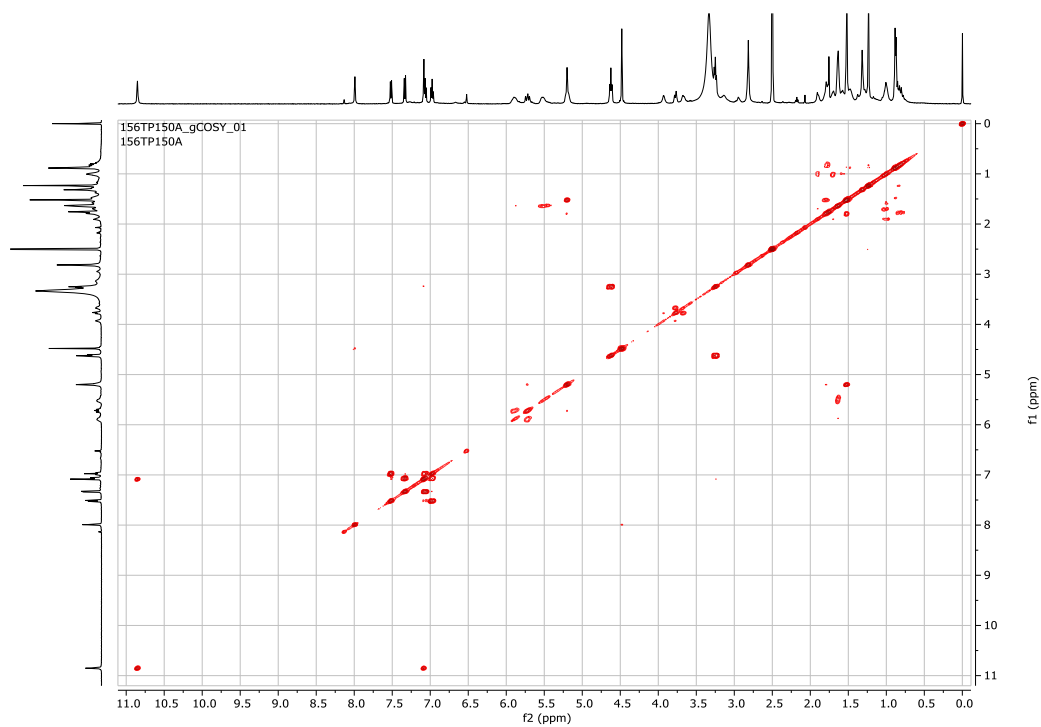


Figure C.132. COSY (500 MHz, DMSO) spectrum of **5h**

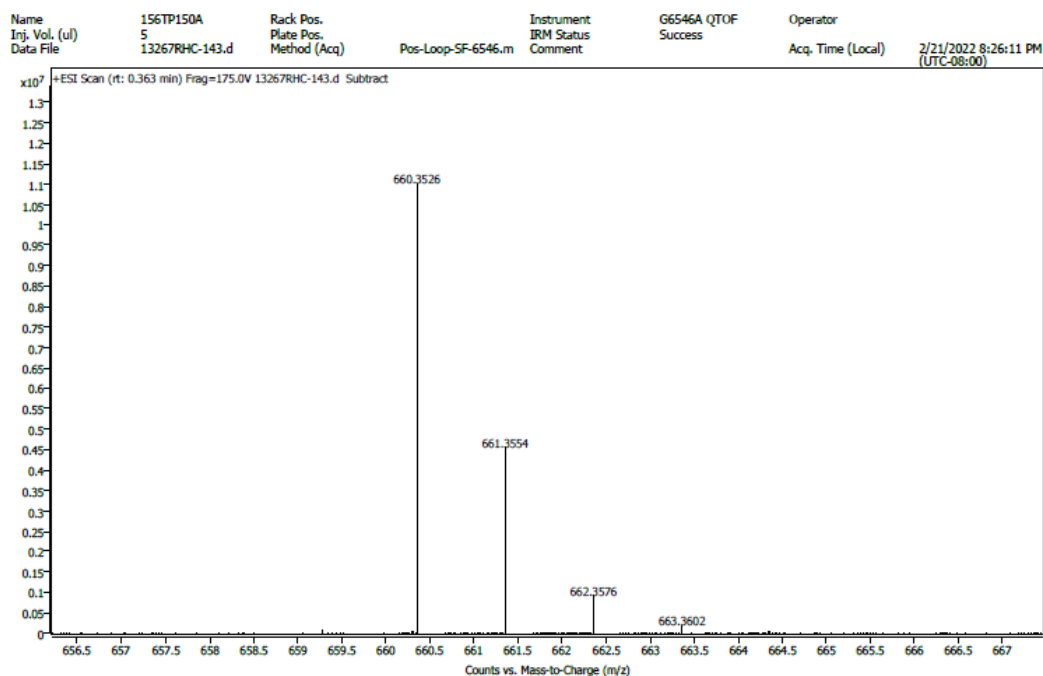


Figure C.133. HRESIMS spectrum of compound **5h**

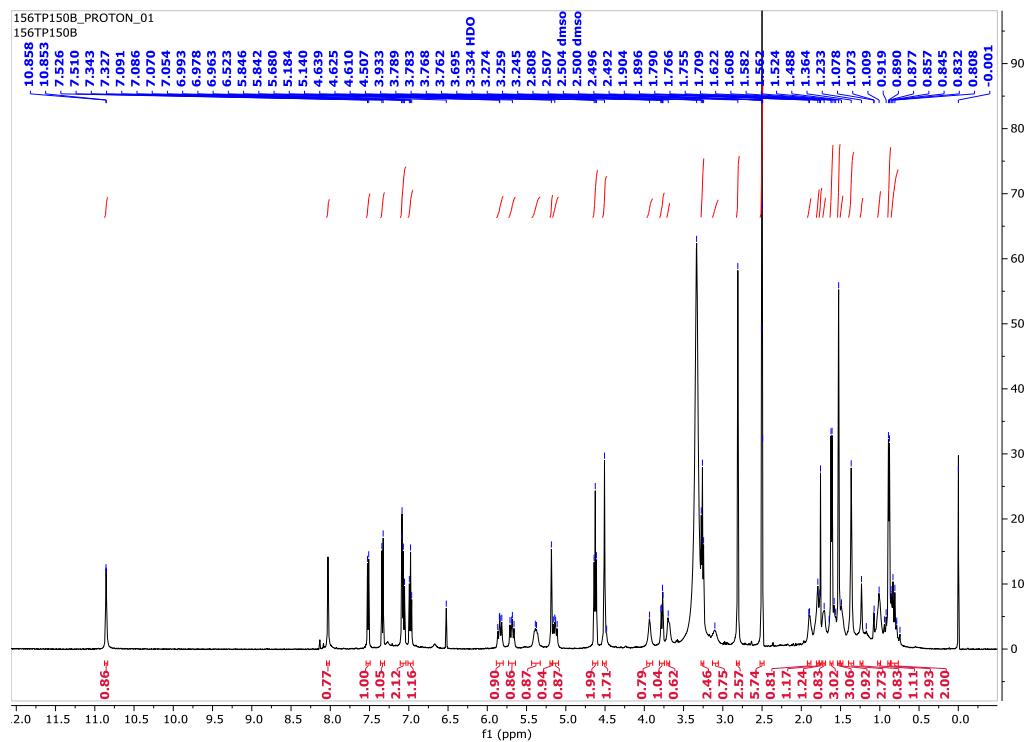


Figure C.134. ^1H NMR (500 MHz, DMSO) of **5h'**

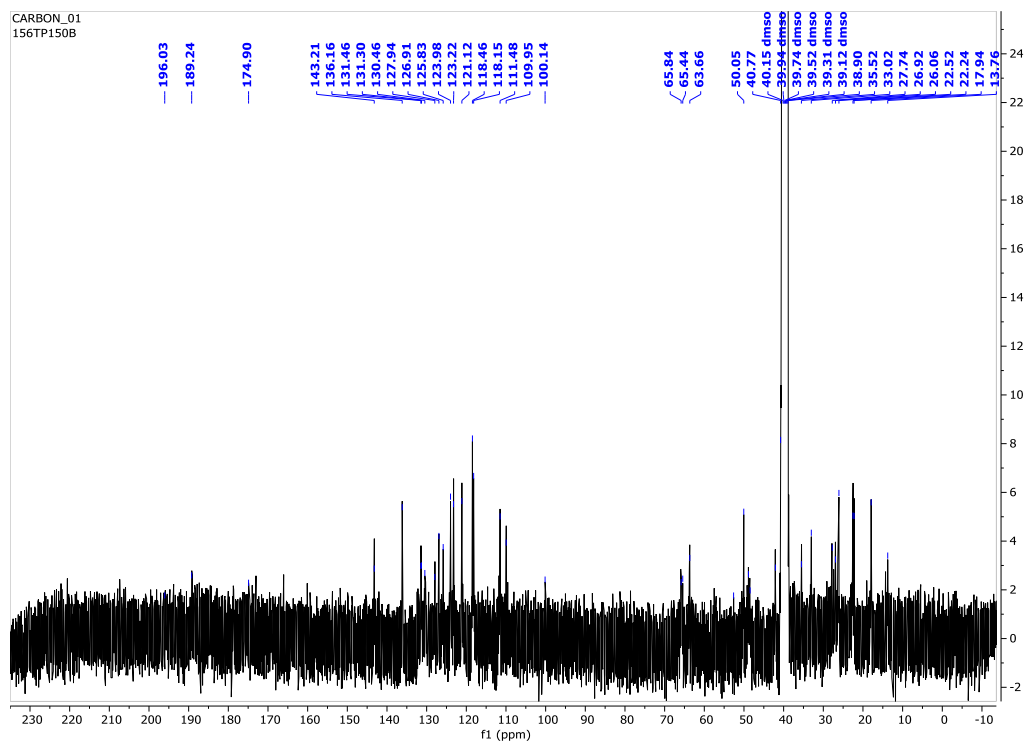


Figure C.135. ^{13}C NMR (400 MHz, DMSO) of **5h'**

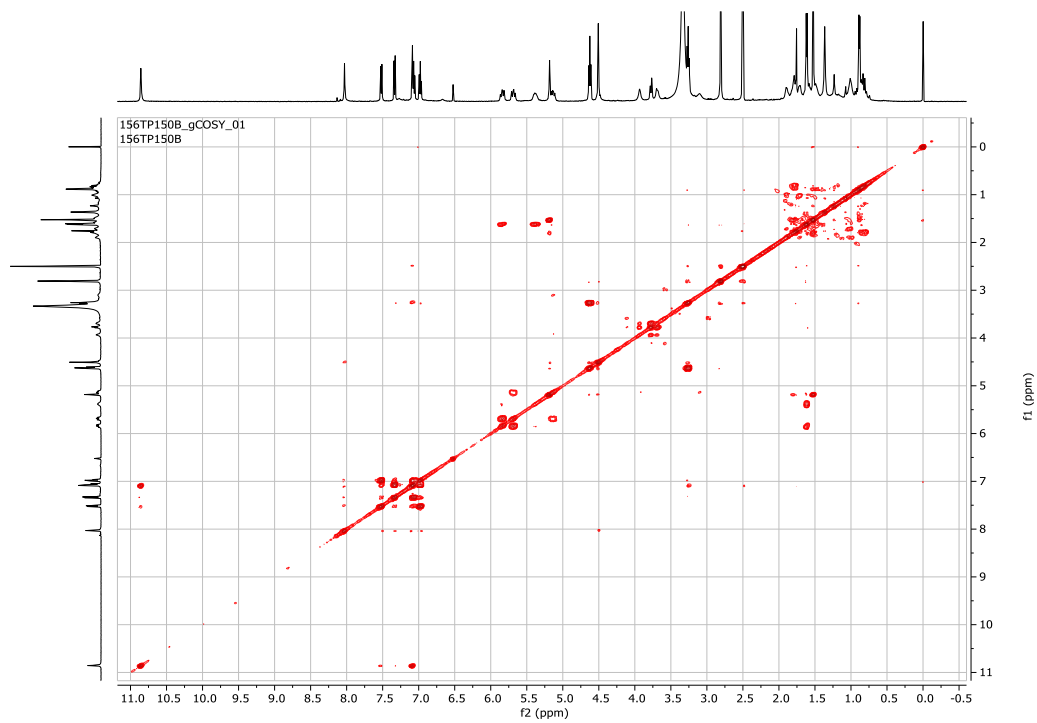


Figure C.136. COSY (500 MHz, DMSO) spectrum of **5h'**

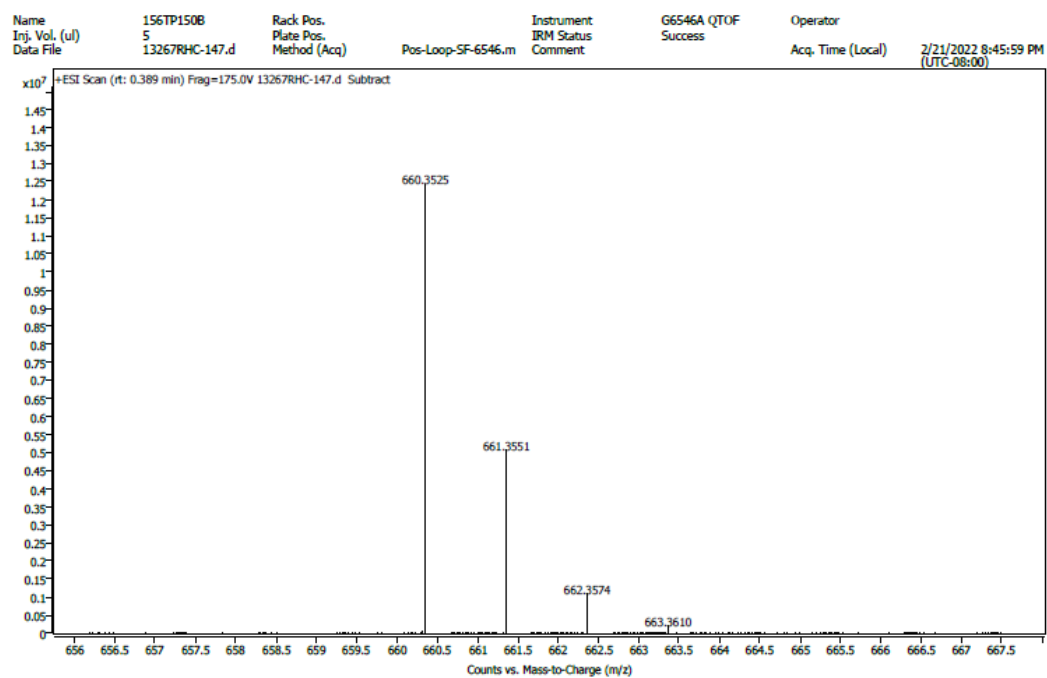


Figure C.137. HRESIMS spectrum of compound **5h'**

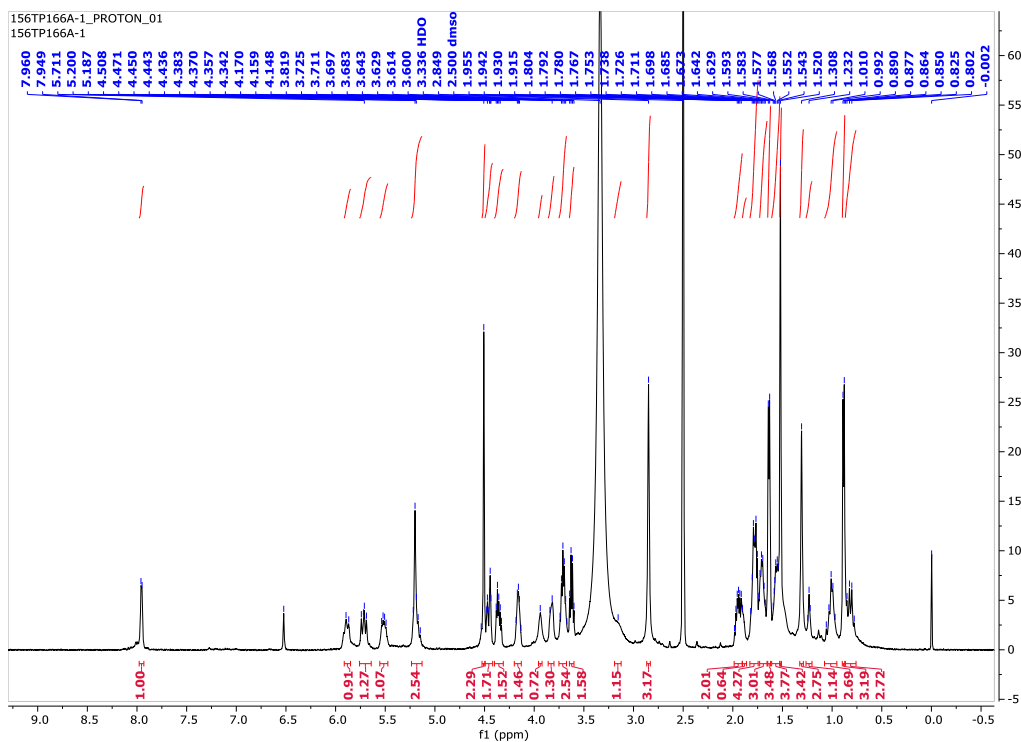


Figure C.138. ^1H NMR (500 MHz, DMSO) of **5i**

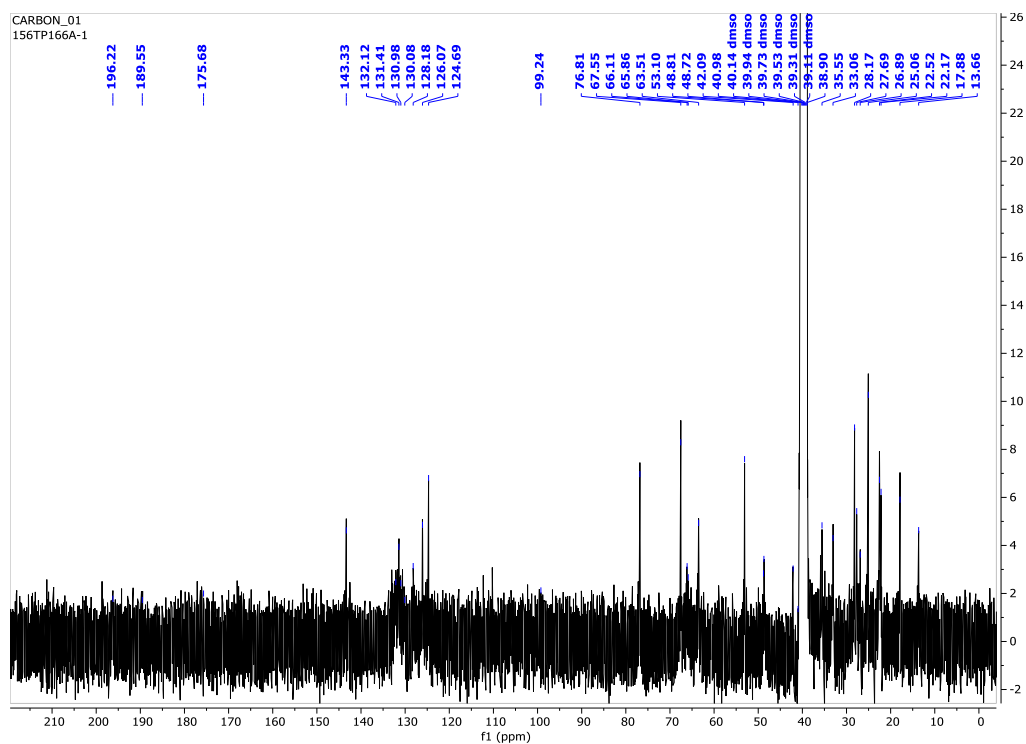


Figure C.139. ^{13}C NMR (500 MHz, DMSO) of **5i**

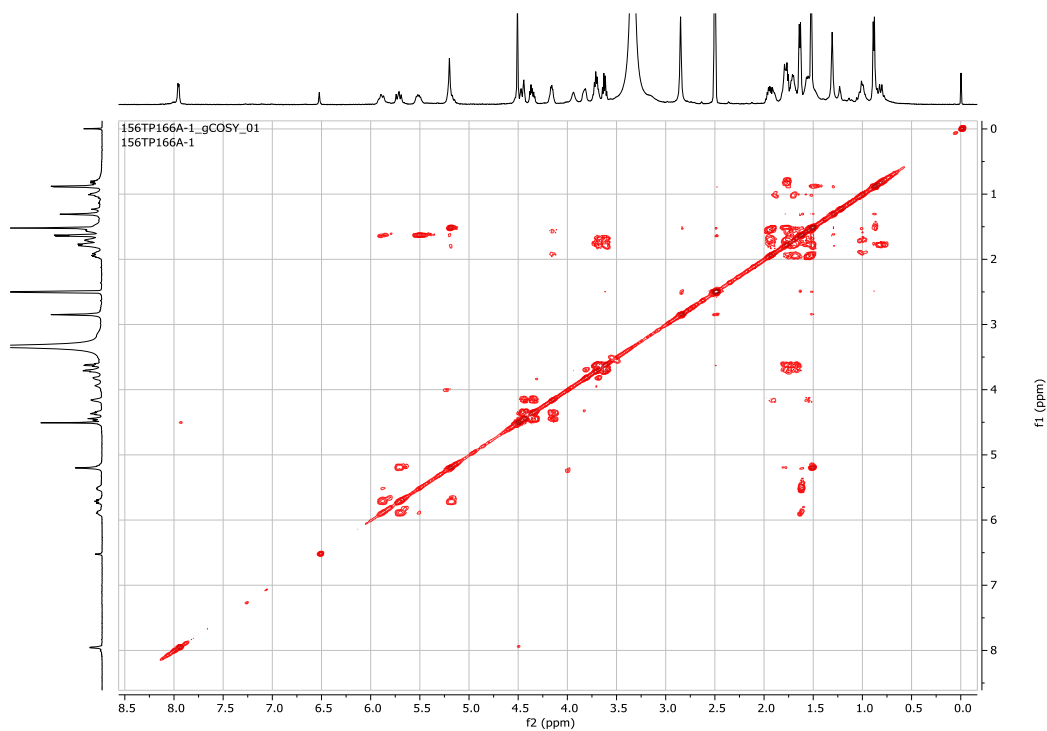


Figure C.140. COSY (500 MHz, DMSO) spectrum of **5i**

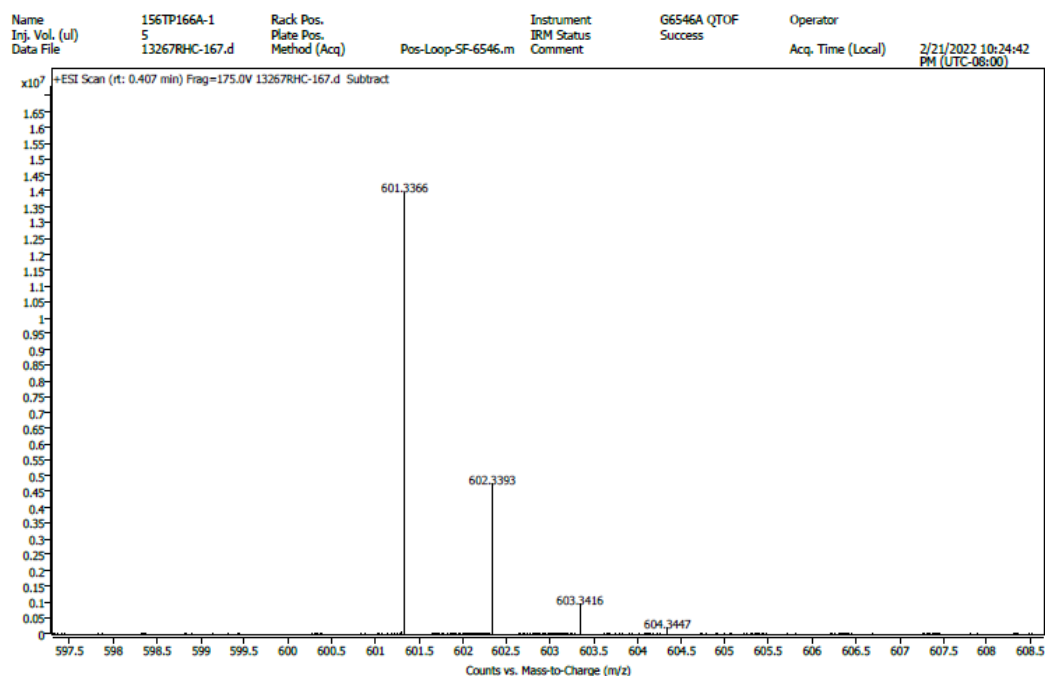


Figure C.141. HRESIMS spectrum of compound **5i**

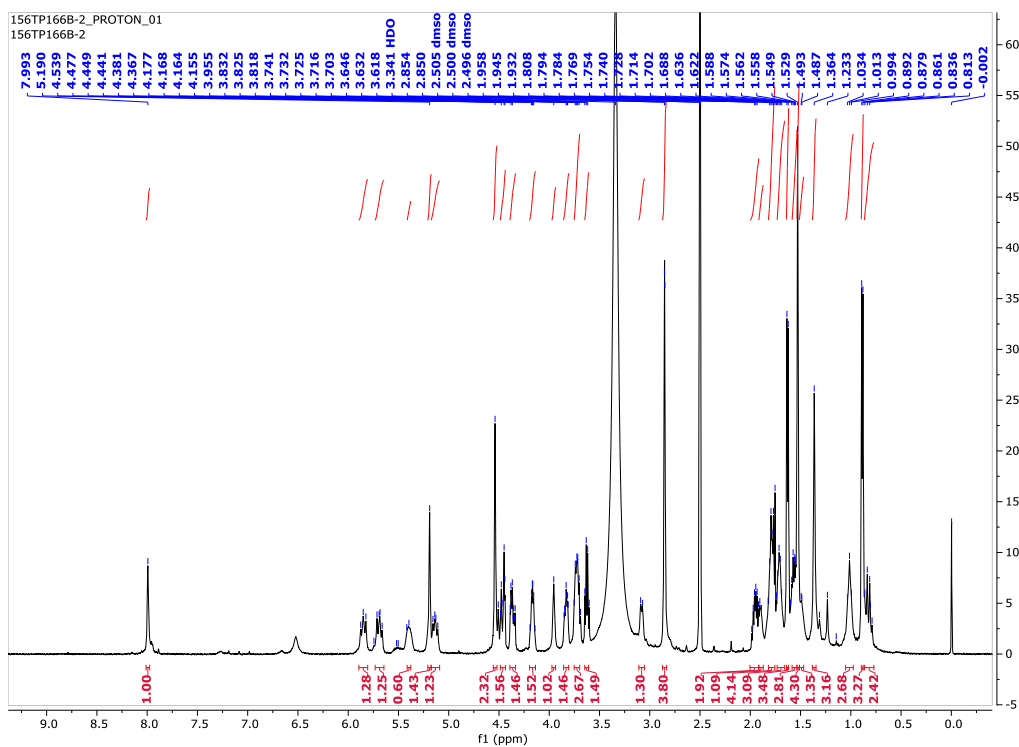


Figure C.142. ^1H NMR (500 MHz, DMSO) of **5i**

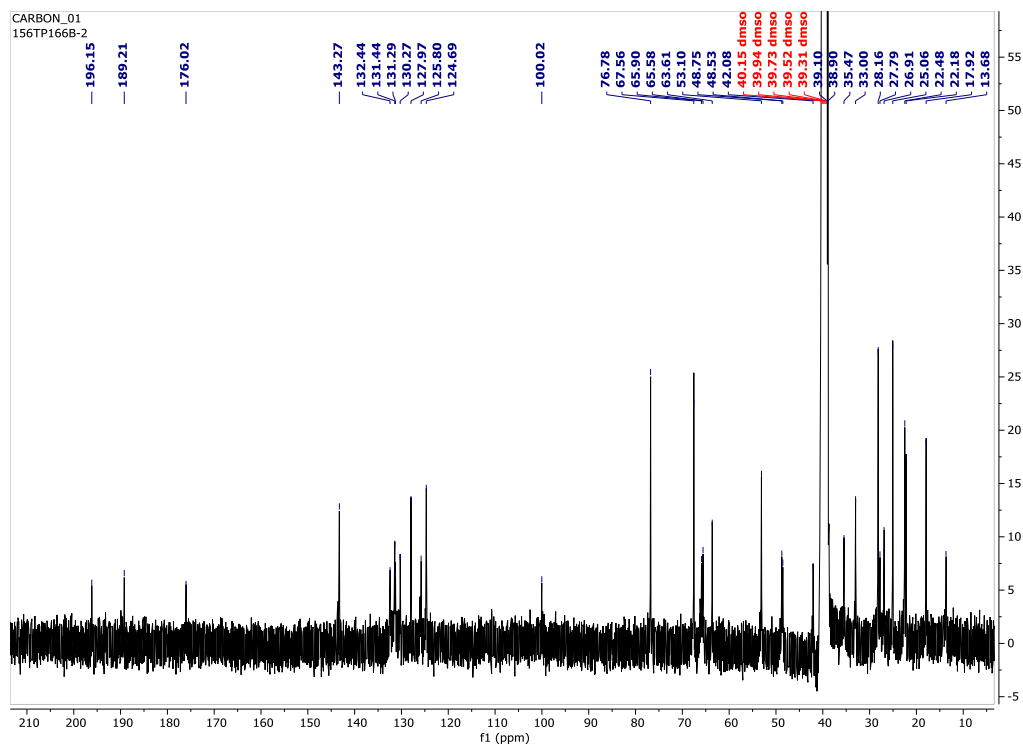


Figure C.143. ^{13}C NMR (400 MHz, DMSO) of **5i'**

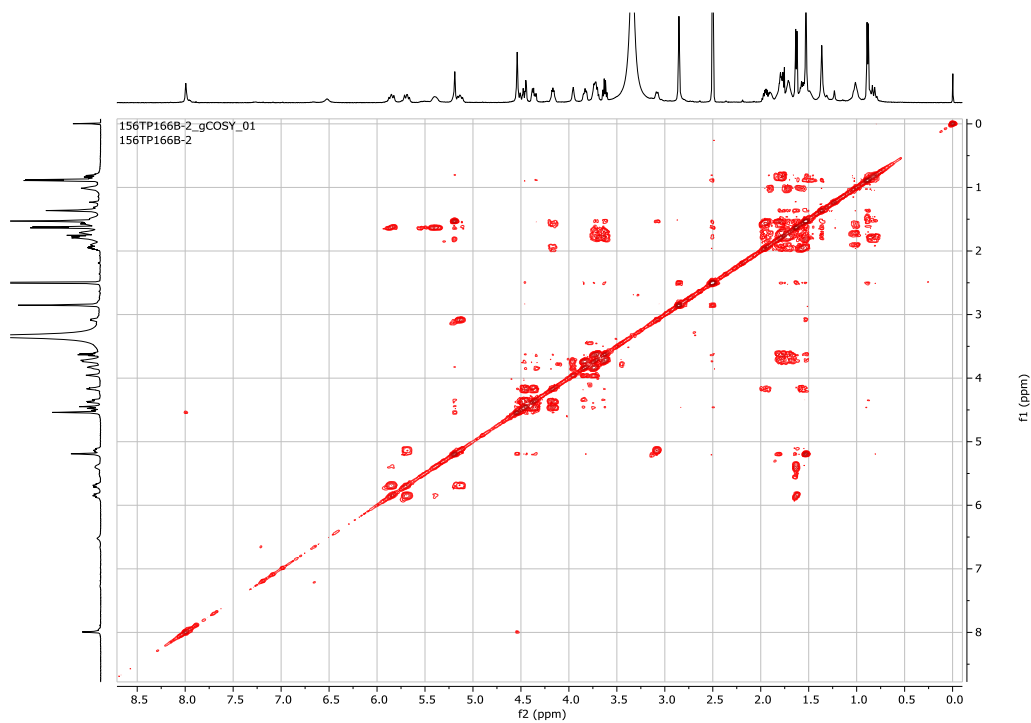


Figure C.144. COSY (500 MHz, DMSO) spectrum of **5i'**

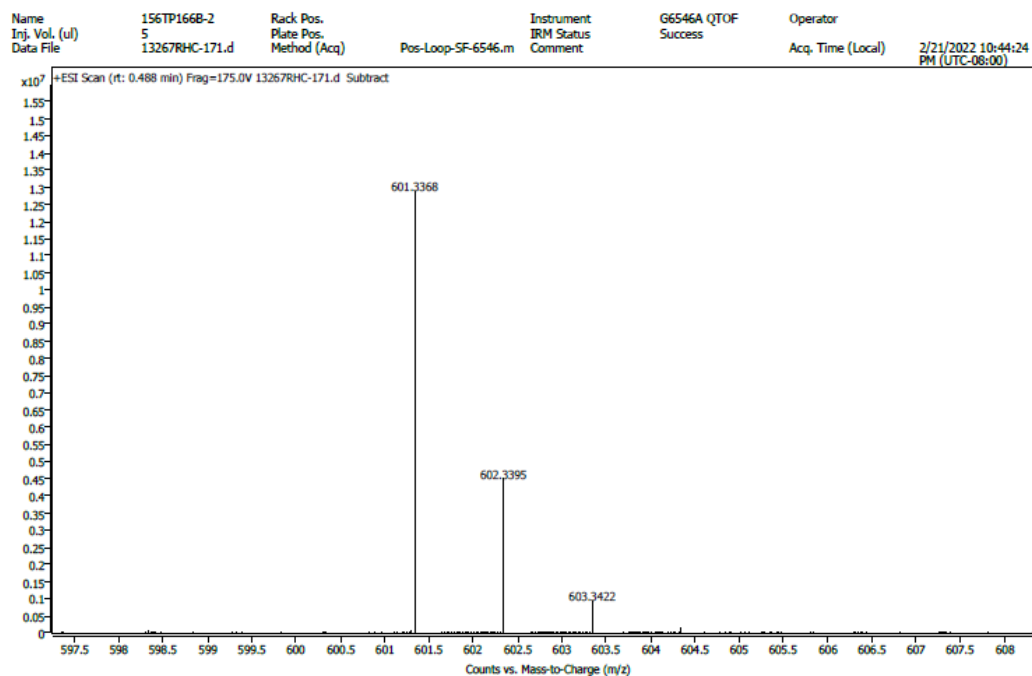


Figure C.145. HRESIMS spectrum of compound **5i'**

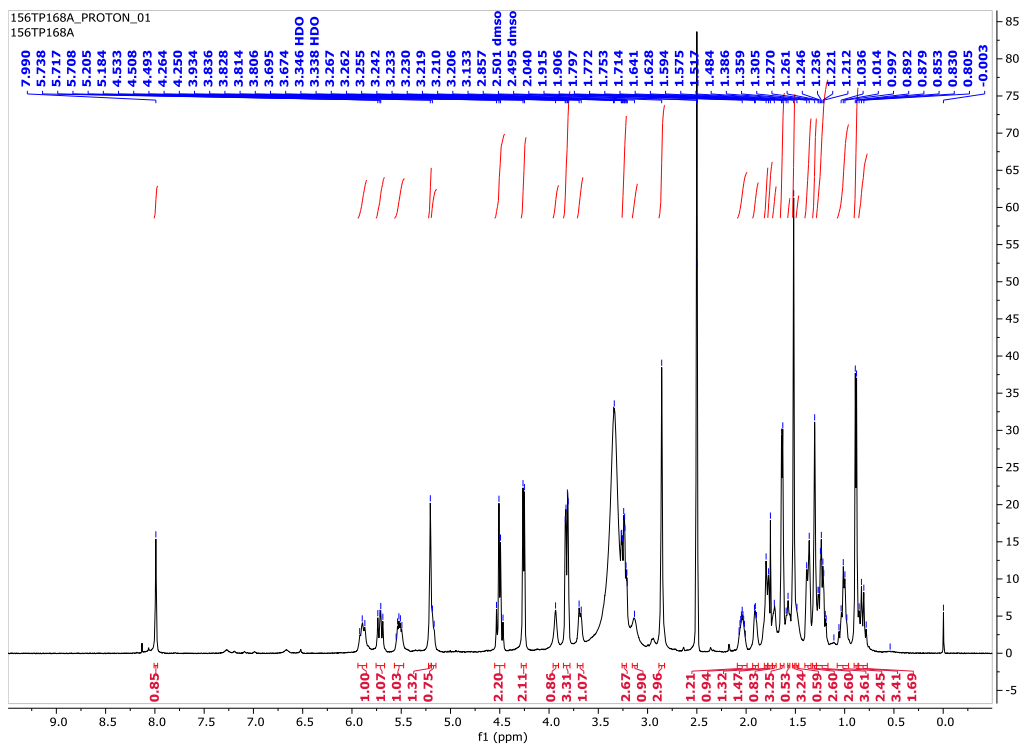


Figure C.146. ^1H NMR (500 MHz, DMSO) of **5j**

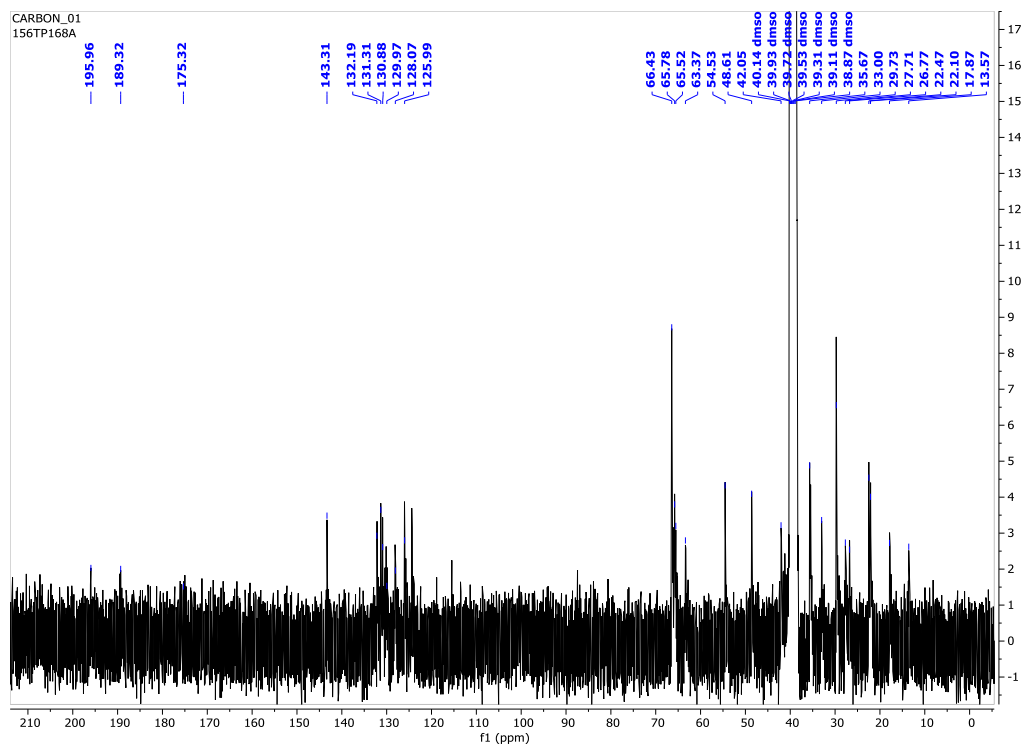


Figure C.147. ^{13}C NMR (400 MHz, DMSO) of **5j**

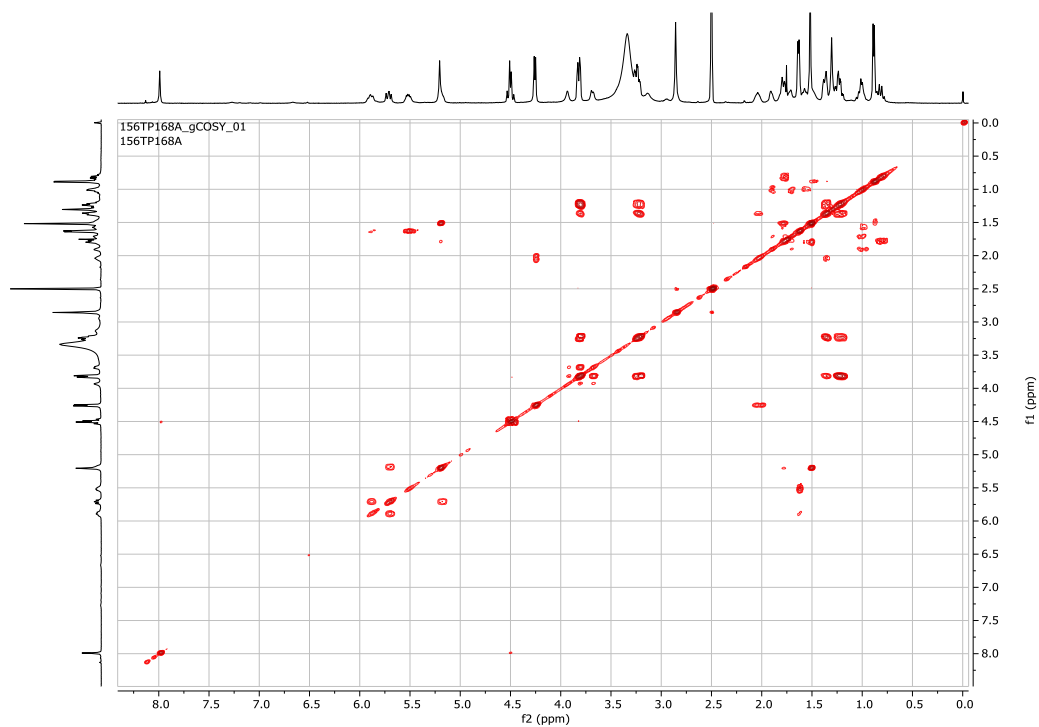


Figure C.148. COSY (500 MHz, DMSO) spectrum of **5j**

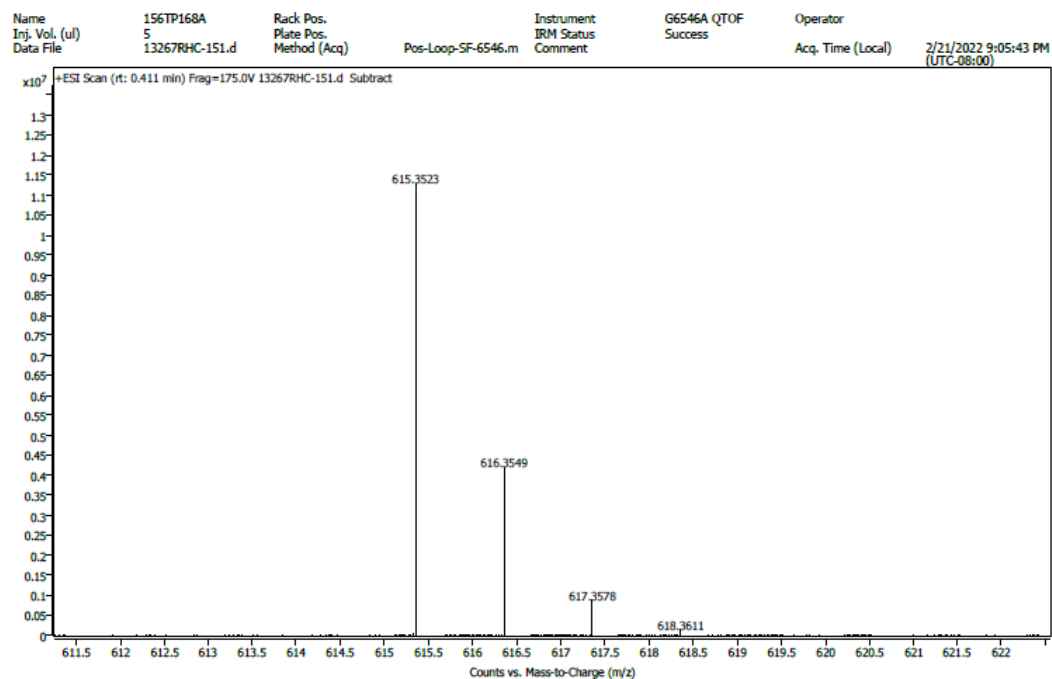


Figure C.149. HRESIMS spectrum of compound **5j**

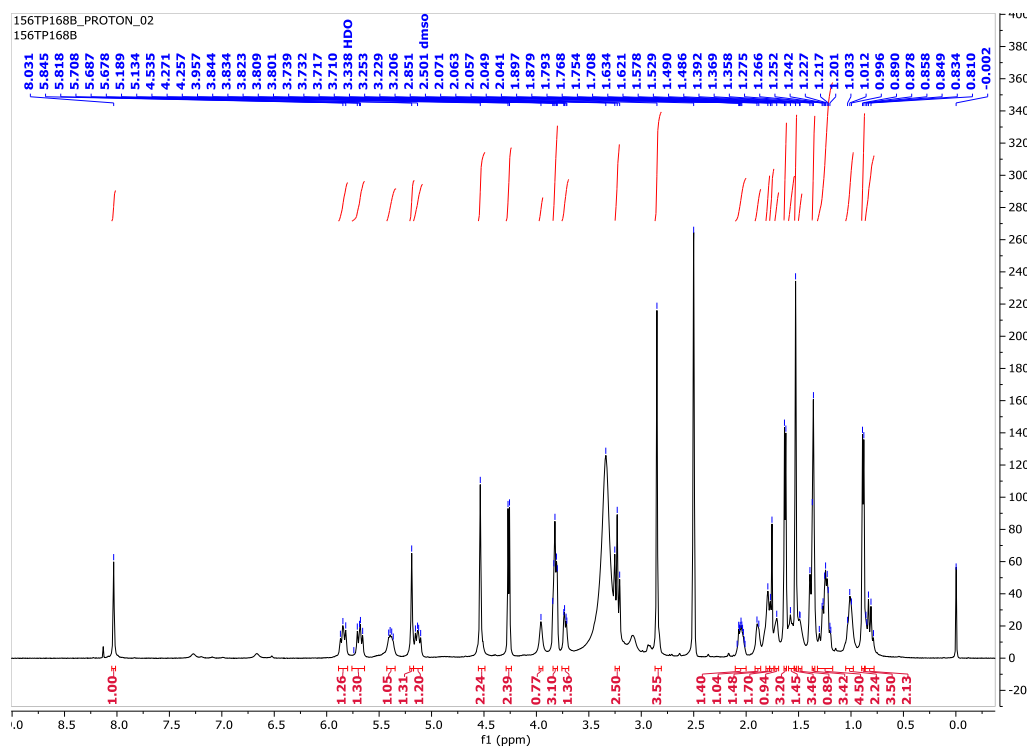


Figure C.150. ^1H NMR (500 MHz, DMSO) of **5j**

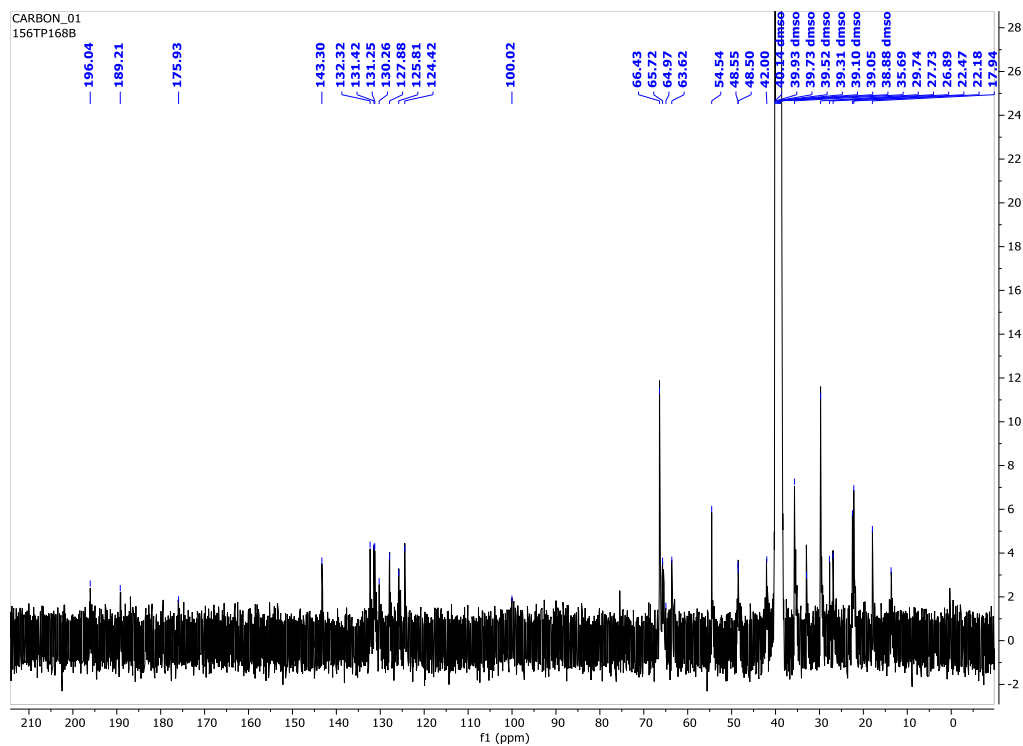


Figure C.151. ^{13}C NMR (400 MHz, DMSO) of **5j'**

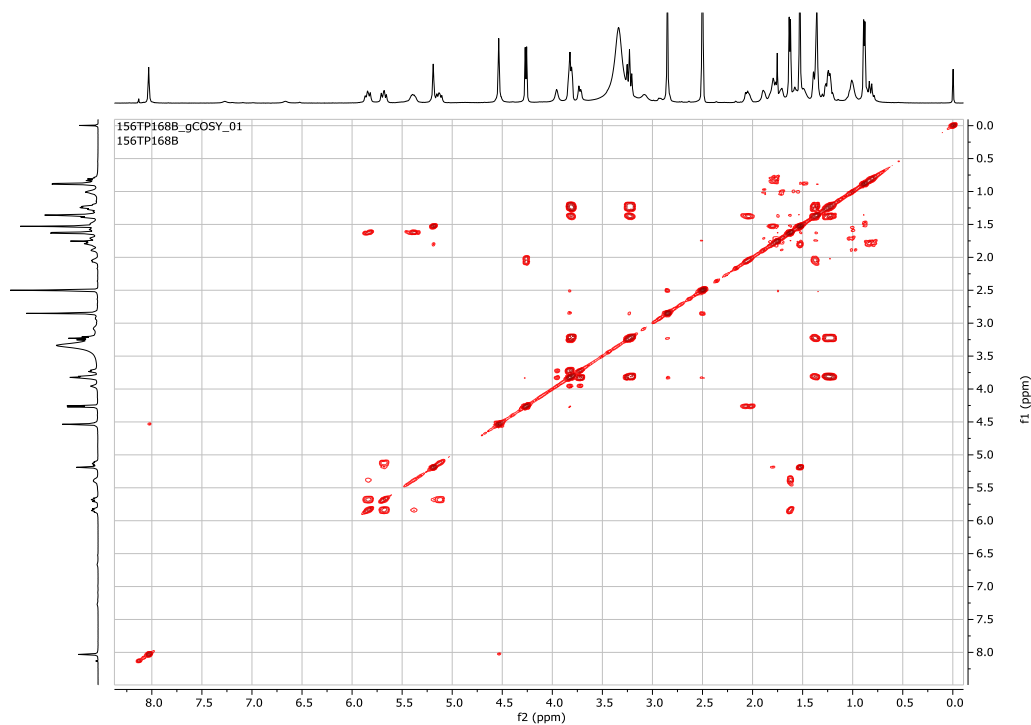


Figure C.152. COSY (500 MHz, DMSO) spectrum of **5j'**

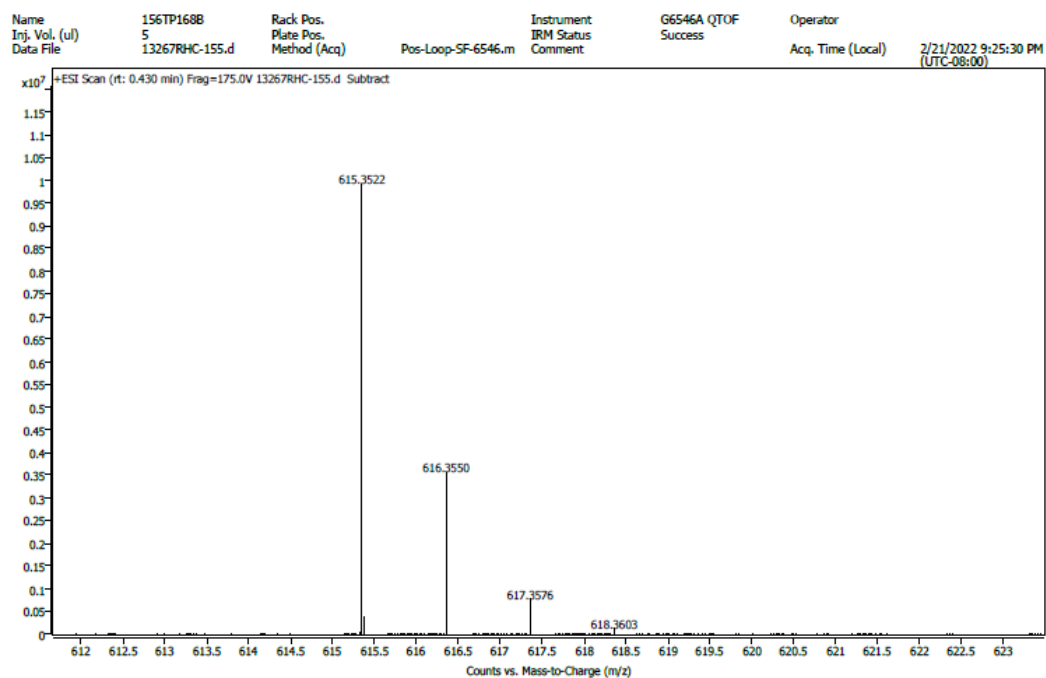


Figure C.153. HRESIMS spectrum of compound **5j'**

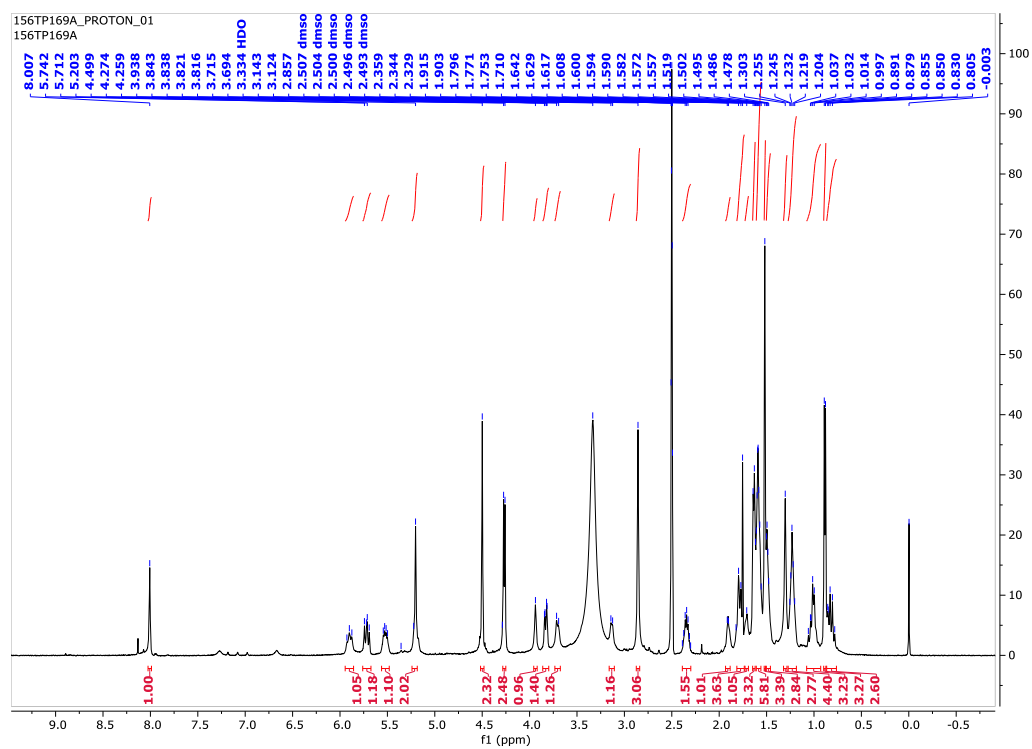


Figure C.154. ^1H NMR (500 MHz, DMSO) of **5k**

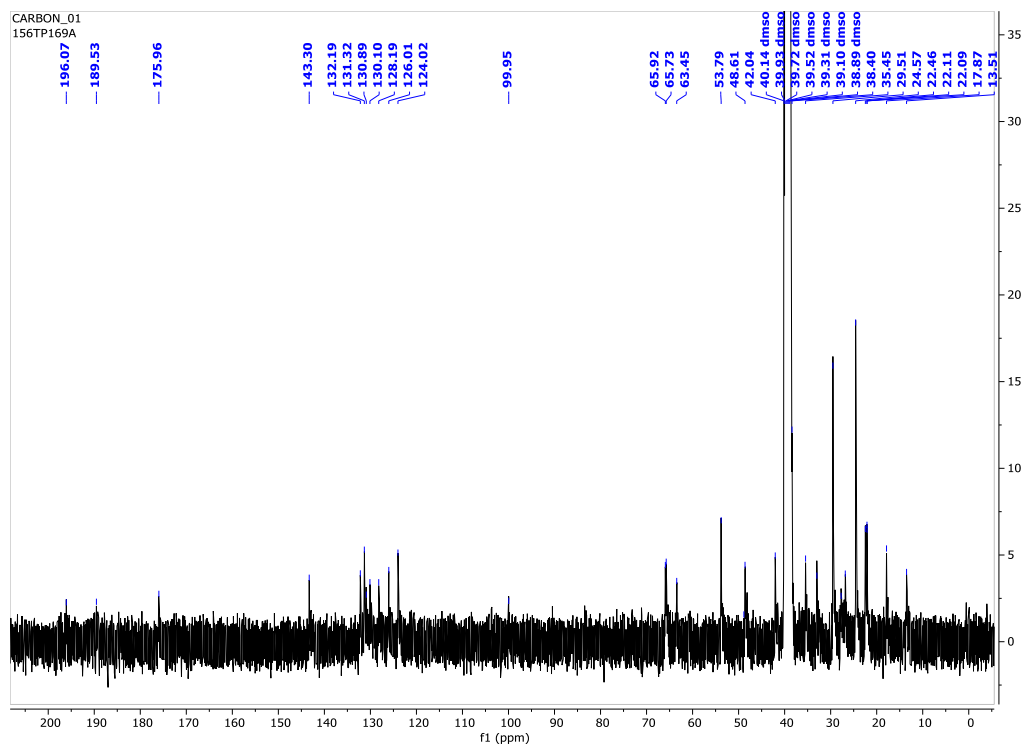


Figure C.155. ^{13}C NMR (400 MHz, DMSO) of **5k**

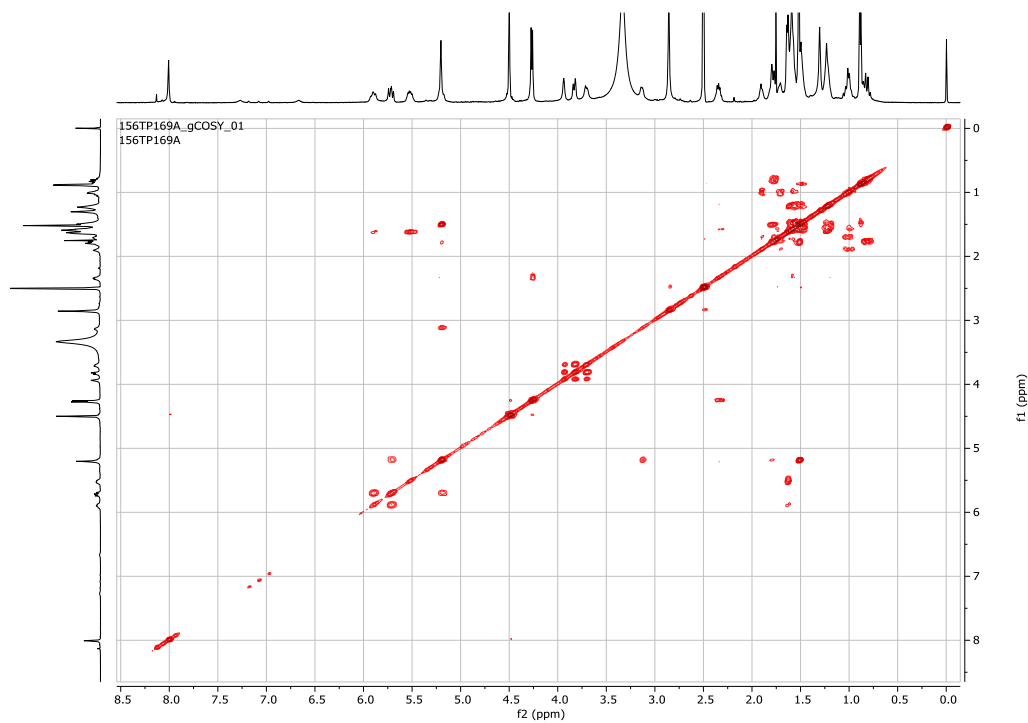


