# Evaluation of secondary metabolites from algae and epibiotic bacteria as allelochemicals and/or inducers of coral larval settlement

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# Evaluation of secondary metabolites from algae and epibiotic bacteria as allelochemicals and/or inducers of coral larval settlement

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**In the marine environment chemical signals play critical roles at every organisational level. One emerging area of interest in the field of Marine Chemical Ecology is the role of bacterially derived chemical signals, in particular for the induction of larval settlement of marine invertebrates. Although bacteria have long been recognised to induce larval settlement, to date no inducer of bacterial origin has been fully characterised. In Chapter 2, I describe the isolation of the bacterium *Pseudoalteromonas* J010 from the surface of crustose coralline algae, which trigger the larval settlement of acroporid corals. In a bioassay-guided fashion, I characterised the metamorphic inducer in this bacterium as tetrabromopyrrole (TBP). TBP rapidly induces larval metamorphosis, however the larvae fail to attach to the substratum when exposed to TBP. The toxic nature of this compound suggests that larval metamorphosis to TBP is a stress response, rather than an evolved response to a habitat specific cue. The production of TBP may provide *Pseudoalteromonas* J010 with an advantage to persist in the highly competitive biofilm environment. To further explore this hypothesis, I screened the allelochemical profile of this strain and characterised further bioactive metabolites, including novel korormicin derivatives and a polybrominated pyrrole with anti-larval, anti-bacterial, anti-fungal and anti-protozoal properties (Chapter 3). Because, TBP did not explain the inductive properties of CCA on coral larval settlement, I addressed the origin and characteristics of inductive cues from CCA (Chapter 4). This resulted in the isolation of purified fractions that readily induced complete larval settlement, including i) low molecular weight organic-soluble compounds identified as glycoglycerolipids and ii) high molecular weight polymeric aqueous-soluble cue(s). These common algal metabolites may explain the highly inductive properties of CCA on acroporid coral larval settlement. I further demonstrated that these settlement inducers can be immobilised, enabling controlled settlement of coral larvae on target surfaces. Targeted larval settlement on substrata is common practise in reef rehabilitation, particularly for techniques based on sexual reproduction of corals. To improve current reef rehabilitation techniques, I explored methods to enhance post settlement survival of settled coral spat (Chapter 5).**


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# NOTES

Chapters Two, Three and Four of this thesis comprise of stand-alone papers that have either been published (2) or about to be submitted (relevant publication details and acknowledgements of my co-authors are given at the start of each chapter). I use the terms ‘we’ and ‘our’ throughout these chapters since each paper has at multiple authors. Wherever possible, chapters are formatted consistently despite different journal requirements. Chapter Three aims for a journal (Marine Drugs) that requires a combined section of Results and Discussion, this format was maintained.

## LIST OF PUBLICATIONS

Penesyan A, Tebben J, Lee M, Thomas T, Kjelleberg S, Harder T, Egan S (2011) Identification of the Antibacterial Compound Produced by the Marine Epiphytic Bacterium *Pseudovibrio* sp. D323 and Related Sponge-Associated Bacteria. Mar Drugs 9:1391-1402

*Tebben J, Tapiolas DM, Motti CA, Abrego D, Negri AP, Blackall LL, Steinberg PD, Harder T (2011) Induction of Larval Metamorphosis of the Coral *Acropora millepora* by Tetrabromopyrrole Isolated from a *Pseudoalteromonas* Bacterium. PLoS One 6:8

Siboni N, Abrego D, Seneca F, Motti CA, Andreakis N, Tebben J, Blackall LL, Harder T (2012) Using Bacterial Extract along with Differential Gene Expression in *Acropora millepora* Larvae to Decouple the Processes of Attachment and Metamorphosis. PLoS One 7:e37774

## CONFERENCE PRESENTATIONS

*Tebben J, Harder T, Tapiolas D, Motti Cherie, Abrego D, Negri A, Blackall L, Steinberg P (2010) Characterization of a bacteria-derived Chemical Signal that triggers Larval Metamorphosis in acroporid Corals [Australian Marine Sciences Association Conference 2010, Wollongong]

Abrego D, Motti C, Siboni N, Tapiolas D, Tebben J, Harder T (2012) Scent of a partner: responses of *Symbiodinium* to coral cues [International Coral Reef Symposium (ICRS) 2012, Cairns]

*Harder T, Tebben J, Tapiolas D, Motti C, Siboni N, Abrego D, Steinberg P (2012) Chemical signaling of coral larval settlement and metamorphosis [ICRS]

Siboni N, Abrego D, Seneca F, Motti C, Andreakis N, Tebben J, Harder T, Blackall L (2012) Gene regulation in coral larvae exposed to metamorphic/settlement cues [ICRS]

*Tebben J, Tapiolas D, Motti C, Abrego D, Siboni N, Steinberg P, Harder T (2012) Chemically-enhanced settlement of acroporid coral larvae on artificial surfaces [ICRS]

*Martina E, Tebben J, Streich C, Steinberg P, Harder T, McDougald D (2012) The marine bacterium *Pseudoalteromonas* sp. J10 produces several toxins that vary in their effects on different heterotrophic protists [International Society for Microbial Ecology Conference, Copenhagen]

Abrego D, Motti C, Siboni N, Tapiolas D, Tebben J, Harder T (2012) Scent of a partner: responses of *Symbiodinium* to coral cues [Ocean Sciences Meeting 2012, Salt Lake City]

***relevant to this thesis**

## ABSTRACT

In the marine environment chemical signals play critical roles at every organisational level. One emerging area of interest in the field of Marine Chemical Ecology is the role of bacterially derived chemical signals, in particular for the induction of larval settlement of marine invertebrates. Although bacteria have long been recognised to induce larval settlement, to date no inducer of bacterial origin has been fully characterised. In Chapter 2, I describe the isolation of the bacterium *Pseudoalteromonas* J010 from the surface of crustose coralline algae, which trigger the larval settlement of acroporid corals. In a bioassay-guided fashion, I characterised the metamorphic inducer in this bacterium as tetrabromopyrrole (TBP). TBP rapidly induces larval metamorphosis, however the larvae fail to attach to the substratum when exposed to TBP. The toxic nature of this compound suggests that larval metamorphosis to TBP is a stress response, rather than an evolved response to a habitat specific cue. The production of TBP may provide *Pseudoalteromonas* J010 with an advantage to persist in the highly competitive biofilm environment. To further explore this hypothesis, I screened the allelochemical profile of this strain and characterised further bioactive metabolites, including novel korormicin derivatives and a polybrominated pyrrole with anti-larval, anti-bacterial, anti-fungal and anti-protozoal properties (Chapter 3). Because, TBP did not explain the inductive properties of CCA on coral larval settlement, I addressed the origin and characteristics of inductive cues from CCA (Chapter 4). This resulted in the isolation of purified fractions that readily induced complete larval settlement, including i) low molecular weight organic-soluble compounds identified as glycoglycerolipids and ii) high molecular weight polymeric aqueous-soluble cue(s). These common algal metabolites may explain the highly inductive properties of CCA on acroporid coral larval settlement. I further demonstrated that these settlement inducers can be immobilised, enabling controlled settlement of coral larvae on target surfaces. Targeted larval settlement on substrata is common practise in reef rehabilitation, particularly for techniques based on sexual reproduction of corals. To improve current reef rehabilitation techniques, I explored methods to enhance post settlement survival of settled coral spat (Chapter 5).

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | degree Celsius |
| µ | micro ($10^{-6}$) |
| A3 | *Pseudoalteromonas* sp. A3 |
| ACN | acetonitrile |
| AHL | N-acyl homoserine lactone |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair(s) |
| C18 | octadecyl carbon chain bonded silica |
| CCA | Crustose coralline algae |
| $CHCl_3$ | Chloroform |
| CMB | Centre for Marine Bio-Innovation |
| Da | dalton |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| ESI | electrospray ionization |
| EtOH | ethanol |
| EtOAc | ethylacetate |
| EPS | Extracellular Polymeric Substances |
| fr | fraction |
| g | gram |
| HCl | hydrochloric acid |
| HPLC | High Performance Liquid Chromatography |
| J010 | *Peseudoalteromonas* sp. J010 |
| k | kilo ($10^3$) |
| L | litre |
| LB10 | Luria-Bertani (medium) |
| LC | Liquid Chromatography |
| m | milli ($10^{-3}$) |
| M | molar |
| MA | Marine agar (Difco 2216) |
| MB | Marine broth (Difco 2216) |

| | |
|---|---|
| MeOH | methanol |
| min | minute |
| MS | Mass Spectroscopy |
| *m/z* | mass-to-charge ratio |
| n | nano ($10^{-9}$) |
| NMR | Nuclear Magnetic Resonance |
| NSS | Nine Salts Solution |
| OD | optical density |
| PCR | Polymerase Chain Reaction |
| QS | quorum sensing |
| $R_f$ | retardation factor |
| RP | reversed phase |
| rt | retention time |
| rpm | revolutions per minute |
| SCUBA | Self Contained Underwater Breathing Apparatus |
| $SiO_2$ | silica |
| TBP | tetrabromopyrrole |
| TFA | trifluoroacetic acid |
| TLC | Thin Layer Chromatography |
| UNSW | University of New South Wales |
| UV | ultra violet |
| wt | weight |

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# CHAPTER ONE

## General Introduction

### *1 Preamble*

In the marine environment chemical signals play critical roles at every organisational level (Hay & Steinberg 1992, Hay 1996, McClintock & Baker 2001, Pohnert et al. 2007, Hay 2009, Paul et al. 2011, Sieg et al. 2011). Early research in the field of Marine Chemical Ecology (MCE) mainly focused on antagonistic interactions between macroorganisms, such as predator–prey and seaweed–herbivore interactions (Hay 1996). In the past ten years, studies of chemically mediated interactions among microorganisms, and between micro- and macro-organisms have increasingly come to the fore (Paul et al. 2011). This is in part due to significant technological advances particularly in the fields of environmental molecular biology biology ("omics") which have highlighted the broad impacts of microorganisms in the marine environment (Giovannoni & Rappe 2000, Simon & Daniel 2011).

More recently these modern sequencing and metagenomic tools have been systematically employed to study associated microbes in and on marine animals and plants, revealing an astounding diversity of host-associated bacterial phyla. In some cases it has been demonstrated that these associations are rather specific to the host and stable over time (Lachnit et al. 2011), raising the question as to whether the functional relationships among the members of these associates are chemically controlled (Paul & Hadfield 2001).

In this thesis, I focused on the identification of chemical signals present on a marine macroalga (a tropical crustose coralline alga or CCA) and its epiphytic biofilm that mediate larval settlement of a marine invertebrate (i.e. the acroporid coral *Acropora millepora*) and

microbial interactions on the algal surface. In the following, I will summarize the main scientific background and objectives that frame this thesis.

## *2 Marine invertebrate larval settlement*

Many marine algae and invertebrates can only thrive in specific habitats. Many of these organisms have a life-cycle with separate planktonic and benthic life stages, linked by a settlement event. Settlement is often a selective process that requires recognition of suitable substrata, and in most cases is steered by sensory reception of settlement cues (Hadfield & Paul 2001, Hadfield 2011). The successful recognition of suitable substrates and habitats ensures that the newly settled propagule can establish itself in a habitat favourable for survival and reproduction (Hadfield & Paul 2001).

This concept has received considerable attention because of the importance of propagule settlement for marine population dynamics and diversity. As larval attachment and metamorphosis into sessile juveniles is generally irreversible (Thorson 1950), the signalling of suitable locations for invertebrate larvae is critical to the population and community dynamics of these organisms.

Crustose coralline algae (CCA) have long been identified as surfaces that mediate settlement of invertebrate larvae belonging to a variety of different phyla (Sebens 1983, Morse et al. 1988, Heyward & Negri 1999, Johnson et al. 1991, Pawlik 1992, Hugget et al 2006). CCA, like any other hard substrates in the marine environment are colonised by bacteria. Bacteria belong to the most abundant organisms in the ocean and are among the first to adhere to immersed surfaces. Once attached, they form a complex three-dimensional structure, referred to as biofilm, which encompasses the microbial community and its extracellular matrix. Bacteria in biofilms are encapsulated in a slimy matrix of extracellular

polymeric substances (EPS) that facilitate adhesion, protection, accumulation of nutrients and community interactions (Wimpenny 2000).

Microbial biofilms have received considerable attention as habitat-specific settlement signals for a wide and phylogenetically diverse array of marine invertebrates (Hadfield 2011). The structure and composition of biofilms and their mediatory effects on larval propagules are dynamic given the constant changes in environmental parameters. The close relationship between habitat characteristics and biofilms presumably reflects key environmental factors near the substratum and thus can act as critical cues to specific environments or habitats (Hadfield 2011).

The ability of invertebrate larvae to differentiate between biofilms of varying age (Szewzyk et al. 1991, Keough and Raimondi 1996), composition (Patel et al. 2003, Lau et al. 2005), density (Maki et al. 1988, Hugget et al. 2006), metabolic activity (Holmstrøm and Kjelleberg 1999), or origin (Qian et al. 2003, Thiyagarajan et al. 2005, Dobretsov and Qian 2006) indicates that biofilms can act as effective cues for settlement. Induction of larval attachment and metamorphosis by monospecies bacterial biofilms can be both bacterial species-specific and cell density dependent, as demonstrated by studies of the marine polychaete, *Hydroides elegans* (Huang & Hadfield 2003). A fatty acid and a simple hydrocarbon in extracts of natural biofilms induced larval settlement in this polychaete (Hung et al. 2009); however, the specific source of these signals, out of the many possible organisms in the biofilm, was not determined. Indeed, despite the long recognised role of marine bacterial biofilms as potent inducers of larval attachment and metamorphosis (Qian et al. 2007), no signaling molecule that induces metamorphosis has yet been characterised from a marine bacterium, severely limiting our capacity to understand the role of bacteria in mediating population and community dynamics of marine invertebrates.

### *3 Coral larval settlement in response to marine biofilms and crustose coralline algae*

Prior studies have hypothesised that coral larvae use bacterial biofilms on CCA as signposts during settlement (Negri et al. 2001, Webster et al. 2004, Erwin et al. 2008). The isolation of an inductive bacterium (*Pseudoalteromonas* sp. A3, Negri et al. 2001) on acroporid coral larval settlement was the seminal example that individual bacteria in complex biofilm communities can be isolated and examined *ex situ* as producers of larval settlement cues.

The first objective of this thesis was to investigate if bacterial induction of larval metamorphosis of coral larvae was chemically mediated, and to isolate and identify putative chemical settlement inducers from cultivable bacterial strains in a bioassay-guided fashion. However, the notion of epiphytic bacteria as the sole source of chemical inducers of coral larval settlement was questioned by Negri et al. (2001), since these authors demonstrated that CCA still induced coral larval settlement after removal of their biofilms. This observation implied that larval settlement cues may derive from the CCA *per se* instead or in addition to their associated microbial biofilms. This implication was supported by studies of Harrington et al. (2004) and Grasso et al. (2011) who demonstrated that organic extracts of CCA induced coral larval settlement. The second objective of this thesis therefore addressed if chemical inducers for coral larval settlement could be isolated and characterised from crustose coralline algae.

### *4 Epiphytic microbial interactions*

The third objective of this thesis was to study the other functions of bioactive secondary metabolites produced by epiphytic bacteria on crustose coralline algae. While larvae may respond quite significantly and strongly to cues produced by algal associated microorganisms, it is debatable whether settlement inducers evolved for that function. Thus it

is of interest to explore other functions of these and other secondary metabolites produced by active strains, both for their own sake and to potentially provide insight to other factors affecting the qualitative or quantitative production of putative settlement inducers.

One function of bacterial metabolites concerns the communication and concerted behavioural traits of bacteria in biofilms to monitor and respond to changes in the environment (Miller & Bassler 2001). Chemical crosstalk within a biofilm is can gauge population density and coordinate gene expression when bacterial densities reach certain thresholds, or quorum. The concentration of small diffusible molecules, e.g. acyl-homoserine lactones (AHLs), increases with higher bacterial cell density (Kaplan & Greenberg 1985). At a certain threshold concentration the AHL is bound by a protein (e.g. LuxR) (Stevens et al. 1994) which eventually cascades into the expression of genes that evoke a change in behaviour. One of the important behavioural traits coordinated by quorum sensing is the production of defense metabolites against other microorganisms and bacterial predators. Examples include the production of antagonistic secondary metabolites that inhibit attachment, growth or survival of competitors or predators (e.g. Long & Azam 2001, Bowman 2007, Givskov & Kjelleberg 2007). These allelochemicals may provide bacteria with a competitive advantage to survive in their niche. A broad range of bacterial phyla, such as members of the genera *Pseudoalteromonas* (reviewed in Bowman 2007), *Pseudovibrio* (Penesyan et al. 2011), *Vibrio* (Matz et al. 2005), *Roseobacter* (Brinkhoff et al. 2004, Bruhn et al. 2005) and *Phaeobacter* (Bruhn et al. 2007) produce highly diverse defence compounds (Bowman 2007, Engel et al. 2002). Consequently, this thesis pursued the chemical analysis of defensive bacterial compounds isolated crustose coralline algae.

## *5. Biotechnological application of larval settlement cues*

It is commonly agreed that coral reefs and associated trophic structures are in decline in many areas of the world (Pandolfi et al. 2003, Bellwood et al. 2004). The main threats for coral reefs have been identified as pollution (Williams 2002, McCulloch et al. 2003), disease (Harvell et al. 2002), and climate change (Gardner et al. 2003, Hughes et al. 2003). Estimates are that effectively 19 % of the original coral reef area worldwide is already lost; 15% is seriously threatened within the next 10 to 20 years; and 20 % is under threat of loss in 20 to 40 years (Wilkinson 2008).

Attempts to counter the decline of coral reef ecosystems through direct intervention have traditionally focused on the propagation of corals. Most rehabilitation projects use asexual fragmentation, nursing and transplanting of coral nubbins (Epstein et al. 2001, Rinkevich 2005, Shaish et al. 2008, Levy et al. 2010, Lirman et al. 2010). Other projects utilised the sexual propagation of corals (Heyward et al. 2002, Hatta et al. 2004, Omori 2005, Baria et al. 2010). Both methods still face various challenges, starting with the cost of the projects. The fragmentation techniques are additionally limited in terms of supply of nubbins, are destructive for the adult colonies and very labour intensive. The sexual propagation techniques, on the other hand, have so far been ineffective because of high post-settlement mortality in the first year after settlement (Babcock & Mundy 1996, Wilson & Harrison 2005). Still, (pending improvements) the sexual reproduction techniques have a great potential because they facilitate a greater genetic diversity than fragmentation, are non-destructive and utilise an almost unlimited supply of gametes during spawning events (Heyward et al. 2002, Boch & Morse 2012).

Coral larval settlement cues from crustose coralline algae identified in this thesis were finally applied in feasibility studies to improve coral reef rehabilitation. The objective of the last chapter of this thesis was to facilitate targeted settlement and improve rehabilitation techniques based on sexual reproduction by enhancing coral post settlement survival.

# CHAPTER 2

## Induction of Larval Metamorphosis of the Coral *Acropora millepora* by Tetrabromopyrrole Isolated from a *Pseudoalteromonas* Bacterium*

***Abstract*:** The induction of larval attachment and metamorphosis of many benthic marine invertebrates is widely considered to rely on habitat specific cues. While microbial biofilms on marine hard substrates have received considerable attention as specific signals for a wide and phylogenetically diverse array of marine invertebrates, the presumed chemical settlement signals produced by the bacteria have to date not been characterized. Here we isolated and fully characterized the first chemical signal from bacteria that induced larval metamorphosis of acroporid coral larvae (*Acropora millepora*). The metamorphic cue was identified as tetrabromopyrrole (TBP) in four bacterial *Pseudoalteromonas* strains among a culture library of 225 isolates obtained from the crustose coralline algae *Neogoniolithon fosliei* and *Hydrolithon onkodes*. Coral planulae transformed into fully developed polyps within 6 h, but only a small proportion of these polyps attached to the substratum. The biofilm cell density of the four bacterial strains had no influence on the ratio of attached vs. non-attached polyps. Larval bioassays with ethanolic extracts of the bacterial isolates, as well as synthetic TBP resulted in consistent responses of coral planulae to various doses of TBP. The lowest bacterial density of one of the *Pseudoalteromonas* strains which induced metamorphosis was 7,000 cells $mm^{-2}$ in laboratory assays, which is on the order of 0.1 –1% of the total numbers of bacteria typically found on such surfaces. These results, in which an actual cue from bacteria has been characterized for the first time, contribute significantly towards understanding the complex process of acroporid coral larval settlement mediated through epibiotic microbial biofilms on crustose coralline algae.

***Keywords*:** Tetrabromopyrrole, coral larval settlement, biofilms, *Acropora millepora,* crustose coralline algae, *Pseudoalteromonas*, A3, larval metamorphosis.

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## *1. Introduction*

Chemical signals play critical roles at every organisational level in marine systems and constitute much of the language of life in the sea (Hay 2009). For many sessile marine invertebrates (e.g. sponges, corals, mussels) chemical signals play a major role in determining the choice of habitats in which juveniles establish themselves. Many benthic marine invertebrates have a motile, planktonic larval phase, and the transition between larvae and the less mobile or immobile benthic stage is marked by a metamorphic event. Because larval attachment and metamorphosis into sessile juveniles is generally irreversible (Thorson 1950), the signalling of suitable locations for settlement by invertebrate larvae is critical to the population and community dynamics of these organisms.

Despite the importance of chemical cues for larval settlement, their actual identities are poorly described, and only very few elicitors have been fully chemically characterized and assigned to a defined natural source (Yvin et al. 1985, Pawlik et al. 1991, Tsukamoto et al. 1999, Swanson et al. 2004, Dreanno et al. 2006). Indeed, to our knowledge only three studies (Pawlik 1986, Swanson et al. 2004, Dreanno et al. 2006) have both characterized chemical cues and supported their ecological role by having verified their production, presence and/or release at ecologically realistic concentrations. This significantly constrains our ability to understand the role such signals play in benthic community dynamics. Moreover, because the induction of larval attachment and metamorphosis of benthic marine invertebrates is widely considered to rely on receptor-mediated processes (Hadfield 2011), our lack of knowledge of cue identity also means that our understanding of the mechanistic processes involved in site recognition and induction of metamorphic cascades is poor.

Marine hard substrata are coated with ubiquitous microbial biofilms, and these have received considerable attention as habitat specific settlement signals for a wide and phylogenetically

diverse array of marine invertebrates (Morse et al. 1988, Johnson et al. 1991, Pawlik 1992, Steinberg & de Nys 2002, Thiyagarajan et al. 2006, Hadfield 2011). Detailed investigations with a marine polychaete, *Hydroides elegans*, a model organism for studying larval responses to biofilms, have shown that stimulation of larval attachment and metamorphosis by monospecies bacterial biofilms is both species-specific and cell density dependent (Huang & Hadfield 2003). A fatty acid and a simple hydrocarbon in extracts of natural biofilms induced larval settlement in *H. elegans* (Hung et al. 2009); however the source of these signals, out of the many possible organisms in the biofilm (complex agglomerates of bacteria, protozoa, and microalgae (Costerton et al. 1995)), was not determined. Thus, despite the long recognized role of marine bacterial biofilms as potent inducers of larval attachment and metamorphosis (Quian et al. 2007), no signaling molecule which induces metamorphosis has yet been characterized from a marine bacterium, severely limiting our capacity to understand the role of bacteria in mediating population and community dynamics of marine invertebrates.

The larvae of tropical hard corals are classic examples of organisms that selectively settle in response to habitat specific cues such as crustose coralline algae (CCA) (Morse et al. 1988) and epiphytic bacterial biofilms associated with these algae (Negri et al. 2001, Webster et al. 2004, Erwin et al. 2008). However, settlement responses of coral larvae to biofilm bacteria vary greatly among bacterial isolates. For example, Negri et al. (2001) found that only one of 20 bacterial isolates from the CCA *Hydrolithon onkodes* (*Pseudoalteromonas* strain A3) induced larval metamorphosis of the abundant reef-building corals *Acropora millepora* and *A. willisae*.

Our aims in this study were to investigate whether bacterial induction of larval metamorphosis of coral larvae was chemically mediated and to isolate and identify putative chemical metamorphic inducers from *Pseudoalteromonas* strain A3 and other biofilm

bacteria in coral reef habitats. This was done by screening 200 bacterial isolates from a highly inductive and widely abundant CCA in the Great Barrier Reef (GBR), *Neogoniolithon fosliei*, in coral larval settlement assays, followed by bioassay guided fractionation of inductive strains in order to characterise any chemical cues of bacterial origin.

The coral *A. millepora* was used in this study because it is an important reef building coral in the GBR and its genome has just been mapped (Wang et al. 2009). Hence, the knowledge of chemical signals responsible for larval metamorphosis in this coral represents a crucial piece of information to elucidate the underlying mechanisms of coral larval settlement on a molecular, cellular, and genomic level in the future.

## *2. Methods*

### 2.1 Larval bioassays

Larval bioassays were performed in sterile 6-well culture plates (IWAKI, 10 ml volume, polystyrene) at 27–28°C. CCA were collected together with corals at both sites typically between 3 and 10 m depth and maintained in flow-through seawater tables. Bioassays were performed with competent larvae 10 d (GBR) or 13 d (WA) post fertilization. Coral larvae (n = 10) were introduced to each well in 10 ml of filtered sea water (0.2 µm, FSW). Larval responses were recorded at 6 and after 24 h under the dissecting microscope and categorized as follows: a) swimming (swimming or crawling, elongated body shape), b) metamorphosis (flattened along the oral-aboral axis with clear radial subdivisions of mesenteries), c) settlement (metamorphosis plus attachment to the dish surface or CCA).

### 2.2 Bacterial isolation from the CCA *Neogoniolithon fosliei*

The top layer (0.5 mm) of three living colonies of *N. fosliei* collected from the Davies reef (GBR) was scraped off with a sterile scalpel and suspended in sterile FSW. The

suspension was vortexed for 10 min and serially diluted 1:10 to $10^{-6}$. A 100 µl aliquot of each dilution was spread-plated in triplicate on 100% and 10% Marine Agar and incubated at 28°C for 72 h. Each distinguishable bacterial morphotype was re-streaked three times to achieve pure strains which were stored in 30% glycerol/70% Marine Broth at − 80°C.

## 2.3 Development and screening of single-strain bacterial films for larval bioassays

Single colonies of each bacterial isolate (n = 200) were inoculated into 6-well culture plates containing Marine Broth and incubated for 12 h at 28°C. The broth was discarded and the resulting biofilms, clearly distinguishable as a turbid layer at the dish surface, were carefully rinsed with FSW (0.2 µm) with a pipette (1 ml) five times. Each bacterial isolate was tested in duplicate both with and without sterile chips of *Porites* sp. (dead skeleton fragments). The Porites sp. chips were added to test for settlement enhancing effects of rugosity and/or calcium carbonate as described by Negri et al. (2001). This screening experiment including all bacterial isolates was repeated twice. *Pseudoalteromonas* strain A3 was used as a positive control (Negri et al. 2001). Well plates without biofilms and sterile *Porites* sp. chips served as negative controls. Larval responses were scored as described above. Treatments containing bacterial isolates that resulted in only swimming larvae were scored as “non-inductive bacterial isolates”, whereas treatments containing mainly metamorphosed and settled larvae were scored as “inductive bacterial isolates”.

## 2.4 Effect of bacterial density in larval bioassays

The effect of bacterial biofilm density on the magnitude of the larval response was investigated with the inductive strain J010. Briefly, ca. ten bacterial colonies were scraped from agar plates and suspended in 15 ml of FSW. This stock suspension was diluted 1:1 with FSW (0.2 µm) in four consecutive steps resulting in five serial dilutions (1, 1:2, 1:4, 1:8,

1:16). To develop bacterial biofilms of different densities on glass cover slips, 500 µl aliquots of each dilution were added to microscope cover slips (n = 12) and incubated at room temperature for 4 h. Biofilm-coated cover slips were carefully rinsed in FSW and used directly in the larval settlement assay (n = 6) and scored after 12 h. The remaining 6 biofilm-coated cover slips obtained from each dilution were stored in FSW (0.2 µm) without larvae for the duration of the bioassay. Subsequently, they were fixed in 10% formaldehyde in FSW, washed with phosphate buffered saline (PBS) and stained with 100 ng $ml^{-1}$ of 4′, 6-diamidino-2-phenylindole in glycerol: water (1:1) for 12 min. Biofilm-coated cover slips were photographed ten times under a fluorescence-microscope. The bacterial cell density was recorded with ImageJ v 1.43 software (Rasband 2010).

## 2.5 Phylogenetic affiliation of bacteria

The 16S rRNA genes of pure bacterial isolates were amplified using colony PCR. The top of a single colony was touched with a sterile toothpick and transferred to an Eppendorf tube with 50 µl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA). Each sample was heated to 95°C for 3 min then held at 4°C and briefly spun in a microfuge to pellet the cell debris. A 0.5 µl aliquot of the supernatant was used as a template for 20 µl PCR reactions containing 12.5 µl Econotaq 2x mastermix, 10 pmol each of 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) primers and 10 µl of deionized water. The PCR protocol included initial denaturation at 94°C for 3 min, 25 cycles of heating (94°C for 30 s, 55°C for 45 s and 72°C for 2 min), followed by a final extension at 72°C for 5 min and storage at 4°C. PCR amplicons were checked for correct size on 1% sodium borate agarose gel (250 V for 10 min) and purified using silica spin columns (DNA concentrator 5, Zymo Research). The amplicon concentration was measured spectrophotometrically. Purified PCR product (10 ng) was sequenced bi-directionally using Big Dye v3.1 (Applied Biosystems)

with modifications to manufacturers' instructions. Each reaction contained 0.5 µl Big Dye Terminator, 0.75 µl 5x buffer, 5 pmol of primer (27F or 1492R) and 10 µl of deionized water. Forward and reverse sequences were combined using CAP3 (Huang 1999). Sequence reads were automatically trimmed to remove poorly resolved base pairs as determined by quality scores. The reverse compliments of these sequences were then aligned to give a consensus sequence. Sequences were checked against the ribosomal database project (RDP) 12 and a species tree constructed using RPDs tree building function with closely related type strains included for comparison.

## 2.6 Bioassay-guided isolation of the metamorphic cue

The metamorphic cue of the inductive bacterial strain J010 was isolated by bioassay-guided fractionation. Two hundred Marine Agar plates were inoculated with a stock culture of J010 and grown for 24 h at 28°C with an inoculation loop. Bacterial colonies were carefully scraped off and the pellet (~20 g bacterial biomass) was extracted twice with ethanol. The combined extract was subjected to chromatography on a reversed phase $C_{18}$-flash vacuum column (5×100 cm) and sequentially eluted with (water/acetonitrile 70:30, acetonitrile, methanol, dichloromethane and hexane). The resulting five fractions were tested in the bioassay. The inductive acetonitrile fraction was concentrated and further purified on a Shimadzu HPLC system consisting of a SCL-10Avp system controller equipped with a LC-10AT pump, a SPD-M10Avp photodiode array detector, a FRC-10A fraction collector, and a SIL-10A auto sampler run on Class-VP software. Separation was achieved by elution with an acetonitrile-water gradient (70% at 0 min, held for 3 min and then rising linearly to 100% at 23 min) at a flow rate of 6 ml $min^{-1}$ from a Luna phenyl-hexyl column (Phenomenex, 250×21.2 mm, 5 µm). The chromatogram was monitored at λ 220 nm. Low resolution mass spectral data of the inductive fractions were also measured by direct infusion on a Bruker

Daltonics Esquire 3000 ion-trap mass spectrometer (MS) with an Apollo electrospray ionization (ESI) source operating in negative mode.

## 2.7 Structure elucidation

Structure elucidation of the isolated metamorphic cue was achieved using Nuclear Magnetic Resonance (NMR) spectroscopy and Fourier Transform Mass Spectrometry (FTMS). $^{1}H$ and $^{13}C$ NMR spectra were acquired employing a Bruker Avance 600 MHz NMR spectrometer with cryoprobe. NMR spectra were referenced to residual $^{1}H$ and $^{13}C$ resonances in the deuterated solvents. Both 1- and 2-dimensional NMR spectra were recorded using standard Bruker pulse sequences. High resolution mass spectra were measured with a Bruker BioApex 47e FTMS fitted with an Analytica of Branford ESI source; ions were detected in negative mode within a mass range *m*/*z* 200–1,000. Direct infusion of fractions and pure compounds was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 120 $\mu$l $h^{-1}$. The instrument was calibrated with methanolic trifluoroacetic acid (0.1 mg $ml^{-1}$).

## 2.8 Chemical synthesis of tetrabromopyrrole and its use in larval bioassays

Tetrabromopyrrole (TBP), the characterized inducer, was synthesized according to Palmer (1967) and purified from the reaction mixture using the same chromatographic conditions described above for the extract of bacterial strain J010. A TBP stock solution of unknown concentration was dried by rotary evaporation in the dark, immediately dissolved in dimethylsuloxide (DMSO), and stored at 4°C until use in bioassays. As TBP is unstable and extremely light sensitive (Palmer 1967), especially as a solid, the preparation of stock solutions of known concentrations was not possible. Instead, a serial dilution (1, 1:10, 1:100, 1:1000 and 1:10000) of the purified synthetic TBP was prepared in DMSO. These dilutions

were tested for the induction of larval metamorphosis (10 larvae per well) with replication (n = 6) in FSW making sure that the final concentration of DMSO in the assay did not exceed 0.01% (v/v). This experiment was performed in the absence and presence of live *H. onkodes* chips.

## 2.9 Effect of growth conditions on induction of larval metamorphosis by isolate J010

Aliquots of a bacterial stock culture of the inductive strains J010 and A3 were each inoculated on four different nutrient agars (1.5% agar) and in four different liquid nutrient media, all of which were duplicated in presence or absence of potassium bromide (0.053 g $l^{-1}$ artificial seawater). This approach tested the effect for growth substrate and the requirement of bromide for these bacteria to induce larval metamorphosis as the main compound of interest (TBP) was brominated. Media were prepared in 4 combinations containing a) glucose (0.5 g $l^{-1}$), yeast (5 g $l^{-1}$), peptone (3 g $l^{-1}$); b.) glucose (0.5 g $l^{-1}$), tryptone (3 g $l^{-1}$); c.) glucose (0.5 g $l^{-1}$), peptone (3 g $l^{-1}$); d.) glucose (0.5 g $l^{-1}$), yeast (5 g $l^{-1}$). Subsequently, 1 mg of bacterial biomass/pellet obtained from each of the different nutrient agars/broth cultures was extracted in 1 ml of ethanol. These extracts (5 µl extract in 10 ml FSW) were tested in larval bioassays and also analysed by HPLC for the presence of TBP. The cell-free broth supernatants were processed through $C_{18}$ SPE mini columns and analysed accordingly by HPLC for the presence of TBP.

## 2.10 Qualitative analysis of tetrabromopyrrole in bacteria closely related to strain J010

A number of *Pseudoalteromonas* species closely related to inductive strain J010 (*Pseudoalteromonas* strain A3, *P. aurantia, P. citrea, P. luteoviolacea, P. piscicida, P. tunicata, P. ulvae,* and *P. undina)* were grown on Marine Agar and extracted as described

above for strain J010. Extracts were processed using $C_{18}$ SPE mini chromatography columns and the eluates analyzed for the presence of TBP by direct infusion on a LTQ Orbitrap nano-ESI mass spectrometer (Thermo) operating in negative high mass resolution mode.

### 2.11 Statistical analysis and data treatment

Most data did not fulfil the conditions of normality and homoscedasticity and could not be improved by transformation. Data were analyzed by permutational analysis of variance in the PERMANOVA routine of PRIMER v6 (Clarke & Gorley 2006). PERMANOVA relies on comparing the observed value of a test statistic (F-ratio) against a recalculated test statistic generated from random permutation of the data. The stated advantage of the permutation approach is that the resulting test is "distribution free" and not constrained by many of the typical assumptions of parametric statistics. PERMANOVA with 999 permutations based on Euclidean distance followed by pairwise comparisons was used to statistically evaluate experimental treatments.

## *3 Results*

### 3.1 Screening and phylogenetic identification of single strain bacterial films

Of 200 distinguishable morphotypes isolated from the CCA *N. fosliei*, three yellow pigmented strains J010 (AN JF309049), J021 (AN JF314511) and J104 (JF314512) induced metamorphosis in 100% of exposed larvae. The other 197 bacterial isolates had no effect on larval metamorphosis, relative to controls, when tested as single strain biofilms, regardless of the presence or absence of sterile chips of *Porites* sp. In the presence of J010, J021 and J104, larvae flattened into discs and displayed obvious septal mesenteries radiating from the central mouth region, indicating a significant developmental event similar to that observed by Negri

et al. (2001). However, most of these newly formed polyps remained floating at the water surface, with very few (max. 10% over all replicates) permanently attached to the dish. An identical response was observed in the presence of *Pseudoalteromonas* strain A3. In contrast, settlement (metamorphosis and attachment) was observed in treatments containing live CCA chips of *N. fosliei* and *H. onkodes.* The potent effect of isolates J010 and J021 on metamorphosis was reproducibly observed after storage and inoculation from the frozen glycerol stock.

Based on their 16S rDNA sequences, isolates J010, J021, and J104 were phylogenetically highly similar to each other (>99.5%) and affiliated most closely with *Pseudoalteromonas citrea* 1373 (GU726872) and *Pseudoalteromonas peptidolytica* (AF007286) (Fig. 2.1). They shared more than 99.3% similarity with *Pseudoalteromonas* strain A3 (Negri et al. 2001). Due to their high similarity J010 was arbitrarily chosen for bioassay-guided isolation of the metamorphic cue with the aim to retrospectively verify the presence of active molecules in other inductive strains.

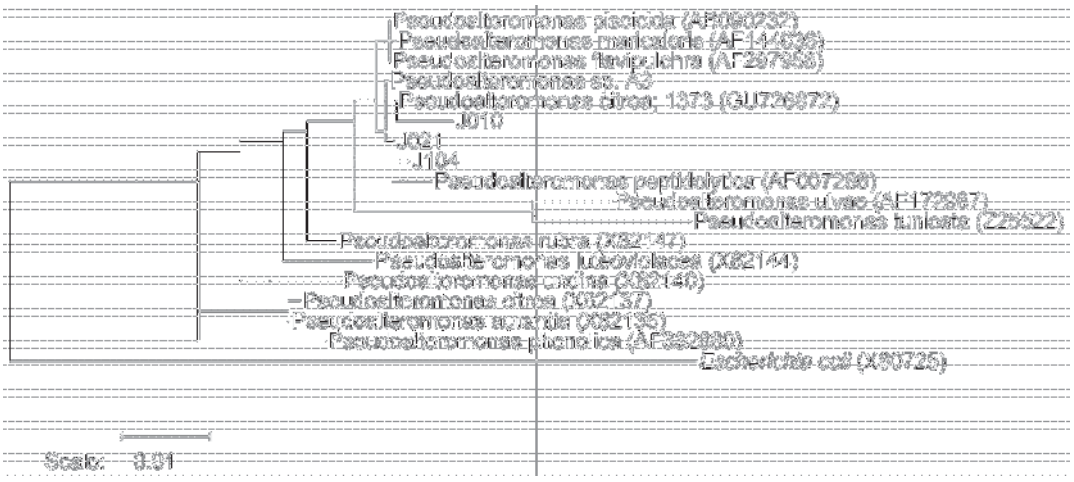


**Figure 2.1**. Phylogenetic tree of bacteria affiliated to the genus *Pseudoalteromonas* based on 16S rRNA gene sequences (5′-prime region, positions 10 to 509 *E. coli* equivalent). Nucleotide distances are based on the maximum likelihood algorithm and the tree generated using the Neighborjoining procedure.

### 3.2 Effect of bacterial biofilm density on larval settlement

There was a progressive and statistically significant effect of the biofilm density of strain J010 on the induction of metamorphosis of coral larvae (F = 34, p = 0.001, permutational ANOVA; Fig. 2.2). The lowest cell density to induce significant levels of metamorphosis was 7200±520 cells $mm^{-2}$ and at this density 50±12% of larvae had undergone metamorphosis (F = 4.2, p = 0.002, pair-wise comparison to the negative control). Bacterial densities of 10,500±680 cells $mm^{-2}$ induced metamorphosis in 100% of larvae.

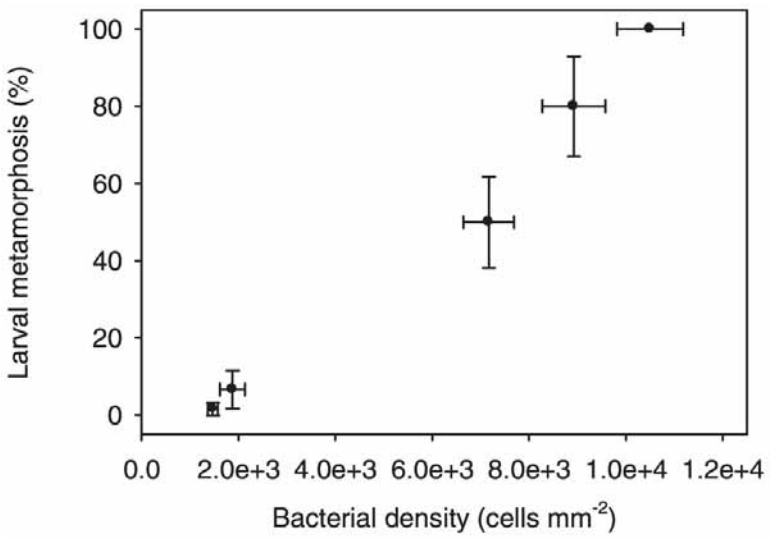


**Figure 2.2**. Percentage of larvae undergoing metamorphosis in response to biofilms of *Pseudoalteromonas* strain J010 at a range of bacterial densities. Each data point represents the mean percentage of metamorphosis (±SE) of 6 replicates containing 10 larvae each, and the mean bacterial density (±SE) of 6 replicates with 10 cell counts in each replicate.

### 3.3 Bioassay-guided isolation of the metamorphic cue

A cue which induced metamorphosis was successfully isolated from the ethanol extract of J010 by bio-assay guided fractionation. The only inductive fraction that triggered 100% of larval metamorphosis eluted with 100% acetonitrile from a $C_{18}$-flash column (Fig.

2.3). This eluate was concentrated and purified by high performance liquid chromatography (HPLC) to give a single active fraction at RT = 21 min (Fig. 2.4).

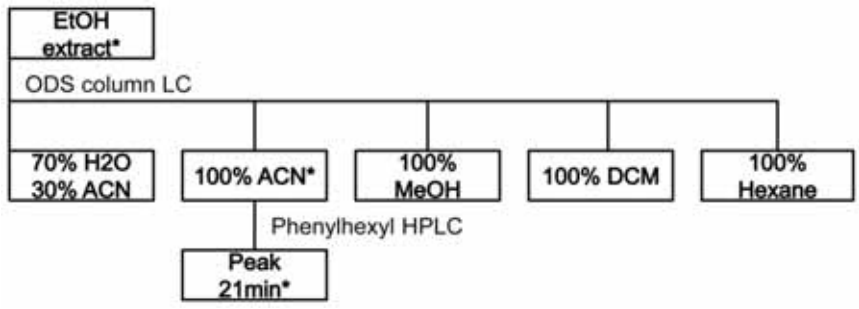


**Figure 2.3**. Isolation of the bacterial metabolite of *Pseudoalteromonas* strains J010 that induced metamorphosis of coral larvae. The asterisk marks 100% larval metamorphosis in bioassays after 6 h.

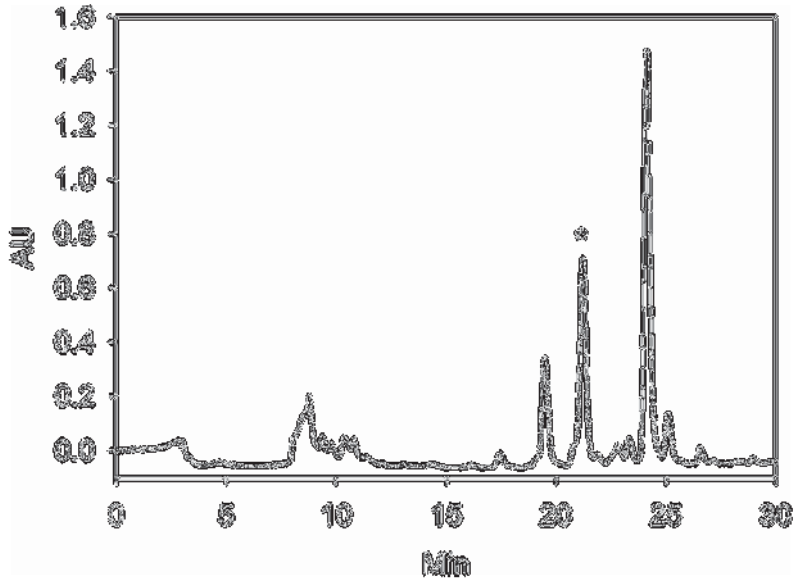


**Figure 2.4**. HPLC chromatogram of the inductive C18-chromatographic fraction of *Pseudoalteromonas* strain J010. The asterisk marks the peak fraction that induced 100% metamorphosis of coral larvae.

### 3.4 Structure elucidation and biological activity of synthetic tetrabromopyrrole

Analysis of the low resolution mass spectrum of the purified metamorphosis inducing cue identified peaks at *m/z* 377.9, 379.9, 381.7, 383.6, 385.6 (1:4:6:4:1) indicative of a tetrabrominated compound. This compound was confirmed as tetrabromopyrrole (TBP) by comparison of the $^{1}H$ and $^{13}C$ NMR and FTMS spectral data (observed *m/z* 377.6771, calculated for $C_4NBr_4^-$, *m/z* 377.6770) with literature values (Andersen et al. 1974). TBP is unstable and extremely light sensitive (Palmer 1967) and contact to air and light was avoided where possible during later isolations from bacterial biofilms.

TBP was chemically synthesized and HPLC of the crude reaction product confirmed the presence of a peak at RT = 21 min, corresponding to that of natural TBP isolated from the ethanol extract of J010. The synthetic TBP was isolated from other reaction products and NMR and MS spectral data were confirmed to be identical to natural TBP.

Synthetic TBP induced metamorphosis of *A. millepora* larvae after 6 h ($F = 48$, $p = 0.001$, permutational ANOVA) (Fig. 2.5 A). The 1:10 and 1:100 dilution of synthetic TBP resulted in the same levels of metamorphosis (~90%) after 6 h and were different from the control (for 1:10; $F = 17$, $p = 0.001$; for 1:100; $F = 20$, $p = 0.001$, pair-wise comparison to the negative control). The stock solution was lethal to 100% of coral larvae after 6 h. The proportion of larvae metamorphosed at each TBP dilution was not affected by the presence of live CCA (*H. onkodes*) after 6 h ($F = 0.7$, $p = 0.545$, 2-way-permutational ANOVA; Fig. 2.5 A, C). There was no further metamorphosis observed following a further 18 h exposure to TBP at each of the dilutions in the absence of live CCA ($F = 0$, $p = 1$, 2-way-permutational ANOVA; Fig. 2.5 A, B) and in the presence of live CCA ($p = 0.991$, 2-way-permutational ANOVA; Fig. 2.5 C, D).

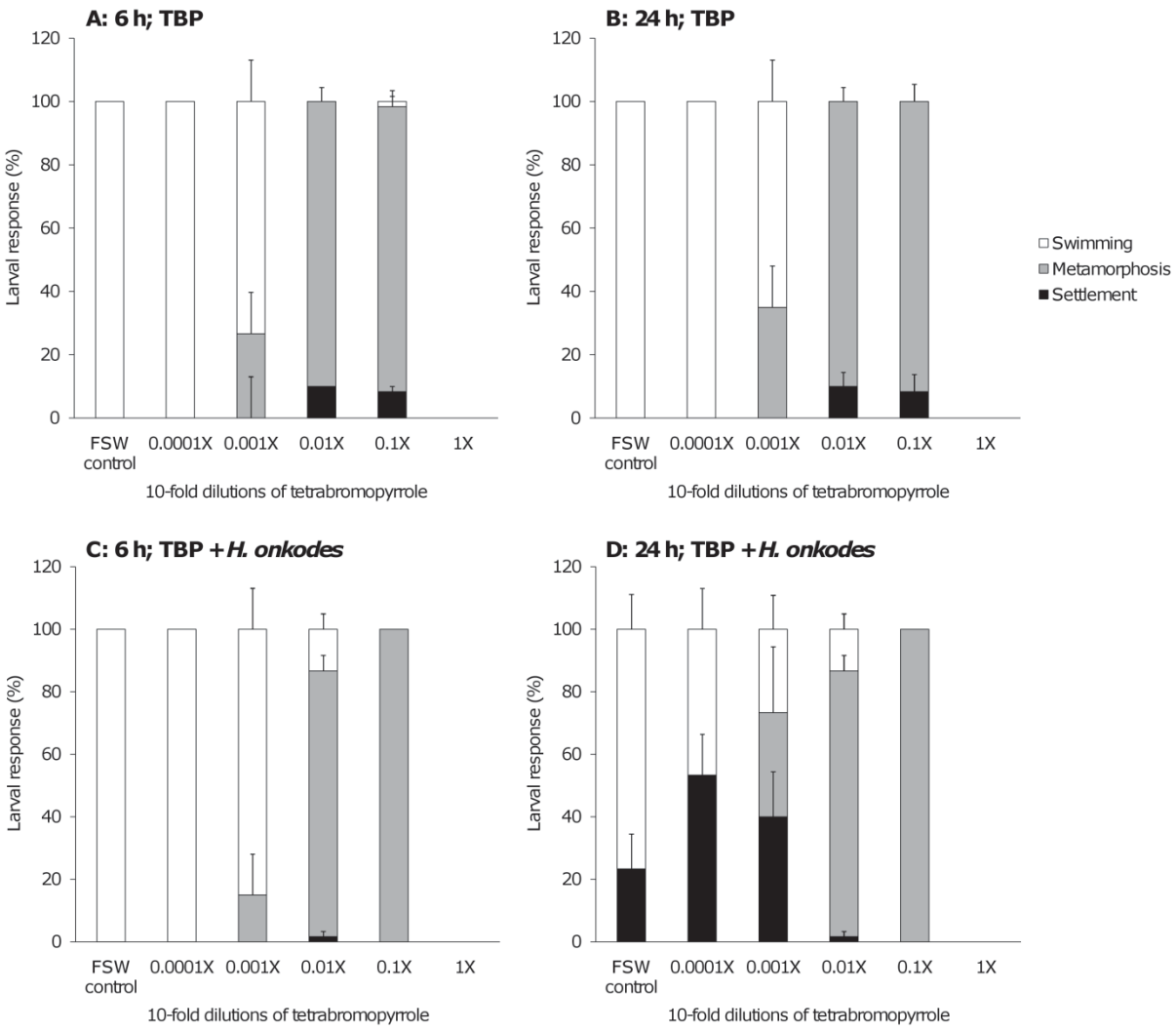


**Figure 2.5**. Different types of larval response (settlement, metamorphosis, swimming) to a 10-fold dilution series of synthetic tetrabromopyrrole in absence (A, B) and presence (C, D) of chips of *Hydrolithon onkodes* after 6 h (A, C) and 24 h (B, D). The control contained FSW only. The 1x concentration was lethal to the larvae. Each value (error bars) represents the mean (±SE) of 6 replicates with 10 larvae in each replicate.

The presence of live CCA chips induced a significant settlement response (metamorphosis+attachment) after 24 h in the TBP dilution of 1:1000 (40±14%) and 1:10,000 (53±13%) as well as in the control (23±11%) (Fig. 2.5 D) compared with the same dilution series after 6 h ($F = 17$, $p = 0.001$, permutational ANOVA; Fig. 2.5 C, D). However, little attachment (<10% was observed in the absence of live CCA after 6 and 24 h (Fig. 2.5 A, B). These results show that larval metamorphosis triggered by TBP generally occurred within 6 h of exposure (Fig. 2.5 A, B), whereas settlement of larvae in response to the CCA *H. onkodes*

took as long as 24 h (Fig. 2.5 D). Only those larvae that had not been triggered to metamorphose after 6 h (by TBP) were subsequently induced to settle in response to live CCA.

### 3.5 Effect of growth conditions on induction of larval metamorphosis by isolate J010

The inductive effect of A3 and J010 was dependent on the type of growth medium (liquid vs. solid) and the presence of potassium bromide (KBr). The nutrient composition of the media did not affect the inductive effect of the ethanolic extracts of J010 as long as this strain was grown on solid media prepared with artificial seawater containing Bromide. In contrast, no larval metamorphosis was observed when cells of J010 or A3 were grown in liquid suspension cultures or in the absence of KBr. The induction of larval metamorphosis by extracts of J010 coincided with the detection of the characteristic HPLC peak for TBP in ethanol extracts of J010 grown on different solid media in the presence of KBr. No such peak was observed from extracts of growth media lacking KBr. Ethanol extracts of J010 grown on media lacking KBr had significantly less yellow pigmentation, did not induce larval metamorphosis and did not contain detectable concentrations of TBP.

### 3.6 Qualitative analysis of TBP in closely related bacteria to J010

The characteristic mass and spectral isotope pattern of TBP (1:4:6:4:1) was only observed in extracts of A3, J010 and J021, and not in extracts from any of the phylogenetically closely related strains *P. aurantia, P. citrea, P. luteociolacea, P. piscicida, P. tunicata, P. ulvae,* or *P. undina*.

## *4. Discussion*

It is well established that bacterial biofilms are widespread inducers of marine invertebrate larval attachment and metamorphosis (Qian et al. 2007, Hadfield 2011). However, the isolation and characterization of the putative inductive chemical signals from bacteria has proved elusive. Here we report the isolation and elucidation of the first chemical signal from a known bacterium that induces transition of planulae into fully developed polyps in the acroporid coral *Acropora millepora*. This metamorphic cue was identified as tetrabromopyrrole (TBP, Andersen et al. 1974) in three *Pseudoalteromonads* isolated from the crustose coralline alga (CCA) *Neogoniolithon fosliei*. This metamorphic cue was also the bacterial metabolite from the closely related *Pseudoalteromonas* strain A3 that induced metamorphosis in *A. millepora* as reported by Negri et al. (2001).

The combined findings of this study and Negri et al. (2001) suggest that only a very few (4 out of 220) bacteria on the CCA *N. fosliei and Hydrolithon onkodes* induce larval metamorphosis in *A. millepora*. The specificity of these bacteria was further highlighted by the finding that TBP was not detected in other closely related *Pseudoalteromonas* strains in this study, although congeners such as *P. luteoviolacea* are known to produce other brominated pyrrole derivatives (Laatsch & Pudleiner 1989). Prior to this study, TBP had only been described in a *Chromobacter* sp. (Andersen et al. 1974). TBP was only produced when the inductive bacteria were grown on surfaces and not when the bacteria were grown in suspension. Such differences in gene transcription are well known for bacteria grown in suspension vs. when grown as biofilms (Sauer et al. 2002).

Despite the rapid metamorphic response of coral larvae to *Pseudoalteromonads* A3, J010, J104 and J021, 90% of the newly formed polyps did not firmly attach, and this was independent of the cell density in the bacterial biofilms. Larval metamorphosis without

attachment has been observed in a phylogenetic diverse range of corals (Vermeij 2009, Erwin & Szmant 2010) and was also described by Negri et al. (2001), who observed that the majority of planulae, despite flattening into discs and developing septal mesenteries radiating from the central mouth region when confronted with a biofilm of *Pseudoalteromonas* strain A3, did not attach. Negri et al. (2001) noticed that up to 50% of the newly formed polyps attached to the dish surface in the presence of the calcareous matrix of the coral *Porites* sp. and hypothesized that the calcareous matrix played a role in the synthesis of additional inducers of larval settlement of strain *Pseudoalteromonas* strain A3. However, we were not able to achieve comparable results with bacterial biofilms from *Pseudoalteromonas* strains A3 and J010 grown on sterile chips of *Porites* sp. skeleton (data not shown). The complete onset of larval attachment and metamorphosis was only observed in the presence of the CCA *N. fosliei* (data not shown) and *H. onkodes*.

The strong metamorphic effect of TBP on coral larvae was further highlighted by studying induction in the presence and absence of live CCA at different time intervals (6 and 24 h, Fig. 2.5). The metamorphic response of larvae to TBP was much faster than the settlement response to CCA only, which typically took 12 to 24 h to induce attachment and metamorphosis. In experiments with both TBP and CCA, only those larvae that had not been triggered to metamorphose after 6 h, such as in the 1:1,000 dilution of synthetic TBP (Fig. 2.5 C), attached and metamorphosed in response to CCA (Fig. 2.5 D). At higher concentrations of TBP (>1:100 dilution) larval attachment was seemingly short-circuited by the fast metamorphic response to TBP (Fig. 2.5 C, D).

These outcomes suggest that complete settlement–larval attachment and metamorphosis–was unlikely to result from a synergistic action of TBP and another, as yet unknown chemical cue. Live CCA are consistently very strong inducers of complete larval settlement in acroporid

corals (our study), and (Heyward & Negri 1999, Erwin & Szmant 2010, Ritson-Williams et al. 2010a), both in the field or in less ecologically realistic Petri dish assays in the laboratory. However, our laboratory assays repeatedly showed that exposure of coral larvae to TBP–by itself and when added in the presence of live and inductive chips of CCA–resulted in unattached polyps. While there may be other as yet unidentified environmental co-factors in the field not present in our experiments which might reverse this effect (and thus result in complete settlement), our results suggest that TBP may not act as an inducer of complete settlement in the field, but is more likely to act either as an antagonistic cue to full settlement or a stress response of the larval organism towards TBP.

The hypothesis that TBP short-circuits the natural settlement response of coral larvae to CCA is consistent with a conceptual model of the induction of larval metamorphosis in hydroids (Leitz 1997). One of the characteristics of this model is that the natural bacterial inducer of metamorphosis can be side-stepped by the external exposure of larvae to a group of neuropeptides (Muller & Leitz 2002), which have been identified as the internal signalling molecules in the larval body (Leitz 1997). One member of the neuropeptide family, Hym-248, also induced larval metamorphosis of *Acropora* spp. within 3–6 h, but the majority of larvae did not attach but rather floated at the water-air interface (Erwin & Szmant 2010). In terms of timing and behaviour, this response was very similar to the one observed to TBP in our study (Fig. 2.5 A). This comparatively fast response was in contrast to the general 12–24 h required for attachment and metamorphosis in response to live CCA (Negri et al. 2001, Ritson-Williams et al. 2010) and suggested that the trigger of larval attachment is either separate from metamorphosis per se, or is bypassed (Iwao et al. 2002).

This concept suggests that TBP may effectively act as an antifouling compound against coral larvae. Interestingly, TBP as a brominated metabolite fits into a category of other well known

and potent defense compounds in the marine environment (Woodin et al. 1993, Nylund et al. 2008). In one case, compounds belonging to the same compound class as TBP (brominated pyrroles) inhibit settlement of barnacle cyprids (Hertiani et al. 2010).

One important caveat to this model of interference of signalling cascades and hence attachment (or indeed to any inference about the ecological effects of TBP) is that although we have repeatedly isolated TBP producing bacteria from two different species of CCA, we currently do not know the densities of the inductive strains on CCA in the field nor the concentrations of TBP *in situ.* Isolate J010 induced significant levels of metamorphosis at cell densities as low as 7,000 cells $mm^{-2}$ (Fig. 2.2), and comparable densities for at least individual genera of bacteria, including *Pseudoalteromonas*, do occur on seaweeds in the field (Bengtsson et al. 2010). Still, the assessment of the ecological role of TBP crucially depends on its natural concentration. Until we have developed targeted and selective molecular and chemical tools to quantify the TBP-producing bacteria as well as the surface concentration of TBP on CCA in the field (e.g. by tools such as fluorescence in-situ hybridization (FISH) or desorption electrospray ionization mass spectroscopy (DESI- MS)), the ecological role of TBP will remain uncertain.

In summary, TBP is the first characterized bacterial inducer of larval metamorphosis in a marine invertebrate. The knowledge of chemical signals for larval attachment and metamorphosis is not only important for the field of marine chemical ecology, but has widespread implications for other fields such as aquaculture (to facilitate settlement of commercially valuable species), biofouling (inhibit settlement of fouling organisms) and reef restoration (rehabilitation of coral reefs *via* targeted larval settlement).

