UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ACCESSING NATURAL PRODUCTS FROM SILENT BIOSYNTHETIC PATHWAYS USING CHEMICAL EPIGENETCS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

DOCTOR OF PHILOSOPHY

By

JON C. HENRIKSON

Norman, Oklahoma

2011

Downloaded from SHAREOK. Persistent Handle URL: https://hdl.handle.net/11244/318924

ACCESSING NATURAL PRODUCTS FROM SILENT BIOSYNTHETIC PATHWAYS USING CHEMICAL EPIGENETCS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

BY

Dr. Robert Cichewicz, Chair

Dr. C. Leroy Blank

Dr. Susan Schroeder

Dr. Bradley Stevenson

Dr. Robert White

© Copyright by JON C. HENRIKSON 2011
All Rights Reserved.

Acknowledgements

This is not just a culmination of the last 5+ years, but a culmination of all the experiences which led to this moment. First, I am grateful to my family, who has always held unwavering support and love even when I was uncertain of myself. To my mother, who showed me unconditional love, patience, and strength even in times of adversity. To my father, who has always demonstrated a fighting spirit, even in a tough battle against cancer for over 25 years. I would also like to thank my brother, sister, and their families for all the encouragement, support, and steadfast belief in me. I am appreciative to Kay and Craig Shandler for treating me as one of their own family members and allowing me the opportunity to come back to work for them while finishing up my bachelor's degree then letting me move forward into this part of my life.

I would like to thank Dr. Cichewicz for allowing me to become a member of his researcher group, and am very grateful for his kindness, patience, and support he has shown during my studies. I greatly appreciate Dr. Hambright for a semester of RA support and allowing me to have an opportunity to work on a unique project with his group. I would like to thank Dr. Schroeder for the opportunity to discuss various NMR experiments early on in my graduate career. I would like to thank Dr. Blank for giving me a sounding board to process and talk things out. In addition, I want express my gratitude to Drs. White and Stevenson, along with previously mentioned professors for all their feedback while being a part of my graduate committee. I am also grateful for all the teachers who have added to my learning throughout the years, and especially want to thank Ms. Mary Cravens (Butcher Elementary) for creating a great environment to learn during my early years of school, Mr. Clarence Howland (Emporia High School) for

sparking an interest in chemistry, and Dr. James Roach (Emporia State University) for convincing me that I had the abilities to take this journey.

I would like to thank the University Of Oklahoma, Department Of Chemistry and Biochemistry, the Department of Education Graduate Assistance in Areas of National Need (GAANN) program, the National Institutes of Health, and the Oklahoma Department of Wildlife Conservation for financial support during my graduate career.

Finally, I would like to thank Drs. Trevor Ellis and Russell Williams for taking the time to teach me while working in the lab as postdocs, as well as their friendships. Also, thanks to Dr. Ellis for his patience as I took a short detour into the realm of synthesis towards the end of my projects. I am grateful to all my friends and colleagues, new and old, for their support and friendship, including Keith, Danny, Matt, Xiaoru, Will, Shawna, Ashley, Katie, Christine, Majed, Rich, Emily, Dr. Easton, Karen, Anne, Jarrod, Mike, Jeff, Adam, Garry, Alexandra, Hannah, and many others.

Table of Contents

Acknowledgements iv

List of Tables vii

List of Figures ix

List of Schemes x

Abstract xi

Chapter 1 Oveview of Natural Products and Methods to Access Silent Biosynthetic Pathways 1

1.1 Natural Products and the Pharmaceutical Industry 1

1.2 Analysis of Fungal Genomes 2

1.3 Mainpulation of Culture Conditions 3

1.4 Heterologous expression as an Approach to Investigating SBPs 10

1.5 Conclusions and Perspectives on Methods for Accessing SBPs 12

Chapter 2 Hypothesis and Chapter Overviews 13

2.1 Hypothesis 13

2.2 Chapter 3: Epigenetic Remodeling of the Fungal Secondary Metabolome 14

2.3 Chapter 4: Chemical Induction of Silent Biosynthetic Pathway Transcription in *Aspergillus niger* 14

2.4 Chapter 5: Analysis of a Class of γ-pyrones and γ-pyridones Isolated from *Aspergillus niger* 15

2.5 Chapter 6: Reassessing the Ichthyotoxin Profile of Cultured Prymnesium parvum (Golden Algae) and Comparing it to Samples Collected from Recent Freshwater Bloom and Fish Kill Events in North America. 16

Chapter 3 Epigenetic remodeling of the fungal secondary metabolome. 17

3.1 Introduction 17

3.2 Results and Discussions 19

3.3 Materials and Methods 23

3.3.1 General Chemical Methods 23

3.3.2 Fungal Test Strains 23

3.3.3 Small Molecule Epigenetic Modifiers 24

3.3.4 Screening Epigenetic Modifers 24

3.3.5 Isolation and Identification of Fungi 25

3.3.6 Fungal Culture Conditions and Initial Screening 27

3.3.7 Culture Comparison and Compound Isolation 28

3.3.8 Lunalide A 29

3.3.9 Lunalide B 29

Chapter 4 Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. 31

4.1 Introduction 31

4.2 Results and Discussions 34

4.3 Materials and Methods 41

4.3.1 Fungal Strain and Growth Condtions 41

4.3.2 Epigenetic modifier screening 42

Chapter 5 Analysis of a class of γ-pyrones and γ-pyridones isolated from *Aspergillus niger* 44

5.1 Introduction 44

5.2 Results and Discussions 45

5.3 Materials and Methods 61

5.3.1 General chemical methods 61

5.3.2 Fermentation, extraction and isolation of nygerone A 62

5.3.3 Nygerone B 63

5.3.4 Density functional theory calculations (DFT) 63

5.3.5 Synthesis of 3-carbmethoxy-6-benzyl-4-pyrone 63

5.3.6 LC-ESIMS analysis of black aspergilli under chemical epigenetic conditions 65

5.3.7 Isolation and purification of *p*-fluoro-nygerone B 67

5.3.8 Analysis of *Aspergillus niger* cultures for *p*-fluoro-Nygerone B under varying culture conditions 68

5.3.9 3-Carbethoxy-6-phenyl-4-pyrone 68

5.3.10 3-Carbmethoxy-6-phenyl-4-pyrone 68

5.3.11 3-Carboxylic-6-phenyl-4-pyrone 68

5.3.12 Pestalamide A 69

5.3.13 Antibacterial assay 69

5.3.14 Antifungal assay 69

Chapter 6 Reassessing the Ichthyotoxin Profile of Cultured *Prymnesium parvum* (Golden Algae) and Comparing it to Samples Collected from Recent Freshwater Bloom and Fish Kill Events in North America 70

6.1 Introduction 70

6.2 Results and Discussions 72

6.2.1 Bioassay-guided ichthyotoxin extraction and compound Isolation 72

6.2.2 Esterase mediated liberation of ichthyotoxic fatty acids 74

6.2.3 Bioassay analysis of fatty acids 76

6.2.4 GC-EIMS assessment of cultured *P. parvum* lipids and Comparison to water from bloom and fish kill sites 76

6.2.5 Further discussions 78

6.3 Materials and Methods 81

6.3.1 General instrumentation and experimental procedures 81

6.3.2 Biological material 81

6.3.3 Extraction and isolation 82

6.3.4 Enzymatic hydrolysis 83

6.3.5 Alkaline hydrolysis 83

6.3.6 GC-EIMS analysis of fatty acids 84

6.3.7 Ichthyotoxicity assay 84

6.3.8 Mammalian cytotoxicity assay 85

References 86

Appendix 119

List of Table

Table 1.1	Number of PKS, NRPS and HPN gene clusters in selected bacteria and fungi	3
Table 4.1	List of epigenetic modifiers and their effects	36
Table 5.1	^{1}H- and ^{13}C-NMR data for nygerone A	46
Table 5.2	Comparison of synthesized ^{1}H- and ^{13}C-NMR data versus publish NMR values for JBIR-86	55
Table 5.3	Comparison of culture conditions for the presence of *p*-fluoro nygerone B	58
Table 5.4	Inhibitory properities of isolated and synthesized γ-pyrones and γ-pyridones	59
Table 6.1	Determination of cell density, stearidonic acid, and ichthyotoxicity of *Prymnesium parvum* samples	79

List of Figures

Figure 3.1 HPLC difference chromatogram of epigenetic treated culture vs. control and planar structures of lunalides A and B 21

Figure 4.1 HPLC difference chromatogram of SAHA treated culture vs. control culture of *Aspergillus niger* 38

Figure 5.1 Analysis of NMR spectroscopic data for fragments of nygerone A 47

Figure 5.2 Key ^{1}H-^{13}C HMBC correlations and stereochemical determination of nygerone A 49

Figure 5.3 Analysis of JBIR-86 and proposed compounds, mean average error, and key ^{1}H-^{13}C HMBC correlations for synthesized compound 52

Figure 5.4 Deviations of DFT calculated versus measured ^{13}C-NMR chemical shifts for JBIR-86 and **48** 54

Figure 6.1 Assesment of ichthyotoxcity and mammalian cell cytoxcity of compounds from hydrolysis GAT 512A 76

Figure 6.2 Library of lipids tested for ichthyotoxic properties 78

List of Schemes

Scheme 5.1 Method utilized to synthesize compound **48** 54

Abstract

Natural products have played a major role in the history of medicine. Despite this history, many view the area of natural products discovery to be tapped dry, thus the focus of drug development companies has shifted towards other avenues to discover new drug leads (*i.e.* combinatorial chemistry). In recent years, there has been an expansion of antimicrobial-resistant organisms accompanied by a lack of new drug entities coming to market. These trends illustrate a need to find new approaches other than the current status quo.

Analyses of microbial genomes show that there are many more secondary metabolite biosynthetic pathways than known secondary metabolites. These silent biosynthetic pathways (SBPs) offer the opportunity to discover new chemistry and the hope of new drug-like entities. Current methodologies to access SBPs fall into one of two categories: culture-dependent methods or molecular-based techniques, which are highlighted in chapter 1. While both approaches have been utilized to access SBPs, there are many deficiencies which inhibit the overall effectiveness of both methods. We offer another approach to bypass these difficulties. Studies of fungal genomes have demonstrated that many secondary metabolite biosynthetic genes are under epigenetic regulation. It is thought that treating fungi with small molecule epigenetic modifiers will upregulate the transcription of SBPs and allow the isolation of new secondary metabolites. This dissertation describes the work done using small molecules epigenetic modifiers to access SBPs and isolate new secondary metabolites. The third chapter involves pilot studies of cultures which were treated with epigenetic modifiers and the resulting isolation of two new polyketide compounds, lunalides A and B. The fourth

chapter follows up this work by demonstrating that the use of epigenetic modifiers results in the transcriptional upregulation of secondary biosynthetic pathways for a well-studied strain of *Aspergillus niger*. This is followed by the isolation of nygerone A from *Aspergillus niger* in the fifth chapter, which also presents a structural revision and bioactivity analysis of a set of γ-pyrones and γ-pyridones. The final chapter diverges from the topic of small molecule epigenetic modifiers and investigates the ichthyotoxicity of *Prymnesium parvum*, an invasive algal species which has transitioned from coastal water regions to local rivers, lakes, and streams.

Chapter 1. Oveview of Natural Products and Methods to Access Silent Biosynthetic Pathways

This chapter has been is adapted from portions of a previously published book chapter.[1]

1.1 Natural Products and the Pharmaceutical Industry

Natural products have had a long, rich history with respect to their importance in the treatment of disease. Since 1980, greater than 60% of drugs developed were derived from natural sources.[2-4] Despite the overwhelming number of drugs and drug leads from natural product sources, pharmaceutical companies appear to believe that new discoveries from natural products have been tapped dry. Thus, companies have moved towards other methods to develop drug leads (*i.e.* combinatorial chemistry).[2, 3]

The completion of the human genome project allowed for new disease targets to be identified for high-throughput bioassays. The development of these bioassays has led to a shift in the approach with which pharmaceutical companies identify new bioactive compounds. Combinatorial chemistry was envisioned as the means to develop large compound libraries to screen for bioactive compounds; however, this approach has not met expectations.[2, 3] In 2004, new active substances from the pharmaceutical companies hit a 24-year low,[2] even with changes in the rationale for creating combinatorial libraries.[5-7] The lack of new drug-like entities has come at a time when there has been dramatic increase in the presence of antimicrobial resistant organisms, which indicates other approaches, outside the current status quo, need to be explored.

1.2 Analysis of Microbial Genomes

The proliferation of fully sequenced microbial genomes during the past decade demonstrated that only a fraction of nature's chemical bounty has been explored. This discovery has sparked significant interest in probing silent biosynthetic pathways (SBPs) for new and bioactive natural products and has led to the rapid development of several innovative technologies for investigating these compounds. Given the substantial scientific interest in this field, several articles about microbial SBPs have been published within the past few years. These publications have provided deeper understanding of the topic.[8-21] The remainder of this chapter focuses on the methodological approaches used to access the products of SBPs.

Further investigations into SBPs illustrate that many microorganisms express only a fraction of their secondary-metabolite-encoding pathways under typical laboratory culture conditions **(Table 1.1).** In each of these cases, the numbers of secondary metabolites observed from laboratory-grown cultures fall short of their genetically encoded biosynthetic potential. Several terms have arisen in the natural products literature as descriptors of this phenomenon. By definition, “silent” biosynthetic pathways include the broad range of suppressive transcriptional, translational, and biosynthetic mechanisms responsible for inhibiting secondary metabolite formation. It is interesting to consider that some secondary-metabolite-encoding pathways that appear functionally silent might actually be transcribed at low levels, resulting in the generation of relatively minor concentrations of their respective biosynthetic products. Consequently, this low-level production could preclude the straightforward detection of a

gene's or gene cluster's associated natural products.[22] In other cases, SBPs have demonstrated complete transcriptional suppression under laboratory culture.[22, 23] Regardless of the cause, SBPs occupy the vast gulf that exists between our knowledge of what microorganisms are genetically able to produce and the natural products that researchers are capable of chemically detecting.

Generally, approaches utilized for exploring SBPs fall into two categories: manipulation of culture-dependent methods or molecular-based techniques. The following sections will take a glimpse into the present state of these two areas.

Table 1.1 Number of polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), and hybrid polyketide synthase/non-ribosomal peptide synthetase (HPN) gene clusters in selected bacteria and fungi.[1]

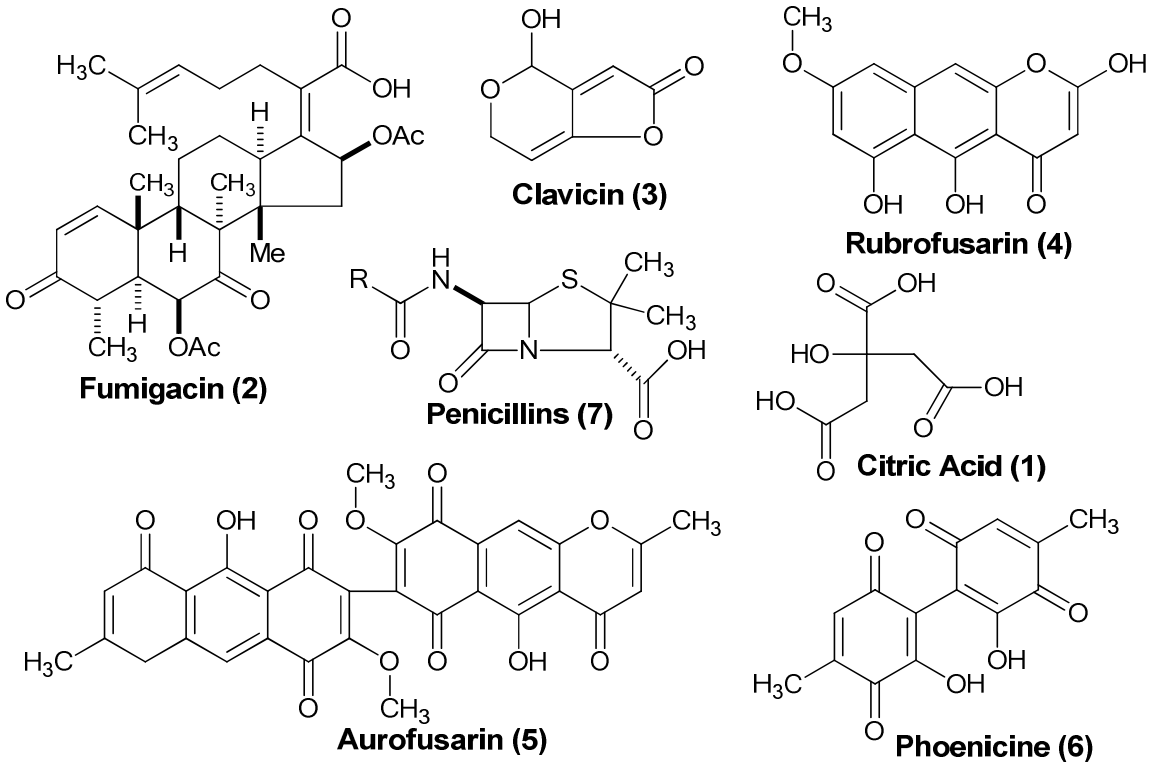
Organism	**Genome size (Mb)**	**Number of gene clusters**			**Reference**
		PKS	NRPS	HPN	
Aspergillus fumigatus Af293	29.4	14	13	1	[24]
Aspergillus nidulans FGSC A4	30.0	27	13	1	[25]
Aspergillus niger ATCC 1015	37.2	31	15	9	[13]
Aspergillus niger CBS 513.88	33.7	34	17	7	[26]
Aspergillus oryzae RIB40	37.0	30	18	0	[27]
Frankia sp. Ccl3	5.4	6	3	1	[28]
Magnaporthe grisea 70-15	37.9	23	6	8	[25]
Mycobacterium tuberculosis H5N1	4.4	11	2	0	[28]
Neurospora crassa N150	38.6	7	2	1	[29]
Nocardia farcinica IFM 10152	6.0	6	7	1	[28]
Penicillium chrysogenum Wisconsin54-1255	32.2	20	10	2	[30]
Salinispora tropica CNB-440	5.2	6	3	4	[28]
Streptomyces avermitilis	9.0	12	4	0	[31, 32]
Streptomyces coelicolor A3(2)	8.7	7	3	0	[33]
Ustilago maydis 521	20.5	3	4	9	[34, 35]

1.3 Manipulation of Culture Conditions

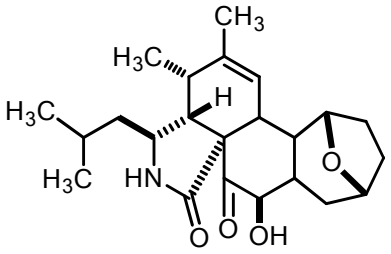
The ability to activate SBPs by altering microbial culture conditions was well known long before researchers were knowledgeable of the functional roles that DNA,

RNA, and proteins played in their production. Early accounts that described altered chemical profiles of microorganisms following culture condition manipulation demonstrated this point. At the turn of the twentieth century, there was significant debate as to the roles that various nutrients and chemical "stimulants" contributed to the phenotypic traits of microbes.[36, 37] While most of these studies focused heavily on features such as cell mass accumulation and spore formation, it was also observed that culture manipulation resulted in significant qualitative and quantitative changes in an organism's chemical composition.[38] For example, the addition of 0.01% $ZnSO_4$ to *Aspergillus niger* was shown to result in a 41% increase in the production of **1**, along with the appearance and greater yields of many other unidentified small organic substances. Similarly, the effects of varying media/culture conditions were observed to appreciably alter the production of pigments and antibiotics including **2** (*Aspergillus fumigatus*),[39] **3** (*Aspergillus clavatus*),[39] **4** and **5** (*Fusarium culmorum*),[40] **6** (*Penicillium phoeniceum*),[41] and **7** (*Penicillium* spp.).[42-47] While this list of examples is by no means exhaustive, it does provide important evidence for understanding the conceptual basis on which modern culture manipulation strategies have been designed.

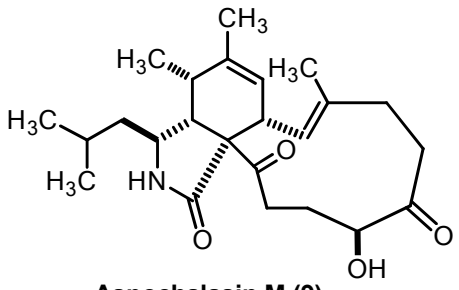
In the last decade, culture condition manipulation to access SBPs was reviewed and presented as the OSMAC (*O*ne *Strain Ma*ny *C*ompounds) method.[48] While previous discussions demonstrate that this concept had been used for decades, the review presented by Bode *et. al.* gives a nice summary of culture alteration methods for the elicitation of new natural products from fungi and other microbial sources.[48] Recently, several new compounds have been characterized from fungi using this methodology. For example, spicochalasin A **(8)** and five new aspochalasins **(9-13)** were isolated from the



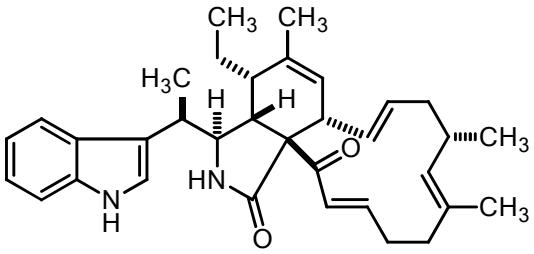
marine derived fungus *Spicaria elegans* when the media conditions were changed.[49] Additionally, compounds **14-16** from *Phomopsis asparagi*,[50] **17-19** from *Daldinia concentric*,[51] and **20** and **21** from *Ascochyta salicornia*[52] represent a set of examples which demonstrated the value of culture manipulations strategies. A significant highlight of the OSMAC method was shown in a study involving *Sphaeropsidales* sp. F-24'707. Varying culture conditions, which included differing culture vessel type, fermentation technique, media formula, and presence or absence of enzyme inhibitors, led to the isolation of 8 new and 6 known spirobisnapthalenes.[53] While these results demonstrate that one can alter growth conditions to trigger the production of SBPs that produce new or hidden natural products, the endless conditions which could be used to access these SBPs represent a significant disadvantage to the process. Additionally, culturing



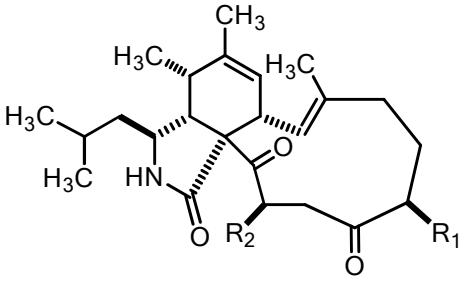
Spicochalasin A (8)



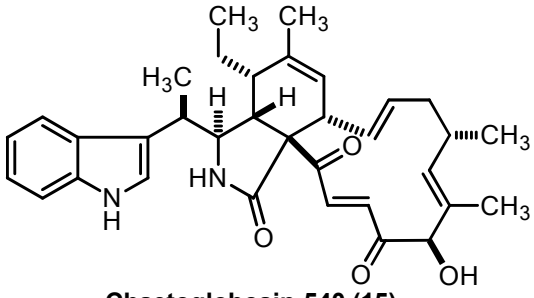
Aspochalasin M (9)



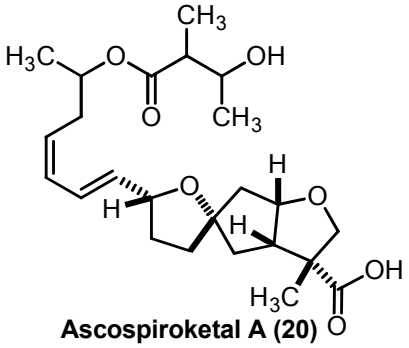
Chaetoglobosin-510 (14)



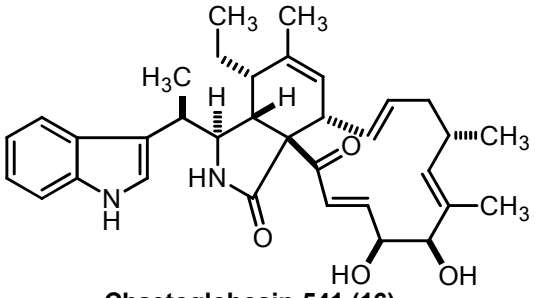
Aspochalasin N (10) R_1 = H, R_2 = Acetonyl
Aspochalasin O (11) R_1 = OH, R_2 = Acetonyl
Aspochalasin P (12) R_1 = H, R_2 = OH
Aspochalasin R (13) R_1 = H, R_2 = H



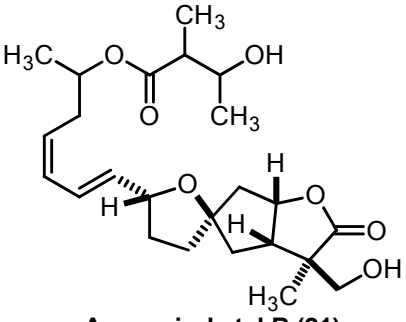
Chaetoglobosin-540 (15)



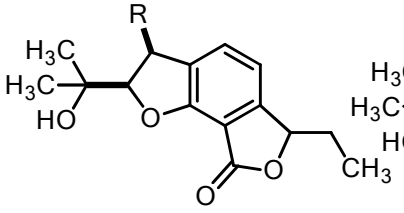
Ascospiroketal A (20)



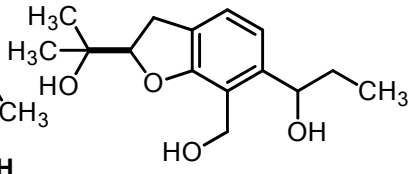
Chaetoglobosin-541 (16)



Ascospiroketal B (21)



Daldinin A (17) R = OH
Daldinin B (18) R = H



Daldinin C (19)

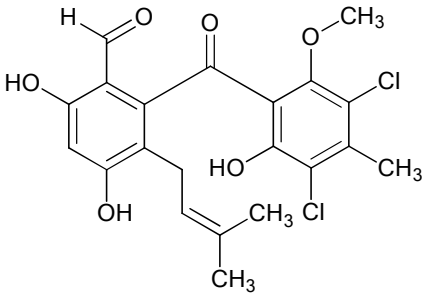
conditions which may be favorable for accessing SBPs of one organism are not guaranteed to be conducive to a different organism.

Another culturing method which has been explored in the attempt to access SBPs is based on the fact that fungi natrually live in complex communities which involve a wide range of chemical interactions. It has been proposed that cohabitating microorganisms might serve as a signal for the production of natural products.[54] Consequently, it was thought that agents capable of triggering SBP production might include a variety of undefined signaling molecules making co-culture a highly promising approach for natural product discovery.[55] Since 1978, the literature is scattered with instances in which mixed fermentation resulted in the increased production of known secondary metabolites.[56-60] What is more interesting are the published accounts which have described new chemistry under co-culture conditions ranging from the mixing of multiple live organisms to the addition of microbial-derived components into cultures.[61-65]

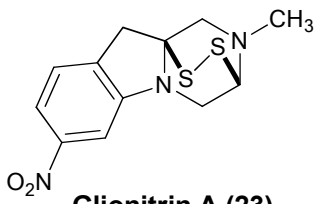
The microbial metabolite pestalone **(22)** was reported in 2001 from a marine-derived *Pestalotia* sp.[61] It is noteworthy that pestalone was not detected from monocultures of the *Pestalotia* sp. However, addition of an unidentified marine bacterium resulted in the production of this metabolite, which was found to possess antimicrobial activity. Other examples in which co-culture conditions produced new secondary metabolites include **23** in a combined culture of a fungal and bacterial strain which were isolated from an abandoned mine;[63] **28** and **29** from a combination of a fungus, *Emericella* sp.,[62] and a bacteria, *Salinispora arenicola*,[62] and **30** and **31** from a combined culture of two endophytic fungi isolated from a mangrove.[64] It should be noted

that in all of these instances, the newly isolated secondary metabolites demonstrated antimicrobial bioactivity.

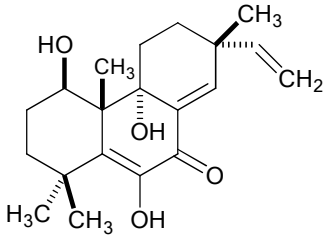
A final example to address is the isolation of libertellenones A-D **(24-27)**. In this instance, Oh *et. al.* were able to isolate this series of secondary metabolites when culturing a marine fungus, *Libertella* sp., with a marine α-proteobacterium.[65] These compounds demonstrated modest bioactivity, with **27** being the most bioactive in the group, which again supports the concepts of co-culturing to access SBPs. However, the authors were also forthright in the work that went into the isolation of these four compounds. During the process, more than 50 fungal strains were co-cultured with a marine bacterium, and only the *Libertella* sp. challenged with marine bacterium showed significant changes in secondary metabolite production by LC-MS when compared to control conditions.[65] These results definitively support the power which co-culturing conditions can provide when accessing SBPs. The small sampling of results demonstrates that often times these isolates have interesting chemistry (*i.e.* presence of halogen **(22)** or nitro-group **(23)**) and bioactivity. As with adjusting media conditions, co-culturing has the potential for accessing SBPs, yet the libertellenone story demonstrates that there are similar drawbacks. The secondary metabolite production of one strain may respond to a certain organism(s), but the next could require completely different organism(s) to be added in order to elicit changes in secondary metabolite production. Ultimately, there is a need for understanding the genome of an organism in order to rationally approach culture dependent methods to access SBPs. Otherwise, the primary factors in accessing SBPs by these methods will remain guesswork.



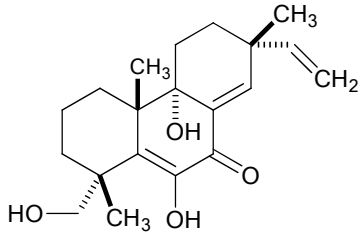
Pestalone (22)



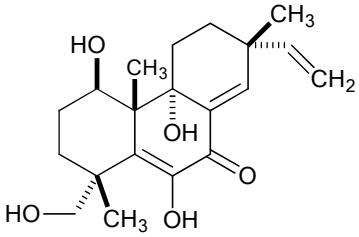
Glionitrin A (23)



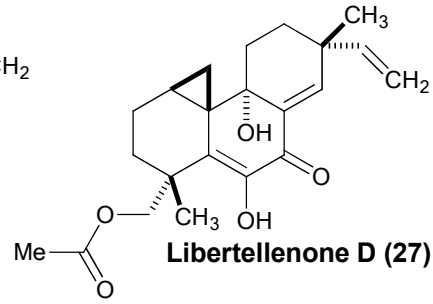
Libertellenone A (24)



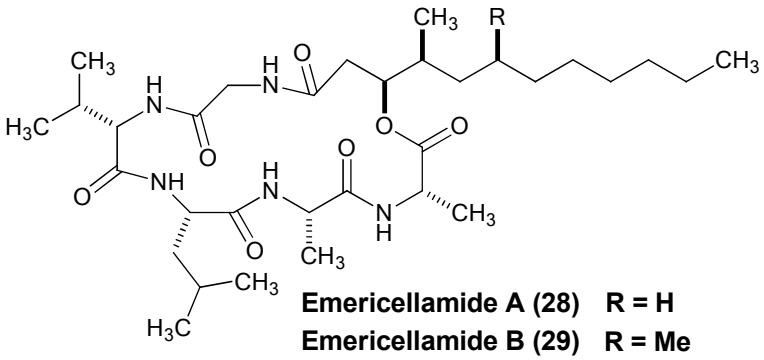
Libertellenone B (25)



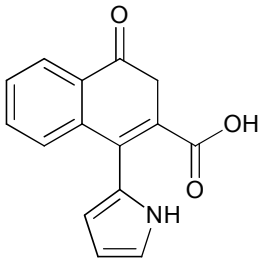
Libertellenone C (26)



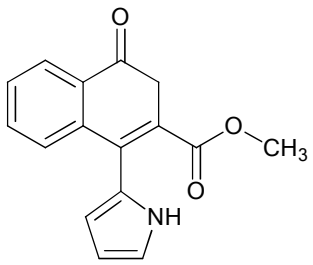
Libertellenone D (27)



Emericellamide A (28) R = H
Emericellamide B (29) R = Me



Marinamide A (29)



Marinamide B (30)

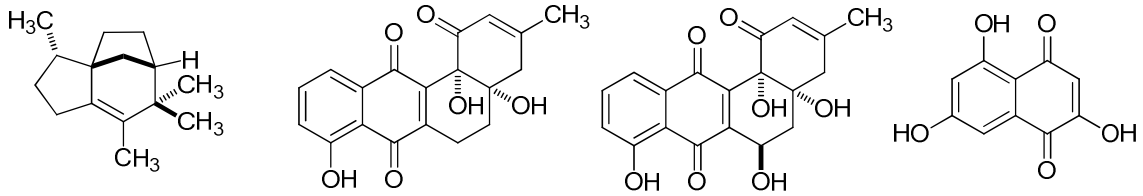
1.4 Heterologous Expression as an Approach to Investigating SBPs

With the access that genome sequencing has granted, additional genomic methodologies have been utilized to explore SPBs. Heterologous expression techniques provide the opportunity to selectively transfer a SBP into a new host possessing a non-transcriptionally suppressed regulatory environment to produce the enzymatic machinery for silent natural product biosynthesis. While this technology has been widely used for investigating non-secondary-metabolite-encoding genes, its application to the study of natural products and SBPs has evolved dramatically in recent years. In 2006, genome scanning by Muller and colleagues led to the discovery of a type III PKS in the myxobacterium *Sorangium cellulosum* for which no corresponding secondary metabolite could be identified.[66] Cloning and subsequent transfection of the type III PKS into *Escherichia coli* and *Pseudomonas* sp. demonstrated that the pseudomonad was able to function as an appropriate host for expression. The transfected *Pseudomonas* sp. culture turned visibly red and HPLC profiling confirmed the presence of a new metabolite. The structure of the compound was determined to be a naphthoquinone, flaviolin **(34),** that is readily generated from the oxidation of the actual biosynthetic precursor product, 1,3,6,8-tetrahydroxynaphthalene.

An important advantage of heterologously expressing SBPs is the opportunity it affords to manipulate biosynthetic genes and enzymes, as well as study secondary metabolite production in a cell-free environment. For example, the Canes group noted a terpene synthase of unknown function in *S. coelicolor* A3[67] and transfected the cloned gene into *E. coli.* Using gene manipulation, the researchers were able to produce a modified His_6-tagged protein that could be readily purified by Ni^{2+} affinity

chromatography.[68] In vitro incubation of the recombinant protein with farnesyl diphosphate in the presence of Mg^{2+} yielded **31**. The importance of this approach is that utilization of the recombinant protein allowed the group to carefully analyze the reaction mechanisms and kinetics of the new terpene synthase, which is generally not possible in a cellular host.

Palmu *et al.* have taken the idea of SBP manipulation one step further, leading to the production of new angucycline analogs.[69] This group observed that both *Streptomyces* sp. PGA64 and *Streptomyces* sp. H021 possessed PKS gene clusters, exhibiting significant homology to each other. Heterologous expression of varying portions of the gene clusters in *Streptomyces lividans* TK24 resulted in the generation of natural products **32** and **33**. These and other examples demonstrate the promise of using heterologous expression systems to manipulate SBPs. The ability to generate unique pools of natural and "unnatural" secondary metabolites presents an exceptional opportunity to identify new drugs and engineer novel chemical scaffolds. Despite its promising features, the routine use of heterologous expression systems for activating SBPs faces a number of significant difficulties. These challenges include the training and development of the necessary skill sets researchers need in heterologous expression, along with new and widely applicable tools (*e.g.*, universal heterologous hosts, novel vectors for routinely handling large gene clusters, etc.) in order to probe the overwhelmingly large number of SBPs that are predicted to exist.



(+)-epi-isozizaene (31) Gaudimycin A (32) Gaudimycin B (33) Flaviolin (34)

1.6 Conclusions and Perspectives on Methods for Accessing SBPs

The investigation of SBPs has advanced significantly in the past 5 years, and it is expected that further advances in genome-based technologies will continue to fuel this trend. Culture-dependent and genomics-driven methods each have unique advantages and disadvantages for studying SBPs. There is the potential that traditional culture-based approaches will have significantly diminished value as part of the repertoire of contemporary SBP research methods. Replacing these approaches will be a variety of new tools, including some of the work discussed in this dissertation, that offer increased speed and efficiency for evaluating SBPs in a high-throughput fashion. Consequently, there may be a revival of the study of microbial natural products as a resource to discover bioactive molecules.

Chapter 2 Hypothesis and Chapter Overviews

2.1 Hypothesis

Natural products have played a crucial role in the drug development process, yet many of the leaders in the pharmaceutical industry have abandoned their natural product research programs. While compelling evidence exists that there are still significant numbers of secondary metabolites left to discover in even well-studied bacterial and fungal strains; the overall methodology to best access these biosynthetic pathways, with respect to time and cost, is still out of reach. Therefore, if we could find a way to directly access these pathways, then a whole new world could be open for discovery in pharmaceuticals as well as other industries.

The work of Keller and colleagues provided enticing evidence for an epigenetic component to the transcriptional regulation of secondary-metabolite-encoding genes and gene clusters.[70-72] Based on this work, the following hypothesis has been proposed for this dissertation: **the use of small molecule epigenetic modifying agents which inhibit histone deacetylase and DNA methyltranferase/histone methyltransferase functions will lead to the transcriptional upregulation of SBPs resulting in the production novel secondary metabolites.** Chapters 3-5 summarize my research and results from the use of epigenetic modifiers, along with further investigations into the propensity of a class of γ-pyrones and γ-pyridinones in black aspergilli are explored through the use of LC-MS. Finally, Chapter 6 diverges into an investigation of ichthyotoxic secondary metabolites produced by an invasive aquatic species, *Prymnesium parvum*. While this project greatly differs from the rest of my work described in this dissertation, the

scientific merits of understanding the toxins and the effects on freshwater ecosystems make it significant and a part of my doctoral research.

2.2 Chapter 3. Epigenetic remodeling of the fungal secondary metabolome.

Genome sequencing has shown that only a small portion of secondary metabolites have ever been observed in laboratory settings. While methods have been developed to access different pathways, major weaknesses in these methods inhibit the overall effectiveness. Molecular based techniques have great potential, but require specialized skills and understanding of the gene sequences. Culture dependent methods are simple and available to everyone, but finding a condition which results in the isolation of a new compound is not straightforward, and is costly and time-consuming.

Ideally, a means to access all the biosynthetic pathways of an organism while in a laboratory is greatly desired. Using inspiration from Keller and colleagues, 12 different strains of filamentous fungi were screened against a panel of small molecule epigenetic modifiers in the culture medium. Extracts of these cultures were subjected to chromatography to determine if there were new and/or enhanced levels of secondary metabolites. The effect a methyltransferase inhibitor, 5-azacytidine, had on a *Diatrype*. sp. was explored in a large scale culture condition, resulting in the isolation of two new secondary metabolites.

2.3 Chapter 4 Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*

The results described in Chapter 3 revealed that the addition of small molecule epigenetic modifiers to 11 of 12 fungal strains resulted in the production of new and/or enhanced levels of secondary metabolites. While these results were enticing, with respect

to our hypothesis, it was still uncertain if the effects of these epigenetic modifiers were actually causing the upregulation of genes in the fungi. To further investigate the extent to which epigenetic modifying agents were able to alter the transcription of secondary-metabolite-encoding pathways, *Aspergillus niger* was treated with suberoylanilide hydroxamic acid or 5-azacytidine, and changes in the transcription of its 31 polyketide synthases (PKS), 15 nonribosomal peptide synthetases (NRPS), and 9 hybrid polyketide synthase-nonribosomal peptide synthetase (HPN) gene clusters were examined.

2.4 Chapter 5 Analysis of a class of γ-pyrones and γ-pyridones isolated from *Aspergillus niger*

Based on the results in the changes in transcription of the PKS, NRPS and HPN gene clusters of *A. niger* under epigenetic modifier conditions, the task of characterizing the new SBP-derived metabolites was undertaken. This led to the identification of the new *N*-phenylpyridine metabolite nygerone A following addition of suberoylanilide hydroxamic acid to the culture medium, which is discussed in this chapter.

During the isolation of nygeone A, a set of compounds were isolated, which when dereplicated, based on NMR and MS data, led to two different classes of compounds. A portion of this chapter focuses on solving the true class of structures to which these metabolites belong in using DFT calculations and total synthesis. In addition, we report the prevalence of this class of compounds in black aspergilli, along with a follow up analysis regarding the nygerone structure and bioassay data for some compounds described in this chapter.

2.6 Chapter 6 Reassessing the Ichthyotoxin Profile of Cultured Prymnesium parvum (Golden Algae) and Comparing it to Samples Collected from Recent Freshwater Bloom and Fish Kill Events in North America.

In the United States, there has been a sharp rise in the number of harmful algal blooms occurring in freshwater systems. Unfortunately, the toxins produced by these algal species are often times poorly characterized or unknown and this presents a significant impediment to the development of effective risk management strategies aimed at protecting humans. One of the most menacing of the freshwater algae, *Prymnesium parvum*, has recently made a surprising transition from coastal waters to inland lakes, rivers, and reservoirs throughout the southern United States. The spread of *P. parvum* and similar algal species into recreational and municipal water supplies is anticipated to have devastating effects on a wide range of ecological and economic systems, as well as human health. Using a bioassay guided fractionation methodology, a series of toxins produced by *P. parvum* grown under both laboratory culture conditions and from recent algal blooms occurring during 2008 and early 2009 were isolated and characterized in this chapter.

Chapter 3. Epigenetic Remodeling of the Fungal Secondary Metabolome.

This chapter is adapted from a publication in Organic and Biomolecular Chemistry.[73]

3.1. Introduction

Fungi are biosynthetically talented organisms capable of producing a wide range of chemically diverse and biologically intriguing small molecules. The majority of scientific interests in fungal natural products have centered on their pharmaceutical applications, roles as mycotoxins, and multifarious ecological functions. Unfortunately, typical fungal fermentation methods such as axenic shake or static cultures on artificially defined media are poor surrogates for mimicking an organism's native habitat. The consequence of these practices is that only a subset of the biosynthetic pathways which encode for secondary metabolite production in fungi are ever expressed, thus limiting prospects for realizing the complete drug discovery potential of these organisms. Moreover, without securing the full range of 'silent'[74] secondary metabolites, opportunities for testing hypothesis-based inquiries regarding their native functions (antibiosis, intra- and inter-species communication, and modulation of diverse biotic responses) remain unattainable. The magnitude of this problem has become markedly apparent with the advent of modern genomic technologies. With the exception of yeast, the number of putative natural product biosynthetic pathways in fungi exceeds the sum total of natural products observed under laboratory culture conditions. Pertinent examples highlighting this phenomenon are found among *Aspergillus* spp.,[75] *Neurospora crassa*,[29] and *Magnaporthe grisea*.[25]

Methods developed for exploring the products of silent secondary metabolic pathways in microorganisms fall into two broad categories:[14] molecular-based techniques and cultivation-dependent approaches. Despite their incredible promise for providing outstanding access to and control over silent biosynthetic pathways, molecular-based methods utilizing heterologous expression systems are limited by problems such as locating and cloning genes, difficulties with gene transformation and inactivation, and host incompatibilities. Alternatively, a variety of cultivation-dependent procedures in which biotic and abiotic culture parameters are manipulated have likewise been proposed. While this methodology has been a mainstay of microbial natural products for decades, the concept was more recently formalized by Zeeck and colleagues[48] and has since been applied by several groups.[76] Unfortunately, this protractive strategy is labor intensive, lacks predictable outcomes, and inflates the workload of natural products screening programs. These difficulties are further compounded by strain-specific variation in the quantity of metabolite production as well as the seemingly capricious behavior of fungi to alter metabolite profiles when re-cultured. Consequently, an alternative methodology that is universally applicable for rationally inducing the expression of silent natural product biosynthetic pathways in fungi is needed.

The discovery of the putative nuclear transcriptional regulator LaeA, which controls secondary metabolite production in *Aspergillus*,[72] has inspired our group to examine global mechanisms by which fungi restrictively manage the production of natural products under laboratory culture conditions. Published fungal genomes demonstrate a propensity for the positioning of many putative natural product biosynthetic gene clusters in the distal regions of chromosomes.[77] Importantly, these

portions of fungal genomes are noted to exist in a heterochromatin state whose constitutive genes are often transcriptionally controlled by epigenetic regulation such as histone deacetylation and DNA methylation. Recently, Keller and colleagues have demonstrated that disruption of histone deacetylase activity (*Δhda*) in *Aspergillus nidulans* led to the transcriptional activation of gene clusters for the production of sterigmatocystin and penicillin.[77] Moreover, extension of these observations to growing cultures of *Alternaria alternate* and *Penicillium expansum* suggested that inhibition of histone deacetylase activity could positively modulate secondary metabolite production. Based on these observations, we hypothesized that small-molecule epigenetic modifiers could be rationally employed for accessing silent natural product pathways and enhancing the native production of fungal secondary metabolites. The development of such an approach would present significant advantages to the natural products research community for controlling the expression of latent biosynthetic pathways and creating new opportunities for novel small-molecule discoveries.

3.3. Results and Discussions

Our initial experiments testing this approach consisted of subjecting a diverse panel of twelve fungi to treatment with a focused library of DNA methyltransferase and histone deacetylase inhibitors in dose dilution series (0.1 μM to 10 mM). Comparative profiling by HPLC, MS, ^{1}H-NMR and TLC demonstrated eleven of the fungi were responsive to one or more epigenetic treatments based on the production of new natural products and/or enhanced accumulation of constitutive secondary metabolites. Furthermore, exposure of selected *Aspergillus*, *Cladosporium*, *Clonostachys*, *Diatrype*, *Penicillium*, and other fungal strains to multiple media types confirmed that many of the

new natural products were exclusively obtained following the use of epigenetic modifying treatments. Cultures treated with amphotericin B, cycloheximide, and 5-fluorouracil did not produce new or enhanced secondary metabolite profiles indicating that epigenetic modifiers impacted fungi in a manner that was functionally distinct from that of a general cytotoxic response. Interestingly, a combination treatment, composed of a DNA methyltransferase inhibitor and histone deacetylase inhibitor, was tested and determined to be only modestly effective due to significant growth restriction and/or generation of metabolite profiles dominated by the effects of a single component in the mixture (*e.g.*, effects of individual compound treatments were not additive as a mixture). In order to probe the nature of metabolic remodeling induced by epigenetic modifier treatment, a fungal isolate from our lab was selected for scale-up studies.

This fungal isolate was obtained from the foregut of a fifth instar luna moth (*Actias luna*; Saturniidae) larva that was cultured on an exclusive diet of sweet gum (*Liquidambar styraciflua* L.; Hamamelidaceae) leaves. Initial characterization of the fungus by analysis of the 26S rRNA gene gave a 93% sequence homology to *Diatrype disciformis*. While control cultures of this *Diatrype* sp. were relatively void of any secondary metabolites, addition of 5-azacytidine triggered a significant change in the organism's metabolic profile (**Figure 3.1A**) based on the HPLC UV chromatographic traces created through the use of background subtraction.

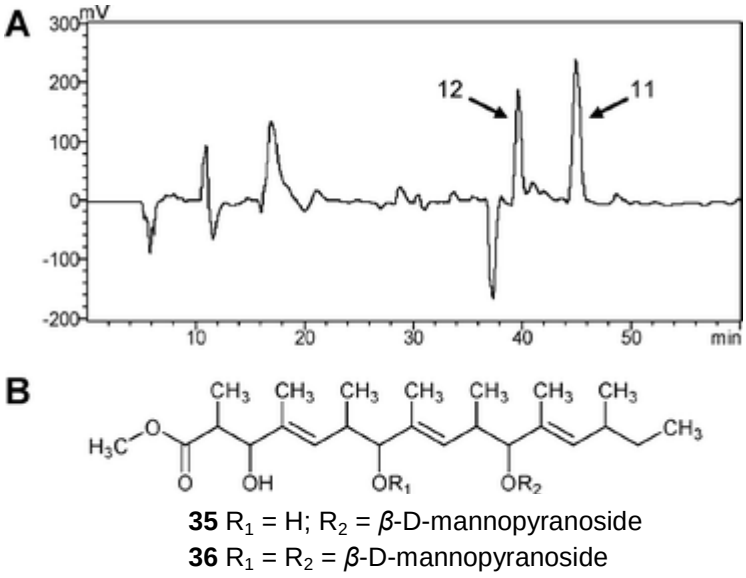


Figure 3.1 A) Difference chromatogram (UV trace at 210 nm) for *Diatrype* sp. Treated with 5-azacytidine. **B)** New glycolsylated polyketides, lunalides A and B, were produced following treatment with the DNA methyltransferase inhibitor. Compounds were characterized by NMR and HRESIMS.

Using the initial analysis as a guide, two metabolites were isolated from the 5-azacytidine treated cultures and were further characterized. Compound **35** was obtained as amorphous solids via HPLC fractionation. Data from HRESIMS exhibited a peak of *m/z* 595.3494 $[M + Na]^+$ indicating the molecular formula of **35** to be $C_{30}H_{52}O_{10}$ (calc. for $C_{30}H_{52}O_{10}Na$, 595.3458). The ^1H and ^{13}C-NMR spectra suggested the presence of nine methyls, two methylenes, and fifteen methines. In addition, four quaternary carbons were observed, one of which was a carbonyl based on its chemical shift and the other three were incorporated into double bonds. HMBC, HSQC, and COSY correlations were used to piece together a polyketide backbone, with a sugar moiety attached at C-12, with distinct anomeric proton (^1H δ 4.37 ppm) and carbon (^{13}C δ 97.5 ppm) chemical shifts. The sugar moiety was determined to be β-mannopyranose based on 1D-NOE correlations and susceptibility to enzymatic hydrolysis with β-mannosidase whereas attempts to remove the sugar residue with α-mannosidase were unsuccessful.

Compound **36** was also obtained as an amorphous solid from HPLC fractionation. Data from HRESIMS exhibited a peak of *m/z* 757.3971 $[M + Na]^+$ indicating the molecular formula of **36** to be $C_{36}H_{62}O_{15}$ (calc. for $C_{36}H_{62}O_{15}Na$, 757.3986). Observing the ^{1}H-NMR spectrum indicated the presence of two anomeric proton signatures (^{1}H δ 4.35 and 4.58 ppm). The proton spectrum, along with the difference in mass data between compounds **35** and **36**, supported that compound **36** had the same polyketide backbone, with an additional β-mannopyranose moiety being present. A combination of 2-D HMBC, HSQC, and COSY data confirmed these suspicions, with the new *β*-mannopyranose substituted at the at the C-8 hydroxyl. Again, the sugar moieties were determined to be *β*-mannopyranose based on enzymatic hydrolysis. Further support as to the identity of the *β*-mannopyranose was obtained by careful comparison of our ^{1}H and ^{13}C-NMR data to that of authentic *D*-mannose. It is interesting to note that one other non-epigenetic culture treatment, elicitation with *E. coli*, resulted in the biosynthesis of **35** and **36** which were otherwise repressed under axenic culture conditions. The results suggest that their production is under specific control of a unique environmental cue.

The success of inducing the production of new natural products from fungi by administering small-molecule epigenetic modifiers indicates that this technique is a very promising and rational approach for the native expression of silent biosynthetic pathways. This method has several significant benefits compared to currently available molecular or culture-dependent techniques. First and foremost, it provides a needed tool for rapidly accessing potential pools of cryptic fungal natural products in their native hosts. Second, this methodology can be readily implemented in most labs without extensive retooling, giving it a wide scope of utilization. Third, this approach will significantly lessen the cost

and effort of acquiring the products of silent secondary metabolic pathways since fungi do not need to be pre-screened using a multitude of culture conditions.

3.2. Materials and Methods

3.2.1 General Chemical Methods. Initial HPLC separation of secondary metabolite mixtures was performed on a Shimadzu preparative HPLC using a SCL-10A VP system controller, SPD-10AV VP UV-VIS detector, LC-6AD pumps, DGU-14A solvent degasser, and FRC-10A programmable fraction collector. Samples were separated over a Phenomenex C_{18} Gemini column (5 μm, 110 Å, 250 × 21.2 mm) with fractions collected at a rate of one per minute. Semi-preparative HPLC was performed on a similar system using LC-10AT VP pumps and a Phenomenex C18 Gemini column (5 μm, 110 Å, 250 × 10 mm). Mass data from electrospray ionization were acquired on a LCT Premier (Waters Corp.) time of flight mass spectrometer. Corrections for exact mass determinations were made automatically with the lock mass feature in the MassLynx data system. A reference compound in an auxiliary sprayer was sampled every third cycle by toggling a 'shutter' between the analysis and reference needles. Samples for mass determination were dissolved in MeOH-H_2O (9:1) and introduced for ionization with a 20 μL loop on an auto injector system. NMR data were obtained on a Varian VNMR spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). All solvents were of ACS grade or better.

3.3.2 Fungal Test Strains. Fungal test strains for initial screening were obtained from the Agricultural Research Services (ARS) Culture Collection (formerly NRRL) and The University of Oklahoma Natural Products Discovery Group (OU NPDG) collections. The OU NPDG collection consists of >1,200 fungi obtained from soil samples and

marine sediments collected from across the United States including Alaska and its coastal waters, Arizona, California and its coastal waters, Indiana, Maine and its coastal waters, Oklahoma, Washington, and Wyoming. All strains were grown on appropriate media and cryopreserved in 15% glycerol at -80 ºC. Fungi selected for this study included the following: *Aspergillus flavus* (ARS), *Aspergillus westerdijkiae* (ARS), *Cladosporium cladosporioides* (OU NPDG), *Clonostachys* sp. (OU NPDG), *Diatrype* sp. (OU NPDG), *Penicillium chrysogenum* (ARS), *Penicillium citrinum* (ARS), *Rhizopus sp.* (OU NPDG), and *Verticillium sp*. (OU NPDG), and three additional marine-derived isolates that have not yet been identified.

3.3.3 Small Molecule Epigenetic Modifiers. Small molecule epigenetic modifiers were selected to include both DNA methyltransferase and histone deacetylase inhibitors. The DNA methyltransferase inhibitors consisted of 5-azacytidine, 5-aza-2′-deoxycytidine, hydralazine, procaine, and procainamide which were all purchase from Sigma. Histone deacetylase inhibitors included sodium butyrate, suberohydroxamic acid, and valproic acid from Sigma and suberoylanilide hydroxamic acid from TRC Biomedical Research. Compounds were prepared in deionized H_2O and filter sterilized (0.22 μM) before being added to fungal cultures.

3.3.4 Screening Epigenetic Modifiers. For MIC determinations, stock cultures were prepared by lawning fungi onto Petri plates and the resulting mycelial mats were submerged in sterile water and mechanically disrupted so as to uniformly disperse cells/spores. Microplates (96-well sterile polystyrene plates, 300 μL well volume, with low-evaporation covers, Corning, Inc.) were loaded with 200 μL of media/well and 1 μL portions of filter sterilized epigenetic compounds dissolved in water or water controls.

Pure compounds were tested in a series of six, ten-fold dilutions ranging in concentration from 0.1 μM to 10 mM. Aliquots of 20 μL fungal cell/spore suspension were used to inoculate each well of the plates. Plates were covered and incubated in a humidified chamber in the dark at 25 ºC. After 3-7 days (dependant upon growth characteristics of each fungal strain), the MIC values of the compounds were determined visually based on turbidity.

The MIC value was assigned as the lowest compound concentration capable of inhibiting fungal growth. Epigenetic modifying agents were tested for their capacity to induce secondary metabolite production by culturing organisms in 25 mL of media, and after 24 h, compounds were added at concentrations 10-fold less than their respective MIC values. The cultures were incubated an additional 6 days at which point 12 mL of MeOH was added to each culture before being partitioned twice against 30 mL of dichloromethane. The combined organic extracts were dried under vacuum and stored at -20 ºC prior to analysis.

3.3.5 Isolation and Identification of Fungi. *Diatrype* sp. was isolated in a three phase process. It was first isolated from the fore gut of a fifth instar *Actias luna* (Saturniidae) larva that was cultured on an exclusive diet of *Liquidambar styraciflua* L. (Hamamelidaceae) leaves. Substrates containing fungi (~500 mg) were mixed with 15 mL sterile water, vortexed vigorously, and two, ten-fold dilutions made. Aliquots (200 μL) of the three suspensions were lawned onto primary isolation plates containing potato-dextrose agar with chloramphenicol to inhibit bacterial growth and cycloheximide to restrict expansion of rapidly growing fungi. Dilution plates were prepared in triplicate and the maintained inverted at 20 ºC. After three days, plates were checked for colony

formation and monitored every other day for three weeks. Emerging colonies exhibiting filamentous character were excised from the primary plate using a sterile probe and transferred to a secondary Petri plate containing potato dextrose agar with chloramphenicol (cycloheximide was omitted). Colonies were selected from primary plates for sub-culturing on secondary plates based on their differential morphologies and time of appearance. Rapidly spreading colonies and those having morphological features identical to isolates already sub-cultured were 'pruned' from the primary plates in order to allow the slower growing and less abundant strains to mature. Secondary plates were maintained upright at 20 ºC and the hyphae of the growing organisms allowed to radiate outward from the point of inoculation. After 1-2 weeks, sterile probes were used to remove small samples from the leading edges of the spreading fungal colonies and transferred to tertiary Petri plates containing potato-dextrose agar (chloramphenicol and cycloheximide were omitted). Plates were incubated upright at 20 ºC for an additional 1-2 weeks after which the fungal cultures were prepared for cryostorage. Sterile probes were used to remove pieces of mycelia and deposited into sterile cryostorage vials containing 15% glycerol in water. The samples were then stored at -80 ºC.

Diatrype sp. was tentatively identified based on analysis of the 300 base pair sequence of the D2 region of the large ribosomal subunit (26S rRNA gene). Analysis of samples was performed under the expert review of Michael G. Sinclair, Ph.D., Mycologist and Laboratory Director, Microcheck, Inc. Briefly, portions of the mycelia mats were scraped from the surface of an agar plate and placed in a sample tube with Prepman reagent (Applied Biosystems) to lyse the cells and liberate DNA. The mixture was boiled and briefly centrifuged to pellet cellular debris. The DNA in solution was

transferred to a fresh tube and PCR reagents added (buffers, primers, and dNTPs) and the 26S rRNA gene amplified in a thermal-cycler. Next, ExoSAP-IT was added to remove unreacted primers and dNTPs. Finally sequencing reagent was added containing fluorescently-tagged ddNTPs yielding 3′ end labeled product and purified. After clean-up, the samples were loaded into a 3130*xl* Genetic Analyzer (Applied Biosystems) for sequencing. The final consensus sequence was compared to a library of 1,113 fungal standards using MicroSeq ID software. The software allowed for comparisons of sample sequences to those stored in the library by weighting mismatches (i.e. purine to purine substitutions are weighted lower than purine to pyrimidine substitutions) thereby providing a percent match score against known fungi. Most fungi that we encounter can be readily identified to the genus level (match score of >95% to known species); however, identification to species is less typical. Most general assignments are then confirmed by morphological/microscopic evaluation according to standard mycological methods.

3.3.6 Fungal Culture Conditions and Initial Screening. Seed cultures of fungi were prepared by inoculating five 25 mL portions of media with agar plugs from Petri plates containing hypae of *Diatrype* sp. The cultures were allowed grow with shaking for seven days at 20 ºC. Scale-up fermentations consisted of sets of ten 1 L Erlenmeyer flasks containing 250 mL of sterile potato-dextrose media that were inoculated with 2 mL of their respective seed cultures. The newly prepared cultures were allowed to incubate with rotary shaking (100 rpm) for 24 h at 25 ºC. Each set of 10 cultures was treated with epigenetic modifying compounds or water blank (control) and returned to further incubate on the shaker for an additional six days. The cultures were harvested by adding

100 mL of MeOH to each flask before being partitioned twice against 300 mL of dichloromethane. The combined organic extracts from replicate flasks were dried under vacuum and stored at -20 ºC prior to analysis. All treatments and controls for each fungus were performed in tandem with test and control cultures randomly interspersed on the shaker.

3.3.7 Culture Comparison and Compound Isolation. For each experiment, sets composed of ten culture flasks (each with 250 mL of potato-dextrose media each inoculated 24 h prior with 1 mL of fungal seed culture) were treated with 5-azacytidine (DNA methyltransferase inhibitor), suberoylanilide hydroxamic acid (histone deacetylase inhibitor), or sterile water blank. Parallel experiments also examined the effects of ten different media conditions on secondary metabolite production **(Table A1)**. Cultures of *Diatrype sp.* were incubated with shaking for 14 days and the contents within each treatment group pooled. An additional experiment was performed on the *Diatrype* sp. by treating growing cultures of the fungus with 1 mL aliquots of autoclaved *Escherichia coli* culture instead of epigenetic modifiers. A 1 L portion of methanol was added to the media-cells mixture and then extracted three times with equal volumes of dichloromethane. The total organic extract from each treatment was injected onto C_{18} preparative HPLC (20-100% acetonitrile-water over 50 minutes) and monitored by dual wavelength UV detection at 210 nm and 254 nm. Difference chromatograms were generated for the 210 nm trace by subtracting the untreated control chromatogram from the treatment groups (Lab Solutions). Peaks phasing upward represent metabolites that were expressed only under epigenetic treatment or produced at enhanced concentrations.

Compounds *Diatrype* sp. were isolated by semi-preparative HPLC with repeated re-injection and separation across C18 using acetonitrile-water gradient conditions.

3.3.8 Lunalide A (35). ^{1}H-NMR (500 MHz, CD_3OD) δ 0.77 (3H, d, *J*=7.0 Hz, H-20), 0.82 (3H, d, *J*=7.5 Hz, H-22), 0.87 (3H, t, *J*=7.5 Hz, H-17), 0.94 (3H, d, *J*=7.5 Hz, H-18), 0.99 (3H, d, *J*=6.5 Hz, H-24), 1.24 (1H, m, H-16a), 1.40 (1H, m, H-16b), 1.59 (3H, d, 1.0 Hz, H-23), 1.64(3H, d, 1.0 Hz, H-19), 1.66 (3H, d, 1.0 Hz, H-21), 2.39 (1H, m, H-15), 2.61 (1H, m, H-3), 2.64 (1H, m, H-7), 2.70 (1H, m, H-11), 3.07 (1H, m, H-5′), 3.36 (1H, m, H-3′), 3.52 (1H, dd, *J*=9.5Hz, 9.5 Hz, H-4′), 3.70 (3H, s, H-1), 3.70 (1H, d, *J*=9.5 Hz, H-8), 3.71 (1H, m, H-6a′), 3.73 (1H, m, H-2′), 3.86 (1H, dd, *J*= 2.5 Hz, 11.5 Hz, H-6b′), 3.95 (1H, d, *J*=10 Hz, H-12), 4.06 (1H, d, *J*=10 Hz, H-4), 4.37 (1H, bs, H-1′), 5.26 (1H, d, *J*=9 Hz, H-14), 5.33 (1H, d, *J*=10 Hz, H-6), 5.38 (1H, d, *J*=9.5 Hz, H-10); ^{13}C-NMR (125 MHz, CD_3OD) δ 10.8 (C-19), 11.2 (C-21 and C-23), 12.7 (C-17), 14.9 (C-18), 17.9 (C-20 and C-22), 21.7 (C-24), 31.5 (C-16), 35.4 (C-11), 35.5 (C-15), 36.8 (C-7), 44.8 (C-3), 52.3 (C-1), 63.0 (C-6′), 68.9 (C-4′), 73.2 (C-2′), 75.8 (C-3′), 78.6 (C-5′), 82.0 (C-4), 84.5 (C-8), 88.9 (C-12), 97.5 (C-1′), 131.8 (C-13), 134.9 (C-10), 35.5 (C-6), 136.2 (C-5 and C-9), 140.8 (C-14), 179.2 (C-2); HRESIMS *m*/*z* $[M + Na]^+$ 595.3494 (calcd for $C_{30}H_{52}NaO_{10}$ 595.3458).

3.3.9 Lunalide B (36). ^{1}H-NMR (500 MHz, CD_3OD) δ 0.77 (3H, d, *J*=7.0 Hz, H-20), 0.81 (3H, d, *J*=7.0 Hz, H-22), 0.87 (3H, t, *J*=7.5 Hz, H-17), 0.93 (3H, d, *J*=6.5 Hz, H-18), 1.00 (3H, d, *J*=6.5 Hz, H-24), 1.24 (1H, m, H-16a), 1.40 (1H, m, H-16b), 1.58 (3H, d, *J*=1.5 Hz, H-19), 1.61(6H, bs, H-21, H-23), 2.40 (1H, m, H-7), 2.60 (1H, m, H-3), 2.68 (1H, m, H-15), 2.74 (1H, m, H-11), 3.03 (1H, m, H-5′), 3.33 (1H, m, H-3′), 3.41 (1H, m, H-5″), 3.49 (2H, m, H-4′ and H-4″), 3.68 (1H, m, H-3″), 3.69 (1H, m, H-6a′),

3.70 (3H, s, H-1), 3.72 (2H, m, H2′ and H2″), 3.78 (1H,dd, *J*=7 Hz, 12 Hz, H-6a″), 3.85 (1H, dd, *J*=2.5 Hz, 12 Hz, H-6b′), 3.89 (1H, dd, *J*=2.5 Hz, 12 Hz, H-6b″), 3.98 (1H, d, *J*=10 Hz, H-8), 4.02 (1H, d, *J*=10 Hz, H-12), 4.08 (1H, d, *J*=10 Hz, H-4),4.35 (1H, bs, H-1′), 4.58 (1H, bs, H-1″), 5.27 (1H, d, *J*=9.5 Hz, H-14), 5.42 (1H, d, *J*=8 Hz, H-6), 5.45 (1H, d, *J*=8.5 Hz, H-10); ^{13}C-NMR (125 MHz, CD_3OD) δ 10.7 (C-19), 11.1 (C-21 and C-23), 12.7 (C-17), 15.0 (C-18), 17.9 (C-20), 18.0 (C-22), 21.7 (C-24), 31.5 (C-16), 35.4 (C-7), 35.5 (C-11 and C-15), 44.8 (C-3), 52.3 (C-1), 63.3 (C-6′ and C-6″), 69.3 (C-4′), 69.5 (C-4″), 73.0 (C-2′), 73.4 (C-2″), 75.0 (C-3″), 75.9 (C-3′), 77.6 (C-5″), 78.9 (C-5′), 82.0 (C-4), 88.4 (C-8), 88.5 (C-12), 97.1 (C-1″), 97.5 (C-1′), 131.8 (C-13), 132.7 (C-9), 134.9 (C-5), 136.4 (C-6), 138.6 (C-10), 141.1 (C-14), 178.3 (C-2); HRESIMS *m/z* [M + Na]$^+$ 757.3494 (calcd for $C_{36}H_{62}NaO_{15}$ 757.3986).

Chapter 4. Chemical Induction of Silent Biosynthetic Pathway Transcription in *Aspergillus niger*

This chapter is adapted from a publication in The Journal of Industrial Microbiology [23]. *The project involved many collaborators in diverse areas such as genomics and evolutionary genetics. The materials and methods along with corresponding results presented in this chapter are my contribution to the research.*

4.1 Introduction

Among the many remarkable discoveries emerging from the genomics era is the revelation that most microorganisms, and in particular, filamentous fungi, possess far greater numbers of gene clusters encoding for the production of secondary metabolites than the numbers of natural products that have been isolated from these same organisms.[15, 20, 21] It is not uncommon for fungi to have dozens of unique gene clusters encoding for previously uncharacterized polyketide (PKS), non-ribosomal peptide (NRPS), and hybrid PKS-NRPS (HPN) natural products.[78-85] These transcriptionally suppressed gene clusters, which are collectively referred to as silent biosynthetic pathways (SBPs), generally remain unexpressed or under-expressed under a variety of laboratory culture conditions. SBPs are anticipated to be a rich source of chemically diverse compounds (*e.g*., novel organic moieties, multifaceted stereochemical features, unique heteroatom incorporation, *etc*.) with outstanding potential for generating novel therapeutic leads. Obtaining the products from transcriptionally suppressed gene clusters requires a new approach for efficiently probing silent biosynthetic pathways in a rational manner.