Figure C.156. COSY (500 MHz, DMSO) spectrum of **5k**

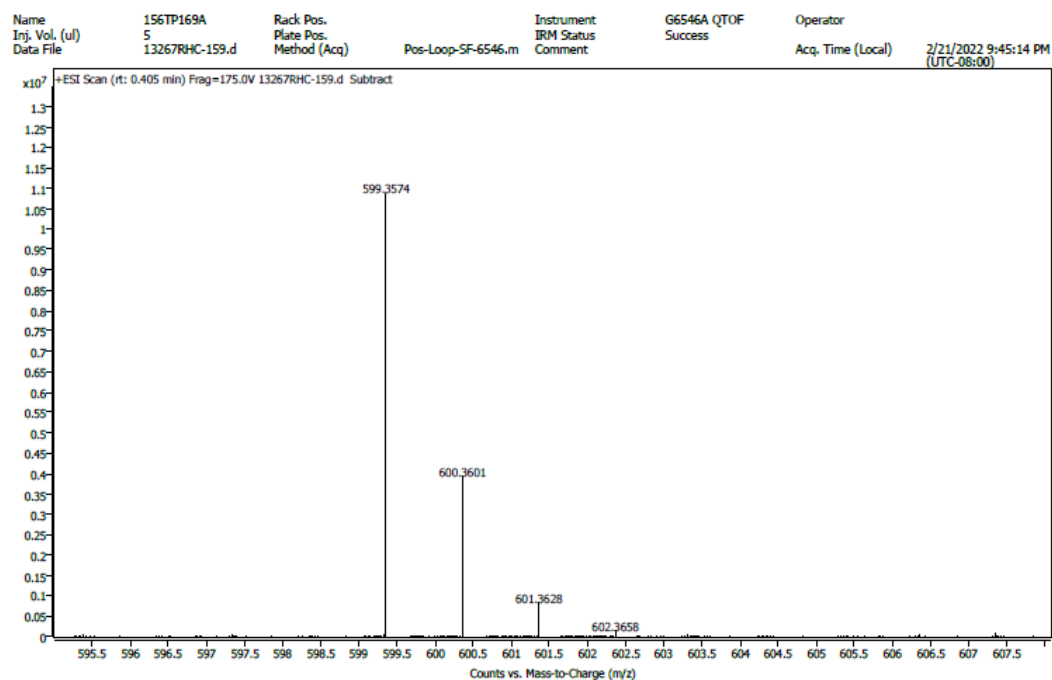


Figure C.157. HRESIMS spectrum of compound **5k**

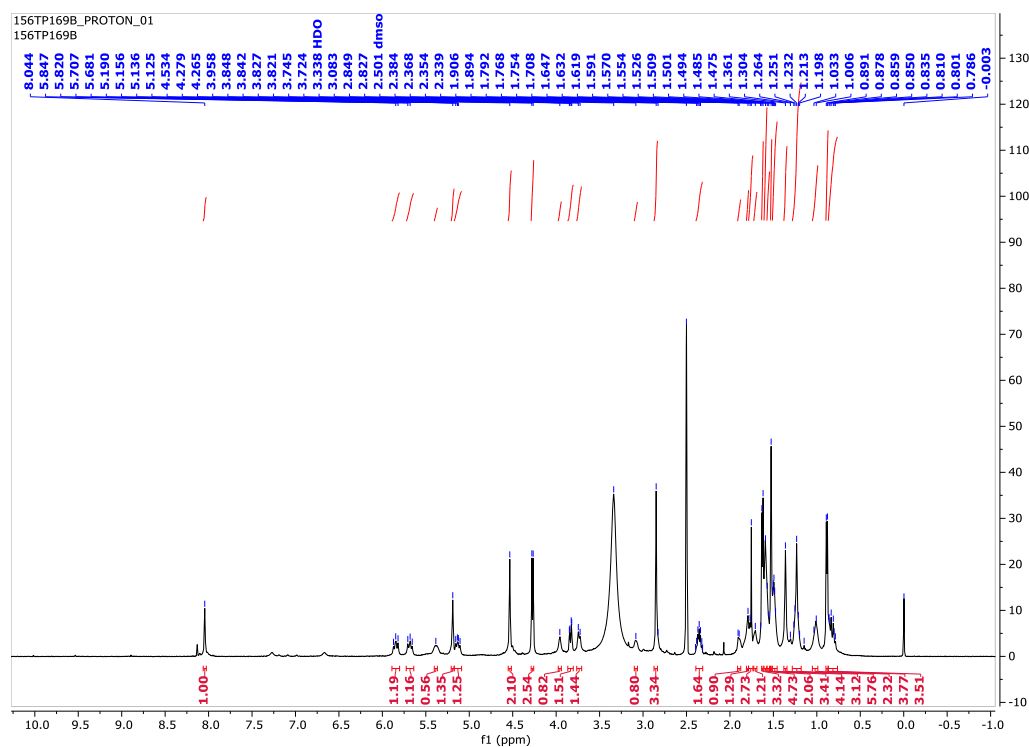


Figure C.158. ^1H NMR (500 MHz, DMSO) of **5k'**

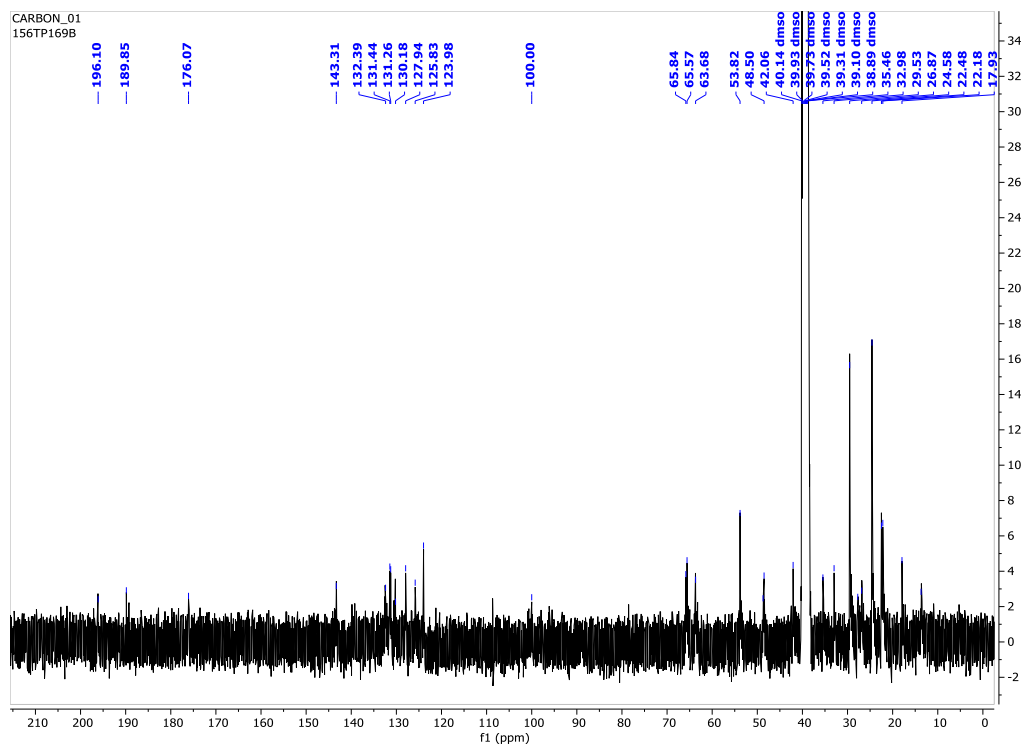


Figure C.159. ^{13}C NMR (400 MHz, DMSO) of **5k'**

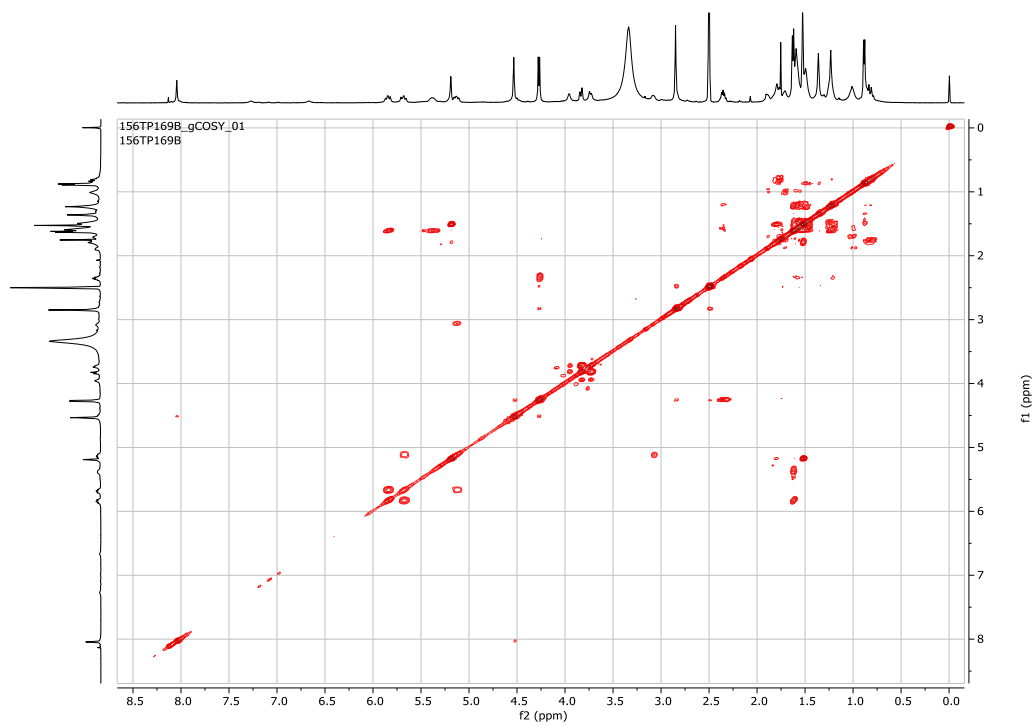


Figure C.160. COSY (500 MHz, DMSO) spectrum of **5k'**

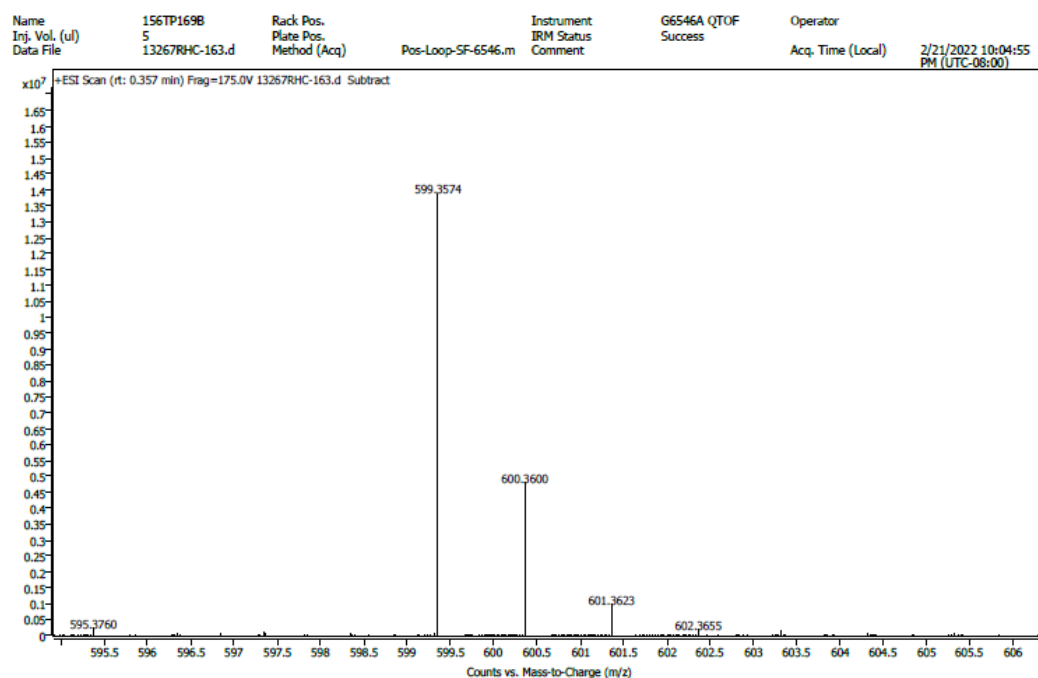


Figure C.161. HRESIMS spectrum of compound **5k'**

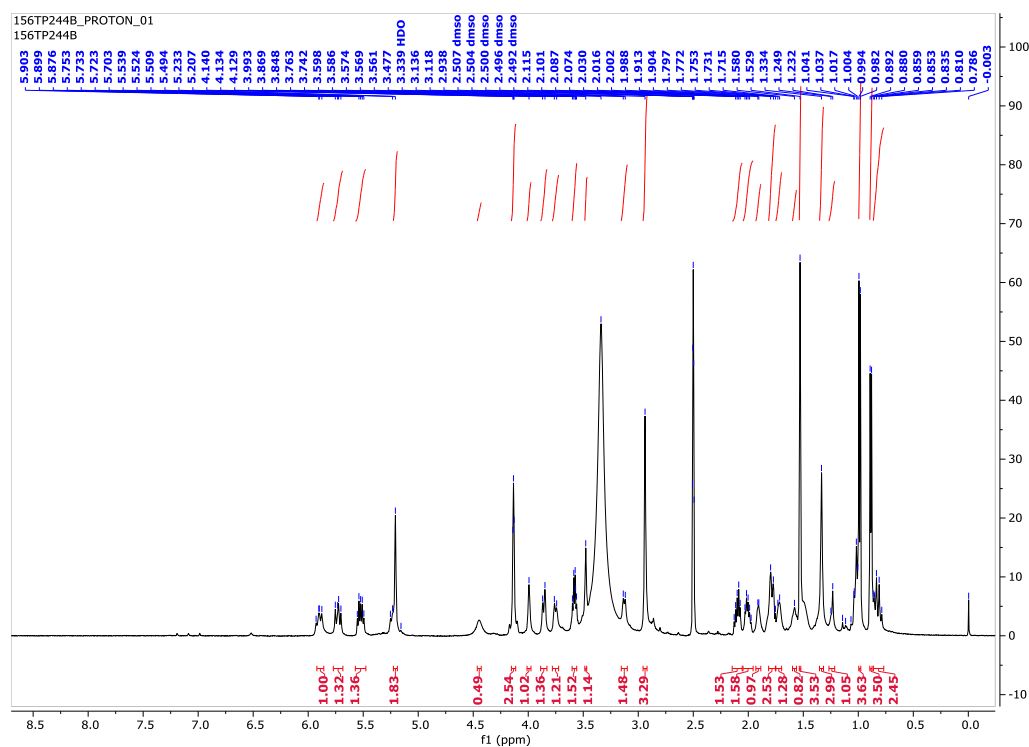


Figure C.162. ^1H NMR (500 MHz, DMSO) of **6a**

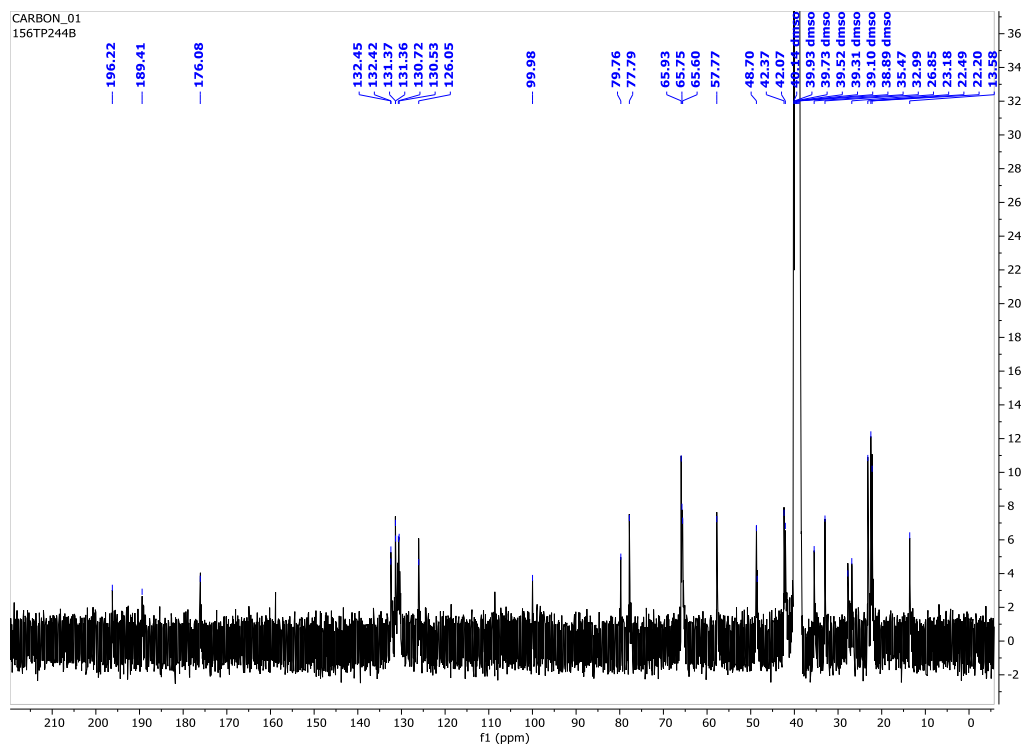


Figure C.163. ^{13}C NMR (400 MHz, DMSO) of **6a**

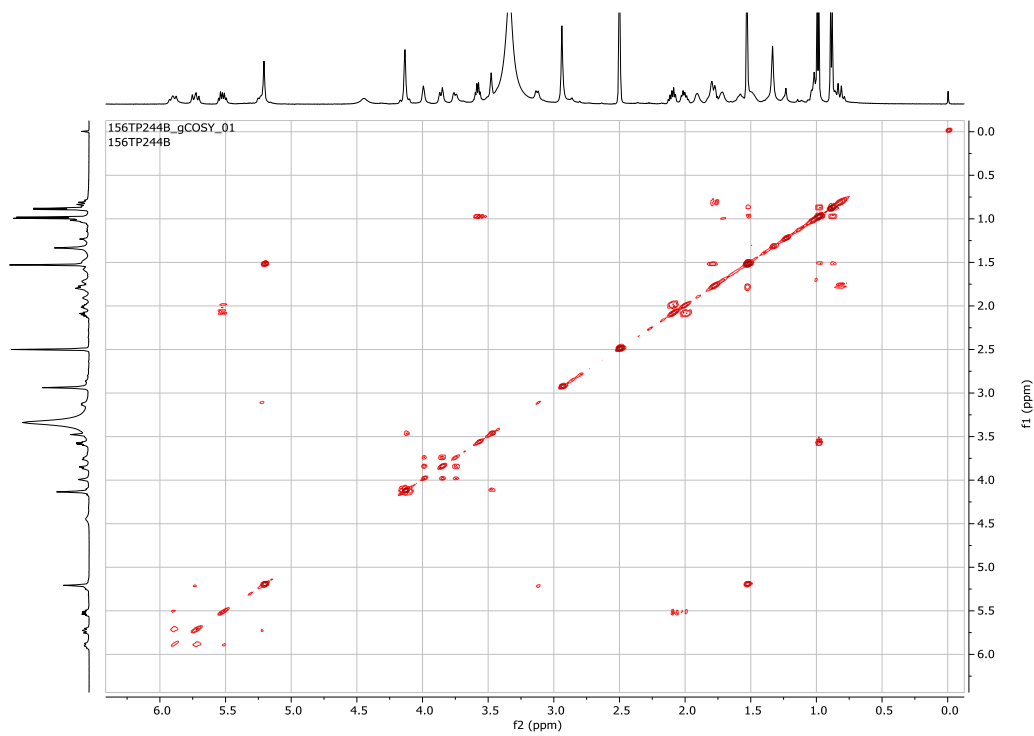


Figure C.164. COSY (500 MHz, DMSO) spectrum of **6a**

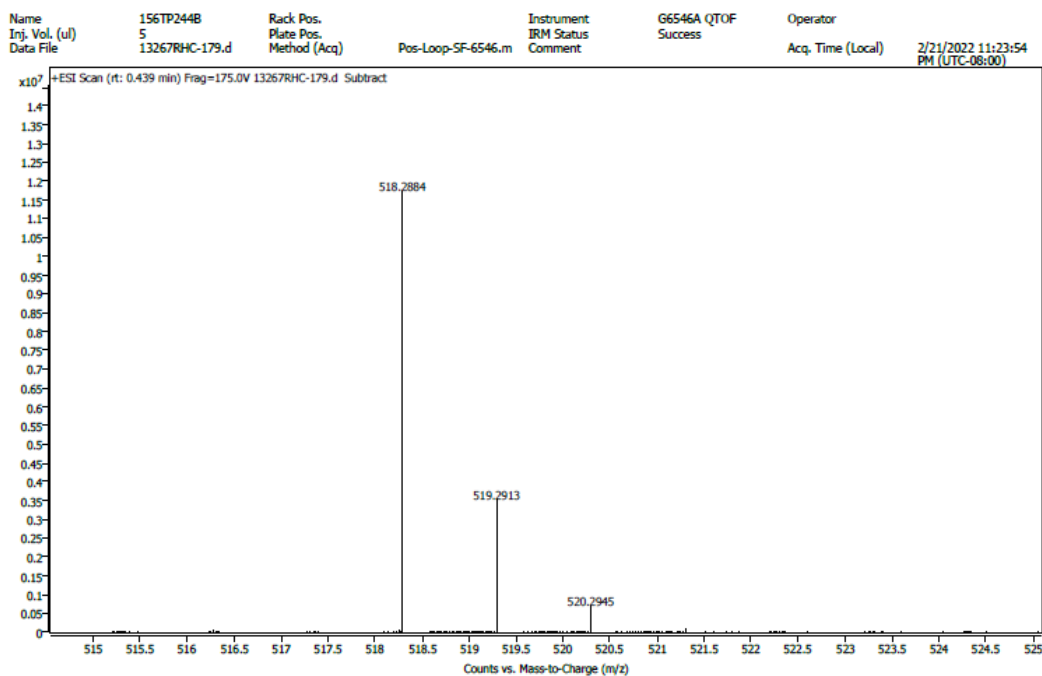


Figure C.165. HRESIMS spectrum of compound **6a**

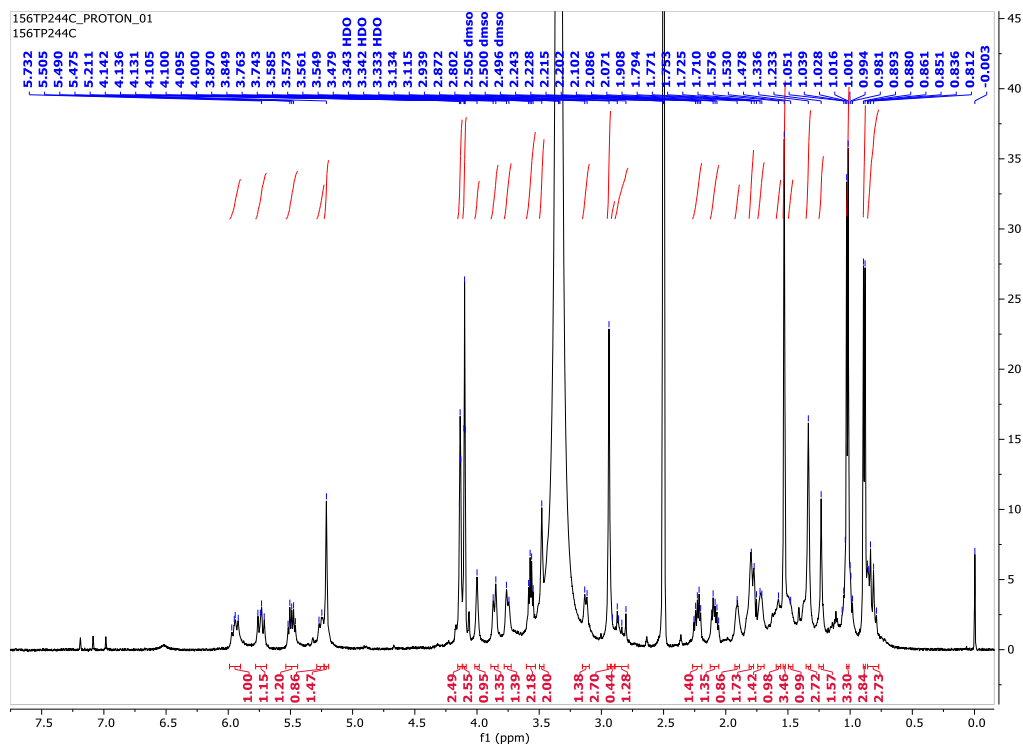


Figure C.166. ^1H NMR (500 MHz, DMSO) of **6b**

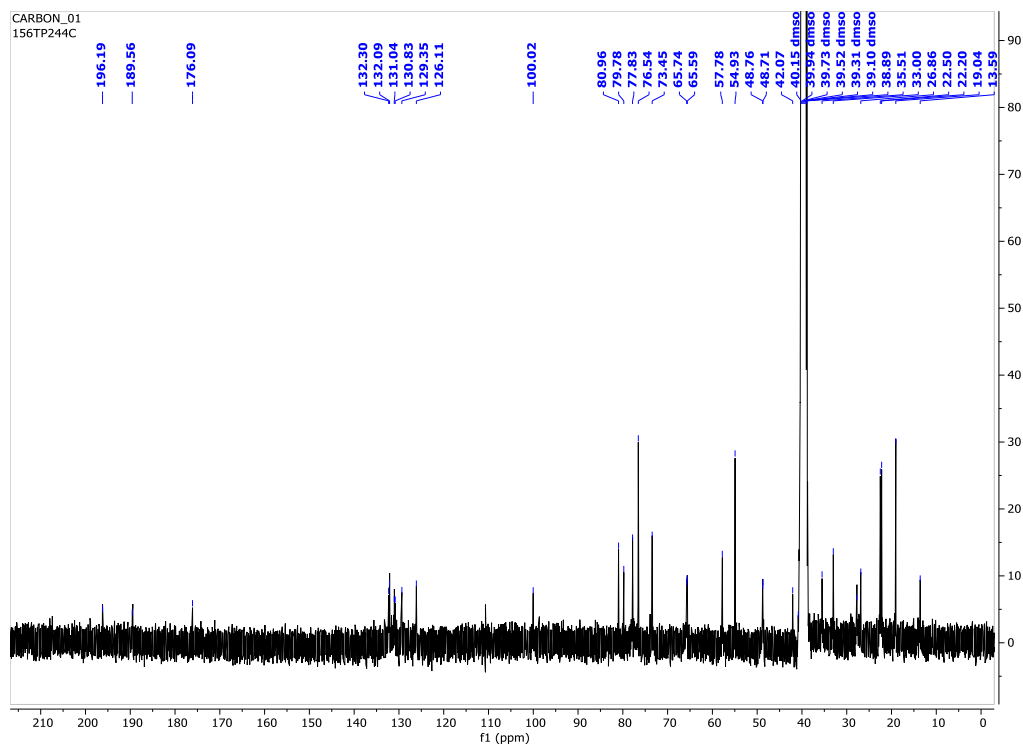


Figure C.167. ^{13}C NMR (400 MHz, DMSO) of **6b**

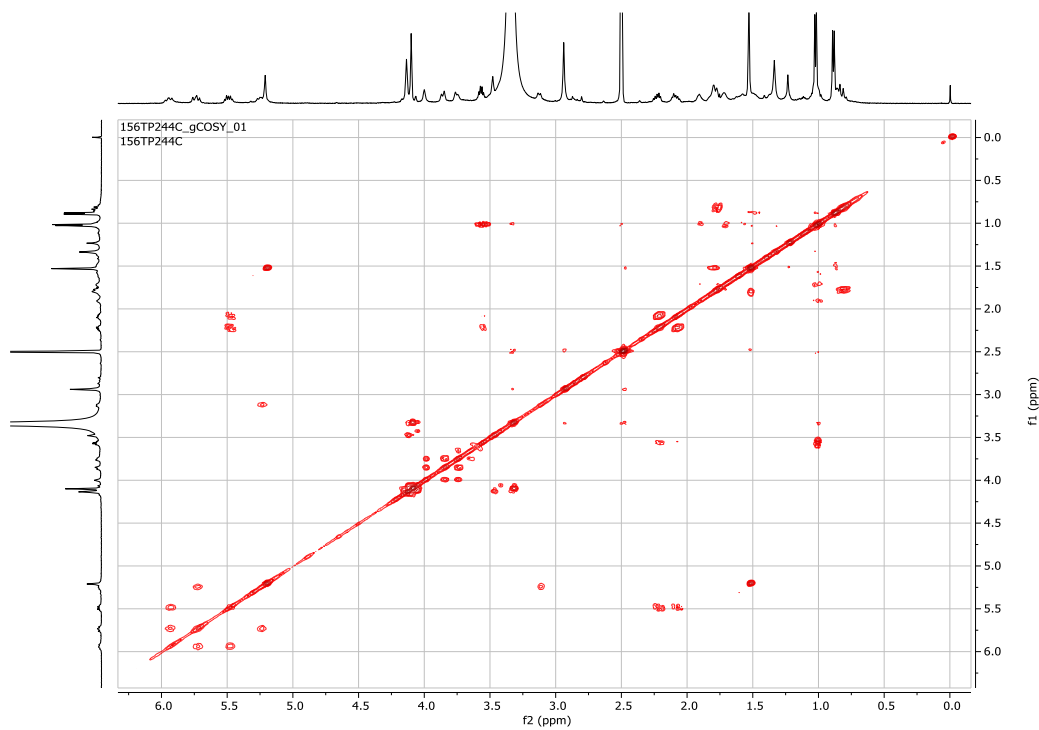


Figure C.168. COSY (500 MHz, DMSO) spectrum of **6b**

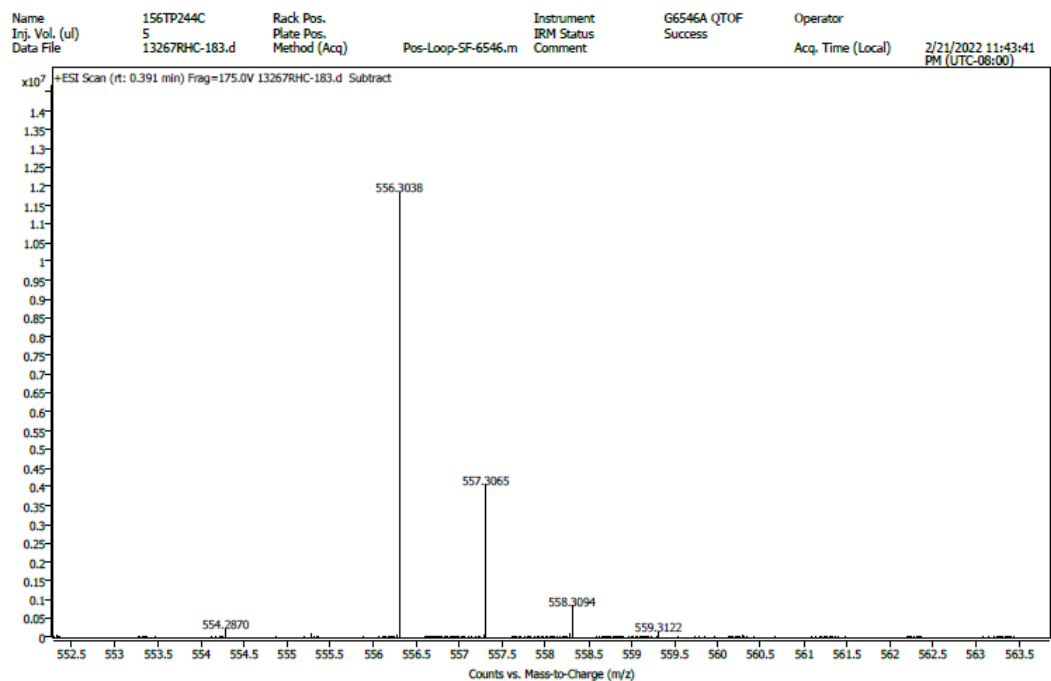


Figure C.169. HRESIMS spectrum of compound **6b**

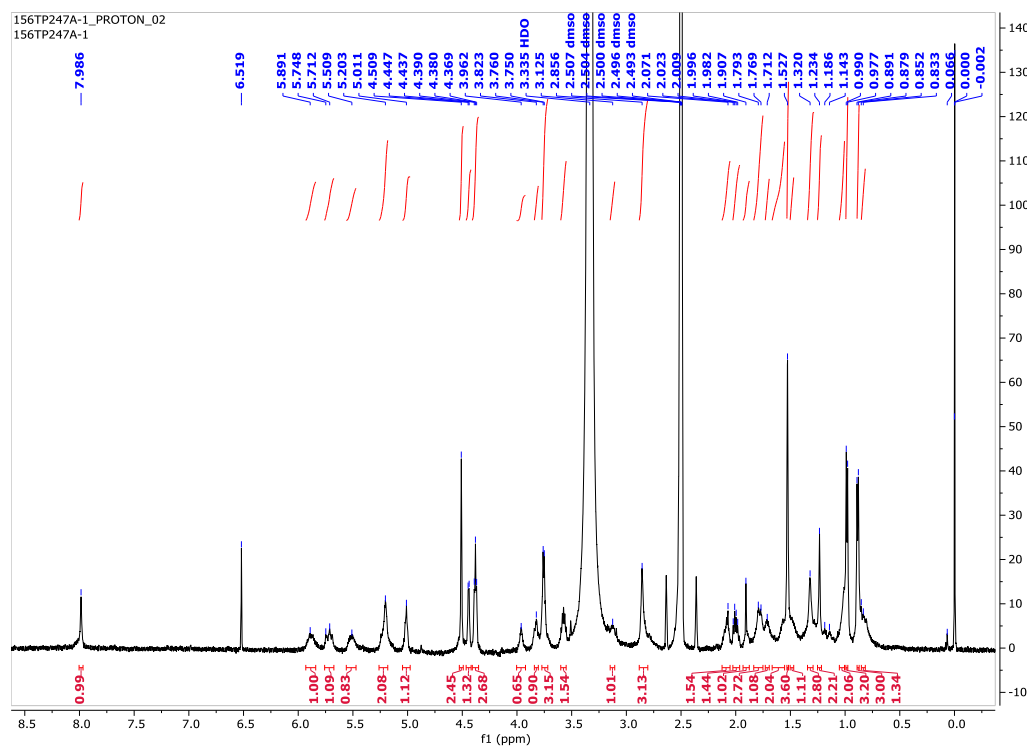


Figure C.170. ^1H NMR (500 MHz, DMSO) of **6c**

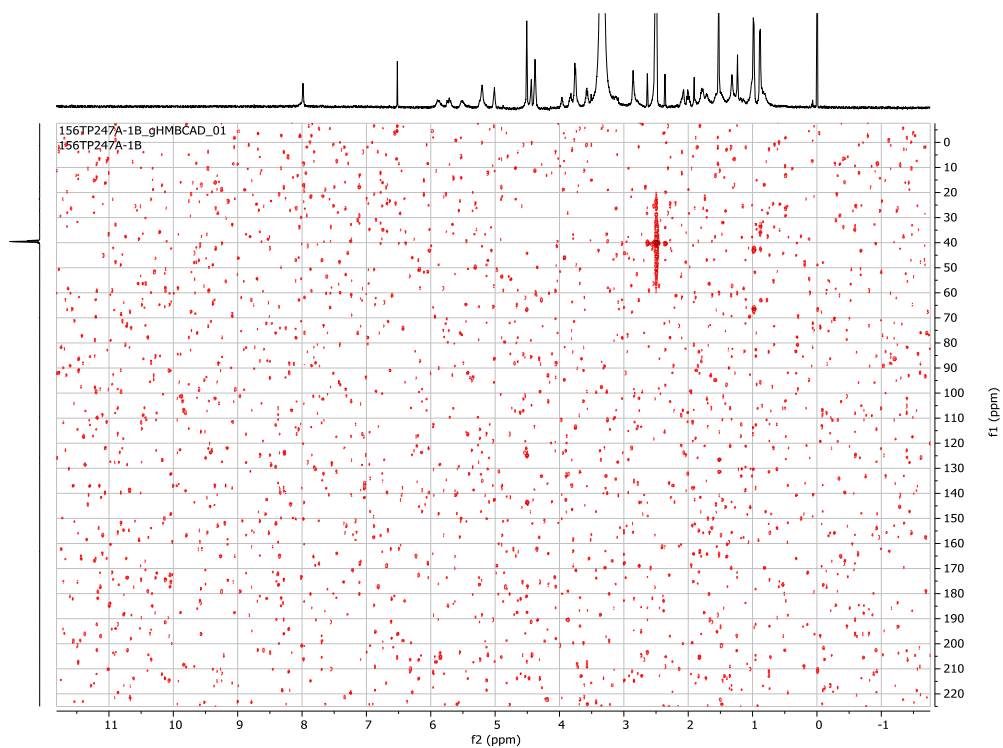


Figure C.171. HMBC (500 MHz, DMSO) spectrum of **6c**

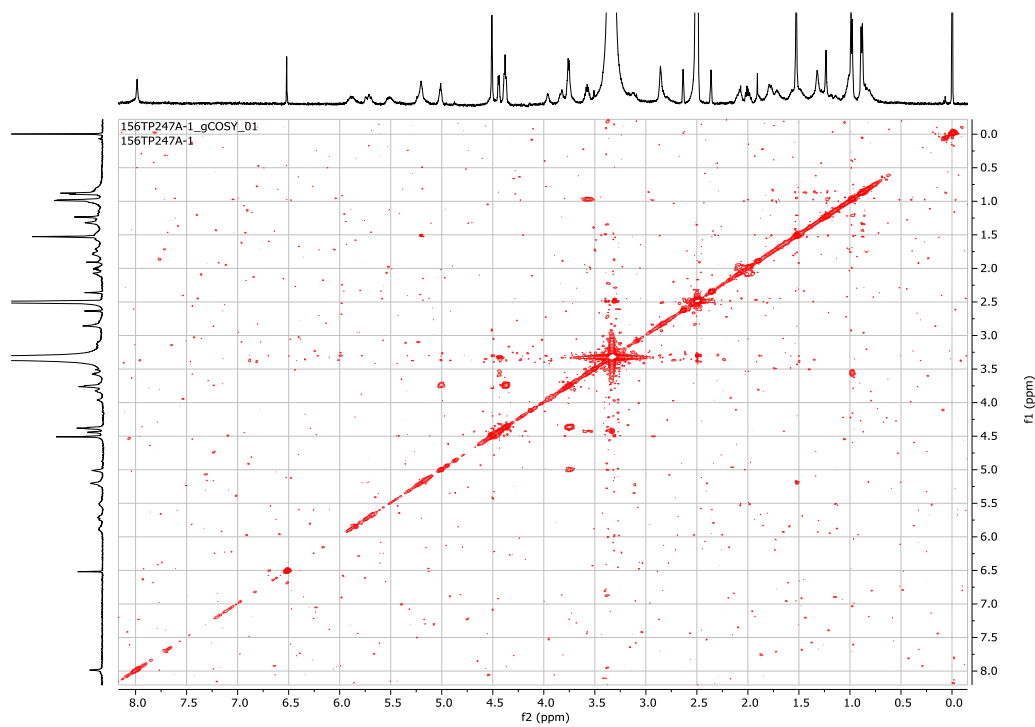


Figure C.172. COSY (500 MHz, DMSO) spectrum of **6c**

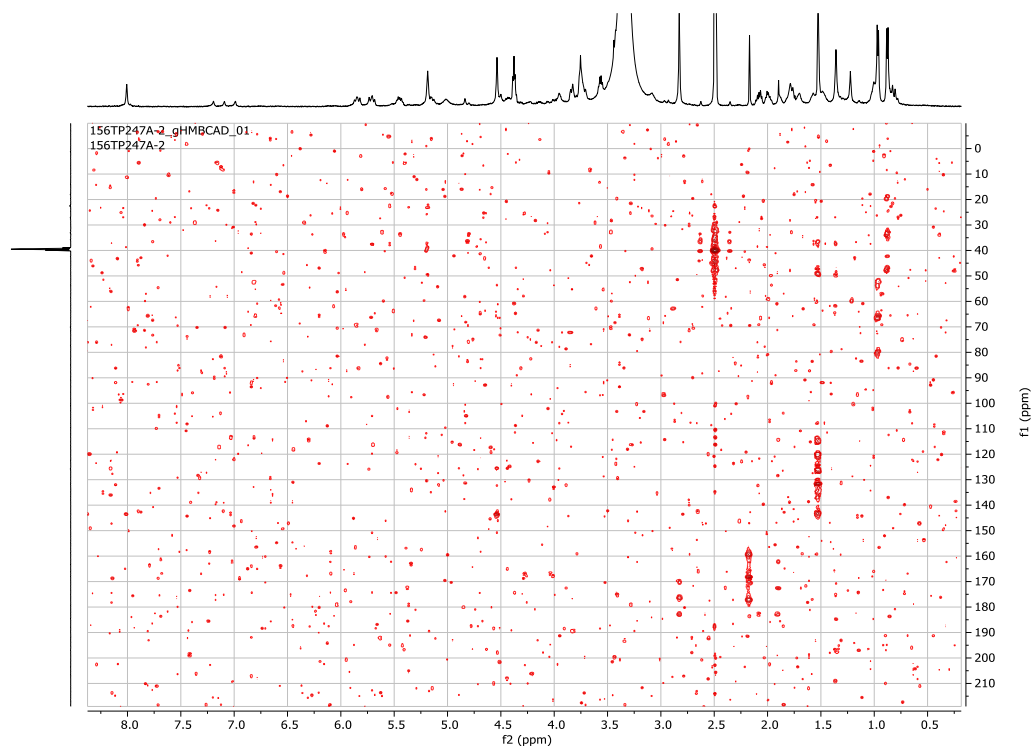


Figure C.175. HMBC (500 MHz, DMSO) spectrum of **6c'**

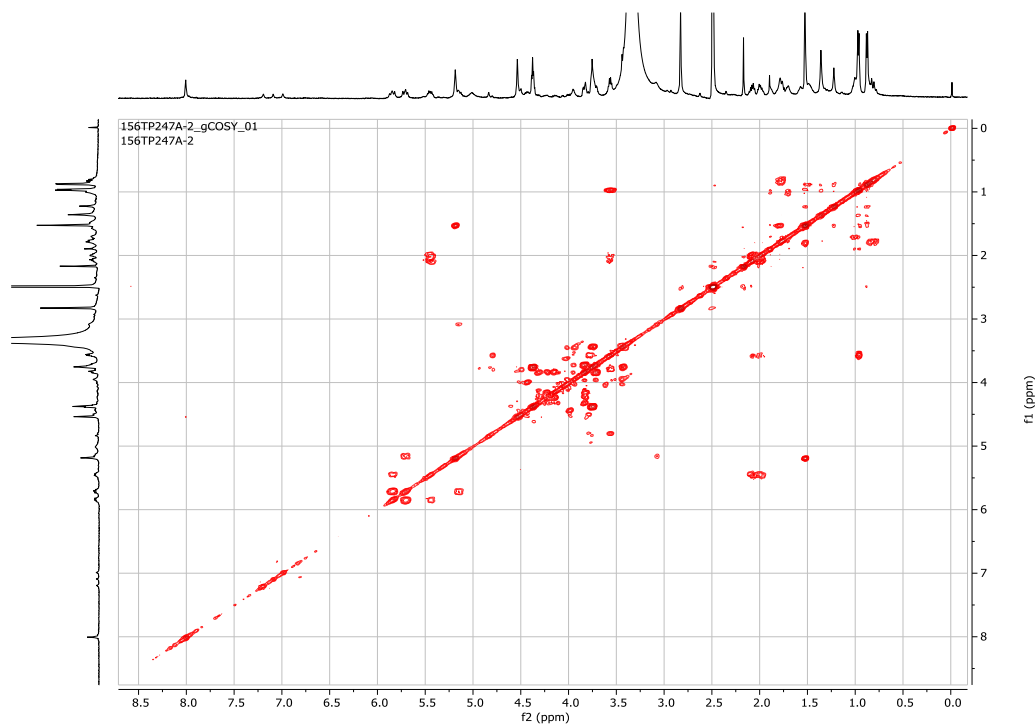


Figure C.176. COSY (500 MHz, DMSO) spectrum of **6c'**

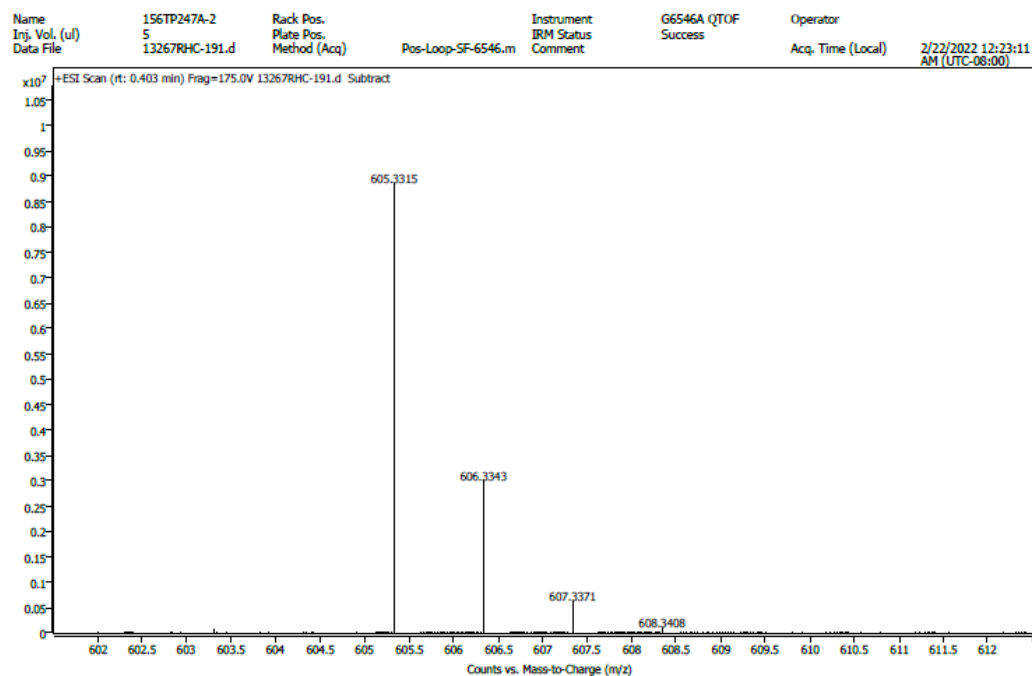


Figure C.177. HRESIMS spectrum of compound **6c'**

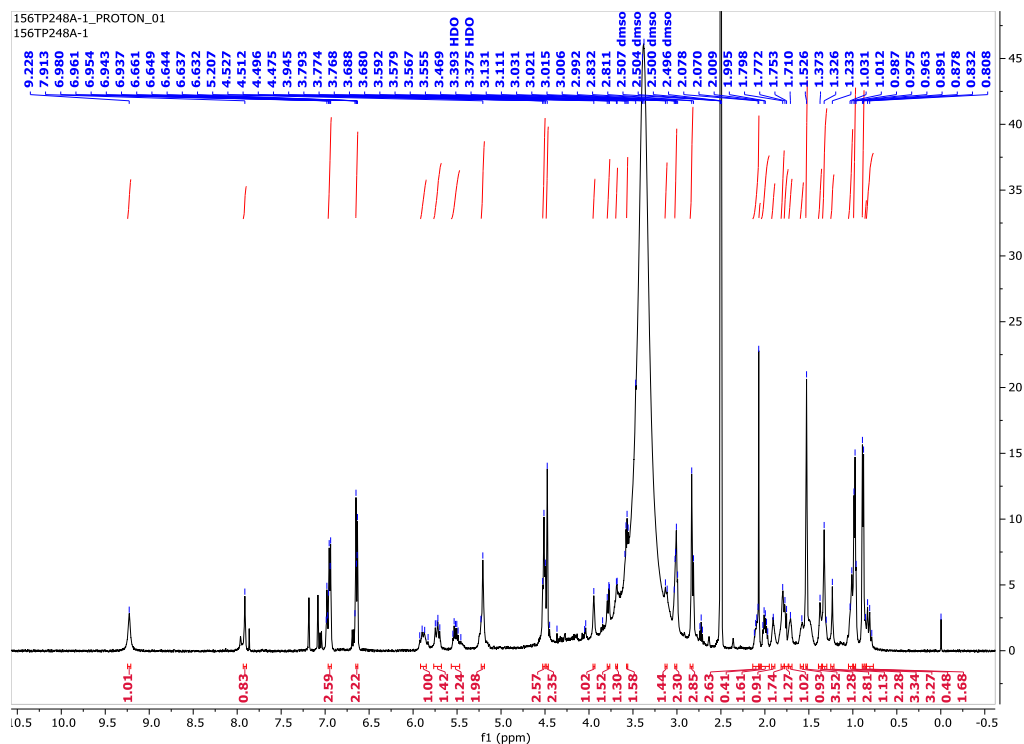


Figure C.178. ^1H NMR (500 MHz, DMSO) of **6d**

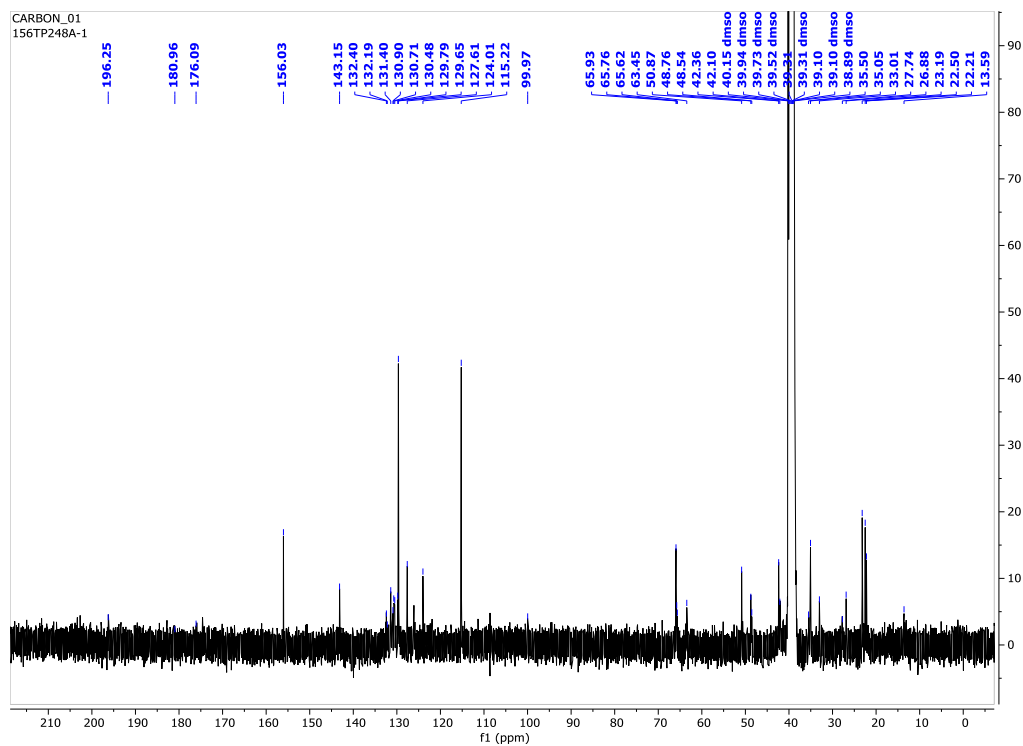


Figure C.179. ^{13}C NMR (500 MHz, DMSO) of **6d**

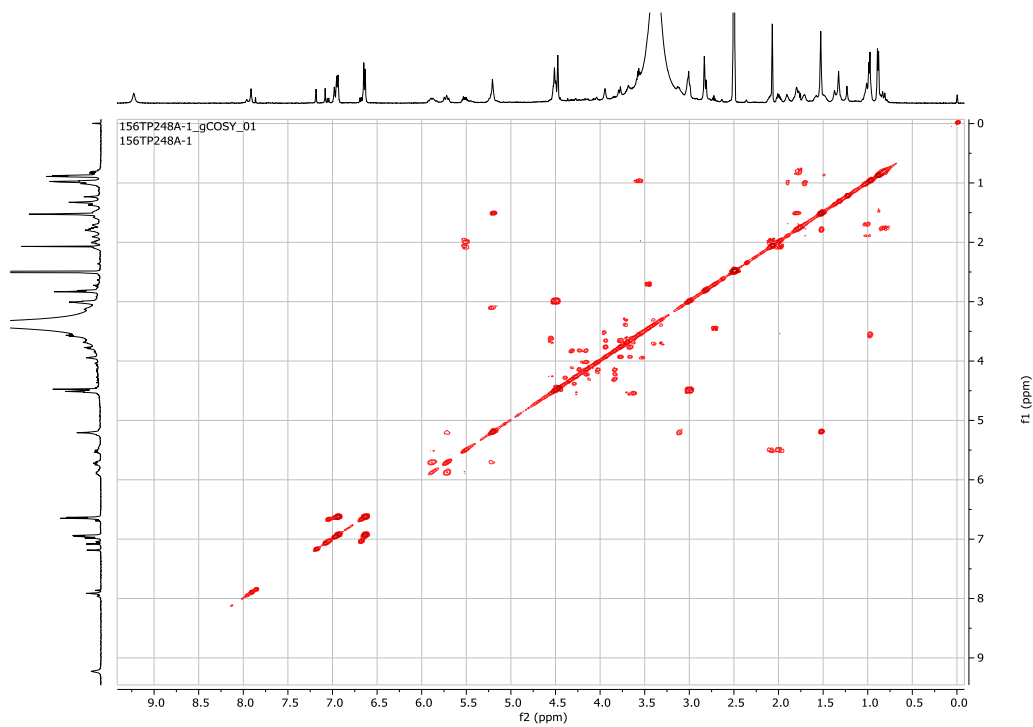


Figure C.180. COSY (500 MHz, DMSO) spectrum of **6d**

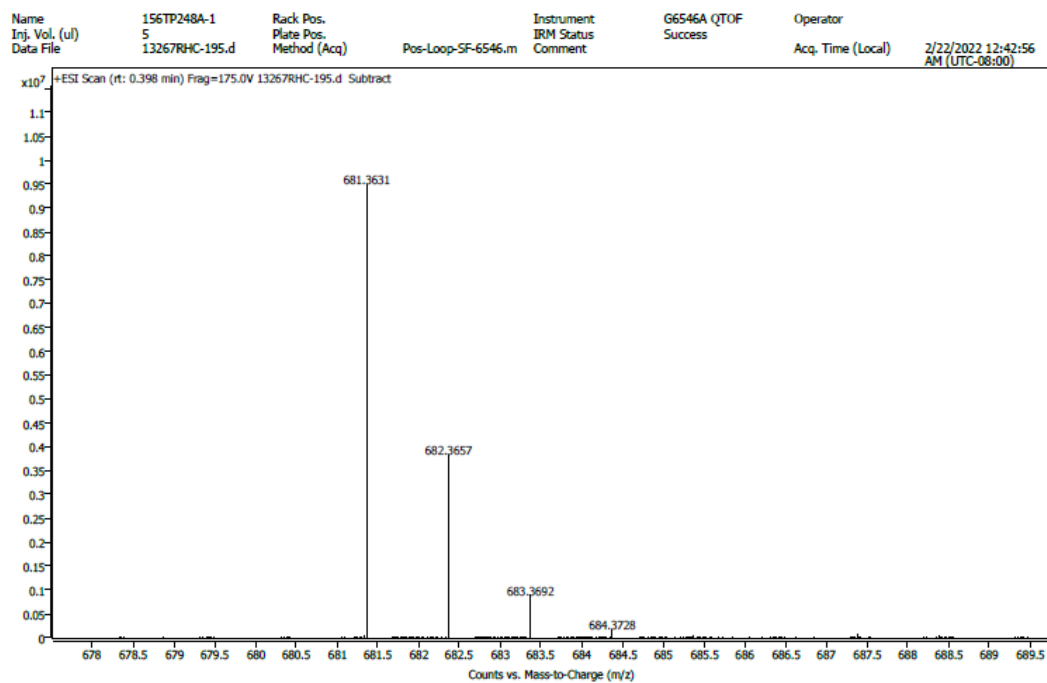


Figure C.181. HRESIMS spectrum of compound **6d**

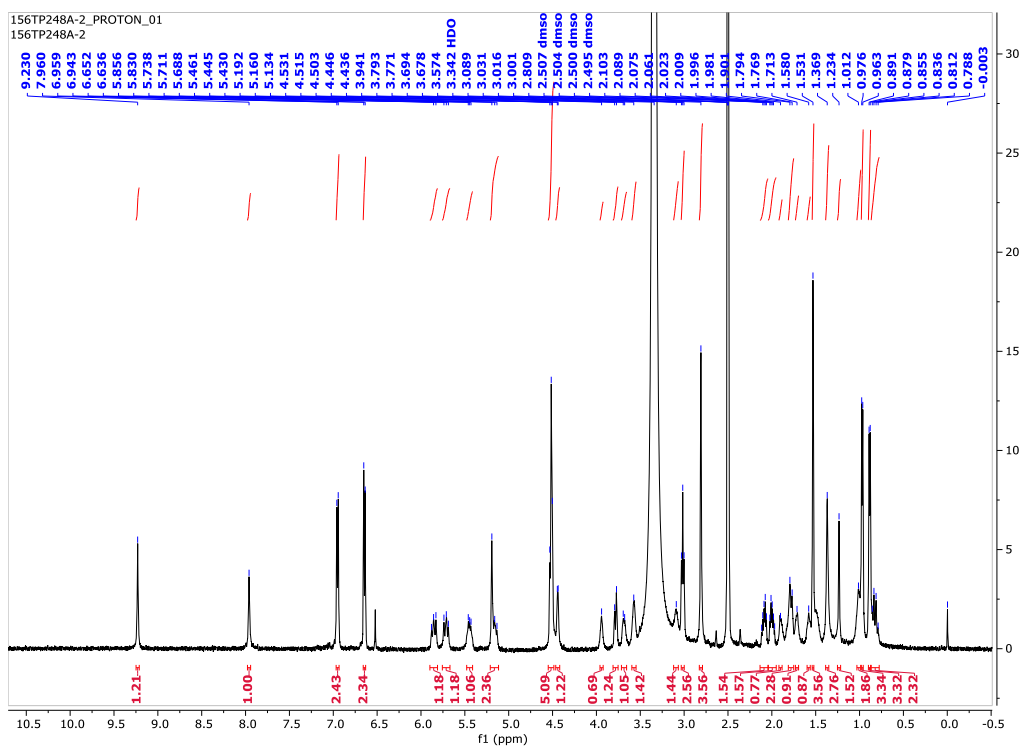


Figure C.182. ^1H NMR (500 MHz, DMSO) of **6d'**