# CHAPTER 3

## A Coralline Algal-associated Bacterium, *Pseudoalteromonas* sp. strain J010, Yields New Korormicins and a Bromopyrrole*

***Abstract*:** The ethanol extract of *Pseudoalteromonas* sp. strain J010, isolated from the surface of the crustose coralline alga *Neogoniolithon fosliei*, yielded thirteen natural products. These included a new bromopyrrole, 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) and five new korormicins G - K (**2** - **6**). Also isolated was the known inducer for coral larval metamorphosis, tetrabromopyrrole, five known korormicins (A – E, previously named 1, 1a-c and 3) and bromoalterochromide A. Structures of the new compounds were elucidated through interpretation of spectra obtained after extensive NMR and MS investigations and comparison with literature values. The anti-bacterial potential of **1** – **6** was assessed. The antiprotozoal activity of compounds from this strain was also assessed and attributed to the previously described metabolites, tetrabromopyrrole and bromoalterochromide A.

***Keywords*:** *Pseudoalteromonas*; korormicin; bromopyrrole, bromoalterochromide

### *1. Introduction*

In the marine environment chemical signals play critical roles at many organisational levels (Hay 2009). Marine chemical signals guide the choice and selection of suitable substrates for motile invertebrate larvae (Pawlik 1992, Qian et al. 2007) during commencement of the benthic, often sessile, mode of life. Research spanning the past six decades has identified that bacteria associated with marine macro-organisms (animals, plants, algae) as a common source of these signals (Hadfield 2011).

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In a previous bioassay-guided study, we identified the bacterial *Pseudoalteromonas* strain J010 (J010) from the surface of crustose coralline algae that triggered larval metamorphosis in acroporid corals. The causative bacterial metabolite for the transition of planula larvae into non-attached primary polyps was identified as tetrabromopyrrole (Tebben et al. 2011). During the course of isolating tetrabromopyrrole from the bacterial extract of J010, other potentially novel bacterial metabolites were encountered including polybrominated pyrrole derivatives and a family of korormicin derivatives. Antimicrobial metabolites belonging to these compound classes have been previously isolated from other Pseudoalteromonads or closely related species (Andersen et al. 1974, Yoshikawa et al. 1997). More generally, pigmented bacteria affiliated with the genus *Pseudoalteromonas* (class Gammaproteobacteria) have gained significant attention during the past decade as producers of a wide range of bioactive compounds (Bowman 2007).

In this study we aimed to deconstruct the NMR and MS spectra of J010 to better understand the metabolic capacity and antibiotic potential of this epiphytic bacterium. This study was motivated by the hypothesis that chemically undefended animate host surfaces, such as crustose coralline algae, may benefit from bioactive secondary metabolites produced by associated surface bacteria against bacterial colonisation by pathogens and fouling bacteria (Engel et al. 2002, Egan et al. 2008). However, the unequivocal distinction between host metabolites and host-associated epibiotic bacterial metabolites often remains unresolved (Kubanek et al. 2003, Piel 2006, Phelan et al. 2012).

Details of the isolation and structural elucidation of the known coral larval metamorphosis cue, tetrabromopyrrole (Andersen et al. 1974, Tebben et al. 2011), five known korormicins (A – E, previously named 1, 1a-c and 3) (Yoshikawa et al. 1997, Yoshikawa et al. 2003) and bromoalterochromide A (Speitling et al. 2007) along with the

new bromopyrrole, 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) and five new korormicins G - K (**2** - **6**) are provided. Their antimicrobial antifungal and antiprotozoal activities are also discussed.

## *2. Methods*

### 2.1. General Experimental Procedures

Reversed-phase $C_{18}$ (RP $C_{18}$) vacuum liquid column chromatography was performed using material from Phenomenex C18 (50 μm). HPLC was undertaken using a Shimadzu SCL-10Avp system controller equipped with a LC-10AT pump, a SPD-M10Avp photodiode array detector, a FRC-10A fraction collector, and a SIL-10A auto sampler operated by Class-VP software. HPLC columns were purchased from Phenomenex (Australia). Compressed gases were supplied by BOC Gases (Townsville, Australia) and were at least 99.99% pure. Purified water was obtained from a Milli-Q water purification system (Millipore, MA, USA). All other solvents used were HPLC grade (Mallinckrodt, MO, USA). All other chemicals were sourced from Sigma-Aldrich (Australia). Low-resolution mass spectral data of the fractions were also measured by direct infusion on a Bruker Daltonics Esquire 3000 ion-trap mass spectrometer (MS) with an Apollo electrospray ionisation (ESI) source. Optical rotations were recorded on a JASCO P-1020 polarimeter. UV spectra were measured using a Shimadzu SPD-M10AVP PDA detector. A Perkin Elmer Spectrum 100 FTIR spectrophotometer was used to record all IR spectra. $^{1}H$ and $^{13}C$ NMR spectra were recorded in either $CDCl_3$, $CD_3OD$ or DMSO-$d_6$ (Cambridge Isotopes Laboratories Inc. USA) using 3 mm Bruker MATCH NMR tubes on a Bruker Avance 600 MHz NMR spectrometer with cryoprobe. NMR spectra were referenced to residual $^{1}H$ and $^{13}C$ resonances in the deuterated solvents. Both 1D and 2D NMR spectra were recorded using standard Bruker pulse

sequences. Accurate mass spectra were measured with a Bruker BioApex 47e FT-ICR mass spectrometer fitted with an Analytica of Branford electrospray source; ions were detected in either negative or positive mode within a mass range *m/z* 200-2000. Direct infusion of samples (0.2 mg m/L) was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 100 μL/h.

## 2.2. Isolation of Bacterial Metabolites

Novel compounds are numbered in Arabic, previously described compounds in Roman letters. Structural analyses is shown in 3.1. Petri dishes (n = 300, 2% marine agar) were inoculated with a stock culture of J010 and incubated for 48 h at 28°C. Bacterial colonies were harvested with a sterile spatula, pooled and the bacterial biomass (25 g) extracted three times with ethanol (EtOH, 150 mL total volume). The combined extract volume was dried *in vacuo,* dissolved in 20 mL of methanol (MeOH), and purified on a MeOH pre-equilibrated flash chromatography column containing C18 bulk medium (Sepra C18-e, 50 μm, Phenomenex, Australia) with a bed volume of 5 x 100 cm. The column was eluted with two column volumes of MeOH. The resulting single fraction was dried *in vacuo*, dissolved in 2 ml of MeOH and further fractionated by HPLC on a Luna Phenyl-Hexyl column (Phenomenex, 250 x 21.2 mm, 5 μm) under gradient elution with $H_2O$ to MeOH at 8 mL/min, gradient elution from 70:30 methanol(MeOH):water(H2O) to 100 % MeOH over 30 mins, followed by 6 mins with 100 % MeOH. Fractions were collected according to the UV peak profile (λ 220 nm). The chromatography yielded (in order of elution) comp. **I** (rt 10.9 mins), **1** (rt 19.25 mins), **II** (rt 20 mins), **III** (rt 20.8 mins), **2** (rt 21.3 mins), **IV** (rt 21.6), **3** (rt 21.6), **4** (rt 21.7), **VI, V** (rt 23.8), **5, VII** (rt 24.8 min) and **6** (rt 25.38 min). All individual compounds were further purified by C18 analytical HPLC (250 x 4.6 mm, 5μ Phenomenex Luna (2) C18; 1 mL/min, gradient elution from 1:1 acetonitrile (ACN): $H_2O$ to 100 % ACN

over 18 mins then held at 100 % ACN for a further 6 mins) and fractions collected according to the UV peak profile (λ 220 nm).

## 2.3. Antibacterial (Disc diffusion) Bioassays

The disc diffusion assay was performed as per Bauer et al. (1966). Briefly, fractions collected during the bioassay-guided fractionation were applied without drying while HPLC-purified compounds were dissolved in suitable organic solvents (**1**, **I, III** in MeOH; **2-6** in $CHCl_3$) at a concentration of 200 µg/mL. Aliquots (20 µL) of these fractions and compounds were evaporated on filter paper discs (d = 6 mm, BioRad). These discs were placed on petri dishes containing Marine Agar or LB agar depending on the target strain. Each petri dish was inoculated with one of the following bacterial strains: the Gram positive *Staphylococcus aureus,* or the Gram negative bacterial strains: J010, *Pseudoalteromonas* strain cp21, *Pseudoalteromonas haloplanctis, Pseudoalteromonas picicida, Pseudoalteromonas undina, Pseudomonas aeruginosa, Vibrio campbellii, Vibrio vulnificus, Vibrio* strain A133, *Vibrio* strain Ap18, *Krokinobacter* strain ub2 and *Shewanella* strain cp20. The fractions and compounds were also tested against the fungus *Candida albicans.* Petri dishes were incubated for 24 h (at the temperature given in table 3.4) after which the growth inhibitory activity of fractions and compounds was assessed. Zones bigger then 8 mm in diameter (2mm zone around the disc) are shown as inhibited by the target compound. by measurement of the inhibitory zone (clearance) around the discs. Blank solvent was used as a negative control.

## 2.4. Antiprotozoal Bioassays

An axenic culture of the ciliate *Tetrahymena pyriformis*(Culture Collection of Algae and Protozoa, Windermere, UK, CCAP 1630/1W) was used for the screening for

antiprotozoal effects as per Matz et al (2005). The bioassay-guided fractionation procedure followed the protocol previously reported for the isolation of metamorphic cues from this bacterium (Tebben et al. 2011 [Chapter 2]). Chromatographic fractions were evaluated for antiprotozoal activity in a 24-well plate bioassay. Fractions were dried under vacuum and suspended in EtOH. Aliquots of these fractions were transferred into 24-well plates and air dried. Nine Salts Solution medium (Väätänen 1976) with $10^3$ flagellates per ml was added to each well resulting in 500 µL final volume per well. Total flagellate numbers and the number of active cells were monitored by direct inspection with an inverted light microscope and compared to the solvent-only control treatment.

## *3. Results and Discussion*

### 3.1 Isolation and Structural Elucidation of Bacterial Metabolites

The NMR and ESI-FTMS of compound **1** established its molecular formula to be $C_{11}H_8ONBr_3$, requiring seven degrees of unsaturation. The IR spectrum suggested the presence of both hydroxyl (3388 $cm^{-1}$) and amine (3271, 2925 $cm^{-1}$) groups. $^1H$ NMR and phase-sensitive multiplicity-edited adiabatic inversion HSQC spectral data (Table 1) showed the presence of a disubstituted double bond ($\delta_C$ 129.9, t, C-8; $\delta_H$ 7.05, d, 8.3, H-8; 115.8, t, C-9; $\delta_H$ 6.81, d, 8.3, H-9) and one methylene ($\delta_C$ 32.5, d, C-6; $\delta_H$ 3.87, s, H-6). Each proton signal integrated for two protons establishing the presence of two disubstituted double bonds and hence symmetry within the molecule. $^1H$-$^1H$ COSY correlations between H-8 and H-9, with a coupling of $J$ = 8.3 indicated a *cis* orientation. HMBC correlations from H-8 to C-10 and H-9 to $\delta_C$ 128.7 (C-7) and the requirement for symmetry established a 1,4-disubstituted benzyl ring. This moiety accounted for four of the seven degrees of unsaturation in **1**. The presence of a hydroxyl moiety on the benzyl ring was determined by the downfield resonance at $\delta_C$ 155.1 (C-10). Further analysis of the HMBC data showed a correlation from H8/12 to

$\delta_C$ 32.5 (C-6) establishing the methylene side chain *para* to the hydroxyl. An amine linkage, forming the second ring, was deduced between C-2 and C-5 based on downfield resonance of C-2 ($\delta_C$ 132.5). A further HMBC correlation from H-6 to $\delta_C$ 131.1 (C-2) and $\delta_C$ 97.8 (C-3) and the isotope distribution requiring three bromine atoms, indicated the presence of a tetrasubstituted pyrrole ring, accounting for all seven degrees of unsaturation and hence the planar structure of **1**, 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol, is as shown.

**Table 3.1**: NMR data (600 MHz and 125 MHz) for 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**)

| **Position** | **1 in DMSO-$d_6$** | | | | **1 in $CDCl_3$** | | | | **1 in $CD_3OD$** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\delta_C$, mult.[a] | $\delta_H$ (*J* in Hz) | gCOSY | gHMBC | $\delta_C$, mult. | $\delta_H$ (*J* in Hz) | gCOSY | gHMBC | $\delta_C$, mult. | $\delta_H$ (*J* in Hz) | gCOSY | gHMBC |
| **NH** | - | | | | - | | | | - | | | |
| **2** | 132.3, qC | | | | 131.1, qC | | | | 133.0, qC | | | |
| **3** | 96.3, qC | | | | 97.8, qC | | | | 97.8, qC | | | |
| **4** | nd[b] | | | | 100.0, qC | | | | 100.5, qC | | | |
| **5** | nd[b] | | | | 100.0, qC | | | | 98.8, qC | | | |
| **6** | 31.8, $CH_2$ | 3.73, s | | 2, 7, 8, 12 | 32.5, $CH_2$ | 3.87, s | | 2, 7, 8, 12 | 32.9, $CH_2$ | 3.87, s | | 2, 7, 8, 12 |
| **7** | 129.2, qC | - | | | 128.7, qC | - | | | 130.8, qC | - | | |
| **8** | 128.8, CH | 6.97, d (8.2) | 9 | 6, 7, 9, 10, 11 | 129.9, CH | 7.05, d (8.3) | 9 | 6, 7, 9, 10, 11 | 130.2, CH | 7.05, d (8.3) | 9 | 6, 7, 9, 10, 11 |
| **9** | 115.3, CH | 6.67, d (8.2) | 8 | 8, 10, 11, 12 | 115.8, CH | 6.81, d (8.3) | 8 | 8, 10, 11, 12 | 116.3, CH | 6.81, d (8.3) | 8 | 8, 10, 11, 12 |
| **10** | 155.9, qC | - | | | 155.1, qC | - | | | 157.0, qC | - | | |
| **OH** | - | | | | - | | | | - | | | |
| **11** | 115.3, CH | 6.67, d (8.2) | 12 | 8, 10, 11, 12 | 115.8, CH | 6.81, d (8.3) | 12 | 8, 10, 11, 12 | 116.3, CH | 6.81, d (8.3) | 12 | 8, 10, 11, 12 |
| **12** | 128.8, CH | 6.97, d (8.2) | 11 | 6, 8, 9, 10, 11 | 129.9, CH | 7.05, d (8.3) | 11 | 6, 8, 9, 10, 11 | 130.2, CH | 7.05, d (8.3) | 11 | 6, 8, 9, 10, 11 |

[a] carbon shifts from HSQC and HMBC

[b] nd = not detected

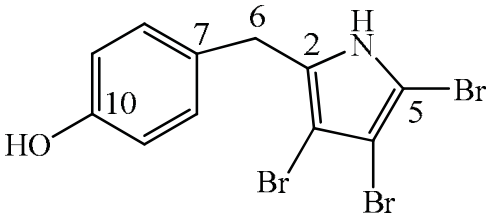
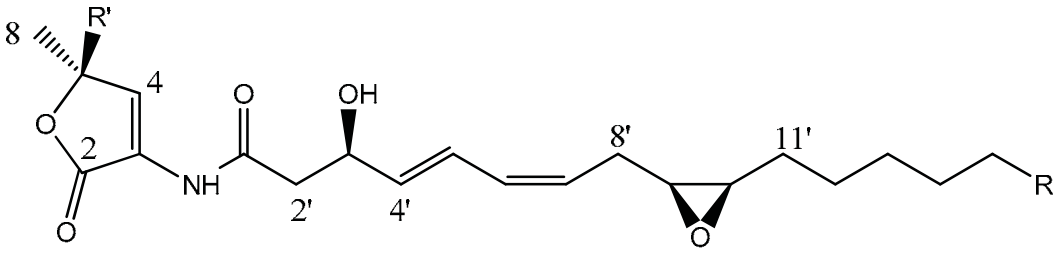
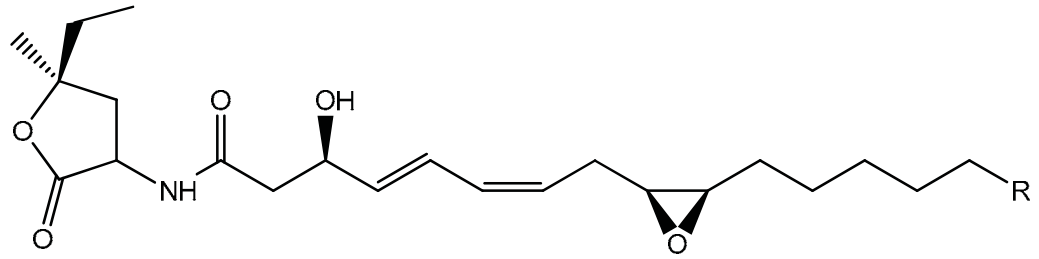


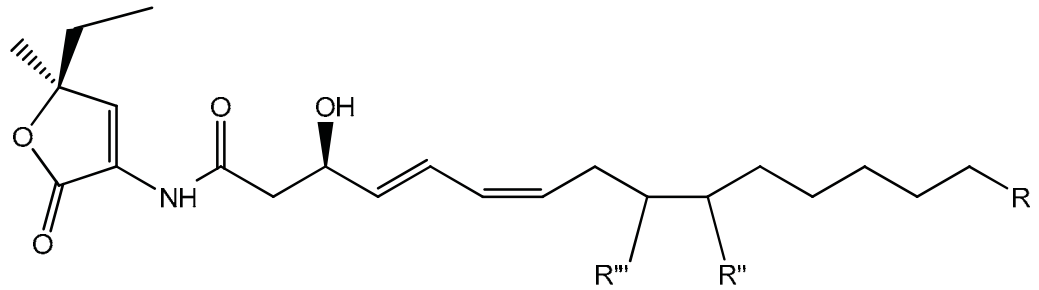

**Figure 3.1.** Structure of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**)



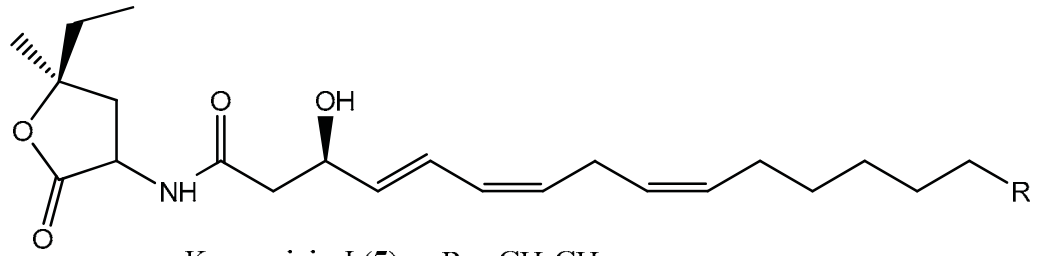

Korormicin A (=1)[a] R = $(CH_2)_2CH_3$ R' = $CH_2CH_3$
Korormicin B (=1a)[a] R = $CH_3$ R' = $CH_2CH_3$
Korormicin C (=1b)[a] R = $CH_2CH_3$ R' = $CH_2CH_3$
Korormicin D (=1c)[a] R = $(CH_2)_3CH_3$ R' = $CH_2CH_3$
Korormicin E (=3)[a] R = $(CH_2)_2CH_3$ R' = $CH_3$




Korormicin G (**2**) R = $(CH_2)_2CH_3$




Korormicin F (=2)[a] R = $(CH_2)_2CH_3$ R'' = Br, R''' = OH
Korormicin H (**3**) R = $(CH_2)_2CH_3$ R'' = OH, R'' '= Cl
Korormicin I (**4**) R = $(CH_2)_2CH_3$ R' '= Cl, R''' = OH




Korormicin J (**5**) R = $CH_2CH_3$
Korormicin K (**6**) R = $(CH_2)_2CH_3$

**Figure 3.2.** Structures of korormicins G- K (**2** – **6**). [a] nomenclature used by Yoshikawa *et al.* (2003)

The $^{13}C$ NMR and ESI-FTMS of the second compound to be isolated from the ethanol extract of *Pseudoaltermonas* sp., **2**, established its molecular formula to be $C_{25}H_{41}O_5N$, requiring six degrees of unsaturation. The IR spectrum suggested the presence of alcohol (3345 br $cm^{-1}$), ester (1734 $cm^{-1}$) and amide (1639 str, 1553 $cm^{-1}$) groups. $^{1}H$ and $^{13}C$ NMR spectral data (Tables 2 and 3) showed that **2** contained two conjugated, disubstituted double bonds ($\delta_C$ 129.3, d, C-4'; 130.0, d, C-5'; $\delta_H$ 5.39, dd, 11.3, 9.4, H-4'; $\delta_H$ 6.07, br t, 11.3, H-5' and 126.4, d, C-6'; 132.0, d, C-7'; $\delta_H$ 6.47, br dd, 14.5, 11.3, H-6'; $\delta_H$ 5.82, br dt, 15.0, 6.7, H-7') and confirmed the presence of two carbonyls ($\delta_C$ 174.3, s, C-2 and $\delta_C$ 172.0, s, C-1'), which accounted for four of the six degrees of unsaturation. Moreover, this information, in combination with the molecular formula, showed the molecule to be bicyclic. $^{1}H$- and $^{13}C$-NMR data (Tables 2 and 3) also indicated the presence of one *sp2* tertiary carbon ($\delta_C$ 85.8, q, C-5), one *sp3* tertiary carbon bonded to a nitrogen ($\delta_C$ 50.0, d, C-3; $\delta_H$ 4.72, ddd, 11.5, 9.2, 5.6 Hz), three oxymethines ($\delta_C$ 64.7, t; $\delta_H$ 5.01, br ddd, 9.4, 8.7, 3.2; $\delta_C$ 55.3, t; $\delta_H$ 3.01, dt, 6.2, 4.2; $\delta_C$ 56.6, t; $\delta_H$ 2.97, dt, 5.9, 4.2), ten methylenes, one singlet methyl ($\delta_C$ 25.9, s, C-8; $\delta_H$ 1.47, s, H-8) and two triplet methyl groups.

The $^{1}H$-$^{1}H$ COSY spectrum of **2** revealed three spin systems. The first from H-3 ($\delta_H$ 4.72, ddd, 11.5, 9.2, 5.6) to NH ($\delta_H$ 6.58, d, 5.6) and $H_a$-4 ($\delta_H$ 2.78, dd, 12.8, 9.2), and the second from $H_2$-6 ($\delta_H$ 1.76, dq, 14.7, 7.5; 1.68, dq, 14.7, 7.5) to $H_3$-7 ($\delta_H$ 1.0, t, 7.5), along with cross-peaks in the HMBC spectrum from $H_a$-4 to C-2 and C-3, and from $H_3$-8 to C-4, C-5 and C-6 confirmed the presence of a 3,5,5-trisubstituted γ-lactone ring. The third spin system in the $^{1}H$-$^{1}H$ COSY spectrum was discerned from $H_2$-2' to $H_3$-18', and included the conjugated double bonds. Further HMBC correlations from $H_2$-2' to C-1' and C-3' located this side chain at the amide carbonyl C-1' attached to the γ-lactone ring, reminiscent of korormicin

(Yoshikawa et al. 1997). Coupling constants of $J_{4'\text{-}5'}$ 11.3 Hz and $J_{6'\text{-}7'}$ 14.5 Hz confirmed the *Z*- and *E*-configurations for each double bond, respectively.

The data so far accounted for two carbonyls, one ring, two conjugated double bonds, and the nitrogen, leaving three oxygenated carbons, two oxygens and one ring unassigned. An ether linkage, forming the second ring, was deduced between C-9' and C-10' based on the $^{13}C$ chemical shifts ($\delta_C$ 55.3, t; $\delta_C$ 56.6, t) and the $^{1}H$-$^{1}H$ COSY correlations from $\delta_H$ 3.01 (dt, 6.2, 4.2, H-9') to $\delta_H$ 3.01 (2.97, dt, 5.9, 4.2, H-10'). This was further supported by a C-O-C stretch at 1103 $cm^{-1}$ in the IR. Therefore by deduction the last unassigned oxygen was a hydroxyl group. As for korormicin (Yoshikawa et al. 1997), the vicinal coupling constant for H-9'/H-10' (4.2 Hz) confirmed the epoxide was of *cis* orientation. Hence the planar structure of **2**, as shown in Figure 2, is similar to korormicin (Yoshikawa et al. 1997) except the γ-lactone ring is fully saturated.

Yoshikawa *et al*. reported the isolation and structural elucidation of six korormicins (korormicin 1, 1a-c, 2 and 3) (Yoshikawa et al. 1997, Yoshikawa et al. 2003). We propose that these korormicins be recognised for nomenclature purposes as korormicins A-F respectively, hence compound **2** is korormicin G.

**Table 3.2**: $^{13}C$ NMR shifts of korormicins G – K (**2** – **6**)

| | **Korormicin G (2)** | **Korormicin H (3)** | **Korormicin I (4)** | **Korormicin I (4)** | **Korormicin J (5)** [d] | **Korormicin K (6)** |
|---|---|---|---|---|---|---|
| **No.** | **$\delta_C$, mult.**[a] | **$\delta_C$, mult.**[b] | **$\delta_C$, mult.**[b] | **$\delta_C$, mult.**[a] | **$\delta_C$, mult.**[a] | **$\delta_C$, mult.**[a] |
| **1** | - | - | - | - | - | - |
| **2** | 174.7, qC | 168.5, qC | 168.5, qC | 168.5, qC | nd [d] | 174.3, qC |
| **3** | 50.0, CH | 125.0, qC | 125.0, qC | 125.0, qC | 50.0, CH | 50.4, CH |
| **4** | 39.9, $CH_2$ | 133.7, CH | 134.0, CH | 134.0, CH | 40.0, $CH_2$ | 40.4, $CH_2$ |
| **5** | 85.8, qC | 87.2, qC | 87.2, qC | 87.2, qC | nd [d] | 85.6, qC |
| **6** | 32.4, $CH_2$ | 31.2, $CH_2$ | 31.1, $CH_2$ | 34.6, $CH_2$ | 32.9, $CH_2$ | 32.8, $CH_2$ |
| **7** | 8.2, $CH_3$ | 8.0, $CH_3$ | 8.0, $CH_3$ | 8.1, $CH_3$ | 8.3, $CH_3$ | 8.4, $CH_3$ |
| **8** | 25.9, $CH_3$ | 23.9, $CH_3$ | 24.1, $CH_3$ | 24.2, $CH_3$ | 25.9, $CH_3$ | 26.2, $CH_3$ |
| **NH** | - | - | - | - | - | - |
| **1'** | 172.0, qC | 170.1, qC | 170.2, qC | 170.2, qC | nd [d] | 171.7, qC |
| **2'** | 42.3, $CH_2$ | 43.9, $CH_2$ | 44.0, $CH_2$ | 43.1, $CH_2$ | 42.7, $CH_2$ | 42.7, $CH_2$ |
| **3'** | 64.7, CH | 63.5, CH | 63.8, CH | 64.6, CH | 64.8, CH | 65.0, CH |
| **OH** | - | - | - | - | - | - |
| **4'** | 129.3 CH | 132.8 CH | 132.2 CH | 129.4, CH | 127.6, CH | 129.0 CH |
| **5'** | 130.0, CH | 128.2, CH | 128.5, CH | 130.7, CH | 130.6, CH | 131.0, CH |
| **6'** | 126.4, CH | 127.8, CH | 127.4, CH | 127.4, CH | 124.0, CH | 124.7, CH |
| **7'** | 132.0, CH | 131.9, CH | 132.5, CH | 132.7, CH | 136.6, CH | 136.4, CH |
| **8'** | 31.1, $CH_2$ | 37.2, $CH_2$ | 37.1, $CH_2$ | 38.2, $CH_2$ | 30.5, $CH_2$ | 30.6, $CH_2$ |
| **9'** | 55.3, CH | 67.0, CH | 72.1, CH | 73.1, CH | 128.3, CH | 126.1, CH |
| **OH** | - | - | - | - | - | - |
| **10'** | 56.6, CH | 71.4, CH | 67.7, CH | 68.0, CH | 129.2 CH | 131.4, CH |
| **11'** | 27.3, $CH_2$ | 33.3, $CH_2$ | 33.7, $CH_2$ | 31.9, $CH_2$ | 27.0, $CH_2$ | 27.2, $CH_2$ |
| **12'** | 29.1[c], $CH_2$ | 25.4, $CH_2$ | 26.3, $CH_2$ | 26.0, $CH_2$ | 25.4, $CH_2$ | 29.1[c], $CH_2$ |
| **13'** | 29.1[c], $CH_2$ | 29.0[c], $CH_2$ | 28.8[c], $CH_2$ | 28.8[c], $CH_2$ | 29.0[c], $CH_2$ | 29.1[c], $CH_2$ |
| **14'** | 29.1[c], $CH_2$ | 29.0[c], $CH_2$ | 28.6[c], $CH_2$ | 28.58[c], $CH_2$ | 28.6[c], $CH_2$ | 29.1[c], $CH_2$ |
| **15'** | 29.1[c], $CH_2$ | 29.0, $CH_2$ | 28.6[c], $CH_2$ | 28.55[c], $CH_2$ | 31.4, $CH_2$ | 29.1[c], $CH_2$ |
| **16'** | 31.6, $CH_2$ | 31.3, $CH_2$ | 31.2, $CH_2$ | 31.7, $CH_2$ | 22.0, $CH_2$ | 31.6, $CH_2$ |
| **17'** | 22.6, $CH_2$ | 22.1, $CH_2$ | 22.1, $CH_2$ | 22.6, $CH_2$ | 13.5, $CH_3$ | 22.6, $CH_2$ |
| **18'** | 13.9, $CH_3$ | 13.9, $CH_3$ | 13.9, $CH_3$ | 13.9, $CH_3$ | . | 14.1, $CH_3$ |

[a] $CDCl_3$

[b] DMSO-$d_6$

[c] interchangeable

[d] No HMBC data was recorded for [c], these carbons remain unassigned

**Table 3.3**: $^{1}$H NMR shifts of korormicins G – K (**2** – **6**)

| | Korormicin G (2) | Korormicin H (3) | Korormicin I (4) | Korormicin I (4) | Korormicin J (5) | Korormicin K (6) |
|---|---|---|---|---|---|---|
| **No.** | **$\delta_H$ (*J* Hz)[a]** | **$\delta_H$ (*J* Hz)[b]** | **$\delta_H$ (*J* Hz)[b]** | **$\delta_H$ (*J* Hz)[a]** | **$\delta_H$ (*J* Hz)[a]** | **$\delta_H$ (*J* Hz)[a]** |
| **1** | - | - | - | - | - | - |
| **2** | - | - | - | - | - | - |
| **3** | 4.72, ddd (11.5, 9.2, 5.6) | - | - | - | 4.70, ddd (11.2, 9.2, 6.1) | 4.70, ddd (11.2, 9.2, 5.6) |
| **4** | 2.78, dd (12.8, 9.2)<br>1.90, dd (12.8, 11.5) | 7.39, s | 7.39, s | 7.37, s | 2.78, dd (12.8, 9.2)<br>1.90, dd (12.8, 11.2) | 2.78, dd (12.9, 9.2)<br>1.90, dd (12.9, 11.2) |
| **5** | - | - | - | - | - | - |
| **6** | 1.76, dq (14.7, 7.5)<br>1.68, dq (14.7, 7.5) | 1.76, q (7.5) | 1.76, q (7.4) | 1.82, q (7.4) | 1.77, dq (14.4, 7.5)<br>1.70, dq (14.4, 7.5) | 1.76, dq (14.7, 7.5)<br>1.69, dq (14.7, 7.5) |
| **7** | 1.0, t (7.5) | 0.76, t (7.5) | 0.76, t (7.4) | 0.90, t (7.4) | 1.00, t (7.5) | 1.00, t (7.5) |
| **8** | 1.47, s | 1.41, s | 1.41, s | 1.50, s | 1.49, s | 1.47, s |
| **NH** | 6.58, d (5.6) | 9.91, br s | 9.88, br s | 8.22, br s | 6.59, d (6.9) | 6.60, d (5.6) |
| **1'** | - | - | - | - | - | - |
| **2'** | 2.51, dd (15.4, 8.7)<br>2.45, dd (15.4, 3.2) | 2.60, dd (14.4, 8.0)<br>2.41, dd (14.4, 4.6) | 2.60, dd (14.4, 8.0)<br>2.41, dd (14.4, 5.2) | 2.63, dd (15.8, 8.7)<br>2.58, dd (15.8, 3.1) | 2.49, dd (15.5, 8.6)<br>2.45, dd (15.5, 3.2) | 2.49, dd (15.3, 8.5)<br>2.45, dd (15.3, 3.5) |
| **3'** | 5.01, br ddd (9.4, 8.7, 3.2) | 4. 85, br dddd (9.2, 8.0, 4.6, 4.1) | 4. 84, dddd (9.1, 8.0, 5.2, 4.7) | 5.06, ddt (9.4, 8.7, 3.1) | 5.01, m | 5.00, ddd (9.4, 8.5, 3.2) |
| **OH** | | 5.16, br d (4.1) | 5.11, d (4.7) | | | |
| **4'** | 5.39, dd (11.3, 9.4) | 5.30, br dd (11.1, 9.2) | 5.28, dd (11.1, 9.1) | 5.41, dd (11.1, 9.4) | 5.35, dd (11.0, 9.4) | 5.33, dd (11.3, 9.4) |
| **5'** | 6.07, br t (11.3) | 5.92, t (11.1) | 5.91, t (11.1) | 6.09, t (11.1) | 6.05, bt t (11.3) | 6.05, br t (11.5) |
| **6'** | 6.47, br dd (14.5, | 6.45, br dd (14.8, 11.1) | 6.41, br dd (14.8, 11.1) | 6.45, br dd (14.9, 11.1) | 6.34, dd (14.9, 11.3, 6.6) | 6.34, br dd (14.7, 11.5) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 11.3) | | | | | |
| **7'** | 5.82, br dt (15.0, 6.7) | 5.71, br dt (14.8, 7.1) | 5.71, br dt (14.8, 7.5, 7.2) | 5.81, dt (14.9, 7.2) | 5.75, dt (14.9, 6.7) | 5.75, br dt (14.7, 6.6) |
| **8'** | 2.36, br dd (7.4, 6.2) | 2.62, m<br>2.45, br dd (15.7, 8.3) | 2.32, br ddd (14.2, 7.2, 6.8)<br>2.28, br ddd (14.2, 7.5, 6.5) | 2.44, br dd (7.2, 6.8) | 2.87, br dd (15.5, 7.5)<br>2.46, br dd (15.5, 7.5) | 2.87, ddd (7.3, 6.6, 1.0) |
| **9'** | 3.01, dt (6.2, 4.2) | 3.95, br ddd (7.8, 4.6, 2.9) | 3.60, dddd (6.8, 6.1, 3.1) | 3.72, ddd (13.9, 6.8, 3.5) | 5.37, dd (7.3) | 5.38, dd (7.3, 6.6) |
| **9'-OH** | - | - | 5.01, br d (6.1) | | - | - |
| **10'** | 2.97, dt (5.9, 4.2) | 3.56, m | 3.91, ddd (9.8, 3.7, 3.1) | 3.91, ddd (9.8, 3.9, 3.5) | 5.47, ddd (10.9, 7.3, 1.5) | 5.48, ddd (10.4, 7.3, 1.0) |
| **10'-OH** | - | 4.95, br d (4.6) | - | - | - | - |
| **11'** | 1.55, m | 1.43, m | 1.76, ddt (14.4, 7.1, 3.7<br>1.66, ddt (14.1, 4.6, 9.8) | 1.82, m<br>1.66, ddt (14.1, 4.6, 9.8) | 2.04, m | 2.05, dt (14.7, 7.3) |
| **12'** | 1.44, m | 1.26, m | 1.45, m<br>1.33, m | 1.43, m<br>1.33, m | 1.39, m | 1.36, m |
| **13'** | 1.28-1.41 | 1.20-1.36 | 1.20-1.36 | 1.20-1.36 | 1.20-1.35 | 1.28-1.41 |
| **14'** | 1.28-1.41 | 1.20-1.36 | 1.20-1.36 | 1.20-1.36 | 1.20-1.35 | 1.28-1.41 |
| **15'** | 1.28-1.41 | 1.20-1.36 | 1.20-1.36 | 1.20-1.36 | 1.24, m | 1.28-1.41 |
| **16'** | 1.30, m | 1.25, m | 1.24, m | 1.26, m | 1.32, m | 1.30, m |
| **17'** | 1.32, m | 1.27, m | 1.27, m | 1.28, m | 0.89, t (7.0) | 1.30, m |
| **18'** | 0.89, t (7.0) | 0.86, t (6.9) | 0.86, t (6.8) | 0.88, t (7.2) | . | 0.89, t (6.9) |

[a] CDCl3

[b] DMSO-$d_6$

[c] interchangeable

The molecular formula of **3** was established as $C_{25}H_{40}O_5NCl$ based on $^{13}C$ NMR (Table 2) and ESI-FTMS spectral data, requiring six degrees of unsaturation. Analysis of the $^{1}H$ and $^{13}C$ NMR spectral data (Tables 2 and 3) of **3** indicated it was similar to that of **2**, with two notable differences. Compound **3** contained a tri-substituted double bond ($\delta_C$ 125.0, q, C-3'; 133.7, t, C-4'; $\delta_H$ 7.39, s, H-4) which, based on HMBC correlations from $\delta_H$ 7.39 to C-2 and C-5, confirmed it to have the same γ-lactone ring present in korormicin A (Yoshikawa et al. 1997). In addition, the $^{13}C$ chemical shifts of C-9' ($\delta_C$ 67.0, t) and C-10' ($\delta_C$ 71.4, t) along with the shift downfield of their respective protons ($\delta_H$ 3.95, br ddd, 7.8, 4.6, 2.9 H-9'; $\delta_H$ 3.60, m, H-10') indicated the epoxide in **2** was no longer present in **3**. Moreover, a $^{1}H$-$^{1}H$ COSY correlation from $\delta_H$ 4.95 (br d, 4.6) to H-10' confirmed the presence of a hydroxyl moiety at C-10' and hence the location of the chlorine at C-9'. Comparison of the 1D NMR data of **3** with korormicin F (Yoshikawa et al. 2003) confirmed the planar structure of **3**, korormicin H, is as shown in Figure 2.

As for **3**, the molecular formula of **4** was determined to be $C_{25}H_{40}O_5NCl$, again requiring six degrees of unsaturation. The $^{13}C$ NMR spectral data (Table 2) of **4** was identical to that of **3**, except the $^{13}C$ chemical shifts of C-9' and C-10' were reversed. $^{1}H$-$^{1}H$ COSY, HSQC and HMBC correlations, and comparison with literature values of korormicin F (Yoshikawa et al. 2003), confirmed the position of the chlorine at C-10' and the planar structure of **4**, korormicin I, to be as shown in Figure 2.

Compound **5** was determined, by NMR and ESI-FTMS to have the molecular formula $C_{24}H_{39}O_4N$. The 1D NMR spectra data (Tables 2 and 3) of **5** showed strong similarity to **1**, with signals diagnostic of the amide substituted γ-lactone ring. Downfield resonances indicating the presence of an isolated double bond ($\delta_C$ 126.7, CH; $\delta_H$ 5.37, dd, 7.3; $\delta_C$ 129.2, $\delta_H$ 5.47, ddd, 10.9, 7.3, 1.5; *cis* geometry) were observed in place of those for the epoxide in

**1**. Mass spectral and $^{13}C$ NMR data also confirmed that **5** had one less methlyene unit than **1**, hence the planar structure of **5**, korormicin J, is as shown.

The final compound isolated in this study, compound **6**, was established as having the molecular formula $C_{25}H_{41}O_4N$ by ESI-FTMS. The 1D and 2D NMR (Tables 2 and 3) for **6** were identical to that of **5**, the only difference being the addition of a methylene unit in the alkyl side chain. The regiochemistry of the three double bonds in both compounds **5** and **6** was determined from their $^1H$ NMR coupling constants, $J_{4',5'}$ (11.0 and 11.3), $J_{6',7'}$ (14.9 and 14.7), and $J_{9',10'}$ (7.3 and 7.3), to be *Z*, *E* and *cis*, respectively. The planar structure of **6**, korormicin K, is as shown in Figure 2.

The complete configuration for the five new korormicins (**2** – **6**) remains unassigned.

### 3.2. Physical properties of novel compounds

**(1)** 4'-((3,4,5-Tribromo-1H-pyrrol-2-yl)methyl)phenol: Pale yellow oil. UV (PDA) $\lambda_{max}$ nm: 222, 279; IR $\nu_{max}$ $cm^{-1}$: 3388 br, 3271, 2925, 1655, 1024, 992, 825; $^1H$ (600 MHz, $CDCl_3$ $CD_3OD$ and DMSO-$d_6$) and $^{13}C$ (125 MHz, $CDCl_3$ and DMSO-$d_6$) NMR data Table 1; (-)-ESI-FTMS *m/z* $[M-H]^-$ 405.8100 (calcd for $C_{11}H_7ONBr_3$ monoisotopic 405.8083).

(**2**) Korormicin G: Colourless oil. $[\alpha]^{24}_D$ -3.5° ($CH_3OH$; *c* 0.84); UV (PDA) $\lambda_{max}$ nm: 232; IR $\nu_{max}$ $cm^{-1}$: 3345, 2914, 1639 str, 1620, 1553, 1388, 1237, 1103, 1044; $^1H$ (600 MHz, $CDCl_3$) Table 2 and $^{13}C$ (125 MHz, $CDCl_3$) NMR data Table 3; (+)-ESI-FTMS *m/z* $[M+ Na]^+$ 458.2863 (calcd for $C_{25}H_{41}O_5NNa$ 458.2877).

**(3)** Korormicin H*:* Colourless oil. $[\alpha]^{24}_D$ -9.5° ($CH_3OH$; *c* 0.23); UV (PDA) $\lambda_{max}$ nm: 232; IR $\nu_{max}$ $cm^{-1}$: 3408, 2963, 2926, 1734, 1635, 1455, 1372, 1234, 1024, 904; $^1H$ (600 MHz, $CDCl_3$ and DMSO-$d_6$) Table 2 and $^{13}C$ (125 MHz, $CDCl_3$ and DMSO-$d_6$) NMR data Table 3.; (+)-ESI-FTMS *m/z* $[M+Na]^+$ 492.2497 (calcd for $C_{25}H_{40}O_5NClNa$ 492.2487).

(**4**) Korormicin I: Colourless oil. $[\alpha]^{24}_{D}$ -9.5° ($CH_3OH$; *c* 0.23); UV (PDA) $\lambda_{max}$ nm: 232; IR $\nu_{max}$ $cm^{-1}$: 3408, 2963, 2926, 1734, 1635, 1455, 1372, 1234, 1024, 904; $^{1}H$ (600 MHz, DMSO-$d_6$) Table 2 and $^{13}C$ (125 MHz, DMSO-$d_6$) NMR data Table 3.; (+)-ESI-FTMS *m/z* $[M+Na]^+$ 492.2499 (calcd for $C_{25}H_{40}O_5NClNa$ 492.2487).

(**5**) Korormicin J: Colourless oil. $[\alpha]^{24}_{D}$ -9.5° ($CH_3OH$; *c* 0.23); UV (PDA) $\lambda_{max}$ nm: 232; IR $\nu_{max}$ $cm^{-1}$: 3408, 2963, 2926, 1734, 1635, 1455, 1372, 1234, 1024, 904; $^{1}H$ (600 MHz, $CDCl_3$) Table 2 and $^{13}C$ (125 MHz, $CDCl_3$) NMR data Table 3.; (+)-ESI-FTMS *m/z* $[M+Na]^+$ 428.2768 (calcd for $C_{24}H_{39}O_4NNa$ 428.2771).

(**6**) Korormicin K: Colourless oil. $[\alpha]^{21}_{D}$ 17° ($CH_3OH$; *c* 0.23); UV (PDA) $\lambda_{max}$ nm: 232; IR $\nu_{max}$ $cm^{-1}$: 3408, 2963, 2926, 1734, 1635, 1455, 1372, 1234, 1024, 904; $^{1}H$ (600 MHz, $CDCl_3$) Table 2 and $^{13}C$ (125 MHz, $CDCl_3$) NMR data Table 3; (+)-ESI-FTMS *m/z* $[M+Na]^+$ 442.2919 (calcd for $C_{25}H_{41}O_4NNa$ 442.2928).

### 3.3 Physical properties of known compounds

**(I)** Bromoalterochromide A: Yellow solid. $^{1}H$-NMR and $^{13}C$-NMR spectral data were consistent with published values (Speitling et al. 2007); (+)-ESI-FTMS m/z $[M+Na]^+$ 866.2611 (calcd for $C_{38}H_{50}O_{10}N_7BrNa^+$ 866.2695, Δ 9 ppm), (-)-ESI-FTMS *m/z* $[M-H]^-$ 842.2761 (calcd for $C_{38}H_{49}O_{10}N_7Br^-$ 842.2730, Δ 4 ppm).

**(II)** Korormicin B (previously 1a): Colourless oil. $^{1}H$-NMR and $^{13}C$-NMR spectral data were consistent with published values (Yoshikawa et al. 2003); (+)-ESI-FTMS *m/z* $[M+Na]^+$ 428.2402 (calcd for $C_{23}H_{35}O_5NNa$ 428.2407, Δ 1 ppm).

**(III)** Tetrabromopyrrole: Brown oil. $^{1}H$-NMR and $^{13}C$-NMR spectral data were consistent with published values (Andersen et al. 1974, Tebben et al. 2011); (-)-ESI-FTMS *m/z* $[M-H]^-$ 377.6771, (calcd for $C_4NBr_4^-$ 377.6770).

**(IV)** Korormicin E (previously 3)

Colourless oil. $^{1}$H-NMR and $^{13}$C-NMR spectral data were consistent with published values (Yoshikawa et al. 2003); (+)-ESI-FTMS *m/z* $[M+Na]^+$ 442.2547 (calcd for $C_{24}H_{37}O_5NNa$ 442.2564, Δ 4 ppm).

**(V)** Korormicin C (previously 1b)

Colourless oil. $^{1}$H-NMR and $^{13}$C-NMR spectral data were consistent with published values [ref]; (+)-ESI-FTMS *m/z* $[M+Na]^+$ 442.2556 (calcd for $C_{24}H_{37}O_5NNa$ 442.2564, Δ 2 ppm).

**(VI)** Korormicin A (previously 1): Colourless oil. $^{1}$H-NMR and $^{13}$C-NMR spectral data were consistent with published values (Yoshikawa et al. 2003); (+)-ESI-FTMS *m/z* $[M+Na]^+$ 456.2708 (calcd for $C_{25}H_{39}O_5NNa$ 456.2720, Δ 3 ppm).

**(VII)** Korormicin D (previously 1c): Colourless oil. $^{1}$H-NMR and $^{13}$C-NMR spectral data were consistent with published values (Yoshikawa et al. 2003); (+)-ESI-FTMS *m/z* $[M+Na]^+$ 470.2874 (calcd for $C_{26}H_{41}O_5NNa$ 470.2877, Δ 1 ppm).

## 3.4 Antibacterial Effects of Bacterial Metabolites

The antimicrobial activities of compounds **1 – 4** and **6** against 13 bacterial strains and one fungal strain are presented in Table 3.4. Compound **5** was insufficient to be tested. Compounds (**1 - 4** and **6**) exhibited antibacterial activities. The strongest antibacterial activity was attributed to TBP that inhibited growth of all strains under investigation. TBP was also the only compound with antifungal activity. Compound **1** was active against the gram positive bacterium, *Staphylococcus aureus*. Interestingly, compound **1** showed broad-spectrum antibacterial activity, however, it was not active against the fungus nor against J010 (from which it was isolated) and another *Pseudoalteromonas* strain cp21 that was isolated from CCA. Bromoalteochromide A did not show any antibacterial or antifungal effect.

**Table 3.4**: Antibacterial and antifungal activities of compounds **1-4, 6** and TBP

| Strains | Growth media | Growth temperature (°C) | Inhibition by compound observed |
|---|---|---|---|
| *Pseudoalteromonas haloplanctis* | MA | 28 | 1-4,6, TBP |
| *Pseudoalteromonas picicida* | MA | 28 | 1-4,6, TBP |
| *Vibrio campbellii* | MA | 28 | 1-4,6, TBP |
| *Vibrio vulnificus* | LB10 | 37 | 1-4,6, TBP |
| *Pseudoalteromonas undina* | MA | 28 | 1-4,6, TBP |
| *Staphylococcus aureus* | LB10 | 37 | 1, TBP |
| *Candida albicans*[#] | MA | 28 | TBP |
| *Pseudoalteromonas* strain. J010* | MA | 28 | TBP |
| *Krokinobacter* strain ub2* | MA | 28 | 1-4,6, TBP |
| *Pseudoalteromonas* strain cp21* | MA | 28 | 4,6, TBP |
| *Shewanella* strain. cp20* | MA | 28 | 1-4,6, TBP |
| *Vibrio* strain A133* | MA | 28 | 1-4,6, TBP |
| *Vibrio* strain Ap18* | MA | 28 | 1-4,6, TBP |

## 3.5 Antiprotozoal Effects of Bacterial Metabolites

The antiprotozoal bioassay-guided fractionation of J010 yielded two active fractions containing TBP and bromoalterochromide A. The active concentration was determined to be 1 $\mu g.mL^{-1}$ for TBP and 50 $\mu g.mL^{-1}$ for bromoalterochromide A. At this concentration all flagellates formed cysts within 5 min of incubation, with cell lyses of all cells occurring within 30 min. None of the novel compounds **1 – 4** or **6** exhibited any toxicity against the protozoa. There was no effect of the solvent controls on grazer numbers in comparison with unmanipulated controls.

## 4. Conclusions

Marine macroalgae often harbour a stable community of host associated microorganisms (Lachnit et al. 2011). This epiphytic conglomerate of microorganisms is distinct from bacteria in the surrounding seawater and inanimate surfaces (Longford et al. 2007, Lachnit et al. 2009, Burke et al. 2011a, Burke et al. 2011b). Several bacterial strains isolated from host algae exhibit antagonistic effects against bacteria, fungi, algal spores and

invertebrate larvae *in vitro* (Egan et al. 2000, Dobretsov et al. 2006, Penesyan et al. 2009, Wiese et al. 2009, Wilson et al. 2010). Chemical screening of these isolates showed that host-associated bacteria produce a wide array of chemically diverse defence compounds (Franks et al., 2001, Skovhus et al. 2007, Matz et al. 2008, Penesyan et al. 2011).

Six new compounds, the unprecedented bromopyrrole, 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) and the novel five korormicins G – K (**2** – **6**), together with the five known compounds korormicins A – E (Yoshikawa et al. 1997, Yoshikawa et al. 2003), one known tetrabrominated pyrrole (Andersen et al. 1974, Tebben et al. 2011) and bromoalterochromide A (Speitling et al. 2007) were isolated from J010 collected from the surface of the crustose coralline alga *N. fosliei*. Although there are many publications detailing the isolation of bromopyrroles from marine sources, including sponges (Keifer et al. 1991, Assmann et al. 2001, Aiello et al. 2006, Grube et al. 2006, Haber et al. 2010) and bacteria, this report shows that investigations are still yielding further new and unprecedented derivatives, and that continued investigations of this chemical class are warranted. The biological and pharmacological properties associated with bromopyrroles including antibacterial/antimicrobial (Yoshikawa et al. 2003, Schillaci et al. 2005, Raimondi et al. 2006), feeding deterrent/antiprotozoal (Scala et al. 2010, Haber et al. 2011) and antineoplastic (Xiong & Pang 2010) activities, are promising leading to the need for more extensive structure-activity relationship studies and evaluation of their mechanism of action.

# Chapter 4

## Settlement Cues for Larvae of the Acroporid Coral *Acropora millepora* from the Crustose Coralline Alga *Hydrolithon onkodes*

***Abstract*:** Chemical signals from crustose coraline algae (CCA) serve as important signposts during larval settlement of many marine invertebrates. This study demonstrates that at least two different chemical compound classes, organic-soluble low molecular weight compounds and water-soluble macromolecular polysaccharide-type polymers, explain larval settlement of the scleractinian coral *Acropora millepora* in response to a highly abundant Great Barrier Reef CCA, *Hydrolithon onkodes*. The organic CCA extracts were repeatedly purified by bioassay-guided fractionation employing different chromatographic conditions during six spawning seasons between 2009 and 2011. The bioactive fractions of *H. onkodes* were dominated by algal membrane glycoglycerolipids, namely sulfoquinovosyl-monoacylglycerol (SQMG) and monogalactosyl-monoacylglycerol (MGMG). Since the chromatographically pure lipids were biologically inactive, another currently unknown factor is likely to be associated with these lipids and renders this complex inductive to coral larval settlement. Another chemically distinct inductive extract was obtained by aqueous extraction of the CCA under high temperature and pressure. The bioactive component in this sample had a molecular mass >100,000 Da and showed properties reminiscent of polysaccharide macromolecules. Chemical larval settlement cues from CCA have long been suggested to play an important role in larval settlement of the coral *A. millepora*. This study narrows the search for these signals to two compound classes, and substantially characterises the small organic molecules. Given their presence on the algal surface and accessibility for surface-exploring coral planulae, these compound classes are logical indicators for the suitability of settlement substrates.

***Keywords:*** Glycoglycerolipids; monogalactose monoacylglycerol (MGMG), sulfoquinovosyl monoacylglycerol (SQMG); polysaccharides; larval settlement cues; *Hydrolithon onkodes*; *Acropora millepora*; coral larvae; crustose coralline algae (CCA)

## *1. Introduction*

A broad range of marine benthic invertebrate larvae settle selectively and choose permanent attachment sites favorable for survival and reproduction (Hadfield & Paul 2001). The process of larval settlement involves two steps, attachment to the substratum followed by metamorphosis into a juvenile benthic invertebrate. Like many other marine invertebrate larvae, competent coral planulae probe the substratum for cues that provide integrated information about the relative attractivenessof the substratum (Harrison & Wallace 1990). While broad environmental factors such as light, salinity, hydrodynamics, sedimentation levels, water depth and substrate orientation influence larval site selection of corals (Rogers et al. 1984, Babcock & Davies 1991, Maida et al. 1994, Mundy & Babcock 1998, Raimondi & Morse 2000, Gleason et al. 2006), the suitability of coral attachment substrates is primarily determined by biochemical factors present on living surfaces. These include certain crustose coralline algae (CCA), including their associated microbial biofilms (Sebens 1983, Morse et al. 1988, Heyward & Negri 1999), and reef biofilms on inanimate surfaces, such as coral rubble (Webster et al. 2004).

While it is clear from numerous studies that cues associated with the surfaces of CCA are highly inductive towards coral larvae, the exact nature of those cues has remained elusive. Indeed this is the case for chemical cues for settlement of invertebrate larvae generally, with only a handful of ecologically relevant cues identified after 40 years of research on the topic (Hadfield and Paul 2001). For corals, much attention has focused on the role of microorganisms in natural biofilms as larval inducers, after it was demonstrated that bio-conditioned terracotta tiles and coral rubble trigger larval settlement of coral planulae (Babcock 1984, Babcock & Heyward 1986). Marine biofilms, particularly their bacterial component, have subsequently been shown to induce larval metamorphosis in several classes of cnidarians, such as hard and soft corals (Morse et al. 1988, Webster et al. 2004),

scyphozoa (Brewer 1978) and hydroids (Leitz & Wagner 1993). However, the components of microbial biofilms responsible for inducing larval settlement remain unclear, either in terms of bacterial species or specific metabolites of those bacteria. No cues have been established as inducers of coral larval settlement, neither for broadcast spawning acroporids (Negri et al. 2001) nor brooding corals (Tran & Hadfield 2011). Furthermore, even the role of those bacteria identified as putative inducers is uncertain; "inductive" *Pseudoalteromonas* bacteria isolated from the surface of CCA trigger metamorphosis of coral planulae but not larval attachment (Negri et al. 2001, Tebben et al. 2011 [Chapter 2]).

Larvae of many members of dominant hard corals, including those in the Acroporidae and Faviidae, settle upon contact with biotic surfaces such as CCA, and with varying degrees of specificity to different species of CCA (Morse et al. 1988, Heyward & Negri 1999, Harrington et al. 2004, Ritson-Williams et al. 2010). As a consequence a separate stream of research (from that of microbial biofilms) focused on the inductive properties of the algae as holobionts (host plus associated bacteria) has emerged. Morse et al. (1988) identified an insoluble inducer of settlement from CCA that was active in the field (Raimondi & Morse 2000) and proposed a common chemosensory mechanism for several Pacific acroporid coral larval species (Morse & Morse 1991). This common inducer or "morphogen" was enzymatically characterised and suggested to be "associated with" or "may itself contain a sulphated glycosaminoglycan" (Morse & Morse 1991). However, the purification and unequivocal structural elucidation of the causative compound or compound class was never pursued and remains speculative.

In a repeat of Morse and Morse's (1999) protocol for producing the coral larval "morphogen", a methanol extraction of the insoluble filter residue of decalcified CCA yielded a crude extract with highly bioactive morphogenetic properties towards *Acropora millepora* larvae (Heyward and Negri 1999). This procedure further simplified by directly

extracting the CCA yielded highly inductive extracts from the CCA *Titanoderma prototypum*, *Hydrolithon onkodes* and *Neogoniolithon fosliei* for larval settlement of two species of acroporid corals (Harrington et al. 2004). The origin of the compounds, whether microbial or algal, remained undetermined in all these studies, though Morse and Morse (1991) argued that the activity associated with glycosaminoglycan was algal cell wall derived.

Given its high solubility in methanol, it would be expected that the CCA-extractable morphogen is chemically different from the glycan-like polymers proposed earlier by Morse & Morse (1991). Recently, Kitamura and colleagues characterised two structurally different inducers for scleractinian coral larval settlement from crude extracts of CCA and coral rubble: the macrodiolide luminaolide, which induces *Lepastrea purpurea* (Kitamura et al. 2009), and 11-deoxyfistularin-3, whose induction of *Pseudosiderastrea tayamai* is amplified by the carotenoids fucoxanthin and fucoxanthinol (Kitamura et al. 2007). The structural differences of these CCA-derived coral larval settlement cues may suggest that different coral phyla have chemosensory specificities for different compounds present in CCA extracts, contrary to the proposed common chemosensory mechanism in broadcast spawning hard corals.

The present study investigated larval settlement of the reef-building coral *Acropora millepora* in response to chemical signals produced by one of the most abundant species of CCA on the Great Barrier Reef, *Hydrolithon onkodes*. Although *A. millepora* is one of the most widely studied coral species with regard to its ecology, genetics (Babcock & Davies 1991, Heyward & Negri 1999, Ayre & Hughes 2000, Kortschak et al. 2003, Berkelmans & van Oppen 2006, Meyer et al. 2009) and larval ecology (Negri & Heyward 1999, Negri et al. 2001, Webster et al. 2004, Harrington et al. 2004, Grasso et al. 2011, Tebben et al. 2011, Siboni et al. 2012), the nature of the chemical cues that trigger larval settlement have remained elusive. Given prior evidence that organic extracts of *H. onkodes* triggered larval

settlement of *A. millepora* (Negri & Heyward 1999, Harrington et al. 2004, Grasso et al. 2011) the present investigation focused on this algal species. A bioassay-guided fractionation strategy coupled with a variety of spectroscopic techniques was applied to isolate and identify the settlement inducers present on *H. onkodes*. Purified fractions containing the coral larval settlement cues were assayed both in solution and immobilised on a substratum in order to test their ability to enhance the inductive nature of otherwise inert surfaces, thereby eliciting settlement and similar to the effect observed on natural CCA chips.