In an effort to obtain new drug leads from silent biosynthetic pathways, epigenetic modifying substances have been identified as promising tools for manipulating the secondary metabolism of fungi. It has been proposed that epigenome-level changes are extensively involved in controlling the transcriptional accessibility of gene clusters encoding for biosynthetic enzymes.[73, 77, 86] Several proteins are required to facilitate chromatin remodeling in fungi and their collective influence on gene expression is quite remarkable.[87-94] Chief among the chromatin interacting proteins are histones. These proteins undergo a wide-range of post-translational modifications[92, 94] with acetylation and methylation being two of the most frequently encountered and well-studied of the histone alterations. While histone deacetylation is generally associated with gene silencing, the effects of methylation vary depending on which histone subtypes and specific amino acid residues are modified within nucleosomal complexes.[87, 95, 96]

A variety of highly specific small-molecule probes are known to selectively inhibit the functions of histone modifying proteins and these compounds have found important roles as molecular tools for studying the epigenome.[96] Several families of histone acetyltransferases are responsible for catalyzing the covalent addition of acetyl groups to histones, while the reverse reaction is carried out by histone deacetylases (HDAC) and sirtuins.[92, 94] The hydroxamate-bearing natural product trichostatin A and its synthetic derivative, suberoylanilide hydroxamic acid (SAHA), are two noteworthy examples of potent HDAC inhibitors that have been successfully used as chemical epigenetic probes in a variety of eukaryotic systems including filamentous fungi.[96]

Another epigenetic modifying agent, 5-azacytidine (5-AZA), was originally developed as an antimetabolite that incorporated into RNA causing a variety of

dysfunctions related to production of tRNAs, rRNAs, and proteins.[97] Further studies have shown that 5-AZA has strong epigenetic modifying properties due to its inhibitory interactions with DNA methyltransferases resulting in hypomethylation of DNA and chromatin restructuring.[96, 98] Interestingly, the epigenetic role of DNA methylation in fungi is still poorly understood and in some cases, the very existence of DNA methylation in these organisms has been questioned.[93, 99] Two proposed explanations for how 5-AZA induces chromatin remodeling in fungi involve 1) inhibition of DNA methylation, which may occur at very low levels or transiently in fungi or 2) inhibition of histone methyltransferase (HMT) function.[100, 101] A HMT inhibitory role for 5-AZA is highly plausible given its structural similarity to other confirmed methyltransferase inhibitors such as methylthioadenosine, sinefungin, and *S*-adenosylhomocysteine.[96] Nevertheless, the ability of 5-AZA to cause distinctive chromatin remodeling effects, even in the absence of detectable changes in DNA methylation, makes this and similar chemical epigenetic probes valuable molecular tools for inducing chromatin remodeling in fungi.

In this study, we screened a suite of epigenome-modifying agents as chemical tools for eliciting the expression of fungal silent biosynthetic pathways. *Aspergillus niger* was selected for this work since three separate genome sequencing initiatives have been completed in recent years[102-104] allowing confirmation of the presence of numerous PKS, NRPS, and HPN gene clusters in this fungus. The genome-wide impacts of the epigenetic modifiers SAHA and 5-AZA on the transcription of PKS, NRPS, and HPN biosynthetic gene clusters in *A. niger* were determined using real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR). These data were compared

to results from two expressed sequence tag (EST) libraries generated with *A. niger* that was grown under a wide variety of culture conditions. In conjunction with this work, we have also addressed the remarkable diversity of PKS and HPN gene clusters in *A. niger* through a phylogenetic analysis of their respective ketosynthase domains. Results emerging from this study strongly support the use of epigenetic modifying agents as an effective methodological approach for upregulating the transcription of secondary-metabolite-encoding biosynthetic gene clusters in fungi.

4.2 Results and Discussions

Comprehensive examination of these and other suspected biosynthetic genes led to the identification of 33 PKS, 15 NRPS, and 9 HPN gene clusters distributed throughout the genome of *A. niger* ATCC 1015.[23] Previously, two expressed sequence tag (EST) libraries have been generated for *A. niger* that was cultured under a wide variety of conditions. The first of these studies utilized wild-type *A. niger* N402 (4732 from the Fungal Genetics Stock Center) resulting in the generation of 12,820 ESTs accounting for a total of 5,108 of the fungus's genes.[105] For this experiment, *A. niger* was grown on seven different carbon sources (glucose, bran, maltose, xylan, xylose, sorbitol, and lactose). Nucleotide BLASTN and protein-nucleotide TBLASTN searches of the EST database provided only a handful of sequences corresponding to homologs for five PKS-, six NRPS-, and zero HPN-containing biosynthetic pathways.[23, 105]

The second library of ESTs was prepared using *A. niger* ATCC 1015 on an even more extensive assortment of media conditions including arabinose, cellulose, corn fiber, glucose, hemicelluloses-protein, lactose, lignins, rapeseed meal, starch-maltose, wheat bran, and xylose along with other carbon-limited and carbon-nitrogen-limited media.[104]

Examination of the ESTs using nucleotide BLASTN and protein-nucleotide TBLASTN searches identified transcripts from 16 PKS- and NRPS-related biosynthetic pathways.[104] Eleven of the 16 ESTs were identical to those observed in the *A. niger* N402 strain. ESTs for the other five secondary-metabolite-encoding genes corresponded to two PKS-, two NRPS-, and one HPN-containing biosynthetic pathway. Interestingly, results of *A. niger* ATCC 1015 grown under a broad range of nutrient conditions revealed that approximately 29% of PKS, NRPS, and HPN gene clusters were expressed.[23]

The results of the EST library studies supported that axenic cultures of *A. niger* generated only a modest portion of its genomically-encoded natural products under *in vitro* culture conditions.[23, 104, 105] Screening of 18 epigenetic modifying agents (five DNA methyltransferase inhibitors (DNMT), seven histone deacetylase inhibitors (HDAC), four SIR2 deacetylase inhibitors, and two histone methyltransferase inhibitors) **(Table 4.1)** was initiated to determine which inhibitors were capable of increasing the number of secondary metabolites that were biosynthesized by fungus. Initially, cultures of *A. niger* ATCC 1015 were treated during early log-phase growth (24 h post-inoculation) with compounds (10-fold steps from 1 nM to 1 mM) in order to determine the effects of treatments and treatment concentrations by visual observation (*i.e.* cell growth, alterations in phenotype, color changes) when compared controls. Five of the treatment conditions had little to no impact on cell growth at the highest dose, so these were eliminated from further analysis in secondary metabolite diversity. Out of the remaining conditions explored, alterations in the culture morphology were visually observed in five treatments (courmarin, dihydrocourmarin, 7,8 dihydroxycourmarin, hydralazine hydrochloride, and suberoylanilide hydroxamic acid) and a pale to strong

Table 4.1 List of epigenetic modifiers and their effects on mycelial growth and secondary metabolite production in A. niger ATCC 1015.

Compound (mode of action)	Effect on cell growth (log [inhibitor], M)[a]					Secondary metabolite diversity[b]	Notes
	-3	-4	-5	-6	-7		
Acetohydroxamic acid (presumed HDAC inhibitor)	++	+++	+++	+++	+++	ND	No significant changes in growth
5-Azacytidine (5-AZA) (DNMT inhibitor)	+	**++**	+++	+++	+++	10	Culture turned pale yellow; new metabolites confirmed by HPLC
5-Aza-2'-deoxycytidine (DNMT inhibitor)	++	**++**	+++	+++	+++	8	Results similar to 5-azacytidine
Benzohydroxamic acid (presumed HDAC inhibitor)	-	**+**	++	+++	+++	8	Strong color change (yellow) at 10 µM
Coumarin (SIR2 deacetylase inhibitor)	-	++	++	+++	+++	ND	Culture phenotype altered (small pellets)
Dihydrocoumarin (SIR2 deacetylase inhibitor)	-	+	**++**	++	+++	11	Culture phenotype altered (small pellets)
7,8-Dihydroxycoumarin (SIR2 deacetylase inhibitor)	++	++	+++	+++	+++	ND	Little toxicity, culture phenotype altered (small pellets)
Eosin Y (histone methyltransferase inhibitor)	-	+	**++**	++	+++	9	Cells exhibited strong discoloration from dye
Eosin Y disodium salt (histone methyltransferase inhibitor)	-	+	**+**	++	+++	10	Cells exhibited strong discoloration from dye
Hydralazine hydrochloride (DNMT inhibitor)	+	**++**	++	+++	+++	11	Culture phenotype altered (small pellets); strong color change (yellow) at 10 µM
Nicotinamide (SIR2 deacetylase inhibitor)	+	**++**	++	+++	+++	10	Strong color change (yellow) at 100 µM
Nicotinic acid hydroxamate (presumed SIR2 deacetylase/HDAC inhibitor)	+	**++**	++	+++	+++	8	Strong color change (yellow) at 100 µM
Procainamide hydrochloride (DNMT inhibitor)	++	++	+++	+++	+++	ND	No significant changes in growth
Procaine hydrochloride (DNMT inhibitor)	++	++	+++	+++	+++	ND	No significant changes in growth
Sodium butyrate (HDAC inhibitor)	**+**	++	++	+++	+++	8	No significant changes in growth
Splitomicin (HDAC/sirtuin inhibitor)	-	**+**	++	+++	+++	7	Poor solubility of splitomicin in aqueous medium
Suberohydroxamic acid (HDAC inhibitor)	**+**	++	+++	+++	+++	4	Culture turned pale yellow
Suberoylanilide hydroxamic acid (SAHA) (HDAC inhibitor)	+	**++**	++	+++	+++	9	Culture phenotype altered; strong color change at 100 µM; new metabolites confirmed by HPLC

[a] Growth of *A. niger* was qualitatively assessed by visual inspection of replicate cultures. Fungal growth was scored as "+++" (growth equivalent to control cultures), "++" (<50% reduction in growth), "+" (≥50% reduction in growth), or "-" (no growth observed). Growth scores indicated in bold and underlined represent the concentration of epigenetic modifier used for assessing secondary metabolite chemical diversity

[b] Chemical diversity was determined as the number of new spots observed from crude culture extracts by silica gel TLC. Plates were developed with toluene-ethyl acetate-acetic acid (50:50:1 by volume). A designation of "ND" indicates that the chemical diversity of that particular treatment was not determined

yellow color appeared in eight of the treatments (5-azacytidine, 5-aza-2'-deoxycytidine, benzohydroxamic acid, hydralazine hydrochloride, nicotinamide, nicotinic acid hydroxamate, syberohydroxamic acid, and suberoylanilide hydroxamic acid).

The extracts of the thirteen (out of 18) remaining treatments were defatted and prepared for TLC and HPLC, giving a simple, yet accurate means for qualitatively comparing the relative secondary metabolite diversity in treatment versus untreated control groups. In most instances, a range of 8 to 11 "new" secondary metabolites could be visualized on TLC plates under UV irradiation at 254 and 365 nm and/or development by sulfuric acid spray reagent. A significant difference in number of new spots on the TLC plate was observed when suberohydroxamic acid treated cultures (4 new TLC spots) were compared to SAHA treated cultures (9 new TLC spots). This was interesting, since the only difference between these two compounds is that SAHA has a phenyl group substituted for one of the terminal hydroxyl groups in suberohydroxamic acid. Further analysis of the extracts was then performed by HPLC, and during this analysis, a dramatic difference in the profile chromatogram of the SAHA treated culture was observed when compared with the control **(Figure 4.1)**. While differences in the HPLC chromatograms of other treated cultures were not as dramatic as SAHA, there were still observed changes. In the end of the analysis, both SAHA and 5-AZA showed good activities resulting in the production of several new secondary metabolites **(Table 4.1)** and they were selected for further investigation through real-time qRT-PCR given their prior successful use in our lab for generating new natural products.[73] The resulting relative fold changes in expression for the PKS, NRPS, and HPN pathways (determined

by real-time qRT-PCR) were more pronounced in SAHA treated cultures over 5-AZA treated cultures.[23] Under the effects of SAHA, expression levels of 23 of 33 PKS, 14 of 15 NRPS and 6 of 9 HPN gene clusters were upregulated.[23] Overall, under SAHA chemical epigenetic conditions with one media source, upregulation of greater than 85% of the PKS, NRPS, and HPN gene clusters was observed, which is a significant increase to the 29% previously reported under a broad range of nutrient conditions.[23]

Whole-genome sequencing data obtained from a variety of fungi demonstrate that these microorganisms harbor large numbers of PKS, NRPS, HPN, and other natural product biosynthetic gene clusters. Most of these pathways remain suppressed under *in vitro* culture conditions meaning that only a fraction of a fungi's biosynthetic potential is generally accessible for drug screening applications. Based on our observations, we conservatively estimate that fungi express less than half of their natural-product-encoding

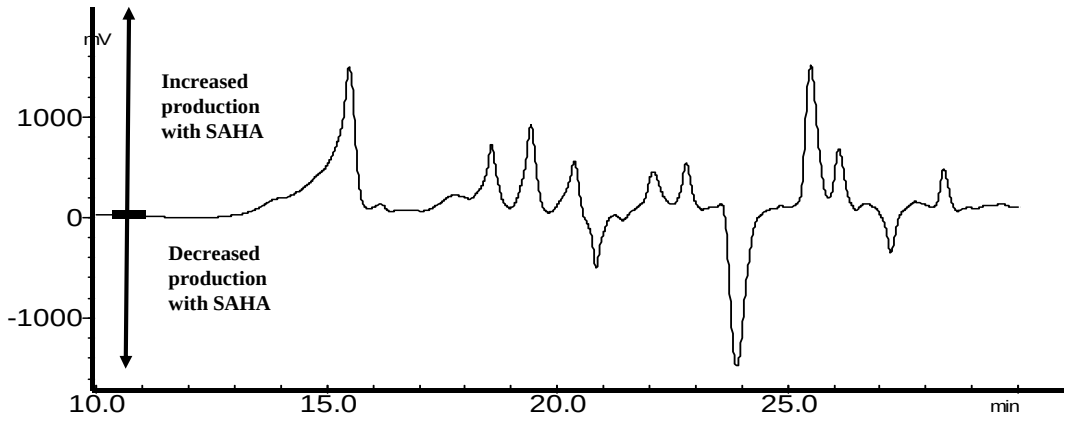


Figure 4.1 HPLC difference chromatogram of SAHA treated versus control cultures observed at a wavelength of 254 nm. Any chromatographic peak above the zero value represents an increased production the in SAHA treated cultures. Any chromatographic peak below the zero value represents a decreased production in the SAHA treated culture. All injections were made at 2 mg of crude extract in 200 µL of MeOH (range of crude extracts were between 9-11 mg).

biosynthetic pathways under standard shake-flask and static culture conditions.

Even *Neurospora crassa*, which has been intensively studied since the 1920s and was considered devoid of any secondary metabolites, has now been shown to possess no less than seven PKS, four NRPS, and several terpene biosynthetic gene clusters that are believed to fulfill a variety of important functions.[80] With the exceptions of fungi from the sub-phyla Saccharomycotina and Taphrinomycotina,[106] most fungi that have been sequenced to date carry a variety of biosynthetic gene clusters encoding for the production of uncharacterized natural products. These compounds are expected to possess a wide range of unique biological activities and therefore have significant value as drug discovery leads.[21, 107, 108] The strict transcriptional suppression of secondary metabolites is conjectured to protect fungi from the potentially deleterious (autotoxic) effects of natural products and their biosynthesis is only elicited when an appropriate environmental trigger is encountered. Based on emerging molecular evidence,[77] we had predicted that the expression of many secondary metabolite pathways is transcriptionally controlled through epigenetic mechanisms.[109, 110] Consequently, we consider the chemical epigenetic manipulation of silent gene clusters to be a highly promising approach for procuring novel drug leads from fungi. This methodology has many significant advantages over currently available molecular-based techniques and cultivation-dependent approaches.[107, 108] Namely, our chemical epigenetic approach offers significantly greater simplicity, broad-spectrum applicability, and an ability to be readily incorporated into modern microbial screening programs.

The precise molecular mechanisms involved in upregulating the expression of silent secondary-metabolite-encoding pathways are likely more complex than what is presumed based on the singular inhibition of specific cellular targets by chemical

epigenetic modifying agents. For example in *Saccharomyces cerevisiae*, the HDAC inhibitor trichostatin A and several HDAC deletion mutants demonstrated a wide variety of transcriptional effects [88]. These included both the upregulation and downregulation of genes involved in cell cycle progression, as well as amino acid and carbohydrate metabolism. Curiously, they noted that the effects of trichostatin A on a subset of *S. cerevisiae* genes were detectable within 15 min of exposure to the inhibitor. This suggests that trichostatin A has other, yet undefined, effects on modifying gene transcription (*e.g.*, direct transcriptional activation).

Similar to our observations, others also have noted that the impact of HDAC inhibitors and HDAC mutants were most pronounced on genes located within the distal regions of fungal chromosomes. For example, Bernstein *et al.*[88] reported that deletion of the yeast HDAC, *RPD3*, preferentially altered the transcription of genes positioned near the termini of chromosomes. Moreover, deletion of the transcriptional regulator *laeA*, whose methyltransferase product was suggested to alter chromatin structure [111], has been shown to decrease the expression of many telomere proximal NRPS gene clusters in *Aspergillus fumigatus*.[72] The dynamics of histone-mediated heterochromatin formation and gene quelling in fungi is proposed to function in a generally linear fashion, exerting an initial silencing influence on the most distally located genes, which progresses proximally over multiple cell division cycles.[112] In view of the complex processes involved in the continual shifting of DNA between euchromatin and heterochromatin states, our data suggest the need for considering the roles of other potential signaling pathways linking outwardly unrelated nuclear and cytoplasmic events to the transcriptional regulation of secondary-metabolite-encoding biosynthetic gene clusters.

For nearly a century, natural products have served as an unrivaled resource in the quest for new drug entities. However, recent concerns and misconceptions in the pharmaceutical industry about the long-term value of natural products as a discovery platform have all but eliminated secondary metabolites from most research and development programs.[113-116] It is likely that the folly of this collective decision will further impair an already flagging drug-discovery pipeline.[2] Fortunately, emerging techniques in the field of natural products discovery, like the genomic and epigenetic methods described in this study, offer an opportunity to reinvigorate the fundamental role of secondary metabolites as a drug discovery tool.

4.3 Materials and Methods

4.3.1. Fungal strain and growth conditions. *Aspergillus niger* ATCC 1015 was obtained from the American Type Culture Collection. Suspensions of mycelia and spores were stored in 15% aqueous glycerol at -80 °C. For experiments, 100 µL samples of the cryopreserved *A. niger* stock cultures were lawned onto potato-dextrose plates (10 g dried potatoes, 5 g dextrose, 1,000 mL DI water, and 20 g agar) and the plates incubated at 25 °C (12 h light/12 dark). Liquid cultures of *A. niger* were prepared by inoculating potato-dextrose broth (10 g dried potatoes, 5 g dextrose, 1,000 mL DI water) with uniform suspensions of spores and mycelia harvested from the potato-dextrose plates. Cultures for compound screening were prepared by adding 100 µL of *A. niger* suspensions to test tubes containing 3 mL of potato-dextrose broth and the tubes were incubated at 25 °C on a rotary shaker (170 rpm). Tube cultures received chemical epigenetic modifier treatments after 24 h and the fungi were incubated an additional six days before being processed. Cultures for total RNA extraction were made by adding 2 mL of *A. niger*

suspensions to 1 L Erlenmeyer flasks containing 250 mL of potato-dextrose broth. Cultures were treated with epigenetic modifiers after incubating for 24 h (25 °C on a rotary shaker at150 rpm) and they were allowed to continue growing for an additional four days before extracting RNA.

4.3.2 Epigenetic modifier screening. Test tube cultures of *A. niger* (pre-incubated for 24 h) were treated with epigenetic modifiers dissolved in H_2O or dimethyl sulfoxide. The solutions of test compounds were filter-sterilized (0.22 µM) before being added to the cultures. After incubating for six days, mycelia growth in each tube was qualitatively scored relative to the vehicle-only controls. Fungal growth was scored as "+++" (growth equivalent to control cultures), "++" (<50% reduction in growth), "+" (≥50% reduction in growth), or "-" (no growth observed).

After scoring all cultures, 3 mL of dichloromethane was added to each tube to extract secondary metabolites. The dichloromethane was removed by aspiration and the organic solvent evaporated *in vacuo*. Prior to analysis, all samples were defatted by partitioning the extracts between aqueous methanol (9:1 MeOH-H_2O) and hexane. After removal of the hexane, the aqueous methanol layers were evaporated *in vacuo* yielding the crude mixtures of secondary metabolites that were used for chemical analyses. Thin layer chromatography (TLC) was performed on the samples by spotting them onto silica gel plates that were developed in toluene-ethyl acetate-acetic acid (50:50:1 by volume). Secondary metabolites were visualized under UV irradiation at 254 and 365 nm and by sulfuric acid spray reagent. Additional visualization of secondary metabolite mixtures was performed on selected extracts by HPLC (C_{18} column using a 10-100% Acetonitrile-H_2O gradient with dual wavelength monitoring at 210 nm and 254 nm) and ^{1}H-NMR

analyses (extracts were dissolved in 650 µL of CD_3OD and spectra generated by averaging 128 scans using a standard ^1H pulse sequence). A list of all the epigenetic modifiers that were screened along with their effects on cell growth and secondary metabolite production are presented in **Table 4.1**.

Chapter 5. Analysis of a Class of γ-pyrones and γ-pyridones Isolated from *Aspergillus niger*

This chapter is adapted from 1) a publication in Organic and Bimolecular Chemistry[86] and 2) a publication that is currently under preparation.

5.1 Introduction

The black aspergilli possess an extraordinarily diverse set of secondary metabolite biosynthetic pathways. In a previous analysis, Nielsen *et. al.* listed 145 metabolites that had been isolated and structurally elucidated from this assemblage of fungi.[117] Further investigation of the genome of one strain from this group, *Aspergillus niger* ATCC 1015, demonstrates the presence of 31 polyketide synthase (PKS), 15 non-ribosomal peptide synthetase (NRPS), and 9 hybrid PKS-NRPS (HPN) gene clusters; yet only 30% of those genes were transcribed under various *in vitro* culture conditions.[23, 104, 105] This demonstrated that there are still many more secondary metabolites to access, isolate, and characterize from *A. niger*.

To address the problem of gene cluster under-expression in laboratory conditions, we demonstrated that treating *A. niger* ATCC 1015 with a small-molecule epigenetic modifier, suberoylanilide hydroxamic acid (SAHA), resulted in a transcriptional upregulation of over 85% of the PKS, NRPS, and HPN gene clusters under a single culture condition.[23] With the ability to access new secondary metabolites, we turned our focus to identifying these compounds. This chapter addresses 1) the elucidation of a new metabolite which was isolated under SAHA-treated epigentic conditions and follow-up analysis regarding this isolated metabolite, 2) the structure revision of published

compounds, and 3) antimicrobial bioactivity of secondary metabolites and semi-synthetic derivatives from *A. niger* ATCC 1015.

5.2 Results and Discussions

Aspergillus niger cultures grown under SAHA epigenetic conditions resulted in the isolation of a unique secondary metabolite, nygerone A (**37**). HRESIMS analysis provided two pseudo-molecular ions at *m*/*z* 419.1542 and 441.1402 corresponding to molecular formulae of $C_{24}H_{23}N_2O_5$ ($[M + H]^+$, calcd 419.1607, −6.5 mmu error) and $C_{24}H_{22}N_2NaO_5$ ($[M + Na]^+$, calcd 441.1426, −2.4 mmu error), respectively. Using these data, a chemical formula of $C_{24}H_{22}N_2O_5$ was proposed for **37** indicating that the fungal metabolite contained a total of 15 double-bond equivalents. Some minor discrepancies between the ^{1}H- and ^{13}C-NMR data for **37 (Table 5.1)** and the MS -derived formula still required reconciliation. For example, integration of the ^{1}H-NMR data collected in methanol-d_4 revealed only 20 hydrogens, but this was rationalized as evidence for the presence of two exchangeable protons in **37**. In addition, the ^{13}C-NMR data provided evidence for 20 unique spins; however, two of these carbons (δ_C 128.9 and 129.0) exhibited significantly greater relative intensities compared to surrounding spins. Therefore, it was surmised that each of these ^{13}C-NMR resonances represented two superimposed carbons. Together, these data offered strong support for $C_{24}H_{22}N_2O_5$ as the chemical formula of **37** which permitted us to proceed with elucidating the metabolite's structure.

Further examination of the 1-D NMR results in combination with data generated from a series of 2-D NMR experiments (^{1}H-^{1}H COSY , ^{1}H-^{1}H NOESY , ^{1}H-^{13}C HSQC , and ^{1}H-^{13}C HMBC) allowed for the establishment of four partial structures (fragments

A-D) for **37 (Figure 5.1)**. Fragment A was determined to contain five aromatic protons [δ_H 6.77 (2H), 7.18 (3H)] belonging to a monosubstituted benzyl group. This was confirmed by ^{1}H-^{13}C HMBC experiment which also gave a strong correlation extending from the aromatic spin at δ_H 6.55 to the benzylic methylene (δ_C 39.4). Similarly, fragment B **(Figure 5.1)** contained five protons in an AA′BB′C system [δ_H 7.06 (2H), 7.42 (2H), and 7.49 (1H)] representative of a monosubstituted phenyl group. Unfortunately, ^{1}H-^{13}C HMBC couplings from the aromatic protons provided no additional evidence as to the identity of the aryl substituent of this fragment.

Table 5.1 ^{1}H and ^{13}C-NMR data for nygerone A **(43)** in $CDCl_3$ at 400 MHz (^{1}H) and 100 MHz (^{13}C)

Position	δ_C	δ_H **(mult., *J* in Hz)**	**HMBC ^{1}H →^{13}C**
[a] Significant overlap occurred between these two sets of resonances.			
C-2	148.2	8.43, 1H (s)	1′, 4, 6, 14
C-3	117.2		
C-4	177.6		
C-5	122.3	6.55, 1H (s)	3, 4, 6, 7, 14 (weak)
C-6	152.0		
C-7	39.4	3.67, 2H (s)	5, 6, 8, 9/13
C-8	134.8		
C-9/13	128.9	6.77, 2H (m)	7, 11
C-10/12	129.0	7.18, 2H (m)[a]	8
C-11	127.7	7.18, 1H (m)[a]	8, 9/13
C-14	163.2		
N-15		12.83, 1H (br s)	17
C-16	173.2		
C-17	42.0	2.86, 1H (dd, 5.1, 17.2); 3.20, 1H (dd, 8.2, 17.2)	16, 18, 19, 20
C-18	35.1	3.05, 1H (m)	16, 17, 19, 20
C-19	178.9		
C-20	17.2	1.27, 3H (d, 7.0)	17, 18, 20
C-1′	140.4		
C-2′/6′	127.0	7.06, 2H (d, 7.6)	1′, 4′
C-3′/5′	130.2	7.42, 2H (t, 7.6)	1′
C-4′	130.5	7.49, 1H (t, 7.6)	2′/6′

Analysis of the protons in fragment C by ^{1}H-^{1}H COSY indicated the presence of an A_2MX_3 spin system which was confirmed by ^{1}H-^{13}C HMBC experiment **(Figure 5.1)**.

Additional $^{2\text{-}3}J_{H\text{-}C}$ correlations extending to two carbonyl resonances (δ_C 173.2 and 178.9) that were positioned at opposing ends of fragment C were also noted. Based on their chemical shifts, both carbonyl groups were presumed to be attached to heteroatoms (Q_1 and Q_2 = N and/or O, **Figure 5.1**); however, the identities of these atoms were not determined at this juncture. Next, we turned our attention to assembling the remaining atoms that comprised fragment D which included six carbons (δ_C 117.2, 122.3, 148.2, 152.0, 163.2, and 177.6) and three heteroatoms (N and/or O). A ^{1}H-^{13}C HSQC experiment showed that two of the carbon atoms (δ_C 122.3 and 148.2) had

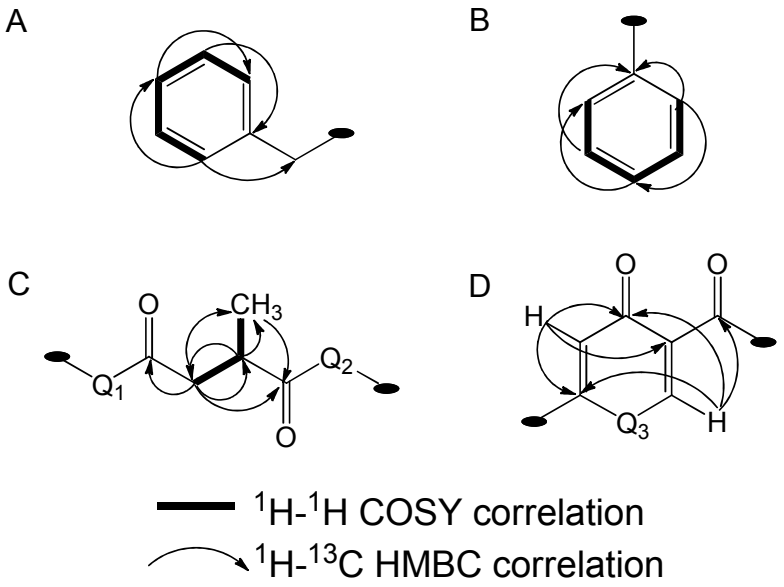


Figure 5.1. NMR spectroscopic data (^{1}H-^{1}H COSY : bold bonds and ^{1}H-^{13}C HMBC : solid curved arrows) used to establish the four partial structures, fragments A-D, for 11. Heteroatoms Q_1, Q_2, and Q_3 were subsequently assigned as N, O, and N, respectively, based on the chemical shifts of adjacent carbon atoms and ^{1}H-^{15}N HMBC correlations as illustrated in **Figure 5.2**.

attached protons (δ_H 8.43 and 6.55, respectively) that appeared as sharp singlet resonances. Two of the other carbons (δ_C 163.2 and 177.6) were determined to be carbonyls based on their downfield shifts. This accounted for two of the five double-bond equivalents in fragment D as well as two hetero-(oxygen) atoms. Similarly, the

chemical shifts for the carbon atoms at δ_C 148.2 and 152.0 suggested that they were both sp^2 hybridized and attached to the remaining heteroatom, Q_3, due to their respective downfield shifts. The identity of Q_3 was determined to be a nitrogen since an oxygen at this position would have resulted in even greater deshielding of the adjacent carbon resonances (i.e. δ_C >160). Our assignment for Q_3 was further confirmed by a ^{1}H-^{15}N HMBC experiment in which the protons in fragment D (δ_H 6.55 and 8.43) exhibited $^{2\text{-}3}J_{H\text{-}N}$ correlations to a nitrogen resonating at δ_N 158.0 **(Figure 5.2)**. The assignments of the carbon atoms at δ_C 117.2 and 122.3 were now simplified since the methine carbons could not be adjacent to one another due to a lack of vicinal coupling between their attached protons. The upfield shifts of these carbons were rationalized to be due to their positions α to a carbonyl, thus fragment D was comprised of a substituted γ-pyridone system.

With working structures proposed for fragments A-D, it was now possible to address the connectivities between these partial structures, as well as clarify the identities of Q_1 and Q_2. Fragment A was joined to the γ-pyridone based on long-range $^{2\text{-}3}J_{H\text{-}C}$ correlations from the benzylic methylene protons (δ_H 6.55, H-7) to carbons at δ_C 122.3 (C-5) and 152.0 (C-6) in fragment D **(Figure 5.1)**. Fragment B was also determined to be attached to fragment D as a result of a $^{3}J_{H\text{-}C}$ correlation from δ_H 8.43 (H-2) to δ_C 140.4 (C-1′). The connection established from the phenyl group through the nitrogen atom yielding a 1-phenylpyridin-4(1*H*)-one system was rather intriguing to us since this unusual substructure has not been previously reported from a natural source. With only two isolated bonds remaining to be joined, it was readily apparent that fragment D was also connected to fragment C through Q_1 or Q_2; however, no long-range coupling could be observed to verify this linkage. Despite this problem, we were able to confirm the

identity of Q_1 based on a $^1J_{H\text{-}N}$ correlation observed in $CDCl_3$ between the proton at δ_H 12.83 (1H, s) and a nitrogen at δ_N 154.8 and an additional $^3J_{H\text{-}C}$ correlation from this proton to the methylene carbon at δ_C 42.0 (C-17). With all of the other heteroatoms accounted for in the molecular formula of **37**, Q_2 was deduced to be the last remaining oxygen atom. We proposed that the connectivity between fragments C and D occurred through the nitrogen atom, rather than the oxygen, since this mono-hydrogenated nitrogen still required one additional covalent bond in order to complete its valence shell.

During the course of our efforts to further optimize $^1H\text{-}^{13}C$ and $^1H\text{-}^{15}N$ HMBC experimental parameters for additional verification of the proposed imide bond in **37**, a doubling of the proton resonances appeared suggesting that the metabolite had begun to degrade. Purification by HPLC yielded two products, nygerone B **(38)** and 2-methylsuccinate **(39) (Figure 5.2)**. The fortuitous appearance of **38** as a hydrolysis product was key to confirming the presence of the imide bond in **37** since the N-15

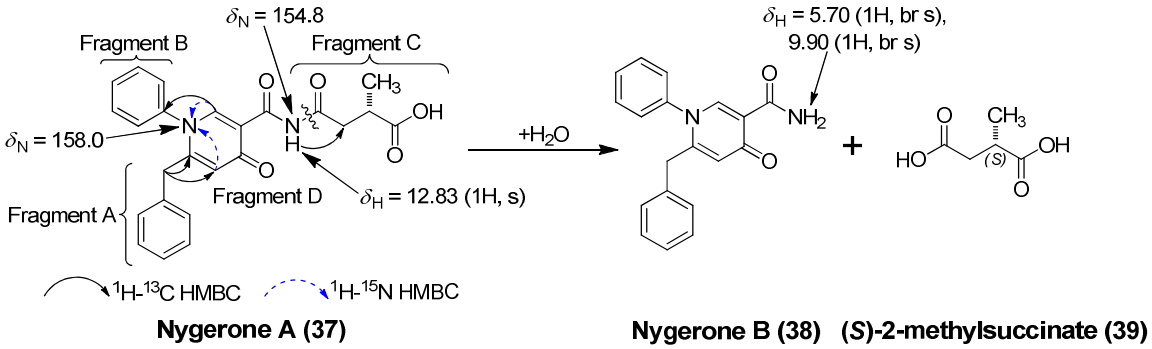
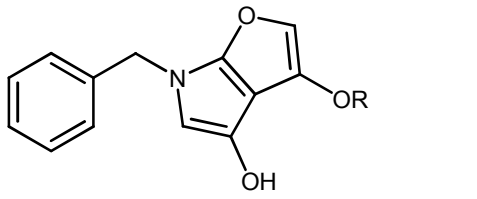
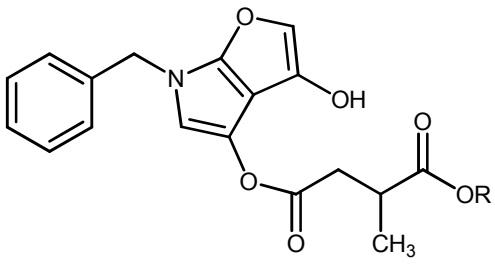


Figure 5.2. Key long-range hetero-nuclear correlation ($^1H\text{-}^{13}C$ HMBC : solid curved arrows and $^1H\text{-}^{15}N$ HMBC : broken curved arrows) and other NMR spectroscopic data used to assemble fragments A-D into 11. The ^{1}H-NMR data of 11 showed a single proton (δ_H 12.83) attached to the imide nitrogen (N-15), while hydrolysis of the N-15 to C-16 bond yielded 12 which displayed two hydrogens attached to the resulting amide nitrogen (δ_H 5.70 and 9.90). The other hydrolysis product, 3, was identified as (*S*)-2-methylsuccinate based on comparisons of its ESIMS , ^{1}H-NMR , and optical rotation data to authentic samples of the *R* and *S* isomers of **39**.

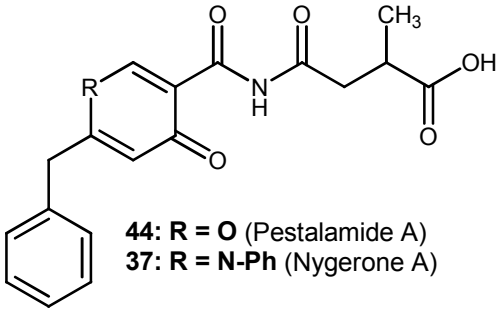
nitrogen atom now exhibited two attached amide protons at δ_H 5.70 (1H, br s) and 9.90 (1H, br s). Interestingly, a similar regio-specific imide hydrolysis process was previously observed as part of the degradation pathway for the quinolactacins from *Penicillium citrinum*.[118] Thus, we were able to rule out an anhydride linkage between fragments C and D and confirm the presence of the imide linkage as illustrated for **37**. With the planar structure of **37** determined, only the absolute configuration of the C-18 asymmetric center remained to be addressed. Polarimetry performed on the purified degradation product **39** gave an optical rotation of $[\alpha]_D^{21}$ −13.3 that was identical to an authentic sample of (*S*)-2-methylsuccinate.



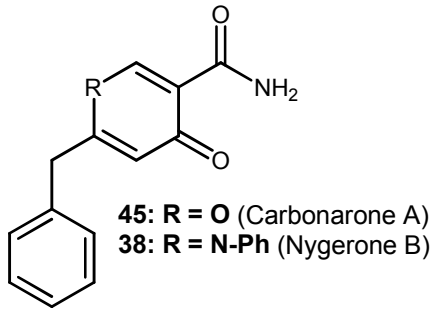
40: R = H (Tensidol A)
41: R = Me (JBIR-86)



42: R = H (Tensidol B)
43: R = Me (JBIR-87)



44: R = O (Pestalamide A)
37: R = N-Ph (Nygerone A)



45: R = O (Carbonarone A)
38: R = N-Ph (Nygerone B)

During the isolation of nygerone A, we detected additional secondary metabolites which displayed similar resonances.[86] The analysis of the ^{1}H- and ^{13}C-NMR data for one of these secondary metabolites demonstrated the presence of a monosubstituted phenyl group (δ_H 7.20-7.38), along with 2 methine protons (δ_H 6.23(s) and δ_H 8.71(s)), and one methylene proton (δ_H 3.87(s)). Key ^{1}H-^{13}C HMBC correlations were observed for the methine protons at δ_H 6.23 and δ_H 8.71 to carbons at δ_C

119.8, 164.3, 169.3, and 178.6 and the methylene proton δ_H at 3.87 to carbons at δ_C 116.5, 128.3, 134.2, and 169.3. This NMR data led to two published compounds, tensidol A **(40)**[119] and carbonarone A **(45)**,[120] which contained the identical ^{1}H- and ^{13}C-NMR data. We conjectured that one of the structures may have been incorrectly elucidated, making a structural revision and unification of the published compounds necessary.

Recently, Takagi *et al.* reported the isolation of two new tensidols, JBIR-86 (**41)** and JBIR-87 (**43)**, which contained a methoxy ester in place of a hydroxy in position 3A of tensidol A **(40)** and methoxy group in place of the acid hydroxyl group of tensidol B **(42)**.[121] Since there were no other structures published in the literature which matched the NMR (^{1}H, ^{13}C, *J*-coupling) and MS data for JBIR-86, we proposed several alternative structural candidates which could fit the published MS and NMR data. Initially, many of the proposed structures were eliminated simply by looking for incompatibilities in theoretical ^{1}H-NMR resonances and *J*-couplings, with particular focus on the presence of the two methine singlets observed at δ_H 6.41 and 8.43 in JBIR-86. After this initial screening and elimination of proposed structures, compounds **46-48 (Figure 5.3A)** were left having the best fit to the methine singlets and rest of the NMR data. Under further scrutiny, compound **46** was eliminated based on the estimated ^{13}C-NMR resonance of position 2 derived from previous reports of ^{13}C-NMR resonance of position 2 of γ-pyridones, aspernigrin A[122] and pestalamide B.[123, 124] In this instance, the expected δ_C would be 15 to 20 ppm upfield from the reported value in JBIR-86. Compound **47** was also eliminated since the methyl group on the proposed *N*-Me moiety would fail to match

the reported resonances for carbon and proton (δC 52.8, δH 3.87). Inspection of **48** provided the best overall fit with the published ^{13}C-NMR resonances at position 2 of the γ-pyrones, carbonarone A[120] and pestalamide A,[123] along with predicted values for the methoxy carbon and proton chemical shifts. However, **48** presented a problem in regards to the fact that the MS data for this compound would be approximately 1 *amu* larger than the published data for JBIR-86. Due to the difficulties that have been presented throughout the structure elucidation history of these compounds,[119, 120, 122, 123, 125] we hypothesized that 1) JBIR-86 was incorrectly elucidated as well, 2) there was an error in the reported mass for JBIR-86, and 3) JBIR-86 actually has the structure of **48**. In order to confirm these hypotheses, we proceeded with computational analyses of **41** and **48** and full synthesis of **48**.

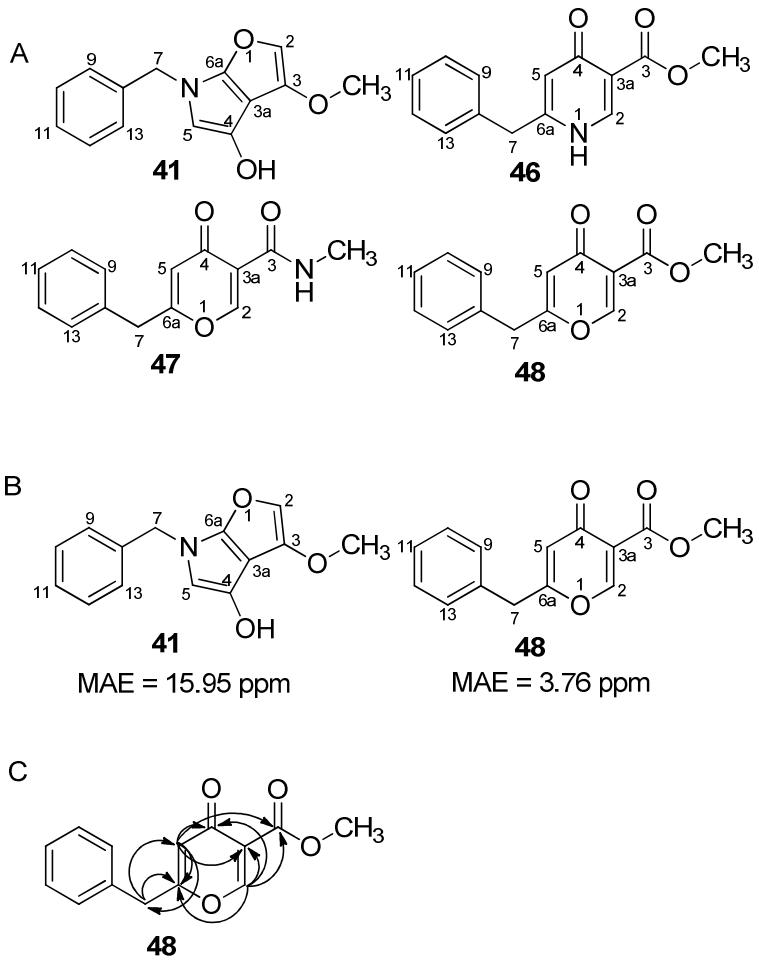


Figure 5.3 A) Compounds **(46-48)** which demonstrated the best initial fit to the NMR data published for 41. B) Numbering scheme utilized for measurements in computational analysis. The mean average error (MAE) was caluculated by $\Sigma(\delta_{calc} - \delta_{exp})$/# of carbons. A smaller MAE value represents a better overall agreement between the computational and published data for ^{13}C-NMR chemical shifts. C) Key ^{1}H-^{13}C HMBC correlations observed in synthesized compound which matched with the literature values of **41**.

Over the last decade, the use of DFT calculations using Gauge-Including Atomic Orbital (GIAO) to theoretically determine ^{13}C-NMR chemical resonances have aided in clarification and correction of structural features in natural products.[126-128] With the success that had been previously reported using density functional theory (DFT) calculations, computations using DFT were utilized for JBIR-86 **(41)** and **48** to test the hypothesis that the tensidol core was incorrectly elucidated. The resulting deviations between calculated and measured ^{13}C-NMR chemical shifts of JBIR-86 and **48** is shown in **Figure 5.4** and demonstrates a dramatic difference in the agreement of the computationally predictive values versus the published ^{13}C-NMR data for JBIR-86 (mean mean absolute error (MAE) was calculated for **41** and **48** (**Figure 5.3B**) found to be 15.95 and 3.76 ppm, respectively). Further analysis of these results demonstrated that both compounds had good calculated ^{13}C-NMR chemical shift agreement where structural similarity was present (*i.e.* the phenyl ring), however, when directly comparing the data for the furopyrrol ring of JBIR-86 and the γ-pyrone ring of **48**, significant deviations were observed. In JBIR-86 at positions C-2, C-3, C-3a, C-4, C-5, and C-6a, labeled in **Figure 5.3B**, the average deviation of the calculated ^{13}C-NMR chemical shifts from published values[121] was 30.2 ppm in the furropyrol ring of JBIR-86, while the average deviation in the same positions of the γ-pyrone ring of **48** was 4.4 ppm. These results strongly suggest that JBIR-86 and other tensidols (**40**, **42**, and **43**), have been

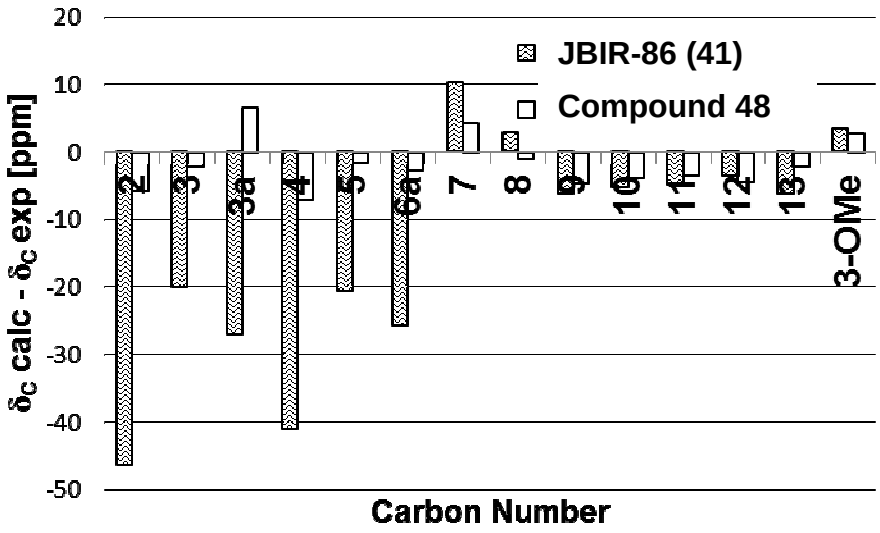
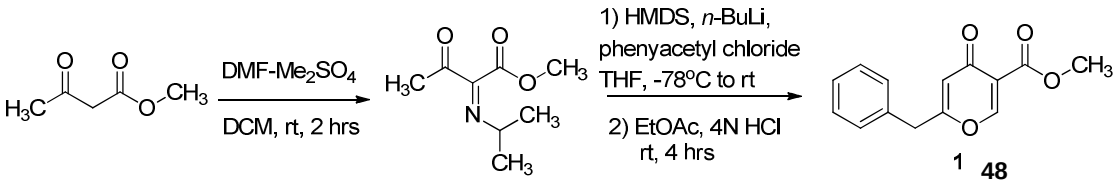


Figure 5.4 Deviations in the calculated versus measured ^{13}C-NMR chemical shifts for **41** and **48.** Differences close to zero demonstrate better agreement of structurally calculated values versus reported ^{13}C-NMR experimental data. Larger differences signify poor agreement between structurally calculation ^{13}C-NMR values and reported ^{13}C-NMR experimental data.

incorrectly assigned, and that the true structure is represented by the γ-pyrone structure shown in **44, 45,** and **48**.

Synthesis of **48** was undertaken using a modified version of a scheme previously utilized by McCombie *et. al.*,[129] as shown in **Scheme 5.1**, to add further support that **48** was the true structure. After purification of the synthesized compound, HRESIMS



Scheme 5.1. Method used to synthesize proposed **48.**

analysis showed a peak with *m/z* at 245.0815 $[M + H]^+$ indicating the molecular formula of $C_{14}H_{12}O_4$ (calc. for $C_{14}H_{13}O_4$, 245.0814). The ^{1}H- and ^{13}C-NMR analysis of the

synthesized compound showed excellent agreement to the published chemical shifts of JBIR-86 **(Table 5.2).** Furthermore, the key correlations from the ^{1}H-^{13}C

Table 5.2 Comparison of experimental ^{1}H- and ^{13}C-NMR of **48** versus the published values of JBIR-86.

	Synthesized Compound 48		**Literature Values for JBIR-86 (41)**	
Position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
2	8.41, s	162.0	8.43, s	162.0
3a		119.3		119.1
4		175.0		175.0
5	6.19, s	116.9	6.21, s	117.3
6a		167.4		167.6
7	3.81, s	39.7	3.83, s	40.0
8		133.9		134.1
9,13	7.22, m	129.1	7.24, m	129.4
10,12	7.35, m	129.1	7.32, m	129.4
11	7.32, m	127.8	7.32, m	128.0
3		163.6		163.9
3-OCH_3	3.85, s	52.7	3.87, s	52.8

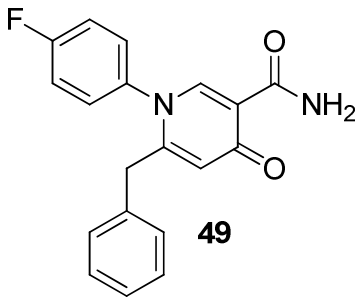
HMBC added additional support that **48** was the correct structure **(Figure 5.3C)**. In compound **48**, long-range couplings from 2-H (δ_H 8.41) and 5-H (δ_H 6.19) were observed to C-3, C-3a, C-4, and C-6a, along with a long-range coupling of the methoxy proton (δ_H 3.85) to C-3. This data aligns precisely with the observed correlations that had been presented with JBIR-86,[121] thus yielding further evidence supporting **48** as the true structure. Extrapolating this data to other tensidol structures, it is apparent that the furopyrrol core of tensidols have been incorrectly elucidated, and that the correct structures contain a γ-pyrone core, as observed in carbonarone A,[120] pestalamide A,[123] and **48**. Even with access to advanced technology and techniques (*i.e.* two-dimensional NMR) which allow for greater structural elucidation power, the importance of thoroughly analyzing the data in order to publish correct structures cannot be forgotten. In this instance, there have been nine subsequent publications that have cited tensidols A **(40)**

and B **(42)**,[117, 121, 130-136] including the structural elucidation of JBIR-86 and JBIR-87, which must now be reevaluated.

Interestingly, the γ-pyrone or γ-pyridinone core shown in these metabolites is relatively new. In a detailed review of secondary metabolites from black aspergilli, it was mentioned that the production of this class of γ-pyrones and γ-pyridones had been shown to be limited to *A. niger* and *Aspergillus tubingensis*.[117] We sought to analyze additional strains of aspergilli for the presence of these compounds under normal and epigentically modified conditions. Using retention time and detection by LC-DAD-ESIMS, the data suggests that these compounds are more prevalent in black aspergilli than originally stated. Based on retention time and mass profiles, pestalamide B was observed in at least one growth condition for each of the 12 aspergilli which were tested. **(Table A2)**. Analysis of the epigentically modified cultures demonstrated neither an increase nor decrease in the number of cultures in which these compounds were present. This indicates that the γ-pyrones and γ-pyridones are produced in these strains under normal culture conditions, and the biosynthetic genes do not appear to be under epigenetic control. Additionally, two other important pieces of information were obtained from this analysis. First, the presence of the methyl succinate moiety of compounds such as pestalamide A and B[123] appears to be play an important role in the biosynthesis of these compounds. As demonstrated, the presence of pestalamide B was always present in the cultures when aspernigrin A was present, however the opposite observation could not be made **(Table A2)**. This data further supports analysis in the review article by Nielsen *et. al.*,[117] indicating that compounds such as **45** and aspernigrin A are most likely artifacts due to hydrolysis of the methyl succinate moiety during the

isolation and purification process, which also matches what was observed during the purification of nygerone A. Interestingly, it is thought that organisms may use the succinate moiety as a means of self-protection from the secondary metabolites being produced.[137] Second, nygerone A **(37)** was only definitively observed in the *A. niger* ATCC 1015 suberoylanilide hydroxamic acid (SAHA) treated cultures. It seemed unusual that only one strain was able to produce nygerone A, so this compound was further explored.

Under further scrutiny of nygerone A, The *N*-phenyl ring of nygerone A was reminiscent of a structural portion of SAHA. Therefore, it was hypothesized that in the course of treating *A. niger* with SAHA to turn on silent pathways,[23] the excess SAHA was catabolized by *A. niger* and incorporated into biosynthesis of nygerone A. To test this, a *para*-flouro SAHA derivative (*p*-F-SAHA) was synthesized **(See Appendix)** and a 50 µM concentration was added to shake-flask cultures of *A. niger* in potato dextrose media. From the crude culture extract of *A. niger* treated with *p*-F-SAHA, LC-ESIMS data showed a molecular ion with an *m/z* of 437.1 $[M + H]^+$, suggesting the presence of nygerone A with fluorine substitution on the *N*-Phenyl ring veryifying the proposed hypothesis. Rapid degradation of this molecular ion occurred during isolation through chromatography which resulted in the loss of the methyl succinate moiety, leaving the fluorinated version of nygerone B. After purification, this new isolate, *p*-fluoro-nygerone B **(49)**, was fully characterized. HRESIMS provided a pseudo-molecular ion at *m*/*z* 323.1192 $[M + H]^+$ (calc. for $C_{19}H_{16}FN_2O_2$, 323.1196). Using this data, a chemical formula of $C_{19}H_{15}FN_2O_2$ was



49

proposed for **49**, which corresponded to the loss of one proton and gain of one fluorine, when compared with nygerone B. Similar ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC correlations were observed when compared to published values of nygerone B, with the exception of one fewer aromatic proton. This was explained by the observation of a ^{19}F-NMR revealed at δ_F = -112.6 ppm. While this demonstrated that *A. niger* was capable of catabolizing excess SAHA and utilizing these components in biosynthetic processes, it still left open the question of whether the epigenetic action of SAHA was necessary for the production of any of the nygerones. To follow up these results, *A. niger* cultures were grown under multiple treatment conditions **(Table 5.3)** to determine if it was possible to observe fluoro-derived nygerones without the presence of *p*-F-SAHA. While both conditions in which the presence of *p*-fluoro aniline (**Table 5.3,** Conditions 3 and 4) showed a lack of fluoro-nygerones by ESIMS, the presence of *p*-fluoro acetanilide (**Table 5.3,** Conditions 5 and 6) yielded the detection of

Table 5.3 Cultures were grown under varying conditions to determine: 1) if suberoyanilide hydroxamic acid was necessary for the production of nygerone-like compounds under amide and free amine conditions and 2) if the amide or free amine alone could produce nygerone-like compounds. A '+' means that this compounds was added to that culture condition, while a '—' means that this compound was not added to that culture condition.

Condition	100 µM *p*-F-SAHA	100 µM *p*-F-acetanilide	100 µM *p*-F aniline	10 µM SAHA	DMSO	Nygerone C Observed?
1	—	—	—	—	+	No
2	+	—	—	—	+	Yes
3	—	—	+	—	+	No
4	—	—	+	+	+	No
5	—	+	—	—	+	Yes
6	—	+	—	+	+	Yes

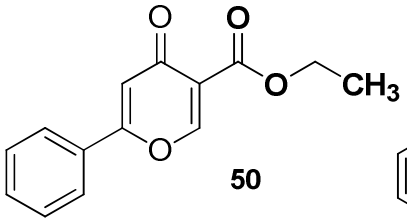
fluoro-nygerones by ESIMS and ^{19}F-NMR. Further inspection through a BLAST search of *A. niger* 1015 showed the presence of gene clusters for an acetamidase and a formamidase which did not have homologs in any of the other *Aspergilli* sp. which were studied during the LC-MS analysis. This may be a potential piece to the puzzle regarding

what allows this strain of *A. niger* the ability to catabolize excess SAHA and use the components in biosynthetic processes or it could simply be coincidental.

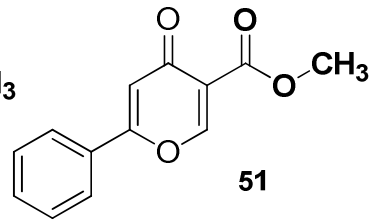
We tested nygerones A and C, pestalamide A, and synthesized γ-pyrones for bioactivity. Initially, compounds **37**, **44**, **48**, **49**, and **50** (**Table 5.4**)[129, 138] were screened against a suite of bacterial and fungal strains. All compounds showed a lack of significant toxicity when tested against the bacterial strains, and only **48** and **50** demonstrated any significant activity against fungal strains between 10 µg/mL to 100 µg/mL. Compound **50** had a slightly better profile of activity, when compared to **48**, sinc activity against *Aspergillus fumigatus*, *Aspergillus flavus*, and *Candida crusei* was observed at 10 µg/mL. The primary differences between the two compounds were that **50** contained an ethoxy and lacked a methylene between the pyrone and phenyl ring, while **48** contained a methoxy and a methylene linker between the pyrone and phenyl ring . A

Table 5.4 Inhibitory properties of γ-pyrone and γ-pyridone compounds when tested against various bacterial and fungal strains based on change in OD600. '—' represents that the compounds were not active at the highest concentration tested (100µg/mL). '+' represents activity observed at 100µg/mL. '++' represents that compounds were active at 10 µg/mL

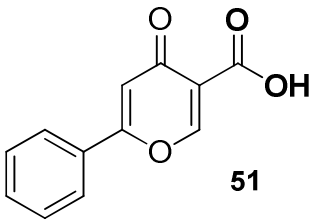
	Compounds						
Microbial Strain	**37**	**44**	**48**	**49**	**50**	**51**	**52**
Acinetobacter baumannii	—	—	—	—	—	NT	NT
Acinetobacter baumannii BAA	—	—	—	—	—	NT	NT
Burkholderia cepacia	—	—	—	—	—	NT	NT
Enterobacter cloacae	—	—	—	—	—	NT	NT
Escherichia coli 10798	—	—	—	—	—	NT	NT
Escherchia coli 11775	—	—	—	—	—	NT	NT
Pseudomonas aeruginosa	—	—	—	—	—	NT	NT
Klebsiella pneumoniae 33495	—	—	—	—	—	NT	NT
Klebsiella pneumonia 51503	—	—	—	—	—	NT	NT
Staphylococcus epidermidis	—	—	—	—	—	NT	NT
Staphylococcus aureus 25293	—	—	—	—	—	NT	NT
Staphylococcus aureus 700787	—	—	—	—	—	NT	NT
Candida albicans	—	—	+	—	+	+	—
Candida glabrata	—	—	+	—	+	+	—
Candida krusei	—	—	+	—	++	++	—
Candida parapsilosis	—	—	+	—	+	+	—
Candida tropicalis	—	—	+	—	+	+	—
Aspergillus fumigatus	—	—	+	—	++	++	—
Aspergillus flavus	—	—	++	—	++	++	—



50



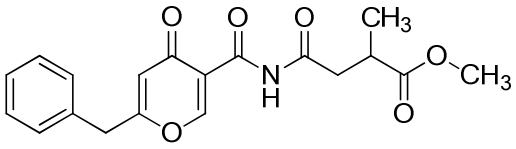
51



51

brief exploration into the structure-activity relationship of the compounds was undertaken by creating derivatives of **50** (described in materials and methods). The results of this assay demonstrated that **51** had a similar range of activity as **50**, however, the acid moiety on **52** resulted in a loss of activity in the compound. This suggests that the activity is dependent on the presence of the carboxylate moiety in **48**, **50**, and **51**, while the presence of a carboxamide (pestalamide A and B and nygerone A) or a carboxylic acid **(52)** results in the loss of bioactivity. In addition, the improved activity of **50** and **51** over **48** suggests that the loss of the methylene between the pyrone and the aromatic rings aids in the potency of the molecules.

This chapter demonstrates the need for exploration and detective work to obtain a deep understanding of scientific data since explanations may be more complex than they first appear. For example, difficulties in the characterization of compounds from this class of γ-pyrones and γ-pyridnones have been observed since they were first isolated.[122, 125, 139] The significant number of correlations in the ring understandably led researchers to incorrectly elucidate the tensidol structures, and would have benefited from other means of support (*i.e.* DFT calculations) to deeply explore all potential structural leads during the elucidation process. The data collected through computations and synthetic methods shows that tensidol's furopyrrol core has been incorrectly assigned, and that the true structure is that of a γ-pyrone core. Since tensidol A and B were isolated and characterized prior to the respective carbonarone A and pestalamide A, precedence



53

suggests that the name of tensidol A and B remain, but the structure is revised to **45** and **44**. In addition, the revisions of JBIR-86 and JBIR-87 are shown in **48** and **53**, respectively. Another example is nygerone A, which was isolated and first thought to be biosynthesized due to the use of small molecule epigenetic modifiers accessing epigenetically controlled gene pathways. However, further evaluations of our initial hypothesis led to a revision in this thought with the realization that *A. niger* is a very biosynthetically talented organism. In turn, nygerone A turned out to be produced due to the ability of *A. niger* to catabolize SAHA and incorporate these molecular components into biosynthetic pathways. This does not diminsh from previous results which illustrate that SAHA upregulates biosynthetic gene pathways under epigenetic control,[23] but demonstrates that additional thought needs to go into the process of isolating the new compounds.

5.3 Materials and Methods

5.3.1 General Chemical Methods. Initial separation of secondary metabolite mixtures was performed by MPLC using HP-20SS resin using a step gradient of 20:80 MeOH-H_2O to 100:0 MeOH-H_2O. Further isolation and purification was performed using preparative and semi-preparative HPLC using Phenomenex C_{18} Gemini columns. ESI-LC-MS data was collected using a Thermo-Finnigan Survery LC system and a Finnigan LCQ Deca mass analyzer. HRESIMS data was obtained from electrospray ionization acquired on a time of flight mass spectrometer. Samples for mass determination were dissolved in MeOH and introduced for ionization with a 20 μL loop on an auto injector system. NMR data were obtained on a Varian NMR spectrometer at

400 MHz (^{1}H) and 100 MHz (^{13}C) or at 500 MHz (^{1}H) and 125 MHz (^{13}C) and processed using VNMRj software. All solvents were of ACS grade or better.

5.3.2 Fermentaion, Extraction, and Isolation of Nygerone A (37).

Cryopreserved sample of *Aspergillus niger* ATCC 1015 was removed and plated on potato-dextrose agar and grown for 14 days. Hyphae were suspended in sterile water and used in a in the inoculation of 28 Fernbach-style flasks, each containing 150 mL of a semi-solid vermiculite-based medium (0.2 grams cornmeal, 0.2 grams steel-rolled oats, 0.2 grams white rice, LB broth (20 g/L)) and treated with 50 μM of SAHA. Cultures were maintained under static conditions (12 h light/12 h dark, 25 °C) for two weeks. Cultures were harvested by adding 500 mL of ethyl acetate to each flask and after 12 h of extraction, the ethyl acetate and aqueous layers were decanted and filtered, the organic layer was removed by partitioning, and the solvent was evaporated yielding 195 mg of ethyl acetate soluble organic matter. The total organic extract was subjected to repeated reversed-phase preparative and semi-preparative HPLC under MeOH-H_2O and Acetonitrile-H_2O gradient conditions providing 1.9 mg of nygerone A (**37)** as an off-white to slightly yellowish amorphous solid. Nygerone A **(37)**: slightly yellow amorphous solid; HRESIMS *m/z* 419.1542 $[M + H]^+$ (calc. for $C_{24}H_{24}N_2O_5$, 419.1607 $[M + H]^+$. See **Table 5.1** for ^{1}H- and ^{13}C-NMR data.

5.3.3 Nygerone B (38): ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 3.66 (2H, s, H-7), 5.70 (1H, br s, N-H_a-15), 6.48 (1H, s, H-5), 6.80 (2H, m, H-9/13), 7.05 (2H, d, J = 7.6 Hz, H-2′/6′), 7.17 (2H, m, H-10/12), 7.18 (1H, m, H-11), 7.40 (2H, t, J = 7.6 Hz, H-3′/5′), 7.47 (1H, d, J = 7.6 Hz, H-4′), 8.42 (1H, s, H-2), 9.90 (1H, br s, N-H_b-15); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C 39.2 (C-7), 118.1 (C-3), 121.8 (C-5), 126.9 (C-2′/6′), 127.5 (C-11),

128.7 (C-9/13), 128.7 (C-10/12), 129.7 (C-3′/5′), 130.0 (C-4′), 134.7(C-8), 140.4 (C-1′), 146.9 (C-2), 150.9 (C-6), 166.0 (C-14), 177.8 (C-4); HRESIMS *m/z* 305.1292 $[M + H]^+$ (calc. for $C_{19}H_{17}N_2O_2$ 305.1290).

5.3.4 Density Functional Theory Calculations (DFT). Full geometry optimization and carbon chemical shift calculations for the proposed **48** and JBIR-86 **(41)** were performed on 8 nodes of the Pentium-4 Xeon54 Quad Core Linux Cluter (running Red Hat Linux) using Gaussian03. Geometry optimization with frequency check and GIAO methods for determining carbon chemical shifts were performed using the basis set, b3lyp/6-31+g(d,p). DFT calculations of tetramethylsilane (TMS) were also performed to determine the carbon chemical shift to be used as a reference. Carbon chemical shifts were referenced by subtracting the calculated δ_C of each carbon from the calculated δ_C of TMS. Analysis of results was performed by comparing the calculated chemical shifts with the published chemical shifts of JBIR-86.[121]

5.3.5 Synthesis of 3-Carbmethoxy-6-benzyl-4-pyrone (48). The general scheme for synthesis of the proposed pyrone followed the previous methodology developed by McCombie *et al.*[129] The enaminone, methyl 2-[(dimethylamino) methylene]-3-oxobutanonte, was synthesized as described in the paper with the modification of using methyl acetoacetate as a starting material instead of ethyl acetoacetate. Methyl acetoacetate (5.4 mL, 0.05 mol) was added to the DMF-Me_2SO_4 adduct in CH_2Cl_2 (75 mL) at 0° C and triethylamine (10 mL) was added over slowly over 3 minutes. The mixture was stirred for 2 hours at room temperature and then washed with aqueous tartaric acid (10% w/v, 100 mL) and water (100 mL × 2) then dried (anhydrous $MgSO_4$). The sample was gravity filtered (Whatman #1 filter paper)

and the filtrate was dried under vacuum. The crude material was subjected to silica gel flash chromoatography (Biotage Isolera) using a $CHCl_3$-ethyl acetate gradient (100:0 to 0:100). The enaminone was then dried under vacuum and further purified by additional flash chromatography using a hexanes-ethyl acetate gradient (90:10 to 0:100). The solvent was dried to give the white solid product methyl 2-(dimethylamino)methylene]-3-oxobutanonte (6.2 g, 72.5%).

To construct the pyrone ring, a solution of hexamethldisilazine in THF was stirred at -78° C and *n*-BuLi in hexanes was added slowly over 2 minutes. After the mixture was cooled, a solution of enaminone (methyl 2-[(dimethylamino)methylene]-3-oxobutanonte) and phenyl acetyl chloride in THF was added over 1 minute. The dry ice-acetone bath was removed and the solution was allowed to warm for 5 minutes. Diethyl ether was added to the reaction, followed by 3N HCl. The solution was allowed to stir rapidly overnight. The following day, the organic phase was washed with saturated sodium bicarbonate (100 mL) and water (100 mL × 2) then dried (anhydrous $MgSO_4$). The sample was gravity filtered (Whatman #1 filter paper) and the filtrate was dried under vacuum. The residue was subjected to silica gel flash chromatography using a CH_2Cl_2-ethyl acetate gradient (100:0 to 0:100). Purification of the γ-pyrone was performed on preparative HPLC (Phenomenex Gemini 5 μm C-18 250 × 20 mm column) using a MeOH-H_2O gradient (50:50 to 100:0) over 60 minutes at a flow rate of 10 mL/minute. The resulting purification resulted in the isolation of a reddish amorphous solid (16 mg, 6%). ^{1}H-NMR (400 MHz, MeOH-d_4): δ 3.84 (2H, s), 3.88 (3H, s), 6.22 (1H, s), 7.23 (2H, m), 7.34 (3H, m), 8.45 (1H, s). ^{13}C-NMR (100 MHz, MeOH-d_4): δ 39.72 (C-7), 52.83 (C-15), 117.22 (C-5), 119.69 (C-3), 128.0 (C-

11), 129.29 (C-10 and 12), 129.35 (C-9 and 13), 134.00 (C-8), 161.77 (C-2), 163.94 (C-14), 167.67 (C-6), 175.15 (C-4). HRESIMS *m/z* 245.0815 $[M + H]^+$ (calc. for $C_{14}H_{13}O_4$, 245.0814).