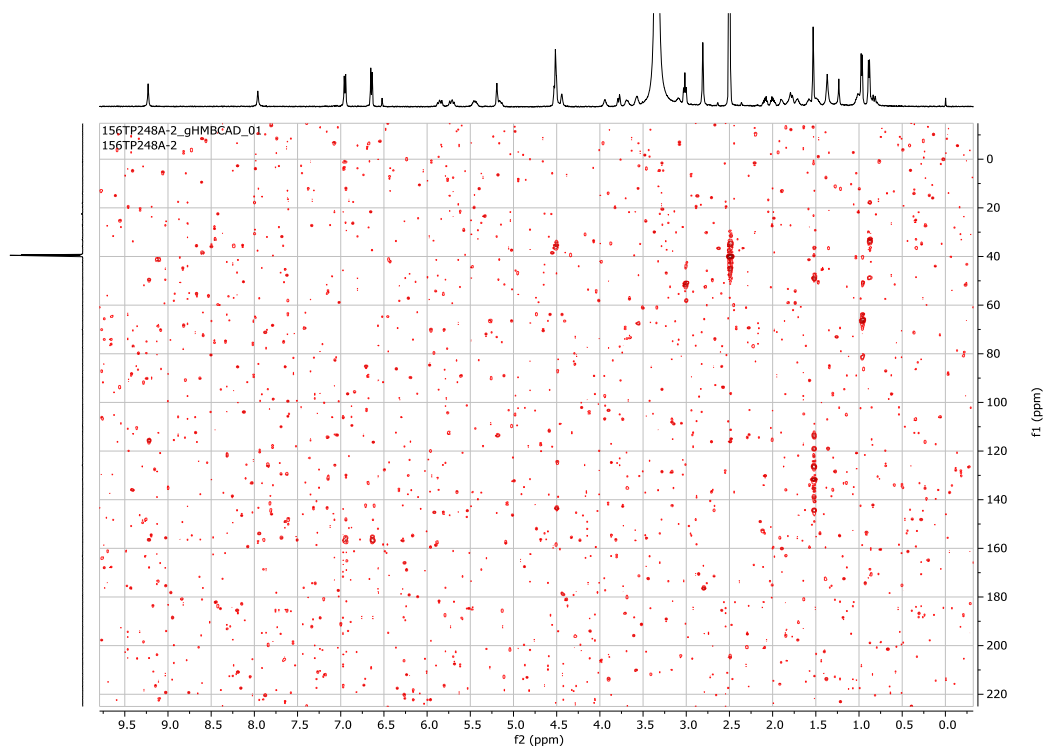


Figure C.183. HMBC (500 MHz, DMSO) spectrum of **6d'**

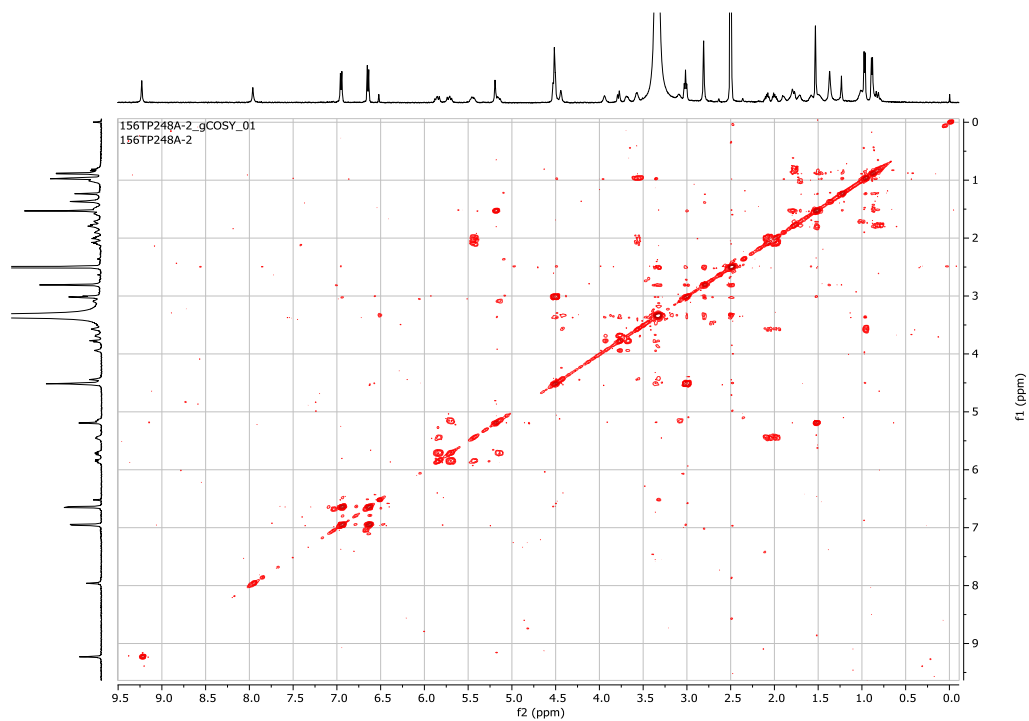


Figure C.184. COSY (500 MHz, DMSO) spectrum of **6d'**

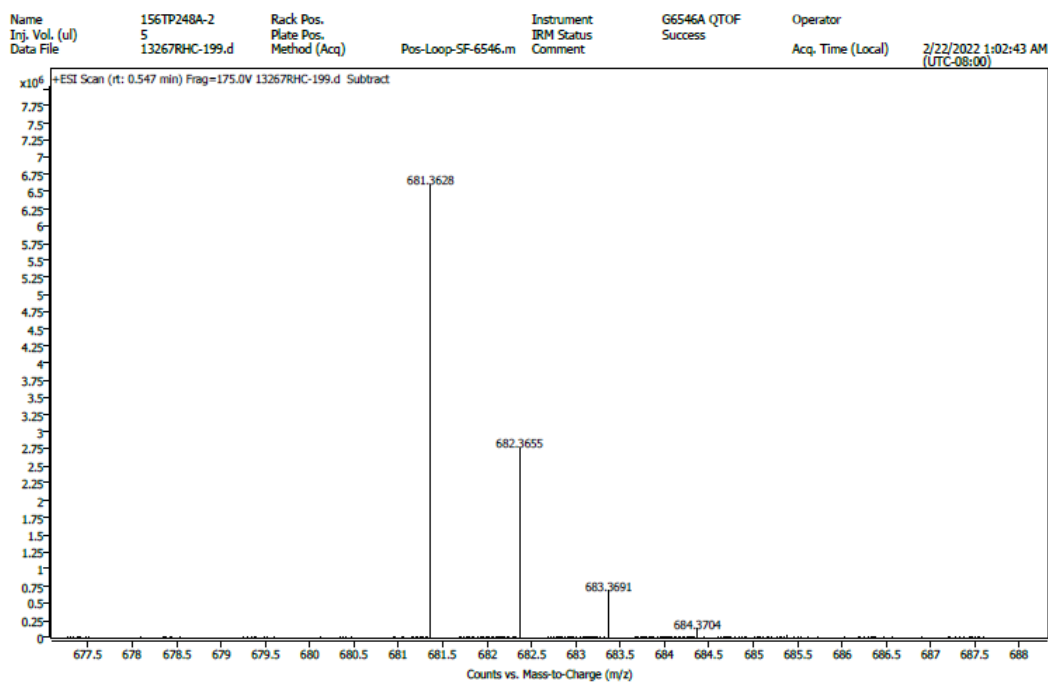


Figure C.185. HRESIMS spectrum of compound **6d'**

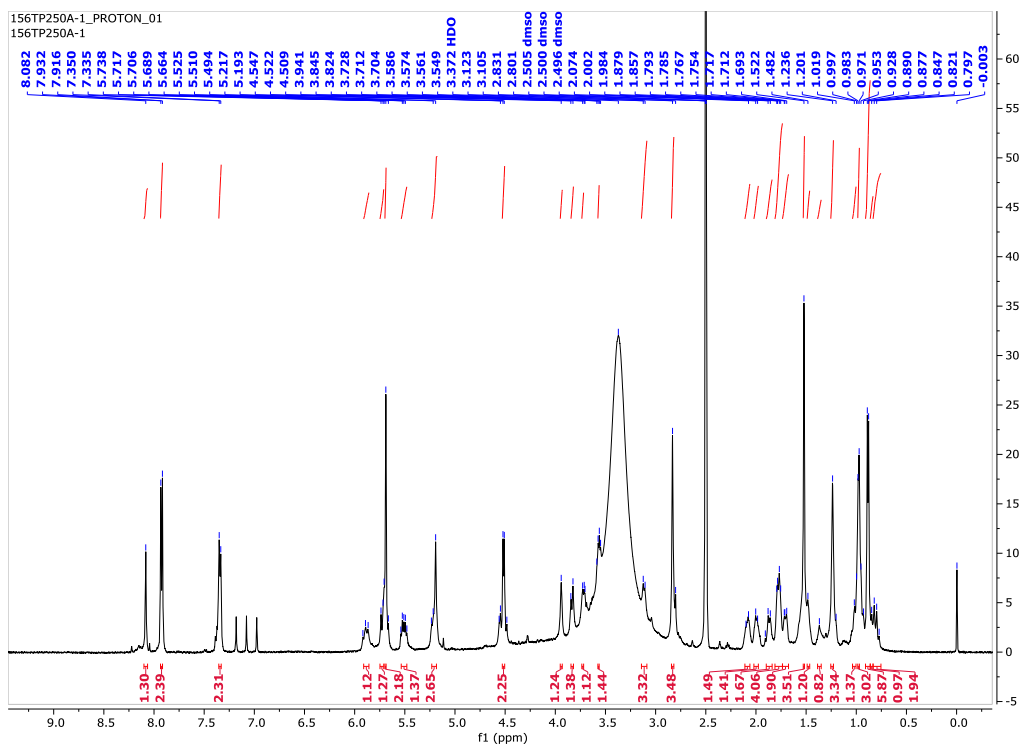


Figure C.186. ^1H NMR (500 MHz, DMSO) of **6e**

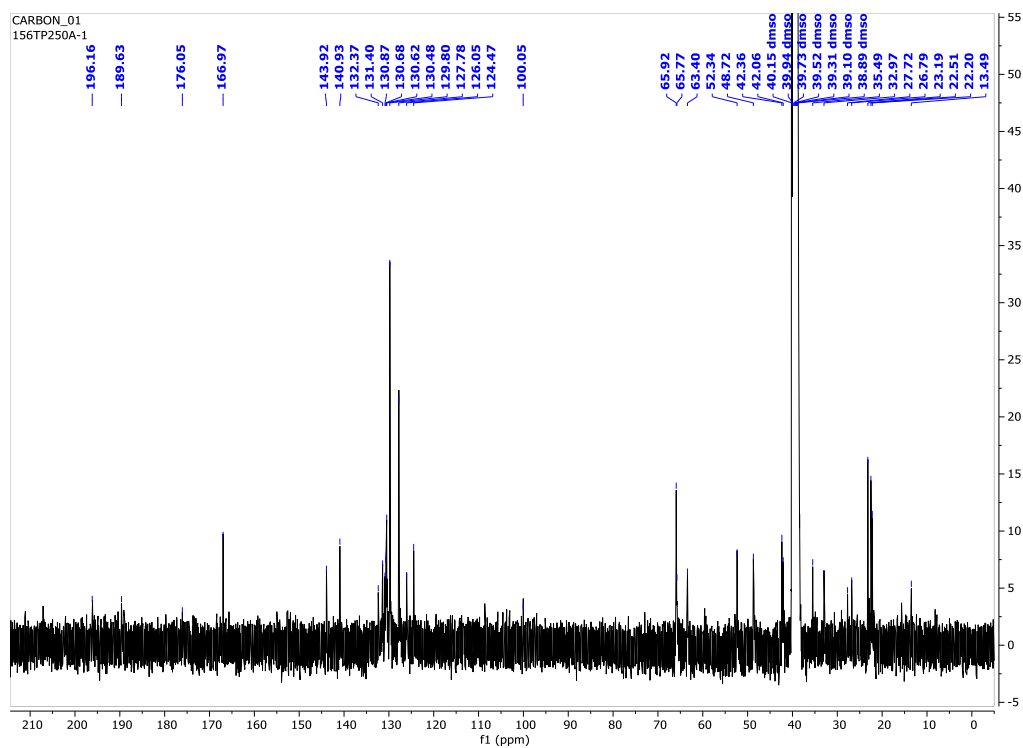


Figure C.187. ^{13}C NMR (400 MHz, DMSO) of **6e**

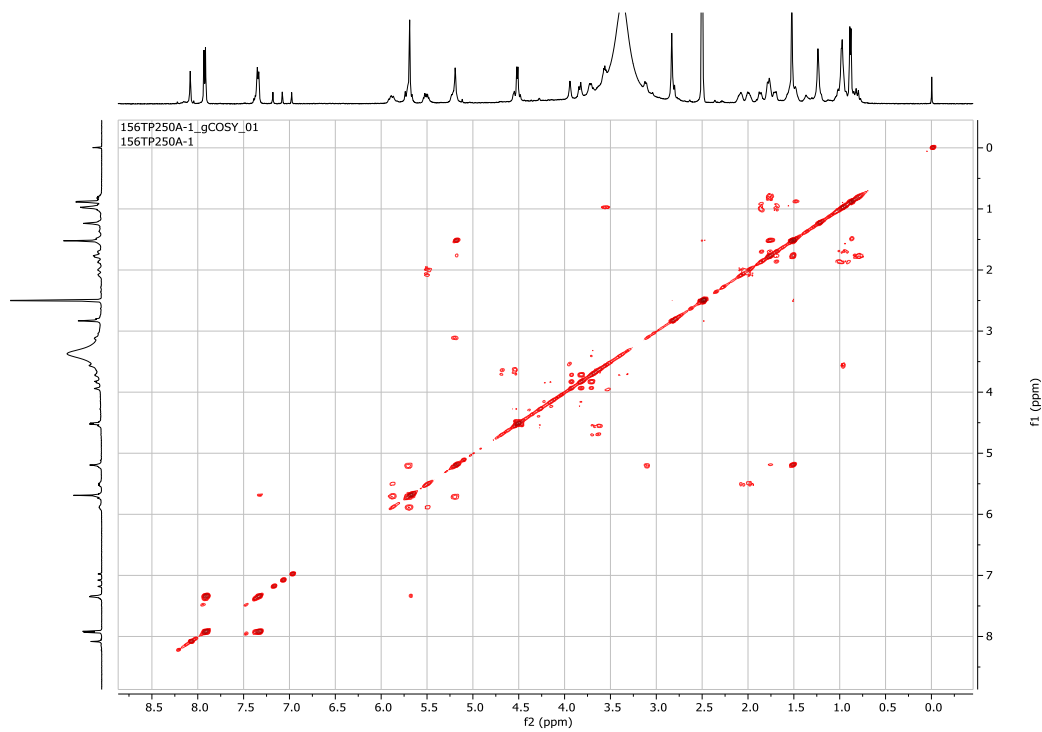


Figure C.188. COSY (500 MHz, DMSO) spectrum of **6e**

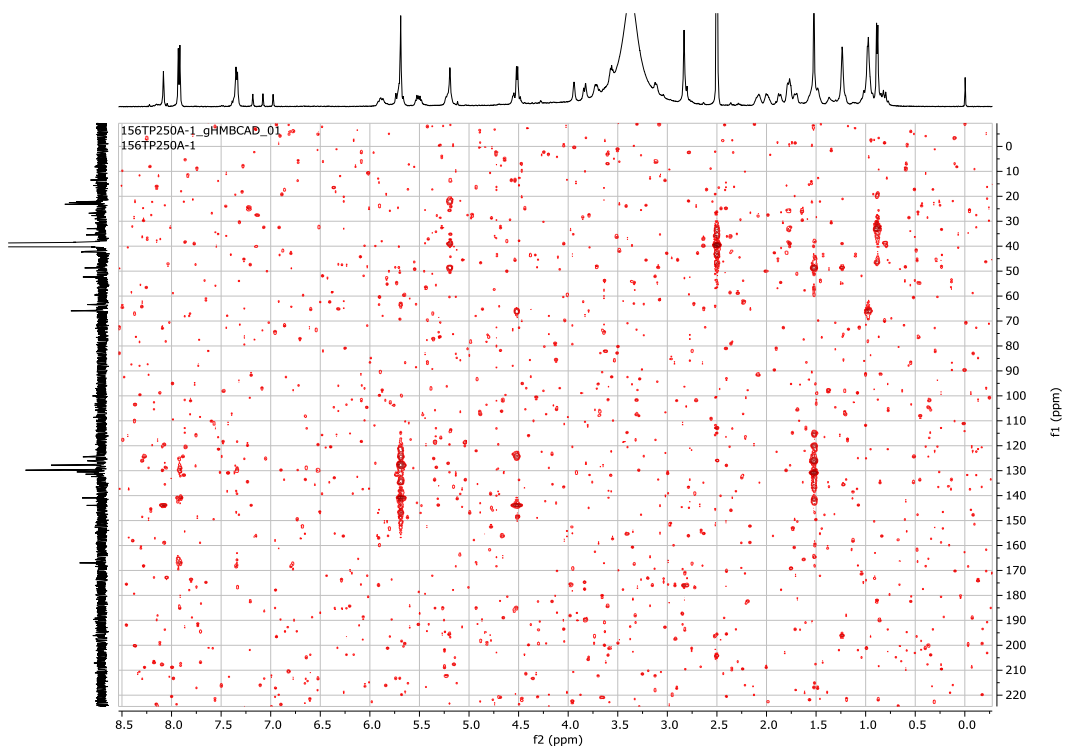


Figure C.189. HMBC (500 MHz, DMSO) spectrum of **6e**

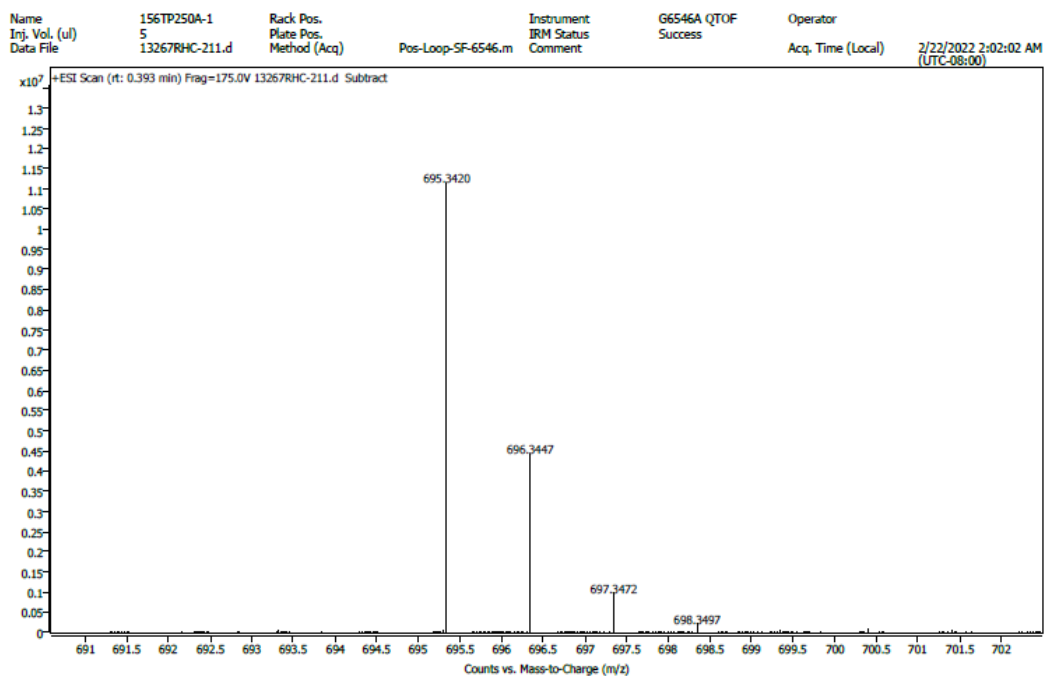


Figure C.190. HRESIMS spectrum of compound **6e**

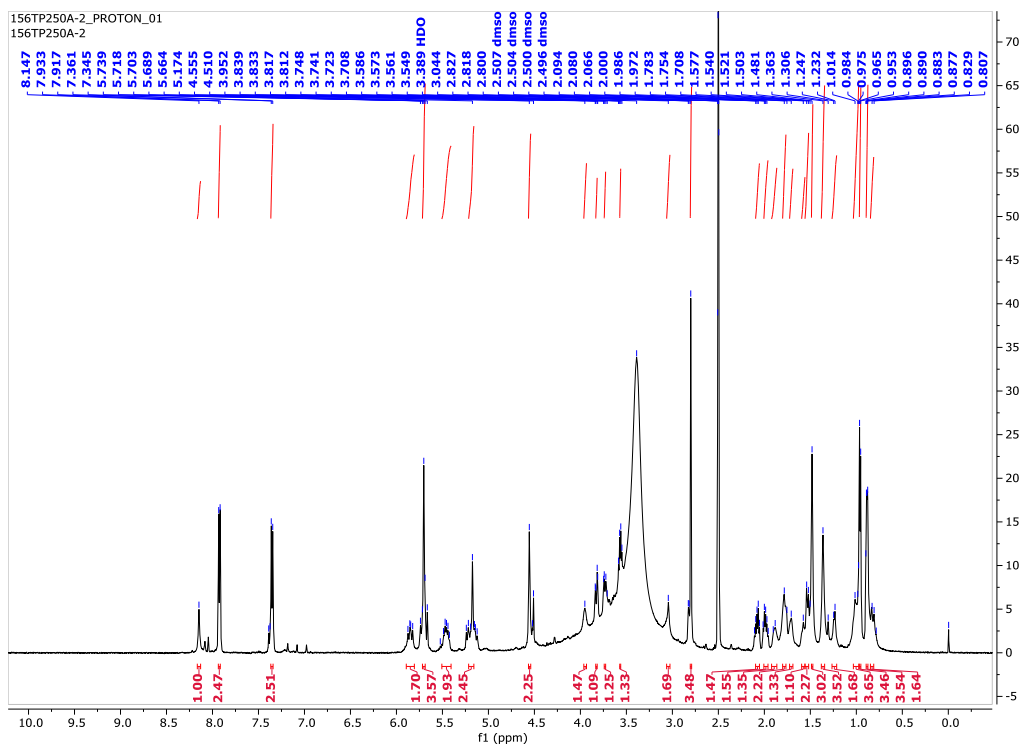


Figure C.191. ^1H NMR (500 MHz, DMSO) of **6e'**

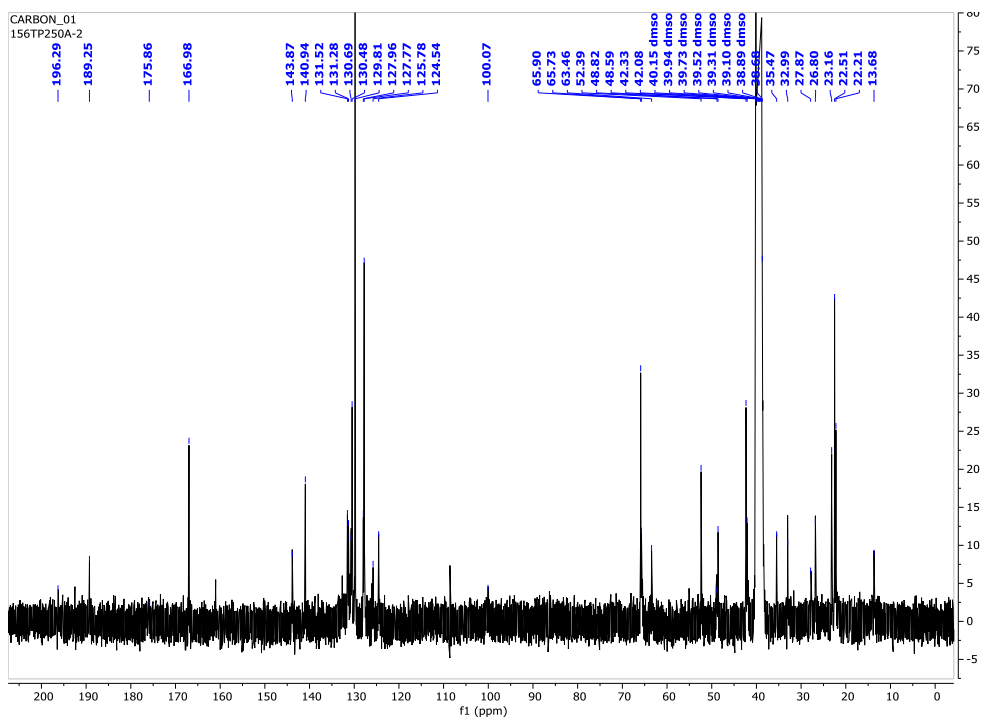


Figure C.192. ^{13}C NMR (400 MHz, DMSO) of **6e'**

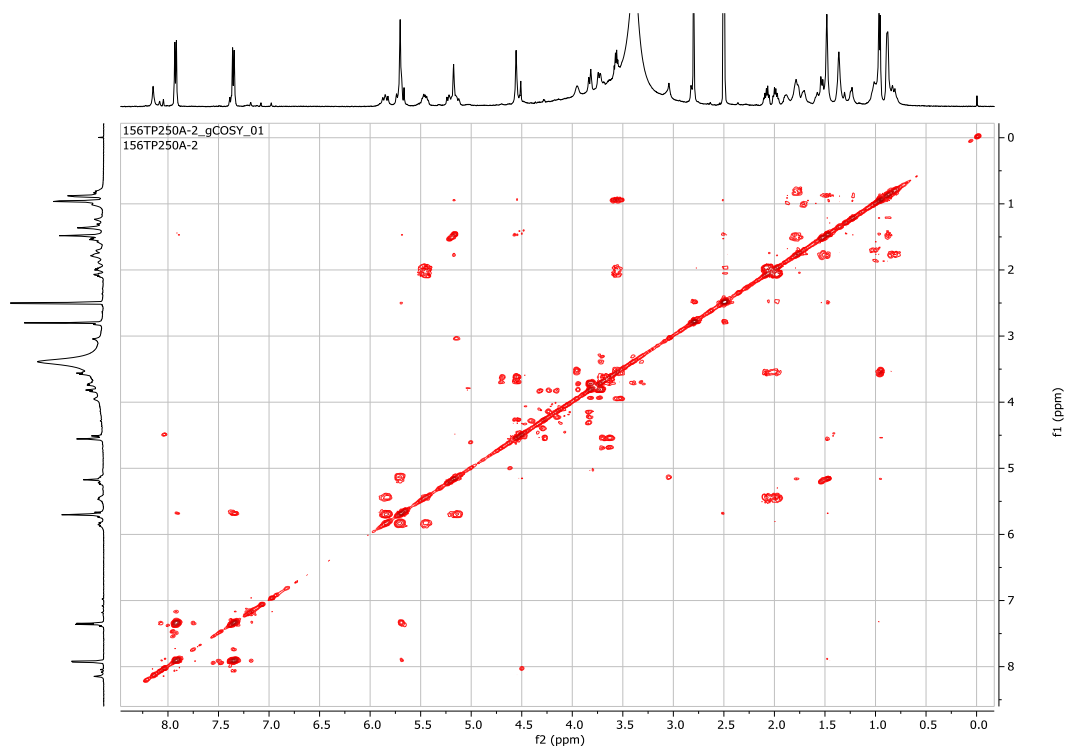


Figure C.193. COSY (500 MHz, DMSO) spectrum of **6e'**

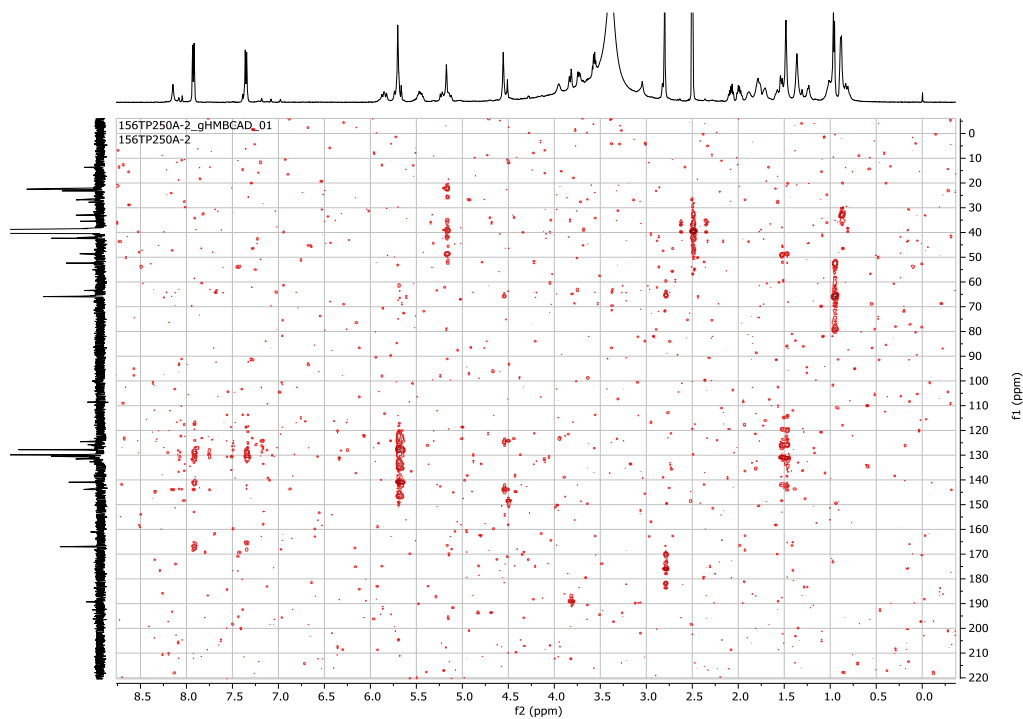


Figure C.194. HMBC (500 MHz, DMSO) spectrum of **6e'**

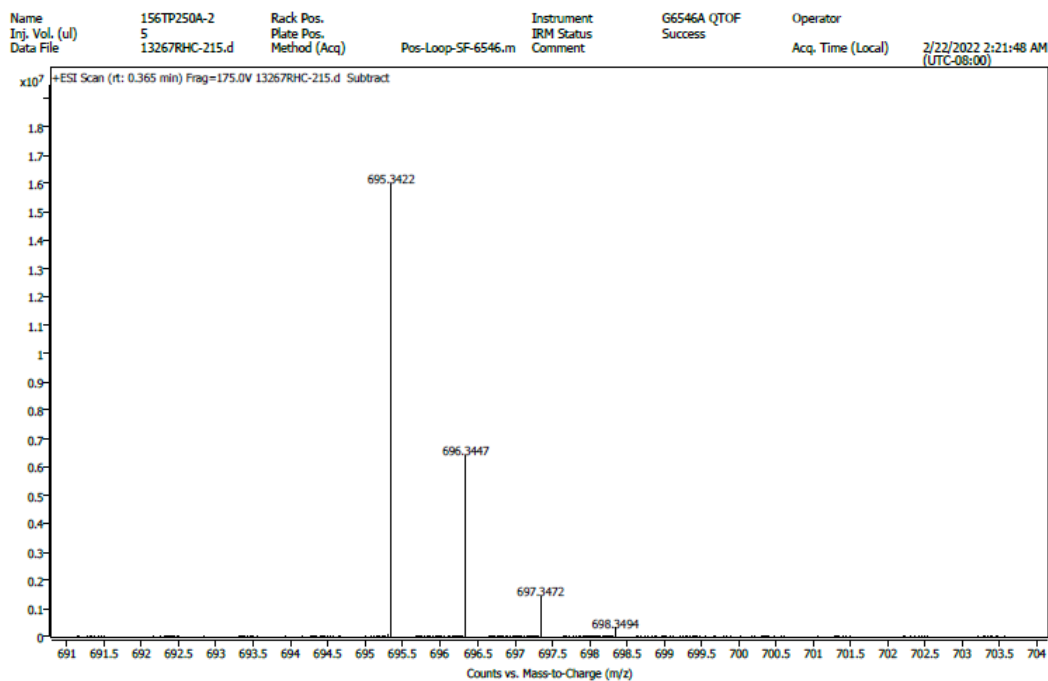


Figure C.195. HRESIMS spectrum of compound **6e'**

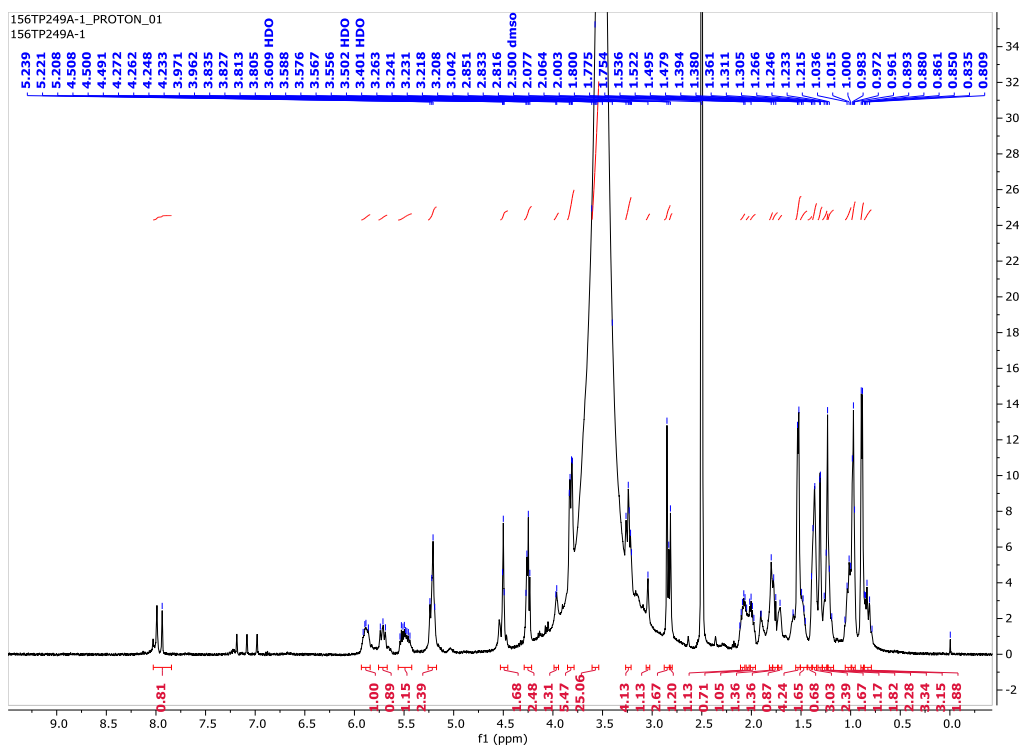


Figure C.196. ^1H NMR (500 MHz, DMSO) of **6f**

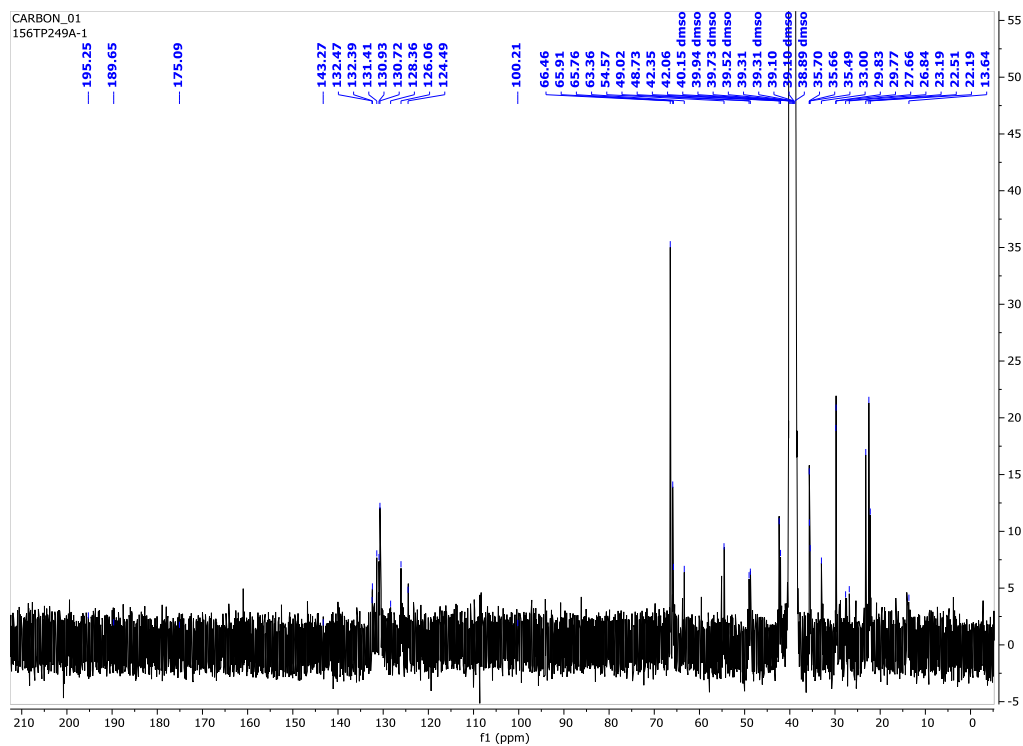


Figure C.197. ^{13}C NMR (400 MHz, DMSO) of **6f**

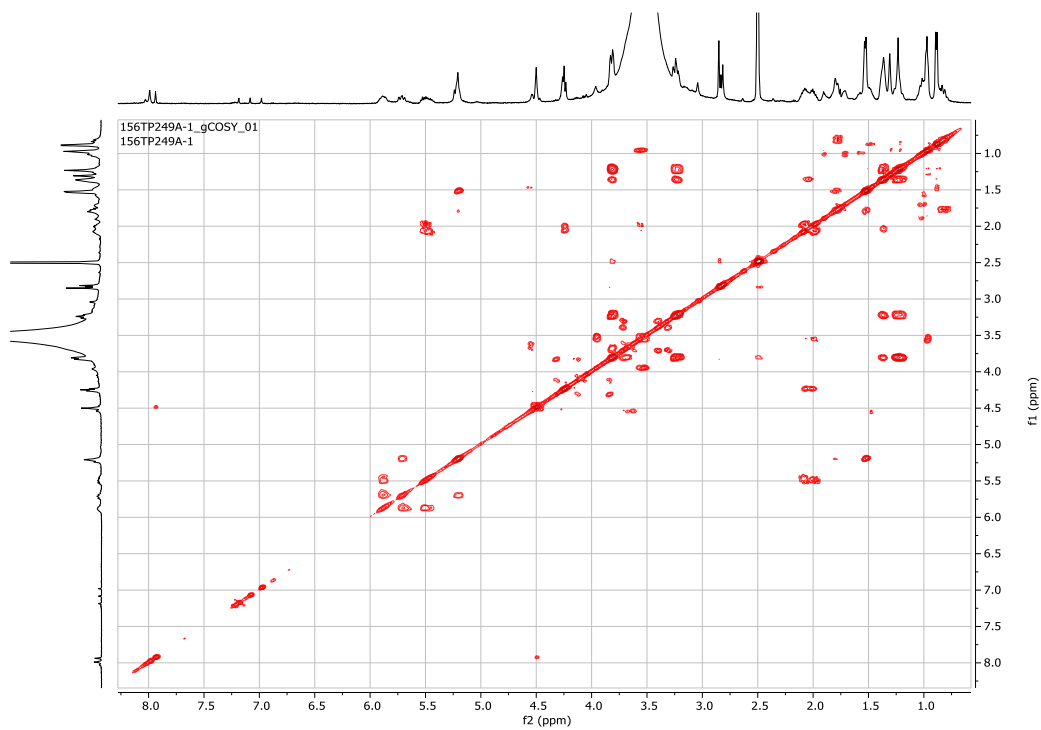


Figure C.198. COSY (500 MHz, DMSO) spectrum of **6f**

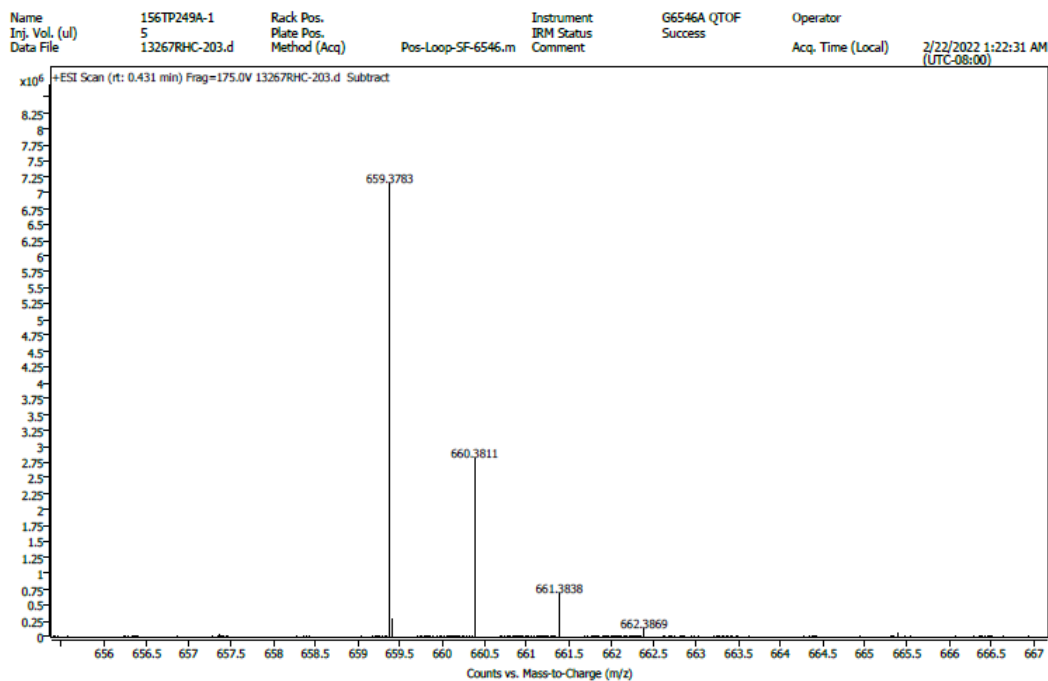


Figure C.199. HRESIMS spectrum of compound **6f**

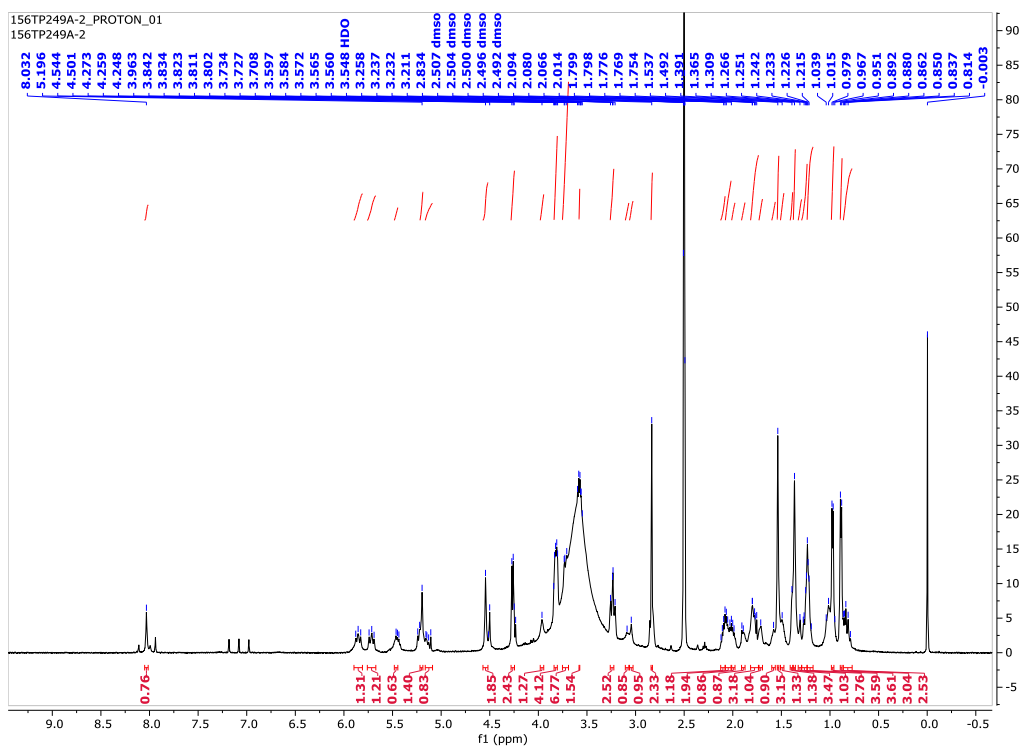


Figure C.200. ^1H NMR (500 MHz, DMSO) of **6f**

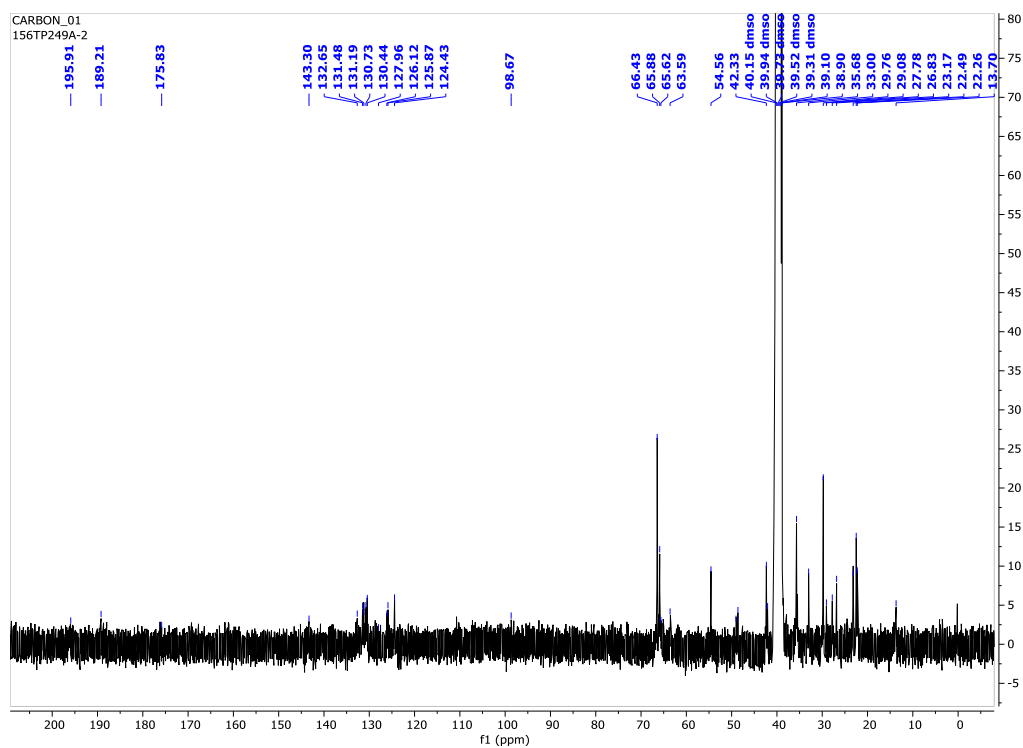


Figure C.201. ^{13}C NMR (400 MHz, DMSO) of **6f'**

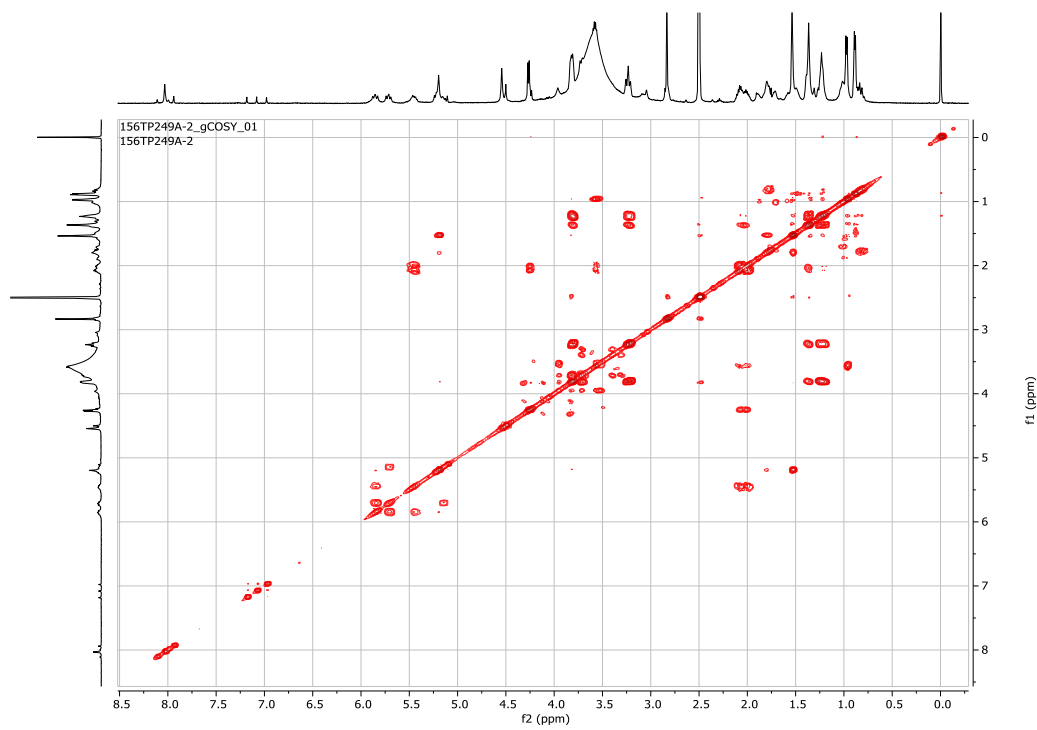


Figure C.202. COSY (500 MHz, DMSO) spectrum of **6f'**

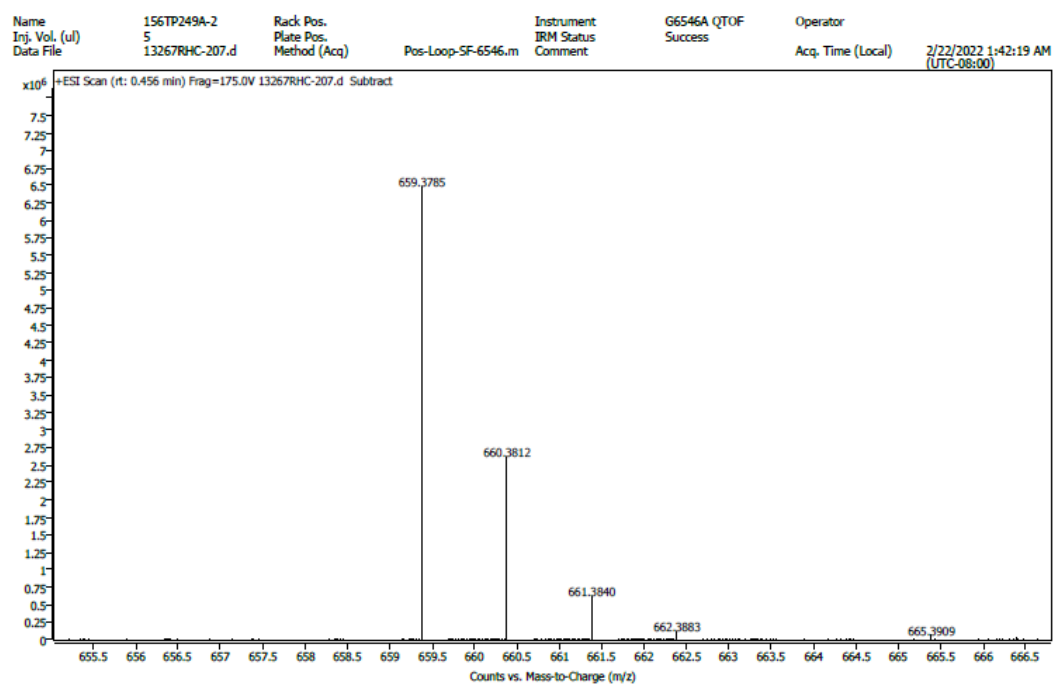


Figure C.203. HRESIMS spectrum of compound **6f'**

Table 5.01. ^1H & ^{13}C (500 MHz) NMR data for compounds **1a** & **1b** in $\text{DMSO-}d_6$

No	1a	1a	1b	1b
1		196.3		197.1
2		48.9		49.2
3	3.51, m	49.3	3.17, m	48.6
4		131.8		131.8
5	5.17, s	126.2	5.16, s	125.8
6	1.62, m	39.0	1.74, m	38.7
7ax	0.82, m	42.6	1.01, m	42.3
7eq	1.77, m		1.67, m	