## *2. Methods*

### 2.1. General experimental procedures

*Spawning and culturing of coral larvae*

Three to five colonies of *Acropora millepora* (Fig. 4.1) were collected by hand 3 - 5 days prior to predicted spawning events from two sites on the Great Barrier Reef (GBR) (November 2009 at Orpheus Island, 18º37' S 146º28' E and December 2009 at Trunk Reef, 18º22' S, 146º47' E, October and November 2010 at Orpheus Island, October 2011 at Orpheus Island, and November 2011 at Pelorus Island 146 29.304 E, 18 33.001'S). Coral brood stocks were transported to laboratories at the Australian Institute of Marine Science (Townsville, QLD) for spawning and larval rearing. CCA were collected along with corals at most sites, cleaned of all other macroscopic organisms and maintained in separate flow-through seawater tables (to serve as live positive CCA controls) or directly extracted as described below. All live specimens were collected under the Australian Institute of Marine Science permit (GBRMPA G09/30237.1). The coral colonies were maintained in outdoor tanks (1000 L) with flow-through seawater at ambient temperature (approximately 28ºC) and isolated in 60 L bins each night a few hours before the predicted spawning. Larval culture

techniques followed Heyward and Negri (1999). After spawning, gametes were gently scooped from the water surface and fertilised in tanks containing FSW (1 µm) for 3 h. Fertilisation was checked microscopically and the embryos were gently washed three times in 50 L FSW (0.2 µm) to remove excess sperm. The cleaned embryos were transferred to 500 L larval rearing tanks with flow-through FSW (0.2 µm) where they were maintained in low densities (~1 larvae $mL^{-1}$).

**Figure 4.1.** *A. millepora* colony, Trunk reef 2009.

*Larval settlement assays*

Larval bioassays were performed in sterile 6- and 12-well culture plates at 27 – 28 °C. Larvae were deemed suitable for settlement assays once they reached competency, as determined by settlement rates higher than 80 % in response to live *H. onkodes* (Fig. 4.2, positive controls), typically 5-7 days post spawning. Larval responses were recorded after 12 h under a dissecting microscope and were categorised as swimming (swimming or crawling, elongated body shape) or settled (metamorphosis plus attachment to the dish surface, settlement substratum, CCA or matrix). CCA extracts and purified compounds were either

dissolved in non-volatile solvents (dimethyl sulfoxide, DMSO) and added directly to plate wells, or in volatile solvents (e.g. methanol, MeOH) and allowed to evaporate in the plate wells. DMSO and MeOH controls (solvents only) were also tested throughout. In contrast to commonly applied procedures, in this study complete drying down of extracts was avoided in order to limit the degradation of potentially unstable compounds. Throughout the separation procedures in this study, extracts, fractions and compounds were generally prepared in a standardised concentrated stock solution in the ratio of 1 kg CCA in 100 mL solvent (note that the CCA was for the most part still encrusted onto rock, thus the ratio of live CCA mass to solvent is largely overstated). Aliquots of these stock solutions were then chromatographically purified. Volumes for each chromatographic step were adjusted such that the original CCA mass/solvent ratio was maintained throughout each isolation sequence. Aliquots of each test solution (fraction or compound) were concentrated or diluted and tested in a concentration range spanning eight orders of magnitude, i.e. from $10^{3}$ to $10^{-4}$ of the original chromatographic volume, unless stated otherwise.

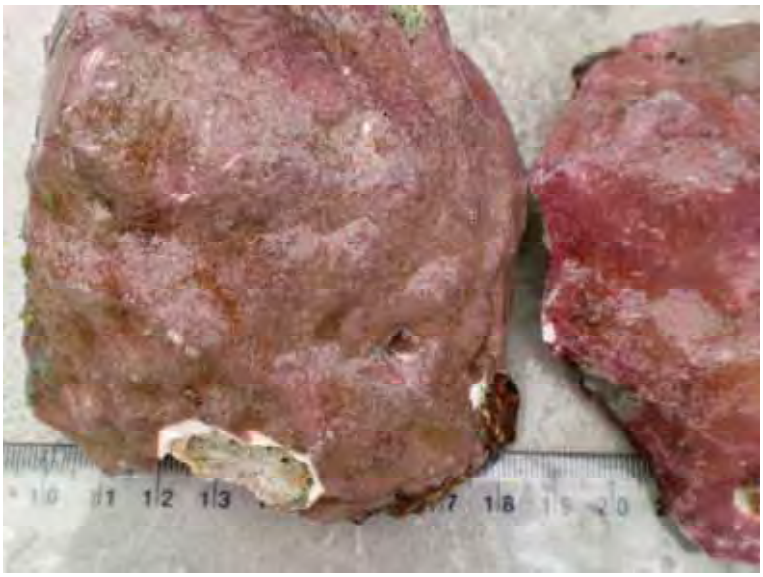


**Figure 4.2.** *H. onkodes* in the laboratory, collected Trunk reef 2009.

*Chromatography*

The larval settlement-inducing compounds present in CCA extracts were purified by iterative bioassay-guided fractionation. The iteration involved subjection bioactive fractions to instrumental analytical chemistry techniques to identify the compound class and main structural features of dominant compounds in these fractions. Based on this knowledge, the downstream chromatographic techniques were optimised to suit the identified compound class and thus further enhance separation of sample constituents. A range of preparative and analytical chromatographic techniques was used to separate extract constituents. Preparative scale vacuum-flash chromatography (VFC) was performed using both normal-phase (Silica gel 60 G, Merck, Germany) and reversed-phase (Silica gel 60 RP-18, 40-63 um, Merck, Germany) methods (in the following referred to as $SiO_2$ VFC and RP-18 VFC, respectively). Analytical normal-phase thin layer chromatography ($SiO_2$ TLC) was performed on Silica gel 60 F254S aluminium backed plates (Merck, Germany). Spots were visualised by illumination under UV light (254 and 365 nm, Spectroline CM 12) and staining with iodine vapour, unless otherwise specified. Sample spots were scraped off with a Merck TLC scraper (Merck, Germany) and eluted in MeOH unless otherwise specified. High-performance liquid chromatography (HPLC) using Luna Phenyl-Hexyl (Phenomenex, 250 x 4.5 mm, 5 µm), Silica (Phenomenex, 250 x 4.6 mm, 5 µm) and Diol (YMC, 150 x 10 mm, 5 µm) columns was performed on a Shimadzu HPLC system consisting of a SCL-10Avp system controller equipped with a LC-10AT pump, a SPD-M10Avp photodiode array detector, a FRC-10A fraction collector, and a SIL-10A autosampler controlled by Class-VP software. Chromatograms were monitored at 220, 254 and 390 nm.

*Spectroscopic analysis*

The semi-purified fractions were analysed by a number of spectroscopic techniques including nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography–mass spectrometry (LC-MS) and Fourier transform mass spectrometry (FT-MS). One- and two-dimensional NMR spectra were recorded in $CD_3OD$, $CDCl_3$ or DMSO-$d_6$ (Cambridge Isotopes Laboratories Inc. USA) using 3 mm Bruker MATCH NMR tubes on a BrukerAvance 600 MHz NMR spectrometer equipped with a cryoprobe. All NMR spectra were referenced to residual $^1H$ and $^{13}C$ resonances in the deuterated solvents ($CD_3OD$, δ 3.31 for $^1H$ and δ 49.0 for $^{13}C$; $CDCl_3$, δ 7.27 for $^1H$ and δ 77.0 for $^{13}C$; DMSO-$d_6$, δ 2.50 for $^1H$ and δ 39.5 for $^{13}C$). Both 1D and 2D NMR spectra were recorded using standard Bruker pulse sequences. The low resolution mass spectra of crude extracts and fractions were measured either via direct injection or following chromatography on an Agilent 1100 series HPLC system (consisting of a degasser, a binary pump, an auto sampler, a column oven and a photodiode array detector) coupled to a Bruker Esquire 3000 quadrupole ion trap LC-mass spectrometer (LC-MS).

Accurate mass spectra of semi-purified fractions were measured using a BrukerBioApex 47e FT-ICR mass spectrometer fitted with an Analytica of Branford electrospray source; ions were detected in either negative or positive mode within a mass range *m/z* 200-2000. Direct infusion of MS samples (0.2 mg m/L) was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 150 μL/h.

*Other materials*

Compressed gases were supplied by BOC Gases (Townsville, Australia) and were at least 99.99% pure. Purified water was obtained from a Milli-Q water purification system

(Millipore, MA, USA). All other solvents used were HPLC grade (Mallinckrodt, MO, USA). *Mucor javanicus* lipase (lyophilised powder, ≥300 units/mg) was obtained from Sigma-Aldrich (Australia). Monogalactose diacylglycerol (MGDG), sufoquinovosyl diacylglycerol (SQDG) and digalagtose diacylglycerol (DGDG) were obtained from Biolipids (UK). All other chemicals were sourced from Sigma-Aldrich (Australia).

## 2.2. Isolation of organic-soluble larval settlement cues

The bioassay-guided fractionation of organic CCA extracts was repeated during six coral spawning seasons (Nov/Dec of 2009, 2010 and 2011). Each fraction separation procedure was optimised based on the partial chemical structure information of bioactive fractions and their chromatographic behaviour identified from the preceding step.

*Chromatographic Method A*

The CCA *H. onkodes* (500 g wet wt) was extracted three times with ethanol (EtOH) under sonication for 80 min (1.5 L total). The EtOH extract was filtered through filter paper (Whatman no. 1), dried by rotary evaporation and subjected to RP-18 VFC (600 x 40 mm) with stepwise elution of water (250 mL), EtOH (250 mL), ethyl acetate (EtOAC, 250 mL) and hexane (250 mL). Each of these extracts was prepared as a stock solution of 50 mL solvent and tested as described above. Aliquots of the bioactive EtOH fraction were further purified by $SiO_2$ TLC with chloroform ($CHCl_3$):MeOH (4:1, v/v). Sample spots were scraped off according to UV (254 and 365 nm) and iodine visualisation, The $SiO_2$ was then extracted with MeOH and the solvent evaporated, although not to completeness. The fractions were redissolved in MeOH in the original aliquot volume applied to the TLC and tested in the bioassay yielding two bioactive $SiO_2$ TLC fractions. Preliminary NMR analyses of these $SiO_2$ TLC fractions revealed glycoglycerolipids as major components (Appendix 12). Based on

this initial structural identification, the chromatographic conditions and specific TLC staining methods were adjusted and optimised for isolation of this chemical compound class.

In order to obtain sample of separated compounds for bioassays and still apply selective but destructive TLC staining techniques, the $SiO_2$ TLC plates were cut vertically, leaving a 1 cm strip on each side of the plate. These strips were sprayed with EtOH-sulphuric acid (60:40, v/v) containing 0.4 % orcinol (Svennerholm 1956) and heated to at least 100 ºC to reveal red and brown lipid-specific sample spots. The $R_f$-values of these sample spots were used as a guide to scrape off fractions of interest from the unstained mid section of the $SiO_2$ TLC plate, which were subsequently eluted in $CHCl_3$, $CHCl_3$:MeOH (1:1) and MeOH, and concentrated by evaporation under a stream of nitrogen. The regions between these spots were also scraped so that the entire chromatographic profile was available for bioassays. Aliquots of these $SiO_2$ TLC fractions were tested in the bioassay and bioactive fractions were further analysed by spectroscopic methods. If the spectroscopic analyses revealed impurities in the samples, they were re-chromatographed by $SiO_2$ TLC under optimised mobile phase conditions. Other TLC surface chemistries, such as cyano, amino, and RP-18 were also trialled but proved either ineffective in separating bioactive compounds or did not yield any bioactive fractions.

*Chromatographic Method B*

*H. onkodes* (1500 g wet weight) was extracted three times with EtOH under sonication for 2 h (5 L total). The EtOH extract was filtered (Whatman no. 1) and dried on RP-18 gel. This material was loaded onto a pre-equilibrated RP-18 VFC (80 x 600 mm), desalted with water (A1, 600 mL) and eluted with EtOH (B2, 600 mL). Fraction B2 was further separated by $SiO_2$ VFC (80 x 600 mm) and three fractions were collected: B3 ($CHCl_3$, 600 mL), B4 (acetone, 600 mL) and B5 (MeOH, 600 mL). Fraction B3 was subsequently

chromatographed on $SiO_2$ TLC with $CHCl_3$:MeOH (4:1), stained and scraped according to the sulphuric acid/orcinol method described above (Svennerholm 1956) to yield five fractions (B6-B10). Fraction B9 was re-chromatographed on $SiO_2$ TLC ($CHCl_3$:MeOH:Water (45:10:1)) to yield compound **1**.

*Chromatographic Method C*

*H. onkodes* (2300 g wet weight) was extracted three times with EtOH under sonication for 2 h (5 L total). The EtOH extract was filtered (Whatman no. 1) and dried on silica gel (Merck, Kieselgel 60, 70–230 mesh) by rotary evaporation. This material was loaded onto a pre-equilibrated SiO2 VFC (80 x 600 mm) and three fractions were collected: C1 ($CHCl_3$, 500 mL), C2 ($CHCl_3$:MeOH (9:1), 500 mL) and C3 (MeOH, 500 mL). The bioactive fractions C1 and C2 were combined and separated by RP-18 VFC (600 x 40 mm) to give C4 (water, 600 mL), C5 (water:MeOH (9:1), 600 mL), C6 (water:MeOH (4:1), 600 mL), C7 (MeOH, 600 mL) and C8 (EtOAc, 600 mL). The bioactive fractions C4 and C5 were pooled and further purified by $SiO_2$ TLC as per Lepage (1967) using $CHCl_3$-acetone-acetic acid-MeOH-water (50:20:10:10:5, v/v). The $SiO_2$ TLC was stained with sulfuric acid/orcinol as described above (Svennerholm 1956) and cut into five individual fractions (C9-C13). As shown by NMR, the bioactive fraction C10 contained compound **1** (Appendix 8) and was further separated on $SiO_2$ TLC with an optimised mobile phase system of $CHCl_3$-acetone-acetic acid-MeOH-water (45:15:10:10:5, v/v) to yield three separate fractions (C11-C14). These fractions were individually purified on $SiO_2$ TLC in $CHCl_3$:acetone:acetic acid:MeOH:water (45:15:10:5, v/v) to yield compounds **2** (Appendix 9), **3** and **4**.

*HPLC Method*

The bioactive $SiO_2$ TLC fractions B7 and C10 were further purified by HPLC. Different column chemistries, such as Luna Phenyl-Hexyl (Phenomenex, 250 x 4.5 mm, 5 µm), Silica (Phenomenex, 250 x 4.6 mm, 5 µm) and Diol (YMC, 150 x 10 mm, 5 µm) were tested for their capacity to further resolve these bioactive compounds and detect other possible impurities.

The phenyl-hexyl column was run with solvent A: Water/MeOH (95/5, v/v), B: MeOH, C: $CHCl_3$/MeOH (95/5, v/v) at a flow rate of 1 ml/min and a gradient of 99% A:1% B to 100%B over 15 mins followed by a second gradient from 100 % B to 100 % C over 10 min followed by 5 mins isocratic elution with 100 % C. The silica column was run with solvent A: $CHCl_3$, B: MeOH/water (95/5, v/v) at a flow rate of 1 ml/min and a gradient from 99% A:1% B to 75% A:25% B over 15 mins, followed by isocratic elution with 100% B for 5 min. The diol column was run as per Yunoki et al. (2009) with solvent A ($CHCl_3$) and solvent B (MeOH:acetone–water–acetic acid (30:60:9:1, v/v/v/v)) with 0.3% triethylamine (pH 4) at a flow rate of 3 ml $min^{-1}$. Fractions were collected according to the peak profile obtained at 220 nm and in one minute time intervals, respectively, avoiding any time gaps between intervals or fractions.

## 2.3. Glycoglycerolipid standards and their enzymatic digestion

Mono- and diacylated glycoglycerolipids were identified as a compound class of interest during the bioassay-guided fractionation in 2011. Therefore, commercially available standards of sulfoquinovosyl diacylglycerol (SQDG), monogalactose diacylglycerol (MGDG) and digalactose diacylglycerol (DGDG) were tested in the bioassay. Since the monoacylated forms of these three lipid standards (MGMG, DGMG and SQMG) were not commercially available, a commercial MGDG (Biolipids, UK) and MGDG isolated from Australian baby

spinach (Woolworths, Australia) was enzymatically hydrolysed. Briefly, spinach (500 g) was extracted with EtOH (500 mL), filtered, concentrated and applied to a $CHCl_3$ pre-conditioned $SiO_2$ VFC (60 x 120 mm). The $CHCl_3$ eluate (500 mL) was dried under vacuum, further separated on $SiO_2$ TLC ($CHCl_3$:MeOH = 3:1, v/v) and purified on $SiO_2$ TLC ($CHCl_3$:MeOH:water = 60:10:1, v/v) to yield 17 mg MGDG. The isolated MGDG (17 mg) and the commercial MGDG (2 mg) standard were hydrolysed with lipase according to procedures described in (Yoshida & Alexander 1983, Murakami et al. 1991, Maeda et al. 2005). Briefly, MGDG was suspended in 0.6 mL of tris buffer (pH 7.6) with 1800 units of lipase (*Mucor javanicus*, Sigma-Aldrich, Australia) and 2.5 mg Triton X-100, and incubated for 12 h at 37°C. The reaction was quenched with acetic acid (0.1 mL) and the reaction mixture extracted with n-butanol. The reaction mixture was separated by analytical $SiO_2$ TLC developed in $CHCl_3$:MeOH (4:1). Due to time constraints related to coral spawning events and larval competency, this procedure could not be repeated for SQDG in order to test its monoacylated analogue.

### 2.4 Aqueous-soluble larval settlement cues and their hydrolysis

Cold aqueous extracts from 2009 and 2010 did not have any activity. However, in 2011 the aqueous extraction method was adapted to liberate polysaccharide compounds (see Results). *H. onkodes* (500 g wet weight) was washed three times in 500 mL Milli-Q water to remove salts and autoclaved under pressure at 121°C for 60 min in 250 mL water. The aqueous extract was decanted and the algae were re-extracted twice with fresh Milli-Q water. The pooled extract was twice filtered (Whatman no. 1, followed by 0.2 μm), frozen at -80°C and lyophilised. The dry extract was taken up in 200 mL water, filtered again and tested in logarithmic serial dilutions covering 10 orders of magnitude (from $10^2$ to $10^{-7}$).

Four 10 mL aliquots of this extract were ultrafiltered (UF) affording four corresponding pairs of size-separated fractions: ultrafiltration residue 1 (UFR1)/ultrafiltration filtrate 1 (UFF1) (Amicon Ultra-15, 10 kDa NMWL), UFR2/UFF2 (Amicon Ultra-15, 30 kDa NMWL), UFR3/ UFF3 (Amicon Ultra-15, 50 kDa NMWL) and UFR4/UFF4 (Amicon Ultra-15, 100 kDa NMWL). The residues (UFR1-4) were washed twice with EtOH (5 mL) to remove any organic-soluble impurities, recovered in 10 mL water and tested in the same serial dilution series as described above.

The bioactive aqueous fraction (>100 kDa, 1 mL) was total-hydrolysed in 50 % trifluoroacetic acid in a sealed vial at 100 °C (oil bath) for 1 h. The reaction mixture was allowed to cool and then dried under vacuum. The dried product was taken up in 1 mL water (the original concentration) and tested in standard dilutions as described above.

### 2.5. Immobilisation of bioactive samples

Two bioactive semi-purified organic cues and two semi-purified aqueous fractions were immobilised in a range of organic matrices to determine if the settlement signals maintained their biological activity while bound to the surfaces. Two $SiO_2$ TLC-purified organic-soluble fractions were prepared from 500 g *H. onkodes* as per method A. The aqueous-soluble fractions UFR1 and UFR4 were also tested. A logarithmic serial dilutions covering 10 orders of magnitude (from $10^2$ to $10^{-7}$) of the bioactive fractions in EtOH was added onto equal amounts of solid paraffin wax (53-57 °C, melting point, Sigma, Australia) and allowed to evaporate. The paraffin was then melted at 55 °C. Likewise, a dilution series of the aqueous ultrafiltrates UFR1 and UFR4 were mixed with aqueous agars to a final concentration of 2 % Agar (NO. 1, OXOID, UK) and 10 % agar (Carrageenan type I, Sigma, Australia), respectively. This mixture was then autoclaved for 20 min at 121 °C. While still liquid, 10 μL aliquots of each of the infused matrices (agar and paraffin) were added to the

base of 6-well plates, allowed to solidify and then tested for their ability to induce settlement (n = 6, 10 larvae per well). The volumes (10 µL) of these cue matrices were the same independent of their cue concentration. The concentration that exhibited greatest bioactivity in this preliminary test was used in a secondary scaled-up study by applying larger volumes of bioactive samples onto larger matrix volumes. This was achieved by painting molten agar and paraffin onto one side of terracotta tiles (5 x 5 cm, n = 30). Controls of the same matrices, containing no cue and using the same volume of applied matrix were also prepared. In order to wash off the non-immobilised settlement cues, the tiles were soaked in 50 L bins containing FSW (0.2 µm) for 3 h, replacing the water in hourly intervals. The settlement substrates were randomly placed into three 500 L tanks (10 substrates per treatment per tank) containing competent *A. millepora* larvae at approximately 1 larva per mL. Larval settlement on these substrates was scored and photographed after 24 h.

### 2.6. Statistics

Extracts, fractions or compounds were classified as 'active' when the most active concentration in the bioassays elicited settlement rate greater than 70 % and "inactive" when the mean settlement of the most active concentration was below 10 %. In most assays larval settlement was binary (high settlement or none) and negative controls rarely showed any settlement. The mean settlement of the most active concentration was recorded in the Results section of this chapter. For assays in which settlement varied more quantitatively, effects of different extracts or treatments were compared by analysis of variance (ANOVA). Cochran's test was used to test for homogeneity of variances. The mean number of settled coral per replicate settlement substrate was analysed by two-way ANOVA with cue matrix type as a fixed factor (nine levels) and Tank as a random factor, orthogonal factor (three levels). ANOVA was followed by post hoc pair-wise comparisons among treatments using Tukey's HSD test (Zar 1996).

## *3. Results*

### 3.1. Separation of organic-soluble bioactive cues

The EtOH extract from different batches of *H. onkodes* collected and extracted over three consecutive years (two spawning events per year from 2009 to 2011) induced consistently high rates of larval settlement. Competent larvae generally settled with rates of 80 to 100 % in 6- and 12-well plates (1 larva per mL). CCA extracts maintained their activity despite long-term storage at -20 °C, i.e. extracts from 2009 and 2010 induced the same rate of settlement as extracts prepared in 2011. Larvae typically did not settle at all in control or solvent control wells.

*Chromatographic Method A*

The bioassay-guided fractionation of the bioactive *H. onkodes* EtOH extract (71.82 ± 7.72 %, mean settlement ± SE, n = 3, Fig. 4.3) yielded two bioactive fractions on $SiO_2$ TLC: fraction A2 ($R_f$ = 0.21, 83.33 ± 12.01 %, mean settlement ± SE, n = 3) and fraction A4 ($R_f$ = 0.95, 70.0 ± 10.0 %, mean settlement ± SE, n = 3). The latter eluted directly below the pigment/solvent front (A5, $R_f$ = 1). The NMR analysis of these $SiO_2$ TLC fractions revealed glycoglycerolipids as major sample components (Appendix 12).

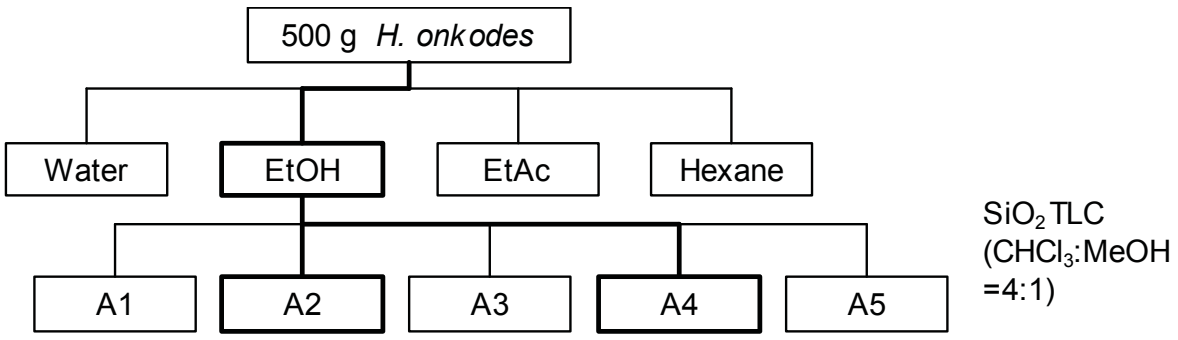



**Figure 4.3.** Bioassay-guided fractionation of *H. onkodes* extract using chromatographic method A ($SiO_2$ TLC = analytical thin layer chromatography on silica gel 60). Thick black line indicate fractions with larval settlement > 70 % (n = 3), thin black lines indicate bioassays with settlement < 10 % or no activity.

The identification of the dominant chemical compound class in the settlement-inducing fractions led to more targeted chromatographic methods for isolation of the settlement inducers, such as method B (Fig. 4.4) and C (Fig. 4.5).

*Chromatographic Method B*

The bioactive EtOH extract of 1500 g of *H. onkodes* (73.49 ± 5.94 %, mean settlement ± SE, n = 3) was desalted by RP-18 VFC and the ensuing EtOH eluate B2 (88.57 ± 5.94 %, mean settlement ± SE, n = 3) further separated on $SiO_2$ VFC (EtOH eluate B3, 100 ± 0 %, mean settlement ± SE, n = 3). The purification of B3 on analytical $SiO_2$ TLC yielded one bioactive fraction (B7, $R_f$ = 0.62, 83.01 ± 1.65 %, mean settlement ± SE, n = 3) that was subsequently re-chromatographed on $SiO_2$ TLC to give a semi-pure bioactive fraction B12 ($R_f$ = 0.51, 85.71 ± 14.28 %, mean settlement ± SE, n = 3) containing **1** (Figure 4.4) as the main constituent.

Although the $^1$H NMR spectrum suggested that **1** was pure, albeit with signals present on the baseline (Appendix 8, Appendix 10), there was a strong blue fluorescence (observed under UV, 365 nm) associated with this fraction. Thus, the dominant fraction contained other co-eluting impurities, albeit in small concentrations. Fraction B12 was further purified on $SiO_2$ TLC ($R_f$ = 0.38) yielding **1**. Although **1** as well as the entire chromatographic range of the plate was eluted and tested as individual samples and as a combined pool over a wide concentration range ($10^4$ to $10^{-7}$), this pooled sample did not induce any settlement of *A. millepora* larvae. This was in contrast to the bioactive fraction B12.

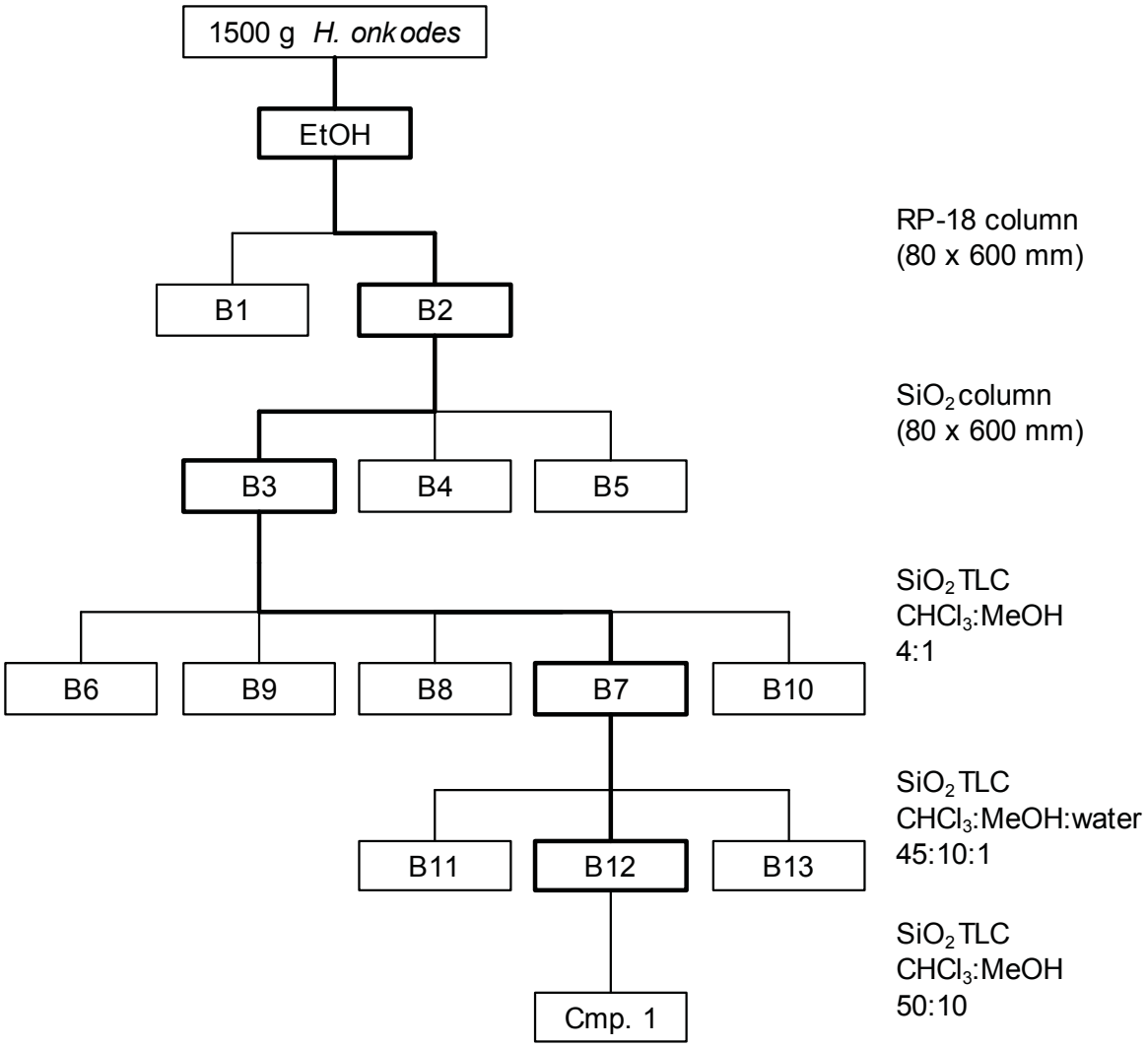



**Figure 4.4.** Bioassay-guided fractionation of *H. onkodes* extract using chromatographic method B ($SiO_2$ column = vacuum flash column chromatography on silica gel G; RP-18 column = vacuum flash column chromatography on C18 reverse phase; $SiO_2$TLC = analytical thin layer chromatography on silica gel 60). Thick black line indicates bioassays with larval settlement >70 % (n=3), thin black lines indicate bioassays with settlement < 10 % or no activity.

*Chromatographic method C*

The bioactive EtOH extract of 2300 g of *H. onkodes* was separated by $SiO_2$ VFC to yield two bioactive fractions C1 (58.33 ± 8.33 %, mean ± SE, n = 3) and C2 (61.48 ± 11.20 %, mean ± SE, n = 3). These fractions were pooled and separated by RP-18 VFC to yield a further two bioactive fractions C6 (100 ± 0 %, mean ± SE, n = 3) and C7 (100 ± 0 %, mean ±

SE, n = 3). Fractions C6 and C7 were also pooled and separated by $SiO_2$ TLC to yield bioactive fractions C10 ($R_f$ = 0.20, 83.71 ± 1.89 %, mean ± SE, n = 3), containing **2** as the main constituent, and the highly pigmented and chromatographically unresolved fraction C13 ($R_f$ = 0.98, 74.44 ± 35.95 %, mean ± SE, n = 3), containing the previously purified **1** (using chromatographic method A) as its main constituent. The NMR analysis of fraction C10 showed a relatively pure compound **2** (Figure 4.5) as the main constituent (Appendix 11) together with other impurities evident as a fluorescent spot that overlapped with the orcinol-red spot on TLC plates. Thus, fraction C10 was further purified by $SiO_2$ TLC using $CHCl_3$:acetone:MeOH:water (45:15:10:10:5) resulting in three fractions with moderate settlement activity identified as C15 ($R_f$ = 0.25, 45.0 ± 22.91 %, mean ± SE, n = 3) with **2** as the main constituent, C16 ($R_f$ = 0.28, 51.85 ± 28.93 %, mean ± SE, n = 3) and C17 ($R_f$ = 0.30, 27.78 ± 27.78 %, mean ± SE, n = 3). These fractions were individually purified on analytical TLC, yielding three compounds (**2** $R_f$ = 0.17 (Appendix 9), **3** $R_f$ = 0.28 and **4**, $R_f$ = 0.31). Similar to chromatographic method B, all chromatographic fractions were tested individually and pooled in a wide concentration range ($10^5$ to $10^{-7}$). None of these fractions showed settlement activity in the bioassays. Some of the less chromatographically resolved fractions (C1, C2, C13) still showed moderate activity in comparison with purified fractions.

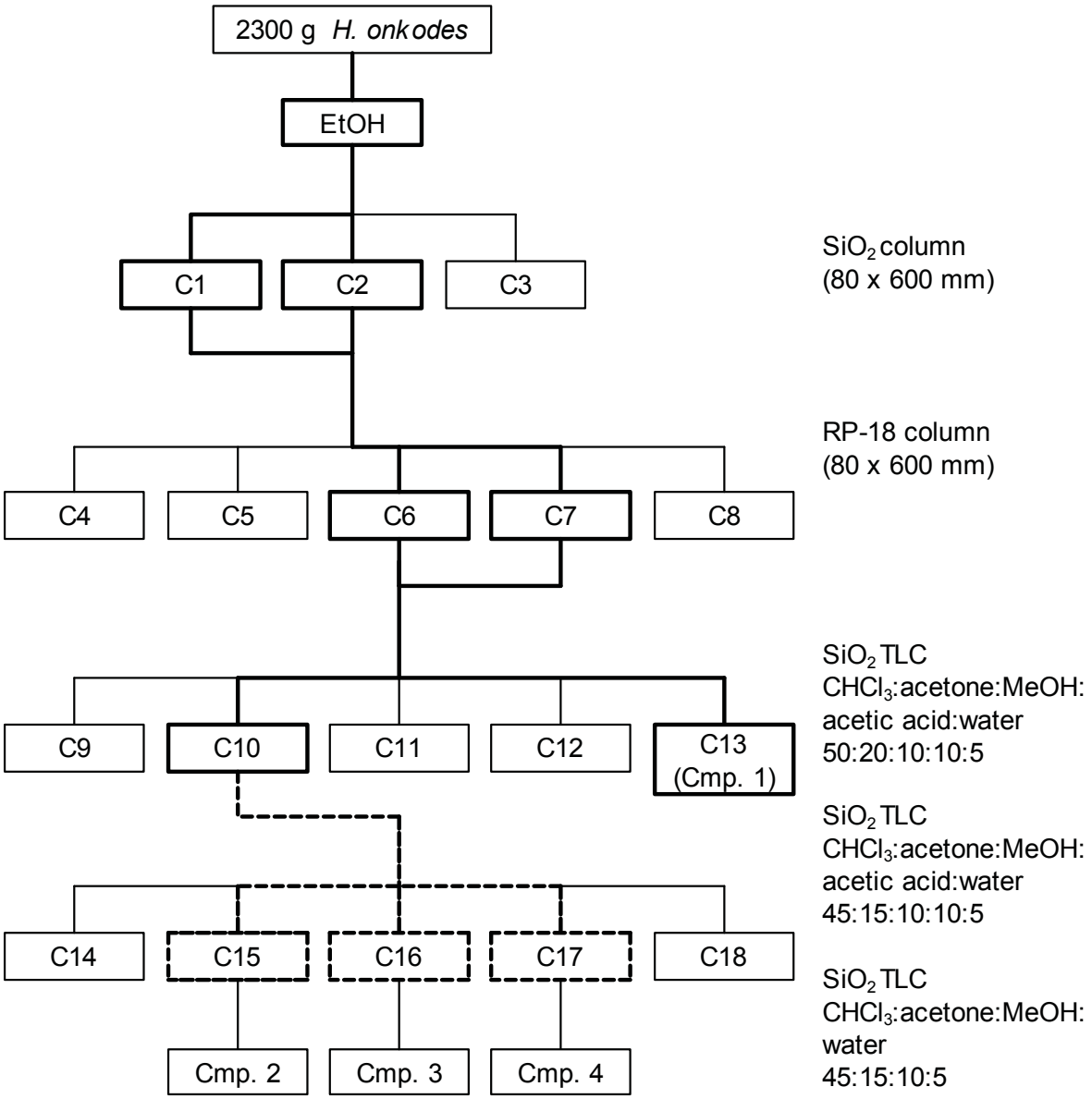



**Figure 4.5.** Bioassay-guided fractionation of *H. onkodes* extract using chromatographic method B ($SiO_2$ column = vacuum flash column chromatography on silica gel G; RP-18 column = vacuum flash column chromatography on C18 reverse phase $SiO_2$TLC analytical thin layer chromatography on silica gel 60). Thick black line indicates bioassays with larval settlement > 70 %, thick dashed line indicates bioassays between 70% and 25 %, thin black lines indicate bioassays with settlement between 0 and 10 % (n = 3).

*3.2. Structural elucidation of compounds of interest*

The molecular formula of **1** was established as $C_{25}H_{42}O_9$ based on $^{13}C$ NMR (Table 2) and ESI-FTMS spectral data, requiring five degrees of unsaturation. The $^{13}C$ NMR signals at $\delta_C$ 104.9, 72.1, 74.5, 69.9, 76.4 and 62.0, and the coupling in the $^1H$ NMR of 7.6 Hz ($J_{1'-2'}$) and 3.3 Hz ($J_{3'-4'}$) indicated the presence of a β-galactopyranose (Kwon 1998). The $^1H$ NMR

and $^{13}C$ NMR spectra of **1** closely resembled those of the commercially available MGDG ($[M+Na]^+$ *m/z* 769.4852, calcd for $C_{43}H_{70}O_{10}Na$ 769.4861) except for the signals on the glycerol moiety. The chemical shifts for the sn-2 carbon were observed upfield for both $^{13}C$ and $^{1}H$ ($\delta_C$ 69.3; $\delta_H$ 4.00, dd 5.7, 4.8) as compared to MGDG ($\delta_C$ 71.5; $\delta_H$ 5.27, m), indicating the sn-2 carbon was not acylated (Sassaki *et al*., 1999). The fatty acid composition in **1** was determined by NMR and (+)-FTMS and indicated the presence of a C16 fatty acid. Further, the $^{1}H$-NMR and $^{13}C$-NMR analyses of **1** in comparison with MGDG and the long-range connectivities observed in the HMBC spectrum between the ester carbonyl 175.3 and $\delta_H$ 4.16 ppm, confirmed the fatty acid side chain was attached at sn-1 on the glycerol moiety. The stereochemistry at sn-2 of the glycerol was determined as 2*S* based on the coupling constants 5.1 Hz ($J_{3\alpha\text{-}2}$) and 4.4 Hz ($J_{3\beta\text{-}2}$), the upfield shift of the sn-1$\alpha$ ($\delta_H$ 4.16, d (4.9); 1$\beta$ $\delta_H$ 4.15, d (6.0)) and comparison with literature values (Kwon et al. 1998, Higara et al. 2008). The geometry of the two double bonds ($\delta_C$ 132.6, 130.4, 129.5 and 128.8) in the fatty acid side chain was determined to be *cis* based on the chemical shifts of the adjacent doubly allylic methylene signals ($\delta_C$ 26.2, $\delta_H$ 2.84, ddd 12.4, 10.4, 5.5, 2H). As a result, the chemical structure of **1** has been determined as (2*S*)-1-*O*-(7,10,13-hexadecatrienoyl)-3-*O*-β-D-galactopyranosyl-sn-glycerol (MGMG, **1**).

The molecular formula of **2** was established as $C_{25}H_{48}O_{11}S$ based on $^{13}C$ NMR (Table 2) and ESI-FTMS spectral data, requiring two degrees of unsaturation. Mass spectral analyses also revealed peaks corresponding to $[M - H]^-$ and the pseudomolecular ion of the Na salt $[M - H + 2Na]^+$, which is reminiscent of sulfonoglycolipids (Al-Fadhli et al. 2006), and accounted for a sulfonic acid moiety ($SO_3H$). The $^{13}C$ NMR signals at $\delta_C$ 98.0, 71.1, 72.7, 72.4, 67.7 and the upfield shift 52.1 (C-6'), and the relative small coupling in the $^{1}H$ NMR of 3.3 Hz ($J_{1'\text{-}2'}$) indicated the presence of a $\alpha$-link glycosyl moiety. The large vicinal coupling constants observed for $J_{2'\text{-}3'}$ (9.5 Hz), $J_{3'\text{-}4'}$ (9.0 Hz) and $J_{4'\text{-}5'}$ (9.0 Hz), confirmed the

glucopyranosyl nature of the sugar moiety. The $^{1}H$ NMR and $^{13}C$ NMR spectra of **2** closely resembled those of SQDG ($[M+Na]^{+}$ *m/z* 839.4955, calcd for $C_{43}H_{76}O_{12}Na$ 839.4950) except for the signals on the glycerol moiety. The signals for sn-2 were shifted upfield for both $^{13}C$ and $^{1}H$ ($\delta_C$ 67.8; $\delta_H$ 4.03, m) as compared to SQDG ($\delta_C$ 71.5; $\delta_H$ 5.31, m), again indicating the sn-2 carbon was not acylated (Sassaki et al. 1999). The fatty acid composition in **2** was determined by NMR (no double bond signals were observed at δ 128.0 to 133.0) and FTMS indicating the presence of a fully saturated C16 fatty acid (palmitic acid, $C_{16:0}$). Further, the $^{1}H$-NMR and $^{13}C$-NMR analyses of **2** in comparison with SQDG and the long range connectivities observed in the HMBC spectrum between the ester carbonyl 175.7 and $\delta_H$ 4.20 ppm (sn-$1_\alpha$ proton in $CD_3OD$), confirmed the fatty acid side chain was attached at position sn-1 on the glycerol moiety. The stereochemistry at sn-2 of the glycerol unit was determined as 2*R* based on the coupling constants 7.2 Hz ($J_{3\alpha\text{-}2}$) and 2.3 Hz ($J_{3\beta\text{-}2}$), the upfield shift of sn-1α ($\delta_H$ 4.07, dd 11.1, 6.1; and sn-1β 4.16, dd 10.9, 3.3) and comparison with literature values (Hiagara et al. 2008). As a result, the chemical structure of **2** has been determined as (2*R*)-1-*O*-(palmitoyl)-3-*O*-α-D-(6'-sulfoquinovosyl)-sn-glycerol (SQMG, **2**).

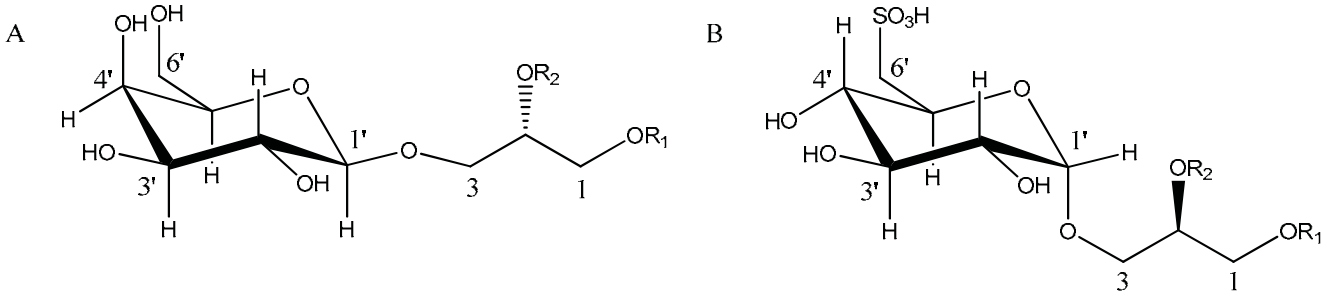



**Figure 4.6.** Chemical structures of A: MGDG (R1 = R2 = acyl side chain), MGMG (**1**, R1 = acyl side chain, R2 = H) and B: SQDG (R1 = R2 = acyl $C_{16:0}$ side chain) and SQMG (**2**, R1 = acyl $C_{16:0}$ side chain, R2 = H).

**Table 4.1**: $^{13}C$ NMR shifts of glycolipids MGDG, SQDG, MGMG (**1**) and SQMG (**2**) in $CD_3OD$

| | **MGMG (1)** | **MGMG (Kwon 1998)** | **MGDG** [a] | **SQMG (C15)** [b] | **SQMG (2)**[c] | **SQMG (Kitagawa 1979)**[d] | **SQDG** [e] |
|---|---|---|---|---|---|---|---|
| **No.** | **$\delta_C$, mult.** | **$\delta_C$, mult.** | **$\delta_C$, mult.** | **$\delta_C$, mult.** | **$\delta_C$, mult.** [a] | **$\delta_C$, mult.** | **$\delta_C$, mult.** |
| **1** | 66.2, $CH_2$ | 66.6, $CH_2$ | 63.6, $CH_2$ | 66.4, $CH_2$ | 64.7, $CH_2$ | 66.4, $CH_2$ | 64.2, $CH_2$ |
| **2** | 69.3, CH | 69.7, CH | 71.5, CH | 69.7, CH | 67.8, CH | 69.2, CH | 71.5, CH |
| **3** | 71.4, $CH_2$ | 71.9, $CH_2$ | 68.4, $CH_2$ | 70.3, $CH_2$ | 68.4, $CH_2$ | 70.0, $CH_2$ | 67.1, $CH_2$ |
| **1'** | 104.9, CH | 105.3, CH | 105.0, CH | 99.9, CH | 98.0, CH | 99.6, CH | 99.8, CH |
| **2'** | 72.1, CH | 72.6, CH | 72.1, CH | 73.5, CH | 71.1, CH | 72.5, CH | 73.4, CH |
| **3'** | 74.5, CH | 74.9, CH | 74.6, CH | 75.0, CH | 72.7, CH | 73.9, CH | 74.5, CH |
| **4'** | 69.9, CH | 70.3, CH | 69.9, CH | 74.7, CH | 72.4, CH | 73.5, CH | 74.9, CH |
| **5'** | 76.4, CH | 76.8, CH | 76.5, CH | 69.7, CH | 67.7, CH | 69.2, CH | 69.8, CH |
| **6'** | 62.0, $CH_2$ | 62.5, $CH_2$ | 62.1, $CH_2$ | 54.0, $CH_2$ | 52.1, $CH_2$ | 53.3, $CH_2$ | 54.1, $CH_2$ |
| **1''** | 175.3, qC | 175.5, qC | 174.6, qC | 175.7, qC | ND, qC | 176.1, qC | 174.7, qC |
| **2''** | 34.6, $CH_2$ | 33.9, $CH_2$ | 34.7, $CH_2$ | 34.8, $CH_2$ | 33.6, $CH_2$ | 34.9, $CH_2$ | 34.7, $CH_2$ |
| **3''** | 25.6, $CH_2$ | 25.9, $CH_2$ | 25.7, $CH_2$ | 25.9, $CH_2$ | 24.4, $CH_2$ | 25.7, $CH_2$ | Not resolved |
| **4''** | 30.2, $CH_2$ | 29.87, $CH_2$ | 30.1, $CH_2$ | 23.6, $CH_2$ | 28.9, $CH_2$ | 30.5, $CH_2$ | 23.5, $CH_2$ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **5''** | 30.2-32.7, $CH_2$ | 30.3, $CH_2$ | Not resolved | | | 30.5, $CH_2$ | Not resolved |
| **6''** | 27.3, $CH_2$ | 28.0, $CH_2$ | Not resolved | | | 30.5, $CH_2$ | Not resolved |
| **7''** | 128.8, CH | 128.2, CH | 30.3, $CH_2$ | - | - | 31.0, $CH_2$ | 30.1, $CH_2$ |
| **8''** | 129.5, CH | 129.0, CH | 27.9, $CH_2$ | | | 31.0, $CH_2$ | 27.9, $CH_2$ |
| **9''** | 26.3, $CH_2$ | 26.5, $CH_2$ | 128.0 CH | | | 31.0, $CH_2$ | 131.3 CH |
| **10''** | 130.4, CH | 129.2, CH | 128.7 CH | | | 31.0, $CH_2$ | 128.8, CH |
| **11''** | 132.6, CH | 129.3, CH | 26.3, $CH_2$ | | | 31.0, $CH_2$ | 26.2, $CH_2$ |
| **12''** | 26.3, $CH_2$ | 26.5, $CH_2$ | 130.5 CH | | | 31.0, $CH_2$ | 128.8, CH |
| **13''** | 128.8, CH | 130.9, CH | 132.2 CH | | | 30.3 | 131.3, CH |
| **14''** | 128.8, CH | 132.8, CH | Not resolved | 30.3, $CH_2$ | 28.9, $CH_2$ | 32.9, $CH_2$ | Not resolved |
| **15''** | 27.9, $CH_2$ | 21.5, $CH_2$ | Not resolved | 23.6, $CH_2$ | 22.2, $CH_2$ | 23.6, $CH_2$ | Not resolved |
| **16''** | 14.2, $CH_3$ | 14.6, $CH_3$ | | 14.3, $CH_3$ | 13.5, $CH_3$ | 14.8, $CH_3$ | |
| **17''** | | | 21.1, $CH_2$ | | | | 21.1, $CH_2$ |
| **18''** | | | 14.3, $CH_3$ | | | | 14.3, $CH_3$ |

[a] standard MGDG (nature of the two side chains was not fully resolved) in $CD_3OD$
[b] semi-purified SQMG in fraction C15 in $CD_3OD$
[c] purified SQMG in $CD_3OD/CDCl_3$, no HMBC data
[d] SQMG in $CD_3OD/CDCl_3$
[e] standard SQDG (nature of the two side chains was not fully resolved) in $CD_3OD$

**Table 4.2**: $^{1}$H NMR shifts of glycolipids MGDG, SQDG, MGMG (**1**) and SQMG (**2**) in $CD_3OD$

| | **MGMG (1)** | **MGMG (Kwon 1998)** | **MGDG[a]** | **SQMG (C15) [b]** | **SQMG (2)[c]** | **SQMG (Takahashi 2002) [d]** | **SQDG [e]** |
|---|---|---|---|---|---|---|---|
| **No.** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** |
| **1** | 4.15, d (6.0)[f]<br>4.16, d (4.9) | 4.13, dd (11.4, 5.9)<br>4.16, dd (11.4, 4.6) | 4.22, dd (12.1, 7.1)<br>4.45, dd (12.1, 2.9) | 4.10, m<br>4.20, dd (13.9, 6.4) | 4.07, dd (11.1, 6.1)<br>4.16, dd (10.9, 3.3) | 3.96, dd (10.7, 6.3)<br>4.03, dd (10.7, 4.4) | 4.18, dd (12.0, 6.9)<br>4.52, dd (12.0, 2.9) |
| **2** | 4.00, dd (5.7, 4.8) | 3.98, m | 5.27, m | 4.09, m | 4.03, m | 3.91, m | 5.31, m |
| **3** | 3.67, dd (10.5, 4.4)<br>3.92, dd (10.5, 5.1) | 3.65, dd (10.5, 4.6)<br>3.90, dd (10.5, 5.2) | 3.74 (10.9, 5.5)<br>3.99 (10.9, 5.5) | 3.40, m<br>4.06, m | 3.36, dd (10.1, 7.2)<br>3.93, dd (10.3, 2.3) | 3.33, dd (10.7, 6.4)<br>3.71, dd (10.7, 4.4) | 3.57, dd (10.8, 6.4)<br>4.11, dd (10.8, 5.3) |
| **1'** | 4.24, d (7.6) | 4.22, d (7.6) | 4.24, d (7.3) | 4.78, d (3.8) | 4.78, d (3.3) | 4.59, d, (3.4) | 4.76, d (3.8) |
| **2'** | 3.55, dd (9.8, 7.7) | 3.53, dd (9.7, 7.6) | 3.51, dd (9.7, 7.3) | 3.42, dd (9.7, 3.8) | 3.45, dd (9.5, 3.1) | 3.18, dd (9.3, 3.4) | 3.40, dd (9.7, 3.8) |
| **3'** | 3.49, dd (9.7, 3.3) | 3.46, dd (9.7, 3.4) | 3.46, dd (9.7, 3.3) | 3.63, t (9.3) | 3.63, m | 3.40, dd (9.3, 9.3) | 3.64, dd (9.3, 9.3) |
| **4'** | 3.85, br d (3.3) | 3.82, dd (3.3, 0.9) | 3.83, dd (3.2, 0.4) | 3.07, br t (9.4) | 3.18, br t (9.0) | 2.96, dd (9.3, 8.8) | 3.08, dd (9.4, 9.4) |
| **5'** | 3.53, m | 3.51, m | 3.51, m (?) | 4.09, m | 3.99, br t (9.0) | 3.88, ddd (8.8, 5.9, 4.9) | 4.07, td (9.6, 1.9) |
| **6'** | 3.74, br t (5.7) | 3.71, dd (11.3, 5.3)<br>3.76, dd (11.5, 6.9) | 3.72, dd (11.3, 5.3)<br>3.76, dd (11.3, 6.9) | 2.92, dd (14.3, 9.4)<br>3.38, m | 2.99, dd (14.5, 7.4)<br>3.36, dd (10.1, 7.2) | 2.61, dd (13.7, 5.9)<br>2.91, dd (13.7, 4.9) | 2.91, dd (14.3, 9.3)<br>3.35, dd (14.3, 2.0) |
| **2''** | 2.36, dt (10.6, 7.5) | 2.35, t (7.4) | 2.32 dd or t (7.3, 2.0) | 2.37, t (7.5) | 2.31, t (7.6) | | 2.33, m |
| **3''** | 1.69, ddd (12.0, 10.3, | 1.62, m | 1.61, dd (12.8, 6.2) | 1.62, br dd (141, | 1.57, dt (14.9, 7.4) | | 1.60, dd (13.6, |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 6.6)<br>1.62, m | | | 7.0) | | | 6.7) |
| **4''** | 1.31-1.37, m | 1.37, m | 1.31-1.33, m | 1.34, m (envelope) | 1.25, m (envelope) | | 1.30-1.35, m |
| **5''** | 1.31-1.37, m | 1.37, m | 1.31-1.33, m | 1.34, m (envelope) | 1.25, m (envelope) | | 1.30-1.35, m |
| **6''** | 2.13, m | 2.09, m | Not resolved | 1.34, m (envelope) | 1.25, m (envelope) | | 1.30-1.35, m |
| **7''** | 5.35-5.39, m | 5.34, m | 1.35-1.44, m | 1.34, m (envelope) | 1.25, m (envelope) | | 1.35-1.38, m |
| **8''** | 5.35-5.39, m | 5.34, m | 2.08, m | 1.34, m (envelope) | 1.25, m (envelope) | | 2.08, m |
| **9''** | 2.84, ddd (12.4, 10.4, 5.5) | 2.80, br t (5.8) | 5.28 – 5.41, m | 1.34, m (envelope) | 1.25, m (envelope) | | 5.38, m |
| **10''** | 5.35-5.39, m | 5.34, m | 5.28 – 5.41, m | 1.34, m (envelope) | 1.25, m (envelope) | | 5.34, m |
| **11''** | 5.35-5.39, m | 5.34,m | 2.82, t (6.0) | 1.34, m (envelope) | 1.25, m (envelope) | | 2.82, br t (6.0) |
| **12''** | 2.84, ddd (12.4, 10.4, 5.5) | 2.80, br t (5.8) | 5.28 – 5.41, m | 1.34, m (envelope) | 1.25, m (envelope) | | 5.38, m |
| **13''** | 5.35-5.39, m | 5.34, m | 5.28 – 5.41, m | 1.34, m (envelope) | 1.25, m (envelope) | | 5.34, m |
| **14''** | 5.35-5.39, m | 5.34, m | Not resolved | 1.67, dt (14.4, 6.7) | 1.57, m | | Not resolved |
| **15''** | 2.08, m | 2.09, m | Not resolved | 1.33, m | 1.25, m | | Not resolved |
| **16''** | 0.98, t (7.6) | 0.97, t (7.6) | Not resolved | 0.90, t (7.1) | 0.83, t (7.0) | | Not resolved |
| **17''** | | | 2.09, m | | | | 2.10, m |
| **18''** | | | 0.98, t (7.5) | | | | 0.99, t (7.5) |

[a] standard MGDG (nature of the two side chains was not fully resolved) in $CD_3OD$
[b] semi-purified SQMG in fraction C15 in $CD_3OD$
[c] purified SQMG in $CD_3OD/CDCl_3$, no HMBC data
[d] SQMG in DMSO-$d_6$/$D_2O$. Note that only the glycosyl and glycerol protons were reported.
[e] standard SQDG (nature of the two side chains was not fully resolved) in $CD_3OD$
[f] 2nd order coupling

**Compound 1 (***MGMG)*

Colourless oil. 1H (600 MHz, CD3OH) and 13C (125 MHz, CD3OH) NMR data Table 1 and 2, respectively; (+)-ESI-FTMS m/z [M+Na]+ 509.2724 (calcd for C25H42O9Na+ 509.2721).

**Compound 2** (SQMG)

Colourless oil. $^{1}H$ (600 MHz, $CD_3OH$) and $^{13}C$ (125 MHz, $CD_3OH$) NMR data Table 1 and 2, respectively; (+)-ESI-FTMS *m/z* $[M - H + 2Na]^+$ 601.2608 (calculated for $C_{25}H_{47}O_{11}SNa_2^+$ 601.2629), (-)-ESI-FTMS *m/z* $[M - H]^-$ 555.2894 (calculated for $C_{25}H_{47}O_{11}S^-$ 555.2845).

### 3.3. Glycolipid standards and digestion

MGDG isolated from spinach (17 mg; (-)-ESI-FTMS *m/z* $[M-H]^-$ 743.4800, calculatedfor $C_{43}H_{67}O_{10}$ monoisotopic 743.4740) and commercially available MGDG (2 mg) were enzymatically degraded with lipase from *Mucor javanicus* to afford the monoacyated derivative MGMG. The crude reaction mixture was analysed by $^{1}H$ NMR (Appendix 12). The $^{1}H$ spectrum was very complex with many overlapping peaks due to the presence of the lipase and the Triton x100. However, the absence of the signals for sn-1 ($\delta_C$ 63.7; $\delta_H$ 4.45, 4.22) and sn-2 ($\delta_C$ 71.3; $\delta_H$ 5.26) of the starting material MGDG and the appearance of signals indicative of sn-1 ($\delta_C$ 66.4; $\delta_H$ 4.16, 4.15) and sn-2 ($\delta_C$ 69.4; $\delta_H$ 4.00) of MGMG indicated that the acyl chain at sn-2 in MGDG had been hydrolysed (Appendix 13). Furthermore, the HMBC revealed a long range coupling from the sn-1 protons at $\delta_H$ 4.16 and 4.15 and from H-2''' ($\delta_H$ 2.36, br t 7.4) to a carbon at $\delta_C$ 175.8 (C1'''), confirming the acyl chain at sn-1 was intact. The $SiO_2$ TLC purification of the reaction mixture with gave one spot ($R_f$= 0.62) that showed the characteristic dark red colour for sugar moieties when

visualised with the orcinol-sulfuric acid reagent and had a $R_f$ of 0.59, similar to MGMG (**1**) isolated from CCA ($R_f$ = 0.61). Neither the crude reaction mixture nor of the purified $SiO_2$ TLC fractions induced larval settlement in the assays.