5.3.6 LC-ESIMS analysis of black aspergilli sp under chemical epigenetic conditions. Freeze-dried spores from thirteen strains of black aspergilli (*Aspergillus aculeatus* NRRL 5094, *Aspergillus japonicus* NRRL 2053, *Aspergillus basiliensis* NRRL 35542, *Aspergillus heteromorphus* NRRL 4747, *Aspergillus carbonarius* NRRL 346, *Aspergillus ibericus* NRRL 35644, *Aspergillus tubingensis* NRRL 4875, *Aspergillus carbonarius* NRRL 369, *Aspergillus parasiticus* NRRL, *Aspergillus ellipticus* NRRL 5120, *Aspergillus niger* NRRL 1956, *Aspergillus niger* NRRL 326, *Aspergillus niger* NRRL 1015) were obtained from the NRRL mycology collection. All strains were grown on appropriate media and cryopreserved in a 15% (v/v) glycerol solution at -80° C. Cryopreserved samples were removed and plated on potato-dextrose agar and grown for 14 days. Hyphae from each aspergilli were suspended in sterile water and used to inoculate 3 1-L Erlenmeyer flasks containing 250-mL of sterile potato-dextrose broth. To each flask for a fungal strain, either 50 μM suberoylanilide hydroxamic acid (dissolved in 50:50 DMSO:H_2O), 50 μM 5-azacytidine (dissolved in 50:50 DMSO: H_2O), or bolus of 50:50 DMSO: H_2O was added. Cultures were grown for 21-days at room temperature under shake flask conditions at 250 rpm. After 21-days, 2% (wt/v) Diaion HP-20 resin was added to each flask and continued to be shaken for 4 hours followed by filtration of the cell mass and resin. The cell mass and resin were collected and then extracted in 2 250-mL aliquots of methanol over a 48-hour period. The methanol extracts were combined and dried

down by rotary evaporation.

Dried extracts were prepared for LC-ESIMS analysis by suspending the extract in a minimal amount of MeOH and then drying to loose C_{18} stationary phase. The dried C_{18} stationary phase, with sample, was then placed in SPE cartridges and rinsed with 5 mL of distilled water followed by 5 mL of methanol, each collected separately, on a vacuum manifold. The separate water and methanol samples were dried in weighed 2 dram vials overnight. The vials containing the dried methanol rinse were weighed and then suspended in a 90:10 MeOH: H_2O solution to give a concentration of approximately 20 mg/mL. A portion of each sample was then pipetted into a 2-mL Ependorff tube and centrifuged at 14,000 RPM for 5 minutes. The crude supernatant in the Ependorff tube was placed in vials and then sampled by LC/MS (Phenomenex Luna 3μm C_{18} 50 x 2 mm column) using a MeOH-H_2O gradient (25:75 isocratic for 3 mintues, 25:75 to 100:0 for 28 minutes, 100:0 for 6 minutes, then re-equilibrated to 25:75 for 3 minutes) at a flow rate of 400 μL/min. The mass spec detector was set to scan a range of *m/z* from 120-1500 mass units. The photodiode array (PDA) detector scanned wavelengths from 200-600 nm, with 3 channels set at 210 nm, 254 nm, and 280 nm. Collected spectra from each sample were analyzed for the presence of known γ-pyrones and γ-pyridinones.

5.3.7 Isolation and Purification of *p*-fluoro-nygerone B (49). Cryopreserved sample of *Aspergillus niger* ATCC 1015 was removed and plated on potato-dextrose agar and grown for 14 days. Hyphae were suspended in sterile water and used in inoculating 20 1-L Erlenmeyer flasks containing 250 mL of sterile potato-dextrose broth (PDB), which were treated with 0.5% (vol/vol) of 50 μM *p*-fluoro-

suberoylanilide hydroxamic acid (*p*-F-SAHA) (dissolved in 50:50 DMSO:H_2O) [Synthesis described in appendix material]. Controls were created by inoculating 4 1-L Erlenmeyer flasks containing 250-mL of sterile PDB and 0.5% (vol/vol) of 50:50 DMSO:H_2O. Cultures were grown on a rotary shaker (150 rpm) for 14 days at room temperature then partitioned three times with an equivalent volume of dichloromethane. The organic phase was collected and dried down by vacuum. The treated and control extracts were generated and processed for LC-ESIMS analysis by methods previously described in **5.3.6**. Further fractionation was performed on the *p*-F-SAHA culture extract by preparative HPLC (Phenomenex Gemini 5 μm C_{18} 250 × 20 mm column) using a MeOH-H_2O gradient (35:65 to 100:0) over 60 minutes at a flow rate of 10 mL/minute, with final purification being performed under varying gradient conditions by semi-preparative HPLC. Purification resulted in the isolation of light-yellow amorphous solid (2 mg). ^{1}H (400 MHz, $CDCl_3$): δ 3.71 (2H, s, H-7), 6.06 (1H, br s, N-H_a-15), 6.61 (1H, s, H-5), 6.83 (2H, m, H-9/13), 7.05 (2H, m, H-2′/6′), 7.10 (2H, m, H-3′/5′), 7.21 (2H, m, H-10/12), 7.23 (1H, m, H-11), 8.49 (1H, s, H-2), 9.89 (1H, br s, N-*Hb*-15). ^{13}C (100 MHz, $CDCl_3$): δ 39.74 (C-7), 116.9 (C-3′/5′), 117.7 (C-3), 121.9 (C-5), 127.7 (C-11), 128.5 (C-9/13), 128.9 (C-10/12 and C-2′/6′), 134.8 (C-8), 146.9 (C-2), 151.3 (C-6), 162.0 (C-1′), 164.1 (C-4′), 166.0 (C-14), 177.7 (C-4). HRESIMS *m/z* 323.1192 $[M + H]^+$ (calc. for $C_{19}H_{16}FN_2O_2$, 323.1196).

5.3.8 Analysis of *A. niger* cultures for nygerone C under varying culture conditions with and without SAHA. Cultures were grown following procedures outlined in **5.3.7**. Six different treatment conditions were utilized as outlined in **Table 5.3** in order to determine what was necessary for the production of **49**. The treated and

control extracts were generated and processed for LC-ESIMS analysis by methods previously described in **5.3.6**.

5.3.9 3-Carbethoxy-6-phenyl-4-pyrone (50). Synthesized and purified based on previous literature methods.[129] The resulting product was a red crystalline solid. HRESIMS *m/z* 245.0816 $[M + H]^+$ (calc. for $C_{14}H_{13}O_4$, 245.0814). ^{1}H-NMR was in agreement with the literature.[129]

5.3.10 3-Carbmethoxy-6-phenyl-4-pyrone (51). Synthesized and purified based on previous literature methods[129], with the use of methyl acetoacetate utilized in place of ethyl acetoacetate. The resulting product was a red crystalline solid. ^{1}H (500 MHz, MeOH-d4): δ 3.86 (3H, s, H-15), 6.94 (1H, s, H-5), 7.53 (2H, m, H-9/11), 7.55 (2H, m, H-10), 7.89 (2H, m, H-8/12). ^{13}C (125 MHz, MeOH- d4): δ 52.3 (C-15), 112.4 (C-5), 118.3 (C-3), 126.2 (C-8/12), 129.6 (C-7), 130.8 (C-9/11 and C-10), 163.0 (C-13), 164.1 (C-6), 166.0 (C-2), 175.6 (C-4). HRESIMS m/z 231.0659 $[M + H]^+$ (calc. for 231.0657, $C_{13}H_{11}O_4$).

5.3.11 3-Carboxylic-6-phenyl-4-pyrone (52). 10 mg of **51** was reacted with 1 *N* NaOH in distilled THF for three hours at room temperature then adjusted to pH 3 with 1 *N* HCl. The resulting solution was partitioned against ethyl acetate, and the resulting organic material was dried down. Purification was performed on preparative HPLC using methods previously described, resulting in 6.2 mg of a yellow amporphous solid. ^{1}H (500 MHz, $(CD_3)_2CO$: δ 6.95 (1H, s, H-5), 7.60 (2H, m, H-9/11), 7.66 (1H, m, H-10), 8.04 (2H, m, H8/12), 9.88 (1H, s, H-2). ^{13}C (125 MHz, $(CD_3)_2CO$): δ 97.6 (C-5), 100.6 (C-3), 126.7 (C-8/12), 129.2 (C-9/11), 130.4 (C-7), 132.8 (C-10), 161.7 (C-13), 167.1 (C-6), 178.5 (C-4), 193.9 (C-2). HRESIMS *m/z* 215.0358 $[M + H]^-$ (calc.

for 215.0350, $C_{12}H_7O_4$).

5.3.12 Pestalamide A (44). HRESIMS *m/z* $[M + Na]^+$ 366.0956 (calcd. for $C_{18}H_{17}NO_6Na$ 366.0954); ^1H- and ^{13}C-NMR were in agreement with published values.[123]

5.3.13 Antibacterial assay. Frozen 20% glycerol stocks of 12 bacterial lines from ATCC **(Table 5.4)** were thawed and diluted into TSB media, 20 µL of stock into 11 mL of media. These stocks were plated to 96-well plates, 100uL per well. Test compounds were diluted in DMSO and added to the wells; final DMSO concentration in the wells was 1%. Plates were shaken orbitally for five seconds and an OD_{600} were taken. Fungal strains were incubated for 24 hours in a humidified air incubator at 37°C. Plates were removed, shaken again, and OD_{600} readings were obtained again. The final OD_{600} was subtracted from the initial OD_{600} to obtain the change in OD_{600}.

5.3.14 Antifungal assay. Frozen 20% glycerol stocks of seven fungal lines from ATCC **(Table 5.4)** were thawed and diluted into YM media, 20 uL of stock into 11 mL of media. These stocks were plated to 96-well plates, 100 uL per well. Test compounds were diluted in DMSO and added to the wells; final DMSO concentration in the wells was 1%. Plates were shaken orbitally for five seconds and an OD_{600} were taken. Fungal strains were incubated for 48 hours in a humidified air incubator at 37°C degrees Celsius. Plates were removed, shaken again, and OD_{600} readings were obtained again. The final OD_{600} was subtracted from the initial OD_{600} to obtain the change in OD_{600}.

Chapter 6. Reassessing the Ichthyotoxin Profile of Cultured *Prymnesium parvum* (Golden Algae) and Comparing it to Samples Collected from Recent Freshwater Bloom and Fish Kill Events in North America

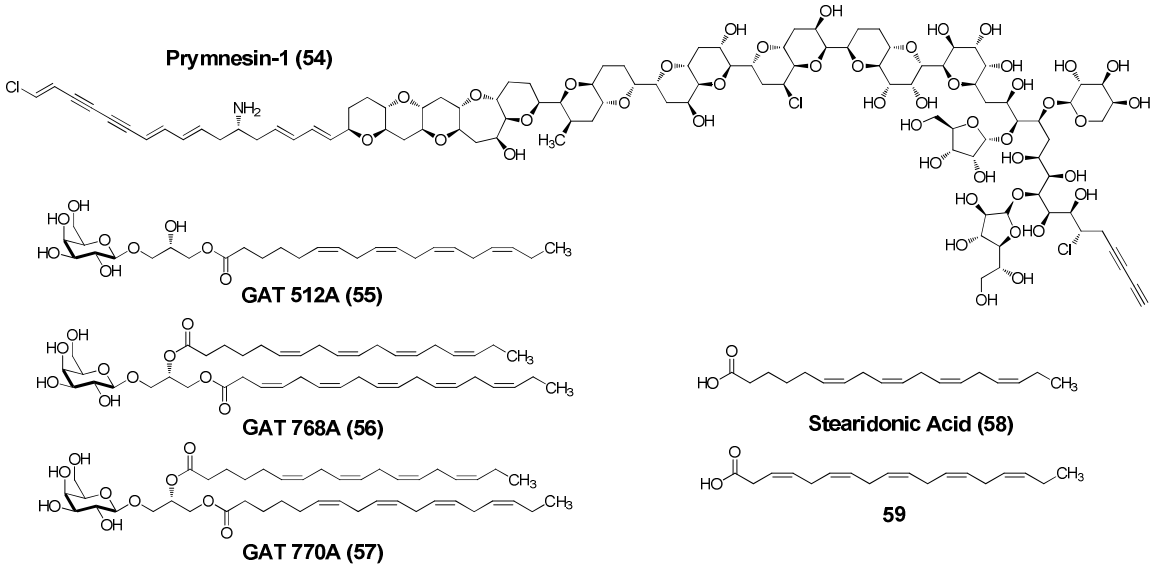
This chapter is adapted from a publication in Toxicon.[140]

6.1 Introduction

The marine haptophyte *Prymnesium parvum* (division Haptophyta, class Prymnesiophyceae),[141] which is commonly referred to as 'golden algae', has played causal roles in scores of massive fish kills in coastal marine and high-to-moderate salinity inland waterways throughout the world.[142-146] In the mid-1980s, the first reported cases documenting the entry of *P. parvum* into North America were marked by multiple large fish kill events in southern Texas, USA.[147, 148] Even more troubling was the realization that *P. parvum* blooms were occurring in moderate-to-low salinity inland lakes, rivers, and reservoirs. This marked a disturbing extension in the range of suitable *P. parvum* habitats[149] causing considerable alarm regarding the ecological and potential human health risks associated with golden algae. Adding to this concern has been the rapid rate of *P. parvum*'s dispersion across the southern United States, and its northerly spread.[150, 151]

Despite its devastating biological effects, the identity of the toxic constituent(s) produced by *P. parvum* has not been definitively established.[152-155] A combination of undefined proteolipids,[156] ceramides,[157] saponins,[158] proteinaceous substances,[159] plasma membrane/plastid components,[159] and proteophospholipids[159] have been credited as

responsible for *P. parvum*'s toxicity. In 1982, Kozaki and colleagues proposed that hemolysin I (a combination of galactoglycerolipids) was the *P. parvum* toxin, but no data substantiating the hypothesis were offered.[160] Later in 1996[161] and 1999,[162] Igarashi and colleagues reported the structure determination of the high molecular weight cyclic polyethers prymnesin-1 ($C_{107}H_{154}Cl_3NO_{44}$) **(62)** and prymnesin-2 ($C_{96}H_{136}Cl_3NO_{35}$)**.** Both of these compounds are potent ichthyotoxins against *Tanichthys albonubes* with LC_{50} values of 8 and 9 nM, respectively.[163] However, none of the prymnesiums have been directly linked to fish kill events and our ongoing LC-ESIMS and toxin isolation studies suggest that these compounds do not accumulate at lethal concentrations under laboratory or natural field conditions.



This study used a bioassay-guided approach to identify some of the ichthyotoxic components in laboratory-grown *P. parvum* cultures. These results were compared to LC-ESIMS and GC-EIMS data obtained for two recent fish kill and bloom events caused by golden algae. Given the increasing levels of human contact with *P. parvum* infested

waters, extracts and pure compounds were also tested against a human cell line. These data support an important toxic role for several polyunsaturated fatty acids in laboratory-cultured *P. parvum*, but do not fully explain the organism's significant ichthyotoxicity at natural bloom sites.

6.2 Results and Discussions

6.2.1 Bioassay-guided ichthyotoxin extraction and compound isolation. We have approached the search for *P. parvum* toxins using an ichthyotoxicity-based bioassay-guided methodology. The fathead minnow (*Pimephales promelas*) has served as a reporter organism for detecting biologically-relevant toxins in golden algae cultures and samples collected at two fish kill sites along the Oklahoma-Texas (Lake Texoma – February 2009) and Pennsylvania-West Virginia (Dunkard Creek – October 2009) borders. Our initial studies applying a modified Kupchan-partitioning scheme to cultured *P. parvum* demonstrated that golden algae toxins accumulated in the organic layer following water-ethyl acetate partitioning. Bioassays performed on the dried and then reconstituted aqueous layer (resolubilized at ×1, ×10 and ×100 its initial concentration) showed that it was devoid of biologically-relevant ichthyotoxic substances. In contrast, the ×1, ×10 and ×100 reconstituted ethyl acetate layer retained the sample's potent ichthyotoxic properties. Notably, this extraction method varies significantly from the elaborate scheme reported for the isolation of intracellular prymnesins.[162]

The ethyl acetate soluble material collected from 50 L of cultured *P. parvum* was subjected to MPLC (HP20SS resin, gradient elution from 30–100% methanol in water followed with a 100% acetone wash). This afforded a single bioactive fraction (100% methanol) that exhibited rapid-onset toxicity against *P. promelas* (note: All fish died

within ~20 min when the extract was reconstituted at ×1 its original concentration). We have observed that fish placed in water taken from highly toxic blooms and lab-grown cultures exhibit similar toxicological symptoms that include excessive mucus production near the gills, hyperventilation, and an impaired righting reflex. These symptoms usually appeared within 10-20 minutes of exposure to toxic water and fish typically ceased visible movement within 20-30 minutes. A similar set of symptoms is reportedly brought on by eicosapentaenoic acid.[164] The ichthyotoxic sample was subjected to preparative-scale HPLC (reversed-phase octadecyl silica gel, gradient from 40-100% acetonitrile in H_2O) yielding a single bioactive fraction. The ^{1}H-NMR spectrum of the toxic fraction indicated that the sample was composed of several metabolites exhibiting spins spanning the region from δ_H 0.5–5.5, which were striking similarities to data reported for galactoglycerolipid and polyunsaturated lipid toxins previously isolated from certain unicellular marine organisms[165-168] including *P. parvum*.[160] Subsequent semi-preparative reversed-phase HPLC performed on a portion of the bioactive sample yielded a series of fractions that exhibited greatly diminished toxicity, which was spread across several consecutive fractions. However, even after recombining the samples, the ichthyotoxic properties of the mixed fractions were substantially reduced (>10-fold loss of toxicity) and all samples were devoid of activity following brief storage (dried and held for < 24 h at 4 ºC). Returning our attention to the parent preparative HPLC fraction, we found that it still retained toxicity and so we set about characterizing the sample's three major components.

Positive-ion HRESIMS of the purified metabolites provided quasi-molecular ions $[M+Na]^+$ at *m/z* 535.2842 (calcd for $C_{27}H_{44}NaO_9$, 535.2883), 791.4678 (calcd for

$C_{45}H_{68}NaO_{10}$, 791.4710), and 793.4801 (calcd for $C_{45}H_{70}NaO_{10}$, 793.4867) for metabolites **55** (GAT 512A), **56** (GAT 768A), and **57** (GAT 770A), respectively. In order to provide a simple naming scheme for compounds **55**, **56**, and **57**, we have proposed the use of the term "GATs" (golden algae toxins) rather than the more lengthy IUPAC nomenclature. Each GAT is assigned a unique identifier number based on its molecular weight, which is followed by a letter code to distinguish isomers from each other. Accordingly, compounds **55**, **56**, and **57** have been termed GAT 512A, GAT 768A, and GAT 770A, respectively. Further data obtained from 1D- (^{1}H and ^{13}C-NMR) and 2D- (COSY, HSQC, and HMBC) NMR (Supplementary Information, Table 1), controlled chemical and enzymatic degradation experiments, and optical rotation measurements of the metabolites and their respective hydrolysis products allowed us to confirm the structures of these compounds. The identities of these compounds were also confirmed by comparisons of our data with values published in the literature for **55**,[169] **56**,[167] and **56**.[167] Surprisingly, **55-57** exhibited no ichthyotoxicity against *P. promelas* (up to 430 µM); however, we did observe modest cytotoxicity for **55** in a human (MDA-MB-435) cancer cell line (IC_{50} 24.2 ± 5.12 µM).

6.2.2 Esterase mediated liberation of ichthyotoxic fatty acids. Further consideration of structures **55-57,** suggested that GATs might function as protoxins since the polyunsaturated *O*-alkyl esters moieties found within these metabolites could be readily hydrolyzed resulting in the release of their corresponding carboxylic acid derivatives. Similar metabolites have been implicated as potent toxins against fish[164] and isolated fish cell lines.[170] We hypothesized that esterases, which are ubiquitous hydrolytic enzymes found in aquatic ecosystems,[171] could readily catalyze the release of

polyunsaturated fatty acids from the **55-57**. We tested this by incubating **55-57** with assorted esterases and this resulted in the liberation of a potent mixture of ichthyotoxins that included stearidonic acid (**58**) and its analog **59**. None of the controls utilizing heat denatured esterases showed any ichthyotoxic properties **(Figure 6.1)**. More importantly, tests performed using purified and commercially available **58** confirmed that the concentration of this fatty acid alone was sufficient to cause rapid-onset toxicity (LC_{50} 21.9 ± 6.3 µM; all fish dead in < 20 minutes at 40 µM) and other distinct pathological features that are typically observed when *P. promelas* is exposed to *P. parvum* cultures.

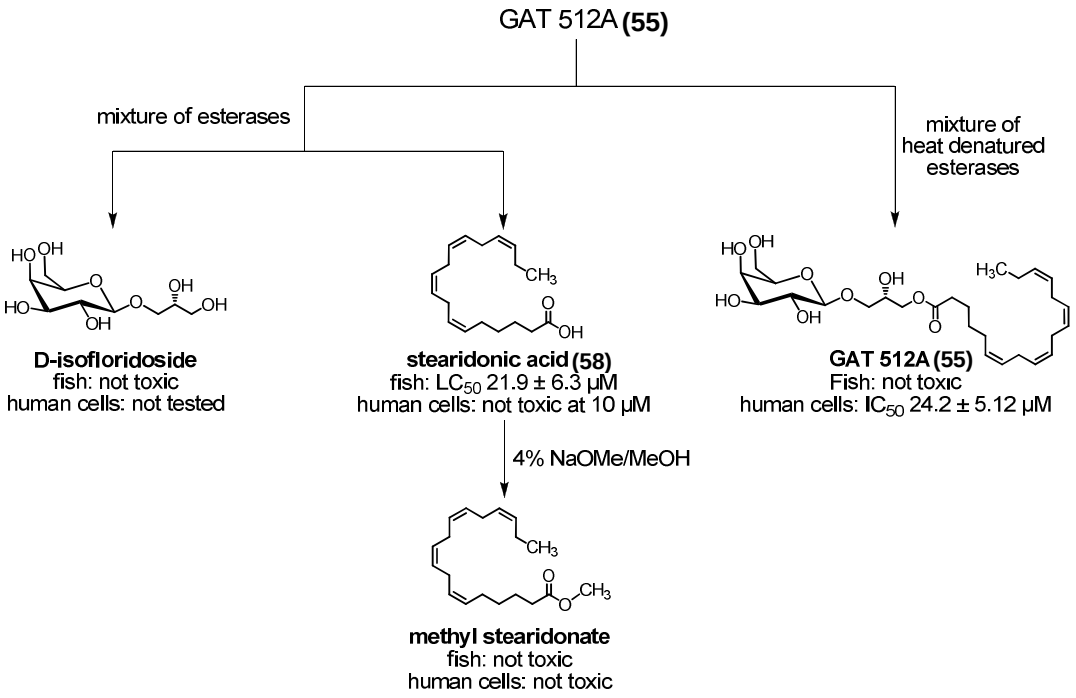


Figure 6.1 Assessment of the ichthyotoxicity and mammalian cell cytotoxicity of compounds obtained from the enzymatic hydrolysis of compound **55** Incubation of **55**with a lipase/esterase mixture resulted in the liberation of D-isofloridoside and **58**, which was ichthyotoxic. Conversion of **58** to its methyl ester derivative in the complete loss of ichthyotoxicity. Incubation of 2 with a heat denatured lipase/esterase mixture yielded unconverted **55**, which was not toxic to fish, but did exhibit modest cytotoxicity toward mammalian cells.

6.2.3 Bioassay analysis of fatty acids. We expanded our testing of **58** to include several of its derivatives (*i.e.*, methyl steridonate, steridonoyl glycine, and stearic acid); however, none of these compounds were toxic to fish at concentrations of ≤ 40 µM **(Figure 6.1)**. An examination of 16 additional lipids led to the identification of several other fatty acids that displayed similar or increased potency against fish (docosahexanoeic acid: LC_{50} 4.7 ± 1.3 µM; eicosapentaenoic acid: LC_{50} 23.6 ± 9.0 µM, arachindonic acid: LC_{50} 9.2 ± 0.8 µM, and pinolenic acid: LC_{50} 18.2 ± 5.9 µM**) (Figure 6.1)**. These results suggest that a combination of a carboxylic acid and ≥2 *cis* double bonds are necessary to confer ichthyotoxic properties to fatty acids. Given the modest size of our lipid library, further speculation regarding the structural features required to impart ichthyotoxic properties to fatty acids is not possible at this time. The cytotoxicity of the lipids was also tested against human cells (MDA-MB-435); however, none of the compounds inhibited cell proliferation at concentrations ≤ 10 µM.

6.2.4 GC-EIMS assessment of cultured *P. parvum* lipids and comparisons to water from bloom and fish kill sites. Tests performed by GC-EIMS on laboratory grown *P. parvum* enabled us to determine that this organism produces a complex suite of lipophilic metabolites. A representative GC-EIMS trace of a lipid profile taken from a 20 day old *P. parvum* culture is provided in **Figure A16**. The bulk of this mixture was found to consist of a combination of saturated and unsaturated C_{16}, C_{18}, C_{20}, and C_{22} compounds. In view of the toxicity data obtained from tests performed with our lipid library **(Figure 6.2)**, we conjecture that in addition to **58**, some of the other polyunsaturated C_{20} and C_{22} metabolites might contribute to the ichthyotoxicity profile of cultured *P. parvum*.

We also assessed the quantity of **58** in cultured *P. parvum* since this compound represented one of the most prevalent (second only to myristic acid) substances in the mixture and we were able to secure authentic samples for analytical comparisons. A seemingly positive correlation was observed between *P. parvum* cell numbers, the concentration of **58** in cultures, and toxicity of culture water against *P. promelas* **(Table 6.1)**. Specifically, we noted that as the cell densities of *P. parvum* cultures approached 10^6 cells/mL, the levels of **58** significantly exceeded the compound's LC_{50} value **(Table 6.1).**

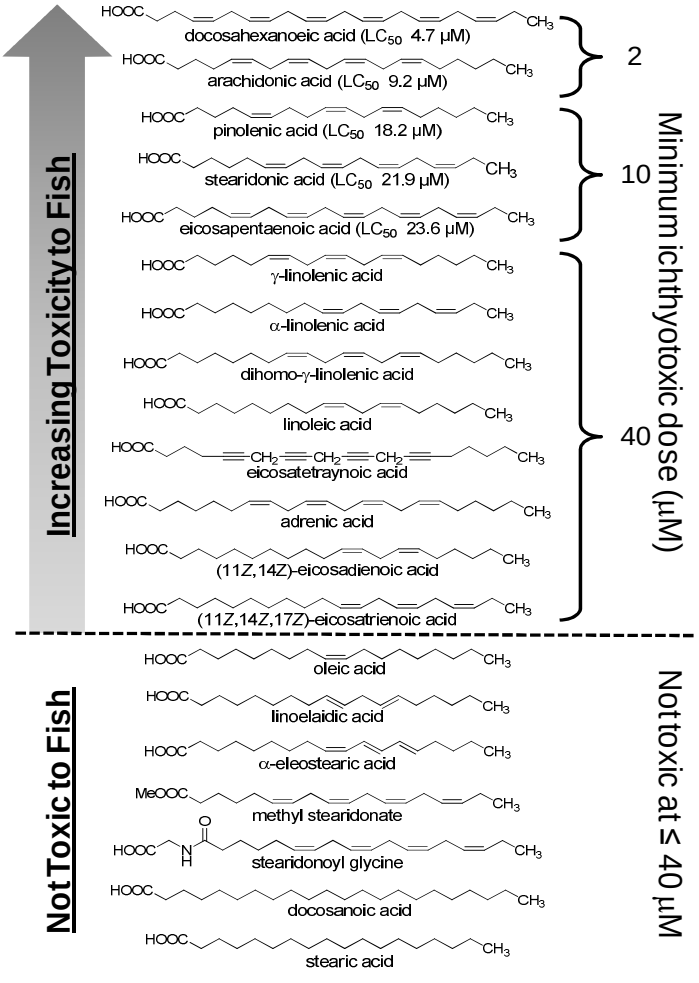


Figure 6.2 Library of lipids tested for ichthyotoxic properties. Compounds are arranged based on their relative potencies against *P. promelas* fry.

Next, we compared the results from cultured golden algae with data from field-collected water samples obtained from recent fish kill/bloom sites. We immediately discerned two substantial differences between laboratory-grown golden algae and field samples taken from recent blooms: 1) the cell density for wild *P. parvum* populations did not reach the same high levels achieved in laboratory cultures and 2) the

concentration of **58** was proportionally reduced. While our analysis of field-collected samples did confirm the presence of **58** at bloom and fish kill events **(Figure A18 and A16)**, its concentrations during these periods failed to reach toxic levels (< 0.5 μM) **(Table 6.1)**. Interestingly, we did not detect **58** at nearby non-bloom sites or in water gathered during non-bloom periods **(Table 6.1** and **Figure A19).** This suggests that **58** may serve as a chemical marker for impeding or ongoing *P. parvum* blooms.

Table 6.1 Determination of the cell density, stearidonic acid concentrations, and ichthyotoxicity of golden algae cultures and water samples collected at recent *P. parvum* associated fish kill and bloom events.

Sample	*P. parvum* cell density ($X10^5$ cells/mL ± SD)	Stearidonic acid content (μM)	Ichthyotoxic (Yes/No)
7 day old laboratory culture	8.2 ± 0.7	5.8 ± 1.8	No
12 day old laboratory culture	13.0 ± 0.4	31.2 ± 7.7	Yes
20 day old laboratory culture	24.0 ± 3.0	95.2 ± 23.7	Yes
Bloom event in Lake Texoma, Lebanon Pool (Feb. 2009)	1.2 ± 0.1	0.06 ± 0.02	Yes
Post bloom in Lake Texoma, Lebanon Pool (June 2009)	Not detected	Not detected	No
Fish kill event in Dunkard Creek drainage, Wana Bridge (Oct. 2009)	5.9 ± 1.5	0.44 ± 0.16	Yes
Non-fish kill area in Dunkard Creek drainage, Upper Beaver Dam (Oct. 2009)	Not detected	Not detected	No

6.2.5 Further Discussions. Despite our many attempts using LC-ESIMS, direct injection ESIMS (positive and negative modes), and bioassay-guided approaches to detect prymnesins (*e.g.*, compound **54**), we were unable to generate conclusive evidence

that these cyclic polyethers meaningfully contribute to the ichthyotoxic properties of laboratory-grown or field samples *P. parvum*. Instead, a suite of uncommon polyunsaturated fatty acids and their conjugated galactoglycerolipid progenitors have emerged as important chemical agents that appreciably contribute to the ichthyotoxic effects of cultured *P. parvum*. This finding is supported by a substantial body of anecdotal evidence and extensive observations concerning the seemingly mixed lipophilic and amphiphilic properties of semipurified and purified *P. parvum* toxins.[154, 156, 158, 172] Moreover, the labile nature of the *P. parvum*'s toxins can be partially explained based on numerous autooxidative degradation processes that polyunsaturated fatty acids can undergo.[173, 174] Even if other ichthyotoxic compounds are biosynthesized by laboratory-grown *P. parvum*, the quantity of toxic fatty acids, alone, are sufficient to render the cultures lethal to fish. Although we are unaware of considerable human health risks attributable to dermal contact, inhalation, or ingestion of galactoglycerolipids and polyunsaturated lipids such as **55-58**, we recommend that caution be used while handling concentrated samples of these substances until further toxicological risk assessment studies have been conducted.

We are confident that prymnesins are not this organism's primary toxin. This assertion is based on several key observations including: 1) prymnesins were not detected by LC-ESIMS in cultured or field-collected water samples where high *P. parvum* cell densities occurred, 2) bioassay-guided isolation methods have not provided any fractions in which ^{1}H-NMR or ESIMS have yielded unequivocal evidence for prymnesins or prymnesin-degradation products, 3) the labile nature of the *P. parvum* toxin is an unlikely characteristic of prymnesins given their described structures and lack of apparent

instabilities. While preliminary FABMS data hint at the presence of high molecular weight (prymnesin-like) compounds in certain *P. parvum* samples (weak quasi-molecular ions have been observed between *m/z* 2,100 and 2,500; data not shown), we have not been able to link the occurrence of these substances with ichthyotoxic effects. Moreover, the quantities of these putative high-molecular weight metabolites fall well below their reported LC_{50} values (*e.g.*, the detection limits for FABMS are generally within the low nanogram to picogram range; the weak intensities of the peaks we have observed would put the concentrations of these compounds at levels that are ≥10–100-fold below their LC_{50} values). Instead, our data support the idea that golden algae produce another, yet uncharacterized ichthyotoxin(s).

The results are particularly significant in view of the increasing frequency with which people are becoming exposed to *P. parvum* infested waters and the current lack of concern regarding human contact with this organism.[175, 176] Although our data provide convincing evidence for certain fatty acids serving as the predominant toxins in laboratory-grown *P. parvum* cultures, we have not yet determined the identity of the ichthyotoxic compound(s) responsible for the growing number of fish kill events in the United States. However, we cannot rule out the possibility that toxic fatty acids may exhibit ichthyotoxic effects when administered chronically to fish at low-dosages. Given the different growth conditions experienced by *P. parvum* in the laboratory versus in nature, it is probable that golden algae produce different toxins under these different conditions. We will continue to investigate methods for preserving the integrity of the labile *P. parvum* toxin (*e.g.*, consideration of pH,[177] free radicals,[164] and possible metal interactions[178]) and we hope to report these findings in due course.

6.3 Materials and Methods

6.3.1 General instrumentation and experimental procedures. HPLC was performed on a Shimadzu preparative instrument using a SCL-10A VP system controller, SPD-10AV VP UV-VIS detector, LC-6AD pumps, DGU-14A solvent degasser, and FRC-10A programmable fraction collector. Samples were separated over a Phenomenex C18 Gemini column (5 μm, 110 Å, 250 × 21.2 mm). Semi-preparative HPLC was performed on a similar system using LC-10AT VP pumps and a Phenomenex C18 Gemini column (5 μm, 110 Å, 250 × 10 mm). TOF-ESIMS data were acquired on a Waters LCT Premier instrument. Corrections for exact mass determinations were made automatically with the lock mass feature in the MassLynx software. Samples for mass determination were dissolved in methanol and introduced for ionization using an auto injector with a 20 μL loop. Samples for LC-MS were analyzed by interfacing the HPLC with the ESIMS instrument. GC-EIMS analyses were carried out on a HP 5890 Series II gas chromatograph and a HP 5971A MS detector using a HP-5 (30 m × 0.32 mm × 0.25 μm) cross-linked 5% PH Me siloxane column. Optical rotation measurements were performed on an Autopol III Automatic Polarmeter (Rudolph Research) at 589 nm and 20º C in water. NMR data were obtained on a Varian VNMR spectrometer at 400 and 500 MHz (^{1}H) and 100 and125 MHz (^{13}C), respectively. All solvents were of ACS grade or better.

6.3.2 Biological material. *Prymnesium parvum* (UTEX LB2927) was purchased from the University of Texas algal collection. Cultures were grown in 1 L Erlenmeyer flasks containing sterilized COMBO media with added salts[179] and adjusted to pH 7.8. The cultures were capped with vented stoppers and bubbled with filtered (0.22 μm) air.

Cultures were maintained under fluorescent grow lights (12 h light/12 h dark photoperiod) until cell densities reached approximately ~2×10^6 cells/mL as determined with a haemocytometer.

6.3.3 Extraction and isolation. Several sample preparation and extraction techniques were tested to identify the best method for extracting the toxic materials. These methods included extraction of freeze dried culture materials, liquid-liquid partitioning of cultures, and extraction of filtered cells. Ultimately, we determined that liquid-liquid partitioning of the whole cultures (medium plus cells) using ethyl acetate was the best method since the ichthyotoxicity profile of the resulting extract was identical to that of *P. parvum* water both in terms of its potency and rapid onset toxicity. The ethyl acetate layers from partitioning against 50 L of *P. parvum* cultures were combined and the organic extract (~30 g) was subjected to gradient MPLC over HP20SS resin (30-100% methanol in water with an acetone wash). The fraction eluting with 100% methanol (680 mg) was found to retain all of the ichthyotoxic activity. Preparative reversed phase HPLC (40-100% acetonitrile in water) yielded a fraction (310 mg) eluting between 75-100% acetonitrile that was ichthyotoxic. ^{1}H-NMR and LC-ESIMS performed on this material showed it contained a variety of polyunsaturated lipids and galactoglycerolipids, but was devoid of any detectable prymnesiums. Fractionation of this material by semi-preparative reversed phase HPLC consistently resulted in sample sets that quickly lost their ichthyotoxic activities. In addition, many of the ^{1}H-NMR spins representing the presumed polyunsaturated lipid components showed diminished intensities. After several failed attempts to stabilize the ichthyotoxic substances, we turned our focus toward purifying the three most abundant substances in the fraction.

This resulted in the purification (semi-preparative HPLC eluted with 65-85% acetonitrile in water) of compounds **55** (75 mg), **56** (3 mg), and **57** (6 mg).

6.3.4 Enzymatic hydrolysis. For each experiment, a sample consisting of 2-3 mg of galactoglycerolipid was dissolved by sonicating the compound in 2 mL of phosphate buffer (pH 7.0) and then adding a lipase/esterase mixture (Sigma lipase basic kit 62327). The sample was incubated at 30 ºC for 36 h after which the reaction mixture was partitioned against dichloromethane. After removal of the solvent from the organic layer *in vacuo*, the remaining residue was resuspended in methanol and immediately analyzed by HPLC for its fatty acid content by comparison of peak retention times to authentic standards. For experiments in which the ichthyotoxicity of hydrolyzed samples were being evaluated, reaction mixtures were not extracted, but instead were directly added to the bioassay vessels containing *Pimephales promelas*. Controls for ichthyotoxicity testing consisted of heat denatured lipase/esterase mixtures.

6.3.5 Alkaline hydrolysis. Compounds (2 mg in 2 mL of methanol) were reacted with 2 mL of 4% (wt./vol.) sodium methoxide in methanol at room temperature for 30 min. Reactions were neutralized by passing over acidic Dowex 50W X 8 ion-exchange resin. The eluents were partitioned between methanol and hexanes and the methanol-soluble phases were subjected to HPLC separation. ^{1}H-NMR and optical rotation data ($[\alpha]^{20}_{D}$ -9.3 (*c* 0.02, water)) were identical to those reported for (2*R*)-3-*O*-[β-D-galactopyranosyl]glycerol.[180] The hexanes soluble material was analyzed by GC-EIMS and the presence of methyl stearidonate was confirmed based on comparisons of the samples retention time and EI fragmentation to an authentic standard.

6.3.6 GC-EIMS analysis of fatty acids. The examination of lipids was performed using a method similar to a process described for the transesterification and GC analysis of lipid mixtures.[181] Laboratory-grown cultures were prepared for fatty acid mixture analysis by lyophilization followed by methanol extraction. After removal of the solvent *in vacuo*, 100 mg samples of the organic extracts were dissolved in 3 mL of methanol-benzene (4:1) and placed in 1 dram vials. Acyl chloride (300 µL aliquots) was added to each vial and the vessels were capped. The mixtures were held at 100 ºC for 1 h with constant stirring. Samples were neutralized by adding 6 mL of 6% K_2CO_3 to each vial. Samples were briefly sonicated and then centrifuged until phase separation occurred. The upper benzene layers were removed and the samples directly submitted to GC-EIMS. For the culture-derived samples, the following GC conditions were employed: oven temperature was 100 ºC, injection port and transfer lines were held at 250 ºC and 280 ºC, respectively. A thermal gradient was applied as follows: held at 100 ºC for 3 minutes followed by a 20 ºC/min gradient to 180 ºC, a second gradient of 3 ºC/min to 225 ºC, and a final gradient of 10 ºC/min to 250 ºC, which was then held for an additional 5 minutes at 250 ºC. Due to the greater complexity of the field-collected samples, we further optimized the GC-EIMS conditions so that samples were held at 100 ºC for 3 min followed by a 20 ºC/min gradient to a final temperature of 280 ºC, which was held for 6 min.

6.3.7 Ichthyotoxicity assay. Determination of the ichthyotoxic properties of *P. parvum* extracts and pure compounds were conducted in accordance with EPA-821-R-02-012 [182] (with the minor modifications noted below) and were approved by the University of Oklahoma Institutional Animal Care and Use Committee (IACUC). Briefly, 90 mL

clear glass jars were loaded with 50 mL of filtered and aged tap water and 10-14 day old *P. promelas* fry (three per jar) were added and allowed to acclimatize for 1 h. The jars were randomized and samples dissolved in 0.5 mL methanol were added to the jars. Controls consisting of vehicle-only were included in each experiment. Fish were maintained under 12 h light/12 h dark photoperiods at 24 ºC. Due to the rapid rate in which the toxin's effects were observed, we monitored fish survivorship at 30 min, 1 h, 24 h and 48 h. We observed that the 1 h mortality counts were typically indistinguishable from observations made at 24 h and 48 h. Each sample was tested in triplicate and the results were expressed as the LC_{50} (the concentration lethal to 50% of fish) ± standard deviation. The LC_{50} values were determined in SigmaPlot v10 (Systat Software Inc) using sigmoidal dose-response regression analyses with variable slope parameters. All fish were euthanized at the conclusion of each experiment.

6.3.8. Mammalian cytotoxicity assay. Evaluations of mammalian cell cytotoxicity were performed as previously described.[183, 184] Briefly, cells were plated in 96-well plates and allowed to adhere and grow for 24 h. Compounds were dissolved in ethanol and cells were incubated with compounds for 48 h. The cells were fixed with TCA and stained with sulforhodamine B. The absorbance was read with a plate reader at 560 nm. Dose response curves were plotted and the IC_{50} values (the concentration required to inhibit cell proliferation by 50%) was calculated for each experiment. Each sample was tested in triplicate in 3-5 independent experiments and results were expressed as the IC_{50} ± standard deviation.

References

1. Cichewicz, R. H.; Henrikson, J. C.; Wang, X.; Branscum, K. M., Strategies for accessing microbial secondary metabolites from silent biosynthetic pathways. In *Manual of Industrial Microbiology and Biotechnology* 3rd Edition ed.; Baltz, R. H.; Davies, J. E.; Demain, A. L., Eds. American Society for Microbiology: Washington DC, **2009**; pp 78-95.
2. Newman, D. J.; Cragg, G. M., Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products* **2007,** *70*, 461-477.
3. Newman, D. J.; Cragg, G. M.; Snader, K. M., Natural products as sources of new drugs over the period 1981−2002. *Journal of Natural Products* **2003,** *66*, 1022-1037.
4. Cragg, G. M.; Newman, D. J.; Snader, K. M., Natural products in drug discovery and development. *Journal of Natural Products* **1997,** *60*, 52-60.
5. Pelish, H. E.; Westwood, N. J.; Feng, Y.; Kirchhausen, T.; Shair, M. D., Use of biomimetic diversity-oriented synthesis to discover galanthamine-like molecules with biological properties beyond those of the natural product. *Journal of the American Chemical Society* **2001,** *123*, 6740-6741.
6. Spring, D. R., Diversity-oriented synthesis; a challenge for synthetic chemists. *Organic & Biomolecular Chemistry* **2003,** *1*, 3867-3870.
7. Gan, Z.; Reddy, P. T.; Quevillon, S.; Couve-Bonnaire, S.; Arya, P., Stereocontrolled solid-phase synthesis of a 90-membered library of indoline-alkaloid-like polycycles from an enantioenriched aminoindoline scaffold. *Angewandte Chemie-International Edition* **2005,** *44*, 1366-1368.

8. Baltz, R. H., Renaissance in antibacterial discovery from actinomycetes. *Current Opinion in Pharmacology* **2008,** *8*, 557-563.

9. Bode, H. B.; Muller, R., The impact of bacterial genomics on natural product research. *Angewandte Chemie-International Edition* **2005,** *44*, 6828-6846.

10. Brakhage, A. A.; Shuemann, S.; Bergmann, S.; Scherlach, K.; Schroeckh, V.; Hertweck, C., Activation of fungal silent gene clusters: A new avenue to drug discovery. *Progress in Drug Research* **2008,** *66*, 3-12.

11. Challis, G. L., Genome mining for novel natural product discovery. *Journal of Medicinal Chemistry* **2008,** *51*, 2618-2628.

12. Challis, G. L., Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology* **2008,** *154*, 1555-1569.

13. Fox, E. M.; Howlett, B. J., Secondary metabolism: regulation and role in fungal biology. *Current Opinion in Microbiology* **2008,** *11*, 481-487.

14. Gross, H., Strategies to unravel the function of orphan biosynthesis pathways: recent examples and future prospects. *Applied Microbiology and Biotechnology* **2007,** *75*, 267-277.

15. Scherlach, K.; Hertweck, C., Triggering cryptic natural product biosynthesis in microorganisms. *Organic & Biomolecular Chemistry* **2009,** *7*, 1753-1760.

16. Schneider, P.; Misiek, M.; Hoffmeister, D., In vivo and in vitro production options for fungal secondary metabolites. *Molecular Pharmaceutics* **2008,** *5*, 234-242.

17. Schumann, J.; Hertweck, C., Advances in cloning, functional analysis and heterologous expression of fungal polyketide synthase genes. *Journal of Biotechnology* **2006,** *124*, 690-703.

18. Singh, S. B.; Pelaez, F., Biodiversity, chemical diversity and drug discovery. In *Natural compounds as Drugs*, Vol. I. Birkhauser: Basel, Switzerland, **2008**; pp 141-147.

19. Van Lanen, S. G.; Shen, B., Microbial genomics for the improvement of natural product discovery. *Current Opinion in Microbiology* **2006,** *9*, 252-260.

20. Wilkinson, B.; Micklefield, J., Mining and engineering natural-product biosynthetic pathways. *Nature Chemical Biology* **2007,** *3*, 379-386.

21. Zerikly, M.; Challis, G. L., Strategies for the discovery of new natural products by genome mining. *ChemBioChem* **2009,** *10*, 625-633.

22. Lee, B. N.; Kroken, S.; Chou, D. Y. T.; Robbertse, B.; Yoder, O. C.; Turgeon, B. G., Functional analysis of all nonribosomal peptide synthetases in *Cochliobolus heterostrophus* reveals a factor, NPS6, involved in virulence and resistance to oxidative stress. *Eukaryotic Cell* **2005,** *4*, 545-555.

23. Fisch, K. M.; Gillaspy, A. F.; Gipson, M.; Henrikson, J. C.; Hoover, A. R.; Jackson, L.; Najar, F. Z.; Wagele, H.; Cichewicz, R. H., Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. *Journal of Industrial Microbiology & Biotechnology* **2009,** *36*, 1199-1213.

24. Galagan, J. E.; Calvo, S. E.; Cuomo, C.; Ma, L. J.; Wortman, J. R.; Batzoglou, S.; Lee, S. I.; Basturkmen, M.; Spevak, C. C.; Clutterbuck, J.; Kapitonov, V.; Jurka, J.; Scazzocchio, C.; Farman, M.; Butler, J.; Purcell, S.; Harris, S.; Braus, G. H.;

Draht, O.; Busch, S.; D'Enfert, C.; Bouchier, C.; Goldman, G. H.; Bell-Pedersen, D.; Griffiths-Jones, S.; Doonan, J. H.; Yu, J.; Vienken, K.; Pain, A.; Freitag, M.; Selker, E. U.; Archer, D. B.; Penalva, M. A.; Oakley, B. R.; Momany, M.; Tanaka, T.; Kumagai, T.; Asai, K.; Machida, M.; Nierman, W. C.; Denning, D. W.; Caddick, M.; Hynes, M.; Paoletti, M.; Fischer, R.; Miller, B.; Dyer, P.; Sachs, M. S.; Osmani, S. A.; Birren, B. W., Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **2005,** *438*, 1105-1115.

25. Dean, R. A.; Talbot, N. J.; Ebbole, D. J.; Farman, M. L.; Mitchell, T. K.; Orbach, M. J.; Thon, M.; Kulkarni, R.; Xu, J. R.; Pan, H. Q.; Read, N. D.; Lee, Y. H.; Carbone, I.; Brown, D.; Oh, Y. Y.; Donofrio, N.; Jeong, J. S.; Soanes, D. M.; Djonovic, S.; Kolomiets, E.; Rehmeyer, C.; Li, W. X.; Harding, M.; Kim, S.; Lebrun, M. H.; Bohnert, H.; Coughlan, S.; Butler, J.; Calvo, S.; Ma, L. J.; Nicol, R.; Purcell, S.; Nusbaum, C.; Galagan, J. E.; Birren, B. W., The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **2005,** *434*, 980-986.

26. Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A. E.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G. J.; Debets, A. J. M.; Dekker, P.; van Dijck, P. W. M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J. M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S. P.; de Groot, P. W. J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P. T. W.; van den Hondel, C. A. M. J. J.; van der Heijden, R. T. J. M.; van der Kaaij, R. M.;

Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J. E. C.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N. M. E.; Ram, A. F. J.; Rinas, U.; Roubos, J. A.; Sagt, C. M. J.; Schmoll, M.; Sun, J. B.; Ussery, D.; Varga, J.; Vervecken, W.; de Vondervoort, P. J. J. V.; Wedler, H.; Wosten, H. A. B.; Zeng, A. P.; van Ooyen, A. J. J.; Visser, J.; Stam, H., Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* **2007,** *25*, 221-231.

27. Machida, M.; Asai, K.; Sano, M.; Tanaka, T.; Kumagai, T.; Terai, G.; Kusumoto, K. I.; Arima, T.; Akita, O.; Kashiwagi, Y.; Abe, K.; Gomi, K.; Horiuchi, H.; Kitamoto, K.; Kobayashi, T.; Takeuchi, M.; Denning, D. W.; Galagan, J. E.; Nierman, W. C.; Yu, J. J.; Archer, D. B.; Bennett, J. W.; Bhatnagar, D.; Cleveland, T. E.; Fedorova, N. D.; Gotoh, O.; Horikawa, H.; Hosoyama, A.; Ichinomiya, M.; Igarashi, R.; Iwashita, K.; Juvvadi, P. R.; Kato, M.; Kato, Y.; Kin, T.; Kokubun, A.; Maeda, H.; Maeyama, N.; Maruyama, J.; Nagasaki, H.; Nakajima, T.; Oda, K.; Okada, K.; Paulsen, I.; Sakamoto, K.; Sawano, T.; Takahashi, M.; Takase, K.; Terabayashi, Y.; Wortman, J. R.; Yamada, O.; Yamagata, Y.; Anazawa, H.; Hata, Y.; Koide, Y.; Komori, T.; Koyama, Y.; Minetoki, T.; Suharnan, S.; Tanaka, A.; Isono, K.; Kuhara, S.; Ogasawara, N.; Kikuchi, H., Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **2005,** *438*, 1157-1161.

28. Udwary, D. W.; Zeigler, L.; Asolkar, R. N.; Singan, V.; Lapidus, A.; Fenical, W.; Jensen, P. R.; Moore, B. S., Genome sequencing reveals complex secondary

metabolome in the marine actinomycete *Salinispora tropica*. *Proceedings of the National Academy of Sciences of the United States of America* **2007,** *104*, 10376-10381.

29. Galagan, J. E.; Calvo, S. E.; Borkovich, K. A.; Selker, E. U.; Read, N. D.; Jaffe, D.; FitzHugh, W.; Ma, L. J.; Smirnov, S.; Purcell, S.; Rehman, B.; Elkins, T.; Engels, R.; Wang, S. G.; Nielsen, C. B.; Butler, J.; Endrizzi, M.; Qui, D. Y.; Ianakiev, P.; Pedersen, D. B.; Nelson, M. A.; Werner-Washburne, M.; Selitrennikoff, C. P.; Kinsey, J. A.; Braun, E. L.; Zelter, A.; Schulte, U.; Kothe, G. O.; Jedd, G.; Mewes, W.; Staben, C.; Marcotte, E.; Greenberg, D.; Roy, A.; Foley, K.; Naylor, J.; Stabge-Thomann, N.; Barrett, R.; Gnerre, S.; Kamal, M.; Kamvysselis, M.; Mauceli, E.; Bielke, C.; Rudd, S.; Frishman, D.; Krystofova, S.; Rasmussen, C.; Metzenberg, R. L.; Perkins, D. D.; Kroken, S.; Cogoni, C.; Macino, G.; Catcheside, D.; Li, W. X.; Pratt, R. J.; Osmani, S. A.; DeSouza, C. P. C.; Glass, L.; Orbach, M. J.; Berglund, J. A.; Voelker, R.; Yarden, O.; Plamann, M.; Seiler, S.; Dunlap, J.; Radford, A.; Aramayo, R.; Natvig, D. O.; Alex, L. A.; Mannhaupt, G.; Ebbole, D. J.; Freitag, M.; Paulsen, I.; Sachs, M. S.; Lander, E. S.; Nusbaum, C.; Birren, B., The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **2003,** *422*, 859-868.

30. van den Berg, M. A.; Albang, R.; Albermann, K.; Badger, J. H.; Daran, J. M.; Driessen, A. J. M.; Garcia-Estrada, C.; Fedorova, N. D.; Harris, D. M.; Heijne, W. H. M.; Joardar, V.; Kiel, J. A. K. W.; Kovalchuk, A.; Martin, J. F.; Nierman, W. C.; Nijland, J. G.; Pronk, J. T.; Roubos, J. A.; van der Klei, I. J.; van Peij, N. N. M. E.; Veenhuis, M.; von Dohren, H.; Wagner, C.; Wortman, J.; Bovenberg, R.

A. L., Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. *Nature Biotechnology* **2008,** *26,* 1161-1168.

31. Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Shinose, M.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M.; Omura, S., Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nature Biotechnology* **2003,** *21,* 526-531.

32. Omura, S.; Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Takahashi, C.; Shinose, M.; Takahashi, Y.; Horikawa, H.; Nakazawa, H.; Osonoe, T.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M., Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites. *Proceedings of the National Academy of Sciences of the United States of America* **2001,** *98,* 12215-12220.

33. Bentley, S. D.; Chater, K. F.; Cerdeno-Tarraga, A. M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Kieser, H.; Harper, D.; Bateman, A.; Brown, S.; Chandra, G.; Chen, C. W.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Huang, C. H.; Kieser, T.; Larke, L.; Murphy, L.; Oliver, K.; O'Neil, S.; Rabbinowitsch, E.; Rajandream, M. A.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders, D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Wietzorrek, A.; Woodward, J.; Barrell, B. G.; Parkhill, J.; Hopwood, D. A., Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **2002,** *417,* 141-147.

34. Bölker, M.; Basse, C. W.; Schirawski, J., *Ustilago maydis* secondary metabolism-from genomics to biochemistry. *Fungal Genetics and Biology* **2008,** *45,* S88-S93.

35. Kamper, J.; Kahmann, R.; Bolker, M.; Ma, L. J.; Brefort, T.; Saville, B. J.; Banuett, F.; Kronstad, J. W.; Gold, S. E.; Muller, O.; Perlin, M. H.; Wosten, H. A. B.; de Vries, R.; Ruiz-Herrera, J.; Reynaga-Pena, C. G.; Snetselaar, K.; McCann, M.; Perez-Martin, J.; Feldbrugge, M.; Basse, C. W.; Steinberg, G.; Ibeas, J. I.; Holloman, W.; Guzman, P.; Farman, M.; Stajich, J. E.; Sentandreu, R.; Gonzalez-Prieto, J. M.; Kennell, J. C.; Molina, L.; Schirawski, J.; Mendoza-Mendoza, A.; Greilinger, D.; Munch, K.; Rossel, N.; Scherer, M.; Vranes, M.; Ladendorf, O.; Vincon, V.; Fuchs, U.; Sandrock, B.; Meng, S.; Ho, E. C. H.; Cahill, M. J.; Boyce, K. J.; Klose, J.; Klosterman, S. J.; Deelstra, H. J.; Ortiz-Castellanos, L.; Li, W. X.; Sanchez-Alonso, P.; Schreier, P. H.; Hauser-Hahn, I.; Vaupel, M.; Koopmann, E.; Friedrich, G.; Voss, H.; Schluter, T.; Margolis, J.; Platt, D.; Swimmer, C.; Gnirke, A.; Chen, F.; Vysotskaia, V.; Mannhaupt, G.; Guldener, U.; Munsterkotter, M.; Haase, D.; Oesterheld, M.; Mewes, H. W.; Mauceli, E. W.; DeCaprio, D.; Wade, C. M.; Butler, J.; Young, S.; Jaffe, D. B.; Calvo, S.; Nusbaum, C.; Galagan, J.; Birren, B. W., Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* **2006,** *444*, 97-101.

36. Steinberg, R. A., A study of some factors in the chemical stimulation of the growth of *Aspergillus niger*. *American Journal of Botany* **1919,** *6*, 357-372.

37. Steinberg, R. A., A study of some factors in the chemical stimulation of the growth of Aspergillus niger. *American Journal of Botany* **1919,** *6*, 330-356.

38. Porges, N., Chemical composition of *Aspergillus niger* as modified by zinc sulphate. *Botanical Gazette* **1932,** *94*, 197-205.

39. Waksman, S. A.; Horning, E. S.; Spencer, E. L., Two antagonistic fungi, *Aspergillus fumigatus* and *Aspergillus clavatus*, and their antibiotic substances. *Journal of Bacteriology* **1943,** *45*, 233-248.

40. Ashley, J. N.; Hobbs, B. C.; Raistrick, H., Studies in the biochemistry of micro-organisms LIII. The crystalline colouring matters of *Fusarium culmorum* (W. G. Smith) sacc. and related forms. *Biochemical Journal* **1937,** *31*, 385-397.

41. Curtin, T.; Fitzgerald, G.; Reilly, J., Production of phoenicine on synthetic media-1. *Penicillium phoeniceum* van beyma - 2. *Penicillium rubrum* gras berger-stoll. *Biochemical Journal* **1940,** *34*, 1605-1610.

42. Calam, C. T.; Hockenhull, D. J. D., The production of penicillin in surface culture, using chemically defined media. *Journal of General Microbiology* **1949,** *3*, 19-31.

43. Davey, V. F.; Johnson, M. J., Penicillin production in corn steep media with continuous carbohydrate addition. *Applied Microbiology* **1953,** *1*, 208-211.

44. Jarvis, F. G.; Johnson, M. J., The role of the constituents of synthetic media for penicillin production. *Journal of the American Chemical Society* **1947,** *69*, 3010-3017.

45. Pirt, S. J.; Righelat.R.C., Effect of growth rate on synthesis of penicillin by *Penicillium chrysogenum* in batch and chemostat cultures. *Applied Microbiology* **1967,** *15*, 1284.

46. Shwartzman, G., Enhanced production of penicillin in fluid medium containing cellophane. *Science* **1944,** *100*, 390-392.

47. Stone, R. W.; Farrell, M. A., Synthetic media for penicillin production. *Science* **1946,** *104*, 445-446.

48. Bode, H. B.; Bethe, B.; Hofs, R.; Zeeck, A., Big effects from small changes: Possible ways to explore nature's chemical diversity. *Chembiochem* **2002,** *3*, 619-627.

49. Lin, Z. J.; Zhu, T. J.; Wei, H. J.; Zhang, G. J.; Wang, H.; Gu, Q. Q., Spicochalasin A and new aspochalasins from the marine-derived fungus *Spicaria elegans*. *European Journal of Organic Chemistry* **2009,** 3045-3051.

50. Christian, O. E.; Compton, J.; Christian, K. R.; Mooberry, S. L.; Valeriote, F. A.; Crews, P., Using jasplakinolide to turn on pathways that enable the isolation of new chaetoglobosins from *Phomospis asparagi*. *Journal of Natural Products* **2005,** *68*, 1592-1597.

51. Shao, H. J.; Qin, X. D.; Dong, Z. J.; Zhang, H. B.; Liu, J. K., Induced daldinin A, B, C with a new skeleton from cultures of the ascomycete *Daidinia concentrica*. *Journal of Antibiotics* **2008,** *61*, 115-119.

52. Seibert, S. F.; Krick, A.; Eguereva, E.; Kehraus, S.; Konig, G. M., Ascospiroketals A and B, unprecedented cycloethers from the marine-derived fungus *Ascochyta salicorniae*. *Organic Letters* **2007,** *9*, 239-242.

53. Bode, Helge B.; Walker, M.; Zeeck, A., Cladospirones B to I from *Sphaeropsidales* sp. F-24′707 by variation of culture conditions. *European Journal of Organic Chemistry* **2000,** 3185-3193.

54. Gloer, J. B., Applications of fungal ecology in the search for new bioactive natural products. In C. P. Kubicek and I. S. Druzhinina (ed.), *The Mycota*. Springer: Berlin, Germany, **2007**; pp 257-283.

55. Burgess, J. G.; Jordan, E. M.; Bregu, M.; Mearns-Spragg, A.; Boyd, K. G., Microbial antagonism: A neglected avenue of natural products research. *Journal of Biotechnology* **1999,** *70*, 27-32.

56. Degenkolb, T.; Heinze, S.; Schlegel, B.; Strobel, G.; Grafe, U., Formation of new lipoaminopeptides, acremostatins A, B, and C, by co-cultivation of *Acremonium* sp. Tbp-5 and *Mycogone rosea* DSM 12973. *Bioscience Biotechnology Biochemistry* **2002,** *66*, 883-886.

57. Slattery, M.; Rajbhandari, I.; Wesson, K., Competition-mediated antibiotic induction in the marine bacterium *Streptomyces tenjimariensis*. *Microbial Ecology* **2001,** *41*, 90-96.

58. Sonnenbichler, J.; Dietrich, J.; Peipp, H., Secondary fungal metabolites and their biological-activities. Investigations concerning the induction of the biosynthesis of toxic secondary metabolites in basidiomycetes. *Biol Chem* **1994,** *375*, 71-79.

59. Yakovleva, E. P.; Sokolova, E. N., Dissociation of *Candida tropicalis* and its capacity for stimulation of levorin synthesis on cultivation with *Actinomyces levoris*. *Antibiot Med Biotek* **1978,** *23*, 199-203.

60. Shin, C. S.; Kim, H. J.; Kim, M. J.; Ju, J. Y., Morphological change and enhanced pigment production of monascus when cocultured with *Saccharomyces cerevisiae* or *Aspergillus oryzae*. *Biotechnol Bioeng* **1998,** *59*, 576-581.

61. Cueto, M.; Jensen, P. R.; Kauffman, C.; Fenical, W.; Lobkovsky, E.; Clardy, J., Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. *Journal of Natural Products* **2001,** *64*, 1444-1446.

62. Oh, D. C.; Kauffman, C. A.; Jensen, P. R.; Fenical, W., Induced production of emericellamides A and B from the marine-derived fungus *Emericella* sp. in competing co-culture. *Journal of Natural Products* **2007,** *70*, 515-520.

63. Park, H. B.; Kwon, H. C.; Lee, C. H.; Yang, H. O., Glionitrin A, an Antibiotic-antitumor metabolite derived from competitive interaction between abandoned mine microbes. *Journal of Natural Products* **2009,** *72*, 248-252.

64. Zhu, F.; Lin, Y., Marinamide, a novel alkaloid and its methyl ester produced by the application of mixed fermentation technique to two mangrove endophytic fungi from the South China Sea. *Chinese Sci Bull* **2006,** *51*, 1426-1430.

65. Oh, D. C.; Jensen, P. R.; Kauffman, C. A.; Fenical, W., Libertellenones A-D: Induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. *Bioorgan Med Chem* **2005,** *13*, 5267-5273.

66. Gross, F.; Luniak, N.; Perlova, O.; Gaitatzis, N.; Jenke-Kodama, H.; Gerth, K.; Gottschalk, D.; Dittmann, E.; Muller, R., Bacterial type III polyketide synthases: Phylogenetic analysis and potential for the production of novel secondary metabolites by heterologous expression in pseudomonads. *Archives of Microbiology* **2006,** *185*, 28-38.

67. Amagata, T.; Tanaka, M.; Yamada, T.; Doi, M.; Minoura, K.; Ohishi, H.; Yamori, T.; Numata, A., Variation in cytostatic constituents of a sponge-derived

Gymnascella dankaliensis by manipulating the carbon source. *Journal of Natural Products* **2007,** *70,* 1731-1740.

68. Lin, X.; Hopson, R.; Cane, D. E., Genome mining in *Streptomyces coelicolor*: Molecular cloning and characterization of a new sesquiterpene synthase. *Journal of the American Chemical Society* **2006,** *128*), 6022-6023.

69. Palmu, K.; Ishida, K.; Mantsala, P.; Hertweck, C.; Metsa-Ketela, M., Artificial reconstruction of two cryptic anglucycline antibiotic biosynthetic pathways. *Chembiochem* **2007,** *8*, 1577-1584.

70. Bok, J. W.; Keller, N. P., LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryotic Cell* **2004,** *3*, 527-535.

71. Keller, N. P.; Turner, G.; Bennett, J. W., Fungal secondary metabolism - from biochemistry to genomics. *Nature Reviews Microbiology* **2005,** *3*, 937-947.

72. Perrin, R. M.; Fedorova, N. D.; Bok, J. W.; Cramer, R. A.; Wortman, J. R.; Kim, H. S.; Nierman, W. C.; Keller, N. P., Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. *PLoS Pathogens* **2007,** *3*, 508-517.

73. Williams, R. B.; Henrikson, J. C.; Hoover, A. R.; Lee, A. E.; Cichewicz, R. H., Epigenetic remodeling of the fungal secondary metabolome. *Organic & Biomolecular Chemistry* **2008,** *6*, 1895-1897.

74. Bergmann, S.; Schumann, J.; Scherlach, K.; Lange, C.; Brakhage, A. A.; Hertweck, C., Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat. Chem. Biol.* **2007,** *3*, 213-217.

75. Nierman, W. C.; Pain, A.; Anderson, M. J.; Wortman, J. R.; Kim, H. S.; Arroyo, J.; Berriman, M.; Abe, K.; Archer, D. B.; Bermejo, C.; Bennett, J.; Bowyer, P.;

Chen, D.; Collins, M.; Coulsen, R.; Davies, R.; Dyer, P. S.; Farman, M.; Fedorova, N.; Fedorova, N.; Feldblyum, T. V.; Fischer, R.; Fosker, N.; Fraser, A.; Garcia, J. L.; Garcia, M. J.; Goble, A.; Goldman, G. H.; Gomi, K.; Griffith-Jones, S.; Gwilliam, R.; Haas, B.; Haas, H.; Harris, D.; Horiuchi, H.; Huang, J.; Humphray, S.; Jimenez, J.; Keller, N.; Khouri, H.; Kitamoto, K.; Kobayashi, T.; Konzack, S.; Kulkarni, R.; Kumagai, T.; Lafton, A.; Latge, J. P.; Li, W. X.; Lord, A.; Majoros, W. H.; May, G. S.; Miller, B. L.; Mohamoud, Y.; Molina, M.; Monod, M.; Mouyna, I.; Mulligan, S.; Murphy, L.; O'Neil, S.; Paulsen, I.; Penalva, M. A.; Pertea, M.; Price, C.; Pritchard, B. L.; Quail, M. A.; Rabbinowitsch, E.; Rawlins, N.; Rajandream, M. A.; Reichard, U.; Renauld, H.; Robson, G. D.; de Cordoba, S. R.; Rodriguez-Pena, J. M.; Ronning, C. M.; Rutter, S.; Salzberg, S. L.; Sanchez, M.; Sanchez-Ferrero, J. C.; Saunders, D.; Seeger, K.; Squares, R.; Squares, S.; Takeuchi, M.; Tekaia, F.; Turner, G.; de Aldana, C. R. V.; Weidman, J.; White, O.; Woodward, J.; Yu, J. H.; Fraser, C.; Galagan, J. E.; Asai, K.; Machida, M.; Hall, N.; Barrell, B.; Denning, D. W., Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **2005,** *438*, 1151-1156.

76. Paranagama, P. A.; Wijeratne, E. M. K.; Gunatilaka, A. A. L., Uncovering biosynthetic potential of plant-associated fungi: Effect of culture conditions on metabolite production by *Paraphaeosphaeria quadriseptata* and *Chaetomium chiversii*. *Journal of Natural Products* **2007,** *70*, 1939-1945.

77. Shwab, E. K.; Bok, J. W.; Tribus, M.; Galehr, J.; Graessle, S.; Keller, N. P., Histone deacetylase activity regulates chemical diversity in *Aspergillus* sp. *Eukaryotic Cell* **2007,** *6,* 1656-1664.