8	1.48, m	33.2	1.42, m	33.2
9ax	1.73, m	35.8	1.77, m	35.8
9eq	0.82, m		0.82, m	
10ax	1.02, m	27.6	0.95, m	27.7
10eq	1.71, m		1.70, m	
11	1.22, m	39.7	1.51, m	39.9
12	1.31, s	14.5	1.28, s	14.0
13	5.18, m	133.6	5.21, m	132.1
14	4.58, m	129.1	5.30, m	128.4
15	3.48, m	76.3	4.03	82.5
16	3.26, m	70.1	3.25, m	70.0
17	0.77, d (6.5)	18.7	0.77, d (6.5)	18.0
18	1.55, s	22.6	1.53, s	22.3
19	0.88, d (6.5)	23.1	0.87, d (6.5)	22.6
2'		174.2		176.7
3'		99.8		99.5
4'		191.2		190.7
5'	3.51, m	64.2	3.51, m	67.4
6'	3.79, m	58.6	3.79, m	59.4
	3.67, m		3.78, m	
N-Me	2.90, s		2.86, s	27.0
7'		27.5		

Table 5.02. ^1H & ^{13}C (500 MHz) NMR data for compounds **2a** & **2b** in $\text{DMSO-}d_6$

No	2a	2a	2b	2b
1		195.4		200.7
2		48.2		62.1
3	1.65, m	54.6		138.2
4		136.0		127.2
5	5.00, s	124.8	1.79, m, 1.90, m	34.1
6	1.69	39.8	1.33, m,	39.5
7ax	0.98, m	43.8	1.25, m	40.1
7eq	1.65		1.32, m	
8	1.42, m	34.0	1.53, m	33.1
9ax	1.68, m	32.0	1.66, m	36.4
9eq	1.01, m		1.28, m	
10ax	0.97, m	27.1	0.78, m	25.7
10eq	1.84, m		1.79, m	
11	1.43, m	37.8	1.53, m	39.6
12	1.17, s	13.5	1.13, s	13.4
13	1.14, m	29.6	2.08, m	24.9
14	1.20, m	31.6	1.93, m	28.1
15	1.20, m	32.5	1.31, m	32.3
16	1.13, m	21.4	1.29, m	23.0
17	0.76, t (7.0)	18.9	0.85, t (7.5)	15.0
18	1.61, s	23.2	1.61, s	24.1
19	0.86, d (6.5)	19.7	0.87, d (7.0)	23.0
2'		179.0		178.1
3'		102.4		101.8
4'		190.5		190.6
5'	3.67, brd	61.2	3.91, d (12)	62.6
6'	3.56, m	59.4	3.80, d (12)	60.3
	3.53, m		3.59, brs	
N-Me	2.79, s	27.3	3.02, s	28.2

Table 5.03. ^1H & ^{13}C (500 MHz) NMR data for compounds **2c** & **2d** in $\text{DMSO-}d_6$

No	2c	2c	2d	2d
1		198.4		198.6
2		60.3		60.1
3	1.29, m	50.4	1.28, m	51.3
4	1.81, m	31.7	1.81, m	30.6
5	1.55, m, 0.69, m	45.4	1.54, m, 0.67, m	47.8
6	1.09, m	32.6	0.94, m	30.7
7ax	1.35, m	44.6	1.53, m	43.9
7eq	0.81, m		1.27, m	
8	1.37, m	30.7	1.28, m	33.9
9ax	1.64, m	35.8	1.62, m	36.2
9eq	0.92, m		0.92, m	
10ax	1.36, m	25.8	1.40, m	25.7
10eq	1.80, m		1.54, m	
11	1.47, m	39.6	1.64, m	40.7
12	1.44, d (5.5)	16.0	1.35, d (5.5)	16.7
13	1.09, m	25.2	1.09, m	25.4
14	1.28, m	27.6	1.22	27.2
15	1.26, m	31.1	1.12	32.7
16	1.13, m	23.2	1.11, m	23.4
17	0.76, t (7.0)	19.5	0.74, t (7.0)	20.1
18	1.15, d (8.0)	19.1	0.79, d (7.0)	22.2
19	0.84, d (6.0)	21.2	0.82, d (6.5)	20.0
2'		179.5		175.1
3'		100.8		100.1
4'		188.7		191.2
5'	3.74, brd	63.2	3.73, d (11.5)	63.6