**Table 4.3**: $^{1}$H NMR shifts of glycoglycerolipid MGMG obtained from lipase digestion of commercial grade MGDG in $CD_3OD$

| | **MGMG (1) isolated from CCA** | **MGMG (Kwon 1998)** | **MGDG[a]** | **MGMG from digest of MGDG (no clean up)** |
|---|---|---|---|---|
| **No.** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** | **$\delta_H$ (*J* Hz)** |
| **1** | 4.15, d (6.0)[f]<br>4.16, d (4.9) | 4.13, dd (11.4, 5.9)<br>4.16, dd (11.4, 4.6) | 4.22, dd (12.1, 7.1)<br>4.45, dd (12.1, 2.9) | 4.14, m<br>4.15 dd (11.0, 5.7) |
| **2** | 4.00, dd (5.7, 4.8) | 3.98, m | 5.27, m | 4.00, dt (10.0, 5.1) |
| **3** | 3.67, dd (10.5, 4.4)<br>3.92, dd (10.5, 5.1) | 3.65, dd (10.5, 4.6)<br>3.90, dd (10.5, 5.2) | 3.74 (10.9, 5.5)<br>3.99 (10.9, 5.5) | 3.91, m<br>Overlapping signals |
| **1'** | 4.24, d (7.6) | 4.22, d (7.6) | 4.24, d (7.3) | 4.24, d (7.5) |
| **2'** | 3.55, dd (9.8, 7.7) | 3.53, dd (9.7, 7.6) | 3.51, dd (9.7, 7.3) | 3.56, m Overlapping signals |
| **3'** | 3.49, dd (9.7, 3.3) | 3.46, dd (9.7, 3.4) | 3.46, dd (9.7, 3.3) | 3.50, m Overlapping signals |
| **4'** | 3.85, br d (3.3) | 3.82, dd (3.3, 0.9) | 3.83, dd (3.2, 0.4) | 3.84, m Overlapping signals |
| **5'** | 3.53, m | 3.51, m | 3.51, m (?) | 3.53, m Overlapping signals |
| **6'** | 3.74, br t (5.7) | 3.71, dd (11.3, 5.3)<br>3.76, dd (11.5, 6.9) | 3.72, dd (11.3, 5.3)<br>3.76, dd (11.3, 6.9) | 3.74, m<br>3.78, m |
| **2''** | 2.36, dt (10.6, 7.5) | 2.35, t (7.4) | 2.32 dd or t (7.3, 2.0) | 2.36 br t (7.4) |
| **3''** | 1.69, ddd (12.0, 10.3, 6.6)<br>1.62, m | 1.62, m | 1.61, dd (12.8, 6.2) | 1.61, m |
| **4''** | 1.31-1.37, m | 1.37, m | 1.31-1.33, m | 1.31-1.33, m |
| **5''** | 1.31-1.37, m | 1.37, m | 1.31-1.33, m | 1.35-1.44, m |
| **6''** | 2.13, m | 2.09, m | Not resolved | 1.35-1.44, m |
| **7''** | 5.35-5.39, m | 5.34, m | 1.35-1.44, m | 1.35-1.44, m |
| **8''** | 5.35-5.39, m | 5.34, m | 2.08, m | 2.08, m |
| **9''** | 2.84, ddd (12.4, 10.4, 5.5) | 2.80, br t (5.8) | 5.28 – 5.41, m | 5.27 – 5.40, m |

| | | | | |
|---|---|---|---|---|
| **10''** | 5.35-5.39, m | 5.34, m | 5.28 – 5.41, m | 5.27 – 5.40, m |
| **11''** | 5.35-5.39, m | 5.34,m | 2.82, t (6.0) | 2.82, t (6.0) |
| **12''** | 2.84, ddd (12.4, 10.4, 5.5) | 2.80, br t (5.8) | 5.28 – 5.41, m | 5.27 – 5.40, m |
| **13''** | 5.35-5.39, m | 5.34, m | 5.28 – 5.41, m | 5.27 – 5.40, m |
| **14''** | 5.35-5.39, m | 5.34, m | Not resolved | |
| **15''** | 2.08, m | 2.09, m | Not resolved | 2.09, m |
| **16''** | 0.98, t (7.6) | 0.97, t (7.6) | Not resolved | 0.98, t (7.5) |
| **17''** | | | 2.09, m | |
| **18''** | | | 0.98, t (7.5) | |

[a] standard MGDG (nature of the two side chains was not fully resolved) in $CD_3OD$
[b] 2$^{nd}$ order coupling

### 3.4 Summary of organic extracts and bioactivity and glycoglycerolipid standards

In summary, all bioactive fractions contained the monoacylated glycoglycerolipids SQMG or MGMG. However, final purification of these fractions resulted in a loss of activity which could not be regained even following recombination of the fractions. NMR analysis revealed impurities at low concentrations in the active fractions. Furthermore, impurities were also evident as fluorescent spots on the TLCs. The commercial standards of MGDG and DGDG did not elicit any settlement activity. SQDG induced a moderate settlement rate of 18.3 ± 12.8% (mean ± SE) at 0.1 µg per ml. The enzymatic product of MGDG, MGMG, did not elicit any settlement activity. It is still unclear whether the monoacylated glycoglycerolipids are the settlement-inducing component within these purified fractions.

### 3.5 Aqueous extract and hydrolysis of aqueous ultrafiltrate

Cold aqueous extracts of CCA (2009, 2010) did not elicit any settlement activity in the bioassays. This result was consistent with that of Morse and Morse (1991) who proposed inducers were insoluble cell wall macromolecules (glycosaminoglycans). However, cold extraction techniques are ineffective for extracting cell wall compounds, especially polysaccharides (Rochas & Lahaye 1989). Thus, the extraction procedure was revisited to extract cell wall associated macromolecules, and in particular aqueous extractions conducted in 2011 were performed under elevated temperature and pressure. In contrast to the cold aqueous extracts, these extracts had significant bioactivity. This bioactive extract could also be obtained with CCA that had previously undergone an exhaustive extraction with organic solvents, clearly demonstrating that the causative settlement signals were insoluble in organic solvents and cold water. The ultrafiltrate of the 10, 30, 50 and 100 kDa, size separation showed that the causative molecule was larger than 100 kDa eliciting high settlement rates

(90 ± 7.5% mean ± SE). The $^{1}$H NMR spectrum of the > 100 kDa filter residue showed broad peaks consistent with those for large polymers.

The fraction containing large polysaccharides was hydrolysed under strong acidic conditions and high temperature to cleave macromolecules under conditions that typically hydrolyse the glycolytic bond. This procedure resulted in the loss of bioactivity and the 1H NMR spectrum of the hydrolysate indicated the presence of smaller polysaccharides and possibly monomeric sugars, verifying that the macromolecular > 100 kDa residue contained a polysaccharide-type component that was causing the larval settlement induction activity..

### 3.6 Immobilization of bioactive fractions

The bioactive organic- and aqueous-soluble fractions were immobilised in a 1:1 ratio of paraffin wax (for A2 and A4) or 2 % agar and 10 % carrageenan (for UFF1 and UFF4). At this ratio, 10 µL of immobilised fractions represented the lowest active threshold concentration that evoked mean larval settlement of 80 % (in 6-well plates, 10 mL). Based on this preliminary result, the same immobilisation ratio of cue to matrix volume was employed in a mesocosm-style trial of these fractions in 500 L tanks. Each of the four fractions was assayed with five replicates, each of which received 1 mL of bioactive extract, resulting in 20 mL combined extract per tank (n = 3).

Larval settlement was significantly higher on all matrices containing immobilised cues in comparison to control surfaces. There was a significant effect of the treatment type on larval settlement (2-way ANOVA, $F_{8,2} = 14.6$, $P < 0.00001$), but no significant effect of the tank location (ANOVA, $F_{2,108} = 1.73$, $P = 0.18$). Larval settlement on the organic fractions immobilised on wax matrices did not differ significantly (A2 14.06 ± 2.73, A4 15.47 ± 2.72, Tukey HSD, $P = 1$), however, they were significantly different from the wax matrix control (0.67 ± 0.67, Tukey HSD, $P_{(A2 \text{ vs. Wax control})} = 0.001$, $P_{(A4 \text{ vs. Wax control})} < 0.0001$). The same

effect was observed for the ultrafiltrates UFF1 and UFF4 immobilised in agar (UFF1 14.53 ± 3.0; UFF4 11.2 ± 1.9 to Agar control 0.2 ± 0.11; Tukey HSD, $P_{(UFF1 \text{ vs. Agar control})} = 0.001$ and $P_{(UFF4 \text{ vs. control})} = 0.027$) and carragenaan (UFF1 21.53 ± 3.09; UFF4 15.8 ± 2.73 to carragenaan control 0.53 ± 0.26; Tukey HSD, $P_{(UFF1 \text{ vs. Car. control})} < 0.0001$ and $P_{(UFF4 \text{ vs. Car. control})} < 0.0001$).

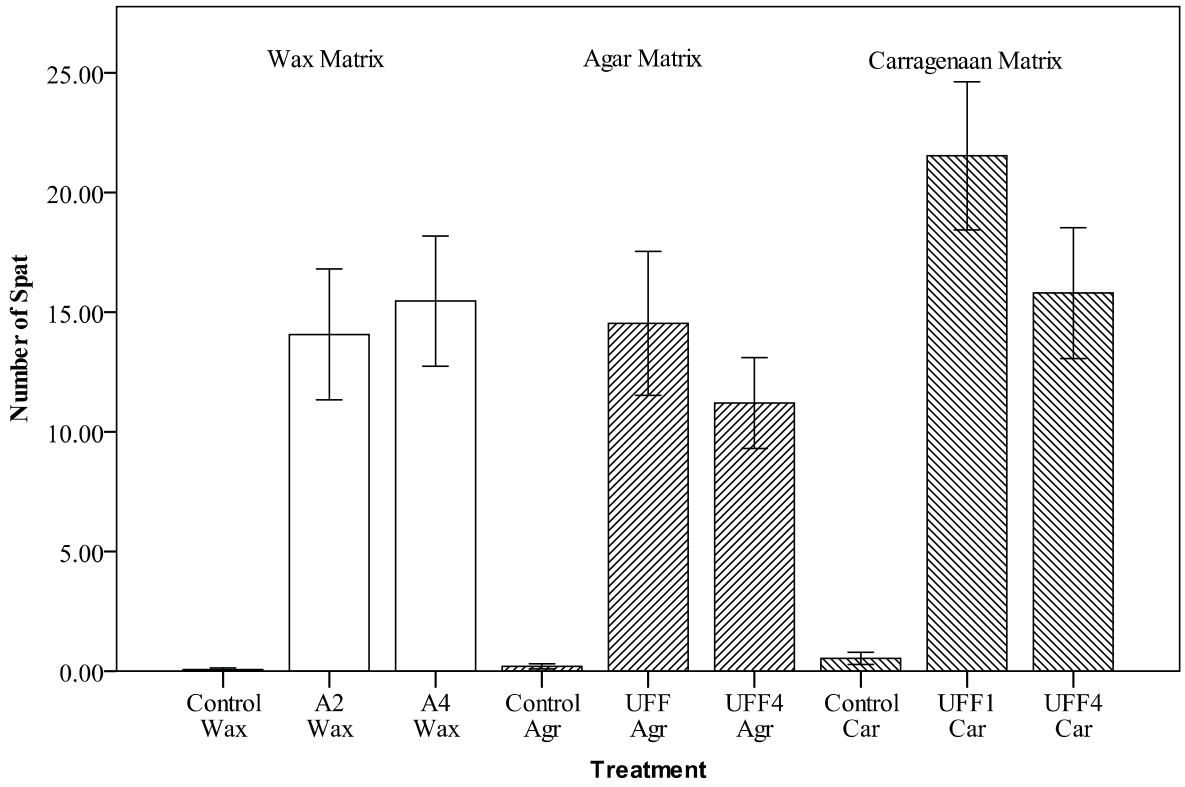



**Figure 4.7**. Immobilisation of *H. onkodes* fractions: Spat number settled on substrates with immobilised cue matrtices and matrices controls. Mean ± SE per replicate (n = 15) of settled spat shown (wax = paraffin wax matricies; A2 & A4 = organic settlement cues as per bioassay-guided fractionation A; Agr = agar matricies; UFF1 = aqueous soluble fraction > 10 kDa; UFF4 = aqueous soluble fraction > 100 kDa; Car = carragenaan matrices).

## *4. Discussion*

Coralline algae occur abundantly on hard substrata in subtidal communities from tropical to polar latitudes and have received particular attention in the literature as specific signposts for larval settlement for a remarkable diversity of marine invertebrates (Hadfield &

Paul 2001). However, despite 40 years of work on the topic (see reviews Hadfield & Paul 2001, Hadfield 2011), to date there has been no fully characterised chemical cue for induction of larval settlement, of either algal or bacterial origin, identified from a CCA. This corresponds to the general paucity of fully identified, ecologically relevant, chemical cues for settlement in the literature from any source (Swanson et al. 2004). In contrast to chemical mediators of other kinds of ecological interactions (e.g., herbivory, antibacterials), characterisation of larval inducers has proved particularly challenging.

In this study, larval settlement cues of the acroporid coral *Acropora millepora* were purified from organic and aqueous extracts of the CCA *Hydrolithon onkodes*. The premise behind this study was that, despite detailed genomic and ecological knowledge of this coral species, the chemical settlement cues that trigger the transition from motile larvae into sessile polyps are unknown. Further, there have been incongruous reports about the general properties of settlement inducers from different biological sources, such that both organic-soluble (Heyward & Negri 1999, Harrington et al. 2004, Kitamura et al. 2007, Kitamura et al. 2009, Grasso et al. 2011) and completely insoluble cues (Morse & Morse 1991) have been shown to evoke larval settlement of scleractinian corals.

In the current study two chemical compound classes were consistently present in settlement-inducing fractions separated from *H. onkodes*, i.e. high molecular weight polymer-like aqueous-soluble cue(s) and low molecular weight organic-soluble compounds identified as glycoglycerolipids. While these cues have been separated from large quantities of CCA, it is possible that they originate from the CCA-associated microflora, but somewhat unlikely given that these compound types have been shown to be mainly of algal origin (Heinz & Tulloch 1969, Critchley & Heinz 1973, Heinz et al. 1974, Ishizuka & Yamakawa 1985, Murakami et al. 1993, Kim et al. 1999).

The exposure of organic extracts of *H. onkodes* to multiple batches of *A. millepora* larvae, repeated with new organisms, resulted in consistently high induction of larvae settlement. Although the organic extract was repeatedly purified under entirely different chromatographic conditions (see Fig. 4.3-6), these different procedures always yielded the same inductive chromatographic fractions dominated primarily by glycoglycerolipids, namely sulfoquinovosyl monoacylglycerol (SQMG) and monogalactosyl monoacylglycerol (MGMG). While the CCA extract contained various other glycoglycerolipids, that either differed in the sugar moiety, the acetylation state and length or substitution of the fatty acid side chain, none of these other glycoglycerolipids fractions evoked larval settlement.

The bioactive MGMG- and SQMG-dominated fractions contained other impurities evident as a fluorescent spot that overlapped with the glycoglycerolipids on TLC plates. Once the chromatographic TLC conditions were optimised to further separate these compounds from the main glycoglycerolipid, the larval settlement-inducing activity was lost. This complete loss of bioactivity was neither related to a concentration effect or quantitative loss of the glycoglycerolipid during the chromatographic procedure, nor did pooling of the separated, purified fractions re-establish the settlement-inducing activity. The same observation was made when the bioactive fraction was processed by HPLC. Neither individual nor pooled HPLC fractions had any biological effect, even though the full polarity range of solvents that yielded these fractions from the VFC was used. As documented in the literature there are many monoacylated glycoglycerolipids varying in the number of sugar moieties as well as in the length and saturation of the fatty acid side chains (Heinz & Tulloch 1969, Critchley & Heinz 1973, Heinz et al. 1974, Ishizuka & Yamakawa 1985, Murakami et al. 1993, Kim et al. 1999) and that these differences can affect mesogenic and lyotropic properties (Milkereit et al. 2004), therefore further effort is required to investigate whether these are factors in the loss of settlement activity.

Despite this consistent loss of bioactivity during the final purification steps, settlement-inducing fractions from organic CCA extracts always coincided with relatively high amounts of glycoglycerolipids in these samples; making this chemical class a primary candidate for further investigation. Thus, the effect of commercially available diacylated structure analogues of MGMG and SQMG was tested. Of these two analogues - monogalactose diacylglycerol (MGDG) and sulfoquinovosyl diacylglycerol (SQDG) - only SQDG evoked low rates of larval settlement (40 ± 36 % mean settlement ± SE). The settlement response to SQDG was significantly lower compared to settlement-inducing fractions that mainly contained SQMG or MGMG (Fig.4.4 fraction B12, Fig. 4.5 fraction C10). As the monoacylated glycoglycerolipids MGMG and SQMG were commercially unavailable, enzymatic digestion of MGDG isolated from spinach was undertaken to yield MGMG (determined by NMR). No larval settlement was observed in response to the hydrolysis product (both reaction mixture and the semi-purified MGMG).

To investigate putative macromolecular cues for coral larval settlement (Morse & Morse 1991) not able to be extracted with organic solvents, a separation procedure for large algal cell wall polysaccharides was undertaken during the 2011 spawning season. A highly inductive water-soluble settlement cue was extracted under elevated temperature and pressure. The bioactive component in this sample had a molecular mass > 100 kDa, and the $^{1}$H NMR spectrum showed broad peaks consistent with those for large polymers. Under conditions that lead to total hydrolysis of polysaccharide-type macromolecule, the formerly bioactive sample was rendered non-active. Subsequent $^{1}$H NMR analysis of the hydrolysate indicated that broad signals had sharpened, significantly improving signal shape and resolution, as expected for smaller polysaccharides and monomeric sugars. The similarity in size and polysaccharide-type polymeric nature of the identified cue and the glycosaminoglycan-type cue identified by Morse and Morse (1991) may suggest that these

cues are structurally similar and share the same origin. We can only speculate at this stage whether the conditions of high temperature and pressure that liberated this cue from CCA may yield compounds similar to those obtained by Morse and Morse (1991) under slow decalcification conditions. Since the macromolecular settlement cue was only discovered in the last two of six spawning seasons, it remains a preliminary result in need of further analyses and comparisons with the protocols published by Morse and Morse (1991) to distinguish between these polymeric coral larval settlement cues.

The results of this study suggest that at least two different chemical compound classes, a macromolecular polysaccharide-type polymer and low molecular weight organic soluble glycoglycerolipids, explain the inductive property of *H. onkodes* on larval settlement of *A. millepora*. This is a novel finding because it is in contrast to previous studies on CCA-derived coral larval settlement cues, all of which resulted in single bioactive compounds of either high (Morse and Morse 1991) or low molecular weight (Kitamura et al. 2007, 2009). Interestingly, the bioactive low- and high-molecular weight fractions identified in this study were quantitatively dominated by compounds usually associated with the cell wall and/or the cell membranes of CCA, such as cell wall polysaccharides and membrane lipids. Given their presence on the algal surface and accessibility for surface-exploring coral planulae, these compound classes are likely appropriately located to function as larval triggers.

A significant unresolved issue for the identification of coral settlement cues is the source of putative inducers, e.g., algal or associated bacteria. For the two classes of compounds of interest here, glycoglycerolipids and large polysaccahrides, the weight of evidence would suggest an algal origin, although the evidence is by no means definitive. *H. onkodes* is still inductive after treatment removing bacterial activity (Negri et al. 2001). Moreover, monoacelatyed glycoglycerols are so far only known from higher plants, seaweeds or cyanobacteria (Heinz & Tulloch 1969, Critchley & Heinz 1973, Heinz et al. 1974, Ishizuka

& Yamakawa 1985, Murakami et al. 1993, Kim et al. 1999). A more thorough understanding of concentrations is also needed, as this may make bacterial origin unlikely (e.g., Swanson et al. 2004 for histamine). The source of the polysaccharides remains to be shown and as the structural information is limited we cannot exclude an origin in the microflora of the CCA, although valid indications for an algal origin have been demonstrated (Negri et al. 2001).

A general caveat for the identification of bioactive compounds in laboratory assays is the scale at which these are performed and the artificial manner in which the target organism is directly exposed to putative cues. Contrary to natural field conditions where larvae encounter variable attractive and unattractive pockets of water and surfaces, traditional settlement assays expose the larvae to chemicals in a confined test volume that may result in artefactual responses. Consequently, the isolated settlement cues were tested under more ecologically relevant conditions. Given the possibility that the entire dose of active fractions that leached in to a water volume < 20 L could exceed the minimum threshold for settlement inducing activity, bioactive fractions were immobilised on test panels and distributed in large vessels of 500 L. Hence, under no circumstances would an active threshold concentration have been reached in a 500 L tank. Therefore, larval settlement on the substrates was deemed a true response to the immobilised cue. Thus, under these conditions, settlement cues were only present on the experimental surfaces and not present in the water column. The immobilised bioactive fractions resulted in targeted site selection and settlement of coral planulae providing further support for the ecological relevance of these CCA-derived cues.

The observation that chemically pure glycoglycerolipds isolated from *H. onkodes*, namely SQMG and MGMG, no longer evoked larval settlement of *A. millepora* does not rule out these compounds as chemical settlement cues per se, but rather suggests that the settlement cue in bioactive chromatographically purified fractions was associated with these glycoglycerolipids. This speculation gains support from the lack of other bioactive fractions

being obtained, together with the inability to re-establish biological activity from pooling chromatographic fractions originating from the one bioactive sample. This consistent and reproducible loss of bioactivity could be explained by irreversible damage to a functionally associated pair of settlement cue(s) upon further chromatographic separation. It is not possible at this stage to provide further structural data explaining this phenomenon owing to insufficient sample quantity (for NMR) of the compounds co-eluting with SQMG and MGMG.

However, the proposed concept of a glycoglycerolipid-associated settlement cue for acroporid coral larvae is in accordance with the some studies demonstrating the induction of larval settlement of other marine invertebrates to this compound class. Takahashi et al. (2002) showed that sea urchin larvae of *Strongylocentrotus intermedius* settled in the presence of glycoglycerolipids isolated by TLC from the green macroalga *Ulvella lens*. Similarly, Schmahl (1985) separated glycoglycerolipids from marine bacteria by TLC that induced settlement of the scyphozoan *Aurelia aurita*. In both studies, the bioactive glycoglycerolipid fractions resulted from a single TLC purification step that was very similar to the one applied in the study presented in this chapter. Given the repeated observation that chromatographically poorly resolved glycoglycerolipids of macroalgal origin always evoked high rates of larval settlement in corals, one can speculate that the bioactive glycoglycerolipid fractions obtained by Takahashi et al (2002) and Schmahl (1985) may contain other essential components that rendered these TLC fractions inductive to larval settlement.

In this context, a study by Williamson et al. (2000) is noteworthy. They proposed that a complex of a glycoglycerolipid (floridoside) with isethionic acid was the inducer of larval settlement of the sea urchin *Holopneustes purpurascens*. Although this result was later disputed by Swanson et al. (2004), who identified a neurotransmitter-type settlement cue for

this sea urchin, it agrees with, and does not *a priori* rule out, the mechanistic perception of a glycoglycerolipid-associated settlement cue identified in the present study.

The glycoglycerolipid class of compounds clearly deserves further attention given that several publications, along with this study, independently isolated bioactive fractions that contained this compound among other unidentified chromatographically co-eluting components. Since sea urchins, scyphozoa and corals share a common settlement substrate of crustose or geniculate coralline algae (Hadfield 2011), the association of larval settlement cues for these phylogenetically different invertebrates with surface available algal membrane glycoglycerolipids raises a parsimonious ecologically meaningful argument. However, as demonstrated here, pure glycoglycerolipids do not act as larval settlement cues, at least not for the corals examined in this study, and thus other unidentified compounds may play a decisive but yet enigmatic role. Despite being the first study to take the purification of coral larval settlement cues to an unprecedented chemical analytical level, any conclusions are speculative as additional bioactive compounds that associate with glycoglycerolipids remained elusive.

# CHAPTER 5

## Early Post-settlement Survival of *Acropora millepora* Coral Spat is Enhanced on Anti-fouling Coated Surfaces*

***Abstract*:** The early post-settlement period is one of the most sensitive stages in the life history of reef building corals. However, relatively few studies have examined the factors that influence coral mortality during this period. Here, the impact of fouling on the survival of newly settled coral spat of *Acropora millepora* was investigated by manipulating the extent of fouling cover on settlement tiles using non-toxic, wax antifouling coatings. Survival of spat on coated tiles was double that on control tiles and there was a significant negative correlation between percentage cover of fouling and spat survival across all tiles types, strongly suggesting a negative effect on the health of coral spat due to competitive interactions with fouling organisms. In addition, an experiment was conducted to examine the effect of introducing fouling obtained adjacent to and distant from dying corals to otherwise healthy coral spat. When healthy coral spat were treated with fouling, there was a significant decrease in spat survival irrespective of tile type and fouling collected adjacent to dying coral spat induced significantly higher levels of mortality than fouling collected distant from dying coral spat. This study suggests that fouling in direct proximity of settled corals has detrimental effects on post settlement survival. These negative effects of fouling organisms on corals may become more pronounced in the future as coastal eutrophication increases and reefs continue to degrade worldwide. As a possible countermeasure against these trends, targeted seeding of coral spat on artificial surfaces in combination with fouling control may be a useful tool for improving the efficiency of sexual reproduction-based coral propagation.

***Keywords*:** *Acropora millepora,* post settlement survival, fouling, reef rehabilitation

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## *1. Introduction*

The early post-settlement period of corals, i.e. the first few weeks and months after pelagic larvae settle and metamorphose, is generally considered a critical life history stage, as newly settled individuals are very vulnerable. Early post settlement mortality is spatially, temporally and taxonomically variable (Dalby and Young 1992, Steele and Forrester 2002). However, most reef building corals exhibit typical type III survival curves (sensu Deevey 1947) of benthic marine invertebrates with high early mortality and increasing probability of survival with age and or size (Babcock and Mundy 1996, Gosselin and Qian 1997, Wilson and Harrison 2005). The extent of mortality during the early post-settlement period can strongly influence the abundance and distribution of adult populations (Vermeij and Sandin 2008). Understanding the factors that influence mortality during this period is therefore important to the effective management and rehabilitation of coral reefs.

Ecological studies of early post-settlement mortality of corals are difficult, because corals of less than one year of age (defined as coral recruits) are usually too small (i.e. <1 cm diameter) to be found *in-situ*. As a result most field studies of early population dynamics omit newly recruited corals, focusing instead on juvenile corals that can be seen with the naked eye, typically >1 cm diameter (Penin et al. 2010). Corals of this size are likely to be at least one year old, and consequently our understanding of the factors that influence the mortality bottleneck in the first year of a coral's life remains limited (Vermeij and Sandin 2008).

Studies of early coral mortality have for the most part relied on monitoring the survival of recently settled corals *in-situ* or *ex-situ* on artificial substrata. Experiments with laboratory-reared coral spat transplanted to the reef have shown that survival of newly settled corals (days to a few weeks after settlement) is typically <15% in the first three to four months (Babcock and Mundy 1996, Wilson and Harrison 2005, Nozawa et al. 2006, Baria et al. 2010). However, direct evidence for the causes of high coral spat mortality is limited and

often constrained by insufficiently frequent sampling (Harriott 1985, Smith 1992, Wilson and Harrison 2005, Vermeij 2006). Known causes of spat mortality include smothering (McCook et al. 2001), sedimentation (Fabricius et al. 2003), chemical warfare (Bak and Borsboom 1984, Gross 2003, Paul and Puglisi 2004), pathogens (Nugues et al. 2004, Smith et al. 2006), and accidental removal by grazing fish or direct predation (Sammarco 1985, Christiansen et al. 2009, Baria et al. 2010, Penin et al. 2010).

Though little studied, biological fouling is also likely thought to be a significant mortality factor for the early life stages of corals (Maida et al 2001). Fouling is the accumulation of micro- (bacteria, fungi, protozoa, etc.) and macro-organisms (algae and animals) on immersed hard substrata. Fouling can impact coral spat in at least two ways, either through competition from adjacent fouling organisms on the substratum – e.g., via overgrowth, shading or allelopathy – or by fouling of the spat themselves by microorganisms or motile propagules of other macroorganisms (McCook et al. 2001). Further, fouling may - by providing a complex physical structure - also enhance passive deposition of inanimate material such as sediment onto coral recruits (Birrell et al. 2008) or provide a refuge for pathogens (Nugues et al. 2004). Mortality of coral planulae is increased in the presence of macroalgae, possibly as a result of increased microbial activity, and spat of the coral *Montipora capitata* settled on surfaces of macroalgae suffer complete mortality (Vermeij et al 2009). However, despite the potentially adverse effects of fouling on young corals, to date, there are no studies that have thoroughly investigated its effect on mortality during the early post settlement period for a scleractinian coral.

As well as being important for our understanding of natural coral mortality, understanding the impact of fouling on early post settlement survival may also have important implications for management and rehabilitation of reefs. Coral reefs are in decline in many areas of the world (Pandolfi et al. 2003, Bellwood et al. 2004) and active

rehabilitation efforts are taking place globally (Edwards 2010). Such efforts typically focus on propagation and transplantation of corals produced by asexual fragmentation (Epstein et al. 2001, Rinkevich 2005). More recently, sexual reproduction of corals has been used to propagate large numbers of coral larvae for enhancing recruitment on small areas of degraded reefs (Heyward et al. 2002) or for settlement and rearing on artificial substrata until corals are large enough to be transplanted (Hatta et al. 2004, Omori 2005, Guest et al. 2010). Control of fouling may significantly enhance the success of both approaches, but in particular that of sexual propagation techniques, because newly settled coral spat are likely to be very vulnerable to mortality due to negative interactions with fouling organisms.

This study investigated the impact of fouling on the early post settlement survival of coral spat of the scleractinian coral *Acropora millepora*, using non-toxic antifouling coatings to modulate the extent of fouling on substrata colonised by laboratory reared corals. Our objectives were to directly investigate the impact of fouling on spat survival, and to work towards a technology that could overcome the bottleneck of spat mortality for reef rehabilitation techniques that rely on sexual reproduction.

## *2. Methods*

***Note: In contrast to the experiments in the previous chapters, all experiments here were performed in Singapore. All Singapore coral reefs show some level of degradation because of sedimentation (due to land reclamation), pollution (one of the largest, busiest harbours in the world) or (likely) due to rising sea surface temperatures. Thus, Singapore was chosen as a potential target area for coral rehabilitation experiments.***

### 2.1. Spawning and culturing of coral larvae

The major annual peak in coral spawning in Singapore occurs during the week following the full moon of March or April (Guest et al. 2005). Five colonies of the scleractinian coral *Acropora millepora* were collected on the day of the full moon (7 April 2012) from Kusu Island (1° 13' 31.9" N, 103° 51' 29.2" E). The colonies were transferred to the Tropical Marine Science Institute (TMSI) at St John's Island and held in outdoor tanks (1800 L) with sand-filtered seawater flow through (SFSW). The colonies were isolated each night two hours prior to the predicted spawning time (21:00 hrs) and kept isolated until 23:00hrs or until spawning occurred. All five colonies spawned on nights 1, 2 and 3 after the full moon (8 to 10 April) between 21:00 hrs and 22:30 hrs. Larval culture techniques followed Heyward and Negri (1999). Gamete bundles were scooped from the water surface immediately after spawning. Sperm and eggs were separated by pouring gamete bundles into a 20 L bucket containing UV-sterilised, 0.2 µm filtered sea water (UV-FSW) through a submerged 100 µm mesh sieve. After 1 h fertilised embryos were removed by surface scooping, washed three times in 50 L of UV-FSW and transferred to larval rearing tanks (500 L) with continuous flow-through of UV-FSW, where they were maintained at low densities ($\leq$ 0.5 larva $mL^{-1}$) until competence. Competency of the larvae was monitored daily with larval bioassays using crustose coralline algae (CCA, ca. 5 $mm^2$, uncharacterised species) in

sterile 6-well culture plates at 28–30ºC. Larvae were settled on tiles when 80% of the larvae settled in response to CCA (6 days post spawning).

### 2.2. Preparation of settlement tiles

Uncoated terracotta tiles (controls) and two terracotta tile treatments with wax anti-fouling coatings (20 × 20 cm in size, n = 16 of each three types) were prepared as settlement substrata. Waxes are generally environmentally benign, and wax anti-fouling coatings are one of the oldest strategies to reduce fouling and epibiotic coverage on artificial structures (since 300 BC, Almeida et al. 2007). Fouling was manipulated with these anti-fouling coatings in preference to other techniques (e.g. manual removal of fouling organisms, toxic anti-fouling paints) because a reduced fouling cover naturally develops on these surfaces and thus does not necessitate other forms of disruptive intervention.

Terracotta tiles were primed with resin (EZ100050P, Ecozean Pty Ltd, Sydney, Australia) to increase wax adhesion and were dip-coated with wax (EZ001-2, Ecozean Pty Ltd, Sydney Australia), hereafter referred to as N-Wax. The same wax was used in a second treatment with an addition of 0.1 % silicone oil (hereafter referred to as S-Wax), as addition of silicone is known to affect antifouling or foul release capacity (Truby et al. 2000, Brady Jr 2001, Kavanagh et al. 2003, Hoipkemeier-Wilson et al. 2004, Nendza 2007).

For the main survival study, control tiles and tiles coated with wax were prepared as follows: 49 micro-wells (5 mm wide and 2 mm deep, 7 × 7 arrays) were drilled into one side each terracotta tile to provide a settlement site for coral larvae. Each micro-well was then filled with 1 μL of powdered CCA in sterile FSW (2:1 v:v) to render these micro-wells inductive to larval settlement. Since the remaining tile area was not biologically conditioned, the aim of this method was to selectively settle coral larvae within the micro-wells. Batch tiles (n = 5) containing wells filled with CCA were placed into tanks with competent coral

larvae (from 6 days post spawning, density <0.5 larvae per mL, 500 L tank volume) for no longer than 24 hours. This procedure resulted in 48 tiles (16 per treatment) containing at least 10 wells with coral spat. Less than 10 % of the total settled spat settled on tile areas next to the designated wells. These spat were ignored for the purpose of the survival study in order to maintain consistency and to facilitate monitoring of survival. The first batch of coral spat was one week old (counting from the day of settlement) at the start of the survival study. Until the start of the survival experiment the substrata with settled spat were kept in clean tanks with UV treated filtered seawater (FSW) to ensure that the initial experimental conditions were the same for all tiles.

### 2.3 Survival studies

Tiles were fully immersed in four shallow constant flow-through (sand filtered) tray tanks (four tiles per treatment and tank, fully dispersed, 2 m x 0. 7 m x 0.1 m length x width x water depth). The tanks were cleaned by brushing immediately before the tiles were introduced to create similar conditions in each tank. Subsequently, the tanks developed a fouling community of macroalgae (brown, green, red and CCA), microalgae, ascidians, polychaetes and anemones. The tanks were not cleaned during the experiment to allow consistent fouling pressure on the experimental tiles. Coral spat survival was scored at the beginning of the experiment followed by weekly intervals over a course of 39 days as follows: The tiles were gently removed from the tanks, placed into seawater trays and spat survival in micro-wells was visually determined under the microscope. Survival was scored on a per well basis, with wells scored as alive when there was at least one living polyp in a well. This was done to avoid confusion over determining individual corals, as they tend to aggregate and bud daughter polyps, and different individuals (spat) that settle in a well can fuse. Thus, it is very difficult to track the survival of individual spat. Micro-wells containing

at least one settled coral were counted as containing an individual to aid regular monitoring in the survival analysis. While this procedure may have resulted in higher survival rates for wells containing more than one coral (assuming density independent mortality) it did not biase any particular treatment as the quantities, densities and frequencies between treatments and replicates did not differ.

### 2.4 Quantification of surface fouling on experimental tiles

The mean percentage of fouling cover on experimental tile surfaces was measured after 39 days in 5 × 5 mm quadrates positioned directly adjacent to the edges of two randomly selected micro-wells per replicate tile. Fouling was estimated via photographs of each quadrate and analysed using the Coral Point Count Software with Excel extensions (CPCe) (Kohler & Gill 2006). Thirty-two points per 25 $mm^2$ quadrate were analysed using a stratified random point count methodology for four columns by four rows and two points per cell. The surfaces were analysed according to two categories: fouled (e.g. macroalgae, microalgae, sediment) and non-fouled (non-fouled coral spat and tile surface with no visible fouling).

### 2.5 Manipulation of the fouling community on coral spat

Fouling was dislodged from the tile surface and added to viable coral spat to directly test for the effects of the fouling community on spat survival,. This experiment was divided into four treatments: in two treatments, individual spat were enclosed by placing a hollow plastic cylinder (7 mm diameter × 7 mm length) onto each micro-well. Fouling was carefully dislodged from a tile surface with a pipette (500 μL) and transferred into the cylinders, covering each spat. Fouling was dislodged from two different tile areas: 1) from micro-wells on the same tile as the healthy recipient spat, but >10 cm away from any other spat

("uncontaminated"); and 2) from micro-wells containing coral spat that showed signs of partial mortality and tissue degradation ("contaminated"). In one treatment individual spat were enclosed by placing a hollow plastic cylinder (7 mm diameter × 7 mm length) onto each micro-well but no fouling was added (procedural control). For a further control treatment neither the cylinder nor fouling was added to the viable test spat (unmanipulated control). These treatments were repeated for all tile surface types (control, N-Wax, S-Wax). A total of five replicate spat, each on independent tiles, were used for each fouling treatment (four levels) and tile type (three levels) (total n = 60). After 5 d each recipient coral spat was scored as dead or alive.

### 2.6 Statistical analyses

Survival of spat over 39 days on three different tile types was analysed by Kaplan–Meier statistics (Lee 1992), comparing treatments (control, N-Wax and S-Wax tiles) using a logrank statistic. Percentage survival of coral spat per replicate tile after 39 days was analysed by two-way ANOVA with tile type as a fixed factor (three levels) and tank as a random factor, orthogonal to tile type (four levels). ANOVA was followed by post hoc pair-wise comparisons among treatments using a post-hoc Tukey's HSD test (Zar 1996). Analysis of fouling was done by analysed by two-way ANOVA as per the survival data. Cochran's test was used to test for homogeneity of variances and where significant heterogeneity was found the data was transformed and retested prior to reanalysis by ANOVA. Percent survival after 39 days and total fouling per tile was compared by regression analysis. Results of the infection experiment were analysed with Chi-squared tests. Initially, tile types were compared with treatments pooled to ascertain whether tile types differed in response. As this was not significant (see Results), a further Chi-squared test was conducted among treatments

with tile types pooled (n = 15) followed by Fisher's exact tests to compare between selected treatments (Sokal and Rohlf 2005).

## *3 Results*

### 3.1 Effect of tile treatment on coral larval settlement

Initial spat densities were analysed by ANOVA to ensure no differences existed in initial spat densities among tile types, which could bias subsequent survival analyses. The average number of spat per wells, the average number of wells containing at least one spat per replicate tile and the total number of spat and number of wells containing at least one spat for the three different substrate types are summarised in Table 5.1. The frequency distribution of spat per well is shown in Figure 5.1. There were no significant differences in any of these metrics among the three tile types at the beginning of the experiment, including the total number of spat per tile (one-way ANOVA, $F_{2, 45} = 0.14$, $P = 0.86$) and the proportion of wells containing at least one living spat per tile (one-way ANOVA, $F_{2, 45} = 1.06$, P = 0.35) The majority of wells had a single spat for all treatments (35% to 36% of wells) and among treatments 69% to 73% of wells had fewer than 3 coral spat (Fig. 5.1) and there was no significant nor in the distribution of spat densities per well among the substrate types (one-way ANOVA, $F_{2,45} = 0.1$, P = 0.9, Figure 5.1).

**Table 5.1**: Metrics for *A. millepora* spat on the three experimental surfaces at the start of the survival experiment.

| | Teracotta control | N-Wax | S-Wax |
|---|---|---|---|
| Average number of spat per well (mean ±SE) | 1.96±0.007 | 1.98±0.007 | 1.82±0.007 |
| Number of wells with spat per tile (mean ±SE) | 29 ±2.9 | 33±2.6 | 27±3.0 |
| total number of spat | 1541 | 1558 | 1322 |
| total wells with spat | 459 | 529 | 439 |

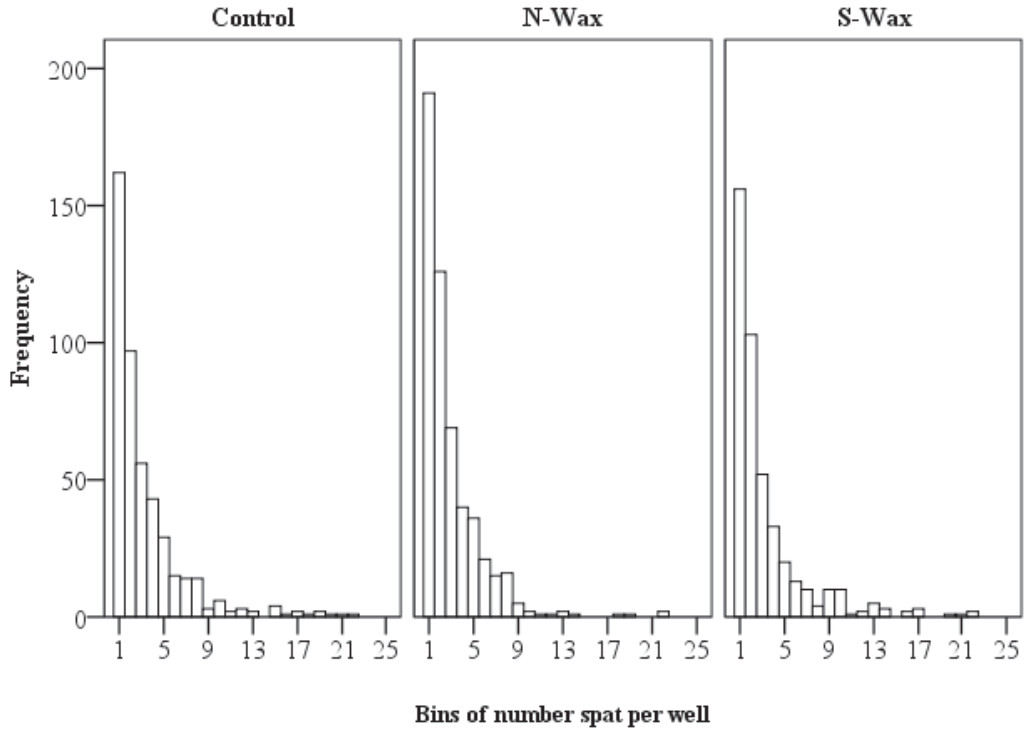


**Figure 5.1.** Frequency distribution of spat per well for the three substratum types (Terracotta control; Wax; Silicone wax)

### 3.2 Survival on different surfaces

After 39 days, the proportion of wells containing at least one live coral spat was 40.4 ± 9.1% (mean ± SE, n = 460) for untreated terracotta tiles, 67.2 ± 5.6% (mean ± SE, n = 529) for N-Wax tiles and 72.3 ± 4.1% (mean ± SE, n = 439) for S-Wax tiles. Comparison of the Kaplan-Meier survival curves over 39 days revealed that mean survival times of 27.9 ± 0.6 days (mean ± SE) for coral spat on untreated terracotta tiles were significantly lower (log-rank test, $P< 0.001$) than those observed on both anti-fouling coatings (31.8 ± 0.5 d for the N-Wax, 36.8 ± 0.2 d for the S-Wax), whereas there was no significant difference in survival times between the wax treatments. At the end of the experimental period (39 days), the majority of surviving coral spat across all treatments had taken up zooxanthellae and many had started to bud daughter polyps, indicating normal development. There was a significant

effect of the tile type on percentage survival after 39 days (two-way ANOVA, $F_{2,3} = 7.9$, P = 0.021) but no significant effect of the tank location (two-way ANOVA, $F_{3,36} = 0.58$, P = 0.65). Survival on N-Wax (Tukey HSD, P = 0.025) and S-Wax (Tukey HSD, P = 0.007) tiles was significantly greater than on control tiles but did not differ between each wax tile type (Tukey's HSD, P < 0.05).

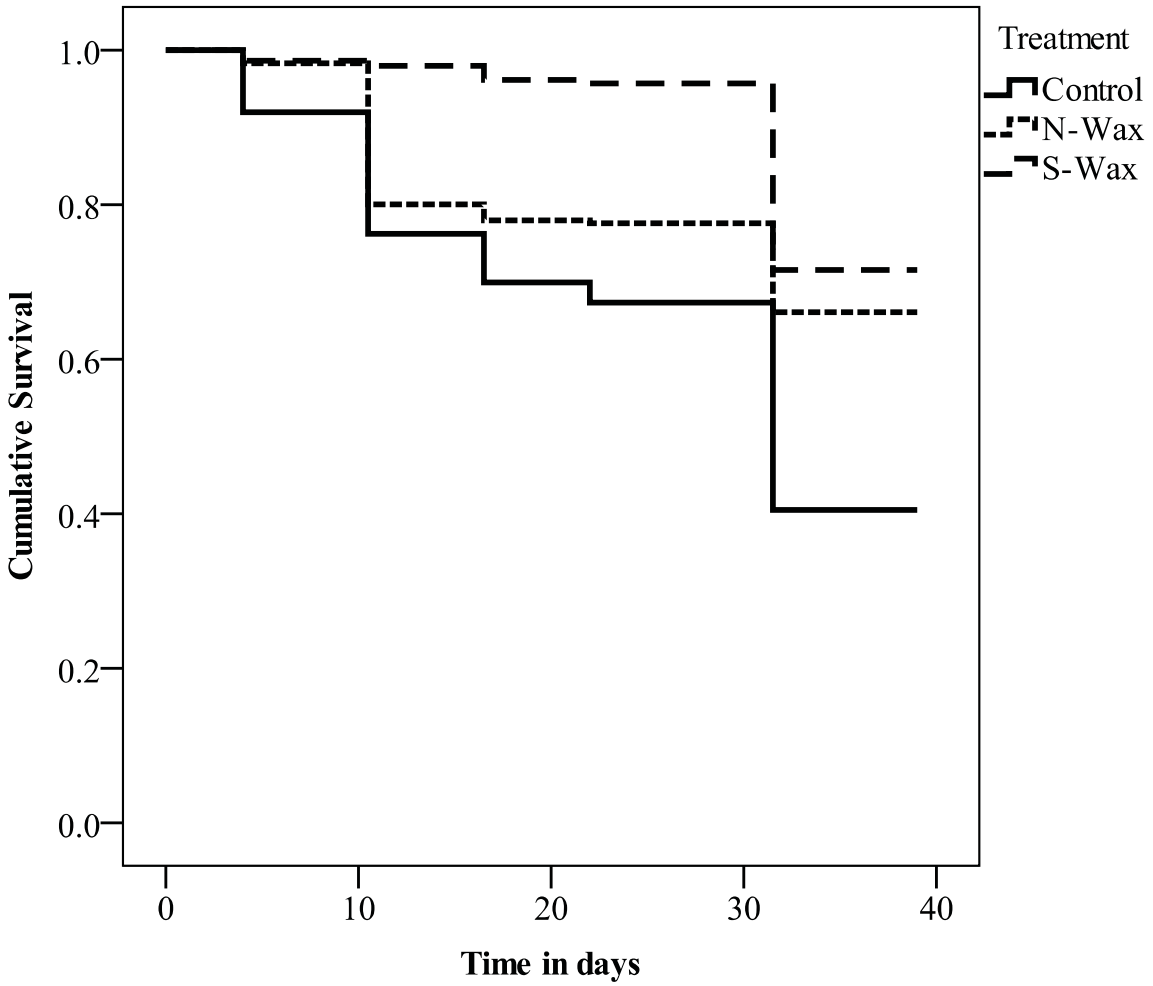


**Figure 5.2.** Cumulative survival of *A. millepora* spat on three substratum types.

**Table 5.2**: Results of two-factor ANOVA for coral spat survivorship. *=P<0.05, **=P<0.01, NS=not significant.

| | df | MS | F | P |
|---|---|---|---|---|
| Substratum type (S) | 2 | 0.4681 | 7.96 | * |
| Tank location (T) | 3 | 0.0339 | 0.45 | NS |
| S x T | 6 | 0.0588 | 0.59 | NS |
| Residual | 36 | 0.0759 | | |
| Total | 47 | | | |

### 3.3 Fouling cover

Percentage cover of fouling was significantly higher on control tiles (85.4 ± 3.9 %, mean ±SE) compared with the N-Wax tiles (41.8 % ± 3.1 %, mean ± SE, Tukey's HSD, P < 0.0001) and S-Wax tiles (35.9 ± 2.5 %, mean ± SE, Tukey's HSD, P < 0.0001), with no significant difference between the two wax types (Figure 5.3, Table 5.3).There was no effect of the tank location (two-way AVOVA, $F_{3,36} = 2$, P = 0.1319) nor of the interaction between tank location and tile type (two-way AVOVA, $F_{6,36} = 71.3$, P = 0.5862).

**Table 5.3**: Results of two-factor ANOVA for fouling on tiles. Data were square root transformed prior to analysis. *=P<0.05, **=P<0.01, NS=not significant.

| | df | MS | F | P |
|---|---|---|---|---|
| Substratum treatment (S) | 2 | 48.93 | 75.29 | ** |
| Tank location (T) | 3 | 1.6494 | 2 | NS |
| S x T | 6 | 0.6499 | 0.79 | NS |
| Residual | 36 | 0.8262 | | |
| Total | 47 | | | |

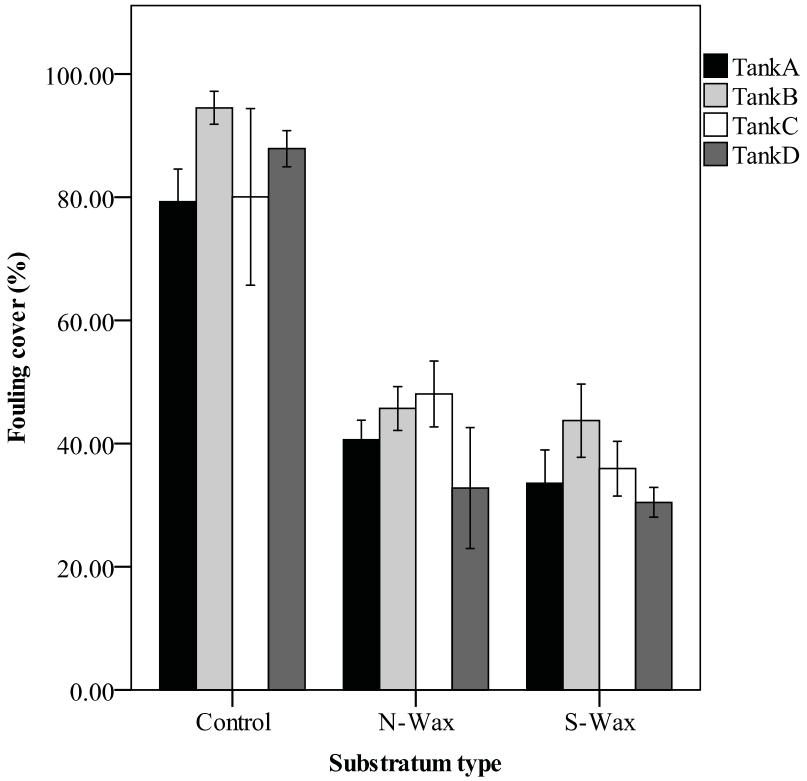


**Figure 5.3.** Percentage fouling cover (percent) on terracotta control, wax anti-fouling coating and silicone wax anti-fouling coating. Data are mean ± SE.

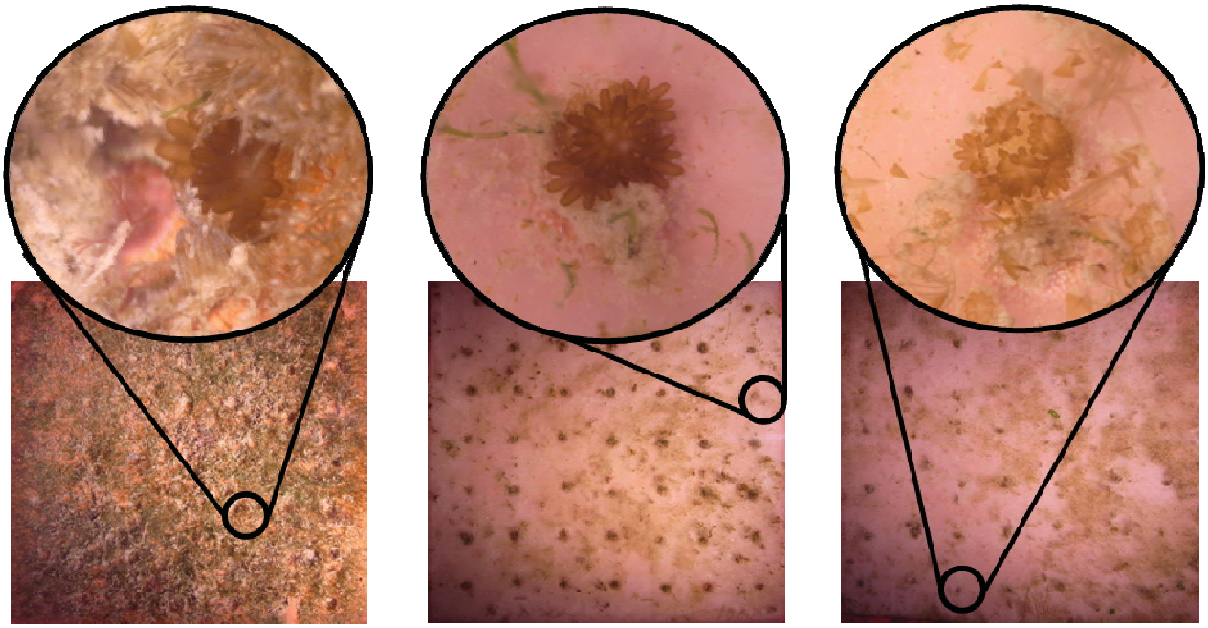

**Figure 5.4.** Representative pictures of the three settlement substrates (terracotta control, wax anti-fouling and silicone wax anti-fouling coating) after 39 days. Square pictures show the entire settlement tile (20 x 20 cm). Round inserts show microscope pictures of spat in the settlement wells (diameter ~ 10 mm).

### 3.4 Spat survival vs. fouling

Regression analysis of spat survival rates versus fouling cover on pooled samples across all tile types resulted in a significant negative influence of fouling on survival (N=47; F=27.06; $p < 0.001$, R2=0.37; Fig. 5.6). Separate analyses of spat survival on each treatment type indicated significant negative effects for control (N=15; F=11.24; $p < 0.005$; R2=0.45) and N-wax treatments (F=6.97; $p < 0.02$; R2=0.33), but there was no significant relationship between spat survival and fouling cover for the S-wax treatment (N=15; F=0.98; $p = 0.34$) (Fig. 5.7).

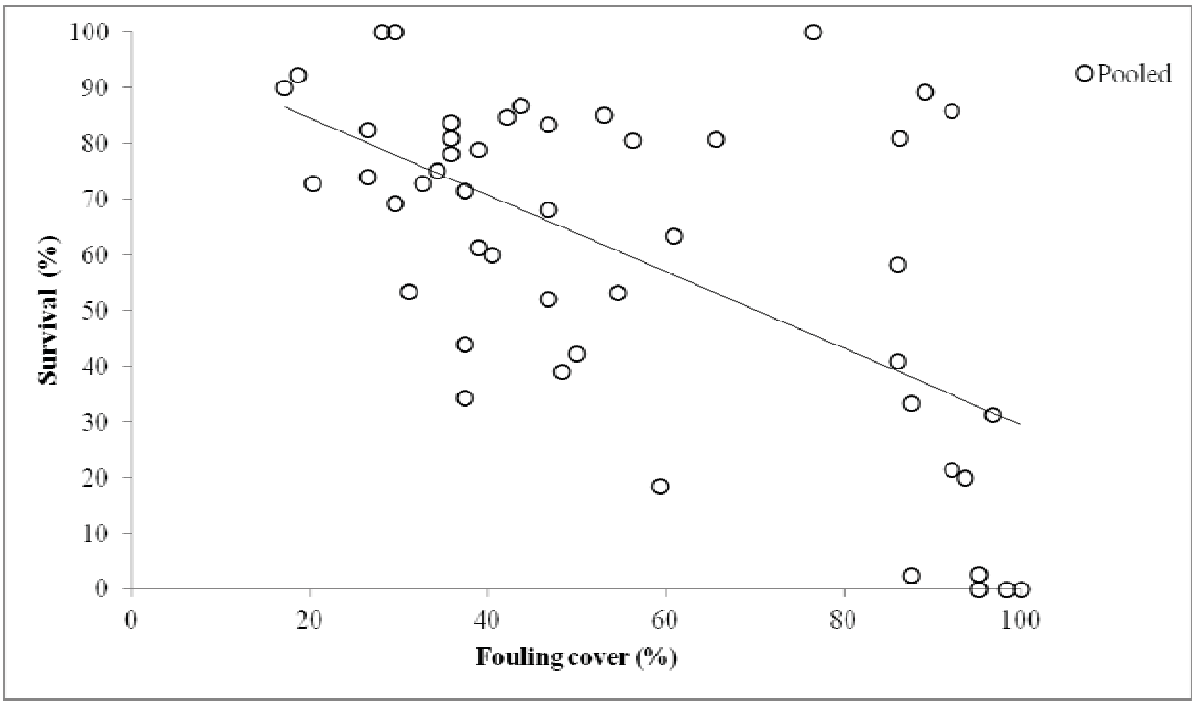


**Figure 5.5.** Percentage survival vs. percent fouling cover pooled across all three tile types. Linear regression statistics are shown in Table 5.4.

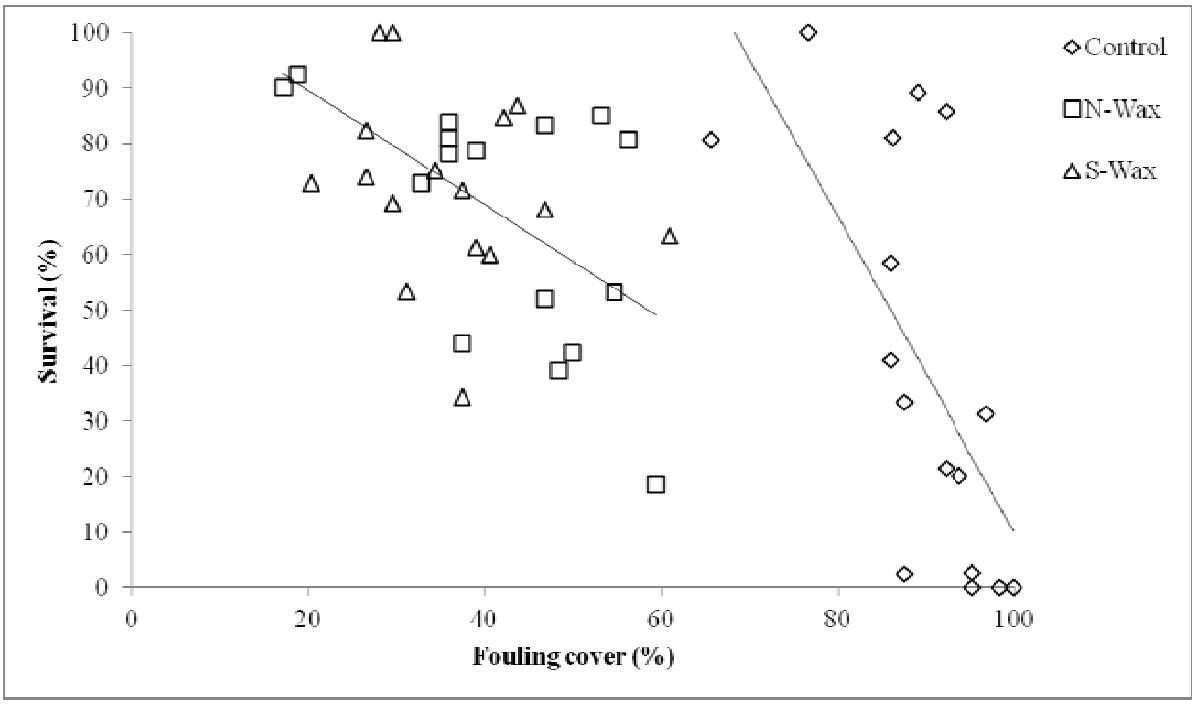


**Figure 5.6.** Percent survival vs. percent fouling for each of the three tile types. Linear regression statistics shown in Table 5.4.