78. Kamper, J.; Kahmann, R.; Bolker, M.; Ma, L.-J.; Brefort, T.; Saville, B. J.; Banuett, F.; Kronstad, J. W.; Gold, S. E.; Muller, O.; Perlin, M. H.; Wosten, H. A. B.; de Vries, R.; Ruiz-Herrera, J.; Reynaga-Pena, C. G.; Snetselaar, K.; McCann, M.; Perez-Martin, J.; Feldbrugge, M.; Basse, C. W.; Steinberg, G.; Ibeas, J. I.; Holloman, W.; Guzman, P.; Farman, M.; Stajich, J. E.; Sentandreu, R.; Gonzalez-Prieto, J. M.; Kennell, J. C.; Molina, L.; Schirawski, J.; Mendoza-Mendoza, A.; Greilinger, D.; Munch, K.; Rossel, N.; Scherer, M.; Vranes, M.; Ladendorf, O.; Vincon, V.; Fuchs, U.; Sandrock, B.; Meng, S.; Ho, E. C. H.; Cahill, M. J.; Boyce, K. J.; Klose, J.; Klosterman, S. J.; Deelstra, H. J.; Ortiz-Castellanos, L.; Li, W.; Sanchez-Alonso, P.; Schreier, P. H.; Hauser-Hahn, I.; Vaupel, M.; Koopmann, E.; Friedrich, G.; Voss, H.; Schluter, T.; Margolis, J.; Platt, D.; Swimmer, C.; Gnirke, A.; Chen, F.; Vysotskaia, V.; Mannhaupt, G.; Guldener, U.; Munsterkotter, M.; Haase, D.; Oesterheld, M.; Mewes, H.-W.; Mauceli, E. W.; DeCaprio, D.; Wade, C. M.; Butler, J.; Young, S.; Jaffe, D. B.; Calvo, S.; Nusbaum, C.; Galagan, J.; Birren, B. W., Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* **2006,** *444*, 97-101.

79. Bölker, M.; Basse, C. W.; Schirawski, J., *Ustilago maydis* secondary metabolism - From genomics to biochemistry. *Fungal Genetics and Biology* **2008,** *45,* S88-S93.

80. Galagan, J. E.; Calvo, S. E.; Borkovich, K. A.; Selker, E. U.; Read, N. D.; Jaffe, D.; FitzHugh, W.; Ma, L.-J.; Smirnov, S.; Purcell, S.; Rehman, B.; Elkins, T.; Engels, R.; Wang, S.; Nielsen, C. B.; Butler, J.; Endrizzi, M.; Qui, D.; Ianakiev, P.; Bell-Pedersen, D.; Nelson, M. A.; Werner-Washburne, M.; Selitrennikoff, C. P.; Kinsey, J. A.; Braun, E. L.; Zelter, A.; Schulte, U.; Kothe, G. O.; Jedd, G.; Mewes, W.; Staben, C.; Marcotte, E.; Greenberg, D.; Roy, A.; Foley, K.; Naylor, J.; Stange-Thomann, N.; Barrett, R.; Gnerre, S.; Kamal, M.; Kamvysselis, M.; Mauceli, E.; Bielke, C.; Rudd, S.; Frishman, D.; Krystofova, S.; Rasmussen, C.; Metzenberg, R. L.; Perkins, D. D.; Kroken, S.; Cogoni, C.; Macino, G.; Catcheside, D.; Li, W.; Pratt, R. J.; Osmani, S. A.; DeSouza, C. P. C.; Glass, L.; Orbach, M. J.; Berglund, J. A.; Voelker, R.; Yarden, O.; Plamann, M.; Seiler, S.; Dunlap, J.; Radford, A.; Aramayo, R.; Natvig, D. O.; Alex, L. A.; Mannhaupt, G.; Ebbole, D. J.; Freitag, M.; Paulsen, I.; Sachs, M. S.; Lander, E. S.; Nusbaum, C.; Birren, B., The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **2003,** *422*, 859-868.

81. Galagan, J. E.; Calvo, S. E.; Cuomo, C.; Ma, L.-J.; Wortman, J. R.; Batzoglou, S.; Lee, S.-I.; Basturkmen, M.; Spevak, C. C.; Clutterbuck, J.; Kapitonov, V.; Jurka, J.; Scazzocchio, C.; Farman, M.; Butler, J.; Purcell, S.; Harris, S.; Braus, G. H.; Draht, O.; Busch, S.; D'Enfert, C.; Bouchier, C.; Goldman, G. H.; Bell-Pedersen, D.; Griffiths-Jones, S.; Doonan, J. H.; Yu, J.; Vienken, K.; Pain, A.; Freitag, M.; Selker, E. U.; Archer, D. B.; Penalva, M. A.; Oakley, B. R.; Momany, M.; Tanaka, T.; Kumagai, T.; Asai, K.; Machida, M.; Nierman, W. C.; Denning, D. W.; Caddick, M.; Hynes, M.; Paoletti, M.; Fischer, R.; Miller, B.; Dyer, P.; Sachs,

M. S.; Osmani, S. A.; Birren, B. W., Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **2005,** *438,* 1105-1115.

82. Nierman, W. C.; Pain, A.; Anderson, M. J.; Wortman, J. R.; Kim, H. S.; Arroyo, J.; Berriman, M.; Abe, K.; Archer, D. B.; Bermejo, C.; Bennett, J.; Bowyer, P.; Chen, D.; Collins, M.; Coulsen, R.; Davies, R.; Dyer, P. S.; Farman, M.; Fedorova, N.; Fedorova, N.; Feldblyum, T. V.; Fischer, R.; Fosker, N.; Fraser, A.; Garcia, J. L.; Garcia, M. J.; Goble, A.; Goldman, G. H.; Gomi, K.; Griffith-Jones, S.; Gwilliam, R.; Haas, B.; Haas, H.; Harris, D.; Horiuchi, H.; Huang, J.; Humphray, S.; Jimenez, J.; Keller, N.; Khouri, H.; Kitamoto, K.; Kobayashi, T.; Konzack, S.; Kulkarni, R.; Kumagai, T.; Lafton, A.; Latge, J.-P.; Li, W.; Lord, A.; Lu, C.; Majoros, W. H.; May, G. S.; Miller, B. L.; Mohamoud, Y.; Molina, M.; Monod, M.; Mouyna, I.; Mulligan, S.; Murphy, L.; O'Neil, S.; Paulsen, I.; Penalva, M. A.; Pertea, M.; Price, C.; Pritchard, B. L.; Quail, M. A.; Rabbinowitsch, E.; Rawlins, N.; Rajandream, M.-A.; Reichard, U.; Renauld, H.; Robson, G. D.; de Cordoba, S. R.; Rodriguez-Pena, J. M.; Ronning, C. M.; Rutter, S.; Salzberg, S. L.; Sanchez, M.; Sanchez-Ferrero, J. C.; Saunders, D.; Seeger, K.; Squares, R.; Squares, S.; Takeuchi, M.; Tekaia, F.; Turner, G.; de Aldana, C. R. V.; Weidman, J.; White, O.; Woodward, J.; Yu, J.-H.; Fraser, C.; Galagan, J. E.; Asai, K.; Machida, M.; Hall, N.; Barrell, B.; Denning, D. W., Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **2005,** *438,* 1151-1156.

83. Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A. E.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G. J.; Debets, A. J. M.; Dekker, P.; van Dijck, P. W. M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J. M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S. P.; de Groot, P. W. J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P. T. W.; van den Hondel, C. A. M. J. J.; van der Heijden, R. T. J. M.; van der Kaaij, R. M.; Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J. E. C.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N. M. E.; Ram, A. F. J.; Rinas, U.; Roubos, J. A.; Sagt, C. M. J.; Schmoll, M.; Sun, J.; Ussery, D.; Varga, J.; Vervecken, W.; van de Vondervoort, P. J. J.; Wedler, H.; Wosten, H. A. B.; Zeng, A.-P.; van Ooyen, A. J. J.; Visser, J.; Stam, H., Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* **2007,** *25*, 221-231.

84. Dean, R. A.; Talbot, N. J.; Ebbole, D. J.; Farman, M. L.; Mitchell, T. K.; Orbach, M. J.; Thon, M.; Kulkarni, R.; Xu, J.-R.; Pan, H.; Read, N. D.; Lee, Y.-H.; Carbone, I.; Brown, D.; Oh, Y. Y.; Donofrio, N.; Jeong, J. S.; Soanes, D. M.; Djonovic, S.; Kolomiets, E.; Rehmeyer, C.; Li, W.; Harding, M.; Kim, S.; Lebrun, M.-H.; Bohnert, H.; Coughlan, S.; Butler, J.; Calvo, S.; Ma, L.-J.; Nicol, R.; Purcell, S.; Nusbaum, C.; Galagan, J. E.; Birren, B. W., The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **2005,** *434*, 980-986.

85. Machida, M.; Asai, K.; Sano, M.; Tanaka, T.; Kumagai, T.; Terai, G.; Kusumoto, K.-I.; Arima, T.; Akita, O.; Kashiwagi, Y.; Abe, K.; Gomi, K.; Horiuchi, H.; Kitamoto, K.; Kobayashi, T.; Takeuchi, M.; Denning, D. W.; Galagan, J. E.; Nierman, W. C.; Yu, J.; Archer, D. B.; Bennett, J. W.; Bhatnagar, D.; Cleveland, T. E.; Fedorova, N. D.; Gotoh, O.; Horikawa, H.; Hosoyama, A.; Ichinomiya, M.; Igarashi, R.; Iwashita, K.; Juvvadi, P. R.; Kato, M.; Kato, Y.; Kin, T.; Kokubun, A.; Maeda, H.; Maeyama, N.; Maruyama, J.-i.; Nagasaki, H.; Nakajima, T.; Oda, K.; Okada, K.; Paulsen, I.; Sakamoto, K.; Sawano, T.; Takahashi, M.; Takase, K.; Terabayashi, Y.; Wortman, J. R.; Yamada, O.; Yamagata, Y.; Anazawa, H.; Hata, Y.; Koide, Y.; Komori, T.; Koyama, Y.; Minetoki, T.; Suharnan, S.; Tanaka, A.; Isono, K.; Kuhara, S.; Ogasawara, N.; Kikuchi, H., Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **2005,** *438*, 1157-1161.

86. Henrikson, J. C.; Hoover, A. R.; Joyner, P. M.; Cichewicz, R. H., A chemical epigenetics approach for engineering the in situ biosynthesis of a cryptic natural product from *Aspergillus niger*. *Organic & Biomolecular Chemistry* **2009,** *7*, 435-438.

87. Grewal, S. I. S.; Bonaduce, M. J.; Klar, A. J. S., Histone deacetylase homologs regulate epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. *Genetics* **1998,** *150*, 563-576.

88. Bernstein, B. E.; Tong, J. K.; Schreiber, S. L., Genomewide studies of histone deacetylase function in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** *97*, 13708-13713.

89. Graessle, S.; Dangl, M.; Haas, H.; Mair, K.; Trojer, P.; Brandtner, E.-M.; Walton, J. D.; Loidl, P.; Brosch, G., Characterization of two putative histone deacetylase genes from *Aspergillus nidulans*. *Biochimica et Biophysica Acta* **2000,** *1492*, 120-126.

90. Peng, W.; Togawa, C.; Zhang, K.; Kurdistani, S. K., Regulators of cellular levels of histone acetylation in *Saccharomyces cerevisiae*. *Genetics* **2008,** *179*, 277-289.

91. Tamaru, H.; Selker, E. U., A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* **2001,** *414*, 277-283.

92. Trojer, P.; Brandtner, E. M.; Brosch, G.; Loidl, P.; Galehr, J.; Linzmaier, R.; Haas, H.; Mair, K.; Tribus, M.; Graessle, S., Histone deacetylases in fungi: Novel members, new facts. *Nucleic Acids Research* **2003,** *31*, 3971-3981.

93. Van West, P.; Shepherd, S. J.; Walker, C. A.; Li, S.; Appiah, A. A.; Grenville-Briggs, L. J.; Govers, F.; Gow, N. A. R., Internuclear gene silencing in *Phytophthora infestans* is established through chromatin remodelling. *Microbiology* **2008,** *154*, 1482-1490.

94. Brosch, G.; Loidl, P.; Graessle, S., Histone modifications and chromatin dynamics: a focus on filamentous fungi. *FEMS Microbiology Reviews* **2008,** *32*, 409-439.

95. Biel, M.; Wascholowski, V.; Giannis, A., Epigenetics - an epicenter of gene regulation: Histones and histone-modifying enzymes. *Angewandte Chemie International Edition* **2005,** *44*, 3186-3216.

96. Cole, P. A., Chemical probes for histone-modifying enzymes. *Nature Chemical Biology* **2008,** *4*, 590-597.

97. Goffin, J.; Eisenhauer, E., DNA methyltransferase inhibitors - state of the art. *Annals of Oncology* **2002,** *13*, 1699-1716.

98. Zhou, Y.; Cambareri, E. B.; Kinsey, J. A., DNA methylation inhibits expression and transposition of the Neurospora Tad retrotransposon. *Molecular Genetics and Genomics* **2001,** *265*, 748-754.

99. Lee, D. W.; Freitag, M.; Selker, E. U.; Aramayo, R., A cytosine methyltransferase homologue is essential for sexual development in *Aspergillus nidulans*. *PLoS ONE* **2008,** *3*, e2531.

100. Wada, H.; Kagoshima, M.; Ito, K.; Barnes, P. J.; Adcock, I. M., 5-Azacytidine suppresses RNA polymerase II recruitment to the SLPI gene. *Biochemical and Biophysical Research Communications* **2005,** *331*, 93-99.

101. Wozniak, R. J.; Klimecki, W. T.; Lau, S. S.; Feinstein, Y.; Futscher, B. W., 5-Aza-2'-deoxycytidine-mediated reductions in G9A histone methyltransferase and histone H3 K9 di-methylation levels are linked to tumor suppressor gene reactivation. *Oncogene* **2006,** *26*, 77-90.

102. Andersen, M. R.; Nielsen, J., Current status of systems biology in aspergilli. *Fungal Genetics and Biology* **2009,** *46*, S180-S190.

103. Baker, S. E., *Aspergillus niger* genomics: past, present and into the future. *Medical Mycology* **2006,** *44*, 17-21.

104. Baker, S. E., Aspergillus niger genomics: Past, present and into the future. *Medical Mycology* **2006,** *44*, S17-S21.

105. Semova, N.; Storms, R.; John, T.; Gaudet, P.; Ulycznyj, P.; Min, X. J.; Sun, J.; Butler, G.; Tsang, A., Generation, annotation, and analysis of an extensive *Aspergillus niger* EST collection. *BMC Microbiol.* **2006,** *6*.

106. James, T. Y.; Kauff, F.; Schoch, C. L.; Matheny, P. B.; Hofstetter, V.; Cox, C. J.; Celio, G.; Gueidan, C.; Fraker, E.; Miadlikowska, J.; Lumbsch, H. T.; Rauhut, A.; Reeb, V.; Arnold, A. E.; Amtoft, A.; Stajich, J. E.; Hosaka, K.; Sung, G.-H.; Johnson, D.; O/'Rourke, B.; Crockett, M.; Binder, M.; Curtis, J. M.; Slot, J. C.; Wang, Z.; Wilson, A. W.; Schuszler, A.; Longcore, J. E.; O/'Donnell, K.; Mozley-Standridge, S.; Porter, D.; Letcher, P. M.; Powell, M. J.; Taylor, J. W.; White, M. M.; Griffith, G. W.; Davies, D. R.; Humber, R. A.; Morton, J. B.; Sugiyama, J.; Rossman, A. Y.; Rogers, J. D.; Pfister, D. H.; Hewitt, D.; Hansen, K.; Hambleton, S.; Shoemaker, R. A.; Kohlmeyer, J.; Volkmann-Kohlmeyer, B.; Spotts, R. A.; Serdani, M.; Crous, P. W.; Hughes, K. W.; Matsuura, K.; Langer, E.; Langer, G.; Untereiner, W. A.; Lucking, R.; Budel, B.; Geiser, D. M.; Aptroot, A.; Diederich, P.; Schmitt, I.; Schultz, M.; Yahr, R.; Hibbett, D. S.; Lutzoni, F.; McLaughlin, D. J.; Spatafora, J. W.; Vilgalys, R., Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature* **2006,** *443*, 818-822.

107. Scherlach, K.; Hertweck, C., Triggering cryptic natural product biosynthesis in microorganisms. *Organic & Biomolecular Chemistry* **2009,** *7*, 1753-1760.

108. Gross, H., Strategies to unravel the function of orphan biosynthesis pathways: recent examples and future prospects. *Applied Microbiology and Biotechnology* **2007,** *75*, 267-277.

109. Henrikson, J. C.; Hoover, A. R.; Joyner, P. M.; Cichewicz, R. H., A chemical epigenetics approach for engineering the *in situ* biosynthesis of a cryptic natural product from *Aspergillus niger*. *Organic & Biomolecular Chemistry* **2009,** *7*, 435-438.

110. Williams, R. B.; Henrikson, J. C.; Hoover, A. R.; Lee, A. E.; Cichewicz, R. H., Epigenetic remodeling of the fungal secondary metabolome. *Organic & Biomolecular Chemistry* **2008,** *6*, 1895-1897.

111. Bok, J. W.; Noordermeer, D.; Kale, S. P.; Keller, N. P., Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. *Molecular Microbiology* **2006,** *61*, 1636-1645.

112. Katan-Khaykovich, Y.; Struhl, K., Heterochromatin formation involves changes in histone modifications over multiple cell generations. *The EMBO Journal* **2005,** *24*, 2138-2149.

113. Lam, K. S., New aspects of natural products in drug discovery. *Trends in Microbiology* **2007,** *15*, 279-289.

114. Koehn, F. E.; Carter, G. T., The evolving role of natural products in drug discovery. *Nature Reviews Drug Discovery* **2005,** *4*, 206-220.

115. Paterson, I.; Anderson, E. A., The renaissance of natural products as drug candidates. *Science* **2005,** *310*, 451-453.

116. Baker, D. D.; Chu, M.; Oza, U.; Rajgarhia, V., The value of natural products to future pharmaceutical discovery *Natural Product Reports* **2007,** *24*, 1225-1244.

117. Nielsen, K. F.; Mogensen, J. M.; Johansen, M.; Larsen, T. O.; Frisvad, J. C., Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Analytical and Bioanalytical Chemistry* **2009,** *395*, 1225-1242.

118. Clark, B.; Capon, R. J.; Lacey, E.; Tennant, S.; Gill, J. H., Quinolactacins revisited: From lactams to imide and beyond. *Organic & Biomolecular Chemistry* **2006,** *4*, 1512-1519.

119. Fukuda, T.; Hasegawa, Y.; Hagimori, K.; Yamaguchi, Y.; Masuma, R.; Tomoda, H.; Omura, S., Tensidols, new potentiators of antifungal miconazole activity, produced by *Aspergillus niger* FKI-2342. *Journal of Antibiotics* **2006,** *59*, 480-485.

120. Zhang, Y. P.; Zhu, T. J.; Fang, Y. C.; Liu, H. B.; Gu, Q. Q.; Zhu, W. M., Carbonarones A and B, new bioactive gamma-pyrone and alpha-pyridone derivatives from the marine-derived fungus *Aspergillus carbonarius*. *Journal of Antibiotics* **2007,** *60*, 153-157.

121. Takagi, M.; Motohashi, K.; Hwang, J.-H.; Nagai, A.; Shin-ya, K., New tensidols, JBIR-86 and JBIR-87, isolated from *Aspergillus* sp. fJ80. *Journal of Antibiotics* **2010,** *63*, 371-373.

122. Ye, Y. H.; Zhu, H. L.; Song, Y. C.; Liu, J. Y.; Tan, R. X., Structural revision of aspernigrin A, reisolated from *Cladosporium herbarum* IFB-E002. *Journal of Natural Products* **2005,** *68*, 1106-1108.

123. Ding, G.; Jiang, L. H.; Guo, L. D.; Chen, X. L.; Zhang, H.; Che, Y. S., Pestalazines and pestalamides, bioactive metabolites from the plant pathogenic fungus *Pestalotiopsis theae*. *Journal of Natural Products* **2008,** *71*, 1861-1865.

124. The chemical shift published for the ^{13}C-NMR of pestalamide B was found to be incorrectly listed in Ding *et. al*.'s article. A revision of this data, with corrected chemical shifts, is published in a table found in the supplemental materials.

125. Hiort, J.; Maksimenka, K.; Reichert, M.; PeroviÄ‡-Ottstadt, S.; Lin, W. H.; Wray, V.; Steube, K.; Schaumann, K.; Weber, H.; Proksch, P.; Ebel, R.; Mäller, W. E. G.; Bringmann, G., New natural products from the sponge-derived fungus *Aspergillus niger*. *Journal of Natural Products* **2004,** *67*, 1532-1543.

126. Rychnovsky, S., 2006-2007 ACS Division of Organic Chemistry Graduate Fellowship Awards. *Org Lett* **2006,** *8*, 5679-5683.

127. White, K. N.; Amagata, T.; Oliver, A. G.; Tenney, K.; Wenzel, P. J.; Crews, P., Structure revision of spiroleucettadine, a sponge alkaloid with a bicyclic core meager in H-atoms. *Journal of Organic Chemistry* **2008,** *73*, 8719-8722.

128. Wipf, P.; Kerekes, A. D., Structure reassignment of the fungal metabolite TAEMC161 as the phytotoxin viridiol. *Journal of Natural Products* **2003,** *66*, 716-718.

129. McCombie, S. W.; Metz, W. A.; Nazareno, D.; Shankar, B. B.; Tagat, J., Generation and in situ acylation of enaminone anions: a convenient synthesis of 3-carbethoxy-4(1H)-pyridinones and -4-pyrones and related compounds. *The Journal of Organic Chemistry* **1991,** *56*, 4963-4967.

130. Zhang, L. X.; Yan, K. Z.; Zhang, Y.; Huang, R.; Bian, J.; Zheng, C. S.; Sun, H. X.; Chen, Z. H.; Sun, N.; An, R.; Min, F. G.; Zhao, W. B.; Zhuo, Y.; You, J. L.; Song, Y. J.; Yu, Z. Y.; Liu, Z. H.; Yang, K. Q.; Gao, H.; Dai, H. Q.; Zhang, X. L.; Wang, J.; Fu, C. Z.; Pei, G.; Liu, J. T.; Zhang, S.; Goodfellow, M.; Jiang, Y. Y.;

Kuai, J.; Zhou, G. C.; Chen, X. P., High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections. *Proc. Natl. Acad. Sci. U. S. A.* **2007,** *104*, 4606-4611.

131. Yamazaki, H.; Omura, S.; Tomoda, H., 6 '-Hydroxy-3 '-methoxy-mitorubrin, a new potentiator of antifungal miconazole activity, produced by *Penicillium radicum* FKI-3765-2. *Chem. Pharm. Bull. 58*, 829-832.

132. Varga, J.; Kocsube, S.; Toth, B.; Frisvad, J. C.; Perrone, G.; Susca, A.; Meijer, M.; Samson, R. A., *Aspergillus brasiliensis* sp nov., a biseriate black *Aspergillus* sp. with world-wide distribution. *Int. J. Syst. Evol. Microbiol.* **2007,** *57*, 1925-1932.

133. Sorensen, L. M.; Lametsch, R.; Andersen, M. R.; Nielsen, P. V.; Frisvad, J. C., Proteome analysis of *Aspergillus niger*: Lactate added in starch-containing medium can increase production of the mycotoxin fumonisin B-2 by modifying acetyl-CoA metabolism. *BMC Microbiol.* **2009,** *9*, 20.

134. Iwatsuki, M.; Uchida, R.; Yoshijima, H.; Ui, H.; Shiomi, K.; Matsumoto, A.; Takahashi, Y.; Abe, A.; Tomoda, H.; Omura, S., Guadinomines, Type III secretion system inhibitors, produced by Streptomyces sp K01-0509. *Journal of Antibiotics* **2008,** *61*, 222-229.

135. Hasegawa, Y.; Fukuda, T.; Hagimori, K.; Tomoda, H.; Omura, S., Tensyuic acids, new antibiotics produced by *Aspergillus niger* FKI-2342. *Chem. Pharm. Bull.* **2007,** *55* (9), 1338-1341.

136. Fukuda, T.; Hasegawa, Y.; Sakabe, Y.; Tomoda, H.; Omura, S., Citrinamides, new potentiators of antifungal miconazole activity, produced by *Penicillium* sp, FKI-1938. *Journal of Antibiotics* **2008,** *61* (9), 550-555.

137. Bowers, A. A.; Walsh, C. T.; Acker, M. G., Genetic interception and structural characterization of thiopeptide cyclization precursors from *Bacillus cereus*. *Journal of the American Chemical Society* **2010,** *132*, 12182-12184.

138. Compound **13** was generated in order to ensure the literature method would produce one of the previously synthesized compounds. This compounds was then submitted to the bioassays along with other compound discussed in this paper.

139. Hiort, J.; Maksimenka, K.; Reichert, M.; Perovic-Ottstadt, S.; Lin, W. H.; Wray, V.; Steube, K.; Schaumann, K.; Weber, H.; Proksch, P.; Ebel, R.; Muller, W. E. G.; Bringmann, G., New natural products from the sponge-derived fungus *Aspergillus niger*. (vol 67, pg 1532, 2004). *Journal of Natural Products* **2005,** *68*, 1821-1821.

140. Henrikson, J. C.; Gharfeh, M. S.; Easton, A. C.; Easton, J. D.; Glenn, K. L.; Shadfan, M.; Mooberry, S. L.; Hambright, K. D.; Cichewicz, R. H., Reassessing the ichthyotoxin profile of cultured *Prymnesium parvum* (golden algae) and comparing it to samples collected from recent freshwater bloom and fish kill events in North America. *Toxicon* **2010,** *55*, 1396-1404.

141. Andersen, R. A., Biology and systematics of heterokont and haptophyte algae. *American Journal of Botany* **2004,** *91*, 1508-1522.

142. Edvardsen, B.; Imai, I., The ecology of harmful flagellates within prymnesiophyceae and raphidophyceae. In *Ecology of Harmful Algae*, Graneli, E.; Turner, J. T., Eds. Springer-Verlag: New York, **2006**; pp 67-79.

143. Guo, M. X.; Harrison, P. J.; Taylor, F. J. R., Fish kills related to *Prymnesium parvum* N Carter (Haptophyta) in the People's Republic of China. *Journal of Applied Phycology* **1996,** *8*, 111-117.

144. Kaartvedt, S.; Johnsen, T. M.; Asksnes, D. L.; Lie, U.; Svendsen, H., Occurrence of the toxic phytoflagellate *Prymnesium parvum* and associated fish mortality in a Norwegian fjord system. *Can. J. Fish. Aquat. Sci.* **1991,** *48*, 2316-2323.

145. Reich, K.; Aschner, M., Mass development and control of the phytoflagellate *Prymnesium parvum* in fish ponds in Palestine. *Pales. J. Bot.* **1947,** *4*, 14-23.

146. Sabour, B.; Loudiki, M.; Oudra, B.; Oubraim, S.; Fawzi, B.; Fadlaoui, S.; Chlaida, M., First report on the occurrence of ichthyotoxic yellow tides of *Prymnesium parvum* Carter (Haptophyceae) in Oued Mellah hypertrophic lake (Morocco). *Revue des science de l'Eau (J. Water Sci.)* **2002,** *15*, 327-342.

147. Lopez, C. B.; Jewett, E. B.; Dortch, Q.; Walton, B. T.; Hudnell, H. K., Scientific assessment of freshwater harmful algal blooms. Interagency working group on harmful algal blooms. In *Hypoxia/Human Health of the Joint Subcommittee on Ocean Science and Technology*, Ed. Washington, D.C., **2008**.

148. Sager, D.; Fries, L.; Singhurst, L.; Southard, G., Guidelines for golden alga *Prymnesium parvum* management options for ponds and small reserviors (public waters) in Texas. Inland Fishers, Texas Parks and Wildlife Department - PWD RP T3200-1404 (1/2007), Texas State Publications, Austin., **2007**.

149. Baker, J. W.; Grover, J. P.; Ramachandrannair, R.; Black, C.; Valenti, T. W.; Brooks, B. W.; Roelke, D. L., Growth at the edge of the niche: An experimental study of the harmful alga *Prymnesium parvum*. *Limnology and Oceanography* **2009,** *54*, 1679-1687.

150. Aguiar, R.; Kugens, P., New and rare chrysophytes from Wyoming and Colorado lakes. *J. Phycology* **2000,** *26*, 1-2.

151. Hargraves, P. E.; Maranda, L., Potentially toxic or harmful microalgae from the northeast coast. *Northeastern Naturalist* **2002,** *9*, 81-120.

152. Kim, Y. S.; Padilla, G. M., Hemolytically active components from *P. parvum* and *G. breve* toxins. *Life Sciences* **1977,** *21*, 1287-1292.

153. Mariussen, E.; Nelson, G. N.; Fonnum, F., A toxic extract of the marine phytoflagellate *Prymnesium parvum* induces calcium-dependent release of glutamate from rat brain synaptosomes. *Journal of Toxicology and Environmental Health-Part A-Current Issues* **2005,** *68*, 67-79.

154. Ulitzur, S., Amphiphatic nature of *Prymnesium parvum* hemolysin. *Biochimica Et Biophysica Acta* **1973,** *298*, 673-679.

155. Ulitzur, S.; Shilo, M., Mode of action of *Prymnesium parvum* ichthyotoxin. *Journal of Protozoology* **1966,** *13*, 332-336.

156. Ulitzur, S.; Shilo, M., Procedure for purification and separation of *Prymnesium parvum* toxins. *Biochimica Et Biophysica Acta* **1970,** *201*, 350-363.

157. Wright, J. L. C.; Tomas, C.; Satake, M.; Alam, N.; Schuster, T., Structures of several new hemolytic toxins from strains of *Prymnesium parvum* isolated from

fish ponds in North Carolina. In *3rd Symposium on Harmful Algae in the US.*, Pacific Grove, CA, **2005**.

158. Yariv, J.; Hestrin, S., Toxicity of extracellular phase of *Prymnesium parvum* cultures. *Journal of General Microbiology* **1961,** *24*, 165-175.

159. Watson, S., Texas Parks and Wildlife Department - Literature review of the microalga *Prymnesium parvum* and its associated toxicity. Texas Parks and Wildlife Department (http://www.tpwd.state.tx.us/landwater/water/environconcners/hab/ga/literature/index.phtml accessed October 18, 2001.

160. Kozakai, H.; Oshima, Y.; Yasumoto, T., Isolation and structural elucidation of hemolysin from the phytoflagellate *Prymnesium par*vum. *Agricultural and Biological Chemistry* **1982,** *46*, 233-236.

161. Igarashi, T.; Satake, M.; Yasumoto, T., Prymnesin-2: A potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. *Journal of the American Chemical Society* **1996,** *118*, 479-480.

162. Igarashi, T.; Satake, M.; Yasumoto, T., Structures and partial stereochemical assignments for prymnesin-1 and prymnesin-2: Potent hemolytic and ichthyotoxic glycosides isolated from the red tide alga *Prymnesium parvum*. *Journal of the American Chemical Society* **1999,** *121*, 8499-8511.

163. Igarashi, T.; Aritake, S.; Yasumoto, T., Biological activities of prymnesin-2 isolated from a red tide alga *Prymnesium parvum*. *Nat. Toxins* **1998,** *6*, 35-41.

164. Marshall, J. A.; Nichlols, P. D.; Hamilton, B.; Lewis, R. J.; Hallegraeff, G. M., Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) to damselfish

(*Acanthochromis polycanthus*): The synergisitic role of reactive oxygen species and free fatty acids. *Harmful Algae.* **2003,** *2*, 273-281.

165. Fu, M.; Koulman, A.; van Rijssel, M.; Lutzen, A.; de Boer, M. K.; Tyl, M. R.; Liebezeit, G., Chemical characterisation of three haemolytic compounds from the microalgal species *Fibrocapsa japonica* (Raphidophyceae). *Toxicon* **2004,** *43*, 355-363.

166. Hiraga, Y.; Kaku, K.; Omoda, D.; Sugihara, K.; Hosoya, H.; Hino, M., A new digalactosyl diacylglycerol from a cultured marine dinoflagellate *Heterocapsa circularisquama*. *Journal of Natural Products* **2002,** *65*, 1494-1496.

167. Kobayashi, M.; Hayashi, K.; Kawazoe, K.; Kitagawa, I., Marine natural-products heterosigma-glycolipid-I, heterosigma-glycolipid-II, heterosigma-glycolipid-III, and heterosigma-glycolipid-IV, 4 diacylglyceroglycolipids possessing omega-3-polyunsaturated fatty-acid residues, from the raphidophycean dinoflagellate H*eterosigma akashiwo*. *Chemical & Pharmaceutical Bulletin* **1992,** *40*, 1404-1410.

168. Stabell, O. B.; Pedersen, K.; Aune, T., Detection and separation of toxins accumulated by mussels during the 1988 bloom of *Chrysochromulina polylepis* in Norwegian coastal waters. *Marine Environmental Research* **1993,** *36*, 185-196.

169. Hiraga, Y.; Shikano, T.; Widianti, T.; Ohkata, K., Three new glycolipids with cytolytic activity from cultured marine dinoflagellate *Heterocapsa circularisquama*. *Natural Product Research* **2008,** *22*, 649-657.

170. Fossat, B.; Porthe-Nibelle, J.; Sola, P.; Masoni, A.; Gentien, P.; Bodennec, G., Toxicity of fatty acid 18:5n3 from *Gymnodinium* cf. mikimotoi: II. Intracellular

pH and KC uptake in isolated trout hepatocytes. *Journal of Applied Toxicology* **1999,** *19,* 275-278.

171. Mudryk, Z. J.; Skorczewski, P., Enzymatic activity and degradation of organic macromolecules by neustonic and planktonic bacteria in an estuarine lake. *Polish Journal of Ecology* **2006,** *54,* 3-14.

172. Shilo, M., Toxins of Chrysophyceae. In *Microbial Toxins, vol. VII.*, Kadis, S.; Ciegler, A.; Ajl, S. J., Eds. Academic Press: New York, **1971**; pp 67-102.

173. Schauenstein, E., Autoxidation of polyunsaturated esters in water - chemical structure and biological activity of products. *Journal of Lipid Research* **1967,** *8,* 417-428.

174. Vanrollins, M.; Murphy, R. C., Autooxidation of docosahexaenoic acid - analysis of 10 isomers of hydroxydocosahexaenoate. *Journal of Lipid Research* **1984,** *25,* 507-517.

175. TPWD, Golden Algae in Texas (document GI-378, 9/07). (Texas Parks and Wildlife Department), accessed October 1, 2009.

176. AGFD, Golden Alga Frequently Asked Questions, http://www.azgfd.gov/temp/golden_alga_faqs.shtml#2, Arizona Game and Fish Department accessed October 1, 2009.

177. Valenti, T. W.; James, S. V.; Lahousse, M. J.; Schug, K. A.; Roelke, D. L.; Grover, J. P.; Brooks, B. W., A mechanistic explanation for pH-dependent ambient aquatic toxicity of Prymnesium parvum carter. *Toxicon* **2010,** *55,* 990-998.

178. Moeller, P. D. R.; Beauchesne, K. R.; Huncik, K. M.; Davis, W. C.; Christopher, S. J.; Riggs-Gelasco, P.; Gelasco, A. K., Metal complexes and free radical toxins produced by *Pfiesteria piscicida*. *Environmental Science & Technology* **2007,** *41*, 1166-1172.

179. Kilham, S. S.; Kreeger, D. A.; Lynn, S. G.; Goulden, C. E.; Herrera, L., COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* **1998,** *377*, 147-159.

180. Oshima, Y.; Yamada, S.-h.; Matsunaga, K.; Moriya, T.; Ohizumi, Y., A monogalactosyl diacylglycerol from a cultured marine dinoflagellate, *Scrippsidla trochoidea*. *Journal of Natural Products* **1994,** *57*, 534-536.

181. Lepage, G.; Roy, C. C., Direct transesterification of all classes of lipids in a one-step reaction. *Journal of Lipid Research* **1986,** *27*, 114-120.

182. U.S.E.P.A., EPA-821-R-02-012 - Methods for Measuring the ACute Toxicity of Effluents and Receiving Waters to Freswater and marine Organisms, 5th edition. Agency, U.S. Environmental Protection Agency. Washington D.C., **2002**.

183. Mooberry, S. L.; Weiderhold, K. N.; Dakshanamurthy, S.; Hamel, E.; Banner, E. J.; Kharlamova, A.; Hempel, J.; Gupton, J. T.; Brown, M. L., Identification and characterization of a new tubulin-binding tetrasubstituted brominated pyrrole. *Molecular Pharmacology* **2007,** *72*, 132-140.

184. Tinley, T. L.; Randall-Hlubek, D. A.; Leal, R. M.; Jackson, E. M.; Cessac, J. W.; Quada, J. C.; Hemscheidt, T. K.; Mooberry, S. L., Taccalonolides E and A: Plant-derived steroids with microtubule-stabilizing activity. *Cancer Research* **2003,** *63* (12), 3211-3220.

Appendix

Appendix Table of Contents

Table A1 Screening of epigenetic modifiers 121

Figure A1 Real time qRT PCR determination of the fold changes in expression of PKS, NRPS, and HPN gene clusters in *A. niger* ATCC 1015 122

Synthesis of *p*-fluoro SAHA 123

Table A2 Results from LC-ESI-MS analysis from 12 different species of aspergilli 124

Table A3 GIAO based calculations of ^{13}C-NMR chemical shifts for **48** and **41** 125

Table A4 ^{1}H- and ^{13}C-NMR data tables for various γ-pyrones and γ-pyridinones from natural and synthetic sources 126

Figure A2 Antibacterial activity of **44** 130

Figure A3 Antifungal activity of **44** 130

Figure A4 Antibacterial activity of **37** 131

Figure A5 Antifungal activity of **37** 131

Figure A6 Antibacterial activity of **49** 132

Figure A7 Antifungal activity of **49** 132

Figure A8 Antibacterial activity of **48** 133

Figure A9 Antifungal activity of **48** 133

Figure A10 Antibacterial activity of **50** 134

Figure A11 Antifungal activity of **50** 134

Figure A12 Antifungal activity of **51** 135

Figure A13 Antifungal activity of **52** 135

Figure A14 Effects of compounds **50**, **51**, and **52** on *C. alibicans* 136

Figure A15 Effects of compounds **50**, **51**, and **52** on *K. pneumonia* 136

Figure A16 Representative GC-EIMS trace showing the lipid profile obtained from a 20 day old *P. parvum* culture 137

Figure A17 Representative GC-EIMS trace showing the lipid profile obtained from water collect at a large *P. parvum* bloom event in Lake Texoma, Lebanon Pool (Oklahoma, USA, Feb. 2009) 138

Figure A18 Representative GC-EIMS trace showing the lipid profile obtained from water collect at a large *P. parvum* fish kill event in the Dunkard Creek drainage, Wana Bridge (Pennsylvania, USA, Oct. 2009) 139

Figure A19 Representative GC-EIMS trace showing the lipid profile obtained from water collect near, but not part of a large *P. parvum* fish kill (control) in the Dunkard Creek drainage, Upper Beaver Dam (Pennsylvania, USA, Oct. 2009) 140

Table A5 NMR data used for the structure determination of compounds **63**, **64**, and **65** 141

Lunalide A NMR spectra 142

Lunalide B NMR spectra 146

Suberoylanilide hydroxamic acid (SAHA) NMR spectra 154

Nygerone A NMR spectra 155

Nygerone B NMR spectra 160

Methyl succinate NMR spectra 163

Nygerone C NMR spectra 164

Pestalamide A/Tensidol B NMR spectra 167

Pestalamide B NMR spectra 170

Carbonarone A NMR spectra 175

Compound 48 NMR spectra 178

Compound 50 NMR spectra 182

Compound 51 NMR spectra 184

Compound 52 NMR spectra 188

GAT 512A NMR spectra 192

GAT 768A NMR spectra 202

GAT 770A NMR spectra 204

Table A1 Screening of epigenetic modifiers against fungal cultures.

	Organism[b]											
Compound[c]	AF	AW	CC	CS	DS	PC1	PC2	RS	VP	I1	I2	I3
A	+	+	+	-	+	+	+	-	+	+	+	+
B	+	+	nt	nt	nt	+	+	nt	nt	nt	nt	nt
C	-	-	nt	nt	nt	-	-	nt	nt	nt	nt	nt
D	+	+	+	-	nt	+	+	-	+	+	-	-
E	-	-	+	-	nt	-	-	-	+	-	-	-
F	-	-	-	-	nt	-	-	-	-	-	-	-
G	+	+	-	+	nt	+	+	-	+	-	-	+
H	+	-	-	+	nt	+	+	-	+	-	-	+
I	-	-	-	+	nt	-	-	-	-	-	-	-
J[d]	+	+	+	-	nt	-	-	-	-	+	-	-
K	-	-	-	-	nt	-	-	-	-	-	-	-
L	-	-	-	-	nt	-	-	-	-	-	-	-
M	-	-	-	-	nt	-	-	-	-	-	-	-

[a]Results described in this table should be interpreted as follows: + (active), - (not active), and nt (not tested); activity was ascribed to those compounds inducing the production of new secondary metabolites not observed under control culture conditions or enhanced levels of constitutively expressed compounds as based on analysis by HPLC, MS, ^{1}H-NMR, or TLC

[b]Fungi used in this study: AF (*Aspergillus flavus*), AW (*Aspergillus westerdijkiae*), CC (*Cladosporium cladosporioides*), CS (*Clonostachys* sp.), DS (*Diatrype* sp.), PC1 (*Penicillium chrysogenum*), PC2 (*Penicillium citrinum*), RS (*Rhizopus* sp.), VP (*Verticillium psalliotae*), I1 (Unidentified isolate 1), I2 (Unidentified isolate 2), and I3 (Unidentified isolate 3).

[c]Compounds used in this study: A (5-azacytidine), B (5-aza-2′-deoxycytidine), C (hydralazine), D (procaine), E (procainamide), F (sodium butyrate), G (suberohydroxamic acid), H (suberoylanilide hydroxamic acid), I (valproic acid), J (5-azacytidine and suberoylanilide hydroxamic acid), K (amphotericin B), L (cycloheximide), and M (5-fluorouracil).

[d]None of the 'active' compound mixture treatments produced metabolites different from those generated following the single administration of either 5-azacytidine or suberoylanilide hydroxamic acid alone. Most fungi exhibited significantly reduced growth when treated with the compound mixture.

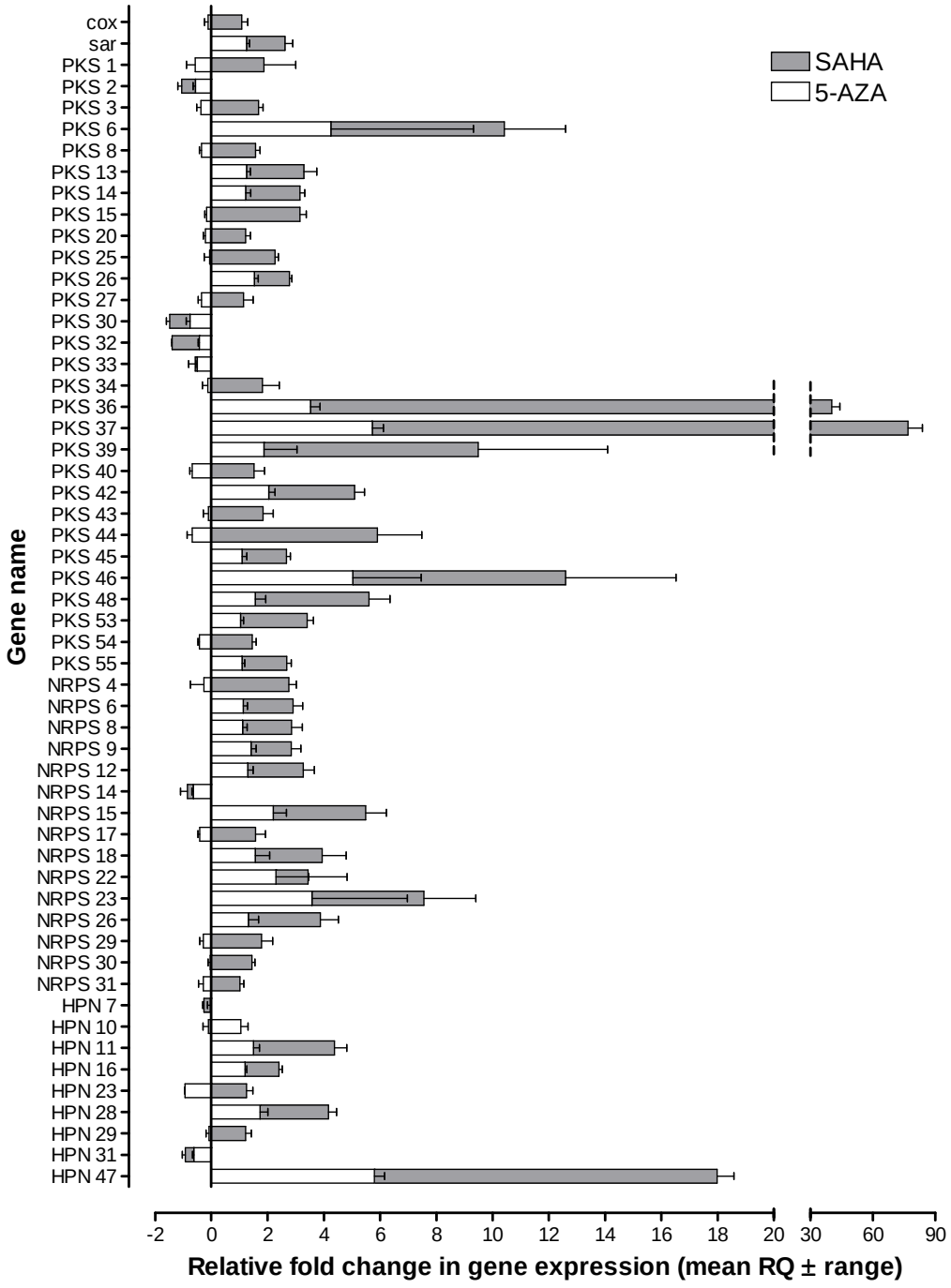
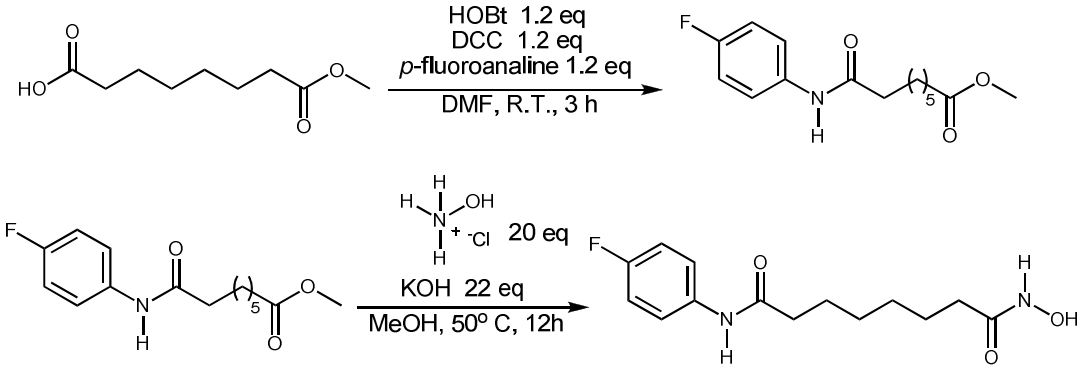


Figure A1 Real-time qRT PCR determination of the fold changes in expression of PKS, NRPS, and HPN gene clusters in *A. niger* ATCC 1015. All data are presented relative to the expression of *actin.* PKSs 12 and 38 are not included since no transcripts were detected for these gene clusters. RQ values were determined using the using the $2^{-\Delta\Delta CT}$ method. Fold changes of <1 were expressed as their corresponding negative fold change to help distinguish genes exhibiting decreased expression

Synthesis of *p*-fluoro-SAHA. The preparation of the fluorine containing SAHA derivative was accomplished by coupling the commercially available suberic acid monomethyl ester with p-fluoroanaline under standard conditions utilizing hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC) in DMF. This reaction was quenched by diluting the reaction mixture with water (1:20 DMF:water) after allowing the reaction to progress for 3 hours at room temperature. The resulting precipitate was purified via flash silica gel chromatography utilizing a hexane, ethyl acetate gradient, providing the suber-*p*-fluoroanilic acid methyl ester in 82% yield. The oxime moiety was introduced via the treatment of the suber-*p*-fluoroanilic acid methyl ester with hydroxylamine hydrochloride in methanol and an excess of potassium hydroxide at 50^{o} C. This mixture was allowed to react for 12 hours before the reaction mixture was diluted with water and neutralized with acetic acid. The resulting precipitate was filtered, washed with water, and dried affording the *p*-fluoro-SAHA derivative in 61% overall yield. Analysis of the product with NMR revealed that the product was obtained in high chemical purity without need for further purification.



Scheme A1 Scheme for synthesizing *p*-fluoro-SAHA

Table A2 Results from LC-ESI-MS analysis from 12 different species of aspergilli, — indicates that the presence of the compounds was not at levels detectable by ESI-MS. + indicates that the presence of the compound at detectable levels by ESI-MS.

Organism	Treatment	Tensidol A/ Carbonarone A	Tensidol B/ Pestalamide A	Aspernigrin A	Pestalamide B	Nygerone A	Nygerone B
A.aculeatus ATCC 5094	Control	—	—	—	+	—	—
	5-Aza	—	—	—	+	—	—
	SAHA	—	+	—	+	—	—
A. japonicas ATCC 2053	Control	—	—	—	+	—	—
	5-Aza	—	—	—	—	—	—
	SAHA	—	—	—	+	—	—
A.brasiliensis ATCC 35542	Control	—	+	—	+	—	—
	5-Aza	—	—	—	+	—	—
	SAHA	—	—	—	+	—	—
A.carbonarius ATCC 346	Control	—	—	—	+	—	—
	5-Aza	—	—	—	+	—	—
	SAHA	+	+	—	+	—	—
A.ibericus ATCC 35644	Control	—	—	—	+	—	—
	5-Aza	—	—	—	+	—	—
	SAHA	—	—	—	+	—	—
A.tubingensis ATCC 4875	Control	—	+	—	+	—	—
	5-Aza	—	+	—	+	—	—
	SAHA	—	+	—	+	—	—
A. carbonarius ATCC 369	Control	—	—	+	+	—	—
	5-Aza	—	—	+	+	—	—
	SAHA	—	—	—	—	—	—
A.parasiticus ATCC 465	Control	+	—	+	+	—	—
	5-Aza	+	—	+	+	—	—
	SAHA	+	—	+	+	—	—
A.ellipticus ATCC 5120	Control	—	—	—	+	—	—
	5-Aza	+	—	—	+	—	—
	SAHA	—	—	—	—	—	—
A.niger ATCC 1956	Control	—	—	—	—	—	—
	5-Aza	—	—	—	—	—	—
	SAHA	—	—	—	+	—	—
A.niger ATCC 326	Control	+	+	—	+	—	—
	5-Aza	+	+	—	+	—	—
	SAHA	—	—	—	—	—	—
A. niger ATCC 1015*	Control	—	+	—	+	—	—
	5-Aza	—	+	—	+	—	—
	SAHA	—	+	—	+	+	—

Table A3 GIAO based Calculations of ^{13}C-NMR chemical shifts for **48** and **41** compared to the reported ^{13}C-NMR chemical shifts

	Compound 48			**JBIR-86 (41)**		
Position	Calculated ^{13}C-NMR Values	Published ^{13}C-NMR Values[a]	$\|\delta_C$ Calc - Exp$\|$ δ_C	Predictive ^{13}C-NMR Values	Published ^{13}C-NMR Values[a]	$\|\delta_C$ Calc - Exp$\|$ δ_C
2	155.92	161.70	5.78	115.24	161.70	46.46
3	161.65	163.90	2.25	143.94	163.90	19.96
3a	126.39	119.80	6.59	92.79	119.80	27.01
4	167.86	175.00	7.14	133.87	175.00	41.13
5	115.63	117.30	1.67	96.74	117.30	20.56
6a	164.94	167.60	2.66	141.80	167.60	25.80
7	44.24	40.00	4.24	50.25	40.00	10.25
8	133.12	134.10	0.98	137.00	134.10	2.90
9	124.81	129.40	4.59	123.30	129.40	6.10
10	125.46	129.30	3.84	124.07	129.30	5.23
11	124.52	128.00	3.48	123.23	128.00	4.77
12	124.77	129.30	4.53	125.68	129.30	3.62
13	127.15	129.40	2.25	123.31	129.40	6.09
OMe	55.50	52.80	2.70	56.21	52.80	3.41

[a] Published 13C-NMR values obtained from publication by Takagi *et. al.*[121].

Table A4 ^{1}H- and ^{13}C-NMR data tables for various γ-pyrones and γ-pyridinones from natural and synthetic sources

	Carbonarone A (52)/ Tensidol A (46)		**Pestalamide A (50)/ Tensidol B (48)**		**Compound 56/ JBIR-86 (47)**		**JBIR-87 (49)/ Proposed Compound 61**	
Position[a]	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.
2 (2)	8.73, s	162.0	8.88, s	164.4	8.45, s	161.8	8.80, s	165.3
3 (3a)		119.3		119.2		119.7		119.5
4 (4)		178.1		178.8		175.2		179.7
5 (5)	6.25, s	116.0	6.44, s	116.5	6.22, s	117.2	6.38, s	116.5
6 (6a)		168.8		170.7		167.7		171.9
7 (7)	3.90, s	39.6	4.06, s	39.6	3.84, s	39.8	4.06, s	40.0
8 (8)		133.7		135.8		134.0		135.9
9,13 (9,13)	7.29, m	129.1	7.38, m	129.7	7.34, m	129.4	7.34, m	130.3
10,12 (10,12)	7.25, m	129.1	7.25, m	130.1	7.23, m	129.3	7.36, m	130.0
11 (11)	7.29, m	127.8	7.29, m	128.3	7.34, m	128.0	7.32, m	128.7
14 (3)		164.0		162.1		163.9		162.7
16 (1′)				173.7				174.4
17 (2′)			2.96, m	42.5			2.90, dd (4.8, 17.9)	42.6
			3.29, dd (8.0, 18.0)				3.16, dd (8.4, 17.9)	
18 (3′)			2.90, m	39.4			2.98, m	36.3
19 (4′)				178.1				177.8
20 (5′)			1.24, d (7.0)	17.4			1.22, d (7.2)	17.4
*N*H-15			11.74, br s				11.72	
(3-OH)								
14-NH_2	5.94, br s							
(3-OH, 4-OH)	9.07, br s							
14-OCH_3					3.88, s	52.83		
(3-OCH_3)								
4′-OCH_3							3.66, s	52.4

[a]Primary positional numbers are based on the scheme utilized for the γ-pyrone structures. Numbers in between () are provided to give context to the tensidol compounds numbering scheme.

Table A4 Continued

Position	Pestalamide B[a]		Nygerone A (51)		Nygerone B (53)		Nygerone C (57)	
	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.
2	8.52, s	145.3	8.43, s	148.2	8.42, s	146.9	8.49, s	146.9
3		117.5		117.2		118.1		117.7
4		178.0		177.6		177.8		177.7
5	6.36, s	120.2	6.55, s	122.3	6.48, s	121.8	6.61, s	121.9
6		154.2		152.0		150.9		151.3
7	4.06, s	39.6	3.67, s	39.4	3.66, s	39.2	3.71, s	39.4
8		137.2		134.8		134.7		134.8
9,13	7.34, m	130.2	6.77, m	128.9	6.80, m	128.7	6.83, m	128.5
10,12	7.37, m	130.2	7.18, m	129.0	7.17, m	128.7	7.21, m	128.9
11	7.20, m	128.7	7.18, m	127.7	7.18, m	127.5	7.23, m	127.7
14		164.8		163.2		166.0		166.0
16				173.2				
17	2.96 dd (5.0, 18.0)		2.86, dd (5.1, 17.2)	42.0				
	3.29 dd (8.0, 18.0)		3.20, dd (8.2, 17.2)					
18	2.90 ddd (5.0, 7.0, 8.0)	43.0	3.05, m	35.1				
19		175.0		178.9				
20	1.12, d (7.0)	17.8	1.27, d (7.0)	17.2				
1′				140.4		140.4		162.0
2′/6′			7.06, t (7.6)	127.0	7.05, d (7.6)	126.9	7.05	128.9
3′/5′			7.42, t (7.6)	130.2	7.40, d (7.6)	129.7	7.10	116.9
4′			7.49, t (7.6)	130.5	7.47, d (7.6)	130.0		164.1
*N*H-15			12.83, br s					
14-NH_2					5.70, br s		6.06, br s	
					9.90, br s		9.89, br s	
4′-F							-112.6[b]	

[a] ^{13}C-NMR Published values for Pestalamide B were incorrectly published, these have been corrected based on the isolation of this compound during the *A. niger* work discussed in Chapter 5.

[b] 19-F NMR chemical is reported in place of a proton at the 4′ position. These cultures were grown with *p*-fluoro-suberoyanlilide hyrdoxamic acid.

Table A4 Continued

Position	Compound 59		Compound 60	
	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.
2	8.82, s	166.0	9.88, s	193.9
3		118.3		100.6
4		175.6		178.5
5	6.94, s	112.4	7.95, s	97.6
6		164.1		167.1
7		129.6		130.4
8,12	7.89, m	126.2	8.04, m	126.7
9,11	7.53, m	130.8	7.60, m	129.2
10	7.55, m	130.8	7.66, m	132.8
13		163.0		161.7
15	3.86, s	52.3		