6'	3.62, m	60.5	3.61, brd	61.2
	3.51, brs		3.61, brd	
N-Me	2.88, s	28.2	2.86, s	27.8

Table 5.04. ^1H & ^{13}C (500 MHz) NMR data for compounds **3a** & **3b** in $\text{DMSO-}d_6$

No	3a	3a	3b	3b
1	3.94, s	60.7	3.93, m	56.0
2		44.4		42.9
3	3.13, m	43.4	3.27, m	71.6
4		123.1		132.6
5	5.27, s	117.8	5.11, s	126.5
6	1.76, m	32.0	1.70, m	38.8
7ax	0.84, m	43.2	0.72, m	42.0
7eq	1.82, m		1.70, m	
8	1.50, m	33.7	1.40, m	32.5
9ax	1.62, m	38.8	1.70, m	35.7
9eq	1.01, m		1.03, m	
10ax	0.83, m	25.1	1.02, m	27.8
10eq	1.86, m		2.04, m	
11	1.61, m	47.9	1.45, m	45.7
12	0.92, m	13.8	0.90, s	14.4
13	5.32, m	122.5	5.46, m	130.9
14	5.81, m	123.6	5.86, m	132.6
15	5.97, m	123.5	6.02, m	130.9
16	5.56, m	119.1	5.56, m	126.5
17	1.72, d (7.0)	18.6	1.69, d (7.5)	17.9
18	1.62, s	21.8	1.49, s	22.1
19	0.93, m	25.3	0.85, d (6.5)	22.6
2'		160.9		175.0
3'		100.5	2.92, t (5.5)	42.0
4'		201.6	4.03, m	42.2
5'	3.62, m	62.1	3.21, m	69.1
6'	3.63, m	64.3	3.48, m	70.5
	3.38, m		3.54, m	
N-Me	2.91, s	27.3	2.73, s	27.4

Table 5.05. ^1H & ^{13}C (500 MHz) NMR data for compounds **4a** & **4b** in $\text{DMSO-}d_6$

No	4a	4a	4b	4b
1		196.0		205.7
2		49.1		57.0
3	3.16, d (4.5)	48.8	2.40, d (9.0)	55.8
4		132.4		133.9
5	5.19, s	126.8	5.17, s	126.1
6	1.79, m	36.8	1.63, m	38.3
7ax	0.80, m	43.8	0.89, m	41.6
7eq	1.77, m		1.65, m	
8	1.48, m	33.0	1.44, m	32.8
9ax	1.69, m	34.4	1.30, m	35.3
9eq	0.99, m		0.90, m	
10ax	1.13, m	28.8	1.33, m	26.6
10eq	1.87, m		1.65, m	
11	1.56, m	38.6	1.50, m	39.4
12	1.29, s	14.8	1.03, s	14.4
13	5.14, m	128.2	5.14, m	131.2
14	5.72, m	134.1	5.92, m	131.4
15	5.88, m	131.8	5.98, m	131.5
16	5.52, m	126.8	5.61, m	127.7
17	1.63, d (7.0)	17.7	1.72, d (6.5)	17.9
18	1.52, s	20.1	1.49, s	22.4
19	0.88, d (6.5)	22.7	0.86, d (6.5)	22.6
2'		173.2		168.7
3'		102.4		126.1
4'		187.0		202.2
5'	4.49, d (12.0)	67.0	2.94, d (13.0)	69.7
6'	4.18, brs	61.5	3.30, d (13.0)	57.0
	4.04, brs			
N-Me	2.89, s	28.5	2.67, s	27.2
7'		170.3		70.0
8'	1.93, s	21.5		131.8
9'			7.28, m	128.4
10'			7.28, m	129.8
11'			6.96, dd (7.5, 3.5)	127.6

Table 5.06. ^1H & ^{13}C (500 MHz) NMR data for compounds **4c** & **4d** in $\text{DMSO-}d_6$

No	4c	4c	4d	4d
1		203.0		201.7
2		50.1		56.0
3	3.29, brs	49.9	2.30, d (9.5)	52.9
4		132.1		131.9
5	5.22, s	127.0	5.16, s	126.1
6	1.77, m	39.9	1.62, m	38.8
7ax	0.86, m	42.5	0.74, m	42.5
7eq	1.83, m		1.74, m	