**Table 5.4**: Linear regression analyses for survival vs. fouling cover (Figures. 5.5, 5.6). NS=not significant.

| | N | F | Significance | $R^2$ |
|---|---|---|---|---|
| Pooled | 47 | 27.06 | < 0.001 | 0.37 |
| Control | 15 | 11.24 | < 0.005 | 0.45 |
| N-Wax | 15 | 6.97 | 0.02 | 0.33 |
| S-Wax | 15 | 0.98 | 0.34 (NS) | |

### 3.5 Infection experiment

There were no significant differences among different tile types in the proportion of spat that died after 5 d when treatments were pooled ($\chi 2 = 0.3383$, $p > 0.05$) (Fig. 5.9). Consequently, tile types were pooled and spat mortality compared among infection treatments. Infection treatments significantly affected proportion of corals that died 5 d post-infection ($\chi 2 = 43.92$, $p < 0.0001$) (Fig. 5.9). All coral spat remained alive in the procedural and the unmanipulated

controls after 5 d. However, for the wells incubated with 'uncontaminated' fouling organisms (sampled at least 10 cm away from any coral spat) 47 % (n = 15) of spat died. All spat in wells incubated with 'contaminated' fouling (sampled in close proximity of spat with partial mortality) died within 5 d. Results for the two fouling infection treatments (uncontaminated vs. contaminated) were significantly different (Fisher's exact test, p = 0.0022), as were those between the uncontaminated fouling and the two control treatments (Fisher's exact test, p = 0.0063).

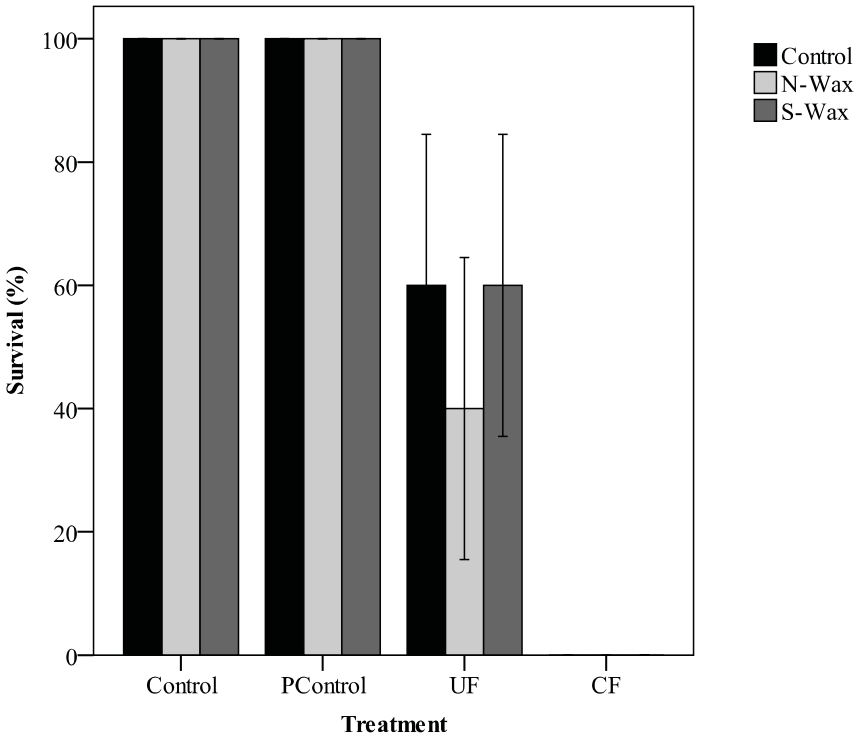


**Figure 5.7** Percent survival (mean ± SE) of spat infected with fouling. Treatments are CF (contaminated fouling); UF (uncontaminated fouling), PControl (procedural control) and control (unmanipulated control))

## *4. Discussion*

During the early post-settlement period, coral spat face a major mortality bottleneck, the extent of which will have implications for the recovery, distribution and abundance of adult populations of reef building corals. Despite this, little is known about natural rates of mortality or about the factors that affect mortality of newly settled spat. In this study we investigated the impact of fouling on survival of newly settled coral spat of *A. millepora ex situ* using antifouling coatings and a fouling addition experiment. These experiments provide one of the few data sets on early post settlement mortality of corals (also see Vermeij et al. 2009), and demonstrate that benthic fouling has a significant impact on the extent of early post-settlement mortality for a scleractinian coral. Our results also suggest that the combined manipulation of larval settlement (via settlement inducers) and fouling (via antifouling coatings) may be useful for restoration efforts based on the use of sexually reproduced larvae.

The *ex situ* experimental approach used in the present study allowed us to control and quantify the extent of fouling on settlement substrata and examine its effect on early post-settlement mortality in the absence of other confounding factors (e.g. grazing, predation, sedimentation). Traditionally, examinations of the early life stages of corals involve random settlement of coral spat on biologically conditioned substrata. Coral spat settlement, however, is spatially very heterogeneous with spat tending to aggregate, fuse and settle on cryptic surfaces of settlement substrata (e.g. edges of tiles). This approach has disadvantages for tracking and quantifying survival; therefore in this study we controlled the spatial distribution of settlement using natural cues, resulting in comparable settlement among treatments and replicates.

The use of environmentally benign anti-fouling coatings was successful in significantly reducing fouling cover on experimental tiles compared to control tiles without the need for disruptive techniques to remove fouling organisms. Both anti-fouling treatments showed a

significantly reduced fouling cover of 42 ± 3 % (N-wax) and 36 ± 3% (S-Wax) in comparison with the control (85 ± 4%). At the same time, survival of coral spat on the anti-fouling coated tiles was significantly greater than that of spat on untreated control tiles (67 ± 6 % N-Wax, 72 ± 4 % S-Wax, 40 ± 9 % control).

Quantitative variation in fouling was significantly negatively correlated to spat survival when pooled across all substrate types, and for spat on control and N-wax tiles (though not on silicon wax tiles). The combined data of average percent survival and fouling cover per tile showed a significant linear relationship between fouling and survival for both the pooled data as well as control and N-wax individually. Although the wax coating with added silicone showed a significant reduction of fouling and significant increase in survival, there was no apparent linear relationship between those two factors. This could be attributed to effects of the silicone additive, which not only affects the fouling density but also may influence other survival-related factors. Because fouling cover co-varied with tile type, the direct effect of the anti-fouling coatings on coral survivorship cannot be completely ruled out. However, when fouling was removed from the settlement tiles and added to healthy coral spat there was a significant decrease in spat survival irrespective of tile type. In addition, the wax coatings are non-toxic (food-grade) and initial settlement was the same across different tile types, this observation suggests that the primary effect of the different tile types on survivorship was likely due to fouling cover modulation. We speculate that the effect of fouling is especially relevant for coral spat, because they may lack many of the defence strategies found in older more established coral colonies (e.g. sweeper tentacles Den Hartog 1977, Chornesky 1983).

Fouling was measured as total cover, without further quantifying the type of fouling. However, our observations indicated that the macro-fouling community was almost entirely comprised of filamentous and micro algae and detritus. The interaction between algae and corals is of profound importance to the health of coral reefs (Nugues and Roberts 2003,

Nugues et al. 2004, Nugues and Bak 2006, Smith et al. 2006, Vermeij et al. 2009, Rasher and Hay 2010, Venera-Ponton et al. 2011). The antagonistic effects of algae on corals range from direct allelopathy (Rasher and Hay 2010) to possible harbouring of pathogens or disruption of coral microbial communities (Smith et al. 2006, Barott et al. 2009) which can inhibition coral recruitment (Connell et al. 1997, Arnold et al. 2010, Hoey et al. 2011, Linares et al. 2012) and lower survival of juvenile and adult corals (Carpenter and Edmunds 2006, Box and Mumby 2007, Linares et al. 2012).

The results of the present study suggest that antagonistic algal–coral interactions may be very relevant for survival of juvenile corals during the early post-settlement period. Survival of coral planulae has been shown to be significantly reduced in the presence of certain macroalgal species, especially when the corals settled on the surface of these alga (Vermeij et al. 2009), and the results from one field study indicate that a reduction in competition with other sessile epibiota can increase survival of settled hard coral spat (Maida et al. 2001).

While the precise mechanisms that lead to increased mortality of coral spat remain unclear, macrofouling may indirectly influence coral spat survival by harbouring microbial organisms or by weakening the coral's resistance to microbial infections (Vermeij et al. 2009). This hypothesis is further supported by this study because we demonstrated that formerly healthy spat had significantly higher rates of mortality, when infected with the fouling community taken in close proximity to dying coral spat (100% mortality), compared to infection with macroscopically identical fouling which had no contact to dying coral spat (47% mortality). This result may suggest that microbial components (e.g. pathogens) in these different macro-fouling communities can greatly affect spat mortality.

An increased abundance of non reef-building benthic fouling organisms - such as fleshy macro-algae and heterotrophic filter feeders- is a characteristic of many degraded reefs (Fabricius 2005). Tropical reefs worldwide have already undergone a phase shift from coral-

to fleshy algal-dominated cover (Done 1992, Hughes 1994, Hughes et al. 2007, Ledlie et al. 2007). This phenomenon is expected to accelerate as coastal eutrophication increases globally (Tilman et al. 2001) and interactions between fouling organisms and corals are likely to become more pronounced and common in the future as reefs continue to degrade worldwide (Pandolfi et al. 2003, Bellwood et al. 2004).

In the context of this increased fouling pressure on degraded reefs, the results of this study may have broader implications for reef rehabilitation. Coral spat reared and settled *ex situ* for subsequent nursery rearing generally have very low survival rates (Guest et al. 2010), making the costs of these techniques prohibitively high. The application of anti-fouling technologies may be able to improve the efficiency of sexual reproduction-based coral propagation by increasing the yield of corals surviving through vulnerable early life history stages. Future studies should identify which fouling-organisms cause high rates of mortality of coral spat in order to facilitate the design of specific anti-fouling technologies that counteract these organisms. Further research is also required to assess whether these techniques can prove effective in the field and if this technique can be used in other rehabilitation efforts, for example by reducing the amount of manual cleaning required during nursery rearing of asexually propagated corals (Shafir et al 2009).

# CHAPTER 6

## General Discussion

The field of Marine Chemical Ecology (MCE) has offered significant insights into the important functions of semiochemicals (chemical compounds that carry a message) (Hay & Steinberg 1992, Hay 1996, McClintock & Baker 2001, Pohnert et al. 2007, Hay 2009, Paul et al. 2011, Sieg et al. 2011). Marine organisms have evolved numerous chemically mediated strategies to enable selection of food, habitat and mates, defence against consumers, pathogens and epibiota, and sexual reproduction. Early research in this field mainly focused on antagonistic interactions between macroorganisms, such as predator–prey and seaweed–herbivore interactions (Hay 1996). However, in the last ten years, studies of chemically mediated interactions among microorganisms, and between micro- and macroorganisms have increasingly come to the fore (Paul et al. 2011).

The initial focus of this thesis, with respect to chemically mediated interactions involving microorganisms, stemmed from the long investigated role of bacterial biofilms as mediators of settlement of larvae of macro-invertebrates (reviewed in Hadfield 2011). Most benthic marine invertebrates have a biphasic life history, in which planktonic larvae return to the benthos to settle, and must select specific sites. Larvae of many species recognise and respond to a variety of specific habitat-based cues ranging from simple tropisms (e.g., light) to very specific chemical cues (Hadfield & Paul 2001), because the consequences of errors in habitat selection are severe for settling larvae. These settlement cues include bacterial biofilms, which are ubiquitous on submerged surfaces. Indeed, bacterial biofilms induce settlement of many phylogenetically diverse marine invertebrates (Morse et al. 1988, Johnson

et al. 1991, Pawlik 1992, Steinberg & de Nys 2002, Thiyagarajan et al. 2006, Hadfield 2011) and have been proposed as "signposts" of appropriate habitats for larvae.

Despite ample evidence (though primarily from laboratory-based assays) of bacterial biofilms as settlement cues for invertebrate larvae (reviewed by Hadfield 2011), to date not a single specific, ecologically realistic, settlement inducer from bacteria has been chemically identified. One possible reason for this paucity of information on bacterially-derived inducers of behaviour is that only a small portion of the bacterial community can be readily cultured under laboratory conditions (Eilers et al. 2000). This has been highlighted by the recent advances in molecular technologies that allow us to qualify and quantify microbial communities (or explore inducers, Huang et al. 2012) on host surfaces at unprecendented levels. Thus the true source of inducers in the field may in fact not be among laboratory strains.

Given this significant knowledge gap, this thesis initially focussed on the chemical ecology of epiphytic bacteria and their role as producers of habitat-specific semiochemicals **(Chapter 2)** for acroporid coral larvae (*Acropora millepora)*. Prior studies have suggested that coral larvae use bacterial biofilms as signposts (Negri et al. 2001, Webster et al. 2004, Erwin et al. 2008) to locate preferred settlement substrates, such as crustose coralline algae (CCA, Morse et al. 1988, Heyward & Negri 1999, Harrington et al. 2004). The first isolation of an inductive bacterial isolate on coral metamorphosis – *Pseudoalteromonas* sp. A3 (A3) - by Negri et al. (2001) supported the notion that CCA-associated bacteria play in important role in site selection of coral larvae.

Building on the findings of Negri et al. (2001), I first investigated the response of *A. millepora* larvae to 200 bacterial isolates obtained from CCA. This screening characterised three Pseudoalteromonads, *Pseudoalteromonas* strain J010 and two others, that induced metamorphosis in larvae of *A. millepora*. The bioactive signal produced by this and the other

inductive bacterial isolates (including A3) was chemically characterised as tetrabromopyrrole (TBP) (Chapter 2; Tebben et al. 2011). However, TBP and all TBP-producing bacterial isolates tested elicited coral larval metamorphosis without attachment, even in the presence of otherwise highly settlement inductive chips of CCA. TBP is a toxic marine natural product, similar to other toxic antagonistic brominated/halogenated metabolites in the marine environment (Andersen et al 1974). As such, TBP may evoke sublethal stress on larvae that may lead to an abnormal response, i.e. metamorphosis without attachment. It has previously been shown that exposure of invertebrate larvae to stress, such as to toxins or high concentrations of neurotransmitters (Morse 1985) can result in metamorphosis, although often incomplete or abnormal (Qian 1999). Therefore, the metamorphic behaviour of coral larvae in the presence of TBP may be a stress response to an isolated toxic marine natural product, presented to larvae in bioassays at unnaturally high concentrations. Regardless of the specific mechanism, results presented in this chapter clearly indicated that TBP is not "the missing (chemical) link" which explains bacteria-mediated larval settlement of corals to CCA and calls into question the generality of bacteria-derived signals as the main semiochemicals for larval settlement in this coral and possibly other corals.

Two hypotheses were generated from the results of **Chapter 2**. First, regarding the chemical ecology of *Pseudoalteromonas* strain J010 and other TBP-producing isolates, the production of TBP might provide these bacteria with an advantage to persist in the highly competitive biofilm environment. To further explore this hypothesis, I screened the allelochemical profile of this strain and characterised further bioactive metabolites in **Chapter 3**. Second, because TBP did not explain the inductive properties of CCA on coral larval settlement, other cues must be associated with these algae. The origin and characteristics of inductive cues from CCA were addressed in **Chapter 4**.

Bacteria produce a range of antagonistic compounds against other microorganisms (Egan et al. 2000, Dobretsov et al. 2006, Penesyan et al. 2009, Wiese et al. 2009, Wilson et al. 2010) that have widespread effects in microbial biofilms (Rao et al. 2005). The bioactivity screen of TBP, other known compounds and novel bioactive metabolites characterised in **Chapter 3**, including korormicin derivatives and a polybrominated pyrrole, provides strong support that J010 is a highly defended bacterium with anti-larval, anti-bacterial, anti-fungal and anti-protozoal properties. These properties may be relevant in competition for space and against predation. Some of these metabolites, particularly TBP and the cytotoxic bromoalterochromides (Speitling et al. 2007) could reach beyond the microbial kingdom boundary and have detrimental effect on invertebrate larvae, such as the ones observed in **Chapter 2**. Preliminary assays indicated that the secondary metabolites identified in **Chapter 3** are also potent quorum quenching agents (data not shown), further supporting the versatile allelochemistry present in J010.

The second hypothesis arising from the results of **Chapter 2** was proposed CCA-derived non-bacterial cues induced complete settlement and metamorphosis of coral larvae. The notion of epiphytic bacteria as the sole source of semiochemicals for induction of larval settlement was tested by Negri et al. (2001). These authors demonstrated that CCA continue to induce coral larval settlement after removal of their biofilms. This observation implied that larval settlement cues may derive from the CCA *per se* instead of their associated microbial biofilms. Thus, **Chapter 4** focussed on the analysis of settlement inducers from whole algal extracts.

Two chemical classes with strong effects on larval attachment and metamorphosis were identified i) high molecular weight polymeric aqueous-soluble cue(s), and ii) low molecular weight organic-soluble compounds identified as glycoglycerolipids. Frustratingly, the glycoglycerolipid fractions lost activity following increasing chromatographic resolution

and purification. These fractions contained very small quantities of unknown compounds that appeared to render the glycoglycerolipids-containing fraction inductive to larval settlement. This association with glycoglycerolipids was not simply an additive association of two independent cues acting together (also see Kitamura et al. 2007), as demonstrated by a range of experiments in which cues were recombined. The results of **Chapter 4** suggest that the settlement cue was made up of a functional association of compounds, of which glycoglycerolipids are a key component. The complete characterisation of this hypothesised association remained unresolved as the coeluting compounds were present at low concentrations that have so far precluded their chemical elucidation, despite extraction of large quantities of CCA.

Nonetheless, the active fractions were always dominated by the same compound classes - polysaccharides and glycoglycerolipids – strongly suggesting these compounds as primary target of interest. These compound classes are ubiquitous algal and cyanobacterial membrane and cell wall components (Heinz & Tulloch 1969, Critchley & Heinz 1973, Heinz et al. 1974, Ishizuka & Yamakawa 1985, Murakami et al. 1993, Kim et al. 1999). The association of these compounds with larval settlement induction is intriguing, as coralline algae induce settlement in a very wide range of invertebrate taxa including, urchins, corals, gastropods and starfish (Morse et al. 1988, Johnson et al. 1991, Pawlik 1992, Steinberg & de Nys 2002, Thiyagarajan et al. 2006, Hadfield 2011).

Interestingly, when the same chemical fractionation as described in Chapter 4 was performed with temperate coralline algae, the resulting fractions showed high settlement activity on larvae of the temperate sea urchin *Holopneustes purpurascens.* It remains open, whether this activity was also due to the same identified compound classes described above, but there are strong indications that these compounds may have broader relevance for other invertebrate phyla. For instance, Williamson et al. (2000) isolated a complex of a

glycoglycerolipid and isethionic acid from coralline algae and proposed this to be the cue for sea urchin larval settlement. Although this result was later refuted (Swanson et al. 2004), it shows that fractions containing compounds with the same chemical terminus and polarity induced larval settlement. Similar results have been shown for scyphozoa (Schmahl 1974) and other sea urchins (Takahashi 2002). These commonalities suggest that glycoglycerolipds, possibly with small, low concentration co-actives, may induce settlement for a broad spectrum of marine organisms.

The use of whole algal extracts in the isolation procedures (**Chapter 4**) does not completely rule out metabolites from bacteria that may have been associated with the algal surface. However, both compound classes are thus far only known from cell wall and/or the cell membranes of CCA, other macroalgae and cyanobacteria (Heinz & Tulloch 1969, Critchley & Heinz 1973, Heinz et al. 1974, Ishizuka & Yamakawa 1985, Murakami et al. 1993, Kim et al. 1999). Given this, and the fact that antibiotic-treated (and even bleached) CCA were still inductive (Negri et al. 2001), my results suggest that the response of coral larvae to CCA are due to algal rather than bacterial metabolites.

In **Chapter 4,** I demonstrated a potential biotechnological application of coral larval settlement cues in the context of reef rehabilitation. Given the increasing concern over the degradation of reefs in many areas of the world (Pandolfi et al. 2003, Bellwood et al. 2004) rehabilitation efforts are taking place globally (Edwards 2010) and improvements to current reef restoration technology is urgently sought. Immobilising and applying the cues outlined in earlier chapters in organic matrices rendered the substrates inductive to larval settlement. Therefore, targeted settlement of corals could prove useful for rehabilitation efforts that utilise sexually reproduced corals to settle and rear coral propagates on artificial substrata until corals are large enough to be transplanted (Hatta et al. 2004, Omori 2005, Guest et al. 2010).

Successfully seeding larvae onto substrates is an incomplete solution as rehabilitation techniques suffer from low efficiency due to high post settlement mortality of coral spat. Should this technology be used in reef rehabilitation, the major bottleneck of early spat mortality still needs to be addressed. One of the factors that might cause these high mortality rates is the negative effect of fouling on spat. In **Chapter 5,** the amount of fouling cover was manipulated by the application of anti-fouling coatings. The survival of coral spat on tiles coated with non-toxic and environmentally benign wax antifouling coatings was about 32 % greater than that of spat on control tiles, and this corresponded to approximately a 40 % increase of fouling on control tiles. This promising result will need to be confirmed in nursery and field trials to show its true potential for future reef rehabilitation efforts.

In summary, this thesis tested hypotheses about the role of bacteria in chemically mediated interactions. I found that one of my major hypotheses, that bacteria produce chemical cues that induce settlement, was not supported, although the putative inducers were active against other bacteria and protozoa. In contrast whole algal (CCA) extracts were highly inductive signals for coral settlement. These signals were immobilised on artificial matricies, rendering these substrates active. I demonstrated that metamorphosis inducing strains J010 and A3 were a particularly rich source of metabolites that did not have the originally proposed function as settlement cues; but the strain possessed antibacterial, antifungal and antiprotozoal properties.

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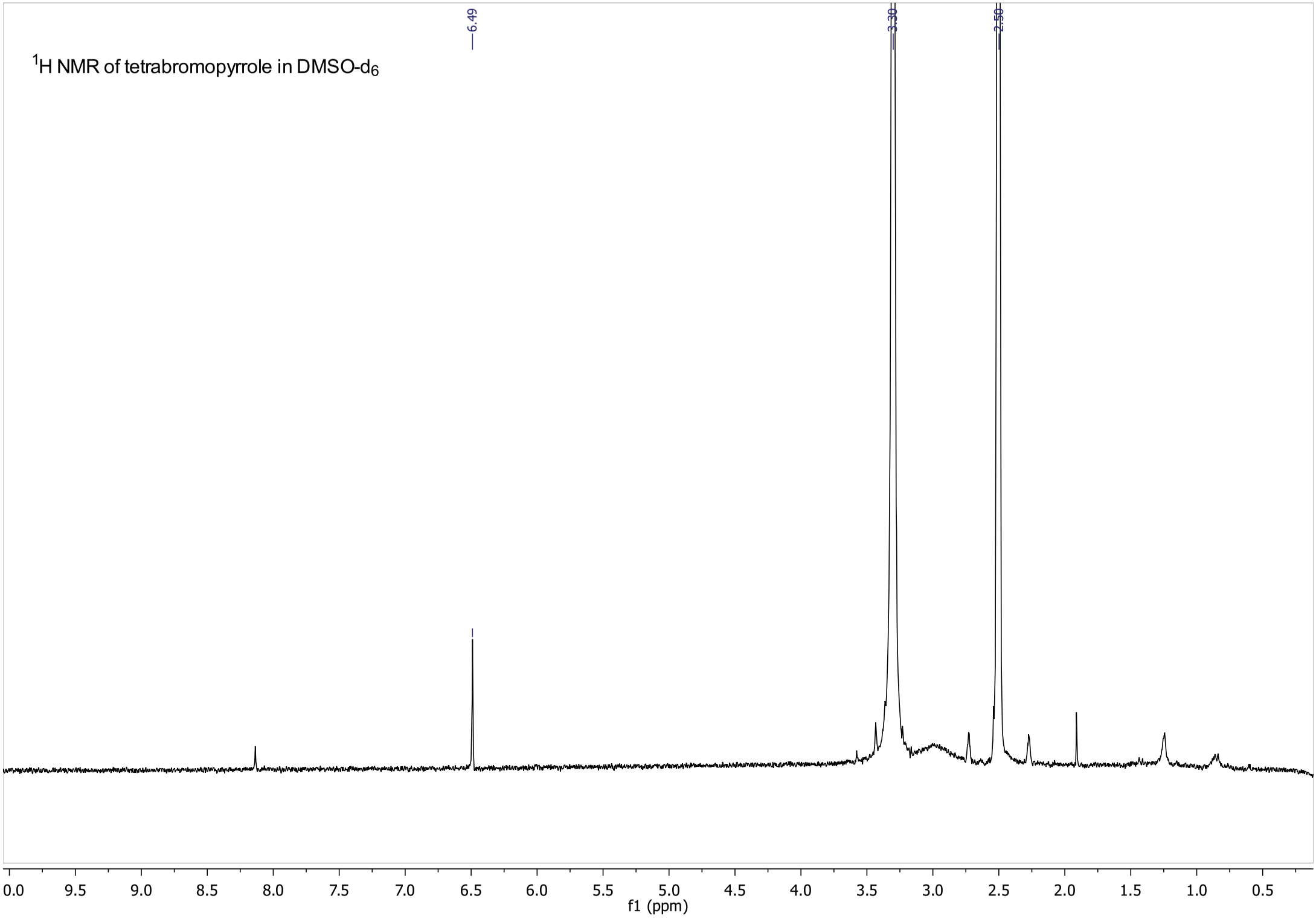
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2.50
0.0
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2.0
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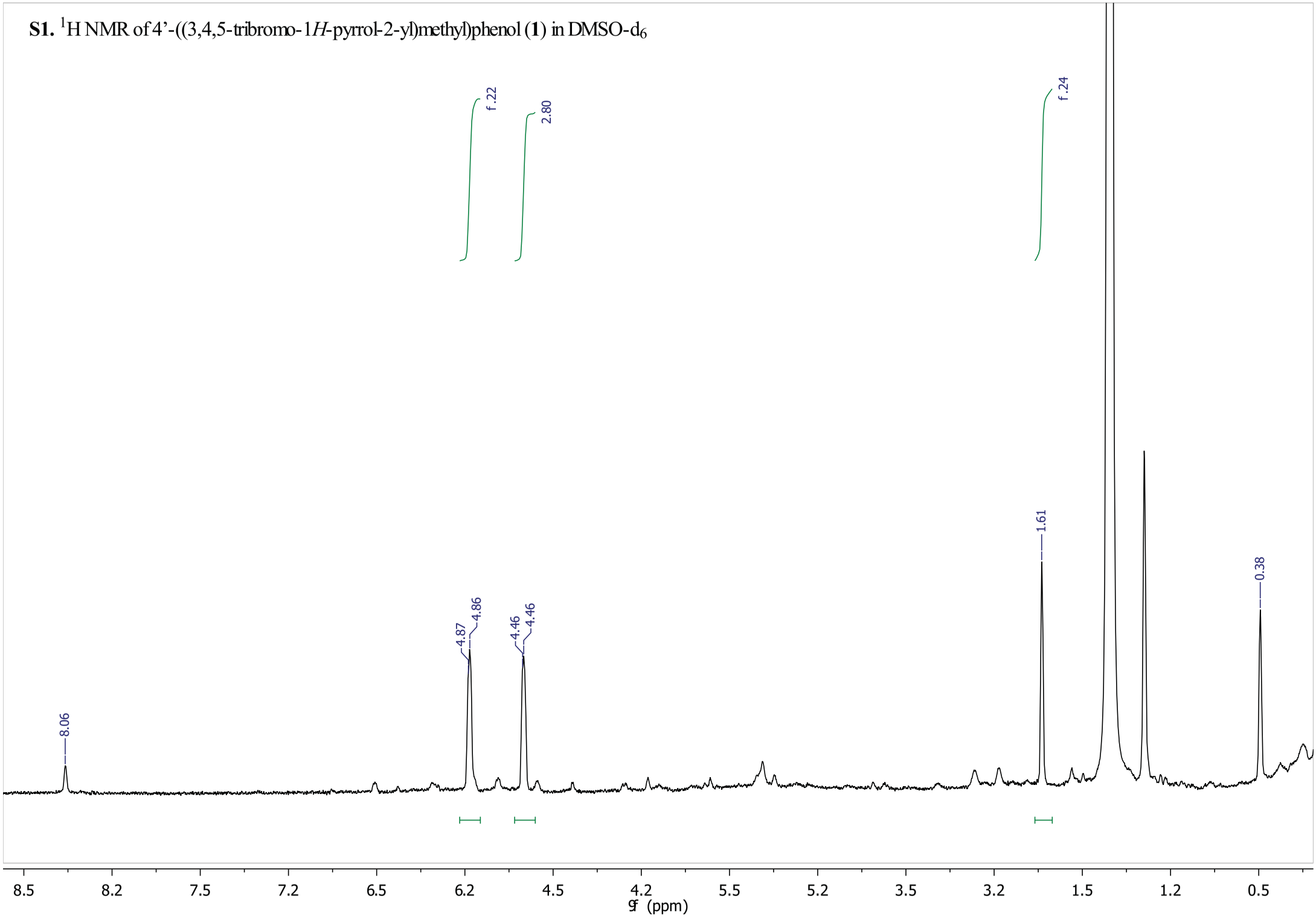
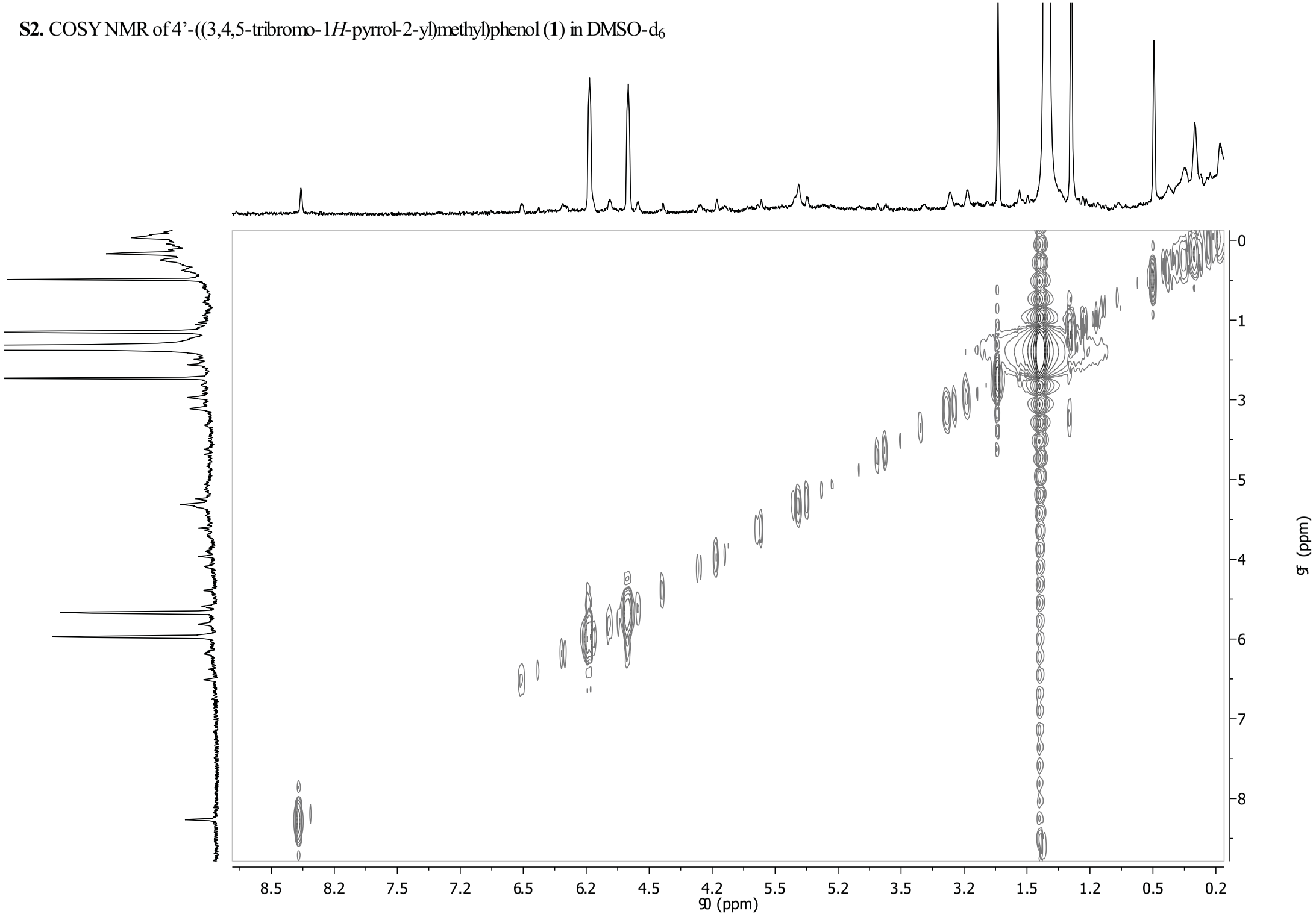
**S1.** $^{1}$H NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in DMSO-$d_6$



**S2.** COSY NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in DMSO-$d_6$



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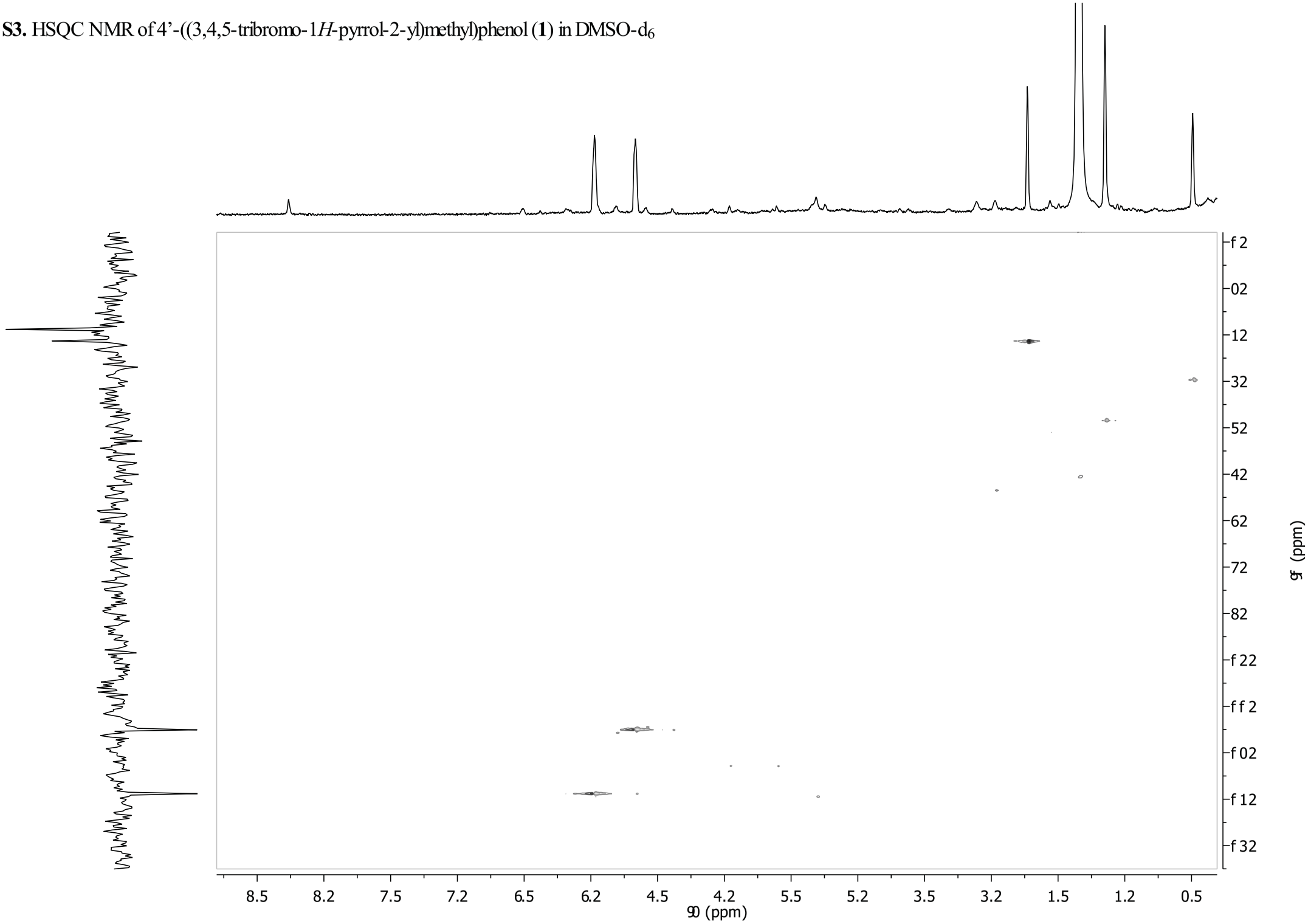
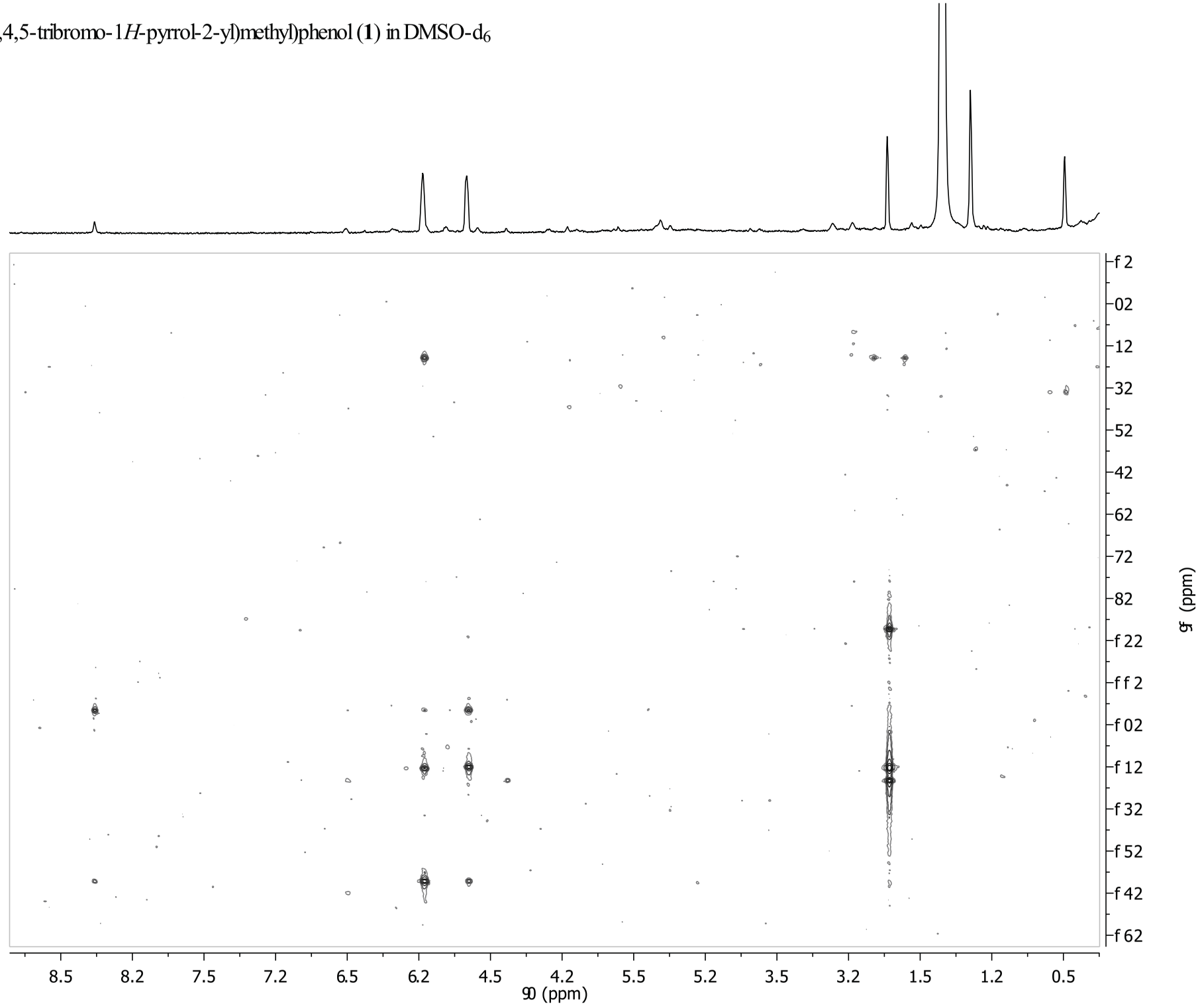
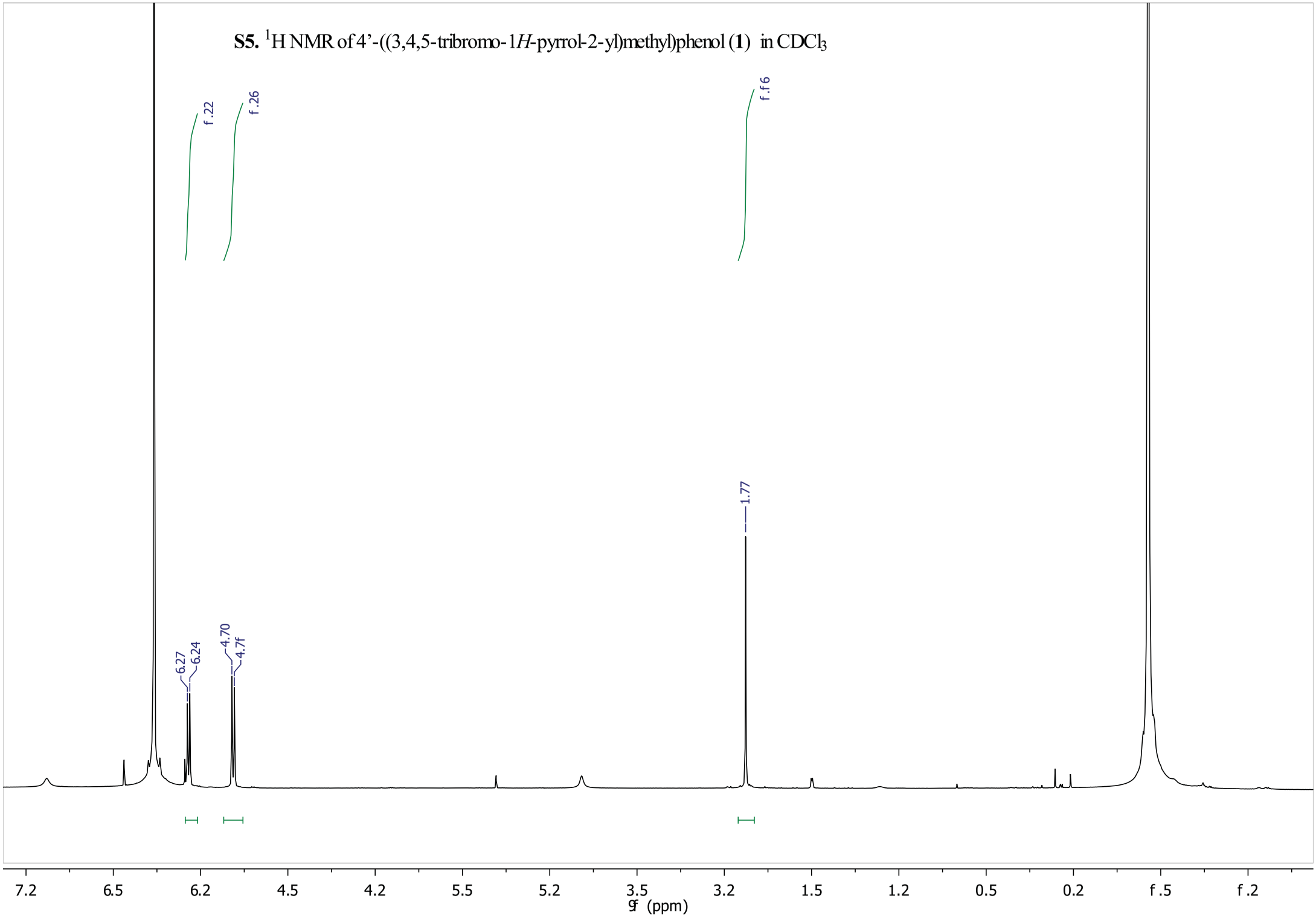


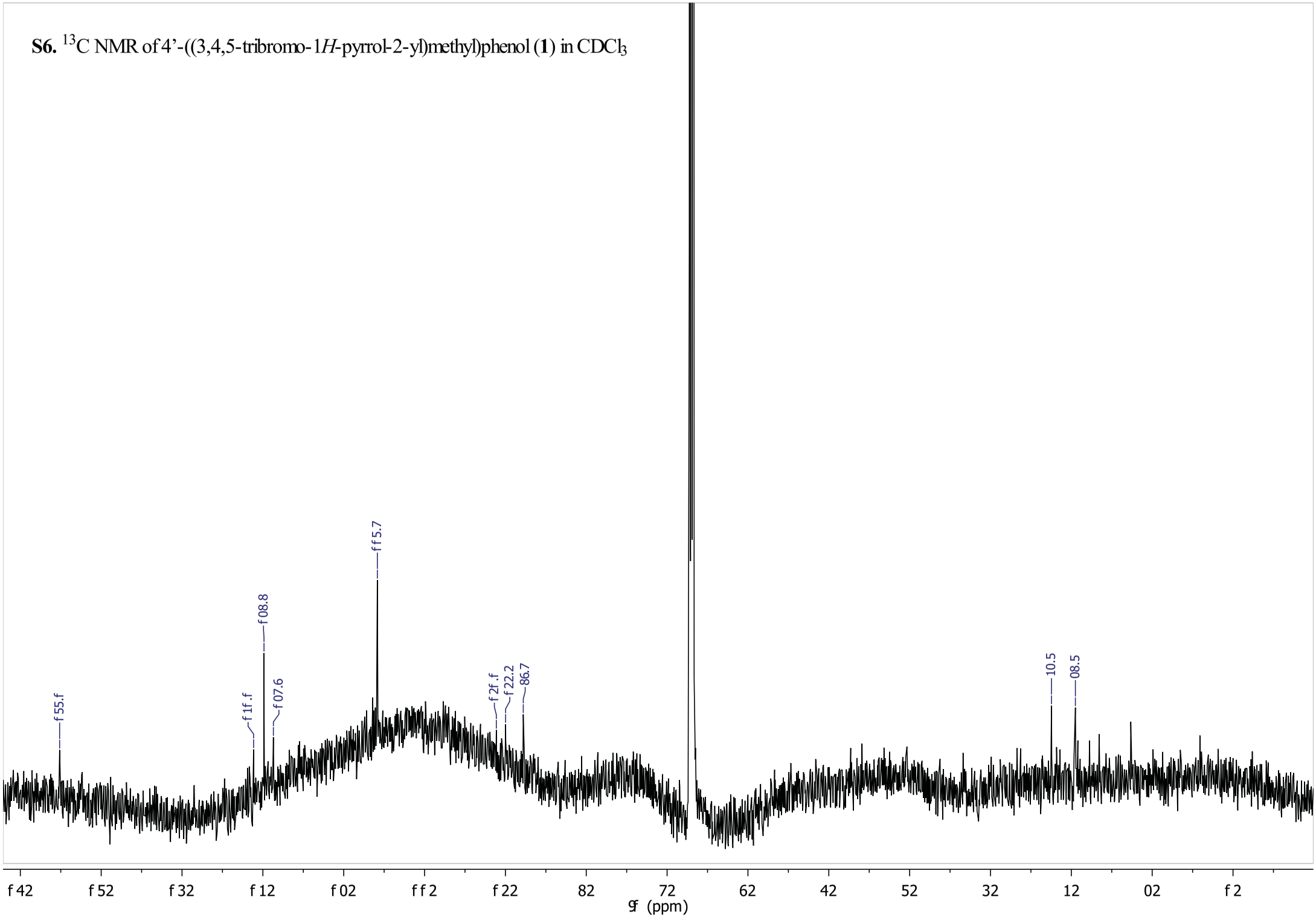
**S4.** HMBC NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in DMSO-$d_6$



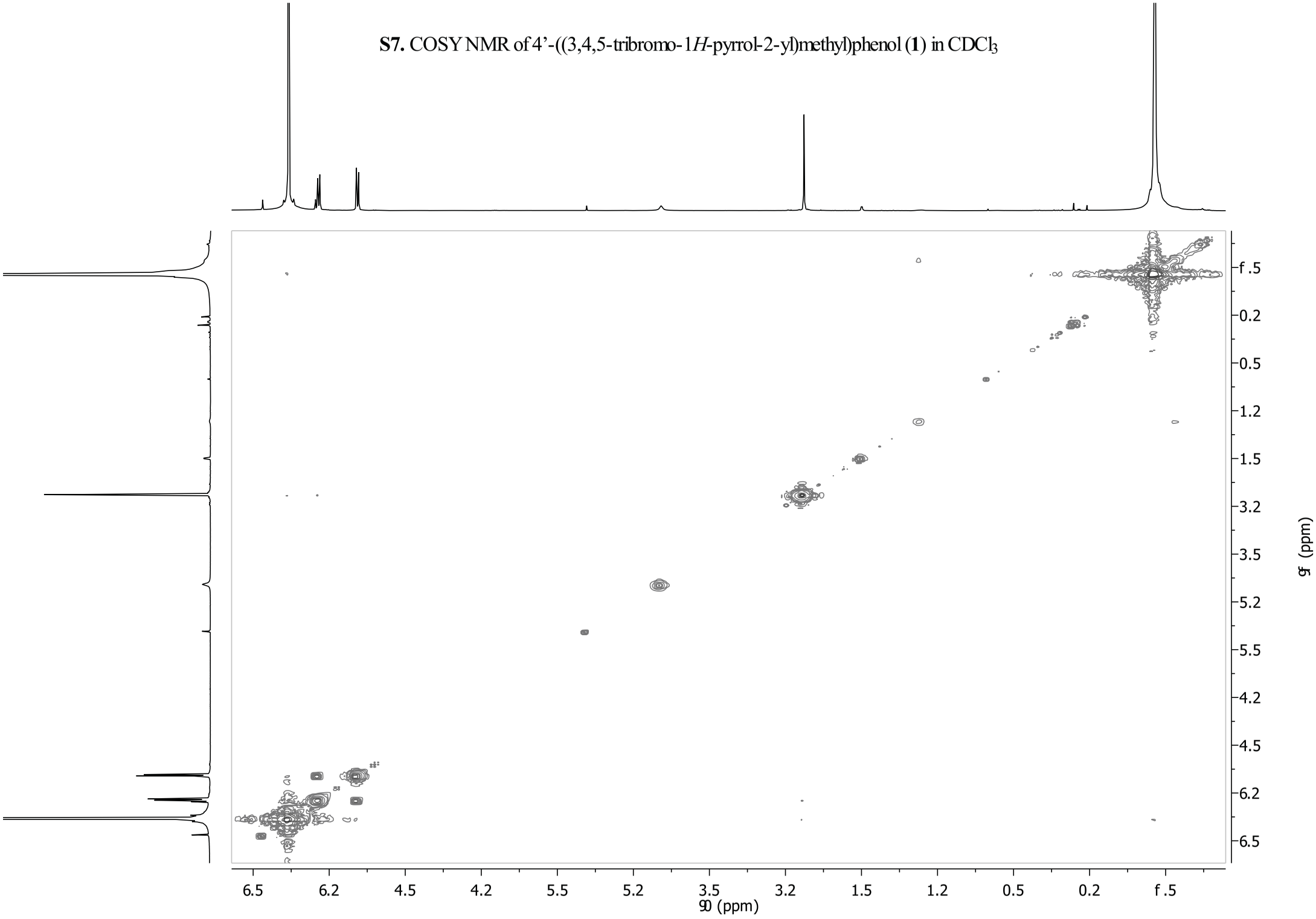
**S5.** $^{1}$H NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CDCl_3$



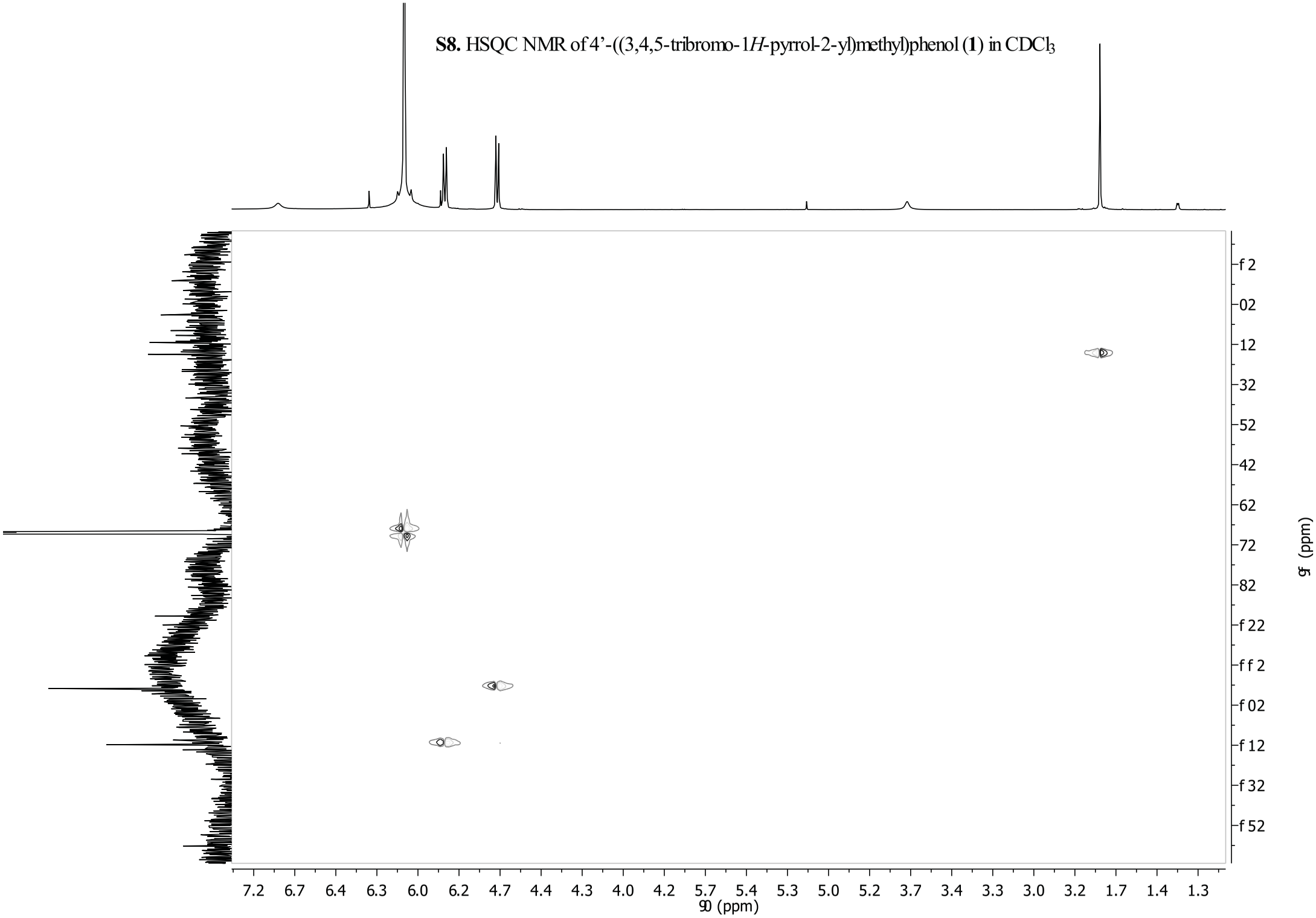
**S6.** $^{13}C$ NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CDCl_3$



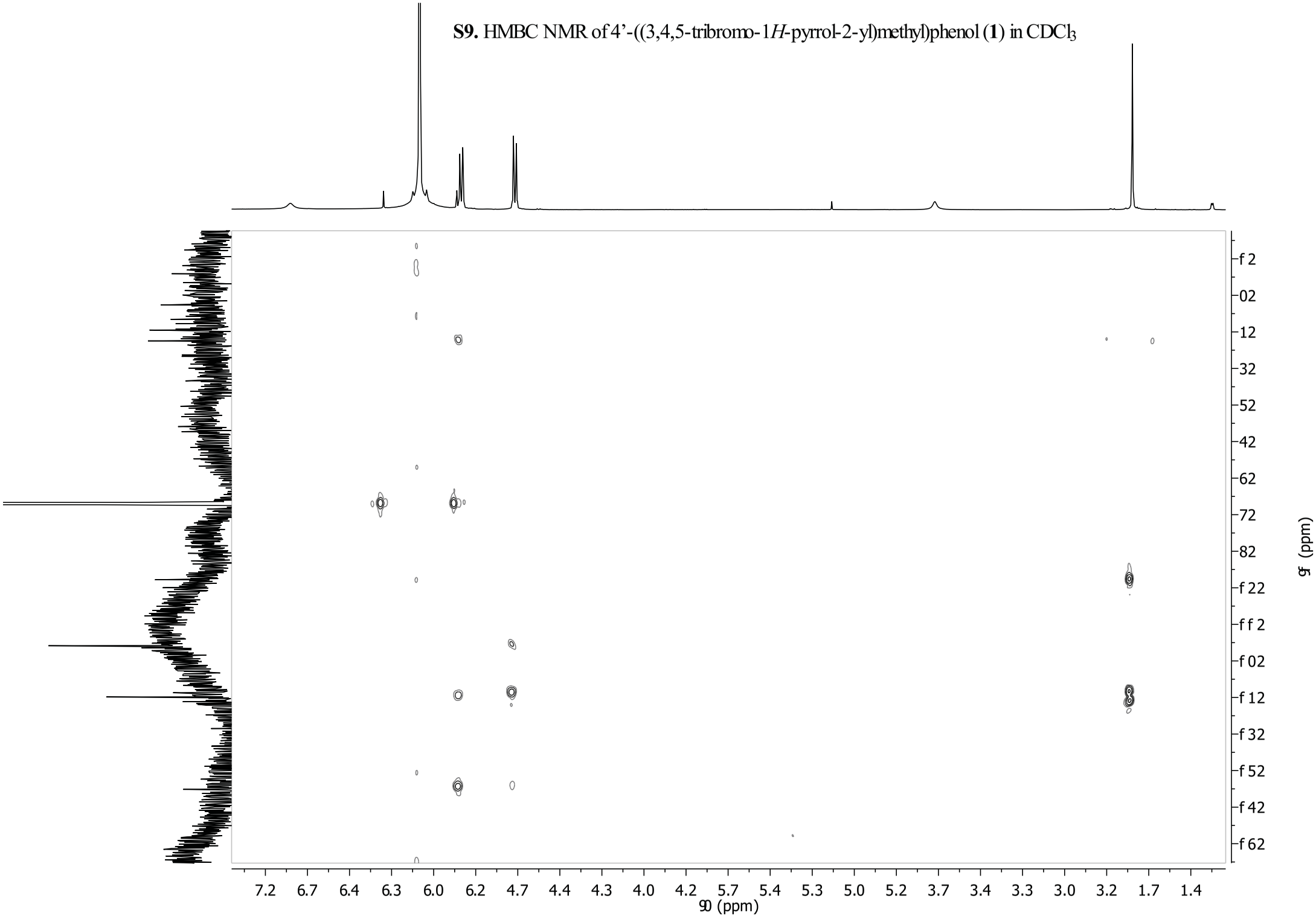
**S7.** COSY NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CDCl_3$