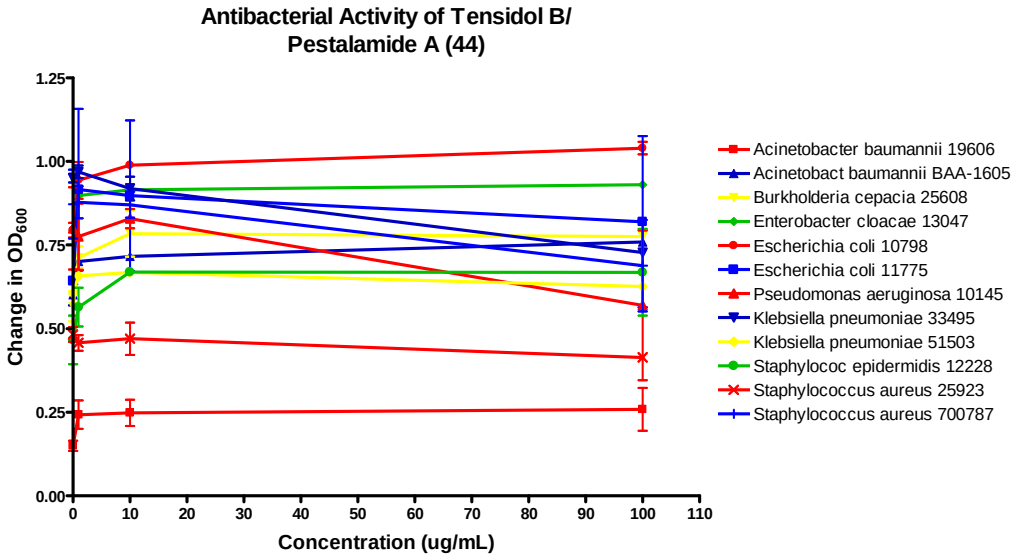


Figure A2 Antibacterial activity of **44**

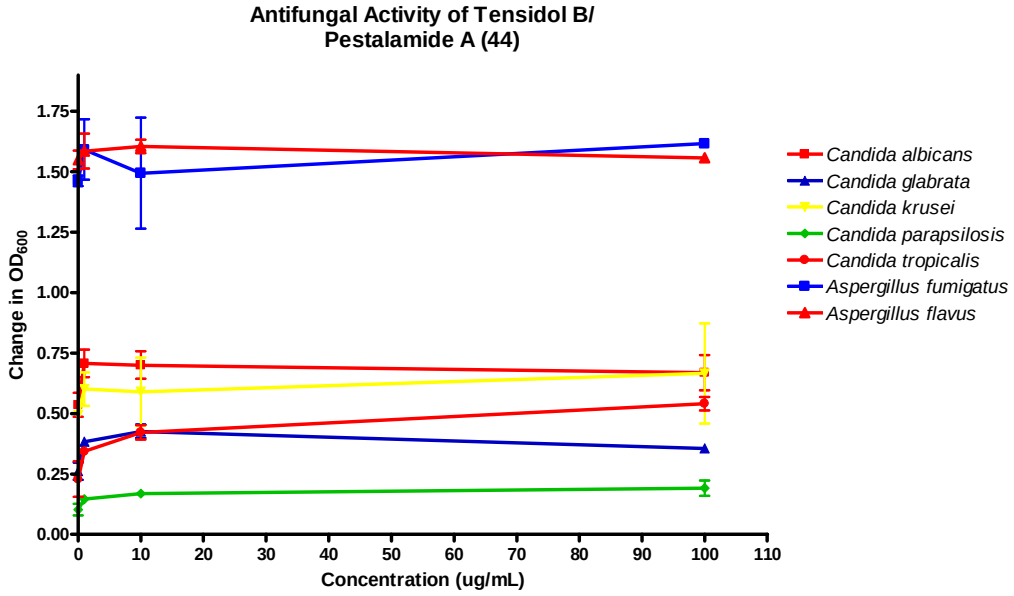


Figure A3 Antifungal activity of **44**

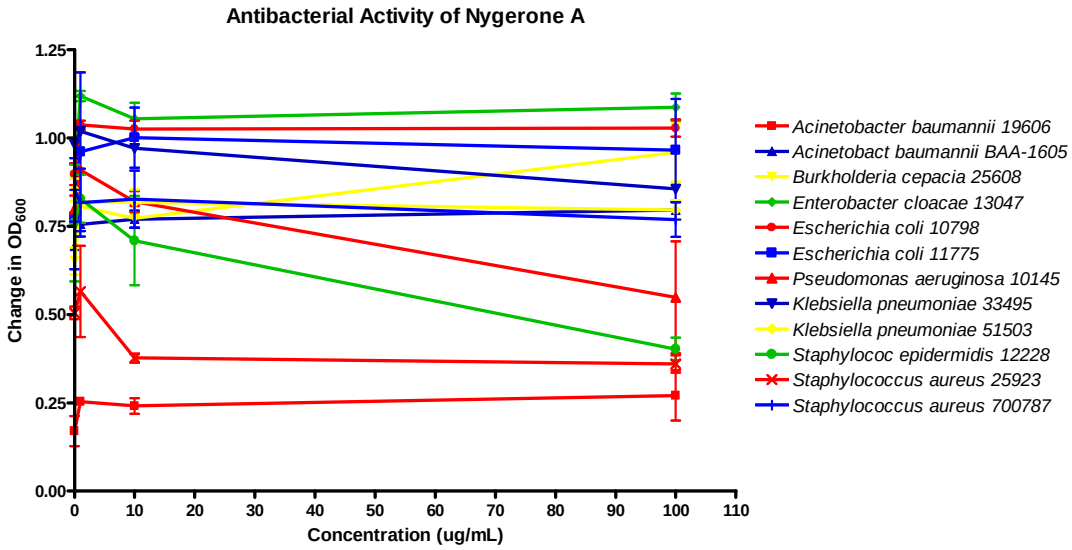


Figure A4 Antibacterial activity of **37**

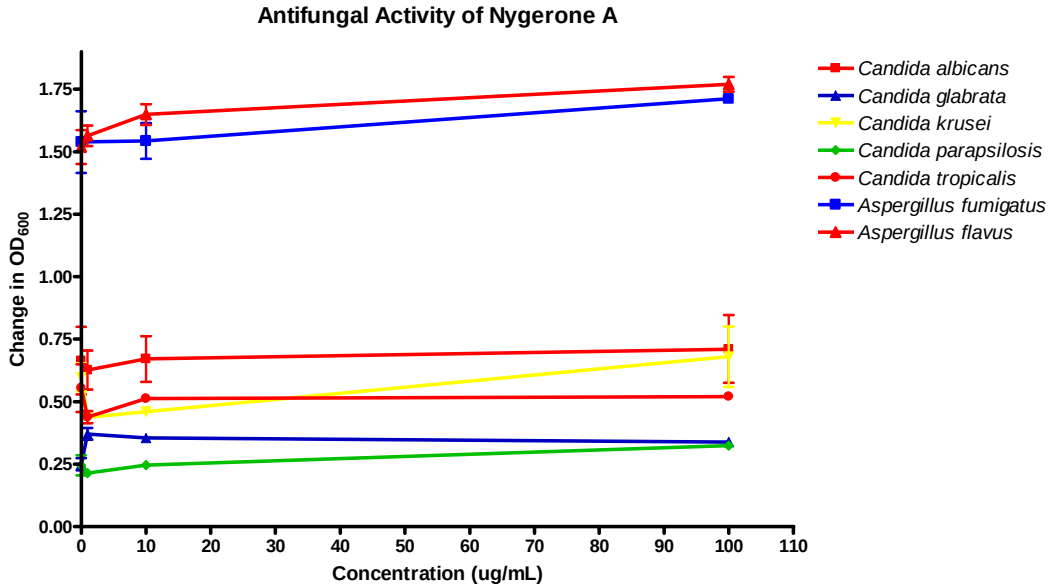


Figure A5 Antifungal activity of **37**

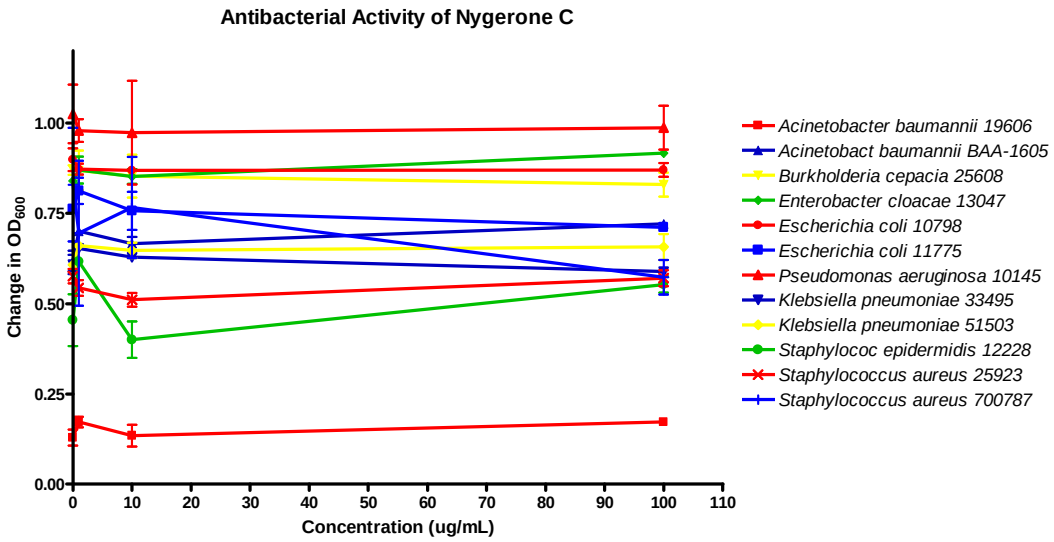


Figure A6 Antibacterial activity of **49**

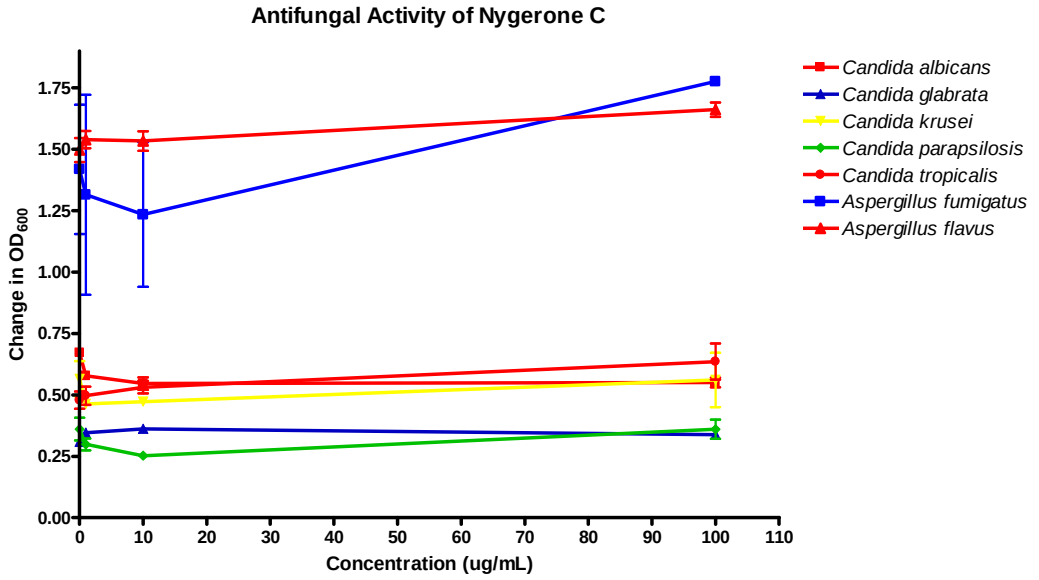


Figure A7 Antifungal activity of **49**

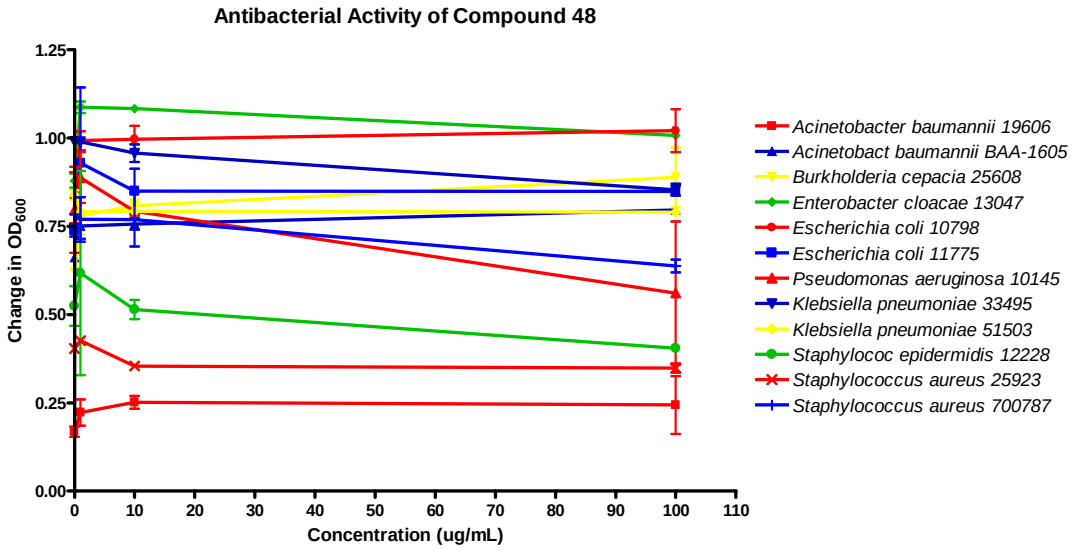


Figure A8 Antibacterial activity of **48**

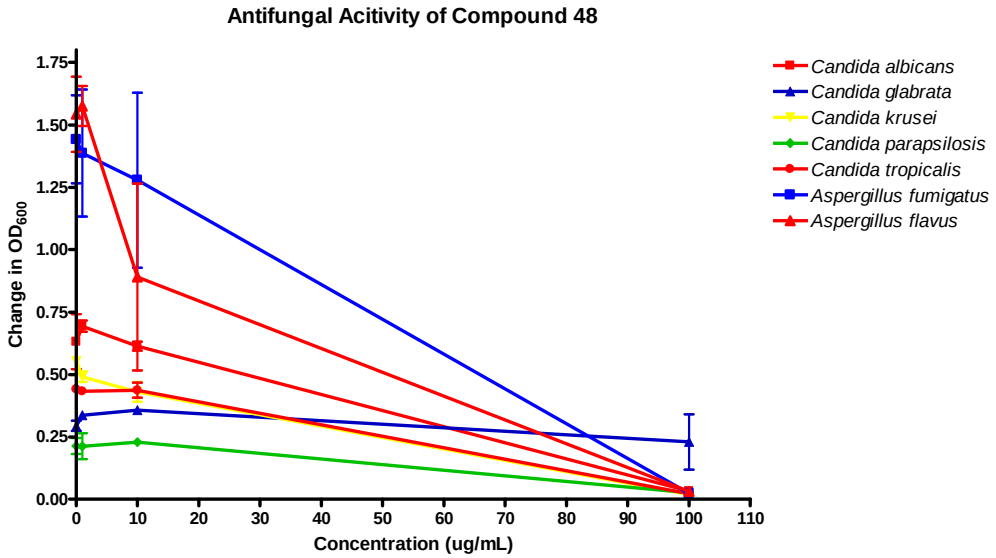


Figure A9 Antifungal activity of **48**

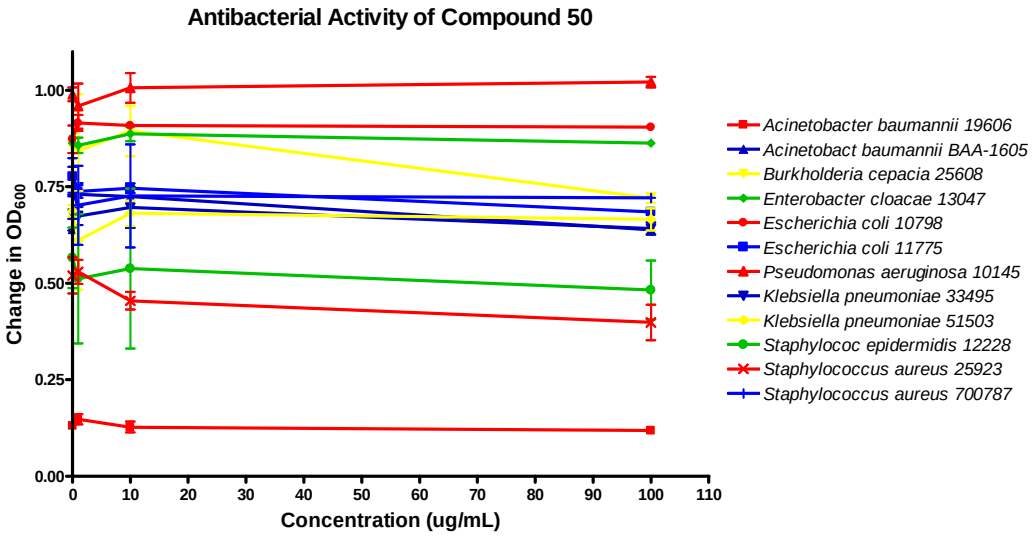


Figure A10 Antibacterial activity of **50**

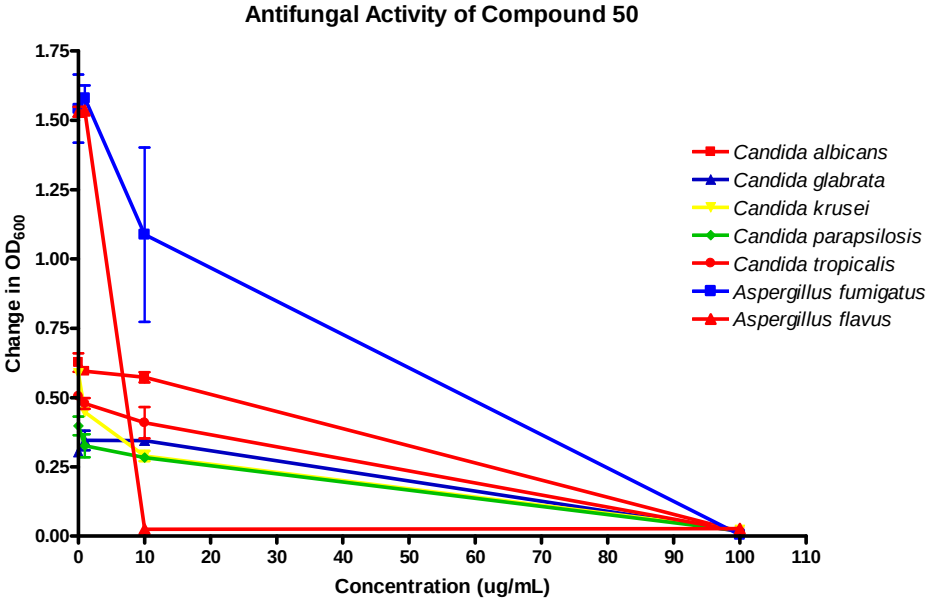


Figure A11 Antifungal activity of **50**

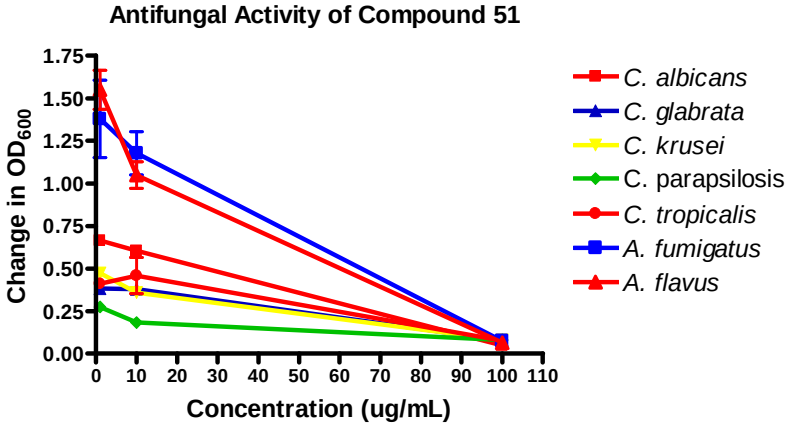


Figure A12 Antifungal activity of **51**

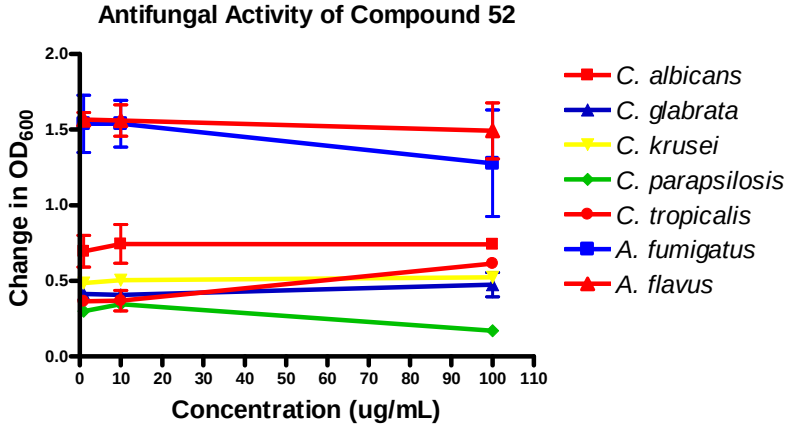


Figure A13 Antifungal activity of **52**

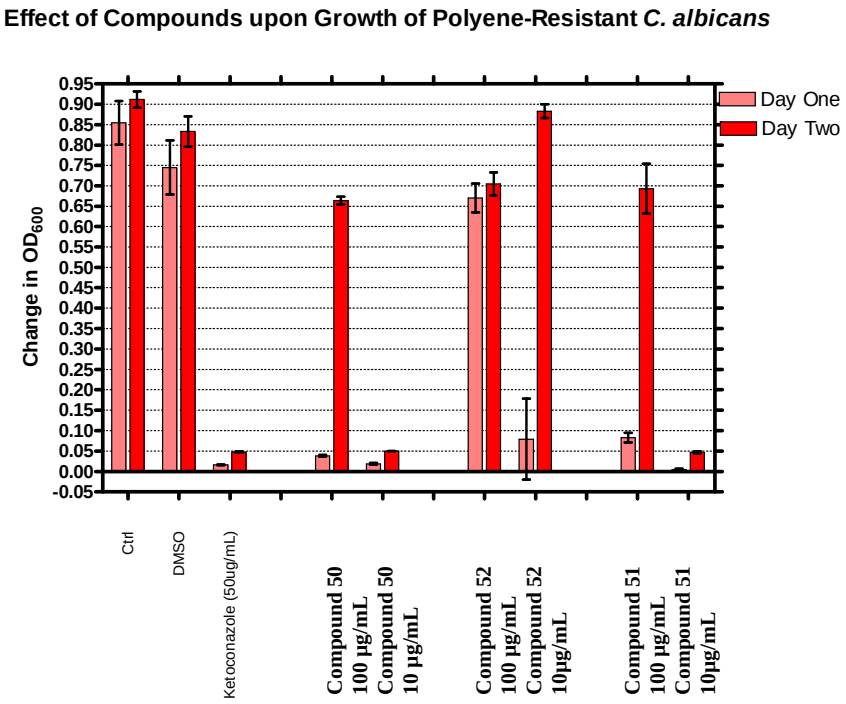


Figure A14 Effects of compounds **50**, **51**, and **52** on *C. alibicans*

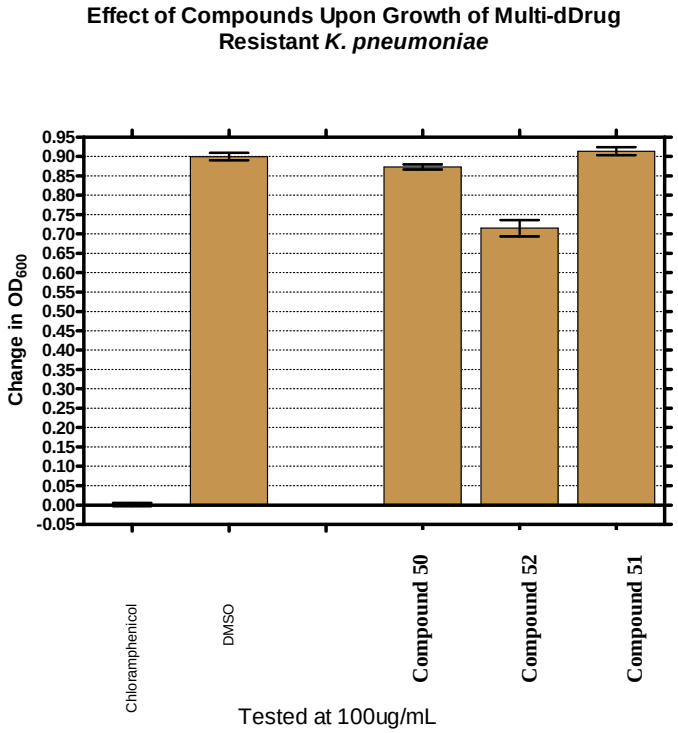


Figure A15 Effects of compounds **50**, **51**, and **52** on *K. pneumoniae*

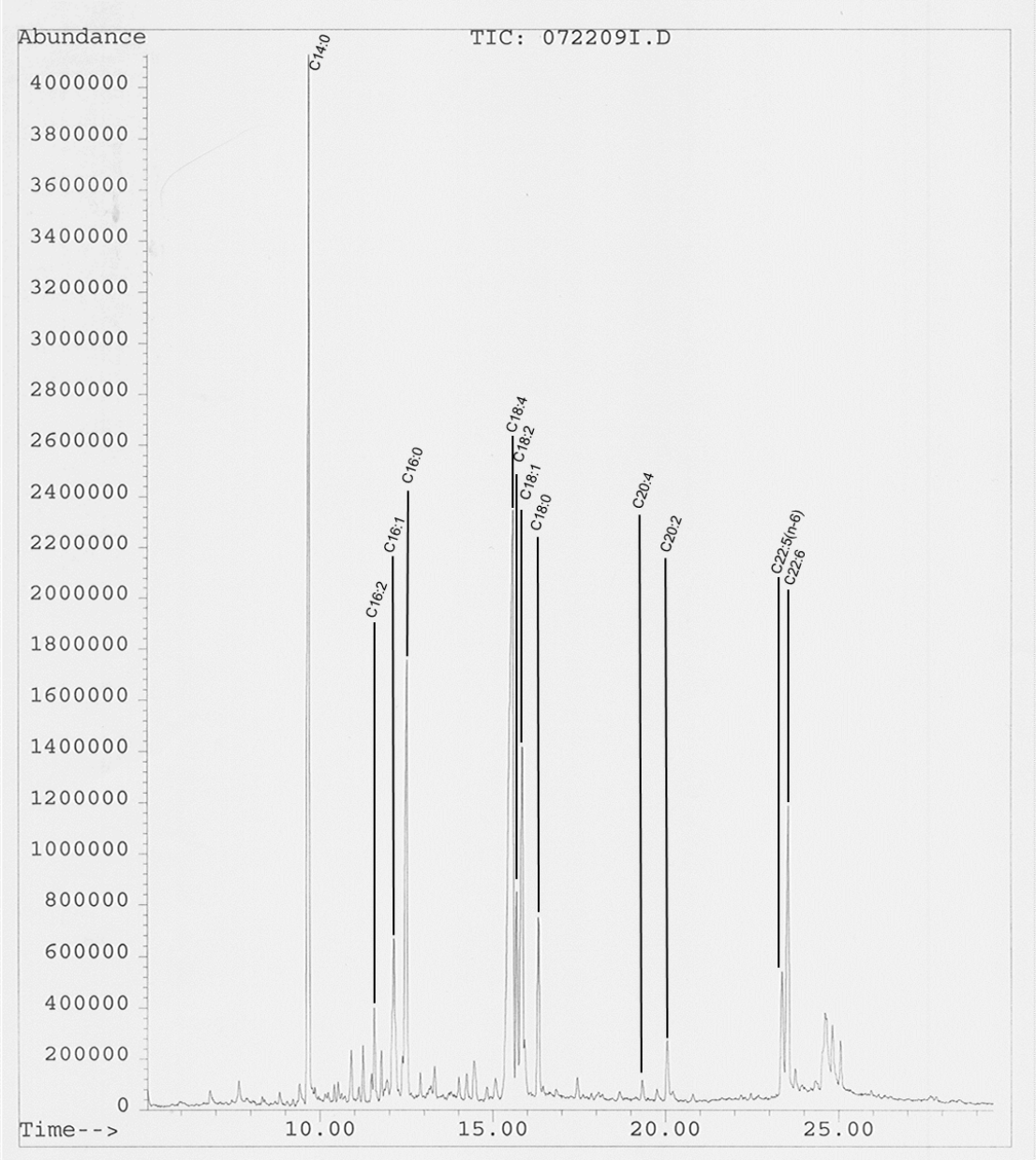


Figure A16 Representative GC-EIMS trace showing the lipid profile obtained from a 20 day old *P. parvum* culture

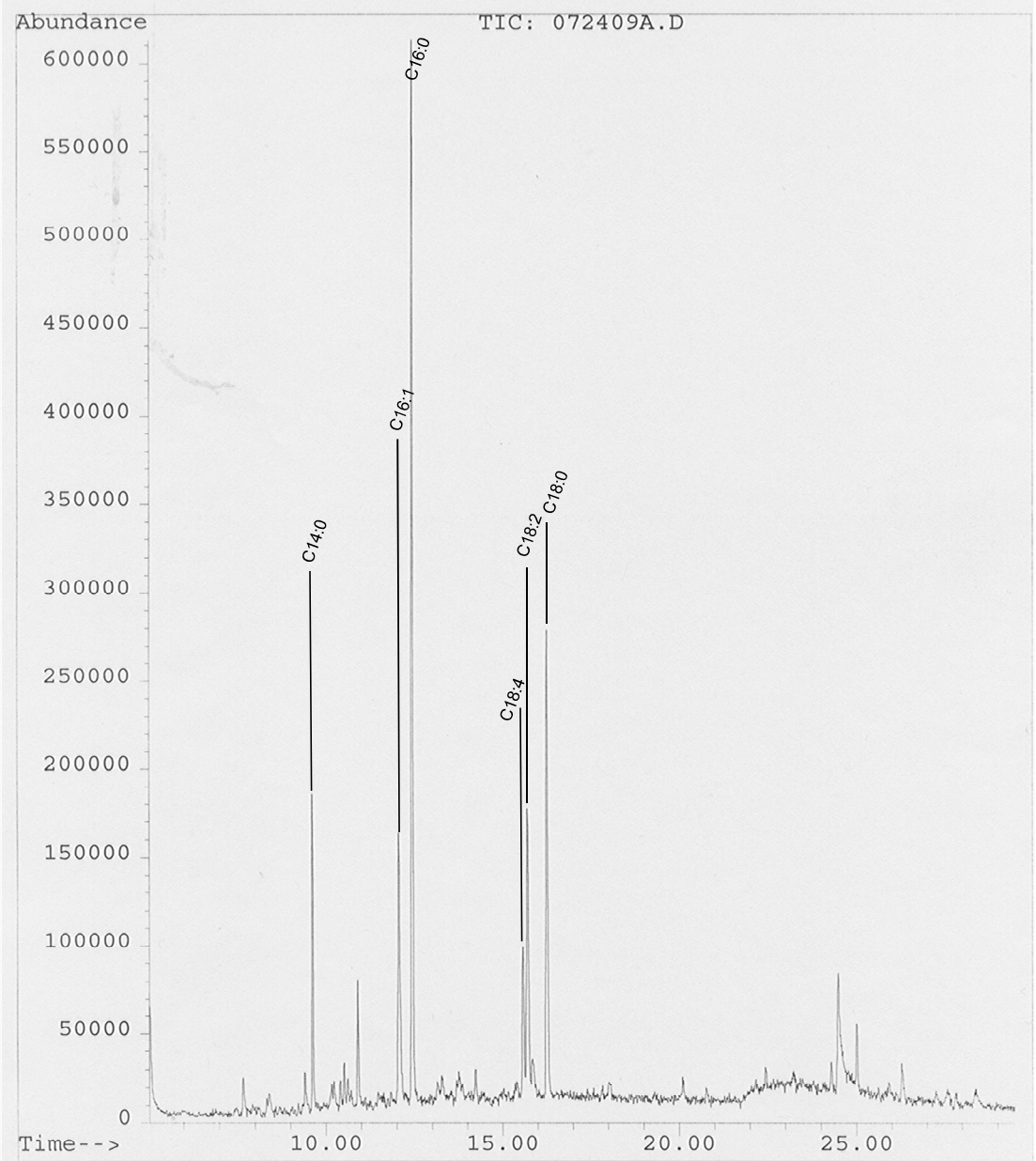


Figure A17 Representative GC-EIMS trace showing the lipid profile obtained from water collect at a large *P. parvum* bloom event in Lake Texoma, Lebanon Pool (Oklahoma, USA, Feb. 2009)

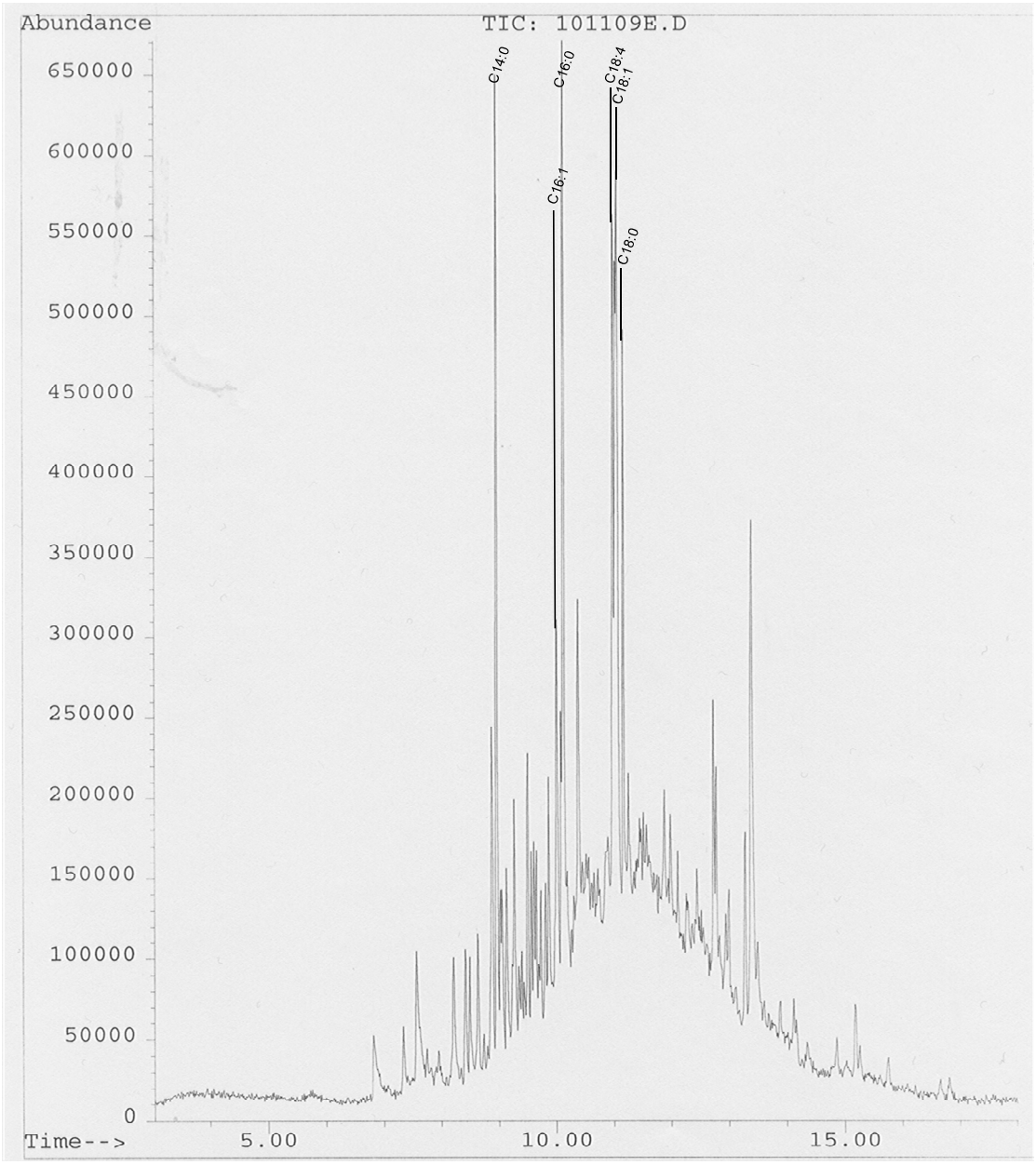


Figure A18 Representative GC-EIMS trace showing the lipid profile obtained from water collect at a large *P. parvum* fish kill event in the Dunkard Creek drainage, Wana Bridge (Pennsylvania, USA, Oct. 2009).

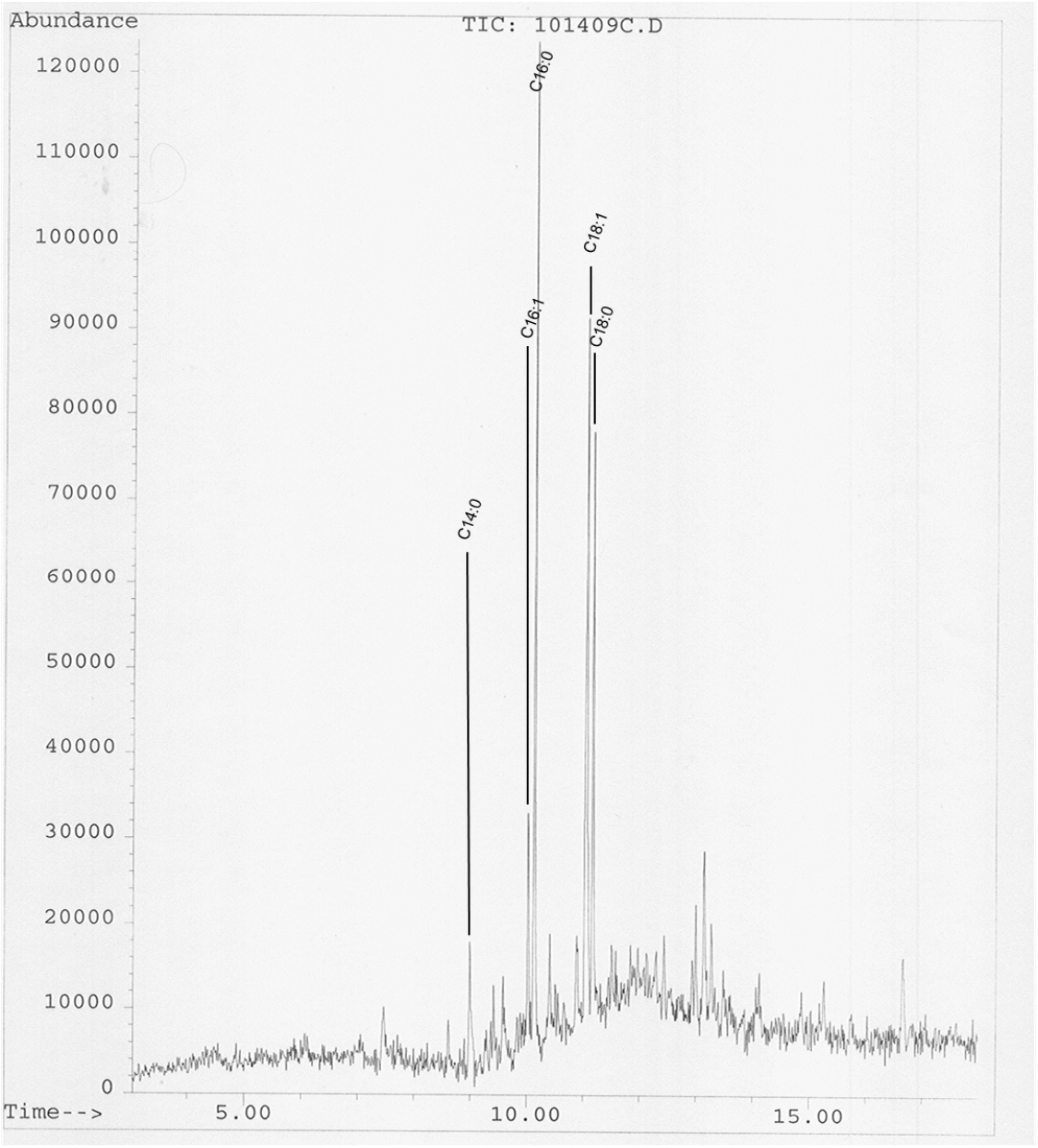
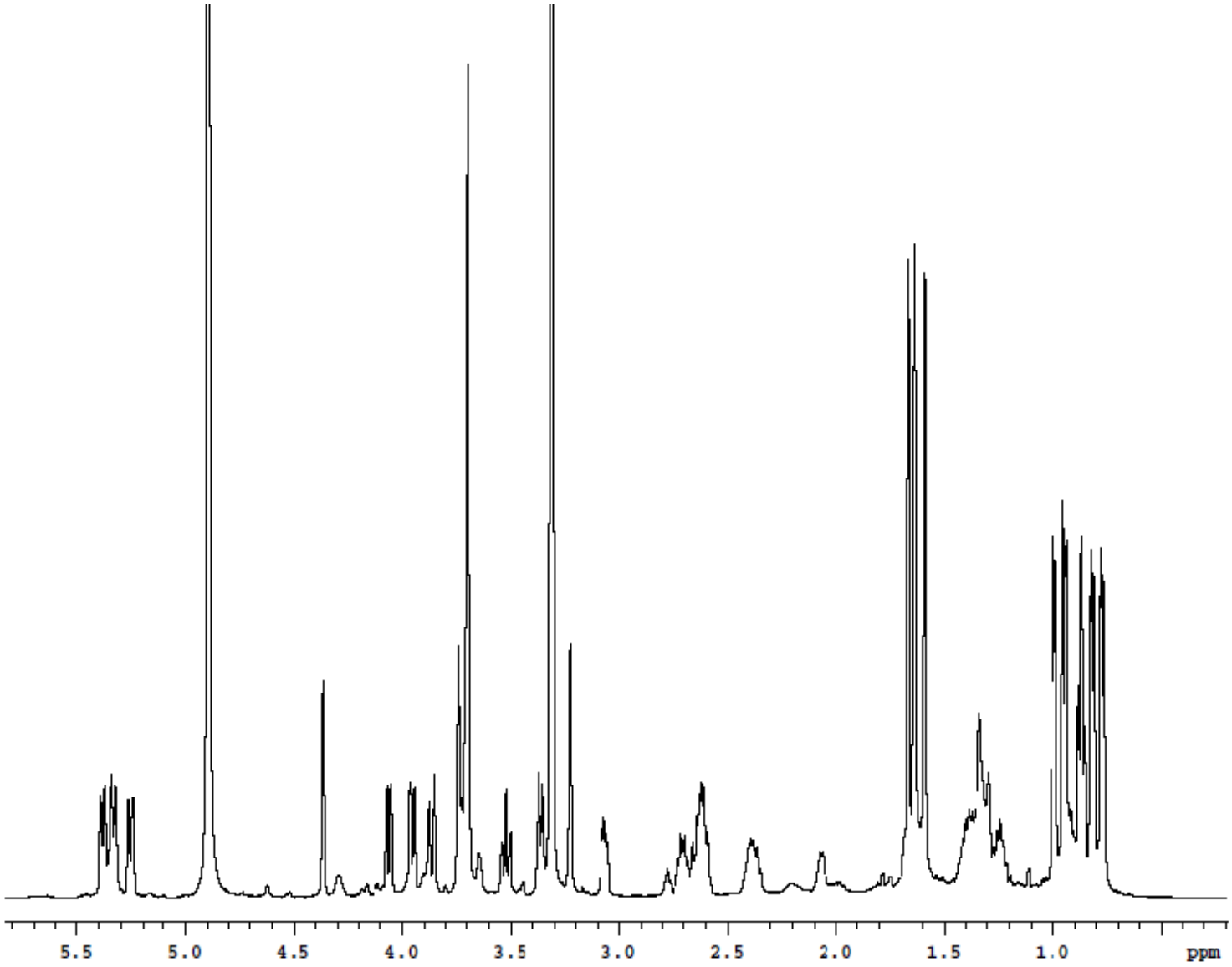


Figure A19 Representative GC-EIMS trace showing the lipid profile obtained from water collect near, but not part of a large *P. parvum* fish kill (control) in the Dunkard Creek drainage, Upper Beaver Dam (Pennsylvania, USA, Oct. 2009).

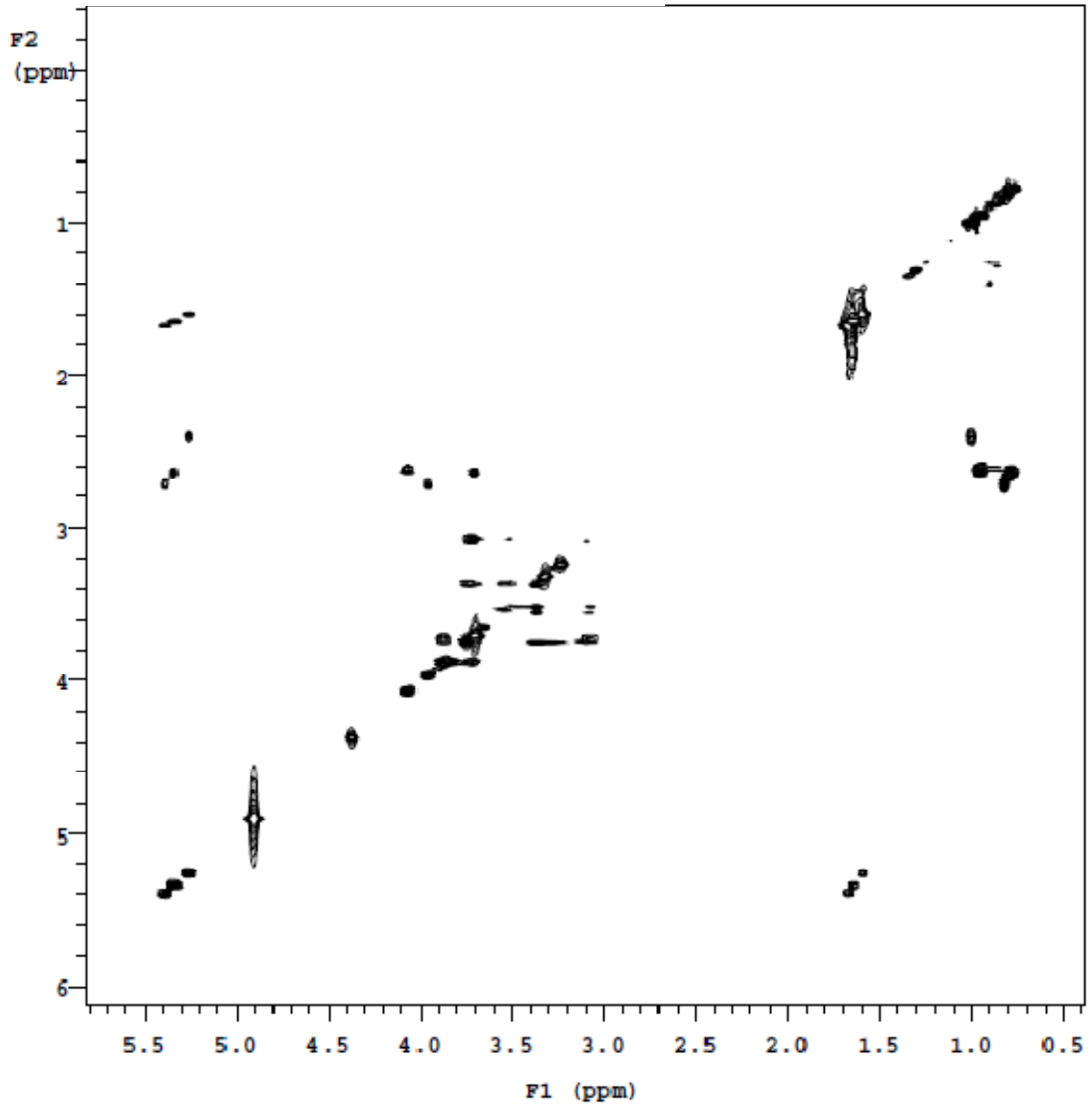
Table A10 NMR data used for the structure determination of compounds **63**, **64**, and **65**. Data were obtained at 500 MHz and 125 MHz for ^{1}H and ^{13} NMR, respectively. Samples were prepared in methanol-d_4.

	Compound 63			Compound 65			Compound 64		
Position	**δ_C**	**δ_H**	**HMBC**	**δ_C**	**δ_H**	**HMBC**	**δ_C**	**δ_H**	**HMBC**
1	66.7	4.15 bd (4.5, 5.9)	2, 3, 1'	61.0	4.20 dd (6.9, 12.1) 4.44 dd (3.1, 12.0)	2, 3, 1'	63.0	4.24 dd (6.7, 12.0) 4.44 dd (3.0, 12.1)	2, 3, 1'
2	68.8	3.99 m	1	70.4	5.29 m		70.4	5.29 m	
3	72.0	3.65 dd (4.6, 10.4) 3.92 dd (5.1, 10.4)	1, 2, 1'''	67.4	3.74 m 3.99 dd (5.2, 10.7)	1, 2, 1'''	67.2	3.76 m 4.00 dd (5.2, 10.8)	1, 2, 1'''
1'	175.5			173.6			171.6		
2'	34.9	2.38 t (7.5)	1', 3', 4'	35.5	2.34 t (7.1)	1', 3', 4'	32.3	3.14 bd (4.8)	1', 3', 4''
3'	25.8	1.66 p (7.4)	3', 4', 5'	25.5	1.63 m		121.0	5.58 m	2'
4'	30.3	1.41 p (7.8)	3', 4', 5'	29.7	1.41 m		131.0	5.58 m	2'
5'	28.0	2.08 m	3', 4'	28.4	2.11 m	4'	25.2	2.84 m	3', 4'
6', 7', 9', 10', 12', 13', 15', 16'	129.2-132.9	5.36 m		127.8-129.2	5.33-5.38		127.8-129.2	5.25-5.35 m	
8', 11', 14'	26.6-26.7	2.83 m		25.2	2.84 m		25.2 m	2.84 m	
17'	21.7	2.06 m	16', 18'	20.2	2.08 m	18'	20.3 m	2.09 m	18'
18'	14.8	0.93 t (7.6)	16', 17'	13.2	0.98 t (7.6)	17'	13.7	0.98 t (7.7)	17'
1''				173.6			173.3		
2''				35.5	2.34 t (7.1)	1'', 3'', 4''	33.5	2.34 t (7.5)	1'', 3'', 4''
3''				25.5	1.63 m		24.3	1.63 m	1'', 2', 4'', 5''
4''				29.7	1.41 m		29.1	1.41 m	2'', 3''
5''				28.4	2.11 m	4''	26.5	2.12 m	3'', 4'
6'', 7'', 9'', 10'', 12'', 13'', 15'', 16'',				127.8-129.2	5.33-5.38 m		127.8-129.2	5.25-5.35 m	
8'', 11'', 14'''				25.2	2.84 m		25.2	2.84 m	
17''				20.2	2.08 m	18''	20.3	2.09 m	18''
18''				13.2	0.98 t (7.6)	17''	13.7	0.98 t (7.7)	17''
1'''	103.9	4.22 d (7.6)	3, 3'''	103.8	4.21 d (7.6)		104.0	4.21 d (7.5)	3, 2'''
2'''	72.3	3.54 m	3'''	71.1	3.48 m	1'''	71.0	3.51 m	1''', 3'''
3'''	74.8	3.47 m	1''', 2''', 4'''	73.4	3.45 dd (3.3, 9.8)	2'''	73.4	3.45 dd (3.1, 9.8)	2'''
4'''	70.4	3.81 bd (2.9)	3''', 2'''	68.8	3.82 m	3''', 5'''	68.8	3.82 m	3''', 5'''
5'''	76.6	3.51 m	4''', 6'''	75.2	3.51 m	4''', 6'''	75.1	3.51 m	4''', 6'''
6'''	62.7	3.73 m	5'''	61.2	3.72 m	5'''	61.2	3.72 m	4''', 5'''