8	1.47, m	34.2	1.42, m	33.6
9ax	1.05, m	36.5	0.73, m	35.3
9eq	1.75, m		0.81, m	
10ax	1.27, m	28.9	1.30, m	29.1
10eq	1.79, m		1.60, m	
11	1.51, m	40.0	1.28, m	40.6
12	1.38, s	14.2	0.90, s	15.3
13	5.26, m	132.3	5.09, m	129.3
14	5.78, m	133.4	5.95, m	133.1
15	5.90, m	131.6	5.93, m	132.4
16	5.57, m	129.3	5.58, m	128.1
17	1.67, d (7.0)	18.1	1.68, d (7.0)	18.2
18	1.55, s	22.5	1.48, s	23.1
19	0.91, s (6.5)	22.7	0.86, d (7.0)	22.9
2'		179.1		173.3
3'		101.2		101.2
4'		196.5		190.0
5'	3.67, m	70.1	4.92, t (6.0)	70.0
6'	3.73, m	67.3	2.62, m	68.3
			2.55, m	
N-Me	2.95, s	27.4	2.90, s	27.4
7'	3.27, s	59.4	3.79, dd (11.0, 3.5)	67.8
8'			3.65, m	119.1
9'			5.29, t (5.0)	138.3
10'				24.8
11'			1.45, s	18.2

Table 5.07. ^1H & ^{13}C (500 MHz) NMR data for compounds **4e** DMSO- d_6

No	4e	4e
1		197.1
2		49.1
3	3.42, brs	48.4
4		131.5
5	5.19, s	126.4
6	1.78, m	38.1
7ax	0.84, m	44.3
7eq	1.79, m	
8	1.47, m	31.9
9ax	1.73, m	34.5
9eq	1.00, m	
10ax	1.32, m	27.8
10eq	1.67, m	
11	1.51, m	39.3
12	1.31, s	13.6
13	5.20, m	131.2
14	5.72, m	132.4
15	5.90, m	131.9
16	5.53, m	128.4
17	1.63, d (7.0)	19.7
18	1.51, s	22.4
19	0.87, d (6.5)	22.7
2'		168.7
3'		99.0
4'		194.3
5'	3.84, brd	66.2
6'	3.73, brs	64.3
	3.46, brs	
N-Me	2.91, s	29.7
7'	4.12, s	66.1
8'	3.49, s	80.0
9'		77.3
10'		
11'		

Table 5.08. ^1H & ^{13}C (500 MHz) NMR data for compounds **5a** & **5a'** in $\text{DMSO-}d_6$

No	5a	5a	5a'	5a'
1		196.6		196.7
2		49.7		48.7
3	3.13, m	48.6	3.08, m	48.6
4		131.3		131.6
5	5.19, s	126.1	5.19, s	125.7
6	1.82, m	38.7	1.81, m	38.7
7ax	0.83, m	42.1	0.85, m	42.1
7eq	1.77, m		1.79, m	
8	1.56, m	32.9	1.52, m	33.1
9ax	1.72, m	35.4	1.72, m	35.5
9eq	0.98, m		0.99, m	
10ax	1.01, m	27.7	1.01, m	27.7
10eq	1.91, m		1.89, m	
11	1.53, m	39.5	1.56, m	39.5
12	1.31, s	13.6	1.36, s	13.7
13	5.20, m	130.1	5.13, m	130.3
14	5.72, m	132.2	5.68, m	132.4
15	5.90, m	131.3	5.85, m	131.4
16	5.52, m	128.2	5.38, m	128.0
17	1.63, d (6.5)	17.8	1.62, d (6.5)	17.9
18	1.52, s	22.1	1.53, s	22.2
19	0.88, d	22.4	0.88, d (6.5)	22.4
2'		176.5		176.4
3'		101.5		99.9
4'		180.5		180.1
5'	3.95, brs	65.7	3.96, brs	65.5
6'	3.83, m	66.5	3.84, m	66.0
	3.70, m		3.72, m	
N-Me	2.85, s	26.9	2.85, s	26.9
7'	4.50, brs	63.5	4.53, brs	63.7
8'		143.6		143.1
9'	8.00, s	124.5	8.00, s	124.5
10'				
11'				
12'				
13'	4.38, t (5.0)	52.3	4.38, t (5.5)	52.1
14'	3.75, t (5.0)	59.9	3.75, t (5.5)	59.9