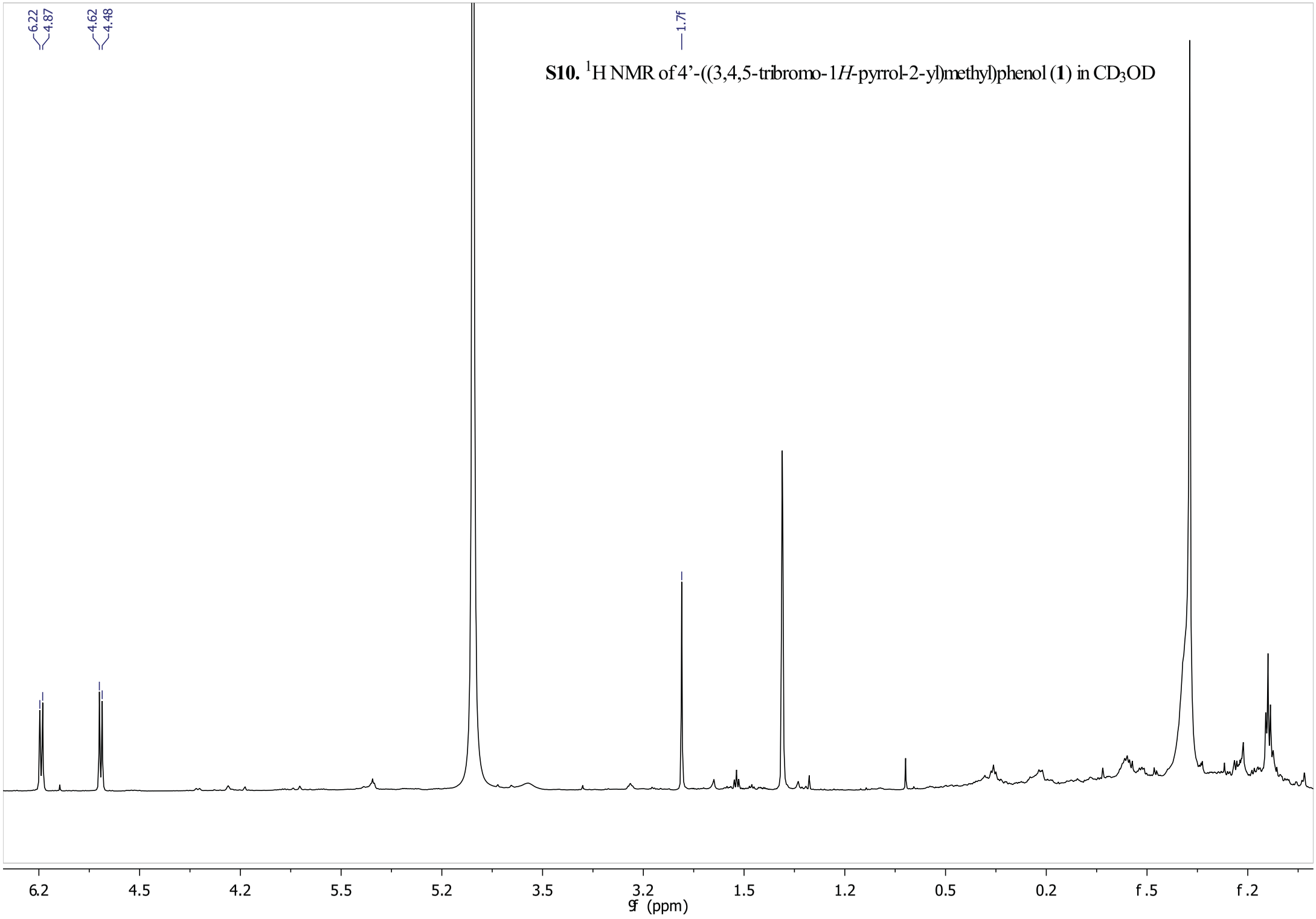


**S8.** HSQC NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CDCl_3$



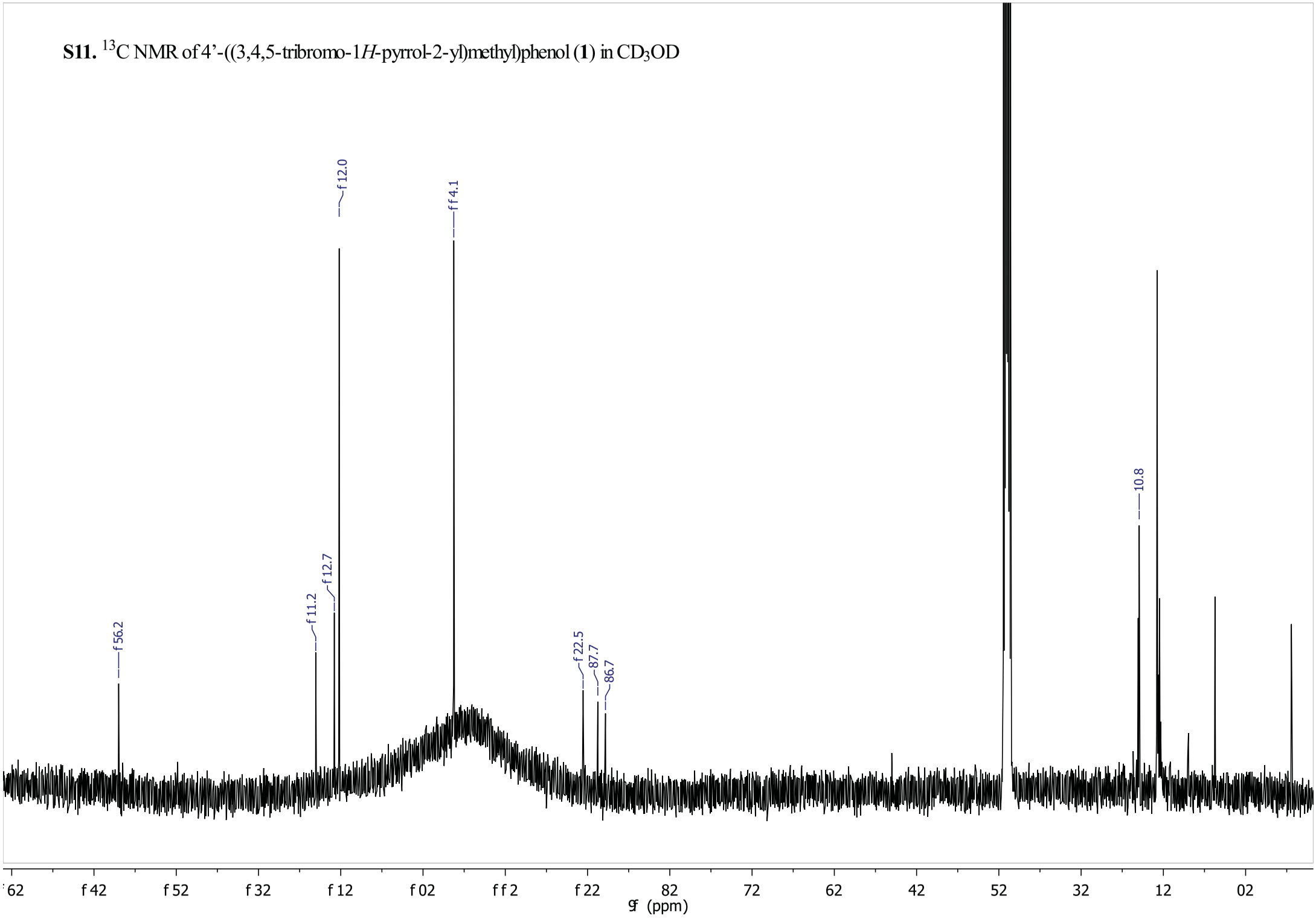
**S9.** HMBC NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CDCl_3$



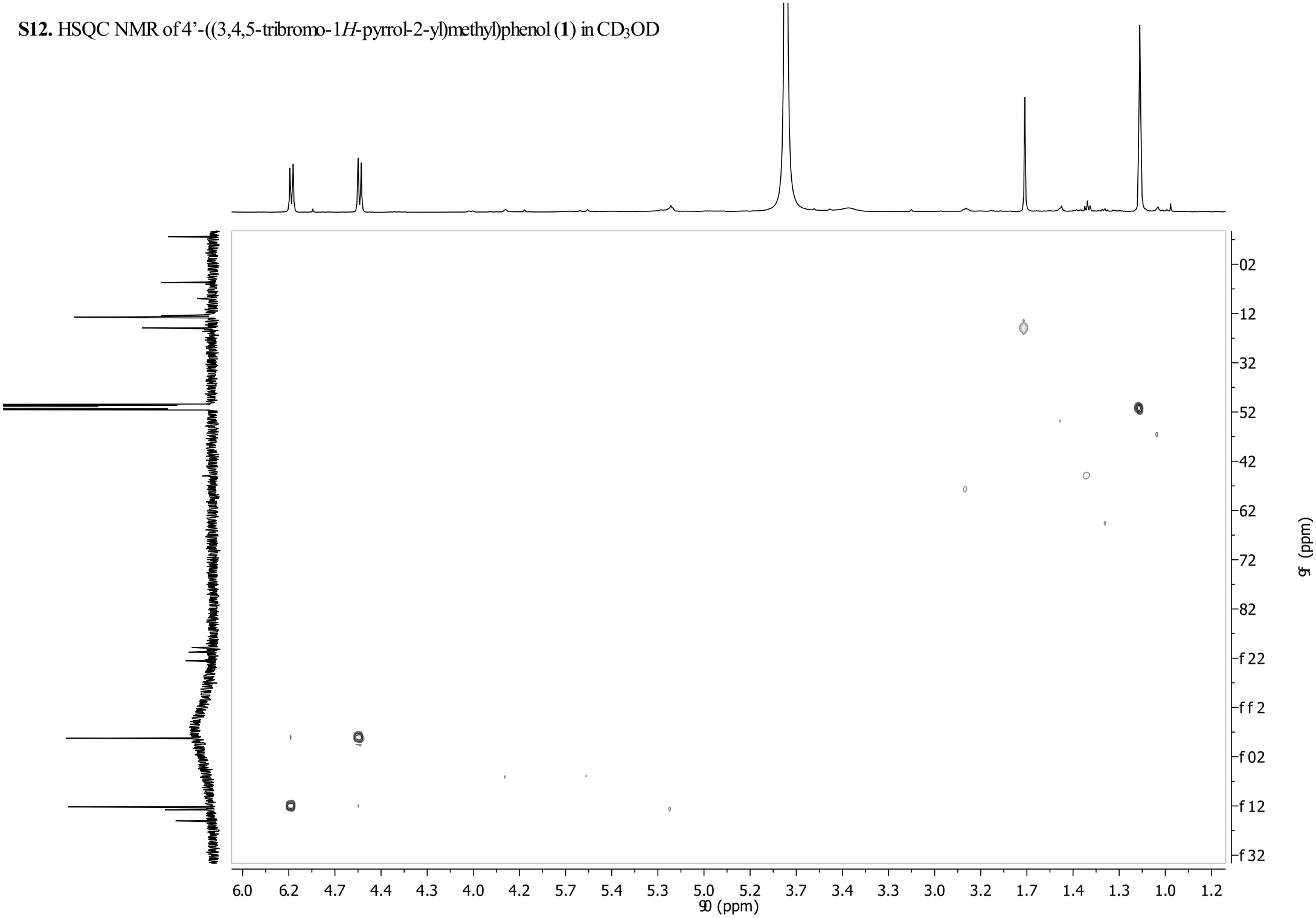



**S10.** $^{1}$H NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CD_3OD$

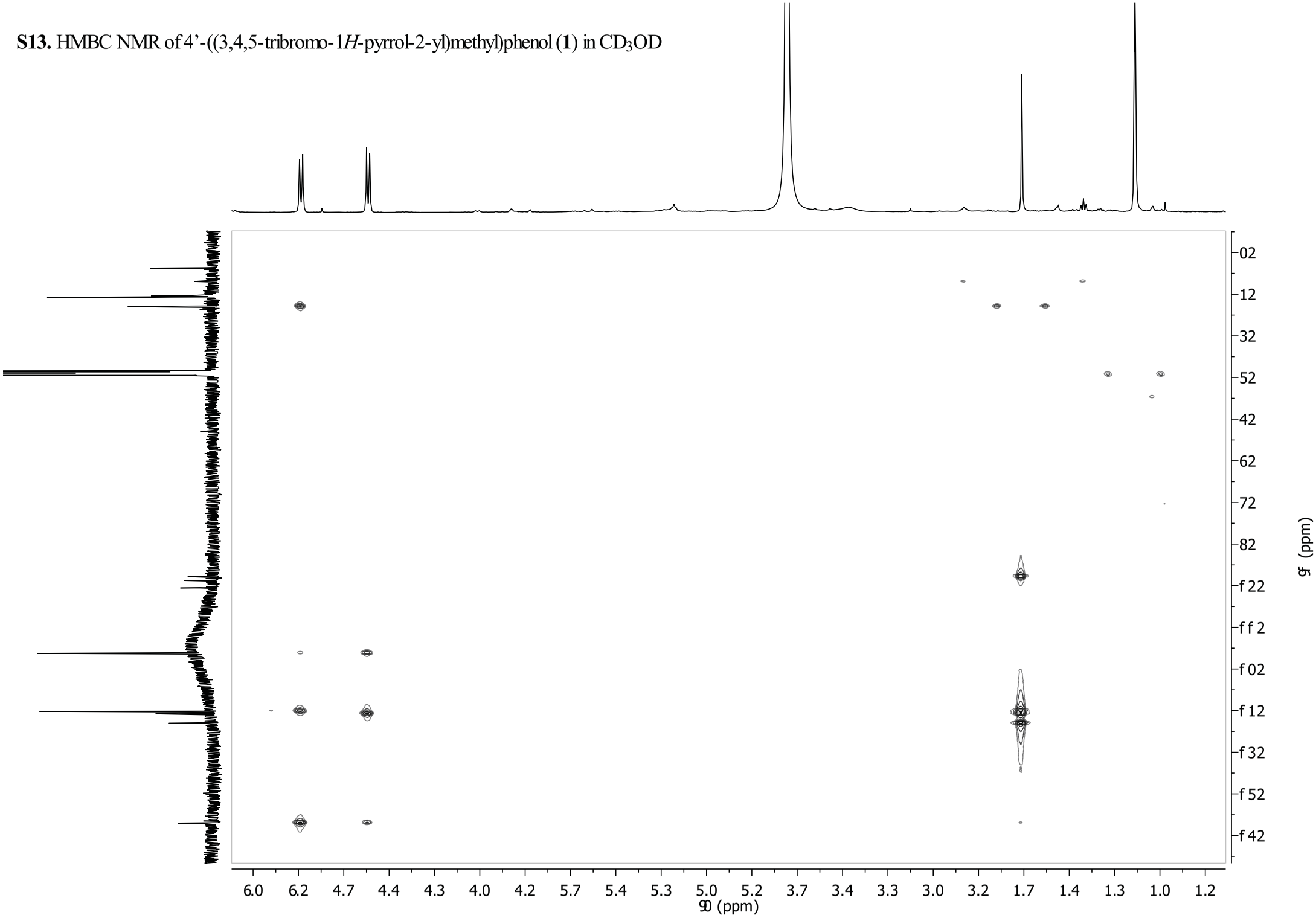
**S11.** $^{13}C$ NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CD_3OD$



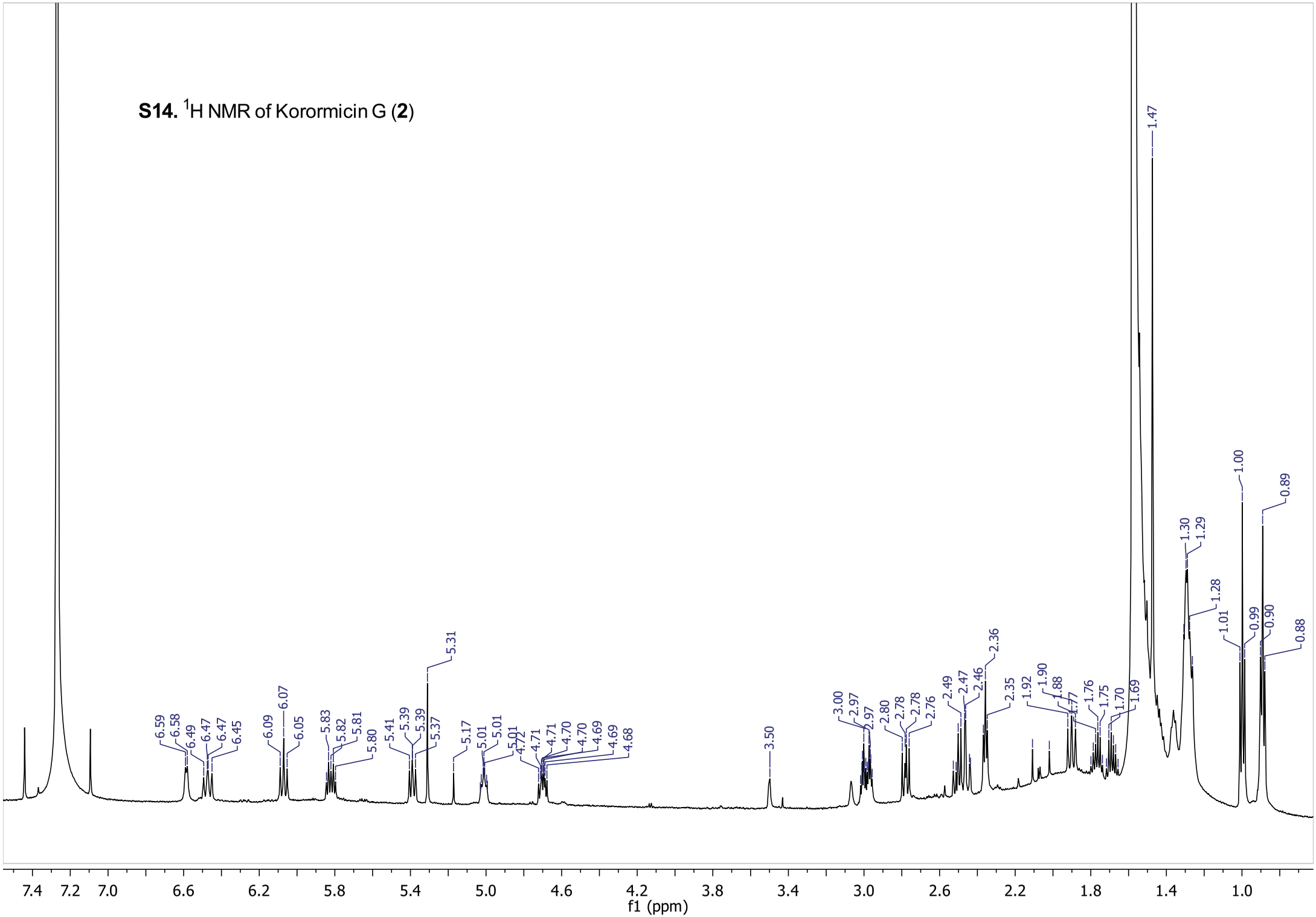
**S12.** HSQC NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CD_3OD$



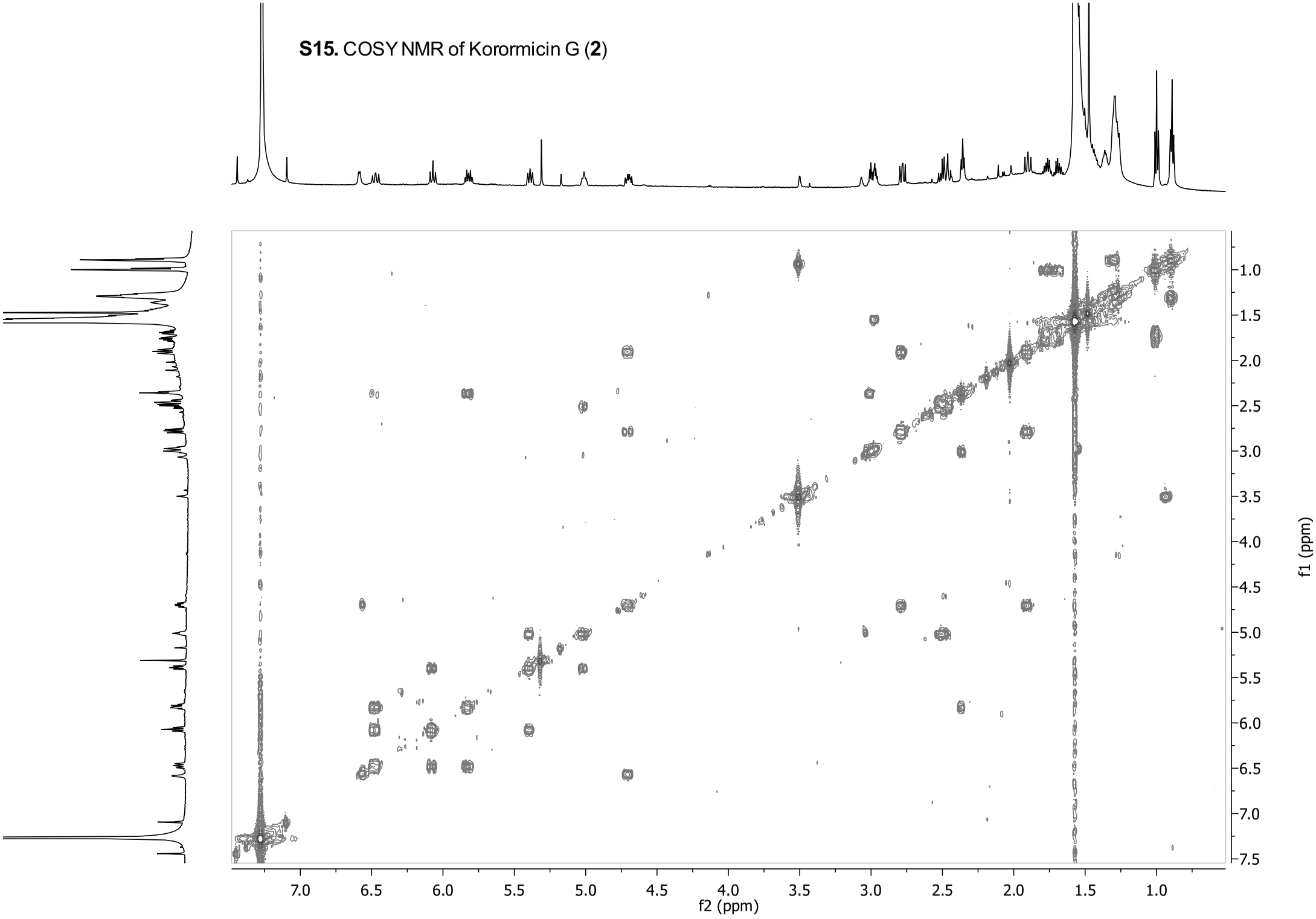
**S13.** HMBC NMR of 4'-((3,4,5-tribromo-1*H*-pyrrol-2-yl)methyl)phenol (**1**) in $CD_3OD$



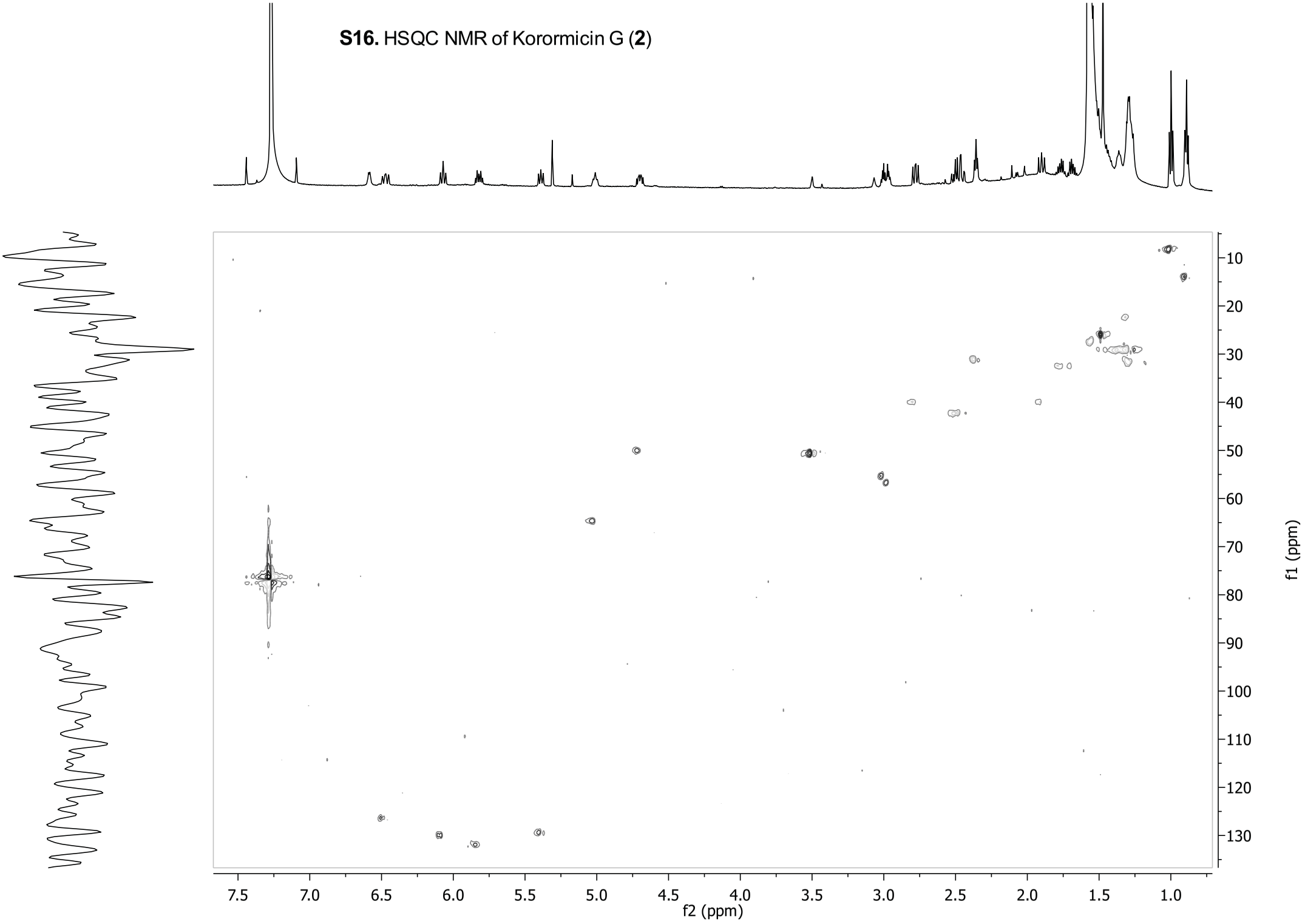
**S14.** $^{1}$H NMR of Korormicin G (**2**)



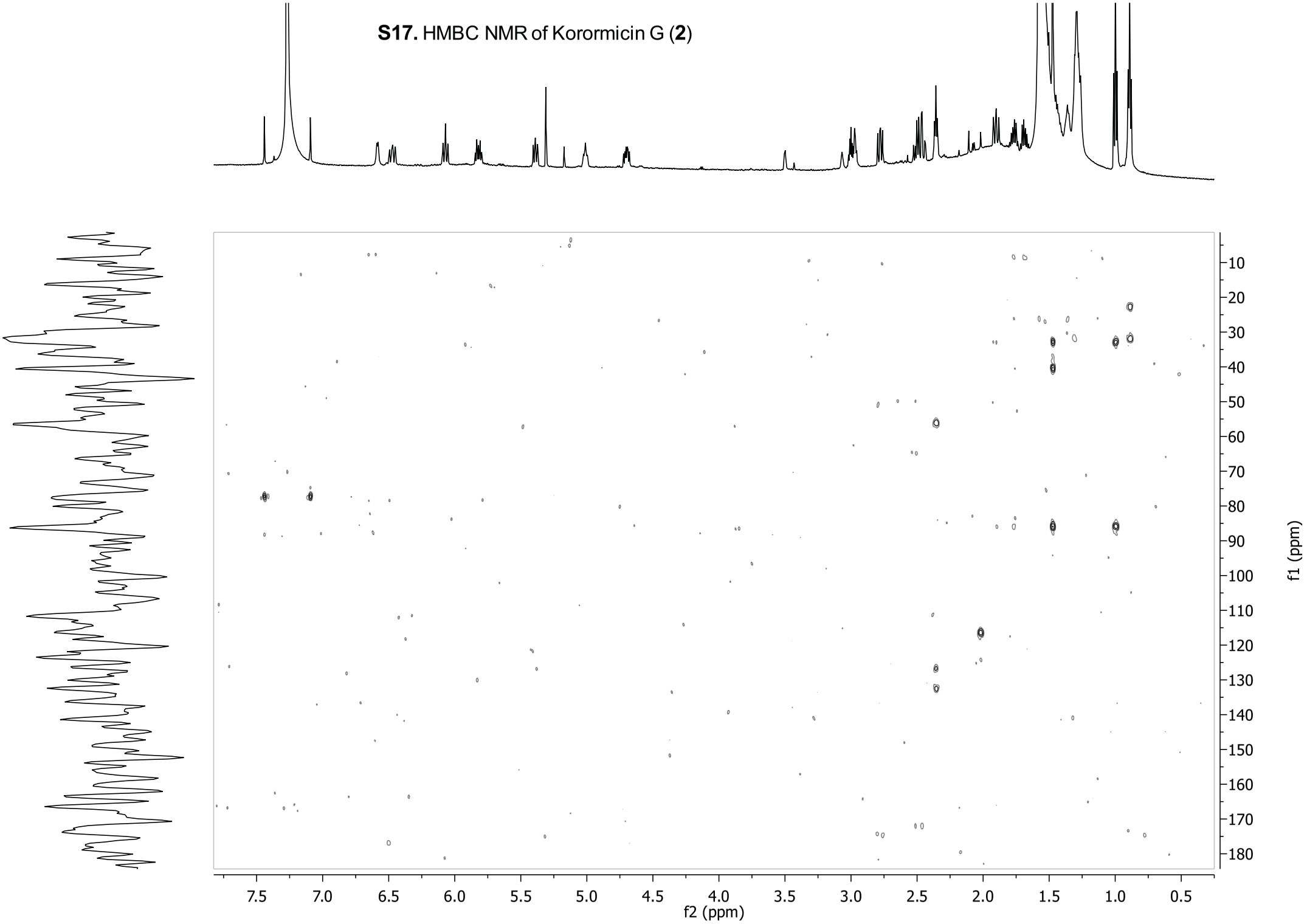
**S15.** COSY NMR of Korormicin G (**2**)



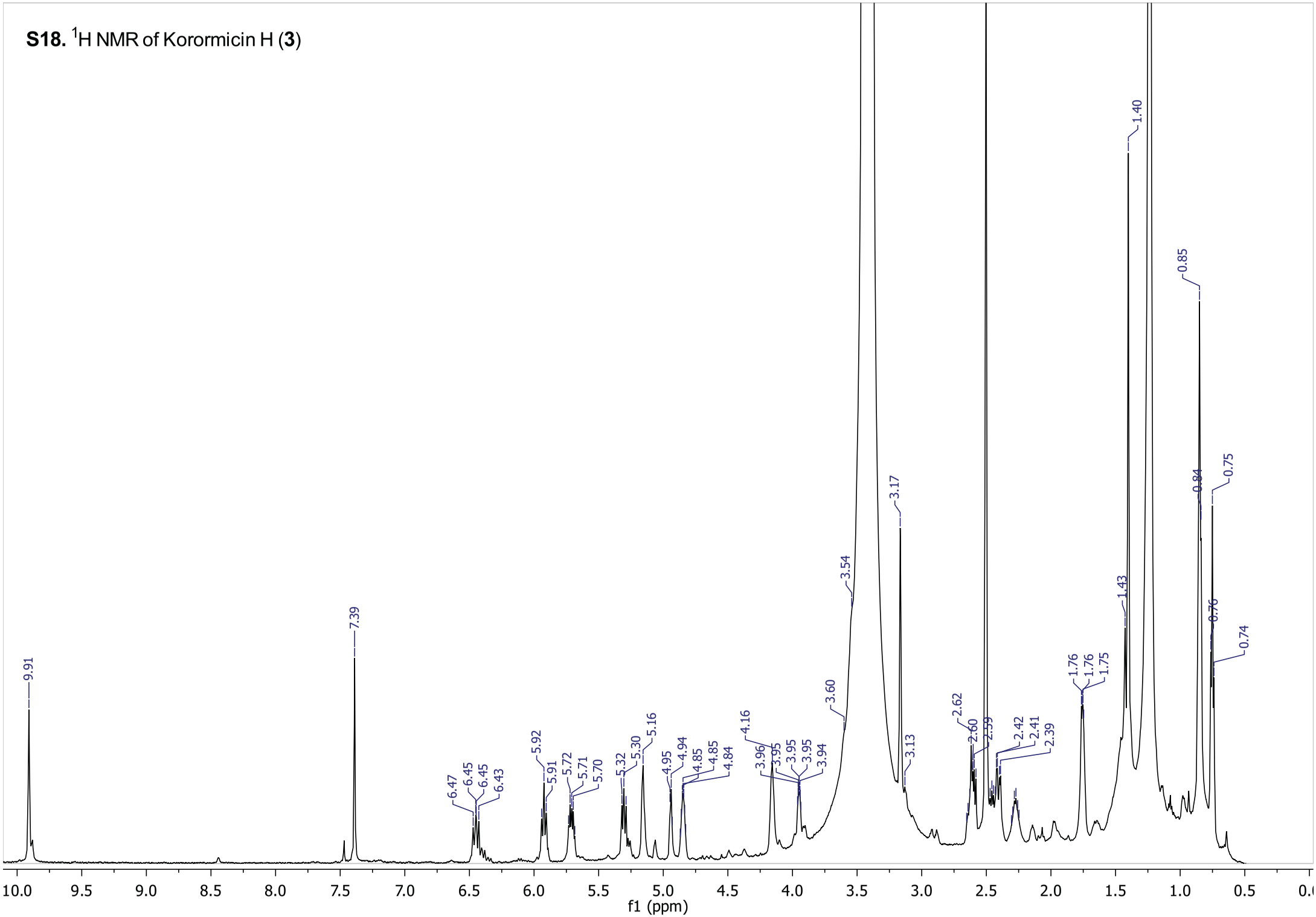
**S16.** HSQC NMR of Korormicin G (**2**)



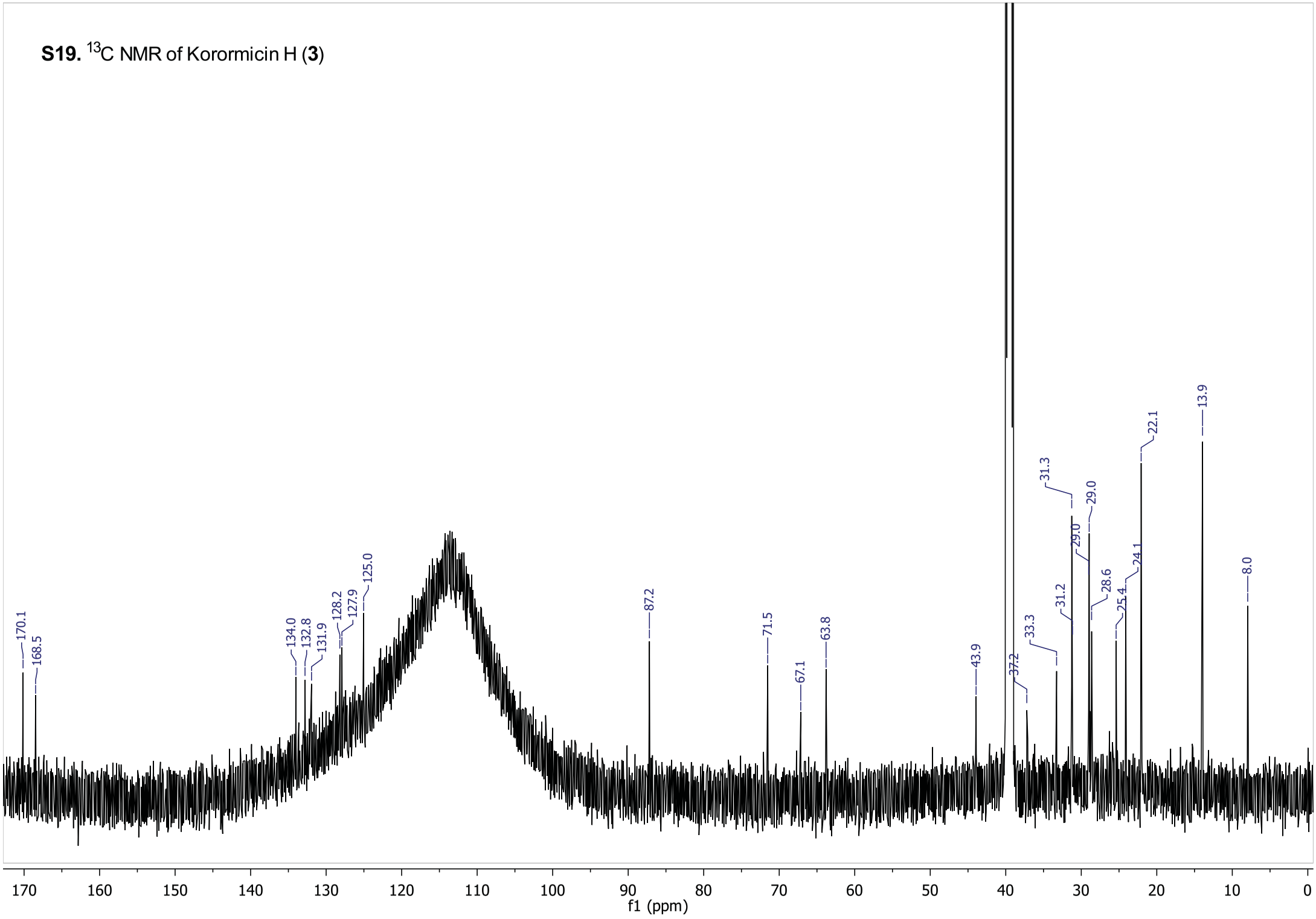
**S17.** HMBC NMR of Korormicin G (**2**)



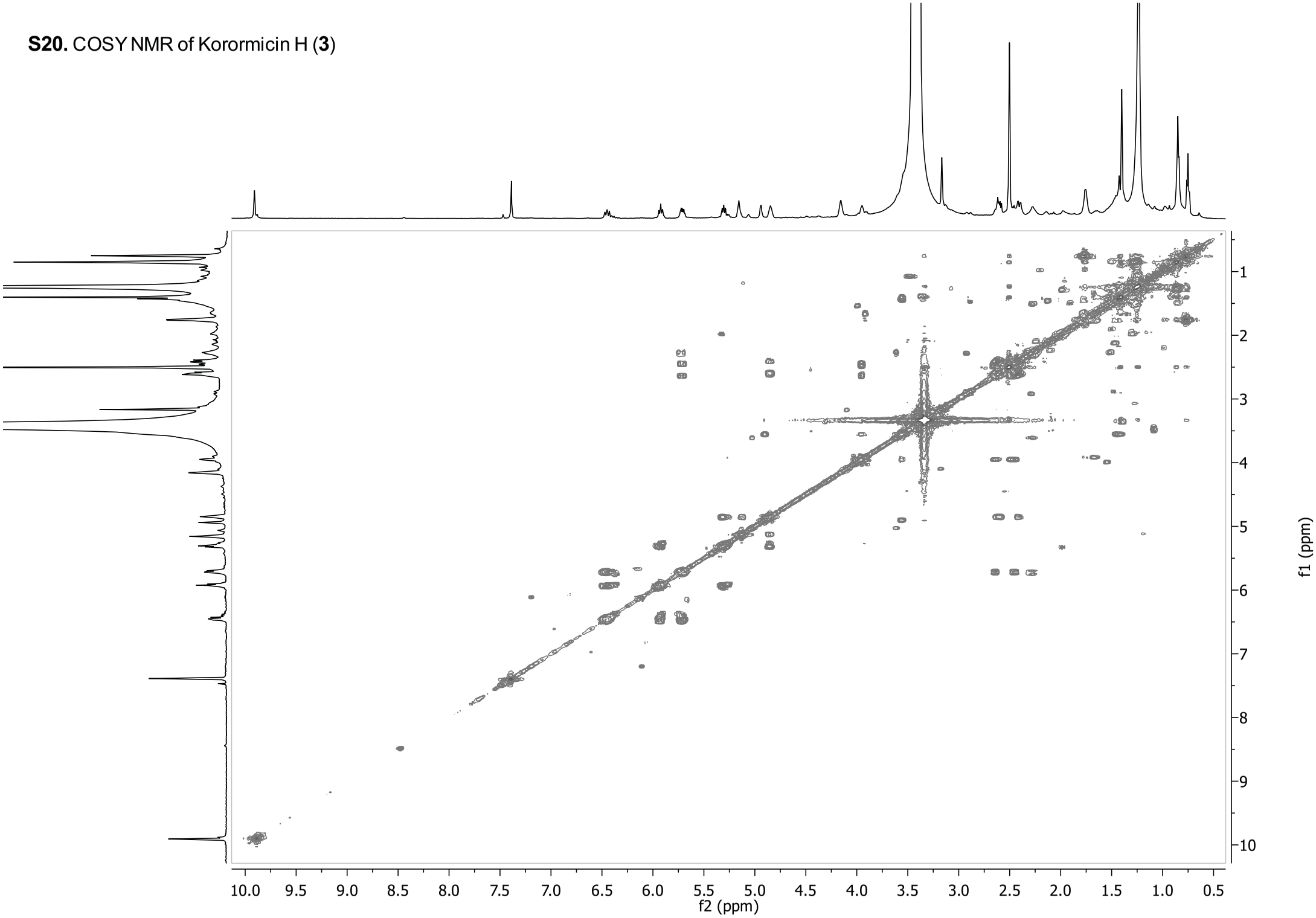
**S18.** $^{1}$H NMR of Korormicin H (**3**)



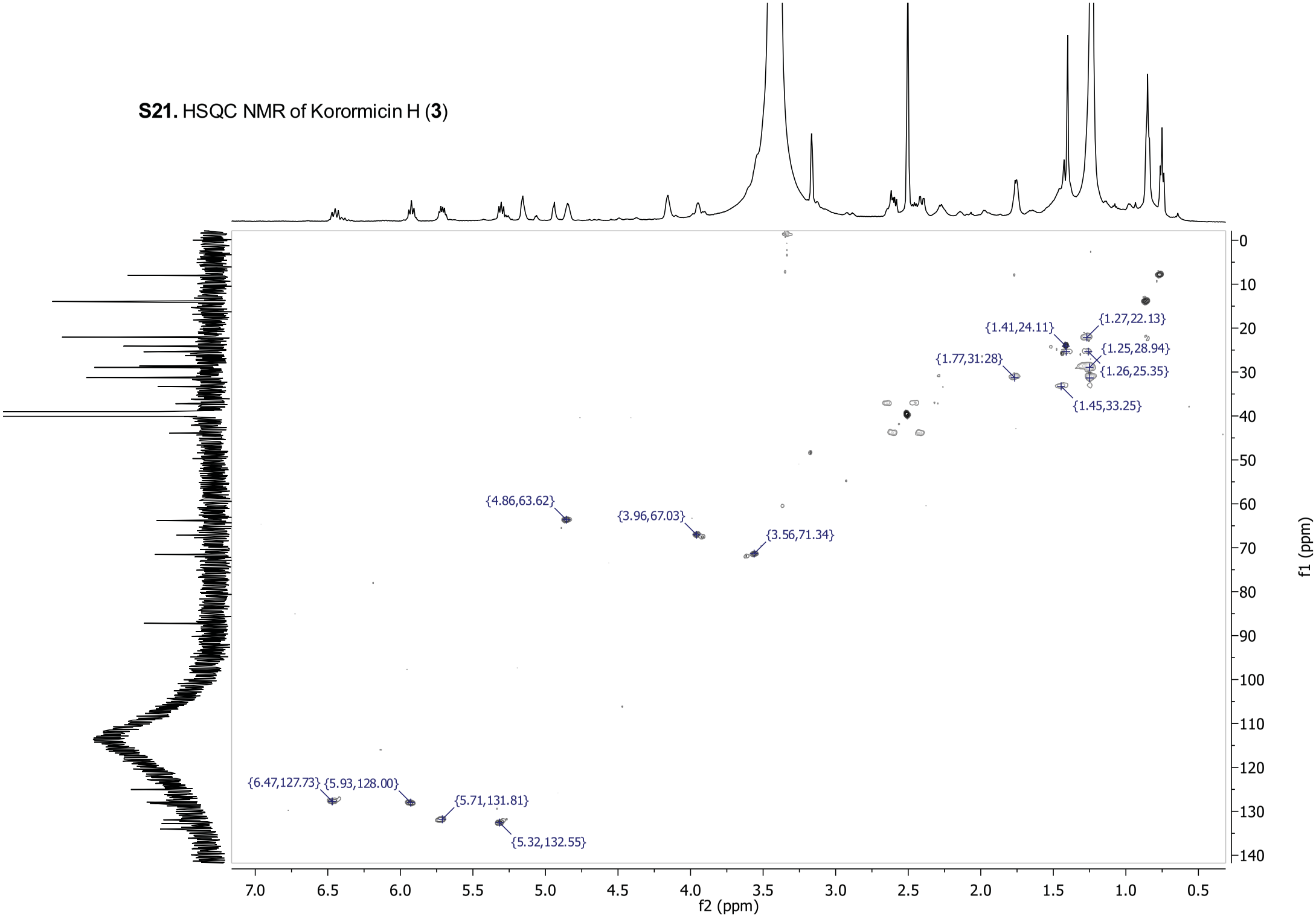
**S19.** $^{13}C$ NMR of Korormicin H (**3**)



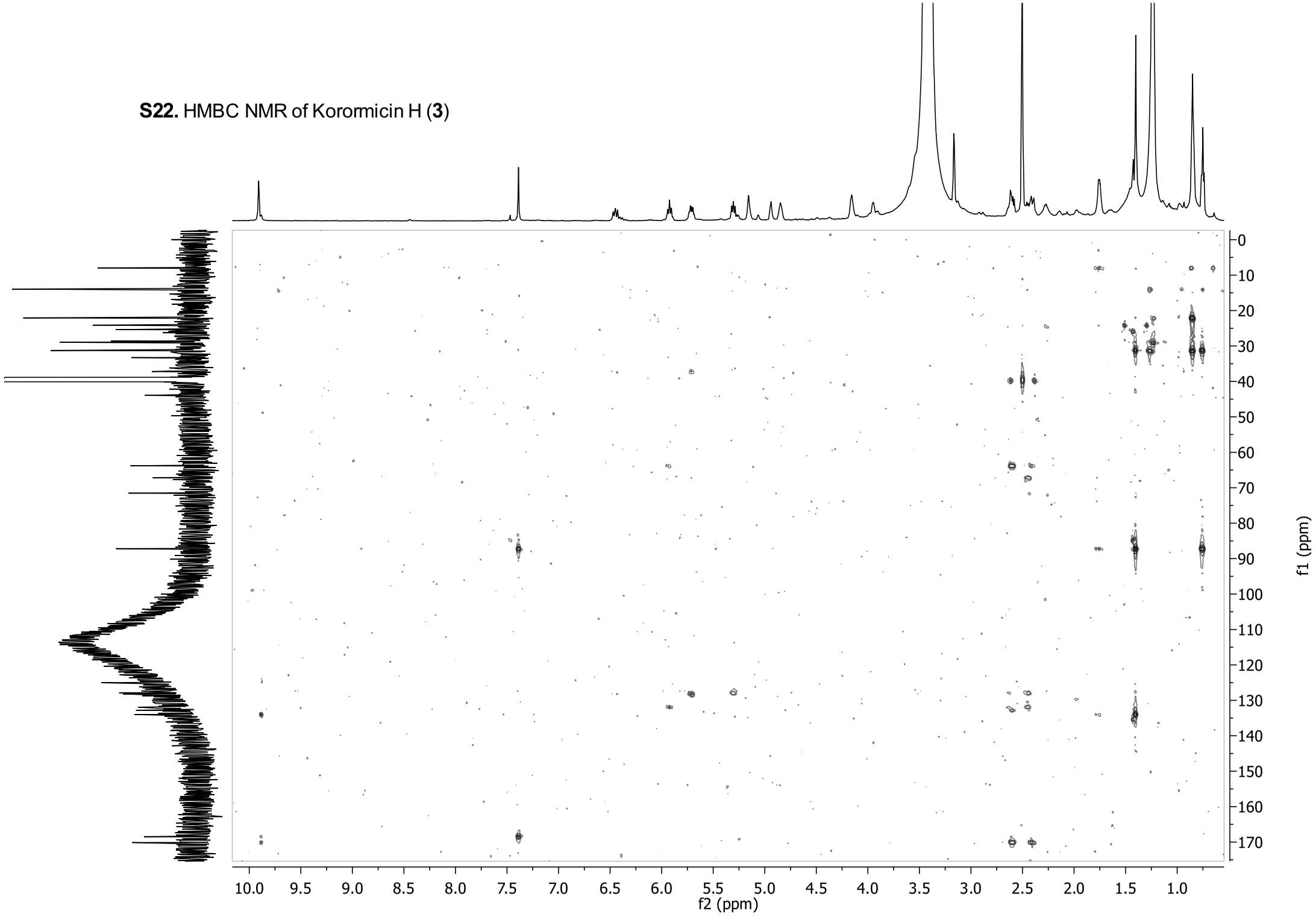
**S20.** COSY NMR of Korormicin H (**3**)



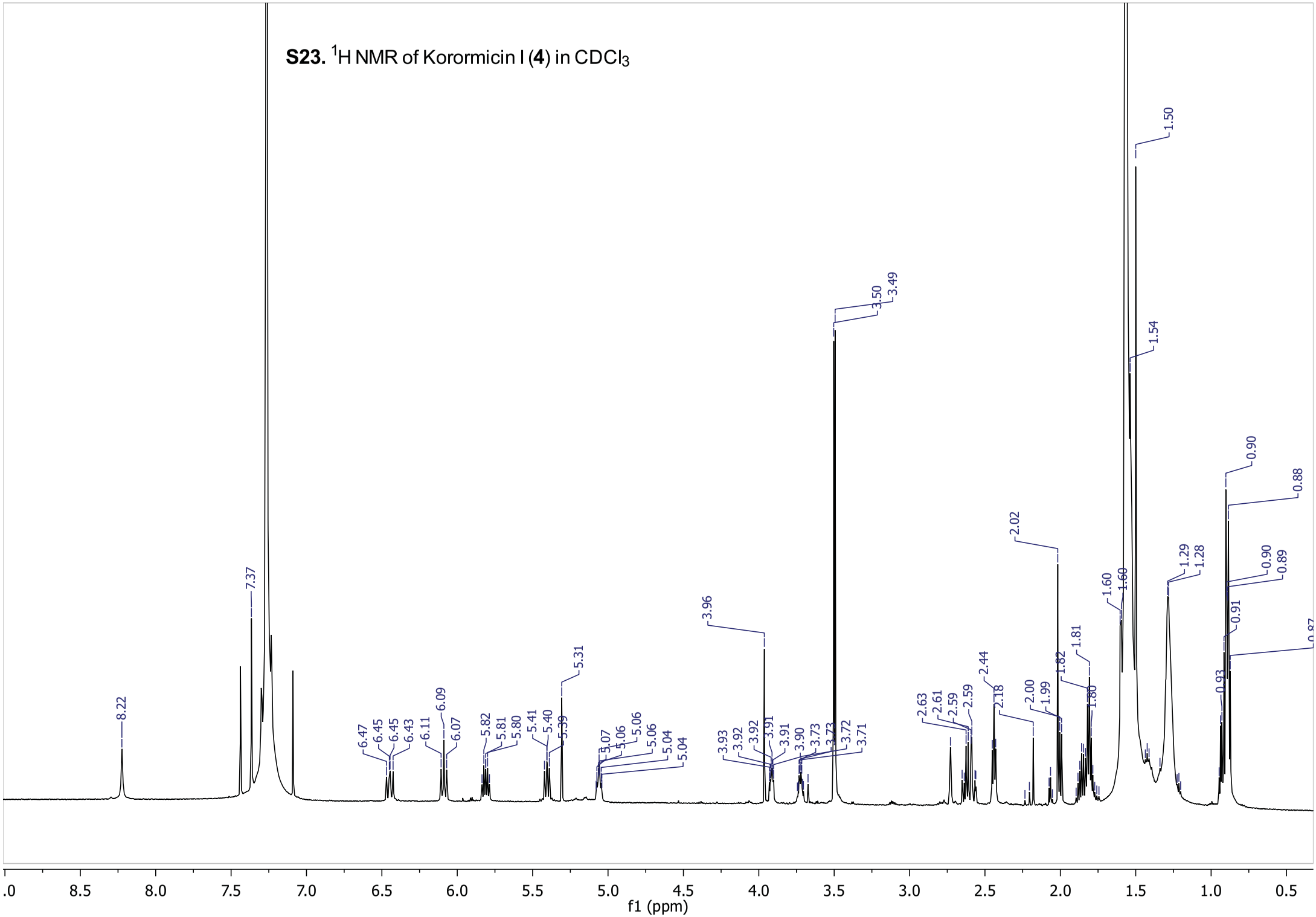
**S21.** HSQC NMR of Korormicin H (**3**)



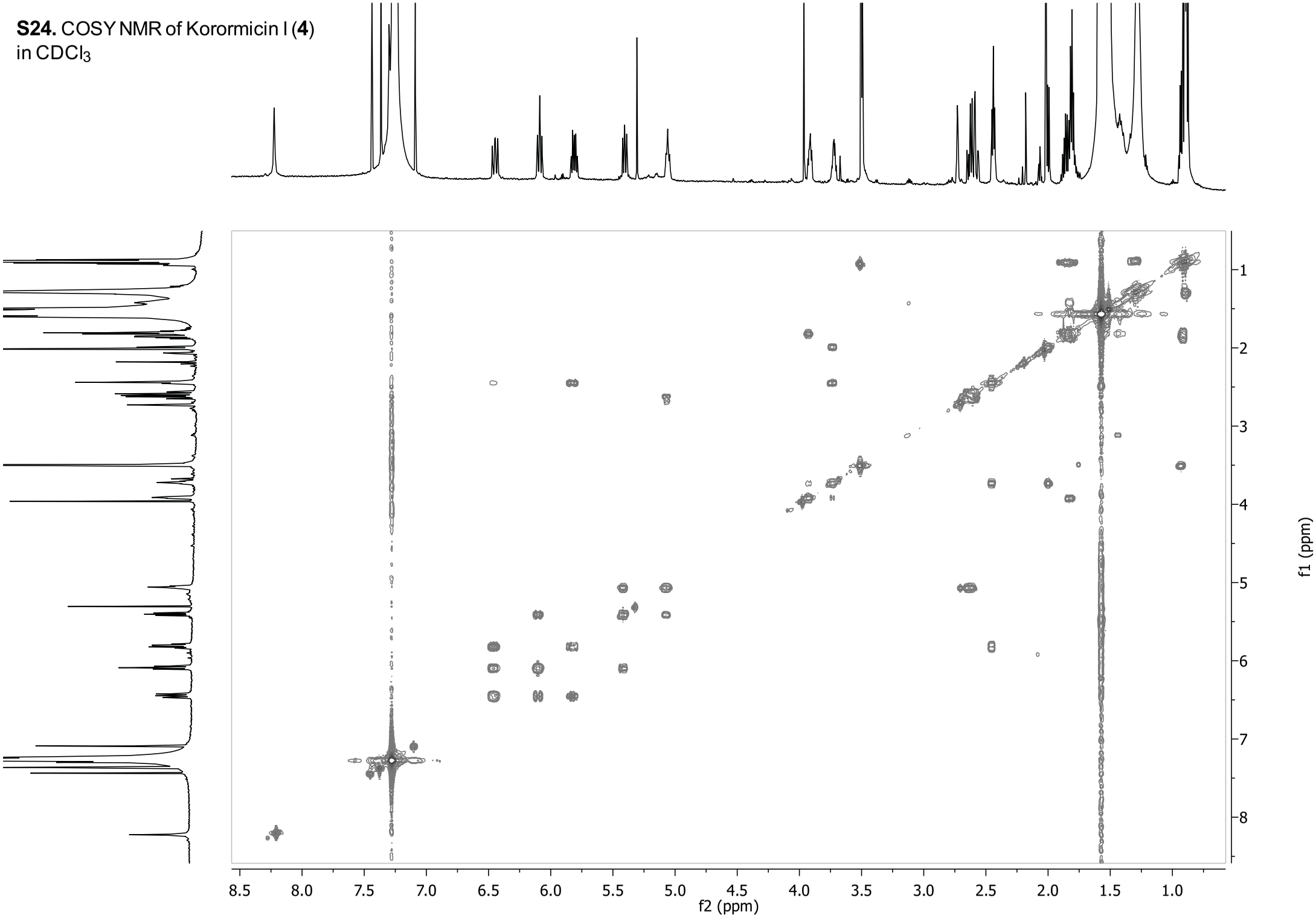
**S22.** HMBC NMR of Korormicin H (**3**)



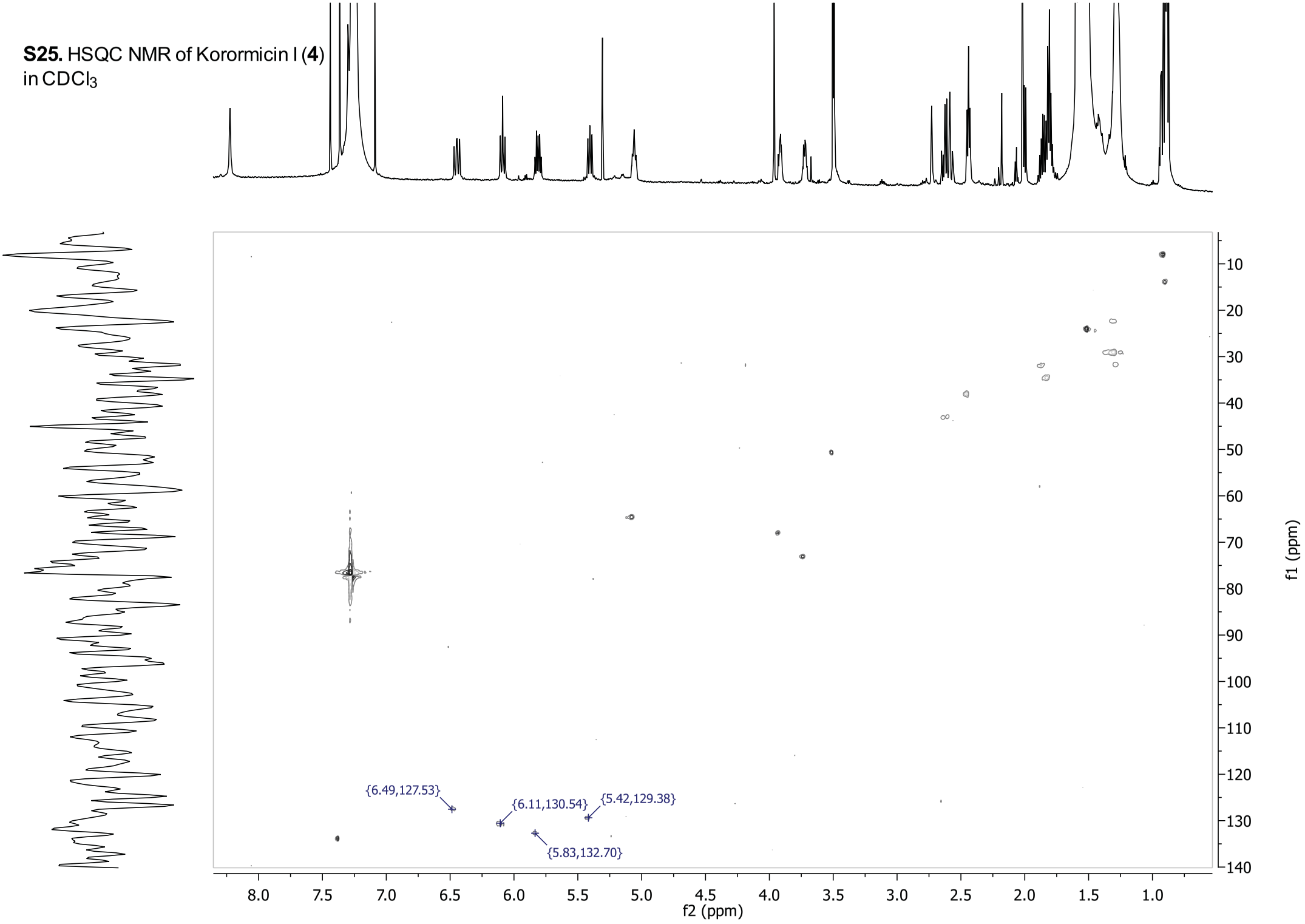
**S23.** $^{1}$H NMR of Korormicin I (**4**) in $CDCl_3$