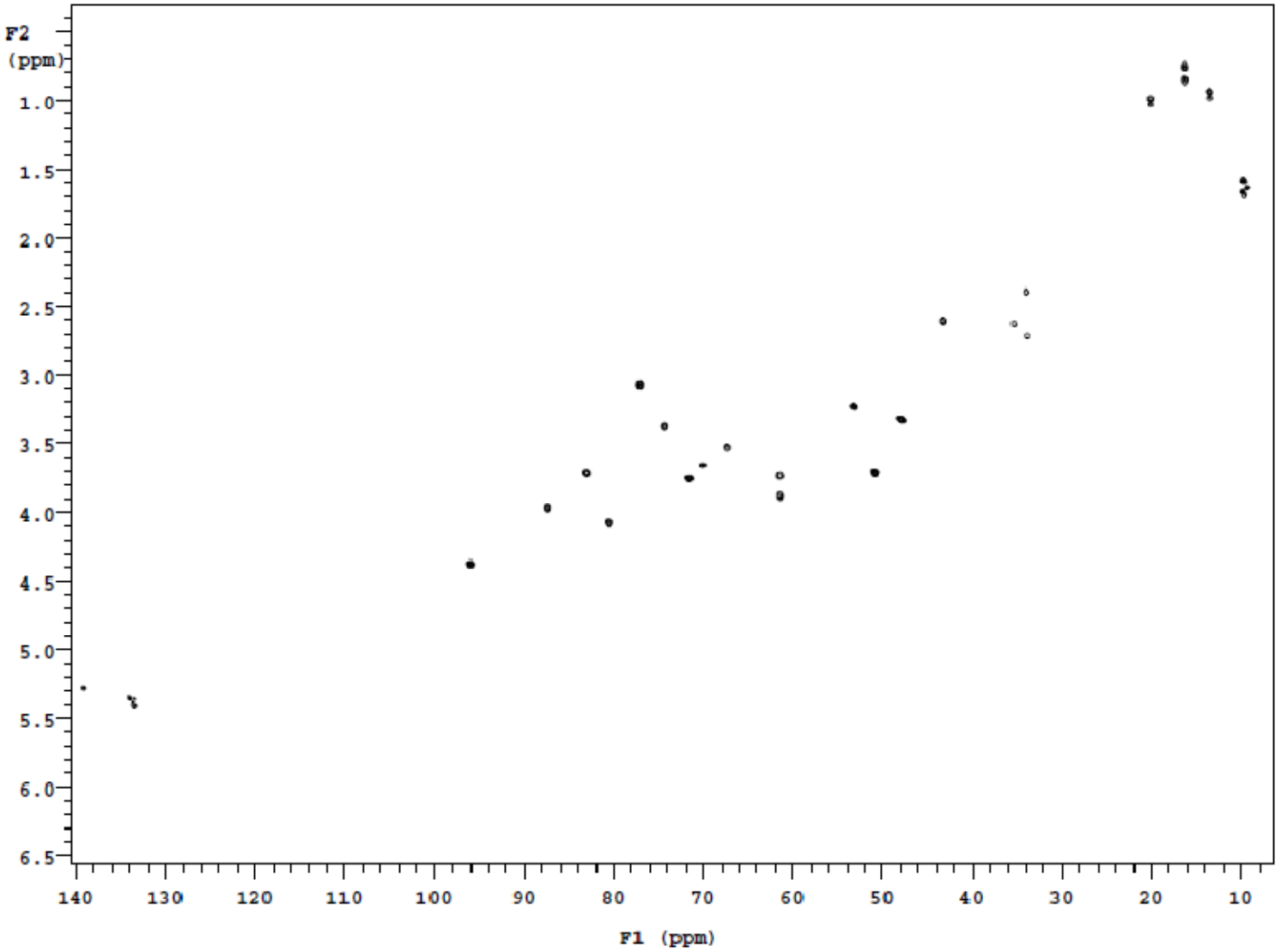
Lunalide A ^{1}H-NMR in CD_3OD



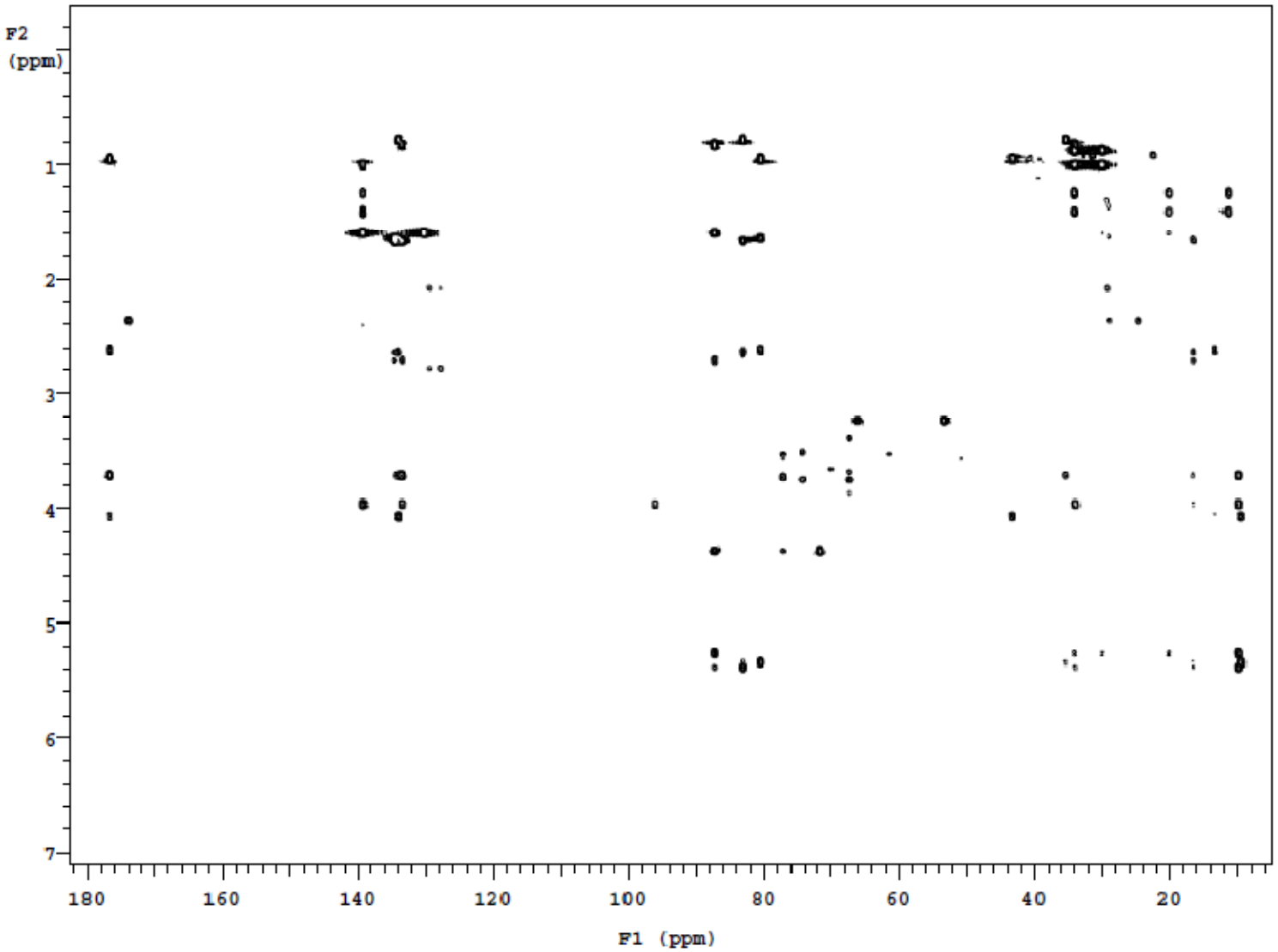
Lunalide A ^{1}H-^{1}H COSY in CD_3OD



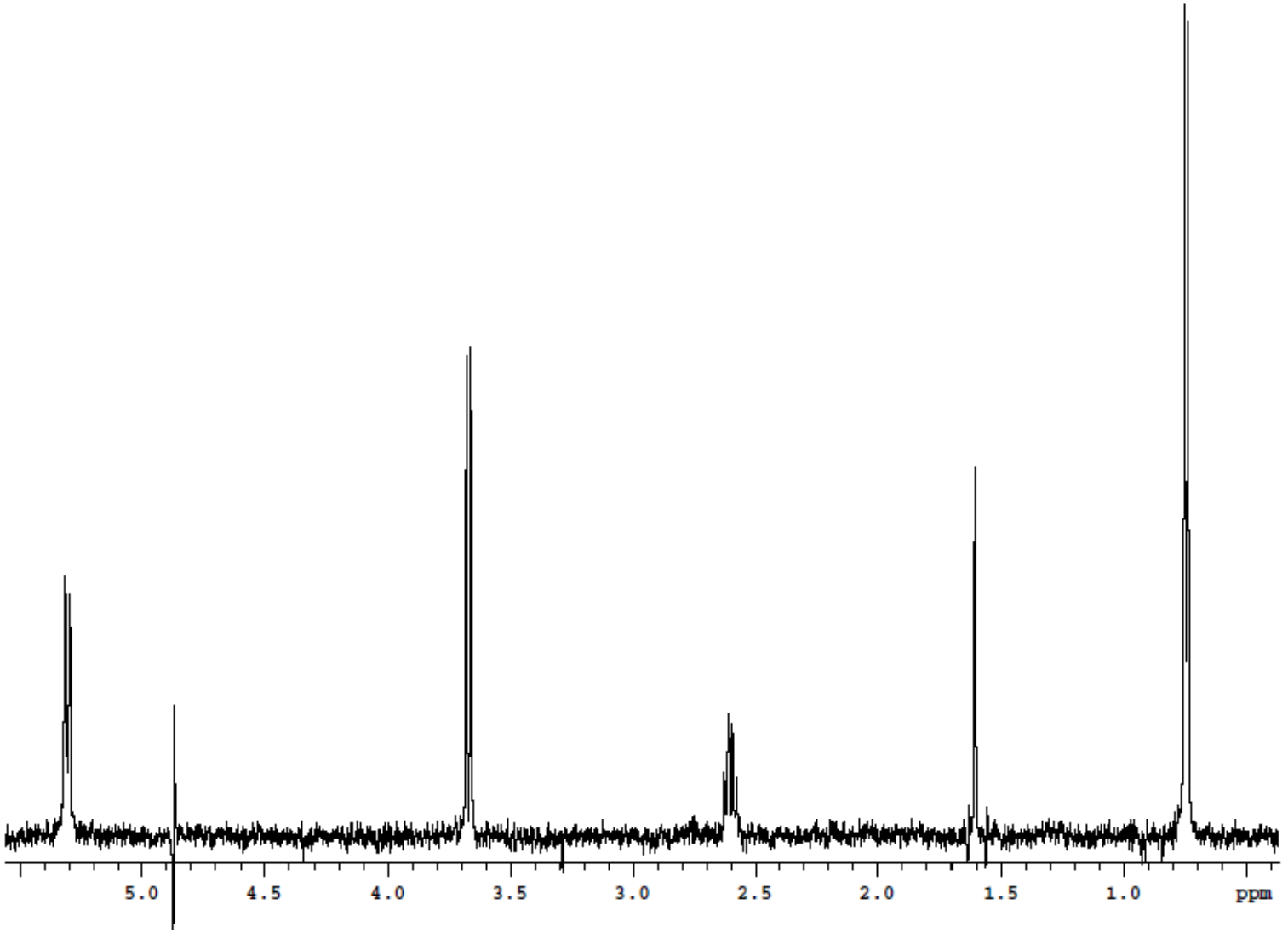
Lunalide A ^{1}H-^{13}C HSQC in CD_3OD



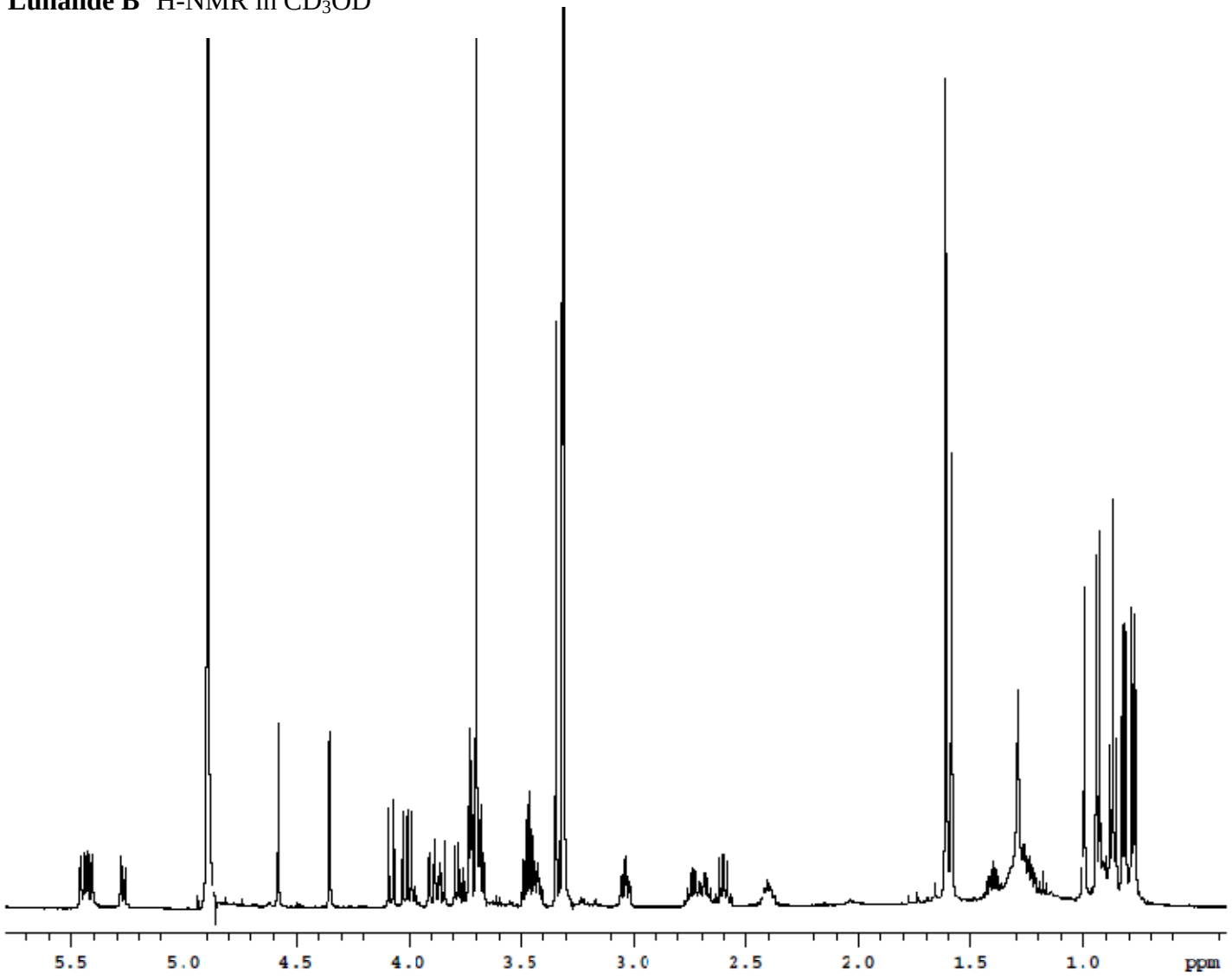
Lunalide A ^{1}H-^{13}C HMBC in CD_3OD



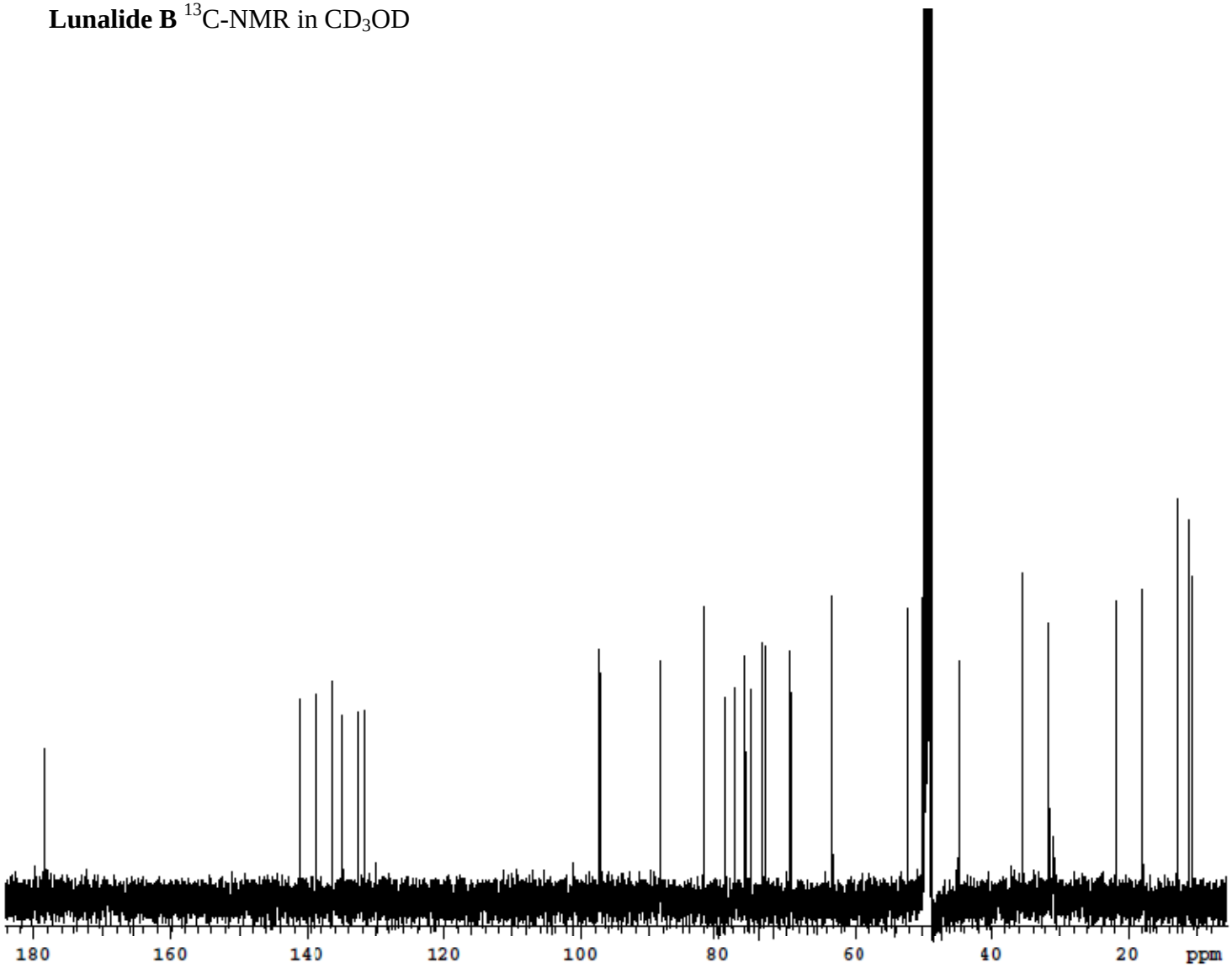
Lunalide A 1D-TOCSY in CD_3OD



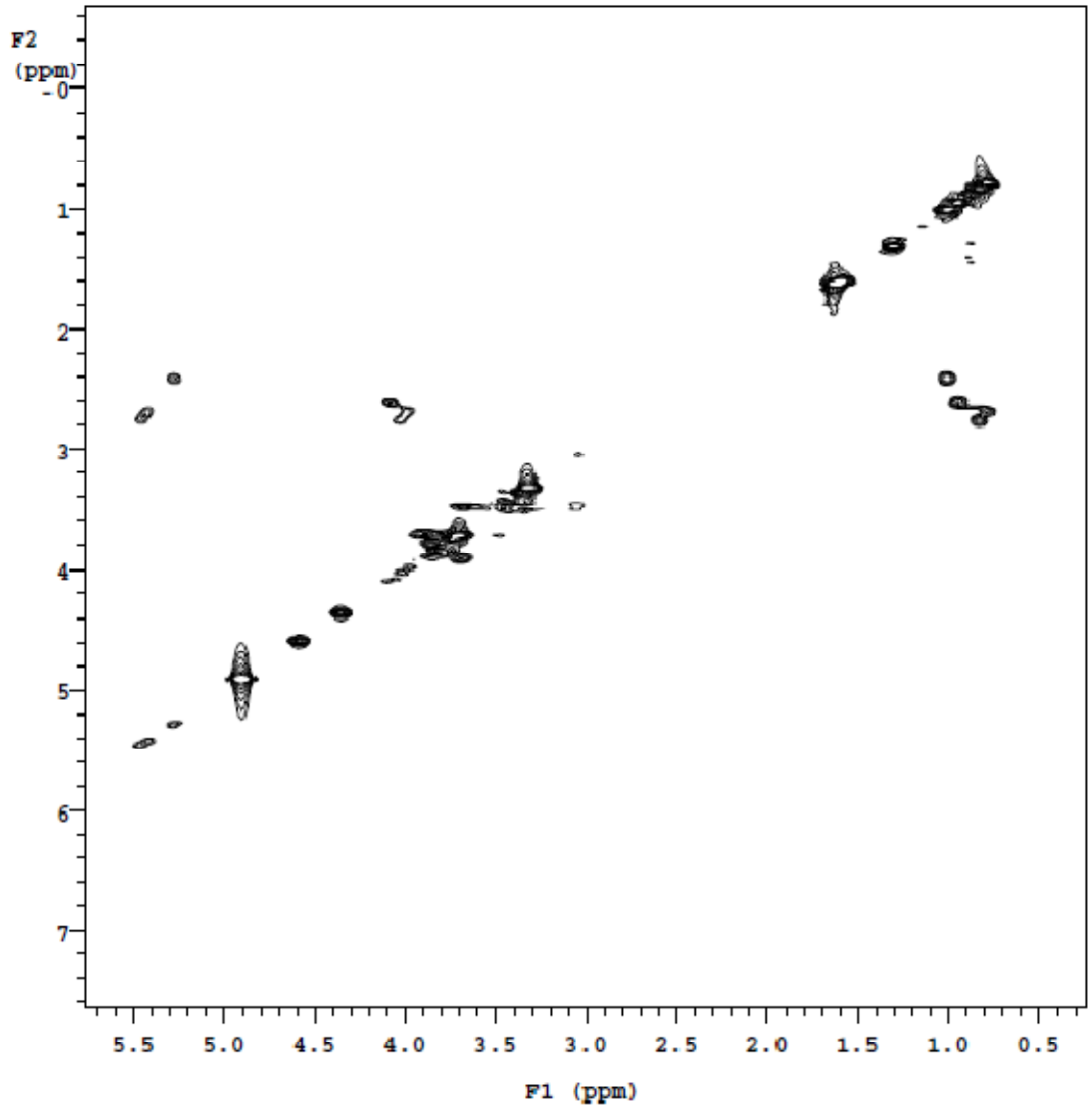
Lunalide B ^{1}H-NMR in CD_3OD



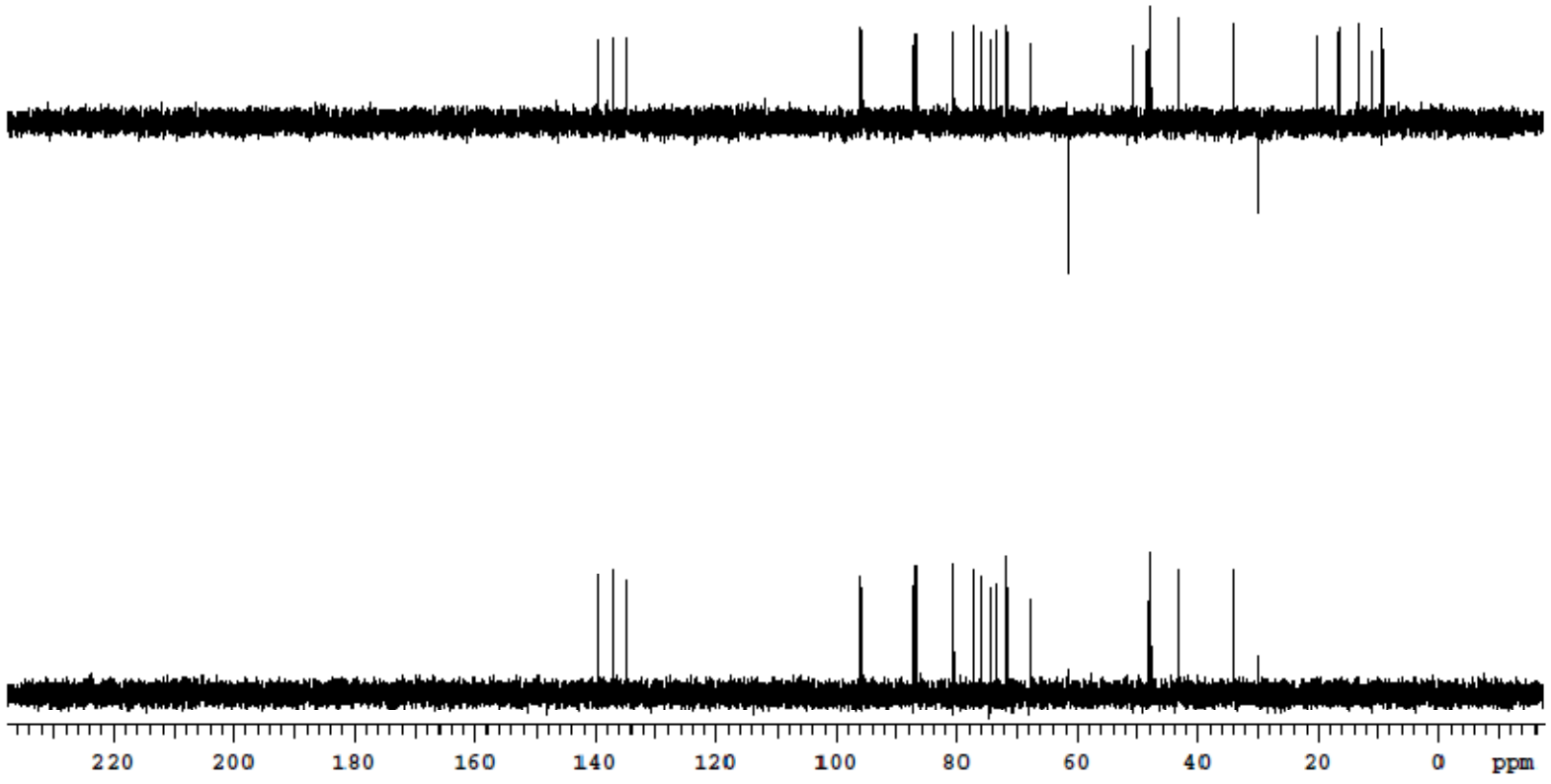
Lunalide B ^{13}C-NMR in CD_3OD



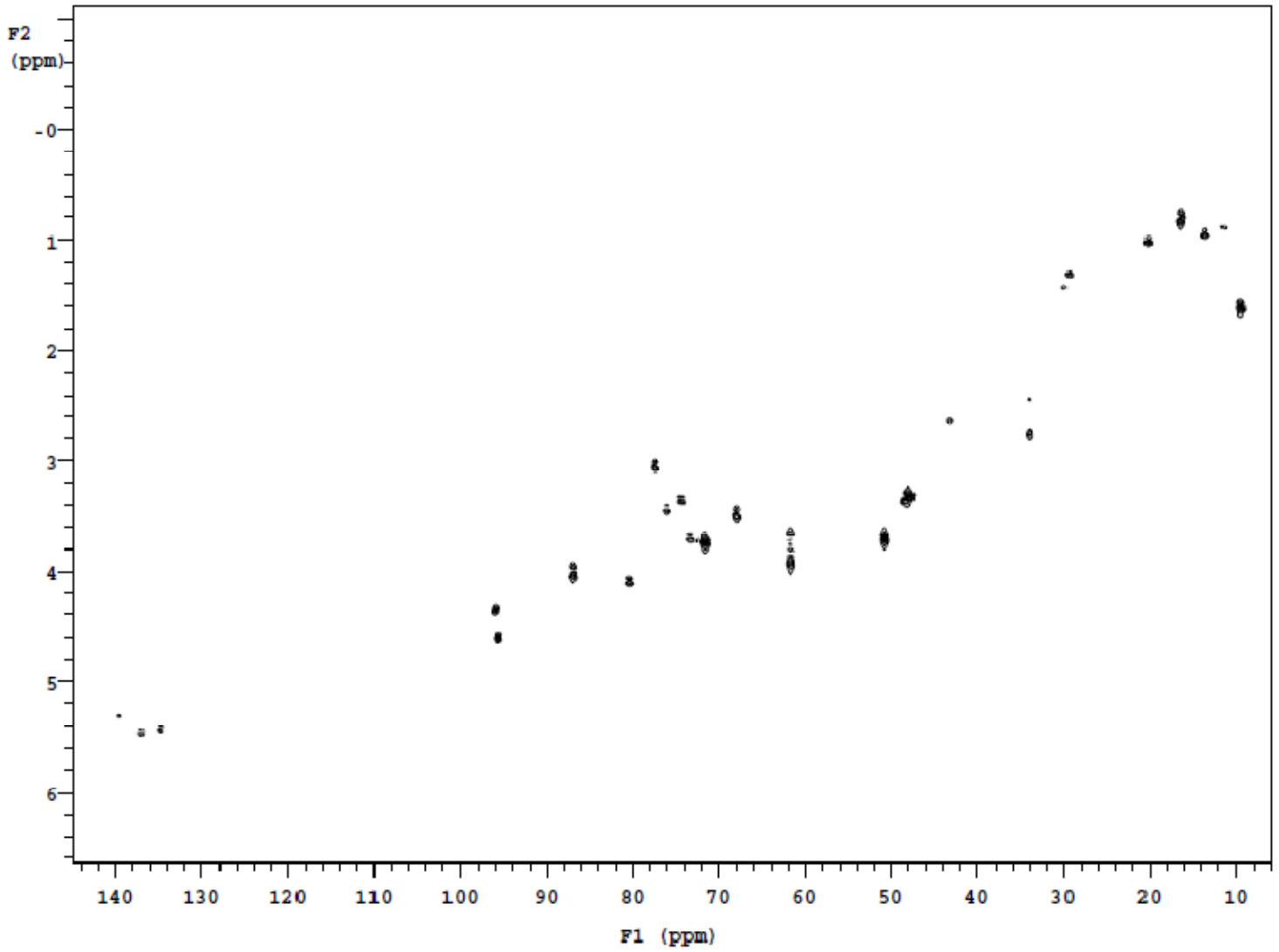
Lunalide B ^{1}H-^{1}H COSY in CD_3OD



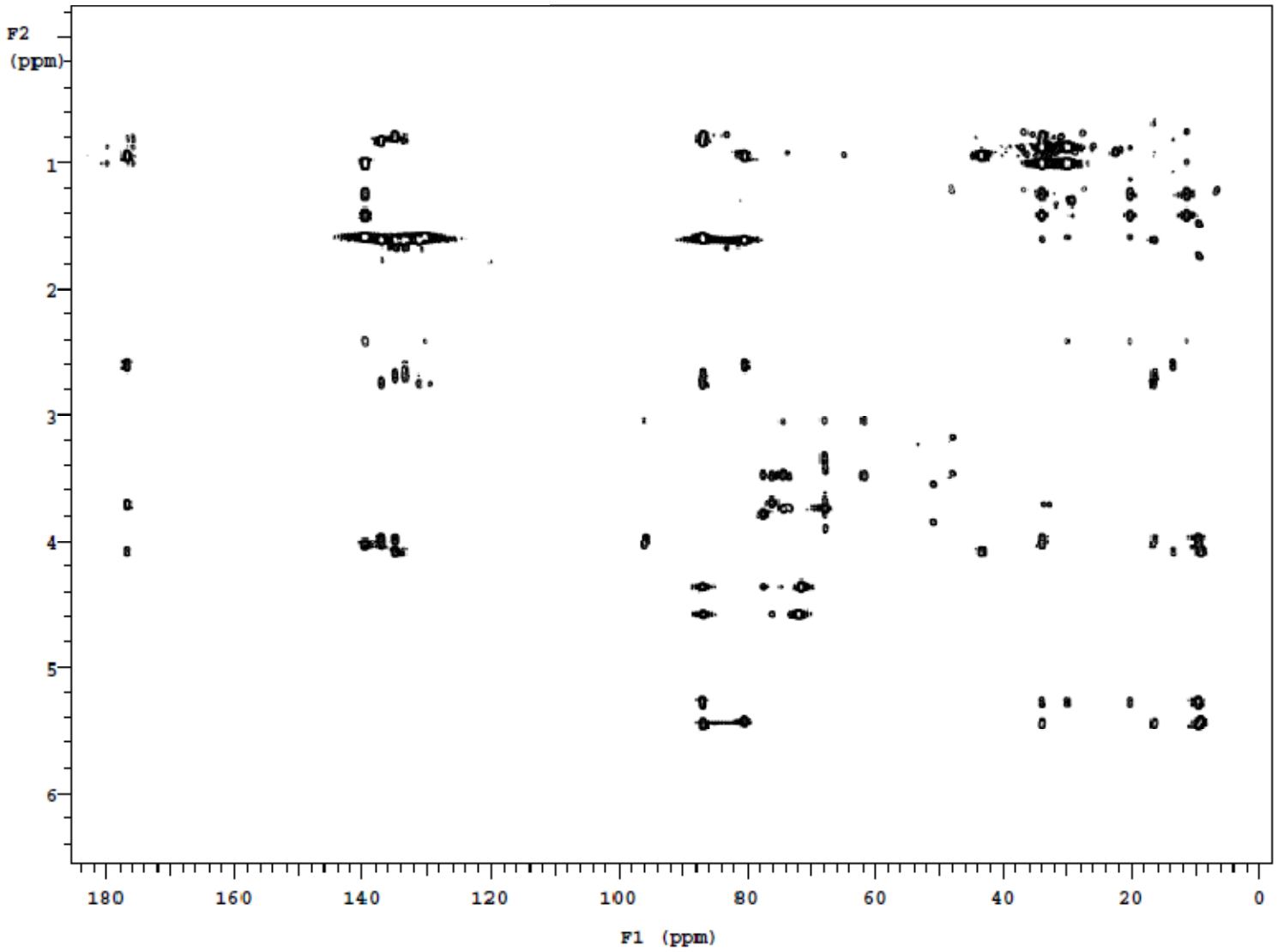
Lunalide B ^{13}C DEPT in CD_3OD



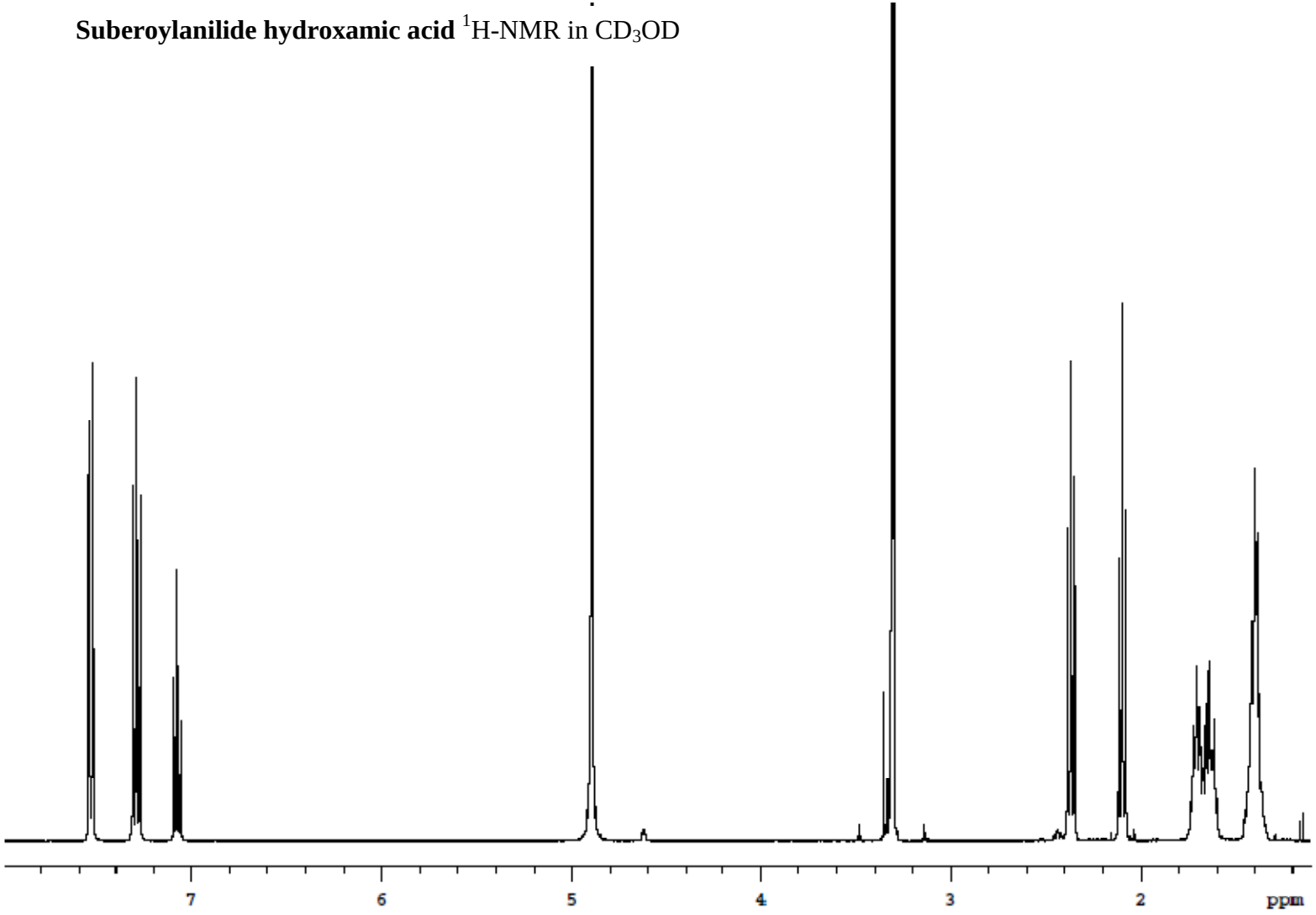
Lunalide B ^{1}H-^{13}C HSQC in CD_3OD



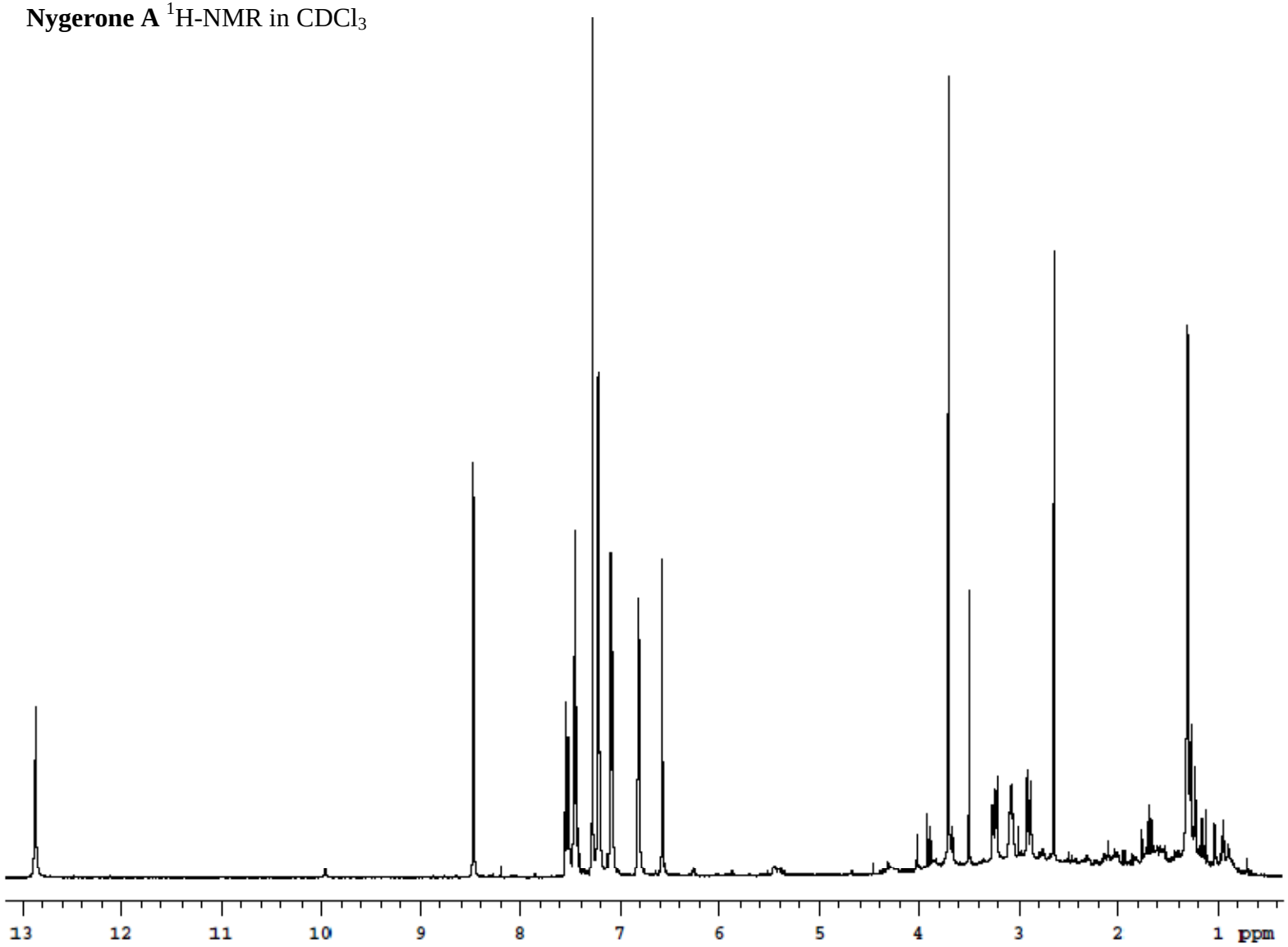
Lunalide B ^{1}H-^{13}C HMBC in CD_3OD



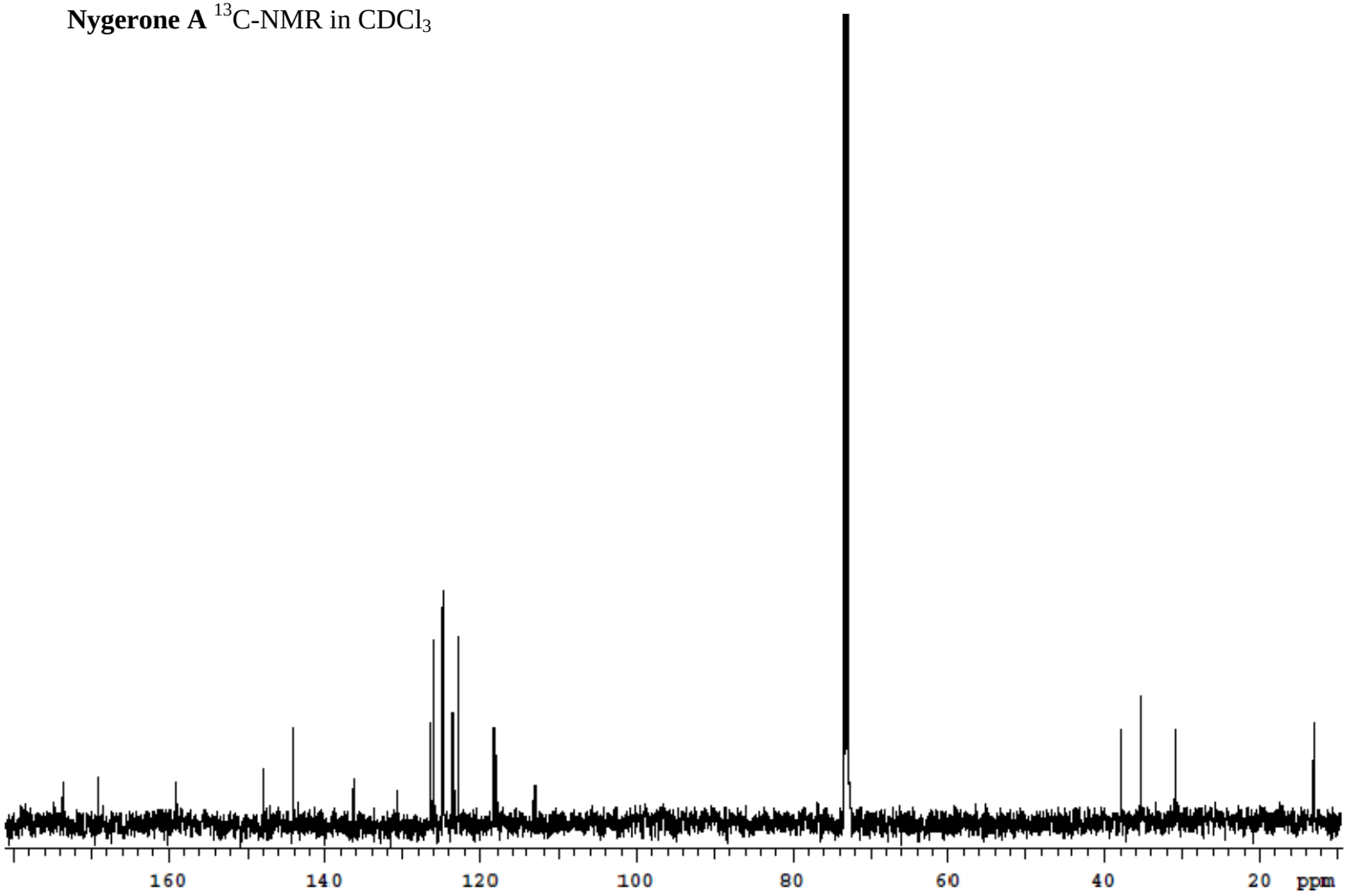
Suberoylanilide hydroxamic acid ^{1}H-NMR in CD_3OD



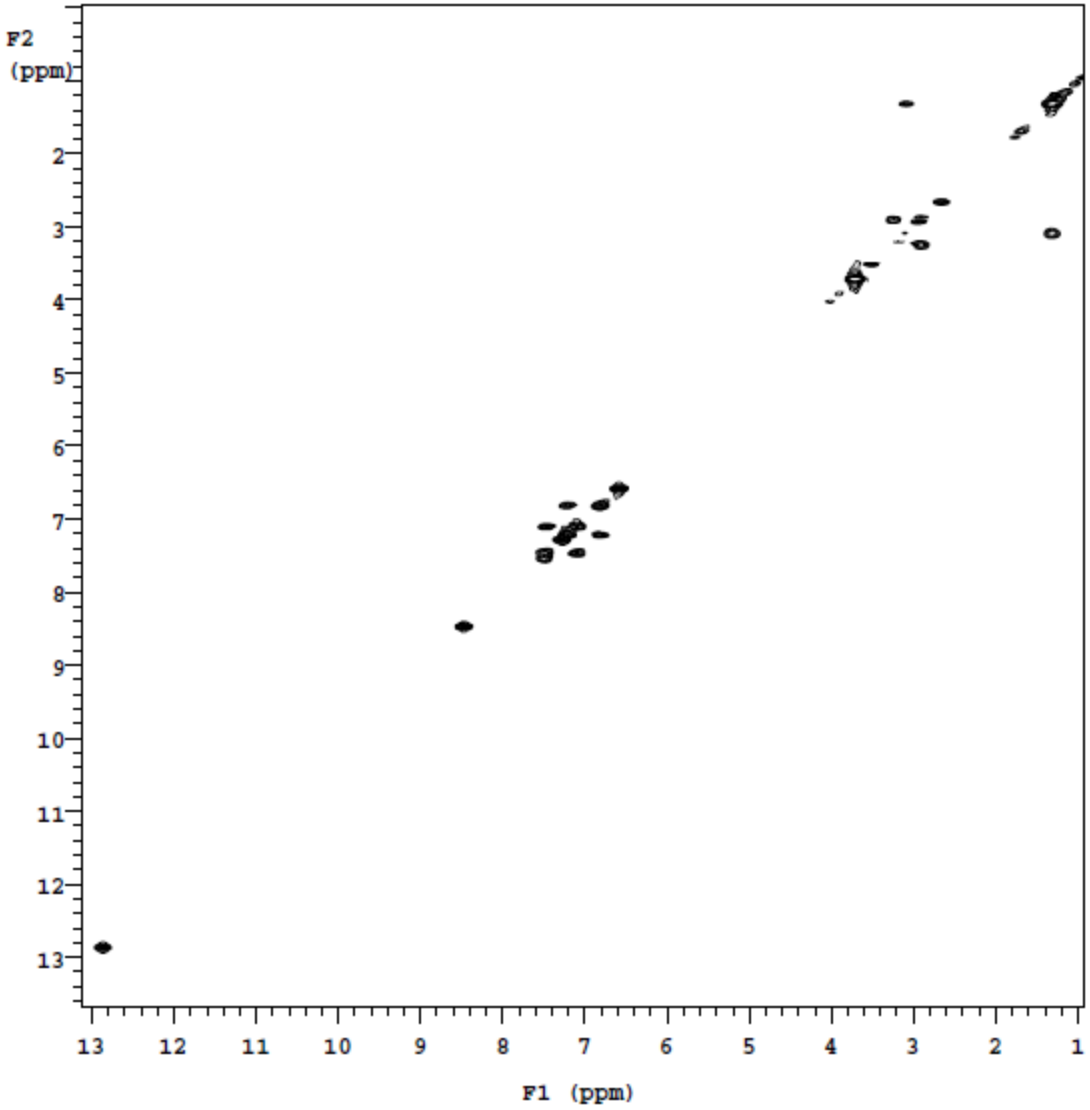
Nygerone A ^{1}H-NMR in $CDCl_3$



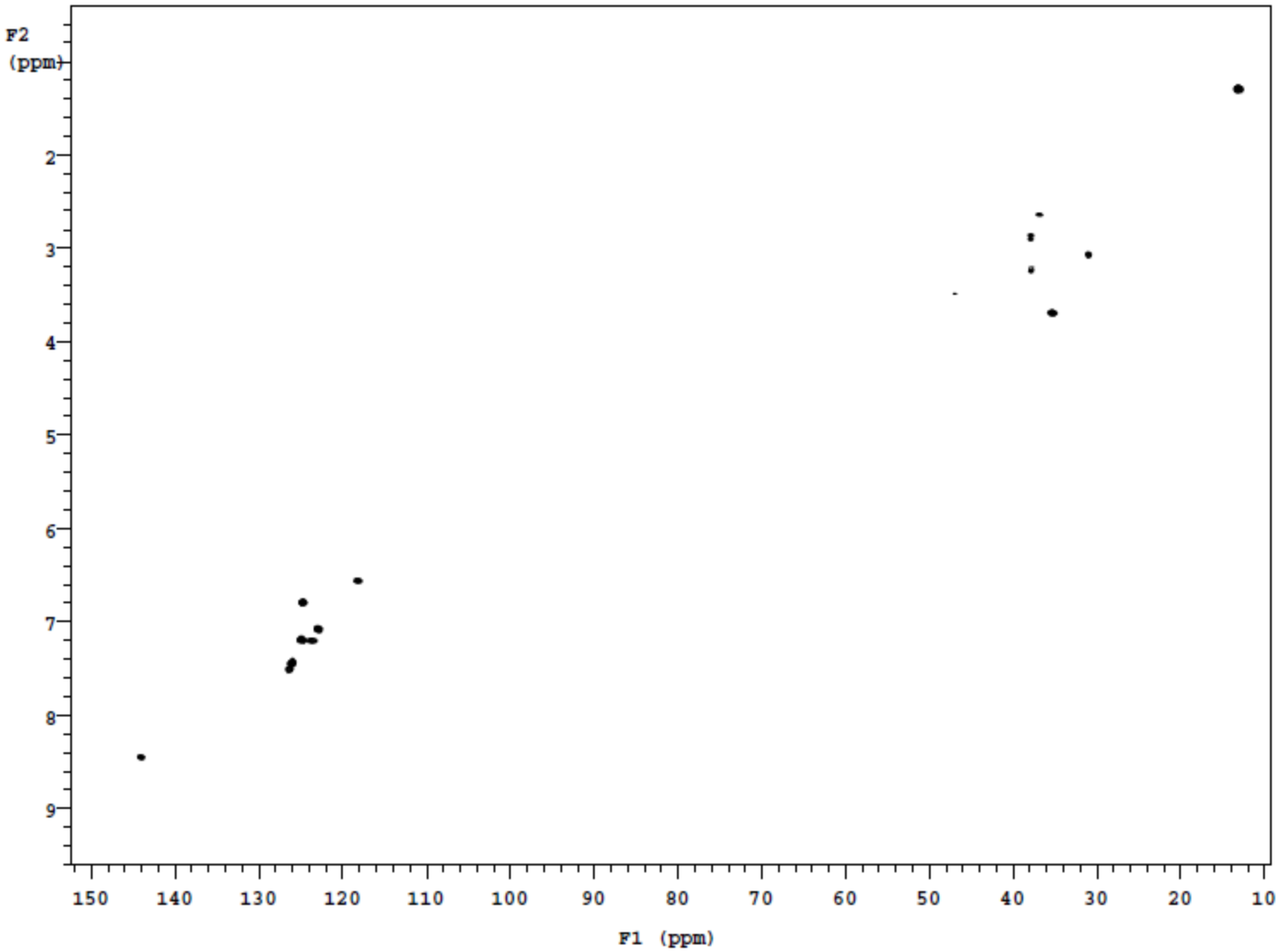
Nygerone A ^{13}C-NMR in $CDCl_3$



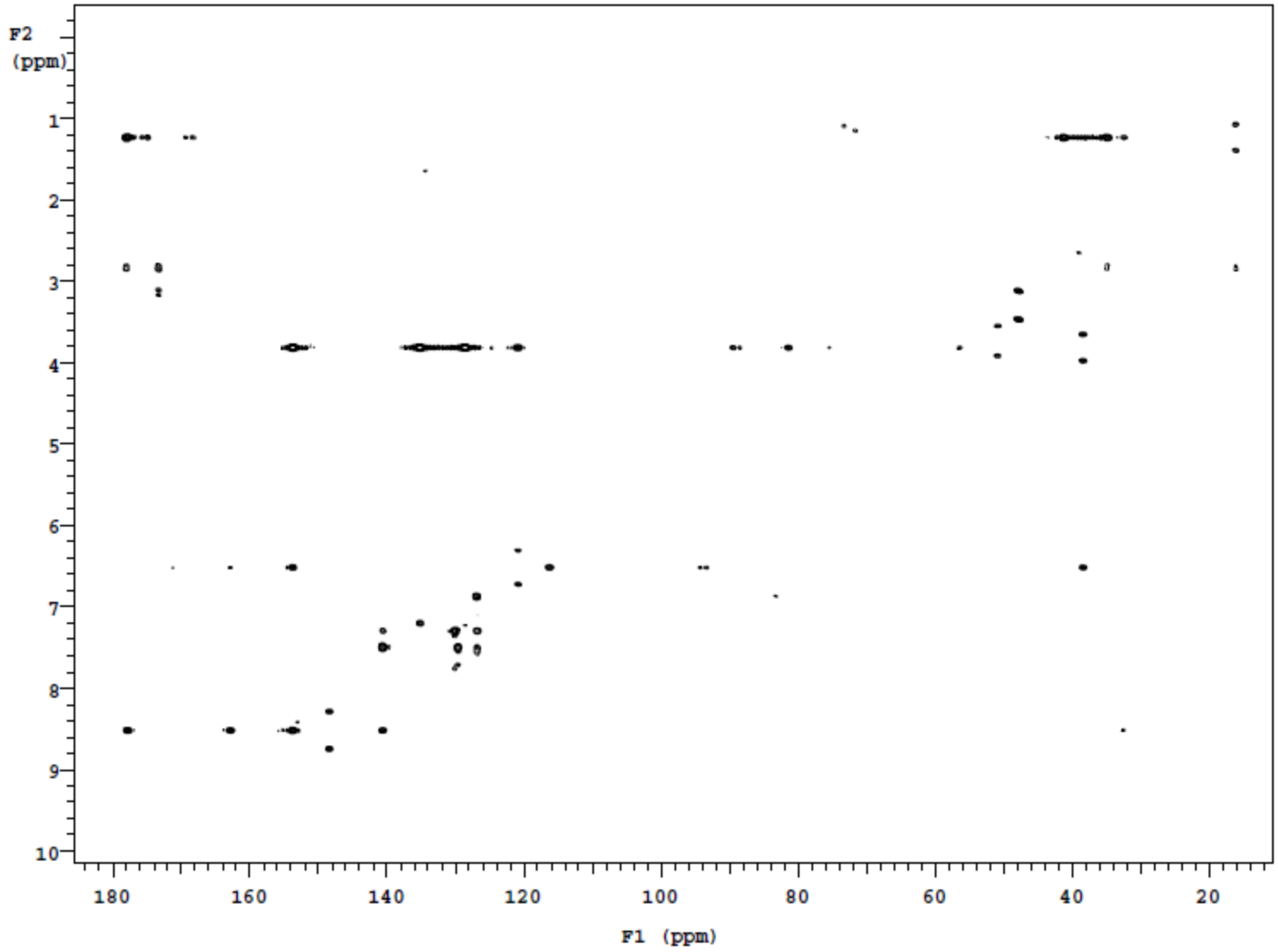
Nygerone A ^{1}H-^{1}H COSY in $CDCl_3$



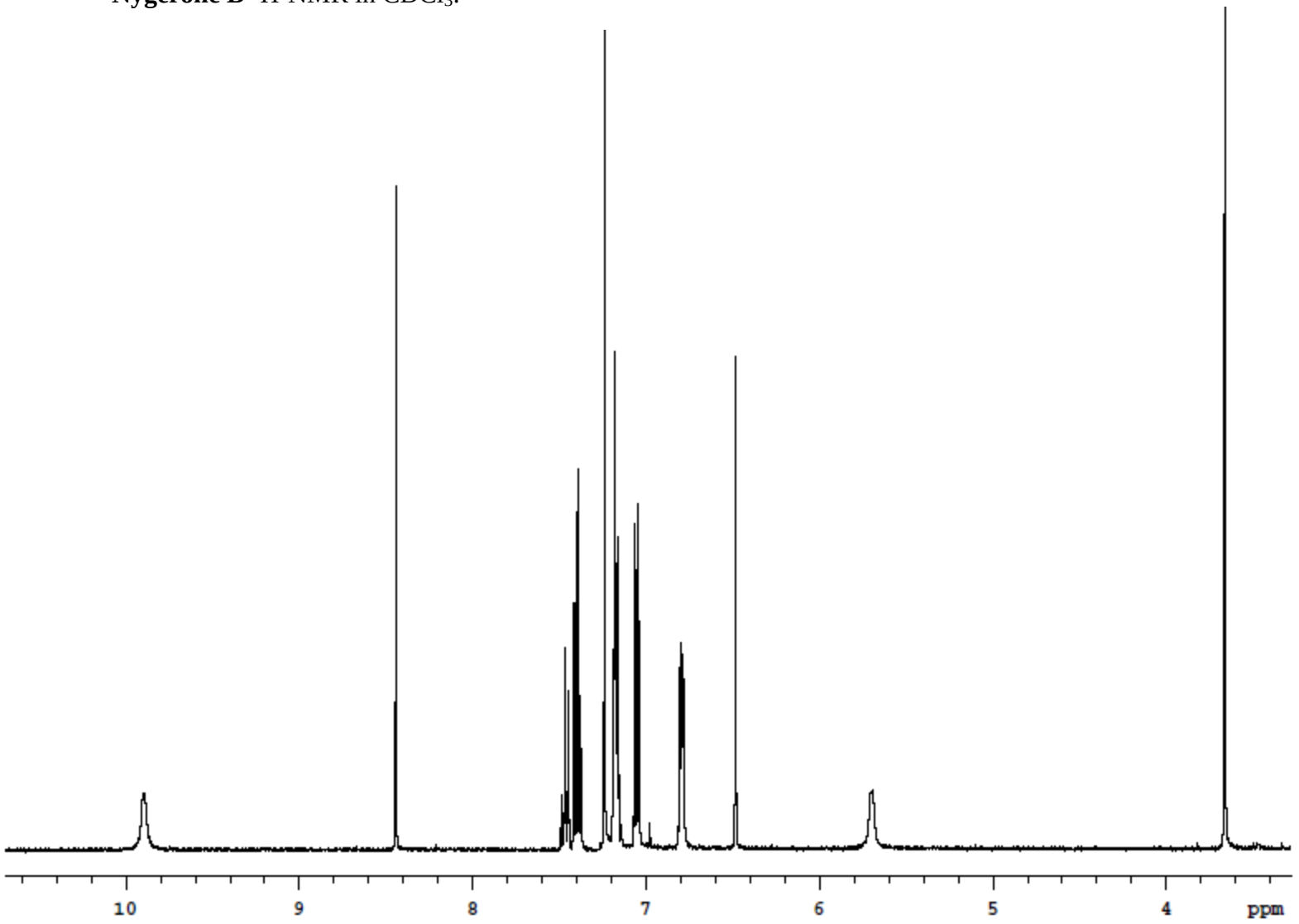
Nygerone A ^{1}H-^{13}C HSQC in $CDCl_3$



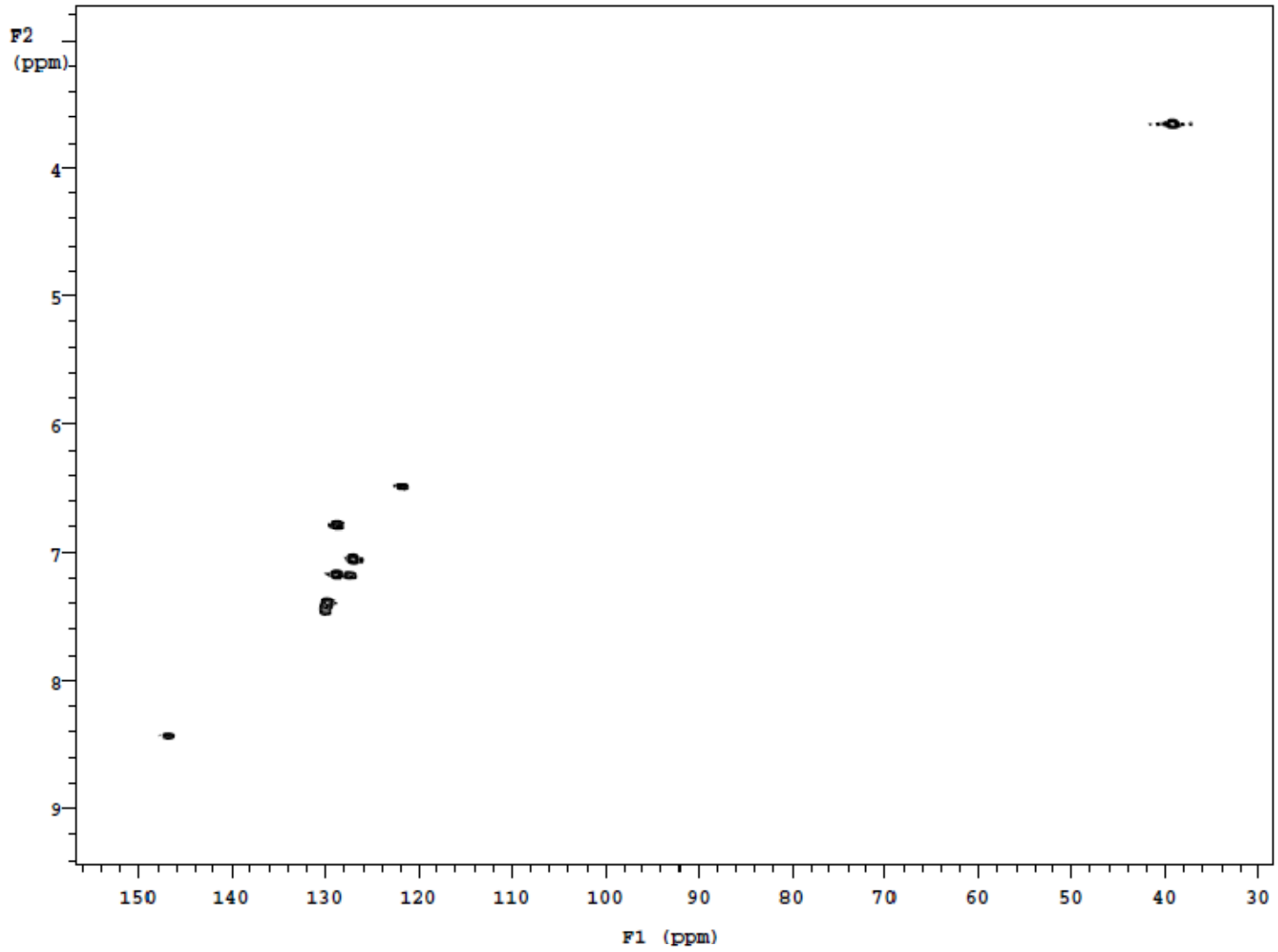
Nygerone A ^{1}H-^{13}C HMBC in $CDCl_3$



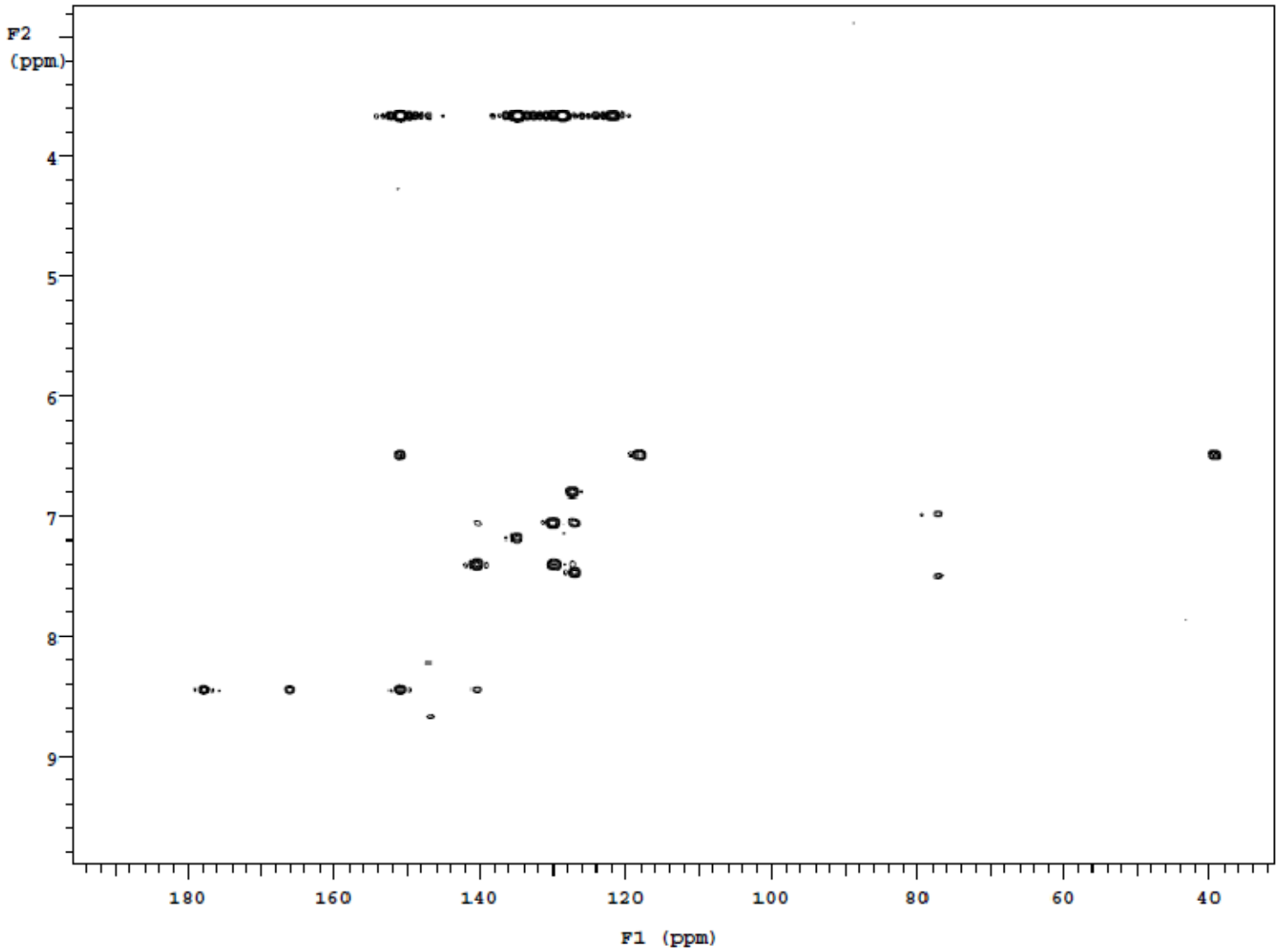
Nygerone B ^{1}H-NMR in $CDCl_3$.



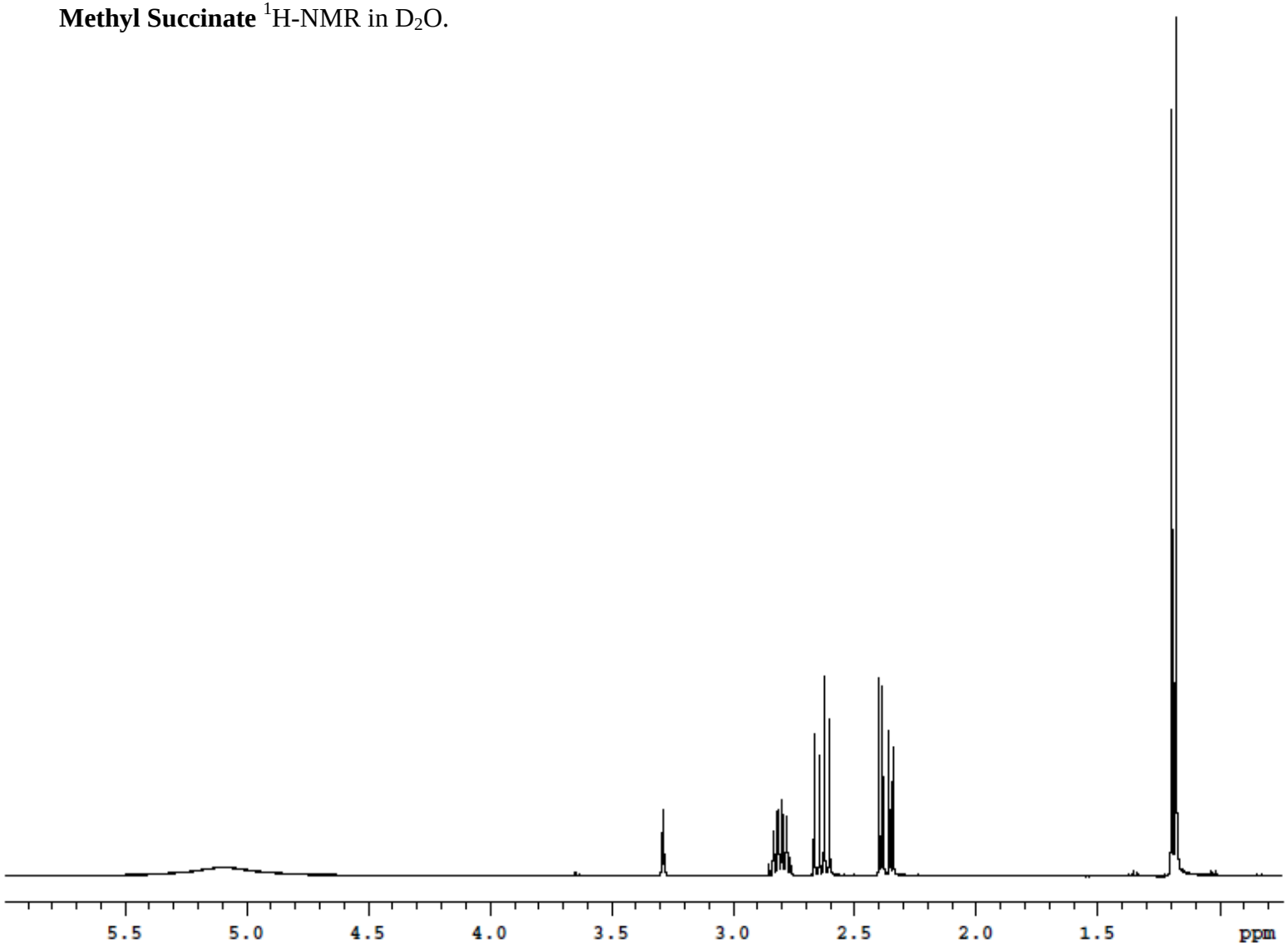
Nygerone B ^{1}H-^{13}C HSQC in $CDCl_3$.



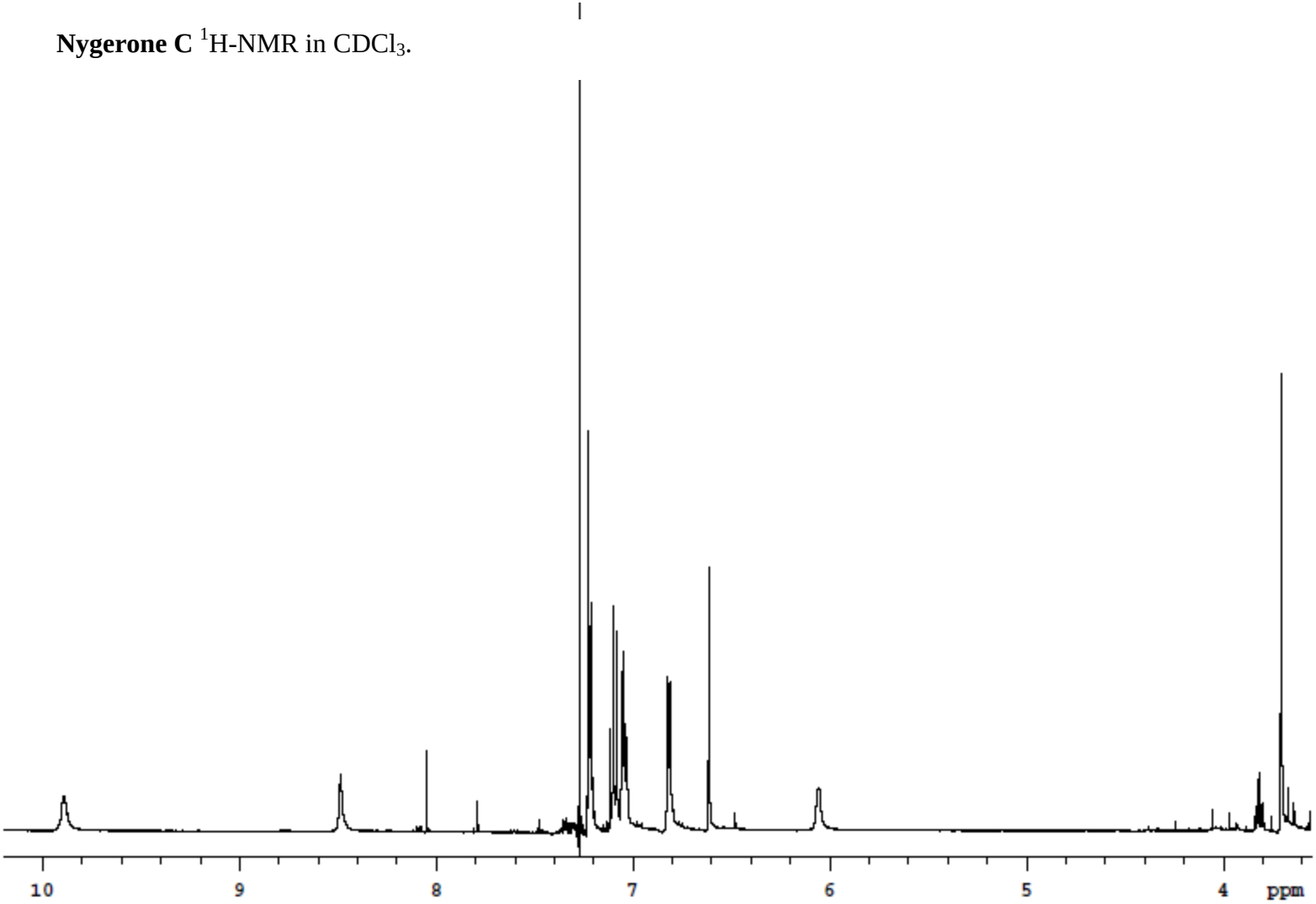
Nygerone B ^{1}H-^{13}C HMBC in $CDCl_3$.



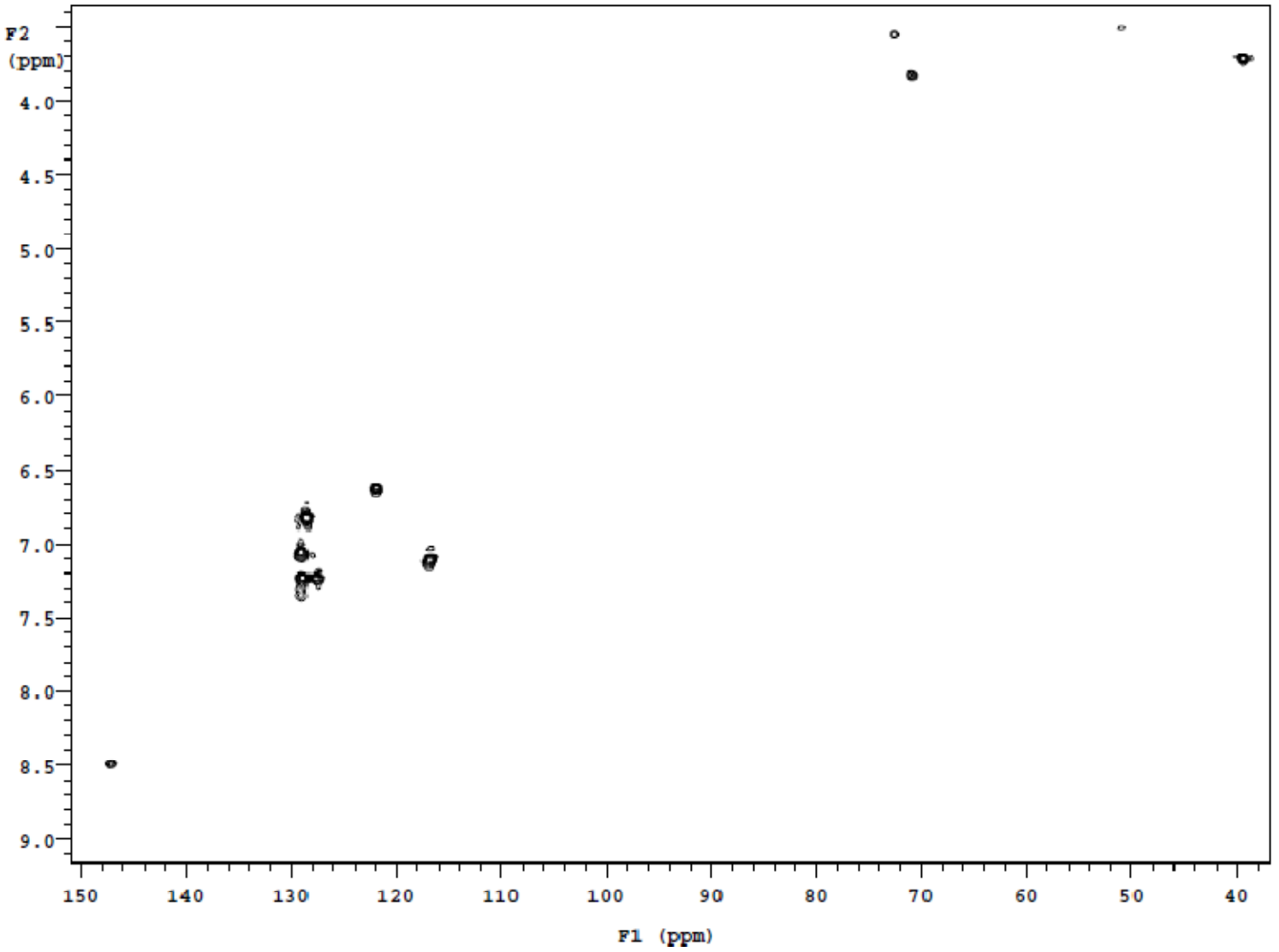
Methyl Succinate ^{1}H-NMR in D_2O.



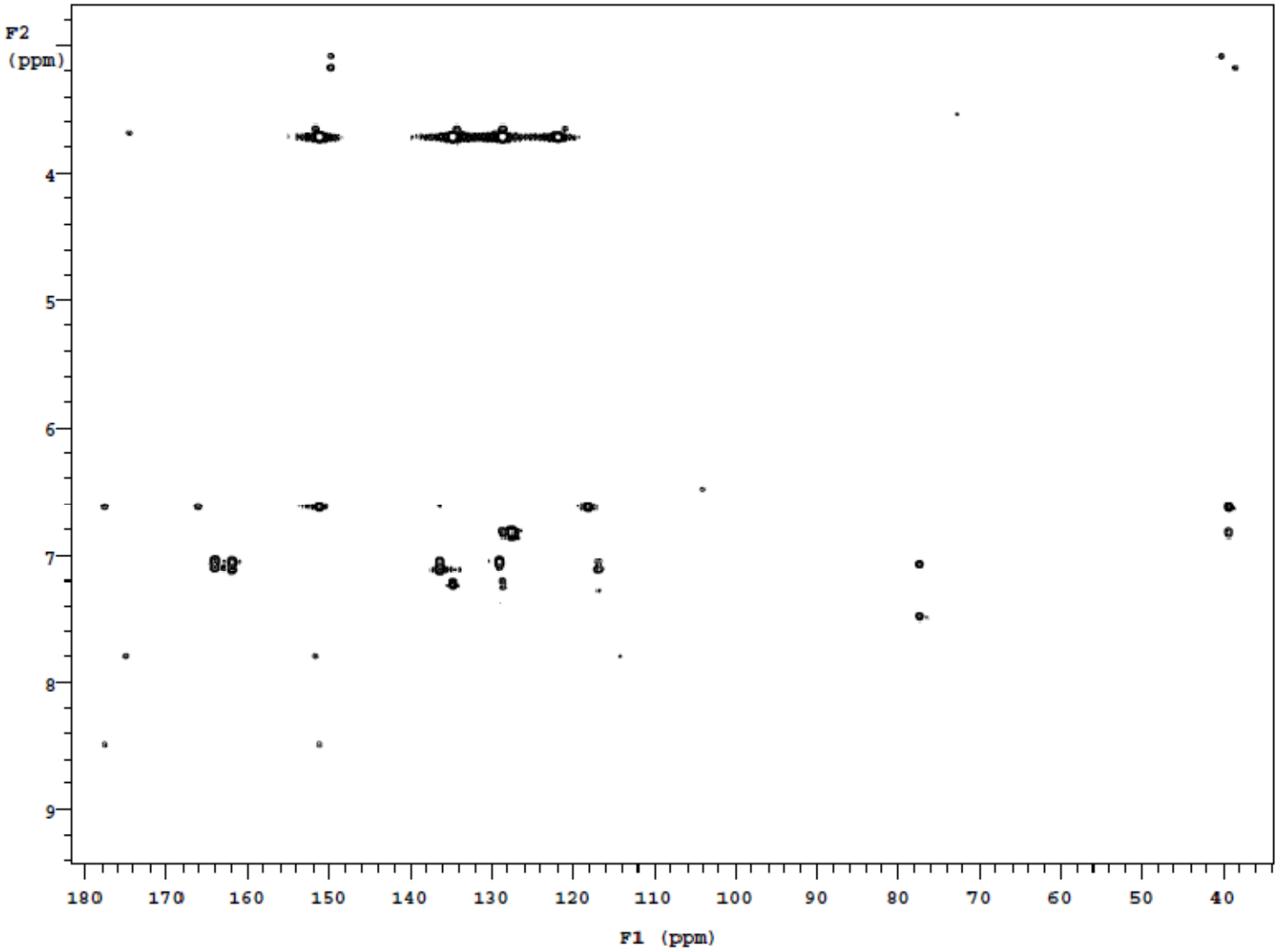
Nygerone C ^{1}H-NMR in $CDCl_3$.



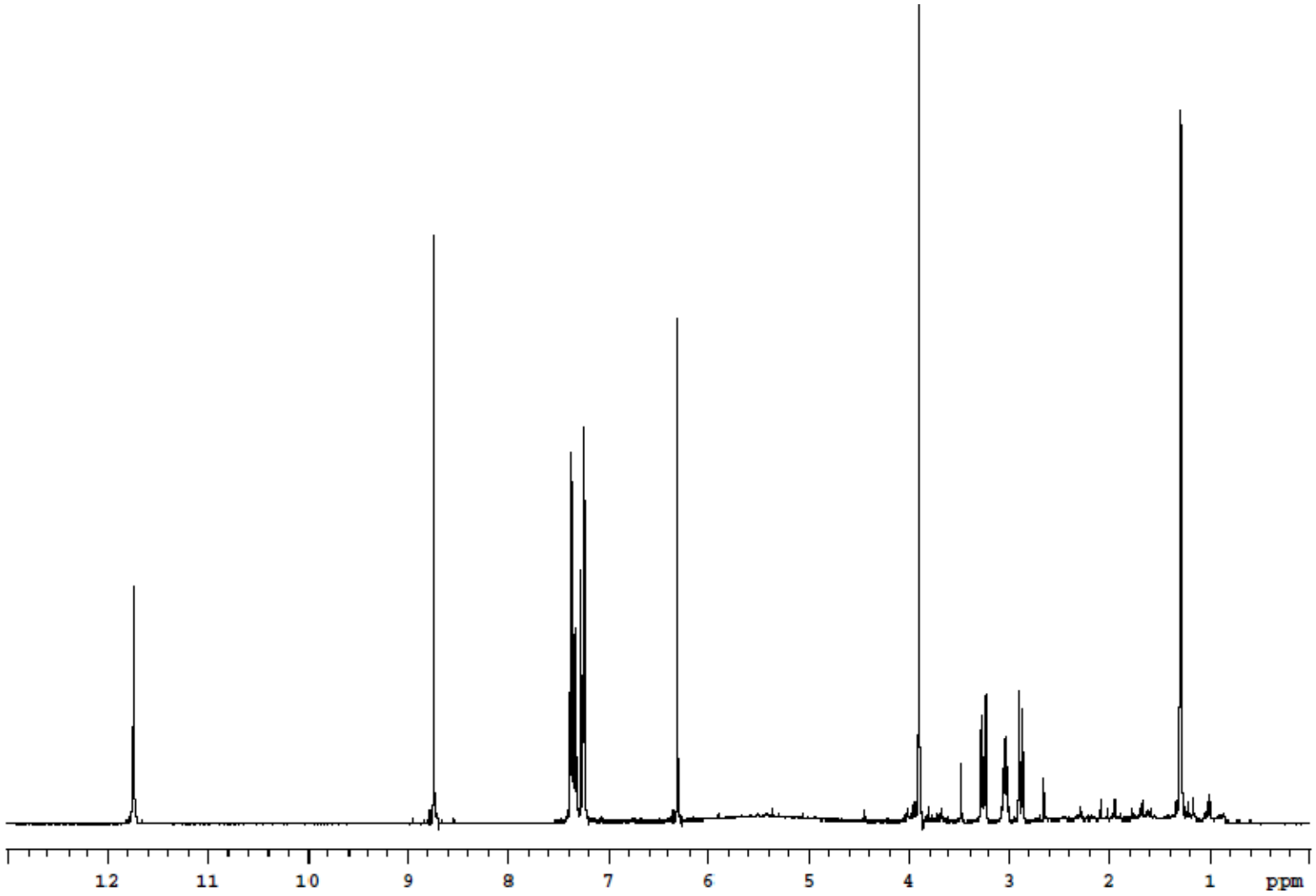
Nygerone C ^{1}H-^{13}C HSQC in $CDCl_3$.



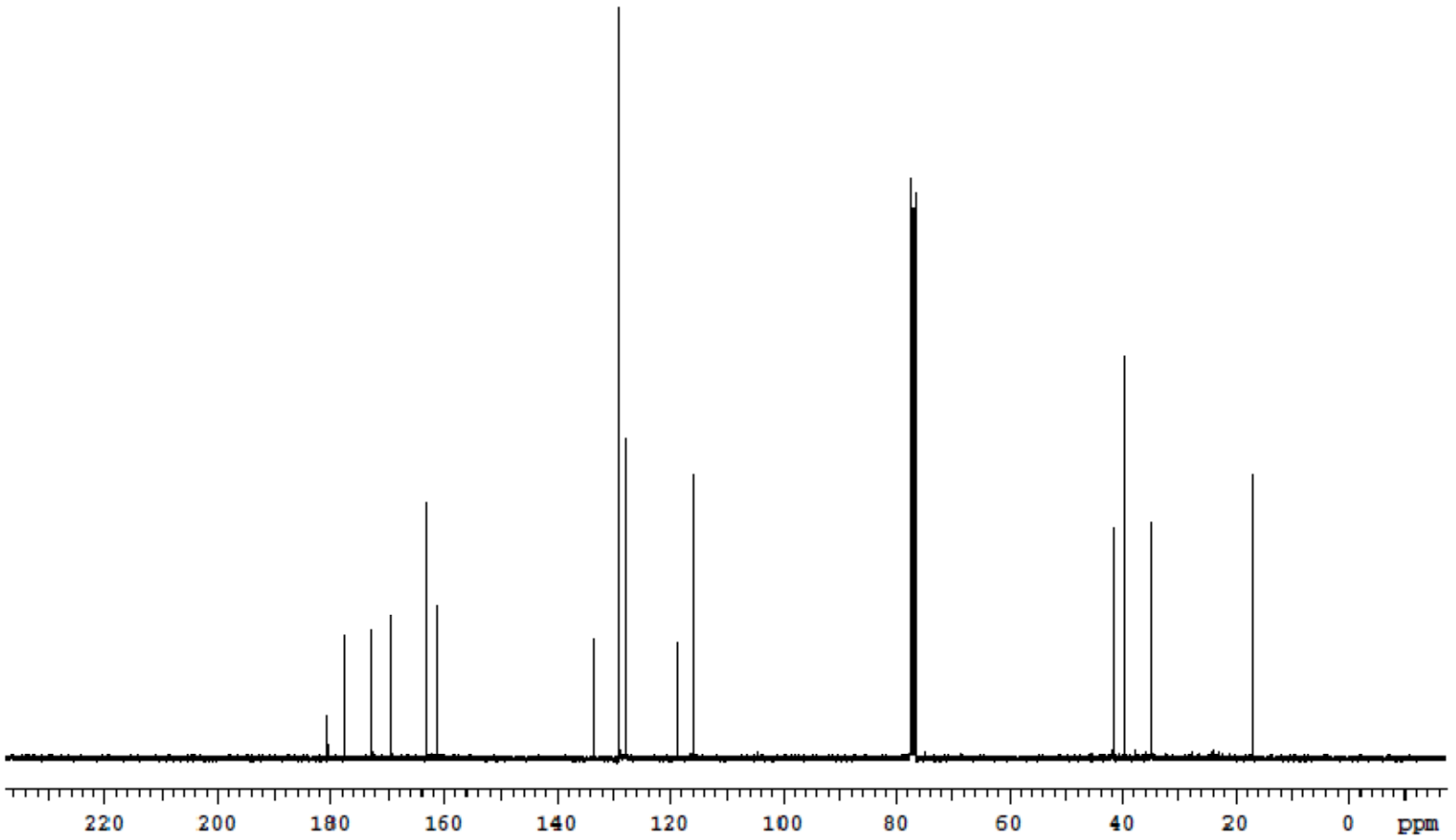
Nygerone C ^{1}H-^{13}C HMBC in $CDCl_3$.



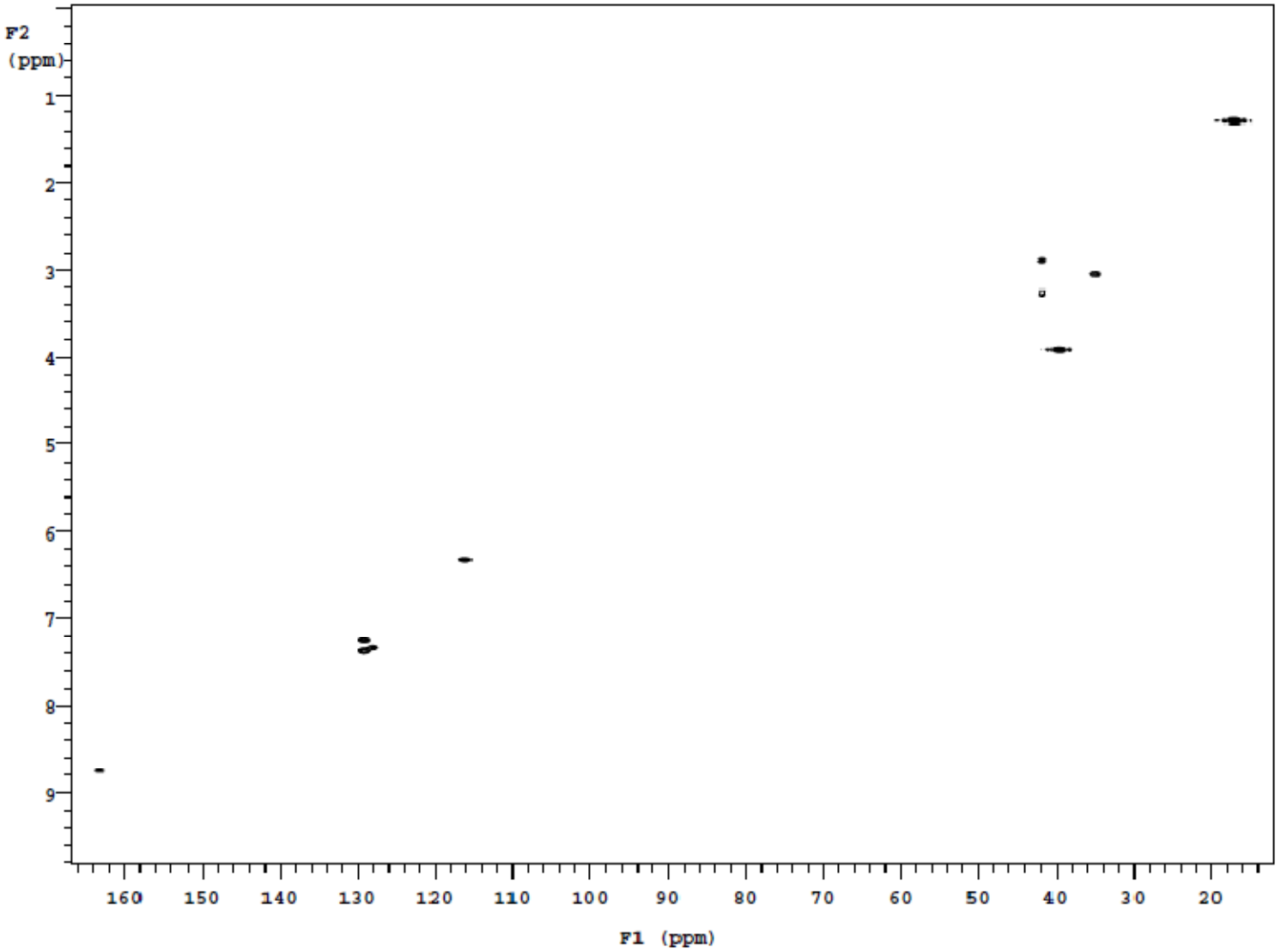
Pestalamide A/Tensidol A ^{1}H-NMR in CD_3OD.