15'				

Table 5.09. ^1H & ^{13}C (500 MHz) NMR data for compounds **5b** & **5b'** in $\text{DMSO-}d_6$

No	5b	5b	5b'	5b'
1		199.2		202.6
2		48.7		48.7
3	3.11, m	48.5	3.32, m	48.5
4		131.0		131.2
5	5.20, s	126.0	5.19, s	125.8
6	1.80, m	38.6	1.78, m	38.3
7ax	0.83, m	42.2	0.84, m	42.0
7eq	1.78, m		1.78, m	
8	1.49, m	32.9	1.50, m	33.1
9ax	1.72, m	35.5	1.70, m	35.3
9eq	0.99, m		1.00, m	
10ax	1.00, m	27.7	1.03, m	27.7
10eq	1.89, m		1.88, m	
11	1.51, m	39.5	1.52, m	39.6
12	1.29, s	13.6	1.35, s	13.8
13	5.16, m	130.2	5.13, m	130.1
14	5.70, m	132.1	5.67, m	132.1
15	5.83, m	131.2	5.83, m	131.3
16	5.51, m	128.0	5.37, m	128.0
17	1.63, d (6.5)	17.8	1.62, d (6.5)	17.9
18	1.51, s	22.1	1.52, s	22.2
19	0.87, d (6.0)	22.5	0.88, d (6.5)	22.5
2'		175.9		177.3
3'	3.92, brs	99.8		98.9
4'	3.70, m	180.8		181.8
5'	3.82, m	65.7	3.94, brs	65.5
6'	2.83, s	66.1	3.73, m	65.9
	4.49, s		3.84, m	
N-Me		26.8	2.85, s	26.9
7'		63.5	4.52, s	63.7
	8.00, s			
8'		143.3		143.3
9'		124.0	8.04, s	124.0
10'				
11'	4.38, t (7.0)			
12'	1.93, m			
13'	3.37, m	46.6	4.39, t (7.0)	46.6
14'		33.0	1.94, m	33.0
15'		57.4	3.39, m	57.4

Table 5.10. ^1H & ^{13}C (500 MHz) NMR data for compounds **5c** & **5c'** in $\text{DMSO-}d_6$

No	5c	5c	5c'	5c'
1		201.0		196.1
2		48.6		49.3
3	3.14, m	48.2	3.08, m	48.5
4		132.2		131.2
5	5.20, s	126.0	5.18, s	125.8
6	1.79, m	38.6	1.80, m	38.9
7ax	0.85, m	42.0	0.84, m	42.0
7eq	1.77, m		1.78, m	
8	1.50, m	32.9	1.50, m	33.0
9ax	1.71, m	35.4	1.71, m	35.4
9eq	0.99, m		1.03, m	
10ax	0.97, m	27.7	0.98, m	27.7
10eq	1.87, m		1.90, m	
11	1.51, m	39.5	1.50, m	39.1
12	1.31, s	14.0	1.36, s	14.0
13	5.18, m	130.0	5.13, m	130.2
14	5.72, m	132.6	5.67, m	132.4
15	5.89, m	131.3	5.84, m	131.4
16	5.52, m	128.2	5.37, m	128.0
17	1.63, d (6.5)	17.8	1.62, d (6.5)	17.9
18	1.52, s	22.1	1.52, s	22.2
19	0.88, d (6.5)	22.4	0.88, d (6.5)	22.5
2'		174.6		174.5
3'		97.4		100.0
4'		180.1		180.4
5'	3.95, brs	65.5	3.95, brs	63.4
6'	3.70, m	66.0	3.73, m	65.9
	3.83, m		3.83, m	
N-Me	2.85, s	26.6	2.85, s	26.6
7'	4.50, s	63.5	4.53, s	63.7
8'		143.6		143.4
9'	8.02, s	123.9	8.04, s	123.8
10'				
11'				
12'				
13'	4.35, m	49.2		
14'	1.82, m	24.5		
15'	1.49, m	29.0		

16'	3.39, m	59.9		
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Table 5.11. ^1H & ^{13}C (500 MHz) NMR data for compounds **5d** & **5d'** in DMSO- d_6