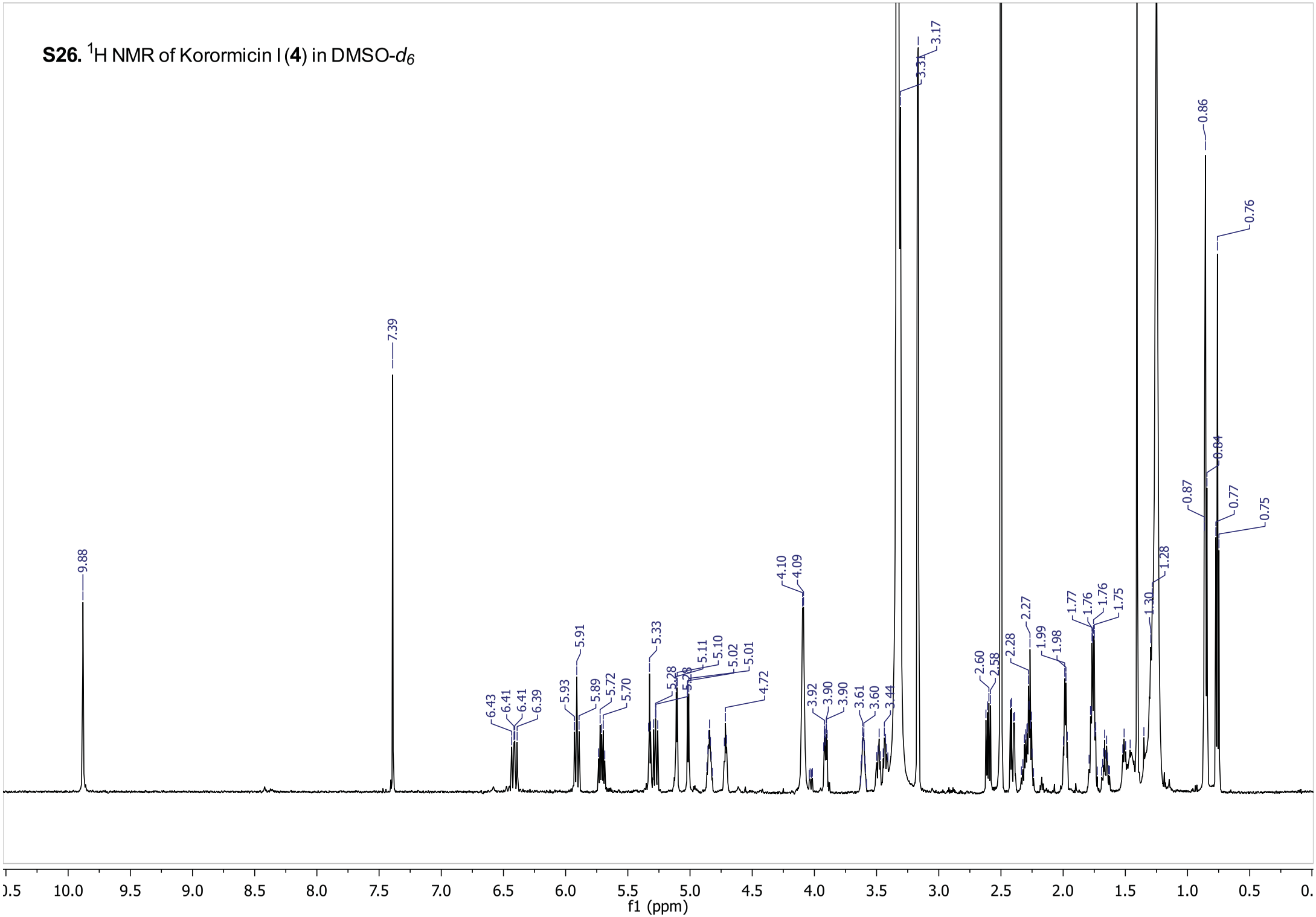
**S24.** COSY NMR of Korormicin I (**4**) in $CDCl_3$



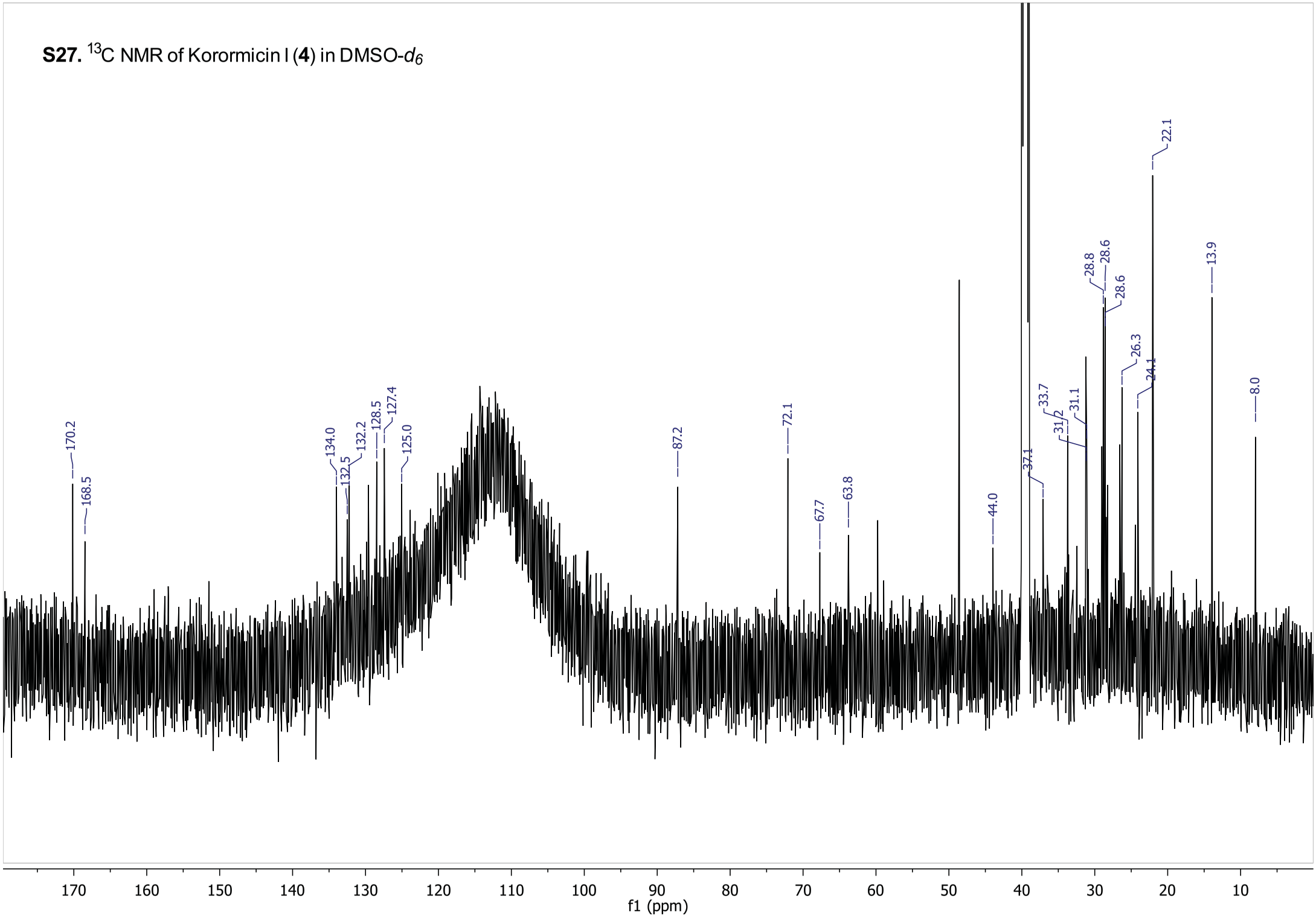
**S25.** HSQC NMR of Korormicin I (**4**) in $CDCl_3$



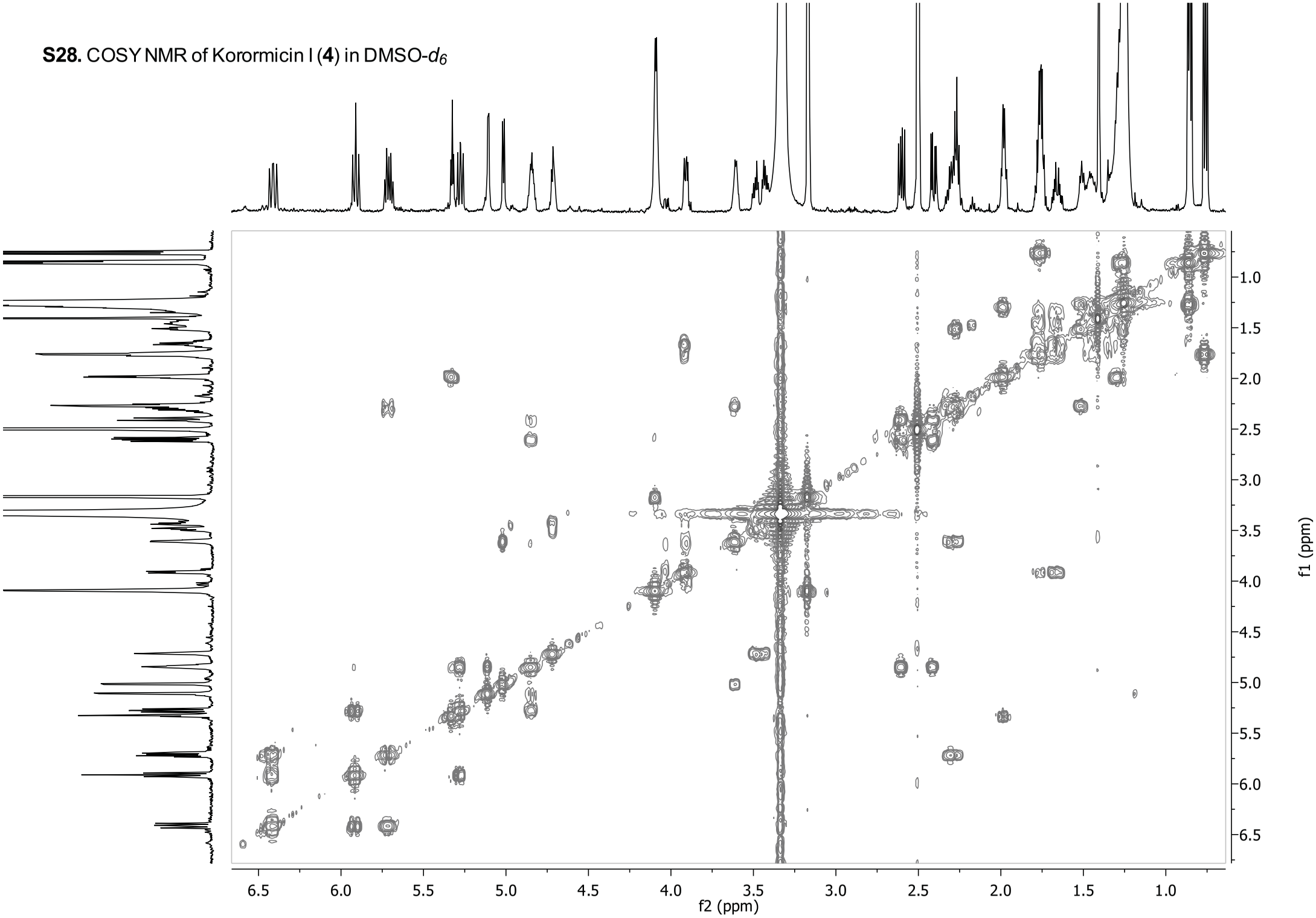
**S26.** $^{1}$H NMR of Korormicin I (**4**) in DMSO-$d_6$



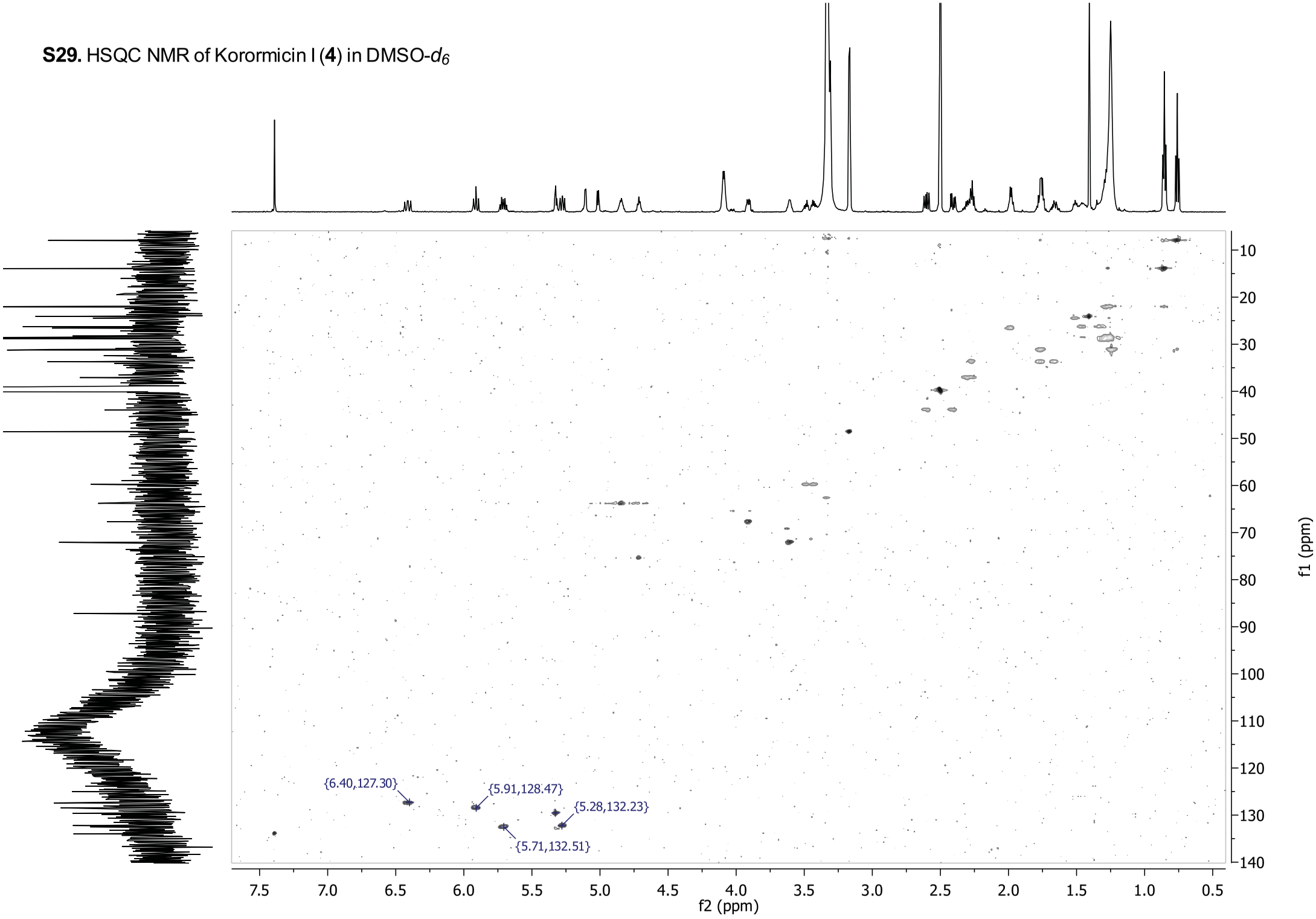
**S27.** $^{13}C$ NMR of Korormicin I (**4**) in DMSO-$d_6$



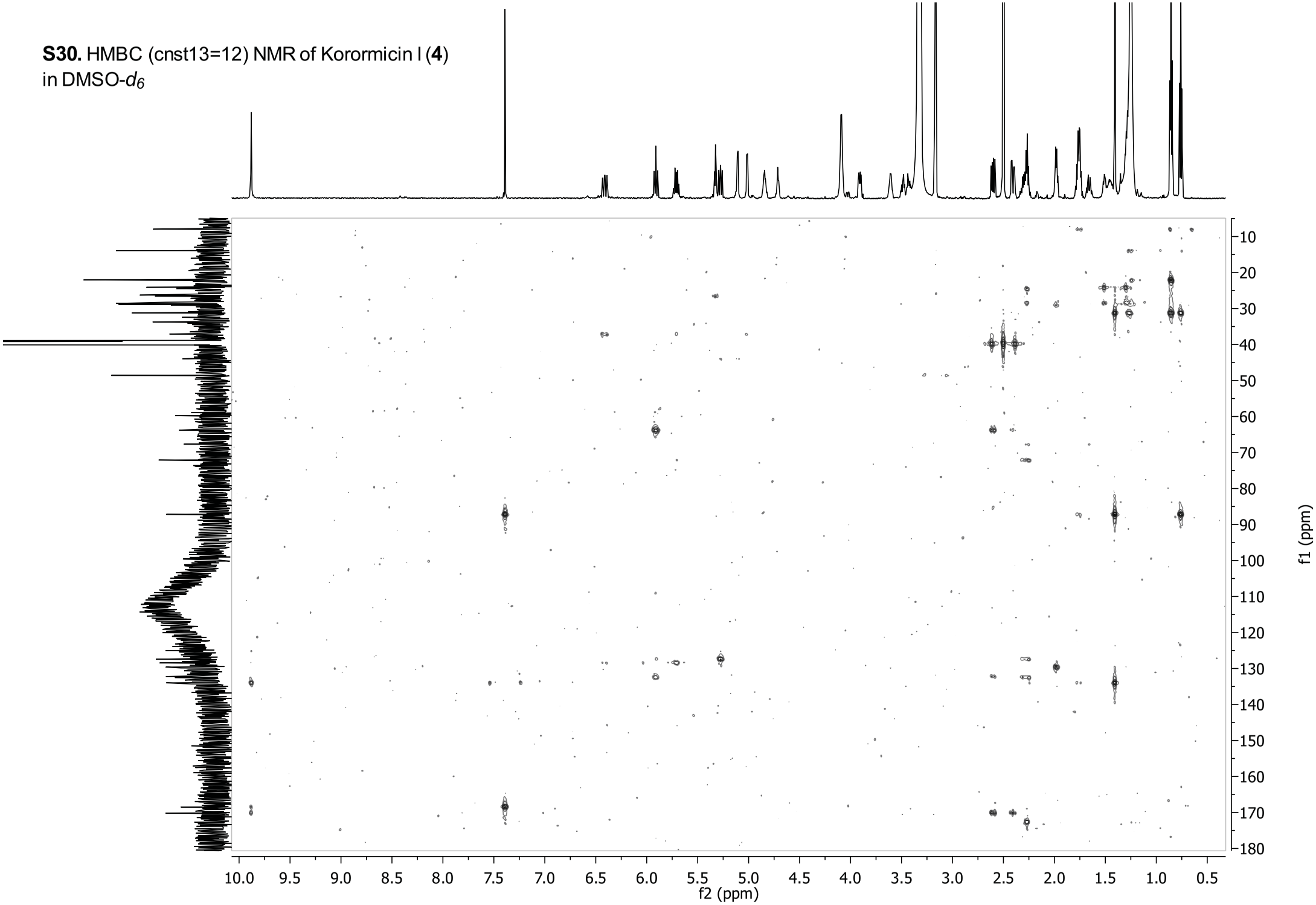
**S28.** COSY NMR of Korormicin I (**4**) in DMSO-$d_6$



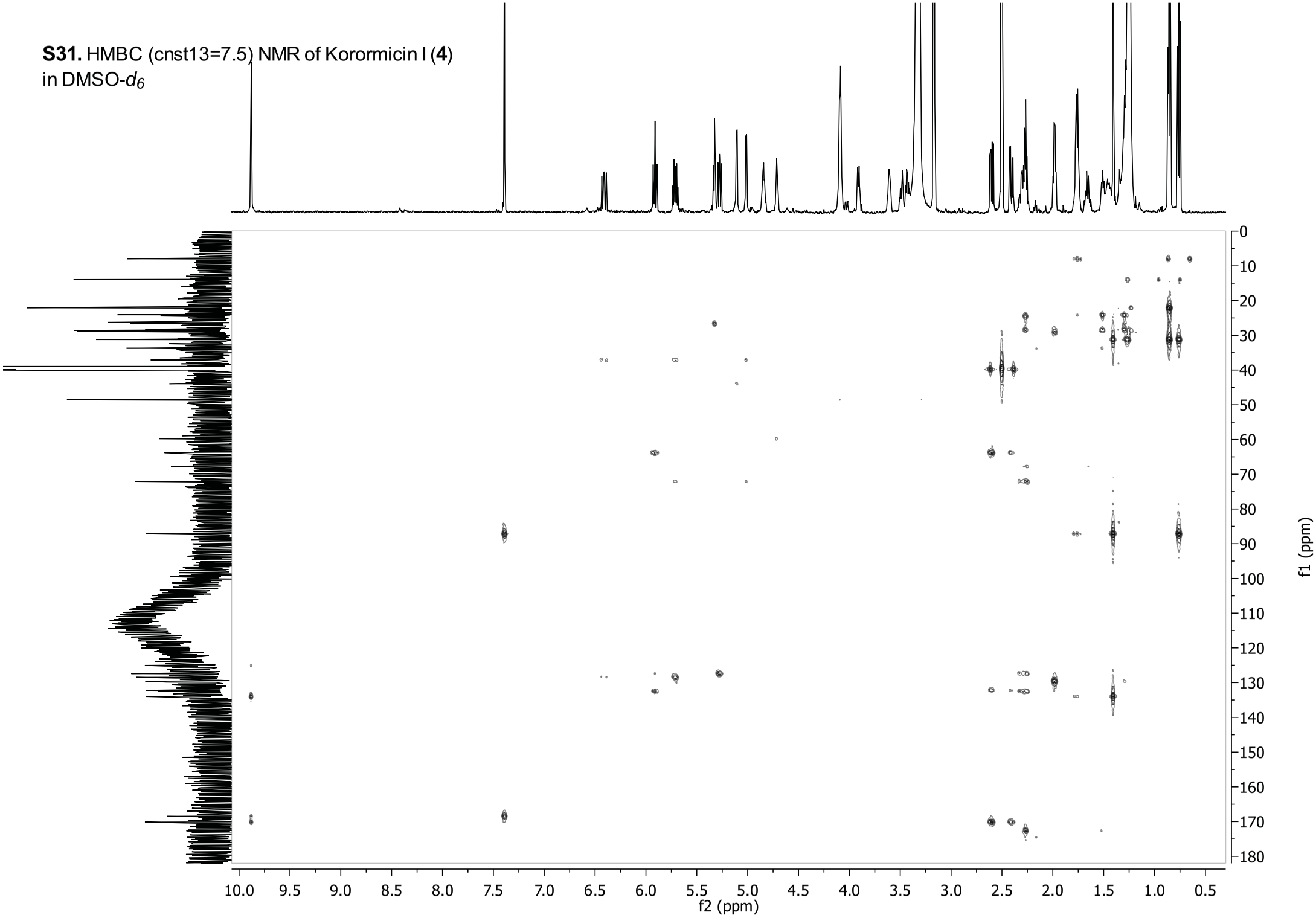
**S29.** HSQC NMR of Korormicin I (**4**) in DMSO-$d_6$



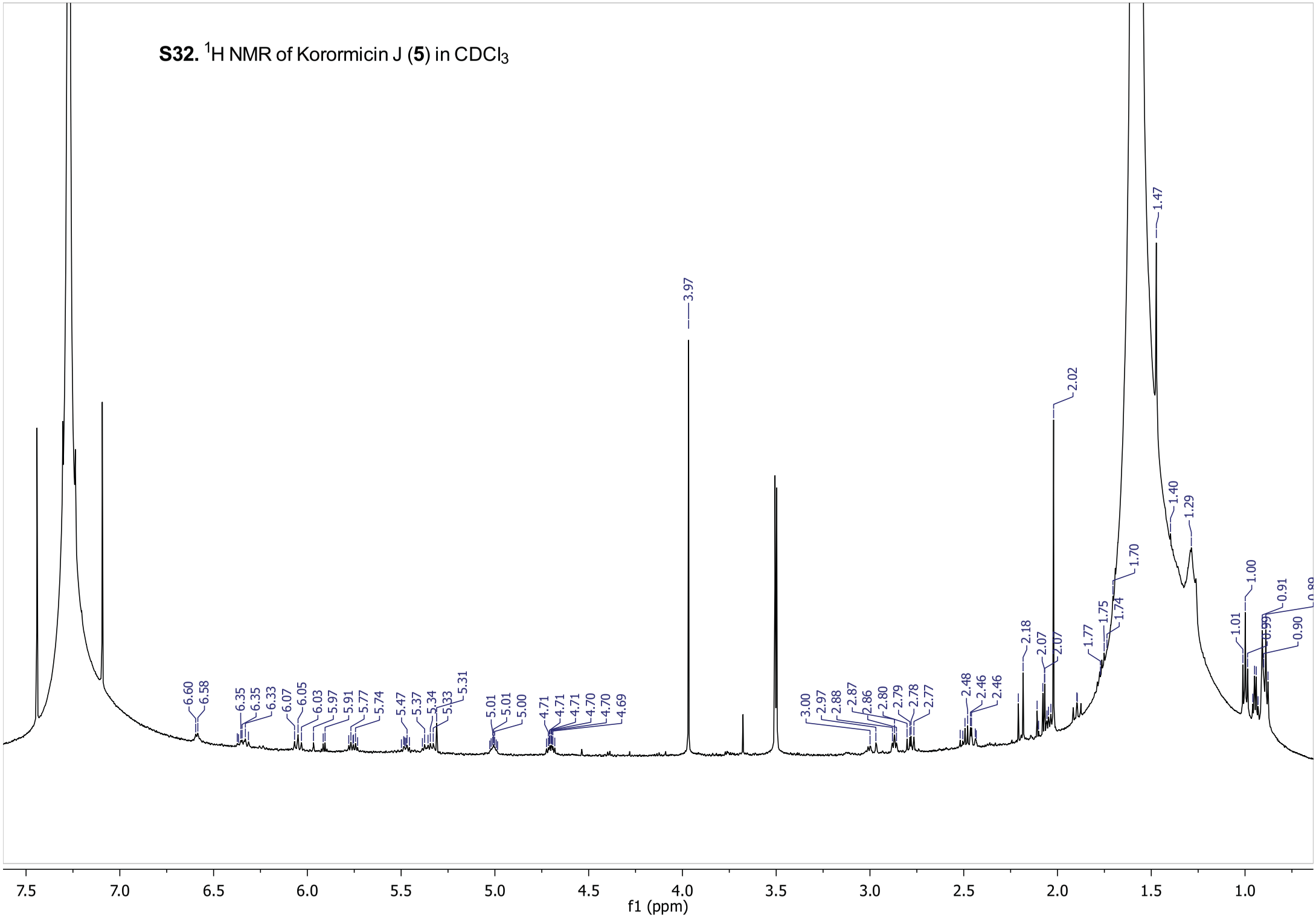
**S30.** HMBC (cnst13=12) NMR of Korormicin I (**4**) in DMSO-$d_6$



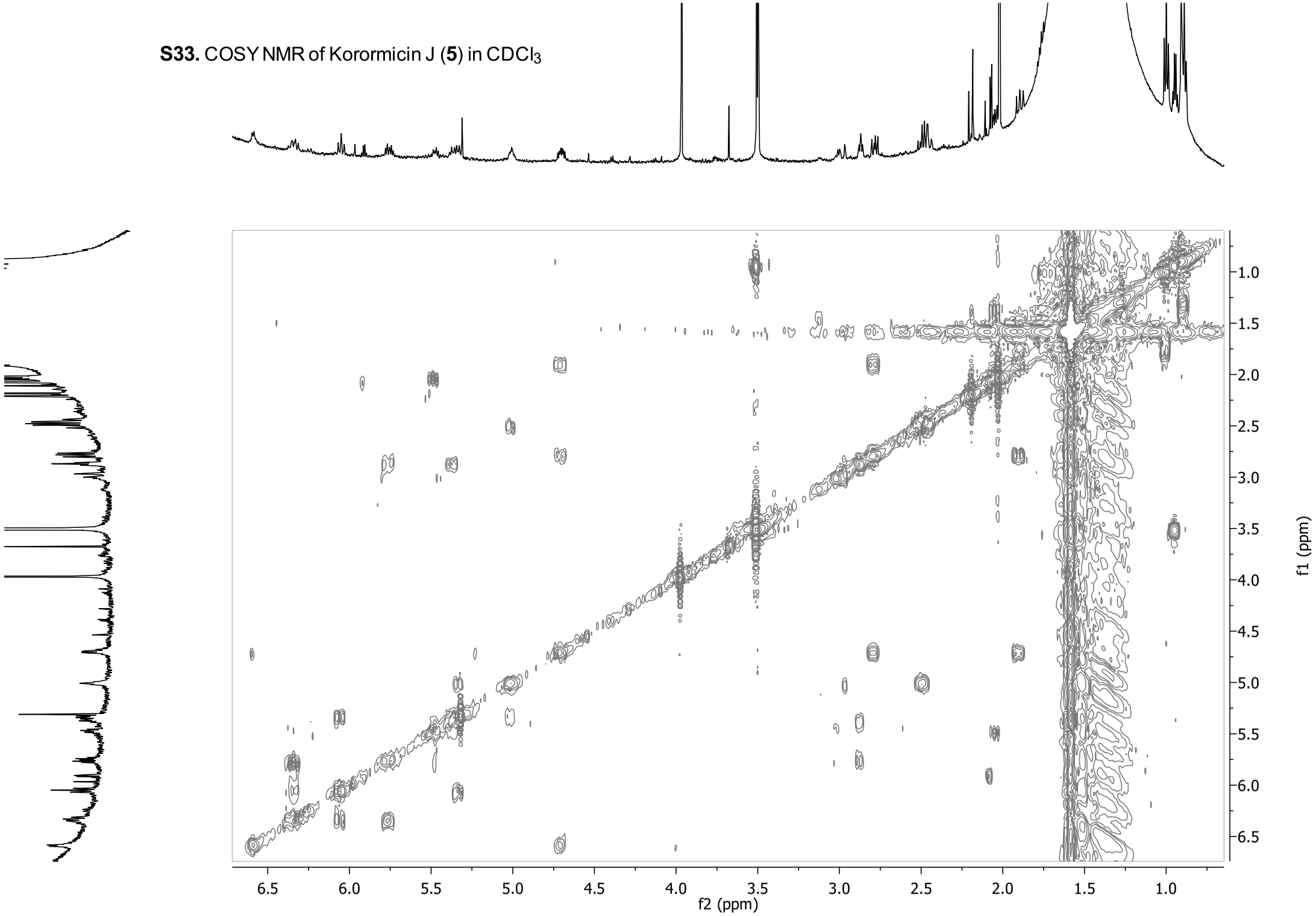
**S31.** HMBC (cnst13=7.5) NMR of Korormicin I (**4**) in DMSO-$d_6$



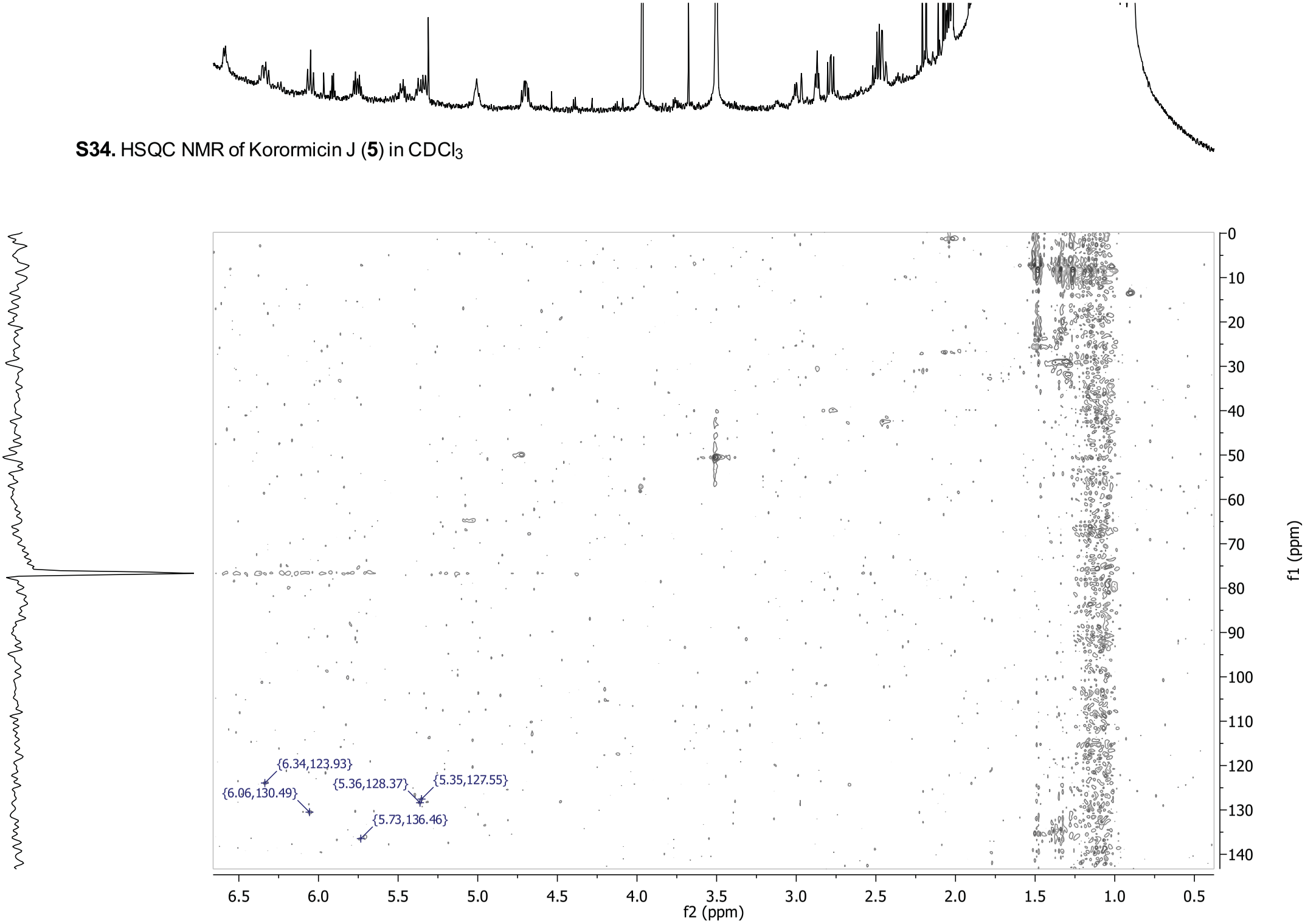
**S32.** $^{1}$H NMR of Korormicin J (**5**) in $CDCl_3$



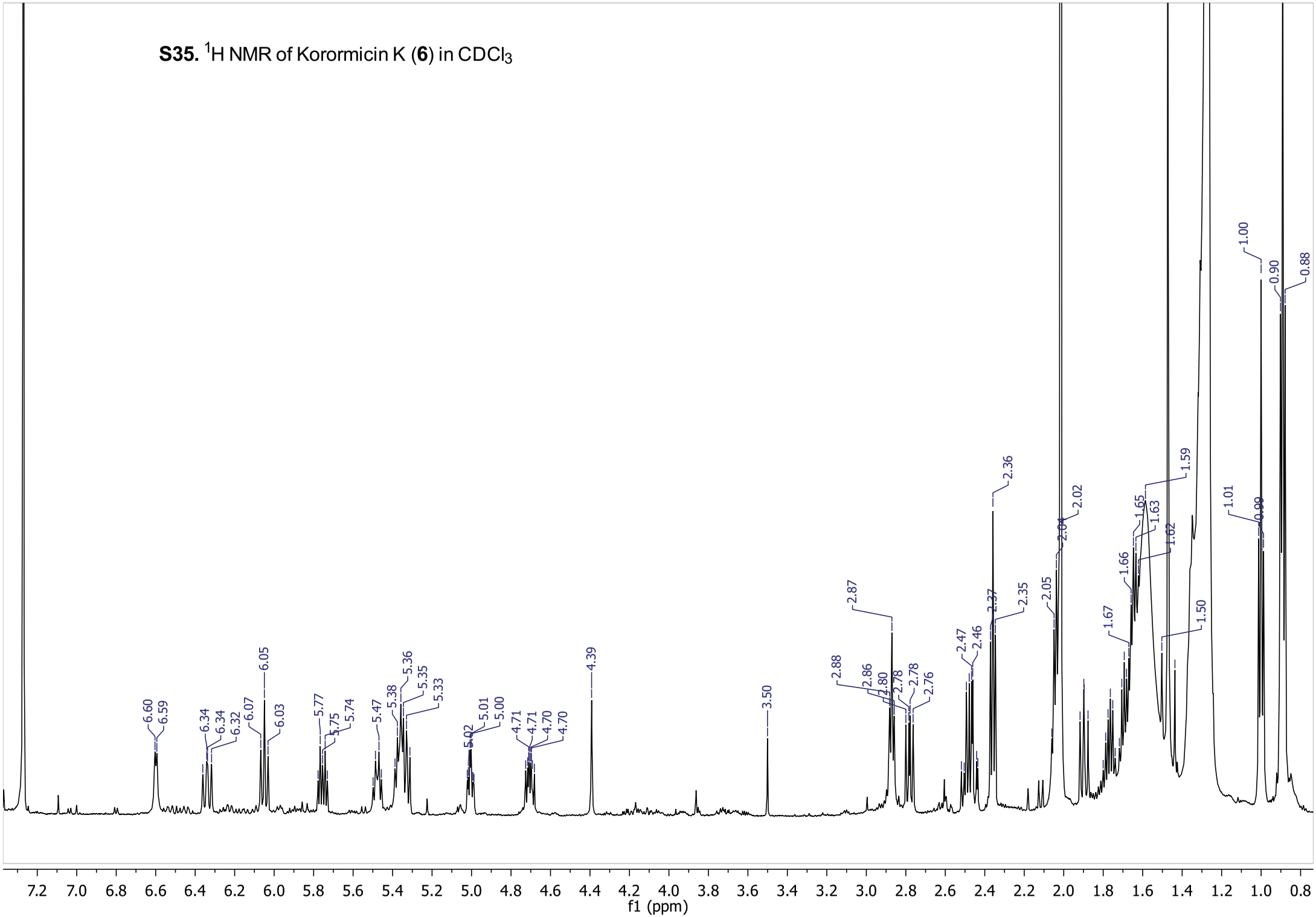
**S33.** COSY NMR of Korormicin J (**5**) in $CDCl_3$



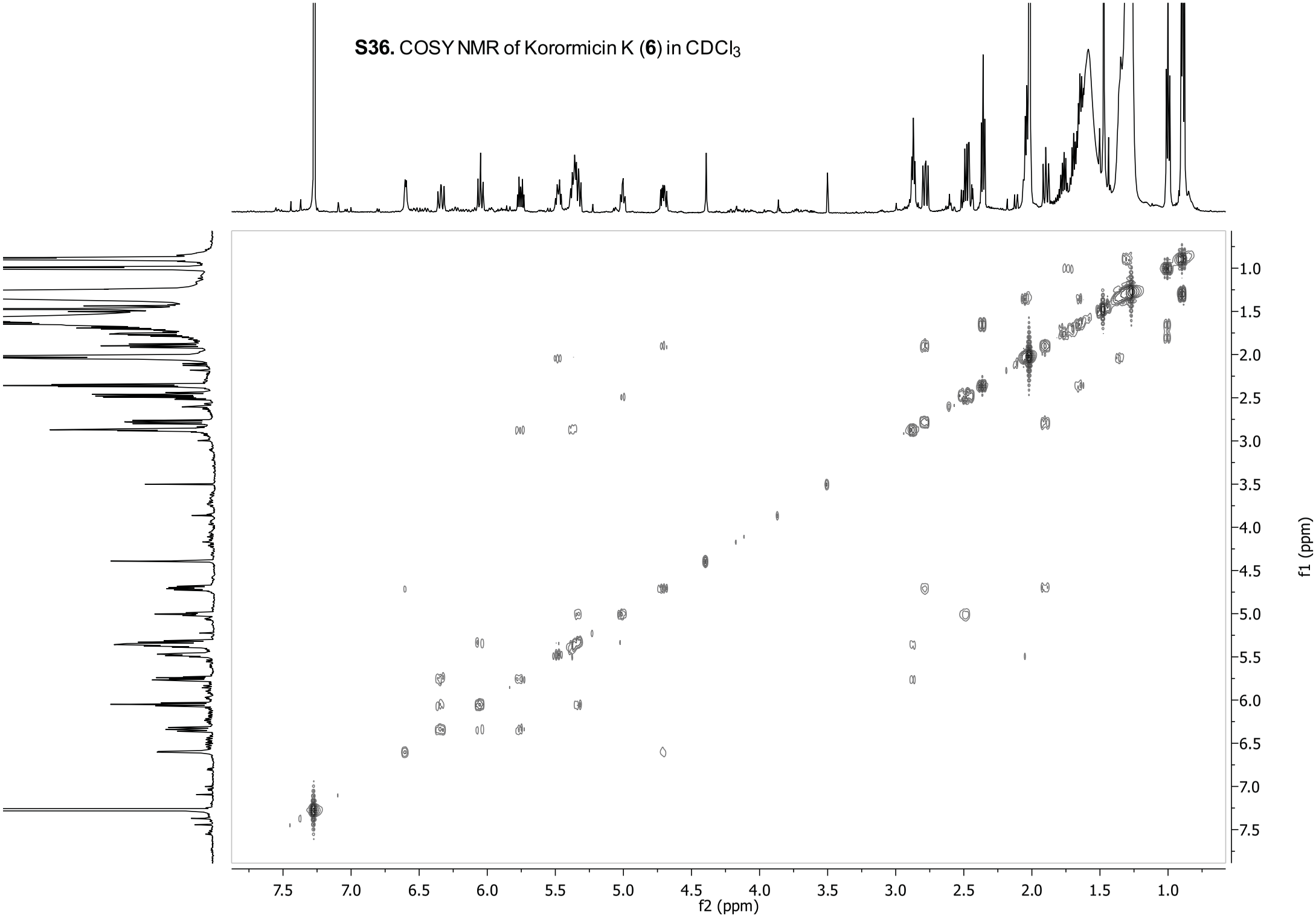
**S34.** HSQC NMR of Korormicin J (**5**) in $CDCl_3$



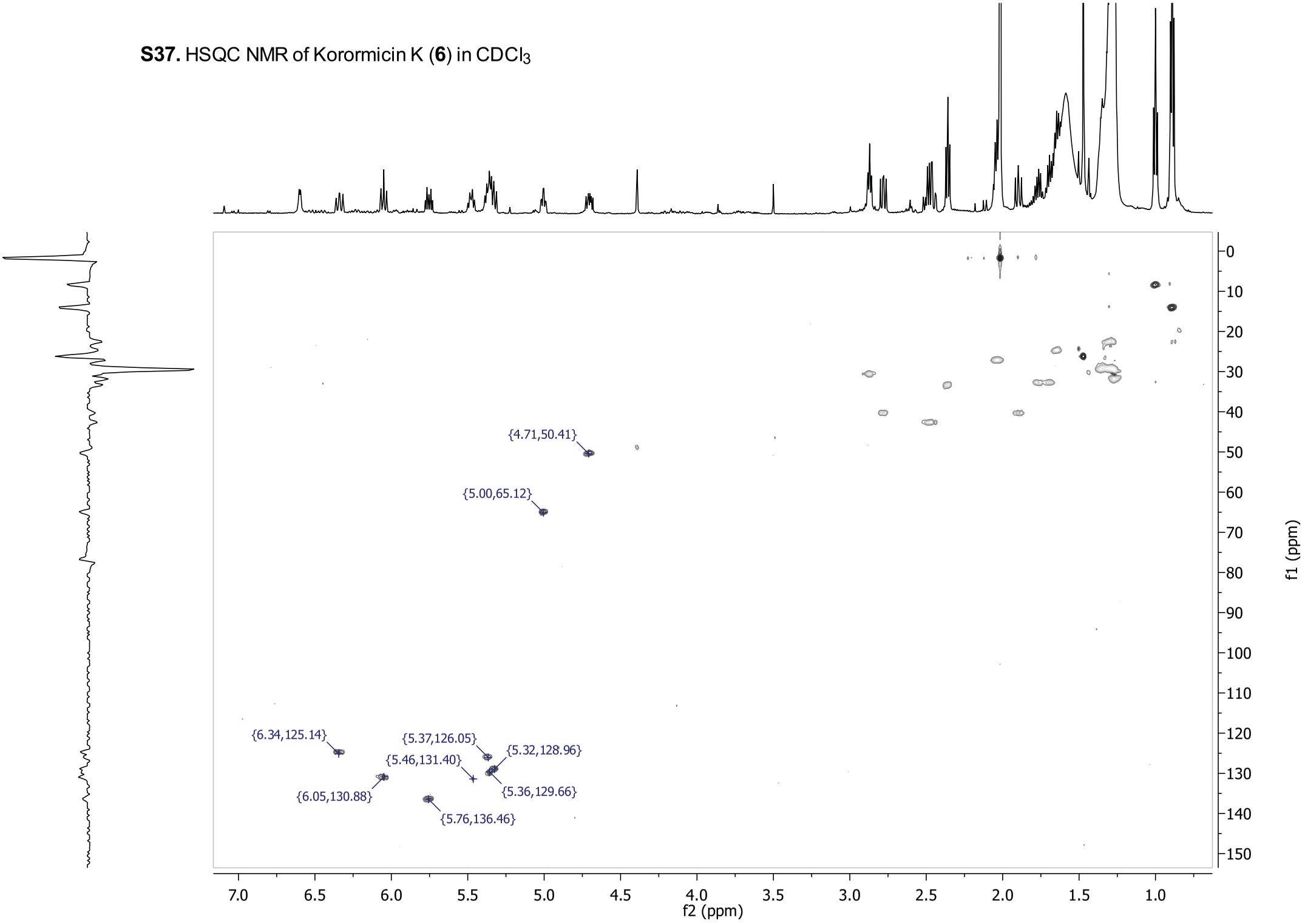
**S35.** $^{1}H$ NMR of Korormicin K (**6**) in $CDCl_3$



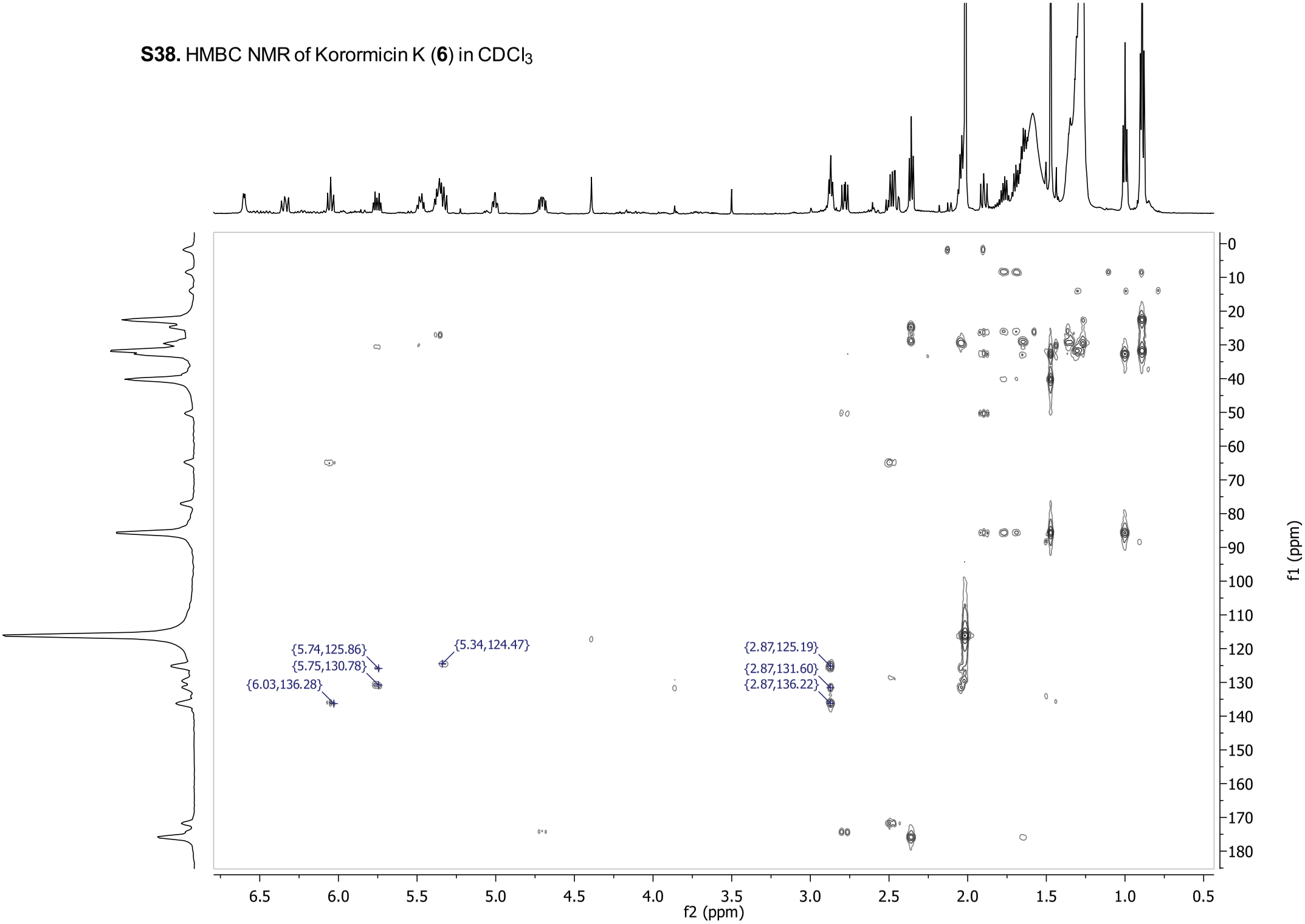
**S36.** COSY NMR of Korormicin K (**6**) in $CDCl_3$

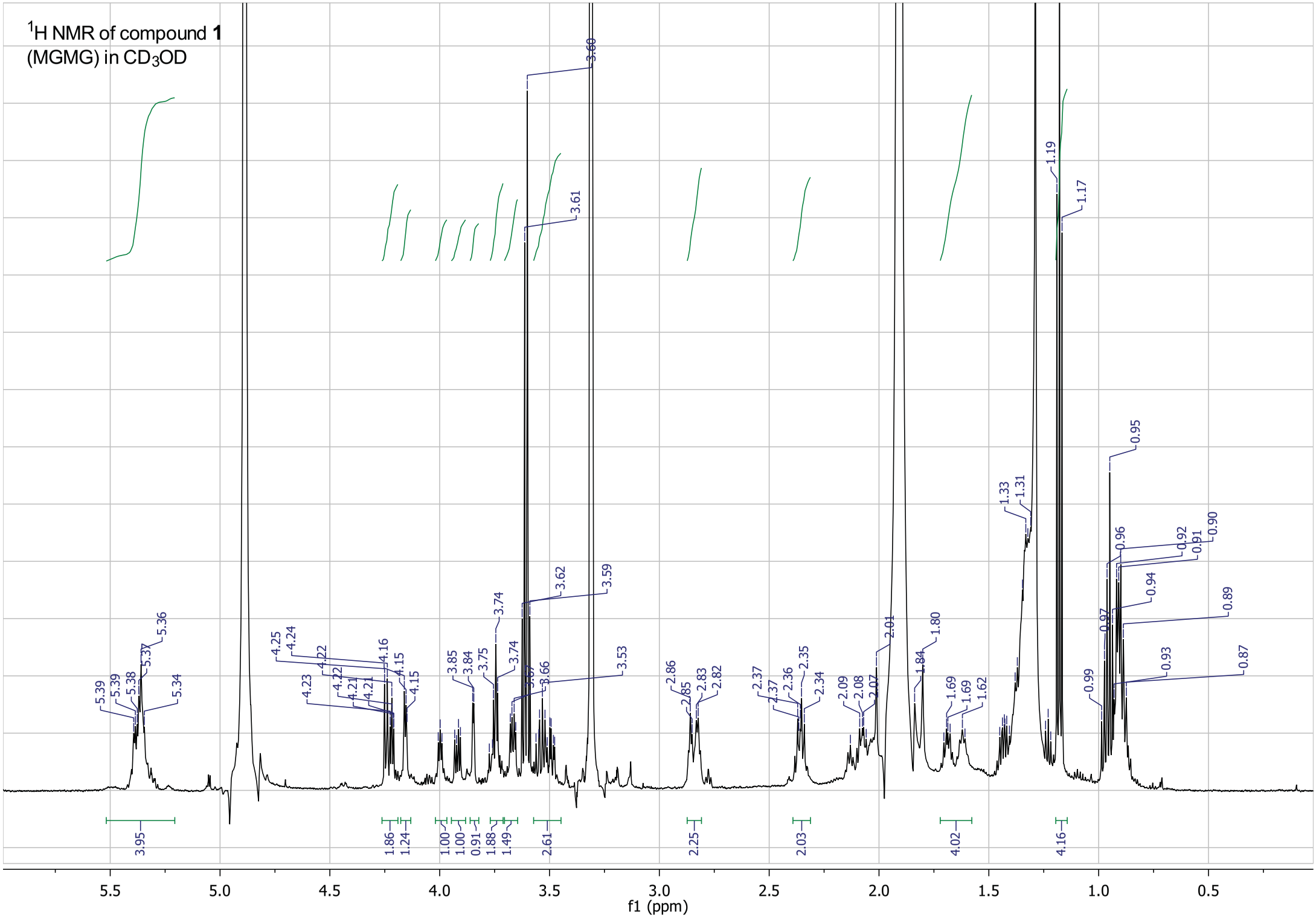


**S37.** HSQC NMR of Korormicin K (**6**) in $CDCl_3$

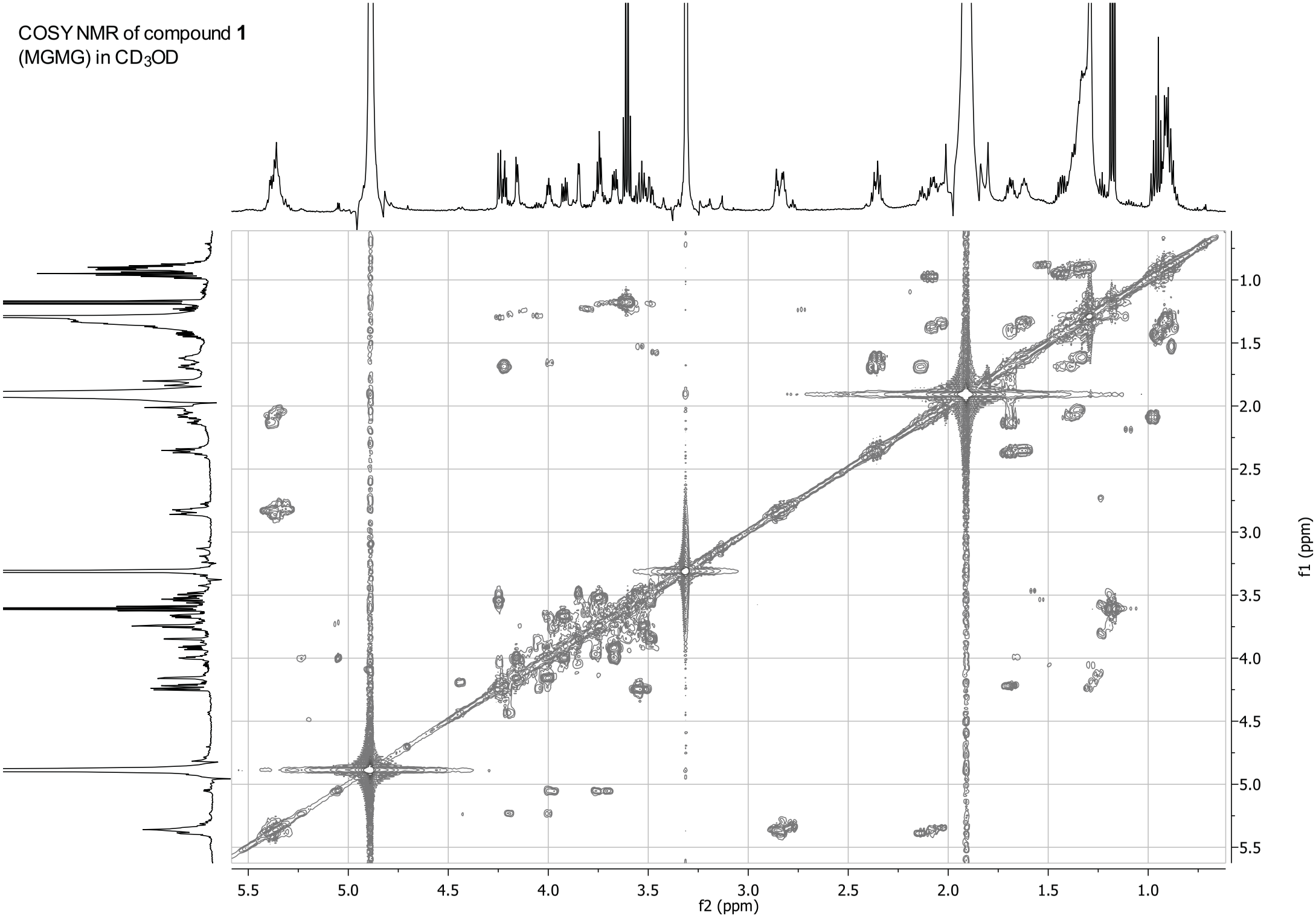


**S38.** HMBC NMR of Korormicin K (**6**) in $CDCl_3$

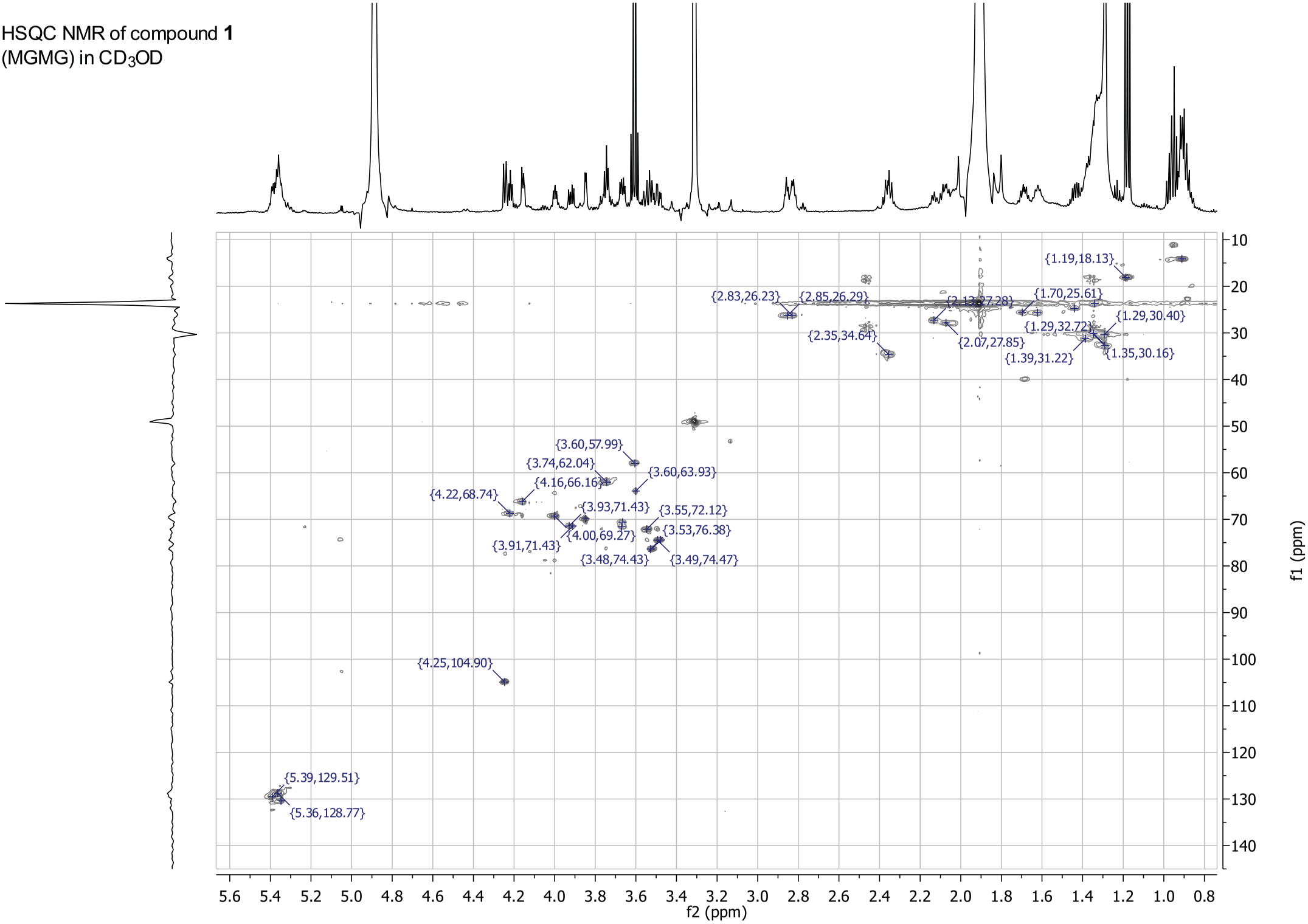



$^1$H NMR of compound **1**
(MGMG) in $CD_3OD$
f1 (ppm)