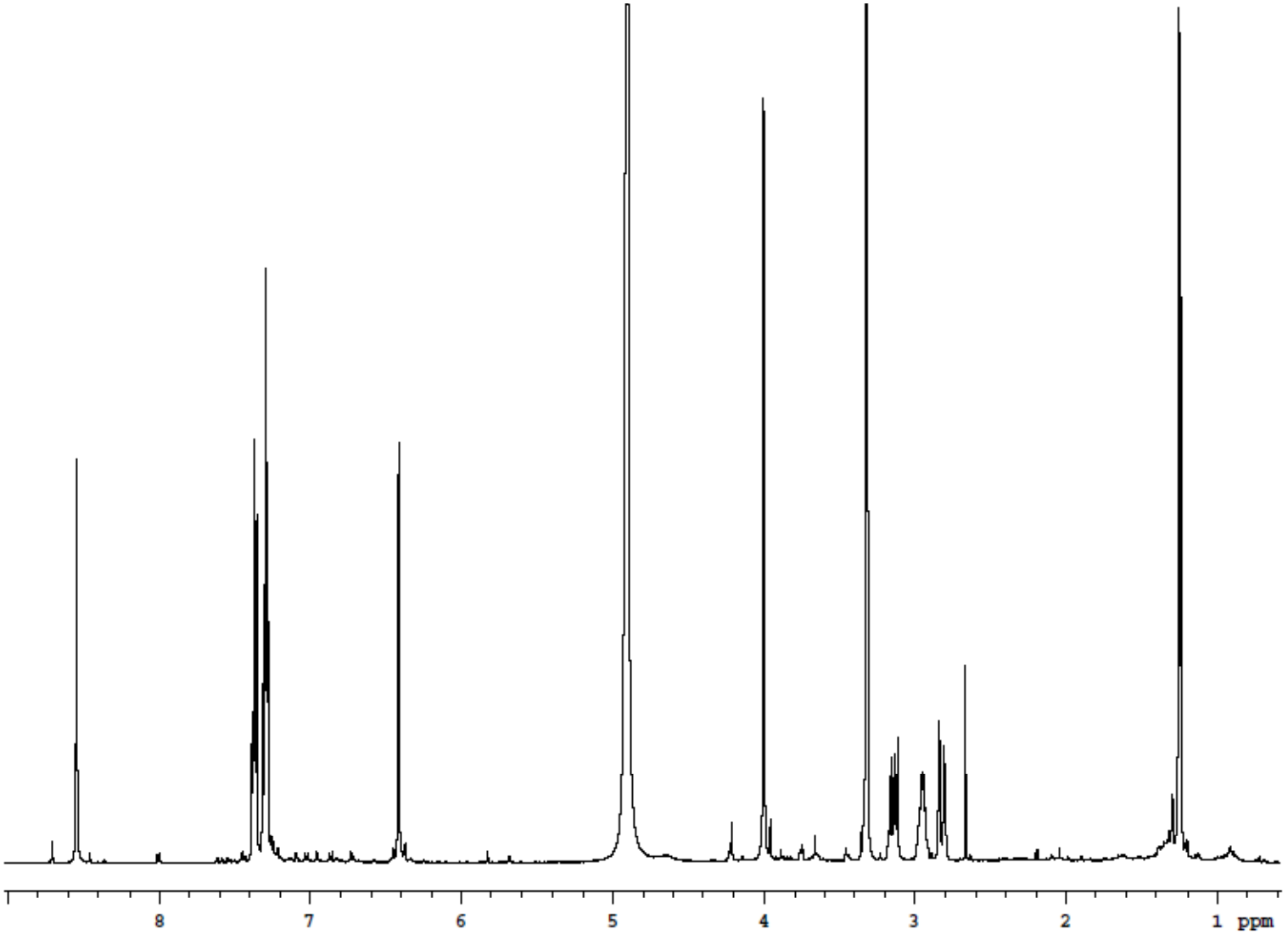
Pestalamide A/Tensidol A ^{13}C-NMR in CD_3OD.



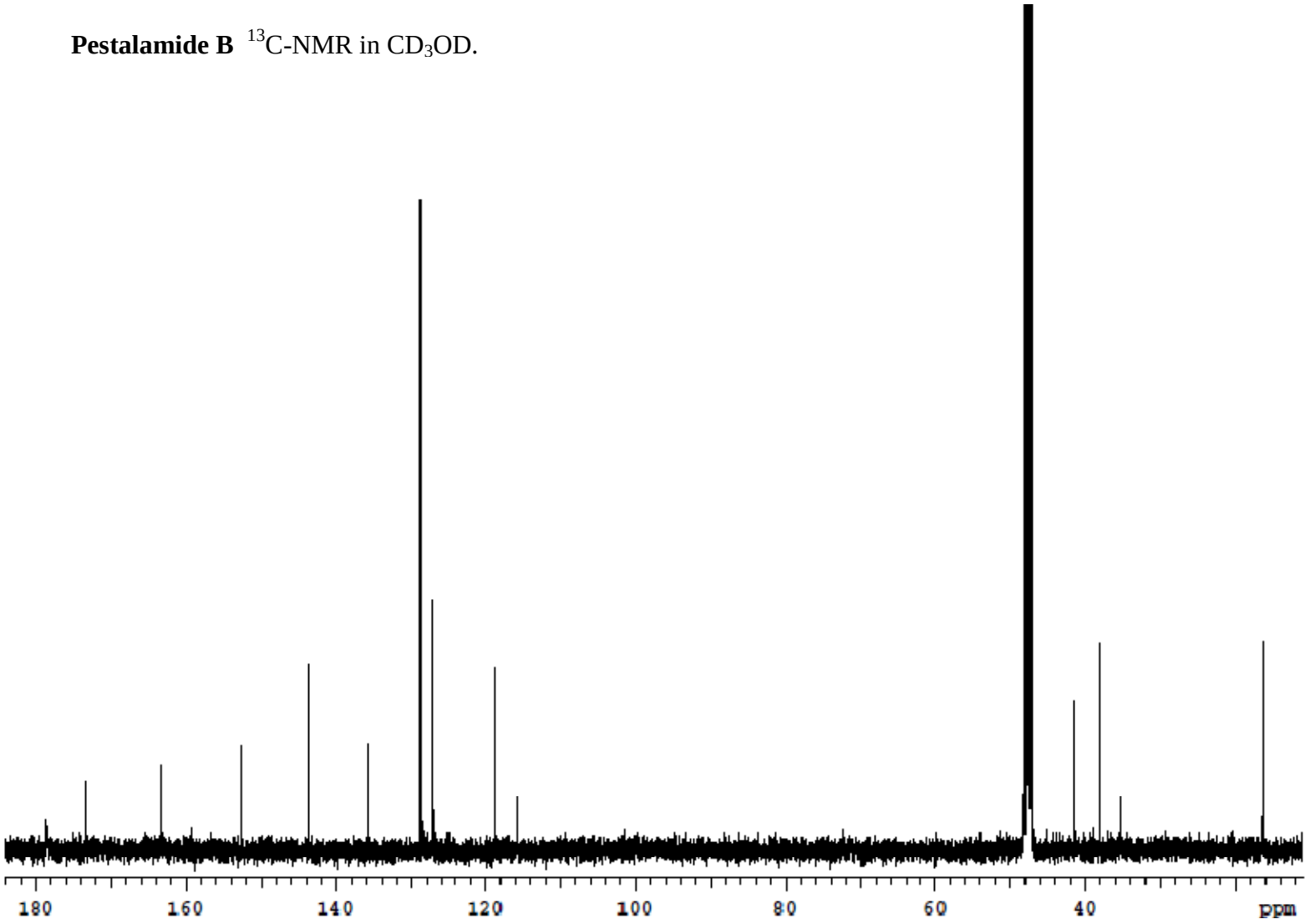
Pestalamide A/Tensidol A ^{1}H-^{13}C HSQC in CD_3OD.



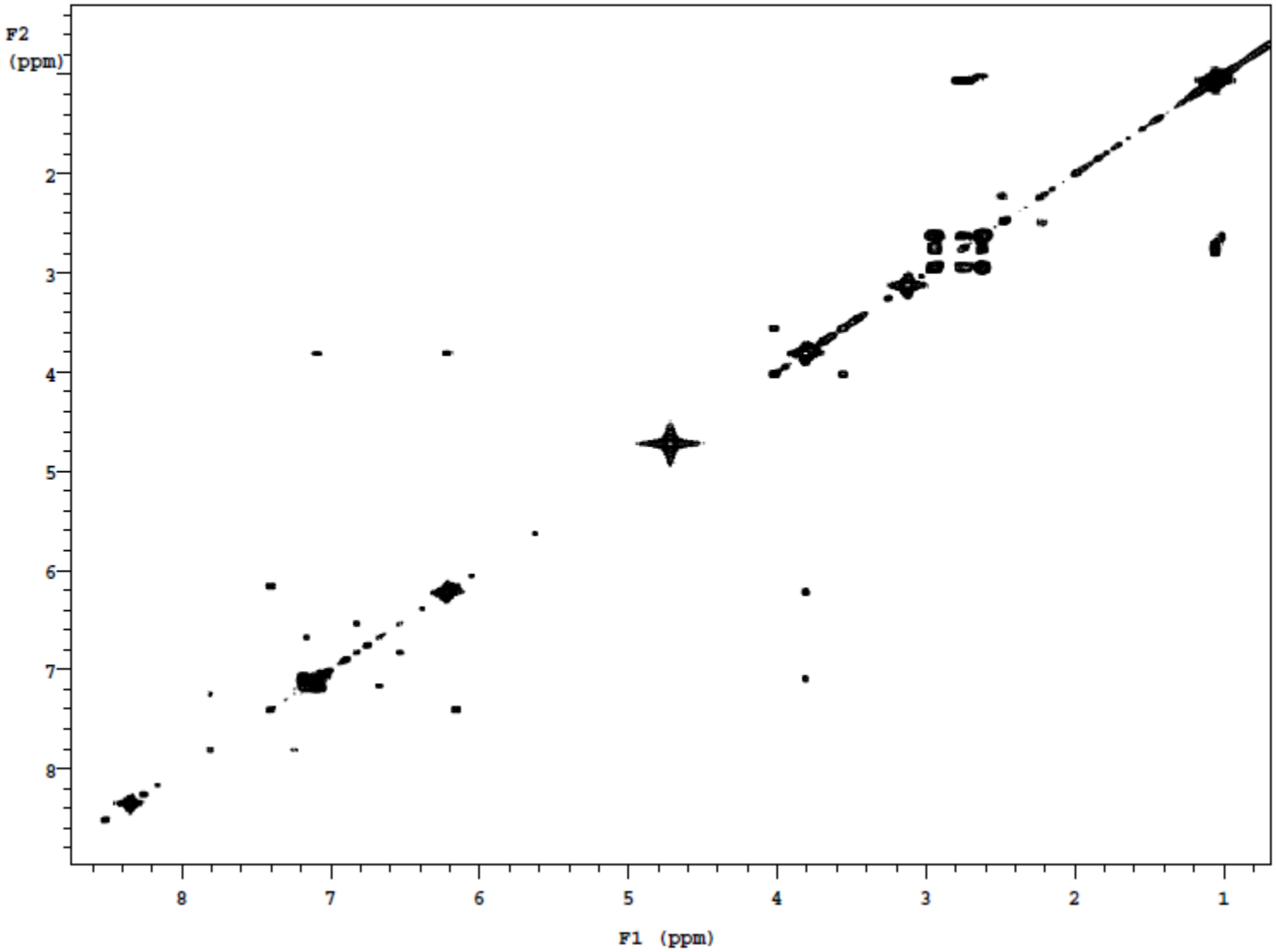
Pestalamide B ^{1}H-NMR in CD_3OD.



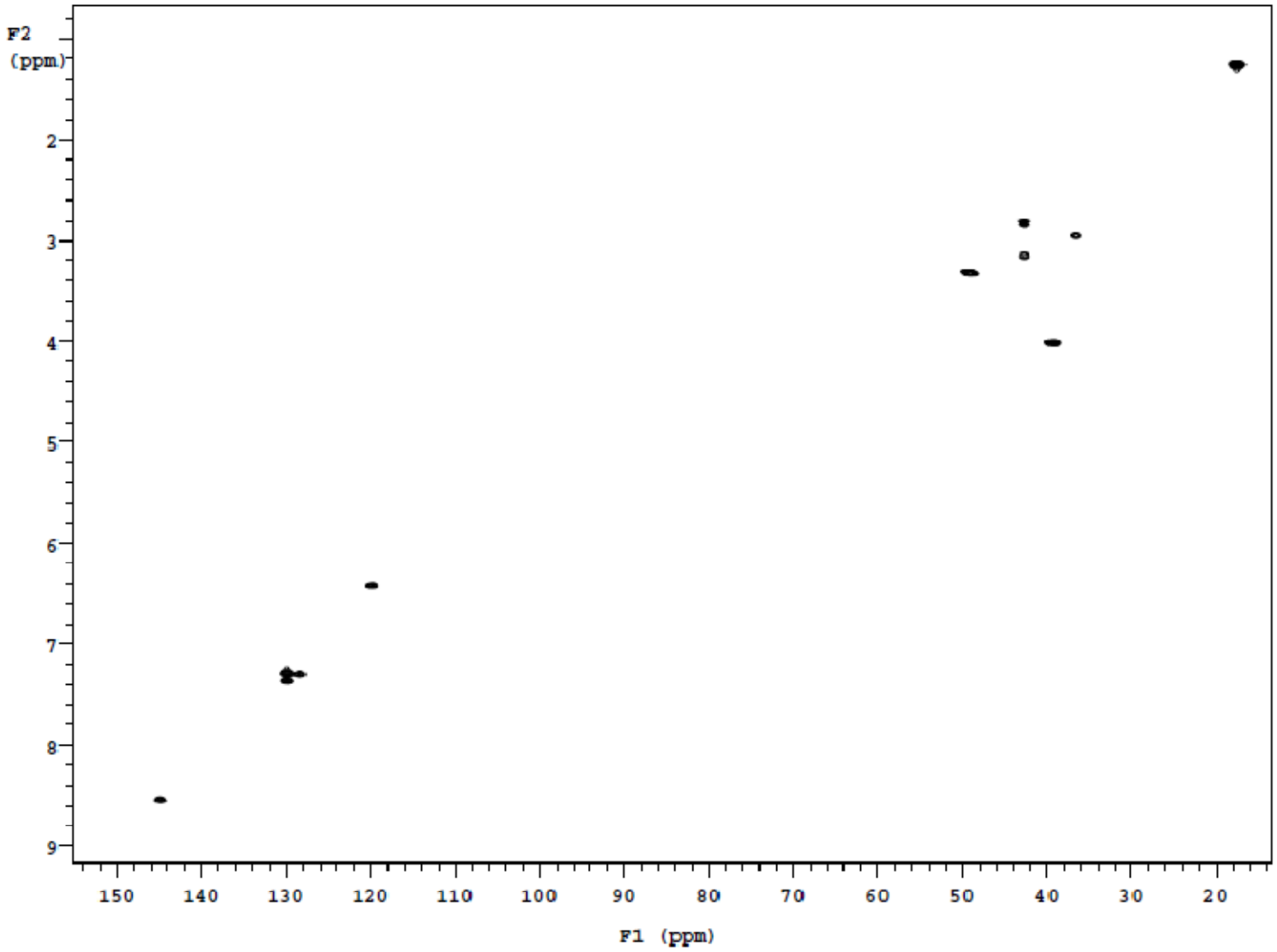
Pestalamide B ^{13}C-NMR in CD_3OD.



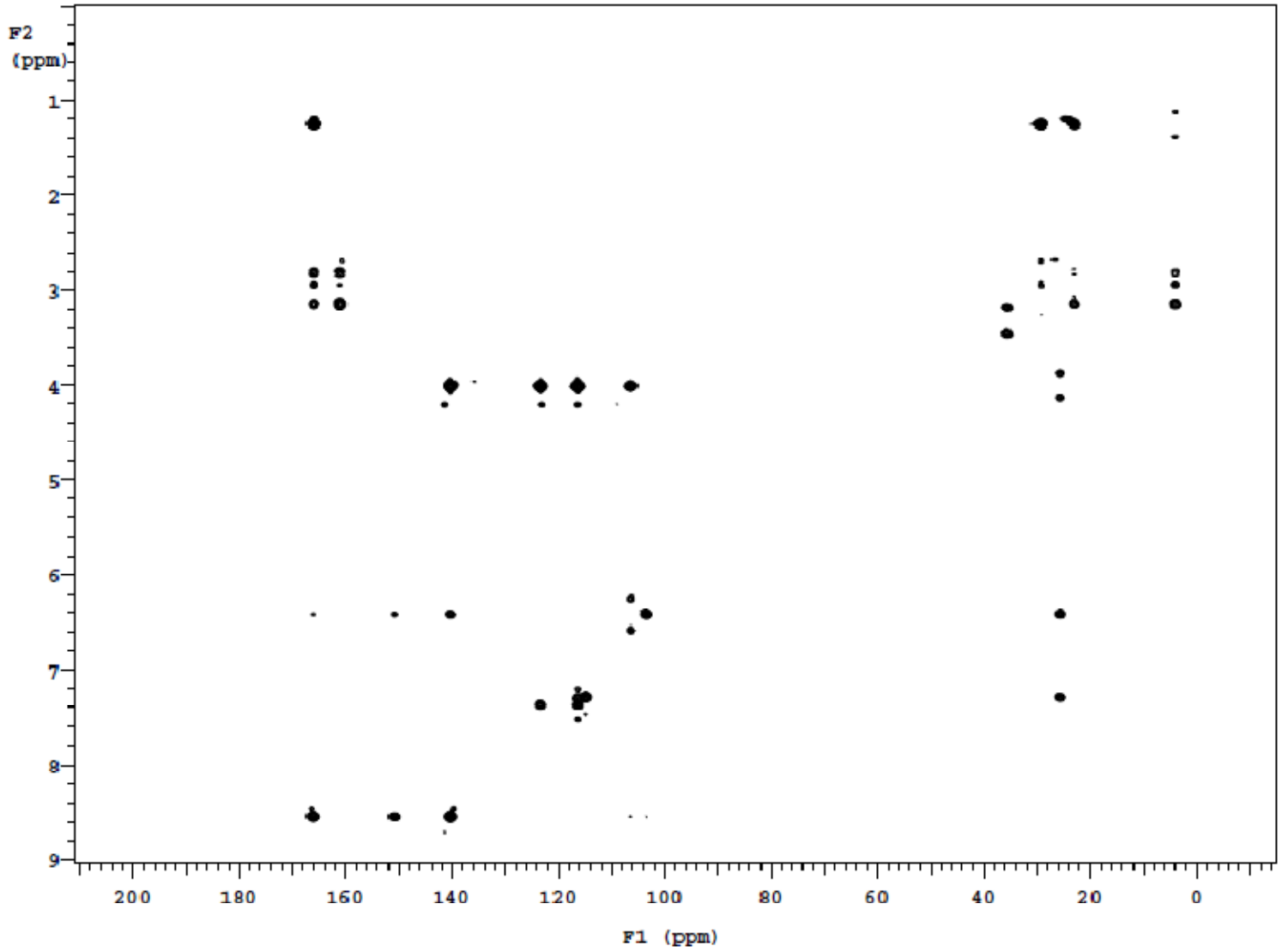
Pestalamide B ^{1}H-^{1}H COSY in CD_3OD.



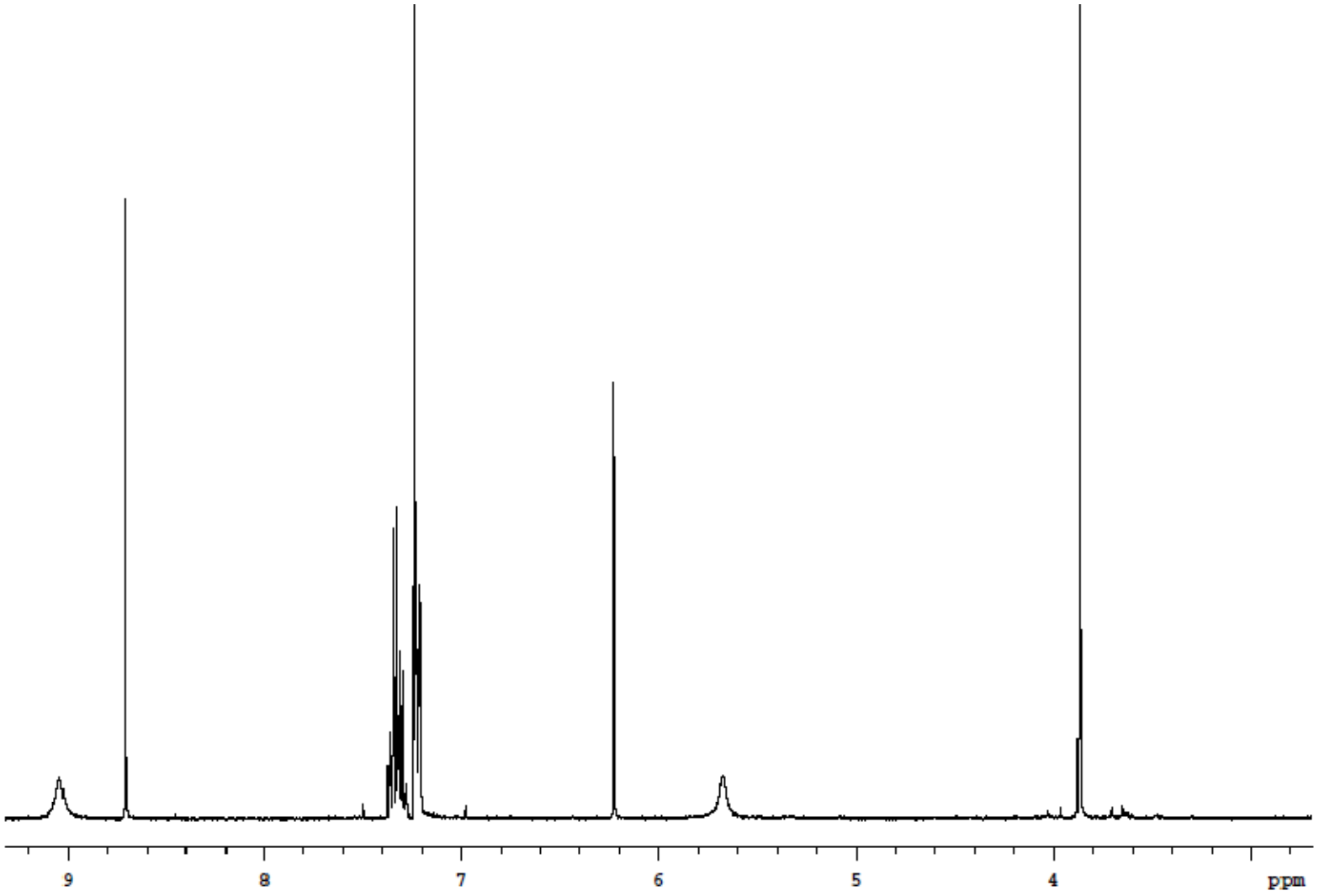
Pestalamide B ^{1}H-^{13}C HSQC in CD_3OD.



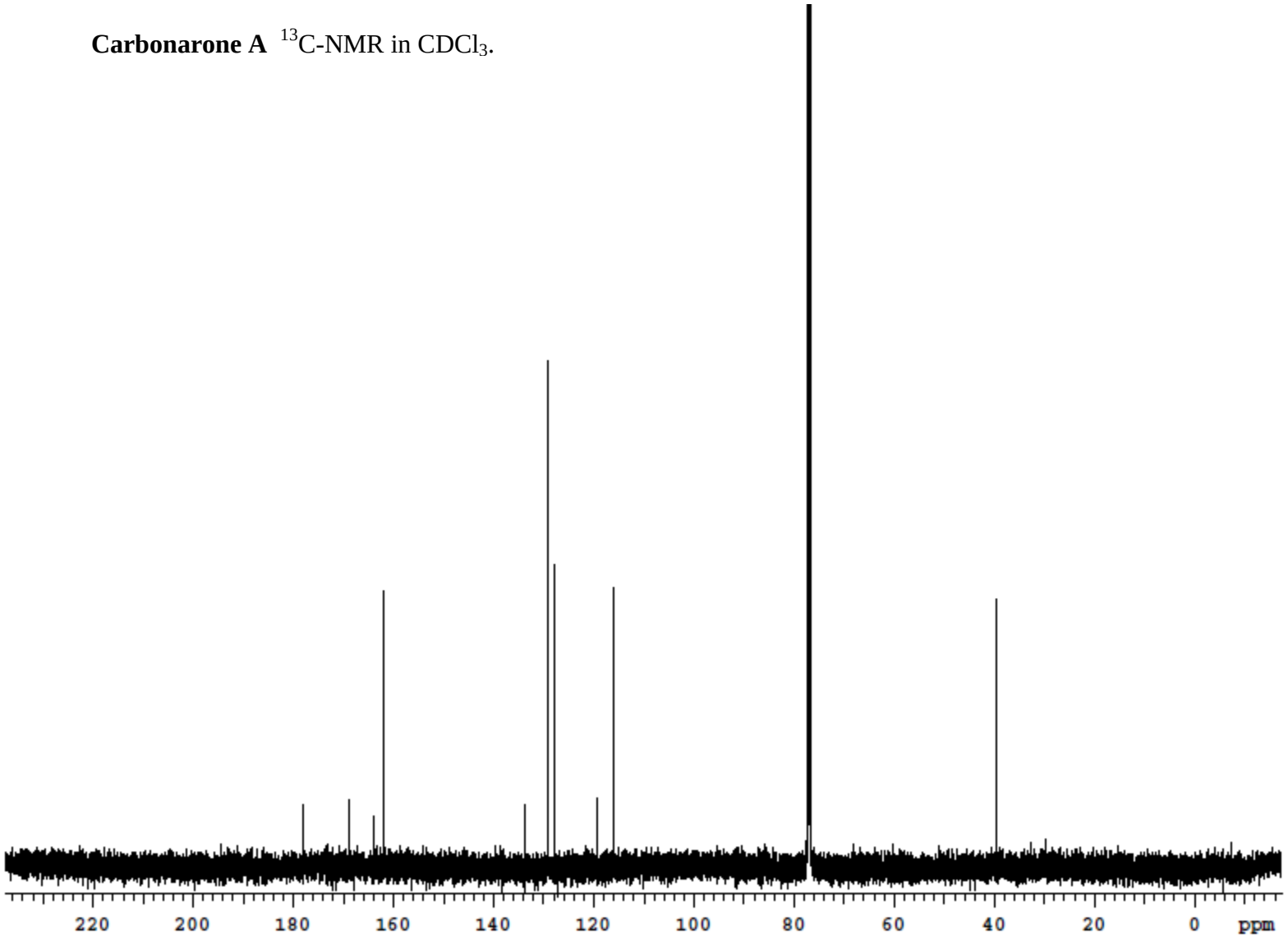
Pestalamide B ^{1}H-^{13}C HMBC in CD_3OD.



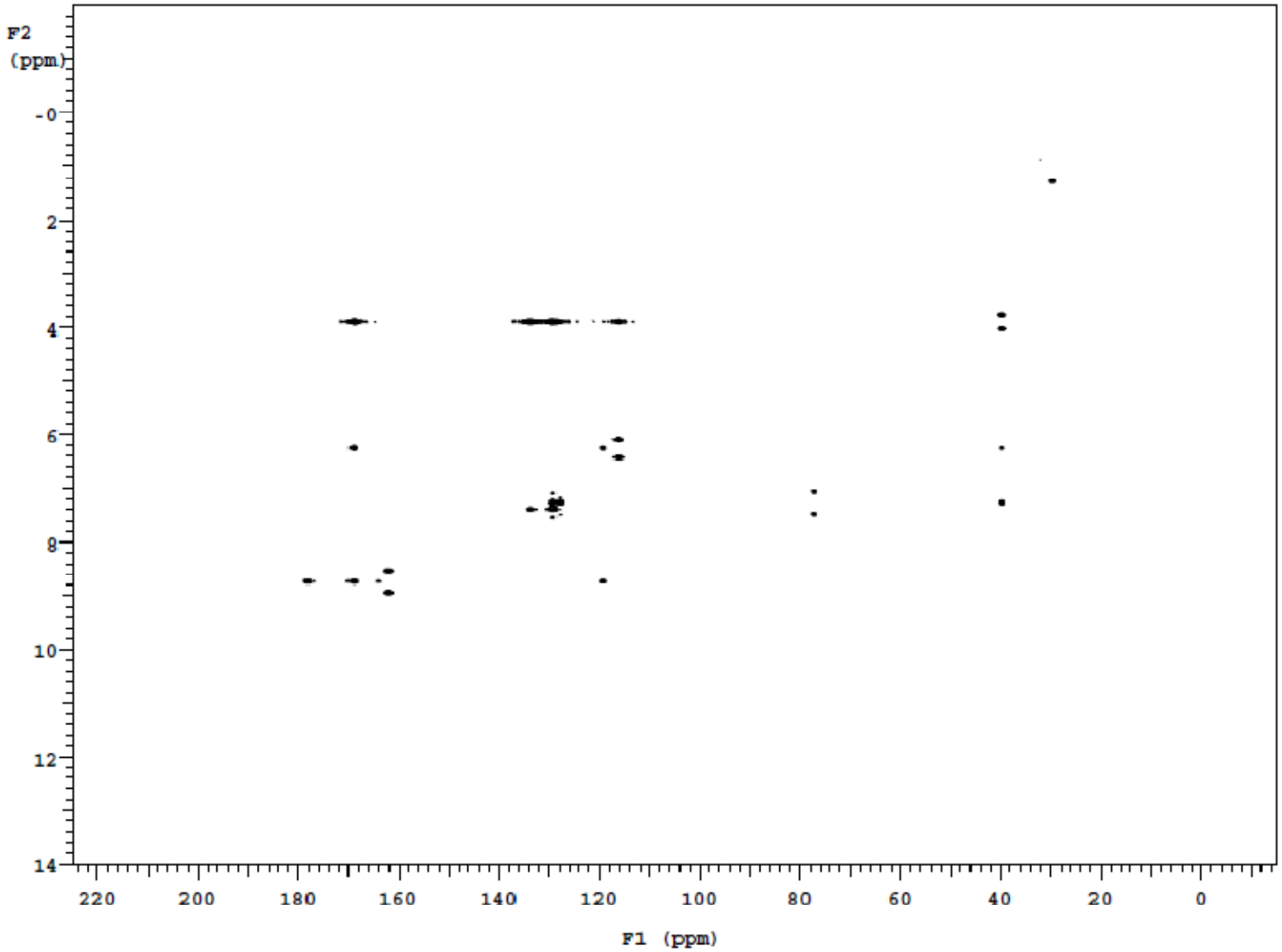
Carbonarone A ^{1}H-NMR in $CDCl_3$.



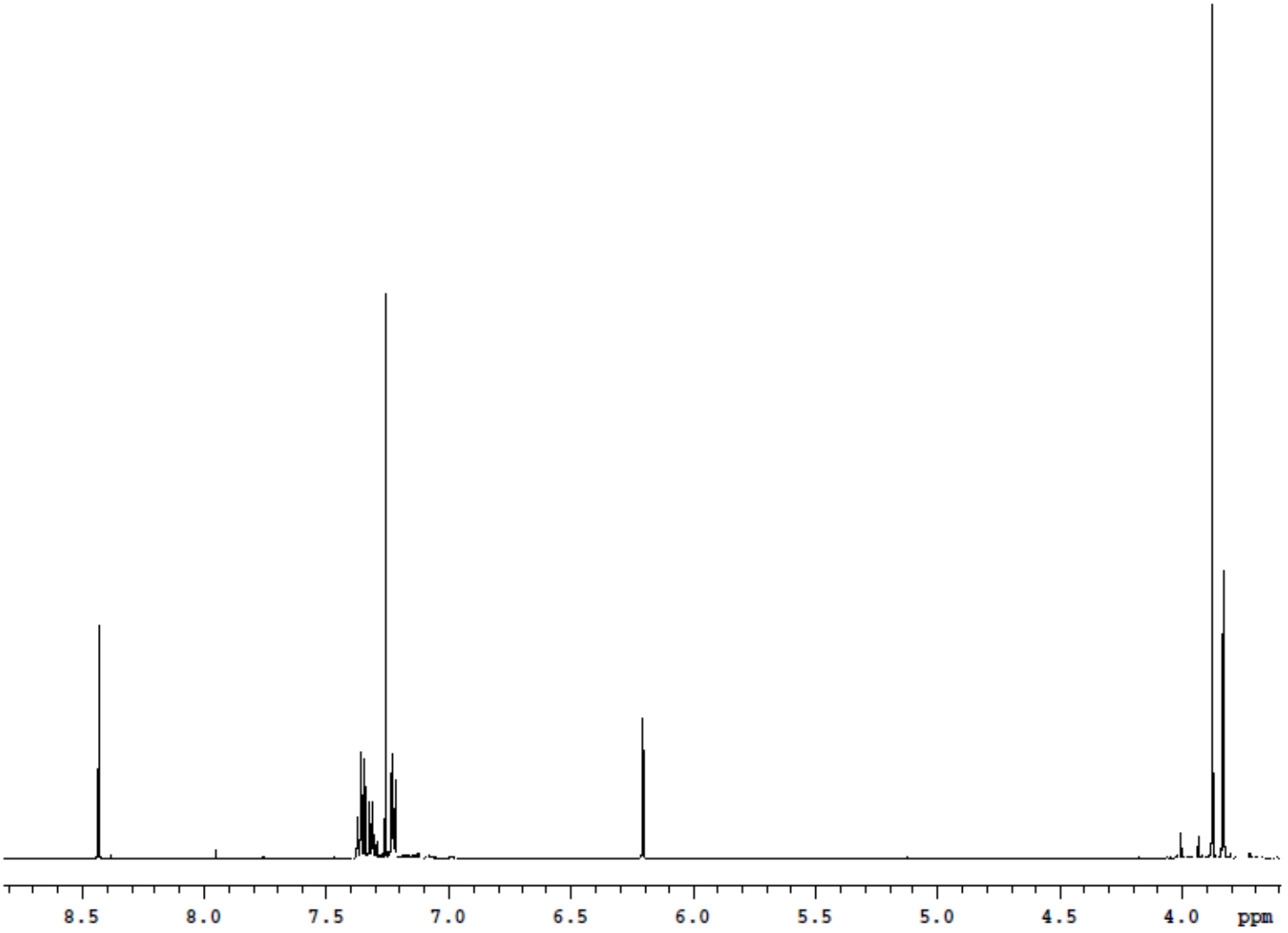
Carbonarone A ^{13}C-NMR in $CDCl_3$.



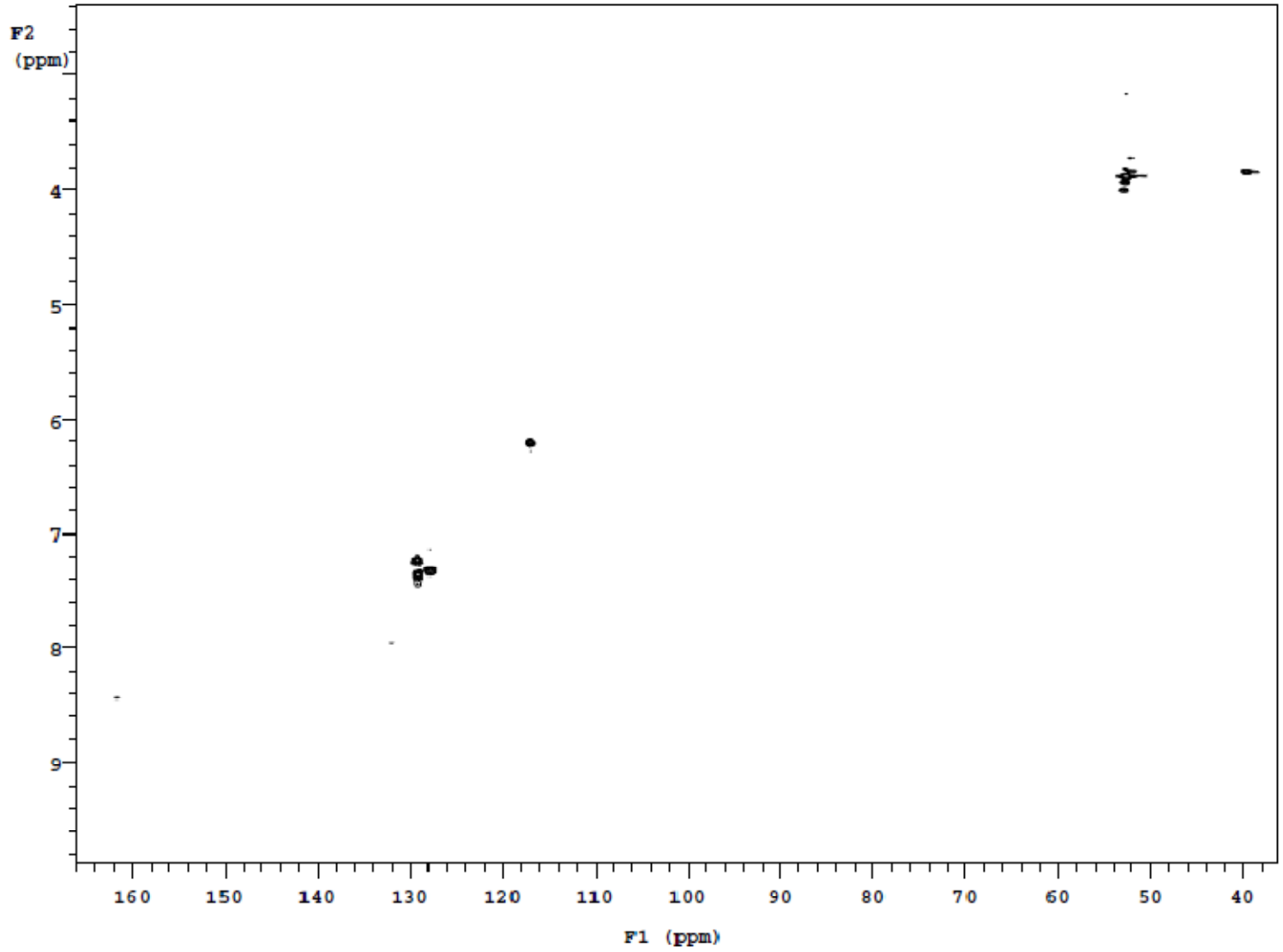
Carbonarone A ^{1}H-^{13}C HMBC in $CDCl_3$.



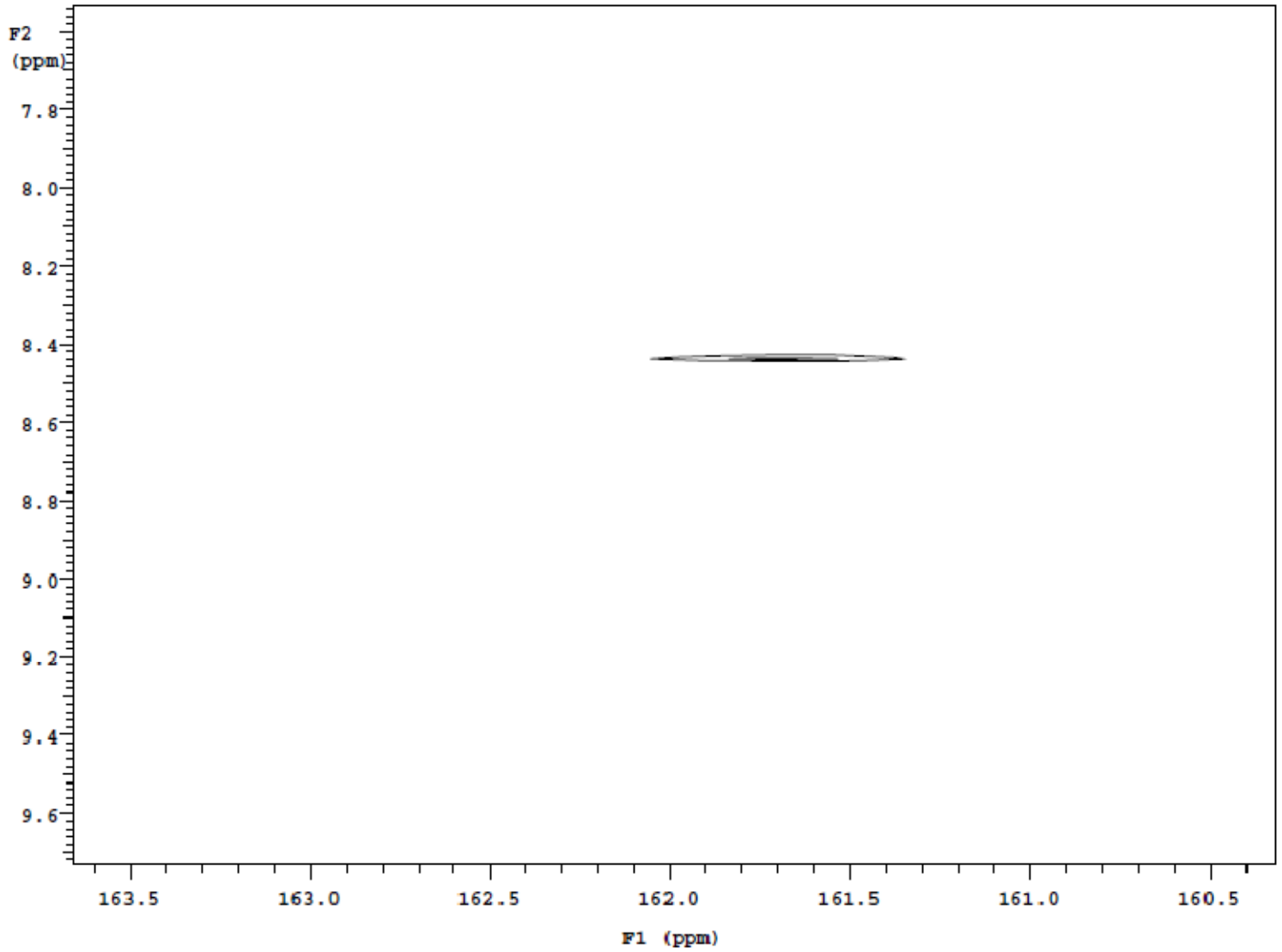
Compound 48 ^{1}H-NMR in $CDCl_3$.



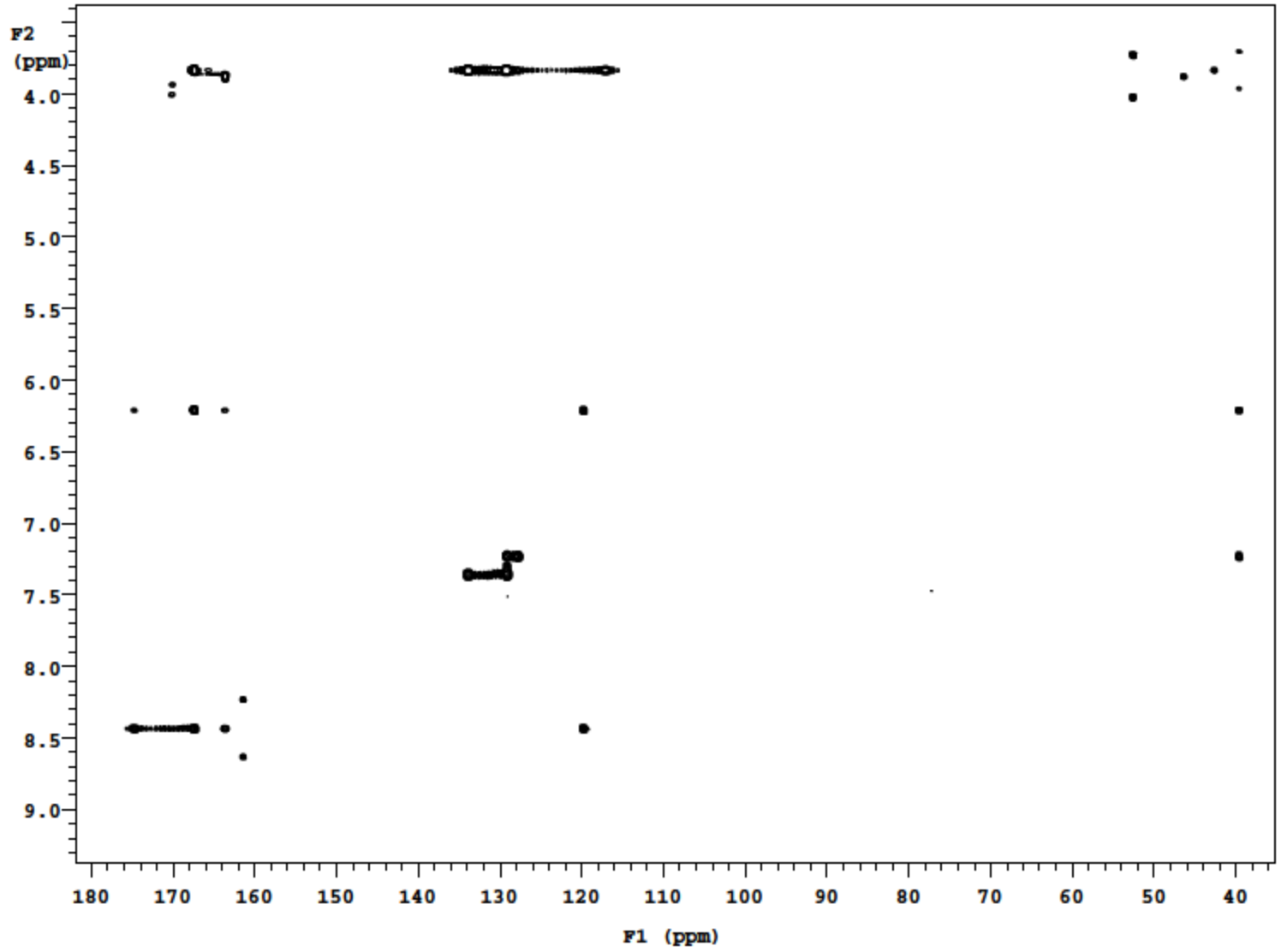
Compound 48 ^{1}H-^{13}C HSQC in $CDCl_3$.



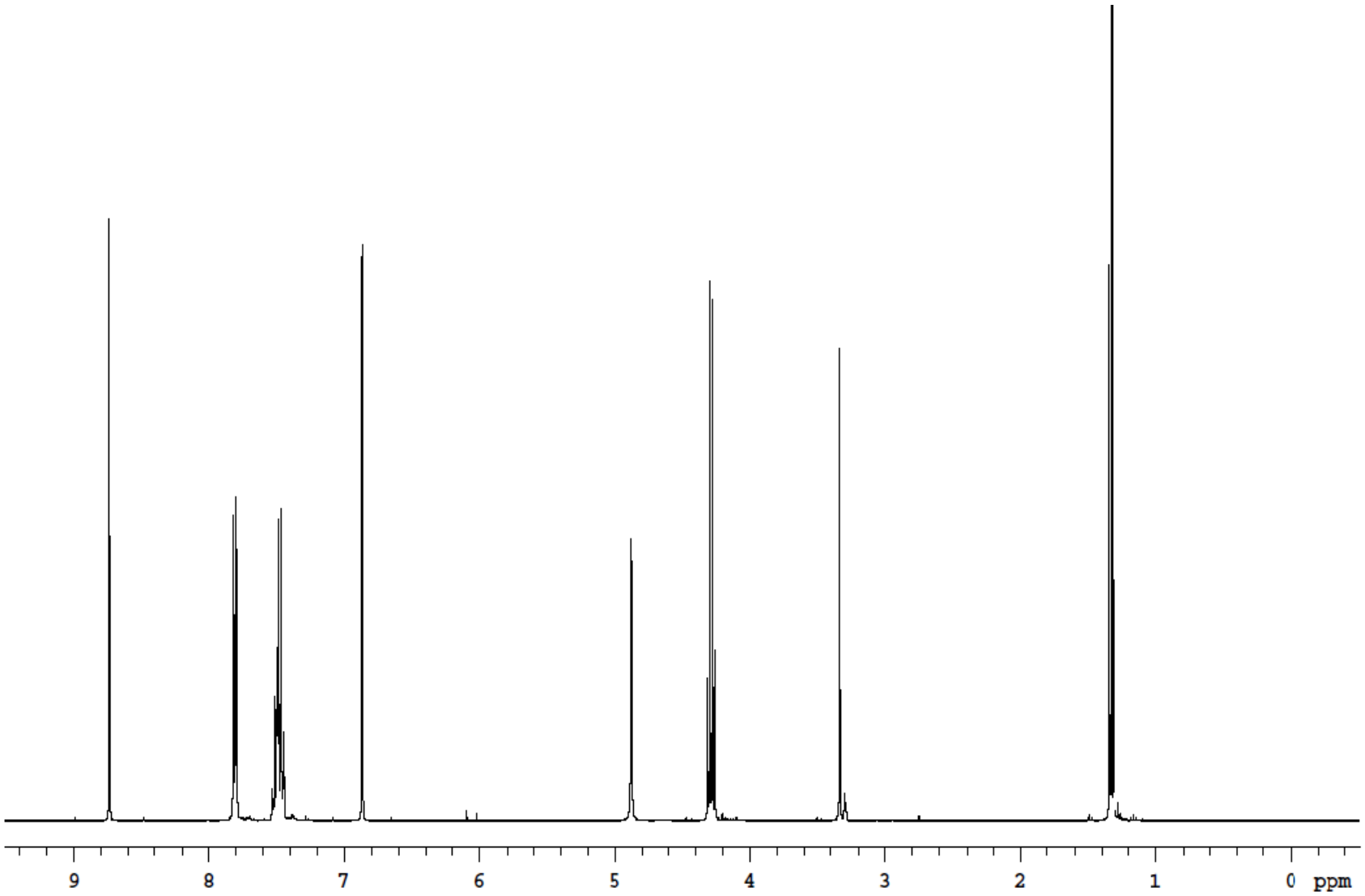
Compound 48 ^{1}H-^{13}C HSQC in $CDCl_3$. Expanded view of correlation of δ_H 8.40.



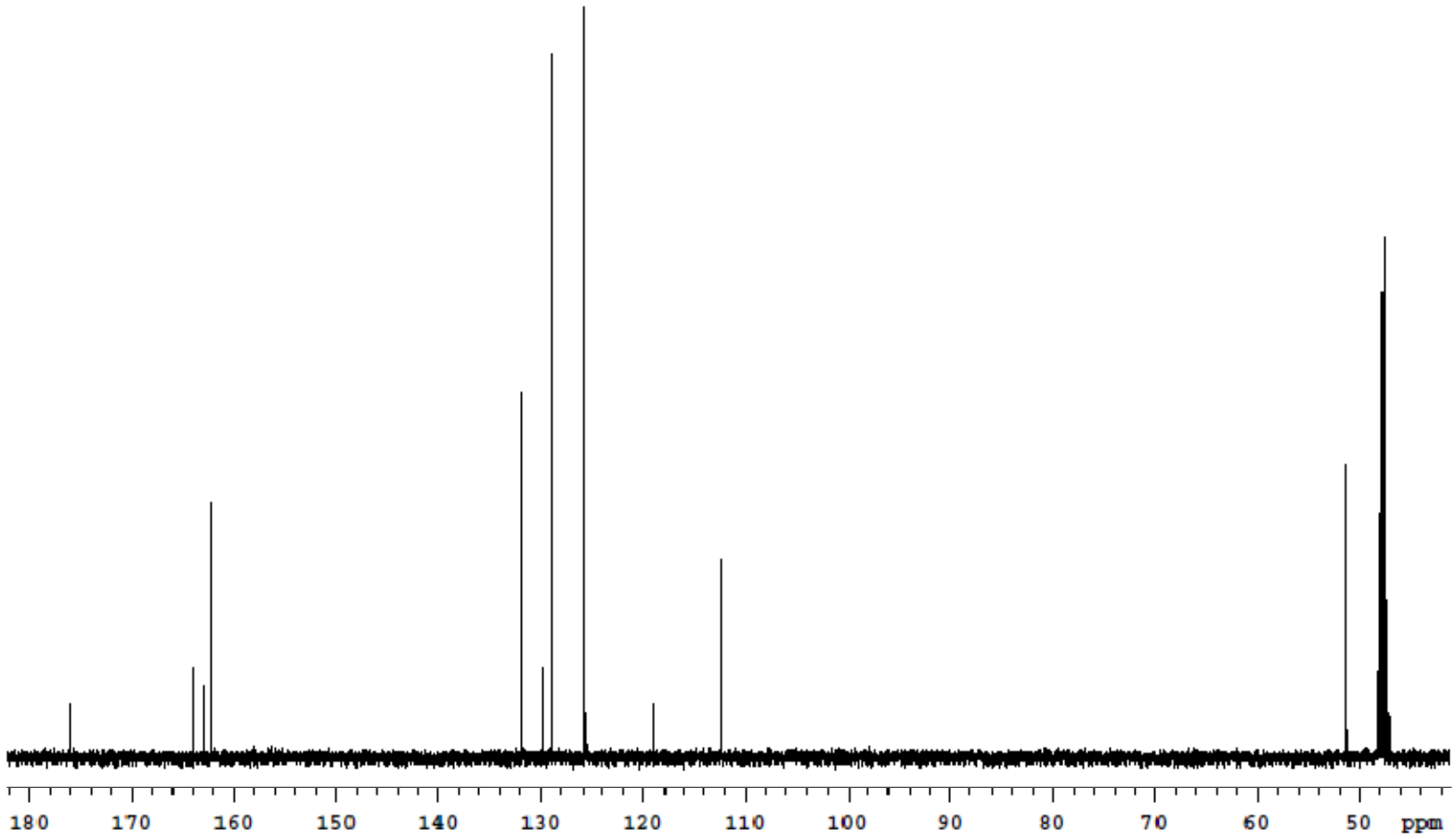
Compound 48 ^{1}H-^{13}C HMBC in $CDCl_3$.