No	5d	5d	5d'	5d'
1		202.0		199.8
2		48.7		48.4
3	3.31, m	48.2	3.09, m	48.0
4		131.2		131.2
5	5.19, s	126.0	5.17, s	126.8
6	1.76, m	38.1	1.78, m	38.9
7ax	0.78, m	42.1	0.79, m	42.1
7eq	1.77, m		1.77, m	
8	1.47, m	33.0	1.48, m	33.0
9ax	1.68, m	35.0	1.68, m	35.5
9eq	1.00, m		1.00, m	
10ax	1.02, m	26.9	0.99, m	26.9
10eq	1.90, m		1.89, m	
11	1.52, m	39.5	1.52, m	39.1
12	1.23, s	14.0	1.35, s	14.1
13	5.17, m	130.3	5.12, m	130.2
14	5.71, m	132.2	5.68, m	132.6
15	5.88, m	131.3	5.83, m	131.6
16	5.50, m	128.2	5.36, m	125.8
17	1.63, d (6.5)	17.8	1.62, d (6.5)	17.9
18	1.52, s	22.1	1.52, s	22.2
19	0.88, d (7.0)	22.5	0.88, d (6.5)	22.5
2'		172.9		176.3
3'		96.9		99.7
4'		182.1		184.9
5'	3.83, m	65.3	3.94, m	65.2
6'	3.70, m	67.0	3.83, m	66.0
	3.65, m		3.72, m	
N-Me	2.83, s	26.9	2.84, s	26.9
7'	4.49, s	63.4	4.53, s	63.7
8'		143.3		143.5
9'	8.01, s	123.8		123.8
10'			8.04, s	
11'				
12'				
13'	4.32, m	49.3	4.32, m	49.3
14'	1.78, m	28.0	1.79, m	28.0
15'	1.19, m	29.4	1.19, m	29.4

16'	1.29, m	21.5	1.28, m	21.5
17'	0.84, m	13.8	0.83, m	13.8

Table 5.12. ^1H & ^{13}C (500 MHz) NMR data for compounds **5e** & **5e'** in DMSO- d_6

No	5e	5e	5e'	5e'
1		197.9		197.6
2		48.6		48.5
3	3.09, m	48.1	3.16, d (5.0)	48.6
4		132.0		131.8
5	5.18, s	126.5	5.09, s	127.2
6	1.80, m	38.3	1.80, m	38.5
7ax	0.80, m	42.6	0.80, m	42.4
7eq	1.79, m		1.76, m	
8	1.47, m	33.4	1.46, m	33.6
9ax	1.70, m	35.7	0.98, m	35.4
9eq	1.00, m		1.70, m	
10ax	0.86, m	27.4	0.84, m	27.6
10eq	1.72, m		1.72, m	
11	1.51, m	39.6	1.51, m	39.4
12	1.23, s	14.2	1.23, s	14.1
13	5.20, m	131.6	5.13, m	131.8
14	5.69, m	133.7	5.60, m	133.4
15	5.86, m	132.8	5.84, m	131.9
16	5.51, m	126.7	5.36, m	126.8
17	1.62, d (6.5)	17.6	1.62, d (6.5)	17.4
18	1.51, s	22.4	1.50, s	22.1
19	0.88, d (6.5)	22.7	0.78, d (6.5)	22.6
2'		172.0		171.8
3'		99.3		97.7
4'		180.3		180.5
5'	3.80, m	66.4	3.83, m	65.6
6'	3.55, m	67.5	3.52, m	67.4
	3.47, m		3.36, m	
N-Me	2.78, s	27.4	2.80, s	27.2
7'	4.49, s	64.2	4.53, m	63.8
8'	8.02, s	144.6		146.0
9'		125.2	8.07, s	126.2
10'				
11'				
12'				
13'	5.48, s	53.2	5.49, m	53.3

14'		130.0		130.0
15'	6.88, s	126.0	6.89, s	126.5
16'		138.3		136.4
17'	6.95, s	126.0	6.95, s	126.1
18'	2.23, s	21.4	2.23, s	21.5

Table 5.13. ^1H & ^{13}C (500 MHz) NMR data for compounds **5f** & **5f'** in $\text{DMSO}-d_6$

No	5f	5f	5f'	5f'
1		198.1		198.7
2		50.0		50.1
3	3.18, m	50.2	3.13, m	50.0
4		131.6		132.6
5	5.22, s	127.1	5.22, s	127.0
6	1.86, m	40.0	1.86, m	40.0
7ax	1.03, m	42.1	1.07, m	43.1
7eq	1.78, m		1.76, m	
8	1.52, m	33.3	1.51, m	34.2
9ax	0.86, m	35.6	0.88, m	36.6
9eq	1.83, m		1.80, m	
10ax	1.06, m	27.6	1.03, m	27.5
10eq	1.96, m		1.97, m	
11	1.56, m	40.5	1.57, m	40.5
12	1.37, s	14.5	1.41, s	14.3
13	5.26, m	131.6	5.19, m	131.7
14	5.78, m	133.6	5.75, m	133.6
15	5.90, m	132.1	5.86, m	132.2
16	5.58, m	129.4	5.45, m	129.1
17	1.66, d (6.5)	18.1	1.64, d (6.5)	18.2
18	1.57, s	22.5	1.56, s	22.5
19	0.90, d (6.5)	22.7	0.91, d (6.5)	22.6
2'		176.6		124.6
3'		102.3		101.2
4'		180.5		177.8
5'	3.83, brd	66.8	3.82, m	66.7
6'	3.75, m	67.8	3.76, m	67.4
	3.72, m		3.78, m	
N-Me	2.89, s	27.6	2.88, s	27.5
7'	4.53, brs	64.3	4.58, brs	64.3
8'		143.1		143.9
9'	7.60, s	125.2	7.65, s	125.2

10'				
11'				
12'				
13'	4.56, t (7.0)	53.0	4.56, t (7.0)	53.1
14'	3.08, t (7.0)	36.0	3.09, t (7.0)	35.9
15'		128.5		129.1
16'	6.95, d (8.0)	130.8	6.96, d (8.5)	130.7
17'	6.70, d (8.0)	116.3	6.70, d (8.0)	116.2
18'		155.8		156.8

Table 5.14. ^1H & ^{13}C (500 MHz) NMR data for compounds **5g** & **5g'** in $\text{DMSO-}d_6$

No	5g	5g	5g'	5g'
1		199.2		200.6
2		49.0		49.0
3	3.33, m	49.4	3.33, m	49.1
4		132.6		131.4
5	5.19, s	126.5	5.17, s	126.4
6	1.79, m	38.6	1.81, m	38.4
7ax	0.82, m	41.2	0.82, m	41.1
7eq	1.78, m		1.80, m	
8	1.47, m	33.7	1.51, m	33.8
9ax	1.70, m	35.4	1.75, m	35.3
9eq	0.99, m		1.00, m	
10ax	1.00, m	27.5	1.20, m	27.7
10eq	1.86, m		1.83, m	
11	1.51, m	39.8	1.48, m	39.1
12	1.23, s	14.2	1.23, s	14.2
13	5.18, m	131.4	5.13, m	131.5
14	5.73, m	133.4	5.67, m	133.6
15	5.87, m	131.6	5.84, m	131.7
16	5.49, m	128.9	5.39, m	128.5
17	1.62, d (6.5)	18.2	1.60, d (6.5)	18.4
18	1.51, s	22.0	1.49, s	22.4
19	0.88, d (6.5)	22.4	0.88, d (6.0)	22.6
2'		172.3		176.5
3'		98.6		96.6
4'		181.8		189.7
5'	3.90, brs	66.7	3.94, brs	66.1
6'	3.81, m	67.7	3.73, m	67.8
	3.69, m		3.83, m	
N-Me	2.81, s	26.4	2.81, s	27.4
7'	4.50, m	64.5	4.54, s	65.1

8'		144.6		144.9
9'	8.08, s	125.1	8.15, s	124.6
10'				
11'				
12'				
13'	5.69, s	36.2	5.70, s	36.6
14'		141.4		141.4
15'	7.37, d (8.0)	128.5	7.35, d (7.5)	128.4
16'	7.92, d (7.5)	131.7	7.92, d (8.0)	131.0
17'		129.4		130.5
18'		168.0		167.3

Table 5.15. ^1H & ^{13}C (500 MHz) NMR data for compounds **5h** & **5h'** in $\text{DMSO-}d_6$

No	5h	5h	5h'	5h'
1		196.0		196.0
2		49.5		48.8
3	3.14, m	42.0	3.10, m	48.4
4	5.20, s	131.3	5.19, s	131.3
5	1.82, m	126.9	1.82, m	126.9
6	0.81, m	38.9	0.82, m	38.9
7ax	1.81, m	42.0	1.80, m	42.0
7eq	1.52, m		1.49, m	
8	0.83, m	32.9	0.85, m	32.9
9ax	1.79, m	35.4	1.80, m	35.5
9eq	1.00, m		1.00, m	
10ax	1.92, m	27.6	1.90, m	27.7
10eq	1.54, m		1.51, m	
11	1.31, s	41.9	1.36, s	40.7
12	5.22, m	14.0	5.14, m	13.7
13	5.70, m	130.1	5.70, m	130.1
14	5.90, m	128.2	5.84, m	132.1
15	5.53, m	132.1	5.40, m	130.9
16	1.64, d (6.5)	131.3	1.61, d (7.0)	127.9
17	1.53, s	17.8	1.51, s	17.9
18	0.88, d (6.5)	22.1	0.88, d (6.5)	22.2
19		22.5		22.5
2'		175.9		174.9
3'		108.5		100.1
4'		189.5		189.2
5'	3.93, brs	65.7	3.93, brs	65.4
6'	3.77, m	65.9	3.79, m	65.8
	3.67, m		3.69, m	

N-Me	2.81, s	26.0	2.81, s	26.0
7'	4.48, s	63.4	4.50, s	63.6
8'		143.2		143.2
9'	7.99, s	128.2	8.03, s	127.9
10'				
11'				
12'				
13'	4.62, t (7.5)	50.0	4.62, t (7.5)	50.0
14'	3.25, t (7.5)	27.6	3.26, t (7.5)	27.7
15'		111.4		111.4
16'	7.08, s	123.9	7.09'	123.9
NH	10.85, s		10.85, s	
17'		136.1		136.1
18'	7.33, d (8.0)	109.9	7.33, d (8.00)	109.9
19'	7.06, t (7.5)	123.2	6.98, t (7.5)	123.2
20'	6.98, t (7.5)	118.4	7.06, t (8.0)	118.4
21'	7.51, d (8.0)	118.1	7.52, d (8.0)	118.1
22'		126.0		125.8

Table 5.16. ^1H & ^{13}C (500 MHz) NMR data for compounds **5i** & **5i'** in $\text{DMSO-}d_6$

No	5i	5i	5i'	5i'
1		196.2		196.1
2		48.8		48.7
3	3.15, m	48.7	3.02, d (9.5)	48.5
4		131.4		131.4
5	5.20, s	126.0	5.19, s	125.8
6	1.78, m	38.9	1.82, m	38.9
7ax	0.80, m	42.0	0.83, m	42.0
7eq	1.77, m		1.79, m	
8	1.51, m	33.0	1.51, m	33.0
9ax	1.01, m	35.5	1.02, m	35.4
9eq	1.72, m		1.73, m	
10ax	1.03, m	27.6	1.03, m	27.7
10eq	1.90, m		1.91, m	
11	1.54, m	40.9	1.54, m	39.1
12	1.30, s	13.6	1.36, s	13.6
13	5.15, m	130.0	5.13, m	130.2
14	5.71, m	132.1	5.69, m	132.4
15	5.89, m	130.9	5.85, m	131.4
16	5.51, m	128.1	5.41, m	127.9
17	1.63, d (6.5)	17.8	1.63, d (7.0)	17.9
18	1.52, s	22.1	1.54, s	22.1
19	0.89, d (6.5)	22.5	0.88, d (6.5)	22.4
2'		175.6		176.0
3'		99.2		100.0
4'		189.5		189.2
5'	3.94, brs	65.8	3.95, brs	65.5
6'	3.83, m	66.1	3.72, m	65.9
	3.68, m		3.62, m	
N-Me	2.85, s	26.8	2.85, s	26.9
7'	4.50, s	63.5	4.54, s	63.6
8'		143.3		143.2
9'	7.95, s	124.6	7.99, s	124.6
10'				
11'				
12'				
13'	4.47, m	53.1	4.46, m	53.1
	4.35, m		4.36, m	
14'	4.16, pent (7.0)	76.8	4.17, pent (6.5)	76.7
15'	1.95, m	67.5	1.95, m	67.5

	1.73, m		1.70, m	
16'	1.80, m	25.0	1.80, m	25.0
	1.68, m		1.56, m	
NH				
17'	3.71, m	28.1	3.72, m	28.1
	3.62, m		3.83, m	

Table 5.17. ^1H & ^{13}C (500 MHz) NMR data for compounds **5j** & **5j'** in $\text{DMSO-}d_6$

No	5j	5j	5j'	5j'
1		195.9		196.0
2		54.5		48.6
3	3.13, m	48.6	3.08, m	48.5
4		131.3		131.4
5	5.21, s	125.9	5.20, s	125.8
6	1.82, m	38.9	1.81, m	39.0
7ax	1.01, m	42.0	1.01, m	42.0
7eq	1.90, m		1.89, m	
8	1.53, m	33.0	1.49, m	32.9
9ax	0.81, m	35.6	0.83, m	35.6
9eq	1.79, m		1.88, m	
10ax	1.00, m	27.7	0.97, m	27.7
10eq	1.90, m		1.91, m	
11	1.50, m	39.6	1.52, m	39.6
12	1.30, s	13.5	1.35, s	13.6
13	5.18, m	130.8	5.14, m	130.2
14	5.72, m	132.1	5.68, m	132.3
15	5.90, m	129.9	5.85, m	131.2
16	5.52, m	128.0	5.40, m	127.8
17	1.63, d (6.5)	17.8	1.63, d (6.5)	17.9
18	1.52, s	22.1	1.52, s	22.1
19	0.88, d (6.5)	22.4	0.88, d (6.0)	22.4
2'	3.93, brs	175.3		175.9
3'	3.82, m	100.1		100.0
4'	3.68, m	189.3		189.2
5'		65.5	3.95, brs	64.9
6'		65.7	3.81, m	65.7
			3.72, m	
N-Me	2.85, s	26.7	2.85, s	26.8
7'	4.50, m	63.3	4.53, s	63.6
8'		143.1		143.3
9'	7.99, s	128.0	8.03, s	127.8

10'				
11'				
12'				
13'	4.25, d (7.0)	52.6	4.26, d (7.0)	54.5
14'	2.05, m	27.7	2.05, m	27.7
15'	1.37 m, 1.38 m	29.7	1.38 m, 1.24 m	29.7
	1.24 m, 1.21 m		1.36 m, 1.28 m	
16'	3.82 m, 3.25 m	66.4	3.82 m, 3.25 m	66.4
	3.80 m, 3.20 m		3.79 m, 3.23 m	
19'	7.06, t (7.5)			
20'	6.98, t (7.5)			
21'	7.51, d (8.0)			
22'				

Table 5.18. ^1H & ^{13}C (500 MHz) NMR data for compounds **5k** & **5k'** in $\text{DMSO}-d_6$

No	5k	5k	5k'	5k'
1		196.0		196.1
2		48.8		48.7
3	3.13, m	48.6	3.07, m	48.5
4		131.3		131.4
5	5.20, s	126.0	5.19, s	125.8
6	1.79, m	38.4	1.79, m	38.4
7ax	1.03, m	42.0	1.00, m	42.0
7eq	1.88, m		1.88, m	
8	1.49, m	33.0	1.51, m	32.9
9ax	0.81, m	35.4	0.82, m	35.4
9eq	1.79, m		1.79, m	
10ax	0.96, m	27.7	0.98, m	27.7
10eq	1.91, m		1.71, m	
11	1.52, m	39.5	1.52, m	39.4
12	1.30, s	13.5	1.36, s	13.7
13	5.18, m	128.1	5.13, m	130.1
14	5.71, m	130.8	5.68, m	132.4
15	5.90, m	130.1	5.84, m	131.2
16	5.52, m	128.1	5.38, m	127.9
17	1.63, d (6.5)	17.8	1.62, d (6.5)	17.9
18	1.51, s	22.1	1.52, s	22.1
19	0.88, d (6.0)	22.4	0.88, d (6.5)	22.4
2'		175.9		176.0
3'		99.9		100.0

4'		189.5		189.9
5'	3.93, brs	65.7	3.95, brs	65.5
6'	3.82, m	65.9	3.83, m	65.8
	3.70, m		3.73, m	
N-Me	2.86, s	26.8	2.85, s	26.8
7'	4.50, s	63.4	4.53, s	63.6
8'		140.3		143.3
9'	8.00, s	124.0	8.04, s	123.9
10'				
11'				
12'				
13'	4.27, d (7.5)	53.7	4.27, d (7.0)	53.8
14'	2.35, sept (7.0)	27.7	2.35, sept (7.0)	27.7
15'	1.59, m	29.5	1.59, m	29.5
16'	1.23, m	24.5	1.23, m	24.5
NH				
17'	1.20, m	24.5	1.21, m	24.5
18'	0.86, m	29.5	0.86, m	29.5

Table 5.19. ^1H & ^{13}C (500 MHz) NMR data for compounds **6a** & **6b** in $\text{DMSO-}d_6$

No	6a	6a	6b	6b
1		196.2		196.1
2		48.5		48.7
3	3.13, m	48.7	3.12, m	48.6
4		131.3		131.0
5	5.20, s	126.0	5.21, s	126.1
6	1.80, m	38.9	1.82, m	38.8
7ax	0.83, m	42.0	0.82, m	42.0
7eq	1.79, m		1.78, m	
8	1.52, m	32.9	1.49, m	33.0
9ax	1.70, m	35.4	1.71, m	35.5
9eq	1.02, m		1.03, m	
10ax	1.03, m	26.8	1.05, m	26.8
10eq	1.89, m		1.89, m	
11	1.51, m	39.2	1.51, m	35.5
12	1.33, s	13.5	1.33, s	13.5
13	5.24, m	130.5	5.24, m	132.3
14	5.72, m	132.4	5.73, m	132.0
15	5.90, m	131.4	5.94, m	130.8
16	5.53, m	130.7	5.49, m	129.3
17	2.01, m	42.3	2.08, m	40.8
	2.10, m		2.23, m	
18	3.58, m	65.7	3.57, m	80.9
19	0.98, d (6.0)	23.1	1.02, d (6.5)	19.0
20	1.52, s	22.2	1.53, s	22.2
21	0.88, d (6.0)	22.5	0.88, d (6.5)	22.5
22			4.10, brs	54.9
23				77.8
24			3.47, s	73.4
1'				
2'		176.0		176.0
3'		99.9		100.0
4'		189.4		189.5
5'	3.99, brs	65.6	3.99, s	65.5
6'	3.85, d (10.5)	57.7	3.85, d (10.5)	57.7
	3.75, d (10.5)		3.75, d (10.5)	
N-Me	2.94, s	27.7	2.94, s	27.7
7'	4.13, brs	65.9	4.13, brs	65.7
8'		79.7		79.7
9'	3.47, s	77.7	3.47, s	76.5

Table 5.20. ^1H & ^{13}C (500 MHz) NMR data for compounds **6c** & **6c'** in $\text{DMSO-}d_6$

No	6c	6c	6c'	6c'
1		197.4		197.6
2		50.1		50.2
3	3.11, m	49.8	3.08, m	49.7
4		131.3		131.6
5	5.20, s	126.6	5.18, s	126.4
6	1.78, m	39.3	1.79, m	39.5
7ax	0.82, m	42.3	0.82, m	42.1
7eq	1.77, m		1.79, m	
8	1.48, m	33.4	1.51, m	33.8
9ax	1.70, m	36.1	1.70, m	36.3
9eq	1.03, m		1.02, m	
10ax	1.00, m	28.2	0.84, m	28.3
10eq	1.89, m		1.83, m	
11	1.58, m	39.5	1.54, m	39.7
12	1.32, s	14.10	1.36, s	14.2
13	5.24, m	130.8	5.15, m	130.6
14	5.71, m	132.5	5.71, m	132.7
15	5.88, m	131.7	5.85, m	131.4
16	5.51, m	130.8	5.44, m	130.6
17	2.00, m	42.9	1.99, m	42.7
	2.07, m		2.07, m	
18	3.57, m	66.6	3.56, m	66.4
19	0.98, d (6.5)	23.8	0.96, d (6.0)	23.4
20	1.52, s	22.5	1.52, s	22.6
21	0.88, d (6.0)	22.7	0.87, d (6.0)	22.7
1'				
2'		176.4		176.5
3'		99.8		100.5
4'		182.7		182.9
5'	3.96, brs	65.8	3.95, brs	64.8
6'	3.82, m	66.7	3.84, m	66.5
	3.73, m		3.71, m	
N-Me	2.85, s		2.82, s	27.6
7'	4.51, s	63.4	4.53, s	63.2
8'		143.7		143.6
9'	7.98, s	124.8	8.00, s	125.6
10'				
11'				
12'				

13'	4.38, m	52.8	4.37, m	52.4
14'	3.76, m	60.4	3.75, m	60.3
15'				

Table 5.21. ^1H & ^{13}C (500 MHz) NMR data for compounds **6d** & **6d'** in $\text{DMSO-}d_6$

No	6d	6d	6d'	6d'
1		196.2		196.7
2		48.5		48.8
3	3.12, m	48.7	3.08, m	49.2
4		131.4		132.2
5	5.20, s	127.6	5.19, s	126.8
6	1.80, m	39.1	1.82, m	39.3
7ax	0.82, m	42.1	0.80, m	42.2
7eq	1.75, m		1.79, m	
8	1.50, m	33.0	1.49, m	33.3
9ax	1.70, m	35.0	1.70, m	35.2
9eq	1.01, m		1.01, m	
10ax	0.84, m	27.7	0.85, m	27.6
10eq	1.80, m		1.78, m	
11	1.52, m	39.3	1.51, m	39.5
12	1.32, s	13.5	1.36, s	13.3
13	5.23, m	130.7	5.15, m	130.5
14	5.72, m	132.4	5.71, m	132.4
15	5.86, m	130.9	5.85, m	130.8
16	5.52, m	130.4	5.44, m	130.6
17	1.99, m	42.3	1.99, m	41.8
	2.09, m		2.08, m	
18	3.57, m	66.9	3.57, m	66.0
19	0.98, d (6.0)	23.1	0.97, d (6.5)	23.2
20	1.52, s	22.5	1.52, s	22.4
21	0.88, d (6.5)	22.2	0.88, d (6.0)	22.1
1'				
2'		176.0		176.4
3'		99.9		100.1
4'		180.9		181.1
5'	3.94, brs	65.6	3.94, brs	64.7
6'	3.78, m	65.7	3.78, m	65.4
	3.68, m		3.68, m	
N-Me	2.83, s	26.8	2.81, s	26.5
7'	4.47, s	63.4	4.52, m	63.1
8'		143.1		143.7

9'	7.92, s	124.0	7.96, s	124.8
10'				
11'				
12'				
13'	4.51, m	50.8	4.51, m	51.4
14'	3.00, m	35.5	3.01, m	35.7
15'		129.7		129.7
16'	6.94, d (8.5)	129.6	6.95, d (8.0)	129.6
17'	6.63, d (8.5)	115.2	6.64, d (8.0)	115.2
18'		156.0		157.1

Table 5.22. ^1H & ^{13}C (500 MHz) NMR data for compounds **6e** & **6e'** in $\text{DMSO}-d_6$

No	6e	6e	6e'	6e'
1		196.1		196.2
2		48.7		48.5
3	3.11, m	48.8	3.04, m	48.8
4		130.7		131.2
5	5.19, s	126.0	5.17, s	127.7
6	1.80, m	38.6	1.80, m	38.6
7ax	0.81, m	42.3	0.83, m	42.3
7eq	1.78, m		1.76, m	
8	1.51, m	32.9	1.51, m	33.0
9ax	1.70, m	35.4	1.70, m	35.4
9eq	0.95, m		1.00, m	
10ax	1.00, m	27.7	0.99, m	27.8
10eq	1.86, m		1.87, m	
11	1.51, m	39.1	1.53, m	39.2
12	1.23, s	13.5	1.36, s	13.6
13	5.21, m	130.8	5.22, m	130.8
14	5.72, m	132.3	5.73, m	130.6
15	5.89, m	131.4	5.85, m	131.5
16	5.51, m	130.4	5.45, m	130.4
17	2.00, m	52.0	1.98, m	52.4
	2.07, m		2.08, m	
18	3.57, m	66.4	3.56, m	66.0
19	0.97, d (6.0)	23.1	0.96, d (6.0)	23.1
20	1.52, s	22.2	1.48, s	22.2
21	0.88, d (6.5)	22.5	0.88, brs	22.5
1'				
2'		176.0		175.8

3'		100.0		100.0
4'		189.6		189.2
5'	3.94, brs	65.7	3.95, brs	65.7
6'	3.83, m	66.0	3.83, m	65.9
	3.72, m		3.73, m	
N-Me	2.83, s	26.7	2.80, s	26.8
7'	4.51, m	63.4	4.55, m	63.4
8'		143.9		143.8
9'	8.08, s	124.4	8.14, s	124.6
10'				
11'				
12'				
13'	5.69, s	56.6	5.70, s	56.4
14'		140.9		141.1
15'	7.34, d (7.5)	127.7	7.35, d (8.0)	127.7
16'	7.92, d (8.0)	130.4	7.92, d (8.0)	130.4
17'		129.8		129.8
18'		167.0		167.2

Table 5.23. ^1H & ^{13}C (500 MHz) NMR data for compounds **6f** & **6f'** in DMSO- d_6

No	6f	6f	6f'	6f'
1		195.2		195.9
2		48.7		48.6
3	3.04, m	49.0	3.04, m	49.0
4		13.3		131.4
5	5.21, s	126.0	5.19, s	126.1
6	1.83, m	39.2	1.82, m	38.9
7ax	0.82, m	42.0	0.82, m	42.0
7eq	1.79, m		1.80, m	
8	1.48, m	33.0	1.48, m	33.0
9ax	1.71, m	35.7	1.70, m	35.6
9eq	0.99, m		1.01, m	
10ax	0.84, m	27.6	0.85, m	27.7
10eq	1.77, m		1.79, m	
11	1.51, m	39.3	1.52, m	39.1
12	1.36, brs	13.6	1.36, brs	13.7
13	5.23, m	130.9	5.13, m	130.7
14	5.72, m	132.4	5.71, m	132.6
15	5.89, m	131.4	5.85, m	131.4
16	5.51, m	130.7	5.45, m	130.7

17	1.99, m	42.3	2.00, m	42.3
	2.07, m		2.09, m	
18	3.56, m	65.9	3.58, m	65.8
19	0.97, d (5.5)	23.2	0.97, d (6.0)	23.1
20	1.52, s	22.1	1.53, s	22.2
21	0.88, d (6.5)	22.5	0.88, d (6.0)	22.4
1'				
2'		175.0		175.8
3'		100.2		98.6
4'		189.6		189.2
5'	3.96, brs	65.7	3.96, brs	65.6
6'	3.82, m	65.9	3.82, m	65.8
	3.71, m		3.72, m	
N-Me	2.85, s	26.8	2.83, s	26.8
7'	4.50, s	63.6	4.54, s	63.5
8'		143.2		143.3
9'	7.94, s	128.3	8.03, s	127.9
10'				
11'				
12'				
13'	4.25, m	54.5	4.27, m	54.5
14'	2.03, m	29.8		29.0
15'	1.39, m 1.26, m	29.7	2.06, m	29.7
	1.35, m 1.17, m		1.38, m 1.23, m	
16'	3.83, m 3.25, m	66.4	1.39, m 1.25, m	66.4
	3.78, m 3.21, m		3.80, m 3.22, m	
			3.82, m 3.26, m	