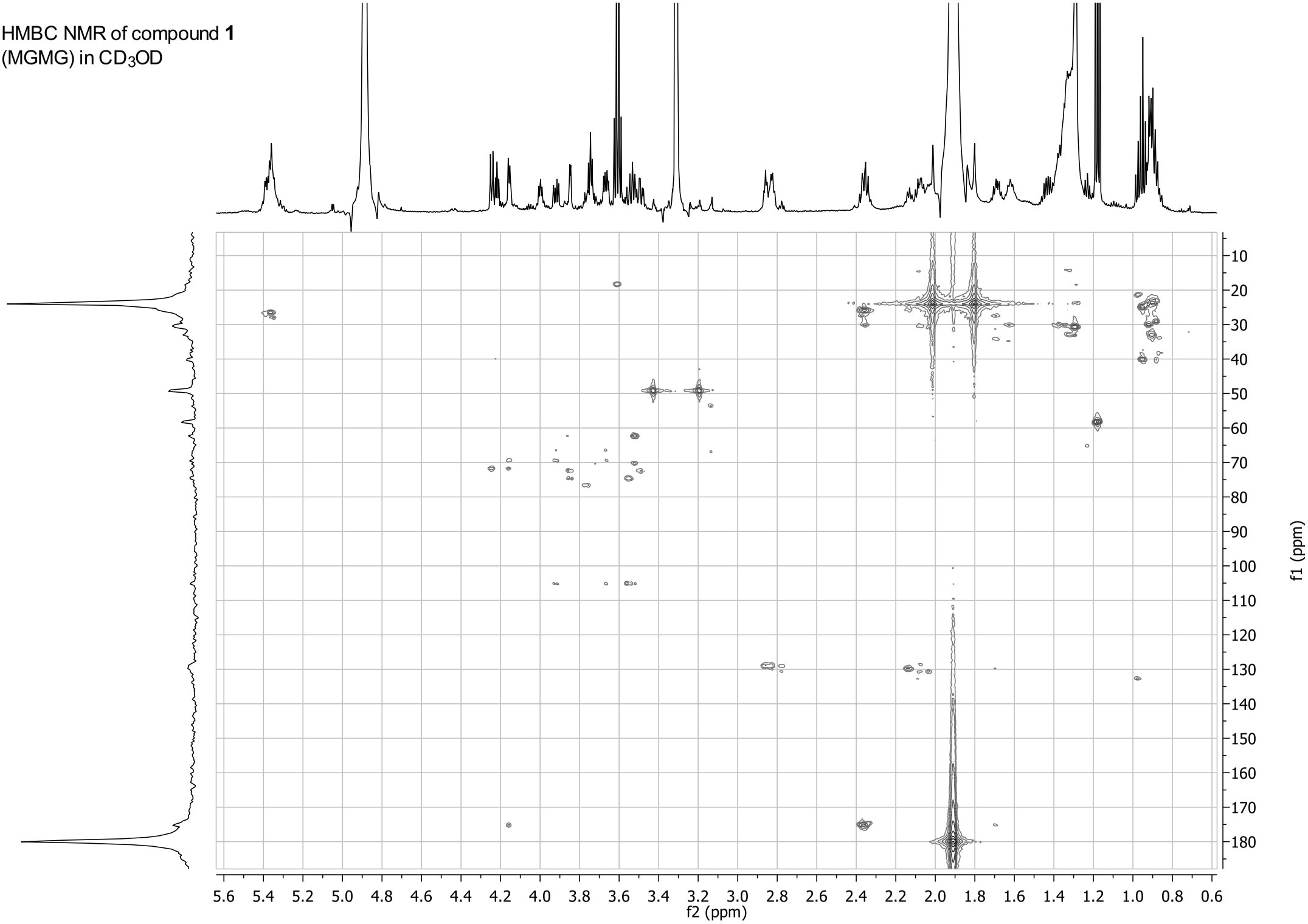
COSY NMR of compound **1** (MGMG) in $CD_3OD$

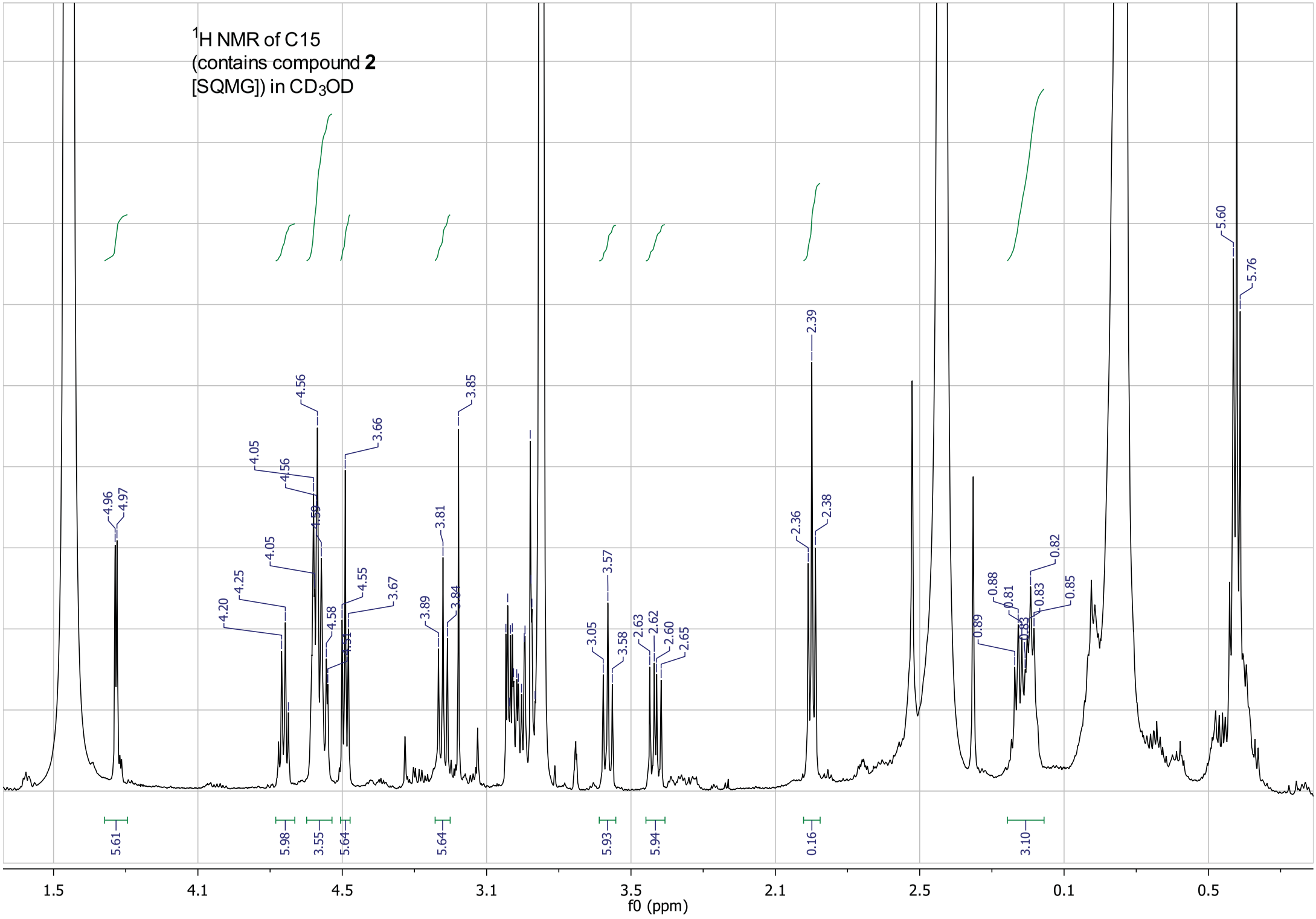


HSQC NMR of compound **1** (MGMG) in $CD_3OD$

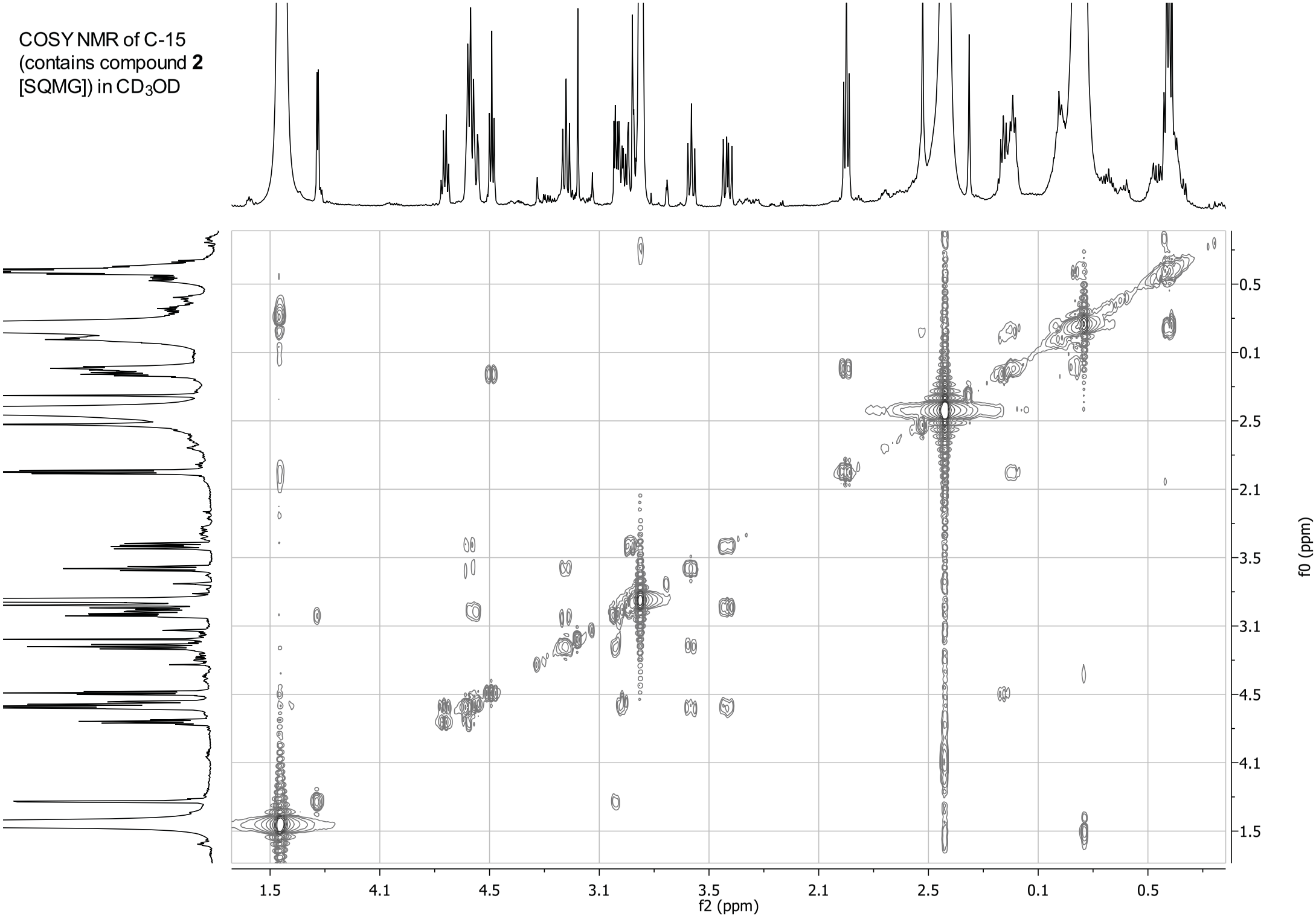


HMBC NMR of compound **1** (MGMG) in $CD_3OD$

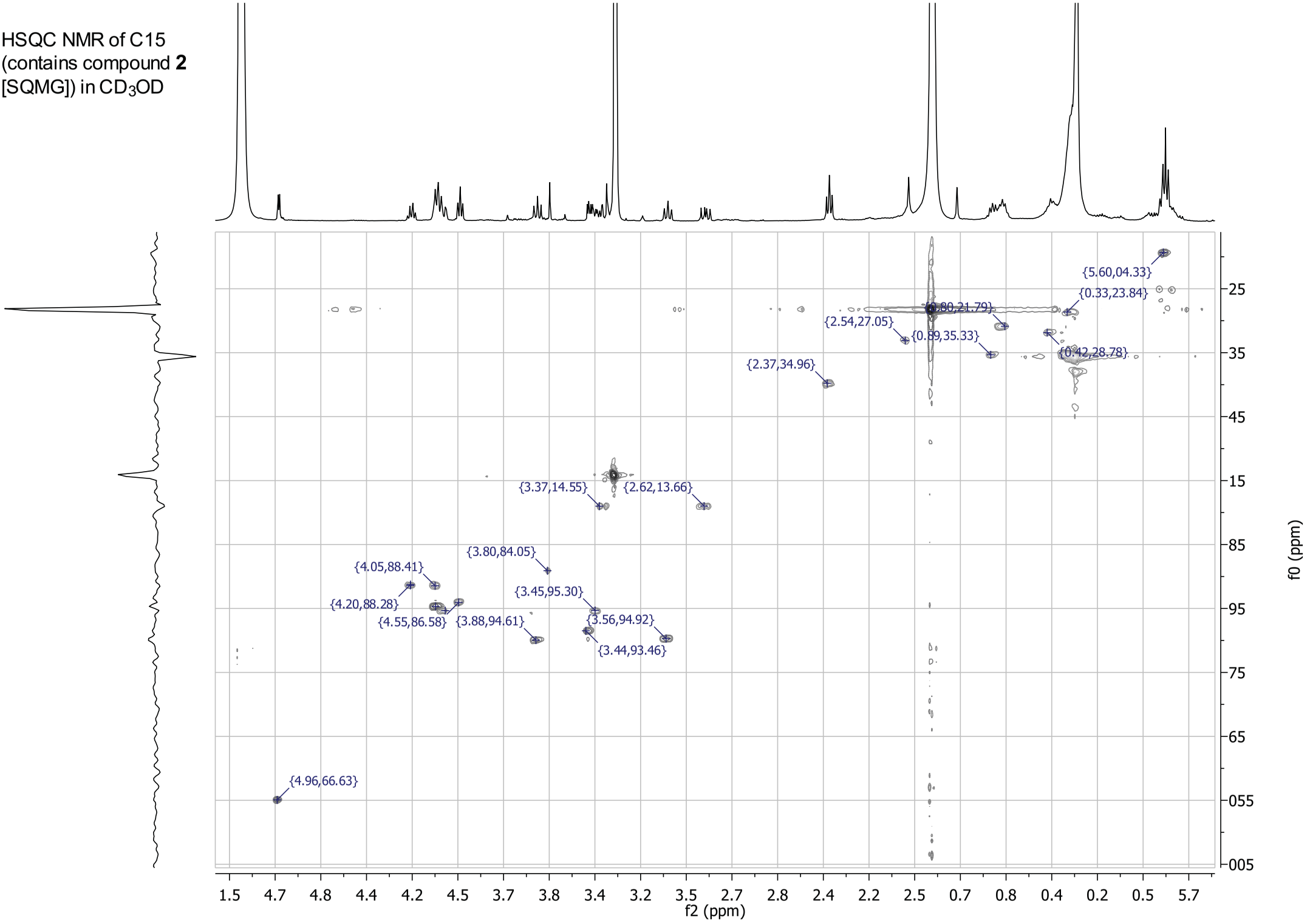



$^1$H NMR of C15
(contains compound **2**
[SQMG]) in $CD_3OD$
5.60
5.76
2.39
4.56
3.85
4.05
3.66
4.96
4.97
4.56
4.05
4.59
3.81
2.36
2.38
4.25
4.20
4.05
4.55
3.67
4.58
3.89
3.84
3.57
3.05
3.58
2.63
2.62
2.60
2.65
0.82
0.88
0.81
0.83
0.85
0.89
5.61
5.98
3.55
5.64
5.64
5.93
5.94
0.16
3.10
1.5
4.1
4.5
3.1
3.5
2.1
2.5
0.1
0.5
f0 (ppm)

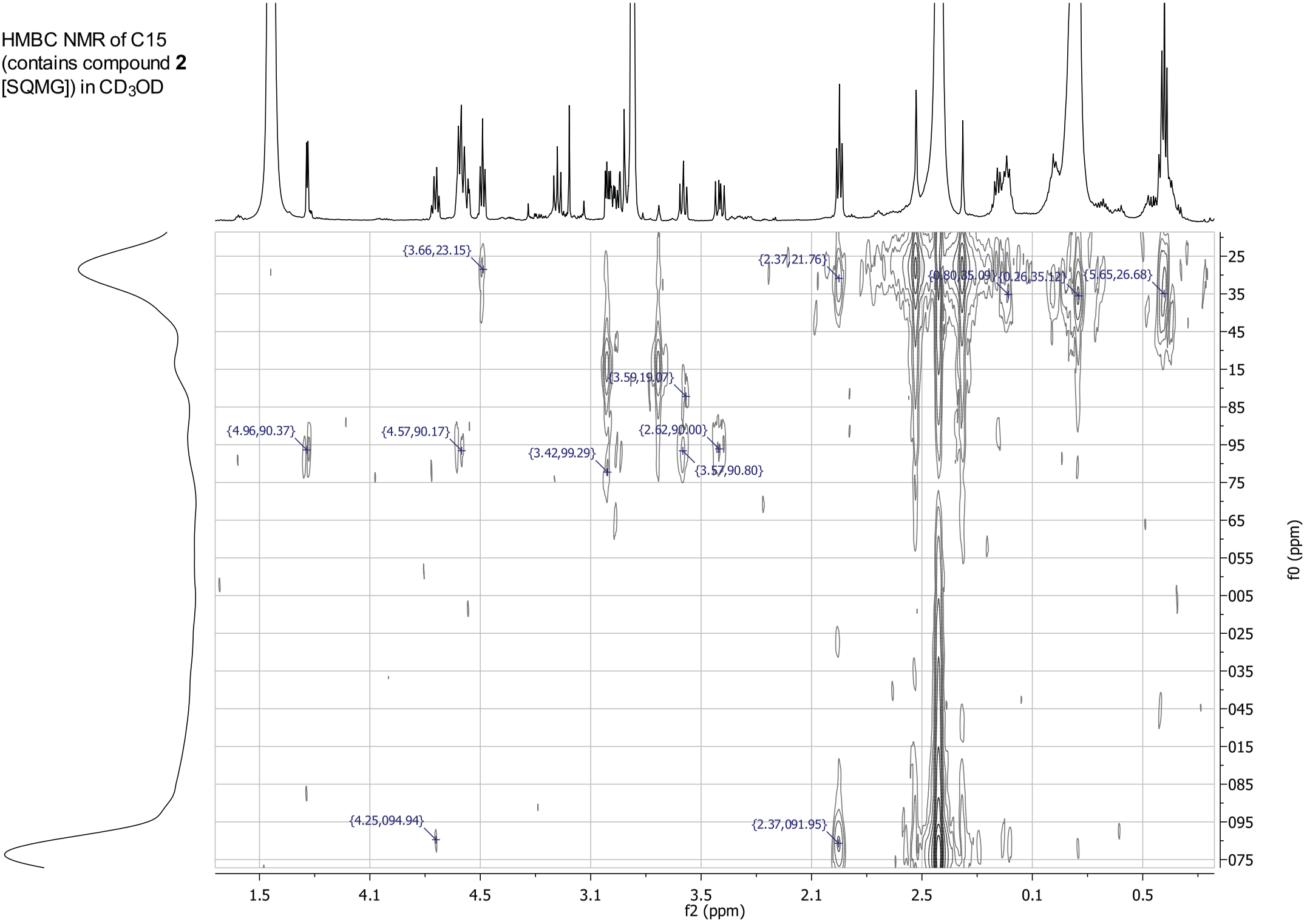
COSY NMR of C-15 (contains compound **2** [SQMG]) in $CD_3OD$

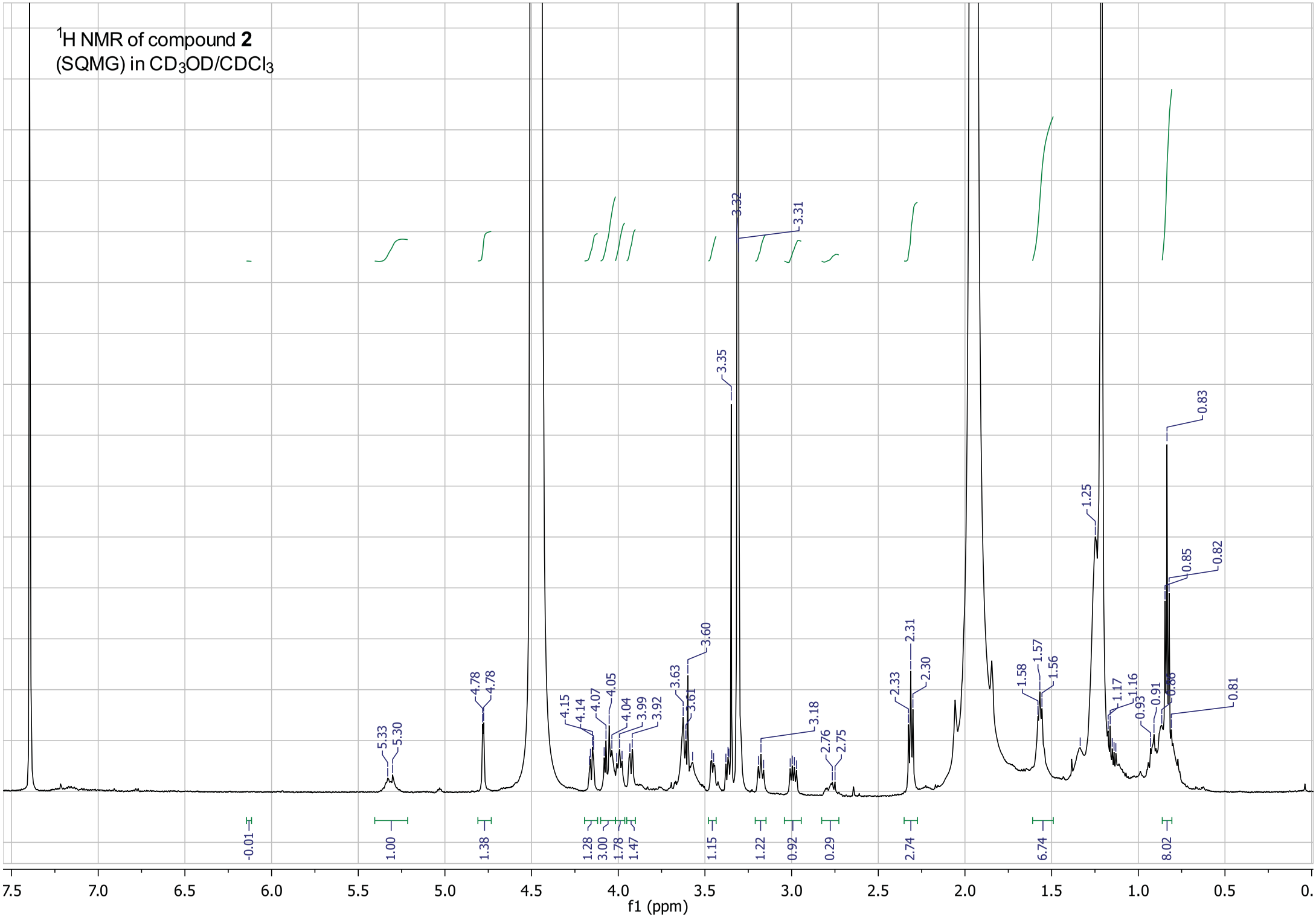


HSQC NMR of C15 (contains compound **2** [SQMG]) in $CD_3OD$

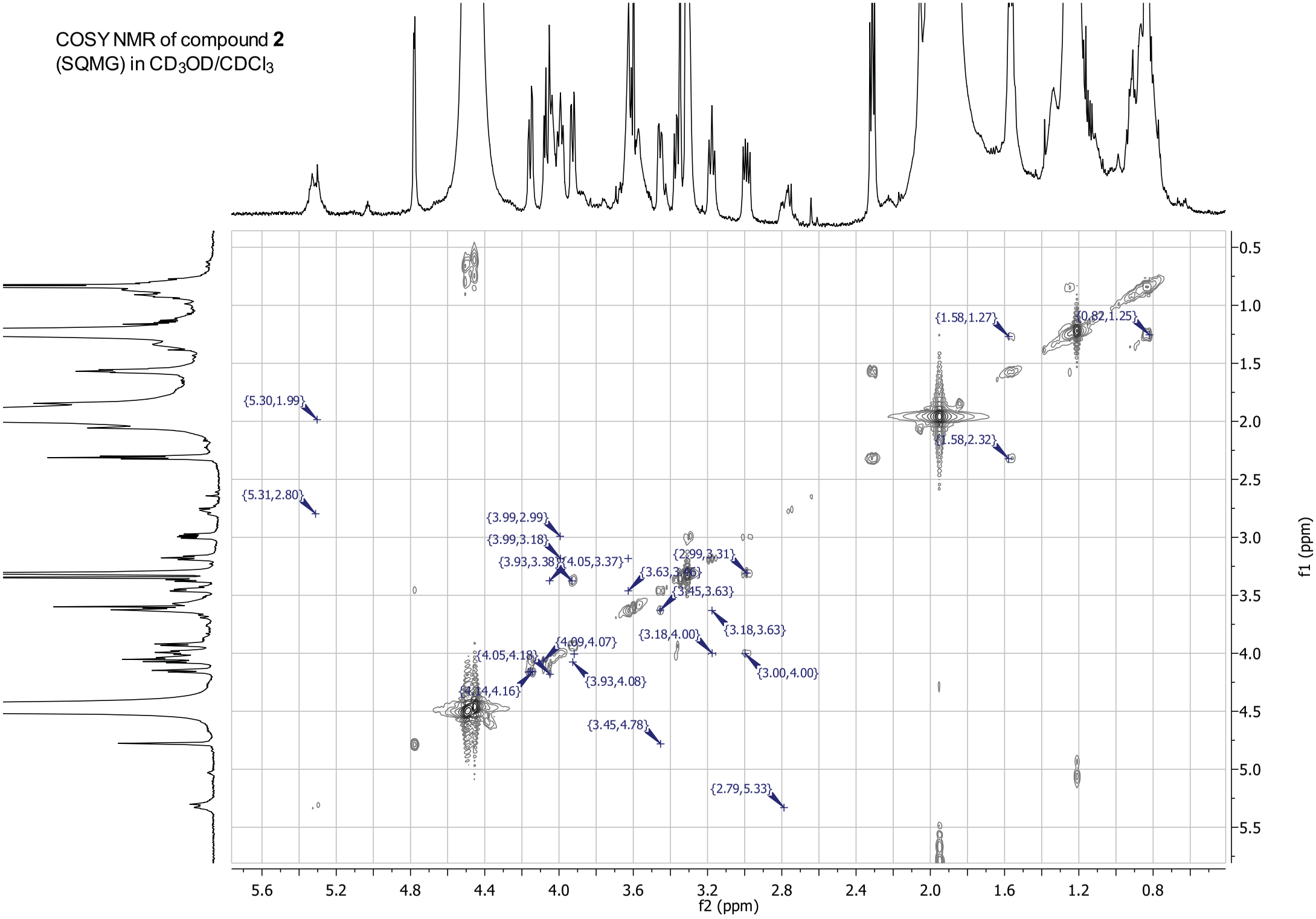


HMBC NMR of C15
(contains compound **2**
[SQMG]) in $CD_3OD$

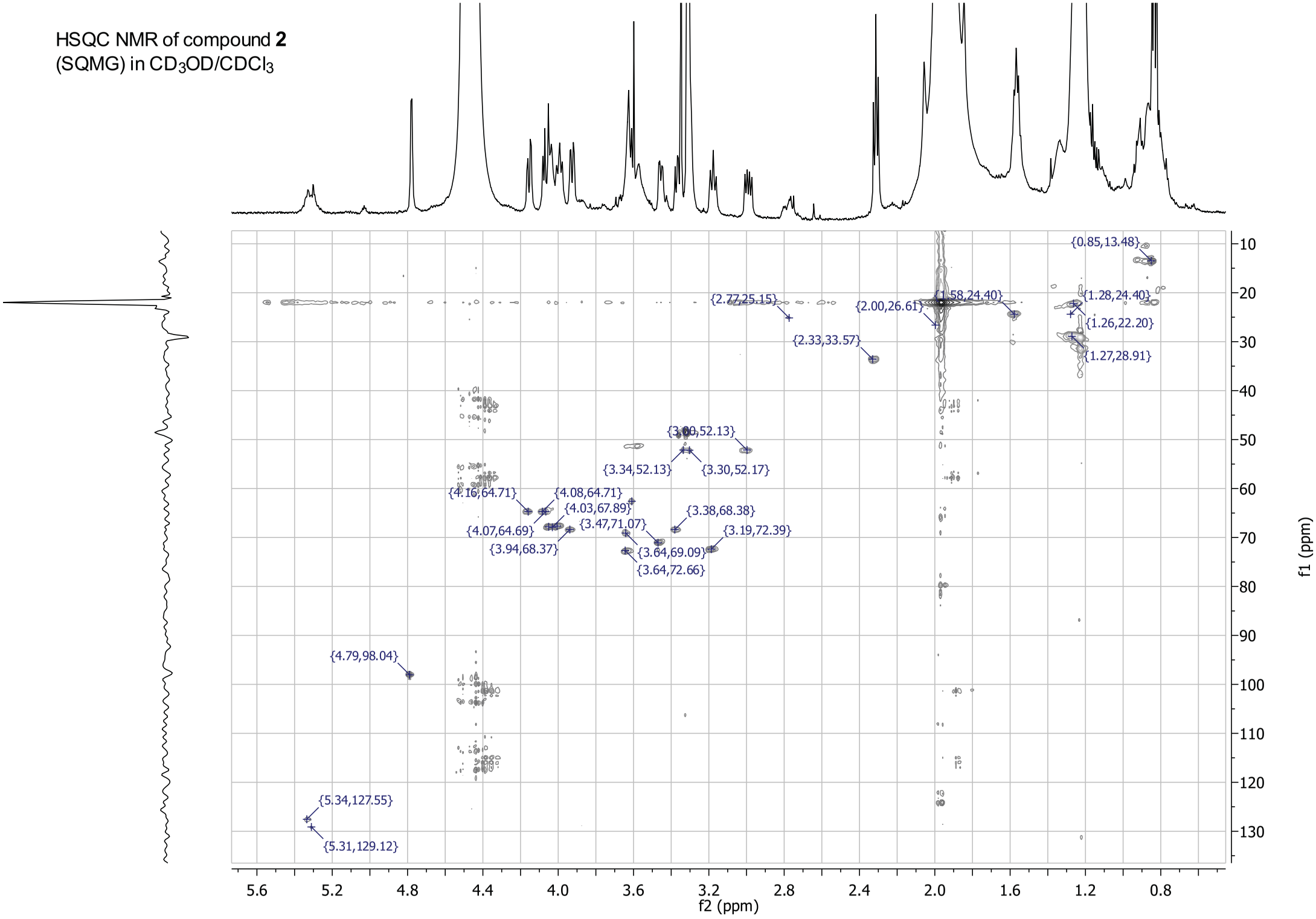


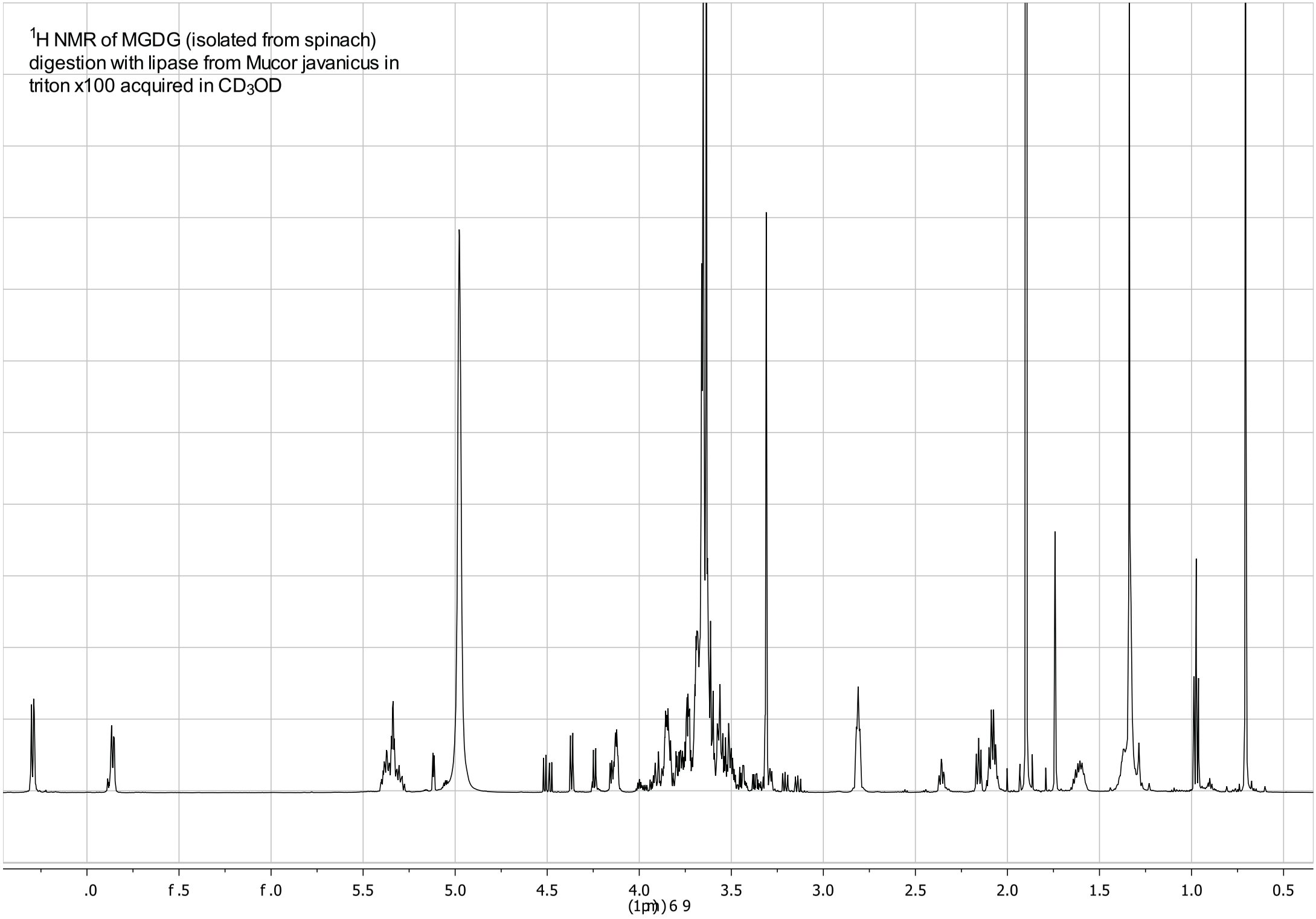

$^1$H NMR of compound **2**
(SQMG) in $CD_3OD/CDCl_3$
3.32
3.31
3.35
0.83
1.25
0.85
0.82
3.60
2.31
4.78
4.78
4.15
4.14
4.07
4.05
4.04
3.99
3.92
3.63
3.61
3.18
2.76
2.75
2.33
2.30
1.58
1.57
1.56
1.17
1.16
0.93
0.91
0.86
0.81
5.33
5.30
-0.01
1.00
1.38
1.28
3.00
1.78
1.47
1.15
1.22
0.92
0.29
2.74
6.74
8.02
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.
f1 (ppm)

COSY NMR of compound **2** (SQMG) in $CD_3OD/CDCl_3$

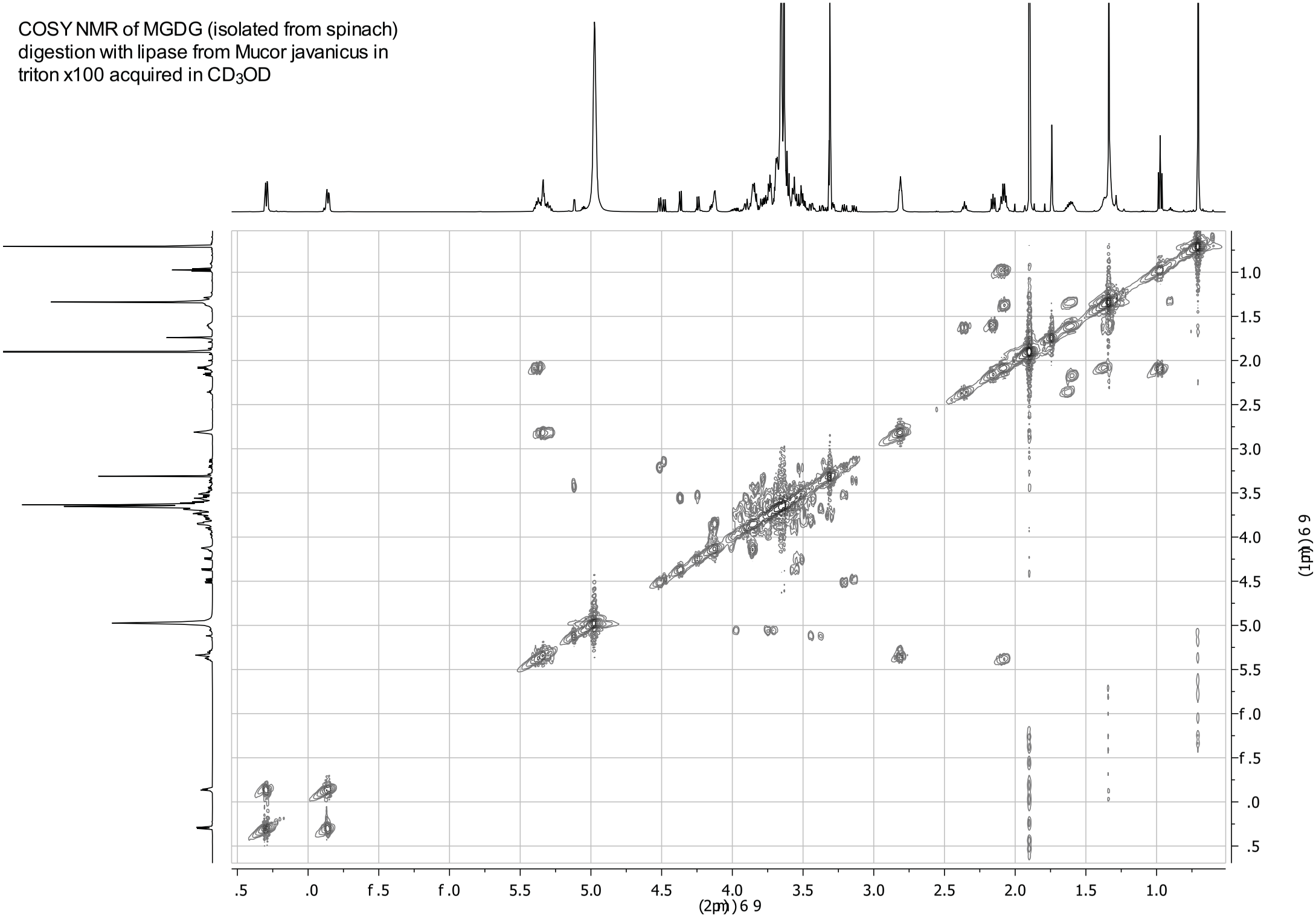


HSQC NMR of compound **2** (SQMG) in $CD_3OD/CDCl_3$

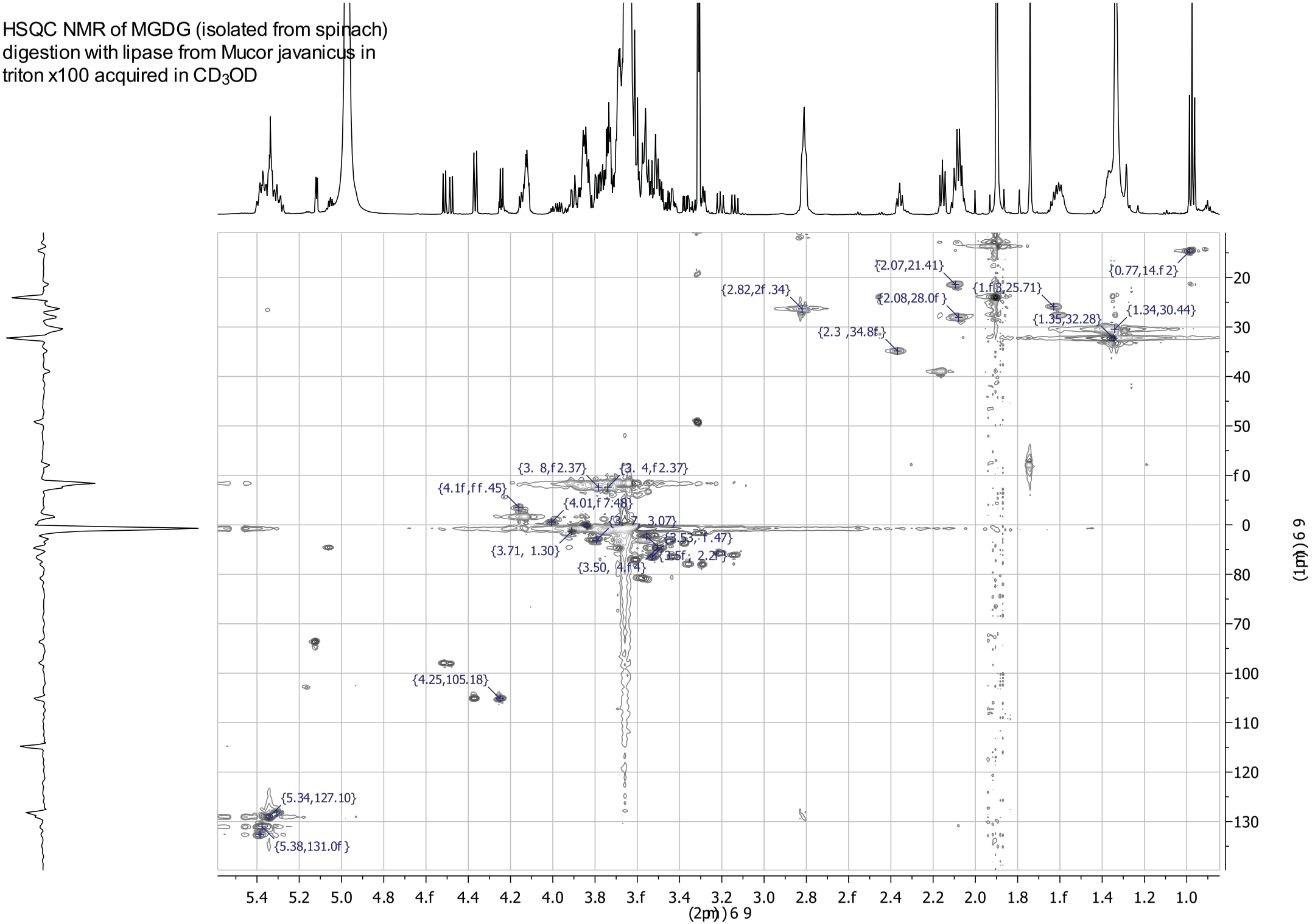



$^1$H NMR of MGDG (isolated from spinach) digestion with lipase from Mucor javanicus in triton x100 acquired in $CD_3OD$
.0
f .5
f .0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
(1pm) 6 9

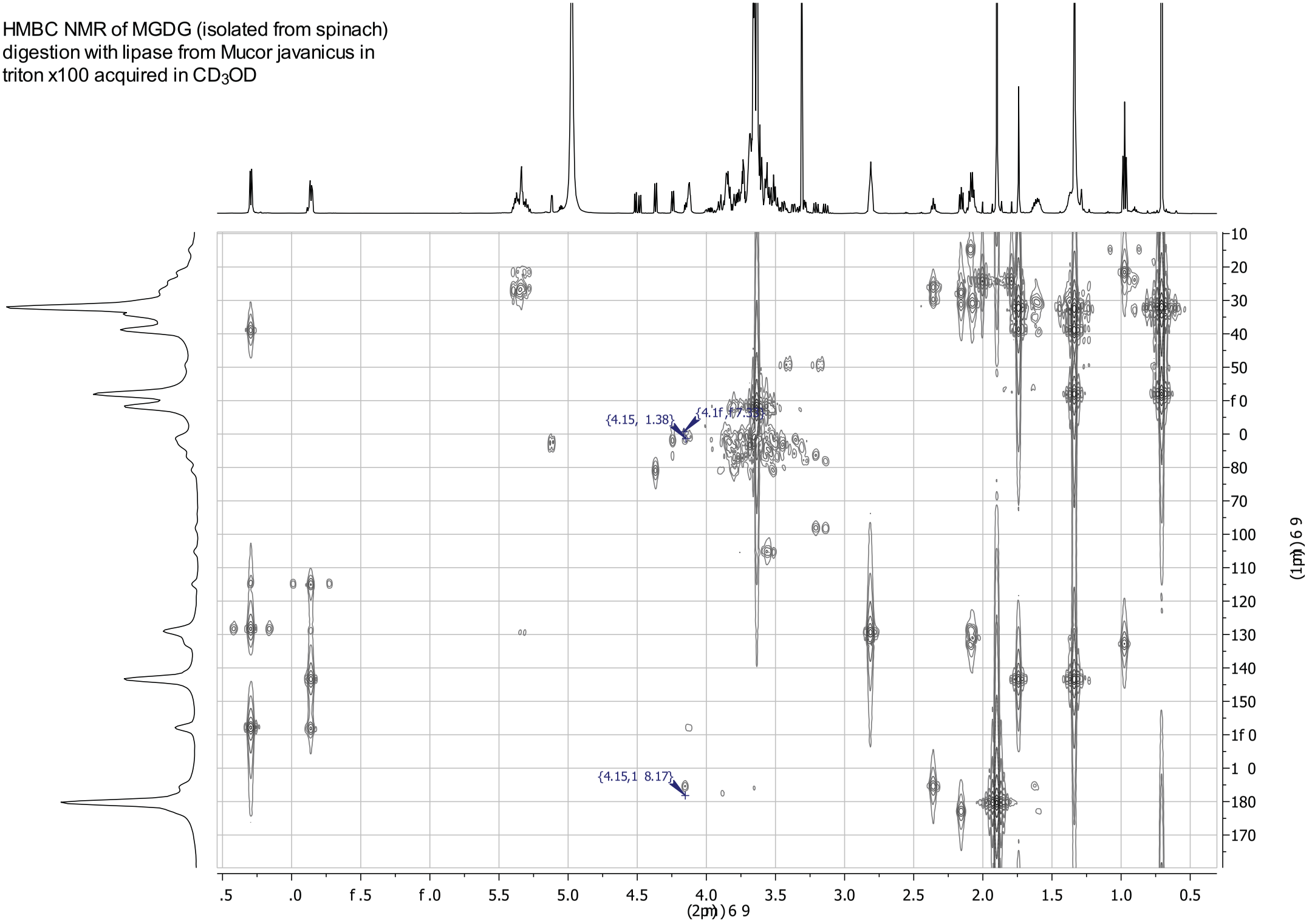
COSY NMR of MGDG (isolated from spinach) digestion with lipase from Mucor javanicus in triton x100 acquired in $CD_3OD$

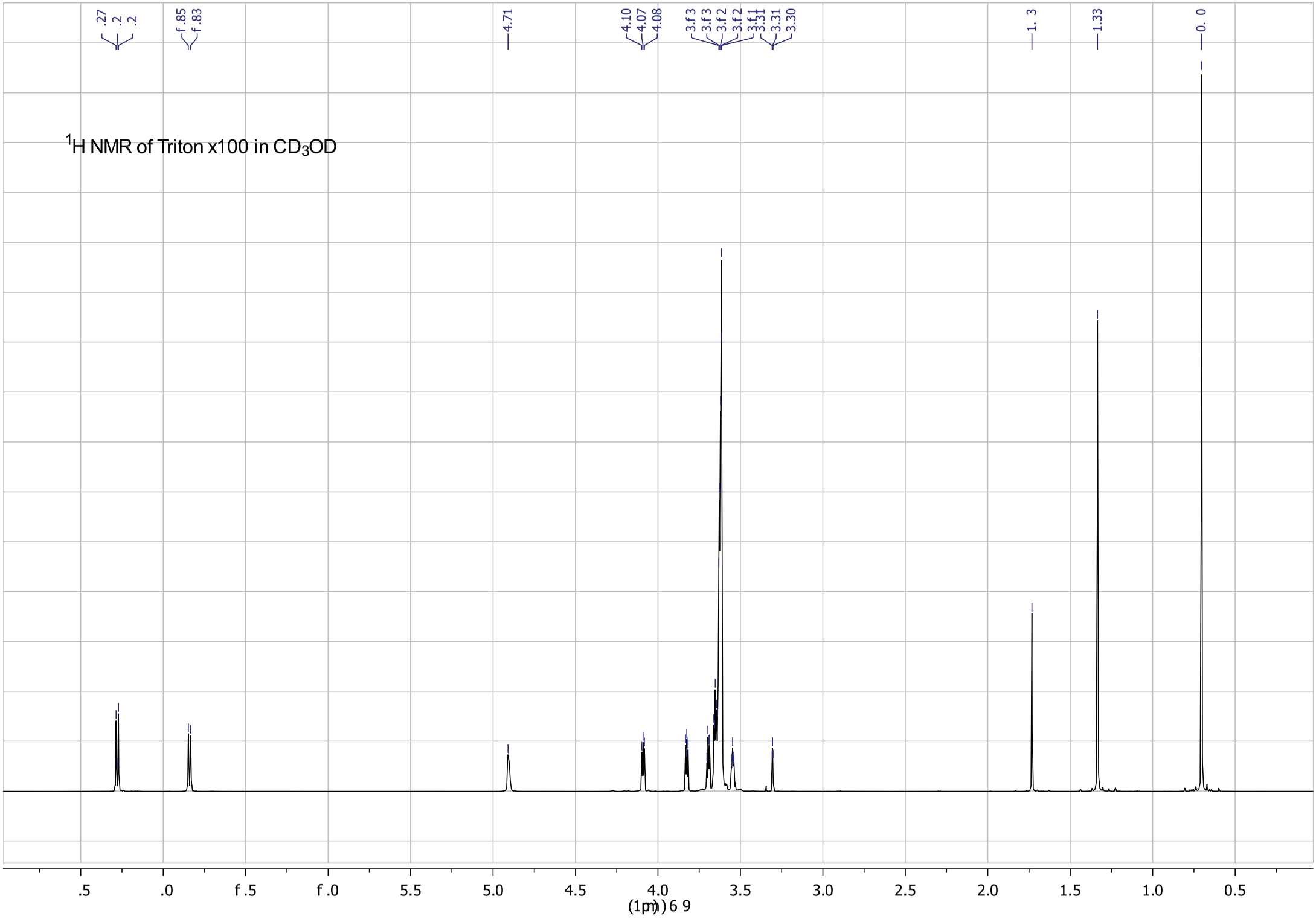


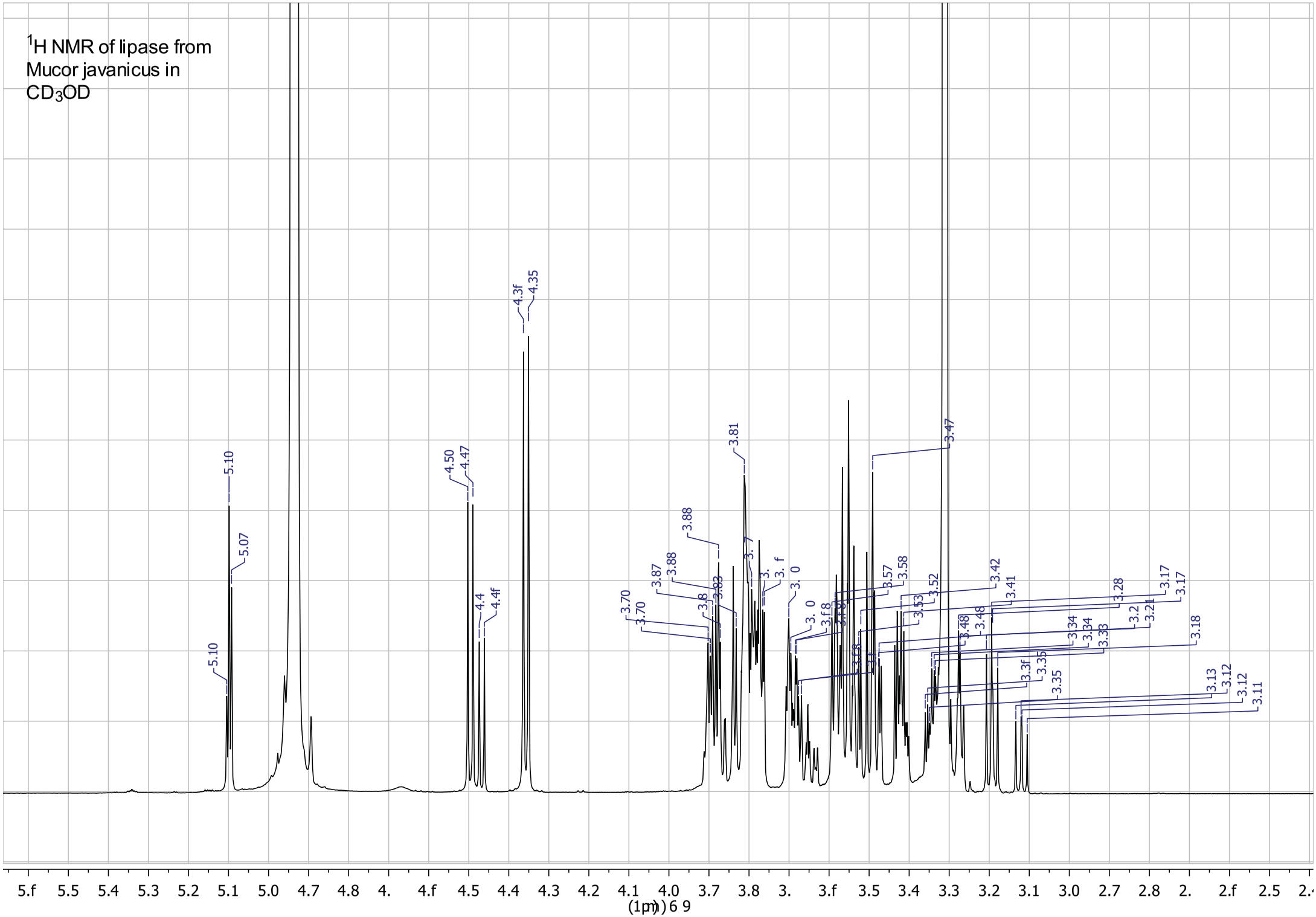
HSQC NMR of MGDG (isolated from spinach) digestion with lipase from Mucor javanicus in triton x100 acquired in $CD_3OD$

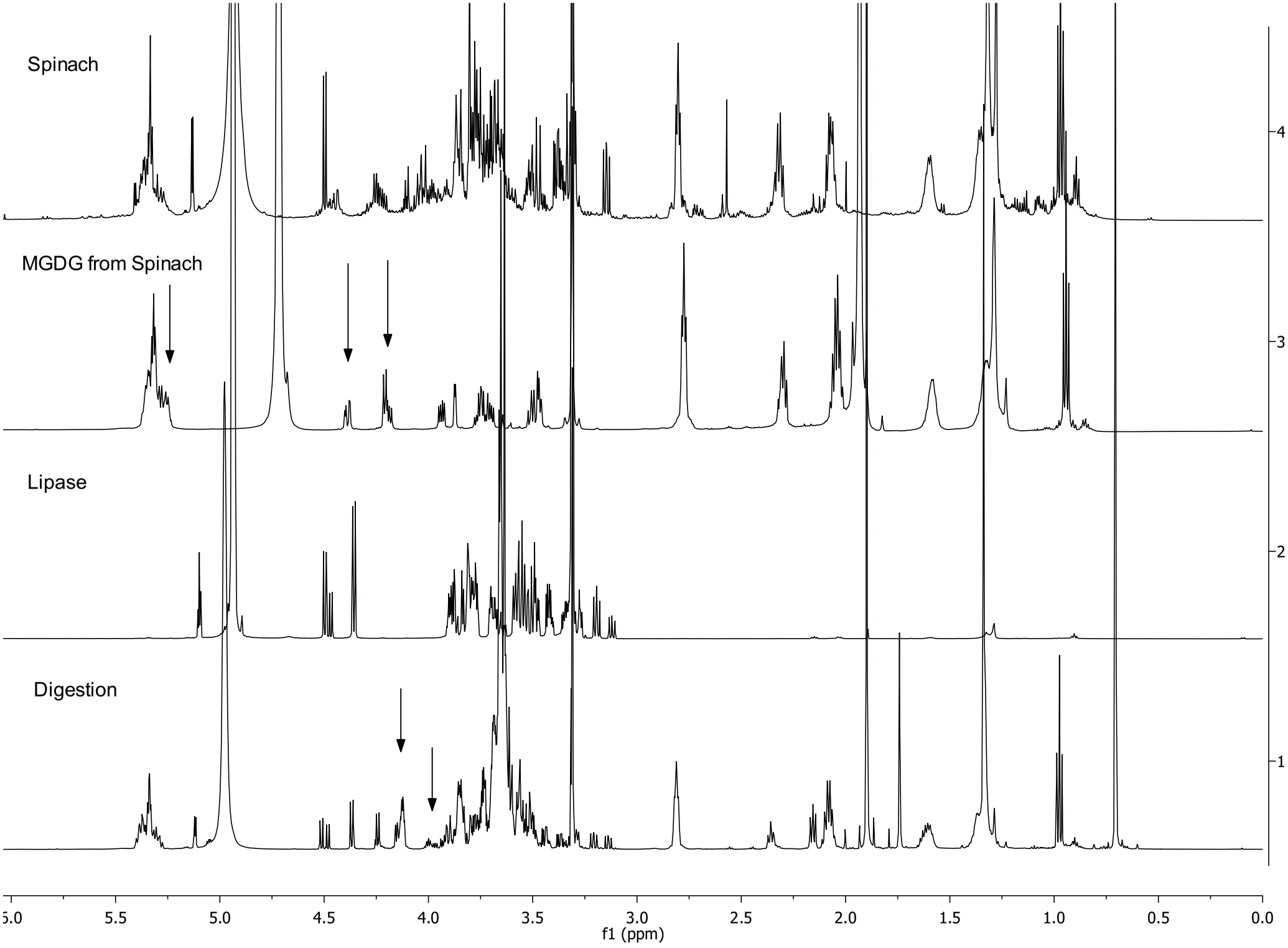


HMBC NMR of MGDG (isolated from spinach) digestion with lipase from Mucor javanicus in triton x100 acquired in $CD_3OD$




.27
.2
.2
f.85
f.83
4.71
4.10
4.07
4.08
3.f3
3.f3
3.f2
3.f2
3.31
3.31
3.30
1. 3
1.33
0. 0
$^1$H NMR of Triton x100 in $CD_3OD$
.5
.0
f.5
f.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
(1pm)


$^1$H NMR of lipase from Mucor javanicus in $CD_3OD$
5.10
5.07
4.50
4.47
4.3f
4.35
3.88
3.81
3.47
3.42
3.41
3.28
3.17
3.18
5.f 5.5 5.4 5.3 5.2 5.1 5.0 4.7 4.8 4. 4.f 4.5 4.4 4.3 4.2 4.1 4.0 3.7 3.8 3. 3.f 3.5 3.4 3.3 3.2 3.1 3.0 2.7 2.8 2. 2.f 2.5
(1pm)


Spinach
MGDG from Spinach
Lipase
Digestion
4
3
2
1
5.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)