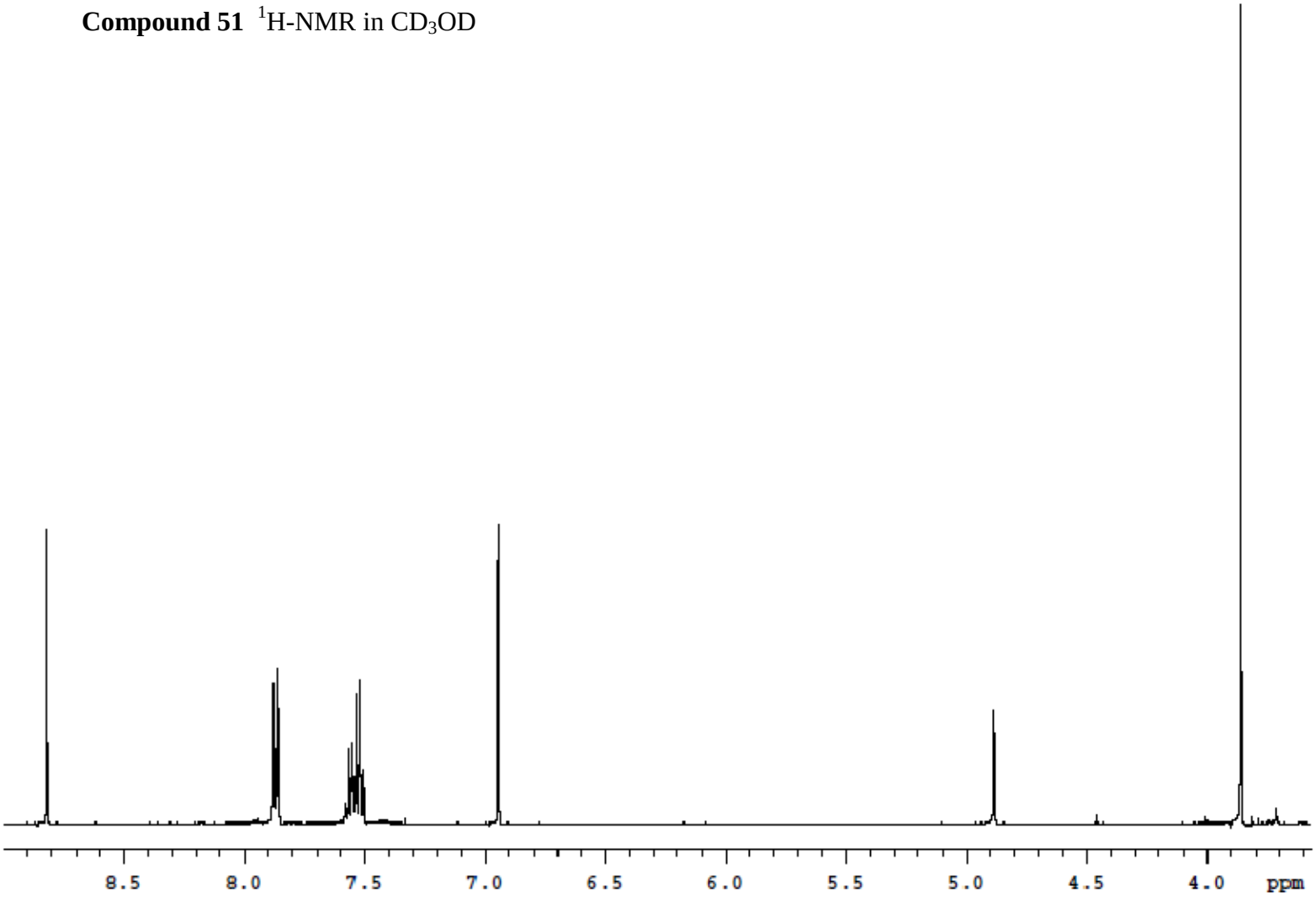
Compound 50 ^{1}H-NMR in CD_3OD



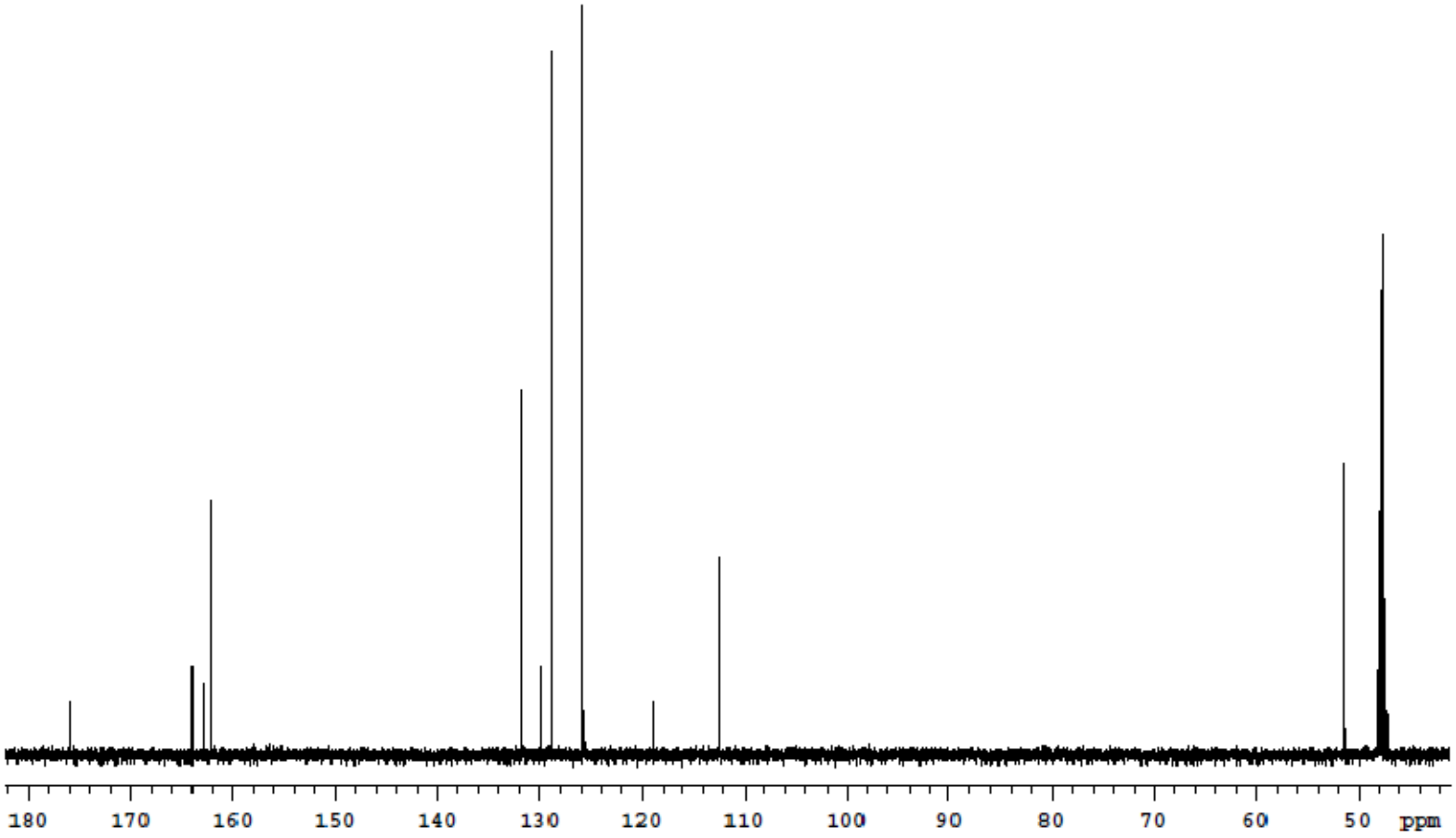
Compound 50 ^{13}C-NMR in CD_3OD



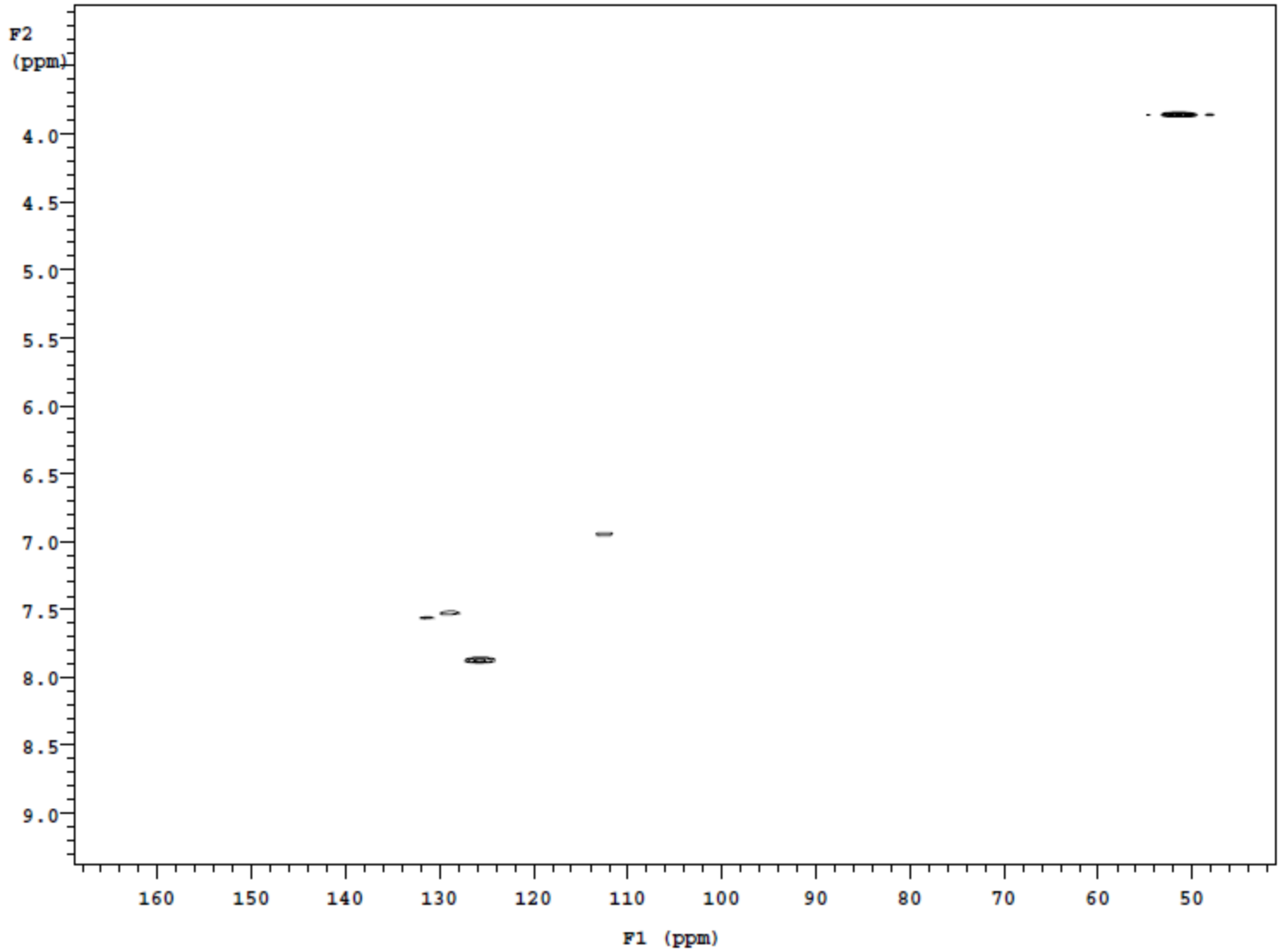
Compound 51 ^{1}H-NMR in CD_3OD



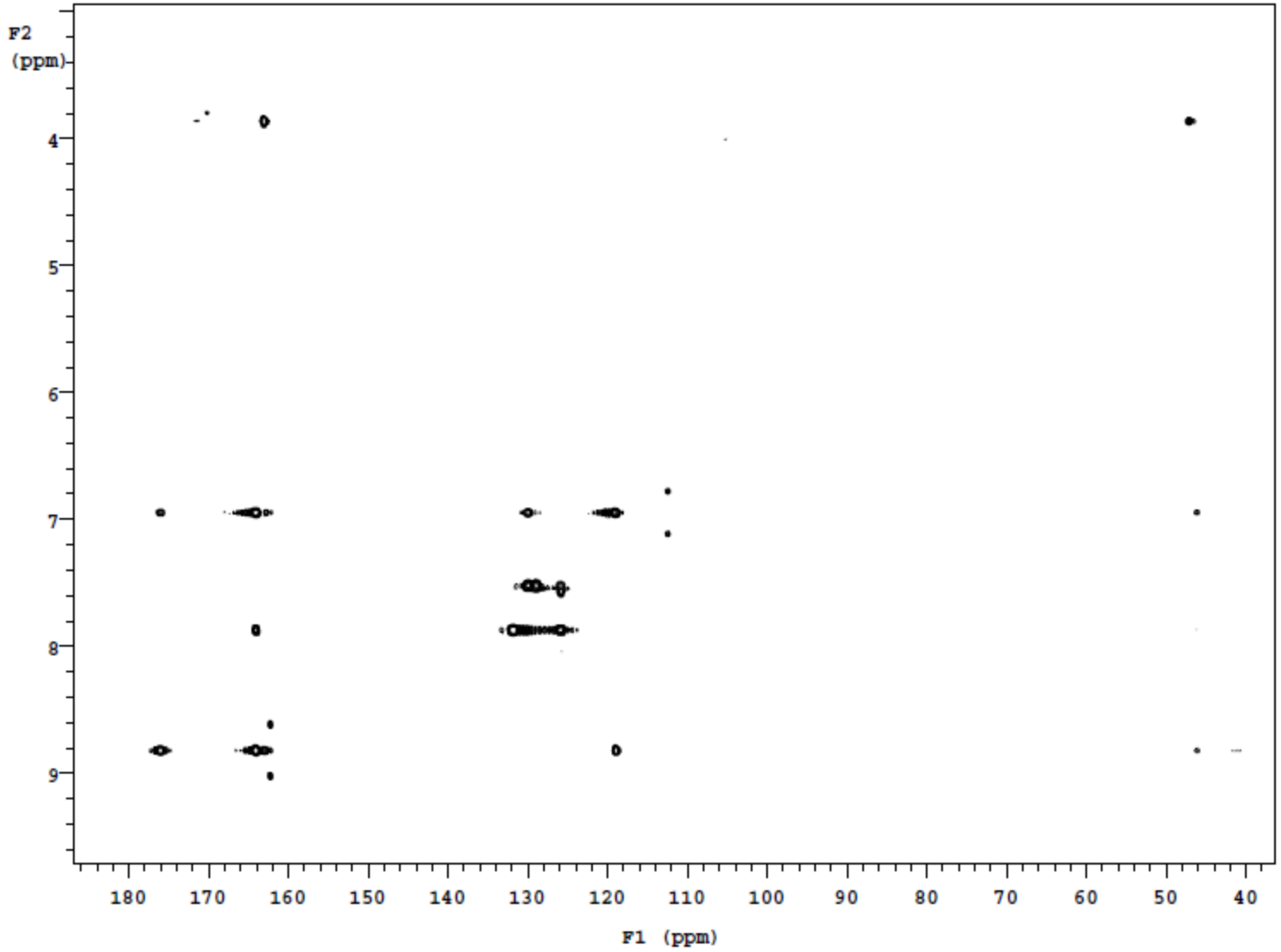
Compound 51 ^{13}C-NMR in CD_3OD



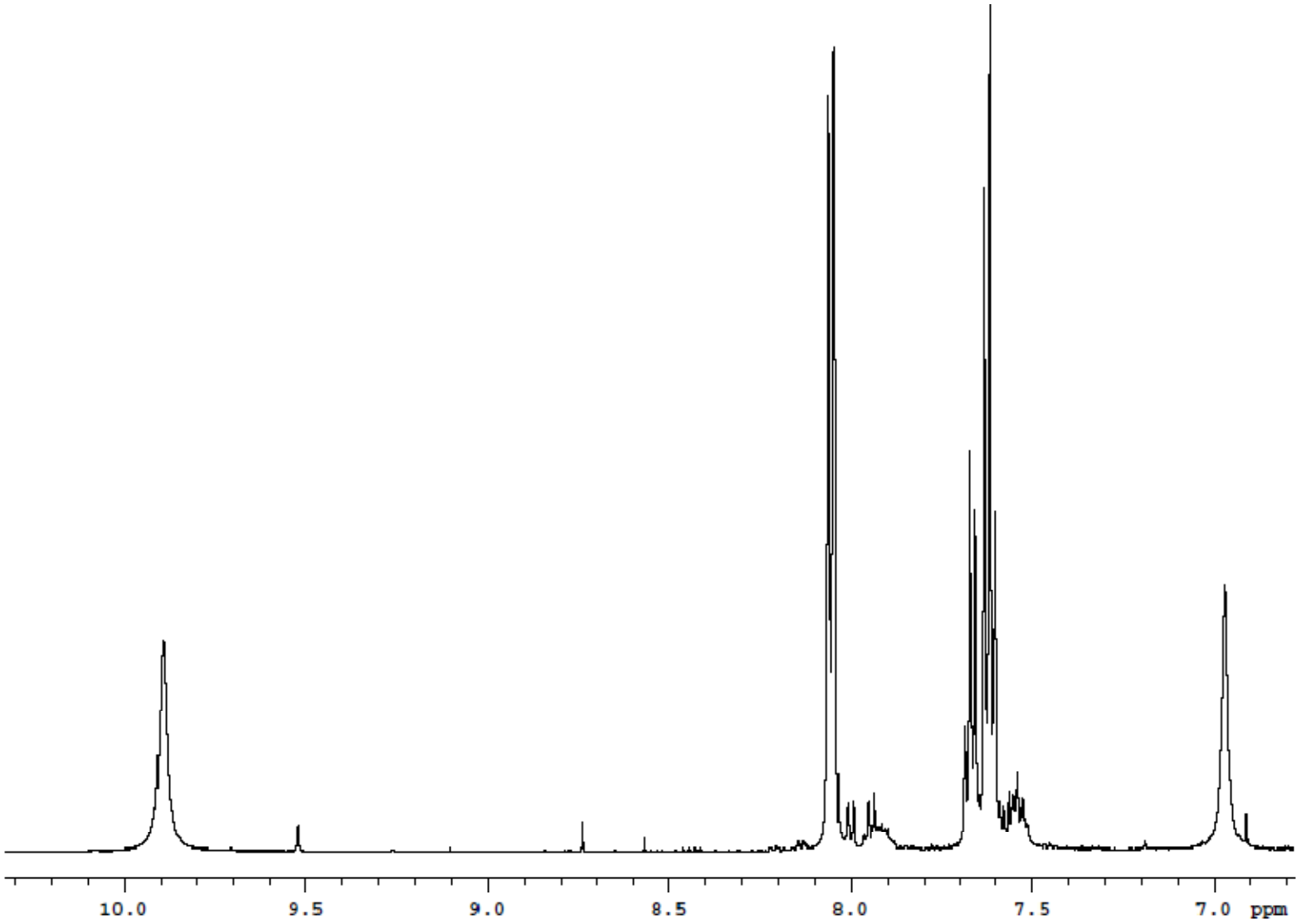
Compound 51 ^{1}H-^{13}C HSQC in CD_3OD



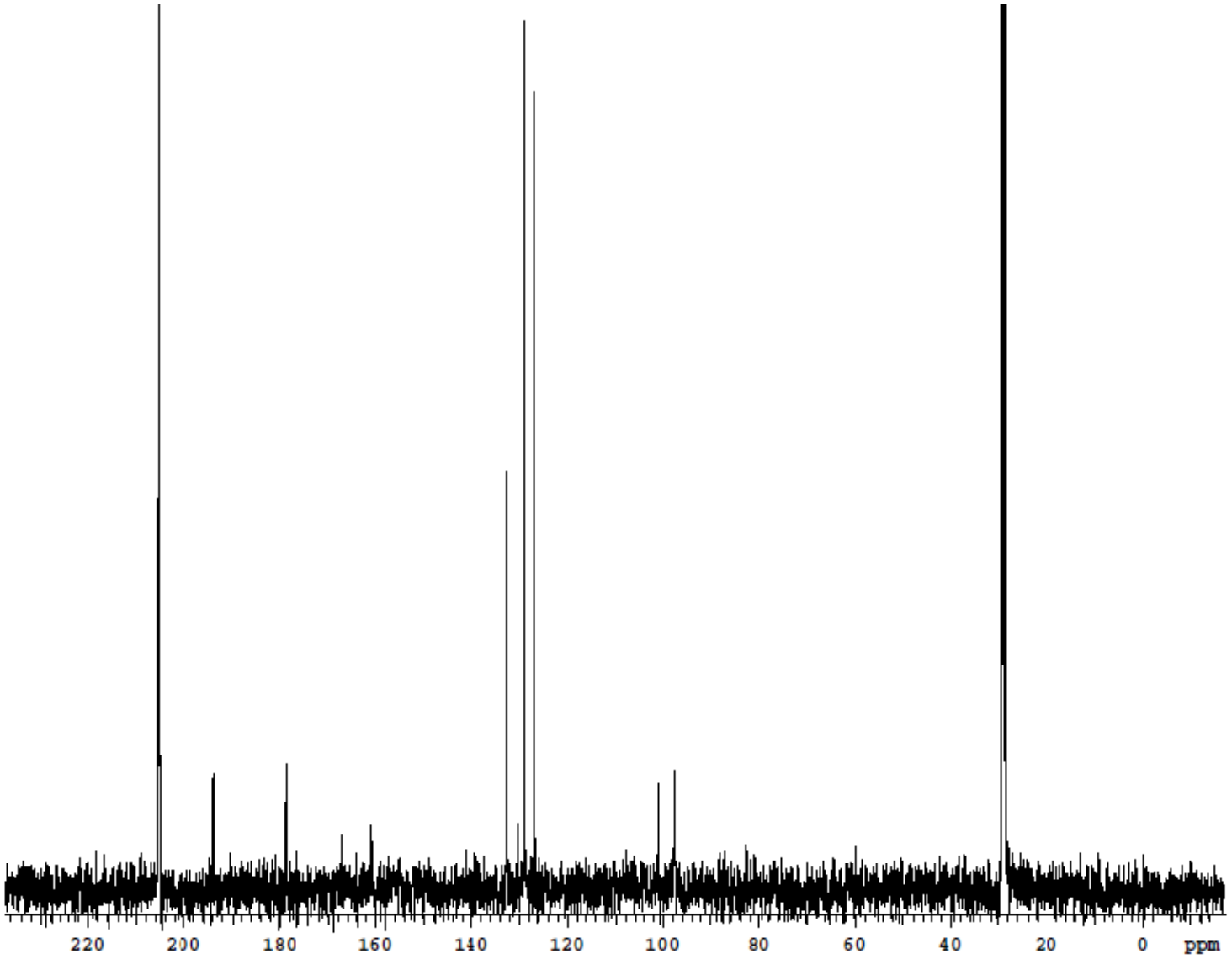
Compound 51 ^{1}H-^{13}C HMBC in CD_3OD



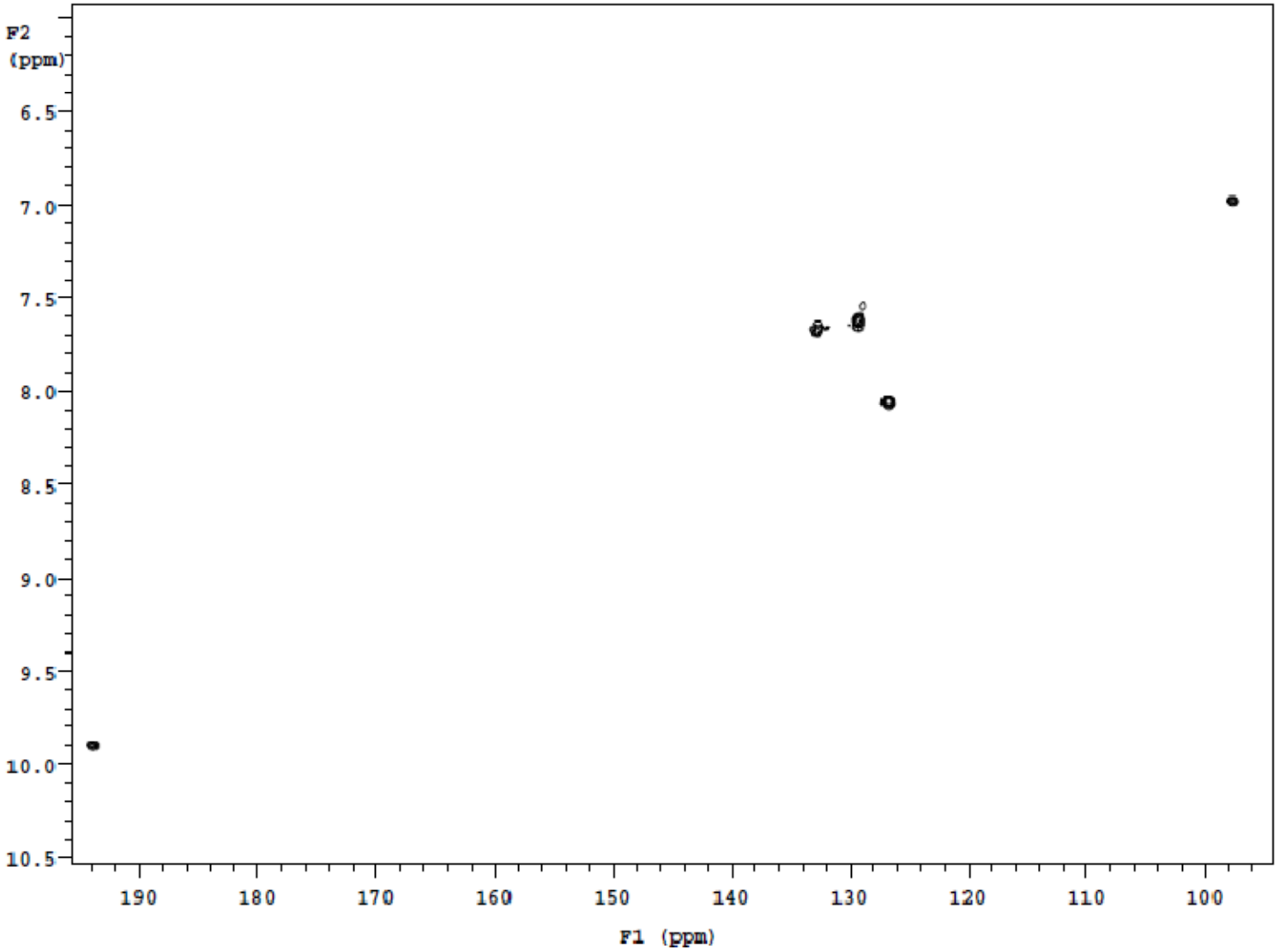
Compound 52 ^{1}H-NMR in (CD3)2CO



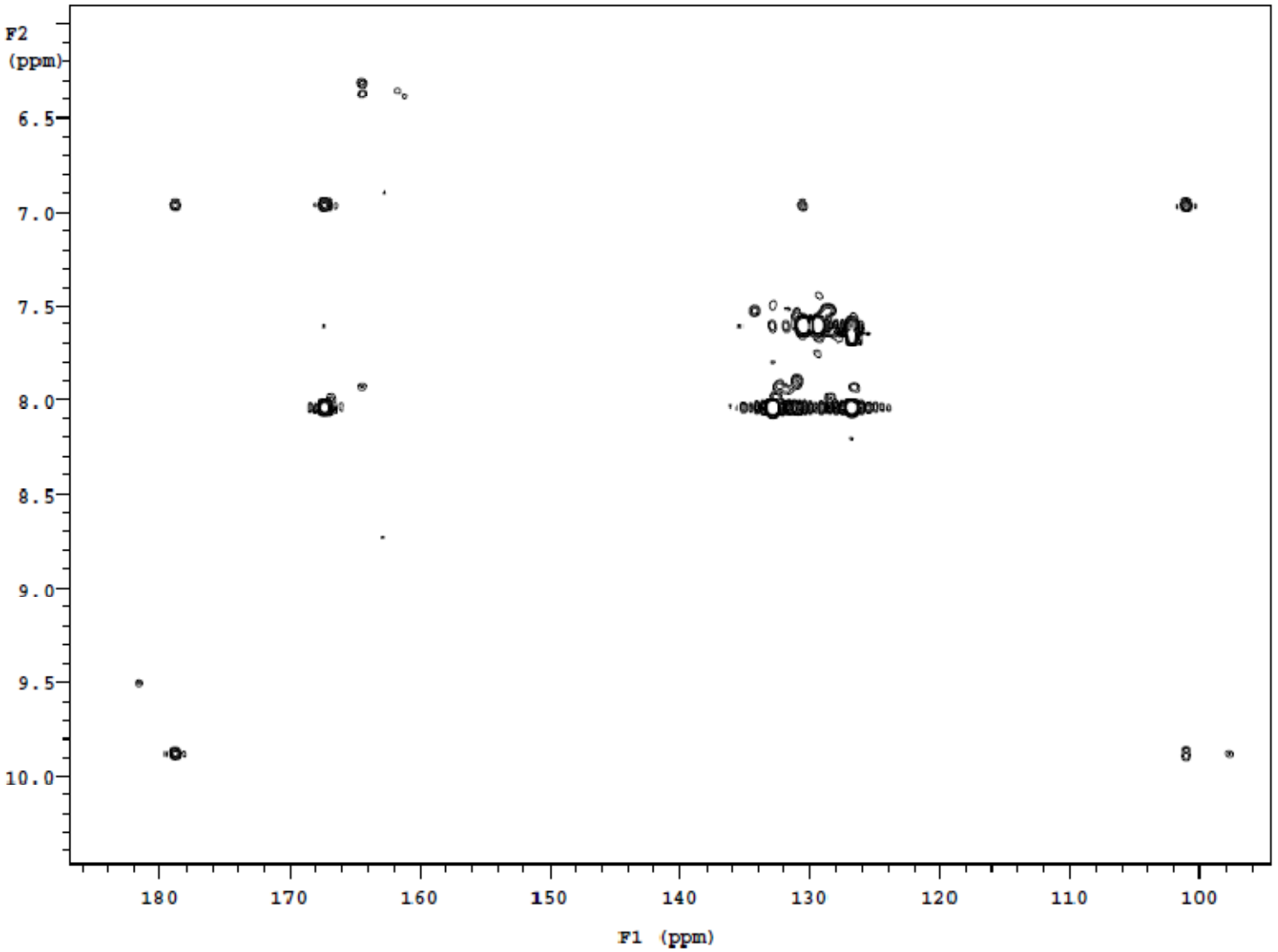
Compound 52 ^{13}C-NMR in (CD3)2CO



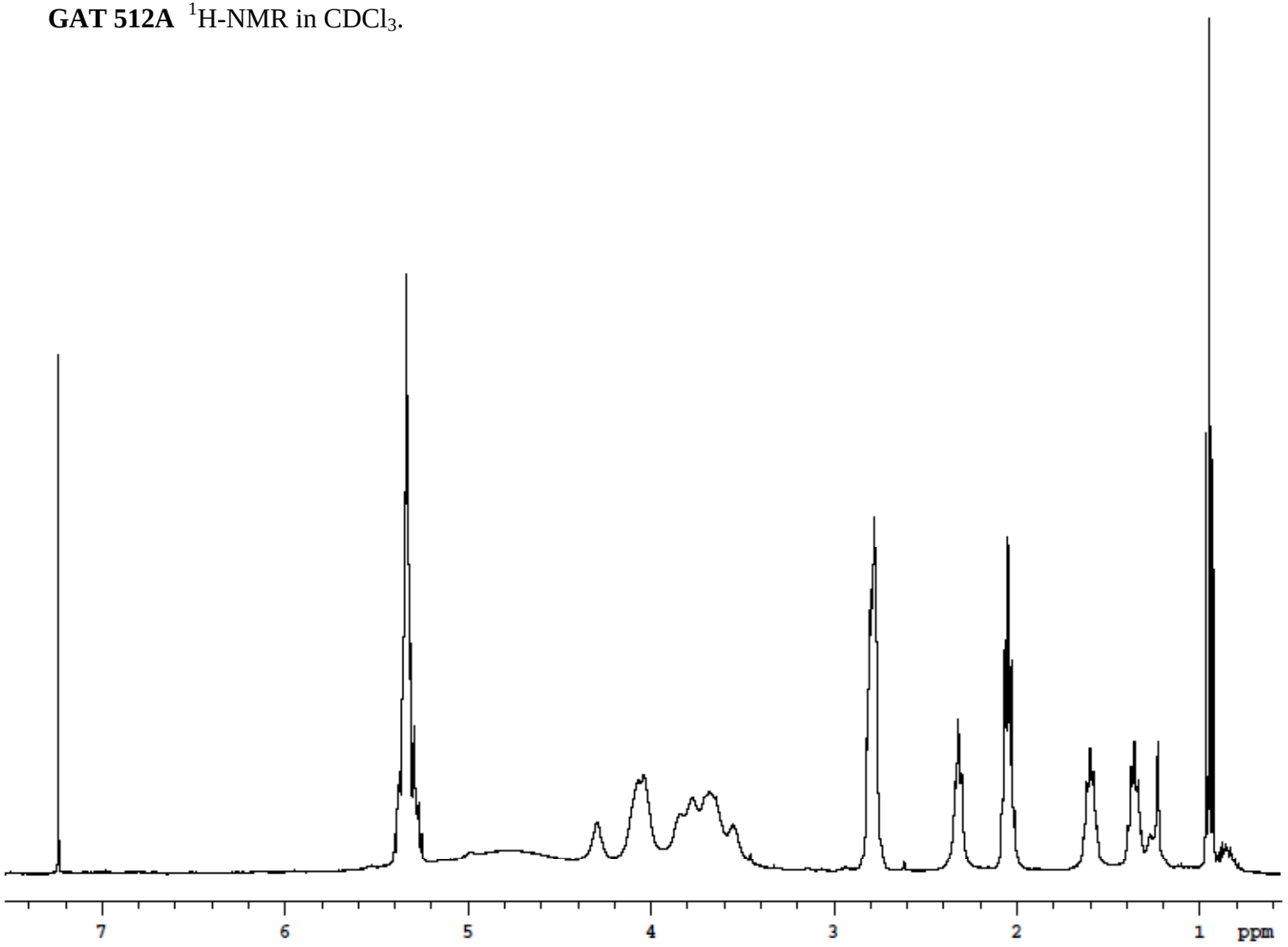
Compound 52 ^{1}H-^{13}C HSQC in (CD3)2CO



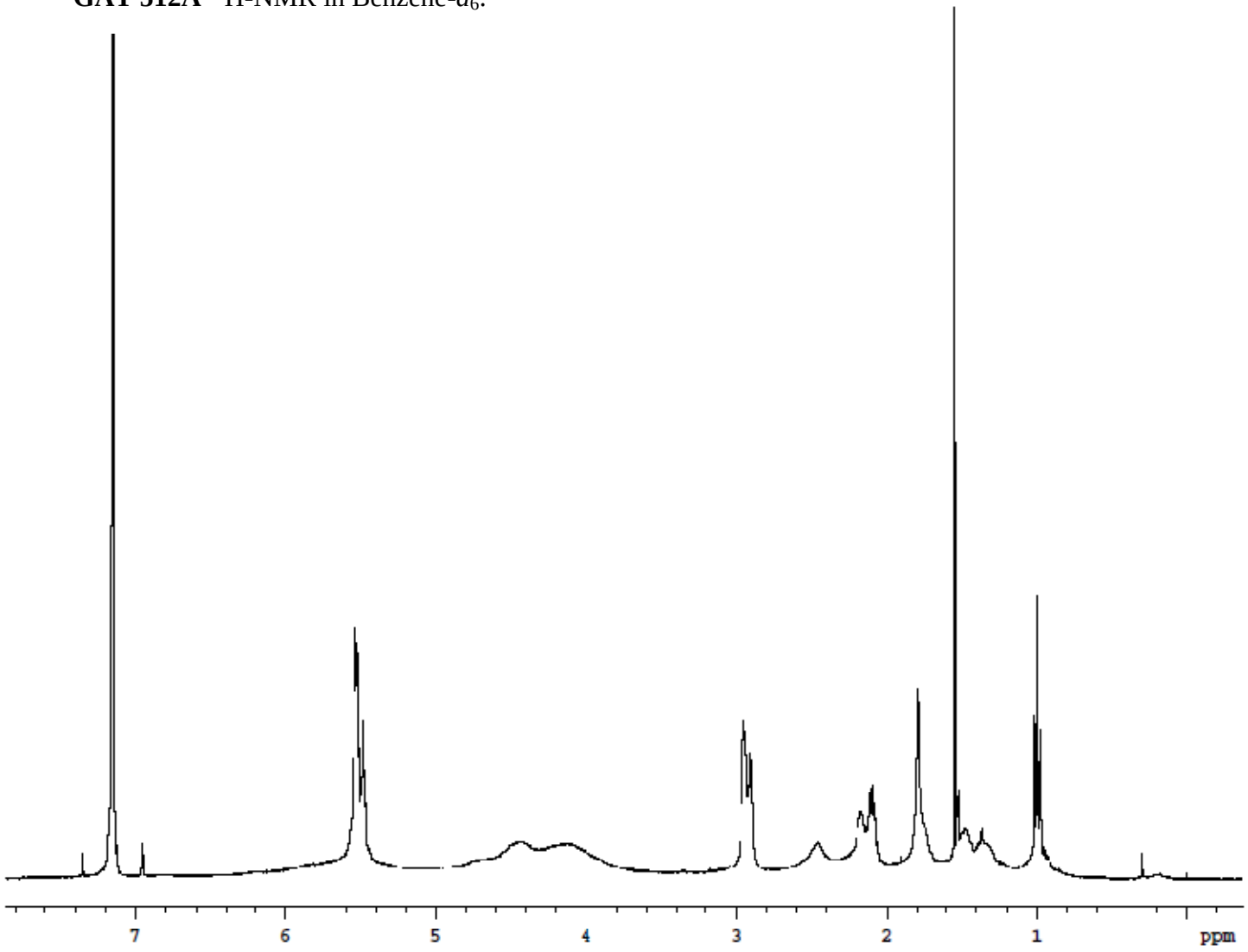
Compound 52 ^{1}H-^{13}C HMBC in (CD3)2CO



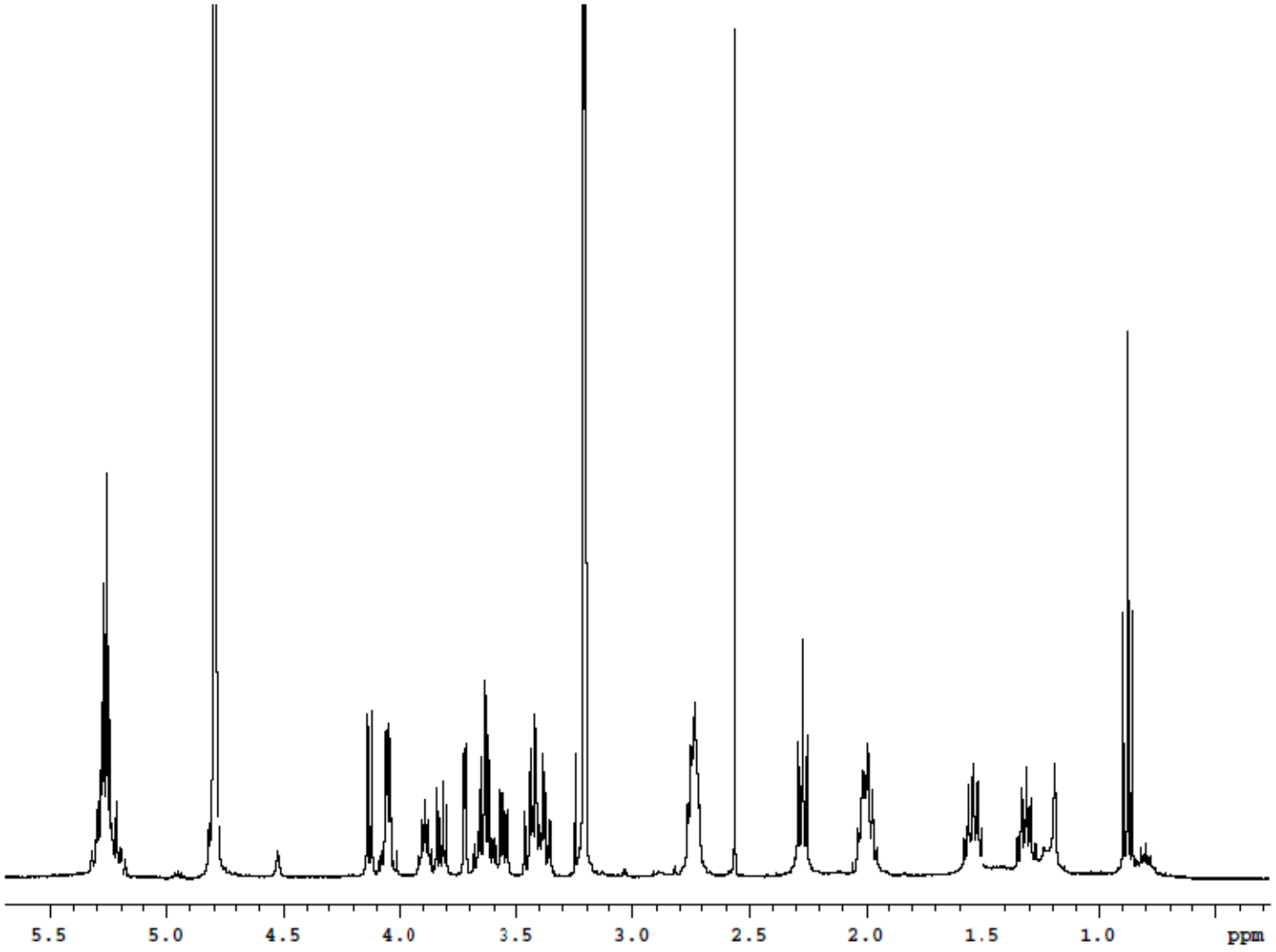
GAT 512A ^{1}H-NMR in $CDCl_3$.



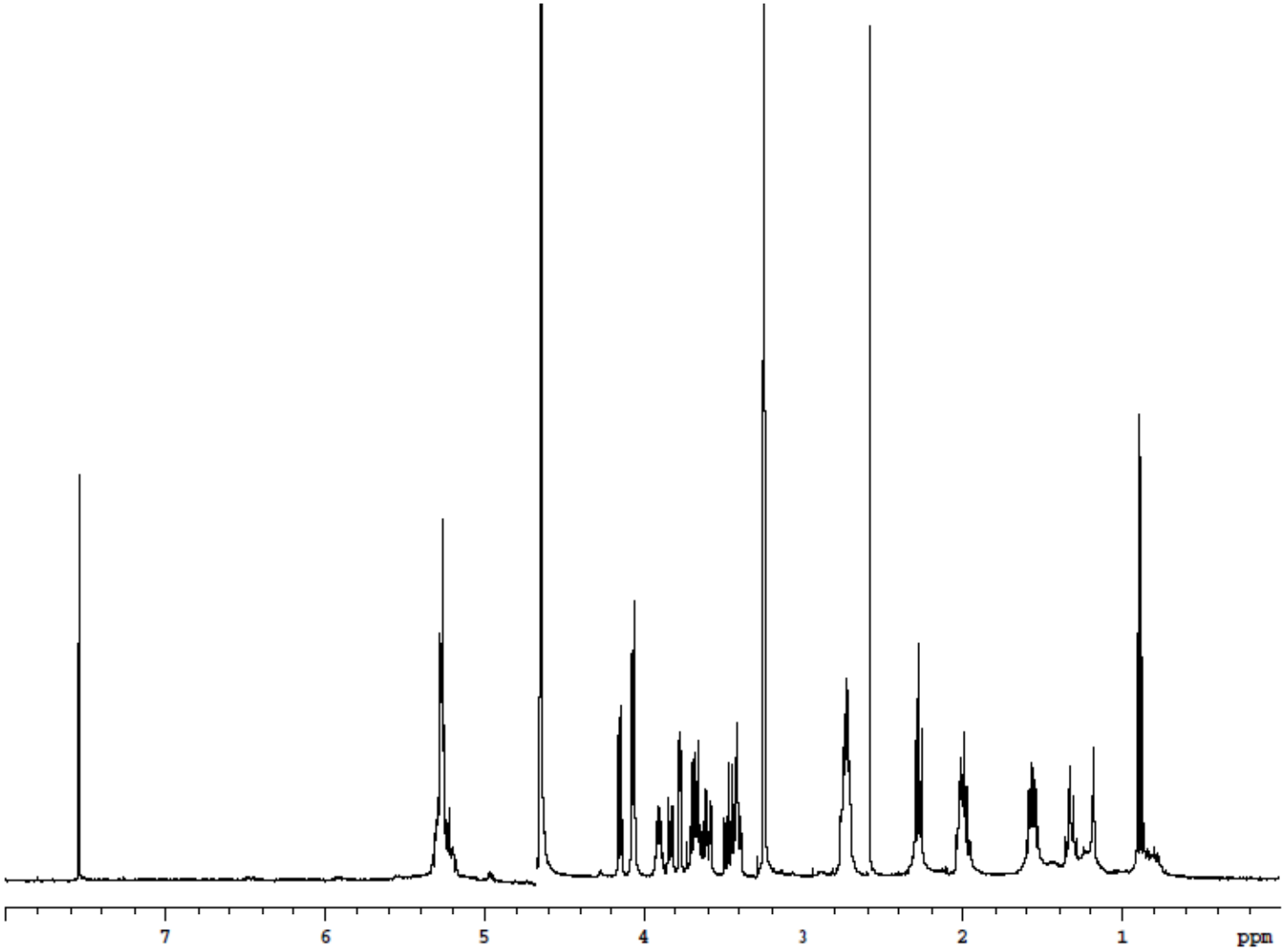
GAT 512A ^{1}H-NMR in Benzene-d_6.



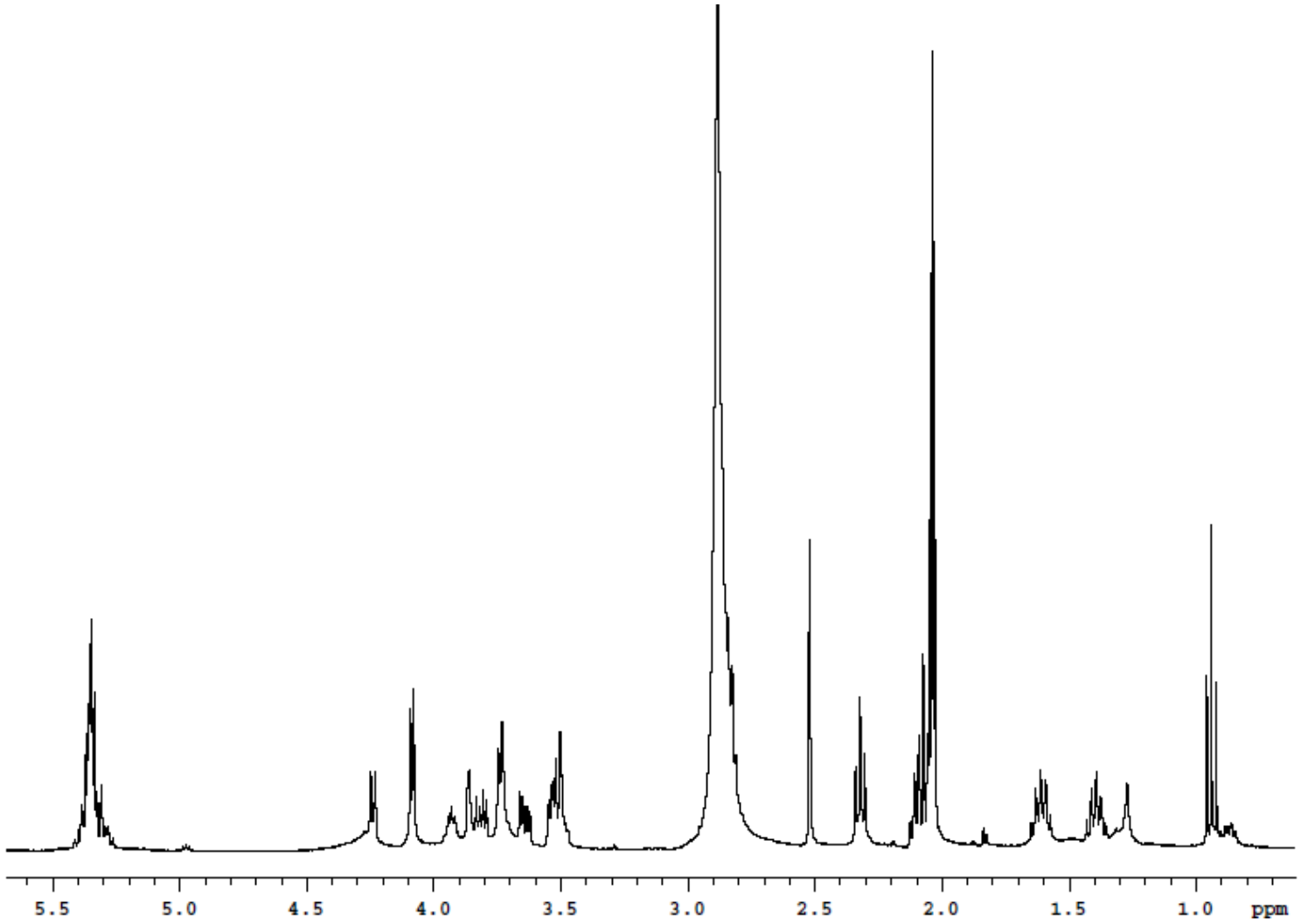
GAT 512A ^{1}H-NMR in CD_3OD.



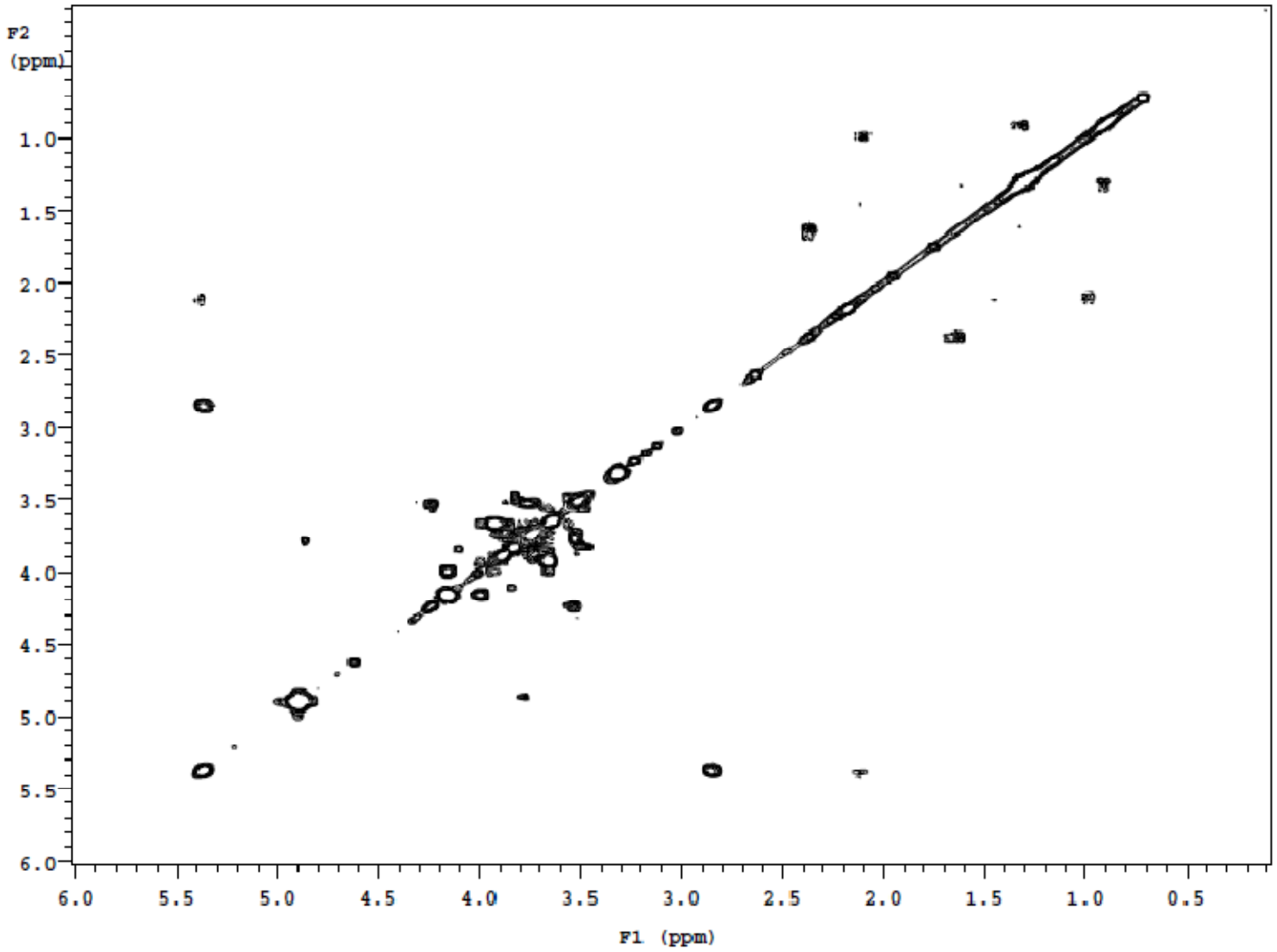
GAT 512A ^{1}H-NMR in 1:1 CD_3OD:$CDCl_3$.



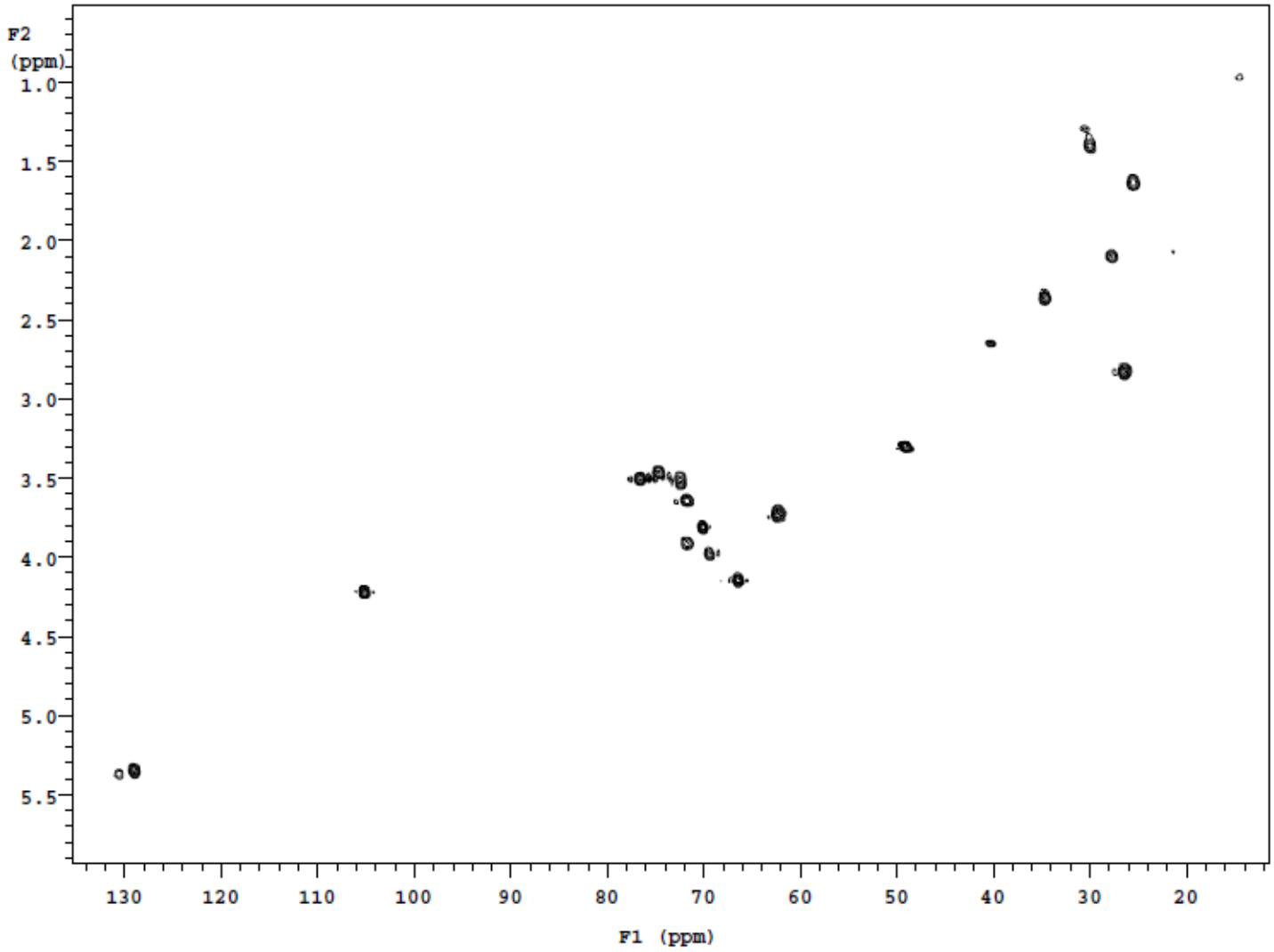
GAT 512A ^{1}H-NMR in Acetone-d_6.



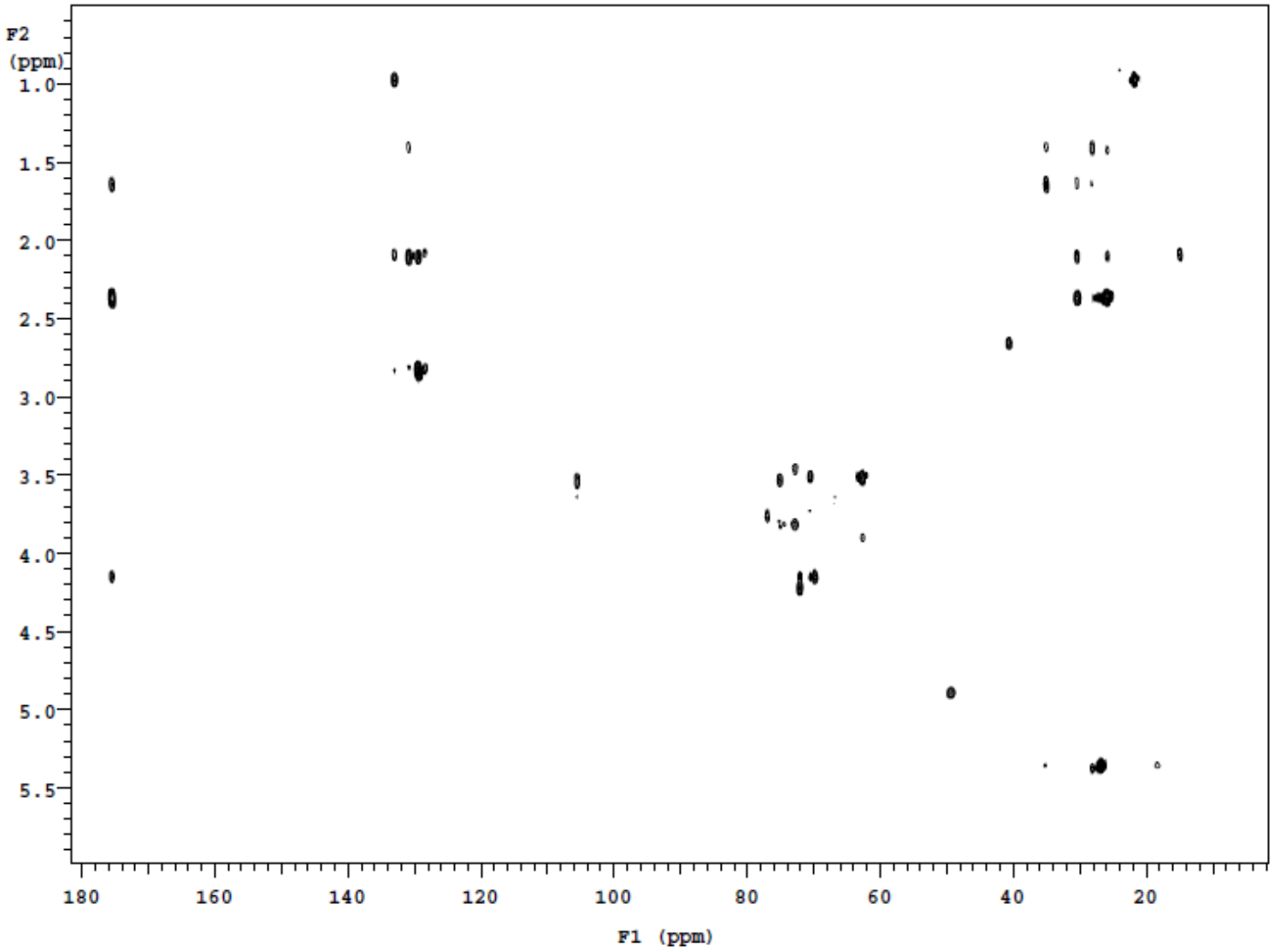
GAT 512A ^{1}H-^{1}H COSY in CD_3OD.



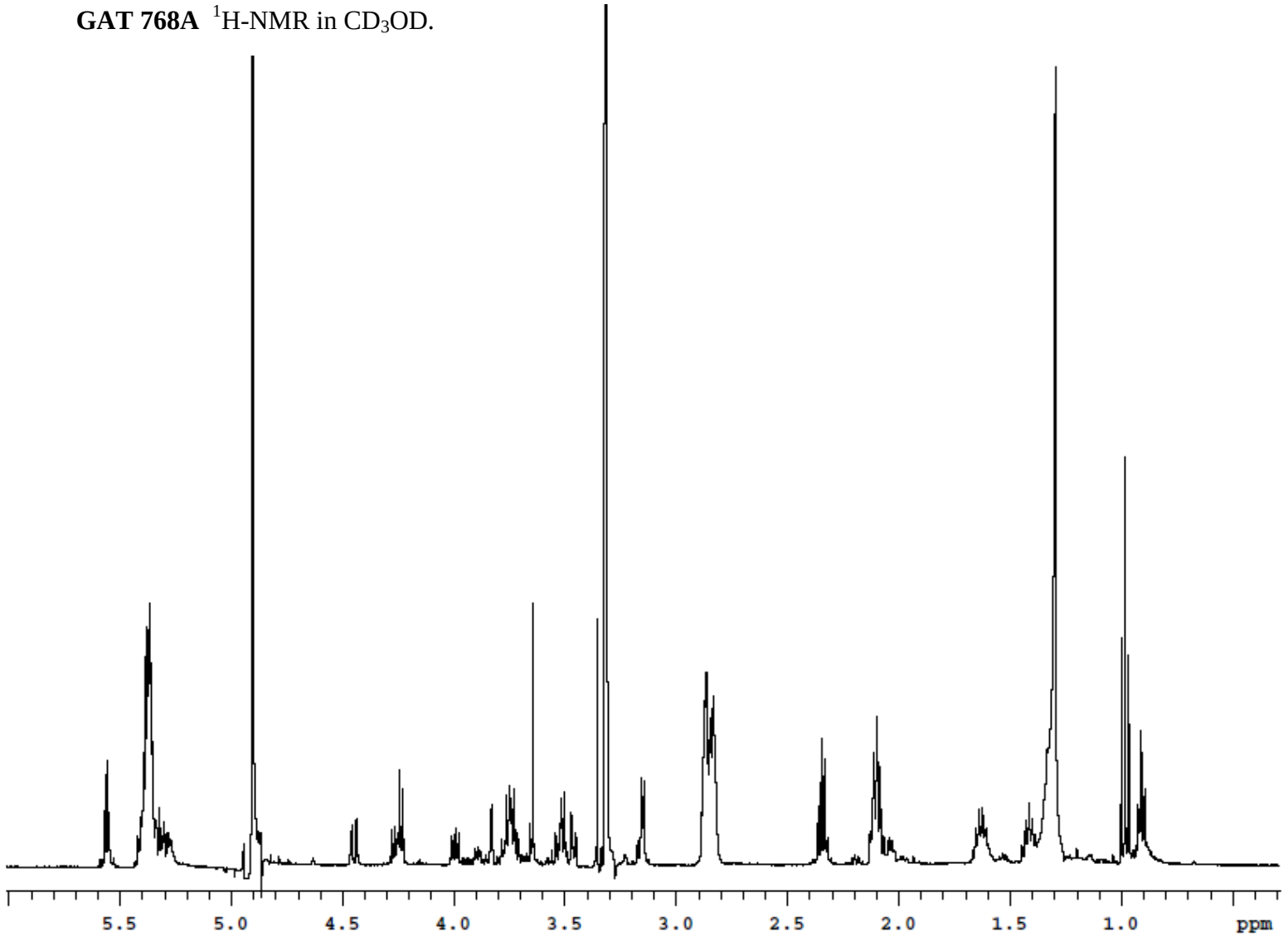
GAT 512A ^{1}H-^{13}C HSQC in CD_3OD.



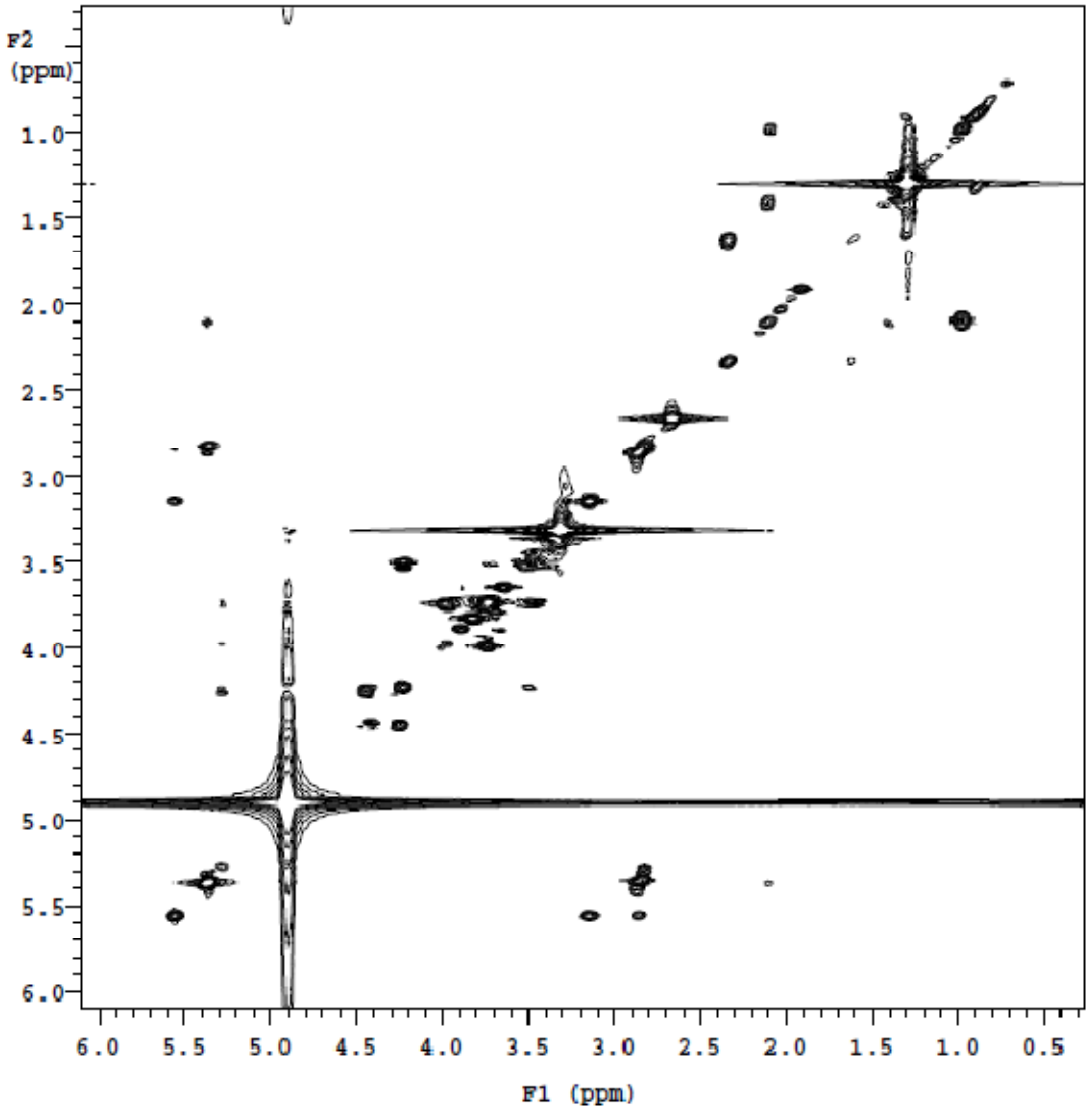
GAT 512A ^{1}H-^{13}C HMBC in CD_3OD.



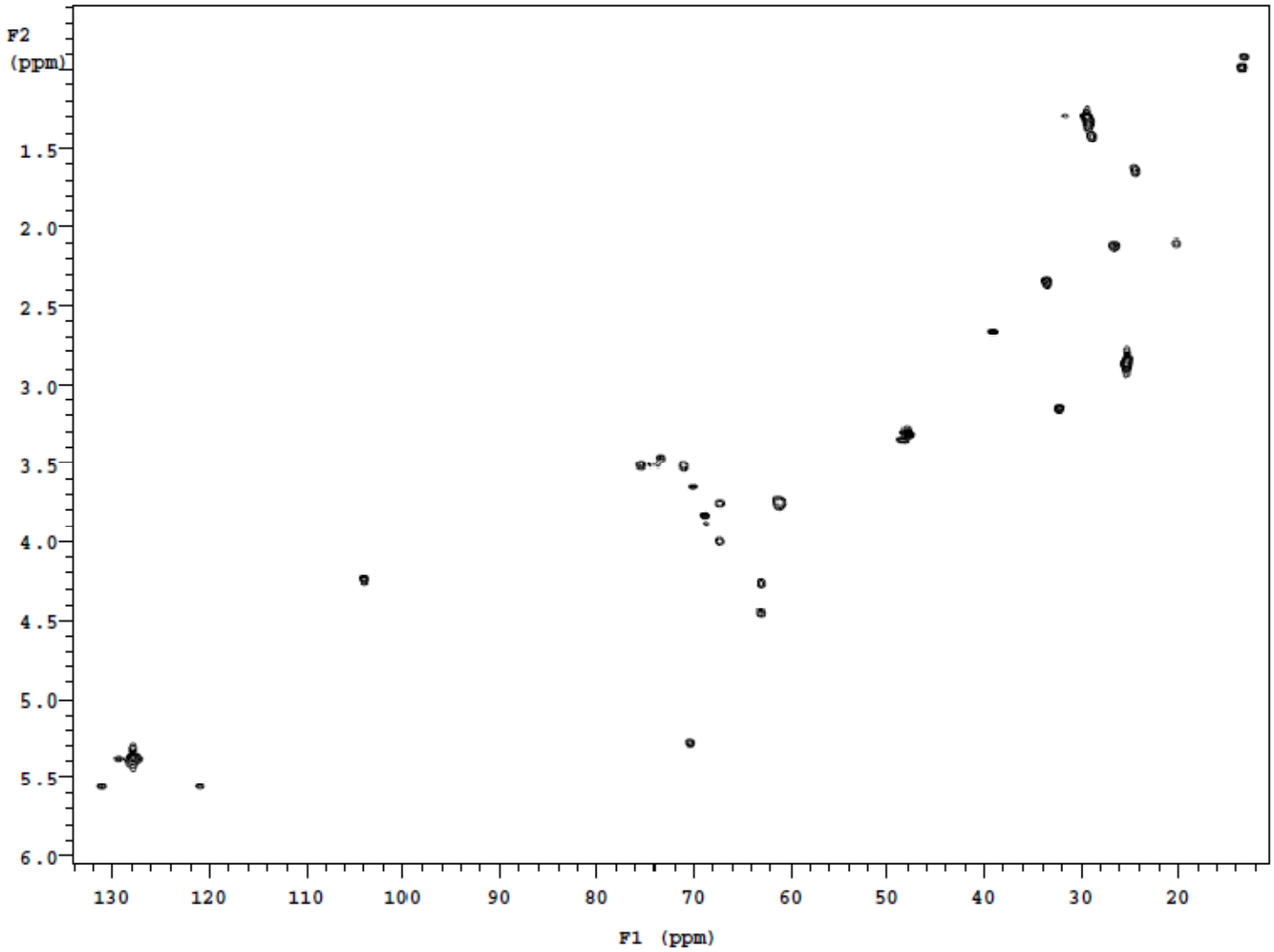
GAT 768A ^{1}H-NMR in CD_3OD.



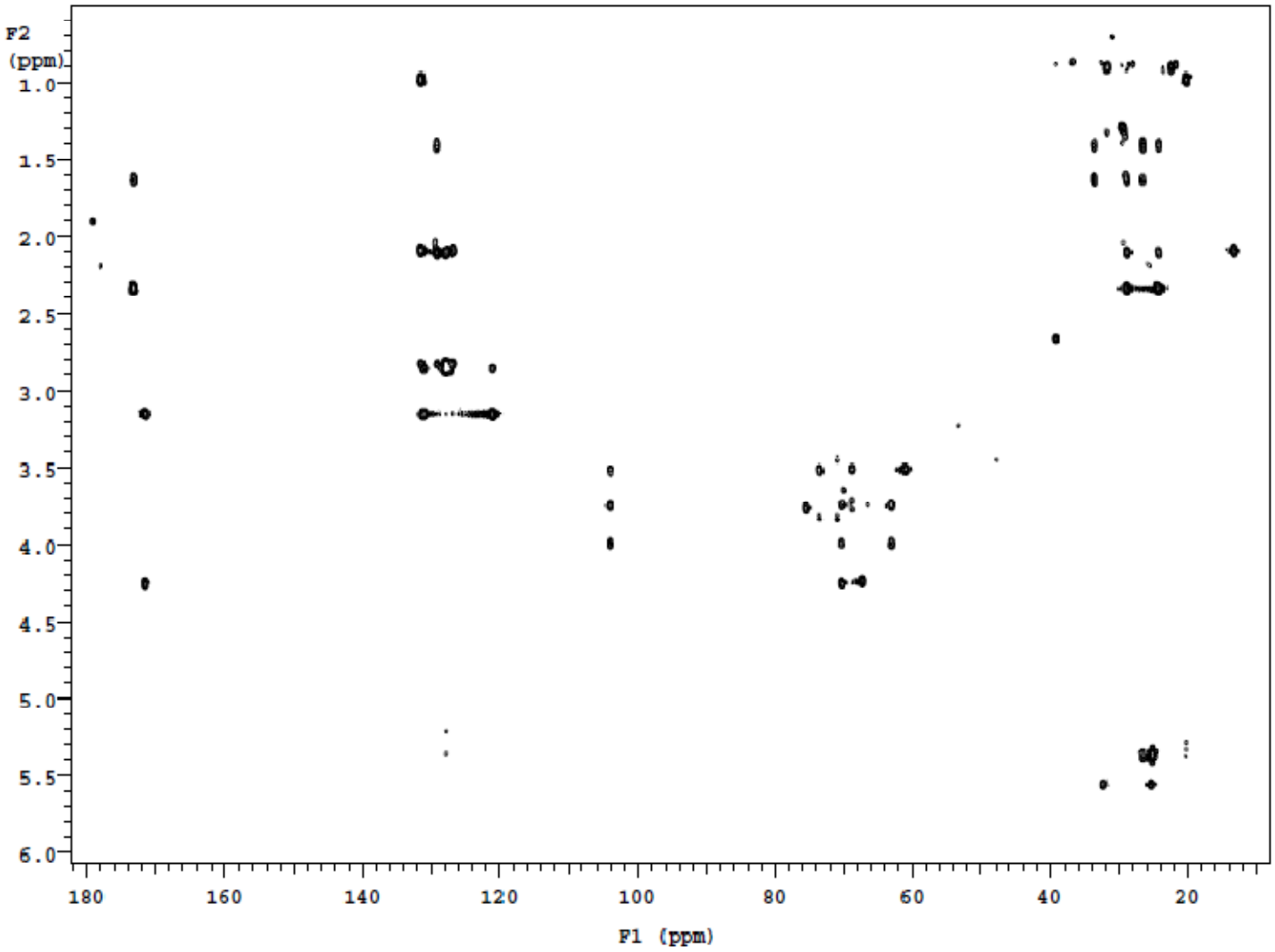
GAT 768A ^{1}H-^{1}H COSY in CD_3OD.



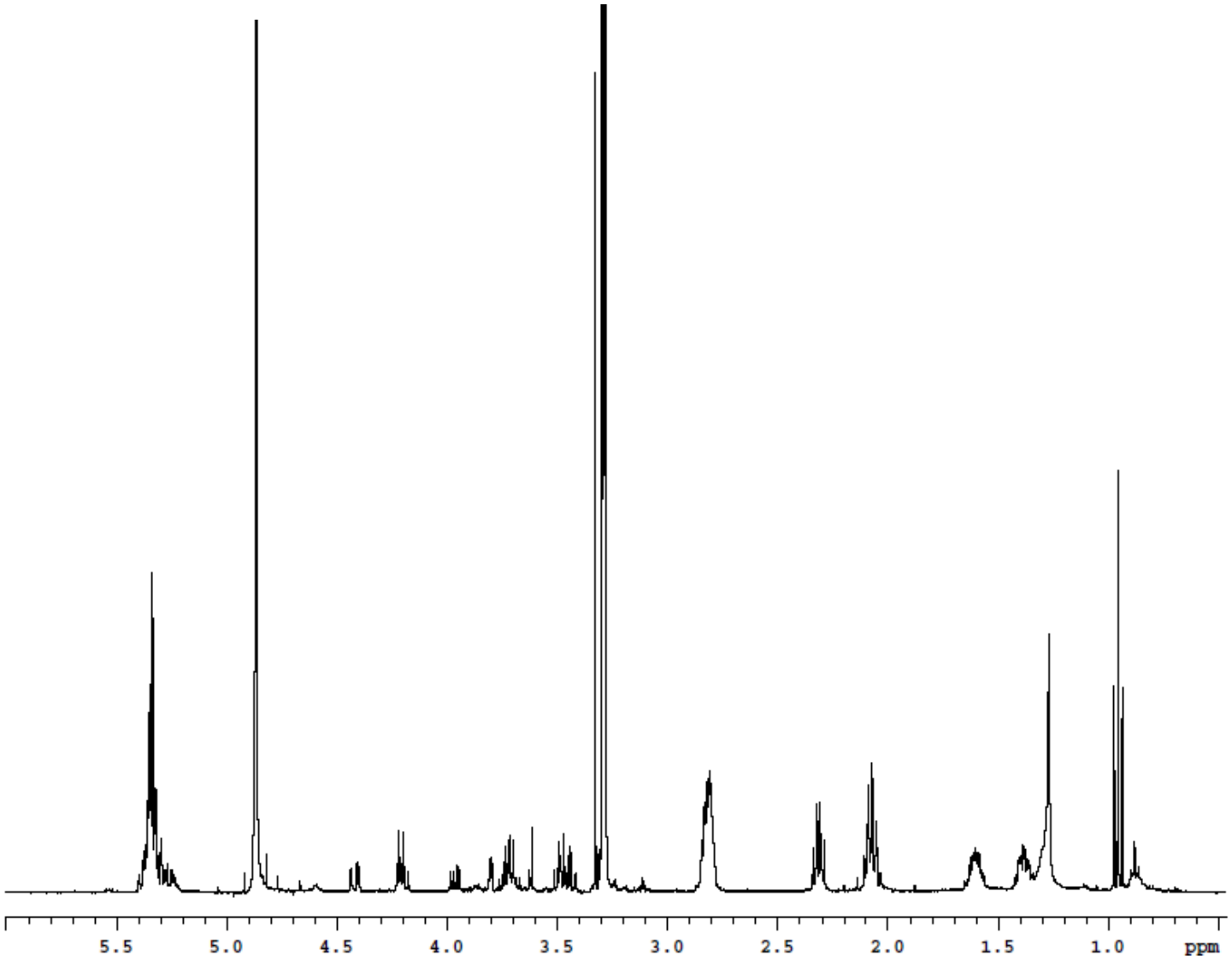
GAT 768A ^{1}H-^{13}C HSQC in CD_3OD.



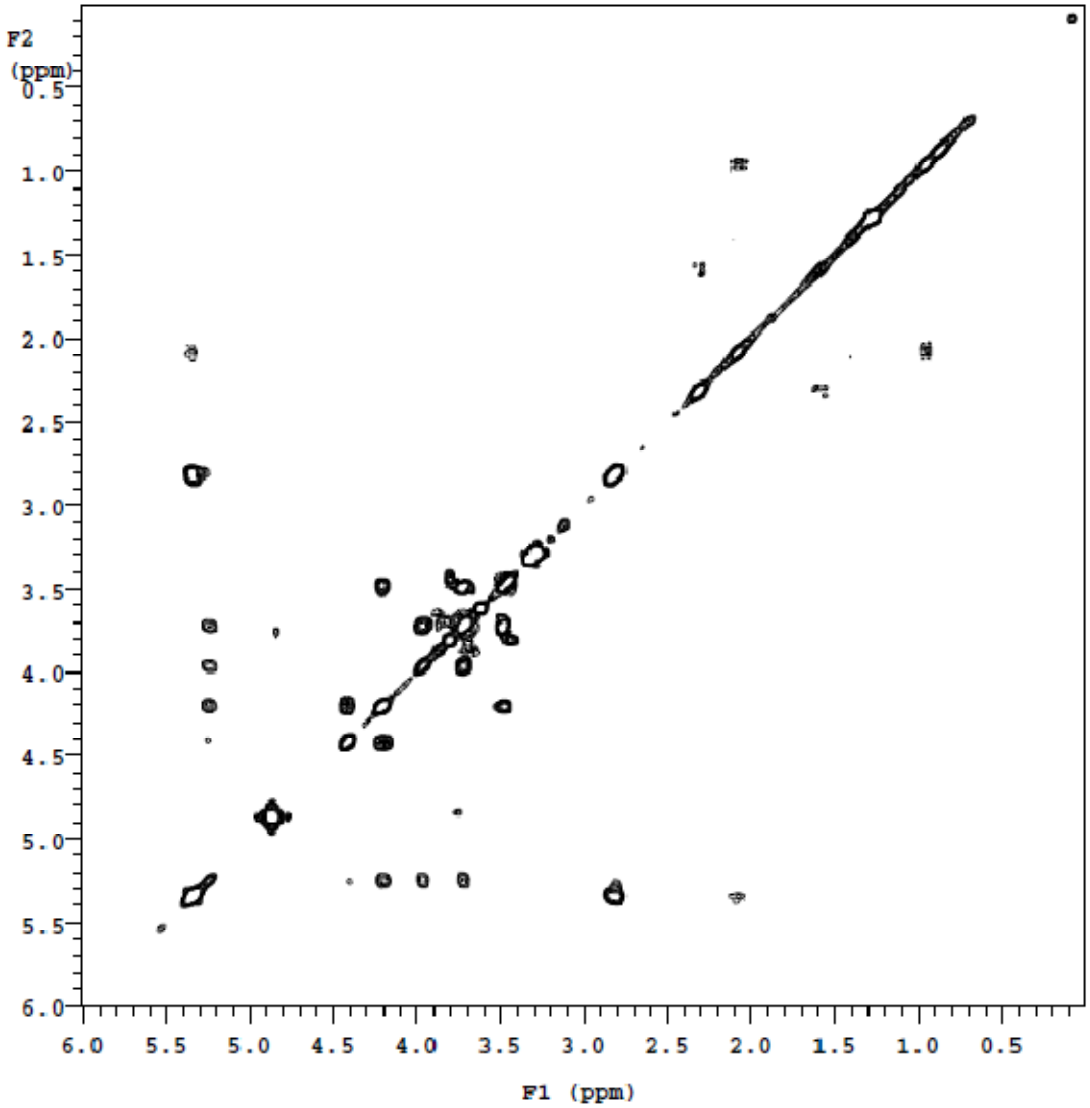
GAT 768A ^{1}H-^{13}C HMBC in CD_3OD.



GAT 770A ^{1}H-NMR in CD_3OD.



GAT 770A ^{1}H-^{1}H COSY in CD_3OD.



GAT 770A ^{1}H-^{13}C HMBC in CD_3OD.

